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Functional knockout of ASIC3 attenuates the exercise pressor reflex in decerebrated rats with ligated femoral arteries

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Kim JS, Ducrocq GP, Kaufman MP. Functional knockout of ASIC3 attenuates the exercise pressor reflex in decerebrated rats with ligated femoral arteries. Am J Physiol Heart Circ Physiol 318: H1316–H1324, 2020. First published April 17, 2020; doi:10.1152/ajpheart.00137.2020.—The exercise pressor reflex arises from contracting muscle and is manifested by increases in arterial pressure, heart rate, and cardiac contractility. In patients with peripheral artery disease, the exercise pressor reflex is exaggerated. This effect is believed to be caused by a metabolite whose concentration is increased when the working muscles are inadequately perfused. Previous work in rats with simulated peripheral artery disease has shown that pharmacological blockade of acid-sensing ion channel 3 (ASIC3), which is found on group III and IV afferents, prevents the exaggeration of the exercise pressor reflex. Blockade of ASIC3, however, may have off-target effects that preclude a conclusion that ASIC3 plays a role in evoking the reflex in rats with simulated peripheral artery disease. In the present experiments performed in decerebrated rats with simulated peripheral artery disease, we compared the exercise pressor reflex in rats with a functional knockout of the ASIC3 (KO) with the reflex in their wild-type counterparts (WT). We found that the exercise pressor reflex in ASIC3 KO rats was significantly lower than the exercise pressor reflex in their WT counterparts (P < 0.05). ASIC3 KO rats demonstrated lower pressor responses to intra-arterial injection of diprotonated phosphate (86 mM; pH 6.0), lactic acid (12 mM; pH 2.85), and capsaicin (0.2 μg; pH 7.2) (P < 0.05). In contrast, both ligated WT and ASIC3 KO rats displayed similar pressor responses to tendon stretch (P > 0.05). We conclude that ASIC3 plays an important role in evoking the exaggerated exercise pressor reflex in rats with peripheral artery disease.

NEW & NOTEWORTHY We used a genetic approach to test the hypothesis that the magnitude of the exercise pressor reflex evoked in ligated ASIC3 KO rats was significantly lower than the magnitude of the exercise pressor reflex evoked in their ligated wild-type (WT) counterparts. The pressor response to contraction in ligated ASIC3 KO rats was significantly smaller than was the pressor response to contraction in ligated WT rats.

diprotonated phosphate; lactic acid; peripheral artery disease; sympathetic nervous system; thin fiber muscle afferents

INTRODUCTION

The exercise pressor reflex is evoked by the contraction of skeletal muscle and is manifested by increases in arterial pressure, heart rate, and cardiac contractility (1, 12, 31, 35). In health the exercise pressor reflex functions to increase arterial blood flow to exercising muscles to match their metabolic to their blood supply (2, 38). The sensory arm of this reflex is composed of group III and IV afferents (13, 31). Group III afferents are responsive to mechanical distortion of their receptive fields (26, 34) and are believed to evoke the mechanical component of the exercise pressor reflex (43), whereas group IV afferents are responsive to metabolic by-products of muscle contraction and are believed to evoke the metabolic component of the reflex (26). Nevertheless, group III afferents can also respond to by-products (40, 41) and, therefore, may participate in evoking the muscle metaboreflex. Although multiple metabolic by-products of muscle contraction are likely to evoke the metabolic component of the reflex, hydrogen ions dissociated from diprotonated phosphate and lactic acid appear to be particularly important. Hydrogen ions stimulate acid-sensing ion channels (ASICs) on the endings of the group III and IV afferents to evoke the metabolic component of the exercise pressor reflex (19, 29, 40, 41, 52).

Functional ASICs are comprised of three subunits that combine to form heterotrimers or homotrimers. In particular, ASIC1, ASIC2, and ASIC3 are expressed in dorsal root ganglion neurons that innervate skeletal muscle (8, 11, 50). ASICs composed of ASIC3 subunits open at pH ~7, have a pH50 of ~6.5 (5, 22) and pass maximum current at a pH ~6 (20, 45, 53). Moreover, their currents are potentiated by the presence of lactate ions (23, 24). ASIC3 are expressed in dorsal root ganglion neurons that innervate skeletal muscles (8, 11, 50).

Peripheral artery disease, which occurs in the arteries perfusing the legs, is caused by atherosclerosis and results in the accumulation of plaque that reduces arterial blood flow to exercising muscles. In patients with peripheral artery disease, the exercise pressor reflex is greater than it is in healthy control subjects (3, 4, 16, 37). In rats, peripheral artery disease can be simulated by ligating one femoral artery and waiting 3 days, after which the exercise pressor reflex evoked from the ligated hindlimb is greater than the exercise pressor reflex evoked from the contralateral freely perfused hindlimb (47). In this preparation arterial blood flow to the hindlimb whose femoral artery is ligated is adequate to meet the metabolic demand of the muscles while the rats are caged, but blood flow is reduced to 20–30% of the level occurring during maximal exercise (39, 46). Likewise, the pressor responses to intra-arterial injection of both lactic acid and capsaicin are greater in rats with ligated femoral arteries than they are in rats with freely perfused hindlimbs (28, 47, 51).

Previously, pharmacological antagonists were used to study the role played by ASICs in evoking the exaggerated exercise pressor reflex in rats with simulated peripheral artery disease. Specifically, the role played by ASIC3 in evoking the exaggerated exercise pressor reflex in ligated rats was determined
by injecting APETX2, a selective ASIC3 antagonist, into the arterial supply of the contracting muscles. Tsuchimochi et al. (48) showed that injection of this agent, which is a toxin extracted from the sea anemone, markedly attenuated the exercise pressor reflex in rats with ligated femoral arteries. Despite the important information provided by this study, pharmacological antagonists frequently have off-target effects. In the case of APETX2, it has been shown to block voltage-gated sodium 1.8 channels (NaV1.8) channels (7). This prompted us to use an alternative genetic approach to validate and strengthen our previous findings that blocking ASIC3 with APETX2 attenuated the exaggerated pressor responses to the static contraction in rats with ligated femoral arteries. We used rats with a functional knockout (KO) of ASIC3 to test the hypothesis that the exercise pressor reflex evoked in ligated ASIC3 KO rats was significantly lower than the exercise pressor reflex evoked in their ligated wild-type (WT) counterparts. We also tested the hypothesis that the pressor responses evoked by lactic acid and H$_2$PO$_4^-$ injections into the hindlimb muscles were significantly lower in ligated ASIC3 KO rats than the pressor responses to lactic acid and H$_2$PO$_4^-$ injections in their ligated WT counterparts. Last, we tested the hypothesis that the pressor responses evoked by tendon stretch and capsacain injection in ligated ASIC3 KO rats were no different than the pressor responses to tendon stretch and capsacain injection in their ligated WT counterparts.

**METHODS**

**Ethical approval.** The Penn State College of Medicine Institutional Animal Care and Use Committee approved all procedures. Adult male Wistar-Kyoto (WKY) rats were used in these experiments ($n = 72$; 250–800 g). Rats were housed in the animal facility (temperature controlled, 24°C) at the Penn State College of Medicine with regular diet and water (ad libitum).

**WT and KO lines.** WKY ASIC3 KO rats were developed by the Gene Editing Rat Resource Center of the Medical College of Wisconsin. The ASIC3 KO strain (WKY-Asic3em6Mcwi, RGDID: 12790599) was generated by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9). This technology deleted 61 base pairs in exon 1 (27, 54), resulting in the truncation of 22 amino acids to create an ASIC3 functional knockout. We bred ASIC3 KO rats by crossing homozygous KOs. The WKY WT rats were purchased from Charles River Laboratories.

**Surgical procedures.** The left iliac and femoral arteries were ligated 72 h before the experiment. To perform the double ligation procedure, we anesthetized the rats with isoflurane (2 to 3%) in oxygen. Using a sterile procedure, we ligated the iliac and femoral arteries with a 6-0 silk suture. Bupivacaine (1 to 2 mg/kg) was administered subcutaneously after surgery for postoperative pain; the rats were allowed to recover in their cages for 72 h before starting the experiment.

On the day of the experiment, rats were anesthetized with 4% isoflurane in O$_2$. After the trachea was cannulated, the isoflurane concentration was reduced to 2% and the lungs were mechanically ventilated with the anesthetic gas mixture. Both carotid arteries and jugular veins were exposed by a ventral midline incision and then cannulated (PE-50) to measure arterial blood pressure and to administer drugs and fluids. Arterial blood pressure was measured (P23 XL; Statham, Oxnard, CA), and heart rate (HR) was calculated from the arterial pressure pulse (Spike 2; Cambridge Electronics Design, Cambridge, UK).

The left triceps surae (the 2 heads of the gastrocnemius, soleus) muscles were isolated, and the calcaneal bone was severed. The severed end of the calcaneal bone and tendon were linked to a force transducer (model FT10; Grass Technologies, Warwick, RI) by a string. The tibial nerve was isolated.

For drug injection studies the left superficial epigastric artery was cannulated (PE-8); the tip of the cannula was advanced so that it was near the junction of the superficial epigastric artery and the femoral artery. In five rats, we performed a lumbar laminectomy, after which we placed a PE-8 catheter intrathecally at L6 and advanced the tip until it reached L1. We identified L6 by finding the indentation between it and S1. The distance between L6 and L1 was next measured, which then dictated the length of the cannula.

The rats were placed in a Kopf customized spinal frame and stereotaxic instrument. A rostral lumbar vertebra and the pelvis were clamped to hold the rat securely in place. Dexamethasone (0.2 mg) was then injected intravenously to control brain swelling. A precollular decerebration was performed, and the forebrain was aspirated as previously described (42). The cranial cavity was packed with gauze to control bleeding. Anesthesia was immediately discontinued after decerebration, and the lungs were mechanically ventilated with room air. A shielded stimulating electrode was placed underneath the tibial nerve, and the leg was covered in saline-soaked gauze to prevent drying. Rats were continuously mechanically ventilated with room air and allowed to stabilize and recover for at least 60 min before initiation of any experimental protocol. All experiments were performed in decerebrate, anesthetized rats. Arterial blood gases and pH were monitored using an automated blood gas analyzer (ABL 80, Radiometer, Brea, CA). $P_{CO_2}$ and arterial pH were maintained within normal physiological limits by adjusting ventilation, adjusting oxygen, or by an intravenous injection of sodium bicarbonate (8.5%). Body temperature was maintained between 36 and 38°C by using a heating pad.

**Experimental protocol.** We measured the pressor responses to static contraction, calcaneal tendon stretch, and chemical stimulation in both WT and ASIC3 KO rats. Regardless of the stimulus employed, we waited 15 min between them. In the static contraction experiments, triceps surae muscle tension was set at 100 g, and baseline was measured for the 30 s. We then contracted the muscle for the 30 s by electrically stimulating the tibial nerve (40 Hz; 0.01 ms). The current applied to the tibial nerve never exceeded two times the motor threshold, which was defined as the minimal current from a single pulse (0.01 ms) that was needed to evoke a twitch from the triceps surae muscles. At the end of every contraction experiment, rats were paralyzed with pancuronium bromide (0.5 mg/kg iv), and the tibial nerve was electrically stimulated with the same parameters that were used to induce muscle contraction. This procedure was performed to confirm that the pressor response to contraction was not due to the electrical activation of afferent nerves. We excluded from the analysis data from three paralyzed rats that displayed a pressor response to electrical stimulation of the tibial nerve. In calcaneal tendon stretch experiments, triceps surae muscle tension was set at 100 g, and baseline was measured for the 30 s. We then stretched the calcaneal tendon for the 30 s by turning the rack and pinion.

We also measured the pressor responses injection to diprotonated phosphate (86 mM; pH 6.0), lactic acid (12 mM; pH 2.85), and capsacain (0.2 µg; pH 7.2) into the superficial epigastric artery. The volume of each injection was 100 µL. The order of stimuli was randomized across rats; as stated above, we waited 15 min between injections. Our rationale for injecting both diprotonated phosphate and lactic acid was that the pH of the 12 mM lactic acid was capable of opening the transient receptor potential vanilloid-1 (TRPV1) channel, whereas the pH of the H$_2$PO$_4^-$ was not (12). At the end of every experiment in which we injected chemicals, we injected Evans blue dye into the superficial epigastric artery (100 µL) to confirm that the injectates reached the triceps surae muscles. We excluded findings from any experiment in which blue dye did not reach this muscle group.
In 11 rats, we determined the effect of injecting APETx2 (100 μg/kg) into the superficial epigastric artery on the pressor responses to static contraction (CTX) and stretch in ligated wild-type (WT) and ligated acid-sensing ion channel-3 (ASIC3) knockout (KO) rats. APETx2 is a toxin extracted from the sea anemone and that antagonizes ASIC3 (15). In these experiments, we ligated the femoral artery but did not ligate the iliac artery, which we did in our other experiments. The reason we did not ligate the iliac artery in these rats was that we found that this ligation as well as ligation of the superficial epigastric artery prevented the hindlimb muscles from contracting when we stimulated the tibial nerve. We speculate that the combined ligation of the iliac artery, the femoral artery, and the superficial epigastric artery reduced collateral blood flow to the hindlimb muscles to the point that these muscles could not contract.

In another set of experiments performed in five ligated WT rats, we compared the pressor responses to injection of capsaicin (0.2 g) into the superficial epigastric artery before and after intrathecal injection of APETx2 (100 μg/kg). The injection volume was 100 μL. In these rats both the left iliac and femoral arteries were ligated. At the end of the

Table 1. Comparisons between ligated WT and ligated ASIC3 KO rats of peak cardioaccelerator responses to 5 stimuli

|                   | Contraction | Tendon Stretch | H$_2$PO$_4^-$ (86 mM) | Lactic Acid (12 mM) | Capsaicin (0.2 μg) |
|-------------------|-------------|----------------|-----------------------|--------------------|-------------------|
| Ligated WT        |             |                |                       |                    |                   |
| ΔHR, beats/min    | 65 ± 34     | 6 ± 2          | 11 ± 6                | 11 ± 3             | 13 ± 2            |
| Baseline HR, beats/min | 393 ± 31 | 351 ± 14       | 445 ± 45              | 463 ± 40           | 401 ± 22          |
| n                 | 13          | 11             | 13                    | 13                 | 10                |
| Ligated ASIC3 KO |             |                |                       |                    |                   |
| ΔHR, beats/min    | 42 ± 35     | 9 ± 3          | 4 ± 2                 | 6 ± 2              | 8 ± 4             |
| Baseline HR, beats/min | 507 ± 68 | 385 ± 11       | 384 ± 31              | 408 ± 36           | 399 ± 50          |
| n                 | 13          | 13             | 13                    | 13                 | 10                |

Values are means ± SE; n, number of rats. There were no significant differences in cardioaccelerator responses between the ligated wild-type (WT) and ligated acid-sensing ion channel-3 (ASIC3) knockout (KO) rats to any of the 5 stimuli. Two ASIC3 KO rats, one each from the contraction and capsaicin injection, were excluded from the analysis due to hypotension, a mean arterial pressure below 70 mmHg. HR, heart rate.
RESULTS

Effect of ASIC3 KO on responses to static contraction. We compared the pressor and cardioaccelerator responses to static contraction between ligated ASIC KO rats (n = 13) and ligated WT rats (n = 13). The peak pressor responses and BPIs to static contraction in ligated WT rats were significantly greater (P = 0.0001 and P = 0.0056, respectively) than the peak pressor responses and BPIs in ligated ASIC3 KO rats (Fig. 1A and C). Exaggerated cardioaccelerator responses were observed in both groups, but there was no significant difference between the two groups of ligated rats (Table 1). The exaggerated pressor responses in ligated WT rats were not a result of differences in contractile force because TTI values were similar in ligated WT and ligated ASIC3 KO rats (Fig. 1E). One ligated ASIC3 KO rat was excluded from the analysis because its baseline mean arterial pressure was below 70 mmHg.

Table 2. Comparisons between ligated WT and ligated ASIC3 KO rats before and after APETx2

|                  | APETx2 Before | APETx2 After |
|------------------|---------------|--------------|
| Ligated WT       |               |              |
| ΔHR, beats/min   | 3 ± 4         | 4 ± 2        |
| n                | 5             | 5            |
| Ligated ASIC KO  |               |              |
| ΔHR, beats/min   | 2 ± 1         | 4 ± 2        |
| n                | 6             | 6            |
| Baseline HR, beats/min | 486 ± 80 | 368 ± 6 |

Values are means ± SE; n, number of rats. There were no significant differences in peak cardioaccelerator responses between the ligated wild-type (WT) and ligated acid-sensing ion channel-3 (ASIC3) knockout (KO) rats to static contraction before and after intra-arterial injection of APETx2 (100 μg/kg). HR, heart rate.
Effect of ASIC3 KO on responses to tendon stretch. We compared the pressor and cardioaccelerator responses to tendon stretch between ligated ASIC3 KO (n = 9) and ligated WT rats (n = 11; Fig. 1B). There was no significant difference between the pressor and cardioaccelerator responses evoked by the tendon stretch between the two groups of rats (Fig. 1 and Table 1).

Effect of APETx2 on the pressor response to contraction. We found that injection of APETx2 (100 μg/kg) into the superficial epigastric artery attenuated the pressor responses to contraction in ligated WT rats (n = 5) but had no effect on the pressor response to contraction in ASIC3 KO rats (n = 6; Fig. 2, A and B). Nevertheless, in one of the six ligated ASIC KO rats tested, APETx2 appeared to reduce the exercise pressor reflex. In the remaining five, APETx2 had no effect on the reflex. There were no significant differences in cardioaccelerator responses between the ligated WT and ligated ASIC3 KO rats to static contraction before and after intra-arterial injection of APETx2 (Table 2). The TTI’s before and after injection of APETx2 were not different for either the ligated WT or ligated ASIC3 KO rats (Fig. 2C).

Effect of ASIC3 KO on responses to chemical stimuli. We compared the pressor and cardioaccelerator responses to the injection of 86 mM diprotonated phosphate, 12 mM lactic acid, and 0.2 μg capsaicin into the superficial epigastric artery between ligated ASIC3 KO rats and ligated WT rats. We found that the peak pressor responses evoked by diprotonated phosphate, lactic acid, and capsaicin were significantly larger (P < 0.01) by 53, 54, and 22%, respectively, in the ligated WT than in the ligated ASIC3 KO rats (Fig. 3, A–C and Table 1). There was no significant difference in the cardioaccelerator responses to the injection of lactic acid (12 mM), H₂PO₄⁻ (86 mM), and capsaicin (0.2 μg) (Table 1). We excluded from the data analysis the responses of one ligated ASIC3 KO rat to capsaicin injection because baseline mean arterial pressure was below 70 mmHg.

Effect of intrathecal injection of APETx2 on the pressor response to capsaicin. We found in ligated WT rats (n = 5) that intrathecal injection of APETx2 (100 μg/kg; 100 μL) had no effect on the pressor and cardioaccelerator responses to capsaicin injection into the superficial epigastric artery (Fig. 4). Injection of Evans blue dye (100 μL) spread from the lumbar spinal cord to the caudal medulla.

DISCUSSION

We found that the exercise pressor reflex evoked by static contraction of the hindlimb muscles was significantly greater in ligated WT rats than it was in ligated ASIC3 KO rats. Likewise, the pressor responses to intra-arterial injections of diprotonated phosphate, lactic acid, and capsaicin were significantly greater in ligated WT rats than they were in ligated ASIC3 KO rats. These findings in a genetically modified rat show that ASIC3 plays a key role in evoking the exaggerated exercise pressor reflex in rats with simulated peripheral artery disease and extend our previous finding that pharmacological antagonists blocking ASIC3 receptors in the hindlimb of ligated genetically intact rats (21, 48) attenuate the exercise pressor reflex.

The validity of our finding that knocking out ASIC3 attenuated the exercise pressor reflex in ligated rats depended on our showing that the CRISPR-induced deletion of 22 amino acids from the ASIC3 protein prevented the channel from functioning. Previously, this task was accomplished by showing that isolated dorsal root ganglion cells innervating the gastrocnemius muscles of freely perfused ASIC3 KO rats did not display desensitization time constants indicative of ASIC3 currents when these cells were exposed to a pH of either 5.0 or 6.0 (27, 54). To supplement these in vitro findings, we now show in vivo that a selective ASIC3 antagonist, APETx2, injected into the arterial supply of the hindlimb had no effect on the exercise pressor reflex in ligated ASIC3 KO rats but markedly attenuated the reflex in their ligated WT counterparts.
The rats used in our experiments were functional global knockouts, meaning that ASIC3 throughout the organism were inactivated. Although we cannot exclude the possibility that knockout of ASIC3 somewhere other than in thin fiber muscle afferents was responsible for the attenuation of the exercise pressor reflex in the ligated rats found in our experiments, we think that this possibility is remote. Specifically, the general consensus appears to be that ASIC3 are found primarily in the peripheral nervous system; in particular, this channel is heavily concentrated in dorsal root ganglion cells (11, 49, 50). In addition, the concentration or even the presence of ASIC3 in the central nervous system is unclear and controversial. For example, there is some evidence that ASIC3 are found in the hypothalamus and some forebrain structures (33), but in our experiments they could play no role because the rats were decerebrated at the midbrain level. Finally, there appears to be no evidence that ASIC3 are found in the vasculature.

In our experiments, capsaicin, injected into the arterial supply of the hindlimb, evoked a larger pressor response in ligated WT rats than did capsaicin injection in ligated ASIC3 KO rats. Although the difference was not large; nevertheless, it was statistically significant. This result was surprising because capsaicin is a TRPV1 agonist and the absence or presence of ASIC3 should not be a factor in the magnitude of the pressor response evoked by this agent. Because our preparation was a global knockout, we were prompted to test the hypothesis that ASIC3 in the spinal cord, which were functionally knocked out in the genetically modified rats used in our experiments, played a role in the central neural circuitry evoking pressor responses to capsaicin injection. We found no support for this hypothesis.

Fig. 4. Peak pressor (A) and cardioaccelerator (B) responses evoked by capsaicin (0.2 μg), injected into the superficial epigastric artery, before and after intrathecal injection of APETx2 (100 μg/kg) in ligated wild-type rats. Numbers in parentheses represent baseline values for mean arterial pressure (MAP) and heart rate (HR). bpm, beats/min.

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Fig. 5. Data are a combination of present results and those previously published from Kim et al. (27). Peak pressor responses (A), blood pressure indexes (BPIs; B), and tension-time indexes (TTIs; C) evoked by static contraction (CTX) in freely perfused and ligated wild-type (WT) and freely perfused and ligated acid-sensing ion channel-3 (ASIC3) knockout (KO) rats. ASIC3 KO rats decreased the pressor responses to static contraction in ligated rats but not in freely perfused rats. Note that the pressor response to static contraction in freely perfused ASIC3 KO rats was not different from that in freely perfused WT rats. In contrast, the pressor response to contraction in ligated ASIC3 KO rats was significantly smaller than that in ligated WT rats. Bars represent means, vertical brackets represent SEs, and numbers in parentheses in A represent baseline blood pressure values, expressed as means ± SE. Horizontal brackets with asterisks connect means that are significantly different: **P < 0.01; ****P < 0.0001. Open and filled circles represent individual data points from freely perfused and ligated WT rats, respectively. Open and filled triangles represent individual data points freely perfused and ligated ASIC3 KO rats, respectively.
because intrathecal injection of APETx2, an ASIC3 antagonist, did not decrease the pressor response to capsaicin in ligated WT rats. We can only speculate as to why ASIC3 KO had a small but persistent effect on the pressor responses to capsaicin in our experiments. Specifically, TRPV1 and ASIC3 are present on 31% of small diameter dorsal root ganglion cells innervating hindlimb muscle in rats (36). Perhaps a coupling effect between the two receptors was removed by the functional knockout of ASIC3 in the rats used in our experiments. Such an effect has been shown between ASIC3 and the purinergic receptor P2X5 (6), so it is not inconceivable that ASIC3 and TRPV1 are coupled also.

Western blots of ASIC protein levels in the L4 and L5 dorsal root ganglia may shed light on why ASIC3 KO had no effect on the exercise pressor reflex in rats with freely perfused hindlimb muscles (27) but markedly attenuated the reflex in rats with ligated femoral arteries (present results). Specifically, ASIC3 protein levels in the L4 and L5 dorsal root ganglia taken from rats with ligated femoral arteries were significantly greater than ASIC3 protein levels in L4 and L5 dorsal root ganglia taken from rats with patent femoral arteries (18, 30). In contrast, ASIC1 protein levels in the L4 and L5 ganglia taken from rats with ligated femoral arteries were significantly less than ASIC1 protein levels in these ganglia taken from rats with patent femoral arteries (18). We interpret these results to indicate that during contraction of freely perfused muscles, the number of ASIC3 on group III and IV afferents is insufficient to make much of a contribution to the elicitation of the exercise pressor reflex (44, 48). Instead, when the contracting muscles are freely perfused, ASIC1a makes an important contribution to the elicitation of the reflex (17). Recently, the potential role of ASICs in evoking the exercise pressor reflex in healthy humans has been demonstrated by Campos et al. (9), who reported that amiloride, a nonselective antagonist to ASICs, epithelial sodium channels, and voltage-gated sodium channels (10), attenuated the exercise pressor reflex. To the extent that this attenuation can be attributed to ASICs, it was most likely caused by blockade of ASIC1a rather than by blockade of ASIC3 (17).

In our experiments, the pressor response to tendon stretch, which evoked the muscle mechanoreflex (43), was the same in ligated ASIC KO rats as it was in ligated WT rats. This finding contrasts with our previous finding that APETx2, a pharmacological antagonist to ASIC3, attenuated the pressor response to stretch in ligated rats (48). The explanation for these contrasting findings may involve the fact that APETx2 can block NaV1.8 at a half maximal inhibitory concentration that is within an order of magnitude of that needed to block ASIC3 (7). This explanation appears consistent with the finding that tendon stretch does not increase muscle lactic acid concentration over its baseline level (43). In addition, the inhibitory effect of APETx2 on NaV1.8 as an explanation for our previous finding that this agent attenuated the pressor responses to tendon stretch (48) is supported by our finding that ASIC blockade with amiloride had no effect on the renal sympathetic nerve response evoked by the mechanoreceptor component of the exercise pressor reflex (32).

ASIC3, in addition to playing a role in evoking the exercise pressor reflex when the arterial blood flow to contracting muscle is compromised, also plays a role in evoking pain arising from damaged muscle. For example, ASIC3 has been shown to be responsible for the hyperalgesia induced by repeated injections of acidic saline (25, 42). Likewise, ASIC3 has been shown to play an important role in evoking post incisional pain as well as thermal hyperalgesia (14). Whether the thin fiber muscle afferents responsible for evoking painful sensations are the same as those responsible for evoking the exaggerated exercise pressor reflex while the muscles are ischemic is not known.

Any conclusions from our findings need to be considered with two limitations in mind. First, we simulated peripheral artery disease in rats by ligating the arterial supply to the hindlimb, after which we waited 72 h to perform our experiments. Peripheral artery disease in humans, however, is caused over years by atherosclerosis in the arteries perfusing the legs. Consequently, the difference between the two may result in differences in channel or receptor expression on group III and IV afferents. Second, ASIC3 may not be the only channel or receptor responsible for the exaggerated exercise pressor reflex in rats with simulated peripheral artery disease. Other channels or receptors contributing to this exaggeration remain to be tested.

The information gained from our experiments in ASIC3 KO rats whose femoral and iliac arteries were ligated for 72 h may shed light on the mechanisms evoking the exaggerated exercise pressor reflex seen in patients with peripheral artery disease (3, 4, 37). Evidence for this conclusion is provided by our finding that functional knockout of ASIC3 in ligated rats prevented the exaggeration of the exercise pressor reflex evoked in ligated WT rats. Moreover, functional knockout of this channel in ligated rats restored the exercise pressor reflex to the same level as that seen in freely perfused WT or ASIC3 KO rats (Fig. 5) (27). Based on our findings, we conclude that ASIC3, stimulated by hydrogen ions, is an important candidate for the cause of the exaggerated pressor response and claudication seen in patients in peripheral artery disease.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
J.S.K. and M.P.K. conceived and designed research; J.S.K. and G.P.D. performed experiments; J.S.K. and M.P.K. analyzed data; J.S.K. and M.P.K. prepared figures; J.S.K. and M.P.K. drafted manuscript; J.S.K., G.P.D., and M.P.K. edited and revised manuscript; J.S.K., G.P.D., and M.P.K. approved final version of manuscript.

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