Original Paper

Proteinase-Activated Receptor-2 Sensitivity of Amplified TRPA1 Activity in Skeletal Muscle Afferent Nerves and Exercise Pressor Reflex in Rats with Femoral Artery Occlusion

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Muscle afferent nerve • TRPA1 • PAR2 • Peripheral arterial disease • Hindlimb ischemia

Abstract

Background/Aims: Limb ischemia occurs in peripheral artery disease (PAD). Sympathetic nerve activity (SNA) that regulates blood flow directed to the ischemic limb is exaggerated during exercise in this disease, and transient receptor potential channel A1 (TRPA1) in thin-fiber muscle afferents contributes to the amplified sympathetic response. The purpose of the present study was to determine the role of proteinase-activated receptor-2 (PAR2) in regulating abnormal TRPA1 function and the TRPA1-mediated sympathetic component of the exercise pressor reflex. **Methods:** A rat model of femoral artery ligation was employed to study PAD. Dorsal root ganglion (DRG) tissues were obtained to examine the protein levels of PAR2 using western blot analysis. Current responses induced by activation of TRPA1 in skeletal muscle DRG neurons were characterized using whole-cell patch clamp methods. The blood pressure response to static exercise (i.e., muscle contraction) and stimulation of TRPA1 was also examined after a blockade of PAR2. **Results:** The expression of PAR2 was amplified in DRG neurons of the occluded limb, and PAR2 activation with SL-NH2 (a PAR2 agonist) increased the amplitude of TRPA1 currents to a greater degree in DRG neurons of the occluded limb. Moreover, FSLLRY-NH2 (a PAR antagonist) injected into the arterial blood supply of the hindlimb muscles significantly attenuated the pressor response to muscle contraction and TRPA1 stimulation in rats with occluded limbs. **Conclusions:** The PAR2 signal in muscle sensory nerves contributes to the amplified exercise pressor reflex via TRPA1 mechanisms in rats with femoral artery ligation. These findings provide a pathophysiological basis for autonomic responses during exercise activity in PAD, which may potentially aid in the development of therapeutic approaches for improvement of blood flow in this disease.

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Introduction

Exercise increases sympathetic nerve activity (SNA) [1, 2], an effect that in turn increases arterial blood pressure (BP), heart rate (HR), myocardial contractility and peripheral vascular resistance. Two mechanisms, namely, central command and the exercise pressor reflex, evoke this exercise-induced increase in SNA. Central command is responsible for a parallel and simultaneous increase in sympathetic and alpha motoneuron discharge [3, 4]. The exercise pressor reflex postulates that thin-fiber afferent nerves (group III & IV) innervating skeletal muscles are activated by contraction-induced mechanical and metabolic stimuli to elicit a reflexive increase in SNA [5, 6].

Peripheral arterial disease (PAD) is atherosclerotic disease with a decrease in blood flow to the arteries of the lower extremities. The most common symptom of this disease is intermittent claudication, which is worsened by intense exercise activity due to muscle ischemia, but subsides at rest when the metabolic demand of the active muscles is low [7]. It was observed that both systolic and diastolic BP rise significantly during walking in patients with PAD compared with those in normal subjects [8]. Furthermore, the exercise pressor reflex plays a crucial role in evoking the exaggerated BP response to walking in PAD patients [9]. Consistently, using a rat model of femoral artery ligation to study PAD in humans [10], prior studies have demonstrated that the SNA and BP responses to static muscle contraction and stimulation of several muscle metabolic receptors are amplified in occluded rats compared with those in control rats [11, 12].

Transient receptor potential channel A1 (TRPA1) is a member of branch A of the transient receptor potential family of nonselective cation channels [13, 14]. This channel is expressed in sensory neurons and is involved in acute and inflammatory pain [13, 14]. TRPA1 acts as a sensory receptor in response to pungent and reactive chemicals such as allyl isothiocyanate (AITC), allicin, cinnamaldehyde, formaldehyde, N-methylmaleimide, and α , β -unsaturated aldehydes [15, 16]. TRPA1 also serves as a sensor of cold temperature and mechanical deformation [17, 18]. In addition to pungent chemicals found in nature, endogenously generated molecules, such as bradykinin, reactive oxygen species, and 4-hydroxynonenal that are produced during inflammation and oxidative stress, can activate TRPA1 [13, 19].

A prior study has demonstrated that intra-arterial injection of AITC, a TRPA1 agonist, into the hindlimb muscle circulation of healthy rats led to increases in SNA and BP via a reflex mechanism [20]. This study also suggested that TRPA1 plays a role in regulating the metabolic component of the exercise pressor reflex, i.e. acid phosphate, bradykinin and arachidonic acid, which are accumulated in exercising muscles, are likely involved in the TRPA1 response to endogenous stimuli. Our published studies have further demonstrated that TRPA1 in thin-fiber muscle afferents plays an important role in the amplified SNA and BP reflex responses observed in rats with femoral artery occlusion [21, 22].

Proteinase-activated receptor-2 (PAR2) has been shown to play a role in regulating TRPA1 currents in sensory neurons as an upstream signal [23]. Co-localization of TRPA1 with PAR2 has been found in rat dorsal root ganglion (DRG) neurons and the activation of PAR2 has been reported to increase TRPA1-induced currents in DRG neurons [23]. One potential mechanism by which TRPA1 plays a role in regulating the SNA and BP responses during the exercise pressor reflex is via PAR2 signaling in skeletal muscle afferent nerves. Thus, in the present study we hypothesized that with hindlimb ischemia expression levels of PAR2 would be amplified resulting in greater amplitude TRPA1 currents in DRG neurons. Blocking PAR2 attenuates exaggeration of SNA and BP responses to static muscle contraction as well as TRPA1 activation following femoral artery occlusion. We also postulated that downstream signal pathways, namely, PLC and PIP2 would be involved in the effects of PAR2 on regulation of TRPA1 currents.

Materials and Methods

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Penn State College of Medicine and complied with NIH guidelines.

Ligation of the Femoral Artery

Male Sprague-Dawley rats (4-6 weeks old) were anesthetized with an isoflurane-oxygen mixture (2-5% isoflurane in 100% oxygen). Then, the femoral artery on one limb was surgically exposed, dissected, and ligated ~3 mm distal to the inguinal ligament as previously described [21, 24]. For the control, the same procedures were performed on the other limb except that a suture was placed below the femoral artery, but it was not tied. For the experiments using the western blotting analysis and patch clamp methods, the limbs in which the femoral arteries were ligated served as the "occluded limbs"; and the other limbs served as the "control limbs". For the BP recording experiment, the rats were divided between those that had the femoral artery ligation ("occluded rats") and those that had sham surgeries on the limb ("control rats"). Seventy-two hours was allowed for recovery before the experiments began.

Examination of PAR2 Expression

Western blotting analysis was employed to examine the protein expression of PAR2. The L4-L6 DRGs from both sides were removed after the rats were anesthetized by an overdose of isoflurane followed by decapitation. Briefly, the concentration of protein in the homogenate was determined using a BCA reagent after the tissues were lysed and centrifuged. PAR2 proteins were loaded onto gel. After electrophoresis, the proteins were electrotransferred onto polyvinylidene difluoride membranes. The membranes were then incubated with the primary antibody rabbit anti-PAR2 (1:500; Abcam). Next, the membranes were washed and incubated with an alkaline phosphatase-conjugated anti-rabbit secondary antibody (1:200; Santa Cruz). The membranes were also incubated with mouse anti- β -actin to show equal loading of the protein. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL kit). The bands recognized by the primary antibody were visualized by exposure of the membrane onto an X-ray film. Then, the film was scanned and the optical density of the bands was analyzed using the Scion Image software (NIH, USA).

Examination of DRG Currents

After the rats were anesthetized by inhalation of an isoflurane-oxygen mixture, the fluorescent retrograde tracer DiI (60 mg/ml; Molecular Probes) was injected into the white portion of the gastrocnemius muscle of both legs to label the muscle DRG neurons, as described in our prior work [25]. The injection volume was 1 μ l, and injection was repeated three times at different locations. The animals were returned to their cages for 4-5 days to permit the retrograde tracer to be transported to the DRG neurons.

The L4-L6 DRGs from both sides of individual animals were removed and transferred immediately into Dulbecco's Modified Eagle's Medium (DMEM) to obtain dissociated DRG neurons [25]. Neurons were then plated onto a 35-mm culture dish and visualized using a combination of epifluorescence illumination and differential interference contrast (DIC, 20-40X) optics on an inverted microscope (Nikon). DiI-positive neurons with < 35 μ m of diameter were patched and recorded at a holding potential of -70 mV. Signals were acquired using pClamp 9.0 and data were analyzed using pClampfit (Axon Inc.).

Drugs stored in the stock solutions were diluted in extracellular solution immediately before being used and were held in a series of independent syringes connected to corresponding fused silica columns. The ends of the parallel columns were connected to a common silica column. The distance from the column mouth to the cell being examined was 100 μ m. Each drug was delivered to the recording chamber by gravity and rapid solution exchange was achieved by controlling the corresponding valve switch.

The effects of PAR2 activation on TRPA1-induced currents were also examined. Fifty μ M of AITC was first applied to DRG neurons of control and occluded limbs for 30 s to obtain a current response. After 3-min washout intervals, PAR2 was activated using SL-NH2 (a PAR2 agonist, 100 μ M) and then TRPA1 currents were evoked by AITC. In this intervention, the respective inhibitors of phospholipase C [PLC, ET-18-OCH3 (ET), 20 μ M] and PKC [GF109203X (GF), 20 μ M] were applied prior to SL-NH2 to examine if these signal pathways were involved in the PAR2 effects on TRPA1 currents. Phosphatidylinositol-4, 5-bisphosphate (PIP2, 10 μ M) was included in the internal patch solution to examine the involvement of PIP2.

A total of 126 DRG neurons from control limbs and 86 DRG neurons from occluded limbs were included in this study. All DRG neurons used in this report were DiI-positive. At the end of each experiment, the gastrocnemius muscle was dissected to confirm that DiI was located in the white portion of the gastrocnemius muscle.

Examination of the Exercise Pressor Reflex

The rats were anesthetized by inhalation of an isoflurane-oxygen mixture and an endotracheal tube was inserted and attached to a ventilator. Polyethylene (PE-50) catheters were inserted into an external jugular vein and the right carotid artery for saline injection and measurement of BP. PE-10 catheters were inserted into the femoral arteries for injection of drugs into the arterial blood supply of the hindlimb muscles. The skin covering the hindlimb muscles was surgically separated from the muscle below to eliminate inputs from cutaneous afferents in the hindlimb. During the experiment, end tidal CO₂, BP and body temperature were monitored and maintained within normal ranges [21, 24].

BP was measured by connecting the carotid arterial catheter to a pressure transducer. Mean arterial pressure (MAP) was obtained by integrating the arterial signal with a time constant of 4 s. Heart rate (HR) was determined from the arterial pressure pulse. Renal SNA (RSNA) was recorded by placing a bipolar electrode under the renal nerve as previously described [21]. The RSNA signal was amplified and made audible via an amplifier (P511, Grass Instruments) with a bandpass filter with a low-cut frequency of 300 Hz and a high-cut frequency of 3 kHz.

A laminectomy was performed to expose the lower lumbar and upper sacral portions of the spinal cord after the rats were placed in a spinal unit (Kopf Instruments) [21]. The spinal roots were exposed and the right L4 and L5 ventral roots were visually identified with the assistance of an anatomical microscope. The peripheral ends of the transected L4 and L5 ventral roots were then placed on bipolar platinum stimulating electrodes. A pool was formed by using the skin and muscle on the back and the exposed spinal region was filled with warmed (37°C) mineral oil.

Decerebration was performed to examine the exercise pressor reflex without the confounding effects of anesthesia. Once the decerebration was complete, anesthesia was removed from the inhaled mixture. A recovery period of 60 min was allowed after decerebration for elimination of the effects of anesthesia from the preparation.

Static muscle contractions in the right hindlimb were performed by electrical stimulation of the L4 and L5 ventral roots (30 seconds, 3-times motor threshold with a period of 0.1 ms at 40 Hz) in the control rats and occluded rats (n=8 in each group). Each static muscle contraction was performed 15 min after arterial injection of saline (control), and 2, 10 and 20 µg/kg of FSLLRY-NH2 (PAR2 antagonist). Twenty minutes were allotted after contraction and before the next injection. Note that RSNA was not examined in this group of experiments due to electrical interference during the ventral root stimulation.

In an additional group, we further determined the effects of blocking PAR2 on the sympathetic and BP responses to activation of TRPA1. Fifteen min after the respective injections of saline, 2, 10 and 20 µg/kg of FSLLRY-NH2, 20 µg/kg of AITC was injected into the arterial blood supply of the hindlimb muscles of the control rats (n=10) and occluded rats (n=12). At least 20 min was allowed between injections. The injected volume was 0.25 ml and the duration of injections was 1 min. In our experiments of muscle contraction and AITC injection, saline and three dosages of FSLLRY-NH2 were applied in a random fashion to minimize the effect of time on the reflex responses.

Statistical Analysis

Experimental data were analyzed using one-way repeated measures analysis of variance (ANOVA). As appropriate, Tukey's post hoc tests were used. All values are presented as the mean ± SEM. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS for Windows version 15.0.

Results

Protein Expression of PAR2

The original bands and averaged data (Fig. 1A) demonstrate that the protein expression levels of PAR2 were augmented in DRG tissues of occluded limbs compared with those in DRG tissues of control limbs (n=5 in each group).

PAR2 and Signal Pathways in Amplifying TRPA1 Response

We further examined whether prior activation of PAR2 could augment the current responses elicited by AITC in muscle DRG neurons of both control and occluded limbs.

Fig. 1. A, Original band and averaged data: protein expression levels of PAR2 were amplified in DRG tissues of occluded limbs compared with those of control limbs. * $P < 0.05$ vs. control limbs. $N = 5$ in each group. B, Original traces: the effects of prior application of $100 \mu\text{M}$ of SL-NH2 on AITC-induced currents in DRG neurons of a control limb and an occluded limb. C, Averaged data: SL-NH2 amplified the amplitude of AITC-activated currents in muscle DRG neurons. * $P < 0.05$ compared with AITC alone. D, The percentage of augmented amplitude of AITC-induced currents by SL-NH2 appeared to be greater in DRG neurons of occluded limbs than that in DRG neurons of control limbs. * $P < 0.05$ vs. control. $N = 10-12$ in each group.

The original traces and (Fig. 1B) averaged data (Fig. 1C) show that activation of PAR2 with SL-NH2 increased the TRPA1 currents. The effect was greater in the DRG neurons of the occluded limb than that in the DRG neurons of the control limb (Fig. 1D). i.e., activation of PAR2 with SL-NH2 (a PAR2 agonist, $100 \mu\text{M}$) increased the TRPA1 currents by $78 \pm 10\%$ in the DRG neurons of the control limb and by $125 \pm 18\%$ in the DRG neurons of the occluded limb ($P < 0.05$ vs. control).

Moreover, we examined the downstream signal pathways that are potentially involved in the effects of PAR2 in regulating TRPA1 currents using DRG neurons obtained from non-occluded limbs. The enhancing effects of PAR2 activation on TRPA1 currents were significantly attenuated by prior application of $20 \mu\text{M}$ of ET, a PLC inhibitor, but not by $20 \mu\text{M}$ of GF, a PKC inhibitor (Fig. 2A). The prior application of $10 \mu\text{M}$ of PIP2 in the internal patch solution also inhibited enhancement of PAR2 on TRPA1 currents (Fig. 2B).

RSNA and BP Responses after Blocking PAR2

Baseline MAPs and HRs were 95 ± 4 mm Hg and 398 ± 15 bpm in the control rats ($n = 8$); and 98 ± 5 mm Hg and 395 ± 12 bpm in the occluded rats, respectively ($n = 8$) ($P > 0.05$, control vs. occlusion for MAP and HR). Fig. 3 shows that compared with saline injection, 10 and $20 \mu\text{g/kg}$ of FSLLRY-NH2 attenuated the BP response to static muscle contraction in the occluded rats ($n = 8$) ($P < 0.05$ vs. saline control). No differences in developed muscle tension were observed between the group with saline and the groups with three different doses of FSLLRY-NH2. Note that FSLLRY-NH2 did not significantly alter the MAP response in control rats ($n = 8$). In both control and occluded rats FSLLRY-NH2 failed to alter the HR response evoked by muscle contraction.

In addition, the RSNA and BP responses to stimulation of TRPA1 were examined. Baseline values for MAP and HR before arterial injections of AITC were 103 ± 6 mmHg and 385 ± 15 bpm in control rats ($n = 10$); and 99 ± 6 mmHg and 392 ± 18 bpm in occluded rats ($n = 12$),

Fig. 2. A, Blocking PLC pathways attenuated amplified TRPA1 currents in DRG neurons induced by activation of PAR2. However, blocking PKC pathways failed to antagonize amplified currents induced by AITC in DRG neurons. Top panel: original traces; and bottom panel: average data. ET-18-OCH₃ (ET, 20 μ M): PLC inhibitor; and GF109203X (GF, 20 μ M): PKC inhibitor. * $P < 0.05$ vs. AITC and SL-NH₂+ET+AITC. N=10-12 in each group. B, PIP₂ (10 μ M) added to the internal patch solution attenuated amplified TRPA1 currents in DRG neurons induced by activation of PAR2. PIP₂: phosphatidylinositol-4,5-bisphosphate. * $P < 0.05$ vs. AITC alone. There were no significant differences in AITC-induced currents for AITC vs. SL-NH₂+AITC with PIP₂. N=10-12 in each group.

Fig. 3. Pressor response to muscle contraction in control rats (n=8) and occluded rats (n=8) after blocking PAR2 using FSLRY-NH₂. There were no significant differences in MAP response evoked by muscle contraction in control rats after FSLRY-NH₂. However, FSLRY-NH₂ attenuated MAP response in occluded rats. * $P < 0.05$ vs. saline control.

There were no significant differences in developed muscle tension among groups.

respectively. There were no significant differences in basal MAP or HR before the injections. Fig. 4 illustrates the effects of increasing concentrations (2, 10, and 20 μ g/kg) of FSLRY-NH₂ injected into the hindlimb muscles on RSNA and MAP responses induced by AITC in control and occluded rats. Arterial injection of 20 μ g/kg of AITC evoked increases in RSNA and MAP in both groups. These responses were amplified in occluded rats compared with those in control rats. FSLRY-NH₂ (10 and 20 μ g/kg) attenuated RSNA and BP responses in

Fig. 4. Sympathetic and pressor responses to activation of TRPA1 by arterial injection of AITC (20 $\mu\text{g/kg}$) with prior blockade of PAR2 using FSLLRY-NH2 in control rats and occluded rats. Note that a higher dose of FSLLRY-NH2 was observed to attenuate sympathetic and pressor responses evoked by AITC in control rats. * $P < 0.05$ vs. saline control. $n = 10$ in control rats and $n = 12$ in occluded rats.

occluded rats ($P < 0.05$ vs. saline control), whereas only 20 $\mu\text{g/kg}$ of FSLLRY-NH2 attenuated RSNA and BP responses in control rats. Note that FSLLRY-NH2 did not alter the HR responses in both groups.

Discussion

A prior study showed that in healthy rats, intra-arterial injection of AITC into the hindlimb muscle circulation led to an increase in SNA via a reflex pathway [20]. This prior study also demonstrated that TRPA1 plays a role in regulating SNA via stimulation of the metabolic component of the exercise pressor reflex. When a similar dosage of AITC was used to stimulate TRPA1 on muscle afferent nerves, and the results of our recent work showed that arterial injection of AITC into hindlimb muscles evoked greater SNA and BP responses in occluded rats than in control rats [21]. Moreover, the higher levels of TRPA1 were induced by femoral artery occlusion in DRG neurons supplying metabolically sensitive C-fiber afferent nerves (group IV), and the AITC-responsive DRG neurons were small and medium size [21]. Consistent with previous findings, the results of the present study showed that AITC produced an increase in the amplitude of inward current responses and that the peak current amplitude induced by AITC was larger in muscle DRG neurons of occluded limbs than in those of control limbs. Notably, activation of the PAR2 signal amplified AITC-currents in DRG neurons.

TRPA1 is responsive to pungent and reactive chemicals such as allyl isothiocyanate (AITC), allicin, cinnamaldehyde, formaldehyde, N-methylmaleimide, and α , β -unsaturated aldehydes [15, 16]. Additionally, TRPA1 is a sensor of cold temperatures and mechanical deformation [17, 18]. An important issue is that endogenous muscle metabolites within ischemic muscles augment responses of TRPA1 in afferent nerves. Specifically, endogenous molecules, such as bradykinin, reactive oxygen species, and 4-hydroxynonenal, produced during inflammation and oxidative stress can activate TRPA1 [13, 19]. Acid phosphate, bradykinin and arachidonic acid, which accumulate in contracting muscles, are specifically considered as potential endogenous stimuli involved in the exercise pressor reflex [20]. Furthermore, our recent studies have reported that blocking the kinin B2 receptor attenuated amplification of SNA and BP responses to stimulation of muscle afferent nerves following femoral artery occlusion and to activation of P2X in muscle afferent nerves [24, 26]. Thus, we speculate that some muscle metabolites and their respective receptors, such

as the bradykinin/kinin B2 receptor, may be involved in the amplified responses of TRPA1 as a modulator after femoral artery occlusion.

One potential mechanism by which TRPA1 plays a regulatory role is via the PAR2 signal pathways in DRG neurons. Co-localization of TRPA1 with PAR2 has been found in rat DRG neurons, and activation of PAR2 has been reported to increase TRPA1-induced currents in DRG neurons [23]. In addition, a prior study has shown that the mRNA expression of PAR2 was increased by 1.9-fold in the adductor muscles of mice whose femoral artery was occluded for a day [27]. Thus, in the present study we examined the expression of PAR2 in the DRG and involvement of PAR2 in modulating TRPA1 currents in DRG neurons innervating the hindlimb muscles. Our data demonstrated that femoral artery occlusion amplified PAR2, and PAR2 stimulation led to greater TRPA1 currents evoked by AITC in DRG neurons of the occluded limbs. This suggests that PAR2 likely plays a role in amplifying current responses in muscle DRG neurons after femoral occlusion. Our data further demonstrate that downstream signal pathways, namely, PLC and PIP2 were involved in the effects of PAR2 regulation of TRPA1 currents. MAPK and PI3K/AKT are also likely involved in the process of PAR2 activation [28]. Consistent with these findings, data from our present study using a whole animal preparation also demonstrated that blocking the PAR2 signal significantly attenuated the BP response evoked by static muscle contraction and SNA and BP responses evoked by TRPA1 stimulation to a greater degree in occluded rats than in control rats. This indicates that upregulation of PAR2 receptors is involved in the exaggerated exercise pressor reflex observed in occluded rats.

Conclusion

Protein expression levels of PAR2 are increased in DRG of the occluded limb, and activation of PAR2 amplifies the amplitude of TRPA1 currents in DRG neurons of the occluded limb to a greater degree. The effects of PAR2 activation are mediated via PLC and PIP2 mechanisms. In addition, blocking the PAR2 signal attenuates SNA and BP responses evoked by muscle contraction and/or TRPA1 stimulation. This suggests that a functional role played by PAR2 in muscle sensory nerves is likely to contribute to the amplified sympathetic responsiveness observed in PAD and that PLC/PIP2 is involved in the sensitization mechanism of PAR2. Overall, our findings support the notion that PAR2 in muscle afferent nerves plays a role in the amplified exercise pressor reflex via the TRPA1 mechanisms when the blood supply to the hindlimb muscles is insufficient as observed in PAD. This pathophysiological basis may aid in the development of potential therapeutic approaches for improvement of blood flow in PAD patients.

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Disclosure Statement

No conflict of interest.

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