Original Paper

TRPA1 Function in Skeletal Muscle Sensory Neurons Following Femoral Artery Occlusion

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Key Words

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

Abstract

Background/Aims: Transient receptor potential channel A1 (TRPA1) is engaged in amplified autonomic responses evoked by stimulation of muscle afferent nerves in rats with experimental peripheral arterial disease. The purposes of this study were to characterize current responses induced by activation of TRPA1 in dorsal root ganglion (DRG) neurons of control limbs and limbs with femoral artery occlusion. **Methods:** DRG neurons from rats were labeled by injecting the fluorescence tracer DiI into the hindlimb muscles and whole-cell patch clamp experiments were performed to determine TRPA1 currents. **Results:** Data show that AITC (a TRPA1 agonist) from the concentrations of 50 μ M to 200 μ M produces a dose-dependent increase of amplitudes of inward current responses. Notably, the peak current amplitude induced by AITC is significantly larger in DRG neurons of ligated limbs than that in control limbs. AITC-induced current responses are observed in small and medium size DRG neurons, and there is no difference in size distribution of DRG neurons between control limbs and ligated limbs. However, femoral occlusion increases the percentage of the AITC-sensitive DRG neurons as compared to control. AITC-induced currents in DRG neurons are significantly attenuated by exposure to 10 μ M of HC-030031, a potent and selective inhibitor of TRPA1, in both control and femoral occlusion groups. In addition, capsaicin (a TRPV1 agonist) evokes a greater increase in the amplitude of AITC-currents in DRG neurons of ligated limbs than that in control limbs. **Conclusions:** A greater current response with activation of TRPA1 is developed in muscle afferent nerves when hindlimb arterial blood supply is deficient under ischemic conditions; and TRPV1 is partly responsible for augmented TRPA1 responses induced by arterial occlusion.

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Introduction

Exercise increases sympathetic nerve activity (SNA) [1, 2], an effect which in turn increases arterial blood pressure (BP), heart rate (HR), myocardial contractility and

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peripheral vascular resistance. Two mechanisms, namely central command and the exercise pressor reflex, evoke this exercise-induced increase in SNA. Central command postulates a parallel and simultaneous increase in sympathetic and alpha motoneuron discharge [3, 4]. The exercise pressor reflex postulates that thin fiber muscle afferent nerves (group III & IV) innervating skeletal muscles are activated by contraction-induced mechanical and metabolic stimuli to elicit a reflex 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. In this disease, the most common symptom 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 in the patients with PAD than in the normal subjects during walking [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 as well as to stimulation of several muscle metabolic receptors are amplified in occluded rats compared with control rats [11, 12]. Nevertheless, the underlying mechanisms by which femoral occlusion augments responsiveness of SNA and BP during activation of muscle mechanically and metabolically sensitive afferents remain to be determined.

Transient receptor potential channel A1 (TRPA1) is a member of branch A of the transient receptor potential (TRP) family of nonselective cation channels. This channel is expressed in the sensory neurons and is involved in acute and inflammatory pain [13-19]. TRPA1 acts as a sensory receptor in response to pungent and reactive chemicals such as allylisothiocyanate (AITC), allicin, cinnamaldehyde, formaldehyde, N-methylmaleimide, and α , β -unsaturated aldehydes [13, 19-21]. TRPA1 also serve as a sensor of cold temperature and mechanical deformation [16, 18, 19, 22-25]. 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, 25-27].

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 [28]. Also, this study has 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 engaged in the role played by TRPA1 as endogenous stimuli. Our recent study has further demonstrated that TRPA1 in thin-fiber muscle afferents plays an important role in engagement in the amplified reflex SNA and BP responses observed in rats with femoral artery ligation [29].

Additionally, TRPA1 is generally found to co-express with TRPV1 receptor [30]. Many agonists i.e. black pepper and garlic extracts have been identified to activate both TRPA1 and TRPV1 receptors, although their potency can vary [31, 32]. Our previous data demonstrate that the magnitude of TRPV1 response induced by capsaicin is greater in DRG neurons of limbs with femoral artery ligation [33]. Accordingly, in this report, we characterized current responses of TRPA1 and further examined the effects of prior activation of TRPV1 on TRPA1 responses in DRG neurons of control limbs and ligated limbs. We hypothesized that under circumstances of chronic muscle ischemia, the amplitude of current responses with stimulation of TRPA1 is greater. We further hypothesized that TRPV1 facilitates TRPA1 currents to a greater degree following muscle ischemia.

Materials and Methods

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Pennsylvania State College of Medicine and complied with the National Institutes of Health (NIH) guidelines.

Labeling DRG Neurons Innervating Hindlimb Muscle

Male Sprague-Dawley rats (4-6 weeks old) were anaesthetized by inhalation of an isoflurane-oxygen mixture (2-5% isoflurane in 100% oxygen). The skin was incised and pulled away from underlying muscle tissue, and the fluorescent retrograde tracer DiI (60 mg/ml) was injected into the white portion of the gastrocnemius muscle [33, 34]. The injection volume of 1 μ l was administered, and injection was repeated three times at different locations. The injection needle was left in the muscle for 5-10 min to prevent leakage of tracer. The skin overlying the muscle was then sutured. The animals were returned to their cages for 4-5 days to permit the retrograde tracer to be transported to DRG neurons.

Ligation of the Femoral Artery

At twenty-four hours prior to the recording experiments, the rats that previously received DiI injections were anaesthetized with an isoflurane-oxygen mixture. Then, the femoral artery on one limb was surgically exposed, dissected, and ligated ~3 mm distal to the inguinal ligament as previously described [33, 35]. The same procedures were performed on the other limb except that a suture was placed below the femoral artery but was not tied; this served as the control.

Examination of DRG Neurons Responsiveness

The rats were anesthetized with an isoflurane oxygen mixture followed by cervical dislocation and decapitation. The L4-6 DRGs were quickly removed and transferred immediately into Dulbecco's modified Eagle's Medium (DMEM). The DRGs were minced, and the ganglion fragments were processed to obtain dissociated DRG neurons as described previously [33, 34]. The cell suspension was centrifuged to remove the supernatant, and the cell pellet was re-suspended in DMEM. The cells were then plated onto a 35-mm culture dish containing pre-coated coverslips.

Next, patch recordings were performed after dissociation [33, 34]. Neurons were first visualized using a combination of epifluorescent illumination and differential interference contrast (DIC, 20-40X) optics on an inverted microscope (Nikon TE2000). Under DIC, images of DiI-positive neurons were displayed on a video monitor. Neurons were patched in the whole-cell configuration and recorded at a holding potential of -70 mV using a MultiClamp 700B amplifier. Seals (1-10 G Ω) between the glass electrode (2-5 M Ω resistance) and the cell were established in a modified Tyrode solution [33, 34]. After the whole-cell configuration was established, all experiments were conducted. Signals were acquired using the pClamp 9.0 software and experimental data were analyzed using the Clampfit software program. Neurons were considered AITC-sensitive if AITC solution elicited an inward current of > 50pA in peak amplitude.

Drugs stored in 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 (inner diameter 200 μ m) [33, 34]. The ends of the parallel columns were connected to a common silica column. The distance from the column mouth to the examined cell was 100 μ m. Cells in the recording chamber were continuously bathed in Tyrode solution. The gravity-fed solutions containing each drug were delivered to the cells by controlling the corresponding valve switch (WP Instruments).

In the first experiment, the effects of TRPA1 agonist on current responses of DRG neurons of control limbs and ligated limbs were examined. Fifty, 100, 200 μ M and 1 mM of AITC were applied onto DRG neurons for a period of 30 s, respectively. In addition, HC-030031 (HC), a TRPA1 antagonist, was applied to examine activation of TRPA1. In this protocol, AITC was first delivered for 30 s and recordings were maintained to observe the full time course of AITC-evoked current responses of DRG neurons. After that, 10 μ M of HC was applied onto DRG neurons for 60s, followed by AITC for 30 s. After 3 min of washout, AITC was delivered alone for 30 s to assess recovery data.

In another experiment, the effects of TRPV1 activation on responses of TRPA1-induced currents were examined. Fifty μ M of AITC was first applied onto DRG neurons of control limbs and ligated limbs for 30 s to obtain current response. After intervals of 3 min washout, 1 μ M of capsaicin was perfused for 2 s. Then, AITC was delivered for 30 s again after the inactivation of capsaicin-elicited current.

A total of 220 DRG neurons from control limbs and 138 DRG neurons from limbs with twenty-four hours of femoral artery ligation 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. Diameters of all neurons recorded were < 40 μ m (small and medium size).

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 were presented as mean $\pm$ 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

TRPA1-Mediated Whole Cell Currents in DRG Neurons

The four concentrations of AITC (50 μ M, 100 μ M, 200 μ M and 1 mM) were used onto muscle DRG neurons to obtain TRPA1 currents (Fig. 1A&B). AITC produced a dose-dependent increase in the amplitudes of inward current responses with the concentrations of 50 μ M to 200 μ M ($P < 0.05$ among three different concentrations). The peak amplitudes of AITC currents induced by 50 μ M to 200 μ M of AITC were 83.1 ± 7.4 pA ($n=15$), 278.4 ± 40.4 pA ($n=20$) and 359.9 ± 65.8 pA ($n=14$), respectively. However, when 1 mM of AITC was applied onto neurons, this did not evoke the greater amplitude of the inward current as compared with the current activated by 200 μ M of AITC (359.9 ± 65.8 pA by 200 μ M of AITC, $n=14$ to 354.6 ± 63.6 pA by 1 mM of AITC, $n=21$, $P > 0.05$ vs. 200 μ M of AITC).

Fig. 1. Characteristics of TRPA1-mediated whole cell currents in DRG neurons: A, Original traces of currents elicited by increasing concentrations of AITC. B, Averaged data show AITC produced a dose-dependent increase in amplitudes of inward current from the concentrations of 50 μ M to 200 μ M. However, 1 mM of AITC failed to augment the amplitude of the inward current as compared with the current activated by 200 μ M AITC. * $P < 0.05$ compared with 50 μ M and 200 μ M AITC.

Effects of Femoral Occlusion on TRPA1 Currents in DRG Neurons

One hundred μ M of AITC was selected to induce TRPA1 current responses in muscle DRG neurons of control limbs and ligated limbs. Fig. 2A shows original traces of currents in DRG neurons innervating hindlimb muscle elicited by 100 μ M of AITC in a control limb and in a limb with femoral artery ligation. When compared with TRPA1 current responses in control limbs, femoral arterial occlusion induced greater peak inward current amplitudes (278.4 ± 40.4 pA in 20 neurons of control limbs vs. 401.1 ± 42.1 pA in 22 neurons of ligated limbs, $P < 0.05$) as shown by average data of Fig. 2B.

In addition, the DRG neurons recorded in control and arterial occlusion are all small and medium size. There was no difference in the average diameter of recorded DRG cells responding to AITC in both groups (28.1 ± 0.9 μ m in control, $n=80$ to 29.1 ± 0.8 μ m in ligation, $n=83$, $P > 0.05$) (Fig. 2C). However, the percentage of the AITC-responsive DRG neurons was greater after arterial occlusion ($58.0 \pm 4.3\%$) than that in the control ($43.1 \pm 5.5\%$, $P < 0.05$ vs. ligation group) (Fig. 2D).

Also, we examined the effects of the inhibitor of TRPA1, HC, [36], on evoked currents by AITC in both control group and femoral ligation group. Note that HC did not elicit any current

Fig. 2. Effects of femoral arterial occlusion on TRPA1-induced currents: A, Original traces of currents elicited by 100 μ M of AITC in DRG neurons of a control limb and a limb with femoral artery ligation. The amplitude of the current in a DRG neuron of the ligated limb is larger than that in a DRG neuron of the control limb.

B, Averaged data show that femoral artery ligation resulted in the greater increase in amplitudes of currents elicited by 100 μ M of AITC. * $P < 0.05$ vs. control. C, Diameters of DRG neurons innervating muscle responding to AITC in control limbs and limbs with femoral arterial occlusion. Averaged data show that there was no significant difference in size of DRG neurons between control and ligation groups. D, Percentage of muscle DRG neurons responsive to 100 μ M of AITC in control and ligation groups. A larger percentage of DRG neurons responsive to AITC were observed after femoral artery ligation compared with control. * $P < 0.05$, vs. control.

Fig. 3. AITC-induced currents in DRG neurons were antagonized by a prior blocking of TRPA1 with 10 μ M of HC-030031 (HC) in both control and ligation groups: A, Typical traces showing the effects of HC on AITC-activated currents. B, Averaged data show that AITC-induced currents in muscle DRG neurons were blocked by prior exposure to 10 μ M of HC. Note that 50 μ M of AITC was used to evoke currents and the antagonizing effects of HC were reversible in both control and ligations groups.

response per se. However, AITC currents in DRG neurons were completely attenuated by exposure to 10 μ M of HC (Fig. 3, A&B). This antagonizing effect of 10 μ M of HC on AITC currents was reversible in both experimental groups (in control: 93.9 ± 11.5 pA before HC to 82.1 ± 11.2 pA after HC, $n=8$, $P > 0.05$; in ligation: 136.6 ± 30.9 pA before HC to 113.3 ± 21.8 pA after HC, $n=8$, $P > 0.05$).

Fig. 4. Prior activation of TRPV1 by capsaicin increases TRPA1 responses to AITC: A, Original traces show the effects of prior application of 1 μ M of capsaicin on AITC-induced currents in DRG neurons of a control limb and a ligated limb. B, Averaged data show that 2 s treatment of capsaicin amplified the amplitude of AITC-activated currents in muscle DRG neurons of both control and ligation group. * $P < 0.05$ compared with before capsaicin. C, The percentage of augmented amplitude of AITC-induced currents by capsaicin appeared to be greater in DRG neurons of ligated limbs as compared with that in DRG neurons of control limbs. * $P < 0.05$ vs. control. AITC was first applied for 30 s to obtain control. After intervals of 3 min washout, 1 μ M of capsaicin was perfused for 2 s. After the inactivation of capsaicin-elicited current, AITC was delivered for 30 s again. Note that the scales in A are applied for AITC-elicited currents.

Prior Activation of TRPV1 Augments TRPA1 Response

We examined whether a prior exposure to 1 μ M of capsaicin could augment the current responses elicited by 50 μ M of AITC in muscle DRG neurons of both control limbs and ligated limbs (Fig. 4 A-C). In AITC-responsive DRG neurons of control, pretreatment of capsaicin increased the amplitude of AITC-activated currents (84.5 ± 7.3 pA before capsaicin to 110.1 ± 9.7 pA after capsaicin, $n=10$, $P < 0.05$) (Fig. 4 A&B). Similarly, in AITC-responsive DRG neurons obtained from limbs with femoral artery ligation, prior application of capsaicin also accentuated the amplitude of the currents induced by 50 μ M of AITC (125.9 ± 16.3 pA before capsaicin to 179.2 ± 16.4 pA after capsaicin, $n=11$, $P < 0.05$) (Fig. 4 A&B). Nevertheless, the percentage of amplified amplitude by capsaicin was significantly higher in DRG neurons of ligated limbs ($43.9 \pm 4.5\%$, $P < 0.05$ vs. control) than that in DRG neurons of control limbs ($29.6 \pm 4.2\%$) (Fig. 4 C). Note that in both control and ligation groups all of the neurons responsive to 50 μ M of AITC were also responding to 1 μ M of capsaicin.

Discussion

Given that DRG cells are the primary sensory innervations to group III and IV fibers afferent nerves, expression and characteristics of sensory receptors (i.e., TRPA1) in DRG neurons are generally examined to study receptor physiology. Previous studies suggest

that the glycolytic muscle plays a major role in reflex muscle responses evoked by static contraction [37]. A study using electrophysiological methods has also demonstrated that DRG neurons with nerve endings in the white portion of the gastrocnemius muscle develop greater inward current responses to metabolic stimulation such as protons and capsaicin (a TRPV1 agonist) [34, 38]. Therefore, in the current report, AITC-induced currents with TRPA1 activation were recorded on rat DRG neurons innervating the white portion of the gastrocnemius muscles identified by retrograde labeling with the fluorescent dye Dil. Furthermore, we characterized TRPA1 currents in muscle DRG neurons of control limbs and ligated limbs.

In general, AITC and cinnamaldehyde are currently used as TRPA1 agonists in experiments [39]. Cinnamaldehyde has been considered to activate only a smaller population of sensory neurons [40]. Therefore, in the current study, we used AITC to evoke TRPA1 current responses in DRG neurons innervating muscle of control limbs and limbs with twenty-four hours of femoral artery occlusion in rats.

AITC was applied from 10 μ M to 1000 μ M sequentially onto the DRG neurons and data of this previous study demonstrated that a value of EC₅₀ for AITC is 173 μ M and the saturating concentration is about 1 mM [41]. Also, a brief application (20–50 s) of AITC at 200 μ M has been shown to induce responses that were readily reversible, but continuous application of higher concentrations (> 500 μ M) of AITC induced a complete desensitization of the current response [41]. Thus, the similar concentrations of AITC were used in our current study. Consistent with these previous findings, results of the present study showed that AITC produced a dose-dependent increase in amplitudes of inward current responses from the concentrations of 50 μ M to 200 μ M. However, when 1 mM of AITC was applied, this failed to augment the amplitude of the inward current as compared with the current activated by 200 μ M of AITC. Moreover, our results showed that AITC-induced currents in DRG neurons were completely attenuated by exposure to HC, a TRPA1 inhibitor; in both control and femoral ligation groups and the antagonizing effect of HC was reversible.

Notably, we found that the peak current amplitude induced by AITC was larger in muscle DRG neurons of ligated limbs than that in control limbs, and that arterial occlusion increased the percentage of the AITC-responsive DRG neurons. In our previous study [29], the protein levels of TRPA1 were amplified in DRG of ligated limbs using western blot analysis, and an increase in the number of TRPA1 expressing DRG neurons supplying metabolically sensitive afferent nerves of C-fiber (group IV) was also observed after femoral ligation using immunocytochemical methods. In accordance with results of this previous study, data of the present study showed that AITC-responsive DRG neurons were all small and medium size and the number of AITC-responsive DRG neurons was increased by femoral occlusion. In addition, the greater peak AITC current amplitude in DRG neurons of ligated limbs was observed in the present study and this was likely due to amplified TRPA1 expression as seen previously [29].

TRPA1 has a similar structure to TRPV1 receptor, but with numerous ankyrin repeats in its amino (N) terminal [42]. TRPA1 has been shown to be co-expressed with TRPV1 [19]. Many agonists have been identified to activate both TRPA1 and TRPV1 receptors [31, 32]. AITC can activate porcine TRPV1 [43]. Note that application of AITC in the micromolar range did not significantly affect currents recorded in TRPV1-transfected HEK293 cells. However, TRPV1 contributes to the DRG neuron responses to 3 mM of mustard oil [44]. Likewise, a previous study has shown that cell membrane staining of TRPA1 increased upon with treatment of 1 μ M of capsaicin [45]. Also, this prior study has demonstrated that activation of TRPV1 by capsaicin that was accompanied with localized calcium influx acutely increased TRPA1 membrane surface expression while TRPV1 levels were unchanged [45]. Our published work showed that a higher density of TRPV1 immunostaining was induced within DRG neurons of rats with femoral artery occlusion when compared with that in control rats, and that the arterial occlusion insult induced greater peak of inward current amplitudes in DRG neurons to capsaicin [33]. In the current study, 1 μ M of capsaicin applied onto DRG neurons augmented the amplitude of current responses elicited by AITC in both experimental

groups. The percentage of amplitude increase by capsaicin was greater in DRG neurons of ligated limbs than that in control limbs. Thus, we speculate that artery occlusion-enhanced expression and responses of TRPV1 in DRG neurons likely induced a greater calcium influx and increased TRPA1 membrane surface expression in engagement in the role played by TRPA1 observed in our current study.

An important issue is what endogenous muscle metabolites within ischemic muscles augment responses of sensory nerves' TRPA1. In addition to pungent chemicals found in nature, endogenously generated molecules such as bradykinin, reactive oxygen species, and 4-hydroxynonenal produced during inflammation and oxidative stress can activate TRPA1 [13, 25, 26]. Acid phosphate, bradykinin and arachidonic acid, which are accumulated in contracting muscles, are considered as potential endogenous stimuli engaged in the exercise pressor reflex [28, 46]. Our study has also reported that femoral artery occlusion amplifies blood pressure responses to stimulation of muscle afferent nerves and increased responses are attenuated by blocking kinin B2 receptor [47]. Thus, we speculate that some muscle metabolites and their respective receptors such as bradykinin/kinin B2 receptor may play a role in engagement in amplified sensory responses of TRPA1 as a modulator after femoral artery occlusion.

Nevertheless, a prior study has shown that in healthy rats, intra-arterially injection of AITC into the hindlimb muscle circulation led to increases in sympathetic activity via a reflex pathway [28]. Also, this prior study has demonstrated that TRPA1 plays a role in regulating sympathetic activity via stimulating the metabolic component of the exercise pressor reflex. When the similar dosages AITC was selected to stimulate TRPA1 on muscle afferent nerves and results of our recent work showed that greater sympathetic and pressor responses were evoked by arterial injection of AITC into the hindlimb muscles in ligated rats than those responses in control rats [29].

PAD can lead to severe limb ischemia. Thus, the classic discomfort syndrome of PAD is termed "intermittent claudication" which is characterized by pain in lower limbs that especially occurs during walking [48]. Note that muscular pain plays a significant component in fibromyalgia and other chronic pain conditions [49]. In general, enhanced expression and function of nociceptive ion channel such as TRPA1 and TRPV1 are critical in regulating pain [50]. In the present study, we observed that response of TRPA1 was increased by femoral occlusion and the effects were amplified by the prior TRPV1 activation. These conditions shed light as to the possible causes of pain associated with PAD. Nonetheless, if these changes reflect in the central terminals of the DRG neurons likely leading to synaptic plasticity still need to be determined.

Conclusion

Results of the current study for the first time demonstrated that DRG current response to activation of TRPA1 is exaggerated following femoral artery occlusion, and that augmented responses are mostly observed in small/medium diameter of DRG neurons innervating the hindlimb muscles. In addition, stimulation of TRPV1 receptors augments TRPA1 responses of DRG neurons to a greater degree after femoral artery occlusion, indicating that TRPV1 is partly responsible for augmented TRPA1 responses. This also suggests that a functional interaction in TRPV1 and TRPA1 in muscle sensory nerves likely contributes to the amplified sympathetic responsiveness observed in PAD. Overall, our findings support the notion that TRPA1 in muscle afferent nerves contributes to augmented sympathetic responsiveness via the metabolic component of the exercise pressor reflex when blood supply to the hindlimb muscles is insufficient as observed in PAD. This pathophysiological basis may further help to aim at a potential therapeutic approach for improvement of blood flow in PAD patients.

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Disclosure Statement

None.

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