Bradykinin B$_2$ receptor contributes to the exaggerated muscle mechanoreflex in rats with femoral artery occlusion

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Lu J, Xing J, Li J. Bradykinin B$_2$ receptor contributes to the exaggerated muscle mechanoreflex in rats with femoral artery occlusion. Am J Physiol Heart Circ Physiol 304: H1166–H1174, 2013. First published February 15, 2013; doi:10.1152/ajpheart.00926.2012.—Static muscle contraction activates the exercise pressor reflex, which in turn increases sympathetic nerve activity (SNA) and blood pressure (BP). Bradykinin (BK) is considered as a muscle metabolite responsible for modulation of the sympathetic and cardiovascular responses to muscle contraction. Prior studies have suggested that kinin B$_2$ receptor contributes to the augmented mechanoreflex activity in rats with 24 h of femoral artery occlusion. First, Western blot analysis was used to examine protein expression of B$_2$ receptors in dorsal root ganglion tissues of control and ligated limbs. Our data show that B$_2$ receptor displays significant overexpression in ligated limbs as compared with control limbs (optical density: 0.94 ± 0.02 in control and 1.87 ± 0.08 after ligation, P < 0.05 vs. control; n = 6 in each group). Second, mechanoreflex was evoked by muscle stretch and the reflex renal SNA (RSNA) and mean arterial pressure (MAP) responses to muscle stretch were examined after HOE-140, a B$_2$ receptors blocker, was injected into the arterial blood supply of the hindlimb muscles. The results demonstrate that the stretch-evoked reflex responses were attenuated by administration of HOE-140 in control rats and ligated rats; however, the attenuating effects of HOE-140 were significantly greater in ligated rats, i.e., after 5 µg/kg of HOE-140 RSNA and MAP responses evoked by 0.5 kg of muscle tension were attenuated by 43% and 25% in control vs. 54% and 34% in ligation (P < 0.05 vs. control group; n = 11 in each group). In contrast, there was no significant difference in B$_1$ receptor expression in both experimental groups, and arterial injection of R-715, a B$_1$ receptors blocker, had no significant effects on RSNA and MAP responses evoked by muscle stretch. Accordingly, results obtained from this study support our hypothesis that heightened kinin B$_2$ receptor expression in the sensory nerves contributes to the exaggerated muscle mechanoreflex in rats with femoral artery occlusion.

two neural mechanisms are suggested to evoke sympathetic nerve and cardiovascular responses during exercise. The first is referred to as the exercise pressor reflex, which is evoked by mechanical and metabolic stimuli that activate thin-fiber muscle afferents in the working muscle (15, 34, 35). Thus the exercise pressor reflex has two functional components, namely the muscle mechanoreflex and metaboreflex. Specifically, mechanical deformation mostly stimulates thinly myelinated group III and muscle metabolites mostly stimulate unmyelinated group IV afferent fibers (15, 17–19). Consequently, the brainstem nuclei that regulate cardiovascular activities are stimulated and sympathetic nerve activity (SNA) and arterial blood pressure (BP) increase (15, 35). The second mechanism is termed central command, which originates in the higher brain responsible for recruiting motor units and is engaged in cardiovascular and respiratory regulation during exercise (12, 57).

Peripheral artery disease (PAD), caused by a restriction of the blood flow in the lower limbs, is typically common in older adults (5, 36, 38). Prior studies have shown that SNA and BP responses during activation of the exercise pressor reflex are augmented in human and rat models with PAD (1, 2, 53). This reflex dysfunction has previously been shown to be mediated, in part, by muscle mechanoreflex overactivity (31). With the use of a rat model of PAD, prior studies have further demonstrated that a number of receptors on muscle afferent nerves contribute to the amplified BP response during the exercise pressor reflex (24, 54, 55). For example, blocking acid sensing ion channels and thromboxane TP receptors, and stimulating μ-opioid receptors can attenuate the augmented pressor response to muscle contraction (24, 54, 55).

A number of metabolites produced in contracting muscles, such as ATP, bradykinin (BK), cyclooxygenase products, lactic acid, and potassium, etc., have been considered as potential stimulants and/or sensitizers of muscle afferents responding to mechanical and metabolic stimuli during the exercise pressor reflex (15, 45). Among those metabolites, BK is an autacoid produced within the interstitium of most tissues and is synthesized from its precursor kininogen after activation of the enzyme, kallikrein. A previous study has shown that kinin B$_2$ receptor, but not B$_1$ receptor, mediates the effects of BK on cardiovascular responses to stimulation of skeletal muscle afferents in anesthetized cats (40). In addition, it is found that muscle BK is increased during exercise and there is a close relationship between the levels of BK and ventilatory response during postexercise ischemia (42). Our recent study further suggests that blocking kinin B$_2$ receptor significantly attenuates SNA response to activation of the muscle mechanoreceptors in rats with heart failure (21). Thus the purpose of this study was to determine the role for BK and its receptors (kinin B$_1$ and B$_2$) in modulating the muscle mechanoreflex in rats with PAD induced by 24 h of femoral artery ligation. Our general hypothesis was that heightened kinin B$_2$ receptor expression in the sensory nerves contributes to the exaggerated peripheral artery disease; blood pressure; sympathetic nerve activity; muscle afferent nerves; static exercise

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muscle mechanoreflex in PAD rats. Specifically, we hypothesized that protein expression of B2 receptors in dorsal root ganglion (DRG) tissues of ligated limbs of rats is upregulated compared with control limbs. In addition, we hypothesized that SNA and BP responses to stimulation of mechanically sensitive muscle afferent nerves evoked by muscle stretch are inhibited to a greater degree in ligated rats than that in control rats with B2 receptor antagonist injected into the arterial blood supply of the hindlimb muscles.

Our previous study demonstrated that 24 h of femoral artery ligation significantly enhanced the SNA and BP responses to muscle stretch, and there were no differences in those responses between rats with 24 h and those with 72 h of femoral artery ligation (31). Also, nerve growth factor (NGF) played a role in regulating expression of kinin B2 receptor (41) and the levels of NGF were significantly increased in the DRG tissues 24 h after femoral artery ligation. However, there were no significant differences in increased NGF between 24 and 48 h after ligation (61). Thus 24 h of ligation was selected for the experiments in this report.

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.

Ligation of Femoral Artery

Under inhalation of an isoflurane-oxygen mixture (2–5% isoflurane in 100% oxygen), the surgical procedures were performed in 54 male Sprague-Dawley rats (6 to 8 wk old) as previously described (28, 58, 61). For the Western blotting experiments, femoral arteries of the right hindlimb of 12 rats were surgically exposed, dissected, and ligated ~3 mm distal to the inguinal ligament; this served as ligated limb. The same procedures were performed on the left hindlimb except that a suture was placed below the femoral artery but was not tied; this served as control limb. For the experiment of SNA and BP recording, 42 rats were equally divided between those that had the right femoral artery ligated and those that had sham surgery on the right hindlimb and served as controls. The ligated rats and control rat were defined in this experiment. For both experiments, 24 h were then allowed for recovery before the experiments began.

Western Blot Analysis

Twelve rats were used to examine expression of kinin B2 and B1 receptors protein in lumbar (L4–6) DRGs of control and ligated limbs. Western blot methods were performed as previously described (28, 30). In brief, DRGs of the rats were removed. All DRG tissues from individual rats were sampled for Western blot analysis. Total protein was then extracted by homogenizing DRG sample in ice-cold radioimmunoprecipitation assay buffer containing 25 mM Tris·HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS with protease inhibitor cocktail kit (Sigma-Aldrich, St. Louis, MO). The lysates were centrifuged at 15,000 g for 15 min at 4°C; the supernatants were collected for measurements of protein concentrations using a bicinchoninic acid assay reagent kit (Pierce Biotech, Rockford, IL) and then stored in −80°C for later use.

After being denatured by heating at 95°C for 5 min in an SDS sample buffer (Cell Signaling Technology, Danvers, MA), the supernatant samples containing 20 μg of protein were loaded onto 4–20% Mini-PROTEAN TGX gels (Bio-Rad Laboratories, Hercules, CA) and then electrically transferred to a polyvinylidene fluoride membrane (GE Water & Process Tech, Trevose, PA). The membrane was blocked in 5% nonfat milk in 0.1% Tween-TBS buffer for 1 h and then incubated overnight with primary antibodies (mouse anti-kinin B2 receptor at 1:200; rabbit anti-kinin B1 receptor at 1:200; Santa Cruz Biotechnology).

After being fully washed, the membrane was incubated with horseradish peroxidase-linked anti-mouse secondary antibody (1:1,000) and anti-rabbit secondary antibody (1:1,000). The immunoreactive proteins were detected by enhanced chemiluminescence system (Cell Signaling Technology). The bands recognized by the primary antibody were visualized by exposure of the membrane onto an X-ray film. The membrane was stripped and incubated with mouse anti-β-actin (Santa Cruz Biotechnology) to show equal loading of the protein in the Western blot analysis. The film was then scanned, and the optical density of B2 receptors was quantitated using the NIH Image software (Bethesda, MD) to determine optical density of B2 receptors.

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Fig. 1. Analysis of kinin B2 and B1 receptors protein expression in control limbs and limbs with 24 h of femoral artery ligation. Western blot assays were performed on dorsal root ganglion tissues from control limbs and ligated limbs. A, top: typical bands. The results of Western blot assays illustrate that optical density of B2 receptor protein is higher in a ligated limb than that in a control limb. Bands of β-actin are used as control for an equal protein loading. B, bottom: representative bands of B1 receptor expression. B, bottom: average data. Significant B2 receptor overexpression was seen in ligated limbs over control limbs. Results represent means ± SE of n = 6. *P < 0.05 compared with control. 2. top: representative bands of B1 receptor expression. B, bottom: average data. Results represent means ± SE of n = 6. No significant difference was observed between control and ligated groups.
density of kinin $B_2$, $B_1$ receptors, and β-actin bands was analyzed using the NIH Scion Image Software.

Examination of Muscle Mechanoreflex

Surgical preparations. Forty-two rats were anesthetized by inhalation of an isoﬂurane–oxygen mixture (2–5% isoﬂurane in 100% oxygen). Note that this was a different group of rats than those used in the Western blot analysis. An endotracheal tube was inserted and attached to a ventilator (Model AWS; Hallowell EMC, Pittsﬁeld, MA). Polyethylene (PE-50) catheters were inserted into an external jugular vein and the common carotid arteries for the purposes of drug administration and measurement of arterial BP, respectively. The femoral artery was isolated in the previously ligated limb/control limb. An incision was made in the artery between the previous suture and the popliteal artery. A catheter (PE-10) was then inserted into the femoral artery, and the tip of the catheter was placed in the popliteal artery for injection of drugs into the arterial blood supply of the hindlimb muscles. As there is collateral flow to maintain limb perfu-

sion, a catheter inserted into the popliteal artery is unlikely to occlude the circulation of the limb being tested. The skin covering the triceps surae muscle and femoral region was surgically separated from the muscle below to eliminate inputs from cutaneous afferents in the hindlimb.

During the experiments, the animals were artiﬁcially ventilated, and tidal CO$_2$ was monitored by a respiratory gas monitor (Model S250; Datex-Ohmeda, Madison, WI) and maintained within normal ranges, as previously described (11, 14, 22, 59). Body temperature was carefully maintained at 37.5–38.5°C by a heating pad and external heating lamps. A continuous infusion of physiological saline was carefully maintained at 37.5–38.5°C by a heating pad and basal BP.

The rats were placed in a spinal unit (Kopf Instruments). A bundle of the renal nerves on the left side was carefully dissected from other connective tissues. A piece of laboratory ﬁlm was placed under the isolated nerves, and two tips of a bipolar electrode used to record neural activity were placed between the nerves and the ﬁlm; these were embedded in a silicone gel. Once the gel hardened, the silicone rubber was ﬁxed to the surrounding tissue with a glue containing α-cyanoacrylate. The skin and muscle tissues in the region of incision were used to form a pool that was ﬁlled with warm (37°C) mineral oil.

The renal SNA (RSNA) signal was ampliﬁed with an ampliﬁer (P511; Grass Instruments) with a band-pass ﬁlter of 300 Hz in low-cut frequency and of 3 kHz in high-cut frequency and recorded as previously described (28, 58). Note that signal-to-noise ratio for the baseline RSNA was examined to validate data obtained.

Decerebration was performed as previously described (11, 28, 58) to avoid the confounding effects of anesthesia on the reﬂex pressor response. A transverse section was made anterior to the superior colliculus and extending ventrally to the mammillary bodies. All brain tissues rostral to the section were removed. After this procedure, the anesthesia was withdrawn from the rats. The calcaneal bone of right hindlimb was cut and its tendon was attached to a force transducer (Grass FT10), and the knee joints were secured by clamping the patellar tendon to a spinal unit. A recovery period of 60 min was allowed before the experiment.

Experimental protocols. The purpose of this experiment was to examine if femoral artery occlusion altered the effects of blocking kinin $B_2$ and $B_1$ receptors on the sympathetic and pressor responses evoked by stimulation of mechanically sensitive muscle afferent nerves. Thus muscle stretch was performed to activate the mecha

noverpppressor component of the exercise pressor reﬂex in control rats and rats whose femoral artery was ligated for 24 h. Note that each muscle stretch was performed 15 min after arterial injection of saline (control and recovery) and each antagonist. Then, 20 min was allowed after stretch and before the next injection. The injected volume was 0.1 to 0.15 ml, and the duration of injections was 1 min. Thus there was a ~36-min resting period between bouts of muscle stretch. In the first group, saline was injected into the arterial line before muscle stretch was evoked to obtain a control. The RSNA, BP, and heart rate (HR)
responses to muscle stretch were then examined. Next, to examine effects of B2 receptors, 5 μg/kg of HOE-140 (a specific B2 receptor antagonist) was administered into the arterial blood supply of hindlimb muscles of control rats and ligated rats (n = 11 in each group) before muscle stretch. After this, saline was arterially given and then the reflex RSNA, BP, and HR responses were examined to obtain a recovery. In the second group, in the same way, in control rats and ligated rats (n = 10 in each group) saline was injected to obtain a control. R-715 (20 μg/kg; a specific B1 receptor antagonist) was given to examine effects of B1 receptors, and a recovery was examined after saline injection. The effective doses of B2 and B1 receptor antagonists were determined based on the previous work from our laboratory and others (7, 9, 21). In this experiment, we attempted to stimulate muscle mechanoreceptors and minimize engagement of other receptors (i.e., pain), although it is unlikely to rule out their effects. Muscle stretch (0.5 kg tension) was produced manually over ~5 s by using a rack and pinion attached to the Achilles’ tendon. Each bout of muscle stretch was maintained for 30 s after 0.5 kg of tension was achieved. The levels of tension and intervention have been reported to generate sufficient RSNA and BP responses in rats (10).

Data acquisition. All measured data of RSNA, BP, and HR were continuously recorded and stored on a computer with PowerLab system (AD instruments, Castle Hill, Australia). Mean arterial pressure (MAP) was obtained by integrating the arterial signal with a time constant of 4 s. HR was calculated on a basis of beat to beat from the arterial pressure pulse. The peak responses of MAP and HR were determined by the peak change from the control value (average over 30 s). RSNA signals were transformed into absolute values, integrated over 1-s interval, and subtracted by 1 s of integrated background noise. To quantify RSNA response to muscle stretch and arterial injection, baseline values were obtained by taking the mean value for the 30 s immediately before each injection and by ascribing the mean value of 100%, and relative change from baseline during the injection was then evaluated.

Data Statistical Analysis

All statistical analyses were performed using SPSS for Windows version 19.0 (SPSS Sci, Chicago, IL). One-way repeated-measures ANOVA was performed to compare variables for RSNA, MAP, and HR, as well as kinin B1 and B2 receptors optical density, followed by Tukey’s post hoc test as appropriate. All values were presented as means ± SE. For all analyses, differences were considered significant at P < 0.05.

RESULTS

Effects of Femoral Occlusion on Protein Levels of B2 and B1 Receptors

Figure 1A shows that femoral artery occlusion significantly increased expression of B2 receptor in DRG. The intensity of the signal in lumbar DRG tissues of ligated limbs was ~1.99-fold greater than that in control limbs (optical density, 1.87 ± 0.08 in occlusion vs. 0.94 ± 0.02 in control, P < 0.05; n = 6 in each group). Figure 1B further shows that no differences were seen in the expression of B1 receptor in DRG of control limbs and ligated limbs (optical density, 1.01 ± 0.03 in occlusion vs. 0.97 ± 0.01 in control, P > 0.05; n = 6 in each group).

Effects of Blocking B2 and B1 Receptors on Responses of RSNA, MAP, and HR during Muscle Stretch

Effects of HOE-140. Baseline MAPs and HRs were 101 ± 5 mmHg; 391 ± 14 beats/min in control rats (n = 11) and 103 ± 4 mmHg; 394 ± 12 beats/min in rats whose femoral artery was ligated for 24 h (n = 11) (P > 0.05, vs. control). Muscle stretch was performed in both groups after each intervention. Typical traces and average data are shown in Figs. 2 and 3. Twenty-four hours of femoral occlusion significantly increased the

Fig. 3. The changes of RSNA, mean arterial pressure (MAP), and HR were measured in response to muscle stretch after B2 receptor inhibition by HOE-140. Average data show that RSNA and MAP responses to muscle stretch were significantly attenuated in both control rats and ligated rats after blocking B2 receptors. Note that the attenuating effects of HOE-140 appeared to be a greater degree in ligated group. The responses of RSNA and MAP returned to their pre-inhibitory state after a recovery period. No significant difference was observed in baseline MAP and HR in control rats and ligated rats. Also, there were no significant differences in signal-to-noise (S/N) ratio for the basal RSNA in control rats (3.5 ± 0.6) and ligated rats (3.6 ± 0.5, P > 0.05 vs. control). The reflex responses to muscle stretch with saline control were higher in ligated rats versus control animals, *P < 0.05 vs. saline control; †P < 0.05, significant differences in changes in RSNA and MAP between control group and ligated group. The number of animals is 11 in each group. Note that the same level of tension was loaded in all interventions.

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responses of RSNA and MAP evoked by muscle stretch with 0.5 kg of muscle tension. No significant differences in the HR response were seen between the two groups. Moreover, Figs. 2 and 3 show RSNA, MAP, and HR responses to muscle stretch after inhibition of B2 receptors with arterial injection of 5 µg/kg of HOE-140 in control rats and ligated rats. HOE-140 significantly inhibited RSNA and MAP responses induced by muscle stretch in control and ligated groups. In control rats, the RSNA and MAP responses were 32 ± 1% and 16 ± 1 mmHg with saline injection and 18 ± 2% and 12 ± 1 mmHg with HOE-140 treatment (P < 0.05, HOE-140 treatment vs. saline control for both RSNA and MAP responses). In ligated rats, the RSNA and MAP responses were 51 ± 2% and 21 ± 1 mmHg with saline injection and 24 ± 2% and 14 ± 2 mmHg with HOE-140 treatment (P < 0.05, HOE-140 treatment vs. saline control for both RSNA and MAP responses). The attenuating effects of HOE-140 on RSNA and MAP responses during muscle stretch returned to their control levels after a recovery period. HOE-140 treatment had no effects on HR responses to muscle stretch in either group.

**Effects of R-715.** There were no significant differences in baseline values for MAP and HR in control rats and ligated rats (n = 10 in each group). Baseline MAPs and HRs were 97 ± 3 mmHg, 384 ± 12 beats/min, in control rats; and 99 ± 2 mmHg, 388 ± 13 beats/min, in ligated rats. Figures 4 and 5 are typical traces and average data demonstrating RSNA, MAP, and HR responses to muscle stretch during each intervention. Before B1 receptor inhibition, significantly higher RSNA and MAP responses were observed in the ligated group versus the control group. No significant HR responses were seen in the two groups during stretch. Blocking B1 receptors with arterial injection of 20 µg/kg of R-715 did not elicit significant changes in RSNA, MAP, and HR responses during muscle stretch in either the control rats or the ligated animals, i.e., in ligated rats, RSNA and MAP responses induced by 0.5 kg of muscle tension were 50 ± 2% and 21 ± 1 mmHg in control and 49 ± 3% and 22 ± 2 mmHg (P > 0.05 vs. control) after 20 µg/kg of R-715.

**DISCUSSION**

The present study has demonstrated that a significant B2 receptor overexpression is observed in the DRG tissues of ligated limbs as compared with control limbs. This result suggests that femoral artery occlusion amplifies expression of B2 receptors in the sensory neurons. In contrast, no significant difference in levels of B1 receptor protein in the DRG was observed between the two conditions, suggesting that femoral artery occlusion is unlikely to change expression of B1 receptors in the sensory neurons. Given that DRG cells are the primary sensory projections to group III and IV fibers afferent nerves, expression and characteristics of sensory receptors in DRG neurons are generally examined to study receptor physiology (4, 25, 27, 56). The receptors in question are found in both the peripheral terminals and cell bodies of sensory DRG neurons. Receptor activity and characteristics of the DRG cell body have been used to reflect activity and characteristics of the receptors located at the nerve endings (4, 25, 27, 56). Accordingly, B2 receptors on the mechanosensitive nerve endings in the muscle interstitium are likely upregulated in this report.
In agreement with the findings of BK receptors expression, our data have further shown that intra-arterial injection of HOE-140, a specific kinin B2 receptor blocker, into the hindlimb circulation significantly attenuates RSNA and BP responses evoked by muscle stretch in both control rats and ligated rats. However, the effects of HOE-140 were greater in ligated rats than they were in control rats. On the other hand, arterial injection of R-715, a B1 receptors blocker, had no effect on RSNA and BP responses to muscle stretch in either experimental group.

During muscle contraction, an increase in the levels of BK (23, 42, 43, 49) stimulates and/or sensitizes muscle afferents responding to contraction, thereby contributing to autonomic function during exercise (8, 16, 40, 50, 51). Recent findings have suggested that femoral artery occlusion augments responsiveness of SNA and BP to stimulation of muscle mechanosensitive and metabosensitive afferents (28, 29, 31, 53, 55, 58, 60, 61). Muscle stretch used in the present experiment is considered to mainly stimulate muscle mechanoreceptors. Thus, based on the data obtained from this study, it is well reasoned that BK sensitizes mechanosensitive muscle afferents via B2 receptors. Due to overexpression of B2 receptors, RSNA and BP responses induced by the mechanoreflex are attenuated to a larger degree in ligated rats when B2 receptors are blocked. Overall, the data suggest that expression of kinin B2 receptor in the sensory nerves is heightened in rats with the hindlimb ischemia, which thereby results in a greater stimulating and/or sensitizing effect on the sympathetic and pressor responses to stimulation of the muscle mechanoreceptor during muscle stretch.

Similarly, our previous study has demonstrated that blocking kinin B2 receptor with HOE-140 can significantly attenuate SNA responses to intermittent muscle contraction (an intervention considered to largely stimulate muscle mechanoreceptors) to a greater degree in rats with heart failure induced by myocardial infarction as compared with control rats (21). Given that the abnormalities in the muscle pressor reflex are mediated primarily by muscle mechanoreflex overactivity in heart failure (22, 26, 46), together with findings of our current study, this suggests kinin B2 receptor is generally engaged in the exaggerated sympathetic responses during stimulation of the muscle mechanoreceptor as the blood flow to active muscle is insufficient.

Both heart failure and PAD can cause skeletal muscle ischemia when muscle is active. Thus there is a possibility that any condition that causes muscle ischemia could evoke such B2 receptor-induced sensitizing effect on skeletal muscle mechanoreceptors. Nonetheless, it should be noted that heart failure is considered as a chronic low flow state compared with femoral artery occlusion-induced hindlimb ischemia employed in the present study. Left ventricular function is largely diminished in rats 6–8 wk after ligation of the coronary artery (11, 21, 26, 46), together with findings of our current study, this suggests kinin B2 receptor is generally engaged in the exaggerated sympathetic responses during stimulation of the muscle mechanoreceptor as the blood flow to active muscle is insufficient.
It is well-known that ischemia can generally induce inflammation, which can provoke the release of BK (41). Thus femoral artery ligation likely increased baseline levels of BK, and this is likely to have contributed to the concomitant upregulation of B2 receptors observed in the present study. In addition, substantial evidence has demonstrated that BK can activate and/or sensitize thin-fiber afferents to mechanical stimulation of numerous organs (6, 32, 33, 37, 39, 52, 62), suggesting that BK is engaged in enhancement of mechanoreceptor sensitivity. As a result, it is reasoned that elevated baseline levels of BK by femoral artery occlusion are likely to lead to sensitization of mechanoreceptors. Nevertheless, whether resting levels of BK in the muscle are greater in the ligated rats than that in the control rats needs to be studied to better clarify this issue.

With regard to amplified expression of B2 receptor in sensory nerves, NGF should be considered as an important player in regulating kinin B2 receptor (41). Our prior study demonstrates that 24 h of femoral ligation increases the levels of NGF in the DRG tissues (61), which is very likely to enhance expression of B2 receptors. Even though the consistent expression of B1 receptors in the DRG tissue was observed in control limbs and ligated limbs, there were no significant differences in B1 receptors in between groups in the present study. Consistently, B1 receptor did not appear to contribute to the exaggerated muscle mechanoreflex in rats with femoral artery occlusion. It is assumed that femoral artery occlusion-evoked NGF specifically modulates B2 receptors in the DRG but not B1 receptors.

Exercise decreases muscle pH of the exercising limb and increases venous level of lactic acid (43, 44). Of note, a prior study (49) has reported that the extent of the release of BK from contracting skeletal muscle was correlated with the magnitude of the decrease in venous pH and the increase in venous lactate. Thus it is speculated that the release of BK is likely to be more in active muscle, thereby leading to upregulation of B2 receptor if femoral artery occlusion induces interstitial pH to a less degree compared with controls. However, a previous study has demonstrated that there are no significant differences in resting levels of intramuscular pH in control limbs and ligated limbs of rats 12 h and 4, 7, and 14 days after the surgery (3). This result is consistent with findings in PAD patients, suggesting that muscle pH is not altered in symptomatic legs (13, 20). These data do not support the idea that B2 receptors in sensory nerves are upregulated via the greater releases of BK by a lower muscle pH in ligated rats.

**Limitations of the Study**

In rats, femoral artery ligation (i.e., 24–72 h) can cause an insufficient low flow directed to the hindlimb and this likely leads to localized muscle ischemia when muscle is active. In contrast, PAD is mainly due to atherosclerotic vascular disease and atherosclerosis develops over time in patients (5, 36, 38), and it is unlikely that acute ligation creates the same conditions. Thus the potential problems in translating this relatively acute rat model of PAD, to the human chronic PAD situation should be noted.

In summary, the data of this report support the hypothesis that kinin B2 receptor, but not B1 receptor, is overexpressed in the DRG tissues of rats after 24 h of femoral artery occlusion. Furthermore, specific inhibition of B2 receptors significantly attenuates the augmented SNA and BP responses to muscle stretch in occluded rats to a greater degree compared with control animals. In addition, the data demonstrate that inhibiting B1 receptors does not significantly affect the muscle stretch-induced SNA and BP responses in either experimental group, suggesting that kinin B1 receptor is unlikely to directly modulate the muscle mechanoreflex. Notably, the role of B2 and B1 receptors in sensitizing muscle mechanoreceptive afferents in a rat model of PAD is congruent with their expression in the sensory nerves. Therefore, the current study has identified kinin B2 receptor as a primary contributor to the over-reactive muscle mechanoreflex observed in PAD.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: J. Lu, J.X., and J. Li conception and design of research; J. Lu performed experiments; J. Lu and J. Li analyzed data; J. Lu, J.X., and J. Li interpreted results of experiments; J. Lu and J. Li prepared figures; J. Lu and J. Li drafted manuscript; J. Lu, J.X., and J. Li edited and revised manuscript; J. Lu, J.X., and J. Li approved final version of manuscript.

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