# Nerve growth factor in muscle afferent neurons of peripheral artery disease and autonomic function

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## Abstract

In peripheral artery disease patients, the blood supply directed to the lower limbs is reduced. This results in severe limb ischemia and thereby enhances pain sensitivity in lower limbs. The painful perception is induced and exaggerate during walking, and is relieved by rest. This symptom is termed by intermittent claudication. The limb ischemia also amplifies autonomic responses during exercise. In the process of pain and autonomic responses originating exercising muscle, a number of receptors in afferent nerves sense ischemic changes and send signals to the central nervous system leading to autonomic responses. This review integrates recent study results in terms of perspectives including how nerve growth factor affects muscle sensory nerve receptors in peripheral artery disease and thereby alters responses of sympathetic nerve activity and blood pressure to active muscle. For the sensory nerve receptors, we emphasize the role played by transient receptor potential vanilloid type 1, purinergic P2X purinoceptor 3 and acid sensing ion channel subtype 3 in amplified sympathetic nerve activity responses in peripheral artery disease.

**Key Words:** acid sensing ion channel subtype 3; exercise pressor reflex; muscle afferents; nerve growth factor; P2X purinoceptor 3; peripheral artery disease; transient receptor potential vanilloid type 1

## Introduction

During the skeletal muscle movement of exercise, sympathetic nervous activity (SNA) increases and this induces enhancement of responses in cardiovascular system including rising myocardial contractility and peripheral vasoconstriction (Victor et al., 1988). Then, the blood pressure (BP) and heart rate (HR) are accelerated as a result. An underneath mechanism, "Exercise Pressor Reflex" (EPR) (Mitchell et al., 1983) has long been considered to attribute to the above-mentioned sympathetic engagement during exercise. Initiating from the terminals of thin fiber afferent embedded in the contracting muscle, the autonomic reflex is evoked (Kaufman and Forster, 1996). As a consequence, the cardiovascular system responds to mechanical deformation and by-product metabolites generated from the contracting muscle (Kaufman and Forster, 1996). Two groups of muscle afferents, group III and group IV, are mainly responsive for detecting and transferring the mechanical deformation and by-product metabolites in the contracting muscle. Mechanoreceptors in group III afferents are predominantly responsible for the mechanical stimuli and mechanoreceptors in group IV afferents for the stimuli by the metabolites during the muscle contraction (Kaufman and Forster, 1996).

In patients with cardiovascular diseases, these reflex mechanisms are different with the healthy population in processing muscle signals via afferent nerve receptors (Sinoway and Li, 2005; Stone and Kaufman, 2015). In peripheral artery disease (PAD) patients, for instance, the SNA, BP and HR are exaggerated in responding to activation of the exercise pressor reflex (Baccelli et al., 1999; Bakke et al., 2007). PAD is one of the most prevalent atherosclerotic diseases that occur in the blood vessels of the lower limbs. Intermittent claudication is one of the most commonly seen clinical symptoms in PAD. Patients with that condition frequently suffer from pain and leg exertion during muscle movement. To relieve the onset of this symptom, patients need to take a break from the continuous physical activity.

A rat model of femoral artery ligation limits the blood supply in the affected lower limb and therefore has been widely applied to study the pathology and molecular mechanism of human PAD. With this model, a number of sensory nerve receptors, such as transient receptor potential vanilloid type 1 (TRPV1), acid sensing ion channel subtype 3 (ASIC3) and P2X purinoceptor 3 (P2X3), have been investigated and the results support their predominant role in up-regulating the SNA and BP responses to static muscle contraction in PAD (**Figure 1**).

## Search Strategy and Selection Criteria

Literatures in the present review were searched from computerized databases including PubMed, Medline, Scopus and Google Scholar. Selection range was original research and literature review papers published within the year of 1980 to 2020. The publication language was limited to English. Search terms included 'nerve growth factor', 'peripheral artery disease', 'femoral artery occlusion', 'peripheral artery occlusion', 'peripheral vascular disease', 'exercise pressor reflex', 'autonomic nerve activity', 'acid sensing ion channel subtype 3', 'muscle afferents', 'P2X purinoceptor 3', 'transient receptor potential vanilloid type 1' and 'hypoxia-inducible factor 1-alpha'. Papers were appraised for the suitability,

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relevance and trustworthiness by the authors. Each original research paper identified clear rationale for the study with a specific research purpose. Data were organized and supported the conclusion. Other than the representative works to support the theoretical background, we specifically focused on the papers published from 2015 to 2020. As a result, a total number of 59 papers were involved in the present reference list and 26 of them were published within 2015 to 2020.

## Sensory Nerve Receptors in Ischemic Muscle of Peripheral Artery Disease

Sensory nerve receptors are essential for the detection of peripheral signals and therefore a vital part during the occurrence of sensory responses such as pain and EPR (Queme et al., 2017). In this review, we focus on discussing the roles of TRPV1, P2X, and ASICs receptors on muscle sensory nerves under the lower limb ischemia situation of PAD (**Figure 1**). Moreover, it is interesting to find from the previous studies that there are several interactions between ASIC and TRPV1, as well as with P2X receptors. In addition, it should be noted that other muscle afferent receptors engaged in the exaggerated EPR of PAD have been also reviewed (Stone and Kaufman, 2015).

### Transient receptor potential vanilloid type 1

The TRPV1 is the receptor for capsaicin, which is a pepper compound and induces the excitability of the neurons (Frias and Merighi, 2016; Jardin et al., 2017). It is predominantly expressed in the metabolite sensitive peripheral thin Aδ and C fiber nerves (groups III and IV) and central sensory nerves in processing afferent signals. The consequences of the TRPV1 stimulation partly depend on its location. For instance, the capsaicin evokes a chemoreflex via the activation of TRPV1 in the C fibers of the pulmonary system (Orr et al., 2017). The activation of TRPV1 receptors in sympathetic nerve system evokes a sympathoexcitatory reflex (Lowin and Straub, 2015; Uchida et al., 2017). To abolish the activity of TRPV1, capsazepine is one of the effective antagonists to attenuate capsaicin-induced TRPV1 activation (Caterina et al., 1997). The afferent type capsazepine effects can be specified to C fiber *in vitro* and *in vivo* (Frias and Merighi, 2016). In addition, inflammation-associated metabolites (lactic acid, $H^+$) activate C fiber afferents with a similar manner as capsaicin (Diaz-Franulic et al., 2016).

Abundant amount of animal studies has shown the role of activated TPRV1 in regulating BP and HR. The BP is raised by 20% following the injection of capsaicin in the arterial supply of the dog hindlimb. When the afferent nerves are dissected, the above-mentioned effect is abolished. Both groups III and IV fibers are responsible for this muscle pressor response since 71% of group IV and 26% of group III muscle afferents are activated when capsaicin is injected (Kaufman et al., 1982). A similar BP response is also observed in rats following the injection of capsaicin in the hindlimb muscle. The effect is mediated via the TRPV1 receptors on muscle sensory afferents (Li et al., 2004; Xing et al., 2008).

Consistent with those previous findings, the SNA and pressor responses are mediated by TRPV1 via a reflex mechanism, and the responses are exaggerated in PAD rats, which are induced by the femoral artery occlusion. This suggests that the sensitivity of TRPV1 receptors is enhanced during the skeletal muscle ischemia condition (Xing et al., 2008). Moreover, evidence has also been reported that: femoral artery occlusion induces upregulation of TRPV1 expression in the dorsal root ganglion (DRG) neurons; and the capsaicin-evoked currents in the isolated DRG neuron are enhanced in rats with the arterial occlusion. Therefore, it can be assumed that the TRPV1 alternation in PAD contributes to the exaggerated sympathetically mediated vasoconstriction, which subsequently leads to decreasing muscle blood flow in PAD. However, in the PAD model with femoral artery occlusion, the usage of TRPV1 blockade does not significantly attenuate the augment BP response to static contraction in hindlimb (Tsuchimochi et al., 2010), which is consistent with the recent findings suggesting that in healthy rats blocking of TRPV1 fails to reduce the exercise pressor reflex (Ducrocq et al., 2019). The TRPV1-induced reflex response is speculated to require a relatively higher $H^+$ (lower pH) than that normally observed in the muscle interstitium during the skeletal muscle ischemia condition. The TRPV1 may therefore not be effectively activated in situations without acidosis.

### P2X purinoceptor 3

The purinergic P2X family is the receptors responsible for ATP and some analogues of ATP can also stimulate P2X3 in the afferent nerves (Burnstock, 2016, 2017). Specifically, the increased ATP in the hindlimb muscles induces the enhancement of blood pressure (Hanna et al., 2002; Li and Sinoway, 2002). In addition, the ATP stimulates mechanoreceptors on muscle afferents' P2Xs and therefore enhances subsequent cardiovascular responses (Li and Sinoway, 2002). The P2X3 receptor is ionotropic and mainly located on primary sensory neurons in processing numerous sensory signals. The primary sensory nerves of groups III and IV muscle afferents mediate an increase in BP after arterial injection of α,β-me ATP, analogues of ATP stimulating P2X3 and P2X2/P2X3 receptors (Hanna and Kaufman, 2004).

It has been demonstrated that the muscle contraction enhances the muscle interstitial ATP levels in both human subjects and animals. Following the occlusion of the blood supply, the ATP is likely to accumulate to a larger degree in the ischemic skeletal muscle than the normal condition. As a result, the higher ATP levels in the muscle interstitium induce the upregulation of P2X receptors on thin fiber afferent nerves and therefore exaggerate the P2X mediated-SNA response. On top of this, following work shows that increases of P2X3 receptors in DRG neurons induced by the femoral artery occlusion lead to the enhanced reflex response to P2X3 stimulation (Liu et al., 2011). The findings of this study indicate that there is a close linkage between the increased P2X3 receptors on muscle sensory nerves and amplified sympathetic response under the condition of PAD. Additional experiments show that, compared with control animals, the peak amplitudes of currents with activation of P2X3 receptors are amplified in DRG neurons of PAD rats (Xing et al., 2013).

### Acid sensing ion channel subtype 3

Acid-sensing ion channels (ASICs) are a group of amiloride-sensitive sodium channels that are expressed in the afferent neurons (De Logu and Geppetti, 2019). They are specifically presented in the nervous system of mammalian species. In general, there are a total amount of six different proteins (ASIC1a, 1b, 2a, 2b, 3 and 4) involved in the ASICs family. Among them, the ASIC3 protein is sensitive to the pH fluctuation and predominantly located in the primary sensory neuron of DRG. In exercising and/or ischemic muscles, the pH value drops and the proton concentration elevates. Once the pH value drops below a certain level, e.g., 6.5–7.0 (Deval et al., 2010), the ASIC3 is activated and therefore contributes to the augmented sympathetic nerve and cardiovascular activity in response to the exercise and/or muscle ischemia condition.

By employing a PAD rat model with femoral artery occlusion, it has been found that the hindlimb skeletal muscle ischemia induces the greater cardiovascular responses to static muscle contraction than in the control group without ischemia insult (Liu et al., 2010). Further studies have shown that the specific blockage of ASIC3 or ASIC1a significantly alleviates thus the amplified reflex pressor response in PAD rats, while there is a modest effect in the control rats without ischemia

condition (Stone and Kaufman, 2015; Kim et al., 2019; Ducrocq et al., 2020). Moreover, it is noted that there are also an up-regulated ASIC3 protein expression and amplified ASIC currents in DRG neurons of PAD rats (Liu et al., 2010; Xing et al., 2012a). This result is consistent with what has been found in a rat model of forelimb ischemia-reperfusion (IR). In this model, the group III and IV muscle afferent is also sensitized, which is accompanied with the enhancement in mRNA expression of ASIC3 and ASIC1. Additionally, in our PAD model with femoral artery occlusion, the stimulation of ASIC3 in muscle afferent nerves by the infusion of lactic acid into the arterial blood supply of hindlimb muscles increases SNA and BP to a greater degree in the PAD rats than the control rats (Liu et al., 2010).

In utilizing the method of whole-cell patch clamp, it has been observed that ASIC3 represents the majority of acid-induced currents in the DRG neurons with nerve endings in the hindlimb muscles with pH under exercise or ischemia situation (Xing et al., 2012b). Additionally, due to the deficiency of blood flow supply, a greater current response with activation of ASIC3 is observed in PAD rats (Xing et al., 2012b). Apart from the pH value, the ASIC3 plays a mediating role during the inflammation-modulated EPR exaggeration. IL-1β, one of the pro-inflammatory cytokines contributing to the sensitization of group III and IV muscle afferent in ischemia situation (Ross et al., 2018a), is postulated to have its effect via up-regulating the expression of ASIC3 in the DRG neurons.

**Interaction between acid sensing ion channel subtype 3 and transient receptor potential vanilloid type 1**

It has been demonstrated that the proton ($H^+$) evokes reflex muscle responses via the stimulation of ASICs including ASIC3 but not TRPV1 (Li et al., 2004). $H^+$ evokes a pressor response, which is attenuated by amiloride, an ASIC blocker, but not by blocking the capsaicin. In addition, by using the resiniferatoxin (RTX) to abolish the muscle afferents containing TRPV1 receptors, both capsaicin and $H^+$-induced responses are blunted (Li et al., 2004). This suggests the probability that ASIC3 is co-localized with TRPV1 receptors on afferent neurons. Another work has also shown that the responsiveness of acidosis and capsaicin is sensitized by each other. This suggests the potential co-existence of ASIC3 and TRPV1 in the DRG neurons (Xing et al., 2008). Another report suggests that TRPV1 and ASIC3 play a coordinated and interactive role in processing muscle afferent response to acid phosphate (Gao et al., 2006). By simultaneously blunting the TRPV1 and ASIC, there is a greater degree of attenuation to the acid phosphate-induced pressor response than the situations with two blockers that are separately administrated (Gao et al., 2006). What is more, BP response to stimulation of TRPV1 receptors is augmented via ASICs during the environment with lower pH (Gao et al., 2007). A recent study further shows that inorganic phosphate potentiates the BP response to acidic stimuli in rats (Ducrocq and Kaufman, 2020).

**Interaction between acid sensing ion channel subtype 3 and P2X receptors**

Previous studies have demonstrated a synergetic effect of acid and ATP in cell culture DRG neurons (Light et al., 2008), as well as in the EPR responses and pain behavior during the ischemia situation (Hayes et al., 2008). The first study conducted by Birdsong et al. (2010) demonstrates the molecular mechanism on the interaction of P2Xs and ASIC3. It suggests that the ATP acts by binding to P2X5 in the sensory neuron and then forms a molecular complex with ASICs. This conclusion is supported by the co-expression of P2X5 and ASIC3 in the DRG neurons, and the evidence that the ASICs current sensitized by ATP remains and sustains high even after removal the ATP. In one of the latest studies, Stephan et al. (2018) reveal that the ASIC3 and P2X3 are not only co-expressed in the rat DRG neuron, but also that the activation of ASIC3 has a unidirectional effect on the P2X3 during activation of the P2X. These data suggest that the ASIC3 and P2X3 are spatially closed with each other and possibly form a cognate receptor during regulation of the sensory neuron activity.

## Nerve Growth Factor Regulates Sensory Nerve Receptors in Ischemic Muscle of Peripheral Artery Disease

Femoral artery occlusion increases the levels of NGF in both the hindlimb muscles and DRG neurons of rats (Xing et al., 2012a). Interestingly, the P2X3 and the ASIC3 have been found to be simultaneously enhanced following treatment of nerve growth factor (NGF) in the DRG neurons (Stephan et al., 2018). Overall, our general notion is that 1) there are increasing protein expression of TRPV1, P2X3 and ASIC3 in DRG and the subsequent amplification of responses following their stimulation in PAD, 2) NGF induces augmented SNA and BP responses via enhancing the expression of the metabolic receptors such as TRPV1, P2X3 and ASIC3 in thin C-fiber afferent neurons (**Figure 1**). A prior study has further tested the hypothesis that femoral artery occlusion increases the levels of sensory nerves' hypoxia-inducible facor-1α (HIF-1α) and augments autonomic responses induced by activation of muscle afferent nerves (Gao and Li, 2013). This is interesting because published work indicates that HIF-1α is likely to regulate the role of NGF.

To perform those studies, several methods have been employed. Specifically, 1) The osmotic minipump used to infuse NGF into the hindlimb muscles; 2) NGF antibody (NGF-Ab) pre-treated to neutralize the effects of NGF in PAD rats; 3) the western blot assay used to examine the protein expression in afferent nerve receptors; 4) the dual immunofluorescence employed to differentiate the DRG neurons from C-fiber or A-fiber; and 5) the whole cell patch clamp performed to investigate the DRG neurons function in terms of TRPV1, P2X3 and ASIC3 currents. In addition, static muscle contraction is designed to evoke two components of exercise pressor reflex: mechano- and metabo-receptors, while the muscle mechanoreceptor is solely activated by the passive muscle stretch. In addition, capsaicin, α,β-me ATP and lactic acid are injected into the arterial blood supply of the hindlimb muscles to stimulate respective muscle metaboreceptors. Thus, both of the separated and combined effects of those components on *in vivo* SNA and BP responses have been determined.

**Nerve growth factor alters current response of dorsal root ganglion neurons to activation of transient receptor potential vanilloid type 1 in distinct phenotypes**

Based on the neurochemical characteristics and responsiveness to the neurotrophic factor, the thin fiber afferent nerves (neurons) are categorized into two types: one class of neurons without the expression of surface carbohydrates binding the plant lectin isolectin B4 (IB4), called IB4-negative afferents; and the other one with the expression of carbohydrates binding with IB4, called IB4-positive afferents. The IB4-negative neurons contain neuropeptides (e.g., calcitonin gene-related peptide and substance P) and express trkA, which is considered as NGF receptors. Thus, their neural survival during postnatal development depends on NGF (Denk et al., 2017; Hefti, 2020). On the other hand, the IB4-positive neurons express receptors for glial cell line-derived neurotrophic factor (GDNF), and are relatively "peptide poor" but express a surface carbohydrate group that binds IB4 (Denk et al., 2017; Kashyap et al., 2018; Coelho et al., 2019). Similar with NGF, the GDNF is also essential for the survival during postnatal development. One of the recent studies suggests that the GDNF plays a dual role in the regulation of both pain sensitivity and cardiovascular responses (Queme et al., 2020).

Accordingly, the research question has been investigated that if the neuronal phenotype such as capsaicin-insensitive sensory neurons can be altered by the NGF. In consistent with our hypothesis, it is shown that there is an exaggerated response with activation of metabolite-sensitive TRPV1 receptors in IB4-positive, and-negative DRG neurons following the femoral artery occlusion (**Figure 2**) (Xing et al., 2009). Moreover, the magnitude of TRPV1 response to capsaicin in IB4-negative DRG neurons is enhanced following the NGF infusion in the skeletal muscle and the NGF addition in the isolated cultured DRG neurons, while the effect is not found in the IB4-positive DRG neurons (**Figure 2**). Therefore, evidence of this study provides answers to our previous mentioned research question by demonstrating that NGF plays a role in the augmented TRPV1 responses and the subsequent sympathetic activity during the condition of muscle ischemia or the blood supply insufficiency in PAD (Xing et al., 2009).

**Nerve growth factor alters blood pressure response to stimulation of P2X and acid sensing ion channel subtype 3**

Compared with the non-infused leg, there is a 1.39 fold increases in P2X3 protein of the DRGs observed following the micro-osmotic pump infusion of NGF into the hindlimb muscle of the healthy rats (**Figure 3**) (Liu et al., 2011). In addition, the pressor response to arterial injection of α, β-me ATP is also significantly enhanced following the NGF infusion (**Figure 3**). Meanwhile, the neutralization of NGF by NGF-Ab effectively attenuates exaggerated BP response induced by α, β-me ATP injection in PAD rats (**Figure 3**) (Liu et al., 2011). Thus, it can be concluded that there is a strong association between the increasing NGF and the upregulation of P2X3 expression in DRG neurons as well as with the ultimate augmentation of SNA and BP responses during the hindlimb ischemia.

The role of NGF on the reflex responses to ASICs activation was examined by injecting lactic into the arterial blood supply of the hindlimb muscles and the SNA and BP responses was recorded. It was also examined in the further experiment that whether this response could be attenuated following the pre-treatment of NGF-Ab in the hindlimb muscles in PAD (Lu et al., 2012). The results show that NGF neutralization significantly attenuates femoral artery occlusion-induced amplified SNA and BP responses evoked by both static contraction and lactic acid, while this effect has not been found following the passive muscle stretch. As a result, it can be concluded that the increased NGF in the skeletal muscle is attributed to the augmented SNA and BP responses in PAD. And this effect is postulated via the stimulation of the chemically sensitive muscle afferent nerves rather than the mechanically sensitive ones (Lu et al., 2012). Meanwhile, the engagement of NGF has also been examined in the process of regulating the ASIC3 in sensory nerves in hindlimb ischemia. Results indicate that the infusion of NGF into the hindlimb muscles significantly increases the ASIC3 protein expression and the NGF-Ab significantly reduces the occlusion-increased ASIC3 protein expression in DRG tissues (**Figure 4**). In addition, in DRG neurons that project C-fiber afferents, there is a selective increase in ASIC3 expression. The evidence therefore supports the hypothesis that NGF plays a role in the up-regulation of ASIC3 expression in thin C-fiber afferent neurons. On top of this, the NGF induces an ASIC3-mediated muscle metaboreflex exaggeration. Based on the evidence of the study, it indicates that the distribution of DRG neurons in C-fiber and A-fiber can be altered by both the hindlimb ischemic condition of PAD and/or NGF intervention (Lu et al., 2012; Xing et al., 2013).

**Nerve growth factor linked to amplified hypoxia-inducible facor-1α in ischemic muscle of peripheral artery disease**

As one of the subunits in HIF-1 family, HIF-1α is sensitive to the change of tissue oxygen and thus its production can be rapidly stimulated during hypoxia to mediate the adaptive response in challenging condition (Balamurugan, 2016; Ivanova et al., 2019). The accumulation intracellular HIF-1α is associated with the expression of several target genes and stabilization of the subsequently produced proteins in protection tissues from damaging by the ischemic insult and infarction process (Pezzuto and Carico, 2018; Zhang et al., 2018; Encinas et al., 2019). Thus, in our previous study, we have examined if there is an increasing HIF-1α expression in sensory neurons of PAD; and if the HIF-1α is participated in the process of the cardiovascular responses enhancement, which is induced by the muscle afferent activation (Gao and Li, 2013).

By using western blot assay, the results from one of our previous studies suggest that, compared with the freely perfused control rats, there is a significantly increased HIF-1α protein expression in DRG neurons of PAD rats (Gao and Li, 2013). It has also been examined whether the intramuscular injection of DMOG (Gao and Li, 2013), which is a prolyl hydroxylase inhibitor for the stabilization or increment of HIF-1α (Milkiewicz et al., 2004), can alter the HIF-1α protein expression in DRG neurons. In consistence with our speculation, compared with control group, the HIF-1α protein expression is significantly increased in lumbar DRG neurons of DMOG injection group. Meanwhile, we examined the role of HIF-1α during the reflex cardiovascular responses evoked by muscle afferent nerves activation. First of all, the arterial BP response in PAD rats with femoral artery occlusion is significantly higher than that in control rats. Interestingly, the injection of DMOG in the control rats does not induce significant difference in the BP and HR responses induced by static muscle contraction. However, when the HIF-1α is inhibited by BAY 87-2243, the exaggerated BP response in PAD rats is significantly attenuated (Gao and Li, 2013). This indicates that the increment of HIF-1α in the DRG neuron may not have an effect on the cardiovascular reflex response. The treatment strategy of HIF-1α inhibition is likely more effective on PAD population in terms of attenuating the EPR responses.

With regard to the relationship between NGF and HIF-1α, there is a strong similarity in terms of the time-course of elevated NGF expression and increased HIF-1α in PAD rats with femoral artery occlusion. This indicates that there is a potential association between NGF and HIF-1α responses in the DRG neurons under the condition of hindlimb skeletal muscle ischemia in PAD. Of interest, it has been reported that neuronal death induced by the deprivation of NGF could be attenuated by addition of prolyl hydroxylases inhibitors (e.g., DMOG) into the cell culture medium with NGF withdrawal. This suggests that the HIF-1α may play a role in regulating the effects of NGF on the neurons (Lomb et al., 2007). Thus, it can be postulated that HIF-1α in the DRG is likely to mediate the NGF-induced augmented muscle metabolic responses after the limb ischemia in PAD.

## Future Perspective

Ischemia induced by the decreasing blood supply in the lower limb muscle, and resultant intermittent claudication is one of the most common symptoms in PAD and it onsets during exercise or daily physical activity, but can be promptly relieved by rest. Since the blood supply to skeletal muscle tissues during exercise is predominantly regulated by the sympathetic nerve system, the major goal of our previous studies has been to determine the role of metabolite sensitive receptors on muscle afferent neurons in regulating the SNA response after limiting blood flow to hindlimb muscle, which is the major etiology characteristic of PAD. In particular, it is more important to understand the molecular mechanisms by which afferent nerves (neurons) affect the symptoms observed in human PAD through the exercise pressor reflex arc. It will be also interesting to study this topic in both sex groups as females and males may possess different level sensitivity to peripheral mechanical and thermal stimuli (Ross et al., 2018b). With this regard, the NGF may also act differently between two

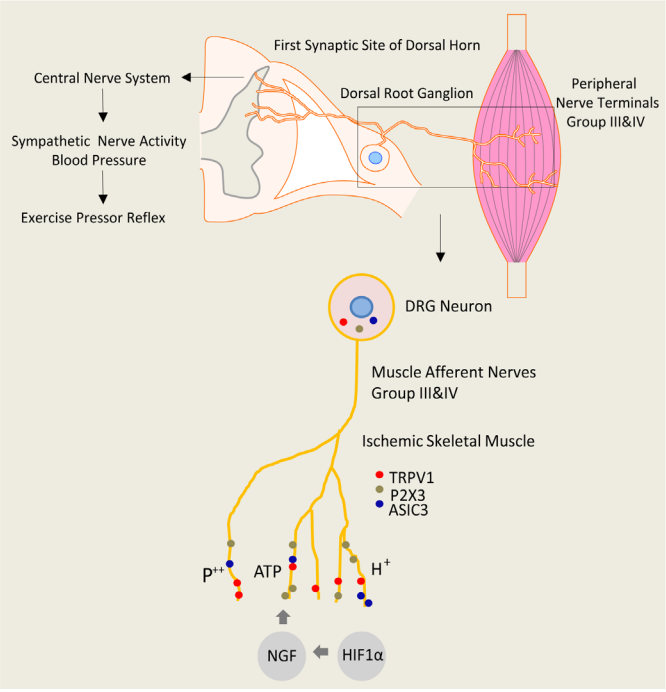


**Figure 1 | Potential molecular mechanisms for the augmented exercise pressor reflex of PAD.**
The major muscle by-products, e.g., acid phosphate, ATP and $H^+$ (lactic acid), are increased in the interstitial space of the exercising muscle. As the receptors for those metabolic products, TRPV1, P2X3 and ASIC3 receptors are sensitized and mediate the muscle reflex responses induced by the metabolites, which likely leads to amplified sympathetic and cardiovascular responses in PAD during exercise. NGF contributes to the enhanced expression and response of TRPV1, P2X3 and ASIC3 and therefore modulates the exaggeration of the exercise pressor reflex in PAD. In addition, ischemic insult induced by the limb ischemia increases the levels of HIF-1α in sensory nerves and HIF-1α likely plays a regulatory role in effects of NGF. Note that in our studies, the animals were under the anesthesia during the experiment. The laminectomy along with a decerebration was performed. The muscle contraction (static exercise) was induced by stimulating the lumbar 4–5 ventral roots to activate both metabo- and mechano-receptors in muscle afferent nerves. Meanwhile, the passive tendon stretch was also utilized to stimulate the mechano-receptors. This process mimics the muscle contraction and stretch during the exercise and activates the peripheral afferent components of the exercise pressor reflex. More importantly, this procedure minimizes the central command effects from the central nerve system, which is unlikely to be performed in human exercise studies. ASIC3: Acid-sensing ion channel 3; ATP: adenosine triphosphate; HIF-1α: hypoxia-inducible facor-1α; NGF: nerve growth factor; P2X3: P2X purinoceptor 3; PAD: peripheral artery disease; TRPV1: transient receptor potential cation channel subfamily V member 1.

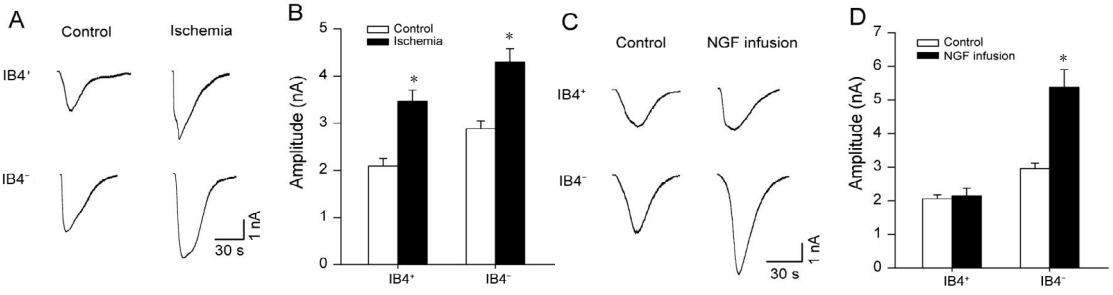


**Figure 2 | Capsaicin-induced currents in response to hindlimb ischemia or 72 hours' NGF infusion on in $IB_4$-positive and IB4-negative DRG neurons.**
(A) Original traces of DRG neuron response to 1 μM capsaicin in control and 24 hours-arterial occlusion. (B) Compared with controls, the 24 hours' femoral artery occlusion enhanced the amplitude of inward currents in both IB4-positive and IB4-negative DRG neurons. (C) Original traces of DRG neuron response to 1 μM capsaicin in the control and 72 hours-NGF infusion. (D) Compared with controls, 72 hours' NGF infusion amplified the peak amplitude of inward currents in IB4-negative DRG neurons but not in $IB_4$-positive DRG neurons. *$P$ < 0.05, *vs.* control group. $IB_4$-positive ($IB_4^+$) refers to neurons with the expression of surface carbohydrates binding the plant lectin isolectin B4 ($IB_4$); and IB4-negative ($IB_4^-$) refers to neurons without the expression of carbohydrates binding with $IB_4$. In (A) and (C), baseline was 0 before current response and the current recovered to baseline after the end of capsaicin application. DRG: Dorsal root ganglion. Reprinted with permission from Xing et al. (2009).

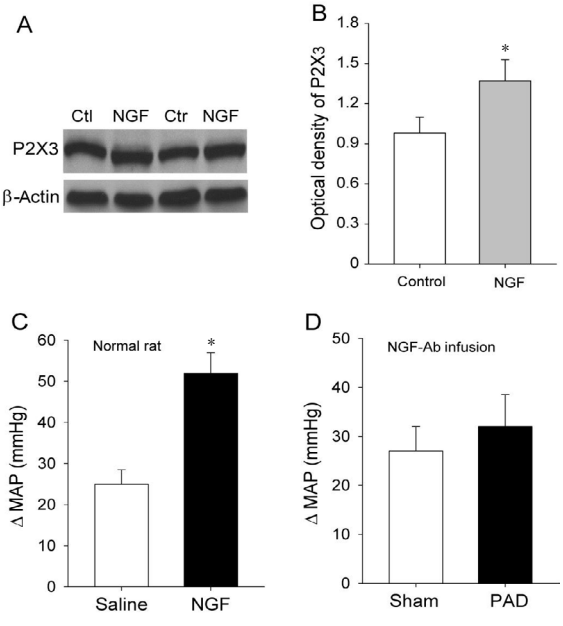


**Figure 3 | P2X3 proteins expression in DRG neurons response to the NGF infusion.**
NGF was continually delivered for 72 hours with a micro-osmotic pump and infused at a rate of 0.25 μg/h into the hindlimb of healthy rats. Saline served as control and was infused into the contralateral limb. Western blot assay was used to assess the P2X3 proteins in bilateral DRGs (L4–6) following the infusion of NGF or saline control. (A) Representative bands of P2X3 expression and β-actin are used for an equal protein loading control. (B) Optical density of P2X3 protein expression in both saline control and NGF infusion group. The optical density in each sample was normalized by the control sample in the respective gels. Data in histograms represent the mean ± SE. The sample size was six in each group. *$P$ < 0.05, *vs.* control group. (C) Blood pressure change in responsive to P2X stimulation following the NGF infusion. To stimulate the P2X receptors, α,β-methylene-ATP (0.125 mM) was injected into arterial blood supply of the muscles of control leg and experimental legs in six healthy rats. Blood pressure changes are presented as means ± SE. *$P$ < 0.05, *vs.* control group. (D) Pressor response to stimulation of P2X following the NGF neutralization in sham control and occluded rats. The injection of α,β-me-ATP (0.125 mM) induced no significant difference in pressor response in both legs. NGF neutralization was processed by injecting 10 μg of NGF-Ab into each leg 24 hours before the experiments. Blood pressure changes are presented as the mean ± SE. Reprinted with permission from Liu et al. (2011). Ctl and Ctr indicate repetitive control samples in the sample gel. DRG: Dorsal root ganglion; NGF: nerve growth factor; P2X3: P2X purinoceptor 3; PAD: peripheral arterial disease.

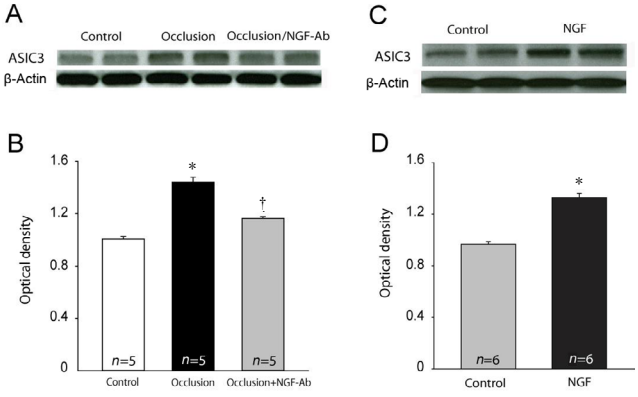


**Figure 4** | **NGF and ASIC3 protein expression.**
(A) Representative bands for ASIC3 expression in control limbs and occluded limbs following treatments with or without NGF-Ab infusion. β-Actin served as equal protein loading control. (B) Optical density of ASIC protein expression in groups with and without NGF-ab infusion. The optical density in each sample was normalized by the control sample in the respective gels. Data in histograms represent means ± SE. (C, D) Representative bands of ASIC3 expression and data of optical density: ASIC3 protein expression with and without NGF infusion. The sample size was six for each group. *$P < 0.05$, *vs*. control group; †$P < 0.05$, *vs*. occlusion group. Reprinted with permission from Lu et al. (2012). ASIC3: Acid-sensing ion channel 3; NGF: nerve growth factor.; NGF-Ab: NGF antibody.

sexes. This may be significant in making individual treatment strategies in patients. To approach to this, therefore, some experimental strategies should be addressed in the future. It may be necessary to perform the similar experiments in conscious animals, by which we can better examine the effect of a certain treatment that improves the exercise pressor reflex function on the claudication and/or exercise incapacity in PAD. Moreover, a restriction of the hindlimb blood flow is simply presented in a rat model of PAD used in our studies completed. Nevertheless, intermittent claudication in human PAD is generally caused by atherosclerotic vascular disease. An animal model that is more representative of human PAD still needs to be developed in the future.

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