P2X3 EXPRESSION IN DRG NEURON AND EFFECTS ON BLOOD PRESSURE RESPONSE FOLLOWING FEMORAL ARTERY OCCLUSION

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Spring 2012

A thesis submitted in partial fulfillment of the requirements for a baccalaureate degree in Accounting with honors in Biology

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ABSTRACT

During exercise, muscle contraction increases sympathetic nerve activity and arterial blood pressure via activation of cardiovascular regulating nuclei in central nerve system. This neural mechanism is termed “Exercise Pressor Reflex”. Femoral artery occlusion augments the sympathetic nerve and pressor responses to static exercise (muscle contraction) and muscle metabolites (which produced in exercising muscles) injected into the arterial blood supply of the hindlimb muscles in rats. The underlying mechanism by which these reflex responses are enhanced after muscle vascular insufficiency is unclear. Purinergic P2X3 receptor has been reported to contribute to the metabolic component of the exercise pressor reflex. It is well known that muscle thin fibers afferent nerves (i.e., group III and IV, also named as A- and C- fibers) mediate the exercise pressor reflex. Additional study shows that femoral artery occlusion increases the levels of P2X3 receptor proteins in sensory neurons-dorsal root ganglion (DRG) neurons. Thus, the purpose of this study was to examine if chronic femoral occlusion would alter the expression of P2X3 in DRG neurons that project C- and/or A- fibers in control rats and in occluded rats. Also, P2X3-mediated blood pressure response was examined after femoral occlusion. In addition, the role played by nerve growth factor (NGF) in regulating blood pressure response to stimulation of P2X3 was examined. The dual immunofluorescence techniques were used to examine co-localization of fluorescent P2X3 and peripherin/NF200 immunoreactivity in the DRG neurons of control rats and occluded rats. Note that peripherin and NF200 are used to label neurons with C-fiber and A-fiber, respectively. The results of this experiment show that P2X3 staining appears in C-fibers of DRG
neurons, but only few P2X3 staining appears in A-fibers of DRG neurons in both control and occlusion groups. Femoral artery occlusion increased expression of P2X3 in a large portion of DRG neurons with C-fiber compared with A-fiber of neurons. Furthermore, the results showed that responses of blood pressure to stimulation of P2X were greater in occluded rats than responses in control rats by injection of α, β-methylene ATP (α, β-me ATP, an agonist to P2X) into the arterial blood supply of the hindlimb muscle. In order to determine response of blood pressure to arterial injection of α, β-me ATP were mainly via P2X3, additional experiments were performed to give a specific antagonist to P2X3 (RO3) prior to α, β-me ATP and then the reflex muscle responses were observed. The data demonstrated that pretreatment of RO3 significantly attenuates blood pressure response evoked by α, β-me ATP in both control rats and occluded rats. Finally, infusion of NGF in the hindlimb muscles of healthy rats increased the pressor response to injection of α, β-me ATP. Likewise, blocking NGF attenuated exaggeration of the reflex response induced by α, β-me ATP in occluded rats. The findings of this study suggest that expression of P2X3 in primary afferent neurons projecting C-fibers are upregulated as the blood supply to the hindlimb is deficient under ischemic conditions, thereby leading to augmentation of the exercise pressor reflex. NGF is likely to be involved in increased reflex pressor response with P2X3 receptors activation observed in hindlimb muscle ischemia.

*Keywords:* P2X3 receptor, muscle afferents, peripheral artery disease, ATP, blood pressure, NGF
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SPECIFIC CONTRIBUTIONS TO RESEARCH

Part of the data used in this thesis was taken from the collaborative work completed in the laboratory at the Penn State Heart & Vascular Institute at Hershey. The collaborating investigators and technical assistant include Drs. Jiahao Liu, Jian Lu, Jihong Xing and Jianhua Li and Ms. Chunying Yang. The general roles played by them and my specific role are summarized as follow.

J. Liu (postdoctoral fellow) made contributions to the western blot experiments and a part of the immunocytochemical study including data collection and analysis and making graphics, and drafting paper. J. Lu (postdoctoral fellow) made contributions to the ELISA methods measuring the levels of nerve growth factor in this study including data collection and analysis, making graphics and drafting paper using his methods. J. Xing (research associate) contributed to experimental designs of the ELISA and immunocytochemical experiments. C. Yang (research technician) contributed to animal preparations and animal care, drug and chemical solution preparations and performing some part of experiments (renal sympathetic nerve recording and subcutaneous insertion of a micro-osmotic pump). J. Li (principal investigator) was responsible for overall administration and overseeing the entire experiments.

My specific role in this project was to perform surgical procedures (tracheotomies, muscle stretch, vessel catheterization, and femoral artery ligation) and experimental protocols to collect cardiovascular data used in the publication and thesis. My additional contributions include: assisting in the design of the cardiovascular experiments and protocols necessary for identifying C- and A-fiber pathways, and role
of RO3 and nerve growth factor in femoral occluded rats. I also participated in
immunocytochemical preparations, data analyses, making graphics of cardiovascular
and immunocytochemical experiments, and drafting and editing the manuscript for
publication.

*** It should be noted that while a portion of the data discussed in this thesis has been
published as a peer-reviewed paper in American Journal of Physiology Heart and
Circulatory Physiology: 301.3 (September 2011), H1070-H1079, the rest of the data is
being prepared for publication.
ACKNOWLEDGEMENTS

I would like to extend my deepest gratitude to Dr. James Marden for his guidance and patience in acting as my advisor and supervisor.

Also, I would like to thank the Schreyer Honors College for giving me the motivation to always strive for excellence.

Additionally, I would like to thank The Heart & Vascular Institute of the Penn State College of Medicine for giving me the opportunity to both take part and contribute to the study presented.

Lastly, I would like to thank Drs. J. Liu, J. Lu, J. Xing, J. Li and Ms. Chunying Yang for their collaborating and assistance in making this study possible.
LIST OF ABBREVIATIONS

α,β-me-ATP: α, β-methylene-ATP

BP: blood pressure

FITC: fluorescein isothiocyanate

DRG: dorsal root ganglion

MAP: mean arterial pressure

NGF: nerve growth factor

NGF-Ab: NGF-antibody

PAD: peripheral arterial disease
Imagine a life where every step you take causes great pain to your legs. Imagine a life where you cannot walk for more than a few minutes without feeling the need to sit down because your legs can no longer support you. Imagine a life where the pain you suffer is so great, it begins to disrupt your sleep.

For over 20 percent of adults over the age of 55 (Campenot et al., 2004b; Ouriel, 2001; Rejeski et al., 2008), they do not have to imagine the life described above. They suffer from peripheral arterial disease (PAD) and the pain they feel is very real. Like other arterial diseases, PAD is caused by narrowed and blocked arteries due to atherosclerosis, which limits the blood supply to the muscle and organs surrounding the affected arteries (Aslam et al., 2009; Campenot et al., 2004b). While advanced cases of PAD can causes damage the legs, arms and vitals organs such as the kidneys, the first clinical sign of PAD is intermittent claudication during exercise (Rejeski et al., 2008). Additionally, previous studies have shown that PAD patients’ autonomic responses are enhanced during exercise and (Baccelli et al., 1999; Bakke et al., 2007) that the exercise pressor reflex, the neural central mechanism responsible for cardiovascular response to exercise (Mitchell et al., 1983), is altered in cardiovascular diseases.

Taking these facts into consideration, the effect of PAD on the exercise pressor reflex was an area of interest in this study. In particular, the Group III and IV muscle afferents, A- and C-fibers, respectively, of the exercise pressor reflex was an area of focus. When engaging in exercise, Group III and IV muscle afferents are stimulated and causes increases in arterial blood pressure (BP) and heart rate (Mitchell et al., 1983). It
has bas been suggested that Group III afferents, A-fibers, are mechanosensitive and Group IV afferents, C-fibers, are metabosensitive (Kaufman *et al.*, 1996; Kaufman *et al.*, 1983; Kaufman *et al.*, 1984a; Kaufman *et al.*, 1984b).

In a previous study using a rat model to mimic intermittent claudication in human PAD (Waters *et al.*, 2004), it was shown that the reflex sympathetic nerve and pressor response to stimulation of metabolically sensitive receptors were greater in rats that underwent femoral artery occlusion (Liu *et al.*, 2010; Tsuchimochi *et al.*, 2010; Xing *et al.*, 2008), suggesting that the metabolite receptors contributed to greater responses. Furthermore, the most recent study on the subject used western blot analysis to show that femoral artery occlusion increases the levels of P2X$_3$ receptor protein in primary afferent neurons/dorsal root ganglion (DRG) neurons (Liu *et al.*, 2011). Consequently, this is likely to lead to the enhanced reflex responses to stimulation of P2X$_3$. Knowing this, it was hypothesized that femoral artery occlusion increases expression of P2X$_3$ receptors in DRG neurons that project C-fibers, the metabolic component of the exercise pressor reflex.

Additionally, the collaborating study showed that blood pressure response to injections of α,β-me ATP into the arterial blood supply of the hindlimb muscles was amplified in occluded rats compared with control rats. α,β-me ATP stimulates P2X subtypes 1-7 (P2X1-7) (Burnstock, 1999; Burnstock, 2001; Burnstock *et al.*, 2004). In order to determine that response of blood pressure to arterial injection of α,β-me ATP were mainly via P2X$_3$, additional experiments were conducted to observe the reflex muscle responses in the current study by pretreatment of a specific antagonist to P2X$_3$ (RO3) prior to α,β-me ATP.
Lastly, prior studies have shown that femoral artery occlusion increases the level of nerve growth factor (NGF) (Xing et al., 2009), a secreted protein that is important for the growth and survival of nerve cells (Campenot et al., 2004b; Wu et al., 2009). Furthermore, it has also been shown that NGF evokes P2X receptor expression in the DRG neurons (Ramer et al., 2001). Due to these facts, it was hypothesized that NGF plays a role in regulating P2X$_3$ response after femoral occlusion.
CHAPTER 2 – METHODS

To determine the P2X$_3$ expression in DRG neurons and effects on blood pressure response in models with femoral artery occlusion, two protocols were used. The first protocol made use of rat models with femoral artery ligation; this protocol will be described in the sections labeled: femoral artery occlusion, fluorescence immunohistochemistry and examination of reflex cardiovascular responses. The second of which consisted of the direct infusion of NGF/NGF-antibody into the hindlimb muscles of healthy rat and occluded rat models; this protocol will be described in the sections labeled: NGF administration, and examination of reflex cardiovascular responses. Additionally, 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 guidelines.

Femoral artery ligation

Previous studies have shown that occlusion of the femoral artery of rat models induces hindlimb vascular insufficiency and muscle ischemia that mimic the symptoms found in PAD patients (Waters et al., 2004). Male Sprague-Dawley rats from the ages of 5-7 weeks were used for femoral artery ligation. After the subject was anesthetized by inhalation of an isoflurane-oxygen mixture, the femoral artery of a randomly chosen hindlimb was surgically exposed, separated from surrounding tissue, and ligated with a suture approximately 3mm away from the inguinal ligament; this limb became the experimental group. To establish the control group, the same procedure was done to the
opposite leg of the subject except the femoral artery ligation; however, a suture was still placed around the femoral artery but not tied.

Immediately after the procedure, subjects were given a 0.05mg/kg SC dose of buprenorphine hydrochloride; this was done every 12 hours until subjects either fully recovered or underwent further procedures. Additionally, subjects were given penicillin potassium twice daily and lactated ringers daily. Subjects had a 24-hour recovery period before reflex cardiovascular responses to ligation were examined.

Fluorescence immunochemistry

To determine the effect of femoral artery occlusion on the level of P2X₃, fluorescence immunostaining procedures were used. After undergoing femoral occlusion procedures, the subject was anesthetized with an isoflurane-oxygen mixture and transcardially perfused sequentially with 200 ml of ice-cold saline containing 1,000 units heparin, 500 ml of 4% freshly prepared ice-cold paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), and 200 ml of 10% sucrose so to preserve targeted proteins. Upon completion of perfusion, the L4-6 DRGs of control and occluded limbs were removed and fixed in 4% paraformaldehyde for 2 hours and stored in 30% sucrose overnight. The processed DRGs were cut into 10 µm of sections using Tissue-Tek.

Each section was then fixed in 4% paraformaldehyde for 10 minutes, permeabilized and blocked with 0.3% Triton X-100 in PBS supplemented with 5% goat serum for 1 hour. Following this, the sections were incubated with rabbit anti-P2X₃ primary antibody overnight at 4°C. After being thoroughly washed in PBS, the sections were incubated with goat anti-rabbit secondary antibody conjugated to fluorescein
isothiocyanate (FITC) for 2 hours at room temperature. The sections were coverslipped and then examined under a fluorescence microscope.

Furthermore, to examine localization of P2X\textsubscript{3} within C-fiber and A-fiber DRG neurons, the sections were incubated with the second primary antibody, mouse anti-peripherin and anti-NF200, overnight. Peripherin and NF200 are used to label neurons with C-fiber and A-fiber, respectively. Briefly, after incubation, the sections were washed and incubated for 1 hour at room temperature with secondary antibody, Alexa Fluor-594 conjugated goat anti-mouse IgM, for 2 hours at room temperature. Then, the sections were washed in PBS, and coverslipped. FITC- and Alexa Fluor-594-labeled DRG neurons were examined.

After all sections were processed for P2X\textsubscript{3} and peripherin/NF200, every fourth section was chosen for immunostaining analysis. Cells with >1.75 times of background intensity were considered to be positive for P2X\textsubscript{3}. The number of P2X\textsubscript{3} positive cells and the total cells was counted in each section followed by calculating the percentage of P2X\textsubscript{3} positive cells vs. total cells. Additionally, to determine the frequency of doubled-labeled cells, the number of total P2X\textsubscript{3} immunostaining and peripherin/NF200 positive neurons was counted in each section. Percentages of double-labeled neurons were calculated: total number of double-labeled cells $\times$ 100 / total number of peripherin/NF200 positive cells.

To minimize the possibility of counting a single DRG neuron more than once, DRG sections were collected on 5 glass slides in series, and tissues from only one of the slides were processed for immunocytochemical analysis.
NGF administration

NGF was administered using a micro-osmotic pump placed subcutaneously in the femoral triangle region with the outlet of the pump 2-3 mm distal to the inguinal ligament of one of the subject’s hindlimbs. The rate of delivery was 0.25µg/hour over 72 hours. The same procedure was performed on the opposite leg of the subjects but with saline instead of NGF; this was the control group.

Examination of reflex cardiovascular responses

Prior to the surgical procedures, subject’s weight was compared to pre-operative weight; a loss of 10 percent or higher eliminated the subject from the test group. Twenty-four hours after femoral artery ligation, subject was anesthetized with an isoflurane-oxygen mixture. While under anesthesia, subject’s right jugular vein and common carotid artery were cannulated to deliver fluids and to connect a pressure transducer for measuring arterial blood pressure, respectively. A catheter was then inserted into both the control and experimental femoral arteries to provide a pathway for the injection of drugs.

Following cannulation, decerebration was performed on the subject by removing all brain tissue above the brainstem. This was done in order to avoid any conflicting effects that the anesthesia might have on the reflex pressor response. After taking the subject off of anesthesia, a 60-minute recovery period was given before the start of the experiment.

To induce muscle pressor reflex in the subject, solutions of 0.125 mM α,β-me ATP were used. Each solution was administered to both the control and experimental
hindlimbs for duration of 1 minute at 20-minute intervals. The volume of solution injected ranged from 0.15-0.25mL depending on the subject’s weight. In order to confirm that the response was due to stimulation of the afferent nerves in the hindlimb, the sciatic nerve was severed after injecting all α,β-me ATP solutions for experiment. A solution of 0.125mM α,β-me ATP was then injected into the femoral artery and blood pressure response was observed.

Furthermore, to determine the specific P2X receptor responsible for response to α,β-me ATP, RO3, a specific antagonist to P2X3, was given prior to an injection of 0.125 mM of α,β-me ATP in both the control and ligated femoral arteries. Then, responses of blood pressure to arterial injection of α,β-me ATP were observed. Lastly, to examine the effects of NGF on pressor response to α,β-me ATP a group of healthy subjects were infused with NGF. To examine the effects of blocked NGF on pressor response, an additional group of subjects were injected with 10µg of NGF-antibody in both control and ligated hindlimbs 24 hours prior to experiments. A solution of 0.125 mM α,β-me ATP was then arterially injected to examine its effect on stimulation P2X.

**Data 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 ± standard error of the mean (SE). For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS for Windows version 15.0.
CHAPTER 3 - RESULTS

P2X₃ immunostaining in DRG neurons that project C- and A-fibers in control rats and occluded rats

Based on the previous findings, we examined the existence of P2X₃ was within the DRG neurons with C- and A-fibers (Figures 1&2). Dual immunofluorescence techniques were used to examine co-localization of fluorescent P2X₃ and peripherin/NF200 immunoreactivity in the DRG neurons of control rats and occluded rats. The appearance of P2X₃ and peripherin/NF200 within DRG neurons is characterized by fluorescent green and red color, respectively.

The photomicrographs show that P2X₃ staining appears in C- and A-fiber of DRG neurons in both control and occlusion groups, and that a large portion of DRG neurons with C-fiber was P2X₃ positive as compared with A-fiber of neurons. Figure 1 further shows that there is a higher amount of C-fiber that contains P2X₃ in occluded rats than in control rats. The percentage of double-labeled neurons with P2X₃ and peripherin was significantly greater in the occluded limbs than that in controls. They were 12 ± 3 % in controls (n=6) and 19 ± 5 % (P < 0.05 vs. control) in the ligation group (n=6) (Figure 3). Figure 2 also demonstrates that few A-fiber of DRG neurons include P2X₃ staining in both control and ligation groups. The percentage of double-labeled neurons with P2X₃ and NF200 was similar in both experimental groups. They were approximately 1 % in controls (n=6) and in the ligation group (n=6, P > 0.05 vs. control) (Figure 3). There were no significant differences in the number of peripherin and NF200-positive DRG neurons between the experimental groups.
Blood pressure responses to arterial injection of $\alpha$, $\beta$-me ATP following 24 hours of ischemia and NGF/NGF-antibody infusion

Figure 4 shows baseline values for mean arterial pressure (MAP) before arterial injections of $\alpha,\beta$-me ATP in control rats and in rats with 24 hours of femoral occlusion with various interventions. The baseline values for MAP and heart rate prior to injections of $\alpha,\beta$-me ATP were 96 ± 6 mmHg and 378 ± 12 beats/minute in control rats ($n = 8$) and 100 ± 9 mmHg and 388 ± 17 beats/minute in rats with 24 hours of femoral occlusion ($n = 9$). There were no significant differences in baseline MAP before each of injections.

Blood pressure response to $\alpha,\beta$-me ATP injected into the arterial blood supply of the hindlimb muscles was enhanced in occluded rats ($n=6$) compared with control rats ($n=8$) (Figure 5). The BP responses were examined after a prior injection of RO3, a specific antagonist to P2X$_3$ in eight control rats and six occluded rats (Figure 5). RO3 (10 mg/kg) injected into the femoral artery attenuated the pressor response evoked by $\alpha,\beta$-me ATP in six of control rats and five of occluded rats. Increases in MAP were 26 ± 6 mmHg in control and 43 ± 8 mmHg in occlusion without the prior RO3, and 10 ± 3 mmHg in control and 16 ± 5 mmHg in occlusion after RO3 ($P <0.05$, vs. without RO3 for both groups).

After infusing NGF into the hindlimb muscles of the subjects, the levels of NGF were 33.2±3.3 ng/g wet weight in control and 47.3±2.7 ng/g wet weight with NGF infusion (Liu et al., 2011). Figure 6 shows that upon injection of 0.125 mM of $\alpha,\beta$-me ATP into the femoral arteries of experimental and control legs of rats, there was a significant increase in BP response in NGF infused leg. Increases in MAP were 25 ± 3
mmHg in control legs and 52 ± 4 mmHg ($P < 0.05$, vs. control) in legs infused with NGF after injecting $\alpha,\beta$-me ATP.

Furthermore, when NGF-antibody was administered into the hindlimb muscles of both the control and occluded legs 24 hours prior to experiments, the effects of femoral occlusion was reduced upon injection of $\alpha,\beta$-me ATP at 0.125 mM (Figure 6). The hindlimb muscles of occluded legs treated with the NGF-antibody produced NGF levels of 34.5±2.7 ng/g wet weight compared to 49.5±4.6 ng/g wet weight without NGF-antibody (Liu et al., 2011). $\alpha,\beta$-me ATP induced no significant difference in BP response in control leg and occluded leg treated with NGF-antibody with an increase of 27 ± 5 mmHg in control legs and 32 ± 7 mmHg in occluded leg (Figure 6).
CHAPTER 4 –DISCUSSION

The purposes of the present study were to determine whether (1) femoral artery occlusion increases expression of P2X<sub>3</sub> receptors DRG neurons that project C-fibers, (2) a specific blockade of P2X<sub>3</sub> attenuates blood pressure response evoked by arterial injection of α,β-me ATP, and (3) NGF was engaged in the role of sensory nerves’ P2X<sub>3</sub> in augmented responses evoked by vascular occlusion of the hindlimb muscle.

First, fluorescence immunohistochemistry was employed to examine expression of P2X<sub>3</sub> in DRG neurons that project C- and A- fibers in control rats and rats with 24 hours of femoral artery ligation. The results of this experiment show that P2X<sub>3</sub> staining appears in C-fibers of DRG neurons, but only few P2X<sub>3</sub> staining appears in A-fibers of DRG neurons in both control and occlusion groups (shown in Figure 1-3). Second, the data demonstrated that pretreatment of RO3, a specific antagonist to P2X<sub>3</sub>, significantly attenuates blood pressure response evoked by α,β-me ATP in both control rats and occluded rats (shown in Figure 5). Third, infusion of NGF in the hindlimb muscles of healthy rats increased the pressor response to injection of α,β-me ATP (shown in Figure 6). Likewise, blocking NGF attenuated exaggeration of the reflex response induced by α,β-me ATP in occluded rats (shown in Figure 6).

Taken together, the findings of this study suggest that expression of P2X<sub>3</sub> in primary afferent neurons projecting C-fibers are upregulated as the blood supply to the hindlimb is deficient under ischemic conditions, thereby leading to augmentation of the exercise pressor reflex. It is also likely that NGF is engaged in amplified pressor response with stimulation of P2X<sub>3</sub> receptors observed in hindlimb muscle ischemia.
Before discussing the results any further, it should be noted that of the seven subtypes of P2X, six are found in sensory neurons with the exception being P2X7. Of these six subtypes, it has been shown that P2X3 had the greatest expression in sensory neurons with P2X3 mRNA expression being two times greater than the expression of P2X2 mRNA (Burnstock, 1999; Burnstock, 2001; Burnstock et al., 2004; Wirkner et al., 2007). It is because of the prominence of P2X3 expression and its role in the pressor reflex that it was the area of focus in the study.

Furthermore, it has been shown that P2X3 expression in DRG neurons is upregulated in the process of pathological responses (Ambalavanar et al., 2005; Banerjee et al., 2009; Xiang et al., 2008; Zhang et al., 2008). Therefore, in the collaborating study, a rat model of intermittent claudication induced by femoral artery ligation was used to examine the levels of P2X3 protein expression in the DRG tissues of occluded and control limbs (Liu et al., 2011). The data demonstrate that P2X3 was increased in DRG neurons of occluded limb 24 and 72 hours after the femoral ligation surgery (Liu et al., 2011). The findings of the present experiment further show that most DRG neurons with C-fiber appear P2X3 positive. Given that C-fiber muscle afferent nerves mediate reflex blood pressure response induced by muscle metabolic stimulation (Kaufman et al., 1996), the data of this experiment suggest that enhancement of P2X3 receptors in DRG neurons plays an important role in regulating the metaboreceptor component of the exercise pressor reflex.

In looking at the effects of blocking P2X3 on the BP response to α,β-me ATP, we saw that there was a significant decrease in cardiovascular response in both control and
ocluded rats injected with RO3, a specific antagonist to P2X\(_3\). This suggests that the P2X\(_3\) subtype plays a significant role in the pressor response.

Previous studies have already suggested that ATP elicits exercise pressor reflex by stimulating muscle afferents’ P2X. The three pieces of evidence that support this include the following. (1) Injection of \(\alpha,\beta\)-me ATP into the arterial blood supply of the hindlimb causes the stimulation of over two-thirds of the Group IV afferents in cats and rats (Hayes et al., 2008; Reinohl et al., 2003). (2) Stimulation of P2X receptors on muscle afferents increases blood pressure via a reflex mechanism (Hanna et al., 2002; Li et al., 2002). (3) Blocking P2X receptors attenuates discharge of group IV afferent fibers as well as blood pressure response during static muscle contraction (Hanna et al., 2004; Hanna et al., 2003; Kindig et al., 2007; McCord et al., 2010).

However, while P2X’s role in pressor reflex has been documented, the focus of this study was on the role of P2X\(_3\). Previous studies have shown that blocking P2X\(_{2/3}\) receptors by injecting A-317491 greatly attenuated the pressor response to arterial injection of \(\alpha,\beta\)-me ATP (McCord et al., 2010). The present study showed that upon blocking P2X\(_3\) via RO3 injection, the pressor response was significantly decreased, which emphasizes the role of P2X\(_3\) in causing greater pressor response in rats with femoral artery ligation.

In examining whether NGF was engaged in the role of sensory nerves’ P2X\(_3\) in augmented responses evoked by vascular occlusion of the hindlimb muscle, an NGF-antibody was employed to isolate the effect of NGF on rats with femoral occlusion. While previous studies have shown that hindlimb ischemia increases endogenous NGF production in skeletal muscle (Emanueli et al., 2002) and that exogenous application of
NGF leads to expression of P2X3 in sensory neurons (Ramer et al., 2001), it has not been established whether endogenous increases in NGF production would lead to P2X3 in sensory neurons. In addition, it has been documented that the level of NGF in DRG tissue of rats significantly increased following 24 and 48 hours of femoral occlusion (Xing et al., 2009). In the present study, after ligating the femoral artery of a rat model and establishing that the NGF level had increased, a NGF-antibody was used to neutralize the endogenous NGF. Upon doing this, the once amplified pressor response to α,β-me ATP in occluded rats was attenuated and had no significant difference to the pressor response of the control. This significant attenuation of pressor response suggests that the endogenous NGF caused by the femoral occlusion did in fact play a role in regulating P2X3 response.

In summary, the present study shows (1) femoral artery occlusion significantly increases expression C-fiber P2X3 in DRG neurons, suggesting that the reflex response is metabolically rooted. (2) Greater increases in arterial blood pressure in rats with femoral occlusion with α,β-me ATP are attenuated after specific blocking afferent’s P2X3 receptors with RO3. (3) NGF increases pressor response induced by stimulation of P2X3 and NGF-antibody attenuates the augmented response seen in occluded rats. In relation to PAD, these findings suggest that a focus on the metabolic role of P2X3 could be the key in addressing treatment options. Additionally, the findings also suggest that NGF is likely to be involved in increased reflex responses with activation of P2X3 receptors observed in hindlimb muscle ischemia.
REFERENCES


Examination of P2X3 within DRG Neurons with C-Fibers

Figure 1 - Co-localization of P2X3 and peripherin. Fluorescence immunohistochemistry was employed to examine double labeling of P2X3 and peripherin. In this experiment, peripherin was used to label DRG neurons that project thin C-fiber. Representative photomicrographs show that P2X3 (green color) and peripherin (red color) staining in DRG neurons of a control rat (top panel) and an occluded rat (bottom panel). Arrows indicate representative cells positive for both P2X3 and peripherin after they were merged. The number of double labeling DRG neurons is greater in occluded rats than that in control rats. Scale bar = 50 µm.
Figure 2 - Co-localization of P2X3 and NF200. Dual fluorescence immunohistochemistry was employed to examine labeling of P2X3 and NF200. Note that NF200 was used to identify A-fiber of DRG neurons. Photomicrographs are representative to illustrate staining of P2X3 (green color) and NF200 (red color) in DRG neurons of a control rat (top panel) and an occluded rat (bottom panel). Few DRG neurons including NF200 staining were seen to be P2X3 positive in control and occluded groups. Scale bar = 50 µm.
Figure 3. *Left panel.* Showing that the percentage of double labeling dorsal root ganglion (DRG) neurons for P2X3 and peripherin is greater in occluded rats than that in control rats. * P < 0.05 vs. control. Six animals were used in each group. *Right panel.* Showing that there are no differences observed in the number of double staining for P2X3 and NF200 in DRG neurons of control and occlusion groups. Note that no differences were observed in the number of peripherin and NF200 positive DRG neurons in control and occlusion groups. NS: no significant difference (P>0.05).
Figure 4. The data show baseline mean arterial pressure in different groups of experiments before injection of $\alpha, \beta$-me ATP. No significant differences were seen among groups.
Figure 5. Blood pressure response to α,β-me ATP injected into the arterial blood supply of the hindlimb muscles was examined and the response was enhanced in occluded rats (n=6) compared with control rats (n=8). Values are means ± SE. # indicates $P < 0.05$, vs. control. In addition, blood pressure response was examined after a prior injection of RO3, a specific antagonist to P2X$_3$. RO3 (10 mg/kg) attenuated the pressor response evoked by α,β-me ATP in six of control rats and five of occluded rats. Values are means ± SE. * indicates $P < 0.05$, vs. without RO3 for both groups.
Blood pressure response to $\alpha,\beta$-me-ATP after NGF infusion and NGF antibody

**Figure 6.** *Left panel:* Effects of NGF infusion on mean arterial pressure (MAP) response to stimulation of P2X. $\alpha,\beta$-me-ATP at 0.125 mM was injected into arterial blood supply of the muscles of control leg and experimental legs in six health rats. The control legs were infused with saline and experimental legs were infused with NGF (0.25µg/h for 72 h of continuous delivery) in the same rats. Values are means ± SE. *$P < 0.05$ compared with saline control.*

*Right panel:* Effects of NGF neutralization on MAP response to stimulation of P2X in sham control and occluded leg of eight rats. No significant difference in blood pressure response was seen in both legs after $\alpha,\beta$-me-ATP at 0.125 mM. 10µg of NGF-Antibody (NGF-Ab) was injected into each leg 24 h prior to experiments. Values are means ± SE. NS: no significant difference ($P > 0.05$).
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RESEARCH EXPERIENCE

2005-2007; 2009-2011 Hershey Medical Center Hershey, PA
Summer Research Student

Worked in a cardiovascular research lab. Performed various surgical procedures for ongoing experiments including tracheotomies, muscle stretch, vessel catheterization, and coronary and femoral artery ligation. Also, I participated in immunohistochemical procedures and echocardiograms. In addition, I helped design the studies that I participated in and collected and analyzed the research data. Furthermore, I contributed to the writing and publication of the research mentioned.

Summer of 2007 Jilin University College of Medicine Jilin, China
Four Week Summer Research Internship

International experience of participating in renal nerve studies at Jilin University. Performed tracheotomies, coronary artery ligation and renal nerve recording on kidneys through surgery of lab mice.

PUBLICATIONS
