The relationship between nitric oxide synthase expression and nitric oxide mediated inhibition of vasoconstriction

by

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Abstract

Nitric oxide (NO) is a ubiquitous signal molecule with many biological functions. NO is produced through the reaction of L-arginine with O_2, which is catalyzed by the family NO synthase (NOS) enzymes. NOS exists in endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoforms and the eNOS and nNOS isoforms are constitutively expressed in many tissues, including skeletal muscle and the endothelium. In the cardiovascular system, NO acts as a vasodilator and an inhibitor of sympathetic vasoconstriction and is important in the regulation of vascular resistance and blood flow response to exercise. Indeed, reduced NOS expression has been linked to vascular dysfunction and the pathogenesis of cardiovascular diseases such as essential hypertension. The relationship between NOS expression and cardiovascular disease suggests that NOS expression is an important determinant of NO bioavailability. Thus, the purpose of this thesis was to investigate the effect of sex, estrogen and exercise training on skeletal muscle NOS expression, as well as the relationship between NOS expression and NO mediated inhibition of sympathetic vasoconstriction. Sprague-Dawley rats (~10 weeks of age; n=49) were utilized. The percentage change of femoral vascular conductance (FVC) in response to sympathetic chain stimulation delivered at 2 and 5 Hz determined at rest and during triceps surae muscle contraction before and after non-selective NOS blockade L-NAME and skeletal NOS expression were measured. nNOS expression was greater in female compared to male rats. However, the greater nNOS expression in females does not appear to be a function of estrogen status as NOS expression was not different between ovary-intact, ovariectomized and ovariectomized-estrogen supplemented female rats. Furthermore, in contrast to our previous findings in male rats, exercise training does not appear to augment NOS expression in female rats skeletal muscle NOS expression was not correlated to the NO-mediated the inhibition of sympathetic vasoconstriction resting and contracting skeletal muscle. These results suggest that

other factors beyond NOS expression modulate NO bioavailability and determine the magnitude of NO dependent sympatholysis. Further study will be required to fully elucidate the mechanism behind the enhanced sympatholysis in females.

Preface

This thesis is an original work by Sixue Liu. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Estrogen and Sympathetic Vascular Control”, Number: Animal Use Protocol 1493, Date: 2014-2018.

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List of Abbreviations

NO- nitric oxide

NOS- nitric oxide synthase

nNOS- neuronal nitric oxide synthase

eNOS- endothelial nitric oxide synthase

iNOS- inducible nitric oxide synthase

OI- ovary intact

OVX- ovariectomized

OVXE- 17β-estradiol supplemented ovariectomized

FVC- femoral vascular conductance

MSNA- muscle sympathetic nerve activity

BH4- tetrahydrobiopterin

O_2^-- superoxide

$ONOO^-$- peroxynitrite

ROS- reactive oxygen species

Chapter 1: Introduction

Introduction

Nitric oxide (NO) is a ubiquitous signal molecule with many biological functions (Bredt & Snyder, 1994; Kobzik, Reid, Bredt, & Stamler, 1994; Kojda & Kottenberg, 1999; Stamler & Meissner, 2001). NO is produced through the reaction of L-arginine with O_2, which is catalyzed by the enzyme NO synthase (NOS). NOS exists in endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) (Delp & O'Leary, 2004) isoforms and the eNOS and nNOS isoforms are constitutively expressed in many tissues, including skeletal muscle (Kobzik et al., 1994) and the endothelium (Furchgott, 1999). In the cardiovascular system, NO acts as a vasodilator and an inhibitor of sympathetic vasoconstriction and is important in the regulation of vascular resistance and blood flow response to exercise (Stamler & Meissner, 2001). Indeed, reduced NOS expression has been linked to vascular dysfunction and the pathogenesis of cardiovascular diseases such as essential hypertension (Loscalzo & Welch, 1995). The relationship between NOS expression and cardiovascular disease suggests that NOS expression is an important determinant of NO bioavailability. Thus, advancing our understanding of factors that regulate NOS expression may enhance our ability to treat and prevent cardiovascular disease.

However, the bioavailability of NO is determined by multiple factors beyond NOS enzyme content and the relationship between NOS enzyme expression and NO function is complex and has not been fully characterized. For example, the availability of substrate and essential co-factors, such as L-arginine and tetrahydrobiopterin (BH_4) influence NO bioavailability (Förstermann, 2010; Katusic & Vanhoutte, 1989). BH_4 is an important cofactor of producing eNOS. Decreased levels of BH_4 have been associated with reduced NO-dependent vascular function (Bevers et al., 2005; Förstermann, 2010; Förstermann & Sessa, 2012) and our

laboratory has shown that acute BH_4 supplementation decreased sympathetic vasoconstrictor responsiveness in resting and contracting skeletal muscle (Jendzjowsky, Just, Jones, & DeLorey, 2014). Perhaps the most important mechanism that influences NO bioavailability is the balance between the production of NO, antioxidant enzyme capacity and the production of reactive oxygen species (ROS), in particular superoxide (O_2^-) (Katusic & Vanhoutte, 1989). The imbalance between the productions of ROS and the antioxidant enzyme activity defined as oxidative stress, leading to the cardiovascular disease. The inactivation of antioxidant enzymes and uncoupling of eNOS resulting in oxidative stress, along with O_2^- production (Higashi, Maruhashi, Noma, & Kihara, 2014). NO readily reacts with O_2^- to produce peroxynitirite ($ONOO^-$), effectively reducing NO bioavailability (Lundberg, Gladwin, & Weitzberg, 2015). Uncouple eNOS leading to the production of O_2^- instead of NO and O_2^- may also oxidize BH_4 (Bevers et al., 2005; Förstermann, 2010). In the lack of BH4, oxygen reduction uncouples from eNOS, thereby converting eNOS to a superoxide-producing enzyme (Li & Forstermann, 2014). Furthermore, during exercise nNOS and eNOS are activated by increased intracellular Ca^{2+} and endothelial shear stress leading to increase NO production (Kojda & Hambrecht, 2005; Stamler & Meissner, 2001). Our lab has also shown that chronic exercise training augments skeletal muscle nNOS expression (Jendzjowsky, Just, & DeLorey, 2014b). Increased NOS expression was associated with enhanced NO-dependent inhibition of sympathetic vasoconstriction, suggesting that the enhanced nNOS-mediated inhibition of vasoconstriction may be a function of nNOS expression/content (Fadel, Zhao, & Thomas, 2003; Jendzjowsky, Just, & DeLorey, 2014b). In summary, NO bioavailability is determined by multiple factors including, NOS enzyme content, substrate and co-factor availability as well as the contractile state of muscle.

Therefore, to advance our understanding of the complex regulation of NO bioavailability and NO-mediated vascular function, this thesis will investigate the influence of sex, estrogen and exercise training on skeletal muscle NOS expression and the relationship between NOS expression and NO-mediated inhibition of sympathetic vasoconstriction. Understanding the determinants of NOS expression and the relationship between NOS expression and NO mediated vascular function will advance our understanding of NO biology and may contribute to the development of therapeutic targets and treatment strategies for vascular dysfunction characterized by altered NO function.

Biological Sex and NOS expression

Sex differences in the regulation of the cardiovascular system have been documented (Hart et al., 2009; Hart & Charkoudian, 2014; Just & DeLorey, 2017; Parker, Kalasky, & Proctor, 2010). Compared to young men, young women tend to have a lower incidence of cardiovascular diseases, including hypertension (Mosca et al., 1997; Narkiewicz et al., 2005). Whether the lower blood pressure in young females is related to NO bioavailability and NOS expression has not been established. However, Hayashi et al. (Hayashi, Fukuto, Ignarro, & Chaudhuri, 1992) reported that the basal release of NO from aortic rings was greater in female compared to male rabbits and it has been reported that eNOS protein expression is greater in brachial and femoral arteries of female compared to male swine (Laughlin et al., 2003). Moreover, our laboratory recently reported that contraction-mediated inhibition of vasoconstriction (i.e. sympatholysis) was enhanced in female compared to male rats and a portion of the enhanced sympatholysis in female rats was NO-dependent (Just & DeLorey, 2017b). However, NOS enzymes were not measured and therefore it is not possible to conclude if the enhanced NO function was the result of greater NOS content or other factors that determine NO bioavailability and function. Whether

biological sex modulates skeletal muscle eNOS and nNOS expression and the relationship between NOS expression and NO-mediated inhibition of vasoconstriction in resting and contracting muscle has not been investigated.

Estrogen status and NOS expression

A decline in estrogen bioavailability has been associated with an increased incidence of cardiovascular disease in post-menopausal females suggesting that reproductive hormones may play a role in vascular function (Hart et al., 2009, 2011). Indeed, cyclical changes in estrogen and progesterone during the menstrual cycle have been shown to alter baroreflex sensitivity and resting muscle sympathetic nerve activity (Minson, Halliwill, Young, & Joyner, 2000) with a rise in estrogen appearing to suppress muscle sympathetic nerve activity (Carter, Fu, Minson, & Joyner, 2013). Moreover, transdermal estrogen replacement therapy has been shown to reduce blood pressure and muscle sympathetic nerve activity in postmenopausal females (Vongpatanasin, Tuncel, Mansour, Arbique, & Victor, 2001). Estrogen also appears to have an impact on NOS expression and NO bioavailability (Fadel et al., 2003; Carl P. Weiner, Knowles, & Moncada, 1994). Reduced estrogen availability has been linked to a reduction in skeletal muscle nNOS expression in ovariectomized rats (Fadel et al., 2003; Carl P. Weiner et al., 1994) and postmenopausal women (Fadel et al., 2004). Additionally, estrogen supplementation has been shown to restore nNOS expression in ovariectomized rats (Fadel et al., 2003).

Exercise training and NOS expression

Regular physical exercise is considered as a cost-effective way to prevent and treat the chronic vascular and cardiovascular disease, including hypertension and vascular dysfunction (Larsen & Matchkov, 2016). Our laboratory has shown that exercise training enhances sympatholysis through an NO-dependent mechanism (Jendzjowsky & Delorey, 2013c;

Jendzjowsky & DeLorey, 2013; Jendzjowsky, Just, & DeLorey, 2014b). Skeletal muscle nNOS expression was greater in exercise-trained compared to sedentary rats, suggesting that increased NOS expression may underlie the enhanced NO dependent sympatholysis (Jendzjowsky, Just, & DeLorey, 2014b). Other labs have also reported a relationship between NOS expression and sympatholysis with impaired inhibition of sympathetic vasoconstriction observed in nNOS-null or nNOS-deficient populations (Grange et al., 2001; Sander et al., 2000; Thomas et al., 1998).

Relationship between NOS expression and NO-dependent inhibition of sympathetic vasoconstriction

Whether NO-mediated sympathetic vasoconstriction in resting and contracting skeletal is a function of skeletal muscle NOS expression has not been established. Our laboratory recently demonstrated that NO derived from nNOS and eNOS inhibit sympathetic vasoconstriction in resting and contracting skeletal muscle (Jendzjowsky & Delorey, 2013b). In resting skeletal muscle, NO derived from eNOS was responsible for approximately 70% of total NO-mediated inhibition, whereas during exercise NO derived from eNOS and nNOS made equal contributions (~50%) to NO mediated inhibition of sympathetic vasoconstriction. These data suggest that that newly derived NO from nNOS may be important to inhibit sympathetic vasoconstriction during exercise (Jendzjowsky & Delorey, 2013b). Furthermore, these data raise questions about the importance of NOS enzyme expression in determining the magnitude of NO mediated inhibition of sympathetic vasoconstriction, particularly during exercise.

Purpose and hypothesis

With this as a background, the purpose of this thesis was to investigate: 1) the effect of biological sex on nNOS and eNOS protein levels in skeletal muscle; 2) the effect of estrogen on

skeletal muscle nNOS and eNOS protein levels; 3) whether exercise training alters skeletal muscle NOS expression in female rats, and; 4) whether the magnitude of NO-mediated inhibition of the sympathetic vasoconstriction in resting and contracting skeletal muscle is a function of skeletal muscle NOS expression.

It was hypothesized that 1) skeletal muscle nNOS and eNOS expression would be greater in female compared to male rats; 2) nNOS and eNOS expression would be related to estrogen status; 3) exercise training would increase nNOS, but not eNOS expression, and; 4) NO-mediated blunting of sympathetic vasoconstriction in resting, but not contracting skeletal muscle would be related to skeletal muscle NOS expression.

Significance

Decreased NO bioavailability has been linked to vascular dysfunction and the development of cardiovascular disease. Therefore, understanding the determinants of NOS expression and the relationship between NOS expression and NO function will advance our understanding of NO biology and contribute to the development of therapeutic targets and treatment strategies for vascular dysfunction characterized by altered NO function.

Chapter 2: The relationship between skeletal muscle nitric oxide synthase expression and nitric oxide mediated inhibition of vasoconstriction

Introduction

Nitric oxide (NO) is a gaseous signal molecule that performs several biological functions (Bredt & Snyder, 1994; Kobzik, Reid, Bredt, & Stamler, 1994; Kojda & Kottenberg, 1999; Stamler & Meissner, 2001). NO is produced through the reaction of L-arginine with O_2, which is catalyzed by the enzyme NO synthase (NOS), of which there are endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoforms (Förstermann & Sessa, 2012; Kone, 2004). In the vasculature, NO acts as a vasodilator and an inhibitor of sympathetic vasoconstriction and is therefore an important regulator of vascular resistance (Jendzjowsky & Delorey, 2013b; Stamler & Meissner, 2001; Thomas & Victor, 1998). The eNOS and nNOS isoforms are constitutively expressed in many tissues, including skeletal muscle (Kobzik et al., 1994) and the endothelium (Furchgott, 1999) and NO derived from both eNOS and nNOS has been shown to contribute to the control of skeletal muscle blood flow (Förstermann & Sessa, 2012).

Reduced NOS expression has been linked to vascular dysfunction and the development of cardiovascular diseases, such as essential hypertension (Loscalzo & Welch, 1995), suggesting that NOS enzyme expression may be an important determinant of NO bioavailability and function. Consistent with that notion, young women have a lower incidence of hypertension (Mosca et al., 1997; Narkiewicz et al., 2005) and eNOS protein expression has been shown to be greater in brachial and femoral arteries of female compared to male swine (Laughlin et al., 2003). Moreover, our laboratory recently reported that contraction-mediated inhibition of vasoconstriction (i.e. sympatholysis) was enhanced in female compared to male rats and a portion of the enhanced sympatholysis in female rats was NO-dependent (Just & DeLorey,

2017b). NOS enzyme expression was not measured and therefore it is not possible to conclude if the enhanced NO function was the result of greater NOS content in female rats. However, the reproductive hormone estrogen may influence NOS expression and NO bioavailability as a decline in estrogen availability has been associated with a reduction in skeletal muscle nNOS expression in ovariectomized rats (Fadel et al., 2003; Carl P. Weiner et al., 1994) and postmenopausal women (Fadel et al., 2004). Additionally, estrogen supplementation has been shown to restore nNOS expression in ovariectomized rats (Fadel et al., 2003). Collectively, these studies suggest that NOS expression may be an important determinant of NO bioavailability and function.

However, NO bioavailability is determined by multiple factors and the relationship between NOS enzyme expression and NO function is complex and has not been fully characterized. Perhaps the most important mechanism, beyond NOS enzyme content, that influences NO bioavailability is the balance between the production of NO, antioxidant enzyme capacity and the production of reactive oxygen species (ROS), in particular superoxide (O_2^-) (Katusic & Vanhoutte, 1989). NO readily reacts with O_2^- to produce peroxynitirite ($ONOO^-$), effectively reducing NO bioavailability. O_2^- may also oxidize BH_4 and uncouple eNOS leading to the production of O_2^- instead of NO (Bevers et al., 2005; Förstermann, 2010). The availability of substrate and essential co-factors, such as L-arginine and tetrahydrobiopterin (BH_4) also influence NO bioavailability (Förstermann, 2010; Katusic & Vanhoutte, 1989). Decreased levels of BH_4 have been associated with reduced NO-dependent vascular function (Bevers et al., 2005; Förstermann, 2010; Förstermann & Sessa, 2012) and our laboratory has shown that acute BH_4 supplementation blunted sympathetic vasoconstrictor responsiveness in resting and contracting skeletal muscle (Jendzjowsky, Just, Jones, et al., 2014). Our lab has also shown that chronic

exercise training augments skeletal muscle nNOS expression (Jendzjowsky et al., 2014). The increased NOS expression was associated with enhanced NO-dependent inhibition of sympathetic vasoconstriction, suggesting that the enhanced nNOS mediated inhibition of vasoconstriction may be a function of nNOS expression/content (Jendzjowsky, Just, & DeLorey, 2014a). However, during an acute bout of exercise NO production increases as nNOS and eNOS are activated by increased intracellular Ca^{2+} in skeletal muscle fibers and shear stress on the endothelium (Kojda & Hambrechat, 2005; Stamler & Meissner, 2001). Our laboratory recently demonstrated that NO derived from nNOS and eNOS inhibit sympathetic vasoconstriction in resting and contracting skeletal muscle (Jendzjowsky & Delorey, 2013b). In resting skeletal muscle, NO derived from eNOS was responsible for approximately 70% of total NO-mediated inhibition, whereas during exercise NO derived from eNOS and nNOS made equal contributions (~50%) to NO mediated inhibition of sympathetic vasoconstriction. These data suggest that newly derived NO from nNOS may be important to inhibit sympathetic vasoconstriction during exercise (Jendzjowsky & Delorey, 2013b). Furthermore, these data raise questions about the importance of NOS enzyme expression in determining the magnitude of NO mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle.

With this as a background, the purpose of this thesis was to investigate: 1) the effect of biological sex on nNOS and eNOS protein levels in skeletal muscle; 2) the effect of estrogen on skeletal muscle nNOS and eNOS protein levels; 3) whether exercise training alters skeletal muscle NOS expression in female rats, and; 4) whether the magnitude of NO-mediated inhibition of the sympathetic vasoconstriction in resting and contracting skeletal muscle is a function of skeletal muscle NOS expression.

It was hypothesized that 1) skeletal muscle nNOS and eNOS expression would be greater in female compared to male rats; 2) nNOS and eNOS expression would be related to estrogen status; 3) exercise training would increase nNOS, but not eNOS expression, and; 4) NO-mediated blunting of sympathetic vasoconstriction in resting, but not contracting skeletal muscle would be related to skeletal muscle NOS expression.

Methods

Animals and animal care

Sprague-Dawley rats were obtained from the institutional animal colony and housed in a 12h-12h light-dark cycle environmentally controlled room (22-24 °C, 40-70% humidity). Food (Lab Diet 5001; PMI Nutrition, Brentwood, MO, USA) and water were provided ad libitum. All experiments were conducted with approval from the University of Alberta Animal Care and Use Committee: Health Sciences, and in accordance with the Canadian Council on Animal Care Guidelines and Policies. A total of 49 rats were utilized in a series of 3 experiments.

Experiment 1: The effect of sex on NOS expression

The vasoconstrictor response to 1 minute of lumbar sympathetic chain stimulation delivered at 2 and 5 Hz in random order was measured at rest and during muscle contraction under control conditions and following NOS blockade (Nω-nitro-L-arginine methyl ester; L-NAME, 10 mg.kg-1, IV) in female (n=7) and male (n=7) Sprague-Dawley rats (~ 8 weeks old). Skeletal muscle tissue was obtained for measurement of NOS enzymes. Sympathetic vasoconstrictor responsiveness and sympatholysis data on these animals has been published (Just & DeLorey, 2017).

Experiment 2: The effect of estrogen on NOS expression

Sprague-Dawley female rats (~ 8 weeks old) were randomly assigned to ovary intact (OI; n=7), ovariectomized (OVX; n=7) or ovariectomized with a 17β-estradiol-replacement (OVXE; n=7). Rats to be ovariectomized were anesthetized by inhalation of isoflurane (3.5% in balance O2) and underwent a bilateral ovariectomy through an abdominal incision. The ovaries were removed following the ligation of the uterus immediately distal to each ovary. The abdominal wall was sutured closed, and a small subcutaneous pocket was made along the rat's abdominal wall. A 90-day timed-release 17β-estradiol pellet (OVXE; 2.5mg pellet-1) or a placebo pellet (OVX; Innovative Research of America, Sarasota, FL, USA) was implanted subcutaneously and the skin was stapled closed. Post-operatively, anti-bacterial/fungal cream (Hibitane; Canada) was applied over the incision site. Post-operative pain management was accomplished by intramuscular Metacam (Boehringer Ingelheim Vetmedica, Inc, Duluth, GA) injections (2-5mg kg^{-1}) immediately following surgery and every 24 hours for 3 days. Rats were then monitored for 10 weeks. The vasoconstrictor response to 1 minute of lumbar sympathetic chain stimulation delivered at 2 and 5 Hz in random order was measured at rest and during muscle contraction under control conditions and following NOS blockade (Nω-nitro-L-arginine methyl ester; L-NAME, 10 mg.kg-1, IV).

Experiment 3: The effect of exercise training on NOS expression

All female rats (~8 weeks old) were habituated to the lab and exercise by walking on a treadmill (Panlab LE8710, Barcelona, Spain) 10 min day^{-1} for 5 days at 10 m min^{-1}, 0° grade. Following familiarization, rats were randomly assigned to a sedentary time-control (n = 7) or heavy-intensity exercise training (n = 7; 40 m min^{-1}, 5° grade) group. Exercise-trained rats trained 5 days $week^{-1}$ for 10 weeks, while sedentary rats were handled and weighed daily. On the

first training day, exercise-trained rats completed 15× 1 min intervals at 40 m min^{-1} 5° grade interspersed with rest periods of equivalent duration. Each subsequent training day, run time was increased while rest time was maintained. This training progression allowed all rats in the exercise-trained group to run continuously for 600 m at the prescribed speed and grade within 11 ± 2 days. This training paradigm is regularly used in our laboratory and has been shown to increase heart mass, heart mass: body mass ratio, soleus citrate synthase activity, and endothelium-dependent vasodilation (Jendzjowsky & Delorey, 2013c; Jendzjowsky & DeLorey, 2012). The vasoconstrictor response to 1 minute of lumbar sympathetic chain stimulation delivered at 2 and 5 Hz in random order was measured at rest and during muscle contraction under control conditions and following NOS blockade (Nω-nitro-L-arginine methyl ester; L-NAME, 10 mg.kg-1, IV).

General methods

Surgical Instrumentation

Following completion of the experimental intervention described above, anesthesia was induced by inhalation of isoflurane (3.5% in balance O_2). The right jugular vein was cannulated and anesthesia maintained with α-chloralose (8-16 mg kg^{-1} h^{-1}) and urethane (50-100mg kg^{-1} h^{-1}). The depth of anesthesia was assessed by the stability of arterial blood pressure, heart rate (HR) and the absence of a withdrawal reflex in response to a painful stimulus (i.e. paw-pinch). Core temperature was monitored by rectal probe and maintained at 36-37ºC by external heating pad (Physitemp, TCAT-2, Clifton, NJ USA). A tracheotomy was performed for the maintenance of a patent airway and to facilitate spontaneous respiration. The left brachial artery was cannulated and connected to a pressure transducer (Abbott, North Chicago, IL USA) for the continuous measurement of arterial blood pressure. The left femoral artery and vein was cannulated for the

delivery of pharmacology. Arterial blood samples were taken at rest and at the end of each contraction bout for the measurement of pH, arterial P_{CO2} and arterial P_{O2} (VetStat; IDEXX Laboratories, Markham, ON, Canada). Blood flow was measured using a flow probe (0.7 V; Transonic Systems, Ithaca, NY USA) placed around the right femoral artery and connected to a flow-meter (T106 Transonic Systems, Ithaca, NY USA). Heart rate was derived from the arterial blood pressure waveform.

Muscle contraction

The right sciatic nerve was exposed and instrumented with a nerve cuff electrode. The triceps surae muscle group was dissected free and attached to a force transducer (Model FT03, Natus Neurology, RI USA) via the calcaneal tendon. Maximal contractile force (MCF) was determined by stimulation of the triceps surae muscle group with 25, 1ms impulses delivered at 100Hz, at 5× the motor threshold (MT). The optimal muscle length for tension development was determined by progressively lengthening the muscle and repeating the nerve stimulation until a plateau in tension (peak-baseline) is observed. Rhythmic contractions of the triceps surae muscles was produced at 60% MCF at 40Hz 0.1ms pulses in 250 ms trains at a rate of 60 trains/minute at ~ 5× MT.

Lumbar sympathetic chain stimulation

To stimulate the lumbar sympathetic chain, a laparotomy was performed and the abdominal aorta and vena cava were temporarily retracted to allow dissection of the lumbar sympathetic chain with a blunt glass pipette. A bipolar silver-wire stimulating electrode was attached on the lumbar sympathetic chain between L3 and L4. The electrodes were embedded and electrically isolated in a rapidly-curing non-toxic silicone elastomer (Kwiksil, WPI, Sarasota, FL USA). The electrodes were connected to a constant current stimulator (Digitimer DS3,

Welwyn City, UK) and electrical stimulation was delivered at 2 and 5Hz to evoke the release of sympathetic neurotransmitters and produce vasoconstriction in the experimental hindlimb.

Euthanasia and Tissue collection

Following the experimental protocol, rats was killed by anesthetic overdose. The heart, soleus, lateral gastrocnemius, and medial gastrocnemius muscles was dissected, weighed and then snap frozen in liquid nitrogen. Samples will be stored at -80°C for further analysis.

Western blot analysis

Snap frozen (100–200 mg) soleus, medial gastrocnemius and lateral gastrocnemius muscles were homogenized in lysis buffer [20 mM Tris (pH 7.4), 5 mM EDTA, 10 mM sodium pyrophosphate tetrabasic, 100 mM sodium fluoride, 1% NP-40] containing 1× Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL, USA). Protein concentrations were determined by bicinchoninic acid assay (Pierce). One hundred micrograms of total protein were separated on an 8% SDS-polyacrylamide gel for about 90 minutes at 110v and then transferred on to nitrocellulose membrane (0.2 μm; Biorad, Hercules, CA, USA) at 100v for 60 minutes. Incubate the blot with 50% blocking solution for a minimum of 60 min with gentle rocking to block non-specific interactions. Membranes were incubated with eNOS or nNOS (1:250; BD Transduction Laboratories, Franklin Lakes, NJ, USA) in the cold room (4°C) for overnight and incubated with GAPDH (1:5000; Novus Biologicals, Littleton CO, USA) at the room temperature for 1 hour. Membranes were subsequently probed with IRDye® 800CW donkey anti-mouse IgG and IRDye® 680RD donkey anti-rabbit IgG secondary antibodies (1:10,000; Li-Cor Biosciences, Lincoln, NE, USA). The protein bands were detected and quantified by the Li-Cor Odyssey imager system v3.0. Results were normalized to GAPDH.

Data analysis

Data were recorded using Chart software (ADInstruments). Arterial blood pressure and femoral artery blood flow (FBF) were sampled at 100 Hz, and femoral vascular conductance (FVC) ($ml \cdot min^{-1} \cdot mmHg^{-1}$) was calculated as FBF divided by mean arterial pressure (MAP).

The change in HR, MAP, FBF, and FVC in response to sympathetic stimulation was calculated as an absolute change and the percentage change in FVC is the accepted metric to assess the magnitude of sympathetic vasoconstrictor responses because the percentage change in FVC accurately reflects percentage changes in resistance vessel radius even across conditions with different baseline levels of vascular conductance. All data are expressed as means ± SD.

Statistical Analysis

The influence of sex on skeletal muscle NOS expression was analyzed by two-way repeated measures ANOVA (gender × muscle). The effect of estrogen on NOS expression was determined by two-way repeated measures ANOVA (estrogen status × muscle). The effect of exercise training on NOS expression was analyzed by two-way repeated measures ANOVA (group × muscle type). The magnitude of NO mediated inhibition of sympathetic vasoconstriction was calculated as the difference in vasoconstrictor responsiveness in the control and NOS blockade conditions. To assess the relationship between NOS expression and NO-mediated inhibition of sympathetic vasoconstriction, linear regression analysis was performed and Pearson's correlation coefficients were calculated. When significant main effects and interactions were detected, a Student-Newman-Keuls post hoc test was performed. A P-value of < 0.05 was considered statistically significant.

Results

Experiment 1: The effect of sex difference on NOS isoform expression

Body mass, heart mass, and soleus and lateral and medial gastrocnemius muscle mass were higher ($P<0.05$) in male compared to female rats (Table 1). The heart mass-to- body mass ratio was lower ($P<0.05$) in male compared to female rats.

Female rats expressed higher ($P<0.05$) nNOS-alpha compared to male rats (Fig.1; panel A). The expression of nNOS-alpha was higher ($P<0.05$) in medial and lateral gastrocnemius muscles compared to the soleus muscle in male and female rats (Fig.1; panel A). nNOS-beta expression was similar ($P>0.05$) in female and male rats, but was lower ($P<0.05$) in the soleus compared to the medial and lateral gastrocnemius muscles in both groups (Fig.1; panel B). eNOS expression was not different ($P>0.05$) in female and male rats, but was higher ($P<0.05$) in the soleus compared to the medial gastrocnemius and lateral gastrocnemius muscles in both groups (Fig.1; panel C). There was no interaction ($P>0.05$) between sex and muscle type on nNOS-alpha, nNOS-beta and eNOS expression.

In resting skeletal muscle, nNOS expression was not correlated with NO mediated inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 2Hz ($r=-0.376$, $P>0.05$; Fig. 2). However, nNOS expression was correlated with NO mediated inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 2Hz in contracting muscle ($r=-0.639$, $P<0.05$) skeletal muscle (Fig.2). nNOS expression was not correlated with NO mediated inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 5Hz in resting or contracting skeletal muscle (Fig, 3). There was also no correlation between skeletal muscle eNOS expression and NO dependent inhibition

of sympathetic vasoconstriction in resting and contracting skeletal muscle at either the 2 (Fig. 3) or 5 Hz (Figure. 3) stimulation frequency.

Table 1. Animal characteristics (Experiment 1.)

Group	n	Body Mass (g)	Heart Mass (g)	Soleus Mass (g)	Lateral Gastrocnemius Mass (g)	Medial Gastrocnemius Mass (g)	Heart Mass-to-Body Mass Ratio (mg/g)
Female	7	286 ± 32	1.00 ± 0.11	0.15 ± 0.02	0.52 ± 0.05	1.06 ± 0.15	3.5 ± 0.3
Male	7	451 ± 92*	1.35 ± 0.22*	0.22 ± 0.06*	0.75 ± 0.19*	1.46 ± 0.28*	3.0 ± 0.2*

All values are means ± SD. A P value < 0.05 was considered statistically significant. * Significant group difference.

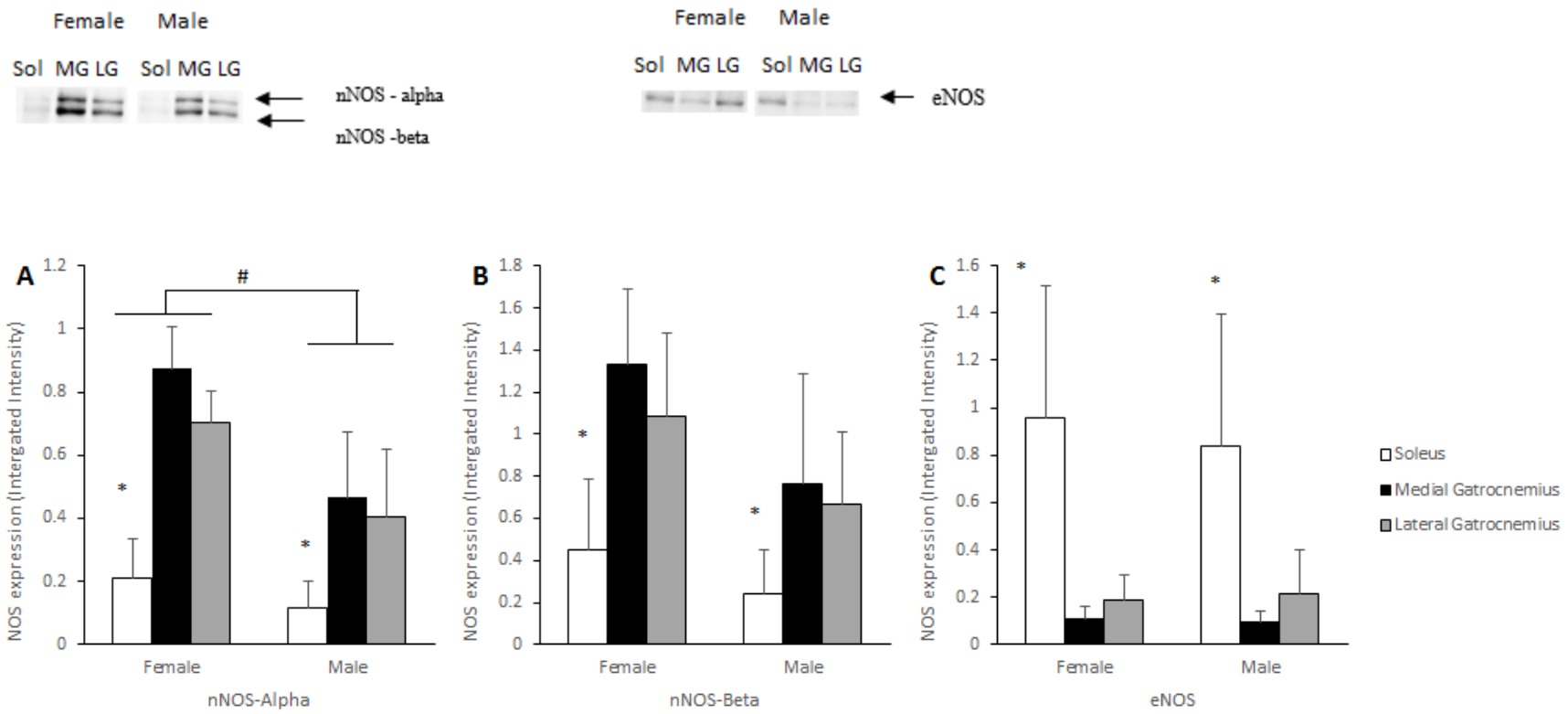

Figure 1. Expression of neuronal NOS-alpha (nNOS-alpha; A), neuronal NOS-beta (nNOS-beta; B) and endothelial NOS (eNOS; C) from soleus muscles (Sol; open columns), medial gastrocnemius muscles (MG; filled columns) and the lateral gastrocnemius (LG; grey columns) in female and male groups. NOS expression was normalized to GAPDH protein level. All values are means ± SD. Significant difference between #groups and *muscles. A value of $P<0.05$ was considered statistically significant.

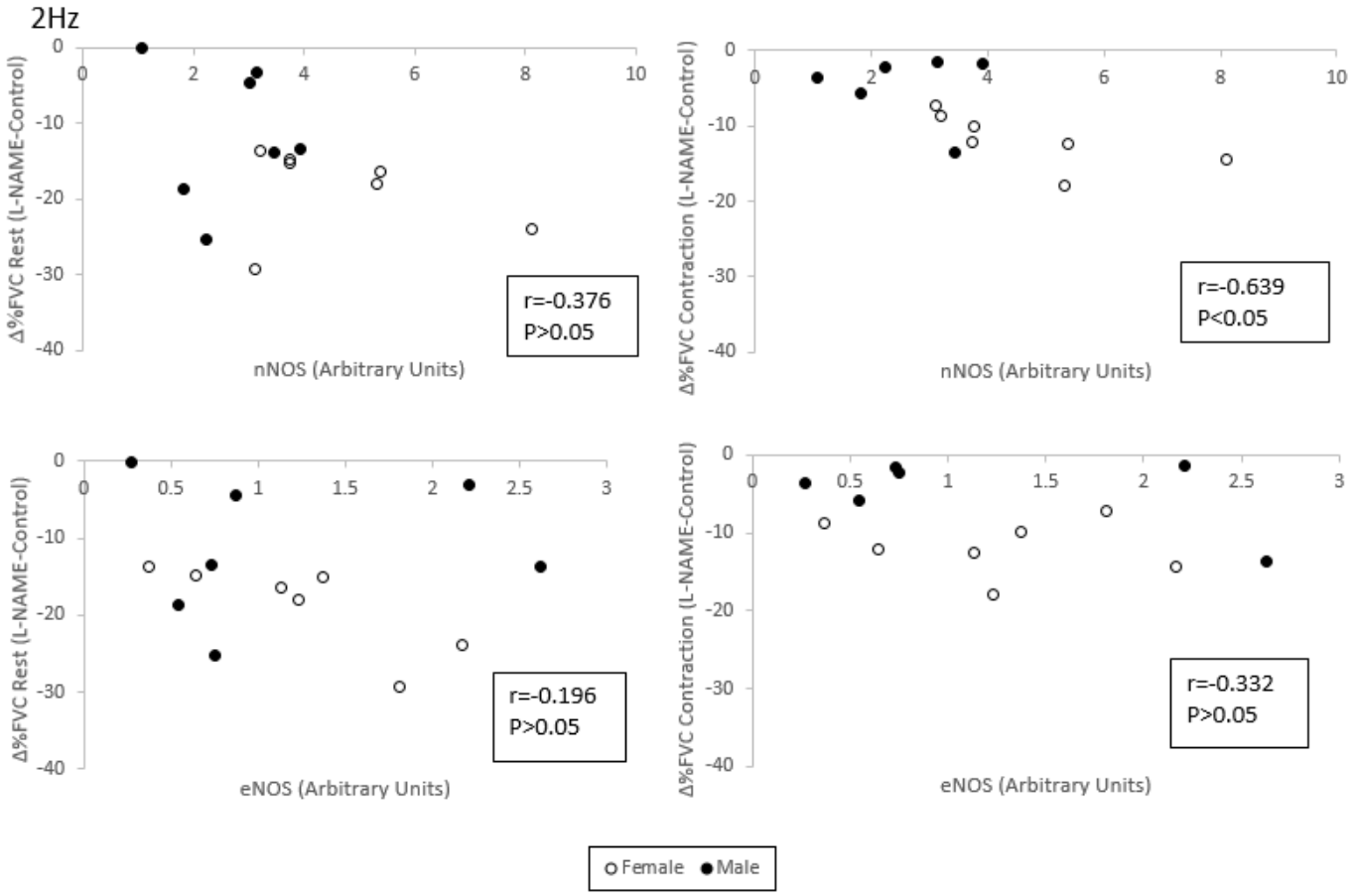

Figure 2. Relationships between skeletal muscle nNOS (upper panels) and eNOS (lower panels) isoform expression and the magnitude of nitric-oxide mediated inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 2Hz in resting (left panels) and contracting (right panels) skeletal muscle. A value of $P<0.05$ was considered statistically significant.

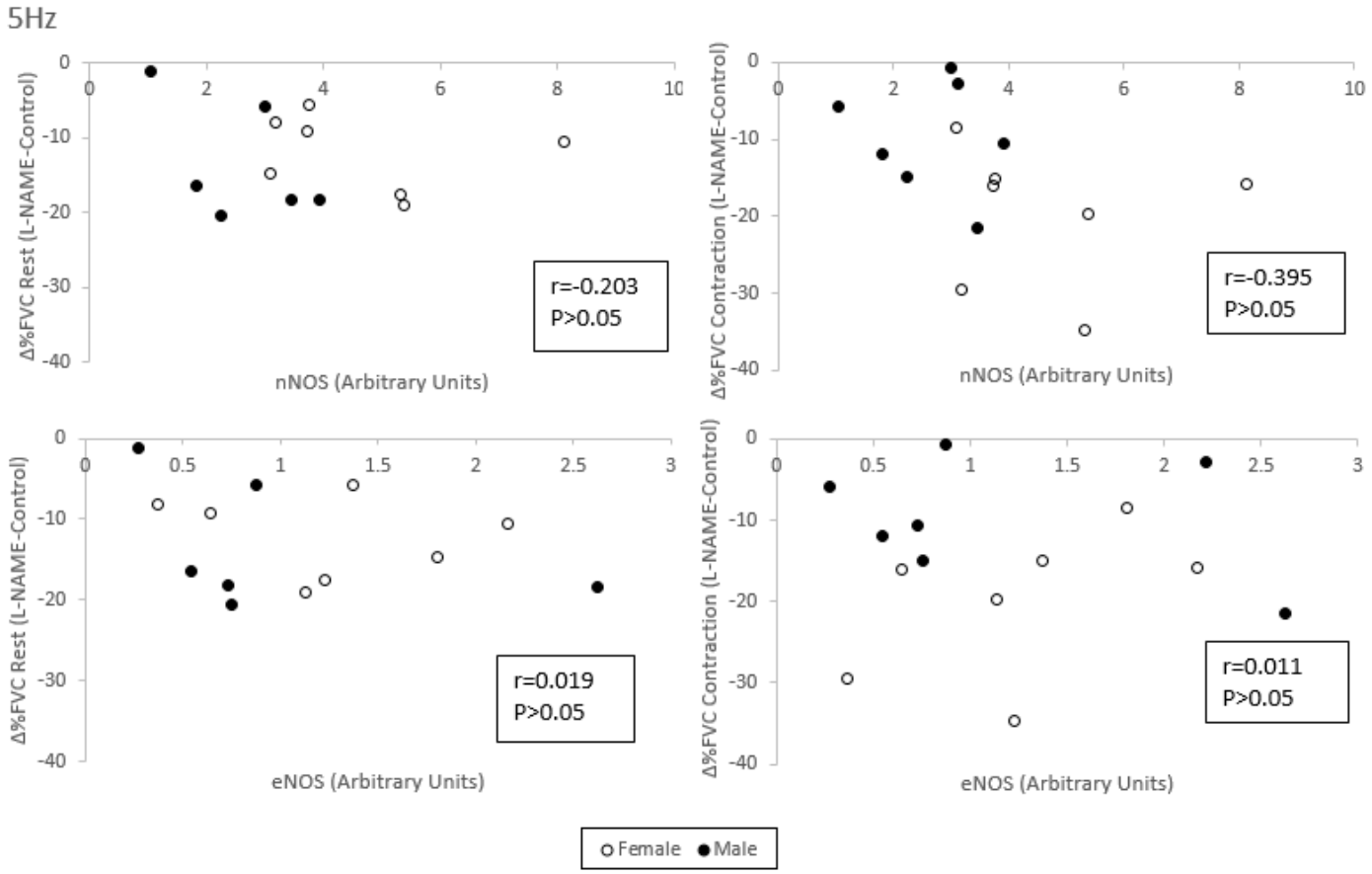

Figure 3. Relationships between skeletal muscle nNOS (upper panels) and eNOS (lower panels) isoform expression and the magnitude of nitric-oxide mediated inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 5Hz in resting (left panels) and contracting (right panels) skeletal muscle. A value of $P<0.05$ was considered statistically significant.

Experiment 2: The effect of estrogen status on NOS isoform expression

Body mass was higher ($P<0.05$) in OVX rats compared to OI and OVXE, and greater in OI rats compared to OVXE rats (Table 2). Heart mass was higher ($P<0.05$) in OVX rats compared to the OVXE rats and was similar ($P>0.05$) in OI and OVXE rats. The soleus and medial and lateral gastrocnemius muscle mass was lower ($P<0.05$) in the OVXE rats and was similar ($P>0.05$) in the OI and OVX rats. Heart mass to body mass ratios were lower ($P < 0.05$) in OVX rats than in OI and OVXE rats.

The expression of nNOS-alpha was not different ($P>0.05$) in OI, OVXE and OVX rats (Fig. 4; panel A) and was similar ($P>0.05$) between muscles. nNOS-beta was higher ($P<0.05$) in the soleus muscles compared with medial gastrocnemius and lateral gastrocnemius muscles, but was similar ($P>0.05$) in OI, OVXE and OVX rats (Fig. 4; panel B). The expression of eNOS was not different between groups, but was higher ($P<0.05$) in soleus muscles compared to the medial and lateral gastrocnemius muscles in all groups (Fig. 4; panel C). There was no interaction ($P>0.05$) between groups and muscle nNOS-alpha, nNOS-beta and eNOS expression.

In OI, OVX and OVXE rats, there was no correlation between nNOS expression and NO mediated inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 2Hz (Fig. 5) or 5Hz (Fig. 6) in resting or contracting skeletal muscle. There was also no correlation between skeletal muscle eNOS and NO dependent inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 2Hz (Fig. 5) or 5Hz (Fig. 6).

Table 2. Animal characteristics (Experiment 2.)

Group	n	Body Mass (g)	Heart Mass (g)	Soleus Mass (g)	Lateral Gastrocnemius Mass (g)	Medial Gastrocnemius Mass (g)	Heart Mass-to-Body Mass Ratio (mg/g)
OI	7	377 ± 12	1.16 ± 0.07	0.22 ± 0.02	0.73 ± 0.08	1.28 ± 0.12	3.07 ± 0.25
OVX	7	456 ± 50*	1.22 ± 0.07	0.21 ± 0.02	0.75 ± 0.06	1.39 ± 0.12	2.69 ± 0.21*
OVXE	7	316 ± 29*†	1.01 ± 0.10*	0.17 ± 0.02*	0.65 ± 0.08†	1.17 ± 0.16†	3.20 ± 0.12†

All values are means ± SD. A P value < 0.05 was considered statistically significant. * Significant group difference. OI, ovary intact; OVX, ovariectomized; OVXE, ovariectomized + 17β-estradiol. * Indicates a statistically significant difference from ovary intact (OI) rats. † Indicates a statistically significant difference from ovariectomized (OVX) rats.

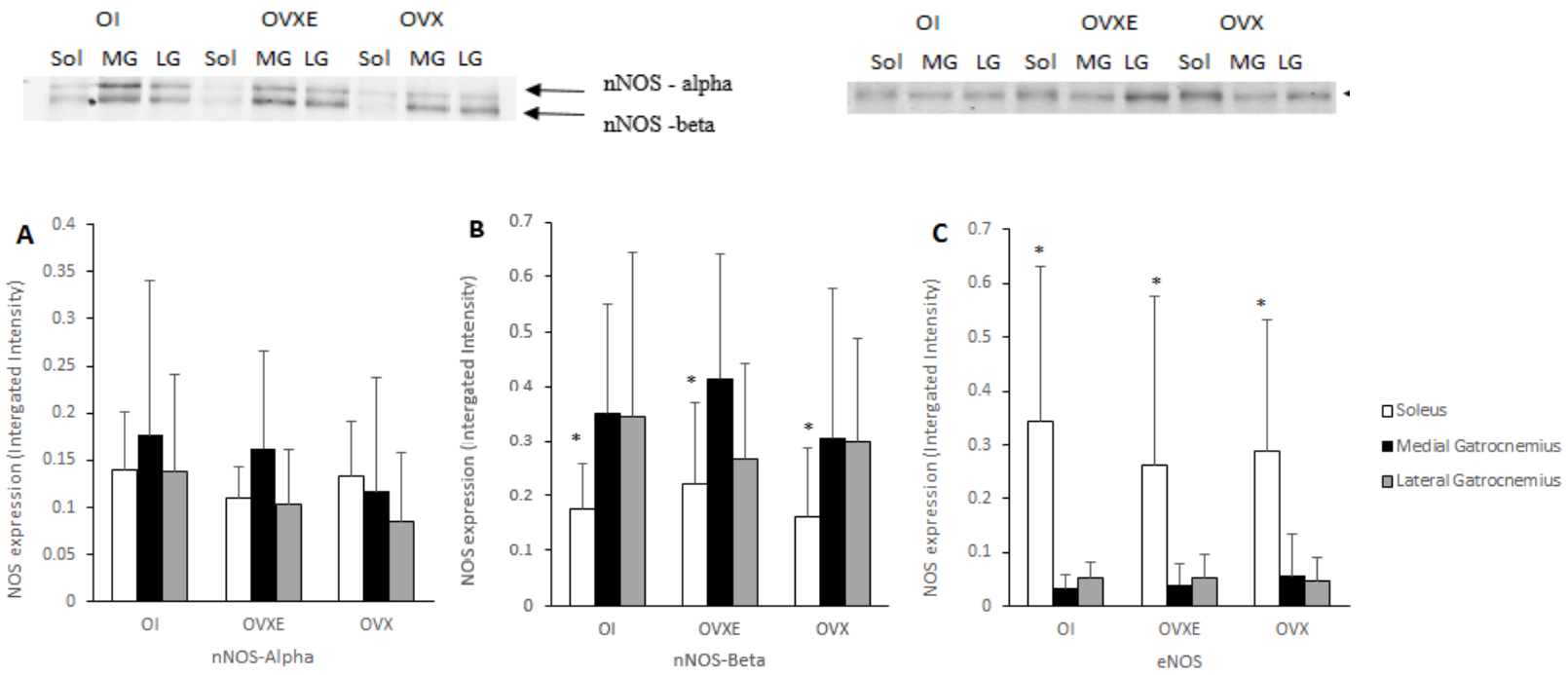

Figure 4. Normalized expression of neuronal NOS-alpha (nNOS-alpha; A), neuronal NOS-beta (nNOS-beta; B) and endothelial NOS (eNOS; C) from soleus muscles (Sol; open columns), medial gastrocnemius muscles (MG; filled columns) and the lateral gastrocnemius (LG; grey columns) in Ovary intact (OI), ovariectomized +17β-Estradiol replacement (OVXE) and ovariectomized (OVX) groups. NOS expression was normalized to GAPDH protein level. All values are means ± SD. Significant difference between #groups and *muscles. A value of $P<0.05$ was considered statistically significant.

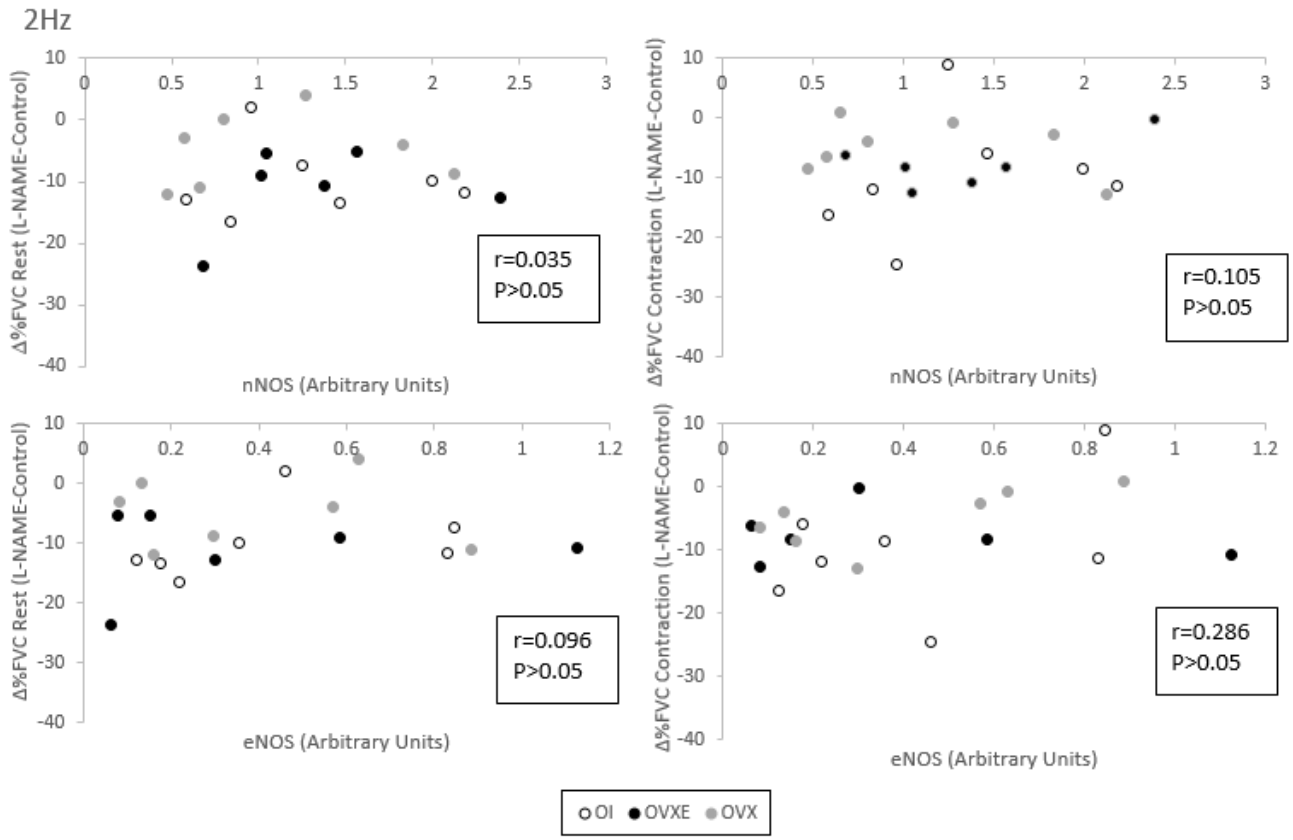

Figure 5. Relationships between skeletal muscle nNOS (upper panels) and eNOS (lower panels) isoform expression and the magnitude of nitric-oxide mediated inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 2Hz in resting (left panels) and contracting (right panels) skeletal muscle. A value of $P<0.05$ was considered statistically significant.

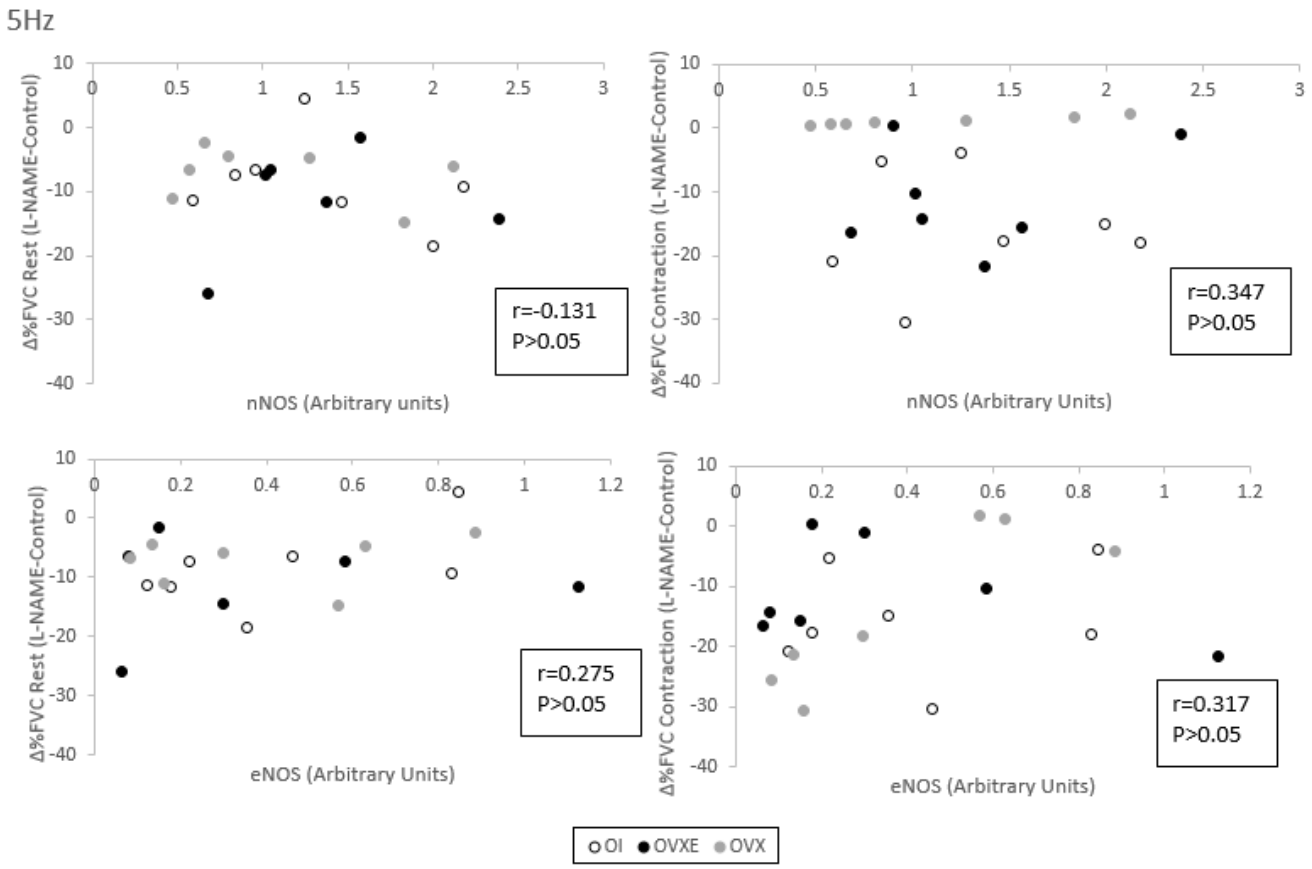

Figure 6. Relationships between skeletal muscle nNOS (upper panels) and eNOS (lower panels) isoform expression and the magnitude of nitric-oxide mediated inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 5Hz in resting (left panels) and contracting (right panels) skeletal muscle. A value of $P<0.05$ was considered statistically significant.

Experiment 3: The effect of exercise training on NOS isoform expression

Body mass and skeletal muscle mass were similar (P>0.05) in sedentary and exercise-trained rats (Table 3). However, heart mass and heart mass to body mass ratios were higher (P<0.05) in the exercise-trained compared to sedentary rats (Table 3).

The expression of nNOS-alpha and nNOS-beta was similar (P>0.05) in exercise-trained and sedentary rats, but was lower (P<0.05) in the soleus muscles compared to the medial and lateral gastrocnemius muscles in both groups (Fig.7; panel A, B). eNOS expression was not different (P>0.05) in exercise-trained and sedentary rats, whereas eNOS expression was higher (P<0.05) in the soleus muscle compared with medial and lateral gastrocnemius muscle in both groups (Fig.7; panel C). There was no interaction between experimental groups and muscle nNOS-alpha, nNOS-beta and eNOS expression.

Skeletal muscle nNOS was not correlated with NO dependent inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 2Hz (Fig. 8) or 5Hz (Fig. 9) in resting or contracting skeletal muscle. There was also no relationship between eNOS expression and NO dependent inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 2Hz (Fig. 8) or 5Hz (Fig. 9) in resting or contracting ($r=0.150, P>0.05$) skeletal muscle.

Table 3. Animal characteristics (Experiment 3.)

Group	n	Body Mass (g)	Heart Mass (g)	Soleus Mass (g)	Lateral Gastrocnemius Mass (g)	Medial Gastrocnemius Mass (g)	Heart Mass-to-Body Mass Ratio (mg/g)
Sedentary	7	377 ± 43	1.16 ± 0.07	0.22 ± 0.02	0.73 ± 0.08	1.28 ± 0.12	3.07 ± 0.25
Exercise	7	387 ± 43	1.24 ± 0.10*	0.21 ± 0.02	0.73 ± 0.06	1.32 ± 0.10	3.23 ± 0.18*

All values are means ± SD. A P value < 0.05 was considered statistically significant. * Indicates a statistically significant difference from exercise rats.

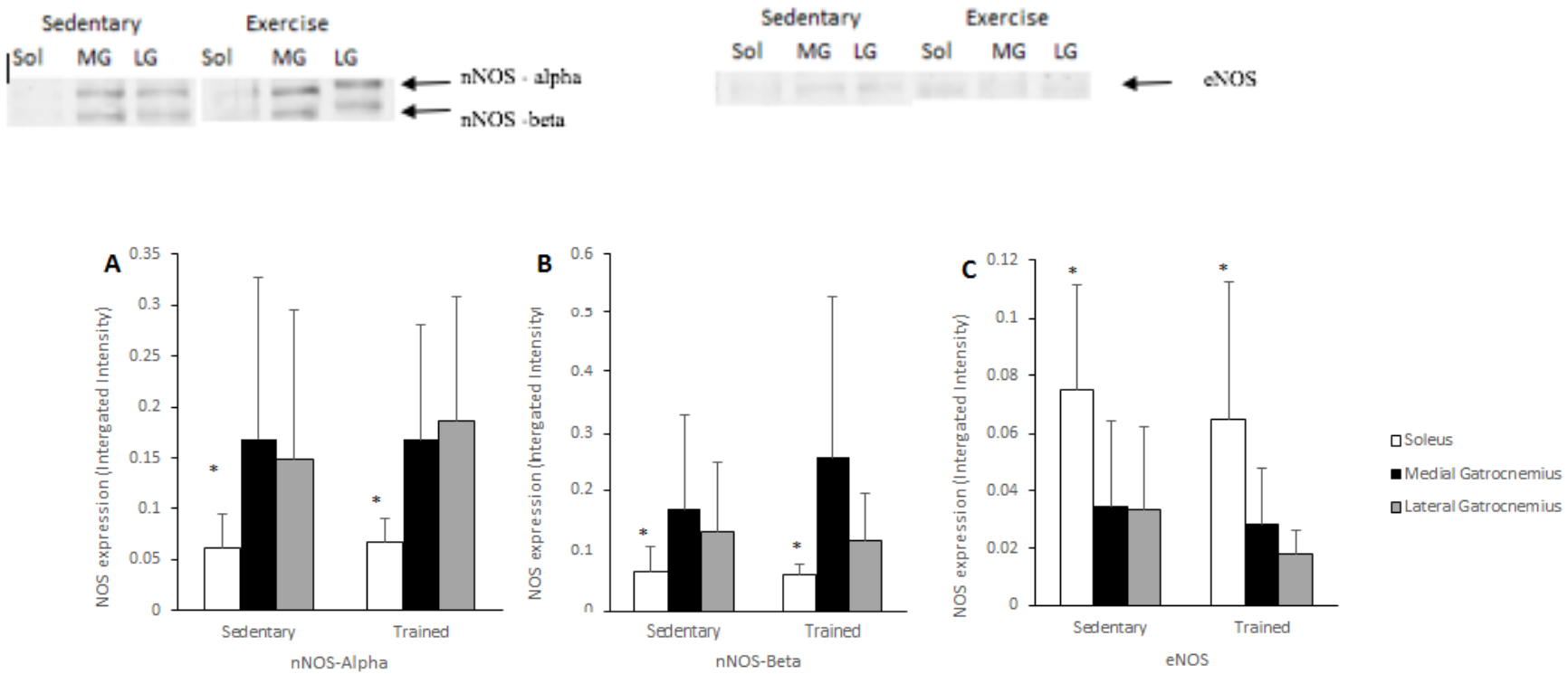

Figure 7. Normalized expression of neuronal NOS-alpha (nNOS-alpha; A), neuronal NOS-beta (nNOS-beta; B) and endothelial NOS (eNOS; C) from soleus muscles (Sol; open columns), medial gastrocnemius muscles (MG; filled columns) and the lateral gastrocnemius (LG; grey columns) in sedentary and exercise-trained groups. NOS expression was normalized to GAPDH protein level. All values are means ± SD. *Significant difference between muscles within a group. A value of $P<0.05$ was considered statistically significant.

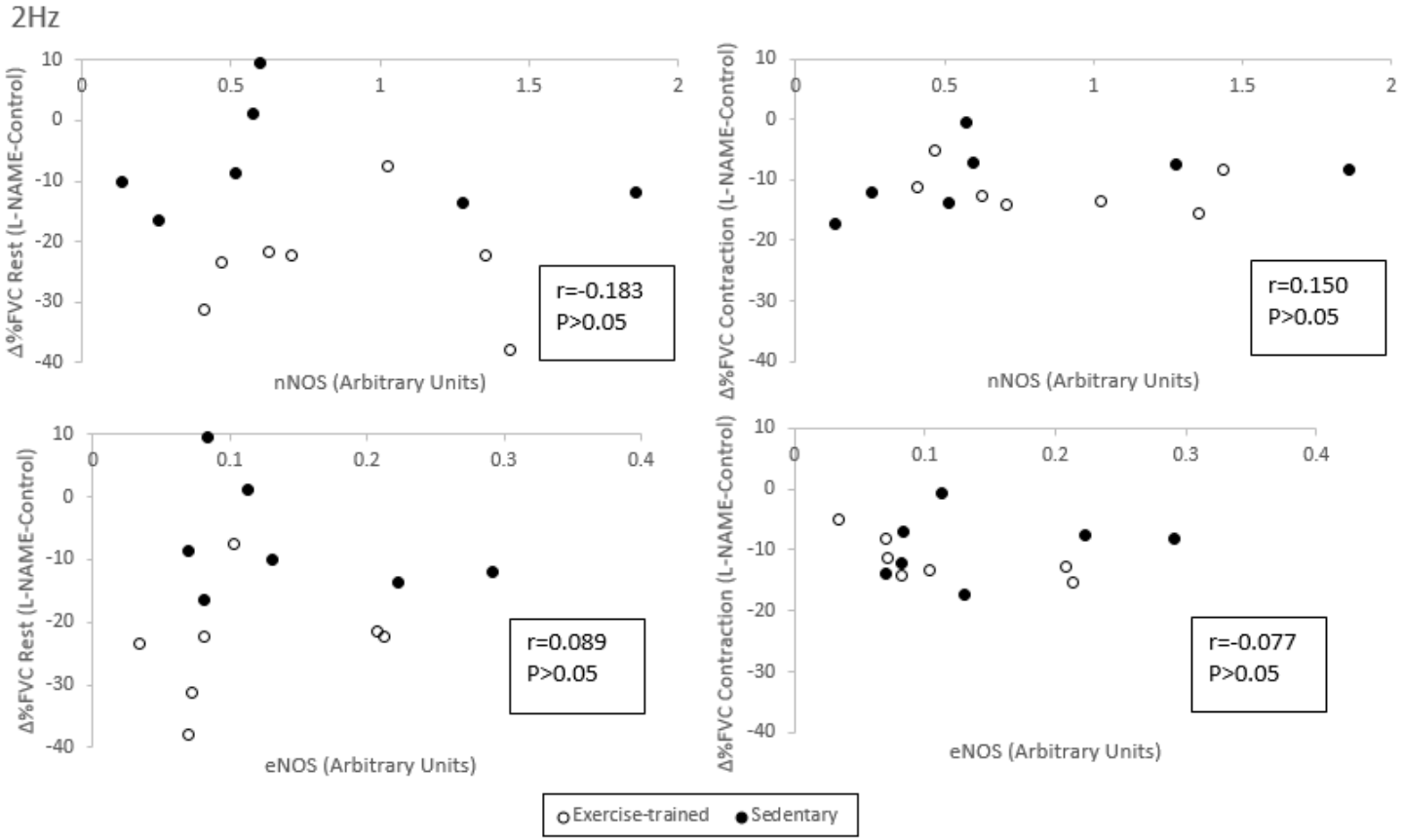

Figure 8. Relationships between skeletal muscle nNOS (upper panels) and eNOS (lower panels) isoform expression and the magnitude of nitric-oxide mediated inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 2Hz in resting (left panels) and contracting (right panels) skeletal muscle. A value of $P<0.05$ was considered statistically significant.

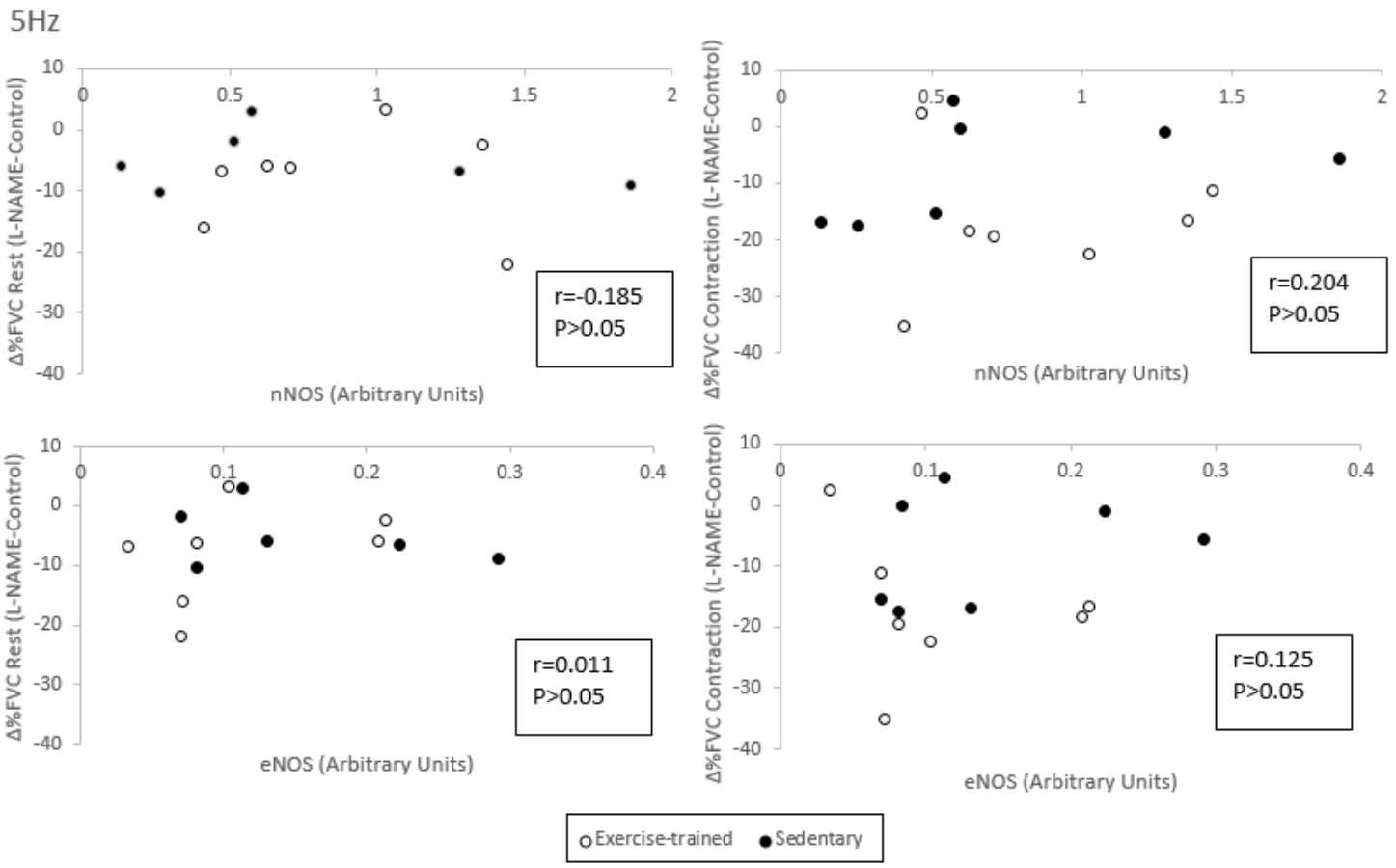

Figure 9. Relationships between skeletal muscle nNOS (upper panels) and eNOS (lower panels) isoform expression and the magnitude of nitric-oxide mediated inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 5Hz in resting (left panels) and contracting (right panels) skeletal muscle. A value of $P<0.05$ was considered statistically significant.

The correlation between the NO dependent inhibition of sympathetic vasoconstriction and total NOS expression

Total NOS expression was not correlated with NO dependent inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 2 or 5Hz in resting or contracting skeletal muscle (Fig. 10)

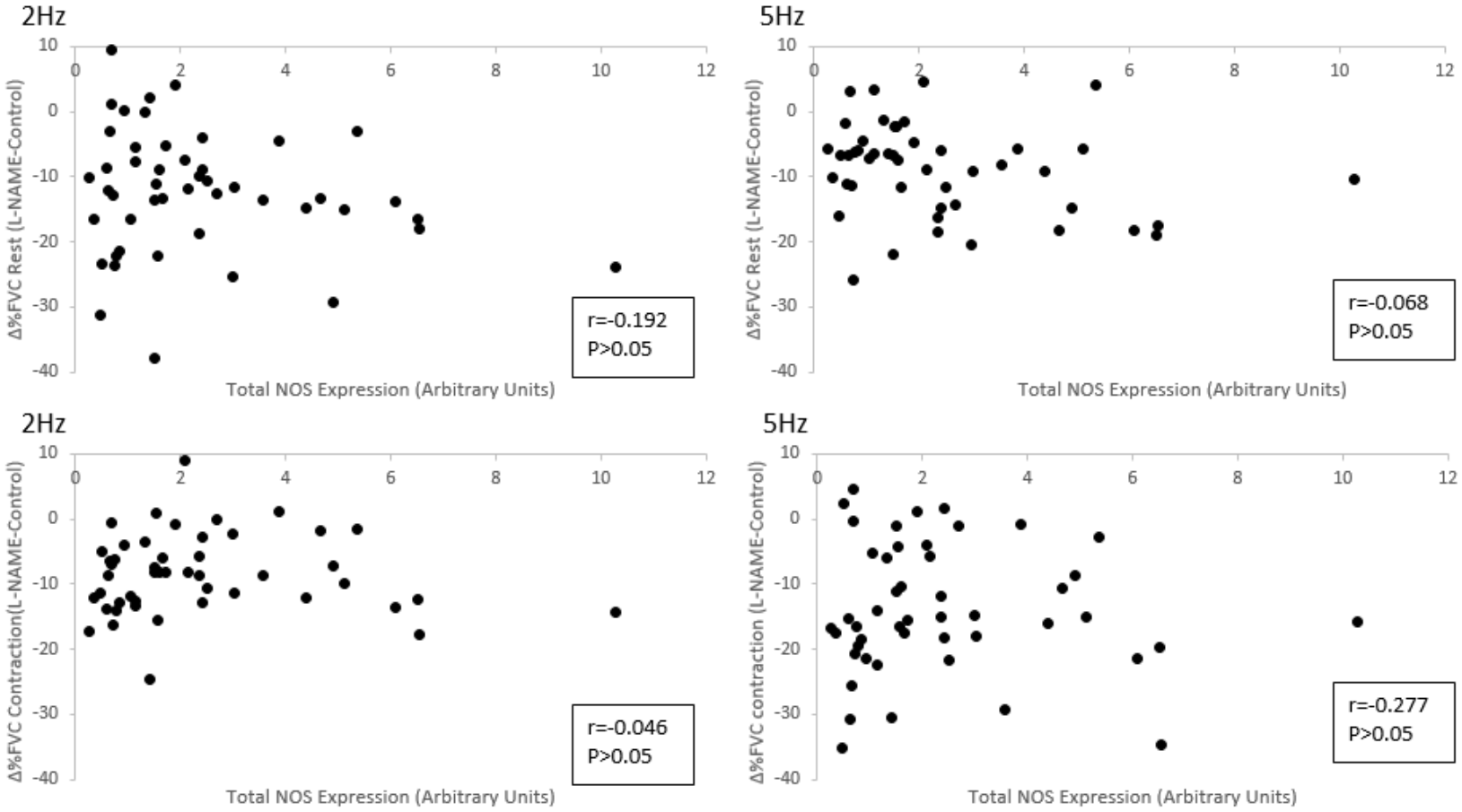

Figure 10. Relationships between total skeletal muscle NOS expression and the magnitude of nitric-oxide mediated inhibition of sympathetic vasoconstriction in response to sympathetic stimulation delivered at 2Hz (left panels) and 5Hz (right panels) in resting (upper panels) and contracting (lower panels) skeletal muscle. A value of $P<0.05$ was considered statistically significant.

Discussion

The purpose of the thesis was to investigate:1) the effect of biological sex on nNOS and eNOS protein levels in skeletal muscle; 2) the effect of estrogen on skeletal muscle nNOS and eNOS protein levels; 3) whether exercise training alters skeletal muscle NOS expression in female rats, and; 4) whether the magnitude of NO-mediated inhibition of the sympathetic vasoconstriction in resting and contracting skeletal muscle is a function of skeletal muscle NOS expression. The important novel findings from this study were that skeletal muscle nNOS expression was greater in female compared to male rats. However, the greater nNOS expression in females does not appear to be a function of estrogen status as NOS expression was not different between ovary-intact, ovariectomized and ovariectomized-estrogen supplemented female rats. Furthermore, in contrast to our previous findings in male rats, (Jendzjowsky, Just, & DeLorey, 2014b) exercise training does not appear to augment NOS expression in female rats skeletal muscle NOS expression was not correlated to the NO-mediated the inhibition of sympathetic vasoconstriction resting and contracting skeletal muscle. These results suggest that other factors beyond NOS expression modulate NO bioavailability and determine the magnitude of NO dependent sympatholysis. Further study will be required to fully elucidate the mechanism behind the enhanced sympatholysis in females.

Effects of sex on NOS expression

Our laboratory recently reported (Just & DeLorey, 2017) that females had a greater ability to inhibit sympathetic vasoconstriction during exercise than males and a portion of the enhanced sympatholysis in females was attributable to NO. Previous studies have reported that basal NO release (Hayashi et al., 1992) and total NO production is greater in premenopausal females compared to males (Forte et al., 1998). Previous studies have also reported elevated

eNOS expression in the kidneys of spontaneously hypertensive female rats compared to male rats and in porcine brachial and femoral arteries. (Sasser, Brinson, Tipton, Crislip, & Sullivan, 2015) In the present study, skeletal muscle nNOS-alpha expression was greater in female than male rats, whereas nNOS-beta and eNOS expression were not different between groups. However, neither individual NOS isoform expression, nor total NOS expression were correlated with NO-mediated inhibition of sympathetic vasoconstriction suggesting that NOS expression may not be the major determinant of enhanced sympatholysis in female rats. The increased in nNOS expression may be functionally important in the control of blood pressure and tissue blood flow in female rats, however further investigation will be required to fully elucidate the mechanisms involved and also to determine the role of augmented nNOS expression in female rats in sympatholysis.

Effects of estrogen on NOS expression

Sex differences in NOS expression may be a function of reproductive hormones and estrogen has been shown to influence NO bioavailability and NOS expression (Fadel et al., 2003; Weiner et al., 1994). For example, estrogen supplementation for ovariectomized rats has been documented to increase the NOS activity in guinea pig skeletal muscle (Weiner et al., 1994). In our current findings, there was no significant difference in skeletal muscle nNOS expression under different estrogen status, including ovary intact, ovariectomized and ovariectomized with estrogen supplementation rats. Collectively, others (Lekontseva, Jiang, Schleppe, & Davidge, 2012) have documented that there was no alteration in nNOS expression in the thoracic aortas of ovary intact, ovariectomized, and ovariectomized with estradiol supplementation rats. Despite nNOS-mediated vasodilation affected by ovariectomized method, the chronical treatment with estradiol would not restore the nNOS. On the contrary, some studies (Ceccatelli et al., 1996;

García-Durán et al., 1999; Pelligrino, Santizo, Baughman, & Wang, 1998; Zhang, Massmann, Mirabile, & Figueroa, 1999) reported that estrogen deficiency has been shown to attenuate the nNOS in the brain, uterus, neutrophils and skeletal muscle. For example, in Fadel's paper (Fadel et al., 2003), they found that OVX rats appear to have a reduction of skeletal muscle nNOS expression and nNOS expression was restored in ovariectomized rats after 60-day estradiol supplementation. These differences might be due to other ovarian hormones are also involved in nNOS regulation. For example, the estrogen and progesterone have been shown to be essential to regulation nNOS and NO-mediated neuronal function in the brain regions (Martini, Pradotto, & Panzica, 2011) and sympathetic vasoconstriction was blunted in resting skeletal muscle of OVX rats chronically treated with progesterone compared with OVX rats. Whereas, the other study (Vedernikov et al., 1997) demonstrated that chronic treatment of OVX rats with sex hormones would not alter the production of NO or the sensitivity of NO in the aortic smooth muscle, indicating that sex hormones may increase the production or release of other endothelial factors such as inhibitory prostanoids and be beneficial against inhibition of endothelial function because of their calcium-channel blocking activity. It's also possible that the OVX group was not completely devoid of estrogen that the skeletal muscle NOS-isoforms expression was not different from rats with ovary intact, estrogen supplementation or ovariectomized. Estrogen can be synthesized in the adrenal glands and in adipose tissue (Simpson, 2003), and this may preserve endothelial NO production. While ovariectomized rats have been shown to lack ER-mediated rapid vasodilation in thoracic aortic rings (Bolego et al., 2005), it is possible that skeletal muscle resistance vessels in ovariectomized through redundancy in vasodilatory mechanisms to maintain normal vascular function.

Further, it's possible that NOS-isoform expression would be affected by the estradiol pellet containing or the concentration of the estrogen. Some studies(Hayashi et al, 1994) suggested that the effect of estrogen on nNOS expression might be associated with the level of hormones. For example, the low concentration ($10^{-10\text{-}7}$M) of 17β-estradiol would enhance the nNOS activity and high concentration ($10^{-6\text{-}5}$M) would attenuate it. Another possible explanation for this would be "timing-hypothesis" of estrogen supplementation, which termed as the effect of estrogen is depending on the time innervation of estradiol supplementation (Pinna, Cignarella, Sanvito, Pelosi, & Bolego, 2008) and estrogen therapy should be intervened as early as possible after menopause (Barrett-Connor, 2007). But how the time of estrogen supplementation affects the vascular function still have not been fully investigated.

Consistent with Fadel *et al* paper (Fadel et al., 2003), skeletal muscle eNOS expression was similar in the OI, OVX and OVXE rats. Further study will be required to investigate the NOS-isoform-mediated inhibition of sympathetic vasoconstriction under different estrogen status in the skeletal muscle.

Effects of Exercise Training on NOS expression

Our lab recently demonstrated that exercise training would enhance sympatholysis is dependent upon training intensity through a NO-dependent mechanism in the skeletal muscle (Jendzjowsky & Delorey, 2013c; Jendzjowsky & DeLorey, 2013; Jendzjowsky, Just, & DeLorey, 2014b; Just & Delorey, 2016). Furthermore, skeletal muscle nNOS expression was greater in exercise-trained compared to sedentary male rats and eNOS was similar in these groups. But above experimental results were based on the male rats, to our knowledge, this is the first paper to investigate whether exercise training alters the skeletal muscle NOS-isoform expression in female rats. In the current study, completion 10 weeks high-intensity exercise training would not

alter nNOS and eNOS expression compared to sedentary female rats. Contrast to our findings, some papers (Laughlin et al., 2003) reported that exercise-trained porcine expressed greater eNOS compared to the sedentary female porcine in the brachial and femoral arteries. But they also investigated that there was no interaction between gender and eNOS expression which consistent with our finding. In the present study, eNOS expression was measured in the homogenate skeletal muscle, not the arteries. It's possible that shear stress was augmented after exercise training to increase eNOS expression, but the mechanism was not fully elucidated. Another study also reported that exercise training and estrogen might play a synergistic role in increasing eNOS expression via upregulation of the eNOS gene (Kojda & Hambrechit, 2005; Mendelsohn, 2000; Moreau & Ozemek, 2017). Chronic estrogen deficiency reduces the ER-α expression (Chakrabarti & Davidge, 2009; Moreau & Ozemek, 2017; Pinna et al., 2008), resulting in impaired ER-α/eNOS signaling (Pinna et al., 2008). They concluded that estrogen and exercise maintain the endothelium function through ER-α/eNOS signaling pathway and may be related to vascular adaptation to exercise-trained postmenopausal female (Moreau & Ozemek, 2017).Thus, further investigation will be required to detect whether aerobic exercise training modify NOS-isoform expression in ovariectomized rats to figure out whether training can restore these vascular functions and how the exercise training affect the female rats in the resting and contracting skeletal muscle in functional study. So that, it's clear to know the role of exercise training in NOS expression in the OVX rats and whether the exercise training would be a potential therapy to restore vascular function for the postmenopausal women.

Consistent with the previous study from our lab (Jendzjowsky, Just, & DeLorey, 2014b; Just, Cooper, & DeLorey, 2016), exercise training augmented NO-dependent inhibition of sympathetic vasoconstriction and exercise training-induced augmented nNOS expression in the

high proportion of Type II, glycolytic muscle fiber. It indicated that the greater nNOS- mediated inhibition of vasoconstriction occurs in the glycolytic (Brooke, Kaiser, & Denver, 1970) compared to oxidative skeletal muscle. In contrast, eNOS expression was higher in the oxidative compared to glycolytic skeletal muscle in exercise-trained and sedentary female rats which consistent with Lau et al study (Lau et al., 2000). That might be due to eNOS preferential expression in the oxidative skeletal muscle and nNOS is less abundant in oxidative slow-twitch fibers (Thomas & Victor, 1998). Thus, exercise training might have adaptions in the skeletal muscle fibers that enhance NO-dependent sympatholysis and estrogen might influence eNOS expression in blood vessels in the oxidative skeletal muscle to improve vascular function. Further study will be required to investigate the role of skeletal muscle fiber types in exercise-trained adaptations in sympatholysis in female rats.

Correlation between NOS expression and NO-mediated inhibition of sympathetic vasoconstriction

A previous study (Fadel et al., 2003) has documented that nNOS expression was highly related to estrogen status and negative related to the sympathetic vasoconstrictor responses in the contracting skeletal muscle. Some studies also concluded that sympatholysis was decreased in postmenopausal women (Fadel et al., 2004) and ovariectomized female rats (Fadel et al., 2003). In the current study, we used the non-selective blockade L-NAME to measure the magnitude of vasoconstriction response to sympathetic stimulation in resting and contracting skeletal muscle and found that there was no relationship between the NO-mediated sympathetic vasoconstriction and NOS-isoform expression in exercise training and estrogen status groups. But the potential mechanisms behind the relationship were still not fully identified. Furthermore, NO bioavailability plays an important role in the regulation of blood pressure, but it`s hard to

measure NO bioavailability directly due to its high solubility, freely diffuses through biological membranes (Tamanini, Basini, Grasselli, & Tirelli, 2002). Thus, we tried to use western blot to measure the NOS protein expressed by the tissues normalized by GAPDH. GAPDH is an essential enzyme to involve in the energy metabolism by producing ATP and pyruvate (Nicholls, Li, & Liu, 2012). The loading control is the protein which expressed consistent at different experimental conditions. In my thesis, GAPDH as the loading control which expressed constantly at different sex, estrogen levels, and exercise training conditions. But western blot was one protocol which just measured the total protein content ends up with in the skeletal muscle so that it still cannot know the protein content which was expressed during the process of exercise and how much protein demand by the tissue. Due to eNOS also expressed in the endothelial to improve vascular function, but in our experiments, we homogenate the skeletal muscle so that the tissue-specific effects on NOS expression would not be investigated. Furthermore, blockade of nNOS activity in the brain causes systemic and pulmonary hypertension and blunts the blood flow (Toda, Ayajiki, & Okamura, 2009). Protein expression reflects the total protein content ends up with in the skeletal muscle, but it cannot predict the enzymatic activity accurately. However, the enzymatic activity reflects the ability to increase the rates of reactions, which depends on enzyme concentration, temperature, the presence of any required cofactors. For example, the enzymatic activity of nNOS and eNOS is regulated by Ca^{2+} and calmodulin (Förstermann & Sessa, 2012). Thus, it is uncertain whether increased NOS-isoforms expression leads to greater NO formation.

Our laboratory (Jendzjowsky, Just, & DeLorey, 2014b) has proven that nNOS-mediated inhibition of sympathetic vasoconstriction in the resting and contracting skeletal muscle. In the current study, these data showed that NO-dependent inhibition of sympathetic vasoconstriction

in the resting and contracting skeletal muscle was not correlated with nNOS and eNOS expression in both exercise-trained and estrogen status group. Thus, it indicated that NOS isoform expression could not definitively reflect the NO bioavailability and NO bioavailability is highly regulated by other factors beyond NOS-isoform expression. The vascular oxidative stress is the main considering reason that attributed to decrease NO bioavailability and reactive oxygen species (ROS) are attributed to inactivate NO (Thomas, Zhang, & Victor, 2001). Excessive ROS production impairs endothelial function by scavenging NO and decreasing NO biosynthesis (Fukai et al., 2000). For example, our lab (Jendzjowsky & Delorey, 2013a) has reported that blunted sympathetic vasoconstriction during O_2^- scavenging was likely mediated by an increased NO-dependent sympathetic vasoconstriction in exercise-trained rats. Moreover, the different modes training will have different results. The acute exercise training would increase ROS production, but chronic endurance exercise training improves the antioxidant defense systems and mitochondrial function (Mankowski, Anton, Buford, & Leeuwenburgh, 2015). Evidence also supported that superoxide levels are greater in the renal cortex of male compared with females spontaneously hypertensive rats (Sullivan, Sasser, & Pollock, 2007). Thus, oxidative stress might affect by exercise training and estrogen status via a NO-mediated system. Thomas et al. (Fadel et al., 2012) have reported heart failure would increase superoxide production in the rat contracting skeletal muscle results in augmented sympathetic vasoconstriction, probably due to superoxide-mediated inactivation of NO. Another study (Fadel et al., 2004) has shown that NO derived from nNOS attenuates α-adrenergic vasoconstriction in the contracting skeletal muscle. The production of O_2^- and NO has been reported to augment in the contracting skeletal muscle (Balon & Nadler, 1994; Reid et al., 1992). But NO and O_2^- undergo a rapid diffusion-limited reaction, excessive O_2^- production in the skeletal muscle might reduce the functional impact of NO by

decreasing NO bioavailability. Thus, combined with our findings, the oxidative stress-mediated NO system is an important mechanism underlying NO-mediated sympathetic vasoconstriction and the regulation of NO bioavailability to modulate the skeletal muscle blood flow and blood pressure.

In the present study, nNOS expression was correlated with NO-dependent inhibition of sympathetic vasoconstriction in the contracting skeletal muscle in response to sympathetic stimulation delivered at 2Hz in female and male groups. However, this phenomenon has not been shown in the resting skeletal muscle and eNOS expression was not correlated with functional data. These data indicated that there might exist different pathways in the resting and contracting skeletal muscle to inhibition of sympathetic vasoconstriction and eNOS and nNOS make a different contribution in the resting and contracting skeletal muscle. From our laboratory previous papers (Jendzjowsky, Just, & DeLorey, 2014b; Just et al., 2016), we tended to use isoform-specific blockade nNOS followed by non-selective blockade to distinguish the relative contribution of NO derived from nNOS and eNOS to the inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle. We found that different NOS-isoforms made a different contribution in the resting and contracting skeletal muscle. For example, NO derived from eNOS made a relatively larger contribution about 65% to the inhibition of sympathetic vasoconstriction in resting skeletal muscle and exercise training increases nNOS-mediated inhibition of sympathetic vasoconstriction in the contracting skeletal muscle (Jendzjowsky, Just, & DeLorey, 2014b; Just et al., 2016). The evidence suggests that contraction-induced an augment of nNOS-mediated NO production (Jendzjowsky, Just, & DeLorey, 2014b; Just et al., 2016). Collectively, nNOS was contributed about 75% to the inhibition of sympathetic vasoconstriction in exercise training and 50% in sedentary male rats.

Another study (Thomas et al., 1998) also reported that nNOS might inhibit sympathetic vasoconstriction in contacting skeletal muscle in the model of nNOS knockout mice. NO production is lower in resting skeletal muscle, but is increased adequately in contracting skeletal muscle and intracellular Ca^{2+} concentration, indicating Ca^{2+} -induced activation of nNOS (Stamler & Meissner, 2001) and may involve cGMP-dependent attenuate of receptor-mediated Ca^{2+} influx into vascular smooth muscle (Thomas et al., 1998). Moreover, nNOS-mediated production of NO was related to the cGMP and relaxation of vascular smooth muscle during muscle contraction (Grange et al., 2001; Lau et al., 2000). The further study still needs to investigate the mechanism of inhibition of sympathetic vasoconstriction in contracting skeletal muscle.

Conclusion

In conclusion, this study demonstrated greater skeletal muscle nNOS expression in female compared to male rats. However, the greater nNOS expression in females does not appear to be a function of estrogen status as NOS expression was not different between ovary-intact, ovariectomized and ovariectomized-estrogen supplemented female rats. Furthermore, in contrast to our previous findings in male rats, (Jendzjowsky, Just, & DeLorey, 2014b) exercise training does not appear to augment NOS expression in female rats skeletal muscle NOS expression was not correlated to the NO-mediated the inhibition of sympathetic vasoconstriction resting and contracting skeletal muscle. These results suggest that other factors beyond NOS expression modulate NO bioavailability and determine the magnitude of NO dependent sympatholysis. Further study will be required to fully elucidate the mechanism behind the enhanced sympatholysis in females.

Chapter 3 General Discussion

Main findings

The purpose of the thesis was to investigate:1) the effect of biological sex on nNOS and eNOS protein levels in skeletal muscle; 2) the effect of estrogen on skeletal muscle nNOS and eNOS protein levels; 3) whether exercise training alters skeletal muscle NOS expression in female rats, and; 4) whether the magnitude of NO-mediated inhibition of the sympathetic vasoconstriction in resting and contracting skeletal muscle is a function of skeletal muscle NOS expression. The important novel findings from this study were that skeletal muscle nNOS expression was greater in female compared to male rats. However, the greater nNOS expression in females does not appear to be a function of estrogen status as NOS expression was not different between ovary-intact, ovariectomized and ovariectomized-estrogen supplemented female rats. Furthermore, in contrast to our previous findings in male rats, (Jendzjowsky, Just, & DeLorey, 2014b) exercise training does not appear to augment NOS expression in female rats skeletal muscle NOS expression was not correlated to the NO-mediated the inhibition of sympathetic vasoconstriction resting and contracting skeletal muscle. These results suggest that other factors beyond NOS expression modulate NO bioavailability and determine the magnitude of NO dependent sympatholysis. Further study will be required to fully elucidate the mechanism behind the enhanced sympatholysis in females.

This study suggested that biological sex modulates skeletal muscle nNOS expression with females having higher levels of nNOS compared to males. This is strong evidence to support the sex difference in the regulation of blood flow and vascular function and female enhances the functional sympatholysis is partly NO-mediated. Moreover, it's possible that sex difference alters the NO derived from nNOS expression to modulate the vascular function.

However, after 10 weeks' ovariectomy, the nNOS and eNOS expression in OVX rats have no difference compared to the ovary intact rats. And the 10 weeks' estrogen supplementations for ovariectomized rats did not alter the NOS expression. Several papers demonstrated that estrogen has a dramatic impact on cardiovascular health and might be toward NO (Chambliss & Shaul, 2002; Miller & Duckles, 2008). Combined with this study, it could suggest that estrogen may have another way to mediate the NO level beyond NOS expression and we still need more experiments to prove this argumentation. Moreover, this study demonstrated that 10 weeks' of heavy intensity treadmill exercise training did not affect NOS expression in the ovary intact female rats. Combined with our previous study (Jendzjowsky, Just, & DeLorey, 2014b), these finding would suggest that exercise training has a various impact on female and male rats, even expressed at different levels in the different muscle types (soleus and gastrocnemius skeletal muscle). For example, in our previous paper (Jendzjowsky, Just, & DeLorey, 2014b), we found that exercise training enhances nNOS expression in male rats. Specifically, nNOS was greater in the gastrocnemius skeletal muscle and eNOS was expressed similar in the oxidative and glycolytic skeletal muscle. In this study, we found that exercise training might have less impact on the healthy female rats and eNOS was expressed greater in the soleus skeletal muscle. Finally, we used the functional data combining with the NOS expression to determine the relationship between the NOS expression and NO-dependent inhibition of sympathetic vasoconstriction. There was no relationship between the NOS expression and NO-dependent inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle. This would suggest that NO is highly regulated by the other factors beyond NOS expression.

Implications

In this study, we focus on the effect of sex difference, estrogen status and exercise training on nNOS and eNOS expression to study the underlying mechanism behind NO-mediated functional sympatholysis. Combined with our previous laboratory study (Just & DeLorey, 2017), it could conclude that the females enhance the functional sympatholysis was NO-mediated and improvement of nNOS expression might be the underlying mechanism. To our knowledge, this is the first study to compare the amount of skeletal muscle NOS-isoform expression in female and male rats. This research could help to establish and compensate the underlying mechanisms of sex difference in the regulation of vascular function. Furthermore, eNOS is the principal source of NO in the vascular wall (Chambliss & Shaul, 2002), but the conclusion we got from this study did not show the difference in skeletal muscle eNOS expression in female and male rats.

Estrogen has been shown to upregulate the NO bioavailability and NOS expression (Fadel et al., 2003; Carl P. Weiner et al., 1994). In contrast to a previous paper (Fadel et al., 2003), we found that the 10 weeks' ovariectomy and estrogen supplementations did no influence on the skeletal muscle nNOS and eNOS expression. A novel aspect of this study was to measure the effect of different levels of estrogen status on NOS expression in three kinds of skeletal muscle. Although there has no significant difference in ovary intact, ovariectomized rats and estrogen supplementation in nNOS and eNOS in the skeletal muscle, the different skeletal muscle expressed different levels of NOS. In the soleus skeletal muscle, eNOS expression was higher compared to the gastrocnemius skeletal muscle, whereas, the glycolytic muscle expressed greater nNOS compared to the oxidative muscle. Thus, skeletal muscle NOS-isoform expression is muscle fiber-type dependent.

Our laboratory has reported that exercise training can improve NO-mediated sympatholysis, which was related to the increased nNOS expression and augment the nNOS-mediated the inhibition of the sympathetic vasoconstriction (Jendzjowsky & Delorey, 2013c; Jendzjowsky, Just, & DeLorey, 2014a, 2014b). It indicated that exercise training has been considered as an effective way to improve the functional sympatholysis in males. The postmenopausal females tend to have a higher risk of cardiovascular disease compared to the young females. And the ovariectomized rats have been shown to reduce the inhibition of sympathetic vasoconstriction. In this study, it's a novel finding that the how the NOS expression affected by exercise training in the female rats and this is an interesting implication for the future studies. If exercise training works, it can be useful in the postmenopausal populations and would be a better therapy than the hormonal therapy without side effects (Hulley et al., 2002; Johnson et al., 2016).

Experimental Considerations and Limitations

The most important purpose of this study was to investigate the effect of sex difference, estrogen status and exercise training on NOS expression. The three subsequence experiments mainly focus on the NOS expression level in the skeletal muscle, which determined by using Western blot. Western blot has been considered as an important method to analyze how much protein content ends up with in many tissues and it's also the main experiment in this study. In this current study, the first step to determine the protein level in the skeletal muscle was to homogenate the three kinds of skeletal muscle. Therefore, drawbacks of experimental protocol in this project include doing the homogenate the skeletal muscle before test the protein content. We did the homogenate in the lysis buffer to break open cells for use in molecular biology experiments. But we directly did homogenate the skeletal muscle and therefore tissue-specific

effects under these three conditions on NOS expression could not be identified. For example, it's hard to distinguish the protein content which was expressed during the process of exercise and how much protein demand by the tissue. It's possible that NOS-isoform expressed from the skeletal muscle fibers, endothelium, smooth muscle, which limited to ensure the capacity of NOS expression. In addition, it`s hard to measure NO bioavailability directly due to its high solubility, freely diffuses through biological membranes (Tamanini, Basini, Grasselli, & Tirelli, 2002).

Future Directions

The present study utilized the Western Blot to measure the NOS-isoforms expression in the skeletal muscle and assess the relationship between the NOS expression and NO-mediated the inhibition of the sympathetic vasoconstriction. However, the present study measured the effect of exercise training on NOS expression in the healthy ovary intact rats, we did not measure how exercise training affects NOS-isoform expression in the ovariectomized rats. In the future, using the Western Blot to measure how exercise training influences the NOS-isoform expression in OVX rats. Aerobic exercise training is considered as an effective and low-cost treatment for improving the vascular function. If exercise training could have a great impact on the NOS expression in OVX rats and augment nNOS expression, it will give a supportive evidence to implicate the exercise training improve the vascular health and decrease the risk of the cardiovascular disease in the postmenopausal females.

In addition, this study would suggest that there have some other factors affect the NO bioavailability beyond the NOS expression. Future studies could investigate the mechanism by the regulation of NO production and bioavailability, discuss which possible factors might be highly associated with the NO bioavailability. For example, BH_4, ROS and SOD involved in the

pathway of the production of NO. A study has been reported that the addition of BH_4 resulted in an increased in NO production in microvascular endothelial cell line (Bevers et al., 2005). A follow-up study could measure how the different level of SOD, BH_4, ROS influence the NO bioavailability in the skeletal muscle, whether these factors are highly related to the NO bioavailability. Therefore, the future directions of this line of study would be the investigation of the effect of exercise training on NOS-isoform expression in OVX rats, as well as the other possible factors might be involved in the regulation of NO bioavailability.

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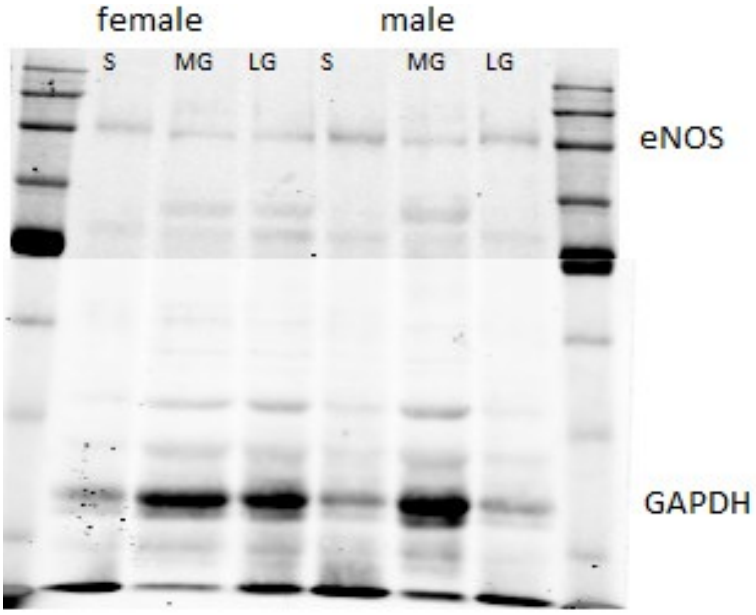
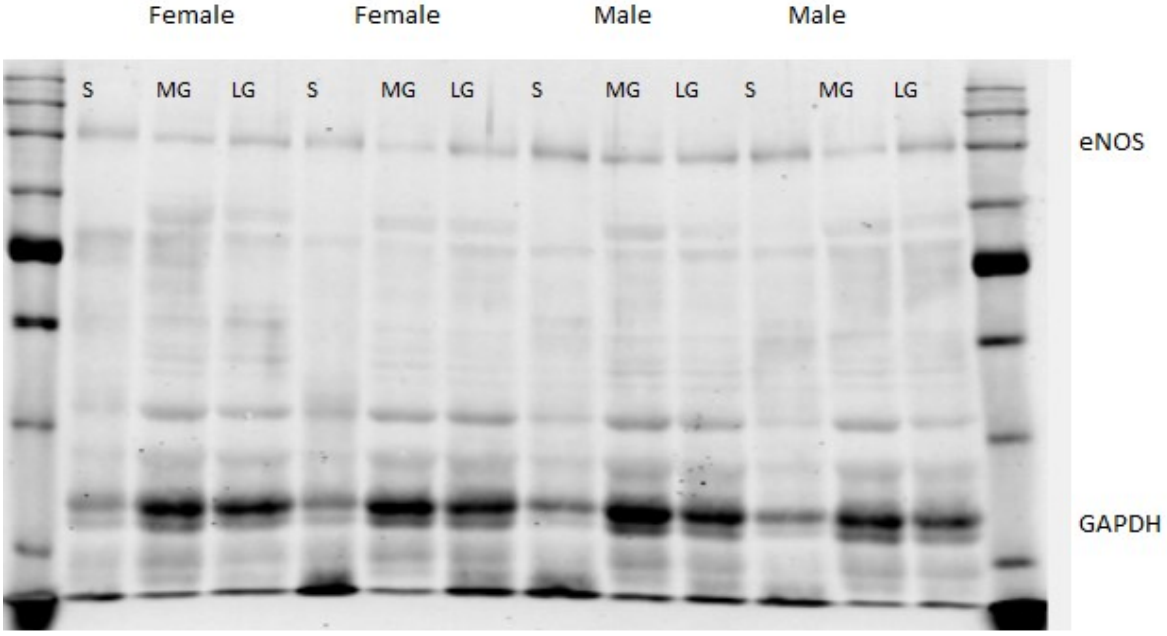
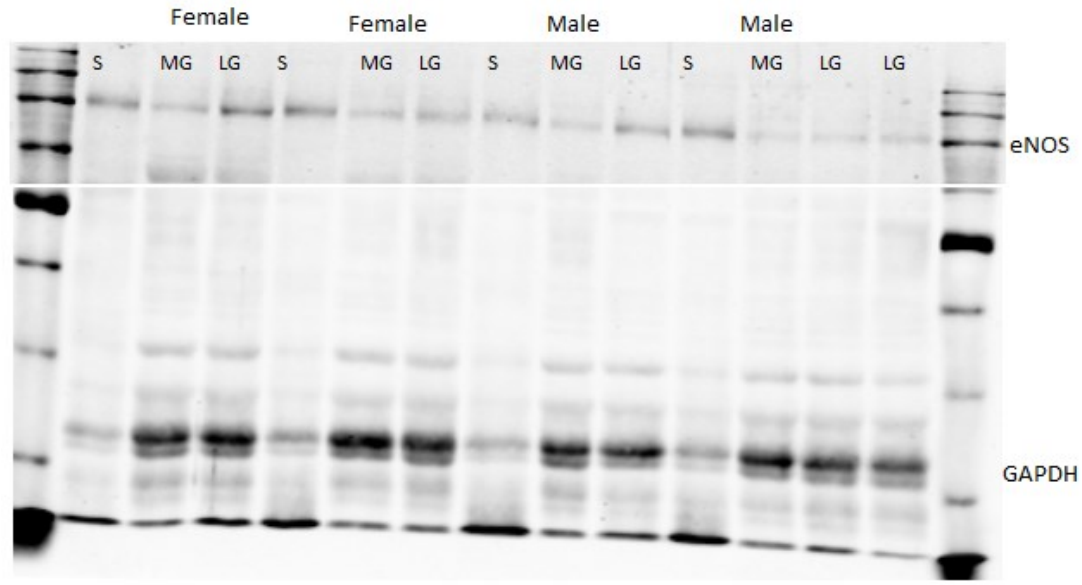
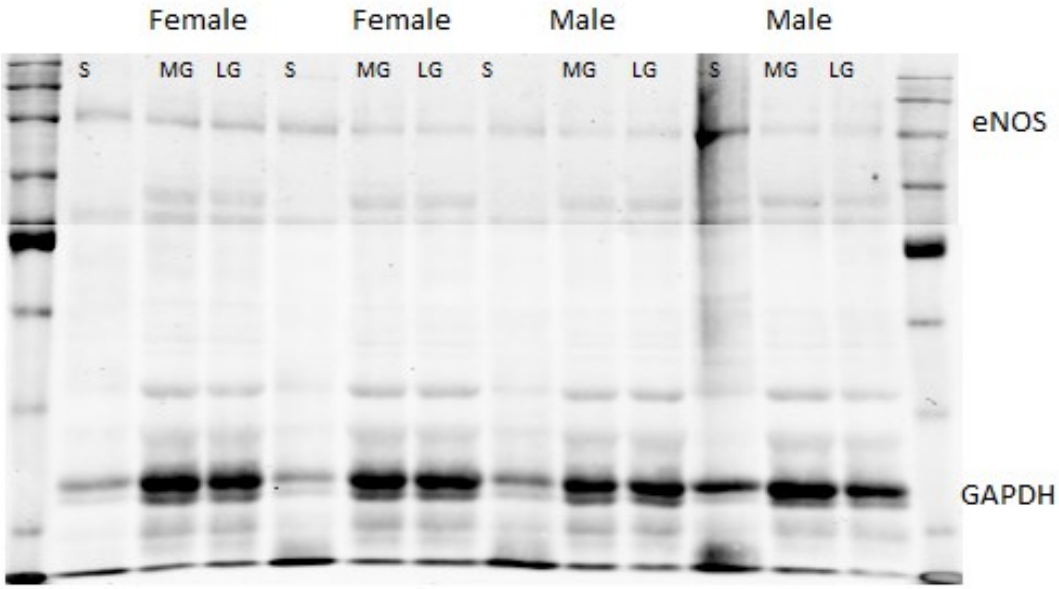
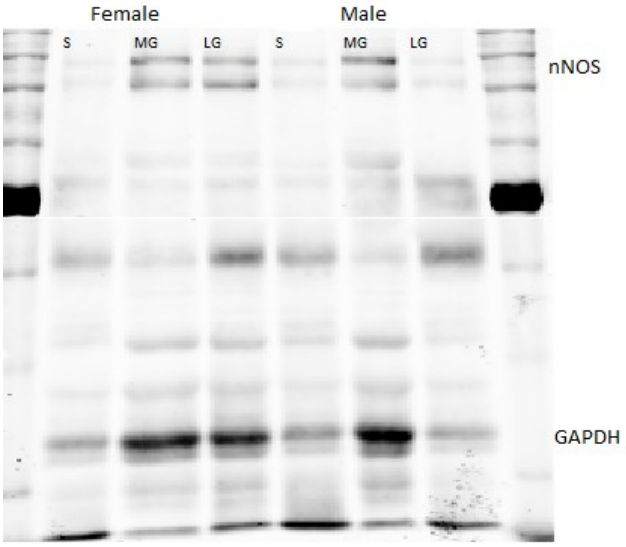
Appendix A: Blots of sex difference on nNOS and eNOS expression.

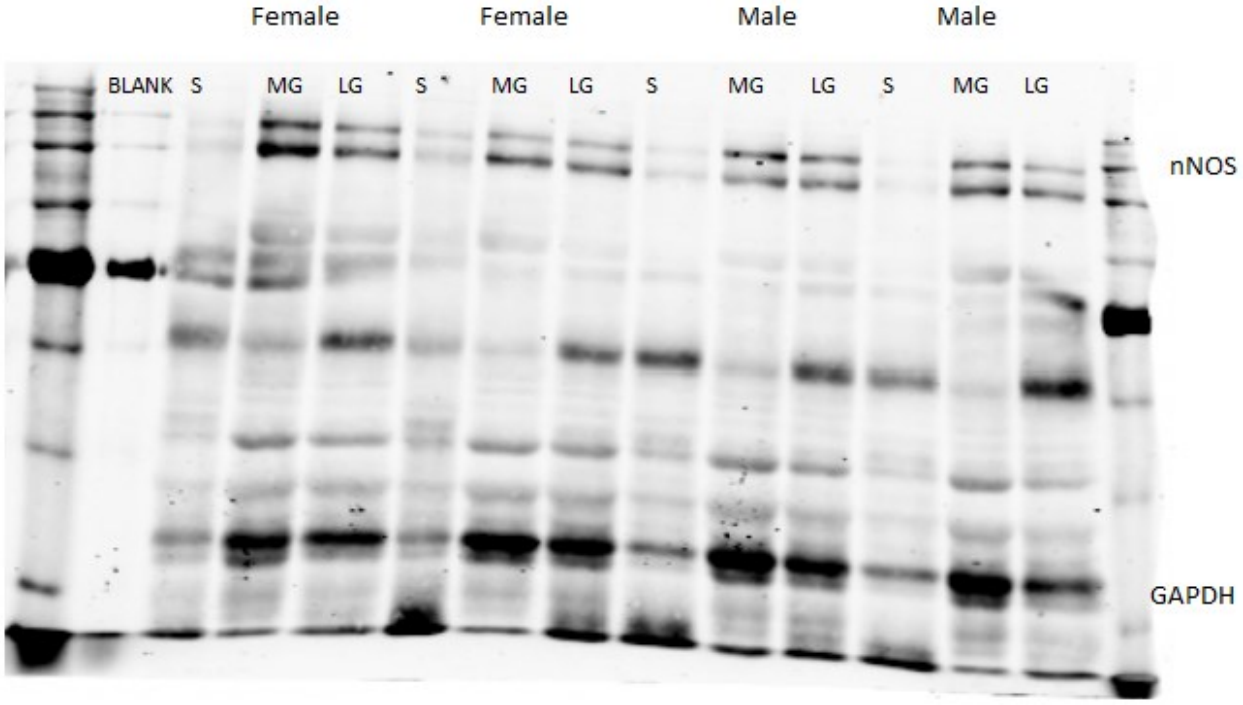


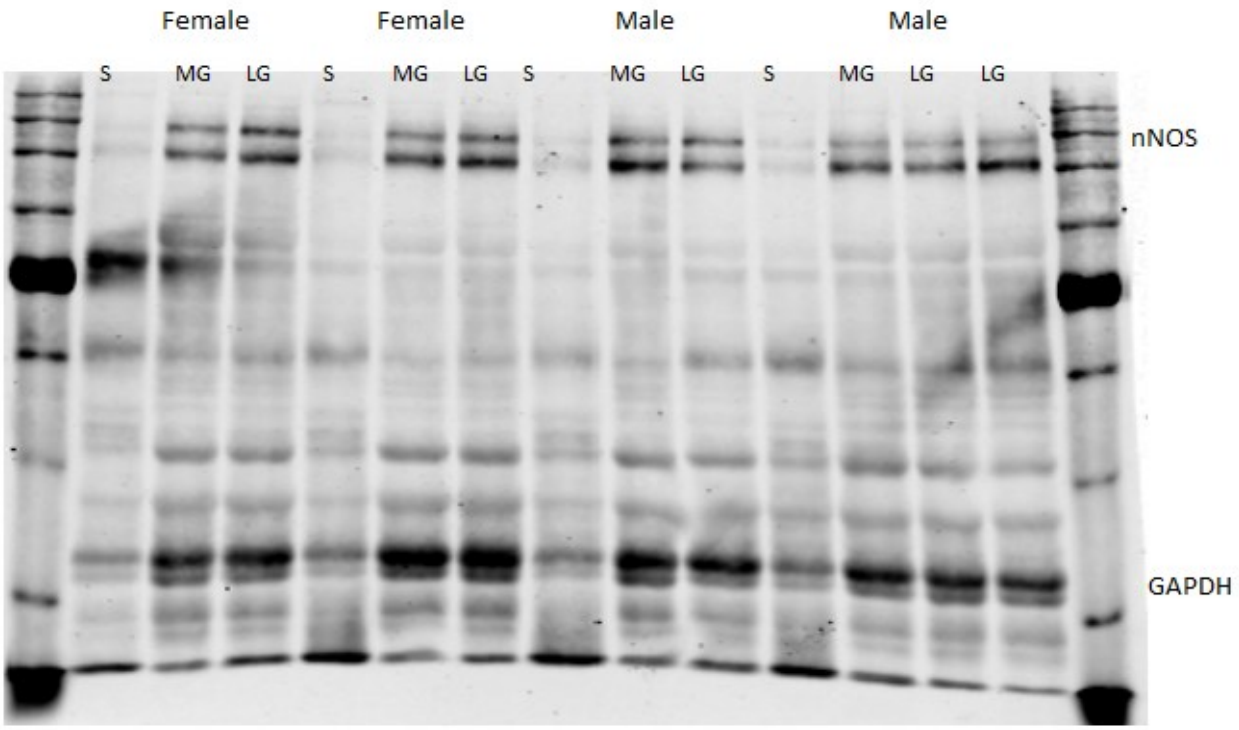


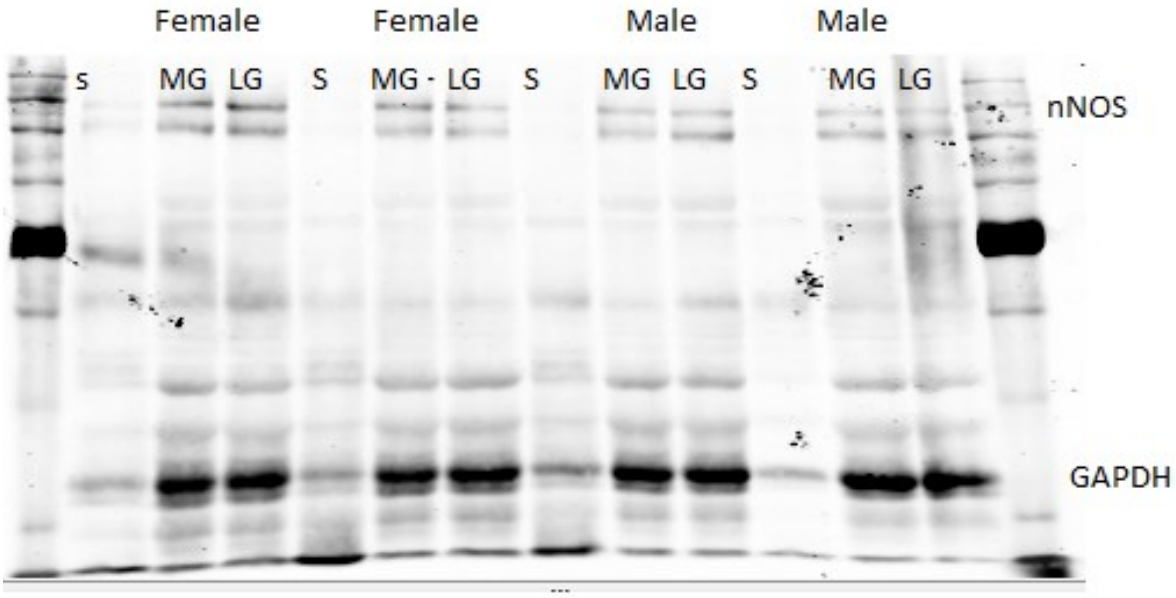

Female
Female
Male
Male
S
MG
LG
S
MG
LG
S
MG
LG
S
MG
LG
LG
eNOS
GAPDH


Female
Female
Male
Male
S
MG
LG
S
MG
LG
S
MG
LG
S
MG
LG
eNOS
GAPDH

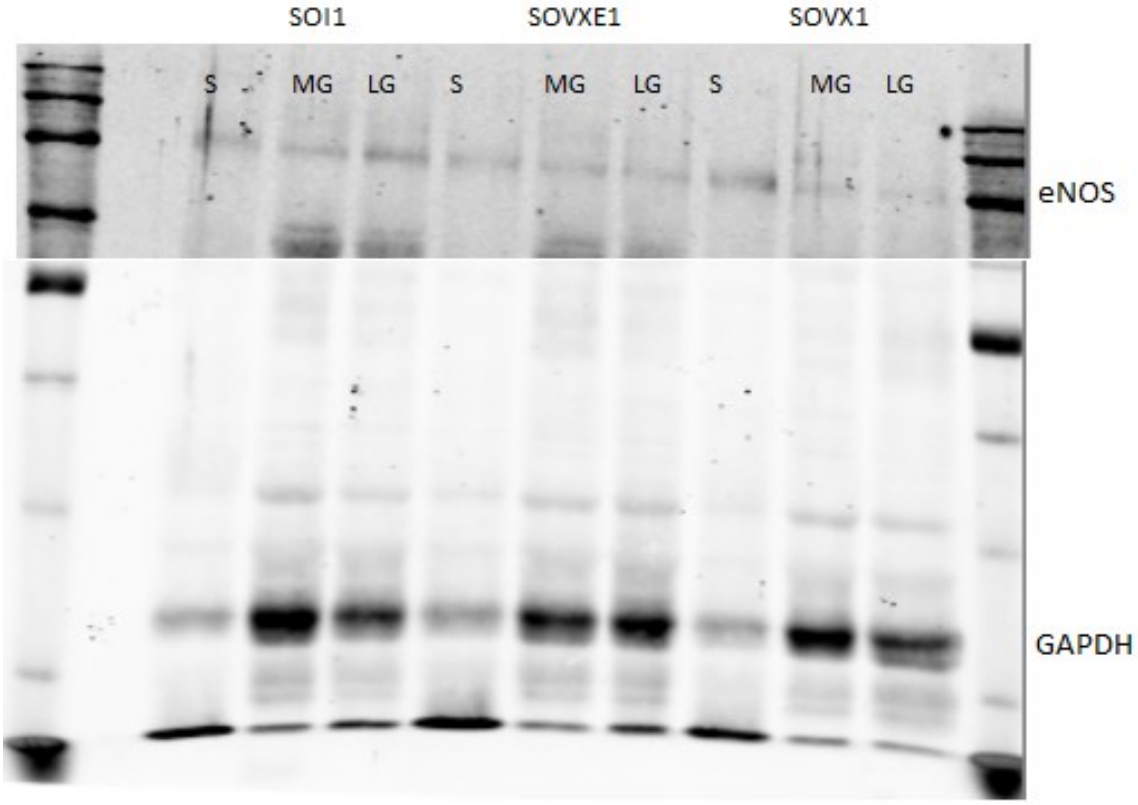

Female
Male
S
MG
LG
S
MG
LG
nNOS
GAPDH

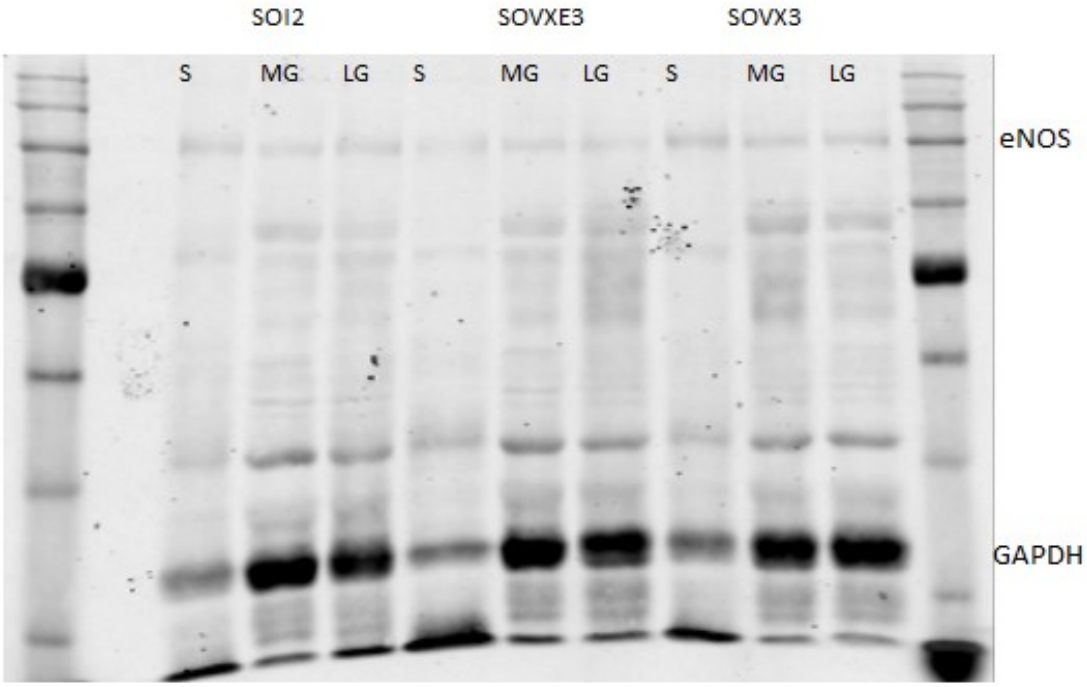

Female
Female
Male
Male
BLANK
S
MG
LG
S
MG
LG
S
MG
LG
S
MG
LG
nNOS
GAPDH

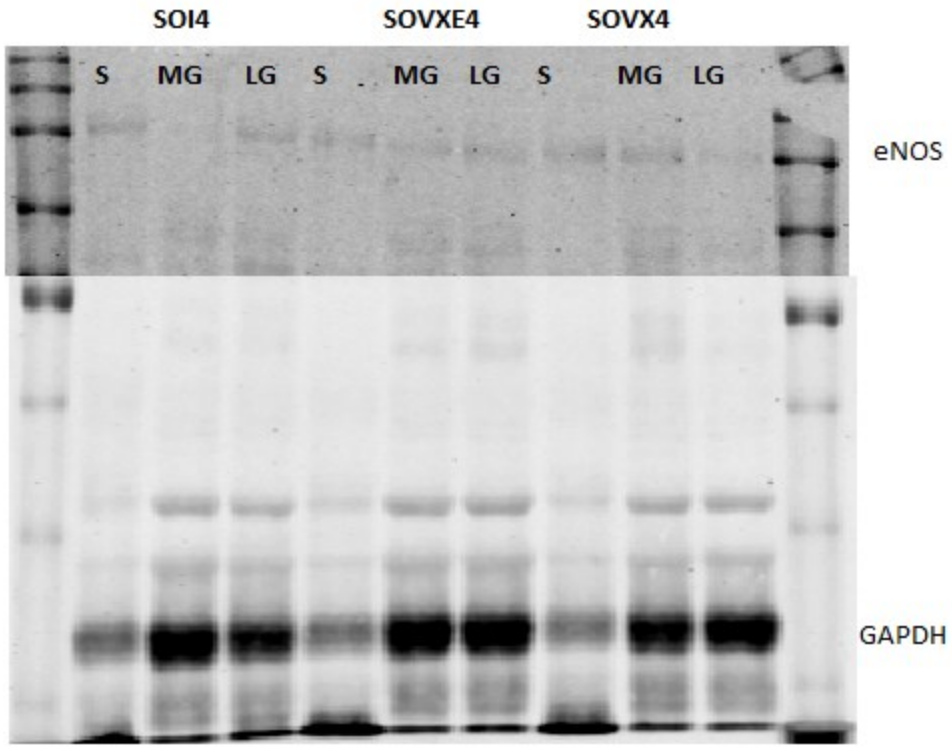

Female
Female
Male
Male
S
MG
LG
S
MG
LG
S
MG
LG
S
MG
LG
LG
nNOS
GAPDH

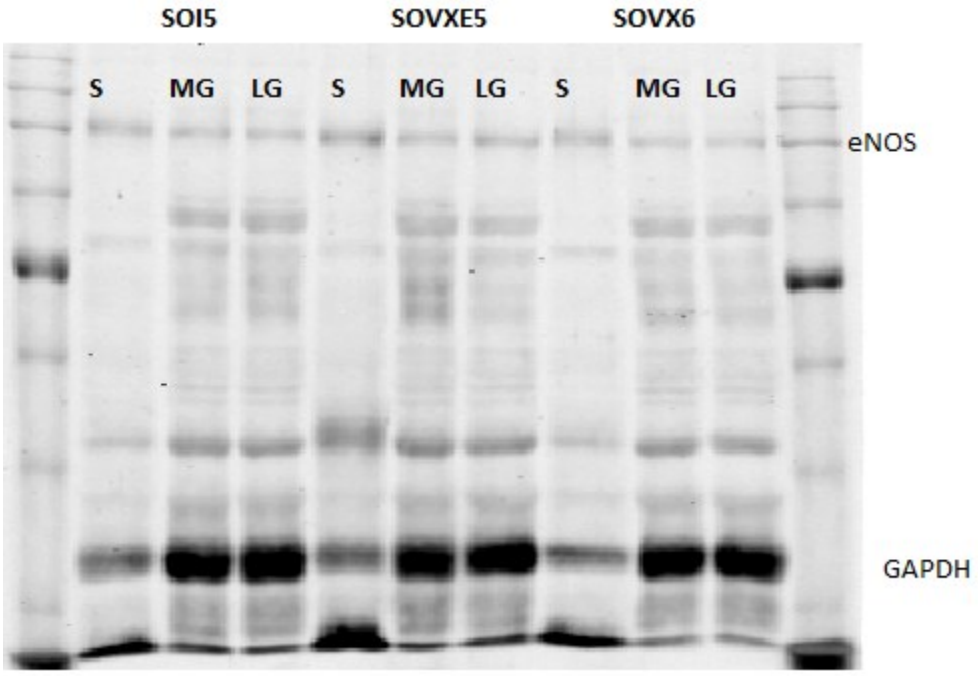

Female
Female
Male
Male
s
MG
LG
S
MG
LG
S
MG
LG
S
MG
LG
nNOS
GAPDH

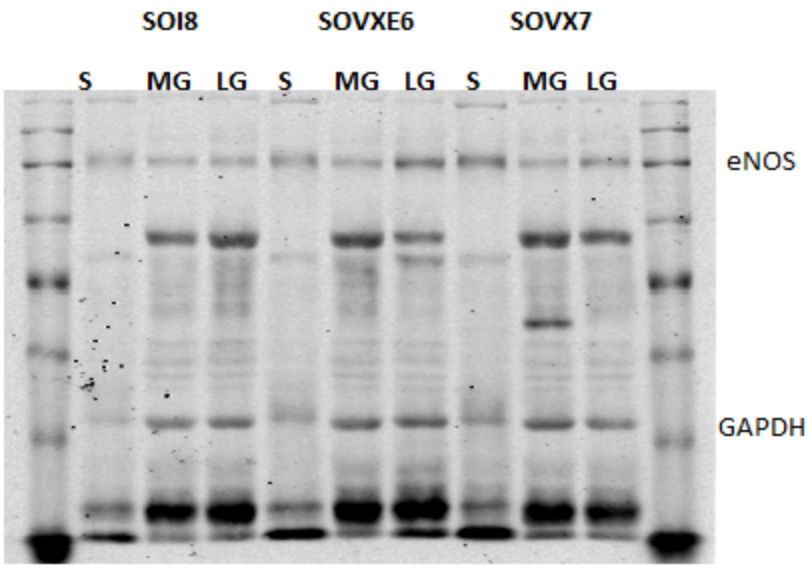
Appendix B: Blots of estrogen on nNOS and eNOS expression.

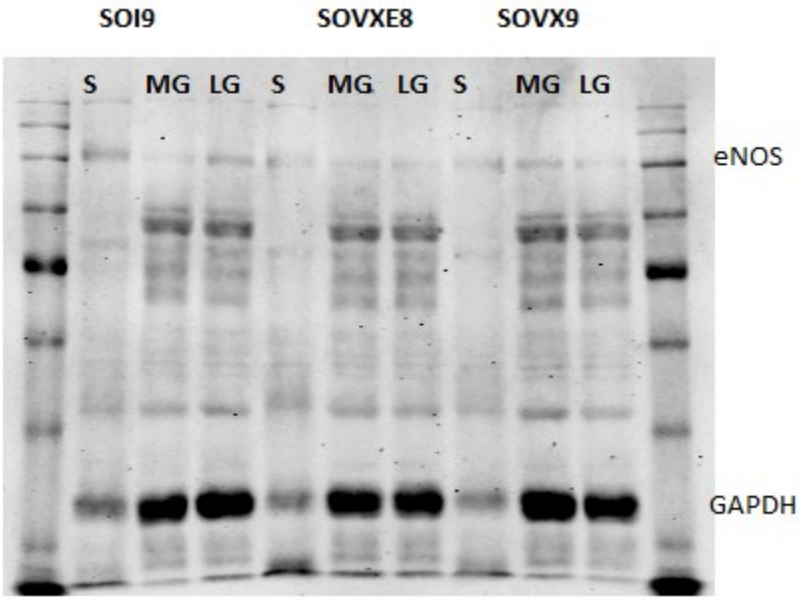


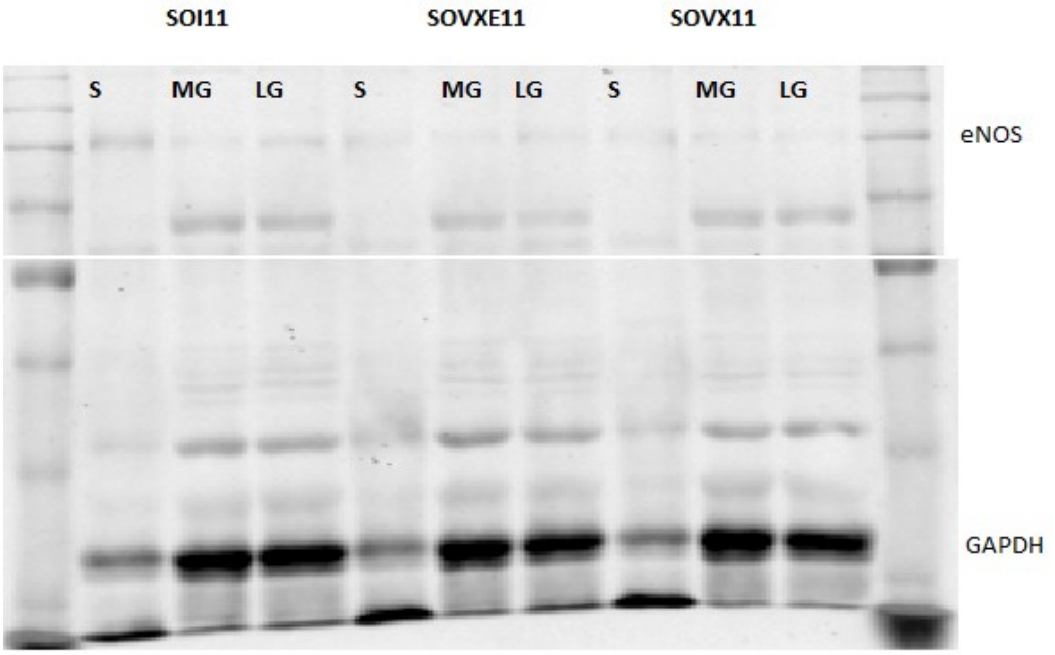


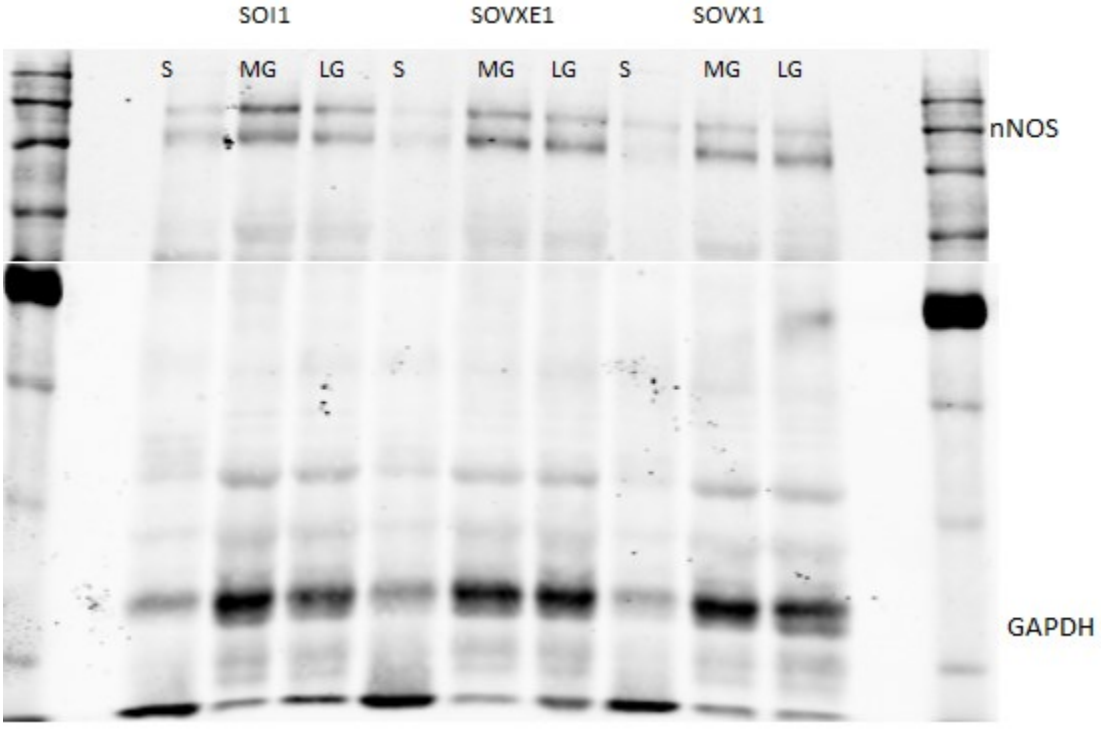

SOI4
SOVXE4
SOVX4
S
MG
LG
S
MG
LG
S
MG
LG
eNOS
GAPDH

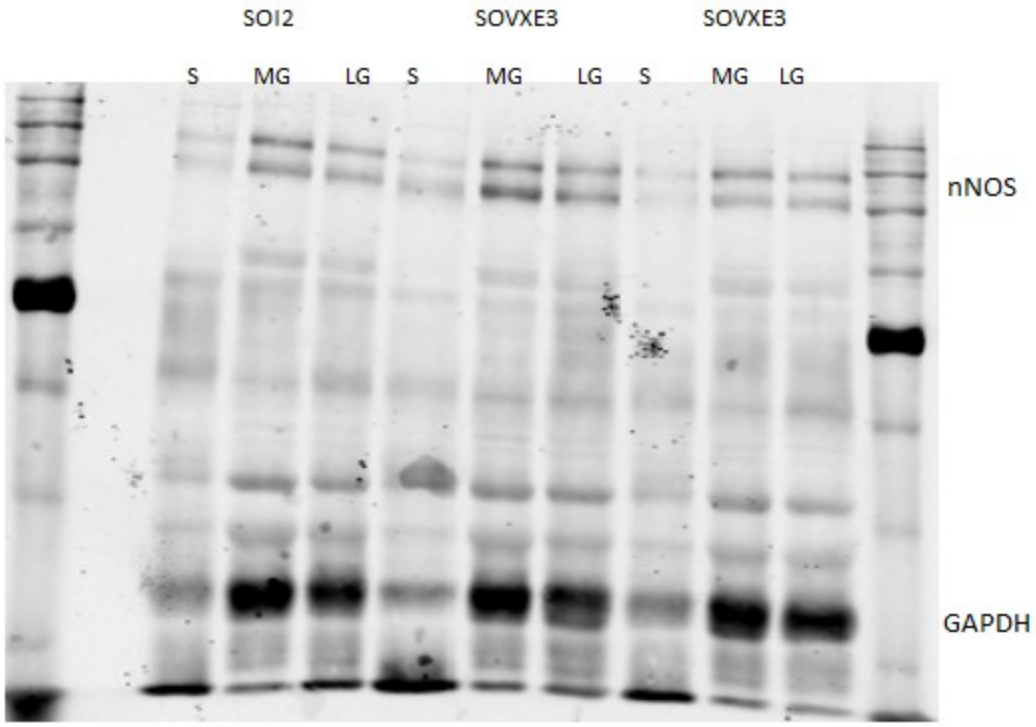

SOI5
SOVXE5
SOVX6
S
MG
LG
S
MG
LG
S
MG
LG
eNOS
GAPDH

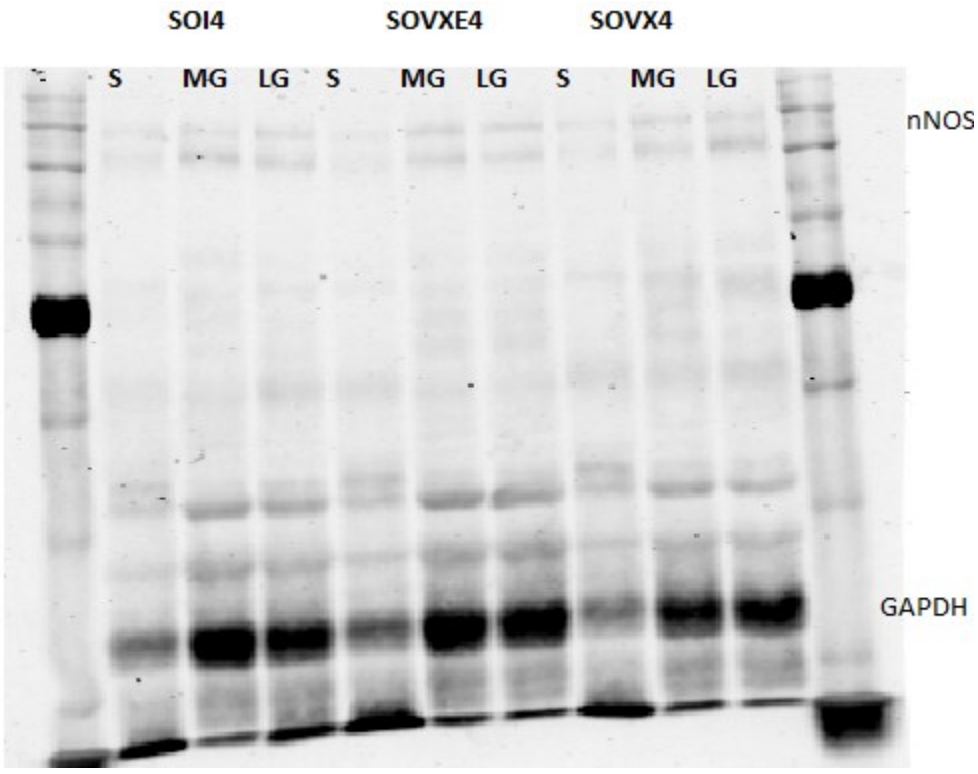

SOI8
SOVXE6
SOVX7
S
MG
LG
S
MG
LG
S
MG
LG
eNOS
GAPDH

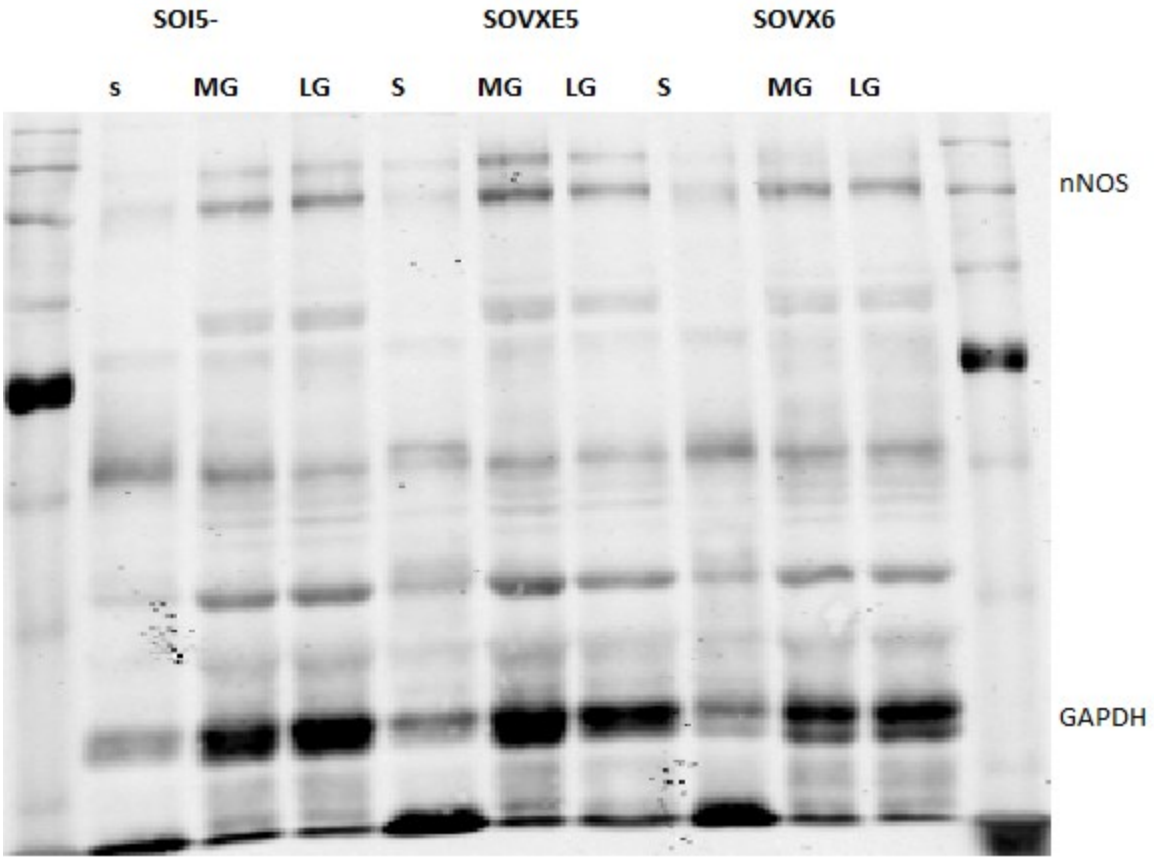

SOI9
SOVXE8
SOVX9
S
MG
LG
S
MG
LG
S
MG
LG
eNOS
GAPDH

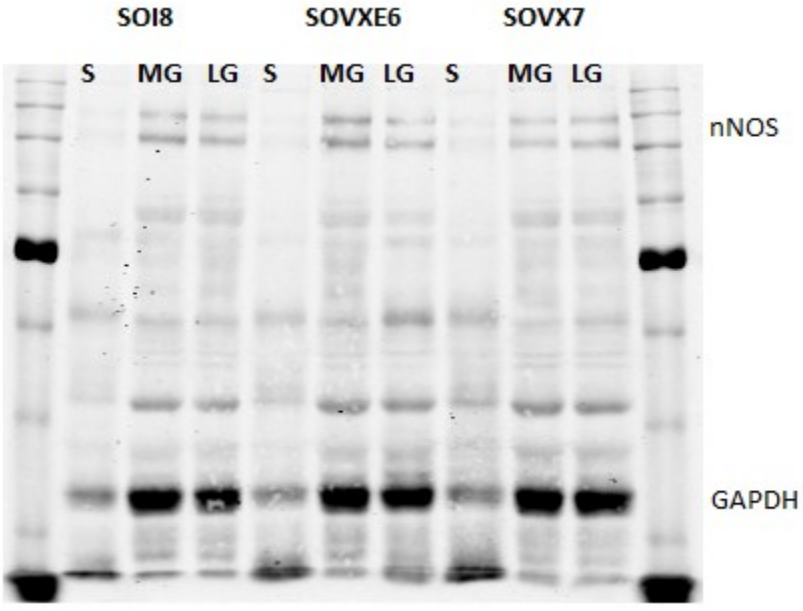

SOI11
SOVXE11
SOVX11
S
MG
LG
S
MG
LG
S
MG
LG
eNOS
GAPDH

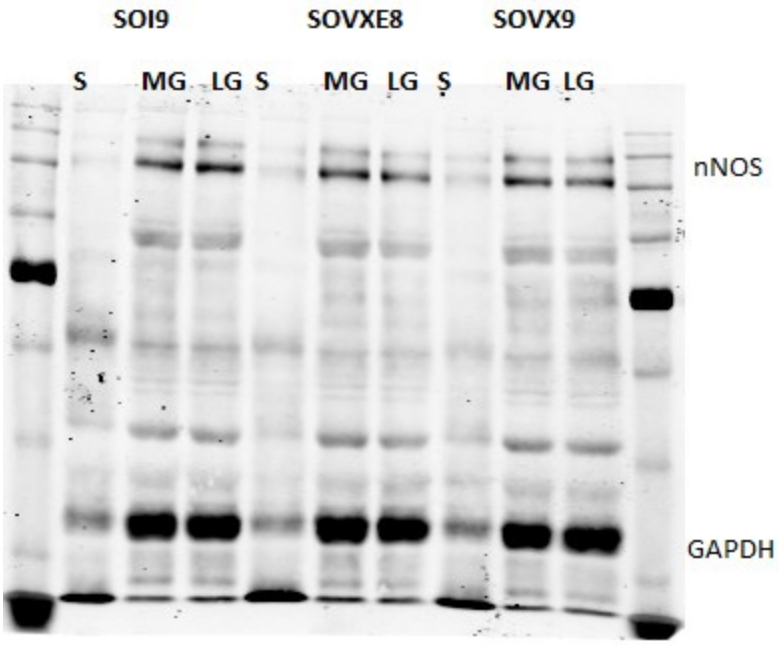

SOI1
SOVXE1
SOVX1
S
MG
LG
S
MG
LG
S
MG
LG
nNOS
GAPDH

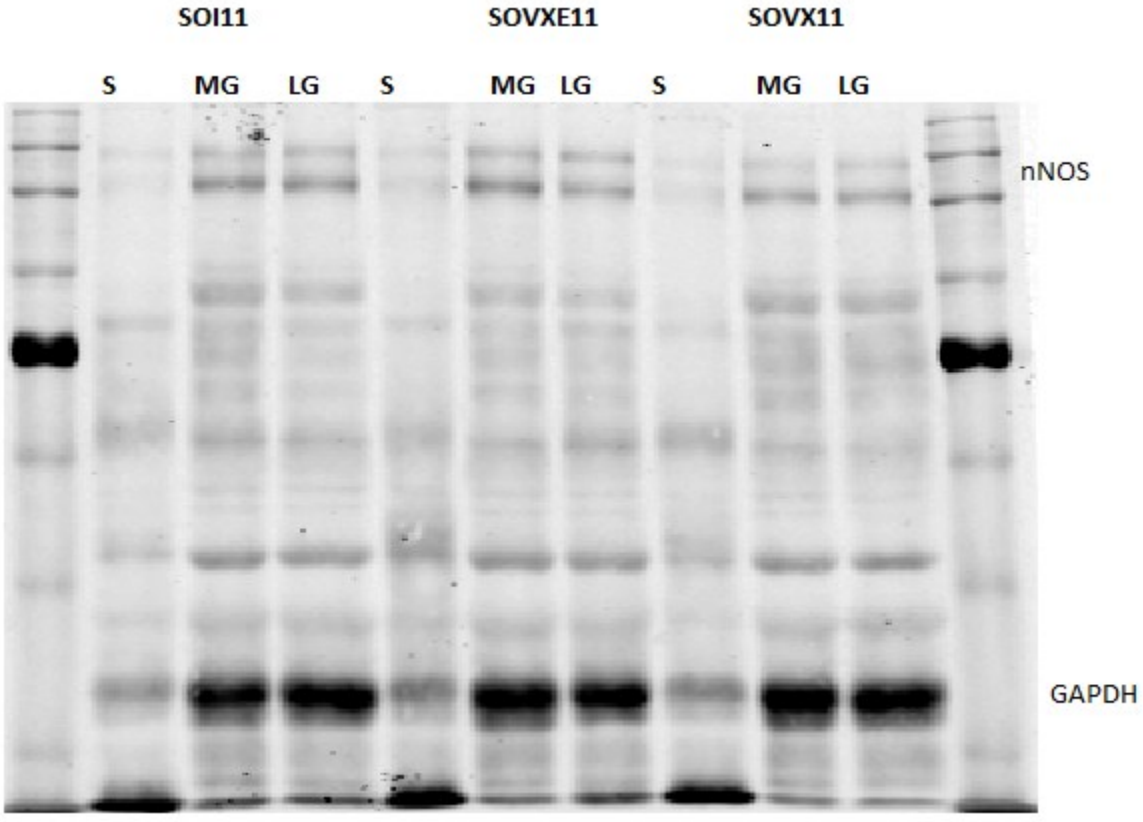

SOI2
SOVXE3
SOVXE3
S MG LG S MG LG S MG LG
nNOS
GAPDH


SOI4
SOVXE4
SOVX4
S MG LG S MG LG S MG LG
nNOS
GAPDH

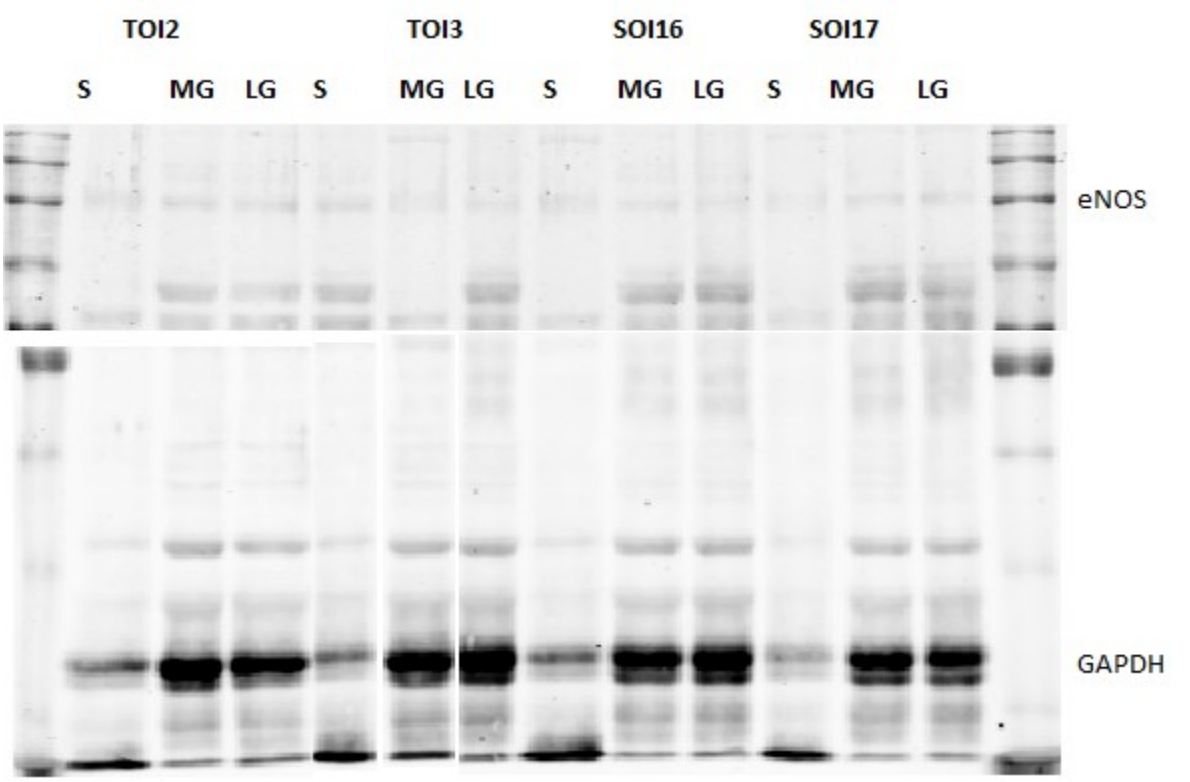

SOI5-
SOVXE5
SOVX6
s
MG
LG
S
MG
LG
S
MG
LG
nNOS
GAPDH

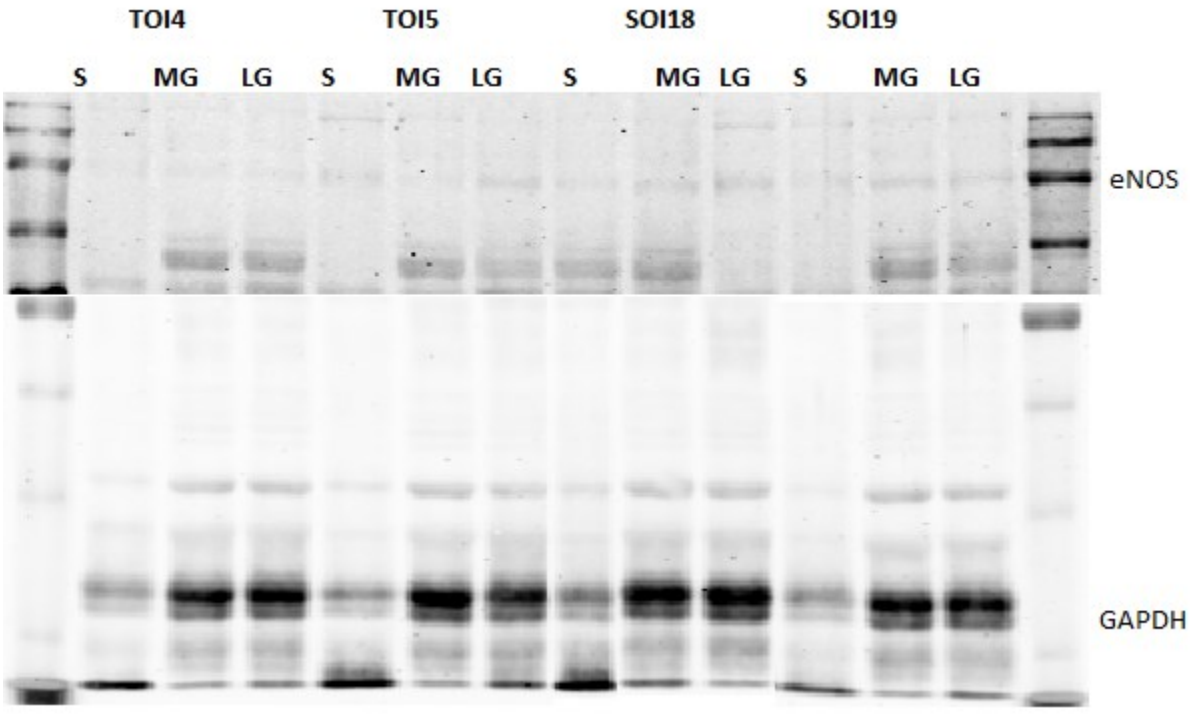

SOI8
SOVXE6
SOVX7
S
MG
LG
S
MG
LG
S
MG
LG
nNOS
GAPDH

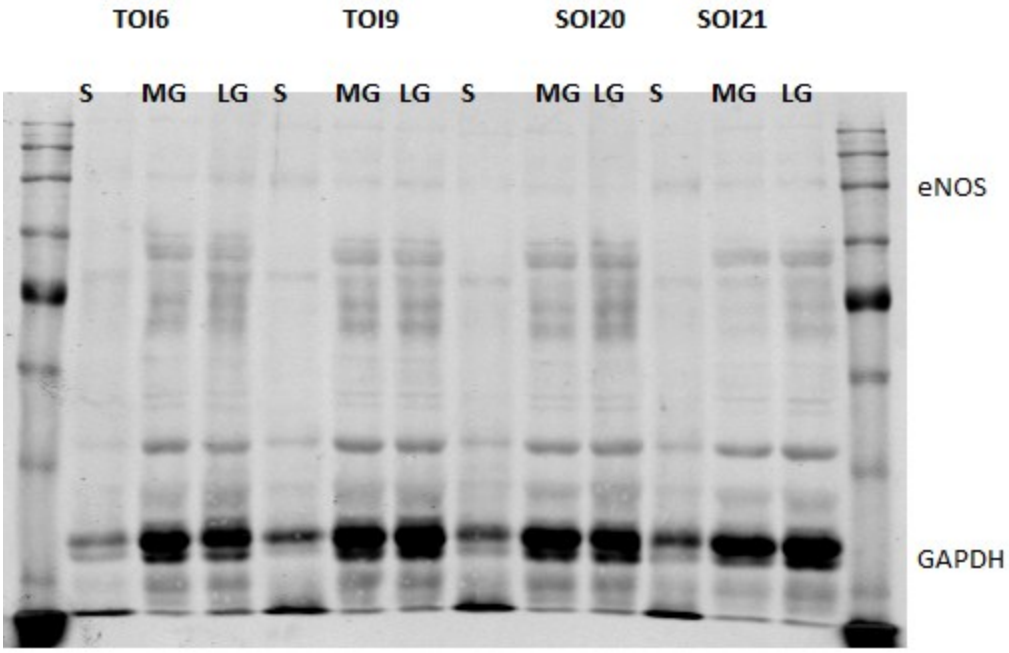

SOI9
SOVXE8
SOVX9
S
MG
LG
S
MG
LG
S
MG
LG
nNOS
GAPDH

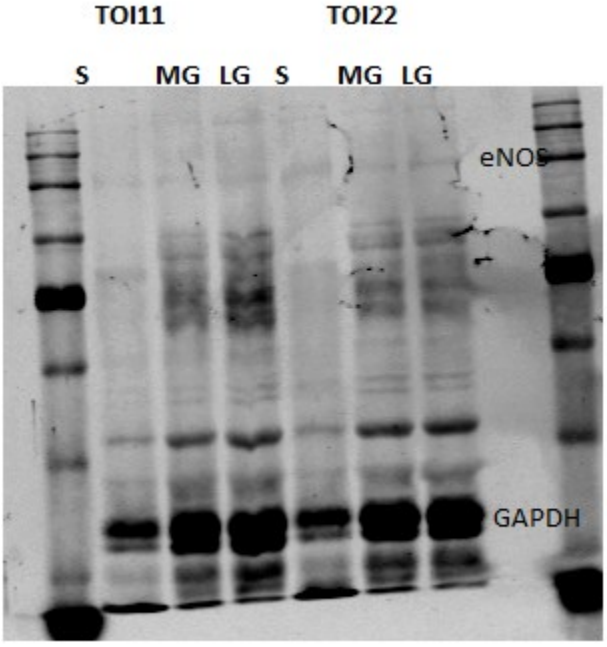

SOI11
SOVXE11
SOVX11
S
MG
LG
S
MG
LG
S
MG
LG
nNOS
GAPDH

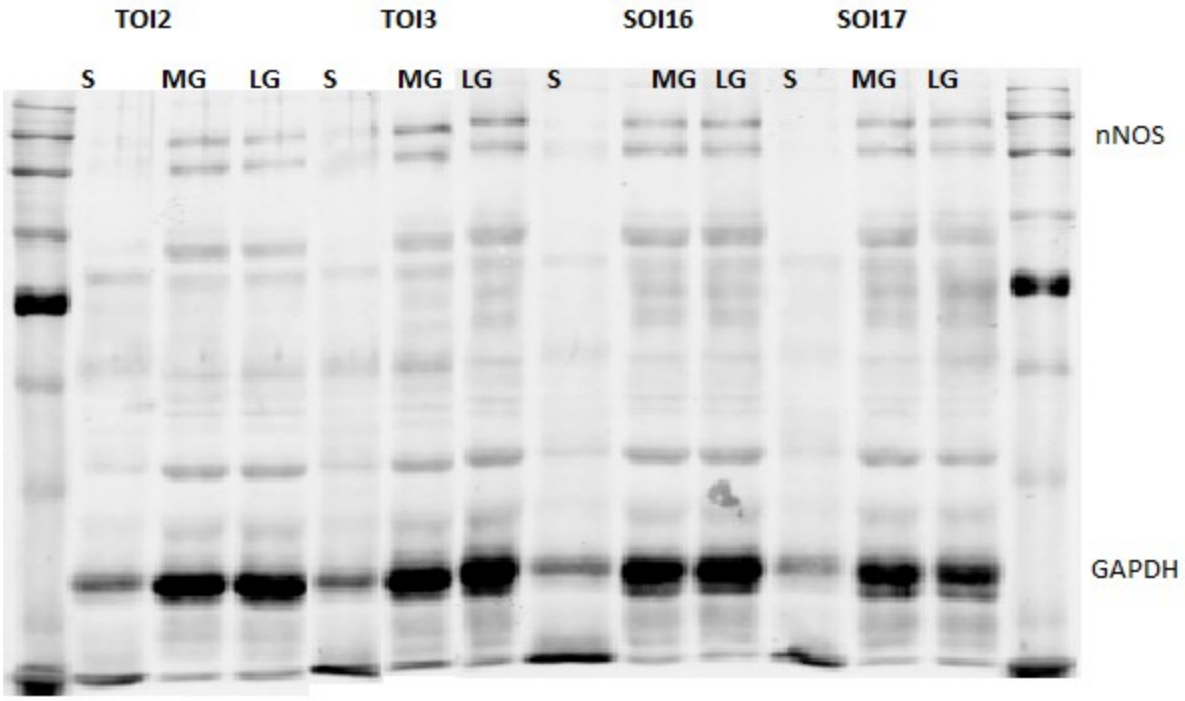
Appendix C: Blots of exercise training on nNOS and eNOS expression.

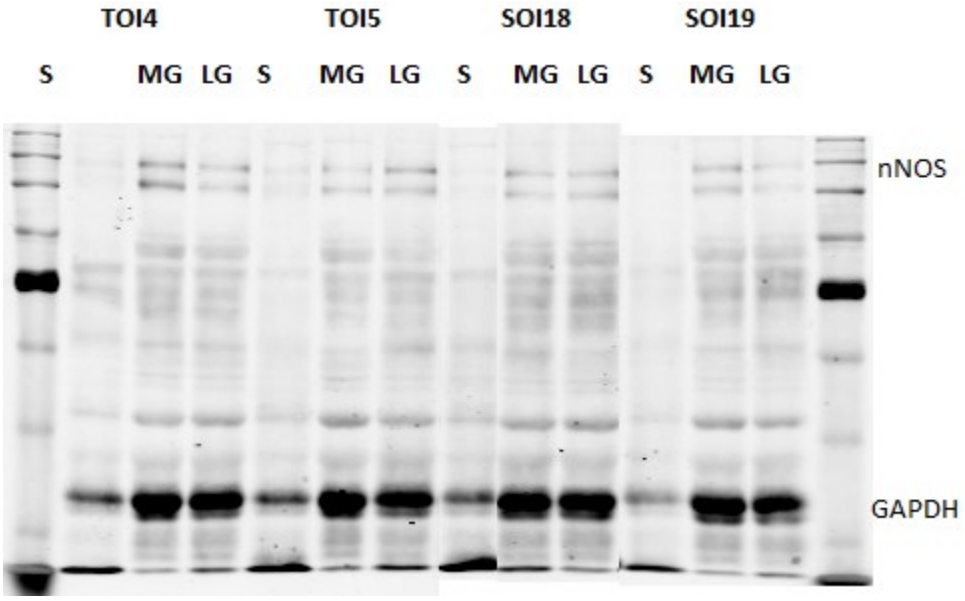


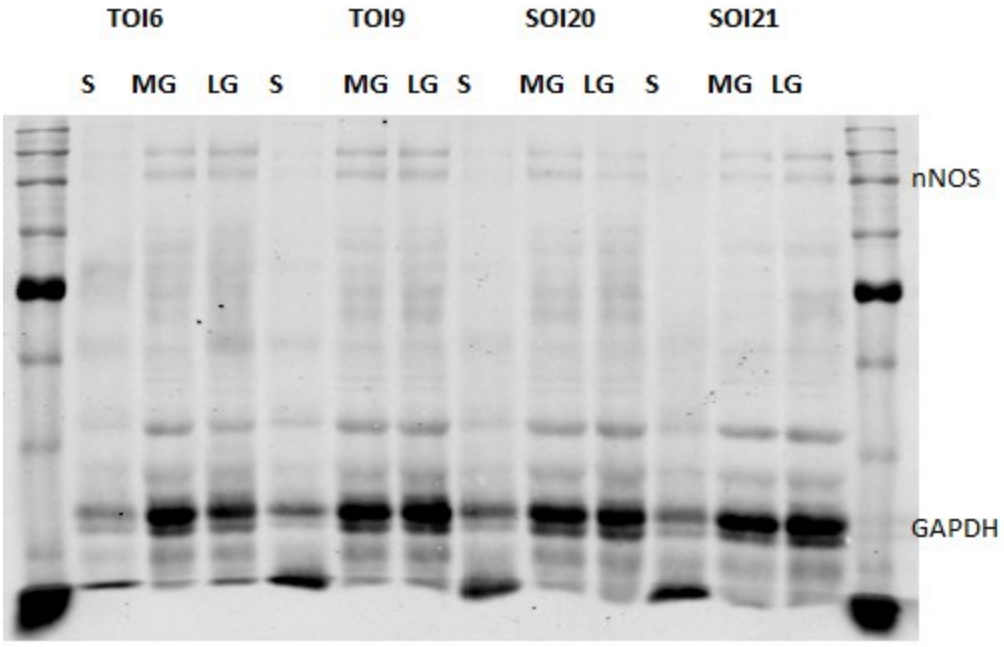


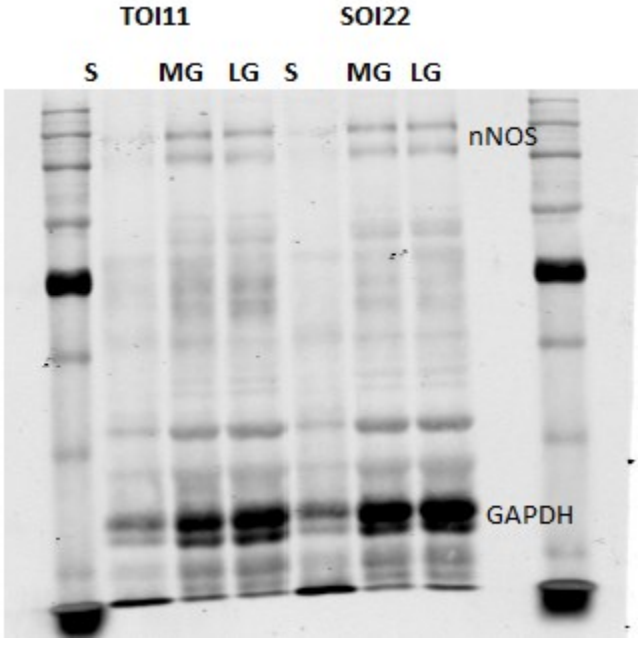

TOI6
TOI9
SOI20
SOI21
S MG LG S MG LG S MG LG S MG LG
eNOS
GAPDH


TOI11
TOI22
S MG LG S MG LG
eNOS
GAPDH


TOI2
TOI3
SOI16
SOI17
S MG LG S MG LG S MG LG S MG LG
nNOS
GAPDH


TOI4
TOI5
SOI18
SOI19
S MG LG S MG LG S MG LG S MG LG
nNOS
GAPDH


TOI6
TOI9
SOI20
SOI21
S MG LG S MG LG S MG LG S MG LG
nNOS
GAPDH


TOI11
SOI22
S MG LG S MG LG
nNOS
GAPDH