Are Sex and Estrogen Modulators of Sympathetic Vascular Control in Resting and Contracting Skeletal Muscle?

by

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Abstract

The purpose of this thesis was to investigate whether sex and estrogen modulate sympathetic neurovascular control in resting and contracting skeletal muscle. This thesis also investigated the hypothesis that the effects of sex and estrogen on neurovascular control would be mediated by the signal molecule nitric oxide (NO).

NO has many biological functions and has been shown to be a potent vasodilator, an inhibitor of sympathetic vasoconstriction in resting and contracting skeletal muscle (sympatholysis) and an inhibitor of efferent sympathetic nerve activity (SNA). NO is produced by the endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoforms of the enzyme NO synthase (NOS). NOS enzyme expression appears to be sensitive to estrogen bioavailability and therefore NO bioavailability and the physiological functions of NO may be modulated by sex and estrogen status.

To investigate the hypothesis that sex and estrogen modulate sympathetic neurovascular control through an NO dependent mechanism an *in vivo* anesthetized rat preparation and vascular pharmacology were utilized in wild-type male and female rats and in female rats that had undergone a surgical procedure to chronically alter estrogen status. Vascular conductance was measured in the rat hindlimb at rest and during muscle contraction. The lumbar sympathetic chain was instrumented to evoke sympathetic vasoconstriction of the hindlimb skeletal muscle vasculature (Study 1 and 2) or record efferent SNA directed to the hindlimb skeletal muscle vasculature (Study 3) at rest and during electrically stimulated muscle contractions of the tricep surae muscle group.

Study 1 investigated the hypothesis that sex modulates sympathetic vascular control, such that sympathetic vasoconstrictor responsiveness would be

blunted and NO-mediated inhibition of sympathetic vasoconstriction would be augmented in female compared to male rats. Muscle contraction-mediated inhibition of sympathetic vasoconstriction (sympatholysis) was augmented in females compared to male rats. NOS blockade reduced sympatholysis in female, but not male rats, suggesting that the enhanced sympatholysis in female rats was partially mediated by NO.

Study 2 investigated the hypothesis that estrogen status alters sympathetic vasoconstrictor responsiveness and modulates the contributions of nNOS- and eNOS derived NO to NO-mediated inhibition of sympathetic vasoconstriction at rest and during muscle contraction. Estrogen status did not alter vasoconstrictor responsiveness or sympatholysis in ovary intact (OI), ovariectomized (OVX) or ovariectomized and estrogen replaced (OVXE) female rats. Selective blockade of nNOS also did not alter vasoconstrictor responsiveness or sympatholysis, suggesting that nNOS derived NO was not involved in NO-mediated inhibition of sympathetic vasoconstriction in female rats, regardless of estrogen status. Subsequent, non-selective NOS blockade reduced sympatholysis in all groups by a similar magnitude, suggesting that eNOS mediated-NO production was responsible for NO-mediated sympatholysis in female rats and was not influenced by estrogen status.

Study 3 addressed the hypothesis that estrogen bioavailability would alter MSNA at rest and during muscle contraction through an NO dependent mechanism. Sympathetic nerve activity was recorded from the lumbar sympathetic chain (LSNA) in OI, OVX and OVXE rats at rest and during muscle contraction before and after non-selective NOS inhibition. Estrogen status did not alter efferent LSNA at rest or during muscle contraction. NOS inhibition elevated blood pressure and reduced efferent LSNA at rest and during muscle

contraction in all groups. However, when blood pressure was normalized (to levels measured before NOS inhibition) by infusion of a vasodilator (hydralazine), LSNA at rest and during contraction were not different from LSNA measured in the control condition. These data suggest that efferent LSNA at rest and in response to muscle contraction was not a function of estrogen status and systemic NOS inhibition does not appear to alter efferent LSNA at rest and during exercise.

This thesis provides evidence that sex modulates sympathetic vascular control and that sex differences in the regulation of sympathetic vasoconstriction in skeletal muscle are partly attributable to sex differences in NO mediated vascular control. Estrogen bioavailability did not alter NO-mediated vascular control or efferent LSNA at rest or in response to exercise. Thus, these data provide critical evidence that challenges the assertion that estrogen modulates NO bioavailability and sympathetic neurovascular control at rest and during exercise through an NO dependent mechanism.

These findings advance our understanding of how biological sex and estrogen influence the integrative control of blood pressure and skeletal muscle vascular resistance at rest and during exercise. The present findings have implication for future studies of vascular control in aging and conditions of altered estrogen status, such as menopause.

Preface

This thesis is an original work by Timothy P. Just, composed of research projects that received ethics approval from the Animal Care and Use Committee of the University of Alberta Research Ethics Board. Project Name: “Estrogen and Sympathetic Vascular Control”; Animal Use Protocol 1493, 2014-2018.

All research studies within this thesis were conceived and designed by Timothy P. Just and Darren S. DeLorey. Both Timothy P. Just and Darren S. DeLorey analyzed data, interpreted the results of the experiments, prepared figures, drafted the manuscript, edited and revised the manuscript, and approved the final version of the manuscript.

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List of Abbreviations

ACh: Acetylcholine

ATP: adenosine triphosphate

EDD: Endothelium-Dependent Vasodilation

eNOS: Endothelial Nitric Oxide Synthase

F: Female

FBF: Femoral Blood Flow

FVC: Femoral Vascular Conductance

FVR: Femoral Vascular Resistance

GPER: G-protein coupled estrogen receptor

HR: Heart Rate

HRT: Hormone Replacement Therapy

iNOS: inducible Nitric Oxide Synthase

L-NAME: N_{ω}-nitro-L-arginine methyl ester

LSNA: Lumbar Sympathetic Nerve Activity

M: Male

MAP: Mean Arterial Pressure

MCF: Maximal Contractile Force

MSNA: Muscle Sympathetic Nerve Activity

MT: Motor Threshold

NE: Norepinephrine

nNOS: Neuronal Nitric Oxide Synthase

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

NPY: Neuropeptide Y

OI: Ovary Intact

OVX: Ovariectomized

OVXE: Ovariectomized with a 17β-estradiol-replacement

PVN: Paraventricular Nucleus

RVLM: Rostral Ventrolateral Medulla

SMTC: *S*-methly-$_L$-thiocitrulline

SNA: Sympathetic Nerve Activity

SNS: Sympathetic Nervous System

Chapter 1

Introduction

The sympathetic nervous system (SNS) is integral to the regulation of peripheral vascular resistance and therefore the control of blood pressure and tissue blood flow at rest and during exercise [1]. At rest, approximately 20% of cardiac output is directed toward skeletal muscle making the skeletal muscle vascular bed an important site for the regulation of total peripheral resistance and blood pressure [2]. During exercise, cardiac output increases and skeletal muscle blood vessels dilate to increase blood flow to skeletal muscle in an exercise intensity dependent manner and at max exercise as much as 85-90% of cardiac output is directed to active skeletal muscles. SNS activity (SNA) increases in response to exercise to augment stroke volume and cardiac output and to regulate peripheral vascular resistance and the distribution of cardiac output [2]. SNA constricts inactive tissue to direct cardiac output to active skeletal muscle. In the vasculature of active skeletal muscle, the SNS produces vasoconstriction that restrains local vasodilation to ensure that a large decrease in peripheral resistance does not occur that could threaten the maintenance of systemic blood pressure during exercise [2, 3]. However, local mechanisms in contracting skeletal muscle partially inhibit SNS-mediated vasoconstriction [3]. This contraction-mediated inhibition of vasoconstriction is known as sympatholysis [4] and our present understanding is that sympatholysis facilitates the distribution of blood flow between and within active skeletal muscle and optimizes the matching of skeletal muscle blood flow and metabolism [2, 3].

Sympathetic regulation of vascular resistance and blood pressure appears to be modulated by biological sex [5, 6]. Young females tend to have

lower resting blood pressure and SNA compared to young males [7]. In response to static handgrip exercise, females have a blunted increase in SNA when compared to males [8, 9]. Furthermore, during low-intensity dynamic exercise the vasoconstrictor response to sympathetic neurotransmitters may be attenuated in females compared to males when females exercise during stages of their menstrual cycle characterized by high hormone levels [10]. These sex differences in neurovascular control may play a role in cardiovascular disease risk progression. Young females have a reduced risk of cardiovascular disease compared to young men [11]. However, with advancing age cardiovascular disease risk appears to accelerate at a faster rate in females compared to males and following the onset of menopause cardiovascular disease risk in females is similar to or exceeds the risk in males [12]. The mechanism(s) underlying sex differences in sympathetic neurovascular control have not been fully elucidated and sex differences in the sympathetic neurovascular regulation during exercise are relatively unexplored. Due to sex differences in estrogen bioavailability and the genomic and non-genomic effects of estrogen on cardiovascular and neurological systems, estrogen has been advanced as a major mediator of sex differences in neurovascular regulation [7, 13, 14]. However, the preponderance of scientific evidence linking estrogen to cardiovascular disease risk is from cross-sectional studies that have correlated changes in estrogen status with cardiovascular disease risk and not from experiments that have directly investigated the role or estrogen [8, 10, 15, 16]. Only one lab has examined the specific impact of altered estrogen bioavailability on sympathetic neurovascular control [17, 18], and these studies support the assertion that augmented estrogen bioavailability blunts sympathetic vasoconstrictor responsiveness [17, 18].

Further, there are few studies that have examined sex-specific sympathetic neurovascular control during exercise [8, 9], and although they lack a mechanistic examination of estrogen's actions, they suggest that augmented estrogen bioavailability reduces efferent SNA at rest and during exercise.

Thus, this thesis aimed to establish if biological sex and estrogen are important mediators of sympathetic neurovascular control at rest and during exercise and examined whether the effects of sex and estrogen on sympathetic neurovascular control were mediated by nitric oxide (NO). To address these hypotheses, an *in vivo* anesthetized rat experimental preparation and vascular pharmacology were utilized in wild-type male and female rats and in female rats that had undergone a surgical procedure to chronically alter estrogen status. In the anesthetized rat, blood pressure and femoral artery blood flow were measured on a beat-by-beat basis and hindlimb vascular conductance was calculated. The lumbar sympathetic chain was instrumented to evoke sympathetic vasoconstriction of the hindlimb skeletal muscle vasculature (Study 1 and 2) or record efferent SNA directed to the hindlimb skeletal muscle vasculature (Study 3) at rest and during electrically stimulated muscle contractions of the tricep surae muscle group. Overall, it was hypothesized that sex and estrogen modulate sympathetic vascular control through effects on NO expression and function.

Sympathetic Vascular Control at Rest and During Exercise

Sympathetic nerve activity arises from spontaneous discharges within the rostral ventrolateral medulla (RVLM) in the brain stem that are independent of input from afferent reflexes [19-21]. Changes in efferent sympathetic nerve activity are dependent on afferent inputs from peripheral neuro-reflexes, including

the arterial and cardiopulmonary baroreceptors, chemoreceptors, and muscle metaboreflex that are received at multiple cell groups, including the paraventricular nucleus (PVN), nucleus of the solitary tract (NTS), and caudal and rostral ventrolateral medullas [21, 22] and integrated within the central nervous system [21]. Short-term blood pressure regulation is largely controlled by central autonomic nuclei which translate afferent arterial baroreceptor activity into appropriate efferent sympathetic activity [23]. Spontaneous increases in arterial blood pressure are sensed by afferent baroreceptors in the aortic arch and carotid arteries which signal sympathoinhibitory reflexes that decrease efferent sympathetic activity (and thus vasoconstrictor tone, myocardial contractility, and heart rate) [24]. Muscle SNA (MSNA) displays cardiac rhythmicity with burst of sympathetic activity occurring during the low pressure diastolic phase of the cardiac cycle [25]. SNS activity can be measured directly from peripheral nerves in humans and animals. SNS activity is typically quantified by measuring the frequency, incidence, amplitude and total activity of sympathetic action potentials [26]. Burst frequency and incidence refer to the number of sympathetic bursts per unit of time and per 100 heart beats, respectively. Measurement of the increase in voltage during a sympathetic burst is an indicator of burst amplitude. Total SNA represents the sum of burst frequency and amplitude and is typically quantified with an integral of nerve activity. In small animal models, such as rats, high heart rates (~400 beats min^{-1}) and high levels of sympathetic nerve activity make identification of individual bursts of sympathetic nerve activity difficult. Thus, quantifying and expressing nerve activity in terms of burst frequency and burst amplitude is challenging and therefore SNA in rodent studies is typically expressed as a total nerve activity [26].

Efferent SNA leads to the release of neurotransmitters that bind to post-synaptic adrenoreceptors and non-adrenergic receptors on vascular smooth muscle to produce vasoconstriction [27]. While NE is the predominant neurotransmitter released from the postganglionic neurons, different frequency patterns of firing cause the co-transmitters adenosine tri-phosphate (ATP) and neuropeptide Y (NPY) to be released as well [28, 29]. Experimental evidence suggests that low-range discharge frequencies favor the release of ATP, low- and mid-range discharge frequencies release mainly NE and ATP, and high discharge frequencies release mainly NPY [30, 31]. After the neurotransmitter is synthesized in the axon, action potentials stimulate the exocytosis of the neurotransmitter into the junctional cleft, where it can diffuse to post-synaptic receptors on the surface of various target tissues. ATP is a purine which acts on P2X receptors of rat arteries to produce vasoconstriction [32]. NE acts mainly by binding to α_1- and α_2-adrenergic receptors, and loses effectiveness as it diffuses away from the receptor, is transported back into the neuron, or is broken down by monoamine oxidase [33]. NPY binds to NPY-Y1 receptors, which appears to act to amplify the effect of post-junctional adrenoreceptor activation by NE [31]. In the skeletal muscle vasculature α_1- and α_2-adrenoreceptors, NPY-Y1, and purinergic P2X receptors have all been shown to contribute to tonic vasoconstriction at rest and during exercise [34].

Exercise presents a unique challenge to the sympathetic nervous and cardiovascular systems to meet the competing demands of blood pressure maintenance and the perfusion of active skeletal muscle. During exercise, muscle blood flow is tightly coupled to muscle oxygen demand and is regulated through the integration of neurogenic, metabolic, and myogenic signals [35].

During dynamic aerobic exercise, muscle blood flow increases in an exercise-intensity dependent manner and ~ 85% of cardiac output can be directed to the skeletal muscle vascular bed during maximal exercise [36]. During isolated muscle mass exercise, it has been shown that the skeletal muscle vasculature has a tremendous capacity to dilate and muscle blood flows as high as ~400 ml · min^{-1} ·100 g^{-1} of muscle have been measured in highly fit endurance athletes [37]. This dilatory capacity suggests that the skeletal muscle vascular bed has the ability to dilate to an extent that would exceed the ability of the pumping capacity of the heart to maintain arterial blood pressure during large muscle mass exercise, such as running, cycling, or swimming [38]. Thus, MSNA increases during exercise in an exercise intensity-dependent manner [39]. In addition to shunting blood from inactive tissues toward active skeletal muscle, the increase in MSNA also produces vasoconstriction in exercising muscle [40] to oppose the robust local vasodilation and maintain systemic arterial blood pressure [3]. The skeletal muscle vascular bed partially blunts the constrictor effects of MSNA during exercise, evidenced by the observation that a sympatho-excitatory stimulus produces less vasoconstriction in contracting compared to resting skeletal muscle [4, 41, 42]. The blunting of the vasoconstrictor response to sympathetic activity in exercising skeletal muscle vascular beds is known as functional sympatholysis [4]. Sympatholysis appears to facilitate the adequate perfusion of exercising muscle while maintaining systemic blood pressure [3] and the magnitude of sympatholysis increases with the intensity of the exercise [43]. Dysfunctional sympatholysis results in elevated vascular resistance, altered blood pressure regulation and may contribute to hypo-perfusion of skeletal muscle [3]. While an exact mechanism that causes the blunting of sympathetic

vasoconstrictor responses is unclear, a decrease in the sensitivity of post-synaptic receptors has been documented [44-46]. It has been hypothesized that metabolites that are produced in response to exercise, including K^+, H^+, adenosine and NO and others may blunt post-synaptic sympathetic receptor responsiveness [47-50] (Figure 1.1).

NO has been shown to inhibit sympathetic vasoconstriction at rest [49, 51-53] and during muscle contraction [43, 54-57]. In animals and humans, NO is produced by the group of NO synthase (NOS) enzymes. NOS exists in endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) isoforms. NOS isoforms (eNOS and nNOS) important to skeletal muscle vascular control are mainly localized in the endothelium [58], smooth muscle sarcolemma, and skeletal muscle [59, 60]. Neuronal NOS is also highly expressed within the central nervous system, where it has been shown to inhibit sympathetic outflow [61]. The iNOS isoform does not appear to be involved in the control of skeletal muscle vascular tone in healthy mammals [62, 63], however expression of iNOS is increased in pathophysiological conditions and iNOS appears to contribute to NO production in disease states, such as Type II Diabetes [64]. NO derived from both eNOS and nNOS has been shown to contribute to the inhibition of sympathetic vasoconstriction in skeletal muscle vascular beds [57, 60, 65, 66].

During exercise, NO production is increased in response to increased endothelial shear stress stimuli (eNOS) and increases in skeletal muscle cytosolic Ca^{2+} concentration (nNOS) [60]. This exercise induced increase in NO has been linked to the inhibition of α_1-adrenoreceptor mediated vasoconstriction in skeletal muscle vascular beds [67]. Some evidence also exists for NO-mediated inhibition of α_2-adrenoreceptors [68, 69], but this is not a universal

finding in skeletal muscle vascular beds [70]. Our understanding of sex differences in NO-mediated inhibition of sympathetic vasoconstriction is incomplete because the available studies have not directly examined sex differences [55, 71] or have studied males [56, 57, 72] or females [54] separately.

Sex Differences in Sympathetic Vasoconstriction

Resting blood pressure is lower in young females compared to males [7] and sex differences in sympathetic neurovascular control may contribute to sex differences in blood pressure regulation. Hart et al, [7] have reported that muscle sympathetic nerve activity (MSNA) is positively and significantly correlated with total peripheral resistance (TPR) in young males, whereas in young females the relationship between resting MSNA and TPR is weaker and not statistically significant. These data suggest that the transduction of MSNA to peripheral vasoconstriction may be blunted in females compared to males. It has been hypothesized that augmented β-adrenoreceptor-meditated vasodilation in young females may inhibit α-adrenoreceptor-mediated vasoconstriction [73]. Indeed, in young females β-adrenoreceptor blockade resulted in a positive relationship between MSNA and TPR, suggesting β-adrenoreceptors reduced vasoconstriction elicited by MSNA [13]. However, infusions of selective α_1 and α_2 -adrenoreceptor agonists produced a similar vasoconstriction in the forearm of young males and females, suggesting that α-adrenoreceptor responsiveness is similar between males and females [10]. The same lab reported no difference between males and females during the infusion of isoproterenol, a β-adrenoreceptor agonist [74]. Lastly, the sympathetic vasoconstrictor response elicited by isometric handgrip exercise was not different between males and females during β-adrenoreceptor blockade [75]. Thus, conflicting evidence

surround sex differences β-adrenoreceptor function suggests other mechanisms may be responsible for sex differences in sympathetic vasoconstrictor responsiveness.

NO has also been shown to inhibit sympathetic vasoconstriction at rest and during exercise. Estrogen has been shown to increase NO bioavailability and NOS expression in many vascular beds [76-80], including skeletal muscle [17]. Thus, NO mediated inhibition of sympathetic vasoconstriction may be influenced by sex and estrogen status.

Estrogen and Sympathetic Vasoconstriction

Circulating estrogen acts directly on endothelial cells and vascular smooth muscle through estrogen-specific receptors (ERα, ERβ) [81] as well as G-protein coupled estrogen receptors (GPER) to regulate gene expression and non-genomic membrane signalling cascades that can stimulate eNOS-dependent NO release [82].

Estrogen receptors are expressed in both females and males, and the activation of estrogen receptors and/or signalling cascades have been shown to effect sympathetic vasoconstrictor responsiveness [83-87]. Estrogen administration to male rats enhanced α-adrenergic-mediated vasoconstrictor responses in mesenteric arteries [83], and in denuded aortic rings of spontaneously hypertensive rats [84], although estradiol-mediated increases in contraction were masked in endothelium intact aortic rings [84]. The mechanism of estrogen-mediated augmentation of vasoconstrictor responsiveness has not been elucidated and may be specific to vessel type, male rats and/or hypertensive rat models. In contrast, ovariectomized female rats with estrogen supplementation had blunted mesenteric artery vasoconstrictor responses to

noradrenaline [85], as well as forearm vasoconstrictor responses in perimenopausal women [86]. In young women, forearm vasoconstrictor responses to norepinephrine were attenuated during periods of high estrogen concentration within the menstrual cycle (luteal phase) when compared to periods of low estradiol concentration (early follicular phase) [87]. The reduction of adrenergic receptor sensitivity by estrogen may be explained by several mechanisms. Activation of smooth muscle estrogen receptors reduce the calcium sensitivity of contractile pathway [88], and estrogen acts to stimulate the production of antagonists to sympathetic nerve transduction [51, 81]. Estrogen may also oppose α-adrenergic vasoconstriction by increasing the sensitivity of vasodilatory β-adrenergic receptors [89]. This has been demonstrated in humans, with the higher sensitivity of β-adrenergic receptors in young females compared to young males [73]. Circulating 17β-estradiol has also been shown to reduce the production of NE by inhibiting tyrosinase [88].

While these data suggest that estrogen blunts sympathetic vasoconstriction in females, research on sex and estrogen-dependent changes in sympathetic vasoconstrictor responsiveness at rest and during exercise is limited and the mechanisms involved have not been fully identified. Indeed, no differences were observed in the vasoconstrictor responsiveness of hindlimb vascular beds during stimulations of the lumbar sympathetic chain in rats with intact ovaries or that had been ovariectomized with and without estrogen replacement when measured at rest [17]. Differences between groups did not emerge until the lumbar sympathetic chain was stimulated during simultaneous hindlimb muscle contractions, with augmented sympathetic vasoconstrictor responsiveness in rats lacking estrogen [17].

Indeed, estrogen has been suggested to augment or provide additional sympatho-inhibitory mechanisms during exercise [10, 17, 18]. Augmented vasoconstrictor responsiveness during exercise was also observed in post-menopausal women when compared to groups who had received estradiol supplementation [18]. During low intensity exercise, young females have blunted vasoconstrictor responsiveness to infusions of clonidine, an α_2-adrenoreceptor agonist, during the high estrogen phase of the menstrual cycle [10]. A potential mechanism for estrogen-mediated alterations in sympathetic vascular control during exercise is augmented bioavailability of NO, a ubiquitous vasodilatory molecule [58], which has been previously implicated in female rats [17].

Estrogen contributes to the regulation of NOS expression in females and a loss of estrogen appears to decrease the bioavailability of NO [90, 91]. Skeletal muscle nNOS expression seems to be especially sensitive to estrogen levels (78). Gastrocnemius muscle nNOS expression was reduced in ovariectomized compared to rats with intact ovaries and ovariectomized rats that had received supplemental estrogen [17], suggesting nNOS to be a suitable target further study. Interestingly, previous work has demonstrated a differential contribution of nNOS and eNOS to the inhibition of evoked sympathetic vasoconstriction in male rats [57]. Therefore, estrogen-mediated changes to NOS-isoform expression may alter sympathetic vascular control in females. However, the specific NOS isoforms involved in an estrogen-mediated blunting of exercising vasoconstriction were not mechanistically investigated [17].

Estrogen and the Regulation of Efferent Sympathetic Nerve Activity

Efferent MSNA is generally reported to be lower in young women than in young men [92-94], with this sex difference in resting MSNA disappearing

between the ages of 40-50 [94, 95]. However, similar resting SNA between young males and females has also been reported [13] despite similar multi-unit nerve recording techniques to other studies [92-94]. This discrepancy may be explained by hormonal fluctuations throughout the menstrual cycle that appear to effect sympathetic outflow [16, 96]. Not all studies of sex differences in sympathetic outflow report controlling for menstrual phase [92, 94, 95] and premenopausal women >40 years of age do not have significantly different SNA than their male counterparts [95], due to age-related increases in SNA [94, 97]. Indeed, studies examining increases in SNA in postmenopausal women when compared to young women are confounded by age-related increases in MSNA that occur in both men and women [97] and changes in progesterone levels [16, 96]. Consistent with an estrogen-mediated blunting of sympathetic outflow, resting MSNA has been shown to increase with age at a faster rate in post-menopausal women compared to men [95], and higher resting MSNA in post-menopausal women compared to young women [98] can be reduced with estradiol supplementation [99].

Estrogen receptors are expressed in multiple central neurones that regulate efferent SNA, including the rostral ventrolateral medulla and the paraventricular nucleus [100]. The injection of estradiol into these brainstem regions blunts the increase in blood pressure and renal SNA in response to phenylephrine infusion [101] and reduces renal SNA in both female [101] and male rats [102]. At the onset of voluntary exercise, feed-forward signaling from central command reduces parasympathetic efferent activity and increases cardiac output through an increase in heart rate (HR). At mild workloads, there is an exercise intensity dependent increase in sympathetic activity mediated by

arterial baroreceptors to maintain blood pressure in the face of skeletal muscle vasodilation. Central command “resets” the position of the arterial baroreflex curve during exercise, and corrects the lower arterial pressure that is suitable during rest to a higher exercising blood pressure utilizing signals from the arterial baroreflex [103]. Chemoreceptors do not generally evoke a large cardiovascular response under dynamic exercise conditions due to the powerful arterial baroreflex, but can help the arterial baroreflex to increase sympathetic nerve activity and thus blood pressure in situations of low blood pressure [104]. The impact on efferent SNA regulation during exercise following altered estrogen and NO bioavailability is also unexplored. Evidence exists for sex differences in sympathetic nerve activity responses to isometric handgrip exercise [8, 9] and chemoreflex activation [105], such that increased levels of estrogen may blunt sympathetic nerve activity increases [15, 106].

The central regulation of MSNA by estrogen may act through a NO-dependent mechanism, as NOS blockade has been shown to increase efferent sympathetic activity [107]. NO is an inhibitor of efferent SNA within the rostral ventrolateral medulla and paraventricular nucleus [100]. Thus, a decrease in NO bioavailability following a decrease in circulating estrogen would potentially augment efferent SNA.

However, no studies to the author’s knowledge have yet examined how estrogen status alters NO-specific effects on efferent SNA *in vivo*.

Summary

Given this background, this thesis aimed to address the overarching hypothesis that sex and estrogen modulate sympathetic neurovascular control and that the modulatory influence of sex and estrogen are mediated by NO. It

was hypothesized in Study 1 that female rats would have blunted sympathetic vasoconstriction at rest and during muscle contraction when compared to males, and that females would have augmented NO-mediated inhibition of sympathetic vasoconstriction.

To determine if estrogen is the major mediator of sex differences in sympathetic neurovascular control, subsequent studies directly investigated the effects of estrogen status on sympathetic vasoconstriction and sympathetic outflow in female rats. Study 2 was performed to address the hypothesis that estrogen modulates nNOS- and eNOS-dependent inhibition of sympathetic vasoconstriction at rest and during muscle contraction. Estrogen deficiency was hypothesized to reduce sympatholysis, while estrogen-replete rats (i.e. ovary intact and ovariectomized with an estradiol replacement) were hypothesized to have intact sympatholysis. This study further hypothesized that NO-mediated sympatholysis would be largely attributable to nNOS, and that nNOS-mediated inhibition of sympathetic vasoconstriction would be significantly reduced in estrogen-deficient rats.

Study 3 was performed to address the hypothesis that reduced estrogen bioavailability would augment MSNA at rest and during muscle contraction. Further, it was hypothesized that only estrogen replete rats would demonstrate an increase in SNA following a reduction in NO bioavailability during a blockade of NOS.

The utilization of an *in vivo* experimental preparation in the following experiments allows for the precise control of muscle contraction intensity as well as the ability to evoke sympathetic vasoconstriction through the release of endogenous neurotransmitters. Further, using selective blockade pharmacology

these experiments can elucidate a mechanistic role for NO in the neurovascular control of skeletal muscle vascular conductance. These experimental strengths are tempered by the use of anesthetized animals, which may blunt efferent SNA and is unable to take into account the role of environmental stimuli (fear, anticipatory exercise responses, etc.) that increase SNA. Further, the use of electrical stimulation to produce muscle contractions alters the normal recruitment of motor units compared to conscious exercise. Regardless, the mechanistic studies that follow contain novel and significant data that contribute to our understanding of sex and estrogen-mediated effects on sympathetic nervous system control of skeletal muscle vasculature at rest and during muscle contraction. These studies have important implications for future studies of vascular health following aging and conditions of altered estrogen status, such as menopause.

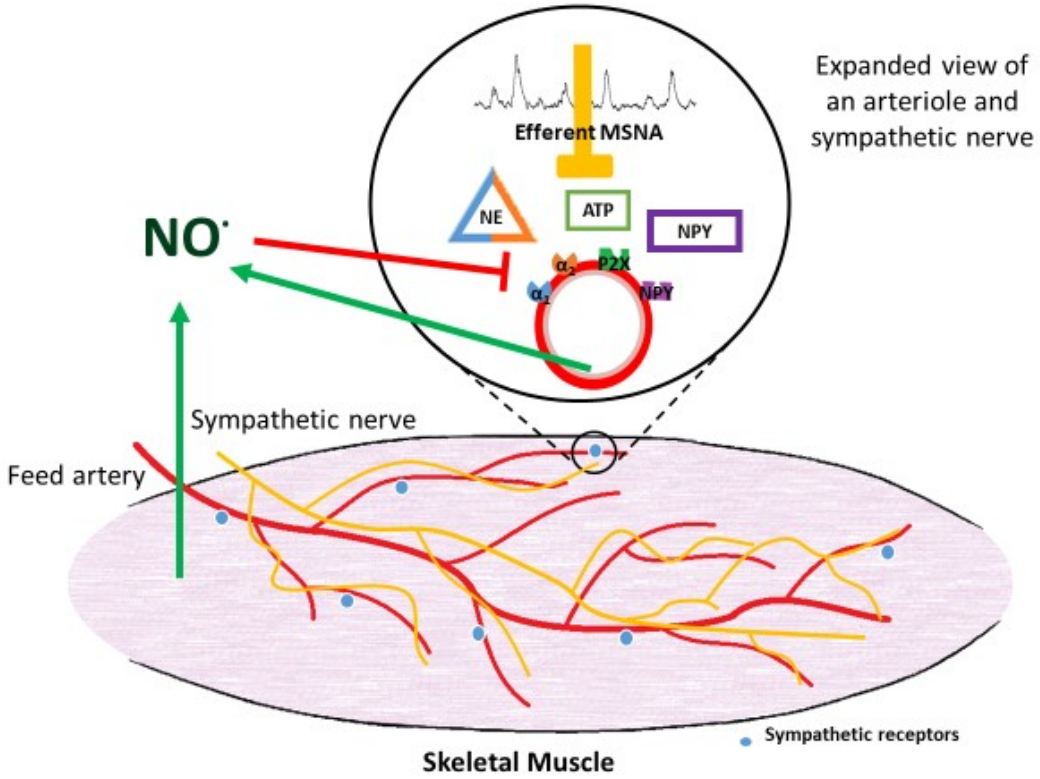

Figure 1.1. The contraction-mediated inhibition of sympathetic vasoconstriction (sympatholysis) has been linked to the release of endogenous vasodilators, such as nitric oxide (NO). During exercise, increased endothelial shear stress and skeletal muscle contraction can increase NO bioavailability, which reduces post-synaptic sympathetic receptor responsiveness, and therefore vasoconstriction. Figure adapted from Just, T.P., et al. *Exerc Sport Sci Rev* 44(4): 137-43, 2016.

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Chapter 2

Sex differences in sympathetic vasoconstrictor responsiveness and sympatholysis

Introduction

Sympathetic nervous system regulation of blood pressure and peripheral vascular resistance differs between males and females [1-8]. Young men exhibit a positive relationship between efferent muscle sympathetic nerve activity (MSNA) and total peripheral resistance at rest that appears to be absent in young females [1]; suggesting that neurovascular transduction and/or sympathetic vasoconstrictor responsiveness may be blunted in females compared to males. Consistent with this notion, blunted calf vasoconstrictor responses to a cold pressor test and isometric handgrip exercise have been reported in young females compared to males [9]. A blunted forearm vasoconstrictor response to injection of noradrenaline has also been reported in young females compared to males [10]. In this investigation, the sex difference in vasoconstrictor responsiveness was abolished by β-adrenoreceptor blockade, suggesting that enhanced β-adrenoreceptor mediated vasodilation may blunt α-adrenoreceptor mediated vasoconstriction in females [10]. However, in contrast, injection of selective α_1- and α_2-adrenoreceptor agonists has been shown to produce similar vasoconstrictor responses in the forearm of females and males [11]. Furthermore, sympathetic vasoconstrictor responses to isometric handgrip were not different between females and males and were not altered by β-adrenoreceptor blockade in either sex, suggesting that β-adrenoreceptor mediated vasodilation may not oppose evoked sympathetic vasoconstriction in

resting skeletal muscle [12]. A recent study has also reported that forearm vasodilation to infusion of the β-adrenoreceptor specific agonist, isoproterenol, was not different between males and females indicating that sex may not influence β-adrenoreceptor vasodilation [13]. Given the conflicting evidence related to sex differences in β-adrenoreceptor function, it is conceivable that other mechanisms may be responsible for differences in sympathetic vasoconstrictor responsiveness between males and females and further investigation in this area appears warranted.

Nitric oxide (NO) has been shown to inhibit sympathetic vasoconstriction in resting and contracting skeletal muscle [14-17]. Estrogen appears to influence NO synthase (NOS) expression and NO bioavailability [18-24]. Indeed, reduced estrogen availability following ovariectomy in rats has been shown to reduce skeletal muscle neuronal NOS expression and contraction-mediated blunting of sympathetic vasoconstriction (i.e. sympatholysis) [22]. Furthermore, estrogen supplementation has been shown to restore NOS expression and sympatholysis in ovariectomized rats [22] and post-menopausal women [25]. The relative abundance of estrogen in females compared to males suggests that females may have greater NO bioavailability and an enhanced potential to inhibit vasoconstriction at rest and during exercise. However, to our knowledge sex differences in NO mediated inhibition of sympathetic vasoconstriction have not been investigated.

Therefore, the purpose of the present study was to determine if sex differences exist in sympathetic vasoconstrictor responsiveness and NO-mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle. We hypothesized that sympathetic vasoconstrictor

responsiveness would be blunted in female compared to male rats at rest and during exercise. We also hypothesized that NO-mediated sympatholysis would be enhanced in female compared to male rats.

Methods

Animal Use

Female (n=10) and male (n=8) Sprague-Dawley rats were obtained from the institutional animal colony (approximately 10 weeks old) 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.

Instrumentation

Anesthesia was induced by inhalation of isoflurane (3.5%, balance O_2) and was subsequently maintained by syringe-pump infusion of a mixture of α-chloralose (8-16 $mg \cdot kg^{-1} \cdot h^{-1}$; Sigma Aldrich, Canada) and urethane (50-100 $mg \cdot kg^{-1} \cdot h^{-1}$; Sigma Aldrich, Canada) through a cannula placed in the right jugular vein. The depth of anesthesia was assessed by absence of a withdrawal reflex to a paw pinch and the stability of hemodynamic variables. A tracheotomy was performed and rats were ventilated (Model 683, Harvard Apparatus, Holliston, MA, USA) with 30% O_2 in balance N_2 to maintain arterial blood gases and acid base status (PaO_2: 90-100mmHg; $PaCO_2$: 39-41mmHg pH: 7.39-7.42). The left carotid artery was cannulated and attached to a pressure transducer (Abbott, North Chicago, IL, USA) for the continuous measurement of arterial blood pressure. The left femoral vein was cannulated for drug delivery and continuous

infusion of heparinized saline via syringe pump (4 units ml^{-1} at 2.5-3 ml hr^{-1} kg^{-1}). Femoral blood flow (FBF) was measured at the right femoral artery with a flow probe (0.7V; Transonic Systems, Ithaca, NY, USA) connected to a flow meter (T106; Transonic Systems). Heart rate (HR) was derived from the arterial blood pressure waveform, and femoral vascular conductance (FVC) was calculated. Core body temperature was monitored by rectal thermistor and body temperature was maintained at 36-37 °C by an external heating pad (TCAT-2, Physitemp, Clifton, NJ, USA).

The right triceps surae muscle group was dissected and attached to a force transducer (FT03, Grass Technologies, Warwick, RI, USA) at the calcaneal tendon. A cuff electrode was placed around the sciatic nerve and muscle contraction was produced by electrical stimulation (Stimulator Panel, Chart software; AD Instruments, Colorado Springs, CO USA). The motor threshold (MT) and the optimal muscle length for tension development were determined. Maximal contractile force (MCF) was determined by stimulation of the triceps surae muscle group with 25 impulses of 1ms duration delivered at 100Hz at 10x the MT. Rhythmic muscle contractions at 60% of MCF were produced by stimulation of the sciatic nerve at 40 Hz, 0.1ms pulses in 250ms trains at a rate of 60 trains·min^{-1} at ~3 times MT.

A midline abdominal incision was performed and the abdominal aorta and vena cava were temporarily retracted to expose the lumbar sympathetic chain. A bipolar silver-wire stimulating electrode was attached to the lumbar sympathetic chain at the level of the L3 and L4 vertebrae and electrically isolated in rapidly curing, non-toxic silicone elastomer (Kwiksil; WPI, Sarasota, FL, USA). An isolated constant current stimulator (DS3; Digitimer, Welwyn Garden City, UK)

was used to stimulate the sympathetic chain and evoke the release of sympathetic neurotransmitters and produce vasoconstriction in the experimental hindlimb.

Following the surgical procedures and an approximately 20-minute stabilization period the following experimental protocol was completed.

Experimental Protocol

The vasoconstrictor response to 1 minute of lumbar sympathetic chain stimulation (1 ms, 1 mAmp pulses) delivered at 2 and 5 Hz in random order was measured at rest and during muscle contraction under control conditions and following non-selective NOS blockade (N_{ω}-nitro-L-arginine methyl ester; L-NAME, 10 $mg \cdot kg^{-1}$, IV). Stimulation frequencies of 2 and 5 Hz were selected to reflect levels of sympathetic nerve activity observed at rest and during conditions of elevated nerve activity (such as in response to exercise or physiological stress) and to evoke frequency-dependent vasoconstrictor responses in the hindlimb vascular bed at rest and during muscle contraction [26-29] . These frequencies of sympathetic nerve stimulation are routinely used in our laboratory and others to investigate neurovascular control in the skeletal muscle vascular bed [16, 30, 31]. Eight minutes of rhythmic muscle contraction was produced and lumbar sympathetic chain stimulation at 2 and 5 Hz was delivered 3 and 6 minutes after the onset of muscle contraction in random order. Control and L-NAME conditions were separated by ~ 60 minutes of recovery.

Following the experimental protocol, rats were killed by anesthetic overdose (α-chloralose and urethane). The heart, soleus, and lateral and medial gastrocnemius muscles were dissected and weighed. The tibia was dissected and tibia length was measured.

Data Analysis

Data were recorded using Chart software (AD Instruments, Colorado Springs, CO USA). Arterial blood pressure and FBF were sampled at 100 Hz and FVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$) was calculated as FBF ÷ mean arterial pressure (MAP). Muscle force production was measured continuously and peak force development was determined for each muscle contraction. To compare force production between groups and experimental conditions a mean of peak contractile forces was calculated between minutes 3-7 (the time period encompassing the sympathetic stimulations) for each contractile bout.

The change in HR, MAP, FBF and FVC in response to sympathetic stimulation was calculated as an absolute change and as a percentage change from the value preceding the sympathetic stimulation in Control and L-NAME conditions. 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 [32, 33]. All data are expressed as mean ± SD.

Statistics

Group differences in body, muscle and heart mass, tibia length, heart:body mass and heart:tibia length ratios and plasma estradiol were determined by t-test. To determine the influence of sex and NO on sympathetic vasoconstrictor responsiveness at rest and during exercise, the vasoconstrictor response to sympathetic stimulation was analyzed by three-way repeated measures ANOVA (group x drug condition x muscle contractile state). Each frequency of sympathetic stimulation was analyzed separately. Two-way

repeated measures ANOVA (Group x Drug condition) was used to determine the effect of sex and NO on baseline hemodynamics, the hemodynamic response to muscle contraction, muscle force production, the magnitude of NO mediated blunting of vasoconstriction and sympatholysis. When significant interactions and main effects were detected, a Student-Newman-Keuls post hoc test was performed. A p-value of < 0.05 was considered statistically significant.

Results

Body mass, heart mass, soleus and lateral and medial gastrocnemius muscle mass were lower ($P < 0.05$) in female compared to male rats (Table 1). The heart mass to body mass ratio was higher ($P < 0.05$) in female compared to males and the ratio of heart mass to tibial length ratio was smaller ($P < 0.05$) in female compared to male rats (Table 1). Plasma estradiol was higher ($P < 0.05$) in females (41 ± 29 $pg \cdot mL^{-1}$) compared to male rats (21 ± 4 $pg \cdot mL^{-1}$).

Resting HR (during anesthesia) was similar ($P > 0.05$) in female and male rats and resting MAP was higher ($P < 0.05$) in female compared to male rats. Resting FBF and FVC were higher in male compared to female rats ($P < 0.05$; Table 2).

Sympathetic vasoconstrictor responsiveness

The MAP, FBF and FVC responses to sympathetic stimulation at rest and during muscle contraction in a representative female rat is shown in Fig 1. Changes in FBF ($mL \cdot min^{-1}$) and FVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$) in response to sympathetic stimulation delivered at 2 and 5 Hz at rest and during contraction are presented in Table 3.

There was an interaction ($P < 0.05$) between the effects of sex and muscle contraction on sympathetic vasoconstrictor responsiveness. At rest the

vasoconstrictor response to sympathetic stimulation delivered at 2Hz was augmented ($P < 0.05$) instead of blunted in female compared to male rats, whereas sympathetic vasoconstrictor responsiveness to sympathetic stimulation delivered at 5Hz was not different ($P > 0.05$) between female and male rats (Fig. 2).

Muscle contraction blunted ($P < 0.05$) sympathetic vasoconstrictor responsiveness in both female and male rats (Fig 2). In contracting muscle, sympathetic vasoconstrictor responsiveness to sympathetic stimulation delivered at 2Hz was similar ($P > 0.05$) in male and female rats, whereas vasoconstriction in response to stimulation delivered at 5Hz was blunted ($P < 0.05$) in female compared to male rats, consistent with our hypothesis. Also consistent with our hypothesis, the magnitude of contraction-mediated inhibition of sympathetic vasoconstriction (sympatholysis) was greater ($P < 0.05$) in female compared to male rats (Fig. 3).

Effect of Nitric Oxide Synthase Blockade

L-NAME increased ($P < 0.05$) resting MAP by a similar ($P > 0.05$) magnitude in male and female rats and decreased ($P < 0.05$) FVC in both male and female rats (Table 2). L-NAME augmented ($P < 0.05$) resting sympathetic vasoconstrictor responsiveness by a similar ($P>0.05$) magnitude in female and male rats (Fig.2). In the presence of NOS blockade, resting sympathetic vasoconstrictor responsiveness in response to sympathetic stimulation delivered at 2Hz was greater ($P < 0.05$) in female compared to male rats, whereas the constrictor response to sympathetic stimulation delivered at 5 Hz was not different ($P > 0.05$) between male and female rats (Fig 2).

During muscular contraction, sympathetic vasoconstrictor responsiveness was not different ($P > 0.05$) between female and male rats in the presence of L-NAME (Fig. 2). Compared to the control condition, NOS blockade did not alter the magnitude of sympatholysis at the 2Hz stimulation frequency in female or male rats. At the 5Hz stimulation frequency, sympatholysis was not altered in male rats, but was reduced ($P < 0.05$) in female rats. In the presence of NOS blockade, sympatholysis was not different in female and male rats (Fig. 3).

Hyperaemic responses to contraction and muscle force production

The increase in FBF and FVC in response to contraction in control conditions was not different ($P > 0.05$) between female and male rats (Table 4) and NOS blockade did not alter the FBF and FVC ($P > 0.05$) response to muscle contraction in either group. Muscle force production was not different ($P > 0.05$) between female and male rats in control (F: 429 ± 58 g; M: 460 ± 57), and L-NAME conditions (F: 437 ± 59; M: 487 ± 77).

Table 2.1. Animal characteristics

Group	Body mass (g)	Heart mass (g)	Soleus mass (g)	Lateral gastrocnemius mass (g)	Medial gastrocnemius mass (g)	Heart-to-body mass ratio (mg:g)	Tibial Length (mm)	Heat Mass: Tibial Length (mg:mm)
Female (n=10)	295 ± 35	1.05 ± 0.12	0.16 ± 0.03	0.53 ± 0.06	1.04 ± 0.13	3.6 ± 0.3	39.4 ± 1.5	26.7 ± 2.4
Male (n=8)	456 ± 86*	1.37 ± 0.21*	0.22 ± 0.06*	0.74 ± 0.18*	1.46 ± 0.26*	3.0 ± 0.2*	41.7 ± 1.5*	33.9 ± 4.3*

All values are mean ± SD. * indicates a significant group difference. A *P*-value < 0.05 was considered statistically significant.

Table 2.2. Basal Hemodynamics

Group	Drug Condition	HR (beats·min^{-1})	MAP (mm Hg)	FBF (mL·min^{-1})	FVC (mL·min^{-1}·mmHg^{-1})
Female	Control	423 ± 29	108 ± 7	3.3 ± 0.5	0.030 ± 0.005
	L-NAME	392 ± 29†	142 ± 1†	2.8 ± 0.8	0.020 ± 0.006†
Male	Control	436 ± 26	96 ± 13*	4.8 ± 1.3*	0.050 ± 0.009*
	L-NAME	413 ± 32	132 ± 16†	3.6 ± 0.7*†	0.027 ± 0.004*†

All values are mean ± SD. Baseline heart rate (HR), mean arterial pressure (MAP), femoral artery blood flow (FBF), and femoral vascular conductance (FVC) in control and nitric oxide synthase blockade conditions (L-NAME) in Female (n=10) and Male (n=8) rats. * indicates a significant difference from the female group within a drug condition. † indicates a significant drug effect within a group. A *P*-value < 0.05 was considered statistically significant.

Table 2.3. Absolute changes in femoral blood flow and vascular conductance in response to sympathetic stimulation at rest and during muscle contraction.

			2 Hz		5 Hz	
Muscle Contractile State	Group	Drug Condition	ΔFBF ($mL \cdot min^{-1}$)	ΔFVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$)	ΔFBF ($mL \cdot min^{-1}$)	ΔFVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$)
Rest	Female	Control	-1.1 ± 0.3	-0.010 ± 0.003	-1.6 ± 0.3	-0.017 ± 0.004
		L-NAME	-1.1 ± 0.4	-0.009 ± 0.003	-1.6 ± 0.6	-0.012 ± 0.004†
	Male	Control	-1.3 ± 0.5	-0.013 ± 0.004	-2.1 ± 0.7	-0.023 ± 0.007
		L-NAME	-1.2 ± 0.6	-0.010 ± 0.003	-2.0 ± 0.9	-0.017 ± 0.005†
Contraction	Female	Control	-0.9 ± 0.4	-0.008 ± 0.003	-1.5 ± 0.6	-0.018 ± 0.005
		L-NAME	-1.2 ± 0.5	-0.013 ± 0.004†‡	-2.6 ± 1.1†‡	-0.026 ± 0.007†‡
	Male	Control	-1.0 ± 0.8	-0.015 ± 0.005*	-3.2 ± 1.4*	-0.039 ± 0.014*‡
		L-NAME	-1.1 ± 0.8	-0.014 ± 0.005‡	-3.6 ± 2.0‡	-0.035 ± 0.013*‡

Absolute changes in femoral artery blood flow (FBF), and femoral vascular conductance (FVC) in response to sympathetic stimulation delivered at 2 and 5 Hz at rest and during muscle contraction in control and nitric oxide synthase blockade (L-NAME) conditions in Female (n=10) and Male (n=8) rats. All values are Mean ± SD. * indicates a significant difference from the female group within a drug and contractile condition. † indicates a significant drug effect within a group and contractile condition. ‡ indicates a significant effect of muscle contraction compared to rest within a group. A *P*-value < 0.05 was considered statistically significant.

Table 2.4. Hemodynamic response to muscle contraction

Group	Drug Condition	HR (beats·min^{-1})	MAP (mmHg)	FBF (mL·min^{-1})	FVC (mL·min^{-1}·mmHg^{-1})
Female	Control	0 ± 10	2 ± 8	4.3 ± 1.2	0.041 ± 0.013
	L-NAME	0 ± 11	-6 ± 4†	5.9 ± 1.7	0.046 ± 0.012
Male	Control	1 ± 18	4 ± 10	6.0 ± 2.0	0.058 ± 0.019
	L-NAME	6 ± 17	-3 ± 8†	6.2 ± 2.7	0.052 ± 0.018

All values are mean ± SD. Absolute change of heart rate (HR), mean arterial pressure (MAP), femoral artery blood flow (FBF), and femoral artery vascular conductance (FVC) in response to muscle contraction at 60% of maximal contractile force in control and nitric oxide synthase blockade conditions(L-NAME) in Female (n=10) and Male (n=8) rats. † indicates a significant drug effect within a group. A *P*-value < 0.05 was considered statistically significant.

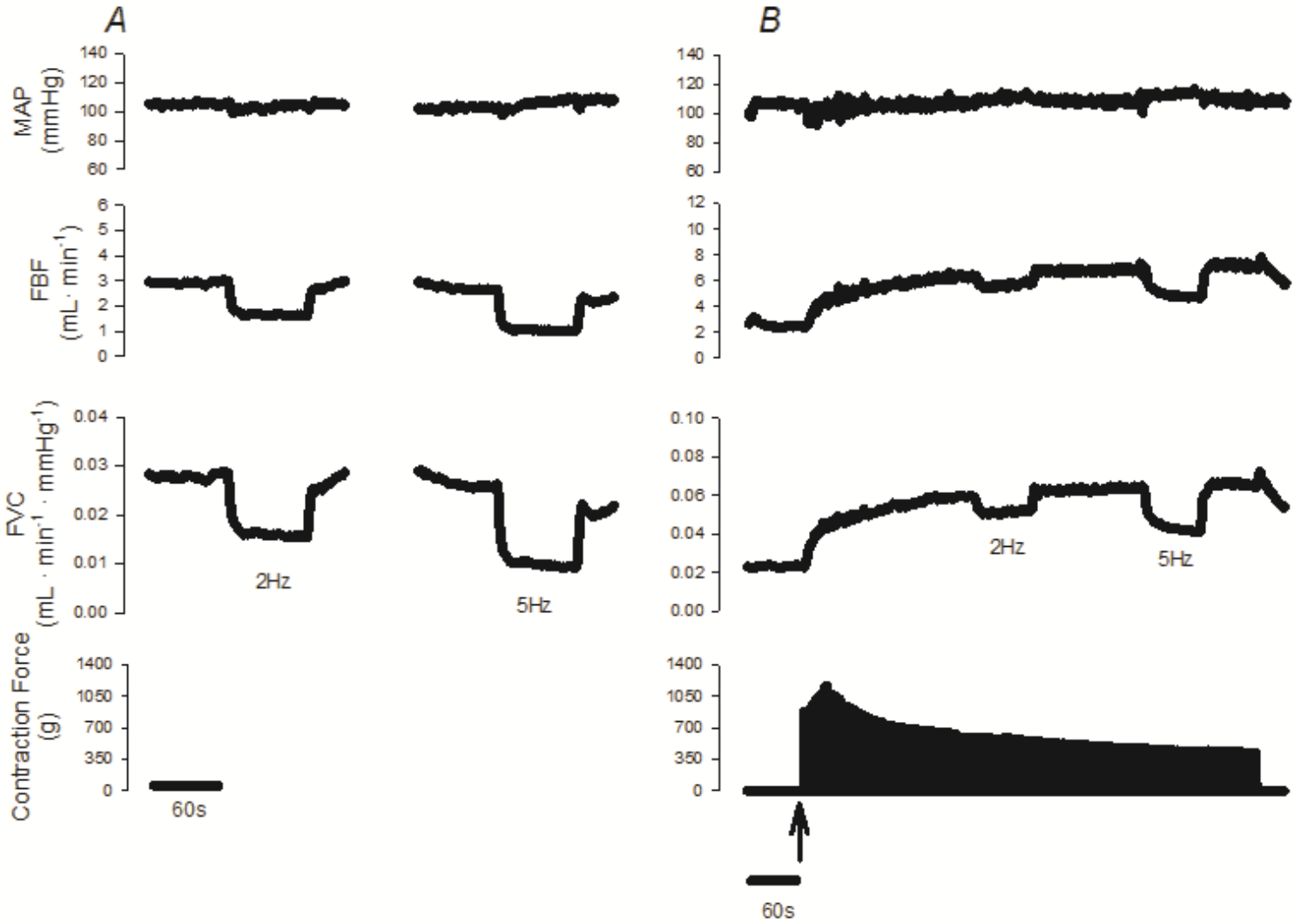

Figure 2.1. Original data from a representative female rat. The response of mean arterial blood pressure (MAP), femoral blood flow (FBF) and femoral vascular conductance (FVC) to lumbar sympathetic chain stimulation delivered at 2 and 5Hz at rest (Panel A) and during skeletal muscle contraction at 60% of maximal contractile force (MCF; B). The onset of contraction is denoted by the arrow.

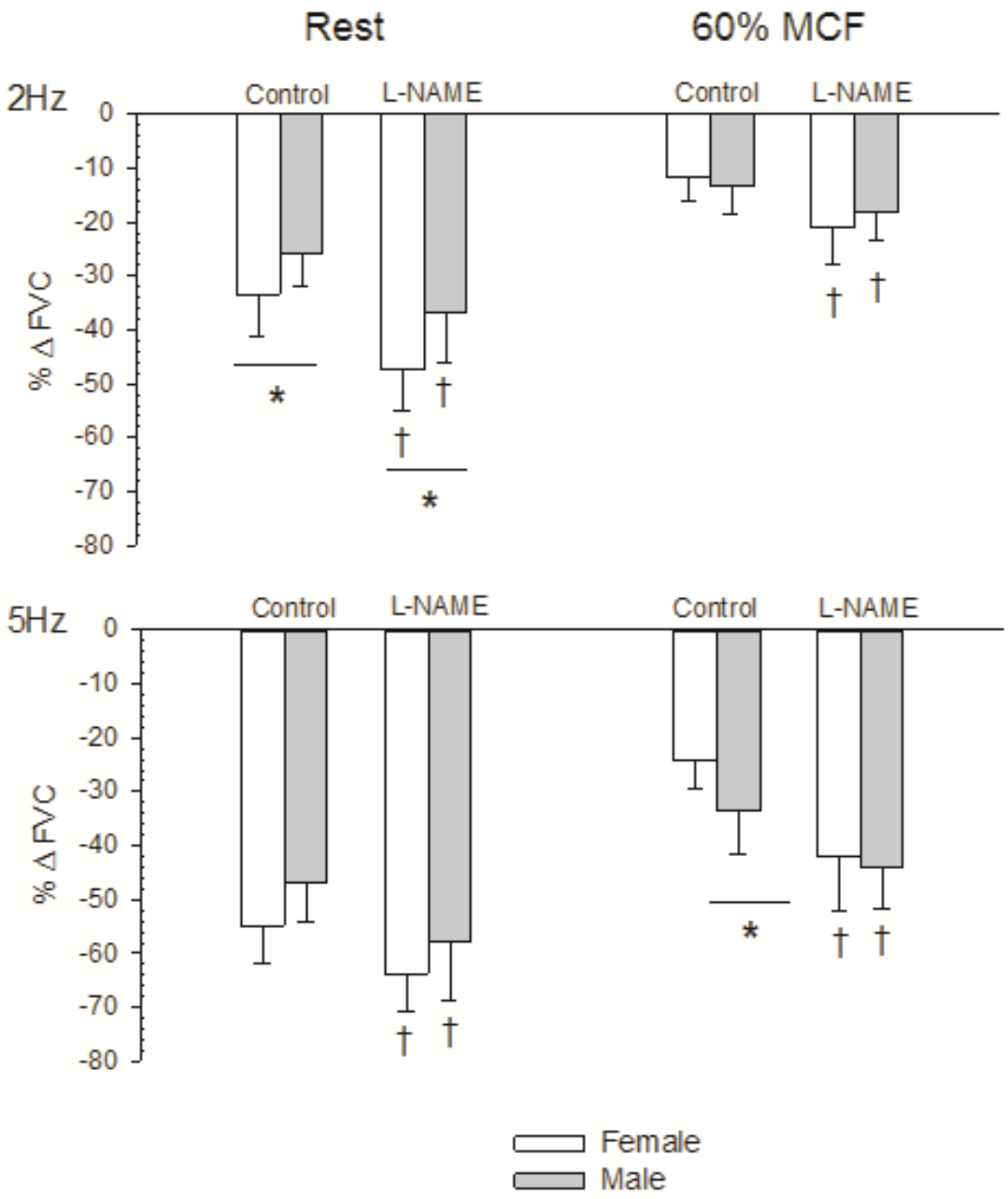

Figure 2.2. The percentage change of FVC in response to stimulation of the lumbar sympathetic chain at 2 and 5Hz in resting and contracting skeletal muscle in female (open bars, n=10) and male (grey bars, n=8) rats in control and NOS blockade (L-NAME; 10 mg kg^{-1} $_{I.V.}$) conditions. Values are means ± SD. * indicates a significant group difference. † indicates a significant drug effect within the group. A *P*-value < 0.05 was considered statistically significant.

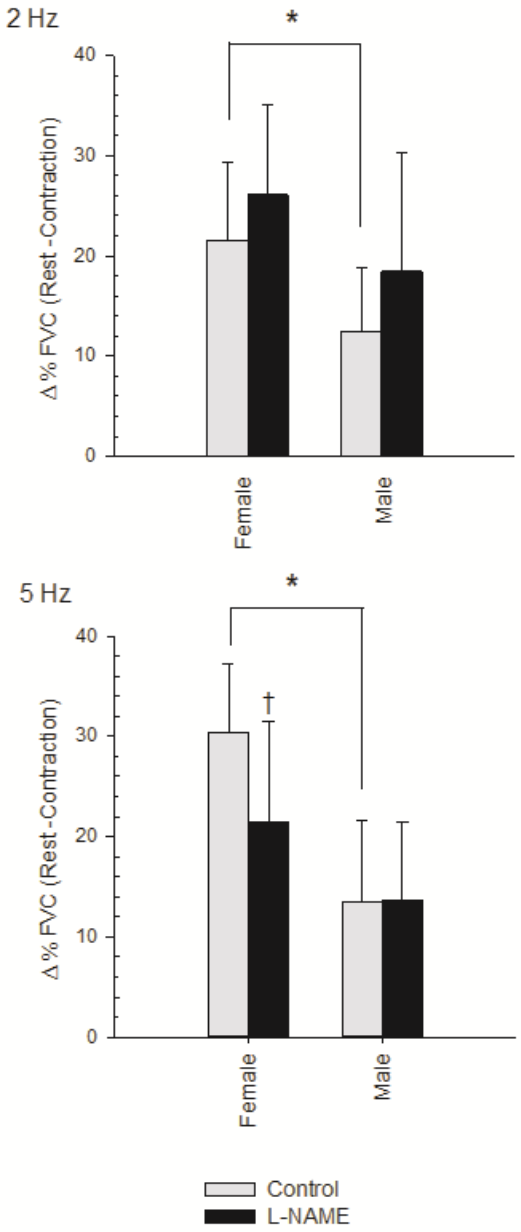

Figure 2.3. The magnitude of sympatholysis expressed as the difference between the decrease in femoral vascular conductance in response to sympathetic stimulation delivered at 2 and 5 Hz at rest and during muscular contraction (Δ%FVC Rest-Contraction) in control (grey bars) and NOS blockade (L-NAME; 10 mg kg^{-1} $_{I.V.}$; black bars) conditions in female (n=10) and male rats (n=8). Values are means ± SD. * indicates a significant group difference. † indicates a significant drug effect within the group. A *P*-value < 0.05 was considered statistically significant.

Discussion

The purpose of this study was to determine whether sex modulates sympathetic vasoconstrictor responsiveness and NO-mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle. We hypothesized that sympathetic vasoconstrictor responsiveness would be blunted in resting and contracting skeletal muscle of female compared to male rats. We also hypothesized that the blunted vasoconstrictor responsiveness in female rats would be mechanistically linked to enhanced NO mediated inhibition of vasoconstriction in female compared to male rats. In contrast to our hypothesis, sympathetic vasoconstrictor responsiveness was augmented in response to low-frequency sympathetic stimulation in female compared to male rats and was not different between sexes in response to high-frequency sympathetic stimulation. In contracting muscle, evoked vasoconstriction to low-frequency sympathetic stimulation was similar in females and males, whereas vasoconstriction in response to high-frequency sympathetic stimulation was blunted in females compared to males. Consistent with our hypothesis, sympatholysis was enhanced in females compared to males. In the presence of NOS blockade, contraction-mediated inhibition of vasoconstriction evoked by high frequency sympathetic stimulation was reduced in female rats and sympatholysis was not different between female and male rats. These data suggest that sex modulates sympathetic vascular control in resting and contracting skeletal muscle and that a portion of the enhanced sympatholysis in female rats was NO dependent.

Sympathetic Vasoconstrictor Responsiveness at Rest

In resting skeletal muscle, sympathetic vasoconstrictor responsiveness to low frequency (2Hz) stimulation of the lumbar sympathetic chain was greater in

female compared to male rats, whereas sympathetic vasoconstrictor responsiveness in response to sympathetic stimulation delivered at a higher (5Hz) frequency was not different between female and male rats. Previous studies in humans have reported similar [11] or blunted [9, 10] vasoconstrictor responsiveness in females compared to males. Intra-arterial infusion of selective α_1- and α_2-adrenoreceptor agonists in the forearm produced a similar vasoconstriction in males and females, suggesting that alpha-adrenoreceptor responsiveness was not different between males and females [11]. In contrast, sympatho-excitation via cold pressor test and isometric handgrip produced smaller increases in calf vascular resistance in females compared to men despite similar increases in efferent sympathetic nerve activity, suggesting that sympathetic vasoconstrictor responsiveness and/or sympathetic vascular transduction was blunted in women compared to men [9]. The contrasting findings between the present study and recent human studies may reflect a species differences in neuro-vascular control or may be a function of differences in the experimental approach and preparation. In the present study, sex differences in sympathetic vasoconstrictor responsiveness were investigated by directly stimulating the lumbar sympathetic chain to evoke the release of endogenous sympathetic neurotransmitters. Sympathetic neurotransmitter release is sensitive to the discharge frequency of sympathetic nerves and in the present study two different frequencies of electrical stimulation were applied to the lumbar chain to simulate the efferent nerve activity that may be observed at rest and in response to physiological stress, such as exercise. Low frequencies of nerve firing have been shown to evoke the release of adenosine triphosphate (ATP) and norepinephrine (NE), whereas higher frequencies appear to evoke the

co-release of NE and neuropeptide Y (NPY) [29, 34, 35]. The augmented constrictor responsiveness to low frequency sympathetic stimulation in female rats may indicate that ATP signalling and/or purinergic receptor responsiveness/density was increased in female compared to male rats. The similar vasoconstrictor response to high frequency sympathetic stimulation in male and females in the present study suggests that sex may not influence NPY mediated vasoconstriction in the skeletal muscle vascular bed. Further studies are necessary to partition the relative contributions of adrenergic and non-adrenergic neurotransmitters and receptors to evoked vasoconstrictor responses in male and female rats to fully elucidate the effect of sex on sympathetic vascular control in the resting skeletal muscle vascular bed.

NOS Inhibition increased sympathetic vasoconstrictor responsiveness at rest in both male and female rats in the present study. Our laboratory has previously reported that NO blunts sympathetic vasoconstriction in resting skeletal muscle in male rats [17] and the present findings extend those observations to female rats (Fig. 2). NO-mediated inhibition of sympathetic vasoconstriction in resting skeletal muscle was similar in male and female rats, suggesting that NO-mediated blunting of vasoconstriction in resting skeletal muscle was similar in male and female rats.

It has been argued that β-adrenoreceptor mediated vasodilation may modulate sympathetic vasoconstriction in females [36]. β-adrenoreceptor blockade has been shown to augment forearm vasoconstriction to noradrenaline in females, but not males, and abolish sex differences in resting vasoconstrictor responsiveness [10]. However, sympathetic vasoconstrictor responses to isometric handgrip were not altered following β-adrenoreceptor blockade in males

or females, suggesting that β-adrenoreceptor mediated vasodilation does not oppose evoked sympathetic vasoconstriction in resting skeletal muscle [12]. Moreover, Limberg et al. [13] have recently reported a similar forearm vasodilation to isoproterenol (β-adrenoreceptor agonist) in males and females, suggesting that sex may not modulate β-adrenoreceptor responsiveness/function. Evoked constrictor responses were not performed in the presence of β-adrenoreceptor blockade in the present study. However, the absence of a blunted vasoconstrictor response to sympathetic stimulation in resting skeletal muscle in female compared to male rats suggests that β-adrenoreceptor mediated opposition to sympathetic vasoconstriction was not augmented in female compared to male rats.

Sympatholysis

Contraction mediated blunting of vasoconstriction (sympatholysis) was enhanced in female compared to male rats in the present study. To our knowledge, this is the first study to report a sex difference in sympatholysis and that sympatholysis is enhanced in females compared to males. Furthermore, the present study indicates that enhanced nitric oxide mediated blunting of sympathetic vasoconstriction underlies a portion of the enhanced sympatholysis in female rats.

NO is enzymatically produced by NO synthase (NOS) and NO derived from the endothelial (eNOS) and neuronal (nNOS) isoforms has been shown to inhibit sympathetic vasoconstriction in resting and contracting skeletal muscle [17, 37]. NOS expression appears to be sensitive to estrogen bioavailability and several studies have reported elevated levels of eNOS and nNOS in females compared to males in a variety of tissues including skeletal muscle arterioles.

[38-43]. Thus, females may be predisposed to NO mediated inhibition of sympathetic vasoconstriction. Consistent with this notion, NOS blockade blunted sympatholysis in female rats in the present study and did not alter sympatholysis in male rats. We have previously reported that NOS blockade did not alter sympatholysis in sedentary male rats [14]. Consistent with the findings from our laboratory, Dinenno and Joyner [44] reported that NOS blockade did not alter sympatholysis during moderate-intensity forearm exercise in adult men. Collectively, these studies suggest that NO may not be required for sympatholysis in males. In contrast in females, NO appears to contribute to contraction mediated blunting of vasoconstriction evoked at high stimulation frequencies, evidenced by the reduction in sympatholysis following NOS blockade in female rats at the 5Hz stimulation frequency in the present study. In agreement with the present findings, Thomas et al [16] have reported that NO inhibits vasoconstriction during exercise in female Sprague-Dawley rats.

The cellular mechanism of NO mediated blunting of sympathetic vasoconstriction has not been established. However, previous studies in female rats have suggested that α_2-adrenorecptors in glycolytic skeletal muscles may be particularly sensitive to metabolic inhibition during contraction and that NO may blunt α_2-adrenoreceptor mediated vasoconstriction [45]. Our laboratory has recently reported that sympatholysis was primarily mediated by inhibition of α_1-adrenoreceptors and that NO was not required for sympatholysis in sedentary males rats [46]. Thus, different sensitivities of post-synaptic α-adrenoreceptors to metabolic inhibition may contribute the observed sex differences in sympatholysis. Similar vasoconstrictor responses to the injection of α_1- and α_2-adrenoreceptor agonists in the exercising forearm have been reported in young

males and females, however these studies were not performed in the presence of NOS blockade and therefore the influence of NO bioavailability on adrenoreceptor responsiveness could not be assessed [11]. Further investigation will be required to isolate sex-specific differences in post-synaptic receptor responsiveness and their relative contribution to the contraction mediated blunting of sympathetic vasoconstriction in the skeletal muscle vascular bed.

Perspectives and Significance

Our lab and others have demonstrated that exercise training can improve NO-mediated sympatholysis [14, 30], and these adaptations can be linked to improvements in nNOS expression and nNOS-mediated NO production [17, 47]. Ovariectomized rats and post-menopausal women have been shown to have a reduced capacity to inhibit sympathetic vasoconstriction during exercise and this has been associated with reduced skeletal muscle nNOS expression [22, 25]. The potential to modify skeletal muscle nNOS protein expression and enhance skeletal muscle vascular control with exercise training, has interesting implications for future studies aimed at optimizing vascular health in post-menopausal women. If effective, aerobic exercise training would be a low-cost therapy to restore vascular function in post-menopausal women that would also avoid the considerable side effects that have been associated with hormone replacement therapy [48, 49].

Conclusion

In conclusion, this study demonstrated that sex modulates sympathetic vasoconstrictor responsiveness in resting and contracting skeletal muscle in a manner that is dependent on the frequency of sympathetic stimulation.

Furthermore, sympatholysis was enhanced in female compared to male rats and the augmented sympatholysis in female rats was partly mediated by NO. Further investigation will be required to fully elucidate the mechanism(s) responsible for the enhanced sympatholysis in females.

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Chapter 3

Estrogen and Nitric Oxide Synthase Isoform Specific Inhibition of Sympathetic Vasoconstriction

Introduction

The sympathetic nervous system (SNS) is essential for the regulation of peripheral vascular resistance and thus the dynamic control of arterial blood pressure and tissue blood flow [1]. In response to exercise, muscle sympathetic nerve activity (MSNA) increases in an exercise intensity-dependent manner [2] and produces tonic vasoconstriction in non-active tissues and active skeletal muscle [1, 3]. In contracting skeletal muscles, the vasoconstrictor response to MSNA is blunted (functional sympatholysis) [4] and the contraction-mediated inhibition of vasoconstriction serves to optimize the distribution of blood flow between and within active skeletal muscles [1]. The vasoactive molecule nitric oxide (NO) has been shown to inhibit vasoconstriction in rats [5-11] and humans [12]. NO is produced by the enzyme NO synthase (NOS) [13] and NO derived from endothelial (eNOS) and neuronal (nNOS) isoforms of NOS have been shown to inhibit constriction in resting and contracting skeletal muscle [5, 6, 8, 11, 14-19]. Indeed, data from our laboratory indicate that NO derived from nNOS and eNOS account for approximately 30% and 70% of NO-mediated blunting of sympathetic vasoconstriction at rest, whereas during exercise NO from each isoform accounts for approximately 50% of NO-mediated inhibition of sympathetic vasoconstriction [20, 21].

Estrogen status appears to influence functional sympatholysis. Fadel et al have reported that sympatholysis is impaired in post- compared to pre-

menopausal women and estradiol replacement restored sympatholysis in post-menopausal women [22]. Similarly, sympatholysis was impaired in ovariectomized rats compared to ovary-intact and ovariectomized rats that had received supplemental estradiol [23]. Non-selective NOS blockade reduced sympatholysis in ovary-intact and estrogen supplemented ovariectomized rats, but had no effect on sympatholysis in ovariectomized rats, suggesting that NO mediated inhibition of sympathetic vasoconstriction may be modulated by estrogen bioavailability [23]. Skeletal muscle nNOS expression was reduced in ovariectomized rats compared to young ovary intact female rats and ovariectomized rats that received estradiol supplementation, and nNOS expression was correlated with plasma estradiol levels [23]. In contrast, skeletal muscle eNOS expression was not dependent on estrogen status [23]. However, other studies have reported estrogen-dependent changes in eNOS expression [24-27] . Overall these data suggest that estrogen bioavailability may modulate NO-mediated inhibition of sympathetic vasoconstriction. However, the effect of estrogen bioavailability on NOS isoform specific sympatholysis has not been directly investigated with selective pharmacology. Changes to sympathetic vascular control and NOS expression have been investigated over a relatively short time period (4 weeks) of altered estrogen bioavailability [22, 23]. Further, data was obtained in a group of women that had been menopausal for a period of 8 ± 2 years and that had either not received hormone replacement therapy (HRT; n=3) or received HRT (n=5) for a time period ranging from 0.5 to 5.5 years [22, 23]. Whether a longer period of reduced estradiol bioavailability exacerbates NO-mediated blunting of sympathetic vasoconstriction is unclear. Prolonged chronic changes in estrogen availability such as seen during menopause may lead to a

progressive vascular dysfunction or alternatively vascular remodelling and /or functional adaptations that may produce different responses than a short-term intervention [28].

Thus, in the present study, we utilized ovary intact (OI), ovariectomized (OVX), and ovariectomized, estradiol supplemented (OVXE) female rats to investigate the hypothesis that chronic changes in estrogen bioavailability modulate sympathetic vasoconstrictor responsiveness and NOS isoform-specific blunting of sympathetic vasoconstriction in resting and contracting skeletal muscle. The following specific hypotheses were tested: 1) nNOS- and eNOS-mediated inhibition of sympathetic vasoconstriction in resting and contracting muscle would be impaired in OVX rats compared to OI rats; 2) estrogen replacement in OVX rats would restore eNOS and nNOS-mediated inhibition of sympathetic vasoconstriction at rest and during muscle contraction.

Methods

Animal Use

Female Sprague-Dawley rats (approximately 8 weeks old) 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.

Ovariectomy and 17β-Estradiol replacement

Rats were randomly assigned to ovary intact (OI; n=10), ovariectomized (OVX; n=8) or ovariectomy and 17β-estradiol-replacement (OVXE; n=11) groups.

Rats to be ovariectomized were anesthetized by inhalation of isoflurane (3.5% in balance O_2) 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 was managed 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.

Experimental Instrumentation

Ten weeks after the surgical procedure, the experiment was initiated by anesthetizing the rats via inhalation of isoflurane (3.5%, balance O_2). A cannula was then placed in the right jugular vein and isoflurane was withdrawn and anesthesia was maintained by syringe-pump infusion of a mixture of α-chloralose (8-16 $mg \cdot kg^{-1} \cdot h^{-1}$; Sigma Aldrich, Canada) and urethane (50-100 $mg \cdot kg^{-1} \cdot h^{-1}$; Sigma Aldrich, Canada). The depth of anesthesia was assessed by absence of a withdrawal reflex to a paw pinch and the stability of hemodynamic variables. A tracheotomy was performed and rats were mechanically ventilated (Model 683, Harvard Apparatus, Holliston, MA, USA) with 30% O_2 in balance N_2 to maintain arterial blood gases and acid base status (PaO_2: 90-100mmHg; $PaCO_2$: 39-41mmHg pH: 7.39-7.42). The left carotid artery was cannulated and attached to a

pressure transducer (Abbott, North Chicago, IL, USA) for the continuous measurement of arterial blood pressure. The left femoral vein was cannulated for drug delivery and continuous infusion of heparinized saline via syringe pump (4 units ml^{-1} at 2.5-3 ml hr^{-1} kg^{-1}). Femoral blood flow (FBF) was measured at the right femoral artery with a flow probe (0.7V; Transonic Systems, Ithaca, NY, USA) connected to a flow meter (T106; Transonic Systems). Heart rate (HR) was derived from the arterial blood pressure waveform, and femoral vascular conductance (FVC) was calculated. Core body temperature was monitored by rectal thermistor and body temperature was maintained at 36-37 °C by an external heating pad (TCAT-2, Physitemp, Clifton, NJ, USA).

The right triceps surae muscle group was dissected and attached to a force transducer (FT03, Grass Technologies, Warwick, RI, USA) at the calcaneal tendon. A cuff electrode was placed around the sciatic nerve and muscle contraction was produced by electrical stimulation (Stimulator Panel, Chart software; AD Instruments, Colorado Springs, CO USA). The motor threshold (MT) and the optimal muscle length for tension development were determined. Maximal contractile force (MCF) was determined by stimulation of the triceps surae muscle group with 25 impulses of 1ms duration delivered at 100Hz at 10x the MT. Rhythmic muscle contractions at 60% of MCF were produced by stimulation of the sciatic nerve at 40 Hz, 0.1ms pulses in 250ms trains at a rate of 60 trains·min^{-1} at ~3 times MT.

A midline abdominal incision was made and the abdominal aorta and vena cava were temporarily retracted to expose the lumbar sympathetic chain. A bipolar silver-wire stimulating electrode was attached to the lumbar sympathetic chain at the level of the L3 and L4 vertebrae and electrically isolated in rapidly

curing, non-toxic silicone elastomer (Kwiksil; WPI, Sarasota, FL, USA). An isolated constant current stimulator (DS3; Digitimer, Welwyn Garden City, UK) was used to stimulate the sympathetic chain and evoke the release of sympathetic neurotransmitters and produce vasoconstriction in the experimental hindlimb.

Following the surgical procedures and an approximately 20-minute stabilization period the following experimental protocol was completed.

Experimental Protocol

The vasoconstrictor response to 1 minute of lumbar sympathetic chain stimulation (1 ms, 1 mAmp pulses) delivered at 2 and 5 Hz in random order was measured at rest and during muscle contraction under control conditions, following selective nNOS blockade (*S*-methly-$_L$-thiocitrulline; SMTC; 0.6 $mg \cdot kg^{-1}$, IV) and following non-selective NOS blockade (N_ω-nitro-L-arginine methyl ester; L-NAME, 10 $mg \cdot kg^{-1}$, IV). The dose of SMTC has been used previously in our laboratory to selectively block nNOS at rest and during exercise [20, 21]. Other laboratories have used similar or lower doses of SMTC to block nNOS in male rats (0.2-2.1 $mg \cdot kg^{-1}$) [29-31], female rats (0.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$) [32], and humans of both sexes (0.2 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) [33, 34]. The dose of L-NAME has been used in our laboratory and others to non-selectively block NOS in male and female rats [5, 8, 35-37] and humans [12]. The effect of SMTC and L-NAME were determined by calculating differences in sympathetic vasoconstrictor responsiveness in control, SMTC and L-NAME conditions [20]. The increase in vasoconstrictor responsiveness following the administration of SMTC was interpreted as inhibition of sympathetic vasoconstriction by NO derived from nNOS. The difference between vasoconstrictor responsiveness in the L-NAME and control

conditions was interpreted as total NO-mediated of vasoconstriction and the difference in vasoconstrictor responsiveness between the SMTC and L-NAME conditions was interpreted as inhibition of sympathetic vasoconstriction by NO derived from eNOS [20].

Stimulation frequencies of 2 and 5 Hz were selected to reflect levels of sympathetic nerve activity observed at rest and during conditions of elevated nerve activity (such as in response to exercise or physiological stress) and to evoke graded vasoconstrictor responses in the hindlimb vascular bed at rest and during muscle contraction [38-41]. These frequencies of sympathetic nerve stimulation are routinely used in our laboratory and others to investigate neurovascular control in the skeletal muscle vascular bed [5, 8, 10]. Eight minutes of rhythmic muscle contraction was produced and lumbar sympathetic chain stimulation at 2 and 5 Hz was delivered 3 and 6 minutes after the onset of muscle contraction in random order. Control, SMTC and L-NAME conditions were separated by ~ 60 minutes of recovery.

Following the experimental protocol, rats were killed by anesthetic overdose (α-chloralose and urethane). The heart, soleus, lateral and medial gastrocnemius muscles were dissected and weighed. The tibia was dissected and tibial length measured.

Plasma Estrogen Levels

Venous blood samples (n=8/group) were drawn into EDTA-coated tubes following the collection of functional data. Samples were immediately centrifuged at 13,000g for 15 minutes at 4°C. The plasma was aliquoted, and immediately stored at -80°C until analysis. Plasma samples were thawed, and 17β-estradiol

levels were measured using a commercially available competitive immunoenzymatic assay (ab108667, Abcam, Cambridge, MA).

Data Analysis

Data were recorded using Chart software (AD Instruments, Colorado Springs, CO USA). Arterial blood pressure and FBF were sampled at 100 Hz and FVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$) was calculated as FBF ÷ mean arterial pressure (MAP). Muscle force production was measured continuously, and peak force development was determined for each muscle contraction. To compare force production between groups and experimental conditions a mean of peak contractile forces was calculated between minutes 3-7 (the time period encompassing the sympathetic stimulations) for each contractile bout.

The change in HR, MAP, FBF and FVC in response to sympathetic stimulation was calculated as an absolute change and as a percentage change from the value preceding the sympathetic stimulation in Control, SMTC and L-NAME conditions. 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 [1, 2]. Comparisons were conducted to examine differences in the percentage change of FVC between groups at rest and during muscle contraction, during Control, SMTC and L-NAME conditions. All data are expressed as mean ± SD.

Statistics

Group differences in body, muscle and heart mass, heart:body mass ratios, and plasma estradiol were determined by one-way ANOVA. The influence

of estrogen and NO derived from nNOS and eNOS on the vasoconstrictor response to sympathetic stimulation at rest and during exercise was analyzed with a three-way repeated measures ANOVA (group x drug condition x muscle contractile state). Each frequency of sympathetic stimulation was analyzed separately. To determine estrogen and nitric oxide effects on baseline hemodynamics, the hemodynamic response to exercise, the difference in percentage change of FVC between rest and muscle contraction (sympatholysis) during Control, SMTC and L-NAME conditions, and muscle force production, a two-way repeated measures ANOVA (Group x Drug condition) was utilized. 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

Body mass was higher ($P < 0.05$) in OVX rats compared to OI and OVXE, and higher ($P < 0.05$) in OI rats compared to OVXE rats (Table 3.1.). Heart mass was similar ($P > 0.05$) in OVX and OI rats, but lower ($P < 0.05$) in OVXE compared to OI and OVX rats (Table 3.1.). The heart mass to body mass ratio was smaller ($P < 0.05$) in OVX rats compared to OI and OVXE rats (Table 3.1.). Tibial length and the heart mass to tibial length ratio were smaller ($P < 0.05$) in OVXE rats compared to OVX and OI rats (Table 3.1.). Medial and lateral gastrocnemius and soleus muscle masses were similar ($P > 0.05$) in OI and OVX rats, whereas muscle mass was lower ($P < 0.05$) in OVXE compared to OI and OVX rats (Table 3.1.). Plasma estradiol was elevated ($P < 0.05$) in OVXE rats compared to OVX and OI rats, and elevated ($P < 0.05$) in OI rats compared to OVX rats (OI, n=8: 31 ± 12 $pg \cdot mL^{-1}$; OVX, n=8: 13 ± 6 $pg \cdot mL^{-1}$; OVXE, n=7: 53 ±

15 pg·mL^{-1}). Resting hemodynamics were similar ($P > 0.05$) in all groups in control conditions (Table 3.2.).

Estrogen and Sympathetic Vasoconstrictor Responsiveness

The response of MAP, FBF and FVC to sympathetic stimulation delivered at rest and during muscle contraction in a representative ovary-intact rat is illustrated in Fig 3.1. The absolute changes in FBF and FVC in response to sympathetic stimulation delivered at 2Hz and 5Hz in resting and contracting skeletal muscle are reported in Table 3.3. At rest, the vasoconstrictor response to sympathetic stimulation at 2Hz and 5Hz was not different ($P > 0.05$) between OI, OVX and OVXE rats (Fig. 3.2.). Muscle contraction blunted ($P < 0.05$) sympathetic vasoconstrictor responsiveness sympathetic stimulation delivered at 2Hz and 5Hz in all groups (Fig. 3.2.). There were no group differences ($P > 0.05$) in the magnitude of vasoconstriction during muscle contraction at either frequency of sympathetic stimulation. The magnitude of contraction-mediated inhibition of sympathetic vasoconstriction (sympatholysis) was also similar ($P > 0.05$) between all groups at both frequencies of sympathetic stimulation (Fig. 3.3.) in control conditions.

Effect of Neuronal Nitric Oxide Synthase Blockade

At rest, selective blockade of nNOS increased ($P < 0.05$; main effect of drug) MAP and FBF in all groups, and did not alter ($P > 0.05$) FVC or HR (Table 3.1.). Following nNOS blockade, sympathetic vasoconstrictor responsiveness at rest was not different ($P > 0.05$) from the response in the control condition in any group (Fig 3.2.).

Vasoconstrictor responsiveness during muscle contraction following nNOS blockade was not different ($P > 0.05$) than in control conditions in any

group (Figure 3.2.). Sympatholysis was also similar ($P > 0.05$) in selective nNOS blockade and Control conditions in all groups (Figure 3.3.).

Effect of Non-Selective Nitric Oxide Synthase Blockade

At rest, non-selective NOS blockade increased ($P < 0.05$; main effect of drug) MAP compared to the Control condition and decreased ($P < 0.05$; main effect of drug) HR, FBF and FVC in all groups compared to the SMTC and Control conditions. Vasoconstrictor responsiveness to sympathetic stimulation delivered at 2Hz was augmented ($P < 0.05$) in OI rats in the presence of L-NAME, whereas vasoconstrictor responsiveness in OVX or OVXE rats was not altered by non-selective NOS blockade. Non-selective NOS blockade augmented ($P < 0.05$) vasoconstrictor responsiveness to sympathetic stimulation delivered at 5Hz by a similar magnitude ($P > 0.05$) in all groups (Figure 3.2.).

During muscle contraction, non-selective NOS blockade, increased ($P < 0.05$) sympathetic vasoconstrictor responsiveness to sympathetic stimulation delivered at 2Hz in OI rats, whereas sympathetic vasoconstrictor responsiveness in OVX or OVXE rats was not altered ($P > 0.05$). Vasoconstriction in response to sympathetic stimulation delivered at 5Hz was increased ($P > 0.05$) by non-selective NOS blockade by a similar magnitude in all groups (Figure 2). Sympatholysis was not altered ($P > 0.05$) by L-NAME in any group at the 2Hz stimulation frequency, whereas L-NAME reduced ($P > 0.05$) sympatholysis at the 5Hz stimulation frequency in OI, OVX and OVXE rats (Figure 3.3.).

Hyperemic Responses to Muscle Contraction and Force Production

The increase in FBF and FVC in response to muscle contraction was not ($P > 0.05$) different between groups in Control, SMTC and L-NAME conditions (Table 3.4.). The increase in FBF and FVC in response to muscle contraction

was larger ($P < 0.05$) following selective nNOS blockade (compared to the Control condition) in all groups (Table 3.4.). The FBF response to muscle contraction during non-selective NOS blockade remained elevated ($P < 0.05$) compared to control conditions, and was not different ($P > 0.05$) than the SMTC condition in all groups. In the presence of L-NAME, the increase in FVC in response to muscle contraction was larger ($P < 0.05$) that the response in the Control conditions but was lower ($P < 0.05$) than the response in the SMTC condition in all groups.

Muscle force production was not different ($P > 0.05$) between groups in Control conditions (OI: 485 ± 67 g; OVX: 524 ± 77 g; OVXE: 463 ± 53 g) SMTC (OI: 499 ± 38 g; OVX: 498 ± 50 g; OVXE: 470 ± 58 g) and L-NAME (OI: 493 ± 35 g; OVX: 496 ± 48 g; OVXE: 465 ± 57 g) did not alter ($P > 0.05$) contractile force in any group.

Table 3.1. Animal Characteristics

Group	Body Mass (g)	Heart Mass (g)	Tibial Length (mm)	Heart Mass (mg) : Body Mass (g)	Heart Mass (mg) : Tibial Length (mm)	Muscle Mass (g)		
						Soleus	Lateral Gastrocnemius	Medial Gastrocnemius
OI	375 ± 20	1.15 ± 0.06	42 ± 1	3.1 ± 0.3	27 ± 2	0.22 ± 0.02	0.70 ± 0.10	1.25 ± 0.14
OVX	446 ± 56*	1.22 ± 0.07	43 ± 1	2.8 ± 0.3*	29 ± 1	0.21 ± 0.02	0.73 ± 0.07	1.36 ± 0.15
OVXE	318 ± 28†*	1.02 ± 0.10*	40 ± 1*†	3.2 ± 0.3†	26 ± 2*†	0.17 ± 0.02*†	0.64 ± 0.06*†	1.12 ± 0.15*†

All values are Mean ± SD. Tissue was obtained from ovary intact (OI; n=10), ovariectomized (OVX; n=8), and ovariectomized, estrogen-replaced (OVXE; n=11) rats. * indicates a statistically significant difference from OI rats. † indicates a statistically significant difference from OVX rats. A *P*-value < 0.05 was considered statistically significant.

Table 3.2. Basal Hemodynamics

Group	Drug Condition	HR (beats·min^{-1})	MAP (mm Hg)	FBF (mL·min^{-1})	FVC (mL·min^{-1}·mmHg^{-1})
OI	Control	390 ± 46	105 ± 9	3.2 ± 0.4	0.030 ± 0.005
	SMTC	391 ± 51	120 ± 5*	4.2 ± 1.2*	0.035 ± 0.011
	L-NAME	364 ± 33*†	137 ± 18 *†	2.9 ± 1.1*†	0.021 ± 0.007*†
OVX	Control	382 ± 29	106 ± 14	4.4 ± 1.2	0.042 ± 0.011
	SMTC	380 ± 23	110 ± 17*	4.3 ± 1.2*	0.039 ± 0.008
	L-NAME	363 ± 48*†	128 ± 25 *†	3.2 ± 1.1*†	0.025 ± 0.007*†
OVXE	Control	382 ± 44	107 ± 12	3.9 ± 0.8	0.037 ± 0.005
	SMTC	380 ± 53	116 ± 16*	4.6 ± 1.2*	0.040 ± 0.011
	L-NAME	350 ± 43*†	132 ± 16*†	3.1 ± 1.0 *†	0.024 ± 0.007*†

Heart rate (HR), mean arterial pressure (MAP), femoral blood flow (FBF) and femoral vascular conductance (FVC) at rest in ovary intact (OI; n=10), ovariectomized (OVX; n=8), and ovariectomized, estrogen-replaced (OVXE; n=11) rats. Values are Mean ± SD. * indicates a statistically significant difference from the control condition (main effect of drug). † indicates a statistically significant difference from SMTC condition (main effect of drug). A *P*-value < 0.05 was considered statistically significant.

Table 3.3. Absolute changes in femoral blood flow and vascular conductance in response to sympathetic stimulation at rest and during muscle contraction.

			2 Hz		5 Hz	
Muscle Contractile State	Group	Drug Condition	FBF ($mL \cdot min^{-1}$)	FVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$)	FBF ($mL \cdot min^{-1}$)	FVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$)
Rest	OI	Control	-1.2 ± 0.5	-0.012 ± 0.005	-1.7 ± 0.5	-0.018 ± 0.005
		SMTC	-1.4 ± 1.0	-0.012 ± 0.009	-1.9 ± 1.0	-0.019 ± 0.009
		L-NAME	-1.4 ± 1.0	-0.11 ± 0.007	-1.7 ± 0.8	0.014 ± 0.006
	OVX	Control	-1.8 ± 0.6	-0.015 ± 0.005	-2.2 ± 0.7	-0.022 ± 0.007
		SMTC	-1.3 ± 0.4	-0.013 ± 0.005	-1.9 ± 0.6	-0.021 ± 0.004
		L-NAME	-1.1 ± 0.4	-0.010 ± 0.004	-1.7 ± 0.5	-0.016 ± 0.005
	OVXE	Control	-1.4 ± 0.3	-0.014 ± 0.003	-1.9 ± 0.4	-0.020 ± 0.003
		SMTC	-1.2 ± 0.6	-0.013 ± 0.004	-2.0 ± 0.9	-0.021 ± 0.009
		L-NAME	-1.2 ± 0.7	-0.010 ± 0.005	-1.7 ± 0.9	-0.014 ± 0.006
Contraction	OI	Control	-1.3 ± 1.2	-0.013 ± 0.008	-1.9 ± 1.4	-0.024 ± 0.010
		SMTC	-1.2 ± 1.4	-0.013 ± 0.010	-1.8 ± 1.6	-0.025 ± 0.011
		L-NAME	-1.7 ± 1.1	-0.016 ± 0.008	-2.8 ± 1.4	-0.028 ± 0.010*
	OVX	Control	-1.5 ± 1.0	-0.011 ± 0.005	-2.4 ± 0.5	-0.025 ± 0.005
		SMTC	-0.9 ± 0.4	-0.010 ± 0.003	-2.9 ± 1.4	-0.031 ± 0.010*
		L-NAME	-1.0 ± 0.8	-0.014 ± 0.006	-3.1 ± 1.8	-0.033 ± 0.015*
	OVXE	Control	-0.9 ± 0.8	-0.009 ± 0.005	-1.3 ± 0.9	-0.019 ±0.007
		SMTC	-0.7 ± 0.8	-0.010 ± 0.006	-1.4 ± 1.5	-0.022 ± 0.013
		L-NAME	-0.7 ± 1.0	-0.011 ± 0.006	-1.8 ± 1.7	-0.024 ± 0.010*

Absolute changes in femoral artery blood flow (FBF), and femoral vascular conductance (FVC) in response to sympathetic stimulation delivered at 2 and 5 Hz at rest and during muscle contraction in control and nitric oxide synthase blockade (L-NAME) conditions ovary intact (OI; n=10), ovariectomized (OVX; n=8), and ovariectomized, estrogen-replaced (OVXE; n=11) rats. All values are Mean ± SD. * indicates a significant difference from the resting (main effect of muscle contraction). A *P*-value < 0.05 was considered statistically significant.

Table 3.4. Hemodynamic response to muscle contraction

Group	Drug Condition	ΔHR (beats·min^{-1})	ΔMAP (mm Hg)	ΔFBF (mL·min^{-1})	ΔFVC (mL·min^{-1}·mmHg^{-1})
OI	Control	-2 ± 7	5 ± 6	5.5 ± 1.6	0.050 ± 0.013
	SMTC	2 ± 9	1 ± 7*	6.9 ± 2.5*	0.060 ± 0.019*
	L-NAME	2 ± 15	-8 ± 5*	5.8 ± 2.5*	0.046 ± 0.014*†
OVX	Control	1 ± 7	7 ± 8	5.1 ± 1.4	0.044 ± 0.012
	SMTC	5 ± 6	4 ± 12*	6.5 ± 1.2*	0.060 ± 0.011*
	L-NAME	2 ± 8	-4 ± 6*†	6.3 ± 1.8*	0.054 ± 0.014*†
OVXE	Control	-1 ± 12	8 ± 11	5.0 ± 1.1	0.044 ± 0.007
	SMTC	0 ± 8	-1 ± 8*	6.4 ± 1.6*	0.057 ± 0.012*
	L-NAME	-2 ± 12	-7 ± 7*†	6.3 ± 1.6*	0.053 ± 0.011*†

Heart rate (HR), mean arterial pressure (MAP), femoral blood flow (FBF) and femoral vascular conductance (FVC) during muscle contraction during control and drug conditions ovary intact (OI; n=10), ovariectomized (OVX; n=8), and ovariectomized, estrogen-replaced (OVXE; n=11) rats. Values are mean ± SD.* indicates a statstically significant difference from control conditions (main effect of drug). † indicates a statstically significant difference from SMTC conditions (main effect of drug). A *P*-value < 0.05 was considered statistically significant.

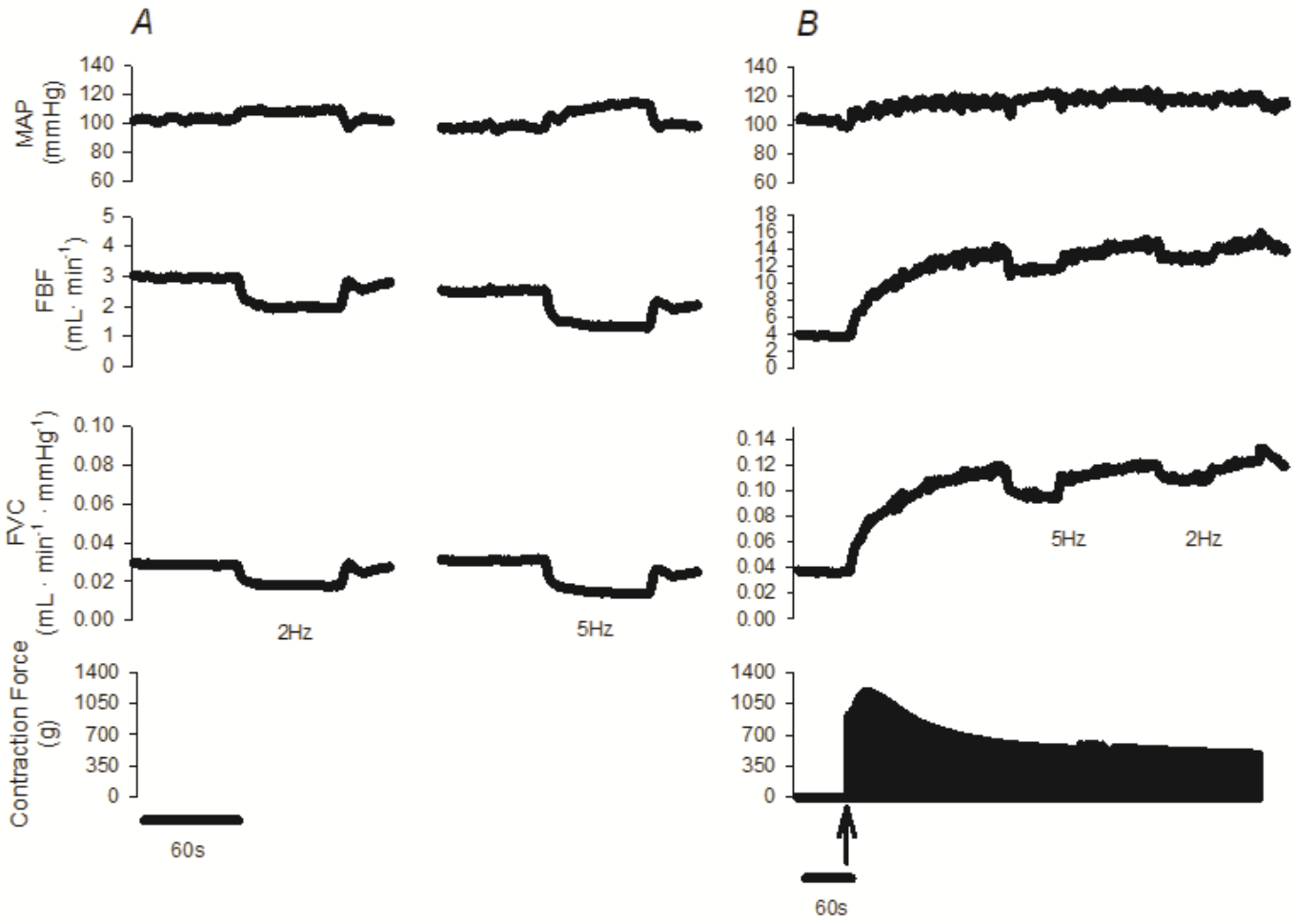

Figure 3.1. A representative ovary intact (OI) rat's responses of mean arterial pressure (MAP), femoral blood flow (FBF), femoral vascular conductance (FVC) to evoked sympathetic vasoconstriction at rest (Panel A) and during muscle contraction (Panel B) during control conditions.

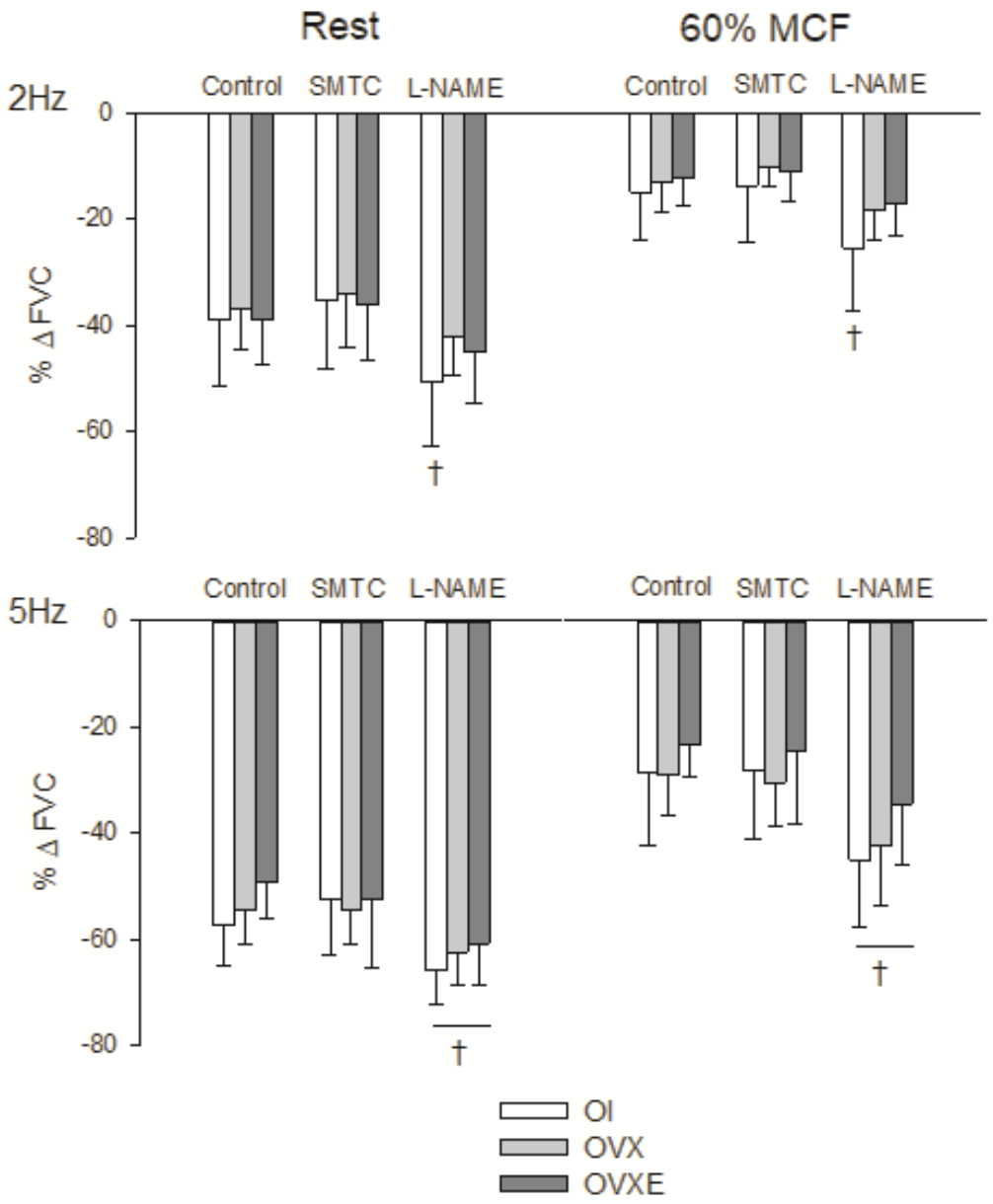

Figure 3.2. The percentage change of femoral vascular conductance (FVC) in response to stimulation of the lumbar sympathetic chain at 2Hz and 5Hz in ovary intact (OI; open bars; n=10), ovariectomized (OVX, light grey bars; n=8), and ovariectomized with estradiol-replaced (OVXE, dark grey bars; n=11) rats before and after sequential inhibition of nNOS (SMTC) and total NOS blockade (L-NAME). Values are Mean ± SD. † indicates a statistically significant difference from the control condition. A *P*-value < 0.05 was considered statistically significant.

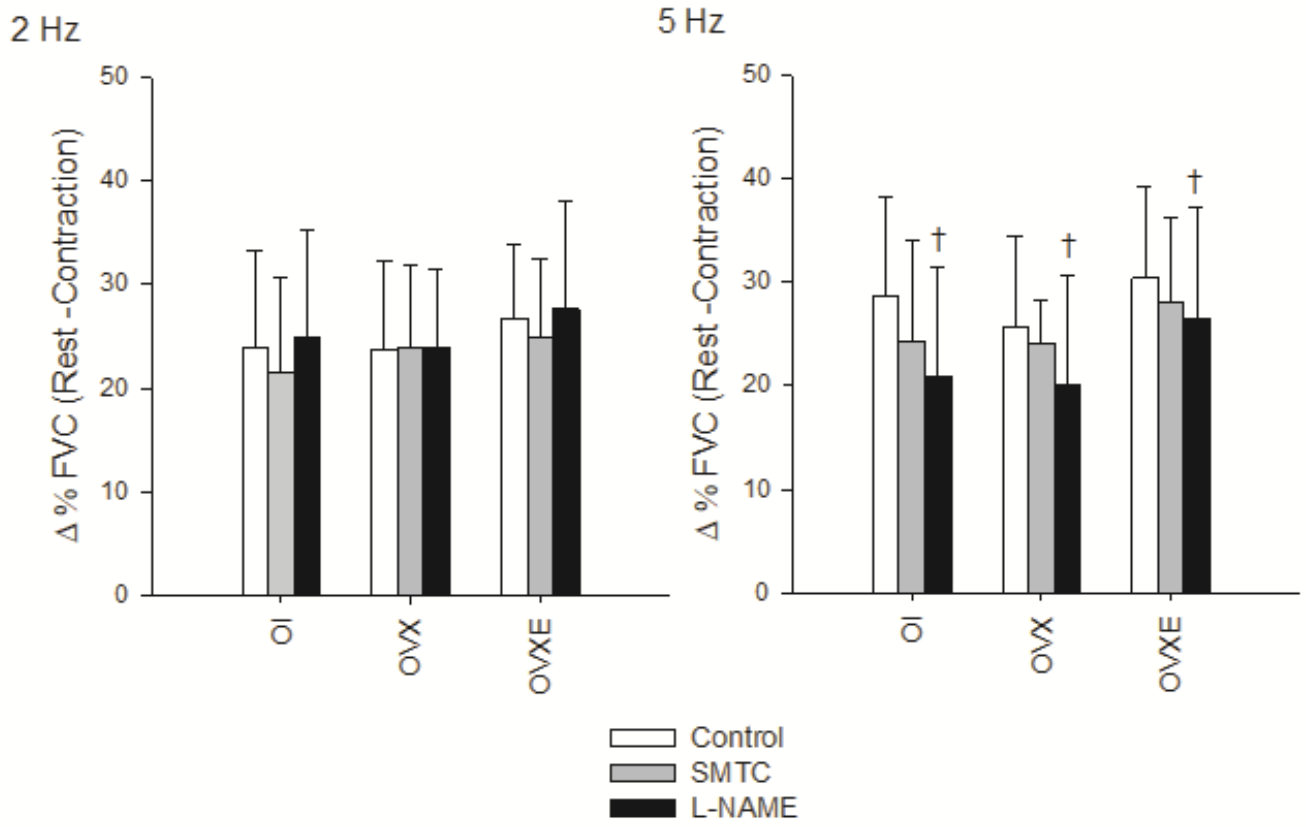

Figure 3.3. The magnitude of sympatholysis expressed as the absolute difference between sympathetic vasoconstrictor responsiveness at rest and during muscular contraction (femoral vascular conductance, Δ%FVC) during 2 Hz and 5 Hz sympathetic stimulation, during control (open bars), nNOS blockade (SMTC; 0.6 mg·kg^{-1} IV, grey bars) and total NOS blockade (L-NAME 10 mg·kg^{-1} IV, black bars) for ovary intact (OI; n=10), ovariectomized (OVX; n=8) and ovariectomized with estradiol-replacement (OVXE; n=11) rats. Values are mean ± SD. † indicates a significant difference from the control condition (main effect of drug). A *P*-value < 0.05 was considered statistically significant.

Discussion

The purpose of the present study was to determine the effect of chronic changes in estrogen status on nNOS- and eNOS-isoform mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle. Sympathetic vasoconstrictor responsiveness at rest or during muscle contraction and sympatholysis were not altered by estrogen status. Furthermore, selective nNOS blockade did not alter sympathetic vasoconstrictor responsiveness in resting or contacting skeletal muscle or sympatholysis regardless of estrogen status. In contrast, non-selective NOS blockade augmented vasoconstriction and reduced sympatholysis in all groups. Collectively, these data in female rats indicate that NO derived from nNOS does not inhibit sympathetic vasoconstriction in resting and contracting skeletal muscle and that NO-dependent sympatholysis is mediated by NO derived from eNOS, regardless of estrogen status.

Estrogen and Sympathetic Vasoconstrictor Responsiveness

A short-term alteration to estrogen status has previously been shown to impact skeletal muscle nNOS expression such that nNOS expression was negatively correlated to plasma estradiol levels [23]. Further NO-mediated blunting of sympathetic vasoconstriction following the administration of non-selective NOS blockade was altered by short-term changes to estrogen status[23]. The lack of NO-mediated blunting of vasoconstriction and a reduction in nNOS expression in ovariectomized rats was interpreted as evidence that estrogen status alters nNOS-mediated sympatholysis [23], but this conclusion was not directly tested with selective pharmacology. Post-menopausal women were found to have impaired functional sympatholysis compared to pre-

menopausal women, and 4 weeks of estrogen supplementation was able to restore the inhibition of sympathetic vasoconstriction during exercise in post-menopausal women [22]. However, the role of NO in the estrogen-mediated restoration of sympatholysis in postmenopausal women was not examined [22]. Our laboratory recently reported that sympatholysis was greater in female compared to male rats and that the enhanced sympatholysis in female rats was partly attributable to augmented NO-mediated inhibition of vasoconstriction [37]. These studies [22, 23, 37] suggested that estrogen facilitates sympatholysis through a NO-dependent mechanism, but did not mechanistically establish NOS isoform specific contributions or the effect of longer alterations of estrogen status. In the present study following 10 weeks of monitoring, ovary intact, ovariectomized, and ovariectomized, estrogen supplemented rats had similar vasoconstrictor responsiveness and sympatholysis at rest and during muscle contraction in control conditions, suggesting that estrogen status does not alter sympathetic vasoconstrictor responsiveness or sympatholysis in female rats.

Few studies have specifically investigated estrogen-status dependent changes in sympatholysis [22, 23]. The available studies have concluded that sympatholysis is reduced in ovariectomized female rats [23] and in postmenopausal women [22], suggesting that estrogen influences the regulation of sympathetic vasoconstriction. An important difference in the present study is the duration of altered estrogen status. It is possible that we observe conflicting results from previous reports [22, 23] of the effect of estrogen-deficiency on sympatholysis from an increased duration of estrogen-deficiency or replacement in the current study. Incongruous results with the role of NO in post-menopausal women [22] may be explained by what has been termed the “timing-hypothesis”

of estrogen replacement, suggesting that the effect of estrogen on the cardiovascular system is altered depending on the time period of estrogen deprivation prior to supplementation [28]. The time course of vascular adaptations to chronic changes in estrogen status have not been clearly established [28, 42]. ERα-expression can change within the human menstrual cycle with corresponding fluctuations in endothelium-dependent vasodilation [43], and endothelial changes happen as early as perimenopause [44]. Following 8 months of estrogen deprivation, estrogen receptor-mediated vasorelaxation was reduced, and was not restored with estradiol supplementation [28]. In the study that demonstrated augmented sympathetic vasoconstrictor responsiveness following menopause, subjects were tested 8 ± 2 years following their last period and the majority of subjects had previously received estrogen replacement therapy for a range of 0.5-5.5 years, thus making if difficult to ascertain the effect of estrogen deficiency from concurrent adaptations with age [22]. Adaptations to estrogen deficiency appear to happen quite quickly, and following a relatively short 4 weeks of estrogen deficiency, a decrease in nNOS expression within hindlimb skeletal muscle of ovariectomized was observed [23]. It has also been well established that estrogen can augment the expression of eNOS [24] in many vascular beds including in aortic endothelial cells [25, 26], HUVEC [26, 27] and brain endothelial cells [27] and is sensitive enough to fluctuate based on menstrual phase in humans [43]. Estrogen has widespread effects on both vasoconstrictor and vasodilator mechanisms, anti-inflammatory effects, reactive oxygen species production and scavenging, and vascular growth [45]. However, the mechanisms by which sympathetic neurovascular control could be affected by structural and genomic adaptations between short-term and long-term

estrogen deficiency is unknown, and underscores the need for additional *in vivo* experiments.

Estrogen's most prominent non-genomic action is through a signaling cascade that mediates NO release through eNOS activation [46]. Augmented estrogen bioavailability did not appear to influence sympatholytic function through non-genomic mechanisms in the current experiment which is supported by previous work that found no restoration of sympatholytic function after an acute infusion of 17β-estradiol [23]. Estrogen does blunt the responsiveness of α-adrenoreceptors to phenylephrine infusion in the mesentery of ovariectomized but estrogen-replaced rats, reducing the EC_{50} but not the maximal vasoconstriction [47]. This response may depend on the vascular bed as no difference was observed in α_1-adrenoreceptor mediated vasoconstriction in the aortic rings of ovariectomized or estradiol-supplemented and ovariectomized rats [48]. Estrogen can be synthesized in the adrenal glands and in adipose tissue [49], and this may have been sufficient to maintain endothelial NO production. While ovariectomized rats have been shown to lack ER-mediated rapid vasodilation in thoracic aortic rings [50], it is possible that resistance vessels in skeletal muscle of ovariectomized rats can preserve normal vascular function through redundancy in vasodilatory mechanisms.

NOS-Isoform Specific Blunting of Sympathetic Vasoconstrictor Responsiveness

Our lab previously reported that NO derived from nNOS mediated a substantial portion of sympatholysis in male rats [20]. To determine the effect of specific NOS isoforms, our pharmacological approach [20] used selective nNOS blockade followed by a non-selective NOS blockade, with the difference between the two drug conditions demonstrating the role of eNOS. The present data

indicate that nNOS is not required for sympatholysis in female rats. The previous study by Fadel et al. (2003) demonstrated similar eNOS expression between ovariectomized, estrogen-replaced and ovary intact rats [23], which is supported in the current study by a lack of estrogen-mediated changes to eNOS-mediated sympatholysis. However, the present findings contrast with an observed decrease in sympatholysis following ovariectomy that was associated with a decrease in skeletal muscle nNOS protein expression [23]. Indeed, only a correlation with skeletal muscle nNOS expression was demonstrated previously [23], and the contribution of NO derived from specific NOS isoforms to the inhibition of vasoconstriction was not investigated. In the current study, our results do not support estrogen dependent alterations in functional sympatholysis. Further, non-selective NOS-blockade augmented vasoconstriction in resting and contracting muscle and decreased sympatholysis in all groups, but there was no effect during selective-nNOS blockade.

Thus, the lack of nNOS-mediated blunting of sympathetic vasoconstriction observed in the present study suggests that augmented NO mediated sympatholysis in females compared to males is in part mediated by an augmented eNOS contribution, although this remains to be directly tested. Our current understanding of the estrogenergic signaling, both genomic and non-genomic, suggest augmented vasodilatory-signaling while the capacity and sensitivity of the sympathetic nervous system to elicit vasoconstriction is blunted. However, discrepancies exist between tissues studied and while estrogen deficiency has been observed to negatively impact skeletal muscle nNOS expression, eNOS expression was not altered in ovariectomized rats [23]. Others have found no changes in eNOS and nNOS expression in the thoracic aortas of

young ovary intact, ovariectomized, and estrogen replaced rats [51]. Thus, the current study furthers the understanding of estrogen- and NO-mediated vascular control by directly testing assumptions made from measurements in isolated vessels or through non-specific blockades of NOS isoforms.

Sympathetic Stimulation Frequency Dependence of Vasoconstrictor Responsiveness

We have previously observed an augmented role for NO-mediated sympatholysis in female rats at high (5Hz) sympathetic stimulation frequencies [37], and others have reported NO-mediated inhibition of sympathetic vasoconstriction in female rats as well [5]. The discharge frequency of sympathetic nerves has been shown to alter neurotransmitter release, with low frequencies of nerve firing evoke the release of adenosine triphosphate (ATP) and norepinephrine (NE), whereas higher frequencies appear to evoke the co-release of NE and neuropeptide Y (NPY) [41, 52, 53]. Nitric oxide appears to contribute to contraction-mediated blunting of vasoconstriction evoked at high stimulation frequencies, evidenced by the reduction in sympatholysis following non-selective NOS blockade at the 5Hz stimulation frequency in the present study. This would suggest a role for NO-mediated inhibition of NPY receptors, which have been identified in distal resistance vessels supplying skeletal muscle in rats [54], although this does not appear to be the case in coronary resistance vessels, where endothelial NO production did not alter NPY-mediated vasoconstriction [55].

Experimental Considerations and Significance

Following menopause the risk of hypertension, ischemic heart disease, and cardiovascular events such as myocardial infarction and stroke are markedly

increased [56, 57]. A decline in estrogen bioavailability is strongly associated with the increased cardiovascular disease risk in post-menopausal women [58]. However, the mechanistic links between estrogen deficiency and elevated cardiovascular disease risk and the time course of vascular adaptations to estrogen deficiency have not been fully elucidated [42, 59]. Our current understanding of the effects of estrogen on neurovascular control relies on cross-sectional studies [60] and short-term interventions [22, 23]. The effects of estrogen in postmenopausal women may be confounded due to 5 out of 8 postmenopausal women having received estrogen replacement therapy for a range of 0.5-5.5 years prior to the onset of the study, and/or already having significant changes to vascular function due to a combined influence of estrogen-deficiency and changes due to increasing age [22]. As the full brunt of estrogen-mediated vascular dysfunction develops over time [28], longer interventions appear necessary to understand the impact of prolonged estrogen deficiency. Indeed, chronological age and post-menopausal status have also been identified as separate risk factors for the development of cardiovascular disease risk [59]. It has been established that 4 weeks of estrogen deficiency reduced nNOS expression and impaired sympatholysis, thus in the present study we utilized a 10-week intervention to investigate the impact of a prolonged change in estrogen status in young animals. We anticipated that a longer intervention would result in progressive vascular dysfunction however in contrast to our hypothesis and data published following a similar intervention for 4 weeks [23] the present study suggests that prolonged chronic changes in estrogen status does not result in impaired sympatholysis or impaired NO-mediated inhibition of sympathetic vasoconstriction.

Relatively little is known about the effect of estrogen on the expression of receptors targeted by the sympathetic nervous system. Estrogen supplementation resulted in significantly blunted mesenteric artery α_1-adrenoreceptor expression [47], as well as downregulation of α_2-adrenoreceptor mRNA in the myometrium of pregnant rats [61] and in the rat hypothalamus [62]. In contrast, α_2-adrenoreceptor expression is augmented in cutaneous artery smooth muscle cells [63]. Estrogen downregulates NPY_1 receptors in cerebral tissue of female rats [64], however it is not known how estrogen affects the subtypes of P2X receptors (P2X1 and P2X4) known to exist in rat arteries [54, 65]. Functionally, estrogen reduced [66] or did not change [48] α_1-mediated vasoconstriction of rat aortic rings. Estrogen-replaced rats had decreased α_1-adrenoreceptor vasoconstriction in mesenteric resistance vessels [67] and EC_{50} to the α-adrenoreceptor agonist phenylephrine, but vasoconstriction was not altered by a non-selective NOS blocker (L-NMMA). No differences in the magnitude of vasoconstriction at rest observed in the current study as well as in a similar experimental preparation [23] suggests β-adrenoreceptor expression and/or sensitivity is not modified by our intervention. More specific studies examining the role for β-adrenoreceptors in females utilizing selective pharmacology appear warranted, given that work in humans suggests that the presence of estrogen in females augments β-adrenoreceptor-mediated vasodilation when compared to males [60].

The lack of an nNOS-mediated change to sympathetic vasoconstrictor responsiveness was contrary to our hypothesis and contrasts with previous studies from our lab in sedentary male rats that demonstrated that NO derived from nNOS was responsible for approximately 30% of total NO-mediated

inhibition of sympathetic vasoconstriction at rest and ~50% of NO mediated inhibition during exercise [20, 21]. The dose of SMTC has been used to selectively block nNOS in previous studies by our lab [20, 21], and is higher than the does of SMTC used by other labs to selectively block nNOS in male rats [29-31], female rats (0.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$) [32], and humans of both sexes (0.2 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) [33, 34]. In previous studies in male rats we utilized changes in blood pressure and intra-arterial infusions of acetylcholine (ACh) to determine the dose of SMTC that would selectively block nNOS without blocking eNOS, the assumption being that selective blockade of nNOS would increase blood pressure without altering eNOS mediated vasodilation to ACh [20, 21]. Subsequent administration of L-NAME would produce a further pressor response and reduce ACh-mediated vasodilation. Unfortunately, infusions of saline produced vasodilation in female rats in the present study. Thus, we were not confident that vasodilation in response to ACh was entirely attributable to NO derived from eNOS and we thus were unable to utilize the vasodilatory response to ACh to demonstrate selective nNOS blockade in the present study. Nonetheless, the pressor response to SMTC in the present study was similar to the pressor response observed in our previous studies [20, 21] and pilot testing of higher doses of SMTC were associated with a much larger pressor response suggesting that the dose of SMTC utilized in the present study selectively blocked nNOS without blocking eNOS.

Lastly, the observation of eNOS as the primary source of NO-mediated sympatholytic function in females has interesting implications for therapeutic exercise interventions. The data from the current study do not support a large NO-mediated role in long-term alterations to estrogen status in female rats.

However estrogen-replete post-menopausal women had augmented endothelial function compared to estrogen-deficient post-menopausal women following an exercise training intervention [68]. In a recent review [69], the authors suggest estrogen likely mediates an exercise-training induced increase in eNOS expression in part by protecting aged arteries from oxidative damage. The ability to modify NO-mediated inhibition of sympathetic vasoconstriction during muscle contraction with exercise training has been previously demonstrated by our lab [6, 21] and others [8], although no female rats were used in these studies. Given the demonstrated role of eNOS-mediated sympatholytic function in the current study and previous work [37], we believe that the interaction of estrogen and exercise training to modify NO bioavailability is a key area for future investigation.

Conclusion

These data suggest that a chronic reduction in estrogen status does not modulate sympathetic vasoconstrictor responsiveness or NO-mediated inhibition of sympathetic vasoconstriction in resting or contracting skeletal muscle.

Furthermore, in female rats NO derived from nNOS does not appear to be necessary for sympatholysis, whereas eNOS mediates NO-dependent sympatholysis, regardless of estrogen status.

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Chapter 4

Lumbar sympathetic nerve activity at rest and during muscle contraction: Effects of estrogen and nitric oxide

Introduction

The sympathetic nervous system is an important regulator of the cardiovascular system. Sympathetic nerve activity (SNA) arises from the rostral ventrolateral medulla, [1] and the level of efferent SNA is determined by the coordination of feedforward “central command” stimuli and feedback reflexes (baro- and chemo-reflex afferents) leading to the release of neurotransmitters [2]. Efferent SNA contributes to the regulation of cardiac output and vascular resistance is therefore integral to the regulation of cardiac output, blood pressure and tissue blood flow at rest and during exercise [3].

Compared to men, young women have lower resting blood pressure, a lower incidence of hypertension and a reduced risk of cardiovascular disease [4-6]. However, with advancing age, rates of cardiovascular disease increase disproportionately in females compared to males, and older females exhibit higher rates of coronary heart disease, stroke and other cardiovascular disease mortality [6]. Coincidentally, MSNA is lower in young women than in young men, but increases at a faster rate with age in women when compared to men and the increase in efferent MSNA has been linked to the increased incidence of CV diseases with aging [7]. One potential mechanism to explain sex differences in the regulation of efferent MSNA and CV disease risk is sex differences in estrogen bioavailability. Estrogen appears to be an important candidate to explain the cardioprotective effect observed in young women in the central

regulation of efferent SNA [5, 8], as premenopausal women have lower MSNA than postmenopausal women [9] and augmented MSNA is reduced in post-menopausal women who receive transdermal estrogen therapy with a corresponding decrease in mean arterial pressure [10]. This evidence suggests estrogen has an important role in the regulation of efferent SNA that may be associated with health outcomes.

Receptors for estrogen are expressed in multiple central nuclei that are responsible for basal and reflex activation of the sympathetic nervous system [11-13]. The activation of estrogen receptors in these autonomic centers reduces central sympathetic outflow [14], and the decrease in efferent sympathetic activity following the injection of estradiol directly on regulatory central nuclei is magnified following a period of estrogen supplementation in ovariectomized rats [14]. However, the actions of estrogen on efferent SNA appear to extend beyond direct effects of binding with estrogen receptors. Estrogen also modulates NO synthase (NOS) expression in the brainstem [13, 15]. Neurons within the rostral ventrolateral medulla (RVLM) generate basal SNA [16], which expresses the endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoforms of NOS [17-19]. NO has emerged as an important regulator of central nervous system control of the circulation [20]: The disruption of endogenous NO production (NOS blockade) in the RVLM of cats produced increases in SNA [21], and increases in mean arterial pressure (MAP) and heart rate in male rats [22]. Further, the injection of a precursor to NO production, L-arginine, into the RVLM of male rats reduced renal SNA [22] and MAP [23]. This NO-dependent blunting of efferent SNA persisted following the removal of baroreflex inputs by sino-aortic baroreceptor denervation NOS in anesthetized [24] and conscious rats [25].

Some labs have found that the inhibition of nNOS within the RVLM decreases SNA, while the inhibition of iNOS augments SNA [17, 18] suggesting NO from nNOS is sympathoexcitatory while NO from iNOS is sympathoinhibitory. Thus, the role of NO on efferent sympathetic outflow is not perfectly clear. However, it is important to note that all but one [25] of the previous studies have been done in male animals, or sex was not specified [21], while the role of NO in the brainstem of females specifically is largely unexplored. As a whole, the evidence suggests that the presence of estrogen may attenuate sympathetic outflow at least partly through a NO-mediated mechanism.

How estrogen and NO interact to control sympathetic outflow *in vivo* at rest and during exercise is also unclear. Very few studies have examined the effect of estrogen on sympathetic vascular control during exercise. In response to exercise, efferent SNA increases in an exercise intensity-dependent manner [26-28] and MSNA produces vasoconstriction that serves to regulate vascular resistance and contributes to the integrated regulation of blood pressure and tissue blood flow [29-31]. Previous studies have observed that women have an attenuated SNA and mean arterial pressure increase during static handgrip exercise when compared to men [32, 33]. The higher bioavailability of estrogen in females has been suggested as the mechanism underlying sex differences in the regulation of SNA during exercise, but evidence is limited [33, 34]. Evidence supporting the hypothesis that estrogen blunts the MSNA response to exercise found that increases in SNA during static handgrip exercise at 30% of maximal voluntary contraction (MVC) were attenuated by high serum estrogen levels compared to 1-4 days after the onset of menstruation (low estrogen) [34]. However, this response was not duplicated in a more recent experiment, that

found SNA response to static handgrip exercise at 40-60% of MVC is unaffected by hormonal phase [33]. Thus, it is unclear whether estrogen modified the response to static exercise.

Therefore, the purpose of this study was to investigate the hypothesis that estrogen status alters SNA at rest and during muscle contraction through a nitric oxide (NO) dependent mechanism. Specifically, we hypothesized that reduced estrogen bioavailability via ovariectomy would increase SNA at rest and during muscle contraction when compared to ovary intact and ovariectomized rats with estradiol replacement. Furthermore, we hypothesized that nitric oxide synthase inhibition would augment SNA at rest and during muscle contraction in rats with a positive estrogen status, but not in ovariectomized rats.

Methods

Animal Use

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.

Female Sprague-Dawley rats (8 weeks old) were housed in university facilities with a 12:12h light-dark cycle, and with temperature and humidity controlled at 22-24°C and 40-70% respectively. Water and rat chow were provided *ad libitum*. Rats were randomly assigned to a treatment group: ovary intact (OI; n=10), ovariectomized (OVX; n=9) or ovariectomized with a 17β-estradiol-replacement (OVXE; n=8), for 4 weeks.

Ovariectomy and 17β-Estradiol replacement

Anesthesia was induced by inhalation of isoflurane (3.5% in balance O_2) and a bilateral ovariectomy was performed in rats in OVX and OVXE groups.

Briefly, rats underwent a bilateral ovariectomy through an abdominal incision. Following the closure of the abdomen a 60-day timed-release 17β-estradiol pellet (OVXE; 1.5mg pellet^{-1}) or a placebo pellet (OVX; Innovative Research of America, Sarasota, FL, USA) was implanted subcutaneously along the abdominal wall 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 4 weeks.

Experimental Preparation

Four weeks after the rats were assigned to their respective groups, anesthesia was induced by inhalation of isoflurane (3.5%, balance O_2) and subsequently maintained by syringe-pump infusion of a mixture of α-chloralose (8-16 mg·kg^{-1}·h^{-1}) and urethane (50-100 mg·kg^{-1}·h^{-1}) in the right jugular vein. Absence of a withdrawal reflex to a paw pinch and the stability of hemodynamic variables ensured proper depth of anesthesia. A tracheotomy was performed and rats were ventilated (Model 683, Harvard Apparatus, Holliston, MA, USA) with a gas mixture of 30% Oxygen and 70% Nitrogen for maintenance of arterial blood gases and acid base status (PaO_2: 88-95mmHg; $PaCO_2$: 39-41mmHg pH: 7.39-7.42). Arterial blood pressure was measured continuously via a cannula that was placed in the left carotid artery and attached to a pressure transducer (Abbott, North Chicago, IL, USA). The left femoral artery and vein were cannulated for drug delivery, and a constant infusion of heparinized saline (4 units ml^{-1}) was delivered intravenously at 2.5-3 ml hr^{-1} kg^{-1}. Femoral blood flow (FBF) was

measured at the right femoral artery with a flow probe (0.7V; Transonic Systems, Ithaca, NY, USA) connected to a flow meter (T106; Transonic Systems). Heart rate (HR) was derived from the arterial blood pressure wave form and femoral vascular conductance (FVC) and femoral vascular resistance (FVR) were calculated. Core body temperature was monitored by rectal thermistor and body temperature was maintained at 36-37 °C by an external heating pad (TCAT-2, Physitemp, Clifton, NJ, USA). The right triceps surae muscle group was dissected and attached to a force transducer (FT03, Grass Technologies, Warwick, RI, USA) at the calcaneal tendon. A cuff electrode was placed around the sciatic nerve and muscle contraction was produced by electrical stimulation (Stimulator Panel, Chart software; AD Instruments, Colorado Springs, CO USA). The motor threshold (MT) and the optimal muscle length for tension development were determined. Maximal contractile force (MCF) was determined by stimulation of the triceps surae muscle group with 25 impulses of 1ms duration delivered at 100Hz at ~5x the MT. Rhythmic muscle contractions at 60% of MCF were produced by stimulation of the sciatic nerve at 40 Hz, 0.1ms pulses in 250ms trains at a rate of 60 trains·min^{-1} at ~3 times MT.

To record lumbar sympathetic nerve activity, a laparotomy was performed and the abdominal aorta and vena cava were temporarily retracted to allow dissection of the L3-L4 lumbar sympathetic chain with a blunt glass pipette. A small piece of paraffin wax paper was placed under the nerve and recording electrodes (TR46SP, Telemetry Research, Auckland, NZ) were placed above the wax paper such that the nerve rested on the electrode as was isolated from the posterior abdominal cavity. The nerve-electrode contact area was then

embedded in a rapidly-curing non-toxic silicone elastomer (Kwiksil, WPI, Sarasota, FL USA). The ground electrode was secured to nearby muscle.

Data was recorded using Chart 7^{TM} data acquisition software (AD Instruments, Colorado Springs, CO USA). Arterial blood pressure, femoral artery blood flow (FBF) were sampled at 100 Hz and femoral vascular conductance (FVC) and heart rate were calculated. The original LSNA signal was amplified with a gain of 1 and fed through a band-pass filter with a band width of 50-2000Hz, and through an integrating network with a time constant of 20ms. This data was recorded using Chart 7^{TM} at a sampling frequency of 1000Hz.

Experimental Protocol

Lumbar sympathetic chain nerve activity (LSNA) was recorded at rest and during muscle contraction in control, non-selective NOS blockade (L-NAME; 10 mg·kg^{-1}), and non-selective NOS blockade with the normalization of blood pressure with hydralazine infusion (0.01-0.06 mg·kg^{-1}·min^{-1}) conditions. Rhythmic muscle contraction was produced for a total of 6 minutes. LSNA responses were analyzed for three 1-minute blocks of resting baseline distributed across a 10-minute time-period that was characterized by hemodynamic stability, and for the last 3 minutes of exercise. The elevation in blood pressure during the L-NAME condition was brought back to baseline levels by a hydralazine infusion to control for arterial baroreflex-mediated decreases in sympathetic nerve activity [35]. Contraction bouts were separated by 60 minutes of rest. The quality of the SNA signal was tested with the SNA baroreflex response, comparing baseline activity to the nadir in SNA activity following a femoral artery bolus infusion of 0.1 mg/kg phenylephrine (PE) and the corresponding spike in MAP. Maximal LSNA was

calculated as the peak 3 seconds of activity following administration of an anesthetic overdose.

Plasma Estrogen Levels

Venous blood samples (n=8/group) were drawn into EDTA-coated tubes following the collection of functional data. Samples were immediately centrifuged at 13,000g for 15 minutes at 4°C. The plasma was aliquoted, and immediately stored at -80°C until analysis. Plasma samples were thawed, and 17β-estradiol levels were measured using a commercially available competitive immunoenzymatic assay (ab108667, Abcam, Cambridge, MA).

Euthanasia and Tissue collection

Following the experimental protocol, rats were killed by anesthetic overdose. The heart, soleus, lateral gastrocnemius, and medial gastrocnemius muscles were dissected, weighed and then snap frozen in liquid nitrogen. The tibia was harvested and the length was measured. Plasma was collected by transferring blood to EDTA coated micropipette tubes which were centrifuged at 15000 rpm for 5 minutes. Samples were stored at -80°C for further analysis.

Data Analysis

Lumbar sympathetic nerve activity was expressed as the root mean square (RMS) of integrated LSNA. The maximum RMS LSNA was recorded approximately 2-3 minutes following anesthetic overdose. Comparisons of RMS LSNA were made as a percentage of maximal RMS LSNA, as well as a percentage of control baseline RMS LSNA. Baseline noise was subtracted from resting, contraction and maximal measurements of LSNA prior to comparison. All data are expressed as mean ± SD.

Statistics

Group differences in body, muscle and heart mass, tibia length, heart mass to body mass and heart mass to tibial length ratios and plasma 17β-estradiol levels were determined by a one-way ANOVA. Differences in LSNA were determined by two-way repeated measures ANOVA (Group x muscle contractile state). When significant main effects and/or interactions were detected, a Student-Newman-Keuls post hoc test was performed. Relationships between LSNA and cardiovascular variables were assessed by Pearson product correlation. A p-value of < 0.05 was considered statistically significant.

Results

Body mass, heart mass, tibial length, soleus and lateral and medial gastrocnemius muscle mass was higher ($P < 0.05$) in OVX rats compared to OI and OVXE rats. Ovary intact rats had higher ($P < 0.05$) body mass, heart mass, tibial length, soleus and lateral and medial gastrocnemius muscle mass compared to OVXE rats. OVX rats had a higher ($P < 0.05$) heart mass to tibial length ratio than OVXE rats. There were no differences ($P > 0.05$) in heart mass to body mass ratio (Table 4.1.).

Plasma Estradiol was higher ($P < 0.05$) in OVXE compared to OVX and OI rats (OI, n=6: 54 ± 16 $pg \cdot mL^{-1}$; OVX, n=8: 34 ± 5 $pg \cdot mL^{-1}$; OVXE, n=6: 140 ± 84 $pg \cdot mL^{-1}$), whereas plasma estradiol levels were not different ($P > 0.05$) between OI and OVX rats.

Resting Hemodynamics

During control conditions, resting HR was higher ($P < 0.05$) in OI than OVXE rats, while resting MAP was similar ($P > 0.05$) in all groups. Resting FBF and FVC were not different ($P > 0.05$) in OI rats compared to OVX and OVXE

rats, whereas resting FBF and FVC were elevated ($P < 0.05$) in OVX rats compared to OVXE rats (Table 4.2.).

NOS blockade increased ($P < 0.05$) MAP similarly ($P > 0.05$) in all groups (Table 2). HR was reduced ($P < 0.05$) in all groups following NOS blockade, and was higher ($P < 0.05$) in OI compared to OVXE rats. L-NAME did not alter ($P > 0.05$) FBF in any group. In the presence of NOS blockade, OVX rats had higher ($P < 0.05$) FBF than OI rats and OVXE rats had lower ($P < 0.05$) FBF than OI rats. Following NOS blockade, Femoral vascular conductance was not different ($P > 0.05$) from the control condition in any group. FVC was lower ($P < 0.05$) in OVXE compared to OVX rats in the presence of L-NAME.

Administration of hydralazine reduced ($P < 0.05$) MAP compared to L-NAME conditions similarly ($P > 0.05$) in all groups, such that MAP was not different ($P > 0.05$) than control conditions (Table 2). Heart rate was increased ($P < 0.05$) compared to L-NAME similarly ($P > 0.05$) in all groups, and was not different ($P > 0.05$) than during control conditions. Ovary intact and OVX rats had reduced ($P < 0.05$) FBF compared to L-NAME and control conditions, while OVXE rats did not have a significant change ($P > 0.05$) in FBF. All groups had significantly reduced ($P < 0.05$) FVC compared to control conditions following hydralazine administration, with no significant ($P > 0.05$) group differences.

Lumbar Sympathetic Nerve Activity at Rest and During Muscle Contractions

Maximal RMS SNA was similar ($P > 0.05$) between groups (OI: 0.553 ± 0.184 V; OVX: 0.511 ± 0.239 V; OVXE: 0.444 ± 0.227 V). Lumbar sympathetic nerve activity (%max LSNA) was not different ($P > 0.05$) between groups at rest. Muscle contraction increased ($P < 0.05$) LSNA compared to resting conditions

similarly in all groups and LSNA was not different ($P > 0.05$) between groups during muscle contraction at 60% of MCF (Figure 4.2.).

Absolute levels of baseline SNA, were also similar ($P > 0.05$) in all groups (OI: 0.075 ± 0.034 V; OVX: 0.073 ± 0.039 V; OVXE: 0.052 ± 0.040 V). Furthermore, there was no difference ($P > 0.05$) in absolute SNA between groups during muscle contraction (OI: 0.104 ± 0.073 V; OVX: 0.093 ± 0.041 V; OVXE: 0.073 ± 0.039 V).

Lumbar sympathetic nerve activity (% max LSNA) was not different between groups ($P < 0.05$) during NOS blockade, but was significantly reduced ($P < 0.05$) during both rest and during muscle contraction in all groups (Figure 4.2.). Muscle contraction increased ($P < 0.05$) LSNA similarly ($P > 0.05$) in all groups.

Following normalization of MAP with hydralazine, resting and exercising LSNA (% max LSNA) was increased ($P < 0.05$) similarly ($P > 0.05$) in all groups. Muscle contraction increased ($P < 0.05$) LSNA in all groups.

Relationships between Sympathetic Nerve Activity and Cardiovascular Variables

In control conditions, resting LSNA and MAP (Figure 4.4.) were not correlated in OI ($r = 0.29$; $P > 0.05$) or OVXE ($r = 0.22$; $P > 0.05$) rats, however LSNA was positively correlated with MAP in OVX ($r = 0.73$; $P < 0.05$) rats. During NOS blockade, LSNA and MAP became positively correlated in OI ($r = 0.69$; $P < 0.05$) and OVXE ($r = 0.82$; $P < 0.05$), but NOS blockade removed a significant relationship between LSNA and MAP in OVX ($r = 0.11$; $P > 0.05$). Following the normalization of blood pressure with hydralazine, LSNA and MAP were not correlated in OI ($r = 0.25$; $P > 0.05$), OVXE ($r = 0.30$; $P > 0.05$) and OVX ($r = 0.59$; $P > 0.05$).

In the control condition, resting FVR was not related ($P > 0.05$) to LSNA in OI (r = 0.24), OVX (r = 0.02), or OVXE (r = 0.10) rats. Following NOS blockade FVR was positively correlated ($P > 0.05$) with LSNA in OVXE (r = 0.76) but not ($P > 0.05$) in OI (r = 0.17) or OVX (r = 0.07) rats. Resting FVR was not correlated ($P > 0.05$) to LSNA in any group following normalization of blood pressure in the NOS blockade condition (OI: r = 0.21; OVX: r = 0.15; OVXE: r = 0.56).

In the control condition, LSNA and MAP during muscle contractions (Figure 4) were not correlated ($P > 0.05$) in OI (r = 0.15) or OVXE (r = 0.33) but was positively correlated ($P < 0.05$) in OVX (r = 0.67). During NOS blockade, LSNA and MAP during muscle contractions were not correlated ($P > 0.05$) in OI (r = 0.42) and OVX (r = 0.47) but was positively correlated ($P < 0.05$) in OVXE (r = 0.79). Following the normalization of blood pressure with hydralazine, SNA and MAP during muscle contractions were not correlated ($P > 0.05$) in any group (OI: r = 0.37; OVXE: r = 0.44; OVX: r = 0.66).

In the control condition, LSNA and FVR during muscle contractions were not correlated ($P > 0.05$) in any group (OI: r = 0.30; OVX: r = 0.36; OVXE: r = 0.04). Following NOS blockade, LSNA and FVR during muscle contractions remained uncorrelated ($P > 0.05$) in all groups (OI: r = 0.01; OVX: r = 0.42; OVXE: r = 0.01). Following the normalization of blood pressure with hydralazine, SNA and FVR during muscle contractions were uncorrelated ($P > 0.05$) in all groups (OI: r = 0.56; OVX: r = 0.06; OVXE: r = 0.54).

Hemodynamics and Force Production of Muscle Contraction

During the control condition, the increase in HR, MAP, FBF, and FVC in response to muscle contraction was not different ($P > 0.05$) between groups (Table 4.3.). Following NOS blockade, the HR and MAP response to muscle

contraction was not altered ($P > 0.05$) in any groups. The FBF response to muscle contraction following NOS blockade in OI and OVXE rats was similar ($P > 0.05$) to control conditions, while OVX rats had increased ($P < 0.05$) FBF during muscle contractions. NOS blockade did not significantly alter ($P > 0.05$) the FVC adaptation to contraction within groups, while OVX rats had an augmented ($P < 0.05$) change in FVC compared to OI rats. The normalization of blood pressure with hydralazine did not change ($P > 0.05$) the HR and MAP response to muscle contraction within groups. The normalization of blood pressure with hydralazine significantly reduced ($P < 0.05$) FBF adaptations to contraction when compared to the NOS blockade condition in all groups, while FBF adaptations were similar ($P > 0.05$) to control conditions in OI and OVXE rats.

In control conditions, average muscle force production was similar ($P > 0.05$) between groups (OI: 402 ± 35 g; OVX: 432 ± 45 g; OVXE: 449 ± 70 g). Administration of drugs did not alter ($P > 0.05$) muscle force production within each group, although OVX rats had higher ($P < 0.05$) force production than OI rats during NOS blockade (OI: 409 ± 38 g; OVX: 461 ± 48 g; OVXE: 449 ± 40 g) and NOS blockade with normalized blood pressure (OI: 389 ± 42 g; OVX: 445 ± 32 g; OVXE: 411 ± 44 g) conditions.

Table 4.1. Animal Characteristics

Group	Body Mass (g)	Heart Mass (g)	Tibial Length (mm)	Heart Mass (mg) : Body Mass (g)	Heart Mass (mg) : Tibial Length (mm)	Muscle Mass (g)		
						Soleus	Lateral Gastrocnemius	Medial Gastrocnemius
OI	322 ± 21	1.09 ± 0.10	40 ± 1	3.4 ± 0.1	28 ± 2	0.17 ± 0.01	0.62 ± 0.05	1.10 ± 0.09
OVX	364 ± 33*	1.22 ± 0.17*	41 ± 1	3.3 ± 0.3	30 ± 4	0.20 ± 0.02*	0.69 ± 0.03*	1.26 ± 0.11*
OVXE	268 ± 19*†	0.94 ± 0.09*†	38 ± 1*†	3.5 ± 0.1	25 ± 2†	0.15 ± 0.02*†	0.54 ± 0.03*†	0.97 ± 0.08*†

All values are mean ± SD. Tissue obtained from ovary intact (OI; n=10), ovariectomized (OVX; n=9), and ovariectomized, estrogen-replaced (OVXE; n=8) rats * indicates a statistically significant difference from OI rats. † indicates a statistically significant difference from OVX. A *P*-value < 0.05 was considered statistically significant.

Table 4.2. Basal Hemodynamics

Group	Drug Condition	HR (beats·min^{-1})	MAP (mm Hg)	FBF (mL·min^{-1})	FVC (mL·min^{-1}·mmHg^{-1})
OI	Control	406 ± 45	107 ± 11	3.1 ± 0.6	0.029 ± 0.004
	L-NAME	366 ± 35‡	139 ± 19‡	3.4 ± 1.0	0.025 ± 0.008
	Hydralazine	394 ± 53	109 ± 12	2.2 ± 0.9‡§	0.021 ± 0.009‡
OVX	Control	388 ± 28	110 ± 10	3.9 ± 1.3	0.036 ± 0.012
	L-NAME	344 ± 29†	130 ± 18‡	4.0 ± 1.1	0.032 ± 0.011
	Hydralazine	372 ± 55	111 ± 9	3.0 ± 1.0‡§	0.028 ± 0.010‡
OVXE	Control	336 ± 34*	102 ± 15	2.4 ± 0.5†	0.025 ± 0.007†
	L-NAME	319 ± 48*‡	124 ± 10†	2.6 ± 1.0†	0.021 ± 0.008†
	Hydralazine	343 ± 60*	104 ± 8	1.9 ± 0.7†	0.018 ± 0.006‡

Heart rate (HR), mean arterial pressure (MAP), femoral blood flow (FBF) and femoral vascular conductance (FVC) at rest during control and drug conditions in ovary intact (OI; n=10), ovariectomized (OVX; n=9), and ovariectomized, estrogen-replaced (OVXE; n=8) rats. Values are mean ± SD. * indicates a statistically significant difference from OI rats. † indicates a statistically significant differences from OVX rats. ‡ indicates a statistically significant difference from control conditions. § indicates a statistically significant difference from L-NAME conditions. A *P*-value < 0.05 was considered statistically significant.

Table 4.3. Hemodynamic response to muscle contraction

Group	Drug Condition	ΔHR (beats·min^{-1})	ΔMAP (mm Hg)	ΔFBF (mL·min^{-1})	ΔFVC (mL·min^{-1}·mmHg^{-1})
OI	Control	0 ± 24	4 ± 10	5.7 ± 1.6	0.050 ± 0.014
	L-NAME	8 ± 10	-3 ± 6	6.0 ± 2.2	0.046 ± 0.017
	Hydralazine	5 ± 20	-9 ± 11	3.9 ± 1.5‡§	0.042 ± 0.016
OVX	Control	-2 ± 22	-8 ± 8	5.7 ± 1.9	0.058 ± 0.013
	L-NAME	4 ± 19	-4 ± 6	7.7 ± 2.6‡	0.061 ± 0.017*
	Hydralazine	4 ± 19	-5 ± 9	6.0 ± 1.9§	0.058 ± 0.011*
OVXE	Control	0 ± 13	-5 ± 16	5.0 ± 1.8	0.052 ± 0.011
	L-NAME	13 ± 29	-6 ± 12	5.8 ± 1.0	0.050 ± 0.010‡
	Hydralazine	-5 ± 15	-9 ± 18	3.9 ± 1.6§	0.042 ± 0.012†§

Heart rate (HR), mean arterial pressure (MAP), femoral blood flow (FBF) and femoral vascular conductance (FVC) during muscle contraction during control and drug conditions in ovary intact (OI; n=10), ovariectomized (OVX; n=9), and ovariectomized, estrogen-replaced (OVXE; n=8) rats. Values are mean ± SD. * indicates a statistically significant difference from OI rats. † indicates a statistically significant differences from OVX rats. ‡ indicates a statstically significant difference from control conditions. § indicates a statstically significant difference from L-NAME conditions. A *P*-value < 0.05 was considered statistically significant.

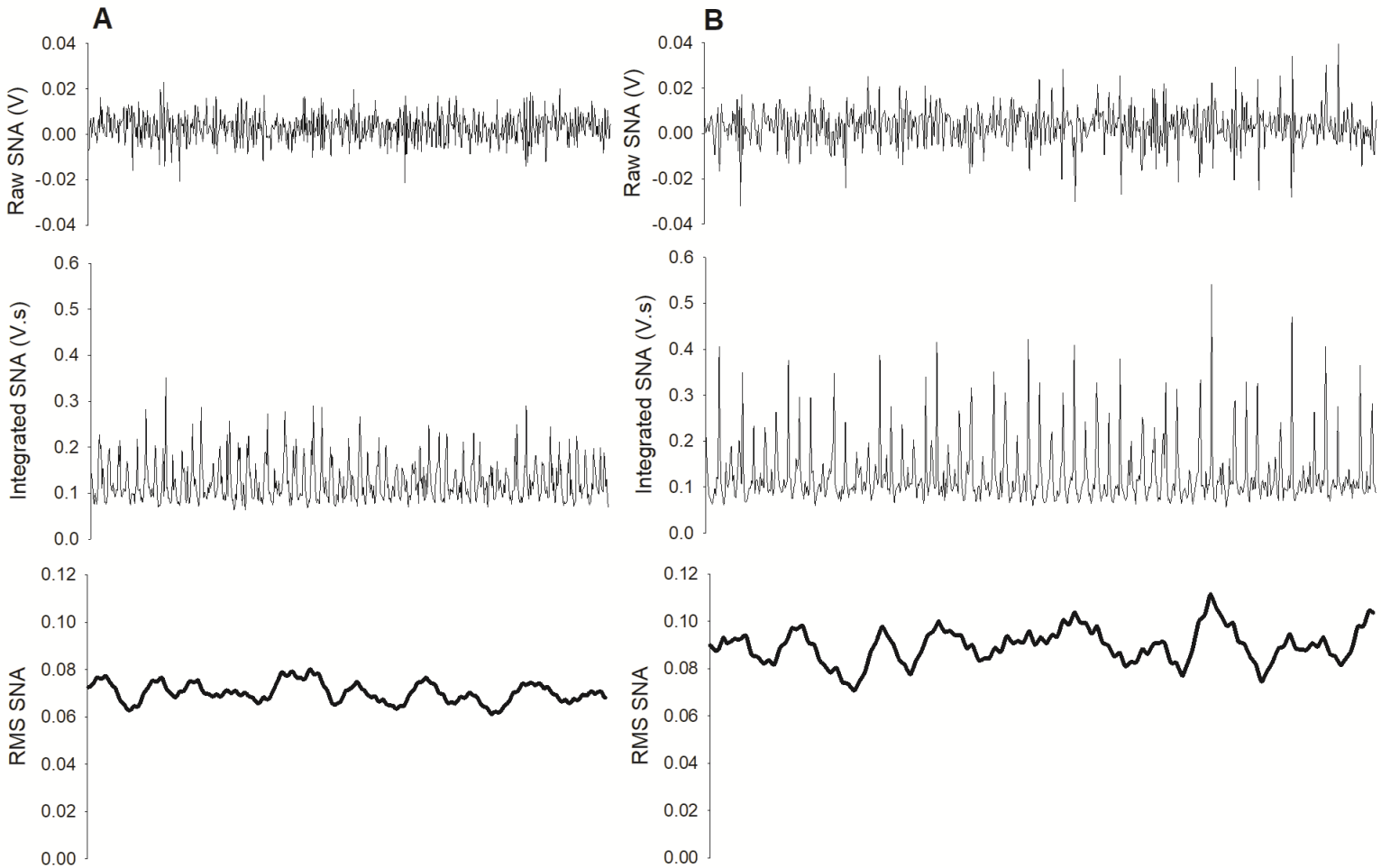

Figure 4.1. Original data tracing with 1 minute of raw (top panel), integrated (middle panel) and root mean square (RMS; bottom panel) lumbar sympathetic nerve activity (LSNA) recorded at rest (A) and during muscle contraction (B) in a representative ovary intact rat in control conditions.

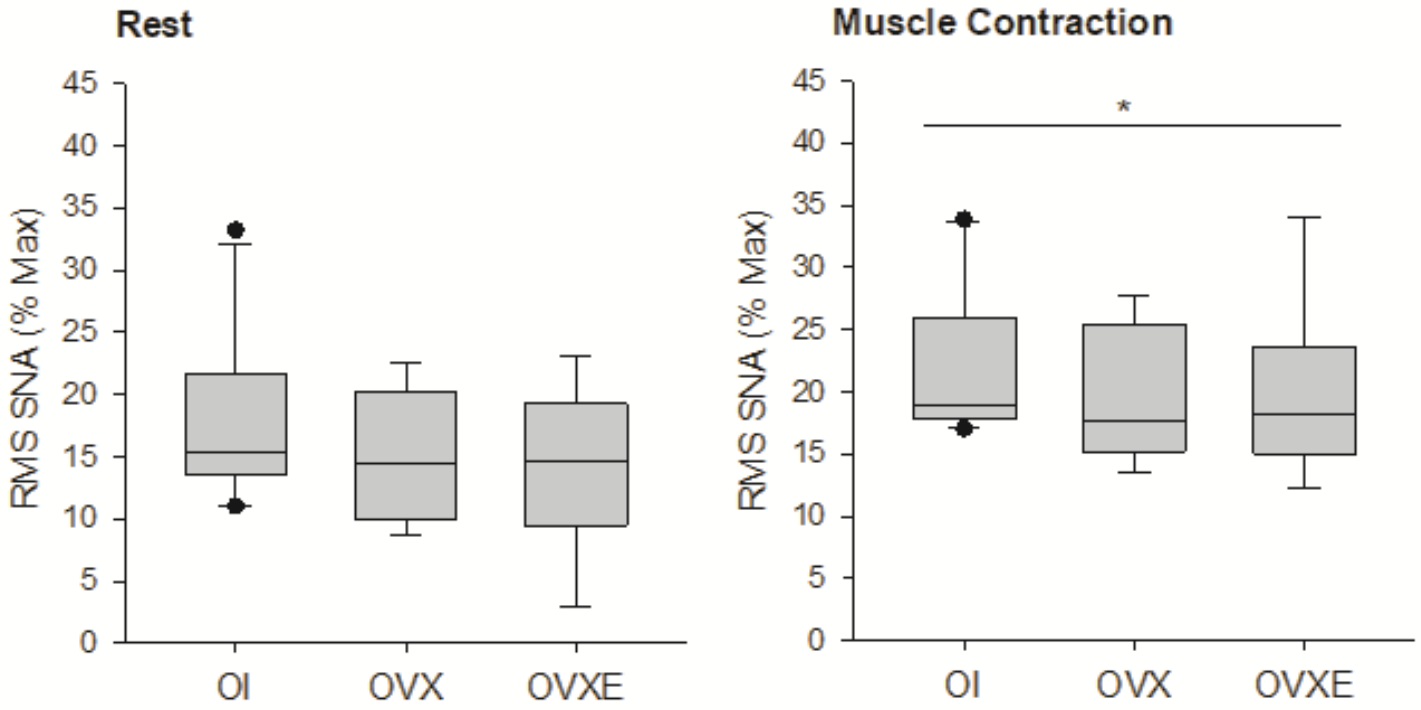

Figure 4.2. Lumbar sympathetic nerve activity (LSNA) at rest and during muscle contraction in control conditions in ovary intact (OI; n=10), ovariectomized (OVX; n=9) and ovariectomized estrogen-replaced (OVXE; n=7) rats. * indicates a main effect of muscle contraction. A *P*-value < 0.05 was considered statistically significant.

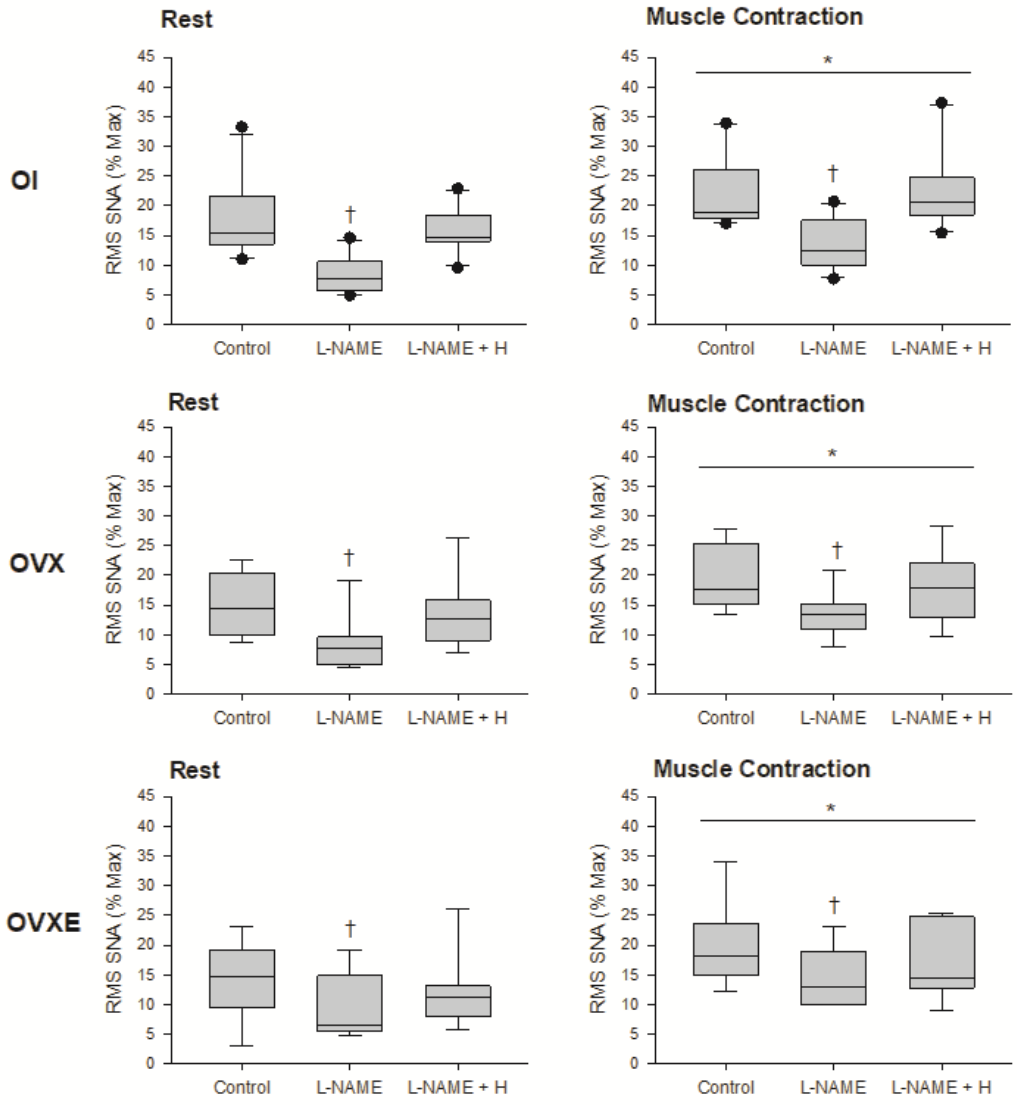

Figure 4.3. Lumbar sympathetic nerve activity (LSNA) at rest and during muscle contraction in ovary intact (OI; n=10), ovariectomized (OVX; n=9) and ovariectomized estrogen-replaced (OVXE; n=7) rats in control, NOS blockade (L-NAME) and NOS blockade + normalization of MAP (L-NAME+H) conditions. * indicates a main effect of muscle contraction. † indicates a statistically significant effect of NOS inhibition. A *P*-value < 0.05 was considered statistically significant.

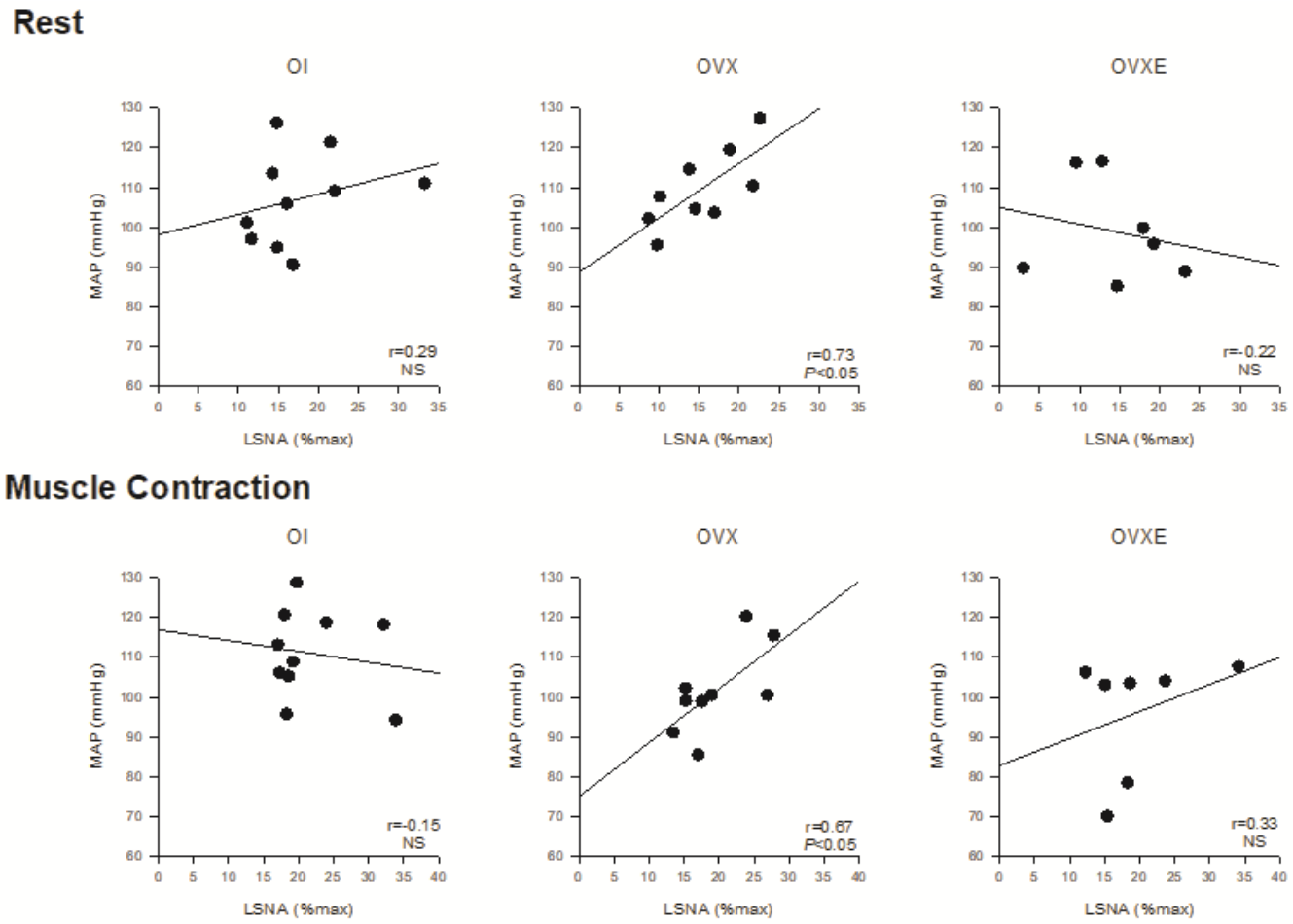

Figure 4.4. The relationship between lumbar sympathetic nerve activity (LSNA; %max) and mean arterial blood pressure (MAP) at rest and during muscle contraction in ovary intact (OI; n=10), ovariectomized (OVX; n=9) and ovariectomized estrogen-replaced (OVXE; n=7) rats in control conditions.

Discussion

The purpose of this study was to determine if estrogen status altered LSNA and NO-mediated inhibition of efferent SNA at rest and during muscle contraction. We hypothesized that SNA would be decreased both at rest and during muscle contraction in rats with intact ovaries and who had received estrogen supplementation when compared with ovariectomized rats. We also hypothesized that non-selective NOS blockade would augment SNA in rats with an estrogen-replete status both at rest and during muscle contraction, while ovariectomized rats would have no change in SNA.

In contrast to our hypothesis, we observed no estrogen-dependent changes in LSNA at rest or during muscle contractions. Furthermore, NOS blockade did not appear to modify efferent LSNA regardless of estrogen-status. This study concludes that short term alterations to estrogen status, and NO-mediated regulation of SNA outflow are not significant modifiers of lumbar SNA at rest or during muscle contraction.

Estrogen and Efferent Sympathetic Nerve Activity at Rest and During Exercise

The RVLM plays a significant role in the regulation of efferent sympathetic nerve activity [2]. The RVLM operates under a significant GABAergic tonic inhibitory signal from the central ventrolateral medulla [36, 37], which receives excitatory input from the nucleus tractus solitarius (NTS) [16, 37, 38]. These baroreflex-dependent nuclei that modify the action of the RVLM can also be influence by higher brain centers in the forebrain and hypothalamus such as the paraventricular nucleus [1]. Measuring efferent SNA directed to the skeletal muscle vascular bed at rest and during muscle contraction in the present study is

a wholistic view of these excitatory and inhibitory influences. Indeed, excitatory glutamate infusions into sub-regions of the RVLM can differentially stimulate the adrenal, renal and lumbar innervating branches of the sympathetic nervous system [39], thus measuring close to the vascular bed of interest we attempt to make conclusions from an integrative perspective.

How estrogen may alter the central regulation of the sympathetic nervous system *in vivo* is still unclear. The present data suggests that a short-term (4 week) change to estrogen status does not significantly alter efferent lumbar SNA directed to skeletal muscle vascular beds at rest or during exercise. In contrast to the present data, animal studies with direct injection of estradiol into brainstem nuclei show a reduction in renal efferent SNA that is enhanced with estradiol supplementation in ovariectomized rats [14]. In humans, muscle sympathetic nerve activity increases with age in both men and women [7], and postmenopausal women have greater MSNA than premenopausal women [9]. Estrogen replacement in post-menopausal women has also been shown to decrease resting sympathetic nerve activity [10, 40]. Sympathetic nerve activity also may fluctuate with hormonal phase, and sympathetic nerve activity has been observed to increase during the surge of estradiol in the mid-luteal phase [41, 42]. This increase in SNA may have been to offset peripheral vasodilatory actions of estrogen, and some indices of an inhibitory role for estrogen occurred when expressed as changes in SNA to changes in estrogen, which were negatively correlated [41]. The present data are supported by a recent study in humans, which indicated no difference in SNA activation to a lower-body negative pressure stimulus in subjects taking hormonal contraception and those who were not [43]. Women in the placebo phase of hormonal oral contraceptive

use had similar MSNA compared to women in the early follicular phase of their natural menstrual cycle [44]. Young women had lower levels of MSNA in the early luteal phase of their menstrual cycle, with high levels of estrogen and progesterone [45], but similar MSNA during the menstrual (low hormone) and follicular phase [34].

Interestingly, ovariectomized rats in the present study had a positive correlation of MAP and LSNA both at rest and during exercise that was not apparent in estrogen replete groups during control conditions. This may be explained by augmented β-adrenoreceptor-mediated vasodilation offsetting sympathetic vasoconstriction in estrogen-replete animals, as observed in young females versus males [46]. However, this role for sex and/or estrogen-mediated β-adrenoreceptor vasodilation has not been consistently demonstrated [47].

The lack of relationship between FVR and LSNA during resting control conditions is supported by findings in humans showing a dissociation of total peripheral resistance and MSNA [48]. Four weeks of estrogen deficiency was not sufficient to alter the relationship between FVR and LSNA in the current study, suggesting that the positive relationship between TPR and MSNA observed in postmenopausal humans [49] is a function of the length of estrogen-deficiency, the use of total peripheral resistance versus FVR, or species dependent.

Studies examining sex and efferent sympathetic outflow during exercise have been limited due to the technical limitations of microneurography techniques and dynamic large-muscle group exercise. One strategy to surpass this limitation is to utilize an inactive limb to measure SNA an extrapolate measured activity to the exercising limb [50]. Others use static muscle

contractions [34] or low contraction intensities to minimize electrode movement [34]. However, SNA increases in an inactive limb have not been consistently demonstrated [51] and may vary due to exercise modality and intensity of contraction. Similar to our results, no difference in sympathetic activation by static handgrip [33] or during low intensity (20% MVC) rhythmic handgrip contractions [34] across low- and high-hormone menstrual phases have been observed in humans. In contrast to the current study, static handgrip exercise increased to MSNA to a greater extent during the menstrual phase than during the follicular phase [34], and females in the early follicular (low hormone) phase had augmented SNA responses to chemoreflex activation compared to the mid-luteal phase [42]. Regardless, it is important to note that isometric small muscle group contractions will act to increase MSNA through different mechanisms than dynamic large muscle group contractions [52]. Isometric contractions elicit a much greater influence of chemoreflex afferent signaling pathways than do dynamic muscle contractions due to the relative ischemic state of isometric exercise [52].

As a whole, fluctuations in estrogen-status are not sufficient to preclude female animals from biomedical research involving the sympathetic nervous system [53], and our results suggest that the in the absence of central command, estrogen-deficiency or supplementation is insufficient to change exercise-induced increases in SNA.

Nitric Oxide and Estrogen-mediated Changes in Efferent Sympathetic Nerve Activity

In the current study, the systemic administration of L-NAME resulted in a pronounced increase in MAP in all groups, demonstrating robust NOS-mediated NO production regardless of NO status. Since the increase in MAP activated baroreflex-mediated decrease in LSNA, as has been observed previously in both conscious rats [54] and rabbits [35]. The role of NO on efferent sympathetic nerve activity at rest and during exercise was also examined upon the normalization of MAP using a constant infusion of hydralazine. Upon MAP normalization, we did not observe any differences in SNA at rest or during contraction when compared to control conditions, nor was there an effect of estrogen status. A similar return to baseline, without exceeding previous values has been observed in conscious animals [35, 55]. These studies support the conclusion that results obtained in anesthetized animals can be extrapolated to conscious animals. However, another experimental approach that normalized blood pressure in conscious rats via the removal of the reflex-mediated attenuation in SNA demonstrated a clear increase in renal SNA following L-NAME administration [25]. As previously noted [25], it is not clear if previous activation of the arterial baroreflex persisted and masked any NO-mediated inhibition of SNA that was present. Regardless, the distinct levels of estrogen in our 3 groups and the lack of difference between them following NOS blockade suggests that 4 weeks of altered estrogen status does not change NO-mediated efferent SNA regulation. However, as previously mentioned, it is possible that renal SNA is regulated in a different manner that lumbar SNA, as lumbar SNA was decreased in male sino-aortic denervated rats following L-NAME administration, while renal SNA was increased [56]. Thus, our results suggest that in female rats with intact baroreceptors, NO plays no significant role in the

integrative regulation of efferent LSNA sympathetic outflow at rest or during exercise, regardless of estrogen status.

The interpretation of the effect of NO on the relationship between LSNA and MAP/FVR in the current study is confounded the pressor effect of L-NAME on baroreceptor mediated decreases on LSNA and by hydralazine-mediated alterations in blood pressure. Regardless NO does not appear to play an influential role, as during the normalization of MAP following NOS blockade OI and OVXE groups had similar relationships between SNA and MAP to control conditions. However, in OVX, the significant positive correlation between LSNA and MAP observed in control conditions was lost during hydralazine infusion at rest and during muscle contractions, suggesting that the systemic blockade of NO production produces a greater upset to neurovascular control during estrogen-deficiency. It could be hypothesized that estrogen-replete rats have the ability to maintain stable sympathetic neurovascular control through unidentified sex hormone-dependent redundant mechanisms.

Conclusion

These data demonstrate that efferent lumbar SNA at rest and during muscle contraction was not altered by estrogen bioavailability, but that ovariectomy may alter the neural control of blood pressure such that MAP is positively correlated with LSNA.

NOS inhibition elevated blood pressure and reduced efferent LSNA at rest and during muscle contraction in all groups. However, when blood pressure was returned to control levels by hydralazine, LSNA increased to control levels

indicating that NO had no inhibitory effect on efferent LSNA and this was not a function of estrogen status.

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Chapter 5: Summary and Conclusions

Summary of Findings

The aim of this thesis was to investigate whether sex and estrogen modulate sympathetic neurovascular control in resting and contracting skeletal muscle, and to address the hypothesis that NO mediates the effects of sex and estrogen on neurovascular control.

The studies in this thesis contribute to our understanding of how sex and estrogen influence sympathetic nervous system control of the skeletal muscle vasculature at rest and during muscle contraction and have important implications for estrogen's role in integrative exercise physiology. A significant sex bias has been observed in basic science research, such that male-only studies comprise the majority of data collected in disciplines including physiology, neuroscience, pharmacology, endocrinology and zoology [1]. To address this issue journals, scientific organizations and major funding agencies are developing guidelines that will require the inclusion of sex as a biological variable in scientific research [2]. The studies in this thesis directly address the need for comparisons of sex as a biological variable and for studies of the physiological effects of female reproductive hormones.

Sex differences in cardiovascular disease risk and in response to drugs are well documented [3], however the reasons behind divergent cardiovascular disease risk profiles in males and females are still unclear. Sex differences in sympathetic vascular control are apparent, such that females appear to have a cardioprotective blunting of sympathetic vasoconstriction when compared to males [4]. An augmented role for vasodilatory β-adrenoreceptors that counteracts increases in SNA with age has been suggested to play a role in the

cardioprotection young females receive [5]. However, conflicting evidence exists that suggests there is no sex difference in β-adrenoreceptor-mediated sympathetic vascular control [6]. Alternative mechanisms to explain this cardioprotection have not been widely explored. Indeed, females have yet to be sufficiently represented in basic science and clinical research for a variety of reasons, including variability due to hormonal fluctuations during menstrual cycles [1].

Estrogen has been implicated as a regulator skeletal muscle blood flow and systemic blood pressure [7], and in postmenopausal women an increased risk of disease characterized by autonomic dysfunction occurs [8]. Estrogen's potential involvement in blood flow and blood pressure regulation suggests that it would also play a major role in regulating the integrated cardiovascular response to exercise, including the distribution of blood flow and control of blood pressure by the sympathetic nervous system [9]. However, only one lab has mechanistically studied estrogen in the context of sympathetic neurovascular control during exercise [10, 11], but examined the role of estrogen in neurovascular control over a relatively short time period of four weeks in a rodent model [10] and in post-menopausal women without controlling for the duration of menopause [11].

The studies in this thesis suggest that despite evidence of sex differences in NO-mediated inhibition of sympathetic vasoconstriction, estrogen is not a major modifier of sympathetic neurovascular control in young female rats.

Study 1 was designed to examine the presence of sex differences in sympathetic vasoconstrictor responsiveness and NO-mediated inhibition of sympathetic vasoconstriction in contracting skeletal muscle. At rest, evoked

vasoconstrictor responses were elevated in female compared to male rats (2Hz). In contracting muscle, sympathetic vasoconstrictor responsiveness was blunted (5Hz) and sympatholysis was augmented in female rats compared to male rats (2Hz and 5Hz). The sex difference in sympatholysis was not present following the inhibition of NOS, and NOS inhibition significantly reduced sympatholysis in female rats. These data demonstrate that sex modulates sympathetic vasoconstrictor responsiveness at rest and during muscle contraction. Furthermore, enhanced sympatholysis in female rats is in part mediated by augmented NO-mediated inhibition of sympathetic vasoconstriction during muscle contraction.

Study 2 examined the mechanistic role of estrogen on NOS isoform-specific NO-mediated inhibition of sympathetic vasoconstriction at rest and during muscle contraction in female rats. Estrogen did not alter sympathetic vasoconstriction at rest or during muscle contraction or sympatholytic capacity. Furthermore, selective blockade of nNOS did not alter sympathetic vasoconstriction or sympatholysis, while complete NOS inhibition reduced sympatholysis in all groups. Thus, these data support the conclusion that eNOS partly mediates sympatholysis in female rats and that this mechanism is not dependent on the estrogen status of young female rats.

Study 3 investigated the hypothesis that estrogen status could alter efferent sympathetic nerve activity in female rats through a NO-dependent mechanism and utilized an experimental model of lumbar sympathetic nerve activity recording. This study found no effect of estrogen status on efferent sympathetic nerve activity at rest or during muscle contraction. Systemic NOS blockade increased mean arterial pressure in all groups, which reduced efferent

sympathetic nerve activity through activation of the baroreflex, while NOS blockade during the normalization of mean arterial pressure returned sympathetic nerve activity to levels observed during the control resting and muscle contraction conditions similarly in all groups. Thus, this study concluded that estrogen status in young female rats did not alter efferent sympathetic nerve activity at rest or during muscle contraction. Furthermore, NO bioavailability was not implicated in the control of efferent sympathetic nerve activity at rest or during muscle contraction regardless of estrogen status.

The findings from this thesis demonstrate that sex modulates sympathetic vascular control, such that females have a sympathetic stimulation frequency-dependent augmentation of sympathetic vasoconstrictor responsiveness while displaying higher sympatholysis that may be partially mediated by NO. Estrogen has previously been advanced as a major mediator of sex differences in neurovascular regulation [4, 5, 7], however data from this thesis does not support estrogen as a major factor in altering sympathetic neurovascular control. Indeed, chronic alterations to estrogen bioavailability did not alter sympathetic vasoconstrictor responsiveness or NO-mediated blunting of sympathetic vasoconstriction in resting or contracting skeletal muscle. Further, manipulation of estrogen status did not alter the regulation of efferent SNA, at rest or during electrically-evoked muscle contractions. These data provide critical evidence that contradicts an assumption that estrogen is the driver of sex differences observed in sympathetic neurovascular control at rest and during exercise.

Experimental Considerations

Estrogen receptors are expressed throughout the cardiovascular system and are involved in genomic and non-genomic processes that impact vascular

endothelial and smooth muscle cells [12], as well as expression within the central nervous system [13, 14]. The effects of estrogen alone have been the subject of thorough review. Both nuclear and non-genomic estrogen signalling play a role in the mitochondrial electron transport chain, myocardial contractility and repolarization, inflammation responses to injury, diabetes and insulin sensitivity, atherosclerosis and infarct size [15]. Further, the impact of NO across the cardiovascular system through regulation of vascular tone and proliferation, as well as factors that may alter NO bioavailability or the source of NO such as age [16] and training status [17, 18], highlight the complexity of NO biology. Conclusions regarding the expression of NOS in target tissues alone are likely therefore too narrow to apply to an intact physiological system. Integrative experiments such as the ones presented in this thesis are necessary to provide practical results to more reductionist studies, and provide direction for fruitful translational studies in humans.

The studies within this thesis utilized anesthetized rats to perform mechanistic *in vivo* experiments on the role of sex, estrogen and nitric oxide in the sympathetic nervous system control of skeletal muscle vascular conductance. These mechanistic studies are not feasible in conscious animals and/or humans. While the experimental preparation allows precise control of muscle force production and the frequency of evoked sympathetic activity and magnitude of sympathetic vasoconstrictor response in an integrative whole animal preparation, it also has limitations. When electrically stimulating the lumbar sympathetic chain to evoke sympathetic vasoconstriction, at rest and during electrically stimulated hindlimb muscle contraction, it is noted that the methodological pattern of delivery appears non-physiological. Stimulation frequencies of 2 and 5 Hz are within the

physiological range of MSNA frequencies at rest and during exercise, but it is difficult to accurately estimate how well they capture each condition. They do produce graded vasoconstrictor responses at rest and during exercise and when used in combination with selective α-adrenoreceptor antagonists, frequency dependent effects on vasoconstriction are observed suggesting that the two frequencies of stimulation evoke the release of neurotransmitters in differing quantities [19, 20]. Efferent sympathetic nerve activity at rest and during exercise is characterized by random bursts of activity, followed by periods of quiescence. This pattern of activity is different in every animal and person and is difficult to approximate in the laboratory. A previous study utilized different patterns of sympathetic nerve stimulation, however the vasoconstrictor response to sympathetic nerve stimulation delivered in a bursting pattern at 20 and 40 Hz was not different than the vasoconstrictor response to sympathetic nerve stimulation delivered continuously at 2Hz [21]. Thus, all things considered the chosen frequencies are approximations of nerve activity in different physiological states and there are obvious strengths and limitations. The present stimulation frequencies are also routinely used in other labs routinely to investigate mechanisms of neurovascular control [18, 22].

To simulate exercise, electrical stimulation of the sciatic nerve produces a non-physiological recruitment of the tricep surae muscle group. Electrical stimulation of motor nerves reverses Henneman's size principle [23], which states that smaller motoneurons will be stimulated first for a given level of synaptic neurotransmitter signaling. Smaller motoneurons generally innervate motor units that include slow-twitch muscle fibers, with larger motoneurons innervating motor units that include fast-twitch (and more fatigable) muscle fibers

[24]. In these experiments, axons with larger diameters are recruited before small diameter axons due to their increased sensitivity to electrical stimulation. Thus, at the onset of muscle contraction muscle fiber recruitment included more fast-twitch fatigable muscle fibers than during voluntary muscle contraction. The control of vascular resistance between muscles of different fiber types has been shown to vary, such that arterioles supplying glycolytic muscle may be more responsive to sympathetic vasoconstrictor influences than arterioles within oxidative muscle [25]. At the same time, studies in humans [26] and animals [27] suggest skeletal muscle composed of predominantly glycolytic fiber types has a higher susceptibility to sympatholysis than predominantly oxidative muscles. Further, NOS expression may also be fiber type dependent, such that fast twitch muscle fibers contain more nNOS than slow twitch muscle fibers [28]. The electrically stimulated muscle activation pattern may therefore alter the distribution of blood flow within contracting skeletal muscle compared to consciously initiated muscle contractions. nNOS plays a role in regulation blood flow to glycolytic muscle fibers during high intensity exercise [29], thus muscle contraction-stimulated vasodilation and the concurrent shear stress-mediated release of NO may have resulted in the measurement of muscle fiber blood supplies that are not normally active during voluntary exercise. However, the studies within this thesis contain an experimental preparation that stimulates the contraction of the entire tricep surae muscle group and thus prevents analysis of specific muscle fiber-type NO contributions to blood flow or inhibition of sympathetic vascular control.

The non-physiological recruitment of fast-twitch fatigable muscle fibers at the onset of exercise in the current study may also impact the hyperemic

response to exercise. However, measurement of sympathetic vasoconstrictor responsiveness and efferent SNA occurred following at least 3 minutes of contraction and after mean arterial pressure, femoral blood flow, and heart rate had achieved a steady state, thus it is unlikely that the pattern of fiber recruitment significantly altered the primary outcome variables. Furthermore, the same pattern of electrical stimulation was utilized in all animals and exercise bouts and each animal acted as its own control to assess the effects of each pharmacological intervention.

In Study 3, LSNA was recorded under anesthesia, and anesthesia effects sympathetic outflow [30]. We selected an anesthetic regimen that has a minimal impact on the autonomic nervous system [30]. Indeed, urethane and α-chloralose have been shown to have limited effects of blood pressure and efferent SNA [30, 31]. Other anesthetics, such as barbituate-based regimens, have been associated with augmented vascular resistance to due heightened sympathetic activity [32]. However, it is still a limited measure when compared to recording nerve activity in conscious animals.

Translational Value

Following menopause, the risk of disease characterized by SNS dysfunction such as hypertension, ischemic heart disease and cardiovascular events such as myocardial infarction and stroke are markedly increased [33, 34], and a decline in estrogen bioavailability is strongly associated with the increased cardiovascular disease risk in post-menopausal women [35]. Post-menopausal estrogen-based therapy reduces the incidence of cardiovascular events and mortality [34]. Hormone replacement therapy (HRT), or estrogen and/or progestin supplementation, is recommended to post-menopausal women to ameliorate the

uncomfortable symptoms of menopause (i.e. Hot flashes) and an attempt to maintain a degree of cardio-protection that seems to be conferred onto younger women when compared to young men [36]. HRT has been shown to improve risk factors associated with hypertension including improved sympathetic baroreflex sensitivity [37] and reduced basal sympathetic efferent outflow [33, 38]. HRT has also been shown to lower resting diastolic and mean blood pressure, exercising diastolic blood pressure, and abolish age related increases in resting diastolic blood pressure at rest [39]. However, HRT is a controversial treatment that may increase risk of coronary heart disease and venous thromboembolism [35, 40, 41]. Given the controversial utility of HRT, it is apparent that determining the mechanisms by which estrogen may modify sympathetic vascular control at rest and during exercise are a priority to establish. A decline in estrogen bioavailability may manifest as autonomic and vascular dysfunction [10, 11, 42], as observed following many years of reduced estrogen bioavailability in postmenopausal women [7]. Thus, this thesis addresses a need for mechanistic studies that are necessary to understand estrogen's role in neurovascular regulation.

Future Directions

A novel finding for future study was observed in the lumbar chain stimulation frequency-dependence of vasoconstrictor responsiveness in Studies 1 and 2. The frequency of sympathetic nerve activity has previously been shown to favor the release of specific neurotransmitters, with low stimulation frequencies favoring ATP and NE and higher stimulation frequencies favoring the co-release of NE and NPY [43-45]. Specific SNS post-synaptic receptor involvement and expression were not measured in the current thesis. However, previous studies have reported that the susceptibility to inhibition may differ between receptor

types [20, 27]. One study found that in female rats, α_2-adrenoreceptors may be more sensitive to metabolic inhibition during muscle contraction through a NO-dependent mechanism [27]. Our own lab reported that in male rats α_1-adrenoreceptors were of primary importance to sympatholytic function, and that NO does not play a significant sympatholytic role in sedentary male rats [20].

Thus, sex differences in post-synaptic adrenoreceptor and non-adrenoreceptor function and expression may exist. In Study 1, a sympathetic chain stimulation frequency-dependent effect of sex on sympathetic vasoconstrictor responsiveness at rest and during muscle contraction was observed. Specifically, female rats were observed to have augmented resting vasoconstriction only during low-frequency sympathetic chain stimulation when compared to male rats. However, during muscle contraction, females had a blunted magnitude of vasoconstriction when compared to male rats at the high-frequency sympathetic chain stimulation. Further, the NO-mediated blunting of sympathetic vasoconstriction appears to be a more important mechanism in female rats at high stimulation frequencies, as inhibition of NOS with L-NAME significantly decreased sympatholysis at the high stimulation frequency in female rats.

Study 2 extends these findings in female rats by examining the specific NOS isoforms that may be the source of the NO that inhibits sympathetic vasoconstriction at high stimulation frequencies. Given the lack of effect of selective nNOS blockade on female rats, but a reappearance of significantly decreased sympatholysis at high stimulation frequencies following non-selective NOS blockade, the source of sympatholytic NO appears to be eNOS.

The results of these studies raise 2 important questions: First, does an augmented expression or responsiveness of specific post-synaptic sympathetic receptors account for the augmented resting vasoconstriction seen at the low stimulation frequency in female rats when compared to male rats; and second, why is NO-mediated sympatholysis augmented in females at high stimulation frequencies?

Since previous work in humans have observed similar [46] or blunted [47, 48] vasoconstriction of post-synaptic α-adrenoreceptors in females compared to males, it could be hypothesized that female rats have augmented expression or responsiveness of type P2X purinergic receptors in skeletal muscle vascular beds that can produce greater vasoconstriction at rest than male rats. Indeed P2X receptors regulate vasoconstriction on distal arterioles and activation of these receptors with selective agonists demonstrated that they are capable of producing substantial changes in vascular resistance [49]. During higher stimulation frequencies, the results presented in this thesis would also support further research to determine if females have an augmented ability to inhibit NPY1 receptor-mediated vasoconstriction during muscle contraction through a mechanism dependent upon eNOS-mediated NO production. Indeed, P2X and NPY1-receptors do produce vasoconstriction during large muscle mass exercise [50, 51] however if sex/estrogen specific mechanisms exist that may alter vascular control in skeletal muscle is not clear. Evidence does exist that ovariectomy augments NPY1 receptor expression and augments NPY-mediated restraint of resting hindlimb vascular conductance in female Sprague-Dawley rats and that NPY-mediated control of skeletal muscle blood flow is different between sexes [52, 53]. However, the relationship between NPY and estrogen is not

conclusive, as others have demonstrated no change in NPY-mediated vasoconstriction of isolated skeletal muscle arterioles from rats who were ovariectomized and ovariectomized with estradiol supplementation [54]. Decreases in vascular conductance induced by NPY-infusion in dogs were not blunted by exercise [55] and blockade of endothelial NO production did not augment NPY-mediated vasoconstriction in the coronary vasculature of mongrel dogs [56], but the sex of the animals was not specified or analyzed as a significant factor in either of these studies and indeed studies thus far have focused on the effect of aging [55, 57].

Thus *in vivo* experiments, like those conducted in this thesis, with the addition of selective blockade of sympathetic adrenoreceptors and non-adrenoreceptors while controlling for age and sex will likely provide valuable insight into sex-dependent sympathetic neurovascular control.

Conclusions

Together, these studies found that divergent sympathetic vasoconstrictor responsiveness between males and females was dependent upon the frequency of lumbar sympathetic chain stimulation and that augmented sympatholysis in females when compared to males is partly mediated through a NO-dependent mechanism. In female rats, the endothelial isoform of NOS was demonstrated to be the likely source of NO that alters on sympathetic vasoconstrictor responsiveness during muscle contraction, while the neuronal isoform of NOS does not play a significant role in the inhibition of sympathetic vasoconstriction regardless of long-term alterations to estrogen status. Estrogen status also has no effect on efferent SNA at rest or during muscle contraction, nor does a reduction NO bioavailability alter efferent SNA.

Studies examining the influence of sex and menopause have reported that estrogen is an important regulator of blood pressure and skeletal muscle blood flow [33, 58, 59]. In contrast to these reports, the data in this thesis suggest that while sex influences vasoconstrictor responsiveness and sympatholysis, chronic changes in estrogen status in young female rats do not alter sympathetic vasoconstrictor responsiveness or efferent LSNA young female rats at rest and during muscle contraction. Thus, these studies provide evidence regarding the role of estrogen in sympathetic vascular control without the confounding influence of vascular aging that occurs in conjunction with normal menopause. These data have important implications for the design of future studies of women's vascular health, and support need for additional studies that the examine the deleterious cardiovascular health effects observed following menopause beyond estrogen-based physiology and to differentiate the effects of vascular aging from the vascular effects of changes in reproductive hormones.

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