Estrogen, Exercise Training, and Nitric Oxide-Mediated Sympatholysis

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

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Abstract

Contraction-mediated inhibition of sympathetic vasoconstriction (sympatholysis) is enhanced in female compared with male rats. Acute pharmacological blockade of nitric oxide (NO) production abolished the sex difference in sympatholysis, indicating that NO-mediated sympatholysis is elevated in female rats. Estrogen has been shown to upregulate expression of the enzyme nitric oxide synthase (NOS), suggesting that estrogen may be an important regulator of NO bioavailability. Indeed, sympatholysis is impaired in estrogen-deficient rodents and humans, and estrogen supplementation appears to restore sympatholysis. NOS is expressed in neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) isoforms and NO derived from eNOS and nNOS has been shown to inhibit vasoconstriction in resting and contracting skeletal muscle in male rats. Moreover, skeletal muscle nNOS expression and nNOS-mediated sympatholysis were enhanced following exercise training in male rats, suggesting that physical activity may modulate NO bioavailability. The reduced sympatholysis reported in estrogen-deficient rats and humans may be due to impaired nNOS function, though the effect of estrogen bioavailability on NOS isoform-specific mediated inhibition of sympathetic vasoconstriction has yet to be investigated. Further, no studies have examined whether exercise training can restore NO-mediated sympatholysis in estrogen-deficient female rats. Therefore, the purpose of this study was to investigate the role of estrogen in NOS isoform-specific mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle of sedentary and exercise-trained female rats. I hypothesized that: 1) sympatholysis would be blunted in sedentary estrogen-deficient rats relative to sedentary control rats; 2) exercise training would increase sympatholysis in estrogen-deficient rats, but not control rats; and 3) the effect of exercise training would be nNOS-dependent. Eight week-old female Sprague-Dawley rats were

familiarized to exercise on a motorized treadmill, then randomly assigned to sedentary ovary-intact (SOI, n=10), sedentary ovariectomized (SOVX, n=9), exercise-trained (10 weeks, 5 days/week, 600m, 40m·min^{-1}, 5% grade) ovary-intact (TOI, n=13), or exercise-trained ovariectomized (TOVX, n=10) groups. Ovariectomy group rats were then anesthetized, and both ovaries surgically removed. Following sedentary behavior or exercise training, all rats were anesthetized and instrumented for measurement of blood pressure and leg blood flow (LBF). Blood pressure was measured at the right carotid artery and mean arterial pressure (MAP) was calculated. LBF was measured at the right femoral artery, and femoral vascular conductance (FVC) was calculated as FVC=LBF÷MAP. The sciatic nerve and lumbar sympathetic chain were fitted with stimulating electrodes, and the left femoral vein was cannulated for drug delivery. Vasoconstriction in response to stimulation of the lumbar sympathetic chain at 2 Hz and 5 Hz was assessed at rest and during muscle contraction at 60% of maximal contraction force. The effect of exercise training, estrogen status, and NO on sympathetic vasoconstrictor responsiveness (% change in FVC) and sympatholysis was assessed by two-way repeated measures (group x drug condition) ANOVA. Resting sympathetic vasoconstrictor responsiveness was not different following selective nNOS blockade ($p>0.05$), but was increased ($p<0.05$, main effect of drug condition) following non-selective NOS blockade compared with the control and selective nNOS blockade conditions at both 2 Hz and 5 Hz. Similarly, during muscle contraction, sympathetic vasoconstrictor responsiveness to 2 Hz sympathetic stimulation was unchanged ($p>0.05$) in the presence of selective nNOS inhibition, but was increased ($p<0.05$, main effect of drug condition) following non-selective NOS blockade compared with control and selective nNOS blockade conditions. At 5 Hz, contracting sympathetic vasoconstrictor responsiveness was increased ($p<0.05$, main effect of drug condition) by selective nNOS blockade, and increased

further ($p<0.05$, main effect of drug condition) by non-selective NOS blockade. The magnitude of sympatholysis at 2 Hz was not different ($p>0.05$) between groups or drug conditions. At 5 Hz, sympatholysis was blunted ($p<0.05$, main effect of drug condition) in the selective nNOS blockade condition, and further reduced ($p<0.05$, main effect of drug condition) following non-selective NOS blockade in all groups. Sympathetic vasoconstrictor responsiveness and sympatholysis were not different ($p>0.05$) following exercise training. In conclusion, sympathetic vasoconstrictor responsiveness in resting and contracting skeletal muscle was not dependent on estrogen bioavailability. Additionally, NO derived from eNOS appears to inhibit sympathetic vasoconstriction at rest and during muscle contraction, whereas NO derived from both eNOS and nNOS are important during high frequency stimulation during muscle contraction, regardless of estrogen bioavailability. Finally, exercise training did not alter sympathetic vasoconstrictor responsiveness and sympatholysis regardless of estrogen bioavailability.

Preface

This thesis is an original work by Breanne S. Collison, composed of a research project 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. The exercise training and surgical protocols and data collection were performed by Timothy P. Just.

Acknowledgements

I owe many thanks to my supervisor, Dr. Darren DeLorey. Your mentorship has been instrumental in my journey from an undergraduate research volunteer to graduate school. I would not be the student I am today without you. Thank you for helping me navigate the challenges of the last two years. Without your guidance, this thesis would not have been possible.

Thank you to Dr. Frances Plane for your assistance as a member of my supervisory committee. I would also like to thank Dr. Paul Kerr for being a member of my examining committee and Dr. Kelvin Jones for chairing the examination.

To my fellow students, especially Daniel Kim, Phil Li, Zack Perala, Scott Thrall, and Justin Duong, thank you for providing feedback, a helping hand, and for making my graduate experience as enjoyable as could be.

Thank you to my family and friends for the support through all of the ups and downs. I could not have made it here without you.

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List of Abbreviations

ACh, acetylcholine

ABP, arterial blood pressure

ATP, adenosine triphosphate

eNOS, endothelial nitric oxide synthase

FVC, femoral vascular conductance

FVR, femoral vascular resistance

HM:BM, heart mass to body mass ratio

HM:TL, heart mass to tibia length ratio

HR, heart rate

iNOS, inducible nitric oxide synthase

LBF, leg blood flow

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

MAP, mean arterial pressure

MCF, maximal contractile force

mRNA, messenger ribonucleic acid

MSNA, muscle sympathetic nervous system activity

MT, motor threshold

NE, norepinephrine

nNOS, neuronal nitric oxide synthase

NO, nitric oxide

NOS, nitric oxide synthase

NPY, neuropeptide Y

NTS, nucleus tractus solitarius

RVLM, rostral ventrolateral medulla

SMTC, *S*-methyl-$_{L}$-thiocitrulline

SOI, sedentary time control ovary intact

SOVX, sedentary time control ovariectomized

TOI, exercise trained ovary intact

TOVX, exercise trained ovariectomized

Chapter 1: Introduction

Introduction

In order to sustain life, vital organs and tissues must receive adequate delivery of oxygen and other substrates to support metabolism (Joyner & Casey, 2015; Mueller et al., 2011). The cardiovascular, respiratory, and autonomic nervous systems work in a coordinated manner to achieve the cardiac output, blood pressure, and vascular resistance necessary to match tissue blood flow to tissue metabolism (Boushel et al., 2002; Buckwalter, Ruble, et al., 1998; Duffy et al., 1999; Kilbom & Wennmalm, 1976; Lott et al., 2001; Maxwell et al., 1998; McAllister, 2003; Rådegran & Calbet, 2001; Saltin et al., 1998). The respiratory system exchanges oxygen and carbon dioxide between the atmosphere and the blood, and the cardiovascular system transports the oxygen and others substrates to the active tissues, and removes metabolic by-products, such as carbon dioxide. Vascular resistance and cardiac output are regulated by the autonomic nervous system to match tissue blood flow to metabolic demand. At rest, the skeletal muscle vascular bed is relatively vasoconstricted, blood flow per unit mass is modest, and the skeletal muscle vasculature is an important contributor to the regulation of systemic vascular resistance. During exercise, skeletal muscle metabolism markedly increases and demand for blood flow and oxygen delivery rises in an exercise-intensity dependent manner. To achieve the increase in skeletal muscle blood flow, cardiac output increases and skeletal muscle blood vessels dilate to direct blood flow to the active muscle. Indeed, skeletal muscle may receive more than 90% of cardiac output during maximal exercise (Joyner & Casey, 2015). The considerable increase in blood flow to the active skeletal muscle tissue must be accomplished while perfusion of other essential organs, such as the brain, and systemic blood pressure are maintained. The maintenance of blood pressure represents a substantial physiological challenge because the capacity of the skeletal

muscle vasculature to vasodilate and receive blood flow far exceeds the capacity of the heart to increase cardiac output. Indeed, it has been estimated that dilation of a large amount of muscle or maximal vasodilation of as little as one third of skeletal muscle in an untrained individual could exceed maximal cardiac output and result in hypotension if skeletal muscle vasodilation was not restrained (Andersen & Saltin, 1985; Joyner & Casey, 2015). Thus, the exercise response requires an increase in cardiac output and tight regulation of peripheral vascular resistance to balance the competing needs of tissue oxygen delivery and maintenance of systemic blood pressure (Joyner & Casey, 2015). In addition to facilitating an increase in cardiac output during exercise, sympathetic nerve activity is also directed towards the periphery, producing vasoconstriction in both inactive and active tissues (Christensen & Galbo, 1983; DiCarlo et al., 1996; Mueller et al., 2011). Vasoconstriction in inactive tissue shunts blood flow towards active muscle, whereas vasoconstriction in active muscle restrains local vasodilation and prevents a profound drop in vascular resistance (DiCarlo et al., 1996; O'Leary et al., 1997). In the active skeletal muscle vasculature, sympathetic vasoconstriction is inhibited by muscle contraction in a process known as sympatholysis. The blunting of vasoconstriction in active tissue modulates local vascular resistance to facilitate the perfusion of active tissues during exercise (Joyner & Casey, 2015). The mechanism(s) by which sympatholysis occurs remain undefined, although several studies have reported that nitric oxide (NO) inhibits sympathetic vasoconstriction in resting and contracting skeletal muscle (Chavoshan et al., 2002; Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b, 2013d; Thomas & Victor, 1998). The formation of NO is catalyzed by the enzyme nitric oxide synthase (NOS), and NOS is expressed in neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) isoforms (Kobzik et al., 1994). NO production is accelerated during exercise (Kobzik et al., 1994; Nakane et al., 1993; Rubanyi et

al., 1986), and NO derived from eNOS and nNOS has been shown to inhibit vasoconstriction in resting and contracting skeletal muscle in male rats (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013d). While NO does contribute to sympatholysis, it is not the only mechanism at play, as NOS inhibition blunts, but does not eliminate, sympatholysis (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b, 2013d).

In female rats, the inhibition of sympathetic vasoconstriction during muscle contraction is enhanced relative to male rats (Cooper et al., 2021; Just & DeLorey, 2017). NOS inhibition eliminated the statistical difference in sympatholysis between male and female rats, suggesting that NO-mediated sympatholysis is enhanced in female rats (Just & DeLorey, 2017), though the mechanism(s) underlying the augmented NO-mediated sympatholysis are unknown. Additionally, elevated β-adrenoreceptor mediated vasodilation in females has been suggested to contribute to this enhanced sympatholysis; however, our laboratory found that acute pharmacological inhibition of β-adrenoreceptors did not alter sympatholysis in male or female rats (Cooper et al., 2021). Estrogen has also been advanced as a putative mechanism for sex differences in vascular regulation, as females have higher circulating estrogen levels than males, and estrogen has been shown to modulate autonomic nervous system activity and vascular resistance (Mendelsohn & Karas, 1999; Vongpatanasin et al., 2001). Indeed, in estrogen-deficient female rats, sympatholysis was impaired compared to female control rats (Fadel et al., 2003). However, sympatholysis was not different between female control and estrogen-replaced rats, suggesting that estrogen bioavailability modulates sympatholysis (Fadel et al., 2003). Similarly, post-menopausal human females had lower sympatholysis than pre-menopausal females, and this difference was abolished following estrogen replacement therapy (Fadel et al., 2004). The authors suggested that higher estrogen bioavailability enhanced sympatholysis by

upregulating NOS expression and increasing NO bioavailability (Fadel et al., 2003, 2004). In estrogen-deficient female rats, nNOS expression was reduced, suggesting that nNOS-mediated inhibition of sympathetic vasoconstriction would also be reduced; however, NO-mediated sympatholysis was not directly assessed in this study, thus insufficient evidence exists to confirm or refute this theory. In summary, our understanding of the mechanisms underlying sex differences in vascular regulation is incomplete. While estrogen has been implicated, further research is needed to firmly establish the extent of its role, as well as the cellular pathways by which it may act.

Chronic exercise training has been shown to cause many cardiovascular adaptations, including reduced blood pressure and enhanced vascular control at rest and during exercise (Thijssen et al., 2009). Research in humans indicates that cardiovascular adaptations to exercise training, such as maximal oxygen consumption, and left ventricular mass and wall thickness, were blunted in females compared to males (Howden et al., 2015). While these data are from a single study, they may indicate that males and females respond differently to a given training paradigm (Diaz-Canestro & Montero, 2019; Howden et al., 2015). Consistent with this notion, the same exercise training program enhanced sympatholysis in male, but not female, rats (Cooper et al., 2021; Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b; Mizuno et al., 2014). Skeletal muscle nNOS expression and nNOS-mediated sympatholysis were increased following exercise training in male rats (Jendzjowsky et al., 2014), indicating that exercise training may modulate NO bioavailability and NO-dependent sympatholysis (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b). Sympatholysis is enhanced in untrained female rats compared to untrained male rats (Cooper et al., 2021; Just & DeLorey, 2017), and NOS blockade abolished the difference between males and females, suggesting that NO bioavailability and/or

NOS expression may be elevated in females relative to males (Just & DeLorey, 2017). Estrogen is known to modulate NOS expression and estrogen may therefore play an important in determining the response to exercise training (Fadel et al., 2003). If true, exercise training may not increase NOS expression or NO bioavailability in females with "normal" levels of estrogen, whereas in females with lowered estrogen bioavailability, such as post-menopausal females or estrogen-deficient rats, exercise training may reverse the estrogen-related loss of nNOS expression and restore sympatholysis. To date, no studies have investigated the effects of exercise training and estrogen bioavailability on NO-mediated sympatholysis.

Regulation of Sympathetic Vasoconstriction

The sympathetic nervous system plays an integral role in the control of vascular resistance and is an important effector for reflex control of blood pressure and the distribution of cardiac output (Joyner & Casey, 2015; Mueller et al., 2011). Efferent sympathetic nervous system activity is controlled in the brainstem, where inputs from various cardiovascular reflexes, as well as higher brain centres, are integrated (Dampney et al., 2002, 2003). The rostral ventrolateral medulla (RVLM) in the brainstem acts as the sympathetic generator, as sympathoexcitatory neurons in the RVLM are tonically active, evidenced by the occurrence of tonic sympathetic activity in the absence of reflex control (Barman & Gebber, 1980). The amount of activity generated by the RVLM is modulated by the nucleus tractus solitarius (NTS), which receives and integrates sensory inputs from various reflex afferents, including the baroreflex, exercise pressor reflex, and chemoreflex, as well as inputs from higher brain centres (Dampney et al., 2002). NTS excitatory neurons either project directly to RVLM, increasing sympathetic outflow, or to the caudal ventrolateral medulla, which in turn projects to and inhibits the RVLM, decreasing sympathetic outflow (Dampney et al., 2002). The excitatory and

inhibitory inputs to the RVLM are integrated to modulate efferent sympathetic nervous system activity, increasing or decreasing it as necessary to maintain homeostasis and to respond to physiological challenges, such as exercise (Dampney et al., 2002). Sympathetic nervous system activity generated in the RVLM is relayed to the periphery by efferent sympathetic nerves, comprised of a cholinergic pre-ganglionic neuron located within the central nervous system and a peripheral post-ganglionic neuron (Dampney et al., 2003). Neurons in the RVLM directly innervate pre-ganglionic sympathetic neurons, which in turn innervate the post-ganglionic sympathetic neurons (Dampney et al., 2002). The primary neurotransmitter released by post-ganglionic sympathetic neurons innervating cardiovascular tissues is norepinephrine (NE), though adenosine triphosphate (ATP) and neuropeptide Y (NPY) are also released (Loewy & Spyer, 1990; Mueller et al., 2011). NE binds to α_1- and α_2-adrenoreceptors, ATP to purinergic P2X receptors, and NPY to NPY receptors on the surface of the vascular smooth muscle cell to cause vasoconstriction (Nielsen, 2015).

During exercise, both the frequency and amplitude of muscle sympathetic nervous system activity (MSNA) increase in an intensity-dependent manner (DiCarlo et al., 1996). Elevated sympathetic nervous system activity serves to increase cardiac output to meet the higher oxygen demand, and also results in systemic vasoconstriction (Christensen & Galbo, 1983; O'Leary et al., 1997). Sympathetic vasoconstriction is necessary to both divert blood flow away from inactive tissues and to restrain blood flow to active skeletal muscle tissues (Andersen & Saltin, 1985; Joyner & Casey, 2015; Mueller et al., 2011; Saltin, 1985; Secher et al., 1977). Despite sympathetic restraint, profound increases in perfusion still occur in the exercising skeletal muscle (Buckwalter, Ruble, et al., 1998; Buckwalter & Clifford, 1999; Clifford & Hellsten, 2004; Lott et al., 2001; O'Leary et al., 1997; Saltin, 1985). The increase in blood flow to the active skeletal

muscle is exercise intensity-dependent, closely matching the increased metabolic demand (Joyner & Casey, 2015). This is possible because sympathetic vasoconstriction is inhibited in contracting skeletal muscle, in a process termed functional sympatholysis (Buckwalter et al., 2001; Buckwalter, Mueller, et al., 1998; Buckwalter & Clifford, 1999; Cooper et al., 2019, 2021; Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b, 2013d; Just & DeLorey, 2017; Mortensen et al., 2014; Mortensen, Mørkeberg, et al., 2012; Mortensen, Nyberg, et al., 2012; Remensnyder et al., 1962; Ruble et al., 2000, 2002; Saltin & Mortensen, 2012). Although this phenomenon was first reported 60 years ago, the underlying cellular mechanisms remain undefined (Remensnyder et al., 1962). Potential mechanisms include the loss of vascular smooth muscle contractile function, blunting of sympathetic neurotransmitter release, altered biological activity of neurotransmitters, and reduced post-synaptic receptor responsiveness (Mueller et al., 2011). Vascular smooth muscle function does not appear to decline during exercise because vasopressin (a non-sympathetic vasoconstrictor) produced similar vasoconstrictor responses at rest and during exercise, indicating preservation of smooth muscle function during exercise (Buckwalter, Taylor, et al., 2004). Tyramine infusion (evokes endogenous release of sympathetic neurotransmitters) produced graded vasoconstriction responses at rest and during incremental intensities of exercise, suggesting that sympathetic neurotransmitters are still released during exercise (Ruble et al., 2002). Indeed, significant attenuation of the vasoconstrictor response to intra-arterial infusion of sympathetic agonists during exercise suggests an important role of the modulation of post-synaptic receptor responsiveness (Buckwalter et al., 2001, 2003; Buckwalter, Hamann, et al., 2004). NO has been advanced as a potential mechanism for this modulation. Indeed, there is evidence that NO inhibits sympathetic vasoconstriction in resting (Jendzjowsky & DeLorey, 2013d; Just & DeLorey, 2017) and contracting skeletal muscle (Chavoshan et al.,

2002; Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b, 2013d; Just & DeLorey, 2017; Mizuno et al., 2014; Thomas & Victor, 1998), though the exact mechanism by which NO inhibits sympathetic vasoconstriction is unknown. The formation of NO is catalyzed by the enzyme nitric oxide synthase (NOS), found in endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) isoforms (Stamler & Meissner, 2001). NO derived from eNOS and nNOS has been shown to be important to skeletal muscle vascular control in healthy individuals (Kobzik et al., 1994; Stamler & Meissner, 2001). During muscular contraction, the formation of NO is accelerated as a result of mechanical and chemical stimuli (Kobzik et al., 1994; Nakane et al., 1993; Rubanyi et al., 1986). While NO appears to be involved in sympatholysis, it is not the only mechanism at play. When NO production is pharmacologically inhibited, sympatholysis is reduced, but not abolished, suggesting that some other mechanism(s) also contribute to sympatholysis (Chavoshan et al., 2002; Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b, 2013d; Just & DeLorey, 2017; Thomas & Victor, 1998).

Estrogen and Sympathetic Vasoconstriction

The current understanding of neural control of the cardiovascular system is based largely on data from studies of men and male animals and rodents. However, several recent studies suggest that biological sex may modulate sympathetic nervous system regulation of blood pressure and skeletal muscle vascular resistance. For instance, young females tend to have lower resting blood pressure and sympathetic nervous system activity than young males (Hart & Charkoudian, 2014; Hogarth et al., 2007; Keir et al., 2020; Narkiewicz et al., 2005; Ng et al., 1993). Further, sympathetic neurovascular transduction and/or post-synaptic receptor responsiveness appears to differ in females. Consistent with this notion, resting MSNA was related to total peripheral resistance (Hart et al., 2009, 2011), and resting calf MSNA was related

to calf vascular resistance (Hogarth et al., 2007) in males but not females. Similarly, in males, resting MSNA was correlated to both mean arterial pressure (MAP) and limb vascular conductance, but no significant relationship was found in females (Robinson et al., 2019). The MSNA response to a resting cold pressor test was also correlated to the MAP and vascular resistance response in males but not females (Coovadia et al., 2022). Sympathetic vasoconstrictor responsiveness also appears to differ between males and females. The vasoconstrictor response to acute resting sympathetic stimulation (via cold pressor test) (Coovadia et al., 2022; Hogarth et al., 2007) and to isometric handgrip exercise was blunted in young females relative to males (Hogarth et al., 2007). Females also had a comparatively smaller vasoconstriction response to the infusion of NE than males (Kneale et al., 2000; Majmudar et al., 2000). In contrast, infusion of selective α_1- and α_2-adrenoreceptor agonists produced similar vasoconstriction responses in males and females (Limberg et al., 2010). Data from our laboratory in rats indicated that resting sympathetic vasoconstrictor responsiveness to sympathetic stimulation was augmented in female rats at 2 Hz, but not different at 5 Hz (Just & DeLorey, 2017). In a subsequent study, sympathetic vasoconstrictor responsiveness was not different at either 2 or 5 Hz (Cooper et al., 2021). In summary, these studies clearly demonstrate that sex modulates neural control of the cardiovascular system at rest and in response to exercise, however the mechanistic basis for these sex based differences are still poorly understood and further investigation in this area is necessary.

Estrogen has been advanced as a putative mechanism for sex differences in cardiovascular regulation, as circulating levels are much higher in young females than in males or post-menopausal females (Fadel et al., 2003, 2004; Wenner et al., 2021). Indeed, autonomic nervous system activity and vascular resistance have been shown to be regulated, in part, by

estrogen (Mendelsohn & Karas, 1999; Vongpatanasin et al., 2001). In contrast to young females, a positive relationship between MSNA and total peripheral resistance does exist in post-menopausal females, suggesting the lack of association in young females may be due to higher levels of circulating sex hormones, particularly estrogen (Hart et al., 2011). Resting (Carter et al., 2013; Minson et al., 2000) and exercising (Carter et al., 2013; Ettinger et al., 1998; Jarvis et al., 2011; Limberg et al., 2010) MSNA was also shown to fluctuate throughout the menstrual cycle, and these fluctuations in MSNA appear to be related to circulating sex hormone levels (Carter et al., 2013; Minson et al., 2000). Moreover, while resting MSNA differs throughout the menstrual cycle, mean arterial pressure did not change, further supporting a role of circulating sex hormones in sympathetic neurovascular transduction (Carter et al., 2013; Minson et al., 2000).

Estrogen can act directly on endothelial and smooth muscle cells via α- estrogen receptors and β-estrogen receptors, as well as indirectly via G-protein coupled estrogen receptors, all of which are found in the male and female human vasculature (Menazza & Murphy, 2016; Mendelsohn & Karas, 1999; Miller & Duckles, 2008). Estrogen has both short- and long-term vascular effects (Knowlton & Lee, 2012). In the short-term, estrogen binding to α-estrogen receptors located on the endothelium stimulates the production of nitric oxide, causing vasodilation (Knowlton & Lee, 2012). Indeed, in endothelial cell cultures, estrogen administration caused a rapid increase in eNOS activity (Caulin-Glaser et al., 1997; Lantin-Hermoso et al., 1997). Likewise, when treated with estrogen, nNOS activity and NO production are increased in isolated human umbilical cord cells (Lekontseva et al., 2011). NOS activity was also augmented in pregnant guinea pigs, and the difference was abolished in the presence of Tamoxifen, an estrogen receptor antagonist (Weiner, Knowles, et al., 1994; Weiner, Lizasoain, et al., 1994). In the long-term, estrogen has also been suggested to indirectly influence

cardiovascular function by modulating NOS expression and subsequently NO bioavailability (Ceccatelli et al., 1996; Chambliss & Shaul, 2002; Fadel et al., 2003; García-Durán et al., 1999; Lekontseva et al., 2011; Pelligrino et al., 1998; Sudhir et al., 1996). Ovariectomized rats demonstrate reduced skeletal muscle nNOS expression compared to non-ovariectomized female rats, which can be reversed with exogenous estradiol replacement (Fadel et al., 2003). Estrogen replacement therapy has also been shown to increase plasma NO and NO biological end-products (Best et al., 1998; Rosselli et al., 1995), as well as neutrophil nNOS expression (García-Durán et al., 1999). These data are corroborated in humans, where the resting vasoconstriction response to NOS inhibition is larger in pre-menopausal females, compared to post-menopausal females and males (Majmudar et al., 2000). Further, after 14 days of estrogen replacement therapy, post-menopausal females exhibited a similar vasoconstriction response to NOS inhibition as pre-menopausal females (Majmudar et al., 2000). The vascular response to the NO donor glyceryl trinitrate was not different between males, pre-menopausal females, and post-menopausal females, or following estrogen replacement therapy, suggesting that the differences in results observed are due to higher NO bioavailability as opposed to increased responsiveness to NO (Majmudar et al., 2000). Similarly, 8 weeks of estrogen supplementation augmented the vasoconstriction response to NOS inhibition in peri-menopausal females (Sudhir et al., 1996).

Estrogen may also play a role in the modulation of resting sympathetic vasoconstrictor responsiveness. In male rat experimental models, estrogen treatment either increased (Colucci et al., 1982) or decreased (Kondo et al., 1980; Shan et al., 1994; Yen & Lau, 2004) sympathetic vasoconstrictor responsiveness. Injection of estradiol decreased the pressor response to NE in conscious male rats (Kondo et al., 1980; Shan et al., 1994), and pre-treatment with estradiol reduced the vasoconstriction response to NE in rat tail artery preparations from male rats (Shan

et al., 1994). Similarly, seven weeks of estrogen treatment decreased sympathetic vasoconstrictor responsiveness to phenylephrine in aortic ring preparations from spontaneously hypertensive male rats (Yen & Lau, 2004). Contrarily, the vasoconstriction response to NE in mesenteric artery preparations from male rats was increased following low-dose estrogen treatment (Colucci et al., 1982). Evidence in female experimental models is also contradictory. Resting MAP (Fadel et al., 2004) and systolic and diastolic blood pressures (Sudhir et al., 1997) were lower following estrogen supplementation in human females. The vasoconstriction response to phenylephrine in mesenteric artery preparations from ovariectomized female rats was blunted following four weeks of estrogen treatment (Zhang & Davidge, 1999). Similarly, eight weeks of estrogen supplementation blunted the forearm vasoconstriction response to NE infusion in perimenopausal human females (Sudhir et al., 1997). Conversely, in post-menopausal females, four weeks of estrogen replacement therapy did not alter the vasoconstriction response (assessed by muscle oxygenation) to lower body negative pressure (Fadel et al., 2004). At rest, sympathetic vasoconstrictor responsiveness was also similar between ovary-intact and ovariectomized rats, with or without chronic estrogen replacement (Fadel et al., 2003). In summary, while some evidence suggests that estrogen is involved in the regulation of resting sympathetic vasoconstrictor responsiveness, its role has not been firmly established.

Circulating estrogen levels may also influence the cardiovascular response to exercise. Females exhibit a blunted MSNA response to isometric handgrip exercise compared to males (Ettinger et al., 1996; Jarvis et al., 2011). The MSNA and blood pressure responses to isometric handgrip exercise were larger in post-menopausal females than in young females, and chronic estrogen replacement therapy reduced both MSNA and blood pressure responses to exercise in post-menopausal females (Wenner et al., 2021). In young females, the vasoconstriction response

to clonidine, an α_2-adrenoreceptor agonist, was reduced during light intensity rhythmic handgrip exercise during the early luteal (high estrogen) phase of the menstrual cycle compared to the early follicular (low estrogen) phase (Limberg et al., 2010). Sympatholysis is also reduced following ovariectomy in female rats, lowering estrogen bioavailability, which can be reversed with estrogen supplementation (Fadel et al., 2003). Further, estrogen supplementation abolished differences in contraction-mediated inhibition of sympathetic vasoconstriction in premenopausal compared to post-menopausal human females (Fadel et al., 2004). Given that females have higher estrogen bioavailability than males, it has been suggested that females may also have a greater ability to blunt sympathetic vasoconstriction compared to males. Indeed, our laboratory has reported that sympatholysis was enhanced in female compared to male rats (Cooper et al., 2021; Just & DeLorey, 2017). In an effort to explain why sympatholysis is higher in female rats, our laboratory has investigated whether NO and/or β-adrenoreceptors contribute to the enhanced sympatholysis in females (Cooper et al., 2021; Just & DeLorey, 2017). While NOS blockade eliminated the statistical difference between males and females, sympatholysis remained greater in female rats (Just & DeLorey, 2017), and β-adrenoreceptor blockade had no effect on the sex difference in sympatholysis (Cooper et al., 2021). Thus, estrogen may contribute to sex differences in sympatholysis via NO-dependent and/or -independent mechanisms.

Exercise Training and Sympathetic Vasoconstriction

Chronic exercise training induces a variety of cardiovascular adaptations, including reduced blood pressure and enhanced vascular control at rest and during exercise (Thijssen et al., 2009). Chronic exercise training may also result in an attenuated sympathetic response to physiological stress, such as exercise (Sothmann et al., 1996). Indeed, following exercise training, sympathetic drive during exercise at a given absolute intensity seems to be decreased in

the trained muscle (Fisher & White, 1999; Ray, 1999; Sinoway et al., 1996; Somers et al., 1992; Winder et al., 1978). Additionally, exercise training may alter vascular responsiveness. Exercise training has been shown to decrease (Delp et al., 1993; Spier et al., 1999; Wiegman et al., 1981), increase (Lash, 1998; McAllister & Laughlin, 1997), or have no effect (Donato et al., 2007; Jasperse & Laughlin, 1999; Sun et al., 1994) on sensitivity to NE in *in vitro* animal experimental models. However, results obtained from *in vitro* experimental models may not reflect integrated whole-body vascular control, and measurements during muscle contraction are not possible. Data from our laboratory in *in vivo* anesthetized rats indicates that the vasoconstriction response to lumbar sympathetic chain stimulation is increased in an exercise training intensity dependent manner at rest (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2012, 2013a, 2013b; Just & DeLorey, 2016). Our laboratory and others have shown that sympatholysis was augmented following exercise training in rats (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b; Just & DeLorey, 2016; Mizuno et al., 2014). One hour of cycle exercise training three to four days per week for eight weeks also augmented sympatholysis in healthy, middle-aged adults (Mortensen et al., 2014). Evidence from our laboratory in an *in vivo* experimental model suggests that this is due in part to an exercise training-induced increase in NO-mediated inhibition of sympathetic vasoconstriction (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b; Just et al., 2016; Just & DeLorey, 2016). Non-selective NOS inhibition blunted sympatholysis in exercise trained but not sedentary rats, indicating that exercise training increased NO-mediated sympatholysis (Jendzjowsky & DeLorey, 2013b). In a subsequent study, selective nNOS inhibition also increased sympathetic vasoconstrictor responsiveness during muscle contraction to a greater extent in exercise trained compared to sedentary rats (Jendzjowsky et al., 2014). The relative contribution of NO derived from nNOS to the inhibition

of sympathetic vasoconstriction during muscle contraction was also greater in exercise trained compared to sedentary rats (Jendzjowsky et al., 2014). Taken together, these findings suggest that exercise training mediated improvements in NO-dependent sympatholysis are mainly attributable to improved blunting of sympathetic vasoconstriction by NO derived from nNOS (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b). This is corroborated by data in arterial preparations where, following exercise training, vascular responsiveness to an exogenous sympathetic agonist was increased in the presence of NOS blockade (Delp et al., 1993; Donato et al., 2007; Spier et al., 1999). Nitric oxide synthase (NOS) expression and NO bioavailability have also been shown to be increased following exercise training (Blanco-Rivero et al., 2013; Gomes et al., 2008; Jendzjowsky et al., 2014; Laughlin et al., 2003; Maeda et al., 2001; McAllister et al., 2005; Song et al., 2009; Spier et al., 2004). However, exercise training has also been shown to have no effect on NOS expression and NO bioavailability (Frandsen et al., 2000; Harris et al., 2008; McConell et al., 2007; Perez et al., 2002). Though not conclusive, current evidence suggests that increases in NO bioavailability as a result of increased eNOS and nNOS protein expression and activity may play a role in exercise training-related enhancement of sympatholysis. To our knowledge, the effect of estrogen bioavailability on cardiovascular adaptations to chronic exercise training has not been investigated.

Purpose

The overall purpose of this study was to examine the role of estrogen bioavailability in the NO-mediated regulation of sympathetic vasoconstriction in resting and contracting skeletal muscle of sedentary and exercise-trained female rats. Specifically, the purpose of this study was to investigate: 1) whether a chronic decrease in estrogen bioavailability via surgical removal of

the ovaries would reduce contraction mediated inhibition of sympathetic vasoconstriction in female rats; 2) the role of NO derived from endothelial and neuronal NOS in the regulation of sympathetic vasoconstriction in ovary intact and ovariectomized female rats; and 3) the effect of exercise training on the regulation of sympathetic vasoconstriction in ovary intact and ovariectomized female rats.

Hypotheses

I hypothesized that: 1) surgical removal of the ovaries (ovariectomy) would impair inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle of sedentary rats; 2) nNOS-mediated inhibition of sympathetic vasoconstriction would be blunted in ovariectomized rats; 3) aerobic exercise training would enhance the inhibition of sympathetic vasoconstriction at rest and during muscle contraction in ovariectomized rats, but have no effect in ovary-intact rats; and 4) aerobic exercise training would enhance the inhibition of sympathetic vasoconstriction in ovariectomized rats in an nNOS-dependent manner.

Significance

While cardiovascular disease risk is relatively low in young females compared to males, risk in females rises rapidly following menopause, matching or exceeding that of males (Appelman et al., 2015; Miller et al., 2013). Cross-sectional data shows that this increased risk coincides with decreased circulating estrogen levels, suggesting that a decline in estrogen bioavailability may play a role in the progression of cardiovascular diseases characterized by elevated sympathetic nervous system activity, particularly hypertension (Appelman et al., 2015; Chambliss & Shaul, 2002; García-Durán et al., 1999; Knowlton & Lee, 2012; Lantin-Hermoso et

al., 1997; Mendelsohn & Karas, 1999; Miller et al., 2013; Sudhir et al., 1997). Estrogen has been shown to modulate both NOS activity (Chambliss & Shaul, 2002; Lantin-Hermoso et al., 1997; Majmudar et al., 2000; Sudhir et al., 1996) and expression (Ceccatelli et al., 1996; Chambliss & Shaul, 2002; Fadel et al., 2003; García-Durán et al., 1999; Laughlin et al., 2003; Lekontseva et al., 2011; Pelligrino et al., 1998; Sasser et al., 2015; Sudhir et al., 1996) and NO is known to inhibit sympathetic vasoconstriction (Chavoshan et al., 2002; Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b, 2013d; Mizuno et al., 2014; Thomas, 2011). Thus, reduced NO-mediated inhibition of sympathetic vasoconstriction as a result of decreased estrogen bioavailability may contribute to chronic increases in blood pressure.

Exercise training may reverse or mitigate the cardiovascular effects of decreased circulating estrogen levels. In estrogen deficient humans and rats, the inhibition of sympathetic vasoconstriction was impaired (Fadel et al., 2003, 2004). nNOS expression was reduced in estrogen deficient rats, suggesting that the blunted inhibition of sympathetic vasoconstriction may be due to decreased NO bioavailability (Fadel et al., 2003). Our laboratory has shown that exercise training upregulates nNOS expression and NO-mediated inhibition of sympathetic vasoconstriction (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b). Thus, exercise training may increase nNOS expression and NO bioavailability in estrogen deficient females, potentially restoring the impaired inhibition of sympathetic vasoconstriction, though this has yet to be studied. If this is the case, exercise training could be used as a low-risk alternative to estrogen therapy to reduce cardiovascular disease risk in post-menopausal females. Unfortunately, cardiovascular adaptations to chronic exercise training in females have received little research attention, despite females appearing to respond differently than males to a similar training stimulus. For example, the response of both absolute and relative maximal oxygen

consumption to a similar exercise training stimulus is greater in males than females (Diaz-Canestro & Montero, 2019). Exercise training has also been shown to enhance sympatholysis in a NO-dependent manner in male rats (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b; Mizuno et al., 2014), but not female rats (Cooper et al., 2021). Beyond these studies, cardiovascular adaptations to exercise training are largely unexplored in females, and the extent to which these adaptations differ in females is unknown.

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Chapter 2: Estrogen, Exercise Training, and Nitric Oxide-Mediated Sympatholysis

Introduction

Nitric oxide (NO) has been shown to inhibit sympathetic vasoconstriction at rest and during muscle contraction (Chavoshan et al., 2002; Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b, 2013d; Thomas & Victor, 1998). Our laboratory has shown that sympatholysis is enhanced in female rats compared with male rats (Cooper et al., 2021; Just & DeLorey, 2017). When nitric oxide synthase (NOS) was inhibited, the difference in sympatholysis between male and female rats was abolished, suggesting that the enhanced sympatholysis observed in females was mediated by NO (Just & DeLorey, 2017). Estrogen has been shown to upregulate NOS expression, indicating that estrogen may contribute to the regulation of NO bioavailability (Ceccatelli et al., 1996; Chambliss & Shaul, 2002; Fadel et al., 2003; García-Durán et al., 1999; Laughlin et al., 2003; Lekontseva et al., 2011; Pelligrino et al., 1998; Sasser et al., 2015; Sudhir et al., 1996), suggesting that the difference in NO-mediated inhibition of sympathetic vasoconstriction between males and females may be attributable to estrogen. Indeed, sympatholysis is reduced in ovariectomized female rats relative to ovary-intact rats, and this difference is abolished with chronic estrogen supplementation (Fadel et al., 2003). Sympatholysis was also blunted in post-menopausal females compared to young females, and estrogen supplementation eliminated the difference between pre- and post-menopausal females (Fadel et al., 2004). Similarly, the blood pressure and MSNA responses to isometric handgrip exercise were larger in post-menopausal females than in young females (Wenner et al., 2021). Estrogen supplementation also blunted the MSNA and blood pressure responses to isometric handgrip exercise in post-menopausal females (Wenner et al., 2021). NOS is expressed in neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) isoforms and NO derived from both

eNOS and nNOS has been shown to inhibit vasoconstriction in resting and contracting skeletal muscle in male rats (Jendzjowsky & DeLorey, 2013d). The reduced sympatholysis reported in estrogen-deficient rats and humans may be due to impaired nNOS function (Fadel et al., 2003, 2004), as estrogen-deficient rats had lower nNOS expression than control rats (Fadel et al., 2003). However, the effect of estrogen bioavailability on NOS isoform specific mediated inhibition of sympathetic vasoconstriction has not been studied.

Chronic exercise training results in a variety of adaptations to the cardiovascular system including enhanced vascular control at rest and during exercise (Hellsten & Nyberg, 2016; Thijssen et al., 2009). Previous research suggests that males and females may respond differently to chronic exercise training (Diaz-Canestro & Montero, 2019; Howden et al., 2015). Indeed, exercise training has been shown to increase sympatholysis in male, but not female, rats (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b; Mizuno et al., 2014). In male rats, skeletal muscle nNOS expression and nNOS-mediated sympatholysis were enhanced following exercise training (Jendzjowsky et al., 2014) suggesting that exercise training may modulate NO bioavailability and NO-dependent sympatholysis (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b). However, the exercise training paradigm that enhanced sympatholysis in male rats did not enhance sympatholysis in female rats (Cooper et al., 2021; Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b). The lack of a training effect in female rats may indicate that females do not respond to exercise training in the same manner as male rats. Indeed, in humans, the adaptation of absolute and relative maximal oxygen consumption, left ventricular mass, and mean wall thickness to a similar exercise training stimulus was smaller in females compared with males (Diaz-Canestro & Montero, 2019; Howden et al., 2015). It is also plausible that exercise training may not upregulate NOS expression in female rats. Just et al reported that sympatholysis

was greater in female compared with male rats and that NOS blockade abolished the difference in sympatholysis (Just & DeLorey, 2017). If female rats possess higher levels of NOS enzymes and/or NO bioavailability than male rats due to higher levels of estrogen bioavailability (Fadel et al., 2003), exercise training may not result in a further increase in NO-dependent sympatholysis in female rats with "normal" estrogen bioavailability. However, in post-menopausal females or estrogen-deficient rats, where estrogen bioavailability is much lower, exercise training may reverse the estrogen-related loss of nNOS expression and subsequent reduction in sympatholysis. No studies have examined whether exercise training can restore NO-mediated sympatholysis in estrogen-deficient female rats.

Therefore, the purpose of this study was to investigate the effect of estrogen bioavailability on NO-mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle of sedentary and exercise trained female rats. It was hypothesized that surgical removal of the ovaries would blunt the inhibition of sympathetic vasoconstriction. Specifically, nNOS-mediated inhibition of sympathetic vasoconstriction would be impaired in ovariectomized rats. We also hypothesized that exercise training would enhance the inhibition of sympathetic vasoconstriction in ovariectomized rats in an nNOS-dependent manner, but would have no effect in ovary-intact rats.

Methods

All experiments were conducted in accordance with the guidelines of the Canadian Council for Animal Care and approved by the University of Alberta Animal Care and Use Committee.

Animals and Animal Care

Female Sprague-Dawley rats (n=42) were housed in an environmentally controlled room (22-24°C, 40-70% humidity) with a 12:12h light-dark cycle. Water and rat chow were provided ad libitum (Lab diet 5001, PMI Nutrition, Brentwood, MO). Animals were obtained from the institutional breeding colony at 8 weeks of age.

Endurance Exercise Training

All rats walked on the treadmill 10 minutes per day for 5 days at 10 $m \cdot min^{-1}$ at a 0% grade to be habituated to the lab environment and to exercise. The rats were then randomly assigned to sedentary time control ovary intact (SOI, n=10), sedentary time control ovariectomized (SOVX, n=9), exercise trained ovary intact (TOI, n=13), or exercise trained ovariectomized (TOVX, n=10) groups. The rats assigned to ovariectomy groups (SOVX and TOVX) were then anesthetized by inhalation of isoflurane (3.5% in balance oxygen). An abdominal incision was made and both ovaries were removed after ligation of the uterus immediately distal to each ovary and the abdominal incision was sutured closed. Anti-bacterial/fungal cream was applied to the abdominal incision post-operatively (Hibitane, Canada). Intramuscular Metacam (Boehringer Ingelheim Vetmedica Inc., Duluth, GA) injections (2-5 $mg \cdot kg^{-1}$) were given immediately following the ovariectomy and every 24 hours for three days for pain management. Sedentary rats (SOI and SOVX) were handled and weighed daily. Exercise trained rats (TOI and TOVX) completed 600 m of treadmill running at 40 $m \cdot min^{-1}$, 5% grade, 5 days per week for 10 weeks. This exercise training paradigm was chosen as our lab has previously demonstrated that it increases heart mass, heart-to-body mass ratio, soleus citrate synthase activity, endothelium-dependent vasodilation, and contraction-mediated inhibition of sympathetic vasoconstriction in male rats (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey,

2012, 2013a, 2013b), as well as heart mass, heart-to-body mass ratio, and heart mass-to-tibia length ratio in female rats (Cooper et al., 2021).

Instrumentation

Approximately 24 hours after the completion of the exercise protocol or sedentary time control, rats were anesthetized and instrumented for data collection. Rats were anesthetized by inhalation of isoflurane (3.5% in balance oxygen). The right jugular vein was cannulated for continuous intravenous administration of alpha-chloralose (8-16 $mg \cdot kg^{-1} \cdot hr^{-1}$) and urethane (50-100 $mg \cdot kg^{-1} \cdot hr^{-1}$) to maintain anesthesia. The right carotid artery was cannulated and fitted to a pressure transducer (Abbott, North Chicago, IL) for the measurement of arterial blood pressure (ABP). Heart rate (HR) was derived from the arterial pressure waveform. Depth of anesthesia was assessed by the stability of ABP and HR, in addition to the absence of a withdrawal reflex to a painful stimulus (i.e., paw pinch) over a 20-minute stabilization period. A tracheotomy was performed to allow mechanical ventilation (small animal ventilator Model 683**,** Harvard Apparatus, Holliston, MA) with 30% O_2 in balance N_2 to maintain arterial blood gases and acid-base status (arterial partial pressures of oxygen and carbon dioxide 90-100 and 39-41 mmHg, respectively; pH 7.39-7.42). Core temperature was monitored by rectal probe and maintained at 37ºC by an external heating pad (TCAT-2, Physitemp, Clifton, NJ).

Blood flow to the right leg was assessed by a transit-time flow probe (7V; Transonic Systems, Ithaca, NY) positioned in the right femoral artery and connected to a flow-meter (T106, Transonic Systems, Ithaca, NY). The left femoral vein was cannulated for the delivery of pharmacology. Femoral vein and carotid artery cannulae were flushed regularly with heparinized saline to maintain patency. Blood clotting was prevented by continuous infusion of heparinized

saline (4 units·mL^{-1}; 2.5-3 mL·hour^{-1}) through the left femoral vein via a syringe pump (K.D. Scientific, Holliston, MA).

Hind-limb Muscle Contractions

The right sciatic nerve was exposed and fitted with a cuff electrode for electrical stimulation. Subsequently, the right triceps surae muscle group was dissected free of all skin and connective tissue, then attached via the calcaneal tendon to a force transducer (FT03, Grass Technologies, Warwick, RI). Electrical stimulation of the right sciatic nerve using Chart 7.2 Software (AD Instruments, Colorado Springs, CO) was performed to produce hind-limb contractions. The triceps surae muscle group was stimulated with 25 1 ms impulses delivered at 100 Hz at 10x motor threshold (MT) for the determination of maximal contractile force (MCF). Nerve stimulation was repeated while progressively lengthening the muscle to determine the optimal muscle length for tension development. Optimal muscle length is achieved when a plateau in force production is observed. The right triceps surae muscle group was stimulated (40 Hz, 0.1 ms pulses in 250 ms trains, at a rate of 60 trains min^{-1} at ∼3× MT) to elicit rhythmic contractions at 60% MCF.

Lumbar Sympathetic Chain Stimulation

A laparotomy was performed, and the aorta and vena cava temporarily retracted to place a bipolar silver-wire stimulating electrode on the lumbar sympathetic chain at the L3/L4 level. The electrode was embedded and electrically isolated in a rapidly curing non-toxic silicone elastomer (Kwiksil, World Precision Instruments, Sarasota, FL). Constant current stimulation was delivered through the electrode using an isolated stimulator (DS3, Digitimer, Welwyn Garden City, UK) at 2 and 5 Hz. These frequencies were selected as they reflect levels of

sympathetic nerve activity at rest and during conditions of high sympathetic activity, such as exercise (Bradley et al., 2003; Hudson et al., 2011; Johnson et al., 2001; Macefield et al., 1994).

Experimental Protocol

Following surgical instrumentation, all rats underwent a 20-minute stabilization period. Subsequently, the vasoconstrictor response to lumbar sympathetic chain stimulation (1 minute of 1 ms, 1 mA pulses, delivered at 2 and 5 Hz, in random order) was measured at rest and during skeletal muscle contraction at 60% MCF in control, selective nNOS inhibition (*S*-methly-$_L$-thiocitrulline, SMTC; 0.6 mg·kg^{-1}, IV), and non-selective NOS inhibition (N_ω-nitro-L-arginine methyl ester, L-NAME; 10 mg·kg^{-1}, IV) conditions. This dose of SMTC has been employed previously in our laboratory to selectively inhibit nNOS at rest and during muscle contraction in male rats (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013d). The same or a lower dose of L-NAME has also been used in our laboratory and others to non-selectively block NOS in male and female rats at rest and during muscle contraction (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b, 2013d; Just & DeLorey, 2017; Mizuno et al., 2014; Thomas & Victor, 1998). Resting sympathetic stimulations were separated by ~2 minutes of recovery to allow restoration of baseline hemodynamic values. Bouts of muscle contraction were 8 minutes in duration, and sympathetic stimulation at 2Hz and 5Hz was delivered at 3 and 6 minutes following the onset of muscle contraction, in random order. Control, SMTC, and L-NAME conditions were separated by ~60 minutes of recovery. Our lab has previously demonstrated that neither the cardiovascular response to sympathetic stimulation, nor muscle force production, are altered over time when bouts of contraction are repeated in this manner (Jendzjowsky & DeLorey, 2013d). Rats were then euthanized by anesthetic overdose and the heart, tibia, and

right triceps surae muscle group were dissected and weighed or measured. The exercise training and surgical protocols and data collection were performed by Timothy P. Just.

Pharmacology

All drugs were obtained from Sigma-Aldrich (Oakville, ON, Canada) and dissolved in 0.9% physiological saline.

Data Analysis

Data were recorded using Chart software (AD Instruments, Colorado Springs, CO). ABP and leg blood flow (LBF, $mL \cdot min^{-1}$) were sampled at 100 Hz. HR and femoral vascular conductance ($FVC = FBF \cdot MAP^{-1}$, $mL \cdot min^{-1} \cdot mmHg^{-1}$) were then calculated. Muscle force production was measured continuously, and peak contractile force for each contraction bout was determined. Average peak contractile force was calculated for contractions from minutes 3 to 7 of each muscular contraction bout (time period encompassing sympathetic stimulations) to compare force production between groups and drug conditions.

The change in HR, MAP, FBF, and FVC in response to sympathetic stimulation were calculated both as an absolute change and as a relative change from the one minute preceding sympathetic stimulation in the control, SMTC, and L-NAME conditions. The magnitude of sympatholysis was calculated as the difference between the percentage change in FVC in response to sympathetic stimulation at rest, and the percentage change in FVC in response to sympathetic stimulation during muscle contraction, within each drug condition. Percentage change in FVC is the accepted metric to measure the magnitude of sympathetic vasoconstriction as the percentage change in FVC accurately reflects changes in resistance vessel radius across a range of baseline conductance levels (Buckwalter & Clifford, 2001; Thomas & Segal, 2004). All data is expressed as mean ± standard deviation.

Statistical Analysis

A two-way repeated measures ANOVA (group x drug condition) was used to assess the effects of exercise training status, estrogen status, and NOS inhibition on baseline hemodynamics, exercise hyperemia, sympathetic vasoconstrictor responsiveness, and the magnitude of sympatholysis. The effect of exercise training status and estrogen bioavailability on body mass, muscle mass, heart mass, heart mass-to-body mass ration, tibia length, and heart mass-to-tibia length ratio was determined by one-way ANOVA. If significant F-ratios were found, Student-Newman-Keuls post-hoc analysis was performed. A p-value less than 0.05 was considered statistically significant.

Results

Animal Characteristics

Body mass was higher ($p<0.05$) in ovariectomized rats (SOVX and TOVX) than in ovary intact rats (SOI and TOI) (Table 1). Body mass was not different ($p>0.05$) between exercise trained and sedentary rats (Table 1). Exercise trained rats (TOI and TOVX) had a larger ($p<0.05$) heart mass than sedentary rats (SOI and SOVX) (Table 1). Heart mass was greater ($p<0.05$) in the SOVX rats than the SOI rats, but was not different ($p>0.05$) between TOI and TOVX rats (Table 1). Exercise training increased ($p<0.05$) heart mass to body mass ratio (HM:BM) in ovary intact rats, but did not alter ($p>0.05$) HM:BM in ovariectomized rats (Table 1). HM:BM ratio was smaller (main effect; $p<0.05$) in ovariectomized rats compared to ovary intact rats (Table 1). Similar to HM:BM, heart mass to tibia length ratio (HM:TL) was increased ($p<0.05$) following exercise training in ovary intact rats, but not ($p>0.05$) in ovariectomized rats, (Table 1). HM:TL was increased ($p<0.05$) in SOVX rats compared with SOI rats, whereas HM:TL was not different ($p>0.05$) between TOVX and TOI rats (Table 1). Exercise training increased ($p<0.05$) soleus and

medial gastrocnemius muscle mass in both ovary intact and ovariectomized rats, as well as lateral gastrocnemius muscle mass in ovariectomized rats (Table 1).

Baseline Hemodynamics

Baseline hemodynamics were measured under anesthesia. Neither estrogen bioavailability nor exercise training altered ($p>0.05$) baseline heart rate (Table 2). In all rats, baseline heart rate was lower ($p<0.0.5$, main effect of drug condition) in the selective nNOS blockade compared with the control condition, and non-selective NOS blockade decreased ($p<0.0.5$, main effect of drug condition) baseline heart rate further (Table 2). Baseline blood pressure was unaffected ($p>0.05$) by estrogen bioavailability or exercise training (Table 2). Baseline blood pressure was increased ($p<0.05$, main effect of drug condition) in the selective nNOS blockade condition compared with the control condition, and further reduced ($p<0.05$, main effect of drug condition) in the non-selective NOS blockade condition (Table 2). There was an interaction between group and drug condition for baseline leg blood flow. Baseline leg blood flow (LBF) in the control condition was higher ($p<0.05$, group x drug condition) in TOVX compared to SOVX and SOI rats, but was not different ($p>0.05$) from TOI rats, and LBF was not different ($p>0.05$) between TOI and SOI rats. Selective nNOS blockade did not alter ($p>0.05$) LBF in any group. Following non-selective NOS blockade, resting LBF was increased ($p<0.05$) compared to the control condition in SOVX rats, and was reduced relative to the control and selective nNOS blockade conditions in TOI rats (Table 2). Baseline femoral vascular conductance (FVC) was greater ($p<0.05$, group x drug condition) in TOVX rats compared to all other groups in the control condition (Table 2). Baseline FVC was not different ($p>0.05$) between groups following selective nNOS blockade, whereas FVC was reduced ($p<0.05$, group x

drug condition) compared to the control and selective nNOS conditions after non-selective NOS blockade in both exercise trained groups, but was not altered ($p>0.05$) in sedentary rats (Table 2).

Sympathetic Vasoconstrictor Responsiveness at Rest

The mean arterial pressure, leg blood flow, and femoral vascular conductance response to lumbar sympathetic chain stimulation at 2 Hz and 5 Hz at rest and during muscle contraction in a representative rat are shown in Figure 1. The percent change in femoral vascular conductance in response to 2 Hz and 5 Hz sympathetic stimulation (sympathetic vasoconstrictor responsiveness) was not different ($p>0.05$) between ovariectomized and ovary-intact rats (Figure 2). Resting sympathetic vasoconstrictor responsiveness also was not different ($p>0.05$) between exercise-trained and sedentary rats (Figure 2). Selective nNOS blockade did not alter ($p>0.05$) resting sympathetic vasoconstrictor responsiveness to sympathetic stimulation at 2 Hz or 5 Hz (Figure 2). However, non-selective NOS blockade augmented ($p<0.05$, main effect of drug condition) sympathetic vasoconstrictor responsiveness to sympathetic stimulation delivered at both 2 Hz and 5 Hz compared with the control and selective nNOS blockade conditions (Figure 2). Changes in LBF ($mL \cdot min^{-1}$) and FVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$) in response to sympathetic stimulation delivered at 2 and 5 Hz at rest are presented in Table 3.

Sympathetic Vasoconstrictor Responsiveness During Muscle Contraction

During muscle contraction, sympathetic vasoconstrictor responsiveness to stimulation of the lumbar sympathetic chain at 2 and 5 Hz was not different ($p>0.05$) between ovariectomized and ovary-intact rats (Figure 3). Sympathetic vasoconstrictor responsiveness was also not different ($p>0.05$) between exercise trained and sedentary rats at both stimulation frequencies (Figure 3). At the 2 Hz stimulation frequency, selective nNOS blockade did not change ($p>0.05$) sympathetic vasoconstrictor responsiveness (Figure 3). However, at 2 Hz, non-selective NOS

blockade increased ($p<0.05$, main effect of drug condition) sympathetic vasoconstrictor responsiveness compared with the control and selective nNOS blockade conditions (Figure 3). At the 5 Hz stimulation frequency, sympathetic vasoconstrictor responsiveness was augmented ($p<0.05$, main effect of drug) by selective nNOS blockade, and increased further ($p<0.05$, main effect of drug) by non-selective NOS blockade (Figure 3). Changes in LBF ($mL \cdot min^{-1}$) and FVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$) in response to sympathetic stimulation delivered at 2 and 5 Hz during muscle contraction are presented in Table 4.

There was no significant effect ($p>0.05$) of estrogen bioavailability or exercise training on the magnitude of sympatholysis at 2 Hz or 5 Hz (Figure 4). At 2 Hz, the magnitude of sympatholysis was not different ($p>0.05$) between drug conditions (Figure 4). Selective nNOS blockade reduced ($p<0.05$, main effect of drug) sympatholysis at 5 Hz compared with the control condition, and non-selective NOS blockade further impaired ($p<0.05$, main effect of drug condition) sympatholysis at 5 Hz (Figure 4). The interaction between group and drug condition was not significant ($p>0.05$); however, visual inspection of the data suggested within group differences for the effect of selective nNOS and non-selective NOS blockade. Thus, to test the specific hypothesis that nNOS-mediated sympatholysis would be enhanced in exercise-trained ovariectomized rats, post hoc analyses were performed. Post-hoc testing revealed that sympatholysis was blunted ($p<0.05$) following selective nNOS blockade in SOI, TOI and TOVX groups. Sympatholysis was not further reduced ($p>0.05$) by non-selective NOS blockade in SOI, TOI, and TOVX groups. In SOVX rats, selective nNOS blockade did not change ($p>0.05$) sympatholysis; however, sympatholysis was blunted ($p<0.05$) in the non-selective NOS blockade condition compared with the control and selective nNOS blockade conditions.

Hyperemic Response to Muscle Contraction

The heart rate response to muscle contraction was unaffected ($p>0.05$) by estrogen bioavailability and exercise training (Table 5). The increase in heart rate in response to muscle contraction was larger ($p<0.05$, main effect of drug condition) following selective nNOS blockade and non-selective NOS blockade compared with the control condition (Table 5). There was a significant interaction between group and drug condition for the blood pressure response to muscle contraction. Ovariectomized rats had a smaller ($p<0.05$, group x drug condition) blood pressure response to muscle contraction than ovary-intact rats in the control condition (Table 5). In ovary-intact rats, the blood pressure response to muscle contraction was decreased ($p<0.05$, group x drug condition) by selective nNOS blockade and non-selective NOS blockade relative to the control condition, regardless of exercise training status (Table 5). The blood pressure response to muscle contraction was decreased ($p<0.05$, group x drug condition) by non-selective NOS blockade, compared to the control and selective nNOS blockade conditions, in TOVX rats but not SOVX rats (Table 5). The increase in LBF in response to muscle contraction was not different ($p>0.05$) between ovariectomized and ovary-intact rats (Table 5). The LBF response to muscle contraction was greater ($p<0.05$, main effect of group) in exercise-trained rats than sedentary rats (Table 5). Compared to the control condition, selective nNOS blockade and non-selective NOS blockade increased ($p<0.05$, main effect of drug condition) the LBF response to muscle contraction to a similar extent in all rats (Table 5). Exercise-trained rats had a larger ($p<0.05$, main effect of group) increase in FVC in response to muscle contraction compared with sedentary rats (Table 5). TOVX rats also had a larger ($p<0.05$, main effect of group) increase in FVC in response to muscle contraction compared to TOI rats (Table 5). Compared to the control condition, the increase in FVC in response to muscle contraction was larger ($p<0.05$, main effect

of drug condition) in both the selective nNOS blockade and non-selective NOS blockade conditions (Table 5).

Muscle force production was not different ($p>0.05$) between or within groups in control (SOI: 515 ± 87 g; SOVX: 492 ± 41 g; TOI: 544 ± 98 g; TOVX: 513 ± 67 g), selective nNOS blockade (SOI: 523 ± 83 g; SOVX: 527 ± 55 g; TOI: 552 ± 97 g; TOVX: 529 ± 74 g), and non-selective NOS blockade (SOI: 513 ± 55 g; SOVX: 504 ± 50 g; TOI: 536 ± 85 g; TOVX: 532 ± 61 g) conditions.

Table 1. Animal characteristics.

Group	Body mass (g)	Heart mass (g)	Tibia length (mm)	Heart mass (mg) : body mass (g)	Heart mass (mg) : tibia length (mm)	Muscle mass (g)		
						Soleus	Lateral gastrocnemius	Medial gastrocnemius
SOI	353 ± 31	1.06 ± 0.09	41.5 ± 0.9	3.03 ± 0.34	25.63 ± 2.15	0.18 ± 0.02	0.63 ± 0.06	1.12 ± 0.09
SOVX	431 ± 35*#	1.16 ± 0.11*	42.5 ± 0.5*	2.69 ± 0.20*#	27.24 ± 2.50*	0.19 ± 0.02	0.68 ± 0.12	1.16 ± 0.07
TOI	376 ± 34	1.26 ± 0.08*$	42.2 ± 0.8*	3.38 ± 0.26*	30.23 ± 1.87*$	0.22 ± 0.03*$	0.70 ± 0.06	1.27 ± 0.10*$
TOVX	451 ± 29*#	1.25 ± 0.09*$	42.7 ± 0.6*	2.79 ± 0.15*#	29.62 ± 2.30*	0.22 ± 0.02*$	0.83 ± 0.08*#$	1.46 ± 0.09*$#

All values are mean ± standard deviation. Measurements were obtained from sedentary time control ovary intact (SOI, n=10), sedentary time control ovariectomized (SOVX, n=9), exercise trained ovary intact (TOI, n=13), and exercise trained ovariectomized (TOVX, n=10) rats. * indicates a statistically significant difference from SOI. # indicates a statistically significant difference from TOI. $ indicates a statistically significant difference from SOVX. A p-value less than 0.05 was considered statistically significant.

Table 2. Baseline hemodynamics.

Group	Drug condition	Heart rate (bpm)	Arterial blood pressure (mmHg)	Leg blood flow ($mL \cdot min^{-1}$)	Femoral vascular conductance ($mL \cdot min^{-1} \cdot mmHg^{-1}$)
SOI	Control	393 ± 35	102 ± 6	3.2 ± 0.6$^{\&}$	0.031 ± 0.005$^{\&}$
	SMTC	369 ± 30*	109 ± 7*	3.8 ± 1.1	0.035 ± 0.011
	L-NAME	339 ± 36*$^{\#}$	131 ± 11*$^{\#}$	3.9 ± 1.0	0.030 ± 0.008
SOVX	Control	379 ± 25	98 ± 10	3.4 ± 0.6$^{\&}$	0.034 ± 0.005$^{\&}$
	SMTC	356 ± 25*	106 ± 11*	4.0 ± 0.9	0.038 ± 0.009
	L-NAME	322 ± 28*$^{\#}$	128 ± 8*$^{\#}$	4.3 ± 1.0†	0.033 ± 0.008
TOI	Control	364 ± 31	108 ± 11	4.2 ± 0.8	0.039 ± 0.008$^{\&}$
	SMTC	349 ± 27	117 ± 11*	4.4 ± 1.4	0.037 ± 0.010
	L-NAME	322 ± 31*$^{\#}$	135 ± 14*$^{\#}$	3.4 ± 1.1†‡	0.025 ± 0.008†‡
TOVX	Control	343 ± 28$^{\$}$	101 ± 14	4.7 ± 1.3	0.047 ± 0.013
	SMTC	337 ± 31*$^{\$}$	110 ± 13*	4.8 ± 1.4	0.043 ± 0.010
	L-NAME	325 ± 24*$^{\#\$}$	126 ± 14*$^{\#}$	4.1 ± 1.6	0.032 ± 0.011†‡

All values are mean ± standard deviation. Measurements were obtained from sedentary time control ovary intact (SOI, n=10), sedentary time control ovariectomized (SOVX, n=9), exercise trained ovary intact (TOI, n=13), and exercise trained ovariectomized (TOVX, n=10) rats. * indicates a statistically significant difference from the Control condition (main effect of drug condition). # indicates a statistically significant difference from the SMTC condition (main effect of drug condition). $ indicates a significant difference from SOI (main effect of group). % indicates a significant difference from SOVX (main effect of group). † indicates a statistically significant difference from the control condition within a group. ‡ indicates a statistically significant difference from the SMTC condition within a group. & indicates a statistically significant difference from TOVX within drug condition. A p-value less than 0.05 was considered statistically significant.

Table 3. Absolute change in leg blood flow and femoral vascular conductance in response to resting sympathetic stimulation at 2 Hz and 5 Hz.

Group	Drug condition	2 Hz		5 Hz	
		Leg blood flow ($mL \cdot min^{-1}$)	Femoral vascular conductance ($mL \cdot min^{-1} \cdot mmHg^{-1}$)	Leg blood flow ($mL \cdot min^{-1}$)	Femoral vascular conductance ($mL \cdot min^{-1} \cdot mmHg^{-1}$)
SOI	Control	-1.4 ± 0.4	-0.014 ± 0.005	-1.8 ± 0.5	-0.020 ± 0.005$^{\$}$
	SMTC	-1.6 ± 0.7	-0.015 ± 0.007	-2.0 ± 0.8	-0.022 ± 0.008
	L-NAME	-1.8 ± 0.9	-0.016 ± 0.007	-2.2 ± 1.0	-0.020 ± 0.008
SOVX	Control	-1.6 ± 0.4	-0.014 ± 0.003	-1.9 ± 0.5	-0.020 ± 0.005$^{\$}$
	SMTC	-1.6 ± 0.3	-0.015 ± 0.003	-2.1 ± 0.5	-0.022 ± 0.004
	L-NAME	-1.8 ± 0.7	-0.015 ± 0.005	-2.4 ± 0.7	-0.021 ± 0.007
TOI	Control	-2.0 ± 0.5	-0.018 ± 0.005	-2.4 ± 0.5	-0.023 ± 0.005$^{\$}$
	SMTC	-1.9 ± 0.7	-0.017 ± 0.006	-2.2 ± 0.7	-0.021 ± 0.006
	L-NAME	-1.8 ± 0.7	-0.014 ± 0.006	-2.0 ± 0.7	-0.017 ± 0.006*$^{\#}$
TOVX	Control	-2.3 ± 0.6	-0.023 ± 0.007	-2.8 ± 0.8	-0.030 ± 0.008
	SMTC	-2.0 ± 0.9	-0.020 ± 0.007	-2.7 ± 1.1	-0.028 ± 0.008
	L-NAME	-2.0 ± 1.1	-0.017 ± 0.008	-2.6 ± 1.2	-0.023 ± 0.009*$^{\#}$

All values are mean ± standard deviation. Measurements were obtained from sedentary time control ovary intact (SOI, n=10), sedentary time control ovariectomized (SOVX, n=9), exercise trained ovary intact (TOI, n=13), and exercise trained ovariectomized (TOVX, n=10) rats. * indicates a statistically significant difference from the Control condition within a group. # indicates a statistically significant difference from the SMTC condition within a group. $ indicates a significant difference from TOVX within drug condition. A p-value less than 0.05 was considered statistically significant.

Table 4. Absolute change in leg blood flow and femoral vascular conductance in response to sympathetic stimulation at 2 Hz and 5 Hz during muscle contraction at 60% of maximal contraction force.

Group	Drug condition	2 Hz		5 Hz	
		Leg blood flow ($mL \cdot min^{-1}$)	Femoral vascular conductance ($mL \cdot min^{-1} \cdot mmHg^{-1}$)	Leg blood flow ($mL \cdot min^{-1}$)	Femoral vascular conductance ($mL \cdot min^{-1} \cdot mmHg^{-1}$)
SOI	Control	-0.8 ± 0.7	-0.009 ± 0.006	-1.0 ± 1.1	-0.019 ± 0.008
	SMTC	-1.0 ± 0.7	-0.012 ± 0.006*	-1.9 ± 1.2*	-0.027 ± 0.008*
	L-NAME	-1.5 ± 1.0	-0.018 ± 0.005*#	-2.6 ± 1.7*#	-0.032 ± 0.010*#
SOVX	Control	-1.5 ± 0.7	-0.014 ± 0.004	-1.7 ± 1.0	-0.025 ± 0.003†
	SMTC	-1.2 ± 1.0	-0.015 ± 0.007*	-2.4 ± 0.8*	-0.031 ± 0.004*†
	L-NAME	-2.0 ± 0.7	-0.021 ± 0.007*#	-3.7 ± 0.7*#	-0.040 ± 0.008*#†
TOI	Control	-1.8 ± 1.0	-0.015 ± 0.006	-2.6 ± 1.3	-0.030 ± 0.010$†
	SMTC	-2.0 ± 1.1	-0.018 ± 0.007*	-3.4 ± 1.8*	-0.039 ± 0.010*$†
	L-NAME	-1.8 ± 1.8	-0.022 ± 0.009*#	-4.0 ± 2.5*#	-0.042 ± 0.015*#$†
TOVX	Control	-2.1 ± 1.4	-0.020 ± 0.009$	-2.5 ± 2.1	-0.036 ± 0.015$
	SMTC	-2.0 ± 1.4	-0.023 ± 0.010*$	-3.2 ± 2.3*	-0.048 ± 0.013*$
	L-NAME	-1.9 ± 1.8	-0.027 ± 0.011*#$	-4.1 ± 2.3*#	-0.054 ± 0.008*#$

All values are mean ± standard deviation. Measurements were obtained from sedentary time control ovary intact (SOI, n=10), sedentary time control ovariectomized (SOVX, n=9), exercise trained ovary intact (TOI, n=13), and exercise trained ovariectomized (TOVX, n=10) rats. * indicates a statistically significant difference from the Control condition (main effect of drug condition). # indicates a statistically significant difference from the SMTC condition (main effect of drug condition). $ indicates a significant difference from SOI (main effect of group). † indicates a significant difference from TOVX (main effect of group). A p-value less than 0.05 was considered statistically significant.

Table 5. Absolute change in heart rate, arterial blood pressure, leg blood flow, and femoral vascular conductance in response to muscle contraction at 60% of maximal contraction force.

Group	Drug condition	Heart rate (bpm)	Arterial blood pressure (mmHg)	Leg blood flow ($mL \cdot min^{-1}$)	Femoral vascular conductance ($mL \cdot min^{-1} \cdot mmHg^{-1}$)
SOI	Control	5 ± 19	10 ± 8	5.2 ± 1.1	0.048 ± 0.007
	SMTC	8 ± 14*	5 ± 6#	6.4 ± 1.1*	0.055 ± 0.07*
	L-NAME	11 ± 9*	0 ± 5#%	7.9 ± 1.1*	0.061 ± 0.007*
SOVX	Control	-5 ± 17	1 ± 10^	5.2 ± 1.1	0.054 ± 0.08
	SMTC	2 ± 15*	0 ± 8	6.4 ± 1.6*	0.063 ± 0.011*
	L-NAME	5 ± 10*	-4 ± 4%	7.1 ± 1.3*	0.059 ± 0.012*
TOI	Control	4 ± 15	7 ± 7	7.2 ± 2.0$†	0.062 ± 0.014$†
	SMTC	7 ± 12*	1 ± 6#	9.1 ± 2.4*$†	0.075 ± 0.014*$†
	L-NAME	13 ± 19*	-5 ± 4#&%	8.9 ± 2.3*$†	0.069 ± 0.017*$†
TOVX	Control	-3 ± 7	0 ± 9^	7.1 ± 1.4$†	0.072 ± 0.010$†‡
	SMTC	4 ± 7*	3 ± 7	9.7 ± 1.7*$†	0.090 ± 0.016*$†‡
	L-NAME	6 ± 12*	-11 ± 6#&	9.7 ± 2.6*$†	0.090 ± 0.017*$†‡

All values are mean ± standard deviation. Measurements were obtained from sedentary time control ovary intact (SOI, n=10), sedentary time control ovariectomized (SOVX, n=9), exercise trained ovary intact (TOI, n=13), and exercise trained ovariectomized (TOVX, n=10) rats. * indicates a statistically significant difference from the Control condition (main effect of drug condition). # indicates a statistically significant difference from the control condition within a group. & indicates a statistically significant difference from the SMTC condition within a group. $ indicates a significant difference from SOI (main effect of group). † indicates a significant difference from SOVX (main effect of group). ‡ indicates a significant difference from TOI (main effect of group). ^ indicates a significant difference from SOI within a drug condition. % indicates a significant difference from TOVX within a drug condition. A p-value less than 0.05 was considered statistically significant.

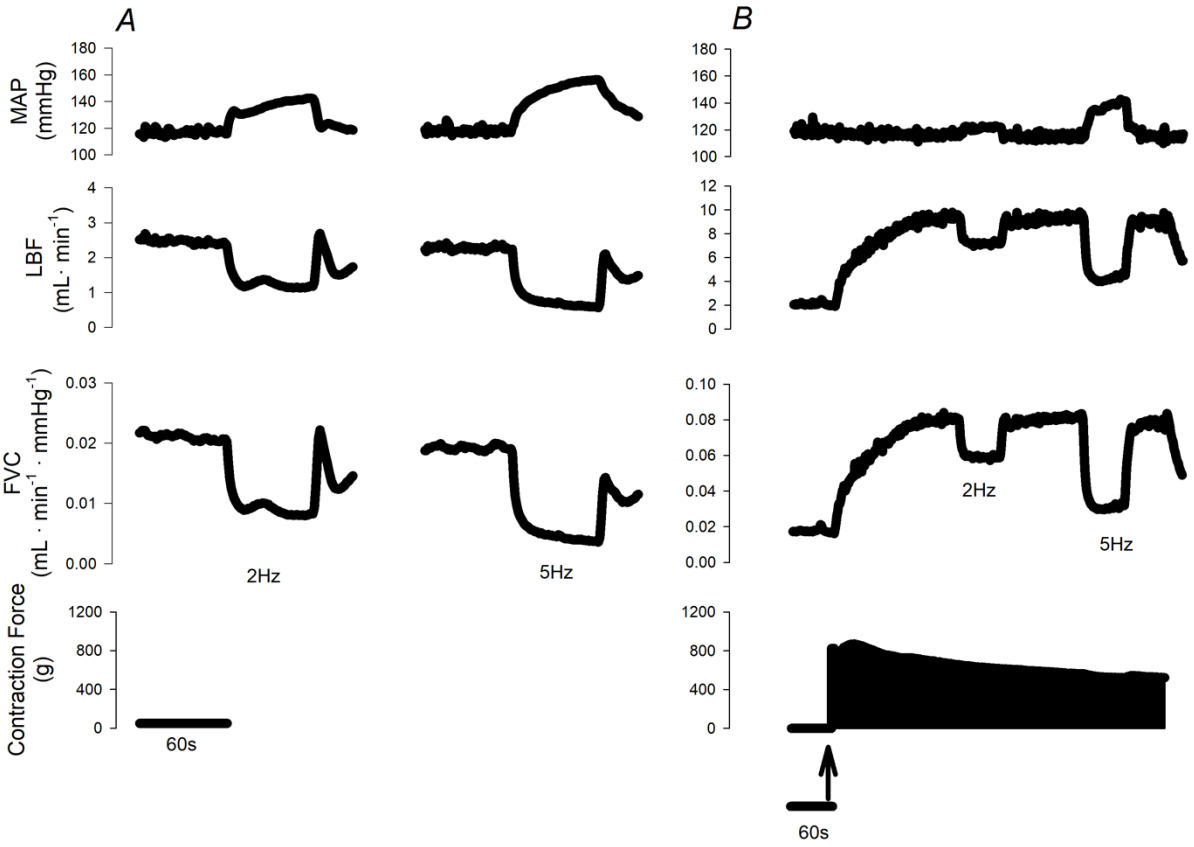

Figure 1. Original data tracing from a representative female rat illustrating the mean arterial pressure (MAP), leg blood flow (LBF), femoral vascular conductance (FVC), and contractile force responses to lumbar sympathetic chain stimulation at 2 Hz and 5 Hz in resting skeletal muscle (panel A) and during skeletal muscle contraction at 60% of maximal contractile force (panel B).

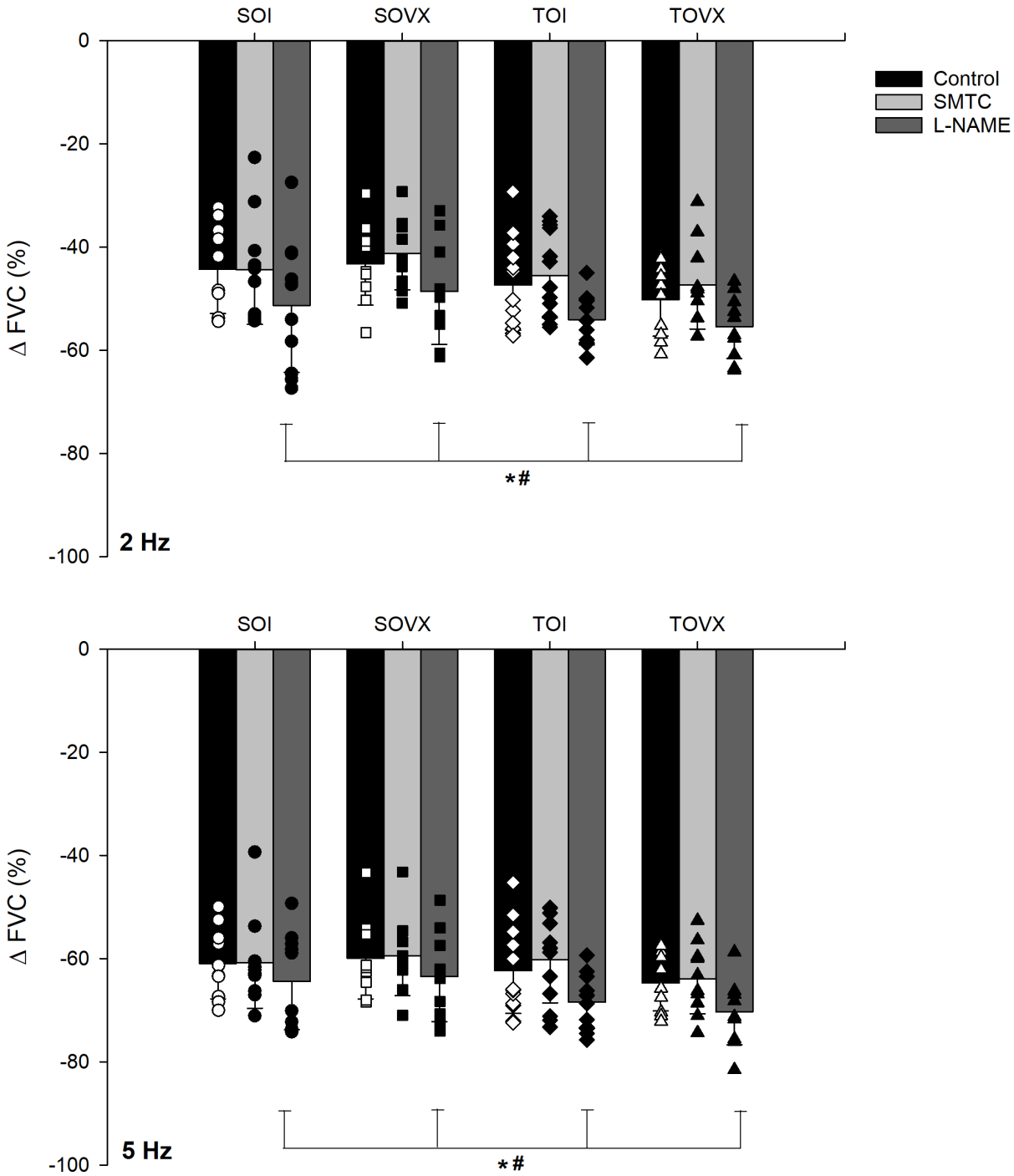

Figure 2. Percentage change in femoral vascular conductance (FVC) in response to resting lumbar sympathetic chain stimulation at 2 Hz (upper panel) and 5 Hz (lower panel) in sedentary ovary intact (SOI, n=10), sedentary ovariectomized (SOVX, n=9), trained ovary intact (TOI, n=13), and trained ovariectomized (TOVX, n=10) rats in control, SMTC, and L-NAME conditions. * indicates a statistically significant difference from the Control condition (main effect of drug condition). # indicates a statistically significant difference from the SMTC condition (main effect of drug condition). A p-value less than 0.05 was considered statistically significant.

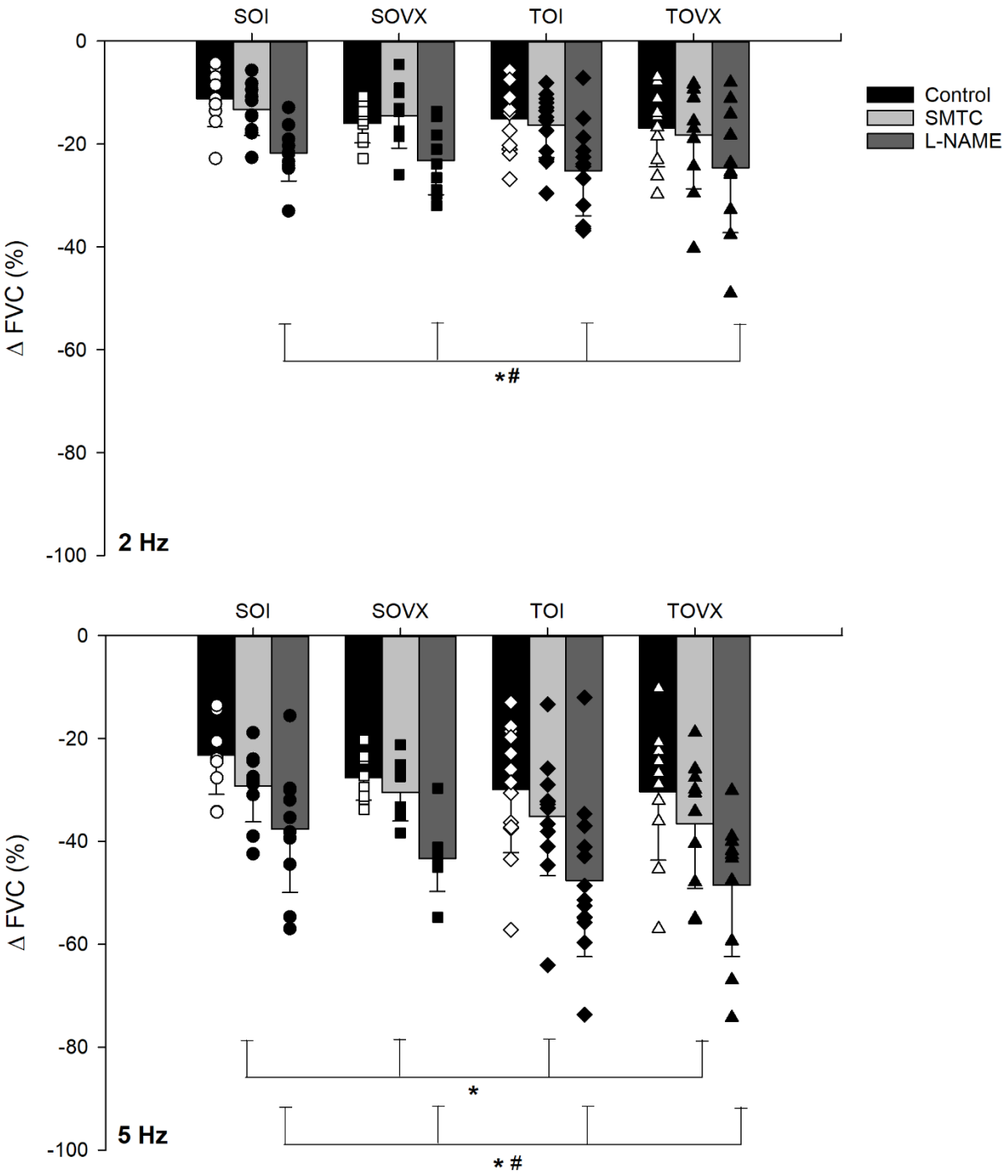

Figure 3. Percentage change in femoral vascular conductance (FVC) in response to lumbar sympathetic chain stimulation at 2 Hz (upper panel) and 5 Hz (lower panel) during muscle contraction at 60% of maximal contraction force in sedentary ovary intact (SOI, n=10), sedentary ovariectomized (SOVX, n=9), trained ovary intact (TOI, n=13), and trained ovariectomized (TOVX, n=10) rats in control, SMTC, and L-NAME conditions. * indicates a statistically significant difference from the Control condition (main effect of drug condition). # indicates a statistically significant difference from the SMTC condition (main effect of drug condition). A p-value less than 0.05 was considered statistically significant.

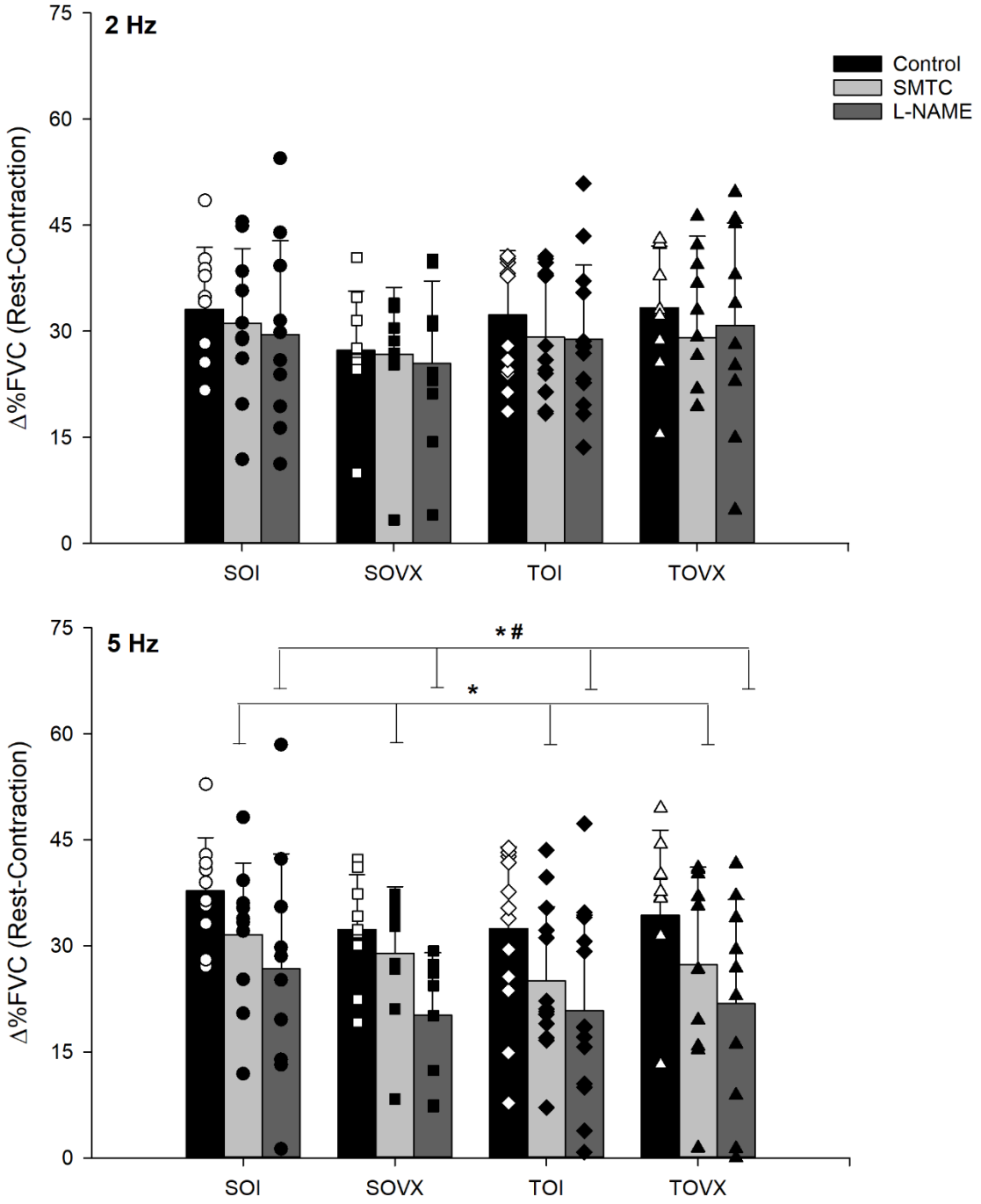

Figure 4. Magnitude of sympatholysis (difference in percentage change in femoral vascular conductance (FVC) in response to lumbar sympathetic chain stimulation at 2 Hz (upper panel) and 5 Hz (lower panel) at rest and during muscle contraction at 60% of maximal contraction force) in sedentary ovary intact (SOI, n=10), sedentary ovariectomized (SOVX, n=9), trained ovary intact (TOI, n=13), and trained ovariectomized (TOVX, n=10) rats in control, SMTC, and L-NAME conditions. * indicates a statistically significant difference from the Control condition (main effect of drug condition). # indicates a statistically significant difference from the SMTC condition (main effect of drug condition). A p-value less than 0.05 was considered statistically significant.

Discussion

The purpose of this study was to investigate the role of estrogen bioavailability in NO-mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle of sedentary and exercise trained female rats. In the present study, neither sympathetic vasoconstrictor responsiveness nor the magnitude of sympatholysis were different between ovary-intact and ovariectomized female rats. Sympathetic vasoconstrictor responsiveness and the magnitude of sympatholysis were also similar in sedentary and exercise trained female rats. Non-selective NOS inhibition (L-NAME) augmented resting sympathetic vasoconstriction responsiveness in all rats. During muscle contraction, sympathetic vasoconstrictor responsiveness to lumbar sympathetic chain stimulation at 5 Hz was increased by selective nNOS blockade (SMTC), and non-selective NOS inhibition augmented sympathetic vasoconstrictor responsiveness further, indicating that NO derived from both nNOS and eNOS is important to the inhibition of sympathetic vasoconstriction by muscle contraction in female rats. At 5 Hz sympathetic stimulation, sympatholysis was blunted in the presence of selective nNOS inhibition, but was not further reduced by non-selective NOS inhibition in sedentary ovary-intact, and exercise-trained ovariectomized and ovary-intact rats. In sedentary ovariectomized rats, selective nNOS inhibition did not affect sympatholysis, and non-selective NOS inhibition reduced sympatholysis. This indicates that nNOS-mediated sympatholysis is impaired in sedentary ovariectomized rats, and that exercise training can restore nNOS-mediated sympatholysis in ovariectomized rats.

Resting Sympathetic Vasoconstrictor Responsiveness

Contrary to our hypothesis, ovariectomy did not augment resting sympathetic vasoconstrictor responsiveness. The effect of estrogen on resting sympathetic vasoconstrictor

responsiveness is controversial. In arterial preparations from male rats, estrogen treatment either increased (Colucci et al., 1982) or decreased (Kondo et al., 1980; Shan et al., 1994; Yen & Lau, 2004) sympathetic vasoconstrictor responsiveness. Five to seven days of low-dose estrogen administration increased the vasoconstriction response to norepinephrine (NE) in mesenteric artery preparations from male rats (Colucci et al., 1982). Contrarily, the pressor response to NE infusion was reduced by estrogen injection in conscious male rats (Kondo et al., 1980; Shan et al., 1994). Estrogen treatment also blunted the vasoconstriction response to NE in rat tail artery preparations from male rats (Shan et al., 1994). Similarly, following seven weeks of estrogen treatment, the vasoconstriction response to phenylephrine was reduced in aortic ring preparations from spontaneously hypertensive male rats (Yen & Lau, 2004). However, when the endothelium was removed, the vasoconstriction response to phenylephrine was increased in the estrogen treated rats (Yen & Lau, 2004). This suggests that an intact endothelium is obligatory for the vascular function of estrogen. Evidence in estrogen replaced females is similarly equivocal. Four weeks of estrogen treatment reduced the vasoconstriction response to phenylephrine in mesenteric artery preparations from ovariectomized female rats (Zhang & Davidge, 1999). In peri-menopausal human females, eight weeks of estrogen supplementation, compared to a placebo, blunted the forearm vasoconstriction response to NE, as well as reducing resting systolic and diastolic blood pressures (Sudhir et al., 1997). Similarly, resting mean arterial pressure was lower following four weeks of estrogen supplementation in post-menopausal females; however, the vasoconstriction response (assessed by changes in muscle oxygenation) to lower body negative pressure did not change (Fadel et al., 2004). At rest, sympathetic vasoconstrictor responsiveness was also similar between ovary-intact and ovariectomized rats, with or without chronic estrogen replacement (Fadel et al., 2003). This is in agreement with our

results, where resting sympathetic vasoconstrictor responsiveness did not differ between ovary-intact and ovariectomized rats. In summary, the effect of estrogen on resting sympathetic vasoconstrictor responsiveness is unresolved, though *in vivo* data in young females does not support a role of estrogen in the regulation of sympathetic vasoconstriction.

In the present study, 10 weeks of exercise training at 40 $m \cdot min^{-1}$, 5% grade, 5 days per week also did not alter resting sympathetic vasoconstrictor responsiveness. To date, the majority of studies investigating the effect of exercise training on sympathetic neurovascular control have been done in male rats and/or isolated arterial preparations. Previous studies in arteries isolated from male rats have produced inconclusive results with the vasoconstrictor response to NE being decreased (Delp et al., 1993; Spier et al., 1999; Wiegman et al., 1981), increased (Lash, 1998), or unchanged (Donato et al., 2007; Jasperse & Laughlin, 1999; Sun et al., 1994) following exercise training. In miniature swine, exercise training augmented the vasoconstrictor response to NE in isolated brachial and femoral arteries (McAllister & Laughlin, 1997). In middle aged men and women, vasoconstriction to tyramine infusion at rest was reduced following exercise training (Mortensen et al., 2014). In contrast, our laboratory has reported that exercise training increased sympathetic vasoconstrictor responsiveness in the resting hindlimb of male rats (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2012, 2013a, 2013b; Just & DeLorey, 2016), but did not alter vasoconstrictor responsiveness in female rats (Cooper et al., 2021). Resting sympathetic vasoconstrictor responsiveness was not different following selective α_2-adrenoreceptor blockade in sedentary male rats, but was reduced in exercise-trained male rats (Jendzjowsky & DeLorey, 2013a). In a subsequent study, selective α_1-adrenoreceptor blockade blunted sympathetic vasoconstriction in resting skeletal muscle in both sedentary and exercise trained male rats, and abolished the difference between sedentary and exercise-trained male rats (Just & DeLorey,

2016). These studies suggest that exercise training augments both α_1- and α_2-adrenoreceptor responsiveness in resting skeletal muscle of male rats. In female rats, resting sympathetic vasoconstrictor responsiveness was not different following four weeks of exercise training (Cooper et al., 2021). The effect of exercise training on α-adrenoreceptor-specific vasoconstriction has not been studied in females, although it appears that exercise training does not alter resting sympathetic vasoconstrictor responsiveness in females. However, this is based upon the present study and one other study, thus more evidence in females is needed to draw a conclusion (Cooper et al., 2021).

Nitric oxide has been shown to inhibit sympathetic vasoconstriction in resting skeletal muscle (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b; Just & DeLorey, 2017) of male and female rats. Estrogen has been implicated in the regulation of NO bioavailability by upregulating NOS expression (Caulin-Glaser et al., 1997; Ceccatelli et al., 1996; Fadel et al., 2003; García-Durán et al., 1999; Lantin-Hermoso et al., 1997; Laughlin et al., 2003; Pelligrino et al., 1998; Sasser et al., 2015; Sudhir et al., 1996; Weiner, Knowles, et al., 1994; Weiner, Lizasoain, et al., 1994), suggesting that females would have higher NO bioavailability. Indeed, in post-menopausal human females, estrogen replacement therapy increased plasma NO and NO biological end-products (Best et al., 1998; Rosselli et al., 1995). Further, skeletal muscle nNOS, but not eNOS, expression was lower in ovariectomized rats compared with ovary-intact and estrogen-replaced ovariectomized rats (Fadel et al., 2003). In peri-menopausal females, eight weeks of estrogen supplementation augmented the reduction in forearm blood flow (measured by venous occlusion plethysmography) in response to acute non-selective NOS inhibition in peri-menopausal females (Sudhir et al., 1996). Similarly, the reduction in forearm blood flow to non-selective NOS inhibition was also larger in pre-menopausal females compared to post-

menopausal females and males, and, after 14 days of estrogen replacement therapy, post-menopausal females exhibited a similar forearm blood flow response to NOS inhibition as pre-menopausal females (Majmudar et al., 2000). Zhang and Davidge investigated the effect of four weeks of estrogen treatment on vasoconstriction in isolated mesenteric arteries from ovariectomized female rats (Zhang & Davidge, 1999). They reported that vasoconstriction to phenylephrine was blunted following four weeks of chronic estrogen treatment. In the presence of the non-selective NOS inhibitor N^{G}-monomethyl-$_{L}$-50 arginine, vasoconstriction was not different between estrogen-treated and control rats, suggesting that estrogen treatment blunted vasoconstriction in a NO-dependent manner (Zhang & Davidge, 1999). In summary, the available evidence indicates that estrogen modulates NOS expression and NO bioavailability, which may result in augmented inhibition of sympathetic vasoconstriction. In the present study, resting sympathetic vasoconstrictor responsiveness (to sympathetic stimulation delivered at 2 Hz and 5 Hz) was not different following selective nNOS inhibition, but was reduced by non-selective NOS inhibition in all rats. This indicates that estrogen bioavailability and exercise training status do not alter resting NO-mediated inhibition of sympathetic vasoconstriction in female rats.

Sympathetic Vasoconstrictor Responsiveness and Sympatholysis in Contracting Muscle

In the present study, sympatholysis and sympathetic vasoconstrictor responsiveness during muscle contraction were not different between ovary-intact and ovariectomized rats. Estrogen has been suggested to modulate sympathetic neurovascular control during exercise (Ettinger et al., 1996; Jarvis et al., 2011; Wenner et al., 2021). Young females exhibit a blunted increase in blood pressure in response to isometric handgrip exercise compared to post-menopausal females (Wenner et al., 2021). The blood pressure response to isometric handgrip

exercise was also attenuated in post-menopausal females following one month of estrogen replacement therapy (Wenner et al., 2021). Vasoconstrictor responsiveness was not measured in this study (Wenner et al., 2021). On the other hand, the blood pressure response to intermittent handgrip exercise was not altered by four weeks of estrogen replacement therapy in post-menopausal females (Fadel et al., 2004). However, in these subjects, estrogen replacement therapy improved sympatholysis, abolishing the difference between young females and post-menopausal females (Fadel et al., 2004). Sympatholysis was reduced in ovariectomized rats relative to ovary-intact and estrogen replaced ovariectomized rats (Fadel et al., 2003). This is in direct contrast to our results, where no differences in sympatholysis were found between ovary-intact and ovariectomized rats. While differences in our results and those in post-menopausal females could be explained by aging and differences between species, the reason for the discrepancy between our findings and those of Fadel et al. (Fadel et al., 2003) is unclear. In the present study, experiments were carried out 10 weeks after ovariectomy, whereas Fadel et al. tested at three to four weeks following ovariectomy. It is possible that physiological systems adapt over time to chronic reductions in estrogen bioavailability, compensating for estrogen-related loss of function through other mechanisms. Thus, the results of Fadel et al. (Fadel et al., 2003) may demonstrate the short-term effects of reduced estrogen bioavailability, whereas our results reflect longer-term adaptions to chronic reductions in estrogen bioavailability. Whether estrogen contributes to the elevated sympatholysis observed in females is undetermined and further study is necessary to establish the role of estrogen in the regulation of exercising sympathetic vasoconstrictor responsiveness.

Exercise training also did not alter contracting sympathetic vasoconstrictor responsiveness or sympatholysis in the present study. Our laboratory and others have previously

demonstrated that sympatholysis is enhanced in male rats following exercise training (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2012, 2013b; Mizuno et al., 2014). However, consistent with the results of the present study, our laboratory found that exercise training had no effect on sympatholysis in female rats (Cooper et al., 2021). In healthy, middle aged adults (four males, four females), eight weeks of cycle exercise training for one hour three to four days per week improved sympatholysis (Mortensen et al., 2014). Sex-based analysis was not performed in this study, therefore conclusions about potential sex differences in sympatholysis cannot be made (Mortensen et al., 2014). Cross-sectional data has also demonstrated that lifelong physical activity can prevent the age-related decline in sympatholysis (Mortensen, Nyberg, et al., 2012). This effect may due in part to a preservation of NO bioavailability (Nyberg et al., 2012). Indeed, exercise training appears to enhance sympatholysis in an NO-dependent manner in male rats (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b; Mizuno et al., 2014). Non-selective NOS inhibition blunted sympatholysis in exercise trained but not sedentary male rats, indicating that exercise training increased NO-mediated sympatholysis (Jendzjowsky & DeLorey, 2013b). In a subsequent study, selective nNOS inhibition had a larger effect in exercise trained compared to sedentary male rats, and the relative contribution of NO derived from nNOS to the inhibition of sympathetic vasoconstriction during muscle contraction was also greater in exercise trained compared to sedentary male rats (Jendzjowsky et al., 2014). The modulation of NOS expression is complex, and the mechanisms by which exercise training upregulates NOS expression are not fully understood. Nuclear factor κB binding to the promoter region of NOS has been implicated in the regulation process, as acute eccentric exercise increased iNOS, eNOS, and nNOS mRNA as well as nuclear factor κB binding (Lima-Cabello et al., 2010). When nuclear factor κB was acutely pharmacologically

inhibited, both NOS mRNA and nuclear factor κB binding were reduced (Lima-Cabello et al., 2010). Hydrogen peroxide also appears to be involved. Indeed, eNOS expression was increased in the aorta and myocardial tissue of mice following three weeks of exercise training, but this effect was abolished in mice overexpressing human catalase, which breaks down hydrogen peroxide (Lauer et al., 2005). In short, exercise training appears to augment sympatholysis in male rats via enhanced NO-mediated sympatholysis as a result of increased NOS expression.

In the present study, selective nNOS inhibition did not alter sympathetic vasoconstrictor responsiveness at the 2Hz stimulation frequency, whereas at the 5Hz stimulation frequency, sympathetic vasoconstrictor responsiveness was augmented in all groups following selective nNOS inhibition. These data suggest that nNOS-mediated blunting of sympathetic vasoconstriction may be dependent upon the frequency of sympathetic stimulation. The type and quantity of sympathetic neurotransmitter released in response to stimulation of the sympathetic chain appears to be frequency-dependent (Bradley et al., 2003). Briefly, high frequencies of sympathetic stimulation (i.e. 5 Hz) evoke the release of larger amounts of neurotransmitter and a proportional increase in NE and neuropeptide Y release, relative to low frequencies (i.e. 2 Hz) of sympathetic stimulation. Functionally, this may result in a different pool of post-synaptic receptors mediating vasoconstriction at each stimulation frequency. NO is believed to blunt sympathetic vasoconstriction by reducing the responsiveness of post-synaptic receptors (Thomas & Victor, 1998). Indeed, it has been argued that NO may inhibit sympathetic vasoconstriction by activating ATP-sensitive K^+ receptors and reducing α_2-adrenergic receptor-mediated influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels (Tateishi & Faber, 1995; Thomas et al. 1997). While investigation of the cellular mechanism responsible for NO-mediated sympatholysis is beyond the scope of this thesis, the evoked release of a distinct pool of

neurotransmitters and at relatively greater concentrations at the higher frequency of sympathetic stimulation may result in greater binding of neurotransmitter to a population of receptors that are particularly susceptible to NO-mediated inhibition. At the 5 Hz sympathetic stimulation frequency, selective nNOS inhibition blunted sympatholysis, and non-selective NOS inhibition decreased sympatholysis further in all groups. A post-hoc test showed that selective nNOS blockade blunted sympatholysis only in the SOI, TOI, and TOVX groups, and had no effect in the SOVX group. Non-selective NOS blockade blunted sympatholysis in all groups, but did not further reduce sympatholysis compared with the SMTC condition in the SOI, TOI, and TOVX groups. This would suggest that the relative contribution of NO derived from nNOS to sympatholysis is reduced in estrogen-deficient rats, but that exercise training can restore nNOS-dependent sympatholysis in these rats. Further, NO derived from eNOS appears to be able to compensate for reduced nNOS-mediated sympatholysis in sedentary ovariectomized rats to maintain the magnitude of sympatholysis. Supporting this notion, non-selective NOS inhibition had a similar effect in all groups. In contrast, our laboratory has shown that non-selective NOS inhibition reduces sympatholysis in exercise trained but not sedentary male rats (Jendzjowsky & DeLorey, 2013b; Just & DeLorey, 2017). This discrepancy between male and female rats may be explained by female rats having inherently higher NO-mediated inhibition of sympathetic vasoconstriction. Given that exercise training was shown to increase sympatholysis in an NO-dependent manner in male rats, exercise training may not further improve the magnitude of sympatholysis in female rats with an already high NO bioavailability. Indeed, exercise training did not alter the magnitude of sympatholysis in female rats in the present study or in a previous study from our laboratory (Cooper et al., 2021). In line with this theory, our laboratory has also previously found that sympatholysis is augmented in sedentary female rats compared to

sedentary male rats (Cooper et al., 2021; Just & DeLorey, 2017), and that a portion of this enhanced sympatholysis was NO-dependent (Just & DeLorey, 2017). However, the mechanism(s) underlying the increased NO-mediated sympatholysis in females remain unknown. While our current findings suggest that estrogen bioavailability may modulate nNOS-mediated sympatholysis in female rats, estrogen bioavailability did not alter the magnitude of sympatholysis or non-selective NO-mediated sympatholysis in female rats. However, without a male experimental group, we cannot conclude whether the enhanced sympatholysis previously reported in female rats (Cooper et al., 2021; Just & DeLorey, 2017) is due to higher estrogen bioavailability.

Exercise Hyperemia

In the present study, the absolute change in leg blood flow and femoral vascular conductance in response to muscle contraction (exercise hyperemia) was not different in ovariectomized compared with ovary-intact rats. Consistent with the present findings, Fadel et al., (Fadel et al., 2003), reported similar limb blood flow during exercise in control, ovariectomized, and estrogen replaced ovariectomized rats. Taken together, these studies suggest that estrogen bioavailability is not an important determinant of the blood flow response to exercise. However, in humans, the blood flow response to moderate-intensity cycling was significantly reduced in post-menopausal compared with pre-menopausal women (Proctor et al., 2003), and the blood flow response to single-leg knee extension exercise was blunted in early peri-menopausal women relative to post-menopausal women (Moore et al., 2012). The reason(s) for these divergent results is not readily apparent, however, species differences and differences in muscle fibre recruitment patterns between voluntary muscle contractions in humans and electrically-evoked muscle contractions in rats may be involved. While reduced exercise

hyperemia following menopause suggests a role of estrogen bioavailability in the control of exercise blood flow, menopause is a complex physiological process, and other factors may influence exercise hyperemia and further investigation will be required to elucidate the role of estrogen in the control of exercise hyperemia.

Exercise hyperemia was greater in exercise trained compared with sedentary rats, regardless of estrogen bioavailability in the present study. This is consistent with previous data from our laboratory where exercise hyperemia was increased in female rats after 4 weeks of exercise training at the same exercise intensity and frequency of training sessions employed in the present study (Cooper et al., 2021). The mechanism(s) responsible for the exercise training mediated increase in the blood flow response to contraction are not readily apparent. However, the lack of changes in sympathetic neuro-vascular control in the present study suggest that a non-neural adaptation may underlie the enhanced exercise hyperemia.

Conclusion

In summary, neither exercise training nor estrogen bioavailability altered sympathetic vasoconstrictor responsiveness at rest or during muscle contraction in female rats. The magnitude of sympatholysis was not different between ovariectomized and ovary-intact female rats; however, nNOS-mediated sympatholysis was impaired in sedentary ovariectomized rats. nNOS-mediated sympatholysis was enhanced following exercise training in ovariectomized rats, suggesting that exercise training can restore the reduced nNOS-mediated sympatholysis observed in sedentary ovariectomized rats. Exercise training did not alter resting or contracting sympathetic vasoconstrictor responsiveness or sympatholysis in ovary-intact female rats.

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Chapter 3: General Discussion

Summary of Main Findings

The aim of this thesis was to investigate the effect of estrogen bioavailability and exercise training on nitric oxide (NO) synthase (NOS) isoform-specific NO-mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle of female rats.

In the present study, resting sympathetic vasoconstrictor responsiveness was not different between ovariectomized and ovary-intact rats. This is in agreement with previous data from Fadel et al. where resting sympathetic vasoconstrictor responsiveness also was not different between ovary-intact and ovariectomized rats, with or without chronic estrogen replacement (Fadel et al., 2003). Similarly, four weeks of estrogen replacement did not alter the vasoconstriction response to lower body negative pressure in post-menopausal women (Fadel et al., 2004). On the other hand, the vasoconstriction response to phenylephrine was reduced in mesenteric arteries from ovariectomized female rats following four weeks of estrogen treatment (Zhang & Davidge, 1999). Eight weeks of estrogen replacement also blunted the vasoconstriction response to norepinephrine in peri-menopausal women (Sudhir et al., 1997). In summary, whether estrogen bioavailability modulates resting sympathetic vasoconstrictor responsiveness is unresolved, though *in vivo* data in young females indicates estrogen bioavailability does not contribute to the regulation of resting sympathetic vasoconstriction.

Further, exercise training did not alter resting sympathetic vasoconstrictor responsiveness, regardless of estrogen bioavailability. This is consistent with previous data from our laboratory, where four weeks of exercise training did not alter resting sympathetic vasoconstrictor responsiveness (Cooper et al., 2021). Based on the present data, and previous

data from our laboratory (Cooper et al., 2021), exercise training does not appear to alter resting sympathetic vasoconstrictor responsiveness in females.

Previous studies by Fadel and colleagues (Fadel et al., 2003, 2004) indicate that circulating estrogen levels may contribute to sympathetic neurovascular control during muscle contraction. In post-menopausal women, sympatholysis was blunted relative to young females; however, four weeks of estrogen replacement therapy restored sympatholysis to levels similar to young females (Fadel et al., 2004). In ovariectomized rats, neuronal NOS (nNOS) expression was reduced, as was sympatholysis (Fadel et al., 2003). In the present study, in the control and non-selective NOS blockade conditions, sympathetic vasoconstrictor responsiveness and the magnitude of sympatholysis were not different between ovary-intact and ovariectomized female rats, regardless of exercise training status. However, nNOS-mediated sympatholysis was reduced in sedentary ovariectomized rats. Exercise training appears to reverse this effect, as there was no difference in nNOS-mediated sympatholysis between exercise-trained ovariectomized rats and exercise-trained ovary-intact rats. Additionally, given that the magnitude of sympatholysis in control and non-selective NOS blockade conditions was not different in sedentary ovariectomized rats, endothelial NOS (eNOS)-mediated sympatholysis appears to compensate for the loss of nNOS-mediated sympatholysis.

Exercise training had no effect on the magnitude of sympatholysis or sympathetic vasoconstrictor responsiveness during muscle contraction in the control condition. This is in agreement with previous data from our laboratory where exercise training also did not alter evoked sympathetic vasoconstriction or the magnitude of sympatholysis in contracting skeletal muscle of female rats (Cooper et al., 2021). On the other hand, sympatholysis has been shown to be augmented by the same exercise training program in male rats (Jendzjowsky et al., 2014;

Jendzjowsky & DeLorey, 2013b; Mizuno et al., 2014), but not female rats (Cooper et al., 2021). This suggests that females may respond differently to a given exercise training stimulus than males. Indeed, in humans, cardiovascular adaptations to exercise training, such as maximal oxygen consumption and left ventricular mass and wall thickness, were blunted in females compared to males (Howden et al., 2015). Exercise training augmented skeletal muscle nNOS expression and nNOS-mediated sympatholysis in male rats (Jendzjowsky et al., 2014), suggesting that exercise training may modulate NO bioavailability and NO-dependent sympatholysis (Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b). Our laboratory has also found that sympatholysis was enhanced in untrained female rats compared to untrained male rats (Cooper et al., 2021; Just & DeLorey, 2017), and the statistical difference between males and females was abolished in the presence of NOS blockade (Just & DeLorey, 2017). This suggests that females may have higher NO bioavailability and NO-mediated sympatholysis than males. Indeed, estrogen has been shown to modulate NOS expression and NO bioavailability (Fadel et al., 2003). Thus, exercise training may not further increase NOS expression and NO-mediated sympatholysis in female rats with "normal" estrogen bioavailability. Supporting this theory, in the present study, selective nNOS and non-selective NOS inhibition had similar effects on sympatholysis in exercise trained and sedentary ovary-intact female rats, suggesting neither eNOS nor nNOS-derived NO bioavailability was different as a result of exercise training.

Experimental Considerations

The rats tested in this study are under anesthesia and muscle contraction is electrically evoked. Thus, this experimental preparation may not be entirely reflective of conscious, voluntary exercise. However, mechanistic studies, such as the present study, are not possible in

conscious animals or humans without the use of highly invasive approaches. During electrically evoked muscle contraction, the influence of neurovascular control mechanisms, such as central command, that are normally present during voluntary contraction are absent. Electrically stimulating muscle contraction also recruits motor units in a non-physiological manner. According to Henneman's size principle, motor units are recruited from smallest motor neuron to the largest, generally progressing from motor units containing primarily slow-twitch oxidative muscle fibers to motor units containing primarily fast-twitch glycolytic muscle fibers. This recruitment pattern is reversed when muscle contraction is electrically evoked, preferentially recruiting the largest motor neurons first, as they are the most sensitive to electrical stimulation. Sympathetic vasoconstrictor responsiveness appears to differ between arterioles supplying primarily oxidative vs glycolytic muscle fibers (Behnke et al., 2011). Sensitivity to selective α_1- and α_2-adrenoreceptor agonists was greater in arterioles supplying low-oxidative fast twitch muscle than in those supplying high-oxidative slow twitch muscle (Behnke et al., 2011). Further, maximal contraction of the gastrocnemius-plantaris muscle (primarily low-oxidative fast twitch) abolished the vasoconstriction response to lumbar sympathetic chain stimulation, whereas vasoconstriction was preserved during maximal soleus muscle (primarily high-oxidative slow twitch) contraction (Thomas et al., 1994). NOS isoform specific NO-mediated inhibition of sympathetic vasoconstriction may also differ between blood vessels supplying different types of muscle fibers. In male rats, nNOS inhibition by SMTC reduced blood flow and vascular conductance during near-maximal treadmill running (recruiting primarily low-oxidative fast twitch muscle fibers), but not light intensity treadmill running (recruiting primarily high-oxidative slow twitch muscle fibers) (Copp et al., 2013). nNOS expression also appears to be greater in low-oxidative fast twitch muscle fibers (Reid, 1998). Indeed, our laboratory has found

that nNOS expression was greater in the medial and lateral gastrocnemius muscles (primarily low-oxidative fast twitch) than the soleus muscle (primarily high-oxidative slow twitch) (Jendzjowsky et al., 2014). Thus, vasoconstrictor responsiveness and NO-mediated sympatholysis may differ between electrically stimulated muscle contraction and voluntary muscle contraction, and our results may not be representative of voluntary exercise. The experimental model used in the present study evokes contraction of the entire triceps surae muscle group, which includes both primarily low-oxidative fast twitch and high-oxidative slow twitch muscle. As such, conclusions about sympathetic vasoconstrictor responsiveness in arterioles supplying different types of muscle fibers cannot be made.

The present study utilized electrical stimulation of the lumbar sympathetic chain to evoke sympathetic vasoconstriction at rest and during muscle contraction. Electrical stimulation of the lumbar sympathetic chain allows for the endogenous release of sympathetic neurotransmitters, which may more faithfully reflect physiological function than infusion of exogenous sympathetic agonists. On the other hand, the stimulation patterns used are non-physiological. Stimulation frequencies of 2 and 5 Hz were chosen as they most closely resemble sympathetic drive at rest and during periods high sympathetic nerve activity, such as exercise (Bradley et al., 2003; Hudson et al., 2011; Johnson et al., 2001; Macefield et al., 1994). In the present study, and in previous studies from our laboratory (Cooper et al., 2019, 2021; Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2012, 2013a, 2013b, 2013d; Just & DeLorey, 2016, 2017) and others (Mizuno et al., 2014; Thomas & Victor, 1998), these stimulation frequencies produced graded sympathetic vasoconstriction responses at rest and during muscle contraction, suggesting that they evoke the release of sympathetic neurotransmitters in differing quantities. However, physiological sympathetic nerve activity typically occurs in random bursts, followed by periods

of inactivity (Christensen & Galbo, 1983; Guyenet, 2006; Robinson et al., 2019). This random pattern is different between individuals and is difficult to replicate in a laboratory setting. In a previous study, our laboratory compared the effect of continuous sympathetic stimulation at 2 Hz and a burst stimulation pattern at 20 and 40 Hz, and found no difference in resting sympathetic vasoconstrictor responsiveness to these stimulation patterns in sedentary or exercise trained male rats (Jendzjowsky & DeLorey, 2013c). Thus, while the stimulation pattern used is non-physiological, it does allow for endogenous release of sympathetic neurotransmitters in quantities reflective of periods of low and high sympathetic nerve activity.

Future Directions

Despite being first reported 60 years ago, the cellular mechanisms responsible for sympatholysis remain unknown (Remensnyder et al., 1962). There is strong evidence that NO is involved in sympatholysis (Buckwalter, Taylor, et al., 2004; Dinenno & Joyner, 2003; Fadel et al., 2003; Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b, 2013d; Just & DeLorey, 2017; Kobzik et al., 1994; Mizuno et al., 2014; Stamler & Meissner, 2001), including the findings from the present study; however, the exact mechanism by which NO inhibits sympathetic vasoconstriction is also unknown. Further, while NO does appear to be involved in sympatholysis, it is not the only mechanism at play. When NO production is pharmacologically inhibited, sympatholysis is reduced, but not abolished, suggesting that some other mechanism(s) contribute to sympatholysis (Chavoshan et al., 2002; Jendzjowsky et al., 2014; Jendzjowsky & DeLorey, 2013b, 2013d; Just et al., 2016; Thomas & Victor, 1998). Other putative mechanisms for sympatholysis merit further study.

Data in *in vitro* experimental preparations and in animal models support sex differences in cardiovascular regulation; however, corroboration in a human model is needed to firmly establish these differences. Females are underrepresented or not included in many areas of research, including cardiovascular physiology research. Indeed, significant gaps remain in our understanding of sex differences in cardiovascular regulation. The mechanism(s) underlying the enhanced sympatholysis observed in female rats have yet to be fully elucidated (Cooper et al., 2021; Just & DeLorey, 2017). In an effort to explain why sympatholysis is higher in female rats, our laboratory has investigated whether NO and/or β-adrenoreceptors contribute to the enhanced sympatholysis in females (Cooper et al., 2021; Just & DeLorey, 2017). While NOS blockade eliminated the statistical difference between males and females, sympatholysis remained greater in female rats, suggesting that factor(s) in addition to NO that contribute (Just & DeLorey, 2017). In a subsequent study, β-adrenoreceptor blockade had no effect on the sex difference in sympatholysis, indicating that β-adrenoreceptors are not responsible for the enhanced sympatholysis in females (Cooper et al., 2021). Our current data suggests that estrogen also does not contribute to this phenomenon. Further study is required to determine the factor(s) other than NO that are responsible for the augmented sympatholysis observed in females, as well as why NO-mediated sympatholysis is enhanced in females.

Conclusion

The purpose of this study was to investigate whether exercise training and estrogen bioavailability alter NOS isoform-specific NO-mediated inhibition of sympathetic vasoconstriction at rest and during muscle contraction. In the present study, estrogen bioavailability did not alter resting or contracting sympathetic vasoconstrictor responsiveness or

the magnitude of sympatholysis. However, nNOS-mediated sympatholysis was blunted in sedentary ovariectomized rats, but not exercise-trained ovariectomized rats. This suggests that exercise training reversed the estrogen-related loss of nNOS-mediated sympatholysis. While nNOS-mediated sympatholysis was impaired in sedentary ovariectomized rats, the magnitude of sympatholysis was not different between any groups, and non-selective NOS inhibition blunted sympatholysis to a similar extent in all groups. This suggests that NO-mediated sympatholysis is not dependent upon estrogen bioavailability, and that NO derived from eNOS can compensate for the estrogen-related loss of nNOS function. Further, in line with previous data from our laboratory (Cooper et al., 2021), exercise training does not appear to alter resting or contracting sympathetic vasoconstrictor responsiveness or the magnitude of sympatholysis in female rats, regardless of estrogen status.

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