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The Effects of Ovariectomy on Neuropeptide Y Neurotransmission in Skeletal Muscle Arterioles

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THE EFFECTS OF OVARIECTOMY ON NEUROPEPTIDE Y
NEUROTRANSMISSION IN SKELETAL MUSCLE ARTERIOLES

THE EFFECTS OF OVARIECTOMY ON NEUROPEPTIDE Y
NEUROTRANSMISSION IN SKELETAL MUSCLE ARTERIOLES

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Kinesiology

By

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May 2011
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ABSTRACT

The purpose of this study was to determine the effects of chronic estrogen supplementation on NPY neurotransmission in gastrocnemius first-order arterioles (G1A) of adult female rats. Female rats (4 mo; n = 30) were ovariectomized (OVX) with a subset (n = 15) receiving an estrogen pellet (OVE; 17 β -estradiol (4 μ g / day)). Following conclusion of the treatment phase (8 weeks), red G1A were excised, placed in a physiological saline solution (PSS) bath, and cannulated with micropipettes connected to albumin reservoirs. A sampling port was placed immediately below the vessel to assess NPY overflow. The contralateral red G1A was homogenized in PSS for dipeptidyl peptidase IV (DPPIV) assay. NPY-mediated vasoconstriction via a Y₁-agonist, [Leu31Pro34]NPY, decreased vessel diameter 44.54 \pm 3.95% as compared to baseline; however, there were no group differences in EC₅₀ (OVE: -8.97 \pm 0.36; OVX: -8.72 \pm 0.20 log M [Leu31Pro34]NPY) or slope (OVE: -1.37 \pm 0.38; OVX: -1.64 \pm 0.31 % baseline / log M [Leu31Pro34]NPY). NPY did not potentiate norepinephrine-mediated vasoconstriction. NPY overflow experienced a slight increase following field stimulation, and significantly increased (p < 0.05) over control conditions in the presence of a DPPIV inhibitor (diprotin A). Estrogen status did not affect DPPIV activity. These data suggest that NPY can induce a moderate decrease in vessel diameter in skeletal muscle G1A, and DPPIV is active in mitigating NPY overflow in young adult female rats. Chronic estrogen supplementation did not influence NPY vasoconstriction, overflow, or its enzymatic breakdown in skeletal muscle G1A.

This dissertation is approved for
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DEDICATION

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INTRODUCTION

The regulation of blood flow throughout the body is a process that utilizes local and systemic control mechanisms to ensure that metabolic demands are fulfilled while, at the same time, maintaining adequate blood pressure for the greater cardiovascular system. The local control mechanisms of the vasculature consist of a finely tuned network of chemical processes that originate primarily from within the endothelium, whereas the systemic control lies within sympathetic innervations in addition to the endocrine stimuli in the blood. It is a coordinated response between these loci that produces constant change in blood vessel diameter, which ensures normal function of biological processes.

The neural control of blood vessel diameter is attributed to the sympathetic nervous system. These sympathetic nerves arise from the spinal cord as pre-junctional nerves, synapse to the post-junctional nerve, and traverse across the blood vessel's adventitial surface (Guyton & Hall, 2006). Norepinephrine, adenosine triphosphate (ATP), and neuropeptide Y (NPY) are the primary neurotransmitters responsible for propagating the neural response from the sympathetic end terminal to the blood vessel. These neurotransmitters work in a coordinated fashion, each possessing their own degree of direct influence on the vessel while also modulating other chemical processes simultaneously. There is evidence to suggest that sympathetic neurotransmission is not uniform between the sexes (Jackson, Milne, Noble, & Shoemaker, 2005a, 2005b). Females may possess more, or less, responsiveness to specific chemical mediators within the sympathetic triad. These differences can be observed in neurotransmitter release, receptor function/activity, enzymatic activity, and cellular signaling.

Neuropeptide Y is found in vascular beds throughout the body, and it possesses many of the same direct and indirect actions as norepinephrine and ATP. Neuropeptide Y is a potent vasoconstrictor, yet its role in vasoconstriction may be more relevant in small caliber vessels such as arterioles (Joshua, 1991; Kim, Duran, Kobayashi, Daniels, & Duran, 1994; Macho, Perez, Huidobro-Toro, & Domenech, 1989; Pernow, 1989) as opposed to large conduit vessels, which exhibit little to no responsiveness to NPY (Grundemar & Hogestatt, 1992; Tsurumaki, Honglan, & Higuchi, 2003; Wahlestedt, Yanaihara, & Hakanson, 1986). Neuropeptide Y can elicit a 2- to 4-fold potentiation of vasoconstriction to norepinephrine in most vascular beds (Abel & Han, 1989; Prieto, Benedito, Simonsen, & Nyborg, 1991). It is plausible that this mechanism is involved during low levels of sympathetic activation as potentiation is observed at nanomolar concentrations of both NPY and norepinephrine. Neuropeptide Y autoregulates its own release as well as modulates the release of norepinephrine and ATP (Lundberg & Stjarne, 1984; Ohhashi & Jacobowitz, 1983; Stjarne, Lundberg, & Astrand, 1986), thus NPY is intimately involved in all facets of sympathetic vasocontrol.

The beneficial effects of 17β -estradiol, herein referred to as estrogen, have been well documented with the most notable being the activation of endothelial nitric oxide synthase (eNOS) (Moriarty, Kim, & Bender, 2006). This provides a secondary stimulus for the generation of nitric oxide, the first being luminal shear stress, which is a direct stimulus for vasodilation. Estrogen modulates sympathetic neurite branching in uterine arteries, possibly due to direct actions on nerve growth factors such as brain derived neurotrophic factor (Krizsan-Agbas, Pedchenko, Hasan, & Smith, 2003). A decrease in neurite density across the blood vessel would mitigate the degree of sympathetic

influence. The cyclical neuritogenesis and neurite degeneration as influenced by estrogen throughout estrous is an example of how estrogen can impact the resultant magnitude of sympathetic neurotransmission. A recent study found postmenopausal women to have higher concentrations of NPY within uterine arteries as compared to premenopausal women (Di Carlo, et al., 2007). This information along with increased protease activity in young females as compared to males (Jackson, et al., 2005b) suggests that estrogen may play a role in sympathetic neurotransmission, but the full scope of that role remains to be fully understood.

There is a disparity in the vascular responses to NPY between males and females with much of the differences attributed to total NPY content, the type of receptor expressed, and the activity of enzymes that breakdown NPY (Jackson, et al., 2005a, 2005b). Estrogen may be the underlying mechanism in the increase in NPY content in the reproductive vasculature following menopause, and it may be responsible, in part, to the differences observed in NPY neurotransmission in large conduit vessels. There is a paucity of literature examining the actions of NPY in sympathetic neurotransmission of skeletal muscle, small caliber resistance vessels. This level of the vasculature is of greater significance as it is at this level where the majority of regulatory control occurs with respect to systemic blood pressure (Segal, 2005). The resistance vasculature is also affected in pathophysiological conditions such as peripheral arterial disease and diabetes (Cersosimo & DeFronzo, 2006), therefore the need for further study is clearly indicated.

The specific aim of the proposed study is to examine the influence of estrogen on NPY release, post-junctional receptor activity, and enzymatic activity of proteases that degrade NPY. It is my belief that estrogen will mitigate the overall stimulus of NPY in

the resistance vessels. These results may explain a portion of the vascular changes that occur in response to the disruption of normal estrogen concentrations during menopause and beyond. In addition, these results may elucidate yet another mechanism of estrogen's cardioprotective function in the vasculature.

Primary Aim: To determine the effects of chronic estrogen supplementation on NPY release, receptor activity, and breakdown in gastrocnemius first-order arterioles.

Hypotheses

1. NPY release following field stimulation will decrease with chronic estrogen supplementation in gastrocnemius first-order arterioles.
2. Chronic estrogen supplementation will decrease the amount of vasoconstriction in response to NPY administration in gastrocnemius first-order arterioles.
3. Chronic estrogen supplementation will decrease the extent at which NPY potentiates adrenergic vasoconstriction in gastrocnemius first-order arterioles.
4. DPPIV activity will be greater in gastrocnemius first-order arterioles with chronic estrogen supplementation.

Limitations

1. Arteriole size was not included in the statistical analysis of the DPPIV activity assay due to the sample medium's interference with the kit components of the protein assay.

REVIEW OF LITERATURE

Neuropeptide Y (NPY) is a 36-amino acid polypeptide that is widely expressed throughout the central and peripheral nervous systems. Neuropeptide Y exhibits neuroendocrine function in the pituitary and hypothalamus, possessing various levels of influence on releasing hormones involved in satiety, reproduction, and development. Neuropeptide Y also acts as a neurotransmitter in central and peripheral nervous tissue owing to its functional duality in the body, and it is in this latter role that NPY exerts a level of influence over vascular regulation.

NPY Structure and Synthesis

A family of polypeptides characterized by a tyrosine residue at the C-terminus was discovered in 1980 by Tatemoto and Mutt (1980). Peptide YY and peptide histidine isoleucine were observed in the gut region with putative influence on pancreatic secretion (Jensen, Tatemoto, Mutt, Lemp, & Gardner, 1981; Tatemoto, 1982a). A polypeptide was also observed in the brain, but it was thought to be peptide YY due to its characteristic N- and C-terminus tyrosine residues (Tatemoto, 1982a). However, while sharing substantial sequence homology with peptide YY, the brain polypeptide was not identical (Tatemoto, 1982b). Thus, the brain polypeptide was designated as a novel neuropeptide, neuropeptide Y (Tatemoto, Carlquist, & Mutt, 1982). In addition to sequence homology with peptide YY (67%), NPY is structurally similar to another gut peptide, pancreatic polypeptide (50%) (see Table 1) (Takeuchi & Yamada, 1985; Tatemoto, 1982b; Tatemoto, et al., 1988). The gene that encodes NPY is found on chromosome 7 (7pter-q22) (Takeuchi, et al., 1986), while the gene for pancreatic polypeptide is located on

chromosome 17. The difference in chromosomal location for peptides of the same family could be attributed to evolutionary genetic translocation (Takeuchi, et al., 1986).

Table 1
 Amino Acid Sequences of NPY, Peptide YY, and Pancreatic Polypeptide

Peptide	Amino Acid Sequence
NPY	Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH ₂
Peptide YY	<u>Tyr-Pro-Ile-Lys-Pro-Glu-Ala-Pro-Gly-Glu-Asp-Ala-Ser-Pro-Glu-Glu-Leu-Asn-Arg-Tyr-Tyr-Ala-Ser-Leu-Arg-His-Tyr-Leu-Asn-Leu-Val-Thr-Arg-Gln-Arg-Tyr-NH₂</u>
Pancreatic Polypeptide	Ala-Pro-Leu-Glu-Pro-Val-Tyr-Pro-Gly-Asp-Asn-Ala-Thr-Pro-Glu-Gln-Met-Ala-Gln-Tyr-Ala-Ala-Asp-Leu-Arg-Arg-Tyr-Ile-Asn-Met-Leu-Thr-Arg-Pro-Arg-Tyr-NH₂

Note. Amino acids underlined are in the same sequential position as the corresponding amino acid in NPY; amino acids in bold are unique to that specific peptide.

Transcription and translation. Prepro-NPY consists of a 97 amino-acid chain with a 28 amino acid signal peptide, followed by the expressed 36 amino acid peptide (NPY) (Minth, Bloom, Polak, & Dixon, 1984). A polymorphism in the signal peptide (Leu7Pro) may influence translocation of prepro-NPY to the endoplasmic reticulum, post-translational modifications, and the expression of mature NPY (Kallio, et al., 2001). This polymorphism has been linked to hypercholesteremia and atherosclerosis in some populations (Karvonen, et al., 2000; Niskanen, et al., 2000). Kaipio, Kallio, and Pesonen (2009) observed premature apoptosis and decreased angiogenesis in response to vascular endothelial growth factor in human umbilical vascular endothelial cells that possessed this polymorphism, which would be an indicator of endothelial dysfunction. However, this polymorphism has also been associated with decreased free fatty acids and insulin concentrations during exercise (Kallio, et al., 2001). While the increase in NPY appears to accompany this polymorphism, it is difficult to surmise a negative or positive relationship in cardiovascular profile. The amino acid chain that follows expressed NPY, C-flanking Peptide Of Neuropeptide Y (CPON), may have physiological function (Allen, Polak, & Bloom, 1985). Immunoreactivity to CPON was detected in the heart, adrenal glands, and kidney. In the brain, CPON immunoreactivity was detected in the hypothalamus, striatum, hippocampus, frontal cortex, and the brain stem. While CPON immunoreactivity appears to mirror NPY concentrations in certain tissues, a physiologically relevant function for CPON has not been identified.

NPY Locations

Brain. NPY is the most abundant peptide of the brain (Adrian, et al., 1983) with NPY immunoreactivity detected at greatest concentrations in the deep layers of the brain

and brain stem (Y. S. Allen, et al., 1983; Sawchenko, et al., 1985). In the limbic system, NPY immunoreactivity was present in the arcuate and paraventricular nuclei of the hypothalamus (Lundberg, Terenius, Hokfelt, & Tatemoto, 1984), the pineal gland, and the pituitary gland (Chronwall, et al., 1985). Neuropeptide Y immunoreactivity was also found in the hippocampus and the dentate gyrus. In the brain stem, cell bodies with NPY immunoreactivity were observed in the periaqueductal gray region, and to a lesser extent, the locus coeruleus, the nuclei of the tractus solitarius (Lundberg, Terenius, et al., 1984), and the superficial laminae of the trigeminal nucleus. In the medulla oblongata, NPY immunoreactivity was abundant in the raphe nuclei of the reticular formation and the dorsal motor nucleus of the vagus (Hokfelt, et al., 1983). In the basal ganglia, high concentrations of NPY immunoreactivity were found in the caudate nucleus and putamen (Adrian, et al., 1983). Hypothalamic NPY is released at circadian intervals with the greatest release occurring in the morning (7:00am) followed by a gradual decline throughout the day (Nicholson, et al., 1983).

Neuropeptide Y can be found in NPYergic (Bai, et al., 1985), adrenergic, and GABAergic (Hendry, et al., 1984) neurons within the brain. The NPYergic neurons arising from the arcuate nucleus contain a rich supply of NPY (Bai, et al., 1985). Removal of the arcuate nucleus resulted in a steep decline in NPY immunoreactivity suggesting that this is the primary origin of NPY immunoreactivity found in the brain. Neuropeptide Y is transported in A₁₋₃ (Bai, et al., 1985; Everitt, et al., 1984), A₄₋₈, A₁₀, A₁₂, A₁₄₋₁₅ (Everitt, et al., 1984), and C₁₋₃ (Everitt, et al., 1984; Sawchenko, et al., 1985) adrenergic neurons. Neuropeptide Y immunoreactivity co-localizes with norepinephrine (A₁₋₂, A₄, A₆) and epinephrine (C₁₋₂) in adrenergic neurons, however trace amounts of

NPY immunoreactivity have been observed in the absence of catecholamines (A₅, A₇, A₈₋₁₀, A₁₂, A₁₄₋₁₅) (Everitt, et al., 1984). Since GABAergic (λ -aminobutyric acid) neurons participate in inhibitory stimuli, NPY found in GABAergic neurons may play a role in facilitating the actions of this neuronal type (Hendry, et al., 1984).

In the brain, NPY exhibits neuroendocrine behavior by modulating the release of hormones involved in protein synthesis, cell growth, reproduction, and eating behavior (Clark, Kalra, Crowley, & Kalra, 1984; J. K. McDonald, Lumpkin, Samson, & McCann, 1985). NPY also has cerebrovascular actions, however this will be discussed in subsequent sections and will receive no further mention here. Neuropeptide Y is a potent stimulus for the feeding response in animal models. Intraventricular injection of NPY resulted in an increased feeding response, and this response was 3-fold greater than the response elicited by pancreatic polypeptide (Clark, et al., 1984). In a separate study by Stanley, Daniel, Chin, and Leibowitz (1985), carbohydrate was the preferred macronutrient following intraventricular injection with smaller differences between control and experimental trials in fat and protein consumption.

Neuropeptide Y possesses stimulatory effects on pituitary release of luteinizing hormone (Kalra & Crowley, 1984; Kerkerian, Guy, Lefevre, & Pelletier, 1985; J. K. McDonald, et al., 1985). In ovariectomized rats with estrogen/progesterone supplementation, intraventricular injection of low concentrations of NPY (0.1-2.0 μ g) resulted in a marked increase in luteinizing hormone concentrations 10 minutes following application (Kalra & Crowley, 1984). Luteinizing hormone-releasing hormone concentrations tend to rise and fall in concert with NPY concentrations, suggesting a modulatory effect for NPY on luteinizing hormone-releasing hormone (Crowley, Tessel,

O'Donohue, Adler, & Kalra, 1985). Progesterone administration causes a substantial, yet transient, increase in NPY and luteinizing hormone-releasing hormone within the median eminence followed by a gradual decline with a concurrent rise in luteinizing hormone concentrations (Crowley, et al., 1985; Kalra & Crowley, 1984). This suggests that NPY release within the hypothalamus is sensitive to progesterone, and that NPY may possibly modulate the effects of luteinizing hormone-releasing hormone and subsequent luteinizing hormone release (Crowley, et al., 1985). Neuropeptide Y may act directly on the anterior pituitary to stimulate the release of luteinizing hormone and follicle-stimulating hormone (FSH) (J. K. McDonald, et al., 1985), however the significance of this mechanism in vivo is unknown. Progressively larger concentrations of NPY (2-5 μ g) depressed circulating concentrations of growth hormone (GH) (J. K. McDonald, et al., 1985).

Adrenal gland. Neuropeptide Y is found in the adrenal glands of many animals with the greatest proportion found in the adrenal medulla (Varndell, Polak, Allen, Terenghi, & Bloom, 1984). There is considerable variability across species in adrenal NPY content with some animals such as mice expressing significant amounts (1244pM/g) and other animals such as dogs expressing very little (68pM/g) (Allen, Adrian, Polak, & Bloom, 1983). In the adrenal cortex, NPY immunoreactivity exists in the varicose nerve fibers that span the zona reticularis and the subcapsular cortical area (Varndell, et al., 1984). In the adrenal medulla, NPY co-localizes with norepinephrine, but not epinephrine, in small (90-120nm) chromaffin granules.

Peripheral vasculature and smooth muscle. Neuropeptide Y is widely expressed in perivascular nerve fibers that form plexuses across blood vessels (Grasby,

Morris, & Segal, 1999) and terminate at the adventitial-tunica media border (Fleming, Gibbins, Morris, & Gannon, 1989). Neuropeptide Y or NPY immunoreactivity has been detected in the gut (Ekblad, Ekelund, Graffner, Hakanson, & Sundler, 1985; Lundberg, Terenius, Hokfelt, & Goldstein, 1983; Sundler, Moghimzadeh, Hakanson, Ekelund, & Emson, 1983), ear (Hieble, Ruffolo, & Daly, 1988), uvea (Terenghi, et al., 1983), throughout the female and male reproductive anatomy (Lundberg, et al., 1983; Owman, et al., 1986; Stjernquist, et al., 1983), kidney (Ballesta, Polak, Allen, & Bloom, 1984), respiratory tract (Uddman, Sundler, & Emson, 1984), heart (Gu, et al., 1984), thyroid gland (Grunditz, Hakanson, Rerup, Sundler, & Uddman, 1984), spleen (Lundberg, et al., 1983), omentum (Edvinsson, et al., 1985), skin (Lundberg, et al., 1983), and sweat glands (Tainio, Vaalasti, & Recharadt, 1986). Neuropeptide Y is found in a variety of nonvascular smooth muscle such as vas deferens (Fried, Terenius, Hokfelt, & Goldstein, 1985; Lundberg, et al., 1983), intestine (Sundler, et al., 1983), lung (Lundberg, et al., 1983), and eye (J. M. Allen, G. P. McGregor, et al., 1983).

Other nonvascular sites. Neuropeptide Y is also found in the submucosal layers of the stomach (Ekblad, et al., 1985), prostate gland, submandibular gland (Lundberg, et al., 1983), fallopian tube, uterus (Owman, et al., 1986), and thyroid gland (Grunditz, et al., 1984).

NPY Storage and Transport

Neuropeptide Y is co-stored along with norepinephrine in the large dense-cored vesicles (80-120nm) of adrenergic neurons (Ekblad, et al., 1984; Fleming, et al., 1989; Fried, Terenius, et al., 1985; Grasby, et al., 1999; Lundberg, et al., 1983; Tainio, et al., 1986). A 50:1 ratio of norepinephrine to NPY exists in these large vesicles, while no

NPY immunoreactivity is observed in the small-type (50nm) vesicles (Fried, Lundberg, & Theodorsson-Norheim, 1985; Fried, Terenius, et al., 1985). Unlike norepinephrine, in which neurons have the enzymatic capability (tyrosine hydroxylase, dopamine β -hydroxylase) for synthesis within the sympathetic end terminal (Fried, Lundberg, et al., 1985), NPY depends (9mm/hour) on axonal transport for reconstitution. Therefore, the discrepancy in norepinephrine:NPY ratios can become more pronounced in distal sympathetic end terminals (Fried, Lundberg, et al., 1985). Reserpine experiments, which deplete the neurotransmitter content of sympathetic end terminals, produced a rebound supracompensation of norepinephrine and dopamine β -hydroxylase (160% and 140%, resp.) that was observed four days following reserpine administration (Dahlstrom, et al., 1986). In comparison, NPY experienced a slight decrease over time in addition to the acute decline (50%) that occurred following reserpine treatment (Dahlstrom, et al., 1986; Morris, Murphy, Furness, & Costa, 1986). The disparity in reconstitution between NPY and norepinephrine supports the putative differences in neurotransmitter synthesis and transport.

Neuropeptide Y is also stored and released from chromaffin granules within the adrenal medulla, thus participating in the systemic pressor response along with norepinephrine and epinephrine (Lundberg, Fried, Pernow, & Theodorsson-Norheim, 1986). Upon adrenal activation, systemic concentrations of NPY can increase 2-fold over normal resting concentrations, while norepinephrine and epinephrine experience a greater increase in circulating concentrations (Lundberg, Fried, Pernow, & Theodorsson-Norheim, 1986). The larger increase in circulating catecholamines is, in part, attributed to larger stored concentrations within the adrenal medulla. In the adrenal medulla of the

cat, NPY storage is 1:2000 to norepinephrine and 1:1500 to epinephrine (Lundberg, Fried, Pernow, & Theodorsson-Norheim, 1986). However, NPY remains elevated in the circulation for a longer duration as compared to norepinephrine and epinephrine following a pressor stimulus.

NPY Release from Peripheral Sympathetic Nerves

Action potentials propagated from the proximal post-junctional nerve result in fusion of the neurotransmitter-containing vesicles to the neural membrane at the synapse and neurotransmitter release via exocytosis. As NPY is co-stored with norepinephrine in large dense-cored vesicles, NPY is also co-released with norepinephrine during the neural action potential (Lundberg, Martinsson, et al., 1985; Pernow, 1988).

NPY Receptors and Vascular Actions

There are six known receptors for NPY, Y₁₋₆ (Balasubramaniam, 1997), although only a few possess vascular actions (Wahlestedt, et al., 1990). In vascular smooth muscle, the Y₁ receptor is located at the cell membrane where it binds with complete NPY₍₁₋₃₆₎. The Y₁ receptor belongs to a class of G-protein coupled receptors, whereby it creates the cellular conditions for vasoconstriction. The Y₂ receptor is primarily located on the sympathetic end terminal although it can be found on vascular smooth muscle in some vascular beds (Tessel, Miller, Misse, Dong, & Doughty, 1993a, 1993b). However, unlike the Y₁ receptor, the Y₂ receptor is capable of binding C-truncated fragments of NPY in addition to binding the full-length peptide.

Y₁ receptor mechanisms. The binding of NPY₍₁₋₃₆₎ to the Y₁ receptor results in the dissociation of an inhibitory G protein, G_i, which inhibits adenylate cyclase activity and subsequent cyclic AMP (cAMP) production (Lobaugh & Blackshear, 1990; Reynolds

& Yokota, 1988). Pertussis toxin, which blocks G_i signaling, abrogates the decrease in adenylate cyclase activity and subsequent vasoconstriction following NPY administration (Andriantsitohaina, Andre, & Stoclet, 1990; Morris, 1991). The fact that pertussis toxin does not effect the contractile mechanisms of vascular smooth muscle suggests that G_i signaling is a primary mechanism behind NPY vasoconstriction (Morris, 1991). While pertussis toxin virtually blocks vasoconstriction in small arterioles (Andriantsitohaina, et al., 1990), there is still vasoconstriction to NPY in larger vessels (Morris, 1991) even in the presence of pertussis toxin. This would suggest that other cellular signaling mechanisms are involved following activation of the Y_1 receptor. Indeed, an increase in intracellular calcium follows Y_1 activation in many vascular beds (Oshita, Kigoshi, & Muramatsu, 1989; Pernow & Lundberg, 1986; Shigeri & Fujimoto, 1993; Tessel, et al., 1993b; Xiong, Bolzon, & Cheung, 1993). Nifedipine, an L-type calcium channel blocker, is successful in blocking a portion of vasoconstriction caused through Y_1 activation (Tessel, et al., 1993b). The L-type calcium channel is voltage sensitive, and is the primary mechanism behind calcium influx in response to cell depolarization of vascular smooth muscle. In cerebral and internal carotid arteries, NPY increased vascular smooth muscle membrane potential 15mV above baseline values (Abel & Han, 1989). Neild (1987) observed depolarization (+16mV over baseline) of rat tail artery similar to the values observed by Abel and Han (1989). The onset of depolarization was slow (5-10 minutes post-application), and a correlation was observed between vasoconstriction and depolarization. The depolarizing effect of NPY is independent of endothelial influences as endothelium removal did not alter the increases in membrane potential following NPY administration (Gustafsson & Nilsson, 1990).

Vascular smooth muscle contraction can also be influenced by the release of calcium from intracellular storage sites. The second messenger that is most associated with the release of intracellular calcium stores is inositol _{1,4,5}-triphosphate (IP₃). Neurotransmitters that utilize IP₃ as a second messenger (e.g. norepinephrine) are capable of producing substantial vasoconstriction. In porcine aortic vascular smooth muscle, NPY administration did not directly increase IP, IP₂, or IP₃ concentrations, but NPY did potentiate angiotensin II-mediated vasoconstriction (Lobaugh & Blackshear, 1990). NPY also increased norepinephrine-stimulated IP₃ production in rat tail artery (Duckles & Buxton, 1994). The ability of NPY to stimulate or influence the upstream enzyme for IP₃ production, phospholipase C, is equivocal. Lobaugh and Blackshear (1990) concluded that phospholipase C activity was unaffected by NPY administration in cultured porcine aortic vascular smooth muscle cells. The absence of changes in phospholipase C activity and IP₃ production was unexpected as NPY increased intracellular calcium in an environment void of extracellular calcium. This would suggest an internal store of calcium susceptible to NPY activation. Shigeri, Nakajima, and Fujimoto (1995) observed nonsignificant increases in IP₃ production in cultured porcine aortic smooth muscle cells. However, their experiments included inhibitors for the various phospholipase C isoforms to determine what, if any, isoforms were susceptible to NPY stimulation. Experiments that inhibited phospholipase C-β abolished the increase in intracellular calcium following NPY administration. Therefore, NPY likely potentiates IP₃ production via activation of phospholipase C-β. While a subsequent increase in IP₃ production following NPY administration is not substantial, it would appear that this pathway is involved in NPY-mediated vasoconstriction via release of calcium from intracellular stores.

ATP-activated potassium channel currents decreased by 43% with NPY administration in rabbit mesenteric artery (Bonev & Nelson, 1996). These channels are inhibited by protein kinase C, which is a downstream signal of phospholipase C. Bonev and Nelson (1996) noted increases in ATP-activated potassium channel currents following inhibition of protein kinase C and phospholipase C activity during NPY stimulation, which would support the notion of phospholipase C activation following Y_1 stimulation. The conglomeration of active second messengers in some tissues could be the result of heterodimeric complexes comprised of multiple receptor subtypes (Pons, et al., 2008). These complexes consist of Y_1 and Y_5 subtypes, and have been classified as $G_{i/o}$ subtype receptors. This heterodimeric complex inhibits adenylate cyclase activity and stimulates phospholipase C activity, which inhibits (cAMP (protein kinase A)) and promotes (IP_3 , diacylglycerol (protein kinase C)) the respective second messengers associated with each enzyme. Certainly, there is still debate as to the functional significance of such multi-receptor complexes in producing meaningful concentrations of IP_3 . Regardless, the Y_1 receptor is recognized as the post-junctional receptor for NPY responsible for stimulating vasoconstriction via activation of L-type and R-type (Pons, et al., 2008) calcium channels.

Y_2 receptor mechanisms. The Y_2 receptor is primarily located on the sympathetic end terminal where it acts to inhibit NPY release in addition to the inhibition of adrenergic and purinergic neurotransmitters (Lundberg, Torssell, et al., 1985). There are vascular beds, however, where Y_2 receptors are expressed on the vascular smooth muscle (McAuley & Westfall, 1992). As a pre-junctional receptor, Y_2 is a G_i -protein coupled receptor where it exerts influence on cellular signaling by inhibiting both

adenylate cyclase activity and N-type calcium channels (R. L. McDonald, Vaughan, Beck-Sickinger, & Peers, 1995; Wahlestedt, et al., 1990). Y₂ receptors have a high affinity for the full length peptide (NPY₍₁₋₃₆₎) in addition to the cleavage products, NPY₍₂₋₃₆₎ and NPY₍₃₋₃₆₎, of aminopeptidase P and dipeptidyl peptidase IV, respectively. Y₂ receptors also exhibit binding affinity for the full-length and truncated fragments of peptide YY and pancreatic polypeptide (Wahlestedt, et al., 1990).

Vascular actions. Neuropeptide Y stimulates vasoconstriction in many vascular beds through Y₁ and Y₂ receptor mechanisms previously discussed. In the cat spleen, NPY caused a dose-dependent increase in perfusion pressure (Lundberg, Anggard, Theodorsson-Norheim, & Pernow, 1984; Lundberg, Fried, Pernow, Theodorsson-Norheim, & Anggard, 1986). The vascular response had a slow onset with the increase in perfusion pressure manifesting 2-5 minutes following NPY administration. The vasoconstriction to NPY can linger over time. In this tissue, the increase in perfusion pressure persisted for 10 minutes following the initial increase (Lundberg, Anggard, et al., 1984). Furthermore, the increase in perfusion pressure was impervious to α - or β -adrenoceptor blockade. In porcine spleen, an increase in NPY-like immunoreactivity following transmural stimulation occurred 2-5 minutes post-stimulation, and the concentration was 4-5 times greater with larger frequencies (20Hz) as compared to small frequencies (2Hz) (Lundberg, Rudehill, Sollevi, Theodorsson-Norheim, & Hamberger, 1986). In rabbit cerebral and internal carotid arteries, NPY produced vasoconstriction that was 21% and 79% to vasoconstriction produced by histamine and norepinephrine (Abel & Han, 1989). Minute vasoconstriction following NPY administration was observed in internal maxillary artery (Lacroix, Stjarne, Anggard, & Lundberg, 1988). In

human studies, forearm blood flow decreased during NPY administration in a dose-dependent fashion. A similar lag in response was observed in these studies (2 minutes between time of administration and changes in blood flow) as was the long-lasting effect (~15 minutes of vasoconstriction) (Clarke, et al., 1991). In human saphenous vein, NPY produced a small amount of vasoconstriction (~ 28% of maximum contraction) (Luu, Chester, O'Neil, Tadjkarimi, & Yacoub, 1992). Neuropeptide Y, along with norepinephrine, is an important neurotransmitter involved in stimulating reflex vasoconstrictor mechanisms in humans (Stephens, Saad, Bennett, Kosiba, & Johnson, 2004). During whole body cooling, Y₁ receptor blockade significantly decreased reflex vasoconstriction, while Y₁ plus α -adrenoceptor blockade abrogated reflex vasoconstriction entirely.

In some vascular beds, NPY fails to cause direct vasoconstriction, but it can potentiate the vasoconstriction caused by other neurotransmitters. In rat tail artery, NPY had no direct vasoconstriction, but did potentiate vasoconstriction to transmural nerve stimulation (Vu, Budai, & Duckles, 1989). Interestingly, NPY did not potentiate vasoconstriction to norepinephrine in this vessel, although it did increase the rate of contraction to norepinephrine as well as transmural nerve stimulation. In rabbit cerebral and internal carotid arteries, NPY was capable of potentiating vasoconstriction to norepinephrine with no change in maximum vasoconstriction (Abel & Han, 1989). In the perfused mesenteric vascular bed, NPY administration produced little vasoconstriction, but potentiated vasoconstriction to norepinephrine and α,β -methylene ATP (Han, et al., 1998). It was concluded that NPY is involved in 30% of the vasoconstriction to

sympathetic nerve stimulation at this level of the vasculature when taking into account the potentiation of vasoconstriction to other neurotransmitters.

The ability of NPY to stimulate vasoconstriction appears to increase as vessel diameter decreases, thus becoming more relevant at the level of the resistance vasculature. In rabbit tenuissimus muscle arterioles, NPY was 10-fold more potent on a molar basis as compared to norepinephrine (Pernow, Ohlen, Hokfelt, Nilsson, & Lundberg, 1987). The magnitude of vasoconstriction was identical (65% and 64%) between NPY and norepinephrine, respectively, in these vessels. As was seen in the perfused spleen, vasoconstriction was long-lasting with constriction observed five minutes following application. There was also a 2-3 minute lag in response between the initial time of administration and the first signs of vasoconstriction. In rat cremaster muscle, NPY-mediated vasoconstriction was approximately 61%, 54%, and 18% of resting diameter in first-, second-, and third-order arterioles (Joshua, 1991). In hamster cheek pouch microvessels, NPY decreased resting vessel diameter by 50% with a lag time of 5 minutes between administration and response, and a long-lasting response up to 20 minutes (Kim, et al., 1994). Neuropeptide Y-mediated vasoconstriction was unaffected by α -adrenoceptor blockade. In human small arteries, NPY was capable of eliciting vasoconstriction equal in magnitude to that of norepinephrine (Pernow, 1989). While the effects of NPY in skeletal muscle arterioles are unknown, the present literature suggests a larger overall response to NPY at the arteriole level.

Proteolytic Enzymes

Dipeptidyl peptidase IV. Dipeptidyl peptidase IV is a proteolytic enzyme that cleaves off the tyrosine-proline residue yielding a C-truncated product, NPY₍₃₋₃₆₎

(Mentlein, Dahms, Grandt, & Kruger, 1993; Mentlein & Roos, 1996). Dipeptidyl peptidase IV has been identified in human umbilical vascular endothelial cells (Zukowska-Grojec, et al., 1998) and it also exists in a soluble form (CD26) (Durinx, et al., 2000). Dipeptidyl peptidase IV activity plays a significant role in modulating NPY neurotransmission in females as inhibition results in a significant decrease in blood flow and vascular conductance in external iliac arteries (Jackson, et al., 2005b). Similar results were observed in vasoconstriction experiments of the rat tail artery using peptidase inhibitors (Glenn, Krause, & Duckles, 1997). Alternative pathways such as angiogenesis are directly influenced by dipeptidyl peptidase IV activity as NPY-mediated angiogenesis depends on the activation of Y_2 receptors, which have binding affinity for C-truncated fragments of NPY. While dipeptidyl peptidase IV appears to possess a greater role in female NPY neurotransmission, the mechanisms that underlie this larger role have yet to be elucidated.

Vascular Effects of Estrogen

Estrogen has many direct and indirect effects on local blood flow and blood pressure with most effects pertaining to vessel dilation. The primary mechanism is activation of endothelial nitric oxide synthase, which produces nitric oxide (cyclic GMP) resulting in vasodilation (Moriarty, et al., 2006). Estrogen also ameliorates the accumulation of free radicals through increased activity of dismutases and peroxidases (Duckles, Krause, Stirone, & Procaccio, 2006; Vina, Borrás, Gomez-Cabrera, & Orr, 2006). In addition to the vasodilating effects, estrogen attenuates endothelium-dependent mechanisms involved in vasoconstriction. Cyclooxygenase products such as prostaglandin H that arise from endothelial cells can be depressed in arteries with

estrogen (Davidge & Zhang, 1998). Other endothelium-derived vasoconstrictive products such as endothelin 1 and angiotensin II, through estrogen's influence on angiotensin converting enzyme, decrease with estrogen (Miller & Duckles, 2008).

Estrogen receptors ($ER\alpha$, $ER\beta$) are expressed on adrenergic, cholinergic, and serotonergic nerves (Vanderhorst, Gustafsson, & Ulfhake, 2005). Removal of estrogen can lead to increases in circulating norepinephrine concentrations and increases in vascular resistance (Miller & Duckles, 2008). Estrogen decreases both the expression of α -adrenergic receptors and the enzymes (tyrosine hydroxylase) responsible for the production of precursors to norepinephrine (Kaur, Janik, Isaacson, & Callahan, 2007; Miller & Duckles, 2008). In rat tail artery segments, estrogen supplementation resulted in decreased force production to norepinephrine (Stice, Eiserich, & Knowlton, 2009). Krizsan-Agbas, Pedchenko, Hasan, and Smith (2003) concluded that estrogen modulates sympathetic activity in uterine tissue by influencing the expression of brain derived neurotrophic factor, which modulates neuritogenesis and neurite degradation. This modulatory influence was recently extended to vascular tissue with estrogen supplementation decreasing nerve growth factor expression in extracerebral blood vessels in addition to the superior cervical ganglia (Kaur, et al., 2007). The effects of estrogen on NPY neurotransmission in the vasculature have not been determined.

The effects of sex on NPY neurotransmission have received attention in large conduit vessels. Females possess less Y_1 receptor expression and total NPY content in the gastrocnemius muscle (whole tissue) than their male counterparts (Jackson, et al., 2005a). Females also experience little change in blood flow to Y_1 receptor blockade in external iliac arteries as compared to males (Jackson, et al., 2005a). A follow-up study

by this same group found Y_2 receptor expression to be greater in the gastrocnemius muscle of females (Jackson, et al., 2005b). Females had a decrease in vascular conductance and blood flow during Y_2 blockade with a return to baseline levels following combined Y_1 - Y_2 blockade. In a separate group of females, inhibition of the proteolytic enzymes that target N-terminal amino acid residues of NPY (Tyr and Tyr-Pro) resulted in a decrease in blood flow and vascular conductance, which suggests that these enzymes are intimately involved in the modulation of NPY neurotransmission in females (Jackson, et al., 2005b). This research lends credence to a sex difference in NPY neurotransmission, but the underlying mechanism or mechanisms responsible for these differences cannot be answered with the present data. The work of Glenn, Krause, and Duckles (1997) suggests that sex steroids, in general, modulate NPY neurotransmission in females. However, there was more potentiation of vasoconstriction to transmural nerve stimulation following NPY administration in control females as compared to ovariectomized animals. This would run counter to what Jackson, Milne, Noble, and Shoemaker (2005b) observed with the decreased Y_1 receptor activity in females as compared to males. However, Glenn, Krause, and Duckles (1997) did note a greater inhibition in vasoconstriction to transmural nerve stimulation following Y_2 receptor stimulation in control animals, which is consistent with the greater expression of Y_2 receptors observed in females (Jackson, et al., 2005b). A weakness of the Glenn, Krause, and Duckles (1997) study was the inability to control estrogen concentrations. While attempts were made to monitor the rat's estrous cycle, the inherent fluctuation of estrogen in intact females increases the experimental variability (error). Little research exists regarding estrogen's impact on NPY neurotransmission in human subjects. Di Carlo et

al. (2007) detected increases in total NPY content of uterine arteries from post-menopausal women. While estrogen was not controlled per se, these results would suggest a possible modulatory role for estrogen with regards to NPY neurotransmission.

Purpose of Study

The purpose of this study is to examine the effects of chronic estrogen supplementation on NPY release, NPYergic receptor actions, modulation of adrenergic vasoconstriction, and proteolytic enzymes that act on NPY. It is expected that chronic estrogen supplementation will decrease NPY release and Y₁ receptor-mediated vasoconstriction. The potentiation effect of NPY on adrenergic vasoconstriction is also expected to attenuate with chronic estrogen supplementation. Lastly, normally active proteolytic enzymes that modulate NPY neurotransmission through enzymatic cleavage of N-terminal amino acid residues will become less active in animals without chronic estrogen supplementation. The present results may possibly elucidate another mechanism of estrogen's direct effect on sympathetic neurotransmission via the modulation of NPY neurotransmission in skeletal muscle arterioles. This would further substantiate estrogen's crucial role in maintaining normal cardiovascular dynamics within the peripheral circulation as well as revealing the physiological changes that occur in skeletal muscle arterioles in the absence of estrogen.

Treatment and Experimental Rationale

Ovariectomy and chronic estrogen supplementation. Estrogen impacts the cardiovascular system through acute (Moriarty, et al., 2006) and chronic mechanisms (LeBlanc, et al., 2009; Stice, et al., 2009). Ovariectomy is a means to remove the variability caused by estrogen, but comparisons to intact animals are fraught with

potential errors pertaining to the estrous cycle. The estrous cycle of a F344 rat is 4-5 days in duration with estrogen fluctuation ranging from approximately 70pg/ml during proestrous to concentrations as low as 15-20pg/ml during estrous (Haim, Shakhar, Rossene, Taylor, & Ben-Eliyahu, 2003). Efforts to 'time' the cycle in this animal model would be tremendously difficult, if not impossible, due to the variable nature of the estrous cycle in this particular breed. The only valid experimental alternative is to control estrogen concentrations by means of a secondary source. The use of estrogen pellets (Innovative Research of America) to maintain an internally controlled environment is a popular and convenient method for experimental control for this type of treatment. While treatment (supplementation) times may vary (Davidge & Zhang, 1998; Kaur, et al., 2007; LeBlanc, et al., 2009; Robbins, Mebane, Ball, Shaffer, & Ness, 2010; Stice, et al., 2009; Yao, et al., 2005; Zhang, Stewart, & Davidge, 2000), chronic changes in the vasculature can be observed within two weeks (Kaur, et al., 2007). Haim et al. (2003) determined that a daily dose of .27 μ g/day would result in an estrogen plasma concentration of 34.6pg/ml, and a daily dose of 1.11 μ g /day would results in an estrogen plasma concentration of 137.8pg/ml in F344 rats. The study by LeBlanc et al. (2009) on F344 rats used a daily dose of 1.67 μ g /day, which produced an estrogen plasma concentration of 41pg/ml. This work was relevant to the proposed project in that it employed microvessel analyses of arterioles. Most importantly, the dose and length of time (6-8 weeks) of the study was sufficient in observing not only changes in protein expression, but also changes in vascular response to agonist/antagonist treatments. In the proposed study, a dose of .25 mg / 60 days, will be used for the estrogen supplementation group, which has a delivery rate of 4.17 μ g /day. The treatment time will range from no

earlier than 6 weeks to no later than 8 weeks. The serum estrogen concentrations (Estradiol EIA kit, Oxford Biomedical, Oxford, MI) and uterine weight will be used to verify estrogen status for each animal at the time of terminal experiment.

Microvessel preparation. In vitro microvessel preparations provide the means to study the microvasculature in isolation. This is a form of technical control thereby limiting confounding elements that are difficult to control otherwise (e.g. systemic influences). Furthermore, this preparation is one of few viable options for the study of microvascular contraction and relaxation in this animal model. Flow probes, which are commonly used to measure blood flow, have yet to be designed for vessels of this size (100-300 μ m). Therefore, this apparatus is a viable and effective tool in measuring microvascular contraction and relaxation. In this study, the amount of contraction will be determined through measurement of the vessel's diameter using video calipers (Colorado Video 307A Horizontal Video Calipers, Boulder, CO), which will be referenced against baseline diameter measurements. While vessel diameter is the measurable event, this variable is synonymous with vasoconstriction.

Field stimulation. Neuropeptide Y release to field stimulation typically occurs at large frequencies (>20Hz), with little to no release observed at small frequencies (2-20Hz) (Lundberg, Rudehill, et al., 1986). This frequency-dependent characteristic of NPY could be attributed, in part, to the large, dense-cored vesicles where NPY is stored (Ekblad, et al., 1984; Fleming, et al., 1989; Fried, Terenius, et al., 1985; Grasby, et al., 1999; Lundberg, et al., 1983; Tainio, et al., 1986). Therefore, a large frequency (60Hz) will be utilized in the present study. While this is a large frequency of stimulation,

instantaneous frequencies that exceed 50Hz have been recorded in sympathetic vasoconstrictor nerves (Macefield, Wallin, & Vallbo, 1994).

Gastrocnemius first-order arteriole. Two (red and white) gastrocnemius first-order arterioles are downstream of the gastrocnemius feed artery. The red arteriole supplies blood to the medial gastrocnemius, which is more oxidative than the lateral gastrocnemius. Therefore, the red arteriole will be used in this study. As a resistance vessel, the arteriole expresses a diverse array of adrenergic (Pernow, et al., 1987), NPYergic (Matsuda, Brumovsky, Kopp, Pedrazzini, & Hokfelt, 2002), and purinergic (Matsuura, Saino, & Satoh, 2004) receptors making it an ideal vessel for the study of sympathetic neurotransmission. Furthermore, blood pressure is maintained at the level of the resistance vasculature, which is capable of accommodating a 100-fold increase in blood flow during periods of maximal oxygen demand (Segal, 2005).

Joshua (1991) observed increases in first-order arteriole vasoconstriction following NPY administration by approximately 39%. Macho et al. (1989) concluded coronary resistance vessels (arterioles) to be highly responsive to NPY with regards to vasoconstriction while noting little responsiveness in large conduit vessels. Neuropeptide Y was 250-fold more potent than norepinephrine in these coronary arterioles. While this research pertained to coronary arterioles, the available literature suggests an inverse relationship between vessel diameter and vessel responsiveness to NPY. Dipeptidyl peptidase IV is also more active in the gastrocnemius first-order arteriole as compared to the femoral artery (unpublished data).

Cumulative concentration response curves. Arteriole vasoconstriction to NPY has been achieved at nanomolar concentrations (10^{-11} - 10^{-7} M) (Joshua, 1991; Kim, et al.,

1994; Pernow, et al., 1987). Therefore, this concentration range for NPY will be encompassed in the NPY concentration response curve performed in this proposal (10^{-11} - 10^{-5} M). The potentiation of norepinephrine vasoconstriction can be elicited at nanomolar concentrations of NPY (10^{-9} - 10^{-7} M) (Abel & Han, 1989; Hieble, et al., 1988; Prieto, et al., 1991; Small, Bolzon, & Cheung, 1992). The greatest concentration (10^{-7} M) of NPY associated with potentiation of vasoconstriction to norepinephrine will be used as it is the most oft reported concentration with measureable effects (Vu, et al., 1989).

Preliminary Data

Neuropeptide Y release has been successfully measured in the gastrocnemius first-order arteriole using the proposed peptide enzyme immunoassay. Figure 1 is NPY release during α_2 -adrenoceptor stimulation and inhibition (n=2). The increase in NPY release following idozoxan (α_2 -adrenoceptor antagonist) is consistent with synergistic effects of adrenergic and NPYergic neurotransmission.

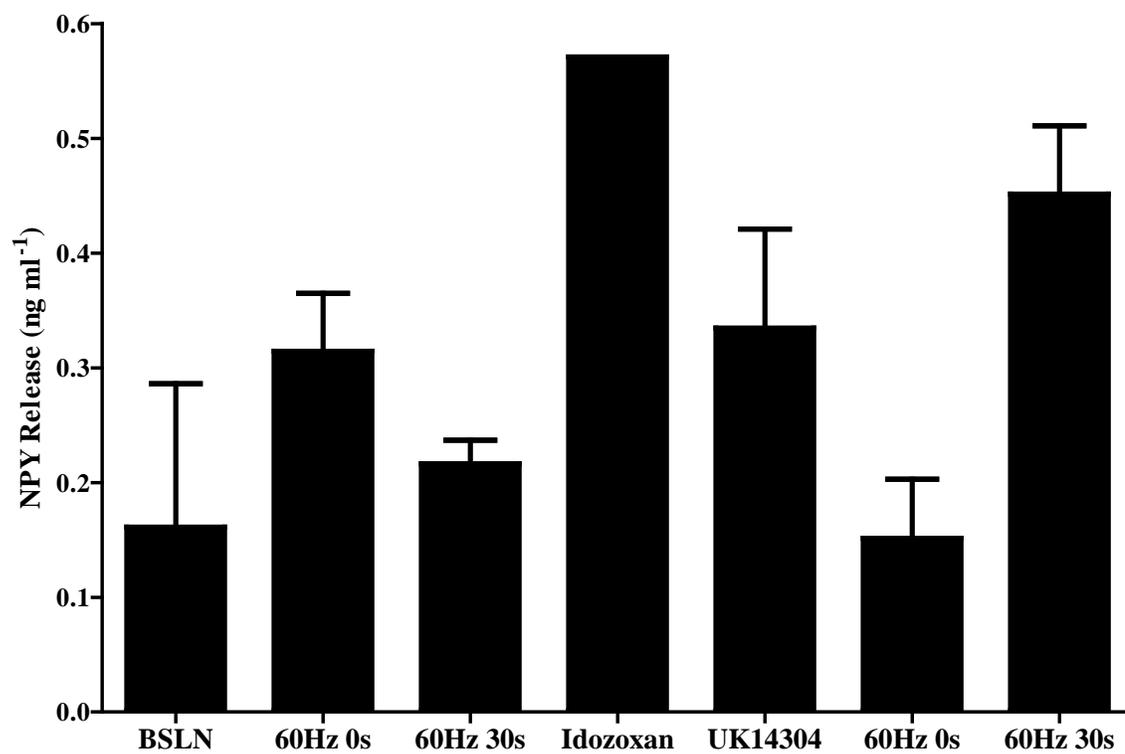


Figure 1. The effects of α_2 -adrenoceptor blockade on NPY release following field stimulation ($n = 2$). NPY release was detectable using the proposed peptide enzyme immunoassay.

Dipeptidyl peptidase IV has been successfully measured in the gastrocnemius first-order arteriole using the synthetic substrate, glycyl-L-proline-4-methoxy-2-naphthylamide. Figure 2 represents dipeptidyl peptidase IV activity in the femoral artery and gastrocnemius first-order arteriole (n=16). Dipeptidyl peptidase IV activity is greater in the arteriole. Figure 3 represents dipeptidyl peptidase IV activity in male and female gastrocnemius first-order arterioles (n=8).

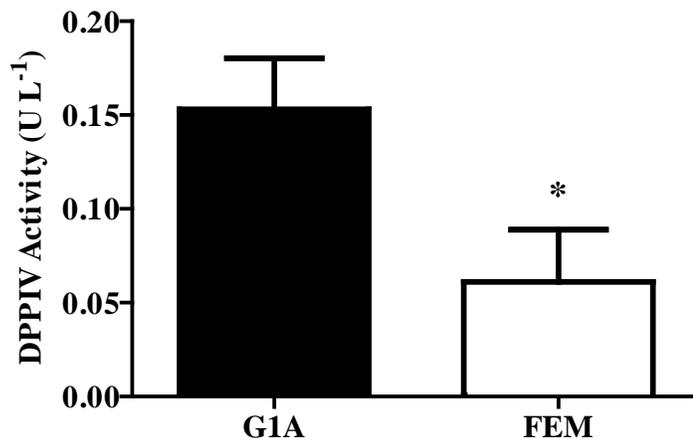


Figure 2. DPPIV activity of gastrocnemius first-order arterioles (G1A: n = 16) and femoral arteries (FEM: n = 16). DPPIV activity was assessed using the substrate, glycy-L-proline-4-methoxy-2-naphthylamide. Bars indicate mean \pm S.E.M. * Significant difference from OVX ($p < 0.05$).

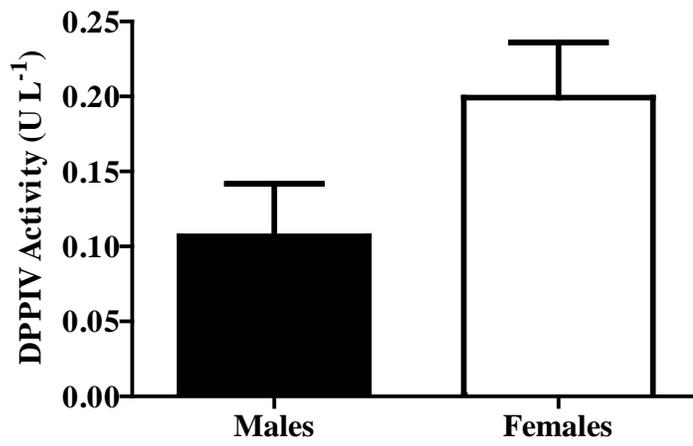


Figure 3. DPPIV activity of male (n = 8) and female (n = 8) gastrocnemius first-order arterioles. Bars indicate mean \pm S.E.M..

METHODS

Animal Model

The Fischer 344 rat (F344) exhibits neutral vascular physiology and is void of hypertensive or other inherent vascular pathological conditions that would preclude detailed vascular study (Harlan Laboratories, n.d.). All female F344 rats will be ovariectomized with 15 rats receiving an estrogen pellet (N=30) (Innovative Research of America, Sarasota, FL) at the time of ovariectomy in order to maintain experimental control over systemic estrogen concentrations. Animal behavior, food and water intake, appearance, and surgical incisions will be monitored for 10 days post-operatively. Records will be kept regarding surgical comments, animal condition, and drug administration (name, dose, route of administration). Animals will be housed in 17" X 10" X 7.5" cages. A 75%/25% mixture of hardwood chip/wood pulp bedding will be used as bedding material. Food and water will be provided *ad libitum*.

Apparatus and Procedure

Ovariectomy and Pellet (17 β -Estradiol) Implantation

Female F344 rats (3-4 months of age) were anesthetized using 40mg/kg IP, sodium pentobarbital. A ventral midline incision was made in the skin caudal to the border of the ribs and cephalad to the pubic symphysis. A subcutaneous tunnel was made lateral to the skin incision and the muscles of the abdominal wall separated to allow access to the abdominal cavity. Using forceps, the periovarian fat was grasped in order to lift and exteriorize the ovary. The fallopian tube and the uterine horn distal to the ovary were clamped and the ovary removed by cutting above the clamped area. Following removal of the ovary, the uterine horn was returned to the abdomen and the process

repeated for the second ovary. The skin incision was closed using one or two sutures or wound clips.

In the chronic estrogen supplementation group, estrogen pellets (.25mg; 60 days) were delivered subcutaneously immediately following ovariectomy. The pellet was implanted in the dorsoscapular region just behind the left ear above the shoulder using a 10-gauge trochar.

The rats were treated with a topical antibiotic when necessary to prevent infection. External sutures were removed 7-10 days following surgery. Animals were housed for 6-8 weeks following ovariectomy to allow for the chronic vascular adaptations associated with estrogen to occur (LeBlanc, et al., 2009).

Terminal Experiment

Six to eight weeks post-ovariectomy, the rats were administered a single IP dose of 0.5-0.8cc of 42.5mg/ml (71mg/kg to 113mg/kg) pentobarbital sodium to induce a deep plane of anesthesia. Depth of anesthesia was determined by lack of response to toe pinching. Following induction, arterioles were dissected out and removed. Arterioles were cannulated with glass pipettes to measure vessel diameter. The process of removing the arterioles took approximately 20 minutes. Blood (5ml) was collected via cardiac puncture. Blood samples were centrifuged for 3 minutes, the supernatant removed, and the serum stored at -80C. Immediately following removal of the vessels, an intracardiac dose of pentobarbital and a pneumothorax was administered. The uterus was removed, trimmed of connective tissue, and weighed.

Vessel chamber. Arterioles were isolated in a refrigerated vessel chamber containing cold (4°C) Krebs-Ringer physiological saline solution (119mM NaCl, 4.7mM

KCl, 2.5mM CaCl₂, 1.2mM MgSO₄, 25mM NaHCO₃, 1.2mM KH₂PO₄, 5.5mM glucose, 2mM glycerol) (Pourageaud & De Mey, 1998) bubbled with 30% O₂, 10% CO₂, 60% N₂. Using 11-0 ophthalmic suture, the arterioles were tied securely to micropipettes in a vessel chamber (Living Systems, Inc., Burlington, VT) and filled with Krebs-Ringer physiological saline solution (described above) containing 1% albumin (pH 7.4, 37°C) (Pourageaud & De Mey, 1998). The bath was filled with Krebs-Ringer physiological saline solution (pH 7.4, 37°C) and transferred to the stage of an inverted microscope (Olympus (CKX41), Melville, NY). Luminal diameter was monitored during equilibration and viability testing (described below) using video calipers (Colorado video 307A Horizontal video calipers, Boulder, CO) and recorded on a computer. The bath was gradually warmed and maintained at 37°C for the equilibration period. Micropipettes were connected to independent reservoir systems. Luminal pressure was initially set at 60cm H₂O, which is a pressure similar to normal in vivo pressure in 1A arterioles (Williams & Segal, 1993). The bath solution was replaced every 15 minutes during equilibration. Arterioles were considered viable if they constricted to phenylephrine (10⁻⁵M) by at least 10% and dilated by at least 20% to acetylcholine (10⁻⁶M) (Schneider, et al., 1994). Maximum vasoconstriction (µm) was assessed by the average vasoconstriction produced by potassium chloride (80mM) prior to, and immediately following, that day's experiments. The arteriole was washed out 5 times, and a calcium-free buffer was added to the bath to measure maximum arteriole dilation (µm).

Field Stimulation. The arteriole was field stimulated using two platinum electrodes connected to an electrically isolated, constant current stimulator (Digitimer

(DS3), Welwyn Garden City, Hertfordshire, UK). In our lab's experience, field stimulation can be repeated up to four times without a loss of vascular tone.

Randomization. The following series described below were randomized to minimize variability due time effects (i.e. the length of time the vessel is maintained in the microvessel bath). The cumulative concentration response curves within Series 3 were randomized to reduce sequence effects.

Series 1: NPY release to field stimulation. The vessel received field stimulation (60Hz, 9mA, 7 impulses), followed by extraluminal sampling (~200µl) immediately after stimulation and 30 seconds post-stimulation. Samples were flash-frozen with liquid nitrogen and stored at -80C. At the conclusion of the first set of experiments, the vessel chamber was washed five times and allowed to equilibrate for 30 minutes before proceeding to the next set of experiments.

Series 2: Y₁ post-junctional receptor sensitivity. A cumulative concentration response curve using the Y₁ specific agonist, [Leu31Pro34]NPY (Bachem, King of Prussia, PA), was performed (10⁻¹¹-10⁻⁵M). Doses were administered at five minute intervals. Data was expressed as a percentage of maximum contraction (µm), percentage of baseline tension, EC50, and slope. Data were analyzed using EC50 and slope. At the conclusion of the second set of experiments, the vessel chamber was washed five times and allowed to equilibrate for 30 minutes before proceeding to the next set of experiments to avoid possible residual effects from the previous experiment.

Series 3: NPY potentiation of adrenergic vasoconstriction. A cumulative concentration response curve for the α-adrenoceptor agonist, norepinephrine (10⁻¹¹-10⁻⁶M), was performed to determine adrenergic responsiveness of the vessel. A

concentration of NPY₍₁₋₃₆₎ (10^{-7} M) capable of eliciting potentiation of norepinephrine vasoconstriction was added to the bath and allowed to incubate for five minutes. A second cumulative concentration response curve for norepinephrine was performed to examine the potentiation effect of NPY on adrenergic vasoconstriction. Data were expressed as a percentage of maximum contraction (μ m), percentage of baseline tension, EC50, and slope. Data were analyzed using EC50 and slope. The vessel bath was washed out and the arteriole allowed to equilibrate as described above.

Series 4: DPPIV activity in gastrocnemius first-order arterioles. The DPPIV inhibitor, diprotin A (1μ M), was added to the bath and allowed to incubate for 20 minutes. The arteriole received field stimulation (60Hz, 9mA, 7 impulses), which was followed by extraluminal sampling ($\sim 200\mu$ l) immediately after stimulation and 30 seconds post-stimulation. Samples were flash-frozen with liquid nitrogen and stored at -80C. Upon completion of all animal experiments, samples were analyzed for NPY content using a peptide enzyme immunoassay (Bachem, King of Prussia, PA).

Arterioles from the contralateral gastrocnemius were homogenized in Krebs-Ringer buffer, centrifuged, and the supernatant removed for DPPIV analysis. DPPIV samples were flash-frozen with liquid nitrogen and stored at -80C. Upon completion of all animal experiments, samples were analyzed for DPPIV and total protein content (Thermo Scientific, Rockford, IL) using spectrophotometric assay. Serum concentrations of 17β -estradiol were determined using an estradiol immunoassay (Estradiol EIA kit, Oxford Biomedical, Oxford, MI).

Peptide enzyme immunoassay (EIA). Vessel bath samples and EIA kit components were brought to room temperature before proceeding with NPY analysis.

The samples were placed in an antibody-coated, 96-well plate with provided standards. Primary antiserum and biotinylated peptide solutions were added and incubated for two hours at room temperature. The samples were washed with an assay buffer and treated with streptavidin HRP solution and incubated for 60 minutes at room temperature. Following a second wash cycle, the substrate was added and the absorbance read at 450nm (Bio-Tek Instruments Inc., Winooski, VT). The concentration of NPY was determined using a normal curve. The assay's minimum detectable NPY concentration is 2 to 3 pg per well or 0.06ng/ml.

Dipeptidyl peptidase IV assay. Whole vessel homogenate samples were brought to room temperature. The samples were then transferred to a 96-well plate and glycyl-L-proline-4-methoxy-2-naphthylamide added (37). The samples were incubated for 20 minutes at 37°C and the reaction stopped using a citrate solution (100mM). The fluorescent signal was read at 340 and 425nm by a FLX800 microplate reader. DPPIV activity was defined as the activity that produces 1µM of 4-methoxy-2-naphthalamine in one minute (Scharpe, et al., 1988).

Protein assay. Whole vessel homogenate samples were brought to room temperature. Samples (150µl) were transferred to a 96-well plate along with a working reagent (150 µl). The plate was placed on a heating block (37°C) and allowed to incubate for 2 hours. The plate was removed from the heating block to equilibrate to room temperature (~10min) before analysis. Absorbance of a colorimetric signal was read at 562nm. Total protein content (µg/ml) was determined using a bovine serum albumin standard curve.

17 β -estradiol assay. The organic phase was separated by vortexing the sample with ethyl ether. The solvent was evaporated using a nitrogen stream, and the remaining residue was diluted in extraction buffer. This sample was added to the microplate along with the enzyme conjugate, shaken, and incubated at room temperature for one hour. Following a wash cycle, the substrate was added with optimal color development (650nm) occurring within 30 minutes of administration. Color development was inversely proportional to the concentration of estradiol.

Statistical Design

It is prudent to minimize the number of statistical tests in order to decrease type I error rate. Therefore, data of similar formats (e.g. cumulative concentration response curves and NPY release) were analyzed together when possible. A multivariate analysis of variance ($\alpha=.05$) was used to analyze the data from series 2 and 3. The EC50 and slope were recorded for NPY, norepinephrine, and NPY+ norepinephrine cumulative concentration response curves. A repeated measures design ($\alpha=.05$) was used to assess differences in data from series 1 and 4. NPY release (ng ml^{-1}) with and without diprotin A (dipeptidyl peptidase IV inhibitor) was recorded at 0 seconds and 30 seconds post-field stimulation. Greenhouse-Geisser's epsilon was used as the criterion (O'Rourke, Hatcher, & Stepanski, 2005) for determining usage of either the univariate ($\epsilon > 0.75$) or multivariate ($\epsilon < 0.75$) case, where appropriate. Dipeptidyl peptidase IV activity (second part of series 4) was analyzed using a one-way analysis of variance design ($\alpha=.05$).

In our lab, we have previously detected differences in NPY release from gastrocnemius 1A arterioles. Based on our prior work, we needed at least 12 animals to achieve power $> .80$ with $\alpha= .05$ ($\Delta=1.83$). Previous analysis of dipeptidyl peptidase IV

activity in our lab did not include the vessel's size in the determination of activity. It was possible that total enzyme activity as determined by the amount of synthetic substrate converted to measurable product was a function of vessel size (i.e. there was more physical dipeptidyl peptidase IV protein in some experiments). Consequently, the standard deviations were higher than desired, which for the present experiment, would have required a larger group size ($n=21$) in order to reach our desired power ($>.80$). Therefore, the protein assay was included in order to normalize the DPPIV activity with respect to the vessel's size (total protein content). Power calculations of subsequent enzyme assay (monoamine oxidase) results using protein quantification to normalize activity required a group size of 12 to meet a power of at least .80 with $\alpha=.05$ ($\Delta=1.87$). NPY-mediated vasoconstriction increases in magnitude as vessel diameter decreases. Joshua (1991) observed decreases of $\sim 39\pm 2\%$ in first-order arteriole diameter following NPY administration. This large effect yields a substantial degree of power ($>.90$). Based on the aforementioned power calculations, this study utilized groups of 15 animals ($N=30$), which was deemed sufficient to detect group differences with power $>.80$; $\alpha=.05$.

RESULTS AND DISCUSSION

Results

Model assumptions for all statistical instruments were examined with follow-up measures performed when necessary. Data considered essential for interpretation are included in the results. Appendix A contains more detailed information regarding data organization, inclusion criteria for statistical analysis, and the handling of model assumptions.

A treatment duration of 8 weeks (56 days) was selected to allow for an adequate amount of exposure time to estrogen in order to detect the presence of physiological adaptations to chronic estrogen exposure. The average treatment time (from OVX/OVE surgery to terminal experiment) was 55.76 ± 0.30 days, and the ages at the time of terminal experiment ranged from 158 days to 224 days. The length of a terminal experiment was 6 hours; thus, a maximum of two experiments could be performed on any given day. The small variation in treatment duration was due, in part, to conflicts that were either unavoidable or beyond control (meetings, equipment failure/damage). A total of 35 rats were ovariectomized, while 17 rats also received the 17β -estradiol pellet. The only death that occurred during the treatment phase was attributed to sodium pentobarbital overdose. No rats exhibited signs or symptoms of surgical complications during the 10-day post-surgical observations, and there were no signs of infection or adverse conditions at the time of the terminal experiment.

Animal Characteristics

Plasma estrogen concentrations were greater in rats with estrogen supplementation (OVE) as compared to rats without estrogen supplementation (OVX;

Figure 4: $F(1, 9) = 7.67, p < 0.05$). The presence of estrogen can produce alterations in phenotype that are readily observable and easy to measure. OVE exhibited less weight gain over the 8-week period (Figure 5: $F(1, 32) = 47.05, p < 0.05$) but had substantially larger uteri as opposed to OVX (Figure 6: $F(1, 26) = 1812.17, p < 0.05$). Figure 7 displays representative uteri from OVE (A and B) and OVX (C and D) rats. The collective statistical effects of measurements related to estrogen supplementation were high (plasma estrogen: $d = 1.68$; body weight: $d = 2.35$; uterine weight: $d = 16.09$). These data suggest a significant difference in estrogen concentrations between OVE and OVX rats.

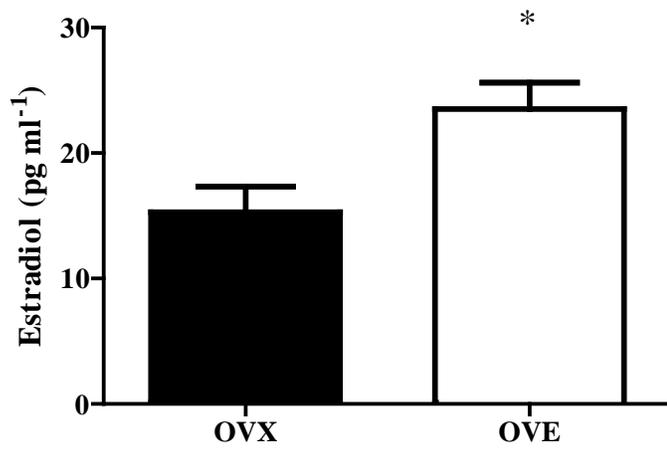


Figure 4. Plasma estradiol concentration of OVX (n = 5) and OVE (n = 6) rats. Estrogen supplementation resulted in an increase in plasma estradiol levels. Bars indicate mean \pm S.E.M. * Significant difference from OVX ($p < 0.05$).

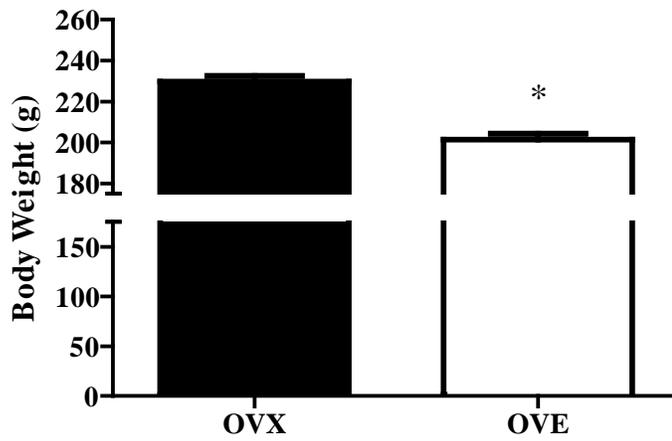


Figure 5. Body weight of OVX (n = 17) and OVE (n = 17) rats. Estrogen supplementation resulted in significantly less body weight. Bars indicate mean \pm S.E.M. * Significant difference from OVX ($p < 0.05$).

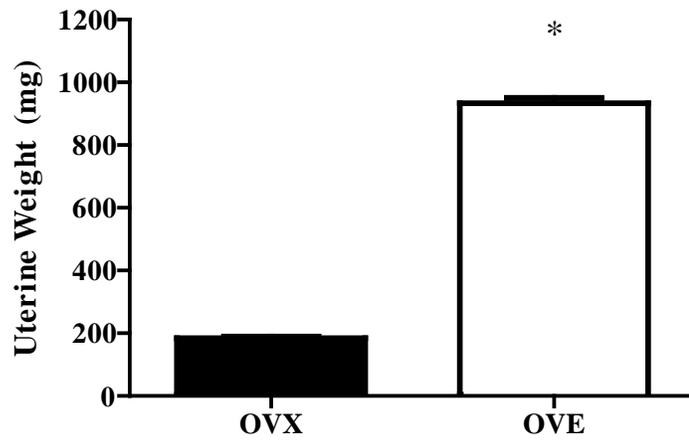


Figure 6. Uterine weight of OVX (n = 14) and OVE (n = 14) rats. Estrogen supplementation resulted in significantly greater uterine weight. Bars indicate mean \pm S.E.M. * Significant difference from OVX ($p < 0.05$).



Figure 7. Representative uteri from OVE (A and B) and OVX (C and D) rats.

Series 1: NPY release to field stimulation

Field stimulation at a high frequency is needed in order to facilitate the exocytosis of large dense-cored vesicles within the sympathetic nerve terminal (Lundberg, Pernow, Franco-Cereceda, & Rudehill, 1987). NPY release increased from 0 to 30 seconds in OVX and OVE rats (Figure 8: $F(1, 21) = 6.44, p < 0.05$). While OVX ($n = 12$) rats experienced slightly greater NPY release at 0 and 30 seconds, the release was not statistically different ($F(1, 21) = 0.05, p < 0.83, D^2 = 0.05$) from OVE ($n = 11$).

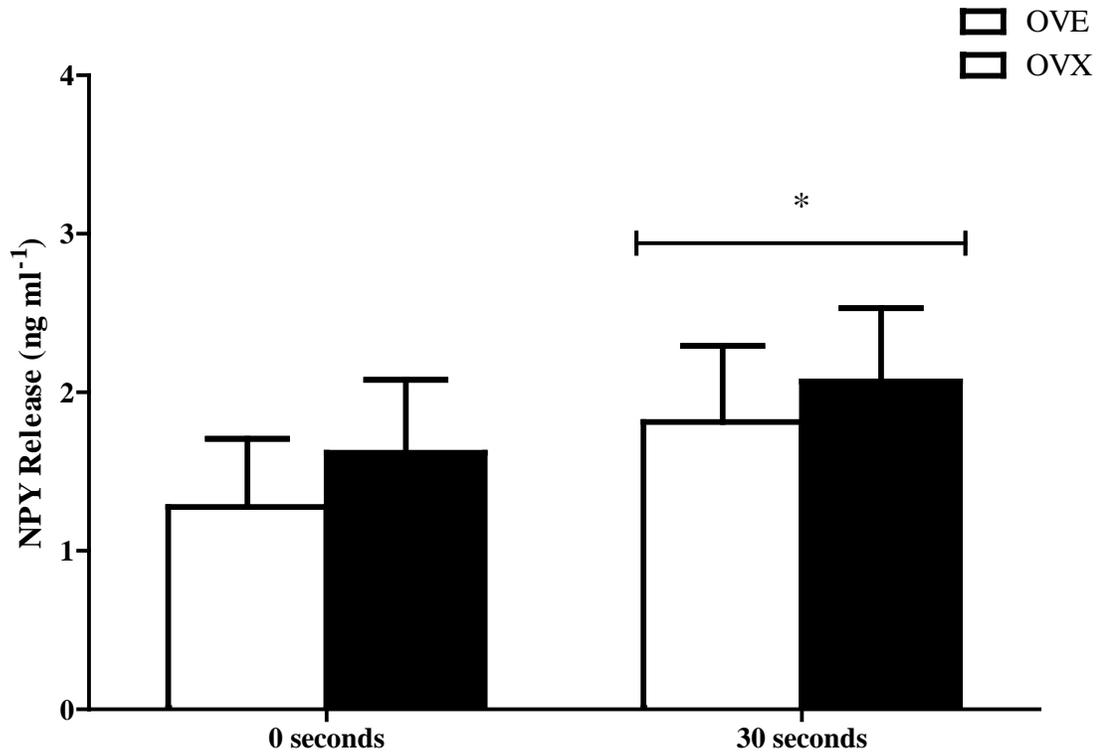


Figure 8. NPY release in gastrocnemius first-order arterioles of OVX (n = 12) and OVE (n = 11) rats. NPY release was greater at 30s following field stimulation for all rats irrespective of estrogen status. Bars indicate mean \pm S.E.M. * Significant difference from 0s ($p < 0.05$).

Series 2: Y₁ post-junctional receptor sensitivity

This series was performed to determine the effects of long-term estrogen supplementation on Y₁-receptor activity in skeletal muscle arterioles. The Y₁-receptor agonist, [Leu31Pro34]NPY, elicited a 45% decrease in vessel diameter of skeletal muscle arterioles. Estrogen status did not affect the maximum amount of Y₁-mediated vasoconstriction ($F(1, 14) = 0.01$, $p = 0.91$; $d = 0.05$) with OVE ($55.07 \pm 5.10\%$; $n = 9$) producing similar magnitudes of vasoconstriction to that observed in OVX ($55.97 \pm 6.25\%$; $n = 7$). The sensitivity of Y₁-receptor actions to cumulative concentrations of [Leu31Pro34]NPY did not differ with respect to estrogen status (Figure 9: $F(2, 13) = 0.84$, $p = 0.45$; $D^2 = 0.46$).

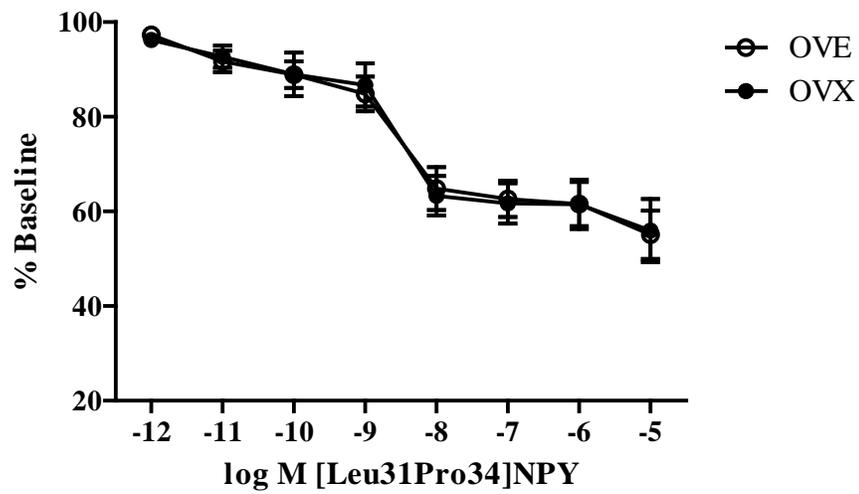


Figure 9. Cumulative concentration response curve for Y₁-receptor agonist. NPY-mediated vasoconstriction was similar in OVX (n = 7) and OVE (n = 9) rats. Bars indicate mean ± S.E.M.

Series 3: NPY potentiation of adrenergic vasoconstriction

NPY potentiates norepinephrine-mediated vasoconstriction through Y_1 -receptor activation. This series was performed to assess the effects of estrogen on this Y_1 -dependent mechanism. OVE (n = 12) did not differ from OVX (n = 13) in norepinephrine-stimulated vasoconstriction (Figure 10: $F(2, 22) = 0.19, p = 0.83; D^2 = 0.06$). Interestingly, neither OVE (n = 11) nor OVX (n = 10) exhibited NPY potentiation of norepinephrine-stimulated vasoconstriction (Figures 11: $F(2, 18) = 1.23, p = 0.32; D^2 = 0.49$).

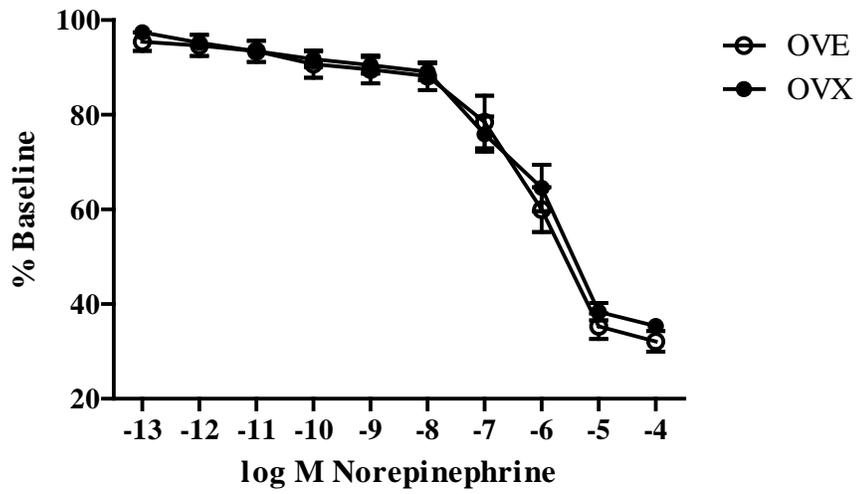


Figure 10. Cumulative concentration response curve for α -adrenergic-receptor agonist. Norepinephrine-mediated vasoconstriction was similar in OVX (n = 13) and OVE (n = 12) rats. Bars indicate mean \pm S.E.M.

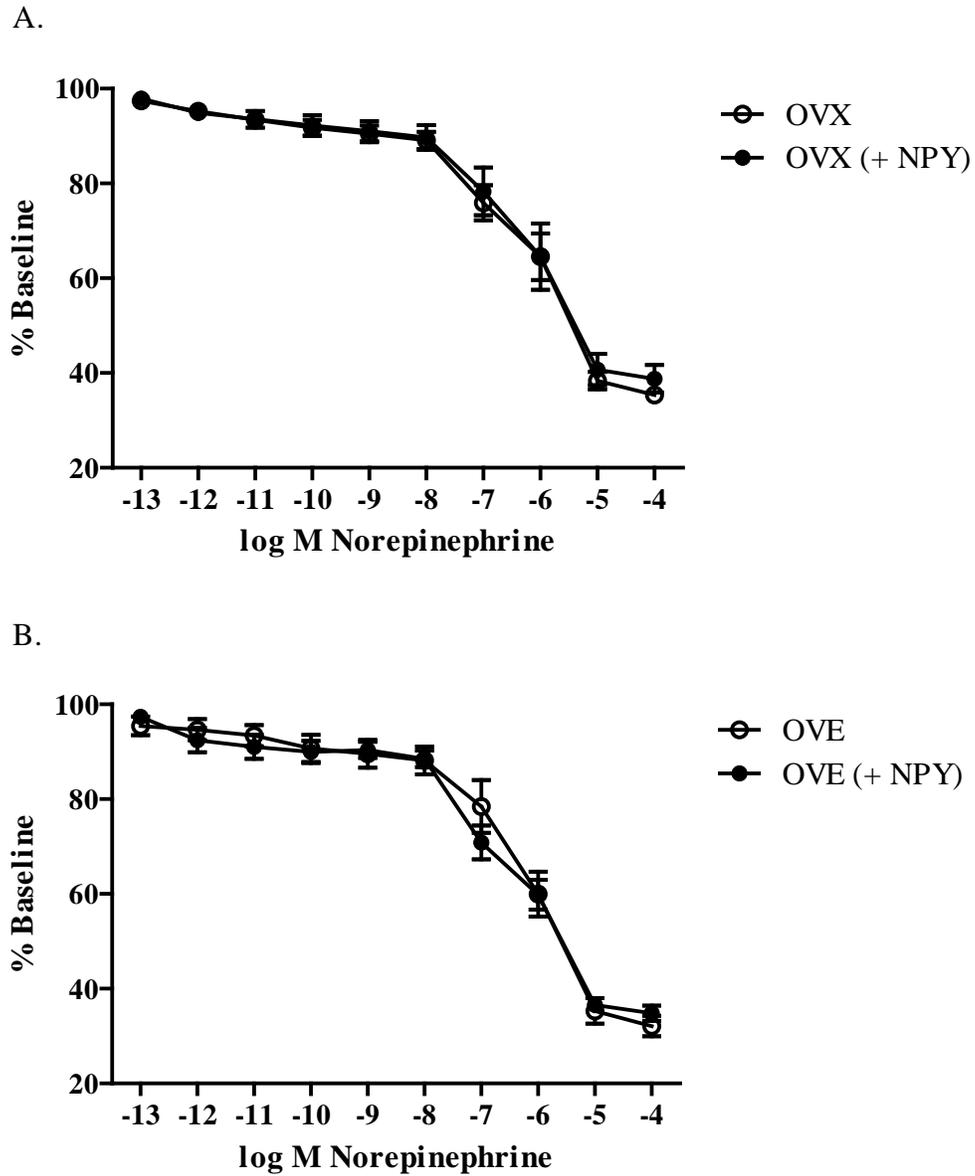


Figure 11. Cumulative concentration response curve for NPY ([Leu31Pro34]NPY) potentiation of α -adrenergic-receptor agonist in OVX (A: n = 10) and OVE rats (B: n = 11). NPY failed to potentiate norepinephrine-mediated vasoconstriction in OVX and OVE rats. Bars indicate mean \pm S.E.M.

Series 4: DPPIV activity in gastrocnemius first-order arterioles

The enzymatic breakdown of NPY can impact the physiological response as only the full-length peptide stimulates vasoconstriction. The DPPIV inhibitor, diprotin A, was added to the vessel bath to determine the effects of DPPIV activity on NPY bioavailability according to estrogen status. DPPIV inhibition resulted in an increase in NPY bioavailability at 0s and 30s following field stimulation in OVX (n = 12) and OVE (n = 11) rats (Figure 12; $F(3, 19) = 4.74, p < 0.05$); however, there were no differences across groups ($F(3, 19) = 1.10, p = 0.37, D^2 = 0.74$).

Whole vessel homogenates were analyzed to directly assess DPPIV activity. DPPIV activity (Figure 13: $F(1, 26) = 0.01, p = 0.94, d = 0.03$) did not differ between OVX (n = 13) and OVE rats (n = 15). Follow-up protein assay was unable to determine differences in total vascular protein of OVX (n = 9) and OVE (n = 9) vessels (see Limitations). DPPIV activity was similar between groups and the proteolytic actions of DPPIV played an integral role in modulating the bioavailability of NPY regardless of estrogen status in this young-adult cohort.

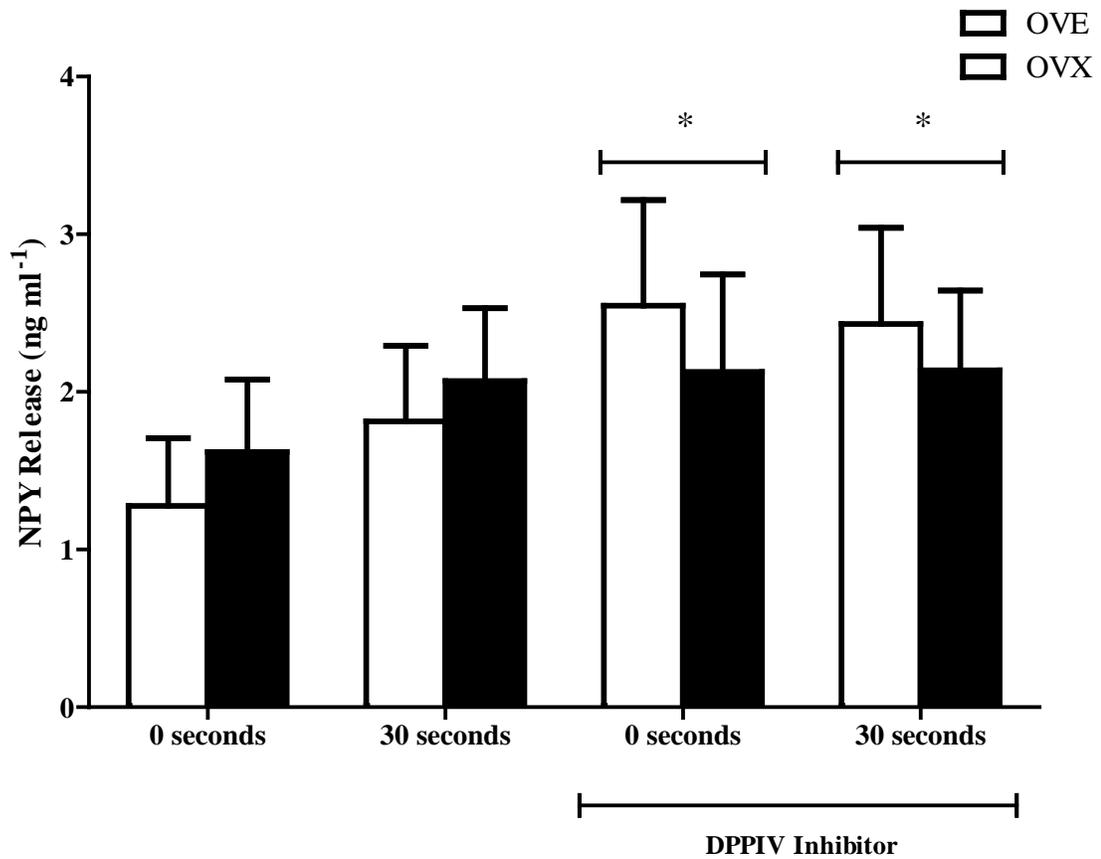


Figure 12. Effects of DPPIV inhibition on NPY release in gastrocnemius first-order arterioles of OVX (n = 12) and OVE (n = 11) rats. NPY release was greater with DPPIV inhibition (diprotin A) at 0s and 30s following field stimulation for all rats regardless of estrogen status. Bars indicate mean \pm S.E.M. * Significant difference from control conditions (0s and 30s, respectively; $p < 0.05$).

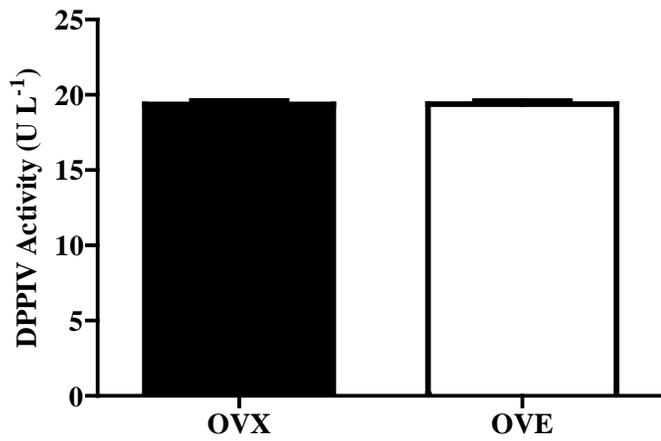


Figure 13. DPPiV activity of OVX (n = 13) and OVE (n = 15) first-order arterioles. Estrogen supplementation did not influence DPPiV activity. Bars indicate mean \pm S.E.M.

Discussion

NPY Vasoconstriction

The efficacy of NPY as a vasoconstrictor depends on the level (artery, arteriole) and location (mesentery, brain, skeletal muscle) of the vasculature under study. Very little is known as to the behavior of NPY in the skeletal muscle arterioles of females. Prior study (Joshua, 1991) of male skeletal muscle arterioles revealed an inverse relationship between the magnitude of vasoconstriction (vessel diameter changes) and vessel size (first-, second-, third-order arterioles). The amount of vasoconstriction observed in female skeletal muscle first-order arterioles was similar to previous measurements of the same vessel type observed in males (Joshua, 1991). The cornerstone of the study was the long-term effect of estrogen on NPY neurotransmission. The available studies on sex-differences (Jackson, et al., 2005a, 2005b) in NPY neurotransmission have suggested that sex hormone status is a likely cause underlying the differences between males and females. The present data fail to support a link with respect to estrogen and Y₁-receptor activity as there were no differences between OVX and OVE groups. While the null hypothesis was not rejected, the results further demonstrate the complexities involved in identifying the factors of influence behind the sex-differences in vascular physiology.

An absence of an estrogen effect is not unprecedented with similar null results having been observed in adrenergic neurotransmission. Stice et al. (2009) observed no differences in adrenergic responsiveness in female aortas of OVE and OVX rats. The aortas of rats without estrogen possessed greater contractile force (developed tension) than rats with estrogen, but the receptor responsiveness according to the adrenergic dose-

response curve did not differ between groups. Females with compromised estrogen profiles may experience vascular smooth muscle thickening (Moreau, et al., 2002; Takahashi, et al., 2004), which is a common clinical sign for increased risk of cardiovascular disease. The nature of microvessel diameter measurement does not allow for an inference as to the amount of contractile force a vessel possesses; therefore, it was unknown if OVX vessels possessed greater contractile force. Y_1 -receptor protein was not quantified during the present study; thus, it is unknown if either of the groups experienced a change in receptor protein with a concurrent change in receptor sensitivity. A recent study by Jackson, Ellis, and Shoemaker (2010) detected estrogen-related differences in Y_1 -receptor expression in white vastus muscle of young mature (2mo) female rats. The increase in post-junctional receptor expression in rats without estrogen was in conjunction with changes in hemodynamics of conduit vessels (external iliac artery) in response to Y_1 -receptor blockade. However, there was a nonsignificant increase in Y_1 -receptor in red vastus of rats with estrogen. These equivocal results may suggest that differences in Y_1 -receptor actions may occur in response to changes in estrogen status, but these changes may be predicated on the type of muscle (red or white) tissue and corresponding blood vessels under study. The present results would support a part of this concept in that Y_1 -receptor actions were identical in the red first-order arterioles of young adult females irrespective of estrogen status. It was concluded that NPY can induce a moderate amount of vasoconstriction in isolated first-order arterioles of young adult females, and estrogen does not impact Y_1 -receptor sensitivity in these vessels.

NPY Potentiation of Adrenergic Vasoconstriction

Neuropeptide Y is well known for its ability to potentiate norepinephrine-induced vasoconstriction at low concentrations (10^{-7} – 10^{-9} M) (Abel & Han, 1989; Han, et al., 1998; Vu, et al., 1989). The type of potentiation can refer to augmentation of the vascular response (vasoconstriction, increases in perfusion pressure) achieved following a neural stimulus (Glenn & Duckles, 1994; Han, et al., 1998), or it can refer to a leftward shift in the dose-response curve of norepinephrine indicating a greater sensitivity to norepinephrine (Abel & Han, 1989). The ability of NPY to potentiate any facet of adrenergic vasoconstriction may depend on other factors such as vessel type and sex. In the present study, OVX and OVE groups experienced a nonsignificant increase to norepinephrine-mediated vasoconstriction following NPY administration. The concentration used, 9.8×10^{-8} M, is within the previously mentioned range of concentrations where NPY potentiates norepinephrine-mediated vasoconstriction. However, the vessels of both groups exhibited vasoconstriction to this concentration of NPY, which could have masked minor influences on adrenergic vasoconstriction.

There are many examples of NPY potentiation of norepinephrine effects on perfusion pressure of whole systems such as the mesentery (Han, et al., 1998; Westfall, et al., 1988). Arterial beds provide a story as to a particular vascular system. However, the potentiation cannot be accurately ascribed to any particular level within the system since the pressure changes represent a collective response to the stimulus (norepinephrine, norepinephrine + NPY). Isolated vessel techniques circumvent the aggregate actions of whole vascular beds to allow for careful examination at specific levels of the vasculature. Thus, while potentiation may occur in some levels of the vasculature, it does not appear to occur in skeletal muscle first-order arterioles.

The existence of multiple pathways for NPY-mediated vasoconstriction may explain the lack of potentiation in norepinephrine vasoconstriction. NPY can influence both potassium and calcium flux via the inhibition of cAMP (Lobaugh & Blackshear, 1990) and the production of IP₃ (Duckles & Buxton, 1994), respectively. Norepinephrine stimulates vasoconstriction through the production of IP₃; therefore, the absence of NPY potentiation of norepinephrine would support the assertion that NPY does not cause a meaningful increase of IP₃ in skeletal muscle arterioles. This is consistent with other studies of arterioles that failed to observe a difference in vasoconstriction following the inhibition of cAMP production (Andriantsitohaina, et al., 1990). It is certainly possible that the ability of NPY to potentiate the effect of norepinephrine is dependent on vessel size as some larger vessels experience increases in IP₃ production following NPY administration (Duckles & Buxton, 1994). It was surmised based on the present results that the cAMP inhibitory mechanism is the predominant mechanism through which NPY-mediated vasoconstriction occurs in skeletal muscle arterioles of females, and that NPY does not elicit a meaningful amount of IP₃ production to potentiate the vasoconstriction of norepinephrine. The similarity of response to NPY in animals with and without estrogen suggests that the Y₁-receptor mechanism was not influenced by estrogen supplementation. NPY was a potent vasoconstrictor of skeletal muscle arterioles in both groups, but it does not affect the vascular response to norepinephrine.

NPY Release

There is little information, if any, pertaining to NPY release from skeletal muscle arterioles. Previous studies of whole skeletal muscle and conduit arteries have detected sex differences in total NPY content of the respective tissues. It has been speculated that

estrogen could contribute to a portion of the differences observed between males and females. In the present study, chronic estrogen supplementation did not affect NPY release at 0 or 30 seconds following field stimulation. These data suggest that chronic estrogen does not influence NPY release in skeletal muscle first-order arterioles of young adult females.

Estrogen affects the expression of many vascular proteins (Miller & Duckles, 2008); therefore, it was plausible that estrogen was an underlying mechanism behind the sex differences observed in total NPY content of various tissues. A follow-up study into the effects of estrogen supplementation on NPY metabolism by Jackson et al. (2010) concluded that rats with estrogen had less NPY content in red and white vastus muscles. The differing results between the present study and the study by Jackson et al. could be attributed to several factors. First, whole muscle homogenate does not discriminate between vessel type (arteries, veins, capillaries, venues); thus, it is difficult to pin down the source behind the differences in NPY content. Whole muscle homogenate will also include blood elements that contain NPY originating from monaural sources (platelets, adrenal gland). These sources may be sensitive to estrogen supplementation, which could affect the expression of NPY. The present study possesses experimental control for extraneous sources of NPY such as those related to blood elements. Therefore, the NPY content recorded is indicative of the NPY release characteristic of an isolated, skeletal muscle first-order arteriole, which appears to be independent of estrogen influence.

While this study was not directly concerned with age, age-related physiological issues in females often encompass dysfunctional release of sex steroids. Recent work by Di Carlo et al. (2007) discovered increases in NPY content of human uterine arteries that

correlated with the different phases of menopause (pre- and post-menopause). These data require a broader context for interpretation as other hormones (progesterone) may also influence vascular NPY content. Ageing, as it relates to vascular physiology, also affects multiple aspects of the vasculature that involve alterations in function to both the endothelium and the vascular smooth muscle (Glenn & Duckles, 1994; Kitlinska, Lee, Movafagh, Pons, & Zukowska, 2002). The present finding indicates that estrogen, in and of itself, does not contribute to a change in NPY release in isolated, skeletal muscle first-order arterioles.

DPPIV Activity

The actions of DPPIV can significantly impact the type and magnitude of response initiated by NPY. Glenn et al. (1997) was one of the first to detect differences in vasoconstriction during peptidase inhibition between sham and ovariectomized females. An elegant study by Jackson et al. (2005) proposed that females possessed a greater level of proteolytic activity than males in order to depress Y_1 -receptor-mediated vasoconstriction. Thus, the hypothesis for estrogen's involvement in the attenuation of NPY-mediated vasoconstriction was developed. The present findings fail to support a link between estrogen and NPY metabolism in skeletal muscle arterioles. While no effect was observed with estrogen supplementation, the results do reveal some interesting developments in our concept of NPY in the vasculature.

DPPIV activity plays a significant role in mitigating NPY availability in resistance vessels of young adult females regardless of estrogen status. The physiological significance of an augmented role for DPPIV in the female resistance vasculature is that females would have less Y_1 -receptor actions, ergo less NPY-mediated vasoconstriction.

Systemic blood pressure is maintained, in part, through the actions of arterioles, which control blood flow into the capillary beds (Segal, 2005). While it is difficult to determine the meaningfulness of DPPIV activity in influencing blood flow through the resistance vasculature, the present results do suggest a significant role for DPPIV in modulating the amount of available NPY.

The stark similarities of DPPIV activity through direct (enzymatic assay) and indirect (NPY assay following DPPIV inhibition) measurements provide evidence to support the idea that estrogen does not affect DPPIV mechanisms in first-order arterioles. Jackson et al. (2010) detected no difference in DPPIV activity with estrogen supplementation in whole tissue homogenate of red and white vastus muscles. The strength of the present study was the DPPIV analysis of isolated first-order arterioles. Thus, DPPIV activity is independent of estrogen influences, and it is active in modulating NPY in first-order arterioles of young adult females.

Practical Implications

The release and metabolism of NPY can have far reaching effects; thus, it is prudent to understand how and under what circumstances these mechanisms associated with NPY metabolism change in the vasculature. A practical concern relevant to cardiovascular health is the increase in blood pressure that accompanies the ageing process (Narkiewicz, et al., 2005; Ng, Callister, Johnson, & Seals, 1993). This is a multifaceted issue consisting of alterations in neurotransmitter release, receptor expression on the vascular smooth muscle, vascular smooth muscle cell proliferation, and endothelial cell function, to name a few. The estrogen-independent conclusions of the present study are not unprecedented. Stice et al. (2009) failed to detect estrogen-related

differences in adrenergic vasoconstriction; however, there were differences with age. Aged females were more sensitive to adrenergic agonists (phenylephrine) as compared to young adult females irrespective of estrogen status. It should be noted that both young and old females without estrogen generated greater amounts of tension as compared to young and old females without estrogen. A greater amount of vascular tension can be a function of vascular smooth muscle cell proliferation. As stated above, vessel tension cannot be extrapolated from vessel diameter. The inability to measure vessel tension along with the inconclusive measurements of total vascular protein makes it difficult to ascertain whether estrogen status influenced vascular smooth cell growth in the present study. It is plausible, however, that estrogen-related differences in blood pressure with age are actually a product of small increases in vascular smooth muscle cell proliferation as opposed to increases in receptor actions of sympathetic neurotransmitters such as NPY.

Correlations between NPY content and menopause status have been observed in human uterine arteries. Menopause marks an onset of dysfunctional release of sex steroids (estrogen, progesterone). It is because of these interrelationships that sex steroids, specifically estrogen, are believed to be crucial in modulating NPY metabolism. The absence of an estrogen effect in the present study would run counter to an estrogen-specific response on NPY metabolism. Perhaps the previous relationships between hypoestrogenism and NPY content is dependent on progesterone or possibly on synergistic effects requiring both sex steroids. Another caveat in the study of sex steroid influence in 'intact' females lies in the enzymatic milieu and form of estrogen present. Some vascular smooth muscle cells express steroid enzymes such as sulfatases and

sulfotransferases, which affect the amount of estrogen in conjugated form (Nakamura, et al., 2003). These enzymes in addition to 17 β -hydroxysteroid dehydrogenase-1 can affect the overall estrogen profile. This profile necessitates careful consideration due to the variable potency of the different forms (estrone, estradiol) of estrogen in the vasculature. It is important to elucidate the underlying mechanisms behind menopause and NPY expression to expand our knowledge into factors related to ageing in females.

NPY is also an angiogenic peptide (Zukowska-Grojec, et al., 1998) with growth factor capabilities that are equivalent to that seen with vascular endothelial growth factor under certain conditions (Kurimoto, et al., 2004). The angiogenic form of NPY is primarily associated with its fragmented metabolites that express an affinity for Y₂ and Y₅ receptors, although the Y₁ receptor also initiates angiogenic mechanisms as well (Movafagh, Hobson, Spiegel, Kleinman, & Zukowska, 2006). Since DPPIV produces these truncated forms of NPY (NPY₃₋₃₆), increased activity of this enzyme at the local vascular level could produce pro-angiogenic processes. Therefore, DPPIV has a dual role in NPY metabolism by decreasing the amount of vasoconstrictive NPY while concurrently increasing the amount of pro-angiogenic NPY. This action could result in both beneficial and deleterious health effects. On one hand, this function could serve to improve overall cardiovascular function by increasing the number of collateral branches from arterioles through angiogenesis and decreasing the amount of vasoconstriction via NPY mechanisms. However, as a growth factor, increased proliferation of vascular smooth muscle cells could lead to changes in the tunica intima/media ratio under certain conditions, which is a risk factor for cardiovascular event.

NPY and estrogen both possess pleiotropic actions in the vasculature. It is this nature that necessitates thorough analysis from multiple angles in order to piece together possible interrelationships between NPY and estrogen. Future studies should include progesterone supplementation in combination with estrogen to better mimic in vivo conditions, which would allow for the identification of possible synergistic effects between hormones. Studies that include peptide/hormone expression (transcripts, prepro/prohormone) in addition to receptor expression along with subtypes would allow for a more comprehensive picture into sex steroid influences on NPY neurotransmission.

Caveats

Ovariectomy of all animals was necessary to ensure experimental control over estrogen concentrations. Surgery and long-term storage can create stress on the animals, which could affect normal physiology. NPY release was elevated in both groups as compared to intact animals of the same age (unpublished data). Therefore, caution should be used when comparing results from the present study to those found in intact females.

The age of animal selected (6 months) represents a young adult female (Turturro, et al., 1999). While this was not an ageing study, some of the research into sex steroids and sympathetic neurotransmission utilize multiple age groups. The present results suggest that long-term estrogen supplementation does not affect NPY release and degradation in young adult females. However, the process of ageing may involve other mechanisms that, combined with estrogen, produce alterations in NPY metabolism that have been observed in other studies (Glenn & Duckles, 1994).

Conclusions

Long-term estrogen supplementation does not affect NPY metabolism in young adult females. The Y_1 -receptor actions are uniform across estrogen and non-estrogen groups with maximal vasoconstriction approximately 55% of control diameter in skeletal muscle first-order arterioles. Estrogen supplementation did not influence NPY release or DPPIV activity. DPPIV plays an important role in attenuating the amount of bioavailable NPY in young adult females.

APPENDIX

Data Analysis

The initial dissertation proposal called for a total of 30 rats (15 / group). A total of 34 rats underwent surgery with half of those receiving the estrogen treatment. While there were a sufficient number of animals for experimental purposes, not all of the animals were used in each experiment. Reasons for animal exclusion on some experiments included equipment failure due to lab damage, drug costs (limited availability), and issues with vessel viability (for microvessel experiments). Information relevant to specific series of experiments are listed below.

Model assumptions ($\alpha = 0.05$) for both the univariate and multivariate case were considered for the respective statistical comparisons. The assumption pertaining to independence of observations was addressed through uniform handling and storage of the animals. Briefly, the animals were of the same strain (Fischer 344 rats) and were purchased from the same provider. Animals were housed under 12:12 hour light:dark cycles, housed in the same facility and room, and consumed the same diet. Ovariectomized (OVX) and ovariectomized + estrogen (OVE) animals shared cages in order to minimize the possibility of 'cage' effects. The previous steps were performed to ensure biological homogeneity with the exception being sex steroid profile, which was manipulated for experimental purposes.

Simple univariate descriptive statistics pertaining to distribution (Shapiro-Wilk, skewness, kurtosis) are provided for each dependent variable in addition to graphical representation (box-and-whisker plots). The following sections provide information regarding the handling of data with regards to adherence to specific model assumptions.

Animal Characteristics

Plasma estradiol concentration. Plasma estradiol was assayed for 11 rats (Figure A1: OVE [n = 6], OVX [n = 5]). Estradiol was normally distributed (Table A1), and the variances were equal.

Body weight. A total of 34 rats were ovariectomized with 17 rats receiving the estrogen treatment (Figure A2). The body weights were normally distributed (Table A2), and the variances were equal.

Uterine weight. The uterine weights were measured for all 34 rats. The normality assumption did not hold due to a few extreme scores in an otherwise narrow distribution. These extreme scores in both groups were attributed to surgical differences across rats. Ovariectomy involves the removal of the ovaries, which requires two sutures for each ovary: one at the uterine horn and one at the adipose tissue on the other side of the uterine horn. The uterine horn and ovary possess a significant amount of adiposity with a high degree of vascular supply to address. Small openings are made in the surrounding adipose tissue to allow for careful suture of the uterine horn only with minimal adipose tissue involved. A minimal amount of adipose tissue is desired when suturing as all tissue distal to the suture will become necrotic, so care is taken to prevent excessive amounts of necrosis. All rats have varying amounts of fat to deal with in and around the reproductive organs, which influences the specific location of the suture around the ovary. These small differences in suture location can affect the overall length of the uterine horn. It was concluded that the small differences in suture location were responsible for the outliers seen in both groups, which caused the deviation away from a normal distribution. Animals that comprised the outliers were removed from both groups

(OVX: # 22 (274 mg), # 27 (258 mg); OVE: # 2 (1330 mg), # 6 (1204 mg), # 12 (1174 mg). This addressed the normality assumption (Figure A3), but variances were not equal. In looking at the data and the magnitude of change in uterine weight with estrogen, it came as no surprise that the variances were not equal. A decision was made to randomly select an observation from the group with the most observations (OVX: rat #31) and remove it from statistical consideration (Glass & Hopkins, 1996). This step produced equal group sizes, which resulted in an analysis that was robust to the homogeneity of variances assumption (Glass & Hopkins, 1996).

Table A1
 Univariate Normality: Descriptive Statistics

Variable	Statistic	OVE	OVX
Plasma Estradiol Concentration	Skewness	1.20	1.28
	Kurtosis	1.30	2.89
	Shapiro-Wilk	0.91	0.84
Body Weight	Skewness	-0.50	0.11
	Kurtosis	-0.55	0.03
	Shapiro-Wilk	0.96	0.99
Uterine Weight	Skewness	-0.13	0.60
	Kurtosis	-1.01	-0.60
	Shapiro-Wilk	0.94	0.93

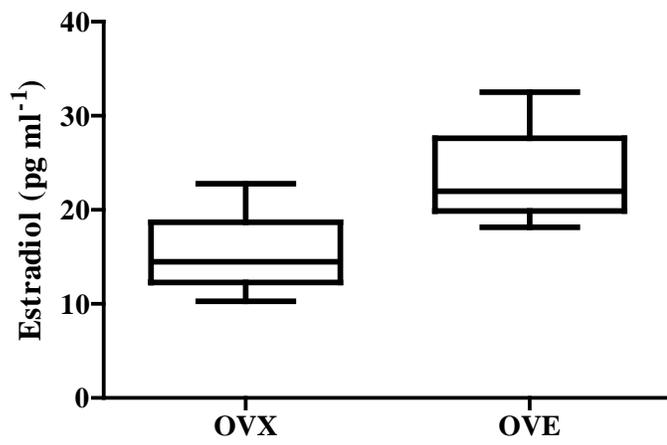


Figure A1. Plasma estradiol concentration box-and-whisker plots for OVX (n = 5) and OVE (n = 6) rats.

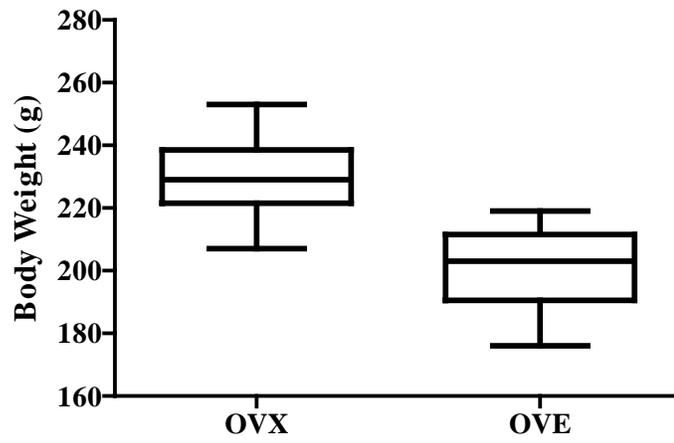


Figure A2. Body weight box-and-whisker plots for OVX (n = 17) and OVE (n = 17) rats.

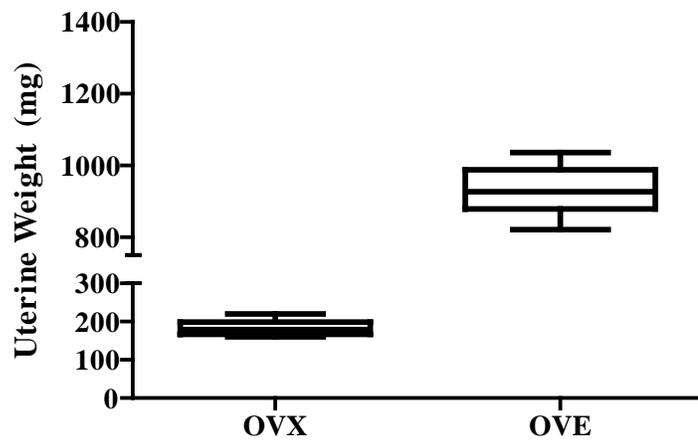


Figure A3. Uterine weight box-and-whisker plots for OVX (n = 14) and OVE (n = 14) rats.

Cumulative Concentration Response Curves

The drug used to assess the activity of NPY's post-junctional receptor, [Leu31-Pro34]NPY, was in limited supply due to cost. Therefore, a limited number of animals ($N = 19$) underwent cumulative concentration response curve testing for NPY with some observations excluded *post hoc* for various reasons (listed below). The omnibus multivariate analysis of variance ($F(6, 9) = 1.39, p = 0.32, D^2 = 3.29$) for the cumulative concentration response curves considered only complete observations, that is to say, only the observations that included the [Leu31Pro34]NPY experiments. Since the omnibus MANOVA consisted of a low number of observations, *post hoc* MANOVAs were performed to include the observations where only norepinephrine or norepinephrine + NPY (NPY potentiation) curves were performed.

MANOVA requires similar assumptions to those associated with the univariate case. Independence of observations was discussed above, which leaves the assumptions of multivariate normality and homogeneity of covariances to address. Univariate normality does not ensure multivariate normality, but it is considered as a prerequisite for multivariate normality (Stevens, 2002). Therefore, univariate normality was assessed for all observations. While multivariate normality is an assumption for multivariate ANOVA, the effect on the alpha level for violation of this assumption may be negligible (Stevens, 2002). The likelihood for violation of the homogeneity of covariances assumption increases with the number of variables included in the design. However, the effects on alpha level with violations of this assumption are tempered when group sizes are similar (large to small: < 1.5) (Stevens, 2002).

A cursory examination of the data revealed the presence of two influential outliers. In the case of rat #16 (OVE), the vessel responded in an atypical fashion. Specifically, vasoconstriction following NPY or norepinephrine administration occurred at one concentration, which yielded high slope values. This atypical response did not exclude the data by itself as there is always variability between vessels during dose response curves. However, there were factors associated with the experimental preparation of this particular vessel that required consideration when interpreting the data. Many of the vessels will have one or more collateral vessels, vessels that branch off of the arteriole, which require a suture in order to pressurize the vessel for experimental use. Suturing the vessel is a trial-and-error process with additional sutures needed if leaks are detected during the equilibration period. Moreover, the amount of branching differs between vessels, so some vessels require a great deal of attention (more sutures) during the preparatory phase of microvessel experimentation. The vessel for rat #16 required many sutures as there were many collateral branches to tie off. Unfortunately, there were a number of leaks discovered during equilibration of this vessel, and the collateral branches were in a position that made tie-off difficult. The leaks were successfully sutured, but the vessel was physically manipulated to a point where normal vascular response could have been compromised. Following careful observation and consideration of the NPY and norepinephrine data, it was decided that this outlier occurred due to excessive manipulation of the vessel during experimentation. The cumulative concentration response curve data from rat #16 was removed from analysis. The second outlier, rat #19 (OVX), had abnormally low resting vessel diameter values. It was noted in the procedural notes that there were a number of bubbles present in the tips

of the vessel. Bubbles near the entry ports of the vessel can interfere with the flow of albumin through the vessel. This causes the vessel to collapse to a certain extent, which may potentially confound measurements of vascular response that utilize vessel diameter as the dependent variable. As the percent of resting baseline values for this animal influenced the normal distribution of NPY and norepinephrine cumulative concentration response curves, it was decided that this outlier also occurred as a result of experimental manipulation and the data was subsequently removed from analysis (Table A2).

NPY cumulative concentration response curve. The EC50 for rat #24 (OVE) was an outlier. Graphpad software was able to obtain the curve fit; however, the curve possessed two concentrations that exhibited substantial responsiveness to NPY. It was decided that a manual curve fit would capture a more accurate representation of the actual response to the drug, which in fact was the case (Graphpad fit: $R^2 = 0.97$; manual fit: $R^2 = 0.98$). The manual fit corrected the outlier, which produced a normal univariate distribution (Figure A4) for the EC50. Rat #23 (OVE) failed to meet inclusion criteria ($R^2 > 0.90$) and was summarily removed from analysis. NPY slope (Figure A5) was normally distributed, and univariate variances were equal for EC50 and slope.

Norepinephrine cumulative concentration response curve. The EC50 for norepinephrine was normally distributed for both groups (Figure A6). The slope for OVE was normally distributed, but the slope for OVX failed to exhibit a normal distribution (Figure A7). All observations were left in the analysis as no justification existed to warrant removal.

Norepinephrine + NPY cumulative concentration response curve. EC50 for norepinephrine + NPY possessed a normal distribution for both groups (Figure A8).

Slope was normally distributed for OVX, but not for OVE (Figure A9). Rat #14 (OVE) had a steep slope (outlier), but there was not sufficient evidence to remove it from analysis. OVX rats #1, Blank, and 1B along with OVE rat # 2 did not undergo norepinephrine + NPY testing due to a shortage of [Leu31Pro34]NPY.

Effect sizes: meaningfulness of the differences. The multivariate effect size for the omnibus MANOVA was large ($D^2 = 3.29$), albeit statistically insignificant. The omnibus MANOVA lacked power (< 0.80) to detect statistically significant differences due to the large number of dependent variables with a relatively small number of observations. While the large effect size was intriguing, the *post hoc* MANOVAs for each drug (EC50 and slope), however, failed to further expose differences between the groups. The moderate effect sizes observed in the NPY ($D^2 = 0.46$) and NPY + norepinephrine ($D^2 = 0.46$) cumulative concentration response curves would necessitate group sizes of approximately 40-50 observations to achieve reasonable statistical power (> 0.80) (Stevens, 2002). The *post hoc* MANOVAs coupled with the graphical representation suggested that the groups were the same over the three cumulative concentration response curves.

Table A2
 Univariate Normality: Descriptive Statistics

Variable	Statistic	OVE	OVX
NPY CCRC			
EC50	Skewness	-1.01	0.59
	Kurtosis	-0.06	-1.32
	Shapiro-Wilk	0.88	0.89
Slope	Skewness	-0.35	-0.20
	Kurtosis	-1.37	-0.96
	Shapiro-Wilk	0.93	0.97
NE CCRC			
EC50	Skewness	-0.70	-0.64
	Kurtosis	-0.90	-0.25
	Shapiro-Wilk	0.89	0.92
Slope	Skewness	-0.57	-0.55
	Kurtosis	-0.93	-1.55
	Shapiro-Wilk	0.90	0.83 *
NPY+NE CCRC			
EC50	Skewness	-1.08	-0.28
	Kurtosis	0.97	-1.63
	Shapiro-Wilk	0.90	0.89
Slope	Skewness	-1.73	-0.94
	Kurtosis	3.12	-0.42
	Shapiro-Wilk	0.80 *	0.86

*p < 0.05.

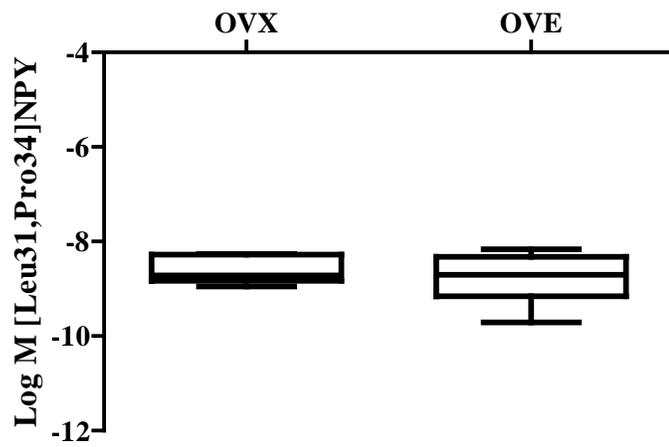


Figure A4. NPY EC50 box-and-whisker plots for OVX (n = 7) and OVE (n = 9) rats.

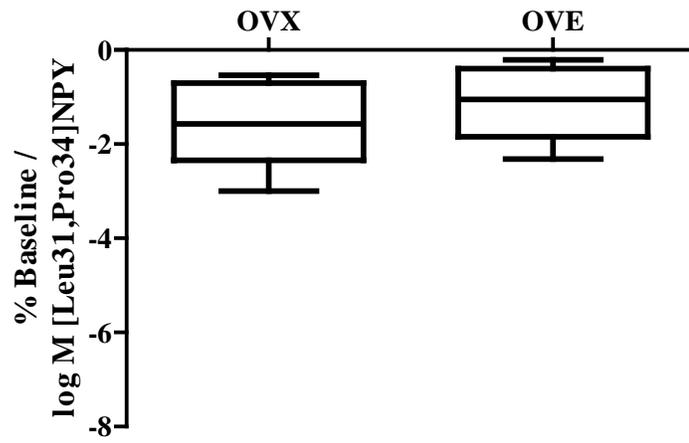


Figure A5. NPY slope box-and-whisker plots for OVX (n = 7) and OVE (n = 9) rats.

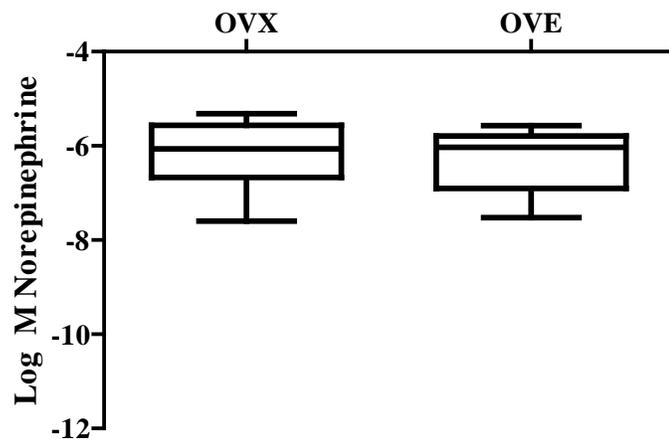


Figure A6. Norepinephrine EC50 box-and-whisker plots for OVX (n = 13) and OVE (n = 12) rats.

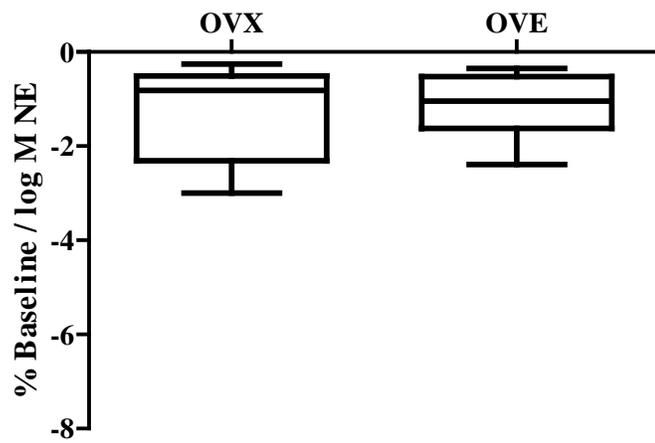


Figure A7. Norepinephrine slope box-and-whisker plots for OVX (n = 13) and OVE (n = 12) rats.

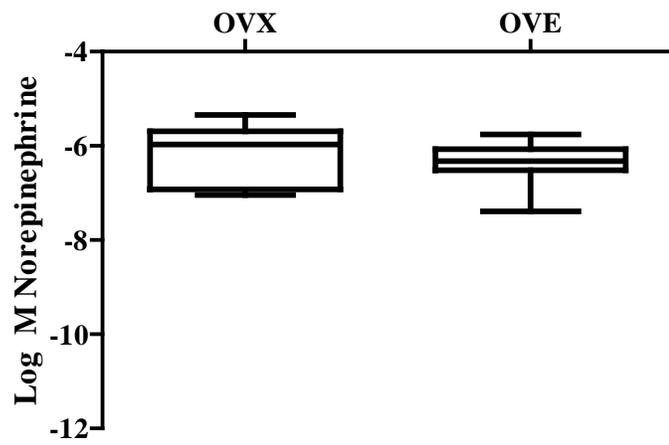


Figure A8. Norepinephrine + NPY EC50 box-and-whisker plots for OVX (n = 10) and OVE (n = 11) rats.

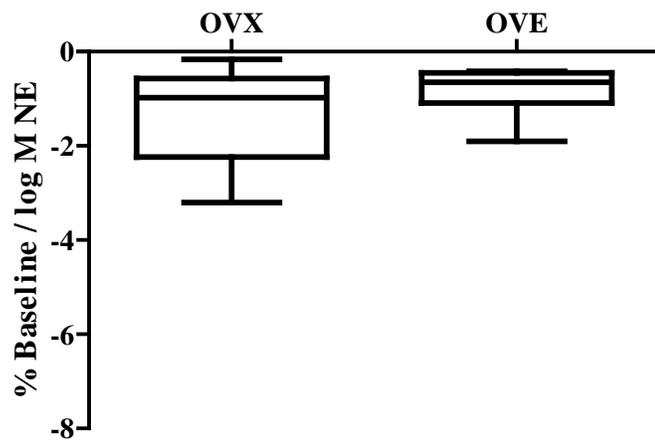


Figure A9. Norepinephrine + NPY slope box-and-whisker plots for OVX (n = 10) and OVE (n = 11) rats.

NPY Release

A repeated measures design was used for the NPY release data. The univariate case is a more powerful design when group sizes are small (Park, Cho, & Ki, 2009), and the assumptions are similar to the previous univariate assumptions with the exception of sphericity. There will be some degree of violation of the sphericity assumption as it is a repeated measures design; thus, it would be expected to find correlations between dependent variables. Severe violations can lead to inflated F values, and possibly, spurious conclusions. Therefore, the Greenhouse-Geisser adjusted F-test was used to protect against type-I error.

It was noted during casual observation of the data that NPY release of both OVX and OVE groups was much higher than those observed in intact animals. The significance of the high concentrations of NPY was that the assay had a maximum range of 10 ng ml^{-1} , and the assay was most sensitive around 1 ng ml^{-1} . Some animals that were otherwise normal possessed NPY concentrations that were beyond the detectable range of the assay. It was speculated that the higher concentrations of NPY were a product of the surgical procedure, long-term storage of the animals, or a combination of both.

Albino rats develop a reddish or pink appearance around their posterior neck and back during long-term storage. It is believed that this color is associated with stress, which can be attributed to the close-quarters storage of the animals. This coloring was not excessive, and there were no signs of abnormal behavior. Animals were never kept in seclusion except following surgery (< 2 days), and the cage size was within acceptable guidelines for animals of this particular size. Nevertheless, long-term storage could have played a role in the high NPY concentrations in both groups. Another cause of the high

NPY concentrations could be due to the surgical procedure itself. Surgery creates a stress on animals; thus, it would be plausible that a portion of the results were a product of the surgical procedure. In either case, all animals received the same treatment, which preserved the veracity of the group comparisons. However, some animals were excluded based off of NPY concentrations that exceeded the assay's concentration range, or in cases where the vessel failed preliminary viability testing.

NPY release (Table A3) was normally distributed for OVX for all time points except the first (0 seconds). OVX data (Figure A10) exhibited a tendency towards positive skewness with a flat distribution with the exception of the 3rd time point (0 seconds with DPPIV inhibition), which was leptokurtic. None of the measures of skewness or kurtosis were considered severe (> 2). OVE data (Figure A11) failed to exhibit a normal distribution for the control time points (0 and 30 seconds) as well as the first time point with DPPIV inhibition (0 seconds). The distributions consisted of a few outliers that positively skewed the mean. Another feature of the OVE data was the narrow distribution, especially with the control time points. Rats #13 and #15 were responsible for the high degree of skewness observed in the OVE group. However, there was nothing about these observations or animal characteristics that merited their removal from the analysis. It was decided, based off a lack of evidence to justify their exclusion, that the observations from rats #13 and #15 should remain in the analysis.

Similar to the cumulative concentration response curves, there was insufficient power to detect statistically significant differences in NPY release between the groups. It would require approximately 35-45 observations per group in order to possess reasonable statistical power (> 0.80) to detect true differences in NPY release (Stevens, 2002) with

the magnitude of effect present ($D^2 = 0.74$). While statistical power was limited in this analysis, the present results do not support a difference in NPY release with respect to estrogen status.

Table A3
Univariate Normality: Descriptive Statistics

Variable	Statistic	OVE	OVX
0 Seconds (Control)	Skewness	2.14	1.03
	Kurtosis	4.08	-0.06
	Shapiro-Wilk	0.66 *	0.85 *
30 Seconds (Control)	Skewness	1.50	0.16
	Kurtosis	3.08	-1.29
	Shapiro-Wilk	0.83 *	0.92
0 Seconds (DPPIV Inhibition)	Skewness	0.94	1.33
	Kurtosis	-0.28	1.56
	Shapiro-Wilk	0.85 *	0.86
30 Seconds (DPPIV Inhibition)	Skewness	0.60	0.28
	Kurtosis	-0.98	-1.32
	Shapiro-Wilk	0.89	0.90

*p < 0.05.

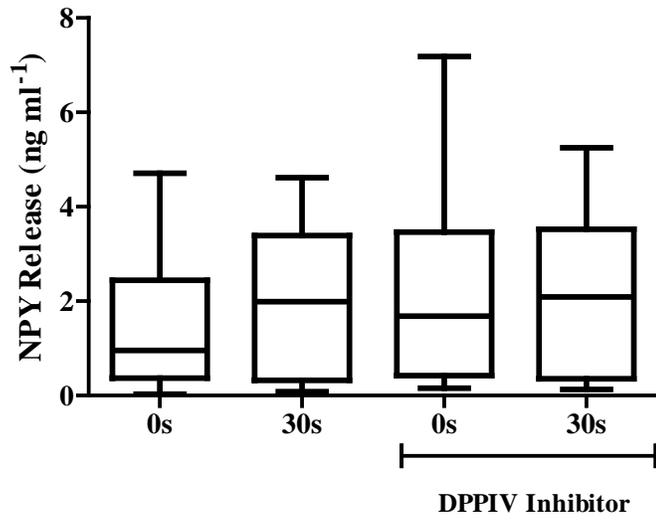


Figure A10. NPY release box-and-whisker plots at control and DPPIV inhibition conditions (OVX: n = 12)

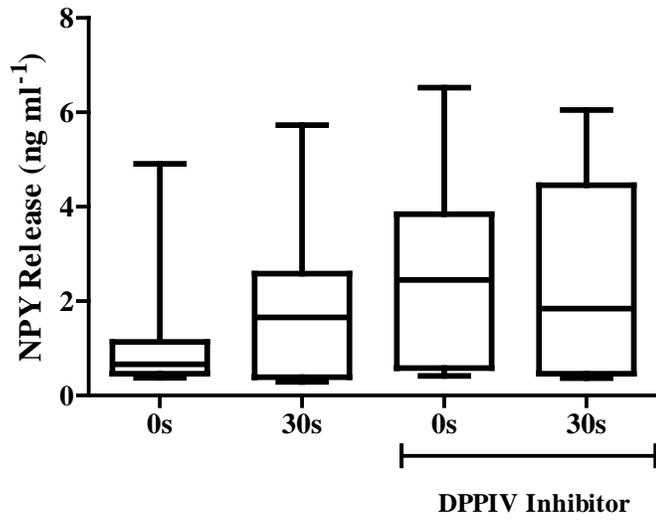


Figure A11. NPY release box-and-whisker plots at control and DPPIV inhibition conditions (OVE: n = 11)

DPPIV Activity

Differences in DPPIV activity were assessed through one-way analysis of variance. OVE and OVX were similar in distribution (Figure A12); however, the OVX group possessed two outliers, which adversely affected normality. Rats #1 and Blank registered substantially lower DPPIV values as compared to the other rats within the group. These differences were attributed to the homogenate composition (saline) of the first two samples, which likely influenced the catalytic rate of the enzyme. These two observations were removed from analysis yielding normal distributions (Table A4) and equal variances.

Table A4
 Univariate Normality: Descriptive Statistics

Variable	Statistic	OVE	OVX
DPPIV Activity	Skewness	-0.43	0.60
	Kurtosis	0.06	0.11
	Shapiro-Wilk	0.97	0.96

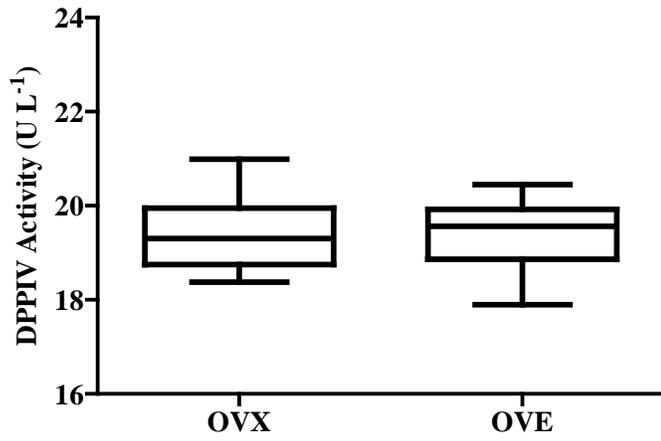


Figure A12. DPPIV activity of whole vessel homogenate (first-order arteriole) box-and-whisker plots for OVX (n = 13) and OVE (n = 15) rats.

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