The Effect of Acute Exercise on Femoral Artery Vasoconstriction: Involvement of Local Vascular Wall Renin-Angiotensin Systems

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

ABSTRACT

During a single bout of aerobic exercise, blood flow is shunted to exercising tissues while blood flow is maintained or reduced to less metabolically active areas (i.e. splanchnic area and non-working muscles). Although increased sympathetic activation and multiple metabolic factors participate in redistributing blood flow during aerobic exercise, the precise mechanism is not entirely known. The renin-angiotensin system (RAS), specifically the local vascular wall RAS, has been hypothesized to participate in the redistribution of blood flow during exercise. This study aimed to investigate whether vascular wall RAS in the femoral arteries (an artery which feeds active tissues during exercise) was altered by acute exercise, and if these vascular RAS alterations led to specific changes in vasomotor function. Male Sprague Dawley rats were exercised on a motorized treadmill for 1h at 21m/min with 15% grade. Immediately following exercise femoral arteries were excised, cleaned of surrounding connective tissue, and vascular RAS was evaluated. There was a decrease in femoral ACE activity (~40%) and expression (~20%) following a single bout of exercise. No change was observed in AT_1 and AT_2 receptor expression. To evaluate the effect of acute exercise and vascular RAS on vessel reactivity, vasomotor properties of the femoral arteries were assessed via vasoconstrictor and vasodilatory dose-response curves. No changes were observed in femoral artery responses to potassium chloride (KCl), signifying that electromechanical coupling was not affected by exercise or RAS pharmacological interventions. However, a significant decrease in maximum phenylephrine (PE) constriction was observed for acutely exercise animals (~13%). Paired with the observed maintenance KCl-mediated constriction, it appears an acute bout of exercise is able to attenuate α-adrenergic receptor-mediated vasoconstriction in the femoral artery. The decrease in

maximum α-adrenergic vasoconstriction may be attributed to vascular RAS. The decrease in ACE activity supports the production of local vasodilating factors. Blocking AT_1 receptors with telmisartan decreased PE constriction in control and exercised animals. Combining AT_1 and AT_2 receptor blockade (with PD123319) eliminated the attenuating effect of telmisartan alone on PE constriction. This data suggests that the attenuating effect of AT_1 receptor blockade, on PE constriction, may depend on AT_2 receptor activation. In addition, combined AT_1 receptor blockade and nitric oxide synthase inhibition eliminated both the lone AT_1 receptor blockade effect and exercise effect on PE constriction. Together, this data suggests that reduced PE constriction following acute exercise, and AT_1 receptor blockade, is dependent on nitric oxide production. Vasodilation to the nitric oxide donor sodium nitroprusside (SNP) was not altered following exercise or RAS pharmacological intervention, signifying no change in signaling downstream of NO production/release. Endothelium-dependent vasodilation to acetylcholine (ACh) was not affected by acute exercise. However, responses to ACh were modulated by RAS pharmacological interventions supporting the responses seen in PE constriction and signifying the participation of vascular RAS in vasomotor function.

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LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
ACh	Acetylcholine
AngI	Angiotensin I
AngII	Angiotensin II
ARBs	Angiotensin receptor blockers
AT_R	Angiotensin Receptors
AUC	area under curve
AVP	arginine vasopressin
B2	bradykinin receptor 2
BSA	bovine serum albumin
CAP	captopril
CON	control
DAG	diacylglycerol
DPI	diphenyleneiodonium
EC_{50}	half maximal effective concentration
eNOS	endothelial nitric oxide synthase
HHL	L-Hip-His-Leu
HL	L-histidyl-L-leucine
HRP	horseradish peroxidase
IP_3	Inositol 1,3,5-triphosphate
IPEx	Immediate post-exercise
KCl	potassium chloride
LIMA	left internal mammry artery
L-NAME	$^{\omega}N$-nitro-L-arginine methyl ester
MAX	maximum
NAD(P)H	nicotinamide adenine dinucleotide phosphate
ND	No drug
NE	Norepinephrine
NO	nitric oxide
NOS	nitric oxide synthase
PD	PD123319
PE	Phenylephrine
PKC	protein kinase C
PPARγ	peroxisome proliferator activated receptor
PWV	pulse-wave velocity
RAS	Renin-Angiotensin System
ROS	rective oxygen species
SD	Sprague Dawley Rat

SDS	sodium dodecyl sulphate
SHR	Spontaneously hypertensive rat
SNP	sodium nitroprusside
SOD	superoxide dismutase
TBS-T	tris buffered saline with Tween 20
TEL	telmisartan
TPR	total peripheral resistance
VSCM	Vascular smooth muscle cells

INTRODUCTION

Renin Angiotensin System (Circulating vs. Local)

It is well known that the renin-angiotensin system (*RAS*) participates in blood pressure regulation and fluid homeostasis [1]. A series of events occurs in the circulation to produce the effector peptide of RAS, Angiotensin II (*AngII*). A decrease in arterial blood pressure stimulates renin release from the kidney. Renin acts on its circulating substrate, hepatic-derived angiotensinogen, to form the decapeptide angiotensin I (*AngI*). AngI is then converted to the active octapeptide AngII by a zinc-containing metalloprotein, angiotensin converting enzyme (*ACE*). AngII can then normalize blood pressure by increasing total peripheral resistance, while also inhibiting water and sodium excretion for long-term stabilization of blood pressure (See Figure 1) [1]. Recent evidence, however, indicates that the traditional view of the RAS as a circulating endocrine system may be an over-simplification. Many tissues (such as blood vessels, brain, and kidney) express renin, angiotensinogen and ACE [2]. Furthermore, in-vitro cell culture work and in-vivo animal studies have identified local tissue production of AngI, AngII and AngII metabolites [1-4]. Thus, expanding our appreciation of local tissue RAS systems will be necessary to fully comprehend pathology associated with RAS.

Local RAS contributes to AngII production within tissues, eliciting physiological responses independent of circulating systemic AngII (i.e. paracrine function) [5]. In fact, AngII synthesis by local RAS can be further categorized as either extrinsic or intrinsic production. Extrinsic formation of AngII occurs primarily at the surface of the blood vessels. ACE, present on the luminal face of vascular endothelial cells, actively converts AngI to AngII, which is either circulated throughout the body for systemic action or sequestered into

the vascular tissue for local action [2]. The basis of intrinsic local RAS relies on the existence of functional RAS components within tissue cells, which produce AngII for local action only [2]. Danser et al. quantified local vascular wall RAS and reported that a substantial fraction of AngII being produced and sequestered did not occur in the plasma as it passed through the vascular bed [6;7]. The extrinsically and intrinsically generated AngII influences local cellular and molecular function and structure via interaction with angiotensin receptors (AT_R) [2;3;8]. Although most known physiological and pathophysiological effects are mediated through AT_1 receptor isoform, evidence suggests that some local action is mediated through the AT_2 isoform.

Vascular Wall RAS

Despite debate over whether renin is expressed within the vasculature, ACE mRNA and protein have been readily found in the endothelium and adventitia (See Figure 2) [9-11]. Furthermore, isolated hindlimb preparations have shown ~50% conversion of AngI to AngII after a single pass through the vessel bed [12] suggesting a robust local vascular ACE capacity. The AngII production in the hindlimb preparations was abolished by the ACE inhibitor captopril, identifying the functional role of vascular wall RAS in AngII production. AT_Rs have also been identified in endothelial and vascular smooth muscle cells (*VSMC*), where locally produced AngII may bind [13]. Thus, growing evidence suggests localization and functionality of RAS components within the blood vessel wall.

Although the blood vessel wall is an effector organ for circulating RAS (AT_1 receptors localized on VSMC mediate vasoconstriction), locally produced AngII can affect various properties of vascular cells. Classically, AT_1 receptors were thought to only mediate

vasoconstriction, in response to systemic or local AngII, through a phospholipase C-dependent mechanism [14-16]. Inositol 1,4,5-trisphosphate (IP_3) signaling leads to an increase in myoplasmic calcium and diacylglycerol (*DAG*) signaling activates protein kinase C (*PKC*), both triggering VSMC contraction. However, AT_1 receptor activation has also been implicated in other cellular events such as: smooth muscle proliferation[17], extracellular matrix formation[18], angiogenesis[19], activation and upregulation of nicotinamide adenine dinucleotide phosphate (*NAD(P)H*) oxidase[20]. The ability of AngII to alter vascular wall growth properties, and function, has motivated cardiovascular disease research to uncover therapeutic interventions involving local vascular wall RAS.

RAS Pharmacology

Several lines of experimental and clinical evidence have documented that disorders of the RAS play a central role in the development and progression of hypertension, as well as many other cardiovascular diseases such as myocardial infarction, stroke, and congestive heart failure[21]. Thus, pharmacological interventions, designed to interfere with RAS, have become widely used for the management of hypertension and other cardiovascular diseases. The earliest drug intervention developed to interfere with RAS aimed to block the enzymatic activity of ACE. Cushman and Ondetti developed the first ACE inhibitor [22] based on purified peptides from the venom of the Brazilian pit viper (*Bothrops jararaca*). A specific sequence of nine peptides from the viper venom, termed teprotide, was shown to lower blood pressure in humans; especially those with high circulating levels of renin[22]. However, the use of the teprotide was limited due to its peptide nature – the nonapeptide had a short biological half-life and required intravenous administration[23]. A breakthrough in ACE

inhibitor development came in 1976 when Cushman, Ondetti and Rubin designed the orally active ACE inhibitor, captopril, using a hypothetical model of the ACE active site, based on carboxypeptidase A (another zinc containing metalloprotein)[23]. Captopril had a high level of oral bioavailability and was effective in lowering blood pressure in renin-dependent experimental models of hypertension[22]. In 1981, the United States Food and Drug Administration approved the use of captopril[23]. Several ACE inhibitors have been developed since 1981 and ACE inhibitors continue to be widely used solely or in combination with other RAS blockers to treat hypertension and various cardiovascular diseases.

For many hypertensive patients angiotensin receptor blockers (*ARBs*), specifically the *sartan* class of drugs that selectively block the AT_1 receptor subtype [24], are commonly prescribed in combination ACE inhibitors in pharmacological treatment regiments. ARBs were developed to completely block the physiological actions of AII via competition at the receptor level. ARBs, in combination with ACE inhibitors, are required for increased interference of RAS since it is known that a significant proportion (up to 40%) of AngII is formed through non-ACE dependent pathways; angiotensinogen is converted to AngII via the enzymes cathepsin G, elastase, and tissue plasminogen activator, or from AngI to AngII by enzymes including chymase[24]. In addition, studies have cited limited tissue penetration of ACE inhibitors, which contributes to persistent AngII formation during ACE inhibitor therapy[25]; indicating a distinct therapeutic role for ARBs in combination with ACE inhibitors. A specific ARB, telmisartan, has gained popularity recently due to its strong binding affinity, a reported long term effect over a 24hr period, and for its prospective use in the management of metabolic syndrome. Large scale clinical studies on telmisartan have

reported substantial antihypertensive effects (reduction of blood pressure values throughout the 24hr average interval) of the drug within 1-2 weeks of initiating therapy[21]. Interestingly, the antihypertensive effect follows the circadian rhythm of patients (measured via ambulatory BP monitoring), allowing protection of the cardiovascular system against the early morning BP rise and its adverse effects on the heart and vital organs[26]. Telmisartan is also capable of activating peroxisome proliferator activated receptor γ (PPARγ), which may be important in the prevention or treatment of metabolic syndrome (a cluster of metabolic and cardiovascular risk factors that predispose to heart attack, stroke, heart failure and sudden cardiac death)[27]. PPARγ activation is known to increase energy expenditure, improved lipid profile[28], increased insulin sensitivity [29], blood pressure reduction[27], and amelioration of the associated pro-inflammatory and pro-atherogenic risk profiles[30]. Therefore, telmisartan may not only be an important drug intervention in the treatment of hypertension, but may also prove to be useful in a variety of cardiovascular complications through its ability to integrate and modulate two major metabolic pathways – one through activation of PPARγ pathway, and the second by selectively blocking the AT_1-dependent pro-inflammatory, pro-atherogenic pathway without opposing the AT_2-dependent anti-inflammatory pathway.

AngII and Vascular NAD(P)H Oxidase

All vascular cell types including endothelial cells, smooth muscle cells, adventitial fibroblasts, and resident macrophages produce reactive oxygen species (*ROS*) [31-33]. ROS is of particular physiological importance since it acts as an inter- and intra-cellular signaling molecule. Under pathological conditions, however, an increased generation of ROS

occurs[34]. An increased ROS load alters nitric oxide (*NO*) bioavailability and redox-sensitive signaling pathways, leading to cellular processes involved in structural remodeling of blood vessels and vascular dysfunction[32]. A major free radical involved in the pathology of vascular dysfunction is superoxide anion[32]. Superoxide anion has an unpaired outer shell electron, which makes the molecule highly reactive, but also unstable and short-lived. It is water soluble and can cross cell membranes through anion channels[35]. The major source of superoxide anion in the vascular wall is non-phagocytic NAD(P)H oxidase in endothelial cells and VSMC [36]. Vascular NAD(P)H oxidase is a multisubunit enzyme comprised of four components: cell membrane p22phox, electron transfer subunit gp91phox, and cytosolic subunits p47phox and p67phox[32]. NAD(P)H oxidase is regulated by many vasoactive agents including AngII[20]. Acute administration of AngII increases NAD(P)H oxidase activity [37]. The subcellular processes involved in AngII-mediated NAD(P)H oxidase activation have been linked to phospholipase A and D, c-Src, RhoA, Rac and phosphatidylinositol 3-kinase[34]. In addition, chronic administration of AngII leads to de novo synthesis of NAD(P)H oxidase subunits[20]. Thus, exposure to high circulating and/or local vascular levels of AngII contributes to increased ROS production via NAD(P)H oxidase.

ROS production and agonist-induced vasoconstriction

A delicate balance between NO production and ROS production exists within the vasculature, contributing to the overall maintenance of vascular reactivity[38]. For example, ROS overproduction has been found to be a contributing mechanism to vasomotor dysfunction in hypertension[39]. When ROS production is increased, as in hypertension, the

shift to a pro-oxidant blood vessel environment disrupts function[40]. In addition to impairing agonist-induced vasodilation, excessive ROS production seems to exaggerate agonist-induced contractile responses in conduit vessels. For example, Miyagawa et al. (2007) reported an amplified contractile response to norepinephrine (*NE*) in the femoral arteries of spontaneously hypertensive rats (*SHR*) [40]. Exaggerated vascular superoxide anion production, mostly accounted for by NAD(P)H, is observed in SHR [32] – suggesting a possible participation of ROS in increasing agonist-induced contractile responses in blood vessels. Indeed, incubation of the aforementioned femoral arteries with diphenyleneiodonium (*DPI*) and apocynin, both NAD(P)H inhibitors, reduced the NE-induced contractions in SHR[40]. Taken together it appears that ROS production, particularly superoxide anion, increases agonist-mediated contractile responses in SHR. In addition, Yang et al. (2002) demonstrated that ROS is involved in endothelium-induced contractions evoked by ACh in SHR aorta [41]. Tiron, a cell-permeable non-enzymatic scavenger of superoxide anions, partly reduced the ACh-induced contractions in SHR aorta. Also incubation of SHR aorta with diethldithiocarbamic acid, which at certain concentrations can inhibit endogenous Cu/Zn superoxide dismutase (*SOD*), led to increased superoxide anion production but not an increase in acetylcholine (*ACh*)-induced contractions in SHR[41]; indicating that superoxide anions per se are not sufficient to evoke contraction, but may form other radicals that exaggerate contraction (i.e. hydroxyl radical). Regardless of the specific mechanism utilized, it seems that ROS has the ability to reduce NO bioavailability and increase agonist induced vasoconstrictory responses.

An interaction exists between AngII and adrenergic receptor stimulated contraction. Several reports indicate that AngII increases vascular reactivity to α-adrenergic stimulation

[42;43]. ACE inhibitor treatment in the drinking water of SD rats has been linked to reduced submaximal aorta contractions to phenylephrine (*PE*)[44]. Lemos et al. (2002) reported reduced in-vitro contractile responses to PE, in the aorta of SD rats, after a 30 minute incubation with the ACE inhibitor *captopril* in the organ baths [45]. In addition, Lemos et al. employed AT_1 receptor blockade, with CV 11974, to further diminish aortic contractile responses to PE; thus signifying a role for AT_1-mediated events, such as increased ROS production, in increased vascular reactivity. Maeso et al. suggested that AT_2 receptors are intricately involved in the ability of AT_1 receptor blockade to attenuate α-adrenergic-stimulated vasoconstriction since a known AT_2 receptor blocker, PD 123319, abolished the attenuating effect of losartan (a specific AT_1 receptor blocker) on PE mediated vasoconstriction [46]. Maeso et al. also reported that the NO synthesis inhibitor N^{ω}-nitro-L-arginine methyl ester (*L-NAME*) reversed the losartan-mediated reduction in PE vasoconstrictor responses[46]; also suggesting mediation by NO in the ability of AT_1 blockade to attenuate PE-mediated vasoconstriction. An interesting relationship exists between AT_2 receptor activation and the NO-cGMP axis [47]. Cosentino et al. (2005) revealed that losartan unmasked AT_2 mediated vasodilation in the thoracic aorta of SHR rats that were administered AngII[48]. AT_2 activation increased NO production which significantly, but not entirely, contributed to the beneficial hemodynamic effects of AT_1 receptor blockade. Cosentino et al. also observed increases in an AngII breakdown product, Ang (1-7). When the corresponding receptor for Ang (1-7), Mas receptor, was concomitantly blocked along with AT_1 and AT_2 receptors the vasoconstrictory attenuating effect of AT_1 blockade was no longer observed[48]. Collectively these research results indicate that tissue AngII can affect α-adrenergic stimulation in the vasculature based on an

intricate balance between AT_1 (ROS production) and AT_2 activation (NO production) within the vascular wall.

Exercise induced changes in ROS production and vascular wall RAS

Research has identified various benefits for performing daily physical activity; among them being improved endothelium-dependent vasodilation[38]. Improvements in vasomotor function, following regular physical activity, are associated with improved NO bioavailability[39]. Adams et al (2005) reported a significant reduction in left internal mammary artery (*LIMA*) expression of NAD(P)H oxidase subunits, and NAD(P)H oxidase derived superoxide anion production, following chronic physical exercise[36]. In addition, downregulation of gp91phox mRNA expression and ROS production occurs in bovine aortic endothelial cells subjected to pulsatile shear stress[49], suggesting that decreased ROS production may improve function following chronic exercise. Exercise causes acute increases in arterial laminar flow, leading to increased pulsatile shear stress, which stimulates the release of nitric oxide and other endothelium-derived relaxing factors to dilate the artery and restore shear stress toward normal[50]. Longer-term increases in flow produce changes in endothelial shape, alignment, and the expression of genes associated with a healthy phenotype including increased endothelial nitric oxide synthase (*eNOS*) expression, and as discussed above, decreased NAD(P)H subunit expression[50]. Thus, increasing evidence suggests that exercise training reduces ROS formation in the vessel wall by downregulating NAD(P)H oxidase activity and subunit expression, which increases NO bioavailability and improves vasomotor function.

AngII induces NAD(P)H activity via AT_1, while AT_2 stimulation counteracts the ROS production mediated through AT_1. Thus, the net effect of AngII depends mainly on the AT_1/AT_2 ratio in VSMC. Adams et al (2005) documented a ~50% decrease in AT_1 receptor protein expression in the LIMA of trained compared to untrained males, whereas AT_2 receptor protein expression remained the same (mRNA expression in trained males was ~5 fold higher than untrained males) [36]. Thus, exercise may alter the AT_1/AT_2 ratio in the vasculature modifying the actions of locally produced AngII. In addition, Kohno et al. discovered lower resting plasma AngII levels following chronic exercise[51], concluding that this was responsible for blunted ROS production, and the blood pressure lowering effect of 10 weeks of training in SHR rats[51]. Thus, it is apparent that chronic exercise training reduces AT_1/AT_2 receptor ratio and circulating AngII, which contributes to the observed improvements in vasomotor function following exercise.

Despite extensive research into chronic aerobic exercise, little is known about the modifications to circulating and vascular wall RAS following a single bout of exercise. During exercise a significant redistribution of tissue blood flow occurs, in which the blood flow to active muscles is increased whereas it remains the same or even decreased in less metabolically demanding areas such as the splanchnic circulation[52]. Although the exercise-induced redistribution of blood flow has been partly attributed to increased SNS and multiple metabolic factors[53], the precise mechanisms are not known. Maeda et al. suggests that tissue production of various vasoconstrictor peptides within the vasculature participate in exercise-induced redistribution of blood flow[53]. For example, they observed an enhancement of endothelin-1 production in the non-exercising muscles of males performing one-legged cycling[54]. Maeda et al. also suggested a role for local RAS in redistributing

blood flow during exercise. In a single bout of exercise (30m/min for 30min, 0% grade) male SD rats showed increased angiotensinogen levels, ACE mRNA expression, ACE protein expression and AII levels in the kidney[53]. The observed enhancement of kidney RAS led the group to conclude that the local RAS is involved in the tissue specific vasoconstriction, aiding blood flow redistribution during acute exercise[53].

Acute exercise also results in a variety of vascular functional alterations such as vasodilation-mediated post-exercise hypotension, increased vascular compliance, and altered vascular sensitivity to vasoactive substances[55-58]. It is well documented that a single bout of dynamic exercise reduces postexercise arterial pressure for several hours[55]. Rao et al. (2002) showed a reduced responsiveness of α-adrenergic receptors post exercise, which contributes to the observed postexercise hypotension[55]. NOS inhibition restored postexercise responses to PE, suggesting NO-induced vasodilation counters the PE induced vasoconstriction. Indeed, human studies have shown increased NO production following exercise[59], indicating a role for NO-mediated vasodilation in post exercise hypotension. Acute exercise has also been shown to increase vascular compliance. The effect of a single 30-min bout of cycling exercise, at 65% of maximal oxygen consumption, on indexes of arterial compliance included reduced aortic and leg pulse-wave velocity (*PWV*) and decreased total peripheral resistance (*TPR*) [56]. The decreases in vascular stiffness may be related to the increased production of NO. NOS inhibition, by L-NMMA administration, was able to elicit increases in leg PWV during acute exercise[57], which suggest that increased production of NO may factor in to the decrease of regional arterial stiffness during a single bout of aerobic exercise. Examination into the effects of acute exercise on vasomotor activity has revealed conflicting results. For example, Howard et al. (1992)

showed that a single bout of exercise reduced maximal contractions to PE in the aorta of New Zealand White Rabbits[58]. Contractile responses to 70mM KCl were unchanged following exercise suggesting that dynamic exercise specifically attenuated α-adrenergic receptor-mediated contraction of VSMC. Patil et al. (1993) also reported diminished maximal vasoconstrictor responses to PE in the hindlimb of acutely exercised SD rats[60]. Surprisingly, L-NAME enhanced maximal PE constrictions in the hindlimb preparations mentioned above, suggesting that NO contributes to the reduction of vasoconstriction following exercise and α-adrenergic receptor stimulation[60]. In contrast, Spier et al. (1999) found that a single bout of exercise did not alter the SD abdominal aorta responsiveness to vasoconstrictor agonists (PE, NE, AVP, KCl)[61]. Thus, the experimental evidence stated above links acute exercise with functional vascular alterations such as vasodilation-mediated post-exercise hypotension and increased vascular compliance. However, the effects of acute exercise on vasomotor properties remain disputed. This disagreement may be related to the type of exercise employed, varied animal species and vascular beds.

Experimental Study Outline

The purpose of this study was to determine whether acute exercise alters vascular RAS activity and if the alterations are associated with distinct vasomotor responses in the femoral arteries of Sprague Dawley rats. Completion of the following 3 studies clarified whether: (1) acute exercise alters femoral vascular wall RAS activity, (2) acute exercise alters vasomotor responses in femoral arteries, and (3) there is a link between altered vasomotor properties and changes in vascular RAS following acute exercise.

Study 1: Measurement of changes in vascular RAS activity following acute exercise

Maeda et al. (2005) reported an increase in kidney RAS activity following a single bout of exercise, which contributed to vasoconstriction of the renal vasculature. Thus, it is possible that local blood flow conditions during exercise (i.e. blood flow is shunted to the working muscles while non-exercising areas, such as the splanchnic area, have decreased flow) are partially dependent upon, or cause alterations to, vascular wall RAS. Pilot studies within our laboratory have determined that ACE protein and activity decrease in thoracic aorta following acute exercise, while increases are observed in kidney tissue. Accordingly, a hypothesis can be made regarding acute exercise and the local femoral artery wall RAS. Since the femoral artery serves active tissues, which require higher blood flow during acute exercise, vascular RAS activity is hypothesized to be reduced either as a response to higher blood flow or to attenuate vasoconstriction and allow for higher blood flow to perfuse the active tissues. To test this hypothesis, femoral arteries were excised immediately following exercise and snap frozen. The alterations in femoral vascular wall RAS were evaluated in exercised rats by assessing vessel wall ACE activity and protein levels, and AT_1 / AT_2 protein expression.

Study 2: Investigation of femoral artery vasomotor responses following an acute bout of exercise

Following an acute bout of exercise, femoral arteries were excised from Sprague Dawley rats and mounted on in-vitro myography baths to assess vasomotor function was assessed. In accordance with the hypotheses made above, regarding vascular RAS and acute exercise, we expected to observe blunted α-adrenergic mediated vasoconstriction and

perhaps enhanced endothelium-dependent vasodilation in the femoral artery following an acute bout of exercise.

Study 3: Examination of the role femoral vascular RAS plays in altered femoral artery vasomotor responses following acute exercise

Femoral arteries were incubated with a series of RAS blockers prior to performing vasoconstrictor and vasodilatory curves. Pre-incubation with captopril, telmisartan, PD123319 and combinations of the three RAS blockers were used to ascertain the functional contribution of specific local vascular RAS components (ACE, AT_1, and AT_2 receptors, respectively) to altered femoral artery vasomotor function following exercise. L-NAME, a nitric oxide synthase inhibitor, was also used in combination with certain RAS blockers to evaluate the possible relationship between NO and vascular RAS in determining femoral vasomotor properties. We hypothesized that vessel wall RAS contributes in agonist induced vasoconstriction, and that the effects will be dependent on NO. Specifically, following acute exercise, we hypothesize that ACEi and AT_1 blockade will blunt α-adrenergic mediated vasoconstriction further than exercise alone in femoral arteries, and that the additional blockade of AT_2 receptors should augment this blunted α-adrenergic mediated vasoconstriction.

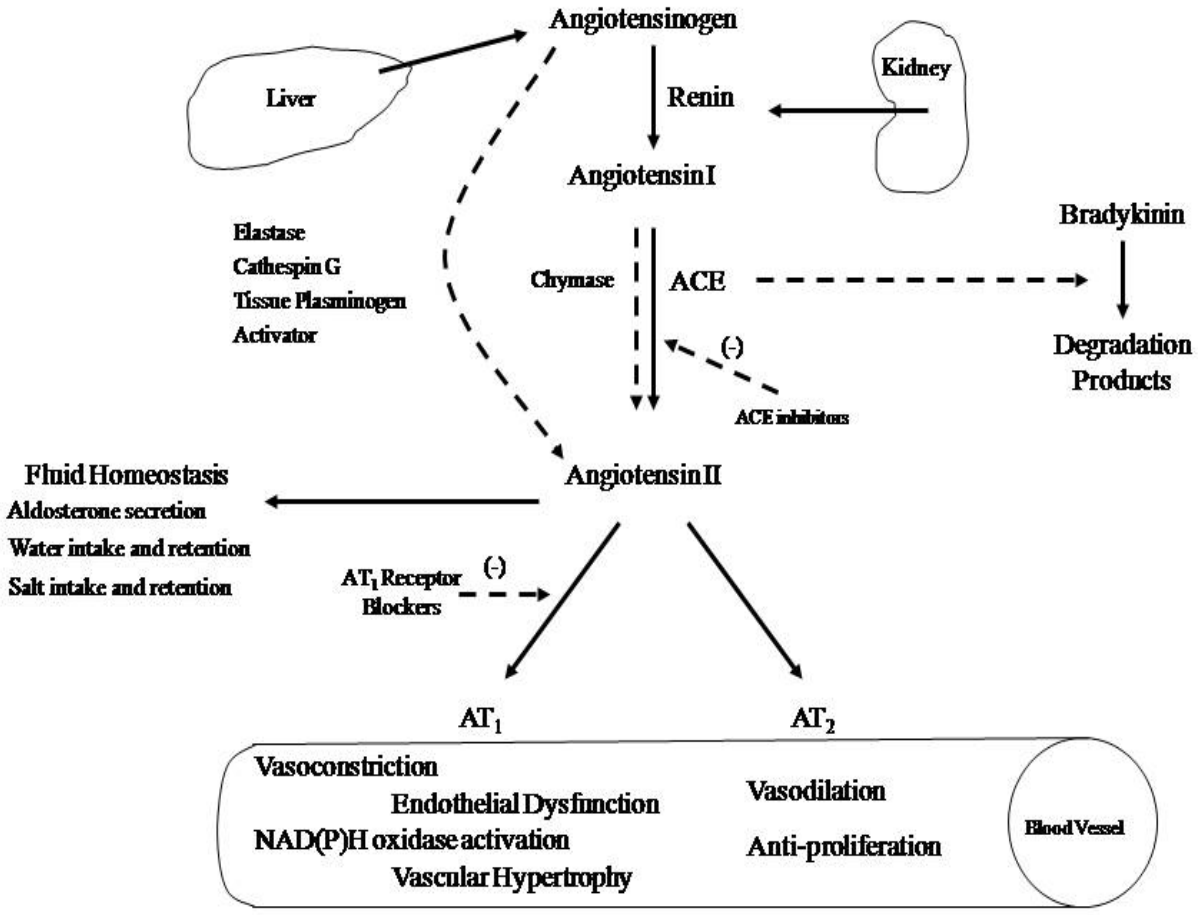

Figure 1: *Circulating Renin-Angiotensin System.* The formation of angiotensin II can arise through both an ACE-dependent pathway and ACE-independent pathway within the vessel wall. Angiotensin II has effects systemically and locally, and can be inhibited by various RAS blockers. Adapted from Rush and Aultman, 2008.

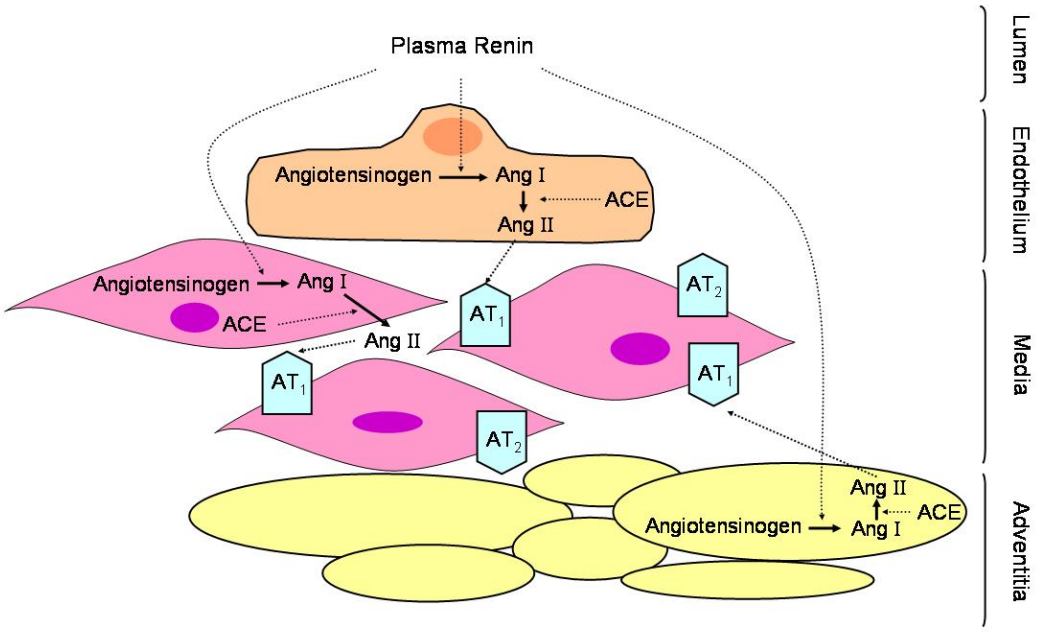

Figure 2: *Local Vascular Wall Renin-Angiotensin System.* ACE-dependent angiotensin II formation has been identified in different layers of the vessel wall. The formation of angiotensin II by the endothelial and adventitial cells may have effects directly on the smooth muscle wall.

METHODS

Animals

18-20wk old male Sprague Dawley rats (SD, n=79) were obtained from an in-house breeding colony (University of Waterloo, ON). Rats were housed at constant air temperature (20–21°C) and humidity (~50%) in a 12:12-h reverse light-dark cycle. Rats had free access to standard 22/5 Rodent Diet lab chow (Harlan, WI) and tap water. The University of Waterloo Animal Care Committee approved all animal-related procedures proposed in this study.

Exercise Protocol

All SD rats were acclimatized to treadmill running 3 days prior to performing the acute exercise challenge. The first two days of acclimatization involved running on a treadmill for 10min (5-10m/min, 0% grade), while the third day (day prior to exercise challenge) allowed the rat to rest. All acutely exercised rats performed the acute exercise challenge in the morning (8-10am) in a dark room. Over the first 10min period, the treadmill speed was ramped up to 21m/min; the speed at which the rat ran for the remaining 50min, at 15% grade. Control (*CON*; n=40) rats were caged and placed on top of the active treadmill to allow for the CON rats to experience similar vibrations and sounds as the exercising rats, while not running on the treadmill. Immediately after the 60min of running elapsed, rats in the CON and immediate post-exercise (*IPEx*; n = 39) group were anesthetized with sodium pentobarbital (90-100 mg/kg body wt).

Surgical Procedure

To draw blood via cardiac puncture, an incision was made along the midline through the sternum to open up the thorax and expose the beating heart. An 18 gauge needle, which was attached to a 20cc syringe, punctured the right ventricle and blood was drawn.

To excise the femoral artery, an incision was made lateral to the midline of the rat opening an area between the leg and abdominal region. Muscle fascia was separated from the leg muscles to expose the femoral vein, artery, and saphenous nerve complex. The fascia connecting the abdominal wall and leg muscles was separated to expose the external iliac artery and vein. The femoral nerve was removed starting from the branch point between the external iliac and femoral nerves. Next, a section of vein, starting from above the branch point to a point where the femoral artery runs deep, was removed. Finally, the femoral artery was removed and placed into a 4°C physiological buffer solution (131.5 mM NaCl, 13.5mM $NaHCO_3$, 11.3mM glucose, 5.0mM KCl, 2.5mM $CaCl_2$, 1.2mM NaH_2PO_4, 1.2mM $MgCl_2$, and 0.025mM EDTA), and cleaned of surrounding fat and connective tissue. Femoral artery rings used for the myography protocols were taken just distal to the branch point between the external iliac and femoral arteries.

Catecholamines

Norepinephrine and epinephrine levels were determined by high pressure liquid chromatography. 3mL of whole blood was collected via cardiac puncture from all rats. 75µL of an antioxidant cocktail (6% EGTA and 9% Glutathione) was present during the collection. The sample was then centrifuged for 10min at 3000g to yield a plasma fraction. The plasma was collected and stored at -80°C until time of assay. The plasma extraction

process was as follows: In a 1.5mL microcentrifuge tube, 1mL of plasma was added to 20mg of acid washed alumina, 400µL 2.0M TRIS + 2% EDTA (pH 8.7), and 50µL of 3,4-Dihydroxybenzylamine. Following inversion for 10min, the alumina inside the microcentrifuge tube was washed with distilled water four times to ensure that the plasma inside the tube was completely rinsed out. Next, 100µL of 0.1M Perchloric Acid was added to the microcentrifuge tube to elute the catecholamines from the alumina. The microcentrifuge was vortexed, then centrifuged at 3000g for 10min. 50µL of the supernatant was injected into the chromatographic column. Catecholamine levels were expressed in pg/mL.

Study 1: Measurement of changes in vascular RAS activity following acute exercise

Plasma and Tissue ACE Activity

Circulating and vascular tissue ACE activity levels were evaluated using a spectrofluorimetric assay adapted from Schwager et al. [62]. A plethora of assays have been employed to examine ACE activity, including early in vitro bioassays and specific HPLC assays, however the protocol employed in this study allows for high-throughput and specificity[62]. A synthetic ACE substrate, L-Hip-His-Leu (*HHL*), was used in this assay. The artificial amino-substituted tripeptide has a free C-terminal end and a non-prolyl residue in the penultimate position akin to physiological substrates of ACE. Once cleaved by plasma or tissue ACE, the remaining L-histidyl-L-leucine (*HL*) can be quantified flourimetrically by forming an adduct with o-phthaldialdehyde.

***Plasma ACE**:* 2mL of whole blood was collected via cardiac puncture from all rats. Whole blood was spun for 10min at 3000g to yield a plasma fraction. Plasma was frozen immediately and stored until time of assay. The assay was completed on a clear 96-well round bottom assay plate. A standard curve was created, in triplicate, with various concentrations (0nM – 12nM) of HL. Next, 30µL of HHL was be added to each well, and the entire plate was incubated at 37°C for 15min. 3µL of sample plasma was added to each well containing HHL, then mixed on an orbital shaker and incubated at 37°C for 30min. After incubation, 177µL of 0.28M NaOH was added to stop the ACE dependent enzymatic reaction. To allow for fluorescence measurements, 15µL of O-phthaldialdehyde (20mg/mL MeOH) was added to each well, to form an adduct with HL, and mixed for 10min at room temperature. 25µL of 3M HCl was then added to each well to stop further fluorescent adduct formation. The plate was mixed well and endpoint fluorescence was measured at an excitation wavelength of 360nm and emission wavelength of 485 nm. Values obtained were expressed as nmol HL and conversion was necessary to achieve the accepted units for plasma ACE activity (expressed as µmol/min/L).

***Tissue ACE**:* ~10mg of femoral artery tissue was homogenized in 10X volume of 0.02M potassium phosphate buffer with 0.1% triton X-100. Samples were then centrifuged for 10min at 3000g with the supernatant being collected and stored at -80°C until time of assay. The ACE activity assay was performed as stated above, with the only exception being the use of the homogenate instead of plasma. Tissue ACE activity was expressed as µmol/min/mg protein.

Plasma AII levels

5mL of whole blood was collected via cardiac puncture from all rats. An inhibitor cocktail was included in the syringe to prevent any ex-vivo generation and/or degradation of AngII [63]. The inhibitor cocktail contained o-phenanthroline 0.44mM, EDTA 25mM, p-hydroxy-mercuribenzoic acid 1mM, and pepstatin A 0.12mM (aforementioned concentrations are in the final sample volume). The blood was then centrifuged at 3000g for 20 minutes at 4°C. Plasma samples were stored at -80°C until time of extraction. AngII was extracted from the plasma prior to using a commercially available AngII enzyme immunoassay (*EIA*) kit (SPI-BIO, France). Briefly, phenyl-bonded cartridges (GE Healthcare) were pre-washed with 1mL methanol, followed by 1mL of water. 2mL of plasma passed through each cartridge, followed by washing with 1mL of water. AngII peptides were eluted from the phenyl cartridge with 0.5mL of methanol, and then spun in a vacuum centrifuge until methanol was evaporated. The pellet was then reconstituted with 0.5mL of buffer provided in the kit (EIA buffer), vortexed and centrifuged at 3000g for 10 min at 4°C. The AngII EIA is based on the immobilization of a specific monoclonal anti-AngII from a 96-well plate. After immunological reaction with AngII, the trapped molecule was covalently linked to the plate by glutaraldehyde via amino groups. After washing and denaturing, AngII reacted with the acethlcholinesterase-labelled mAb, which is used as a tracer. The AChE tracer acts on Ellman's reagent to form a yellow compound, which can be quantified by spectrophotometry. Known concentrations of AngII were used to develop a standard curve. The amount of tracer bound to the well is proportional to the amount of AngII. AngII concentration in the plasma was expressed in pg/mL.

Immunoblot Analyses

Femoral artery tissue was homogenized in lysis buffer (20mM HEPES, 10mM NaCl, 1.5mM MgCl, 1mM DTT, 20% Glycerol, 0.1% Triton X-100), and centrifuged at 4000g for 10min at 4°C. The supernatant was collected and protein concentrations were obtained via BCA protein assay reagents using bovine serum albumin (*BSA*) as a standard. The remaining homogenate was stored at -80°C. These samples were optimally diluted to 1µg/µL with lysis buffer and sodium dodecyl sulphate (*SDS*) buffer containing DTT. After denaturing samples at 95°C for 5min, 30µL of the diluted sample (30µg of protein) was separated on a SDS-polyacrylamide gel (7.5%). Each sample was then transferred to a polyvinylidene difluoride (*PVDF*) membrane (40min at 25V). The membrane was treated overnight with blocking buffer containing 10% skim milk in tris buffered saline with 0.05% Tween20 (*TBS-T*). Membranes were sequentially exposed to a primary antibody (contained in a 10% skim milk solution) for 1 h at room temperature, three wash sequences with TBS-T, and a horseradish peroxidase (*HRP*)-conjugated secondary antibody for 1h. After the reaction, the membrane was washed 5 times with TBS-T and combined with enhanced chemiluminescence solution (GE Healthcare). Signals were detected on the Syngene gel documentation system. Primary antibodies applied included: polyclonal anti-ACE antibody (H-170), AT_1 (N-10), and AT_2 (H-143) receptor antibodies (1 : 200 dilution with blocking buffer; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies applied included: anti-rabbit (sc-2304) and goat anti-rabbit (sc-2004) immunoglobulin (1 : 2500 dilution with blocking buffer; Santa Cruz Biotechnology).

Study 2: Investigation of femoral artery vasomotor responses following an acute bout of exercise

Myography Protocol

The purpose for performing myography on the femoral arterial segments in this study was to quantify vasomotor function. Femoral arteries were excised from CON of IPEx male SD rats, cleaned of surrounding fat and connective tissue, and cut into 2mm ring segments. Excised femoral artery rings were mounted on single wire myography systems (Model 310A, v2.03; Danish Myo Technology, Denmark). Due to size limitations in the femoral artery (internal diameter = 150μm), single wire myographs were employed (these myographs are able to investigate vessels with internal diameters as small as 100μm). Thus, only two femoral artery rings could be used from each animal due to a limited number of single myographs available. These femoral artery rings were held by two adjacent 30μm stainless steel wires running through the vessel lumen. One wire was fixed to a micrometer to allow for adjustment of vessel lumen diameter and wall tension, while the other wire was linked to a force transducer. Femoral rings were immersed in a 10mL bath containing 37°C physiological buffer solution (131.5 mM NaCl, 13.5mM $NaHCO_3$, 11.3mM glucose, 5.0mM KCl, 2.5mM $CaCl_2$, 1.2mM NaH_2PO_4, 1.2mM $MgCl_2$, and 0.025mM EDTA) aerated with 95%O_2 - 5%CO_2 air. Isometric tension, in response to vasoactive compounds, was recorded at 100Hz sampling rate via a PowerLab system (AD Instruments, Boulder Springs, Colorado).

Identification of Femoral Artery Optimal Length

In vitro vasomotor function has been assessed in our laboratory on a number of vessel beds and rat species; however, myography protocols specified for the femoral arteries of SD

rats have not been previously developed. Thus, a straightforward resting length tension relationship of the vessel was determined to find an optimal resting length (L_0) for all future protocols to employ. Determining L_o requires administration of 60mM KCl, a dosage that elicits voltage-mediated vasoconstriction, at various resting tensions until the developed isometric tension has reached a plateau. More specifically, femoral were mounted, set to a resting tension of 100mg, and administered 60mM KCl. After a washout period, the vessel resting tension was increased by 50mg to 150 and, again, the vessel was subjected to 60mM KCl. This procedure was repeated (i.e. increasing resting tension by 50mg and administration of 60mM KCl) until a plateau or decrease in developed isometric tension had occurred. Discovering the optimal length for these femoral artery rings eliminated ~2h from future extensive myography protocols involving vasoconstrictory and vasodilatory agonists, as well as in-bath drug interventions. From pilot work completed in the laboratory I have determined that the L_0 of SD rat femoral arteries to be used in this study translated to a resting tension of 300mg; a number which was used in studies throughout my thesis work.

Assessment of electromechanical coupling and receptor-mediated vasoconstriction

To assess electromechanical coupling, in exercised and non-exercised male SD rats, dose-response curves to KCl were performed. Freshly excised femoral artery ring segments were set to the pre-determined L_0 and subjected to increasing amounts of KCl (10,20,30,40,50,60,70,80,90,100; all in mM). As developed isometric tensions plateaued at each concentration, subsequent KCl concentrations were administered until 100mM KCl was reached. After a washout period, α-adrenergic receptor-mediated vasoconstriction was assessed. PE was introduced into the tissue bath in increasing concentrations from 10^{-9}M to

10^{-4}M. After the developed isometric tension had reached a plateau at a given concentration, the subsequent concentration was administered until 10^{-4}M PE. The following administration pattern was followed: 10^{-9}M, $10^{-8.5}$M, 10^{-8}M, 10^{-7}M, $10^{-6.5}$M, 10^{-6}M, $10^{-5.5}$M, 10^{-5}M, $10^{-4.5}$M, 10^{-4}M.

Assessment of endothelium-dependent and independent vasodilation

Prior to assessing endothelium-dependent vasodilation via ACh dose-response curves, vessels were set to L_0 and pre-constricted with a concentration of PE that elicited ~75-80% maximal KCl contraction. These constriction conditions have been pre-determined in our laboratory to elicit vasoconstriction lasting at least 60min in quiescent rings (sufficient time needed to obtain an ACh dose-response curve) and it allows for decreases in developed isometric tension to be evaluated. Cumulative doses of ACh (10^{-9}M to10^{-4}M) were introduced at half Log M concentrations. To evaluate endothelium-independent vasodilation, rings were administered a maximal concentration of the NO donor sodium nitroprusside (*SNP*); thus, after completion of the ACh dose-response curve, a 10^{-4}M dose of SNP was administered to the femoral vessels which caused the vessels to fully dilate from the pre-determined PE pre-constriction dosage.

Study 3: Examination of the role that femoral vascular RAS plays in altered femoral artery vasomotor responses following acute exercise

To observe the involvement of local vascular RAS in femoral vasomotor function following acute exercise, femoral artery rings were subjected to various pharmacological treatments to manipulate specific components of the local RAS. Pharmacological treatment was applied to randomly selected rings and allowed to incubate for 30min prior to

performing the various dose-response curves. The drugs treatments used in study 3 are as follows: (i) no drug; *ND*, (ii) 10^{-5}M of the ACE inhibitor captopril; *CAP* (iii) 10^{-5}M of the AT_1 blocker telmisartan; *TEL* (iv) 10^{-5} of the AT_2 blocker PD123319; *PD* (v) 10^{-5}M captopril and 10^{-5}M telmisartan; *CAP+TEL* (vi) 10^{-5}M telmisartan and 10^{-5} PD123319; *TEL+PD* (vii) 10^{-5}M telmisartan and 10^{-4} of the NOS inhibitor L-NAME; *TEL+ L-NAME* (viii) 10^{-4} L-NAME; *L-NAME*.

Statistics

Vasoconstrictor dose-response curve data was measured as developed tension (peak constriction – resting tension) in milligrams. Vasodilator curve data was measured as peak % dilation [(pre-constriction tension – tension at peak dilation)/(pre-constriction tension) X 100%]. Dose-response curves were fit into a sigmoidal model with a bottom boundary of 0 (GraphPad Prism, v5.0a, San Diego, CA). Curve characteristics (maximal constriction/dilation, EC_{50} and area under the curve) were obtained from each femoral artery ring based on the fit sigmoidal curve. Rings pre-incubated with L-NAME alone or in combination with other drugs had a non-sigmoidal response when subjected to increasing amounts of ACh. Thus, the EC_{50} curve parameter from the ACh dose-response curve was not recorded for L-NAME rings; however, the max dilation and area under the curve was recorded and plotted.

The curve characteristics were compared with a two-way ANOVA (exercise vs. drug pre-incubation, $\alpha=0.05$) using SAS statistical software (v9.1, SAS Institute Inc., Cary, NC). Tukey's HSD post-hoc test ($\alpha=0.05$) was employed when significant differences were obtained. When required, curve characteristics for both control and exercised animals were

analyzed using a one-way ANOVA (vs. drug pre-incubation, α=0.025). Tukey's HSD post-hoc test (α=0.025) was employed when significant differences were obtained in the one-way ANOVA. For rat characteristics, ACE activity, catecholamines, plasma AngII, and Western blot data, Student's T-test for independent means was used (α=0.05). Values are expressed as means ± s.e.m. wherever appropriate.

RESULTS

Sprague Dawley Rat Characteristics and Exercise Protocol Confirmation

There were no age differences recorded between control and acutely exercised SD rats. In addition, no differences we observed between CON and IPEx SD rats in terms of body mass, left ventricular weight, right ventricular weight and kidney weight (Table 1). Exercise caused a significant increase in norepinephrine (Figure 3) and epinephrine (Figure 4) levels for SD rats. Norepinephrine rose approximately 87% in IPEx (n = 12) animals compared to CON animals (n = 14) ($p = 0.04$). Epinephrine rose approximately 162% in IPEx (n = 15) animals compared to CON (n = 14) animals ($p = 0.02$).

Plasma ACE activity and AngII levels

Plasma AngII levels were measured via a commercially available AngII enzyme immunoassay kit (Figure 5). Plasma AngII levels were ~94% higher following exercise when compared to control levels ($p = 0.03$). IPEx: 169.18 ± 32.63 pg/mL vs. CON: 97.20 ± 14.78 pg/mL. Plasma ACE activities were measured via a spectrofluorimetric assay adapted from Schwager et al.[62]. Plasma ACE activity (Figure 6) decreased by ~10% following acute exercise when compared to control ($p = 0.03$). CON: 0.89 ± 0.02 μmol/min/L vs. IPEx: 0.80 ± 0.03 μmol/min/L.

Femoral ACE activity

Tissue ACE activities were measured (Figure 7), in excised femoral arteries from CON animals (n = 11) and IPEx animals (n = 11), by a spectrofluorimetric assay adapted from Schwager et al.[62]. Femoral ACE activity decreased after the acute exercise protocol

by ~20% ($p = 0.04$). CON: 0.74 ± 0.05 μmol/min/g protein vs. IPEx: 0.59 ± 0.05 μmol/min/g protein.

Immunoblot Analyses

ACE (Figure 8), AT_1 (Figure 9) and AT_2 (Figure 10) receptor protein levels were measured in the femoral arteries of CON and IPEx animals via Western blotting. Femoral ACE protein decreased after acute exercise by ~39% following the acute exercise protocol (CON: 1.09 ± 0.26 AU vs. IPEx: 0.66 ± 0.14 AU, $p = 0.047$). Femoral AT_1 protein levels did not change following exercise (CON: 2.32 ± 0.27 AU vs. IPEx: 2.07 ± 0.16 AU, $p = 0.44$). Femoral AT_2 protein levels did not change following exercise (CON: 2.49 ± 0.26 AU vs. IPEx: 2.32 ± 0.27 AU , $p = 0.65$).

Assessment of electromechanical coupling – KCl dose-response curves

To assess electromechanical coupling, in exercised and non-exercised male SD rats, dose-response curves to KCl were performed. Three curve characteristics were used to identify any possible differences due to exercise or drug pre-incubations (Table 2A). These curve characteristics were the maximum vasoconstriction to KCl (MAX), half maximal effective concentration (EC_{50}), and area under the curve (AUC). No significant main effect of exercise (Figure 11) or interaction effects were observed for MAX, EC_{50} and AUC. However, a main effect of drug was observed for EC_{50} ($p < 0.001$). Post-hoc tests revealed that pre-incubation with the nitric oxide synthase inhibitor L-NAME alone, and in combination with AT_1 receptor blocker, telmisartan, caused a decrease in EC_{50} (Figure12, Tables 2B/C).

Assessment of α-adrenergic receptor-mediated vasoconstriction

Receptor-mediated vasoconstriction was assessed via dose-response curves to the α-adrenergic receptor specific agonist PE. Three curve characteristics were used to identify any possible differences due to exercise or pre-drug incubations (Table 3). These curve characteristics were the maximum vasoconstriction to PE (MAX), the half maximal effective concentration (EC_{50}), and area under the curve (AUC) (Tables 5/6). Main effects of exercise ($p = 0.01$) and drug pre-incubation ($p < 0.001$) were observed for MAX. There was a ~13% reduction in MAX following the acute exercise protocol when collapsed over all drug pre-incubation groups (Figure 13, CON: 2022.00 ± 66.85 mg vs. IPEx: 1766.82 ± 70.40 mg). Similar to MAX, there were significant main effects of exercise ($p = 0.003$) and drug pre- incubation ($p < 0.001$) for AUC. AUC decreased ~17% following exercise (CON: 3061.21 ± 121.21 AU vs. IPEx: 2536.69 ± 127.64 AU). A main effect of pre-drug incubation for EC_{50} was observed ($p = 0.04$), however Tukey's HSD post-hoc test revealed no differences in sensitivity to PE ($p>0.05$).

Although interaction effects of drug pre-incubation and exercise were not statistically significant for MAX and AUC in the two-way ANOVA ($p = 0.10$, $p=0.19$), apparent differences observed in the plotted data and a p-value close to significance warranted further investigation. Thus, a one-way ANOVA was performed for each exercise condition (CON and IPEx). To ensure true effects of MAX were being observed, a Bonferroni corrected alpha of 0.025 (0.05 / # of comparisons) was used to evaluate significance for the one-way ANOVA and Tukey's HSD post-hoc tests. Both CON ($p=0.02$) and IPEx ($p < 0.001$) groups displayed significant pre-drug incubation effects (Table 4/5). For simplicity, the following

sections address the changes in MAX only, since effects of drug pre-incubations are mirrored in AUC.

A) Effect of individual RAS blocker pre-incubation on PE maximal vasoconstriction

The maximal response to PE after exercise and no drug pre-incubation was ~18% lower compared to control animals with no drug pre-incubation (Tables 6/7). AT_1 blockade with telmisartan decreased MAX for CON by ~22% ($p = 0.015$) and decreased MAX for IPEx by ~31% ($p = 0.016$). An interesting observation was noted, where AT_1 blockade in CON elicited the same MAX as IPEx with no pre-drug incubation ($p = 0.75$) (Figure 14). ACE inhibition with captopril had no effect on MAX for CON ($p = 0.69$), but neared statistical significance in IPEx animals ($p = 0.04$) (Figure 15). In addition, AT_2 receptor blockade with PD123319 had no further effect on MAX for both CON ($p = 0.56$) and IPEx animals ($p = 0.83$) (Figure 16).

B) Effect of combined RAS blocker pre-incubation on PE maximal vasoconstriction

Combined ACE inhibition, with captopril (CAP), and AT_1 receptor blockade, with telmisartan (CAP+TEL), decreased MAX for CON by ~29% ($p = 0.01$) and ~30% for IPEx ($p = 0.01$) when compared to ND (Figure 17). CAP+TEL was, however, no different from TEL pre-incubation alone for both CON ($p = 0.61$) and IPEx (0.95). Interestingly, CAP+TEL was significantly lower from CAP alone in CON animals ($p = 0.01$), but not in IPEx animals ($p = 0.10$).

Combined AT_1 and AT_2 receptor blockade (TEL+PD) significantly decreased MAX in CON animals ($p = 0.02$), but not in IPEx animals ($p = 0.97$). TEL+PD was not significantly different from TEL alone in CON animals ($p = 0.75$), while TEL+PD increased MAX by ~31% in IPEx animals ($p = 0.025$) when compared to TEL alone. Interestingly, the MAX elicited by TEL alone for CON is no different from TEL+PD in IPEx animals ($p = 0.76$) (Figure 18).

C) Effect of nitric oxide synthase inhibition on PE maximal vasoconstriction

NOS activity was inhibited by pre-incubation with 10^{-4} N^{ω}nitro-L-arginine methyl ester (L-NAME), for 30min, prior to the construction of PE dose-response curves. L-NAME incubation had no effect on MAX in CON animals ($p=0.99$), while L-NAME increased MAX by ~29% in IPEx animals ($p = 0.002$) when compared to ND. Interestingly, the MAX elicited by CON with no pre-drug incubation was not significantly different from MAX elicited by L-NAME in IPEx ($p = 0.15$) (Figure 19).

Combined NOS inhibition and AT_1 receptor blockade eliminated the attenuating effects of TEL alone for IPEx animals ($p < 0.001$) and CON animals ($p = 0.02$. A special note, there is no difference observed in MAX PE elicited by TEL+L-NAME between CON and IPEx ($p = 0.84$) (Figure 20).

Assessment of endothelium-dependent vasodilation – ACh dose-response curves

Endothelium-dependent vasodilation was assessed via dose-response curves of the muscarinic receptor agonist ACh. Rings were subjected to a $10^{-5.5}$ dose of phenylephrine to cause a pre-constriction level equal to ~75-80% max constriction from which the dilatory

curve could be produced. Three curve characteristics were used to identify any possible differences due to exercise or pre-drug incubations (Table 8). These curve characteristics were the maximum vasodilation to ACh (MAX), the half maximal effective concentration (EC_{50}), and area under the curve (AUC) (Table 9/10). There was a main effect of drug on MAX ($p < 0.001$), EC_{50} ($p = 0.02$), and AUC ($p < 0.001$). There was no main effect of exercise (Figure 21) or interaction effects observed for MAX, EC_{50}, or AUC. For simplicity, the following sections will highlight the differences in pre-drug incubation on EC_{50} and MAX compared to no drug pre-incubation, since AUC changes reflect those seen in MAX.

A) Effect of individual RAS blocker pre-incubations on ACh vasodilation

The only individual RAS drug pre-incubation that was able to alter MAX was the AT_1 receptor blocker telmisartan (Figure 22, $p = 0.01$). CAP and PD alone were not able to alter MAX (Figure 23, $p = 0.38$ and Figure 24, $p=0.71$). The only individual RAS drug pre-incubation that was able to alter EC_{50} was the AT_2 receptor blocker PD123319 (Figure 24, $p = 0.03$). CAP and TEL pre-incubations did not alter EC_{50} ($p = 0.20$, $p = 0.12$).

B) Effect of combined RAS blocker pre-incubations on ACh vasodilation

ACE inhibition in combination with AT_1 receptor blockade (CAP+TEL) did not alter MAX, EC_{50}, or AUC any differently than TEL alone (Figure 25, $p > 0.05$). Combined AT_1 and AT_2 receptor blockade (TEL+PD) decreased MAX compared to AT_1 blockade alone (Figure 26, $p = 0.05$). MAX elicited by TEL+PD pre-incubation was not significantly different from ND pre-incubation ($p = 0.52$). EC_{50} was not affected by any combined RAS drug pre-incubations.

C) Effect of nitric oxide inhibition on ACh vasodilation.

EC_{50} was unable to be calculated for ACh dose-response curves with L-NAME or TEL+L-NAME pre-incubation due to the flat curve shape. MAX was decreased significantly NOS inhibition. L-NAME decreased MAX by ~83% (Figure 27, $p < 0.001$) and TEL+L-NAME decreased MAX by ~90% (Figure 28, $p < 0.001$).

Assessment of endothelium-independent vasodilation to SNP

Endothelium-independent vasodilation was assessed by administering a maximal dose (10^{-4}) of the NO donor SNP. This maximal dose was administered following the completion of the ACh dose-response curve. As seen in figure 29, endothelium-dependent vasodilation was not statistically different across all exercise and drug pre-incubation conditions ($p>0.05$).

Table 1: *Sprague Dawley Rat Characteristics*

Characteristic	***CON***	***n***	***IPEx***	***n***	***p-value***
Age (wks)	19.01±0.21	40	19.10±0.20	39	0.992
Body Mass (g)	453.32±6.52	40	450.99±5.43	39	0.785
LV weight (g)	1.43±0.03	39	1.41±0.02	39	0.691
RV weight (g)	0.23±0.01	39	0.25±0.02	39	0.361
Kid weight (g)	0.94±0.01	39	0.91±0.02	39	0.236

Values are stated as means ± s.e.m. CON, control animals; IPEx, immediately post-exercise animals; LV, left ventricle; RV, right ventricle; Kid, kidney. p-value obtained by Student's T-test for independent means.

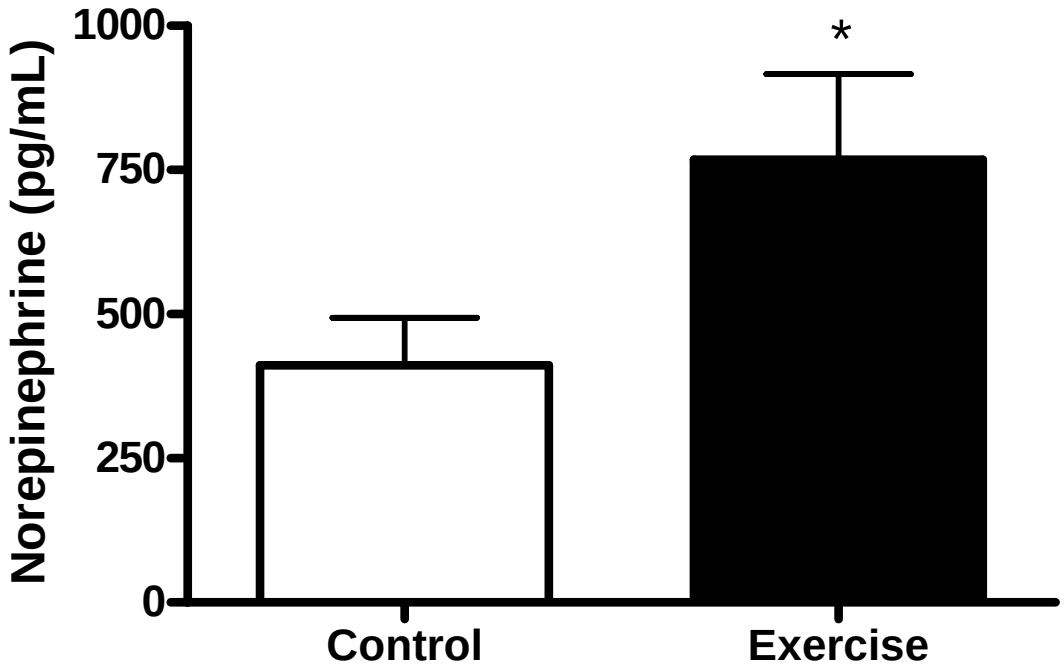

Figure 3: *Norepinephrine levels increase after acute exercise.* Norepinephrine levels were obtained via high pressure liquid chromatography. SD rats that underwent the acute exercise protocol had increased levels of norepinephrine (~87%; $p = 0.04$) compared to control animals. IPEx: 767.73 ± 148.28 pg/mL vs. CON: 411.18 ± 82.85 pg/mL. Values are expressed as means ± s.e.m., n = 12-15 per exercise condition. p-value obtained by Student's T-test for independent means.

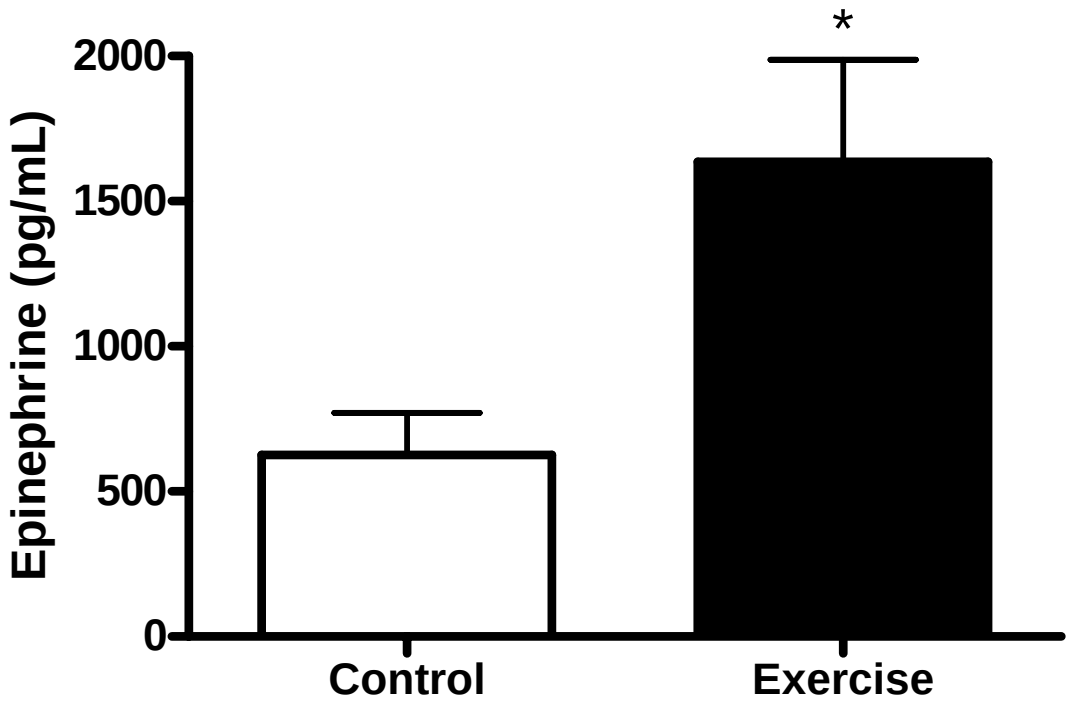

Figure 4: *Epinephrine levels increase after acute exercise.* Eprinephrine levels were obtained via high pressure liquid chromatography. SD rats that underwent the acute exercise protocol had increased levels of epinephrine (~162%; $p = 0.02$) compared to control animals. IPEx: 1635.3 ± 352.01 pg/mL vs. CON: 625.02 ± 145.09 pg/mL. Values are expressed as means ± s.e.m., n = 12-15 per exercise condition. p-value obtained by Student's T-test for independent means.

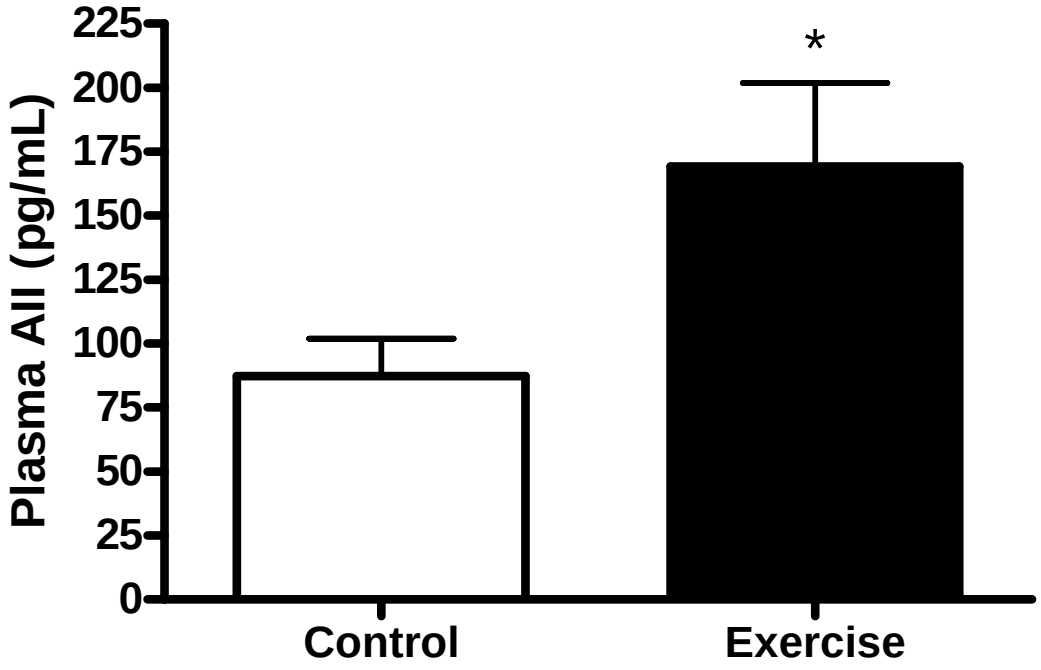

Figure 5: *Plasma angiotensin II levels increase following acute exercise.* Plasma AngII levels were measured via a commercially available AngII enzyme immunoassay kit (SPI-BIO, France). Plasma AngII levels were ~94% higher following exercise when compared to control levels ($p = 0.03$). IPEx: 169.18 ± 32.63 pg/mL vs. CON: 97.20 ± 14.78 pg/mL. Values are expressed as means ± s.e.m., n = 18-19 per exercise condition. p-value obtained by Student's T-test for independent means.

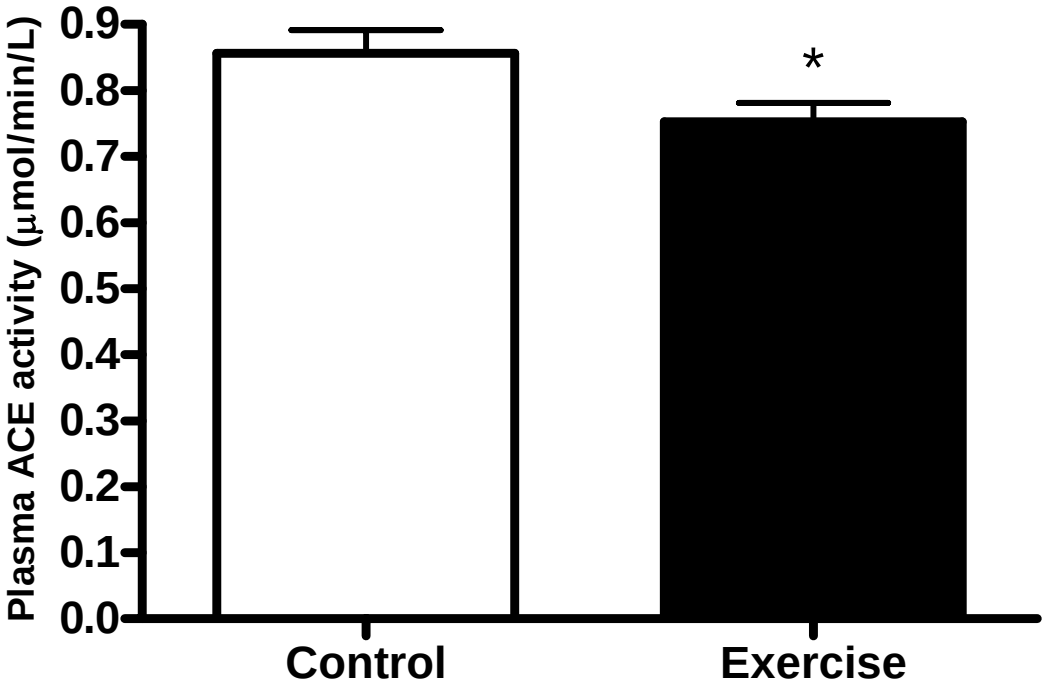

Figure 6: *Plasma ACE activity is reduced following acute exercise.* Plasma ACE activities were measured via a spectrofluorimetric assay adapted from Schwager et al. (2006). Plasma ACE activity decreased by ~10% following acute exercise when compared to control ($p = 0.03$). CON: 0.89 ± 0.02 µmol/min/L vs. IPEx: 0.80 ± 0.03 µmol/min/L. Values are expressed as means ± s.e.m., n = 13-15 per exercise group. p-value obtained by Student's T-test for independent means.

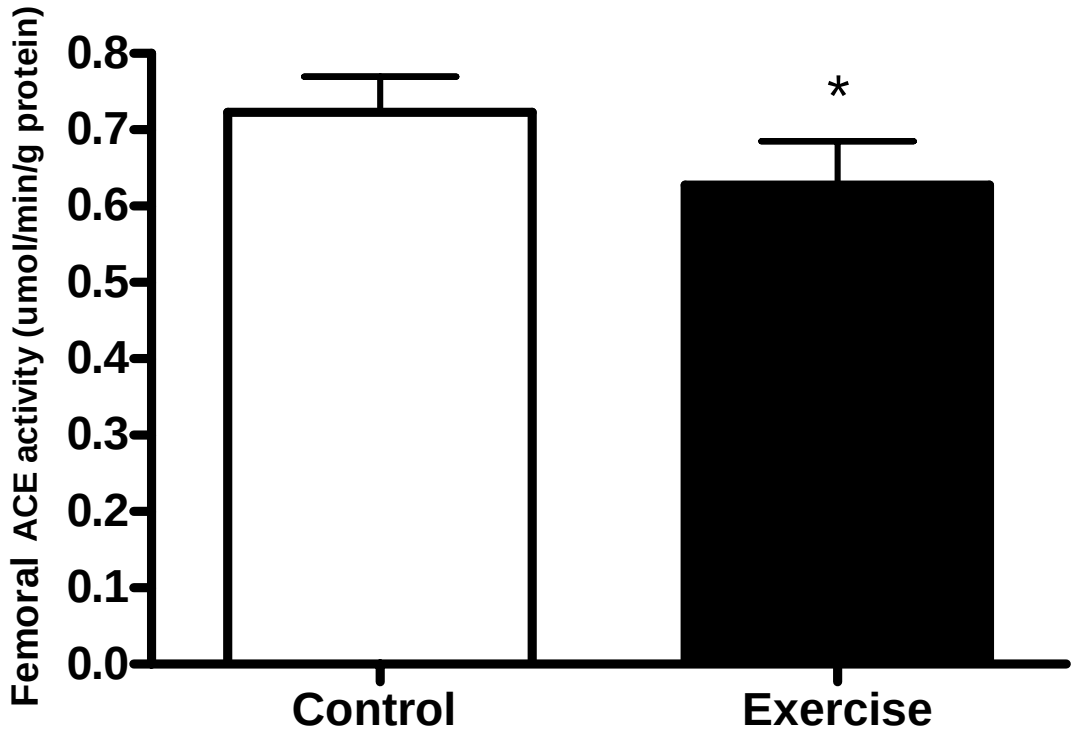

Figure 7: *Femoral ACE activity decreases following acute exercise.* Tissue ACE activities were measured, in excised femoral arteries from CON animals (n = 11) and IPEx animals (n = 11), by a spectrofluorimetric assay adapted from Schwager et al. (2006). Femoral ACE activity decreased after the acute exercise protocol by ~20% (p = 0.04). CON: 0.74 ± 0.05 μmol/min/g protein vs. IPEx: 0.59 ± 0.05 μmol/min/g protein. Values are expressed as means ± s.e.m. p-value obtained by Student's T-test for independent means.

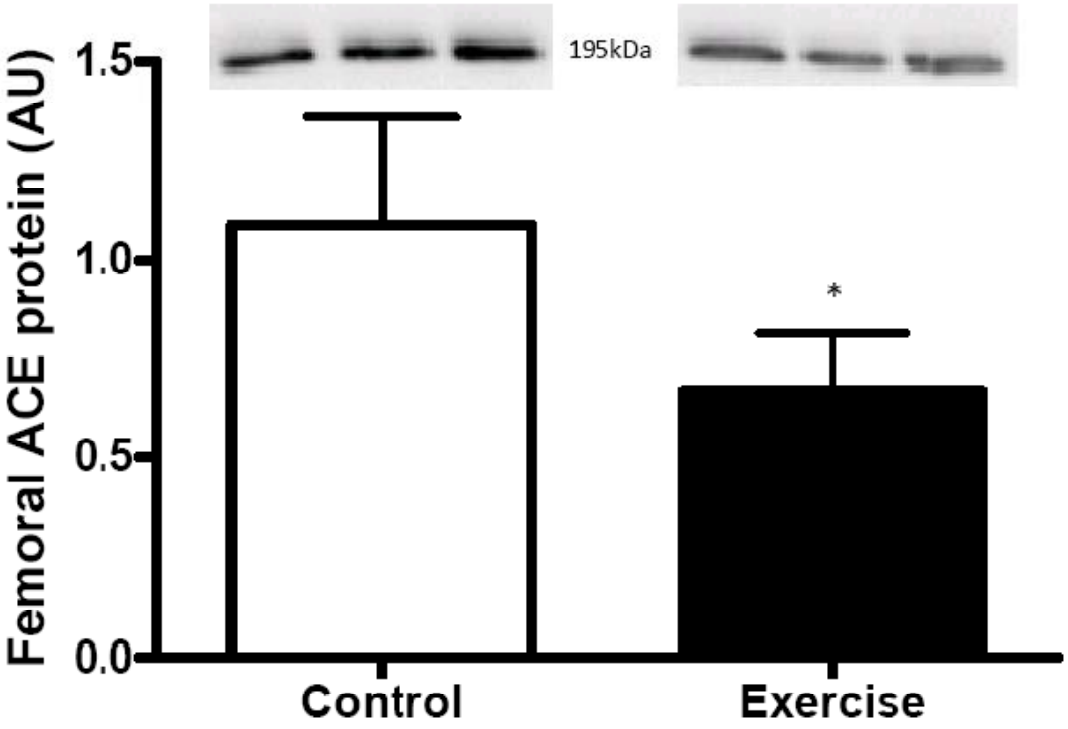

Figure 8: *Femoral ACE protein levels decrease following acute exercise.* Femoral ACE protein levels were measured via Western blot analyses. Femoral ACE protein decreased after acute exercise by ~39% following the acute exercise protocol ($p = 0.047$). CON: 1.09 ± 0.26 AU vs. IPEx: 0.66 ± 0.14 AU. Values are expressed as means ± s.e.m.. p-value obtained by Student's T-test for independent means.

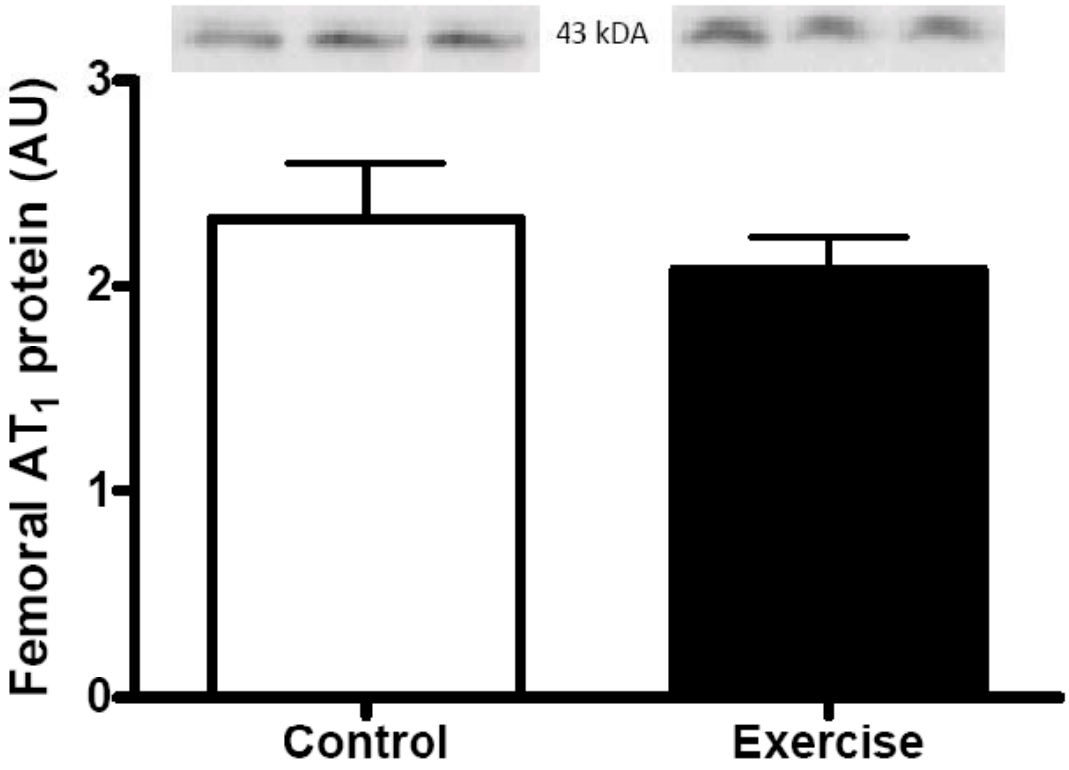

Figure 9: *Femoral AT_1 protein levels are unchanged following acute exercise.* Femoral AT_1 protein levels were measured via Western blot analyses. Femoral AT_1 protein levels did not change following exercise ($p = 0.44$). CON: 2.32 ± 0.27 AU vs. IPEx: 2.07 ± 0.16 AU. Values are expressed as means ± s.e.m.. p-value obtained by Student's T-test for independent means.

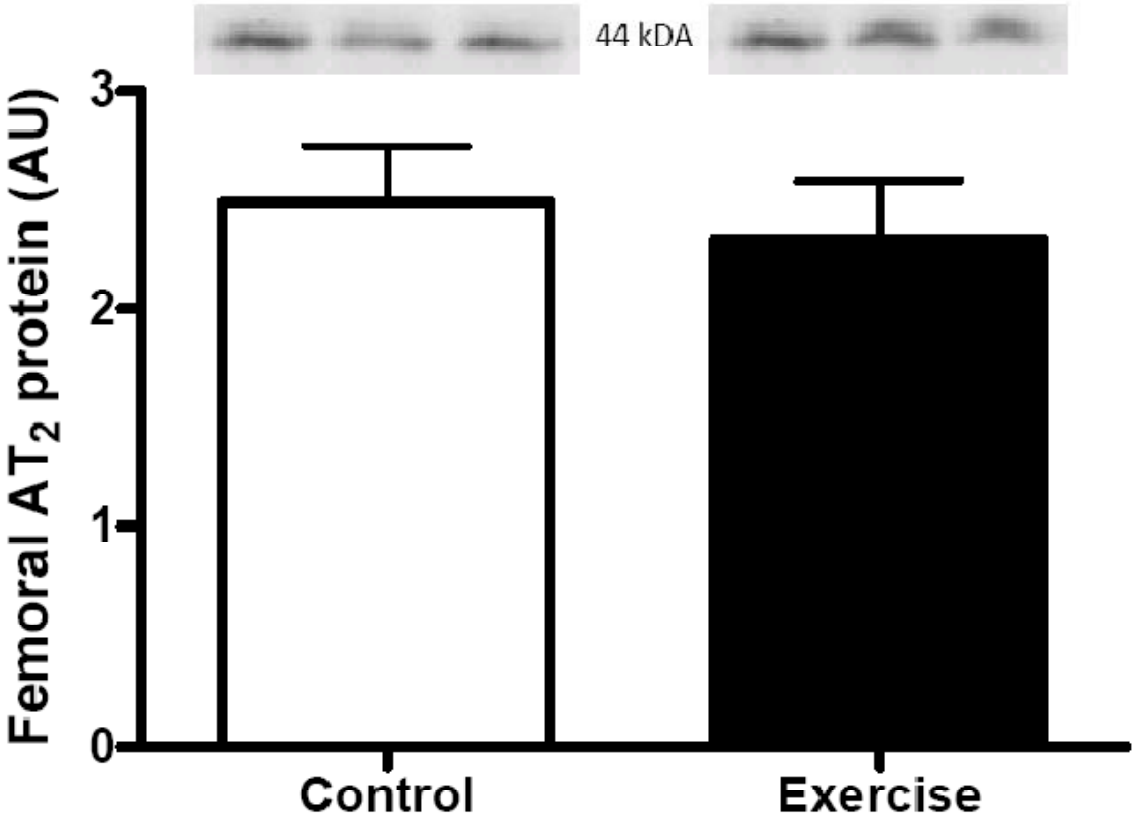

Figure 10: *Femoral AT2 protein levels are unchanged following acute exercise.* Femoral AT_2 protein levels were measured via Western blot analyses. Femoral AT_2 protein levels did not change following exercise ($p = 0.65$). CON: 2.49 ± 0.26 AU vs. IPEx: 2.32 ± 0.27 AU. Values are expressed as means ± s.e.m.. p-value obtained by Student's T-test for independent means.

Table 2: *Electromechanical coupling to potassium chloride (KCl)*

A

KCl	***p-values***		
	Drug	Exercise	Drug*Exercise
MAX (mg)	0.96	0.25	0.82
EC_{50} (mM)	<0.001	0.22	0.08
AUC (AU)	0.52	0.49	0.53

B

KCl	***ND*#*** ***(n=21)***	***CAP*#*** ***(n=13)***	***TEL*#*** ***(n=10)***	***PD*#*** ***(n=10)***	***L-NAME*** ***(n=11)***
EC_{50} (mM)	46.10 ± 1.13	48.77 ± 1.43	46.31 ± 1.68	45.48 ± 1.77	40.21 ± 1.51

C

KCl	***CAP+TEL*#*** ***(n=11)***	***TEL+PD*#*** ***(n=10)***	***TEL+L-NAME*** ***(n=12)***
EC_{50} (mM)	45.47 ± 1.51	45.42 ± 1.58	38.66 ± 1.53

A *p-values obtained from two-way ANOVA*. Main effect of drug on Log EC_{50}. No main effect of drug on KCl max constriction (MAX) and area under curve (AUC). No main effect of exercise on MAX, Log EC_{50}, and AUC. No interaction effects seen in MAX, Log EC_{50}, and AUC. MAX: max constriction, AUC: area under curve, AU: arbitrary units. **B** *Log EC_{50} values from single drug conditions.* **C** *Log EC_{50} values from combination drug conditions.* Log EC_{50} values of ND, CAP, TEL, PD, CAP+TEL, and TEL+PD were significantly different from L-NAME and TEL+L-NAME values ($p<0.05$). No difference was observed between L-NAME and TEL+L-NAME ($p>0.05$). * significantly different from L-NAME. # significantly different from TEL+L-NAME.

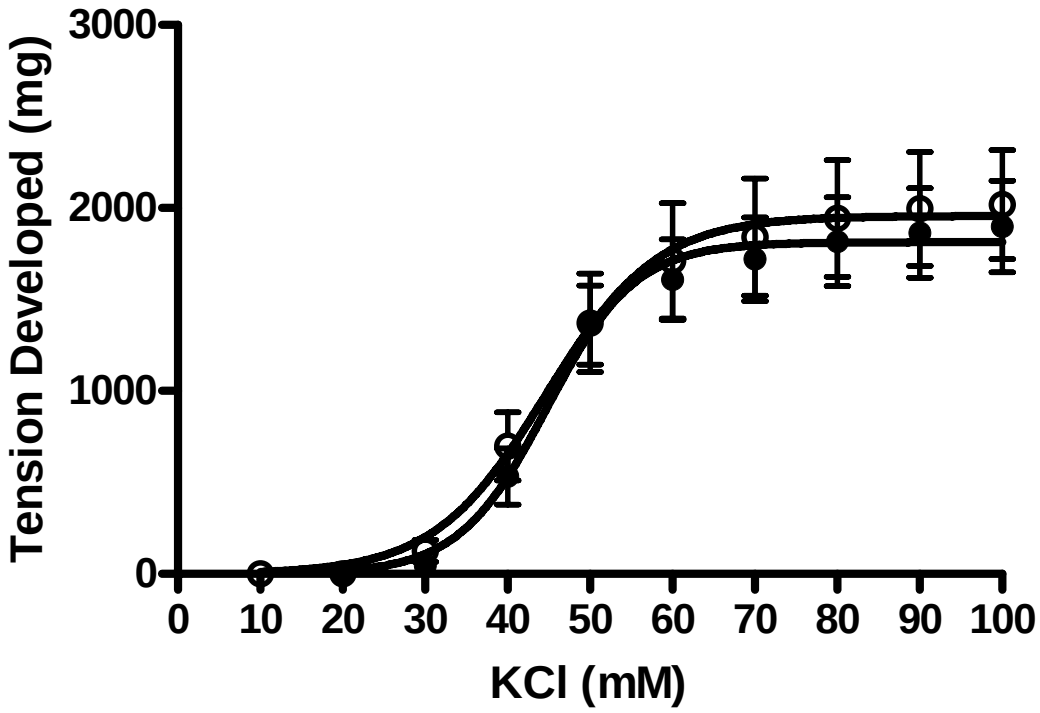

Figure 11: *No differences in CON and IPEx KCl dose-response curves.* Femoral artery rings from CON (•, n=52) and IPEx animals (○, n=43) were mounted on single wire myographs and set to the pre-determined L_0 = 300mg. Differences between CON and IPEx electromechanical coupling was assessed via a KCl dose-response curve. No differences were seen between CON and IPEx electromechanical coupling (exercise main effect, p = 0.25). Curve characteristics were (1) MAX (CON: 1859.66 ± 73.87mg vs. IPEx: 1983.79 ± 77.29mg), (2) EC_{50} (CON: 43.26 ± 0.75 mM vs. IPEx: 44.60 ± 0.78mM), (3) AUC (CON: 104579.79± 4342.39 AU vs. IPEx: 108892 ± 4537.94 AU). Values are expressed as means ± s.e.m. p-value obtained from two-way ANOVA.

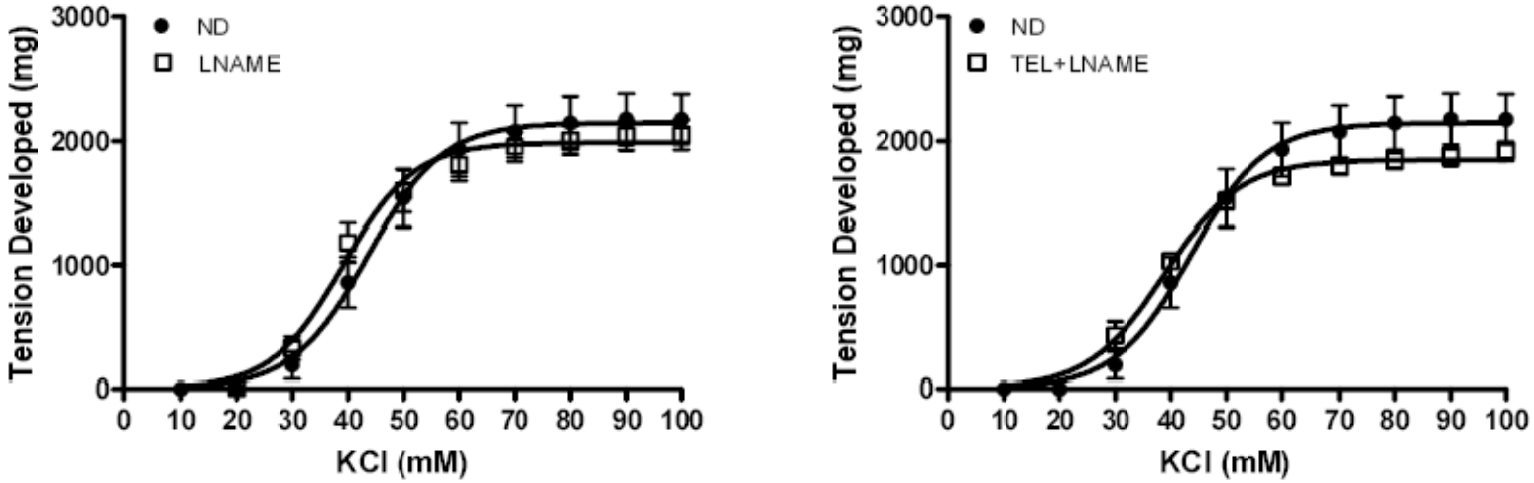

Figure 12: *Log EC_{50} values for L-NAME and TEL+L-NAME are different compared to all other drug conditions.* The left panel shows the KCl dose-response curves for the nitric oxide synthase inhibitor L-NAME (n=11) and no drug (n=22) conditions. The Log EC_{50} value, or concentration to half of max constriction, for L-NAME is significantly different from ND ($p<0.05$). The right panel shows the KCl dose-response curves for the TEL+L-NAME (n=12) and no drug conditions (n=22). The Log EC_{50} value for TEL+L-NAME is significantly different from ND ($p<0.05$). Note: The ND condition was used to represent all other drug conditions without L-NAME for clarity purposes. ND was not significantly different than CAP, TEL, PD, CAP+TEL, TEL+PD ($p>0.05$). p-values were obtained from Tukey's HSD post-hoc tests performed after two-way ANOVA.

Table 3: *Phenylephrine dose-response curve – p-values from two-way ANOVA*

PE	***p-values***		
	Drug	Exercise	Drug*Exercise
MAX (mg)	<0.001	0.01	0.10 ^
EC_{50} (LogM)	0.04	0.12	0.92
AUC (AU)	<0.001	0.003	0.19 ^

p-values obtained from two-way ANOVA. Main effect of drug and exercise on MAX PE constriction was significant. No main effect of exercise on EC_{50}. However, drug main effect was observed for EC_{50}. Main effects of drug and exercise on AUC were significant. ^Drug*Exercise interaction effect was not significant (p = 0.10, 0.19), but was further investigated via one-way ANOVA, see text for details.

Table 4: *Phenylephrine dose-response curve – p-values from one-way ANOVA for MAX PE*

	p-value (vs. drug condition)
CONTROL	0.02
EXERCISE	<0.001

p-values obtained from one-way ANOVA performed for CON and IPEx groups evaluating differences due to drug condition. Both CON and IPEx groups have significant drug effect. A Bonferroni-adjusted alpha was used ($\alpha = 0.025$).

Table 5: *Phenylephrine dose-response curve – p-values from one-way ANOVA for AUC*

	p-value (vs. drug condition)
CONTROL	<0.001
EXERCISE	<0.001

p-values obtained from one-way ANOVA performed for CON and IPEx groups evaluating differences due to drug condition. Both CON and IPEx groups have significant drug effect. A Bonferroni-adjusted alpha was used ($\alpha = 0.025$).

Table 6: *Phenylephrine dose-response curve characteristics for control animals*

PE	*ND* *(n=13)*	*CAP* *(n=8)*	*TEL* *(n=5)*	*PD* *(n=5)*	*L-NAME* *(n=5)*	*CAP+TEL* *(n=5)*	*TEL+PD* *(n=5)*	*TEL+ L-NAME* *(n=8)*
MAX (mg)	2169.1±119.9	2256.2±161.9	1688.3±224.3	2323.3±224.3	2168.4±200.6	1527.4±200.6	1588.4±200.6	2288.5±158.6
EC_{50} (LogM)	-5.51±0.19	-5.30±0.23	-5.43±0.13	-5.45±0.17	-5.67±0.12	-5.42±0.53	-5.56±0.11	-5.56±0.46
AUC (AU)	3332±217	3188±288	2450±407	3485±407	3649±364	2246±364	2561±364	3579±288

Values are listed as means ± s.e.m. Max constriction, EC_{50} and area the under curve values to phenylephrine are listed. These values correspond to control animals under various pre-drug incubation conditions.

Table 7: *Phenylephrine dose-response curve characteristics for acute exercise animals*

PE	*ND* *(n=8)*	*CAP* *(n=5)*	*TEL* *(n=5)*	*PD* *(n=5)*	*L-NAME* *(n=6)*	*CAP+TEL* *(n=6)*	*TEL+PD* *(n=5)*	*TEL+ L-NAME* *(n=5)*
MAX (mg)	1777.4±139.8	1630.3±165.4	1227.3±165.4	1829.0±184.9	2488.2±150.9	1240.2±150.9	1769.4±165.4	2345.8±184.9
EC_{50} (LogM)	-5.29±0.26	-5.14±0.58	-5.36±0.21	-5.48±0.12	-5.52±0.16	-5.31±0.21	-5.41±0.12	-5.63±0.23
AUC (AU)	2254±307	1900±364	1699±364	2751±407	3869±332	1667±332	2298±364	3856±407

Values are listed as means ± s.e.m. Max constriction, EC_{50} and area the under curve values to phenylephrine are listed. These values correspond to exercise animals under various pre-drug incubation conditions.

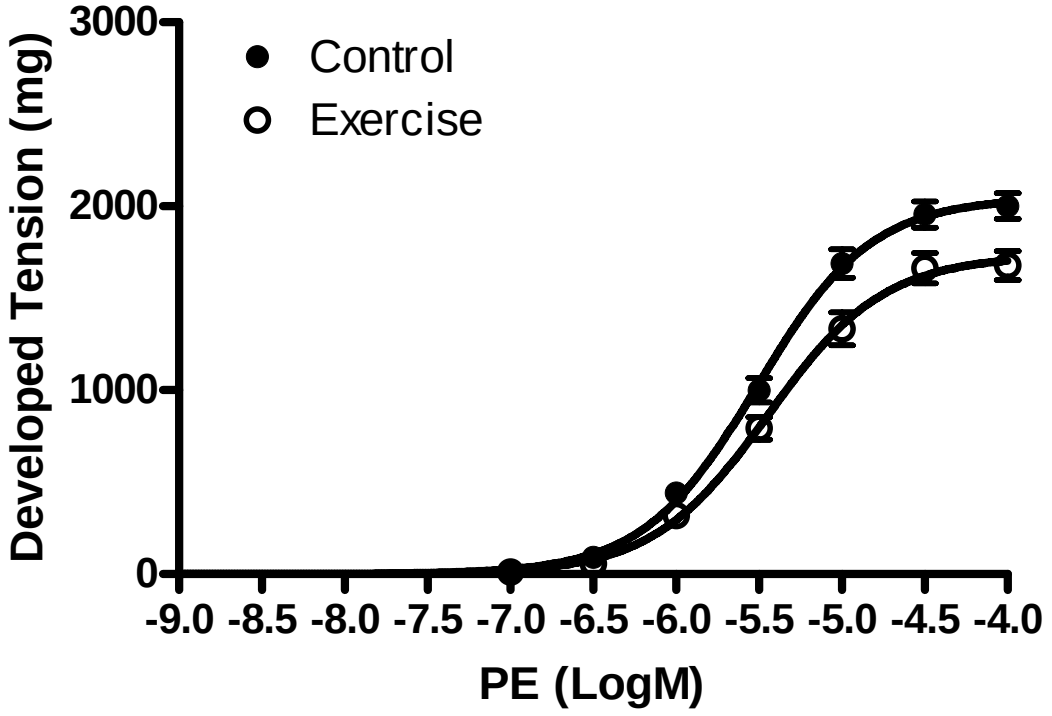

Figure 13: *Acute exercise effect on max α-adrenergic receptor-mediated constriction.* Femoral artery rings from CON and IPEx animals were mounted on single wire myographs and set to the pre-determined L_0 = 300mg. Differences between CON (n=53) and IPEx (n=42) α-adrenergic receptor-mediated contraction was assessed via a PE dose-response curve. A significant difference were observed between CON and IPEx phenylephrine constriction (exercise main effect, p = 0.01). Curve characteristics obtained were (1) MAX (CON: 2022.00 ± 66.85 mg vs. IPEx: 1766.82 ± 70.40 mg), (2) EC_{50} (CON: -5.49±0.04 LogM vs. IPEx: -5.39±0.04 LogM), (3) AUC (CON: 3061.21±121.21 AU vs. IPEx: 2536±127.64 AU). Values are expressed as means ± s.e.m. p-value obtained from two-way ANOVA.

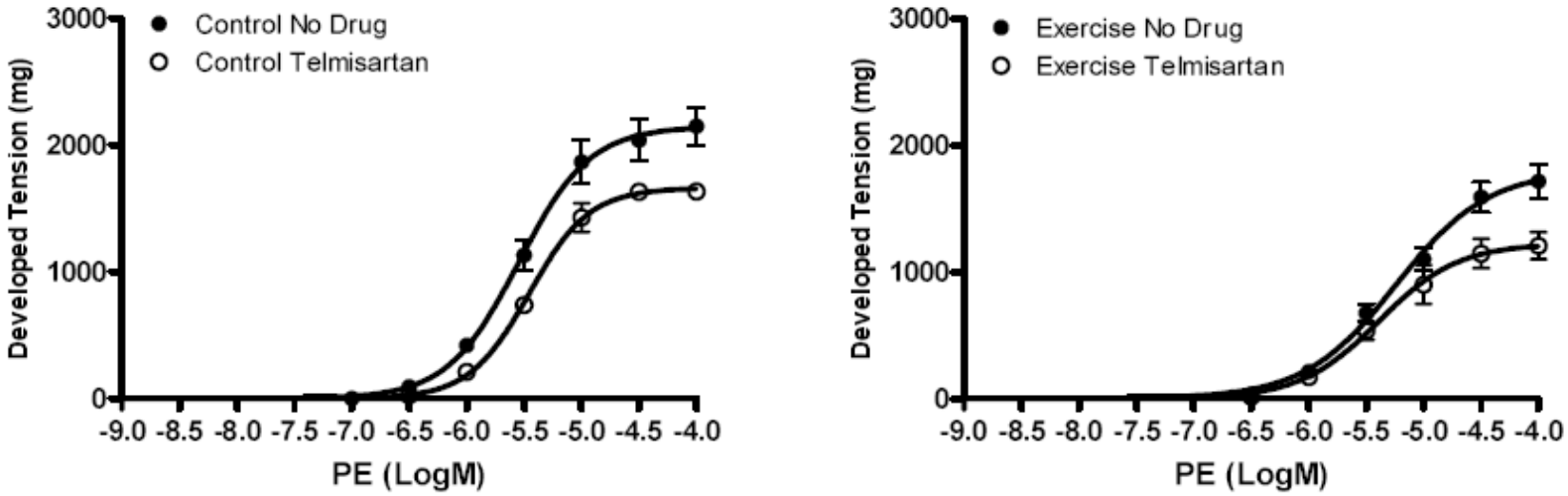

Figure 14: *Effect of the AT_1 receptor blocker telmisartan on max α-adrenergic receptor-mediated constriction.* Femoral arteries from CON (n=5) and IPEx (n=5) animals were pre-incubated with 10^{-5} telmisartan (TEL) for 30min prior to the construction of phenylephrine (PE) dose-response curves. For clarity purposes, the left panel illustrates the TEL effect on control animals and the right panel displays the TEL effect on exercise animals. Main effect of drug ($p = 0.01$). p-value obtained from two-way ANOVA. Tukey's HSD post-hoc test revealed significant differences between TEL and ND ($p<0.05$).

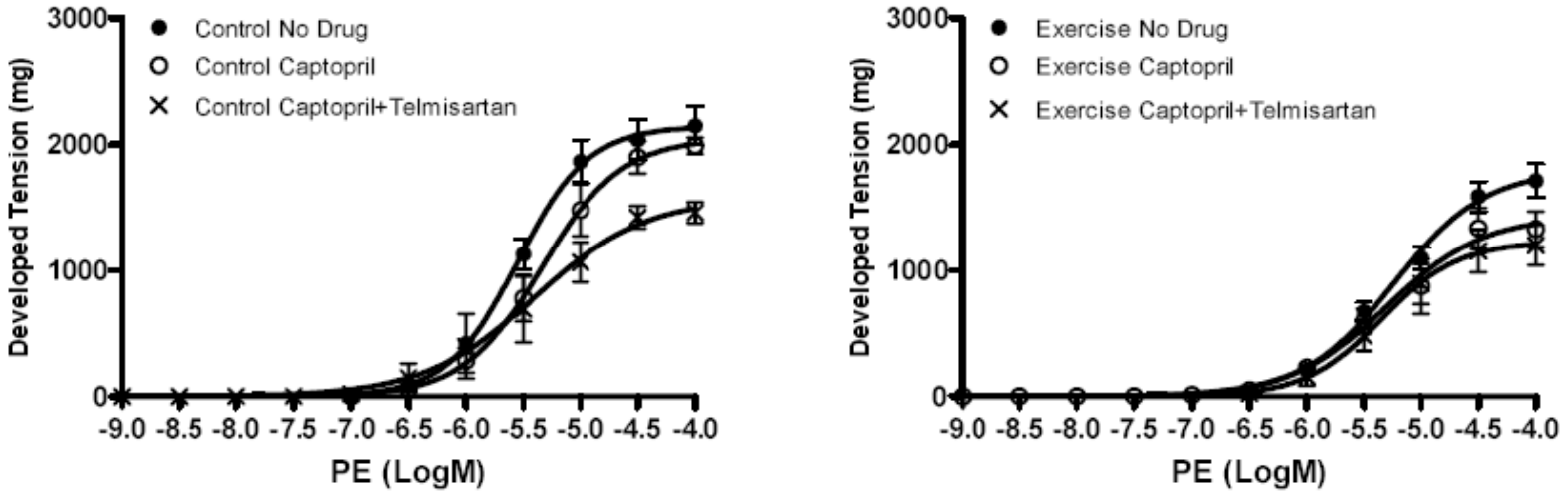

Figure 15: *No effect of the ACE inhibitor captopril on max α-adrenergic receptor-mediated constriction.* Femoral arteries from CON (n=8) and IPEx (n=5) animals were pre-incubated with 10^{-5} captopril (CAP) for 30min prior to the construction of phenylephrine (PE) dose-response curves. For clarity purposes, the left panel illustrates the CAP effect on control animals. CAP is not significantly different from the ND condition ($p = 0.68$) in CON animals, however co-incubation with 10^{-5} telmisartan (CAP+TEL, n=5) is significantly lower than ND alone ($p = 0.01$). The right panel displays the CAP effect on exercise animals. CAP appears to be different, however with the adjusted p-value, it did not reach statistical significance when compared to the ND condition ($p = 0.04$). In IPEx animals, however co-incubation with 10^{-5} telmisartan (CAP+TEL, n=6) is significantly lower than ND alone ($p = 0.02$). p-values obtained from one-way ANOVA with a Bonferroni corrected alpha = 0.025.

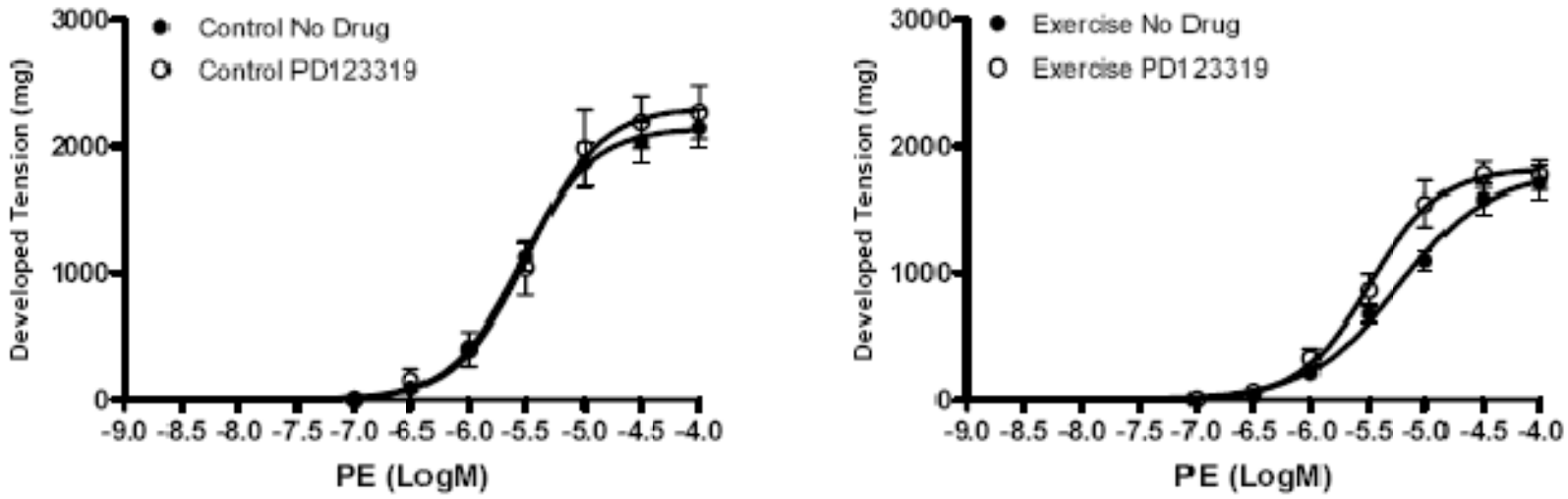

Figure 16: No effect of the AT_2 receptor blocker PD123319 on max α-adrenergic receptor-mediated constriction. Femoral artery rings from CON and IPEx animals were pre-incubated with 10^{-5} PD123319 (PD) for 30min prior to the construction of phenylephrine (PE) dose-response curves. For clarity purposes, the left panel illustrates the PD effect on control animals and the right panel displays the PD effect on exercise animals. Main effect of drug ($p = 0.01$). p-value obtained from two-way ANOVA. Tukey's HSD post-hoc test revealed no significant differences between PD and ND ($p>0.05$).

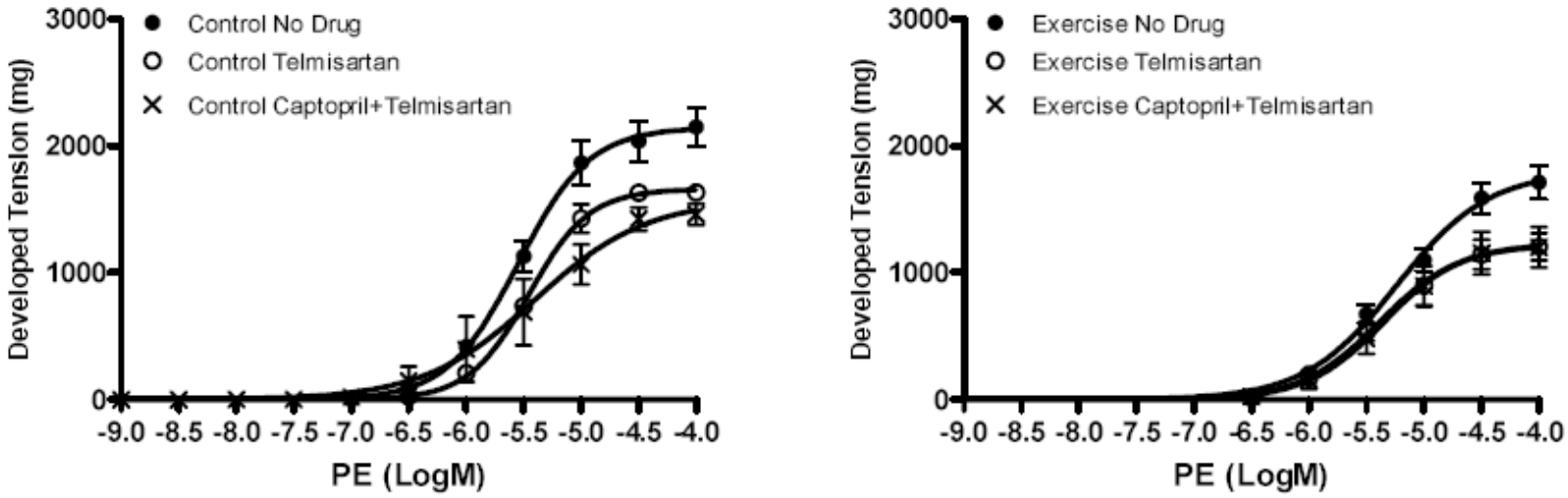

Figure 17: *The effect of combined ACE inhibitor and AT_1 receptor blocker on max α-adrenergic receptor-mediated constriction.* Femoral artery rings from CON and IPEx animals were pre-incubated with 10^{-5} telmisartan (TEL) alone or in combination with 10^{-5} captopril (CAP+TEL) for 30min prior to the construction of phenylephrine (PE) dose-response curves. The left panel illustrates that, for CON animals, there is no further attenuation of MAX PE with CAP+TEL pre-incubation compared to TEL ($p = 0.59$). The right panel illustrates that, for IPEx animals, there is no further attenuation of MAX PE with CAP+TEL pre-incubation compared to TEL ($p = 0.96$). p-values are obtained from one-way ANOVAs performed on both CON and IPEx groups, with a Bonferroni adjusted alpha ($\alpha = 0.025$).

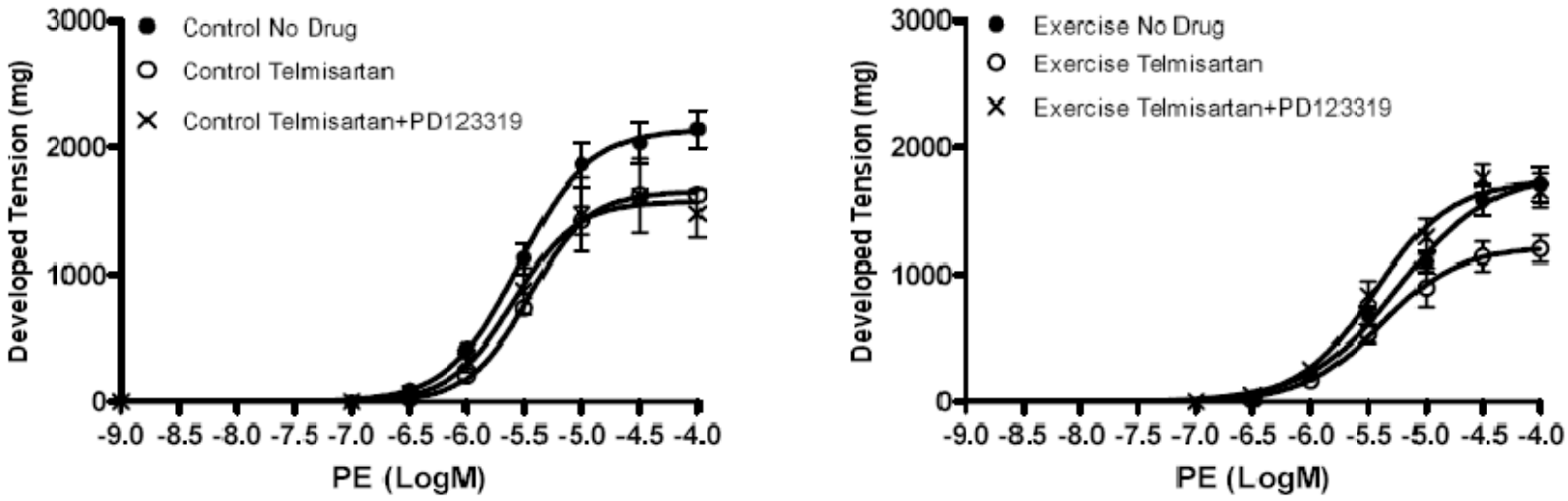

Figure 18: *The effect of combined AT_1 and AT_2 receptor blockade on max α-adrenergic receptor-mediated constriction.* Femoral artery rings from CON and IPEx animals were pre-incubated with 10^{-5} telmisartan (TEL) alone or in combination with 10^{-5} PD123319 (TEL+PD) for 30min prior to the construction of phenylephrine (PE) dose-response curves. The left panel illustrates that, for CON animals, there is no difference in MAX PE between TEL+PD pre-incubation compared to TEL ($p = 0.75$). The right panel illustrates that, for IPEx animals, the attenuated MAX PE with TEL pre-incubation was eliminated with TEL+PD ($p = 0.02$). p-values are obtained from one-way ANOVAs performed on both CON and IPEx groups, with a Bonferroni adjusted alpha ($\alpha = 0.025$).

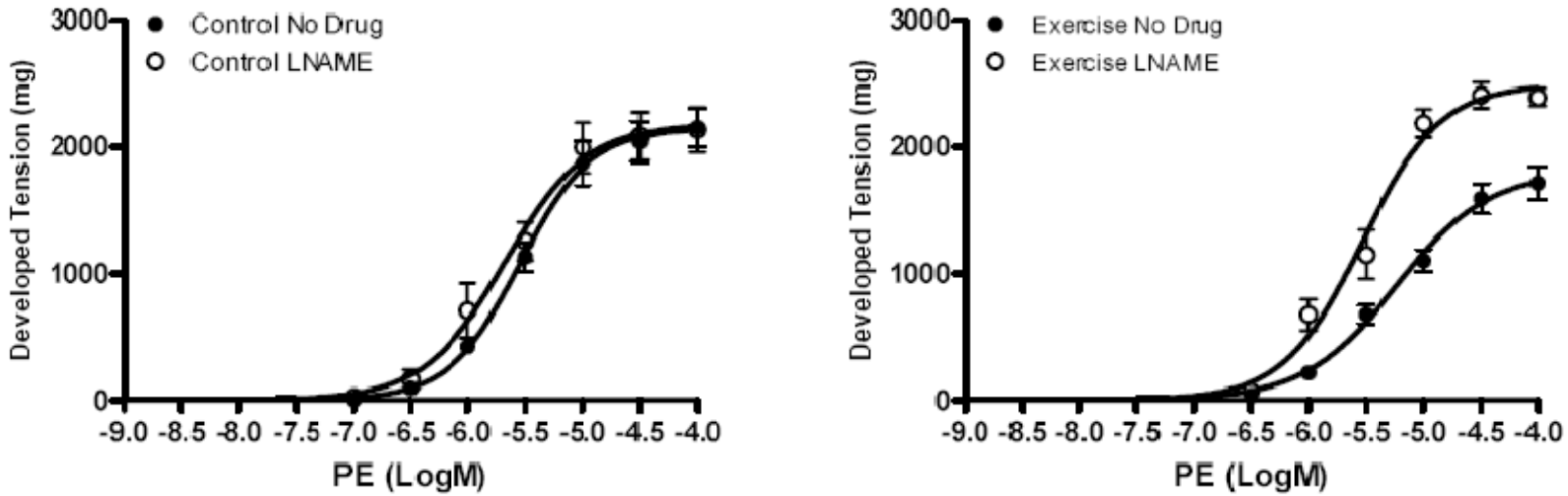

Figure 19: *The effect of nitric oxide synthase (NOS) inhibition on max α-adrenergic receptor-mediated constriction.* Femoral arteries from CON (n=5) and IPEx (n=6) animals were pre-incubated with 10^{-4} N^{ω}-nitro-L-arginine methyl ester (L-NAME) for 30min prior to the construction of phenylephrine (PE) dose-response curves. For clarity purposes, the left panel illustrates the L-NAME effect on control animals and the right panel displays the L-NAME effect on exercise animals. There is no L-NAME effect on MAX PE for CON animals ($p = 0.99$), while a significant L-NAME effect occurs for IPEx animals ($p = 0.002$). p-values are obtained from one-way ANOVAs performed on both CON and IPEx groups, with a Bonferroni adjusted alpha ($\alpha = 0.025$).

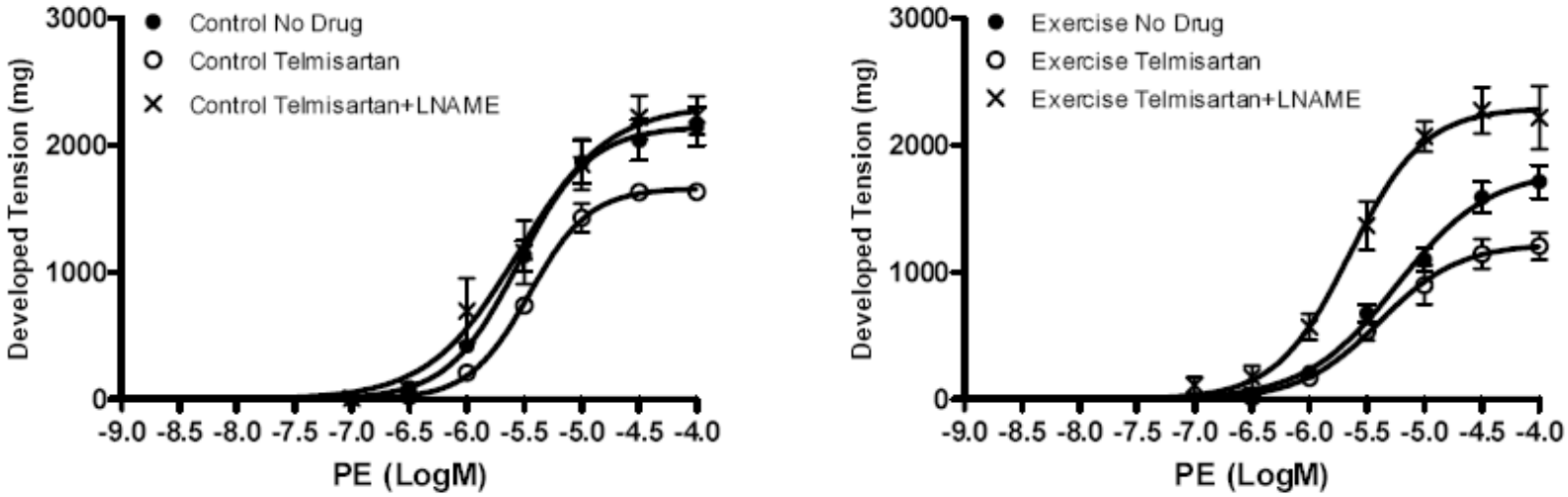

Figure 20: *Effect of Combined AT_1 blockade and nitric oxide synthase (NOS) inhibition on max α-adrenergic receptor-mediated constriction.* Femoral artery rings from CON and IPEx animals were pre-incubated with 10^{-5} telmisartan (TEL) alone or in combination with 10^{-4} N^{ω}-nitro-L-arginine methyl ester (TEL+L-NAME) for 30min prior to the construction of phenylephrine (PE) dose-response curves. The left panel displays CON PE curves, while the right panel displays IPEx PE curves. For CON animals, the combination of TEL+L-NAME eliminates the attenuating effect of TEL on MAX PE ($p = 0.02$). There is no difference in MAX PE between TEL+L-NAME and ND for CON animals (0.56). For IPEx animals, the combination of TEL+L-NAME eliminates the attenuating effect of TEL on MAX PE (<0.001). The MAX PE elicited by TEL+L-NAME is significantly higher than ND for IPEx animals ($p = 0.02$). A special note, there is no difference observed in MAX PE elicited by TEL+L-NAME between CON and IPEx ($p = .84$) p-values are obtained from one-way ANOVAs performed on both CON and IPEx groups, with a Bonferroni adjusted alpha ($\alpha = 0.025$).

Table 8: *Endothelium-dependent relaxation to acetylcholine (ACh)*

ACh	***p-values***		
	Drug	Exercise	Drug*Exercise
MAX (% dilation)	<0.001	0.33	0.77
EC_{50} (LogM)	0.02	0.37	0.09
AUC (AU)	<0.001	0.57	0.45

p-values obtained from two-way ANOVA. Main effect of drug on maximum dilation (MAX), EC_{50}, and area under curve (AUC). No main effect of exercise across curve characteristics. No interaction effect seen across curve characteristics. on KCl max constriction (MAX) and area under curve (AUC). AU: arbitrary units.

Table 9: *Acetylcholine dose-response curve characteristics for control animals*

ACh	*ND* *(n=13)*	*CAP* *(n=8)*	*TEL* *(n=5)*	*PD* *(n=5)*	*L-NAME* *(n=5)*	*CAP+TEL* *(n=5)*	*TEL+PD* *(n=5)*	*TEL+ L-NAME* *(n=8)*
MAX (%)	67.83±5.66	60.79±7.49	99.98±10.59	71.99±10.59	9.91±9.47	95.39±9.47	79.80±9.47	9.66±7.85
EC_{50} (LogM)	-6.59±0.07	-6.68±0.10	-6.86±0.14	-6.19±0.14	-	-6.86±0.13	-6.40±0.12	-
AUC (AU)	185.6±14.40	201.76±19.05	292.02±26.94	164.73±26.94	30.66±24.10	275.32±24.09	200.78±24.10	28.75±19.05

Values are listed as means ± s.e.m. % of maximum dilation, EC_{50} and area the under curve values to ACh are listed. These values correspond to control animals under various pre-drug incubation conditions.

Table 10: *Acetylcholine dose-response curve characteristics for acute exercise animals*

ACh	*ND* *(n=8)*	*CAP* *(n=5)*	*TEL* *(n=5)*	*PD* *(n=5)*	*L-NAME* *(n=6)*	*CAP+TEL* *(n=6)*	*TEL+PD* *(n=5)*	*TEL+ L-NAME* *(n=5)*
MAX (%)	75.52±8.00	78.83±9.47	98.94±9.47	74.72±10.59	14.54±8.64	97.33±9.47	72.00±9.47	9.56±10.59
EC_{50} (LogM)	-6.66±0.11	-6.83±0.12	06.75±0.12	-6.52±0.14	-	-6.50±0.12	-6.70±0.12	-
AUC (AU)	222.20±20.36	231.32±24.10	279.60±24.09	205.78±26.94	41.78±21.99	218.40±24.10	202.72±24.10	30.65±26.94

Values are listed as means ± s.e.m. % of maximum dilation, EC_{50} and area the under curve values to acetylcholine are listed. These values correspond to exercise animals under various pre-drug incubation conditions.

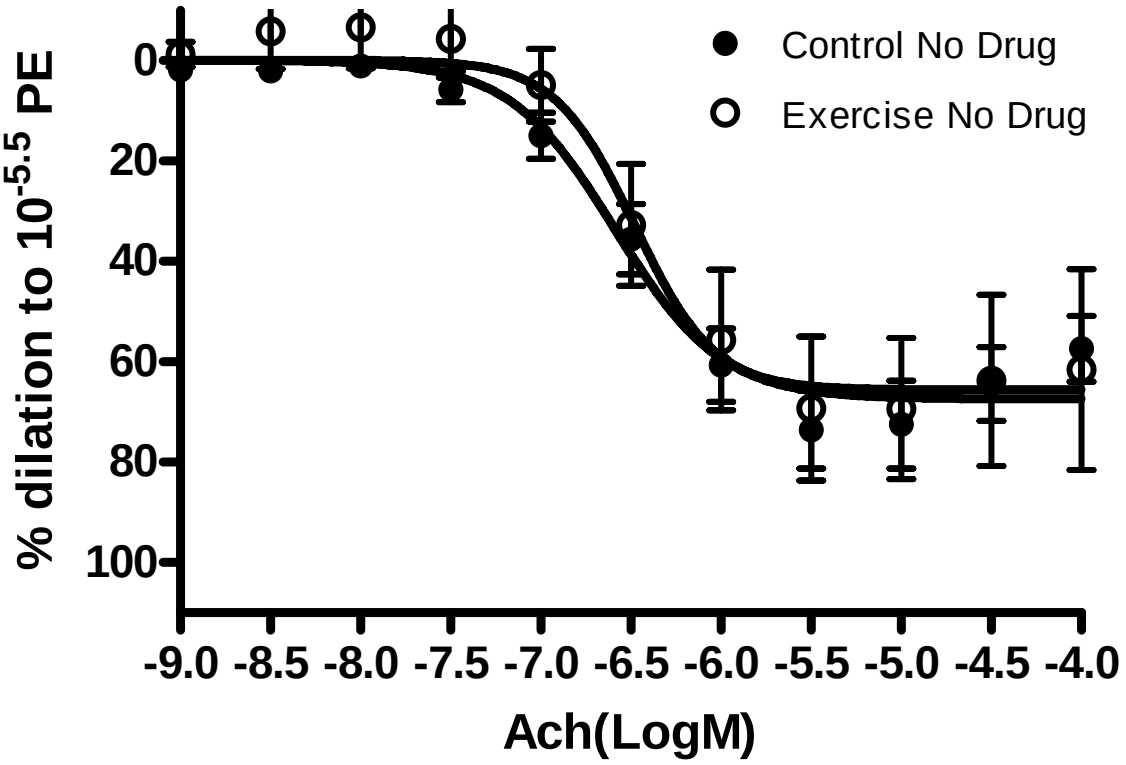

Figure 21: *No exercise effect observed in max endothelium-dependent vasodilation to acetylcholine.* Femoral artery rings from CON (•, n=52) and IPEx animals (○, n=43) were mounted on single wire myographs and set to the pre-determined L_0 = 300mg. Rings were subjected to a $10^{-5.5}$ dose of phenylephrine to cause a pre-constriction level equal to ~75-80% max constriction. From the pre-constricted state, femoral artery rings were subjected to increasing doses of acetylcholine (ACh) to cause endothelium-dependent vasodilation. There was no significant effect of exercise on ACh dose response curve characteristics MAX ($p = 0.33$), EC_{50} ($p = 0.37$), AUC ($p = 0.57$) (MAX; maximum dilation, EC_{50}, area under curve (AUC)). p-values were obtained from two-way ANOVA.

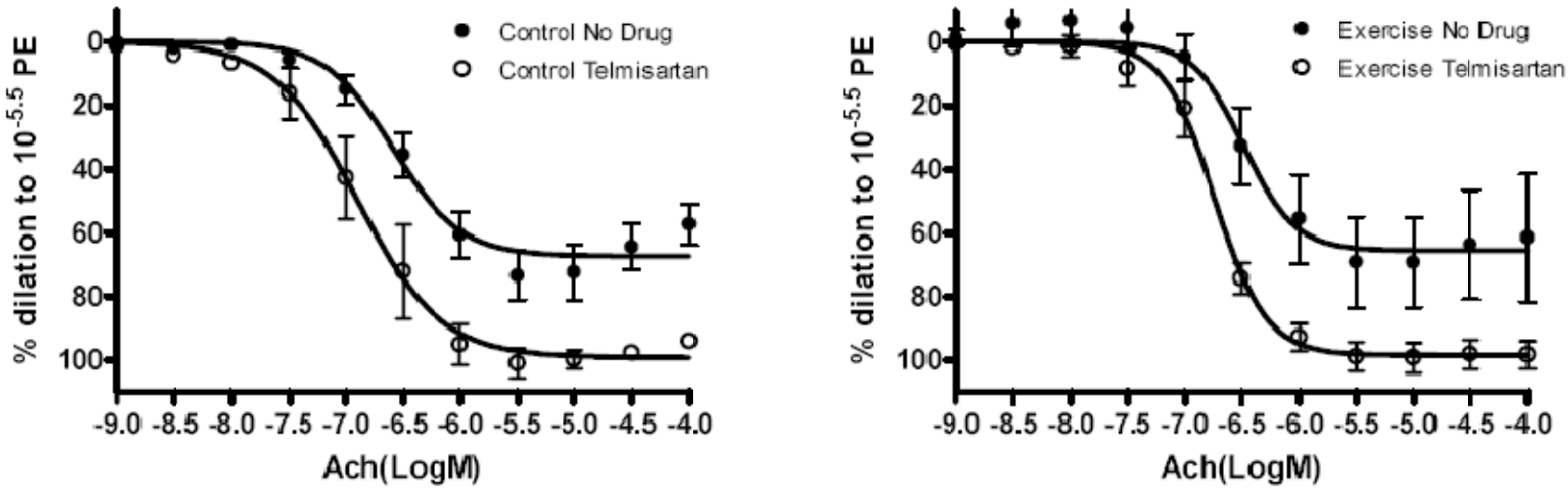

Figure 22: *Increased max endothelium-dependent vasodilation to acetylcholine with AT_1 receptor blockade.* Femoral arteries from CON (n=5) and IPEx (n=5) animals were pre-incubated with 10^{-5} telmisartan (TEL) for 30min prior to the construction of acetylcholine (ACh) dose-response curves. For clarity purposes, the left panel illustrates the TEL effect on control animals and the right panel displays the TEL effect on exercise animals. Main effect of drug ($p < 0.001$). p-value obtained from two-way ANOVA. Tukey's HSD post-hoc test revealed significant differences between TEL and ND ($p<0.05$).

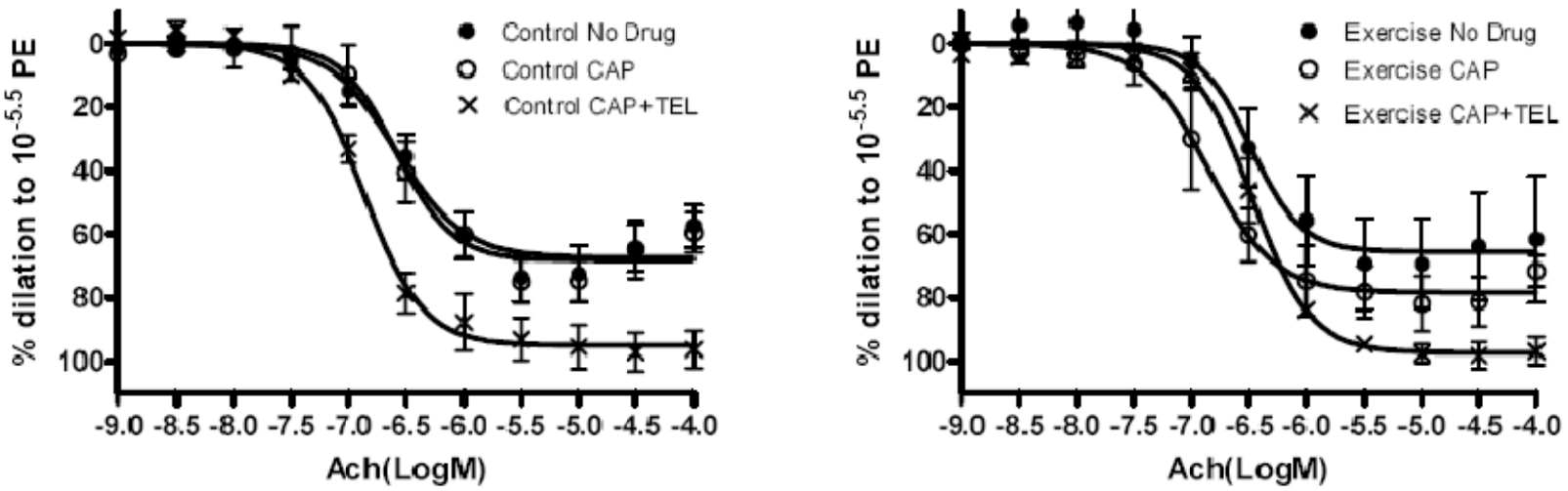

Figure 23: *No effect of ACE inhibition on max endothelium-dependent vasodilation to acetylcholine.* Femoral arteries from CON (n=8) and IPEx (n=5) animals were pre-incubated with 10^{-5} captopril (CAP) for 30min prior to the construction of acetylcholine (ACh) dose-response curves. For clarity purposes, the left panel illustrates the CAP effect on control animals and the right panel displays the CAP effect on exercise animals. Main effect of drug ($p < 0.001$). p-value obtained from two-way ANOVA. Tukey's HSD post-hoc test revealed no significant differences between CAP and ND ($p>0.05$).

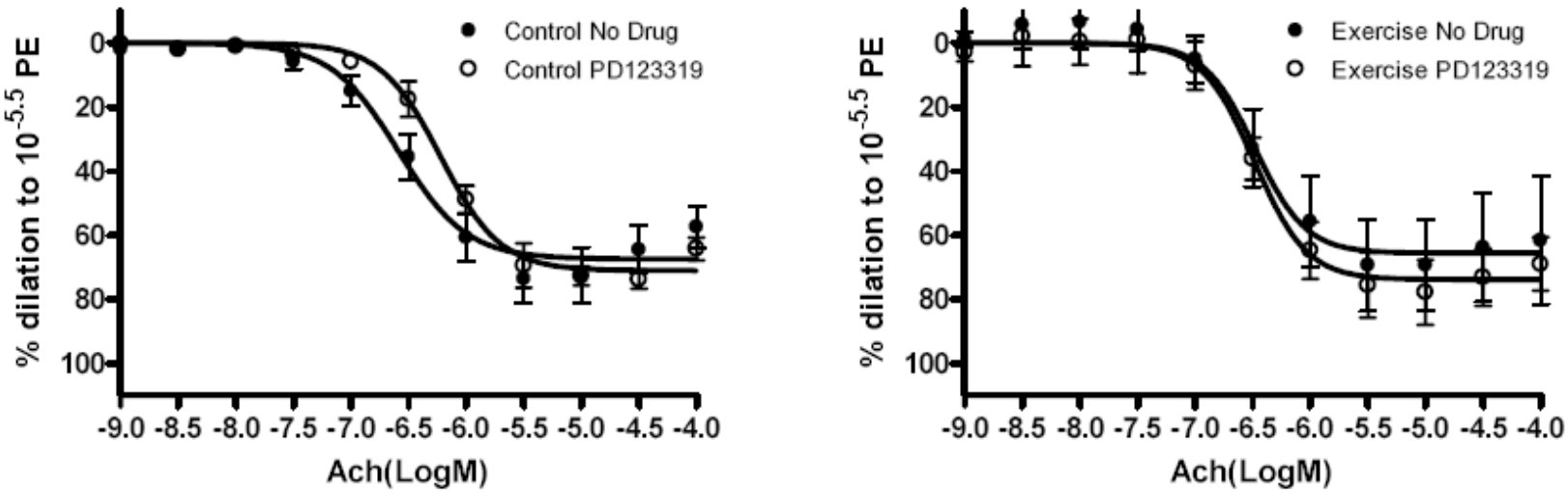

Figure 24: *Effect of AT_2 receptor blockade on endothelium-dependent vasodilation to acetylcholine*. Femoral arteries from CON (n=5) and IPEx (n=5) animals were pre-incubated with 10^{-5} PD123319 (PD) for 30min prior to the construction of acetylcholine (ACh) dose-response curves. For clarity purposes, the left panel illustrates the PD effect on control animals and the right panel displays the PD effect on exercise animals. Main effect of drug on MAX dilation and AUC ($p < 0.001$). Tukey's HSD post-hoc test revealed no significant differences between PD and ND ($p>0.05$). However, a difference was observed in the EC_{50} of PD treated rings (main effect, $p = 0.02$. Tukey's post-hoc test revealed a difference between PD and ND ($p>0.05$)).

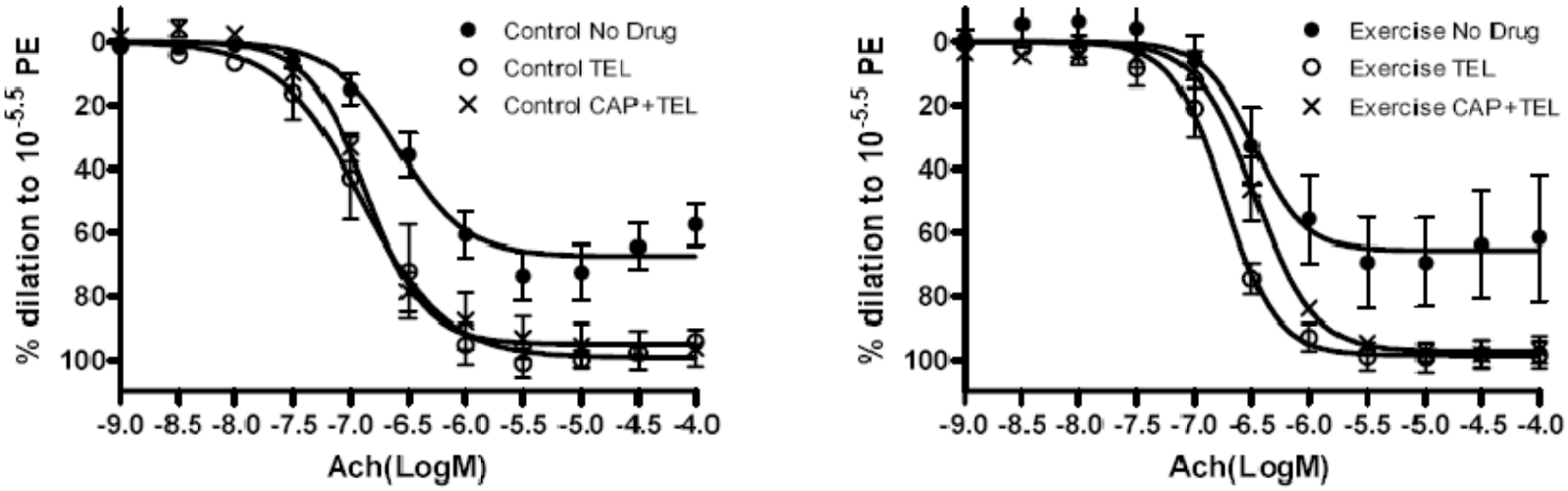

Figure 25: *Effect of combined ACE inhibition and AT_1 receptor blockade on max endothelium-dependent vasodilation to acetylcholine.* Femoral artery rings from CON and IPEx animals were pre-incubated with 10^{-5} telmisartan (TEL) alone or in combination with 10^{-5} captopril (CAP+TEL) for 30min prior to the construction of acetylcholine (ACh) dose-response curves. The left panel illustrates that, for CON animals, there is no further increase in MAX ACh with CAP+TEL pre-incubation compared to TEL. The right panel illustrates that, for IPEx animals, there is no further increase of MAX ACh with CAP+TEL pre-incubation compared to TEL. p-value for drug effect on MAX ($p<0.001$) was obtained from two-way ANOVAs, but Tukey's HSD post-hoc test revealed no significant difference between TEL and CAP+TEL groups ($p>0.05$).

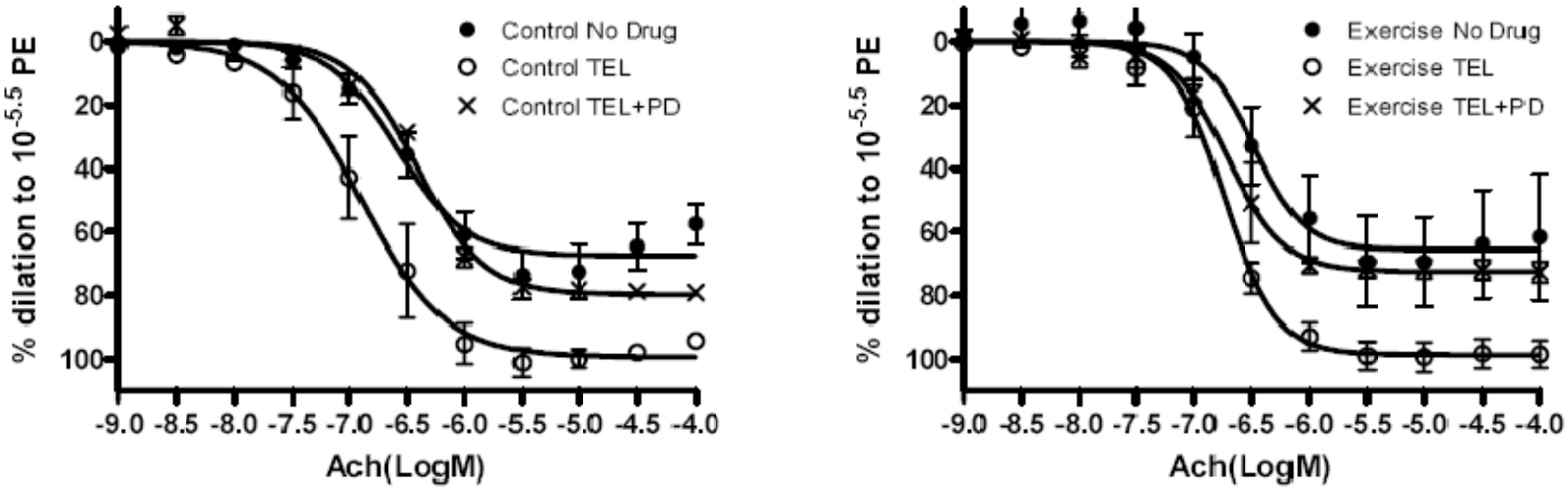

Figure 26: *Effect of combined AT_1 and AT_2 receptor blockade on max endothelium-dependent vasodilation to acetylcholine.* Femoral artery rings from CON and IPEx animals were pre-incubated with 10^{-5} telmisartan (TEL) alone or in combination with 10^{-5} PD123319 (TEL+PD) for 30min prior to the construction of acetylcholine (ACh) dose-response curves. The main effect of drug pre-incubation on MAX ACh was $p<0.001$. Tukey's HSD post-hoc tests revealed a significant difference between TEL and TEL+PD pre-incubations on MAX ACh ($p<0.05$). The left panel illustrates the TEL vs. TEL+PD differences in CON animals, while the right panel displays the TEL vs TEL+PD differences for IPEx animals. p-values were obtained via two-way ANOVA.

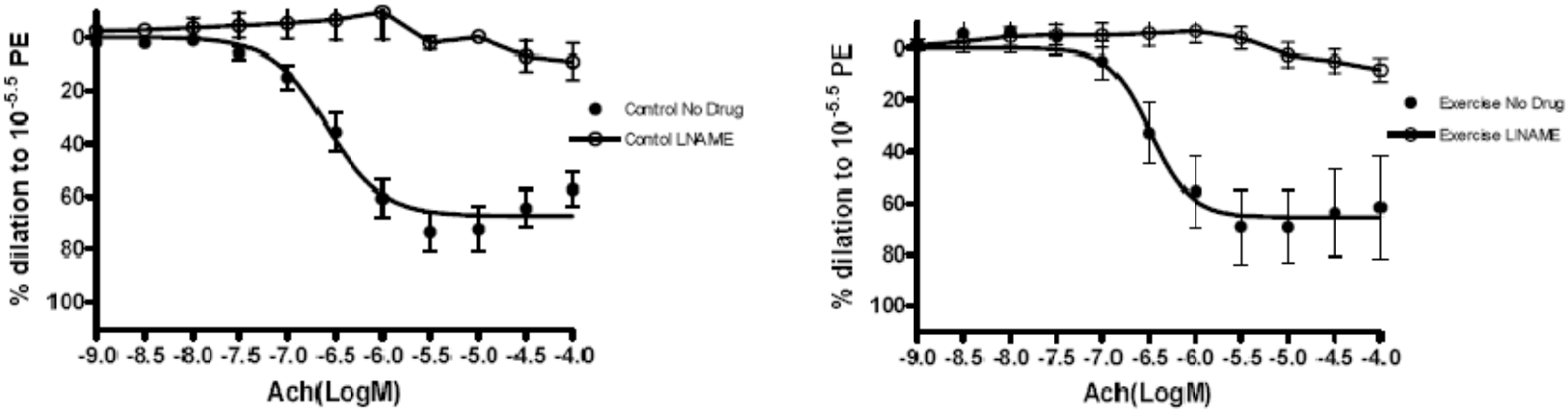

Figure 27: *The effect of nitric oxide synthase (NOS) inhibition on max endothelium-dependent vasodilation.* Femoral arteries from CON (n=5) and IPEx (n=6) animals were pre-incubated with 10^{-4} N^{ω}-nitro-L-arginine methyl ester (L-NAME) for 30min prior to the construction of acetylcholine (ACh) dose-response curves. For clarity purposes, the left panel illustrates the L-NAME effect on control animals and the right panel displays the L-NAME effect on exercise animals. There is a significant L-NAME effect on MAX ACh for CON and IPEx animals ($p<0.05$). p-value is obtained from Tukey's HSD post-hoc test performed following a two-way ANOVA (main effect of drug, $p<0.001$).

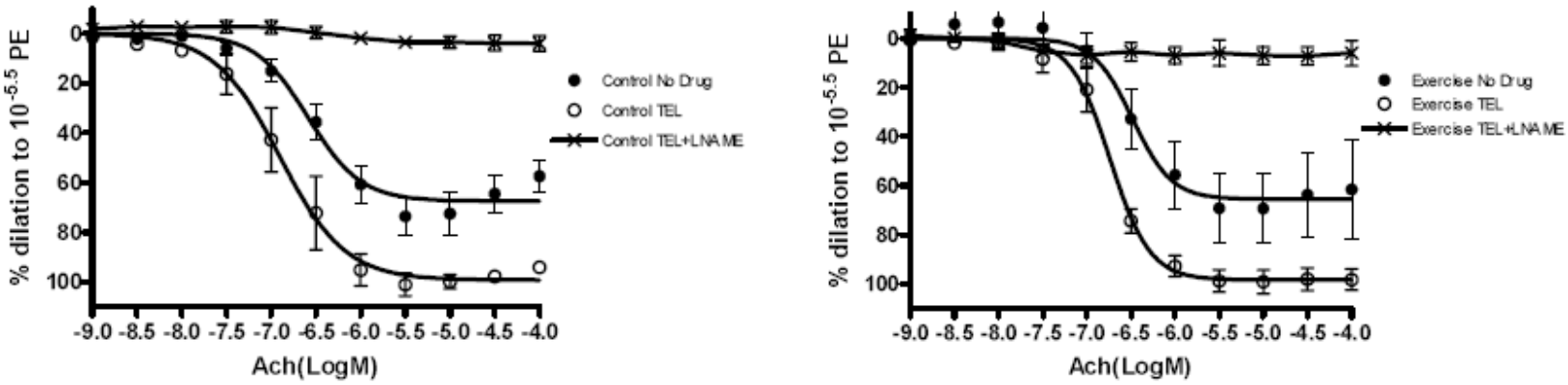

Figure 28: *Effect of Combined AT_1 blockade and nitric oxide synthase (NOS) inhibition on max endothelium-dependent vasodilation.* Femoral artery rings from CON and IPEx animals were pre-incubated with 10^{-5} telmisartan (TEL) alone or in combination with 10^{-4} N^{ω}-nitro-L-arginine methyl ester (TEL+L-NAME) for 30min prior to the construction of acetylcholine (ACh) dose-response curves. The left panel displays CON PE curves, while the right panel displays IPEx PE curves. For CON and IPEx animals, the combination of TEL+L-NAME appears to eliminate the increased MAX Ach effect of TEL ($p<0.05$). A special note, there is no difference observed in MAX PE elicited by TEL+L-NAME between CON and IPEx ($p>0.05$) p-values are obtained from Tukey's HSD post-hoc test following a two-way ANOVA (main effect of drug $p<0.001$).

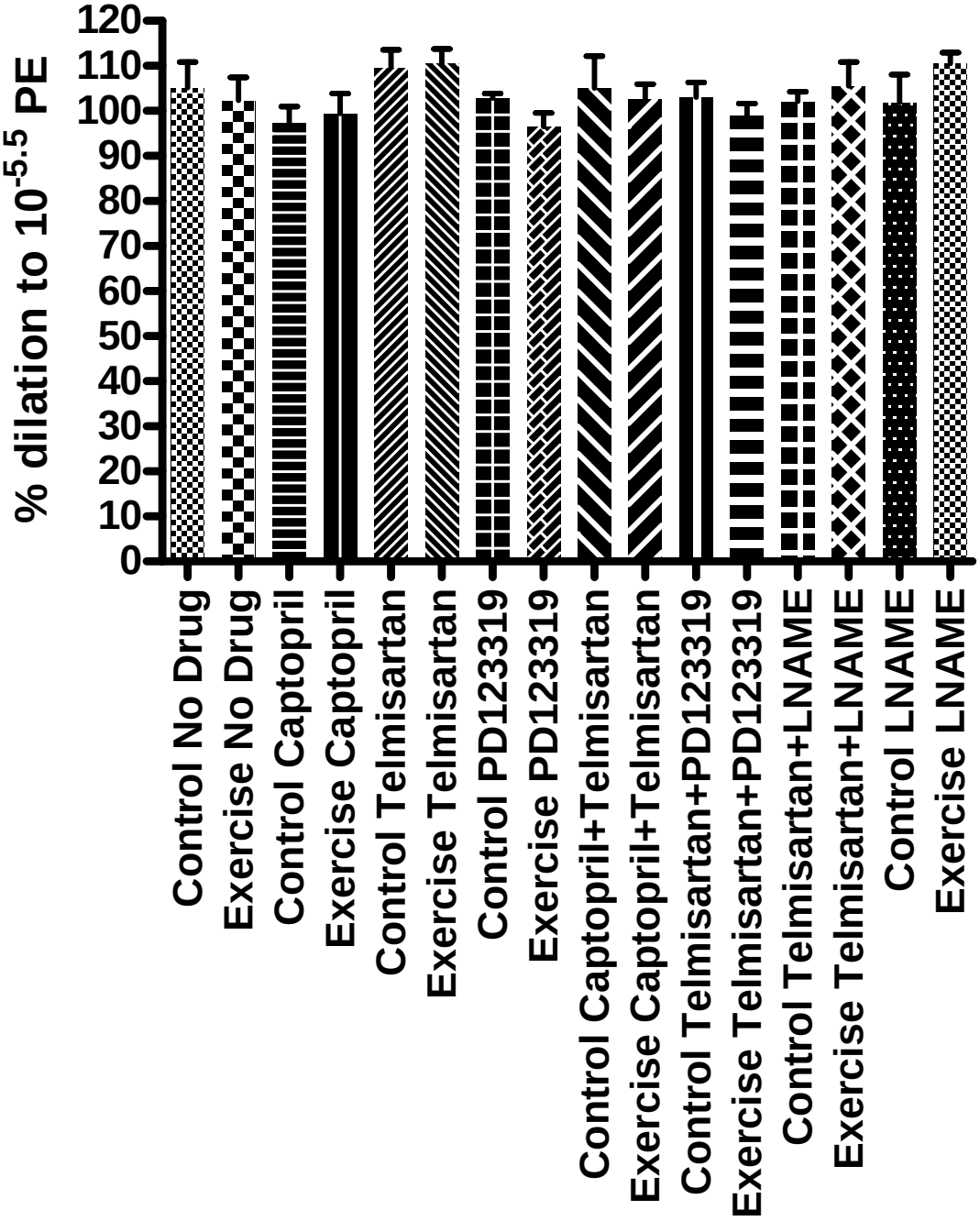

Figure 29: *No changes in endothelium-independent vasodilation across exercise and drug pre-incubation conditions.*

DISCUSSION

Acute exercise induced alterations in circulating RAS

Modifications to circulating and vascular RAS components, stemming from chronic exercise training, have been previously researched[36;51]. However, little is known about the alterations to circulating and vascular wall RAS following a single bout of exercise. This study evaluated several RAS components from the plasma (representing the systemic RAS) and femoral artery (representing the vascular wall RAS) of 18-20wk Sprague Dawley rats. Treadmill running for 1h, at a speed of 21m/min and 15% grade, increased plasma AngII levels by approximately 94%. Increases in plasma AngII following exercise have been observed in other species, including humans[64]. Exercise can stimulate AngII production through several mechanisms. AngII is known to regulate not only blood pressure, but also fluid and electrolyte homeostasis. Although sweating during exercise helps regulate core temperature, excessive sweating leads to a loss of sodium and fluid volume. To counteract the fluid and electrolyte loss, AngII levels increase which give rise to increasing levels of aldosterone[65]. Aldosterone stimulates water and salt intake to maintain appropriate fluid and electrolyte balance during exercise. Another mechanism, involving sympathetic activation during exercise, leads to an increase in plasma AngII. The kidney receives both sympathetic and parasympathetic fibres, but sympathetic neurons are more prominent[66]. Sympathetic stimulation of the kidney, specifically the juxtaglomerular apparatus, leads to the secretion of renin. Secreted renin enters the circulating blood where it is converted, ultimately, to AngII. Sympathetic activation was identified clearly in our study via the rise in catecholamine levels. Norepinephrine and epinephrine rose approximately 87% and 162%, respectively.

Although ACE catalyzes the conversion of AngI to AngII in the circulation, an approximate 10% decrease in plasma activity levels was observed after exercise in this study. The observation of decreased plasma ACE activity seems to conflict with the rise in AngII following acute exercise. It may be possible that, following exercise, AngII production within the circulation is directed mainly by non-ACE dependent mechanisms. A significant proportion (~40%) of AII is formed by non-ACE dependent pathways, either from angiotensinogen by the enzymes cathepsin G, elastase, and tissue plasminogen activator, or from angiotensin I by enzymes including chymase[24]. In addition, a negative feedback mechanism occurs in the circulation during high exposure to AngII, whereby ACE production is halted to prevent further increases in AngII. Therefore, it may be also possible that ACE enzymatic activity is reduced to further oppose the rise in AngII following exercise. This possibility becomes more apparent when femoral ACE activity and protein levels are discussed.

Acute exercise induced alterations in femoral vascular wall RAS

Current research suggests that tissue production of various vasoconstrictor peptides within the vasculature may participate in exercise-induced redistribution of blood flow[53;54] . Maeda et al. observed enhancements in kidney RAS following acute exercise, including increased angiotensinogen levels, ACE mRNA expression, ACE protein expression and AII levels, and suggested that these RAS alterations may play a role the redistribution of blood flow. Thus, we expected to observe decreases in ACE activity and expression following a bout of exercise in our 18-20wk old rats. Indeed, a ~20% decrease in femoral ACE protein expression and a ~40% decrease in femoral ACE activity was observed

following acute exercise. However, no changes were observed in AT receptor expression following the acute bout of exercise. The decrease in femoral ACE expression and activity observed in this study seems to follow the hypothesis that vascular RAS plays an important role in the redistribution of blood flow during exercise. A decrease in ACE activity would presumably lead to decreased AngII production and less vasoconstriction; although, some characteristics of the ACE enzyme must be considered before jumping to these conclusions. ACE is able to produce AngII and breakdown the vasodilatory peptide bradykinin. Thus, the exercise induced decrease in femoral ACE could potentially prevent AngII production and potentiate vasodilatory responses in the localized region. In addition, the majority of ACE exists as an ectoenzyme anchored in the endothelial membrane[67]. ACE can be 'shed' from the membrane, by a class of proteases known as secretases, and circulate as soluble ACE[24]. Very few studies have identified whether ACE secretase activity is altered following an acute bout of exercise[68;69]. However, an interesting discovery of ACE shedding resulting from additional shear stress experienced at the surface-fluid interface during exercise was made by Rieder et al. (1997). This group observed a decreased ACE activity in bovine pulmonary artery endothelial cells following 8 hours of shearing (20 dyne/cm^2)[70]. Thus, the decreased femoral ACE activity we observed could potentially be an artifact of increased shedding due to increased shear stress produced during exercise. Investigation of femoral artery vasomotor function following exercise with RAS pharmaceutical intervention, discussed later, will help clarify whether this reduction in ACE expression and activity is functionally important.

The vascular AT_1/AT_2 receptor ratio is a very important determinant of local AngII action[36]. A key benefit of regular physical activity may include a reduction in AT_1/AT_2

receptor ratio, whereby less negative AT_1 receptor-mediated events occur and more positive AT_2 receptor counteraction is uncovered. This study revealed that no change occurs in AT_1 and AT_2 receptor expression, and thus AT_1/AT_2 ratio following a single bout of exercise (at least immediately after). Therefore vascular events mediated by AT receptors, such as vasomotor activity, should be unaltered so long as AngII levels and AT_1 receptor signaling remain the same.

Acute exercise, femoral RAS and vasoconstriction

The advantage of using an in-vitro preparation to study alterations in vascular responsiveness following exercise is that potentially confounding neural, humoral, and metabolic influences present in-vivo can be eliminated[64]. This apparent advantage also permits the study of local vascular RAS isolated from the circulating systemic RAS. KCl dose-response curves were completed to investigate whether exercise or RAS drug pre-incubations would alter electromechanical coupling, and thus the overall ability of the vessel to contract. The maximal responses to KCl were not significantly different between CON and IPEx. Spier et al. (1999) also observed no changes in SD rat KCl constriction following treadmill exercise for 1h at 30m/min[61]. There were also no differences in maximal KCl responses with all drug pre-incubations. Hence, any effects seen in PE vasoconstriction would be a result of isolated effects of exercise or vascular RAS on α-adrenergic receptor or the signaling pathway initiated by the receptors.

In this study, a significant decrease in maximum PE constriction was observed for acutely exercise animals when collapsed over all drug conditions (~13%) and in the no drug pre-incubation condition (~18%). These findings, paired with the observed maintenance of

maximum constriction to KCl mentioned above, indicate that an acute bout of exercise is able to attenuate α-adrenergic receptor-mediated vasoconstriction. The lowered vasoconstrictor responsiveness of the arterial vasculature has been high debated, especially in the realm of whether it ultimately contributes to post-exercise hypotension[61]. Spier et al. (1999) did not observe any changes in SD abdominal aortic sensitivity or maximal contraction to PE. However, Howard et al. (1992) have shown that PE-evoked tension of aortic ring segments is diminished after a single bout of exercise in rabbits[58]. In addition, studies on SD rats are consistent with this notion of reduced adrenergic receptor responsiveness (termed sympatholysis), suggesting that it could carry forward into the post-exercise period and contribute to post-exercise hypotension[55;60]. Both authors considered it a possibility that ineffective transduction of sympathetic outflow into vascular resistance could result from competing influences at the level of the arterial smooth muscle, such as the release of local vasodilator substances. Although we cannot extend our findings to post-exercise hypotension (blood pressure measurements were not made) we can definitely comment on the in-vitro vasomotor experiments observed in this study.

The decreases in femoral ACE expression and activity may help explain the observed reduction in maximum PE constriction following exercise. Decreased ACE activity would presumably lead to a reduced production of AngII. Local AngII is known to facilitate adrenergic transmission in rat mesenteric arteries[71]. Decreased production of AngII can have profound effects since less AT_1 receptor-mediated events would occur. Mainly, reduced AT_1-mediated ROS production would occur; AT_1 is known to activate NAD(P)H oxidase[41] leading to the production of superoxide anion and other ROS[34]. Blockade of AT_1 receptors has been shown to diminish contractile responses to PE in the aorta of SD

rats[45] and SHR rats[46]. Reduced maximum constriction to PE during AT_1 receptor blockade has been replicated in our study of femoral arteries. Thus, lowered AT_1 receptor stimulation following acute exercise (due to the decreased ACE-dependent AngII production) could contribute to the reduced PE-mediated constriction observed in this study. Another possible link between post-exercise decreases in ACE activity and PE-mediated constriction exists through the vasodilator bradykinin. Shear stress activates the local kallikrein-kinin system in the arterial wall, which has powerful vasodilator properties mainly through the peptide bradykinin[44]. The role of bradykinin in vasomotion depends highly on its rate of production by kininogenases and degradation by ACE. Although ACE is most known for its participation in AngII production, the active enzymatic site can also cleave bradykinin at the penultimate amino acid and inactivate it[67]. Thus, increased bradykinin-mediated vasodilation and reduced ACE activity during both ACE inhibitor treatment or exercise (as indicated in this study) may explain the observation of reduced PE-mediated vasoconstriction.

Whether or not decreased ACE activity is responsible for the reduced PE-mediated vasoconstriction, the exercise effect observed in this study is NO-dependent. When IPEx femoral rings were pre-incubated with the NOS inhibitor L-NAME alone, or in combination with other drugs, the attenuated PE constriction disappeared. In addition, L-NAME pre-incubations elicited similar maximum PE constrictions in IPEx animals resembled CON animals. Hemodynamic shear stress on the arterial wall is known to be an important modulator of NO release[38]. Increments of blood flow through arteries in-situ[60] or in-vitro[72] can induce immediate vasodilation that is reduced by NOS inhibitor or abolished by removal of the endothelium. As mentioned above, decreased ACE activity can reduce NO

degradation or promote NO production. Decreased AT_1 receptor stimulation by local AngII increases NO bioavailability due to lower rates of AT_1-mediated ROS quenching of NO. Decreased ACE activity also reduces bradykinin degradation; activation of bradykinin receptor B2 phosphorylates eNOS[48], which leads to NO production. A crosstalk between bradykinin receptor B2 and RAS exists. Both the AT_2 receptor and the Mas receptor for Ang(1-7) are able to signal and activate the B2 receptor[48]. This study revealed no changes to AT_2 expression following exercise. In addition, blockade of the AT_2 receptor with PD123319 following exercise had no significant effect. However, the combination of AT_1 and AT_2 blockade eliminated the attenuating effect of isolated AT_1 blockade in the exercise animals suggesting that the AT_2 effects are unmasked or perhaps sensitized only during exercise and AT_1 blockade (figure 18) – which coincides with the literature on AT receptor blockade[45;46]. The specific contribution of Ang(1-7) to post-exercise reductions in max PE constriction was not studied, although the vasodilator peptide has been shown to augment α-adrenergic constriction[45;48].

Acute exercise, femoral RAS and vasodilation

SNP Dose-response curves were completed to evaluate whether exercise or RAS drug pre-incubations modulated signaling downstream of NO. No differences in maximum dilation to SNP were observed across all exercise and drug conditions. Thus, the responses of femoral artery rings to ACh are not likely due to alterations in signaling downstream of NO, but rather are endothelium dependent.

ACh dose-response curves were created to evaluate the effect of exercise and RAS drug pre-incubations on endothelium-dependent vasodilation. Both sensitivity and maximal

responses to ACh were not different after a single bout of exercise. Studies have evaluated the time course of endothelium adaptation to exercise. Delp and Laughlin (1997) reported that enhanced endothelium-dependent dilation in the aorta of male SD rats was seen after four weeks of training[73]. Haram et al. (2006) also reported no changes in ACh-mediated dilation immediately following exercise in female SD rats[74]. This study displays that no change in femoral artery endothelium-dependent vasodilation occurs immediately after a single bout of exercise.

Some important facts about the femoral artery were discovered from the effect of various drug pre-incubations on ACh-mediated vasodilation. First, whether or not the animal was exercised, L-NAME significantly reduced the ACh-mediated vasodilation. After NOS inhibition, the femoral arteries were only able to dilate about 10% from the PE pre-constricted state. These results suggest that the major component of endothelium-dependent vasodilation in the femoral arteries, as with other conduit arteries, is mediated by NO. Shi et al. (2006) also reported the predominance of NO in femoral artery endothelium-dependent vasodilation[75]. Second, the femoral artery reacts similarly to AT receptor blockade as the aorta and other conduit vessels. When femoral arteries were pre-incubated with telmisartan, an AT_1 receptor blocker, vasodilation to ACh was enhanced. This is in agreement with other studies performed on SD aorta[76] and SHR aorta[48], whereby the authors attributed the effect of AT_1 blockade to enhanced AT_2 activation. This unmasking of AT_2 dilation during AT_1 blockade was observed in this study, as the improved vasodilation vanished with combined AT_1 and AT_2 receptor blockade. Lastly, when the femoral arteries of CON animals were pre-incubated with the ACE inhibitor captopril, no significant changes in ACh-mediated dilation were observed. This leaves the possibility that local AngII production in

the femoral artery is moderately dictated by non-ACE dependent mechanisms; however, this postulation cannot be valid without actual measurement of tissue AngII with and without ACE inhibition.

LIMITATIONS

Due to technical limitations local tissue AngII production could not be measured, only implied. The commercially available AngII enzyme immunoassay kit used in this study requires 2mL of sample to properly extract AngII peptides. This volume was obtained for plasma samples; however for tissue homogenate, pooling of multiple samples would have been required (one entire femoral artery yields ~150μL of homogenate with 3μg/μL protein concentration). The amount of AngII peptide would be untraceable if it was gathered from a single homogenate. Other studies have evaluated tissue AngII levels by infusing radio-labeled AngI and measuring the radio-labeled AngII[77]. The process requires SepPak extraction and high-perfromance liquid chromatography separation. In addition, losses in AngII peptides have been reported to be ~20-30% in tissue homogenate after extraction and separation[77]. Thus, due to unfamiliar and imprecise methods, tissue AngII production was implied from the myography data.

Another limitation of this study was the inability to determine whether the alterations in femoral RAS were responsible for increasing blood flow to the hind-limbs during exercise. Blood flow following acute exercise was not measured in this study, however vasomotor function was assessed. The advantage of using an in-vitro preparation to study alterations in vascular responsiveness following exercise is that potentially confounding neural, humoral, and metabolic influences present in-vivo can be eliminated[64]. Thus, the data acquired from the various RAS drug conditions can be used to explain the role of femoral RAS plays in vascular reactivity.

CONCLUSION

The aim of this study was to examine whether a single bout of exercise alters vascular RAS in the femoral artery, and if these alteration would lead to a functional change in the vasomotor properties of the vessel. The main findings of this study are:

1) A single bout of exercise reduced the femoral artery ACE expression and activity.
2) A single bout of exercise attenuated the α_1-adrenergic receptor-mediated vasoconstriction, but this effect was abolished with NOS inhibition.
3) The reduction in α_1-adrenergic receptor-mediated vasoconstriction may be linked to the decreased activity of femoral ACE, which supports the production of local vasodilatory factors.
4) A single bout of exercise did not alter femoral artery endothelium-dependent vasodilation to acetylcholine or vascular smooth muscle sensitivity to exogenous NO.
5) Femoral artery vasodilation was modulated by AT_1 receptor blockade, whereby AT_1 blockade unmasked AT_2 receptor-mediated events.
6) For α_1-adrenergic receptor-mediated vasoconstriction, only exercised animals displayed sensitivity of AT_2 receptor activation during AT_1 receptor blockade.
7) The effect of AT_1 receptor blockade displays NO sensitivity, whereby NOS inhibition eliminated the attenuating effect of AT_1 blockade in PE-mediated vasoconstriction and the additional vasodilation elicited by AT_1 blockade in ACh-mediated vasodilation.

FUTURE DIRECTIONS

To confirm the findings of this study, vasculature serving tissues undergoing less metabolic activity during exercise should be studied – for example the mesenteric or renal artery. Increases in ACE activity would be expected in these vessels after exercise and no reduction should be observed when PE dose-response curve are completed. Increases in tissue ACE protein have been reported in the kidney following exercise[53]. Our laboratory has also determined that ACE activity is increased in the renal artery following exercise.

Investigating changes to ACE shedding and secretase activity following exercise would provide further insight into the mechanisms by which vascular RAS effects vasomotor function. This study showed that femoral ACE protein and activity decreased following a single bout of exercise. However, the contribution of secretase or shear-induced shedding of ACE was not determined. ACE shedding may be an exciting branch of exercise physiology to investigate to fully comprehend the function of vascular RAS during exercise. In addition, the contributions of Ang(1-7) and the bradykinin B2 receptor to vasomotor function following exercise should investigated. An abundance of new research regarding RAS has focused on the interesting vasodilatory properties of Ang(1-7)[24;45;48]. Although this study did not measure the importance of Ang(1-7), it is possible that it contributed to function effects of exercise. This study recognized that the attenuation of PE responses following exercise was NO dependent. AT_2 receptor mediated NO production and reduced AT_1 receptor activity was partially responsible for the decrease in maximum PE constriction. Thus, it is possible that NO production via Ang(1-7) or B2 receptors was participating in the exercise effect observed in this study.

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