EXERCISE PRESSOR REFLEX DYSFUNCTION IN HYPERTENSION

APPROVED BY SUPERVISORY COMMITTEE

Scott A. Smith, PhD.

Jere H. Mitchell, MD.

Robert Eberhart, PhD.

Mario Romero-Ortega, PhD.

Charles Chuong, PhD.

ACKNOWLEDGEMENTS

This project was supported by a Ruth L. Kirschstein National Research Service Award for Individual Predoctoral Fellowships to Anna K. Leal from the National Institute of Health. The project was also supported by grants from the American Heart Association and the National Institute of Health to Dr. Scott A. Smith as well as the Lawson & Rogers Lacy Research Fund in Cardiovascular Diseases to Dr. Jere H. Mitchell.

I would like to express my sincerest gratitude to my mentor, Dr. Scott A. Smith, for his quidance and expertise throughout the completion of my graduate studies. I am also forever grateful to Dr. Jere H. Mitchell for giving me the opportunity to be a part of the Weinberger Laboratory and for introducing me and all exercise physiologists to the exercise pressor reflex. In addition, I wish to express thanks to all the past and current Weinberger Laboratory members, including Dr. Mary G. Garry, Dr. Maurice A. Williams, Martha Romero, Maggie Robledo, Julius Lamar, Marilyn Gardner, Dr. Megan N. Murphy, Richard Newcomb, Jack Squiers, and Kate Squiers for their technical, intellectual, and moral support. I am also appreciative of my committee members, Dr. Robert Eberhart, Dr. Mario Romero-Ortega, and Dr. Charles Chuong, for taking the time to guide and encourage my research career.

Finally and most importantly, I would like to thank my family, including my fairy godfathers, for always having my back and never letting me give up or settle for less than my best. I am truly the luckiest girl in the world!

EXERCISE PRESSOR REFLEX DYSFUNCTION IN HYPERTENSION

by

Anna Katherine Leal, M.S.

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

August, 2009

EXERCISE PRESSOR REFLEX DYSFUNCTION IN HYPERTENSION

Anna Katherine Leal, M.S.

The University of Texas Southwestern Medical Center at Dallas, 2009

Scott A. Smith, Ph.D.

The exercise pressor reflex and its components, the muscle mechanoreflex and the metaboreflex, are overactive in hypertension. The mechanoreflex and metaboreflex are feedback mechanisms originating in skeletal muscle that increase mean arterial pressure (MAP) and heart rate (HR) during exercise. In hypertensive individuals, mechanoreflex and metaboreflex overactivity can cause dangerous elevations in MAP and HR during physical activity, creating risks for adverse cardiac events. Mechanoreflex (predominantly group III) and metaboreflex (predominantly group IV) afferent fibers, which are activated by mechanical stress and the metabolic byproducts of working muscle, respectively, project to the nucleus tractus solitarius (NTS) in the brainstem. Within this nucleus, nitric oxide (NO) is produced from L-arginine via the enzymatic activity of nitric oxide synthase (NOS). Brainstem NO has been shown to modulate exercise pressor reflex-driven changes in MAP and HR. Therefore, we hypothesized that a decrease in NO production/availability within the NTS is involved in mediating both mechanoreflex and metaboreflex dysfunction in

hypertension. To test this, we microdialyzed a NOS inhibitor, L-nitro-arginine methyl ester (L-NAME), and the NO precursor, L-arginine, into the NTS of normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats to experimentally alter endogenous NO production during preferential activation of mechanically and metabolically sensitive skeletal muscle afferents. Passive hindlimb muscle stretch was the maneuver used to simulate mechanoreflex activation while metabolically sensitive afferents were activated by hindlimb intra-arterial capsaicin injections. Capsaicin binds to transient potential 1 (TRPv1) receptors, which are primarily localized to group IV afferents. We found that blocking NO production via L-NAME within the NTS of normotensive WKY rats recapitulates the exaggerated cardiovascular response elicited by both mechanically and metabolically sensitive afferent neurons in hypertension. Importantly, we demonstrated that experimentally increasing NO production within the NTS of hypertensive SHR rats partially corrects the enhanced cardiovascular response to activation of both mechanically and metabolically sensitive afferent neurons. These findings provide evidence that a decrease in NO production/availability within the brainstem contributes to mechanoreflex and metaboreflex dysfunction in hypertension. Future utilization of this research could lead to effective treatment options for hypertensive individuals, allowing them to engage in physical activity without the associated hemodynamic risks.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Book Chapter

Smith, S.A., **A.K. Leal**, C.N. Young, and P.J. Fadel. Neural mechanisms of cardiovascular control during exercise in health and disease. In: *Recent Advances in Cardiovascular Research: From Sleep to Exercise.* Research Signpost, 2009 (in press).

Articles

Smith, S.A., M.A. Williams**, A.K. Leal**, J.H. Mitchell, and M.G. Garry. Exercise pressor reflex function is altered in spontaneously hypertensive rats. Journal of Physiology, 577.3: 1009-1020, 2006.

Leal, A.K., M.A. Williams, M.G. Garry, J.H. Mitchell, and S.A. Smith. Evidence for functional alterations in the skeletal muscle mechanoreflex and metaboreflex in hypertensive rats. American Journal of Physiology, 295: H1429-H1438, 2008.

Leal, A.K., J.H. Mitchell, and S.A. Smith. A role for nitric oxide within the nucleus tractus solitarius in the development of muscle mechanoreflex dysfunction in hypertension. American Journal of Physiology, submitted, 2009.

Smith, S.A., M.A. Williams, **A.K. Leal**, M.N. Murphy, J.H. Mitchell, and M.G. Garry. Pharmacological blockade of the skeletal muscle TRPv1 receptor reduces exercise pressor reflex activity in rats. submitted, 2009.

Leal, A.K., J.H. Mitchell, and S.A. Smith. Increasing nitric oxide within the brainstem attenuates the exaggerated cardiovascular response to simulated mechanoreflex activation in hypertension. American Journal of Physiology, in preparation, 2009.

Leal, A.K., J.H. Mitchell, and S.A. Smith. Altered circulatory control by metabolically sensitive skeletal muscle afferent fibers in hypertension: a role for central-acting nitric oxide. American Journal of Physiology, in preparation, 2009.

Abstracts

Leal, A.K., M.A. Williams, M.G. Garry, J.H. Mitchell, and S.A. Smith. The pressor response to activation of mechanically and metabolically sensitive skeletal muscle afferent fibers is exaggerated in hypertension. *FASEB Journal,* Abst # 474.14, 20(4): A770, 2006.

Leal, A.K., M.A. Williams, M.G. Garry, J.H. Mitchell, and S.A. Smith. Increasing nitric oxide within the nucleus tractus solitarius attenuates skeletal muscle mechanoreflex overactivity in hypertension. *FASEB Journal*, Abst # 753.13, 21(6): A892-A893, 2007.

Leal, A.K., J.H. Mitchell, and S.A. Smith. A role for brainstem nitric oxide in the development of muscle mechanoreflex overactivity in hypertension. *Medicine and Science in Sports and Exercise,* Volume 40:5 Supplement, 2008.

Leal, A.K., R. Newcomb, J. Squiers, and S.A. Smith. Exercise pressor reflex dysfunction in hypertension: a role for nitric oxide synthase (NOS) within the nucleus tractus solitarius (NTS). *FASEB Journal*, *Abst # 957.20,* 2008.

Leal, A.K., S.A. Smith, M.A. Williams, and J.H. Mitchell. Pharmacological blockade of the TRPv1 receptor in skeletal muscle attenuates the exercise pressor reflex in rats. *The Physiologist,* Abst #4.18, 51(6):33, 2008.

Leal, A.K., J.H. Mitchell, and S.A. Smith. Exaggerations in skeletal muscle metaboreflex activity are attenuated by increasing nitric oxide production within the brainstem of spontaneously hypertensive rats. *FASEB Journal,* 2009 (in press).

Hawkins, M.N., J. Squiers, **A.K. Leal,** J.H. Mitchell, and S.A. Smith. Neuronal nitric oxide synthase (nNOS) and c-Fos expression in the nucleus tractus solitarius (NTS) of normotensive and hypertensive rats. *FASEB Journal,* 2009 (in press).

Leal, A.K., J.H. Mitchell, and S.A. Smith. Alterations in central-acting nitric oxide contribute to the abnormal skeletal muscle metaboreflex in hypertension. *Medicine and Science in Sports and Exercise,* 2009 (in press).

Hawkins, M.N., **A.K. Leal,** J.H. Mitchell, and S.A. Smith. Decreasing superoxide within the nucleus tractus solitarius partially corrects skeletal muscle mechanoreflex overactivity in hypertension. *Medicine and Science in Sports and Exercise,* 2009 (in press).

LIST OF TABLES

LIST OF FIGURES

Chapter I Figure

Chapter IV Figure

Chapter V Figure

LIST OF ABBREVIATIONS

4V	fourth ventricle
ABP	arterial blood pressure
aCSF	artificial cerebrospinal fluid
Ang II	angiotensin II
ANOVA	analysis of variance
ASIC	acid-sensitive ion channel
ATP	adenosine triphosphate
B2	bradykinin 2
BH_4	tetrahydrobiopterin
bpm	beats per minute
CB1	cannabinoid 1
CC	central command
CO	cardiac output
Con	control
CS	calamus scriptorus
cVLM	caudal ventrolateral medulla
D-NAME	D-nitro-arginine methyl ester
DAA	diaminoanthraquinone
DBP	diastolic blood pressure
EAA	excitatory amino acid
ECF	extracellular fluid
eNOS	endothelial nitric oxide synthase

EPR	exercise pressor reflex
GABA	gamma-aminobutyric acid
Hex	hexamethonium
HR	heart rate
HT	hypertensive
IAA	inhibitory amino acid
IML	intermediolateral
iNOS	inducible nitric oxide synthase
L-arg	L-arginine
L-cit	L-citrulline
L-NAME	L-nitro-arginine methyl ester
MAP	mean arterial pressure
mmHg	millimeters Mercury
MMR	muscle mechanoreflex
MVC	maximum voluntary contraction
NAD(P)H	nicotinamide-adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NT	normotensive
NTS	nucleus tractus solitarius
Phent	phentolamine
PRU	peripheral resistance unit

rec	recovery
ROS	reactive oxygen species
rSNA	renal sympathetic nerve activity
rVLM	rostral ventrolateral medulla
SAD	sinoaortic denervation
SHR	spontaneously hypertensive rat
SNA	sympathetic nerve activity
SV	stroke volume
TPR	total peripheral resistance
TRPv1	transient receptor potential vanilloid 1
WKY	Wistar-Kyoto

LITERATURE REVIEW

Hypertension and the Cardiovascular Response to Exercise

Hypertension is a medical condition affecting roughly 1 in 3 adults in the United States, according to the American Heart Association. Hypertension is characterized by chronic elevations in mean arterial pressure caused by a systolic pressure above 140 mmHg and a diastolic pressure over 90 mmHg. This disease state stresses the heart and vasculature and can lead to stroke, heart attack, heart failure, arterial aneurism, and renal failure(16, 34, 40, 53, 82, 121, 141, 164). Hypertension and its effects can be life threatening; even moderate elevations in mean arterial pressure have been linked to shortened life expectancy(34). Therefore, treatment of hypertension is critically important.

A non-pharmacological treatment option is exercise. Exercise has been shown to lower baseline blood pressure in rats and humans(2, 14, 64, 84, 89, 120, 129, 130). In experiments involving spontaneously hypertensive rats (SHR) low-intensity exercise training lowered heart rate and cardiac output, resulting in decreased basal blood pressure(151). For example, 20 weeks of low-intensity exercise training in SHR animals resulted in resting blood pressures that were, on average, 60% lower than that of their sedentary SHR counterparts and the difference was significant from the third week of exercise(45). In yet another experiment with aged SHR rats, it was shown that just six weeks of voluntary low-intensity treadmill running significantly lowered baseline blood pressure when compared to their sedentary counterparts(143). In humans, a number of experiments have shown that mild to moderate dynamic exercise over a period of three months of more lowers blood pressure in men and women with essential hypertension(2, 6, 9, 14, 32, 61, 63, 64, 84, 120, 129, 130). In fact, individuals with essential hypertension have enjoyed 8-10 mmHg reductions in systolic blood pressure and 7-8 mmHg reductions in diastolic blood pressure as consequence of a regular exercise regimen(66, 85, 105).

There are two types of exercise: static and dynamic. Static (isometric) exercise consists of muscle contraction where the muscle fibers do not shorten

and the joint is stationary. During static exercise, stroke volume (the amount of blood pumped out of each ventricle with each contraction) remains constant or decreases minimally (Figure 1). Total peripheral resistance varies little and blood flow to the active muscle is reduced because of sympathetically-mediated vasoconstriction and compression of the vessels by the working muscle(29, 91). A slight increase in heart rate (the number of beats per minute) caused by the sympathetic nerve activity increases cardiac output in an attempt to keep the active muscle perfused. However, the larger cardiac output is primarily redirected to skin and non-active muscle. Cardiac output, or the volume of blood pumped out of each ventricle per minute, is the product of stroke volume and heart rate (CO=SV*HR). Due predominantly to the increased cardiac output, mean arterial pressure rises. Mean arterial pressure is determined by the product of cardiac output and total peripheral resistance (MAP=CO*TPR)(29, 91).

In contrast, dynamic exercise consists of rhythmic contractions that change the muscle length and joint angle and the cardiovascular response to this type of exercise is different than that of static exercise. During dynamic exercise, increased sympathetic nerve activity causes a rise in heart rate and stroke volume leading to a large increase in cardiac output (Figure 1). In contracting skeletal muscle, blood vessel dilation increases vascular conductance so that more blood can perfuse the active muscle(29, 91). Vasoconstriction in the viscera, skin, and non-active muscle shunts blood away from these areas and allows blood flow to the heart, brain, and active muscle tissue to be maintained. Venous return is augmented due to the activation of the skeletal muscle pump, increasing end diastolic volume. This mechanism, along with the increase in heart rate, contributes to the large increase in stroke volume(91, 123, 124). However, local vasodilation of active muscle causes total peripheral resistance to decrease, leading to only a slight increase in mean arterial pressure over time(91).

While consistent exercise has been shown to lower baseline systolic and diastolic blood pressure, there is cause for concern(66, 85, 105). During exercise in normotensive individuals most cardiovascular parameters increase. Systolic blood pressure (blood pressure during left ventricular contraction) increases,

diastolic blood pressure (blood pressure during left ventricular dilatation) increases or stays the same, and mean arterial pressure (= diastolic pressure + 1/3 (systolic – diastolic pressure)) and heart rate also increase(91). However, individuals with hypertension see large increases in diastolic, systolic, and mean arterial pressures, as well as heart rate during exercise(5, 60, 81, 131). In one of the first experiments showing exercise causes an increased pressor response in hypertension, men performed static handgrip for 3 minutes at 30% maximal voluntary contraction. The study showed that during exercise, the increase in systolic blood pressure was significantly greater in borderline hypertensive, mildly hypertensive, and markedly hypertensive individuals compared to the normotensive controls (Figure 2)(5). In another experiment focusing on dynamic exercise, steady state cycling at 10 Watts caused larger increases in both systolic and diastolic blood pressure in hypertensive than normotensive individuals (Figure 3)(131). These large increases in mean arterial pressure and heart rate in response to both static and dynamic exercise in hypertension create risks for stroke, arrhythmias, myocardial infarction, and cardiac infarct(44, 54, 83, 94, 96, 111). As exercise training is known to improve cardiovascular health, it is important to establish why exercise causes this enhanced cardiovascular response in hypertension so that the benefits of training can be realized and the risks associated with physical activity in hypertension can be reduced(65).

The Neural Inputs that Control the Cardiovascular Response to Exercise

The cardiovascular adjustments to exercise are mediated by three inputs: central command, the arterial baroreflex, and the exercise pressor reflex. All are candidates for causing the augmented cardiovascular response to exercise in hypertension. These three inputs are integrative and they direct changes in the heart, resistance and capacitance vessels, and the adrenal gland. The combination of these effects is to alter heart rate, stroke volume, and total peripheral resistance in order to control blood pressure (Figure 4).

Central command is a feed-forward neural pathway thought to originate in higher brain centers that simultaneously activates locomotor neurons in skeletal

muscle and cardiovascular neurons in the brainstem(31, 52, 67, 166). The exact origin of central command is unknown, but animal and human studies have identified the motor cortex, insular cortex, and the mesencephalic and hypothalamic locomotor regions of the brain as possible candidates(15, 97, 98, 157, 160, 161). In separate experiments, electrical stimulation of the hypothalamic locomotor region in the cat and electrical stimulation of the mesencephalic locomotor region in the decerebrate rat have caused locomotion and, in a parallel fashion, activation of cardiovascular areas in the brain(7, 158). At the onset of exercise, central command causes a decrease in parasympathetic nerve activity which reduces vagal tone and increases heart rate causing cardiac output to rise(99). Additionally, at high exercise intensities, central command influences the sympathetic nerve activity response to exercise(153).

Baroreceptors are unencapsulated free nerve endings within the internal blood vessel walls of the carotid arteries and aorta that quickly respond to changes in blood pressure on a beat-to-beat basis by initiating negative feedback reflexes through autonomic neural activity(1, 127). The baroreflex stabilizes blood pressure by altering heart rate, stroke volume, and total peripheral resistance(132). Briefly, when arterial pressure rises, the vessel walls are stretched and the baroreceptors increase their firing frequency. Their firing inhibits sympathetic outflow and increases parasympathetic outflow from the medulla. This neural activity decreases heart rate, cardiac output, and total peripheral resistance, which lowers the blood pressure (Figure 5). In response to hypotension, the baroreceptors decrease their firing frequency which causes an increase in sympathetic nerve activity and a decrease in parasympathetic nerve activity ultimately leading to increases in total peripheral resistance and blood pressure (Figure 5)(1, 25). During exercise, the baroreceptors are reset to function around the higher blood pressures invoked by physical activity without a change in sensitivity(19, 48, 86). This resetting is caused by both central command and the exercise pressor reflex(27, 28, 101, 117, 119, 125, 137)**.** It is possible that the central command-baroreceptor interactions occur in the nucleus tractus solitarius within the brainstem because projections from the insular cortex that innervate

locomotor regions within the hypothalamus and mesencephalon have terminal fields that overlap baroreceptor afferent neurons within this region of the brainstem(116).

The Exercise Pressor Reflex and its Circulatory Effects

The exercise pressor reflex, first described by Alam and Smirk in 1937, is a feedback mechanism originating in skeletal muscle that has the ability to alter arterial pressure and heart rate during exercise(3, 92). During physical activity when skeletal muscle contracts, exercise pressor reflex activation is mediated by two afferent components: the mechanoreflex and the metaboreflex (Figure 6). The mechanoreflex consists of predominantly mechanically sensitive group III (A-δ fibers) afferent neurons while the metaboreflex consists of predominantly metabolically sensitive group IV (C fibers) afferent neurons(57-59, 142, 162). Both the mechanoreflex and metaboreflex afferent neurons synapse within medullary sites in the brainstem and work to accentuate blood pressure, heart rate, and ventilation through increases in sympathetic nerve activity and a withdrawal of parasympathetic nerve activity(56, 90, 92).

Afferent Limb of the Exercise Pressor Reflex

The afferent arm of the exercise pressor reflex elicits a cardiovascular response to exercise through activation of skeletal muscle receptors associated with the mechanoreflex and metaboreflex. Thinly-myelinated group III afferent neurons are predominantly activated by mechanical stimuli of skeletal muscle such as pressure and stretch(142, 162). Thus, the mechanoreflex responds to physical activity at the onset of muscle contraction. Unmyelinated group IV afferent fibers, which are primarily chemically-sensitive, are activated by metabolites produced by working skeletal muscle(57, 58). It should be noted, however, that the exercise pressor reflex afferent neurons exhibit some polymorphism and there are a small portion of group III fibers that are metabolically activated while a small portion of group IV fibers are mechanically stimulated(56).

The receptor mechanisms mediating mechanoreflex activation in skeletal muscle are not well described. Stimulation of the mechanoreflex is thought to be mediated by stretch-sensitive receptors predominantly anatomically located on group III afferent fibers terminating in collagen tissue between skeletal fibrocytes (Figure 7)(4). These receptors have been shown to be pharmacologically antagonized during muscle contraction by the trivalent lanthanide gadolinium(39, 136). For example, it has been demonstrated in cats that group III mechanically-sensitive afferent fibers fire significantly less during hindlimb skeletal muscle contraction and passive stretch when gadolinium is administered intra-arterially into the limb(39). In addition, pre-treatment of the limb with gadolinium markedly reduces the reflex-induced mean arterial pressure response to hindlimb contraction in both cats and rats(39, 136). Despite these compelling results, the receptor or receptors responsible for activating mechanically sensitive afferent fibers in skeletal muscle have yet to be fully characterized.

Metaboreflex activation is thought to signal an oxygen deficit to contracting muscle caused by reduced blood flow during exercise. This theory is supported by the finding that group IV afferent fibers have been shown to fire 4-10 seconds after the onset of contraction and increase their discharge rate steadily until contraction ceases(58, 59). In addition, group IV afferent fibers terminate in the walls of capillaries and venules within skeletal muscle advantageously positioned to detect metabolic changes within the surrounding tissue (Figure 8)(4). However, the chemical substances and receptors mediating the metaboreflex activation are unknown. Group IV fibers have been shown to be activated by a number of chemical substances known to be byproducts of muscle work as well as exogenous substances. Substances eliciting a pressor response in humans and animals by arterial infusion include lactic acid, glucose, capsaicin, diprotonated phosphate, potassium, bradykinin, prostaglandins, hydrogen ions, and adenosine, to name a few(21, 24, 37, 70, 122, 133, 145, 146, 152). Experimental evidence can be found to support and refute the involvement of each of these substances to some extent. Most likely, activation of the metaboreflex is not mediated by just one metabolic by-product of exercise but

rather a combination of several metabolic substances. In some of the most compelling research to date, mouse dorsal root ganglion neurons innervating skeletal muscle responded best to combinations of protons, adenosine triphosphate (ATP), and lactate at physiological concentrations(77). Clearly, the manner in which these endogenous chemicals work to stimulate the metaboreflex remains incompletely understood.

In a similar fashion, much research has been directed at determining the receptor or receptors responsible for activating the chemically-sensitive afferent fibers of the metaboreflex. For example, the ATP-gated ion-channel receptor $P2X_3$ has been shown to be localized exclusively on small diameter afferents(12, 71). Recently, it has been shown in cats that intra-arterial injection of α,β-methylene ATP (a $P2X_1$ and $P2X_3$ agonist) causes an increase in mean arterial pressure and heart rate via activation of the $P2X_3$ receptor on group IV afferent neurons(37). In the rat, however, data has shown that afferent neurons expressing the $P2X_3$ receptor rarely project to skeletal muscle(10). Another substance known to elicit increases in mean arterial pressure and heart rate in dogs, cats, and rats when injected into the arterial supply of skeletal muscle is capsaicin, the main pungent chemical found in hot peppers(39, 57, 139). While this chemical is not endogenous, it is known to selectively bind to transient receptor potential vanilloid 1, or TRPv1. TRPv1 receptors have been shown to be primarily localized to group IV afferent neurons(35, 88). Recent data in rats has suggested that the TRPv1 receptor contributes importantly to activation of the exercise pressor reflex during muscle contraction. However, studies in the cat suggest the TRPv1 receptor is not involved in metaboreflex activation(62, 69). Evidence to support and refute the potential involvement of other receptors in metaboreflex activation has also been reported. These receptors include, but are not limited to, the acid-sensing ion channel receptor (ASIC), the bradykinin receptor B2 and the cannabinoid receptor CB1 (26, 69, 103). As is the case with the chemical activators of this reflex, it is likely that stimulation of multiple chemically-sensitive receptors is required for the full expression of the muscle metaboreflex. A current study found a combination of ASIC, $P2X_5$ and/or $P2X_4$, and TRPv1

receptors on muscle-innervating sensory afferents worked together to detect muscle metabolites(77). Clearly, more research is needed to definitively determine the skeletal muscle receptors mediating metaboreflex activation during exercise.

Central Processing of the Exercise Pressor Reflex

The first synapse of most group III and IV afferents occurs within the dorsal horn of the spinal cord, specifically, to Rexed's laminae I, II, V, and X(55). Lamina VI, in the rostral portion of the sacral cord, also has dense projections of small diameter muscle afferents(104). Within the superficial dorsal horn of the spinal cord, these small diameter afferents impinge on both ascending neurons and interneurons(75). While the mapping of the spinal pathways through which group III and IV afferent neurons transmit their signals to the brainstem is incomplete, recent studies in rats using the anterograde tracer biotinylated dextran amine have shown neurons in the superficial laminae of the cervical spinal cord (including group III and IV fibers) project bilaterally to the cuneate nucleus, the nucleus tractus solitarius, the lateral reticular nucleus, and the caudal and rostral ventrolateral medulla within the medulla oblongata of the brainstem(30, 114). Specifically, neurons projecting from the dorsal horn to the caudal and medial subnuclei of the nucleus tractus solitarius have been found to play roles in cardiovascular and exercise pressor reflex processing(30, 79).

Areas within the brain that have been identified as transmitting neuronal activity along the exercise pressor reflex arc are the nucleus tractus solitarius, the caudal and rostral ventrolateral medulla, the lateral tegmental field, and the ventromedial region of the rostral periaqueductal gray(13, 49, 50, 73, 74). Neurons within these brainstem regions have been shown to be activated during muscle contraction and in contrast, neurons in the nucleus ambiguous have been shown to be inhibited during muscle contraction(49). Importantly, studies showing peripheral input from skeletal muscle modulates neuronal activity within the nucleus tractus solitarius (NTS) have suggested that this brainstem region is a central site of integration for the exercise pressor reflex(110).

Efferent Limb of the Exercise Pressor Reflex

As mentioned, the exercise pressor reflex induces a cardiovascular response to exercise via increases in sympathetic nerve activity and withdrawal of parasympathetic nerve activity(56). From the brainstem, vagal motor neurons, which are the primary parasympathetic efferents, travel via central preganglionic neurons originating in the nucleus ambiguous to postganglionic neurons in or near the walls of the heart(20). Sympathetic efferent neurons project from the brainstem to sympathetic preganglionic neurons in the interomediolateral cell columns of the spinal cord. From here, the sympathetic efferents synapse on the paravertebral chain ganglia and these neurons, in turn, innervate postganglionic neurons that travel to the heart and regional vasculature(20). Augmentations in sympathetic nerve activity to the heart cause increases in both heart rate and contractility leading to larger cardiac outputs during exercise(91, 92, 162). In addition, release of norepinephrine to receptors within arterial and venous adventitia produces vasoconstriction in inactive or active skeletal muscle, depending on the type of exercise being performed. Together, the higher cardiac output and increased peripheral vascular tone lead to elevations in blood pressure(41, 93). This insures that the heart is able to adequately pump blood to the exercising muscle(100).

Rat Model of Exercise

Our lab has developed a decerebrate rat model of exercise in order to preferentially activate the exercise pressor reflex without input from central command and with the option to eliminate arterial baroreflex input(134). In brief, this preparation preferentially activates the exercise pressor reflex through hindlimb skeletal muscle contraction via spinal ventral root stimulation. By directly stimulating the cut, distal end of the ventral root, static contraction of the triceps surae muscles can be induced without direct activation of afferent neurons (Figure 9A). In anesthetized animals, static contraction via ventral root stimulation produces a depressor and bradycardic response, opposite of that seen in humans during exercise(38). Therefore, rats in our preparation are rendered

insentient through pre-collicular decerebration and anesthesia is discontinued (Figure 9B). This procedure allows both blood pressure and heart rate to increase in response to static contraction(38, 134). Additionally, by removing the portions of the brain from which central command originates, its influence is eliminated in this preparation. Further, the rat model of exercise allows the option to eliminate baroreceptor input from the carotid arteries through a well-defined sino-aortic barodenervation procedure(138).

The rat model of essential hypertension used in our laboratory is the spontaneously hypertensive rat (SHR), which develops high blood pressure around 5-10 weeks(102). The Wistar Kyoto (WKY) rat has been established as its normotensive control and was bred from the same colony as SHR(80). SHR rats and individuals with essential hypertension appear to have a polygenic predisposition for hypertension that can be influenced by environmental factors, such as diet, stress, and physical activity(23, 47). Importantly, young, pre-hypertensive (3-4 week-old) SHR rats have increased sympathetic nerve discharge to the heart and vasculature as do pre-hypertensive individuals(23). This sympathetic overactivity may be causative as it pre-dates the onset of hypertension in both man and SHR. However, the increased sympathetic activity is also partially responsible for the progress and maintenance of the disease in both species(155). In addition, pre-hypertensive SHR have increased cardiac outputs, while adult, hypertensive SHR have normal or decreased cardiac outputs. Peripheral resistance is initially normal in young SHR, but increases as hypertension is established. These circulatory changes are also seen in humans who develop essential hypertension(36). Finally, humans with established hypertension have a normal baroreflex with decreased sensitivity, as do adult SHR rats(108). These similarities make the SHR rat one of the most common models for essential hypertension in man.

Hypertension and the Exercise Pressor Reflex

As previously mentioned, the cardiovascular response to exercise is exaggerated in hypertension. Increases in mean arterial pressure and heart rate are

significantly greater in hypertensive individuals compared to normotensive individuals in response to both static and dynamic exercise(5, 60, 131). These augmented increases in the cardiovascular response to exercise are partly mediated by the exercise pressor reflex. Studies from our lab in male (SHR) have shown that preferentially activating the exercise pressor reflex through hindlimb contraction causes increases in mean arterial pressure and heart rate that are significantly greater than the increases seen in normotensive (WKY) animals at maximal and submaximal contraction intensities (Figure 10A)(138). The responses were greater in SHR than in WKY rats over a range of contraction strengths, suggesting that all physical activity, including low-intensity, is more strenuous on the circulatory system in hypertension. Also, the slope of the relationship between the pressor response and muscle work intensity was significantly greater in SHR rats, showing the exercise pressor reflex to be more sensitive in hypertension. In addition, the exercise pressor reflex overactivity seen in hypertensive rats was related to exercise intensity with the change in mean arterial pressure caused by hindlimb contraction increasing as developed muscle tension increased. Furthermore, the changes in mean arterial pressure were positively correlated to baseline blood pressures in both hypertensive and normotensive animals. It is also important to note that the changes in mean arterial pressure in response to exercise pressor reflex activation were mediated by the sympathetic nervous system. When the exercise pressor reflex was activated by contraction before and after systemic infusion of the ganglionic blocker hexamethonium and sympathetic blocker phentolamine in SHR rats, the pressor response to contraction was almost completely abolished, suggesting that the reflex regulates cardiovascular hemodynamics predominantly via increases in sympathetic nerve activity (Figure 10B)(138).

Integration of Multiple Neural Inputs during Exercise

While our data suggested the exaggerated circulatory response to exercise in hypertension could be attributed to an overactive exercise pressor reflex, the other two neural inputs that control the cardiovascular response to exercise also

need to be considered. In order to preserve the sensitivity of the baroreflex as it buffers changes in mean arterial pressure and heart rate, the baroreceptors are reset by both central command and the exercise pressor reflex to operate around higher pressures during exercise (86, 106, 107). However, it has been well established that baroreflex sensitivity is reduced in hypertension(89, 115, 138). Furthermore, the exercise pressor reflex is known to buffer baroreflex activity, therefore it is possible that a diminished baroreflex is responsible for the increased cardiovascular response to exercise in hypertension(138, 159). In order to investigate this possibility, we tested exercise pressor reflex function during electrically-induced static muscle contraction in baro-intact and barodenervated SHR and WKY animals(138). While both the mean arterial pressure and heart rate responses to contraction significantly increased in barodenervated WKY rats, these circulatory changes were significantly less than those produced in baro-intact SHR rats (Figure 11)**.** Additionally, the increases in mean arterial pressure and heart rate occurring in SHR animals in response to contraction were further enhanced when these animals were likewise barodenervated, suggesting that the baroreflex does maintain the ability to buffer changes in blood pressure in hypertension. As a result, it was concluded that the exaggerated response to exercise in SHR is primarily attributed to an overactive exercise pressor reflex.

Mechanoreflex and Metaboreflex Activity in Hypertension

Having established that the exercise pressor reflex is overactive in hypertension, the next logical step was to test both components of the reflex (the mechanoreflex and metaboreflex) for dysfunction. In order to preferentially activate the mechanoreflex, the triceps surae muscles were passively stretched to produce a developed muscle tension similar to that seen during ventral root stimulation (Figure 12). This maneuver is known to primarily activate mechanically sensitive group III afferent fibers. Using the passive hindlimb stretch procedure, studies from our lab showed that both the pressor and heart rate response to activation of mechanically-sensitive muscle afferent fibers are enhanced in hypertensive rats compared to normotensive rats (Figure 13)(69). The

increases in both heart rate and mean arterial pressure due to mechanoreflex activation were significantly greater in hypertensive rats compared to age-matched (14-20 weeks old) normotensive rats over a range of developed tensions produced by hindlimb muscle stretch (Figure 14). Linear regression analysis showed the changes in mean arterial pressure and heart rate in response to passive stretch were positively correlated to the stretch intensities in both WKY and SHR rats (Figure 14A & C). However, in the graph illustrating changes in mean arterial pressure in response to passive stretch, the steeper slope for the SHR animals provide evidence that the mechanoreflex is more sensitive in hypertension (Figure 14A)(69).

In order to preferentially activate the metaboreflex, capsaicin, a TRPv1 receptor agonist, was injected into the hindlimb arterial supply (Figure 15). It is well established that the pungent chemical capsaicin selectively binds to transient potential vanilloid 1 (TRPv1) receptors which are located predominantly on group IV, metabolically sensitive afferent fibers(88). Therefore, the cardiovascular responses elicited by intra-arterial capsaicin injections are due to the activation of group IV afferent fibers, which are known to mediate metaboreflex activity. Hindlimb intra-arterial capsaicin injections caused dose-related increases in mean arterial pressure and heart rate in both SHR and WKY rats (Figure 16)(69). However, there were significantly greater increases in mean arterial pressure in SHR compared to WKY rats at 14-20 weeks of age, especially at the two highest capsaicin concentrations (0.3 and 1.0 μg/100 μL). Changes in heart rate were much more variable. To substantiate that the injected capsaicin was causing the cardiovascular responses by binding to TRPv1 receptors, capsazepine, a chemical known to be a selective, competitive antagonist to the TRPv1 receptors, was injected simultaneously with capsaicin. The addition of the TRPv1 antagonist to the capsaicin hindlimb intra-arterial injections effectively reduced the group IV afferent fiber-mediated increases in mean arterial pressure (Figure 17). From these experiments we were able to conclude that the exercise pressor reflex is exaggerated in hypertension and that the exaggerations in the circulatory response are mediated by both the mechanoreflex and the metaboreflex.

Potential Mechanisms of Exercise Pressor Reflex Dysfunction in Hypertension: A Role for Brainstem Nitric Oxide

To briefly summarize, in hypertension, exercise induces an exaggerated cardiovascular response by greatly increasing mean arterial pressure and heart rate. Data from our laboratory has provided evidence that the exercise pressor reflex and both its components (the mechanoreflex and metaboreflex) mediate, in part, this exaggerated cardiovascular response(69). However, the mechanism of this exercise pressor reflex dysfunction remains undetermined. Central processing in the brainstem is of particular interest because current research has established the regulatory role of nitric oxide within the brainstem on exercise pressor reflex function(76, 135). Sensory afferents activated by the exercise pressor reflex synapse within the dorsal horn of the spinal cord and then project to the NTS of the medulla oblongata within the brainstem(51, 55, 78, 114). While these afferents also synapse within other brainstem nuclei, functional, electrophysiological, and neuroanatomical evidence suggests that the NTS is the most important site for exercise pressor reflex sensory processing (Figure 18)(110, 148, 149).

Within the NTS, there are many neurotransmitters and neuromodulators involved in the exercise pressor reflex arc that are known to be altered in hypertension. The nitric oxide pathway is of particular interest because the activity of medullary neurons that receive and process sensory information from group III and IV afferents can be modulated by its endogenous production(72, 128, 150). Within the NTS, L-arginine is oxidized by nitric oxide synthase (NOS) to produce nitric oxide (NO) and L-citrulline (Figure 19)(95). This centrally-produced NO in the NTS has been shown to tonically inhibit sympathetic outflow from the medulla as well as modulate cardiovascular reflexes(17, 42, 68, 109, 113, 118, 126, 165). Additionally, experiments in rats and cats have shown that NO within the NTS can decrease mean arterial pressure, heart rate, and renal sympathetic nerve activity during rest and exercise(72, 76, 140, 150, 154). Studies from our laboratory have shown that sympathetically mediated-increases in mean arterial pressure caused by activation of the exercise pressor reflex were

attenuated when NO production was experimentally increased in the NTS of normotensive cats (Figure 20)(135).

Given the role of NO within the NTS in exercise pressor reflex sensory processing and the role of NO in regulating vasomotor tone, it seems plausible that the L-arginine-NO pathway in the NTS may be impaired and responsible for the exercise pressor reflex dysfunction seen in hypertension(76, 128). Specifically, sensory processing of exercise pressor reflex mechanically and metabolically sensitive afferents may be altered due to NO impairment within the NTS in hypertension. It has been shown that methylated arginines, which are elevated in hypertension, can pharmacologically inhibit NOS(8, 46). Also, the superoxide anion reactive oxygen species (ROS) is known to inactivate NO and form peroxynitrite through the action of nicotinamide-adenine dinucleotide phosphate (NAD(P)H) oxidase (Figure 21). NAD(P)H oxidase activity is increased in hypertensive individuals due to the increased physical stress and/or the presence of angiotensin II, which has also been shown to be increased in the NTS of SHR rats compared to WKY(33, 87). In fact, research has shown that there is a higher levels of angiotensin II and its AT_1 receptors within the NTS of SHR rats compared to normotensive WKY rats(109, 140). It seems possible that reductions of NO production and/or availability within the NTS could cause the exaggerated sympathetically mediated-increases in mean arterial pressure and heart rate observed during exercise pressor reflex activation in hypertensive rats(138).

One cause of an L-arginine-NO pathway impairment could be decreases in the expression/activity of any of the three NOS isoforms present within the NTS, which are endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and neuronal nitric oxide synthase (nNOS) proteins. As mentioned previously, NOS protein is the enzyme involved in NO production. In normotensive WKY rats, immunostaining for NOS-positive neurons within the NTS found NOS immunoreactivity to be highly restricted to the medial subnucleus, which is known to receive input from both baroreflex and exercise pressor reflex afferents(68). However, to date, studies describing NOS

expression/activity within the brainstem of hypertensive rats have been conflicting. Some studies in hypertensive rats have shown eNOS and nNOS expression and activity within the medulla to be decreased during infancy, but significantly increased in adulthood compared to normotensive controls(112, 118, 156). Other studies show basal levels of NOS expression and activity are decreased in the NTS of adult hypertensive rats compared to their normotensive controls(22, 113). Functional studies have demonstrated in normotensive and hypertensive cats and rats that NOS suppression within the NTS causes increases in resting mean arterial pressure, heart rate, and sympathetic nerve activity(18, 147, 163). Specifically, in cats it was shown that reducing tonically-released NO within the NTS by microinjection of a NOS inhibitor significantly increased mean arterial pressure and heart rate(163). On the other hand, increasing NOS protein levels within the NTS has been shown to significantly decrease basal mean arterial pressure and heart rate in normotensive and hypertensive rats(43, 144). Finally, one study found adult SHR rats' blood pressure was more responsive to central-acting NO than their normotensive counterparts, but interventions that modulated the activity of NOS had less of an effect, leading the authors to conclude that there is less NO within the brainstem of SHR rats(11). The contradictory data on NOS activity and expression within the NTS of hypertensive animals and its effect on blood pressure illustrate the need for more comprehensive experiments.

Hypothesis and Specific Aim

Based on research from our laboratory as well as others, we hypothesize that the exaggerated exercise pressor reflex, as well as its mechanically and metabolically sensitive components, observed in hypertension is mediated by changes in NO activity within the NTS. To test this hypothesis, we performed experiments using age-matched (over 20 weeks old) male WKY and SHR rats to:

Specific Aim. **Determine the contribution of alterations in NO availability/activity within the NTS to exercise pressor reflex dysfunction in**

hypertension; specifically its mechanically and metabolically sensitive components.

For the beneficial effects of exercise to be realized to a greater extent in hypertension, the mechanism of exercise pressor reflex dysfunction in this disease must be determined. This knowledge may lead to novel treatments that could potentially increase exercise tolerance in hypertensive patients and reduce the risks associated with exercise in affected individuals.

References

1. Aicher SA and Randich A. Antinociception and cardiovascular responses produced by electrical stimulation in the nucleus tractus solitarius, nucleus reticularis ventralis, and the caudal medulla. *Pain* 42: 103-119, 1990.
2. Akinpelu AO. Responses of the African hypertensive to exercise training: preliminary observations. *J Hum Hypertens* 4: 74-76, 1990.
3. Alam M and Smirk FH. Observations in man upon a blood pressure raising reflex arising from the voluntary muscles. *J Physiol* 89: 372-383, 1937.
4. Andres KH, During MV, and Schmidt RF. Sensory innervation of Achilles tendon by group III and IV afferent fibers. *Anat Embyol (Berl)* 172: 145-156, 1985.
5. Aoki K, Sato K, Kondo S, Pyon C, and Yamamoto M. Increased response of blood pressure to rest and handgrip in subjects with essential hypertension. *Jpn Circ J* 47: 802-809, 1983.
6. Baglivo HO, Fabregues G, Burrieza H, Esper RC, Talacio M, and Esper RJ. Effect of moderate physical training on left ventricular mass in mild hypertensive persons. *Hypertension* 15: I153-I156, 1990.
7. Bedford TG, Loi PK, and Crandall CC. A model of dynamic exercise: the decerebrate rat locomotor preparation. *J App Physiol* 72: 121-127, 1992.
8. Benjafield AV and Morris BJ. Association analyses of endothelial nitric oxide synthase gene polymorphisms in essential hypertension. *Am J Hypertens* 13: 994-998, 2000.
9. Blumenthal JA, Siegal WC, and Appelbaum M. Failure of exercise to reduce blood pressure in patients with mild hypertension. *JAMA* 266: 2098-2104, 1991.
10. Bradbury EJ, Burnstock G, and McMahon SB. The expression of $P2X_3$ purinoceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. *Molecular and Cellular Neuroscience* 12: 256-268, 1998.
11. Cabrera CL, Bealer SL, and Bohr DF. Central depressor action of nitric oxide is deficient in genetic hypertension. *Am J Hypertens* 9: 237-241, 1996.

12. Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, and Wood JN. A P2X purinoceptor expressed by a subset of sensory neurons. *Nature* 377: 428-431, 1995.
13. Ciriello J and Calaresu FR. Lateral reticular nucleus: a site of somatic and cardiovascular integration in the cat. *Am J Physiol* 233: R100-R109, 1977.
14. Cononie CC, Graves JE, Pollock ML, Phillips MI, Sumners C, and Hagberg JM. Effect of exercise training on blood pressure in 70- to 79-yr-old men and women. *Med Sci Sports Exerc* 23: 505-511, 1991.
15. Critchley HD, Corfield DR, Chandler MP, Mathias CJ, and Dolan RJ. Cerebral correlates of autonomic cardiovascular arousal: a functional neuroimaging investigation in humans. *J Physiol* 523: 259-270, 2000.
16. DeArtinano AA and Gonzalez VL-M. Endothelial dysfunction and hypertensive vasoconstriction. *Pharmacol Res* 40: 113-124, 1999.
17. Dias ACR, Vitela M, Colombari E, and Mifflin SW. Nitric oxide modulation of glutamatergic, baroreflex, and cardiopulmonary transmission in the nucleus of the solitary tract. *Am J Physiol Heart Circ Physiol* 288: 256-262, 2005.
18. Eshima K, Hirooka Y, Shigematsu H, Matsuo I, Koike G, Sakai K, and Takeshita A. Angiotensin in the nucleus tractus solitarii contributes to neurogenic hypertension caused by chronic nitric oxide synthase inhibition. *Hypertension* 36: 259-263, 2000.
19. Fadel PJ, Ogoh S, Watenpaugh DE, Wasmund W, Olivencia-Yurvati A, Smith ML, and Raven PB. Carotid baroreflex regulation of sympathetic nerve activity during dynamic exrecise in humans. *Am J Physiol Heart Circ Physiol* 280: H1383-H1390, 2001.
20. Fadel PJ, Smith SA, and Gallagher KM. Neural mechanisms influencing baroreflex resetting during exercise. *Recent Res Devel Physiol* 2, 2004.
21. Fallentin N, Jensen BR, Bystrom S, and Sjogaard G. Role of potassium in the reflex regulation of blood pressure during static exercise in man. *J Physiol* 451: 643-651, 1992.
22. Ferrari MFR and Fior-Chadi DR. Differential expression of nNOS mRNA and protein in the nucleus tractus solitarii of young and aged Wistar-Kyoto and spontaneously hypertensive rats. *J Hypertens* 23: 1683-1690, 2005.
23. Folkow B. Central neurohumoral mechanisms in spontaneously hypertensive rats compared with human essential hypertension. *Clin Sci and Mol Med* 48: 205s-214s, 1975.
24. Fontana GA, Pantaleo T, Bongianni F, Cresci F, Lavorini F, Guerra CT, and Panuccio P. Prostaglandin synthesis blockade by ketoprofen attenuates respiratory and cardiovascular responses to static handgrip. *J App Physiol* 78: 449-457, 1995.
25. Fox SI. *Human Physiology*. New York: WCB/McGraw-Hill, 1999.
26. Fuster V, Alexander RW, and O'Rourke RA. *Hurst's The Heart*. New York: McGraw-Hill Medical Publishing Division, 2004.
27. Gallagher K, Fadel P, Stromstad M, Ide K, Smith S, Querry R, Raven P, and Secher N. Effects of exercise pressor reflex activation on carotid baroreflex function during exercise in humans. *J Physiol* 533: 871-880, 2001.

28. Gallagher K, Fadel P, Stromstad M, Ide K, Smith S, Querry R, Raven P, and Secher N. Effects of partial neuromuscular blockade on carotid baroreflex function during exercise in humans. *J Physiol* 533: 861-870, 2001.
29. Gallagher KM, Raven PB, and Mitchell JH. Classification of Sports and the Athlete's Heart. In: *The athlete and heart disease: Diagnosis, evaluation, & management*, edited by Williams RA. Philadelphia, PA: Lippincott Williams & Wilkins, 1999, p. 9-21.
30. Gamboa-Esteves FO, Tavares I, Almeida A, Batten TFC, McWilliam PN, and Lima D. Projection sites of superficial and deep spinal dorsal horn cells in the nucleus tractus solitarii of the rat. *Brain Res* 921: 195-205, 2001.
31. Goodwin GM, McCloskey DI, and Mitchell JH. Cardiovascular and respiratory responses to changes in central command during isometric exercise at constant muscle tension. *J Physiol* 226: 173-190, 1972.
32. Gordon NF, Scott CB, and Levine BD. Comparison of single versus multiple lifestyle interventions: are the antihypertensive efects of exercise training and diet-induced weight loss additive? *Am J Cardiol* 79: 763-767, 1997.
33. Griendling KK, Sorescu D, and Ushio-Fukai M. NAD(P)H oxidase. *Circ Res* 86: 494-501, 2000.
34. Group NHBPEPW. National high blood pressure education program working group on prevention of hypertension. *Arch Intern Med* 153: 186-208, 1993.
35. Guo A, Vulchanova L, Wang J, Li X, and Elde R. Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the $P2X_3$ purinoceptor and IB4 binding sites. *Eur J Neurosci* 11: 946-958, 1999.
36. Hallback M and Weiss L. Mechanisms of spontaneous hypertension in rats. *Med Clinics of N America* 61: 593-609, 1977.
37. Hanna RL, Hayes SG, and Kaufman MP. α, β Methylene ATP elicits a reflex pressor response arising from muscle in decerebrate cats. *J App Physiol* 93: 834-841, 2002.
38. Hayashi N. Exercise pressor reflex in decerebrate and anesthetized rats. *Am J Physiol* 284: H2026-H2033, 2003.
39. Hayes SG and Kaufman MP. Gadolinium attenuates exercise pressor reflex in cats. *Am J Physiol* 280: 2153-2161, 2001.
40. Heron E, Chemla D, Megnien J-L, Pourny J-C, Levenson J, Lecarpentier Y, and Simon A. Reactive hyperemia unmasks reduced compliance of cutaneous arteries in essential hypertension. *J App Physiol* 79: 498-505, 1995.
41. Hill JM, Adreani CM, and Kaufman MP. Muscle reflex stimulates sympathetic postganglionic efferents innervating triceps surae muscles of cats. *Am J Physiol* 271: H38-H43, 1996.
42. Hironaga K, Hirooka Y, Matsuo I, Shihara M, Tagawa T, Harasawa Y, and Takeshita A. Role of endogenous nitric oxide in the brain stem on the rapid adaptation of baroreflex. *Hypertension* 31: 27-31, 1998.
43. Hirooka Y, Sakai K, Kishi T, Ito K, Shimokawa H, and Takeshita A. Enhanced depressor response to endothelial nitric oxide synthase gene transfer into the nucleus tractus solitarii of spontaneously hypertensive rats. *Hypertens Res* 26: 325-331, 2003.

44. Hoberg E, Schuler G, Kunze B, Obermoser AL, Hauer K, Mauther HP, Schlierf G, and Kubler W. Silent myocardial ischemia as a potential link between lack of premonitoring symptoms and increased risk of cardiac arrest during physical stress. *Am J Cardiol* 65: 583-589, 1990.
45. Horta PP, deCarvalho JJ, and Mandarim-de-Lacerda CC. Exercise training attenuates blood pressure elevation and adverse remodeling in the aorta of spontaneously hypertensive rats. *Life Sciences* 77: 3336-3343, 2005.
46. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, and Fishman MC. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 377: 196-197, 1995.
47. Hubner N and Ganten D. Genetics in arterial hypertension-clinical and experimental aspects. *Herz* 20: 309-314, 1995.
48. Ichinose M, Saito M, Fujii N, Ogawa T, Hayashi K, Kondo N, and Nishiyasu T. Modulation of the control of muscle sympathetic nerve activity during incremental leg cycling. *J Physiol* 586: 2753-2766, 2008.
49. Iwamoto GA and Kaufman MP. Caudal ventrolateral medullary cells responsive to static muscular contraction. *J App Physiol* 62: 149-157, 1987.
50. Iwamoto GA, Kaufman MP, Botterman BR, and Mitchell JH. Effects of lateral reticular nucleus lesions on the exercise pressor reflex in cats. *Circ Res*, 1982.
51. Iwamoto GA, Waldrop TG, Kaufman MP, Botterman BR, Rybicki KJ, and Mitchell JH. Pressor reflex evoked by muscular contraction: contributions by neuraxis levels. *J App Physiol* 59: 459-467, 1985.
52. Johansson JE. Ueber die Einwirkung der Muskelthatigkeit auf die Athmung und die Hertzhiitigkeit. *Skand Arch Physiol* 5: 20-66, 1895.
53. Joint National Committee on Prevention D, Evaluation, and Treatment of High Blood Pressure. Sixth report of the Joint National Committee on detection, evaluation, and treatment of high blood pressure. *Arch Intern Med* 157: 2413-2446, 1997.
54. Kahn JF. The static exercise-induced arterial hypertension test. *Presse Medicine* 20: 1067-1071, 1991.
55. Kalia M, Mei SS, and Kao FF. Central projections from ergoreceptors (c fibers) in muscle involved in cardiopulmonary responses to static exercise. *Circ Res* 48: I48-I62, 1981.
56. Kaufman MP and Forster HV. Reflexes controlling circulatory, ventilatory and airway responses to exercise. In: *Section 12, Exercise: Regulation and Integration of Multiple Systems*. Bethesda, MD: Am Physiol Soc, 1996, p. 381-447.
57. Kaufman MP, Iwamoto GA, Longhurst JC, and Mitchell JH. Effects of capsaicin and bradykinin on afferent fibers with endings in skeletal muscle. *Circ Res* 50: 133-139, 1982.
58. Kaufman MP, Longhurst JC, Rybicki KJ, Wallach JH, and Mitchell JH. Effects of static muscular contraction on impulse activity of groups III and IV afferents in cats *J App Physiol* 55: 105-112, 1983.

59. Kaufman MP, Waldrop TG, Rybicki KJ, Ordway GA, and Mitchell JH. Effects of static and rhythmic twitch contractions on the discharge of group III and IV muscle afferents. *Card Res* 18: 663-668, 1984.
60. Kazatani Y, Hamada M, Shigematsu Y, Hiwada K, and Kokubu T. Beneficial effect of a long-term antihypertensive therapy on blood pressure response to isometric handgrip exercise in patients with essential hypertension. *American Journal of Therapy* 2: 165-169, 1995.
61. Keleman MH, Effron MB, Valenti SA, and Stewart KJ. Exercise training combined with antihypertensive drug therapy. *JAMA* 263: 2766-2771, 1990.
62. Kindig AE, Heller TB, and Kaufman MP. VR-1 receptor blockade attenuates the pressor response to capsaicin but has no effect on the pressor response to contraction in cats. *Am J Physiol* 288: H1867-H1873, 2005.
63. Koga M, Ideishi M, Matsusaki M, Tashiro E, Kinoshita A, Ikeda M, and al e. Mild exercise decreases plasma endogenous digitalis like substance in hypertensive individuals. *Hypertension* 19: S231-S236, 1992.
64. Kokkinos PF, Narayan P, Colleran J, Pittaras A, Notargiacomo A, and al e. Effects of regular exercise on blood pressure and left ventricular hypertrophy in African-American men with severe hypertension. *N Engl J Med* 333: 1462-1467, 1995.
65. Kokkinos PF, Narayan P, Fletcher RD, Tsagadopoulos D, and Papademetriou V. Effects of aerobic training on exaggerated blood pressure response to exercise in African-Americans with severe systemic hypertension treated with indapamide + verapamil + enalapril. *Am J Cardiol* 79: 1424-1426, 1997.
66. Kokkinos PF and Papademetriou V. Exercise and hypertension. *Coronary Artery Dis* 11: 99-102, 2000.
67. Krogh A and Lindhard J. The regulation of respiration and circulation during the initial stages of muscular work. *J Physiol* 47: 112-136, 1913.
68. Lawrence AJ, Castillo-Melendez M, McLean KJ, and Jarrott B. The distribution of nitric oxide synthase-, adenosine deaminase- and neuropeptide Y-immunoreactivity through the entire rat nucleus tractus solitarius. Effect of unilateral nodose ganglionectomy. *J Chem Neuroanat* 15: 27-40, 1998.
69. Leal AK, Williams MA, Garry MG, Mitchell JH, and Smith SA. Evidence for functional alterations in the skeletal muscle mechanoreflex and metaboreflex in hypertensive rats. *Am J Physiol Heart Circ Physiol* 295: H1429-H1438, 2008.
70. Leal AK, Williams MA, Garry MG, Mitchell JH, and Smith SA. The pressor response to activation of mechanically and metabolically sensitive skeletal muscle afferent fibers is exaggerated in hypertension. *FASEB Journal* 20: A770, 2006.
71. Lewis C, Neidhart S, Holy C, North RA, Buell G, and Surprenant A. Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. *Nature* 377: 432-435, 1995.
72. Lewis SJ, Ohta H, Machado B, Bates JN, and Talman WT. Microinjection of S-nitrosocysteine into the nucleus tractus solitarii decreases arterial pressure and heart rate via activation of soluble guanylate cyclase. *Eur J Pharmacol* 202: 135-136, 1991.

73. Li J, Hand GA, Potts JT, Wilson LB, and Mitchell JH. c-Fos expression in the medulla induced by static muscle contraction in cats. *Am J Physiol* 272, 1997.
74. Li J and Mitchell JH. c-Fos expression in the midbrain periaqueductal gray during static muscle contraction. *Am J Physiol* 279: H2986-H2993, 2000.
75. Li J and Mitchell JH. Role of NO in modulating neuronal activity in superficial dorsal horn of spinal cord during exercise pressor reflex. *Am J Physiol* 283: H1012-H1018, 2002.
76. Li J and Potts JT. NO formation in nucleus tractus solitarii attenuates pressor response evoked by skeletal muscle afferents. *Am J Physiol* 280: H2371-H2379, 2001.
77. Light AR, Hughes RW, Zhang J, Rainier J, Liu Z, and Lee J. Dorsal root ganglion neurons innervating skeletal muscle respond to physiological combinations of protons, ATP, and lactate mediated by ASIC, P2X, and TRPV1. *J Neurophysiol* 100: 1184-1201, 2008.
78. Light AR and Perl ER. Re-examination of the dorsal root projection to the spinal dorsal horn including observations on the differential termination of coarse and fine fibers. *J Comp Neurol* 186: 117-132, 1979.
79. Lin L-H, Cassell MD, Sandra A, and Talman WT. Direct evidence for nitric oxide synthase in vagal afferents to the nucleus tractus solitarii. *Neuroscience* 84: 549-558, 1998.
80. Louis WJ and Howes LG. Genealogy of the spontaneously hypertensive rat and Wistar-Kyoto strains: implications for studies of inherited hypertension. *J Cardiovasc Pharmacol* 16: S1-S5, 1990.
81. Lund-Johansen P. Twenty-year follow-up of hemodynamics in essential hypertension during rest and exercise. *Hypertension* 18: III54-61, 1991.
82. MacMahon S, Peto R, Collins R, Godwin J, Cutler J, Sorlie P, Abbott R, Neaton J, Dyer A, and Stamler J. Blood pressure, stroke, and coronary heart disease. Part 1, prolonged differences in blood pressure: prospective observational studies corrected for the regression dilution bias. *The Lancet* 335: 765-774, 1990.
83. Manolis AJ, Beldekos D, Hatzissavas J, Foussas S, Cokkinos D, Bresnahan M, Gavras I, and Gavras H. Hemodynamic and humoral correlates in essential hypertension: Relationship between patterns of LVH and myocardial ischemia. *Hypertension* 30: 730-734, 1997.
84. Martin JE, Dubbert PM, and Cushman WC. Controlled trial or aerobic exercise in hypertension. *Circulation* 81: 1560-1567, 1990.
85. Medicine ACoS. Physical activity, physical fitness and hypertension [position stand]. *Med Sci Sports Exerc* 25: i-x, 1993.
86. Melcher A and Donald DE. Maintained ability of carotid baroreflex to regulate arterial pressure during exercise. *Am J Physiol* 241: H838-H849, 1981.
87. Meyer JM, Felten DL, and Weyhenmeyer JA. Levels of immunoreactive angiotensin II in microdissected nuclei from adult WKY and SH rat brain. *Clin Exp Hypertens A* 11: 103-117, 1989.
88. Michael GJ and Priestley JV. Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its down regulation by axotomy. *J Neurosci* 19: 1844-1854, 1999.

89. Minami N, Yoshikawa T, Kataoka H, Mori N, Nagasaka M, Kurosawa H, Kanazawa M, and Kohzuki M. Effects of exercise and beta-blocker on blood pressure and baroreflexes in spontaneously hypertensive rats. *Am J Hypertens* 16: 966-972, 2003.
90. Mitchell JH. Neural control of the circulation during exercise. *Med Sci Sports Exerc* 22: 141-154, 1990.
91. Mitchell JH, Haskell WL, and Raven PB. *J Am Coll Cardiol* 24: 864-866, 1994.
92. Mitchell JH, Kaufman MP, and Iwamoto GA. The exercise pressor reflex: Its cardiovascular effects, afferent mechanisms, and central pathways. *Ann Rev Physiol* 45: 229-242, 1983.
93. Mittelstadt SW, Bell LB, O'Hagan KP, and Clifford PS. Muscle chemoreflex alters vascular conductance in nonischemic exercising skeletal muscle. *J App Physiol* 77: 2761-2766, 1994.
94. Mittleman M and Siscovick D. Physical exertion as a trigger of myocardial infarction and sudden cardiac death. *Cardiol Clin* 14: 263-270, 1996.
95. Moncada S and Higgs A. The L-arginine-nitric oxide pathway. *New Eng J Med* 329: 2002-2012, 1993.
96. Mundal R, Kjeldsen SE, Sandvik L, Erikssen G, Thaulow E, and Erikssen J. Exercise blood pressure predicts mortality from myocardial infarction. *Hypertension* 27: 324-329, 1996.
97. Nowak M, Holm S, Biering-Sorensen F, Secher NH, and Friberg L. "Central command" and insular activation during attempted foot lifting in paraplegic humans. *Human Brain Mapping* 25: 259-265, 2005.
98. Nowak M, Olsen KS, Law I, Holm S, Paulson OB, and Secher NH. Command-related distribution of regional cerebral blood flow during attempted handgrip. *J Appl Physiol* 86: 819-824, 1999.
99. O'Leary DS. Autonomic mechanisms of muscle metaboreflex control of heart rate. *J App Physiol* 74: 1748-1754, 1993.
100. O'Leary DS, Robinson ED, and Butler JL. Is active skeletal muscle functionally vasoconstricted during dynamic exercise in conscious dogs? *Am J Physiol* 272: R386-R391, 1997.
101. Ogoh S, Wasmund WL, Keller DM, A OY, Gallagher KM, Mitchell JH, and Raven PB. Role of central command in carotid baroreflex resetting in humans during static exercise. *J Physiol* 543: 349-364, 2002.
102. Okamoto K and Aoki K. Development of a strain of spontaneously hypertensive rats. *Jpn Circulation J* 27: 282-293, 1963.
103. Pan HL, Stebbins CL, and Longhurst JC. Bradykinin contributes to the exercise pressor reflex: mechanism of action. *J App Physiol* 75: 2061-2068, 1993.
104. Panneton WM, Gan Q, and Juric R. The central termination of sensory fibers from nerves to the gastrocnemius muscle of the rat. *Neuroscience* 134: 175-187, 2005.
105. Papademetriou V and Kokkinos PF. The role of exercise in the control of hypertension and cardiovascular risk. *Curr Opin Nephrol Hypertens* 5: 459-462, 1996.

106. Papelier Y, Escourrou P, Gauthier JP, and Rowell LB. Carotid baroreflex control of blood pressure and heart rate in men during dynamic exercise. *J App Physiol* 77: 502-506, 1994.
107. Papelier Y, Escourrou P, Helloco F, and Rowell LB. Muscle chemoreflex alters carotid sinus baroreflex response in humans. *J App Physiol* 82: 577-583, 1997.
108. Paton JF, Dickinson CJ, and Mitchell G. Harvey Cushing and the regulation of blood pressure in giragge, rat, and man: introducing 'Cushing's mechanism'. *Exp Physiol* 94: 11-17, 2009.
109. Paton JFR, Deuchars J, Ahmad Z, Wong LF, Murphy D, and Kasparov S. Adenoviral vector demonstrates that angiotensin II-induced depression of the cardiac baroreflex is mediated by endothelial nitric oxide synthase in the nucleus tractus solitarii of the rat. *J Physiol* 531, 2001.
110. Person RJ. Somatic and vagal afferent convergence on solitary tract neurons in cat: electrophysiological characteristics. *Neuroscience* 30: 283-295, 1989.
111. Pickering TG. Pathophysiology of exercise hypertension. *Herz* 12: 119-124, 1987.
112. Plochocka-Zulinska D and Krukoff TL. Increased gene expression of neuronal nitric oxide synthase in brain of adult spontaneously hypertensive rats. *Brain Res Mol Brain Res* 48: 291-297, 1997.
113. Pontieri V, Venezuela MK, Scavone C, and Michelini LC. Role of endogenous nitric oxide in the nucleus tractus solitarii on baroreflex control of heart rate in spontaneously hypertensive rats. *J Hypertens* 16: 1993-1999, 1998.
114. Potts JT, Lee SM, and Anguelov PI. Tracing of projection neurons from the cervical dorsal horn to the medulla with the anterograde tracer biotinylated dextran amine. *Aut Neurosci* 98: 64-69, 2002.
115. Potts JT and Li J. Interaction between carotid baroreflex and exercise pressor reflex depends on baroreceptor afferent input. *Am J Physiol* 274: H1841-H1847, 1998.
116. Potts JT and Mitchell JH. Rapid resetting of carotid baroreceptor reflex by afferent input from skeletal muscle receptors. *Am J Physiol* 275: H2000-H2008, 1998.
117. Potts JT, Shi XR, and Raven PB. Carotid baroreflex responsiveness during dynamic exercise in humans. *Am J Physiol* 265: H1928-H1938, 1993.
118. Qadri F, Arens T, Schwarz EC, Hauser W, Dendorfer A, and Dominiak P. Brain nitric oxide synthase activity in spontaneously hypertensive rats during the development of hypertension. *J Hypertens* 21: 1623-1624, 2003.
119. Querry RG, Smith SA, Stromstad M, Ide K, Raven PB, and Secher NH. Neural blockade during exercise augments central command's contribution to carotid baroreflex resetting. *Am J Physiol* 280: H1635-H1644, 2001.
120. Rogers MW, Probst MM, Gruber JJ, Berger R, and Boone JB. Differential effects or exercise training intensity on blood pressure and cardiovascular responses to stress in borderline hypertensive humans. *J Hypertens* 14: 1375-1399, 1996.

121. Roman MJ, Pickering TG, Pini R, Schwartz JE, and Devereux RB. Prevalence and determinants of cardiac and vascular hypertrophy in hypertension. *Hypertension* 26: 369-373, 1995.
122. Rotto DM and Kaufman MP. Effect of metabolic products of muscular contraction on discharge of group III and IV afferents. *J App Physiol* 64: 2306-2313, 1988.
123. Rowell LB. *Human Cardiovascular Control*. New York: Oxford University Press, 1993.
124. Rowell LB. *Human Circulation Regulation During Physical Stress*. New York: Oxford University Press, 1986.
125. Rowell LB and O'Leary DS. Reflex control of the circulation during exercise: chemoreflexes and mechanoreflexes. *J Appl Physiol* 69: 407-418, 1990.
126. Ruggiero DA, Mtui EP, Otake K, and Anwar M. Central and primary visceral afferents to nucleus tractus solitarii may generate nitric oxide as a membrane-permeant neuronal messenger. *J Comp Neurol* 361: 51-67, 1996.
127. Sagawa K. Baroreflex control of systemic arterial pressure and vascular bed. In: *Handbook of Physiology. The Cardiovascular System*. Bethesda, MD: Am Physiol Soc, 1983, p. 453-496.
128. Sander M, Chavoshan B, and Victor RG. A large blood pressure-raising effect of nitric oxide synthase inhibition in humans. *Hypertension* 33: 937-942, 1999.
129. Seals DR and Reiling MJ. Effect of regular exercise on 24-hour arterial pressure in older hypertensive humans. *Hypertension* 18: 583-592, 1991.
130. Seals DR, Silverman HG, Reiling MJ, and Davy KP. Effect of regular aerobic exercise on elevated blood pressure in postmenopausal women. *Am J Cardiol* 80: 49-55, 1997.
131. Seguro C, Sau F, Zedda N, Scano G, and Cherchi A. Arterial blood pressure behavior during progressive muscular exercise in subjects with stable arterial hypertension. *Cardiologia* 36: 867-877, 1991.
132. Sheehan D, Mulholland JH, and Shafiroff B. Surgical anatomy of the carotid sinus nerve. *Anat Record* 80: 431-442, 1941.
133. Sinoway LI, Smith MB, Enders B, and Leuenberger U. Role of diprotonated phosphate in evoking muscle reflex responses in cats and humans. *Am J Physiol* 267: H770-H778, 1994.
134. Smith SA, Mitchell JH, and Garry MG. Electrically induced static exercise elicits a pressor response in the decerebrate rat. *J Physiol* 537: 961-970, 2001.
135. Smith SA, Mitchell JH, and Li J. Independent modification of baroreceptor and exercise pressor reflex function by nitric oxide in nucleus tractus solitarius. *Am J Physiol* 288: 2068-2076, 2005.
136. Smith SA, Mitchell JH, Naseem RH, and Garry MG. Mechanoreflex mediates the exaggerated exercise pressor reflex in heart failure. *Circulation* 112: 2293-2300, 2005.
137. Smith SA, Querry RG, Fadel PJ, Gallagher KM, Stromstad M, Ide K, Raven PB, and Secher NH. Partial blockade of skeletal muscle somatosensory afferents attenuates baroreflex resetting during exercise in humans. *J Physiol* 551: 1013-1021, 2003.

138. Smith SA, Williams MA, Leal AK, Mitchell JH, and Garry MG. Exercise pressor reflex function is altered in spontaneously hypertensive rats. *J Physiol* 577: 1009-1020, 2006.
139. Smith SA, Williams MA, Mitchell JH, Mammen PPA, and Garry MG. The capsaicin-sensitive afferent neuron in skeletal muscle is abnormal in heart failure. *Circulation*, 2005.
140. Song K, Kurobe Y, Kanehara H, Okunishi H, Wada T, Inada Y, Nishikawa K, and Miyazaki M. Quantitative localization of angiotensin II receptor subtypes in spontaneously hypertensive rats. *Blood Press Suppl* 5: 21-26, 1994.
141. Stamler J, Stamler R, and Neaton J. Blood pressure, systolic and diastolic, and cardiovascular risks. *Arch Intern Med* 153: 598-615, 1993.
142. Stebbins CL, Brown B, Levin D, and Longhurst JC. Reflex effect of skeletal muscle mechanoreceptor stimulation on the cardiovascular system. *J App Physiol* 65: 1539-1547, 1988.
143. Sun M-W, Qian F-L, Wang J, Tao T, Gup J, Wang L, Lu A-Y, and Chen H. Low-intensity voluntary running lowers blood pressure with simultaneous improvement in endothelium-dependent vasodilation and insulin sensitivity in aged Spontaneously Hypertensive Rats. *Hypertens Res* 31: 543-552, 2008.
144. Tai MH, Hsiao M, Chan JY, Lo WC, Wang FS, Liu GS, Howng SL, and Tseng CJ. Gene delivery of endothelial nitric oxide synthase into nucleus tractus solitarii induces biphasic response in cardiovascular functions of hypertensive rats. *Am J Hypertens* 17: 63-70, 2004.
145. Tallarida G, Baldoni F, Peruzzi G, Brindisi F, Raimondi G, and Sangiorgi M. Cardiovascular and respiratory chemoreflexes from the hindlimb sensory receptors evoked by intra-arterial injection of bradykinin and other chemical agents in the rabbit. *J Pharmacol Exp Ther* 208: 319-329, 1979.
146. Tallarida G, Peruzzi G, and Raimondi G. The role of chemosensitive muscle receptors in cardiorespiratory regulation during exercise. *J Auton Nerv Syst* 30: S155-S161, 1990.
147. Talman WT and Dragon DN. Transmission of arterial baroreflex signals depends on neuronal nitric oxide synthase. *Hypertension* 43: 820-824, 2004.
148. Toney GM and Mifflin SW. Time-dependent inhibition of hindlimb somatic afferent inputs to nucleus tractus solitarius. *J Neurophysiol* 72: 63-71, 1994.
149. Toney GM and Mifflin SW. Time-dependent inhibition of hindlimb somatic afferent transmission within nucleus tractus solitarius: an *in vivo* intracellular recording study. *Neuroscience* 68: 445-453, 1995.
150. Tseng C-J, Liu H-Y, Lin H-C, Ger L-P, Tung C-S, and Yen M-H. Cardiovascular effects of nitric oxide in the brain stem nuclei of rats. *Hypertension* 27: 36-42, 1996.
151. Veras-Silva AS, Mattos KC, Gava NS, Brum PC, Negrao CE, and Krieger EM. Low-intensity exercise training decreases cardiac output and hypertension in spontaneously hypertensive rats. *Am J Physiol* 273: H2627-H2631, 1997.

152. Victor RG, Bertocci LA, Pryor SL, and Nunnally RG. Sympathetic nerve discharge is coupled to muscle cell pH during exercise in humans. *J Clin Invest* 82: 1301-1305, 1988.
153. Victor RG, Secher NH, Lyson T, and Mitchell JH. Central command increases muscle sympathetic nerve activity during intense isometric exercise in humans. *Circ Res* 76: 127-131, 1995.
154. Vitagliano S, Berrino L, D'Amico M, Maione S, Vovellis VD, and Rossi F. Involvement of nitric oxide in cardiorespiratory regulation in the nucleus tractus solitarius. *Neuropharmacology* 35: 625-631, 1996.
155. Waki H, Gouraaud SS, Maeda M, and Paton JF. Specific inflammatory condition in nucleus tractus solitarii of the SHR: movel insight for neurogenic hypertension? *Autonomic Neuroscience: Basic and Clinical* 142: 25-31, 2008.
156. Waki H, Murphy D, Yao ST, Kasparov S, and Paton JFR. Endothelial NO synthase activity in nucleus tractus solitarii contributes to hypertension in spontaneously hypertensive rats. *Hypertension* 48: 644-650, 2006.
157. Waldrop TG, Eldridge FL, Iwamoto GA, and Mitchell JH. Central neural control of respiration and circulation during exercise. In: *Handbook of Physiology. Exercise: Regulation and Integration of Multiple Systems.* Bethesda: Am. Physiol. Society, 1996.
158. Waldrop TG, Henderson MC, Iwamoto GA, and Mitchell JH. Regional blood flow responses to stimulation of the subthalamic locomotor region. *Resp Physiol* 64: 93-102, 1986.
159. Waldrop TG and Mitchell JH. Effects of barodenervation on cardiovascular responses to muscle contraction. *Am J Physiol* 249: H710-H714, 1985.
160. Williamson JW, McColl R, Mathews D, Mitchell JH, Raven PB, and Morgan WP. Brain activation by central command during actual and imagined handgrip under hypnosis. *J App Physiol* 92: 1317-1324, 2001.
161. Williamson JW, McColl R, Mathews D, Mitchell JH, Raven PB, and Morgan WP. Hypnotic manipulation of effort sense during dynamic exercise: cardiovascular responses and brain activation. *J Appl Physiol* 90: 1392-1399, 2001.
162. Williamson JW, Mitchell JH, Olesen HL, Raven PB, and Secher NH. Reflex increases in blood pressure induced by leg compression in man. *J Physiol* 475: 351-357, 1994.
163. Wu WC, Wang Y, Kao LS, Tang FI, and Chai CY. Nitric oxide reduces blood pressure in the nucleus tractus solitarius: a real time electrochemical study. *Brain Res Bull* 57: 171-177, 2002.
164. Yoshihara F, Nishikimi T, Yoshitomi Y, Nakasone I, Abe H, Matsuoka H, and Omae T. Left ventricular structural and functional characteristics in patients with renovascular hypertension, primary aldosteronism and essential hypertension. *Am J Hypertens* 9: 523-528, 1996.
165. Zanzinger J, Czachurski J, and Seller H. Inhibition of basal and reflex-mediated sympathetic activity in the RVLM by nitric oxide. *Am J Physiol Reg Int Comp Physiol* 268: R958-R962, 1995.

166. Zuntz N and Geppert J. Ueber die natur der normalen atemreize und den ort ihrer wirkung. *Arch Gen Physiol* 38: 337-338, 1886.

Figures

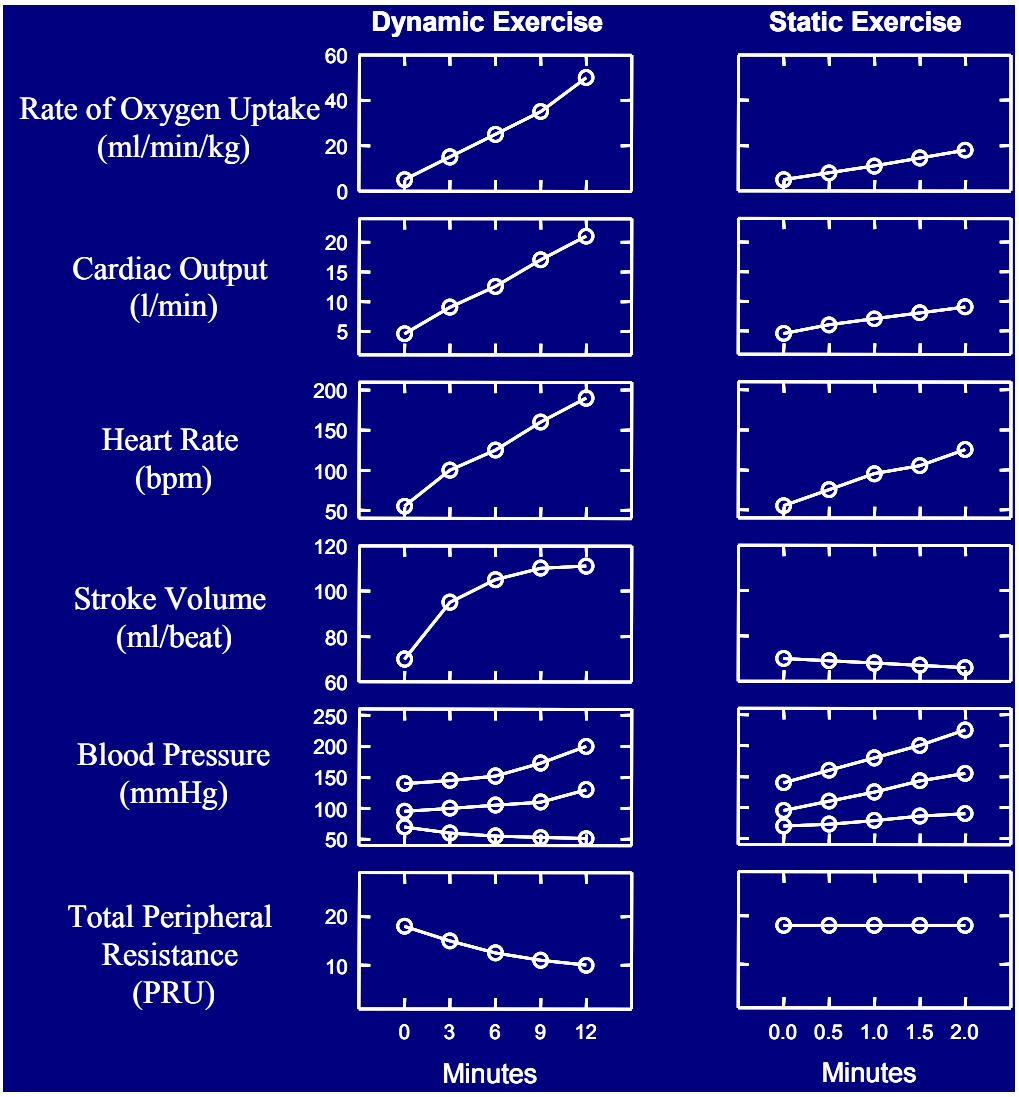

Figure 1: The Circulatory Response to Exercise. During dynamic exercise, cardiac output (stroke volume X heart rate) rises due to increases in both stroke volume and heart rate. Total peripheral resistance decreases to allow blood to oxygenate the working muscles. These changes cause mean blood pressure (cardiac output X total peripheral resistance) to increase minimally. In contrast, during static exercise, cardiac output increases only slightly due to an increase in heart rate. Stroke volume decreases or stays constant while total peripheral resistance stays the same as the working muscle compresses blood vessels and sympathetic nerve activity increases to cause vasoconstriction. All of this causes a steady increase in mean blood pressure over a period of time, predominantly due to the increase in cardiac output. Source: *J Am Coll Cardiol*. 24: 864-866, 1994.

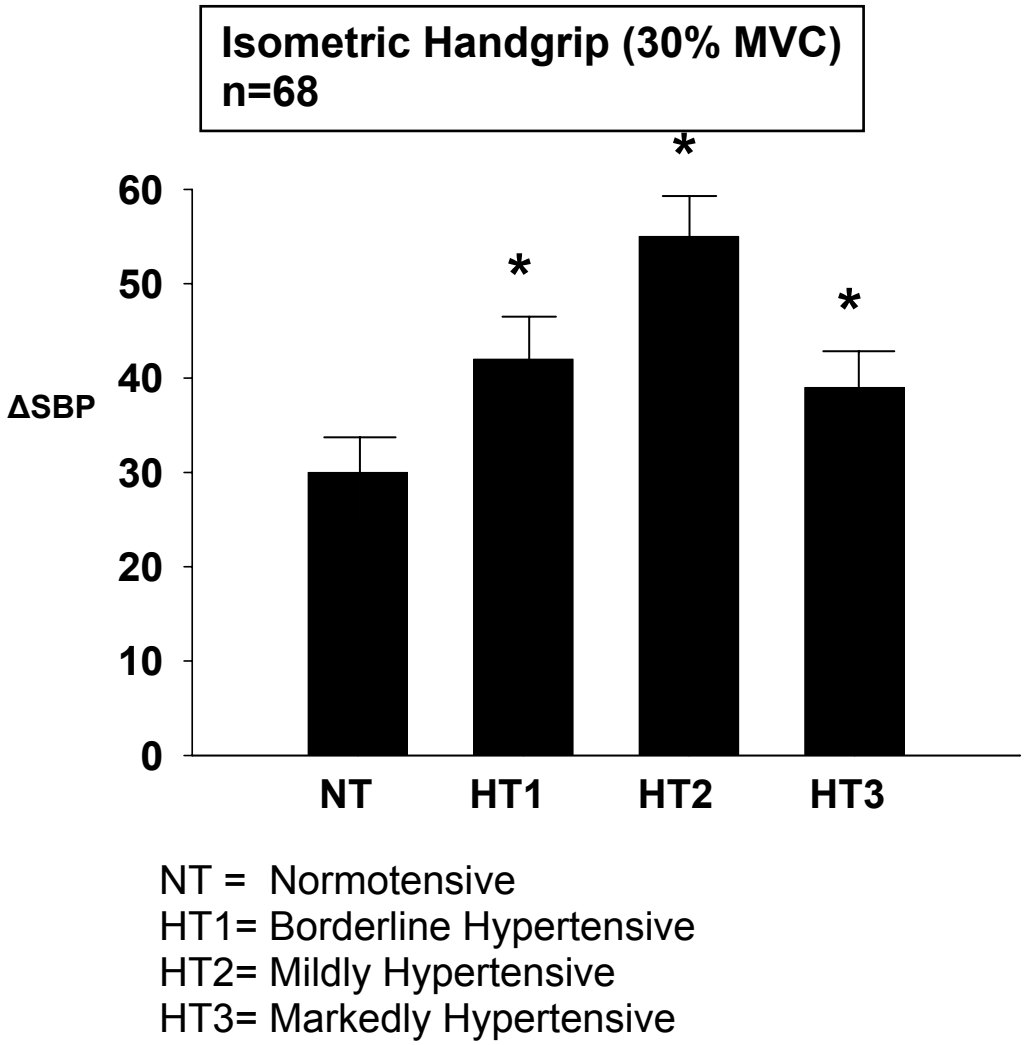

Figure 2: The Pressor Response to Static Exercise is Exaggerated in Hypertension. Borderline hypertensive (HT1), mildly hypertensive (HT2), and markedly hypertensive (HT3) men performing isometric handgrip for 3 minutes at 30% maximal voluntary contraction (MVC) experienced increases in systolic blood pressure (SBP) that were significantly greater than the normotensive (NT) controls. *Significance from NT. ($P<0.05$). Source: *Jpn Circ J* 47: 802-809, 1983.

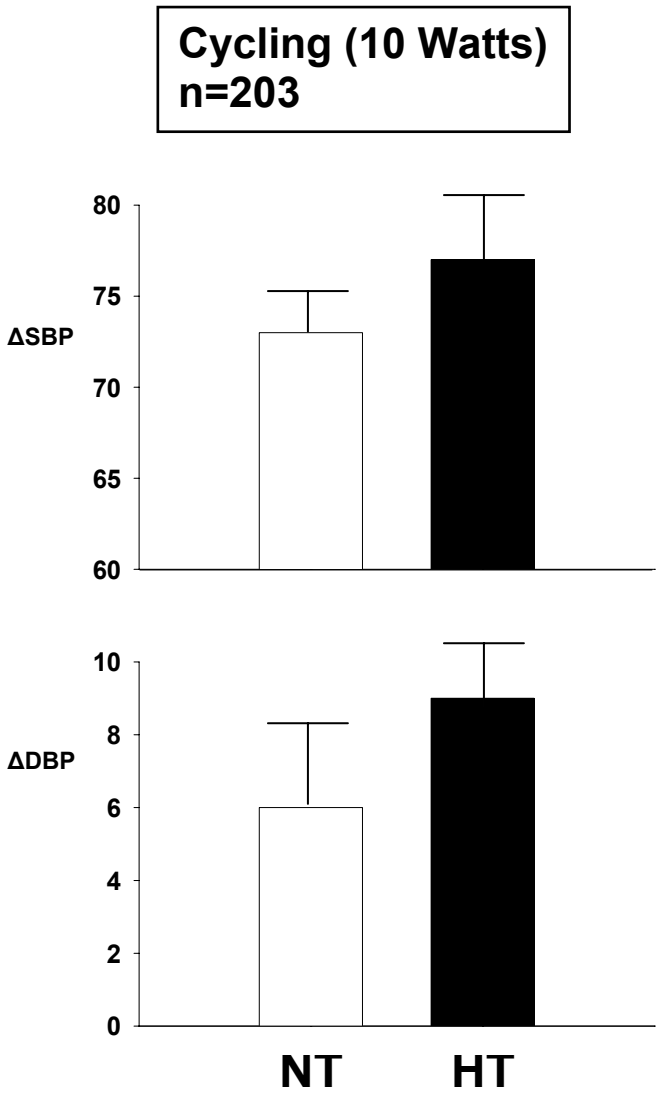

Figure 3: The Pressor Response to Dynamic Exercise is Exaggerated in Hypertension. Hypertensive (HT) individuals who performed dynamic exercise, steady state cycling at 10 Watts, experienced larger increases in both systolic blood pressure (SBP) and diastolic blood pressure (DBP) than normotensive (NT) controls. Source: *Cardiologia* 36: 867-877, 1991.

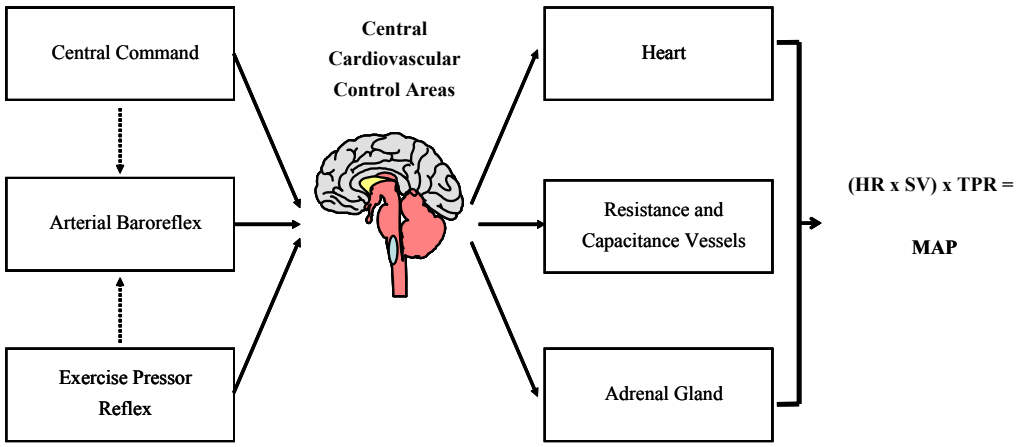

Figure 4: The Neural Inputs that Control the Cardiovascular Response to Exercise. The cardiovascular adjustments to exercise are mediated by three inputs: central command, the arterial baroreflex, and the exercise pressor reflex. These three mechanisms are integrative and they direct changes in the heart, resistance and capacitance vessels, and the adrenal gland. The combination of these effects is to alter heart rate (HR), stroke volume (SV), and total peripheral resistance (TPR) in order to control mean arterial pressure (MAP).

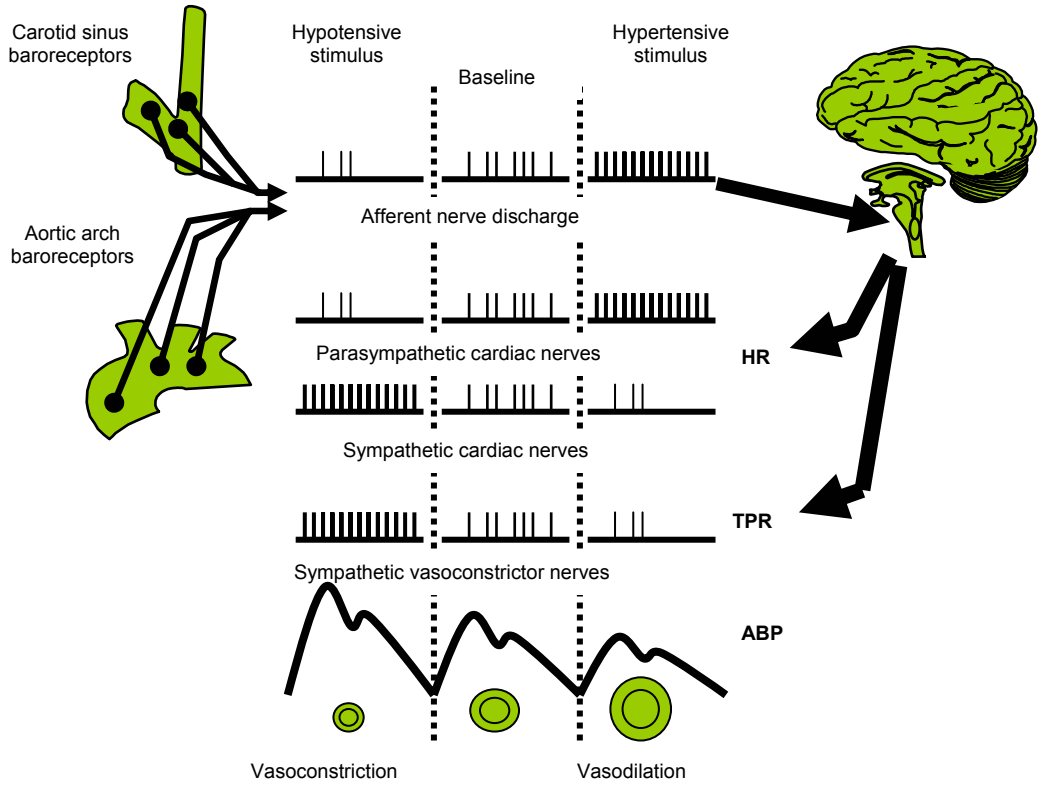

Figure 5: The Baroreflex Response to Pressure Stimuli. During a hypotensive stimulus, baroreceptors in the carotid sinus and aortic arch decrease their afferent nerve discharge in comparison to basal levels. Subsequently, this causes a decrease in parasympathetic nerve activity to the heart and an increase in sympathetic nerve activity to the heart and vasculature. Heart rate (HR) increases and vasoconstriction leads to an increase in total peripheral resistance (TPR), causing a rise in arterial blood pressure (ABP). In response to a hypertensive stimulus, afferent nerve discharge from the baroreceptors increases compared to baseline. This causes an increase in parasympathetic nerve activity to the heart and decreases in sympathetic nerve activity to the heart and vasculature. This combination of efferent activity decreases HR and TPR in order to vasodilate blood vessels and normalize ABP.

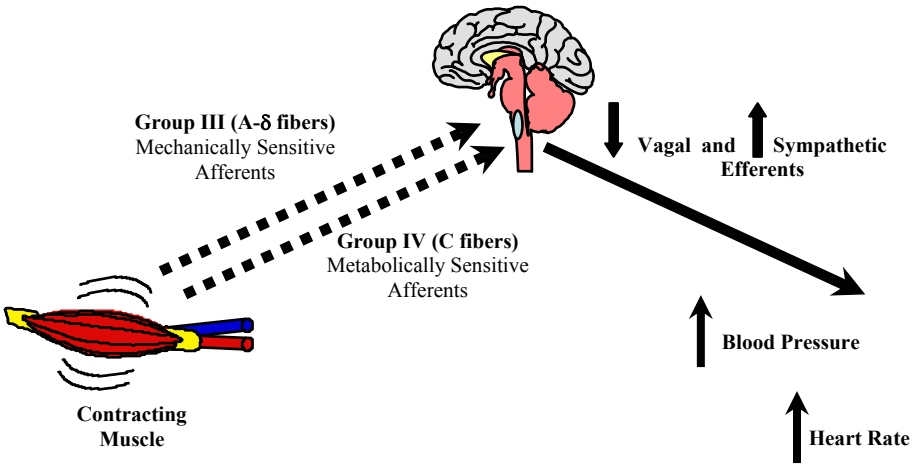

Figure 6: The Exercise Pressor Reflex and its Circulatory Effects. The exercise pressor reflex is a feed-back reflex originating in skeletal muscle that consists of two afferent arms. The mechanoreflex is composed of group III fibers that are mostly mechanically sensitive and the metaboreflex is composed of unmyelinated group IV fibers that are predominantly metabolically sensitive. Mechanoreflex and metaboreflex afferents synapse within the spinal cord and then project to the brainstem. From here, efferents project to the heart and vasculature where a decrease in parasympathetic nerve activity and an increase in sympathetic nerve activity causes blood pressure and heart rate to rise in response to exercise.

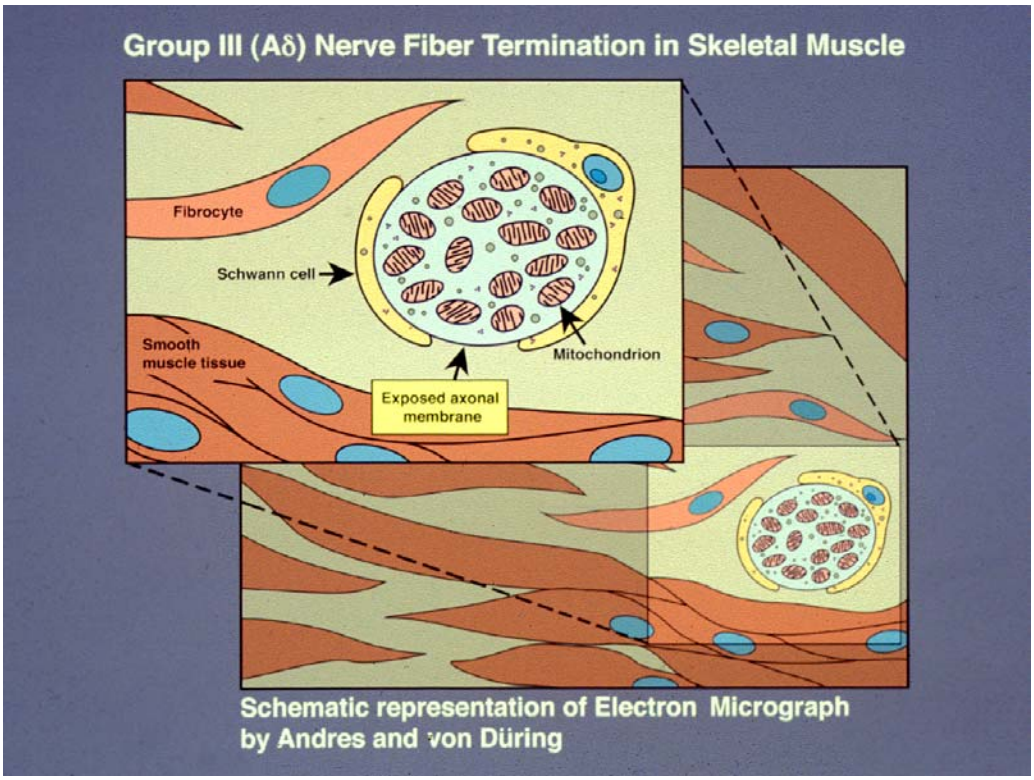

Figure 7: Schematic of Group III Afferent Nerve Termination Site. Mechanically sensitive receptors mediating the muscle mechanoreflex are anatomically located on myelinated group III small-diameter afferent fibers that terminate in collagen tissue between skeletal fibrocytes.

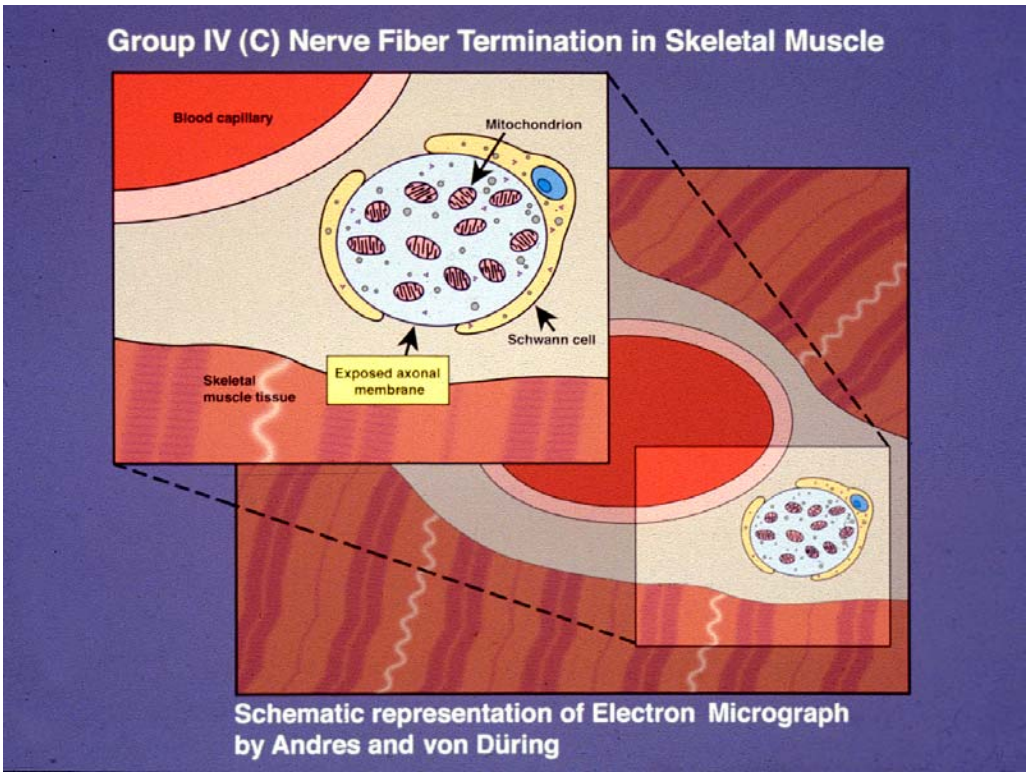

Figure 8: Schematic of Group IV Afferent Nerve Termination Site. Metabolically sensitive receptors mediating the muscle metaboreflex are anatomically located on unmyelinated group IV small-diameter afferent fibers that terminate in the walls of capillaries and venules within skeletal muscle. They are positioned to detect oxygen mismatches between blood and the surrounding muscle tissue.

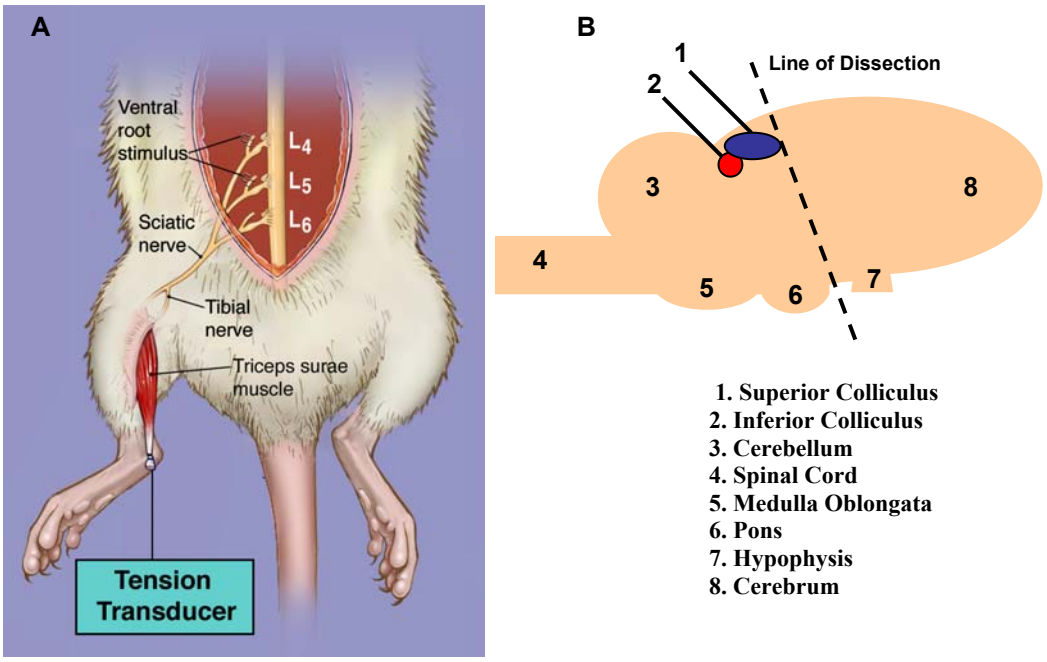

Figure 9: Rat Model of Exercise. (A) To isolate and study the exercise pressor reflex, a laminectomy is performed and ventral roots L_4 through L_6 are isolated and sectioned. Then ventral roots L_4 and L_5 are placed on bipolar electrodes. Electrical stimulation of these two ventral roots causes contraction of the triceps surae muscles and activates both components of the exercise pressor reflex. A tension transducer is attached to the Achilles' tendon for tension measurement of the hindlimb. **(B)** All animals undergo pre-collicular decerebration, which eliminates central command input, and anesthesia is discontinued prior to experimentation. The line of dissection is rostral to the superior colliculus and pons.

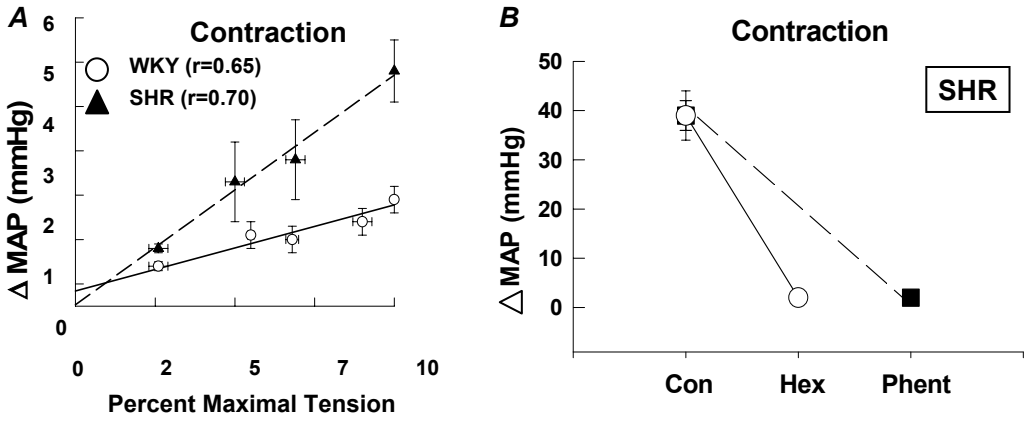

Figure 10: The Pressor Response to Contraction is Exaggerated in Hypertensive Rats. (A) Data from our lab shows the mean arterial pressure (MAP) response to electrically-induced static contraction is significantly greater in SHR (n=9) rats compared to normotensive WKY (n=10) rats at maximal and submaximal work intensities. The slope of the relationship is significantly greater in SHR, showing the exercise pressor reflex to be more sensitive in hypertension. **(B)** This graph shows changes in MAP in response to hindlimb contraction before (Con) and after systemic infusion of the ganglionic blocker hexamethonium (Hex) and sympathetic blocker phentolamine (Phent) in SHR rats. The pressor responses to contraction were almost completely abolished, therefore the changes in MAP in response to exercise pressor reflex activation were sympathetically-mediated. Source: *J Physiol* 577: 1009-1020, 2006.

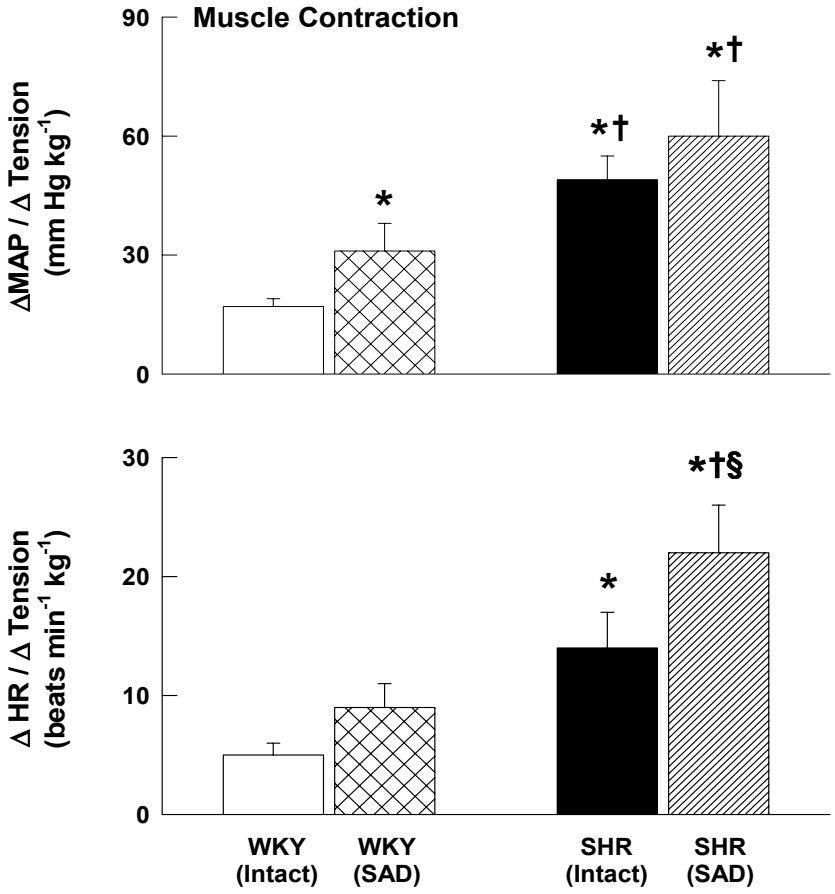

Figure 11: The Exaggerated Response to Exercise in SHR is Primarily Attributed to an Overactive Exercise Pressor Reflex. Exercise pressor reflex function was tested during muscle contraction in baro-intact (Intact) and barodenervated (SAD) SHR and WKY animals. While the changes in mean arterial pressure (MAP) and heart rate (HR) were significantly increased in SAD WKY rats, these circulatory changes were significantly less than those produced in Intact SHR rats. Additionally, the increases in MAP and HR occurring in SHR animals in response to contraction were further enhanced when the animals were barodenervated, suggesting that the baroreflex maintains the ability to buffer changes in blood pressure in hypertension. *P<0.05 compared to Intact WKY. †P<0.05 compared to SAD WKY. §P<0.05 compared to Intact SHR. Source: *J Physiol* 577: 1009-1020, 2006.

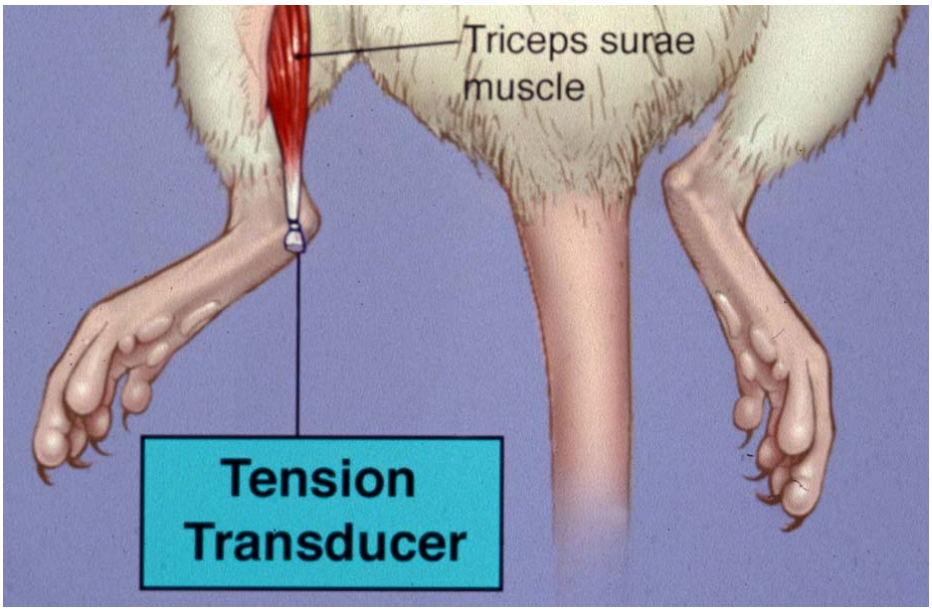

Figure 12: Surgical Preparation Used to Activate the Mechanoreflex. To preferentially activate the mechanoreflex, a rack and pinion system was attached to the Achilles' tendon to passively stretch the triceps surae muscles.

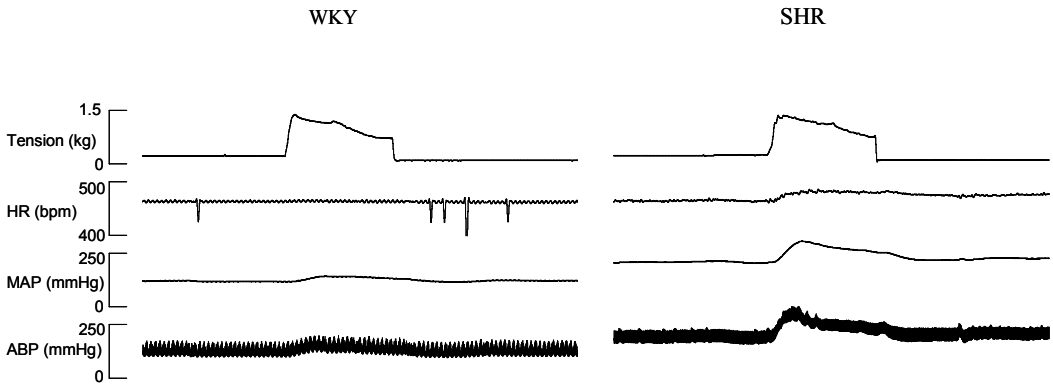

Figure 13: Characteristic Cardiovascular Response to Passive Stretch in Representative WKY and SHR Rats. In response to passive hindlimb muscle stretch of the same tension, mean arterial pressure (MAP) and heart rate (HR) were consistently larger in hypertensive compared to normotensive animals. Source: *Am J Physiol,* 295: H1429-H1438, 2008.

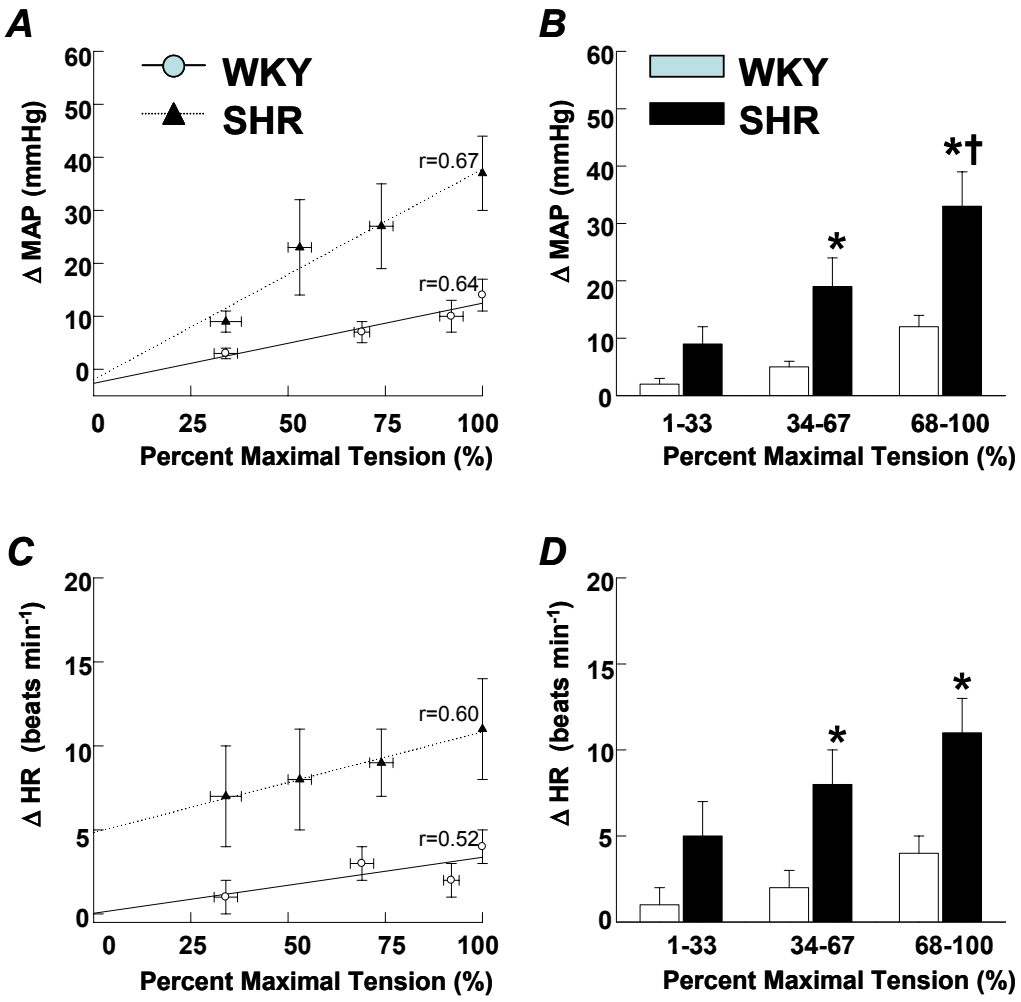

Figure 14: The Circulatory Response to Mechanoreflex Activation is Enhanced in Hypertensive Rats. (A & B) Preferentially activating the muscle mechanoreflex through passive muscle stretch produced significantly greater increases in mean arterial pressure (MAP) in SHR rats compared to WKY over a range of stretch intensities. While pressor responses for SHR and WKY were positively correlated to tension development ($P<0.01$), in graph A, the steeper slope for the SHR animals provides evidence that the mechanoreflex is more sensitive in hypertension. **(C & D)** Increases in heart rate (HR) were significantly greater in SHR compared to WKY rats over a range of developed hindlimb tensions. Linear regression analysis showed the changes in HR in response to passive stretch were positively correlated to the stretch intensities in both WKY and SHR rats ($P<0.01$). All data points within a group were used to determine the correlation coefficient (r) and regression slope. *$P<0.05$ compared with WKY. †$P<0.05$ compared with all preceding lower levels of tension development. Source: *Am J Physiol,* 295: H1429-H1438, 2008.

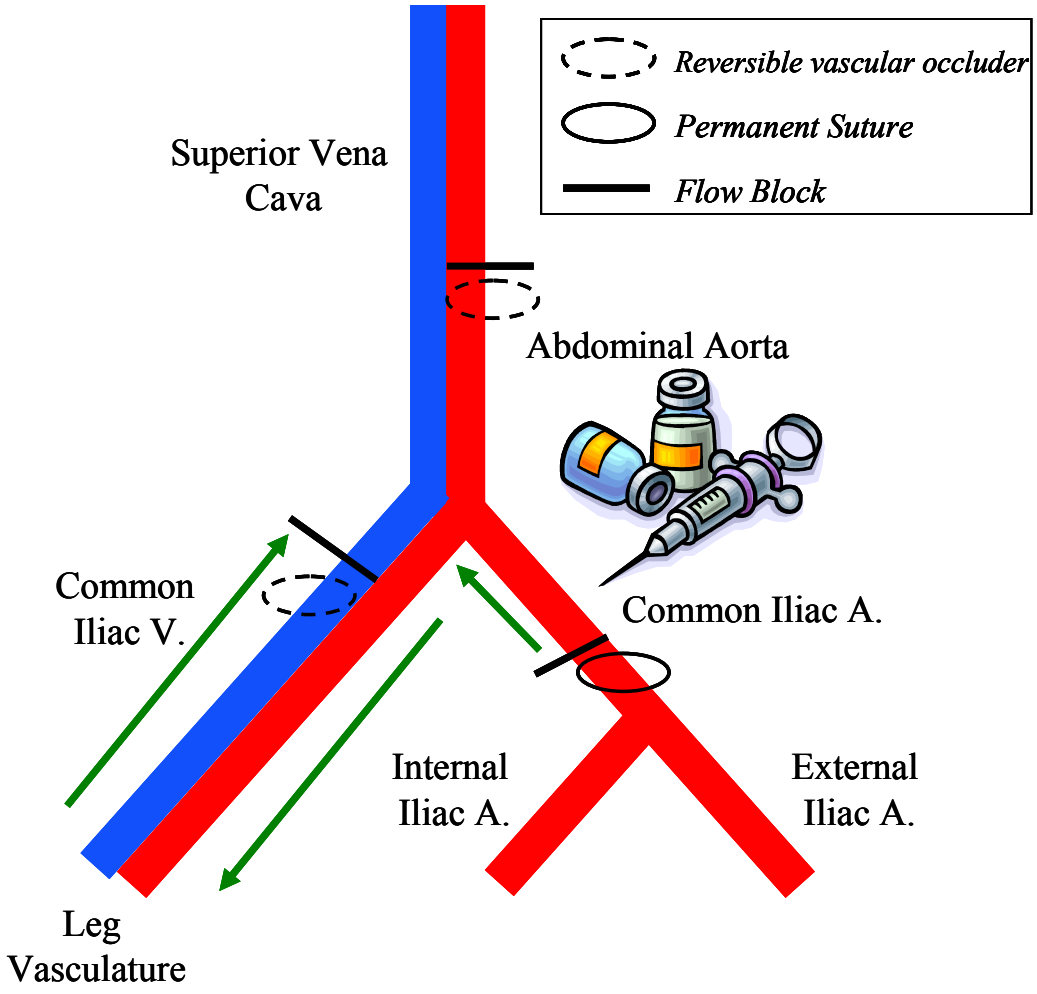

Figure 15: Surgical Preparation Used to Activate the Metaboreflex. This schematic illustrates the surgical preparation used to selectively activate group IV afferent neurons. First, the left common iliac artery is cannulated with the tip advanced to the bifurcation of the abdominal aorta in order to inject drugs into the right hindlimb. A reversible ligature is tied around the right common iliac vein and this allows the injected drug to be trapped within the right hindlimb arterial supply.

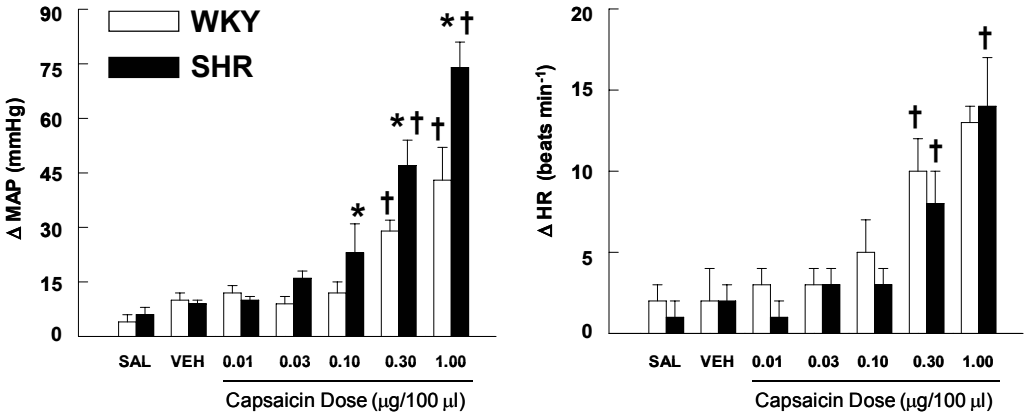

Figure 16: The Pressor Response to Metaboreflex Activation is Accentuated in Hypertensive Rats. When the metaboreflex was preferentially activated by intra-arterial capsaicin injections of varying doses, there were dose-related increases in mean arterial pressure (MAP) and heart rate (HR) in both SHR and WKY rats. The pressor responses in SHR rats were significantly greater than those of WKY rats, however the HR responses were more variable with no significant difference between the two groups. *P<0.05 compared with WKY. †P<0.05 compared with all preceding lower doses of capsaicin. §P<0.05 compared with all doses of capsaicin of <0.30 μg/100 μl. Source: *Am J Physiol,* 295: H1429-H1438, 2008.

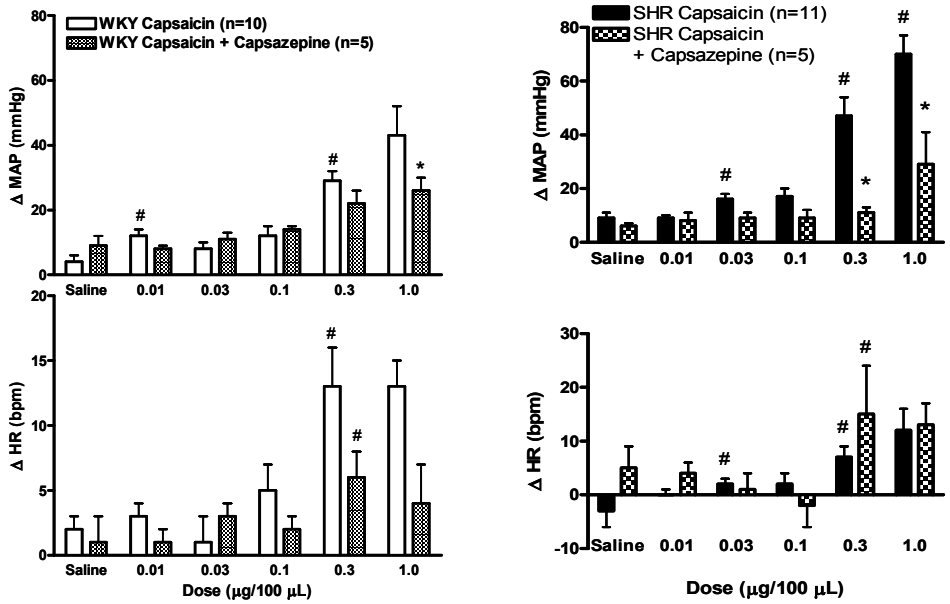

Figure 17: Capsaicin Increases Mean Arterial Pressure by Activating TRPv1 Receptors in WKY and SHR Rats. These graphs show changes in mean arterial pressure (MAP) and heart rate (HR) in response to graded doses of capsaicin in the absence and presence of capsazepine in WKY and SHR rats. Introduction of capsazepine, a TRPv1 antagonist, into the hindlimb blocked the effect of capsaicin, suggesting that the pressor responses were mediated by activation of the TRPv1 receptor located exclusively on Group IV afferent neurons. *P<0.05 compared with trials in which capsaicin was only injected. #P<0.05 compared with preceding capsaicin or capsaicin plus capsazepine response. Source: *Am J Physiol,* 295: H1429-H1438, 2008.

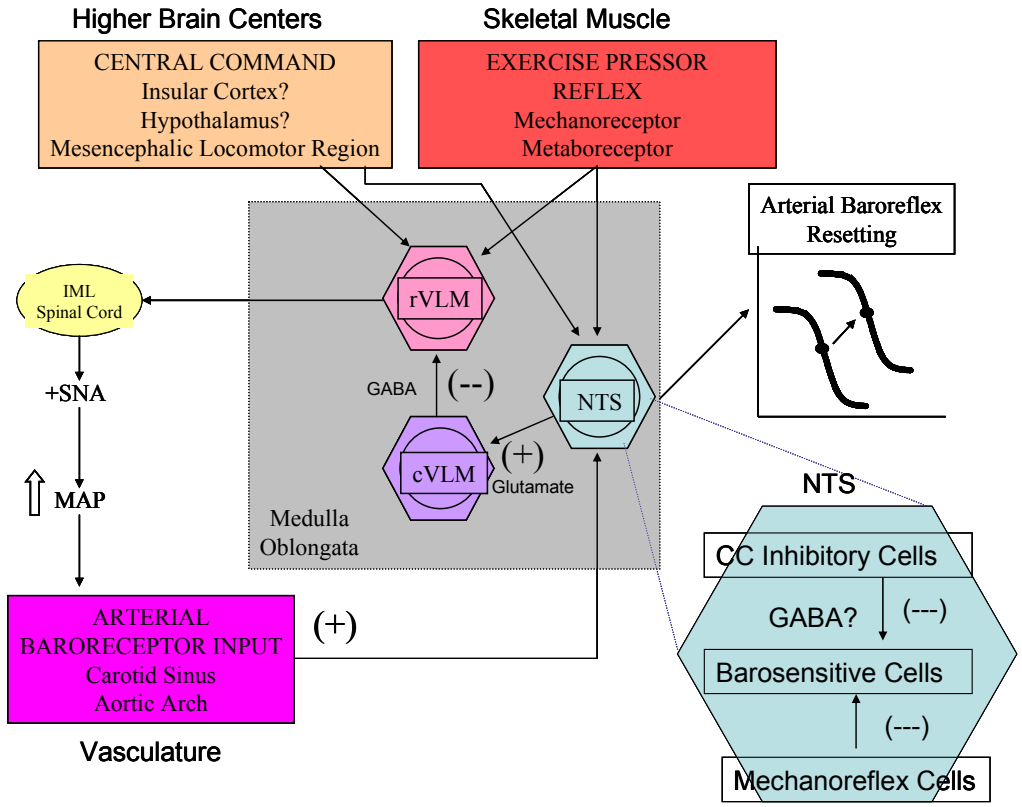

Figure 18: Diagram of Plausible Neural Pathways between Arterial Baroreceptors, Central Command, and the Exercise Pressor Reflex During Exercise. Arterial baroreceptor afferents project to the nucleus tractus solitarius (NTS), where exercise pressor reflex afferents also synapse and excite NTS neurons. The exercise pressor reflex also may alter neuronal function within the rostral ventrolateral medullar (rVLM) to control cardiovascular responses. Evidence suggests that central command (CC) afferent fibers synapse within the rVLM and NTS. It has been proposed that there are distinct CC and group III afferent neuron populations within the NTS. These cells have been shown to inhibit barosensitive neuron populations within the NTS possibly via the neurotransmitter GABA. During exercise, neurons from the NTS excite caudal ventrolateral medulla (cVLM) neurons, which send inhibitory projections to the rVLM. From the rVLM, efferents synapse in the intermediolateral cell columns (IML) of the spinal cord and then travel with sympathetic neurons to raise sympathetic nerve activity (SNA) and mean arterial pressure (MAP). Source: *Recent Res Devel Physiol* 2, 2004.

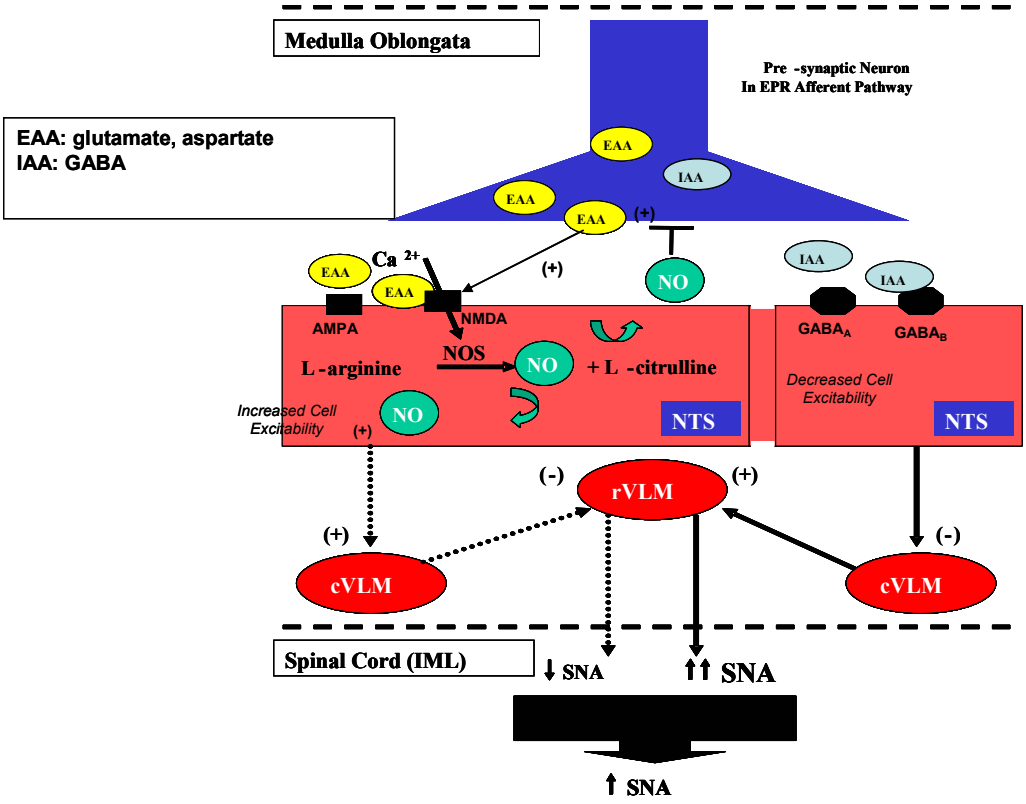

Figure 19: Simplified Illustration of the Synaptic Interactions between Skeletal Muscle Afferent Terminals and NTS Neurons. Activation of exercise pressor reflex afferent neurons induces the release of excitatory (EAA) and inhibitory (IAA) amino acids. One population of NTS neurons inhibited by IAA increases sympathetic nerve activity (SNA). In a separate group of NTS neurons, EAA binding to NMDA receptors induces an influx of Ca^{2+}. Calcium activates nitric oxide synthase (NOS) via calmodulin and nitric oxide (NO) is formed. NO has been reported to have two actions: i) NO is generated postsynaptically and increases NTS cell excitability. As a result, caudal ventrolateral medullary (cVLM) neurons are activated which subsequently inhibit sympathetic motor neurons in the rostral ventrolateral medulla (rVLM); ii) NO diffuses out and acts as a retrograde messenger stimulating the release of EAA from the presynaptic neuron. There is glutamate-mediated NO production and NO-mediated glutamate release in the NTS. This suggests a positive feedback loop; the net result being to decrease SNA. Efferent SNA is determined by the balance between forces favoring increases in SNA and those favoring decreases in SNA. During exercise, the balance is shifted towards an increase in SNA. Intermediolateral cell columns, IML; gamma-aminobutyric acid, GABA.

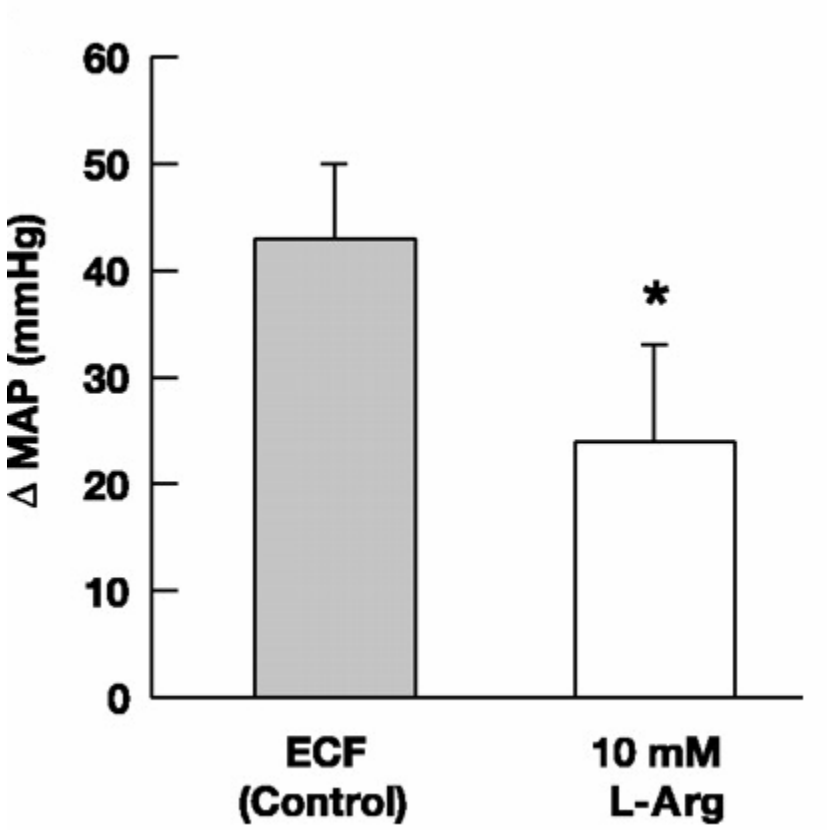

Figure 20: Effects of the NO Precursor, L-arginine, on the Pressor Response to Static Muscle Contraction. Graph shows mean arterial pressure (MAP) response to contraction during the individual dialysis of artificial extracellular fluid (ECF) and L-arginine (L-Arg) into the NTS of decerebrated cats. There was a significant attenuation in the pressor response to contraction when NO was experimentally increased in the NTS. The amount of tension developed during contraction (~4.0 kg) was not different between trials. *P<0.05 compared to control. Source: *Am J Physiol Heart Circ Physiol* 288: 2068-2076, 2005.

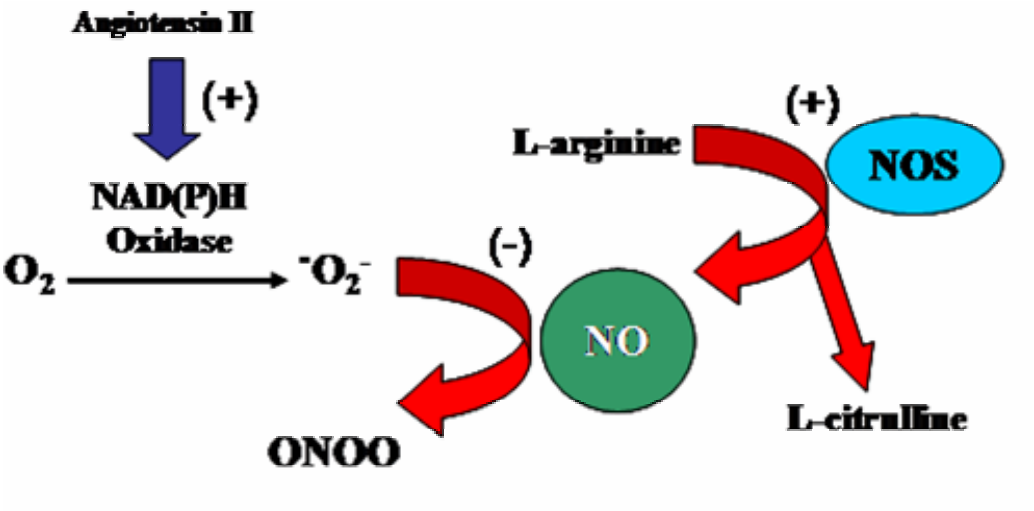

Figure 21: Schematic Illustration of Factors that Affect NO Production and/or Availability in the NTS. NO production is dependent on NOS. Reductions in NOS activity or expression potentially decrease the amount of NO produced in hypertension. Reactive oxygen species, such as superoxide (.O2-), inactivate NO forming peroxynitrite (ONOO). Within the brain, production of superoxide is dependent on NAD(P)H oxidase activity. Evidence suggests that NAD(P)H oxidase activity is increased in hypertension. This increased NAD(P)H oxidase activity is likely driven by Angiotensin II, which is also shown to be increased in hypertension. The increased generation of superoxide via an Angiotensin II/NAD(P)H oxidase mechanism potentially reduces the NO available for biological activity within the NTS.

RESEARCH DESIGN and METHODS

General Procedures

The following experimental techniques were used in age-matched groups of SHR and WKY animals as specified in the specific aim. Rats were anesthetized with isoflurane gas (2-3%) in pure oxygen, intubated, and mechanically respirated (Harvard Apparatus) for the duration of the experiment. Both carotid arteries and a jugular vein were catheterized (PE-50, polyethylene tubing) for pressure transducer readings and administering of fluids, respectively. To maintain fluids and stabilize the animals when necessary, 1 M $NaHCO_3$ was continuously infused intravenously at a rate of 2 mL/hr. In addition, arterial blood gases and pH were measured throughout experimentation using an automated blood gas analyzer (Model ABL 5, Radiometer). Also, 0.15 mg dexamethasone was given intramuscularly in the left hindlimb to minimize edema. Body temperature was maintained between 36.5 and 38.0°C by an isothermal pad (Deltaphase). Animals were held in stereotaxic head units (Kopf Instruments) during pre-collicular decerebration and experimental procedures. Decerebration effectively eliminated input from central command and rendered the animal insentient. Briefly, holes were drilled into the parietal skull and the bone superior to the central sagittal sinus removed. Dura mater was cut away and aspiration of the cerebrum performed. Once the superior and inferior colliculi were within view, a pre-collicular section was made and the transectioned forebrain was aspirated. To minimize bleeding, small pieces of oxidized regenerated cellulose (Ethicon, Johnson & Johnson) were placed on the internal skull surface and the cranial cavity was packed with cotton. Immediately after pre-collicular transection, anesthesia was discontinued and all animals were allowed to stabilize for at least one hour. At the conclusion of testing procedures, animals were humanely euthanized. The heart, lungs, and triceps surae were excised and wet weights obtained. Tibial length was also measured to assess heart mass / tibial length ratios.

Exercise Pressor Reflex Testing

The exercise pressor reflex was activated via ventral root stimulation. Electrically induced static contraction of the triceps surae muscles of the hindlimb via ventral root stimulation was used to activate the exercise pressor reflex at maximal intensities. A laminectomy was performed to expose the spinal cord and the dura was cut away to reveal the lower lumbar roots (L_2-L_6). The ventral and dorsal roots were separated. Then the L_4 and L_5 ventral roots, which stimulate the triceps surae muscles, were sectioned in order to control the efferent neural activity to the right hindlimb. Bipolar platinum electrodes were placed around the cut peripheral ends and all exposed neural tissue was immersed in warm mineral oil. Animals were secured in a customized spinal frame (Kopf Instruments) by clamps placed on rostral lumbar vertebrae. The pelvis was stabilized with steel posts within the frame and the exercising limb was fixed in one position using clamps attached to the tibial bone. Then the calcaneal bone was sectioned and the Achilles' tendon connected to a force transducer (Grass Instruments, FT10) for the measurement of muscle tension. Electrical stimulations were performed with a Grass Instruments S88 stimulator. Using constant current stimulation (1-3 times motor threshold, 0.1 ms pulse duration, 40 Hz), 30 second contractions were produced. Hindlimb contraction activates both the mechanically and metabolically sensitive components of the exercise pressor reflex. This testing procedure ensures that all changes in cardiovascular parameters are the result of reflex activation and not from direct electrical stimulation of the hindlimb muscle afferents.

Simulated Mechanoreflex Activation

The mechanoreflex was selectively activated by passively stretching the triceps surae muscles of the hindlimb. This technique is a valid procedure to manipulate the muscle mechanoreflex because no metabolites are produced by the muscle. Using electrical stimulation of spinal ventral roots, the triceps surae muscles of the hindlimb were contracted at maximal intensities to establish peak

levels of tension development. Using a calibrated 9.5 mm rack and pinion system (Harvard Apparatus), preferential activation of mechanically sensitive afferent fibers was induced by passively stretching the triceps surae muscles of the hindlimb for 30 seconds. The amount of developed hindlimb muscle tension during passive stretch was matched to that produced during maximal muscle contraction (roughly 1200 g). This maneuver allowed the mechanoreflex to be preferentially activated over a range of work intensities, if necessary.

Simulated Metaboreflex Activation

The metaboreflex was preferentially activated by injecting a known concentration (0.3 μg/100 μL) of capsaicin into the hindlimb arterial supply. As previously mentioned, capsaicin is known to bind to TRPv1 receptors which are a selective marker of group IV afferent fibers(1). To verify that the cardiovascular response to capsaicin was mediated by stimulation of the TRPv1 receptor, capsaicin injections were given with and without the competitive TRPv1 antagonist capsazepine (100 μg/100 μL). In order to administer capsaicin and its antagonist to chemically-sensitive muscle receptors on group IV afferent within skeletal muscle, the circulation of the right hindlimb was isolated. A catheter (PE-10, polyethylene tubing) was placed in the left common iliac artery with its tip advanced to the abdominal aorta. To limit drug delivery to the right hindlimb, a reversible vascular occluder was placed around the common iliac vein emptying the right hindlimb. Drugs were then injected directly into the arterial supply of the right hindlimb via the right common iliac artery. Selective activation of metabolically-sensitive muscle receptors using pharmacological agents is a valid technique to manipulate the muscle metaboreflex. Additionally, previous findings from our laboratory have shown that the pressor response elicited by capsaicin injections is caused by activation of metabolically sensitive afferent fibers and is not a pain response, as blood pressure rises when capsaicin is injected into a skinned leg's arterial supply.

In all physiologic experiments, baseline as well as reflex changes in mean arterial pressure, heart rate, and developed tension were recorded. Baseline values for each measured variable were obtained over a 30 second period prior to reflex activation. The greatest change in each variable from this baseline in response to reflex activation was taken as the peak value. A 15 minute recovery period between each maneuver was employed.

Brainstem Microdialysis

Animals were held in a stereotaxic head unit (Kopf Instruments). A limited occipital craniotomy was performed to expose the dorsal surface of the brainstem and microdialysis probes (Bioanalytical Systems, model CMA 11, 0.24 mm outer diameter, 1 mm membrane tip) were stereotaxically positioned unilaterally within the NTS at an area known to receive projections from exercise pressor reflex afferent fibers (coordinates: 0.5 mm lateral to the obex and 0.5 mm below the dorsal medullary surface)(2, 3). Probes were continuously perfused at a rate of 2.5 µl/min with either artificial cerebral spinal fluid (0.2 % bovine serum albumin, 0.1 % bacitracin, and the following ions (in mM): 6.2 K^+, 134 Cl^-, 2.4 Ca^{2+}, 150 Na^+, 1.3 P^-, 13 HCO_3^- and 1.3 Mg^{2+}) buffered to a pH of 7.4 (control) or an experimental substance (i.e. L-arginine, D-arginine, L-NAME, or D-NAME). To verify probe placement, Evans blue dye was dialyzed into the NTS at the conclusion of experimentation. Then the brainstem was excised and fixed in 10 % phosphate buffered formalin and stored at 4^0C. Medullary tissue was blocked, and 40 µm sections were cut serially using a cryostat (Cambridge Instruments). Sections were placed on coated slides and examined to establish the neuroanatomical location of probe placement. The perfusion area of the probe was verified by the distribution of the dye.

In microdialysis experiments, two-point dose response curves were constructed for each drug used. In addition, each concentration of drug was dialyzed for a minimum of 45 minutes prior to reflex testing. As a control, reflex testing was also

assessed during the dialysis of artificial cerebrospinal fluid before and after dialysis of the drug of interest.

Statistical Analysis

In physiological experiments, all cardiovascular and contractile force data were acquired, recorded, and analyzed using data acquisition software (Spike 2, version 3, Cambridge Electronic Design, Ltd) for the CED micro 1401 system (Cambridge Electronic Design Ltd). Data was analyzed by means of paired and unpaired t-tests or analysis of variance (ANOVA) with Student Newman-Keuls multiple comparison tests employed as appropriate. The significance level was set at $P<0.05$. All statistical analysis was performed using Sigma Stat for Windows (SPSS Inc.)

References

1. Kaufman MP, Iwamoto GA, Longhurst JC, and Mitchell JH. Effects of capsaicin and bradykinin on afferent fibers with endings in skeletal muscle. *Circ Res* 50: 133-139, 1982.
2. Li J. Nitric oxide synthase (NOS) coexists with activated neurons by skeletal muscle contraction in the brainstem of cats. *Life Sciences* 71: 2833-2843, 2002.
3. Paxinos G and Watson C. *The Rat Brain in Stereotaxic Coordinates*. Orlando, FL: Academic, 1986.

A ROLE FOR NITRIC OXIDE WITHIN THE NUCLEUS TRACTUS SOLITARIUS IN THE DEVELOPMENT OF MUSCLE MECHANOREFLEX DYSFUNCTION IN HYPERTENSION

Running Title – Brainstem NO in MMcR dysfunction in hypertension

Anna K. Leal, MS[1]; Jere H. Mitchell, MD[2] and Scott A. Smith, PhD[3]

Departments of Biomedical Engineering[1], Internal Medicine[2] and Physical Therapy[3]

University of Texas Southwestern Medical Center

Dallas, Texas, USA 75390-9174

Corresponding Author:

Anna K. Leal, MS
University of Texas Southwestern Medical Center
Southwestern Allied Health Sciences School
Department of Biomedical Engineering
5323 Harry Hines Boulevard
Dallas, Texas 75390-9174
214-648-9188 (office)
214-648-3566 (facsimile)
anna.leal@utsouthwestern.edu

Abstract

The muscle mechanoreflex (MMcR), a mechanism originating in skeletal muscle that increases mean arterial pressure (MAP) and heart rate (HR) when mechanically sensitive afferents are activated is exaggerated in hypertension. MMcR afferent fibers project to the nucleus tractus solitarius (NTS) in the brainstem. The enzymatic activity of nitric oxide synthase (NOS) is responsible for the production of nitric oxide (NO), which has been shown to modulate cardiovascular reflex-driven responses. Therefore, we hypothesized that MMcR dysfunction in hypertension is mediated by a decrease in NO production/availability in the NTS. To test our hypothesis, we microdialyzed L-NAME, a NOS inhibitor, into the NTS of normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive (SHR) rats to block endogenous NO production while stimulating mechanically sensitive afferent fibers in muscle. We found that blocking NO production within the NTS in normotensive rats recapitulates the exaggerated cardiovascular response elicited by MMcR activation in hypertension. In addition, blocking NO production within the NTS in SHR animals further increases the enhanced cardiovascular response to passive hindlimb muscle stretch. Finally, the MMcR elicits an exaggerated circulatory response to exercise in hypertension via the sympathetic nervous system. These findings provide evidence that NO production/availability within the brainstem contributes to MMcR dysfunction in hypertension. Future utilization of this research could allow hypertensive individuals to engage in physical activity without the associated hemodynamic risks.

Key Words: blood pressure, heart rate, exercise

Introduction

Hypertensive individuals experience exaggerated increases in mean arterial pressure (MAP), heart rate (HR), and systemic resistance during physical activity(3, 38). This augmented circulatory response is potentially dangerous and may increase the risk for stroke, arrhythmias, and myocardial infarction during exercise(11, 14, 29). Additionally, these elevations in MAP and HR have been shown to occur during both static and dynamic exercise(3, 38). Thus it is important to understand the mechanism responsible for this cardiovascular hyperexcitability in hypertension.

To this end, data from our laboratory has shown that the exercise pressor reflex (EPR) partially contributes to the exaggerated cardiovascular response to exercise over a range of work intensities(42). The EPR is a neural drive originating in skeletal muscle that elicits an increase in MAP, HR, and ventilation when skeletal muscle contracts by increasing sympathetic nerve activity and withdrawing parasympathetic nerve activity(2, 15, 24, 25). EPR activation is mediated by two afferent components: the mechanoreflex and the metaboreflex(16, 17). The metaboreflex is thought to signal a mismatch between oxygen supply and demand to the working muscle. It is stimulated when by-products of skeletal muscle metabolism activate predominantly unmyelinated group IV afferent fibers, which are primarily chemically sensitive (23)Kaufman, 1982 #74}. The other component of the EPR, the muscle mechanoreflex (MMcR), is activated at the onset of muscle contraction in response to mechanical stimuli of skeletal muscle such as pressure and stretch(43, 50). Stimulation of the MMcR is mediated by stretch-sensitive receptors predominantly located on thinly-myelinated group III afferent neurons terminating in collagen tissue between skeletal fibrocytes(23)Kaufman, 1983 #30; Andres, 1985 #272}. Recent evidence from our laboratory suggests that the abnormal EPR control of MAP and HR in hypertension is partially mediated by an overactive MMcR(19). It is important that the underlying mechanisms of MMcR dysfunction are understood so that hypertensive individuals may enjoy the benefits of physical activity without the associated hemodynamic risks.

In the brainstem, MMcR afferent neurons synapse within the nucleus tractus solitarius (NTS) of the medulla oblongata(32). Functional, electrophysiological and neuroanatomical evidence suggests that the NTS is a major sensory nucleus and a central site of cardiovascular regulation during adaptive behaviors such as exercise(28, 47, 48). Specifically, electrophysiological studies have shown activation of MMcR afferent fibers modulates neuronal activity within the NTS(12, 13). Because the NTS has been found to play a significant role in the cardiovascular response to exercise and MMcR processing, we chose to focus on this nucleus as a potential site for MMcR dysfunction in hypertension.

While many neurotransmitters and neuromodulators within the NTS may be involved in processing EPR input, as well as its MMcR component, current research has established a regulatory role for nitric oxide (NO). Further, accumulating evidence suggests that the NO pathway within the NTS is altered in hypertension(22, 40).Within the NTS, L-arginine is oxidized by nitric oxide synthase (NOS) to produce NO and L-citrulline(26). This centrally-produced NO has been shown to tonically inhibit sympathetic outflow from the medulla as well as modulate reflex-driven hemodynamic responses(36, 51). Studies from our laboratory have shown that sympathetically mediated-increases MAP caused by activation of the EPR are attenuated when NO production is experimentally increased in the NTS of normotensive cats(40). These experiments provided evidence that NO contributes to EPR regulation in the central nervous system.

Given the role of NO within the NTS in EPR sensory processing, impairment in the L-arginine-NO pathway in the NTS is a plausible candidate for the development of the MMcR dysfunction seen in hypertension(22). The purpose of this investigation was, therefore, to determine the contribution of NO within the NTS to MMcR dysfunction in hypertension. Given the role of NO within the brainstem in controlling sympathetic outflow as well as the cardiovascular response to exercise, we hypothesize that MMcR dysfunction in hypertension is mediated by a decrease in NO production/availability in the NTS. To test this hypothesis, we performed microdialysis in the NTS of normotensive Wistar-

Kyoto (WKY) rats and spontaneously hypertensive (SHR) rats to block endogenous NO production while stimulating mechanically sensitive afferent fibers in skeletal muscle. Determining the central mechanisms that contribute to MMcR dysfunction in hypertension will provide invaluable insight and may lead the development of treatment options that could allow hypertensive individuals to safely participate in a variety of physical activities.

Materials and Methods

Subjects

Experiments were performed in 20 SHR and 25 WKY age-matched (14-20 week old) male rats (Harlan, Indianapolis, IN). Animals were housed in standard rodent cages on 12-h light-dark cycles and were given food and water *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas at Southwestern Medical Center. In addition, all studies were conducted in accordance with the United States Department of Health and Human Services National Institutes of Health Guide for the Care and Use of Laboratory Animals.

General Surgical Procedures

Rats were anesthetized with isoflurane gas (2-3%) in pure oxygen, intubated, and mechanically respirated (Harvard Apparatus) for the duration of the experiment. To minimize edema, 0.15 mg dexamethasone was given intramuscularly in the left hindlimb(46). Both carotid arteries and a jugular vein were catheterized (PE-50, polyethylene tubing) for blood pressure readings and administering of fluids, respectively. To maintain fluids and stabilize the animals, 1 M $NaHCO_3$, 5% dextrose Ringers solution was infused intravenously at a rate of 2 mL/hr when necessary(35). In addition, arterial blood gases and pH were measured throughout experimentation using an automated blood gas analyzer (50 μL blood samples; Model ABL 5, Radiometer) to ensure variables were maintained within physiological ranges (arterial Po_2 of >80 Torr; arterial PCO_2 of 35-45 Torr; pH, 7.3-7.4). Body temperature was maintained between 36.5 and 38.0°C by an isothermal pad (Deltaphase). Animals were held in a stereotaxic

head unit (Kopf Instruments) and a pre-collicular decerebration was performed. Briefly, holes were drilled into the parietal skull and the bone superior to the central sagittal sinus removed. Dura mater was cut away and the cerebrum was aspirated. Once the superior and inferior colliculi were within view, a pre-collicular section was made and the transectioned forebrain was aspirated rendering the animal insentient. To minimize cerebral bleeding, small pieces of oxidized regenerated cellulose (Ethicon, Johnson & Johnson) were placed on the internal skull surface and the cranial cavity was packed with cotton. Immediately after pre-collicular transection, inhaled anesthesia was discontinued and the animal was allowed to stabilize for one hour.

Mechanoreflex Activation

Preparation. Mechanically sensitive afferent fibers in skeletal muscle were selectively activated by passively stretching the triceps surae muscles of the hindlimb(43). This technique is a valid procedure to preferentially stimulate stretch sensitive afferent fibers because no metabolites are produced by the muscle. The pelvis was stabilized with steel posts within the frame, and the right hindlimb was fixed in one position using clamps attached to the tibial bone. The gastrocnemius and soleus (ie. triceps surae) muscles were isolated, and the calcaneal bone cut. Lastly, the Achilles' tendon was connected to a force transducer (Grass Instruments, FT10), allowing the measurement of muscle tension.

Experimental protocol. Using a calibrated 9.5 mm rack and pinion system (Harvard Apparatus), preferential activation of mechanically sensitive afferent fibers was induced by passively stretching the triceps surae muscles of the hindlimb for 30 seconds. The amount of developed hindlimb muscle tension during passive stretch was matched to that known to be produced during maximal hindlimb muscle contraction in WKY and SHR rats (approximately 1200 g)(19, 42). During separate trials, triceps surae muscles were stretched a minimum of two times with a 15-minute recovery period between each stretch. Before all maneuvers, hindlimb muscles were preloaded by stretching to 70-100 g of tension.

Microdialysis Procedures

Preparation. Animals were held in a stereotaxic head unit (Kopf Instruments). A limited occipital craniotomy was performed to expose the dorsal surface of the brainstem and microdialysis probes (Bioanalytical Systems, model CMA 11, 0.24 mm outer diameter, 1 mm membrane tip) were stereotaxically positioned unilaterally within the NTS at an area known to receive projections from MMcR afferent fibers (coordinates: 0.5 mm lateral to the obex and 0.5 mm below the dorsal medullary surface)(27, 33). Probes were continuously perfused at a rate of 2.5 μl/min with artificial cerebral spinal fluid (aCSF) buffered to a pH of 7.4. aCSF contained 0.2 % bovine serum albumin, 0.1 % bacitracin, and the following ions (in mM): 6.2 K^+, 134 Cl^-, 2.4 Ca^{2+}, 150 Na^+, 1.3 P^-, 13 HCO_3^- and 1.3 Mg^{2+}. After the probe was inserted, the preparation was allowed to stabilize for a minimum of one hour.

Experimental protocol. The circulatory response to activation of stretch sensitive afferent fibers was obtained during the individual administration of aCSF (control), 1mM L-NAME, 5 mM L-NAME, and aCSF (recovery). In these experiments, the microdialysis probe was placed ipsilateral to the stretched muscle. Furthermore, the concentrations of dialysate used were carefully chosen, as these have been shown to alter the reflex control of MAP and HR. aCSF and L-NAME were dialyzed a minimum of 45 minutes before stretching the muscle. During dialysis of each substance, two reproducible responses were obtained with a minimum of 15 minutes between events. In additional control experiments in SHR (n=4) and WKY (n=4) animals, mechanically sensitive afferent fibers were activated before and after the microdialysis of D-NAME (5 mM), the inactive isomer of L-NAME.

Validation of probe placement. To verify probe placement, Evans blue dye was dialyzed into the NTS at the conclusion of experimentation. Then the brainstem was excised and fixed in 10 % phosphate buffered formalin and stored at 4^0C. Medullary tissue was blocked, and 40 μm sections were cut serially using a cryostat (Cambridge Instruments). Sections were placed on coated slides and

examined to establish the neuroanatomical location of probe placement. The perfusion area of the probe was verified by the distribution of the dye.

Corollary Experiment

The EPR induces increases in MAP and HR predominantly via increased activation of the sympathetic nervous system. Therefore, we reasoned that the circulatory response to passive hindlimb muscle stretch should be abolished by sympathetic blockade if mediated by the MMcR. To ascertain the involvement of the sympathetic nervous system in the hemodynamic response to muscle stretch, the MMcR testing procedure was employed before and after sympathetic blockade in a subset of WKY (n=4) and SHR animals (n=4). In both groups, the triceps surae muscles were stretched to maximal tension development as previously described. The ganglionic blocking agent hexamethonium (30 mg kg^{-1}) was administered intravenously and the passive stretch maneuver performed.

Morphological Measurements

At the conclusion of testing procedures, animals were humanely euthanized with intravenous injection of saturated potassium chloride. The heart and lungs were excised and wet weights obtained. Tibial length was also measured to assess heart mass / tibial length ratios.

Data Acquisition

In all physiologic experiments, baseline as well as reflex changes in MAP, HR, and developed tension were recorded. Baseline values for each measured variable were obtained over a 30 second period prior to reflex activation. The greatest change in each variable from this baseline in response to reflex activation was taken as the peak value.

Statistical Analysis

All cardiovascular and contractile force data were acquired, recorded, and analyzed using data acquisition software (Spike 2, version 3, Cambridge Electronic Design, Ltd) for the CED micro 1401 system (Cambridge Electronic Design Ltd). Data was analyzed by means of paired and unpaired t-tests or analysis of variance (ANOVA) with Student Newman-Keuls multiple comparison

tests employed as appropriate. The significance level was set at $P<0.05$. All statistical analysis was performed using Sigma Stat for Windows (SPSS Inc.)

Results

Characterization of Hypertensive Model

Morphometric and hemodynamic baseline data for WKY and SHR animals are presented in Table 1. Ratios of heart weight to both body weight and tibial length were significantly greater in SHR than WKY. However, lung weight/ body weight was not different between the two groups, suggesting that the SHR rats were not in heart failure as a large lung weight/body weight is indicative of pulmonary edema. Baseline MAP was significantly higher in SHR than WKY animals, but baseline HR data was not statistically different.

Activation of MMcR Afferent Fibers Elicits an Exaggerated Cardiovascular Response in Hypertension

MMcR activation caused significant increases in MAP and HR from baseline in both groups of animals. As previously reported, passive stretch of the hindlimb to a developed tension of approximately 1200 g caused a significantly greater increase in both MAP and HR in SHR compared to WKY animals. The rise in MAP in SHR rats (37 ± 3 mmHg) was significantly greater than that seen in the WKY group (19 ± 2 mmHg). The increase in HR was also significantly exaggerated in SHR (12 ± 2 bpm) compared to WKY animals (6 ± 1 bpm).

Microdialysis of L-NAME in the NTS Partially Recapitulates the Exaggerated Cardiovascular Response to MMcR Activation in Hypertension

Microdialysis of 1mM L-NAME into the NTS significantly increased the MAP and HR response to MMcR activation in SHR animals (Figure 1). In normotensive WKY animals, inhibiting NOS activity within the NTS with L-NAME increased the MAP response to passive muscle stretch from 14 ± 2 mmHg to 20 ± 4 mmHg and the HR response from 4 ± 2 bpm to 12 ± 4 bpm. In hypertensive SHR animals, microdialysis of 1 mM L-NAME further increased the

MAP and HR responses to MMcR activation from 40 ± 6 mmHg and 9 ± 2 bpm to 61 ± 8 mmHg and 18 ± 5 bpm, respectively. When the passive stretch procedure was repeated during the dialysis of aCSF at the end of the experimental protocol, the circulatory responses returned to pre-L-NAME levels in both groups of animals.

Increasing the concentration of L-NAME from 1 mM to 5 mM had an excitatory effect on the cardiovascular response to MMcR activation in normotensive WKY animals (Figure 2). The MAP response to MMcR activation in WKY rats increased by 5 ± 3 mmHg when 1 mM L-NAME was dialyzed into the NTS. Subsequently, microdialysis of the higher dose of L-NAME (5 mM) into the NTS increased the MAP response to MMcR activation by 12 ± 5 mmHg, suggesting that NOS blockade is greater at the higher dose (Figure 3A). Specifically, when the hindlimb muscle was passively stretched during NTS L-NAME (5 mM) dialysis in WKY rats, MAP and HR increased to 32 ± 5 mmHg and 10 ± 1 bpm, respectively. These hemodynamic responses match those that occurred in response to MMcR activation in hypertensive SHR animals during NTS dialysis of aCSF (37 ± 3 mmHg and 12 ± 2 bpm). We were therefore able to reproduce the exaggerated cardiovascular response to MMcR activation evidenced in hypertension by inhibiting the enzymatic activity of NOS in the NTS of normotensive rats.

In hypertensive SHR rats, the MAP and HR responses to MMcR activation during NTS dialysis of 5 mM L-NAME was 55 ± 7 mmHg and 15 ± 2 bpm, respectively (Figure 2). Further, dialyzing 5 mM L-NAME into the NTS during MMcR activation significantly increased the pressor response to stretch in both groups of animals. However, there was no concentration-dependant change in the MAP response to MMcR activation in SHR animals when the concentration of dialyzed L-NAME was increased to 5 mM (Figure 3B). Specifically, the MAP response to MMcR activation increased by 21 ± 7 mmHg when 1 mM L-NAME was dialyzed into the NTS and 17 ± 6 mmHg when 5 mM L-NAME was perfused.

Microdialysis of D-NAME into the NTS had no Effect on the Cardiovascular Response to MMcR Activation

As a control experiment, MMcR activation was repeated in WKY and SHR animals during the dialysis of D-NAME (5 mM), the inactive isoform of L-NAME, within the NTS. The dialysis of D-NAME into the NTS had no effect on the hemodynamic response to MMcR activation in both groups of animals. Specifically, in normotensive WKY rats, increases in MAP and HR to MMcR activation were 23 ± 2 mmHg and 8 ± 2 bpm, respectively during the dialysis of aCSF and 20 ± 4 mmHg and 6 ± 1 bpm during the dialysis of D-NAME (Figure 4). In hypertensive SHR rats, increases in MAP and HR to MMcR activation were 42 ± 8 mmHg and 14 ± 4 bpm, respectively during the dialysis of aCSF and 45 ± 9 mmHg and 14 ± 4 bpm during the dialysis of D-NAME (Figure 5).

Effects of Sympathetic Blockade on MMcR Activation in Hypertensive Rats

Administration of hexamethonium, a ganglionic blocker, was used to inhibit sympathetic nerve activity before and after MMcR activation. Ganglionic blockade almost completely abolished the MAP and HR response to MMcR activation in both WKY and SHR groups (Figure 6). This data confirms that the pressor and tachycardia responses to MMcR activation are mediated by the sympathetic nervous system.

Verification of Probe Placement

To verify probe placement, Evans blue dye was dialyzed at the end of experimentation for 40 minutes. A representative example of dye distribution marking the probe perfusion area is shown in Figure 7. The microdialysis site was within 500 μm of the calamus scriptorus and lateral dye spread was approximately 400 μm. The dye area was restricted to the medial, dorsomedial, and commissural subdivisions of the caudal NTS, where EPR afferents have been shown to synapse(21, 27, 32).

Discussion

Using a decerebrate rat model, we recently provided evidence supporting the concept that MMcR activation partially mediates the exaggerated cardiovascular response to exercise manifest in hypertension(19). This study expanded our knowledge of MMcR dysfunction in hypertension with three major findings, which are: (i) blocking NO production within the NTS in normotensive rats recapitulates the augmented cardiovascular response elicited by MMcR activation in hypertension, (ii) blocking NO production within the NTS in hypertensive rats further enhances the exaggerated cardiovascular response caused by MMcR activation, and (iii) the MMcR elicits an exaggerated circulatory response to exercise in hypertension via the sympathetic nervous system. These findings support our hypothesis that a reduction in NO production/availability within the NTS plays a significant a role in MMcR dysfunction in hypertension.

Blocking NO within the NTS Produces an Exaggerated Circulatory Response to MMcR Activation

The most interesting finding is that we were able to reproduce an increase in MAP and HR that was similar to that seen during MMcR activation in hypertension when L-NAME was dialyzed into the NTS of normotensive rats. This suggests that the bioavailability of NO within the NTS is involved in the processing of MMcR afferent fiber input and subsequently, the cardiovascular response elicited by MMcR activation. This is consistent with data that has shown brainstem NO is able to modulate cardiovascular reflexes and to affect sympathetic outflow from the medulla(10, 22, 40, 52). Our findings are also corroborated by experiments demonstrating that NO within the NTS is involved in EPR processing, of which the MMcR is a component(22, 40).

It is of significance that L-NAME dialyzed into the NTS produced a concentration-dependant increase in the pressor and tachycardia responses to MMcR activation in normotensive WKY rats. While microdialysis of 1 mM L-NAME into the NTS raised the pressor and tachycardia response to MMcR

activation in WKY rats, it was not until the larger concentration (5 mM) of L-NAME was dialyzed into the NTS that the cardiovascular response to MMcR activation in hypertension was fully recapitulated. This suggests that blocking NOS enzymatic activity with 5 mM L-NAME may decrease the amount of NO in the NTS to similar basal levels present in the SHR NTS. Further, the finding that MMcR-mediated pressor and tachycardia responses in SHR rats were not sensitive to the concentration of L-NAME dialyzed into the NTS provides evidence that there is a finite amount of NOS within the NTS of SHR rats that can be blocked by L-NAME. This supports our hypothesis that there is a decrease in NO bioavailability within the NTS of hypertensive animals. While there has been much research performed trying to quantify the amount of NOS and NO in the brainstem of normotensive and hypertensive rats, to date the results have been conflicting.

NO and NOS within the Hypertensive Brainstem

A decrease in NO bioavailability within the hypertensive brainstem could be attributed to several factors. One cause of an L-arginine-NO pathway impairment could be decreases in the expression/activity of the NOS isoforms present within the NTS. Unfortunately, studies to date describing NOS expression/activity within the brainstem of hypertensive rats have been conflicting. Some studies in hypertensive rats have shown NOS expression and activity within the medulla to be decreased during infancy, but significantly increased in adulthood compared to normotensive controls(30, 34, 49). However, other studies show basal levels of NOS expression and activity are decreased in the NTS of adult hypertensive rats compared to their normotensive controls(4, 31). Data from our experiments support the idea that basal NOS levels or activity are decreased in the NTS of SHR rats because there was not a dose-dependant increase in the pressor response to MMcR activation when differing doses of L-NAME were dialyzed into the NTS. The contradictory data on NOS activity and expression within the NTS of hypertensive animals and its effect on blood pressure illustrate the need for more comprehensive experiments.

Another possible explanation of decreased NO within the brainstem involves reactive oxygen species, such as superoxide. The superoxide anion is known to inactivate NO and form peroxynitrite through the enzymatic action of nicotinamide-adenine dinucleotide phosphate (NAD(P)H) oxidase. NAD(P)H oxidase activity is increased in hypertensive individuals due to the increased physical stress and/or the presence of angiotensin II (6). Angiotensin II, a peptide that regulates sympathetic outflow, has also been shown to be increased in the brainstem of hypertensive rats(39, 45). In fact, research has shown that there is a higher density of angiotensin II receptors (subtype: AT_1) within the NTS of SHR rats compared to normotensive WKY rats(7). Additionally, all the substrates and enzymes necessary for Ang-II production and activity have been identified in brainstem nuclei important to cardiovascular control(37). Therefore, the concentration of NO within the NTS may be reduced by the generation of superoxide via an Ang-II/NAD(P)H oxidase mechanism. Reductions in NO availability via this mechanism could mediate the elevations in MAP and HR characteristic of MMcR dysfunction in hypertension. In support of this concept, recent studies have demonstrated that reactive oxygen species levels are increased in specific autonomic nuclei important to cardiovascular regulation within the brainstem of SHR as compared to WKY(18, 44). It is most likely that both NOS levels and reactive oxygen species are involved in regulating the amount of available NO within the brainstem in hypertension. In the future, it will be important to definitively quantify the amount of NO present within the brainstem of hypertensive individuals and to fully describe the mechanisms responsible for its bioavailability so that a better understanding of its role in modulating MMcR activity can be reached.

Limitations

Several limitations could affect the interpretation of the results of the current study. First, we performed unilateral microdialysis during MMcR activation due to spatial constraints within the rat brainstem and to reduce surgical trauma within the medulla to preserve the functional and structural integrity of the brainstem.

However, it has been established that in the rat, mechanically-sensitive afferents project bilaterally to the NTS(5, 32). With this limitation in mind, much research has been performed using unilateral microinjections and microdialysis demonstrating the technique is a valid procedure to alter the circulatory responses to activation of cardiovascular reflexes(20, 31, 40).

Another limitation of the present study concerns the technique used for MMcR activation. Passive muscle stretch, the method we used to activate mechanically sensitive afferent fibers, may mimic the same procedure by which group III afferent fibers are activated during physiological exercise. For example, it has been shown in cats that only a portion (25-75 %) of group III afferent fibers with receptive fields in the triceps surae muscles that respond to muscle contraction also respond to passive stretch of the hindlimb(1, 8). However, both contraction and passive muscle stretch produce similar effects on afferent discharge rate, as well as increases in MAP and HR in these animals(8, 9). In addition, hindlimb stretch has been shown to increase renal sympathetic efferent discharge rates and the same efferents responsive to stretch were also activated by hindlimb contraction(8). Finally, gadolinium, a known blocker of mechanosensitive channels, affectively abolishes group III afferent discharge and the concomitant increases in MAP and HR that occur in response to passive muscle stretch(8, 41). As such, this technique is commonly used at activate group III afferent fibers.

Lastly, it is important to recognize that the MMcR is just one component of the EPR. In the future it will be necessary to determine how altering NO activity within the brainstem affects the hemodynamic response to activation of metabolically sensitive afferent fibers. We recently presented evidence that the metaboreflex contributes to the exaggerated cardiovascular response mediated by the EPR in hypertension. Therefore knowledge of the central mechanisms and neuromodulators involved in metaboreflex processing is critical for effective treatment of EPR dysfunction in hypertension.

Conclusions and Clinical Significance

To summarize, we have provided evidence supporting the concept that NO production/availability within the brainstem contributes to MMcR dysfunction in hypertension. We have also shown that MMcR activation elicits an exaggerated circulatory response to exercise in hypertension via the sympathetic nervous system and we speculate that the increased sympathetic drive is partially caused by a decrease in medullary NO in hypertension. In the future, it will be important to learn how augmenting NO production within the NTS affects the circulatory response to MMcR activation. Hopefully, this will provide insight into possible treatment options for hypertensive individuals allowing them to engage in physical activity without the associated hemodynamic risks.

Acknowledgements

This research was supported by grants from the National Institutes of Health (HL-094075 to A.K. Leal and HL-088422 to S.A. Smith) and the Lawson & Rogers Lacy Research Fund in Cardiovascular Diseases (to J.H. Mitchell). The authors thank Martha Romero and Julius Lamar, Jr. for their expert technical assistance.

References

1. Adreani CM, Hill JM, and Kaufman MP. Responses of group III and IV muscle afferents to dynamic exercise. *J App Physiol* 82: 1811-1817, 1997.
2. Alam M and Smirk FH. Observations in man upon a blood pressure raising reflex arising from the voluntary muscles. *J Physiol* 89: 372-383, 1937.
3. Aoki K, Sato K, Kondo S, Pyon C, and Yamamoto M. Increased response of blood pressure to rest and handgrip in subjects with essential hypertension. *Jpn Circ J* 47: 802-809, 1983.
4. Ferrari MFR and Fior-Chadi DR. Differential expression of nNOS mRNA and protein in the nucleus tractus solitarii of young and aged Wistar-Kyoto and spontaneously hypertensive rats. *J Hypertens* 23: 1683-1690, 2005.
5. Gamboa-Esteves FO, Tavares I, Almeida A, Batten TFC, McWilliam PN, and Lima D. Projection sites of superficial and deep spinal dorsal horn cells in the nucleus tractus solitarii of the rat. *Brain Res* 921: 195-205, 2001.
6. Griendling KK, Sorescu D, and Ushio-Fukai M. NAD(P)H oxidase. *Circ Res* 86: 494-501, 2000.
7. Gutkind JS, Kurihara M, Castren E, and Saavedra JM. Increased concentration of angiotensin II binding sites in selected brain areas of spontaneously hypertensive rats. *J Hypertens* 6: 79-84, 1988.

8. Hayes SG and Kaufman MP. Gadolinium attenuates exercise pressor reflex in cats. *Am J Physiol* 280: 2153-2161, 2001.
9. Hayes SG, Kindig AE, and Kaufman MP. Comparison between the effect of static contraction and tendon stretch on the discharge of group III and IV muscle afferents. *J Appl Physiol* 99: 1891-1896, 2005.
10. Hironaga K, Hirooka Y, Matsuo I, Shihara M, Tagawa T, Harasawa Y, and Takeshita A. Role of endogenous nitric oxide in the brain stem on the rapid adaptation of baroreflex. *Hypertension* 31: 27-31, 1998.
11. Hoberg E, Schuler G, Kunze B, Obermoser AL, Hauer K, Mauther HP, Schlierf G, and Kubler W. Silent myocardial ischemia as a potential link between lack of premonitoring symptoms and increased risk of cardiac arrest during physical stress. *Am J Cardiol* 65: 583-589, 1990.
12. Iwamoto GA and Kaufman MP. Caudal ventrolateral medullary cells responsive to static muscular contraction. *J App Physiol* 62: 149-157, 1987.
13. Iwamoto GA, Waldrop TG, Kaufman MP, Botterman BR, Rybicki KJ, and Mitchell JH. Pressor reflex evoked by muscular contraction: contributions by neuraxis levels. *J App Physiol* 59: 459-467, 1985.
14. Kahn JF. The static exercise-induced arterial hypertension test. *Presse Medicine* 20: 1067-1071, 1991.
15. Kaufman MP and Forster HV. Reflexes controlling circulatory, ventilatory and airway responses to exercise. In: *Section 12, Exercise: Regulation and Integration of Multiple Systems*. Bethesda, MD: Am Physiol Soc, 1996, p. 381-447.
16. Kaufman MP, Longhurst JC, Rybicki KJ, Wallach JH, and Mitchell JH. Effects of static muscular contraction on impulse activity of groups III and IV afferents in cats *J App Physiol* 55: 105-112, 1983.
17. Kaufman MP, Waldrop TG, Rybicki KJ, Ordway GA, and Mitchell JH. Effects of static and rhythmic twitch contractions on the discharge of group III and IV muscle afferents. *Card Res* 18: 663-668, 1984.
18. Kishi T, Hirooka Y, Kimura Y, Ito K, Shimokawa H, and Takeshita A. Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats. *Circulation* 109: 2357-2362, 2004.
19. Leal AK, Williams MA, Garry MG, Mitchell JH, and Smith SA. Evidence for functional alterations in the skeletal muscle mechanoreflex and metaboreflex in hypertensive rats. *Am J Physiol Heart Circ Physiol* 295: H1429-H1438, 2008.
20. Lewis SJ, Ohta H, Machado B, Bates JN, and Talman WT. Microinjection of S-nitrosocysteine into the nucleus tractus solitarii decreases arterial pressure and heart rate via activation of soluble guanylate cyclase. *Eur J Pharmacol* 202: 135-136, 1991.
21. Li J and Mitchell JH. Role of NO in modulating neuronal activity in superficial dorsal horn of spinal cord during exercise pressor reflex. *Am J Physiol* 283: H1012-H1018, 2002.
22. Li J and Potts JT. NO formation in nucleus tractus solitarii attenuates pressor response evoked by skeletal muscle afferents. *Am J Physiol* 280: H2371-H2379, 2001.

23. McCloskey DI and Mitchell JH. Reflex cardiovascular and respiratory responses originating in exercising muscle. *Journal of Physiology* 224: 173-186, 1972.
24. Mitchell JH. Neural control of the circulation during exercise. *Med Sci Sports Exerc* 22: 141-154, 1990.
25. Mitchell JH, Kaufman MP, and Iwamoto GA. The exercise pressor reflex: Its cardiovascular effects, afferent mechanisms, and central pathways. *Ann Rev Physiol* 45: 229-242, 1983.
26. Moncada S and Higgs A. The L-arginine-nitric oxide pathway. *New Eng J Med* 329: 2002-2012, 1993.
27. Paxinos G and Watson C. *The Rat Brain in Stereotaxic Coordinates*. Orlando, FL: Academic, 1986.
28. Person RJ. Somatic and vagal afferent convergence on solitary tract neurons in cat: electrophysiological characteristics. *Neuroscience* 30: 283-295, 1989.
29. Pickering TG. Pathophysiology of exercise hypertension. *Herz* 12: 119-124, 1987.
30. Plochocka-Zulinska D and Krukoff TL. Increased gene expression of neuronal nitric oxide synthase in brain of adult spontaneously hypertensive rats. *Brain Res Mol Brain Res* 48: 291-297, 1997.
31. Pontieri V, Venezuela MK, Scavone C, and Michelini LC. Role of endogenous nitric oxide in the nucleus tractus solitarii on baroreflex control of heart rate in spontaneously hypertensive rats. *J Hypertens* 16: 1993-1999, 1998.
32. Potts JT, Lee SM, and Anguelov PI. Tracing of projection neurons from the cervical dorsal horn to the medulla with the anterograde tracer biotinylated dextran amine. *Aut Neurosci* 98: 64-69, 2002.
33. Potts JT, Paton JFR, Mitchell JH, Garry MG, Kline G, Anguelov PT, and Lee SM. Contraction-sensitive skeletal muscle afferents inhibit arterial baroreceptor signalling in the nucleus of the solitary tract: role of intrinsic GABA interneurons. *Neuroscience* 119: 201-214, 2003.
34. Qadri F, Arens T, Schwarz EC, Hauser W, Dendorfer A, and Dominiak P. Brain nitric oxide synthase activity in spontaneously hypertensive rats during the development of hypertension. *J Hypertens* 21: 1623-1624, 2003.
35. Quintin L, Gillon JY, Saunier CF, and Ghignone M. Continuous volume infusion improves circulatory stability in anesthetized rats. *J Neurosci Meth* 30: 77-83, 1989.
36. Ruggiero DA, Mtui EP, Otake K, and Anwar M. Central and primary visceral afferents to nucleus tractus solitarii may generate nitric oxide as a membrane-permeant neuronal messenger. *J Comp Neurol* 361: 51-67, 1996.
37. Sakai K and Sigmund CD. Molecular evidence of tissue renin-angiotensin systems: a focus on the brain. *Curr Hypertens Rep* 7: 135-140, 2005.
38. Seguro C, Sau F, Zedda N, Scano G, and Cherchi A. Arterial blood pressure behavior during progressive muscular exercise in subjects with stable arterial hypertension. *Cardiologia* 36: 867-877, 1991.

39. Senanayake PD, Moriguchi A, Kumagai H, Ganten D, Ferrario CM, and Brosnihan KB. Increased expression of angiotensin peptides in the brain of transgenic hypertensive rats. *Peptides* 15: 919-926, 1994.
40. Smith SA, Mitchell JH, and Li J. Independent modification of baroreceptor and exercise pressor reflex function by nitric oxide in nucleus tractus solitarius. *Am J Physiol* 288: 2068-2076, 2005.
41. Smith SA, Mitchell JH, Naseem RH, and Garry MG. Mechanoreflex mediates the exaggerated exercise pressor reflex in heart failure. *Circulation* 112: 2293-2300, 2005.
42. Smith SA, Williams MA, Leal AK, Mitchell JH, and Garry MG. Exercise pressor reflex function is altered in spontaneously hypertensive rats. *J Physiol* 577: 1009-1020, 2006.
43. Stebbins CL, Brown B, Levin D, and Longhurst JC. Reflex effect of skeletal muscle mechanoreceptor stimulation on the cardiovascular system. *J App Physiol* 65: 1539-1547, 1988.
44. Tai M-H, Wang L-L, Wu KLH, and Chan JYH. Increased superoxide anion in rostral ventrolateral medulla contributes to hypertension in spontaneously hypertensive rats via interactions with nitric oxide. *Free Radical Biology & Medicine* 38: 450-462, 2005.
45. Teruya H, Muratani H, Takishita S, Sesoko S, Matayoshi R, and Fukiyama K. Brain angiotensin II contributes to the development of hypertension in Dahl-Iwai salt-sensitive rats. *J Hypertens* 13: 883-890, 1995.
46. Tian G and Duffin J. Spinal connections of ventral-group bulbospinal inspiratory neurons studied with cross-correlation in the decerebrate rat. *Exp Brain Res* 111: 178-186, 1996.
47. Toney GM and Mifflin SW. Time-dependent inhibition of hindlimb somatic afferent inputs to nucleus tractus solitarius. *J Neurophysiol* 72: 63-71, 1994.
48. Toney GM and Mifflin SW. Time-dependent inhibition of hindlimb somatic afferent transmission within nucleus tractus solitarius: an *in vivo* intracellular recording study. *Neuroscience* 68: 445-453, 1995.
49. Waki H, Murphy D, Yao ST, Kasparov S, and Paton JFR. Endothelial NO synthase activity in nucleus tractus solitarii contributes to hypertension in spontaneously hypertensive rats. *Hypertension* 48: 644-650, 2006.
50. Williamson JW, Mitchell JH, Olesen HL, Raven PB, and Secher NH. Reflex increases in blood pressure induced by leg compression in man. *J Physiol* 475: 351-357, 1994.
51. Zanzinger J, Czachurski J, and Seller H. Effects of nitric oxide on sympathetic baroreflex transmission in the nucleus tractus solitarii and caudal ventrolateral medulla in cats. *Neurosci Lett* 197: 199-202, 1995.
52. Zanzinger J, Czachurski J, and Seller H. Inhibition of basal and reflex-mediated sympathetic activity in the RVLM by nitric oxide. *Am J Physiol Reg Int Comp Physiol* 268: R958-R962, 1995.

Table

	WKY	SHR
n	25	20
Body weight, g	351±7	402±8*
Heart weight/body weight, mg/g	2.9±0.1	3.4±0.1*
Lung weight/body weight, mg/g	5.8±0.5	6.9±0.4
Heart weight/tibial length, mg/mm	26.1±0.7	34.5±0.6*
MAP, mmHg	103±5	146±7*
HR, beats/min	413±14	419±9

Table 1. Morphometric characteristics and baseline hemodynamics. Data are means ± S.E.M. MAP, mean arterial pressure; HR, heart rate. * Significantly different from WKY. $P<0.05$.

Figures

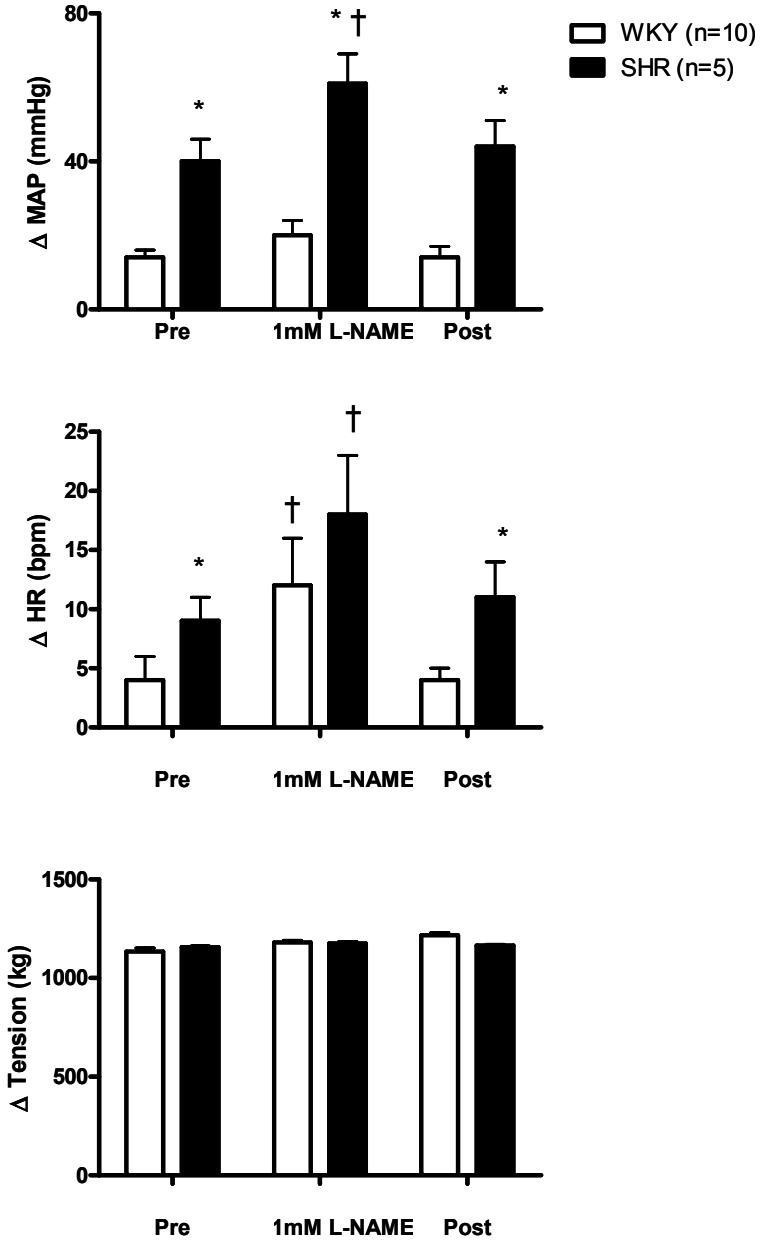

Figure 1. Cardiovascular responses to activation of mechanically sensitive afferent fibers during 1 mM L-NAME dialysis in WKY and SHR animals. Passive stretch of hindlimb skeletal muscle induced increases in MAP and HR that were significantly greater in SHR as compared to WKY rats at maximal levels of tension development. In addition, dialysis of 1mM L-NAME into the NTS significantly increased MAP and HR responses to hindlimb muscle stretch in SHR animals. * Significantly different from WKY rats. † Significantly different from Pre aCSF response. $P<0.05$.

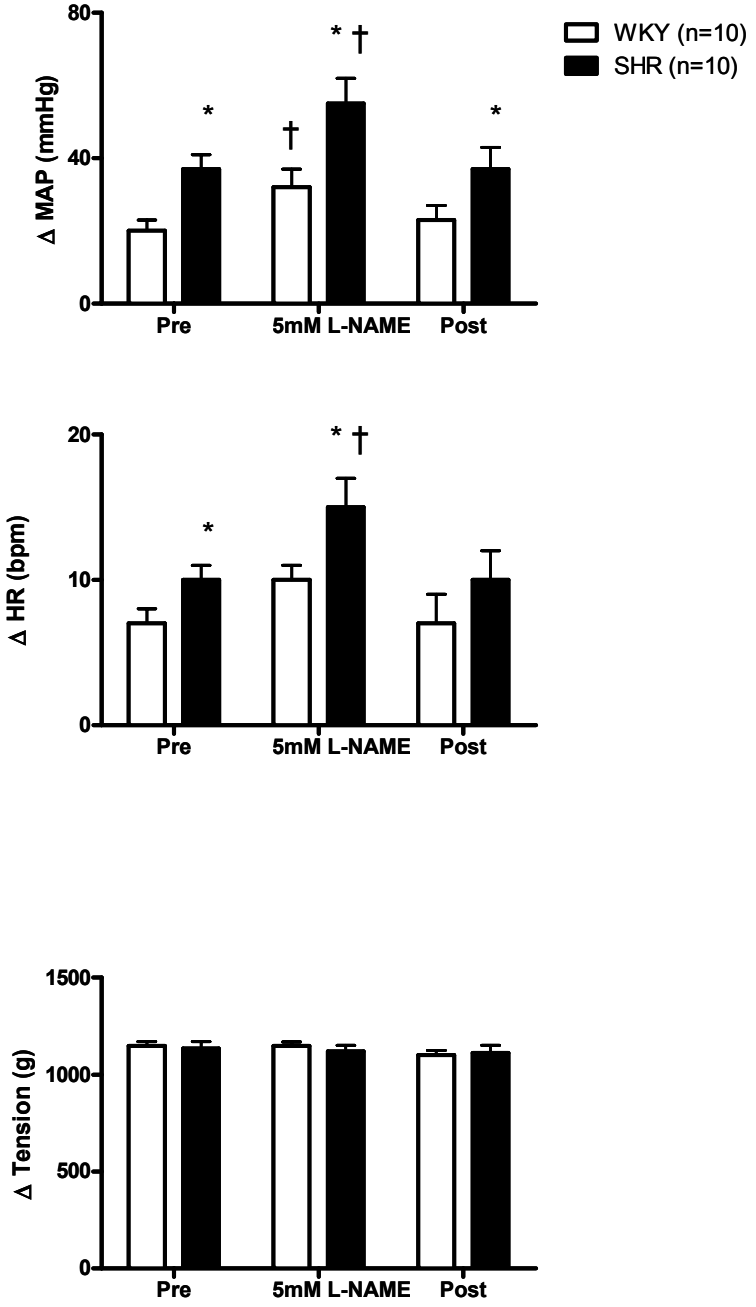

Figure 2. Cardiovascular responses to activation of mechanically sensitive afferent fibers during 5 mM L-NAME dialysis in WKY and SHR animals. Passive stretch of hindlimb skeletal muscle during dialysis of 5 mM L-NAME caused significant increases in MAP in SHR and WKY. In addition, the HR response to hindlimb muscle stretch in SHR animals also significantly increased during dialysis of L-NAME. * Significantly different from WKY rats. † Significantly different from Pre aCSF response. $P<0.05$.

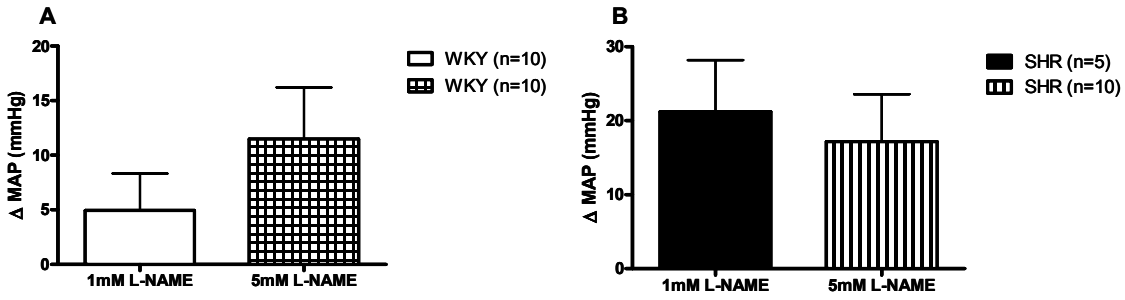

Figure 3. MAP response to activation of mechanically sensitive afferents during dialysis of 1 mM and 5 mM L-NAME in WKY and SHR animals. (A) The dialysis of L-NAME into the NTS of normotensive rats seems to have a concentration-dependant effect on the pressor response to passive hindlimb muscle stretch. **(B)** Increasing the concentration of L-NAME being dialyzed into the NTS of hypertensive rats had no effect on the pressor response to activation of mechanically sensitive afferent neurons.

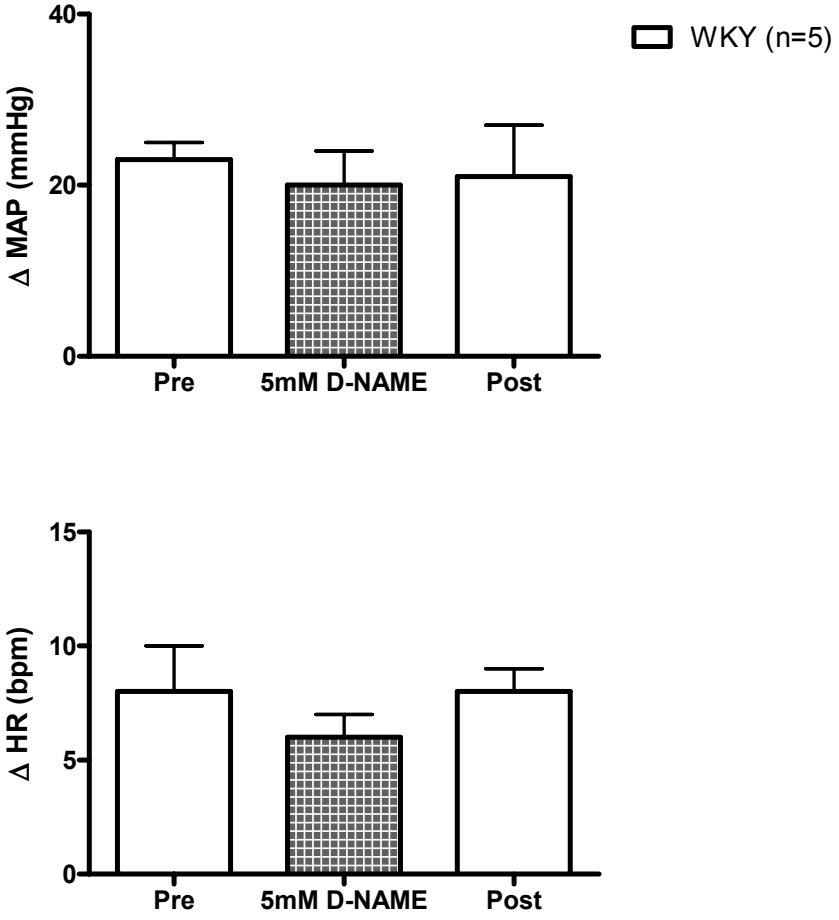

Figure 4. Cardiovascular responses to activation of mechanically sensitive afferent fibers during dialysis of D-NAME in WKY animals. Dialysis of the inactive isomer D-NAME had no effect on the cardiovascular response to passive stretch of hindlimb skeletal muscle.

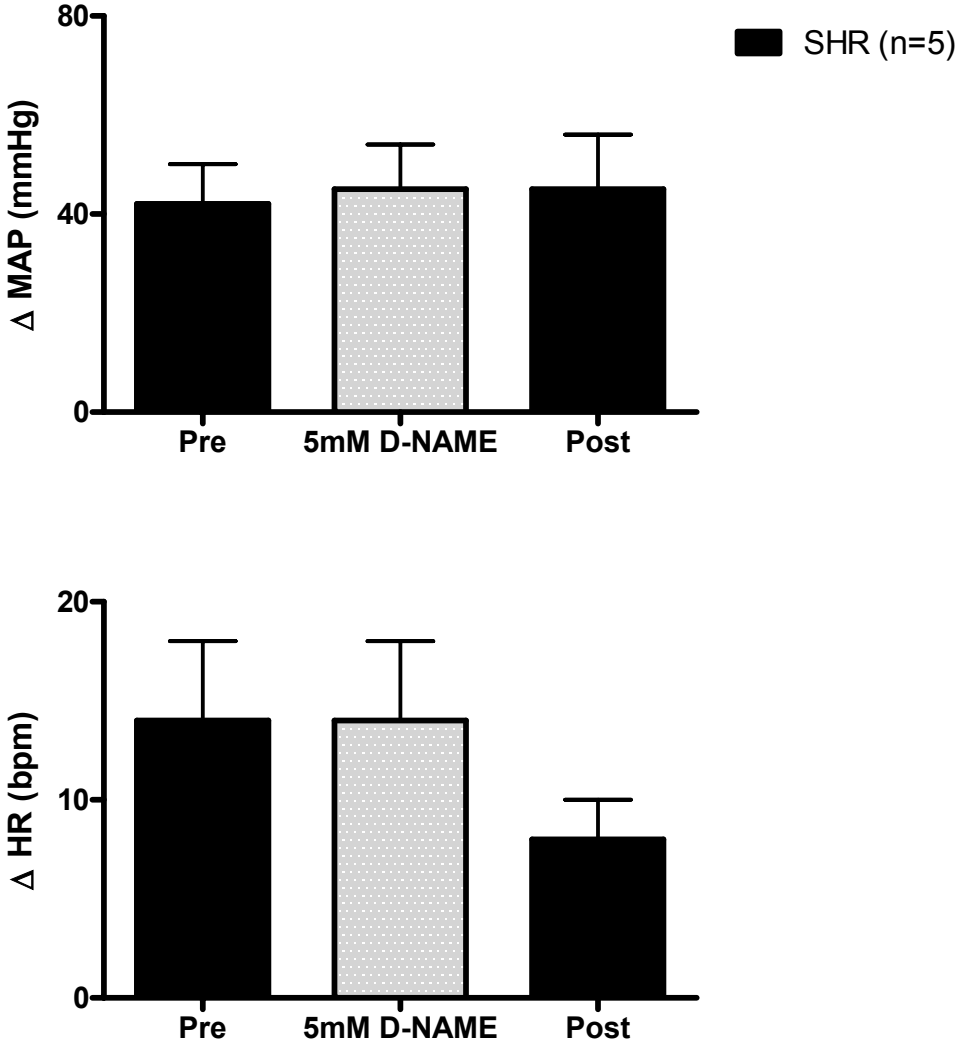

Figure 5. Cardiovascular responses to activation of mechanically sensitive afferent fibers during dialysis of D-NAME in SHR animals. Dialysis of the inactive isomer D-NAME had no effect on the cardiovascular response to passive stretch of hindlimb skeletal muscle.

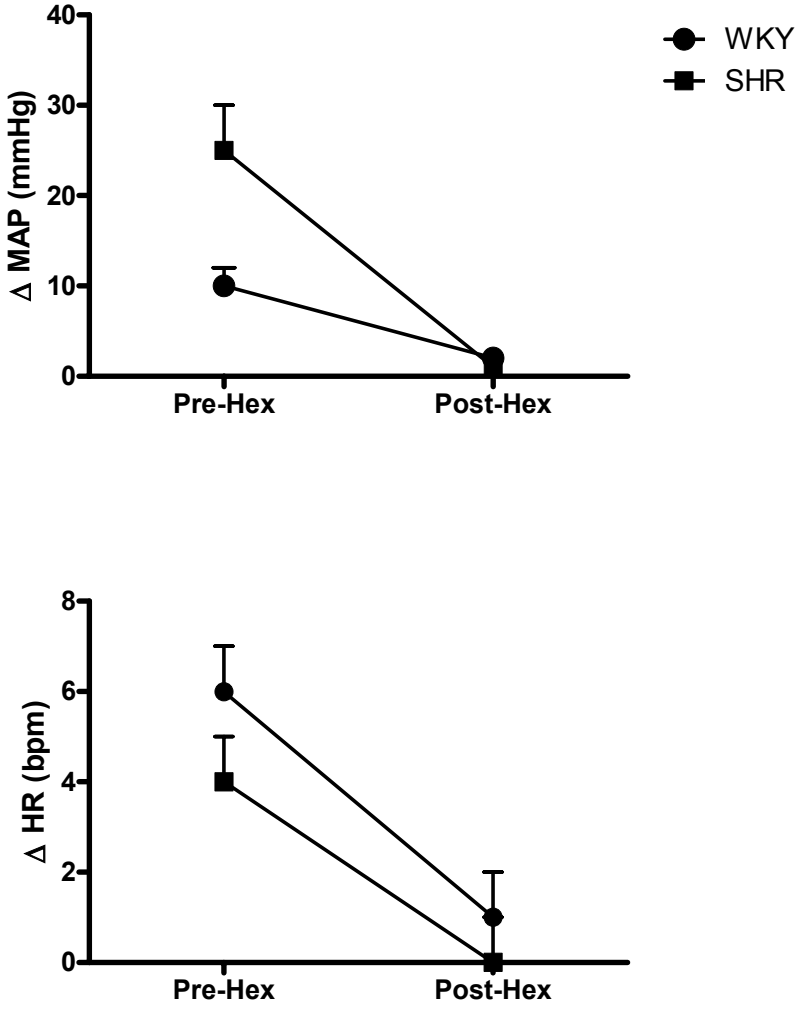

Figure 6. Ganglionic blockade with hexamethonium in WKY and SHR animals. Hexamethonium treatment almost completely abolished the MAP and HR response to passive hindlimb muscle stretch in both WKY and SHR groups.

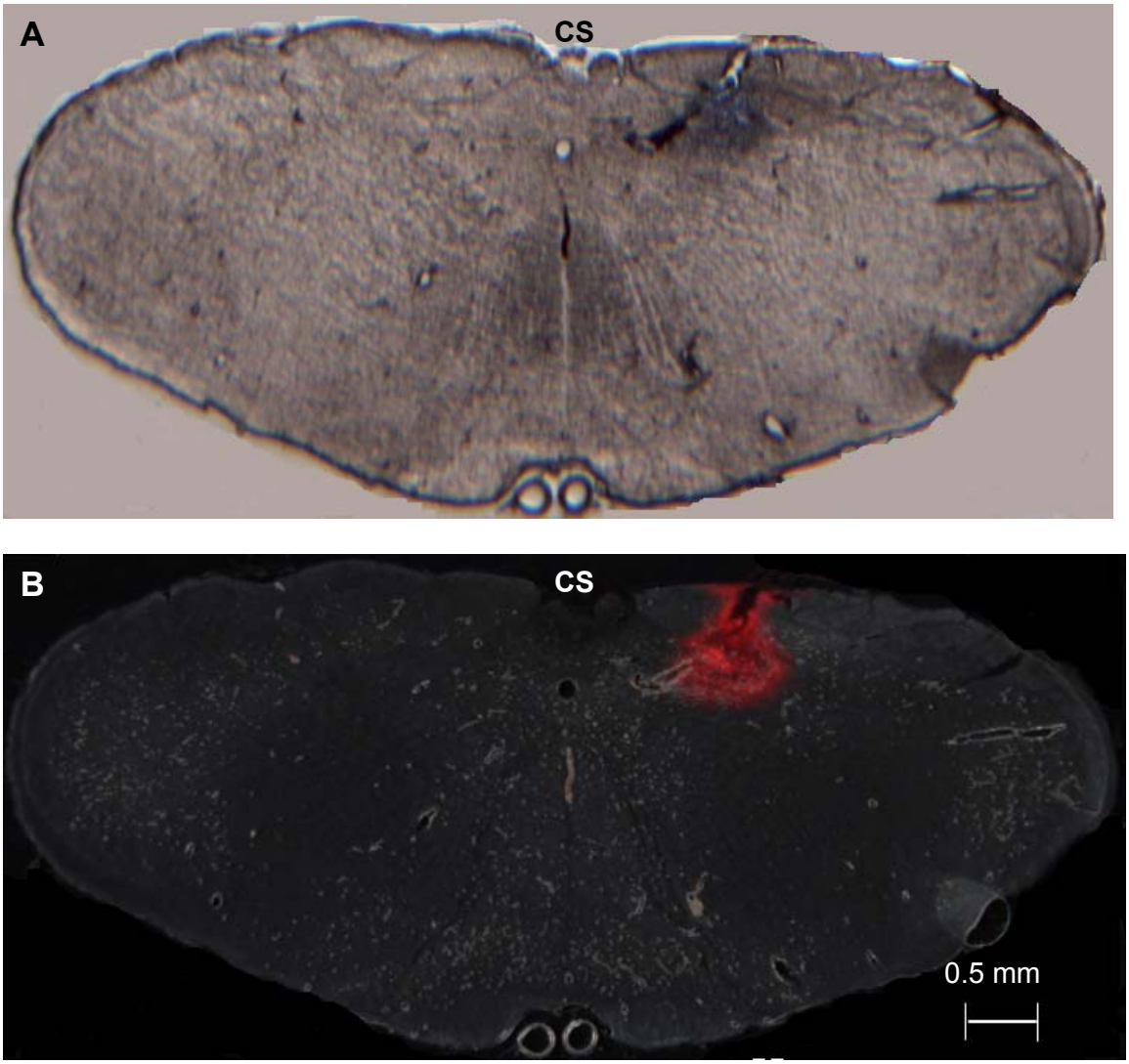

Figure 7. Representative example of dye distribution in the NTS.
(A) Photomicrograph of brain slice marking the probe perfusion area with Evans blue dye. The microdialysis probe was placed 0.5 mm lateral to midline and 0.5 mm below the medullary surface. The dye spread is detected in the NTS within 0.5 mm of the calamus scriptorus (CS). Magnification, 1.25X. **(B)** Same photomicrograph of brain tissue with dye spread identified by red fluorescence. Minimal structural damage was caused by placement of the dialysis probe. Magnification, 1.25X.

INCREASING NITRIC OXIDE WITHIN THE BRAINSTEM ATTENUATES THE EXAGGERATED CARDIOVASCULAR RESPONSE TO SIMULATED MECHANOREFLEX ACTIVATION IN HYPERTENSION

Running Title – Medullary NO attenuates MMcR dysfunction in hypertension

Anna K. Leal, MS[1]; Jere H. Mitchell, MD[2] and Scott A. Smith, PhD[3]

Departments of Biomedical Engineering[1], Internal Medicine[2] and Physical Therapy[3]

University of Texas Southwestern Medical Center

Dallas, Texas, USA 75390-9174

Corresponding Author:

Anna K. Leal, MS
University of Texas Southwestern Medical Center
Southwestern Allied Health Sciences School
Department of Biomedical Engineering
5323 Harry Hines Boulevard
Dallas, Texas 75390-9174
214-648-9188 (office)
214-648-3566 (facsimile)
anna.leal@utsouthwestern.edu

Abstract

The muscle mechanoreflex (MMcR) originates in skeletal muscle and responds to activation of mechanically sensitive afferent fibers by increasing mean arterial pressure (MAP) and heart rate (HR). This feedback mechanism is exaggerated in hypertension, producing large increases in MAP and HR. MMcR afferent neurons project to the nucleus tractus solitarius (NTS) in the medulla, where nitric oxide (NO) is produced from the precursor L-arginine. Blocking NO production within the NTS of normotensive rats has been shown to recapitulate the exaggerated cardiovascular elicited by the MMcR in hypertension. Therefore, we hypothesized that experimentally increasing NO production/availability in the NTS of hypertensive rats would partially correct MMcR dysfunction. To test our hypothesis, we microdialyzed L-arginine, the NO precursor, into the NTS of normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive (SHR) rats to increase endogenous NO production while simulating MMcR activation via passive hindlimb muscle stretch. Data shows that increasing NO production in SHR rats partially corrects the enhanced cardiovascular response to simulated MMcR activation. In addition, microdialysis of L-arginine into the NTS has a greater absolute effect on the MMcR-mediated cardiovascular response in hypertensive SHR rats than in normotensive WKY rats. This suggests that increasing NO bioavailability within the NTS may help attenuate MMcR dysfunction in hypertension. These findings could lead to treatment options that help control MMcR dysfunction in hypertensive individuals.

Key Words: blood pressure, mechanoreflex, exercise

Introduction

Hypertension is a medical condition affecting roughly 1 in 3 adults in the United States, according to the American Heart Association. This disease state stresses the heart and vasculature; even moderate elevations in mean arterial pressure (MAP) have been linked to shortened life expectancy(15, 34, 62, 71). Regular exercise, a non-pharmacological treatment option, has been shown to lower baseline blood pressure in animals and humans(3, 9, 26, 36, 39, 60, 64, 65). Unfortunately, the hemodynamic response to exercise is augmented in hypertension, producing larger than normal increases in heart rate (HR) and MAP, exacerbating already elevated baseline levels of these variables(5, 67). These augmented increases have the potential to cause stroke, arrhythmias, and myocardial infarction during or immediately after the exercise bout, limiting the safety of exercise prescription(20, 21, 35, 41, 45, 51).

During exercise, contracting muscle reflexively increases MAP, HR, and ventilation through a neural feedback pathway termed the exercise pressor reflex(4, 40). The muscle mechanoreflex (MMcR), an afferent component of the exercise pressor reflex, increases MAP and HR at the onset of muscle contraction primarily via activation of mechanically sensitive afferent neurons(23, 24, 37, 72, 85). These small-diameter sensory neurons are predominantly group III (A-δ) afferent fibers that respond to stretch and pressure. Once activated, signals from these fibers are processed within the nucleus tractus solitarius (NTS) of the brainstem(12, 23, 54, 72, 85). The efferent arm of the MMcR elevates MAP and HR through increases in sympathetic nerve activity and withdrawal of parasympathetic nerve activity(22).

Our laboratory recently provided evidence that the MMcR contributes to the exaggerated circulatory response to exercise in hypertension and that sympathetic nerve activity mediates this exaggerated response(28, 70). While many factors could alter MMcR activity in hypertension, one logical candidate for the development of MMcR dysfunction is its processing within the NTS. Within the NTS, L-arginine is oxidized by nitric oxide synthase (NOS) to produce nitric oxide (NO)(44). Research has shown that NO within the NTS can modulate

exercise pressor reflex-mediated sympathetic outflow from the medulla(19, 47, 53, 63). Data from our laboratory lends support to this concept as we demonstrated that experimentally increasing NO production within the NTS of cats significantly attenuated the cardiovascular response to exercise pressor reflex activation(69). In addition, we recently showed that blocking endogenous NO production in the NTS significantly increased the cardiovascular response to MMcR activation in both normotensive and hypertensive rats(27). Interestingly, we were able to recapitulate the exaggerated increases in MAP and HR in response to MMcR activation when the NOS-inhibitor L-NAME was microdialyzed into the NTS of normotensive rats. This not only provides evidence that endogenous NO in the NTS is involved in the sympathetically-mediated cardiovascular response to MMcR activation but also raises the possibility that alterations in the L-arginine/NO pathway within the NTS may be partially responsible for the MMcR dysfunction in hypertension. Therefore, the purpose of this study was to further elucidate the function of NO within the NTS and expand on its contribution to MMcR dysfunction in hypertension.

Given our recent finding that blocking NO production within the NTS of normotensive rats reproduces the exaggerated cardiovascular response to MMcR activation observed in hypertension, we hypothesized that experimentally increasing NO production/availability in the NTS of hypertensive rats would partially correct MMcR dysfunction. In order to test our hypothesis, we used microdialysis techniques to deliver the NO precursor L-arginine to the NTS of hypertensive rats while simultaneously activating mechanically sensitive afferent neurons associated with the MMR in the hindlimb. Further characterization of NO's involvement in the central processing of the MMcR in hypertension will be important to developing viable treatment options for individuals with hypertension, making it safer for them to participate in beneficial physical activities.

Materials and Methods

Subjects

Experiments were performed in 28 spontaneously hypertensive rats (SHR) and 28 normotensive Wistar-Kyoto (WKY) age-matched (14-20 week-old) male rats (Harlan, Indianapolis, IN). Animals were provided food and water *ad libitum* and housed in standard rodent cages on 12-h light-dark cycles. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas at Southwestern Medical Center. In addition, all studies were conducted in accordance with the United States Department of Health and Human Services National Institutes of Health Guide for the Care and Use of Laboratory Animals.

General Surgical Procedures

Rats were anesthetized with isoflurane gas (2-3%) in pure oxygen, intubated, and mechanically respirated (Harvard Apparatus) for the duration of the experiment. To minimize edema, 0.15 mg dexamethasone was given intramuscularly in the left hindlimb(77). Cannulations of the carotid arteries and a jugular vein (PE-50, polyethylene tubing) allowed for blood pressure readings and administering of fluids, respectively. Arterial blood gases and pH were measured throughout experimentation using an automated blood gas analyzer (50 μL blood samples; Model ABL 5, Radiometer) to ensure variables were maintained within physiological ranges (arterial Po_2 of >80 Torr; arterial PCO_2 of 35-45 Torr; pH, 7.3-7.4). When needed, 1 M $NaHCO_3$, 5% dextrose Ringers solution was infused intravenously at a rate of 2 mL/hr to stabilize the animal(59). During experimentation, body temperature was maintained between 36.5 and 38.0°C by an isothermal pad (Deltaphase). Animals were held in a stereotaxic head unit (Kopf Instruments) during pre-collicular decerebration and all acute experimental procedures. Briefly, the decerebration procedure was conducted by drilling burr holes into the parietal skull and the bone superior to the central sagittal sinus removed. The cerebrum was aspirated until the superior and inferior colliculi were within view. At this point, a pre-collicular section was made and the transectioned forebrain was aspirated rendering the animal insentient. To minimize cranial

bleeding, small pieces of oxidized regenerated cellulose (Ethicon, Johnson & Johnson) were placed on the internal skull surface and the cranial cavity was packed with cotton. Inhalant anesthesia was immediately discontinued after decerebration.

Simulated Muscle Mechanoreflex Activation

Preparation. To selectively activate the mechanically sensitive afferent fibers, the triceps surae muscles of the hindlimb were passively stretched(72). This is a common technique used to simulate MMcR activation as the procedure does not generate muscle metabolites. Animals were secured in a customized spinal frame that allowed the pelvis to be stabilized with steel posts. The right hindlimb was fixed in position by clamping the tibial bone. Finally, the triceps surae muscles were isolated, and the calcaneal bone cut. Then the cut end of the Achilles' tendon was connected to a force transducer (Grass Instruments, FT10), allowing for measurement of hindlimb muscle tension.

Microdialysis Procedures

Preparation. After decerebration, a limited occipital craniotomy was performed to expose the dorsal surface of the brainstem. Microdialysis probes (Bioanalytical Systems, model CMA 11, 0.24 mm outer diameter, 1 mm membrane tip) were stereotaxically positioned unilaterally within the NTS at an area known to receive projections from MMcR afferent fibers (coordinates: 0.5 mm lateral to the obex and 0.5 mm below the dorsal medullary surface)(27, 30, 49, 55). The positioned probe was continuously perfused at a rate of 2.5 µl/min with artificial cerebral spinal fluid (aCSF) buffered to a pH of 7.4. aCSF contained 0.2 % bovine serum albumin, 0.1 % bacitracin, and the following ions (in mM): 6.2 K^+, 134 Cl^-, 2.4 Ca^{2+}, 150 Na^+, 1.3 P^-, 13 HCO_3^- and 1.3 Mg^{2+}. After decerebration and probe insertion, the animal was allowed to stabilize for a minimum of one hour.

Experimental Protocol

Using a calibrated 9.5 mm rack and pinion system (Harvard Apparatus), preferential activation of the MMcR was induced by passively stretching the triceps surae muscles for 30 seconds. The amount of developed hindlimb muscle

tension during passive stretch was matched to the amount produced during maximal hindlimb muscle contraction in WKY and SHR rats (roughly 1200 g)(28, 70). The triceps surae muscles were stretched a minimum of two times with a 15 minute recovery period between each trial. Before all stretch maneuvers, hindlimb muscles were preloaded by stretching to 70-100 g of tension.

The circulatory response to mechanoreflex activation was obtained during the individual administration of aCSF (control), as well as during the dialysis of 1 μM, and 10 μM L-arginine. In these experiments, the microdialysis probe was placed ipsilateral to the hindlimb muscles being stretched. All dialysates were dialyzed a minimum of 45 minutes before simulated MMcR activation, thus ensuring sufficient time for delivery of chemical substances(32). During dialysis of each substance, two reproducible responses were obtained with a minimum of 15 minutes between events. As a control, MMcR function was examined during the dialysis of D-arginine (1 μM and 10 μM) in both SHR (n=8) and WKY (n=8) groups. D-arginine is the inactive enantiomer of L-arginine.

Morphological Measurements and Validation of Probe Placement

At the conclusion of testing procedures, animals were humanely euthanized. The heart, lungs, and triceps surae were excised and wet weights obtained. Tibial length was also measured to assess heart mass / tibial length ratios. Additionally, the brainstem was excised and fixed in 10 % phosphate buffered formalin and stored at 4^0C. Medullary tissue was blocked, and 40 μm sections were cut serially using a cryostat (Cambridge Instruments). Sections were placed on coated slides and examined to establish the neuroanatomical location of probe placement.

Data Acquisition

In all physiologic experiments, baseline as well as reflex changes in MAP, HR, and developed tension were recorded. Baseline values for each measured variable were obtained over a 30 second period prior to reflex activation. The greatest change in each variable from this baseline in response to reflex activation was taken as the peak value.

Statistical Analysis

All cardiovascular and contractile force data were acquired, recorded, and analyzed using data acquisition software (Spike 2, version 3, Cambridge Electronic Design, Ltd) for the CED micro 1401 system (Cambridge Electronic Design Ltd). Data was analyzed by means of paired and unpaired t-tests or analysis of variance (ANOVA) with Student Newman-Keuls multiple comparison tests employed as appropriate. The significance level was set at $P<0.05$. All statistical analysis was performed using Sigma Stat for Windows (SPSS Inc.)

Results

Characterization of Hypertensive Model

Morphometric and hemodynamic baseline data for WKY and SHR animals are presented in Table 1. Ratios of heart weight to both body weight and tibial length were significantly greater in SHR than WKY. However, lung weight/ body weight was not different between the two groups, suggesting that the hypertensive animals were not in heart failure and/or suffering from pulmonary edema. Baseline MAP was significantly higher in SHR (134 ± 6 mmHg) than WKY (95 ± 5 mmHg) animals, but baseline HR data was not different.

Mechanically Sensitive Afferent Fibers Mediate the Overactive Cardiovascular Response to Exercise in Hypertension

Passive hindlimb stretch caused significant increases in MAP and HR from baseline in both SHR and WKY animals. However, MMcR activation caused a significantly greater increase in both MAP and HR in SHR compared to WKY animals. The rise in MAP in SHR rats (59 ± 5 mmHg) was significantly greater than that seen in the WKY group (21 ± 3 mmHg). The increase in HR was also significantly exaggerated in SHR (14 ± 2 bpm) compared to WKY animals (8 ± 1 bpm). The tension produced by the triceps surae muscles in each group was not significantly different, averaging 1136 ± 11 g for WKY and 1161 ± 13 g for SHR.

Increasing NO Production within the NTS of Normotensive and Hypertensive Rats Significantly Attenuates the Cardiovascular Response to Simulated MMcR Activation

Brainstem microdialysis of 1 μM L-arginine, an NO precursor, into the NTS of hypertensive rats significantly attenuated the pressor and tachycardic response to simulated MMcR activation (Figure 1). For example, MAP increased by 64 ± 6 mmHg before delivering NO to the NTS and by 42 ± 6 mmHg after L-arginine delivery during simulated MMcR activation. The HR response to simulated MMcR activation was 12 ± 2 bpm before dialysis of L-arginine into the NTS and 8 ± 1 bpm after. In addition, increasing NO production in the NTS of normotensive WKY rats also had an inhibitory affect on the cardiovascular response to simulated MMcR activation (Figure 1). Before L-arginine dialysis, the pressor and HR responses to hindlimb stretch were 25 ± 4 mmHg and 9 ± 2 bpm, respectively. After brainstem NO was experimentally increased, the cardiovascular responses were decreased to 15 ± 3 mmHg and 5 ± 2 bpm. Hindlimb muscle tension development was not different across groups.

As a control, the inactive isomer D-arginine (1 μM) was dialyzed into the NTS of WKY and SHR animals and simulated MMcR activation was repeated. D-arginine in the brainstem had no affect on the circulatory response to simulated MMcR activation in either group of animals. Specifically, in WKY animals, simulated MMcR activation caused increases in MAP and HR of 29 ± 10 mmHg and 6 ± 4 bpm before D-arginine dialysis and 26 ± 9 mmHg and 5 ± 2 bpm after (Figure 2). In SHR animals, simulated MMcR activation caused increases in MAP and HR of 53 ± 12 mmHg and 9 ± 3 bpm before D-arginine dialysis and 52 ± 14 mmHg and 8 ± 3 bpm after (Figure 3). Hindlimb muscle tension development was similar in all animals, averaging 1111 ± 18 g for WKY and 1132 ± 27 g for SHR.

Increasing the concentration of L-arginine (10 μM) being dialyzed into the NTS also had a significant inhibitory affect on the hemodynamic response to simulated MMcR activation. In WKY rats, the increases in MAP and HR went from 15 ± 3 mmHg and 6 ± 1 bpm before L-arginine dialysis to 8 ± 3 mmHg and 3 ± 1 bpm after (Figure 4). In SHR rats, the absolute decreases were even larger.

In the hypertensive group, increases in MAP and HR caused by hindlimb stretch were 53 ± 7 mmHg and 16 ± 3 bpm before NO was experimentally increased in the NTS and 40 ± 6 mmHg and 11 ± 2 bpm after (Figure 4). Hindlimb muscle tension development was not different across groups. As before, the dialysis of 10 μM D-arginine, the inactive enantiomer of L-arginine, did not affect the cardiovascular response to simulated MMcR activation in WKY (Figure 5) or SHR (Figure 6) groups. Tension developed by the triceps surae muscles was similar for all animals, averaging 1190 ± 7 g in WKY and 1189 ± 8 g in SHR.

L-arginine Displays a Trend for Biphasic Dose Response in WKY and SHR Groups

The attenuation of the pressor response due to simulated MMcR activation was greater in SHR rats compared to WKY animals during the dialysis of both concentrations (1 and 10 μM) of L-arginine. Further, L-arginine appeared to produce a biphasic dose response in both groups of animals, shown in Figure 7. For WKY animals, the pressor response to simulated MMcR activation was decreased by 8 ± 6 mmHg compared to the control response during the dialysis of 1 μM L-arginine. When 10 μM L-arginine was dialyzed into the NTS, the reduction in the MMcR-mediated MAP response was 5 ± 3 mmHg when compared to the control response. These decreases were more pronounced in the hypertensive group. For SHR rats, the pressor response to simulated MMcR activation was attenuated by 22 ± 9 mmHg compared to the control response during the dialysis of 1 μM L-arginine. Increasing the concentration of L-arginine to 10 μM only lowered the pressor response to simulated MMcR activation by 12 ± 7 mmHg in this same group.

Verification of Probe Placement

To verify probe placement, brainstems were excised from the rats and fixed and sliced into 40 μm sections. The slices were then examined in order to visualize probe placement. A schematic of the NTS with probe placement for 5 representative animals is shown in Figure 8. All of the probes were placed

ipsilateral to the stretched hindlimb and were restricted to the medial, dorsomedial, and commissural subdivisions of the NTS, where MMcR afferents have been shown to project(12, 31, 49, 54, 55).

Discussion

In the current investigation, we experimentally increased NO within the NTS and selectively engaged the MMcR in normotensive and hypertensive rats(27). This study produced two major findings concerning MMcR dysfunction in hypertension, which are: (i) increasing NO production in SHR animals partially corrects the exaggerated cardiovascular response to simulated MMcR activation and (ii) the microdialysis of L-arginine into the NTS had a greater absolute effect in hypertensive SHR animals than normotensive WKY animals. In addition, the inactive enantiomer D-arginine in the NTS has no effect on the cardiovascular response to exercise, providing evidence that the L-arginine/NO pathway was activated during L-arginine microdialysis. These findings support our hypothesis that experimentally increasing NO production/availability in the NTS of hypertensive rats will partially correct MMcR dysfunction.

Brainstem NO and Sympathetic Outflow

Our data suggests that increasing NO within the brainstem in hypertensive rats partially corrects the exaggerated cardiovascular response to simulated MMcR activation. It has been previously established that the cardiovascular response to simulated MMcR activation is mediated by the sympathetic nervous system. Therefore, it is logical to suggest that NO within the NTS works to modulate sympathetic outflow during activation of mechanically sensitive afferent fibers. It is our contention that the amount of NO available for biologic activity is reduced in hypertension. As a result, the sympathetic response mediated by the MMcR is exaggerated. Data from the current study supports this contention.

Evidence for Possible Impairment of NO Production within the NTS in Hypertension

In the current study, we found the absolute decrease in the pressor response to simulated MMcR activation during microdialysis of L-arginine into the NTS was greater in hypertensive rats when compared to their normotensive counterparts. This suggests that NO availability or production may be impaired within the NTS of hypertensive animals. The reasons for this impairment are not presently clear. NO deficiency could be caused by an impairment in the L-arginine/NO pathway in hypertension. One possibility is a decrease in the expression or activity of NOS enzyme, which catalyzes the reaction of NO from L-arginine. To date, studies quantifying NOS expression/activity within the brainstem of adult hypertensive rats have been inconclusive, with some showing NOS expression and activity within the medulla to be significantly increased and others reporting basal levels of NOS to be decreased in the NTS when compared to normotensive controls(11, 52, 53, 57, 81). Independent of NOS, other factors such as the presence of reactive oxygen species, known to be increased in specific autonomic nuclei important to cardiovascular regulation within the brainstem of hypertensive rats, could reduce NO availability(25, 74). Superoxide inactivates NO and forms peroxynitrite through the enzymatic action of nicotinamide-adenine dinucleotide phosphate (NAD(P)H) oxidase, which is increased in hypertensive individuals(13). Angiotensin II, a peptide found to be increased in the brainstem of hypertensive rats, has been shown to modulate NAD(P)H oxidase activity, further decreasing the levels of active NO(68, 76). Therefore, it is possible that the availability of endogenous NO within the NTS is reduced through the generation of peroxynitrite via an Angiotensin II/NAD(P)H oxidase mechanism. Most likely, all of these factors are work in concert to have a negative impact on NO production and activity within the brainstem of hypertensive individuals.

NO Affects Biphasic Dose Responses in Multiple Species and Physiological Systems

Our data appeared to display a biphasic dose response to NO microdialysis. The lower concentration (1 μM) of L-arginine dialyzed into the NTS in both groups of animals had a greater inhibitory effect on the pressor response to simulated MMcR activation than did the larger concentration (10 μM). This concentration-dependant phenomena has been shown to occur in other tissues such as bone, the central nervous system, and myocardium where lower doses of NO are protective while higher concentrations reverse these positive effects(6-8, 10, 33, 42, 43, 50, 56). Regardless of the NO donor or precursor used, NO has been shown to exert regulatory influences in a biphasic dose-dependant manner in species such as birds, mice, rats, frogs, rabbits, and humans(1, 8, 38, 66, 73, 79). For example, it has been shown in contracting ventricular myocytes that lower doses of NO have positive ionotropic effects and higher doses induce negative ionotropic effects(42). More importantly, within the central nervous system, NO donors have demonstrated a biphasic dose response on basal levels of and the release of excitatory amino acids in the ventral hippocampus of Wistar rats(66). These observations could explain why central-acting NO has been shown to be both neuroprotective as well as neurotoxic when looking at convulsant behaviors(6, 10, 33, 43, 50, 56). Based on these findings, it seems likely that NO has the ability to mediate both excitatory and inhibitory functions based on its concentration and location within brainstem nuclei(82, 84).

Several possible explanations for the biphasic dose response of NO exist. The first is that NO acts as a negative feedback regulator of its own synthesis(61). Several studies have shown that at high concentrations, NO donors are able to inhibit NOS activity and decrease NO production(14, 61). This inhibition of the L-arginine/NO pathway has been shown to be concentration dependent and reversible(14, 61). Yet another reason for the biphasic dose response of NO is that the molecule has been shown to competitively inhibit oxygen binding to cytochrome P450-linked proteins(1). The enzymatic activity of cytochrome P450s is diverse; they are able to metabolize multiple endogenous and exogenous

substrates and can catalyze multiple reactions. For example, in the mitochondrial electron chain, high concentrations of NO have been shown to inhibit oxygen binding on the P450 enzyme, thus reducing hormone synthesis(1). However, at stimulatory concentrations, NO acted as an electron donor, thus forming NAD(P)H, which supplies the mitochondrial electron chain with accessory proteins and promotes hormone synthesis(1). Of course, in hypertension, NAD(P)H provides another method of NO reduction via formation of superoxide, as previously mentioned. Therefore, the mechanism of the NO biphasic NO response likely involves multiple molecules and is dependent on the cellular location of the reaction.

Limitations

One limitation of the current study is that we were not able to quantify the amount of NO being produced by dialysis of the two concentrations of L-arginine. While techniques to measure NO exist, they can be unreliable and difficult to implement *in vivo*(16, 48). In addition, common methods of NO detection such as the quantification of nitrates and nitrites as well as L-citrulline are better indicators of NOS activity and not specific for NO production(46, 75). When assessing NO concentrations in neural tissue, multiple challenges arise. Sub-nanomolar concentrations of NO have been shown to be effective at modulating neurotransmission within the NTS(82, 84). Further, the half life of NO is only 6-10 seconds in biological systems, therefore its method of action is most likely a quick, highly dynamic process(46, 83). Finally, as mentioned previously, NO may exert different effects on different brainstem nuclei, therefore NO detection methods may require cellular and sub-cellular resolution(83). For these reasons, we chose to dialyze the inactive isomer D-arginine and record the cardiovascular response to simulated MMcR activation as a control experiment. This method allowed us to experimentally confirm that L-arginine increased NO production within the NTS because simulated MMcR activation during D-arginine microdialysis had no effect on the changes in MAP or HR in all rats tested.

Finally, microdialyzing L-arginine into the brainstem is commonly used and has been shown to be a valid method to increase NO production(32, 69).

It is important to note that the method used for simulated MMcR activation, passive muscle stretch, does not activate group III mechanically-sensitive afferent fibers the same way that physiological exercise does. In cats, it has been demonstrated that static muscle contraction and passive hindlimb stretch activate separate, but overlapping, populations of group III afferent fibers(2, 17, 18). However, both contraction and passive muscle stretch produce similar effects on neuronal discharge rate and hemodynamic parameters in these animals(17, 18). In addition, hindlimb stretch has been shown to increase discharge activity in the same efferent neurons responsive to hindlimb contraction(17). As such, passive muscle stretch is a common technique for MMcR activation.

It is of note that evidence supporting brainstem NO as a neurotransmitter that modulates sympathetic outflow has been derived from studies in which NO levels were altered within the NTS and changes in basal hemodynamics occurred(29, 58, 78, 80, 86, 87). In our experiments, however, baseline blood pressure and heart rate were not affected by the two concentrations of L-arginine dialyzed into the NTS of normotensive or hypertensive rats. An explanation for these differences is the method and duration of NO donor delivery, as well as the concentrations and type of NO precursors being given. While we did not see changes in basal MAP and HR in our experiments, the studies showing increased NO within the NTS lowers baseline blood pressure, HR, ventilation rate, and renal sympathetic nerve activity only confirm that NO is a powerful inhibitor of medullary sympathetic outflow and has great therapeutic potential in hypertension(29, 78, 80, 87).

Conclusions and Clinical Significance

In conclusion, we have provided evidence that increasing NO production/availability within the NTS may help attenuate MMcR dysfunction in hypertension. The finding that NO within the NTS decreases the cardiovascular response to MMcR activation may lead to therapeutic treatments that allow

individuals with hypertension to participate in a range of physical activities without the risk for an adverse cardiac event.

Acknowledgements

This research was supported by grants from the National Institutes of Health (HL-094075 to A.K. Leal and HL-088422 to S.A. Smith) and the Lawson & Rogers Lacy Research Fund in Cardiovascular Diseases (to J.H. Mitchell). The authors thank Martha Romero and Julius Lamar, Jr. for their expert technical assistance.

References

1. Adams JS and Ren S-Y. Autoregulation of 1,25-dihydroxyvitamin D synthesis in macrophage mitochondria by nitric oxide. *Endocrinology* 137: 4514-4517, 1996.
2. Adreani CM, Hill JM, and Kaufman MP. Responses of group III and IV muscle afferents to dynamic exercise. *J App Physiol* 82: 1811-1817, 1997.
3. Akinpelu AO. Responses of the African hypertensive to exercise training: preliminary observations. *J Hum Hypertens* 4: 74-76, 1990.
4. Alam M and Smirk FH. Observations in man upon a blood pressure raising reflex arising from the voluntary muscles. *J Physiol* 89: 372-383, 1937.
5. Aoki K, Sato K, Kondo S, Pyon C, and Yamamoto M. Increased response of blood pressure to rest and handgrip in subjects with essential hypertension. *Jpn Circ J* 47: 802-809, 1983.
6. Baggetta G, Iannone M, Scorsa AM, and Nistic G. Tacrine-induced seizures and brain damage in LiCl-treated rats can be prevented by N^w-nitro-L-arginine methyl ester. *Eur J Pharmacol* 213: 301-304, 1992.
7. Calabrese EJ. Nitric oxide: biphasic dose response. *Crit Rev Toxicol* 31: 489-501, 2001.
8. Chae H-J, Park R-K, Kang J-S, Shin H-S, Kim S-C, Chung H-T, Son D-W, Ko K-I, Kim J-B, Park Y-C, and Kim H-R. Effect of stem cell factor, interleukin-6, nitric oxide and transforming growth factor-B on the osteoclast differentiation induced by 1 α,25-$(OH)_2D_3$ in primary murine bone marrow cultures. *Pharmacol Toxicol* 82: 223-229, 1998.
9. Cononie CC, Graves JE, Pollock ML, Phillips MI, Sumners C, and Hagberg JM. Effect of exercise training on blood pressure in 70- to 79-yr-old men and women. *Med Sci Sports Exerc* 23: 505-511, 1991.
10. DeSorro G, DiPaola ED, Sarro AD, and Vidal MJ. L-arginine potentiates excitatory amino acid-induced seizures elicited by the deep prepiriform cortex. *Eur J Pharmacol* 230: 151-158, 1993.
11. Ferrari MFR and Fior-Chadi DR. Differential expression of nNOS mRNA and protein in the nucleus tractus solitarii of young and aged Wistar-Kyoto and spontaneously hypertensive rats. *J Hypertens* 23: 1683-1690, 2005.

12. Gamboa-Esteves FO, Tavares I, Almeida A, Batten TFC, McWilliam PN, and Lima D. Projection sites of superficial and deep spinal dorsal horn cells in the nucleus tractus solitarii of the rat. *Brain Res* 921: 195-205, 2001.
13. Griendling KK, Sorescu D, and Ushio-Fukai M. NAD(P)H oxidase. *Circ Res* 86: 494-501, 2000.
14. Griscavage JM, Rogers NE, Sherman MP, and Ignarro LJ. Inducible nitric oxide synthase from a rat alveolar macrophage cell line is inhibited by nitric oxide. *J Immunol* 151: 6329-6337, 1993.
15. Group NHBPEPW. National high blood pressure education program working group on prevention of hypertension. *Arch Intern Med* 153: 186-208, 1993.
16. Halbach OvBu. Nitric oxide imagine in living neuronal tissues using fluorescent probes. *Nitric Oxide* 9: 217-228, 2003.
17. Hayes SG and Kaufman MP. Gadolinium attenuates exercise pressor reflex in cats. *Am J Physiol* 280: 2153-2161, 2001.
18. Hayes SG, Kindig AE, and Kaufman MP. Comparison between the effect of static contraction and tendon stretch on the discharge of group III and IV muscle afferents. *J Appl Physiol* 99: 1891-1896, 2005.
19. Hironaga K, Hirooka Y, Matsuo I, Shihara M, Tagawa T, Harasawa Y, and Takeshita A. Role of endogenous nitric oxide in the brain stem on the rapid adaptation of baroreflex. *Hypertension* 31: 27-31, 1998.
20. Hoberg E, Schuler G, Kunze B, Obermoser AL, Hauer K, Mauther HP, Schlierf G, and Kubler W. Silent myocardial ischemia as a potential link between lack of premonitoring symptoms and increased risk of cardiac arrest during physical stress. *Am J Cardiol* 65: 583-589, 1990.
21. Kahn JF. The static exercise-induced arterial hypertension test. *Presse Medicine* 20: 1067-1071, 1991.
22. Kaufman MP and Forster HV. Reflexes controlling circulatory, ventilatory and airway responses to exercise. In: *Section 12, Exercise: Regulation and Integration of Multiple Systems*. Bethesda, MD: Am Physiol Soc, 1996, p. 381-447.
23. Kaufman MP, Longhurst JC, Rybicki KJ, Wallach JH, and Mitchell JH. Effects of static muscular contraction on impulse activity of groups III and IV afferents in cats *J App Physiol* 55: 105-112, 1983.
24. Kaufman MP, Waldrop TG, Rybicki KJ, Ordway GA, and Mitchell JH. Effects of static and rhythmic twitch contractions on the discharge of group III and IV muscle afferents. *Card Res* 18: 663-668, 1984.
25. Kishi T, Hirooka Y, Kimura Y, Ito K, Shimokawa H, and Takeshita A. Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats. *Circulation* 109: 2357-2362, 2004.
26. Kokkinos PF, Narayan P, Colleran J, Pittaras A, Notargiacomo A, and al e. Effects of regular exercise on blood pressure and left ventricular hypertrophy in African-American men with severe hypertension. *N Engl J Med* 333: 1462-1467, 1995.

27. Leal A, Mitchell J, and Smith S. A role for nitric oxide within the nucelus tractus solitarius in the development of muscle mechanoreflex dysfunction in hypertension. *Am J Physiol* submitted, 2009.
28. Leal AK, Williams MA, Garry MG, Mitchell JH, and Smith SA. Evidence for functional alterations in the skeletal muscle mechanoreflex and metaboreflex in hypertensive rats. *Am J Physiol Heart Circ Physiol* 295: H1429-H1438, 2008.
29. Lewis SJ, Ohta H, Machado B, Bates JN, and Talman WT. Microinjection of S-nitrosocysteine into the nucleus tractus solitarii decreases arterial pressure and heart rate via activation of soluble guanylate cyclase. *Eur J Pharmacol* 202: 135-136, 1991.
30. Li J. Nitric oxide synthase (NOS) coexists with activated neurons by skeletal muscle contraction in the brainstem of cats. *Life Sciences* 71: 2833-2843, 2002.
31. Li J and Mitchell JH. Role of NO in modulating neuronal activity in superficial dorsal horn of spinal cord during exercise pressor reflex. *Am J Physiol* 283: H1012-H1018, 2002.
32. Li J and Potts JT. NO formation in nucleus tractus solitarii attenuates pressor response evoked by skeletal muscle afferents. *Am J Physiol* 280: H2371-H2379, 2001.
33. Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HSV, Sucher SJ, Loscalzo J, Singel DJ, and Stamler JS. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 364: 626-632, 1993.
34. MacMahon S, Peto R, Collins R, Godwin J, Cutler J, Sorlie P, Abbott R, Neaton J, Dyer A, and Stamler J. Blood pressure, stroke, and coronary heart disease. Part 1, prolonged differences in blood pressure: prospective observational studies corrected for the regression dilution bias. *The Lancet* 335: 765-774, 1990.
35. Manolis AJ, Beldekos D, Hatzissavas J, Foussas S, Cokkinos D, Bresnahan M, Gavras I, and Gavras H. Hemodynamic and humoral correlates in essential hypertension: Relationship between patterns of LVH and myocardial ischemia. *Hypertension* 30: 730-734, 1997.
36. Martin JE, Dubbert PM, and Cushman WC. Controlled trial or aerobic exercise in hypertension. *Circulation* 81: 1560-1567, 1990.
37. McCloskey DI and Mitchell JH. Reflex cardiovascular and respiratory responses originating in exercising muscle. *Journal of Physiology* 224: 173-186, 1972.
38. Mery P-F, Pavoine C, Belhassen L, Pecker F, and Fischmeister R. Involvement of cGMP-inhibited and cGMP-stimulated phosphodiesterases through guanylyl cyclase activation. *J Biol Chem* 268: 26286-26295, 1993.
39. Minami N, Yoshikawa T, Kataoka H, Mori N, Nagasaka M, Kurosawa H, Kanazawa M, and Kohzuki M. Effects of exercise and beta-blocker on blood pressure and baroreflexes in spontaneously hypertensive rats. *Am J Hypertens* 16: 966-972, 2003.
40. Mitchell JH, Kaufman MP, and Iwamoto GA. The exercise pressor reflex: Its cardiovascular effects, afferent mechanisms, and central pathways. *Ann Rev Physiol* 45: 229-242, 1983.

41. Mittleman M and Siscovick D. Physical exertion as a trigger of myocardial infarction and sudden cardiac death. *Cardiol Clin* 14: 263-270, 1996.
42. Mohan P, Brutsaert DL, Paulus WJ, and Sys SU. Myocardial contractile response to nitric oxide and cGMP. *Circ Res* 93: 1223-1229, 1996.
43. Mollace V, Baggetta G, and Nistic G. Evidence that L-arginine possesses proconvulsant effects mediated through nitric oxide. *NeuroReport* 2: 869-872, 1991.
44. Moncada S and Higgs A. The L-arginine-nitric oxide pathway. *New Eng J Med* 329: 2002-2012, 1993.
45. Mundal R, Kjeldsen SE, Sandvik L, Erikssen G, Thaulow E, and Erikssen J. Exercise blood pressure predicts mortality from myocardial infarction. *Hypertension* 27: 324-329, 1996.
46. Nagano T. Practical methods for detection of nitric oxide. *Luminescence* 14: 283-290, 1999.
47. Paton JFR, Deuchars J, Ahmad Z, Wong LF, Murphy D, and Kasparov S. Adenoviral vector demonstrates that angiotensin II-induced depression of the cardiac baroreflex is mediated by endothelial nitric oxide synthase in the nucleus tractus solitarii of the rat. *J Physiol* 531, 2001.
48. Paton JFR, Lonergan T, Deuchars J, James PE, and Kasparov S. Detection of angiotensin II mediated nitric oxide release within the nucleus of the solitary tract using electron-paramagnetic resonance (EPR) spectroscopy. *Autonomic Neuroscience: Basic and Clinical* 126-127: 193-201, 2006.
49. Paxinos G and Watson C. *The Rat Brain in Stereotaxic Coordinates*. Orlando, FL: Academic, 1986.
50. Penix LP, Davis W, and Subramanian S. Inhibition of NO synthase increases the severity of kainic acid-induced siezures in rodents. *Epilepsy Res* 18: 177-184, 1994.
51. Pickering TG. Pathophysiology of exercise hypertension. *Herz* 12: 119-124, 1987.
52. Plochocka-Zulinska D and Krukoff TL. Increased gene expression of neuronal nitric oxide synthase in brain of adult spontaneously hypertensive rats. *Brain Res Mol Brain Res* 48: 291-297, 1997.
53. Pontieri V, Venezuela MK, Scavone C, and Michelini LC. Role of endogenous nitric oxide in the nucleus tractus solitarii on baroreflex control of heart rate in spontaneously hypertensive rats. *J Hypertens* 16: 1993-1999, 1998.
54. Potts JT, Lee SM, and Anguelov PI. Tracing of projection neurons from the cervical dorsal horn to the medulla with the anterograde tracer biotinylated dextran amine. *Aut Neurosci* 98: 64-69, 2002.
55. Potts JT, Paton JFR, Mitchell JH, Garry MG, Kline G, Anguelov PT, and Lee SM. Contraction-sensitive skeletal muscle afferents inhibit arterial baroreceptor signalling in the nucleus of the solitary tract: role of intrinsic GABA interneurons. *Neuroscience* 119: 201-214, 2003.
56. Przegalinski E, Baran L, and Siwanowicz J. The role of nitric oxide in the kainate-induced seizures in mice. *Neurosci Lett* 170: 74-76, 1994.

57. Qadri F, Arens T, Schwarz EC, Hauser W, Dendorfer A, and Dominiak P. Brain nitric oxide synthase activity in spontaneously hypertensive rats during the development of hypertension. *J Hypertens* 21: 1623-1624, 2003.
58. Qadri F, Carretero OA, and Scicli AG. Centrally produced neuronal nitric oxide in the control of baroreceptor reflex sensitivity and blood pressure in normotensive and spontaneously hypertensive rats. *Jpn J Pharmacol* 81: 279-285, 1999.
59. Quintin L, Gillon JY, Saunier CF, and Ghignone M. Continuous volume infusion improves circulatory stability in anesthetized rats. *J Neurosci Meth* 30: 77-83, 1989.
60. Rogers MW, Probst MM, Gruber JJ, Berger R, and Boone JB. Differential effects or exercise training intensity on blood pressure and cardiovascular responses to stress in borderline hypertensive humans. *J Hypertens* 14: 1375-1399, 1996.
61. Rogers NE and Ignarro LJ. Constitutive nitric oxide synthase from cerebellum is reversibly inhibited by nitric oxide formed from L-arginine. *Biochemical and Biophysical Research Communications* 189: 242-249, 1992.
62. Roman MJ, Pickering TG, Pini R, Schwartz JE, and Devereux RB. Prevalence and determinants of cardiac and vascular hypertrophy in hypertension. *Hypertension* 26: 369-373, 1995.
63. Ruggiero DA, Mtui EP, Otake K, and Anwar M. Central and primary visceral afferents to nucleus tractus solitarii may generate nitric oxide as a membrane-permeant neuronal messenger. *J Comp Neurol* 361: 51-67, 1996.
64. Seals DR and Reiling MJ. Effect of regular exercise on 24-hour arterial pressure in older hypertensive humans. *Hypertension* 18: 583-592, 1991.
65. Seals DR, Silverman HG, Reiling MJ, and Davy KP. Effect of regular aerobic exercise on elevated blood pressure in postmenopausal women. *Am J Cardiol* 80: 49-55, 1997.
66. Segieth J, Getting SJ, Biggs CS, and Whitton PS. Nitric oxide regulates excitatory amino acid release in a biphasic manner in freely moving rats. *Neurosci Lett* 84: 427-434, 1995.
67. Seguro C, Sau F, Zedda N, Scano G, and Cherchi A. Arterial blood pressure behavior during progressive muscular exercise in subjects with stable arterial hypertension. *Cardiologia* 36: 867-877, 1991.
68. Senanayake PD, Moriguchi A, Kumagai H, Ganten D, Ferrario CM, and Brosnihan KB. Increased expression of angiotensin peptides in the brain of transgenic hypertensive rats. *Peptides* 15: 919-926, 1994.
69. Smith SA, Mitchell JH, and Li J. Independent modification of baroreceptor and exercise pressor reflex function by nitric oxide in nucleus tractus solitarius. *Am J Physiol* 288: 2068-2076, 2005.
70. Smith SA, Williams MA, Leal AK, Mitchell JH, and Garry MG. Exercise pressor reflex function is altered in spontaneously hypertensive rats. *J Physiol* 577: 1009-1020, 2006.
71. Stamler J, Stamler R, and Neaton J. Blood pressure, systolic and diastolic, and cardiovascular risks. *Arch Intern Med* 153: 598-615, 1993.

72. Stebbins CL, Brown B, Levin D, and Longhurst JC. Reflex effect of skeletal muscle mechanoreceptor stimulation on the cardiovascular system. *J App Physiol* 65: 1539-1547, 1988.
73. Suzuki S, Takeshita A, Imaizumi T, Hirooka Y, Yoshida M, Ando S, and Nakamura M. Biphasic forearm vascular responses to intraarterial arginine vasopressin. *J Clin Investigation* 84: 427-434, 1989.
74. Tai M-H, Wang L-L, Wu KLH, and Chan JYH. Increased superoxide anion in rostral ventrolateral medulla contributes to hypertension in spontaneously hypertensive rats via interactions with nitric oxide. *Free Radical Biology & Medicine* 38: 450-462, 2005.
75. Tarpey MM, Wink DA, and Grisham MB. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. *Am J Physiol Regul Integr Comp Physiol* 286: R431-R444, 2003.
76. Teruya H, Muratani H, Takishita S, Sesoko S, Matayoshi R, and Fukiyama K. Brain angiotensin II contributes to the development of hypertension in Dahl-Iwai salt-sensitive rats. *J Hypertens* 13: 883-890, 1995.
77. Tian G and Duffin J. Spinal connections of ventral-group bulbospinal inspiratory neurons studied with cross-correlation in the decerebrate rat. *Exp Brain Res* 111: 178-186, 1996.
78. Tseng C-J, Liu H-Y, Lin H-C, Ger L-P, Tung C-S, and Yen M-H. Cardiovascular effects of nitric oxide in the brain stem nuclei of rats. *Hypertension* 27: 36-42, 1996.
79. VanUffelen BE, Zee JVd, Koster BMd, VanSteveninck J, and Elferink JG. Sodium azide enhances neutophil migration and exocytosis: involvement of nitric oxide, cyclic GMP and calcium. *Life Sci* 63: 645-657, 1998.
80. Vitagliano S, Berrino L, D'Amico M, Maione S, Vovellis VD, and Rossi F. Involvement of nitric oxide in cardiorespiratory regulation in the nucleus tractus solitarius. *Neuropharmacology* 35: 625-631, 1996.
81. Waki H, Murphy D, Yao ST, Kasparov S, and Paton JFR. Endothelial NO synthase activity in nucleus tractus solitarii contributes to hypertension in spontaneously hypertensive rats. *Hypertension* 48: 644-650, 2006.
82. Wang DH. The vanilloid receptor and hypertension. *Acta Pharmacologica Sinica* 3: 286-294, 2005.
83. Wang S, Paton JFR, and Kasparov S. The challenge of real-time measurements of nitric oxide release in the brain. *Autonomic Neuroscience: Basic and Clinical* 126-127: 59-67, 2006.
84. Wang Y and Wang DH. A novel mechanism contributing to development of Dahl salt-sensitive hypertension: role of the Transient Receptor Potential Vanilloid Type 1 *Hypertension* 47: 609-614, 2006.
85. Williamson JW, Mitchell JH, Olesen HL, Raven PB, and Secher NH. Reflex increases in blood pressure induced by leg compression in man. *J Physiol* 475: 351-357, 1994.
86. Zanzinger J, Czachurski J, and Seller H. Effects of nitric oxide on sympathetic baroreflex transmission in the nucleus tractus solitarii and caudal ventrolateral medulla in cats. *Neurosci Lett* 197: 199-202, 1995.

87. Zanzinger J, Czachurski J, and Seller H. Inhibition of basal and reflex-mediated sympathetic activity in the RVLM by nitric oxide. *Am J Physiol Reg Int Comp Physiol* 268: R958-R962, 1995.

Table

	WKY	SHR
n	28	28
Body weight, g	338±6	370±6*
Heart weight/body weight, mg/g	2.9±0.1	3.3±0.1*
Lung weight/body weight, mg/g	6.8±0.3	7.3±0.3
Heart weight/tibial length, mg/mm	25.6±0.7	31.4±0.6*
MAP, mmHg	95±5	134±6*
HR, beats/min	432±9	424±11

Table 1. Morphometric characteristics and baseline hemodynamics. Data are means ± S.E.M. MAP, mean arterial pressure; HR, heart rate. * Significantly different from WKY. $P<0.05$.

Figures

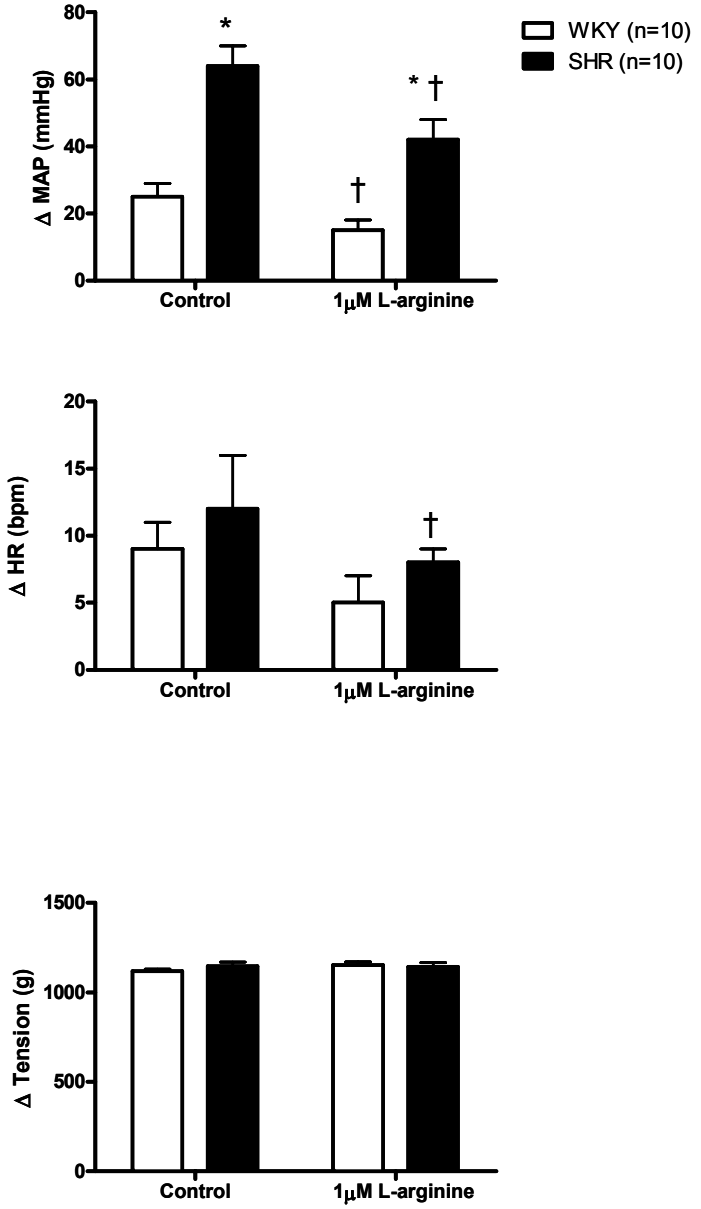

Figure 1. Cardiovascular responses to activation of mechanically sensitive afferent fibers during 1 μM L-arginine dialysis in WKY and SHR animals. Passive stretch of hindlimb skeletal muscle during dialysis of 1 μM L-arginine induced significant decreases in MAP in both SHR and WKY animals and a significant decrease in HR in SHR rats at maximal levels of tension development. In addition, the pressor response to hindlimb muscle stretch was significantly greater in SHR animals compared to WKY. * Significantly different from WKY rats. † Significantly different from Control response. $P<0.05$.

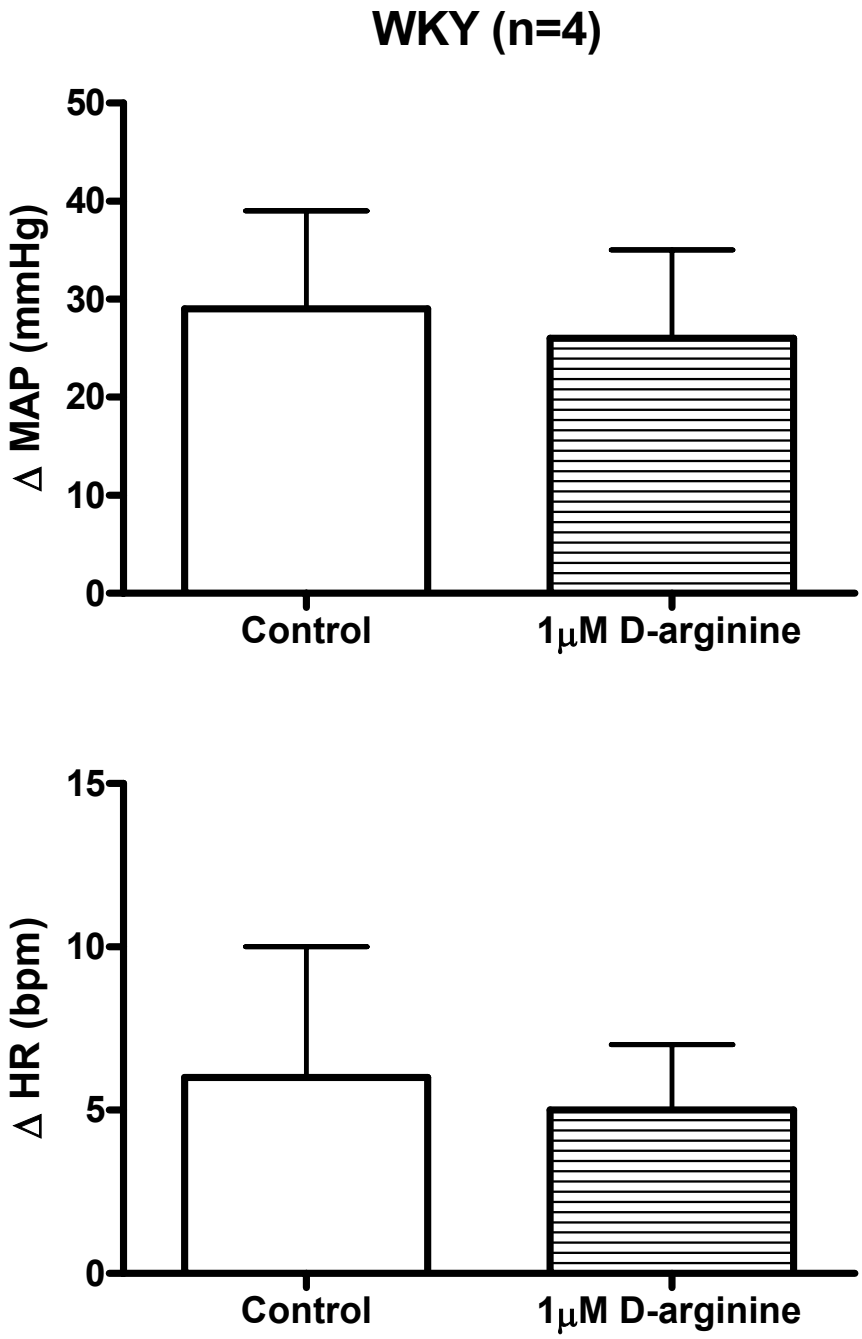

Figure 2. Cardiovascular responses to activation of mechanically sensitive afferent fibers during dialysis of D-arginine in WKY animals. Dialysis of the inactive isomer D-arginine had no effect on the MAP and HR response to passive stretch of hindlimb skeletal muscle.

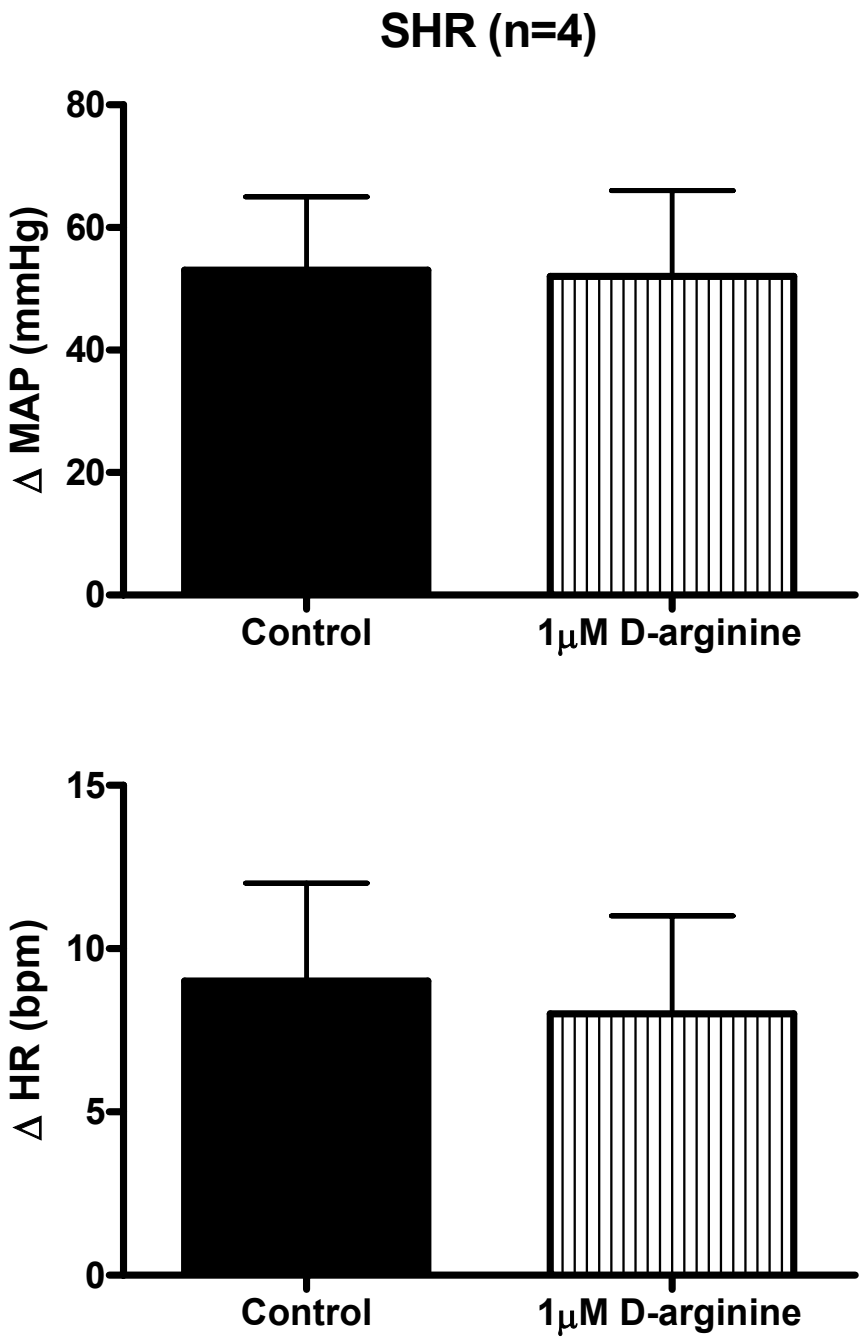

Figure 3. Cardiovascular responses to activation of mechanically sensitive afferent fibers during dialysis of D-arginine in SHR animals. Dialysis of the inactive isomer D-arginine had no effect on the MAP and HR response to passive stretch of hindlimb skeletal muscle.

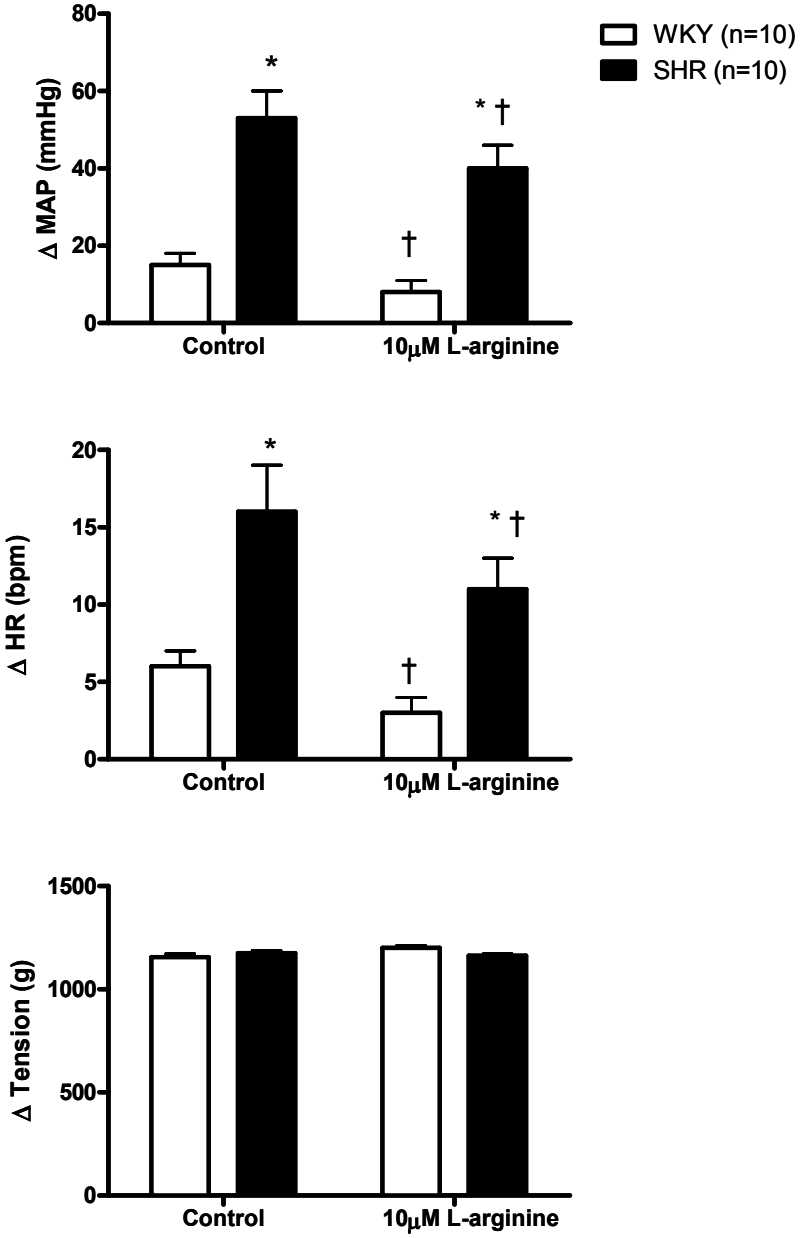

Figure 4. Cardiovascular responses to activation of mechanically sensitive afferent fibers during 10 μM L-arginine dialysis in WKY and SHR animals. Passive stretch of hindlimb skeletal muscle during dialysis of 10 μM L-arginine caused significant decreases in MAP and HR in both SHR and WKY animals. In addition, the MAP and HR responses to hindlimb muscle stretch were significantly greater in SHR rats compared to WKY. * Significantly different from WKY rats. † Significantly different from Control response. $P<0.05$.

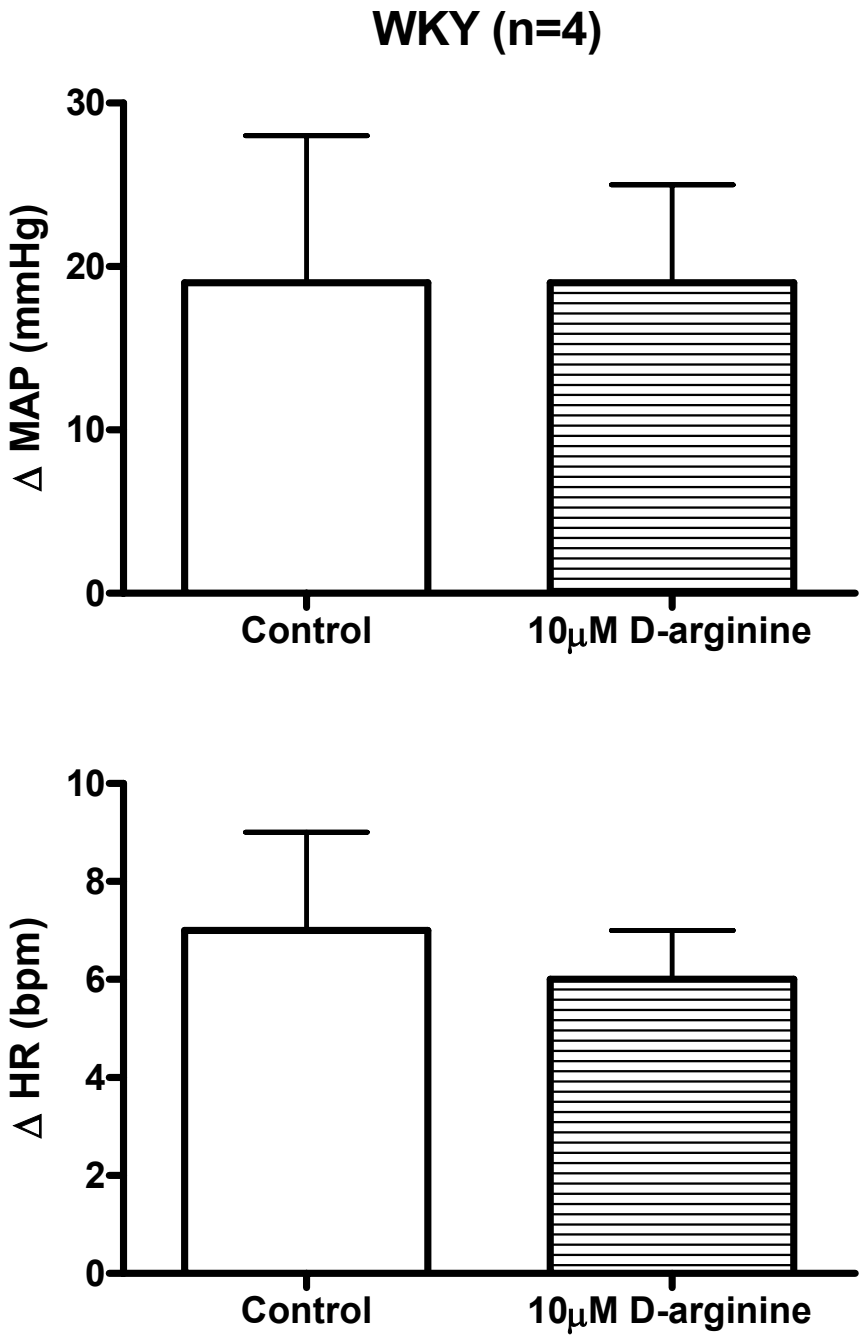

Figure 5. Cardiovascular responses to activation of mechanically sensitive afferent fibers during dialysis of D-arginine in WKY animals. Dialysis of the inactive isomer D-arginine had no effect on the MAP and HR response to passive stretch of hindlimb skeletal muscle.

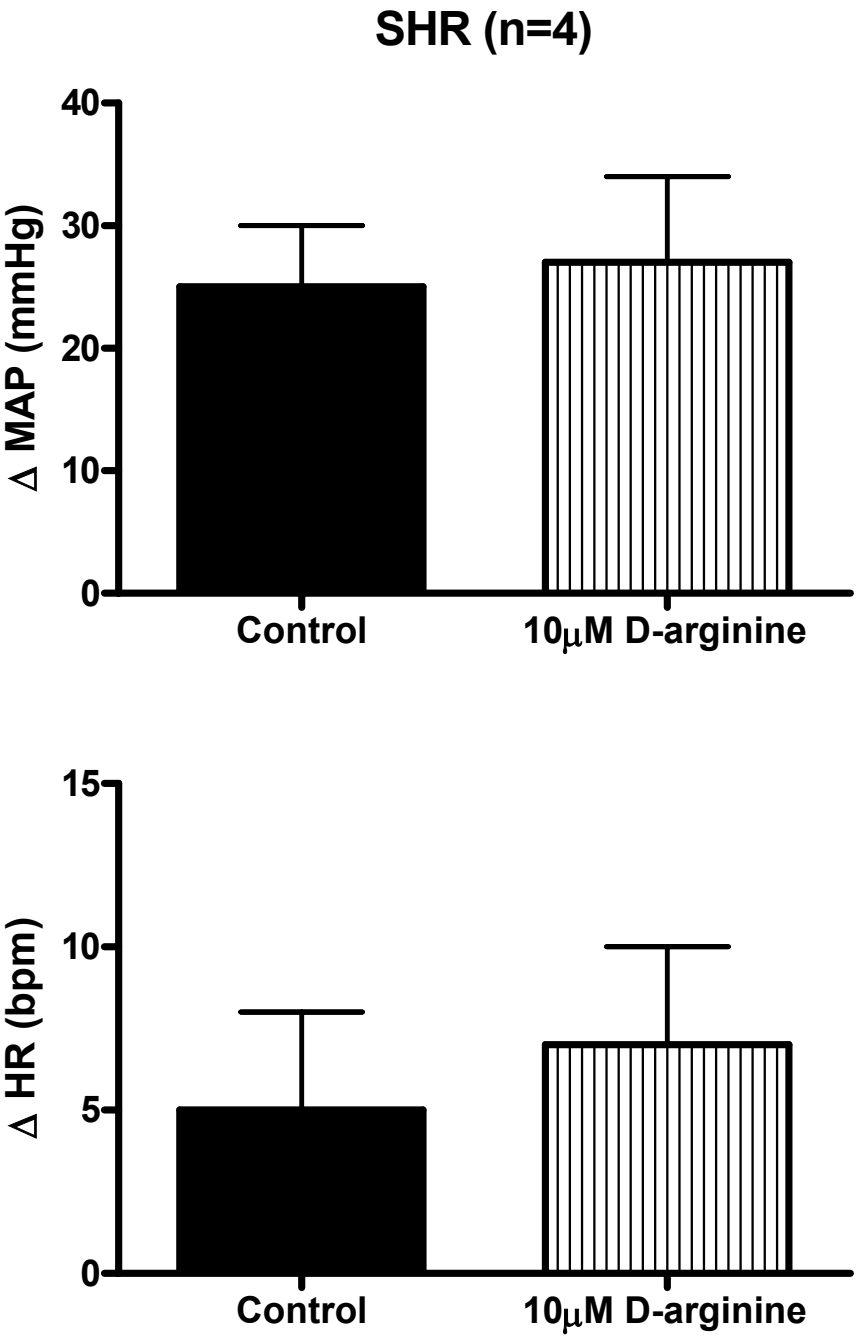

Figure 6. Cardiovascular responses to activation of mechanically sensitive afferent fibers during dialysis of D-arginine in SHR animals. Dialysis of the inactive isomer D-arginine had no effect on the MAP and HR response to passive stretch of hindlimb skeletal muscle.

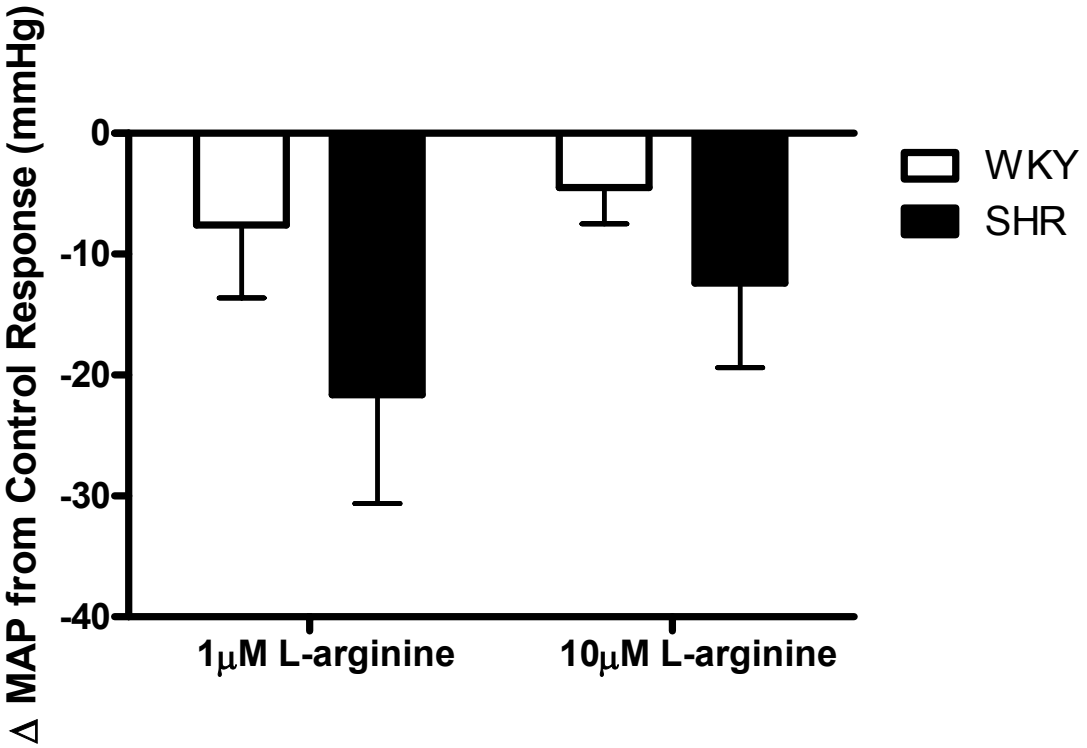

Figure 7. L-arginine displays a biphasic dose response in WKY and SHR groups. Attenuation of the pressor response to passive hindlimb muscle stretch appears optimal during microdialysis of 1 μM L-arginine in both SHR and WKY groups.

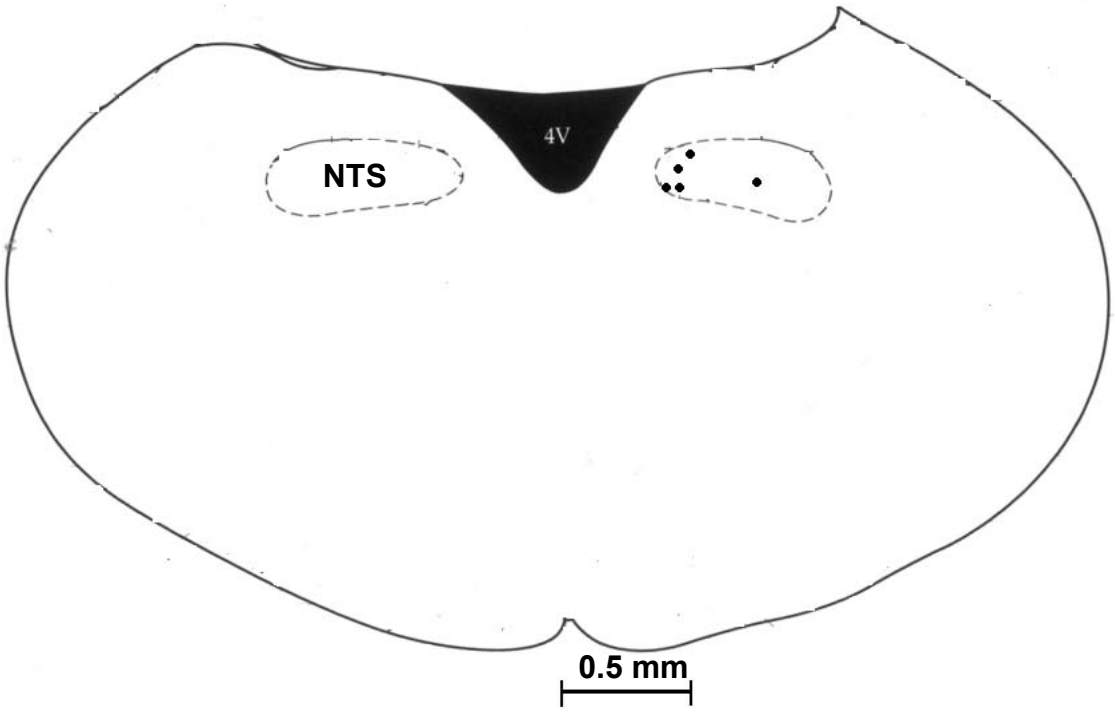

Figure 8. Microdialysis of probe placement in five representative animals. Probe was placed 0.5 mm lateral to the fourth ventricle (4V) and 0.5 mm below the medullary surface. Probe placement within the nucleus tractus solitarius (NTS) was identified in excised medullary tissue after experimentation and is shown by dots in schematic.

ALTERED CIRCULATORY CONTROL BY METABOLICALLY SENSITIVE SKELETAL MUSCLE AFFERENT FIBERS IN HYPERTENSION: A ROLE FOR CENTRAL-ACTING NITRIC OXIDE

Running Title – Role of NO in metaboreflex dysfunction in hypertension

Anna K. Leal, MS[1]; Jere H. Mitchell, MD[2] and Scott A. Smith, PhD[3]

Departments of Biomedical Engineering[1], Internal Medicine[2] and Physical Therapy[3]

University of Texas Southwestern Medical Center

Dallas, Texas, USA 75390-9174

Corresponding Author:

Anna K. Leal, MS
University of Texas Southwestern Medical Center
Southwestern Allied Health Sciences School
Department of Biomedical Engineering
5323 Harry Hines Boulevard
Dallas, Texas 75390-9174
214-648-9188 (office)
214-648-3566 (facsimile)
anna.leal@utsouthwestern.edu

Abstract

The muscle metaboreflex, a feedback mechanism originating in skeletal muscle, increases mean arterial pressure (MAP) and heart rate (HR) in response to activation of metabolically sensitive afferent fibers. This reflex is overactive in hypertension, producing exaggerated increases in MAP and HR in response to exercise. Metaboreflex afferent fibers project to the nucleus tractus solitarius (NTS) in the brainstem where nitric oxide (NO) is produced via the enzymatic activity of nitric oxide synthase (NOS). NO is involved in metaboreflex central processing and has been shown to modulate reflex-driven changes in MAP and HR. Therefore, we hypothesized that NO production/availability is involved in mediating metaboreflex dysfunction in hypertension. To test our hypothesis, we microdialyzed L-NAME, a NOS inhibitor, and L-arginine, the NO precursor, into the NTS of normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive (SHR) rats while stimulating metabolically sensitive afferent fibers in muscle. We found that blocking NO production within the NTS in WKY and SHR rats increased the cardiovascular response to activation of metabolically sensitive afferents, with the effect being greater in SHR animals. Importantly, increasing the amount of endogenous NO within the NTS of SHR rats normalized the exaggerated cardiovascular response to activation of metabolically sensitive afferent neurons. These findings suggest that a decrease in NO production/availability within the brainstem contributes to metaboreflex dysfunction in hypertension. This research could form the basis of treatment options for hypertensive individuals, allowing them to engage in more beneficial physical activity.

Key Words: blood pressure, heart rate, exercise

Introduction

The cardiovascular response to physical activity is partially mediated by the exercise pressor reflex, a feedback mechanism originating in skeletal muscle that responds to contracting muscle by increasing mean arterial pressure (MAP), heart rate (HR), and ventilation(1, 37). This reflex is composed of two afferent components; the muscle mechanoreflex and the metaboreflex. The mechanoreflex consists of predominantly mechanically sensitive group III afferent neurons that are sensitive to muscle stretch and pressure while the metaboreflex consists of predominantly metabolically sensitive group IV afferent neurons that are activated by the metabolites produced by working skeletal muscle(20-22, 35, 51, 55). Metaboreflex activation is thought to signal an oxygen deficit to contracting muscle caused by reduced blood flow during exercise. This theory is supported by the finding that group IV afferent fibers do not begin to fire until 4-10 seconds after the onset of contraction, increasing their discharge rate steadily until contraction ceases(21, 22). In addition, group IV afferent fibers terminate in the walls of capillaries and venules within skeletal muscle advantageously positioned to detect metabolic changes within the surrounding tissue(3). Both the mechanoreflex and metaboreflex work together to increase cardiovascular parameters during exercise and ensure active muscle is sufficiently oxygenated during the exercise bout.

In hypertensive individuals, exercise produces exaggerated increases in MAP and HR. The exercise pressor reflex has been implicated as one mechanism that is responsible for this augmented response(4, 23, 47, 49). A previous study from our laboratory provided evidence that both afferent arms of the exercise pressor reflex, the mechanoreflex and metaboreflex, may mediate this hyper responsiveness(28, 49). In addition, we have recently shown that the mechanoreflex dysfunction manifest in hypertension is partially caused by alterations in the function of brainstem nitric oxide (NO), a neurotransmitter involved in exercise pressor reflex central processing(26, 27).

Both mechanoreflex and metaboreflex afferent neurons project to the nucleus tractus solitaries (NTS) within the medulla oblongata(13, 34, 42). In this

nucleus, L-arginine is oxidized by nitric oxide synthase (NOS) to produce NO and L-citrulline(38). Research has shown that within the NTS, NO has the ability to modulate the sympathetically-mediated cardiovascular response to exercise pressor reflex activation in cats(8, 18, 25, 32, 39, 41, 44, 46, 48, 60). Specifically, NO production reduces the extent to which sympathetic outflow is increased by the exercise pressor reflex. We recently demonstrated that blocking the endogenous production of NO in the NTS in normotensive Wistar-Kyoto (WKY) rats recapitulated the exaggerated cardiovascular response to muscle mechanoreflex activation seen in spontaneously hypertensive (SHR) rats(27). In addition, we experimentally increased NO production by dialyzing the NO precursor, L-arginine, into the NTS of hypertensive rats(26). This procedure significantly attenuated the increases in MAP and HR in response to mechanoreflex activation in the hypertensive animals(26). While these findings provided evidence that the mechanism of mechanoreflex overactivity in hypertension is partly due to alterations in brainstem neurotransmission, similar conclusions cannot yet be drawn for the muscle metaboreflex. Therefore, the goal of this investigation was to examine the role of brainstem NO in mediating the circulatory response to activation of metabolically sensitive afferent fibers in hypertension.

Given our previous findings that endogenous NO within NTS is partially responsible for muscle mechanoreflex dysfunction in hypertension, we hypothesized that central-acting NO is also involved in mediating the exaggerated cardiovascular response to metaboreflex activation in hypertensive rats. The purpose of the current study was to perform microdialysis to block endogenous NO production within the NTS in normotensive WKY rats and determine if an exaggerated metaboreflex response, as seen in hypertension, could be reproduced. We next performed microdialysis to deliver the NO precursor, L-arginine, to the NTS in hypertensive SHR rats in an attempt to normalize the accentuated cardiovascular response to metaboreflex activation. Determining the involvement of NO in the central processing of the muscle metaboreflex is important to the

development of affective treatments for exercise pressor reflex dysfunction in hypertension.

Materials and Methods

Subjects

Experiments were performed in 44 Wistar-Kyoto (WKY) and 42 spontaneously hypertensive (SHR) age-matched (14-20 week old) male rats (Harlan, Indianapolis, IN). Animals were housed in standard rodent cages on 12-h light-dark cycles and were given food and water *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas at Southwestern Medical Center. In addition, all studies were conducted in accordance with the United States Department of Health and Human Services National Institutes of health Guide for the Care and Use of Laboratory Animals.

General Surgical Procedures

Rats were anesthetized with isoflurane gas (2-3%) in pure oxygen, intubated, and mechanically respirated (Harvard Apparatus) for the duration of the experiment. To minimize edema, 0.15 mg dexamethasone was given intramuscularly in the left hindlimb(53). Both carotid arteries and a jugular vein were catheterized (PE-50, polyethylene tubing) for blood pressure readings and fluid administration, respectively. When animals needed stabilization, 1 M $NaHCO_3$, 5% dextrose Ringers solution was infused intravenously at a rate of 2 mL/hr(45). To ensure all physiological variables were maintained within a healthy range (arterial Po_2 of >80 Torr; arterial PCO_2 of 35-45 Torr; pH, 7.3-7.4), arterial blood gases and pH were measured throughout experimentation using an automated blood gas analyzer (50 μL blood samples; Model ABL 5, Radiometer). In addition, body temperature was maintained between 36.5 and 38.0°C by an isothermal pad (Deltaphase). Animals were held in a stereotaxic head unit (Kopf Instruments) during pre-collicular decerebration and experimental procedures. Briefly, during the decerebration procedure, burr holes were drilled into the

parietal skull and the bone superior to the central sagittal sinus was removed. The dura mater was cut and the cerebrum was aspirated until the superior and inferior colliculi were within view. A pre-collicular section was made and the transectioned forebrain was aspirated rendering the animal insentient. To minimize bleeding, small pieces of oxidized regenerated cellulose (Ethicon, Johnson & Johnson) were placed on the internal skull surface and the cranial cavity was packed with cotton. Immediately after pre-collicular transection, anesthesia was discontinued and the animal was allowed to stabilize for one hour.

Microdialysis Procedures

Animals were held in a stereotaxic head unit (Kopf Instruments). A limited occipital craniotomy was performed to expose the dorsal surface of the brainstem and a microdialysis probe (Bioanalytical Systems, model CMA 11, 0.24 mm outer diameter, 1 mm membrane tip) was stereotaxically positioned unilaterally within the NTS at an area known to receive projections from exercise pressor reflex afferent fibers (coordinates: 500 μm lateral to the calamus scriptorus and 500 μm below the dorsal medullary surface)(31, 40, 43). Probes were continuously perfused at a rate of 2.5 μl/min with artificial cerebral spinal fluid (aCSF) buffered to a pH of 7.4. aCSF contained 0.2 % bovine serum albumin, 0.1 % bacitracin, and the following ions (in mM): 6.2 K^+, 134 Cl^-, 2.4 Ca^{2+}, 150 Na^+, 1.3 P^-, 13 HCO_3^- and 1.3 Mg^{2+}. After the probe was inserted, the preparation was allowed to stabilize for a minimum of one hour.

Activation of Metabolically Sensitive Afferent Fibers

Afferent fibers associated with the metaboreflex were preferentially activated by injecting a known concentration (0.3 μg/100 μL) of capsaicin into the hindlimb arterial supply. This procedure is an accepted way to activate the afferent neurons known to drive the metaboreflex, as capsaicin binds to transient receptor vanilloid 1 (TRPv1) receptors, which are primarily localized to group IV afferent fibers(20, 28). In order to administer capsaicin to chemically sensitive muscle receptors on group IV afferents, the circulation of the right hindlimb was

isolated. A catheter (PE-10, polyethylene tubing) was placed in the left common iliac artery with its tip advanced to the abdominal aorta. To limit drug delivery to the right hindlimb, a reversible vascular occluder was placed around the common iliac vein emptying the right hindlimb. Drugs were then injected directly into the arterial supply of the right hindlimb via the right common iliac artery. Selective activation of metabolically sensitive muscle receptors using pharmacological agents has been shown to be a valid technique to manipulate the metabolically sensitive muscle afferents(17, 20, 50).

Experimental Protocol

All capsaicin injections (100 μL in volume) were performed over a period of 30 seconds followed by injection of 200 μL of saline to ensure all infusate went into the leg. Upon injection into the right common iliac artery, the reversible ligature was pulled for 2 minutes to allow the drug to locally circulate. Capsaicin (0.3 μg/100 μL) injections were administered a minimum of two times with a 15-minute recovery period between each trial.

The circulatory response to activation of metabolically sensitive afferent fibers was obtained during the individual administration of aCSF (control), 1 mM and 5 mM of the nonspecific NOS inhibitor, L-nitro-arginine methyl ester (L-NAME), and 1 μM and 10 μM of the NO precursor, L-arginine. In these experiments, the microdialysis probe was placed ipsilateral to the leg being injected with capsaicin. Furthermore, the concentrations of dialysates used were carefully chosen, as these have been shown to alter the reflex control of MAP and HR(26, 27). aCSF, L-NAME, and L-arginine were dialyzed a minimum of 45 minutes before activation of metabolically sensitive afferents as this time period has been shown to be sufficient for effective substance delivery(33). During dialysis of each substance, two reproducible responses were obtained with a minimum of 15 minutes between events. As a control, reflex testing was also assessed during the dialysis of aCSF after dialysis of the drug of interest. In additional control experiments in SHR (n=9) and WKY (n=9) animals, metabolically sensitive afferent function was examined before and after the

microdialysis of D-NAME (5 mM) and D-arginine (10 μM), the inactive isomers of L-NAME and L-arginine, respectively.

Validation of Probe Placement

To verify probe placement at the conclusion of experimentation, the brainstem was excised and fixed in 10 % phosphate buffered formalin and stored at 4^0C. Medullary tissue was blocked, and 40 μm sections were cut serially using a cryostat (Cambridge Instruments). Sections were placed on coated slides and examined to establish the neuroanatomical location of probe placement.

Morphological Measurements

At the conclusion of testing procedures, animals were humanely euthanized. The heart, lungs, and triceps surae were excised and wet weights obtained. Tibial length was also measured to assess heart mass / tibial length ratios.

Data Acquisition and Statistical Analysis

In all physiologic experiments, baseline as well as reflex changes in MAP and HR were recorded. Baseline values for each measured variable were obtained over a 30 second period prior to reflex activation. The greatest change in each variable from this baseline in response to reflex activation was taken as the peak value.

All cardiovascular data was acquired, recorded, and analyzed using data acquisition software (Spike 2, version 3, Cambridge Electronic Design, Ltd) for the CED micro 1401 system (Cambridge Electronic Design Ltd). Data was analyzed by means of paired and unpaired t-tests or analysis of variance (ANOVA) with Student Newman-Keuls multiple comparison tests employed as appropriate. The significance level was set at $P<0.05$. All statistical analysis was performed using Sigma Stat for Windows (SPSS Inc.)

Results

Characterization of Hypertensive Model

Morphometric and baseline hemodynamic data for WKY and SHR animals are presented in Table 1. Ratios of heart weight to both body weight and tibial length were significantly greater in SHR than WKY. In addition, the lung weight to body weight ratio was greater in SHR thank WKY. Importantly, baseline MAP was significantly higher in SHR than WKY animals, but baseline HR data was not statistically different.

Microdialysis of L-NAME in the NTS Partially Recapitulates the Exaggerated Cardiovascular Response to Activation of Metabolically Sensitive Afferents in Hypertension

Intra-arterial capsaicin injections caused significant increases in MAP and HR from baseline in both groups of animals. As previously demonstrated, the rise in MAP in SHR rats was significantly greater than that seen in the WKY group. However, increases in HR were similar in both WKY and SHR groups.

Microdialysis of 1mM L-NAME into the NTS significantly increased the pressor response to intra-arterial capsaicin injections in both WKY and SHR animals (Figure 1). In normotensive WKY animals, inhibiting NOS activity within the NTS with L-NAME increased the MAP response to passive muscle stretch from 31 ± 3 mmHg to 43 ± 3 mmHg and the HR response from 5 ± 1 bpm to 6 ± 1 bpm. The data shows blocking NOS enzymatic activity within the NTS increases the pressor response to group IV afferent fiber activation in normotensive animals, partially recapitulating the exaggerated response observed in hypertension. In hypertensive SHR animals, microdialysis of 1 mM L-NAME further enhanced the exaggerated MAP and HR responses. The control circulatory response to hindlimb capsaicin injections were 53 ± 7 mmHg and 9 ± 3 bpm while the MAP and HR responses during dialysis of L-NAME significantly increased to 76 ± 10 mmHg and 18 ± 5 bpm, respectively. When the intra-arterial capsaicin injections were repeated during dialysis of aCSF at the end of the

experimental protocol, the circulatory responses returned to pre-L-NAME levels in both groups of animals.

Interestingly, increasing the concentration of L-NAME from 1 mM to 5 mM did not have a dose-dependant effect on the cardiovascular response to activation of metabolically sensitive afferents in normotensive WKY or hypertensive SHR animals. The MAP response to activation of metabolically sensitive afferent fibers in WKY rats increased, from the control response, by 12 ± 2 mmHg when 1 mM L-NAME was dialyzed into the NTS, while microdialysis of the higher dose of L-NAME (5 mM) increased MAP from the control response by 8 ± 4 mmHg (Figure 2A). In hypertensive SHR rats, as in the WKY group, there was not a concentration-dependant increase in the MAP response when the concentration of dialyzed L-NAME was increased to 5 mM. Specifically, the MAP response to activation of metabolically-sensitive afferents increased, from the control response, by 19 ± 6 mmHg when 1 mM L-NAME was dialyzed into the NTS and by 17 ± 9 mmHg when 5 mM L-NAME was perfused (Figure 2B).

As a control experiment, intra-arterial capsaicin injections were repeated in WKY and SHR animals during the dialysis of D-NAME (5 mM), the inactive isoform of L-NAME. The dialysis of D-NAME into the NTS had no effect on the hemodynamic response to activation of group IV afferent neurons in either WKY (Figure 3) or SHR (Figure 4) groups.

Increasing NO Production within the NTS of Hypertensive Rats Normalizes the Cardiovascular Response to Intra-arterial Capsaicin Injections

Brainstem microdialysis of 1 μM L-arginine, an NO precursor, into the NTS of WKY and SHR rats attenuated the pressor response to activation of metabolically sensitive skeletal muscle afferents (Figure 5). Hindlimb capsaicin injections in the SHR animals, produced increases in MAP and HR of 55 ± 6 mmHg and 6 ± 1 bpm, respectively before L-arginine microdialysis. After NO was experimentally increased within the NTS, the MAP and HR responses were lowered to 42 ± 5 mmHg and 5 ± 1 bpm, respectively. These cardiovascular responses were similar to that seen in normotensive animals during dialysis of

aCSF. Increasing NO production in the NTS of normotensive WKY rats also had an inhibitory affect on the cardiovascular response to activation of group IV afferent neurons. Before L-arginine dialysis, the pressor and HR responses to hindlimb capsaicin injections were 38 ± 6 mmHg and 6 ± 2 bpm, respectively. After brainstem NO was experimentally increased, the cardiovascular responses were reduced to 30 ± 5 mmHg and 4 ± 1 bpm. Increasing the concentration of L-arginine (10 μM) being dialyzed into the NTS did not produce a dose-dependant inhibitory effect on the hemodynamic response to activation of metabolically sensitive afferent fibers in WKY rats. This lack of a dose-dependant effect was also demonstrated in the SHR group (Figure 6).

In the control experiment, the dialysis of 10 μM D-arginine, the inactive enantiomer of L-arginine, did not affect the cardiovascular response to activation of metabolically sensitive afferent fibers in WKY (Figure 7) or SHR (Figure 8) groups.

Verification of Probe Placement

To verify probe placement, brainstems were excised from the rats, fixed and sliced into 40 μm sections. The slices were then examined microscopically to visualize probe placement. Two photomicrographs of successive brainstem slices from a representative animal are shown in Figure 9. The probe track is clearly visible in both slices, and is located within 500 μm of the fourth ventricle. This area of the NTS has been shown to receive metaboreflex afferent fiber projections(13, 32, 40, 42, 43). All of the probes were placed ipsilateral to the hindlimb that received capsaicin injections.

Discussion

A recent study from our laboratory provided evidence that metabolically sensitive afferent neurons partially mediate the exaggerated cardiovascular response to exercise manifest in hypertension(28). The current experiments were designed to investigate the possible central mechanisms involved in this metaboreflex overactivity. This research produced two major findings, which are:

(i) blocking NO production within the NTS increases the pressor response elicited by group IV afferent fibers in normotensive rats and (ii) increasing NO production within the NTS via L-arginine in hypertensive rats normalizes the pressor response mediated by metabolically sensitive afferent neurons. These findings support our hypothesis that endogenous NO within the NTS plays a significant role in generating the exaggerated pressor response to metaboreflex activation in hypertension.

Blocking NO within the NTS in Normotensive Rats Recapitulates the Exaggerated Pressor Response to Hindlimb Capsaicin Injections Evident in Hypertension

The most important finding is that we were able to reproduce, in a normotensive population of animals, the exaggerated increases in MAP that occur when metabolically sensitive afferent fibers are activated in hypertension. Dialysis of 5 mM L-NAME into the NTS of normotensive rats during hindlimb capsaicin injections increased the pressor response to values observed in their hypertensive counterparts. This suggests that endogenous NO within the NTS acts as a neuromodulator during activation of metabolically sensitive afferent neurons and normally buffers the reflex-induced cardiovascular response. These results are similar to those reported during the dialysis of L-NAME into the NTS during muscle mechanoreflex activation. In that study, we were able to recapitulate the mechanoreflex overactivity evident in hypertension by blocking NO within the NTS of normotensive rats(27). It is interesting to note that dialysis of L-NAME into the NTS of hypertensive rats had a greater effect on the absolute increases in MAP and HR mediated by activation of metabolically sensitive fibers than in normotensive rats. One explanation of this finding is that blocking endogenous NO within the NTS releases the inhibition on medullary sympathetic outflow produced by NO(8, 18, 46, 59). This allows the already-exaggerated sympathetic nervous system in hypertensive rats to produce a more robust hemodynamic response to metaboreflex activation(2, 6, 9-11, 14, 58).

Another important finding is that L-NAME dialyzed into the NTS did not have a concentration-dependant effect on the pressor and tachycardic responses to

hindlimb capsaicin injections in normotensive WKY or hypertensive SHR rats. This data differs from the results produced when L-NAME was dialyzed into the NTS of normotensive rats during simulated mechanoreflex activation. In that study, L-NAME produced a concentration-dependant increase in the pressor and tachycardic responses to simulated muscle mechanoreflex activation(27). It is possible that metabolically sensitive neurons are less sensitive to L-NAME as has been shown previously in cats(56). Concerning hypertensive rats, in the simulated muscle mechanoreflex study as well as this one, increasing the concentration of L-NAME had no significant effect on the circulatory responses to activation of metabolically sensitive afferent neurons(27). It is possible that in hypertensive rats there is a finite or diminished amount of NOS within the NTS that can be blocked by L-NAME. This could partially explain why the metaboreflex produces an exaggerated circulatory response in hypertension and supports the hypothesis that there is a decrease in NO bioavailability within the NTS of hypertensive animals.

NO bioavailability within the hypertensive brainstem is affected by several factors and any or all of them could contribute to a shortage of this neuromodulator. A reduction in the expression/activity of NOS within the NTS could have a negative impact on NO formation as NOS is the enzyme responsible for catalyzing the NO reaction(12, 38, 41). Another factor affecting NO bioavailability is the presence of reactive oxygen species, an example of which is the superoxide anion. Superoxide is known to inactivate NO and form peroxynitrite(15). Additionally, studies have found that reactive oxygen species levels are increased in specific autonomic nuclei important to cardiovascular regulation within the brainstem of SHR as compared to WKY rats(24, 52). In all probability, both a decrease in NOS levels and an increase in reactive oxygen species affect endogenous NO levels within the brainstem of hypertensive individuals. Future studies should quantify the amount of NO and NOS present within the brainstem of hypertensive individuals in order to better understand how these molecules affect medullary sympathetic outflow.

Increasing NO within the NTS of Hypertensive Rats Normalized the Pressor Response to Activation of Metabolically Sensitive Afferent Neurons

The dialysis of L-arginine into the NTS of SHR rats produced increases in MAP during hindlimb capsaicin injections that were not exaggerated, but rather similar, to those seen in normotensive WKY animals. These results suggest that NO within the NTS is reduced in hypertension and is partly responsible for the overactive metaboreflex. This data is consistent with our previous finding that increasing NO in the NTS of hypertensive rats decreased the pressor response to muscle mechanoreflex activation(26). However, unlike our previous findings concerning the mechanoreflex, changing the concentration of the L-arginine being dialyzed did not have an effect on the cardiovascular response to hindlimb capsaicin injections. It is possible that L-arginine, the NO precursor, is not as effective because its actions are dependant on the enzyme NOS(38). A different method of increasing NO, such as administering an NO donor and not a precursor, may modulate the circulatory response to metaboreflex activation more than L-arginine in SHR rats.

It is also possible that metabolically sensitive group IV afferent neurons respond differently to NO than mechanically sensitive group III afferent fibers. Group III and group IV neurons have distinct terminals within the NTS. Therefore, it is possible that these different types of neurons could have varying sensitivities to NO(13, 34, 42). In support of this postulate, it has been shown in the paraventricular nucleus that the effects of NO are highly localized and concentration-dependant(54). Within the paraventricular nucleus, NO has exerted both excitatory and inhibitory responses depending on the micro-domains in which it was acting(5, 7, 19, 30). The same could be true for NO within the NTS. Given the lipophilic quality of this gaseous molecule and its short duration of action, it is possible that NO produces slightly different responses when acting on chemically or mechanically sensitive afferent fibers.

Limitations

It is appropriate to acknowledge several limitations of the current study. First, injecting capsaicin into the hindlimb arterial supply does not mimic the physical act of exercise, which activates the metaboreflex through the generation of muscle metabolites. However, capsaicin has been shown to bind to TRPv1 receptors, which are primarily localized to group IV afferent fibers(16, 36). Unmyelinated group IV afferent fibers are known to mediate the metaboreflex(20, 21). Therefore, it follows that the binding of intra-arterial capsaicin to the TRPv1 receptors excites group IV afferent neurons and elicits a cardiovascular response that is mediated by the same afferent fibers that are activated during exercise. What's more, capsazepine, a selective TRPv1 inhibitor, has been shown to block the circulatory response to intra-arterial capsaicin injections, confirming that capsaicin is indeed binding to group IV nerve fibers(28). It is realized, however, that in the future it will be important to repeat these microdialysis experiments in a rat model of exercise, that allows for isolation of the metaboreflex.

Another limitation of the current study is that we performed unilateral microdialysis while the NTS is a bilateral nucleus. We used unilateral microdialysis due to spatial constraints within the rat brainstem and in order to reduce surgical trauma. Intra-arterial capsaicin injections, however, were given in the ipsilateral hindlimb and it has been well established that in the rat, metabolically-sensitive afferents project predominantly to the unilateral NTS(13, 42). Further, studies involving activation of cardiovascular reflexes utilizing unilateral microinjections and microdialysis techniques have demonstrated reliable and reproducible results(29, 41, 48, 57).

Conclusions and Clinical Significance

In conclusion, this study demonstrated that endogenous NO within the brainstem is able to modulate the hemodynamic response to activation of metabolically sensitive afferents in normotension and hypertension. We showed that by blocking NO production in the NTS of normotensive rats we could recapitulate the exaggerated circulatory response elicited by the metaboreflex in

hypertension. In addition, we found that increasing NO production in the brainstem of hypertensive rats normalized the augmented pressor response to hindlimb capsaicin injections. Collectively, these findings provide evidence that alterations in central-acting NO contribute significantly to the development of muscle metaboreflex overactivity in hypertension.

Acknowledgements

This research was supported by grants from the National Institutes of Health (HL-094075 to A.K. Leal and HL-088422 to S.A. Smith) and the Lawson & Rogers Lacy Research Fund in Cardiovascular Diseases (to J.H. Mitchell). The authors thank Martha Romero and Julius Lamar, Jr. for their expert technical assistance.

References

1. Alam M and Smirk FH. Observations in man upon a blood pressure raising reflex arising from the voluntary muscles. *J Physiol* 89: 372-383, 1937.
2. Anderson EA, Sinkey CA, Lawton WJ, and Mark AL. Elevated sympathetic nerve activity in borderline hypertensive humans: evidence from direct intraneural recordings. *Hypertension*: 177-183, 1989.
3. Andres KH, During MV, and Schmidt RF. Sensory innervation of Achilles tendon by group III and IV afferent fibers. *Anat Embyol (Berl)* 172: 145-156, 1985.
4. Aoki K, Sato K, Kondo S, Pyon C, and Yamamoto M. Increased response of blood pressure to rest and handgrip in subjects with essential hypertension. *Jpn Circ J* 47: 802-809, 1983.
5. Bains JS and Ferguson AV. Angiotensin II neurotransmitter actions in paraventricular nucleus are potentiated by a nitric oxide synthase inhibitor *Regul Pept* 50: 53-59, 1994.
6. Biaggioni I. Sympathetic control of the circulation in hypertension: lessons from autonomic disorders. *Curr Opin Nephrol Hypertens* 12: 175-180, 2003.
7. Carvajal JA, Thompson LP, and Weiner CP. Chorion-induced myometrial relaxation is mediated by large-conductance Ca^{2+}-activated K^{+} channel opening in the guinea pig. *Am J Obstet Gynecol* 188: 84-91, 2003.
8. Dias ACR, Vitela M, Colombari E, and Mifflin SW. Nitric oxide modulation of glutamatergic, baroreflex, and cardiopulmonary transmission in the nucleus of the solitary tract. *Am J Physiol Heart Circ Physiol* 288: 256-262, 2005.
9. Esler M. The sympathetic system and hypertension. *Am J Hypertens* 13: 99S-105S, 2000.
10. Esler M, Lambert G, and Jennings G. Increased regional sympathetic nervous activity in human hypertension: causes and consequences. *J Hypertens* 8 (suppl 7): S53-S57, 1990.

11. Esler M, Rumantir M, Kaye D, Jennings G, Hastings J, Socratous F, and Lambert G. Sympathetic nerve biology in essential hypertension. *Clin Exp Pharmacol Physiol* 28: 986-989, 2001.
12. Ferrari MFR and Fior-Chadi DR. Differential expression of nNOS mRNA and protein in the nucleus tractus solitarii of young and aged Wistar-Kyoto and spontaneously hypertensive rats. *J Hypertens* 23: 1683-1690, 2005.
13. Gamboa-Esteves FO, Tavares I, Almeida A, Batten TFC, McWilliam PN, and Lima D. Projection sites of superficial and deep spinal dorsal horn cells in the nucleus tractus solitarii of the rat. *Brain Res* 921: 195-205, 2001.
14. Grassi G, Colombo M, Seravalle G, Spaziani D, and Mancia G. Dissociation between muscle and skin sympathetic nerve activity in essential hypertension, obesity, and congestive heart failure. *Hypertension* 31: 64-67, 1998.
15. Griendling KK, Sorescu D, and Ushio-Fukai M. NAD(P)H oxidase. *Circ Res* 86: 494-501, 2000.
16. Guo A, Vulchanova L, Wang J, Li X, and Elde R. Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the $P2X_3$ purinoceptor and IB4 binding sites. *Eur J Neurosci* 11: 946-958, 1999.
17. Hayes SG and Kaufman MP. Gadolinium attenuates exercise pressor reflex in cats. *Am J Physiol* 280: 2153-2161, 2001.
18. Hironaga K, Hirooka Y, Matsuo I, Shihara M, Tagawa T, Harasawa Y, and Takeshita A. Role of endogenous nitric oxide in the brain stem on the rapid adaptation of baroreflex. *Hypertension* 31: 27-31, 1998.
19. Horn T, Smith PM, McLaughlin BE, Bauce L, Marks GS, Pittman QJ, and Ferguson AV. Nitric oxide actions in paraventricular nucleus: cardiovascular and neurochemical implications. *Am J Physiol* 266: R306-R313, 1994.
20. Kaufman MP, Iwamoto GA, Longhurst JC, and Mitchell JH. Effects of capsaicin and bradykinin on afferent fibers with endings in skeletal muscle. *Circ Res* 50: 133-139, 1982.
21. Kaufman MP, Longhurst JC, Rybicki KJ, Wallach JH, and Mitchell JH. Effects of static muscular contraction on impulse activity of groups III and IV afferents in cats *J App Physiol* 55: 105-112, 1983.
22. Kaufman MP, Waldrop TG, Rybicki KJ, Ordway GA, and Mitchell JH. Effects of static and rhythmic twitch contractions on the discharge of group III and IV muscle afferents. *Card Res* 18: 663-668, 1984.
23. Kazatani Y, Hamada M, Shigematsu Y, Hiwada K, and Kokubu T. Beneficial effect of a long-term antihypertensive therapy on blood pressure response to isometric handgrip exercise in patients with essential hypertension. *American Journal of Therapy* 2: 165-169, 1995.
24. Kishi T, Hirooka Y, Kimura Y, Ito K, Shimokawa H, and Takeshita A. Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats. *Circulation* 109: 2357-2362, 2004.
25. Lawrence AJ, Castillo-Melendez M, McLean KJ, and Jarrott B. The distribution of nitric oxide synthase-, adenosine deaminase- and neuropeptide Y-immunoreactivity through the entire rat nucleus tractus solitarius. Effect of unilateral nodose ganglionectomy. *J Chem Neuroanat* 15: 27-40, 1998.

26. Leal A, Mitchell J, and Smith S. Increasing nitric oxide within the brainstem attenuates the exaggerated cardiovascular response to simulated mechanoreflex activation in hypertension. *Am J Physiol* in preparation, 2009.
27. Leal A, Mitchell J, and Smith S. A role for nitric oxide within the nucelus tractus solitarius in the development of muscle mechanoreflex dysfunction in hypertension. *Am J Physiol* submitted, 2009.
28. Leal AK, Williams MA, Garry MG, Mitchell JH, and Smith SA. Evidence for functional alterations in the skeletal muscle mechanoreflex and metaboreflex in hypertensive rats. *Am J Physiol Heart Circ Physiol* 295: H1429-H1438, 2008.
29. Lewis SJ, Ohta H, Machado B, Bates JN, and Talman WT. Microinjection of S-nitrosocysteine into the nucleus tractus solitarii decreases arterial pressure and heart rate via activation of soluble guanylate cyclase. *Eur J Pharmacol* 202: 135-136, 1991.
30. Li DP, Chen SR, Finnegan TF, and Pan HL. Signalling pathway of nitric oxide in synaptic GABA release in the rat paraventricular nucleus. *J Physiol* 554: 100-110, 2004.
31. Li J. Nitric oxide synthase (NOS) coexists with activated neurons by skeletal muscle contraction in the brainstem of cats. *Life Sciences* 71: 2833-2843, 2002.
32. Li J and Mitchell JH. Role of NO in modulating neuronal activity in superficial dorsal horn of spinal cord during exercise pressor reflex. *Am J Physiol* 283: H1012-H1018, 2002.
33. Li J and Potts JT. NO formation in nucleus tractus solitarii attenuates pressor response evoked by skeletal muscle afferents. *Am J Physiol* 280: H2371-H2379, 2001.
34. Lin L-H, Cassell MD, Sandra A, and Talman WT. Direct evidence for nitric oxide synthase in vagal afferents to the nucleus tractus solitarii. *Neuroscience* 84: 549-558, 1998.
35. McCloskey DI and Mitchell JH. Reflex cardiovascular and respiratory responses originating in exercising muscle. *Journal of Physiology* 224: 173-186, 1972.
36. Michael GJ and Priestley JV. Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its down regulation by axotomy. *J Neurosci* 19: 1844-1854, 1999.
37. Mitchell JH, Kaufman MP, and Iwamoto GA. The exercise pressor reflex: Its cardiovascular effects, afferent mechanisms, and central pathways. *Ann Rev Physiol* 45: 229-242, 1983.
38. Moncada S and Higgs A. The L-arginine-nitric oxide pathway. *New Eng J Med* 329: 2002-2012, 1993.
39. Paton JFR, Deuchars J, Ahmad Z, Wong LF, Murphy D, and Kasparov S. Adenoviral vector demonstrates that angiotensin II-induced depression of the cardiac baroreflex is mediated by endothelial nitric oxide synthase in the nucleus tractus solitarii of the rat. *J Physiol* 531, 2001.
40. Paxinos G and Watson C. *The Rat Brain in Stereotaxic Coordinates*. Orlando, FL: Academic, 1986.

41. Pontieri V, Venezuela MK, Scavone C, and Michelini LC. Role of endogenous nitric oxide in the nucleus tractus solitarii on baroreflex control of heart rate in spontaneously hypertensive rats. *J Hypertens* 16: 1993-1999, 1998.
42. Potts JT, Lee SM, and Anguelov PI. Tracing of projection neurons from the cervical dorsal horn to the medulla with the anterograde tracer biotinylated dextran amine. *Aut Neurosci* 98: 64-69, 2002.
43. Potts JT, Paton JFR, Mitchell JH, Garry MG, Kline G, Anguelov PT, and Lee SM. Contraction-sensitive skeletal muscle afferents inhibit arterial baroreceptor signalling in the nucleus of the solitary tract: role of intrinsic GABA interneurons. *Neuroscience* 119: 201-214, 2003.
44. Qadri F, Arens T, Schwarz EC, Hauser W, Dendorfer A, and Dominiak P. Brain nitric oxide synthase activity in spontaneously hypertensive rats during the development of hypertension. *J Hypertens* 21: 1623-1624, 2003.
45. Quintin L, Gillon JY, Saunier CF, and Ghignone M. Continuous volume infusion improves circulatory stability in anesthetized rats. *J Neurosci Meth* 30: 77-83, 1989.
46. Ruggiero DA, Mtui EP, Otake K, and Anwar M. Central and primary visceral afferents to nucleus tractus solitarii may generate nitric oxide as a membrane-permeant neuronal messenger. *J Comp Neurol* 361: 51-67, 1996.
47. Seguro C, Sau F, Zedda N, Scano G, and Cherchi A. Arterial blood pressure behavior during progressive muscular exercise in subjects with stable arterial hypertension. *Cardiologia* 36: 867-877, 1991.
48. Smith SA, Mitchell JH, and Li J. Independent modification of baroreceptor and exercise pressor reflex function by nitric oxide in nucleus tractus solitarius. *Am J Physiol* 288: 2068-2076, 2005.
49. Smith SA, Williams MA, Leal AK, Mitchell JH, and Garry MG. Exercise pressor reflex function is altered in spontaneously hypertensive rats. *J Physiol* 577: 1009-1020, 2006.
50. Smith SA, Williams MA, Mitchell JH, Mammen PPA, and Garry MG. The capsaicin-sensitive afferent neuron in skeletal muscle is abnormal in heart failure. *Circulation*, 2005.
51. Stebbins CL, Brown B, Levin D, and Longhurst JC. Reflex effect of skeletal muscle mechanoreceptor stimulation on the cardiovascular system. *J App Physiol* 65: 1539-1547, 1988.
52. Tai M-H, Wang L-L, Wu KLH, and Chan JYH. Increased superoxide anion in rostral ventrolateral medulla contributes to hypertension in spontaneously hypertensive rats via interactions with nitric oxide. *Free Radical Biology & Medicine* 38: 450-462, 2005.
53. Tian G and Duffin J. Spinal connections of ventral-group bulbospinal inspiratory neurons studied with cross-correlation in the decerebrate rat. *Exp Brain Res* 111: 178-186, 1996.
54. Wang S, Paton JFR, and Kasparov S. The challenge of real-time measurements of nitric oxide release in the brain. *Autonomic Neuroscience: Basic and Clinical* 126-127: 59-67, 2006.

55. Williamson JW, Mitchell JH, Olesen HL, Raven PB, and Secher NH. Reflex increases in blood pressure induced by leg compression in man. *J Physiol* 475: 351-357, 1994.
56. Wilson LB. Spinal modulation of the muscle pressor reflex by nitric oxide and acetylcholine. *Brain Res Bulletin* 53: 51-58, 2000.
57. Wu WC, Wang Y, Kao LS, Tang FI, and Chai CY. Nitric oxide reduces blood pressure in the nucleus tractus solitarius: a real time electrochemical study. *Brain Res Bull* 57: 171-177, 2002.
58. Yamada Y, Miyajima E, Tochikubo O, Matsukawa T, and Ishii M. Age-related changes in muscle sympathetic nerve activity in essential hypertension. *Hypertension* 13: 870-877, 1989.
59. Zanzinger J, Czachurski J, and Seller H. Effects of nitric oxide on sympathetic baroreflex transmission in the nucleus tractus solitarii and caudal ventrolateral medulla in cats. *Neurosci Lett* 197: 199-202, 1995.
60. Zanzinger J, Czachurski J, and Seller H. Inhibition of basal and reflex-mediated sympathetic activity in the RVLM by nitric oxide. *Am J Physiol Reg Int Comp Physiol* 268: R958-R962, 1995.

Table

	WKY	SHR
n	44	42
Body weight, g	337±4	369±6*
Heart weight/body weight, mg/g	2.8±0.04	3.3±0.04*
Lung weight/body weight, mg/g	5.9±0.2	6.7±0.2*
Heart weight/tibial length, mg/mm	25.2±0.5	30.8±0.6*
MAP, mmHg	105±4	146±7*
HR, beats/min	439±8	435±11

Table 1. Morphometric characteristics and baseline hemodynamics. Data are means ± S.E.M. MAP, mean arterial pressure; HR, heart rate. * Significantly different from WKY. $P<0.05$.

Figures

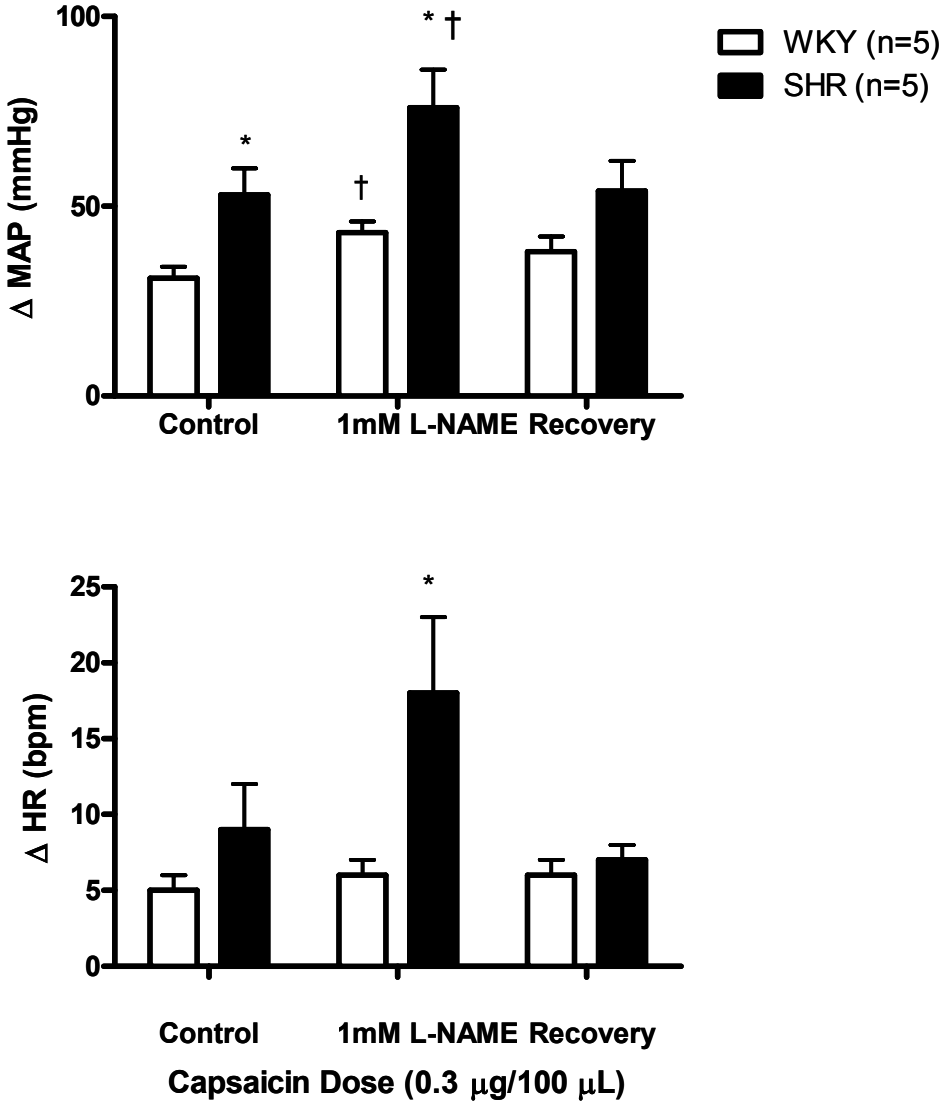

Figure 1. Cardiovascular responses to activation of metabolically sensitive afferent fibers during 1 mM L-NAME dialysis in WKY and SHR animals. Hindlimb intra-arterial capsaicin injections induced increases in MAP that were significantly greater in SHR as compared to WKY rats. In addition, dialysis of 1mM L-NAME into the NTS significantly increased the pressor response to capsaicin injections in both WKY and SHR and the HR response in SHR animals. * Significantly different from WKY rats. † Significantly different from Control and Recovery responses. $P<0.05$.

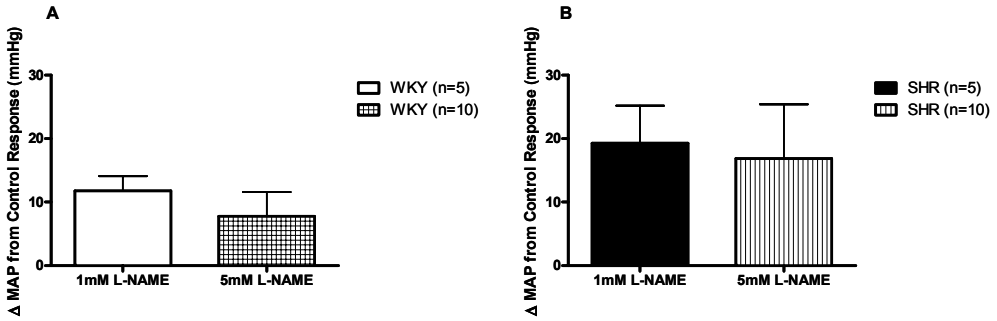

Figure 2. Increase in pressor response during dialysis of 1 mM and 5 mM L-NAME compared to control response in WKY and SHR animals. The dialysis of L-NAME into the NTS of WKY rats (Panel A) and SHR animals (Panel B) did not have a concentration-dependant effect on the pressor response to hindlimb intra-arterial capsaicin injections.

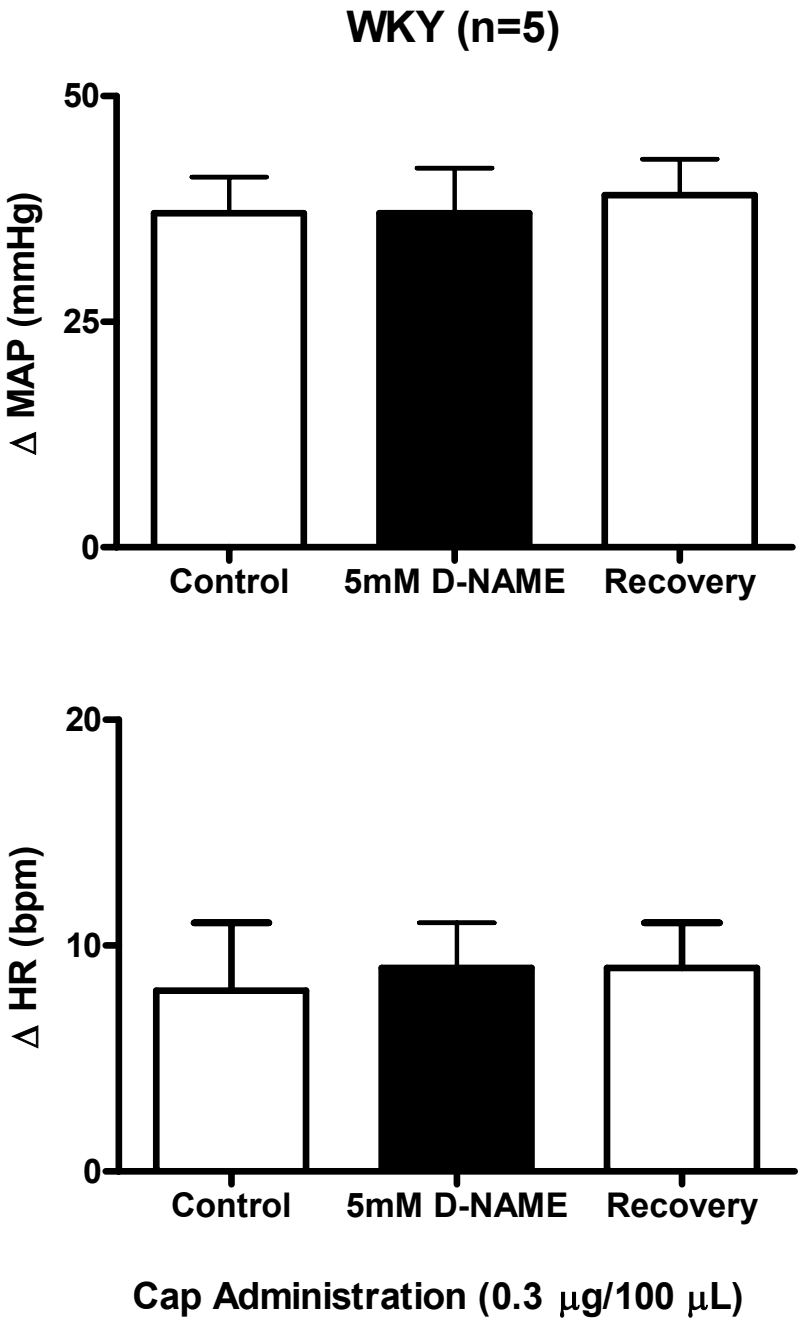

Figure 3. Cardiovascular responses to activation of metabolically sensitive afferent fibers during dialysis of D-NAME in WKY animals. Dialysis of the inactive isomer D-NAME had no effect on MAP or HR during hindlimb intra-arterial capsaicin injections.

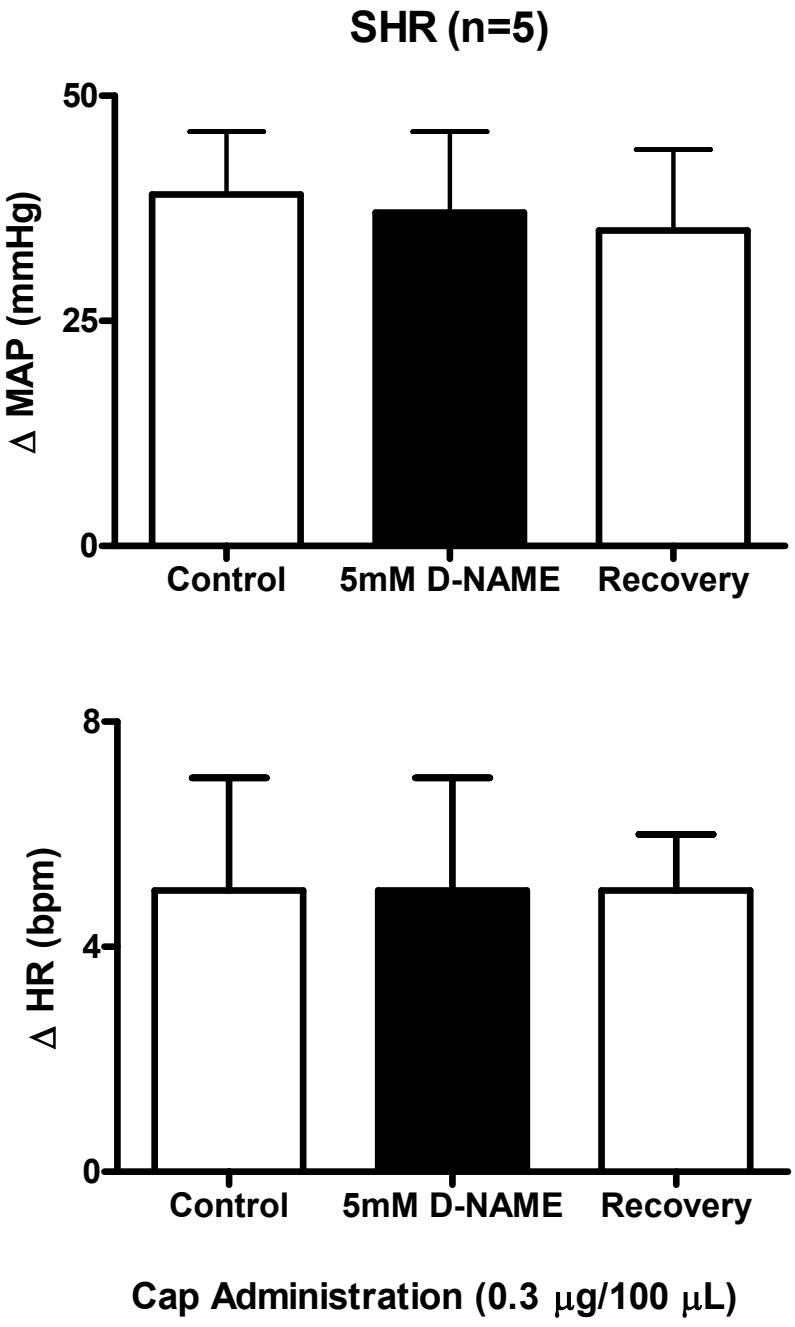

Figure 4. Cardiovascular responses to activation of metabolically sensitive afferent fibers during dialysis of D-NAME in SHR animals. Dialysis of the inactive isomer D-NAME had no effect on MAP or HR during hindlimb intra-arterial capsaicin injections.

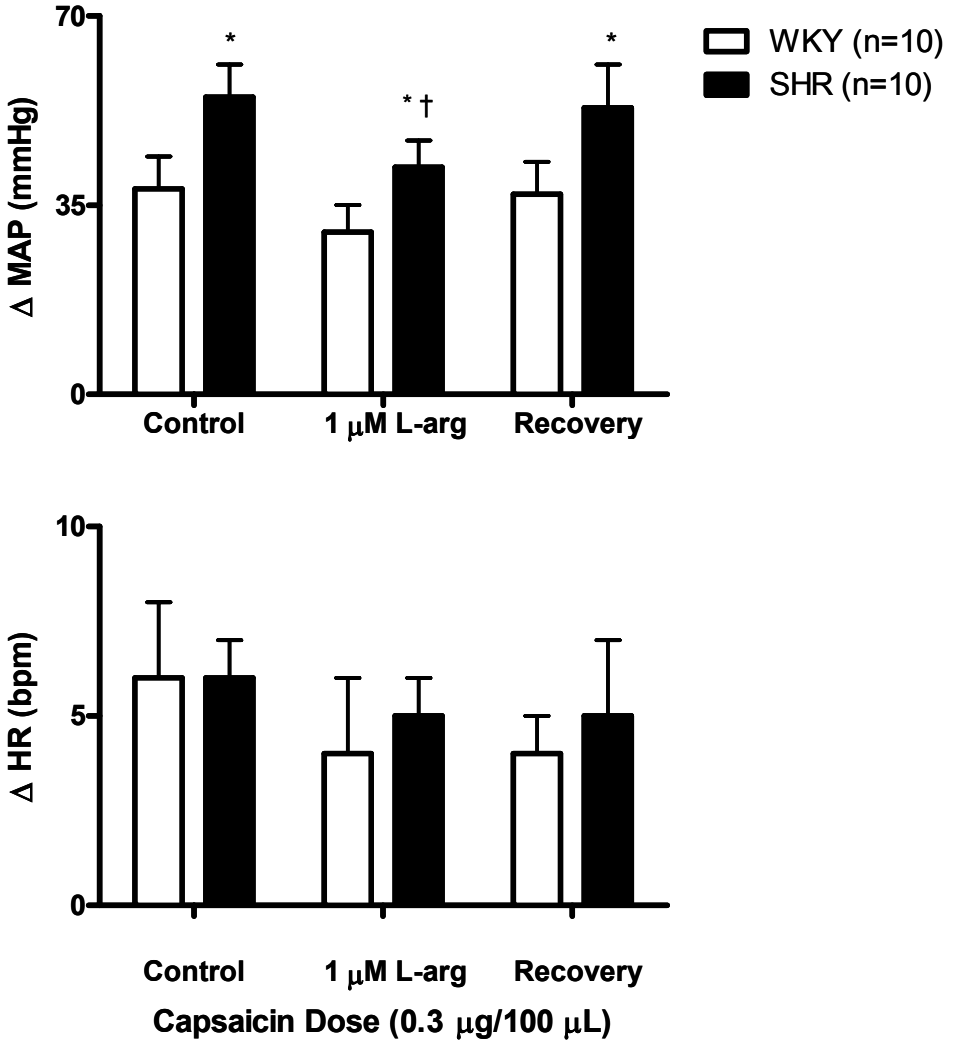

Figure 5. Cardiovascular responses to activation of metabolically sensitive afferent fibers during 1 μM L-arginine dialysis in WKY and SHR animals. Dialysis of 1 μM L-arginine during intra-arterial capsaicin injections induced a significant decrease in MAP in SHR animals. In addition, the pressor response to activation of metabolically sensitive afferent neurons was significantly greater in SHR animals compared to WKY. * Significantly different from WKY rats. † Significantly different from Control and Recovery responses. $P<0.05$.

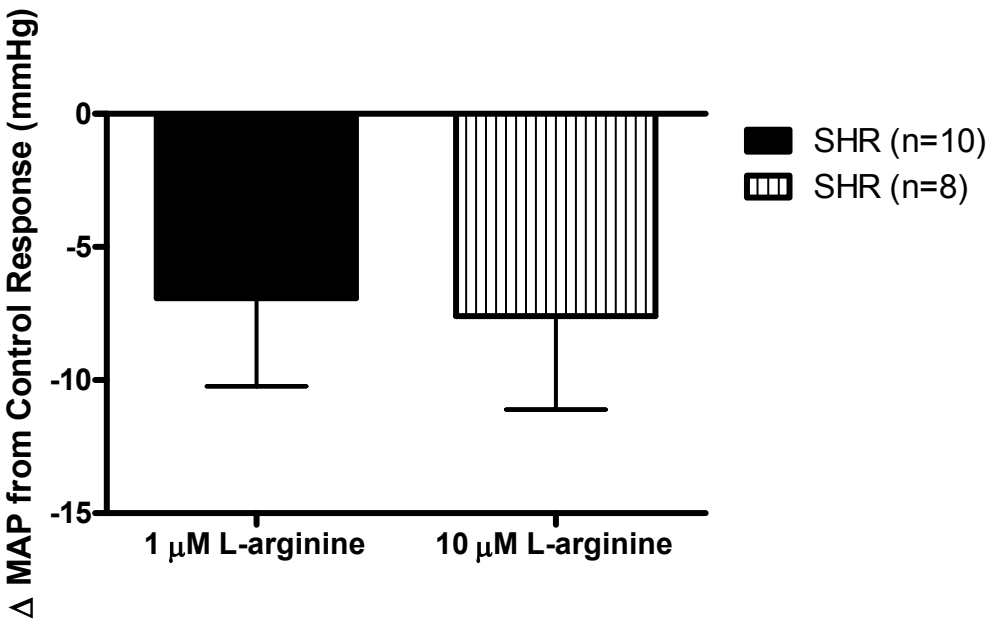

Figure 6. Decrease in pressor response during dialysis of 1 μM and 10 μM L-arginine compared to control response in SHR animals. Increasing the concentration of L-arginine to from 1 μM to 10 μM did not have a concentration-dependant effect on the pressor response to hindlimb capsaicin injections in hypertensive rats.

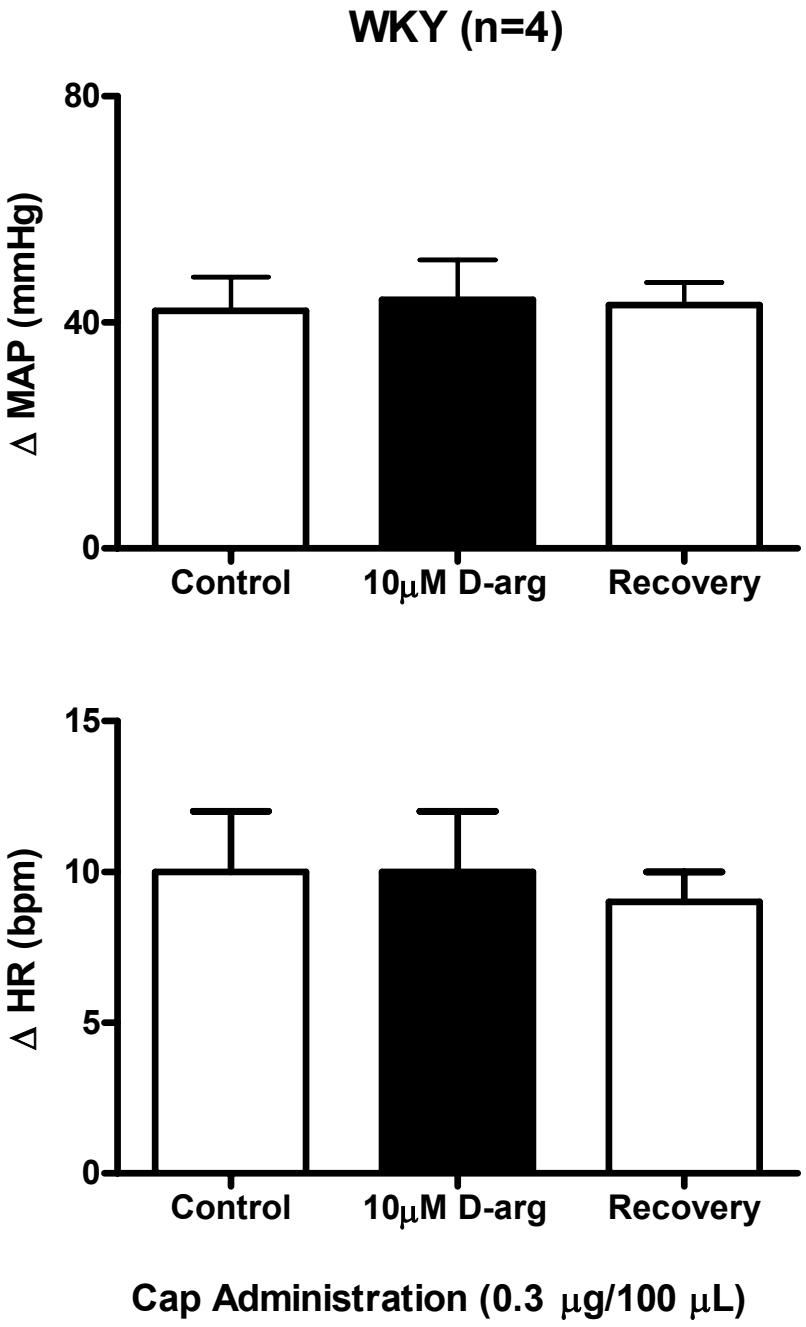

Figure 7. Cardiovascular responses to activation of metabolically sensitive afferent fibers during dialysis of D-arginine in WKY animals. Dialysis of the inactive isomer D-arginine had no effect on MAP or HR during hindlimb intra-arterial capsaicin injections in either group of animals.

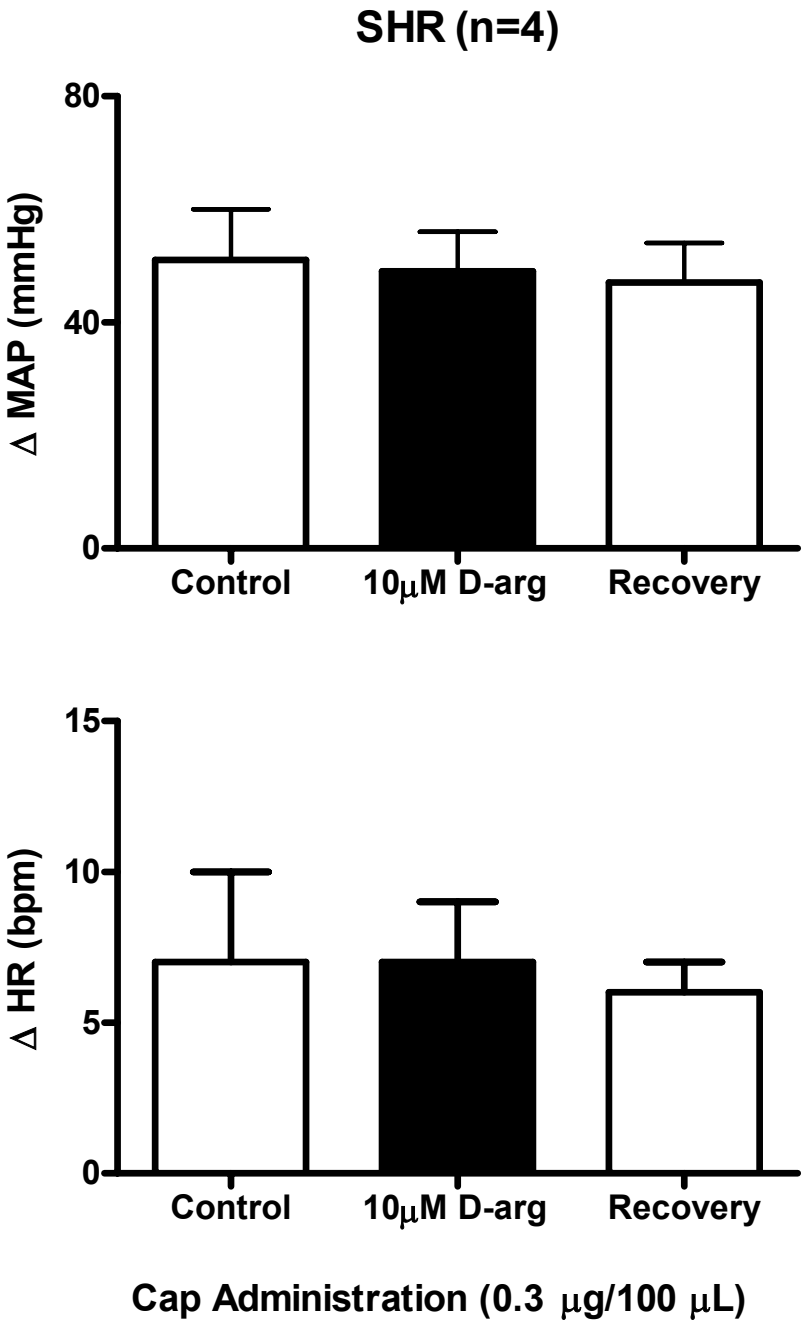

Figure 8. Cardiovascular responses to activation of metabolically sensitive afferent fibers during dialysis of D-arginine in SHR animals. Dialysis of the inactive isomer D-arginine had no effect on MAP or HR during hindlimb intra-arterial capsaicin injections in either group of animals.

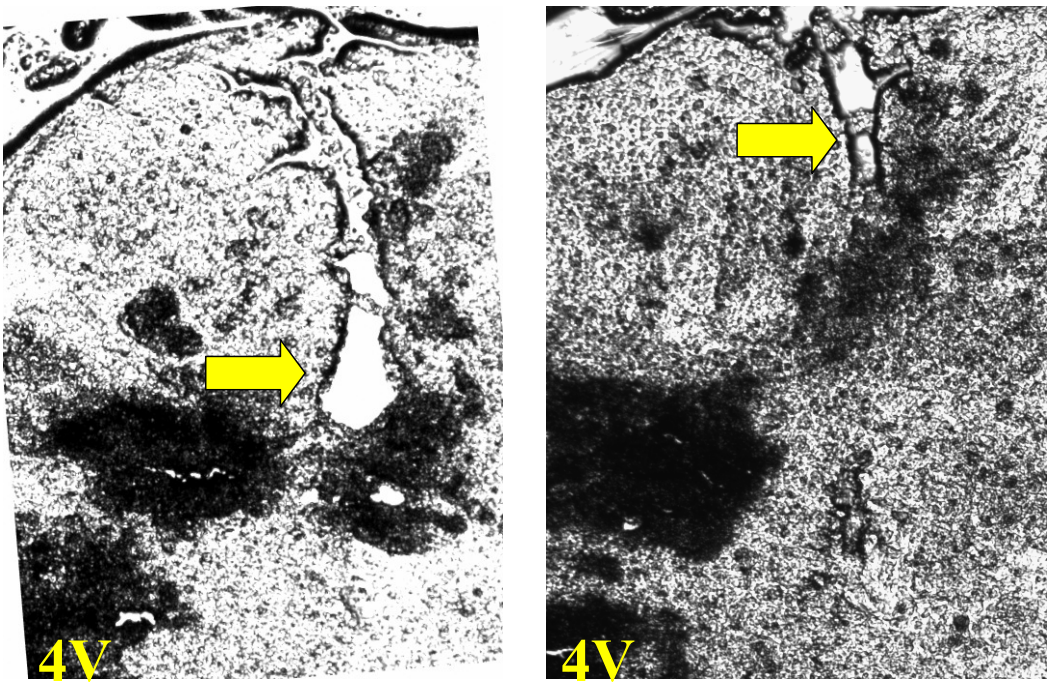

Figure 9. Microdialysis probe placement in one representative animal. Probe was placed 0.5 mm lateral to the fourth ventricle (4V) and 0.5 mm below the dorsal medullary surface. Photomicrographs (4x) of two consecutive brain slices show the probe track within the NTS. Minimal structural damage was caused by placement of the dialysis probe (arrow).

CONCLUSIONS

The current studies were performed to address the specific aim of determining the contribution of alterations in NO availability/activity within the NTS to exercise pressor reflex dysfunction in hypertension, specifically the dysfunction of its mechanically and metabolically sensitive components. In order to do this, we performed microdialysis within the NTS to block endogenous NO production and delivered an NO precursor during selective activation of mechanically and chemically sensitive afferent neurons. Given the role of NO within the brainstem in controlling sympathetic outflow as well as the cardiovascular response to exercise, we hypothesized that both mechanoreflex and metaboreflex dysfunction in hypertension could be partially attributed to a decrease in NO production/availability in the NTS. Further, we hypothesized that experimentally increasing NO production/availability in the NTS of hypertensive rats would partially correct mechanoreflex and metaboreflex dysfunction. A summary of all experimental conclusions follows.

Simulated Mechanoreflex Activation Studies

Simulated activation of the mechanoreflex by passive hindlimb stretch during dialysis of the nonspecific NOS inhibitor L-NAME demonstrated that blocking NO production within the NTS in normotensive rats recapitulates the augmented cardiovascular response elicited by mechanoreflex activation in hypertension. We were able to reproduce an increase in MAP and HR that was similar to that seen during mechanoreflex activation in hypertension when L-NAME was dialyzed into the NTS of normotensive rats. Additionally, blocking NO production within the NTS in hypertensive rats further enhanced the exaggerated cardiovascular response caused by mechanoreflex activation. Corollary experiments showed that the exaggerated circulatory response induced by mechanoreflex activation in hypertension was mediated by the sympathetic nervous system. It is possible that exaggerated sympathetic nerve activity in the SHR is partially responsible for the effectiveness of both L-arginine and L-NAME at altering the circulatory response to mechanoreflex activation. This

could explain why both dialysates, which act through NOS, produced results even though we speculate NOS protein is downregulated in SHR. These studies confirmed the importance of NO and its role as a neuromodulator of sympathetic outflow from the brainstem.

Next, when L-arginine was dialyzed into the NTS of hypertensive SHR rats in order to experimentally increase NO and the mechanoreflex was selectively engaged, the exaggerated cardiovascular response to mechanoreflex activation was partially corrected. Further, the microdialysis of L-arginine into the NTS had a greater absolute effect in hypertensive SHR animals than normotensive WKY animals. As a control, we dialyzed the inactive enantiomer D-arginine in the NTS of both groups of animals during mechanoreflex activation. We found that D-arginine had no effect on the cardiovascular response to exercise, providing evidence that the L-arginine/NO pathway was activated during L-arginine microdialysis.

Taken together, these findings support our hypothesis that a reduction in NO production/availability within the NTS plays a significant a role in mechanoreflex dysfunction in hypertension. In addition, experimentally increasing NO production/availability in the NTS of hypertensive rats partially corrected mechanoreflex dysfunction. This finding provides evidence that there is a decrease in NO bioavailability within the NTS of hypertensive animals.

Simulated Metaboreflex Activation Studies

Microdialysis of L-NAME, a nonspecific NOS inhibitor, into the NTS of normotensive and hypertensive rats increased the pressor response to selective activation of group IV afferent fibers, which are associated with the muscle metaboreflex. Further, the effect was greater in hypertensive SHR rats than in normotensive WKY rats. In addition, we were able to reproduce the exaggerated increases in MAP that occur when metabolically sensitive afferent fibers are activated in hypertension. Dialysis of L-NAME into the NTS of normotensive WKY rats during hindlimb capsaicin injections increased the pressor response to values observed in their hypertensive SHR counterparts. These findings support

our hypothesis that endogenous NO within the NTS plays a significant role in the exaggerated pressor response to metaboreflex activation in hypertension. Finally, when the bioavailability of NO was increased within the NTS via dialysis of L-arginine, the pressor response mediated by metabolically sensitive afferent neurons was normalized in hypertensive SHR rats.

Clinical Significance

To summarize, we have provided evidence that NO production/availability within the brainstem contributes to mechanoreflex and metaboreflex dysfunction in hypertension. These studies demonstrated that endogenous NO within the brainstem is able to modulate the hemodynamic response to activation of mechanically and metabolically sensitive afferents in normotension and hypertension. We showed that by blocking NO production in the NTS of normotensive rats we could recapitulate the exaggerated circulatory response elicited by both the mechanoreflex and metaboreflex in hypertension. We also showed that mechanoreflex activation elicits an exaggerated circulatory response to exercise in hypertension via the sympathetic nervous system and we speculate that the increased sympathetic drive is partially caused by a decrease in medullary NO in hypertension. Additionally, we found that increasing NO production/availability in the brainstem of hypertensive rats attenuated mechanoreflex dysfunction as well as normalized the augmented pressor and tachycardic responses to hindlimb intra-arterial capsaicin injections, a maneuver known to selectively activate metabolically sensitive afferent neurons.

These studies provide insight into the central processing of exercise pressor reflex afferents within the NTS and describe a role for the neuromodulator NO in controlling brainstem sympathetic outflow in hypertension. It is our hope that translation of these findings will lead to treatment options for hypertensive individuals that allow them to participate in a range of physical activities without the risk for an adverse cardiac event.

SUGGESTIONS FOR FUTURE RESEARCH

Future Studies

The current experiments have not only established that NO is involved with the central processing of mechanoreflex and metaboreflex activation, but also identified NO as a cause of EPR dysfunction in hypertension. By decreasing NO production in the NTS of normotensive rats, we were able to produce the exaggerated cardiovascular response to both mechanoreflex and metaboreflex activation evidenced in hypertension. In addition, experimentally increasing NO within the NTS of hypertensive rats during activation of mechanically and metabolically sensitive afferent fibers normalized the exaggerated increases in MAP and HR. While these studies adequately tested our hypotheses and provided insight into the mechanisms of EPR dysfunction in hypertension, future experiments are necessary to address current limitations and provide a more focused view of mechanoreflex and metaboreflex central processing in health and hypertension.

A limitation of the current study is that the techniques employed to activate mechanically and metabolically sensitive afferent fibers, while commonly used, may not completely mimic what occurs during physiological exercise. Therefore, in the future it will be important to repeat the microdialysis protocol during hindlimb muscle contraction. This rat model of exercise, recently developed by our laboratory, activates both the mechanoreflex and metaboreflex component of the EPR via hindlimb muscle contraction caused by ventral root stimulation(53). Briefly, a laminectomy is performed to expose the spinal cord and the dura is cut away to reveal the lower lumbar roots (L_2-L_6). Then the ventral and dorsal roots are separated. Next the L_4 and L_5 ventral roots, which stimulate the triceps surae muscles, are sectioned in order to control the efferent neural activity to the right hindlimb. Bipolar platinum electrodes are placed around the cut peripheral ends and all exposed neural tissue is immersed in warm mineral oil. Animals are secured in a customized spinal frame by clamps placed on rostral lumbar vertebrae and the pelvis is stabilized with steel posts. After the exercising limb is fixed in one position using clamps attached to the tibial bone,

the calcaneal bone is sectioned and the Achilles' tendon connected to a force transducer for the measurement of muscle tension. Electrical stimulations are then performed using a stimulator. This technique activates both the mechanically and metabolically sensitive components of the EPR(53).

In addition, it is possible to isolate the mechanoreflex and metaboreflex during hindlimb contraction in the rat model of exercise. Studies have shown that mechanically-sensitive afferent fibers can be preferentially blocked by pharmacologically antagonizing the stretch-sensitive receptors localized to predominantly group III afferent fibers. These receptors have been shown to be pharmacologically blocked during muscle contraction by the trivalent lanthanide gadolinium(24, 54). For example, it has been demonstrated in cats that the activity of group III mechanically-sensitive afferent fibers is significantly attenuated during contraction and passive stretch of hindlimb skeletal muscle when gadolinium is introduced into the arterial supply of the hindlimb(24). In addition, pre-treatment of the limb with gadolinium has also been shown to markedly reduce the reflex-induced MAP response to hindlimb contraction in both cats and rats(24, 54). The use of intra-arterial gadolinium during hindlimb contraction provides a way to determine the contribution of the muscle mechanoreflex to the EPR. With regard to the muscle metaboreflex, metabolically sensitive receptors can be supra-stimulated during EPR activation by performing ischemic muscle contraction or post exercise circulatory occlusion(2, 6, 15, 23, 49, 62). In order to occlude the circulation during or after hindlimb contraction in rats and cats, both the iliac artery and vein ipsilateral to the contracting triceps surae muscles must be reversibly tied with suture(23). When the sutures are retracted, the blood flow to the contracting muscle is arrested and metabolically sensitive receptors located on afferent fibers mediating the EPR are hyperstimulated. Ischemic contraction provides an even greater mismatch of oxygen supply and demand to the working hindlimb muscle. Studies using post exercise circulatory occlusion have shown that the increase in MAP is maintained even after the muscle is relaxed(6, 23, 34, 49). In addition, discharge activity from renal sympathetic efferents is significantly increased during ischemic contraction and maintained during post

exercise circulatory occlusion(23, 34, 62). Combining the use of these isolation techniques with hindlimb muscle contraction during brainstem microdialysis will provide a more direct method to activate afferent neurons associated with the muscle mechanoreflex and metaboreflex as well as allow quantification of the contributions of the two components to total EPR responsiveness.

A useful addition to the current study would be measurement of sympathetic nerve activity, which is known to increase during activation of the EPR and its components(25, 33, 43). Further, sympathetic nerve activity in hypertensive rats has been shown to be increased compared to normotensive counterparts(5, 13, 64). Therefore, it is likely that the heightened cardiovascular response to stimulation of the EPR and its components in hypertension is mediated by the altered regulation of sympathetic activity. A common way of measuring sympathetic nerve activity in the rat is by recording renal sympathetic nerve activity (rSNA). In order to measure rSNA, a kidney is exposed using a retroperitoneal approach. With the aide of a stereomicroscope, the renal nerve branches are isolated from the surrounding tissue and a piece of insulating film is placed beneath the isolated nerve branch. Next, two stainless steel electrodes are positioned between the nerve branch and the insulating film and secured with a drop of silicone gel. The muscle and skin are then closed using suture. Nerve activity is amplified by a differential preamplifier with a bandpass filter of 100-3000 Hz. The amplified output is full-wave rectified and integrated with a time constant of one second(37, 42). Recording rSNA activity during EPR activation combined with brainstem microdialysis would provide an additional parameter to quantify the effect of NO on central processing during EPR, mechanoreflex, and metaboreflex activation.

While microdialysis of L-arginine is commonly used in the brainstem and is an accepted method to increase NO, it would be beneficial if future studies not only recorded the cardiovascular effect of brainstem NO to reflex activation, but also quantified the amount of NO being produced by L-arginine. Because NO is an easily diffusible lipophilic gas that is dependant on NOS activity, it is difficult to detect(44). Complicating detection further is the fact that NO is very unstable

with a half-life of only 6-10 seconds *in vivo* due to its reactions with oxygen and water to form nitrite and nitrate, nitrogen dioxide, and dinitrogen trioxide(12, 18). Despite these difficulties, methods for NO detection *in vivo* exist, such as chemiluminescence, the Griess method, electron paramagnetic resonance spectroscopy (spin trapping), microelectrodes, HPLC measurement of L-citrulline, and fluorescent probes(10, 19, 45, 66). Unfortunately, high costs, low sensitivity, and the actual feasibility of measuring NO within the NTS of rats make many of these detection methods impossible to implement in the current study(65). However, the use of the fluorescent probe 1,2-diaminoanthraquinone (DAA, Figure 1) seems the best option due to its high sensitivity, specificity, and accuracy and the ability to distribute it in the brainstem of rats(19). DAA is a nonfluorescent molecule until NO reacts with the aromatic amino groups of DAA at neutral pH and in the presence of oxygen to form a condensed triazole ring. The first step of this reaction is nitrosylation of an amino group, most probably by dinitrotrioxide, which is formed through the reaction of NO with oxygen. The final product, H-anthra-[1,2d]-[1,2,3]-triazole-6,11-dione, produces a red-fluorescent precipitate that is water-insoluble(19). This ensures the precipitate will not diffuse in the tissue and allows the fluorescence pattern to be examined in fixed tissue(19). Thus far, DAA has been utilized *in vitro* in cultured rat hippocampal neurons and in rat brain slices(8, 20, 52). In these studies, DAA in artificial cerebrospinal fluid was used to incubate the neurons while NOS inhibitors and NO donors were added simultaneously. Neurons from these studies demonstrated an increased fluorescence when incubated with NO donors and L-arginine and no fluorescence when incubated with NO scavengers and NOS inhibitors(8, 20, 52).

The use of DAA as an NO fluorescent probe in future rat brainstem microdialysis studies is viable because the probe does not have to be loaded into cells and it does not distinguish between intracellular and extracellular NO, so it can be used for spatial imaging(19). This is important because NO is an easily diffusible molecule that can easily pass out of neurons after its production. In addition, DAA has already been used successfully in rat neuronal tissue and has

not been shown to be neurotoxic(19, 20). DAA has also not been found to react with reactive oxygen species or hydrogen peroxide. Finally, DAA is more sensitive to NO than to peroxynitrite, a product of NO and oxygen, and therefore does not induce background fluorescence(19). A disadvantage of using DAA as a fluorescent probe are that the NO/DAA reaction is irreversible, meaning fluorescence builds up progressively making it difficult to assess a dynamic process, such as NO release. In addition, to date no information has been published on the impact of catecholamines on DAA-induced fluorescence(65).

In conjunction with the current study, I propose performing bilateral microdialysis of DAA for 30 minutes into the NTS of WKY and SHR animals. The right NTS will also receive either L-NAME (1 and 5 mM) or L-arginine (1 and 10 μM) simultaneously with the fluorescent probe. The contralateral NTS will receive DAA alone and this will serve as a control, providing information on baseline NO production in both normotensive and hypertensive rats. At the end of microdialysis, animals will then be transcardially perfused and the fixed tissue slices will be examined with a fluorescence microscope for NO quantification. A possible concern is that this procedure does not take into consideration the different diffusion properties of DAA, L-NAME, and L-arginine, meaning the fluorescence may not accurately describe the complete area of NO production caused by microdialysis of L-arginine. However, these experiments would provide information about the amount of endogenous NO being produced in the NTS of normotensive and hypertensive rats as well as confirm the effectiveness of microdialysis of L-NAME and L-arginine in blocking and increasing NO production, respectively.

There are three NOS isoforms; inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). While all three isoforms have been identified in autonomic nuclei important to cardiovascular regulation in the rat brainstem, the expression and activity of each may differ in normotensive and hypertensive rats(7, 11, 36). If the use of the fluorescent probe DAA demonstrates that the nonspecific NOS inhibitor L-NAME does not provide sufficient inhibition of NO or if we want to selectively antagonize a specific NOS isoform, many

inhibitors with variable selectivity for each NOS isoform are available. The selective nNOS antagonist, 1-(2-trifluoromethylphenyl)-imidazole, the selective eNOS antagonist, L-*N*(5)-(1-iminoethyl)ornithine and the selective iNOS antagonist, aminoguanidine can be used, to name a few. The efficacy of these selective inhibitors within the brainstem has been established previously(3, 29, 30). Elucidating the role of each NOS isoform to the control of muscle reflex function in hypertension is important to our understanding of the pathogenesis of this disorder. This will become increasingly important in the future as information concerning the expression of each NOS isoform within the NTS increases. The microdialysis of selective NOS antagonists during EPR activation may help identify which NOS isoform or isoforms is responsible for NO production during EPR activation and provide information on how specific NOS isoform activity differs in health and hypertension.

As mentioned previously, NOS expression within the NTS is known to be altered throughout the progression of hypertension and there are conflicting reports on the amount of iNOS, eNOS, and nNOS protein in SHR and WKY brainstem tissue(14, 46-48, 63). Therefore, we interrogated iNOS, eNOS, and nNOS protein expression in 20 week-old SHR and WKY animals. Immunohistochemical staining of both iNOS and nNOS proteins within the NTS suggested that their expression was downregulated in SHR compared to WKY animals (Figures 2 and 3, respectively). However, the amount of eNOS protein present within the NTS of the SHR animal seemed similar to the amount seen within the NTS of the WKY animal (Figure 4). As NOS is required to produce NO, a downregulation of any of its isoforms could reduce endogenous levels of NO within the NTS, thus contributing to the development of EPR dysfunction in hypertension. While this pilot data supports the hypothesis that NOS expression is altered in hypertension and contributes to EPR dysfunction, more experiments are needed to confirm our findings.

In order to confirm that NTS neurons involved in EPR processing are producing NO during mechanoreflex and metaboreflex activation, it must be determined whether NOS expression is present in neurons excited by the EPR.

The expression of the early response gene c-Fos has been widely used as a marker of EPR-induced neuronal activity within the brain(39-41). In previously collected pilot data, we probed the NTS for c-Fos expression in response to activation of the EPR. Within the NTS, c-Fos was expressed in response to activation of the EPR via static muscle contraction but was absent in animals in which no exercise was performed (Figure 5). In a corollary experiment, we preferentially activated the mechanoreflex for one hour in a WKY and SHR animal and then simultaneously probed for both c-Fos and nNOS expression within the NTS(22). Expression of c-Fos and nNOS was clearly present within the same NTS neurons of WKY and SHR rats. However, the expression of nNOS was markedly reduced in the hypertensive rat(22) (Figure 6). The findings are consistent with our previous nNOS immunohistochemistry data. Further, this data supports the concept that NO production via nNOS within NTS neurons excited by the mechanoreflex may be decreased in hypertension and likely contributes to the development of mechanoreflex dysfunction in this disease.

In the future, more experiments need to be performed combining the techniques of immunohistochemistry for specific NOS isoforms and c-Fos expression during EPR, mechanoreflex, and metaboreflex activation in order to anatomically probe NTS neurons shown to be activated by the EPR for NOS protein expression. Briefly, primary antibodies for nNOS, eNOS, and iNOS will be used to interrogate protein expression within the NTS of SHR and WKY. We will evaluate NOS expression in animals after one hour of EPR, mechanoreflex, or metaboreflex activation. Subsequently, medullary brainstem tissue will be stained immunohistochemically for both NOS and c-Fos expression. It should be noted that a one-hour period of exercise has been reported to be sufficient for the induction of c-Fos expression within the brain(40, 41).

It is possible that eNOS immunohistochemistry will demonstrate that protein expression of this isoform is upregulated in the SHR brainstem. This is because a mechanism referred to as eNOS uncoupling may be occurring in hypertensive animals(67). eNOS uncoupling refers to the discrepancy between eNOS protein levels and NO production and is caused by a switch in the

enzymatic activity of eNOS to generate superoxide rather than NO. eNOS consists of a flavin-containing reductase domain, a heme-containing oxygenase domain, and a regulatory calmodulin-binding linker sequence. When calcium/calmodulin binds, NADPH-derived electrons flow from the reductase domain to the oxygenase domain through tetrahydrobiopterin (BH_4, a required cofactor for NOS enzymes). The reduction of iron facilitates oxygen binding to form a ferrous-dioxygen complex. This complex subsequently reduces to water and a heme iron (IV)-oxo species, which oxidizes L-arginine to NO and L-citrulline(17, 32, 51, 55). BH_4 both stabilizes and donates electrons to the ferrous-dioxygen complex in the oxygenase domain, thus activating eNOS and initiating NO synthesis(27, 50, 61). In vascular disease states, when BH_4 is absent, the ferrous-dioxygen complex dissociates to form superoxide, as shown in Figure 7(59, 60). In hypertension, eNOS uncoupling within the vasculature and iNOS uncoupling has been demonstrated(1, 9, 38). However, eNOS uncoupling within the brainstem in hypertension may have a profound effect on NO bioavailability because not only is NO production reduced, but superoxide production is increased, leading to further reductions in endogenous NO and greatly affecting reflex-mediated sympathetic outflow from the medulla.

If eNOS protein is upregulated in the SHR brainstem, we will investigate the bioavailability of BH_4 through pharmacological supplementation. This technique has been shown to not only improve endothelial dysfunction by increasing NO production and decreasing superoxide generation, but also lower blood pressure(9, 26). In rodents, BH_4 has been shown to be effective while given orally in both food and water(31, 38) I propose to supplement the drinking water of SHR and WKY rats with BH_4 (10-20 mg/kg per day) for four weeks(26, 31). At the end of this treatment period, EPR, mechanoreflex, and metaboreflex activation will be performed with and without microdialysis of L-arginine. If NOS uncoupling is partially responsible for EPR dysfunction, BH_4 treatment should help normalize the response the EPR, mechanoreflex, and metaboreflex activation. In addition, the microdialysis of L-arginine during reflex testing should further attenuate the cardiovascular response as L-arginine increases NO

production via the enzymatic activity of NOS. These studies will not only allow us to determine if NOS uncoupling is occurring, but also provide important data regarding the effectiveness of NOS at generating NO in BH_4-treated SHR animals.

It is well known that reactive oxygen species (ROS), such as superoxide anion, convert NO into peroxynitrite(28, 58). The multi-subunit enzyme NAD(P)H oxidase catalyzes the reduction of molecular oxygen to form superoxide(4). The activity of NAD(P)H oxidase is increased by angiotensin II (Ang-II), a peptide that regulates sympathetic outflow, and enhanced superoxide production within the central nervous system is a result(16, 68). Therefore, the concentration of NO within the NTS may be reduced by the generation of superoxide via an Ang-II/NAD(P)H oxidase mechanism. In support of this concept, recent studies have demonstrated that ROS levels are increased in specific autonomic nuclei important to cardiovascular regulation within the brain stem of SHR rats as compared to WKY animals(35). Further, bilateral microinjection of superoxide dismutase mimetics (the enzyme responsible for the breakdown of superoxide) within specific brain stem autonomic nuclei has been shown to decrease both MAP and sympathetic nerve activity in SHR animals(35, 56). To determine the effects of endogenously produced superoxide on EPR activity, we dialyzed the superoxide dismutase mimetic tempol (30 μM) into the NTS of WKY and SHR animals(21). In both normotensive and hypertensive rats, tempol significantly reduced the pressor response to activation of the mechanoreflex during hindlimb passive muscle stretch (Figure 8). Likewise, the MAP response to activation of the muscle metaboreflex during intra-arterial administration of capsaicin in the hindlimb was attenuated in normotensive and hypertensive animals after tempol treatment (Figure 9). In both sets of experiments, the magnitude of the decrease in the pressor response to reflex activation was significantly larger in SHR as compared to WKY. Despite the larger attenuation of MAP, the reflex-induced pressor response in tempol treated-SHR treated was still greater than the pressor response elicited in untreated WKY(21). This pilot data suggests that superoxide within the NTS modulates

EPR activity in both WKY and SHR and that decreasing superoxide levels within the NTS can partially correct EPR dysfunction in hypertension. In addition, the data support the concept that increases in superoxide production within the NTS contribute to the exercise pressor reflex dysfunction that develops in hypertension.

In the future, more studies are needed to confirm these findings. Using the microdialysis procedure, we will deliver the superoxide dismutase mimetic tempol into the NTS of both WKY and SHR rats during selective activation of the EPR, mechanoreflex, and metaboreflex. By dialyzing tempol, we will be able to effectively inhibit superoxide activity. As a corollary experiment, we will experimentally increase the production of ROS within the NTS of WKY and SHR animals by utilizing the superoxide generating solution xanthine/xanthine oxidase. Xanthine oxidase catalyzes the production of superoxide from xanthine(57). In WKY, experimentally increasing the availability of NO within the NTS by inhibiting superoxide activity directly through superoxide breakdown should attenuate the cardiovascular response to activation of the EPR, mechanoreflex and metaboreflex while in SHR, enhancing NO availability using this technique should partially correct the hemodynamic abnormalities mediated by each reflex. Conversely, we expect increasing superoxide production via the microdialysis of a xanthine/xanthine oxidase solution to augment the cardiovascular response to muscle reflex activation in both WKY and SHR animals. Collectively, these findings would support the hypothesis that muscle reflex overactivity in hypertension is mediated, in part, by the increased generation of superoxide within the NTS.

In conclusion, these studies will provide further evidence regarding the mechanisms by which medullary NO mediates the central processing of the EPR in hypertension. These suggested experiments will activate the EPR in more physiological ways as well as provide a more complete description of the effects of EPR activation on cardiovascular control. In addition, these studies will add to the current data by demonstrating the role of specific NOS isoforms, as well as reactive oxygen species, on NO production and availability within the NTS.

Importantly, these future studies will determine the amount of NOS expression and activity within the NTS. Finally, the use of the fluorescent probe DAA will confirm the effectiveness of the microdialysis procedure in altering NO availability within the NTS.

It is acknowledged that hypertension is a complex disease with many influences. Other factors such as genetics and environment play huge roles in the pathophysiology and maintenance of this disease. To that end, the EPR and its central processing is only one mechanism that contributes to altered blood pressure states in hypertensive individuals. To better understand the development of hypertension and its regulation, it would be beneficial in the future to develop a mathematical model that integrates central command, the arterial baroreflex, and the EPR in order to predict the subsequent changes in MAP and HR during exercise.

References

1. Abou-Donia M, Daniels C, Nichol C, and Viveros H. *Regulation of adrenocortical guanosine triphosphate cyclohydrolase and tetrahydrobiopterin in normal and spontaneously hypertensive rats*. New York, NY: Walter de Gruter and Co, 1983.
2. Alam M and Smirk FH. Observations in man upon a blood pressure raising reflex arising from the voluntary muscles. *J Physiol* 89: 372-383, 1937.
3. Ally A, Phattanarudee S, Kabadi S, Patel M, and Maher T. Cardiovascular responses and neurotransmitter changes during static muscle contraction following blockade of inducible nitric oxide synthase (iNOS) with the ventrolateral medulla. *Brain Res* 1090: 123-133, 2006.
4. Babior B. NADPH oxidase. *Curr Opin Immunol* 16: 42-47, 2004.
5. Biaggioni I. Sympathetic control of the circulation in hypertension: lessons from autonomic disorders. *Curr Opin Nephrol Hypertens* 12: 175-180, 2003.
6. Bonde-Petersen F, Rowell L, Murray R, Blomqvist G, White R, Karlsson E, Campbell W, and Mitchell J. Role of cardiac output in the pressor response to graded muscle ischemia in man. *J Appl Physiol* 45: 574-580, 1978.
7. Chan JYH, Wang S, and Chan SHH. Differential roles of iNOS and nNOS at rostral ventrolateral medulla during experimental endotoxemia in the rat. *Shock* 15: 65-72, 2001.
8. Chen X, Sheng C, and Zheng X. Direct nitric oxide imaging in cultured hippocampal neurons with diaminoanthraquinone and confocal microscopy. *Cell Biol Int* 25: 593-598, 2001.

9. Cosentino F, Patton S, d'Uscio L, Werner E, Werner-Felmayer G, moreau P, Malinski T, and Luscher T. Tetrahydrobiopterin alters superoxide and nitric oxide release in prehypertensive rats. *J Clin Invest* 101: 1530-1537, 1998.
10. Davies I and Zhang X. Nitric oxide selective electrodes. *Methods Enzymol* 436: 63-95, 2008.
11. Dawson T, Bredt D, Fotuhi M, Hwang P, and Snyder S. Nitric oxide synthase and neuronal NADPH diaphorase are identified in brain and peripheral tissues. *Proc Natl Acad Sci USA* 88: 7797-7801, 1991.
12. Edelman GM and Gally JA. Nitric oxide: linking space and time in the brain. *Proc Natl Acad Sci USA* 89: 11651-11652, 1992.
13. Esler M. The sympathetic system and hypertension. *Am J Hypertens* 13: 99S-105S, 2000.
14. Ferrari MFR and Fior-Chadi DR. Differential expression of nNOS mRNA and protein in the nucleus tractus solitarii of young and aged Wistar-Kyoto and spontaneously hypertensive rats. *J Hypertens* 23: 1683-1690, 2005.
15. Freund P, Rowell L, Murphy T, Hobbs S, and Butler S. Blockade of pressor response to muscle ischemia by sensory nerve block in man. *Am J Physiol Heart Circ Physiol* 236: H433-H439, 1979.
16. Gao L, Wang W, Li Y, Schultz H, Liu D, Cornish K, and Zucker I. Superoxide mediates sympathoexcitation in heart failure: roles of angiotensin II and NAD(P)H oxidase. *Circ Res* 95: 937-944, 2004.
17. Govers R and Rabelink TJ. Cellular regulation of endothelial nitric oxide synthase. *Am J Physiol Renal Physiol* 280: F193-F206, 2001.
18. Hakim T, Sugimori K, Camporesi E, and Anderson G. Half-life of nitric oxide in aqueous solutions with and without haemoglobin *Physiol Meas* 17: 267-277, 1996.
19. Halbach OvBu. Nitric oxide imagine in living neuronal tissues using fluorescent probes. *Nitric Oxide* 9: 217-228, 2003.
20. Halbach OvBu, Albrecht D, Heinemann U, and Schuchmann S. Spatial nitric oxide imaging using 1,2-diaminoanthraquinone to investigate the involvement of nitric oxide in long-term potentiation in rat brain slices. *Neuroimage* 15: 633-639, 2002.
21. Hawkins M, Leal A, Mitchell J, and Smith S. Decreasing superoxide within the nucleus tractus solitarius partially corrects skeletal muscle mechanoreflex overactivity in hypertension. *Med Sci Sports Exerc* in press, 2009.
22. Hawkins M, Squiers J, Leal A, Mitchell J, and Smith S. Neuronal nitric oxide synthase (nNOS) and c-Fos expression in the nucleus tractus solitarius (NTS) of normotensive and hypertensive rats. *FASEB Journal* in press, 2009.
23. Hayes S and Kaufman M. MLR stimulation and exercise pressor reflex activate different renal sympathetic fibers in decerebrate cats *J Appl Physiol* 92: 1628-1634, 2002.
24. Hayes SG and Kaufman MP. Gadolinium attenuates exercise pressor reflex in cats. *Am J Physiol* 280: 2153-2161, 2001.
25. Hill JM, Adreani CM, and Kaufman MP. Muscle reflex stimulates sympathetic postganglionic efferents innervating triceps surae muscles of cats. *Am J Physiol* 271: H38-H43, 1996.

26. Hong H, Hsiao G, Cheng T, and Yen M. Supplementation with tetrahydrobiopterin suppresses the development of hypertension in spontaneously hypertensive rats. *Hypertension* 38, 2001.
27. Hurshman A, Krebs C, Edmondson D, Huynh B, and Marletta M. Formation of a pterin radical in the reaction of the heme domain of inducible nitric oxide synthase with oxygen. *Biochemistry* 38: 15689-15696, 1999.
28. Infanger D, Sharma R, and Davisson R. NADPH oxidases of the brain: distribution, regulation, and function. *Antioxidants Redox Signaling* 8: 1583-1596, 2006.
29. Ishide T, Nauli S, Maher T, and Ally A. Cardiovascular responses and neurotransmitter changes following blockade of nNOS within the ventrolateral medulla during static muscle contraction. *Brain Res* 977: 80-89, 2003.
30. Ishide T, Preuss C, Maher T, and Ally A. Neurochemistry within the ventrolateral medulla and cardiovascular effects during static exercise following eNOS antagonism. *Neurosci Res* 52: 21-30, 2005.
31. Kase H, Hashikabe Y, Uchida K, Nakanishi N, and hattori Y. Supplementation with tetrahydrobiopterin prevents the cardiovascular effects of angiotensin II-induced oxidative and nitrosative stress. *J Hypertens* 23: 1375-1382, 2005.
32. Katusic ZS. Vasular endothelial dysfunction: does tetrahydrobiopterin play a role? *Am J Physiol Heart Circ Physiol* 281: H981-H986, 2001.
33. Kaufman MP and Forster HV. Reflexes controlling circulatory, ventilatory and airway responses to exercise. In: *Section 12, Exercise: Regulation and Integration of Multiple Systems*. Bethesda, MD: Am Physiol Soc, 1996, p. 381-447.
34. Kindig AE, Hayes SG, and Kaufman MP. Purinergic 2 receptor blockade prevents the responses of group IV afferent to post-contraction circulatory occlusion. *J Physiol* 578: 301-308, 2007.
35. Kishi T, Hirooka Y, Kimura Y, Ito K, Shimokawa H, and Takeshita A. Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats. *Circulation* 109: 2357-2362, 2004.
36. Kishi T, Hirooka Y, Sakai K, Shigematsu H, Shimokawa H, and Takeshita A. Overexpression of eNOS in the RVLM causes hypotension and bradycardia via GABA release. *Hypertension* 38: 896-901, 2001.
37. Koba S, Yoshida T, and Hayashi N. Renal sympathetic and circulatory responses to activation of the exercise pressor reflex in rats. *Exp Physiol* 91.1: 111-119, 2006.
38. Landmesser U, Dikalov S, Price S, McCann L, Fukai T, Holland S, Mitch W, and Harrison D. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 111: 1201-1209, 2003.
39. Li J, Hand G, Potts J, and Mitchell J. Identification of hypothalamic vasopressin and oxytocin neurons activated during the exercise pressor reflex in cats. *Brain Res* 752: 45-51, 1997.

40. Li J, Hand GA, Potts JT, Wilson LB, and Mitchell JH. c-Fos expression in the medulla induced by static muscle contraction in cats. *Am J Physiol* 272, 1997.
41. Li J and Mitchell JH. c-Fos expression in the midbrain periaqueductal gray during static muscle contraction. *Am J Physiol* 279: H2986-H2993, 2000.
42. Miki K, Kosho A, and Hayashida Y. Method for continuous measurements of renal sympathetic nerve activity and cardiovascular function during exercise in rats. *Exp Physiol* 87.1: 33-39, 2002.
43. Mitchell JH, Kaufman MP, and Iwamoto GA. The exercise pressor reflex: Its cardiovascular effects, afferent mechanisms, and central pathways. *Ann Rev Physiol* 45: 229-242, 1983.
44. Moncada S and Higgs A. The L-arginine-nitric oxide pathway. *New Eng J Med* 329: 2002-2012, 1993.
45. Paton JFR, Lonergan T, Deuchars J, James PE, and Kasparov S. Detection of angiotensin II mediated nitric oxide release within the nucleus of the solitary tract using electron-paramagnetic resonance (EPR) spectroscopy. *Autonomic Neuroscience: Basic and Clinical* 126-127: 193-201, 2006.
46. Plochocka-Zulinska D and Krukoff TL. Increased gene expression of neuronal nitric oxide synthase in brain of adult spontaneously hypertensive rats. *Brain Res Mol Brain Res* 48: 291-297, 1997.
47. Pontieri V, Venezuela MK, Scavone C, and Michelini LC. Role of endogenous nitric oxide in the nucleus tractus solitarii on baroreflex control of heart rate in spontaneously hypertensive rats. *J Hypertens* 16: 1993-1999, 1998.
48. Qadri F, Arens T, Schwarz EC, Hauser W, Dendorfer A, and Dominiak P. Brain nitric oxide synthase activity in spontaneously hypertensive rats during the development of hypertension. *J Hypertens* 21: 1623-1624, 2003.
49. Rowell L, Hermansen L, and Blackmon J. Human cardiovscular and respiratory responses to graded muscle ischemia. *J Appl Physiol* 41: 693-701, 1976.
50. Schmidt P, Lange R, Gorren A, Werner E, Mayer B, and Andersson K. Formation of a protonated trihydrobiopterin radical cation in the first reaction cycle of neuronal and endothelial nitric oxide synthase detected by electron paramagnetic resonance spectroscopy. *J Biol Inorg Chem* 6: 151-158, 2001.
51. Schmidt TS and Alp NJ. Mechanisms for the role of tetrahydrobiopterin in endothelial function and vascular disease. *Clinical Science* 113: 47-63, 2007.
52. Schuchmann S, Albrecht D, Heinemann U, and Halbach OvBu. Nitric oxide modulates low-Mg^{2+}-induced epileptiform activity in rat hippocampal-entorhinal cortex slices. *Neurobiology of Diseases* 11: 96-105, 2002.
53. Smith SA, Mitchell JH, and Garry MG. Electrically induced static exercise elicits a pressor response in the decerebrate rat. *J Physiol* 537: 961-970, 2001.
54. Smith SA, Mitchell JH, Naseem RH, and Garry MG. Mechanoreflex mediates the exaggerated exercise pressor reflex in heart failure. *Circulation* 112: 2293-2300, 2005.
55. Stroes E, Hijmering M, van Zandvoort M, Wever R, Rabelink TJ, and van Faassen EE. Origin of superoxide production by endothelial nitric oxide synthase. *FEBS Lett* 438: 161-164, 1998.

56. Tai M-H, Wang L-L, Wu KLH, and Chan JYH. Increased superoxide anion in rostral ventrolateral medulla contributes to hypertension in spontaneously hypertensive rats via interactions with nitric oxide. *Free Radical Biology & Medicine* 38: 450-462, 2005.
57. Thomas G, Zhang W, and Victor R. Impaired modulation of sympathetic vasoconstriction in contracting skeletal muscle of rats with chronic myocardial infarctions. *Circ Res* 88: 816-823, 2001.
58. Thomas G, Zhang W, and Victor R. Nitric oxide deficiency as a cause of clinical hypertension. *JAMA* 285: 2055-2057, 2001.
59. Vasquez-Vivar J, Kalyanaraman B, and Martasek P. The role of tetrahydrobiopterin in superoxide generation from eNOS: enzymology and physiological implications. *Free Radical Res* 37: 121-127, 2003.
60. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters B, Karoui H, Tordo P, and KA Pritchard J. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci USA* 95: 9220-9225, 1998.
61. Vasquez-Vivar J, Martasek P, Whitsett J, Joseph J, and Kalyanaraman B. The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. *Biochem J* 362: 733-739, 2002.
62. Victor R, Bertocci L, Pryor S, and Nunnally R. Sympathetic nerve discharge is coupled to muscle cell pH during exercise in humans. *J Clin Invest* 82: 1301-1305, 1988.
63. Waki H, Murphy D, Yao ST, Kasparov S, and Paton JFR. Endothelial NO synthase activity in nucleus tractus solitarii contributes to hypertension in spontaneously hypertensive rats. *Hypertension* 48: 644-650, 2006.
64. Wallin BG and Charkoudian N. Sympathetic neural control of integrated cardiovascular function: insights from measurement of human sympathetic nerve activity. *Muscle & Nerve* 36: 595-614, 2007.
65. Wang S, Paton JFR, and Kasparov S. The challenge of real-time measurements of nitric oxide release in the brain. *Autonomic Neuroscience: Basic and Clinical* 126-127: 59-67, 2006.
66. Weissman B and Gross S. Measurement of nitric oxide and nitric oxide synthase. *Current Protocols in Neuroscience*, 1998.
67. Xia Y, Tsai AL, Berka V, and Zweier JL. Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J Biol Chem* 273: 25804-25808, 1998.
68. Zimmermann M, Lazatigues E, Lang J, Sinnayah P, Ahmad I, Spitz D, and Davisson R. Superoxide mediates the actions of angiotensin II in the central nervous system. *Circ Res* 91: 1038-1045, 2002.

Figures

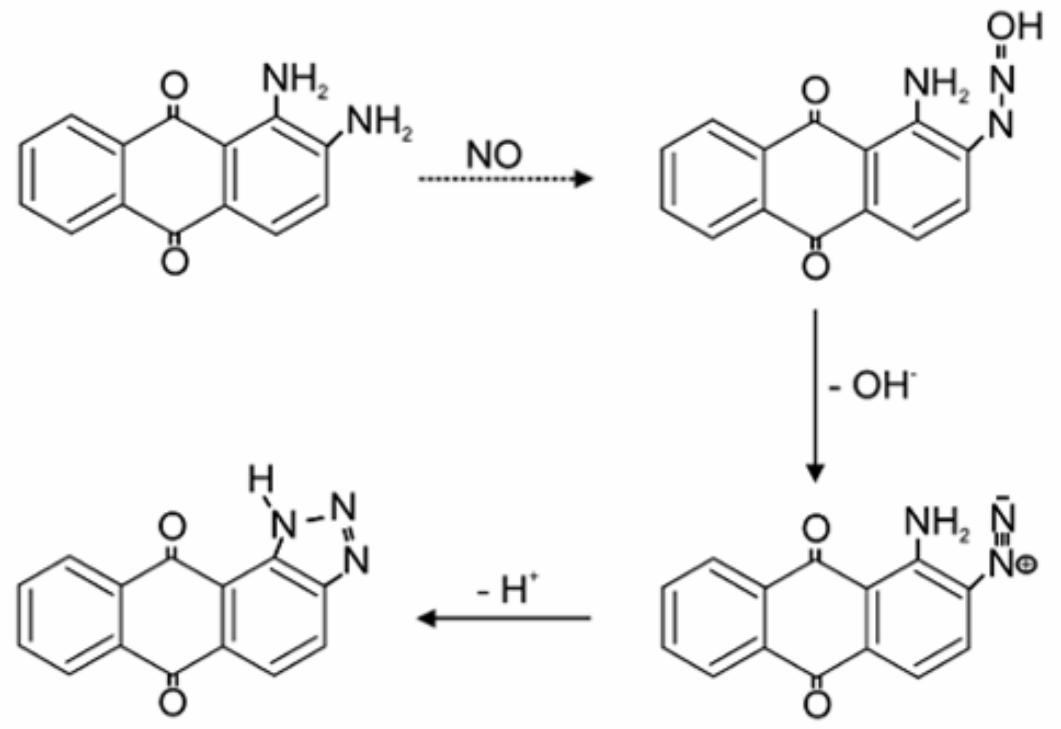

Figure 1. The Nitric Oxide Fluorescent Probe 1,2-Diaminoanthraquinone. Diaminoanthraquinone is a nonfluorescent molecule until NO reacts with its aromatic amino groups to form a condensed triazole ring. The first step of this reaction is nitrosylation of an amino group, most probably by dinitrotrioxide, which is formed through the reaction of NO with oxygen. The final product, H-anthra-[*1*,2d]-[*1*,*2*,3]-triazole-6,11-dione, produces a red-fluorescent precipitate that is water-insoluble. Source: *Nitric Oxide* 9: 217-228.

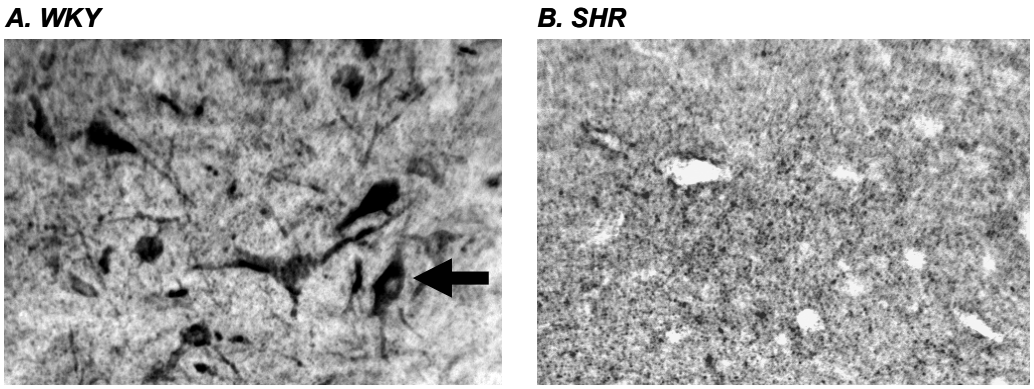

Figure 2. Photomicrograph of Inducible Nitric Oxide Synthase Immunoreactivity in the NTS. Positive protein expression, demarcated by arrow, seemed greater in normotensive (A, WKY) than in hypertensive (B, SHR) animals. Magnification: 10x.

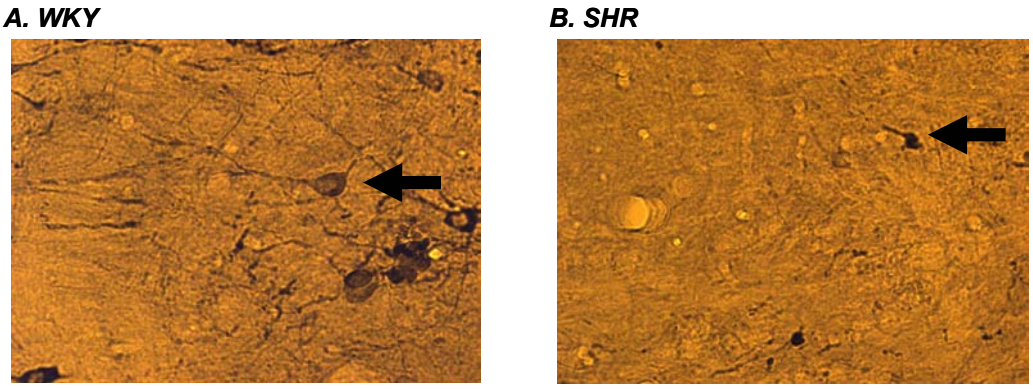

Figure 3. Photomicrograph of Neuronal Nitric Oxide Synthase Immunoreactivity in the NTS. Positive protein expression, demarcated by arrows, seemed greater in normotensive (A, WKY) than in hypertensive (B, SHR) animals. Magnification: 10x.

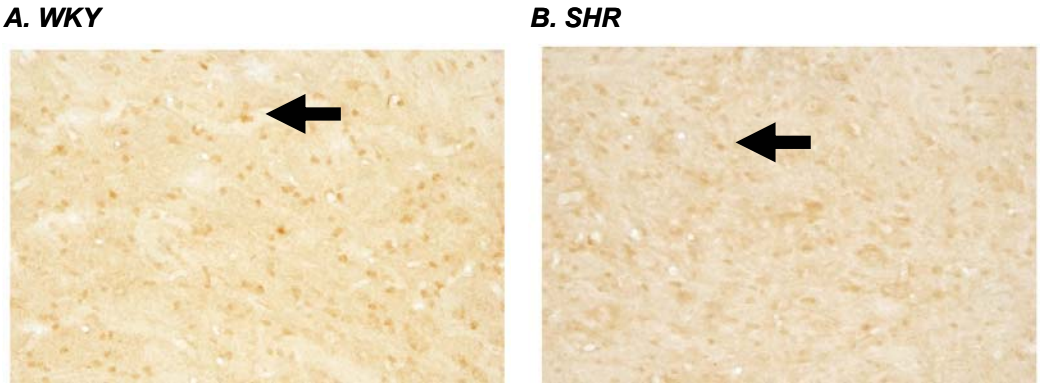

Figure 4. Photomicrograph of Endothelial Nitric Oxide Synthase Immunoreactivity in the NTS. Positive protein expression, demarcated by arrows, seemed similar in normotensive (A, WKY) and hypertensive (B, SHR) animals. Magnification: 10x.

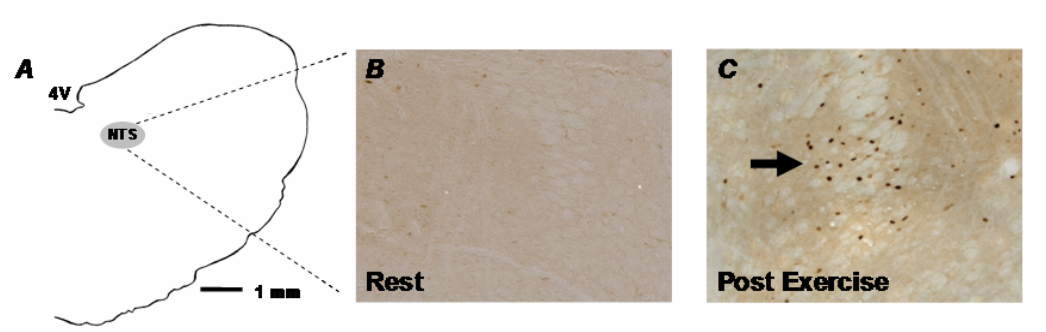

Figure 5. c-Fos Expression within the NTS of a Representative WKY Animal after EPR Activation. (A) Diagram of a medullary brain section showing the NTS region stained for c-Fos expression. **(B)** Photomicrograph of the NTS region (20x) stained immunohistochemically for c-Fos in a sham animal under resting conditions. **(C)** Photomicrograph of the NTS region (20x) stained immunohistochemically for c-Fos in an animal postexercise. 4V, fourth ventricle. Arrow demarcates an example of positive protein expression.

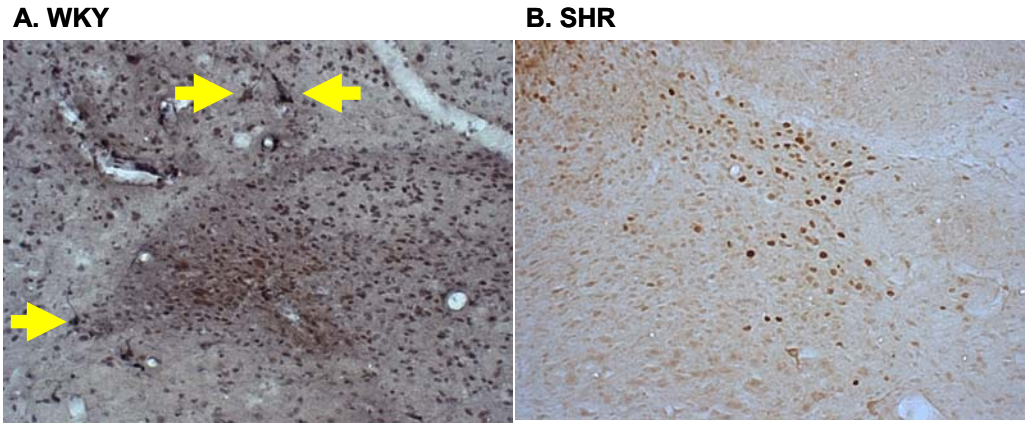

Figure 6. c-Fos and nNOS Expression within the NTS of a Representative WKY and SHR Animal after Mechanoreflex Activation Photomicrographs (20x) of NTS tissue in WKY (A) and SHR (B) rats after one hour of passive hindlimb muscle stretch. c-Fos expression is evident in the NTS of WKY and SHR brainstem tissue, however cells positive for nNOS protein can only be seen in normotensive WKY NTS. Arrows demarcate neurons with positive c-Fos and nNOS expression.

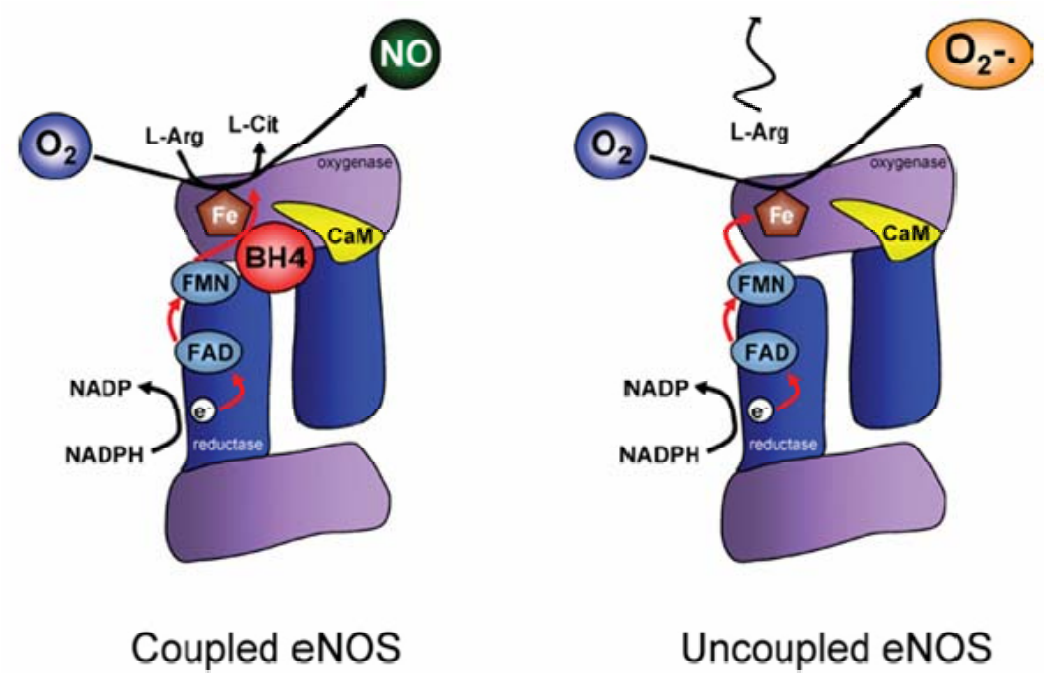

Figure 7. Schematic Illustrating the Differences in Coupled and Uncoupled eNOS. When calcium/calmodulin binds to coupled eNOS, NADPH-derived electrons (e-) flow from the FAD and FMN in the reductase domain to a ferrous-dioxygen complex in the oxygenase domain through tetrahydrobiopterin (BH4). This is coupled with the reduction of molecular oxygen and the oxidation of L-arginine to NO and L-citrulline. When BH4 is absent, as in uncoupled eNOS, the ferrous-dioxygen complex dissociates to form superoxide (O2-.). CaM, calmodulin; Fe, ferrous-dioxygen complex; FAD and FMN, flavins; L-arg, L-arginine; L-Cit, L-citrulline. Source: *Clinical Science* 113: 47-63.

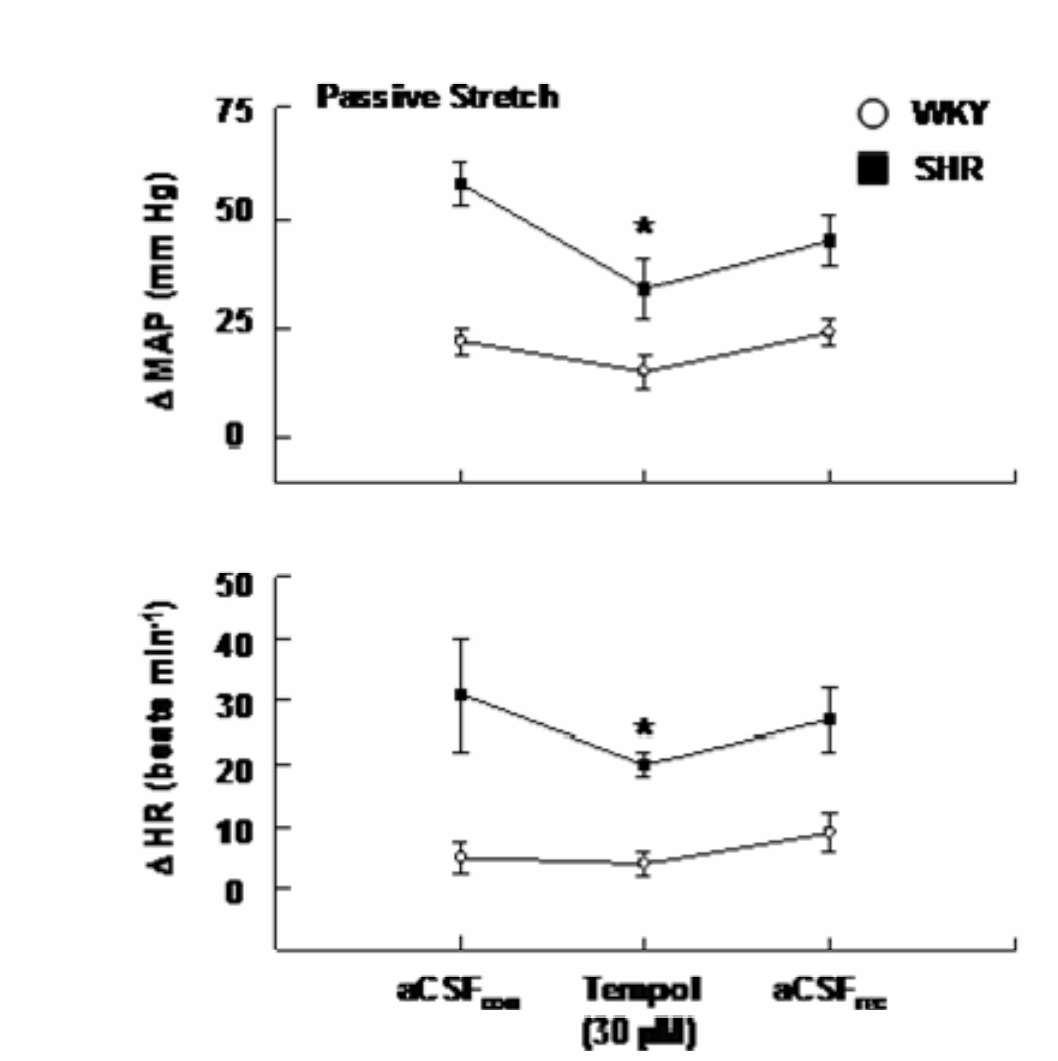

Figure 8. Affect of Tempol on Mechanoreflex Activation in WKY and SHR Rats. Microdialysis of the superoxide dismutase mimetic tempol within the NTS attenuated the MAP and HR response to passive muscle stretch in SHR (n=4) and WKY (n=3) animals. aCSF, artificial cerebrospinal fluid; con, control; rec, recovery. The change in muscle tension in response to passive muscle stretch was not different between groups. *Indicates significantly different from aCSF trial ($P<0.05$).

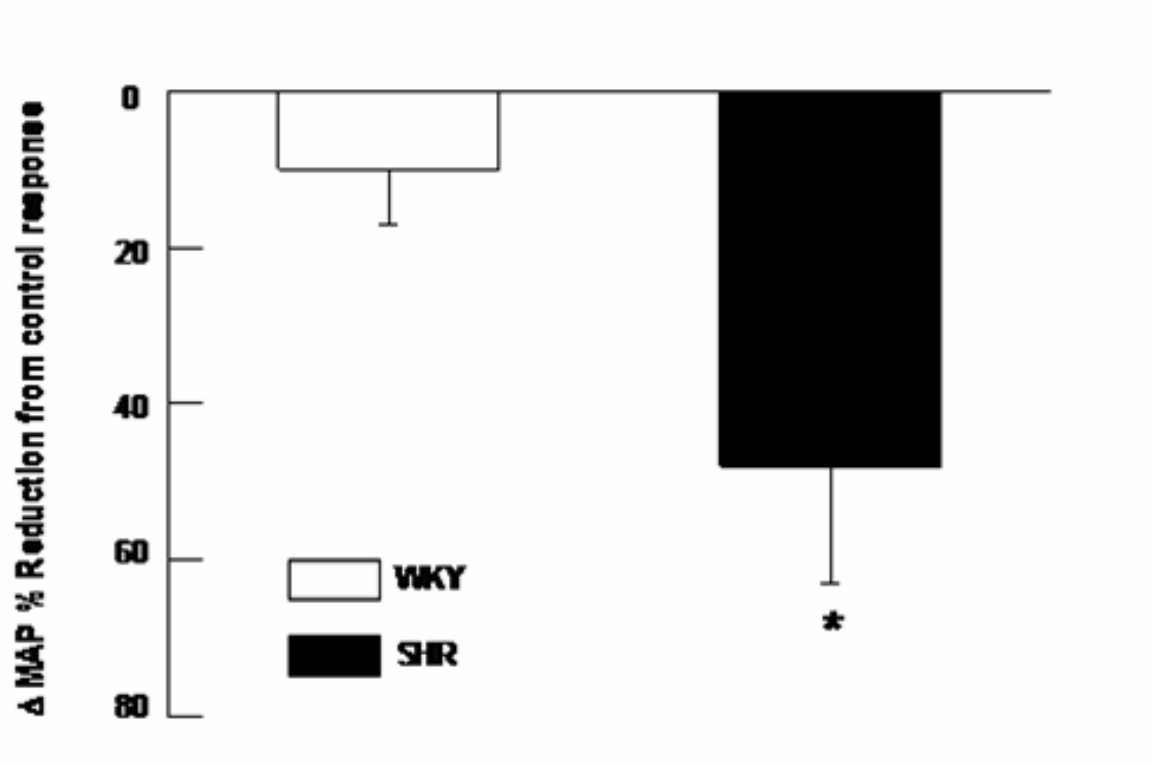

Figure 9. Affect of Tempol on Metaboreflex Activation in WKY and SHR Rats. Microdialysis of the superoxide dismutase mimetic tempol within the NTS attenuated the pressor response to activation of group IV afferent fibers in both WKY (n=4) and SHR (n=5) animals. However, the tempol-induced reduction in the pressor response to capsaicin was significantly greater in SHR. *Indicates significantly different from WKY ($P<0.05$).