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MECHANISMS BY WHICH ACUTE ET_B RECEPTOR ACTIVATION AFFECT THE AUTONOMIC NERVOUS SYSTEM IN THE CONTROL OF BLOOD PRESSURE

By

Yanny Lau Phillips

A DISSERTATION

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ABSTRACT

MECHANISMS BY WHICH ACUTE ET_B RECEPTOR ACTIVATION AFFECT THE AUTONOMIC NERVOUS SYSTEM IN THE CONTROL OF BLOOD PRESSURE IN THE RAT

By

Yanny Lau Phillips

It is well known that the autonomic nervous system plays an important role in the regulation of blood pressure. Increased sympathetic activity has been linked to the development of hypertension in both human and animal models of the disease. This study focused on a new rat model of hypertension where blood pressure was increased by in vivo activation of endothelin type B receptors (ETBR)s. This is achieved by infusion of the specific receptor agonist, sarafotoxin 6c (S6c). The mechanism by which S6c causes hypertension is not clear, but evidence points to the systemic veins as a likely target. Preliminary studies indicate that S6c infusion may also increase sympathetic nervous activity (SNA). It is not clear whether this action of S6c is directly on the veins or mediated indirectly through the sympathetic nervous system. The primary purpose of the experiments described here was to identify mechanisms by which ETBR receptor activation affects autonomic regulation of blood pressure.

Using a combination of histological, surgical and pharmacological techniques, I tested three hypotheses: 1) S6c acts on the ETBR in veins to cause venoconstriction, leading to blood volume shifts into the thoracic region; 2) S6c

acts on ETBR in sympathetic ganglia to increase the production of superoxide anions; 3) S6c acts on ETBR in the brain to increase SNA.

Overall, the data support hypothesis 1 and 2 as likely mechanisms by which ETBR activation affects autonomic nervous control of blood pressure. Based on my findings, I contend that *in vivo* ETBR activation primarily involves peripheral venoconstriction to increase blood pressure. Increased venous return to the heart would consequently raise cardiac output and centralize blood volume from the extrathoracic vasculature to the cardiothoracic region. Increased blood volume to the heart would also cause decreased sympathetic nervous system activity due to activation of cardiopulmonary receptors.

The results of my work highlight a potentially important hemodynamic mechanism by which acute ETBR activation may lead to the pathogenesis of hypertension, and illustrate appropriate autonomic nervous system responses to those hemodynamic changes. Furthermore, this study suggests an important contribution of veins to the development of high blood pressure. The roles of reactive oxygen species and the sympathetic nervous system in chronic hypertension produced by systemic ETBR activation remain to be established.

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2006

To my parents, Duncan and Lily, for giving up some many of your dreams so that I can pursue mine.

To my husband, Shaun, for being my constant light in the forest...you fill up my senses, too.

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LIST OF ABBREVIATIONS

AP	area postrema
Ang II	angiotensin II
ATP	adenosine 5'-triphosphate
CHF	congestive heart failure
CO	cardiac output
CSF	cerebrospinal fluid
CVLM	caudal ventrolateral medulla
CVO	circumventricular organ
d	day
DAG	1,2-diacylglycerol
DHE	dihydroethidine
DOCA	deoxycorticosterone acetate
ECE	endothelin converting enzyme
ET	endothelin
ET_A	endothelin type A receptor
ET_B	endothelin type B receptor
ETBR	endothelin type B receptor
GFR	glomerular filtration rate
h	hour
HR	heart rate
dβh	dopamine-beta-hydroxylase
ICC	immunocytochemistry

icv	intracerebroventricular
IMG	inferior mesenteric ganglia
ip	intraperitoneal
IP3	inositol (1,4,5) trisphophate
IR	immunoreactivity
iv	intravenous
KA	kainic acid
MCFP	mean circulatory filling pressure
MAP	mean arterial pressure
ME	median eminence
min	minute
NO	nitric oxide
NTS	nucleus tractus solitarius
O_2^-	superoxide anion
OVLT	organum vasculosum lateral terminalis
PBS	phosphate buffered saline
PE	phenylephrine
PKC	protein kinase C
PIP2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PVN	paraventricular nucleus
RPF	renal plasma flow
S6c	sarafotoxin 6c

SHR	spontaneously hypertensive rats
SV	stroke volume
SNA	sympathetic nervous activity
SON	supraoptic nucleus
SFO	subfornical organ
TPR	total peripheral reistance
VE	volume expansion
5-HT	5 hydroxytryptamine

CHAPTER 1

GENERAL INTRODUCTION

Hypertension

High blood pressure or hypertension remains a common and serious problem in the United States and worldwide despite efforts in the recognition and treatment of the disease over the past decades. Currently, about 65 million adults (18 years and older) in the United States have the disease, which is up from 50 million just a decade ago (US Census bureau 2000). According to National Health and Nutrition Examination Surveys (NHANES), one in four Americans will develop hypertension during their lifetimes. Many of these individuals will go undiagnosed, as hypertension is a lifelong condition that is usually asymptomatic for many years, and is thus called the 'silent killer'. The operational definition of hypertension offered by the Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure (JNC) is a systolic blood pressure of over 140 mmHg and/or a diastolic blood pressure of over 90 mmHg. However, in the venerable words of Sir George Pickering, "There is no dividing line. The relationship between arterial pressure and mortality is quantitative; the higher the pressure, the worse the prognosis." Uncontrolled hypertension may lead to many cardiovascular and renal pathologies that include coronary artery disease, stroke, congestive heart failure (CHF), glomerulonephritis and end stage kidney failure. Hypertension is an insidious disease that kills thousands of Americans each year and millions globally. Since the cause is unknown in the majority of cases, treatment for hypertension to date is heuristic and not very successful. New approaches to treating or preventing hypertension are needed.

Primary or essential hypertension has no known cause and constitutes as much as 90-95% of all hypertension (American Heart Association). The other 5-10% is called secondary hypertension because the observed chronic high blood pressure is secondary to an identifiable underlying abnormality. Some of the underlying causes of secondary hypertension includes abnormal function of the renin-angiotensin system, pheochromocytoma, primary aldosteronism, chronic kidney disease, aortic coarctation and Cushing's syndrome (Gordon et al, 1994; Chobanian et al, 2003).

Though the bulk of hypertensive cases do not have definitive etiologies, multiple hypotheses have been generated. It is unlikely that any single underlying defect starts the hemodynamic course toward sustained hypertension. Rather, hypertension is believed to be the end result of interactions between several different patterns of genotypic predisposition and environmental factors affecting the various cardiovascular regulatory systems (Kostis et al, 2005).

Veins in Hypertension

Blood pressure (BP) is primarily determined by cardiac output (CO) and total peripheral resistance (TPR). CO reflects the pressure required for the heart to pump blood volume through the circulatory beds and is the product of the stroke volume (SV) and heart rate (HR), while TPR is a measure of contractile activity in the smooth muscles of the arterial vasculature. Hypertension can be initiated by increases in either CO or TPR, but a common pattern in human essential

hypertension is an early increase in CO followed by a gradual return of CO to normal and an increase in TPR (Lund-Johansen, 1994).

$$MAP = CO \times TPR$$

$$CO = SV \times HR$$

Compliance is the ability of arteries and veins to distend with increasing transmural pressure. Vascular capacitance, which is the total blood volume throughout the physiological range of transmural distending pressures, is greatly affected by compliance (Rothe, 1993). Compared to arteries, veins have thinner walls, bigger cross-sectional area and higher compliance owing to their structural composition of greater collagen and less smooth muscle (Shepherd and Vanhoutte, 1975). The smooth muscle cells provide tone during contraction, while the fibrous proteins give rise to the vessel's viscoelastic properties. Venules are 60 times more distensible than arterioles due to smaller amounts of elastin in their structural composition.

Venous smooth muscle tone is controlled by both the distending blood volume and the vessel's compliance and is measured by a variety of methods including mean circulatory filling pressure (MCFP) (Rothe, 1993). MCFP is the best measure of total vascular compliance (primarily venous) and represents the pressure obtained once the heart is stopped and pressures from all points of the arterial and venous circulation are allowed to equilibrate. Together with blood

volume, MCFP is an index of venous capacitance (Yamamoto et al, 1981). Venous tone is a strong determinant of CO by affecting cardiac filling, which in turn influences arterial blood volume and systemic blood pressure (Greenway, 1983; Rothe, 1983, 1993). Hence, veins are the primary capacitance system in the body and alterations in venomotor tone can greatly affect CO and blood pressure.

Small veins and venules hold most (85%) of the body's blood volume (Rothe, 1983; Milnor, 1990; Haase and Shoukas, 1992), most of which is located within the splanchnic circulation (Pang CC, 2000). The splanchnic region, consisting of the liver, pancreas, spleen and intestines, is highly compliant and densely innervated by sympathetic nerves (Rothe, 1983; Rowell, 1990).

Blood flow to the splanchnic organs derives from the abdominal aorta via celiac, superior mesenteric and inferior mesenteric arteries. The veins in the splanchnic region normally hold a volume of blood equal to a quarter of the CO, making it the most important capacitance bed in the circulation (Greenway, 1983; Safar and London, 1987; Pang, 2000). Increased venous smooth muscle contraction would cause blood volume to redistribute from the periphery and the splanchnic circulation back to the heart, causing an upsurge in venous return and consequently increased cardiac filling and cardiac output, an autoregulatory adjustment in peripheral arterial resistance, thus resulting in greater arterial blood pressure. Therefore, alterations in the venomotor tone of splanchnic vasculature can have far reaching hemodynamic consequences. In animal studies, venomotor tone is reported to be elevated in deoxycorticosterone

acetate (DOCA)-salt (Yamamoto et al, 1983) and spontaneously hypertensive (Martin et al, 1998; Trippodo et al, 1981) rat models. Futhermore, dogs with angiotensin II induced hypertension and aldosterone induced hypertension also show elevated MCFP (Pan and Young, 1982; Young et al, 1980). Clinically, patients that have borderline hypertension show decreased venous compliance and a central redistribution of blood volume (Mark, 1984). Additionally, patients suffering from orthostatic hypotension resulting from autonomic insufficiency have been successfully treated with a drug that constricts the splanchnic veins (Lamarre-Cliché and Cusson, 1999).

The Autonomic Nervous System

The peripheral autonomic nervous system is comprised of two opposing branches, the sympathetic and parasympathetic nervous systems, which are vital to cardiovascular regulation, as well as a third branch, the enteric nervous system, which innervates the splanchnic organs and controls gastrointestinal function (Langley, 1921).

The sympathetic nervous system arises from preganglionic neurons in the intermediolateral columns of the spinal cord that extend from the thoracic to the lumbar spinal segments and synapse on postganglionic neurons housed in sympathetic ganglia, mainly in the paravertebral and prevertebral ganglia. The paravertebral ganglia consist of 22 pairs and are also called sympathetic chain ganglia because they lie on either side of the spinal cord and connected by nerve trunks forming a chain (Burnstock 1986). The prevertebral ganglia, on the other

hand, are much fewer in number, consisting only of the celiac, superior mesenteric and inferior mesenteric ganglia (IMG) and are found on the ventral surface of the vertebral column near the abdomen and pelvis. Postganglionic fibers from the prevertebral sympathetic ganglia innervate pelvic and abdominal viscera (Burnstock, 1986). Activation of nicotinic acetylcholinergic receptors on postganglionic neurons which synapse on neuroeffector cells, causes the release of norepinephrine (NE). The effects of increased sympathetic nervous activation are mediated primarily by α and β adrenergic receptors on the effector organs, although other transmitters also participate.

The parasympathetic system has a craniosacral distribution and mainly consists of the cranial nerves that originate from preganglionic neurons in the brain and their postganglionic neurons located on or nearby the effector tissue. Of particular importance is the 10^{th} cranial nerve called the vagus which carries afferent and efferent fibers that provide vital function to areas including the heart, diaphragm, and viscera. Cell bodies of vagal sensory fibers lie predominantly in the nodose ganglia. Parasympathetic neurotransmission is similar to the sympathetic nervous system with the exception that postganglionic axon terminals cause the release of acetylcholine that bind to either nicotinic or muscarinic receptors in the effector organs.

The sympathetic and parasympathetic nervous systems function in an opposing manner to control the internal physiological environment, including metabolism, heart rate and blood pressure. The sympathetic nervous system is associated with moment-to-moment activation, which is sometimes referred to as

the 'flight or fight' response where heart rate is accelerated and blood pressure is increased. Because the parasympathetic limb is more concerned with conservation of energy and maintenance of bodily functions, its role is sometimes referred to as 'rest and digest.' Parasympathetic activation slows the heart and decreases blood pressure.

Hyperactivity of the sympathetic nervous system has been implicated as a primary element in the pathogenesis of hypertension as well as in its maintenance in both human and experimental animal models of the disease. Increased activity of neurons in several brain regions enhances firing of neurons in the anterior horn of the spinal cord (de Champlain, 1990) which, in turn, leads to the elevated activity in postganglionic neurons. Efficiency of transmission in the prevertebral ganglia has been reported to be potentiated in some forms of hypertension (Anderson et al, 1989; Aileru et al 2004). Patients in early stages of the disease, also called "borderline" hypertension, have increased CO and HR, associated with increased sympathetic and decreased parasympathetic activities (Julius, 1994; Wyss, 1993). Elevated sympathetic nervous activation also has been implicated in many hypertension related morbidities and mortality (Brooke and Julius, 2000). Moreover, drugs that reduce SNA have proven especially beneficial in the treatment of hypertensive patients with signs of autonomic imbalance (Brooke and Julius, 2000). In addition, plasma catecholamines have been reported to participate in both the development of hypertension by stimulating pressor mechanisms and its maintenance via vascular smooth muscle hypertrophy (Yu et al, 1996). It has also been observed that increased

α adrenergic receptor activation in the early morning, as reflected by elevated forearm vascular resistance, correlates well with the increased prevalence of sudden death, stroke and other cardiovascular morbidities that occur at those hours (Kaplan, 1998).

The sympathetic nervous system is the most important determinant of venomotor tone, especially in the splanchnic venous bed (Hainsworth, 1990; Ozono et al, 1991; Rothe, 1993; Shoukas and Bohlen, 1990). Browning et al (1999) found that sympathetic neurons innervating arteries and veins differ in their location in the ganglia and in their electrophysiological properties. Furthermore, differences in the localization of sympathetic postganglionic neurons that innervate arteries and veins have been reported (Hsieh et al, 2000). Luo et al (2003) observed a differential response in sympathetic neurotransmission of mesenteric arteries and veins to hypertension. Collectively, these studies suggest differential sympathetic neural control of arteries and veins.

Sympathetic activation of the splanchnic veins are integral to the minute to minute regulation of venoconstriction and can decrease blood volume up to 60% in that region, shifting blood volume into the heart, resulting in blood pressure increase (Rothe, 1983; Karim and Hainsworth, 1976; Greenway, 1983). Since the splanchnic venous bed is the primary vascular capacitance in circulation, (Furness et al, 2001; Hainsworth; 1990; Rothe, 1986), factors that modulate it can have profound consequences for cardiovascular function.

Increased sympathetic venomotor tone has been shown to lower venous capacitance in hypertension in animal models as well as human patients (Albrecht et al, 1975; Frohlich and Pfeffer, 1975; Julius, 1988; Martin et al, 1998; Noresson et al, 1979). Clinical evidence highlighting the physiological importance of sympathetic venoconstriction is appreciated in patients with orthostatic hypotension associated with autonomic dysfunction (Smit et al, 1999). In orthostatic hypotension, venous return is impaired leading to an excessive fall in blood pressure upon standing erect, resulting in tachycardia, dizziness and loss of consciousness. Patients with neuromediated syncope have also been reported to have abnormal venous function (Manyari et al, 1996). Low et al (1994) reported that in postural orthostatic tachycardia syndrome, sympathetic nervous activity (SNA) is impaired in veins, most likely in the splanchnic region. Abnormal venous function has also been implicated in the pathogenesis of chronic fatigue syndrome (Streeten, 2001). The importance of sympathetic control of veins is further supported in the clinical treatment of the orthostatic disorders. Orthostatic hypotension can be effectively treated either by drugs that target splanchnic venoconstriction (Lamarre-Cliché and Cusson, 1999) or by direct compression of the capacitance beds in the abdominal cavity (Denq et al, 2001). Epidural anesthetics have been demonstrated in rabbits to increase vascular capacitance by inhibiting sympathetic venomotor tone in the splanchnic bed (Hogan et al, 1995). Moreover, venomotor tone can be dampened by drugs that interfere with SNA, such as α adrenergic blocker, and preload reducing venodilators like nitroglycerin and sodium nitroprusside (Pang, 2001). Thus, one

mechanism by which the sympathetic nervous system controls blood pressure is through the maintenance of venous tone and this has been implicated as a primary precursor of hypertension.

Baroreceptor Reflex system

Baroreceptors are the principal modulators of sympathetic and parasympathetic activity during acute changes in blood volume or pressure (Thrasher, 2004). Cardiopulmonary baroreceptors and arterial baroreceptors of the aortic arch and carotid sinus respond to stretch by relaying afferent neural signals to the brain via branches of the glossopharyngeal and vagus nerves, cranial nerves IX and X, respectively, and terminate on barosensitive neurons of the nucleus of the solitary tract (NTS) (Badoer et al, 1994; Hines et al 1994). The NTS sends excitatory projections to the caudal portion of the ventral lateral medulla (CVLM), referred to as the ventral depressor area (Willette et al, 1987), which in turn provides tonic inhibition of sympathetic premotor neurons in the rostral portion of the ventral lateral medulla (RVLM), resulting in sympathoinhibition (Minson et al, 1997; Sved et al, 2000). Stretch signals from mechanoreceptors inhibit sympathetic and stimulate parasympathetic efferent activity, while plasma volume depletion causes a decrease in receptor stretch to reduce the afferent stimuli, thus decreasing parasympathetic activity and increasing sympathetic activity. Normally, the baroreceptor reflexes respond to increased blood volume/pressure with a reduction in HR by sympathoinhibition and vagal stimulation. In hypertension, these reflexes are reset, such that a given BP increase elicits less compensatory decrease in HR (Chapleau et al,

1995). Floras et al (1988) reported that baroreceptors of hypertensive patients have less sensitivity than normotensives when stimulated with plasma norepinephrine and pressor changes.

Recent studies suggest that baroreceptor reflexes themselves may participate in the long-term regulation of blood pressure (Malpas, 2004). Lohmeier et al (2000) showed that chronic (5 day) Ang II infusion into dogs causes decreased renal SNA, mediated by the baroreceptor reflex. They also reported that chronic Ang II treatment effected a sustained neural activation of central nervous system components of the baroreflex pathway as reflected in Fos-like immunoreactive staining (Lohmeier et al, 2002). Abnormal baroreceptor function facilitates vasopressin release from the posterior pituitary and stimulates renal release of renin (Dibona and Swain, 1985; Haanwinkel et al, 1995; Norsk, 1996), factors which promote long term increases in blood pressure. Taken together, these data suggest that the baroreceptor reflex system may participate in the long-term control of blood pressure and may be involved in the genesis, as well as maintenance, of hypertension.

Endothelin System

ET synthesis

Endothelin (ET) was first described as a smooth muscle constricting factor two decades ago (Hickey et al, 1985) and has since been identified, isolated, cloned and characterized by Dr. Masashi Yanagisawa in 1988, who gave this protein its present name. This 21 amino acide peptide is a very potent vasoconstrictor secreted by a wide variety of cells. ET family peptides are formed by cleavage of 174 amino acids from the 212 amino acid pre-pro-endothelin by specific endopeptidases, resulting in a 38 amino acid Big ET (Figure 1). Big ET is subsequently converted to ET by an endothelin converting enzyme (ECE; Yanagisawa et al, 1988; Parissis et al, 2001) of which 2 types have been identified, ECE-1 and ECE-2. ECE-1 is membrane-bound and operates at neutral pH while ECE-2 acts in the intracellular environment where it is more acidic (Rubanyi and Polokoff, 1994)

The ET family consists of 3 isoforms, ET-1, ET-2, and ET-3, encoded by 3 distinct genes. They differ in their chemical structure, potency of smooth muscle effect (Inoue et al, 1989) and distribution (Shriffrin, 1999). ET is produced and active in almost all tissues with variations in the distribution of the different isoforms (Goraca, 2002). ET-1 is the most important biological isoform produced in endothelial cells (Yanagisawa et al, 1988; Inoue et al, 1989) and vascular smooth muscle (Hahn et al, 1990). It has also been reported to be produced by neurons and astrocytes in the brain (Davenport and Battistini, 2002). ET-2 is produced mainly in kidney and intestines. ET-3 is most abundant in the central

nervous system and has been implicated in the development of neuronal function (Furuya et al, 2001). ET-1 and ET-3 peptides are also synthesized and secreted from rat postganglionic sympathetic neurons, where they are believed to modulate neurotransmission (Damon, 1998).

ET affects blood pressure

Studies of ET in animal models and in humans have suggested that these peptides are involved in vascular physiology and disease. Plasma levels of ET-1 are higher after a myocardial ischemic event in both animals and humans (Battistini et al, 1993). Furthermore, in rats, intravenous infusion of ET-1 has been shown to reduce coronary blood flow by more than 90% (Kurihara et al, 1989). Direct infusion of ET-1 into the ventricles of the brain increased blood pressure as well as catecholamine secretion, an effect which further maintained the rise in blood pressure (Ouchi et al, 1989). Plasma ET concentrations have been found to be up to four-fold higher in patients suffering from congestive heart failure (CHF) (Wei et al, 1994). In fact, it has been suggested that plasma ET measurements may have prognostic value in treating CHF patients (Omland et al, 1994). Macquin-Mavier et al (1989) demonstrated that ET-1 induced bronchoconstriction in the guinea pig suggesting a role in the pathogenesis of pulmonary disease. This view is supported by the fact that asthma patients have elevated ET-1 levels in alveolar fluid (Mattoli et al, 1991). In addition, ET-1 has been reported to decrease both the renal plasma flow (RPF) and glomerular filtration rate (GFR) through vasoconstriction of the glomerular afferent and

efferent arterioles (Badr et al, 1989). These effects of ET-1 to lower RPF and GFR both act to increase blood pressure.

How ET contributes to the development of hypertension is not entirely clear, however, it has been reported that some hypertensive patients have elevated plasma levels of the peptide (Ergul et al, 1996). Moreover, hypertensive patients have a more exaggerated vasoconstrictor response to ET-1 treatment than their normotensive counterparts (Cardillo et al, 1997). ET-1 has been reported to be increased in gestating women with preeclampsia (Branch et al, 1991). In addition, normotensive patients given a systemic ET receptor blocker developed peripheral vasodilation and hypotension (Haynes et al, 1996). In rats, intravenous (iv) administration of ET has been demonstrated to provoke a sustained increase in blood pressure (Mortensen and Fink, 1990). ET also has central actions as Ouchi et al (1989) showed that an intracerebroventricular (icv) infusion of ET-1 induced a dose-dependent increase in blood pressure.

The effects of all three isoforms of ET in humans are mediated by two receptors subtypes, ET_A and ET_B, that have been isolated and cloned from mammalian tissue (Inoue et al, 1989; Arai et al, 1990). Potentially, a third subtype, ET_C, exists and has been found to be specific for ET-3, however, this receptor has only been detected in Xenopus frogs (Tukawa 1993).

ET receptors

ET receptors belong to the rhodopsin superfamily of G-protein coupled putative seven transmembrane domain receptors (Sakurai et al, 1990; Alexander et al, 2001; Davenport 2000). ET activation causes vasoconstriction by increasing intracellular calcium levels via modulation of both dihydropyridine receptors and the phospholipase C cascade (PLC; Sakurai et al, 1990; Resink et al, 1988). All three endogenous ET isomers, especially ET-3, bear similar structure and sequence homology to a family of 21 amino acid peptides isolated from the venom of the snake, *Atractaspis engadensis,* called sarafotoxins (Figure 3) (Sokolovsky, 1992; Goraca 2002).

The rates of ligand-receptor dissociation vary among the three different ET isoforms (Galdron et al, 1989; Devesly et al, 1990) as well as from different animal species and tissues (Galdron et al, 1991). However, they all share the unique property of having a slow rate of dissociation and near irreversibility of binding (Hirata et al, 1988). Hence, effects of ET are prolonged, but the half-life of ET in circulation is extremely short (Anggard et al, 1989). ET receptors are differentially expressed in many tissues and organs of the body, including the cardiomyocytes in the heart, brain blood vessels and parenchyma, adipocytes, as well as vasculature and collecting tubules of the kidney (Luscher et al, 1993; Kedzierski and Yanagisawa, 2001).

ET_A receptors are primarily located on vascular smooth muscle where they mediate the direct vasoconstrictor action of ET (Arai et al, 1990). Thus the main action of ET_A receptor activation on vasculature is contractile; however, this

can vary by animal species and vascular region. Other cardiovascular effects thought to be mediated by ET_A receptors include smooth muscle cell proliferation and hypertrophy (Shriffin, 1995).

ET_B receptors (ETBR) are also present on vascular smooth muscle (Davenport et al, 1993) but they are predominantly expressed on endothelial cells that line the body's vasculature and affect the release of endothelial relaxing factors, NO and prostacyclin (de Nucci et al, 1988; Clozel et al, 1992; Hirata et al, 1993; Batra et al, 1993) to cause transient vasodilation. In addition, vascular ETBR promote the clearance of plasma ET-1 from circulation (Ozaki et al, 1995; Dupuis et al, 1996; Davenport, 2000). These findings suggest a beneficial role of ETBR activation in countering hypertension. Interestingly, the activation of ETBR by ET-3 has vital importance in the migration and development of neural crest cells (Baynash, 1994). ETBR knockout mice have aganglionic megacolon (Hosada et al, 1994), a condition which parallels Hirschsprung's disease in humans. Patients with hereditary Hirschsprung's disease are associated with ETBR mutations (Puffenberger et al, 1994).

In contrast to endothelial ETBR, activation of VSMC ETBR produces direct vasoconstriction (Burke et al 2000). Moreover, this latter constrictor effect seems to be relatively selective for veins versus arteries. In animal and human studies, there is functional evidence of ETBR-mediated vasoconstriction in the veins (Moreland et al, 1994; Seo et al, 1994; Sumner et al, 1992). *In vitro* studies indicate that ETBR agonists produce little or no contraction of isolated arteries, but marked contraction of veins (Thakali et al, 2004). In a previous study, we

demonstrated that acute *in vivo* activation of the ETBR produces a sustained increase in blood pressure (Lau et al 2005). Furthermore, Strachan et al (1995) showed that selective ETBR stimulation *in vivo* produced constriction of human dorsal hand veins. Whether the constrictor or relaxant action of ET-1 is the predominant physiological effect on blood vessels varies depending upon vascular bed and animal model.

ET in the Central Nervous System:

The functional role of ET within the central nervous system has not been fully elucidated, however, there is compelling evidence that ET participates in the central control of blood pressure and volume possibly by direct modulation cardiorespiratory centers and through the release of hormones (Kedzierski and Yanagisawa, 2001). ET-1 stimulates the secretion of arginine vasopressin (AVP) from the posterior pituitary (Shichari et al, 1989), a hormone known to be physiologically important for maintaining body fluid homeostasis. Other hormones stimulated by ET administration include growth hormone, thyrotropin, luteinizing hormone and follicle-stimulating hormone (Kanyicksa et al, 1991; Levin, 1995).

One line of evidence to support the notion of ET having a role in central cardiovascular regulation is simply the finding that components of the ET system are localized in the brain regions known to be important for modulating the cardiovascular and renal systems. ET peptides, especially ET-3, have been reported in the PVN and SON (Lee et al. 1990; Yoshizawa et al, 1990), as well

as in neurons and glial cells of the cerebellum (MacCumber et al, 1990). The ETBR has been found in the cerebellum at various stages of fetal life where they are thought to participate in brain development (Elshourbagy et al, 1992; Levin, 1997; Furuya et al. 2001) and also in peripheral ganglia (Kobayashi et al, 1993). It has been suggested that ETBRs found in astrocytes may mediate ET's potential role in stimulating DNA synthesis (Levin et al, 1992). ET-1 and ET-3 can activate the sodium-potassium-chloride transporter at capillary-endothelial junctions of the brain suggesting that they may participate in the maintenance of the blood brain barrier (Vigne et al, 1994).

A higher concentration of ET is detected in the CSF than in plasma (Yamaji et al, 1990). One explanation is that ET may be acting as a hormone or neuromodulator in the circulating CSF to convey fluid homeostatic signals to regions of the brain involved in central autonomic control of various effector systems that maintain blood pressure and body fluid balance (Kuwaki et al, 1997; Kedzierski et al, 1999; Rossi 2003). Infusion of ET into the brain icv produced increased blood pressure and plasma catecholamines; both effects were blocked with α adrenergic antagonists (Ouchi et al, 1989), as well as produced cardiorespiratory changes (Kuwaki et al, 1997). One mechanism by which ET in the CSF may exert its effects is possibly by acting at discrete brainstem nuclei and neurons located in circumventricular organs (CVOs), where there is no blood brain barrier, to increase blood pressure by stimulating central sympathetic nervous activity (SNA). ET may also play a crucial role in development. As mentioned above, the interaction of ET-3 and ETBR is vitally important for

development of tissue derived from migration of neural crest cells, such as enteric neurons as evidenced clinically in Hirshsprung's disease (Hosada et al, 1994; Kuwaki et al, 1997).

Superoxide anions and nitric oxide in hypertension

Reactive oxygen species (ROS) commonly referred to as oxygen free radicals are oxygen molecules that have an unpaired electron (Campese et al, 2004). Superoxide is formed by the actions of NADPH oxidase which is a five-subunit enzyme that transfers electrons from NADPH to molecular oxygen. The role of superoxide anion (O_2^-) and especially its interaction with nitric oxide (NO) has received much attention in hypertension and cardiovascular disease (Nakazono et al, 1991). In several animal models of hypertension, O_2^- is increased in the vasculature (Sedeek et al, 2003; Somers et al, 2000) and can act as a vasoconstrictor (Auch-Schwelk, 1989; Consentino et al, 1994). Furthermore, angiotensin II, a vasoconstrictive factor, has been shown to stimulate O_2^- production via upregulation of NADPH oxidase in rats (Griendling et al, 1994; Rajagopalan 1996). And in the central nervous system, O_2^- can serve as a signaling molecule mediating the effects of neuroactive substances, like angiotensin II, to increase blood pressure (Zimmerman et al, 2002). Endogenous O_2^- has also been shown to affect vasomotor tone in human vessels (Hamilton et al, 1997). Mehta et al (1994) demonstrated increased O_2^- generation in human essential hypertension and its reversal using celiprolol, a ß-adrenergic antagonist.

O_2^- is a biological intermediate in the breakdown of oxygen to form ATP during aerobic metabolism. Under normal conditions, reactive O_2^- is reduced by superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2). However, excessive O_2^- can scavenge and react with NO to form peroxynitrite ($ONOO^-$), thereby reducing the bioavailability of NO (Rubyani and Vanhoutte, 1986).

NO, an endogenous vasodilator produced by the vascular endothelium first described in 1980 by Furchgott and Zawadski as endothelium-derived relaxing factor (EDRF), has been shown to regulate vascular tone (Vallance et al, 1989; Angus et al, 1992). In addition, NO causes renal vasodilatation and consequently, diuresis and natriuresis (Salom et al, 992). These actions would tend to be beneficial for lowering blood pressure; thus, a reduction in NO is one way in which O_2^- may contribute to hypertension. Grunfeld et al (1995) found using lucigenin chemiluminescence that the amount of excess O_2^- could exactly account for the reduced bioavailability of NO in the aortas of spontaneously hypertensive rats (SHR).

Though recent studies have suggested augmented ROS production (e.g. O_2^-) as one mechanism by which endothelin may cause hypertension (Diederich et al, 1994; Galle et al, 2000; Sedeek et al, 2003), little is known about the effect of O_2^- on the peripheral sympathetic nervous system and vice versa. However, compelling evidence suggests a link between the two factors. The ability of O_2^- to alter β adrenergic function has been reported in ferret hearts, an effect which is reversed with SOD treatment (Liang et al, 2000). More importantly, recent work by Dai et al (2004) found that sympathetic neurons in peripheral ganglia contain

O_2^-, and that higher amounts exist in sympathetic ganglia of DOCA-salt rats. The same group also demonstrated stimulation of ETBR *in vitro* increases O_2^- levels in sympathetic ganglia, suggesting that ETBR-mediated O_2^- production may be involved in sympathetic nervous activation, by controlling the effectiveness of ganglionic neurotransmission.

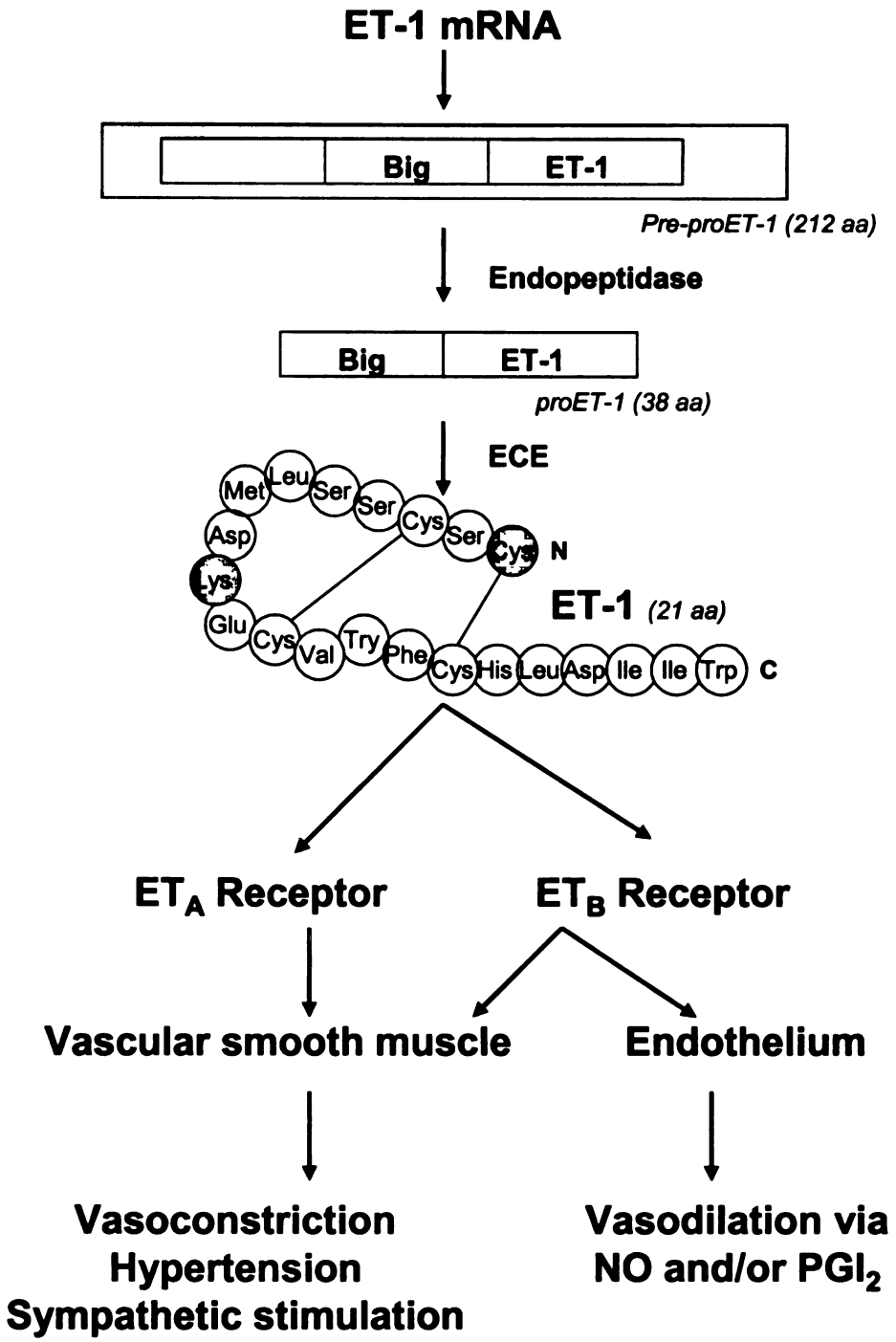


Figure 1. Schematic illustrating the biosynthetic pathway of ET-1 and its effects on the cardiovascular system. ECE=endothelin converting enzyme, NO = nitric oxide; PGI2 = prostacyclin.

CHAPTER 2

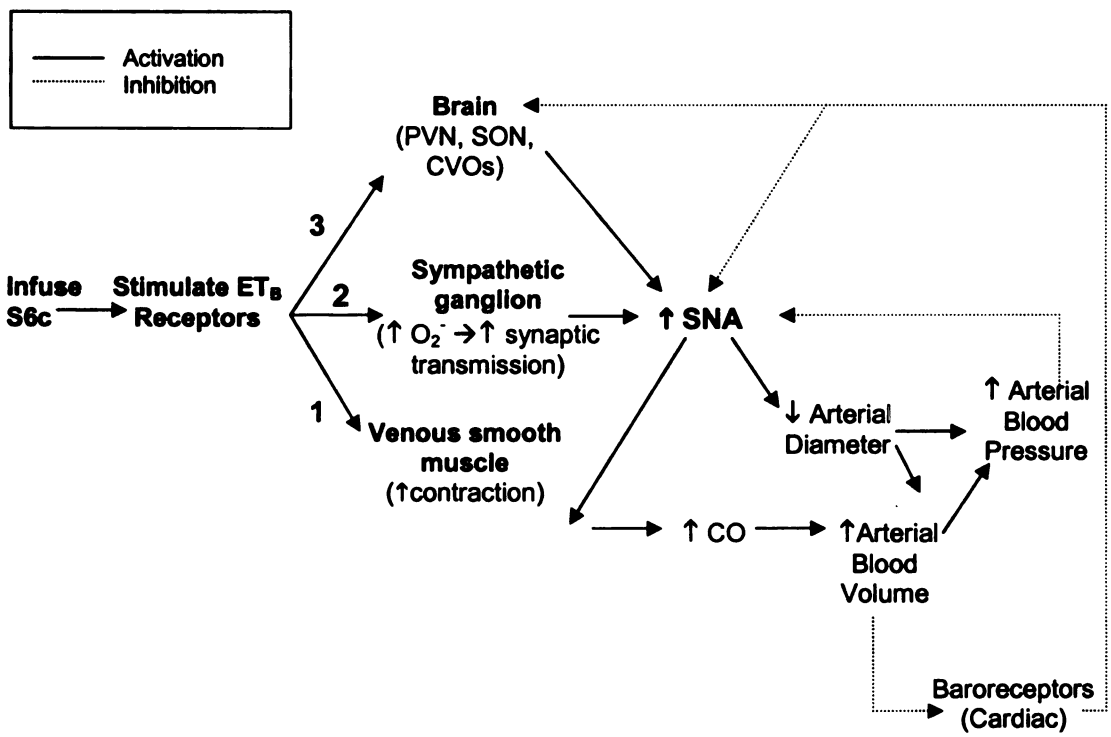
HYPOTHESES AND SPECIFIC AIMS

Overall hypotheses

Hypertension is common condition that afflicts 30% of Amercans and is a major risk factor for stroke, coronary artery disease, and heart failure. Traditionally, hypertensive research has focused on arterial function; however, accumulating evidence indicate altered venous function as also an important contributing factor (Johnson et al, 2000). Blood pressure is a product of the total peripheral resistance, which is regulated largely by the quality of small arteries and arterioles, and cardiac output, which depends on heart rate, contractility, and venous capacitance (Rothe, 1993). Veins, especially in the splanchnic circulation, hold most of the body's blood volume and therefore venoconstriction greatly distress capacitance function and augment blood pressure. Maintaining the homeostasis of blood pressure involves a complex interplay between the many organ systems. Recent evidence indicate that the sympathetic nervous system, endothelin, and superoxide anion production all increase venomotor in hypertension. This study uses a rat model of hypertension where blood pressure is increased by activation of the endothelin type B (ET_B) receptor, which selectively constricts the veins but not arteries. This is achieved by intravenous infusion of the specific receptor agonist, sarafotoxin 6c (S6c). Though venoconstriction is one effect of S6c, it is not clear whether this is a direct action of S6c on venous smooth muscle, or an indirect response mediated through the sympathetic nervous system. **The goal of this project was to identify mechanisms by which ET_B receptor activation affects autonomic regulation of blood pressure.** The proposed experiments in this study will address three

possible mechanisms by which S6c may affect sympathetic nervous activity to the vasculature.

Figure 2. Overall hypothesis of the project.



ET_B receptor activation by S6c affects autonomic nervous control of blood pressure ...

1. by directly constricting veins, leading to volume shifts into the thoracic region and causing arterial or cardiopulmonary reflex activation.
2. by acting on sympathetic ganglia to increase ganglionic transmission, possibly by increasing O_2^- levels.
3. by acting on ET_B receptors in the brain to increase sympathetic activity.

The specific aims are:

Specific Aim 1: Determine whether ET_B receptor activation causes hypertension by acting directly on the veins, and changes in sympathetic nervous system activity during S6c infusion are primarily due to reflexes activated by the hemodynamic response to venoconstriction.

- Compare the pattern of neuronal activation identified by Fos immunohistochemistry by 2 h S6c infusion to isotonic volume expansion.
- Establish the contribution of cardiopulmonary receptors to the pattern of neuronal activation caused by ET_B receptor activation.
- Compare the pattern of neuronal activation in 2 h S6c infused rats to those that receive 5 day S6c infusion.

Specific Aim 2: Determine whether ET_B receptor activation increases O2- levels in sympathetic ganglia and assess the contribution of ganglionic neurotransmission to the acute pressor response.

- Compare superoxide anion generation in prevertebral sympathetic ganglia of S6c infused rats to normotensive saline control rats.
- Evaluate the contribution of pressor effects on O_2^- production by direct alpha adrenergic stimulation.
- Evaluate the contribution of central input to pressor effects and sympathetic ganglionic O_2^- production: Ganglionic blockade.
- Evaluate the contribution of direct activation of post-ganglionic neurons to the pressor effect of S6c by adrenergic blockade.

Specific Aim 3: Determine whether ETBRs are expressed in central nervous system regions that have been shown to be important in the regulation of blood pressure and blood volume. This would support the possibility that changes in autonomic nervous function during systemic ETBR stimulation result from direct actions of S6c on the brain.

CHAPTER 3

Part I

Central nervous system Fos expression during acute ET_B receptor activation

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Introduction

The central nervous system is critical to the initiation and integration of various physiological responses to peripheral hemodynamic changes. For example, in response to increased blood volume, neurohumoral and behavioral compensatory mechanisms work in concert to restore homeostasis, including inhibition of sympathetic outflow and thirst, increased natriuresis and urination (Dibona and Sawin 1985; Norsk 1996).

Endothelins (ET)s are a family of 21-amino-acid peptides (ET-1, ET-2, ET-3) well known to act powerfully in the peripheral vasculature as cardiovascular regulators. ET's potent vasoconstrictor and vasopressor actions are mediated by two heterotrimeric G-protein coupled receptors, ET_A and ET_B. The ET_A receptor is expressed in vascular smooth muscle cells (VSMCs) of blood vessels and is typically involved in vasoconstrictor effects of ET-1 (Davenport et al 1995). The ET_B receptor (ETBR), on the other hand, is expressed on both endothelial cells and VSMCs (D'Orlean-Juste et al, 2002) and has dual pressor and depressor effects depending on its tissue location. Activation of the endothelial ETBR causes the release of vasodilatory factors including nitric oxide and prostacyclin (Gomez-Alamillo et al 2003; De Nucci et al 1998) and has also been implicated as a clearance receptor for circulating ET-1 (Dupuis et al 1996). Both of these actions lead to vasodilation. In contrast, activation of VSMC ETBR produces direct vasoconstriction (Burke et al, 2000). This constrictor effect is much more prominent in veins than in arteries. In vitro studies indicate that ETBR agonists produce little or no contraction of isolated arteries, but marked

contraction of veins (Thakali et al, 2004). In a previous study, we demonstrated that *in vivo* activation of the ETBR produces a sustained 2 h increase in blood pressure (Lau et al 2005). In this model of acute hypertension produced by infusion of a specific ETBR agonist, we also observed decreased blood volume, increased sodium and water excretion without alterations in thirst, heart rate or plasma electrolyte concentration (unpublished data), effects that are consistent with volume redistribution from veins to arteries. Taken together, these studies suggest that venoconstriction is one mechanism by which ETBR activation increases arterial blood pressure. We propose that increased constriction of veins following activation of the ETBRs raises blood pressure by shifting blood volume from the peripheral extrathoracic veins to the central circulation. One line of evidence to support this hypothesis is the fact that ETBR agonists have been shown to increase mean circulatory filling pressure (MCFP), which is a measure of venomotor tone (Johnson et al 2001). Venous tone is a strong determinant of CO by affecting cardiac filling, which in turn influences arterial blood volume and systemic blood pressure (Greenway, 1983; Rothe, 1983, 1993). Increasing venous return into the thoracic cavity would result in transiently augmented right atrial pressure and cardiac output (CO), effectively redistributing a greater fraction of total blood volume to the arterial side of circulation. Subsequent reactive increases in arterial resistance vessel tone would be necessary to maintain elevated arterial pressure. Central redistribution of blood volume is a hemodynamic phenomenon that has been reported in early human essential hypertension (Schneider et al, 1995). In animal studies, increased venomotor

tone has been reported in various models of experimental hypertension including the spontaneously hypertensive rat (Martin et al, 1998) and the deoxycorticosterone acetate (DOCA)-salt hypertensive rat (Yamamoto et al, 1983; Johnson et al, 2001). Clinically, patients with borderline hypertension show decreased venous compliance as well as blood volume redistribution (Schneider et al 1995). Together, these studies suggest the possible importance of venoconstriction to the development of hypertension.

Recent studies have used the protein product of the immediate early gene *cfos* to identify hypothalamic forebrain and medullary hindbrain nuclei that are activated by fluctuations in plasma volume (Narváez et al 1993, Badoer et al 1997, Randolph et al 1998, Potts et al 2000, Godino et al 2005). Fos immunohistochemistry reveals synaptic excitation in central nervous system neurons and has been used widely to assay brain activity in situ in response to many cardiovascular stimuli (Badoer et al 1994, Badoer et al 1997, Curtis et al 1999, Dampney et al 1995, Potts et al 2000, Potts et al 1997, Godino et al 2005, Gottlieb et al 2005, Penny et al 2005, Lohmeier et al 2002, Li et al 1998). Among these, Randolph and colleagues (1998) reported a distinct neuronal pattern of increased Fos expression caused by acute isotonic volume expansion (VE). The VE technique involved volume loading the circulatory system to produce increased central venous pressure. The rise in cardiothoracic blood volume distends the atria and ventricles, stimulating cardiopulmonary mechanoreceptors, which is signaled to the brain by baroreceptor fibers. Some of the brainstem and forebrain nuclei identified in that study include the caudal portion of the

ventrolateral medulla (CVLM), the nucleus of the solitary tract (NTS), parvocellular neurons in the paraventricular nucleus (PVN), the supraoptic nucleus (SON) and the perinuclear zone (PNZ) of the SON. Both CVLM and NTS are part of brainstem afferent pathways and are critical to the integrity of the baroreflex (Willette et al, 1984; Agarwal et al, 1989; Li et al, 1991; Blessing, 1997). Primary afferent stretch receptor endings carried by the ninth and tenth cranial nerves synapse on NTS neurons which then excite the CVLM. The PVN and SON play pivotal roles in the regulation of sympathetic outflow, oxytocin and vasopressin release as well as initiate the thirst response (Haselton et al, 1994). The PVN is well known to be important for the autonomic response to volume load (Deering and Coote, 2000).

In the present study, we examined the central nervous system Fos response to in vivo ETBR activation using the specific ETBR agonist sarafotoxin 6c (S6c). In particular, we focused on brain pathways elucidated by the VE protocol reported in Randolph et al (1998). We measured the neuronal activation in the PVN, SON, PNZ, RVLM, CVLM, and NTS. We reasoned that observing an activation pattern similar to VE would strongly support the hypothesis that ETBR activation involves peripheral venoconstriction and blood volume centralization.

Methods:

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Portage, ME) weighing 250-300g were housed in temperature- and humidity-controlled rooms with a 12:12-h light-dark and had ad libitum access to distilled water and pelleted rat chow (Harlan/Teklad 8640 Rodent Diet). The experimental protocol was approved by the Michigan State University All University Council on Animal Use and Care.

Catheterization

Sodium pentobarbital anesthetized rats (50mg/kg ip) were chronically instrumented with catheters made of silastic rubber and polyvinyl tubing inserted into the femoral artery and vein for continuous hemodynamic measurements and drug delivery respectively. Both catheters were subcutaneously tunneled through the dorsal side of the animal exiting between the scapula and secured by a plastic harness connected to a stainless steel spring. After catheterization, rats were then individually housed in standard stainless steel metabolic cages. The distal end of the spring containing both arterial and venous catheters was attached to an exterior clamp outside the cage via a hydraulic swivel, allowing continuous access to catheters without direct handling or disturbance to the animal. Ticarcillin (10 mg/kg; SmithKine Beecham Pharmaceuticals, Philadelphia, PA) and enrofloxacin (2 mg/kg; Bayer) were administered daily via the venous catheter to prevent bacterial infection. Both catheters were flushed

daily with heparinized saline (100u/ml; Sigma) to maintain patency. Mean arterial pressure (MAP) and heart rate (HR) were measured from the arterial catheter with a TXD-300 pressure transducer linked to a digital BPA-200 Blood Pressure Analyzer (Micro-Med, Louisville, Kentucky).

Experimental protocol

After allowing 2-3 days for surgical recovery, catheterized rats were divided into three experimental groups. One group received intravenous infusions of isotonic saline at 0.01 ml/min for 120 minutes (control; N=11), while another group also received isotonic saline but at a volume equal to 10 % of their body weight for the first 10 min followed by 0.5 ml/min for 110 min as described in Randolph et al 1998 (VE; N=11). The third group received an infusion of S6c (American Peptide, Sunnydale, CA) at a rate of 5 pmol/kg/min for 120 minutes (S6c; N=16). All infusions were performed in unanesthetized, conscious, unrestrained rats. MAP was recorded throughout the protocol. At the end of infusion, rats were immediately sacrificed with an overdose of sodium pentobarbital (100mg/kg iv) and transcardially perfused with 0.1 M PBS followed by 4% phosphate buffered paraformaldehyde solution. After perfusion, brains were dissected and postfixed with 4% paraformaldehyde for 24 hours and stored in 30% sucrose.

Fos Immunohistochemistry

Fixed brains encased with tissue freezing medium (Optimal Cutting Temperature compound, TissueTek) were cut into 35 μm sections with a cryostat and collected into 12 well cell culture plates filled with 0.1 M PBS as free-floating sections.

After PBS washes, brain slices were incubated with 0.3% hydrogen peroxide (Sigma) in distilled water for 30 minutes at room temperature then rinsed in PBS for 30 min. Sections were then incubated with a blocking solution consisting of 3% normal goat serum (NGS; Vector Labs, Burlingame, CA), 0.25% Triton X 100 and 0.1 M PBS for 2 h at room temperature. After blocking, brain slices were reacted with rabbit polyclonal anti-Fos antibody (Santa Cruz Biotech, Santa Cruz, Ca) diluted 1:1000 in 0.1 M PBS/ 3% NGS overnight at 4° C. Sections were rinsed three times in PBS for 10 min prior to incubation in 1:500 biotinylated goat anti-rabbit IgG (Vector labs, Burlingame, CA) for 2 hours at room temperature. The tissue was then incubated with an avidin-biotin peroxidase reagent (ABC-Vectastain Elite, Vector Labs, Burlingame, CA) for 1 h. After three rinses in PBS, brain sections were reacted with a nickel 3,3'-diaminobenzidine solution (Nickel-DAB, Vector labs), which produced a dark brown stain. No immunoreactivity was observed in control brain slices incubated without primary antibody.

Double-immunostaining protocol:

After processing brain sections for Fos immunohistochemistry, some forebrain slices were stained for oxytocin immunofluorescence while hindbrain slices were co-labeled with anti-dopamine-beta-hydroxylase antibody in order to localize the

brain regions of interest and characterize the cell type that has been activated. Forebrain slices were incubated for five days with a mouse anti-oxytocin antibody (Cunningham lab) at a dilution of 1:1000. Sections were then rinsed and reacted with a CY3 fluorescence conjugated secondary antibody against mouse (0.1 μg/ml, Jackson ImmunoResearch,West Grove, PA). Hindbrain slices were similarly stained for anti-dopamine beta hydroxylase (DBH) immunoreactivity (Chemicon). Fos immunostained sections were first incubated in a mouse anti-DBH antiserum (1:500) for three days. Then sections were treated with ABC reagent and then with biotinylated goat anti-mouse secondary antibody (1:200) for 1 h. Finally, sections were reacted with VIP chromogen (Vectastain, Vector Labs) which produces a light red stain. Subsequently, after extensive rinsing, both forebrain and hindbrain sections were mounted on gel-coated slides, dehydrated in an alcohol and xylene series and coverslipped with Permount mounting medium (Fisher Scientific).

Histological analysis

The number of Fos positive nuclei in each brain region as demarcated by the rat atlas (Paxinos and Watson 2nd ed, 1986) and corroborated by either DBH or oxytocin immunoreactive staining was visually quantified under a light microscope. Each brain region was represented by the average of three sample slices (anterior, middle and posterior). Control rats served as a negative control, while VE groups provided a positive comparison for the S6c treatment group. Quantification of Fos was performed by the same investigator for all brain

samples. Microscope slide identifications were obfuscated by double layered laboratory tape and randomly coded by another person unconnected with this study, and was finally revealed only once all counts had been completed.

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM) and were analyzed with Prism 3.0 Software (GraphPad, Inc). Two-way ANOVA followed by post hoc test was performed to examine differences in variables among groups of rats while Student's t-test was used to compare two groups. A *P* value ≤ 0.05 was considered statistically significant.

Results

Hemodynamic

Both VE and S6c infusion produced a significant increase in blood pressure, compared to control rats, although S6c rats had a greater increase (Figure 3). Moreover, within the first 20 minutes of VE infusion, the MAP decreased slightly before rebounding. The difference between initial and final MAPs of S6c and VE animals was 15.3 ± 1.9 mmHg and 10.9 ± 4.2 mmHg, respectively while in control rats MAP decreased 2.7 ± 2.1 mmHg. No significant differences in heart rate were observed during the 2 h infusion in any group.

Fos expression

Control rats with saline infusions at a rate of 0.01 ml/min for 120 minutes showed sparse Fos activation in all counting regions and provided a baseline standard for comparison (Figure 6; Table 1).

In the hindbrain, VE and S6c infusion induced a significant increase in Fos positive nuclei in the NTS and CVLM, but in not the RVLM (Figure 4). Fos positive neurons were sparsely distributed throughout the rostral-caudal extent of the NTS but were highly concentrated commissurally at the level of the central canal (Bregma -13.80mm). Bilateral Fos counts of the NTS were taken at that level as well as anteriorly and posteriorly, and averaged to render a mean value. Fos counts of the CVLM (Bregma -14.30mm) showed significant increases after S6c and VE infusion, while modest increases in Fos were observed in the RVLM (Bregma -12.72mm) for all three groups (Figure 6). DBH immunostaining facilitated the identification of the brain regions and also characterized the phenotype of Fos activated medullary neurons. A majority of Fos positive neurons in the NTS and CVLM of VE animals were not DBH-immunoreactive, however, S6c infusion induced a more heterogeneous distribution as about 50% of the activated neurons were colocalized with DBH. Among the few Fos activated neurons in the RVLM, 30% of them were DBH positive (Table 1).

In the PVN (Bregma -1.80mm), significant increases in Fos were observed in S6c and VE rats compared to control rats, with the S6c group showing a greater increase (Figure 5). Unlike VE, which caused Fos activation in mostly parvocellular PVN neurons, 76% of cells activated following S6c infusion were

oxytocinergic. VE and S6c treatment both induced a significant increase in Fos positive nuclei in the SON (Bregma -1.40mm). Most of the SON neurons that were activated were immunostained with oxytocin (Figure 6, Table 1).

Among the three treatment groups, S6c showed the greatest Fos induction in all brain regions except for the RVLM where it was slightly lower than VE (Figure 6).

Discussion

Fos is a general marker of neuronal activity and its expression can be induced by many different stimuli, including stress (Andrews et al, 1987; Gubits and Fairhurst, 1988). Therefore, to minimize nonspecific staining, we took extra measures to ensure that rats were not disturbed throughout protocol. This was accomplished by the implantation of permanent catheters for the delivery of drugs as well as continuous hemodynamic measurements. Moreover, proper control animals were instituted to provide baseline standards for comparison (e.g. light, water intake, anesthesia).

In the present study, we used the Fos technique to explore central nervous system activation in response to ETBR stimulation in vivo. The objective was first to determine whether S6c infusion evoked Fos expression, and if so how this compared with the pattern induced by VE. We found that systemic infusion of S6c evoked a discrete pattern of Fos activation in the forebrain and brainstem highly analogous but not identical to that generated by acute VE.

A significant increase in the number of Fos-positive neurons was observed in the PVN of both S6c and acute VE rats. Double labeling of the Fos positive cells with anti-oxytocin antibody revealed differences in the distribution of neuronal activation. VE stimulated mostly parvocellular neurons, consistent with previous findings (Randolph et al 1998); however, although S6c infusion increased Fos immunoreactivity in this portion of the PVN, the stimulus activated more magnocellular neurons. The parvocellular subnucleus in the PVN is well known to be activated during acute VE and is associated with inhibition of renal sympathetic discharge (Karim et al, 1972; Linden and Kappagoda, 1982; Badoer et al 1997; 1998; Haselton and Vari, 1998). Following direct chemical stimulation of PVN neurons in the anesthetized rabbit, Deering and Coote (2000) found decreased renal sympathetic nerve activity with compensatory increases in adrenal, splanchnic and cardiac sympathetic outflows. Decreased activity of renal sympathetic nerves has been shown to participate in the diuresis and natriuresis that is initiated in response to VE (Dibona and Sawin, 1985; Lovick et al, 1993; Haselton et al, 1994). However, it has been argued that the above effects may instead be a consequence of atrial natriuretic peptide (ANP) and antidiurectic hormone (ADH) release (Kaufman and Stelfox, 1987), both events which are modulated by the release of oxytocin from magnocellular portion of the PVN. The series of hemodynamic events initiated by acute VE includes increased right atrial pressure, central blood volume, and cardiac output (Ricksten et al, 1981; Anderson et al, 1986; Pettersson et al, 1988). Pyner et al (2001), using balloon inflation of the right atrium, confirmed that specifically right

atrial distention is the stimulus that caused inhibition of the renal sympathetic nerve activity as well as discrete Fos induction in the parvocelluar PVN neurons. Furthermore, stimulation of vagally innervated cardiac afferents which mediate atrial stretch information may similarly alter PVN activity (Lovick and Coote, 1989). There is evidence that activated neurons in the parvocellular subnucleus may be inhibitory interneurons within the PVN that project to the kidney as electrophysiological stimulation of cardiac receptors has been shown to cause inhibition of spinally projecting PVN neurons (Lovick and Coote, 1988). Taken together, the above studies indicate that right atrial stretch following VE stimulates cardiac receptors, leading to the activation of parvocellular PVN neurons, which in turn causes suppression of sympathetic nerve activity. Thus increased Fos expression in the parvocellular subnucleus of the PVN following S6c infusion provides compelling evidence that ETBR activation produces right atrial stretch and further suggests that the overall circulatory response to ETBR activation is dependent at least in part on autonomic responses to volume shift.

Magnocellular neurosecretory neurons of the PVN and SON project to the neurohypophyseal terminals in the pituitary gland, where oxytocin and vasopressin are stored and released into circulation (Antunes-Rodrigues et al 2004). In our study, a great number of PVN neurons activated by S6c were immunostained with oxytocin indicating the participation of neuroendocrine mechanisms in the modulation of ETBR activation, though acute VE also activated a small percentage of oxytocinergic PVN neurons. It is well known that oxytocin is a key modulator in the control of body fluid and cardiovascular

homeostasis. Oxytocin increases sodium excretion from the kidneys, induces natriuresis and restores fluid balance in response to volume load (Verbalis et al, 1991). Indeed, administration of oxytocin is associated with a fall in mean arterial pressure in both humans and animal studies (Petty et al, 1985; Peterssen et al, 1996). Maier et al (1998) showed that injection of oxytocin icv decreased blood pressure, while inhibition of its synthesis raised blood pressure. Recent reports suggest that the mechanism by which oxytocin exerts its renal and cardiovascular effects is through the actions of atrial natruretic peptide (ANP) a potent natriuretic hormone released from the atria (De Bold et al, 1981; Haanwinkel et al, 1995; Gutkowska et al, 2000) that is associated with increased glomerular filtration, reduced tubular reabsorption of electrolytes as well as suppression of aldosterone and vasopressin release and the effects of the renin-angiotensin system (Jamison et al, 1992). Volume expansion has been shown to cause ANP release, important in the induction of natriuresis and diuresis, which in turn acts to reduce the increased blood volume (Haanwinckel et al, 1995). Central administration of endothelin 3 (ET-3), which has relatively high affinity for the ETBR (Inuoe et al 1989; Schriffrin EL, 1999; Masaki T, 2004) has also been shown to stimulate ANP release as well as induce natriuresis (Antunes-Rodrigues et al 1993). In that study, water loaded rats received ET-3 injection into the anteroventrolateral third ventricle (AV3V), a site many consider to be critical for the central control of volume regulation (Baldissera et al, 1989; Johnson et al, 1996), produced a rise in plasma ANP release as well as a dose dependent natriuresis (Antunes-Rodrigues et al, 1993). However, iv injection of

the same dose did not elicit the same response. Our finding that Fos positive nuclei were colocalized with magnocellular PVN neurons correlates with data showing increase plasma oxytocin release in response to VE (Haanwinkel et al 1995; Godino et al 1995) and is consistent with the study by Godino et al (2005) demonstrating increased Fos activation in particular magnocellular subnuclei located in the medial PVN associated with oxytocin release but not in vasopressinergic subnuclei of the lateral PVN. Although we did not perform specific immunoreactive labeling of AVP neurons, previous studies reported the suppressed activation of AVP neurons in response to VE (Randolph et al 1998). However, there is evidence that ET increases arterial blood pressure as well as cause the release of AVP *in vivo* (Martin and Haywood, 1992; Rossi et al, 1997; Yamamoto et al, 1991) and *in vitro* (Shichari et al, 1989; Rossi NF 1993, 1995). Our analysis of immunostained brain sections revealed Fos activation primarily in the medial subnucleus of the PVN where there is a preponderance of oxytocinergic cells while the AVP dense subnucleus in the lateral portion of the PVN is sparsely activated (Godino et al 2005).

Our finding that Fos is highly expressed in parvocellular as well as magnocellular neurons of the PVN may reflect the non-specific nature of the stimulus since S6c infusion caused a significant increase in mean arterial pressure (MAP) which may activate arterial baroreceptors as well as cardiopulmonary receptors. The greater number of Fos positive neurons following S6c compared to VE suggests that the S6c infusion may have activated a separate population of neurons in addition to those Fos positive nuclei induced

by VE. This also suggests the possibility that the response to ETBR activation involves multiple mechanisms of action.

We also found significant Fos immunoreactivity in the SON of VE and S6c rats. These activated neurons were predominantly colocalized with oxytocinergic neurosecretory cells, as revealed by double-labeling protocol. Our finding is consistent with previous reports (Randolph et al, 1998; Godino et al, 2005).

Following VE, Fos immunoreactivity was significantly increased in the NTS and CVLM, confirming earlier results first reported by Randolph et al (1998) and again by Godino et al (2005). Infusion with S6c caused an even greater Fos response than that produced by VE in both NTS and CVLM. Another novel finding reported here is that S6c infusion induced activation of catecholaminergic neurons. We found that roughly half the Fos positive cells within these brainstem regions were double-labeled for dopamine β hydroxylase (DBH) which differ from a previous report that VE activated neurons within the brainstem do not colocalize with DBH (Cunningham et al, 2001). A recent study by Godino et al (2005) using a different volume expansion protocol also observed a high degree of tyrosine hydroxylase colocalization with Fos positive neurons in both regions. The authors in the latter study infer that both cardiopulmonary and arterial baroreceptors are stimulated by the VE protocol which would be consistent with the observed pattern of activation. The NTS receives input from vagal afferents carried by the 9^{th} and 10^{th} cranial nerves that convey both baroreceptor as well as cardiac receptor information (Badoer et al, 1994; Hines et al 1994). In the primary baroreflex circuit, central barosensitive afferents synapse onto second

order neurons in the NTS that in turn send excitatory projections onto neurons in the CVLM, which then project to sympathetic premotorneurons of the RVLM (Minson et al, 1997) via GABAergic inhibitory interneurons (Dampney, 1994). Both the NTS and CVLM contain catecholaminergic neurons known to be important for the integration of cardiovascular reflexes and fluid balance consistent with our findings. Vasomotor neurons of the CVLM are also barosensitive and can regulate blood pressure and sympathetic nerve activity, via inhibition of the RVLM (Dampney et al, 1995). Consistent with previous reports, we did not observe significant neuronal activation in the RVLM (Randolph et al, 1998; Godino et al, 2005).

Our results showing the pattern of Fos expression caused by VE are in agreement with previous findings that specific hypothalamic and medullary nuclei are activated. In earlier studies, acute isotonic VE significantly increased the number of Fos positive nuclei in the PVN, SON, NTS and CVLM among other brain regions (Randolph et al 1998; Godino et al 2005). Consistent with previous reports, we found similarly increased Fos responses in the PVN, SON, NTS and CVLM. Consequently, the data are consistent with our view that ETBR activation follows similar hemodynamics to the acute volume expansion model, where a rise in peripheral blood volume leads to increase right atrial pressure and central blood volume, greater cardiac output and stroke volume. The increase in blood pressure and shift in blood volume, specifically right atrial distention, is relayed to the NTS and CVLM via baroreceptors and/or cardiac receptors (Columbari et al, 1997, Cunningham et al, 2002).

In our hemodynamic measurements, a significant increase in MAP was observed in both S6c and VE treatment rats. Contrary to earlier reports that MAP remained unchanged during VE infusion (Randolph et al, 1998; Godino et al 2005), our VE data show a moderate increase in blood pressure preceded by transient hypotension lasting 20 min post-infusion. We have no explanation for the difference between our study and the earlier ones. It has been suggested that the transient hypotension may be due to the release of oxytocin and ANP peptides in immediate response to the increased volume load (Godino et al, 2005). The activation of oxytocinergic cells in the PVN and SON as revealed by our double-labeling studies supports this view. The greatest increase in MAP was observed in S6c infused rats, which correspond to our observation that S6c infusion evoked the most robust increase in Fos expression. Thus we cannot discount that the pattern and/or degree of neuronal activation in the S6c rats may be a reflection of blood pressure as well as blood volume increase. Reflex bradycardia has been reported in previous VE experiments (Randolph et al, 1998; Godino et al 2005). Though there was a slight drop in heart rate in VE rats during the first ten min of infusion, we did not observe significant changes in any of the three treatment groups.

In conclusion, we demonstrated for the first time neuronal activation caused by acute ETBR stimulation *in vivo*. As discussed, increased blood volume to the thorax like that caused by VE is accompanied by a neural response in specific areas of the brain well known to regulate blood pressure and fluid homeostasis and this response was indicated with a Fos protein marker.

ETBR activation by 2 h S6c infusion significantly increased Fos expression in the PVN, SON, NTS and CVLM, brain regions associated with sympathoinhibition, vasopressin and oxytocin release. Our results indicate that the pattern of brain activation during ETBR stimulation is very similar to that caused by VE and fits in with our hypothesis that *in vivo* ETBR activation involves peripheral venoconstriction and blood volume centralization to cause increased blood pressure. We contend that venoconstriction caused by acute *in vivo* ETBR activation increases venous return to the heart and consequently raises cardiac output and centralization of blood volume from the extrathoracic vasculature to the cardiothoracic region, resulting in hypertension. This blood volume redistribution would also serve to produce decreased sympathetic nervous system activity due to activation of cardiopulmonary receptors and baroreceptors.

Alternatively, circulating S6c may bind to ETBR in the brain to increase neuronal activation and consequently, blood pressure. It is uncertain whether Fos activation with ETBR activation is an effect of volume redistribution or perhaps the result of direct activation of central neurons or pathways. There is compelling evidence that ET participates in the central control of blood pressure and volume possibly by direct modulation of cardiorespiratory centers and through the release of hormones (Kedzierski and Yanagisawa, 2001). Injection of ET-1 into the brain causes the release of AVP (Shichari et al, 1989; Martin and Haywood, 1992; Rossi et al, 1997; Yamamoto et al, 1991). Moreover, components of the ET system have been localized to brain regions known for cardiovascular regulation (Lee et al, 1990; Yoshizawa et al, 1990; MacCumber et

al, 1990; Furuya et al, 2001). However, it is unlikely that blood-borne S6c crosses the blood brain barrier to exert its effects on central ETBR as S6c is structurally analogous to ET-1, which is a polar peptide known to not cross the capillary-endothelial junction. Administering a radioactive tracer tagged mixed receptor agonist intravenously, Aleksic et al (2001) found low radioactivity in the brain indicating that the agonist did not cross the blood brain barrier. Furthermore, Hartz et al (2004) showed that 1-2 h exposure to ET-1 or to S6c acting through ETBRs reduced P-glycoprotein function, decreasing transport at the barrier. Although there are conflicting reports since ET-1 and ET-3 can activate the sodium potassium chloride transporter at capillary-endothelial junctions of the brain suggesting that they may participate in the maintenance of the blood brain barrier (Vigne et al, 1994). Furthermore, Narushima et al (2003) reported that injection of ET-1 into the brain increased permeability.

To rule out the possibility that the observed Fos activation is due to direct binding of plasma S6c on central ETBRs, we would first ascertain whether ETBRs are present in these specific brain nuclei and pathways by immunohistochemical assay or *in situ* autoradiographic ET-binding. Furthermore, we can determine whether circulating S6c can affect the expression of ETBR in these brain regions using western blot analysis for quantification, which would support a role for S6c to cross the blood brain barrier and act on brain ETBR. Finally, we can determine whether blockade of efferent pathways following central ETBR activation by S6c can inhibit the hypertensive effect of S6c. Abolishing the pressor response to systemic S6c infusion following central

blockade would strongly oppose our hypothesis that *in vivo* ETBR activation involves peripheral venoconstriction and blood volume centralization to cause increased blood pressure.

In recent years, a rat model of transgenic expression of the ETBR has been developed (Gariepy et al, 1998). The spotting lethal rat which is a naturally occurring rodent model of Hirschsprung disease carries a deletion in the ETBR gene. Rats homozygous for this mutation exhibit coat color spotting and congenital intestinal aganglionosis which result from migration failure of the neural crest-derived epidermal melanoblasts and enteric nervous system precursors to fully colonize the skin and intestine and have a postnatal median survival of 21.5 days, dying from intestinal obstruction (Ikadai et al, 1979; Hosada et al, 1994; Gariepy et al, 1996; Nagahama et al, 1985). Gariepy et al (1998) demonstrated that targeted transgenic expression of ETBR using the human DBH promoter to colonize ENS precursors prevented the intestinal defect and premature mortality in these homozygous mutant rats. The resultant transgenic ETBR rat model displays normal ETBR expression in neurons but not in smooth muscle cells. This differential ETBR expression would provide a powerful tool to study the effects of acute S6c infusion on pressor response and, consequently, the pattern of Fos expression. Since ETBR transgenic rats do not have functional ETBR receptors in the veins, pressor changes following S6c infusion would not be attributed to peripheral venoconstriction. Furthermore, the absence of blood pressure increase in this transgenic rat model would eloquently strengthen our hypothesis.

	Number of Fos positive nuclei		
Brain Region	**Control**	**VE**	**S6c**
Caudal ventrolateral medulla	2.2 ± 0.5 (35.2%)	6.7 ± 1.3* (18%)	9.8 ± 1.1* (54.2%)
Nucleus tractus solitarius	3.6 ± 1.3 (32.8%)	15.3 ± 2.1* (18.4%)	18.5 ± 2.3* (59.6%)
Paraventricular nucleus	4.5 ± 1.4 (34.4%)	13.1 ± 1.9* (23.5%)	18.9 ± 2.1* (76%)
Rostral ventrolateral medulla	3.2 ± 0.9 (14.5%)	5.5 ± 0.9 (29.7%)	5.1 ± 0.6 (32.8%)
Supraoptic nucleus	3.1 ± 1.3 (54%)	9.0 ± 1.8* (77.3%)	10.4 ± 1.4* (88.9%)

Table 1. Average number of Fos positive nuclei in each brain region represented as mean ± SEM. Number in ( ) show % of Fos positive neurons that are immunostained with either anti-oxytocin or anti-dβh antibodies. * significance $P<0.05$

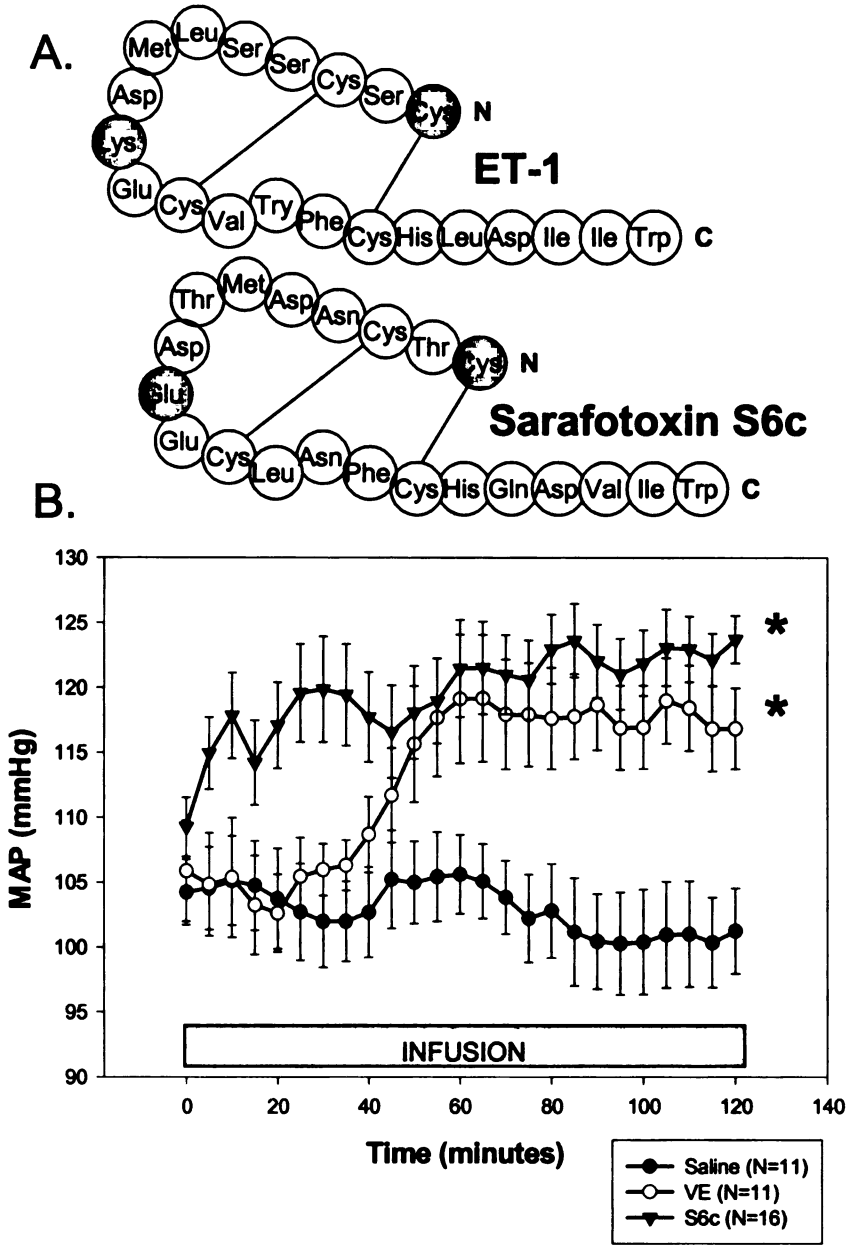

Fig 3. Acute S6c infusion increased MAP. Structure of Sarafotoxin 6c (S6c) bears strong sequence homology to ET-1 (B). MAP was measured for the 2 hour duration of treatment (B). No differences were observed in the final MAP level between VE (N=11) and S6C (N=16) rats. Both VE and S6C groups had a significantly higher MAP at the end of treatment compared with initial measurements, while CON (N=11) decreased slightly ($p<0.05$). Data are presented as means $\pm$ SE. * = statistically significant, $P<0.05$.

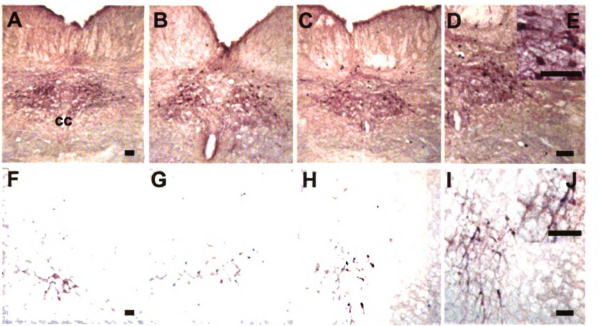

Fig 4. Representative photomicrographs of rat brain slices show colocalization of Fos immunoreactivity and dopamine beta hydroxylase in the NTS (A-E) and CVLM (F-J) of Control (A,F), VE (B,G) and S6c (C-J) rats. High magnification images reveal double labeled neurons (D-J). cc, central canal. Scale bar = 100 μm.

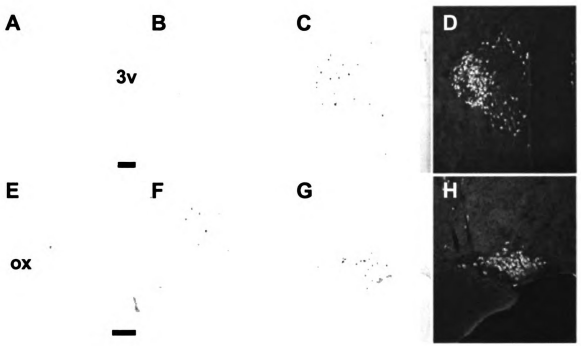

Fig 5. Photomicrographs show Fos immunoreactivity in the PVN (A-D) and SON (E-H) of Control (A,E), VE (E,F) and S6c (C,G) rats. Representative fluorescent photomicrograph of oxytocin double-labeled PVN and SON of S6c infused rats (D,H). 3v, third ventricle; ox, optic chiasm. Scale bar = 100 μm.

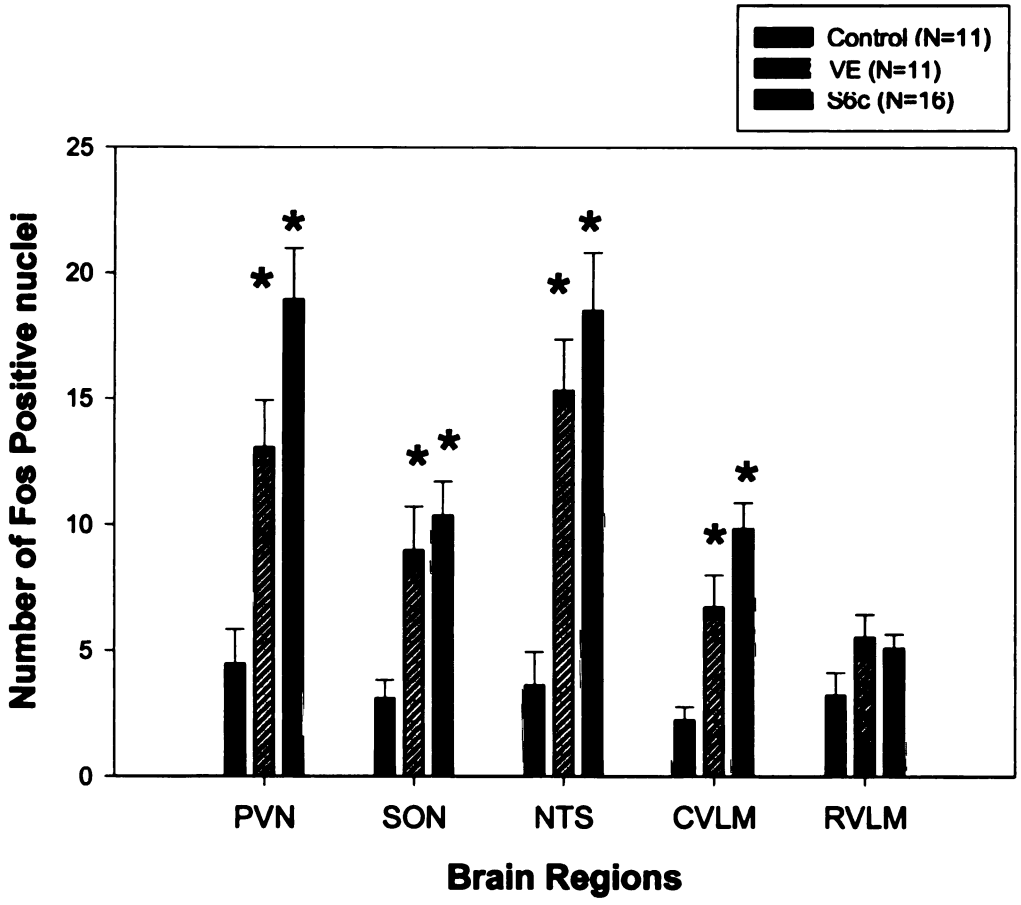

Fig 6. Fos immunoreactivity after 2h infusion protocol. S6c and VE infused rats had significantly more Fos positive neurons in all counting brain regions than control animals gradually infused with isotonic saline. Data are presented as average number of Fos-immunoreactive cells in each brain region. * = statistically significant, $P<0.05$.

Chapter 3

Part II

Central autonomic response to acute in vivo ET_B receptor activation is dependent on cardiopulmonary afferents: nodose ganglia deafferentation by kainic acid

INTRODUCTION

We provided evidence (Part I) that one mechanism by which acute ET_B receptor (ETBR) activation through infusion of S6c increases blood pressure is through venoconstriction and blood volume redistribution towards the cardiothoracic region, indicated by stimulation of brain regions known to be activated by the volume expansion (VE) protocol. In Part I of this study, we reported that Fos immunocytochemistry (ICC) technique revealed a distinct pattern of neuronal activation common to both VE and S6c treatment. These brain regions include the caudal portion of the ventrolateral medulla (CVLM), the nucleus of the solitary tract (NTS), the paraventricular nucleus (PVN) and the supraoptic nucleus (SON). However, the mechanism by which blood volume redistribution causes increased neuronal activity in these brain regions remains unknown.

Cardiopulmonary receptors and arterial baroreceptors respond to stretch by relaying afferent neural signals to the NTS in the brain to affect sympathetic nervous activity (SNA). Though both baroreceptor systems are well known to modulate blood pressure increases induced by acute VE, there is great discrepancy in the contribution of each depending on animal species and protocol (Badoer et al, 1997, 1998; Potts et al 2000; Columbari et al 1997). Cunningham et al (2002) reported that the central nervous system response to acute volume expansion primarily involved input from cardiac afferents in the heart. Cardiac afferent fibers are comprised of both mechanoreceptors and chemoreceptors that travel to vasomotor centers in the brainstem via the vagus

nerve and terminate in the NTS (Berthoud and Neuhuber, 2000; Kashihara et al, 2003). The ascending sympathetic control pathway from the NTS projects to the CVLM which in turn sends inhibitory projections to the RVLM (Willette et al, 1984; Agarwal et al, 1989; Li et al, 1991; Blessing, 1997). **Our objective in Part II of Specific Aim I was to determine whether the cardiac afferent system similarly contributes to the pattern of neuronal activation caused by ETBR stimulation.** To achieve this goal, we abolished input from cardiac afferents by bilateral chemical denervation of the nodose ganglia.

The nodose ganglion, also called the inferior vagal node, is the distal vagal ganglion containing perikarya of vagal afferents from cardiac receptors, abdominal visceral receptors, pulmonary receptors and aortic arch baroreceptors (Palkovits and Zaborsky, 1977; Kummer et al 1992; Hopkins et al, 1989). Therefore, lesioning the nodose ganglia would remove input from the cardiac afferents. Kainic acid (KA), which is a neuroexcitotoxin that acts at glutamatergic sites, causes selective destruction of sensory neurons in mixed peripheral nerves (Schwartz et al, 1978; Lewis et al, 1990; Wallick et al, 2002). When directly superfused onto the nodose ganglia, KA produces degeneration of the somata of vagal afferent neurons, while sparing passing axons from efferent neurons (Lewis et al, 1990; Wallick et al, 2002). We used the KA deafferentation method described by Lewis and colleagues (1990) to destroy the cardiac vagal afferent nerve cell bodies in the nodose ganglia while leaving intact the preganglionic parasympathetic axons that pass through the ganglia (as described in the Lewis et al, 1990 protocol) to permanently denervate cell bodies of cardiac sensory

neurons. Bezold-Jarisch reflex testing, which involves direct activation of cardiopulmonary chemosensitive vagal afferent C fibers mediated by 5-hydroxytryptamine (5-HT_3) serotonergic receptors (Verberne and Guyenet, 1992; Whalen et al, 2000; Kashihara et al, 2003), and postmortem hematoxylin-eosin staining of the nodose ganglia provided both physiological and histological verification of cardiac afferent destruction. Selective cardiopulmonary deafferentation with KA allowed us to examine the central nervous system response to blood volume redistribution induced by ETBR activation without the potentially confounding effects of surgical transection of the vagus nerve, which may alter blood pressure and cardiac reflexes as well as cause death.

As described in detail in Part I, acute ETBR activation evoked a discrete pattern of Fos activation in the forebrain and brainstem that is analogous to that generated by acute VE. Briefly, we saw increased Fos immunoreactivity in the SON, PVN, NTS and CVLM of S6c infused rats, however, no differences were observed in the RVLM. If cardiac afferents are the primary modulators following ETBR activation, deafferentation of the nodose ganglia should abolish the augmented Fos response in those brain regions.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Portage, ME) weighing 250-300g were housed in temperature- and humidity-controlled rooms with a 12:12-h light-dark and had ad libitum access to distilled water and pelleted rat chow (Harlan/Teklad 8640 Rodent Diet). The experimental protocol was approved by the Michigan State University All University Council on Animal Use and Care.

Surgery

Under a dissecting microscrope, the left and right nodose ganglia of anesthetized (sodium pentobarbital, 50 mg/kg ip) rats were revealed with a midline incision below the cricoid cartilage (Figure 7). Ultrafine atraumatic microforceps (Fine Science Tools, Switzerland) were used to divest the ganglia of surrounding fascia, separating them from the carotid sheath. Care was taken to not puncture the adjacent carotid artery and not disrupt the superior laryngeal nerve which lies just inferior to the ganglion. Then, a small piece of parafilm was placed beneath each exposed ganglion to minimize spreading of the chemical onto surrounding nerve bundles. Nodose ganglia were superfused with 2 μl of 0.47 nmol/ml KA (Sigma-Aldrich, St. Louis, MO) for 3 minutes as any excess KA was removed with a sterile cotton swab. Sham rats also followed this protocol with the only exception of receiving an equivalent superfusion of saline instead of KA. Incisions were immediately sutured (Ethicon) and rats were allowed to recover on

heating pads under careful observation before being housed individually in home cages. Attention was paid to ensure that animals did not exhibit ptosis of the eyes, which would suggest superior cervical ganglion involvement.

Catheterization

Following a weeklong recovery period, both sham and KA rats were anesthetized for catheterization surgery as described in Methods section of Part I. Catheters were flushed twice a day with heparinized saline (100u/ml; Sigma) to maintain patency and rats received ticarcillin (10 mg/kg; SmithKine Beecham Pharmaceuticals, Philadelphia, PA) and enrofloxacin (2 mg/kg; Bayer) daily to prevent bacterial infection.

Cardiopulmonary Reflex testing

The Bezold-Jarisch reflex bradycardia technique was performed to assess cardiopulmonary baroreflex function in both sham operated and KA-treated rats. The Bezold-Jarisch reflex, elicited by intravenous injections of 5 hydroxytryptamine (5-HT; Sigma, St. Louis, MO, USA) at 2, 4, 8 mg/kg given at 3 minute intervals, consists of an immediate and transient drop in HR in sham rats with intact cardiopulmonary afferent systems. This is followed by a gradual, longer-lasting decrease in blood pressure

Baroreceptor reflex testing

Sham and KA rats were also assessed for arterial baroreceptor reflex integrity. The pressor agent, phenylephrine (Sigma), and the depressor agent sodium nitroprusside (Sigma) were injected at incremental doses of 1, 5, 25 μg/kg and 2, 4, 8 μg/kg, respectively, given at 3 minute intervals to stimulate the arterial baroreceptors and elicit reflex-mediated changes in HR.

Experiment

Successfully deafferentated rats showing a reduction in Bezold-Jarisch reflex bradycardia received a 2 h infusion of either isotonic saline at 0.01 ml/min or S6c (5pmol/kg/min). MAP and HR were continuously monitored throughout the 2 h protocol. Rats were immediately sacrificed after infusion and transcardially perfused with 4% paraformaldehyde (described in Methods of Part I). The nodose ganglia were dissected and post-fixed in the same 4% paraformaldehyde fixative for 24 h prior to storage in 30% phosphate buffered sucrose cryoprotectant solution. Then, transverse sections of the paraffin-imbedded ganglia were serially cut with a 10 μm and thaw-mounted onto gel-coated Superfrost Plus microscope slides. After drying, hemotoxylin & eosin histology was performed on nodose ganglion sections. Following staining, ganglia were coverslipped using Permount. Fixed brains were coronally sectioned on a cryostat at 35 μm and stored in a 12-well culture plate filled with 0.1M PBS solution. Immunohistochemistry for Fos was executed using the same protocol as previously described (see Methods of Part I). Sections were

examined using light microscopy to identify ganglionic cells and quantify Fos positive neurons in the brain (described in Methods section of Part I).

Statistical analysis

All data were presented as mean ± SEM and were analyzed with Prism 3.0 Software (GraphPad, Inc). Differences in mean HR of baroreceptor reflex and cardiopulmonary reflex activities were analyzed using Student's t-test to compare slopes. Two-way ANOVA followed by post hoc test was performed to examine differences in variables among groups of rats. A P value ≤ 0.05 was considered statistically significant.

Results

Cardiopulmonary reflex testing

Figure # shows reflexly mediated reductions in HR in response to incremental doses of 5-HT (2, 4, 8 μg/kg) in KA (N=11) and sham control rats (N=7). The Bezold-Jarisch reflex, shown as a sharp drop in HR in sham rats with an intact cardiopulmonary afferent system, was significantly blunted in KA treated rats with bilateral nodose ganglionectomies (Figure 8). KA treatment resulted in a significant reduction in the slope of the HR dose-response curve compared to sham controls. In KA treated rats the slope was -6.0 ± 2.2 compared to -34.2 ± 2.0 in sham rats. Therefore, the baroreflex gain was reduced by an average of 82.5%.

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Baroreceptor reflex testing

The mean pressure-HR reflex relationships obtained from KA treated rats (N=11) and sham operated control rats (N=7) show no significant differences between the two treatments in response to either pressor or depressor agents (Figure 9). Phenylephrine (10, 25, 50 μg/kg) induced a dose-dependent decrease in HR while sodium nitroprusside (2, 4, 8 μg/kg) caused a dose-dependent increase in HR in both groups of animals. Though linear regression analysis showed a slightly reduced HR reflex in KA rats, these differences were not statistically significant. This indicates that arterial baroreceptor function was preserved after KA treatment.

Histology

Hematoxylin and eosin staining of fixed nodose ganglion slices shows that in comparison to control sham operated rats, after KA treatment there is degeneration of nodose ganglionic neurons (Figure 10). The ganglia of KA treated rats had noticeably fewer neurons, which were replaced by increased fibrous tissue. Some of the remaining neurons show signs of piknosis. No histological differences were observed in axons of KA and sham treated rats.

Hemodynamic

No differences were observed in the resting MAP and HR between KA-treated and sham rats.

MAP increased significantly in both KA treated and sham control rats receiving S6c infusion. With S6c infusion, the mean difference between final and initial MAP of sham and KA rats was 15.1 ± 4.8 mmHg and 21.4 ± 2.7 mmHg, respectively (Figure 11). Though the pressor response of KA rats was slightly higher than sham rats, the difference was not statistically significant. In contrast, there was no increase in MAP in either group of rats receiving saline infusion. Saline-infused sham rats had a mean MAP increase of 2.7 ± 1.3 mmHg, while the MAP decreased 2.4 ± 6.5 mmHg in KA rats receiving saline (Figure 11). Thus, KA deafferentation did not impair blood pressure responses to ETBR activation. No significant differences in HR response to S6c infusion were observed in any of the groups.

Fos expression

Very few Fos activated neurons were observed in any brain region examined in sham operated rats receiving saline infusion (Figure 12). Similarly, saline-infused KA treated rats also showed only sparse Fos activation. No significant difference was observed between the KA and sham groups receiving saline infusion. Therefore, these two groups each served as negative controls to provide baseline profiles for Fos expression.

S6c infusion induced a significant increase in Fos positive nuclei in the brainstem of sham control rats (Figure 12). Fos positive neurons were found to be distributed in the commissural NTS at the level of the central canal (Bregma -13.80mm). Fos counts of the NTS were taken bilaterally at three different levels

corresponding to anterior, middle and posterior portions and averaged to render a mean value. In the CVLM (Bregma -14.30mm), S6c infusion in sham rats caused a significant increase in Fos expression compared to controls. These results are consistent with previous data from Part I. Also consistent with previous results, the RVLM (Bregma -12.72mm) was not activated by S6c infusion (Figure 12).

Double-labeling the NTS and CVLM brain slices with DBH showed that roughly half of Fos activated neurons in the NTS and CVLM induced by S6c infusion were DBH positive (Figure 13).

In the forebrain, a significant increase in Fos was observed in the PVN (Bregma -1.80mm) of S6c infused sham rats compared to control rats (Figure 14). Though oxytocin-immunostaining was not performed in this portion of the study, Fos immunoreactivity was distributed predominantly in the medial portion of the PVN associated with oxytocinergic neurons. S6c treatment also caused a significant increase in Fos positive nuclei in the SON (Bregma -1.40mm) (Figure 14).

In short, sham operated rats that received a 2h S6c infusion showed robust Fos expression in the PVN, SON, NTS and CVLM consistent with earlier findings (Part I).

In KA treated rats, S6c infusion did not produce a significant increase in Fos expression in the PVN, CVLM and NTS. In the PVN, some cells were activated, particularly in the medial portion corresponding to the location of oxytocinergic neurons. Though KA treatment reduced the number of Fos

positive neurons in the SON induced by S6c infusion, the reduction was not significant compared to S6c-infused sham rats.

Therefore, KA deafferentation blocked the increase in Fos expression produced by S6c infusion in the PVN, CVLM and NTS with lesser reductions in the SON. No significant increase in Fos expression was observed in the RVLM of KA treated receiving S6c infusion.

DISCUSSION

The objective of the present study was to evaluate the contribution of cardiac afferents to the central nervous system Fos response induced by systemic ETBR activation. We found that cardiac deafferentation reduced/abolished the increase in neuronal Fos expression in the brainstem and forebrain following ETBR activation that we have hypothesized to be caused by blood volume (Randolph et al, 1998; Cunningham et al 2000; Godino et al, 2005) and centralization of blood volume (Part I).

We used the excitotoxin induced deafferentation method described by Lewis et al, (1990) to denervate the cardiopulmonary receptors. Kainic acid (KA) was used to selectively destroy vagal afferent neurons within the nodose ganglia with minimal injury to parasympathetic efferent axonal fibers. This method has been used successful in dogs (Wallick et al, 2002) and rats (Lewis et al, 1990). We verified the deafferentation using the Bezold-Jarisch reflex to assess cardiopulmonary afferent function and performed histological analysis to confirm the extent of the damage in hemotoxylin & eosin stained dissected nodose

ganglia. The Bezold-Jarisch reflex normally elicits a reflex mediated bradycardia from stimulation of 5-hydroxytryptamine (5-HT_3) serotonergic receptors in the heart of animals with intact cardiopulmonary chemosensitive afferents (Lewis et al, 1990; Verberne and Guyenet, 1992; Whalen et al, 2000; Kashihara et al, 2003). In KA treated rats, we observed a significant reduction in the Bezold-Jarisch reflex HR response compared to control sham operated rats. However, baroreceptor reflex assessment with pressor and depressor agents revealed relatively unaffected HR reflex function. This is in contrast to the study by Lewis et al (1990) in which the arterial baroreflex was also affected by kainic acid deafferentation. Though kainic acid presumably destroyed neurons in the nodose ganglia that carry both aortic as well as cardiopulmonary information to the brain (Portalier and Vigier, 1979), afferents from carotid sinus and petrosal ganglion (Ruiz-Pesine et al 1995) should not have been affected by kainic acid superfusion onto the nodose ganglion. Our finding that arterial baroreflex function is maintained after KA treatment is consistent with data from a study by Wallick et al (2002) who measured renal sympathetic nerve activity in dogs with bilateral carotid occlusion to illustrate intact carotid baroreflex function. Taken together, our data demonstrate that activity of vagal cardiopulmonary receptors was significantly reduced by kainic acid superfusion on the nodose ganglia, without affecting adjacent ganglia and/or nerves. Moreover, histological analysis of the nodose ganglia revealed dramatic degeneration and loss of neurons in KA treated rats compared to their normal counterparts, further highlighting the success of the deafferentation. This finding reinforces the precision of the

neuroexcitotoxin denervation method to selectively destroy only sensory neurons in mixed peripheral nerves.

Our results showed that KA deafferentation did not affect blood pressure responses to ETBR activation as both KA treated and sham rats had significantly and similarly elevated blood pressure during S6c compared to saline infusion. Destruction of cardiopulmonary receptors impairs the afferent limb of the cardiopulmonary reflex; however, the parasympathetic efferent nerves appeared to be largely intact. Therefore, we would not expect impairment of the blood pressure response to S6c. Operation of the cardiopulmonary reflex does not appear to affect the MAP or HR response to S6c infusion.

The number of Fos-positive neurons was significantly increased in the PVN of sham control rats receiving S6c, consistent with our earlier findings. KA deafferentation blocked this increase in Fos expression in the PVN. Our data are consistent with studies that reported increased Fos immunoreactivity in the PVN during volume expansion (Randolph et al, 1998) was abolished by cardiac nerve block with intrapericardial procaine (Cunningham et al 2002). As discussed in the previous chapter (Part I), the PVN is comprised of parvocellular and magnocellular subdivisions that are equally important in the regulation of cardiovascular and fluid homeostasis. The parvocellular PVN is activated during acute VE and right atrial distention and is associated with inhibition of renal sympathetic discharge (Pyner et al, 2001; Karim et al, 1972; Linden and Kappagoda, 1982; Badoer et al 1997; 1998; Haselton and Vari, 1998). The magnocellular PVN on the other hand is associated with neuroendocrine

mechanisms in the modulation of blood pressure through the release of oxytocin and vasopressin (Antunes-Rodrigues et al 2003). Oxytocin increases sodium excretion from the kidneys, induces natriuresis and restores fluid balance in response to volume load (Verbalis et al, 1991) and has been shown to decrease blood pressure in both humans and animals models (Petty et al, 1985; Peterssen et al, 1996; Maier et al 1998. In part I of our study, we found that Fos is highly expressed in parvocellular as well as magnocellular neurons of the PVN which suggests involvement of both arterial baroreceptors as well as cardiopulmonary receptors in response to S6c infusion.

We found that S6c infusion is associated with a significant increase in Fos immunoreactivity in the SON of sham rats as shown previously (Part I). However, unlike the PVN, KA denervation did not significantly attenuate the increase in Fos response to S6c infusion, though the amount of Fos was diminished compared to sham rats. In a previous study, Cunningham et al (2002) found that cardiac nerve block by intrapericardial procaine also did not significantly affect the Fos response to acute volume expansion in the SON in contrast to other regions of the central nervous system. Both that report and our findings suggest that changes in neuronal activity in the SON during blood volume shifts do not require input from cardiopulmonary afferents. Instead, arterial baroreceptors may contribute to the Fos response in SON. We showed previously that almost all Fos positive neurons activated by either S6c or VE within the SON were oxytocinergic, consistent with plasma oxytocin data from VE studies (Haanwinkel et al, 1995; Godino et al, 2005). Oxytocin cells in the SON

may be activated to modulate the increase in blood pressure signaled by intact arterial baroreceptors in the carotid sinus.

Fos immunoreactivity was significantly increased in the NTS and CVLM following S6c infusion in sham rats, consistent with our previous data. DBH double-labeling revealed that both catecholaminergic cells and non-catecholaminergic cells were activated. KA deafferentation severely attenuated the Fos response in both populations of NTS and CVLM neurons. Both NTS and CVLM are part of the primary afferent pathway that relay volume and pressure information from the cardiopulmonary and arterial baroreceptors (Badoer et al, 1994; Hines et al 1994). Barosensitive afferents project to the NTS which in turn send excitatory projections to the CVLM. Activated CVLM neurons decrease sympathetic nervous activity through inhibitory projections to the RVLM (Minson et al, 1997). Consistent with previous data, there was no measurable neuronal activation change in RVLM neurons in all four groups. In the absence of reflexly induced sympathoinhibition from an intact cardiopulmonary afferent system, the pressor response to S6c should be larger in KA rats. Though, we saw a slightly greater magnitude of blood pressure increase in KA rats during the 2h S6c infusion, the final MAP did not indicate statistically significant differences from sham rats.

The pattern of Fos expression caused by S6c infusion here is in agreement with our previous findings that specific hypothalamic and medullary nuclei are activated (Part I). In sham operated rats, S6c infusion significantly increased the number of Fos positive nuclei in the PVN, SON, NTS and CVLM.

Cardiac deafferentation by KA injection into the nodose ganglion attenuated, but did not abolish the Fos response to S6c in the PVN, CVLM, and NTS. KA treated rats receiving S6c infusion had slightly greater Fos response in the PVN, CVLM, and NTS and significantly more Fos positive neurons in the SON than rats receiving saline. Thus, cardiac deafferentation did not completely abolish the Fos increase in response to S6c stimulation. This suggests that the central nervous system response to ETBR activation is mediated by more than one pathway, as cardiac denervation only partially blocked the Fos response. It is very likely that intact arterial baroreceptor function contributed. To evaluate the contribution of arterial baroreceptors and facilitate the study of vagal cardiopulmonary reflexes in isolation from reflexes mediated by these arterial baroreceptors, sinoaortic denervation can be performed in all groups of animals prior to KA deafferentation.

Alternatively, the persistence of Fos activation in the brain following cardiopulmonary deafferentation may be attributed to the administration of KA. Using KA as a chemoconvulsant, Sylveira et al (1998) found that KA triggered seizures and induced a long sustaining neuronal activation indicated by Fos-like immunoreactivity (FLI) in limbic and brainstem nuclei. They found that KA treatment significantly increased FLI in NE secreting neurons. Furthermore, Kasof et al (1995) reported that following treatment with KA there was a protracted expression of Fos in the central nervous system lasting 2-3 days. However unlikely the occurrence, there is a small possibility that part of the neuronal response attributed to S6c infusion results from KA contamination into

the brain. In our protocol, the utmost care was taken to ensure a localized lesion. Prior to KA application, each vagus nerve containing the nodose ganglion was desheathed and separated from the internal carotid artery while parafilm was used beneath the exposed ganglion to minimize spreading of the chemical onto surrounding nerve bundles and vasculature. To rule out the possibility that KA itself caused the Fos increase after cardiopulmonary deafferentation, we can alter our protocol by administering another neuroexcitoxin in place of KA such as *N*-methyl-D-aspartic acid or α-amino-3-5hydroxy-4-isoxazolepropionic acid (Lewis et al, 1990). Alternatively, another method of cardiopulmonary deafferentation can be employed such as left ventricular deafferentation (Minisi and Cersley, 1994), intrapericardial procaine or lidocaine infusion (Cunningham et al, 2002; Minisi et al, 1998).

Based on our present data, we conclude that cardiopulmonary receptor activation is the primary but not exclusive cause of the central nervous system response to systemic ETBR activation. Because cardiac afferents have been found to be critical in the modulation of the neural response to acute volume expansion (Cunningham et al, 2002), our finding that ETBR activation also stimulates these receptors further supports our hypothesis that *in vivo* ETBR activation involves peripheral venoconstriction and blood volume centralization to cause increased blood pressure.

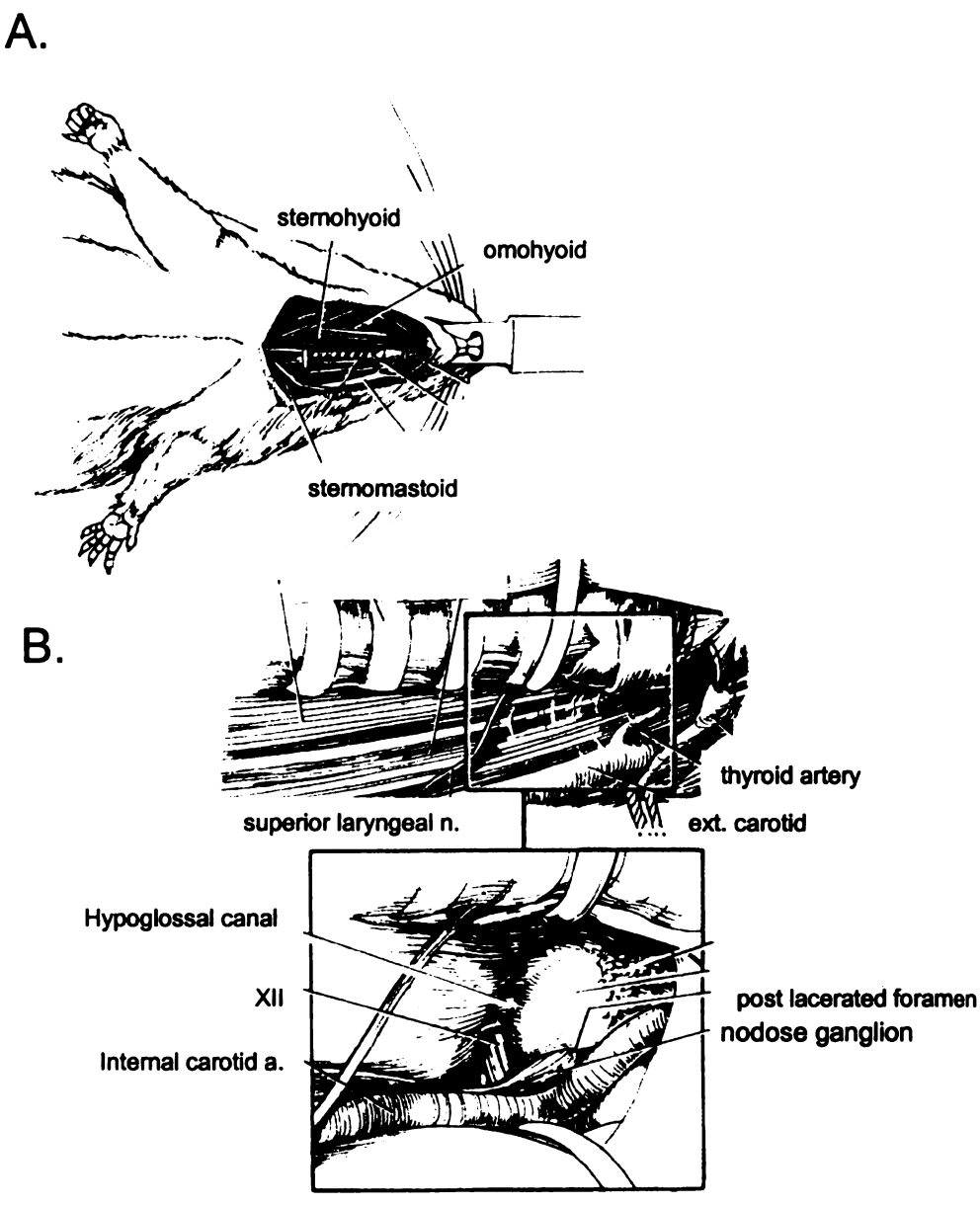

Fig 7. Illustration of the kainic acid deafferentation procedure. First, midline incision is made to expose the trachea at the level of the cricoid cartilage (A). Then, under a dissecting microscope, the internal carotid artery and nodose ganglion are exposed (B). *Images from Norgren and Smith, 1994.*

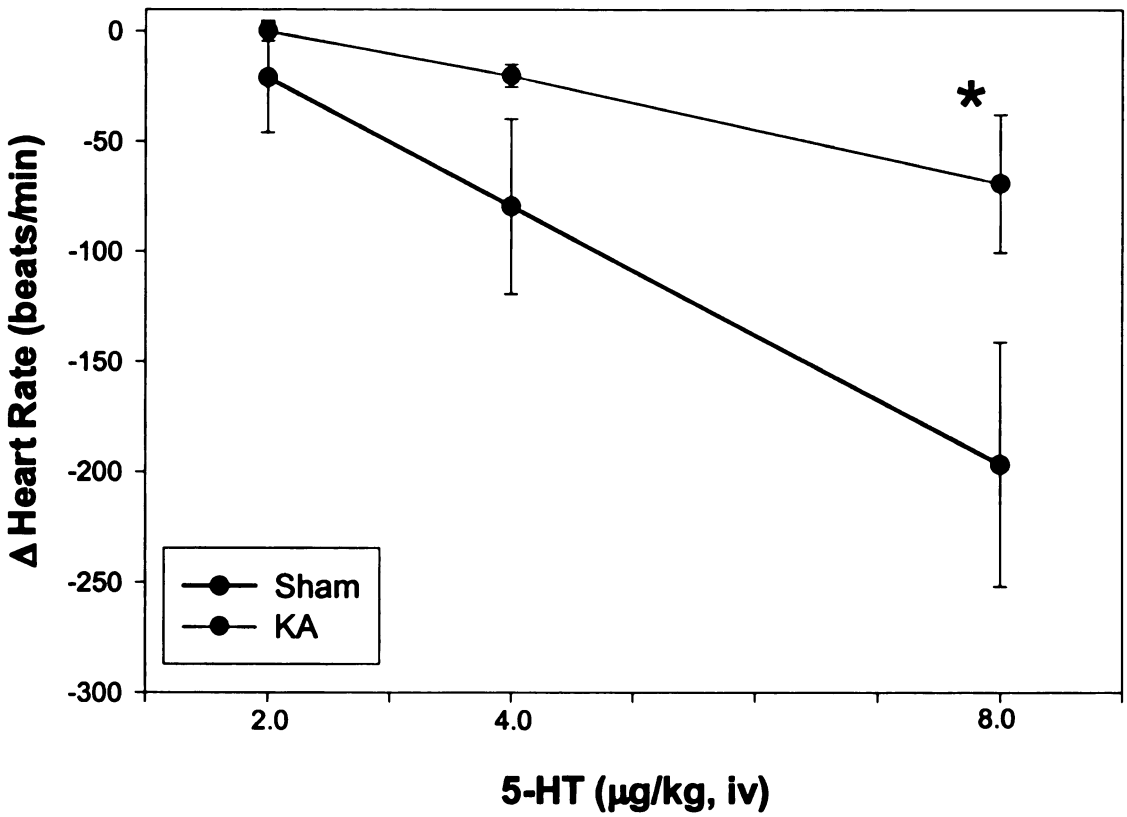

Fig 8. The Bezold-Jarisch reflex (BJR) is elicited by iv injections of 5 hydroxytryptamine (5-HT) at 2, 4, 8 μg/kg given at 3 minute intervals. The BJR, shown as a sharp drop in HR in sham rats with an intact cardiopulmonary afferent system, was significantly blunted in KA treated rats with bilateral nodose ganglionectomies. KA treatment resulted in a significant reduction in the slope of the HR dose-response curve compared to sham controls. In KA treated rats the slope was -6.0 $\pm$ 2.2 compared to -34.2 $\pm$ 2.0 in sham rats. Therefore, the baroreflex gain was reduced by an average of 82.5%. *= significance, $P<0.05$.

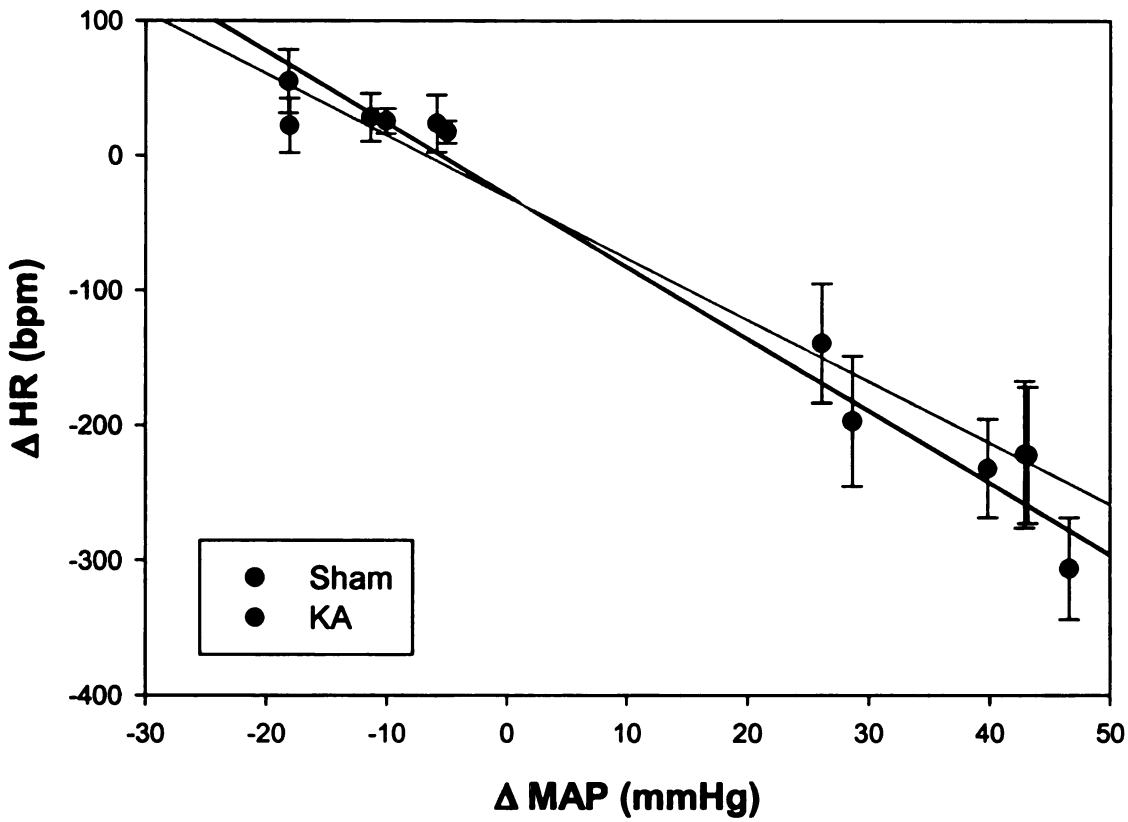

Fig 9. The mean pressure-HR reflex relationships obtained from KA treated rats (N=11) and sham operated control rats (N=7) show no significant differences between the two treatments in response to either pressor or depressor agents. Phenylephrine (10, 25, 50 μg/kg) induced a dose-dependent decrease in HR while sodium nitroprusside (2, 4, 8 μg/kg) caused a dose-dependent increase in HR in both groups of animals.

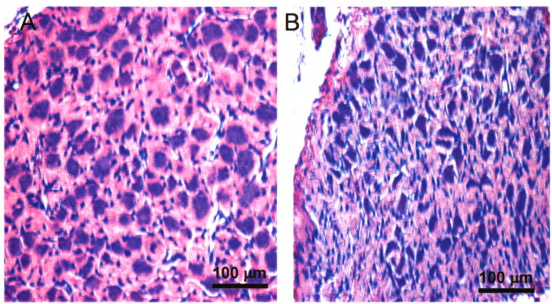

Fig 10. Images in this dissertation are presented in color. Light micrographs showing hematoxylin and eosin staining in the nodose ganglion 7 days after sham (A) and kainic acid (KA) deafferentation (B). The ganglia of KA treated rats had noticeably fewer neurons, which were replaced by increased fibrous tissue. Some of the remaining neurons show signs of piknosis. No histological differences were observed in axons of KA and sham treated rats.

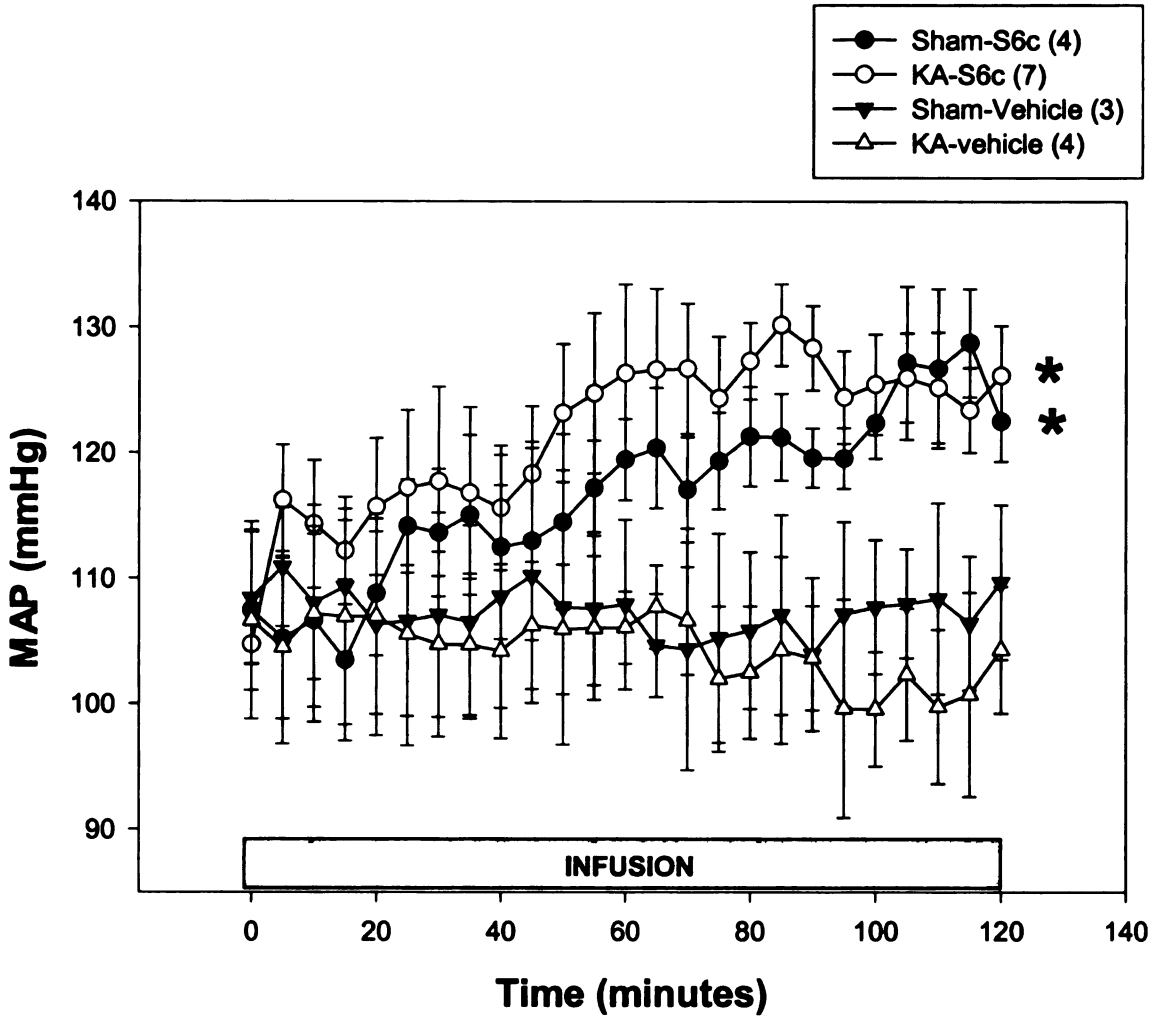

Fig 11. MAP increased significantly in both KA treated and sham control rats receiving S6c infusion. With S6c infusion, the mean difference between final and initial MAP of sham and KA rats was 15.1 ± 4.8 mmHg and 21.4 ± 2.7 mmHg, respectively. Vehicle-infused sham rats had a mean MAP increase of 2.7 ± 1.3 mmHg, while the MAP decreased 2.4 ± 6.5 mmHg in KA rats receiving saline vehicle. * significance, $P<0.05$.

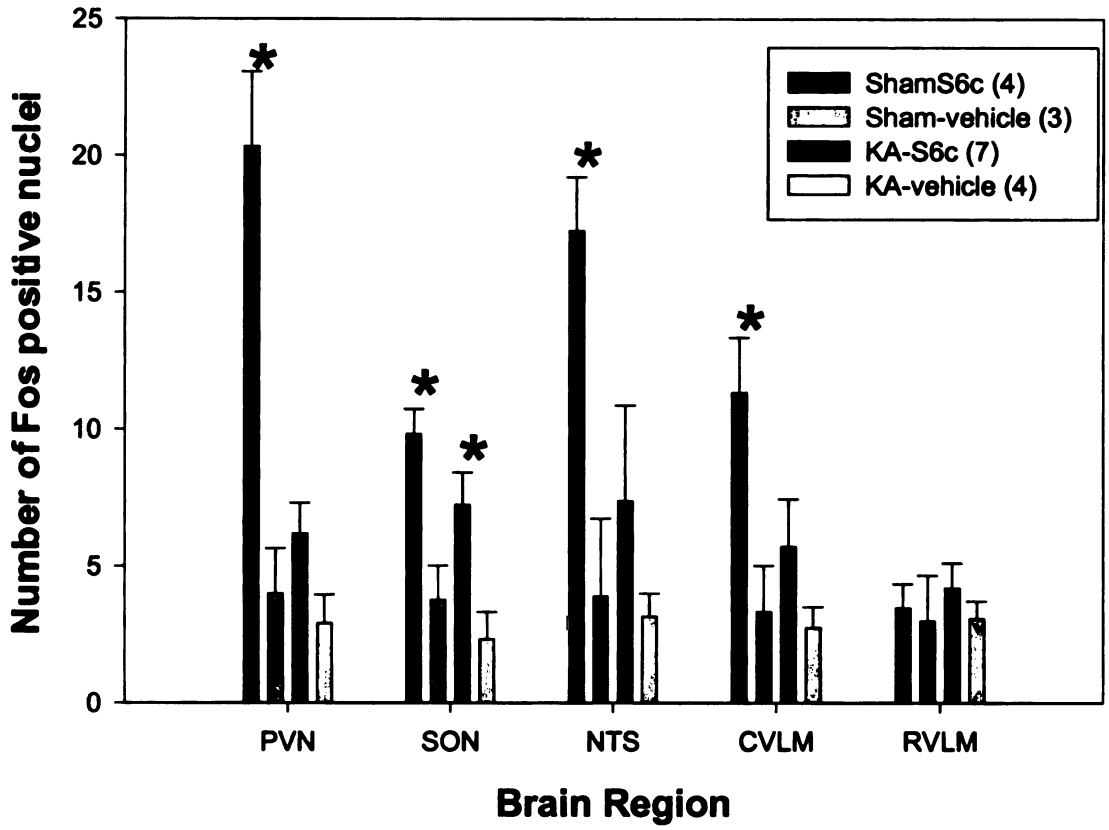

Fig 12. Number of Fos positive nuclei in KA and sham rats receiving S6c or saline infusion for 2h. KA deafferentation blocked the increase in Fos expression produced by S6c infusion in the PVN, CVLM and NTS but not in the SON. * significance, $P < 0.05$.

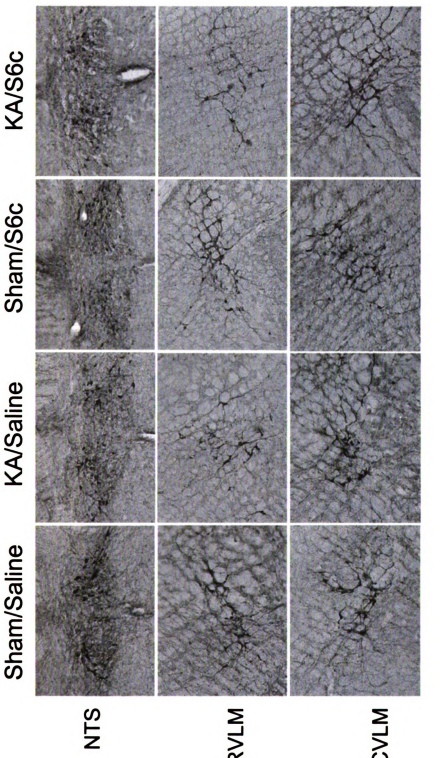

Fig 13. Photomicrographs of Fos immunohistochemisty in KA and sham hindbrain slices after S6c and saline infusion.

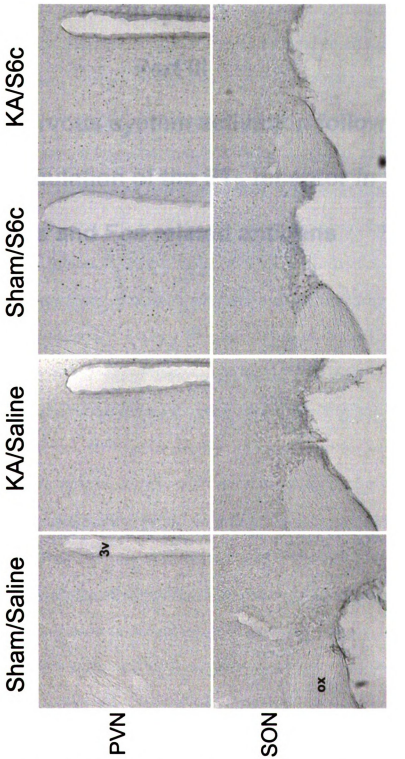

Fig 14. Photomicrographs of Fos immunohistochemisty in the forebrain of KA and sham operated rats after S6c and saline infusion. ox, optic chism; 3v, third ventricle

Chapter 3

Part III

Central nervous system activation following chronic stimulation of the ET_B receptor *in vivo*: Fos and Fos related antigens

INTRODUCTION

We demonstrated that short-term (2 h) in vivo stimulation of ET_B receptors induced a rise in mean arterial pressure (MAP) accompanied by increased neuronal activity in the paraventricular nucleus (PVN), supraoptic nucleus (SON), caudal portion of the ventral lateral medulla (CVLM) and nucleus of the solitary tract (NTS)—brain regions associated with regulation of body fluid volumes and sympathetic nervous system activity. The present study examined neuronal activation following long-term (5 day) stimulation of the ET_B receptor (ETBR) to determine whether the pattern of brain activity seen during acute hypertension persists in chronic hypertension. A 5 day infusion of the selective ETBR agonist, sarafotoxin 6c (S6c) produces sustained activation of ETBRs resulting in hypertension (*unpublished data*) while a 2 h infusion elicits a transient increase in blood pressure. Previous findings in our lab suggest that there may be a neurogenic component to the hypertension maintained by a chronic 5 day activation of ETBRs (*unpublished data*). In that study, the decrease in MAP produced by pretreatment of rats with the ganglionic blocker, trimethaphan, during the last 2 days of S6c infusion was significantly greater than the drop in blood pressure after 1-3 days of S6c infusion (Figure 15), suggesting that the initial response to S6c involved direct constriction of the vasculature while the later response to long term S6c infusion was mediated at least in part by a neurogenic mechanism, possibly increased sympathetic nervous activation (SNA). In angiotensin II induced hypertension, it has been suggested that hypertension exists in two phases, an acute and chronic phase, which may

involve differential contributions of the brain and vasculature (McCubbin et al, 1965; Wong et al, 1991; Lever et al, 1992; Li et al, 1998). For example, Li et al (1998) found different brain regions were activated depending on the duration of hypertension, possibly due to the combined effects of reflex mechanisms stimulated by vascular actions of angiotensin II and direct activation of brain pathways. We hypothesized that a similar situation might exist for S6c-induced hypertension. **Thus, the goal in this study was to characterize the pattern of Fos activation in chronic 5 day S6c infused rats.**

In the present study, we attempted to identify the hypothalamic forebrain and medullary brainstem regions in the central nervous system that are activated in response to chronic stimulation of ETBR. Specifically, we focused on brain regions previously shown to be affected by two-hour ETBR activation and that are important in the regulation of blood volume and sympathetic output--the NTS, CVLM, RVLM, PVN and SON. We used immunorectivity (ir) for the protein product of the c-fos gene, Fos, as well as other Fos-related proteins FosB, Fos related antigen (Fra)-1 and Fra-2, collectively referred to as Fos-Like (Li)-ir as a marker of central nervous system activation (Lohmeier et al, 2003). In neurons, the Fos protein is induced immediately following synaptic activation, peaking 90 minutes post stimulation (Morgan and Curran, 1991) and is well established as a useful tool in mapping central nervous system activation in response to blood pressure and other physiological stimuli (Badoer et al., 1994; Dampney et al, 1995; Li and Dampney, 1994; Graham et al., 1995; Miura et al., 1994; Potts et al., 1997). Fos related proteins, FosB, FRA-1 and FRA-2, have a slow onset

and much longer duration (Morgan and Curran, 1992) and have been shown to be valuable in identifying longer-term CNS changes in response to chronic stimulation. Fos and Fos-related proteins have been previously used to study sustained neuronal activation produced by chronic Ang II hypertension (Li et al, 1998), obesity hypertension (Lohmeier et al, 2003) and long-term isotonic volume expansion (Howe et al, 2004). Therefore, Fos-Li immunohistochemistry was presumed to be a useful assay for neuronal activity after chronic ETBR activation by 5 day S6c infusion.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Portage, ME) weighing 250-300g were housed in temperature- and humidity-controlled rooms with a 12:12-h light-dark and had ad libitum access to distilled water and pelleted rat chow (Harlan/Teklad 8640 Rodent Diet). The experimental protocol was approved by the Michigan State University All University Committee on Animal Use and Care.

Catheterization

Rats were catheterized as previously described (Part I, II). Briefly, sodium pentobarbital anesthetized rats (50mg/kg ip) were chronically instrumented with catheters made of silastic rubber and polyvinyl tubing into the femoral artery and vein for continuous blood pressure and heart rate measurements and drug delivery respectively. After catheterization, rats were then individually housed in standard stainless steel metabolic cages. Ticarcillin (10 mg/kg; SmithKine Beecham Pharmaceuticals, Philadelphia, PA) and enrofloxacin (2 mg/kg; Bayer) were administered daily via the venous catheter to prevent bacterial infection. Both catheters were flushed daily with heparinized saline (100u/ml; Sigma) to maintain patency. Mean arterial pressure (MAP) and heart rate (HR) were measured from the arterial catheter with a TXD-300 pressure transducer linked to a digital BPA-200 Blood Pressure Analyzer (Micro-Med, Louisville, Kentucky).

Experimental protocol

After allowing 3 recovery days for catheterization surgery, rats were divided into 2 experimental groups. Initially, both groups received intravenous infusions of isotonic saline at 0.01 ml/min for 2 days. On the third day, one group continued receiving saline (control; N= 4), while the second group received an infusion of S6c (American Peptide, Sunnydale, CA) at a rate of 5 pmol/kg/min for 5 days. (S6c; N=6). All infusions were performed in unanesthetized, conscious, unrestrained rats. MAP was recorded throughout the protocol. At the end of infusion on the seventh day, rats were immediately sacrificed with an overdose of sodium pentobarbital (100mg/kg iv) and transcardially perfused with 0.1 M PBS followed by 4% phosphate buffered paraformaldehyde solution. After perfusion, brains were dissected and postfixed with 4% paraformaldehyde for 24 hours and stored in 30% sucrose.

Fos Immunohistochemistry

Dissected brains were encased with tissue freezing medium (Optimal Cutting Temperature compound, TissueTek) and were cut into 35 μm sections with a cryostat and collected into 12 well cell culture plates filled with 0.1 M PBS as free-floating sections. After PBS washes, brain slices were incubated with 0.3% hydrogen peroxide (Sigma) in distilled water for 30 minutes at room temperature then rinsed in PBS for 30 min. Sections were then incubated with a blocking solution consisting of 3% normal goat serum (NGS; Vector Labs, Burlingame, CA), 0.25% Triton X 100 and 0.1 M PBS for 2 h at room temperature. After

blocking, brain slices were reacted with rabbit polyclonal anti-Fos antibody for the detection of c-Fos, Fos B, Fra-1, Fra-2 proteins (sc-253; Santa Cruz Biotech, Santa Cruz, CA) diluted 1:500 in 0.1 M PBS/ 3% NGS for 48 h at 4° C. Sections were rinsed 3 times in PBS for 10 min prior to incubation in 1:400 biotinylated goat anti-rabbit IgG (Vector labs, Burlingame, CA) for 2 hours at room temperature. The tissue was then incubated with an avidin-biotin peroxidase reagent (ABC-Vectastain Elite, Vector Labs, Burlingame, CA) for 1 h. After 3 rinses in PBS, brain sections were reacted with a nickel 3,3'-diaminobenzidine solution (Nickel-DAB, Vector labs), which produced a dark brown stain.

We did not observe specific immunoreactivity in control brain slices incubated without primary antibody, although the background staining was a little higher than the previously used purified c-Fos antibody (sc-52; Santa Cruz Biotech, CA).

Double-immunostaining protocol:

Hindbrain slices containing the CVLM, RVLM and NTS were co-labeled with mouse anti-dopamine-beta-hydroxylase monoclonal antibody (DBH; Chemicon, Temecula, CA) in order to more accurately localize the brain regions of interest. Fos immunostained sections were first incubated in a mouse anti-DBH antiserum (1:500) for 3 days. Then sections were treated with ABC reagent and then with biotinylated goat anti-mouse secondary antibody (1:200) for 1 h. Finally, sections were reacted with VIP chromogen (Vectastain, Vector Labs) which produces a light red stain. Subsequently, after extensive rinsing, both sections were

mounted on gel-coated slides, dehydrated in an alcohol and xylene series and coverslipped with Permount mounting medium (Fisher Scientific).

Histological analysis

Histological analysis was performed as described in Part I and II. We used the Paxinos and Watson (1986) atlas as well as hindbrain DBH-ir to demarcate brain regions for quantification of Fos positive nuclei.

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM) and were analyzed with Prism 3.0 Software (GraphPad, Inc). Two-way ANOVA followed by post hoc testing was performed to examine differences in variables among groups of rats, while Student's t-test was used to compare two groups. A *P* value ≤ 0.05 was considered statistically significant.

RESULTS

Hemodynamic

Chronic in vivo S6c infusion produced a significant increase in blood pressure, compared to control rats (Figure 16). MAP of S6c infused rats increased 24.8 ± 4.6 mmHg between the start of active infusion on day 3 to the end of infusion at day 7, whereas the MAP of control rats decreased 3.2 ± 3.4 mmHg. No

significant changes in heart rate were observed during the 7 days of infusion in either group.

Fos expression

Relative to control rats which showed only sparse activation in all brain regions of interest, there was differential activation of brain regions in rats receiving S6c infusion (Figure 19). In the forebrain, S6c infusion significantly increased in Fos-Li expression in the SON (Bregma -1.40mm) with lesser increases in the PVN (Bregma -1.80mm) (Figure 17). Though there was an increase of Fos-Li in the PVN, it was not statistically significant. In the brainstem, S6c infusion significantly increased Fos in the RVLM (Bregma -12.72mm) but not the NTS (Bregma -13.80mm). Fos-Li immunoreactivity in the CVLM (Bregma -14.30mm) was similar in both control and S6c treated groups (Figure 18).

DISCUSSION

We previously investigated the central nervous system effects of acute ETBR activation. A 2 h infusion of the selective ETBR agonist, S6c into conscious, catheterized rats produced an immediate increase in blood pressure that was sustained throughout the duration of the drug treatment. This was accompanied by neuronal activation as indicated by Fos immunohistochemistry in central vasomotor neurons of the baroreflex pathway as well as in brain regions integral to cardiovascular and fluid homeostasis. Preliminary experiments in this laboratory (Lau and Fink, 2005) showed that chronic peripheral infusions of S6c, like chronic ET-1 infusion (Mortensen et al, 1990), causes a sustained increase in MAP without apparent sodium and water retention. In fact, chronic (5 day) ETBR activation results in a sustained increase in blood pressure, despite also producing a significant diuresis and natriuresis (*abstract*). ETA receptor antagonism did not diminish the resultant hypertension (*abstract*) but receptor antagonism by a mixed ET_A/ ET_B receptor antagonist abolished this effect (*unpublished data*) confirming that the sustained blood pressure increase is produced by the chronic activation of ETBR.

The mechanism of how ETBR stimulation causes hypertension is not fully understood. However, we have evidence (Part I) that in the acute situation, activation of the ETBR on vascular smooth muscle cells by two-hour S6c infusion produces direct venoconstriction. We previously confirmed that constriction of the peripheral extrathoracic veins may cause hypertension by centralizing blood volume to the heart thereby increasing blood pressure. Though venoconstriction

is one effect of ETBR activation, studies also indicate that S6c infusion may increase SNA. Recent evidence demonstrated that S6c may act on ETBR in peripheral sympathetic ganglia to increase SNA through the production of superoxide anions (Dai et al, 2003; Lau et al, 2005). Alternatively, S6c may act on ETBR in the brain (Garrido et al,1997; Yamamoto et al, 2004) to cause sympathoexcitation centrally. It is not clear whether the S6c-induced venoconstriction is a direct action of the systemic veins or if it is mediated indirectly through the peripheral or central sympathetic nervous system.

In the present study we chronically stimulated ETBR by 5 day intravenous infusions of S6c and examined regions previously shown to be affected by acute S6c infusion, and also to be activated by blood volume increases (Cunningham et al, 2000; Randolph et al, 1998; Godino et al, 2005). We confirmed our earlier findings that chronic S6c infusion produces a significant increase in blood pressure, and also found evidence that both venoconstriction and sympathoexcitation contribute to hypertension caused by systemic ETBR stimulation.

In the acute S6c infusion model, we found that the number of Fos positive cells were augmented significantly in the PVN, SON, NTS, and CVLM, but not in the RVLM. Though many immunohistochemical assays of neuronal activity have been performed to investigate long-term physiological changes using the antibody to the protein product, *c-fos*, its expression shows rapid onset and short duration, peaking 90 to 120 minutes after stimulation with subsequent degradation, generally disappearing in 3-4 hours (Morgan and Curran, 1991;

Herdegen and Leah, 1998; Miyata et al, 2001). Therefore, we used Fos-Like immunoreactivity (FLI), which comprises antibodies generated against Fos as well as FosB and Fos related antigens (Fra)-1, Fra-2 to examine CNS responses to S6c infusion over 5 days.

There was a significant increase in FLI in the SON of rats receiving chronic S6c infusion compared to control infusion, consistent with our findings from acute S6c infusion. Cardiopulmonary deafferentation did not significantly alter the Fos response in the SON to acute S6c stimulation, suggesting that the mechanism by which SON neurons were triggered did not involve an increase in cardiothoracic blood volume (Part II). More likely, the pressor effects of S6c infusion were responsible for the increased Fos as evidence by the activation of predominantly oxytocinergic neurons. Oxytocin either through direct effects or through the activation of atrial natriuretic peptide (Haanwinkel et al, 1995; Gutkowska et al., 1997, 2000) works powerfully to restore fluid balance and cardiovascular homeostasis (Chriguer et al, 2003; Verbalis et al, 1991). Oxytocin is associated with a fall in mean arterial pressure in both humans and animal studies (Petty et al, 1985; Peterssen et al, 1996; Maier et al, 1998).

Chronic S6c infusion did not significantly increase the level of FLI in the PVN, although there was a greater amount of neuronal activation following 5 day S6c than control infusion. The PVN is integral to sympathetic outflow and neurohumoral control (Badoer, 2001). Previously, we reported that 2h S6c infusion significantly increased Fos in mostly oxytocinergic magnocellular but also parvocellular neurons of the PVN. This effect was significantly attenuated

by cardiopulmonary deafferentation, indicating that the response is primarily volume dependent (Cunningham et al, 2002). The two subdivisions of the PVN differentially react to perturbations in cardiovascular inputs. Increased activity of parvocellular PVN neurons is associated with inhibition of renal sympathetic discharge (Karim et al, 1972; Linden and Kappagoda, 1982; Badoer et al 1997; 1998; Haselton and Vari, 1998) while activaton of oxytocinergic neurons also decrease blood pressure and increase excretion of sodium and water (Chriguer et al, 2003; Verbalis et al, 1991). Our current results show that the ability of acute S6c to induce activation in the PVN does not persist during long-term stimulation, and is thus unlikely to contribute to chronic S6c-induced hypertension.

No significant increase in FLI in the NTS was observed following chronic S6c infusion indicating that activation in this vasomotor region evidenced in the acute S6c infusion protocol did not persist in chronic hypertension. The NTS neurons are the terminal site for baroreceptor input from both cardiopulmonary as well as arterial baroreptors (Badoer et al, 1994; Hines et al 1994). Acute S6c infusion produced a presumably arterial baroreceptor mediated activation of inhibitory neurons within the CVLM, which mediate reflex inhibition of sympathetic premotor neurons of the RVLM (Minson et al, 1997). Our previous findings that both NTS and CVLM were activated by 2h S6c infusion with concomitant lack of increased activity in the RVLM support this concept. Moreover, our current findings show that chronic S6c stimulation induced a transition from activation in the NTS and CVLM to the RVLM. Neurons in the

RVLM are normally active and generate tonic excitatory signals to the spinal sympathetic preganglionic fibers that regulate sympathetic outflow to peripheral circulation (Dampney 1994). In our present study, there was a significant activation of RVLM neurons after chronic S6c infusion. Upregulation of RVLM neurons offers a plausible explanation for the increase in SNA. Signaled by changes in the firing rate of cardiopulmonary and arterial baroreceptors, neurons in the NTS influence the firing of RVLM neurons and SNA. Inactivation of the NTS and CVLM would presumably result in withdrawal of sympathoinhibition and thus, a more sustained excitation in the RVLM leading to a greater sympathetic output. This hypothesis is consistent with the report that chronic Ang II infusion produced increased blood pressure and activation of RVLM neurons that was baroreceptor-independent (Li et al, 1998). Furthermore, in spontaneously hypertensive rats (SHR), RVLM neurons and the sympathetic motor neurons in the spinal cord where they project both have elevated increases in baseline Fos expression (Minson et al, 1996).

Our finding that RVLM but not NTS or CVLM neurons were activated by chronic hypertension is consistent with the profile of neuronal activation reported in chronic Ang II hypertension (Li et al, 1998) where Ang II is systemically infused for either 2h or 18 h. Our findings further support the notion that baroreceptors reset in the direction of pressure change, which presumably results in a rightward shift in threshold during chronic blood pressure elevation (Thrasher 2005; Chapleau et al 1991). However, a report by Lohmeier et al (2003) found that activation of NTS and CVLM neurons during acute baroreflex stimulation by

obesity hypertension persists in chronic obesity hypertension, suggesting that baroreflex function may contribute to long-term regulation of body fluid volume and arterial pressure.

Chronic ETBR activation by 5 day intravenous S6c infusion significantly increased neuronal activity in the SON and the sympathetic premotor RVLM with lesser increases in the PVN and NTS. Sympathoinhibitory neurons of the CVLM were not activated, in contrast to acute ETBR activation. The reverse was observed in the acute ETBR protocol, where NTS and CVLM neurons were highly activated but not the RVLM. This pattern of neuronal activation indicated by FLI is consistent with our hypothesis that chronic S6c infusion may excite brain regions known to be associated with sympathetic nervous system activation.

It is unclear how circulating S6c results in excitation of the RVLM and increased SNA. Conceivably, S6c may bind to ETBRs in the central nervous system to directly influence sympathoexcitation. The presence of ETBRs has been demonstrated in sympathetic premotor RVLM neurons (Chapter 5) which may be activated by S6c. However, it is not known whether circulating S6c can cross the blood brain barrier to reach central ETBRs. RVLM neurons may also be activated indirectly via the PVN-RVLM axis (Guyenet; 2006; Brooks et al, 2005; Coote, 2005; Stocker et al, 2005). ETBRs are found to be abundantly expressed in the subfornical organ (SFO) and the organum vasculosum lamina terminalis (OVLT), which are circumventricular organs that do not have a blood brain barrier (Yamamoto et al, 1997; Chapter 5). Plasma S6c may potentially act

on ETBRs in the SFO and OVLT projecting to autonomic PVN neurons (Ferguson et al, 1984; Lind 1985; Miselis 1982; Weiss et al, 1990), which in turn innervate the brainstem RVLM (Guyenet 2006; Coote, 2005). PVN neurons have also been shown to make direct projections to sympathetic preganglionic neurons in the spinal cord (Bennorach, 2005; Brooks et al, 2005; Coote, 2005). Activation of the PVN-RVLM axis may contribute to the sympathoexcitation following systemic S6c infusion.

The results from this study suggest that chronic stimulation of the ETBR generates a pattern of brain activation different from that observed in acute hypertension. Acute *in vivo* ETBR activation produces a pattern of neuronal activation that supports our hypothesis of a mechanism involving peripheral venoconstriction and blood volume centralization to raise blood pressure, while the activation pattern following chronic S6c infusion is more consistent with the view that ETBR activation maintains blood pressure through sympathetically mediated mechanisms. This indicates that during acute ETBR activation, there is minimal contribution of SNA and the hypertension is initially mediated by S6c acting on vascular smooth muscle cells to produce venoconstriction. In contrast, after 5 days of S6c infusion, SNA is the predominant mechanism sustaining the blood pressure increase. Based on the present findings, it is tempting to draw the conclusion that development of hypertension produced by ETBR activation involves venoconstriction while the maintenance of chronic hypertension is dependent on SNA.

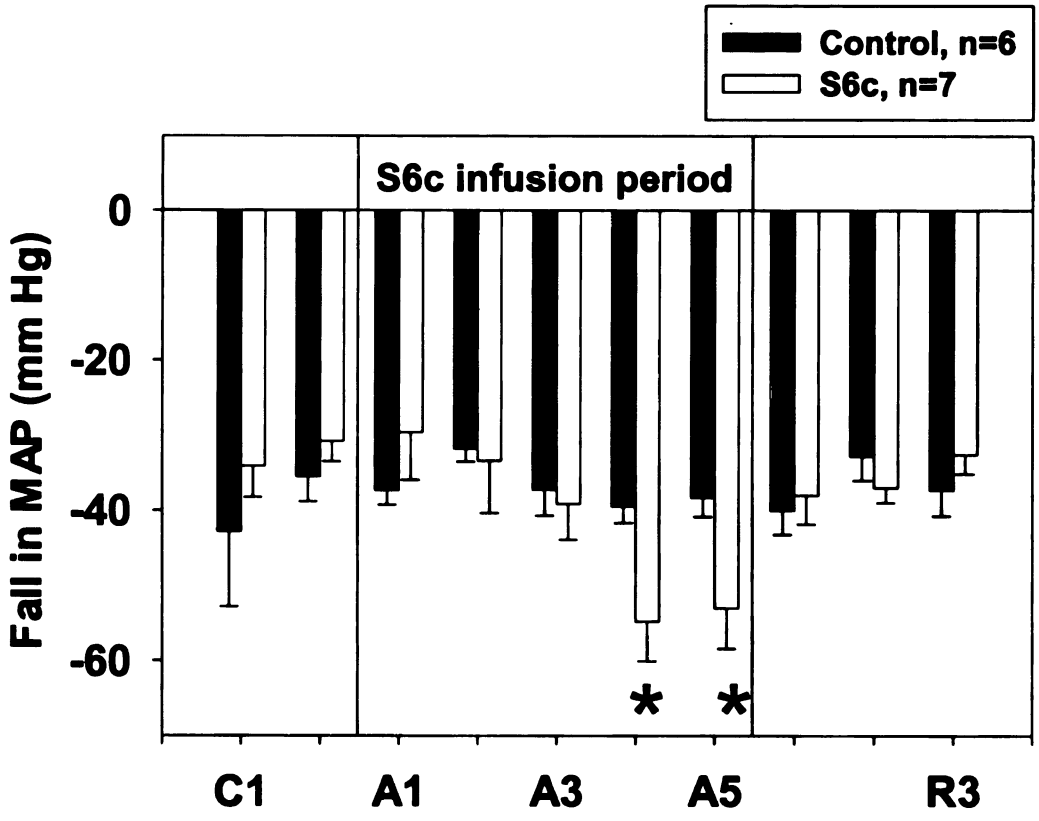

Fig 15. Depressor responses to ganglion blockade with trimethaphan suggest that there may be a neurogenic component to the hypertension maintained by a chronic 5 day activation of ETBRs. The decrease in MAP produced by pretreatment of rats with the ganglionic blocker trimethaphan, during the last 2 days of S6c infusion (A4-A5) was significantly greater than the drop in blood pressure after 1-3 days of S6c infusion (A1-A3), suggesting that the initial response to S6c involved direct constriction of the vasculature while the later response to long term S6c infusion was mediated at least in part by a neurogenic mechanism, possibly increased sympathetic nervous activation.

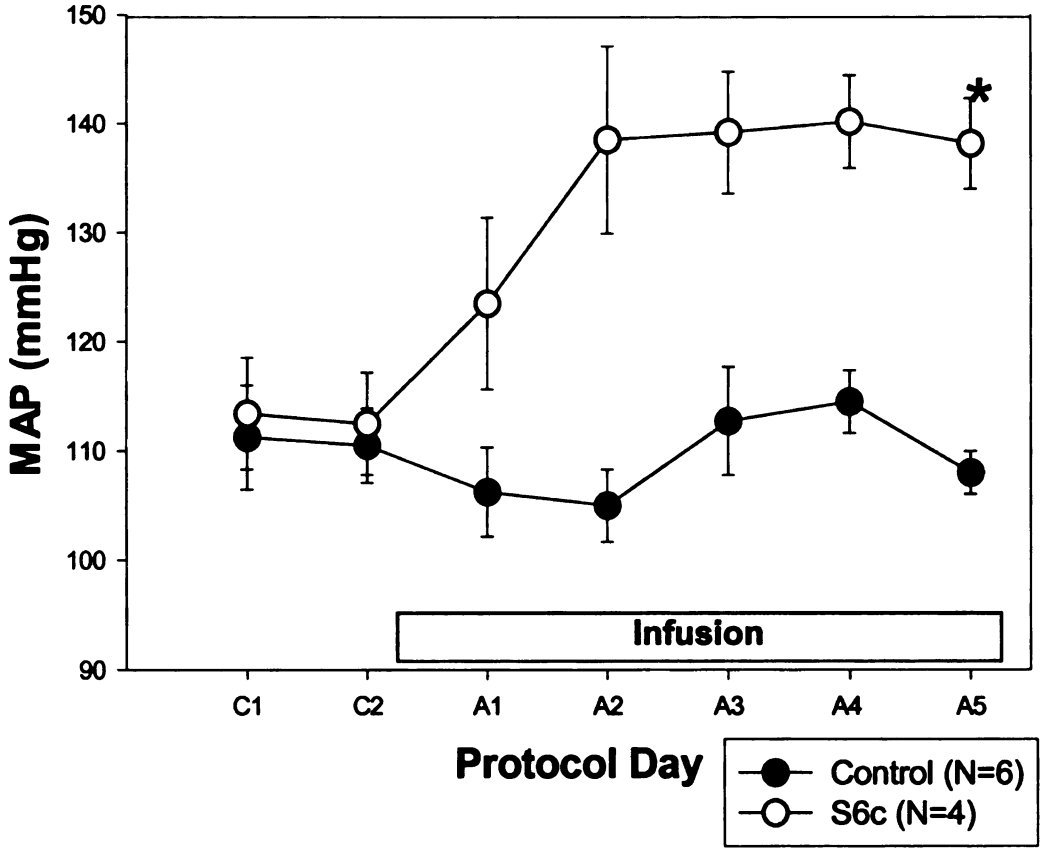

Fig 16. Chronic *in vivo* S6c infusion produced a significant increase in blood pressure, compared to control rats. MAP of S6c infused rats increased 24.8 ± 4.6 mmHg between the start of active infusion (A1) to the end of infusion (A6), whereas the MAP of control rats decreased 3.2 ± 3.4 mmHg. * significance, $P<0.05$.

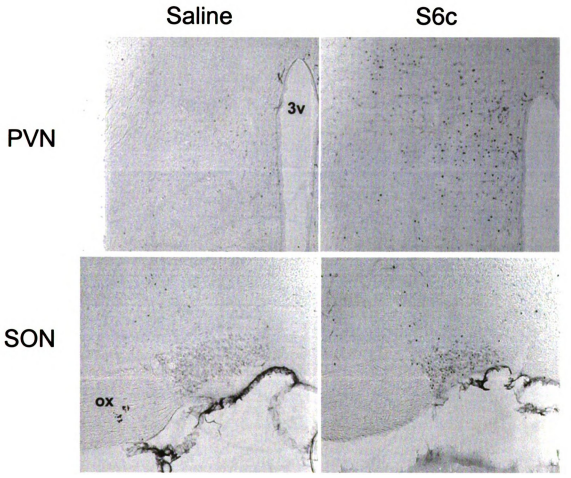

Fig 17. Representative photomicrographs of Fos immunohistochemistry in the PVN and SON of chronic (5 day) S6c and saline infused rats. ox, optic chiasm; 3v, third ventricle

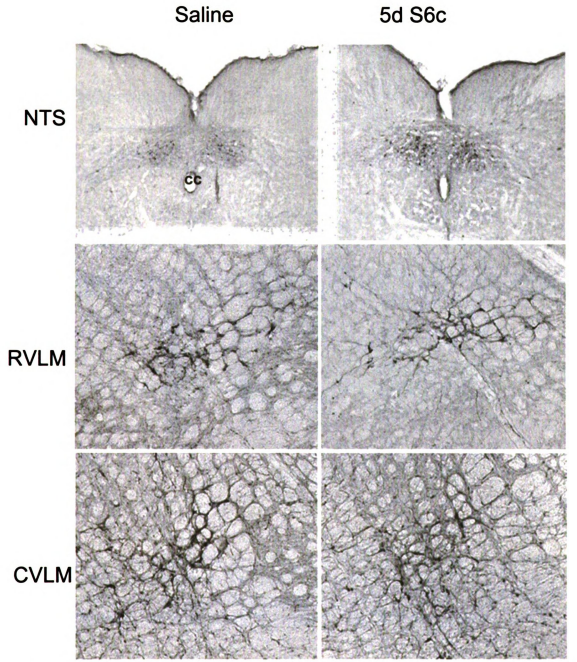

Fig 18. Representative photomicrographs showing increased Fos expression in hindbrain after 5d S6c infusion. The level of Fos-Like immunoreactivity was significantly higher in the RVLM, but not the CVLM or NTS in S6c infusion. Double labeling with dopamine b hydroxylase provided more accurate localization of brain regions. cc, central canal

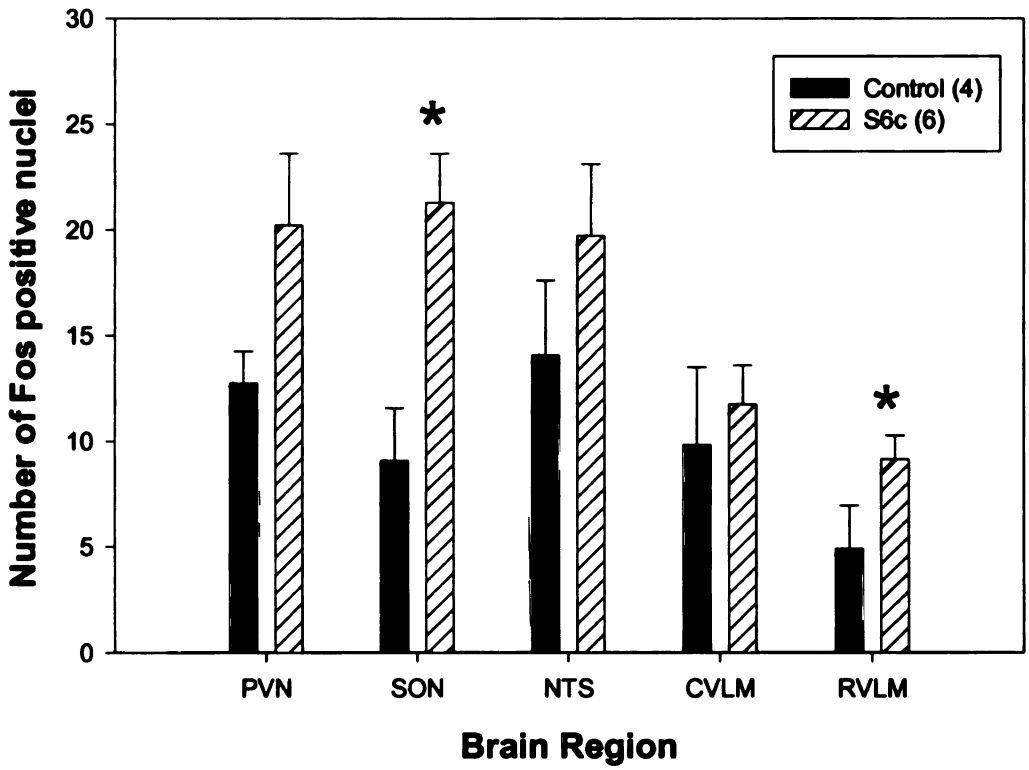

Fig 19. The number of Fos positive nuclei after 5 day infusion of S6c or saline. S6c infusion significantly increased in Fos-Li expression in the SON and RVLM. Though there was an increase of Fos-Li in the PVN, it was not statistically significant. *significance, $P<0.05$.

CHAPTER 4

Part I

Activation of ET_B receptors increases superoxide levels in sympathetic ganglia in vivo

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INTRODUCTION

Hypertension caused by numerous genetic and neurohumoral factors is associated with higher amounts of reactive oxygen species (ROS) in blood vessels, brain and kidneys; examples include angiotensin II-mediated hypertension, DOCA-salt hypertension, mineralocorticoid hypertension, aortic banding induced hypertension, renovascular hypertension and endothelin-induced hypertension (Beswick et al, 2001; Bouloumie et al, 1991; Grunfeld et al, 2003; Heitzer et al, 1999; Higashi et al, 2002; Landmesser et al, 2003; Mollnau et al, 2002; Rajagopalan et al, 1996; Somers et al, 2000). The best characterized ROS in tissues of hypertensive individuals is superoxide anion (O_2^-). Reduction in O_2^- formation can lower blood pressure in some experimental models of hypertension (Beswick et al, 2001, Chen et al, 2001, Duffy et al, 1999; Onuma and Nakanishi, 2004; Schnackenberg and Wilcox, 1999), suggesting that increased production of ROS is an etiologic factor in hypertension. O_2^- can increase blood pressure by several mechanisms. In the vasculature O_2^- causes vasoconstriction, in part by inducing endothelial cell dysfunction (Cai and Harrison, 2000). Increased O_2^- in the kidney is associated with enhanced tubular reabsorption of sodium and water (Majid and Nishiyama, 2002). In key brain regions, increased O_2^- leads to increased sympathetic nervous system activity (SNA) (Campese et al, 2004; Zanzinger and Czachurski, 2000; Zimmerman et al, 2002; 2004). The focus of the work to be reported here, however, is on the peripheral sympathetic nervous system. We previously presented evidence that

O_2^- enhances peripheral sympathetic neurotransmission and that this action is accentuated in rats with DOCA-salt hypertension (Xu et al, 2001; 2002).

The DOCA-salt model of experimental hypertension depends in part on the activity of the endothelin (ET) system (Lariviere et al, 1993). Considerable evidence indicates that ET can both stimulate the formation of O_2^- (Li et al, 2003; Majid and Nishiyama, 2002) and increase SNA (Boarder et al, 1991; Damon, 1998; Giaid et al, 1989; Tabuchi et al, 1989). Furthermore, several studies have found that antioxidants (Duffy et al, 1999; Onuma and Nakanishi, 2004; Schnackenberg and Wilcox, 1999; Xu et al, 2004) or a reduction in SNA (Mortensen, 1999) can attenuate the hypertensive effects of ET. A critical finding for the present study was the observation by Dai et al (2004) that sympathetic neurons in peripheral ganglia contain O_2^- and that the content of O_2^- is significantly increased in ganglia from DOCA-salt rats. They went on to test for a possible influence of ET on O_2^- production by sympathetic ganglia. Although ET generally increases O_2^- levels by stimulating the ET_A receptor subtype (Callera et al, 2003), Dai *et al* (2004) showed that ET increases O_2^- levels in sympathetic ganglia by activating ET_B receptors. They also reported that the expression of ET_B receptors is higher in ganglia from hypertensive DOCA-salt rats than in normotensive control rats. Their findings suggest that ET may increase SNA in DOCA-salt hypertension through an action at ET_B receptors located on cell bodies of post-ganglionic sympathetic neurons.

We and others have shown that infusion of the selective ET_B receptor agonist sarafotoxin 6c (S6c) into conscious rats results in an increase in blood

pressure (Lau et al, 2004; Moreland et al, 1994). Though ET_B receptors are known to cause transient hypotension by the release of vasodilatory peptides, nitric oxide and prostacyclin (Gomez-Alamillo et al, 2003), they also function as clearance receptors to remove circulating ET-1 (Fukuroda et al, 1994). Blockade of ET_B receptors increases blood pressure presumably by decreasing bioavailability of ET-1 in the circulation, thus potentiating activation of ET_A receptors (Just et al, 2005; Pollock, 2000; Reinhart et al, 2002). The present study was designed to determine whether activation of ET_B receptors *in vivo* increases O_2^- levels in sympathetic ganglia. To this end, we infused S6c into rats and measured the amount of O_2^- production in sympathetic ganglia using the dihydroethidium oxidative fluorescence method. To test whether any changes in O_2^- levels in ganglia are a consequence of hypertension or of direct ET_B receptor activation in ganglia, we also examined superoxide production in response to elevated blood pressure induced by the α adrenergic agonist phenylephrine (PE).

MATERIALS AND METHODS

Animals

Adult, male Sprague-Dawley rats (200-350g; Charles River Laboratories, Portage, ME) were assigned to either of two experimental protocols: in vivo or in vitro. All animals were fed standard rat chow and had *ad libitum* access to both food and water. Animal procedures were in accordance with the institutional guidelines of the Michigan State University

In Vivo Studies

In rats under sodium pentobarbital (50 mg/kg, ip) anesthesia, catheters were positioned in the abdominal aorta via the left femoral artery for continuous hemodynamic monitoring and in the femoral vein for drug administration. Rats were then housed in standard stainless steel metabolic cages for the duration of the study. Free ends of the catheters exited the cage through a stainless steel tether connected to the rat by a plastic harness around the thorax. After 2-3 days of surgical recovery, rats were subjected to one of three different treatments: they received iv infusions of either 1) the specific ET_B receptor agonist sarafotoxin 6c (S6c; 5 pmol/kg/min; American Peptide, Sunnydale, CA), 2) isotonic saline at 0.01 ml/min (control), or 3) the alpha-adrenoceptor agonist phenylephrine (PE; 10 μg/kg/min; Sigma-Aldrich Corp, St. Louis, MO) for 2 h. Blood pressure measurements were obtained continuously throughout the protocol without disturbing the animal. Immediately after systemic infusion, animals were euthanized with pentobarbital (100 mg/kg iv) and the inferior mesenteric ganglion (IMG) was excised for superoxide measurement.

In Vitro Studies

Animals were sacrificed with a lethal dose of sodium pentobarbital (100 mg/kg, ip) and their IMG immediately harvested. To evaluate whether in vitro administration of agonist to the ganglia might affect levels of O_2^-, isolated IMG were incubated with varying concentrations of PE (1 μM to 100 μM) for 30 min at 37°C. Another set of IMG were treated with S6c (10^{-8} mol/L; as described in Dai

et al, 2004) for 30 min at 37°C to serve as positive control, while negative control IMG received no treatment.

Superoxide assay

Ganglionic O_2- production was assessed by oxidative dihydroethidium fluorescence method as previously described (10). In brief, IMG were incubated with the oxidant sensitive probe dihydroethidine (DHE; 2 μmol/L; Molecular Probes) for 45 min at 37°C. The levels of O_2- were assayed by measuring the fluorescence signal intensity resulting from intracellular oxidation of the DHE to fluorescent ethidium by O_2^-. The fluorescent intensity is proportional to O_2^- levels. The fluorescent signal (excitation: 514 nm; emission: 560 nm) was measured with a confocal microscope and analyzed using ImageJ Software (U. S. National Institutes of Health, Bethesda, MD). Because DHE fluorescence measurements only provide semi-quantitative information, the assay was performed on control groups of animals alongside the treatment groups for every experiment using the same parameters, i.e. animals were sacrificed and tissue harvested at (approximately) the same time, on the same day, using the same reagents, on the same microscope and software—thus, providing a baseline standard for each comparison. Larger cells (20-35 μm) were identified as neurons while smaller cells in the periphery (5-10 μm) were identified as glia.

Data Analysis

All data were expressed as mean ± SEM. Statistical significance was assessed with one-way ANOVA with Tukey's *post-hoc* test using Prism 3.0 Software (GraphPad, Inc). Paired t-tests were used to compare blood pressure values before and after treatment. A value of $P<0.05$ was considered significant.

RESULTS

Effect of in vivo S6c infusion on MAP and O_2^- production

S6c infusion for two hours in conscious rats significantly increased blood pressure. MAP (the difference between the 2 hr value and the initial value) increased 26.6±1.7 mmHg in the S6c treated rats (N=6) and 3.6±6.0 mmHg in control rats (N=5) (Figure 20). S6c infusion also significantly augmented O_2^- levels in both neurons and glial cells of the IMG when compared to control rats. DHE fluorescence measured by average pixel intensity in the ganglionic neurons and surrounding glial cells was 96.7% and 160% greater in S6c than in control rats, respectively (Figure 21).

Effect of increased MAP on O_2^- production

To determine if the alteration in O_2^- levels observed in rats receiving S6c was a direct effect of ET_B receptor activation on sympathetic ganglia or an indirect consequence of hypertension, in a separate study rats received either S6c, PE (at a dose chosen to mimic the pressor response to S6c) or isotonic saline treatments. MAP increased 29.9±0.1 mmHg in S6c, 31±1.2 mmHg in PE and

1.7±1 mmHg in control rats (Figure 22). As observed in the previous experiment, in vivo infusion of S6c increased the DHE fluorescence intensities of ganglionic neurons and surrounding glial cells significantly greater than control rats, 215.5% and 197.6%, respectively. Fluorescence intensities of ganglia from PE rats were also significantly greater than controls, 137.7% in neurons and 104.6% in glia, but significantly lower than in ganglia from S6c rats (Figure 23).

Effects of PE on ganglionic cells

To determine whether PE acts directly on ganglia to increase O_2^- levels, we incubated freshly dissociated inferior mesenteric ganglia from normal rats with either PE at 1 μM and at 100 μM or with S6c at 10^{-8} M. Results (Figure 24) show that PE has little direct effect *in vitro* on O_2^- levels in sympathetic ganglia, whereas S6c produced a large increase.

DISCUSSION

The main new finding of this study is that activation of ET_B receptors *in vivo* increases O_2^- levels in sympathetic ganglia. Our observation is consistent with an earlier report that ET peptides stimulate O_2^- production in sympathetic ganglion neurons *in vitro* by activating ET_B receptors (Dai et al, 2004). In the current study we also confirmed that previous finding.

The ability of ET_B receptor activation to increase O_2^- levels in sympathetic ganglia *in vivo* may be due to both direct and indirect mechanisms. Experiments performed in sympathetic ganglia *in vitro* show that ET can increase O_2^- levels by stimulating NAD(P)H oxidase (Dai and Kreulen, *in press*). This mechanism also appears to account for the increased O_2^- levels measured in sympathetic ganglia from DOCA-salt rats (Dai and Kreulen, *in press*). In the current study we did not test whether activation of NAD(P)H oxidase contributes to elevated O_2^- levels in rats receiving acute infusions of S6c.

Growing evidence points to the possibility that hypertension per se can increase O_2^- levels in various tissues (DeLano et al, 2005; Ungvari et al, 2003, 2004), although other studies indicate hypertension is not invariably associated with increased O_2^- levels (Rajagopalan et al, 1996). Therefore, to test the hypothesis that S6c increases O_2^- levels in sympathetic ganglia in part by elevating blood pressure, we infused PE (10 μg/kg/min) into conscious rats to produce an increase in blood pressure similar to that observed during S6c infusion. Additional rats received either S6c or saline infusions in order to allow direct comparison of DHE fluorescence with the three stimuli. The results

confirmed our previous experiment showing that DHE fluorescence intensities of ganglionic neurons and surrounding glial cells were significantly greater in rats receiving S6c than in control rats. Interestingly, PE infusion also produced O_2^- levels that were significantly greater than those observed in saline control animals. It is important to note however that they remained significantly less than those found in S6c infused animals. To determine if PE has any direct effect on superoxide anion levels, we performed an additional study in freshly dissociated rat inferior mesenteric ganglionic neurons and glial cells *in vitro*. We found that application of PE did not induce a significant increase in superoxide anion levels in either neurons or glial cells. We conclude that an acute increase in blood pressure alone can cause elevated O_2^- levels in sympathetic ganglia, although it is possible that some other physiological response to PE infusion is responsible. Overall then these data indicate that S6c infusion *in vivo* may increase O_2^- levels in sympathetic ganglia by both direct (stimulation of ET_B receptors on neurons and glia) and indirect (pressure-dependent) mechanisms. The indirect mechanism may play a predominant role.

Our findings demonstrate for the first time that *in vivo* ET_B receptor activation increases O2- anion levels in sympathetic ganglia. Although the actions of O2- in the vasculature, kidney and brain have been well described, its role in the peripheral sympathetic nervous system is less well characterized. We previously presented evidence that O_2^- enhances peripheral sympathetic neurotransmission and that this action is accentuated in rats with DOCA-salt hypertension (Xu et al, 2001, 2002). Others have confirmed that finding in

spontaneously hypertensive rats (Shokoji et al, 2003). They suggested that a potential mechanism of O_2^- action on sympathetic neurotransmission is by affecting voltage-gated potassium channels in sympathetic nerve fibers (Shokoji et al, 2004). Another possibility is that O_2^- may reduce the bioavailability of nitric oxide in sympathetic ganglia (Ceccatelli et al, 2001). Nitric oxide can alter potassium currents in sympathetic ganglion neurons (Browning et al, 1998) and the effects of nitric oxide on ganglionic neurotransmission are generally inhibitory (Quinson et al, 2000). Furthermore, it has been shown that activation of ET_B receptors in sympathetic ganglia causes an increase in nitric oxide which acts to inhibit nicotinic transmission through the ganglion (Yamada et al, 1999). Generation of O_2^- in response to ET_B receptor activation might then moderate the inhibitory action of nitric oxide, i.e. enhance neurotransmission through the ganglion.

Alternatively, prolonged oxidative stress due to elevated O_2^- levels in sympathetic ganglia could impair ganglionic transmission by hastening apoptosis of post-ganglionic sympathetic neurons (Jordan et al, 1995; Tammariello et al, 2000). Currently, however, there is no evidence for decreased neurotransmission through sympathetic ganglia in animals with DOCA-salt or other forms of ET dependent hypertension.

Elevated superoxide (O_2^-) anion concentrations in sympathetic ganglia may participate in the pathogenesis of endothelin (ET) dependent hypertension by facilitating nicotinic neurotransmission through the ganglion. Sympathetic

ganglia represent a potential target for antioxidant-based therapy of hypertension and other cardiovascular diseases.

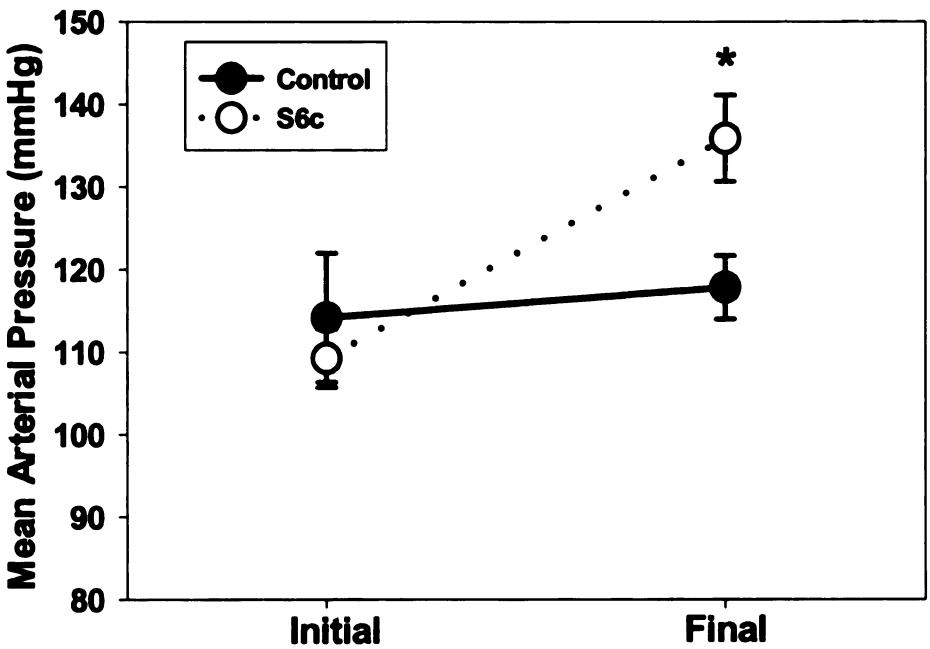


Fig 20. S6c infusion for two hours in conscious rats significantly increased blood pressure. MAP increased 26.6±1.7 mmHg in the S6c treated rats (N=6) and 3.6±6.0 mmHg in control rats (N=5). * = statistically significant, $P<0.05$

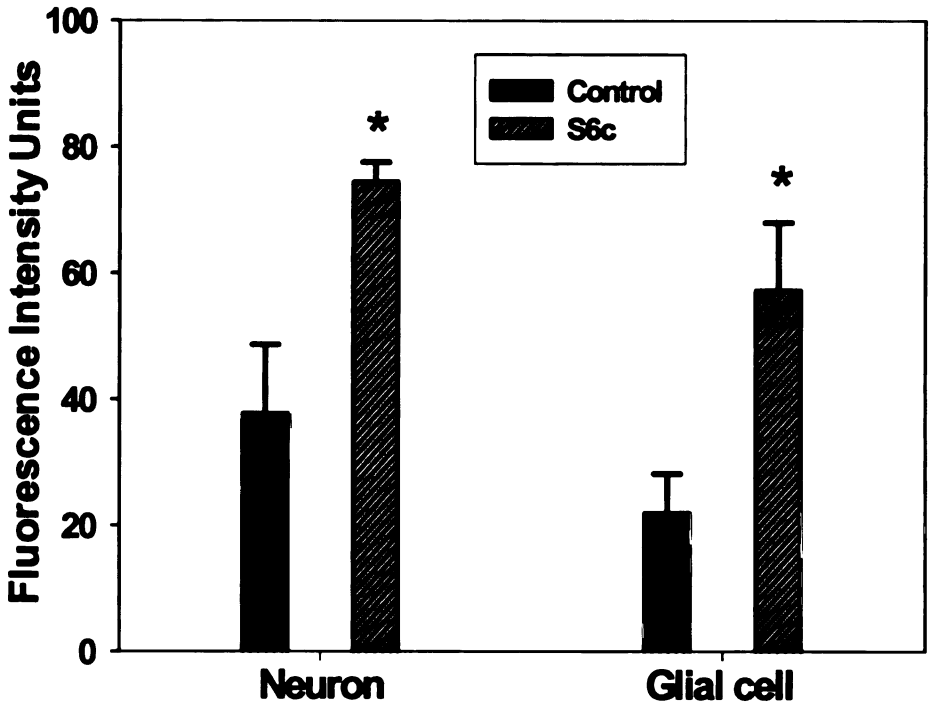


Fig 21. O_2- levels in IMG of S6c and control rats. DHE flourescence intensities of neurons and glial cells were quantified and the mean values are shown, which were 96.7% and 160% greater in S6c (N=6) than in control rats (Control; N=5), respectively. *=statistically significant, $P<0.05$

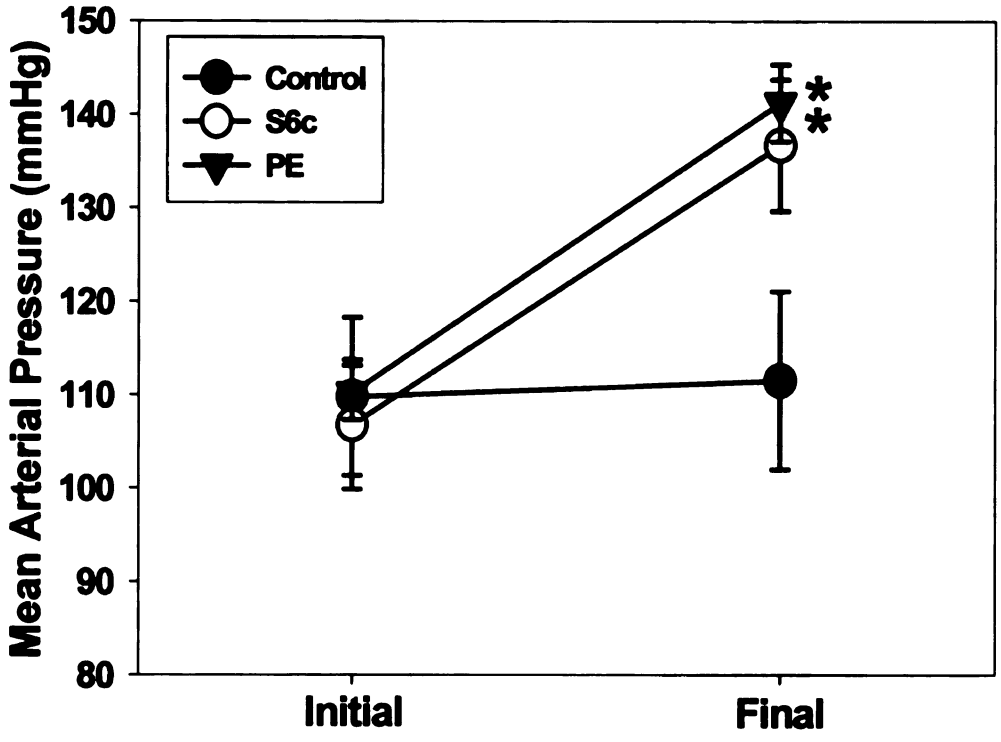


Fig 22. Two hour MAP measurement during systemic infusion. To determine if the alteration in O_2- levels observed in rats receiving S6c was a direct effect of ET_B receptor activation on sympathetic ganglia or an indirect consequence of hypertension, in a separate study rats received either S6c (N=5), PE (N=5) or isotonic saline treatments (N=5). MAP increased 29.9±0.1 mmHg in S6c, 31±1.2 mmHg in PE and 1.7±1 mmHg in control rats. *=statistical significance, $P<0.05$

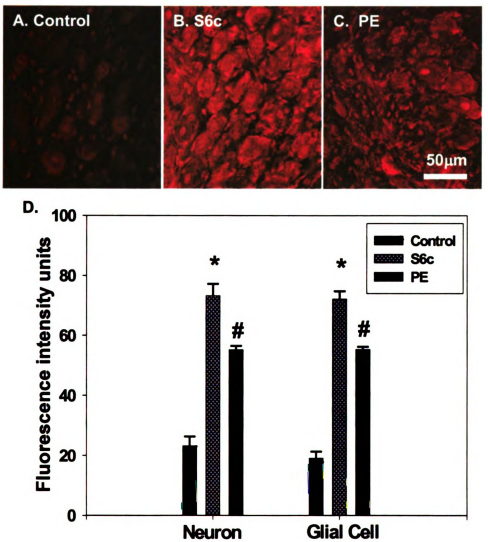


Fig 23. Confocal photomicrographs of O_2- expression in rat IMG following 2 h infusions of (A) isotonic saline, (B) S6c, or (C) PE. D, In vivo infusion of S6c increased the DHE fluorescence intensities of ganglionic neurons and surrounding glial cells significantly greater than control rats, 215.5% and 197.6%, respectively (*) while fluorescence intensities of ganglia from PE rats were also significantly greater than controls, 137.7% in neurons and 104.6% in glia, but significantly lower than in ganglia from S6c rats (#). Bar=50μm. $P<0.05$.

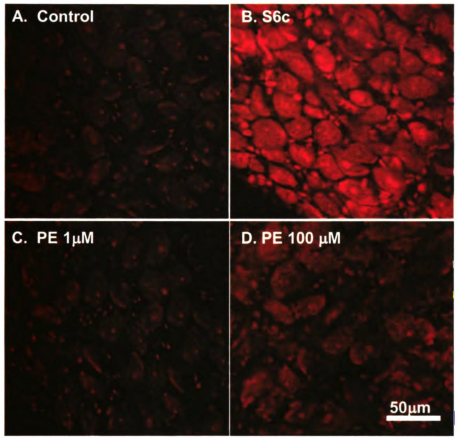


Fig 24. ET_B receptor activation but not PE elevates O2- production in IMG in vitro. Confocal fluorescent photomicrographs show superoxide expression in freshly dissociated IMG following (A) isotonic saline, (B) S6c, (C) 1μM PE and (D) 100μM PE. Bar = 50μm

CHAPTER 4

Part II

ET_B receptor activation increases blood pressure and sympathetic ganglionic O_2^- production in the presence of ganglionic and adrenergic blockade

INTRODUCTION

Reactive oxygen species, especially superoxide (O_2-) anions, have been increasingly implicated in the pathogenesis of hypertension both in experimental animal models (Kerr et al, 1999; Lerman et al, 2001; Wilcox 2002) as well as clinically (Touyz and Schiffrin 2001; Romero and Reckelhoff 1999). Elevated O_2- production has been found in deoxycorticosterone acetate (DOCA) salt hypertensive rats (Dai et al, 2004; Wu et al, 2001; Somers et al, 2000), spontaneously hypertensive rats (SHR) and stroke-prone SHR (Zalba et al 2000; Kerr et al 1999) and angiotensin (Ang) II hypertension (Nishiyama et al, 2001; Mollnau et al, 2002; Rajagopalan et al, 1996). The superoxide dimutase (SOD) mimetic, tempol, which scavenges O_2-, has been shown to be effective in lowering blood pressure and renal vascular resistance in SHR (Schnackenberg and Wilcox, 1999) and significantly decreased urinary excretion of 8-isoprostanglandin F2α (Schnackenberg et al, 1998; Schnackenberg and Wilcox, 1999), a marker of oxidative stress. Tempol decreased vascular O_2- production and lowered MAP in Ang II hypertension (Nishiyama et al, 2001). Chronic treatment with tempol has been reported to decrease blood pressure and prevent renal injury in DOCA salt hypertensive rats (Beswick et al 2001) as well as inhibit vascular remodeling in salt-loaded stroke-prone SHR (Park et al, 2002). Furthermore, recent reports indicate that the antihypertensive effect of tempol is at least partly mediated by inhibition of the sympathetic nervous system (Xu et al, 2001, 2004; Shokoji et al 2003).

The sympathetic nervous system is crucial to the regulation of blood pressure and its hyperactivity has been implicated in the pathogenesis of hypertension (Mark 1996; Esler 2000; Wyss 1993). Zanzinger and Czachurski (2000) reported that microinjection of SOD, an enzyme that removes O_2- radicals, into the rostral ventral lateral medulla (RVLM), which is the vasomotor center regulating sympathetic nervous activation (SNA), produced tonic inhibitory effects on baseline sympathetic tone, suggesting that sympathoexcitation by O_2- contributes significantly to basal SNA. Recent studies show that systemically administered tempol also exerts parts of its antihypertensive effect through actions on the sympathetic nervous system. In DOCA salt rats, administration of tempol by intravenous infusion but not by intracerebroventricular injection decreased MAP and heart rate and reduced renal SNA, effects not mediated by the quenching of nitric oxide bioavailability (Xu et al 2001). The finding that tempol directly inhibited peripheral SNA, possibly through inhibition of sympathetic neurotransmission, suggests that O_2- exerts its hemodynamic effects through facilitation of peripheral sympathetic neuroeffector transmission.

The potent vasoconstrictor, endothelin (ET), has been shown to increase O_2- production in the vasculature (Li et al, 2003) and can also increase SNA (Boarder and Marriott, 1991; Damon 1998; Giaid et al 1989; Tabuchi et al, 1989). Dai et al (2003) found that ET acting on endothelin type B (ET_B) receptors (ETBR) increases O_2- in postganglionic sympathetic neurons innervating the splanchnic circulation. In a previous study, we presented evidence that *in vivo* stimulation of ETBR induced an acute rise in MAP accompanied by increased

O_2^- production in prevertebral sympathetic postganglionic neurons and surrounding glial cells. The mechanism by which ETBR activation increases O_2^- production and blood pressure is unclear. Recent studies suggest that elevated O_2^- production by ETBR activation in sympathetic ganglia may increase blood pressure (Xu et al, 2001; 2004). Our objective in the present study was to determine if ET induced elevations in local O_2^- concentration facilitates nicotinic neurotransmission through sympathetic ganglia, thereby increasing sympathetic activity and blood pressure. We used chlorisondamine (CHL), a long-acting nicotinic acetylcholinergic receptor antagonist, to block central input to autonomic ganglia. CHL has been shown to be effective in producing long term , non-competitive ganglionic blockade (Chadman and Woods, 2004; Wang et al, 2005). Furthermore, we examined whether the acute hypertension during ETBR activation is caused by increased sympathetic innervation of the vasculature via catecholamine release at the neuroeffector junction. We measured acute blood pressure responses to ETBR stimulation in the absence of peripheral sympathetic effects on the vasculature and heart by concomitant use of phentolamine and propranolol to block both α and β adrenergic receptors peripherally (Muntzel et al 1997).

MATERIALS AND METHODS

Animals

We used adult, male Sprague-Dawley rats (200-350g; Charles River Laboratories, Portage, ME) for all aspects of this study. All animals were fed standard rat chow and had *ad libitum* access to both food and water. Animal procedures were in accordance with the institutional guidelines of the Michigan State University

Catheterization

In rats under sodium pentobarbital (50 mg/kg, ip) anesthesia, catheters were placed in the abdominal aorta via the left femoral artery for continuous hemodynamic monitoring and in the femoral vein for drug administration. Rats were then housed in standard stainless steel metabolic cages for the duration of the study. The free end of the spring containing both arterial and venous catheters was attached to an exterior clamp outside the cage via a hydraulic swivel, allowing continuous access to catheters without direct handling or disturbance of the animal. Ticarcillin (10 mg/kg; SmithKine Beecham Pharmaceuticals, Philadelphia, PA) and enrofloxacin (2 mg/kg; Bayer) were administered daily via the venous catheter to prevent bacterial infection. Both catheters were flushed daily with heparinized saline (100u/ml; Sigma) to maintain patency. Mean arterial pressure (MAP) and heart rate (HR) were measured from the arterial catheter with a TXD-300 pressure transducer linked to a digital BPA-200 Blood Pressure Analyzer (Micro-Med, Louisville, Kentucky).

Chlorisondamine

After 3 days of surgical recovery, rats were assigned to one of three treatments: 1) 2h iv infusion of the specific ETBR agonist sarafotoxin 6c (S6c; 5 pmol/kg/min; American Peptide, Sunnydale, CA) only, 2) chlorisondamine (CHL; 5 mg/kg iv; Tocris, Ellisville, MO), followed by 2h S6c infusion, 3) CHL followed by 2h isotonic saline infusion (0.01 ml/min). Hemodynamic measurements were obtained continuously throughout the protocol without disturbing the animal. Immediately after systemic infusion, animals were euthanized with pentobarbital (65 mg/kg iv) and the inferior mesenteric ganglion (IMG) was excised for superoxide measurement as described previously (Part I). Briefly, these dissected ganglia were incubated with DHE (0.2 μmol/L; Molecular Probes) for 40 min before being mounted onto microscope slides and visualized under confocal microscopy. The fluorescence intensity level is proportional to the amount of O_2-. Microscope images were analyzed using ImageJ software (NIH) to measure fluorescence intensity of individual cells.

αβ– adrenergic blockade

Catheterized rats were subjected to one of three treatments: they received 1) a single bolus injection of αβ adrenergic antagonists (AB) consisting of phentolamine (5 mg/kg iv; Sigma-Aldrich Corp, St. Louis, MO) and propranolol (3 mg/kg iv; Sigma), 2) AB injection followed by 2h S6c infusion, 3) AB injection followed by 2h isotonic saline infusion. BP and HR were measured throughout

the protocol. At the end of infusion, all groups were euthanized with a lethal dose of sodium pentobarbital (65 mg/kg iv).

Data Analysis

All data were expressed as mean ± SEM. Statistical significance was assessed with one-way ANOVA with Tukey's *post-hoc* test using Prism 3.0 Software (GraphPad, Inc). Paired t-tests were used to compare blood pressure values before and after treatment. A value of $P<0.05$ was considered significant.

RESULTS

Chlorisondamine on blood pressure and O_2^- production

Administration of CHL iv decreased MAP by 37.5 $\pm$ 2.4 mmHg. After allowing 10 min for blood pressure to stabilize, either S6c or saline was infused. Rats that received S6c only (without CHL pretreatment) (N=4) increased blood pressure 39.9 $\pm$ 5.9 mmHg. Subsequent S6c (N=5) and saline (N=5) infusions following CHL increased MAP 56.7 $\pm$ 2 mmHg and 11.32 $\pm$ 4.4 mmHg, respectively (Figure 25).

Both S6c only and CHL-S6c infusion significantly augmented O_2^- levels in both neurons and surrounding satellite cells of the rat IMG when compared to CHL-saline (control) infusion. DHE fluorescence measured by average pixel intensity in the ganglionic neurons and satellite cells were 296.3% and 337.7%

greater than controls respectively in S6c-only group, and 294.9% and 324.9% respectively in CHL-S6c group (Figure 26).

Effect of αβ adrenergic antagonists on blood pressure

Figure 27 shows the effect of combined αβ adrenergic antagonists (AB) on ET dependent MAP. Administration of adrenergic blockade AB caused an initial transient surge in MAP in all three treatment groups followed by a more prolonged depressor response lasted almost the duration of subsequent S6c or saline infusion. AB injection decreased MAP by an average of 25 ± 5.1 mmHg. In the AB only treatment group, MAP gradually increased back to baseline blood pressures after 90 min. Similarly, blood pressures of rats that received a 2h infusion of isotonic saline after AB pretreatment also increased 15 ± 9.1 mmHg to within baseline levels by 90 min. Infusion of S6c for 2 h following AB pretreatment increased MAP 43.3 ± 3.8 mmHg.

DISCUSSION

The objective of the present study was to determine if elevated local O_2- anion concentration produced by the activation of ETBRs facilitates nicotinic neurotransmission through sympathetic ganglia, thereby increasing SNA and the subsequent release of catecholamines from the neuroeffector junction to raise blood pressure. Previously, we demonstrated that *in vivo* ETBR activation increases O_2- anion levels in sympathetic ganglia and speculated that generation of O_2^- in response to ETBR activation might then enhance sympathetic neurotransmission through the ganglion. Studies in DOCA salt hypertension as well as SHR suggest that O_2^- facilitates peripheral sympathetic neuroeffector transmission (Xu et al, 2001; 2004; Shokoji et al, 2003), possibly by acting on voltage-gated potassium channels in sympathetic nerve fibers (Shokoji et al, 2004). Another possible mechanism is that O_2^- may react with nitric oxide (NO) to form peroxynitrate, reducing the bioavailability of NO in peripheral sympathetic ganglia (Ceccatelli et al, 1984). NO has been shown to exert an inhibition on nicotinic transmission of ganglionic neurons (Quinson et al, 2000). Yamada et al (1999) reported that increased NO by activation of ETBR causes presynaptic inhibition on ganglionic neurotransmission. Hence, decreased NO may enhance the excitability of sympathetic ganglia, although recent data indicate that effects of vascular O_2- on blood pressure and SNA are NO-independent (Xu et al, 2002, 2004; Fink et al, 2000).

Our current findings confirm results of our previous study showing significantly increased O_2- production and blood pressure following ETBR

activation by intravenous S6c infusion. Chlorisondamine (CHL), a nicotinic receptor antagonist, was used to evaluate the effect of central autonomic input on ganglionic O_2- and MAP following ETBR activation. A novel finding is that the blood pressure response and increased O_2- production during ETBR activation persisted in the presence of the ganglion blockade. Rats that received S6c after pretreatment with chlorisondamine had a pressor response that was even greater in magnitude than those that did not receive CHL. These two treatment groups produced similar increases in ganglionic O_2- levels, suggesting that neither the acute pressor effects of S6c nor the associated oxidative stress in the ganglia are caused by alterations in nicotinic neurotransmission. Since ETBR activation increases blood pressure and sympathetic ganglionic O_2- production in the absence of nicotinic ganglionic neurotransmission, we conclude that S6c does not cause hypertension by binding to central ETBR to produce sympathetic nervous activation.

The increase in ganglionic O_2- may be a reflection of the pressor response itself since hypertension in many forms have been associated with its production (Ungvari et al, 2004), including angiotensin II-mediated hypertension (Rajagopalan et al, 1996), DOCA-salt hypertension (Dai et al, 2004; Beswick et al 2001; Somers et al, 2000), aortic banding induced hypertension (Boulomie et al, 1997; Ungvari et al, 2003, 2004) renovascular hypertension (Higashi et al, 2002; Heitzer et al, 1999) and endothelin-induced hypertension. In an earlier study, we found that acute hypertension caused by phenylephrine infusion also produced an increase in ganglionic O_2- levels. These studies support our

conclusion that O_2- does not play a causative role in the blood pressure response to acute ETBR activation via increased sympathetic neurotransmission.

Conversely, elevated local O_2- concentration may instead impair sympathetic neurotransmission (Tammariello et al 2000; Jordan et al, 1995). It is well known that reactive oxygen species are integral to programmed cell death. Tammariello et al (2000) reported that NADPH oxidase, which transfers electrons from NADPH to molecular oxygen to produce O_2- (DeLeo and Quinn, 1996), increases oxidative stress and apoptosis in sympathetic neurons. Furthermore, superoxide dismutase (SOD), which removes O_2-, has been shown to delay or inhibit sympathetic neuronal apoptosis (Jordan et al, 1995; Greenlund et al, 1995). Overall, these studies suggest that prolonged increase in O_2- levels in sympathetic ganglia could inhibit neurotransmission by prompting apoptosis in postganglionic neurons. The vast majority of literature on neuronal cell death in the autonomic nervous system is confined to embryonic sympathetic or sensory ganglia or PC-12 cells during development. Though adult neurons are not normally associated with the cellular process of apoptosis, studies on mental degenerative disease models provided some evidence to the contrary. In several forms of familial amyotrophic lateral sclerosis (ALS), apoptosis in adult motor neurons has been found to be associated with mutations in SOD type 1 (Deng et al, 1993; Rosen et al, 1993). Using a rat ALS model, Martin et al (2005) reported that apoptosis in adult motor neurons is mediated by NO. Furthermore, apoptotic cell death has been observed in sympathetic neurons in brains of Alzheimer's disease patients (Pedraza et al, 2005). Chan et al (2005) found that

endotoxin-induced apoptosis in sympathetic premotor neurons of the RVLM in adult rats is associated with O_2- and NO dependent mitochondrial signaling. Taken together, these data support a possible role for O2- in modulating cell death in sympathetic ganglionic neurons.

We also tested the hypothesis that ETBR activation directly activates post-ganglionic sympathetic neurons innervating the vasculature to produce vasoconstriction and hypertension. We found that combined α and β adrenergic receptor blockade with phentolamine and propranolol did not prevent the acute blood pressure response to S6c infusion. This finding is consistent with the CHL data indicating that the pressor response to acute ETBR activation does not require sympathetic activation.

We proposed and confirmed in Specific Aim I that one mechanism whereby acute in vivo ETBR activation exerts its hypertensive effects involves peripheral venoconstriction and blood volume centralization. This point is highlighted by another study from our laboratory suggesting that the initial response to acute S6c infusion involves direct constriction of the vasculature smooth muscle cells while the response to long-term S6c infusion was mediated by a neurogenic mechanism, possibly increased sympathetic nervous activation (unpublished data) (Figure 15).

Collectively, our results show that ETBR activation increases blood pressure and sympathetic ganglionic O_2^- production in the absence of nicotinic ganglionic neurotransmission. These findings further support our hypothesis in

Specific Aim 1 that direct venoconstriction by S6c infusion is the primary mechanism whereby acute ETBR activation produces hypertension.

Though this is the most likely conclusion of our data, there are other possible explanations. Our finding that increased blood pressure and superoxide production persisted after nicotinic and adrenergic blockade may be attributed to substances other than acetylcholine (ACh) and NE acting at synaptic and neuroeffector junctional sites, respectively. Accumulating evidence shows that a vast majority of neurons in the peripheral nervous system contain more than one neurotransmitter with potential consequence at the effector site (Bartfai et al, 1988; Elfvin et al, 1993). Following stimulation of the preganglionic neuron, the corelease of a host of other non-adrenergic, non-cholinergic chemical messengers such as peptides and purines has been demonstrated that may function as primary transmitters, cotransmitters, or neuromodulators. Some of these substances that are shown to have potential transmitter function include ATP, vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY).

The coexistence of ATP and ACh in cholinergic vesicles (Dowdall et al, 1974) and the corelease of ATP with NE in adrenergic nerve terminals innervating the vasculature (Sneddon and Westfall, 1984) have long been known. ATP and its metabolite, adenosine, may have a significant function in synaptic transmission by acting on different subtypes of purinergic receptors (Burnstock,1969; 1986), which are differentially expressed on arterial and venous smooth muscle cells (Galligan et al, 2001).

There is also evidence that adenosine can act as a neuromodulator in the release of NE since administration of adenosine receptor antagonists have been shown to increase plasma NE and DBH concentrations (Crubeddu et al, 1975; Yoneda et al, 1990).

The peptide, VIP, has been detected in peripheral autonomic neurons alongside ACh, suggesting the possibility of cotransmission. Though VIP has not been reported in preganglionic nerve fibers that synapse on postganglionic neurons in the sympathetic nervous system, its colocalization with ACh has been demonstrated in parasympathetic fibers that innervate blood vessels and cholinergic sympathetic neurons that innervate sweat glands (Lindh and Hokfelt, 1990). The role of VIP and ACh working synergistically to stimulate vasodilation and sphincter relaxation has been implicated (Fahrenkrug, 1998).

Following sympathetic nerve stimulation, the corelease of NPY and NE have been demonstrated (Grundemar and Hakanson, 1994; Wahlestedt and Reis, 1993). In the periphery, NPY has been found in sympathetic nerve fibers and is involved in the maintenance of vascular tone. It has potent and prolonged vasoconstrictor action and appears to work synergistically with NE (Zhao et al, 2006; Racchil et al, 1999).

Other candidate peptides including enkephalins, substance P, somatostatin, gonadotopin-releasing hormone, cholecystokinin, calcitonin gene-related, galanin, may also be involved. Physiological experiments have shown that enkephalins may presynaptically inhibit cholinergic transmission in sympathetic ganglia (Lindh and Hokfelt, 1990).

To determine whether the pressor response induced by circulating S6c following ganglionic and/or adrenergic blockade is attributed to these non-adrenergic, non-nicotinic candidate substances, we can assess each substance with the following four measures: 1) determine the presence of the substance in the preganglionic/postganglionic nerve fiber by autoradiographic localization or immunocytochemistry; 2) determine if the substance is present in the venous or perfusion effluent after preganglionic stimulation. 3) determine whether exogenous application of the substance can elicit the same pressor response as S6c in the presence of ganglionic and/or adrenergic blockade; 4) determine whether specific antagonism of the substance can abolish the blood pressure increase induced by S6c.

Although, the actions of ACh and NE still provide the essential constitution of synaptic neurotransmission in the autonomic nervous system while the above substances only account for a minor fraction of neurotransmission, nevertheless, these candidate substances may potentially contribute and should be taken into consideration.

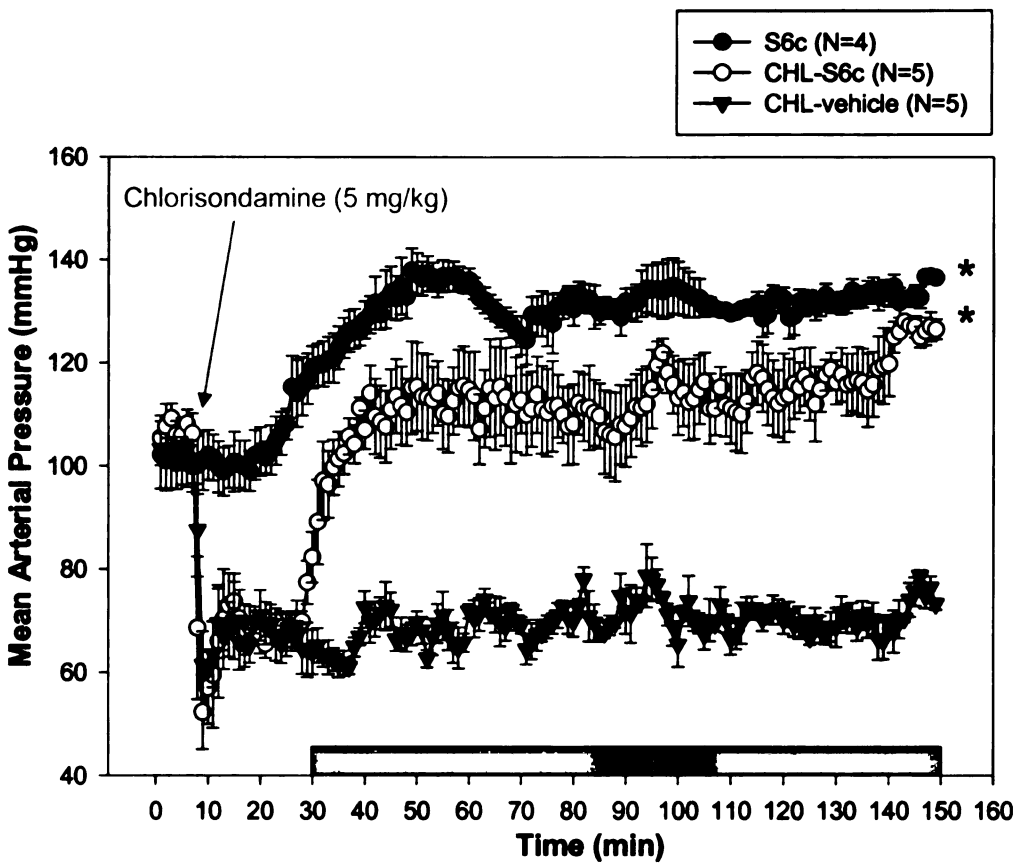


Fig 25. Effect of chlorisondamine pretreatment on MAP of rats receiving S6c infusion. Sprague-Dawley rats were infused with either isotonic saline or 5 pmol/kg/min S6c for 2 hours after CHL bolus (5 mg/kg iv). Initial and final MAP are recorded. After 2 hours of infusion, S6c increased blood pressure 39.9 $\pm$ 4mmHg, while CHL lowered MAP 37.5$\pm$ 4 mmHg. Subsequent S6c and vehicle infusions following CHL increased MAP 56.7 $\pm$ 0.06 mmHg and 11.32 $\pm$ 0.7 mmHg, respectively * = significance, $P<0.05$.

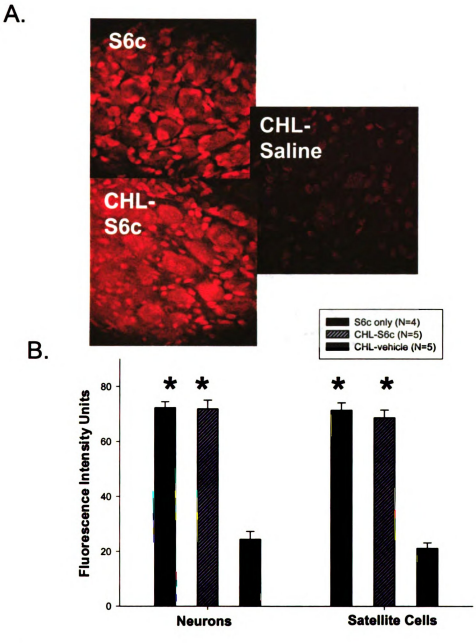


Fig 26. Superoxide generation in IMG following infusion and stained with dihydroethidine (DHE). Fluorescence confocal photomicrographs, showing in situ O2- detected in rat IMG(A). The DHE fluorescence intensities of ganglionic neurons and surrounding glial cells were significantly greater in both S6c and CHL-S6c rats compared to CHL vehicle infused rats (B). S6c infusion alone increased O2- in neurons and satellite cells by 293.3% and 336.3%, respectively. S6c infusion following CHL treatment increased O2- 294.9% in neurons and 324.9% in surrounding satellite cells compared to vehicle infusion. Bar = 25 μm, * = statistically significant, $P<0.05$.

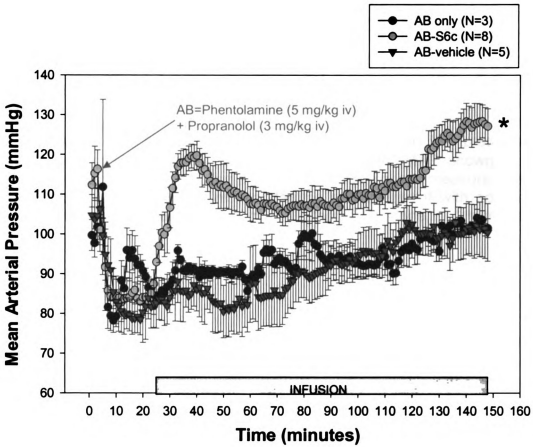


Fig 27. Effect of combined αβ adrenergic antagonists (AB) on S6c induced hypertension. Administration of AB caused an initial transient surge in MAP in all three treatment groups followed by a more prolonged depressor response lasted almost the duration of subsequent S6c or saline infusion. AB injection decreased MAP by an average of 25 ± 5.1 mmHg. In the AB only treatment group, MAP gradually increased back to baseline blood pressures after 90 min. Similarly, blood pressures of rats that received a 2h vehicle infusion after AB pretreatment also increased 15 ± 9.1 mmHg to within baseline levels by 90 min. Infusion of S6c for 2 h following AB pretreatment increased MAP 43.3 ± 3.8 mmHg.

	No Pretreatment	Chlorisondamine	α and β Adrenergic Blockade
Vehicle	2.7 ± 2.1	11.32 ± 0.7	15 ± 9.1
S6c	39.9 ± 4 *	57.7 ± 0.1 *	43.3 ± 3.8 *

Table 2. Acute S6c infusion increased blood pressure in the presence of ganglionic and combined α and β adrenergic blockade. MAP shown as the difference between the final and the initial value in mmHg was measured for the 2 hour duration of treatment. Chlorisondamine and treatment with adrenergic blockers induced a greater increase in MAP than S6c alone. * significance $P<0.05$

Chapter 5

Central nervous system distribution of ET_B receptors: an immunohistochemical survey

INTRODUCTION

There is compelling evidence that supports a functional role for endothelin (ET) in the brain. Central nervous system (CNS) ET could be involved in the modulation of cardiorespiratory centers and the release of hormones that control fluid volume and blood pressure (Kuwaki et al, 1997). Mature ET and its precursors, ECE, and ET_A and ET_B receptors (ETBR)s all are detected at strategic sites in the CNS, especially those controlling autonomic functions (Kuwaki et al, 1997; Kedzierski and Yanagisawa, 1999). It has been demonstrated that ET acts directly on both neurons and glia, and exhibits neurotransmitter-like activity (Yoshizawa et al, 1989; Kuwaki et al, 1997). In addition, ET is released by primary explants of hypothalamic neurons (Rossi, 2003).

A higher concentration of ET is detected in the cerebrospinal fluid (CSF) than in plasma. One explanation may be that ET is acting as a hormone or neuromodulator in the circulating CSF to convey signals to regions of the brain involved in central autonomic control of various effector systems that maintain blood pressure and body fluid balance (Kuwaki et al, 1997; Kedzierski et al, 1999; Rossi 2003). ET in the blood or CSF may exert its actions by affecting neurons located in circumventricular organs (CVO)s, where there is a deficient blood brain barrier. Intracerebroventricular (icv) infusion of ET into the brain results in increased blood pressure and heart rate accompanied by cardiorespiratory changes (Kuwaki et al, 1997). ET may also play a crucial role

in development. It has been demonstrated that the interaction of ET-3 and ET_B is vitally important for development of tissue derived from migration of neural crest cells, such as enteric neurons as evidenced clinically in Hirshsprung's disease (Kuwaki et al, 1997).

ET receptors have been localized in the central nervous system. Studies using radiolabeled ET suggest the presence of ET receptors not only on blood vessels, but also on neural elements (Masaki et al, 1991; Simonson et al, 1990). Yamamoto et al (1997) reported dense immunoreactive labeling of ETBRs in the median eminence (ME) and organum vasculosum lateral terminalis (OVLT), both of which are CVOs. The same group also found light immunostaining in the ventral and periventricular regions of hypothalamus. In a recent study, Garrido and Israel (2004) identified dense ET binding sites in the subfornical organ (SFO) and ME, where ET stimulates the phosphoinositide (PI) signaling pathway through binding to ETBR.

Taken together, these studies show that ETBR are localized in the vasculature as well as the neural elements of the central nervous system, where they may play an important role in central autonomic control of blood pressure, neuromodulation and development. **The purpose of the present study was to determine if ETBR are localized in brainstem and hypothalamic forebrain regions involved in autonomic regulation of fluid homeostasis and blood pressure.**

Relevant areas we examined include the paraventricular nucleus (PVN) and supraoptic nucleus (SON), which play crucial roles in the regulation of

sympathetic outflow, oxytocin and vasopressin release as well as initiate the thirst response (Koizumi and Yamashita, 1978; Jhamandas et al, 1989; Jerova et al, 1993; Haselton et al, 1994), the nucleus tractus solitarius (NTS), caudal ventral lateral medulla (CVLM) and rostral ventral lateral medulla (RVLM). Both CVLM and NTS are part of brainstem afferent pathway and are critical to the integrity of the baroreflex (Willette et al, 1984; Agarwal et al, 1989; Li et al, 1991; Blessing, 1997). Primary afferent stretch receptor endings carried by the cranial nerves IX and X synapse on NTS neurons which then excite the CVLM. Activated CVLM neurons decrease sympathetic nervous activity through inhibitory projections to central vasomotor neurons of the RVLM (Minson et al, 1997). Other regions of particular focus were the CVOs, including the SFO, OVLT, ME and AP. The CVOs are midline structures bordering the 3rd and 4th ventricles and are unique areas of the brain that are outside the blood-brain barrier. These blood-brain barrier-deficient areas are recognized as important sites for communicating with the CSF and between the brain and peripheral organs via blood-borne substances. The PVN, SON, NTS, SFO, ME, OVLT, and AP are all important in maintaining blood pressure and body fluid homeostasis. Furthermore, we have shown previously that acute and chronic administration of the ETBR agonist S6c causes neuronal activation in any of these brain sites. The presence of ETBR there would suggest that one way in which circulating ET exerts its effects on the autonomic regulation of blood pressure is by acting directly on central receptors in the brain.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Portage, ME) weighing 250-300g were housed in temperature- and humidity-controlled rooms with a 12:12-h light-dark and had ad libitum access to distilled water and pelleted rat chow (Harlan/Teklad 8640 Rodent Diet). The experimental protocol was approved by the Michigan State University All University Council on Animal Use and Care.

ET_B receptor Immunocytochemistry

Rats were sacrificed with a lethal dose of sodium pentobarbital (100mg/kg ip) and immediately transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde solution. After perfusion, brains were dissected and postfixed in 4% paraformaldehyde prior to cryoprotection in 30% phosphate buffered sucrose solution. Brains were then encased in OCT freezing media and sectioned on a cryostat at 35 μm. Brain sections were serially collected as free floating slices onto 0.1 M PBS filled 12-well cell culture plates.

Sections were first incubated in 0.2% hydrogen peroxide 0.1M PBS solution to remove endogenous peroxidase for 30 min. Following 3 washes, they were incubated in a blocking solution consisting of 7.5% normal goat serum (NGS; Vector Labs, Burlingame, CA) and 0.25% Triton X 100 in 0.1M PBS for 1 h at room temperature. Sections were then incubated with a rabbit polyclonal

antibody raised against the 3rd cytoplasmic domain of ETBR (Alomone Labs, Jerusalem, Israel) diluted 1:200 in 3% NGS/0.25% Triton X100/ 0.1M PBS solution for 1 hour at room temp. Sections were rinsed in 0.1M PBS solution containing 3% NGS and subsequently incubated with the biotinylated goat anti-rabbit antibody (1:400; Vector labs) for 1h. After three rinses, the tissue was then incubated with an avidin-biotin peroxidase reagent (ABC-Vectastain Elite, Vector Labs) for 45 min. Sections were thoroughly washed in PBS and reacted with a 0.0125% 3,3'-diaminobenzidine solution containing 0.05% nickel ammonium sulfate (Nickel-DAB, Vector labs) for 7 min which produced a dark brown stain. Processed brain sections were mounted onto gel-coated slides and coverslipped with Permount. No immunoreactivity was observed in control brain slices incubated without primary antibody.

Dopamine β hydroxylase Immunocytochemisty

Adjacent series of hindbrain brain slices were immunolabeled for dopamine beta hydroxylase protein (DBH; Chemicon) to help identify brain regions. Sections were first incubated in a mouse anti-DBH antiserum (1:500) for three days. Then sections were treated with ABC reagent and then with biotinylated goat anti-mouse secondary antibody (1:200) for 1 h. Finally, sections were reacted with VIP chromogen (Vectastain, Vector Labs) which produces a light red stain. Subsequently, after extensive rinsing, both forebrain and hindbrain sections were mounted on gel-coated slides, dehydrated in an alcohol and xylene series and coverslipped with Permount mounting medium (Fisher

Scientific). We used a rat atlas to localize brain regions (Paxinos and Watson, 1989).

Histological analysis

Slides were viewed and analyzed under a Leica light microscope for presence of immunoreactivity. Areas of particular interest in this study include the PVN, SON, ME, SFO, CVLM, NTS, and AP. We also examined immunostaining in the hippocampus and cerebellum as controls for positive staining.

RESULTS

The ETBR antibody is targeted against residues 298-314 of rat ETBR, putatively located in the third intracellular loop (Alomone Labs data sheet). Western blotting of the antibody with and without immunizing peptide preabsorption demonstrated antibody specificity (Alomone Labs) (Figure 28).

The highest densities of ETBR binding sites was observed in the granule cells of the hippocampus (Figure 29), the Purkinje and molecular layers of the cerebellum (Figure 30) and the ME (Bregma -2.12mm to -3.30mm) (Figure 31) . Immunostaining with ETBR antibody also revealed dense staining in the SFO (Bregma -0.80mm to –1.40mm), AP (Bregma -13.68mm to -14.08mm) and OVLT (Bregma 0.20mm to -0.30mm) (Figures 32-33).

There was moderate immunoreactivity in the SON (Bregma -0.80mm to -1.80mm), however, the staining was not distributed equally throughout the rostro-caudal extent of the nucleus (Figure 34). Most of the staining was distributed in the anterior-middle portion of the SON. Similarly in the brainstem, immunostaining in the RVLM (Bregma -11.60mm to -12.80mm), CVLM (Bregma –13.68mm to -14.60mm) and NTS (Bregma -13.68 to -14.60mm), while present, was sparse (Figure 35-37). No appreciable immunoreactivity was discerned in the PVN (Bregma -1.80mm to -2.56mm) (Figure 38) although different concentrations of Triton-x-100 detergent from 0.1% to 1.0% triton were used in an attempt to enhance staining.

The staining pattern in the ME reveals dense immunoreactivities in the fibers emanating from the neurons. Fibrous staining was observed in other brain regions as well (OVLT, NTS, CVLM, RVLM, AP), however, none as apparent as seen in the ME.

High magnification provided evidence of ETBR immunoreactivities in the cell nucleus (Figure 29) as well as in neuronal processes (Figure 31). Counterstaining or co-labeling with glial marker was not performed in this study. Therefore, the phenotype of ETBR positive cells cannot be determined. However, the staining pattern does not indicate primarily vascular ETBR immunoreactivity.

DISCUSSION

In this study, we confirmed that endothelin type B receptor (ETBR) expression is not limited to the vascular system but rather is localized in distinct central nervous system regions including hypothamic and brainstem nuclei known to be important in central vasoregulatory and cardiovascular control.

The highest densities of ETBR immunostaining were found in the pyramidal cell layers in the CA1-CA3 region and the granular layer of hippocampal dentate gyrus. Dense ETBR expression was also observed in the cerebellular Purkinje cells and cells in the molecular layer, which contains dendritic arbors of Purkinje neurons as well as stellate and basket inhibitory interneurons which form GABAergic synapses onto Purkinje cell dendrites. These data support the findings of autoradiographic studies that localized heavy radioactive ET-1 binding in the cerebellum and hippocampus (Niwa et al, 1991; Jones et al, 1989; Koseki et al, 1989; Kohzuki et al, 1990). Moreover, we confirmed previous studies using *in situ hybridization* (Tsaur et al, 1997) and immunocytochemistry (Furuyu et al, 2001) to localize cerebellar ETBR distribution. Furuyu et al (2001) reported dense staining in the Bergman's glia but found no ETBR immunoreactivities in the granule cell layer, contrary to our findings. This discrepancy may be caused by different characteristics of antibodies. In that report, the antibody was generated against residues 420-442 of the human ETBR, which recognizes a different epitope than the antibody in our experiment. Differences in the antigenic determinant site may result in variations in staining patterns. Immunoreactivities in the hippocampus and

cerebellum provided a basis for positive comparison for staining in other brain regions.

Lee et al (1990) examined endothelin (ET) binding sites in the human brain by an *in situ hydridization* technique and found the highest density of ET-1 mRNA in the cytoplasm of magnocellular neurons in the hypothalamic PVN and SON which controls the release of oxytocin and vasopressin from the pituitary (Antunes-Rodrigues et al 2003), suggesting a central cardiovascular regulatory role for ET. In our immunocytochemistry study, we found moderate density of cells in the SON that expressed ETBR protein, confirming previous data but no discernible staining in the PVN. Lack of immunoreactivity in the PVN does not indicate absence of ETBRs in that region. Rather, it may reflect antibody specificity and/or penetrance in the PVN. Varying concentrations of Triton-x-100 detergent was used to perforate tissue and provide better receptor-ligand exposure. However, no improvement was observed in these protocol modifications. ETBR immunoreactivity in the SON supports a central role for the ET system in the control of blood pressure and fluid balance and is consistent with studies showing increased neuronal activation in that nuclei after icv administration of ET (Zhu and Herbert, 1996).

In the brainstem, low but detectable ETBR immunoreactive densities were observed in the NTS, CVLM and RVLM. Autoradiographic studies have documented the presence of ET binding sites in the NTS and RVLM (Koseki et al, 1989; Kuwaki et al, 1994). The CVLM and NTS are part of brainstem afferent pathway (Willette et al, 1984; Agarwal et al, 1989; Li et al, 1991; Blessing, 1997)

and together with the RVLM (Minson et al, 1997) are integral to central baroreflex modulation of sympathetic nervous activation. Presence of ETBRs in these brain regions suggests a possible functional role for central ETBR to affect sympathetic nervous activation.

Among the circumventricular organs (CVOs) examined, we found the highest density of ETBR immunostaining in the ME. The ME, located in the basal hypothalamus ventral to the third ventricle and adjacent to the arcuate nucleus is not a sensory CVO, but rather one of the most important regions in the hypothalamus for regulation of the pituitary gland. All hypophysiotrophic hormones converge at the ME before they are conveyed to the pituitary gland (Cottrell and Ferguson, 2004). We observed ETBR immunoreactivity in the cell nuclei and dendritic or axonal processes throughout the entire extension of the ME. Immunohistochemical labeling of the fibers may suggest ETBR synthesis or assembly in these processes. Yamamoto et al (1997) reported a similar pattern of ETBR immunostaining in the ME that was colocalized with luteinizing hormone releasing hormone (LHRH)-secreting cells, suggesting that endothelin affects LHRH fibers in the ME via ETBRs. Double labeling of the ETBR positive cells with oxytocin and/or vasopressin by the same authors produced no colocalization suggesting that ETBR do not directly mediate the release of these neuropeptides. Moreover, the involvement of ETBRs in the phosphoinositide signaling cascade in the ME has been reported (Garrido and Israel, 2004).

We also observed ETBR immunoreactivities in sensory CVOs that are critical regulators of body fluid balance and cardiovascular function. The sensory

CVOs link both humoral and neural inputs conveying cardiovascular information and then integrate and initiate appropriate physiological responses (Cottrell and Ferguson, 2004). In our study, dense ETBR immunoreactivities were observed in the forebrain SFO and OVLT and hindbrain AP. Our findings support ET binding data from other researchers. With the use of selective ETBR agonists and antagonists, Garrido and Israel (2004) reported that the SFO contains dense ET binding sites and is involved in ET-induced phophoinositide signaling mediated through the ETBR, indicating a functional role for these receptors in the brain. In the current study, we confirmed the presence of ETBR expression in the SFO. The SFO hangs on the dorsal wall of the third ventricle and makes direct and indirect connections with the PVN and SON, which in turn communicate with the ME and motor nuclei of the autonomic nervous system (Ferguson et al, 1984; Lind 1985; Miselis 1982; Weiss et al, 1990). It has been reported the SFO neurons express the highest density of angiotensin binding, suggesting the SFO plays a critical role in signaling the level of circulating angiotensin II to the brain to influence cardiovascular function. Our finding that ETBRs are localized in the SFO suggests that the renin-angiotensin system may exert some of its regulatory effects on blood pressure via its interaction with the endothelin system. Angiotensin II regulates endothelin synthesis in the kidney (Sasser et al, 2002) and chronic angiotensin II induced hypertension can be attenuated by an ET_A/ET_B receptor antagonist (Herizi et al, 1998; Sasser et al, 2002). However, the influence of ET-1 on actions of angiotensin II may be mediated by the endothelin type A receptor subtype (Ballew and Fink, 2001)

instead of ETBR (Ballew and Fink, 2001). Although, Zeng et al (2005) showed that in the proximal tubules of the kidney angiotensin receptors can regulate ETBRs by increasing cell surface membrane ETBR expression, an effect that is impaired during hypertension.

In the OVLT, high ETBR immunoreactivity was observed, consistent with previous immunohistochemistry reports (Yamamoto et al, 1997; Yamamoto and Uemura 1998). The OVLT is located in the anteroventral tip of the third ventricle (AV3V) and contains osmoreceptors that stimulate thirst and vasopressin secretion and also receives information from baroreceptors in the heart. Lesions of the AV3V area attenuates the pressor response to many forms of experimental hypertension (Haywood et al, 1983; Gordan et al, 1982; Whalen et al, 1999; Catelli et al, 1988; Goto et al, 1982) and stimulation of the OVLT has been reported to increase blood pressure and SNA (Mangiapane and Brody, 1987), suggesting that the OVLT may play a role in cardiovascular regulation.

Radiolabeled ET binding implicates the presence of ET receptors in the AP (Koseki et al, 1989; Kuwaki et al 1994), however the receptor subtype has heretofore been unknown. We have now demonstrated the presence of ETBRs in the AP. The AP is located just dorsal to the NTS and is an important site for regulating the sensitivity of cardiovascular control centers to incoming bareceptor signals (Cox et al, 1990). Hasser and Bishop (1990) reported that the AP affects baroreflex control of the sympathetic nervous system. Furthermore, the AP is influenced by many hormones that affect cardiovascular function, including angiotensin II (Guan et al, 2000), vasopressin (Carpenter et al, 1988), atrial

natriuretic peptide (Bianchi et al, 1986) and endothelin (Jones et al, 1989; Koseki et al, 1989; Kuwaki et al 1994). Our finding that ETBRs are localized in the AP further support a role for ET to regulate cardiovascular function via the brain.

Though several immunohistochemical studies of ETBR have examined various tissues including the brain, there is no clear consensus concerning their distribution pattern. Primary antisera raised against ETBR purified from bovine lung is immunoreactive in the blood vessels of the brain, cerebellum and adrenal gland (Hagiwara et al, 1993), while antibody against human ETBR shows binding in the pulmonary capillaries and hepatic stellate cells (Fukushige et al, 2000; Muramatsu et al, 1999). Furthermore, antisera raised against different partial amino-acid sequences of the same species can result in variations in tissue susceptibility. Polyclonal antibody raised against the carboxyl terminus of rat ETBR corresponding to residues 425-439 was immunoreactive to fibrous axonal processes but not somata or dendritic processes of cells in the ME and OVLT and was not immunoreactive to blood vessels in the rat (Yamamoto et al, 1997). We used a commercially available antibody raised against residues 298-314 of rat ETBR (Alomone) and observed nuclear staining as well as dense immunoreactivities in proximal and distal cellular processes in the ME, OVLT, SFO and AP and detectable immunoreactivities in the SON, NTS, CVLM and RVLM. Immunostaining of endothelial cells in peripheral blood vessels by this antibody has also been demonstrated (Watts lab data).

We have shown in a previous study that *in vivo* ETBR receptor activation by intravenous infusion of the specific ETBR agonist, S6c, increases blood

pressure as well as stimulates neurons in discrete regions of the central nervous system known to be important in the regulation of blood pressure and blood volume. It is not known whether circulating S6c can reach ETBR in the brain; or whether activation of brain ETBR by circulating S6c can increase arterial pressure. ET-1, which bears strong homology to S6c, has been shown not to cross the blood-brain barrier (Koseki et al 1989). Since CVOs do not have a blood brain barrier and are thought to couple circulating chemical signals with neural networks that mobilize various effector systems such as sympathetic outflow, vasopressin release, water and salt intake. Therefore, we speculate that plasma S6c may be able to act on ETBRs in CVOs. Our finding of positive ETBR immuonstaining in key CVOs including the SFO, OVLT and AP supports our hypothesis.

In summary, our findings confirmed previous reports of ETBR localization in the cerebellum, hippocampus, ME, OVLT and SFO. In addition, we demonstrated specific ETBR immunoreactivities in the NTS, CVLM and RVLM, brainstem nuclei that are part of the baroreflex arc. ETBR expression was also found in the SON and AP. These results are consistent with the involvement of central ETBRs in the regulation of blood pressure and fluid balance. Furthermore, the finding that ETBRs are located in the brain, especially in the circumventricular organs, which play a critical role in relaying information between blood, cerebrospinal flood and central nervous system neurons, supports our hypothesis that circulating S6c may affect central ETBR to produce increases in SNA and blood pressure.

Conversely, the presence of ETBR in the central nervous system may be acting in the capacity of clearance receptors instead of causing sympathoexcitation. Though ETBRs are known to cause transient hypotension by the release of vasodilatory peptides, NO and prostacyclin (Gomez-Alamillo et al, 2003), in the periphery, they also function as clearance receptors to remove circulating ET-1 (Fukuroda et al, 1994). Blockade of ETBR increases blood pressure presumably by increasing bioavailability of ET-1 in the circulation, thus potentiating activation of ET_A receptors (Just et al, 2005; Pollock, 2000; Reinhart et al, 2002). In the central nervous system, mature ET and its precursors as well as ECE have all been detected, suggesting the central regulation of ET production (Kuwaki et al, 1997; Kedzierski and Yanagisawa, 1999). The finding of ETBR in the brain may suggest a clearance function to modulate the level of central ET.

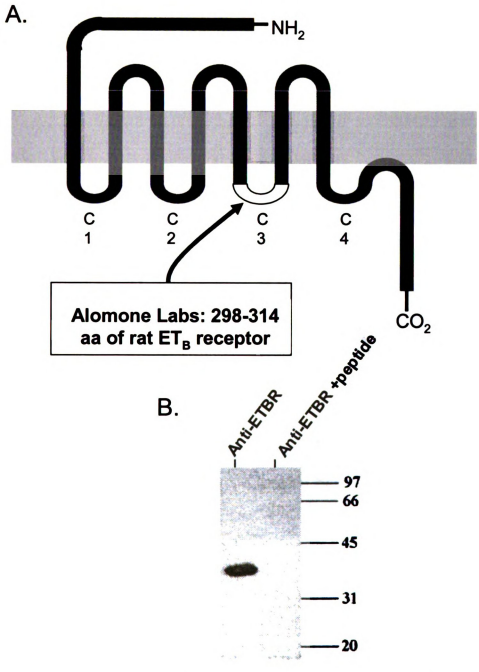


Fig 28. Rat ET_B receptor (ETBR). Structure of seven transmembrane ETBR and the putative epitope location for the Alomone antibody in the 3rd intracellular loop (A). Western blotting of rat brain membranes with Anti-ETB antibody and antibody preabsorbed with the ETBR peptide antigen (B).

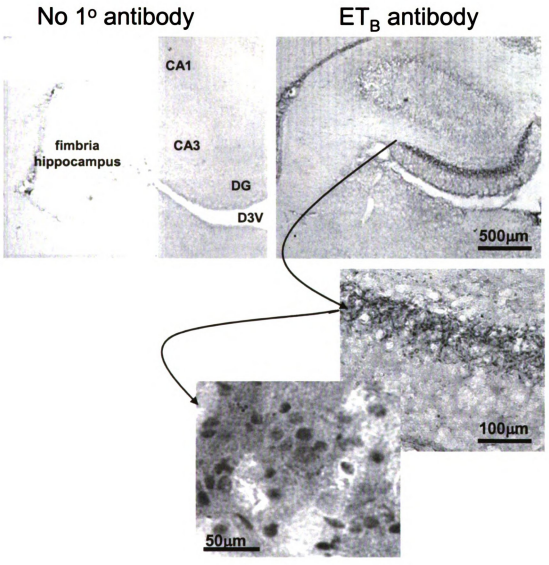


Fig 29. Photomicrograph showing ETB receptor immunoreactivity in the hippocampal granule cells. High magnification showed robust nuclear staining. DG, dentate gyrus; D3V, dorsal third ventricle; CA1-CA3, hippocampal fields.

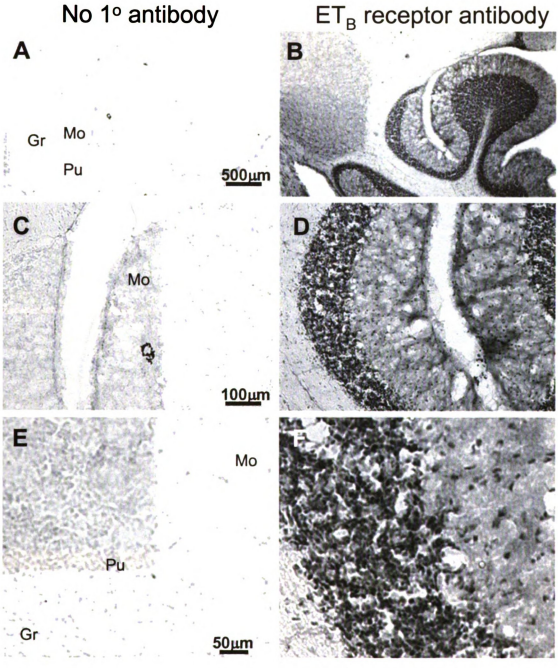


Fig 30. Photomicrograph of ETB receptor expression in the Purkinje and molecular layers of the cerebellum. Gr, granule cell layer; Mo, molecular cell layer; Pu, Purkinje cell layer.

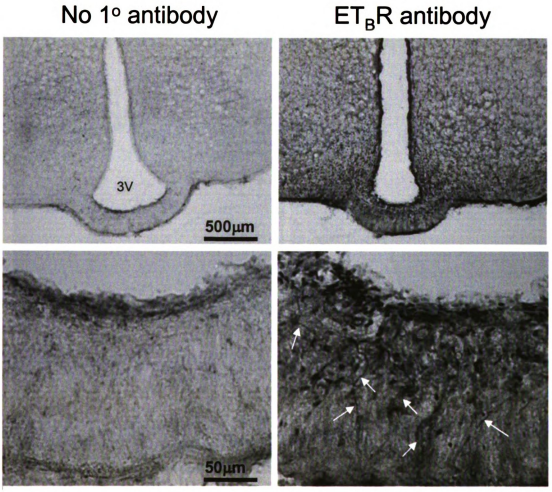


Fig 31. Photomicrograph of ETB receptor expression in the median eminence. High magnification shows immunostaining in the cell nuclei as well as in dendritic/axonal processes. Arrow indicate staining in the neuronal processes. 3v, third ventricle.

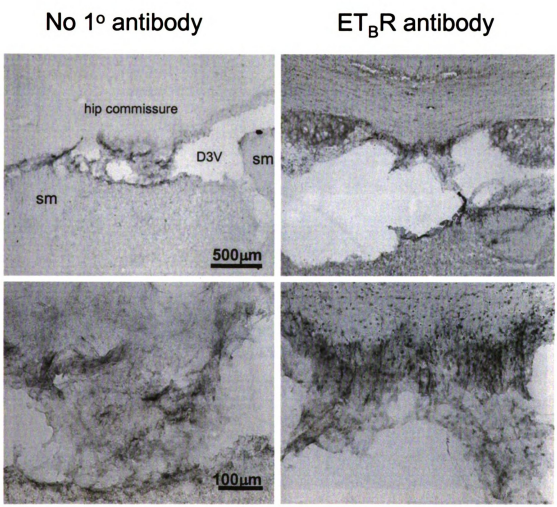


Fig 32. Photomicrograph of ETB receptor immunoreactivity in the subfornical organ (SFO). High magnification shows high density of ETB receptors in the SFO. Hip commissure, hippocampal commissure; D3V, dorsal third ventricle; sm, stria medullaris.

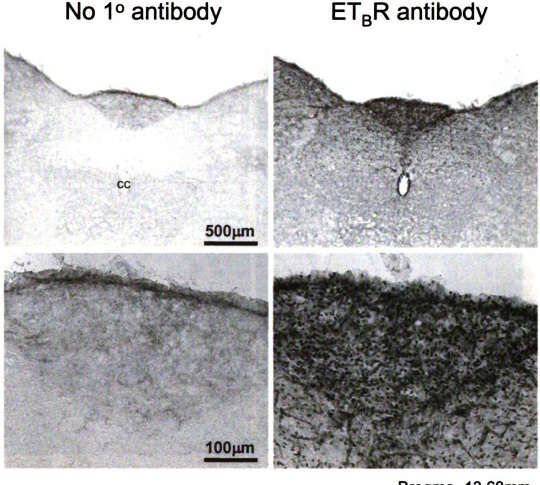


Fig 33. Photomicrograph of ETB receptor immunoreactivity in the area postrema (AP). High magnification shows high density of ETB receptors in the AP. cc, central canal.

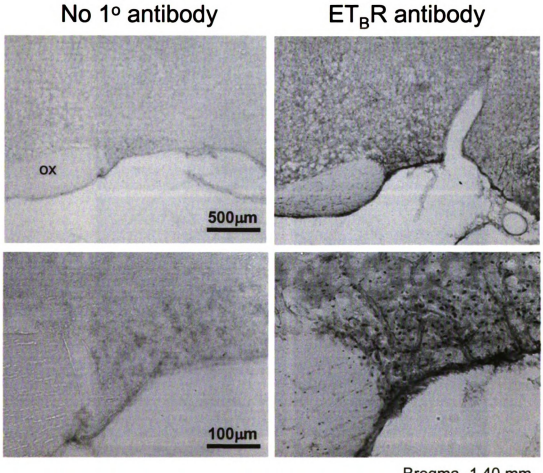


Fig 34. Photomicrograph of ETB receptor immunoreactivity in the supraoptic nucleus tractus solitarius. High magnification shows dense immunostaining. ox, optic chiasm.

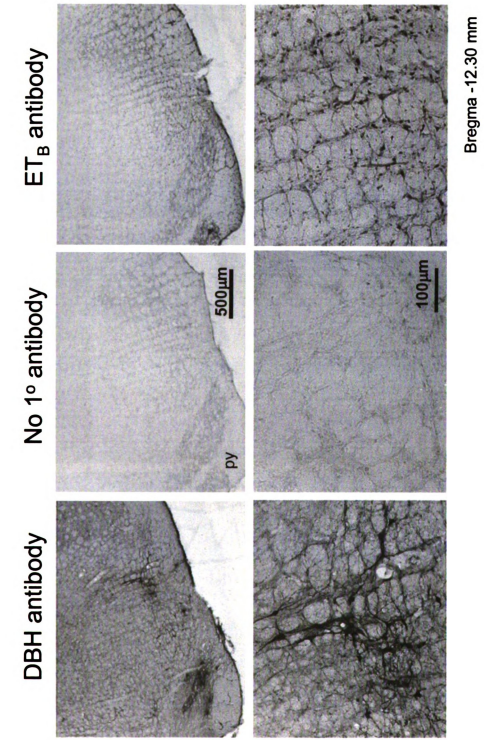


Fig 35. Light micrographs of ETB receptor immunoreactivity in rostral ventrolateral medulla (RVLM) slices of rats infused with S6c or saline. Py, pyramidal tract.

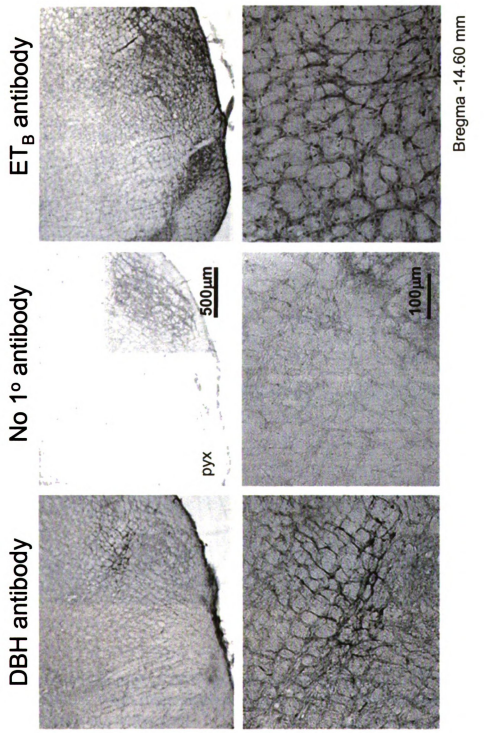
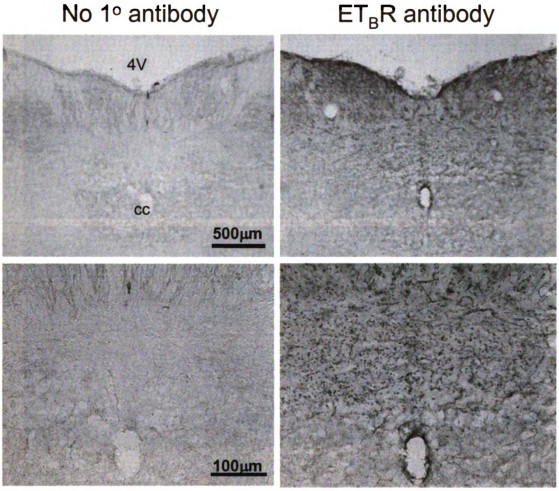


Fig 36. Light micrographs of ETB receptor immunoreactivity in caudal ventrolateral medulla (CVLM) slices of rats infused with S6c or saline. pyx, pyramidal decussation.



Bregma -14.30 mm

Fig 37. Photomicrograph of ETB receptor immunoreactivity in the nucleus tractus solitarius. High magnification shows sparse immunostaining. 4V, fourth ventricle; cc, central canal.

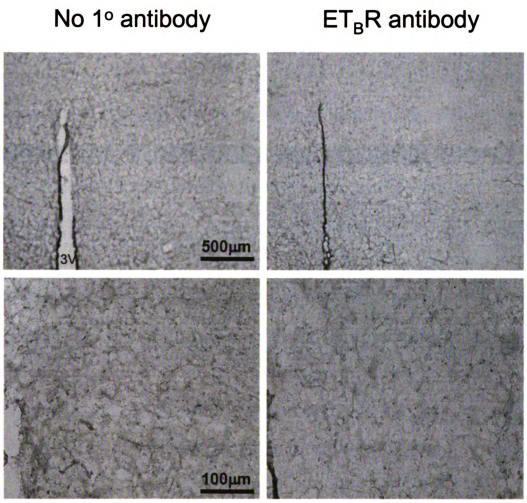


Fig 38. No ETB receptor immunoreactivity was observed in the PVN. 3v, third ventricle.

CHAPTER 6

GENERAL SUMMARIES AND CONCLUSIONS

GENERAL SUMMARIES

The main objective of this project was to identify mechanisms by which systemic ET_B receptor (ETBR) activation affects autonomic regulation of blood pressure. I used a rat model of hypertension where blood pressure is increased by iv infusion of the agonist, sarafotoxin 6c (S6c), to activate ETBRs which selectively constricts veins. With this animal model, I tested three possible mechanisms by which S6c may affect sympathetic nervous activity to the cardiovascular system. The following are summaries of major findings from each proposed experiment.

Specific Aim 1

ETBR stimulation using the specific agonist S6c causes venoconstriction. This effect may contribute to S6c-induced hypertension. Venoconstriction should produce a redistribution of blood volume towards the cardiothoracic region, thereby stimulating cardiac baroreceptors in a manner resembling that observed with blood volume expansion. Resulting neural responses include inhibition of vasopressin release, decreased sympathetic nerve activity and reduced thirst. Blood volume expansion (VE) studies in rats identified brain areas that participate in these neural responses (nucleus of the solitary tract, NTS; caudal ventrolateral medulla, CVLM; paraventricular nucleus, PVN; supraoptic nucleus, SON). Using the nuclear protein, Fos, as a marker of neuronal activation, we tested whether ETBR stimulation activates the same brain areas as VE by constricting extrathoracic veins and redistributing blood volume into the

cardiothoracic region. We also determined if the increase in Fos that is generated following S6c infusion is dependent on the activation of cardiopulmonary afferents in the heart. In addition, we compared the pattern of neuronal activation in rats exposed to S6c for 2h to that in rats receiving long-term S6c infusion (5 days).

We found that S6c infusion significantly increased the number of Fos-positive cells in the NTS, CVLM, SON and PVN as compared to normotensive control rats. This pattern of activation is very similar to that caused by VE according to previous reports (Randolph et al, 1998; Godino et al, 2002) and our own VE experiments, although we observed less consistent activation of SON and PVN. Double labeling of the Fos positive cells in the PVN and SON with anti-oxytocin antibody revealed differences in the distribution of neuronal activation. In the PVN, VE stimulated mostly parvocellular neurons; however, although S6c infusion increased Fos immunoreactivity in this portion of the PVN, the stimulus activated more magnocellular neurons. Activated neurons in the SON of VE and S6c rats were predominantly colocalized with oxytocinergic neurosecretory cells. In the brainstem, we found that roughly half of the Fos positive cells within the NTS and CVLM were double-labeled for dopamine β hydroxylase (DBH). In contrast to VE, where only non-catecholaminergic neurons where activated (Randolph et al, 1998), ETBR stimulation triggered both norepinephrine (NE)- secreting and non NE-secreceting cells. MAP was significantly increased in VE and S6c rats in comparison to control animals. The difference between initial and final MAPs of S6c and VE animals was 15.3 $\pm$ 1.9

mmHg and 10.9 ± 4.2 mmHg, respectively while in control rats MAP decreased 2.7 ± 2.1 mmHg. No significant differences in heart rate were observed during the 2 h infusion in any group. The results from this study suggest that ETBR activation causes hemodynamic changes similar but not identical to VE.

Both VE and *in vivo* ETBR stimulation similarly increased neuronal activity in brain regions important for the maintenance of cardiovascular homeostasis. Cunningham et al (2002) reported that the central nervous system response to VE primarily involved input from cardiopulmonary afferents in the heart. The second goal of Specific Aim I was to likewise establish the contribution of cardiac receptors to the pattern of neuronal activation caused by ETBR activation. To achieve this goal, we reduced/abolished input from cardiopulmonary receptors in rats by bilateral kainic acid (KA) deafferentation of the nodose ganglia, the distal cranial ganglion of the vagus nerve which provides sensory innervation to the heart and other viscera. Subsequent physiological and histological tests confirmed the success of the chemical lesions. The Bezold-Jarisch reflex which measures cardiopulmonary baroreflex function elicited by intravenous injections of 5 hydroxytryptamine was significantly blunted in KA treated rats. However, arterial baroreceptor function was not affected. Moreover, hematoxylin and eosin staining of fixed nodose ganglion slices revealed severe degeneration of ganglionic neurons following KA treatment.

Cardiopulmonary deafferentation did not impair the pressor response to ETBR activation. Blood pressures of both KA and sham rats increased significantly during S6c infusion. With S6c infusion, the mean difference between

final and initial MAP of sham and KA rats was 15.1 ± 4.8 mmHg and 21.4 ± 2.7 mmHg, respectively. In contrast, there was no increase in MAP in either group of rats receiving saline vehicle infusion. We compared the pattern of neuronal activation following S6c infusion in these chemically denervated KA rats to sham and found that KA deafferentation significantly blocked the Fos increase in the PVN, CVLM and NTS. However, KA treatment did not disrupt the S6c induced Fos increase in the SON. The finding that disrupting these afferents attenuated Fos expression following S6c infusion suggests that cardiopulmonary baroreceptors play a major role in the central response to ETBR activation.

We examined the sites of neuronal activation following long-term (5 day) stimulation of the ETBRs to determine whether the brain pattern of increased activity by acute hypertension persists in chronic hypertension. We used Fos-Like immunohistochemistry (FLI ICC) to localize the expression of Fos and Fos-related antigens as a marker of central nervous system activation. Relative to control animals, S6c significantly increased FLI expression in the SON and rostral ventrolateral medulla (RVLM), a sympathetic premotor nucleus, with lesser increases in the PVN and NTS. Fos-Li expression in the CVLM was similar in both S6c and control groups. Our results confirmed that chronic stimulation of the ETBR causes sustained hypertension and produces a pattern of brain activation different from acute hypertension. In contrast to the prominent activation of medullary baroreflex neurons following 2h S6c infusion, 5 day S6c produced the most robust increase in neuronal activity in brain regions associated with sympathoexcitation, consistent with our anticipated results.

However, the NTS, a major center in the relay of baroreceptor signals, also showed increased FLI expression. This finding supports mounting evidence that baroreceptors play a role in the long-term regulation of blood pressure (Dibona and Sawin, 1985; Haanwinckel et al, 1995; Lohmeier et al, 2002).

Based on the above data, we conclude that one mechanism by which S6c causes hypertension is through increasing cardiothoracic blood volume. We think that venoconstriction caused by acute ETBR activation increases venous return to the heart, consequently resulting in a centralization of blood volume from the extrathoracic vasculature to the cardiothoracic region and then to the arterial vasculature, resulting in hypertension. This blood volume redistribution would also serve to produce decreased sympathetic nervous system activity due to activation of cardiopulmonary baroreceptors, as evidenced by the increase in Fos expression in medullary neurons of the central baroreflex circuitry following S6c infusion, which was abolished after KA disruption of the nodose ganglia. Furthermore, we conclude that chronic stimulation of the ETBR is associated with a pattern of brain activation different from that observed during acute hypertension, suggesting a different mechanism in the maintenance of chronic versus acute endothelin dependent hypertension. One way that chronic S6c infusion increases neuronal activity in the RVLM and possibly SNA may be through the withdrawal of sympathoinhibitory input from the NTS and CVLM, so that tonic excitation of RVLM neurons produces sustained SNA. Alternatively, circulating S6c may bind to ETBR in RVLM directly (Chapter 5) or indirectly

through circumventricular organ (CVO)-mediated activation of the PVN-RVLM axis (Guyenet 2006; Brooks et al, 2005; Coote, 2005; Stocker et al, 2005).

Specific Aim 2

Endothelin induced hypertension may be mediated by the production of superoxide (O_2^-) anions. Dai et al (2004) reported that ET stimulated O_2^- production in sympathetic ganglion neurons in vitro by activating ETBRs and speculate that the increase O_2^- may directly modulate sympathetic neuroeffector transmission, resulting in heightened sympathetic nervous activity and increased vasoconstriction, both leading to the development of hypertension. As described in the proposal, Specific Aim 2 tests whether the activation of the ETBR would similarly elevate O_2^- levels in the sympathetic ganglia *in vivo*. Elevated O_2^- production in the sympathetic nervous system may contribute to increased excitability of sympathetic neurons and vasoconstriction. Ganglionic O_2^- production was assessed by oxidative dihydroethidium (DHE) fluorescence method in the inferior mesenteric ganglia (IMG) of S6c and saline infused. As described previously in the proposal, DHE reacts with O_2^- (primarily) to produce the red fluorescent ethidium marker, whose intensity is proportional to the amount of O_2^- present. Compared to controls, S6c infusion induced a significantly greater increase in O_2^- production in both neurons and surrounding satellite cells of the IMG. The DHE fluorescence intensity in the ganglionic neurons and satellite cells were 96.7% and 160% greater in S6c than in control rats, respectively.

Growing evidence indicated the possibility that hypertension per se can increase O_2^- levels in various tissues although other studies showed that hypertension is not invariably associated with increased O_2^- levels (Rajagopalan et al, 1996). Therefore, to test the hypothesis that S6c increases O_2^- levels in sympathetic ganglia in part by elevating blood pressure, we infused the α adrenergic agonist phenylephrine (PE) into conscious rats to produce an increase in blood pressure similar to that observed during S6c infusion. Additional rats received either S6c or saline infusions in order to allow direct comparison of DHE fluorescence with the three stimuli. In vivo infusion of S6c increased the DHE fluorescence intensities of ganglionic neurons and surrounding glial cells significantly greater than control rats, 215.5% and 197.6%, respectively. The results confirmed our previous experiment. Interestingly, PE infusion also produced O_2^- levels that were significantly greater than those observed in saline control animals; however they remained significantly less than those found in S6c infused animals. Compared to controls, fluorescence intensities of ganglia from PE rats were 137.7% in neurons and 104.6% in satellite cells greater.

To determine if PE has any direct effect on superoxide anion levels, we performed an additional study in freshly dissociated rat inferior mesenteric ganglionic neurons and glial cells *in vitro*. We found that application of PE did not induce a significant increase in O_2^- levels in either neurons or satellite cells. Our data show that an acute increase in blood pressure alone can cause elevated O_2^- levels in sympathetic ganglia, although it is possible that some other

physiological response to PE infusion is responsible. Overall then these data indicate that S6c infusion *in vivo* may increase O_2^- levels in sympathetic ganglia by both direct (stimulation of ETBRs on neurons and glia) and indirect (pressure-dependent) mechanisms.

We speculate further that elevated O_2^- concentrations in sympathetic ganglia may participate in the pathogenesis of ETBR dependent hypertension by facilitating nicotinic neurotransmission through the ganglion either by increasing preganglionic nerve activity or neurotransmission. To test this, we evaluated the effect of ganglionic blockade on MAP, HR and ganglionic O_2^- production during S6c infusion. We used chlorisondamine (CHL, 5 mg/kg iv), a long-acting nicotinic cholinergic receptor antagonist, to block central input to autonomic ganglia. Shortly after administration, CHL lowered MAP significantly and decreased HR. Despite pretreatment with CHL, the blood pressure of rats receiving S6c infusion increased significantly compared with rats receiving vehicle infusion. MAP increased 56.7 $\pm$ 1.97 mmHg and 11.3 $\pm$ 4.4 mmHg, respectively. Moreover, the magnitude of MAP increase following S6c infusion was significantly greater in rats pretreated with CHL than rats receiving S6c only, which increased 39.9 $\pm$ 5.9 mmHg. The DHE fluorescence intensities of ganglionic neurons and surrounding satellite cells were significantly greater in both S6c and CHL-S6c rats compared to CHL saline infused rats. Fluorescence intensities in the ganglionic neurons and satellite cells were 296.3% and 337.7% greater than controls respectively in S6c-only group, and 294.9% and 324.9% respectively in CHL-S6c group. Clearly, hypertension and increased O_2^-

production following ETBR activation persist in the presence of ganglionic blockade. Based on these findings, we conclude that neither the acute pressor effects of S6c nor the associated oxidative stress in ganglia are caused by alterations in nicotinic neurotransmission.

Since ETBR activation increases blood pressure and sympathetic ganglionic O_2^- production in the absence of nicotinic ganglionic neurotransmission, it is unlikely that S6c causes hypertension by binding to central ETBRs to modulate sympathetic nervous activation, as was proposed in Specific Aim 3. From the outcome of the previous experiment, we hypothesized that S6c may bind directly to ETBR on sympathetic postganglionic neurons, thereby increasing their activity and the release of catecholamines (NE and epinephrine). The resultant hypertension would be due to binding of NE and Epi to receptors in effector tissues. To test this, phentolamine (5 mg/kg iv) and propranolol (3 mg/kg iv) were administered to block peripheral α and β adrenergic receptors prior to S6c infusion. Combined adrenergic receptor blockade lowered MAP by an average of 25 $\pm$ 5.1 mmHg. S6c infusion was still able to increase MAP in the presence of combined adrenergic receptor blockade. Infusion of S6c for 2 h following adrenergic blockade increased MAP 43.3 $\pm$ 3.8 mmHg. Overall, from these results we conclude that acute intravenous S6c infusion causes hypertension primarily by direct venoconstriction as proposed in Specific Aim 1.

Specific aim 3

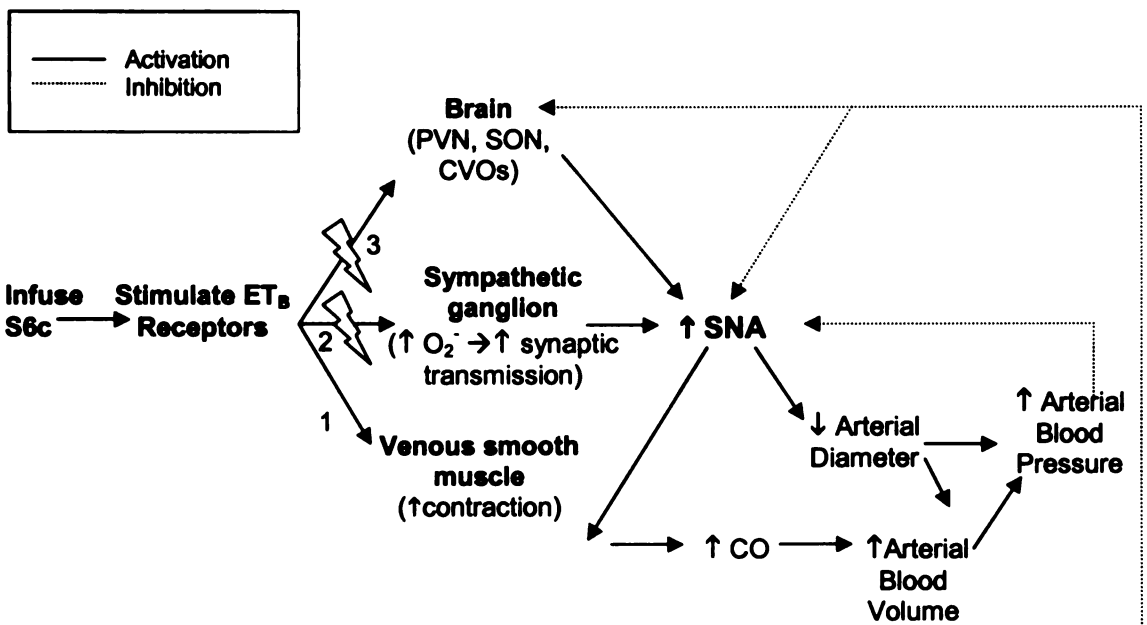
ETBRs are localized in the vasculature as well as the neural elements of the central nervous system, where they may play an important role in central autonomic control of blood pressure, neuromodulation and development (Garrido and Israel, 2004; Yamamoto et al, 1997). The purpose of Specific Aim 3 was to determine if ETBRs are localized in brainstem and central nervous system regions involved in autonomic regulation of fluid homeostasis and blood pressure. The presence of ETBR there would suggest that one way in which ET or S6c exerts their effect on the autonomic regulation of blood pressure is by acting directly on receptors in the brain. Brain sections were processed for ICC with a rabbit polyclonal IgG raised against the third cytoplasmic domain of ETBR, then stained with the Ni-DAB chromogen and later visualized under brightfield light microscopy. We observed the highest densities of ETBR binding sites in the granule cells of the hippocampus, the Purkinje and molecular layers of the cerebellum and the ME. Immunostaining with ETBR antibody also revealed dense staining in the SFO, AP and OVLT. Moderate immunoreactivity was observed in the SON, RVLM, CVLM and NTS. However, we found no appreciable immunoreactivity in the PVN. High magnification provided evidence of ETBR immunoreactivities in the cell nucleus as well as in neuronal processes.

In this study, we confirmed previous reports of ETBR localization in the cerebellum, hippocampus, ME and OVLT (Yamamoto and Uemura, 1998). In

addition, we found dense ETBR immunoreactivities in key hypothalamic and brainstem nuclei and sensory circumventricular organs, suggesting a functional role for ETBR in the central nervous system. However, the fact that hypertension and increases in superoxide anion production still persisted in the absence of any central autonomic input (Specific Aim 2) precludes the hypothesis that circulating S6c acts on central ETBRs to increase SNA and blood pressure. The activation of brain ETBRs may serve other physiological functions such as removal of central ET-1 (Fukuroda et al, 1994).

OVERALL CONCLUSIONS AND PERSPECTIVES

The overall goal of my dissertation was to assess how acute in vivo ETBR activation/venoconstriction affects the neural control of blood pressure. We contend that venoconstriction is the main potential mechanism by which acute *in vivo* ETBR activation produces hypertension. Increased venous return to the heart would consequently raise cardiac output and centralize of blood volume from the extrathoracic vasculature to the cardiothoracic region. Increased blood volume to the heart would also serve to produce decreased sympathetic nervous system activity due to activation of cardiopulmonary receptors.



ET_B receptor activation by S6c affects autonomic nervous control of blood pressure ...

1. by directly constricting veins, leading to volume shifts into the thoracic region and causing arterial or cardiopulmonary reflex activation.
2. by acting on sympathetic ganglia to increase ganglionic transmission, possibly by ncreasing O_2^- levels.
3. by acting on ET_B receptors in the brain to increase sympathetic activity.

Perspectives

It appears that hypertension produced by *in vivo* activation of ETBRs is initially characterized by direct constriction of venous vascular smooth muscle cells effecting redistribution of blood volume to the heart. At this stage, autonomic nervous system acts as a "brake" on the pressor actions of S6c, due to engagement of cardiopulmonary and arterial baroreceptors. Continuing ETBR activation allows progression to a stage where the increased SNA may become a more important determinant of the elevated arterial pressure. How this transition occurs and the mechanisms (central or peripheral) responsible for sympathetic activation by ETBR stimulation remains to be elucidated. Nevertheless, my work emphasizes the importance of the often neglected interactions between autonomic nervous system activity and vascular capacitance in overall control of circulation.

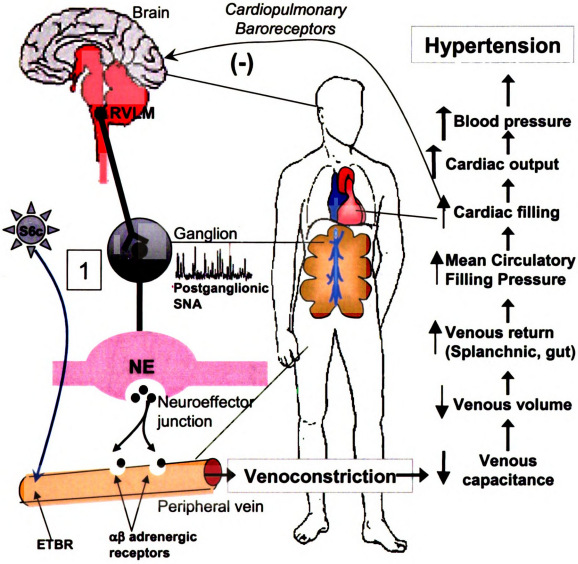


Fig 39. Schematic diagram summarizing mechanism 1 involved in the pressor response to acute in vivo ETBR activation by S6c. Stimulation of ETBRs on venous smooth muscle cells causes venoconstriction and blood volume redistribution into the thoracic cavity resulting in a greater cardiac output and arterial blood pressure. Increased blood volume to the heart would also cause decreased sympathetic nervous system activity due to activation of cardiopulmonary receptors.

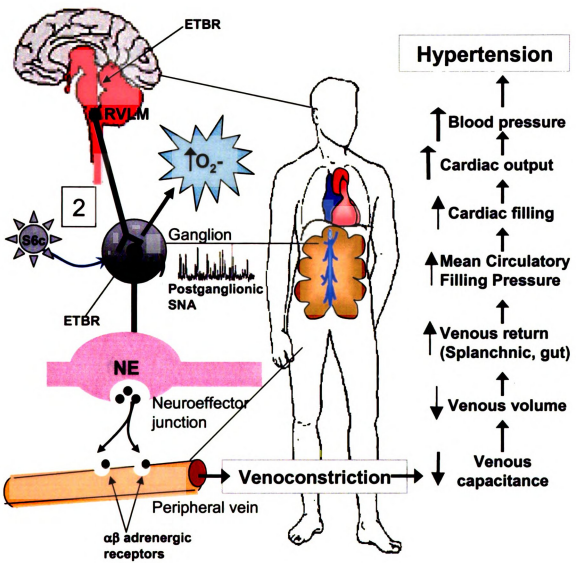


Fig 40. Schematic diagram summarizing mechanism 2 in the pressor response to acute *in vivo* ETBR activation by S6c. Stimulation of ETBRs on postganglionic neurons increased ganglionic O2- production and may lead to increased SNA by facilitating nicotinic neurotransmission through the ganglion either by increasing preganglionic nerve activity or neurotransmission. However, results from chlorisondamine and combined α and β adrenergic receptor blockade experiments preclude this hypothesis in mediating acute S6c infusion.

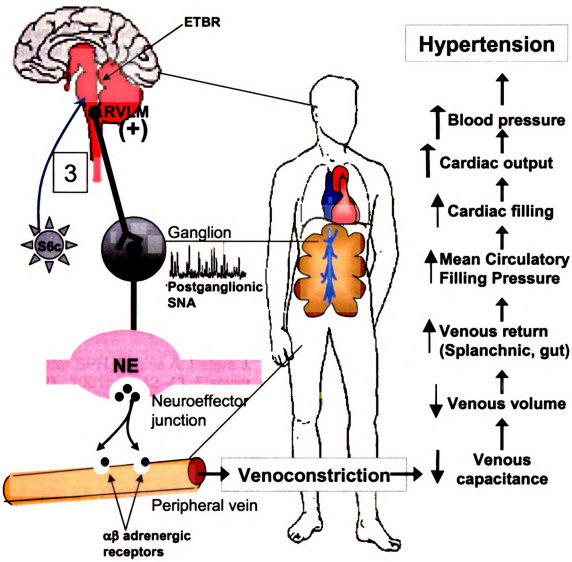


Fig 41. Schematic diagram summarizing mechanism 3 in the pressor response to acute *in vivo* ETBR activation by S6c. ETBR expression was found in brainstem and central nervous system regions involved in autonomic regulation of fluid homeostasis and blood pressure. The presence of ETBR in these nuclei, especially in the circumventricular organs, suggests that S6c may exert its effect on the autonomic regulation of blood pressure by acting directly on receptors in the brain. However, the fact that the effects of S6c still persisted in the absence of any central autonomic input precludes this hypothesis as a likely mechanism mediating acute S6c infusion.

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