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THE EFFECTS OF CHRONIC ACTIVATION OF ENDOTHELIN ETB RECEPTORS ON BLOOD PRESSURE, VENOMOTOR TONE, NEUROHUMORAL ACTIVITY, AND OXIDATIVE STRESS

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Melissa Wei Li

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Major Professor's Signature

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By

Melissa Wei Li

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ABSTRACT

THE EFFECTS OF CHRONIC ACTIVATION OF ENDOTHELIN ETB RECEPTORS ON BLOOD PRESSURE, VENOMOTOR TONE, NEUROHUMORAL ACTIVITY, AND OXIDATIVE STRESS

By

Melissa Wei Li

Activation of endothelin ETB receptors (ETBRs) modulates blood pressure by affecting several different physiological systems. The stimulation of ETBRs is known to cause transient arterial relaxation, a marked increase in renal excretion of sodium and water, and a clearance of endogenous endothelin-1 (ET-1). It is reasonable to speculate that the depressor and natriuretic effects induced by ETBR activation would cause blood pressure to decrease. Our lab, however, revealed for the first time that chronic activation (5 days) of ETBRs using intravenous infusion of the ETBR selective agonist sarafotoxin 6c (S6c) in rats caused a seemingly paradoxical increase in arterial pressure (*S6c-induced hypertension*).

I proposed the hypothesis that S6c-induced hypertension is mediated by direct venoconstriction, increased sympathetic activity to the splanchnic region mediated by elevated ROS levels in sympathetic ganglia, and increased release of vasodilating neurotransmitters from sensory nerves.

Most studies of hypertension have focused on altered regulation of the arterial vasculature. It is true that arterial resistance is elevated in hypertension. However, there is also a decrease in venous capacitance. Unfortunately, there

have been few studies of the mechanisms causing constriction of veins in hypertension. Since S6c is a selective venoconstrictor, venoconstriction may mediate S6c-induced hypertension. Since the splanchnic veins are the main capacitance region of the circulation, I focused on the effects of ETBR activation in the splanchnic bed in my thesis project. In addition, celiac ganglionectomy (CGX) attenuated S6c-induced hypertension, indicating that sympathetic innervation participates in this hypertension model as well. My data also show that there was an increased neuronal, but not vascular, superoxide level in S6c-induced hypertension. Systemic treatment of antioxidant (tempol) lowered neuronal oxidative stress and blood pressure, suggesting that S6c-induced hypertension is partially mediated by sympathoexcitation to the splanchnic organs driven by increased oxidative stress in sympathetic ganglia. In ETBR-deficient transgenic (Tg) rats, ETBRs are not functional in endothelium, smooth muscle, or renal tubules. The only place where ETBRs are functional is where dopamine-ß-hydroxylase is expressed. When CGX Tg wildtype (+/+) received S6c, the increase in blood pressure was attenuated.

The results of my work provide strong evidence that S6c-induced hypertension is mediated by increased splanchnic sympathetic activity at least partially driven by increased neuronal superoxide level. Therefore, my studies provide more information on how oxidative stress and sympathetic innervation contribute to blood pressure regulation.

To my grandparents, Zhiqin Xiang and Zuoting Liu, for their endless love.

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To my husband, Zetian Mi, and my daughter Allie, for completing my life.

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Also, I would say thanks to all lab members, Helen (who's also a close friend and gave me valuable input), Barb, Andrew, Sachin, Hannah, and Bridget. I enjoyed spending time with you all.

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was born. Without their help, I can not imagine how I could handle my research and family at the same time.

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LIST OF ABBREVIATIONS

BBB	blood brain Barrier
CGRP	calcitonin gene-related peptide
CGX	celiac ganglionectomy
CO	cardiac output
CON	Control
CVO	circumventricular organ
DOCA	deoxycorticosterone acetate
ENS	enteric nervous system
EPI	epinephrine
ET	endothelin
ET_AR	endothelin type A receptor
ET_BR	endothelin type B receptor
HR	heart rate
IMG	inferior mesenteric ganglia
ip	intraperitoneal
iv	intravenous
MCFP	mean circulatory filling pressure
MAP	mean arterial pressure
NADPH	nicotinamide adenine dinucleotide phosphate
NE	norepinephrine

NO	nitric oxide
NOS	nitric oxide synthase
O_2^-	superoxide anion
PBS	phosphate buffered saline
S6c	sarafotoxin 6c
sc	subcutaneous
sl	spotting lethal
SNS	sympathetic nervous system
TTX	tetrodotoxin
VSMC	vascular smooth muscle cell

FREQUENTLY USED DRUGS AND CHEMICALS

Sarafotoxin 6c	selective ETB receptor agonist
Heparin	anticoagulant
Ticarcillin	antibiotic
Enrofloxacin	antibiotic
Tylenol	pain reliever
Sodium pentobarbital	anesthetic
Dihydroethidium	oxidant sensitive fluorogenic probe
Tetrodotoxin	sodium channel blocker
Tempol	antioxidant
Norepinephrine	endogenous adrenal receptor agonist

COMMONLY USED SOLUTIONS

Krebs' physiological saline solution (1L)

Sodium chloride	68.3g
Potassium chloride	3.55g
Calcium chloride	3.65g
Magnesium chloride	2.45g
Sodium phosphate, monobasic	1.65g
Sodium bicarbonate	2.1g
Glucose	2.0g

0.1 M Phosphate buffered saline (1L)

Monobasic monohydrate	3.85g
Dibasic anhydrous	10.2g
Sodium chloride	9.0g

pH=7.4

CHAPTER 1

GENERAL INTRODUCTION

1. Hypertension as a serious disease

The definition of hypertension is systolic blood pressure of 140 mmHg or higher, or diastolic blood pressure of 90 mmHg or higher, or both. In the US alone, there are about 73 million people who are hypertensive. That is, almost one in every three American adults has high blood pressure (American Heart Association). Hypertension can damage the blood vessels and is associated with other disease conditions, such as stroke, heart failure, heart attack, kidney failure, and vision problems. Therefore, it is very important to study hypertension and discover the causes of this disease. My research was designed to help understand the causes of hypertension, and therefore find improved treatments for this serious disease.

2. Blood pressure regulation

To understand hypertension, we need to understand the physiology of blood pressure regulation. As Figure 1 shows, mean arterial pressure (MAP) is the product of cardiac output (CO) and total peripheral resistance (TPR). CO is the product of stroke volume (SV) and heart rate (HR). SV is determined by both ventricular preload and inotropy (force of cardiac contraction). And the former is determined by blood volume and venous compliance. For TPR, it is mainly determined by vascular structure and vascular function. Neurohumoral factors are one of the most important groups of factors affecting blood pressure regulation, since the changes in these factors may alter renal sodium/water excretion, venous compliance, inotropy, HR, and TPR, thereby regulating MAP

through multiple pathways. Some of the factors regulating arterial blood pressure are discussed below.

2.1 Humoral factors

2.1.1 The endothelin system

2.1.1.1 Discovery and synthesis

In 1985, an endothelial cell-derived vasoconstrictor polypeptide was found (Hickey, Rubanyi et al. 1985). Later the substance was isolated by Yanagisawa *et al.* and it was named endothelin (ET), since it was originally found to be generated by vascular endothelial cells (Yanagisawa, Kurihara et al. 1988). This 21-amino acid peptide has been shown to be one of the most potent endogenous vasoconstrictors known. There are 3 family members of this group of mammalian vasoactive peptides: ET-1, ET-2, and ET-3, which are encoded by 3 different genes in human and rat tissues (Inoue, Yanagisawa et al. 1989). ET-2 is very similar to ET-1, but ET-3 is different from ET-1 at 6 out of 21 positions (Goraca 2002). There are two essential disulphide bonds in the structure of ETs: one between amino acids 1 and 15, and the other between amino acids 3 and 11. As Figure 2 shows, the preproform of ET is a 203-amino acid peptide, from which 164 amino acids is cleaved by dibasic endopeptidase and carboxypeptidases, generating 39-amino acid big ET (Seidah, Day et al. 1993; Xu, Emoto et al. 1994). Then big ET is converted to ET by endothelin converting enzyme (ECE) (Xu, Emoto et al. 1994).

The ETs are generated by various tissues *in vivo*, including kidney, lung, central nervous system, pituitary and peripheral endocrine tissues, and placenta

(Hemsen and Lundberg 1991); (Hemsen, Gillis et al. 1991; Stojilkovic and Catt 1992). ET-1 is the most important one among the three isoforms, since it is the main isoform in human cardiovascular regulation. It is the most ubiquitous and powerful vasoactive peptide which can exert long-lasting constrictor effects on blood vessels (Davenport 2002). Therefore, it is the best characterized among the three. ET-1 is mainly generated and released continuously from the vascular endothelial cells *in vivo* (Yanagisawa 1994). The released ET-1 induces vascular blood vessels to constrict, therefore maintaining basal vascular tone (Haynes and Webb 1994). Upon external stimuli, ET-1 is also released from Weibel-Palade bodies (the endothelium-specific storage granules), leading to additional vasoconstriction (Russell, Skepper et al. 1998). Therefore, ET-1 acts primarily as a paracrine hormone, as opposed to a circulating hormone (Levin 1995). To a less extent, ET-1 is also produced by epithelial and smooth muscle cells (Miller, Pelton et al. 1993), mast cells (Ehrenreich, Burd et al. 1992), mesangial cells, neurons, glial cells, and liver cells (Levin 1996). ET-2 is primarily synthesized in the kidney and intestine. It has been reported to be a vasoconstrictor in human blood vessels (Maguire and Davenport 1995). Mature ET-3 circulates in the plasma (Matsumoto, Suzuki et al. 1994). Endothelial cells do not produce ET-3 and the principal source of ET-3 has not been identified yet. ET-3 is unique in that it has different binding affinities for the two endothelin receptors.

2.1.1.2 Endothelin receptors

Two types of endothelin receptors have been cloned: endothelin receptor type A (ETAR) (Arai, Hori et al. 1990) and endothelin receptor type B (ETBR)

(Arai, Hori et al. 1990; Sakurai, Yanagisawa et al. 1990). These two subtypes show 59% similarity in amino acid composition. Human and rat ETARs are 91% similar. There is a 12% difference in ETBRs between human and rat (Davenport 2002). Both types are G protein-coupled rhodopsin-type receptors with 7 transmembrane domains (Sakurai, Yanagisawa et al. 1992; Alexander, Mathie et al. 2007). The signal transduction pathways engaged upon activation of ET receptors usually involve phospholipase C (PLC) (Bousso-Mittler, Kloog et al. 1989; Vigne, Lazdunski et al. 1989), phospholipase A2 (PLA_2) (Bousso-Mittler, Kloog et al. 1989; Stojilkovic, Iida et al. 1991), phospholipase D (PLD) (MacNulty, Plevin et al. 1990; Konishi, Kondo et al. 1991), or Na^+/H^+ exchange (Simonson, Wann et al. 1989; Battistini, Filep et al. 1991; Kramer, Smith et al. 1991).

The dissociation rates of receptor-ligand complexes in the ET system are isoform-dependent (Galron, Kloog et al. 1989; Devesly, Phillips et al. 1990) and species-dependent (Galron, Bdolah et al. 1991). These differences could be due to different modes of binding (Galron, Kloog et al. 1989). However, one unique characteristic of ET binding is that the rate of dissociation is extremely slow in various tissues. For example, the binding is almost irreversible in vascular smooth muscle cells (VSMCs) from rats (Hirata, Yoshimi et al. 1988). ET receptor activation induces long-lasting physiological effects, while the half-life of circulating ET is very short (Anggard, Galton et al. 1989). This seeming paradox is at least partially due to slow dissociation rate of ET from its receptors.

ETARs have the same binding affinity for ET-1 and ET-2. On the other hand, ET-3 has little or no affinity for ETARs at physiological concentrations

(Sakurai, Yanagisawa et al. 1992). ETARs are mainly located at VSMCs and cardiac muscle cells. They mediate vasoconstriction on ET-1 binding (Miller, Pelton et al. 1993). They also mediate cellular proliferation as a growth factor (Bobik, Grooms et al. 1990; Luscher, Oemar et al. 1993).

ETBRs have the same affinity for the three isoforms of ETs (Arai, Hori et al. 1990; Sakurai, Yanagisawa et al. 1990). ETBRs are present on endothelial cells, VSMCs, kidney, liver, etc. The activation of ETBRs has multiple functions. Activation of receptors on vascular endothelium can produce vasodilation by the release of endothelial cell derived vasodilators, such as nitric oxide and prostaglandins (Wright and Fozard 1988; Warner, de Nucci et al. 1989; Rubanyi and Polokoff 1994). Activation of ETBRs in the kidney leads to a marked increase in renal excretion of sodium and water by inhibiting sodium reabsorption in the renal tubules and water reabsorption in the collecting duct (Kohan, Padilla et al. 1993; Plato, Pollock et al. 2000). Whereas, renal vasoconstriction is induced by the stimulation of ETBRs in the rat (Clozel, Gray et al. 1992; Cristol, Warner et al. 1993). In addition, ETBRs act as clearance receptors for endogenous ET-1 (Ozaki, Ohwaki et al. 1995; Dupuis, Goresky et al. 1996; Berthiaume, Yanagisawa et al. 2000). Recently, it has also been found that ETBR activation constricts veins but only weakly constricts resistance arteries (Moreland, McMullen et al. 1992; Gray, Loffler et al. 1994; Johnson, Fink et al. 2002). Another *in vitro* study shows that ETBR activation leads to increased oxidative stress in sympathetic ganglia (Dai, Galligan et al. 2004). Acute activation of ETBRs *in vivo* induces a sustained blood pressure increase (Lau,

Galligan et al. 2006) and increased superoxide levels in sympathetic ganglia (Dai, Galligan et al. 2004; Lau, Galligan et al. 2006). Therefore, the multifunctional nature of ETBRs makes studying their roles in physiological and pathological conditions complicated.

Although ETBRs are present in both arterial and venous vasculatures, they are differentially expressed and functional in arteries and veins. For example, both ETBR mRNA and protein densities are higher in veins than in arteries (Watts, Fink et al. 2002). Furthermore, although ETBRs are present in both arteries and veins, they are not functional in most arterial beds, including large arteries and small arteries (Johnson, Fink et al. 2002; Watts, Fink et al. 2002). On the other hand, veins are constricted by S6c, a selective ETBR agonist, in a concentration-dependent manner, indicating that ETBRs are functional in veins. Additionally, it has been shown that S6c-induced constriction in ET-1 pretreated veins is the same as in untreated vessels, indicating that venous ETBRs do not desensitize to ET-1 (Thakali, Fink et al. 2004). On the contrary, ETARs on arteries and veins desensitize to ET-1. This may explain why the hypertension is maintained while ETARs are densensitized to a increased circulating ET-1 level in an animal model of hypertension (Nguyen, Parent et al. 1992; Fujita, Matsumura et al. 1995; Laurant and Berthelot 1996; Watts, Fink et al. 2002).

2.1.1.3 Endothelin-1 in physiological conditions

ET-1 acts at multiple locations in biological systems under physiological conditions. ET-1 participates in maintaining basal tone of vascular smooth

muscles (Haynes, Ferro et al. 1995). Resistance arteries and capacitance veins are especially sensitive to ET-1 (Cocks, Broughton et al. 1989). Intravenous administration of ET-1 induces a transient and fast vasodilation, followed by a prolonged vasoconstriction (Yanagisawa, Kurihara et al. 1988), which in turn elevates systemic blood pressure by increasing total peripheral resistance (Rubanyi and Polokoff 1994). ET-3 can produce similar effects in a dose-dependent manner (Mortensen, Pawloski et al. 1990). The initial vasodilation is regulated through ETBRs on vascular endothelium by the release of endothelial cell derived vasodilators (Wright and Fozard 1988; Warner, de Nucci et al. 1989; Rubanyi and Polokoff 1994). ET-1-induced vasoconstriction is mediated by both ETARs and ETBRs located in VSMCs (Haynes, Strachan et al. 1995). ET-1 has powerful positive inotropic effects on the heart *in vitro* (Ishikawa, Yanagisawa et al. 1988). Coronary exposure to ET-1 may result in ventricular arrhythmias and myocardial ischemia (Ezra, Goldstein et al. 1989). A significant amount of ET-1 is generated by the collecting duct cells from inner medulla in the rat (Kohan and Fiedorek 1991). Both acute and chronic intracerebroventricular administration of ET-1 increases mean arterial blood pressure and heart rate through elevation of central sympathetic activity (Ouchi, Kim et al. 1989; Matsumura, Abe et al. 1991; Nishimura, Takahashi et al. 1991), indicating that ET-1 has a central pressor action. ET-1 also plays a role in the peripheral nervous system. Studies have demonstrated that ET binding sites are present in the baroreceptor and chemoreceptor afferent pathways. Afferent activity of baroreceptors and chemoreceptors can be affected by ETs (Spyer, McQueen et al. 1991). In human

arteries, endothelin-1 potentiates the effects of other vasoconstrictor hormones, such as serotonin and norepinephrine, suggesting that ET-1 amplifies the effects of sympathetic nervous system activity (Yang, Richard et al. 1990).

2.1.1.4 Endothelin in hypertension

Plasma concentrations of ET-1 in experimental models of hypertension are not increased unless malignant hypertension is present (Suzuki, Miyauchi et al. 1990; Kohno, Murakawa et al. 1991). Elevated circulating ET levels have been reported in some forms of human hypertension (Kohno, Yasunari et al. 1990; Shichiri, Hirata et al. 1990; Yoshibayashi, Nishioka et al. 1991). Several experimental models of hypertension have shown activated endothelin system, such as the deoxycorticosterone acetate (DOCA) salt hypertensive rats (Lariviere, Day et al. 1993; Lariviere, Thibault et al. 1993; Li, Lariviere et al. 1994), DOCA salt–treated spontaneously hypertensive rats (SHR) (Schiffrin, Lariviere et al. 1995), Dahl salt–sensitive rats (Doucet, Gonzalez et al. 1996), and 1-kidney 1-clip Goldblatt hypertensive rats. The vasoconstriction caused by ET-1 is more sensitive in hypertensive rats than in controls (Tomobe, Miyauchi et al. 1988; Suzuki, Miyauchi et al. 1990). In patients with essential hypertension, an increased vasoconstrictor response to ET-1 was reported, indicating enhanced vascular endothelin vasoconstrictor activity (Cardillo, Kilcoyne et al. 1999). In addition, ET-1-induced vasoconstriction of glomerular arterioles causes a decrease in glomerular filtration rate, renal plasma flow, and sodium excretion, as well as a significant increase in renal vascular resistance (Lopez-Farre, Montanes et al. 1989). Furthermore, in SHR rats less ET-1 is produced in the

renal medulla, where ET-1 mediates renal excretion of sodium and water through ETBRs, resulting in water/sodium retention and hypertension (Kitamura, Tanaka et al. 1989; Hughes, Cline et al. 1992). The results from experimental models are consistent with those from patients with essential hypertension, since these patients show less urinary excretion of endothelin than control subjects. ECE inhibitors are able to lower blood pressure in hypertensive rats (McMahon, Palomo et al. 1991; McMahon, Palomo et al. 1993; Nishikibe, Tsuchida et al. 1993; Pollock and Opgenorth 1993; Douglas, Gellai et al. 1994; Veniant, Clozel et al. 1994; Fujita, Matsumura et al. 1996). Endothelin receptor antagonists have also been shown to have anti-hypertensive effects (Nishikibe, Tsuchida et al. 1993; Douglas, Gellai et al. 1994; Fujita, Matsumura et al. 1996). Moreover, data from experiments in the dog indicate that the anti-endothelin therapies by ECE inhibitors and ET receptor blockade are mediated by independent pathways, since the effects induced by different categories of agents are additive (Donckier, Massart et al. 1997). Together these data indicate that ET-1 is involved in the pathogenesis of hypertension.

2.1.2 Catecholamines

Catecholamines are a group of hormones widely distributed in mammalian tissues. They include norepinephrine (NE), epinephrine (EPI), and dopamine. EPI is released by the adrenal medulla and it performs its functions mainly as a circulating hormone (Elliot 1905). EPI is a neurotransmitter of central nervous system (CNS) (Hokfelt, Fuxe et al. 1974), as well as of autonomic nervous system (Loewi 1936). A small portion of NE is released from adrenal medulla.

The majority of NE in the circulation is spillover from sympathetic nerves innervating blood vessels (Silverberg, Shah et al. 1978). NE is the primary neurotransmitter of mammalian sympathetic nervous system (Von Euler 1946). Dopamine is a precursor of NE and EPI. It is localized with them in various tissues (Bell 1983; Kopin 1985; Pierpont, DeMaster et al. 1985). It is an important central transmitter (Carlsson, Fuxe et al. 1966). It is involved in peripheral nervous regulation as well (Yorikane, Kanda et al. 1986; Bell 1987).

Tyrosine is the amino acid precursor for the biosynthesis of catecholamines (Axelrod 1968). It is transported into sympathetic neurons or chromaffin cells, and hydroxylated by thyrosine hydroxylase (TH), forming dopa (Chirigos, Greengard et al. 1960). This step is rate-limiting in the synthesis of NE and thus TH is critical in regulating NE synthesis. Then aromatic 1-amino acid decarboxylase transformed dopa into dopamine through the reaction of decarboxylation. Dopamine enters a chromaffin granule in adrenal medulla or a vesicle in sympathetic neurons. Upon the entrance, dopamine is hydroxylated on the ß position to form NE, by the enzymatic activity of dopamine-ß-hydroxylase. Finally, EPI is converted from NE by the enzyme phenylethanolamine methyltransferase (Axelrod 1962).

As a critical sympathetic neurotransmitter, the majority of NE is synthesized and stored in the vesicles of sympathetic nerves. Upon an action potential, there is an influx of Ca^{2+} into the nerve terminal. Then the vesicles containing NE fuse to the plasma membrane and NE is released from the vesicles by the process of exocytosis. The neurotransmitter then activates α- and

β-adrenergic receptors in the membrane of postjunctional cells. The termination of NE is achieved in part by two uptake systems: uptake 1 and 2. For uptake 1, the released NE in the junctional space is taken back into the nerves by high affinity transporters on the plasma membrane of sympathetic neurons. For uptake 2, NE binds to postjunctional vascular smooth muscle cells, where it is taken up by the corticosterone-sensitive extraneuronal monoamine transporter. The amount of released NE is tightly regulated by not only NE itself (via α2-adrenergic receptors on cell membrane of non-neuronal cells), but also many other mediators such as EPI, serotonin , histamine and acetylcholine (El-Etri, Ennis et al. 1999; Kulkarni, Opere et al. 2006). Normally, most of the NE released by sympathetic nerves is taken up by the nerves where it is metabolized. A small amount of NE, however, diffuses into the blood and circulates throughout the body. With a high sympathetic nerve activation, the amount of NE entering the blood increases significantly (Axelrod and Kopin 1969).

The release of sympathetic neurotransmitters, especially NE, is an index of sympathetic nerve activity (Doyle and Smirk 1955; Wallin, Delius et al. 1973). Measurement of NE spillover can be used for chronic studies of sympathetic nervous system activity in awake animals (Sano, Way et al. 1989). Additionally, this technique is feasible in human beings by the application of central venous catheters (Esler, Jennings et al. 1984; Hasking, Esler et al. 1986; Esler, Jennings et al. 1988). Although systemic NE spillover is very useful to indicate whole body neural tone, it can not be used to predict local sympathetic function in an organ-

specific manner, since NE is not evenly distributed among all organs (Korner 1971). Therefore, regional sampling of NE is a more precise method to quantify local sympathetic nervous activity (Robertson, Johnson et al. 1979). The actions of NE on VSMCs and myocytes of the myocardium are through the activation of β-adrenergic receptors, and α1- and α2-adrenergic receptors, resulting in vasodilation, vasoconstriction, and inotropy, depending on receptors and locations (Philipp, Brede et al. 2002; Brede, Nagy et al. 2003).

2.1.3 Calcitonin gene-related peptide

Capsaicin is a derivative of vanillyl amide (8-methyl-N-vanillyl-6-nonenamide) with a molecular weight of 305.32. It is a primary ingredient in the red hot chili peppers of the genus *Capsicum* (Holzer 1991). In the late 19th century, Hogyes was the first to report that the pungent action of an extract from *Capsicum* was mediated through sensory nerves (Hogyes 1878). Later on, capsaicin was shown to have a chronic blocking effect on sensory receptors (Jancso 1968) and destroy some sensitive nerves at a very high concentration (Jancso 1955). This group of nerves is termed "capsaicin-sensitive sensory nerves" (CSSNs). They are usually C fibers (with unmyelinated axons) and A fibers (with thinkly myelinated axons) (Maggi 1993).

The cardiovascular system is widely innervated by CSSNs (Mazzocchi, Malendowicz et al. 1992). CSSNs play important roles in blood pressure regulation by releasing vasoactive neuropeptides and therefore modulating peripheral resistance (Deng and Li 2005). Calcitonin gene-related peptide (CGRP), is a major neurotransmitter in CSSNs (Bell and McDermott 1996). It is a

37-amino acid neuropeptide found by cloning of the calcitonin (CT) gene in 1983 (Rosenfeld, Mermod et al. 1983). CGRP is generated by alternative splicing of the primary RNA transcript of the CT/CGRP gene (Rosenfeld, Amara et al. 1981). There are two isoforms of CGRP: α-CGRP and β-CGRP. α-CGRP is synthesized in neuronal tissues almost exclusively (Breimer, MacIntyre et al. 1988) and is widely distributed in the central nervous system and peripheral nervous system in both animals and humans (Oh-hashi, Shindo et al. 2001). The β-CGRP gene is believed to have arisen by gene duplication and these two isoforms exhibit nearly identical biological activities (Wimalawansa 1996). CGRP is synthesized in dorsal root ganglia where the cell bodies of sensory afferent neurons reside. These sensory nerves terminate peripherally on blood vessels and other tissues (Wimalawansa 1996). It is distributed at a higher density around blood vessels than on the heart (Wimalawansa 1996).

CGRP is a very potent vasodilator (Brain, Williams et al. 1985). Studies have shown that it is 100 to 1000 times more potent than substance P or acetylcholine. It has been suggested that CGRP may play an important role in regulating peripheral vascular tone and regional organ blood flow under physiological conditions (DiPette, Schwarzenberger et al. 1989). As to pathological conditions, It has been shown that systemic administration of CGRP lowers blood pressure in spontaneously hypertensive rats (Dipette and Wimalawansa 1995) and DOCA-salt rats (Supowit, Zhao et al. 1997) through vasodilation. Therefore, CGRP may attenuate blood pressure increase as an endogenous depressor substance. It plays a significant role in the initiation,

development, and maintenance of hypertension (Deng and Li 2005). Furthermore, ET-1 has been shown to induce the release of CGRP, most likely through an ETAR-based mechanism (Wang and Wang 2004).

2.1.4 Others

There are many other hormones that are important in blood pressure regulation. For example, the renin-angiotensin-aldosterone system (RAAS) plays an important role in regulating blood volume and vascular resistance, which in turn affects arterial pressure. Vasopressin (arginine vasopressin, AVP; antidiuretic hormone, ADH) is a peptide hormone formed in the hypothalamus, then transported via axons to, and released from, the posterior pituitary. It regulates extracellular fluid volume by affecting renal water excretion, leading to a decrease in urine formation, an increase in blood volume, cardiac output, and finally arterial pressure. On the other hand, some hormones favor blood pressure decrease, such as arterial natriuretic peptide (ANP) and brain-type natriuretic peptide (BNP). They not only dilate blood vessels, but also induce natriuresis and diuresis.

2.2 Vascular function

Vascular resistance is determined by both the structure and the function of blood vessels. Due to the differences between arteries and veins, each blood vessel type is discussed separately. Additionally, the splanchnic region, a critical bed in blood pressure regulation, is discussed.

2.2.1 Arterial bed

Arteries take blood away from the heart. Their walls are thick and enclose a small lumen. Aorta and large arteries mainly serve to distribute blood, while the small arteries and arterioles are the main vessels involved in blood pressure regulation as well as blood flow in various organs. These vessels are highly innervated by autonomic nerves (especially sympathetic nerves), and respond to changes in nerve activity and circulating hormones by constriction or dilation. These vessels are referred to as resistance vessels. Changes in the diameter of these resistance vessels lead to changes in total peripheral resistance and blood pressure.

2.2.2 Venous bed

Veins take blood to the heart. Their walls are thin and enclose a large lumen. They are not as elastic as arteries, because their walls contain mainly connective tissue and less smooth muscle than arteries. Veins and venules function as the primary capacitance vessels of the body, since most of the blood is stored in these vessels. Both experimental results (De Michele, Cavallotti et al. 1991) and clinical data (Fitzpatrick, Hinderliter et al. 1986) show that altered physiological functions of veins can contribute to the development and maintenance of "arterial" diseases. Unfortunately, much less experimental attention has been paid to veins, compared to arteries. Veins are usually viewed as simple, passive "conduits" for return of blood to the heart. In fact, they are complex physiological entities and important in overall function of the cardiovascular system (Rothe 1983). Veins contain about 70% of the blood volume, approximately 75% of which is in small veins and venules (Rothe 1983)

(Figure 3a). As such, they are the most important blood reservoir in the circulation.

Vascular compliance is defined as "the ratio of a change in volume to the concomitant change in transmural distending pressure" (Rothe 1993). It is a quantitative measure of the elasticity of a vascular bed. Systemic venous compliance is at least 30 times greater than that of arterial compliance in most species of mammals, including rats and humans (Pang 2001). Vascular capacity is the total blood volume held in the circulation at a specific pressure, as a sum of unstressed and stressed blood volumes (Shoukas and Sagawa 1973). Vascular capacitance is the relationship between contained volume and distending pressure of a segment of the vasculature. Since it is a relationship, capacitance can not be described by a simple number (Rothe 1993). Because the compliance of veins is much larger than that of arteries, and the cross-sectional area of venules is larger than that of arterioles, venules are frequently regarded as the primary capacitance component of the overall vascular tree. Therefore, total vascular capacitance is almost entirely determined by the structural and functional properties of veins. Constriction of veins can be very important in blood pressure regulation, since it leads to a redistribution of some stored blood from peripheral veins to the heart and then into the arterial circulation. As a result, the cardiac filling pressure and/or cardiac output increase (Halliwill, Minson et al. 1999) and increased arterial pressure occurs. There are several methods available to measure total body venous tone (constriction degree of veins). Mean circulatory filling pressure (MCFP), the effective driving force for venous return to

the heart, can be used as an index of venomotor tone and it is commonly used in experimental work. MCFP is usually, but not always, increased in hypertension (Martin, Rodrigo et al. 1998). The main factors determining MCFP are blood volume, venous smooth muscle structure and constrictor tone (Yamamoto, Trippodo et al. 1980). Safar *et al.* has indicated that blood volume is not changed, or even reduced, in established hypertension (Safar and London 1987). So either structural or functional changes of veins, or both, account for the increased MCFP.

2.2.3 The splanchnic bed – a critical bed for regulation of vascular capacitance

The splanchnic region, including liver, spleen, pancreas, small and large intestines, is the most important vascular bed to consider in studies concerning the role of veins in hypertension.

2.2.3.1 Veins and venules in the splanchnic bed

The splanchnic vascular bed is very compliant, especially the veins. In addition, the vein and venules in the splanchnic region account for most of the active capacitance responses of the circulation (Greenway and Lautt 1986; Rothe 1986; Rothe 1993). Moreover, this bed holds about 33% of the total blood volume (Figure 3b) (Greenway and Lister 1974). So the splanchnic veins act as the biggest modifiable reservoir of blood in the circulation.

Decreased venous capacitance associated with hypertension has been documented in animal models (Fink, Johnson *et al.* 2000) and humans (Schobel, Schmieder et al. 1993). This finding is especially evident in the splanchnic bed,

due to changes in smooth muscle activity (functional changes) and/or alterations in connective tissue content and/or organization of vasculatures (structural changes). The decrease in venous capacitance may lead to a blood shift from peripheral vascular beds to arterial circulation (Ricksten, Yao et al. 1981). As a result, the blood pressure increases. Additionally, Miura reported that TPR, renal vascular resistance, and hepatosplanchnic vascular resistance, were all increased in the hepatosplanchnic bed before the occurrence of vascular resistance elevation in other areas at the earliest stage of development in human hypertension (Sugawara, Noshiro *et al.* 1997), indicating the important role of the splanchnic circulation in blood pressure regulation and hypertension development.

2.2.3.2 Differential sympathetic innervation: arteries vs. veins

The splanchnic bed is richly innervated, especially by sympathetic nerves. Therefore, sympathetic control over the splanchnic vasculature may play a critical role in blood pressure regulation. The compliance and capacitance of the innervated splanchnic bed are altered in response to sympathetic stimulation or inhibition. The splanchnic sympathetic nerves projecting to the splanchnic bed arise from prevertebral and paravertebral ganglia. In rats, the cell bodies of a great majority of sympathetic postganglionic neurons innervating the splanchnic bed are located in two prevertebral ganglia: celiac ganglion and superior mesenteric ganglion (Trudrung, Furness et al. 1994; Hsieh, Liu et al. 2000; Quinson, Robbins et al. 2001). These two ganglia are very close to each other in

the rat and are usually termed the "celiac plexus" (Chevendra and Weaver 1991; Furness, Koopmans et al. 2000).

Although sympathetic activation constricts both arteries and veins, the sympathetic neurons innervating arteries and veins are differentially located in the ganglia and they show different electrophysiological properties, indicating there is a differential sympathetic neural control of mesenteric arteries and veins (Browning, Zheng et al. 1999). These results are consistent with the data from studies on the neuroeffector mechanisms in arteries and veins (Hottenstein and Kreulen 1987). Sympathetic nerve stimulation to veins produces a slow depolarization and contraction mediated by norepinephrine acting at α1-adrenergic receptors. On the other hand, in small mesenteric arteries and arterioles, electrical stimulation evokes short latency, short-duration excitatory junction potentials and contractions mediated by ATP acting at P2X receptors (Gitterman and Evans 2001).

Veins are more sensitive to vasoconstrictor effects of sympathetic nerve stimulation than arteries under physiological conditions (Kreulen 1986) and pathological conditions (Luo, Hess et al. 2003). Quantitatively speaking, sympathetic nerve activity (SNA) is the most critical factor controlling venoconstriction, especially in the splanchnic circulation (Shoukas and Bohlen 1990; Ozono, Bosnjak et al. 1991). Sympathetic activation of the splanchnic veins can reduce blood volume by up to 60%, redistributing blood from veins into the heart and thereby increasing blood pressure (Karim and Hainsworth 1976; Greenway 1983). The membrane potential of venous smooth muscle cells in

some hypertension models, such as SHRs, is relatively depolarized compared to that in normotensive rats. This change is due to increased sympathetic tone to veins in SHRs (Willems, Harder et al. 1982). Therefore the splanchnic SNA may have more influence on veins than on arteries in blood pressure regulation.

2.2.3.3 Increased splanchnic SNA in hypertension

The sympathetic nervous system (SNS) plays an important role in hypertension development in animal models (Wyss 1993; Cabassi, Vinci et al. 2002) and in humans (Anderson, Sinkey et al. 1989; Weber 1993; Esler, Rumantir et al. 2001; DeQuattro and Feng 2002; Zhu, Poole et al. 2005). The SNS can elevate blood pressure by increasing cardiac output and peripheral vascular resistance. It is generally found that sympathetic effects on the cardiovascular system are augmented in hypertension (Nestel 1969; Egan, Panis et al. 1987; Somers, Anderson et al. 1993; Esler and Kaye 1998). This could be caused by increased sympathetic nerve density, high sympathetic nerve firing rates, epinephrine cotransmission, norepinephrine transporter dysfunction, increased release of norepinephrine from the neuroeffector junction, increased end organ responsiveness to sympathetic neurotransmitters, or a combination of the above (Esler, Rumantir et al. 2001). Increased sympathetic neurotransmission has been documented in experimental hypertension models, such as DOCA-salt hypertension (Bouvier and de Champlain 1986) and spontaneous hypertension (Ekas and Lokhandwala 1981). Increased plasma norepinephrine (NE) levels are sometimes measured in these models, suggesting an enhanced neurotransmitter release from sympathetic nerves.

Clinically, antihypertensive drugs that reduce SNA may be the best treatment for hypertensive patients with autonomic imbalance (Brook and Julius 2000). Moreover, the SNS in the splanchnic bed is particularly important in blood pressure regulation, since the splanchnic SNS innervates the majority of resistance and capacitance vessels. The activation of the splanchnic SNS may increase total peripheral resistance, reduce vascular capacitance, and elevate blood pressure. Our lab and others show that blood pressure is significantly lowered when most splanchnic sympathetic innervation is removed (Kregel and Gisolfi 1989; King, Osborn et al. 2007). In humans, the removal of celiac plexus was performed as a treatment for human essential hypertension before antihypertensive medications were available (Heuer 1936; Martin 1938; Weiss 1939; Peet, Woods et al. 1940).

3. Oxidative stress

3.1 Introduction

Oxidative stress is a state where there is a disturbance in the prooxidant-antioxidant balance in tissues in favor of prooxidant species, leading to potential oxidative damage (Sies 1991). Reactive oxygen species (ROS) are a variety of oxygen-containing molecules that are byproducts of cellular metabolic processes under physiological conditions, including superoxide anion ($O2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl ion ($OH^{\cdot}$). Superoxide production is mediated by several enzymes including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, and nitric oxide synthase (NOS) (Wilcox 2002). In addition to direct effects, $O_2^{\cdot -}$ affects tissues indirectly by generating

other ROS. It can be converted, via enzyme superoxides dismutase (SOD), into H_2O_2, which is then converted into water by catalase and glutathione peroxidase, or into $OH^{\cdot}$ by interacting with reduced transition metals (Taniyama and Griendling 2003).

3.2 ROS in hypertension

Under physiological conditions, ROS are present at low concentrations. Originally, ROS were simply thought to be byproducts of some biological reactions taking place in multiple cellular components such as mitochondria (Balaban, Nemoto et al. 2005), peroxisomes (Schrader and Fahimi 2004), and cytochrome P-450 (Gottlieb 2003; Gonzalez 2005). However, emerging evidence has supported that ROS act as cellular signaling molecules (Zimmerman and Davisson 2004; Mueller, Laude et al. 2005; Bedard and Krause 2007). They achieve signaling functions by participating in phosphatase inhibition (Lee, Kwon et al. 1998; Wu, Hardy et al. 2003; Goldstein, Mahadev et al. 2005; Kwon, Qu et al. 2005), kinase activation (Griendling, Sorescu et al. 2000; Han, Kim et al. 2003; Furst, Brueckl et al. 2005), ion channel regulation (Hidalgo, Bull et al. 2004; Tang, Santarelli et al. 2004), and calcium signaling (Wang, Takeda et al. 1999; Cheranov and Jaggar 2006; Granados, Salido et al. 2006). Increased ROS levels have been found in blood vessels, kidneys and other tissues in many experimental models of hypertension, including DOCA-salt (Somers, Mavromatis et al. 2000), Dahl salt-sensitive (Swei, Lacy et al. 1999), angiotensin II hypertension (Kawada, Imai et al. 2002), and lead-induced hypertension (Vaziri, Liang et al. 1999). ROS generation also is elevated in hypertensive patients

(Touyz and Schiffrin 2001). Under pathological conditions, ROS may impair endothelium-dependent vasorelaxation (Somers, Mavromatis et al. 2000), induce VSMC growth (Griendling, Minieri et al. 1994; Zafari, Ushio-Fukai et al. 1998), elevate sympathetic nervous system activity (Campese, Ye et al. 2004), alter renal function, and raise deposition of extracellular matrix proteins. All of the above effects may contribute to pathophysiological changes, such as vascular and organ damages, in a variety of cardiovascular diseases (Harrison 1997). Accumulating evidence indicates that ROS play a role in the development and maintenance of hypertension (Touyz and Schiffrin 2004). Increased tissue ROS levels appear to cause hypertension, because blocking ROS generation or increasing their removal lowers blood pressure (Kopkan and Majid 2005; Sullivan, Pollock et al. 2006; Baumer, Kruger et al. 2007).

About 20% of the oxygen consumed by the body goes to the nervous system. Therefore, the nervous system is an important source of ROS. Emerging evidence has shown that ROS plays an important role in neuropathogenesis of cardiovascular diseases (Peterson, Sharma et al. 2006). Extensive research has been carried out to discover the role of ROS in the central nervous system (CNS) (Kishi, Hirooka et al. 2004; Lee, Shoji et al. 2004; Lu, Helwig et al. 2004; Wang, Anrather et al. 2004; Gao, Wang et al. 2005; Peterson, Sharma et al. 2006). Excessive production of ROS in the brain has been reported in many experimental models of hypertension (Zimmerman and Davisson 2004). The increased ROS levels in CNS may be crucial in the pathogenesis of hypertension (Griendling, Minieri et al. 1994; Zimmerman, Lazartigues et al. 2004). NAD(P)H

oxidase seems to be a major enzyme generating ROS in the brain. ROS plays an important role as a key mediator in the central effect of angiotensin II on cardiovascular regulation. Mechanisms of redox imbalance in CNS may be involved in the pathogenesis of hypertension (Peterson, Sharma et al. 2006). In addition, oxidative stress is important in the peripheral nervous system. Oxidative stress has been shown to inhibit baroreceptor activity, contributing to baroreceptor dysfunction in atherosclerosis (Li, Mao et al. 1996). Tempol, an antioxidant, lowers renal sympathetic nerve activity, heart rate, and mean arterial pressure in rats (Xu, Fink et al. 2001). The administration of diethyldithio-carbamic (DETC), an SOD inhibitor, increased renal superoxide levels (Zou, Li et al. 2001; Makino, Skelton et al. 2002) and peripheral sympathetic nerve activity (Shokoji, Fujisawa et al. 2004). Moreover, increased neuronal superoxide levels in an acute hypertension model are partially due to direct ETBR activation in sympathetic ganglia (Lau, Galligan et al. 2006).

3.3 Approaches to measure oxidative stress

ROS are highly reactive and relatively instable (half-life in seconds). Therefore it is difficult to measure ROS levels directly in biological systems. Measurements of ROS are mainly performed by indirect methods. Usually some "sensor" molecules are applied to target tissues. These sensors are oxidized by ROS in various cellular elements, such as lipids, protein, or DNA (Freeman and Crapo 1982; Pryor and Godber 1991; Hirota, Murata et al. 1999; Bowie and O'Neill 2000). These oxidized sensors bring out luminescent or fluorescent signals to reflect the concentrations of ROS.

Lucigenin assay has been used extensively for $O_2^{\cdot -}$ detection in many biological systems (Griendling, Minieri et al. 1994; Guyton, Liu et al. 1996; Rajagopalan, Kurz et al. 1996; Irani, Xia et al. 1997; Li, Zhu et al. 1998; Skatchkov, Sperling et al. 1999), due to the access to intracellular sites, small cellular toxicity, and $O_2^{\cdot -}$ selectivity (Tarpey and Fridovich 2001). This assay is based on the mechanism that lucigenin (Luc^{2+}) can be reduced to $LucH^{\cdot +}$, which is then oxidized by $O_2^{\cdot -}$ and elicit an unstable dioxetane component yielding light during the spontaneous decomposition to its stable-state electronic configuration (Faulkner and Fridovich 1993). It has recently been shown that several enzymatic systems *in vitro* may reduce Luc^{2+} even without the presence of $O_2^{\cdot -}$, generating $LucH^{\cdot +}$, which is then oxidized automatically (Liochev and Fridovich 1997). Therefore lucigenin is able to be chemically active in a "redox cycle" fashion. This is a major drawback in the use of this chemiluminescent substrate in the detection of $O_2^{\cdot -}$ (Liochev and Fridovich 1997).

Dihydroethidium (DHE) is a cell permeant compound. It can be oxidized by $O_2^{\cdot -}$ with a two-electron transfer, resulting in the formation of the fluorophore ethidium bromide which is DNA-binding (Benov, Sztejnberg et al. 1998). The reaction is relatively selective for $O_2^{\cdot -}$, since the oxidations induced by other oxidants, such as H_2O_2, $ONOO^-$, or hypochlorous acid, are minimal. The evaluation of intracellular fluorescent ethidium can be performed by flow cytometry (Carter, Narayanan et al. 1994). The oxidation can also be visualized with digital imaging microfluorometry (Bindokas, Jordan et al. 1996; Li, Fink et al. 2003; Dai, Galligan et al. 2004; Lau, Galligan et al. 2006). Unlike lucigenin, DHE

does not generate "artificial" $O_2^{\cdot -}$ through redox cycling. However, there are some limitations to the application of DHE as a quantitative detector for the production of $O_2^{\cdot -}$ (Benov, Sztejnberg et al. 1998). One is that cytochrome *c* is also able to oxidize DHE. This is critical when cytochrome *c* is released into the cytosol under certain conditions (Green and Reed 1998). In addition, quantification of $O_2^{\cdot -}$ production using DHE is semi-accurate because of its capacity to increase rates of $O_2^{\cdot -}$ dismutation to H_2O_2 (Benov, Sztejnberg et al. 1998). Last, a control is always required for comparison when the oxidation is evaluated by fluorescent images. Therefore, it is not a very convenient assay.

Other methods for $O_2^{\cdot -}$ detection include reduction of ferricytochrome *c* (Babior, Kipnes et al. 1973; Matsubara and Ziff 1986; Shingu, Yoshioka et al. 1989), adrenochrome Formation (Boveris 1984), cyanide-resistant oxygen consumption (Hassan and Fridovich 1979), etc. Additionally, H_2O_2 can be measured by horse radish peroxidase–linked assays (Boveris 1984), dichlorofluorescein fluorescence (Paul and Sbarra 1968), and intermediate complex (Sies 1981).

4. S6c-induced hypertension

4.1 Sarafotoxins

Sarafotoxins are a family of peptides that show a high degree of similarity in amino acid sequence of ETs. They were discovered in the venom of the Israeli burrowing asp, *Atractaspis engadensis* (Kochva, Viljoen et al. 1982; Takasaki, Tamiya et al. 1988). They are a group of four (sarafotoxin 6a, 6b, 6c, 6d) 21-amino-acid peptides, with a similar structure to ETs'. The sequence similarity

between these two families varies from 52-67% (Kochva, Bdolah et al. 1993). Both families have two disulfide bonds, a hydrophobic C-terminal, and three polar charged side chains (Sokolovsky 1992).

Among sarafotoxins, sarafotoxin 6c (S6c), a member of sarafotoxin family is very unique in that it is a selective agonist for ETBRs (Ambar, Kloog et al. 1989; Williams, Jones et al. 1991). S6c shows approximately the same binding affinity to ETBRs as ETs (Kloog, Bousso-Mittler et al. 1989; Wollberg, Bdolah et al. 1991), while its binding affinity to ETARs is much smaller than ET-1 and ET-2, which are ETARs selective binding ligands (Galron, Kloog et al. 1989; Williams, Jones et al. 1991). Therefore, S6c can be used as a selective ETBR agonist for pharmacological studies.

4.2 Pharmacology of S6c

Since S6c is a selective agonist for ETBRs, the pharmacological actions induced by S6c are mainly mediated through ETBRs. S6c has been shown to constrict isolated mesenteric veins in rats. On the other hand, S6c-induced arterial constriction in mesenteric bed is negligible (Johnson, Fink et al. 2002). Additionally, no constriction was demonstrated in isolated human mesenteric arteries (Clozel, Gray et al. 1992). Therefore, *in vitro* results indicate that S6c is a selective venoconstrictor.

Systemic *in vivo* pharmacology studies of S6c can not be performed in humans, due to ethical issues. However, a scientist was accidentally bitten by a Burrowing Asps *Atractaspis engadensis,* whose venom contains sarafotoxins,

during milking. He reported a rapid increase in blood pressure (Kurnik, Haviv et al. 1999). Several studies of local intravenous infusion of S6c to human hand veins have been conducted (Strachan, Haynes et al. 1995; Strachan, Crockett et al. 2000). All the data indicate that S6c causes veins to constrict *in vivo* although it is not as potent as ET-1.

In early 90s, Clozel *et al.* performed short-term infusions of S6c into intact rats and reported the following significant findings (Clozel, Gray et al. 1992). 1) Upon S6c infusion, there was a transient blood pressure decrease lasting a couple of minutes after the infusion was begun. Then blood pressure was increased for over 30 minutes. 2) S6c significantly lowered mesenteric blood flow, indicating vasoconstriction in the splanchnic region. As I mentioned earlier, they also showed that S6c did not cause direct arterial constriction. 3) The pressor effect was not due to ETBR activation in the central nervous system, release of catecholamines from the adrenal medulla, or increased plasma levels of ET-1. This is the earliest publication focused on S6c-induced cardiovascular effects in whole animals. My data are quite consistent with the above results. This earlier study provided critical support for the possibility of a hypertension model produced by chronic infusion of S6c. My studies were among the first to characterize this new model.

4.3 A novel hypertension model: chronic activation of ETBRs by S6c

As discussed in the section above on endothelin, ETBR activation causes different results at different locations. Global activation leads to a transient depressor response by release of endothelial cell derived vasodilators, diuresis,

and natriuresis, all of which should produce a decrease in blood pressure (Rubanyi and Polokoff 1994; Schiffrin 1998; Pollock, Allcock et al. 2000). To our surprise, studies from our laboratory showed that five-day infusion (5 pmol/kg/min, iv) of S6c into instrumented normal rats caused a marked and sustained increase in mean arterial pressure (S6c-induced hypertension) (Fink, Li et al. 2007). We believe venoconstriction contributes to S6c-induced hypertension, because as described earlier, *in vitro* S6c has been shown to constrict veins from most vascular regions but have little effect on most arteries (Evans, Cobban et al. 1999; Johnson, Fink et al. 2002; Perez-Rivera, Fink et al. 2005; Savoia and Schiffrin 2007). Furthermore, chronic administration of other relatively specific venoconstricting drugs also can increase arterial pressure (Rothe 1983; Greenway and Lautt 1986; Hainsworth 1986; Mortensen, Pawloski et al. 1990; Rothe 1993). Decreased venous capacitance has been documented in animal models (Manning, Coleman et al. 1979; Ricksten, Yao et al. 1981; Ackermann and Tatemichi 1983; Fink, Johnson et al. 2000) and in humans with hypertension (Ulrych 1976; Mark 1984; Safar and London 1985). Veins in the splanchnic region are probably the most important target for S6c and other venoconstrictors, since they account for most of the active capacitance responses of the circulation (Greenway and Lautt 1986; Rothe 1986; Rothe 1993). Veins in the splanchnic region hold about 33% of the total blood volume (Greenway and Lister 1974) and maximal activation can reduce their volume up to 60%, redistributing blood from the abdomen into the heart and thereby

increasing cardiac output and blood pressure (Karim and Hainsworth 1976; Greenway 1983).

It is likely, however, that other mechanisms contribute to S6c-induced hypertension. As reviewed earlier, the sympathetic nervous system plays an important role in hypertension development in both animal models (Wyss 1993; Cabassi, Vinci et al. 2002) and in humans (Anderson, Sinkey et al. 1989; Weber 1993; Zhu, Poole et al. 2005). My results show that partial removal of sympathetic innervation to the splanchnic bed attenuates S6c-induced hypertension, supporting the idea that sympathetic nerves are at least partially involved in S6c-induced hypertension (Li and Fink 2005). Additionally, as described above, increasing evidence has shown that ROS can function as cellular signaling molecules (Bedard and Krause 2007). Increased tissue ROS levels can cause hypertension, because blocking ROS generation or increasing their removal lowers blood pressure (Kopkan and Majid 2005; Sullivan, Pollock et al. 2006; Baumer, Kruger et al. 2007). Recent reports indicate that ETBR activation increases oxidative stress in sympathetic ganglia *in vitro* (Dai, Galligan et al. 2004) and *in vivo* (Lau, Galligan et al. 2006). Combined with the importance of sympathetic nervous system, it is possible that ROS signaling could enhance ganglionic neurotransmission and thereby increase sympathetic activity, although the precise effects of increased ROS levels in sympathetic ganglia are unknown. Therefore, it is reasonable to speculate that increased superoxide generation in sympathetic ganglia is involved in S6c-induced hypertension.

My research was designed to identify mechanisms of hypertension caused by sustained activation of ETBRs, with focuses on venous function, sympathetic nerve activity, and oxidative stress in the splanchnic bed of the rat.

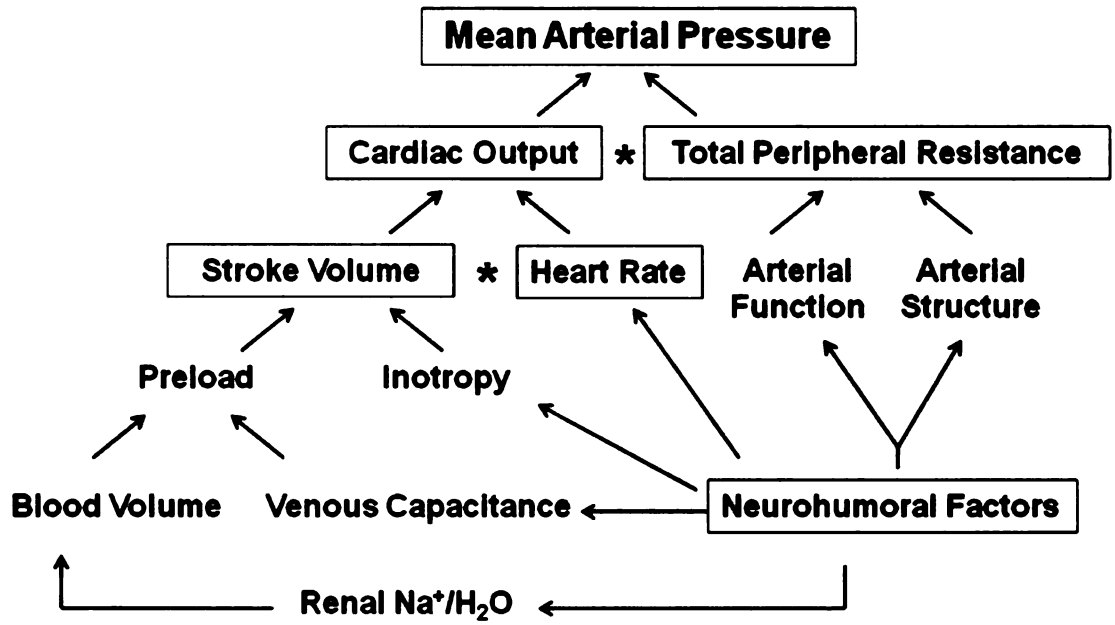

Figure 1: Factors mediating blood pressure. Details are discussed in GENERAL INTRODUCTIONS.

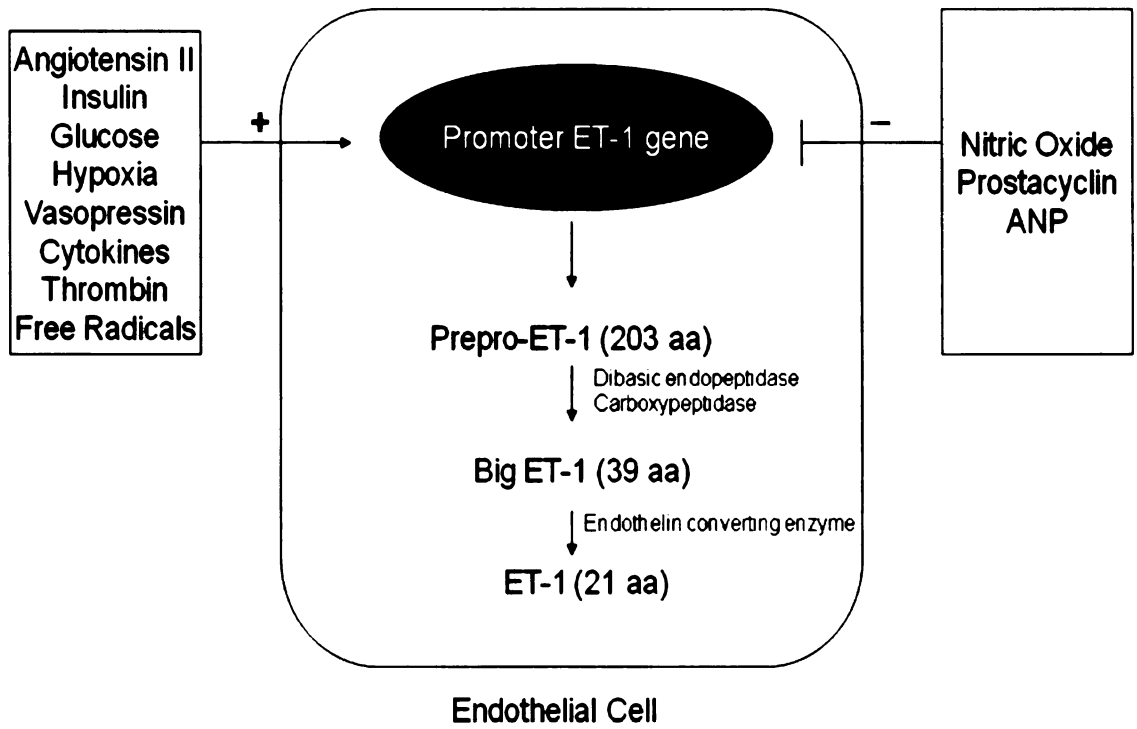

Figure 2: The proposed preteolytic processing pathway for conversion of preproendothelin to endothelin-1. The preproform (203-amino acid) of ET-1 is converted to 39-amino acid big ET-1 by dibasic endopeptidase and carboxypeptidases. Big ET-1 is then converted to 21-amino acid ET-1 by endothelin converting enzyme (Rubanyi and Botelho 1991). Many factors may stimulate or inhibit the expression of ET-1.

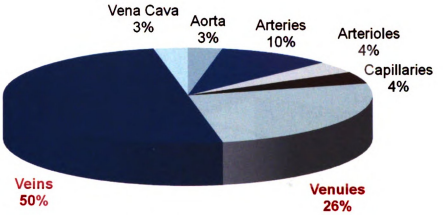

Figure 3a: Blood distribution in various blood vessels. Over 75% of total blood is stored in veins and venules.

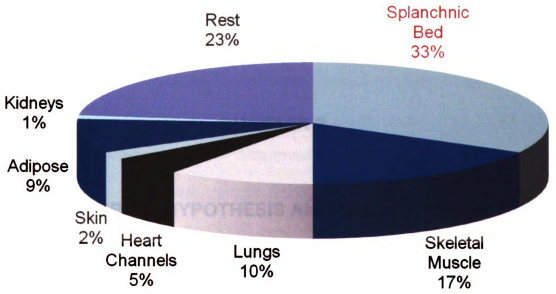

Figure 3b: Blood volume distribution in various regions. About one third of total blood volume is stored in the splanchnic bed.

CHAPTER 2

OVERALL HYPOTHESIS AND SPECIFIC AIMS

Recently, our lab has published a new hypertension model: sarafotoxin 6c-induced hypertension (Fink, Li et al. 2007). When conscious rats received S6c intravenously for 5 days, there was a significant increase in blood pressure and the hypertension was sustained at that level during the infusion period. It is believed that venoconstriction is involved in S6c-induced hypertension, because *in vitro* S6c has been shown to constrict veins in most vascular regions but very few arteries. Moreover, there is evidence indicating that ETBR activation increases sympathetic nervous system activity. This action also may contribute to S6c-induced hypertension. Recent data revealed that ETBR activation increases oxidative stress in sympathetic ganglia. On the other hand, vasodilators released from sensory nerves, such as CGRP may counterbalance the increase in blood pressure. The objective of this work was to identify neural mechanisms underlying S6c-induced hypertension. I proposed the hypothesis that S6c-induced hypertension is mediated by direct venoconstriction, increased sympathetic activity to the splanchnic region mediated by elevated ROS levels in sympathetic ganglia, and increased release of vasodilating neurotransmitters from sensory nerves. Figure 4 shows the overall hypothesis of the project.

Specific Aim I

Celiac ganglionectomy (CGX) was extensively used in my thesis project. The purpose of this specific aim was to evaluate the effectiveness of CGX in reducing the density and function of the splanchnic sympathetic innervation.

Specific Aim I-1: To test the hypothesis that CGX causes a nearly complete loss of sympathetic innervation to mesenteric arteries and veins.

Protocol I-1: Glyoxylic acid staining allows the sympathetic nerves to be visualized. Therefore, this technique was used to determine the distribution and density of sympathetic nerves innervating mesenteric arteries and veins.

Protocol I-2: Another way to visualize sympathetic nerve density is tyrosine hydroxylase staining, since sympathetic nerves contain tyrosine hydroxylase, a key enzyme for the synthesis of catecholamines.

Protocol I-3: Norepinephrine (NE) concentrations in tissues, including vessels and organs, were measured using high performance liquid chromatography as a more quantitative index of sympathetic innervation density.

Specific Aim I-2: To test the hypothesis that contractile responses to sympathetic nerve stimulation is significantly lowered by CGX in the splanchnic vasculatures.

Protocol I: CGX effectiveness was evaluated at a functional level. The sympathetic nerves on mesenteric arteries and veins from CGX rats were stimulated by two parallel wire electrodes linked to an electrical stimulator. The percentage of blood vessel constriction was measured as an indicator of sympathetic neurotransmission.

Specific Aim II

The focus of this specific aim was to identify if sympathetic activity and oxidative signaling in the splanchnic sympathetic ganglia are involved in S6c-induced hypertension.

Specific Aim II-1: To test the hypothesis that S6c-induced hypertension is in part by increased sympathetic nervous system activity.

Protocol I: Circulating NE level is an indicator of global sympathetic nerve activity (SNA). So plasma NE concentrations were measured to determine if there is an elevated SNA in S6c-induced hypertension.

Protocol II: CGX is an effective procedure to remove most splanchnic sympathetic innervation. So it was used in S6c-induced hypertension to determine if increased sympathetic activity to the splanchnic organs is a critical mechanism causing hypertension in this model.

Specific Aim II-2: To test the hypothesis that reactive oxygen species levels in neurons and vessels are increased with chronic activation of ETBRs.

Protocol I: Dihydroethidium (DHE), a fluorescent dye, was used to measure superoxide levels in various tissues, including blood vessels and sympathetic ganglia, after either 1 day or 5 days of S6c infusion.

Protocol II: Lucigenin chemiluminescence is a quantitative method to measure superoxide concentrations. Again, a time course study was carried out to test if increased levels of ROS occur in blood vessels in a time-dependent manner in S6c-induced hypertension.

Specific Aim II-3: To test the hypothesis that increased superoxide levels in sympathetic ganglia or blood vessels contribute to hypertension development in S6c-induced hypertension.

Protocol I: Tempol (4-hydroxy 2,2,6,6,-tetramethyl peperidine 1-oxyl), a superoxide dismutase mimetic, was used to test if increased blood pressure in S6c-infused rats requires increased tissue levels of superoxide. Both hemodynamic parameters and superoxide levels in sympathetic neurons were evaluated.

Specific Aim III

Endothelin-B receptor (ETBR) transgenic spotting lethal rat model (Tg (sl/sl)) is a unique animal model to study the roles of ETBRs in sympathetic nerves. In this model, ETBRs are not functional in endothelium, renal tubular

cells, or vascular smooth muscles. They function only at the places where dopamine-ß-hydroxylase is expressed, including sympathetic nerves. These rats are hypertensive. The aim of this specific aim was to discover the mechanisms underlying the hypertension in Tg (sl/sl) rats, with a focus on the sympathetic nerves. Since Tg (+/+) rats have 70 more copies of the ETBR gene than their control strain, Wistar Kyoto (WKY) rats (the genetic background of these transgenic rats) were also used to determine the differences between natural wildtype and transgenic wildtype.

Specific Aim III-1: To confirm others' finding that the circulating ET-1 levels in Tg (sl/sl) are higher than in Tg (+/+) rats.

Protocol I: Endothelial ETBRs function as endogenous "clearance receptors" for ET-1. In Tg (sl/sl) rats, ETBRs are not functional in endothelium. Therefore, circulating ET-1 in Tg (sl/sl) can not be cleared as much as in normal rats. An increased ET-1 level has been reported by several labs. Blood pressure could be increased by a higher ET-1 concentration through endothelin A receptor activation. Therefore ET-1 in the circulation was measured by a quantitative sandwich enzyme immunoassay.

Specific Aim III-2: Numerous studies have shown that elevated sympathetic nerve activity may mediate hypertension. The goal of this specific aim was to test

The hypothesis that hypertension in Tg (sl/sl) rats is mediated by increased sympathetic activity.

Protocol I a: Sympathetic nerve density was evaluated by glyoxylic acid staining which makes sympathetic nerves visible.

Protocol I b: A quantitative way to determine sympathetic nerve density is to measure norepinephrine (NE) concentrations in tissues. For this study, NE levels were evaluated by high performance liquid chromatography.

Protocol II: Vasoconstrictor responses to the sympathetic nerve activation were compared in tertiary mesenteric arteries and veins from Tg (sl/sl) and Tg (+/+) rats.

Specific Aim IV

This specific aim was to test the hypothesis that S6c-induced hypertension is caused at least in part by activation of ETBRs in sympathetic neurons.

Specific Aim IV-1: To test the hypothesis that S6c infusion causes hypertension through activation of ETBRs in sympathetic neurons.

Protocol I: Hemodynamic parameters, including systolic blood pressure, diastolic blood pressure, mean arterial pressure, pulse pressure, heart rate, and activity, were compared in Tg (+/+) and Tg (sl/sl) rats, during S6c infusion.

Specific Aim IV-2: To test the hypothesis that ETBRs in the splanchnic sympathetic nervous system play an important role in the development and the maintenance of S6c-induced hypertension.

Protocol I: After a recovery from CGX for two weeks, hemodynamic responses were compared in Tg (+/+) and Tg (sl/sl) rats as in Specific Aim IV-1.

Specific Aim V

There is good evidence suggesting that the sensory nervous system plays a role in blood pressure regulation. The focus of this specific aim was to determine the involvement of sensory nerves in S6c-induced hypertension.

Specific Aim V-1: To test the hypothesis that the sensory nervous system plays a role in S6c-induced hypertension.

Protocol I: Capsaicin-treated neonatal rats are characterized by loss of capsaicin-sensitive sensory nerves. These rats were treated with S6c, followed by hemodynamic measurements. The role of the sensory nervous system was determined by the idea of "loss of function".

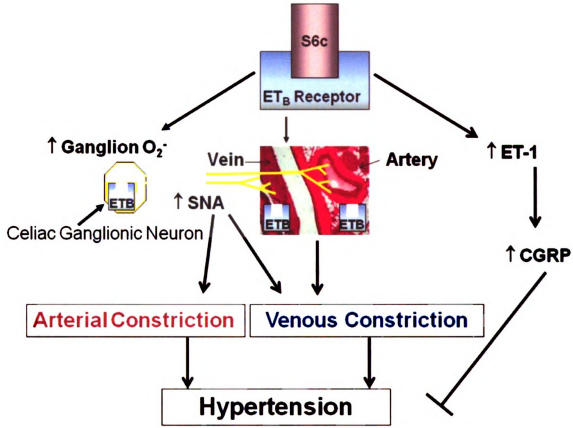

Figure 4: Overall hypothesis of the project. ETBR activation in the splanchnic bed increases venoconstriction directly, and sympathetic nerve activity via increased ganglionic oxidative stress. Both effects contribute to increased arterial blood pressure. ETBR activation leads to enhanced CGRP release from sensory nerves, which attenuates the blood pressure increase by causing local vasodilation.

CHAPTER 3

GENERAL METHODS

1. Animals

All animal protocols were approved by the All University Committee on Animal Use and Care of Michigan State University.

1.1 Sprague-Dawley rats and WKY rats

Normal male Sprague-Dawley rats (300-325 g, Charles River, Portage, MI, USA) or/and WKY rats (male: 300-325 g; female: 210-250 g, Charles River) were used. Before the experiments, all rats were housed 2 or 3 per cage in a temperature and humidity controlled room with a 12h on/12 off light cycle. Pelleted rat chow (8640 Rodent Diet; Harlan/Teklad) and water were given ad libitum.

1.2 Transgenic spotting lethal (Tg (sl/sl)) rats

Rats carrying transgenic gene of ETBRs whose expression is driven by the human dopamine-β-hydroxylase (*DβH*) promoter were generated. The transgenic rats were crossed with the spotting lethal rats to breed transgenic spotting lethal (Tg (sl/sl)) animals that carry functional ETBRs only regulated by *DβH* promoter, as previously described (Gariepy, Williams et al. 1998). Two pairs of transgenic (Tg) heterozygous ETBR-deficient rats (two males and two females, Tg (sl/+)) were sent to Michigan State University from University of Michigan animal facility. After each pair's mating, baby rats were born and nursed for three weeks. The newborn rats were genotyped immediately post-weaning by polymerase chain reaction on DNA extracted from tail biopsy specimens. Experiments were started when the animals were approximately 12 weeks of age.

1.3 Neonatal capsaicin-treated rats

Pregnant Wistar female rats (Charles River Laboratories Inc, Wilmington, Massachusetts, USA) were ordered and housed. On the first or second day after birth, new born rats were injected with capsaicin (50 mg/kg, 5 mg/ml in 5% ethanol, 5% Tween 80 in saline, subcutaneously). Control rats received vehicle solution (5% ethanol, 5% Tween 80 in saline) of the same volume. The injections were given while rats were anesthetized by ether. After nursing for three weeks, male and female rats were separated and weaned. Male rats were used in the experiments. Pelleted rat chow (8640 Rodent Diet; Harlan/Teklad) and water were given ad libitum.

2. Vascular catheterization

Either sodium pentobarbital (30-50 mg/kg plus 0.4 mg atropine sulfate, i.p.) or isoflurane was used for anesthesia. One polyvinyl catheter with a silicone rubber tip was inserted into the abdominal aorta via a femoral artery for drawing blood samples and hemodynamic measurements. A similar catheter was placed into a femoral vein for drug administration. Free ends of the catheters were passed through a stainless steel spring tether attached to a plastic harness fixed around the chest of the rat. Rats were allowed to recover consciousness on a heated pad under constant observation. When they became conscious, they were placed in individual cages allowing continuous access to both catheters without handling or otherwise disturbing the rats. Acetaminophen was given for 3 days after the surgery to relieve surgical pain. Arterial lines were filled with a

heparin-saline solution and occluded when not in use. Rats were allowed at least three days for recovery after the surgery before measurements were begun.

3. Radiotelemetry implantation

3.1 *In intact rats*

An opening was cut in left thigh area. A pocket was made by separating the skin and muscle in the abdomen region. The rediotelemetry transmitter's body was placed subcutaneously in the abdomen area. The tubing of the transmitter was pulled to the left thigh and the tip of the tubing was inserted into the abdominal artery through left femoral artery. Every animal was placed in an individual cage above a heating pad for recovery. Then they were transferred to the telemetry animal room, where each cage was put on a radiotelemetry receiver (RPC-1, Data Sciences International). After a recovery for one week, transmitters were turned on for recording.

3.2 In celiac ganglionectomized rats

Transmitter implantation was performed right after CGX was completed. The body of the transmitter was placed in the abdominal cavity. Several sutures were made to secure the position of the transmitter's body. The catheter of the transmitter was pulled subcutaneously to the opening in the left thigh area. Catheterization was carried out in the similar way as introduced above in intact animals. Animals rested ten days for recovery.

4. Hemodynamic measurements

4.1 By external blood pressure analyzer

Arterial pressure was determined by connecting the arterial catheter to a low-volume displacement pressure transducer (TXD-300, Micro-Med, Louisville, Kentucky, USA) linked to a digital pressure monitor (BPA-200 Blood pressure Analyzer, Micro-Med), which measures systolic, mean, and diastolic pressures, and heart rate every 0.5 second (sample rate = 1000 Hz). The transducer was zeroed at mid-chest level of rats in a typical crouched posture and was calibrated every day against a column of water. Hemodynamic parameters were recorded for 30 minutes each morning between 10:00 AM - 12:00 PM without handling or otherwise disturbing the rat sitting quietly in its home cage. All data were averaged minute-by-minute and saved using a computerized data acquisition system (DMSI-400 System Integrator, Micro-Med).

4.2 By telemetry

The signals from transmitters were monitored by radiotelemetry receivers (RPC-1, Data Sciences International) which were connected to a data exchange matrix. Data, including systolic pressure, diastolic pressure, mean arterial pressure, pulse pressure, heart rate, and activity, were collected every 10 minutes during the entire period of the experiments. Data acquisition and analysis were performed by custom software Dataquest ART 4.0 (Data Sciences International).

5. Minipump implantation

S6c solution was made and transferred into micro-osmotic pumps (Alzet, Model 1007D). A small opening was made between the scapulae and a pocket was opened for the subcutaneous implantation of the minipump with the delivery

port oriented towards the lower body. The drug was delivered at the rate of 30 pmol/kg/min.

6. Celiac ganglionectomy (CGX)

The surgery was conducted on a heating pad to maintain the animals' body temperature. The abdomen was opened through a midline laparotomy. The small intestine was gently moved aside from the midline and covered with saline-soaked gauze. The celiac ganglion area was exposed. Cotton swabs were used to rub off the fat covering the celiac plexus and main vessels (eg. aorta, celiac artery, and mesenteric artery) in the vicinity. Visible nerves around the three arteries were removed as completely as possible by stripping. The small intestine was put back to the original position. A ticarcillin solution was applied to the organs and the abdomen was closed in two layers with interrupted sutures. For sham rats, the small intestine was moved out of the body. Only fat was rubbed off and nerves stayed intact. Surgical recovery was monitored with the rats on a heated table. After the rats awakened, they were fed with normal food and tap water for one week during recovery from surgery. Acetaminophen was given for 3 days after the surgery to relieve surgical pain. We have shown previously that this procedure results in effective denervation of the splanchnic organs, as indicated by > 90% depletion of norepinephrine content of liver, spleen and small intestine, while causing only limited denervation of the kidneys [King and Fink, 2007].

7. Inferior mesenteric ganglionectomy (IMGX)

The procedure was similar to that described for CGX. The only difference was that inferior mesenteric ganglion, instead of celiac ganglion, was removed using fine scissors. Nerves around the ganglion and blood vessels were stripped as well.

8. *In vitro* preparation of mesenteric vessels

Small intestine was removed and placed in oxygenated (95% O_2, 5% CO_2) Krebs physiological saline. A segment of ileum with associated mesenteric vessels was removed and pinned flat in a dish. A section of third-order mesenteric vessel was separated and transferred to a small recording bath chamber and pinned flat. Surrounding tissue around the vessel was cleaned by fine scissors and forceps. The chamber was mounted on the stage of an inverted microscope (Olympus CK-2; Leco, St Joseph, Michigan, USA). The vessel was superfused with 37°C Krebs solution at a flow rate of 7 ml/min for 20 min, and allowed to relax to a stable resting diameter.

9. Video monitoring of vessel diameter

The output of a black-and-white video camera (Hitachi model KP-111; Yokohama, Japan) attached to the microscope was fed to a PC Vision Plus frame-grabber board (imaging Technology Inc., Woburn, MA) mounted in a personal computer. The video images were analyzed using computer software DiamTrack (Adelaide, Australia). The digitized signal was converted to an analog output (DAC-02 board; Keithley Megabyte, Tauton, MA) and fed to a chart recorder (EZ Graph; Gould Inc., Cleveland, OH) for a record of vessel diameter. Diameter changes as small as 1.8 μm could be resolved.

10. Transmural stimulation of perivascular nerves

Two parallel silver/silver chloride wire electrodes were positioned parallel with the longitudinal axis of mesenteric vessels. The electrodes were linked to an electrical stimulator (Grass Medical Instruments, S48). For each stimulation frequency, a train of 30 stimuli were given, with stimulus duration of 0.5 ms and at the voltages ranging from 70 to150 volts. The neurogenic nature of the constriction induced by electrical stimulation was confirmed by showing a complete blockade of constriction at 20 Hz by applying tetrodotoxin (TTX, 0.3 μM). The tissue was discarded if the constriction at 20 Hz was not completely blocked by TTX. The peak constriction at each given frequency was recorded. Measurements were made using DiamTrack software.

11. Plasma sampling

Blood samples (0.5 ml or 1 ml, depending on the studies) were drawn from the arterial or venous catheter over twenty-five μl of EGTA/glutathione solution into ice-chilled plastic syringes and transferred to ice-chilled plastic tubes. Blood samples were centrifuged at 10,000 g for 5 minutes at 4°C. Plasma was separated and stored in a -80°C freezer for later assays.

12. Plasma catecholamine measurements

Plasma NE was measured by a radioenzymatic assay based on the technique proposed by Peuler and Johnson (Peuler and Johnson, 1977). The principle of this method is the conversion of NE and EPI to tritiated normetanephrine and metanephrine in the presence of catechol-O-methyltransferase and tritiated S-adenosylmethionine as a labeled methyl donor.

After purification through a series of organic extractions, tritiated normetanephrine was separated from the derivatives of dopamine by oxidation with sodium periodate. Tritiated vanillin then was counted in a liquid scintillation spectrometer.

13. DHE fluorescence

Dihydroethidium (DHE), an oxidative fluorescent dye, was used to measure superoxide ($O_2^{\cdot -}$) levels in mesenteric arteries, mesenteric veins, and inferior mesenteric ganglion (IMG) (Miller, Gutterman et al. 1998); (Millette, de Champlain et al. 2000). Rats were sacrificed with pentobarbital sodium (ip). Mesenteric vessels and IMG were removed from each rat. Blood vessels were placed into oxygenated Krebs buffer at 4°C, dissected free of loosely adhering tissue, and cut into 3- to 4-mm-wide ring segments. Unfixed frozen ring segments were cut into 25-µm-thick sections using a cryostat and placed on a glass slide. DHE ($5*10^{-6}$ mol/l) was topically applied to each tissue section. Slides were incubated in a light-protected humidified chamber at 37°C for 60 minutes for vessels and 45 minutes for IMG. Fluorescent images were observed with an Olympus Fluoview laser scanning confocal microscope mounted on an Olympus BW50WI upright microscope, equipped with krypton/argon lasers. A 488-nm argon laser line was used to excite DHE fluorescence, which were detected with a 585-nm long-pass filter. Fluorescence was quantified by software Image J. Individual cells (sympathetic neurons in inferior mesenteric ganglia and vascular smooth muscle cells in blood vessels) were individually selected. The intensity of red fluorescence in the selected area was expressed as a number. The actual

intensity was the difference between the value of selected area and the value of background. Five to ten cells were selected in each image. The average value of all the selected cells represented the fluorescent density of the target tissue in one animal.

14. Lucigenin-enhanced chemiluminescence

Vascular $O_2^{\cdot -}$ was quantified by lucigenin chemiluminescence. Isolated vessel segments were cleaned and incubated for 30 minutes in modified Jude Krebs buffer at 37°C in the presence of 10 mM diethyldithiocarbamate. Vessels were transferred to small tubes which contained 5 μM lucigenin in modified Krebs-HEPES buffer and incubated for 10 minutes at 37°C in the dark. After incubation, tubes were put into a luminometer (TD-20e; Turner Designs, Sunnyvale, CA). Luminescence measurements were integrated for 30s periods. Ten repeated measurements were then averaged. After 10 cycles, the cell-permeant $O_2^{\cdot -}$ scavenger tiron (10 mM) was added, and 15 more cycles were read. The last 8 values, which were maximally reduced, were averaged. Data were calculated as the change in the rate of luminescence per minute per milligram of tissue of values before and after tiron and then converted to $O_2^{\cdot -}$ (nmol · min^{-1} · mg $tissue^{-1}$) (Cifuentes, Rey et al. 2000); (Li, Fink et al. 2003).

15. Glyoxylic acid staining

Tertiary mesenteric arteries and veins were removed and placed in normal PBS. After surrounding fat was trimmed off, blood was flushed out with PBS (0.1 M, pH 7.4) through a 30 ga. hypodermic needle and syringe. Three mesenteric arteries and three mesenteric veins were randomly selected in each rat and five

rats were used. Blood vessels were incubated in 2% glyoxylic acid solution (in 0.2 M phosphate buffer, pH=7) for 5 minutes at room temperature. Blood vessels were then mounted on a microscope slide and placed in an oven (80°C) for 5 minutes. After being heated, blood vessels were mounted in mineral oil and cover-slipped. Catecholamine fluorescence was viewed by a fluorescence microscope (Nikon Eclipse TE 2000-U) equipped with a filter set, UV2E/C (excitation filter, 340-380 nm and emission, 435-485 nm).

16. Immunohistochemical staining of tyrosine hydroxylase and CGRP

Mesenteric arcades were pinned in a Sylgard-lined petri dish. A 30-gauge hypodermic needle filled with PBS (PBS, 0.01 M, pH 7.2) was used to flush blood out of mesenteric vessels by cannulating the primary artery or vein. The tissues were immersed in Zamboni fixative (4% formaldehyde, 2% picric acid in 0.1 M phosphate buffer, pH 7.4) at 4 °C for 24 hours. The tissues were then washed three times at 10-min intervals with dimethyl sulfoxide followed by 3 washes at 10-min intervals with PBS. Arteries and veins were then dissected from mesenteric fat and incubated overnight with primary antibody. We used a mouse anti-tyrosine hydroxylase (TH) antibody (1:200 dilution in PBS, Calbiochem, San Diego, Ca) to localize immunoreactivity for TH and a rabbit polyclonal anti-CGRP antibody (1:200 in PBS, Chemicon, Temecula, CA) for CGRP. After incubation with primary antibody, tissues were washed 3 times in PBS and then incubated with Cy3 AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG (1:200 dilution in PBS; Jackson Immunoresearch Laboratories, West Grove, PA) and goat anti-rabbit fluorescein isothiocyanate (1:40 dilution in PBS; Sigma-Aldrich, St. Louis,

MO) for 1 hour at room temperature. The tissues were washed 3 times with PBS and then mounted on microscope slides and coverslipped using buffered glycerol (pH 8.6). Fluorescent images were acquired by a confocal microscope.

17. Measurement of norepinephrine levels in mesenteric vessels

Mesenteric arcades were pinned in a Sylgard-lined petri dish. A 30-gauge hypodermic needle filled with PBS (PBS, 0.01 M, pH 7.2) was used to flush blood out of mesenteric vessels by cannulating the primary artery and vein. Fat around vessels was cleaned. Three pieces of one-centimeter-long segments of tertiary mesenteric arteries and veins were collected, put into 0.1N perchloric acid solution, and stored at -80°C for later measurement. NE level was measured by high performance liquid chromatography (HPLC) as previously described (Luo, Hess et al. 2003). Briefly, tissues were thawed and sonicated for membrane disruption. Twenty-five µl supernatant was injected into a C-18 reverse-phase analytical column, which was coupled to a single colorimetric electrode-conditioning cell in series with dual-electrode analytical cells. The content of NE was determined by comparing maximal heights to those of standards in the same day. NE concentrations in vessels were expressed as NE (in picogram)/protein (in grams). The protein content of blood vessels was measured by using folin phenol reagent (Lowry, Rosebrough et al. 1951).

18. Norepinephrine measurement in the splanchnic organ tissues

The splanchnic organs were harvested, weighed, frozen, and stored at -80°C. Perchloric acid was added to the collected tissues (0.1 M perchloric acid solution, 2ml/0.5g). Then the tissues were homogenized. The homogenate was

centrifuged at the rate of 12000 rpm for 15 minutes at 4°C. The supernatant was collected. The supernatant was transferred into new tubes, followed by another centrifuge at the same speed and temperature for 10 minutes. The final supernatant was collected by a syringe. About 0.3 ml of the final supernatant was placed in top compartment of a 30 KD filtration tube and it was centrifuged twice at 13000 rpm for 5 minutes at 4°C. The filtrate was placed into a clean microtube and NE content was measured by HPLC, as described in NE measurement in mesenteric vessels. NE concentration in organs was expressed by NE (in picogram)/tissue (in grams).

19. Plasma endothelin-1 measurement

Plasma ET-1 level was measured by a quantitative sandwich enzyme immunoassay using an ELISA kit (Human Endothelin-1 Immunoassay,QET00B, R & D Systems, Minneapolis, MN). A 96-well microplate pre-coated with a monoclonal antibody specific for ET-1 was provided in the kit. One hundred µl standards or EDTA-plasma was pipetted into each well. The plate was then covered and incubated for 1.5 hours at room temperature on a horizontal orbital microplate shaker set at 500 rpm. Any ET-1 in the standards or samples was bound by the monoclonal antibody. After the incubation, each well was washed four times to eliminate any unbound substances. Two hundred µl enzyme-linked monoclonal antibody specific for ET-1 was added to each well. The plate was covered and incubated for 3 hours on the same shaker at room temperature. Each well was again washed four times to remove any unbound antibody-enzyme reagent. Then two hundred µl enhanced luminol/peroxide substrate

solution was added to each well, followed by an incubation for 30 minutes at room temperature on the benchtop. The microplate was wrapped by aluminum foil for light protection. Light was produced in proportion to the amount of ET-1 bound in the initial step. A microplate luminometer was used to measure the intensity of the light omitted.

20. Animal Euthanasia

Rats were euthanized by administration of sodium pentobarbital (100 mg/kg, ip.), which is a commonly used method for euthanasia. This approach is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

21. Analysis of the data and statistics

Data were shown as means ± standard error. A p value less than or equal to 0.05 was considered statistically significant. Different comparison methods were used depending on the studies. The data were analyzed by GraphPad InStat and Prism. The details are described in each chapter.

CHAPTER 4

THE EFFECTS OF CELIAC GANGLIONECTOMY ON SYMPATHETIC INNERVATION TO THE SPLANCHNIC ORGANS IN THE RAT

INTRODUCTION

The splanchnic vascular bed (liver, kidneys, spleen, and small and large intestines) holds about one third of the total blood volume (Greenway and Lister 1974; Greenway 1983). Therefore the splanchnic vasculature acts as the biggest single reservoir of blood in the circulation. Small veins and venules are considered as "capacitance vessels" since they are responsible for most of the active capacitance responses of the circulation (Greenway and Lautt 1986; Rothe 1986; Rothe 1993). A decreased venous capacitance in hypertension has been reported in experimental models (Ferrario, Page et al. 1970; Smith and Hutchins 1979) and humans (Schobel, Schmieder et al. 1993). This decrease in venous capacitance will lead to a blood shift from peripheral vascular beds to the heart and ultimately in part into the arterial circulation (Ricksten, Yao et al. 1981). As a result, blood pressure increases. It has also been reported that hepatosplanchnic vascular resistance is increased before the occurrence of vascular resistance increases in other vascular beds during the development of human hypertension (Sugawara, Noshiro et al. 1997). This suggests that increased arterial and venous tone in the splanchnic region may be important factors causing hypertension.

The sympathetic nervous system plays an important role in hypertension development both in animal models (Wyss 1993; Cabassi, Vinci et al. 2002) and in humans (Anderson, Sinkey et al. 1989; Weber 1993; Zhu, Poole et al. 2005). Generally, it is found that sympathetic effects on the cardiovascular system are augmented in hypertension (Nestel 1969; Egan, Panis et al. 1987; Somers,

Anderson et al. 1993; Esler and Kaye 1998). This could be caused by increased sympathetic nerve density, high sympathetic nerve firing rates, epinephrine contransmission, norepinephrine transporter dysfunction, increased release of norepinephrine from the neuroeffector junction, increased end organ responsiveness to sympathetic neurotransmitters, or a combination of the above (Esler, Rumantir et al. 2001).

Stimulation of the sympathetic nerves induces arterial and venous constrictions (Brooksby and Donald 1971; Furness and Marshall 1974; Karim and Hainsworth 1976; Ozono, Bosnjak et al. 1989). This sympathetic regulation is very critical in cardiovascular hemodynamics by controlling resistance, capacitance, blood flow, and blood pressure in physiological conditions (Mellander 1960; Brooksby and Donald 1971; Rothe 1983; Pang 2001) and hypertension (Somers, Anderson et al. 1993; Johansson, Elam et al. 1999; Schlaich and Esler 2003; Schlaich, Lambert et al. 2004). The vascular bed in the splanchnic region, including arteries and veins, is densely innervated by the sympathetic nervous system. Sympathetic nerve activity (SNA) is a very critical factor controlling vasoconstriction in the splanchnic circulation (Shoukas and Bohlen 1990; Ozono, Bosnjak et al. 1991). For example, a norepinephrine spillover study has shown that a big fraction of total body sympathetic outflow goes to mesenteric organs (Aneman, Eisenhofer et al. 1996). Thus, increased SNA to the splanchnic circulation may be a common component in the development and maintenance of hypertension.

Sympathetic nerves project to the splanchnic blood vessels via the prevertebral and paravertebral ganglia. In rats, the cell bodies of a great majority of the sympathetic postganglionic neurons innervating the splanchnic bed are located in two prevertebral ganglia: the celiac ganglion and superior mesenteric ganglion (Trudrung, Furness et al. 1994; Hsieh, Liu et al. 2000; Quinson, Robbins et al. 2001). These two ganglia are very close to each other in the rat and are usually termed "the celiac plexus" (Chevendra and Weaver 1991; Furness, Koopmans et al. 2000). Since sympathetic activity through the celiac plexus is an important pathway in blood pressure regulation, a surgical removal (celiac ganglionectomy, CGX) can help to reveal the roles of the splanchnic sympathetic innervation in blood pressure regulation. In the study, I evaluated the changes in the splanchnic sympathetic innervation and function after CGX in the rat.

In addition, the sensory nerves and the sympathetic nerves are closely localized in animals (Galligan, Costa et al. 1988; Lindh, Haegerstrand et al. 1988; Davies and Campbell 1994) and humans (Levanti, Montisci et al. 1988; Del Fiacco, Floris et al. 1992). The sensory nervous system plays important roles in blood pressure regulation. Some vasoactive neuropeptides, such as calcitonin gene-related peptide (CGRP), is released from some sensory nerves (Deng and Li 2005). CGRP-containing sensory nerves terminate peripherally on blood vessels at a higher density around blood vessels than on the heart (Wimalawansa 1996).

Finally, since regeneration of sympathetic nerves has been reported after ganglionectomy (Hill, Hirst et al. 1985; Yamada, Terayama et al. 2006), the evaluation was performed at different points in time after the surgery. Other experiments were carried out to discover the origin of the reinnervation that was observed after CGX.

METHODS

Animals

Normal male Sprague-Dawley rats (300-325 g, Charles River, Portage, MI, USA) were used in the experiments.

The following methods were used in current study.

- Celiac ganglionectomy (CGX);
- Inferior mesenteric ganglionectomy (IMGX);
- Glyoxylic acid staining;
- Immunohistochemical staining of tyrosine hydroxylase and CGRP;
- Measurement of norepinephrine levels in mesenteric organs;
- Measurement of norepinephrine levels in mesenteric vessels;
- *In vitro* preparation of mesenteric vessels;
- Video monitoring of vessel diameter.

Protocol

After CGX, rats were allowed a two-week-recovery. All experiments were started after the recovery period.

Statistical analyses

Data were shown as means ± standard error. Differences between groups were evaluated by a two-way ANOVA followed by a post hoc test (Bonferroni's). A p value less than or equal to 0.05 was considered statistically significant.

RESULTS

Sympathetic nerve staining

Both glyoxylic acid staining (Figure 5a and 5b) and tyrosine hydroxylase staining (Figure 6) demonstrated that most sympathetic nerves were no longer visible two weeks after CGX was performed. CGRP-containing sensory nerves were absent as well (Figure 6). Figure 5 shows that new nerves started to appear on mesenteric arteries and veins five weeks after the surgery, and even more so ten weeks later.

Norepinephrine (NE) content measurement

Figure 7 illustrates NE levels in the splanchnic organs, including kidneys, liver, spleen, and small intestine. It shows that NE concentrations in all splanchnic organs were significantly decreased in CGX rats, compared to those in sham rats, two weeks after the surgery.

Figure 8 shows NE content in mesenteric arteries and veins. The data demonstrate that NE levels were significantly lowered two weeks after CGX.

Electrical stimulation

Figure 9 shows contractile responses to transmural stimulation of perivascular nerves on mesenteric vessels. The figure clearly demonstrates that neurogenic constriction was largely abolished by CGX in both veins and arteries.

Nerve regeneration

Figure 5 shows that reinnervation began around five weeks after CGX. Reinnervation was also revealed by NE measurements (Figure 10 for splanchnic organs, Figure 11 for mesenteric vessels). Sympathetic nerve density started to increase in mesenteric vessels and most splanchnic organs. IMG was postulated to be a possible origin of the reinnervation. The data showed that NE concentrations in organs and vessels from CGX rats were not significantly different from those from CGX and IMGX rats, at the same time point, indicating that IMG did not prevent the nerve regeneration following CGX.

DISCUSSION

Some global sympathetic denervation methods have been available for several decades, such as 6-hydroxydopamine (6-OHDA) (Hokfelt, Jonsson et al. 1972; Evans, Heath et al. 1979) and guanethidine (Evans, Heath et al. 1979) treatments. Both of the two techniques disrupt sympathetic innervation systemically, without a specific target. On the other hand, bilateral celiac ganglionectomy (CGX) specifically removes most splanchnic sympathetic neurons. As early as 1930s, CGX was performed as a treatment for human essential hypertension (Heuer 1936; Martin 1938; Weiss 1939; Peet, Woods et al. 1940). The surgery was reported to lower blood pressure, with a partial or complete sympathetic blockade in 87% of the patients, indicating the involvement of splanchnic sympathetic innervation in blood pressure regulation in humans. Animal studies also suggest that CGX effectively lowers mean arterial pressure in physiological and pathological conditions (Kregel and Gisolfi 1989; King, Osborn et al. 2007). Therefore, the celiac plexus, including the celiac ganglion and superior mesenteric ganglion, is a critical component of overall sympathetic regulation of blood pressure. Removal of this plexus, i.e. CGX, can be used to investigate the roles of splanchnic sympathetic nerves in cardiovascular regulation.

In current study, I evaluated the effectiveness of CGX in denervating splanchnic vessels and organs by studying structural changes of the nerves via two histological methods, glyoxylic acid staining of catecholamine-containing nerves and tyrosine hydroxylase (TH) staining. The vascular bed in the

splanchnic region was expected to show significantly lower innervation density after CGX. Consistent with the expectation, staining shows that the sympathetic nerves innervating mesenteric vessels (arteries and veins) are almost completely absent two weeks after CGX. The data are in accordance with other groups' findings (Hill, Hirst et al. 1985; Galligan, Costa et al. 1988; Yamada, Terayama et al. 2006). They all reported effective sympathetic denervation after small intestines were extrinsically denervated by nerve crushing, CGX, or nerve freezing. Although Hill claimed a partial degeneration after two weeks of the surgery, Yamada showed no regeneration even ninety days after CGX. The variability may be due to differences in denervation techniques.

In addition to qualitative examination by images, we quantified sympathetic nerve density by measuring norepinephrine (NE) contents in the splanchnic organs and mesenteric vessels via high performance liquid chromatography (HPLC). Our data show that CGX significantly lowered NE concentrations in the organs, compared to those in organs from sham-operated rats. The results are consistent with previous studies showing that chemical sympathectomy, by 6-OHDA, or alpha-methyl-tyrosine, or a combination of both, leads to a major NE content depletion in mice and rats (Williams, Peterson et al. 1981; Bellinger, Felten et al. 1989). Among all the splanchnic organs, the decrease in NE levels was the greatest in the spleen. The data are concordant with others showing that over 85% of splenic NE is lowered in rats by CGX (Bellinger, Felten et al. 1989). Combined, the results indicate that there is an extremely rich innervation by the sympathetic nerves in the spleen and CGX is

an effective surgery to denervate most sympathetic nerves in the splanchnic organs, especially in the spleen. Similarly, there was an almost complete loss of norepinephrine in mesenteric arteries and veins, indicating a marked decline in sympathetic nerve density after CGX. Hence this study further confirmed that CGX is a successful procedure to remove most sympathetic innervation to the splanchnic vascular bed.

The next step to validate the effectiveness of CGX was to look at the sympathetic nerves at the functional level. Previous functional studies of CGX mainly focused on single neurons (Hill, Hirst et al. 1985) or measuring electrophysiological parameters (Marlett and Code 1979). I applied direct electrical transmural stimulation to perivascular nerves on mesenteric vessels. This method is more integrative in showing the effects of sympathetic denervation on vasculature at the tissue, rather than the cellular, level. Our results showed that two weeks after CGX, mesenteric veins and arteries did not respond to electrical stimulation at a frequency as high as 10 Hz. A very small constriction was observed at very high frequencies (20 and 30 Hz), which usually do not occur under physiological conditions. Therefore, the functional study indicates that CGX largely eliminates sympathetic activity to arteries and veins in the splanchnic region at two weeks after surgery.

It is worth mentioning that CGX does not impair sympathetic input only to the intestine, but also other splanchnic organs. For example, approximately 66% of the sympathetic supply to kidneys originates from the paravertebral ganglia in rats while 20% of originates from the prevertebral ganglia (Ferguson, Ryan et al.

1986; Sripairojthikoon and Wyss 1987; Chevendra and Weaver 1991). In addition, numerous studies have reported that the renal innervation is critical in many hypertension models (Liard 1977; Katholi, Naftilan et al. 1980; Winternitz, Katholi et al. 1980; Norman and Dzielak 1982; Osborn and Camara 1997; Jacob, Clark et al. 2005). Therefore, a concern could be raised that any cardiovascular effects of CGX are mainly due to renal denervation, since our data show that some renal nerves are removed during the CGX. However, a colleague in our lab has performed bilateral renal denervation (RDX) and CGX, separately, in angiotensin (Ang) II salt hypertension (King, Osborn et al. 2007). The data showed that RDX does not attenuate the blood pressure increase induced by Ang II treatment, while CGX significantly decreases Ang II-mediated hypertension. This study shows that CGX can have important cardiovascular actions independent of any effects on renal sympathetic nerve activity.

In addition, the sensory nerves and the sympathetic nerves are closely localized in animals (Galligan, Costa et al. 1988; Lindh, Haegerstrand et al. 1988; Davies and Campbell 1994) and humans (Levanti, Montisci et al. 1988; Del Fiacco, Floris et al. 1992). Our immunochemistry results revealed that calcitonin gene-related peptide (CGRP)-containing sensory nerves were removed during the CGX procedure. CGRP is a very potent vasodilator (Brain, Williams et al. 1985) and is mainly found in dorsal root ganglia where the cell bodies of sensory afferent neurons exist. The afferent neurons terminate peripherally on blood vessels and other tissues (Wimalawansa 1996). It has been suggested that CGRP may play an important role in regulating peripheral vascular tone and

regional organ blood flow under physiological conditions (DiPette, Schwarzenberger et al. 1989; Vaishnava and Wang 2003). As to pathological conditions, several labs have shown that systemic administration of CGRP lowers blood pressure in spontaneously hypertensive rats (Dipette and Wimalawansa 1995) and DOCA-salt rats (Supowit, Zhao et al. 1997). Therefore, it is possible that partial removal of the sensory nerves in the splanchnic bed may affect cardiovascular regulation after CGX. In order to test this idea, neonatal capsaicin-treated rats were used. In this rat model, capsaicin-sensitive nerves are destroyed right after birth (Gamse 1982; Holzer-Petsche and Lembeck 1984). Our preliminary data (not shown here) indicate that CGX did not affect the overall sensory nerve regulation of hemodynamic parameters in ETBR activation-induced hypertension (Fink, Li et al. 2007), in spite of the partial absence of the nerves. This could be due to upregulation of CGRP receptors or a higher binding affinity to these receptors after CGX.

Furthermore, attention should be paid to nerve regeneration after CGX, since sympathetic postganglionic neurons show regrowth after transplantation (Olson and Malmfors 1970). Also, after chemical or surgical sympathectomy, regeneration of sympathetic postganglionic nerves innervating mesenteric vessels has been reported (Hill, Hirst et al. 1985; Galligan, Costa et al. 1988; Yamada, Terayama et al. 2006). Since nerve regeneration possibly leads to reinnervation and return of neuroeffector transmission, it's a critical issue to test if the effectiveness of CGX is time-dependent. Therefore, I examined reinnervation of the sympathetic postganglionic neurons after CGX. Glyoxylic acid staining

showed that two weeks after CGX sympathetic nerves were almost completely removed. By five weeks, a small portion of the sympathetic innervation had returned, while the density of innervation was almost fully restored by ten weeks. Assay of tissue norepinephrine content also demonstrated that innervation levels were higher in the splanchnic organs twelve weeks after CGX than after two weeks. The results indicate a regeneration of sympathetic nerves after CGX. Therefore, CGX is a time-sensitive procedure and experiments involving CGX must be performed in a timely manner to avoid the confounding effects due to nerve regeneration.

Sympathetic postganglionic nerves projecting to the splanchnic organs originate from the celiac ganglion, superior mesenteric ganglion (celiac plexus), and inferior mesenteric ganglion (IMG) (Hsieh, Liu et al. 2000; Quinson, Robbins et al. 2001). While the celiac plexus provides most of the sympathetic supply to the upper part of the digestive system, the IMG primarily innervates the lower digestive tract (furness and Costa 1987). Reinnervation of the splanchnic organs was observed in our CGX rats. A possible source of these nerves is the IMG. Reinnervation of the sympathetic postganglionic nerves after CGX in mice has been reported to be derived from the IMG (Yamada, Terayama et al. 2006). Therefore I performed both CGX and inferior mesenteric ganglionectomy (IMGX) in the rat to investigate the role of IMG in nerve regeneration after CGX. Norepinephrine measurements two weeks after the surgeries showed that IMGX and CGX together did not further lower norepinephrine concentrations in splanchnic organs and mesenteric vessels, compared to those in the organs and

tissues of rats undergoing CGX alone. Similar results were observed twelve weeks after the surgeries. Our results indicate that the IMGX is not a major source of sympathetic reinnervation in the splanchnic organs after CGX in the rat. More studies are necessary to discover the origin of the splanchnic reinnervation after CGX in rats.

Prolonged diarrhea was reported after CGX in dogs (Dayton, Schlegel et al. 1984). We did not observe any loose feces in our CGX or CGX and IMGX rats, indicating CGX rats have normal intestinal water/electrolyte absorption. This is consistent with other groups' conclusions (Teitelbaum, Sonnino et al. 1993; Sarr, Walters et al. 1994; Duininck, Libsch et al. 2003). The animals survived the surgeries well and they showed no signs of illness. Previous studies also show that bilateral splanchnic nerve section does not cause any changes in behavior, food intake, abdominal fat, body weight, brown adipose tissue weight, abdominal organ weight, plasma leptin concentration, or hypothalamic neuropeptide Y level, compared to shams (Furness, Koopmans et al. 2001). Additionally, a recent study shows that sodium intake is not affected by CGX in rats fed 2% NaCl (Osborn, Guzman et al. 2007).

Conclusions and limitations

Increasing evidence indicates that the sympathetic nervous system, especially in the splanchnic region, may be critical in blood pressure regulation in humans and animals (normotensive and hypertensive). A selective denervation of sympathetic nerves in the splanchnic bed, such as CGX, would be a useful procedure to determine the roles of the splanchnic sympathetic nervous system

in many physiological and pathological conditions. The current study characterized CGX, with a focus on qualification/quantification of the splanchnic sympathetic innervation at structural and functional levels. My results indicate that CGX is an effective procedure to remove the sympathetic innervation to the splanchnic bed within one month after the surgery. CGX, therefore, could be used in any studies designed to determine if the splanchnic sympathetic nervous system is involved in physiological or pathophysiological processes.

The current study, however, has several limitations. First of all, all the experiments were *in vitro* studies. Some *in vivo* studies, such as monitoring hemodynamic parameters (blood pressure, heart rate, total peripheral resistance, etc.), could be performed in CGX rats to identify the cardiovascular effects in conscious animals. Secondly, my data suggest that CGX is a time-sensitive procedure and nerve regeneration may occur after CGX. Therefore, functional studies, such as electrical stimulation, should be carried out to examine the effectiveness of CGX at different points in time. It is expected that after a certain period of time (maybe 2 months) following CGX vascular responses to electrical stimulation is bigger than right after recovery. Additionally, blood pressure could be monitored continuously after CGX to determine if sympathetic nerve regeneration would affect blood pressure regulation. Last, the origin of nerve regeneration after CGX remains unclear. A retrograde tracer could be used to discover the specific source of reinnervating fibers.

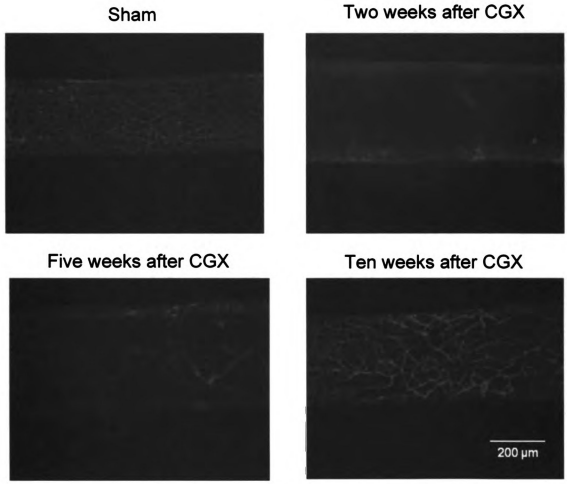

Figure 5a: Glyoxylic acid staining of mesenteric arteries. The blue fluorescence represents the presence of catecholamines. The scale bar applies to all pictures.

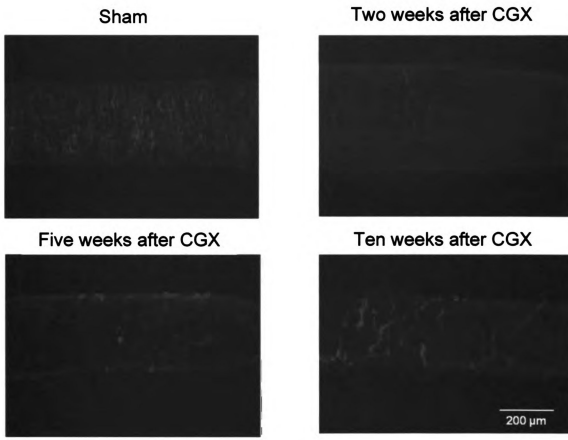

Figure 5b: Glyoxylic acid staining of mesenteric veins. The blue fluorescence represents the presence of catecholamines. The scale applies to all pictures.

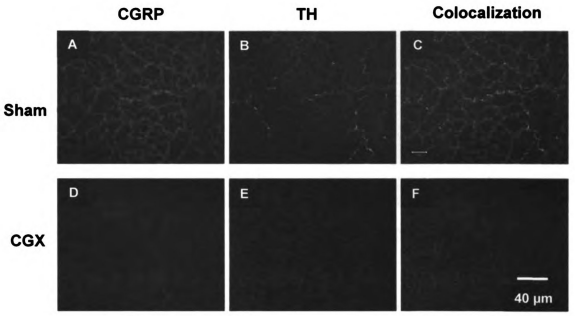

Figure 6: Tyrosine hydroxylase staining and calcitonin gene-related peptide (CGRP) staining of mesenteric arteries. The green fluorescence shows the presence of tyrosine hydroxylase, a key enzyme for catecholamine synthesis. The red fluorescence indicates the presence CGRP, a peptide released from sensory neurons. The figures show that CGX effectively removed both sympathetic nerves and CGRP-containing nerves.

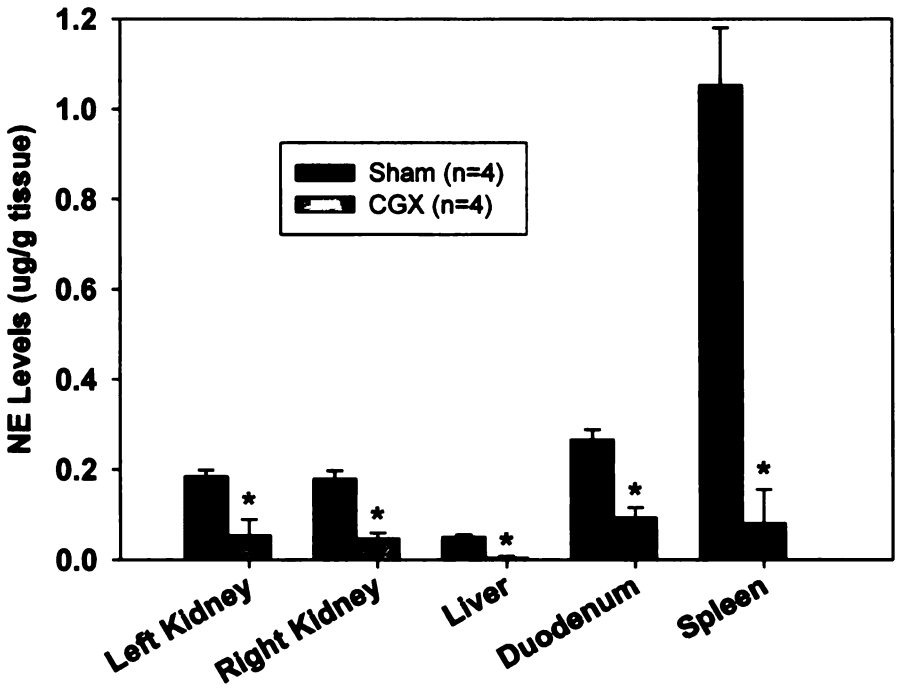

Figure 7: Norepinephrine (NE) concentrations in splanchnic organs two weeks after CGX. *: Significant difference from sham rats ($P<0.05$).

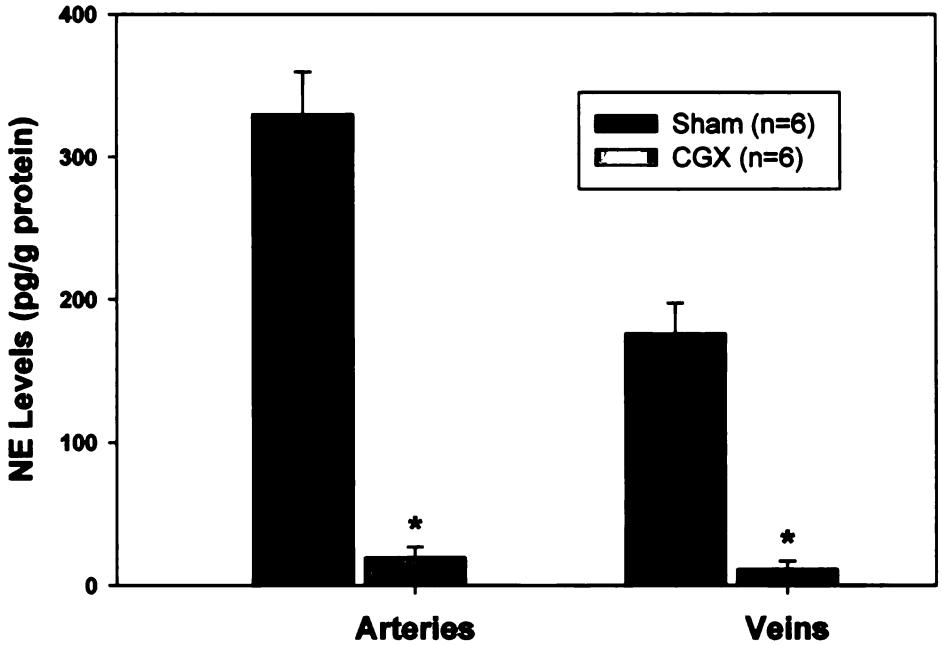

Figure 8: Norepinephrine concentrations in mesenteric arteries and veins. NE levels in mesenteric arteries and veins from CGX rats were significantly lower than those from sham-operated rats, two weeks after the surgery. IMGX did not further reduce sympathetic nerve density. *: Significant difference from sham rats ($P<0.05$).

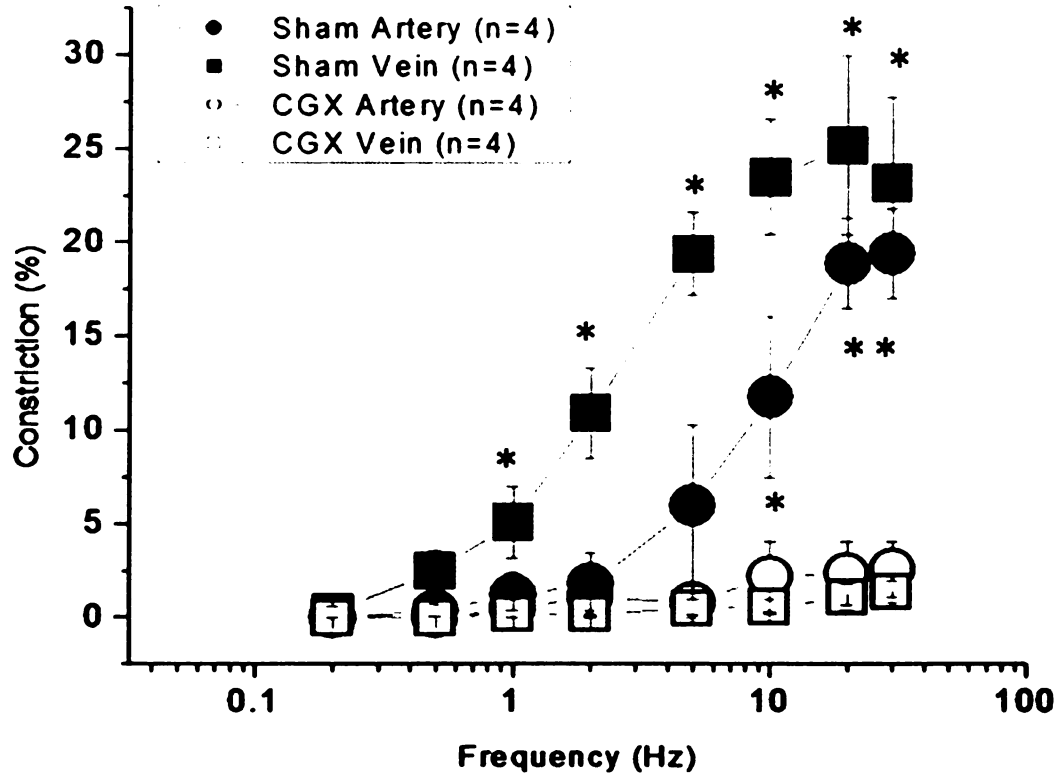

Figure 9: Constriction of mesenteric vessels in response to electrical stimulation. The constriction in response to electrical stimulation was virtually abolished in mesenteric vessels from CGX rats. *: Significant difference from the same type of vessel in CGX rats ($P<0.05$).

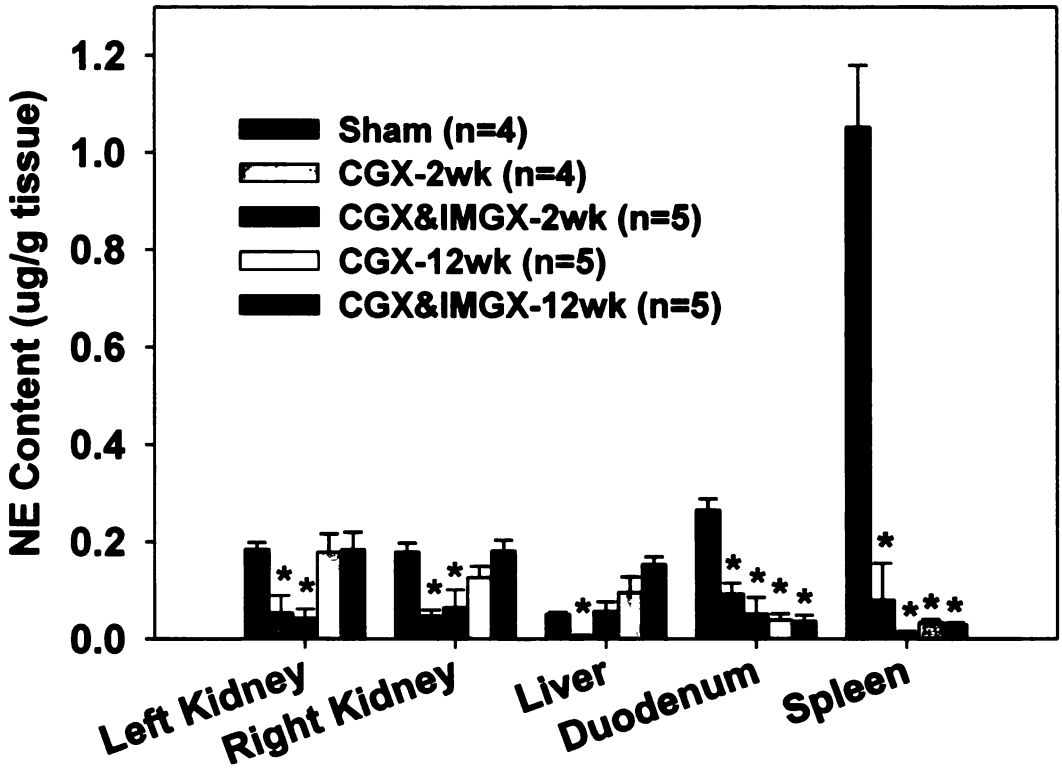

Figure 10: Norepinephrine concentrations in splanchnic organs from rats with sham-operation, CGX, or CGX and IMGX. The animals were sacrificed two weeks or twelve weeks after the surgery/surgeries. *: Significant difference from sham rats ($P<0.05$).

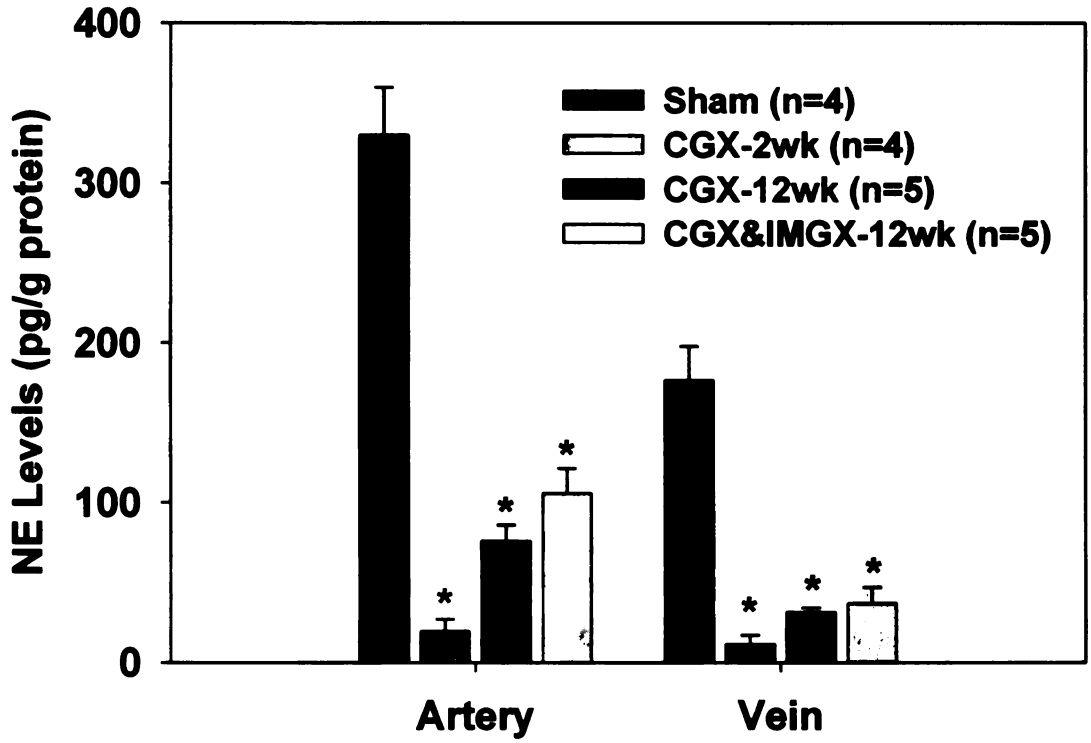

Figure 11: Norepinephrine concentrations in mesenteric arteries and veins from rats with sham-operation, CGX, or CGX and IMGX. The animals were sacrificed two weeks or twelve weeks after the surgery/surgeries. *: Significant difference from sham rats ($P<0.05$).

CHAPTER 5

INCREASED SUPEROXIDE LEVELS IN GANGLIA AND SYMPATHOEXCITATION ARE INVOLVED IN SARAFOTOXIN 6C-INDUCED HYPERTENSION

INTRODUCTION

We recently reported that chronic activation of ETBRs using intravenous infusion of the ETBR selective agonist, sarafotoxin 6c (S6c), in rats causes an increase in arterial pressure (S6c-induced hypertension) (Fink, Li et al. 2007). This was surprising in light of the fact that the two best-described physiological responses to ETBR activation—release of vasodilators from endothelial cells, and increased renal sodium and water excretion— should lead to a fall in arterial pressure (Rubanyi and Polokoff 1994; Schiffrin 1998; Pollock, Allcock et al. 2000). We believe venoconstriction contributes to S6c-induced hypertension, because *in vitro* S6c has been shown to constrict veins from most vascular regions but to have little effect on most arteries (Evans, Cobban et al. 1999; Johnson, Fink et al. 2002; Perez-Rivera, Fink et al. 2005; Savoia and Schiffrin 2007). Furthermore, chronic administration of other relatively specific venoconstrictors drugs also can increase arterial pressure (Rothe 1983; Greenway and Lautt 1986; Hainsworth 1986; Mortensen, Pawloski et al. 1990; Rothe 1993). Decreased venous capacitance has been documented in animal models (Manning, Coleman et al. 1979; Ricksten, Yao et al. 1981; Ackermann and Tatemichi 1983; Fink, Johnson et al. 2000) and in humans with hypertension (Ulrych 1976; Mark 1984; Safar and London 1985). Veins in the splanchnic region are probably the most important target for S6c and other venoconstrictors, since they account for most of the active capacitance responses of the circulation (Greenway and Lautt 1986; Rothe 1986; Rothe 1993). Veins in the splanchnic region hold about 33% of the total blood volume (Greenway and Lister 1974) and

maximal activation can reduce their volume up to 60%, redistributing blood from the abdomen into the heart and thereby increasing cardiac output and blood pressure (Karim and Hainsworth 1976; Greenway 1983).

It is likely, however, that other mechanisms contribute to S6c-induced hypertension. Reactive oxygen species (ROS) are a variety of oxygen-containing molecules that are by-products of cellular metabolic processes under physiological condition, including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl ion ($OH^{\cdot}$). Superoxide production is mediated by several enzymes including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, and nitric oxide synthase (NOS) (Wilcox 2002). Recently, increasing evidence has shown that ROS can function as cellular signaling molecules (Bedard and Krause 2007). They achieve this signaling function by participating in phosphatase inhibition (Lee, Kwon et al. 1998; Wu, Hardy et al. 2003; Goldstein, Mahadev et al. 2005; Kwon, Qu et al. 2005), kinase activation (Griendling, Sorescu et al. 2000; Han, Kim et al. 2003; Furst, Brueckl et al. 2005), ion channel regulation (Hidalgo, Bull et al. 2004; Tang, Santarelli et al. 2004), and calcium signaling (Wang, Takeda et al. 1999; Cheranov and Jaggar 2006; Granados, Salido et al. 2006). Increased ROS levels have been found in blood vessels, kidneys and other tissues in many experimental models of hypertension, including renovascular (Lerman, Nath et al. 2001), DOCA-salt (Somers, Mavromatis et al. 2000), Dahl salt-sensitive (Swei, Lacy et al. 1999), and lead-induced hypertension (Vaziri, Liang et al. 1999). ROS generation also is elevated in hypertensive patients (Touyz and Schiffrin 2001). Increased tissue

ROS levels can cause hypertension, because blocking ROS generation or increasing their removal lowers blood pressure (Kopkan and Majid 2005; Sullivan, Pollock et al. 2006; Baumer, Kruger et al. 2007). Recent reports indicate that ETBR activation increases oxidative stress in sympathetic ganglia *in vitro* (Dai, Galligan et al. 2004) and *in vivo* (Lau, Galligan et al. 2006). Although the precise effects of increased ROS levels in sympathetic ganglia are not known, it is possible that ROS signaling could enhance ganglionic neurotransmission and thereby increase sympathetic activity. Therefore, we tested the hypothesis that increased superoxide generation in vascular tissues or sympathetic ganglia is involved in S6c-induced hypertension.

METHODS

Animals

Normal male Sprague-Dawley rats (300-325 g, Charles River, Portage, MI, USA) were used in these experiments.

The following methods were used in current study.

- Vascular catheterization;
- Hemodynamic measurements by external blood pressure analyzer;
- Celiac ganglionectomy (CGX);
- Dihydroethidium fluorescence;
- Lucigenin-enhanced chemiluminescence;
- Plasma sampling;
- Plasma norepinephrine assay.

Protocols

S6c-infusion

For S6c-treated rats, saline vehicle (3.47 μl /min) was infused intravenously for two days (control period, C). Then the animals were treated with S6c (5 pmol/kg/min, iv) for five days (infusion period, E), followed by a three-day-infusion of saline only (3.47 μl /min, recovery period, R). Sham rats received saline only (3.47 μl /min) for ten consecutive days. Hemodynamic parameters were measured every day.

Trimethaphan study

Trimethaphan (15 mg/kg, iv) was given daily to S6c-treated and sham rats. The peak fall in blood pressure after trimethaphan injection (generally within 2-5 minutes) was recorded as an index of sympathetic pressor activity.

Plasma NE measurement

S6c-infused and sham rats were used in this study. Blood samples were collected on the 2nd control day, the 3rd and the 5th day of infusion period, and the 2nd recovery day.

CGX study

Celiac ganglionectomy and sham-operation were performed 10 to 14 days prior to beginning the study. Rats were then subjected to S6c infusion as described above.

Superoxide studies in S6c-induced hypertension

For S6c-treated rats, saline (3.47 µl/min) was infused intravenously for two days (control period, C). Then the animals were treated with S6c (5 pmol/kg/min, iv). Tissues were collected after S6c infusion for one day or five days. Sham rats received saline for seven consecutive days. Animals were sacrificed in pairs of S6c-treated and saline-treated rats.

The effect of tempol treatment on S6c-induced hypertension

After the recovery from catheterizations of femoral artery and vein, and a two-day control period, one group of rats were infused with saline while drinking water containing tempol tempol (4-hydroxy 2,2,6,6,-tetramethyl peperidine 1-oxyl, a superoxide dismutase mimetic, 1 mmol/L). Each water bottle was wrapped with

aluminum foil to protect the drug from light exposure. The tempol solution was prepared fresh every day. The second group received S6c (5 pmol/kg/min) and the third group received S6c while drinking tempol solution. S6c and tempol were removed after a 5-day-infusion in group 2 and 3, followed by saline infusion (recovery period).

DHE staining in S6c-treated rats with tempol treatment

One group of rats was given water containing tempol (1 mmol/L) immediately after catheterization surgery. After six days of tempol treatment, rats received S6c (5 pmol/kg/min, iv) infusion for one day while they continued to receive tempol. A second group of animals were infused with saline only (3.47 µl/min, iv) for seven days and did not receive tempol treatment. A third group of rats was treated with saline for six days, then received S6c (5 pmol/kg/min, iv) for one day. For DHE staining, one animal from each group was sacrificed and tissues were collected from all three rats and studied together.

Statistical analyses

The effect of CGX on MAP was evaluated by Student *t* test compared to the average of baseline MAP in intact rats. Within-group differences over time were assessed by a one-way ANOVA and posthoc multiple comparisons with Tukey's test (GraphPad Instat 3). Between-group differences were evaluated using the protected least significant difference test. Superoxide levels in lucigenin studies were reported in the unit of nmol/min/mg protein. DHE staining was quantified by taking readings from three to five individual sympathetic neurons or vascular smooth muscle cells in each rat. The fluorescence density of the

measured cells was the difference between the absolute number in arbitrary fluorescence units and the value of background. Control rats were used for comparison. The ratio of fluorescence density from treated rats to that from controls was used for statistical comparisons. When comparing 2 groups, the appropriate Student *t* test was used. Analysis of variance followed by Tukey post hoc test was performed when comparing 3 or more groups. In all cases, a *P* value less than or equal to 0.05 was considered statistically significant. A $P<0.05$ was considered significant. All of the results are presented as mean ± SE.

RESULTS

Acute ganglion blockade in S6c-induced hypertension

Figure 12a shows mean arterial pressure (MAP) changes during 5-day S6c or vehicle infusion. MAP increased significantly during S6c infusion while it did not change in vehicle-treated rats (average of 123.8 ± 1 vs 106.7 ± 2 mmHg). Figure 12b shows acute depressor responses to ganglion blockade with trimethaphan. Infusion of S6c produced sustained hypertension, whereas no changes in blood pressure were observed in vehicle-treated rats. No differences in depressor responses to ganglion blockade were found in vehicle and S6c-treated rats during the control period or during the first 3 days of the infusion period. However, on days 4 and 5 of the infusion period, the response to trimethaphan was significantly larger in S6c-treated rats than in vehicle-treated control animals. Termination of S6c treatment was associated with a return of trimethaphan responses to control period values.

Plasma norepinephrine levels in S6c-induced hypertension

Mean arterial pressure (MAP) increased significantly in S6c-infused rats and the increase was sustained during the infusion period. MAP did not change in vehicle-treated animals (average of 127.4 ± 1 vs. 99.6 ± 2 mmHg, Figure 13a). Plasma norepinephrine concentrations (Figure 13b) did not change in either vehicle-treated rats or S6c-treated rats relative to their respective control period values, nor were differences between vehicle- and S6c-treated rats significant at any time during the study.

S6c-induced hypertension in celiac ganglionectomized rats

Figure 14 shows the effect of CGX on S6c-induced hypertension. Ganglionectomy alone slightly decreased resting arterial pressure, although not significantly. The increase in pressure during S6c treatment, however, was markedly less in CGX than in sham-operated rats. In fact, during the last three days of S6c infusion, arterial pressure in CGX rats returned to values not significantly different from those measured during the control period.

Superoxide levels in mesenteric vessels of S6c-treated rats

DHE staining showed an apparent increase in superoxide level in smooth muscle cells from mesenteric arteries and veins from rats receiving S6c, compared to vessels from rats receiving saline only (Figure 15a), but the difference was not statistically significant (Figure 15b). Similarly, lucigenin-enhanced chemiluminescence measurements (Figure 16) indicated that although the mean values of superoxide levels were increased in blood vessels from the

rats treated with S6c, the differences were not statistically significant. The basal level of superoxide in veins was significantly higher than in arteries.

Superoxide levels in inferior mesenteric ganglion of S6c-treated rats

Figure 17a shows DHE staining of inferior mesenteric ganglion (IMG) from rats receiving saline for 7 days, or 1- or 5-day infusions of S6c. More intense fluorescence was observed in IMGs from rats treated with S6c for either 1 day or 5 days, compared to those treated with saline. In addition, more fluorescence was observed in IMG from rats receiving S6c for 5 days than in those receiving S6c for 1 day. Quantitative analyses revealed that these differences were statistically significant (Figure 17b).

Effects of tempol on arterial pressure and ganglion superoxide in S6c-treated rats

Figure 18 demonstrates that tempol treatment prevented a significant rise in blood pressure in rats receiving S6c, but had no effect in control rats. Figure 19a shows DHE fluorescence in IMG of rats treated with saline, S6c, and S6c plus tempol. Quantification revealed that superoxide levels in IMG from rats treated with S6c and tempol were significantly lower than those in IMG from rats treated with S6c alone (Figure 19b).

DISCUSSION

The data presented here suggest that the sympathetic nervous system contributes to S6c-induced hypertension. For example, depressor responses to the ganglion blocker trimethaphan became gradually larger during a 5-day S6c infusion. This finding is generally accepted as evidence for elevated sympathetic activity to the cardiovascular system (Biaggioni and Robertson 2002), although other explanations are possible (Biaggioni and Robertson 2002; Degoute 2007). Therefore, plasma norepinephrine levels were measured as another index of sympathetic nervous system activity. However, plasma norepinephrine concentrations were not elevated during S6c infusion. This could be due to the insensitivity of this method for detecting increased sympathetic nervous system activity (Aneman, Eisenhofer et al. 1996; Peaston and Weinkove 2004). Alternatively, it could indicate that sympathetic activity is increased in a regionally specific way in S6c-induced hypertension. As discussed in the Introduction, there is evidence that venoconstriction participates in S6c-induced hypertension. Since the splanchnic veins and venules are the main capacitance segments of the circulation, we speculated that sympathetic nerves to the splanchnic vascular bed may be activated in S6c-induced hypertension. The celiac plexus contains the cell bodies of most sympathetic neurons innervating the splanchnic organs. Therefore, surgical celiac ganglionectomy (CGX) was performed to determine if sympathetic nerves in the splanchnic region play a role in blood pressure responses to S6c infusion. The results showed that in celiac ganglionectomized rats S6c increased blood pressure significantly for the first two days of the five

day infusion period. But subsequently pressure returned to levels not significantly higher than pre-infusion control period values, despite continued S6c administration.

Collectively, these findings support the idea that S6c-induced hypertension is partially dependent on increased activity of the sympathetic nerves (with cell bodies in the celiac ganglion plexus) supplying the splanchnic organs. These nerves control smooth muscle activity in the arteries and veins of the intestine, stomach, pancreas, mesentery, liver and other organs. They account for a substantial fraction of norepinephrine released from the peripheral sympathetic nervous system (Hottenstein and Kreulen 1987; Stjarne, Bao et al. 1994), and their activation leads to both increased systemic vascular resistance and decreased capacitance (Mark 1984; Hainsworth 1986; Pang 2001). As noted earlier, activation of the splanchnic sympathetic nerves can redistribute blood from compliant peripheral veins into the central circulation and thereby increase blood pressure (Karim and Hainsworth 1976; Greenway 1983).

It's not clear why an influence of the sympathetic nervous system on blood pressure in S6c-induced hypertension was only demonstrable 3 or more days after starting S6c infusion. One possibility is that the early increase in blood pressure is non-neurogenic and causes a baroreflex mediated reduction in sympathetic activity until significant resetting of the reflex occurs. This could be tested by performing S6c infusion in animals with sino-aortic denervation. Another question is: If splanchnic sympathetic nerve activity is increased during S6c infusion, why isn't this reflected in elevated peripheral plasma concentrations

of norepinephrine? The likely explanation is that norepinephrine released from splanchnic sympathetic nerves is efficiently cleared from the circulation by the liver (Aneman, Eisenhofer et al. 1995; Eisenhofer, Aneman et al. 1995). This idea could be tested using regional norepinephrine spillover measurements in S6c-infused rats.

Under physiological conditions, ROS are present at low concentrations and act as signaling molecules regulating the growth and function of vascular smooth muscle cells (Rao and Berk 1992; Cosentino, Sill et al. 1994). Under pathological conditions, ROS may impair endothelium-dependent vasorelaxation (Somers, Mavromatis et al. 2000), induce vascular smooth muscle cell growth (Zafari, Ushio-Fukai et al. 1998), elevate sympathetic nervous system activity (Campese, Ye et al. 2004), alter renal function (Han, Lee et al. 2005), and cause increased deposition of extracellular matrix proteins (Ha and Lee 2003). All of these effects may contribute to vascular and organ damage, and other pathophysiological changes, in a variety of cardiovascular diseases (Harrison 1997; Griendling 2004; Touyz and Schiffrin 2004; Brandes and Kreuzer 2005). Accumulating evidence indicates that ROS play physiological and pathophysiological roles in hypertension (Touyz and Schiffrin 2004).

Most studies indicate that endothelin-family peptides stimulate ROS generation in blood vessels and other tissues by acting on ETA subtype receptors (ETARs) (Callera, Touyz et al. 2003; Ozdemir, Parlakpinar et al. 2006). However, endothelin-induced superoxide production in sympathetic neurons *in*

vitro is mediated by ETBRs (Dai, Galligan et al. 2004). Furthermore, acute activation of ETBRs *in vivo* in conscious rats produces increased superoxide concentrations in sympathetic ganglia (Lau, Galligan et al. 2006). Therefore, we tested the hypothesis that chronic infusion of S6c also would increase superoxide in sympathetic ganglia. We made measurements in the inferior mesenteric ganglion (IMG), a prevertebral ganglion providing innervation to splanchnic blood vessels, small intestine, colon, rectum, etc. (Szurszewski 1981), because fluorescent imaging is easier in this ganglion than in the celiac plexus. In order to assess a possible effect on blood vessels of chronic exposure to S6c *in vivo*, superoxide levels in mesenteric arteries and veins also were determined. Because evidence reported above suggested that the mechanisms of S6c-induced hypertension might differ during the early and later stages, we measured superoxide levels in rats treated with S6c for 1 or 5 days.

Both DHE staining and lucigenin-enhanced chemiluminescence measurements revealed that superoxide levels were not significantly increased in either arteries or veins from rats treated with S6c for 1 or 5 days. This result is consistent with previous work (discussed above) indicating vascular superoxide production in response to endothelin peptides is mediated by ETARs. Interestingly, the basal level of superoxide in veins was significantly higher than in arteries, possibly due to a higher nitric oxide production in arteries which may interact with superoxide to lower the level, compared to veins (Szasz, Thakali et al. 2007). Lucigenin was not used to determine ROS levels in ganglia because the method lacks sufficient sensitivity for very small amounts of tissue. However,

DHE staining showed that superoxide levels were significantly increased in sympathetic ganglia from S6c-treated rats. Moreover, the increase was time-dependent. That is, superoxide levels were higher in sympathetic neurons from rats receiving S6c for 5 days versus 1 day.

With this evidence of increased ROS in sympathetic ganglia in S6c-induced hypertension, we designed an experiment to address the cause-and-effect relationship between increased oxidative ROS and elevated blood pressure. Tempol, a superoxide dismutase mimetic (Schnackenberg, Welch et al. 1998); (Shokoji, Nishiyama et al. 2003), was used to test if increased blood pressure in S6c-infused rats required increased tissue levels of superoxide. Our results confirmed previously published studies showing that tempol does not affect blood pressure in normotensive rats (Sullivan, Pollock et al. 2006). However, tempol attenuated blood pressure elevations in S6c-infused rats, suggesting that increased tissue concentrations of superoxide contribute importantly to S6c-induced hypertension. To determine possible target tissues for the actions of tempol, we measured superoxide levels in sympathetic neurons from rats treated with S6c, with and without tempol. These results showed that superoxide was lower in a prevertebral ganglion of rats treated with tempol compared to those treated with S6c alone, although levels were not completely normalized. These results are consistent with the idea that tempol ameliorated S6c-induced hypertension by reducing ROS levels in sympathetic ganglia.

How do ROS in sympathetic ganglia affect post-ganglionic sympathetic nerve activity? Although there is no direct evidence in sympathetic ganglia, in

other neuron types ROS have been shown to increase cell excitability by inhibiting calcium-activated potassium channel (Soto, Gonzalez et al. 2002; Zucker and Gao 2005). Therefore, we hypothesize that in S6c-induced hypertension, increases in superoxide content in the prevertebral sympathetic ganglia cause higher post-ganglionic nerve discharge and constriction of splanchnic blood vessels. We further hypothesize that tempol decreases blood pressure in S6c-induced hypertension by reducing ROS levels in sympathetic ganglia and thereby post-ganglionic sympathetic nerve activity. Previous studies have shown that tempol is, in fact, sympathoinhibitory in hypertensive rats (Fujita, Ando et al. 2007; Han, Shi et al. 2007). A caveat to this conclusion, however, is that tempol also has other pharmacological effects which could attenuate hypertension, for example, opening large conductance, calcium activated potassium channels (Xu, Jackson et al. 2006).

In summary, the data presented here are consistent with the idea that hypertension produced by chronic activation of ETBRs in rats is due to both neurogenic and non-neurogenic mechanisms. The neurogenic mechanism has a delayed onset and probably results from changes in sympathetic activity to the splanchnic vascular bed. Although it is not known if chronic selective activation of ETBRs occurs under normal circumstances, the model illuminates the possible importance of both venoconstriction and the splanchnic sympathetic nerves in the development of hypertension.

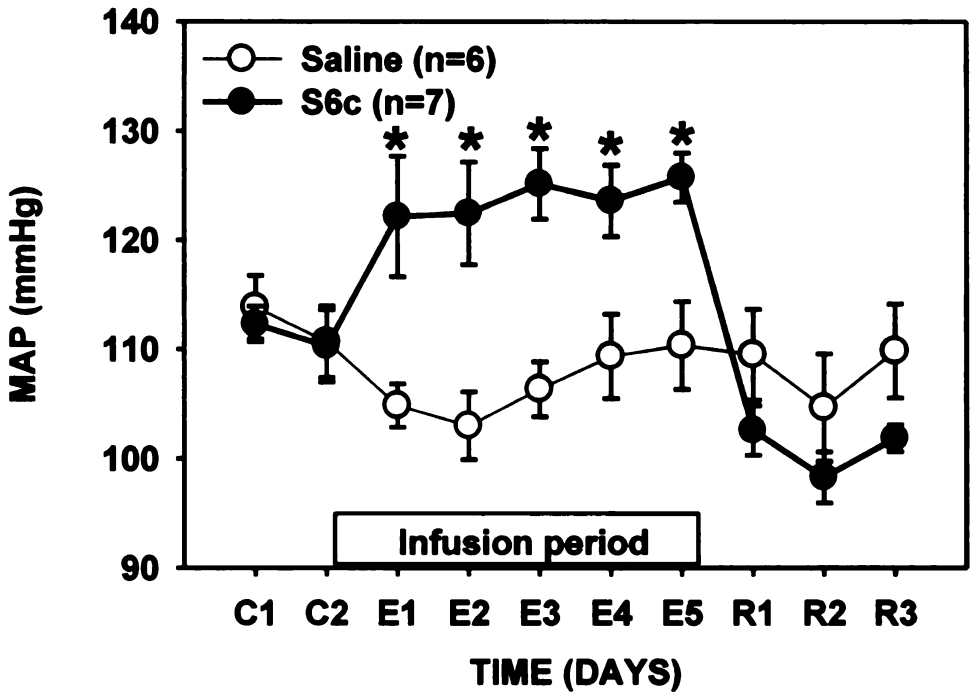

Figure 12a: The depressor responses to ganglion blockade with trimethaphan before (C1-C2, C=control), during (E1-E5, E=infusion), after (R1-R3, R=Recovery) continuous S6c infusion (5 pmol/kg/min, iv) or vehicle into rats. Figure 1a shows mean arterial pressure (MAP). MAP significantly increased during S6c-infusion period and it did not change with vehicle infusion. *: a statistical difference ($p<0.05$) from the control period.

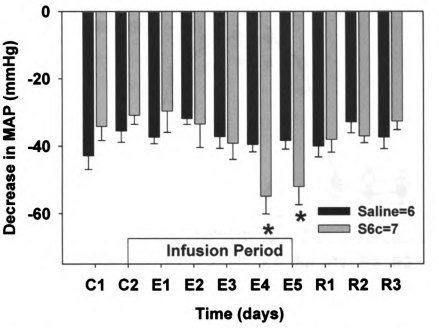

Figure 12b: Changes in MAP in S6c- or vehicle-infused animals with a bolus injection of trimethanphan (15 mg/kg, iv), a ganglionic blocker, during infusion period. MAP decrease was significantly bigger in S6c-treated rats than in vehicle-treated rats on the fourth and the fifth day. Numbers in parentheses means the number of rats in each group. *: a statistical difference ($p<0.05$) from control rats on the same day.

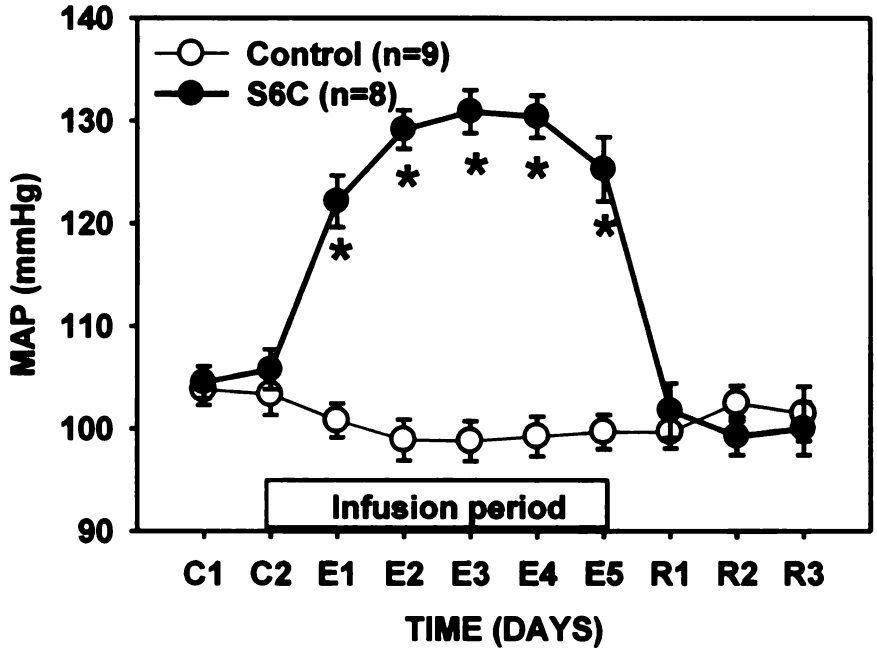

Figure 13a: Mean arterial pressure (MAP) in rats before (C1-C2, C=control), during (E1-E5, E=infusion), after (R1-R3, R=recovery) continuous S6c infusion (5 pmol/kg/min, iv) or vehicle. MAP significantly increased during S6c-infusion period and it did not change with vehicle infusion. *: a statistical difference ($p<0.05$) from the control period.

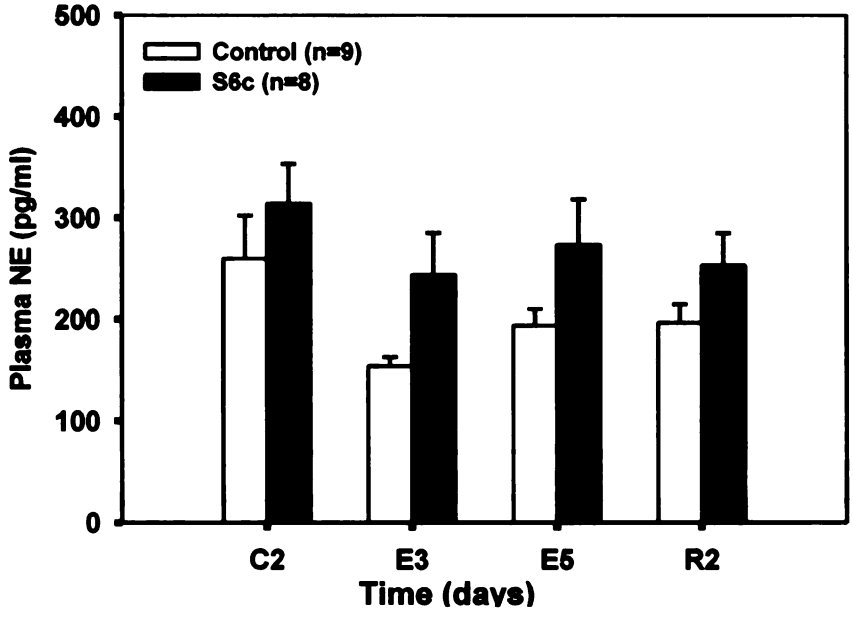

Figure 13b: Average (± SEM) plasma norepinephrine (NE) concentrations in rats receiving continuous S6c infusion (5 pmol/kg/min, iv) or vehicle according to the same protocol shown in Figure 1. Plasma samples were collected on the second control day (C2), the third and the fifth infusion days (E1 and AE), and recovery day 2 (R2). Plasma NE levels did not increase during S6c-infusion period, although MAP significantly elevated.

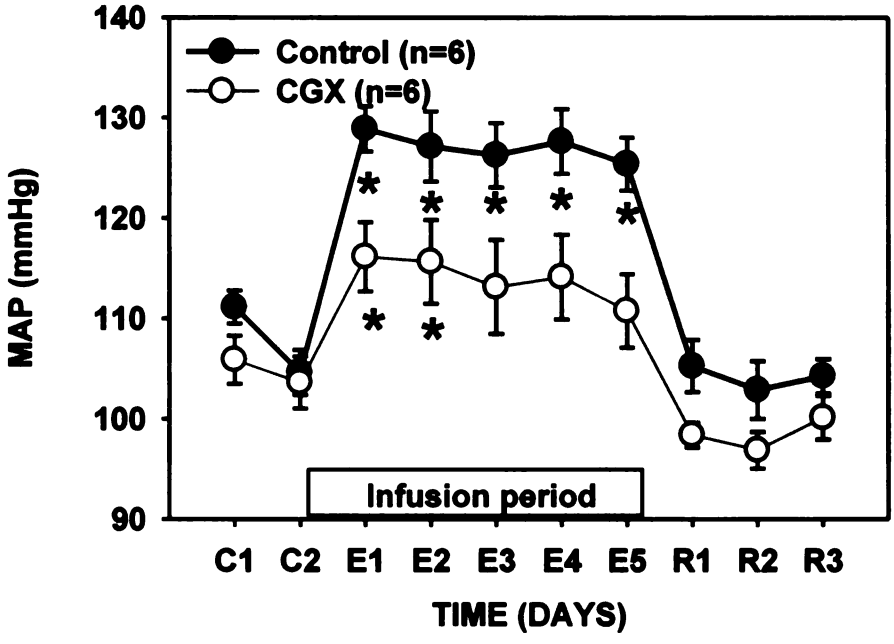

Figure 14: Average mean arterial pressure (MAP) before (day 1-2, control period), during (day 3-7, infusion period), and after (day 8-10, recovery period) continuous S6c infusion (5 pmol/kg/min, iv) into celiac ganglionectomized (CGX) and sham-operated rats. MAP significantly increased during infusion period in sham-operated rats. It increased significantly only in the first two days of infusion period in CGX-operated rats.*: a statistical difference ($p<0.05$) from control period.

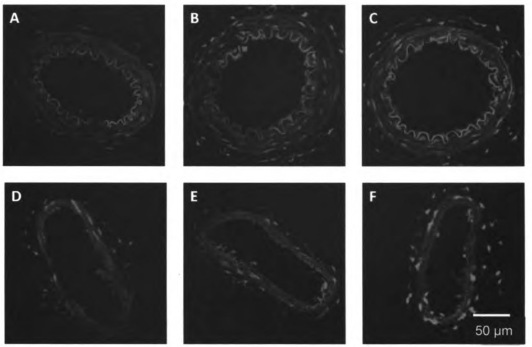

Figure 15a: Representative pictures showing superoxide levels in smooth muscle cells from superior mesenteric arteries (SMA) and veins (SMV) in control and S6c-infused rats, measured by DHE. $O_2^{\cdot-}$ levels, indicated by DHE fluorescence intensity in confocal images of smooth muscle cells, are higher in S6c-treated rats than in sham rats. A, SMA from a sham rat; B, SMA from a rat receiving S6c for one day; C, SMA from a rat receiving S6c for five days; D, SMV from a sham rat; E, SMV from a rat receiving S6c for one day; F, SMV from a rat receiving S6c for five days. The scale bar in F applies to all panels.

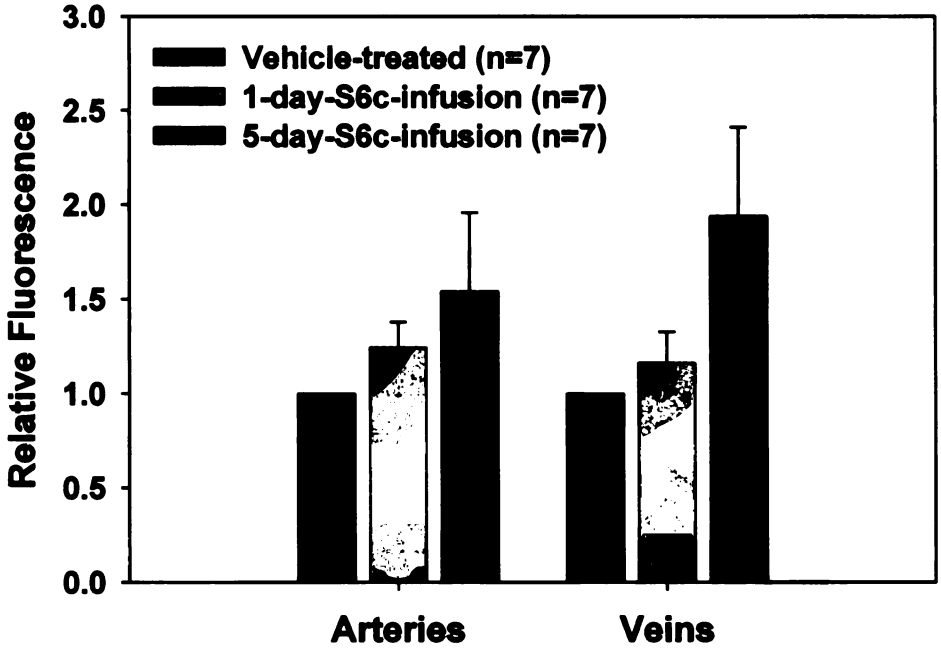

Figure 15b: Quantification of mean fluorescence intensity of smooth muscle cells in SMA and SMV (n=7 in each group). Fluorescence of smooth muscle cells was not significantly greater in rats receiving S6c for either one day or five days compared with shams'.

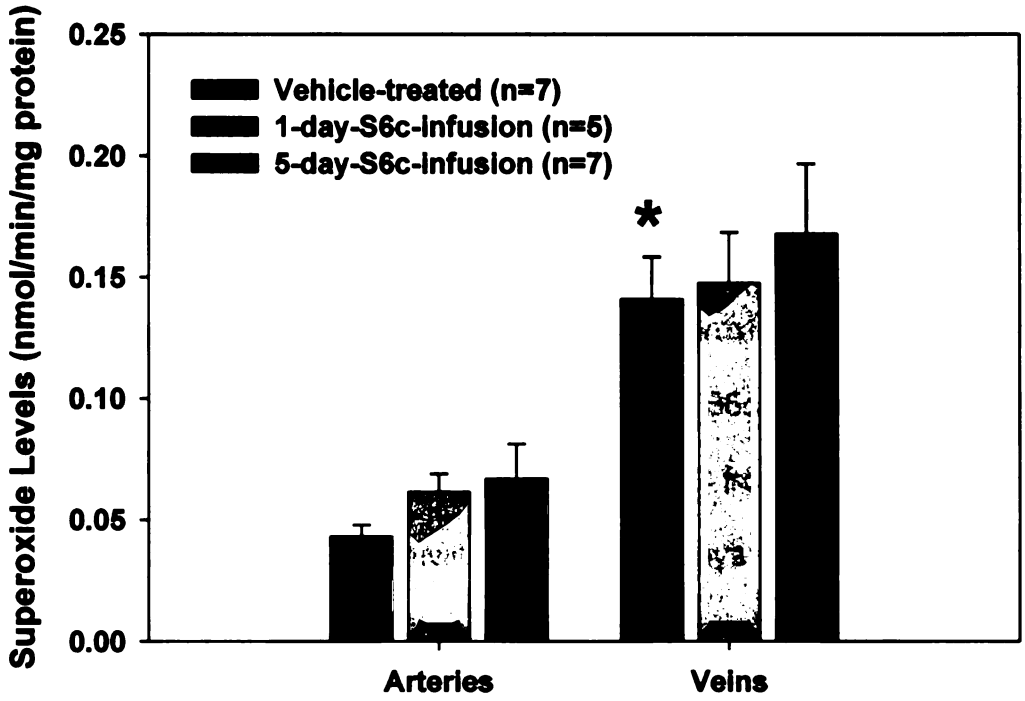

Figure 16: Superoxide levels in superior mesenteric arteries and veins from control and S6c-infused rats, measured by lucigenin-enhanced chemiluminescence. Rats were either treated with S6c for 1 day or 5 days. Superoxide levels were not significantly increased with S6c treatment. Basal superoxide level was higher in veins than in arteries. *: a statistical difference ($p<0.05$) from control rats.

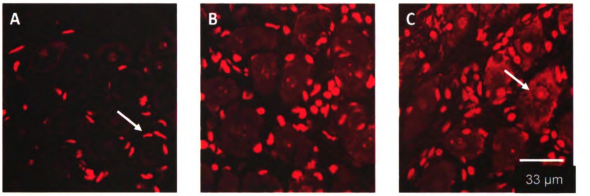

Figure 17a: Representative pictures showing superoxide levels in inferior mesenteric ganglion (IMG) neurons from control and S6c-treated rats. A, IMG from a sham rat (the arrow points to a glial cell); B, IMG from a rat receiving S6c for one day; C, IMG from a rat receiving S6c for five days (the arrow points to a neuron); D, Comparison of mean fluorescence intensity of S6c-treated neurons to sham neurons (n=7 Sham rats; n=7 S6c-treated rats). The scale bar in C applies to all panels.

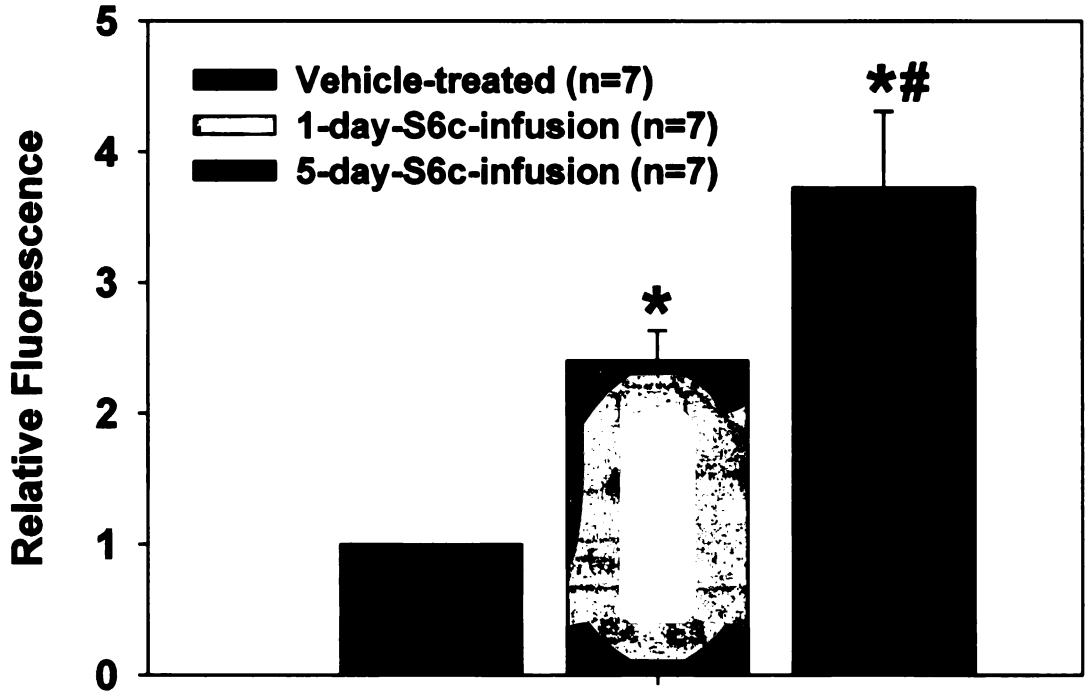

Figure 17b: Quantification of mean fluorescence intensity of sympathetic neurons from S6c-treated and saline-treated rats (n=7 in each group). The fluorescence density was significantly higher in S6c-treated rats (one day or five day-infusion) than that in control rats. Superoxide level increased in a time-dependent manner. The level from rats with five day-infusion was higher than that from rats with one day-infusion. *: Fluorescence of IMG neurons was significantly ($P<0.05$) greater in ganglia from rats receiving S6c for both 1 day or 5 days compared with shams'; #: Fluorescence of IMG neurons was significantly ($P<0.05$) greater in ganglia from rats treated with S6c for 5 days compared with those treated for 1 day.

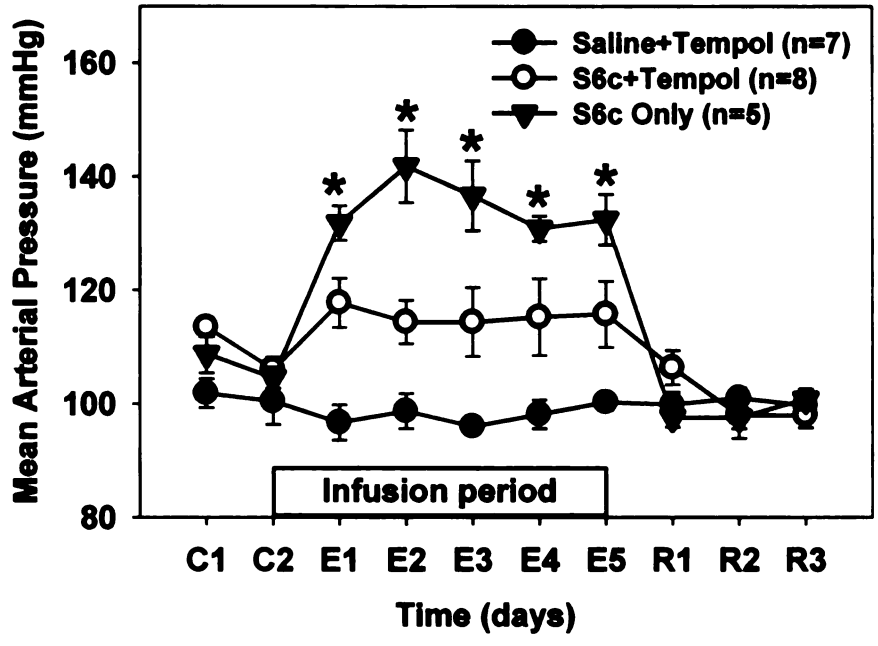

Figure 18: Average (± SEM) mean arterial pressure (MAP) before (C1-C2, C=control), during (E1-E5, E=infusion), and after (R1-R3, R=recovery) continuous S6c infusion (5 pmol/kg/min, iv) or vehicle into rats, while tempol was given through drinking water (1 mmol/l). A third group of animals were on S6c, while drinking normal distilled water. Numbers in parentheses means the number of rats in each group. MAP significantly increased in S6c-infused rats without tempol treatment. The rats with dual treatments of S6c and tempol did not show a significant increase in MAP. Tempol did not change MAP in vehicle-treated rats. *: a statistical difference ($p<0.05$) from control period.

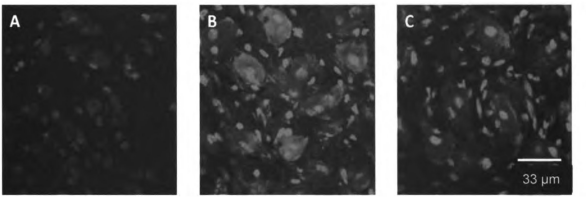

Figure 19a: Superoxide levels in inferior mesenteric ganglion (IMG) neurons from control and S6c-treated rats. A, IMG from a sham rat; B, IMG from a rat receiving S6c for one day; C, IMG from a rat receiving S6c for one day while drinking tempol water (1 mmol/l); The scale bar in C applies to all panels.

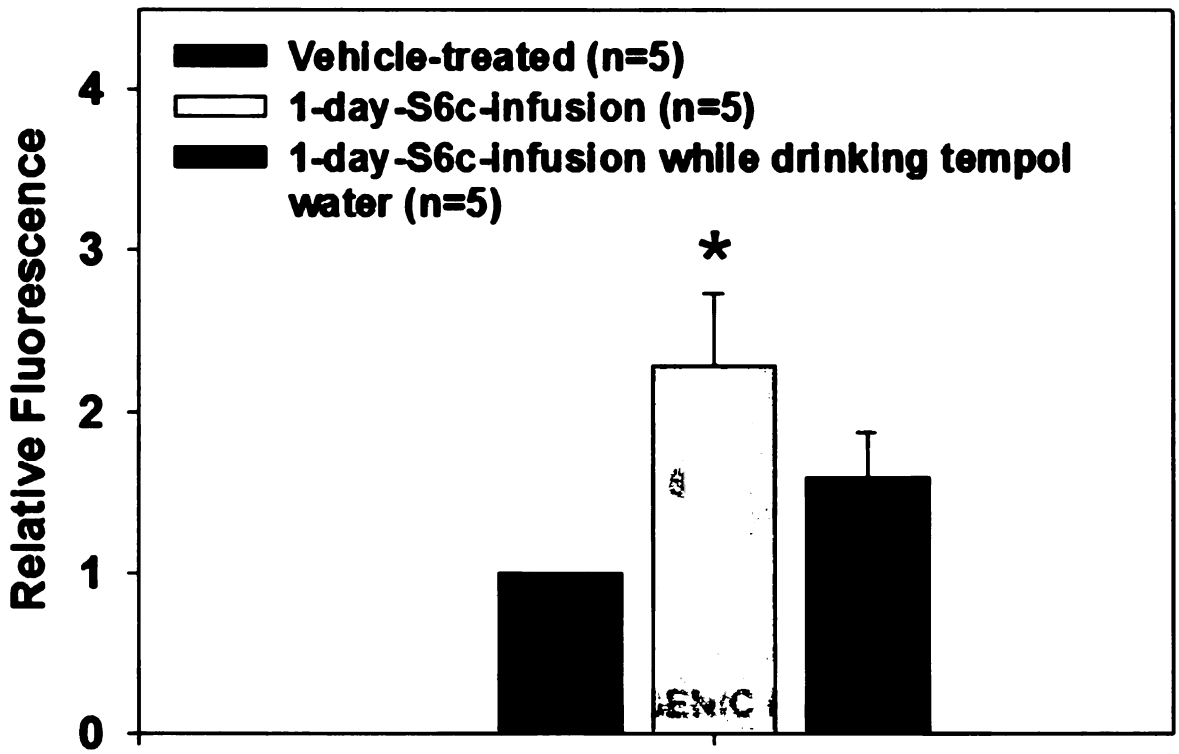

Figure 19b: Quantification of mean fluorescence intensity of S6c-treated smooth muscle cells to sham smooth muscle cells in SMA and SMV (n=5 in each group). *: Fluorescence of IMG neurons was significantly ($P<0.05$) greater in ganglia from rats receiving S6c alone compared with shams'.

CHAPTER 6

CHARACTERIZATION OF ENDOTHELIN-B RECEPTOR-DEFICIENT TRANSGENIC RATS

INTRODUCTION

Endothelin type B receptors (ETBRs) are present and functional in various tissues, such as endothelial cells, vascular smooth muscle cells (VSMCs), kidney and liver. Activation of ETBRs on vascular endothelium can produce vasodilation through the generation of endothelial cell derived vasodilators, such as nitric oxide and prostaglandins (Wright and Fozard 1988; Warner, de Nucci et al. 1989; Rubanyi and Polokoff 1994). Activation of ETBRs in the kidney leads to a marked increase in renal excretion of sodium and water by inhibiting sodium reabsorption in the renal tubules and water reabsorption in the collecting duct (Kohan, Padilla et al. 1993; Plato, Pollock et al. 2000). In addition, ETBRs act as endogenous "clearance receptors" for ET-1 (Ozaki, Ohwaki et al. 1995; Dupuis, Goresky et al. 1996; Berthiaume, Yanagisawa et al. 2000). On the other hand, renal vasoconstriction is induced by the stimulation of ETBRs in the rat (Clozel, Gray et al. 1992; Cristol, Warner et al. 1993); (Clozel, Gray et al. 1992; Cristol, Warner et al. 1993). Recently, it has also been found that ETBR activation contracts veins from most vascular beds but only weakly constricts most non-renal resistance arteries (Moreland, McMullen et al. 1992; Gray, Loffler et al. 1994; Johnson, Fink et al. 2002). ETBR activation also leads to increased oxidative stress in splanchnic sympathetic ganglia (Dai, Galligan et al. 2004). Acute (2-hour) activation of ETBRs *in vivo* induces a sustained blood pressure increase (Lau, Galligan et al. 2006) and elevates oxidative stress level in sympathetic ganglia (Dai, Galligan et al. 2004; Lau, Galligan et al. 2006). Therefore, the

multifunctional nature of ETBRs makes studying their roles in physiological and pathological conditions complicated.

A mutation of the ETBR gene in humans causes Hischsprung's disease, which leads to aganglionic megacolon and pigment abnormalities (Puffenberger, Hosoda et al. 1994). The spotting lethal (sl) rat (with a background related to the WKY rat) is an animal model for human Hischsprung's disease (Gariepy, Cass et al. 1996; Gariepy, Williams et al. 1998). In this model, there is a natural 301 base pair deletion which spans the exon 1-intron 1 junction in the ETBR gene. The deletion leads to an abnormal ETBR mRNA lacking the coding sequence for the receptor's transmembrane domain. No expression of functional ETBRs is detected in tissues from homozygous sl/sl rats. The animals show intestinal aganglionosis and color spotting. This phenotype is lethal and the rat usually dies at the age of one month due to the intestinal defect. In order to rescue the (sl/sl) rats, the human dopamine-β-hydroxylase (*DβH*) promoter was used to direct transgenic (Tg) expression of ETBR to colonizing precursors of the enteric nervous system (Gariepy, Williams et al. 1998). The *DβH*- ETBR transgene compensates for deficiency of endogenous ETBRs in these rats. Therefore the intestinal obstruction is prevented. This transgenic rescue generates Tg (sl/sl) rats with ETBRs that are expressed and functional in adrenergic tissues, including the locus ceruleus, adrenal medulla, and sympathetic ganglia. Unlike in normal animals, ETBRs are not functional in endothelium, renal tubules, or vascular smooth muscles in Tg (sl/sl) rats.

In Tg (+/+) rats, not only endogenous but extra copies of the ETBR gene (by *DβH*- ETBR transgene) are present. Therefore, WKY rats (the background of these transgenic rats) were used as a more natural wildtype control to evaluate strain differences. Gender differences have been reported in various hypertension models (Mallik R. Karamsetty 2004; Reckelhoff 2005). Specifically, gender differences in blood pressure regulation have been reported in Tg (sl/sl) rats (Sullivan, Pollock et al. 2006). The differences are possibly mediated by disparities in plasma ET-1 in the circulation. In addition, the Tg (sl/sl) rats are reported to be salt-sensitive. That is, the animals show elevated blood pressure on high salt diet (Gariepy, Ohuchi et al. 2000; Taylor, Gariepy et al. 2003). Others, however, have reported that systolic blood pressure of Tg (sl/sl) rats is significantly higher than that of controls even on normal salt diet (Elmarakby, Dabbs Loomis et al. 2004).

The specific goal of current study was to characterize this unique rescued transgenic rat model, with an emphasis on neurohumoral mediation (NE and ET-1), gender differences (female vs. male), and strain differences (WKY vs. Tg (+/+) vs. Tg (sl/sl)) in hemodynamic regulation. The overall goal of the work was to better understand the role of the ETBRs in arterial pressure regulation.

METHODS

Animals

Transgenic endothelin type B receptor-deficient rats (Male: 300-325 g; Female: 200-225g) and Wistar Kyoto (WKY) rats (with a related genetic background to the transgenic rats) were used in these experiments. All rats were bred at the animal facility of Michigan State University.

The following methods were used in current study.

- Plasma endothelin-1 measurement;
- Norepinephrine measurement in mesenteric vessels;
- Norepinephrine measurement in organ tissues;
- Glyoxylic acid staining;
- *In vitro* preparation of mesenteric vessels;
- Video monitoring of vessel diameter;
- Transmural stimulation of perivascular nerves;
- Hemodynamic measurement by radiotelemetry.

Statistical analyses

Differences were assessed by a 1-way ANOVA and posthoc multiple comparisons with Tukey's test (GraphPad Instat 3). A $P<0.05$ was considered significant. All of the results are presented as mean±SE.

RESULTS

Blood pressure

Continuous radiotelemetry recording shows (Figure 20), in each gender, mean arterial pressure (MAP) in Tg (sl/sl) rats was significantly higher than that in Tg (+/+) rats (Male: 124 ± 2 vs. 114 ± 1 mmHg; Female: 129 ± 2 vs. 105 ± 1 mmHg; $P<0.05$). In addition, MAP of male Tg (+/+) rats was higher than that of females ($P<0.05$) and male WKY ($P<0.05$).

Plasma ET-1 concentration

Plasma ET-1 levels in Tg (sl/sl) rats were higher than those in Tg (+/+) animals for both genders (Male: 5.2 ± 0.9 vs. 3.6 ± 0.1pg/ml; Female: 7.3 ± 1.4 vs. 3.6 ± 0.4 pg/ml; $P<0.05$) (Figure 21). Also, circulating ET-1 in male Tg (sl/sl) rats was significantly lower than in females ($P<0.05$).

Glyoxylic acid staining

Mesenteric tertiary arteries and veins from male animals were used for glyoxylic acid staining. Figure 22 shows sympathetic nerve distribution on the vessels. There was no visible difference in nerve density among Tg (+/+), Tg (sl/+), and Tg (sl/sl) rats, in either arteries or veins.

NE content measurements

In male rats (Figure 23a), NE concentration in Tg (+/+) arteries was higher than that of male WKY ($P<0.05$). In female rats (Figure 23b), no significant difference was found in NE content of mesenteric arteries or veins among three groups of rats.

Figure 24 shows NE concentrations in splanchnic organs, including kidneys, liver, spleen, and small intestine, from female WKY, Tg (+/+), and Tg (sl/sl) rats. There were no significant differences between any groups.

Electrical stimulation

Mesenteric arteries and veins from male Tg (+/+), Tg (sl/+), and Tg (sl/sl) were collected for this experiment. Figure 25a (for arteries) and Figure 25b (for veins) show that mesenteric vessels constricted in response to electrical stimulation in a frequency-dependent manner. For each vessel type, there were no differences in vasoconstrictor responses among the three groups of rats. Additionally, as it has been shown previously (Luo, Hess et al. 2003), veins were more sensitive than arteries to electrical stimulation.

DISCUSSION

For both male and female animals, resting blood pressure in Tg (sl/sl) rats was significantly higher than that in Tg (+/+) rats. This hypertension was also reported by other labs in both genders of intact Tg (+/+) rats (Taylor, Gariepy et al. 2003; Sullivan, Pollock et al. 2006) or sham-operated rats of DOCA-salt hypertension model (Kawanishi, Hasegawa et al. 2007). However the data remain controversial, since these animals were reported to be normotensive elsewhere (Perry, Molero et al. 2001). Our results are consistent with those in most research labs. More importantly, the blood pressures were measured by radiotelemetry, while others performed hemodynamic measurements by tail-cuff method, which does not monitor the highly dynamic nature of blood pressure as accurately as telemetry (Kurtz, Griffin et al. 2005; Kurtz, Griffin et al. 2005).

My data confirm the finding from other groups that there is a significant increase in circulating ET-1 levels in Tg (sl/sl) rats, compared to Tg (+/+) rats (Perry, Molero et al. 2001; Sullivan, Pollock et al. 2006). So a higher ET-1 level in the circulation may lead to a blood pressure increase via endothelin type A receptor (ETAR) activation. In fact, it has been demonstrated that hypertension induced by high salt diet in Tg (sl/sl) rats can be completely abolished by ETAR blockade, suggesting that the hypertension is caused entirely by endothelin acting at ETARs (D'Angelo, Pollock et al. 2005).

Until now, most characterization studies of Tg (sl/sl) rats have been focused on the vascular and renal ET systems, while sympathetic innervation has received little attention. The sympathetic nervous system, especially in the

splanchnic bed, plays an important role in hypertension development and maintenance (Anderson, Sinkey et al. 1989; Weber 1993; Wyss 1993; Cabassi, Vinci et al. 2002; Zhu, Poole et al. 2005); (Karim and Hainsworth 1976; Greenway 1983). It is generally found that sympathetic effects on the cardiovascular system are elevated in hypertension (Nestel 1969; Egan, Panis et al. 1987; Somers, Anderson et al. 1993; Esler and Kaye 1998).

Therefore, the splanchnic sympathetic nervous system was studied to test if the hypertension in Tg (sl/sl) rats is mediated via sympathetic hyperinnervation. This was accomplished by studying structural changes of the nerves via histological method, glyoxylic acid staining of catecholamine-containing nerves. Staining of sympathetic nerves innervating mesenteric arteries and veins showed that the nerve density was the same in Tg (+/+) and Tg (sl/sl) animals, suggesting that the hypertension in Tg (sl/sl) rats was not due to a difference in the density of sympathetic innervation. Sympathetic nerve density also was quantified by measuring norepinephrine (NE) contents in the splanchnic organs (kidneys, liver, spleen, and small intestine) and mesenteric vessels via high performance liquid chromatography (HPLC). The data revealed that the sympathetic nerve densities in the splanchnic organs and mesenteric vessels were comparable between Tg (+/+) and Tg (sl/sl) rats. Furthermore, the results from functional studies of sympathetic nerves were consistent with the data from nerve density experiments. That is, the nerve responses to electrical stimulation were similar in arteries and veins from these two groups of rats.

Mean arterial pressure of male Tg (+/+) rats was higher than that of male WKY rats. One piece of evidence in my study could be an explanation: the norepinephrine concentration in mesenteric arteries from male Tg (+/+) was significantly higher than that from WKY rats. Higher regional catecholamine level may be an indicator of a higher sympathetic activity in that vascular region. The activation of the sympathetic nerves innervating resistance arteries may lead to a direct vasoconstriction and blood pressure increase (Weber 1993; Wyss 1993).

Additionally, the splanchnic sympathetic ganglia in Tg (+/+) rats ETBRs have 70 more copies of the ETBR gene than normal WKY rats (Gariepy, Williams et al. 1998). More experiments, such as chronic S6c infusion into WKY rats and calcium imaging in celiac ganglionic neurons with ETBR activation, need to be done to elucidate the differences in sympathetic ganglion function between WKY and Tg (+/+) rats. If sympathetic neurons from Tg (+/+) rats respond to S6c more than those from WKY, this transgenic rat strain may represent an interesting model to study the effects of ETBRs in the sympathetic ganglia without the need for exogenous activation of the receptors with S6c.

Gender differences in blood pressure regulation have been reported in this rat model (Sullivan, Pollock et al. 2006). The data in current study support these findings. In addition, in human saphenous veins, the binding capacity for ETARs in males is higher than that in females (Ergul, Shoemaker et al. 1998). This could help explain why the MAPs are comparable in Tg (sl/sl) between the two genders, even with a higher ET-1 concentration in the circulation in the females. Therefore, ETAR densities need to be measured in Tg (sl/sl) rats to test if

different receptor densities make a contribution to the difference in resting blood pressure between the two genders. In the future, more research should be carried out to study gender differences in Tg (sl/sl) rats, either through surgical approaches (oophorectomy or orchidectomy) or drug treatment (estrogen or androgen or hormone blockers).

Collectively, my data show that there are no differences in the splanchnic sympathetic innervation at structural and functional levels between WKY, Tg (+/+), and Tg (sl/sl) rats, indicating a successful genetic rescue in Tg (sl/sl). My results confirm the findings of others that plasma ET-1 levels are higher in both male and female Tg (sl/sl) rats. The main reason for the hypertension in Tg (sl/sl) rats seem to be augmented ETAR activation because of their high circulating endothelin-1 level. Still, the possibility of the splanchnic sympathetic nervous system's involvement in Tg (sl/sl) hypertension can not be excluded, since no publications have shown studies of neural regulation in hemodynamic regulation of these transgenic rats by means of direct neural blockade. CGX could be performed in Tg (sl/sl) rats to test the hypothesis that the splanchnic sympathetic innervation contributes to the hypertension. If the results support that hypothesis, more studies need to be done to reveal the relationship between ETAR activation and the sympathetic nervous system in the splanchnic bed.

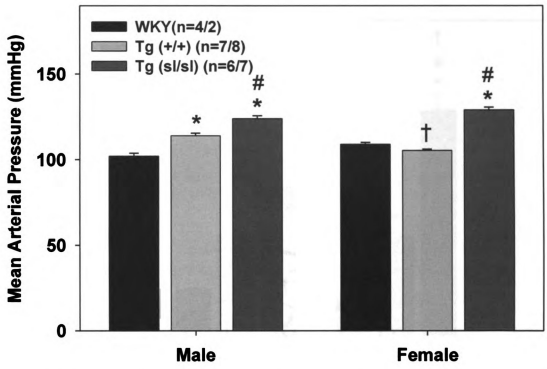

Figure 20: Mean arterial pressure of male and female WKY, Tg (+/+) and Tg (sl/sl) rats. *: significant difference from WKY rats for each gender; #: significant difference from Tg (+/+) rats for each gender; †: significant different from male Tg (+/+) rats.

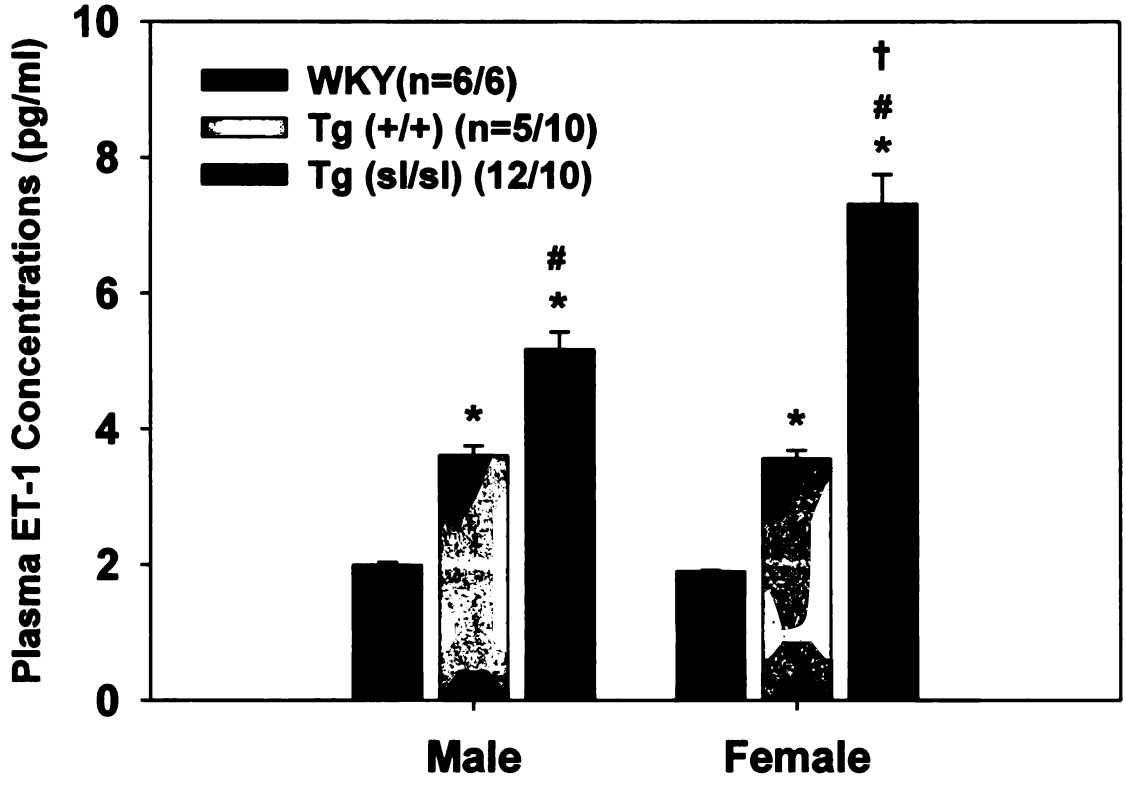

Figure 21: Plasma ET-1 concentrations in male and female WKY, Tg (+/+) and Tg (sl/sl) rats. *: significant difference from WKY in each gender; #: significant difference from Tg (+/+) rats in each gender; †: significant difference from male Tg (sl/sl) rats.

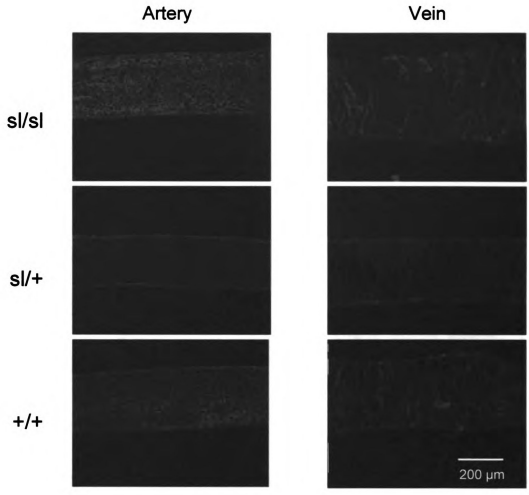

Figure 22: Glyoxylic acid staining of mesenteric arteries and veins from Tg (sl/sl), Tg (sl/+), and Tg (+/+) rats. The blue fluorescence represents the presence of catecholamine. The scale bar applies to all pictures.

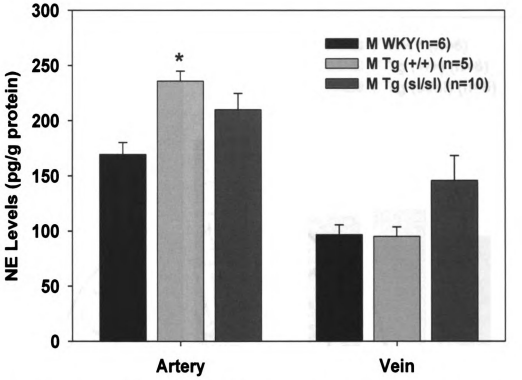

Figure 23a: NE levels in mesenteric artery and vein from male WKY, Tg (+/+), and Tg (sl/sl) rats. The concentration of NE in artery from Tg (+/+) rats was significantly higher than that from WKY rats.

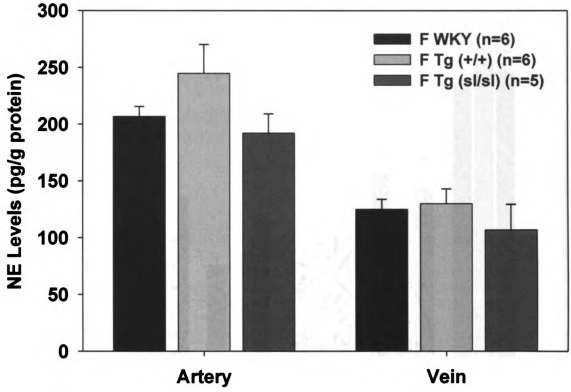

Figure 23b: NE levels in mesenteric artery and vein from male WKY, Tg (+/+), and Tg (sl/sl) rats. There was no significant difference between any two groups for either blood vessel type.

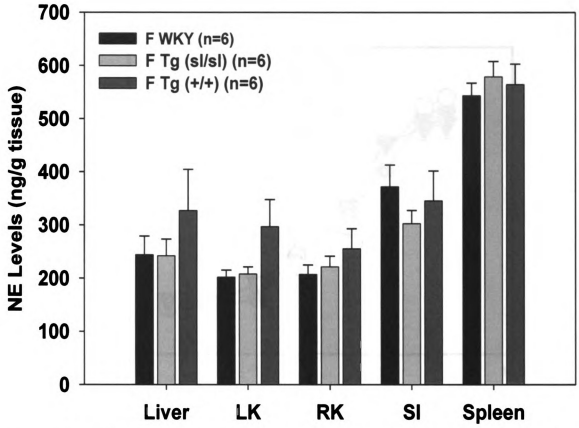

Figure 24: NE levels in splanchnic organs from female WKY, Tg (sl/sl), and Tg (+/+) rats. No significant difference was shown between any two groups for any organ. LK: lift kidney; RK: right kidney; SI: small intestine.

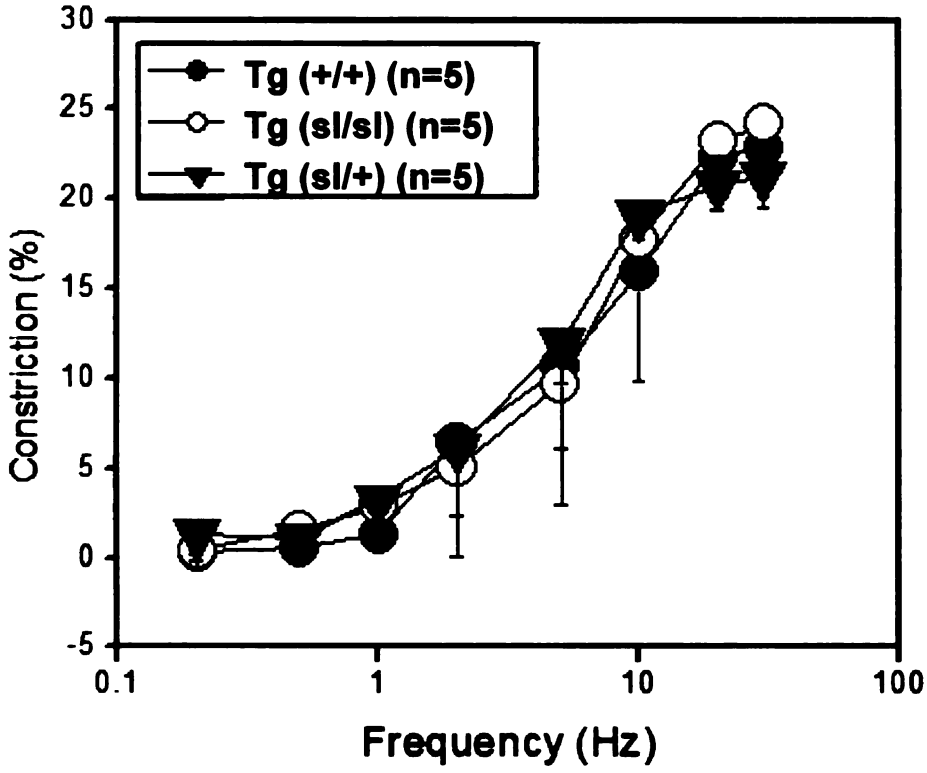

Figure 25a: Comparison of frequency–response curves for neurogenic constrictions of arteries from Tg (+/+), Tg (sl/sl), and Tg (sl/+) rats. No significant difference in frequency-dependent response was shown.

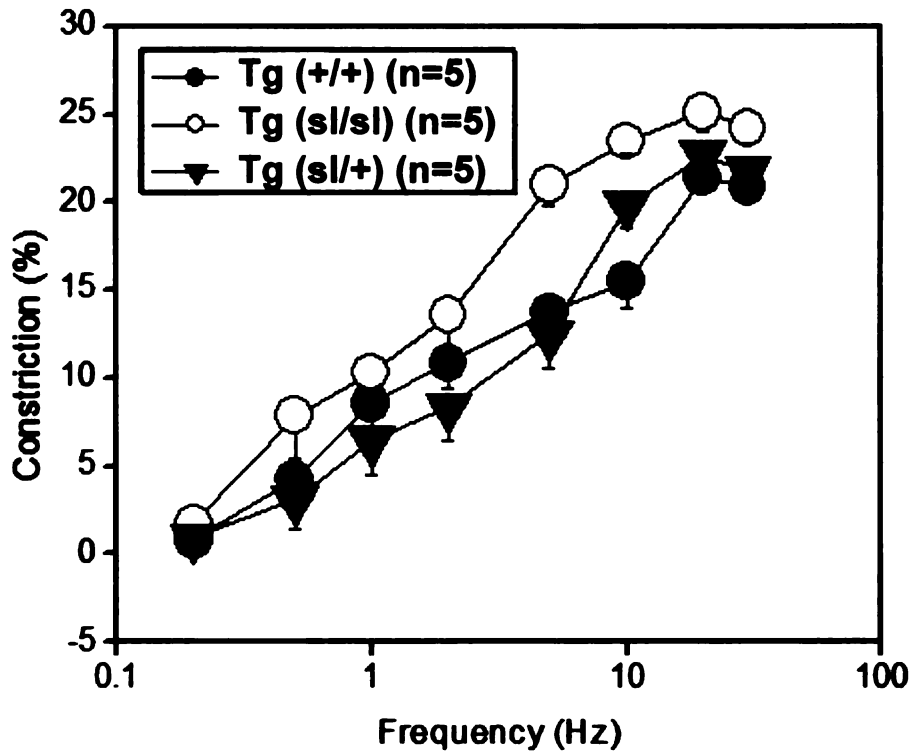

Figure 25b: Comparison of frequency–response curves for neurogenic constrictions of veins from Tg (+/+), Tg (sl/sl), and Tg (sl/+) rats. No significant difference in frequency-dependent response was shown.

CHAPTER 7

MECHANISMS OF HYPERTENSION INDUCED BY ENDOTHELIN B RECEPTOR (ETBR) ACTIVATION: STUDIES IN ETBR-DEFICIENT RATS

INTRODUCTION

We have recently established a novel hypertension model: S6c-induced hypertension (Fink, Li et al. 2007). In this model, endothelin type B receptors (ETBRs) are chronically activated by the infusion of sarafotoxin 6c (S6c), a selective ETBR agonist, into conscious rats. Most physiological responses mediated by ETBRs cause decreased blood pressure (Rubanyi and Polokoff 1994; Schiffrin 1998; Pollock, Allcock et al. 2000), e.g. vasodilators released from endothelial cells, and enhanced excretion of sodium and water by the kidneys. Therefore, the mechanisms underlying S6c-induced hypertension are unclear. However, because S6c constricts veins from most vascular regions but has much less effect on arteries (Strachan, Haynes et al. 1995; Strachan, Crockett et al. 2000; Johnson, Fink et al. 2002; Alexander, Mathie et al. 2007), we hypothesized that one mechanism of S6c-induced hypertension could be direct venoconstriction.

Additionally, sympathetic nerve activity is the most critical factor controlling venoconstriction, especially in the splanchnic circulation (Shoukas and Bohlen 1990; Ozono, Bosnjak et al. 1991). Sympathetic activation of the splanchnic veins can reduce blood volume up to 60%, redistributing blood from veins into the heart and thereby increasing blood pressure (Karim and Hainsworth 1976; Greenway 1983). Therefore, it is interesting that ETBRs are found on the sympathetic neurons (Takimoto, Inui et al. 1993; Pomonis, Rogers et al. 2001), and can affect their function (Takimoto, Inui et al. 1993(Yamada, Kushiku et al.

1999; Isaka, Kudo et al. 2007). One effect of ETBR activation in sympathetic ganglia is increased levels of reactive oxygen species (ROS). For example, superoxide levels are increased in sympathetic neurons from prevertebral ganglia (innervating the splanchnic organs) when they are treated with S6c *in vitro* (Dai, Galligan et al. 2004). Furthermore, acute intravenous infusion of S6c into conscious rats increases superoxide levels in prevertebral sympathetic ganglia (Lau, Galligan et al. 2006). Because ROS can function as signaling molecules in neurons and thereby affect neuronal activity (Dai, Galligan et al. 2004); (Zimmerman and Davisson 2004; Sun, Sellers et al. 2005; Infanger, Sharma et al. 2006), S6c could cause hypertension by increasing post-ganglionic sympathetic activity. In fact we recently produced evidence that S6c-induced hypertension depends in part on ROS-driven sympathetic activation to the splanchnic region (Li, Dai et al. 2007). Nevertheless, previous work has not allowed a clear delineation of the overall importance of ETBRs on sympathetic neurons to S6c-induced hypertension.

In humans, a mutation of the gene of ETBR causes Hischsprung's disease, characterized by aganglionic megacolon and pigment abnormalities (Puffenberger, Hosoda et al. 1994). The spotting lethal (sl) rat is a naturally occurring animal model of human Hischsprung's disease (Gariepy, Cass et al. 1996; Gariepy, Williams et al. 1998). In this rat model, there is a 301-bp deletion which spans junction of the exon 1-intron 1 junction in the ETBR gene. The deletion leads to the result of an abnormal ETBR mRNA lacking the coding

sequence for the receptor's transmembrane domain. No functional ETBRs are detected in tissues from homozygous sl/sl rats. The animals show intestinal aganglionosis and color spotting. They usually die at the age of one month due to congenital intestinal aganglionosis. In order to rescue the (sl/sl) rats, the human dopamine-β-hydroxylase (*DβH*) promoter was used to direct transgenic (Tg) expression of ETBR to colonizing precursors of the enteric nervous system (Gariepy, Williams et al. 1998). The *DβH*- ETBR transgene compensates for deficiency of endogenous ETBRs in these rats. Therefore the intestinal obstruction is prevented. This transgenic rescue generates Tg (sl/sl) rats with ETBRs that are expressed and functional in adrenergic tissues, including the locus ceruleus, adrenal medulla, and sympathetic ganglia. Unlike in normal animals, ETBRs are not functional in endothelium, renal tubules, or vascular smooth muscles in Tg (sl/sl) rats. Therefore, this unique animal model can be used to investigate the roles of ETBRs in a tissue-specific manner. In Tg (+/+) rats, not only endogenous but extra copies of ETBR gene (by *DβH*- ETBR transgene) are present. In previous sections of my thesis, I characterized S6c-induced hypertension, including the effects on oxidative stress in blood vessels and sympathetic ganglia; showed the effects of CGX on splanchnic nerve density and function; and described basal blood pressure, and splanchnic sympathetic nerve density and function in Tg (sl/sl) and Tg (+/+) rats. Here I tested the hypothesis that if S6c-induced hypertension is due in part to the activation of ETBRs on the sympathetic neurons.

METHODS

Animals

Male transgenic ETBR-deficient rats (300 – 325 g) were used in the experiments.

The following methods were involved in this study.

- Celiac ganglionectomy (CGX);
- Radiotelemetry implantation;
- Minipump implantation;
- Hemodynamic measurements by telemetry.

Experimental protocols

S6c only treatment

This protocol was used for intact rats (one week after transmitter implantation) and celiac ganglionectomized rats (three weeks after transmitter implantation). Baseline data were recorded for three days as a control period. S6c solution was infused using micro-osmotic pumps (Alzet, Model 1007D). The drug was delivered at the rate of 30 pmol/kg/min, *sc*. After seven days, the pump was removed and an additional four days of recording was obtained.

S6c and tempol treatment

When the initial S6c infusion study was completed in normal rats, transmitters were turned off. After ten days transmitters were turned on again, and three days of control period data were collected. Then all rats were give tempol (4-hydroxy 2,2,6,6,-tetramethyl peperidine 1-oxyl, a superoxide dismutase

mimetic) in the drinking water at a concentration of 1 mmol/l. Each water bottle was wrapped with aluminum foil for light protection. Tempol-containing drinking water was freshly prepared every day. After the rats received tempol for three days, S6c was infused for five days, as described above. Tempol treatment was then terminated while S6c infusion continued for two additional days. Finally, S6c infusion was stopped by removing the pumps and rats were monitored for a four day recovery period.

Statistical analyses

The effect of CGX on MAP during the control period was evaluated by Student *t* test compared to the average of baseline MAP in intact rats. Between-group differences over time was assessed by a 2-way ANOVA and posthoc multiple comparisons with Bonferroni's test. A $P<0.05$ was considered significant. All of the results are presented as mean±SE.

RESULTS

Effects of S6c infusion in Tg (+/+) and Tg (-/-) rats

As shown in Figure 26, mean arterial pressure (MAP) was significantly higher in Tg (sl/sl) rats (124 ± 2 mmHg) than in Tg (+/+) rats (114 ± 1 mmHg) during the control period. During the first three days of S6c treatment, MAP increased significantly in Tg (+/+) rats (~30 mmHg). Subsequently MAP decreased to a level not significantly higher than during the control period. When S6c infusion was terminated, however, MAP fell significantly below control period values for two days. In Tg (sl/sl) rats, S6c did not significantly affect MAP at any time during the infusion period; termination of infusion also was without effect. Baseline heart rates (HR) were not different in Tg (+/+) (311 ± 4 bpm) and Tg (sl/sl) (318 ± 4 bpm) rats (Figure 27). In Tg (+/+) rats, HR declined significantly on the first day of S6c infusion, then gradually returned to pre-infusion values. When S6c infusion was terminated, HR was significantly increased on the first two days of the recovery period. The infusion of S6c had no effect on HR in Tg (sl/sl) rats.

Effects of tempol treatment on S6c-induced hypertension

Tempol treatment alone did not change MAP (Figure 28) or HR (Figure 29) in either Tg (sl/sl) or Tg (+/+) rats. S6c infusion during tempol treatment increased MAP in Tg (+/+) rats but not Tg (sl/sl) rats. Ceasing tempol treatment during continued S6c infusion did not affect MAP in either group of the rats. MAP decreased significantly in Tg (+/+) but not Tg (sl/sl) rats when S6c was

withdrawn. Tempol treatment had no effect on basal HR in either Tg (+/+) or Tg (sl/sl) rats, nor did it modify responses to S6c infusion (Figure 29).

Effects of celiac ganglionectomy (CGX) on S6c-induced hypertension

In Tg (+/+) rats, CGX caused a significant decrease in basal MAP compared to sham-operated controls (Figure 30a; intact rats are the same as shown in Figure 26). In Tg (sl/sl) rats CGX also decreased basal MAP, but the difference was not statistically significant (Figure 30b; intact rats are the same as shown in Figure 28). S6c infusion increased MAP significantly for only two days in CGX Tg (+/+) rats, then MAP returned to levels no different from those of the control period. Unlike in intact Tg (+/+) rats, termination of S6c infusion did not affect MAP in CGX Tg (+/+) rats. S6c infusion in CGX Tg (sl/sl) rats produced no change in MAP throughout the study. CGX had no effect on basal HR in either group of rats, not did it modify HR responses to S6c infusion (Figure 31).

DISCUSSION

The transgenic (Tg) spotting lethal (sl) rat is a unique animal model to study the roles of ETBRs in blood pressure regulation. Although heterozygous ETBR-deficient mice (Ohuchi, Kuwaki et al. 1999) and Tg (sl/sl) mice (Kapur, Sweetser et al. 1995) are available, their small size makes physiological studies difficult. On the other hand, in Tg (sl/sl) rats (Gariepy, Williams et al. 1998) numerous aspects of cardiovascular system function have already been characterized (Gariepy, Ohuchi et al. 2000; Sullivan, Pollock et al. 2006). For our purposes, a key attribute of these rats is their failure to express functional ETBRs in endothelial cells, renal tubular cells, and vascular smooth muscle cells. But because the "rescue" transgene is linked to a dopamine-β-hydroxylase promoter, robust ETBR expression occurs in sympathetic neurons and other cells expressing high levels of this gene. Thus they offer an ideal opportunity to isolate cardiovascular effects of ETBR activation mediated by the sympathetic nervous system. It is already known that Tg (sl/sl) rats exhibit a pressor response to acute activation of ETBRs with high doses of S6c (Pollock, Portik-Dobos et al. 2000). The goal of the current study was to determine if chronic infusion of lower doses of S6c would increase MAP in Tg (sl/sl) rats, as a way of clarifying the role of the sympathetic nervous system in this new model of hypertension.

The baseline MAP of Tg (sl/sl) rats was ~ 10 mmHg higher than that of Tg (+/+) rats. This finding is consistent with the results of other studies (Gariepy, Ohuchi et al. 2000; Sullivan, Pollock et al. 2006).There are several possible reasons for the higher blood pressure in Tg (sl/sl) rats. First, ETBRs function as

endogenous ET-1 "clearance receptors", i.e. circulating ET-1 is removed from blood by ETBRs located on endothelial cells. Tg (sl/sl) rats lack this function, so have higher circulating levels of ET-1 (Sullivan, Pollock et al. 2006). This could cause elevated blood pressure through activation of ETA receptors (Elmarakby, Dabbs Loomis et al. 2004). Second, hypertension in Tg (sl/sl) rats is salt-sensitive (Gariepy, Ohuchi et al. 2000; Elmarakby, Dabbs Loomis et al. 2004; Ohkita, Wang et al. 2005) and is attenuated by diuretic therapy (Gariepy, Ohuchi et al. 2000). Furthermore, selective knockout of ETBRs in the collecting duct of the kidney results in hypertension (Ge, Bagnall et al. 2006). Thus, hypertension in Tg (sl/sl) rats may be due to renal sodium and water retention. Third, there is limited evidence that increased sympathetic activity can contribute to hypertension in Tg (sl/sl) rats, at least during high salt intake (Ohkita, Wang et al. 2005). We did not investigate this question directly in the current study. But increased sympathetic input to the splanchnic region via the celiac ganglion is not critical for hypertension in Tg (sl/sl) rats, since CGX reduced MAP to approximately the same degree in Tg (+/+) and Tg (sl/sl) rats. Finally, tempol treatment also had no effect on blood pressure in hypertensive Tg (sl/sl) rats. This was somewhat surprising, since hypertension in Tg (sl/sl) rats may be driven by increased circulating ET-1 (Elmarakby, Dabbs Loomis et al. 2004), and endothelin-dependent hypertension in one study was attenuated by tempol treatment (Sedeek, Llinas et al. 2003). However, other work suggests that oxidative stress has only a limited role in endothelin-dependent hypertension (Elmarakby, Loomis et al. 2005; Sullivan, Pollock et al. 2006).

Early studies on cardiovascular responses to acute S6c infusion revealed sympathetically mediated constriction of the mesenteric circulation (Clozel, Gray et al. 1992). We have found evidence that sympathetic activation—especially in the splanchnic region— secondary to increased ganglionic superoxide levels contributes to chronic S6c-induced hypertension in Sprague-Dawley rats (Li, Dai et al. 2007). Thus, we hypothesized that S6c would induce hypertension in Tg (sl/sl) rats by activating ETBRs and increasing ROS levels in the sympathetic ganglia. Two results from the current study suggest that this mechanism is not operative in Tg (+/+) or Tg (sl/sl) rats. First, contrary to what was observed in our previous work in normal Sprague-Dawley rats (Li, Dai et al. 2007), tempol did not attenuate S6c-induced hypertension in either Tg (+/+) or Tg (sl/sl) rats. There are at least two possible technical reasons that could explain this difference. One reason may be the strain difference. The transgenic rats were derived from the Wistar Kyoto (WKY) strain, while all of our previous work was in Sprague-Dawley animals. Another difference is that, in the current study, S6c was given by subcutaneous infusion at a dose (30 pmol/kg/min) calculated to produce similar blood pressure changes to those we observed previously using intravenous infusion (5 pmol/kg/min). Thus it is possible that the strength of activation of ETBRs differed in the two protocols (even though the increases in arterial pressure were similar). Another possibility is that overexpression of the ETBR transgene in the sympathetic neurons alters their ability to respond to S6c with increased generation of ROS. Finally, plasma clearance of ET-1 is impaired in Tg

(sl/sl) rats, so it is possible that ganglionic ETBRs are downregulated by high circulating concentrations of ET-1 in these rats.

A second piece of evidence arguing against an important effect of S6c on the sympathetic activity is that although MAP increased significantly during chronic S6c infusion in Tg (+/+) rats, no change in MAP was observed in Tg (sl/sl) rats. The obvious interpretation of this result is that in normal rats S6c must be able to activate ETBRs in non-adrenergic tissue to cause chronic hypertension. As discussed earlier, a possible site for this hypertensive effect of S6c is vascular smooth muscle in veins. Stimulation of ETBRs in other most tissues (endothelial cells, renal epithelial cells) should lower blood pressure. However, another possible target is renal arterioles, where ETBR stimulation produces direct vasoconstriction and reduced renal blood flow (Clozel, Gray et al. 1992); (Gellai, DeWolf et al. 1994; Pollock, Jenkins et al. 2005). Nevertheless, it also is possible that ETBRs on sympathetic neurons are simply non-functional in Tg (+/+) and Tg (sl/sl) rats, as discussed above. Data from experiments exploring the effects of CGX on S6c-induced hypertension suggest that this may be the case.

Our results demonstrated that when Tg (sl/sl) rats were treated with S6c, there was no change in blood pressure. So it seems that the sympathetic activation is not involved in Tg (sl/sl) rats upon S6c infusion, which was to our surprise and quite contrary to our previous findings. Some unpublished results (not shown here) from the Kreulen laboratory indicate that ETBRs are actually not functional in the splanchnic sympathetic neurons of adult Tg (sl/sl) rats. As

we noted earlier, circulating ET-1 level is high in Tg (sl/sl) rats, so it is possible that ETBRs are somehow downregulated by a high ET-1 concentration in the circulation. Since ETBRs must be functional during the development of enteric nervous system in Tg (sl/sl) rats, it is possible that ETBRs may have a greater role in S6c-induced hypertension in younger rats. This idea remains to be tested.

In additional experiments, we removed celiac ganglia (CGX) to further test if the splanchnic sympathetic innervation is critical for S6c-induced hypertension. The increase in blood pressure during S6c infusion was less in CGX Tg (+/+) rats than that in intact Tg (+/+) rats, suggesting that the splanchnic sympathetic nervous system makes a contribution to the hypertension. Interestingly, we also found that MAP increase during the second half infusion period was less than that in the first half. Our previous data show that direct venoconstriction mainly mediates S6c-induced hypertension in the first 2-3 days, then neuronal regulation gradually becomes more important. In support of this idea, current study demonstrated that CGX attenuated S6c-induced hypertension in Tg (+/+) rats, most markedly, in the later stage of the infusion period. Collectively, our previous and present results are very consistent in showing that the splanchnic sympathetic nerves are partially involved in S6c-induced hypertension, especially during the maintenance phase.

Perspectives

Most studies on ETBRs have been focused on the receptor's beneficial effects via the mechanisms of vasodilation. Our lab is one of the few groups that have carried out *in vivo* experiments with a focus on the vasoconstrictor effects

induced by ETBR activation. Our results indicate that S6-induced hypertension is indeed mediated through ETBR activation, since S6c does not change MAP in Tg (sl/sl) rats. Furthermore, the development and the maintenance of S6c-induced hypertension are attenuated when the majority of the splanchnic sympathetic innervation is removed. Accumulating evidence indicates that the sympathetic nervous system (SNS) may be an important mediator in experimental models and human hypertension. My data not only support the above finding but suggest that the SNS in the splanchnic bed, at least partially via ETBR activation, is a critical neural component in blood pressure regulation. Therefore, ETBRs in the splanchnic SNS could be a therapeutic target for the treatment of hypertension.

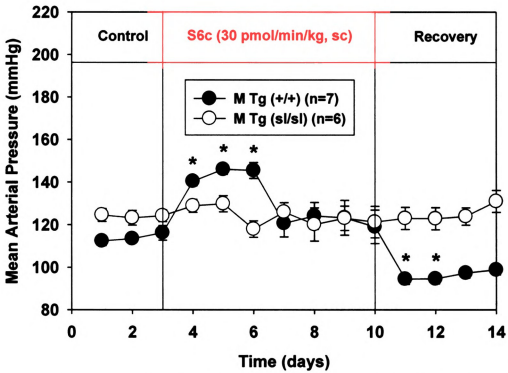

Figure 26: Mean arterial pressure (MAP) before (day 1-3), during (day 4-10), after (day 11-14) continuous S6c infusion (30 pmol/kg/min, sc.). MAP was significantly higher in Tg (sl/sl) rats than in Tg (+/+) rats during the control period In Tg (+/) rats. MAP significantly increased upon S6c infusion in the first three days. It decreased significantly on the first two days of the recovery period upon S6c removal. In Tg (sl/sl) rats, MAP did not change during the infusion or recovery period, compared to the control period. Asterisks indicate a statistical difference ($p<0.05$) from the last day of the control period.

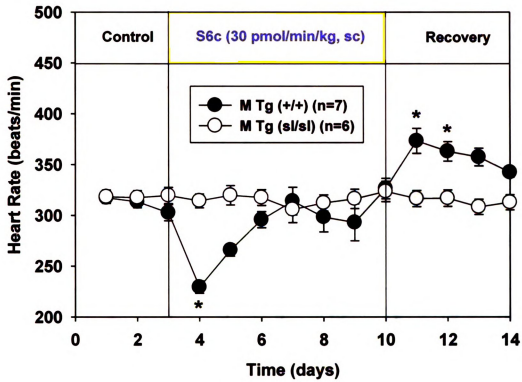

Figure 27: Heart rate (HR) before (day 1-3), during (day 4-10), after (day 11-14) continuous S6c infusion (30 pmol/kg/min, sc.). In Tg (+/) rats, HR significantly decreased on the first day of S6c infusion. It increased significantly on the first two days of recovery period upon S6c removal. In Tg (sl/sl) rats, HR did not change during the infusion or recovery period, compared to the control period. Asterisks indicate a statistical difference ($p<0.05$) from the last day of the control period.

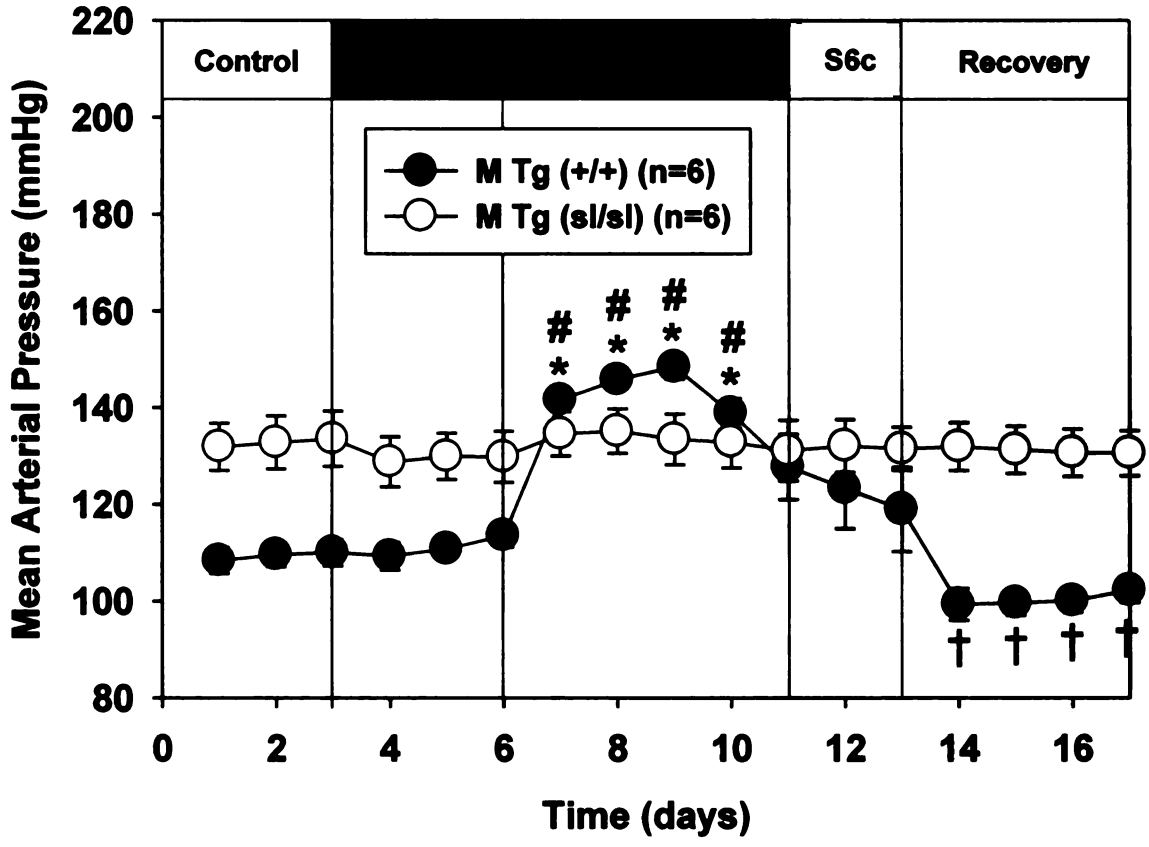

Figure 28: Mean arterial pressure (MAP) during the control period (day 1-3), tempol treatment period (day 4-6), tempol treatment with S6c infusion (30 pmol/kg/min, sc.) period (day 7-11), S6c infusion period (day 12-13), and recovery period (day 14-17). In Tg (+/) rats, MAP did not change with tempol treatment alone. It significantly increased during the whole period of S6c infusion while the rats were drinking tempol solution. It decreased significantly in the recovery period. In Tg (sl/sl) rats, MAP did not change during the infusion or recovery period, compared to the control period. Asterisks stand for a statistical difference ($p<0.05$) from the last day of the control period. Pound signs represent significant difference from the last day of tempol treatment period. Cross signs indicate significant difference from the last day of tempol treatment.

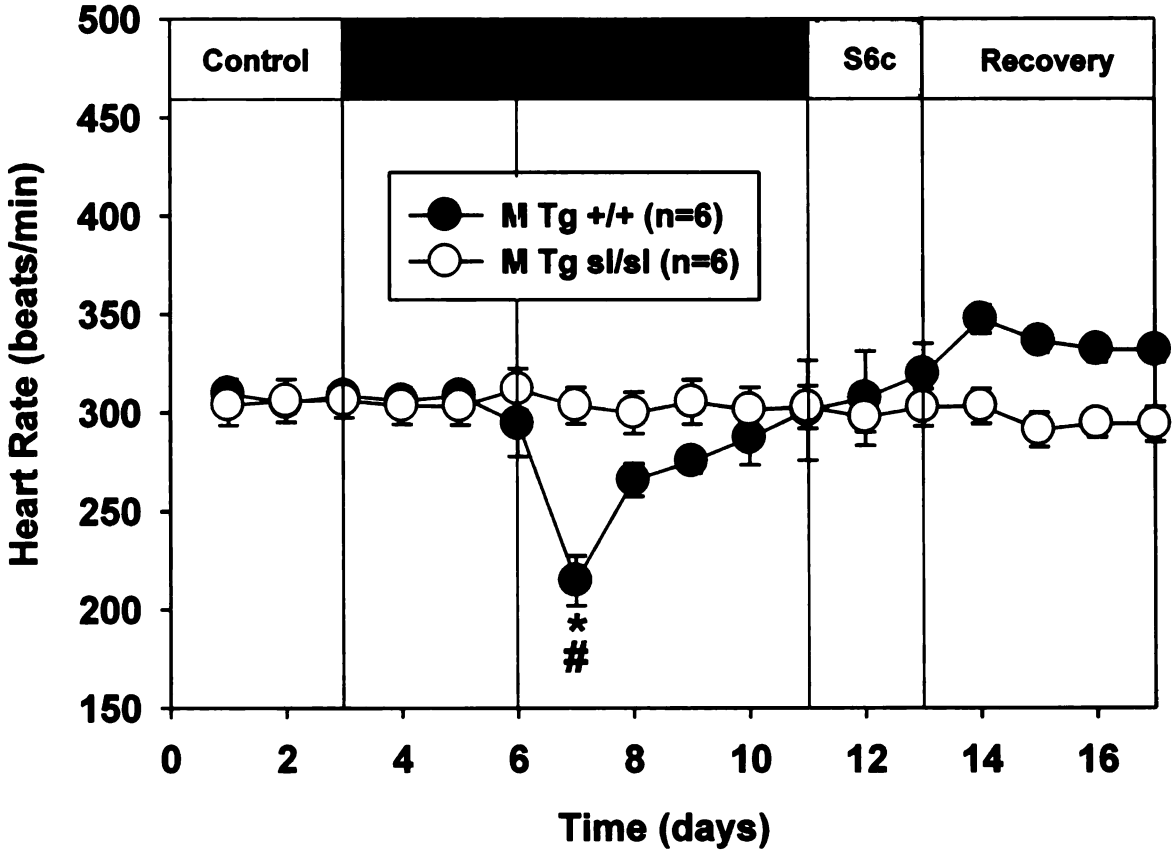

Figure 29: Heart rate (HR) during the control period (day 1-3), tempol treatment period (day 4-6), tempol treatment with S6c infusion (30 pmol/kg/min, sc.) period (day 7-11), S6c infusion period (day 12-13), and recovery period (day 14-17). In Tg (+/+) rats, HR significantly dropped on the first day of S6c infusion (day 7). In Tg (sl/sl) rats, HR did not change during the infusion or recovery period, compared to the control period. Asterisk stands for a statistical difference ($p<0.05$) from the last day of control period. Pound sign represents a significant difference from the last day of the tempol treatment period.

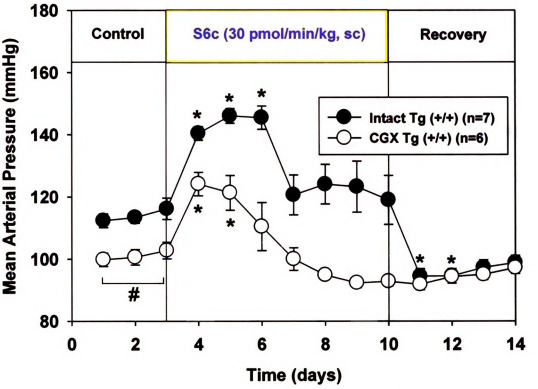

Figure 30a: Mean arterial pressure (MAP) before (day 1-3), during (day 4-10), after (day 11-14) continuous S6c infusion (30 pmol/kg/min, sc.) in Tg (+/+) rats. CGX significantly lowered resting blood pressure. In CGX Tg (+/+) rats, MAP significantly increased upon S6c infusion on the first two days, compared to the control period. Asterisks indicate statistical difference ($p<0.05$) from the last day of the control period. Pound signs indicate statistical difference from intact Tg (+/+) rats on the same day.

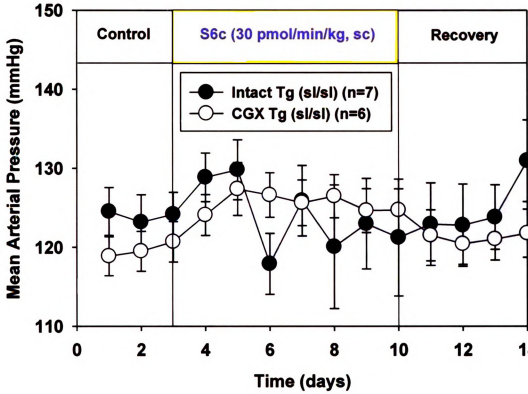

Figure 30b: Mean arterial pressure (MAP) before (day 1-3), during (day 4-10), after (day 11-14) continuous S6c infusion (30 pmol/kg/min, sc.) in Tg (sl/sl) rats. CGX did not change resting blood pressure of Tg (sl/sl) rats. There was no significant difference in MAP between Tg (+/+) and Tg (sl/sl) rats during the infusion and recovery periods.

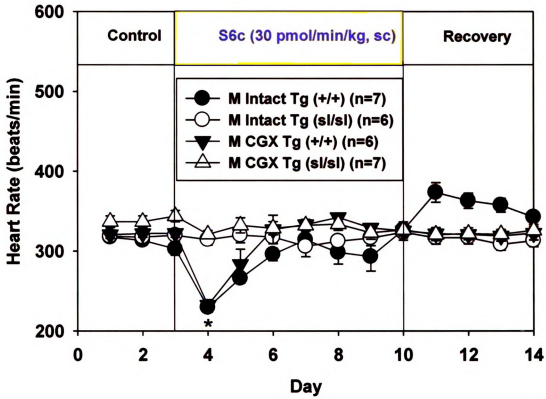

Figure 31: Heart rate (HR) before (day 1-3), during (day 4-10), after (day 11-14) continuous S6c infusion (30 pmol/kg/min, sc.) in intact and CGX Tg rats (+/+ and sl/sl). HR significantly decreased upon S6c infusion on the first day in intact and CGX Tg (+/+) rats, compared to the control period . In Tg (sl/sl) rats, HR did not change during the infusion or recovery period. Asterisk indicate a statistical difference ($p<0.05$) from the last day of the control period.

CHAPTER 8

SENSORY INNERVATION IN S6C-INDUCED HYPERTENSION

INTRODUCTION

There is good evidence suggesting that the sensory nervous system plays a role in blood pressure regulation. As discussed in the **GENERAL INTRODUCTION**, calcitonin gene-related peptide (CGRP) is a very potent vasodilating neuropeptides. CGRP is primarily synthesized in dorsal root ganglia, where sensory neuron bodies are located. These sensory neurons terminate on many cardiovascular tissues, including blood vessels (Li and Wang 2003). CGRP has been reported to innervate almost all vascular beds (Dipette and Wimalawansa 1995; Wimalawansa 1996). Also, CGRP receptors are present throughout the arterial system (Wimalawansa and MacIntyre 1988). CGRP affects blood pressure regulation mainly by increasing water and sodium excretion secondary to elevated renal blood flow and glomerular filtration rate (Shekhar, Anand et al. 1991). Activation of CGRP receptors leads to reduced vascular resistance in the kidney (Howden, Logue et al. 1988; Jager, Muench et al. 1990) and relaxation of afferent arterioles in the glomeruli (Sanke, Hanabusa et al. 1991). CGRP is released from perivascular sensory nerve terminals that are capsaicin-sensitive (Holzer and Maggi 1998). A sensory-motor denervation model is available in rats (Holzer-Petsche and Lembeck 1984). In this model, the neonatal rats are treated with capsaicin and capsaicin-sensitive sensory nerves are impaired right after birth. Thereby the release of CGRP is inhibited.

It has been shown that ET-1 induces CGRP release by acting on ETA receptors (ETARS) (Wang and Wang 2004). In S6c-induced hypertension, since ETBRs are mainly occupied by S6c, the "clearance receptor' function of ETBRs

to remove endogenous ET-1 from the circulation may be less effective. Therefore, plasma ET-1 level may be increased and thereby causes the release of CGRP through activation of ETARs. Neonatal capsaicin-treated rats were used to explore the role of sensory nerves and CGRP in S6c-induced hypertension.

METHODS

Animals

Male neonatal capsaicin-treated rats were used in this study.

The following methods were used in current chapter.

- Hemodynamic measurements by telemetry;
- Minipump implantation.

Experimental protocol

S6c-infusion

Baseline data were recorded for three days as a control period. Then animals were weighed for preparation of S6c solution. S6c solution was made and transferred into micro-osmotic pumps (Alzet, Model 1007D). A small opening was made between the scapulae and a pocket was created for the subcutaneous implantation of the mini-pump with the delivering site toward the lower body. The drug was delivered at the rate of 30 pmol/kg/min. After an infusion for seven days, the pumps were taken out, followed by additional recording for four days, as a recovery period.

Statistical Analyses

Due to the small sample size (n=2) of this study, the statistical analyses were not performed.

RESULTS

Resting mean arterial pressure (MAP) in vehicle-treated rats was lower than that in capsaicin-treated rats (Figure 32). With S6c treatment, MAP

increased in both groups, then it gradually dropped back to baseline level. Upon the removal of S6c, MAP did not change compared to that of the last infusion day.

Figure 33 shows that the resting heart rate (HR) in vehicle-treated rats was higher than that in capsaicin-treated rats. HR decreased upon S6c infusion and gradually increased. Statistical analyses were not performed on these data because of the small number of animals studied.

DISCUSSION

The difference in resting pressure between the two groups of rats suggests a successful capsaicin treatment, since it has been shown that sensory nerves counterbalance the prohypertensive effects of several neuro-hormonal systems to maintain normal blood pressure (Vaishnava and Wang 2003). In capsaicin-treated rats, capsaicin-sensitive sensory nerves degenerate right after birth. The release of some neuropeptides, such as CGRP, from sensory nerves, is attenuated in capsaicin-treated rats, due to the neural degeneration. These peptides are potent vasodilators as well as diuretic and natriuretic factors. As a result of loss of these functions in capsaicin-treated rats, hypertension occurs.

The data suggest that sensory nerves do not play a major role in S6c-induced hypertension, since the increase in MAP during S6c infusion was the same in vehicle- and capsaicin-treated rats.

HR results showed similar baroreceptor reflex mediated blood pressure change in both animals. So the baroreflex was not impaired by neonatal capsaicin treatment.

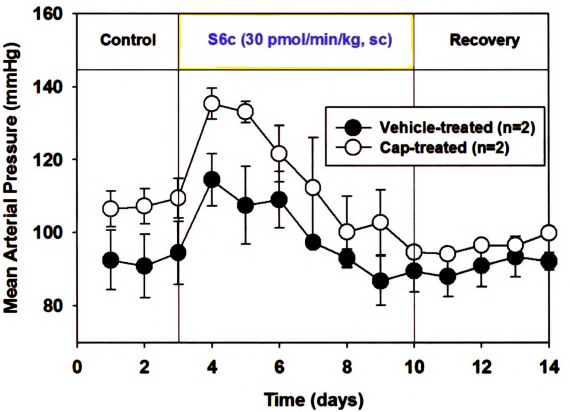

Figure 32: Mean arterial pressure (MAP) before (day 1-3), during (day 4-10), after (day 11-14) continuous S6c infusion (30 pmol/kg/min, sc.) in neonatal capsaicin- and vehicle-treated rats. The average resting blood pressure of capsaicin-treated animals was higher than that of vehicle-treated ones. S6c elevated MAP in a similar way in the two groups.

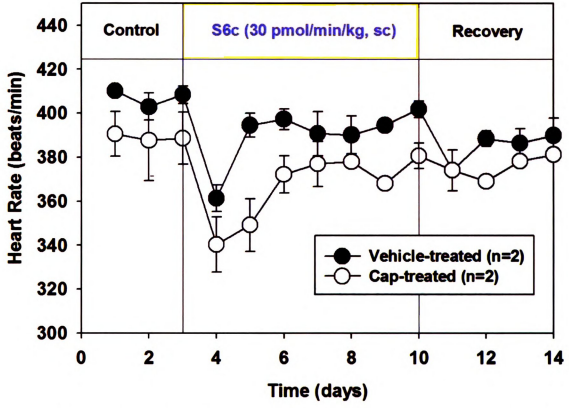

Figure 33: Heart rate (HR) before (day 1-3), during (day 4-10), after (day 11-14) continuous S6c infusion (30 pmol/kg/min, sc.) in neonatal capsaicin- or vehicle-treated rats. The average resting HR of capsaicin-treated animals was lower than that of vehicle-treated ones. S6c reduced HR to a similar degree in the two groups.

CHAPTER 9

GENERAL CONCLUSIONS AND DISCUSSION

A major focus of our laboratory is to test the hypothesis that veins mainly located in the splanchnic bed play an important role in blood pressure regulation. In support of this idea our lab recently developed a novel experimental hypertension model: S6c-induced hypertension (Fink, Li et al. 2007). In this model, sarafotoxin 6c (S6c) is infused into conscious rats, and induces a sustained hypertension by activating ETB subtype endothelin receptors. Previous data from our lab and others indicate that S6c-induced hypertension could be mediated by direct venoconstriction, but that activation of the sympathetic nervous system (especially to the splanchnic region) also may play a role (as reviewed earlier). The main goal of my thesis project was to investigate neural mechanisms underlying S6c-induced hypertension, using both *in vivo* and *in vitro* approaches. The major conclusions from my research, and the results supporting them, are summarized below.

Specific Aim I

The sympathetic nervous system is important in blood pressure regulation, and regionally-specific increases in the sympathetic nerve activity occur during the development of hypertension. Therefore, the sympathetic activation in key tissues may be an important cause of hypertension. Sympathetic neurons innervating the splanchnic organs may be especially critical, because the splanchnic vascular resistance is increased early in hypertension development. In addition, sympathetically mediated decreases in the splanchnic vascular capacitance may account for the central redistribution of blood volume that

occurs early in hypertension. The celiac ganglionic plexus contains the majority of the sympathetic neurons that innervate the splanchnic organs and tissues. Celiac ganglionectomy (CGX) involves surgical removal of the celiac ganglionic plexus, and has been used by ourselves and others to study the roles of the splanchnic sympathetic innervation in cardiovascular regulation. In the current study I characterized the short-term (two-week) and long-term (five-week and ten-week) effects of CGX in rats on the splanchnic sympathetic nerve structure and function. In the short-term, norepinephrine concentrations in whole splanchnic organs and mesenteric arteries and veins were significantly decreased by CGX. Immunohistochemistry and glyoxylic acid staining showed an almost complete loss of the typical sympathetic innervation of mesenteric arteries and veins. Constrictor responses of mesenteric arteries and veins to sympathetic nerve stimulation were abolished by CGX. However, the effects of CGX were time-dependent, since significant regeneration of sympathetic nerves was observed five weeks after surgery. The inferior mesenteric ganglion had minimal impact on this reinnervation process. Additionally, *in vivo* studies demonstrated that the splanchnic sympathetic innervation is important in normal blood pressure regulation, based on the finding that CGX significantly lowers resting blood pressure in normal Sprague Dawley (SD) rats. To summarize, CGX is an effective means to impair sympathetic input to the splanchnic organs, but the effect of the procedure is not permanent.

Specific Aim II

This study was performed to determine if S6c-induced hypertension is caused by increased generation of reactive oxygen species (ROS) and/or activation of the sympathetic nervous system. The model employed was continuous 5-day infusion of S6c into male Sprague-Dawley rats. No changes in superoxide anion levels in arteries or veins were found in hypertensive S6c-treated rats. However, the superoxide levels were increased in sympathetic ganglia from S6c-treated rats. In addition, superoxide levels in ganglia increased progressively the longer the animals received S6c. Treatment with the anti-oxidant tempol attenuated S6c-induced hypertension and decreased superoxide levels in ganglia. Acute ganglionic blockade lowered blood pressure more in S6c-treated rats than in vehicle-treated rats. Although plasma norepinephrine (NE) levels were not increased in S6c hypertension, surgical ablation of the celiac ganglionic plexus, which provides most of the sympathetic innervation to the splanchnic organs, significantly attenuated hypertension development. Collectively, the data suggest that a regional, as opposed to global, increase in sympathetic nerve activity is involved in S6c-induced hypertension. S6c-induced hypertension is partially mediated by sympathoexcitation to the splanchnic organs driven by increased oxidative stress in prevertebral sympathetic ganglia.

Specific Aim III

ETBR-deficient transgenic (Tg) spotting lethal (sl/sl) rats show elevated blood pressure, compared to Tg (+/+) control rats. The goal of this specific aim was to characterize this rat model, with a focus on sympathetic innervation. An

increased plasma ET-1 level was found in Tg (sl/sl) rats, suggesting a possible pathway for blood pressure elevation through ETAR activation by ET-1 binding. Sympathetic nerve density was evaluated by glyoxylic acid staining and no difference was demonstrated between Tg (sl/sl) and Tg (+/+) rats. NE concentrations in mesenteric arteries and veins were not significantly different between the two groups of rats in both genders, except that NE level in Tg (sl/sl) animals was higher than that in Tg (sl/sl) controls in mesenteric artery from male rats. Similarly, no difference in NE concentrations was shown in the splanchnic organs. Finally, an electrical stimulation study was carried out as a functional test of sympathetic influences on the splanchnic vascular bed. The vasoconstrictor response to the sympathetic stimulation was comparable in Tg (sl/sl) and Tg (+/+) rats. All the data suggest that hypertension in Tg (sl/sl) rats is not likely due to differential sympathetic innervation to the splanchnic blood vessels. Therefore, the hypertension is more likely caused by increased ET-1 concentration in the circulation. Data from another group support this finding, since hypertension in Tg (sl/sl) fed with high salt diet was completely abolished by ETAR blocker (D'Angelo, Pollock et al. 2005).

Specific Aim IV

Since S6c constricts most veins, and other relatively selective venoconstrictors can increase arterial pressure, we proposed that venoconstriction is one mechanism of S6c-induced hypertension. It has also been found, however, that S6c infusion may increase sympathetic activity to the

splanchnic region by increasing oxidative stress in prevertebral ganglia. This specific aim was to determine if S6c-induced hypertension is caused primarily by direct constriction of veins and/or activation of the sympathetic nervous system. The model employed was a continuous 7-day infusion of S6c into male ETBR-deficient transgenic (Tg) spotting lethal (sl/sl) and control Tg (+/+) rats. I used the Tg (sl/sl) rat because it does not express functional ETBRs in most tissues, except for sympathetic neurons (and other tissues with high dopamine-ß-hydroxylase activity). S6c induced a significant blood pressure increase in Tg (+/+) rats, while BP was not changed in Tg (sl/sl) rats, suggesting that S6c-induced hypertension is mediated in part by direct venoconstriction. Treatment with an antioxidant tempol did not affect the response to S6c in either strain. The splanchnic sympathetic denervation by chronic celiac ganglionectomy significantly attenuated S6c-induced hypertension in Tg (+/+) but not Tg (sl/sl) rats. I conclude that 1) ETBRs on the sympathetic neurons of Tg (sl/sl) and Tg (+/+) rats may function differently than those of wild-type rats, and 2) S6c-induced hypertension is mediated by both direct venoconstriction and the splanchnic sympathetic activation.

Specific Aim V

My limited preliminary data suggest that sensory innervation does not play a major role in S6c-induced hypertension, based on the finding that there is no difference in blood pressure responses to S6c infusion between capsaicin-treated and vehicle-treated rats. Also, baroreceptor reflex is not impaired in

neonatal capsaicin-treated rats, since heart rate significantly decreased in response to blood pressure increases in capsaicin-treated rats.

The above findings are summarized in Figure 34.

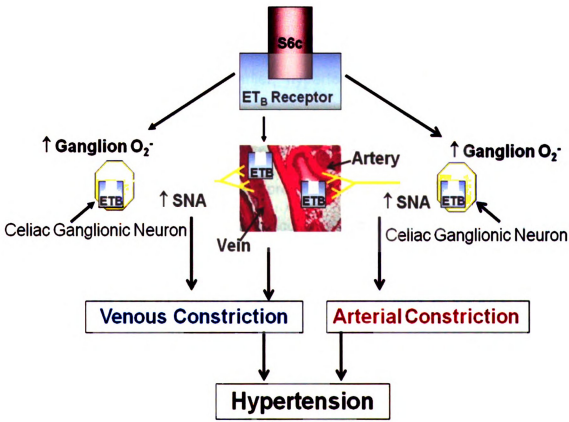

Figure 34: Summary of the main findings of this research project.

GENERAL CONCLUSIONS

Taken together, my results provide evidence that supports the following conclusions.

ETB receptor activation-induced hypertension is an important model of hypertension.

S6c-induced hypertension is a unique hypertension model, because: a. S6c is a selective venoconstrictor and S6c-induced activation of ETBRs leads to hypertension. As I mentioned earlier, hypertension has been viewed as an "arterial" disease and most research has been on arteries while veins have received much less attention. This new hypertension model shows that veins could play an important role in blood pressure regulation; b. ETBRs are located on sympathetic neurons, as well as vascular smooth cells. Stimulation of ETBRs induces sympathetic activation. My studies, along with findings from other groups, indicate that increased sympathetic nerve activity is involved in S6c-induced hypertension. Therefore, S6c-induced hypertension is a model that involves both vascular and neuronal mechanisms.

Increased splanchnic sympathetic nerve activity is a cause of hypertension.

My studies support the idea that increased splanchnic sympathetic nerve activity (SNA) is a cause of hypertension. When celiac ganglia, a plexus containing most neurons innervating the splanchnic organs, is surgically removed (CGX), S6c-induced hypertension is attenuated, indicating that splanchnic nerve

activity partially mediates the hypertension. In addition, superoxide levels in prevertebral sympathetic ganglia are higher in S6c-treated rats than in vehicle-treated rats. So my results are consistent with other groups' data, since many studies have shown that elevated oxidative stress cause increased SNA (Campese, Ye et al. 2004). ROS have been shown to be involved in the development of hypertension by increasing peripheral and central SNA. Possible mechanisms are either nitric oxide (NO)-dependent where decreased productions/availability of NO leads to a reduction in NO-mediated sympathoinhibitory effects (Campese, Ye et al. 2004; Zucker 2006; Zhang, Yu et al. 2008), or NO-independent where tempol directly regulate calcium channel and/or potassium channel activities (Xu, Jackson et al. 2006; Zucker 2006; Chen, Patel et al. 2007).

ROS, however, may exert their cardiovascular effects through a nonneural pathway. For example, ROS induce oxidative damages in DNA, lipids, and proteins in VSMCs and vascular endothelial cells, possibly resulting in vascular dysfunction and endothelial dysfunction (Ballinger, Patterson et al. 2000). Therefore, tempol does not lower blood pressure exclusively by decreasing SNA. An approach to clarifying this issue is proposed in **FUTURE STUDIES**.

FUTURE STUDIES

It remains controversial if intravenously administered S6c exerts its effects partially by binding to central ETBRs. On one hand, it is unlikely that blood-borne S6c passes the blood brain barrier (BBB), since its structural analogue ET-1 does not cross the capillary-endothelial junction due to the electrical polarity. When mice were given a radioactive tracer with an unselective ETAR/ETBR antagonist for *in vivo* ET receptor labeling, the radioactivity was very low in the brain, indicating that the antagonist does not cross the BBB (Aleksic, Szabo et al. 2001). On the other hand, it has been shown that ET-1 regulates P-glycoprotein (a critical component of the BBB protecting the CNS from neurotoxic agents) function by binding to ETBRs, but not ETARs (Hartz, Bauer et al. 2004). In addition, Na/K/Cl cotransporter at rat brain capillary endothelial cells could be activated by ET-1 and ET-3, suggesting a possible role of ETs in the BBB (Vigne, Lopez Farre et al. 1994). Furthermore, circumventricular organs (CVOs) with an incomplete BBB in the brain directly sense the concentrations of various compounds, especially peptide hormones, in the bloodstream. These organs could be possible action sites of S6c to regulate blood pressure. Previous results in our lab and others support this idea by the evidence of ETBR presence in several key CVOs (Yamamoto, Suzuki et al. 1997). Additional studies should be carried out to elucidate the involvement of central ETBRs in S6c-induced hypertension. For example, radio-labeled S6c could be intravenously infused and radioactivity in the brain, especially in CVOs, could be measured to determine if S6c binds to ETBRs in the CNS. If it is true, ETBR

antagonists could be administered to specific CVOs in S6c-induced hypertension, followed by hemodynamic studies, to verify if S6c regulates the cardiovascular system at least partially by activating ETBRs in the brain.

In sympathetic ganglia from Tg (+/+) rats, there are 70 more copies of the ETBR gene than normal WKY rats, the natural wildtype. My data demonstrate that the resting blood pressure of WKY rats is significantly lower than that of Tg (+/+) rats. More experiments, such as chronic S6c infusion into WKY rats and calcium imaging in celiac ganglionic neurons with ETBR activation, need to be done to elucidate the differences in the function of sympathetic ganglia between WKY and Tg (+/+) rats. If sympathetic neurons from Tg (+/+) rats respond to S6c more than those from WKY, this transgenic rat strain may represent an interesting model to study the effects of ETBRs in sympathetic ganglia without the need for exogenous activation of the receptors with S6c.

In my thesis project, S6c-induced hypertension model was performed in two ways. The model initially involved giving Sprague-Dawley (SD) rats S6c at the rate of 5 pmol/min/kg intravenously and hemodynamic parameters were measured by external blood pressure analyzer. In the second stage of the project, the model was performed using osmotic minipumps delivering S6c (at the rate of 30 pmol/min/kg, *sc.*) and a continuous hemodynamic measurement by radiotelemetry. The animals in these experiments were mainly ETBR transgenic and WKY rats. So more work should be done to calibrate the S6c-induced hypertension model, induced by subcutaneous S6c administration and monitored by telemetry. For instance, SD rats could be used to confirm that similar results

from WKY rats could be reproduced in SD rats. Similarly, oxidative stress studies were performed in SD rats. Experiments in WKY rats should be carried out to determine if there is any strain difference.

In addition, alternative methods could be used to evaluate sympathetic nerve activity (SNA) in the splanchnic bed in S6c-induced hypertension. Non-hepatic splanchnic NE spillover, for example, is an accurate way to examine the splanchnic SNA.

Although my data suggest that systemic antioxidant treatment lowers neural superoxide level and arterial pressure, the cause-effect relationship between oxidative stress and hypertension remains controversial (Pollock 2005). A direct way to find out the relationship between these two phenomena is to locally deliver a gene regulating oxidative stress in a specific target tissue. So for S6c-induced hypertension, a virus vector carrying superoxide dismutase (SOD) gene could be introduced into the celiac ganglia, followed by a hemodynamic study with S6c infusion and superoxide evaluation. The advantage of this procedure is that $O_2^{\cdot -}$ level can be reduced in a more specific way in the target vascular region. If increased $O_2^{\cdot -}$ in the celiac ganglion contributes to blood pressure elevation in the S6c-induced hypertension model, increasing SOD activity should have an antihypertensive effect. The rats treated with SOD gene transfer and S6c should show a smaller increase in blood pressure than rats treated with S6c and sham-vector. Superoxide concentrations in celiac ganglionic neurons from rats given S6c should be lower in rats that are treated with SOD gene transfer. This type of gene transfer study may help to elucidate the cause-

effect relationship between oxidative stress and hypertension. The limitations of this method would be possible immune response to viruses and difficulty of strictly localizing gene delivery.

It has been shown that NO generation in various vasculatures is higher in females than in males in humans (Herman, Robinson et al. 1997) and animals (Hayashi, Fukuto et al. 1992). Renal NO synthase (NOS) activity is higher in female Tg (sl/sl) rats than that in males (Neugarten, Ding et al. 1997; Taylor, Gariepy et al. 2003). In addition, estrogen replacement therapy significantly increased renal NOS levels in oophorectomized rats (Neugarten, Ding et al. 1997). On the other hand, androgen administration has been shown to be associated with increased atherosclerosis in animals (Herman, Robinson et al. 1997). Therefore, the above results indicate that female hormones play a protective role in cardiovascular regulation, while male hormones may have deleterious effects on the cardiovascular system. Possible mechanisms include the effects of these hormones on renin-angiotensin system (Ellison, Ingelfinger et al. 1989; Gallagher, Li et al. 1999; Dubey, Oparil et al. 2002), on vasoconstrictors (James, Sealey et al. 1986; van Kesteren, Kooistra et al. 1998), and on oxidative stress (Dantas, Tostes et al. 2002; Reckelhoff 2005).

Gender differences should be studied further in the future. I used both male and female transgenic rats in my studies, since gender differences in blood pressure regulation have been reported in this rat model (Sullivan, Pollock et al. 2006). My preliminary data support these findings. In the future, more research should be carried out to study gender differences in Tg (sl/sl) rats, either through

surgical approaches (oophorectomy or orchidectomy) or drug treatment (estrogen or androgen or hormone blockers). Additionally, in human saphenous veins, the density of ETARs in females is higher than that in males (Ergul, Shoemaker et al. 1998). So with the same ET-1 concentration in the circulation, ET-1 would induce more vasoconstriction in men than in women, which may lead to a higher blood pressure in men. Therefore, ETAR density could be measured in Tg (sl/sl) rats to test if the different receptor densities make a contribution to the difference in resting blood pressure between the two genders.

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