ALTERATIONS OF SYMPATHETIC NEUROEFFECTOR MECHANISMS AND THE UNDERLYING LOCAL MODULATORY MECHENISMS ASSOCIATED WITH MESENTERIC ARTERIES AND VEINS IN DEOXYCORTICOSTERONE ACETATE (DOCA)- SALT HYPERTENSIVE RATS

By

Min Luo

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Abstract

ALTERATIONS OF SYMPATHETIC NEUROEFFECTOR MECHANISMS AND THE UNDERLYING LOCAL MODULATORY MECHENISMS ASSOCIATED WITH MESENTERIC ARTERIES AND VEINS IN DEOXYCORTICOSTERONE ACETATE (DOCA)- SALT HYPERTENSIVE RATS

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The aim of my dissertation project was to test the hypothesis that alterations in neurotransmitter release from sympathetic nerves and postsynaptic reactivity of the vascular smooth muscle cells for the neurotransmitters are different in arteries and veins from DOCA-salt hypertensive rats. These alterations are at least partly due to the functional changes of the presynaptic α2-adrenergic receptors and to the effects of ET-1. The key findings of my research are as follows:

1. The mechanisms underlying sympathetic neuroeffector transmission to arteries and veins from normotensive rats are different as indicated by: i) different neurotransmitters mediating sympathetic neuroeffector transmission to arteries and veins. ii) Enhanced NE release relative to basal levels in veins compared to arteries.

2. NE release is increased from sympathetic nerves to arteries and veins from DOCA-salt hypertensive rats, which might be due to the impaired function of presynaptic α2-adrenergic receptors and enhanced NE uptake activity.

The alterations in sympathetic neuroeffector mechanisms in arteries and veins in DOCA-salt hypertension are also different as indicated by: i) In DOCA-salt arteries, the presynaptic ET_B receptors are activated which could also contribute to the increased NE release. ii) In DOCA-salt veins, the postsynaptic ET_B receptors mediate a facilitatory effect on sympathetic neuroeffector transmission which could contribute to the increased venomotor tone, whereas the facilitatory effects mediated by postsynaptic ET_A receptors observed in sham arteries were removed in DOCA-salt arteries presumably due to the down-regulation of the ET_A receptors in DOCA-salt hypertension.

To my parents, Pu-Eng and Guan-Ying Luo for your uttermost love and support

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LIST OF ABBREVIATIONS

α_1R	α1-adrenergic receptor
α_2R	α2-adrenergic receptor
ACE	Angiotensin Converting Enzyme
CGRP	Calcitonin Gene-Related Peptide
CNS	Central Nervous System
Cy^3	Indocarbocyanine
DAG	*sn*1, 2-Diacylglycerol
DβH	Dopamine β–Hydroxylase
DOCA	Deoxycorticosterone Acetate
DOPA	Dihydroxyphenylalanine
EC50	Half-maximal concentration
EFS	Electrical Field Stimulation
EJC	Excitatory Junction Current
EJP	Excitatory Junction Potential
Emax	Maximum Response
ENaC	Epithelial Na+ channel
ET	Endothelin
FITC	Fluorescein Isothiocyanate
HPLC	High Pressure Liquid Chromatography
HR	Heart Rate

IMA	Inferior Mesenteric Artery
IMG	Inferior Mesenteric Ganglia
IMV	Inferior Mesenteric Vein
IP3	Inositol 1,4,5, -Triphosphate
LDCV	Large Dense Core Vesicles
MAO	Monoamine Oxidase
MAP	Mean Arterial Pressure
MCFP	Mean Circulatory Filling Pressure
NGS	Normal Goat Serum
NE	Norepinephrine
NET	Norepinephrine Transporter
NLA	N^G-nitro-l-arginine
NO	Nitric Oxide
NPY	Neuropeptide Y
OCT1	Organic Cation Transporter 1
OCT2	Organic Cation Transporter 2
OCTs	Organic Cation Transporters
PBS	Phosphate Buffer Saline
PE	Phenylephrine
P2R	P2 receptor
RAS	Renin-Angiotensin System
RFLP	Restriction Fragment Length Polymorphism
SCG	Superior Cervical Ganglia

SDCV	Small Dense Core Vesicles
SEJCs	Spontaneous Excitatory Junctional Currents
S_{50}	Half- maximal Frequency
SHR	Spontaneous Hypertensive Rat
SNS	Sympathetic Nervous System
S6a, S6b, S6c, S6d	Sarafotoxins 6a, Sarafotoxins 6b, Sarafotoxins 6c, Sarafotoxins 6d
TH	Tyrosine Hydroxylase
TPR	Total Peripheral Resistance
TTX	Tetrodotoxin
WKY	Wistar-Kyoto
2K1C	Two-kidney, One clip
1K1C	One-kidney, One clip

Chapter 1

INTRODUCTION

1. Hypertension

Cardiovascular disease is the leading cause of death in western countries, accountable for the annual death of nearly 1 million people in the United States. Hypertension afflicts approximately 50 million Americans (Burt et al., 1995). Although hypertension itself does not cause life-threatening symptoms, it is one of the most important risk factors for cardiovascular diseases including congestive heart failure, stroke, atherosclerosis and renal failure (Burt et al., 1995). These complications associated with hypertension are due to the fact that its late stage hypertension can cause cardiac hypertrophy, endothelial dysfunction and renal injury (Raij, 1998).

1.1. Essentail vs. Secondary hypertension

Hypertension is a disease, which is difficult to define. Nevertheless, hypertension is a pathphysiological condition when systolic and diastolic pressures are $\geq$ 140 mmHg and 90 mmHg, respectively (National Institute of Health, 1997). One category of hypertension is secondary hypertension which has identifiable causes such as endocrine disorders (aldosteronism), renal diseases (renal failure, renal fibrosis) and mechanical vascular abnormalities (coarctation of the aorta). Hypertension of this kind can often be cured by

correcting its original cause (Lilly, 1993). 95% of hypertensive subjects fall into the other category called essential hypertension which has unknown causes (Lilly, 1993; Fink, 1998). Treatments mainly serve to control the elevation of blood pressure rather than to cure the disease. The difficulty in finding the causes for essential hypertension lies in the fact that essential hypertension is a multifactorial disease with heterogenous etiology involving combinations of abnormalities in the blood pressure regulating systems (Staessen et al., 2003).

1.2. Etiology of essential hypertension

There is considerable evidence suggesting that genetic factors play a role in the incidence of essential hypertension. For example, the prevalence of hypertension among the races is not evenly distributed with the higher incidence in African Americans compared with Caucasian (Burt et al., 1995; Whelton et al., 1994). There is also a familial aggregation of the disease demonstrated by the significantly higher rate of elevated blood pressure among the first-degree relatives of hypertensives than the general population and by greater concordance between monozygotic twin than dizygotic twins (Burt et al., 1995; Whelton et al., 1994). Genetic studies indicate that there are many genetic alterations of the blood pressure controlling components (Hunt and Williams, 1994; Luft, 2002). The most established genetic alterations in the blood pressure controlling components involve those regulating sodium and water balance such as the epithelial Na+ channel (ENaC) of the renal collecting duct (Warnock,

1999) and kallikrein, the major enzyme for the synthesis of kinin which regulates renal vascular tone (Katori and Majima, 1996).

It is generally believed that essential hypertension results from an interaction of environmental and genetic factors (Hamet et al., 1998). Environmental factors are the major contributors to modification of genetic influence as they can influence phenotype through the stimulation of gene expression or via an interaction with gene products (Hamet et al., 1998). High salt intake, stress and obesity, have been recognized to make an individual susceptible to the development of hypertension or advance the clinical onset of hypertension (Staessen et al., 2003). For example, a high salt intake alters the expression of the renin gene, which ultimately will have an impact on blood pressure level (Hamet et al., 1998). Furthermore, subjects with a family history of hypertension have a greater increase in blood pressure when they are exposed to mental and physical stresses than do subjects of normotensive descent with matching basal blood pressure levels (Widgren et al., 1992). Obesity, insulin resistance and dyslipidemia are metabolic conditions that appear to be involved in the pathogenesis of essential hypertension as body weight and blood pressure are often closely correlated based on the results from population studies (Whyte, 1956; Hamet et al., 1998).

2. Hemodynamics of blood pressure

Arterial blood pressure is the product of total peripheral resistance (TPR) and arterial blood volume. Peripheral resistance is mainly determined by arterial

tone whereas arterial blood volume is determined by cardiac output as well as secretion of sodium and water (Guyton, 1991). Cardiac output is determined by stroke volume and heart rate. While the heart provides the pumping pressure and rate, venous return determines end-diastolic filling pressure and stroke volume (Guyton, 1991). Venous return is determined by venomotor tone. Mean circulatory filling pressure (MCFP) represents the pressure that drives venous return and is an index of whole body integrated vascular tone and blood volume (Yamamoto et al., 1980, Guyton, 1991).

As shown by hemodynamic studies on human and animals in the initial stages of hypertension there is a high cardiac output and normal TPR (Lund-Johansen, 1994). As the hypertension progresses to a chronic state, the contribution to the rise of blood pressure by cardiac output is reduced whereas that by TPR increases (Lund-Johansen, 1989; Coleman and Hall, 1993). Chronic hypertension is therefore characterized by elevated resistance to blood flow through small arteries in most vascular beds (Folkow, 1982).

3. Regulation of blood pressure

Maintainance of normal blood pressure involves a complex interplay among the nervous, endocrine, renal and cardiovascular systems.

The central nervous system controls blood pressure by adjusting the autonomic outflow to the cardiovascular system. The paraventricular nucleus (Kenney et al., 2003), area postrema (Hasser et al., 2000), rostral and caudal ventral lateral medulla (Colombari et al., 2001) are involved through direct or

indirect connection with the autonomic nervous system. One well-known example is the arterial baroreceptor reflex, a powerful and rapidly acting reflex serving as a protective mechanism to maintain blood pressure homeostasis in the presence of acute blood pressure changes (Dampney et al., 2002). The autonomic nervous system directly regulates the cardiovascular system by primarily controlling vascular tone and cardiac output (Dampney et al., 2002).

The kidney maintains sodium and water balance and controls the intravascuclar blood volume through a mechanism called pressure-natriuresis (McDonough et al., 2003). The kidney excretes more sodium and water when the blood pressure rises so that blood volume is reduced and blood pressure returns to the normal range (McDonough et al., 2003). Kidney function is regarded as a long-term mechanism for blood pressure regulation (Hall et al., 1986). In hypertension, pressure-natriuresis is desensitized and a greater rise of blood pressure is required for kidney to execute this function (Guyton et al., 1972a).

There are various hormonal factors that act on the cardiovascular system to regulate blood pressure including angiotensin II (Hall, 1991) and vasopressin (Abboud et al., 1990). Angiotensin II causes vasoconstriction, aldosterone release, sodium and water retention, and sympathoexcitation (Hall, 1991; Ferrario, 1990). Vasopressin, also known as the antidiuretic hormone, causes water retension (Abboud et al., 1990). The cardiovascular system can also secrete a number of vasoactive factors such as endothelin (ET)-1, nitric oxide (NO), and atrial naturetic peptide (ANP) (Shepherd and Katusic, 1991; Nicholls et al., 1987; Luscher, 1990).

These above systems interact with one another, forming a delicate blood pressure controlling mechanism. Hence, maintenance of blood pressure homeostasis is an integrated function of those systems.

4. The role of sympathetic nervous system (SNS) in long-term control of blood pressure.

There is little disagreement that hyperactivity of the SNS occurs in the early stages of hypertension as indicated by elevated plasma NE concentration, increased total body NE spill over and increased sympathetic nerve traffic (Julius et al., 1988; Esler et al., 1989; Izzo, 1998). Increased SNS activity causes increased arterial and venous constriction with a resulting increase in systemic blood pressure. For example, activation of α–adrenergic receptors causes increased vascular resistance and volume retention (through renal vasoconstriction). Meanwhile, activaton of β-adrenergic receptors causes increased cardiac output and renin release that consequently activate the renin-angiotensin system (RAS), a mechanism known to reinforce SNS activity (Ferrario, 1990). Accordingly, early hypertension is characterized by increased cardiac output as a result of hyperactivity of cardiac sympathetic nerves (Julius et al., 1988). Therefore, it is generally agreed that hyperactivity of the SNS is a critical initiating factor for hypertension development. However, whether the SNS plays a substantial role in long-term blood pressure control in humans remains controversial (Izzo, 1998).

One view holds that the SNS plays little if any role in maintaining elevated BP chronically (Izzo, 1998). The notion that SNS hyperactivity observed in earlier phases of hypertension disappears chronically is based on two observations: 1) during established hypertension cardiac output is often not increased whereas increased vascular resistance accompanied by structural changes is the hallmark abnormality. Therefore, during the chronic phase, structural changes such as vascular hypertrophy instead of hyperactivity of the SNS are the sustaining force for maintenance of high vascular resistance. 2) The plasma NE level is often normal in established hypertension. For example, only about 30% of hypertensive patients displayed plasma NE values greater than those found in the normotensive population (Grassi, 1998).

An opposite view holds that there is sustained activation of the SNS in chronic hypertension, which plays an important role in maintaining high blood pressure (Mark, 1996; Leenen, 1999; Izzo, 1998). This notion is based on clinical observations suggesting that the true hallmark abnormality in hypertension is a dynamic blend of high cardiac output and systemic vascular resistance (Izzo, 1998). Whether cardiac output or vascular resistance is predominantly elevated is dependent on the relative proportion of β-receptors (primarily cardiac) and α-receptors (primarily systemic vascular) stimulation. For example, when hypertensives are in the supine position there is a predominant stimulation of α-receptors and elevated systemic resistance is the prevalent abnormality. However, when they are at an upright posture elevated cardiac output is the

prevalent abnormality (Izzo et al., 1990). A declining function of β-receptors with aging is also suggested to contribute to a seemingly decreased or normal cardiac output as hypertension progresses in elderly patients (Izzo, 1998).

Although there are some reports that plasma NE level is normal in established hypertension, others reported that men with untreated sustained essential hypertension for more than 5 years showed increased supine venous and arterial plasma catecholamines as well as an increased difference between these two levels, indicating an increased sympathetic nerve activity in chronic essential hypertension (Kjeldsen et al., 1981). Furthermore, plasma NE is not always an appropriate means to assess SNS activity in the setting of increased systemic BP as the level of circulating NE depends on not only the release of NE from the nerve terminals, but also on plasma clearance of NE (Grassi, 1998). In studies using arteriolar dilators to normalize BP for 6-8 weeks in essential hypertension, plasma NE doubled (Izzo et al., 1987). These results suggest that SNS hyperactivty present in established hypertension has been suppressed by the chronic BP elevation and was unmasked when BP was lowered to normal levels (Izzo et al., 1987). Therefore, unaltered plasma NE level may not necessarily mean that SNS activity is not increased.

There is also evidence for increased SNS activity to specific organs in chronic hypertension. For example, elevated cardiac and renal sympathetic activity was demonstrated in patients with essential hypertension (Esler et al., 1989). Increased peripheral sympathetic nerve firing rates to the leg muscles in humans has also been demonstrated (Grassi, 1998). These observations support

an ongoing role of the SNS in chronic hypertension. The strongest evidence supporting an important role of the SNS in chronic hypertension perhaps is the consistent antihypertensive effect exerted by adrenergic receptor blockers (van Zwieten, 1996). For example, in young hypertensives, β-blockade alone is extremely effective (Frisk-Holmberg et al., 1981). Furthermore, central sympatholytic drugs or combined α and β adrenergic receptor blockade lower BP in all forms of early and late essential hypertension (Campese et al., 1980; Izzo, 1998).

5. Treatments of hypertension in humans

As mechanisms underlying hypertension development are poorly understood, rather than curing hypertension, current therapeutic approaches serve to lower blood pressure and hopefully prevent other cardiovascular diseases secondary to hypertension.

There are six classes of drugs used to treat hypertension: 1) diuretics represendted by thiazides. Diuretics are recommended to be the first drugs of choice for initial antihypertensive therapy due to the affordability and efficacy of these drugs in treating hypertension (Gavras et al., 1997). The hypotensive effects of diuretics are attributable to blood volume depletion as a result of decreased sodium-reabsorption and increased water secretion in the kidney. However, the exact mechanism underlying the long-term antihypertensive effect of diuretics is unknown (Pepper, 1999). Diuretics also cause a number of metabolic side effects including hypokalemia, dehydration and hyponatremia

(Pepper, 1999). 2) Calcium channel blockers. By blocking calcium entry into the cytoplasm of vascular and cardiac smooth muscle cells, calcium channel blockers decrease vascular tone, cardiac contractility and cardiac electrical conductivity, leading to decreased peripheral resistance and cardiac output and hence decreased blood pressure (Pepper, 1999). There are two subclasses of calcium channel blockers: dihydropyridines (e.g., nifedipine, nicardipine) and nondihydropyridines (e.g., verapamil and diltiazem). Dihydropyridines are more potent as vasodilators, whereas the nondihydropyridines are more noted for their cardiac effects (Pepper, 1999). 3) Angiotensin converting enzyme (ACE) inhibitors represented by captapril. ACE inhibitors block the production and thus the various cardiovascular actions of angiotensin II including vasoconstriction, sympathoexcitation, and sodium and water retention. Accordingly, ACE inhibitors lower blood pressure by decreasing peripheral resistance and blood volume. ACE inhibitors are effective as monotherapy in about 50 percent of unselected patients (Gavras et al., 1997). 4) Peripherally-acting sympatholytics act at the level of sympathetic neuroeffector junction to inhibit sympathetic neuroeffector mechanisms. For example, guanethidine and reserpine were used to treat hypertension by depleting vesicular stores of NE. 5) Direct vasodilators including nitro-compounds and hydralazine (Gavras et al., 1997). Nitro-compounds cause vasodilation by stimulating cellular guanylate cyclase and hydralazine cause vasodilation by inhibiting pharmacomechanical coupling as well as causing membrane hyperpolarization (Kreye, 1984). The side effects of vasodilators include reflex tachycardia and fluid retention (Gavras et al., 1997). 6) Adrenergic

receptor blockers. There are several subclasses that block different subtypes of the adrenergic receptors as follows:

A: β-adrenergic receptor blockers. One of the mechanisms by which β-adrenergic receptor blockers lower blood pressure is that they decrease cardiac contractility and thus the cardiac output (Koch, 1981). β-adrenergic receptor blockers also decrease the release of renin from juxtaglomerular cells in the kidney (Karlberg, 1983). Most of the adverse effects of these drugs are caused by the non-selective blockade of β-adrenergic receptors in various organs. For example, adverse effects related to β_1 blockade in the heart include bradycardia, congestive heart failure, and cardiac conduction abnormalities, whereas those related to β_2 blockade include bronchospasm and sexual dysfunction (Gavras et al., 1997). B: Non-selective α-adrenergic receptor blockers. They lower blood pressure by blocking vascular α adrenergic receptors mediating vasoconstriction. As the feedback inhibition of NE release from sympathetic nerve terminals mediated by the prejunctional α2-adrenergic receptors were also blocked by the non-selective adrenergic receptor blockers, they permit excessive release of NE. This is why phentolamine, a non-selective adrenergic blocker, causes palpitations and tachycardia. Hence, non-selective adrenergic blockers are used primarily in the management of hypertension secondary to pheochromocytoma (Frishman et al., 1999). C: Selective α1-adrenergic receptor blockers. Prazosin is one example of this class. Selective α1-adrenergic receptor blockers usually do not produce tachycardia and palpitation as they do not interfere with the negative-feedback mechanism that controls the release of NE (Ram and Kaplan,

1979). Therefore, the 1993 report of the Joint National Committee included the selective α1-adrenergic receptor blockers as candidates for monotherapy (Frohlich, 1993). However, these drugs can cause substantial adverse effects such as dizziness, orthostatic hypotension and sodium and water retention (Gavras et al., 1997). D: α2-adrenergic receptor agonists. Clonidine, the α2 adrenergic receptor agonist has been used as centrally acting sympatholytic to decrease sympathetic tone as they promote the inhibitory effects mediated by prejunctional α2-adrenergic receptors (Mathew and Parker, 1971). Mental depression is among the side effects caused by this class of drugs (Gavras et al., 1997).

6. Experimental hypertension models

A number of animal models of hypertension have been developed to address the role of these various blood pressure regulating systems in the development of hypertension. Since the different models have a different etiology, it is conceivable that the choice of a model significantly influences the outcome of experiments done using these different models.

6.1. Genetic hypertension models

Genetic models of hypertension are produced by selective inbreeding of normotensive strains of animals. They are characterized by spontaneous elevation of blood pressure with aging (Kurtz et al., 1994). The specific

abnormalities that initiate genetic hypertension are unclear (Bohr and Dominizak, 1991).

The Spontaneous Hypertensive Rat (SHR), generated from the Wistar-Kyoto (WKY) normontensive strain, is the most widely used genetic strain of hypertensive animals (Okamato and Aoki, 1963). Despite that SHRs are relatively resistant to changes in sodium intake in the developmental stages of hypertension, blood pressure in SHRs in established hypertension is augmented by salt loading (Yamori et al., 1981). There is also evidence for an increase in MCFP and cardiac output in the early stage of hypertension development in SHRs although sodium and extracellular fluid volume are not elevated (Trippodo et al., 1981; Martin et al., 1998). Futhermore, increases in MCFP and TPR are maintained at the established stage of hypertension development in SHRs (Martin et al., 1998).

The other commonly studied genetic model of hypertension in rats is the Dahl salt-sensitive strain. The Dahl salt- sensitive and corresponding salt-resistant strains were developed from normotensive Sprague-Dawley rats. In contrast to the SHR, both genetic and environmental factors are involved in the development of hypertension in the Dahl salt-sensitive strain (Friedman, 1988). For example, increased dietary sodium is required for the rapid and full development of the blood pressure elevation in Dahl salt-sensitive rats (Friedman, 1988). The most established genetic alteration in Dahl salt- sensitive strain involves the renin gene. For example, a restriction fragment length polymorphism (RFLP) in the renin gene was found between Dahl salt-sensitive

and Dahl salt-resistant rats. Furthermore, the renin RFLP cosegregated with blood pressure as the dose of the renin allele from Dahl salt-sensitive rat directly correlates with the increment in blood pressure (Rapp et al., 1989).

6.2. Renal models of experimental hypertension

Hypertension based on renal etiology is the most common form of human secondary hypertension (Lilly, 1993). Therefore, renal models of experimental hypertension are relevant to studies of the basis for renal-dependent human hypertension. Renal models of hypertension use blockade of renal blood flow via stenosis of the renal arteries and /or reduction of renal mass. The two-kidney, one clip (2K1C) and the one-kidney, one clip (1K1C) renovascular models of hypertension are the most commonly used models. Hypertension in this model is initiated by stenosis of the renal artery (Goldblatt et al., 1934), which is performed by a placing silver clip around the renal artery causing a partial occlusion of blood flow. In 2K1C, the renal artery supplying one kidney is clipped and the contralateral kidney is left intact. In the 1K1C, the renal artery supplying one kidney is clipped and the contralateral kidney is removed. Renal renin levels and peripheral renin activity in 2K1C are elevated indicating an important role for the renin-angiontensin system in the development of this form of hypertension. In the 1K1C model, renin levels and peripheral activity are reduced as the source of renin production is reduced. In the 2K1C model of hypertension, there is an elevation of blood pressure that depends on an increased TPR with a reduction in cardiac output, while the 1K1C model shows

an increase in TPR with a small increase in cardiac output (Thurston, 1994). In both models there is an elevation in MCFP (Yamamoto et al., 1981; Edmunds et al., 1989).

6.3. Neural models of hypertension

The central nervous system (CNS) is involved in regulating blood pressure and hence has been implicated in the pathophysiology of hypertension. For example, the CNS is involved in DOCA-salt hypertension (Gomez Sanchez, 1991) Specifically neural models of experimental hypertension with a genetic basis include the stroke-prone spontaneous hypertensive rat models. There are also models that involve surgical or pharmacological manipulations of critical CNS centers for blood pressure regulation such as the paraventricular nucleus, nucleus tractus solitarus or the rostral ventral medulla (Reis, 1981). Each of these manipulations is designed to address the role played by each brain center in the regulation of blood pressure. Models focusing on the role of peripheral nervous system in blood pressure regulation can be produced by sinoatrial denervation to remove baroreceptor reflex (Trindade and Krieger, 1984) and degeneration of peripheral sensory nerve fibers to remove afferent inputs to the nervous system (Wang et al., 1998).

7. DOCA-salt hypertension

7.1 Salt intake and salt sensitivity

As noted before, environmental and genetic factors contribute to the incidence of essential hypertension. Salt intake and salt sensitivities are examples of the environmental and genetic factors associated with hypertension, respectively. Epidemiological studies have demonstrated a positive correlation between dietary sodium intake and the prevalence of hypertension. For example, the prevalence of hypertension in primitive societies with very low dietary salt intakes is low (Page et al., 1974). Furthermore, studies performed in the 1980's on humans suggested a threshold relation between the amount of salt intake and the incidence of hypertension (Intersalt, 1988; Kaplan, 1993). Those societies with daily sodium intakes of less than 60 mmol fail to show a rise in arterial pressure with aging (Kaplan, 1993).

The incidence of hypertension in individuals challenged with high salt intake also depends upon whether those individuals are salt- sensitive (Intersalt, 1988 and Kaplan, 1993). Salt sensitivity of blood pressure is defined as a 10% or greater increase in blood pressure after salt loading compared with the blood pressure at the time of low-salt intake (Weinberger, 1993). Salt-sensitivity is more prominent in older, black people as well as in those with high sympathetic nervous system activity or renal insufficiency (Guyton et al., 1964; Weinberger, 1993).

7.2 Etiology of salt sensitive hypertension

The exact etiology of salt- sensitive hypertension is unknown although it seems to involve abnormalities in renal, neural, hormonal and genetic mechanisms controlling blood pressure.

The kidneys have been suggested to play a central role in the pathogenesis of sensitivity of blood pressure to salt intake. Renal causes of salt-sensitivity include defects in renal plasma flow and increased tubular reabsorption of sodium (Campese, 1994; Hamet et al., 1998).

Potential neural mechanisms for high salt sensitivity involve stimulation of the SNS by high salt intake. Studies suggests that salt-sensitive people lack the appropriate baroreceptor response in responding to increase in blood pressure as high salt levels have been shown to sensitize the baroreflex in salt-resistant, but not salt-sensitive patients (Trimarco et al., 1991). This lack of baroreceptor response is likely to be accountable for the blood pressure elevation in those salt-sensitive patients when challenged with high salt intake. Hormonal factors like the ANP (Melo et al., 2000), kallikrein-kinin system (Katori et al., 2001) and renal dopamine (Jose et al., 2003) have all been considered to play a role in the salt-sensitivity of hypertension.

Genetic studies showed that the effect of salt intake on blood pressure is mediated through interactions with specific genetic loci (Hamet et al., 1998). Those loci implicated in the genetic control of salt sensitivity include the renin gene on rat chromosome 13 and the *Gca* gene at chromosome 2 which codes for ANP receptor A (Hamet et al., 1998).

7.3. Etiology of DOCA- salt hypertension

DOCA-salt hypertension is a model in which hypertension is produced by administration of high levels of a mineralocorticoid (DOCA) and salt intake accompanied by surgical uninephrectomy (Schenk and McNeill, 1992). The mineralocorticoid promotes sodium and water re-absorption in the collecting tubule of the nephron (Schenk and McNeill, 1992). It acts on the type 1-adrenococorticosteroid receptor and enhances the permeability of renal tubules to sodium via an amiloride-sensitive Na^+-H^+ exchanger and activation of a serosal sodium pump (Schenk and McNeill, 1992).

Sodium retention and a subsequent volume expansion may contribute to the initiation of hypertension although this is not likely to be the sole mechanism by which blood pressure rises (Schenk and McNeill, 1992). Rather, increased sodium levels can alter neurohormonal mechanisms to contribute to the initiation and/or maintenance of hypertension (Ferrario et al., 1987).

Altered neural mechanisms could include an attenuated baroreceptor reflex and increased peripheral autonomic discharge to the cardiovascular system. Sympathetic drive to the cardiovascular system is elevated as indicated by increased circulating catecholamine levels (Bouvier et al., 1986) and sympathetic nerve responses to various stimuli in DOCA-salt hypertensive animals (Takeda et al., 1988a). Central catecholaminergic depletion attenuated DOCA-salt hypertension as indicated by a normalization of blood pressure in hypertensive animals injected with 6-OHDA intracerebroventricularly (Lamprecht et al., 1977). Sympathetic nerve fiber activity has been shown to be elevated in

DOCA-salt treated rats as reflected in an enhanced splanchnic sympathetic nerve output in response to electrical stimulation of the hypothalamus (Takeda, 1988 a).

Hormonal mechanisms including Angiotensin II, vasopressin, catecholamines and ET-1 involved in control of autonomic function via peripheral or central actions are altered in DOCA-salt hypertension (Schenk et al., 1992; Schiffrin, 1998). Activity of the brain RAS is increased, which could increase peripheral sympathetic discharges and vasopressin release (Itaya et al., 1986). Vascular reactivity to vasopressin has been implicated to be enhanced in DOCA-salt treated animals (Lariviere et al., 1988).

In DOCA-salt hypertensive animals, cardiac output and stroke volume were significantly elevated without concomitant increases in heart rate (Ueno et al., 1988). It is likely that increased cardiac output is due to an expanded blood volume and an augmented venous return (Ueno et al., 1988). Other studies of DOCA-salt hypertension in dogs attribute the elevation in arterial pressure to an increase in both total peripheral resistance and cardiac output (Schenk and McNeill, 1992). In addition, DOCA-salt hypertension causes characteristic changes in vascular structure that contribute to the overall pathology of the DOCA-salt state (Schenk and McNeill, 1992)

Thus, this model is relevant to salt-sensitive human hypertension and permits studies of changes in neural and hormonal mechanisms secondary to adrenal steroid excess (Schenk and McNeill, 1992).

8. The mesenteric vascular bed

The splanchnic circulation receives 25% of cardiac output and is the largest capacitance bed (Folkow, 1982 and Greenway, 1983). It consists of the blood supply to the gastrointestinal tract, liver, spleen, and pancreas. The splanchnic circulation is a hemodynamically important vascular region for its contribution to peripheral resistance and its reflex capacitance control to secure cardiac filling (Daugirdas, 2001). There are three major branches coming from the abdominal aorta that supply blood the visceral organs: celiac, superior mesenteric and inferior mesenteric artery. The celiac artery supplies the stomach, spleen, duodenum and the liver (Rosenblum et al., 1997). The superior mesenteric artery is the largest of all the aortic branches and carries over 10% of the cardiac output. It supplies the small intestine and part of the large intestine (Rosenblum et al., 1997). The mesenteric vascular bed originating from the superior mesenteric artery consists of arcades of blood vessels with a gradient of vessel caliber in which the largest blood vessels is most distal to (first order) and the smallest is most proximal to the intestinal wall (third or fourth order). The inferior mesenteric artery supplies the caudal part of large intestine (Rosenblum et al., 1997).

8.1. The arterial system

Arterial structure: The arterial wall consists of three layers: the innermost intima layer, the media layer and the outermost adventitia layer. The intima is comprised of a single layer of endothelial cells embedded in an

extracellular matrix (Burton, 1954). The endothelial cells serve a dual function in the control of vascular tone. They release relaxing factors such as nitric oxide and adenosine and constricting factors such as endothelin (Rossi et al., 2001). The media consists of smooth muscle cells and elastic lamina (Burton, 1954). Elastic tissue allows the wall of an artery to be distended by the sudden thrust of blood from the heart and then to contract again, which helps force the blood forward. Therefore, the elastic tissue tends to smooth pressure pulses and lessen the velocity of the blood. The smooth muscle cells are innervated by sympathetic nerve terminals which can cause constrictions of the smooth muscle cells (Nilsson, 1985). Constrictions of the smooth muscle decrease the vessel caliber and thereby increase intravascular pressure. In addition, the blood vessels receive dense innervation from sensory neurons located in the dorsal root ganglia (Wang et al., 1998). Sensory nerves release the vasodilator neuropeptides calcitonin gene-releated peptide (CGRP) and substance P in response to local stimuli (Wang et al., 1998). The adventitia is the most variable layer, containing primarily loose connective tissues, nerves, and small nutrient blood vessels in the larger arteries (Burton, 1954; Levick, 2000).

The actual composition of each of these layers varies with the type of blood vessels. Large, conduit arteries such as superior mesenteric artery (with internal diameter around 1.2 mm) are typically referred to as elastic arteries because of their high ratio of elastic lamina to smooth muscle cells (Burton, 1954; Levick, 2000). As branches of superior mesenteric artery are traced distally, the relative amount of elastic tissue decreases and the relative amount of smooth

muscle increases in the media layer. At the level of vessels with internal diameter around 150-200 μm, the resistance to blood flow starts to become prominent (Burton, 1954; Levick, 2000). The smallest arteries arterioles (<100 μm) have a media layer composed almost entirely of smooth muscle and are particularly contractile. It is through the activity of the arterioles that blood is shunted from one part of the circulation to another and hence they are perhaps the most important governors of the peripheral resistance to blood flow and therefore of systemic blood pressure (Schiffrin, 1992).

Structural changes associated with hypertension: Hypertension is associated with altered structure and mechanical properties of resistance arteries (Schiffrin, 1992). Structural changes in vascular smooth muscle cells in hypertension take up two forms: remodeling and growth. Remodeling is a rearrangement of normal sized cells whereas growth is either an increased smooth muscle cell mass due to increased protein synthesis (hypertrophy) or cell replication (hyperplasia). As remodeling involves rearranging of existing cells and extracellular matrix around a lumen without invoking a growth response, it leads to increased media to lumen ratio, but not increased media width or cross sectional area (Heagerty et al., 1993). In essential hypertension, the increased media / lumen ratio of small arteries due to remodeling could contribute to increased vascular resistance (Folkow, 1982; Aalkjaer et al., 1987). Although remodeling occurs in arteries from SHRs, growth due to hyperplasia appears to play a large role in the alteration of vascular structure (Heagerty et al., 1993).

Whether the altered vascular structure is a cause or a consequence of the increased blood pressure is not clear. On the one hand, thickening of the vascular wall could be a secondary adaptation produced by the elevation of blood pressure as such changes in large arteries can be prevented by antihypertensive treatment. On the other hand, altered structure could contribute to blood pressure elevation by increasing vascular resistance. Studies on mesenteric and renal arteries of SHR showed that wall thickening of the vasculature occur at prehypertensive stage and were present even under conditions where the blood pressure has been normalized. These observations suggest that some structural alterations in the blood vessels observed in hypertension are blood pressure independent and could be of etiological importance in the initiation of hypertension (Lee and Smeda, 1985).

In DOCA-salt hypertension, morphological changes of the resistance arteries occur within 2 weeks of elevation of blood pressure. These structural changes include narrowing of the lumen and external diameters, and increases in media width and media cross-sectional area. These structural changes were found to correlate with an increase ET-1 mRNA (Schiffrin et al., 1996). In addition, treatment of DOCA-salt hypertensive rats with the combined ET_A/ET_B endothelin antagonist bosentan caused a complete regression of vascular hypertrophy. The effects by bosentan were much less marked in lowering blood pressure. These results suggest that in DOCA-salt hypertension over expression of vascular ET-1 produces vascular hypertrophy independently of blood pressure elevation (Schiffrin et al., 1995).

Structural changes induced by ET-1 are likely to be initiated by vasopressin. A number of studies indicate that vasopressin is required for DOCA-salt treatment to exert its vascular effects and vasopressin acts in part by enhancing ET production. For example, vasopressin receptor antagonism attenuated DOCA-salt induced increases in media-lumen ratio and expression of the prepro ET-1 gene (Intengan et al., 1998.). These findings were consistent with abnormal vasopressin levels and function in this model. Furthermore, DOCA-salt treatment in the absence of endogenous vasopressin, for example in homozygous vasopressin-deficient Brattleboro rats, failed to alter vascular structure, ET expression, or wall component stiffness and resulted in less blood pressure elevation (Intengan et al., 1999).

8.2. Venous system

The venous system has a similar architecture to that of the arterial system. However, compared with arteries, the vessel wall of veins is much thinner with lesser connective tissue and less layers of smooth muscle (Burton, 1954; Levick, 2000). Hence, veins have a greater lumen diameter, a smaller media width, and consequently a smaller media-lumen ratio compared with their corresponding arteries (Burton, 1954; Levick, 2000). Due to its structural properties the venous system provides a significant capacitance function (Monos et al., 1995). 60-80% of the circulating blood volume is found in the systemic veins and venules, particularly vessels with a caliber <400 μm (Hainsworsth, 1986). Vascular capacitance is defined as the change in intravascular pressure

as a result of a unit change in intravascular volume (Guyton et al., 1972b). As pressure in the right atrial is provided by blood redistrubted from the venous system to the heart, a reduction in venous capacitance will shift blood from the peripheral vascular beds towards the heart and increase right atrial pressure. According to the Frank-Starling mechanism, the increased right atrial pressure results in increased end diastolic filling pressure, myocardial stretch and more forceful constriction. In this way, augmented venous return leads to higher stroke volume and cardiac output (Guyton et al., 1972b).

In the splanchnic circulation, vascular capacitance is a function of vein structure and venous smooth muscle activity (venomotor tone). There are a number of factors that can affect venomotor tone including myogenic activity, circulating and locally produced vasoactive hormones and innervation of veins by the sympathetic nervous system. It is generally believed that the sympathetic nervous system is the most important factor to regulate venomotor tone as it has a constant and the greatest quantitative regulation of venomotor tone, especially in the critical splanchnic circulation (Monos et al., 1995). Venomotor tone can be assessed by measurement of MCFP, the pressure that exists across the entire circulation when the heart is stopped. MCFP represents the major driving force for venous return to the heart (Johnson et al., 2001).

Capacitance of the venous system in animals with established hypertension is decreased (Safar and London, 1985, 1987). A reduction in venous capacitance has therefore been implicated in the etiology of hypertension. In SHR, Goldblatt 1K1C renovascular hypertensive rats, and

humans with essential hypertension, venous tone is elevated early in development of hypertension and remains elevated into established hypertension (Safar and London, 1987; Schobel et al., 1993, Yamamoto, et al., 1981; Martin et al., 1998). It is not clear, however, whether increases in venous tone are an adaptive response to maintain cardiac output in the presence of increased resistance for ventricular ejection (Folkow, 1982; safar and London, 1987) or an active initiating factor participating in hypertension development.

8.3. Differences in structural and mechanical properties between arteries and veins

Arteries and veins serve distinct functions in the circulation, i.e. resistance vs. capacitance (Burton, 1954; Levick, 2000). These different functions result from differences in their structural properties. Arterial walls are comprised of substantial layers of smooth muscle providing efficient adjustment to blood pressure changes. As the muscle layer of venous wall is thinner compared with arteries, veins are more capable of accommodating changes in blood volume (Burton, 1954; Levick, 2000). Morphological studies on canine mesenteric artery and vein found that secondary arteries contain a greater number of smooth muscle cells within the media and have 5 to 10 times thicker smooth muscle layer compared to the corresponding veins (Yamboliev et al., 2002). Moreover, there is a tighter arrangement of smooth muscle cells in arteries as the arterial smooth muscle cells have smaller intercellular gaps compared to veins. This tighter arrangement suggests that arterial walls have closer intercellular coupling

and more efficient production of force during muscle constriction, which provides a greater mechanical resistance to blood flow compared to veins (Yamboliev et al., 2002). As veins have less smooth muscle cell, the wall tension or the maximal force generated by constriction is much less compared with arteries (Nilsson, 1985).

Despite that there is more total protein in the smooth muscle layer of arteries compared with veins, the fractions of major contractile proteins such as actin, myosin heavy and light chains, and calponin were found to be similar in mesenteric artery and vein (Yamboliev et al., 2002). The only differences observed were as follows: the arteries express a higher amount of the actin-binding protein caldesmon than the vein and α isoform of tropomyosin predominates in the artery while the β isoform predominates in the vein (Yamboliev et al., 2002). Although it remains to be determined whether such differences in those accessory proteins account for the different function of arteries and veins, thicker and more tightly assembled smooth muscle layer rather than the profile of the major contractile proteins is the likely cause for a higher mechanical potential of the mesenteric artery compared to vein (Yamboliev et al., 2002).

Descending down the mesenteric tree, there is a gradient of decreasing wall thickness and increasing density of sympathetic innervation in arteries, but not veins (Nilsson, 1985; Yamboliev et al., 2002). Furthermore, as the artery becomes smaller, the contractile response to single electrical stimulation becomes more rapid (Nilsson, 1985). This difference reflecting a differential

organization in neurotransmission with regard to the vessel size was much less marked among the veins (Nilsson, 1985). Hence, compared to the corresponding arteries small veins tend to have slower twitches (Nilsson, 1985). This difference in contractile properties of arteries and veins perhaps underlie their different hemodynamic function e.g., resistance vs. capacitance.

9. Sympathetic innervation of arteries and veins

9.1. Sympathetic nerves innervate both mesenteric arteries and veins

Sympathetic nerves directly innervate the cardiovascular system and have a profound control over its function. Sympathetic nerves arise from para and preverterbral ganglia. The paravertebral ganglia, also termed the sympathetic chain or trunk, are a series of ganglia which lie in a line lateral and parallel to the vertebral bodies of the spinal column. The ganglia are interconnected to each other and extend from the base of the skull to the sacrum. Paravertebral ganglia supply the thoracic organs (lung and heart), skin, sweat glands, head, neck and skeletal muscle. The prevertebral ganglia are associated with the unpaired visceral arteries and located in front of the vertebral column and cemented on the aorta. They are divided into the celia ganglion, superior mesenteric ganglion and inferior mesenteric ganglion and they innervate the blood vessels in splanchnic circulation (Hsieh et al., 2000). Studies using retrograde tracing identified the ganglionic origins of sympathetic nerves innervating the mesenteric artery and vein at the ileal-cecal junction in the order of decreasing importance as follows:

celiac, superior mesenteric, paravertebral and inferior mesenteric ganglia (Hsieh et al., 2000).

Over the adventitial surface of the blood vessels, sympathetic nerves are paravascular nerve bundles coursing along the longitudinal axis of the vessels. Rather than innervating the blood vessels they travel along these nerve bundles to reach and innervate other targets farther a long the blood vessels or to other effector tissues. For example, paravascular nerves travel along mesenteric blood vessels to supply the arterioles inside the gastrointestinal tract. Near the adventitial-medial border nerve terminals are present as a perivascular plexus to make close contact with vascular smooth muscle cells. This close neuromuscular contact occurs at varicosities where nerve terminals are enlarged and transmitters are stored. This is the site of sympathetic neuroeffctor transmission. In mesenteric arteries and arterioles from rat and guinea pig, there is a close apposition (<100 nm) between sympathetic varicosities and smooth muscle cells, which demonstrates the ultrastructural morphology of a specialized neuromuscular junction (Luff et al., 1987; Luff and Mclachlan, 1989). In terminal arterioles with thin smooth muscle layer (1-2 smooth muscle cells) nearly every smooth muscle cell is innervated by one varicosity. In larger arterioles and arteries where the smooth muscle cell layer is thicker, only the smooth muscle cells located at the adventitial/medial border are directly innervated (Luff et al., 1987; Luff and Mc Lachlan, 1989). Smooth muscle cells on the blood vessels are coupled through low resistance gap junctions, through which electrical and chemical signals spread (Watts et al., 1994).

The specialized neuromuscular junctions were also observed in small mesenteric veins of the guinea pig intestine with a similar frequency to that occurring in mesenteric arteries (Klemm et al., 1993). The appearance and arrangement of sympathetic nerves associated with arteries and veins are similar although nerve density in veins is generally less than that in the adjacent arteries (Nilsson, 1985). These structural data indicate that sympathetic nerves are arranged and positioned to exert significant control of the diameters of mesenteric veins and arteries and arterioles.

Sympathetic nerves exert primary control over venomotor tone (Monos et al., 1995). It is generally believed that sympathetic nerves have the greatest quantitative importance in minute-to-minute regulation of venomotor tone (Shoukas and Bohlen, 1990; Monos et al., 1995). Sympathetic nerve stimulation can reduce intestinal blood volume by up to 60% leading to a significant re-distribution of blood from the splanchnic veins. *In situ* recordings of membrane potential of mesenteric veins in anesthetized rats have shown that blockade of neural input to the veins causes a membrane hyperpolarization (Lombard et al., 1982), suggesting that there is tonic neural input causing smooth muscle depolarization and constriction.

Sympathetic nerves also control arterial tone, particularly tone of the small arteries (Nilsson, 1985). Nerve mediated constriction of arteries and veins increases peripheral resistance (Ekas and Lokhandwala, 1980) and cardiac output, respectively (Guyton et al., 1972b). Thus, by controlling the two functional

parameters of blood pressure: peripheral resistance and cardiac output, the sympathetic nervous system plays an important role in blood pressure regulation.

9.2. Differential control of the sympathetic constriction of mesenteric arteries and veins

As noted before, sympathetic innervation of arteries and veins is similar with regard to the appearance and arrangement of the nerves on the blood vessels. However, substantial evidence indicates that there is differential sympathetic neural control of mesenteric arteries and veins. Firstly, studies intended to determine the neuronal sources of sympathetic nerves innervating the inferior mesenteric artery (IMA) and inferior mesenteric vein (IMV) suggested that there are different groups of sympathetic neurons innervating arteries and veins (Browning et al., 1999). The evidence includes: 1) retrogradely labeled neurons were never found to project to both the IMA and the IMV (Browning et al., 1999). Furthermore, neurons projecting to the IMA were located predominantly in the central areas of the IMG whereas those supplying the IMV were located more peripherally in IMG (Browning et al., 1999); 2) neurons projecting to the IMV are larger than those to the IMA (Browning et al., 1999); 3) although both groups of neurons fire tonically, neurons projecting to the IMA were more likely than those to the IMV to display inward rectification when they were hyperpolarized (Browning et al., 1999).

Secondly, the synaptic mechanism of neurotransmission may also differ between arteries and veins. Although exogenous ATP and NE contract both

mesenteric arteries and veins, in studies using intracellular recording methods in isolated mesenteric blood vessels from guinea pigs, it was shown that transmural electrical stimulation of veins and arteries produced slow depolarizations mediated by NE (Hottenstein and Kreulen, 1987). However in arteries, but not veins, electrical stimulation evoked an excitatory junction potential (EJP, a short latency and short duration membrane depolarization) mediated by ATP (Evans and Surprenant, 1992).

Thirdly, veins are more sensitive to the vasoconstrictor effect of sympathetic nerve stimulation than arteries as frequency-response curves for nerve stimulation- induced constriction in veins are to the left of the curves from arteries (Kreulen, 1986; Hottenstein and Kreulen, 1987). The threshold and half-maximal frequencies for slow depolarization were also lower in veins compared to arteries, which probably is due to the fact that resting membrane potentials of venous smooth muscle cells are less negative than the arterial smooth muscle cells (Kreulen, 1986). Therefore, venous smooth muscle cells are closer to threshold for constrictions and it also may require less depolarization for veins to contract than arteries.

10. Sympathetic neuroeffector mechanisms in arteries and veins

Sympathetic nerves control mesenteric vascular tone by releasing vasoconstricting neurotransmitters to cause vascular constrictions. Upon the propagation of action potential to nerve terminals a voltage-sensitive calcium

channel is activated to permit calcium entry, which initiates a cascade of events eventually leading to the exocytosis of transmitters from synaptic vesicles (Fon and Edwards, 2001). Sympathetic tone is reflected by the number of nerve impulses traveling along the nerve terminals per unit time, namely the impulse frequency. The higher the frequency, the more the transmitters will be released. Studies investigating the discharge pattern of perivascular sympathetic nerve fibers *in vivo* have found that neuronal activity occurs in regular short bursts of high frequency firing rather than long sustained trains (Johnson and Gilbey, 1996). This result implies that electrical stimulation of sympathetic nerves with a single train of relatively high frequency is more relevant to nerve impulses under physiological conditions.

According to size and electron density of the core of the synaptic vesicles, there are two major categories of synaptic vesicles in sympathetic nerve terminals: large dense core vesicles (LDCV: external diameter 80-120 nm) and small dense core vesicles (SDCV, some small vesicles may not have a dense core). LDCVs mainly contain peptidergic neurotransmitters such as neuropeptide Y (NPY) and are <20% of the vesicles in the nerve terminals (Stjarne, 2001). Transmitter release from LDCV occurs at extrasynaptic sites and is triggered by high frequency stimulation. SDCVs mainly contain NE and are > 80% of the vesicles in the nerve terminals. Transmitter release from SDCV occurs at the active zone of the prejunctional membrane of the nerve terminal and is triggered by lower stimulation frequencies (Stjarne, 2001).

Sympathetic control of vascular tone also relies on the reactivity of the postjunctional receptors for the transmitters. Reactivity of the postjunctional receptors is determined by the number and binding affinity of the receptors and by their capacity to activate specific second messenger pathways (De Champlain, 1990).

10.1. Adrenergic transmission

Synthesis of NE: The first step in the synthesis of NE is the conversion of tyrosine to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH) (Thoenen, 1972). This is the rate-limiting step in the synthesis of NE. DOPA is then decarboxylated to form dopamine. After taken up into the synaptic vesicles by monoamine transporter, dopamine is converted to NE by side-chain hydroxylation through the action of dopamine-β-hydroxylase (DβH). DβH is localized within the synaptic vesicles where NE is stored and released via exocytosis (Thoenen, 1972).

Metabolism of NE: Released NE is subject to several possible fates: 1) metabolism including oxidative deamination by monoamine oxidase (MAO), methylation by catechol *o*-methyltransferase and sulfoconjugation by phenol sulfotransferase (Esler et al., 1990). 2) diffusion into the plasma. Of NE released by sympathetic nerves throughout the body, perhaps 10-20% overflows into the circulation (Esler et al., 1990). NE in the interstitial fluid enters the circulation passively by diffusion down a concentration gradient (Esler et al., 1990). 3)

reuptake into sympathetic nerves where it is either packaged into the synaptic vesicles for re-release or metabolized by MAO (Esler et al., 1990).

Uptake of NE: There are two transporters for NE uptake with neuronal or extraneuronal locations. NE uptake into the nerve terminals is performed by the neuronal transporter, the so-called uptake-1 (Eisenhofer, 2001). The neuronal transporter is located at the sympathetic nerve terminals and it has12 membrane-spanning domains (Eisenhofer, 2001). Reuptake by this transporter is responsible for rapid termination of NE action in the synaptic cleft, accounting for almost 90% of the overall activity of the uptake system. Actions of this transporter can be blocked by cocaine (Eisenhofer, 2001).

Extraneuronal NE transporters, the so-called uptake-2, are much more diversely localized including vascular endothelium and smooth muscle cells. It has been established that uptake-2 are organic cation transporters (OCTs), including the classic corticosterone-sensitive transporter with a broad tissue distribution and two other transporters (OCT1 and OCT2) localized to the liver, kidney, and intestine (Eisenhofer, 2001). Important for clearance of NE from the plasma, their activity is responsible for about 5% of total uptake (Eisenhofer, 2001). Hence, NE transporters (NET) function as part of integrated systems where NE synthesis, release, uptake, and metabolism are regulated in a coordinated fashion in response to the demands placed on the system (Esler et al., 1988).

Assessment of sympathetic nerve activity: There are a number of methods that have been used to assess sympathetic nerve activity. For example, measurement of the changes in mean arterial pressure (MAP) and heart rate (HR) after ganglionic blockade is used to estimate overall sympathetic nerve activity (Deka-Starosta et al., 1989). Recording of renal or splanchnic nerve activity provides a more direct measurement (Deka-Starosta et al., 1989). More specifically, sympathetic nerve activity can be assessed at a regional level by measurement of NE spill over rate (Esler et al., 1985) at an organ or tissue level by measurement of NE content (Dae et al., 1989; Lorton, 1997), turnover rate (Takahashi et al., 1993) and NE release using amperometry (Dugast et al., 2002).

Tissue NE content generally reflects the steady state NE store in sympathetic nerves. Decreased NE content suggests there is reduced NE store that is due to an increased NE release. Therefore, tissue NE content is inversely related to the activity of sympathetic nerves innervating the tissue (Lorton, 1997). This is a simple one-time approach.

NE turnover rate can be measured by monitoring the decline of NE tissue content over time after treatment with NE synthesis-blocking drugs, such as α methylparatyrosine (McNamara and Murray, 2001). As NE synthesis is stopped, NE content in the nerve terminals will only be affected by NE release. Therefore, the rate of decline in NE content correlates with the transmitter releasing activity of the sympathetic nerves. The faster the rates of decline in NE content, the higher the sympathetic nerve activity (Takahashi et al., 1993).

NE released from sympathetic nerve terminals into the interstitial fluid can enter the venous circulation by diffusion down a concentration gradient. This is called spillover of NE into circulation (Esler et al., 1988). NE spillover into the venous circulation results in a higher plasma NE concentration in the venous circulation compared to the arterial circulation. Hence, differences between these two concentrations reflect the spillover rate. There are a number of factors that can affect the spillover rate including regional blood flow, activity of the uptake systems, metabolism and diffusion flow to the circulation. However, in general, spill over rate is approximately proportional to the rate of sympathetic nerve firing (Esler et al., 1988). This relationship provides the experimental justification for the use of measures of regional NE spill over to plasma in the estimation of regional sympathetic nervous activity (Esler et al., 1988).

Postjunctional action of NE: NE acts at metabotropic α– adrenergic receptors on vascular smooth muscle cells to cause constriction. The constrictions mediated by α–adrenergic receptors are slowly developing and long lasting as the receptors are G-protein coupled metabotropic receptors (Docherty, 1998). The intracellular mechanism involves the activation of Gq protein and production of IP_3 via a phosphoinositide pathway that leads to release of calcium from the intracellular calcium store (Docherty, 1998).

There are two subtypes for α adrenergic receptors: α1 and α2 subtypes (Docherty, 1998). The binding affinities of NE for α1- adrenergic receptors were much higher compared to α2- adrenergic receptors. For example, the binding

affinities of dog mesenteric arteries and veins for α1-adrenergic receptors were 10 fold higher than for α2-adrenergic receptors (Shi et al., 1990). In addition, a much higher density of α2-adrenergic compared to α1-adrenergic receptor binding sites is required for a contractile response (Shi et al., 1990). A large reserve for postjunctional vascular α1-adrenergic receptors, but not for α2-adrenergic receptors might be accountable for the much higher maximum responses produced by α1-adrenergic receptor agonists compared to α2-adrenergic receptor agonists in canine saphenous vein (Ruffulo and Zeid, 1985).

In arteries, only α1-adrenergic receptors are functionally active, but in veins α2-adrenergic receptors may also be functional. Ligand binding studies demonstrated that both α1-and α2-adrenergic receptors are present on the vascular smooth muscle cells of both mesenteric arteries and veins. However, in mesenteric arteries, only α1-adrenergic receptors mediate NE- induced constrictions. For example, prazosin, an α1-adrenergic receptor antagonist, completely inhibited NE- induced constrictions in arteries (Shi et al., 1989). Similarly, in isolated rat mesenteric resistance vessels prazosin was three orders of magnitude more potent than yohimbine, the α2-adrenergic receptors antagonist, in inhibiting NE-mediated calcium influx, intracellular calcium release and smooth muscle contraction in these vessels (Cauvin and Malik, 1984). However, in mesenteric veins from dogs and cats, α2-adrenergic receptors also mediate NE-induced constrictions. For example, dog mesenteric veins, but not arteries responded to an α2-adrenergic receptor agonist (Shi et al., 1990). In canine mesenteric veins, UK 14,304 and clonidine, the α2-adrenergic receptor

agonists, produced contractile responses (Shimamoto and Daniel, 1992; Shoji et al., 1983). Phenylephrine (PE), an α1-adrenergic receptor agonist, was a more potent agonist in the canine mesenteric artery than in the vein while UK 14,304 exhibited the opposite profile of activity (Itoh and Rajfer, 1987).

Studies done on dog mesenteric artery and vein showed that the binding affinity for prazosin was lower in mesenteric veins compared with mesenteric arteries whereas the maximum binding was similar, suggesting that the total amount of α1-adrenergic receptors expressed on venous and arterial smooth muscle cells is similar although the affinity for the α1-adrenergic receptors is lower in veins (Shi et al., 1989).

10.2. Purinergic transmission

Metabolism: ATP plays an important role in sympathetic neuroeffector transmission as a co-transmitter with NE. ATP is synthesized in mitochondria in accordance with energy demands. Therefore, compared with NE synthesis, ATP synthesis is perhaps much less tightly related to nerve activity. Hence, activity of ATP as a neurotransmitter is mainly regulated by mechanisms responsible for the termination of ATP actions. Nucleotidases that degrade ATP are a regulatory mechanism for terminating the action of ATP as a neurotransmitter. There are at least two kinds of nucleotidase mediating the degradation of ATP: membrane-bound ecto-nucleotidase particulate and releasable nucleotidase soluble (Westfall et al., 2002). Ecto-nucleotidase is localized at the nerve terminal membrane and is constitutively active, whereas the release of soluble

nucleotidase is concomitant with ATP release and thus its action nerve activity-dependent (Westfall et al., 2002).

Postjunctional action: ATP released from sympathetic nerve terminals binds with its receptors localized at the smooth muscle cells to induce constriction. Vascular receptors for ATP are P2 receptors, which are present on both mesenteric artery and vein (Hirst and Jobling, 1989). There are two subtypes for P2 receptors: P2X and P2Y. P2X receptors are ligand-gated ionotropic receptors that are non-selectively permeable to cations including sodium, potassium and calcium. P2X receptors mediate depolarization of smooth muscle cells causing opening of voltage-dependent calcium channels and provides an additional source of calcium influx (Benham and Tsien, 1987). The rise in the cytosolic level of calcium triggers the contraction of smooth muscle. P2Y receptors are metabotropic G-protein coupled receptors, which mediate constriction by inducing calcium release from intracellular calcium stores (Benham and Tsien, 1987).

The receptor mechanism mediating ATP-induced contraction of smooth muscle is different in arteries and veins. Constriction of the mesenteric artery from rats caused by exogenously applied ATP was mediated by P2X receptors (Galligan et al, 2001, Benham and Tsien, 1987, Gitterman and Evans, 2000), whereas constrictions of the mesenteric vein from both rats (Galligan et al, 2001) and guinea pigs (Mutafova-Yambolieva, 2000) were mediated by P2Y receptors.

10.3. Co-transmission in the sympathetic nervous system

ATP and NE are co-released from sympathetic nerves and mediate vasoconstriction (Sneddon and Burnstock, 1984; von Kugelgen and Starke, 1985). The early evidence suggesting that ATP and NE are co-transmitters released from sympathetic nerves was obtained from studies showing that pretreatment with 6-hydroxydopamine to destroy sympathetic nerves abolished the fast and slow phases of the constriction of the guinea pig vas deferens mediated by ATP and NE, respectively (Fedan et al., 1981).

The notion that ATP and NE are stored and released from the same synaptic vesicle originally came from studies showing that adrenal medulla chromaffin cells released ATP and NE in a constant molar ratio indicating co-release of ATP and NE from the same secretory granule (Todorov et al., 1996). Studies of ATP and NE release from sympathetic nerve terminals innervating the rat tail artery supported this notion. In those studies, NE release was measured by continuous amperometry as well as by the slow depolarizations. ATP release was measured by its postjunctional effects, EJPs. The evidence supporting co-release includes: 1) activation of prejunctional β-adrenergic receptors increases both electrically evoked ATP and NE release (Brock et al., 1997); 2) varying Ca^{2+} concentration and applying Ca^{2+} channel blockers results in similar effects on NE and ATP release (Brock and Cunnane, 1999).

In contrast, studies on sympathetic neuroeffector transmission to the smooth muscle cells of the guinea pig vas deference suggest that ATP and NE are stored in separate synaptic vesicles and released via an independent

mechanism. Evidence supporting separate release mechanisms includes: 1) a temporal disparity between ATP and NE release. The early transient twitch- like constriction of the smooth muscle cells is mediated by ATP and reflects the early and transient release of ATP, whereas the following tonic constriction represents a later phase of NE release (Todorov et al., 1996). 2) Although prejunctional α2-receptors seemed to regulate both ATP and NE release, they may be regulated by different subsets of prejunctional α2-receptors as the reduction caused by α2 agonists, xylazine and clonidine, on ATP release was greater compared to that in NE release (Westfall et al., 1996); 3) endogenously released NE had a greater influence on its own release than on that of ATP as pretreatment with the α2 receptor antagonists idazoxan and yohimbine produced a substantial increase in the overflow of NE and a lesser increase in the overflow of ATP (Westfall et al., 1996); 4) NE release may be more dependent on calcium influx through N-type channels, whereas ATP release is coupled to calcium entry through P-type channels. This conclusion is based on the results that the N-type calcium channel antagonist omega-conotoxin reduced the electrical field stimulation (EFS)-evoked release of NE to a greater extent than ATP, while the P-type calcium channel antagonist omega-agatoxin did the reverse (Westfall et al., 1996).

ATP modulates NE release: In addition to its postjunctional action on smooth muscle cells, ATP and its metabolites can modulate NE release by acting at prejunctional P2 receptors on sympathetic nerve terminals. For example,

exogenous ATP enhanced synaptosomal NE release via P2X receptors and inhibited NE release via P2Y receptors from cardiac sympathetic nerve terminals (Sesti et al., 2002). ATP-induced release of NE depends on influx of calcium from the extracellular space through both voltage-gated calcium channels and probably the P2X receptor itself (von Kugelgen et al., 1999).

10.4. NPY

It is known that NPY is localized in LDCVs of sympathetic nerve terminals and can be released upon long duration nerve stimulation (Potter, 1988). Using long trains of stimulation, NPY has also been shown to mediate part of the contractile response to electrical field stimulation (Phillips et al., 1998). However, studies done by Gitterman showed that co-application of prazosin, the α1-adrenergic receptor antagonist and suramin, P2 receptor antagonist, abolished neurogenic contractile responses in mesenteric artery of different sizes. These results clearly demonstrated that NPY does not contribute to the constrictor response in mesenteric vascular bed (Gitterman and Evans, 2001). Therefore, NPY is more likely to function as a neuromodulator in mesenteric vascular beds (Gitterman and Evans, 2001).

11. Size dependency of sympathetic control of mesenteric blood vessels

Comparative studies on sympathetic neuroeffector mechanisms in mesenteric blood vessels with various sizes strongly suggest that there are size

related differences in several aspects of sympathetic neuroeffector transmission including density of sympathetic innervation, proportion of ATP and NE transmission and cellular mechanisms underlying ATP-induced smooth muscle constriction.

11.1. Difference in sympathetic innervation

In studies using TH and DβH to stain for the sympathetic nerves innervating mesenteric blood vessels of different size it was found that as arteries becomes smaller, the density of the sympathetic nerves increases (Nilsson, 1985). This gradient of sympathetic innervation may contribute to the differential responses of those blood vessels to sympathetic nerve stimulation. For example, responses to a single electrical stimulation are more rapid in the smaller artery compared to the bigger artery (Nilsson, 1985). These differences were not related to the properties of the vascular smooth muscle as responses to direct electrical activation of the smooth muscle in all vessels were quite rapid. Furthermore, the maximal neurogenic responses correlate with innervation density, being greatest in the small arteries and least in the large arteries.

11.2. Difference in the proportion of co-transmission

It was shown that the relative roles of ATP and NE in mediating sympathetic control of arterial tone in the rat mesenteric bed depend on the diameter of the vessel. As the size of the blood vessel increases, the contribution by ATP to sympathetic neuroeffector transmission decreases. For example, the

purinergic component of sympathetic transmission dominates in small-medium arteries (3rd – 6th order) while constrictions in large arteries (1st order) are almost entirely adrenergic (Gitterman and Evans, 2000). A similar proportion of purinergic constriction has also been seen in other arteries when similar short trains of stimulation were used (Evans and Cunnane, 1992; Warland and Burnstock, 1987). The much smaller purinergic component of constriction in large vessels is probably due to a lower density of sympathetic innervation in large arteries (Luff and McLachlan, 1989) and a lower sensitivity of P2X receptors in large mesenteric arteries (Gitterman and Evans, 2000).

11.3. Difference in cellular mechanisms underlying the P2X receptor mediated smooth muscle contraction

Cellular mechanisms underlying P2X receptor-mediated constriction of mesenteric arteries are different depending on the vessel size. In large arteries P2X receptor mediated constriction is sensitive to the L-type calcium channel blocker nifedipine (Bulloch et al., 1991; Omote et al., 1989). By contrast, in small resistance arteries of the guinea-pig submucosa (Galligan et al., 1995) nifedipine as well as cadmium, the non-selective calcium channel blocker, had no effect on contractile responses mediated by P2 receptors. Furthermore, cyclopiazonic acid which blocks calcium release from intracellular calcium store had no effect on P2X receptor mediated responses in small arteries (Gitterman and Evans, 2000). These data suggest that for small arteries all the calcium required for constriction enters directly though the P2X receptor channel whereas in large

arteries calcium entry through calcium channel and activation of intracellular calcium stores are required for the P2X receptor mediated constriction (Gitterman and Evans, 2000)

12. Local modulatory mechanisms for sympathetic neuroeffector transmission

Sympathetic nerve activity is not exclusively dependent on impulse frequency. As a matter of fact, transmitter-releasing activity of the sympathetic nerves is under modulation by multiple autoreceptors and heteroreceptors localized at the nerve terminal. These modulatory receptors can either enhance or attenuate transmitter release during sympathetic stimulation (De Champlain, 1990). Together, these modulatory effects optimize sympathetic stimulation to ensure that local sympathetic functions are integrated with those of other circulating hormones under various conditions. Accordingly, altered sympathetic drive in DOCA-salt hypertension could be due to altered local modulation on sympathetic neuroeffector transmission to the vascular system (De Champlain, 1990).

12.1. Prejunctional α2-adrenergic receptors on sympathetic nerves modulate sympathetic nerve activity.

Prejunctional α2-adrenergic receptors located at the sympathetic nerve terminals are autoreceptors. They are activated by released NE and inhibit further NE release, thus mediating feedback inhibition of NE release (Langer, 1980). These receptors are metabotropic receptors coupled to a heterotrimeric G-protein. Activation of prejunctional α2-adrenergic receptors causes dissociation of the β, γ subunits from the G-protein. The dissociated β, γ subunits interact directly with the pore-forming subunits of N- and P/Q-type calcium channels, thus reducing the channels' open probability and inhibit calcium entry into the cytoplasm. Inhibition of calcium entry eventually leads to inhibition of NE release (Starke, 2001). Mechanisms other than modulation of calcium entry have also been reported to underlie the inhibition of NE release by prejunctional α2-adrenergic receptors. These potential mechanisms include regulation of cytoplamic calcium homeostasis and interference with the transmitter releasing process such as transporting and docking of the synaptic vesicles at the active zone (Starke, 2001).

Studies on sympathetic neuroeffector transmission to guinea pig vas deferens suggest that regulation of NE release by α2-adrenergic receptors is frequency dependent. At lower frequencies (8 Hz), α2-adrenergic receptors inhibit NE release and modulate the composition of the co-transmitters. As the frequency of stimulation increases (above 8 Hz), the autoinhibition of the release of NE is overridden and the amount of NE relative to ATP increases. Increased release of NE caused an enhanced ATP mediated constriction via a postjunctional mechanism. These results suggest that the prejunctional α2-

adrenergic receptors may function as a sensor that "reads" the frequency of action potentials and converts that information into discrete neurochemical messages with varying proportions of co-transmitters. Some studies showed that α2-adrenergic receptors also regulate ATP release (Westfall et al., 1996; Bobalova et al., 2001a).

Increased sympathetic nerve activity in experimental hypertension suggests that there are alterations in baroreflex function or in local mechanisms modulating sympathetic neuroeffector transmission (De Champlain, 1990). Data from several studies indicate that prejunctional α2-adrenergic receptor function is impaired in hypertensive animals. For example, intravenously administered yohimbine, an α2-adrenergic receptor antagonist, increased plasma NE levels in sham normotensive, but not in DOCA-salt hypertensive rats (De Champlain, 1990). Furthermore, yohimbine potentiated nerve stimulation-evoked NE release into the mesenteric vasculature in normotensive rats, but not in DOCA-salt hypertensive rats (Tsuda et al., 1989). The increased NE release found in portal vein and caudal artery of SHRs was due to a decrease in the functional activity of prejunctional α2-adrenoceptor (Westfall et al., 1986).

12.2. Altered function of peripheral catecholamine transporters may be involved in disturbances of the autonomic nervous system in hypertension (Eisenhofer, 2001).

In chronically hypertensive animals such as SHR, neuronal and extraneuronal uptake of NE is enhanced in the blood vessel wall (Vanhoutte et

al., 1980). For example, the uptake of ^{3}H-NE was greater in tail arteries from SHR as compared to those from normotensive rats. Cocaine, an uptake-1 blocker, had a greater potentiating effect on responses to NE and electrical stimulation in tail artery from adult SHR than in normotensive rats. The magnitude of inhibition on uptake by cocaine was greater in SHR (Webb and Vanhoutte, 1981). Furthermore, studies on young SHR showed that uptake of ^{3}H NE in tail arteries was significantly higher compared to that in normotensive rats, demonstrating an enhanced uptake of NE prior to the full development of hypertension (Zsoter and Wolchinsky, 1983). Studies on mesenteric arteries (200 μm) also showed that there was a 44% increase in neuronal NE uptake in vessels from SHR in the established phase of hypertension (Whall et al., 1980). Studies on NE uptake in humans with essential hypertension yielded similar results. For example, there was an enhanced leftward shift of the NE sensitivity with cocaine in isolated subcutaneous resistance vessels from the hypertensives suggesting an enhanced NE uptake in essential hypertension (Aalkjaer et al., 1987). Studies on DOCA-salt hypertension, however, produced somewhat different results. For example, there was no difference in uptake activity in perfused caudal arteries between 3-week-DOCA-salt hypertensive rats and normotensive rats (Longhurst et al., 1988). Yet, *in vivo* studies showed that plasma NE level of DOCA-salt rats increased in the presence of neuronal uptake blockade, clearly demonstrating that neuronal uptake of NE was not defective in DOCA-salt hypertension (Drolet et al., 1989).

12.3. ET-1 modulates sympathetic nerve activity

In addition to changes in receptor subtypes and their responsiveness, some neuromodulators including ET-1 may cause the alterations in sympathetic neuroeffector mechanisms in DOCA-salt hypertensive animals (Rubanyi and Polokoff, 1994).

13. ET-1 and its receptor subtypes

13.1 Synthesis and bioactivity

The endothelin family consists of three isoforms of ETs: ET-1, ET-2 and ET-3 and four sarafotoxins (S6a, S6b, S6c and S6d, isolated from snake venom). Endothelin converting enzyme-1, a metalloendopeptidease, cleaves the precursor peptides of ET to form the final 21 amino acid peptide, ET. ET-1 comprises 80% of total ET formation and is perhaps the most biologically active member of the family. ET-1 in the vascular system is mainly synthesized and secreted from the endothelial cells of the blood vessels.

Release of ET-1 appears to be directly connected to its *de novo* synthesis as there is no evidence that ET-1 is stored in endothelial cells (Rubanyi and Polokoff, 1994). The synthesis and activity of ET-1 is regulated by various factors derived from endothelial cells and smooth muscle cells including vasoactive substances, cytokines and growth factors (Rubanyi and Polokoff, 1994). For example, in addition to inhibiting the activity of ET-1, endothelium-derived

vasodilator, NO, also inhibits production of ET-1. Smooth muscle cells also release diffusible factors that suppress the production of ET-1 and/or proteases associated with the degradation of ET-1 (Rubanyi and Polokoff, 1994).

Secretion of ET-1 is polarized as 80% of the total amount of ET-1 is secreted toward the underlying smooth muscle cells (Rubanyi and Polokoff, 1994). The dynamics of synthesis and secretion of ET-1 are well-suited for ET-1 to be an important mediator of vasoconstriction. ET-1 produces prolonged contractile responses. Because of its high vasoconstrictor potency and long-lasting actions, the continuous release of small amounts of ET could contribute to the maintenance of vascular tone (Rubanyi and Polokoff, 1994). Other cardiovascular effects of ET-1 include activation of both the SNS and RAS and increased mitogenesis (Rubanyi and Polokoff, 1994).

13.2. Signal transduction mechanisms

ET-1 causes constrictions of arteries and veins by binding with its receptors on smooth muscle cells (Rubanyi and Polokoff, 1994). ET receptors are found in the vascular wall, kidney, and heart, suggesting that ET-1 has a widespread influence on cardiovascular function (Rubanyi and Polokoff, 1994). There are two classes of receptors for ET-1 in the cardiovascular system. ET_A receptors are expressed by vascular smooth muscle cells and ET_B receptors are expressed by the endothelium and smooth muscle cells of some vascular beds (Gellai et al., 1996; Rubanyi and Polokoff, 1994). The ET_A and ET_B receptor

subtypes are both G-protein coupled receptors with seven membrane-spanning domains and a relatively long extracellular N terminal.

Activation of ET receptors on vascular smooth muscle cells stimulates phospholipase C and thus the hydrolysis of phosphatidylinositol, which results in the formation of inositol 1,4,5, -triphosphate (IP3) and *sn*1, 2-diacylglycerol (DAG). IP3 and DAG cause mobilization of calcium from intracellular calcium stores. ET-1 also directly depolarizes the smooth muscle cells to activate voltage-gated L-type calcium channels to permit calcium entry. Therefore, ET-1 induced vascular smooth muscle contraction involves the increase in cytoplasmic calcium concentration by facilitaion of calcium influx and mobilization of intracellular calcium stores (Rubanyi and Polokoff, 1994).

ET_B receptors in the endothelium mediate vasodilatation by stimulating the production and release of vasodilator substances such as NO and prostacyclin (Rubanyi and Polokoff, 1994). In veins and some large caliber arteries, ET_B receptors are expressed on the smooth muscle cells and mediate vasoconstriction (Gellai, 1996; Johnson et al., 2002). Although the ET_B receptors expressed on the vascular smooth muscle cells are functionally different from those expressed on the endothelium, no molecular distinction has ever been made between these two receptors (Johnson et al., 1999).

13.3. ET-1 and DOCA-salt hypertension

Intravenous infusion of ET-1 causes elevation of systemic arterial blood pressure in both humans and experimental animals (Mortensen and Fink, 1990;

Brunner, 1998). Blockade of ET receptors with acute intravenous administration of the mixed ET_A/ET_B receptor antagonist TAK-044 in normotensive subjects caused a decrease in systemic blood pressure (Haynes et al., 1996). These results suggest that ET-1 is involved in regulation of blood pressure.

ET-1 plays an important role in the development and maintenance of DOCA-salt hypertension. This notion is supported by the observations that there is overexpression of ET-1 in DOCA-salt hypertensive rats (Day et al., 1995) and chronic treatment with a mixed ET_A/ET_B receptor antagonist decreased blood pressure to a normal level (Doucet et al., 1996). The mechanisms underlying ET-1's role in DOCA-salt hypertension may be due to its potent and long-lasting vasoconstricting effects.

ET-1 is a more potent constrictor of veins than arteries (Warner, 1990 and Leppaluoto, 1992; Johnson et al., 2002). This increased potency may be related to the differences in expression of ET receptor subtypes and receptor density (Johnson et al., 2002). For example, S6c, the selective ET_B receptor agonist, produces constriction of mesenteric veins, but not the mesenteric arteries from the rats, suggesting that functional ET_B receptors are expressed on smooth muscle cells of veins, but not arteries (Johnson et al., 2002). Studies *in vivo* show that intravenous administration of ETs increase central venous pressure, venous resistance and MCFP (Waite and Pang, 1992; Yamamato et al., 1980). Studies *in vitro* show that ET-1 is found in the post arterial bed, i.e., capillaries and veins (Hemsen, 1991). In DOCA-salt hypertension, ET-1 induced constriction was reduced in aorta, but maintained in vena cava (Watts et al.,

2002). Similar finding were obtained from studies on mesenteric blood vessels where ET-1 induced constriction was decreased in mesenteric arteries, but maintained in mesenteric veins from DOCA-salt rats (Johnson et al., 2002).

Studies showed that both an increase and decrease in venous pressure was accompanied by an increase in ET-1 production, suggesting that ET-1 plays a role in the long-lasting adaptations of veins to changes in blood volume (Serneri et al., 1995). Exogenously applied ET-1 also caused increased MCFP in rats (Waite and Pang, 1992). In humans, increased venous pressure caused by blood volume expansion stimulates ET-1 production causing venoconstriction and decreased venous compliance (Serneri et al., 1995). These results led to the postulate that in DOCA-salt hypertension, elevated ET-1 production due to increased venous pressure would cause prolonged venoconstriction as DOCA and high salt cause sodium and water retention and volume expansion. This could lead to enhanced cardiac output (Guyton et al., 1972b).

13.4. ET-1 modulates sympathetic nerve activity.

ET-1 and its receptors are present in the CNS (Nakagomi, et al., 2000), sympathetic ganglia (Hemsen and Lundberg, 1991) and sympathetic nerve terminals (Power et al., 1989). ET-1 modulates sympathetic neuroeffector transmission to arteries (Zhang et al., 1996) and veins (Waite and Pang, 1992). Its action can be prejunctional since ET receptors have been localized to cholinergic nerve terminals in smooth muscle of the trachea (Fernandes et al., 1998) and at sympathetic nerve endings in human and porcine coronary arteries

(Power et al., 1989). ET-1 facilitated the purinergic components of sympathetically mediated constrictions of rabbit saphenous artery (Mutafova-Yambolieva and Radomirov, 1994). Furthermore, it was shown that ET-1 inhibited prejunctional NE release from sympathetic nerves and postjunctionalally it increased the responsiveness of α adrenergic receptors to catecholamines in rat mesenteric arteries (Tabuchi et al., 1990). Therefore, ET can also act via a postjunctional mechanism. Using isolated rat tail artery *in vitro*, superfusion of ET-1 or S6c (ET_B receptor agonist) with a subthreshold contractile concentration significantly reduced NE and ATP overflow caused by electric field stimulation. This effect was blocked by the selective ET_B receptor antagonist, suggesting that the ET_B receptors may be important in prejunctional neuromodulation of sympathetic tone by ET-1 to the blood vessels (Mutafova-Yambolieva and Westfall, 1998).

The interactions between ET-1 and sympathetic neuroeffector transmission is bi-directional. Transmitters released by sympathetic nerves can also modulate ET-1's vasoconstrictor effects. Studies on conscious rats have shown that verapamil, which increases sympathetic nerve activity by decreasing mean arterial pressure and activating the baroreceptor reflex, facilitated ET-1 induced venoconstriction. This facilitation was blocked by hexamethonium, the nicotinic acetylcholine receptor antagonist (Waite and Pang, 1992).

14. Summary of the general introduction and issues to be resolved

Mechanisms of sympathetic transmission differ in arteries and veins. Therefore, characterization of sympathetic neuroeffector mechanisms including identifying the neurotransmitters as well as the postjunctional receptor subtypes and reactivities for the transmitters in arteries and veins in mesenteric circulation will further elucidate the difference in neural mechanisms controlling arterial and venous tone. There is elevated activity of the SNS in DOCA-salt hypertension. Despite that vascular peripheral resistance mainly resides in small arteries and arterioles, the majority of studies have focused on medium and large arteries with comparatively little research investigating small resistance arteries (Evans and Surprenant, 1992; Phillips et al., 1998). As there is a vessel size- related difference in mechanisms of sympathetic neuroeffector transmission to mesenteric arteries (Nilsson, 1985; Gitterman and Evans, 2001), findings obtained from studies on large and medium arteries are not applicable to the small arteries and arterioles.

ATP is a co-transmitter of NE from sympathetic nerves associated with the vascular system (Burnstock and Sneddon, 1985) and a few studies showed that there are alterations in purinergic transmission associated with mesenteric arteries in hypertension. For example, there was enhanced purinergic transmission in mesenteric arteries from SHRs as EJPs evoked by single electrical stimuli in mesenteric arteries from SHR are increased compared to those from normal rats with similar passive electrical properties of the vascular smooth muscle (Brock and Van Helden, 1995). Furthermore, there was a potentiated response to ATP in SHR (Naito et al., 1998). However, as no study

has been done to assess potential changes in purinergic component of the sympathetic neuroeffector transmission to arteries from DOCA-salt hypertensive animials it is unclear whether similar changes occur in DOCA-salt hypertension.

Although venomotor tone was known to be elevated in DOCA-salt hypertensive rats (Fink et al., 2000), very little studies have been done on changes in mechanisms of sympathetic control of venous tone in hypertension. For example, there has been no direct assessment of changes in transmitter release in mesenteric veins in DOCA-salt hypertension. Furthermore, no study has been done to investigate the NE uptake activity of the sympathetic nerves associated with mesenteric veins in DOCA-salt hypertension.

Data from several studies indicate that prejunctional α2-adrenergic receptor function is impaired in hypertensive animals (De Champlain, 1990; Moreau et al., 1995; Tsuda et al., 1989). Prejunctional α2-adrenergic receptors associated with sympathetic nerves also regulate NE release in veins (Bobalova and Mutafova-Yambolieva, 2001a; Foucart et al., 1987). The few studies of the function of α2-adrenergic receptors associated with veins in hypertension, however, have yielded controversial results. Increased NE release and attenuated yohimbine effect on NE release occur in the portal vein of SHRs. However, in the same study it was shown that in portal veins from DOCA-salt hypertensive and 1K1C hypertensive rats there was not an increase in NE release or an attenuation of the yohimbine effect on NE release (Westfall et al., 1986). As the portal vein makes only a small contribution to total vascular capacitance, this blood vessel may not be an appropriate model to study

hypertension associated with changes in the function of peri-venous sympathetic nerves.

ET-1 acting at ET receptors modulates sympathetic neuroeffector mechanisms associated with arteries (Zhang et al., 1996) and veins (Waite and Pang, 1992) via both a prejunctional and postjunctional mechanism. However, no study has been done to determine whether ET receptors are localized at the sympathetic nerves innervating the vascular system. Furthermore, it was shown that veins produced a more potent and sensitve response than arteries to ET-1 (Johnson et al., 2002). In addition, functional ET_B receptors are expressed on smooth muscle cells of the veins, but not the arteries in mesenteric vascular bed (Johnson et al., 2002). However, no study has been done to examine whether the modulatory effects by subpressor ET-1 on sympathetic neuroeffector transmission to mesenteric arteries and veins differ. As the most potent vasoconstrictor substance known, ET-1 has been shown to play an important role in the development of DOCA-salt hypertension (Day et al., 1995; Doucet et al., 1996; Johnson et al., 2001). However, no study has been done to examine whether the modulatory effects by subpressor ET-1 on sympathetic neuroeffector transmission to arteries and veins alter in DOCA-salt hypertension.

Chapter 2

HYPOTHESES AND SPECIFIC AIMS

Overall hypothesis

Alterations in neurotransmitter release from sympathetic nerves and postjunctional reactivity of the vascular smooth muscle cells for the neurotransmitters are different in arteries and veins from DOCA-salt hypertensive rats. These alterations are at least partly due to functional changes of the prejunctional α2-adrenergic receptors and to the effects of ET-1.

DOCA-salt hypertension is a well-characterized model of experimental hypertension which is associated with increased sympathetic nerve activity. This model can be used to study the neurohormonal mechanisms secondary to adrenal steroids excess in hypertension. This study has the following specific aims:

Specific aim 1: What are the alterations in neurotransmitter release from sympathetic nerves and postjunctional reactivity to the transmitters in mesenteric arteries and veins from DOCA-salt hypertensive rats?

Specific aim 2: Do prejunctional α2-adrenergic receptors modulate transmitter release from sympathetic nerves in arteries and veins and are those modulatory effects altered in DOCA-salt hypertension?

Specific aim 3: Does ET-1 modulate transmitter release from sympathetic nerves and the postjunctional reactivity of arteries and veins to the neurotransmitter and are those modulatory effects altered in DOCA-salt hypertension?

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CHAPTER 3

Differential alterations in sympathetic neuroeffector mechanisms in mesenteric arteries and veins in DOCA-salt hypertensive rats

Min Luo[4], Margaret C. Hess[1], Gregory D. Fink[1,4], L. Karl Olson[2], Jennifer Rogers[2], David L. Kreulen[2,3,4] Xiaoling Dai[4] and James J. Galligan[1,4]

Departments of Pharmacology and Toxicology[1], Physiology[2], Ophthalmology and Neurology[3] and The Neuroscience Program[4], Michigan State University, East Lansing, MI 48824

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INTRODUCTION

DOCA-salt hypertension is initiated, in part, by salt retention promoted by treatment with the mineralocorticoid and uninephrectomy. Thus, this model is relevant to salt-sensitive human hypertension and permits studies of the neural and hormonal mechanisms associated with elevated blood pressure (Schenk and McNeill, 1992). Changes in the function of the sympathetic nervous system occur in DOCA-salt hypertension. Circulating NE and epinephrine levels are increased in DOCA-salt hypertensive rats suggesting that there is increased release of NE from sympathetic nerves and epinephrine from the adrenal gland in these animals (Bouvier, et al., 1986). This result is supported by studies done *in vitro* where it was shown that stimulation of sympathetic nerves associated with mesenteric arteries from DOCA-salt rats released more NE than those obtained from normotensive rats (Tsuda et al., 1989). Increased release of NE from peri-arterial sympathetic nerves in DOCA-salt rats is due, at least in part, to a disruption in the function of prejunctional α2-adrenergic autoreceptors (De Champlain, 1990). There are also postjunctional changes in arterial smooth muscle that result in enhanced neurogenic constrictions in tissues from DOCA-salt rats. Large mesenteric arteries from DOCA-salt rats are more sensitive to the contractile effects of exogenously applied NE (Perry and Webb, 1988; Longhurst et al., 1989; Suzuki et al., 1994) although other studies showed that the sensitivity to adrenergic agonists did not change in caudal artery (Hermsmeyer et al., 1982) and the binding affinity of α1-adrenergic receptors in mesenteric arteries is reduced (Meggs et al., 1988).

The studies summarized above focused on sympathetic nerves and adrenergic receptors in arteries. However, sympathetic nerves supply veins and venomotor tone is controlled largely by sympathetic nerve activity (Monos et al., 1995). In human hypertension and in SHRs, sympathetic neural input to veins is increased. It has also been shown that venomotor tone is elevated in DOCA-salt rats and this is largely due to the increased sympathetic tone in veins (Fink et al., 2000). Detailed studies of the mechanisms underlying increases in sympathetic input to veins in hypertension have not been done. These studies would be important because the sympathetic neurons innervating arteries and veins differ in their location in the ganglia and in their electrophysiological properties (Browning et al., 1999), suggesting that there is differential sympathetic neural control of mesenteric arteries and veins. This suggestion is supported by data from studies of the neuroeffector mechanisms in arteries and veins. Intracellular electrophysiological studies of mesenteric veins showed that sympathetic nerve stimulation produces a slow depolarization and constriction mediated by NE acting at α1-adrenergic receptors (Hottenstein and Kreulen, 1987; Van Helden, 1988). In small mesenteric arteries and arterioles, electrical stimulation evokes short latency, short duration EJPs and constrictions mediated by ATP acting at P2X receptors (Ramme et al, 1987; Evans and Surprenant, 1992; Gitterman and Evans, 2001).

Changes in neuroeffector transmission to veins in hypertension are important because veins are more sensitive to the vasoconstrictor effects of sympathetic nerve stimulation than arteries (Kreulen, 1986; Hottenstein and

Kreulen, 1987). Furthermore, the membrane potential of venous smooth muscle cells in SHRs is depolarized compared to that found in normotensive rats. This change is due to increased sympathetic tone to veins in SHRs (Willems et al., 1982), but these studies did not establish if there was increased release of NE or if there was increased venous reactivity to NE. Human forearm veins obtained as biopsy specimens from normotensive and hypertensive subjects show several hypertension associated disturbances in neuroeffector transmission including decreased contractile responses to α1-adrenergic receptor agonists, but enhanced responses to nerve stimulation (Sudhir et al., 1990). This previous work indicates that there are changes in sympathetic neuroeffector transmission to veins in hypertension, but the details of these changes are unclear.

Identification of the mechanisms controlling venomotor tone in hypertension is important because venous compliance is reduced in hypertensive subjects (Safar and London, 1987). Decreased venous compliance is most marked in peripheral vessels, producing a redistribution of blood toward the heart (Schobel et al., 1993). Decreased compliance of splanchnic veins is particularly notable in hypertension (Nyhof et al., 1983) and, as the splanchnic circulation is the largest capacitance bed, changes in mesenteric vasomotor tone will impact overall hemodynamics (Greenway, 1983).

The data summarized above indicate that there is differential sympathetic neural control of arteries and veins and that there maybe changes in sympathetic neural control of both arteries and veins in hypertension. The present study used an *in vitro* approach to examine sympathetic nerve-mediated constrictions of

mesenteric arteries and veins from sham-operated normotensive (sham) and DOCA-salt hypertensive rats in order to test this hypothesis.

METHODS AND MATERIALS

Animals

All experiments were done using Sprague-Dawley rats from Charles River, Inc (Portage, MI). Upon arrival at the animal care facility, animals were maintained according to standards approved by the Michigan State University All-University Committee on Animal Use and Care. All experimental procedures were carried out in accordance with the "Guiding Principles in the Care and Use of Animals" of the American Physiological Society. Rats were acclimatized for 2-3 days prior to entry into any experimental protocol. Pelleted rat chow (Harlan/Teklad 8640 Rodent Diet) and water were provided *ad libitum*. Rats were housed in temperature and humidity controlled rooms with a 12 hours on / 12 hours off light cycle.

Preparation of DOCA-salt rats

Male Sprague-Dawley rats weighing 175-200 grams were anesthetized with sodium pentobarbital (50 mg/kg i.p.). The skin over the left flank (lateral abdominal wall) was shaved and prepared with an iodine-based antiseptic. A 1.5 cm vertical incision was made through the skin and underlying muscle just caudal to the rib cage. The left kidney was exteriorized and removed after ligation of the renal artery, vein and ureter with 4-0 silk sutures. The muscle and skin layers were closed separately with 4-0 silk and 4-0 monofilament nylon sutures, respectively. A 3 x 1.5 cm rectangle area between the shoulder blades of the

back was shaved and disinfected for s.c. DOCA implantation under a 1 cm incision. The skin was closed with 4-0 nylon sutures. DOCA implants (600 mg/kg) were prepared by mixing deoxycorticosterone acetate in silicone rubber resulting in a giving dose of 200 mg/kg. Sham-operated rats underwent left kidney removal only. Surgery was performed on a heated pad and rats recovered in a heated box. Antibiotics (enrofloxacin, 5 mg/kg s.c.) and an analgesic (butorphanol tartrate, 2 mg/kg, s.c.) were administered immediately after surgery. After recovery the rats were housed under standard conditions for 4 weeks. DOCA-implanted rats received standard pelleted rat chow and salt water (1 % NaCl + 0.2 % KCl) *ad libitum*, while sham rats received standard pelleted rat chow and tap water *ad libitum*. Systolic blood pressure was measured using the tail-cuff method four weeks after surgery. Rats with systolic blood pressure equal to or higher than 150 mmHg were considered hypertensive.

Measurement of vasoconstriction *in vitro*

Rats were killed using a lethal pentobarbital injection (50 mg, i.p). The small intestine was removed and placed in oxygenated (95% O_2, 5% CO_2) Krebs' solution of the following composition (mM): NaCl 117; KCl 4.7; $CaCl_2$ 2.5; $MgCl_2$ 1.2; $NaHCO_3$ 25; NaH_2PO_4 1.2; glucose 11. The ileal segment was placed in a petri dish and the mesentery was stretched gently and pinned flat. A section of mesentery close to the ileal wall was carefully cut free from the intestine and the mesentery was transferred to a small silicone elastomer-lined recording bath (2.5 ml volume). The arteries and veins (100 – 300 μm diameter)

were exposed by carefully cutting away surrounding adipose and connective tissues. The recording bath containing the preparation was mounted on the stage of an inverted microscope (Olympus CK-2) and superfused continuously with warm (36 ^{o}C) Krebs' solution at a flow rate of 7 ml/min. The output of a black and white video camera (Hitachi, KP-111) attached to the microscope was fed to a PCVision Plus frame-grabber board (Imaging Technology Inc., Woburn MA) mounted in a personal computer. The sampling rate was 10 Hz and changes in blood vessel diameter of 0.5 μm could be resolved. The video images were analyzed using Diamtrak® software. The digitized signal was converted to an analog output (DAC-02 board, Keithley Metrabyte) and which was fed to an analog to digital converter (Labmaster 125) and a second computer running Axotape software (version 2.0, Axon Instruments, Inc. Foster City CA) for a permanent record of changes in vein diameter. Analog signals were sampled at 33 Hz and data were stored on the computer hard drive for subsequent display and analysis.

Drug application

Drugs were applied using a system of 3-way stopcocks so that the superfusing Krebs' solution could be changed to one containing a known concentration of drug. The flow rate was 9 ml/minute and it took one minute for drugs to reach the tissue. NE, PE and ATP concentration- response curves were constructed using non-cumulative addition of each agonist concentration. Each agonist concentration was applied for 2 minutes and there was a 20 minute

interval between application of each agonist concentration. Tissues were washed continuously with normal Krebs' solution between each dose. Antagonists were applied for a minimum of 20 minutes prior to testing their effect on agonist- or nerve-mediated responses.

Transmural stimulation of perivascular nerves

Two parallel silver/silver chloride wire electrodes connected to a Grass Instruments stimulator (S88) were placed perpendicular to the longitudinal axis of mesenteric tissues. Parameters for nerve stimulation were: 30 stimuli, stimulus duration 0.5 ms, frequency 0.2 – 30 Hz, voltage 60-140 volts. The neurogenic origin of constrictions caused by electrical stimulation was verified in each preparation by initially demonstrating that a constriction caused by 20 Hz stimulation was blocked by tetrodotoxin (TTX, 0.3 μM). Preparations in which the initial 20 Hz constriction was not blocked by TTX were discarded. The peak constriction and in some experiments the duration of vasoconstriction caused by nerve stimulation was measured. Measurements were made using Axotape software and on-screen cursors.

Statistical analysis

Agonist-induced constrictions were measured in μm and are expressed as a percentage of the initial resting diameter of the blood vessel. For NE and PE concentration-response curves, EC_{50} and Emax were calculated from a least

squares fit of individual agonist concentration-response curves using the following logistic function (Origin 5.0 Microcal Software Inc., Northampton, MA):

$$Y = \{(Ymin - Emax)/[1 + (X / EC_{50})^{m}]\} + Emax$$

Where "Ymin " is the minimum response (constrained to 0), "E_{max}" is the maximum response, "EC_{50}" is the half maximal effective concentration and "m" is the slope factor. For nerve stimulation frequency-response curves, the half maximal effective stimulation frequency (S50) and Emax were calculated from curves obtained in individual tissues using the following function (Origin 5.0 software):

$$Y = Emax\ X / (S50 + X)$$

Where "X" is the stimulation frequency tested and "y" is the peak response amplitude. All data are expressed as the mean ± S.E.M and "n" values refer to the number of animals from which the data were obtained. Differences between groups were assessed by the Kruskal-Wallis and Dunn's multiple comparisons tests and Students' t-test.

Drugs

All drugs were obtained from Sigma Chemical Company (St. Louis, MO).

Results

General

Data were obtained from 68 sham rats and 74 DOCA rats. Mean systolic blood pressure from sham and DOCA-salt rats was 118 ± 1 mmHg and 179 ± 3 mmHg, respectively ($P < 0.05$). The mean weight of sham rats was 462 ± 6 g and the mean weight of DOCA-salt rats was 322 ± 9 g ($P < 0.05$). The inner diameter of mesenteric arteries from sham and DOCA-salt rats was 141 ± 7.4 μm, 149 ± 8.0 μm ($P > 0.05$), respectively. The diameter of mesenteric veins from sham rats was 214 ± 9 μm and the diameter of mesenteric veins from DOCA-salt rats was 228 ± 10 μm ($P > 0.05$).

Comparison of frequency response curves from sham and DOCA-salt arteries and veins

Transmural stimulation caused frequency-dependent constrictions of mesenteric arteries and veins (Fig. 3.1). There were no differences between the frequency-response curves obtained in sham and DOCA-salt veins as the mean E_{max} and S_{50} values obtained in these tissues were similar (Table 3.1). However, frequency-response curves from sham and DOCA-salt veins were both shifted to the left of the curves obtained from sham arteries (Fig. 3.1, Table 3.1). The E_{max} obtained in DOCA-salt arteries was significantly greater than that occurring in sham arteries and there were no differences between the frequency

response curves obtained in veins and those obtained in DOCA-salt arteries (Fig. 3.1, Table 3.1).

Increased adrenergic contribution to neurogenic constrictions in DOCA-salt arteries

To test for contributions of ATP and NE to neurogenic constrictions in mesenteric blood vessels, frequency-response curves in sham and DOCA-salt arteries were constructed in the absence and presence of prazosin (0.1 μM), an α1- adrenergic receptor antagonist and PPADS (10 μM), a P2 receptor antagonist. Prazosin blocked constrictions caused by exogenously applied NE (10 μM, the response was 49 ± 5% before prazosin treatment and 0 ± 0% after prazosin treatment, n=8), but it did not change the frequency-response curves in sham arteries (Fig. 3.2A). The E_{max} was 19 ± 5% before prazosin treatment and 14 ± 2% after prazosin treatment (n=7, $P > 0.05$); the S_{50} value was 11 ± 1 Hz before prazosin treatment and 9 ± 1 Hz after prazosin (n=7, $P > 0.05$). Subsequent addition of PPADS completely blocked the neurogenic responses in sham arteries (Fig. 3.2A). PPADS alone greatly reduced neurogenic constrictions in sham arteries and therefore it was not possible to calculate E_{max} and S_{50} values reliably. However, before PPADS, 5, 10 and 20 Hz stimulation caused constrictions of 8 ± 2%, 20 ± 6% and 26 ± 4% respectively. After PPADS treatment these values were 0.7 ± 0.7%, 4 ± 2% and 9 ± 5% (n=5; $P < 0.05$ at each stimulation frequency).

Prazosin markedly inhibited neurogenic responses in DOCA-salt arteries (Fig. 3.2B). In these studies, the E_{max} was 25 ± 2% before prazosin treatment and 8 ± 2% after prazosin treatment (n=6, $P < 0.05$); the S_{50} was 8 ± 1 Hz before prazosin treatment and 14 ± 4 Hz after prazosin treatment (n=6, $P > 0.05$). The response remaining in the presence of prazosin was blocked by combined application of PPADS and prazosin (Fig. 3.2B). PPADS alone had little effect on responses in DOCA-salt arteries. In these studies, the control E_{max} was 34 ± 5% before PPADS treatment and 22 ± 6% after PPADS treatment (n=6, $P > 0.05$); the S_{50} was 7 ± 2 Hz before PPADS treatment and 8 ± 4 Hz after PPADS treatment (n=6, $P > 0.05$). PPADS blocked P2 receptors in DOCA-salt arteries as constrictions caused by ATP (0.1 mM) were blocked by PPADS. The control ATP constriction was 43 ± 9% before PPADS treatment and 13 ± 3% after PPADS treatment ($P < 0.05$). These data demonstrate that there was an increase in the adrenergic contribution to neurogenic constrictions in DOCA-salt arteries compared to that occurring in sham arteries.

When the frequency-response curves for nerve stimulation in the presence of prazosin from sham and DOCA-salt arteries were compared, the maximum response and half maximal frequency were found not to be different ($p>0.05$). This result suggests that there is no change of purinergic component in arterioconstriction in DOCA-salt arteries.

Neurogenic constrictions are blocked by prazosin in sham and DOCA-salt veins

Similar studies to those described above were done in sham and DOCA-salt veins and it was found that prazosin completely blocked neurogenic constrictions in both types of tissues (Fig. 3.3).

Reactivity to α1-adrenergic and P2 receptor stimulation is not altered in DOCA-salt arteries

The increased adrenergic component to the neurogenic response in DOCA-salt arteries could be due to an increase in NE release or an increase in reactivity of DOCA-salt arteries to the stimulatory effects of nerve-released NE. To address this issue, concentration-response curves for NE, the α1-adrenergic receptor agonist, PE, and the α2-adrenergic receptor agonist, UK 14,304, were obtained in arteries from sham and DOCA-salt rats. NE and PE were approximately equipotent in contracting sham and DOCA-salt arteries and the E_{max} values were also similar in these tissues (Fig 3.4, Table 3.2). UK 14,304 (0.1-10 μM) did not contract sham or DOCA-salt arteries (Fig 3.5). Furthermore, there was no difference between concentration-response curves for ATP obtained in sham and DOCA-salt arteries. The ATP Emax value was 57 ± 4% in sham arteries (n=11) and this value was 47± 5% in DOCA-salt arteries (n=8, P>0.05). The ATP EC_{50} was 0.1± 0.02 μM in sham arteries and 0.2 ± 0.08 μM in DOCA-salt arteries (P>0.05). Taken together, these data indicate that α1-adrenergic receptors mediate adrenergic vasoconstrictions in sham and DOCA-

salt mesenteric arteries and that there is no change in reactivity of DOCA-salt arteries to α1-adrenergic or P2 receptor stimulation.

Inhibition of catecholamine uptake potentiates responses in DOCA-salt veins

Cocaine (10 μM) and corticosterone (10 μM) treatment prolonged the duration of the constriction caused by 5 and 10 Hz stimulation in DOCA-salt, but not sham veins (Table 3.3). These frequencies were chosen for study as they caused submaximal and maximum responses in sham and DOCA-salt veins.

Table 3.1: Maximum response (Emax) and half maximum stimulation frequency (S_{50}) in arteries and veins from sham and DOCA-salt rats. All data are expressed as the mean ± S.E.M and "n" values refer to the number of animals from which the data were obtained. $^{\Psi}$indicates significantly different from the S_{50} in sham and DOCA-salt veins ($P < 0.05$). $^{\#}$indicates significantly different from the Emax in DOCA-salt arteries and DOCA-salt veins ($P<0.05$).

	Artery		Vein	
	Sham (n=14)	DOCA-salt (n=13)	Sham (n=22)	DOCA-salt (n=24)
S_{50} (Hz)	$9.6 \pm 1.2^{\Psi}$	7.4 ± 0.9	4.9 ± 0.9	5.4 ± 0.7
Emax (%)	$21.9 \pm 3.1^{\#}$	31.3 ± 3.3	27.7 ± 3.1	36.0 ± 3.4

Table 3.2: Emax and EC_{50} values of the concentration-response curves for NE and PE in arteries from sham and DOCA-salt rats. All data are expressed as the mean ± S.E.M and "n" values refer to the number of animals from which the data were obtained. There were no difference in Emax and EC_{50} between sham and DOCA-salt arteries.

	Artery	
NE	Sham (n=9)	DOCA-salt (n = 10)
EC_{50} (μM)	5.4 ± 2.5	4.7 ± 1.0
Emax (%)	52 ± 3.8	57 ± 4.0
PE	Sham(n = 6)	DOCA-salt(n = 6)
EC_{50} (μM)	1.5 ± 0.8	3.2 ± 1.1
Emax (%)	47± 1.7	45± 3.5

Table 3.3: Duration (seconds) of neurogenic responses caused by 5 and 10 Hz stimulation of veins in the absence (Control) and presence of cocaine (10 μM) and corticosterone (10 μM). All data are expressed as the mean ± S.E.M and "n" values refer to the number of animals from which the data were obtained. *indicates significantly different from the duration in control sham and DOCA-salt veins at the same stimulation frequencies ($P < 0.05$).

	Sham (n=3)		DOCA-salt (n=4)	
Stimulation Frequency	Control	Cocaine/ corticosterone	Control	Cocaine/ corticosterone
5 Hz	60 ± 9	51 ± 17	120 ± 51	271 ± 36*
10 Hz	58 ± 10	68 ± 20	73 ± 7	288 ± 17*

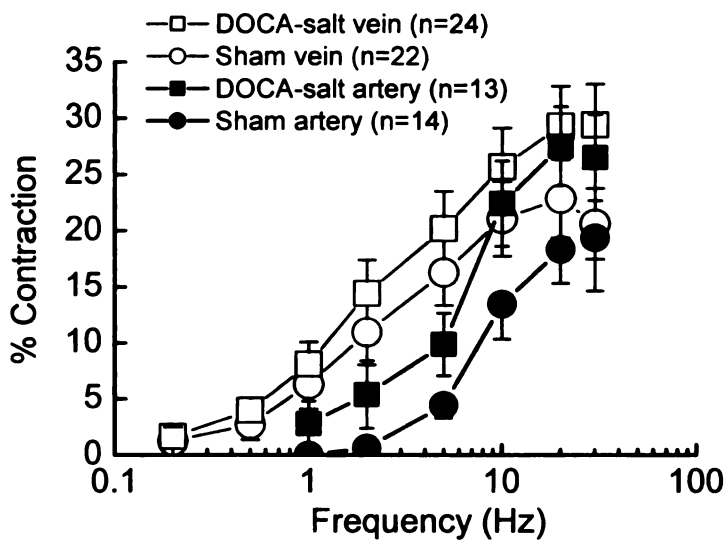

Fig. 3.1: Comparison of frequency response curves for neurogenic constrictions of arteries and veins from sham and DOCA-salt rats. The frequency-response curves from sham and DOCA-salt veins were shifted significantly to the left of those obtained from sham arteries. There was no difference between the frequency-response curves from DOCA-salt arteries, DOCA-salt veins and sham veins.

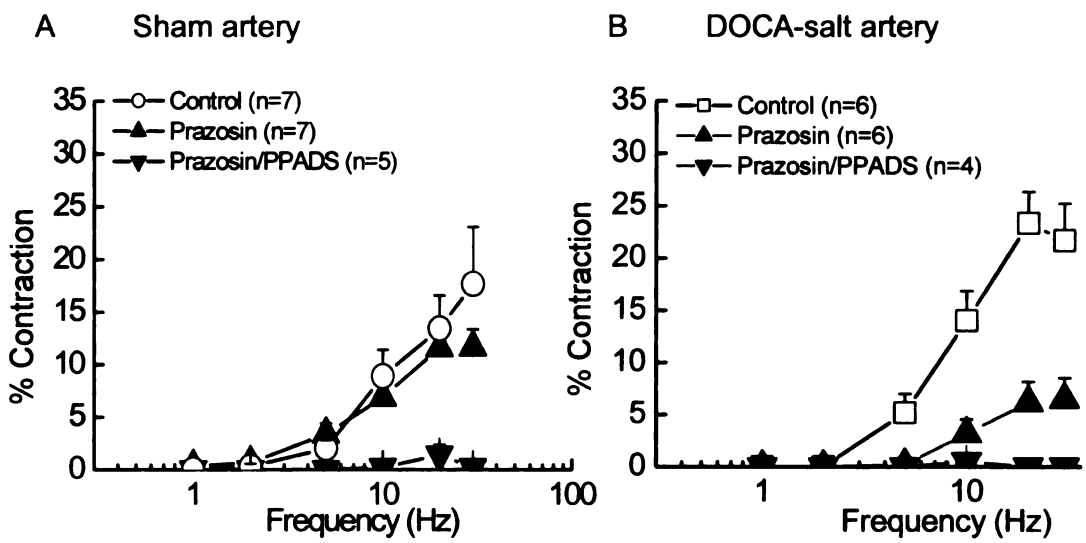

Fig. 3.2: Contributions of α1-adrenergic and P2 receptor-mediated components to neurogenic constrictions of arteries from sham and DOCA-salt rats. (A) Frequency-response curves before and after sequential application of prazosin and then PPADS in sham arteries. Prazosin alone had no effect while PPADS greatly reduced constrictions in sham arteries. (B) Frequency-response curves before and after sequential application of prazosin and them PPADS in DOCA-salt arteries. Prazosin alone greatly reduced constrictions in DOCA-salt arteries while combined application of prazosin and PPADS completely blocked the neurogenic responses.

Sham and DOCA-salt veins

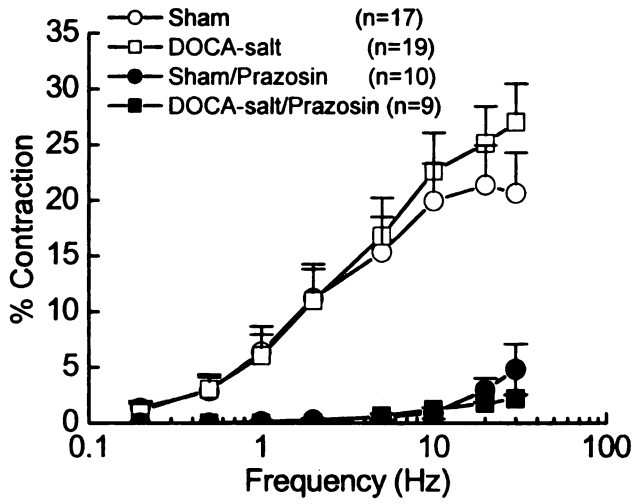

Fig. 3.3: Frequency-response curves for neurogenic constrictions in sham and DOCA-salt veins before and after prazosin (0.1 μM) treatment. Prazosin blocked the constriction caused by nerve stimulation in both sham and DOCA-salt veins.

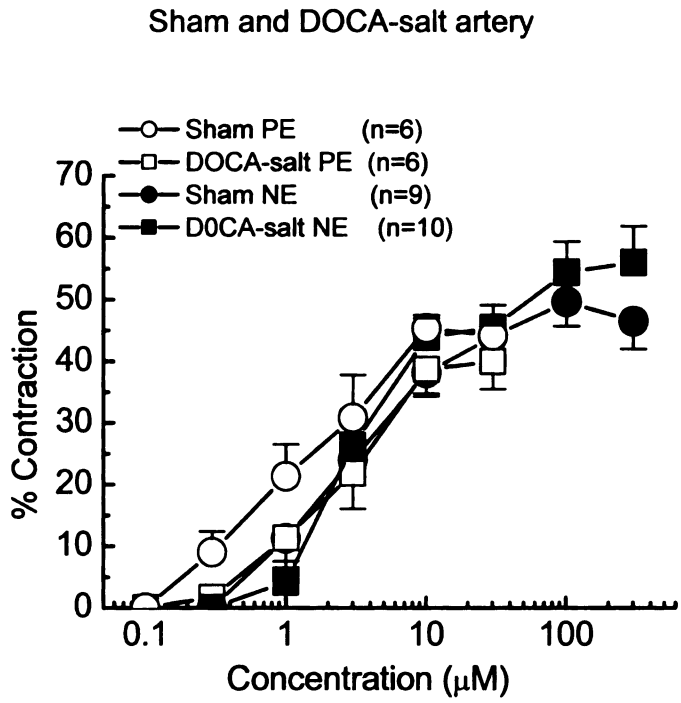

Fig. 3.4: Comparison of constrictions caused by adrenergic receptor agonists in arteries from sham and DOCA-salt rats. Concentration-response curves for NE from sham and DOCA-salt arteries were not different. Concentration-response curves for PE were similar in arteries from DOCA-salt and sham rats.

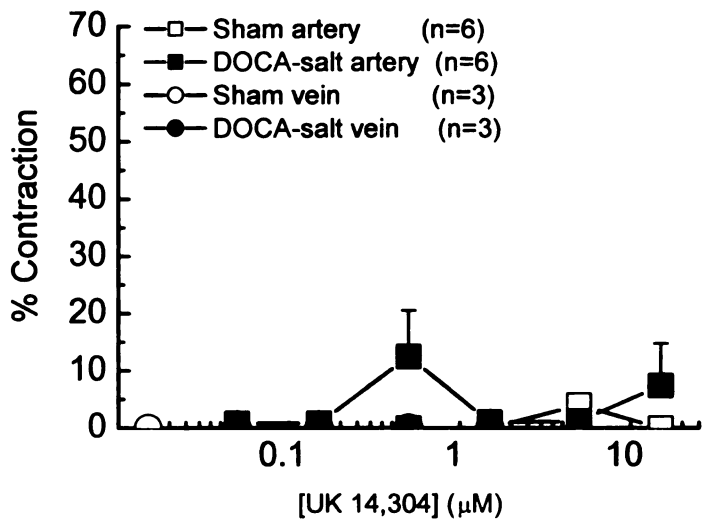

Fig. 3.5: Concentration-response curves for UK 14,304 in arteries from sham and DOCA-salt rats. UK14, 304 did not contract mesenteric arteries, suggesting that α_2 adrenergic receptors are not expressed by mesenteric blood vessels.

Discussion

Neurogenic responses in mesenteric arteries

NE and ATP are co-transmitters released by sympathetic nerves associated with most arteries (Kennedy, 1996). Studies by Gitterman and Evans (2001) showed that the P2 receptor antagonist, suramin, inhibits (60–80%) neurogenic constrictions of mesenteric arteries (< 300 μm diameter) from normotensive rats indicating that ATP is the dominant vasoconstrictor transmitter in these vessels. This result was confirmed in the present study where it was shown that the neurogenic responses of sham mesenteric arteries (≤ 300 μm) were greatly reduced by PPADS, a P2 receptor antagonist. My study also showed that prazosin did not alter frequency-response curves from sham arteries. However, co-application of prazosin and PPADS completely blocked neurogenic responses in sham arteries. Therefore, NE makes only a minor contribution to sympathetic neuroeffector transmission to small mesenteric arteries in normotensive rats. Conversely, neurogenic constriction of small mesenteric arteries from DOCA-salt rats were greatly reduced by prazosin indicating that NE makes a major contribution to sympathetic neuroeffector mechanisms in small mesenteric arteries from DOCA-salt hypertensive rats. This is a novel finding and indicates that DOCA-salt hypertension is associated with a change in neuroeffector mechanisms in small mesenteric arteries.

In DOCA-salt arteries, the E_{max} caused by nerve stimulation was greater than that occurring in sham arteries. This result suggests that sympathetic neuroeffector mechanisms was potentiated in DOCA-salt arteries, which could be due to increased transmitter release (a prejunctional mechanism) or increased sensitivity of vascular smooth muscle to sympathetic neurotransmitters (a postjunctional mechanism). My data show that concentration-response curves for NE in DOCA-salt arteries were not different from those obtained in sham arteries. This result differs from previously published work that showed increased α1-adrenergic receptor reactivity of arteries from DOCA-salt rats (Perry and Webb, 1988; Longhurst et al., 1989; Suzuki et al., 1994). Differences in the methods used to assess vascular reactivity could account for the difference in results obtained in my study and by these other investigators. Longhurst et al. (1988) and Suzuki et al. (1994) studied changes in perfusion pressure of the entire mesenteric vascular bed, whereas Perry and Webb (1988) measured agonist-induced increases in isometric force of arterial strips. I measured diameter changes in unpressurized blood vessels. However, the discrepancy between the data from previous work and my own data may also be due to differences in the size of the mesenteric arteries studied. Perry and Webb (1988) studied large mesenteric arteries (1 mm o.d.) while Suzuki et al. (1994) studied the main branches of the superior mesenteric arterial trunk (~0.5 mm o.d.). Longhurst et al. (1988) studied perfusion pressure in the whole mesenteric vascular bed and changes perfusion pressure would be affected by changes in

the diameter of the larger mesenteric arteries. I studied small mesenteric resistance arteries (<200 μm diameter). Gitterman and Evans (2001) have shown that the relative proportion of purinergic and adrenergic contributions to sympathetically-mediated arterial constriction changes with the size of the mesenteric arteries. They showed that the adrenergic component increases and the purinergic component decreases as the diameter of the artery increases. NE is the principal vasoconstrictor in mesenteric arteries ≥500 μm in diameter while ATP is the predominant neurogenic constrictor in the small mesenteric arteries (Gitterman and Evans, 2001). My study also showed that in small (<200 μm diameter) mesenteric arteries, NE plays a minor role in sympathetic neuroeffector mechanisms in normotensive rats. Therefore, reactivity of the α1-adrenergic receptor in the smaller arteries may be unaffected by DOCA-salt hypertension because NE is not normally the predominant vasoconstrictor transmitter in these blood vessels. My data also show that concentration-response curves for ATP in DOCA-salt arteries were not different from those obtained in sham arteries (see also Galligan et al., 2001). Therefore, changes in sensitivity to nerve-released ATP or NE are unlikely to account for the increased neurogenic constriction in DOCA-salt arteries. It is also unlikely α2 adrenergic receptor upregulation in small mesenteric arteries accounts for the increased neurogenic constriction in DOCA-salt tissues. This conclusion is based on data showing that clonidine or UK 14,304 did not contract small mesenteric arteries from sham or DOCA-salt rats.

Previous studies showed that there is increased release of NE from sympathetic nerves in DOCA-salt hypertension (Tsuda et al., 1989; de Champlain, 1990). Although NE release was not measured directly in the present study, the reduced NE content in DOCA-salt arteries (Luo et al., 2003) suggests that there was increased release of NE from sympathetic nerves in DOCA-salt rats leading to store depletion. This result and conclusion is similar to that reported previously in studies of DOCA-salt rats (Crabb et al., 1980; Longhurst et al., 1989). Increased NE release could contribute to the increased neurogenic constrictions and also could stimulate increased NET expression in DOCA-salt arteries. Though the mechanism underlying this increased adrenergic neurotransmission remains unclear, dysfunction of α2-adrenergic autoreceptors in DOCA-salt hypertension (Tsuda et al., 1989; de Champlain, 1990), which under normal conditions inhibit NE release, might be a cause.

Neurogenic responses in mesenteric veins

This study is unique in that I examined sympathetic constrictions of veins in order to assess changes that might be associated with hypertension. The present study and previous work (Kreulen, 1986; Hottenstein and Kreulen, 1987) showed that veins are more sensitive than arteries to nerve stimulation as frequency-response curves in veins are to the left of those obtained in arteries. This conclusion was based on the observation that the S_{50} was lower in sham and DOCA-salt veins (~5 Hz) compared with sham arteries (~10 Hz). Studies on sympathetic nerve activity in rat tail artery and veins show that the dominant firing

frequencies are between 2 and 6 Hz (Johnson and Gilbey, 1998). Similarly, the frequency of synaptic activity of presumed vasoconstrictor neurons in rat superior cervical ganglia (SCG) is 3 Hz (McLachlan et al., 1997). Therefore, if the *in vivo* frequency of nerve activity in sympathetic nerves associated with mesenteric blood vessels is similar to that in tail vessels and in the SCG, then increases in sympathetic nerve activity would produce greater constrictions of veins than arteries. These data also suggest that increases in sympathetic nerve activity would increase venous tone before changing arterial tone. The splanchnic circulation holds one-third of total blood volume and this store resides largely in veins (Greenway, 1983). As the sympathetic nervous system is the principal mechanism for controlling venomotor tone (Monos et al., 1995), small increases in sympathetic nerve activity would increase venous return and cardiac stroke volume. Therefore, at low levels of sympathetic nerve activity, vascular-based increases in systemic blood pressure could be due, in part, to an increase in venomotor tone.

Neurogenic constrictions of veins from sham and DOCA-salt rats are mediated by NE acting at α1-adrenergic receptors as they are blocked by prazosin (Luo et al., 2003). Furthermore, NE-induced constrictions of veins are mimicked by PE, an α1-receptor selective agonist, but not by the α2-receptor selective agonists, clonidine or UK 14,304 (Luo et al., 2003). NE and PE concentration-response curves from DOCA-salt veins were shifted to the right of those obtained in sham veins (Luo et al., 2003). Similar decreases in NE sensitivity occur in hand veins taken from human hypertensive subjects (Sudhir

et al., 1990). Decreased NE sensitivity could be due to downregulation of α1-adrenergic receptors in DOCA-salt veins caused by the increased sympathetic nerve activity known to occur in DOCA-salt hypertension (De Champlain, 1990; Fink et al., 2000). However, I found that neurogenic responses were not different between sham and DOCA-salt veins. Maintenance of nerve-mediated responses in the presence of decreased postjunctional sensitivity could indicate that more NE is released from nerves in DOCA-salt veins. This hypothesis was supported by data showing that NE content was lower in DOCA-salt compared to sham veins (Luo et al., 2003) as increased NE release would lead to store depletion. In DOCA-salt veins, there was also an increase in NET activity as inhibition of NE uptake by combined application of cocaine and corticosterone caused a leftward shift in concentration-response curves to exogenous NE (Luo et al., 2003) and prolonged neurogenic constrictions in DOCA-salt, but not sham veins. There was also an increase in the amount of NET protein in DOCA-salt veins (Luo et al., 2003). A similar upregulation of NE uptake also occurs in SHRs (Zsoter and Wolchinsky, 1983) and in human hypertensive subjects (Aalkjaer et al., 1987). Taken together these data suggest that there is increased release of NE from sympathetic nerves in DOCA-salt veins, which results in lower levels of stored NE and increased NET levels. Direct measurements of NE release *in vivo* and *in vitro* will be required to substantiate this suggestion.

Summary and conclusions

In summary, NE mediates sympathetic neuroeffector mechanisms by acting at α1-adrenergic receptors in mesenteric veins while ATP, acting at P2X receptors, is the dominant vasoconstrictor transmitter in small mesenteric arteries from normotensive rats. This result supports the proposal that there is differential sympathetic neural control of arteries and veins (Dehal et al., 1992; Browning et al., 1999). In DOCA-salt rats, neurogenic responses in mesenteric arteries are potentiated due to increased adrenergic neurotranmission. In mesenteric veins from DOCA-salt rats there is no change in neurogenic responses, but there is a decrease in responses to exogenous NE due to an increase in NET levels in DOCA-salt veins (Luo et al., 2003). Therefore, it is likely that NE release from perivascular sympathetic nerves in DOCA-salt hypertension is increased, which stimulates an upregulation of NET levels in blood vessels (Luo et al., 2003). The increased venomotor tone known to occur in DOCA-salt rats *in vivo* (Fink et al., 2000) must be due to factors other than an intrinsic increase in reactivity of mesenteric veins to neurally-released NE. Locally acting or circulating substances are likely to interact with sympathetic nerves in the control of venomotor tone in DOCA-salt hypertension.

Chapter 4

Impaired function of α2 adrenergic autoreceptors on sympathetic nerves associated with mesenteric arteries and veins in DOCA-salt hypertension

Min Luo, Gregory D. Fink, Keith J. Lookingland, John A. Morris, James J. Galligan

Department of Pharmacology & Toxicology and the Neuroscience Program

Michigan State University, East Lansing, MI 48824 USA

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Introduction

There is increased activity of the sympathetic nervous system in DOCA-salt hypertension (De Champlain, 1990; Schenk and McNeill, 1992). Evidence supporting this conclusion includes: the presence of increased circulating catecholamine levels in DOCA-salt hypertensive rats (De Champlain, 1990; Foucart S., et al., 1987), greater decreases in arterial blood pressure caused by ganglionic blockade with hexamethonium in DOCA-salt rats compared to sham normotensive rats (Fink et al., 2000); and normalization of blood pressure in DOCA-salt hypertensive rats following central catecholamine depletion after 6-hydroxydopamine treatment (Lamprecht et al., 1977).

Increased sympathetic nerve activity in DOCA-salt hypertension suggests that there may be alterations in the local mechanisms that modulate sympathetic neuroeffector mechanisms (Schenk and McNeill, 1992; Westfall et al., 1986). α2 adrenergic autoreceptors located on sympathetic nerve terminals mediate feedback inhibition of NE release (Langer, 1980). Data from several studies indicate that prejunctional α2-adrenergic receptor function is impaired in hypertensive animals. For example, intravenously administered yohimbine, an α2 adrenergic receptor antagonist, increased plasma NE levels in sham normotensive, but not in DOCA-salt hypertensive rats (De Champlain, 1990; Moreau et al., 1995). Furthermore, yohimbine potentiated nerve stimulation-evoked NE release into the mesenteric vasculature in normotensive rats, but not in DOCA-salt hypertensive rats (Tsuda et al., 1989). As nerve stimulation would

activate periarterial and perivenous nerves, the source of NE in these studies was unclear.

ATP and NE are co-transmitters released by sympathetic nerves associated with arteries (Bobalova and Mutafova-Yambolieva, 2001a; Bobalova and Mutafova-Yambolieva, 2001 b; Burnstock et al., 1985; Gitterman and Evans, 2001). In normotensive rats, ATP is the dominant neurotransmitter in small mesenteric arteries (Fink et al., 2000; Luo et al., 2003); however, in arteries from DOCA-salt hypertensive rats NE is the major sympathetic neurotransmitter (Luo et al., 2003). This increased adrenergic component in sympathetic neuroeffector transmission to DOCA-salt arteries is not likely to be due to an increase of postjunctional reactivity to NE as the dose-response curves for exogenous NE and phenylepherine were not different between sham and DOCA-salt arteries (Luo et al., 2003). Increased adrenergic transmission is, therefore, likely due to increased NE release. In addition, it was shown that sympathetic arterioconstriction was increased in DOCA-salt arteries (Luo et al., 2003). This potentiation appears to be attributable to increased NE release as there was no change in postjunctional reactivity of DOCA-salt arteries to exogenous NE and ATP (Luo et al., 2003). Whether ATP release is altered in DOCA-salt arteries is yet to be determined.

Sympathetic nerves exert primary control over venomotor tone (Langer, 1980) and NE is the neurotransmitter mediating neurogenic constrictions of mesenteric veins from sham and DOCA-salt rats (Luo et al., 2003). Prejunctional α2-adrenergic receptors associated with sympathetic nerves also regulate NE

release in veins (Bobalova and Mutafova-Yambolieva, 2001b; Foucart et al., 1987). The few studies of the function of α2-adrenergic receptors associated with veins in hypertension, however, have yielded controversial results. Increased NE release and attenuated yohimbine effect on NE release occur in the portal vein of SHRs. However, in the same study it was shown that in portal veins from DOCA-salt hypertensive and 1K1C hypertensive rats there was not an increase in NE release or an attenuation of the yohimbine effect on NE release (Westfall et al., 1986). As the portal vein makes only a small contribution to total vascular capacitance, this blood vessel may not be an appropriate model to study hypertension associated with changes in the function of peri-venous sympathetic nerves.

Veins are more sensitive to the vasoconstrictor effect of sympathetic nerve stimulation than arteries as frequency-response curves caused by nerve stimulation obtained from veins are to the left of those from arteries (Hottenstein and Kreulen, 1987; Kreulen 1986; Luo et al., 2003). Accordingly, small increases in sympathetic nerve activity would increase venous tone before changing arterial tone. As 25% of the blood volume is in the splanchnic circulation, increases in mesenteric venomotor tone will lead to an increase in venous return and cardiac output. These changes will have a profound impact on overall hemodynamics (Greenway, 1983). Therefore, mesenteric veins are an appropriate system to study hypertension-associated changes in sympathetic nerve function.

Our previous studies showed that there is maintained *in vitro* neurogenic venoconstriction in the presence of decreased postjunctional reactivity to

exogenously applied NE. These data indicate that there must be increased NE release in mesenteric veins from DOCA-salt rats (Luo et al, 2003). In addition, the steady state stores of NE in DOCA-salt arteries and veins were reduced, presumably due to increased NE release in DOCA-salt rats (Luo et al., 2003). Clearly, direct measurements of NE release in mesenteric arteries and veins are needed to substantiate these suggestions.

The present study examined changes in sympathetic neurotransmitter release in DOCA-salt hypertension and the function of α2-adrenergic receptors in regulating ATP and NE release from periarterial nerves, and NE release from perivenous nerves. I also assessed changes in α2-adrenergic receptor function in DOCA-salt hypertension.

METHODS

Animals

See Chapter 3 Methods.

Preparation of DOCA-salt rats

See Chapter 3 Methods.

Tissue preparation

Rats were killed using a lethal sodium pentobarbital injection (50 mg, i.p). The intestine was removed and placed in oxygenated (95% O_2, 5% CO_2) Krebs' solution of the following composition (mM): NaCl (117); KCl (4.7); $CaCl_2$ (2.5); $MgCl_2$ (1.2); $NaHCO_3$ (25); NaH_2PO_4 (1.2); glucose (11) in a silicone elastomer-lined petri dish. The mesenteric bed was stretched gently and pinned flat. Mesenteric arteries or veins from about 75% of the mesentery bed were dissected out by carefully cutting away surrounding adipose and connective tissues. Blood vessels were transferred to a small silicone elastomer-lined recording bath (1.0 ml volume). The recording bath containing the preparation was superfused continuously with warm (36 ^{o}C) Krebs' solution at a flow rate of 7-8 ml/min. The preparation was equilibrated for 1 hour before beginning experiments.

Drug application

Drugs were applied using a system of 3-way stopcocks so that the superfusing Krebs' solution could be changed to one containing a known concentration of drug. It took one minute for drugs to reach the tissue. Agonists or antagonists were applied for 30 minutes prior to testing their effects on transmitter release.

Transmural stimulation of perivascular nerves

Two parallel silver/silver chloride wire electrodes connected to a Grass Instruments stimulator (S88, Quincy, MA) were used for transmural stimulation (30 stimuli, stimulus duration 0.5 ms, frequency 10 Hz, voltage 120-140 volts). We showed previously that 10 Hz causes maximum constrictions in mesenteric arteries and veins (Luo et al., 2003). Two conditioning trains of stimulation were applied at 20-minute intervals for ATP measurement and one conditioning train of stimulation for NE measurement. Nerve-mediated ATP and NE release was stable following the conditioning trains of stimulation (Bobalova and Mutafova-Yambolieva, 2001a). The flow of the superfusing solution was stopped during nerve stimulation and the solution (1 ml) was quickly withdrawn from the bath by using a 1 ml syringe. Flow was restarted immediately after sample withdrawal. Superfusate was then processed for high performance liquid chromatography (HPLC) measurement of ATP and NE. Basal levels of ATP or NE were measured in superfusate samples collected before nerve stimulation.

Measurement of adenine nucleotides and adenosine by HPLC with fluorescence detection

Our measurements of ATP release include ATP and its metabolites: ADP, AMP and adenosine. Superfusate samples (500 μl) were analyzed using HPLC in conjunction with fluorescence detection (Levitt B, et al., 1984). Briefly, chloroacetaldehyde (25 μl) was added to superfusate samples, which were then heated for 40 min at 80 °C in an oven (Baxter, Scientific Products) to produce the fluorescent derivative 1, N^6-ethenopurine analogs. The derivatization reaction was stopped by cooling the samples on ice. Separation of derivatized ATP, ADP, AMP and adenosine was achieved using a C-18 column (Biophase ODS, 5 μm, 250 X 4.6 mm, Bioanalytical Systems) and a gradient system in which the concentration of mobile phase buffer B was increased from 0 to 100% in 10 min and maintained at 100% for 5 min followed by a decrease to 0% in 5 min. The column then was re-equilibrated with 100% mobile phase A for 10 min. Mobile phase A consisted of 0.1 mol/l KH_2PO_4 (pH = 6.0) and mobile phase B consisted of 0.1 mol/l KH_2PO_4 with 25% methanol (pH = 6.0). Fluorescence signals were measured using a programmable fluorescence detector (RF-10Axl, Shimadzu, Japan) at an excitation wavelength of 300 nm and emission wavelength of 420 nm. Fluorescence signals were integrated using Gilson 712 software (Gilson Medical Electronics, Middleton, WI). Individual adenine nucleotides and adenosine were identified by retention times established using known standards. The standard curves for each individual adenine nucleotide and adenosine were constructed by using standards dissolved in Krebs' solution and plotted using

peak height as dependent variables and the standard as the independent variable. The amounts of individual adenine nucleotides and adenosine were then calculated using linear standard curves. Total ATP overflow was a summation of measured ATP plus its metabolites: ADP, AMP and adenosine.

Measurement of NE by HPLC with electrochemical detection

Aliquots of the collected superfusate (50 μl) were acidified with phosphoric acid (pH = 2.6), filtered through a 0.22 μM Cameo 3N syringe filter and injected into an isocratic HPLC system equipped with a C-18 reverse-phase analytical column (5-μm spheres, 250 x 4.6 nm, Biophase ODS, Bioanalytical Systems). The HPLC column was coupled to a single coulometric electrode-conditioning cell in series with dual-electrode analytical cells (ESA). The conditioning electrode potential was set at +0.4 V; the analytical electrodes were set at +0.12 V and -0.31 V, respectively, relative to the reference electrodes. The HPLC mobile phase consisted of 1.0 mol/L phosphate-citrate buffer, pH = 2.65, with 0.1 mmol/L EDTA, 0.0475% sodium octylsulfate, and 5% methanol. The amount of NE in the samples was determined by comparing peak heights (determined by a Hewlett Packard Integrator, Model 3393A) with those obtained from standards run on the same day.

Statistical analysis

All data are expressed as the mean ± S.E.M and "n" values refer to the number of animals from which the data were obtained. Differences between

groups were assessed by analysis of variance and Kramer Tukey multiple comparisons test or Students' t-test using GraphPad InStat version 3.0 for Windows 95 (GraphPad Software, San Diego CA). $P < 0.05$ is considered significant.

Drugs

All drugs were obtained from Sigma Chemical Company (St. Louis, MO).

RESULTS

Nerve-mediated ATP release was not changed in DOCA-salt compared with sham arteries

ATP release caused by consecutive periods of nerve stimulation was normalized to the basal release prior to nerve stimulation. Normalized ATP release increased at the third stimulation after two conditioning stimulations and there was a positive correlation between the basal release and stimulation-induced ATP release caused by the third stimulation (Table 4.1). Normalized ATP release caused by the third stimulation was not different from that caused by the fourth stimulation (Fig.4.1). The normalized release caused by the third stimulation was used as the release before drug treatment and that caused by the fourth period of stimulation was used to assess release after drug treatment. Basal ATP release prior to nerve stimulation and normalized ATP release were not different between sham and DOCA-salt arteries (Table 4.1). TTX blocked ATP release caused by nerve stimulation. The normalized release in sham arteries before TTX treatment was 2.9 ± 0.7 and after TTX treatment was 1.1 ± 0.09 ($n = 4$, $P < 0.05$). The normalized release before TTX treatment in DOCA-salt arteries was 4.7 ± 1.5 and after TTX treatment was 0.6 ± 0.1 ($n = 5$, $P < 0.05$).

Increased NE release in DOCA-salt arteries and veins

Nerve stimulation produced TTX-sensitive NE release in arteries and veins. NE release caused by consecutive nerve stimulation was normalized to the basal release prior to nerve stimulation. Normalized NE release was not different during the first, second and third stimulation (Fig.4.2). There was a positive correlation between the basal levels and the stimulation-induced NE release by the second stimulation in both arteries and veins (Table 4.2). Therefore, normalized release caused by the second stimulation was taken as the release before drug treatment and that caused by the third stimulation was used to assess release after drug treatment. The normalized release in sham arteries before TTX treatment was 1.6 ± 0.1 and after TTX treatment was 0.7 ± 0.1 ($n = 4$, $P < 0.05$). The normalized release before TTX treatment in DOCA-salt arteries was 4.1 ± 1.4 and after TTX treatment was 0.7 ± 0.2 ($n = 4$, $P < 0.05$). The normalized release in sham veins before TTX treatment was 4.6 ± 0.3 and after TTX treatment was 0.7 ± 0.3 ($n = 4$, $P < 0.05$). The normalized release before TTX treatment in DOCA-salt veins was 4.4 ± 0.7 and after TTX treatment was 1.3 ± 0.5 ($n = 4$, $P < 0.05$). Basal NE release was not different between sham and DOCA-salt arteries or between sham and DOCA-salt veins (Table 4.2). Normalized NE release caused by nerve stimulation was compared between arteries and veins from sham and DOCA-salt hypertensive rats. The normalized NE release was increased in both DOCA-salt arteries and veins compared with their sham counterparts (Table 4.2). In addition, normalized NE release in sham veins was greater than that in sham arteries (Table 4.2).

α2 adrenergic autoreceptor function is impaired in arteries from DOCA-salt rats

To determine whether α2 adrenergic autoreceptors regulate ATP release, the effects of the α2 adrenergic receptor antagonist, yohimbine (1 μM), and the agonist, UK 14,304 (10 μM), on ATP release in arteries from sham rats were studied. In sham arteries, yohimbine increased ATP release caused by nerve stimulation whereas UK 14,304 decreased ATP release caused by nerve stimulation (Fig. 4.3A). In DOCA-salt arteries, the normalized ATP release before yohimbine treatment was not different from the release after yohimbine treatment (Fig.4.3B). Similarly, ATP release before UK 14,304 treatment was not different from the release after UK 14,304 treatment in DOCA-salt arteries (Fig. 4.3B).

α2 adrenergic autoreceptors associated with mesenteric arteries regulate NE release and are impaired in DOCA-salt hypertensive rats

Yohimbine increased NE release caused by nerve stimulation in sham arteries (Fig. 4.4A), but it did not alter evoked NE release in DOCA-salt arteries (Fig. 4.4B).

α2 adrenergic autoreceptors are impaired in veins from DOCA-salt rats

We assessed the function of α2 adrenergic autoreceptors associated with veins by examining the effect of yohimbine (1μM) on NE release. NE release caused by nerve stimulation before and after yohimbine treatment was compared in sham and DOCA-salt rats. Yohimbine increased NE release in sham veins

(Fig. 4.5A), but failed to alter NE release in DOCA-salt veins (Fig. 4.5B). Conversely, UK 14,304, decreased NE release in sham (Fig. 4.5A), but not DOCA-salt veins (Fig.4.5B).

Table 4.1: ATP released is not altered in DOCA-salt arteries. Data are the basal ATP release, nerve stimulation-evoked release (stimulated release) and evoked release normalized to basal levels (normalized release) during the third stimulation period in sham and DOCA-salt arteries. *indicates significantly different from basal release ($P < 0.05$). There were no differences between basal release and normalized release between sham and DOCA-salt arteries. "r" is the correlation coefficient for the relationship between basal and stimulated release. Both correlation coefficients are different from zero ($P < 0.05$).

	Artery	
	Sham (n=18)	DOCA-salt (n=19)
Basal release (ng /ml)	2.7 ± 0.4	3.9 ± 1.0
Stimulated release (ng/ ml)	6.9 ± 1.0*	11.4 ± 2.4*
Normalized release (fold increase)	2.9 ± 0.3	4.1 ± 0.5
r	0.59	0.71

Table 4.2: NE release from peri-arterial and peri-venous nerves is increased in DOCA-salt hypertension. Data are basal NE release, nerve stimulation-evoked release (stimulated release), and evoked release normalized to basal levels (normalized release) in sham and DOCA-salt arteries & veins. * indicates significantly different from basal release ($P < 0.05$). # indicates significantly different from normalized release in sham counterparts ($P < 0.05$). ¶indicates significantly different from release in sham artery ($P<0.05$). "r" is the correlation coefficient for the relationship between basal and stimulated release. All correlation coefficients are different from zero ($P < 0.05$).

	Artery		Vein	
	Sham (n=17)	DOCA-salt (n=15)	Sham (n=16)	DOCA-salt (n=18)
Basal release (pg/ml)	1331 ± 243	2673 ± 931	611 ± 100¶	477± 83
Stimulated release (pg/ml)	1705 ± 284*	7148 ± 1799*	1764 ± 490*	4624 ± 1850*
Normalized release (fold increase)	1.5 ± 0.2	3.9 ± 0.7#	2.9 ± 0.5¶	8.4 ± 2.8#
r	0.90	0.90	0.70	0.82

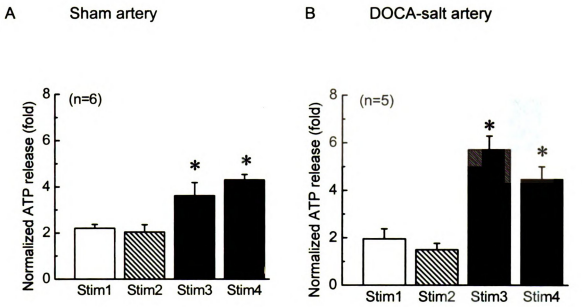

Fig.4.1: Stimulation-dependent increase in ATP release from sham and DOCA-salt arteries. ATP release caused by consecutive stimulations was normalized to basal levels in arteries. Bars labeled Stim1, Stim 2, Stim 3, and Stim 4 represent release caused by the first, second, third and fourth stimulation periods respectively. In sham (A) and DOCA-salt arteries (B), Stim1 and Stim2 were not different from each other and Stim3 and Stim4 were not different from each other. *indicates significantly different from Stim2 ($P < 0.05$). Data are presented as mean + SE and were analyzed by one-way analysis of variance with Tukey-Kramer multiple comparisons test.

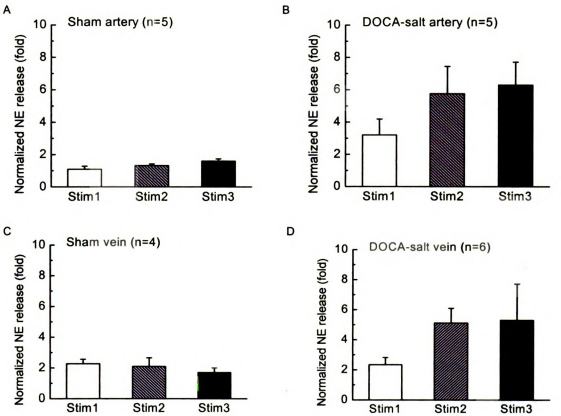

Fig. 4.2: NE release was stable during consecutive periods of nerve stimulation. NE release caused by consecutive stimulations was normalized to basal levels in sham arteries (A), DOCA-salt arteries (B), sham veins (C) and DOCA-salt veins (D). Bars labeled Stim1, Stim 2, and Stim 3 represent release caused by the first, second, and third periods of nerve stimulation, respectively. Stim1, Stim2, and Stim3 were not different from each other in any preparation. Data are mean + SE and were analyzed by one-way analysis of variance and Tukey-Kramer multiple comparisons test.

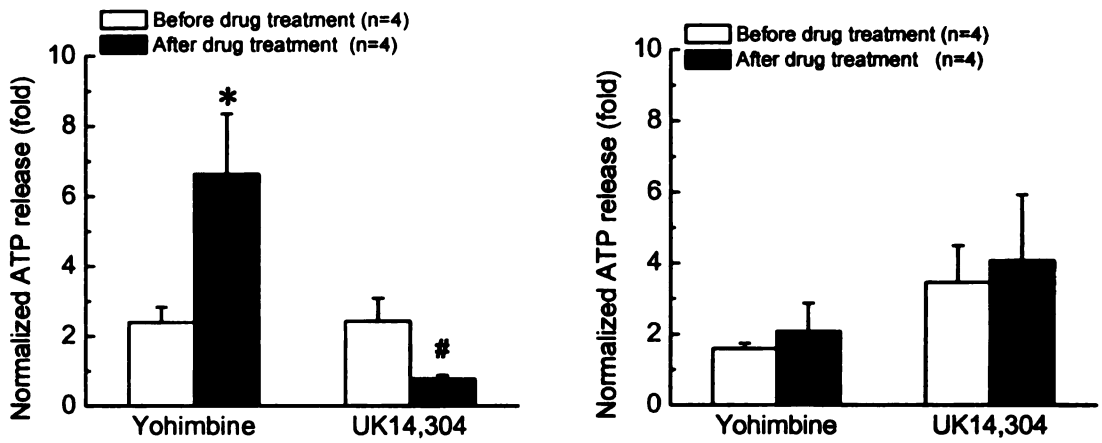

Fig. 4.3: α2-adrenergic receptors modulate ATP release in sham, but not DOCA-salt arteries. A: ATP release caused by nerve stimulation is compared before and after yohimbine or UK 14,304 treatment in sham arteries. *indicates significantly different from release before yohimbine treatment. #indicates significantly different from release before UK 14,304 treatment. B: ATP release caused by nerve stimulation is compared before and after yohimbine or UK 14,304 treatment in DOCA-salt arteries. Data are presented as mean + SE.

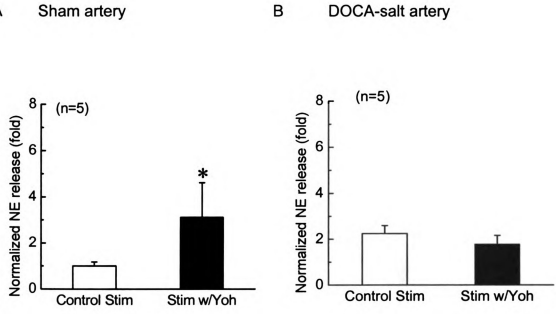

Fig. 4.4A: NE release caused by nerve stimulation is potentiated by yohimbine treatment in sham arteries. *indicates significantly different from release before yohimbine treatment. B: NE release caused by nerve stimulation was not altered by yohimbine treatment in DOCA-salt arteries. Data are presented as mean + SE.

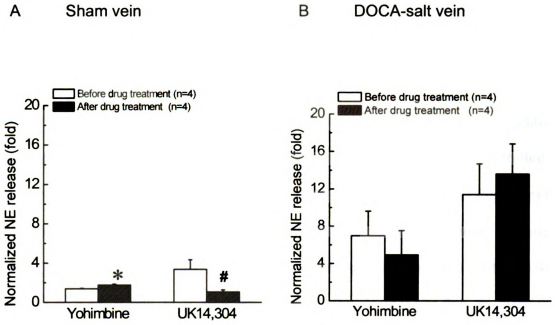

Fig. 4.5A: NE release caused by nerve stimulation is increased by yohimbine and decreased by UK 14,304 in sham veins. *indicates significantly different from release before yohimbine treatment. #indicates significantly different from release before UK 14,304 treatment. B: NE release caused by nerve stimulation was not altered by yohimbine or UK 14,304 treatment in DOCA-salt veins. Data are mean + SE.

DISCUSSION

Increased sympathetic tone in veins in hypertension

In human hypertension and in SHRs, sympathetic neural input to veins is increased (Willems et al., 1982). Furthermore, the membrane potential of venous smooth muscle cells in SHRs is depolarized compared to that found in normotensive rats, a change that is due to increased sympathetic tone to veins in SHRs (Willems et al., 1982). It has also been shown that venomotor tone is elevated in DOCA-salt hypertensive rats and this is largely due to increased sympathetic input to veins (Fink et al., 2000), which will cause decreased venous compliance. In hypertension, decreased venous compliance occurs in peripheral vessels and this change is most prominent in splanchnic veins causing redistribution of blood toward the heart, leading to increased cardiac output (Moreau et al., 1995). The shift of blood from the venous side of the circulation to the arterial side will result in increased arterial blood pressure (Nyhof et al., 1983). There are a number of changes that could account for increased sympathetic tone to veins in hypertension. My study is the first to directly measure NE release caused by nerve stimulation in mesenteric veins from hypertensive rats. I showed that more NE is released during nerve stimulation in DOCA-salt veins than in sham veins. This result provides one potential mechanism underlying the increased venomotor tone found in DOCA-salt hypertensive rats (Fink et al., 2000).

We normalized nerve stimulation-induced ATP and NE release to their basal levels to minimize differences in release that might be due to hypertension-induced structural changes in blood vessels. For example, hypertrophy of the smooth muscle of arteries occurs in DOCA-salt hypertension (Schenk and McNeill, 1992). Therefore, it would be problematic for interpretation of the results to normalize the stimulation-induced release to tissue weight or total protein. The correlation between basal release and nerve stimulation-induced ATP and NE release suggests that the basal release of ATP and NE is from nerve terminals. This result also justifies normalization of stimulation-induced release to that occurring under basal conditions. Unaltered basal levels of ATP and NE in arteries and veins from DOCA-salt hypertensive rats suggest that the overall number of nerve terminals associated with mesenteric arteries and veins is not changed in DOCA-salt rats. Therefore, the increased release most likely results from the increased transmitter release per nerve terminal rather than from hyperinnervation of those blood vessels. Upregulation in NE release from nerve terminals in DOCA-salt rats differs from changes known to occur in SHRs where it has been shown that there is an increased density of sympathetic nerve fibers in the vasculature (Cassis et al., 1985; Tabei et al., 1995).

There is more NE release from peri-venous nerves per stimulus compared to peri-arterial nerves as NE release caused by nerve stimulation normalized to basal levels is greater in veins than in arteries from sham rats. This result could explain in part why veins are more sensitive to the contractile effects of sympathetic nerve stimulation than arteries (Hottenstein and Kreulen,

1987; Luo et al., 2003). However, postjunctional factors could also contribute to increased venous sensitivity to sympathetic neural input. For example, venous smooth muscle cells have a less negative resting membrane potential compared with the arterial smooth muscle cells (Hottenstein and Kreulen, 1987).

The greater increase of NE release in veins compared to arteries during nerve stimulation may be related to the different functional properties of sympathetic nerves innervating arteries and veins (Browning, et al., 1999). The notion that sympathetic nerves innervating arteries and veins is different was based on the following evidence: 1) in arteries, stimulation of sympathetic nerves elicits excitatory junction potentials mediated by ATP followed by a slow depolarization mediated by NE whereas in veins only slow depolarizations mediated by NE occur (Evans et al., 1992; Hottenstein and Kreulen, 1987); 2) sympathetic neurons innervating mesenteric arteries and veins differ in their localization in prevertebral ganglia and in their electrophysiological properties (Browning et al., 1999). My data showing a greater increase of NE release during sympathetic nerve stimulation in veins compared with arteries provides another important example of the difference in the functional properties of sympathetic nerves supplying arteries and veins.

α2 adrenergic autoreceptors on peri-venous nerves are impaired in DOCA-salt rats

Yohimbine increased NE release in sham, but not DOCA-salt veins, whereas UK 14,304 decreased NE release in sham, but not DOCA-salt veins.

These results suggest that the function of prejunctional α2-adrenergic receptors associated with mesenteric veins is impaired in DOCA-salt hypertensive rats. This differs from previous data demonstrating integrity of the autoinbihitory function of α2-adrenergic receptors in portal veins from DOCA-salt rats (Westfall et al., 1986). This difference in results could be attributed to the difference in vascular beds studied. Although the portal vein is linked to the mesenteric circulation, it serves a much smaller capacitance function compared with mesenteric veins and is less likely to be affected by hypertension.

α2 adrenergic autoreceptors on peri-arterial nerves regulate ATP release and are impaired in DOCA-salt rats

Nerve stimulation with the parameters used in the present study produces vasoconstriction that can be blocked by TTX (Luo et al., 2003) and ATP release measured here was TTX-sensitive. Therefore, I concluded that ATP release caused by electrical stimulation is nerve-mediated. ATP is a co-transmitter released with NE in the vascular system (Bobalova et al., 2001a; Bobalova et al., 2001b; Burnstock et al., 1985; Gitterman and Evans, 2001), particularly in small mesenteric arteries where it plays a major role in mediating sympathetic neurotransmssion (Fink et al., 2000; Luo et al., 2003). My study showed that α2-adrenergic receptors regulate ATP release as yohimbine increased, whereas UK 14,304 decreased, ATP release in sham arteries. These results are consistent with previous studies that showed that yohimbine (1 μM) increased the overflow of ATP caused by electrical field nerve stimulation (Bobalova et al., 2001b).

Taken together these data indicate that ATP and NE release, at least that caused by 10 Hz stimulation, are regulated in a similar manner.

My study is the first to directly measure nerve stimulation-induced ATP release from sympathetic nerves in DOCA-salt hypertensive rats. My study showed that ATP release is not altered in mesenteric arteries from DOCA-salt rats. This result is consistent with studies on ATP release from sympathetic nerves in the isolated kidney from SHRs where it was shown that ATP release was not altered compared to that occurring in Wistar Kyoto rats (Bohmann et al., 1995). In DOCA-salt arteries, α2 adrenergic receptor function is impaired as yohimbine or UK 14,304 did not change ATP release, but this does not result in an increase in ATP release. It is possible that changes in other mechanisms regulating ATP release may compensate for impaired function of α2-adrenergic receptors in hypertension (Cheung, 1989). For example, it has been shown that the inhibitory effects of prejunctional prostaglandin E2 receptors on sympathetic neurotransmitter release are enhanced in SHRs (Rump et al., 1990).

I have shown previously that ATP mediates neurogenic constrictions of small mesenteric arteries from sham rats while both ATP and NE mediate neurogenic constrictions in arteries from DOCA-salt rats (Luo et al., 2003). It was concluded that the increased adrenergic component of neurogenic constriction is due to increased NE release rather than an increase in postjunctional reactivity as constrictor responses to NE or phenylepherine are not different between sham and DOCA-salt arteries (Luo et al., 2003). The present study showed directly that NE release caused by nerve stimulation was

increased in DOCA-salt arteries, further demonstrating an alteration of sympathetic neuronal function in DOCA-salt hypertension. Impaired function of prejunctional α2-adrenergic receptors is likely to account for increased NE release in DOCA-salt arteries as NE release was increased when α2-adrenergic receptors were blocked by yohimbine in sham arteries. This result is consistent with studies showing a failure of yohimbine to increase plasma NE level (De Champlain, 1990; Moreau et al., 1995) and nerve stimulation–induced NE release by yohimbine in the mesenteric vasculature of DOCA-salt rats (Langer, 1980; Tsuda et al., 1989).

Summary and Conclusion

There is a greater increase of nerve stimulation-induced NE release in mesenteric veins compared with arteries from sham rats, which indicates a greater release of NE per nerve terminal. This result provides further support for the hypothesis that sympathetic nerves with different functional properties innervate arteries and veins. NE release caused by sympathetic nerve stimulation is increased in mesenteric arteries and veins from DOCA-salt hypertensive rats although ATP release is not altered in arteries. Prejunctional α2-adrenergic receptors on sympathetic nerves regulate ATP release in sham arteries and NE release in sham arteries and veins. Function of these receptors is impaired in DOCA-salt arteries and veins, which would contribute to increased nerve stimulation-induced NE release and increased peripheral arterial and venomotor tone in DOCA-salt hypertension (Fink et al., 2000; Luo et al., 2003).

CHAPTER 5

Modulatory effects of ET receptor activation on sympathetic neuroeffector transmission to mesenteric arteries and veins in DOCA-salt hypertension

Introduction

ET-1 modulates the activity of the sympathetic nervous system and it is a potent vasoconstrictor (Rubanyi and Polokoff, 1994; Schiffrin, 2001). For example, ET-1 has modulatory effects on the responses to sympathetic nerve stimulation of various vascular systems including the rat mesenteric arteries (Tabuchi et al., 1990), rabbit saphenous arteries (Mutafova-Yambolieva and Radomirov, 1994) and dog mesenteric arteries (Zhang et al., 1996). There are three potential mechanisms by which ET-1 acting at its receptors can modulate sympathetic neuroeffector mechanisms. The first mechanism is prejunctional where ET-1 affects transmitter release. For example, ET-1 inhibits NE release from rat and dog mesenteric arteries (Tabuchi et al., 1990; Zhang et al., 1996). However, others showed that ET-1 could both inhibit and increase NE and ATP release in isolated rat-tail artery and the effect is dependent on the concentration of ET-1 (Mutafova-Yambolieva et al., 1998). Therefore, it remains controversial whether the effect mediated by ET-1 on transmitter release is inhibitory or facilitatory. The second mechanism is postjunctional where ET-1 changes the postjunctional reactivity of the blood vessels to stimulation by neurotransmitters. For example, ET-1, at subcontractile concentrations, potentiates contractile responses to NE in human coronary arteries (Yang et al., 1990c), dog mesenteric arteries (Zhang et al., 1996) and rat mesenteric arteries (Tabuchi et al., 1990). The third is a mixed prejunctional and postjunctional mechanism by which ET-1

can both alter transmitter release and postjunctional reactivity to the neurotransmitters (Tabuchi et al., 1990; Zhang et al., 1996).

There are two subtypes of receptors for ET-1 in the cardiovascular system. In arteries, ET_A receptors are expressed on vascular smooth muscle cells and ET_B receptors are expressed on endothelium. ET_A receptors mediate vasoconstriction by releasing IP_3 and mobilizing intracellular calcium. ET_B receptors in the endothelium mediate vasodilation by releasing NO and prostacyclin (Rubanyi and Polokoff, 1994). In veins and in the aorta ET_B receptors are also localized to smooth muscle cells where they mediate vasoconstriction (Moreland et al., 1992; Davenport et al., 1995; Johnson et al, 2000). This conclusion is supported by data showing that S6c, a selective ET_B receptor agonist, contracts mesenteric veins, but not arteries from rats, suggesting that functional ET_B receptors are expressed on smooth muscle cells of mesenteric veins, but not arteries (Johnson et al., 2002). In addition, ET-1 is a more potent constrictor of veins than arteries (Warner et al., 1990 and Leppaluoto et al., 1992; Johnson et al., 2002). This increased potency may be related to the differences in expression of ET receptor subtypes and receptor density (Johnson et al., 2002).

Modulation by ET-1 on sympathetic neuroeffector transmission to the vascular system changes in hypertension (Tabuchi et al., 1990). For example, in isolated mesenteric arteries from SHR and WKY rats, the enhancement of the responses to NE by a subpressor dose of ET-1 was attenuated in SHR compared to WKY rats. The inhibitory effect of ET-1 on NE release was also

attenuated in SHR compared to WKY rats (Tabuchi et al., 1990). A number of studies suggest that ET-1 plays an important role in the development of DOCA-salt hypertension. For example, there is overexpression of ET-1 in DOCA-salt hypertensive rats (Day et al., 1995; Yu et al., 2002). ET-1 production in cultured endothelial cells from DOCA-salt hypertensive rats was up-regulated (Takada et al., 1996). Furthermore, chronic treatment with a mixed ET_A/ET_B receptor antagonist decreased blood pressure to a normal level (Doucet et al., 1996). The elevated sympathetic nerve activity in DOCA-salt hypertension could be due to altered local modulation on sympathetic neuroeffector transmission to the vascular system (De Champlain, 1990). Therefore, the role played by ET-1 in the development of DOCA-salt hypertension could be due to its function as a potent vasoconstrictor as well as a modulator for the sympathetic nervous system. However, no study has been done to assess the potential alteration of ET's modulatory effects on sympathetic neuroeffector transmission to the vascular system in DOCA-salt hypertension. My previous study demonstrated that purinergic transmission dominates sympathetic neuroeffector mechanisms in sham arteries whereas in DOCA-salt arteries there is increased adrenergic transmission due to the increased NE release (Luo et al., 2003). It was also demonstrated that sympathetic nerve activity associated with veins from DOCA-salt rats is increased as indicated by increased NE release, which results in decreased postjunctional reactivity to NE (Luo et al., 2003). Whether altered modulation by ET-1 on sympathetic neuroeffector transmission to mesenteric arteries and veins contribute to those alterations is unknown.

The present study tested the hypothesis that ET-1 modulates sympathetic neuroeffector transmission to mesenteric arteries and veins and those modulatory effects by ET-1 are altered in DOCA-salt hypertension. The following studies were done to test the above hypothesis: 1) Measurement of the effect of exogenous ET-1 on sympathetic neuroeffector transmission to arteries and veins; nerve stimulation evoked constriction of arteries and veins were compared before and after application of an ET receptor agonist. 2) Assess postjunctional effects of ET-1. Responses of arteries and veins to exogenous ATP and NE were compared before and after the application of ET receptor agonist. 3) The postjunctional effect of ET-1 was subtracted from the overall effect in order to determine the magnitude of the prejunctional effects of ET-1. As a selective ET_A receptor agonist is not available, the effect mediated by ET_A receptor on sympathetic neuroeffector mechanisms can be indirectly measured assessed by comparing the effects of a by non-selective ET receptor agonist, ET-1, and the effects of the selective ET_B receptor agonist, S6c. The changes in the modulatory effects associated with DOCA-salt hypertension can be studied by comparing those effects on mesenteric arteries and veins from sham and DOCA-salt rats.

There are ET binding sites on cardiac nerve terminals (Davenport et al., 1989) and paravascular nerves (Power et al., 1989). However, those reports did not determine whether those nerves were sympathetic nerves. Nor is there any report with regard to the expression of ET receptors on sympathetic nerve terminals innervating mesenteric blood vessels. ET binding sites are widely

distributed on the vascular smooth muscle cells of various blood vessels including conductance and resistance arteries, arterioles and veins (Power et al., 1989; Davenport et al., 1989). However, it is not clear which receptor subtype is expressed on the vascular smooth muscle cells. Therefore, another goal of the present studies was to identify the location of ET receptors in the mesenteric vascular bed with regard to postjunctional or prejunctional location using immunohistochemical methods. Ultrastructural studies have shown that sympathetic nerve endings have close contact with smooth muscle cells of arteries and veins (Luff et al., 1987). Immunohistochemical studies have localized the NE synthesizing enzymes: TH in perivascular nerves associated with mesenteric veins and arteries (Furness and Marshall, 1974). Co-staining of ET receptors and TH, a marker for sympathetic nerves, would demonstrate if ET receptors are localized on the sympathetic nerves.

Methods and materials

Animals

See Chapter 3 Methods.

Preparation of DOCA-salt rats

See Chapter 3 Methods.

Measurement of vasoconstriction *in vitro*

Rats were killed using a lethal pentobarbital injection (50 mg, i.p). The small intestine was removed and placed in oxygenated (95% O_2, 5% CO_2) Krebs' solution of the following composition (mM): NaCl 117; KCl 4.7; $CaCl_2$ 2.5; $MgCl_2$ 1.2; $NaHCO_3$ 25; NaH_2PO_4 1.2; glucose 11. The ileal segment was placed in a petri dish and the mesentery was stretched gently and pinned flat. A section of mesentery close to the ileal wall was carefully cut free from the intestine and the mesentery was transferred to a small silicone elastomer-lined recording bath (2.5 ml volume). The arteries (4° and 5° order) and corresponding veins were exposed by carefully cutting away surrounding adipose and connective tissues. The recording bath containing the preparation was mounted on the stage of an inverted microscope (Olympus CK-2) and superfused continuously with warm (36 ^{o}C) Krebs' solution at a flow rate of 7 ml/min. The output of a black and white video camera (Hitachi, KP-111) attached to the microscope was fed to a PCVision Plus frame-grabber board (Imaging Technology Inc., Woburn MA)

mounted in a personal computer. The sampling rate was 10 Hz and changes in blood vessel diameter of 0.5 μm could be resolved. The video images were analyzed using Diamtrak® software. The digitized signal was converted to an analog output (DAC-02 board, Keithley Metrabyte) and which was fed to an analog to digital converter (Labmaster 125) and a second computer running Axotape software (version 2.0, Axon Instruments, Inc. Foster City CA) for a permanent record of changes in vein diameter. Analog signals were sampled at 33 Hz and data were stored on the computer hard drive for subsequent display and analysis.

Drug application

Drugs were applied using a system of 3-way stopcocks so that the superfusing Krebs' solution could be changed to one containing a known concentration of drug. The flow rate was 8 ml/minute and it took one minute for drugs to reach the tissue. NE and ATP concentration-response curves were constructed using non-cumulative addition of each agonist concentration. Each agonist concentration was applied for 2 minutes and there was a 20 minute interval between application of each agonist concentration. Tissues were washed continuously with normal Krebs' solution between each dose. Agonists were applied for a minimum of 30 minutes prior to testing their effect on agonist- or nerve-mediated responses.

Transmural stimulation of perivascular nerves

Two parallel silver/silver chloride wire electrodes connected to a Grass Instruments stimulator (S88) were placed perpendicular to the longitudinal axis of mesenteric tissues. Parameters for nerve stimulation were: 30 stimuli, stimulus duration 0.5 ms, frequency 0.2 – 30 Hz, voltage 60-140 volts. The neurogenic origin of constrictions caused by electrical stimulation was verified in each preparation by initially demonstrating that a constriction caused by 20 Hz stimulation was blocked by TTX (0.3 μM). Preparations in which the initial 20 Hz constriction was not blocked by TTX were discarded. The peak constriction and in some experiments the duration of vasoconstriction caused by nerve stimulation was measured. Measurements were made using Axotape software and on-screen cursors.

Immunohistochemical localization of ET_B receptors in arteries and veins

1) Tissue fixation: Mesenteric tissues were taken from sham and DOCA-salt hypertensive rats. Blood was flushed from blood vessels by injecting phophate buffer saline (PBS) solution into the lumen of the blood vessels with a 30 G ½ inch needle. The tissues were fixed with 4% paraformaldehyde for 10 minutes and washed with 0.01 M PBS 3 times and 10 minutes each time at room temperature. Blood vessels were then completely dissected out from the mesenteric fat and connective tissue.

2) Primary antibody incubation: Mesenteric arteries and veins from sham and DOCA-salt hypertensive rats were divided into 4 groups by incubating with: i) primary antibody for ET_B receptor (Alamone Laboratories, Jerusalem, Israel;

raised from rabbit, kept at –20 °C and diluted to 1:200 in 0.01 M PBS (pH 7.2); ii) primary antibodies for ET_B receptor and TH (Calbiochem, San Diego, CA; raised in mouse and diluted to 1:200 in 0.01 M PBS, pH 7.2); iii) primary antibody for ET_B receptor preincubated with control antigen for 1 hour; iv) no primary antibody. The blood vessels were incubated with antisera for 24 hours at room temperature.

3) Secondary antibody incubation: After washing with 0.01 M PBS 3 times at 10 minutes each time, blood vessels were incubated in the secondary antibody for 1 hour in a sealed humidified chamber. Each of the four groups was incubated with two secondary antibodies from different species which bind to the primary antibody for ET_B receptors or TH: indocarbocyanine (Cy3) and fluorescein isothiocyanate (FITC) conjugated IgG (Jackson Immunoresearch, PA) diluted (1:200 and 1:40 respectively) in 0.01 M PBS containing 1% normal goat serum (NGS).

4) Mounting and observation of the blood vessels under fluorescence microscope. After the blood vessels were washed with 0.01 M PBS 3 times and 10 minutes each time, they were mounted with buffered glycerol (pH 8.0) to slides coverslipped for fluorescence microscopy. Staining in the groups incubated with primary antibody for ET_B receptors was considered positive when the other two control groups (no primary antibody or primary antibody preincubated with control antigen) showed negative staining. For the group incubated with primary antibodies for both TH and ET_B receptors, negative cross reactivity was assured as the group incubated with primary antibody for TH

(raised from mouse) alone does not demonstrate Cy3 (anti-rabbit) fluorescence and the group incubated with primary antibody for ET_B receptors (raised from rabbit) alone does not demonstrate FITC (anti-mouse) fluorescence. Furthermore, co-staining of TH and ET_B receptors was examined using a confocal microscopy (Leica Microsystems, Heidelberg, Germany). Images in this dissertation are presented in color.

Statistical analysis

Agonist-induced constrictions were measured in μm and are expressed as a percentage of the initial resting diameter of the blood vessel. For NE and ATP concentration-response curves, EC_{50} and E_{max} were calculated from a least squares fit of individual agonist concentration-response curves using the following logistic function (Origin 5.0 Microcal Software Inc., Northampton, MA):

$$y = \{(Y_{min} - E_{max})/[1 + (x / EC_{50})^{m}]\} + E_{max}$$

Where "Y_{min} " is the minimum response (constrained to 0), "E_{max}" is the maximum response, "EC_{50}" is the half maximal effective concentration and "m" is the slope factor. For nerve stimulation frequency-response curves, the half maximal effective stimulation frequency (S_{50}) and E_{max} were calculated from curves obtained in individual tissues using the following function (Origin 5.0 software):

$$y = E_{max} X / (S_{50} + X)$$

where "X" is the stimulation frequency tested and "y" is the peak response amplitude. All data are expressed as the mean ± S.E.M and "n" values refer to

the number of animals from which the data were obtained. Differences between groups were assessed using Students' t-test.

Drugs

All drugs were obtained from Sigma Chemical Company (St. Louis, MO).

Results

Modulatory effects of ET receptor activation on sympathetic neuroeffector transmission to mesenteric arteries in DOCA-salt hypertension

1. ET-1 facilitated the nerve- mediated vasoconstriction of sham arteries, but not DOCA-salt arteries.

To determine the overall effects mediated at ET receptors on sympathetic neuroeffector transmission to arteries, responses to nerve stimulation from sham and DOCA-salt arteries before and after the application of ET receptor agonists were compared. ET-1 with a low concentration (3 pM) increased the Emax of the frequency-response curves caused by nerve stimulation of sham arteries and had no effects on that of DOCA-salt arteries (Fig. 5.1, Table 5.1). Similarly, ET-1 with subcontractile concentration (300 pM) increased the Emax of the frequency-response curves caused by nerve stimulation obtained from sham arteries and had no effects on that of DOCA-salt arteries (Fig. 5.2, Table 5.2). An ET_B receptor agonist, S6c (30 pM), had no effects on the nerve-mediated response of arteries from sham and DOCA-salt rats (Table 5.3). However, when the concentration of S6c increased to 100 pM, it decreased the S_{50} of the frequency-response curves of DOCA-salt arteries, but not sham arteries (Table 5.3).

2. ET-1 decreased the reactivity to ATP and increased the reactivity to NE of sham arteries, but not DOCA-salt arteries

To determine the postjunctional effects mediated by ET receptor on sympathetic neuroeffector transmission to arteries, the reactivity of arteries from sham and DOCA-salt rats to exogenous ATP before and after the application of ET receptor agonists were compared. ET-1 (3 pM) increased the EC_{50} of the concentration-response curves for ATP from sham arteries, but had no effects on that from DOCA-salt arteries (Table 5.1). Consistently, ET-1 with subcontractile concentration (300 pM) increased the EC_{50} of the concentration-response curves for ATP from sham arteries, but had no effect on DOCA-salt arteries (Table 5.2). Conversely, ET-1 (300 pM) decreased the EC_{50} of the concentration-response curves for NE from sham arteries, but not DOCA-salt arteries (Table 5.2). S6c (30 pM) had no effect on concentration-response curves for ATP of arteries from either sham and DOCA-salt rats. The EC_{50} (mM) of the ATP concentration-response curves from sham arteries before and after ET-1 application were 0.1 $\pm$ 0.04 and 0.1$\pm$ 0.03, respectively (n=4, P>0.05). The Emax (%) of the concentration-response curves from sham arteries before and after ET-1 application were 47.4 $\pm$ 5.1 and 40.7$\pm$ 2.5, respectively (n=4, P>0.05). The EC_{50} (mM) of the concentration-response curves from DOCA-salt arteries before and after ET-1 application were 0.45 $\pm$ 0.2 and 0.3 $\pm$ 0.1, respectively (n=6, P>0.05). The Emax (%) of the concentration-response curves from DOCA-salt arteries before and after ET-1 application were 49 $\pm$ 9.9 and 34 $\pm$ 5.7, respectively (n=6, P>0.05).

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Modulatory effects of ET receptor activation on sympathetic neuroeffector transmission to mesenteric veins in DOCA-salt hypertension

1. *S6c facilitated nerve-mediated vasoconstriction of mesenteric veins from DOCA-salt hypertensive rats.*

Responses to nerve stimulation from sham and DOCA-salt veins before and after the application of ET receptor agonists were compared. ET-1 (3 pM) had no effect on the response to nerve stimulation of veins (Table 5.4). S6c had no effect on the maximum response to nerve stimulation of sham veins (Table 5.5, Fig. 5.3A), but increased the maximum response of DOCA-salt veins (Table 5.5, Fig. 5.3B). There was no difference in the S_{50} of the response to nerve stimulation before and after the application of S6c (Table 5.5).

2. ET-1 and S6c increased the sensitivity of adrenergic receptors in mesenteric veins from DOCA-salt hypertensive rats. The reactivity of veins to exogenously applied NE before and after the application of ET receptor agonists was compared in sham & DOCA-salt veins to determine their postjunctional effects of ET receptors on veins. Both ET-1 (Table 5.4) and S6c decreased the EC_{50} of concentration-response curves to NE from DOCA-salt veins (Table 5.5 and Fig. 5.4.B) and had no effects on those of sham veins (Table 5.5 and Fig. 5.4.A).

Immunohistochemical staining of ET_B receptors

Arteries from sham and DOCA-salt rats incubated with primary antibody for ET_B receptors demonstrate positive staining on sympathetic nerves as there was co-staining from with primary antibodies for TH and ET_B receptors. Arteries incubated with primary antibody for ET_B receptors preincubated with blocking peptide or with no primary antibody demonstrated negative staining (Fig. 5.5 and Fig. 5.6). Therefore, ET_B receptors were localized to sympathetic nerves in arteries.

Veins from sham and DOCA-salt rats incubated with primary antibody for ET_B receptors demonstrate positive staining on smooth muscle cells that is not co-localized with TH staining. Veins from sham and DOCA-salt rats incubated with primary antibody for ET_B receptors preincubated with the blocking peptide or with no primary antibody demonstrated negative staining (Fig. 5.7 and Fig. 5.8). Therefore, ET_B receptors were localized on the smooth muscle cells in veins. Images in this dissertation are presented in color.

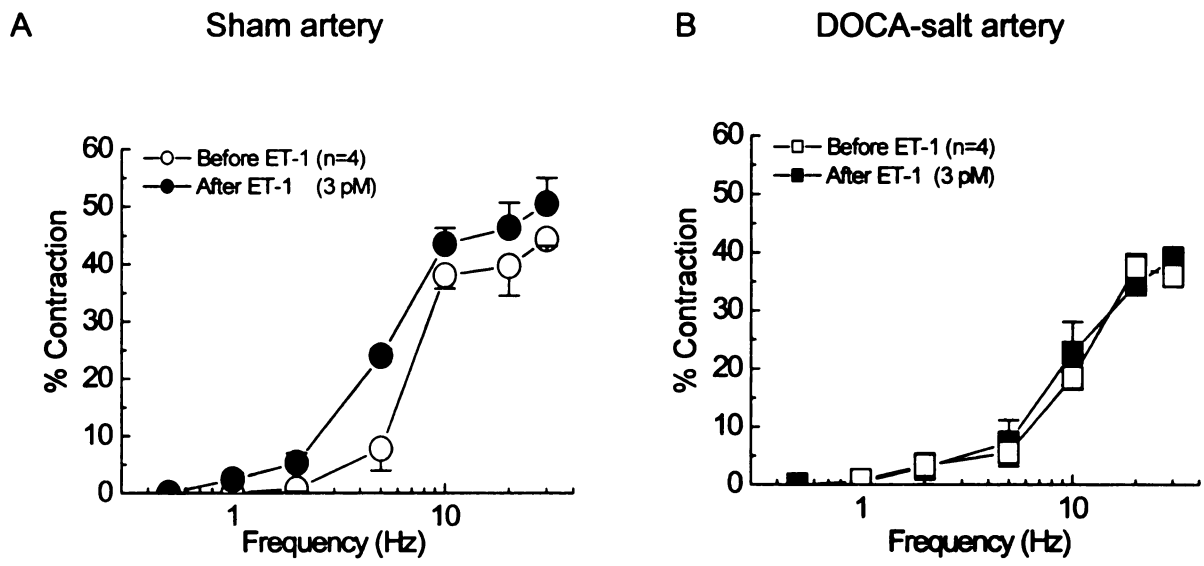

Fig. 5.1 A: Frequency-response curves caused by nerve stimulation from sham arteries before and after the application of ET-1. Emax of the frequency-response curves obtained after the application of ET-1 is greater bigger than that obtained before the application of ET-1. B: Frequency-response curves caused by nerve stimulation from DOCA-salt arteries before and after the application of ET-1. There was no difference in Emax and S_{50} between concentration-response curves obtained before and after the application of ET-1. All data are expressed as the mean + S.E.M and "n" values refer to the number of animals from which the data were obtained.

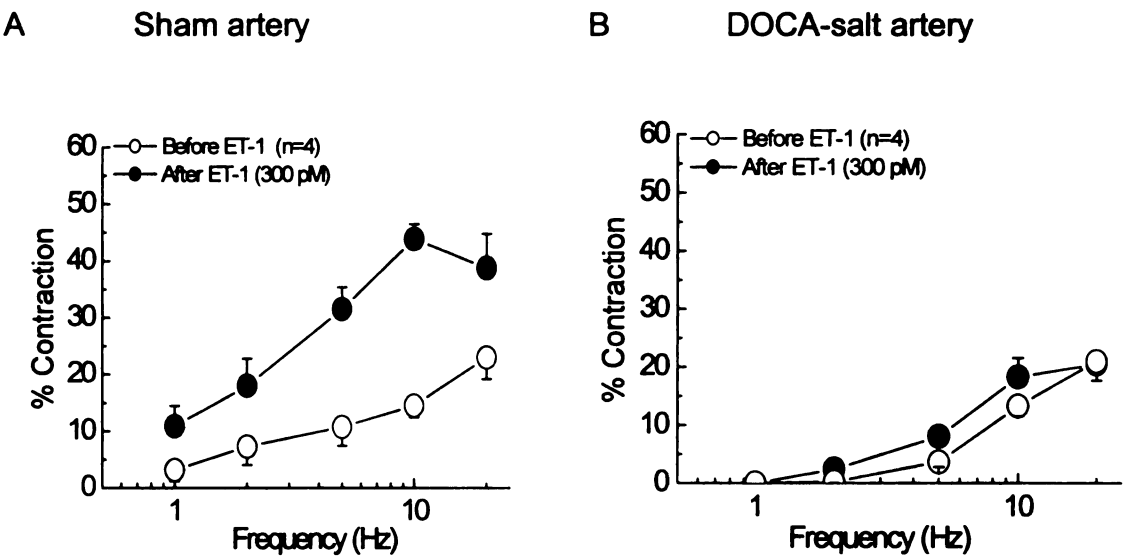

Fig. 5.2 A: Frequency-response curves caused by nerve stimulation from sham arteries before and after the application of ET-1. The Emax of the frequency-response curves obtained after the application of ET-1 is greater compared with that obtained before the application of ET-1. B: Frequency-response curves caused by nerve stimulation from DOCA-salt arteries before and after the application of ET-1. There was no difference in Emax and S_{50} between concentration-response curves obtained before and after the application of ET-1. All data are expressed as the mean + S.E.M and "n" values refer to the number of animals from which the data were obtained.

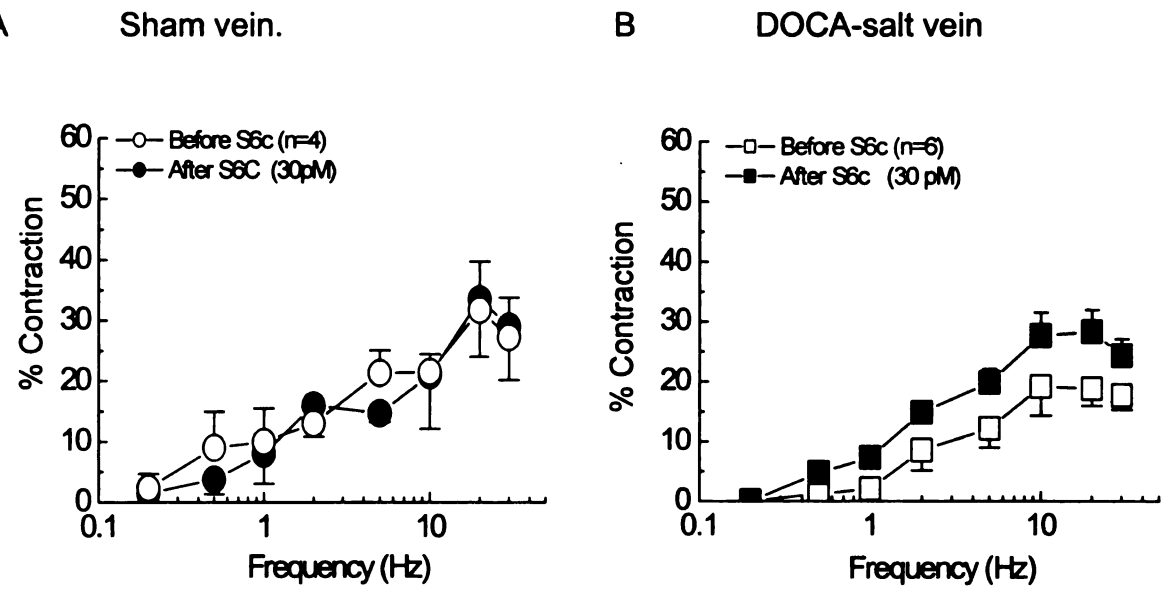

Fig. 5.3 A: Frequency-response curves caused by nerve stimulation from sham veins before and after the application of S6c. There was no difference in Emax and S_{50} of the frequency-response curves obtained before and after the application of S6c. B: Frequency-response curves caused by nerve stimulation from DOCA-salt veins before and after the application of S6c. The Emax of the frequency-response curves obtained after the application of S6c is greater compared with that obtained before the application of S6c. All data are expressed as the mean + S.E.M and "n" values refer to the number of animals from which the data were obtained.

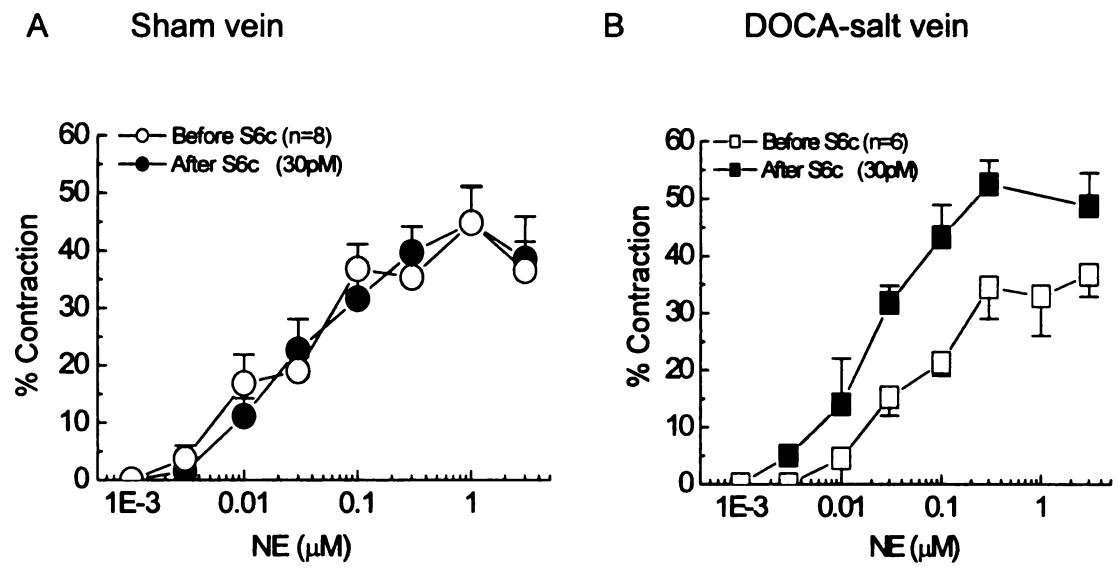

Fig. 5.4 A: Concentration-response curves for NE from sham veins before and after the application of S6c. There was no difference in concentration-response curves obtained before and after the application of S6c. B: Concentration-response curves for NE from DOCA-salt veins before and after the application of S6c. The EC_{50} of the concentration-response curves obtained after the application of S6c is smaller compared with that obtained before the application of S6c. All data are expressed as the mean + S.E.M and "n" values refer to the number of animals from which the data were obtained.

Sham artery

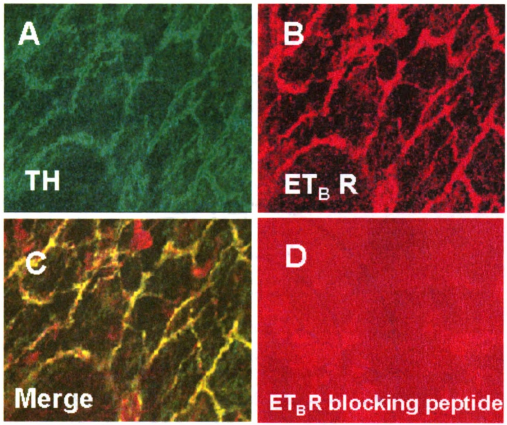

Fig. 5.5: Pictures taken from confocal fluorescence microscopy showing ET_B receptors (ET_BR, Cy3 conjugated secondary antibody) and TH (FITC conjugated secondary antibody) are co-stained on arteries from sham rats (A, B, C). Incubation with ET_B receptor antibody preincubated with control antigen (D) produces no staining on the nerves.

DOCA-salt artery

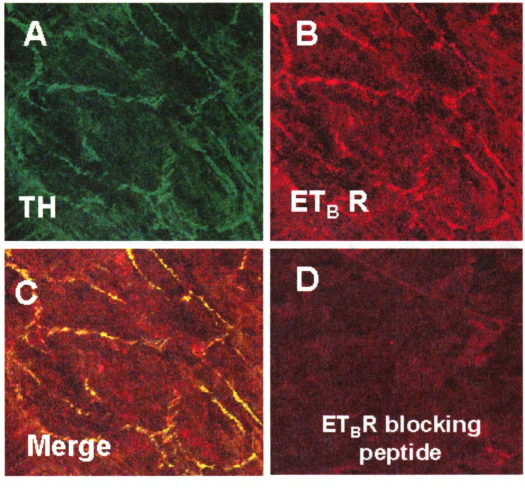

Fig. 5.6: Confocal images showing ET_B receptors and tyrosine hydroxylase (TH) are co-stained on arteries from DOCA-salt hypertensive rats (A, B, and C). Incubation with ET_B receptor antibody preincubated with control antigen produced no staining on the nerves (D).

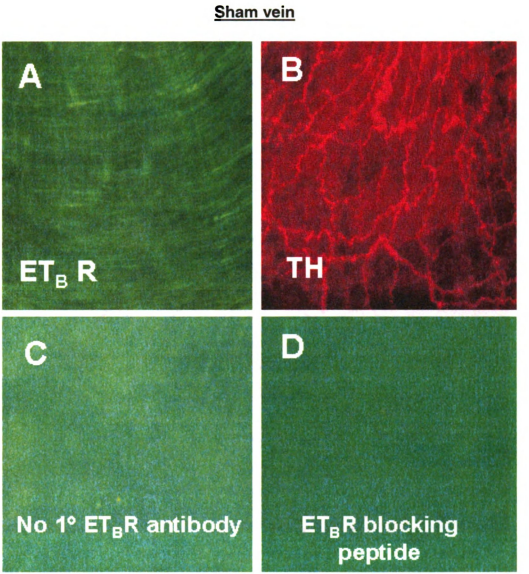

Fig. 5.7: ET$_B$ receptors staining on veins from sham rats. The ET$_B$ receptor staining at the smooth muscle cells (A) was different from TH staining at sympathetic nerves (B). Omitting the ET$_B$ receptor antibody (C) or incubation with ET$_B$ receptor antibody preincubated with control antigen (D) produces no staining on the smooth muscle cells.

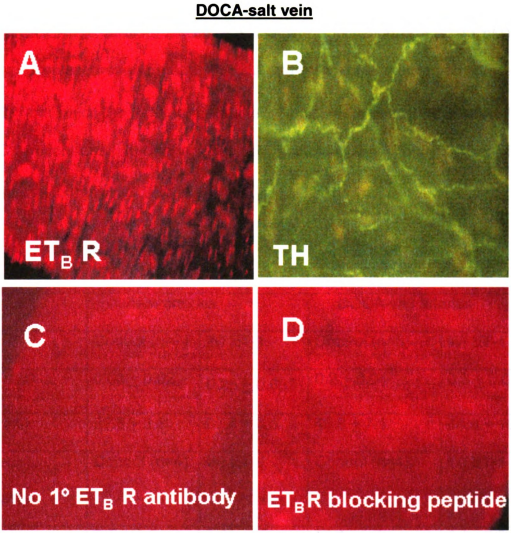

Fig. 5.8: Stain ET_B receptors on veins from DOCA-salt rats. The ET_B receptor staining at the smooth muscle cells (A) was different from TH staining on sympathetic nerves (B). Omitting the ET_B receptor antibody (C) or incubation with ET_B receptor antibody preincubated with control antigen (D) produced no staining on the smooth muscle cells.

Table 5.1: Constrictions caused by nerve stimulation and exogenous ATP from sham & DOCA-salt arteries before and after the application of ET-1 (3 pM). All data are expressed as the mean ± S.E.M and "n" values refer to the number of animals from which the data were obtained. *indicates significantly different from the EC_{50} in control sham arteries ($p<0.05$). ¶indicates significantly different from the Emax in control sham arteries ($p<0.05$).

	Sham arteries		DOCA-salt arteries	
ATP	Control (n=7)	W/ ET-1	Control (n=6)	W/ ET-1
EC_{50} (mM)	0.08 ± 0.02	0.25 ± 0.05*	0.2 ± 0.04	0.3 ± 0.06
Emax (%)	61.3 ± 3.8	67.4 ± 6.3	46.8 ± 7.1	44.8 ± 10.6
Nerve stimulation	Control (n=4)	W/ ET-1	Control (n=4)	W/ ET-1
S_{50} (Hz)	7.1 ± 1.3	5.3 ± 0.5	11.1± 0.6	9.9± 2.3
Emax (%)	44.9 ± 5.6	52.4 ± 5.1¶	41.3± 2.6	40.5± 4.2

Table 5.2: Constrictions caused by exogenous ATP and NE from sham & DOCA-salt arteries before and after the application of ET-1 (300 pM). All data are expressed as the mean ± S.E.M and "n" values refer to the number of animals from which the data were obtained. ¶indicates significantly different from the EC_{50} in control sham arteries ($p<0.05$). *indicates significantly different from the Emax in control sham arteries ($p<0.05$).

	Sham arteries		DOCA-salt arteries	
ATP	Control (n=5)	W/ ET-1	Control (n=4)	W/ ET-1
EC_{50} (mM)	0.05 ± 0.02	0. 13 ± 0.02¶	0.18 ± 0.09	0.16 ± 0.06
Emax (%)	68.2 ± 5.3	54.6 ± 16.2	62.3 ± 9.2	55 ± 11.7
NE	Control (n=4)	W/ ET-1	Control (n=4)	W/ ET-1
EC_{50} (μM)	5.3 ± 2.6	1.0 ± 0.3 ¶	3.4 ± 1.2	1.6 ± 0.6
Emax (%)	61.5 ± 10.7	60 ± 10.9	59.3 ± 9.3	52.1 ± 6.2
Nerve stimulation	Control (n=4)	W/ ET-1	Control (n=4)	W/ ET-1
S_{50} (Hz)	11.5 ± 6.9	3.9 ± 1.7	8.4 ± 0.7	9.6 ± 2.4
Emax (%)	29.2 ± 2.5	53.4 ± 7.9*	21 ± 3.3	30 ± 6.6

Table 5.3: Constrictions caused by nerve stimulation from sham & DOCA-salt arteries before and after the application of S6c (30 pM and 100 pM). All data are expressed as the mean ± S.E.M and "n" values refer to the number of animals from which the data were obtained. * indicates significantly different from the S_{50} in control DOCA-salt arteries ($p<0.05$).

	Sham arteries		DOCA-salt arteries	
Nerve stimulation	Control (n=4)	W/ S6c (30 pM)	Control (n=4)	W/ S6c (30 pM)
S_{50} (Hz)	6.8 ± 1.5	15.8 ± 6.8	8.2± 2.1	9.9± 0.7
Emax (%)	33 ± 8.1	35 ± 6.8	35± 3.8	32± 2.9
Nerve stimulation	Control (n=4)	W/ S6c (100 pM)	Control (n=4)	W/ S6c (100 pM)
S_{50} (Hz)	5.1 ± 0.9	5.3 ± 1.1	10.2 ± 2.1	4.6 ± 2.1*
Emax (%)	37 ± 6.8	38 ± 8.8	33.3 ± 5.0	33.3 ± 2.0

Table 5.4: Responses to nerve stimulation and to exogenous NE from sham & DOCA-salt veins before and after the application of ET-1 (3 pM). All data are expressed as the mean ± S.E.M and "n" values refer to the number of animals from which the data were obtained. *indicates significantly different from the EC_{50} in the control DOCA-salt veins ($p<0.05$).

	Sham veins		DOCA-salt veins	
NE	Control (n=5)	W/ ET-1	Control (n=4)	W/ ET-1
EC_{50} (μM)	0.057± 0.03	0.054 ± 0.02	0.27 ± 0.07	0.04 ± 0.016*
Emax (%)	60 ± 2.5	65 ± 2.7	35.1 ± 4.3	44.4 ± 8
Nerve stimulation	Control (n=4)	W/ ET-1	Control (n=5)	W/ ET-1
S_{50} (Hz)	5.9 ± 4.0	4.3± 1.9	4.9 ± 0.8	4.5 ± 0.7
Emax (%)	31± 17	33± 11	40 ± 11	43 ± 8

Table 5.5: Responses to nerve stimulation and to exogenous NE from sham & DOCA-salt veins before and after the application of S6c (30 pM). All data are expressed as the mean ± S.E.M and "n" values refer to the number of animals from which the data were obtained. *indicates significantly different from the EC_{50} in the control DOCA-salt veins ($p<0.05$). ♣ indicates significantly different from the Emax in control DOCA-salt veins ($p<0.05$)

	Sham veins		DOCA-salt veins	
NE	Control (n=8)	W/ S6c (n=8)	Control (n=6)	W/ S6c
EC_{50} (μM)	0.04 ± 0.01	0.03 ± 0.01	0.15 ± 0.01	0.02 ± 0.01*
Emax (%)	46 ± 4.5	48 ± 5.1	48 ± 5.1	54 ± 6.3
Nerve stimulation	Control (n=4)	W/ S6c	Control (n=6)	W/ S6c
S_{50} (Hz)	3.2 ± 1.4	6.1 ± 0.8	3.4 ± 1.6	2.2 ± 0.6
Emax (%)	31 ± 7.0	36 ± 9.4	22.1 ± 1.6	32.9 ± 2.9 ♣

DISCUSSION

ET_A receptors mediate facilitation of sympathetic vasoconstriction to sham arteries.

A low concentration (3 pM) of ET-1 potentiated neurogenic responses to nerve stimulation of sham arteries as it increased the Emax of the frequency-response curves. ET-1 at a subcontractile concentration (300 pM) potentiated responses to nerve stimulation in sham arteries as it increased the Emax of the frequency-response curves to nerve stimulation. Furthermore, the potentiation caused by ET-1 at 300 pM is greater than that by ET-1 at 3 pM. The potentiation by ET-1 on contractile responses to sympathetic nerve stimulation is consistent with the results obtained from other studies (Tabuchi et al., 1990; Mutafova-Yambolieva and Radomirov, 1994; Zhang et al., 1996). For example, ET-1 (300 pM) enhanced the pressor responses to nerve stimulation of the rat mesenteric arteries (Tabuchi et al., 1990). Yet, to exert facilitatory effect on the responses of dog mesenteric arteries to sympathetic nerve stimulation, the concentration of ET-1 was raised to 1 nM (Zhang et al., 1996). This result suggests that different concentrations are required for ET-1 to exert potentiating effects on sympathetic neuroeffector transmission to the mesenteric arteries from animals of different species.

ET-1 (300 pM) also increased the reactivity of sham arteries to NE as it decreased the EC_{50} for NE. This result is similar to that reported in other studies demonstrating a potentiating effect on the reactivity of mesenteric arteries to

exogenous NE by ET-1 with similar concentrations (Tabuchi et al., 1990; Zhang et al., 1996). Studies done on human mammary arteries also demonstrated a facilitation of the constrictions to NE by ET-1 with the same concentration (300 pM, Yang et al., 1990). Therefore, the facilitatory effect mediated by ET-1 on sympathetic neuroeffector transmission to sham arteries is likely to be via the postjunctional mechanism involving a potentitation of the reactivity of the adrenergic receptors. Conversely, ET-1 decreased the reactivity of sham arteries to ATP as it increased the EC_{50} of the concentration-response curves for ATP. This is different from the result obtained from studies using rabbit saphenous artery that showed a potentiated effect on the responses to exogenous ATP by ET-1 (Mutafova-Yambolieva and Radomirov, 1994). This discrepancy may be related to the different vascular bed used. Taken together, these results indicate that ET-1 has a differential modulatory effect on the reactivity of P2 and adrenergic receptors. Furthermore, the facilitatory effect mediated by ET-1 on postjunctional reactivity is mediated by ET_A receptors as it was shown that there was no functional expression of ET_B receptors on the arterial smooth muscle cells (Johnson et al., 2002). My study was unable to demonstrate positive staining of ET_B receptors on the arterial smooth muscle cells and any effect of S6c on responses of arteries to exogenous ATP.

S6c had no effect on the responses of sham arteries to nerve stimulation. This result is similar to that obtained from other studies showing that the modulatory effect caused by ET-1 on dog mesenteric arteries was mediated by ET_A receptors (Zhang et al., 1996). However, S6c potentiated the responses of

DOCA-salt arteries to nerve stimulation as it decreased the S_{50} of the frequency-response curves of DOCA-salt arteries caused by nerve stimulation. My study also demonstrated positive staining for ET_B receptors at the sympathetic nerve terminal innervating the mesenteric arteries. Therefore, it appears that in DOCA-salt arteries ET_B receptors located at the sympathetic nerve terminal facilitate sympathetic neuroeffector transmission to DOCA-salt arteries. Yet, ET-1 had no effect on the vasoconstrictions of DOCA-salt arteries caused by nerve stimulation as there was no difference in frequency-response curves caused by sympathetic nerve stimulation from DOCA-salt arteries obtained before and after the application of ET-1. Combined together, these results suggest that ET_A receptors must mediate an inhibitory effect on sympathetic neuroeffector transmission to DOCA-salt arteries to counteract the facilitatory effect mediated by ET_B receptors. This notion is supported by other studies showing that ET-1 acting at ET_A receptors inhibited NE release from sympathetic nerves innervating mesenteric arteries from dog (Zhang et al., 1996). Furthermore, ET-1 had no effect on the postjunctional reactivity of DOCA-salt arteries to ATP and NE as there was no difference in both concentration-response curves for NE and ATP obtained before and after the application of ET-1. The disappearance of modulatory effects by ET-1 on DOCA-salt arteries is likely due to the desensitization of the ET receptors on the arterial smooth muscle cells from DOCA-salt rats as indicated by a reduced ET-1 induced constrictions of mesenteric arteries from DOCA-salt rats (Johnson et al., 2002). This decreased reactivity is likely a result of the increased level of circulating ET-1 level in DOCA-

salt hypertensive animals (Day et al., 1995;Yu et al., 2002). Hence, the facilitatory effect by ET-1 on sympathetic neuroeffector transmission to sham arteries with normal ET receptors suggest that increased ET-1 level would enhance the sympathetic constriction of arteries. This effect would contribute to the elevation of blood pressure, and likely occurs during the development of DOCA-salt hypertension when the ET receptors are not yet desensitized.

ET_B receptors mediate facilitation of sympathetic vasoconstriction of DOCA-salt veins via a postjunctional effect.

ET_B receptors are differentially expressed in arteries and veins as they are expressed on the smooth muscle cells of the veins and on the sympathetic nerves of the arteries. The present study used subcontractile concentrations of ET-1 (3 pM) and S6c (30 pM) based on the results obtained from studies by Johnson et al., (2002). These concentrations are also similar to endogenous levels of ET-1 (Rubanyi and Polokoff, 1994). S6c potentiated the responses to nerve stimulation of DOCA-salt veins, but not sham veins as it increased the E_{max} of the frequency-response curves of DOCA-salt veins to nerve stimulation. S6c also increased the reactivity of DOCA-salt veins, but not sham veins to NE as it decreased the EC_{50} of the concentration-response curves for NE. Similarly, ET-1 increased the reactivity of DOCA-salt veins to exogenously applied NE (Table 5.3.). Those results combined together suggest that ET-1 acting at ET_B receptors potentiates sympathetic neuroeffector transmission to veins from DOCA-salt rats at least partly by increasing postjunctional reactivity to NE. However, ET-1 had

no effects on nerve-mediated response of DOCA-salt veins. Possibly, inhibitory prejunctional ET_A receptors are activated by ET-1 in DOCA-salt veins to counteract the facilitatory postjunctional ET_B receptors. The reactivity of ET receptors is maintained in DOCA-salt veins (Johnson et al., 2002), suggesting that there is no functional change of the ET receptors *per se*. Therefore, the emergence of a facilitatory effect on sympathetic neuroeffector transmission to DOCA-salt veins by S6c can attribute to factors other than a functional change of the ET receptors such as the coupling of the intracellular signaling pathways downstream from the ET and adrenergic receptors.

Studies *in vitro* found that ET-1 was in the post arterial bed, i.e., capillaries and veins (Hemsen and Lundberg, 1991) and ET-1 binding sites were distributed in the veins (Power et al., 1989; Davenport et al., 1989). Studies *in vivo* showed that intravenous administration of ETs increased central venous pressure, venous resistance and MCFP (Waite and Pang, 1990; Yamamato et al., 1980). In DOCA-salt hypertension, ET-1 induced constriction was reduced in rat aorta (Watts et al., 2002) and mesenteric arteries (Johnson et al., 2002), but maintained in vena cava (Watts et al., 2002) and mesenteric veins (Johnson et al., 2002). The venous system plays an important role in blood pressure regulation by serving a capacitance function. In DOCA-salt rats there is an elevated sympathetic drive to the venous system as indicated by a greater decrease of the MCFP by hexamethonium, a blocker of sympathetic ganglionic transmission, on DOCA-salt rats compared to the control rats (Fink, et al., 2000). Elevated sympathetic drive to the venous system would increase venous return

and cardiac output, which will lead to elevated blood pressure (Guyton et al., 1972b). Although ET-1 and the sympathetic nervous system control venoconstriction (Waite and Pang, 1992), and ET_A antagonist did no alter the effect of hexamethonium on MCFP. Therefore endogenous ET-1 doesn't modulate sympathetic venoconstriction through ET_A receptors (Fink et al., 2000). This result is consistent with the result obtained from the present study suggesting that ET_B receptors located at the venous smooth muscle cells mediating the facilitatory effect caused by ET-1 on sympathetic neuroeffector transmission to the veins from DOCA-salt rats. Hence, the modulatory effects by ET-1 acting at ET_B receptors on sympathetic neuroeffector transmission to veins indicated by the present study are likely to contribute to the MCFP elevation observed in DOCA-salt rats (Fink et al., 2000).

Summary and Conclusion

The present study suggests that ET-1 acting at ET_A receptors facilitate sympathetic neuroeffector transmission to mesenteric arteries from sham rats, which could raise blood pressure in the development of hypertension. This facilitation by ET-1 was removed in DOCA-salt hypertension possibly due to the desensitization of ET_A receptors. My study also showed that ET-1 acting at ET_B receptors facilitate sympathetic transmission to veins from DOCA-salt rats via a postjunctional mechanism. This facilitation is likely to contribute to the increased MCFP observed in DOCA-salt rats (Fink et al., 2000).

CHAPTER 6

General discussion

1. Differential sympathetic neuroeffector transmission to arteries and veins from normotensive rats

1.1. Different neurotransmitters mediating sympathetic neuroeffector transmission to small mesenteric arteries and veins

My study showed that ATP is the dominant neurotransmitter mediating sympathetic constriction of the small mesenteric arteries, whereas in mesenteric veins NE is the transmitter mediating smooth muscle contraction. A recent study using confocal fluorescence microscopy to visualize Ca^{2+} transients in perivascular nerves and adjacent smooth muscle cells from rat mesenteric small arteries in response to electrical field stimulation with both low (< 1 Hz) and high frequencies (10 Hz) demonstrated that the local Ca^{2+} transients are due to activation of P2X receptors by ATP released from sympathetic perivascular nerves. Consistent with my results, these studies provided further evidence that purinergic components mediate a component of sympathetic neuroeffector mechanisms in small mesenteric arteries (Lamont and Wier, 2002; Lamont et al., 2003). The purinergic component predominates during the early phases of a train of stimulation (< 20 s) as in my study where the train length was 3 s. When the stimulation persists beyond that duration and last for 3 minutes, a slowly rising force and asynchronous propagating Ca^{2+} waves resulting from the release of NE acting at α1-adrenergic receptors became dominant (Lamont et al., 2003).

Therefore, it appears that NE dominates sympathetic neuroeffector mechanisms during the later part of a long train of stimulation. Yet, studies investigating the discharge pattern of perivascular sympathetic nerve fibers innervating the tail artery from normal rats *in vivo* suggest that neuronal activity occurs in regular short bursts of high frequency firing rather than long sustained trains (Johnson and Gilbey, 1996). Hence, prolonged impulses of sympathetic nerves are more likely to occur under unique physiological or pathophysiogical conditions such as essential hypertension when nerve activity is enhanced.

Results obtained from my studies suggest that: 1) as constrictions of small arteries and arterioles are the major source of peripheral resistance, ATP plays a role in controlling peripheral resistance and thus blood pressure regulation under normal conditions. ATP causes a multitude of cardiovascular effects such as positive inotropy in the heart, platelet aggregation, release of endothelial factors, and growth stimulation of vascular smooth muscle cells (Erlinge, 1999); 2) differential sympathetic neural control of arteries and veins (Dehal et al., 1992; Browning et al., 1999), which may be one mechanism underlying the different function performed by arteries and veins in mesenteric vascular bed (eg. resistance vs. capacitance).

1.2. Enhanced NE release relative to basal levels in veins compared to arteries

My study showed that basal release of NE was strongly correlated to stimulation-induced release of NE in arteries and veins, suggesting that basal

release of NE comes from the nerve terminals. Other studies using continuous amperometry demonstrated that there was spontaneous release of NE from nerve terminals (Brock et al., 2000). These results suggest that basal release of NE measured in my study was due to the spontaneous release of NE from sympathetic nerve terminals. Furthermore, my study showed that there is a lower basal release of NE in sham veins compared to sham arteries. This is consistent with other reports that sympathetic innervation of veins is generally less than arteries (Nilsson, 1985).

My study also showed that there was more NE release measured as normalized release per impulse of nerve stimulation in veins compared to arteries. This provides further evidence supporting the notion that sympathetic nerves supplying veins and arteries are different (Kreulen, 1986; Hottenstein and Kreulen, 1987). In addition, greater release of NE per impulse of nerve stimulation in veins compared to arteries could partly account for the observation that veins are more sensitive to nerve stimulation than arteries (Kreulen, 1986; Hottenstein and Kreulen, 1987; Luo et al., 2003).

1.3. Veins are more sensitive than arteries to nerve stimulation.

As frequency response curves caused by nerve stimulation of veins are shifted to the left of arteries, veins are more sensitive than arteries to nerve stimulation as shown previously (Kreulen, 1986; Hottenstein and Kreulen, 1987). The potential causes accountable for this phenomenon are discussed below.

In small mesenteric arteries, ATP is the dominant neurotransmitter mediating sympathetic smooth muscle constraction whereas in mesenteric veins NE is the transmitter mediating venous smooth muscle contraction. NE is about 20 fold more potent than ATP in contracting arteries (Luo et al., 2003). Given that the concentrations of the neurotransmitter at the neuroeffector junction depend the firing frequency of the nerve terminals, it would require higher frequency to release enough ATP to produce the constrictions of the same amplitude as that caused by NE. Accordingly, by having ATP instead of NE as the dominant transmitter under physiological conditions small mesenteric arteries have a capacity to resist changes and fluctuations in neural input compared to their corresponding veins.

There is a greater release of NE per impulse in veins compared to arteries. Studies by Nilsson (1985) showed that the density of sympathetic innervation of mesenteric veins was less than arteries. Furthermore, innervation density is associated with maximum responses to nerve stimulation as maximum responses from veins are smaller than arteries (Nilsson, 1985). Therefore, the greater release of NE per impulse in veins compared to arteries is not likely due to the difference in innervation density of arteries and veins. Rather, it is due to the difference in functional properties of sympathetic nerves innervating veins and arteries. Different functional properties of sympathetic nerves innervating arteries and veins are demonstrated by the following results.

Although it was reported that there is ATP release from sympathetic nerves innervating mesenteric veins in responding to nerve stimulation (Bobalova

et al., 2001a; Bobalova et al., 2001b), it does not prove that ATP mediates sympathetic neuroeffector transmission to mesenteric veins. It is possible that the released ATP functions as a neuromodulator provided that it is released from the synaptic vesicles. My studies demonstrated that in veins NE is the sole neurotransmitter as prazosin completely blocked the venoconstriction caused by nerve stimulation. This result is also consistent with other studies showing that NE mediats slow depolarization of venous smooth muscle cells leading to the contractions (Evans and Surprenant, 1992; Hottenstein and Kreulen, 1987; Luo et al., 2003). However, in arteries from normotensive rats, ATP is the dominant transmitter mediating sympathetic neuroeffector transmission. Sympathetic neurons innervating mesenteric arteries and veins differ in their localization in prevertebral ganglia and in their electrophysiological properties (Browning et al., 1999). There is a greater postjunctional sensitivity to NE in veins compared to arteries. This is supported by results showing that veins are more sensitive to exogenous NE than arteries as the EC_{50} for NE from veins is smaller than that from arteries (Luo et al., 2003). This greater sensitivity of veins to NE compared to arteries indicates that there is a difference in the postjunctional mechanisms mediating NE-induced smooth muscle contractions. This greater postjunctional sensitivity to NE in veins could be due to the more depolarized membrane potential of venous smooth muscle cells compared to arterial smooth muscle cells (Kreulen, 1986) or more effective electrical coupling of venous smooth muscle cells (Nilsson, 1985).

Studies on dog mesenteric artery and vein showed that the binding affinity for prazosin was lower in veins compared with arteries whereas the maximum binding was similar, suggesting that the total amount of α1-adrenergic receptors expressed on venous and arterial smooth muscle cells is similar whereas the affinity of the α1-adrenergic receptors is lower in veins (Shi et al., 1989). Therefore, the greater postjunctional sensitivity to NE in veins is unlikely be due to the differences in density or binding affinity of the adrenergic receptors as the affinity is lower in veins. These results suggest that factors involved in the intracellular signaling pathway downstream to the receptors play an important role in determining venous sensitivity to adrenergic stimulation.

As veins are more sensitive than arteries to sympathetic nerve stimulation, similar increases in sympathetic nerve activity would increase venous tone before changing arterial tone. The splanchnic circulation holds one-third of total blood volume and this store resides largely in veins (Greenway, 1983). As the sympathetic nervous system is the principal mechanism for controlling venomotor tone (Monos et al., 1995), small increases in sympathetic nerve activity would increase venous return and cardiac stroke volume. Therefore, at low levels of sympathetic nerve activity, vascular-based increases in systemic blood pressure could be due, in part, to an increase in venomotor tone.

2. Alterations in sympathetic neuroeffector mechanisms in arteries and veins in DOCA-salt hypertension

2.1. Alterations in sympathetic neuroeffector mechasnims in arteries in DOCA-salt hypertension

2.1a. Unaltered basal release of ATP and NE in DOCA-salt arteries

The basal release of ATP and NE were correlated to nerve stimulation-induced release of ATP and NE, respectively suggesting that basal release is spontaneous release of those neurotransmitters. Many studies showed that there is spontaneous release of ATP from sympathetic nerve terminals. For example, spontaneous excitatory junctional currents (SEJCs) mediated by spontaneously released ATP from sympathetic nerves were recorded in rat mesenteric arteries (Astrand and Stjarne, 1989). SEJCs and EJCs as a result of spontaneous and evoked release of ATP, respectively from sympathetic nerves innervating the guinea-pig vas deferens were identical in amplitude and time course (Brock and Cunnane, 1988). Furthermore, spontaneous Ca^{2+} transients were observed in smooth muscle cells of rat mesenteric small arteries and are caused by the actions of spontaneously released ATP from sympathetic nerve terminals (Lamont and Wier, 2002).

SEJCs produced by spontaneously released transmitters were unaffected by TTX, suggesting that spontaneous release does not require action potentials (Brock and Cunnane, 1988; Astrand and Stjarne, 1989). Furthermore, my result

showing that basal NE release is not altered in DOCA-salt arteries despite elevated sympathetic nerve activity supports the notion that basal release is nerve activity independent. In addition, spontaneous release per nerve terminal is likely to be derived from single quanta (Astrand and Stjarne, 1989; Stjarne et al., 1994). Accordingly, the magnitude of spontaneous release per nerve terminal would depend upon the quanta size and hence the overall spontaneous release at the neuromuscular junctions would depend upon both the quantal size and innervation density. My study showed that the basal release of ATP and NE are not different between sham and DOCA-salt arteries. These results suggest that the spontaneous release of ATP and NE and thus the innervation density of mesenteric arteries is not altered in DOCA-salt hypertension assuming that quantal size is not altered. Studies on mesenteric arteries from SHRs yielded similar results as spontaneous release of ATP measured by EJPs was not different between SHRs and WKYs (Brock and Van Helden, 1995).

2.1b. Increased nerve stimulation-induced release of NE, but not ATP

My study directly demonstrated that nerve stimulation-induced release of NE was increased in DOCA-salt arteries compared to sham arteries, indicating that there is an alteration of sympathetic neuronal function in DOCA-salt hypertension. This result is consistent with a previous result obtained from our laboratory showing that NE content was reduced in DOCA-salt arteries (Luo et al., 2003). This result and conclusion is also similar to those reported previously in studies of DOCA-salt rats (Crabb et al., 1980; Tsuda et al., 1989). For

example, significant decreases in the NE content of the mesenteric artery, renal artery and cardiac tissue were found in DOCA-salt rats (Crabb et al., 1980). Electrical stimulation of sympathetic innervation caused a significantly greater release of NE into the mesenteric vasculature of DOCA-salt hypertensive rats than in age-matched normotensive controls (Tsuda et al., 1989). As ATP release was not altered in DOCA-salt arteries, increased NE release is likely to account for the increased neurogenic contractions of smooth muscle in DOCA-salt arteries.

Augmentation of purinergic transmission to mesenteric arteries (Brock and Van Helden, 1995) and vas deferens (Guitart et al., 2002; Naito et al., 1998) occurs in tissues from SHRs. However, my data indicate that there was no alteration in nerve mediated ATP release or postjunctional reactivity to ATP in small mesenteric arteries from DOCA-salt rats. This conclusion is based on the results showing that nerve stimulation-induced ATP release and reactivity to exogenous ATP was not altered in DOCA-salt arteries. The discrepancy between my result and the results obtained from studies on SHR is likely due to the different hypertension model used for the studies. Nevertheless, this is the first study that has assessed ATP release from sympathetic nerve terminals in the mesenteric vascular bed in DOCA-salt hypertension.

2.1c. Increased noradrenergic component of neurogenic constrictions in arteries from DOCA-salt rats

My study showed that there is an increase in the contribution of NE to sympathetic neuroeffector mechanisms in DOCA-salt arteries as prazosin-induced inhibition of neurogenic constrictions was greatly increased in DOCA-salt arteries. This is a novel finding, indicating that DOCA-salt hypertension is associated with a change in the function of sympathetic nerves fibers associated with small mesenteric arteries.

Changes in postjunctional reactivity are not likely to contribute to the increased adrenergic component as the response of DOCA-salt arteries to exogenous NE is not altered. However, unaltered reactivity to exogenous NE does not necessarily indicate that there is no change in affinity or density of the α1–adrenergic receptors. Postjunctional reactivity depends on not only the density and affinity of the receptors, but also the cellular capacities to activate the specific second messenger pathways (De Champlain, 1990). It was shown that there is dissociation between receptor density, affinity and reactivity to exogenous agonists. For example, there was increased density of mesenteric α1-adrenergic receptor binding sites with a reduced affinity in mesenteric arteries from DOCA-salt rats. The increased density of α1-adrenergic receptors did not correlate with the vascular responses to electrical nerve stimulation and exogenous agonists (Meggs et al., 1988). Similarly, an augmented vascular sensitivity in mesenteric arteries from DOCA-salt hypertensive rats to NE co-exists with unaltered adrenergic receptor affinity (Perry and Webb, 1988). These results suggest that additional factors participate in the vascular response to adrenergic stimulation in this model including an alteration in a post-receptor activation event.

It is also unlikely α2- adrenergic receptor upregulation in small mesenteric arteries and direct activation of these receptors accounts for the increased neurogenic smooth muscle contractions in DOCA-salt tissues. This conclusion is based on data showing that clonidine or UK 14,304 did not directly contract small mesenteric arteries from sham or DOCA-salt rats.

2.1d. Potentiated neurogenic constrictions in mesenteric arteries

Constrictions caused by exogenous ATP and NE were not altered in DOCA-salt arteries, suggesting that the postjunctional reactivity to these neurotransmitters are maintained. Therefore, the potentiated neurogenic constrictions caused by nerve stimulation in DOCA-salt arteries compared to sham arteries must result from increased NE release, as ATP release was not altered.

Combined together, these data findings suggest that there is a potentiated adrenergic transmission in arteries in DOCA-salt hypertension. These results also confirm the notion that there is increased sympathetic nerve activity in DOCA-salt hypertension.

2.2. Alterations in sympathetic neuroeffector mechanisms in veins in DOCA-salt hypertension

2.2a. Increased NE release in DOCA-salt veins

There were maintained neurogenic constrictions in the presence of decreased postjunctional reactivity and increased NE release. The maintained neurogenic constriction is based on an increased release of NE from sympathetic nerves associated with mesenteric veins from DOCA-salt hypertensive rats. NE release from sympathetic nerves is increased according to the following evidence: i) NE content was lower in DOCA-salt veins due to a depletion of sympathetic NE stores (Luo et al., 2003); ii) direct measurements on the superfusate obtained from the blood vessels showed that nerve stimulation-induced NE release is increased in DOCA-salt veins. This result provides a potential mechanism underlying the increased venomotor tone found in DOCA-salt hypertensive rats (Fink et al., 2000). This was the first study that directly measured NE release in mesenteric veins and showed that NE release is increased in veins from DOCA-salt hypertensive rats.

My study also demonstrated that the neurogenic constrictions of DOCA-salt veins are maintained whereas a previous study from our laboratory showed that there was decreased postjunctional reactivity to NE (Luo et al., 2003). Considering that neurogenic constrictions depend on both the amount of transmitter release and postjunctional reactivity. The increased nerve mediated NE release demonstrated in my study provides direct evidence that sympathetic nerve activity is elevated in DOCA-salt veins. Reduced postjunctional reactivity results in the failure for the neurogenic constrictions of veins to increase despite the increased NE release.

2.2b. Increased NE uptake activity

My study demonstrated that NE uptake is increased in DOCA-salt veins, which may occur in response to the increased NE release and hence provides a compensatory mechanism to offset the potential for augmented postjunctional actions of NE. This is the first study that assessed NE uptake activity in mesenteric vascular beds in DOCA-salt hypertension. As the decreased postjunctional reactivity is reversed when NET uptake is blocked, it suggests that the decreased postjunctional reactivity is due to the decreased availability of NE as a result of enhanced NET uptake activity. Therefore, the increased NE release results in enhanced NET uptake activity, which leads to decreased postjunctional reactivity. Similar findings were obtained from studies on mesenteric and tail arteries in SHRs (Webb and Vanhoutte, 1981; Whall et al., 1980; Zsoter and Wolchinsky, 1983). However, studies on caudal arteries of DOCA-salt hypertension indicate that there was no change in uptake activity (Longhurst et al., 1988). In addition, *in vivo* studies suggest that neuronal uptake of NE was not altered in DOCA-salt hypertension (Drolet et al., 1989). Therefore, it appears that there are vascular bed-dependent alterations of NE uptake activity in DOCA-salt hypertension.

The increased uptake of NE usually results from enhanced release and/or synthesis of the neurotransmitter (Houchi et al., 1993), which could serves as a compensatory mechanism for the increased NE release in DOCA-salt rats. However, the precise mechanism underlying the enhanced NE uptake activity remains to be elucidated. It could be due to a direct action of NE on NET or

through indirect mechanism. For example, it was shown that enzyme activities such as Na^+-K^+-ATPase and Mg^{2+}-activated ATPase are increased in mesenteric arteries of hypertensive animals (Gardner and Laing, 1965; Oka and Angrist, 1967; Ooshima, 1973). Since NE uptake is activated by Na^+-K^+-and Mg^{2+}-dependent ATPase, their enhanced activities could account for the increased NE uptake in DOCA-salt hypertension.

2.3. Sympathetic nerve activity is increased in both arteries and veins from DOCA-salt hypertensive rats.

2.3a. Increased NE release is due to the facilitated transmitter-releasing process in DOCA-salt arteries and veins.

In my studies, the same nerve stimulation parameters were used to cause neurogenic constrictions of sham and DOCA-salt arteries and veins. Therefore, the increased NE release observed in DOCA-salt arteries and veins is not due to increased firing frequency of the sympathetic nerves associated with these blood vessels which could occur *in vivo*. Instead, changes in the transmitter-releasing process at the axonal terminal level must account for this phenomenon. These changes could include: i) more effective electrical coupling due to changes in electrical properties of the axonal plasma membrane; ii) increased NE synthesis and storage; iii) increased size of the readily releasable pool of NE. These changes could partly result from attenuated inhibitory effects by autoreceptors and enhanced facilitatory effects by heteroceptors.

Consistent with the antihypertensive effects of drugs that block adrenergic receptors, these results support the notion that NE plays an important role in the pathogenesis of hypertension. Increased NE release in DOCA-salt arteries and veins could contribute to the increased neurogenic constrictions of the blood vessels. Increased NE release in arteries and veins from DOCA-salt hypertensive rats also suggests that sympathetic nerve activity is increased during the maintenance stage of hypertension. Thus, it favors the hypothesis that sympathetic nervous system not only plays an important role in the initiation of hypertension, but also the maintenance of DOCA-salt hypertension.

2.3b. NE acts directly at α1-adrenergic receptors in mesenteric arteries and veins from sham and DOCA-salt rats.

My study demonstrated that only α1-adrenergic receptors directly mediate NE-induced constrictions of arteries and veins, which is consistent with other studies (Shi et al., 1989; Cauvin and Malik, 1984). However, others have shown that in canine mesenteric veins α2-adrenergic receptors contribute significantly to NE-induced constrictions (Shi et al., 1990; Shoji et al., 1983; Itoh et al., 1987). My study clearly demonstrated that direct activation of α2-adrenergic receptors by its selective agonist, UK 14,304, does not cause significant constriction of veins. Therefore, if α2-adrenergic receptors are expressed by rat small mesenteric arteries and veins, their function is not clear. The discrepancies between results obtained from those studies and my study suggest that there are species-dependent

differences in the expression or function of the postjunctional α2-adrenergic receptors.

2.4. Differences in alterations of sympathetic neuroeffector transmission to arteries and veins in DOCA-salt hypertension

The major difference in alterations of sympathetic neuroeffector transmission to arteries and veins are as follows: i) neurogenic constriction of veins is maintained, but neurogenic constriction of arteries is potentiated from DOCA-salt hypertensive rats; ii) postjunctional reactivity of DOCA-salt arteries is not changed while in DOCA-salt veins postjunctional reactivity is decreased.

Although neurogenic constrictions of veins are not elevated, the increase in nerve-mediated NE release in veins indicates that the activity of sympathetic nerves associated with veins is increased. Therefore, sympathetic neuroeffector transmission to mesenteric arteries and veins is potentiated in DOCA-salt hypertensive rats. Nevertheless, the fact that neurogenic constrictions are maintained in DOCA-salt veins, but potentiated in DOCA-salt arteries does suggest that there is a temporal disparity in alterations of sympathetic neuroeffector transmission to arteries and veins. This temporal disparity could reflect the distinct role played by the venous and arterial circulation in the development of DOCA-salt hypertension. There is no doubt that at the early stage of DOCA-salt hypertension, sympathetic nerve activity is elevated (Julius et al., 1988; Esler et al., 1989; Izzo, 1998). Considering that the decreased

reactivity of postjunctional α1-adrenergic receptors occurs secondary to the increased transmitter release, at the early stage of hypertension development when the receptor reactivity is not yet decreased neurogenic constrictions of veins must be potentiated. The elevated neurogenic constrictions of veins would in increased cardiac output that is known to occur in early stage of hypertension (Lund-Johansen, 1994). Therefore, it also supports the notion that veins as the capacitance vessels play an important role in the initiation of hypertension whereas arteries providing the peripherial resistance are important in maintaining the high blood pressure (Lund-Johansen, 1989; Coleman and Hall, 1993).

3. Alterations in local modulatory mechanisms of sympathetic neuroeffector transmission to arteries and veins in DOCA-salt hypertensive rats

3.1. Alterations in prejunctional adrenergic autoreceptors in arteries and veins in DOCA-salt hypertensive rats

3.1a. Prejunctional adrenergic autoreceptors associated with mesenteric arteries regulate ATP and NE release and are impaired in DOCA-salt hypertensive rats.

My study demonstrated that α2-adrenergic receptors regulate ATP release. This result is consistent with the results obtained from other studies showing that

yohimbine increased the overflow of ATP caused by electrical field nerve stimulation (Westfall et al., 1996; Bobalova and Mutafova-Yambolieva, 2001a). The regulation by α2-adrenergic receptors of ATP release indicates that ATP and NE release are regulated in a similar manner. However, the result that α2-adrenergic receptors regulate both ATP and NE release does not provide evidence supporting the notion that ATP and NE are stored in the same synaptic vesicles. Release of ATP and NE may be differentially regulated by prejunctional α2-adrenergic receptors. For example, the reduction of ATP release caused by the α2 agonists, xylazine and clonidine, was greater than the reduction in NE release (Westfall et al., 1996). Furthermore, pretreatment with the α2 receptor antagonists, idazoxan and yohimbine, produced a substantial increase in NE overflow and a lesser increase in ATP overflow (Westfall et al., 1996). Therefore, although prejunctional α2-adrenergic receptors regulate both ATP and NE release, they may be regulated by different subsets of prejunctional α2-adrenergic receptors or the same α2-adrenergic receptors my couple to different inhibitory mechanisms linked to NE and ATP release.

3.1b. Function of prejunctional adrenergic autoreceptors associated with mesenteric arteries is impaired in DOCA-salt hypertensive rats.

My study demonstrated that the function of prejunctional adrenergic autoreceptors associated with mesenteric arteries is impaired in DOCA-salt hypertensive rats as yohimbine had inhibitory effects on NE release in sham arteries, but not DOCA-salt arteries. This result is consistent with other studies

showing that prejunctional α2-adrenergic receptor function is impaired in hypertensive animals. For example, intravenously administered yohimbine, an α2-adrenergic receptor antagonist, increased plasma NE levels in sham normotensive, but not in DOCA-salt hypertensive rats (De Champlain, 1990). Furthermore, yohimbine potentiated nerve stimulation-evoked NE release into the mesenteric vasculature in normotensive rats, but not in DOCA-salt hypertensive rats (Tsuda et al., 1989). The increased NE release found in portal vein and caudal artery of SHR rats was due to a decrease in the functional activity of prejunctional α2-adrenergic receptors (Westfall et al., 1986).

As my study also showed that nerve stimulation-induced NE release was increased in DOCA-salt arteries, it is possible that the impairment of the prejunctional α2-adrenergic receptors is responsible for the increased NE release in DOCA-salt arteries. Therefore, the impaired function of prejunctional α2-adrenergic receptors could contribute to the increased sympathetic nerve activity in experimental hypertension (De Champlain, 1990).

In DOCA-salt arteries, function of the prejunctional α2-adrenergic receptors is impaired. However, ATP release is not increased in DOCA-salt arteries, which suggest that ATP release is also regulated by other factors such as the prejunctional P1-receptors (Langer, 1980). Alterations in those regulatory factors may have counteracted the impaired function of α2-adrenergic receptors in DOCA-salt hypertension to maintain ATP release at a normal level. For example, changes in local mechanism modulating ATP release were proposed to be considered in SHR (Cheung, 1989). Furthermore, the unaltered ATP release

compared to an increased NE release appears to favor the notion that ATP and NE may not be co-released from the sympathetic nerve terminals (Westfall et al., 1996), at least in mesenteric arteries from DOCA-salt rats.

3.1c. Prejunctional adrenergic autoreceptors associated with mesenteric veins are impaired in rats from DOCA-salt hypertension.

My study demonstrated that the function of prejunctional α2-adrenergic receptors associated with mesenteric veins is impaired in DOCA-salt hypertensive rats. This is the first study that assessed the function of prejunctional α2-adrenergic receptors in mesenteric veins in DOCA-salt hypertension. The impaired function of α2-adrenergic receptors could account for the increased NE release observed in DOCA-salt veins.

The mechanism underlying the impaired function of the α2-adrenergic autoreceptor function remains to be elucidated. Attenuated function of the α2-adrenergic autoreceptors will cause increased NE release, which in turn can desensitize the receptors and lead to further NE release. Thus, a positive feedback cycle forms and leads to the impairment of the α2-adrenergic autoreceptor function (De Champlain, 1989). The impaired function of the α2-adrenergic autoreceptors could also be due to the imbalance between two prejunctional modulatory mechanisms: the inhibitory α2-adrenergic receptor and the facilitatory β-adrenergic receptor as in DOCA-salt hypertension there co-

exists an attenuation of the α2-adrenergic receptor function and an enhancement of β-adrenergic receptor function (De Champlain, 1990).

The impaired function of the prejunctional α_2-adrenergic receptors indicate that there could be a down-regulation of receptor function, which could be due to a decreased receptor density and/or receptor affinity. There could also be a functional impairment of the signal transduction pathways linked to the prejunctional α2-adrenergic receptors. There are a few studies suggesting that sodium ion affects the reactivity of prejunctional α2-adrenergic receptors. For example, increased dietary sodium intake decreases the affinity of brainstem α2-adrenergic receptors (Gavras, 1986). Furthermore, Limbird et al. (1982) reported that increased sodium concentration inhibits the binding of α-adrenergic receptor agonists *in vitro*. These results suggest that sodium loading can decrease the responsiveness of α2-adrenergic receptors and this could be a mechanism by which sodium loading contributes to hypertension development. Nevertheless, little is known of the precise mechanisms by which sodium affects the reactivity of α2-adrenergic receptors (Limbird et al., 1982).

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In conclusion, prejunctional α2-adrenergic receptors on sympathetic nerves regulate ATP release in sham mesenteric arteries and NE release in sham arteries and veins. These receptors are impaired in DOCA-salt arteries and veins, which might account for the increased NE release. The potentiated neurogenic responses of arteries (Luo et al., 2003) and the increased venomotor

tone in DOCA-salt hypertensive rats (Fink, et al., 2000) could be attributable to the increased NE release in arteries and veins, respectively. Therefore, DOCA-salt hypertension is associated with an alteration of neuronal function.

3.2. Alterations in modulatory effects mediated by ET receptors in arteries and veins from DOCA-salt hypertensive rats

3.2a. Alterations in modulatory effects mediated by ET receptors in arteries from DOCA-salt hypertensive rats

1) ET-1 acting at postjunctional ET_A receptors differentially modulates postjunctional α1-adrenergic receptors and P2X receptors.

My study demonstrated that subcontractile ET-1 presumably acting at postjunctional ET_A receptors decreased the reactivity of sham arteries to ATP and increased the reactivity to NE. This result indicates that ET-1 acting at ET_A receptors differentially modulates P2X receptors and α1-adrenergic receptors as these two receptors mediate the constrictions caused by ATP and NE respectively in arteries (Galligan et al., 2001; Luo et al., 2003). The differential modulation mediated by ET_A receptors is perhaps related to the different intracellular signaling mechanisms linked to the P2X receptors and α1-adrenergic receptors activation. For example, P2X receptors are ligand-gated ionotropic receptors and non-selectively permeable to cations including calcium. The depolarization of vascular smooth muscle cells mediated by P2X receptors also

cause opening of voltage-dependent calcium channels and provides an additional source of calcium influx (Benham and Tsien, 1987). The rise in the cytosolic level of calcium triggers the contraction of smooth muscle. The α1-adrenergic receptors are metabotropic G-protein coupled receptors. The intracellular mechanism involves the activation of Gq protein and production of IP_3 via a phosphoinositide pathway that leads to release of calcium from the intracellular calcium store (Docherty, 1998). Similarly, ET_A receptors are also G-protein coupled receptors. Activation of vascular ET_A receptors stimulates phospholipase C and subsequent hydrolysis of phosphatidylinositol, which results in the formation of IP3 and DAG and calcium release from intracellular stores (Rubanyi and Polokoff, 1994). Thus, ET_A and α1-adrenergic receptors are linked to the same intracellular pathway to induce vascular constrictions. Therefore, pretreatment with subcontractile ET-1 acting at ET_A receptors could activate and prime the common intracellular signaling pathways, which leads to the amplification of the signals produced by the subsequent adrenergic receptors activation. This could be the mechanism underlying the facilitatory effect of the adrenergic receptor sensitivity by ET-1 acting at ET_A receptors. This hypothesis is supported by results obtained from studies on rabbit aorta (Henrion and Laher, 1993). These studies demonstrated that potentiation by subcontractile ET-1 (100 pM) of the sensitivity of the rabbit aorta to NE was unaffected by removal of the endothelium or changes in Ca^{2+} entry, but was prevented by protein kinase C inhibitors, suggesting that ET-1 increases the sensitivity to NE by activating protein kinase C-dependent mechanisms. However, the mechanism by which the

ET_A receptors decreased the sensitivity of P2X receptors is unknown. It may be due to inhibition of the P2X receptors by molecules produced in response to the ET_A receptors activation.

2) ET-1 facilitates sympathetic neuroeffector transmission to sham, but not DOCA-salt arteries, which could be due to the down-regulation of the postjunctional ET_A receptors in DOCA-salt hypertension.

My study demonstrated that ET-1 presumably acting at postjunctional ET_A receptors potentiated sympathetic neuroeffector transmission to sham arteries and this effect was not observed in DOCA-salt arteries. Furthermore, ET-1 altered the postjunctional reactivity of sham arteries to ATP and NE and these effects were not observed in DOCA-salt arteries. The disappearance of modulatory effects by ET-1 on DOCA-salt arteries is likely due to the down-regulation of the ET_A receptors as indicated by a reduced ET-1 induced constrictions of mesenteric arteries from DOCA-salt rats (Johnson et al., 2002). It was also found that binding of endothelium denuded mesenteric artery to ET-1 and subsequent accumulation of IP3 induced by ET-1 application was significantly lower in DOCA-salt rats compared to normal rats, suggesting that decreased ET receptor density results in decreased activation of ET receptors (Nguyen et al., 1992). The down regulation of the ET_A receptors is likely a result of increased vascular ET-1 production in DOCA-salt rats (Lariviere et al., 1993; Day et al., 1995;Yu et al., 2002). Hence, the result that ET-1 facilitates sympathetic neuroeffector transmission to sham arteries infers that increased

ET-1 production would enhance the sympathetic constriction of arteries and leads to blood pressure elevation. This would occur when the ET receptors are not yet down regulated as down-regulation of the receptors occurs secondary to increased vascular ET-1 production (Lariviere et al., 1993).

3) Facilitatory prejunctional ET_B receptors are activated to overcome the inhibitory prejunctional ET_A receptors and likely contribute to the increased NE release in DOCA-salt arteries.

My study demonstrated that S6c, the ET_B receptor agonist, potentiated, via a prejunctional mechanism, sympathetic neuroeffector transmission to DOCA-salt arteries, but not sham arteries. This result suggests that prejunctional ET_B receptors mediate a facilitatory effect on transmitter release in DOCA-salt arteries. This facilitatory effect mediated by prejunctional ET_B receptors in DOCA-salt arteries could be due to the functional activation of the receptors in DOCA-salt hypertension. An alternative explanation is that the facilitatory effect is unmasked in DOCA-salt arteries due to the impaired function of inhibitory endothelial ET_B receptors. This hypothesis is supported by the result showing that indomethacin and NLA, the inhibitors for vasodilators produced by endothelial ET_B receptor activation, enhanced ET-1-induced constrictions of sham arteries whereas constrictions of arteries from DOCA-salt rats were unaffected by these two drugs (Johnson et al., 2002). This result, however, could also simply reflect a generally impaired function of the endothelial cells in DOCA-

salt hypertension rather than a specific impairement of the endothelial ET_B receptors.

However, ET-1, the non-selective ET_A and ET_B receptor agonist, had no effect on sympathetic neuroeffector transmission to DOCA-salt arteries. Taken together, these results suggest that ET_A receptors mediate an inhibitory effect whereas ET_B receptors a facilitatory effect on transmitter release in DOCA-salt arteries. The notion that ET-1 acting at ET_A receptors to inhibit transmitter release is based on results obtained from a number of studies showing that in normal animals ET-1 inhibits NE release from sympathetic nerve terminals in mesenteric vascular bed (Zhang et al., 1996; Tabuchi et al., 1990) and non vascular tissues including rat and guinea pig vas deference (Wiklund et al., 1990; Wiklund et al., 1991) and stomach (Nakamura et al., 2003). Some of those studies identified that the receptor subtype mediating this inhibitory effect of ET-1 is the ET_A receptor (Zhang et al., 1996). Hence, although the effect of ET-1 on co-transmitter release remains to be established, my study does support that in sham arteries ET_A receptors mediates an inhibitory effect on transmitter release whereas in DOCA-salt arteries this inhibitory effect is overcome by a facilitatory effect mediated by ET_B receptors. The activation of a facilitatory prejunctional ET_B receptors leading to the disinhibition of transmitter release could in part account for the increased NE release in DOCA-salt arteries.

3.2b. Alterations in modulatory effects mediated by ET receptors in veins from DOCA-salt hypertensive rats

1) ET_B receptors mediate facilitation of sympathetic constriction of DOCA-salt veins via a postjunctional effect.

My study demonstrated that ET_B receptors mediate a facilitatory effect on sympathetic neuroeffector transmission to DOCA-salt veins at least partly by increasing the postjunctional reactivity to NE. It was shown that there is no functional change of the postjunctional ET_B receptors in veins from DOCA-salt rats as the reactivity of DOCA-salt veins to S6c was not altered (Johnson et al., 2002). Therefore, the facilitatory effect mediated by ET_B receptors on sympathetic neuroeffector transmission to DOCA-salt veins must be attributed to factors other than functional change of the vascular ET_B receptors.

The α1-adrenergic receptors and ET_B receptors both are metabotropic G-protein coupled receptors. Vascular constrictions caused by the α1-adrenergic receptors and ET_B receptors both involve production of IP_3 via a phosphoinositide pathway leading to release of calcium from the intracellular calcium store (Docherty, 1998; Rubanyi and Polokoff, 1994). In DOCA-salt veins intracellular signaling pathways linked to ET_B and α1-adrenergic receptors activation could be somehow coupled so that the activation of ET_B receptors could lead to the enhancement of the signal transduction associated with the α1-adrenergic receptor activation. The coupling of these two signaling pathways seems to be a

change associated with DOCA-salt hypertension as the modulation did not occur in veins from sham rats.

The facilitatory effect mediated by postjunctional ET_B receptors in DOCA-salt veins is also likely due to the unmasking of this effect by the impaired function of inhibitory endothelial ET_B receptors. Dysfunction of the endothelial ET_B receptors in DOCA-salt veins is demonstrated by the findings that NLA, the blocker for vasodilators produced by endothelial ET_B receptors activation, enhanced S6c-induced constrictions of sham veins, but not DOCA-salt veins (Johnson et al., 2002). This finding, however, could also simply reflect a generally impaired function of the endothelial cells in DOCA-salt hypertension rather than a specific impairement of the endothelial ET_B receptors.

2) The facilitatory effect mediated by ET_B receptors on sympathetic neuroeffector transmission to DOCA-salt veins could contribute to the increased MCFP.

Veins are more sensitive than arteries to ET-1 (Johnson et al., 2002). Studies *in vivo* showed that intravenous administration of ETs increased central venous pressure, venous resistance and MCFP (Waite and Pang, 1990; Yamamato et al., 1980). ET-1-induced constriction of hand veins of human subjects with essential hypertension is greater compared to normotensives (Haynes et al., 1994). Furthermore, ET-1 induced- venoconstriction correlated positively with mean arterial pressure in the hypertensive subjects, but not in the normotensives. In DOCA-salt hypertension, ET-1 induced smooth mscule

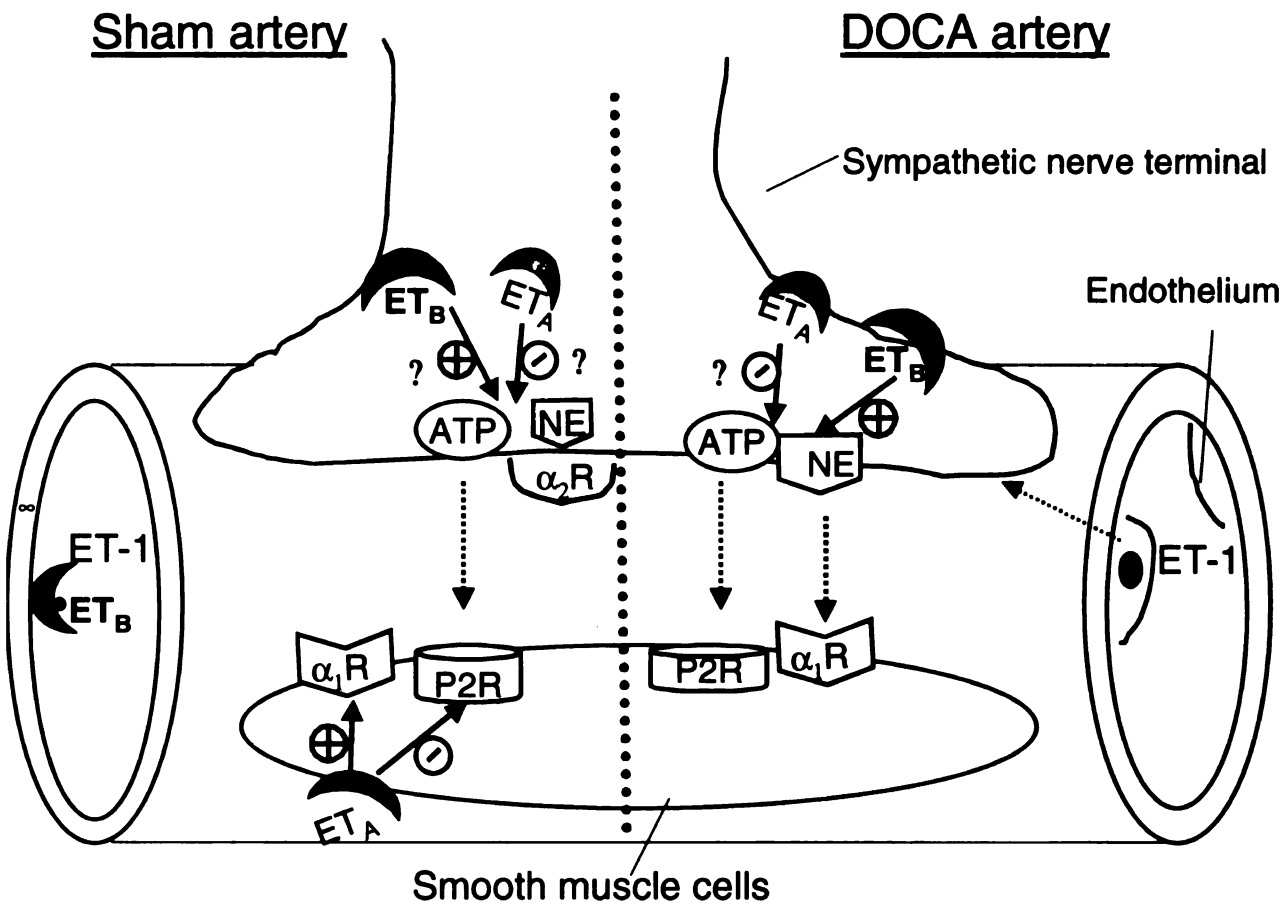
contraction was reduced in rat aorta (Watts et al., 2002) and mesenteric arteries (Johnson et al., 2002), but maintained in vena cava (Watts et al., 2002) and mesenteric veins (Johnson et al., 2002). Furthermore, ET-1 exerts a greater effect on venomotor tone *in vivo* in DOCA-salt rats compared to normal rats, and these data are consistent with a role for ET-1 in DOCA-salt hypertension (Fink et al., 2000)

In DOCA-salt rats there is an elevated sympathetic drive to the venous system as indicated by a greater decrease of the MCFP via blockade of sympathetic ganglionic transmission in DOCA-salt rats compared to the normal rats (Fink et al., 2000). The elevated sympathetic drive and venomotor tone observed in DOCA-salt rats (Fink et al., 2000) can attribute to the facilitatory effects mediated by ET_B receptors on sympathetic neuroeffector transmission to veins demonstrated by my study. Similar to my result, it was shown that sympathetically mediated venoconstriction was substantially potentiated by ET-1 in hypertensive, but not normotensive subjects (Haynes et al., 1994). The venous system plays an important role in blood pressure regulation by serving the capacitance function. Elevated sympathetic drive to the venous system would increase venous return and cardiac output, which will lead to elevated blood pressure (Guyton et al., 1972b).

3.2c. Differential alterations of modulation by ET receptors on sympathetic neuroeffector transmission to arteries and veins

My study demonstrated that ET receptors mediate a differential modulation of sympathetic neuroeffector transmission to arteries and veins, which lies in the following aspects: 1) ET_B receptors are differentially expressed in arteries and veins as they are expressed on the smooth muscle cells of the veins and on the sympathetic nerves of the arteries. 2) In arteries, ET-1 acting at ET_A receptors facilitates sympathetic neuroeffector mechanisms in sham rats, which could contribute to the elevation of peripheral resistance and hence hypertension development. This facilitation by ET-1 was removed in DOCA-salt hypertension possibly due to the down-regulation of the vascular ET_A receptors in the maintenance stage of DOCA-salt hypertension. In veins, ET-1 acting at postjunctional ET_B receptors facilitates sympathetic transmission in DOCA-salt rats. This facilitation is likely to contribute to the increased venomotor tone observed in DOCA-salt rats (Fink et al., 2000).

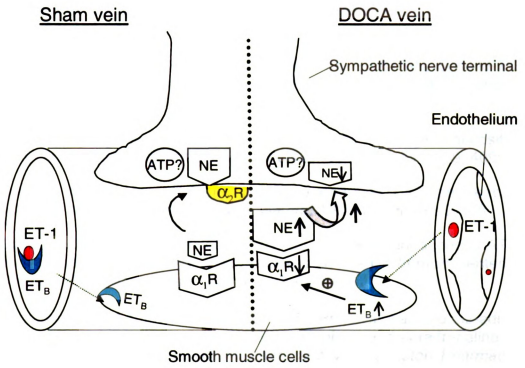
Sympathetic neuroeffector transmission to arteries



In sham arteries, ATP acting at P2X receptors functions as the dominant neurotransmitter. NE release is inhibited by prejunctional α2-adrenergic autoreceptors. ET-1 acting at postjunctional ET_A receptors exerts an inhibitory effect on P2 receptors and a facilitatory effect on α1-adrenergic receptors. The latter effect could be the mechanism by which subcontractile ET-1 potentiated sympathetic neuroeffector transmission to sham arteries.

In DOCA-salt arteries, NE release is increased due to the impaired function of presynaptic α2-adrenergic receptors, which could account for the increased adrenergic component in sympathetic neuroeffector transmission as well as the potentiated sympathetic constriction. ATP release is maintained. Postjunctional ET_A receptors are down regulated possibly due to the increased ET-1 production. A facilitatory effect mediated by prejunctional ET_B receptors for transmitter release is unmasked due to the impaired function of inhibitory endothelial ET_B receptors producing vasodilators, which could also contribute to the increased NE release. Images in this dissertation are presented in color.

Sympathetic neuroeffector transmission to veins



In sham veins and DOCA-salt veins, NE acting at the α1-adrenergic receptor is the neurotransmitter. NE release is increased in DOCA-salt veins, which stimulates the up-regulation of NE uptake system. This increased NE release is due to the impaired function of the prejunctional α2-adrenergic autoreceptors. The postjunctional ET_B receptors mediates a potentiating effect on sympathetic transmission to DOCA-salt veins by increasing the postjunctional reactivity to NE, which could contribute to the increased venomotor tone of DOCA-salt veins. Images in this dissertation are presented in color.

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