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Neurohumoral Control of Splanchnic Circulation in DOCA-Salt Hypertensive Rats

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NEUROHUMORAL CONTROL OF SPLANCHNIC CIRCULATION IN DOCA-SALT HYPERTENSIVE RATS

By

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ABSTRACT

NEUROHUMORAL CONTROL OF SPLANCHNIC CIRCULATION IN DOCA-SALT HYPERTENSIVE RATS

By

Hong Wang

Most studies of the etiology of hypertension have focused on altered regulation of the arterial vasculature. While arterial resistance is elevated in hypertension, there is also a decrease in venous capacitance. Enhanced venous smooth muscle contractile activity appears to be an important factor accounting for decreased systemic venous capacitance in hypertension. There have been few studies of the mechanisms causing constriction of veins in hypertension. The goal of my thesis work is to identify the mechanisms responsible for increased venoconstriction in DOCA-salt model of hypertension.

Existing evidence suggests that there is both increased endothelin-1 (ET-1) and sympathetically mediated venoconstriction in DOCA-salt rats. However, it is not clear if this is due to increased ET-1 levels and/or sympathetic activity in the splanchnic vasculature, the main capacitance bed of the circulation. My thesis work tested the overall hypothesis that ET-1 levels and/or sympathetic nerve activity in the splanchnic vasculature was increased in DOCA-salt hypertensive rats.

The results of my work provide strong evidence that higher endothelial cell formation of ET-1 is not likely responsible for enhanced ET-1 mediated splanchnic venoconstriction in DOCA-salt rats *in vivo*. When combined with the fact that venous

smooth muscle contractile responsiveness to ET-1 is not increased in DOCA-salt rats, the combined data suggest that the ability of ET receptor antagonists to reduce venoconstriction in DOCA-salt rats *in vivo* results from actions of the drugs on a non-vascular target, i.e. ET-1 may exert indirect control of venous tone in vivo, for example, via the modulation of sympathetic neurotransmission.

By estimation of nonhepatic splanchnic norepinephrine spillover rate, my work provides direct evidence that there is increased splanchnic sympathetic activity in DOCA-salt hypertension, and altered local modulation of sympathetic neurotransmission, including impaired presynaptic α2-adrenoceptor function and inhibitory effect of ET-1, contribute to sympathetic activation in this model of hypertension.

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LIST OF ABBREVIATIONS

DOCA .....deoxycorticosterone acetate

ET-1 .....endothelin isoform 1

i.p .....intraperitoneal

i.v .....intravenous

MAP.....mean arterial pressure

SBP .....systolic blood pressure

DBP.....diastolic blood pressure

SNS .....sympathetic nervous system

SNA.....sympathetic nerve activity

VSMC .....vascular smooth muscle cell

NE .....norepinephrine

NA.....norepinephrine

EPI.....epinephrine

NO.....nitric oxide

MCFP.....mean circulatory filling pressure

vWF.....von Willebrand Factor

PBS .....physiological buffered saline

S6C.....sarafotoxin 6C

DHPG.....dihydroxylphenylglycol

MHPG.....3-methoxy-4-hydroxylphenylglycol

DMI.....desipramine

CHAPTER 1

GENERAL INTRODUCTION

1. Hypertension

Hypertension is defined as a sustained elevated blood pressure (systolic blood pressure of 140 mmHg or greater and/or diastolic blood pressure of 90 mmHg or greater). Hypertension is a major risk factor for stroke, heart failure, coronary artery disease, renal failure and other conditions.

2. Regulation of blood pressure

Maintaining the homeostasis of blood pressure involves a complex interplay between the nervous, endocrine, renal and cardiovascular systems. The autonomic nervous system directly regulates the cardiovascular system by controlling vascular tone and cardiac strength. The kidney controls sodium and water balance and thus the blood volume. The endocrine system releases hormonal factors to act on the cardiovascular system, such as the renin-angiotensin system, vasopressin, and epinephrine. The cardiovascular system itself can regulate blood pressure by secreting a number of vasoactive factors including endothelin-1, nitric oxide (NO), atrial natriuretic factor. These systems interact with each other and form a complicated and delicate blood pressure controlling system.

Different mechanisms are responsible for the short-term and long-term control of blood pressure. Short-term regulation (time frame of seconds to minutes) is dominated by the baroreceptor mechanism. Changes in mean arterial pressure are sensed by the baroreceptors and processed by the vasomotor centers in the medulla which differentially regulate sympathetic and parasympathetic nervous system output. From the long-range point of view (hours to days) the control of fluid balance by the kidney, adrenal cortex,

and central nervous system, with maintenance of a constant blood volume, is generally considered to be of the greatest importance.

3. Determinants of blood pressure

Mean arterial pressure is determined by systemic vascular resistance and cardiac output. Peripheral vascular resistance is regulated largely by the caliber of small arteries and arterioles. Cardiac output depends on heart rate, cardiac contractility, and the amount of blood returning to the heart, which in turn depends on venous and venular capacitance, and blood volume.

3.1 Arterial microvasculature

3.1.1 The small arteries, arterioles, and capillaries account for 90% of vascular resistance

The larger arteries have a low resistance to blood flow and function primarily as conduits. However, as arteries approach the organ they perfuse, they divide into many small arteries both just outside and within the organ. These smallest of arteries and the arterioles of the microcirculation constitute the resistance blood vessels; together they regulate about 90% of the total vascular resistance. Contraction and relaxation of the smooth muscle in the walls of small arteries and arterioles cause changes in vessel diameter, which, in turn, influence blood flow.

3.1.2 Regulation of microvascular resistance

The vascular smooth muscle cells around arterioles respond to a wide variety of physical and chemical stimuli, altering the diameter and resistance of the microvessels.

3.1.2.1 Myogenic regulation

Vascular smooth muscle is able to contract rapidly when stretched and conversely, to reduce actively developed tension when passively shortened. A vessel's ability to constrict when intravascular pressure is increased or dilate as the pressure is decreased is called myogenic regulation. The most important benefit of myogenic regulation is that blood flow and downstream pressure can be indirectly regulated when the arterial pressure is too high or low for appropriate tissue perfusion. The myogenic response is rapidly activated (within 2 seconds) by changes in arterial and venous pressure. This allows rapid modifications in microvascular resistance before appreciable changes in the tissue chemical and metabolic environment occur.

3.1.2.2 Tissue metabolites

Blood flow is also governed by the metabolic activity of the tissue. Any intervention that results in an O_2 supply that is inadequate for the requirements of the tissue prompts the formation of vasodilator metabolites. These metabolites are released from the tissue and act locally to dilate the resistance vessels. Many substances have been proposed as mediators of metabolic vasodilation, such as adenosine, lactic acid, CO_2 and hydrogen ions.

3.1.2.3 Endothelial cells are capable of releasing chemicals that cause relaxation and constriction of arterioles

The endothelium regulates local vascular tone through release of vasodilator (e.g. NO) and vasoconstrictor substances. Endothelin-1 (ET-1), synthesized by endothelial cells, is a very potent vasoconstrictor. It causes arterial contraction by binding to ETA receptors on the smooth muscle cells (More details on the endothelin system are provided later).

3.1.2.4 The sympathetic nervous system regulates blood pressure and flow by constricting the microvessels

The sympathetic nervous system (SNS) plays a critical role in the maintenance of physiological homeostasis in general, and arterial blood pressure in particular under basal (resting) conditions and in response to acute stress. Post-ganglionic sympathetic neurons innervating the heart and resistance vessels help control cardiac output, arterial blood pressure and regional vascular conductance, thus ensuring the proper perfusion of vital organs. Sympathetic nervous system stimulation of epinephrine release from the adrenal medulla contributes importantly to the regulation of cardiovascular function as well as energy metabolism.

Although the microvasculature uses local control mechanisms to adjust vascular resistance based on the physical and chemical environment of the tissue and vasculature, the dominant central regulatory system is the sympathetic nervous system. The arterial pressure is monitored moment-to-moment by the baroreceptor system, and the brain adjusts the cardiac output and systemic vascular resistance as needed via the sympathetic

and parasympathetic nervous systems. Sympathetic nerves communicate with the resistance vessels and venous system through the release of norepinephrine onto the surface of smooth muscle cells in vessel walls.

3.2 Venous microvasculature

3.2.1 Venous compliance and capacitance

Blood vessels are elastic, and they expand when the blood in them is under pressure. The degree to which a distensible vessel expands when it is filled with fluid is determined by the distending pressure and its compliance. Compliance (c) is defined by the equation:

$$C = \Delta V / \Delta P$$

Where ΔV is the change in volume and ΔP is the change in distending pressure. The distending pressure is equal to the pressure inside the vessel minus the pressure outside the vessel; this is called the transmural pressure. The more compliant a structure, the greater the change in volume for a given transmural pressure change.

Venules are much thinner and contain much less elastin tissue than arterioles. Because of these structural differences, venules are up to 60 times more distensible than arterioles.

Vascular capacitance is the volume of blood contained in the vascular tree at a given transmural distending pressure. The compliance of veins is far larger than any other part of the systemic circulation. In addition, venules have a cross-sectional area that is far larger than their arteriole counterparts. Consequently, venules have the capacity to store 3-4 times more blood than arterioles. Thus, venules are often described as the capacitance

section of the vascular bed and usually contain approximately 2/3 of the body's total blood volume. Vascular capacitance is therefore determined almost entirely by the structure and smooth muscle constrictor activity of veins.

3.2.2 Methods to estimate venoconstriction in vivo

A variety of methods are available to estimate venoconstriction. The best approach so far in experimental animals in vivo is by measurement of mean circulatory filling pressure (MCFP). MCFP is defined as the mean vascular pressure that exists after a cessation in cardiac output and redistribution of blood, so that all pressures are the same throughout the system (187). It represents the effective driving force for venous return of blood to the heart. MCFP is affected by both blood volume and vascular capacitance (almost entirely venous). A change in MCFP provides a uniquely useful index of a change in overall venous smooth muscle tone if the blood volume is not concomitantly changed.

3.2.3 Factors regulating venous compliance

The walls of veins contain variable amount of fibrous connective tissue and smooth muscle. Venous compliance thus is affected by 1) alterations in salt and water content of the venous wall (172) and alteration in connective tissue and/or smooth muscle mass 2) or contraction of smooth muscle in the vein wall (venoconstriction or increased venomotor tone).

Venous tone in different vascular beds is regulated in different ways depending on their nature (189, 244). Cutaneous venous tone is regulated by the thermoregulatory

system. Skeletal veins play an important role, especially during exercise, as a muscle pump. Splanchnic venous tone largely contributes to mobilization of blood volume by sympathetic nerve stimulation (82, 188).

3.2.3.1 Sympathetic nervous system

3.2.3.1.1 Synthesis, action and fate of norepinephrine at sympathetic neuroeffector junctions (Fig. 1)

Sympathetic nerves travel along arteries and veins and nerves are found in the adventitia. Capillaries receive no innervation.

NE synthesis and storage take place in the vesicles of the varicosity. An action potential causes an influx of Ca++ into the nerve terminal, with subsequent fusion of the vesicle with the plasma membrane and exocytosis of NE. NE is the main neurotransmitter, although neuropeptide Y and ATP also are released (212) (245). The transmitter then activates α- and β-adrenergic receptors in the membrane of the postjunctional cell. NE that penetrates into these cells (a process termed uptake2) is rapidly inactivated by catechol-O-methyltransferase to normetanephrine (NMN). In some organs, the most important mechanism for termination of the action of NE in the junctional space is active reuptake into the nerve (a process termed uptake 1) by high affinity transporters on the plasma membrane of sympathetic neurons. The amount of NE released is tightly regulated by negative feedback mechanisms involving not only NE itself (via α2-adrenergic receptors) but also many other mediators such as EPI, serotonin, histamine and acetylcholine.

The effects of NE at the postjunctional level, i.e. vascular smooth muscle cells of blood vessels and myocytes of the myocardium, involve β-adrenergic receptors, as well as α1- and α2-adrenergic receptors. While the former are involved in vasodilation (β2-adrenergic receptors) and inotropy (β1-adrenergic receptors), the latter mediate vasoconstriction of the blood vessel wall.

3.2.3.1.2 Differential sympathetic neural control of arteries and veins

The sympathetic neurons innervating arteries and veins differ in their location in the ganglia and in their electrophysiological properties (17), suggesting that there is a differential sympathetic neural control of mesenteric arteries and veins (95). 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 contraction mediated by norepinephrine acting at α1-adrenergic receptors (93). In small mesenteric arteries and arterioles, electrical stimulation evokes short latency, short-duration excitatory junction potentials (EJPs) and contractions mediated by ATP acting at P2X receptors (77).

3.2.3.1.3 SNS and venoconstriction

Sympathetic nerves have the greatest quantitative importance in minute-to-minute regulation of venoconstriction, especially in the splanchnic circulation (81) (216) (155). Sympathetic nerve stimulation can reduce intestinal blood volume by up to 60%, leading to a significant redistribution of blood from the splanchnic veins. This change in volume

distribution can have a profound effect on overall cardiovascular functions including blood pressure (81) (109) (65).

3.2.3.2 Humoral factors

There are many other influences on venous smooth muscle activity besides sympathetic input. These include tissue metabolites, pO2, pCO2, ions, osmolarity, myogenic factors and circulating hormones. Veins also exhibit significant myogenic tone when distended by increased intraluminal pressure. Enothelium-derived vasodilators such as prostanoids, NO affect venoconstriction, but in general their effects are less pronounced than in arteries. A number of studies implicate endogenous ET-1 in the control of venomotor activity by binding to both ETA and ETB receptors on the smooth muscle cells.

3.2.3.3 Endothelin system

3.2.3.3.1 Isoforms and function

In 1988, Yanagisawa et al (267) discovered a 21-amino acid peptide, subsequently named endothelin (ET), in the supernatant of cultured porcine aortic endothelial cells. The ET family consists of three distinct peptides (ET-1, -2 and -3), all with very similar structure (267). In addition, 31-residue ETs have also been identified (113). ET-1 is the most important subtype in the vasculature (266). It comprised 21 amino acid residues with free amino- and carboxy-termini. The fmy cysteine residues of this peptide form two intrachain disulfide bonds (between positions 1-15 and 3-11). Its molecular weight was estimated to be 2492 from the sequence data.

ET-1 is one of the most potent vasoconstrictors known, being nearly 2 logs more potent in this function than comparable amounts of norepinephrine (3). In addition to its cardiovascular effects, ET-1 is involved in embryonic development (118), bronchoconstriction (240), prostate growth (251), carcinogenesis (120), and gastrointestinal (184, 252) (119) and endocrine function (185) (106).

3.2.3.3.2 ET-1 and venoconstriction

Evidence indicates that ET-1 is an important determinant of venous tone (23) (87) (222) (268). Exogenous endothelin causes potent and sustained venoconstriction in experimental animals (250) (171) and in humans (175) (88). This effect is mediated by both ETA and ETB receptor subtypes, whereas arterial constriction to ET-1 is largely through ETA receptor activation (27). Stimulation of ETB receptors on endothelial cells can cause release of vasodilators that oppose venoconstriction (170) (219). In intact rats, exogenous ET-1 is a particularly potent constrictor of the mesenteric/splanchnic circulation, an effect mediated by both ETA and ETB receptors (73). On the other hand, endogenous ET-1 has only modest effects on basal, resting venoconstriction in experimental animals (64) (104) or humans (88), as inferred from responses to pharmacological antagonists. However, substantial evidence suggests a role for ET-1 in the regulation of venoconstriction in response to cardiovascular stresses and in a variety of disease states where decreased vascular capacitance plays a part (104) (210).

3.2.4 Splanchnic veins and venules are the main vessels contributing to total vascular capacitance

The splanchnic circulation consists of those vascular beds perfused by the celiac, superior mesenteric and inferior mesenteric arteries. Smaller arteries branch off to supply the various structures of the GI wall. The hepatic artery is a major branch of the celiac artery that provides oxygenated blood to the liver. The stomach receives blood from branches of the celiac artery. The celiac trunk also gives rise to splenic artery that provides blood to the spleen. The superior mesenteric artery provides blood to the small intestine, pancreas and the proximal region of the large bowel. The remainder of the colon is perfused by the inferior mesenteric artery. The blood draining the vascular beds perfused by the major splanchnic arteries empties into a system of veins that join to form the portal vein. The portal vein ultimately collects the blood from the spleen and the G.I. tract and delivers it to the liver where it arborizes into a series of smaller and smaller vessels that ultimately deliver blood to the hepatic sinusoids. The liver receives a dual blood supply. The portal vein drains the splanchnic circulation and provides 75% of the total blood flow. The hepatic artery provides the remaining 25%. In the hepatic microcirculation, the partially deoxygenated blood carried by the low pressure, portal vein joins the well oxygenated blood derived from the high pressure, hepatic artery. Blood draining the liver empties into the hepatic vein, which subsequently flows into the inferior vena cava.

The splanchnic bed is a major reservoir of blood in the body. The organs perfused by the splanchnic vasculature receive 25% of the cardiac output at rest. So the splanchnic veins hold the largest amount of blood within the vascular system, i.e. the splanchnic veins and venules are the main vessels contributing to total vascular capacitance. Venoconstriction especially in this region can cause a huge change in the distribution of

blood, allowing more vital functions to be supported with the available cardiac output. Factors regulating the venomotor activity in the splanchnic vasculature are critical in determining active changes in total vascular capacitance.

4. Vascular capacitance and compliance in deoxycorticosterone acetate (DOCA) – salt hypertensive rats

4.1 Deoxycorticosterone acetate (DOCA) – salt hypertensive rats

This is a hypertension model with suppressed renin activity due to water and sodium retention. This method of inducing hypertension is important because it allows an examination of the neural and hormonal contribution to hypertension secondary to adrenal steroid excess, which may be relevant to understanding of essential hypertension (200).

4.2 Increased vascular resistance in hypertension

The hallmark hemodynamic change in established hypertension is increased vascular resistance. For the typical increase in resistance that increases arterial blood pressure by 30-50 mmHg, the arteriolar vasculature need constrict only 4-7%. In addition, there is evidence of an increased incidence of temporary and, perhaps, permanent closure of small arterioles, which would also elevate resistance. The vascular smooth muscle cells of small arteries are hypertrophied, but hypertrophy of these cells around arterioles is minor. Many factors contribute to increased vascular resistance in hypertension, including alterations in neural and hormonal inputs and myogenic tone.

4.3 Reduced total systemic and venous compliance

Because the hallmark hemodynamic change in established hypertension is increased vascular resistance, it is not surprising that the common interest in the study of blood pressure control is on arteries. However, in human subjects with established hypertension, total systemic and venous compliance also are reduced (207) (198) (144). In several models of experimental hypertension in animals, such as spontaneously hypertensive rats, one and two-kidney Goldblatt renal hypertensive rats, and dogs made hypertension by angiotensin II, aldosterone, and reduced renal mass (181), MCFP is elevated even when blood volume and cardiac output are not increased, suggesting decreased venous compliance. Decreased venous compliance is most marked in peripheral vessels, and is particularly notable in splanchnic veins (166). An important function of decreased venous capacitance and/or compliance in established hypertension is to provide higher filling pressures to hypertrophied, less compliant cardiac ventricles in order to maintain normal cardiac output (207) (198). Arterial vasoconstriction in the splanchnic bed also contributes to decreased vascular capacitance by allowing passive recoil of splanchnic veins. As the splanchnic circulation is the largest capacitance bed, changes in mesenteric vasomotor tone will impact overall hemodynamics including blood pressure (81).

Reduced compliance of the extrathoracic venous system has also been documented in DOCA–salt hypertensive rats (64). However, only few studies have directly addressed venous capacitance function in DOCA-salt hypertension. Although most investigators report that in established DOCA-salt hypertension, there is normal cardiac output and increased total peripheral resistance (270), it is important to note that

hypertension develops fully even when total peripheral resistance is prevented from increasing (97). This underlies the importance of cardiac output regulation in the development of hypertension in this model.

4.4 Factors mediating increased venoconstriction in hypertension

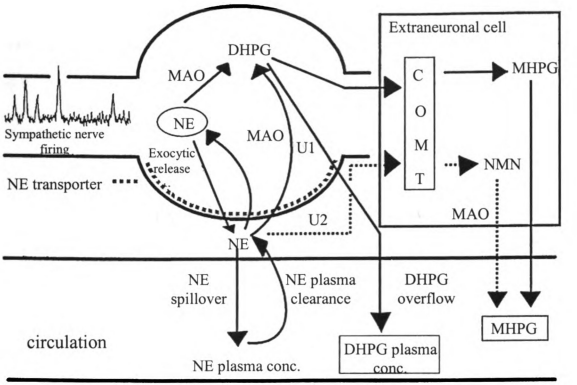
Blood volume and capacitance of the venous system are the major determinants of MCFP (263). In established hypertension, blood volume is either normal or reduced (198). Thus, elevated MCFP in hypertension is caused by structural changes in the venous wall (143), increased venous smooth muscle contractile activity, or both. Relative little is known about the mechanisms responsible for venous changes in hypertension. Evidence of structural abnormalities in veins exists for hypertensive humans (198) (143) and some experimental models of hypertension, eg. spontaneously hypertensive rats (181). Recent studies in my group showed that the passive distensibility and myogenic tone were significantly decreased in DOCA-salt small mesenteric veins, but not in small mesenteric arteries compared to sham rats (253). Vascular remodeling was present in DOCA-salt small mesenteric arteries and veins (253). Therefore, structural changes in the venous wall contribute to the increased venoconstriction in DOCA-salt hypertension.

Increased sympathetic venoconstriction contributes to reduced venous capacitance and compliance in established hypertensive human subjects and experimental animals. Increased sympathetic tone to mesenteric veins was demonstrated in SHR and rats with reduced renal mass. In DOCA-salt hypertensive rats, my lab found that ganglion blockade caused a larger decline in the MCFP than in sham rats (64), suggesting increased sympathetic venoconstriction in DOCA-salt rats. Both increased sympathetic

discharge rate and enhanced neuroeffector transmission can be contributing factors. A major goal of my study is to identify the mechanism of increased sympathetic venoconstriciton in DOCA-salt hypertension.

Because ET-1 is one of the most potent vasoconstrictors known in vivo, a pathophysiological role for the peptide as a mediator of hypertension has been postulated. ET-1 has a prominent role in the pathogenesis of DOCA-salt hypertension (201). A number of studies implicate that ET-1 is important in the control of venoconstriction by binding to ETA and ETB receptors. Venoconstriction produced by endogenous ET-1 acting on ET_A and ET_B receptors is enhanced in DOCA-salt hypertension, since both selective ETA antagonism and combined ETA/B antagonism are effective in decreasing the MCFP of DOCA-salt rats (104). No significant change occurs in sham rats. Another major goal of my study is to identify the mechanism of enhanced ET-1 mediated venoconstriction in DOCA-salt hypertension.

Figure 1: Schematic representation of NE overflow from sympathetic nerves to the circulation (NE Spillover). NE is removed from the circulation (plasma NE clearance) through neuronal uptake (U1) into sympathetic nerves and uptake into extraneuronal cells (U2). DHPG is an intraneuronal metabolite of NE. Plasma DHPG levels are determined mainly by MAO in sympathetic nerves, net leakage of NE from vesicles into the axoplasm, reuptake of released NE, and extraneuronal O-methylation of DHPG catalyzed by COMT. MHPG in plasma is derived from multiple sources, including 1) deamination of NMN after its cellular uptake, 2) deamination of NMN after cellular uptake and intracellular O-methylation of NE, 3) O-methylation of DHPG after its uptake from the circulation, and 4) O-methylation of DHPG after its uptake from the interstitial fluid but before its entry into the circulation. In the presence of impairment of U1, production of DHPG is reduced and that of MHPG is increased, although the majority of DHPG production derives from NE leaking from the sympathetic vesicles, independent of NE reuptake. MAO indicates monoamine oxidase; COMT, catechol-*o*-methyltransferase; DHPG, dihydroxylphenylglycol ; MHPG, 3-methoxy-4-hydroxylphenylglycol; NMN, normetanephrine (192).


Extraneuronal cell
DHPG
MAO
MHPG
C
O
M
T
NE
MAO
U1
Sympathetic nerve firing
Exocytic release
NE transporter
NMN
MAO
U2
NE
NE spillover
NE plasma clearance
DHPG overflow
MHPG
circulation
DHPG plasma conc.
NE plasma conc.

CHAPTER 2

HYPOTHESES AND SPECIFIC AIMS

Existing evidence suggests that there is both increased endothelin-1 and sympathetically mediated venoconstriction in DOCA-salt rats. However, it is not clear if venoconstriction is due to increased ET-1 levels and/or sympathetic activity in the splanchnic vasculature, the main capacitance bed of the circulation. **The aim of my work was to test the hypothesis that ET-1 levels and/or sympathetic nerve activity in the splanchnic vasculature is increased in DOCA-salt hypertensive rats (as illustrated in Fig. 2).**

Specific Aim I

The focus of this specific aim was to test the hypothesis that ET-1 levels and/or sympathetic activity in the splanchnic vasculature is increased in rats in the established phase of DOCA-salt hypertension (4-5 week).

Specific Aim I-1: Previous studies in my lab have shown that venoconstriction produced by endogenous ET-1 acting on ET_A and ET_B receptors is enhanced in vivo in DOCA-salt hypertension. The contractile responses of veins to ET-1, however, were unchanged in vitro. Therefore, enhanced ET-1 mediated venoconstriction in vivo could be due to greater splanchnic vascular formation of ET-1 in DOCA-salt hypertension. This aim tested the hypothesis that there was higher ET-1 concentration around the splanchnic venous smooth muscle cells of rats in the established phase of DOCA-salt hypertension.

Protocol I-a: I measured venous vascular ET-1 content to determine if local concentrations of ET-1 are higher in DOCA-salt rats.

Protocol I-b: Since most ET-1 in blood vessels is presumably located within endothelial cells, I investigated further the ET-1 levels affecting the splanchnic veins in vivo using ET-1 concentration in splanchnic venous blood as an index, which is presumed to be in equilibrium with ET-1 concentration in the interstitial fluid of venous smooth muscle cells. And I used the difference between inflowing arterial ET-1 concentration and outflowing portal venous ET-1 concentration as an index of net non-hepatic splanchnic release (nNHSR) of ET-1.

Protocol II-a: Plasma ET-1 level is determined by a combination of factors and clearance by endothelial ETB receptors is the most important mechanism for removing ET-1 from blood. The aim of this study was to determine if altered ETB receptor-mediated plasma clearance of ET-1 contributes to circulating ET-1 level and nNHSR of ET-1 in DOCA-salt hypertension.

Protocol II-b: My functional studies suggest impaired ETB receptor-mediated plasma clearance of ET-1 in the splanchnic bed of DOCA-salt rats. The aim of this study was to identify the underlying mechanism by examining the expression of endothelial ETB receptors in small mesenteric blood vessels of DOCA-salt and sham rats using double label immunofluorescence and cofocal microspy.

Protocol III: Endothelin converting enzyme is a rate-limiting step in ET-1 generation. The change in nNHSR of ET-1 due to inhibition of ECE should provide more direct manifestation of ET-1 generation in the splanchnic bed. The aim of this study was to

estimate ET-1 synthesis in the splanchnic vasculature using the difference between the net NHSR of ET-1 in rats treated only with ETB receptor antagonist and that in rats treated with both ETB receptor antagonist and ECE inhibitor as an index.

Protocol IV: There appears to be a relatively small amount of ET peptide in blood and tissue in relation to the concentration of ET receptors. The binding of ET-1 to its receptors is particularly tight and ET-1 remains bound to ETA receptors even after their internalization. The aim of this study was to test the possible role of ETA receptor binding in the plasma clearance of ET-1 in the splanchnic bed of DOCA-salt rats.

Specific Aim I-2: There is substantial evidence for increased sympathetic input to the cardiovascular system in DOCA-salt hypertension. The non-hepatic splanchnic circulation accounts for ~50% of total peripheral sympathetic outflow in humans and experimental animals. This aim was to test the hypothesis that there was increased sympathetic activity to the splanchnic bed in conscious DOCA-salt rats using estimated non-hepatic splanchnic NE spillover as an index.

Protocol I: The aim of this study was to test the hypothesis that there was increased plasma NE levels and estimate nonhepatic splanchnic spillover of NE in DOCA-salt hypertensive rats.

Protocol II: Prejunctional α_2-adrenoceptors mediate strong inhibition of NE release from sympathetic terminals. Changes in prejunctional regulation of sympathetic nerves

associated with veins in DOCA-salt rats remain to be fully characterized. The aim of this study was to test the hypothesis that prejunctional α_2-adrenoceptor inhibition of NE release was dysfunctional in sympathetic nerve endings associated with splanchnic vasculature in DOCA-salt hypertensive rats.

Protocol III: NET is critical in central modulation of sympathetic tone and autonomic regulation of peripheral hemodynamics by reducing NE concentrations in the neuroeffector junction and preventing the resultant deleterious effects on cardiovascular tissues. The rate of spillover is also dependent on the adequacy of neuronal uptake. The aim of this study was to test the hypothesis that neuronal NE reuptake was altered in the splanchnic vasculature in DOCA-salt hypertensive rats.

Protocol IV: An interaction between reuptake and α_2-receptors has been found to be present in certain vascular beds so as to maintain a largely constant effector response. The aim of this study was to test the hypothesis that the interaction between prejunctional α_2-adrenoceptor and neuronal transmitter reuptake was altered in the splanchnic vasculature in DOCA-salt hypertensive rats.

Protocol V: The modulatory effect of ET-1 on sympathetic neurotransmission has been reported. The aim of this study was to test the hypothesis that the modulatory effect of ET-1 on sympathetic neurotransmission was altered in the splanchnic vasculature in DOCA-salt hypertensive rats.

Specific Aim II

Venous capacitance and compliance are reduced in established hypertension, but it is uncertain whether this is a cause or a consequence of hypertension. Existing evidence suggests that there is both increased endothelin-1 and sympathetically mediated venoconstriction in early DOCA-salt rats. **The focus of this specific aim was to test the hypothesis that ET-1 levels and/or sympathetic activity in the splanchnic vasculature was increased in the early stage of DOCA-salt hypertension (4-5 day).**

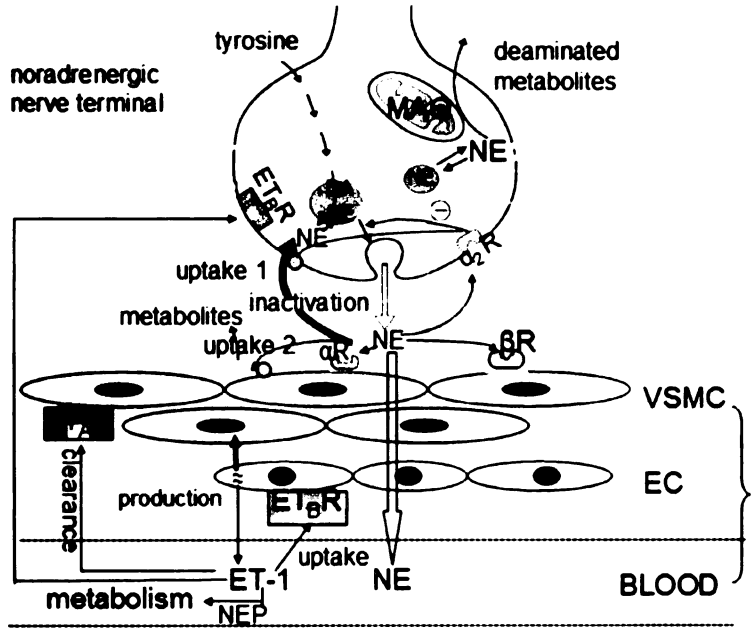
Specific Aim II-1: I tested the hypothesis that sympathetic nerve activity was increased in the splanchnic vasculature of early DOCA-salt hypertensive rats.

Specific Aim II-2: I tested the hypothesis that there was impaired prejunctional α2-adrenoceptor inhibition of NE release in the splanchnic bed of early DOCA-salt hypertensive rats.

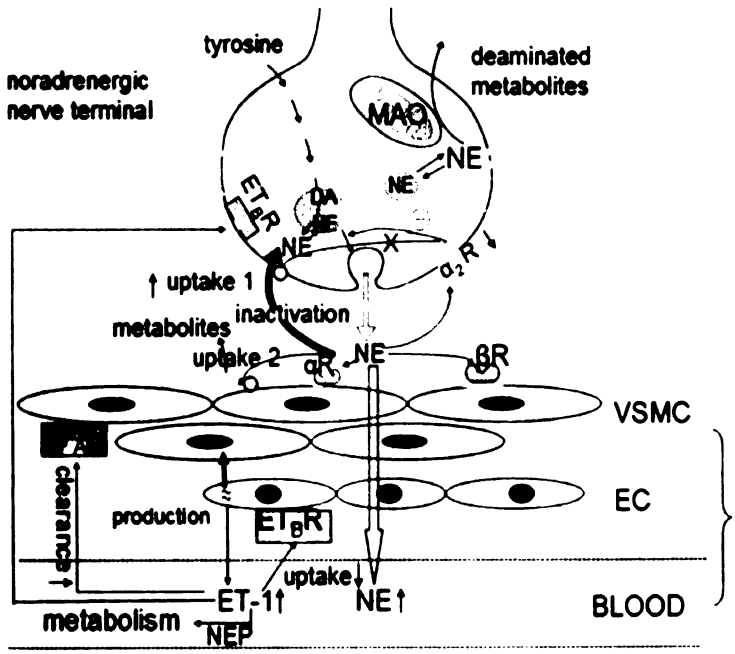
Specific Aim II-3: I tested the hypotheses that there was increased ET-1 formation and impaired ETB receptor mediated uptake activity in the splanchnic vasculature of early DOCA-salt hypertensive rats.

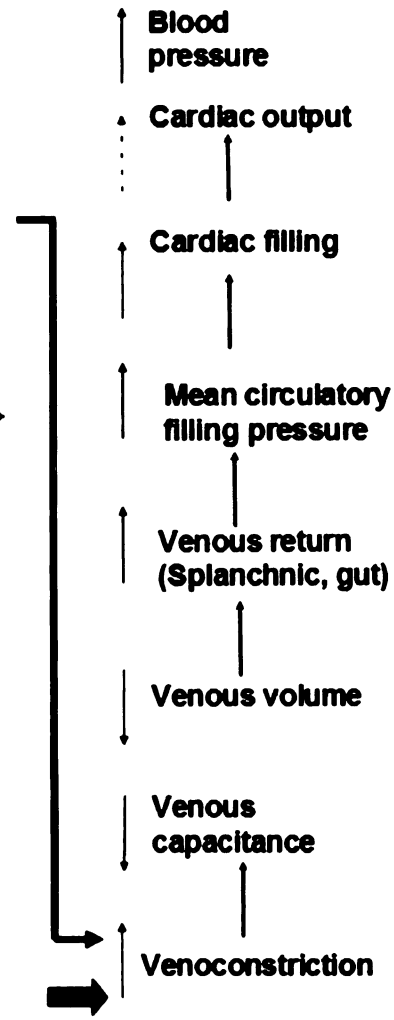
Figure 2: Schematic representation of overall hypothesis. I tested the hypothesis that there was increased ET-1 levels and/or sympathetic nerve activity in the splanchnic vasculature of rats in the established and early phase of DOCA-salt hypertension. In specific aim I-1, protocol I tested splanchnic vascular and venous plasma ET-1 levels. I also used the portal venous aortic plasma ET-1 concentration difference as an index of nNHSR of ET-1. Protocol II focused on determining if altered ETB receptor mediated plasma clearance of ET-1 contributed to circulating ET-1 level and nNHSR of ET-1. Protocol III aimed at estimating ET-1 synthesis in the splanchnic vasculature using the difference between the net NHSR of ET-1 in rats treated only with ETB receptor antagonist and that in rats treated with both ETB receptor antagonist and ECE inhibitor as an index. Protocol IV tested the possible role of ETA receptor binding in the plasma clearance of ET-1 in the splanchnic bed. In Specific aim I-2, protocol I tested the hypothesis that there was increased plasma NE level and estimated nonhepatic splanchnic spillover of NE in DOCA-salt rats. Protocol II aimed at determining if there was impaired prejunctional α2-adrenoceptor function in sympathetic nerve endings associated with DOCA splanchnic vasculature that contributed to increased NE release. Protocol III tested if there was altered neuronal NE reuptake in the DOCA splanchnic vasculature. Protocol IV tested the existence of an interaction between prejunctional α2-adrenoceptor function and NET activity. Protocol V tested the hypothesis that the modulatory effect of ET-1 on sympathetic neurotransmission was altered in DOCA splanchnic vasculature. Specific aim II tested some the above hypotheses in early stage of DOCA-salt hypertensive rats.

sham



DOCA-salt





CHAPTER 3

GENERAL METHODS

1. Animals

All experiments were performed in male Sprague-Dawley rats (Charles River Laboratories) that weighed 175 to 225 grams at the beginning of the study. Until the time of catheterization, rats were housed 2 or 3 per cage in a temperature and humidity controlled room with a 12h on/12h off light cycle. Pelleted rat chow and water were given ad libitum (8640 Rodent Diet; Harlan/Teklad).

1.1 Preparation of DOCA-salt and sham rats

The left kidney was exteriorized and removed under sodium pentobarbital anesthesia (50 mg/kg, i.p.). DOCA-salt rats received a DOCA pellet (200 mg) implanted SC and they received 1% NaCl and 0.2% KCl in their drinking water. Sham-operated rats (sham) underwent uninephrectomy but drank tap water. Three weeks after surgery, arterial blood pressure was measured using the tail cuff method. Rats with a mean systolic blood pressure of >150 mmHg were considered hypertensive. Sham rats generally had a mean systolic blood pressure $\leq$ 125mmHg.

1.2 Surgery of rats used in chapter 4 and 5

Approximately 4 weeks after DOCA implantation or sham surgery, catheters were placed in each rat. Anesthesia was produced with sodium pentobarbital (30 to 50 mg/kg, i.p.). Catheters with silicone rubber tips were inserted into the abdominal aorta via a femoral artery. Another catheter was positioned in the portal vein via a side branch for sampling blood (Fig. 3). A third catheter was placed into a femoral vein for drug administration. The rats were allowed to recover on a heated pad for 30 min before the start of study. In chronically instrumented rats, the ends of all 3 catheters were tunneled

subcutaneously to the neck, where they exited the rat inside a stainless steel spring secured to the body with a harness around the rat's thorax. Injections of enrofloxacin (5 mg/kg, i.v.) were given for bacterial prophylaxis. Rats were allowed to recover consciousness on a heated pad under constant observation. They were then placed in stainless steel metabolism cages under loose tethering allowing continuous access to all catheters without handling or otherwise disturbing the rat. Enrofloxacin (5 mg/kg, i.v.) was administered to all rats for a three day period after surgery. Vascular catheters were filled with heparin saline solutions when not in use and flushed daily. Experiments were started after at least 4 days of surgical recovery, as previous work in my lab indicated that normal food and saline intake in DOCA-salt rats typically did not recover to pre-surgery amount until 4 days. Blood samples were taken (with or without drug intervention) with at least a one day interval separating experiments. In both anesthetized and conscious groups, blood samples were collected simultaneously from arterial, portal venous sites into ice-chilled plastic tubes containing EDTA (or EGTA and reduced glutathione for NE experiments) and centrifuged at 10,000g for 15 min at 4°C. Plasma was separated and stored at –80°C until used.

1.3 Surgery of rats used in chapter 6

Catheterization and uninephrectomy were performed (as described earlier) simultaneously on normal rats. Enrofloxacin (5 mg/kg, i.v.) was administered to all rats for a three-day period after surgery. Vascular catheters were filled with heparin saline solutions when not in use and flushed daily. 4 days after surgery, rats were selected randomly to receive DOCA pellet implantation SC or sham operation. Both DOCA-salt

and sham rats received 1% NaCl and 0.2% KCl in their drinking water. Blood samples were collected simultaneously from arterial, portal venous sites for ET-1 or NE, EPI measurement on the 4th day after DOCA or sham surgery. One drug intervention was given on the 5th day and blood samples were taken again.

2. Hemodynamic measurements

Arterial pressure of anesthetized rats was determined by connecting the arterial catheter to Powerlab data acquisition system (ADInstruments Inc., CO). In the conscious group, arterial pressure was determined by connecting the arterial catheter to a low-volume displacement pressure transducer (TXD-300, Micro-Med, Louisville, Kentucky, USA) linked to a digital pressure monitor (BPA-200 Blood Pressure Analyzer, Micro-Med), which derived systolic, mean and diastolic pressures and heart rate every 0.5s (sampling rate=1000Hz). All values were averaged minute-by-minute and saved using a computerized data acquisition system (DMSI-200/4 system Integrator, Micro-Med).

3. Plasma ET-1 measurement

ET-1 content was measured by Quantitative Sandwich Enzyme Immunoassay technique. It is a 5-hmy solid phase ELISA. A monoclonal antibody specific for ET-1 has been pre-coated onto a microplate (R&D Systems, Inc.). 100 μl standards/EDTA plasma was pipetted into each well. The plate was covered and incubated for 1.5 hmys at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm. Any ET-1 present was bound by the immobilized antibody. Each well was washed fmy times to eliminate any unbound substances. 200 μl enzyme-linked monoclonal antibody specific

for ET-1 was added to each well. The plate was covered and incubated for 3 hmys at room temperature. Each well was again washed fmy times to remove any unbound antibody-enzyme reagent. 200 μl enhanced luminol/peroxide substrate solution was added to each well. Incubation was for 30 min at room temperature on the benchtop. Light was produced in proportion to the amount of ET-1 bound in the initial step. A microplate luminometer was used to measure the intensity of the light omitted. Minimum detectable concentration of ET-1 was 0.16 pg/ml. Some cross-reactivity and interference was observed with hBig ET-2 (0.010%), pBig ET-39 (0.019%), hBig ET-38 (0.019%), bBig ET-39 (0.018%), rBig ET-39 (0.020%), sarafotoxin (0.490%), hET-3 (7.8%) and hET-2 (27.4%). Interassay coefficients of variation is 7.1%. Intraassay coefficients of variation is 2.1%.

4. Plasma catecholamine measurement

Plasma NE and epinephrine were measured by radioenzymatic assay (REA) which was based on the method described by Peuler and Johnson (177). The principle of this technique is based on the conversion of NE and EPI to tritiated normetanephrine and metanephrine in the presence of catechol-O-methyltransferase and tritiated S-adenosylmethionine as a labeled methyl donor. After purification through a series of organic extractions, tritiated normetanephrine is separated from the derivatives of dopamine by oxidation with sodium periodate. Tritiated vanillin thus formed is counted in a liquid scintillation spectrometer.

5. Portal blood flow measurement

After midline laparotomy, portal vein was exposed and lightly separated. Ultrasound transit-time flow probe (Transonic systems Inc. Ithaca, NY) was positioned around the portal vein. Acoustic gel (Transonic systems Inc. Ithaca, NY) was applied in order to achieve proper ultrasonic contact between probe and vessel and obtain satisfactory acoustic signal. During the experiments, probe was connected to a HT207 dual channel flow meter (Transonic Systems Inc.). Portal venous blood flow (ml/min) was measured after a 30 min stabilization period.

6. Estimate of non-hepatic splanchnic (NHS) spillover of NE

NHS NE spillover was estimated based on two assumptions: 1) Portal venous blood flow of DOCA-salt rats equals that of sham rats. 2) NHS fractional extraction (FX) of NE (fractions of NE removed from plasma during passage through NHS organs) equals NHS FX of epinephrine.

$S_R = [(NE_o - NE_i) + (NE_i \times FX)] \times Q \times (1 - Hct)$

NE_i: concentration of NE in inflowing plasma (pg/ml)

NE_o: concentration of NE in outflowing plasma (pg/ml)

Q: regional blood flow

Hct: hematocrit

FX: fractional extraction of NE (i.e. fraction of NE removed from plasma during passage through mesenteric organs)

Fractional extraction of epinephrine $= (EPI_i - EPI_o) / EPI_i$

EPI_i: concentration of EPI in inflowing plasma (pg/ml)

EPI_o: concentration of EPI in outflowing plasma (pg/ml)

7. Radiotracer method

The radiotracer method allows the estimation of regional sympathetic nervous activity from measurement of the organ-specific NE spillover rate.

7.1 Radiotracer infusion

Tritium-labeled NE ([^{3}H]-NE; [levo-ring-2,5,6-^{3}H]NE, 40-60 Ci/mmol, 1 mCi/ml, PerkinElmer, MA) was diluted with 500 µl of 0.2 M acetic acid plus 50 µl of sodium sulfite (100 mg/ml), 350 µl of reduced glutathione (6 mg/ml) qsp 10ml with 0.9% saline and was infused at 27 µl/min intravenously (femoral vein), i.e. 0.06 µCi/kg/min with the pump for 90 min (rate at which blood pressure levels will not be modified in either sham or DOCA-salt rats) into catheterized DOCA-salt or sham rats. The radiotracer infusion was started at least 30 min before blood sampling to ensure attainment of steady-state plasma [^{3}H]-NE concentration. Arterial and portal venous blood samples were obtained to measure [^{3}H]-NE and endogenous NE. We are still working on the technique to separate tritiated NE from its metabolites (mainly dihydroxylphenylglycol) with the help of another lab. Ideally, timed collection of the [^{3}H]-NE in the eluant leaving the HPLC detection cell enables the separation. [^{3}H]-NE can then be quantified by liquid scintillation counting. Plasma concentrations of endogenous NE will be determined by REA as described earlier.

8. Immunofluorescence localization and quantification of endothelial ET_B receptors

Paraffin embedded sections of Methacarn (or known as Carnoy's) (consists of 60% methanol, 30% chloroform and 10% glacial acetic acid) fixed mesenteric blood vessels (including both superior mesenteric artery and vein and small mesenteric arteries and veins from DOCA-salt and sham rats) were deparaffinized and rehydrated. Antigen retrieval was performed to recover antigenicity by soaking the sections in Vector Labs antigen unmasking solution and heating in a microwave for 2 minutes. After wash with PBS, sections were incubated with rabbit anti-ETB receptor antibody (Alomone labs, Jerusalem, Israel) at a dilution of 1: 50 in 0.01 M PBS with 1% Triton X-100 and sheep anti-rat vWF antibody (Enzyme Research Laboratories, South Bend, IN) at a dilution of 1:200 in 0.01 M PBS with 1% Triton X-100 overnight at 4 °C in a humidified chamber. Unbound primary antibody was washed away using phosphate buffered saline and after washing, sections were incubated with cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, PA) at a dilution of 1: 200 in 0.01 M PBS with 1% Triton X-100 and cy5-conjugated donkey anti-sheep IgG (Jackson Immunoresearch, PA) at a dilution of 1:50 for 2h at room temperature in a humidified chamber. Unbound secondary antibodies were washed away and sections were mounted with AquaPerm mounting medium (Thermo Electron Corporation, PA). The slides were coverslipped and sealed with nail polish. The slides were stored in dark at 4 °C until observed using a confocal fluoresence microscope.

The fluorescence intensity of ETB receptors in endothelial cells was quantified using Image J (software from NIH, downloaded from internet) using the fluorescence staining

of von Willebrand Factor as an endothelial cell marker. The mean fluorescence intensity of ETB receptors from 3-9 individual endothelial cells was plotted and compared between DOCA-salt and sham rats.

Images in this thesis are presented in color.

9. Statistical analysis of data

All data were given as mean ± SEM. Differences in variables between rats were compared by ANOVA followed by Bonferroni's test to correct the error rate for multiple comparisons. Differences in variables within rats (i.e. over time) were compared using paired-t tests or repeated measures ANOVA followed by Bonferroni's test. $P \leq 0.05$ was regarded as significant.

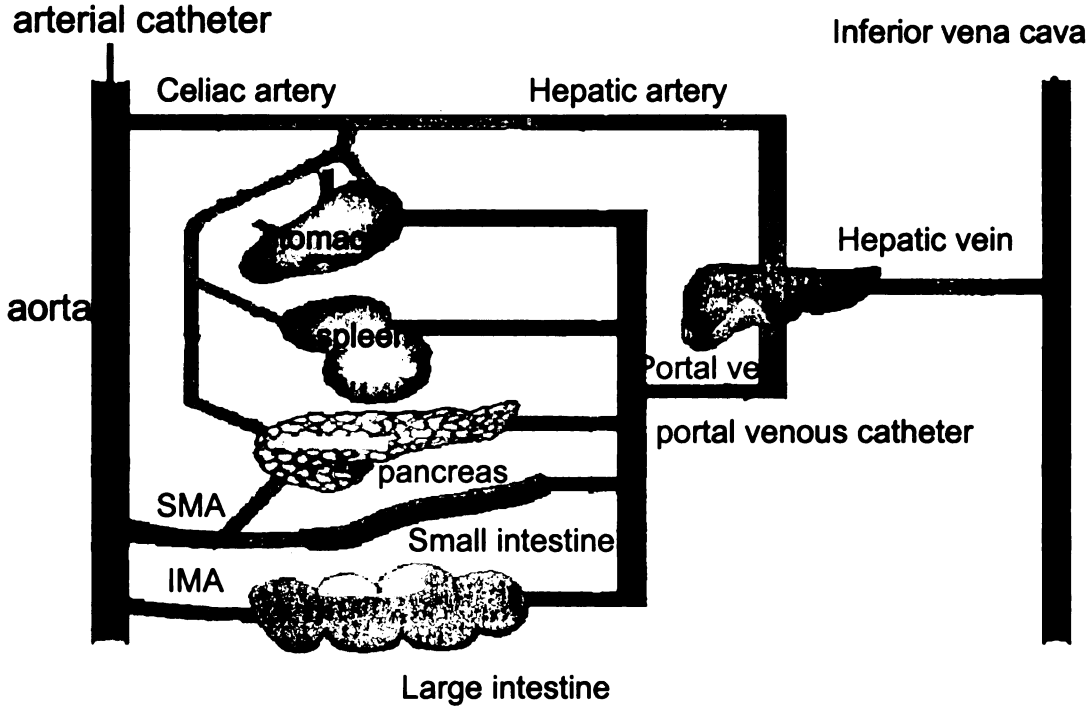


Figure 3: Schematic representation of splanchnic circulation and the positions of the arterial catheter and portal venous catheter.

CHAPTER 4

ENDOTHELIN-1 CONCENTRATION AROUND THE SPLANCHNIC VENOUS SMOOTH MUSCLE CELLS OF RATS IN THE ESTABLISHED PHASE OF DOCA-SALT HYPERTENSION

Introduction

Role of ET-1 in human hypertension and experimental models of hypertension in animals

ET-1 is a powerful vasoconstrictor, has mitogenic properties, and induces inflammatory responses. These effects contribute to altered vascular reactivity and to vascular remodeling, important processes in blood pressure elevation. Therefore, a pathophysiological role for the peptide as a mediator of hypertension has been postulated. Unlike experimental models of hypertension, such as DOCA-salt hypertensive rats (127, 128), Dahl salt-sensitive rats (25), aldosterone-salt hypertensive rats (173), one kidney one clip Goldbaltt hypertensive rats (226), stroke prone spontaneously hypertensive rats (236), salt loaded spontaneously hypertensive rats (201, 236), in which ET-1 has been implicated to be important in the pathophysiology of hypertension, the role ET-1 played in a normal cardiovascular homeostasis and in mild essential hypertension in humans is unclear. Plasma concentrations of ET-1-like immunoreactivity have been reported to be normal or slightly elevated in patients with mild essential hypertension (204) (199). However, increasing evidence suggests that ET-1 may be important in certain groups of essential hypertensive patients, including severe and/or malignant hypertension (128), pulmonary hypertension (76), hypertension in African Americans (49), salt-sensitive hypertensives , low renin hypertension (129, 174), pre-eclampsia (83), and those with obesity (129) and insulin resistance (271). In all of these conditions, plasma immunoreactive ET levels are elevated and tissue ET-1 expression is increased. Data from clinical trials using combined ETA/B receptor blockers have already demonstrated significant blood-pressure-lowering effects (117). Thus, targeting the endothelin system

has important therapeutic potential in the treatment of hypertension, particularly by contributing to the prevention of target organ damage and the management of cardiovascular disease.

ET-1 increases total peripheral resistance in DOCA-salt rats by contracting arteries and arterioles.

ET-1 has a prominent role in DOCA-salt hypertension (201). There is blood pressure lowering upon administration of endothelin antagonists (130). Endothelin receptor antagonists also regressed vascular growth and inflammation, and improved endothelial dysfunction (130). DOCA-salt rats treated with endothelin antagonists were protected from stroke and renal injury.

One important effect of ET-1 in DOCA-salt hypertension is to increase total peripheral resistance by contracting arteries and arterioles (270). Most studies have shown, however, that arterial plasma levels of ET-1 are not increased in DOCA-salt rats (161, 162, 203, 223, 247). Nonetheless both preproendothelin-1 (preproET-1) mRNA expression and immunoreactive ET-1 content of aorta and mesenteric arteries are increased in DOCA-salt hypertensive rats (28, 127, 128, 203), suggesting that one mechanism of ET-1 induced arterial constriction in hypertension is increased levels of the peptide around arterial smooth muscle. A caveat to that conclusion is raised by findings that contractions to ET-1 are significantly reduced in arteries of DOCA-salt rats when compared with those of sham rats (103). Thus, increased local production of ET-1 by arteries may be countered by decreased arterial responsiveness, and it remains unclear how ET-1 increases vascular resistance in DOCA-salt hypertension.

There is enhanced ET-1 mediated venoconstriction in DOCA-salt hypertension.

The demonstration of the involvement of the endothelin system in the development and maintenance of hypertension in DOCA-salt hypertensive rats has stimulated investigations into a potential role of this system at the venous site of the circulation, especially because in-vitro studies have consistently shown that the maximal vasoconstrictor response to, and potency of, ET-1 in veins is greater compared to that in corresponding arteries (23, 105) (190) (182). The venous system is an important, yet infrequently considered part of the circulation. Hypertensive humans and experimental animals also exhibit decreased systemic vascular capacitance (181, 198). This is primarily the result of changes in the structure or vasoconstrictor activity of extra-thoracic veins, especially those in the splanchnic bed (198). Reduced vascular capacitance contributes to the hemodynamics of hypertension by contributing—along with blood volume—to maintenance of "effective blood volume", a critical determinant of venous return of blood to the heart and thus cardiac output (198). In experimental animals, information about venous tone in vivo can be derived from the MCFP (as introduced earlier). Similar to vascular resistance, MCFP is increased in practically all forms of hypertension (132) including DOCA-salt hypertensive rats (104). As blood volume in hypertension is not increased but, in contrast, is usually slightly reduced, the increase in MCFP in hypertension must be caused by a decrease in the compliance of the venous system, which in turn may be a consequence of an increase in venous tone, of structural venous changes or of the combination of these two effects (197). Endogenous ET-1 appears at least partly responsible for that augmented venoconstriction, since ET

receptor antagonists lower MCFP in DOCA-salt hypertensive but not normotensive rats (104).

Chapter 4 – Part I

1) Total vascular ET-1 content in mesenteric veins of DOCA-salt hypertensive rats

Rationale: Two possible mechanisms could explain increased ET-1 mediated venoconstriction: higher levels of the peptide around venous smooth muscle cells and/or enhanced responsiveness of venous smooth muscle to ET-1. It has been shown that vena cavae and small mesenteric veins from DOCA-salt hypertensive rats do not exhibit increased reactivity to ET-1 *in vitro* (103, 254). Therefore, I hypothesized that higher ET-1 mediated venoconstriction in DOCA-salt rats was caused by increased ET-1 concentrations around venous smooth muscle. First, I measured vascular ET-1 in the superior and small mesenteric veins of hypertensive DOCA-salt and normotensive sham rats. Since ET-1 is primarily synthesized de novo by vascular endothelial cells, I expected vascular ET-1 content to be representative of local vascular production of the peptide.

Experimental Procedures: Approximately 4 weeks after DOCA implantation or sham surgery, rats were euthanized by sodium pentobarbital overdose (100mg/kg, i.p., Sigma, MO) and segments of thoracic aorta, thoracic vena cava, abdominal vena cava were removed and dissected free of fat. The complete mesenteric vascular bed was removed and placed in a Petri dish full of ice cold Kreb's solution. The small intestine was stretched gently and pinned flat. Superior mesenteric artery and vein, small mesenteric arteries and veins (150-250 μm in diameter) were dissected free of fat and collected separately. The tissues were snap-frozen in liquid nitrogen and stored at -80°C until extraction of ET-1 was performed. On the day of extraction, tissues were homogenized with a Polytron in 1ml 1M acetic acid containing 10 μg/ml pepstatin A (ICN

pharmaceuticals, Costa Mesa, CA) and immediately heated to 100°C for 10 min. After chilling, the homogenate was centrifuged at 14,000g for 30 min at 4°C. The supernatant was dried in a Speed-Vac and then reconstituted in 300 µl calibrator diluent (R&D systems, Minneapolis, MN). The reconstituted sample was stored at -80°C until measurement of ET-1 was performed. Vascular ET-1 content was measured by quantitative sandwich enzyme immunoassay technique. Cross reactivity of the antibodies with some of the other peptides was mentioned in General Methods.

Results: ET-1 content was normalized using tissue weight. I found that 1) consistent with previous published result (28, 127, 128, 202), there was significantly higher ET-1 content in aorta and superior mesenteric artery of DOCA-salt rats than sham rats; 2) ET-1 content was higher in small vessels than in larger vessels in both DOCA-salt and sham rats; 3) there was no significant difference in the ET-1 content in veins of any size between DOCA-salt and sham rats (Fig. 4).

2) Venous plasma ET-1 concentration and net non-hepatic splanchnic release (nNHSR) of ET-1

Rationale: Since most of the ET-1 in the blood vessels is presumably localized within endothelial cells (72), I was not sure if my findings above indicate that the ET-1 concentrations at the surface of venous smooth muscle cells are the same in DOCA-salt and sham rats. So I conducted additional experiments to measure portal venous plasma ET-1 level.

In contrast to circulating hormones, ET acts primarily as a local hormone in an autocrine and paracrine fashion. Plasma concentrations are typically very low even in pathological states (67). More importantly, ET has been shown to be secreted primarily (80%) into the basolateral compartment (i.e., abluminally) and not into the apical compartment (luminally) (20%) (249). Thus, plasma concentrations may not be representative of locally released ET-1 at its site of action. However, the portal vein drains venous blood from all of the splanchnic organs except the liver. Portal venous ET-1 concentration therefore should reflect splanchnic venous ET-1 concentration, which is presumed to be in equilibrium with ET-1 concentration in interstitial fluid of vascular smooth muscle cells. Therefore, studies on plasma ET-1 levels are still helpful to determine the direction of differences in locally released ET-1 at its site of action between DOCA-salt and sham rats. Because part of portal venous plasma ET-1 is derived from arterial blood entering the splanchnic bed, it was also necessary to measure arterial plasma ET-1 level. Thus, I used the portal venous-aortic plasma ET-1 concentration difference as an index of net non-hepatic splanchnic release (NHSR) of ET-1. I presumed also that most ET-1 in plasma is derived from vascular endothelial cells. The liver was excluded from these experiments because of the difficulty of sampling blood from the hepatic venous drainage in the rat. In addition, portal vein sampling prevents interference of substantial hepatic extraction (35) with the measurement of splanchnic release of ET-1.

Experimental Procedures: I did a preliminary study using anesthetized rats. Catheters were inserted into the abdominal aorta, portal vein and femoral vein as introduced in

General Methods. Additional studies were performed in conscious rats because anesthesia and acute surgery can affect hormone synthesis and release. In both anesthetized and conscious groups, 0.5 ml blood samples were collected simultaneously from arterial and portal venous catheters into ice-chilled plastic syringes and transferred into ice-chilled plastic tubes containing EDTA. Blood samples were centrifuged at 10,000g for 5min at 4°C. Plasma was separated and stored at -80°C until assayed using ELISA.

Results: The average mean arterial pressure (MAP) of DOCA-salt rats was significantly higher than that of sham rats in either anesthetized or conscious group (Fig. 5). The MAP difference between anesthetized DOCA-salt and sham rats, however, was much smaller than that between conscious DOCA-salt and sham rats.

Plasma ET-1 concentration was significantly higher in portal vein than in aorta in anesthetized sham and DOCA-salt rats, indicating nNHSR of ET-1. However, there was no significant difference in portal venous plasma ET-1 level between sham and DOCA-salt rats or in aortic plasma ET-1 level between the two groups (Fig. 6A). There was no correlation between plasma ET-1 concentrations and blood pressure levels of DOCA-salt rats.

Plasma ET-1 concentration also was significantly higher in portal vein than in aorta in conscious sham and DOCA-salt rats. However, there was no difference in portal venous plasma ET-1 level between conscious sham and DOCA-salt rats or in aortic plasma ET-1 levels between the two groups (Fig. 6B).

Both the aortic and portal venous plasma ET-1 concentrations were significantly lower in conscious sham rats compared with anesthetized sham rats. In conscious DOCA-salt rats, the portal venous plasma ET-1 concentration was significantly lower than that of anesthetized DOCA-salt rats (Fig. 6A, 6B). The aortic plasma ET-1 concentration was lower in conscious DOCA-salt rats, but not quite significant (P=0.066), compared with anesthetized DOCA-salt rats.

The net NHSR of ET-1 was estimated using portal venous aortic plasma ET-1 concentration difference. There was no significant difference in the nNHSR of ET-1 between DOCA-salt and sham rats in either anesthetized or conscious group (Fig. 7A, 7B). The basal nNHSR of ET-1 was significantly lower in conscious sham rats than in anesthetized sham rats. In conscious DOCA-salt rats, the nNHSR of ET-1 was lower, but not significantly so (P=0.057), compared with anesthetized DOCA-salt rats.

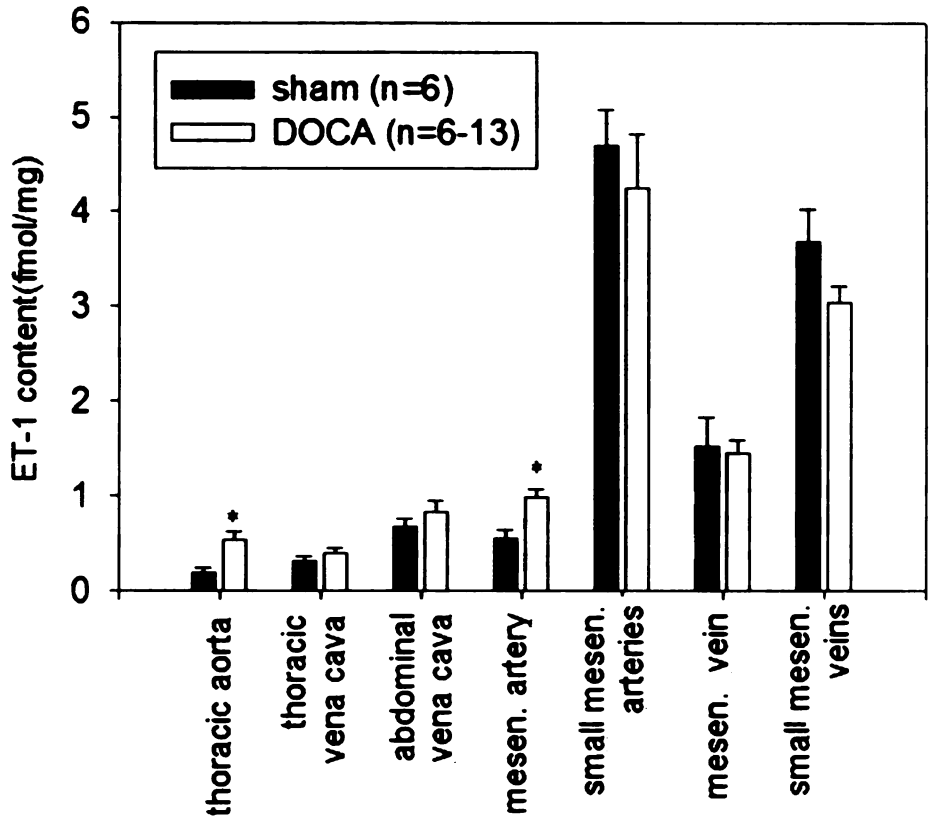


Figure 4: Vascular ET-1 content of DOCA-salt and sham rats. Bars indicate mean ET-1 content in blood vessels from sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between sham and DOCA-salt rats.

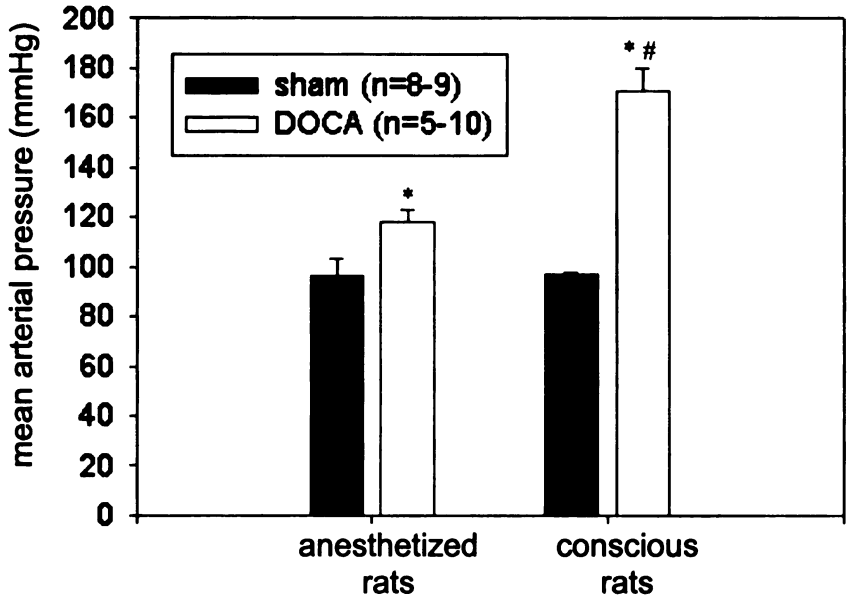


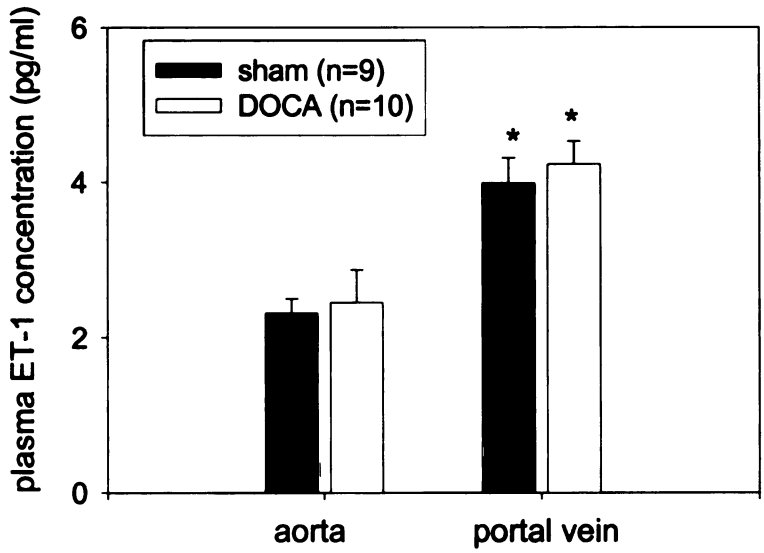
Figure 5: Mean arterial pressure of anesthetized and conscious DOCA-salt and sham rats. Bars indicate mean arterial pressure of anesthetized sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between sham and DOCA-salt rats. # indicates a significant difference ($P<0.05$) between anesthetized and conscious DOCA-salt rats.

Figure 6:

A: Aortic and portal venous plasma ET-1 level in anesthetized DOCA-salt and sham rats. Bars indicate mean aortic or portal venous plasma ET-1 concentration in anesthetized sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between portal venous and aortic plasma ET-1 concentration.

B: Aortic and portal venous plasma ET-1 level in conscious DOCA-salt and sham rats. Bars indicate mean aortic or portal venous plasma ET-1 concentration in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between portal venous and aortic plasma ET-1 concentration. #s indicate a significant difference ($P<0.05$) between conscious and anesthetized rats.

A


plasma ET-1 concentration (pg/ml)
6
4
2
0
sham (n=9)
DOCA (n=10)
*
*
aorta
portal vein

B

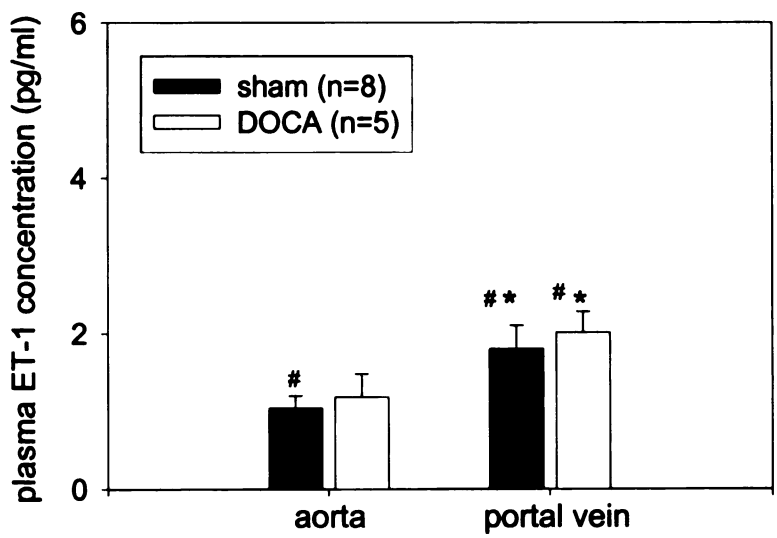
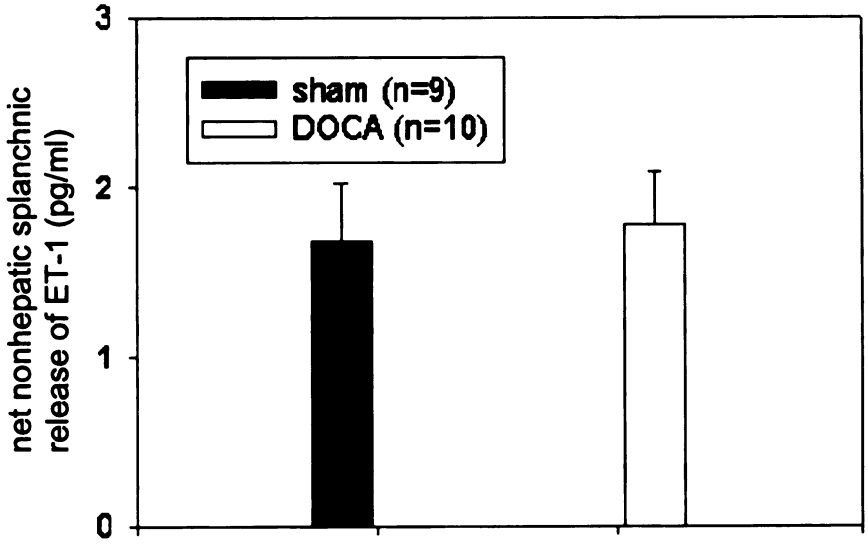

plasma ET-1 concentration (pg/ml)
6
4
2
0
sham (n=8)
DOCA (n=5)
#
#*
#*
aorta
portal vein

Figure 7:

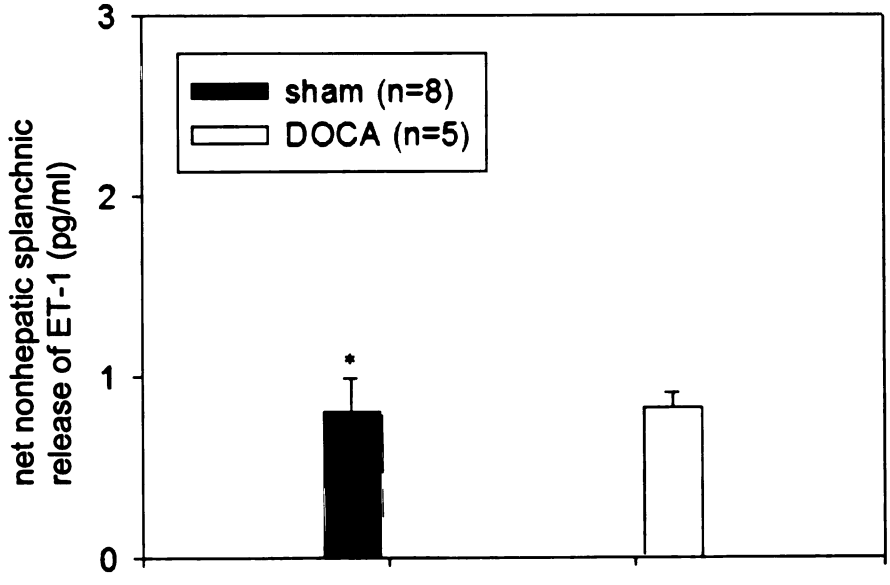
A: Net nonhepatic splanchnic release of ET-1 in anesthetized DOCA-salt and sham rats. Bars indicate mean net nonhepatic splanchnic release of ET-1 in anesthetized sham and DOCA-salt rats. Brackets indicate SEM.

B: Net nonhepatic splanchnic release of ET-1 in anesthetized DOCA-salt and sham rats. Bars indicate mean net nonhepatic splanchnic release of ET-1 in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between anesthetized and conscious sham rats.

A


sham (n=9)
DOCA (n=10)
net nonhepatic splanchnic release of ET-1 (pg/ml)
0
1
2
3

B


sham (n=8)
DOCA (n=5)
net nonhepatic splanchnic release of ET-1 (pg/ml)
*
0
1
2
3

Discussion for Chapter 4—Part I

1) ET-1 content of mesenteric arteries and veins

ET-1 content of blood vessels is determined by both local vascular production, and reuptake and storage, of the peptide. These processes occur predominantly but not exclusively in endothelial cells. Total vascular content of ET-1 thus represents a rough index of local concentrations of the peptide available to cause vasoconstriction *in vivo*. Little is know about the status of tissue ET-1 in hypertensive patients. From gluteal subcutaneous biopsies, Schiffrin et al demonstrated that the prepro-ET-1 gene is overexpressed in the endothelium of small arteries of moderate to severe hypertensive patients, in contrast with mild hypertensive patients and normotensive subjects (202). This may contribute to downregulation of endothelin receptors and lead to blunted responses to ET-1, which has been demonstrated in isolated human small arteries from hypertensive patients (89).

Measurements of splanchnic vascular ET-1 content in my study produced novel findings. First, on the basis of tissue weight, veins have higher ET-1 content than arteries. This may reflect a higher ratio of endothelial cells to other cell types in veins versus arteries, or a greater ET-1 synthetic capacity of venous compared to arterial endothelial cells. However, more ET-1 binding to venous smooth muscle ETA receptors and further ground down into the supernatant remains to be a possibility too. Second, smaller blood vessels had higher ET-1 content than larger vessels. This may reflect the relative amount of endothelial cells in my samples (especially in arteries), but also is consistent with an important role for ET-1 in control of vascular resistance and capacitance, since these physiological functions are mainly served by smaller sized vessels in the intact

circulation. Third, I found that vascular ET-1 content was significantly greater in DOCA-salt rats only in the aorta and superior mesenteric artery. Significantly increased ETA receptor number and decreased contractile reponse to ET-1 have also been found in DOCA aorta and superior mesenteric artery (154, 254) (104). The combined results suggest dysfunction of ETA receptors. Upregulation of the vascular endothelin system may contribute to cellular processes associated with vascular remodeling and to increased stiffness of conduit arteries in hypertension (142, 201).

Finally, I found that there was no significant difference in ET-1 content in veins of any size between DOCA-salt and sham rats. This suggests that production of ET-1 by venous endothelial cells is not increased in DOCA-salt hypertensive rats. This is consistent with my previous finding of unchanged preproET-1 mRNA level in mesenteric veins in DOCA-salt rats (103). Although it has been found that cultured rat vascular smooth muscle cells can synthesize/secret ET-1 in response to a variety of stimuli, it is suggested (84) that in vivo expression of the gene for prepro ET-1 in SMC may depend on the existing and/or past integrity of the blood vessel wall (e.g. endothelial desquamation, phenotypic modulation of constituent SMC, and local production of growth factors). It remains to be determined whether expression and synthesis of ET-1 by VSMC represent a physiological and/or pathophysiological axis of regulation.

Steady-state vascular content of ET-1, however, may not be an accurate indicator of local formation of ET-1 around venous capacitance vessels. This is because released ET-1 in vivo is subjected to rapid tissue binding, reuptake and metabolism. The kinetics of ET-1 disposition are only beginning to be understood (35, 36).

2) ET-1 level in venous blood and nNHSR of ET-1

Therefore, I conducted additional experiments designed to evaluate the rate of ET-1 release by the non-hepatic splanchnic vasculature of normotensive and DOCA-salt hypertensive rats *in vivo*. Although ET-1 release by endothelial cells is reported to be polarized towards vascular smooth muscle (80%) compared to blood (20%) (249), I reasoned that venous blood (portal vein) draining the splanchnic bed (except the liver) would yield a reliable estimate of differences in ET-1 production and release by splanchnic vessels. Because part of portal venous plasma ET-1 is derived from arterial blood entering the splanchnic bed, it was also necessary to measure arterial plasma ET-1 levels. Thus, in analogy to other studies (2, 24), I used the difference between arterial and portal venous ET-1 plasma concentrations as an index of net NHS release of ET-1. Previous studies demonstrated that arterial plasma levels of ET-1 are not increased in DOCA-salt rats. My results confirmed this finding in both anesthetized and conscious DOCA-salt rats (Fig. 6A, 6B). No studies have been reported on splanchnic venous plasma ET-1 level or nNHSR in this model of hypertension. I hypothesized that both portal venous plasma ET-1 concentration and nNHSR of ET-1 would be higher in DOCA-salt rats compared to sham rats. This would indicate that the endothelial cells in the splanchnic vasculature of DOCA-salt rats release more ET-1 *in vivo*; and that venous smooth muscle cells of the splanchnic bed are exposed to higher ET-1 concentrations.

I found that the arterial plasma ET-1 level is not significantly higher in DOCA-salt rats, i.e. similarly as in sham rats, plasma levels of circulating ET-1 in DOCA-salt rats are too low to produce cardiovascular effects. The low plasma levels of ET-1 may be attributable to rapid clearance of circulating ET-1 (about 80% of the radioactivity was

cleared from the circulation within the first minute of bolus injection of [^{125}I] ET-1), a slow rate of ET-1 production and/or preferential release of ET-1 to vascular smooth muscles.

I also found that portal venous plasma ET-1 level was significantly higher than aortic plasma ET-1 level in both anesthetized hypertensive DOCA-salt rats and normotensive sham rats, indicating net non-hepatic splanchnic release of ET-1 in both groups of animals. However, there was no difference in portal venous plasma ET-1 level, or in nNHSR of ET-1 between the two groups. This provides the first *in vivo* evidence that endothelial cells of the splanchnic vasculature of DOCA-salt rats do not produce more ET-1 compared to those of sham rats. However, as mentioned earlier, plasma ET-1 level is determined by a combination of factors including production rate, metabolism, clearance and regional blood flow. Blood flows to the stomach, small and large intestines are not affected by DOCA-salt treatment (97, 264), so disparities in regional blood flow are not likely to contribute to my findings. Some studies suggest that plasma ET levels are also regulated by renal clearance of ET-1. Increased plasma ET levels could be secondary to the impairment of renal clearance. It has been found that there is lower urinary ET excretion in patients with salt-sensitive hypertension in which it has been known that there is increased circulating ET level (235). However, opposite opinions also exist suggesting that at least in human, plasma ET-1 and urinary ET-1 represent two distinct systems regulated by different control mechanisms. The ET-1 normally present in the urine is more likely to be of renal origin (210). In rats injected with ^{125}I-ET-1, labeled endothelin is detectable only in trace amount in the urine (5). The relationship between plasma ET-1 and urinary ET-1 has not been clarified in DOCA-salt hypertensive rats.

Another mechanism that could influence venous plasma ET-1 level and nNHSR of ET-1 in DOCA-salt rats is altered ET_B receptor-mediated uptake activity. For example, enhanced endothelial cell production and release of ET-1 in the splanchnic vasculature of DOCA-salt rats might not be apparent with my *in vivo* measurements if there is also increased removal of ET-1 from plasma by reuptake into endothelial cells. I tested this possibility in my later studies.

3) Anesthetized versus conscious rats

My preliminary studies were performed on anesthetized rats after acute surgery for catheter implantation. The MAP differences between DOCA-salt and sham rats were much less than when they were conscious (probably reflecting the important role of the sympathetic nervous system in regulation of blood pressure in DOCA-salt hypertension). Further studies were then performed on conscious rats to avoid the effects of surgery and anesthesia on sympathetic nerve activity, body fluid status and hormone synthesis or release. Rats also were in a normal crouched posture instead of lying on their back when blood was sampled. This is a potentially significant factor when considering vascular capacitance function. I saw significantly lower plasma ET-1 concentration and nNHSR of ET-1 in conscious rats, presumably reflecting less stress or anesthesia induced effects on ET-1 formation, metabolism, or uptake.

Chapter 4 – Part II

1) ETB receptor-mediated clearance of plasma ET-1 in the splanchnic bed of DOCA-salt hypertensive rats

Rationale: Plasma ET-1 level is determined by a combination of factors including production rate, metabolism and clearance. Previous studies indicate that clearance by endothelial ET_B receptors is the most important mechanism for removing ET-1 from blood (19, 191, 213, 217).

ETB receptors are abundant on endothelial cells, vascular smooth muscles and parenchymal cells of the lung and kidney. ET-1 is rapidly cleared from the circulation mainly through the lungs (~80%), kidneys or liver, probably at least in part in a receptor-mediated fashion like many other peptide hormones including angiotensin II and atrial natriuretic peptide. The rapid clearance of ET-1 from the circulation may play a role in protecting the systemic circulation from the vasoconstrictor actions of ET-1 (217). At the low concentrations occurring physiologically, the major ET-1 clearing sites appear to reside in the lungs. When pulmonary binding capacity is saturated by ET-1, other vascular beds may take over the function of clearing endothelin from the circulation (217).

To test the possibility that a functional alteration in ET_B receptors might affect the venous plasma ET-1 concentration and net nonhepatic splanchnic release of ET-1 in DOCA-salt hypertension, I measured the aortic and portal venous plasma ET-1 concentrations and calculated portal venous-aortic plasma ET-1 concentration difference after selective ET_B receptor blockade in both anesthetized and conscious rats.

Experimental Procedures: Catheters were inserted into abdominal aorta, portal vein and femoral vein as introduced in General Methods. In the anesthetized group, rats were allowed to recover from surgery on a heated pad for 30 min before blood sampling. A192621, a selective ET_B receptor antagonist (256), was then given (12 mg/kg, i.v., Abbott Laboratories, IL) and blood samples were collected again 30 min later. It has been shown by previous studies in my lab (104) that this dose of A192621 is effective at blocking ET_B receptors and causes a significant increase in MAP in DOCA-salt hypertensive rats. The increase reaches maximum at 30 minutes after A192621 administration. In rats to be studied while conscious, blood samples (with and without A192621 treatment) were taken with at least a one day interval separating experiments.

Results:

i. Effects of selective ETB receptor antagonist on MAP

Administration of A192621 caused a significant increase in the mean arterial pressure in both anesthetized DOCA-salt and sham rats (Fig. 8A). A192621 also caused a significant increase in the mean arterial pressure in conscious sham rats, but not in conscious DOCA-salt rats (Fig. 8B).

ii. Total ET_B receptor mediated plasma clearance of ET-1

A192621 caused a significant increase in the aortic plasma ET-1 concentration in both anesthetized (Fig. 9A) and conscious (Fig. 9B) sham and DOCA-salt rats. There was no significant difference in the aortic plasma ET-1 concentrations between sham and DOCA-salt rats after A192621 treatment under anesthesia or in the conscious state. The

magnitude of change in aortic plasma levels of ET-1 before and after treatment also was not significantly different between sham and DOCA-salt rats.

30 min after A192621 treatment, the portal venous plasma ET-1 concentration was significantly and similarly increased in both anesthetized and conscious sham and DOCA-salt rats (Fig. 10A, 10B).

iii. ET_B receptor mediated plasma clearance of ET-1 in the NHS vasculature

After A192621 treatment, there was a significant increase in the nNHSR of ET-1 in anesthetized sham, but not anesthetized DOCA-salt rats (Fig. 11A).

In conscious animals, after ET_B receptor antagonist treatment, nNHSR of ET-1 was significantly increased in sham rats, but remained unchanged in DOCA-salt rats (Fig. 11B).

Data from both anesthetized and conscious rats suggest impaired ETB receptor-mediated clearance of plasma ET-1 in the splanchnic bed of DOCA-salt hypertensive rats.

2) <u>Mechanisms of impaired ETB receptor-mediated clearance of plasma ET-1 in the splanchnic bed of DOCA-salt hypertensive rats</u>

Rationale: Possible mechanisms underlying the decreased uptake activity of ET_B receptor specifically in the splanchnic vasculature of DOCA-salt rats include: a decrease in overall endothelial cell function; a decreased density of endothelial ET_B receptors; a lower binding affinity of ET_B receptors for ET-1; or altered post-receptor ET-1

disposition mechanism. I tested the possibility that there were fewer ETB receptors in the endothelial cells of DOCA-salt hypertensive rats.

Experimental Procedures: I examined the expression of ETB receptors in the endothelial cells of cross sections from small mesenteric arteries and veins of DOCA-salt and sham rats using double label immunofluorescence and cofocal microscopy. Von Willebrand Factor (vWF) was used as an endothelial cell marker and its distribution pattern was used as an index of endothelial cell injury. The fluorescence intensity of ETB receptors in endothelial cells was quantified using Image J as an index of the total number of endothelial ETB receptors. Images in this thesis are presented in color.

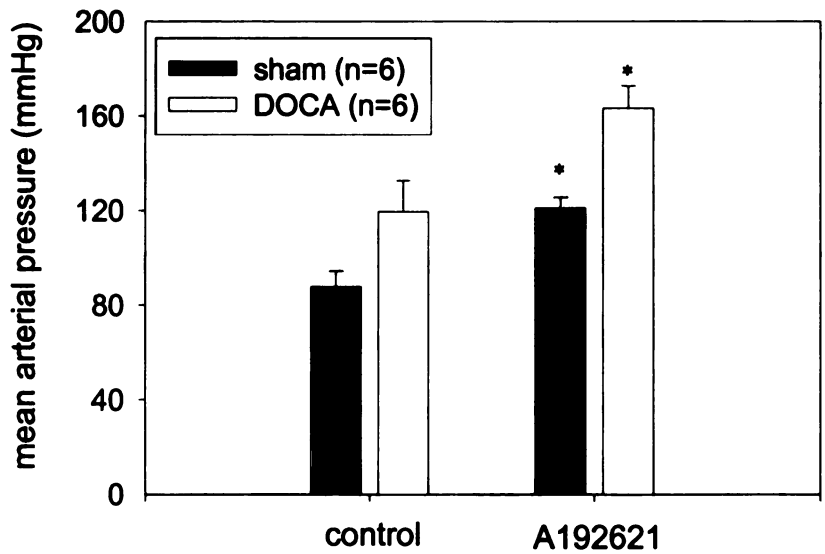
Results: There was no significant change in the distribution pattern of vWF between DOCA-salt and sham tissue. Although the average fluorescence intensity of endothelial ETB receptors was significantly higher in sham small mesenteric veins than sham small mesenteric arteries, there was no significant difference between DOCA-salt and sham tissues (Fig. 12, Fig. 13).

Figure 8:

A: Mean arterial pressure responses to i.v. bolus selective ETB receptor antagonist administration in anesthetized DOCA-salt and sham rats. Bars indicate mean arterial pressure before or after A192621 treatment in anesthetized sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between mean arterial pressure before and after drug treatment.

B: Mean arterial pressure responses to i.v. bolus selective ETB receptor antagonist administration in conscious DOCA-salt and sham rats. Bars indicate mean arterial pressure before or after A192621 treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between mean arterial pressure before and after drug treatment. # indicates a significant difference ($P<0.05$) between DOCA-salt and sham rats.

A


mean arterial pressure (mmHg)
200
160
120
80
40
0
sham (n=6)
DOCA (n=6)
*
*
control
A192621

B

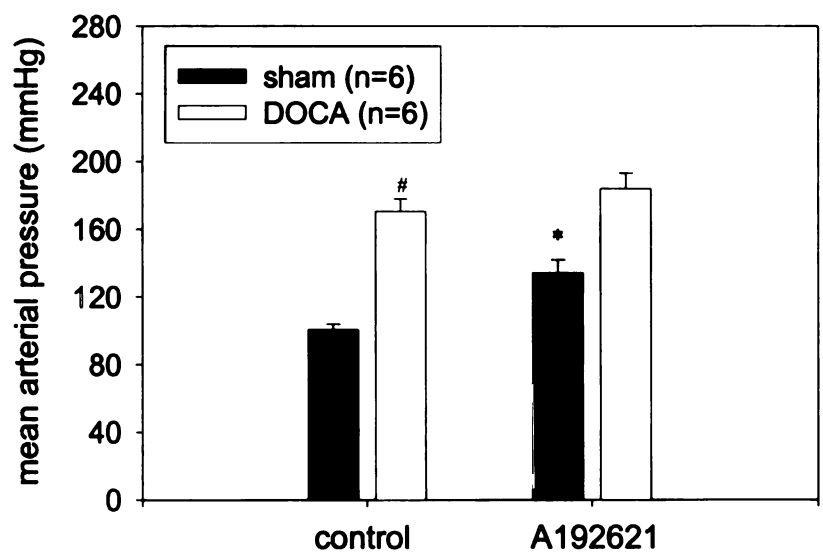
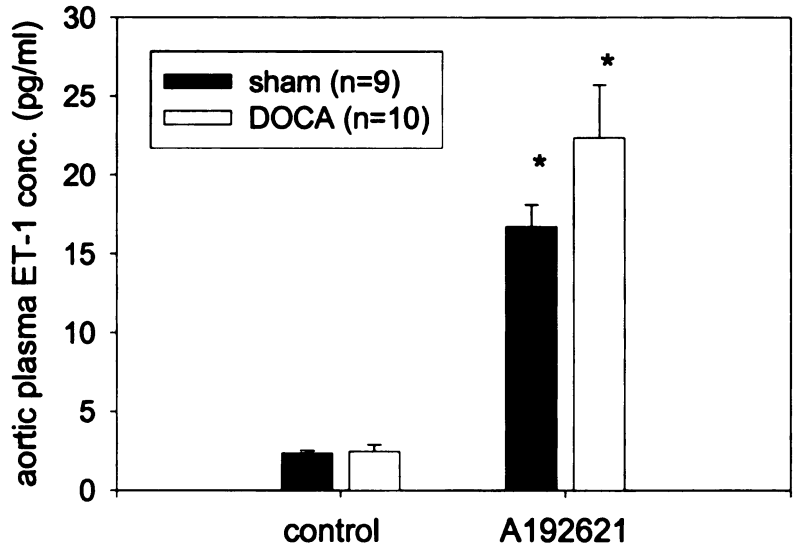

mean arterial pressure (mmHg)
280
240
200
160
120
80
40
0
sham (n=6)
DOCA (n=6)
#
*
control
A192621

Figure 9:

A: Effects of i.v. bolus selective ETB receptor antagonist administration on aortic plasma ET-1 level in anesthetized DOCA-salt and sham rats. Bars indicate mean aortic plasma ET-1 concentration before or after drug treatment in anesthetized sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between mean aortic plasma ET-1 concentration before and after drug treatment.

B: Effects of i.v. bolus selective ETB receptor antagonist administration on aortic plasma ET-1 level in conscious DOCA-salt and sham rats. Bars indicate mean aortic plasma ET-1 concentration before or after drug treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between mean aortic plasma ET-1 concentration before and after drug treatment. # indicates a significant difference ($P<0.05$) between conscious and anesthetized rats.

A


aortic plasma ET-1 conc. (pg/ml)
30
25
20
15
10
5
0
sham (n=9)
DOCA (n=10)
*
*
control
A192621

B

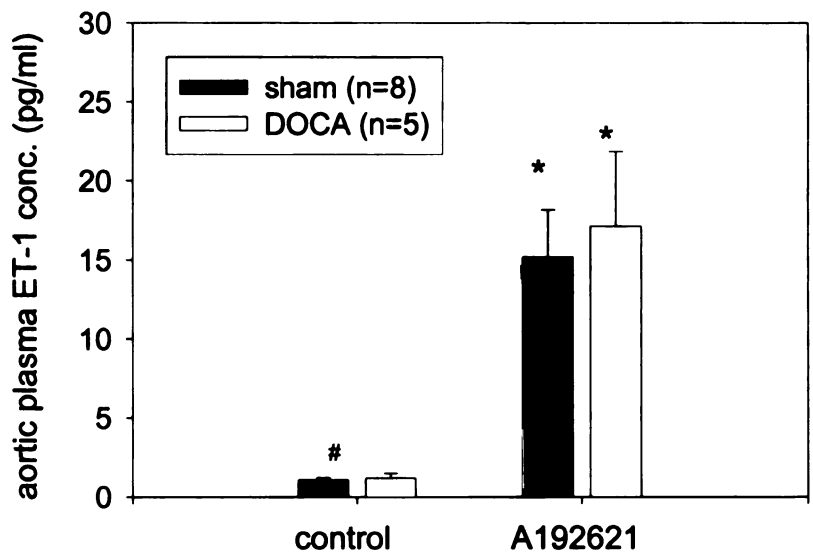
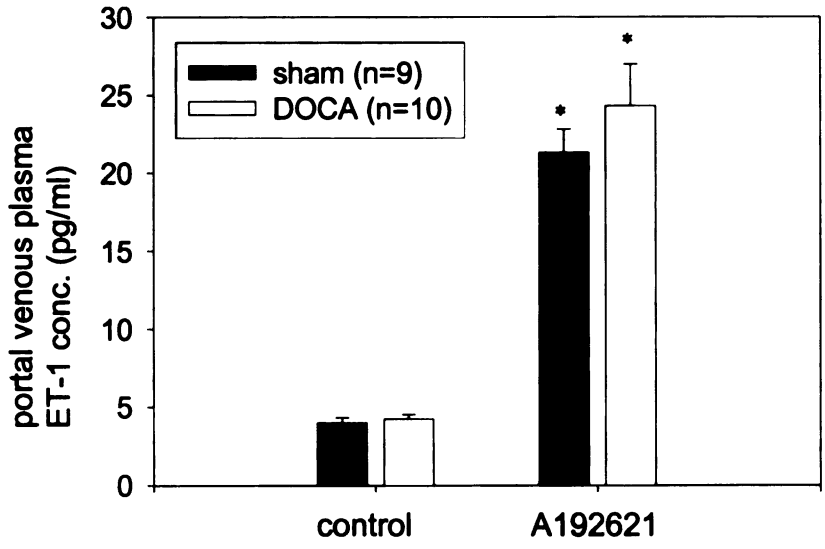

aortic plasma ET-1 conc. (pg/ml)
30
25
20
15
10
5
0
sham (n=8)
DOCA (n=5)
#
*
*
control
A192621

Figure 10:

A: Effects of i.v. bolus selective ETB receptor antagonist administration on portal venous plasma ET-1 level in anesthetized DOCA-salt and sham rats. Bars indicate mean portal venous plasma ET-1 concentration before or after drug treatment in anesthetized sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between mean portal venous plasma ET-1 concentration before and after drug treatment.

B: Effects of i.v. bolus selective ETB receptor antagonist administration on portal venous plasma ET-1 level in conscious DOCA-salt and sham rats. Bars indicate mean portal venous plasma ET-1 concentration before or after drug treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between mean portal venous plasma ET-1 concentration before and after drug treatment. #s indicate a significant difference ($P<0.05$) between conscious and anesthetized rats.

A


sham (n=9)
DOCA (n=10)
portal venous plasma
ET-1 conc. (pg/ml)
30
25
20
15
10
5
0
*
*
control
A192621

B

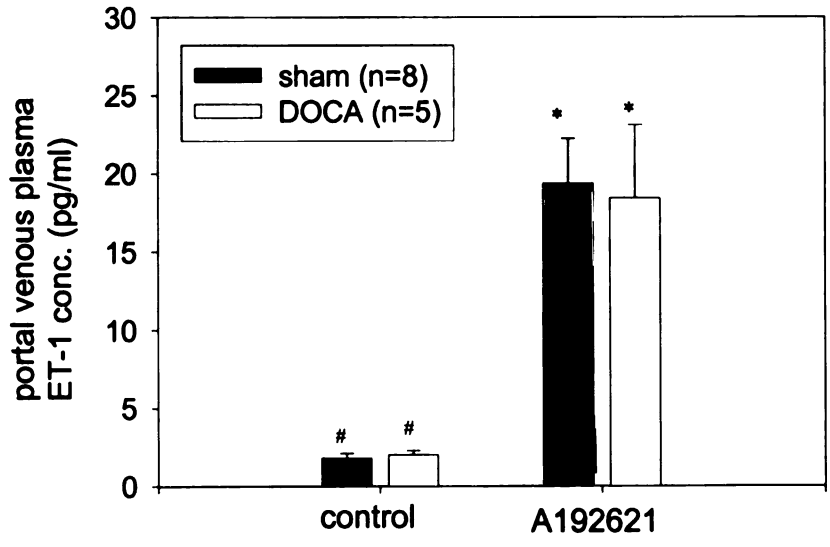
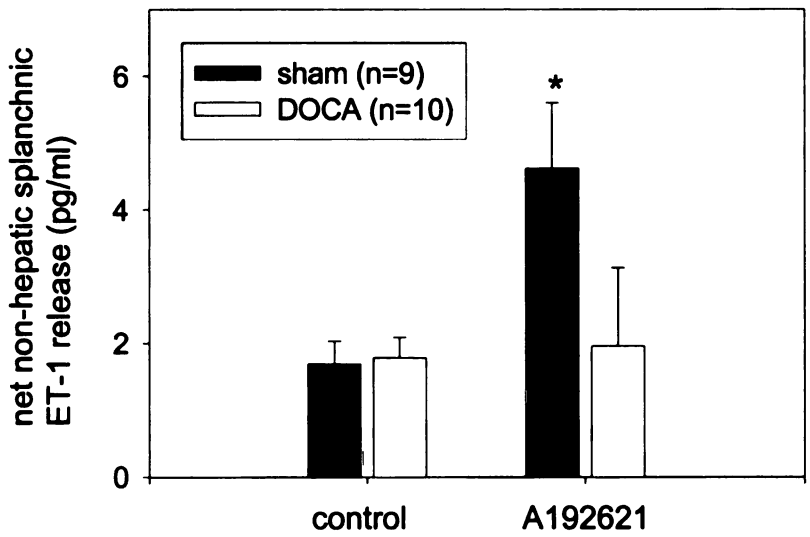

sham (n=8)
DOCA (n=5)
portal venous plasma
ET-1 conc. (pg/ml)
30
25
20
15
10
5
0
#
#
*
*
control
A192621

Figure 11:

A: Effects of i.v. bolus selective ETB receptor antagonist administration on net NHSR of ET-1 in anesthetized DOCA-salt and sham rats. Bars indicate mean net non-hepatic splanchnic ET-1 release before or after drug treatment in anesthetized sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between mean net non-hepatic splanchnic ET-1 release before and after drug treatment.

B: Effects of i.v. bolus selective ETB receptor antagonist administration on net NHSR of ET-1 in conscious DOCA-salt and sham rats. Bars indicate mean net non-hepatic splanchnic ET-1 release before or after drug treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between mean net non-hepatic splanchnic ET-1 release before and after drug treatment. # indicates a significant difference ($P<0.05$) between conscious and anesthetized rats.

A


sham (n=9)
DOCA (n=10)
net non-hepatic splanchnic ET-1 release (pg/ml)
0
2
4
6
*
control
A192621

B

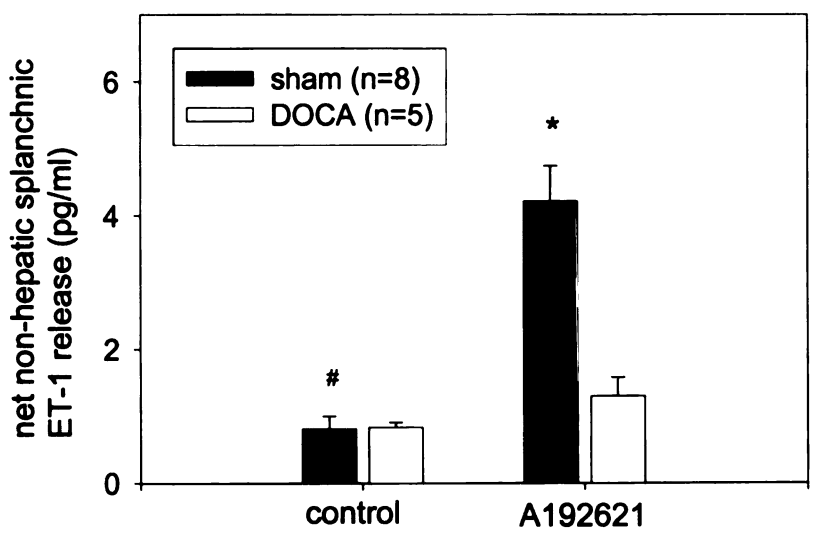
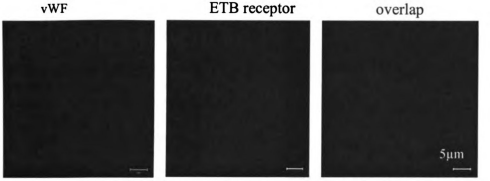
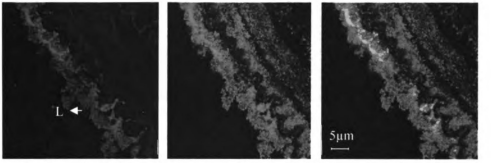

sham (n=8)
DOCA (n=5)
net non-hepatic splanchnic ET-1 release (pg/ml)
0
2
4
6
#
*
control
A192621

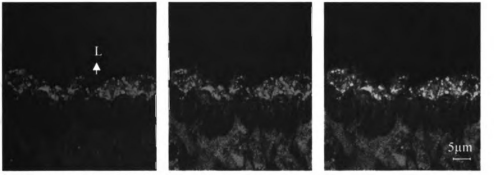
Figure 12: Representative immunofluorescence staining of vWF and ETB receptors of cross sections of DOCA and sham small mesenteric blood vessels.



without primary antibody



cross section of sham small mesenteric vein



cross section of DOCA small mesenteric vein

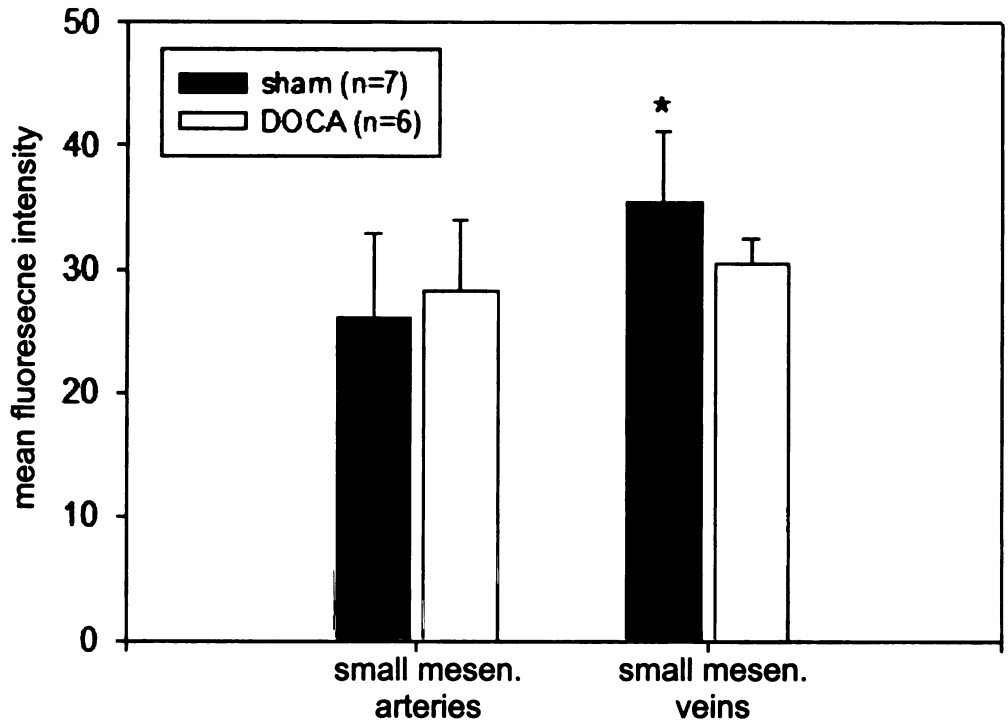


Figure 13: Quantification of the immunofluorescence intensity of ETB receptors. Bars indicate mean immunofluorescence intensity of endothelial ETB receptors in sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between different blood vessels in the same group.

Discussion for Chapter 4 – Part II

1) ET-1 clearance by ET_B receptors

ET-1 is rapidly removed from circulating blood, and current evidence suggests that the ET_B receptor is very important for that process. ET-1 binds almost irreversibly to the ET_B receptor under physiological conditions (229). Direct transport of ET_B receptor-associated ET-1 to lysosomes for degradation may serve as the main mechanism for clearance of plasma ET-1 (14). To determine whether altered endothelial ET_B receptor mediated uptake activity contributes to venous plasma ET-1 level and nNHSR of ET-1, I measured the portal venous plasma ET-1 concentration and portal venous-aortic plasma ET-1 concentration difference in the presence of the selective ET_B receptor antagonist A192621.

ETB antagonist treatment significantly increased the MAP in anesthetized DOCA-salt and sham rats, possibly due to the blockade of plasma clearance of ET-1. However, although ETB receptor antagonist treatment also significantly increased the MAP of conscious sham rats, it didn't cause any significant change in the MAP of conscious DOCA-salt rats. This could be due to better physiological compensation, less increase in plasma ET-1 and/or decreased vascular response to ET-1 in conscious DOCA-salt rats.

I found a significant increase in portal venous-aortic plasma ET-1 difference in sham, but not DOCA-salt rats, after acute blockade of ET_B receptors. Thus, in sham rats there is significant clearance of ET-1 from the splanchnic circulation through uptake mediated by ET_B receptors. The uptake activity of endothelial ET_B receptors in the splanchnic vasculature of DOCA-salt rats, however, was significantly decreased (or even

absent). Interestingly though the whole body clearance of ET-1 mediated by ET_B receptor (predominantly pulmonary), which is reflected by the aortic plasma ET-1 concentration difference before and after A192621 treatment, was unchanged in DOCA-salt rats. These novel results reveal a regionally specific defect in ET_B receptor mediated plasma clearance of ET-1 in the splanchnic vasculature of DOCA-salt rats.

Despite the absence of ETB receptor-mediated clearance of plasma ET-1 in the splanchnic circulation of DOCA-salt rats, there is not an increase in the circulating ET-1. It may reflect that the splanchnic bed plays a minor role in the plasma clearance of ET-1. The relative role of splanchnic circulation in the plasma clearance of ET-1 can be tested in the future by measuring the radiolabelled ET-1 trapping by different tissues. Another possibility is that there is less ET-1 production in DOCA splanchnic vasculature.

Comparing the data from anesthetized rats and conscious rats, I found that the change in aortic plasma ET-1 level, and in nNHSR of ET-1 after ET_B receptor blockade, was greater in conscious versus anesthetized rats. This indicates that ET_B receptor mediated clearance of ET-1 was decreased in anesthetized and surgically prepared rats. The decreased uptake activity of ET_B receptors in anesthetized rats may be due to desensitization of endothelial ET_B receptors caused by higher circulating ET-1 level, or to a lower cardiac output (and tissue blood flow) under anesthesia. However, data from both conscious and anesthetized rats provide consistent evidence that the uptake activity of endothelial ET_B receptors in DOCA-salt rats is impaired in the splanchnic vasculature, while remaining unchanged in the whole body.

One of the weaknesses of this study is that I didn't measure the possible differential influence of ETB receptor antagonist on the portal venous blood flow of DOCA-salt and sham rats. It was reported that A192621 didn't cause any significant change in the portal venous blood flow of normal rats (31). No similar studies have been done in DOCA-salt hypertensive rats.

2) Mechanism and impact of reduced ET_B receptor mediated plasma clearance of ET-1

ETB receptors are localized in both endothelial cells and vascular (mainly venous) smooth muscle cells. It is not clear if smooth muscle ETB receptors also play a role in the clearance of plasma ET-1. So in my study, I only considered the possible mechanisms contributing to the impaired uptake activity mediated by endothelial ETB receptors.

Previous results indicate immunoreactive ET_B receptor number tends to be higher in vena cava compared to aorta, but no difference was found in the vena cavae of sham versus DOCA-salt rats (254). However, that study did not distinguish between endothelial and smooth muscle ET_B receptors; these probably differ in their ability to clear ET-1 from the circulation. Possible differences between large and small veins also were not considered. In my study, I found that there was no significant difference in the fluorescence intensity of ETB receptors in the endothelial cells between DOCA-salt and sham tissue. The lack of difference in the distribution pattern of vWF in the endothelial cells between DOCA-salt and sham rats suggests the structure of the DOCA-salt endothelium is intact. Western blot analyses from Dr.Watts lab showed that there was no

significant difference in the protein level of ETB receptors between DOCA-salt and sham small mesenteric blood vessels. Although that result represents the protein level of ETB receptors in the entire blood vessel, the result is consistent with mine, i.e. both suggest that the impaired ETB receptor-mediated uptake activity in the splanchnic bed of DOCA-salt rats is not due to fewer ETB receptor on endothelial cells.

One of the limitations of this study is that it is difficult to separately quantify the fluorescence intensity of ETB receptors in the membrane versus ETB receptors inside the endothelial cells, which would, otherwise, give us an estimate of the relative number of active ETB receptors and internalized, inactive ETB receptors. Unaltered total number of ETB receptors in the endothelial cells of DOCA-salt rats doesn't necessarily mean unaltered number of active ETB receptors. It is possible that there are fewer active ETB receptors in the endothelial cells of DOCA-salt rats and that contributes to the impaired ETB receptor-mediated uptake activity. Theoretically, the cellular localization of ETB receptors can be studied by taking different fractions of endothelial cells by supercentrifugation and testing the protein level of ETB receptors in different fractions using western blot analysis. However, it may be hard techniqualy in terms of collecting enough endothelial cells fractions.

In terms of other possible mechanisms underlying the decreased uptake activity of ET_B receptor specifically in the splanchnic vasculature of DOCA-salt rats, some possibilities are:

1) a decrease in overall endothelial cell function

Previous studies compared the overall endothelial cell function between DOCA-salt and sham small mesenteric blood vessels using acetylcholine induced vasorelaxation

(103). No significant difference was found, suggesting that the overall endothelial cell function is unchanged in DOCA-salt small mesenteric blood vessels.

2) a lower binding affinity of ET_B receptors for ET-1

No radioligand binding assay has been done to provide direct evidence for the binding affinity of ETB receptors for ET-1 in DOCA-salt and sham rats. Functional studies (103) showed that the dose response curve of small mesenteric veins to S6C, a selective ETB receptor agonist, is not significantly different between DOCA-salt and sham rats, suggesting that the binding affinity of ETB receptors for ET-1 is unchanged in DOCA-salt rats.

3) altered post-receptor ET-1 disposition mechanisms

It has been suggested (14) (Fig. 14) that both ETA and ETB receptors are rapidly internalized upon agonist stimulation. This process is dependent on G-protein coupled receptor kinases (GRKs), arrestin, dynamin, and clathrin (Agonist-activated receptors are phosphorylated by GRKs, followed by binding of arrestin and initiation of receptor internalization through a clathrin/dynamin-dependent mechanism). Internalized ETA and ETB are directed to Rab 5 positive early endosomes. From this location, the two receptor subtypes are targeted to different intracellular fates. The ETA receptor is directed to the recycling compartment and subsequently reappears at the plasma membrane. The ETB receptor is directed to lysosomes for degradation. That is suggested to be the mechanism by which ETB receptor mediates the clearance of plasma ET-1. There may be something wrong with one or multiple steps involved in the ETB receptor internalization pathway in DOCA-salt rats that leads to the impaired uptake activity of ETB receptors.

In any case, the physiological significance of reduced splanchnic ET-1 plasma clearance by ET_B receptors is not known, i.e. it is uncertain if the decreased uptake activity of ET_B receptor in the splanchnic vasculature affects the ability of ET-1 to control vascular capacitance in DOCA-salt rats. Further work is required to address this question.

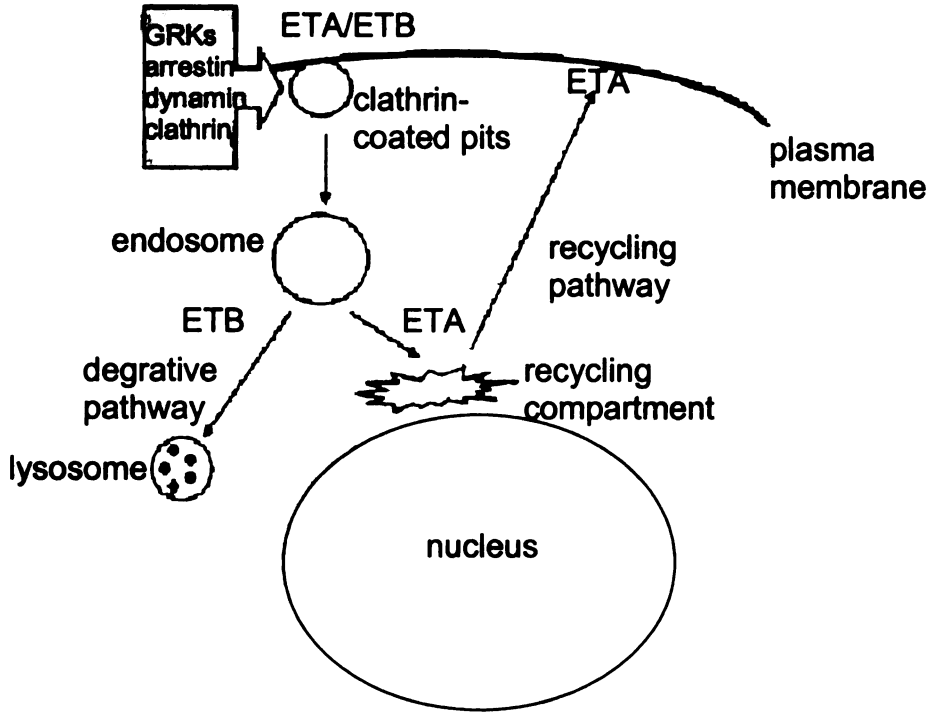


Figure 14: Model for regulation and intracellular trafficking of the ET receptors (14)

Chapter 4 – Part III

ET-1 synthesis in the splanchnic vasculature of DOCA-salt rats

Rationale: ET-1 is derived from preproendothelin-1. The translation of preproendothelin mRNA results in the formation of a 212-amino acids preproendothelin peptide, which is cleaved by a furin convertase (33) to the 38-amino acid peptide big ET-$1_{1\text{-}38}$ (91). Once formed, big ET-1 is processed to ET-$1_{1\text{-}21}$ through cleavage of the Trp21-Val22 bond by ET-converting enzyme-1 (ECE-1), which exists in 4 isoforms (a, b, c, and d) (259) and by ECE-2 (205) (214) (208) (241) (48) and chymase (258). ECEs are localized in endothelial (259) and smooth muscle cells (208) (153), cardiomyocytes (186) (186) (114), and macrophages (69) (153). ECEs belong to the metalloprotease family (259), share functional and structural similarity with neutral endopeptidases (NEPs) and Kell blood group proteins (241) (48) (180), and are partially inhibited by phosphoramidon (146). ECE-1 expression is regulated through protein kinase C-dependent mechanisms (239), ETB receptors (160), the transcription factor ets-1(167), and cytokines (269).

Increased expression of ECE-1 has been found in failing human myocardium (156), renal medullary area of DOCA-salt rats (96), but not in DOCA blood vessels (234). Even though overexpression of ECE-1 itself does not lead to systemic hypertension (233), the enzyme can be a crucial rate-limiting factor in the production of mature ET-1 in vivo.

It has been found that CGS compounds, which are vasopeptidase inhibitors that simultaneously inhibit ECE and NEP, can dose-dependently increase plasma big ET-1 level, decrease the pressor effect of big ET-1 in conscious rats (47) and reduce plasma ET-1 to normal levels in rats with chronic heart failure (34). CGS compound has also been found to reduce blood pressure in conscious unrestrained normotensive rats,

spontaneously hypertensive rats (The magnitude of the antihypertensive effects was greater in SHRs than in normotensive rats) (4) and human without reflex increases in heart rate (34).

In my studies mentioned in Chapter 4-part I and II, I measured vascular ET-1 content, plasma ET-1 level before and after blockade of the major process affecting circulating ET-1 level—ETB receptor mediated uptake, in order to get an estimate of the formation of ET-1 by the splanchnic vasculature of DOCA-salt and sham rats. However, since ECE is a rate-limiting step in ET-1 generation, the change in nNHSR of ET-1 due to inhibition of ECE should provide more direct manifestation of ET-1 generation in the splanchnic bed. I first treated my rats with ETB receptor antagonist to provide higher basal level of ET-1. Then the rats were treated with a dual ECE/NEP inhibitor. The difference between the net NHSR of ET-1 in rats treated only with ETB receptor antagonist and that in rats treated with both ETB receptor antagonist and ECE inhibitor can be used as an index of net NHS ET-1 synthesis.

Experimental Procedures: After cannulation surgery, rats were given A192621 (12mg/kg, i.v.) first to block ET-1 uptake and 30 minutes later, rats were treated with CGS26393 (3mg/kg, i.v.), a dual ECE-1 and NEP inhibitor. Portal venous and aortic blood samples were taken simultaneously 1 hmy later. Plasma ET-1 levels were measured.

Results: 30 min after surgery, the average MAP of sham rats was 87.8 ± 6.5 mmHg. It was increased to 121.0 ± 4.6 mmHg 30min after A192621 treatment. 60 min after

CGS26393 treatment, the average MAP was 89.0 ± 6.9 mmHg. 30 min after surgery, the average MAP of DOCA-salt rats was 119.6 ± 13.0 mmHg. It was increased to 163.3 ± 9.5 mmHg 30min after A192621 treatment. 60 min after CGS26393 treatment, the average MAP was 133.1 ± 12.7 mmHg. I didn't find, however, a significant effect of CGS26393 on the net NHSR of ET-1 in either DOCA-salt (nNHSR of ET-1 was 3.21 ± 0.9 pg/ml with ETB antagonist treatment and 4.83 ± 1.3 pg/ml with ETB antagonist + CGS 26393 treatment) or sham rats (nNHSR of ET-1 was 4.43 ± 1.1 pg/ml with ETB antagonist treatment and 2.52 ± 1.3 pg/ml with ETB antagonist + CGS 26393 treatment).

Discussion for Chapter 4 - Part III

ECE is the rate-limiting step in ET-1 generation. ECE inhibition is a very important and attractive target for the inhibition of the ET system for treating cardiovascular diseases where the ET system plays a pathogenic role, e.g. essential hypertension, chronic heart failure (248). A number of non-selective and selective ECE-1 inhibitors have been identified and are currently in early-phase clinical studies. Results from early studies are promising, particularly in essential hypertension.

In my studies, I found that ETB receptor antagonist significantly increased the MAP in both anesthetized DOCA-salt and sham rats, which was consistent with what I saw in my previous studies. 60 min after ECE inhibition (i.e. 90 min after ETB receptor blockade), the MAP in both DOCA-salt and sham rats were not significantly different from the basal level. It was not clear if this was due to the anti-hypertensive effect of the ECE/NEP inhibitor I used or just the effect of ETB receptor antagonist on blocking the clearance of ET-1 started to wear off. However, it is more likely to be the latter because in my later studies, in vivo bolus injection of same dose of CGS 26393 failed to cause a significant decrease in the mean arterial pressure in conscious DOCA-salt rats.

CGS 26393 also failed to cause any significant change in either the nNHSR of ET-1, nor the aortic or portal venous plasms ET-1 levels in either DOCA-salt or sham rats. When combined with the fact of lack of significant effect on MAP, both data suggest that a higher dose of ECE inhibitor may be required in future studies.

However, another possibility exists. ECE-independent pathways also contribute to ET-1 production. In ECE-1 knockout mice, tissue levels of ET-1 are reduced by only one-third (265). Indeed, chymase generates ET-$1_{1\text{-}21}$ (258). In addition, 2 novel ET-$1_{1\text{-}21}$-

forming enzymes, a non-ECE metalloprotease and a vascular smooth muscle cell chymase, have been cloned. Therefore, activation of alternative ET-1 production pathway could also be used to explain the lack of change in the nNHSR of ET-1 in my study.

Chapter 4 – Part IV

Possible role of ETA receptor binding in the plasma clearance of ET-1 in the splanchnic bed of DOCA-salt rats

Rationale: In contrast to other peptides, there appears to be a relatively small amount of ET peptide in blood and tissue in relation to the concentration of ET receptors. Besides, the binding of ET-1 to its receptors is particularly tight and ET-1 remains bound to ETA receptors even after their internalization (67). These facts lead to the hypothesis that ETA receptors may also function as a clearance receptor, and the binding of ET-1 to ETA receptors in the vascular smooth muscle cells may affect the measurement of nNHSR of ET-1. To test the possibility that ETA receptor binding contributes to the nNHSR of ET-1, I compared the nNHSR of ET-1 after either combined ETA and ETB receptor blockade with that after selective ETB receptor antagonism alone. The difference was used as an index of the amount of ET-1 bound to ETA receptors in the splanchnic vasculature of normotensive and DOCA-salt hypertensive rats. nNHSR of ET-1 in rats treated with selective ETA receptor antagonist was also calculated to further identify the role of ETA receptors in the clearance of plasma ET-1.

Experimental Procedures: Two sets of experiments were performed. Both were done in anesthetized rats.

1) A182086, a mixed $ET_{A/B}$ receptor antagonist, was given (12 mg/kg, i.v., Abbott Laboratories, IL) to rats 30 minutes after recovery from catheterization as described in General Methods. Aortic and portal venous blood samples were collected before and 1 hmy after drug treatment. The dose of A182086 was shown to be efficacious in

preliminary studies, in which antagonist was tested against acute pressor actions of ET-1 (data not shown). Published studies also indicate that this dose is effective in blocking both receptor subtypes in rats (256). The nNHSR of ET-1 after A182086 treatment was compared with that after selective ET_B receptor antagonist A192621 treatment. The difference was used as an index of the amount of ET-1 bound to ET_A receptors in the splanchnic vasculature.

2) Atrasentan, a selective ETA receptor antagonist, was given (1mg/kg, i.v.) to rats 30 minutes after recovery from catheterization as described in General Methods. Aortic and portal venous blood samples were collected before and 1 hmy after drug treatment. Aortic and portal venous plasma ET-1 levels were measured. The increase in aortic plasma ET-1 level and nNHSR of ET-1 caused by atrasentan was compared with those caused by selective ETB antagonist and combined ETA/B antagonist.

Results:

1) A182086 treatment did not cause a significant change in mean arterial pressure in either DOCA-salt or sham rats (data not shown). A182086 caused a significantly greater increase in the aortic plasma ET-1 concentration in both DOCA-salt and sham rats than the selective ETB receptor antagonist. There was no significant difference in the magnitude of increase between DOCA-salt and sham rats (Fig. 15A). A182086 also significantly increased the net NHSR of ET-1 in both groups (Fig. 15B) and the magnitude of increase was not significantly different between the two groups.

2) Atrasentan significantly decreased the MAP of DOCA-salt (from 120.3 ± 7.9 mmHg to 88.6 ± 8.0 mmHg), but not sham rats (from 101.5 ± 6.8 mmHg to 117.7 ± 8.5 mmHg).

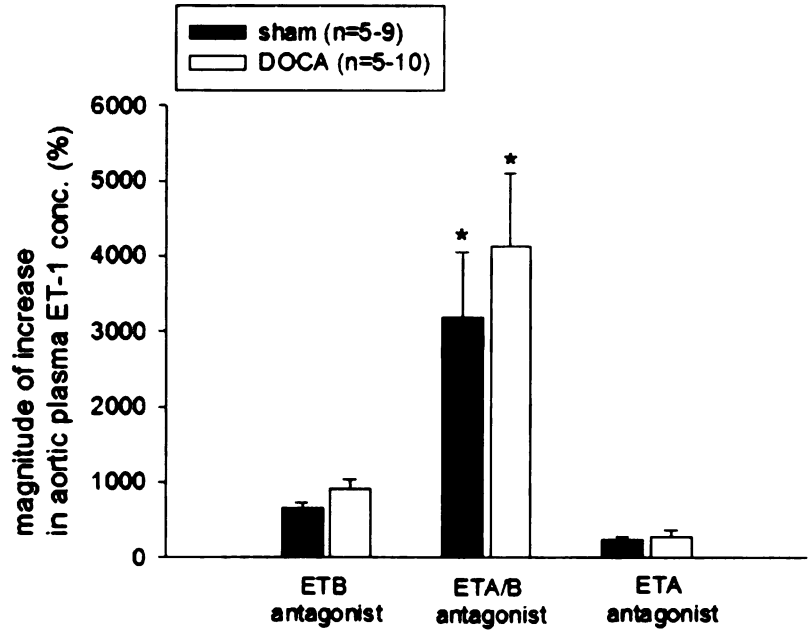
Atrasentan caused a small increase in the aortic plasma ET-1 level and the nNHSR of ET-1 in both DOCA-salt and sham rats (Fig. 15A, 15B). There was no significant difference in the magnitude of increase between the two groups.

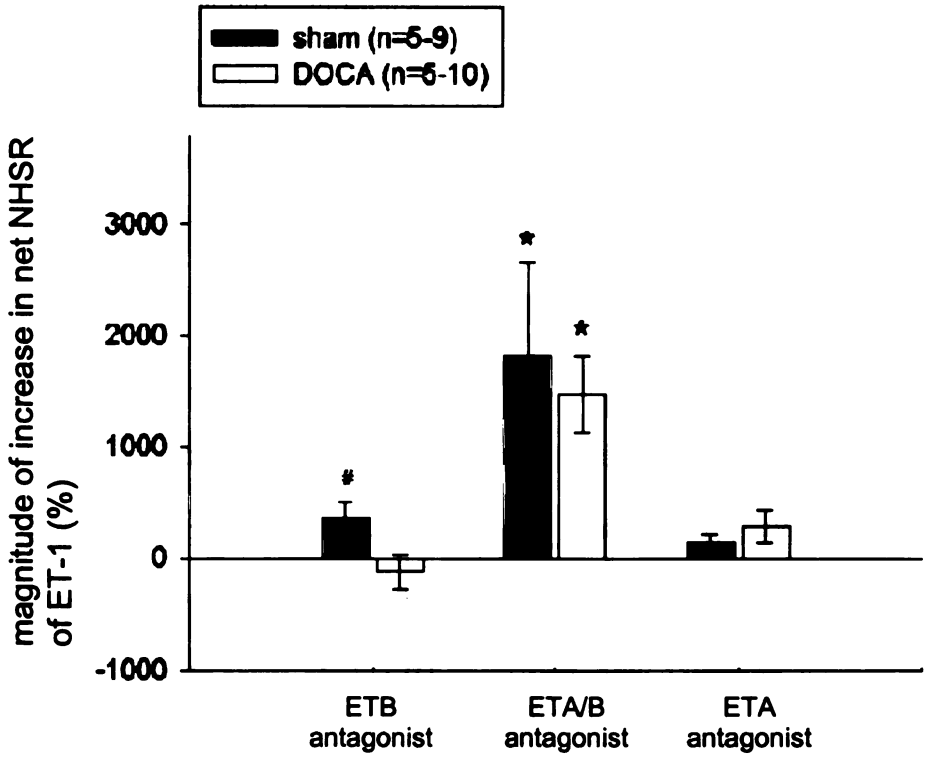
Figure 15:

A: Effects of selective ETB receptor antagonist, combined ETA/B receptor antagonist and selective ETA receptor antagonist on aortic plasma ET-1 levels in anesthetized DOCA-salt and sham rats. Bars indicate the magnitude of increase in aortic plasma ET-1 level with different drug treatments in anesthetized sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between different drug treatments.

B: Effects of selective ETB receptor antagonist, combined ETA/B receptor antagonist and selective ETA receptor antagonist on estimated net NHSR of ET-1 in anesthetized DOCA-salt and sham rats. Bars indicate the magnitude of increase in net NHSR of ET-1 with different drug treatments in anesthetized sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between different drug treatments. # indicates a significant difference ($P<0.05$) between DOCA-salt and sham rats.

A


sham (n=5-9)
DOCA (n=5-10)
magnitude of increase in aortic plasma ET-1 conc. (%)
6000
5000
4000
3000
2000
1000
0
*
*
ETB antagonist
ETA/B antagonist
ETA antagonist


sham (n=5-9)
DOCA (n=5-10)
magnitude of increase in net NHSR of ET-1 (%)
3000
2000
1000
0
-1000
#
*
*
ETB antagonist
ETA/B antagonist
ETA antagonist

Discussion for Chapter 4 – Part IV

Combined ET_A and ET_B receptor blockade caused a significant increase in aortic and portal venous plasma ET-1 concentrations in both DOCA-salt and sham rats. The magnitude of that increase was roughly two times higher than that seen in rats with ET_B receptor blockade alone. This suggests that in both DOCA-salt and sham rats, a significant amount of ET-1 is removed from the circulation by ET_A receptor binding and is consistent with a previous hypothesis (67) that functional ET_A receptors also act as "clearance" receptors. Combined ETA and ETB receptor blockade also caused a much higher increase in the net NHSR of ET-1 in both group of rats compared with the increase caused by ETB receptor blockade alone, suggesting that ETA receptors also contribute to the plasma clearance of ET-1 in the splanchnic circulation. Although selective ETB antagonism caused a significant higher increase in the nNHSR of ET-1 in sham rats than DOCA-salt rats, there was no significant difference in the magnitude of increase in the nNHSR of ET-1 between sham and DOCA-salt rats after the treatment of combined ETA/B antagonist. These suggest that there may be more ETA receptor binding in the splanchnic bed of DOCA-salt rats.

To further understand the role of ETA receptors in the plasma clearance of ET-1, I treated my DOCA-salt and sham rats with selective ETA receptor antagonist. What I expected to see is a great increase in the aortic plasma ET-1 concentration, representing the huge amount of ET-1 bound to ETA receptors in the systemic circulation. And ideally, the increase in aortic plasma ET-1 concentration caused by ETA antagonist plus the increase caused by ETB antagonist should be close to the increase caused the combined ETA and ETB receptor antagonist. Surprisingly, in my study, I found that

selective ETA antagonist only caused a small increase in the aortic plasma ET-1 concentration in both DOCA-salt and sham rats. The magnitude of increase in aortic plasma ET-1 level with combined ETA and ETB antagonism is much greater than the sum of increase in aortic plasma ET-1 level with selective ETB and ETA antagonism, suggesting that systemic plasma ET-1 clearance by ETA receptors is different in the presence and absence of ETB receptors.

In terms of the effect of selective ETA receptor antagonist on the nNHSR of ET-1, similarly, I would expect to see a huge increase in the nNHSR of ET-1 in both DOCA-salt and sham rats, representing the huge amount of ET-1 bound to ETA receptors in the splanchnic vasculature. I also expected to see greater increase in DOCA-salt rats compared with sham rats because there appears to be more ETA receptor bindings in DOCA splanchnic bed according to this study. And again, surprisingly, selevtive ETA receptor antagonist only caused a small increase in the nNHSR of ET-1 in both DOCA-salt and sham rats. The increase is not significantly greater in DOCA-salt rats. This suggests that it is the same in the splanchnic vasculture that the plasma clearance of ET-1 by ETA receptors is different in the presence and absence of ETB receptors.

My data suggest that the effects of combined receptor antagonist were not consistent with simple additive receptor blockade. Plasma ET-1 is not cleared from the circulation via direct and additive activation of ETA and ETB receptors. One possible explanation could be that there is an important interaction between ETA and ETB receptors in the plasma clearance of ET-1. For example, functional ETB receptors may have an inhibitory effect on the clearance activity of ETA receptors. Findings in other in vitro studies have also suggested the existence of an interaction between the ETA and ETB receptors

including vascular (both arteries and veins) (152) (131) and nonvascular preparations (71) (70). The mechanism behind the proposed interaction between ETA and ETB receptors is not understood but may involve interactions at the receptor level or through second messengers (70). Biochemical studies using cells transfected with different endothelin receptor subtypes to examine receptor and second-messenger interactions should provide valuable insight into the mechanism behind the proposed receptor "cross talk".

My studies suggest that there may be more ETA receptor binding in the splanchnic bed of DOCA-salt rats. However, it is not consistent with previous in vitro studies in other group (152) (154) showing that ET_A receptor binding was reduced, whereas binding affinity was not altered in membranes of both large and small mesenteric arteries from DOCA-salt compared with sham rats. The decrease in ET_A receptor binding was not due to reduced expression of ET_A receptors, since protein levels were elevated in vessels from DOCA-salt rats compared with sham rats (154). The finding that ET_A receptor protein was elevated while maximum binding was decreased indicates that some of the ET_A receptors are modified in a manner that will reduce binding. No similar studies have been performed in mesenteric veins. Future in vitro studies in the ETA receptor binding using mesenteric veins can provide basis for further comparison of ETA receptor binding in the whole splanchnic vasculture between DOCA-salt and sham rats.

Discussion for Chapter 4

Previous studies in my lab have provided evidence for increased venoconstriction in conscious DOCA-salt hypertensive rats using repeated measurements of blood volume and MCFP, an in vivo index of venous smooth muscle contractile activity (64). Endogenous ET-1 appears at least partly responsible for that augmented venoconstriction, since ET receptor antagonists lower MCFP in DOCA-salt hypertensive but not normotensive rats (104).

Two possible mechanisms could explain increased ET-1 mediated venoconstriction: higher levels of the peptide around venous smooth muscle cells and/or enhanced responsiveness of venous smooth muscle to ET-1. It has been shown that vena cava and small mesenteric veins from DOCA-salt hypertensive rats do not exhibit increased reactivity to ET-1 *in vitro* (103) (254). Therefore, it was logical to conclude that higher ET-1 mediated venoconstriction in DOCA-salt rats is caused by increased ET-1 concentrations around venous smooth muscle. Measurement of preproET-1 gene expression — as an index of ET-1 synthesis —in small veins, however, revealed no differences between sham and DOCA-salt rats (103). That finding does not support the hypothesis that increased venomotor activity in DOCA-salt rats is caused by local overproduction of ET-1 by veins.

Veins in DOCA-salt rats could be exposed to higher concentrations of ET-1 by mechanisms other than increased venous endothelial cell synthesis. For example, increased production and release into blood of ET-1 from upstream arterial endothelial cells could affect venous smooth muscle cell activity directly (112), or provide "excess" ET-1 to be taken up into venous endothelial cells for subsequent release. To test these

possibilities, I made two kinds of measurements to estimate ET-1 concentrations in the biophase around venous smooth muscle cells: total vascular ET-1 content and venous plasma ET-1 concentration. Measurements were made in the splanchnic vascular bed, since that region represents the most important capacitance bed in the circulation. I also estimated net non-hepatic splanchnic release of ET-1 by determining the difference between ET-1 concentrations in inflowing arterial and outflowing portal venous blood. Plasma ET-1 level is determined by a combination of factors including production rate, metabolism and clearance. To test the possibility that a functional alteration in ET_B receptors might affect the venous plasma ET-1 concentration in DOCA-salt hypertension, I measured the portal venous-aortic plasma ET-1 concentration difference after selective ET_B receptor blockade in both anesthetized and conscious rats. I also measured the portal venous-aortic plasma ET-1 concentration difference after combined ETA/B and selective ET_A receptor blockade in order to test the potential role of ETA receptor in the plasma clearance of ET-1.

My studies found that neither ET-1 content of venous capacitance vessels nor portal venous plasma ET-1 concentration is higher in DOCA-salt versus sham rats. Furthermore, estimated nNHSR of ET-1 is not higher in DOCA-salt versus sham rats even when major processes removing ET-1 from the circulation are blocked. Collectively the results provide strong evidence that higher endothelial cell formation of ET-1 is not likely responsible for enhanced ET-1 mediated splanchnic venoconstriction *in vivo*. When combined with the fact that venous smooth muscle contractile responsiveness to ET-1 is not increased in DOCA-salt rats, the data suggest that the ability of ET receptor antagonists to reduce venoconstriction in DOCA-salt rats *in vivo* results from actions of

the drugs on a non-vascular target. I speculate that the enhanced venoconstrictor effect of ET-1 could be mediated by an action of ET-1 on the sympathetic nervous system. A number of studies have shown prejunctional modulation of sympathetic neurotransmission by ET-1 in vitro (158), e.g. Intrarenal arterial infusion of ET-1 inhibited norepinephrine release induced by renal sympathetic nerve stimulation in anesthetized dogs (147). Localization of ET-1 receptors on the nerve terminals have been reported (60). Neurotransmission in sympathetic ganglia also can be modulated by ET-1 (238) (26). A previous study in my lab (104) found that pretreatment of DOCA-salt hypertensive rats with an ET_B receptor antagonist produced greater declines in MCFP following ganglionic blockade than after ganglionic blockade alone. These findings suggest that ET-1 acting at ET_B receptors may provide an important contribution to venoconstriction through modulation of sympathetic tone to the veins. The ET_B antagonist may have facilitated NE release from sympathetic nerve terminals on veins, accounting for the significantly larger response of MCFP to subsequent ganglion blockade. This action presumably was more prominent in DOCA-salt rats because of their higher pretreatment levels of endogenous vascular ET-1.

The decrease in venous capacitance in hypertension might be mediated by other mechanisms, such as changes in venous wall structure, or other influences on venous smooth muscle activity besides ET-1 and sympathetic input, such as reactive oxygen species.

CHAPTER 5

SYMPATHETIC NEUROTRANSMISSION IN THE SPLANCHNIC BED OF RATS IN THE ESTABLISHED PHASE OF DOCA-SALT HYPERTENSION

Introduction

1. Autonomic innervation of the cardiovascular system

The sympathetic nervous system plays an important part in regulating cardiovascular homeostasis. Catecholaminergic neurons releasing NE are widely distributed in the brain, but are located in particular in the medulla and pons (66). The hypothalamus, and parts of the limbic system including the amygdala, receive projections from these brainstem noradrenergic nuclei. Electrophysiologic and anatomical experiments carried out in animals provide evidence of a connection between pressor noradrenergic hypothalamic and brainstem centers and sympathetic preganglionic neurons in the thoracolumbal cord (99, 242).

Autonomic outflow from the brainstem is divided principally into sympathetic and parasympathetic (vagal) branches. Efferent fibers of sympathetic nerves travel along the nerve column into sympathetic ganglia. These ganglia use acetylcholine as a neurotransmitter where activity is transmitted to noradrenergic neurons which innervate the heart, vasculature and many other organs of the body.

Celiac, superior mesenteric, and inferior mesenteric ganglia provide axons that are distributed with the three major gastrointestinal arteries arising from the aorta. The celiac ganglion innervates the stomach, liver, pancreas, duodenum, and the first part of the small intestine; the superior mesenteric ganglion innervates the small intestine; and the inferior mesenteric ganglion innervates the descending colon, sigmoid colon, rectum, urinary bladder, and sexual organs.

2. Increased sympathetic nervous system activity in human hypertension

Evidence drawn from a number of smyces, including electrophysiological and neurochemical techniques, provides compelling evidence that overactivity of the sympathetic nervous system is commonly present in younger patients with essential hypertension. In borderline and established hypertension, nerve firing rates in postganglionic sympathetic fibers passing to skeletal muscle blood vessels are increased. There is also increased spillover of the sympathetic neurotransmitter NE from the heart and kidneys, providing evidence of stimulated sympathetic outflow to these organs (1, 58, 80, 260). The increased cardiac and renal sympathetic nerve firing provides a plausible mechanism for the development of hypertension, through the regulatory influence of the sympathetic nervous system on renin release, glomerular filtration rate, and renal tubular sodium reabsorption, and on cardiac growth and pump performance (51). The specific causes of the increased sympathetic activity in essential hypertension remain largely unknown, although genetic influences are evident and behavioral and lifestyle factors appear to be involved. There is growing evidence of an underlying disturbance in CNS monoaminergic control of sympathetic outflow (57), which may perhaps be the common mediating mechanism of peripheral sympathetic activation with stress, obesity, and physical inactivity. In hypertensive patients, NE spillover from the brain on average is higher than in healthy subjects, as is the overflow into the internal jugular veins of dihydroxylphenylglycol (DHPG, the intraneuronal metabolite of NE) and 3-methoxy-4-hydroxylphenylglycol (MHPG, the extraneuronal metabolite of NE) (62). In patients with an increased spillover of NE and its metabolites from the brain, peripheral sympathetic activity is increased (62).

3. Sympathetic nerves and adrenergic receptors in arteries of hypertensive human subjects and animal models

Changes in the function of the sympathetic nervous system occur in DOCA-salt hypertension. Circulating norepinephrine and epinephrine levels are increased in DOCA-salt hypertensive rats, suggesting that there is an increased release of norepinephrine from sympathetic nerves and epinephrine from the adrenal gland in these animals (12). 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 norepinephrine than those obtained from normotensive rats (237). Increased release of norepinephrine from periarterial sympathetic nerves in DOCA-salt rats is due, at least in part, to a disruption in the function of prejunctional α2-adrenergic autoreceptors (29). 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 norepinephrine (133, 176), although other studies showed that the sensitivity to adrenergic agonists did not change in caudal artery (90), and the binding affinity of α1-adrenergic receptors in mesenteric arteries is reduced (149).

4. Sympathetic nerves and adrenergic receptors in veins of hypertensive human subjects and animal models

Venoconstriction is controlled largely by sympathetic nerve activity (155). 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 (116) (138).

In human hypertension and in spontaneously hypertensive rats (SHRs), sympathetic neural input to veins is increased: 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 (221). 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 (257). It has also been shown that hexamethonium caused a significantly larger decline in the MCFP in DOCA-salt than sham rats, suggesting that increased sympathetic venoconstrictor activity is an important mediator of increased venoconstriction in DOCA-salt hypertension. This previous work indicates that there are changes in sympathetic neurotransmission to veins in hypertension, but the mechanism of these changes are unclear.

5. Causal mechanisms leading to sympathetic augmentation in hypertension are poorly understood

Although there is growing evidence that essential hypertension and many hypertensive animal models are neurogenic (1, 80), and are initiated and sustained by overactivity of the sympathetic nervous system, the precise causal mechanisms leading to sympathetic augmentation in hypertension are still poorly understood. Among others,

possible mechanisms leading to sympathetic augmentation in hypertension include increased sympathetic nerve firing rate, increased NE release from the nerve terminals, impaired prejunctional α2 adrenoceptor feedback inhibition, altered neuronal NE reuptake (53, 193), and facilitation of NE release by neurohumoral factors such as ET-1.

It is important to identify the mechanisms since the increase in sympathetic activity is a mechanism for both initiating and sustaining the blood pressure elevation. Sympathetic nervous activation also confers specific cardiovascular risk. Stimulation of the sympathetic nerves to the heart promotes the development of left ventricular hypertrophy and contributes to the genesis of ventricular arrhythmias and sudden death. Sympathetically mediated vasoconstriction in skeletal muscle vascular beds reduces the uptake of glucose by muscle, and is thus a basis for insulin resistance and consequent hyperinsulinemia(50) (107, 140).

Chapter 5 – Part I

NE release from the sympathetic nerves associated with the splanchnic vasculature of anesthetized and conscious DOCA-salt rats.

1) Plasma NE concentration and NHS spillover of NE in anesthetized and conscious DOCA-salt rats

Rationale: There is substantial evidence for increased sympathetic input to the cardiovascular system in DOCA-salt hypertension (29). The splanchnic vasculature is richly innervated by sympathetic nerve fibers. Non hepatic splanchnic (NHS) circulation accounts for ~50% of total peripheral sympathetic outflow in humans (39) and experimental animals (2) (38). Sympathetic nerve stimulation can reduce intestinal blood volume by up to 60%, leading to a significant redistribution of blood from the splanchnic veins. This change in volume distribution will have a profound effect on overall cardiovascular functions including blood pressure (81) (65, 109). Net production of NE by the NHS circulation has been documented in normotensive rats (74), but not in DOCA-salt hypertensive rats.

In mesenteric veins of spontaneouly hypertensive rats or reduced renal mass hypertensive rats, membrane potentials were less negative compared to veins in control rats. Adrenergic blockade in hypertensive rats caused a hyperpolarization of venous smooth muscle membrane potential to a level similar to that measured in sham rats (257). Recent studies by my group also found more depolarized mesenteric venous smooth muscle membrane potential in DOCA-salt rats than sham rats, and adrenergic blockade hyperpolarized the venous smooth muscle membrane potential to a level similar to that

measured in sham rats. These data suggest that in hypertensive rats there is a tonic elevation of sympathetic input to mesenteric veins. Previous studies in my group (138) showed that in both sham and DOCA rats, mesenteric veins are more sensitive than arteries to nerve stimulation. Therefore increases in sympathetic nerve activity are likely to have a larger effect on veins than arteries for a given change in nerve activity. Although there was no significant difference in the frequency response curves obtained for contraction of DOCA and sham veins, DOCA veins were less sensitive to exogenous NE. This suggests that the sympathetic nerves may release more NE to compensate for the decrease in postjunctional sensitivity in DOCA-salt hypertension. DOCA-salt arteries are sensitized to the contractile effects of nerve stimulation. This could be due to increased transmitter release from sympathetic nerves or increased sensitivity of vascular smooth muscle to sympathetic neurotransmitters. The observation that there is no increase in sensitivity to exogenous NE in arteries indicates that there may be an increase in transmitter release. This suggestion is supported by the result in arteries showing that the increased response may be due to the combined contractile actions of nerve released NE and ATP in DOCA arteries while ATP alone mediates contractions in sham arteries.

Although there is indirect evidence for increased sympathetic nerve activity to the splanchnic circulation in DOCA-salt hypertensive rats (100, 145, 215), no direct measurements of the net production of NE by the NHS circulation have been reported. In my study, portal venous plasma NE concentration (NEpv), which should reflect splanchnic venous NE concentration, was measured. I also used the difference in plasma NE concentration between the portal venous blood and arterial blood (NEpv-NEa) as a

measure of net NHS release of NE, which is an index of splanchnic sympathetic nerve activity.

Both the portal venous plasma NE level and the net NHS release of NE, however, are just rough indice of splanchnic sympathetic nerve activity. NE is concurrently taken up and released from tissue beds. As a consequence, tissue NE release and removal can't be assessed by measurements of plasma NE and arteriovenous differences alone. Therefore, NHS NE spillover, which represents the rate of entry of locally released NE into portal venous plasma, was estimated as introduced in General Methods based on two assumptions: 1) Portal venous blood flow of DOCA-salt rats equals that of sham rats (264). 2) NHS fractional extraction (FX) of NE (fractions of NE removed from plasma during passage through NHS organs) equals NHS FX of epinephrine (13, 16, 22, 92).

Of the NE released by sympathetic nerves throughout the body, perhaps 10-20% overflows into the circulation (Fig. 1). The bulk of the remainder is disposed of by reuptake back into the nerves. NE escaping into the interstitial fluid of individual organs enters the circulation passively by diffusion down a concentration gradient. Possible determinants of this rate of NE spillover include factors such as regional blood flow and the adequacy of neuronal NE uptake, but in general the overflow of NE is approximately proportional to the rate of sympathetic nerve firing. The existence of this proportionality between the rate at which NE released from sympathetic nerves spills into the venous drainage of individual organs and the rate of sympathetic nerve firing provides the foundation for the use of NE overflow measurements as an index of sympathetic nerve firing rates in inaccessible sites, such as the sympathetic nerves of splanchnic organs.

Experimental Procedures: Initial experiments were performed in 4-5 week DOCA-salt and same age sham rats under urethane anesthesia (1.2 g/kg i.p.). Silicone rubber tipped catheters were placed into abdominal aorta via a femoral artery and portal vein via a small branch of the portal vein after laparotomy. 1 ml blood samples were collected simultaneously from arterial and portal venous catheters. Plasma NE and epinephrine were measured using a radioenzymatic assay. NHS spillover of NE was estimated as introduced in General Methods. Sampling of blood from the portal venous and aortic sites offers an advantage over conventional measurements in hepatic venous and arterial plasma. A substantial proportion of total body sympathetic outflow is directed towards splanchnic organs. The liver, however, removes approximately 57% and 32% of circulating NE and EPI, respectively (42) (115). Sampling before the liver avoids the interference of the efficient hepatic extraction of NE on the observation of substantial splanchnic organ NE spillover.

Further experiments were perfomed using conscious chronically tethered rats to avoid the influence of anesthesia and surgical stress on plasma catecholamine levels.

Results:

1) Neither the plasma NE level nor the estimated NHS spillover of NE is higher in anesthetized DOCA-salt rats than sham rats

I found that plasma NE level in portal vein equals that in aorta in anesthetized DOCA-salt and sham rats, indicating no net NHS production of NE. Furthermore, there was no significant difference in portal venous plasma NE concentration between DOCA-salt and sham rats, and surprisingly, higher aortic plasma NE concentration in sham rats (Fig. 16). This may be at least partly explained by the inhibitory effect of anesthesia on sympathetic nerve activity, especially in DOCA-salt rats, reflected by the much lower MAP in anesthetized DOCA-salt rats compared with that of conscious DOCA-salt rats (Fig. 17). I estimated NHS spillover of NE and found no significant difference between anesthetized DOCA-salt and sham rats (Fig. 18).

Data from anesthetized rats suggest that the sympathetic nerve activity is not increased either globally or in the splanchnic bed of DOCA-salt rats.

2) Both the plasma NE level (aortic and portal venous) and estimated NHS spillover of NE are significantly increased in conscious DOCA-salt rats versus sham rats

There is significantly higher aortic plasma NE level in conscious DOCA-salt rats than sham rats (Fig. 19). Portal venous plasma NE level is also significantly increased in DOCA-salt rats (Fig. 19). DOCA-salt rats have significantly greater estimated NHS spillover of NE than sham rats (Fig. 20). But the aortic plasma NE level and NHS spillover of NE in DOCA-salt rats are unrelated to arterial blood pressure and heart rate.

There is no significant difference in the estimated fractional extraction of NE in the splanchnic bed between DOCA-salt (approximately 42%) and sham rats (approximately 25%).

In both DOCA-salt and sham rats, there is significant higher EPI level in the aortic plasma than portal venous plasma (81 ± 19 pg/ml in sham aortic plasma; 46 ± 12 pg/ml in sham portal venous plasma; 89 ± 16 pg/ml in DOCA aortic plasma; 46 ± 7 pg/ml in DOCA portal venous plasma). However, there is no significant difference in either aortic or portal venous plasma EPI level between DOCA-salt and sham rats.

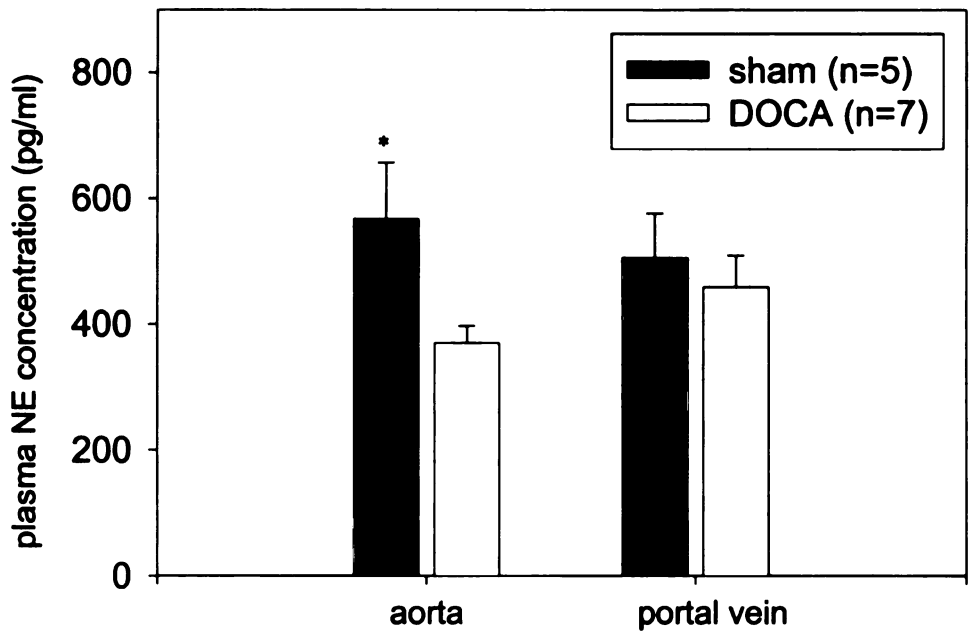


Figure 16: Aortic and portal venous plasma NE level in anesthetized DOCA-salt and sham rats. Bars indicate mean aortic or portal venous plasma NE concentration in anesthetized sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between sham and DOCA-salt rats.

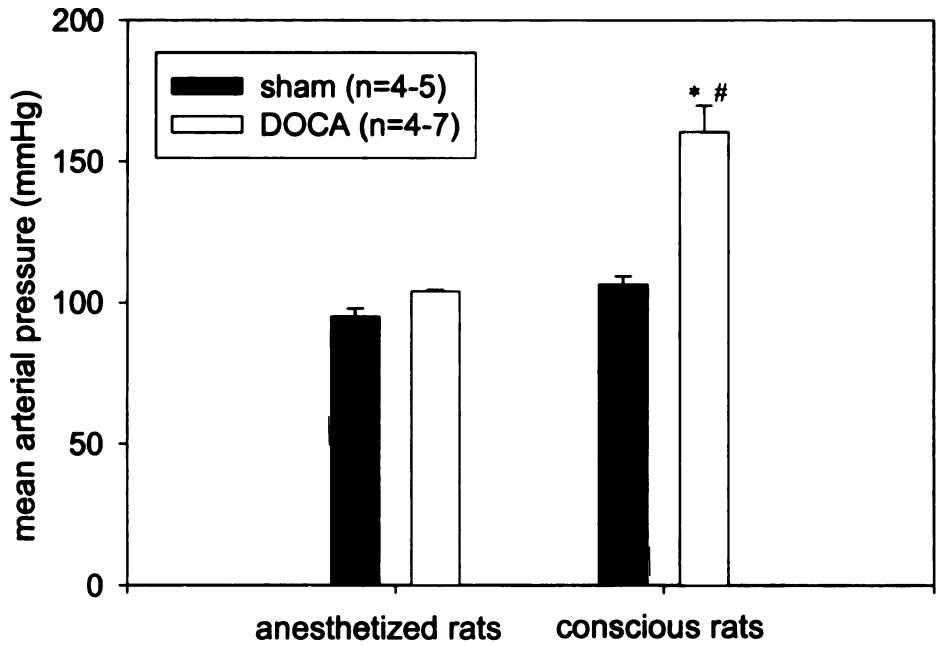


Figure 17: Mean arterial pressure of anesthetized and conscious DOCA-salt and sham rats. Bars indicate mean arterial pressure in anesthetized and conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between sham and DOCA-salt rats. # indicates a significant difference between conscious and anesthetized DOCA-salt rats.

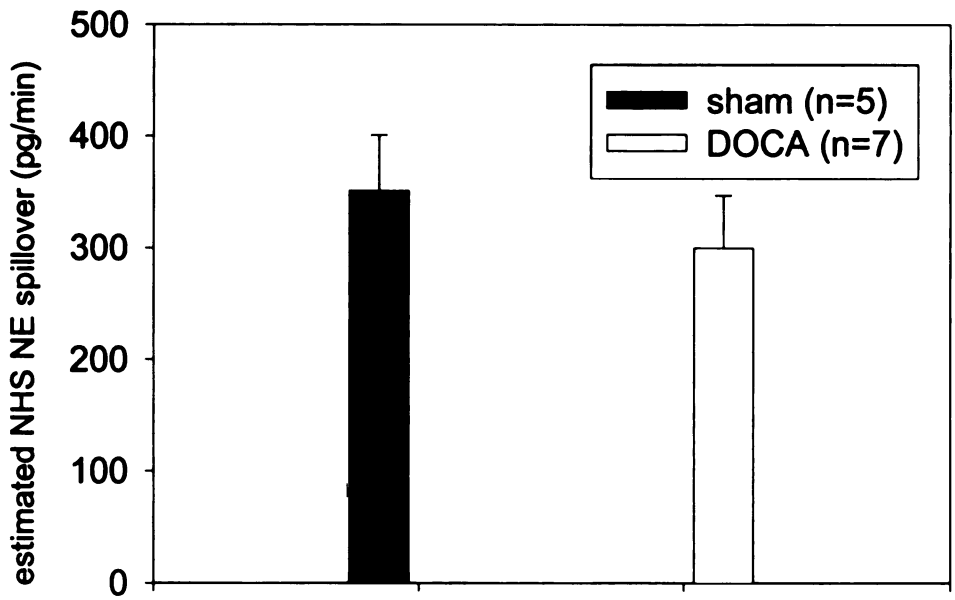


Figure 18: Estimated nonhepatic splanchnic spillover rate of NE in anesthetized DOCA-salt and sham rats. Bars indicate estimated NHS spillover rate of NE in anesthetized sham and DOCA-salt rats. Brackets indicate SEM.

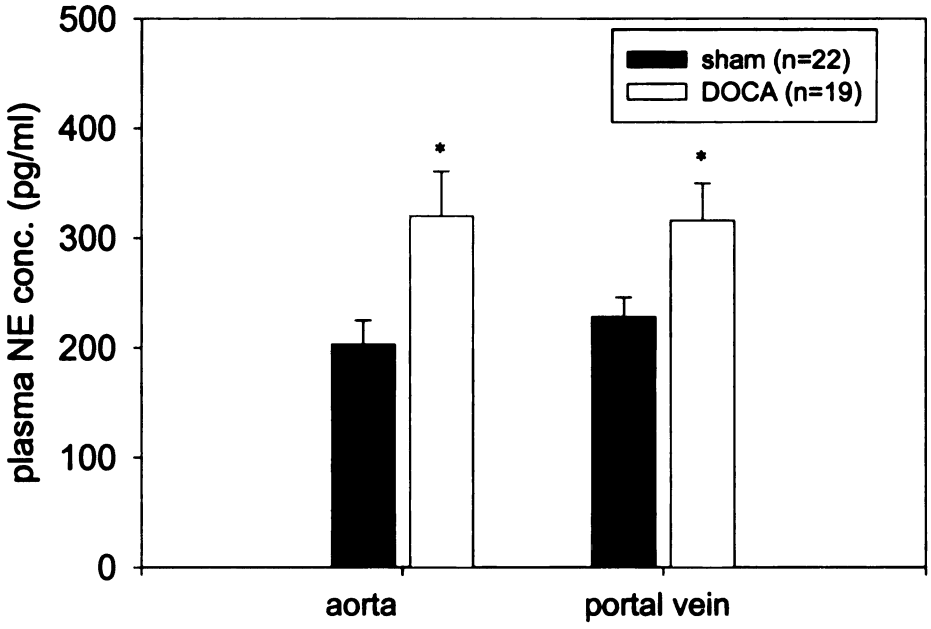


Figure 19: Aortic and portal venous plasma NE level in conscious DOCA-salt and sham rats. Bars indicate mean aortic or portal venous plasma NE concentration in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between sham and DOCA-salt rats.

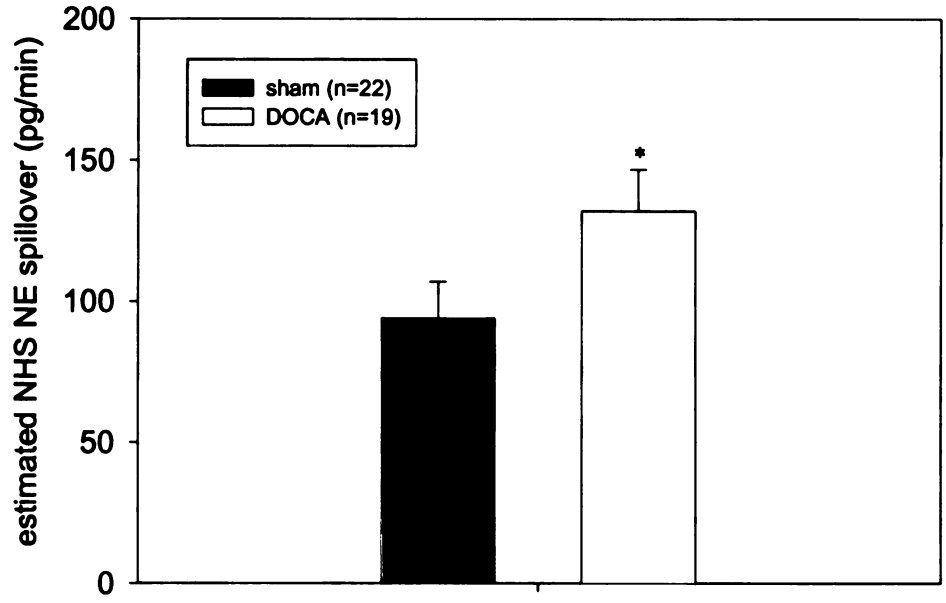


Figure 20: Estimated nonhepatic splanchnic spillover rate of NE in conscious DOCA-salt and sham rats. Bars indicate mean estimated NHS NE spillover rate in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between sham and DOCA-salt rats.

Discussion for Chapter 5-Part I

Numerous experimental and clinical studies, using various approaches to evaluate the release of sympathetic transmitters and to obtain sympathetic nerve microneurographic recordings, have shown clearly that sympathetic system activity and reactivity is increased in experimental models of hypertension, including DOCA-salt hypertensive rats. Consistent with published results (12), I found increased aortic plasma NE level in conscious DOCA-salt rats, suggesting systemic sympathetic hyperactivity in DOCA-salt rats. What needs to be mentioned here is that I did similar studies in anesthetized rats and I were not able to find either increased arterial or portal venous plasma NE level in DOCA-salt rats, although previous studies by de Champlain et al (12) found significantly higher baseline NE level in anesthetized DOCA-salt rats than in the respective normotensive controls.

In essential hypertension, there is increased cardiac and renal NE spillover and unchanged hepatomesenteric NE spillover (122) (54, 192, 194, 195). Increased cardiac (68) and renal sympathetic tone (110, 111) has also been reported in DOCA-salt hypertension. However, non-hepatic splanchnic NE spillover has never been measured before. I estimated the spillover of NE from the splanchnic bed to portal venous plasma and found it was significantly increased in DOCA-salt rats compared with sham rats. This difference was not a consequence of hemodynamic influences on transmitter washout to the circulation, as my later studies showed that portal blood flows were similar in the two groups. There is also a significantly increased portal venous plasma NE level in DOCA-salt rats than sham rats. Since the portal vein drains venous blood from the splanchnic organs, I used portal venous plasma NE concentration as an index of splanchnic

sympathetic nerve activity. As I expected, I saw significantly increased portal venous plasma NE level in DOCA-salt rats. The increased portal venous plasma NE level and increased NHS NE spillover suggest that there is increased splanchnic sympathetic nerve activity in DOCA-salt rats. In the future, total body NE spillover can be measured by the radiotracer method (introduced in more detail later) and the ratio of NHS NE spillover to total body NE spillover can be used as an index of the contribution of increased NE spillover in the splanchnic bed to the increase in total body NE spillover in DOCA-salt rats.

The origin of this sympathetic activation still remains largely unknown. NE release from sympathetic nerve endings is modulated pre-junctionally by adrenergic receptor mechanisms (125). Moreover, 80-90% of neuronally released NE is taken back up by the sympathetic nerve endings through an active reuptake (reuptake 1) mechanism (55). NE release from the nerve terminal can be facilitated by other neurohumoral factors like ET-1, Ang II and EPI. Thus, in addition to increased sympathetic nerve firing, changes in either or all of these modulatory mechanisms in DOCA-salt rats could confound the interpretation of plasma NE level and regional spillover measurements. For example, a decrease in α2 prejunctional inhibition of peripheral NE release would serve to augment the amount of NE released per unit sympathetic nerve discharge and result in an overestimation of SNS activity based on plasma NE spillover. It has been suggested that an increased rate of sympathetic nerve firing, faulty NE reuptake into the sympathetic nerves of the heart and prejunctional augmentation of NE release by EPI contribute to the increased spillover of NE from the kidneys and heart to plasma in essential hypertension (193). Increased sympathetic nerve firing rate to the skeletal

muscle has also been reported in DOCA-salt rats. It is well documented in the spontaneously hypertensive rat that there is an increase in the density of sympathetic innervation. This possibility in DOCA-salt hypertension is still under investigation by my group. The increased arterial and portal venous plasma NE level in DOCA rats is not likely due to the facilitation of sympathoneural NE release by EPI through stimulation of prejunctional α2-adrenoceptors located at the sympathetic nerve terminals. As in my study, although the aortic plasma EPI level was significantly higher than portal venous plasma EPI level in both DOCA-salt and sham rats, neither the aortic nor the portal venous plasma EPI level in DOCA-salt rats is significantly higher than that of sham rats. This is not the same as de Champlain's result showing increased arterial EPI level in DOCA-salt rats (12). It has been suggested by other lab that the enhancement of NE release by EPI is absent in rat mesentery (196).

The increased portal venous plasma NE level in conscious DOCA-salt rats is not likely due to less NE extraction by the mesenteric organs, as the estimated NHS fractional extraction of NE is the same in DOCA-salt and sham rats.

In my later studies, I tested some of the other possibilities contributing to sympathetic nervous system activation in the splanchnic bed of DOCA-salt hypertension.

Sympathetic nerve system release of EPI from the adrenal medulla contributes importantly to the regulation of cardiovascular function as well as energy metabolism. Similar to what has been found in essential hypertension (122) (192, 193, 195, 209), there appears to be normal adrenal medullary secretion of epinephrine in DOCA-salt rats, as there was no significant difference in the aortic plasma epinephrine level between DOCA-salt and sham rats. However, the interpretation of plasma EPI levels as a measure

of secretion from the adrenal medulla is not straightforward given the possibility of DOCA treatment related changes in clearance. Although the fractional extraction of epinephrine I obtained from my study appears to be unchanged in DOCA-salt rats, tracer methodology, i.e. infusion of tritium labeled epinephrine should also be employed to study plasma epinephrine kinetics.

Limitations:

1) Estimated NE spillovers are also dependent on removal processes that intervene between sites of release and measurement in the bloodstream (43). Because of highly efficient neuronal uptake, only a small fraction of NE released by sympathetic nerves throughout the body escapes into the bloodstream. So spillover is not equal to release.

2) It is not possible to know from which sympathetic nerves in the splanchnic bed the released NE is derived, and in what proportions. So my measurements did not directly reflect release of NE from sympathetic nerves to splanchnic veins.

However, it has been shown in dog (7) and guinea pig (8) that release of neurotransmitters in mesenteric veins exceeds release of neurotransmitters in mesenteric arteries, although both vessels demonstrate similar density of sympathetic neuronal terminals (218). In both sham and DOCA-salt rats, mesenteric veins are more sensitive than arteries to the vasoconstrictor effect of sympathetic nerve stimulation, as frequency-response curves caused by nerve stimulation obtained from veins are to the left of those from arteries. Accordingly, small increases in sympathetic nerve activity would increase venous tone before changing arterial tone and increases in sympathetic nerve activity are likely to have a larger effect on veins than arteries for a given change in nerve activity.

Chapter 5 – Part II

Prejunctional α2-Adrenoceptor Inhibition of NE Release in Sympathetic Nerve Endings Associated with Splanchnic Vasculature in Conscious DOCA-Salt Rats

Rationale: Prejunctional α_2-adrenoceptors mediate strong inhibition of NE release from sympathetic terminals, constituting an important autoregulatory system for sympathetic tone. Several mechanisms by which this inhibition of NE release may be mediated have been described. These include activation of inwardly rectifying K^+ channels, inhibition of N-type voltage-gated Ca^{++} channels, and acceleration of Na^+/H^+ exchange (18). Prejunctional regulation of transmitter release by α_2-adrenoceptors is altered in some experimental models of hypertension (21) (220). Either impaired (237) or unaltered (45) α_2-adrenoceptor prejunctional negative feedback system in the isolated mesenteric artery of DOCA-salt animals have been reported. Changes in prejunctional regulation of sympathetic nerves associated with veins in DOCA-salt rats remain to be fully characterized. Luo et al (137) showed that nerve stimulation caused a greater increase in the release of NE over basal level in DOCA veins compared to sham veins. The α2-adrenoceptor agonist UK14304 inhibited stimulation induced NE release in sham, but not DOCA veins. So in vitro studies suggest that prejunctional α2-adrenoceptors are dysfunctional in DOCA veins. However, no studies have been reported that were performed to directly measure NE release from the splanchnic bed in conscious rats. In my study, I evaluated the functioning of the prejunctional α_2-adrenoreceptor negative feedback mechanism controlling NE release by measuring the changes in NE overflow following α_2-adrenoreceptor agonist or antagonist treatment.

Experimental Procedures: Experiments were performed in 4-5 week DOCA-salt and same age sham rats. After sodium pentobarbital anesthesia (50 mg/kg, i.p.), silicone rubber tipped catheters were placed into abdominal aorta via a femoral artery and portal vein via a small branch of the portal vein after laparotomy. The third catheter was inserted into a femoral vein. I set up the rats as introduced in the General Methods. The rats were treated with either α_2-adrenoceptor antagonist yohimbine (0.5 mg/kg, i.v.) or agonist clonidine (10 μg/kg, i.v.) after at least fmy days of recovery from anesthesia and surgical stress. 30 min after drug administration, 1 ml blood samples were collected simultaneously from arterial and portal venous catheters. Plasma NE and EPI were measured using a radioenzymatic assay. NHS spillover of NE was estimated as introduced in General Methods.

Results:

1) Effects of α2-adrenoceptor antagonist yohimbine

Acute treatment with yohimbine didn't cause any significant change in the MAP in sham rats. Yohimbine caused a small, but significant decrease in the MAP of DOCA-salt rats (Fig. 21). Yohimbine significantly increased the heart rate in both sham (from 369 ± 14 to 405 ± 16 beats/ min) and DOCA-salt rats (from 363 ± 12 to 406 ± 14 beats/min).

Yohimbine significantly increased aortic (Fig. 22) and portal venous plasma NE concentrations in both sham and DOCA-salt rats (from 209 ± 23 to 467 ± 76 pg/ml in sham aortic plasma; from 226 ± 33 to 446 ± 66 pg/ml in sham portal venous plasma; from 339 ± 63 to 589 ± 113 pg/ml in DOCA aortic plasma; from 315 ± 50 to 568 ± 92 pg/ml in DOCA portal venous plasma). There was no significant difference in the

magnitude of increase between DOCA-salt and sham rats (144 ± 44% and 99 ± 47% in sham and DOCA-salt aortic plasma; 79 ± 26% and 105 ± 36% in sham and DOCA-salt portal venous plasma). Yohimbine also significantly increased the aortic and portal venous plasma EPI concentrations in sham rats (from 73 ± 22 to 236 ± 88 pg/ml in aortic plasma; from 37 ± 11 to 104 ± 34 pg/ml in portal venous plasma). However, yohimbine didn't cause any significant change in either aortic or portal venous plasma EPI level in DOCA-salt rats (from 104 ± 32 to 105 ± 27 pg/ml in aortic plasma; from 50 ± 12 to 65 ± 15 pg/ml in portal venous plasma).

Yohimbine didn't cause any significant change in the estimated NHS fractional extraction of NE in either group (from 25 ± 11% to 42 ± 11% in sham rats; from 42 ± 4% to 31 ± 6% in DOCA-salt rats). Yohimbine significantly increased the NHS NE spillover in sham rats, but not in DOCA-salt rats (Fig. 23).

2) Effects of α2-adrenoceptor agonist clonidine

Acute treatment of clonidine didn't cause any significant change in the MAP and heart rate of sham rats. Clonidine didn't have a significant effect on the heart rate of DOCA-salt rats (from 372 ± 18 beats/min to 361 ± 20 beats/min) either. However, clonidine significantly decreased the MAP of DOCA-salt rats (Fig. 24).

Clonidine didn't significantly change the aortic plasma NE concentrations in either sham or DOCA-salt rats (Fig. 25). Clonidine significantly decreased the portal venous plasma NE concentration only in sham (from 233 ± 26 pg/ml to 140.5 ± 35 pg/ml), but not DOCA-salt rats (from 336 ± 52 pg/ml to 354 ± 119 pg/ml). Clonidine didn't cause any significant change in plasma EPI levels in sham rats (from 62.2 ± 11 to 130 ± 35 pg/ml in aortic plasma; from 39 ± 8 to 72 ± 21 pg/ml in portal venous plasma). Clonidine

significantly increased the portal venous plasma EPI level in DOCA-salt rats (from 87.6 ± 25 to 125.1 ± 35 pg/ml in aortic plasma; from 46 ± 10 to 73 ± 16 pg/ml in portal venous plasma).

Clonidine didn't cause any significant change in the NHS FX of NE in either group (from 18 ± 20% to 39 ± 10% in sham rats; from 38 ± 7% to 30 ± 7% in DOCA-salt rats). Clonidine significantly decreased the NHS NE spillover in sham, but not DOCA-salt rats (Fig. 26).

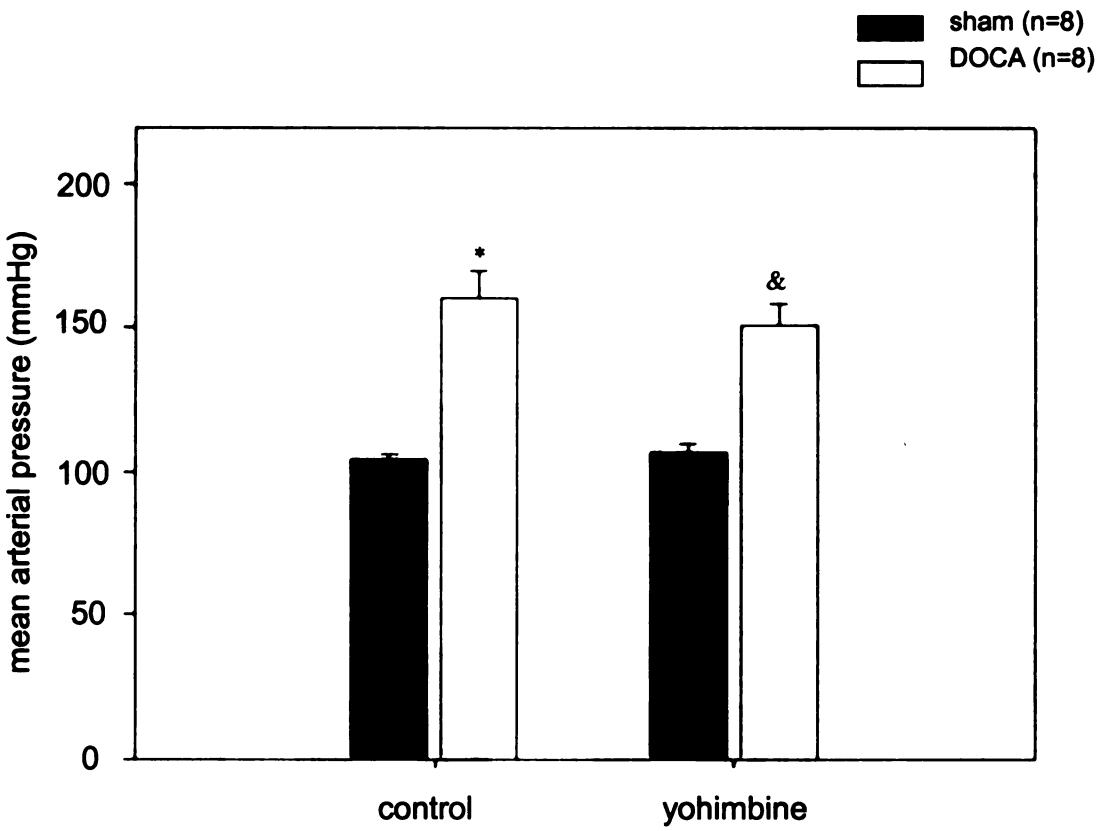


Figure 21: Mean arterial pressure responses to i.v. bolus yohimbine administration in conscious DOCA-salt and sham rats. Bars indicate mean arterial pressure before or after yohimbine treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between sham and DOCA-salt rats. & indicates a significant difference ($P<0.05$) between before and after drug treatment.

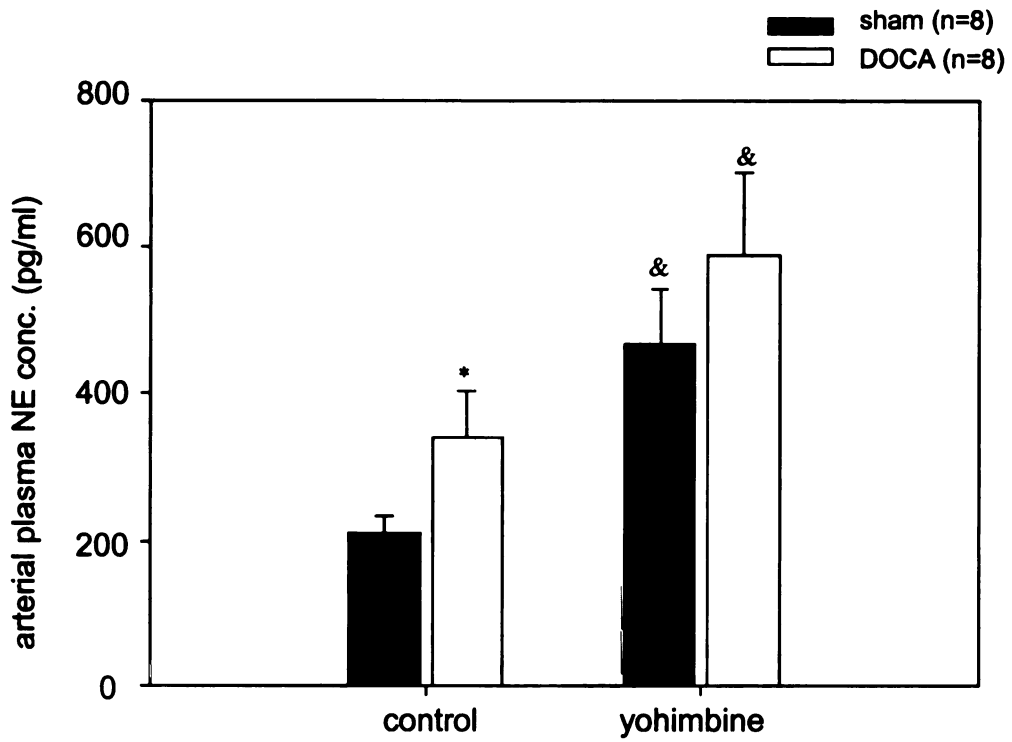


Figure 22: Effects of i.v. bolus yohimbine administration on aortic plasma NE level in conscious DOCA-salt and sham rats. Bars indicate mean arterial plasma NE concentration before or after yohimbine treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between sham and DOCA-salt rats. &s indicate a significant difference ($P<0.05$) between before and after drug treatment.

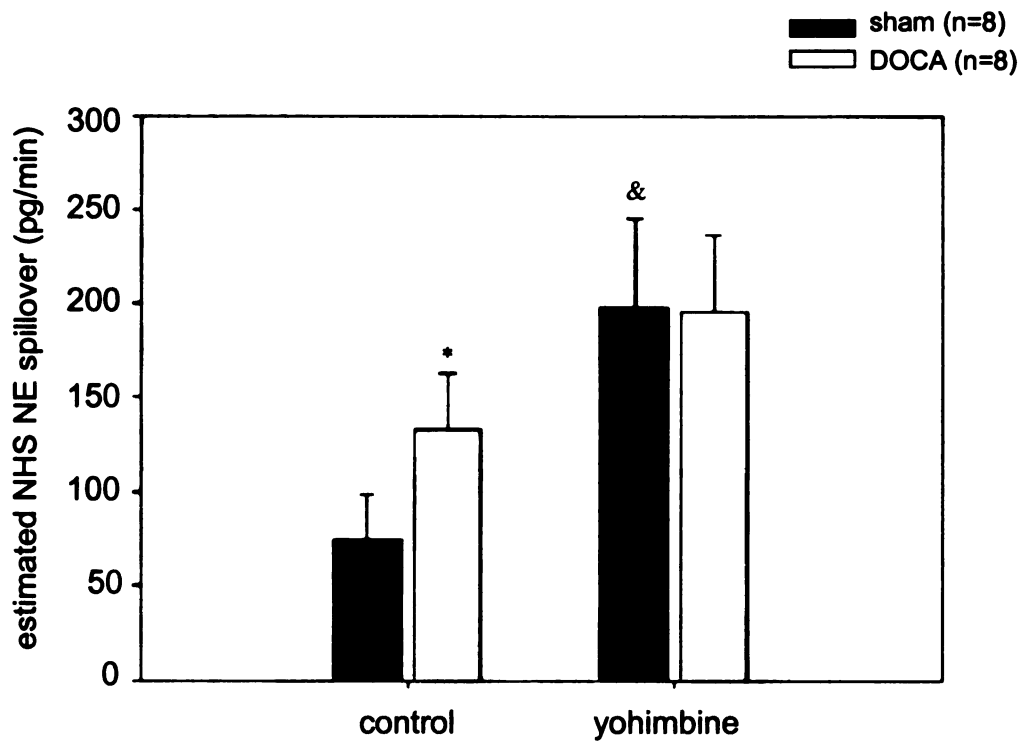


Figure 23: Effects of i.v. bolus yohimbine administration on estimated NHS spillover rate of NE in conscious DOCA-salt and sham rats. Bars indicate mean estimated NHS NE spillover rate before or after yohimbine treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between sham and DOCA-salt rats. & indicates a significant difference ($P<0.05$) between before and after drug treatment.

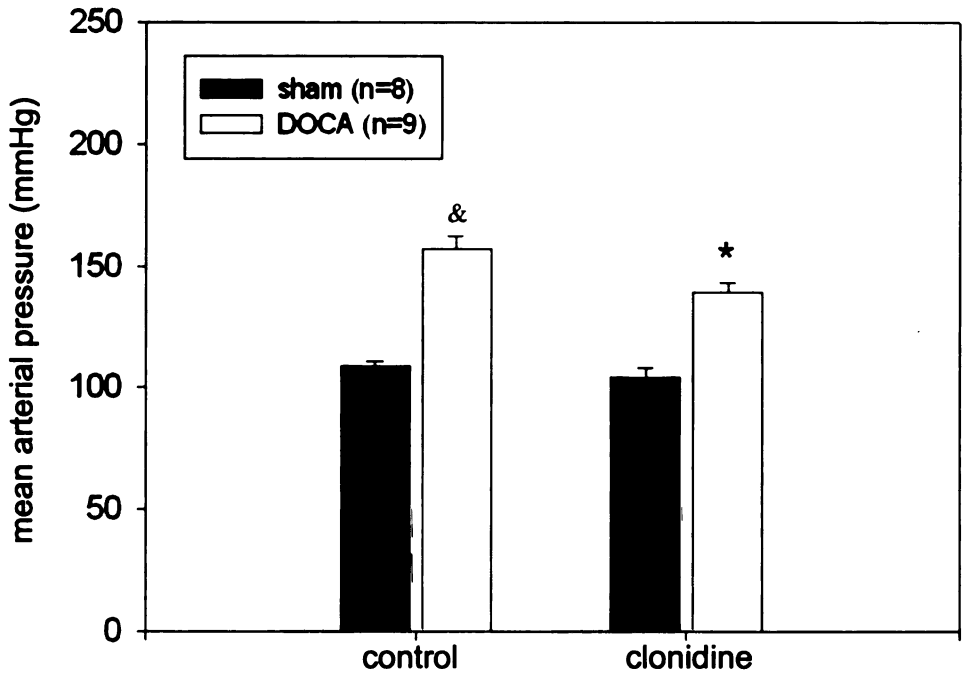


Figure 24: Mean arterial pressure responses to i.v. bolus clonidine administration in conscious DOCA-salt and sham rats. Bars indicate mean arterial pressure before or after clonidine treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between before and after drug treatment. & indicates a significant difference ($P<0.05$) between sham and DOCA-salt rats.

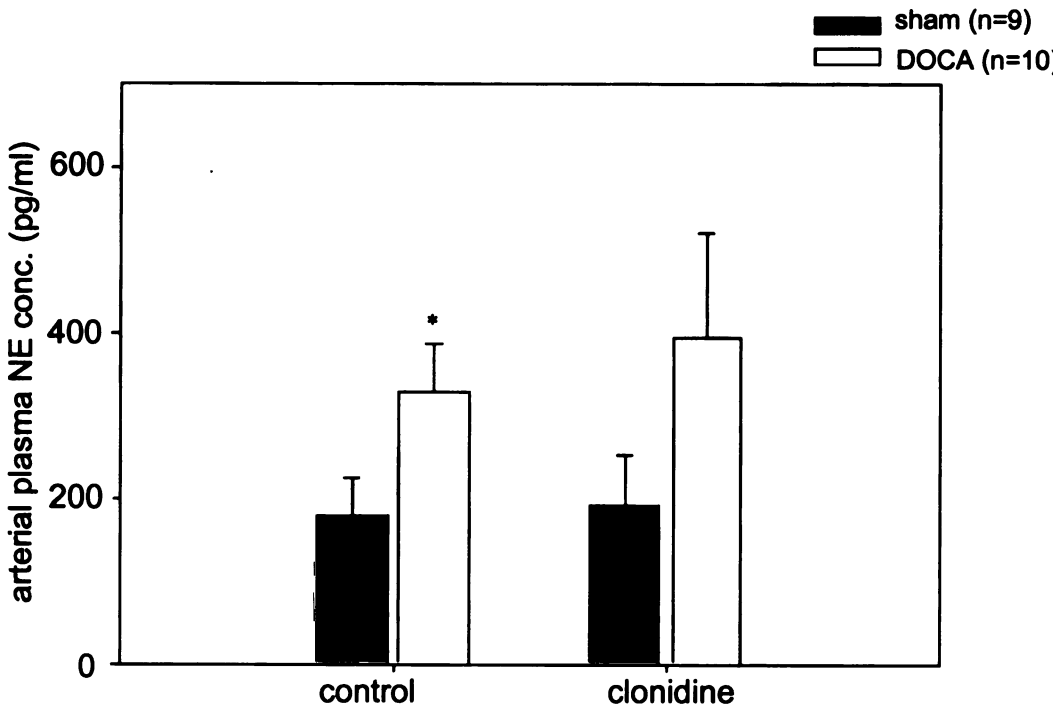


Figure 25: Effects of i.v. bolus clonidine administration on aortic plasma NE level in conscious DOCA-salt and sham rats. Bars indicate mean arterial plasma NE concentration before or after clonidine treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between sham and DOCA-salt rats.

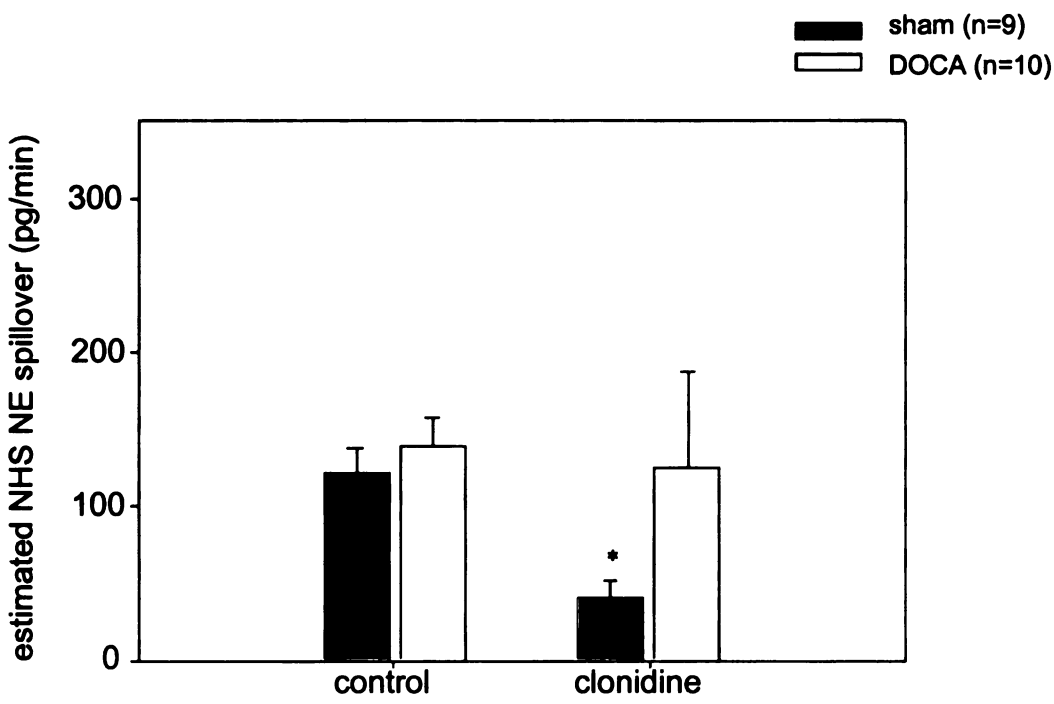


Figure 26: Effects of i.v. bolus clonidine administration on estimated NHS spillover rate of NE in conscious DOCA-salt and sham rats. Bars indicate mean estimated NHS NE spillover rate before or after clonidine treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between before and after drug treatment.

Discussion for Chapter 5-Part II

Prejunctional α2-adrenoceptors are activated by NE that is released upon neural membrane depolarization thus limiting further release of neurotransmitters (126). Data from several studies indicate that prejunctional α2-adrenoceptor function is impaired in hypertensive animals. In my study, I tested the hypothesis that there was impaired α2-adrenoceptor function in the systemic and splanchnic circulation in conscious DOCA-salt rats in vivo by comparing the effects of α2-adrenoceptor antagonist and agonist on plasma NE level and the estimated NHS NE release between DOCA-salt and sham rats.

I found that in sham rats, the administration of yohimbine produced no significant change in MAP despite the important increase in plasma NE level. The lack of change in blood pressure could be due to the blockade of postjunctional α2-adrenergic receptors that mediate vasoconstriction (243). In DOCA-salt rats, however, the decrease in blood pressure induced by yohimbine could result from amplified postjunctional α2-adrenoceptor blockade even there was a simultaneous increase in NE release.

Yohimbine significantly increased the systemic plasma NE concentration in both DOCA-salt and sham rats, suggesting that the systemic prejunctional α2-adrenoceptor regulation of NE release is normal in DOCA-salt rats. However, yohimbine didn't cause any significant change in the plasma EPI level in DOCA-salt rats, suggesting impaired prejunctional α2-adrenoceptor regulation of EPI release from adrenal gland. My finding is not consistent with previous studies by De Champlain J et al showing that intravenously administered yohimbine increased plasma NE levels in conscious normotensive but not DOCA-salt hypertensive rats (30, 157). However, they also found decreased function of the prejunctional α2-adrenergic autoinhibitory mechanism at the

adrenal medulla in DOCA-salt rats, reflected by lack of significant effect of yohimbine on plasma EPI level (157).

In my study, I also found that yohimbine only significantly increased the estimated NHS NE spillover in sham, but not DOCA-salt rats, suggesting that there is impaired prejunctional α2-adrenoceptor function in the splanchnic bed of DOCA-salt rats. This is consistent with previous in vitro studies by Luo et al (137) showing that yohimbine, increased NE release in sham, but not DOCA mesenteric arteries and veins. An alternative explanation could be that under basal conditions the prejunctional α2-adrenoceptors do not thave a dominanting influence on the transmitter release in the splanchnic vasculature of DOCA-salt rats.

I also treated my rats with clonidine as an alternative way to look at α2-adrenoceptor function. I found that clonidine only significantly decreased the portal venous plasma NE level in sham rats. It didn't cause a significant change in either arterial or portal venous plasma NE level in DOCA-salt rats. Clonidine significantly decreased the estimated NHS spillover of NE in sham rats, but failed to cause any significant change in that of DOCA-salt rats. So data from the clonidine experiment also suggest that there is impaired α2-adrenoceptor function in the splanchnic bed of DOCA-salt rats. In another hypertension model, conscious male stroke-prone spontaneously hypertensive rats (SPSHR), it was reported that i.v. administration of clonidine (5 μg) decreased splanchnic nerve activity significantly (136). The different effect of clonidine on the splanchnic bed of DOCA-salt rats and SPSHR may be related to the different hypertension mechanisms underlying these two hypertension models.

In conclusion, both yohimbine and clonidine studies suggest that in DOCA-salt rats, there is impaired activity of the prejunctional α2-adrenergic negative feedback mechanism regulating the release of NE from the sympathetic nerve fibers associated with the splanchnic vasculature under basal conditions. This regulatory mechanism also seemed impaired at the level of the adrenal medulla under basal conditions. These alterations could contribute at least in part to elevate the plasma levels of NE and net NHS NE spillover in DOCA-salt rats. One possible mechanism underlying these alterations could be α2-adrenoceptor desensitization, perhaps due to high basal sympathetic activity in DOCA-salt rats (9). Impairment of α2 receptor mediated prejunctional negative feedback mechanism because of reduced expression of the α2 receptor subtype A has been reported in spontaneously hypertensive rats (272).

α2-adrenoceptor mediated neuromodulation plays a greater role in veins than in arteries(7). Quantitative differences in α2-adrenoceptor mediated neuromodulation in the splanchnic blood vessels may participate to differing contributions of arteries and veins to the control of blood flow and volume distribution in splanchnic circulation. Besides the impaired α2 inhibitory mechanism, enhanced sensitivity of facilitatory prejunctional ß2 mechanisms have also been reported in DOCA-salt rats (11). There is an impairment of the functional reciprocal balance between the a and ß prejunctional receptors (11). Since DOCA-salt hypertension seems to depend on the sympathetic nervous system, these abnormalities may act together in facilitating the release of sympathetic neurotransmitters, therefore sustaining an increased sympathetic tone and reactivity which, in turn, may help develop and maintain DOCA-salt hypertension.

It is important to keep in mind, though, that with chronically increased NE release into the neuroeffector junction that exceeds the capacity of the neuron to return NE to prejunctional vesicles, there can be a net neuronal loss of NE. This loss is considered a feature of severe progressive heart failure (40, 150). Tissue NE depletion has also been reported in DOCA-salt hypertension (138) (94).

Limitation: It can be argued that yohimbine and clonidine are acting on the central nervous system to produce the change in plasma NE level observed in normotensive rats, and that the lack of effect of yohimbine and clonidine on splanchnic NE release in DOCA-slat rats may reflect an alteration in central regulatory mechanisms. My study does not permit one to distinguish between the central and peripheral effects of yohimbine and clonidine. However, two studies carried out in cats (179) and in rabbits (228) demonstrated that yohimbine, at a similar dose to the one used in my study, acts mainly in the periphery to produce its increase in plasma NE levels. No similar data are available for clonidine.

Chapter 5 – Part III

Neuronal NE uptake activity in the splanchnic vasculature of anesthetized and conscious DOCA-salt rats.

Rationale: Recent identification of a syndrome of orthostatic intolerance (OI) (an autonomic disorder characterized by excessive tachycardia, minimal blood pressure changes, and increased NE after assuming a standing position) resulting from a defect in norepinephrine uptake has renewed clinical interest in enzymes and transporters involved in the synthesis and metabolism of NE. A dominant negative mutation in human norepinephrine transporter (NET) reduces its surface expression via heterologous oligomerization (85), thus rendering a major fraction of the normal transporter inert and reducing NE reuptake in affected heterozygotes (211).

NET is critical in central modulation of sympathetic tone and autonomic regulation of peripheral hemodynamics by reducing NE concentrations in the neuroeffector junction (10) and preventing the resultant deleterious effects on cardiovascular tissues. The amount and type of neurotransmitter removed at any given site varies (37). NET removes not only residual NE but, a major portion of surplus dopamine as well (20). Peripherally, NET is perhaps most important in the heart (59, 78). In humans, approximately 92% of cardiac NE removal occurs via NET, with the remainder eliminated by exraneuronal carriers and circulatory dissipation (40). So the rate of spillover is also dependent on the adequacy of neuronal uptake, not solely on the rate of NE release. At a given rate of sympathetic nerve firing, the capacity for neuronal reuptake of NE influences rates of NE overflow. This has been shown experimentally, with electrical stimulation of the sympathetic nerve supply, in a range of individual

organs (108) (124, 261, 262) and for the body as a whole in the pithed rabbit (227). The magnitude of the effect of pharmacological neuronal NE uptake (U1) block on NE overflow differs between different organs and tissues, depending on the importance of U1 in NE disposition in a particular site (6, 168). Typical neurochemical consequences of neuronal NE reuptake impairment are facilitation of NE spillover from sympathetic nerves to plasma, reduced clearance of NE from plasma by the body as a whole and by individual organs, and alteration in the pattern of NE metabolites due to reduction in neuronal metabolism and augmentation of extraneuronal metabolism (41, 53, 59, 79).

Neuronal uptake of NE was found to be normal in cardiac and vascular tissue in young spontaneously hypertensive rats (SHR) (151) and increased in resistance arteries of adult SHR (41). Impaired neuronal NE reuptake in lean patients with essential hypertension perhaps is due to dysfunction of the NE transporter (193). In vitro studies suggest reduced neuronal reuptake of NE in the heart of DOCA-salt rats partly due to reduced NET 80 kD subunit protein level (255). Luo M et al (138) found that there was an upregulation of NE transporter activity in the splanchnic vasculature of DOCA-salt rats as neuronal and extraneuronal NE uptake blockers, cocaine and corticosterone, prolonged neurogenic responses in veins from DOCA-salt but not sham rats. There was a significant increase in NET-immunoreactivity in arteries, veins and sympathetic ganglia when compared to levels in the tissues from sham rats. No in vivo studies have been done to study the functioning of NET and the relative importance of neuronal uptake in the disposition of released NE in the splanchnic bed of DOCA-salt rats. It is likely that norepinephrine release from perivascular sympathetic nerves in DOCA-salt hypertension is increased (as I found in my study), which stimulates an upregulation of NET levels in

blood vessels. The latter may serve to reduce NE levels in the neuroeffector junction, thereby providing a mechanism to partially offset the potential deleterious effects of excess and unregulated NE in DOCA-salt rats. In addition, this adaptive response may prevent the global neuronal depletion of NE.

To test if there is altered NET activity that contributes to plasma NE level in DOCA-salt rats, I estimated the NHS spillover of NE and NHS FX of NE in the presence of the neuronal uptake inhibitor, desipramine, in anesthetized and conscious DOCA-salt and sham rats.

Experimental procedures: Initial experiments were performed in 4-5 week DOCA-salt and sham rats under urethane anesthesia (1.2 g/kg, i.p.). Silicone rubber tipped catheters were placed into abdominal aorta via a femoral artery and portal vein via a small branch of the portal vein after laparotomy. The third catheter was inserted into a femoral vein. 30 min after recovery, the rats were treated with desipramine (2mg/kg, i.v.). 30 min later, 1 ml blood samples were collected simultaneously from arterial and portal venous catheters. Plasma NE and epinephrine were measured using a radioenzymatic assay. NHS spillover of NE was estimated as introduced in General Methods.

Additional studies were performed using couscious rats which were treated with desipramine (2mg/kg, i.v.) after at least fmy days of recovery from anesthesia and surgical stress.

Results: In anesthetized rats: Desipramine didn't cause any significant change in the mean arterial pressure in either DOCA-salt (Fig. 27) (from 93.7 ± 9.1 mmHg to 91.8 ±

6.1 mmHg) or sham rats (from 95.4 ± 1.7 mmHg to 87.3 ± 6.1 mmHg). Acute blockade of neuronal NE uptake significantly increased the aortic and portal venous plasma NE level in both sham and DOCA-salt rats (Fig. 28) (from 567.2 ± 89.9 to 824.3 ± 75.8 pg/ml in sham aortic plasma; from 504.6 ± 71.7 to 878.9 ± 82.7 pg/ml in sham portal venous plasma; from 370.0 ± 26.7 to 891.2 ± 93.0 pg/ml in DOCA aortic plasma; from 458.9 ± 50.0 to 710.7 ± 59.8 pg/ml in DOCA portal venous plasma). The magnitude of increase was significantly higher in the aortic plasma of DOCA-salt rats than in that of sham rats. Desipramine caused a significant decrease in the estimated NHS FX of NE only in sham rats (Fig. 29). Desipramine didn't cause a significant change in the estimated NHS spillover in either group (Fig. 30).

In conscious rats: Desipramine significantly decreased the MAP in DOCA-salt rats (Fig. 31) (from 164.3 ± 5.2 mmHg to 154.2 ± 6.1 mmHg), but not in sham rats (from 102.5 ± 2.2 mmHg to 108.8 ± 3.2 mmHg). Desipramine caused a significant increase in the aortic plasma NE level in sham rats, but not DOCA-salt rats (Fig. 32). Desipramine didn't cause any significant change in the estimated NHS FX of NE (Fig. 33) and the estimated NHS NE spillover (Fig. 34) in either DOCA-salt or sham rats.

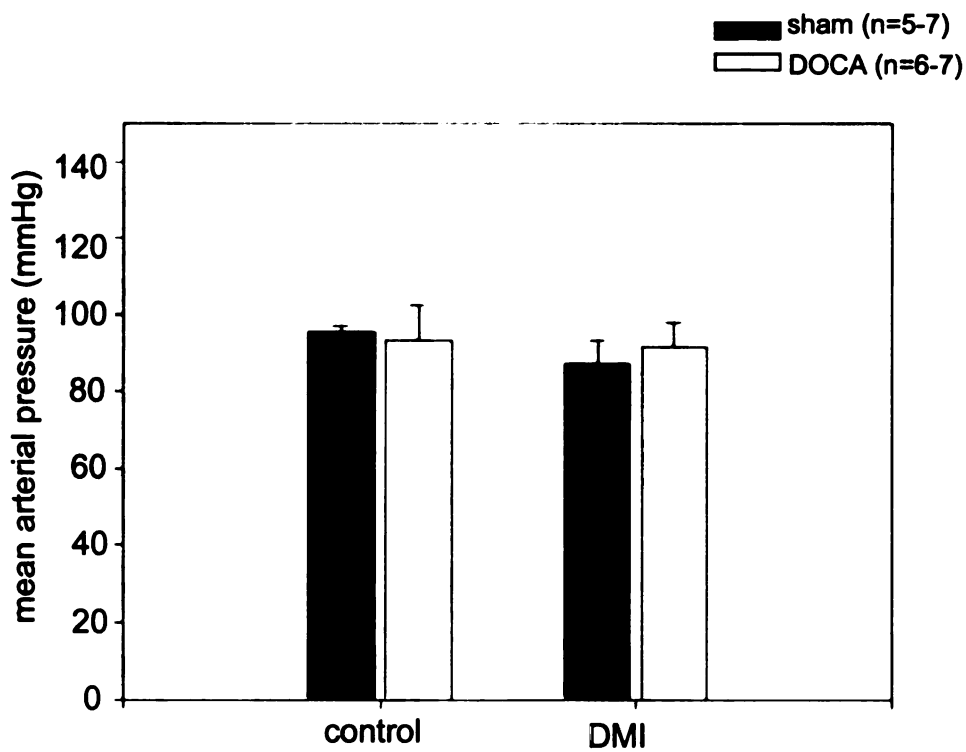


Figure 27: Mean arterial pressure responses to i.v. bolus desipramine administration in anesthetized DOCA-salt and sham rats. Bars indicate mean arterial pressure before or after DMI treatment in anesthetized sham and DOCA-salt rats. Brackets indicate SEM.

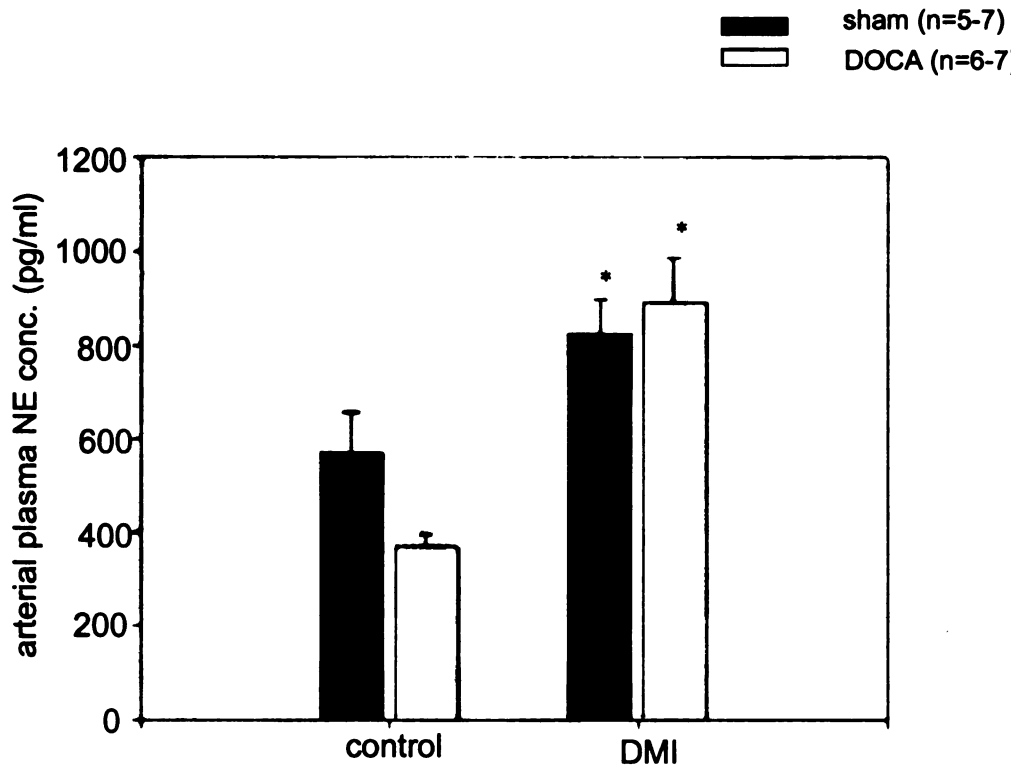


Figure 28: Effects of i.v. bolus desipramine administration on aortic plasma NE level in anesthetized DOCA-salt and sham rats. Bars indicate mean arterial plasma NE concentration before or after DMI treatment in anesthetized sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) before and after drug treatment.

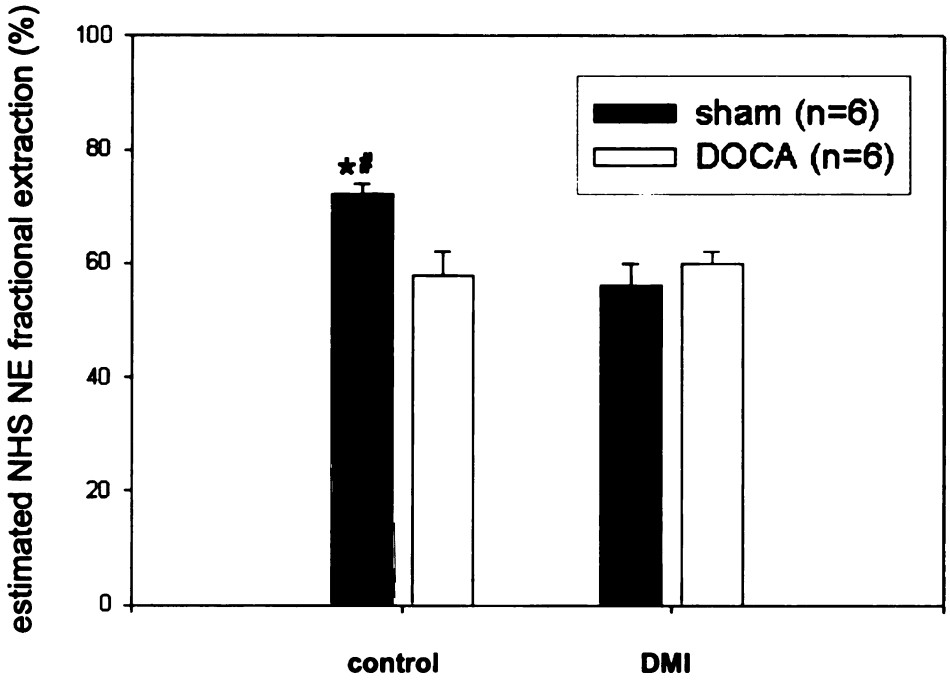


Figure 29: Effects of i.v. bolus desipramine administration on estimated NHS fractional extraction of NE in anesthetized DOCA-salt and sham rats. Bars indicate mean estimated NHS NE fractional extraction before or after DMI treatment in anesthetized sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between sham and DOCA-salt rats. # indicates a significant difference ($P<0.05$) between before and after drug treatment.

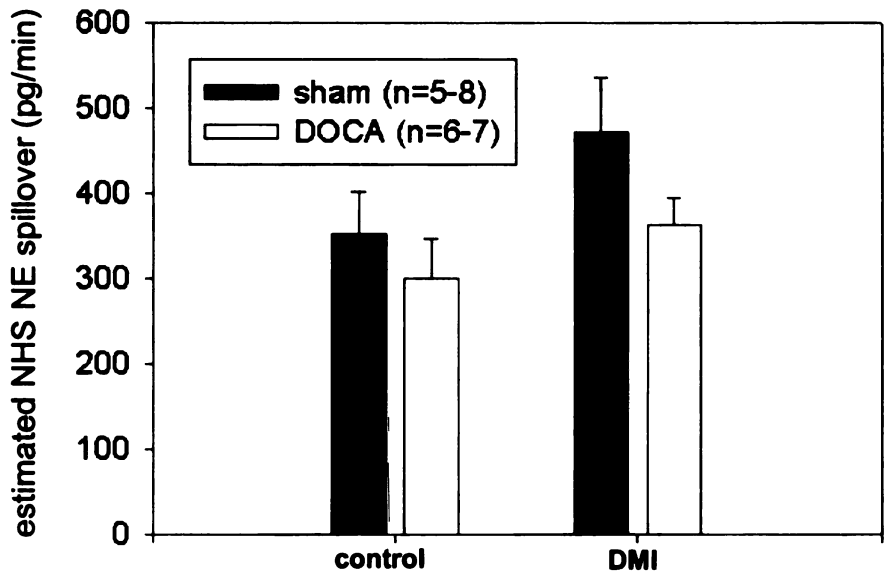


Figure 30: Effects of i.v. bolus desipramine administration on estimated NHS spillover rate of NE in anesthetized DOCA-salt and sham rats. Bars indicate mean estimated NHS NE spillover rate before or after DMI treatment in anesthetized sham and DOCA-salt rats. Brackets indicate SEM.

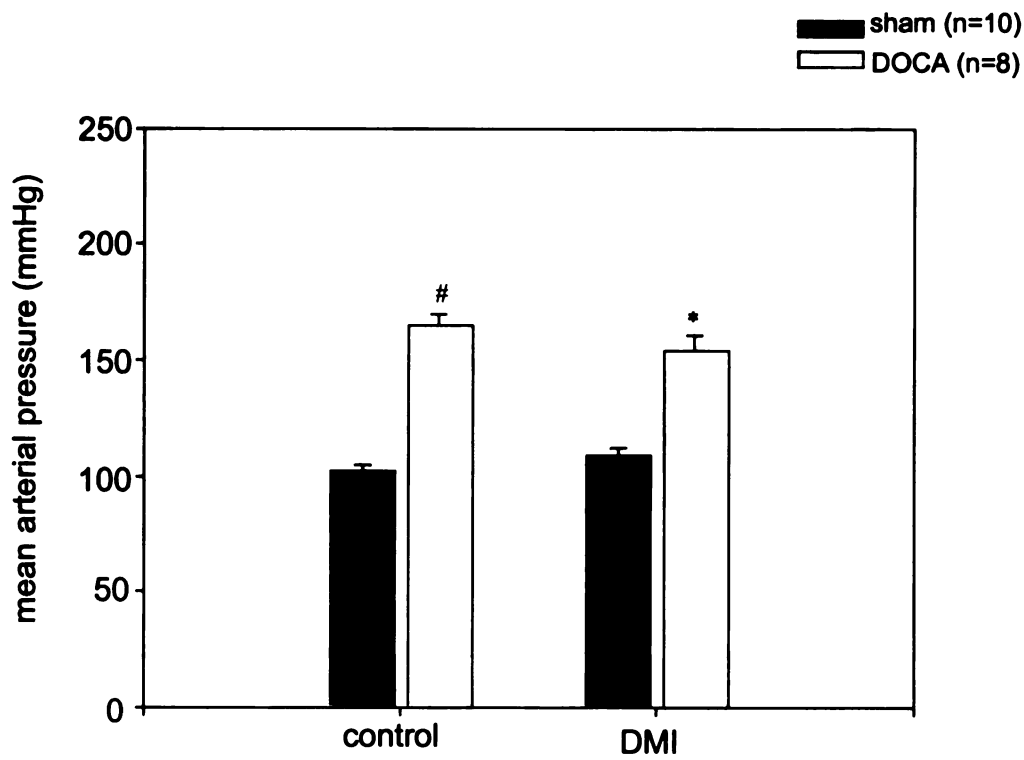


Figure 31: Mean arterial pressure responses to i.v. bolus desipramine administration in conscious DOCA-salt and sham rats.Bars indicate mean arterial pressure before or after DMI treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. # indicates a significant difference ($P<0.05$) between DOCA-salt and sham rats. Asterisk indicates a significant difference ($P<0.05$) between before and after drug treatment.

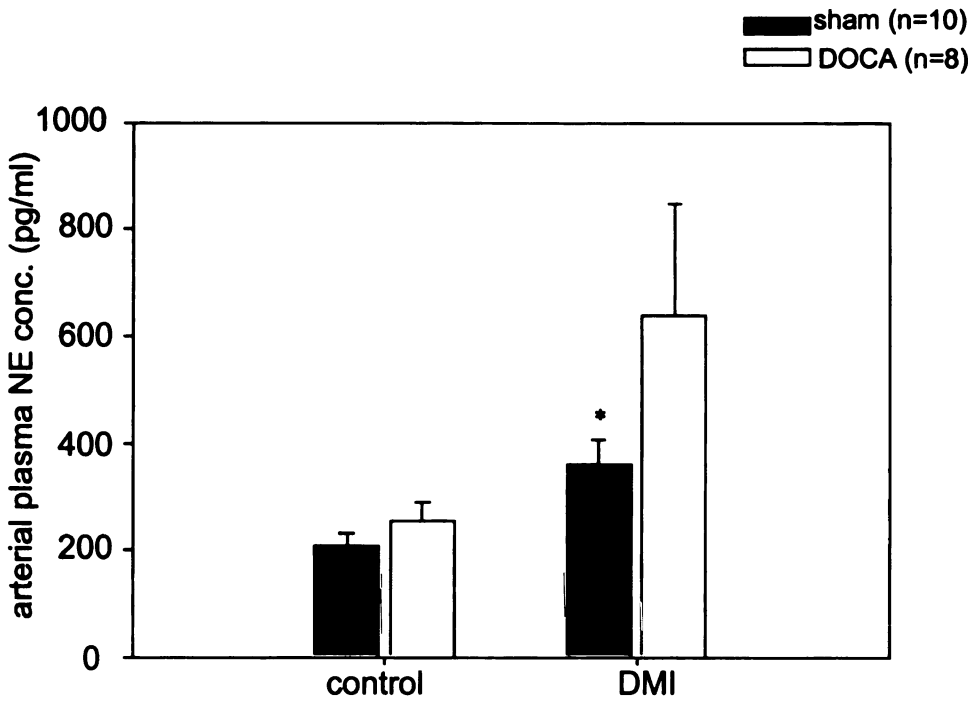


Figure 32: Effects of i.v. bolus desipramine administration on aortic plasma NE level in conscious DOCA-salt and sham rats. Bars indicate mean arterial plasma NE concentration before or after DMI treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) before and after drug treatment.

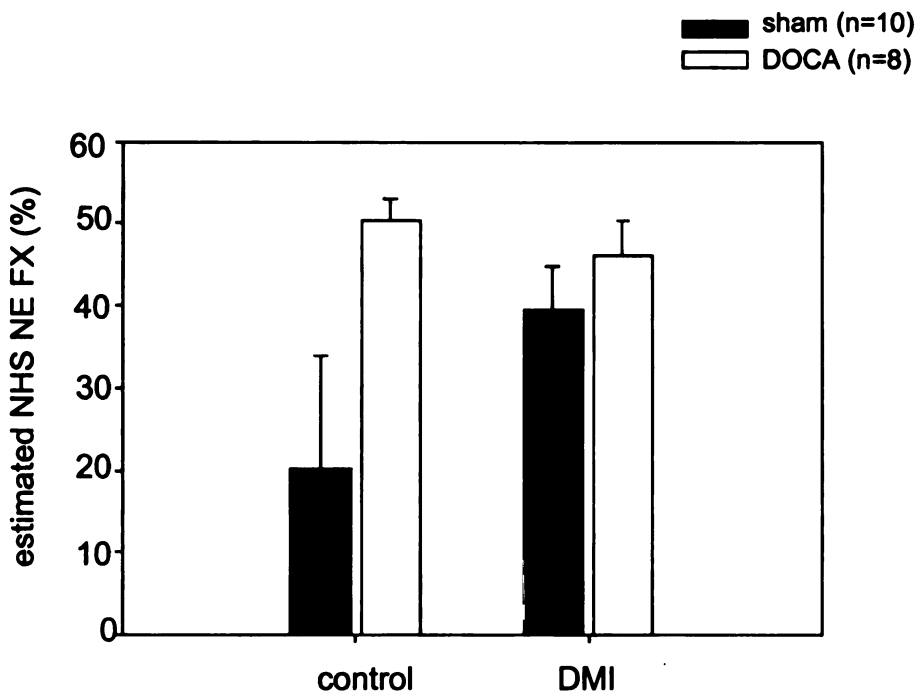


Figure 33: Effects of i.v. bolus desipramine administration on NHS fractional extraction of NE in conscious DOCA-salt and sham rats. Bars indicate mean estimated NHS NE fractional extraction before or after DMI treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM.

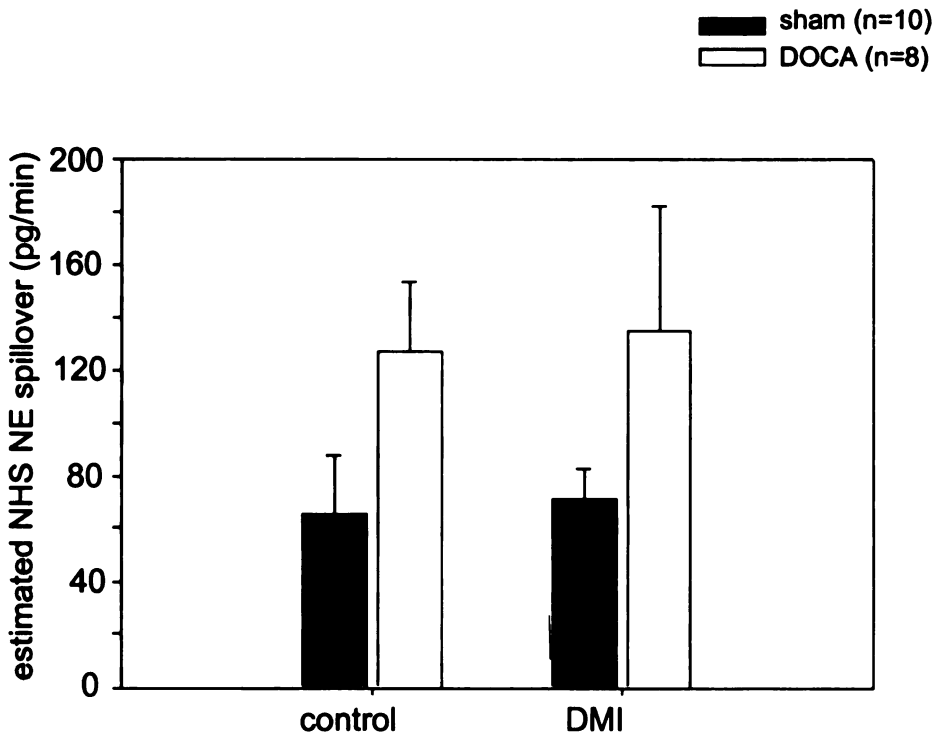


Figure 34: Effects of i.v. bolus desipramine administration on estimated NHS spillover rate of NE in conscious DOCA-salt and sham rats. Bars indicate mean estimated NHS NE spillover rate before or after DMI treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM.

Discussion for Chapter 5-Part III

The lack of effects of desipramine on the NHS FX and spillover of NE in conscious rats is not likely due to inadequate dose, as same dose significantly increased the plasma NE levels in both anesthetized DOCA-salt and sham rats. In the future, plasma DHPG, the intraneuronal metabolite of NE, can be measured to confirm that I gave my rats a sufficient dose of desipramine (decreased plasma DHPG is expected).

My results may suggest that neuronal uptake accounts for a very small proportion of NE/EPI removal from the splanchnic circulation. However, the effects of pharmacological neuronal uptake block on the rate of NE spillover, with systemic administration of the blocking drug desipramine, is complicated by the fact that sympathetic nerve firing rates fall (55), presumably due to an action of the drug within the central nervous system (53, 225). Decreased NE clearance after DMI administration leads to increased levels of NE in the brain stem and to increased stimulation of inhibitory α2a receptors, resulting in decreased sympathetic outflow. DMI-induced sympathoinhibitory effects of increased NE in the brain stem can predominate over the functional elevation of NE stimulation of peripheral targets (183). Attenuated baroflex function has also been observed in DMI-treated rats (183). The attenuation in sympathetic outflow and to suppression of the baroreflex obscure or prevent the expression of the peripheral manifestations (such as the NHS NE spillover rate I was looking for in my study) of impaired NE uptake. The change in NE spillover rate could be a consequence of this fall in nerve firing rate plus uptake blockade. Actually, it has been reported that intravenous desipramine dose-dependently inhibited splanchnic sympathetic nerve discharge (-64 +/- 3% after 4 mg/kg iv DMI) in urethane-anesthetized

rats (98). It has been suggested that after i.v. desipramine, blockade of neurotransmitter reuptake increased NE concentrations at sympathoeffector junctions so as to offset the fall in SNA, so that there was little change in NE spillover (44). Changes in systemic NE concentrations will provide some insight into the magnitude of this effect in my experiments. Unfortunately, currently there are no drugs available that only specifically block neuronal uptake of NE and have no sympathoinhibitory effect.

A paper published in J Nucl Cardiol in 2003 showed an alternative way to evaluate the activity of NET (75). In this study, the authors were interested in the NET activity in the heart of heart failure patients. They injected I-123 metaiodobenzylguanidine (MIBG), which is a guanethidine congener, into human subjects through an indwelling catheter and the conjugate count images of the thorax were acquired using a gamma camera. The images were entered into a dedicated computer for quantitation of I-123 MIBG. Activity of NET can be assessed from uptake of I-123 MIBG because radiolabeled preparations of MIBG have been shown to mimic uptake of tritiated NE into the storage vesicles by NET and provides a marker for sympathetic nerve activity and activity of the transporter. A similar experimental design could be used in my study. However, it is not clear if it would be more difficult to acquire satisfactory images from gut in animals compared with from heart in human subjects.

Limitation: Alterations in the function of the NET could be a result of one or several changes. For example, there could be a change in the amount of transporter protein inserted in the membrane or there could be a change in the transport capacity of the transporter or there could be a change in the way the protein is regulated by intracellular

signaling mechanism. The spillover measurement does not discriminate among the various alternative cellular and molecular changes that might occur in the transporter.

Chapter 5 – Part IV

Interaction between Prejunctional α2-adrenoceptor and Neuronal Transmitter Reuptake in DOCA-Salt Hypertensive Rats

Rationale: It has been reported previously that in the mesenteric vessels where the effector response is normally influenced by the combined activity of α_2-adrenoceptors and uptake, failure of one mechanism increases the activity of the other, so as to maintain a largely constant effector response (163). An interaction between reuptake and α_2-receptors is present in the vas deferens of the mouse and rat, guinea-pig atrium, the spleen and various blood vessels from the dog (135). Patients with genetic impairment of the prejunctional α_{2c} adrenergic receptor have augmented activity of the norepinephrine uptake-1 transporter (75). The mechanism is not delineated but may be related to increased NE substrate in the symaptic cleft. No similar study has ever been done in the splanchnic vasculature of DOCA-salt rats.

Experimental Procedures: Experiments were performed in 4-5 week DOCA-salt and same age sham rats. After sodium pentobarbital anesthesia (50 mg/kg, i.p.) anesthesia (1.2 g/kg i.p.), silicone rubber tipped catheters were placed into abdominal aorta via a femoral artery and portal vein via a small branch of the portal vein after laparotomy. The third catheter was inserted into a femoral vein. I set up the rats as introduced in the General Methods. After at least fmy days of recovery from anesthesia and surgical stress, the rats were treated with yohimbine (0.5mg/kg, i.v.), 10min later the rats were given desipramine (2mg/kg, i.v.). 30 min after drug administration, 1 ml blood samples were

collected simultaneously from arterial and portal venous catheters. Plasma NE and EPI were measured using a radioenzymatic assay. NHS spillover of NE was estimated as introduced in General Methods.

Results: Yohimbine+DMI treatment didn't cause any significant change in the MAP of either sham (Fig. 35) (from 101.2 ± 3.0 to 94.2 ± 2.8 mmHg) or DOCA-salt rats (from 164.1 ± 10.2 to 167.1 ± 4.6 mmHg). Yohimbine + DMI treatment caused a significant increase in the arterial plasma NE concentration in sham rats (Fig. 36). The increase was greater than that caused by DMI and yohimbine treatment alone, but not significantly so. Yohimbine+DMI treatment didn't cause any significant change in the arterial plasma NE concentration in DOCA-salt rats (Fig. 36). Yohimbine+DMI significantly increased the estimated NHS NE spillover in sham rats and the magnitude of increase was not significantly different than that caused by yohimbine and DMI alone. Yohimbine+DMI didn't cause any significant change in the NHS NE spillover in DOCA-salt rats (Fig. 37).

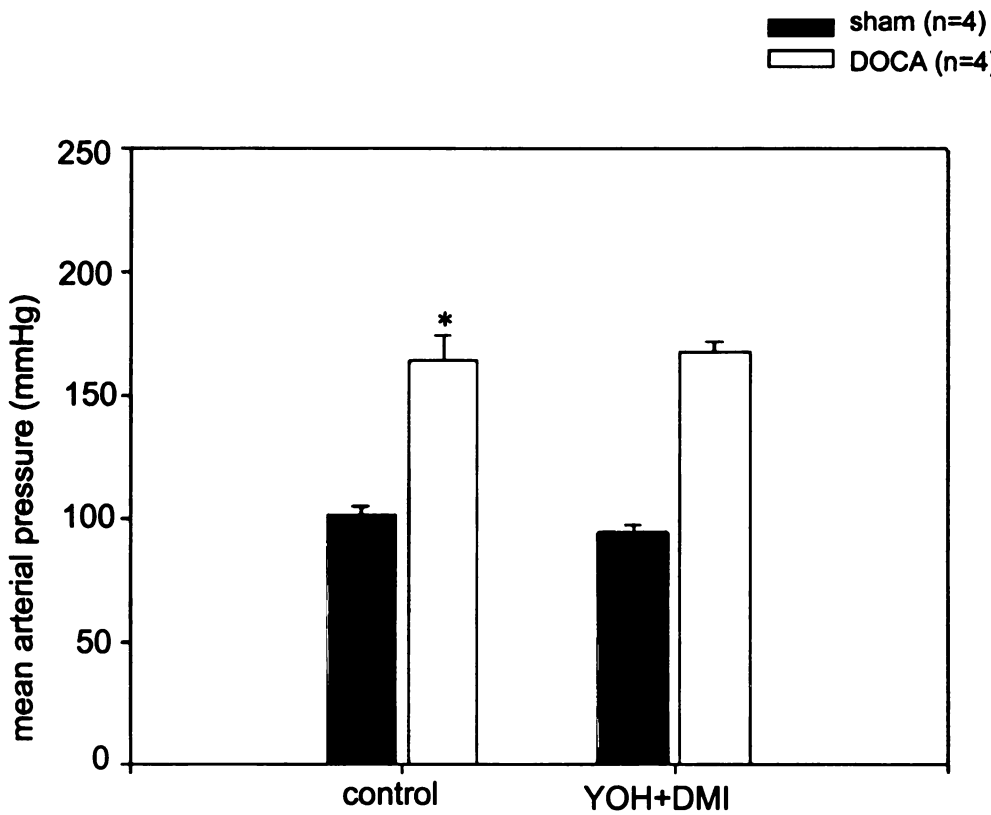


Figure 35: Mean arterial pressure responses to i.v. bolus yohimbine+desipramine administration in conscious DOCA-salt and sham rats. Bars indicate mean arterial pressure before or after YOH+DMI treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between DOCA-salt and sham rats.

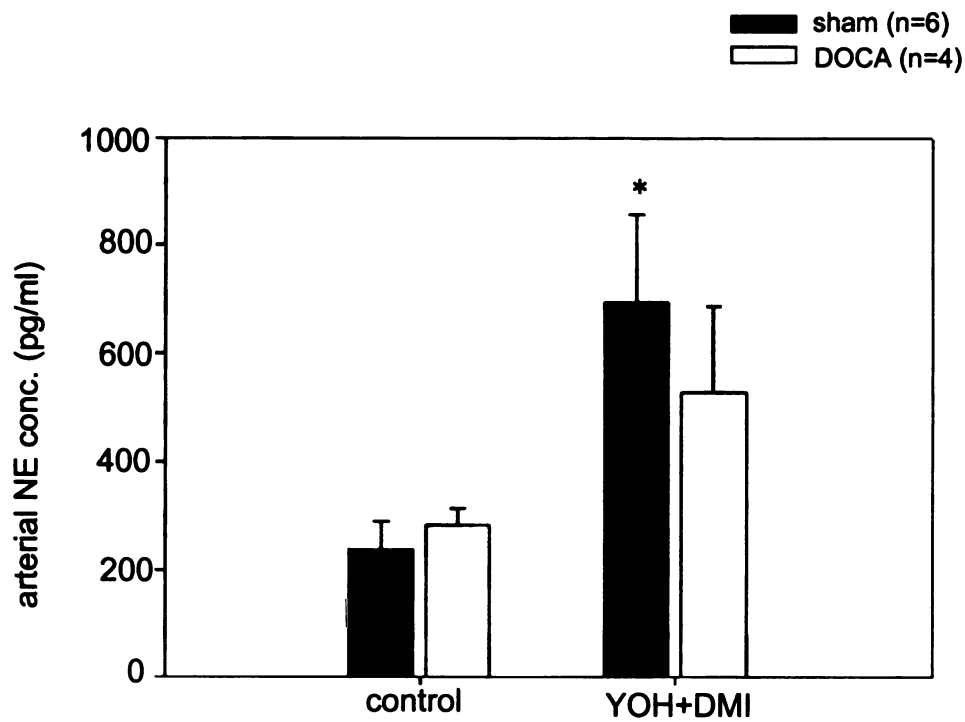


Figure 36: Effects of i.v. bolus yohimbine+desipramine administration on aortic plasma NE level in conscious DOCA-salt and sham rats. Bars indicate mean arterial plasma NE concentration before or after YOH+DMI treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) before and after drug treatment.

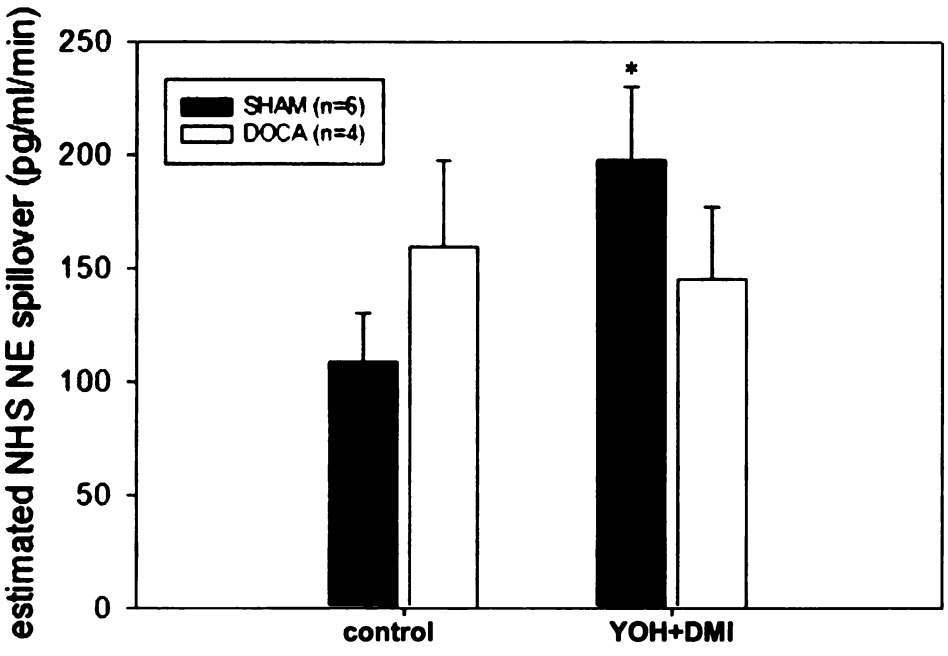


Figure 37: Effects of i.v. bolus yohimbine+desipramine administration on estimated NHS spillover rate of NE in conscious DOCA-salt and sham rats. Bars indicate mean estimated NHS NE spillover rate before or after DMI treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) before and after drug treatment.

Discussion for Chapter 5-Part IV

In my previous study, DMI alone didn't cause any significant change in the estimated NHS NE spillover in either DOCA-salt or sham rats. In this study, after α2-adrenoceptor blockade by i.v. yohimibine, NE spillover was increased in response to i.v. DMI in sham rats. This may reflect the increased NET activity after the failure of prejunctional α2 autoinhibiton. Besides, DMI has two distinct actions: it blocks neuronal reuptake by direct actions on nerve endings and reduces SNA by actions of raised intrasynaptic NE concentrations on inhibitory α2-adrenoceptors within the CNS. Therefore, blockade of α2-adrenoceptors may block the central sympathoinhibitory effect of desipramine. And subsequently, the increase in NE concentration at sympathoeffector junctions would not be offset by a fall in SNA. In contrast to what happened in sham rats, yohimbine+DMI didn't cause any significant change in the estimated NHS NE spillover in DOCA-salt rats. This could be due to impairment of prejunctional α2-adrenoceptors function in the splanchnic bed of DOCA-salt rats. It could also reflect the absence of an important functional interaction between the two mechanisms.

Chapter 5 – Part V

Modulation of Sympathetic Neurotransmission by Endothelin-1 in vivo in DOCA-Salt Hypertensive Rats

Rationale: I speculate based on the results of my endothelin study discussed earlier in Chapter 4 that the enhanced venoconstrictor effect of ET-1 could be mediated by an action of ET-1 on the sympathetic nervous system. A number of studies have shown prejunctional modulation of sympathetic neurotransmission by ET-1 in vitro (158), e.g. Intrarenal arterial infusion of ET-1 inhibited norepinephrine (NE) release induced by renal sympathetic nerve stimulation in anesthetized dogs (147). This neuroinhibitory effect of ET-1 was mediated through activation of ET_B receptor mechanisms (224). Localization of ET-1 receptors on the nerve terminals have also been reported (60). Neurotransmission in sympathetic ganglia also can be modulated by ET-1 (238). Superoxide is elevated in prevertebral sympathetic ganglia in DOCA-salt hypertension, and ET-1 is a potent stimulus for the elevation of superoxide levels in sympathetic ganglia, an effect that may be mediated by the upregulation of ETB receptors (26). Superoxide production evoked by ET-1 may play a role in the increased sympathetic excitability in DOCA-salt hypertension (26). Increased circulating ET-1 level was found in ganglionectomized DOCA-salt hypertensive rats (63). A previous study in my lab (104) found that pretreatment of DOCA-salt hypertensive rats with an ET_B receptor antagonist produced greater declines in MCFP following ganglionic blockade than after ganglionic blockade alone. These findings suggest that ET-1 acting at ET_B receptors may provide an important contribution to venoconstriction through modulation of sympathetic tone to the veins. The ET_B antagonist may have facilitated NE release from sympathetic

nerve terminals on veins, accounting for the significantly larger response of MCFP to subsequent ganglion blockade. This action presumably was more prominent in DOCA-salt rats because of their higher pretreatment levels of endogenous vascular ET-1. Furthermore, in hypertensive patients, ET-1 has been shown to potentiate sympathetically mediated venoconsctriction (87).

No studies have been performed to directly measure splanchnic NE release in vivo to evaluate the effect of ET_B receptor blockade on sympathetic input to the splanchnic bed in DOCA-salt hypertensive rats. In my study, I treated rats with an ETB receptor antagonist and measured aortic and portal venous plasma catecholamine levels.

Experimental Procedures: The selective ET_B receptor antagonist, A192621 was given (12 mg/kg, Abbott Laboratories, IL) via a femoral venous catheter to conscious DOCA-salt and sham rats at least 4 days after recovery from catheterization as described earlier. Blood samples were collected simultaneously from the arterial and portal venous catheters 30 min after drug treatment. Plasma NE, EPI levels were measured using REA. NHS spillover of NE was estimated.

Results: As I saw in my previous studies, A192621 significantly increased the MAP in conscious sham, but not conscious DOCA-salt rats (Fig. 38). A192621 caused a significant increase in the arterial plasma NE level in sham rats. A192621 tended to decrease the arterial plasma NE level in DOCA-salt rats, however, the decrease was not significant (Fig. 39). The ETB receptor antagonist didn't cause any significant change in the estimated NHS NE spillover in either DOCA-salt or sham rats (Fig. 40).

ETB antagonist treatment caused an approximately 5-7 fold increase in both the aortic and portal venous plasma EPI levels in sham rats (Fig. 41). No significant change in either aortic or portal venous plasma EPI level was observed in DOCA-salt rats.

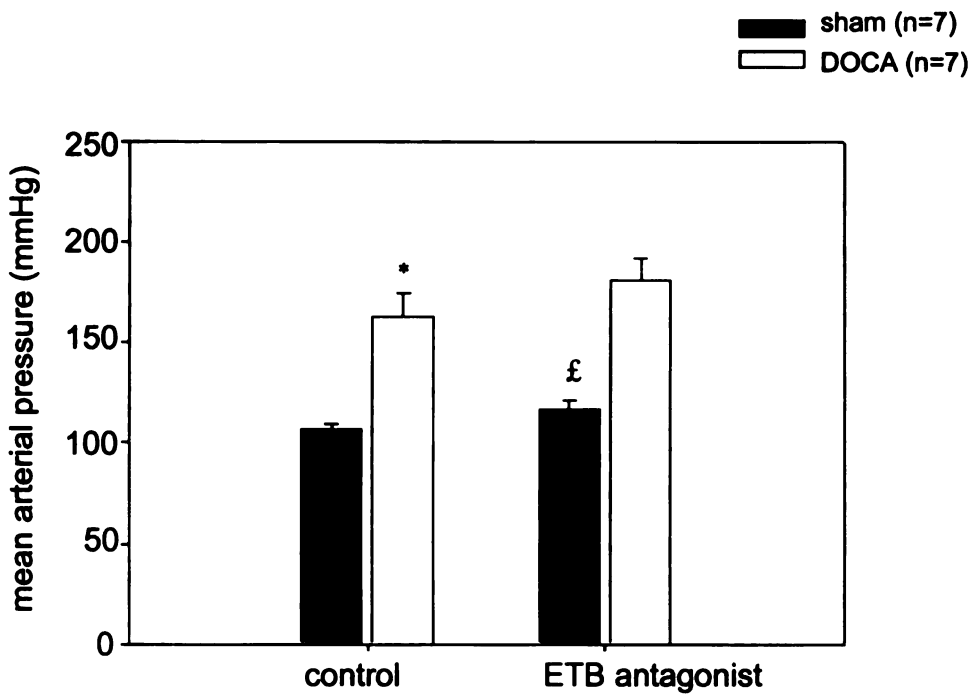


Figure 38: Mean arterial pressure responses to i.v. bolus A192621 administration in conscious DOCA-salt and sham rats. Bars indicate mean arterial pressure before or after selective ETB receptor antagonist treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between DOCA-salt and sham rats. £ indicates a significant difference ($P<0.05$) between before and after drug treatment.

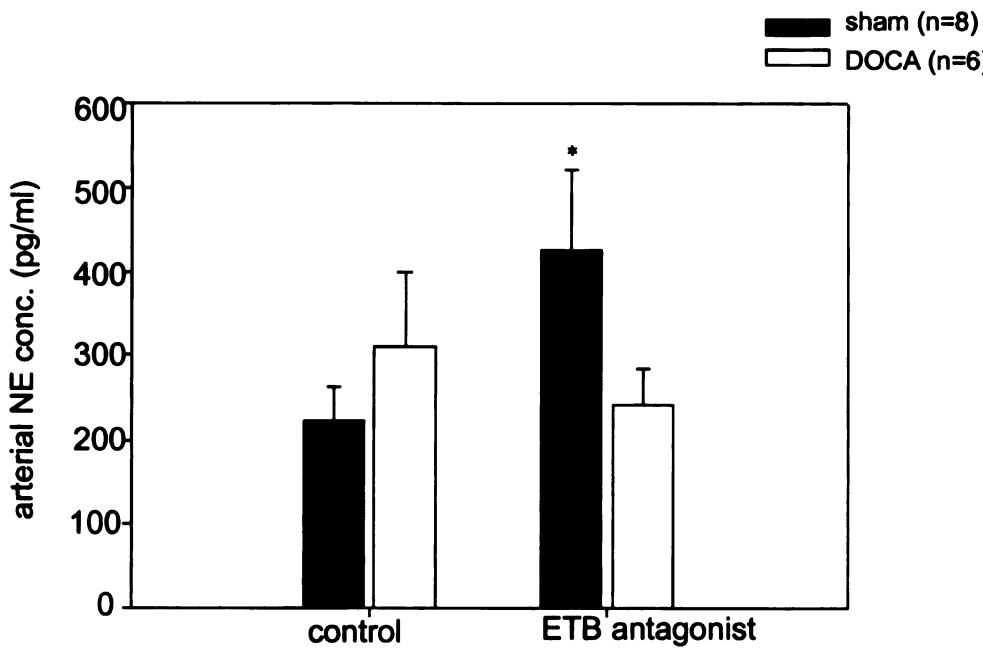


Figure 39: Effects of i.v. bolus A192621 administration on aortic plasma NE level in conscious DOCA-salt and sham rats. Bars indicate mean arterial plasma NE concentration before or after selective ETB receptor antagonist treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) before and after drug treatment.

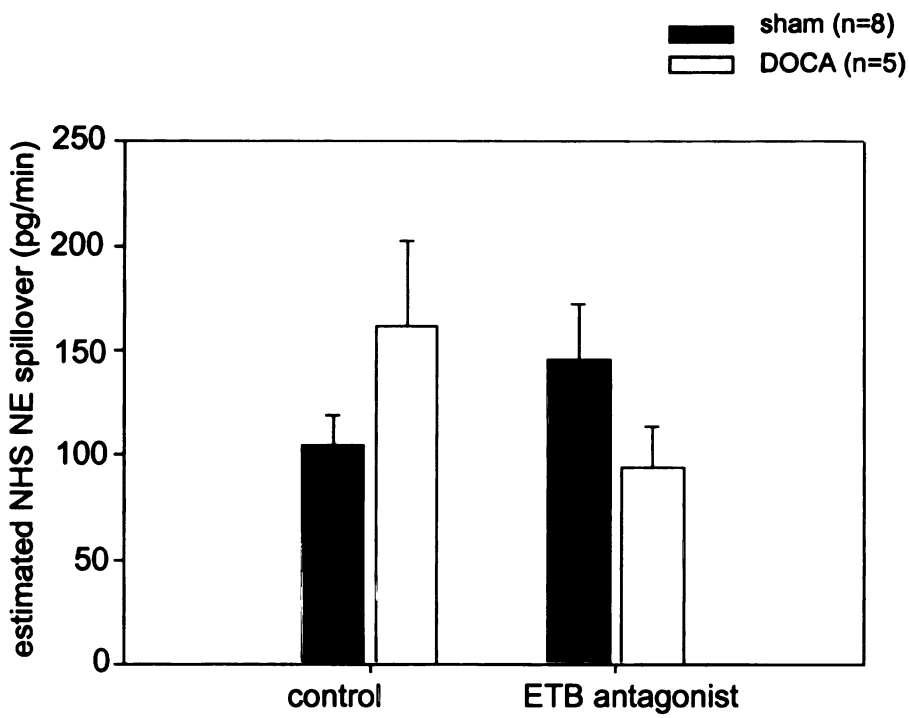


Figure 40: Effects of i.v. bolus A192621 administration on estimated NHS spillover rate of NE in conscious DOCA-salt and sham rats. Bars indicate mean estimated NHS NE spillover rate before or after selective ETB receptor antagonist treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM.

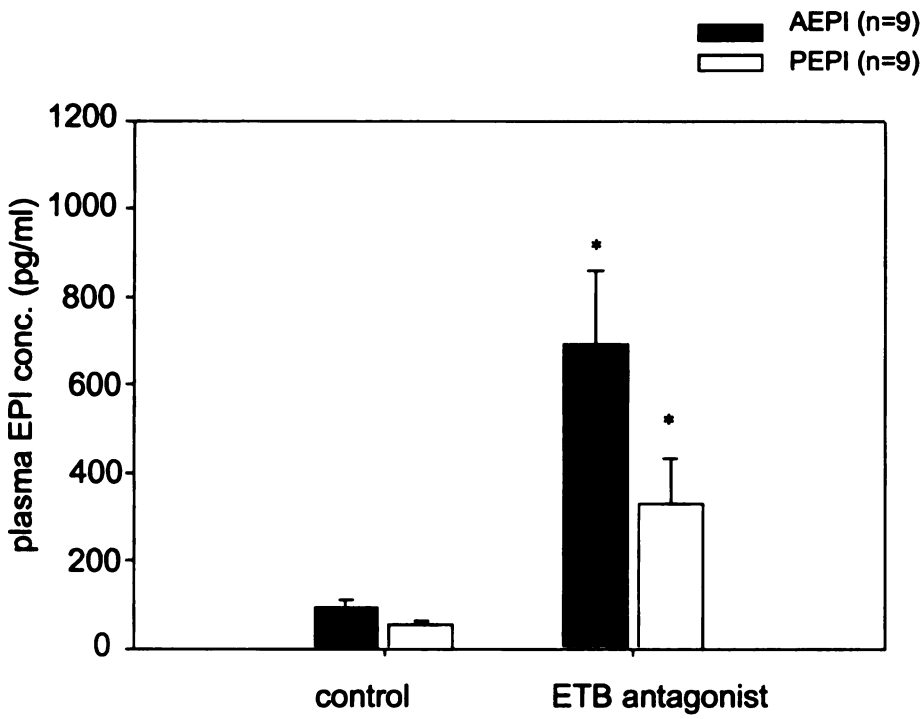


Figure 41: Effects of i.v. bolus A192621 administration on plasma EPI level in conscious sham rats. Bars indicate mean arterial plasma EPI concentration before or after selective ETB receptor antagonist treatment in conscious sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) before and after drug treatment.

Discussion for Chapter 5-Part V

A192621 didn't cause any significant change in the arterial plasma NE level in DOCA-salt rats as it did in sham rats, suggesting that the global inhibitory effect of ET-1 on sympathetic nerve activity via ETB receptors is impaired in DOCA-salt rats, which, may consequently suggest a relative 'facilitatory' effect of ET-1 on SNA. ETB receptor antagonist treatment didn't cause any significant change in the estimated NHS NE spillover in either DOCA-salt or sham rats, suggesting the lack of an inhibitory effect of ET-1 on SNA in the splanchnic bed.

It is noteworthy that the ETB antagonist caused an approximately 5-7 times increase in both the aortic and portal venous plasma EPI levels in sham rats, suggesting a strong inhibitory effect of ET-1 on adrenal medullary secretion of EPI. This inhibitory effect of ET-1, however, is missing in DOCA-salt rats because ETB antagonist treatment didn't cause any significant change in either aortic or portal venous plasma EPI levels. The modulatory effect of ET-1 on adrenal medullary EPI release has been reported before in control rats (123). The same group also found that infusion of exogenous ET-1 into an isolated, perfused adrenal gland preparation resulted in an increase in the basal release of NE and EPI in DOCA rats, suggesting the functional integrity of this modulatory effect in DOCA-salt rats. The difference between their result and mine may reside in the fact that my studies were perfomed in whole animals.

Discussion for Chapter 5

Consistent with previous reports, I found increased SNA in DOCA-salt hypertension. Two primary mechanisms may contribute to the increase in peripheral SNA in DOCA-salt hypertension under resting conditions: 1) reduced tonic baroreflex inhibition of 'normal' central SNS outflow; and 2) a primary increase in CNS-generated sympathetic nerve discharge. Both arterial and cardiopulmonary baroreflexes tonically inhibit central SNS outflow in humans (139). Thus one possibility is that this tonic inhibition lessens in DOCA-salt hypertension, allowing progressively greater levels of SNS activity to peripheral tissues (Rowe & Troen, 1980). It has been reported that the arterial and/or cardiopulmonary baroreflex control of renal SNS activity was reduced in DOCA-salt hypertension (230). In several clinical contexts, most notably cardiac failure and essential hypertension, Esler and colleagues (62, 121) have demonstrated the possible importance of projections of noradrenergic neurons to the forebrain in generating elevated levels of peripheral SNS activity. Increased central nervous system NE turnover (into the subcortical venous drainage) (reflecting increased noradrenergic activity) has been reported in patients with primary hypertension. Increased overflow of endogenous NE from paraventricular nucleus of the hypothalamus (PVH) was found in conscious spontaneously hypertensive rats (178). However, both decreased hypothalamus NE turnover (68) and unchanged overflow of endogenous NE from PVH nucleus (178) have been reported in DOCA-salt rats, suggesting a reciprocal relationship between the activities of central and peripheral noradrenergic fibers (159). The reduced turnover of NE in the hypothalamus may be secondary to the elevated blood pressure, reflecting an

attempt to decrease noradrenergic activity, a possibly compensatory change in actively trying to counteract the rise in blood pressure in DOCA-salt rats (68).

My study is the first to estimate NHS NE spillover in DOCA-salt hypertensive rats. I found that the estimated NHS NE spillover was increased in DOCA-salt hypertensive rats, suggesting increased splanchnic sympathetic nerve activity. This is of particular importance as sympathetic nerve stimulation can enhance splanchnic venoconstriction, reduce intestinal blood volume by up to 60%, leading to a significant redistribution of blood from the splanchnic veins. This change in volume distribution will have a profound effect on overall cardiovascular functions including blood pressure (81) (65, 109).

The increased sympathetic nerve activity in DOCA-salt hypertension suggests that there may be alterations in the local mechanisms that modulate sympathetic neurotransmission. Possibilities include impaired neuronal reuptake, impaired prejunctional α2-adrenoceptor function, facilitation of NE release by other neurohumoral factors such as EPI, and ET-1. I designed a series of in vivo studies to test these possibilities and found that there was impaired prejunctional α2-adrenoceptor function in the splanchnic vasculature of DOCA-salt rats. This would serve to augment the amount of NE released per unit sympathetic nerve discharge. ET-1 has an inhibitory effect on systemic NE release in sham rats. That inhibitory effect is missing in DOCA-salt rats systemically. My results concerning the neuronal uptake activity in the splanchnic bed are hard to explain because of the influence of the central sympathoinhibitory effect of the neuroal uptake inhibitor I used on the regional sympathetic ouflow. However, the result may also reflect that there is little neuronal uptake occuring in the splanchnic bed.

My studies have been focused on the sympathoadrenal system under basal (resting) conditions. Experiments aimed at determining the functional integrity of NET and prejunctional α2-adrenoceptor inhibition mechanism of DOCA-salt hypertension in face of hyperactivation of the sympathoadrenal system (by stimuli such as haemorrhage) can be performed in the future. These observations will be important considering that the NET activity and the negative feedback mechanism are probably maximally activated during the reflex hyperactivation of the sympathetic nervous system. These experiments should help us gain insight into the augmented sympathoadrenal adjustments to acute stress (e.g. haemorrhage) and possible DOCA-salt hypertension- related differences in regional SNS regulatory mechanisms as well as epinephrine secretion from the adrenal medulla compared with sham rats.

Plasma concentration of NE is commonly used as an index of SNA. Several important caveats limit the validity of plasma NE levels as an index of sympathetic "tone". Multiple factors determine the proportionality between the rate of neuronal release of NE and plasma NE concentration. Plasma NE concentration is determined by both the rate of diffusion of NE from the various nerve junctions into the plasma (the apparent release rate or spillover rate) and the rate of removal (clearance) from the plasma by the processes of uptake, metabolism and urinary excretion. Thus, a rise in plasma NE concentration could result from either an increase in spillover rate with no change in clearance or a decrease in clearance with no change in spillover rate. In like fashion, a decrease in plasma NE concentration could result from a decrease in spillover rate and/or an increase in clearance. For example, normal circulating catecholamines were found in the presence of markedly increased muscular sympathetic nerve activity in

pulmonary arterial hypertension (246), further underscoring their limited usefulness as indicators of sympathetic nervous system activity.

My estimate of NHS spillover of NE provides a better index of splanchnic regional sympathetic nerve function. However, it is still an estimated value based on the two assumptions mentioned in General Methods. One assumption is that there is no EPI release from the splanchnic bed and the fractional extraction of NE equals that of EPI. However, it has been reported by Esler et al (56) that at rest, epinephrine was released from the heart in older men despite the fact that adrenal medullary secretion of epinephrine was reduced. For this reason, I am developing the radiotracer methods described by Esler et al (1979) (52) for the measurement of NE clearance and spillover rate. Since both clearance of NE from the bloodstream and the rate of NE entry into the bloodstream (spillover) determine plasma NE levels (spillover = clearance X arterial plasma NE level), Esler et al used the specific activity (SA) of plasma [^{3}H] NE measured during intravenous infusion of [^{3}H] NE to estimated NE clearance from and NE spillover into the systemic circulation. Increased dilution of the tracer with endogenous NE from inflowing to outflowing plasma reflects local release of transmitter and provides the basis of NE spillover measurements (Fig. 42). I also did preliminary studies to test the second assumption (Portal venous blood flow is the same in sham and DOCA-salt rats) on which my estimated NHS spillover in previous studies was based. I found that there was no significant difference in the portal blood flow between DOCA-salt (18.6 ± 3.56 ml/min, n=3) and sham rats (25.78 ± 3.13 ml/min, n=3), which was consistent with pusblished results (97) (264), suggesting that DOCA treatment didn't significantly change the portal

blood flow, and the increase in NHS spillover in DOCA-salt rats was not due to decreased portal blood flow.

In the future, radiotracer methods can be used in conscious chronically tethered rats with different drug interventions to provide a more precise assessment of NHS spillover of NE and a better guide to overall sympathetic nervous tone in the splanchnic bed of DOCA-salt hypertensive rats. However, successful measurements of various plasma NE metabolites (DHPG, MHPG) require separation and quantification techniques feasible for small volume samples, which is still a challenge for our group.

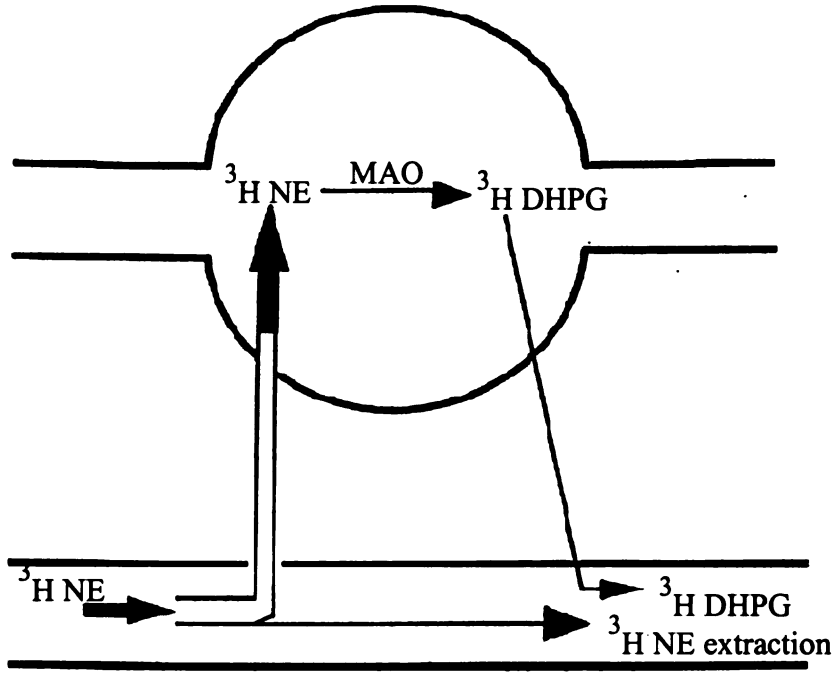


Figure 42: Schematic of splanchnic processing of tritiated NE (^{3}H NE). The majority of [^{3}H] NE is removed from plasma via a clearance mechanism that involves neuronal uptake. Within sympathetic nerves, [^{3}H] NE is metabolized into [^{3}H]DHPG by monoamine oxidase (MAO), with some subsequent release into the venous circulation (192).

CHAPTER 6

PRELIMINARY STUDIES ON SYMPATHETIC NEUROTRANSMISSION AND ENDOTHELIN-1 IN THE SPLANCHNIC BED OF DOCA-SALT RATS IN THE EARLY DEVELOPMENTAL STAGE

Introduction

The pathogenesis of essential hypertension is still uncertain, but genetic factors and the sympathetic nervous system are likely to be involved. Sympathetic nerve activity and hormonal circulatory control mechanisms, however, are affected by blood pressure itself. Hence, early functional changes are best investigated in normotensive subjects at risk to develop hypertension, such as normotensive offspring of hypertensive parents.

Neural adrenergic factors have long been hypothesized to be important in the initiation of high blood pressure. Higher level of plasma NE (134) (102) and overall rate of spillover of NE to plasma (61) have been found in offspring of hypertensive parents. Although resting muscle sympathetic nerve activity (measured by microneurography) appears to be normal (86), more pronounced increases in muscle sympathetic nerve activity and blood pressure in response to mental stress was noticed in offspring of hypertensive than in offspring of normotensive parents (164). The possible association between predisposition to essential hypertension, plasma NE level and two polymorphisms of the gene for tyrosine hydroxylase (Tetranucleotide TCAT repeat and Val81Met polymorphisms) were studied and the studied TH polymorphisms do not appear to be associated with a family history of essential hypertension (102). However, decreased baroreceptor sensitivity has been found in offsprings of hypertensive parents (169).

To investigate the pathogenesis of DOCA-salt hypertension, I used DOCA-salt rats in the early developmental stage (4-5 days). Fewer studies have been done compared with human subjects. However, it has been found that central attenuation of baroreflex precedes the development of hypertension in DOCA-salt treated rats (231).

Chapter 6 – Part I

NE release and Prejunctional α2-Adrenoceptor function in the sympathetic nerves associated with the splanchnic vasculature of conscious DOCA-salt rats in the early developmental stage.

Rationale: Venous capacitance is reduced in established DOCA-salt hypertension, but it is uncertain whether this is a cause or a consequence of hypertension. It has been found that the baseline radial arterial distensibility and compliance were comparable in offspring of hypertensive and normotensive parents (32). However, there were also studies showing that there was increased carotid arterial stiffness in children with a parental history of hypertension (148) and decreased brachial arterial compliance in still-normotensive offspring of hypertensive parents (15). When the function of veins in the early stage of hypertension was examined, venous distensibility was found to be decreased in normotensive young men with hypertensive relatives (101). Previous studies in my lab showed that MCFP is increased early (before blood pressure rose) in DOCA-salt hypertension, and it is not due to intravascular blood volume expansion. This indicates that structural and/or functional vascular alterations precede a distinct rise in blood pressure, suggesting that venous changes could be a cause of elevated blood pressure. Pharmacological studies in the early stage of hypertension in DOCA-salt rats (7-14 days after DOCA implantation) demonstrated an increased MCFP dependent on both endogenous ET-1 and sympathetic neurogenic venoconstriction. This suggests that ET-1 and SNS contribute to increased venoconstriction early in the development of DOCA-salt hypertension. The mechanisms are still unclear. No studies about the reactivity to NE of blood vessles from early DOCA-salt hypertensive rats had been

performed. In this study I tested the hypotheses that 1) the enhanced sympathetic nerve mediated venoconstriction occurring early during the development of DOCA-salt hypertension could be attributable to increased NE release from the splanchnic bed. 2) Impaired prejunctional α2-adrenoceptor function contributes to this increased NE release.

Experimental Procedures: All rats used in this study were uninephrectomized and drank 1% NaCl/0.2% KCl solution throughout the protocol. After sodium pentobarbital anesthesia (50 mg/kg, i.p.), silicone rubber tipped catheters were placed into abdominal aorta via a femoral artery and portal vein via a small branch of the portal vein after laparotomy. The third catheter was inserted into a femoral vein. I set up the rats as introduced in the General Methods. After at least fmy days of recovery from anesthesia and surgical stress, hemodynamic measurements (HR, SBP, DBP, MAP) were made on two control days (C1 and C2). After measurements were completed on day C2, rats either received a DOCA patch (200mg/kg, s.c. DOCA) or sham implant surgery (SHAM) under methohexital sodium (Brevital sodium) anesthesia (9 mg/kg, i.v.). Blood samples were collected from arterial and portal venous catheters on the 4th day (A4) after DOCA or sham surgery. On the 5th day (A5), rats were treated with prejunctional α2-adrenoceptor antagonist yohimbine (0.5 mg/kg, i.v.) and arterial and portal venous blood samples were taken 30 min after drug treatment. Plasma NE and EPI levels were measured using a radioenzymatic assay and net NHS spillover of NE was estimated. The reason that I was interested in what happened in rats 4 and 5 days after DOCA or sham implant surgery was that based on my previous studies, the MAP stared to increase on the 4th day after

DOCA implantation. My goal was to evaluate the change in sympathetic neurotransmission accompanying the increase in BP.

Results: I found that the mean arterial pressure was significantly higher in DOCA-salt rats (122.0 ± 2.5 mmHg) at 4 days than sham rats of the same age (113.2 ± 3.9 mmHg) (Fig. 43). There was no significant difference in the arterial plasma NE concentration (Fig. 44) (365 ± 40 pg/ml in sham; 411 ± 110 pg/ml in DOCA) and the estimated NHS NE spillover (Fig. 45) (119 ± 39 pg/min in sham; 64 ± 29 pg/min in DOCA) between 4-day sham and DOCA-salt rats.

Yohimbine caused a significant decrease in the MAP in DOCA-salt (Fig. 43) (from 120 ± 4 to 104.2 ± 4 mmHg) but not sham rats, which was similar to what I saw in established DOCA-salt and sham rats. Although yohimbine significantly increased the arterial plasma NE concentration only in sham rats (Fig. 46) (from 365 ± 40 to 1113 ± 210 pg/ml), it caused a significant increase in the estimated NHS NE spillover in both groups of rats (Fig. 47) (from 119 ± 39 to 499 ± 83 pg/min in sham, from 65 ± 33 to 326 ± 51 pg/min in DOCA).

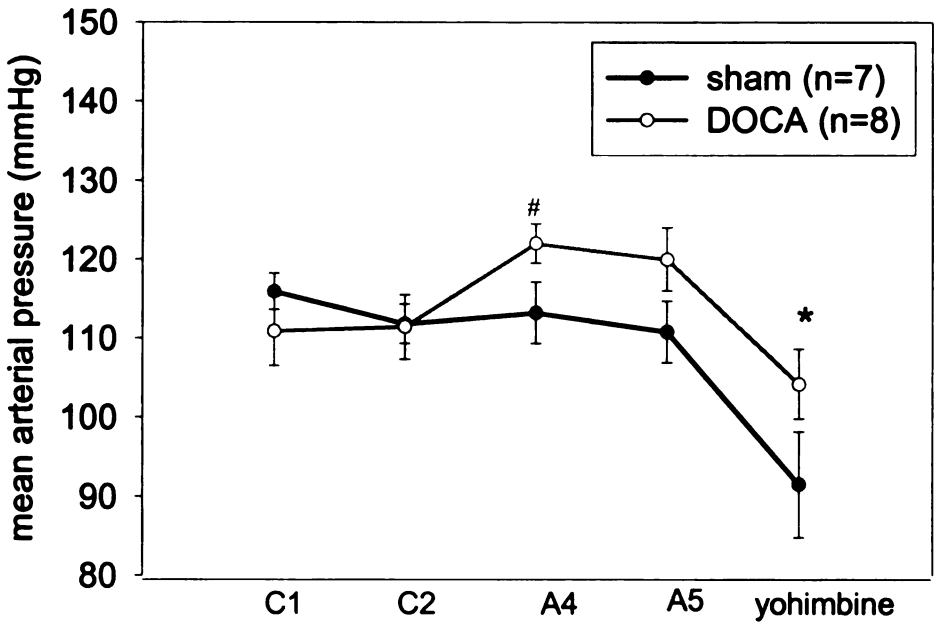


Figure 43: Mean arterial pressure of conscious early DOCA-salt and sham rats and MAP response to i.v. bolus yohimbine administration. Dots indicate mean arterial pressure in control days, 4 and 5 days after DOCA implantation (sham surgery) and MAP response to yohimbine treatment. Brackets indicate SEM. # indicates a significant difference ($P<0.05$) between sham and DOCA-salt rats. Asterisk indicates a significant difference ($P<0.05$) before and after drug treatment.

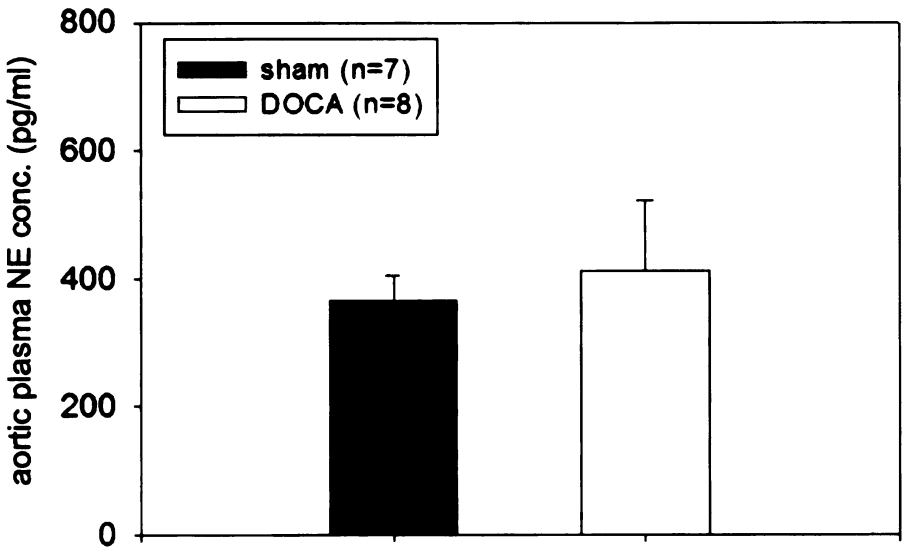


Figure 44: Aortic and portal venous plasma ET-1 level in conscious early DOCA-salt and sham rats. Bars indicate mean arterial plasma NE concentration in conscious 4-day sham and DOCA-salt rats. Brackets indicate SEM.

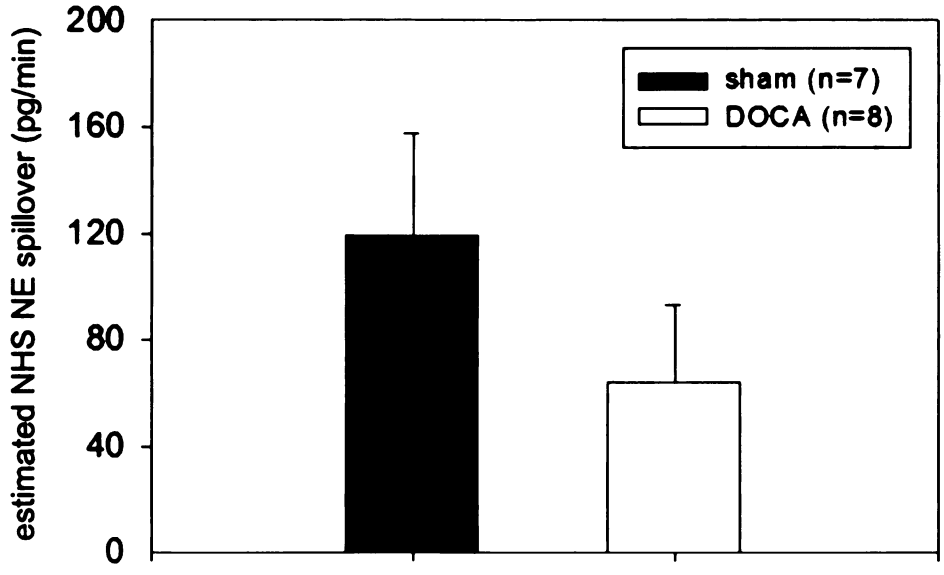


Figure 45: Estimated nonhepatic splanchnic spillover rate of NE in conscious early DOCA-salt and sham rats. Bars indicate mean estimated NHS NE spillover rate in conscious 4-day sham and DOCA-salt rats. Brackets indicate SEM.

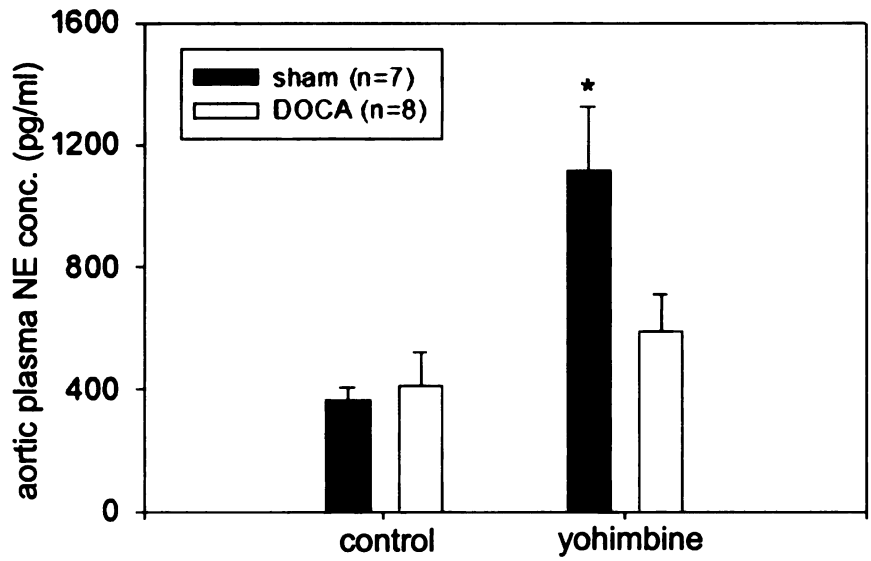


Figure 46: Effects of i.v. bolus yohimbine administration on aortic plasma NE level in conscious early DOCA-salt and sham rats. Bars indicate mean arterial plasma NE concentration before or after yohimbine treatment in conscious early sham and DOCA-salt rats. Brackets indicate SEM. Asterisk indicates a significant difference ($P<0.05$) between before and after drug treatment.

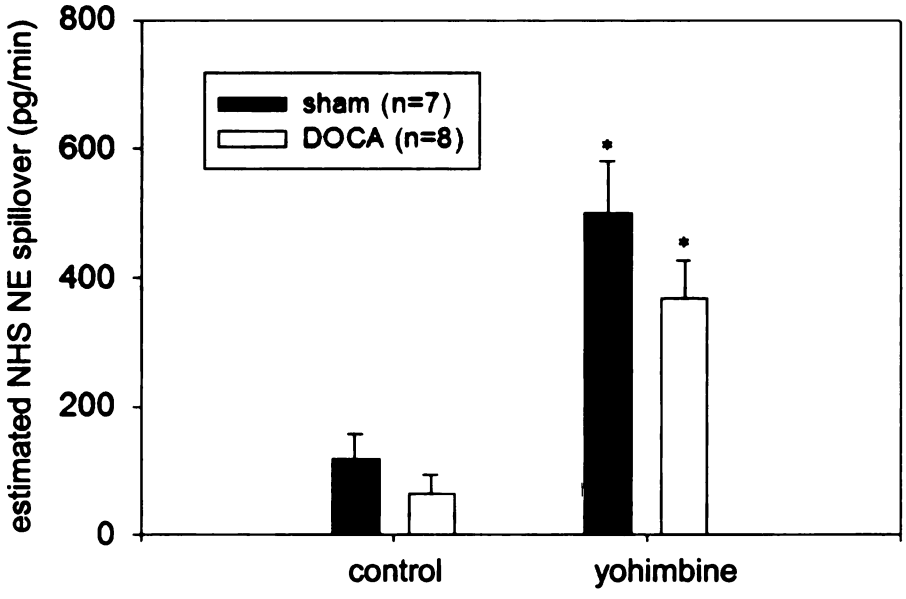


Figure 47: Effects of i.v. bolus yohimbine administration on estimated NHS spillover rate of NE in conscious early DOCA-salt and sham rats. Bars indicate mean estimated NHS NE spillover rate before or after yohimbine treatment in conscious early sham and DOCA-salt rats. Brackets indicate SEM. Asterisks indicate a significant difference ($P<0.05$) between before and after drug treatment.

Discussion for Chapter 6-Part I

My studies suggest unchanged SNA both globally and in the splanchnic bed of DOCA-salt rats at 4-day. Although yohimbine significantly increased the arterial plasma NE concentration only in sham rats, it caused a significant increase in the estimated NHS NE spillover in both groups of rats, suggesting that the function of α2-adenoceptor, although unchanged in the splanchnic bed, is impaired systemically.

A hyperreactivity of the SNS has long been suspected in the development of hypertension, but most studies have yielded inconsistent results. Besides the numerous studies showing increased SNA mentioned in the Introduction, there are also studies suggesting that there is no enhanced sympathetic activity or heightened cardiovascular responsiveness among normotensive young adults who are familially predisposed to essential hypertension (141). In addition to methodological problems, it is likely that the activity of the SNS changes during the natural history of high blood pressure. For example, as suggested by Noll et al. (165), it is likely that in normotensive individuals with a genetic background for hypertension, muscle sympathetic nerve activity is increased only in response to factors such as stress, becomes increased even under unstimulated conditions in borderline hypertension, and eventually returns to normal levels in established hypertension. Therefore, on the basis of my results and those of the literature, it is possible that in the early stage of DOCA-salt hypertension, splanchnic sympathetic nerve activity is normal under basal condition, maybe increased only in response to factors such as stress, and becomes increased even under unstimulated conditions in established hypertension.

Therefore, although my studies didn't find increased sympathetic nerve activity either globally or in the splanchnic bed under baseline conditions, further studies testing the response of SNS to certain stimuli should be performed, as dysfunction of this system id more likely to occur during stress. It was found (165) that the activity of the sympathetic nervous system and plasma norepinephrine levels were increased during mental stress only in offspring of hypertensive parents, whereas the response to hypoxia was similar in offspring of hypertensive and normotensive parents, suggesting an abnormal regulation of the sympathetic nervous system to certain stressful stimuli in offspring of hypertensive parents. On the basis of this study, the response of SNS to various stimuli may need to be tested in the future.

Chapter 6 – Part II

Net NHS release of ET-1 in conscious DOCA-salt rats in the early developmental stage.

Rationale: Increased plasma ET-1 production has been demonstrated in young, untreated, essential hypertensive patients with no signs of target-organ damage, suggesting that ET may play a role in the early phases of development of hypertension in some subsets of patients (206). In normotensive offspring of hypertensive parents, handgrip exercise produced increased ET-1 plasma levels in offspring of hypertensive parents and resulted in a sustained ET-1 release into the bloodstream during recovery compared with offspring of normotensive parents (232). Mental stress significantly increased plasma endothelin level in offspring of hypertensive parents (165). Pharmacological studies in the early stage of hypertension in DOCA-salt rats (7-14 days after DOCA implantation) by my group demonstrated an increased MCFP dependent on both endogenous ET-1 and sympathetic neurogenic venoconstriction. There are few studies on the mechanisms contributing to enhanced ET-1 mediated venoconstriction in early DOCA-salt rats. It was found that there was no significant difference in the reactivity to ET-1 between DOCA-salt and sham small mesenteric veins (Dr. Galligan). To test the possibility that higher endothelial formation of ET-1 contributes to increased ET-1 mediated venoconstriction, I performed preliminary studies to measure aortic and portal venous plasma ET-1 level and estimated net NHS release of ET-1 in rats 4th day after DOCA or sham surgery. As I mentioned before, ET-1 is a locally released vascular regulator that at very low concentrations enhances contractions to norepinephrine and at higher concentrations has potent direct vasoconstrictor properties. In experimental

animals, endothelin stimulates sympathetic outflow. If as I concluded from my previous studies that ET-1 increased venoconstriction indirectly in DOCA-salt rats by modulation of SNA, alterations in the local release of ET-1 could alter the activity and vascular effects of the SNS. I also treated rats with selective ETB receptor antagonist A192621 on the 5th day to see if the impaired ETB receptor mediated plasma clearance of ET-1 occurs early in the development of hypertension.

Experimental Procedures: Methods similar to those described earlier in Chapter 6-Part I were used to assess net NHS ET-1 release.

Results: I was not able to continue the experiment after I collected samples from three DOCA-salt and sham rats because of the lack of supply of A192621.

General Conclusions and Discussion

The work covered by this thesis is part of the project funded by NIH Program Project Grant which compares the different hemodynamic regulation of arteries and veins and aims at proving the novel idea that functional and/or structural changes in veins, especially small capacitance veins and venules are important in the pathogenesis of DOCA-salt hypertension. I examined the regulation of veins because the venous system is an important influence on cardiac output, and has been reported to be abnormal in early hypertension (46).

My work focused mainly on identifying the mechanisms by which endothelin-1 and sympathetic nervous system increase venomotor activity in DOCA-salt hypertension in in vivo whole animal level.

Results from present studies suggest that (corresponding to numbers in the bottom half part of Fig. 48):

A. ET-1 concentration around venous smooth muscle cells is not increased in DOCA-salt hypertensive rats. Evidence includes: 1. venous vascular ET-1 content is not increased in DOCA-salt rats; venous preproET mRNA level is not increased in DOCA-salt rats; 2. there is not a higher portal venous plasma ET-1 level and higher net NHS release of ET-1 in DOCA-salt rats despite impaired ETB receptor mediated plasma clearance of ET-1; 3. ETA receptors play an important role in the plasma clearance of ET-1 both globally and in the splanchnic bed. Although I have indirect evidence for more ETA receptor binding in the splanchnic bed of DOCA-salt rats, currently there is no direct evidence to support this. I also found impaired ETB receptor mediated plasma

clearance of ET-1 in DOCA splanchnic vasculature, shown as number 4 in Fig. 48. However, the physiological significance is not clear.

B. Systemic sympathetic nerve activity and sympathetic drive to the splanchnic bed are increased in DOCA-salt hypertensive rats. Evidence includes: *1.* plasma NE levels (both aortic and portal venous plasma) are significantly increased in DOCA-salt rats; the estimated NHS spillover of NE is significantly increased in DOCA-salt rats.

C. There is alteration in local modulation of sympathetic neurotransmission in DOCA-salt hypertensive rats. Evidence includes: prejunctional α2-adrenoceptors function is impaired in the splanchnic bed of DOCA-salt rats (shown as number *2* in Fig. 48); the global inhibitory effect of ET-1 on sympathetic nerve activity via ETB receptors is impaired in DOCA-salt rats (shown as number *3* in Fig. 48). My efforts to identify the functional integrity of NET (shown as ? in Fig. 48) and the interaction between prejunctional inhibitory autoreceptor and neuronal uptake (shown as ? in Fig. 48) were complicated by the complex properties of the neuronal uptake inhibitor I used. No better drug choice is available currently.

Collectively my results provide strong evidence that higher endothelial cell formation of ET-1 is not likely responsible for enhanced ET-1 mediated splanchnic venoconstriction in DOCA-salt rats *in vivo*. When combined with the fact that venous smooth muscle contractile responsiveness to ET-1 is not increased in DOCA-salt rats, the combined data suggest that the ability of ET receptor antagonists to reduce venoconstriction in DOCA-salt rats *in vivo* results from actions of the drugs on a non-

vascular target. i.e. ET-1 may exert indirect control of venous tone in vivo, for example, via the modulation of sympathetic neurotransmission.

Despite increasing evidence for an important contribution of neurogenic mechanisms to DOCA-salt hypertension, the precise causal mechanisms leading to sympathetic augmentation in DOCA-salt hypertension remain unclear. The present study provides direct evidence that there is increased splanchnic NE spillover and suggests that impaired prejunctional α2-adrenoceptor function and inhibitory effect of ET-1 both contribute to splanchnic sympathetic activation in hypertension. My finding of an increased sympathetic activity and altered local modulation of sympathetic neurotransmission was obtained from estimated NHS NE spillover. Further studies using the radiotracer method will provide more precise estimate of sympathetic nerve activity and therefore further test the notion that splanchnic sympathetic overactivity plays an important role in the underlying causes of DOCA-salt hypertension.

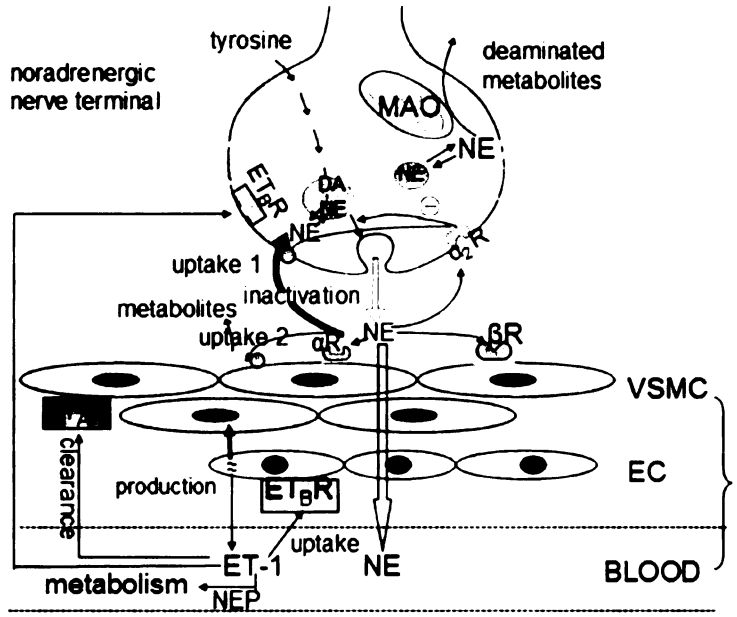
It is worth mentioning that studies of the roles of endothelin-1 and sympathetic nervous system in the regulation of venoconstriction in DOCA-salt rats are complicated by the fact that the effects of these two systems on the arterial system and kidney also contribute significantly to the high blood pressure. It has been found by my group that changes in venous function may play a particularly important role in a novel model of experimental hypertension in rats produced by chronic intravenous infusion of the selective ETB receptor agonist sarafotoxin 6C (S6C), as actions of S6C on arteries (endothelium-mediated vasodilation) and the kidney (marked diuresis and natriuresis) should produce hypotension intead of hypertension. Studies of the two systems in this

model may help better elucidate the underlying important roles of the two systems in mediating venoconstriction in the development and maintenance of hypertension.

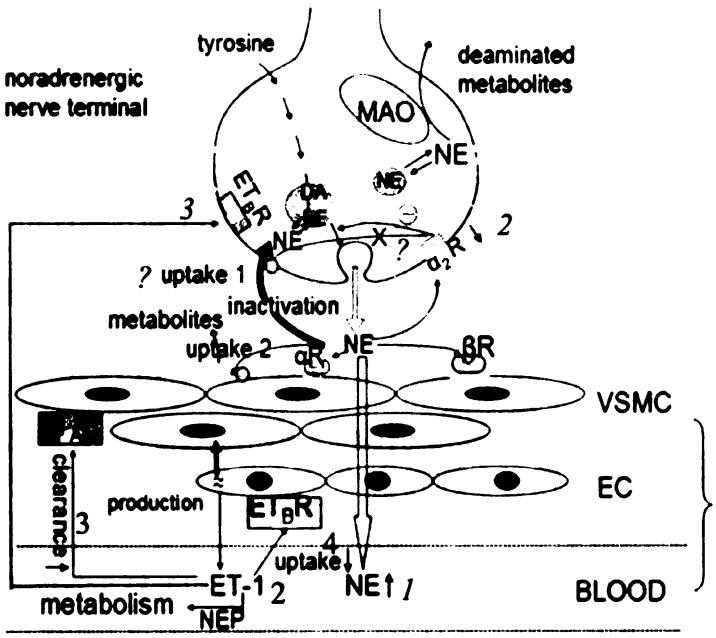
I excluded the liver from my experiments because of the difficulty of sampling blood from the hepatic venous drainage in the rat and the potential interference of hepatic ET-1 and NE extraction with the measurement of splanchnic release. However, it is important to note that liver is an important capacitance vascular bed. As I mentioned before, while adrenergic venoconstriction occurs in a number of vascular beds, splanchnic venules account for nearly 50% of the blood that is mobilized to the systemic circulation. Within the splanchnic vascular bed, the hepatic venules/veins represent the major smyce of blood mobilization. The hepatic venous system contains about the same amount of blood as the venules/veins in skeletal muscle and twice the volume stored in the intestine. However, hepatic venules/veins can mobilize nearly 65% of their stored blood during sympathetic activation, while their counterparts in skeletal muscle can displace less than 30% of the stored blood. Indeed, the hepatic venous system mobilizes an amount of blood following sympathetic activation that is equivalent to the combined contributions of the intestine and skeletal muscle. The greater sensitivity of hepatic venules/veins to sympathetic activation reflects either more adrenergic innervation, a higher density of adrenergic receptors, or both. Therefore, future studies may need to take the liver into account.

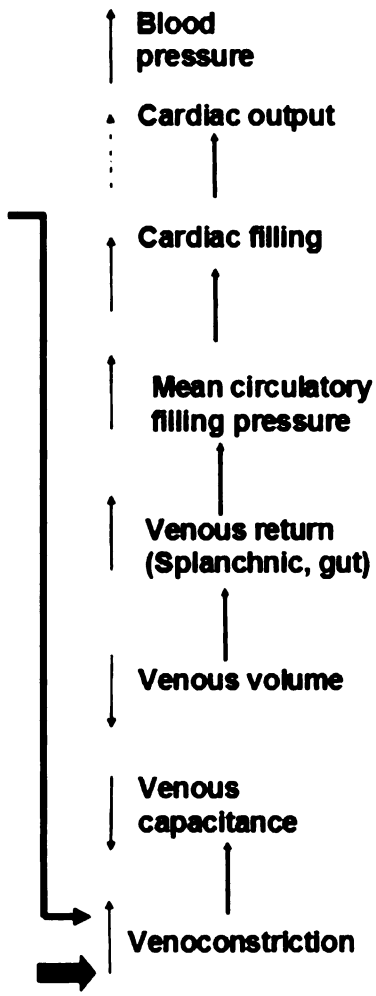
Figure 48: Schematic representation of experimental results, explained in detail in General Conclusion and Discussion.

sham



DOCA-salt





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