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ALTERED VENOCONSTRICTOR MECHANISMS IN DEOXYCORTICOSTERONE ACETATE-SALT HYPERTENSION: THE ROLE OF ENDOTHELINS

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Ronald Joseph Johnson

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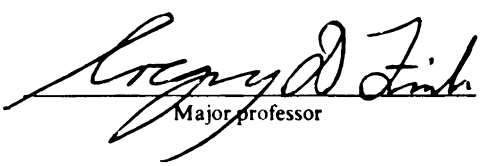

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ALTERED VENOCONSTRICTOR MECHANISMS IN DEOXYCORTICOSTERONE ACETATE-SALT HYPERTENSION: THE ROLE OF ENDOTHELINS

By

Ronald Joseph Johnson

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ABSTRACT

ALTERED VENOCONSTRICTOR MECHANISMS IN DEOXYCORTICOSTERONE ACETATE-SALT HYPERTENSION: THE ROLE OF ENDOTHELINS

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Hypertensive humans exhibit an elevated venomotor tone (integrated venous smooth muscle activity). This also has been observed in several animal models of hypertension, particularly the salt-sensitive forms. Alterations in venous function have therefore been implicated in the etiology of salt-sensitive hypertension. Evidence suggests that increased production of the vasomodulatory peptide endothelin-1 (ET-1) may be an important factor in the development of salt-sensitive hypertension in humans and animal models, and that venous tone may be a target for ET-1 induced alterations in cardiovascular function. Therefore, the aim of my research project was to test the overall hypothesis that the endothelial cell-derived peptide ET-1 contributes to the maintenance of salt-sensitive hypertension by increasing venomotor tone in the deoxycorticosterone acetate-salt (DOCA-salt) model of experimental hypertension in the rat. To test this hypothesis I devised a series of specific aims to evaluate the effects of endothelins on veins in DOCA-salt hypertensive rats. Based on key findings in studies conducted in vivo and in vitro, I propose that increased activation of the endothelin system in DOCA-salt hypertension in the rat produces two significant effects on venomotor tone which affect hypertension development. First, direct actions of ET-1 on vascular smooth

muscle ET_A receptors increases venomotor tone in DOCA-salt hypertension in the rat. The increase in venomotor tone is not due to altered responsiveness of the venous smooth muscle to ET-1. Second, the elevated venomotor tone observed in DOCA-salt hypertension due to increased sympathetic input to the veins is modulated by neuroinhibitory actions of ET-1 acting at ET_B receptors. Activation of the venous smooth muscle ET_B receptor by ET-1 may also contribute to the development of DOCA-salt hypertension; however, further studies are required. Based on findings in my thesis, I conclude that ET-1 contributes to the maintenance of DOCA-salt hypertension in the rat, in part, by increasing venomotor tone. Further, this role of ET_A and ET_B receptors has important implications for the use of balanced ET_A/ET_B versus selective ET_A receptor antagonism in several cardiovascular diseases including heart failure and hypertension.

To my parents, Evelyn and Albert Johnson

who have always been there for me.

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LIST OF ABBREVIATIONS

AA	arachiodonic acid
ACE	angiotensin converting enzyme
APP	arterial plateau pressure
AVP	arginine vasopressin
BV	blood volume
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanlyl monophosphate
CHF	congestive heart failure
CNS	central nervous system
CO	cardiac output
CVP	central venous pressure
DAG	diacylglycerol
DOCA-salt	deoxycorticosterone acetate-salt
EC50	effective concentration 50
ECE	endothelin converting enzyme
EDHF	endothelium derived hyperpolarizing factor
Emax	maximal response
ERK	extracellular signal-regulated kinase
ET	endothelin
ET-1	endothelin-1
ET-2	endothelin-2
ET-3	endothelin-3
HCT	hematocrit
HR	heart rate
IP3	inositol 1,4,5-triphosphate
[Ca2+]i	intracellular calcium
i.p.	Intraperitoneal
i.v.	intravenous
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
MAP	mean arterial pressure
MABP	mean arterial blood pressure
MAPK	mitogen activated protein kinase
MCFP	mean circulatory filling pressure
NE	norepinephrine
NLA	Nω-nitro-L-arginine
NO	nitric oxide
NOS	nitric oxide synthase
1K1C	one kidney, one-clip
OD	outer diameter

PGH2	prostaglandin H_2
PGI2	prostacyclin
PKC	protein kinase C
PLA2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PNS	peripheral nervous system
PV	plasma volume
S6a	sarafotoxin 6a
S6b	sarafotoxin 6b
S6c	sarafotoxin 6c
S6d	sarafotoxin 6d
s.c.	subcutaneous
SHR	spontaneously hypertensive rat
TPR	total peripheral resistance
2K1C	two-kidney, one-clip
TXA2	thromboxane
VPP	venous plateau pressure
VSM	vascular smooth muscle
VSMC	vascular smooth muscle cell

Chapter 1

INTRODUCTION

I. *Hypertension*

A. *Epidemiology*

In most Westernized countries, cardiovascular disease is the leading cause death in adults, as well as a major source of morbidity, loss of income and social disruption. In the United States alone, nearly 1 million people (43 % of all deaths) die annually from cardiovascular disease (Whelton et al., 1994). Many cardiovascular diseases are common and more severe in humans with elevated blood pressure, including stroke, coronary artery disease, atherosclerosis, congestive heart failure, diabetes, retinal and renal disease. While several modifiable risk factors are involved in the development of cardiovascular disease, high blood pressure or hypertension is the most important (Burt et al., 1995). Hypertension is usually accompanied by other risk factors, however, and seldom occurs alone (Anderson et al., 1991). Currently, the Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure defines hypertension as a sustained systolic blood pressure greater than 140 mmHg and/or a diastolic pressure above 90 mmHg (National Institutes of Health, 1997). Pharmacologic intervention in hypertension has reduced the risk of cardiovascular disease, however, the prevalence of blood pressure related cardiovascular disease underlies

the continued need for research in order to understand and better treat hypertension.

B. *Primary versus secondary forms of hypertension*

Approximately 5-10 % of hypertensive individuals have an identifiable cause such as renal (parenchymal disease, renal artery stenosis, tumors), mechanical (coarctation of the aorta) or endocrine (tumors, aldosteronism) abnormalities (Lilly, 1993). These individuals suffer from *secondary hypertension*. Management of secondary hypertension often involves surgical intervention and can be curable. The vast majority of hypertensives (90-95 %), however, have blood pressure values which are merely at the upper end of the normal distribution of blood pressures for their population (Fink, 1998). This form of hypertension is known as *primary* or *essential hypertension* and is due to an as yet unidentifiable cause (or causes). Although the etiology of essential hypertension is currently unknown, what is clear is that hypertension is a multifactorial disease which appears to involve a genetic component. Support for this comes from a strong familial aggregation of the disease and the lack of even distribution of prevalence among the races (Burt et al., 1995; Whelton et al., 1994). A significant research effort aimed at determining those genes responsible for blood pressure regulation and hypertension is currently underway. The determination of blood pressure is the result of several genetically controlled factors interacting with the environment and each other. Identification of the specific gene (or genes) at a molecular level that are associated with an

observed hypertension phenotype allows for the determination of the genetic defect responsible for that phenotype. Currently, the phenotypes for which there is considerable evidence for a genetic involvement and a consistent relationship with hypertension are alterations in the endothelium sodium channel, angiotensinogen, angiotensin converting enzyme, kallikrein levels, angiotensin II receptor and sodium-lithium countertransport (Hunt and Williams, 1994).

Regulation of blood pressure is under several levels of control in the body, involving different organs and organ systems. Breakdown or defects in blood pressure regulators can be genetic in origin or acquired. Further, environmental influences may accelerate abnormalities advancing the clinical onset of hypertension. Factors in blood pressure regulation which have been implicated in the development of essential hypertension, either individually, or acting in concert with other defects include: abnormalities of the central nervous system (Brody et al., 1991), baroreceptors (Zanchetti and Mancia, 1991), adrenal glands (Ely et al., 1997), kidneys (Guyton, 1991b) and blood vessels (Safar and London, 1987; Martin et al., 1998). Essential hypertension is therefore best described as a syndrome, rather than a single disease entity, as varying combinations of abnormalities in the above blood pressure regulators can produce subsets or subpopulations of hypertensives, each with differing pathophysiology, but all sharing the common physical finding of elevated blood pressure (Lilly, 1993).

C. *Animal models of experimental hypertension*

As discussed above, the etiology of hypertension is heterogeneous, existing as primary (essential) hypertension and secondary forms. Further, the pathophysiology of essential hypertension is also heterogeneous resulting in subsets or subpopulations of hypertensives which vary according to renin status, sympathetic nervous system function, etc. As a result, a number of animal models of experimental hypertension have been developed to further our understanding of both primary and secondary hypertension. Experimental hypertension can be produced by genetic, renal, adrenal and neural methods, and by the use of molecular biologic techniques.

1. *Genetic models of experimental hypertension*

Genetic models of experimental hypertension share one thing in common, that being they all show the spontaneous development of elevated blood pressure with aging. These models may differ, however, in genetics, cellular changes or neurohumoral mechanisms (Kurtz et al., 1994). Therefore, whereas the investigator-imposed initiating event is clear in other forms of experimental hypertension such as mineralocorticoid and renal experimental hypertension, the specific abnormality or abnormalities that initiates genetic hypertension are still a matter of conjecture and differ in the various genetic strains of rats (Bohr and Dominiczak, 1991).

Genetic models of experimental hypertension arose from selective

inbreeding of normotensive strains of rats. The most commonly studied strain of genetically hypertensive rat is the spontaneous hypertensive rat (SHR) which was generated from the Wistar-Kyoto normotensive strain (Okamoto and Aoki, 1963). The SHR is a normal-renin model being relatively resistant to changes in sodium intake during the development stages of hypertension, however, blood pressure in established hypertension is augmented by salt loading (Yamori et al., 1981). Although exchangeable sodium and extracellular fluid volume are not elevated in SHR, there is evidence for increased mean circulatory filling pressure (MCFP) which is associated with an increased cardiac output (CO) noted early in the development of hypertension in SHR (Trippodo et al., 1981). MCFP represents the pressure for venous blood return to the heart, and is an index of whole body integrated venomotor tone and blood volume (Yamamoto et al., 1980; Guyton, 1991a). As hypertension progresses in the SHR model, increases in total peripheral resistance (TPR) and MCFP are maintained compared to normotensive Wistar-Kyoto rats (Martin et al., 1998).

2. *Renal models of experimental hypertension*

Hypertension of renal etiology is the most common form of human secondary hypertension (Lilly, 1993). Therefore, renal models of experimental hypertension may best represent animal counterparts to secondary forms of hypertension in humans. In 1934, Goldblatt and his colleagues pioneered the development of a renal artery stenosis model of experimental hypertension, notably the two-kidney,

one-clip (2K1C) and the one-kidney, one-clip (1K1C) renovascular models of experimental hypertension (Goldblatt et al., 1934). In the 2K1C model of hypertension, studies show that an elevation of blood pressure depends on an increased TPR with a reduction in CO while the 1K1C model shows an increase in TPR with a small increase in CO (Thurston, 1994). Both models show elevations in MCFP (Yamamoto et al., 1981; Edmunds et al., 1989). Studies also show that peripheral renin activity and renal-renin levels in the clipped kidney (silver clips placed around the renal artery producing stenosis) are significantly decreased when a contralateral nephrectomy is performed (1K1C), but elevated when the contralateral kidney is left in place (2K1C) (Regol et al., 1962; Koletsky et al., 1967). Both models have been developed and used for study in several animal species, however, the rat and the dog are most commonly studied. Soon after Goldblatt's initial work others showed that persistent hypertension could be achieved by coarctation of the aorta between the renal arteries or by wrapping both kidneys in a cellophane wrap creating a perinephric hypertension. Other renal models of experimental hypertension include the reduced renal mass salt-sensitive model of renal parenchymal hypertension and the angiotensin-salt model of experimental hypertension.

3. *Adrenal models of experimental hypertension*

Excess production of mineralocorticoids, glucocorticoids or catecholamines results in elevation in blood pressure. Excess catecholamine production by the

adrenal medulla resulting in secondary hypertension is seen in the clinical disease pheochromocytoma. Excess glucocorticoid production (cortisol, corticosterone) as seen in Cushing's syndrome also produces a modest hypertension probably by a multifactorial mechanism (Kenyon and Morton, 1994). Perhaps the most common adrenal model of experimental hypertension studied is the deoxycorticosterone-salt model which most closely resembles Conn's syndrome in humans. Typically, this experimental model of hypertension is produced by surgical uniphrectomy followed by administration (implants or injection) of excess mineralocorticoids (deoxycorticosterone versus aldosterone) and salt (diet versus drinking water). Deoxycorticosterone derivatives containing acetate and pivalate are preferred based on their longer half-lives. Mineralocorticoids, acting through renal type 1 adrenocorticosteroid receptors in the collecting tubule of the nephron, enhance permeation of renal tubules to sodium ions via an amiloride-sensitive Na^+-H^+ exchanger and activation of a serosal sodium pump (Garty, 1986). This model is therefore a renin-independent, salt-sensitive form of experimental hypertension. Blood pressure rises into the hypertensive range within a few weeks in deoxycorticosterone acetate-salt (DOCA-salt) hypertensive rats, and if left unchecked will become malignant resulting in weight loss and end-organ damage. Mineralocorticoid effects also include potentiation of vasoconstrictors both peripherally and centrally (Kenyon and Morton, 1994).

4. *Neural models of experimental hypertension*

The central nervous system (CNS) plays a key role in the maintenance of blood pressure and hence is implicated in the pathophysiology of hypertension. Although many of the pathological conditions associated with hypertension occur in the periphery, growing evidence suggests that the CNS responds to these peripheral events via humoral and afferent inputs (Brody et al., 1991). The involvement of the CNS in the expression of other forms of experimental hypertension is clear, however, several specifically neural models of experimental hypertension also exist. These models can be genetic in origin eg. stroke-prone spontaneous hypertension rat, or non-genetic neural models of experimental hypertension which usually involve surgical manipulations of critical CNS centers such as the periventricular region, nucleus tractus solitarus or the rostral ventrolateral medulla. Hypertension studies examining the peripheral nervous system often employ sinoatrial denervation (baroreceptor removal) or ganglion blockade as a model.

5. *Other models of experimental hypertension*

Recently, a great deal of interest has been generated in molecular techniques and their application to hypertension research. The use of antisense oligonucleotides targeted to knock out key mRNA and the use of whole animal transgenic technology are two examples. One of the major limitations of existing genetic models of hypertension is that the hypertensive strains differ from their

normotensive counterpart strains throughout several regions of their genomes besides those regions containing genes regulating blood pressure (St. Lezin et al., 1992). Therefore, researchers have begun to develop recombinant inbred strains and congenic strains from existing models of experimental hypertension which offer the advantage of being able to identify critical chromosome segments and hence genetic loci affecting blood pressure (St. Lezin et al., 1991).

D. *Hemodynamics of essential hypertension*

The major determinants of blood pressure are CO and TPR. Therefore alterations in blood pressure must involve changes in these functional parameters. Most hemodynamic studies perfomed in young mild or borderline human essential hypertensives have described a high CO in the presence of a normal TPR in the initial stages of hypertension development (Lund-Johansen, 1994). Findings in human studies are supported by similar results in studies using animal models of experimental hypertension in the rat, notably the renovascular models and the SHR model (Ledingham et al., 1963; Smith and Hutchins, 1979). Both human and animal studies show that with time and establishment of hypertension, however, that the contribution made by CO to chronic elevation in blood pressure is reduced while TPR contributions increase (Lund-Johansen, 1994). Chronic hypertension is therefore marked by elevated resistance to blood flow through small arteries in most vascular beds (Folkow, 1982). Another virtually invariable finding in established hypertension is decreased capacitance of the venous system (Safar and London,

1985; 1987). A reduction in venous capacitance has therefore been implicated in the etiology of hypertension (Safar and London, 1985; Edmunds et al., 1989).

It is not clear whether increases in venous tone participate in hypertension development or are compensatory mechanisms in established hypertension serving to facilitate cardiac filling and thereby maintain CO in the face of decreased ventricular compliance and increased resistance to ventricular ejection (Folkow, 1982; Safar and London, 1987). In humans with essential hypertension venous tone has been reported to be elevated early in development of hypertension and remain elevated into established hypertension (Safar and London, 1985; Schobel et al., 1993). Similar findings have been demonstrated in SHR and Goldblatt 1K1C renovascular hypertensive rats (Yamamoto et al., 1981; Martin et al., 1998). In DOCA-salt hypertensive rats, MCFP was not elevated until the fifth week of treatment suggesting that elevated venous tone may not be involved in the developmental stages but may be important in the later stages of this form of hypertension (Yamamoto et al., 1983). Therefore, early increases in venous tone resulting in enhancement of CO may be an important initiating factor in some forms of human and animal hypertension while increases in venous tone occurring in later stages of hypertension may be important in disease progression and maintenance of elevated blood pressure.

II. *Veins and the venous system*

It is now well recognized that veins are not purely passive conduits for blood flow. Important physiological functions related to the veins include regulated capacity functions, maintenance of the filling pressure of the heart and synthesis of biologically active substances in the vascular wall (Monos et al., 1995). The venules also play an important role in controlling the transport processes between the interstitial and the intravascular space, and as postcapillary flow resistors (Grega et al., 1988).

It has been established that 60-80% of the circulating blood volume is found in the systemic veins and venules, particularly vessels with a caliber <400 μm (Hainsworth, 1986). Veins are some 30-60 times more compliant than arteries. Therefore, the venous system, particularly the critical splanchnic circulation, provides a significant reservoir or capacitance function for the body (Monos et al., 1995). Multiple factors regulate vascular capacitance including the structure of veins, blood volume and venous smooth muscle activity (venomotor tone) (Introduction, Figure 1.1). Because veins have limits of distensibility, increases in venous blood volume can have significant effects on cardiac filling pressure (preload) and blood pressure. Further, direct sympathetic nervous system innervation of veins and circulating and locally produced vasoactive hormones also affect preload and blood pressure through their actions on the venous smooth muscle, and resultant venomotor tone. Current dogma is that sympathetic nerves have the greatest quantitative importance in minute-to-minute regulation of

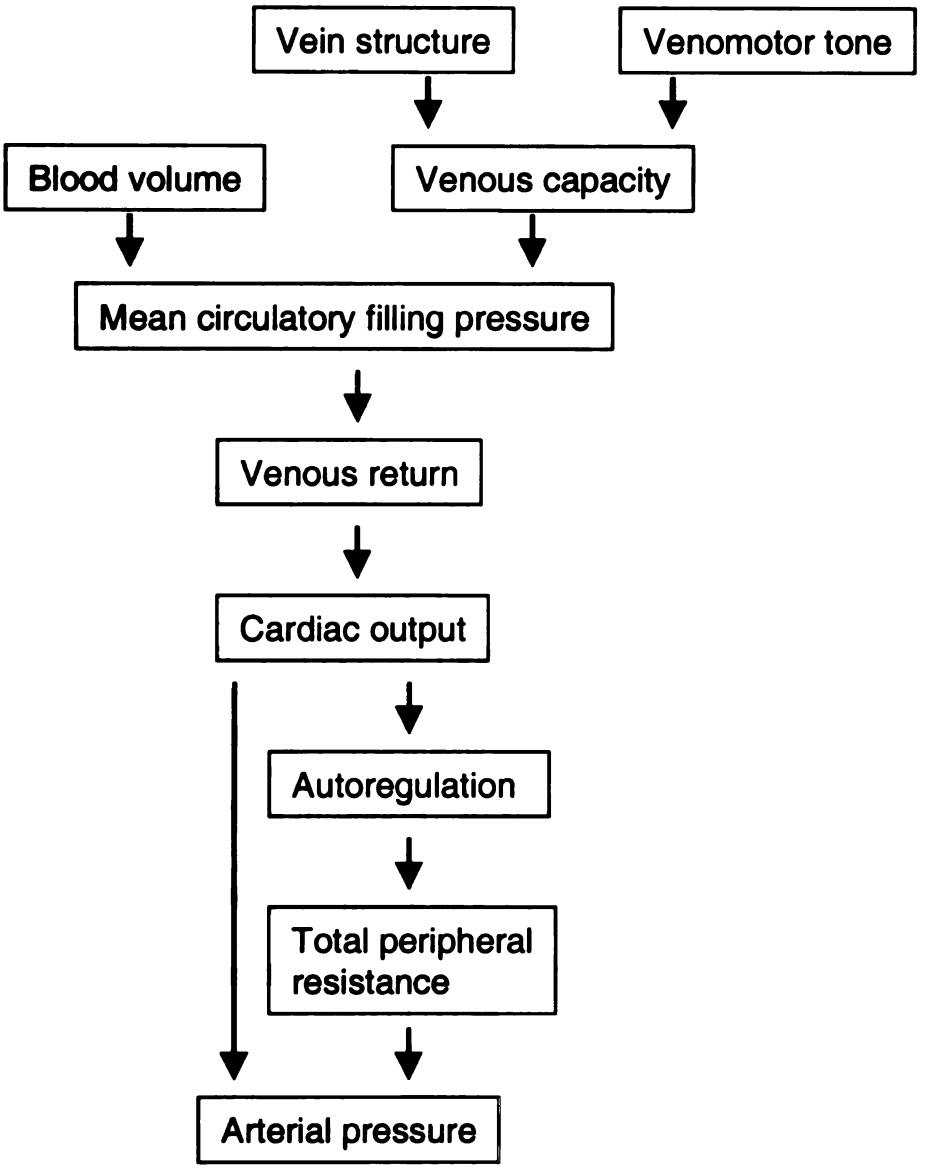

Figure 1.1: Relationship between systemic filling pressure and arterial pressure.

venomotor tone (Shoukas and Bohlen, 1990; Monos et al., 1995). Therefore, the capacitance function of the venous system and resultant pressure for blood return to the heart are regulated directly by venous wall properties, venous blood volume and the prevailing venomotor tone. The pressure for venous blood return to the heart which has already been defined as MCFP can be determined experimentally as the systemic pressure obtained after brief circulatory arrest and immediate equilibration of arterial and venous pressures (Guyton, 1991a). Venous return to the right heart dynamically maintains cardiac filling pressure, providing adaptation to changing CO requirements. An increase in MCFP results in shifting of blood away from the peripheral vascular beds towards the heart increasing right atrial filling and pressure during diastole. This results in increased myocardial stretch and a more forceful contraction (Frank-Starling mechanism). In this way augmented MCFP leads to higher stroke volume and CO. The MCFP may then characterize this steady-state venocardiac coupling, and makes clear the importance of veins in regulating CO and circulatory homeostasis (Rothe, 1993).

III. *Vascular endothelin system*

A. *Endothelin isopeptide family*

The endothelin (ET) family currently consists of three ET isoforms (ET-1, ET-2 and ET-3) encoded by three distinct genes (Inoue et al., 1989a) and four highly homologous cardiotoxic peptides known as the sarafotoxins (S6a, S6b, S6c and

S6d) which can be isolated from the venom of certain snakes (Takasaki et al., 1988; Landan et al., 1991). Endothelin family members contain 21 amino acids with free carboxy and amino termini, and two intrachain disulfide (cys^1-cys^{15} and cys^3-cys^{11}) bridges which generate a loop-like structure to the peptides (Yanagisawa et al., 1988a). ET-1 binding sites as well as functional responses have been demonstrated in several lower vertebrates including amphibians, fish and reptiles suggesting that ETs have been conserved during evolution in vertebrates (Zigdon-Arad et al., 1992).

B. *Biosynthesis and secretion of endothelins*

Isoforms of ETs are produced in many tissues and cell types (Rubanyi and Polokoff, 1994). Regarding the cardiovascular system, endothelial cells of either venous (Inoue et al., 1988a; 1988b) or arterial (Dohi et al., 1992) origin appear to exclusively produce ET-1. Some studies have shown that vascular smooth muscle cells (VSMC) are also capable of producing ET-1 (Resink et al., 1990). Release of ET-1 by cultured endothelial cells has been shown to occur preferentially toward the basal side implying that endothelins act on endothelial cells and underlying smooth muscle in a local autocrine and paracrine manner (Wagner et al., 1992). Support for this comes from studies showing plasma ET-1 concentrations in healthy individuals which are too low to produce vasoconstriction based on results of ET-1 vascular contraction studies in vitro (La and Reid, 1995). Regulation of ET-1 synthesis is thought to occur largely at the level of gene transcription, with de novo

peptide production and release occurring in response to endothelial cell stimulation from cytokines and growth factors, vasoactive substances, hypoxia and low shear stresses (Rubanyi and Polokoff, 1994). Although ET-1 can be identified within endothelial cells, it remains unclear whether intracellularly stored peptide is an important source for rapid hormone release (Webb and Strachan, 1998).

In endothelial cells, ET-1 is first expressed as a precursor peptide known as prepro-ET-1 (203 to 212 amino acids depending on species) (Yanagisawa and Masaki, 1989). Prepro-ET-1 is converted to an intermediate form called bigET-1 (proET-1; 38 or 39 amino acids depending on species) by proteolytic cleavage from dibasic pair-specific endopeptidases and carboxypeptidases. The final processing step involves conversion of bigET-1 to the biologically active form, mature ET-1 (21 amino acids), by the endothelin converting enzyme (ECE) (Yanagisawa et al., 1988b; Takahashi et al., 1993). The most physiologically important ECE in vivo appears to be the endothelial membrane-bound form which is a neutral metalloproteinase belonging to the same enzyme family as angiotensin-converting enzyme (ACE) (Opgenorth et al., 1992). Recent studies, however, show that VSMC's isolated from human umbilical vein also possess ECE activity (Maguire et al., 1997).

C. *Endothelin receptor subtypes*

Autoradiographical studies have shown that distribution of [^{125}I]ET-1 binding sites is widespread with regards to species and major organ sites (Koseki et al.,

1989). The existence of multiple ET binding sites was verified subsequent to cloning of similar, yet distinct, G-protein-coupled seven-transmembrane receptors from bovine and rat lung cDNA libraries, notably the ET_A (Arai et al., 1990) and ET_B (Sakurai et al., 1990) receptor subtypes, respectively. Functional studies, however, suggest that the ET_B receptor in blood vessels may be further subtyped into a putative ET_{B1} and ET_{B2} receptor, but to date no support from molecular studies exists that these are distinct proteins (Douglas et al., 1995). Finally, a proposed ET_C receptor has been identified and cloned from the dermal melanophores of *Xenopus laevis* (Karne et al., 1993). Currently, no mammalian homolog for this receptor subtype has been identified.

Both the ET_A and ET_B receptors are found in vascular tissue. The ET_A receptor can be located to the vascular smooth muscle (VSM) where it mediates vasoconstriction with a rank order agonist potency of ET-1>ET-3 (Moreland et al., 1992). The ET_{B1} receptor is found on the vascular endothelium where it mediates release of vasodilator substances such as the nitric oxide synthase (NOS) product nitric oxide (NO), the cyclooxygenase product prostacyclin (PGI_2) and possibly an as yet uncharacterized endothelial-derived hyperpolarizing factor (EDHF) (Fozard and Part, 1992; Nakashima and Vanhoutte, 1993). Activation of the ET_{B1} receptor may also result in the generation of vasoconstrictors such as thromboxane (TxA_2) (Ito et al., 1991; Spatz et al., 1993). The ET_{B2} receptor (contractile ET_B receptor), like the ET_A receptor, is also found on the VSM in certain vascular beds where it mediates vasoconstriction (Sumner et al., 1992). The ET_B receptor (ET_{B1} and ET_{B2}

receptor) shows equal affinity for all endothelin isoforms (Sakurai et al., 1990). Further subtypes of contractile ET_A and ET_B receptors have been proposed based on differing ET binding characteristics, making the exact role of the different endothelin receptor subtypes in VSM unclear (Karaki et al., 1994). In addition, several studies have proposed the presence of ET_A receptors on cultured endothelial cells obtained from brain microvessels and aorta, however, their role is not known (N'Diaye et al., 1997; Spatz et al., 1997).

The distribution of ET receptor subtypes in vascular tissue varies considerably with animal species and between vascular beds. However, vasoconstrictor responses to ET-1 in arteries are largely mediated by ET_A receptors (Moreland et al., 1992; Sumner et al., 1992; Cardell et al., 1993). In contrast, in veins, contractions to ET-1 involve the ET_{B2} receptor (Moreland et al., 1992; Sumner et al., 1992). It has therefore been postulated that the ET_A receptor is the major subtype mediating vasoconstriction in the arterial or high pressure side of the cardiovascular system, while contractile ET_B receptors are located principally in low pressure systems such as the pulmonary circulation and venous system (Moreland et al., 1994; Davenport et al., 1995).

Characterization of ET receptor subtypes in blood vessels has been accomplished with ET receptor subtype specific agonists and antagonists. ET-1 is a mixed or non-selective agonist for all ET receptor subtypes and shows similar affinities for the ET_A and ET_B receptors; however, ET-1 does show significantly greater affinity (100 fold) than ET-3 at the ET_A receptor (Moreland et al., 1992).

Currently, there are no selective ET_A receptor agonists. Several selective ET_B receptor agonists are available with the most reliable being S6c (Williams et al., 1991). Other selective ET_B peptide agonists include 4Ala-ET-1 (10-21), BQ-3020 and IRL-1620 (Rubanyi and Polokoff, 1994). High affinity selective antagonists (K_d= pM-nM) for ET_A and ET_B receptors are currently available (Polokoff and Rubanyi, 1994). Endothelin receptor antagonists are classified as competitive and reversible, but, like ET receptor agonists, their association with ET receptors is slow (Webb and Strachan, 1998). Further, ET agonists and antagonists dissociate from receptor sites very slowly making their binding more appropriately classified as pseudoirreversible (Waggoner et al., 1992).

D. *Endothelin signal transduction mechanisms*

ET-mediated responses in blood vessels are accomplished through the production of numerous second messengers including inositol phosphates, diacylglycerol (DAG), arachidonic acid (AA), intracellular calcium ($[Ca^{2+}]_i$) and the cyclic nucleotides; cyclic adenosine monophosphate (cAMP) and cyclic guanlyl monophosphate (cGMP). In turn, these second messengers result in vascular responses through activation of kinases and phosphatases. Currently, there is no general consensus regarding differences between ET_A and ET_B receptor-mediated signaling mechanisms in vascular tissues (Douglas and Ohlstein, 1997). This statement must be tempered, however, by the paucity of data examining ET-receptor subtypes and their signal transduction pathways. Also, limited studies

have examined ET-signaling pathways using vessels of venous origin, while virtually no studies have employed VSMC's isolated from veins for study.

1. *VSM signal transduction*

In vascular smooth muscle, ET-1 binding to ET_A and ET_B receptors activates phospholipase C (PLC) which in turn hydrolyzes phosphatidyl inositol to form the second messengers inositol 1,4,5-trisphosphate (IP_3) and DAG resulting in Ca^{2+} release from intracellular calcium stores and protein kinase C (PKC) activation, respectively (Rubanyi and Polokoff, 1994). ET-1 also stimulates phospholipase D (PLD) and phospholipase A_2 (PLA_2) activity which lead to a sustained production of DAG and the generation of cyclooxygenase products (PGH_2 ,PGI_2 and TxA_2), respectively (La and Reid, 1995; Fleming et al., 1997).

The use of fluorescent indicator dyes (eg. Fura-2, Quin-2) and calcium isotopes ($^{45}Ca^{2+}$) has demonstrated that ET-induced contraction of VSM is accompanied by an increase in $[Ca^{2+}]_i$ (Sudjarwo et al., 1995; Wallnofer et al., 1989). In most preparations the rise in $[Ca^{2+}]_i$ consists of an IP_3 generated rapid, transient, initial spike in $[Ca^{2+}]_i$ which is due to release of Ca^{2+} from intracellular calcium stores, and a secondary sustained phase of $[Ca^{2+}]_i$ rise which is dependent on the transmembrane influx of calcium through membrane channels (Rubanyi and Polokoff, 1994). Complete understanding of the ion channels through which extracellular calcium enters the VSMC is presently lacking. Further, studies examining ET-receptor subtypes and contributions from extracellular calcium influx

versus intracellular calcium release from intracellular stores are currently lacking.

Typically, the production of second messengers such as those mentioned result in the rapid or short term actions of ETs, namely vasoconstriction. ETs are also capable of long term or nuclear control of cellular proliferation and differentiation within the VSM. ET-1 induced activation of nuclear signaling involves various protein tyrosine kinases such as the mitogen activated protein kinases (MAPK), specifically the extracellular signal-regulated kinase (ERK) subfamily of MAPK's (Wang et al., 1993). Long term signaling by ETs in blood vessels are thought to be important in physiological and pathophysiological remodeling of arteries, however, studies in veins are lacking (Schiffrin, 1995a).

2. Endothelium signal transduction

Signal transduction by ETs in endothelial cells appears to rely on similar pathways to the VSMC in achieving ET-mediated effects. Activation of endothelial ET_{B1} receptors results in the generation of PLC and mobilization of intracellular calcium stores as well as the activation of cation channels (Fleming et al., 1997; Hirata et al., 1993). The nature of the membrane channels mediating calcium influx are unclear. Endothelial cells lack voltage-operated Ca^{2+} channels which leaves receptor-operated, second messenger or G-protein coupled channels as possibilities for calcium influx (Wang and Breeman, 1997). ET-induced activation of endothelial NOS relies largely on Ca^{2+}-calmodulin while the generation of the cyclooxygenase products PGI_2 and TxA_2, and possibly EDHF, appear to rely on

PLA_2 production as the rate limiting step (Fleming et al., 1997). NO and PGI_2 liberation by endothelial cells result in VSM relaxation through increases in VSMC cGMP and cAMP levels, respectively (Shimakowa and Vanhoutte, 1997). Increases in NO also negatively regulate the production of ET-1 by increases in endothelial cGMP levels (Boulanger and Luscher, 1990; La and Reid, 1995).

E. *Cardiovascular actions of endothelins*

1. *ETs and vasomodulation*

ETs exert a wide range of biological actions both cardiovascular and noncardiovascular in origin (Rubanyi and Polokoff, 1994). Classically, ETs are viewed as potent vasomodulatory peptides. In the inital in vitro studies leading to the discovery of ET-1, the concentration of ET-1 producing half-maximal contraction (EC_{50}) in porcine coronary artery was reported to be $3x\ 10^{-10}$ M making ET-1 approximately 100-fold more potent than angiotensin II, which was previously the most potent endogenous vasoconstrictive peptide known (Yanagisawa, 1988b). These findings raised the natural question of a physiological role for ETs in blood pressure maintenance. An ET-dependent vascular tone has been demonstrated in healthy human subjects by their response to the acute intravenous (i.v.) administration of the mixed ET_A/ET_B receptor antagonist TAK-044 which lowered systemic blood pressure slightly and increased forearm blood flow (Haynes et al., 1996). However, unequivocal proof of a physiological role for ETs in blood

pressure maintenance is currently lacking.

Intravenous infusion of ET-1 in vivo results in a biphasic response in blood pressure consisting of an initial transient decrease followed by a sustained increase in arterial pressure (Yanagisawa et al., 1988a, 1988b; DeNucci et al., 1988). The inital depressor response is due to vasodilation as a result of release of endothelial-derived dilator substances such as NO, PGI_2 and possibly EDHF (Nakashima andVanhoutte, 1993; DeNucci et al., 1988). Suppport for this comes from in vitro ET studies where removal of the endothelium or application of the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) and the cyclooxygenase inhibitor indomethacin in combination, rather than individually, abolishes ET-induced vasodilation and thus suggests that ET-1 mediated relaxation is due to both NO and cyclooxygenase products derived from the endothelium (Mehta et al., 1992). The pressor response is a result of direct activation of contractile ET_A and/or ET_B receptors on the VSM (DeNucci et al., 1988; Takase et al., 1995) with possible contributions from endothelium-derived production of TxA_2 and reduction in dilator release (Armstead et al., 1989). The transient vasodilation to ETs has been shown to preferentially occur at low doses of ET-1 (10^{-11} to 10^{-9} M) while higher concentrations result in contraction or pressor responses (Mehta et al., 1992). It has been suggested therefore that endothelial ET receptors have a higher sensitivity to ET-1 compared to VSMC's, which may be the result of a post-receptor event affecting efficacy, as both the ET_A and ET_B receptors have similar affinities for ET-1 (Mehta et al., 1992). Nevertheless, it is likely that the net result of ETs on vessel tone will depend on the

concentration of ET in the vascular bed and the distribution and density of ET receptor subtypes present (Rubanyi and Polokoff, 1994).

Vasoconstriction to ETs has been studied in many vascular beds both in vivo and in vitro from several animal species, including humans. Although the majority of studies have been performed in arteries, several studies have examined the effects of ETs in veins. Studies in vivo show that i.v. administration of ETs increase central venous pressure (CVP), venous resistance and MCFP (Waite and Pang, 1990; 1992; Yamamoto et al., 1980). Studies in vitro consistently show that efficacy (ie. maximal responses) and potency (EC_{50} values) of ET-induced contractions in veins are greater compared with results from corresponding arteries (Cocks et al., 1989; Haynes et al., 1991; Riezebos et al., 1994). Intravenous infusion of ET-1 (5 pmol/min) into human forearm vessels in vivo also resulted in significantly enhanced contractions in dorsal hand veins versus corresponding arteries (Haynes et al., 1991). Taken together these findings suggest that veins are more sensitive to the contractile effects of ETs than arteries, possibly due to differences in ET receptor subtypes, receptor density, receptor signaling mechanisms or ET-1 production levels in veins compared to arteries.

2. *Peripheral nervous system effects of ETs*

Experimental evidence suggests that ET-1 potentiates the actions of norepinephrine (NE) on postjunctional adrenergic VSM sites while inhibiting NE release from prejunctional sympathetic nerve terminals found on the VSM. Less is

known regarding the effects of ETs on parasympathetic neurons or neuroeffector mechanisms, but studies suggest that ETs also inhibit presynaptic release of neurotransmitter from cholinergic nerves (Wiklund et al., 1989). In isolated perfused rat mesenteric arteries, infusion of ET-1 (1pM to 100 pM) attenuated vasoconstriction to periarterial nerve stimulation and inhibited [^{3}H]-NE release in a dose-dependent manner (Nakamura et al., 1989). However, in the same study higher doses of ET-1 (300 pM to 10 nM) enhanced the pressor response to nerve stimulation suggesting that low ET-1 concentrations acted prejunctionally to inhibit sympathetic neurotransmission while higher concentrations of ET-1 acted postjunctionally to potentiate noradrenergic transmission. ET-1 (1 nM) superfused in isolated canine mesenteric veins enhanced contractile responses to the selective alpha$_2$-adrenoceptor agonist UK-14304, an effect which was blocked by the selective alpha$_2$-adrenoceptor antagonist rauwolscine (100 nM), but was not sensitive to the selective alpha$_1$-adrenoceptor antagonist prazosin (100 nM) (Shimamoto et al., 1992). The authors concluded that ET-1 enhanced responses to UK-14304 through amplification of postjunctional alpha$_2$-adrenoceptor-mediated responses. Using isolated rat tail artery in vitro, superfusion of ET-1 (1-30 nM) or S6c (1-300 pM) significantly reduced NE and ATP overflow to electric field stimulation, an effect which was blocked by the selective ET_B antagonist BQ-788 (Mutafova-Yambolieva and Westfall, 1997). These findings suggest that the ET_B receptor may be important in ET-1 presynaptic neuromodulation of sympathetic tone to the blood vessels.

In conscious instrumented rats, cumulative i.v. bolus of ET-1 (80 pmol/kg to 1.94 nmol/kg) reduced heart rate (HR) and increased mean arterial pressure (MAP) and MCFP in a dose-dependent manner (Waite and Pang, 1992). In the presence of either hexamethonium or phentolamine ET-1 effects on MCFP were abolished suggesting ET-1 elevated venous tone through an increase in sympathetic nerve activity and the activation of alpha adrenoceptors (Waite and Pang, 1992). These findings suggest that hemodynamic responses following ET-1 administration can involve alterations in sympathetic tone to blood vessels.

3. *Central nervous system effects of ETs*

Experimental evidence suggests that ETs are localized in the CNS, and modulate several CNS functions, however ETs do not cross the blood brain barrier (Kadel et al., 1990). Therefore, ETs may also be regarded as neuropeptides. Using RNA blot hybridization, the hypothalamus and striatum of human brain were found to have the highest density of cells expressing preproET-1 mRNA (Lee et al., 1990). Radiolabeling of specific ET-1 binding sites with ^{125}I-ET-1 in human brain revealed the highest levels of labeling in the dentate gyrus, cerebellum, caudate and temporal cortex with lower levels of labeling occurring in the spinal cord (Jones et al., 1989). Quantitative receptor autoradiography, also using ^{125}I-ET-1, revealed high concentrations of specific binding in rat brain nuclei that are involved in CNS regulation of the cardiovascular system including nuclei of the anteroventral hypothalamus, the subfornical organ and the supraoptic nuclei (Calvo et al., 1990;

Banasik et al., 1991).

The systemic and regional circulatory effects of centrally administered ET-1 have been shown to be mediated through the ET_A receptor (Gulati et al., 1992; Rebello et al., 1995). Intracerebroventicular microinjection of ET-1 (30 pmol/kg) in rats produced significant pressor as well as vasoconstrictor responses (Siren and Feurerstein, 1989). Microinjection of ET-1 into the area postrema (Ferguson and Smith, 1990), the cerebral ventricles of conscious rats (Kawano et al., 1989; Yamamoto et al., 1991) and conscious rabbits (Matsumura et al., 1991) resulted in similar findings. These same studies revealed parallel increases in plasma arginine vasopressin (AVP), adrenocorticotropic hormone, catecholamines and renal sympathetic nerve activity along with increases in mean arterial blood pressure following microinjection of ET-1. Further, ganglion blockade (eg. hexamethonium) or the administration of a V_1-vasopressin receptor antagonist (eg. TMe-AVP) or an α1-adrenergic receptor antagonist (eg. prazosin) attenuated or completely blocked the observed pressor response (Kawano et al., 1989; Yamamoto et al., 1991; Matsumura et al., 1991). These findings suggested that central administration of ET-1 results in sympathoadrenal and AVP systems activation which mediate the central pressor response observed. Finally, systemic ET-1 (50-100 pmol) administration to anesthetized rats activated vasopressin and oxytocin secreting neurons in the supraoptic and paraventricular nucleus as well as activated subfornical neurons which have projections to the paraventricular nucleus of the hypothalamus (Wall and Ferguson, 1992). In the same study, ET-1

had minimal effects on vasopressin and oxytocin releasing neurons in rats in which the subfornical organ was lesioned, suggesting systemic ET-1, which does not cross the blood brain barrier and therefore could not act directly on paraventricular and supraoptic neurons, acted on these neurons via the subfornical organ which lacks a blood brain barrier.

4. *Cardiac effects of ETs*

In human heart tissue sections, using in situ hybridization and radiolabeling with ^{125}I-ET-1 and selective ET receptor antagonists, ET_A and ET_B mRNA and receptors have been localized to the atrial and ventricular myocardium, the conducting system and endocardial cells (Molenaar et al., 1993). The direct actions of ETs on the heart include action potential prolongation and positive inotropic and chronotropic effects. The ET_A receptor subtype appears to mediate the majority of the effects of ET-1 on the rat myocardium (Hilal-Dandan et al., 1994), however, studies examining ET receptor subtype contributions to ET-1 actions on the heart are currently lacking.

Much of the effects of ETs on heart rate have been reported following systemic administration of ET-1 in vivo to intact animals, leaving interpretation of results challenging due to reflex effects of sympathetics on the heart and vasculature. However, ET-1 did cause a dose-dependent increase in rate of beating of isolated, spontaneously beating guinea pig atrial myocytes (Ishikawa et al., 1988). Positive inotropic effects of ET-1 as evidenced by an increased extent

of ventricular cardiomyocyte shortening have been demonstrated in rat (Kelly et al., 1990) and human (Qiu et al., 1992) isolated ventricular myocytes and rat isolated perfused hearts (Firth et al., 1990). The positive inotropic effects of ET-1 are associated with prolongation of the total duration of contraction and retardation of relaxation which is also characteristic of α1-adrenoceptor-mediated modulation of isometric contractions (Takanashi and Endoh, 1991). ETs effects on the electrical properties of the myocardium may account for the peptide's proarrhythmic actions which can be observed following intracoronary or systemic administration, or in vitro (Burrel et al., 2000).

IV. *Endothelins and hypertension*

A. *Endothelins and animal models of experimental hypertension*

To propose a role for ETs in the pathophysiology of hypertension it is necessary to demonstrate either that the levels of the peptide are enhanced (increased production or reduced degradation), or that ET-induced vasoconstrictor responses are potentiated. Most importantly selective ET receptor antagonists should lower arterial blood pressure in hypertensive animals (or humans). Several models of experimental hypertension have been employed to study the potential role of ETs in hypertension. Based on findings in these studies it can be concluded that the endothelin system is activated particularly in the salt-sensitive forms of experimental hypertension (Schiffrin, 1999).

1. *ETs and salt-sensitivity in hypertension*

Subsets of essential hypertensive humans show a salt-related progression of disease (Tobian, 1991). Further, it has been shown that reduction in dietary salt can significantly lower both systolic and diastolic blood pressure in some essential hypertensives (Taubes, 2000), hence the term "salt-sensitive hypertension". Several forms of salt-dependent experimental hypertension such as the 1K1C (Sventek et al., 1996), reduced renal mass (Potter et al., 1997), DOCA-salt (Schiffrin et al., 1996) and Dahl (Kassab et al., 1997) salt-sensitive models also show ET-1 involvement in their development. Increased salt intake has been shown to be necessary to produce sustained hypertension during chronic ET-1 infusion in instrumented rats (Mortensen and Fink, 1992), but an increased salt intake does not appear to consistently alter vascular ET-1 formation (Michel et al., 1993; Ishimitsu et al., 1996; Oh et al., 1997). The relationship between salt-sensitivity and ET-1 in hypertension is, therefore, not fully understood. Nevertheless, compelling evidence suggests that a relationship does exist between ET-1 and salt intake in salt-sensitive forms of hypertension. Currently, the DOCA-salt model of experimental hypertension provides the most convincing evidence of a role for ETs in the pathophysiology of hypertension. Further discussion, therefore, will focus on that model.

2. *Vascular production of ETs in experimental hypertension*

Elevation of plasma concentrations of ET-1 in animal models of experimental hypertension, like human essential hypertension, has not been unequivocably established. Perhaps the most convincing evidence for increased plasma immunoreactive ET-1 levels in animal models of hypertension appears to be in the malignant forms of hypertension, such as those produced by DOCA-salt administration (Kohno et al., 1991). In conscious rats following i.v. administration of ET-1 (0.4 nmol) arterial plasma levels of ET-1 declined slowly when compared to normotensive controls, suggesting clearance of ETs may be altered in DOCA-salt hypertension (Yokokawa et al., 1990). Since ETs are thought to possess a paracrine mode of action, the relative importance of plasma ET-levels may be questionable (Wagner et al., 1992). In a recent study, ET-1 production in cultured endothelial cells was increased significantly in cells obtained from DOCA-salt hypertensive versus sham normotensive cells (Takada et al., 1996).

Several studies have demonstrated that vascular expression of the preproET-1 gene and ET-1 peptide production are enhanced in experimental hypertension. In the aorta and mesenteric arteries of DOCA-salt hypertensive rats, an early increase in content or levels of immunoreactive ET-1, as well as ET-1 prepromRNA abundance, was demonstrated in the vascular endothelial cells (Lariviere et al., 1993a; 1993b). The same studies showed that vascular ET-1 content was approximately 100-fold that obtained in plasma, suggesting vascular measurement of ETs may provide a better estimate of ET production than plasma.

Similar findings were shown early in 1K1C hypertensive rats, but not until later in the 2K1C model of renovascular hypertension and not at all in the SHR model (Lariviere et al., 1993a; Sventek et al., 1996). The authors concluded that enhancement of expression of the ET-1 gene in blood vessels of hypertensive rats may occur in the absence of exposure to DOCA and salt, and that ET-1 gene overexpression in experimental hypertension occurs early in non-renin-dependent volume-expanded models such as the 1K1C or the DOCA-salt hypertensive rat, but only in the progressively non-renin-dependent late phase of the initially renin-dependent volume-contracted 2K1C hypertensive rat (Sventek et al., 1996).

3. *Effects of ETs on blood vessels in experimental hypertension*

Several in vitro studies have examined the vasoconstrictor effects of ETs in blood vessels obtained from animal models of hypertension, however, these studies are limited to either conduit or resistance arteries. Results of these studies are mixed, with findings of reduced, normal and enhanced vascular responses to ETs in differing models of experimental hypertension (Vanhoutte, 1993; Rubanyi and Polokoff, 1994). In contrast, contractile responses to ET-1 in arteries from DOCA-salt hypertensive rats (Hagen and Webb, 1984; Nguyen et al., 1992; Giulumian et al., 1998) and Dahl salt-sensitive (d'Uscio et al., 1997) hypertensive rats are usually reduced compared to responses in arteries from sham normotensive rats. These findings may be explained by increased vascular ET-1 production and ET receptor down-regulation as the density of ET-receptors as well as signal transduction (IP_3

and DAG production, and calcium transients) are both reduced in arteries from DOCA-salt hypertensive rats compared to normotensive control rats (Nguyen et al., 1992; Fluckiger et al., 1992). Yet, in both the DOCA-salt and Goldblatt 2K1C renovascular models contractile-responses to ET-1 can be enhanced (De Carvalho et al., 1990). Attenuation of ET-mediated release of vasorelaxant factors may provide an explanation for the contradiction in findings between these studies (Wu and Bohr, 1990). Another explanation is that ET-mediated vascular remodeling in these vessels compensates for direct ET-1 contractile responses resulting in their augmentation when compared to normotensive controls (Schiffrin et al., 1996). ETs, as well as other vasoconstrictor peptides, possess mitogenic and hypertrophic properties (Hirata et al., 1989; Schiffrin et al., 1996). Hypertrophy of the vascular media of arteries of DOCA-salt hypertensive rats is prominent (Schiffrin, 1999). Therefore, elevations in systemic blood pressure in the DOCA-salt model could be a function of vasoconstriction and vascular hypertrophy.

Endothelins can interact with the neuroeffector junction in animal models of hypertension in addition to their direct effects on vessels. Application of subpressor doses of ET-1 (1 pM-100 pM) attenuated the pressor responses in mesenteric resistance arteries to periarterial sympathetic nerve stimulation significantly less in hypertensive SHR versus normotensive WKYsham rats (Tabuchi et al., 1990). In the same study, application of a subpressor dose of ET-1 (100 pM) enhanced the pressor response to exogenous NE, however, responses in WKY normotensive sham rats were greater than hypertensive SHR. Results contradicting the above

findings were obtained in a study using rat thoracic aorta which showed application of ET-1 (30 nM) potentiated the contractile responses to NE (1 nM-100 nM) in arteries from SHR, but not normotensive controls (Zerrouk et al., 1997). The same study also showed that this potentiation could be abolished by application of indomethacin and not Nω-nitro-L-arginine (NLA). Differences in vascular origin may explain, in part ,some of the above conflicting results. Clearly, further work is needed in this area, particularly in veins.

4. *Effects of ET receptor antagonists in experimental hypertension*

Pharmacologic antagonism of either ET_A or both ET_A and ET_B receptors with selective and non-selective ET-receptor antagonists, respectively, has been shown to slow hypertension development during DOCA-salt treatment (Schiffrin et al., 1997a), and to decrease blood pressure in rats with established DOCA-salt (Bird et al., 1995), reduced renal mass (Potter et al., 1997) or Dahl (Kassab et al., 1997) salt-sensitive hypertension. On the other hand, in a similar study early treatment with the selective ET_A receptor antagonist ABT-627 in DOCA-salt rats attenuated both systolic blood pressure elevations and vascular hypertrophy of the aorta while the selective ET_B receptor antagonist A-192621 exaggerated both systolic blood pressure and vascular remodeling (Matsumura et al., 1999). The authors concluded that selective ET_A receptor antagonism, but not selective ET_B receptor blockade, may be useful in the treatment in hypertension. Support for a role for the

ET_A receptor in DOCA-salt hypertension also comes from studies showing improvement in coronary vascular function in DOCA-salt hypertensive rats treated with the selective ET_A receptor antagonist A-127722 (Giulumian et al., 1998). In other models of experimental hypertension, namely hypertensive 1K1C and 2K1C rats or young (4 week old) and adult SHR rats, chronic oral treatment with the ET_A/ET_B receptor antagonist bosentan resulted in no significant changes in blood pressure or vascular hypertrophy or remodeling (Li et al., 1996; Li and Schiffrin 1995a; 1995b). These results suggest vascular hypertrophy and remodeling may be important in DOCA-salt hypertension development, but not in SHR hypertension development (Sciffrin et al., 1996).

B. *Endothelins and human essential hypertension*

Endothelins have been implicated in several cardiovascular diseases, including essential hypertension, pulmonary hypertension, hypertension of gestational origin, coronary restenosis, atherosclerosis, congestive heart failure (CHF), myocardial ischemia, subarachnoid hemorrhage, diabetic vasculopathy, migraine, endotoxic shock, cerebral vasospasm and Raynaud's phenomenon (Rubanyi and Polokoff, 1994). ET plasma levels are usually normal in hypertensive humans (Davenport et al., 1990; Kohno et al., 1990), however, some studies show circulating levels of ET-1 are increased (2 to 10-fold) in several cardiovascular diseases including CHF (Battistini et al., 1993) , severely hypertensive patients and in black hypertensives (Ergul et al., 1996). Salt-sensitive hypertensive patients

often have low plasma renin activity and demonstrate an exaggerated rise in plasma ET levels in association with elevated plasma catecholamines following sodium depletion (Elijovich et al., 1999). This suggests a relationship between the sympathetic system, salt-sensitivity, and the reactivity of the endothelin system that may contribute to elevated blood pressure in these individuals (Schiffrin, 1999).

Molecular and functional studies have provided further support of a role for ETs in the pathophysiology of essential hypertension. Using in situ hybridization with ET-1 RNA antisense probes, Schiffrin showed significant labeling in endothelial cells of small arteries (obtained from gluteal subcutaneous fat) from moderate to severe esssential hypertensives when compared to normotensive controls (Schiffrin et al., 1997b). The authors concluded that overexpression of the ET-1 gene could play a role in blood pressure elevation and perhaps in the pathogenesis of vascular hypertrophy.

Changes in ET-1 plasma levels, mRNA expression, ET-receptor subtypes or density, or functional responses to ETs in specific cardiovascular disease development do not establish a cause-effect relationship. The development of ECE inhibitors and ET-receptor specific antagonists and the demonstration of experimental efficacy with their use, however, has established ETs as valid therapeutic targets (Douglas and Ohlstein, 1997). Although several ET-receptor antagonists are currently available for experimental study, limited human clinical trials using ET-receptor antagonists have been conducted. To date, the primary therapeutic indication for ET-antagonists has been CHF. In one study the non-

selective ET_A/ET_B antagonist bosentan was administered to male CHF patients currently on conventional digoxin, Ca^{2+} channel blockers and nitrate therapy, but no ACE inhibitors (Kiowski et al., 1995). In this study, acute i.v. administration of 100 mg bosentan improved systemic hemodynamics, reducing systemic and pulmonary arteriolar tone, indicative of venous vasodilation. Bosentan has also been used in mildly hypertensive humans where it was shown to be equieffective to treatment with the ACE inhibitor enalapril at lowering blood pressure and blunting reflex neurohumoral vasoconstrictor activation (Krum et al., 1998). Finally, in hypertensive patients using forearm blood flow as a measure of vascular resistance it was demonstrated that selective ET_A receptor antagonism, but not selective ET_B blockade, increased forearm blood flow (Cardillo et al., 1999). However, the same study showed that combined intraarterial injection of both ET antagonists in combination resulted in a greater vasodilator response compared to ET_A receptor blockade alone.

C. *Endothelins and veins and hypertension*

Research on the pathophysiological role of ET-1 in hypertension has focused on the resistance vessels because of the exceptional potency and duration of action of ET-1 as an arterial constrictor in vivo and in vitro (Schriffin, 1998). What is less appreciated is that in most studies, in vitro, ET-1 has proven to be significantly more potent as a venoconstrictor than as an arterial constrictor (Cocks et al., 1989). Furthermore, decreased venous pressure and vascular filling both are strong stimuli

for endogenous ET-1 formation in humans and animals (Serneri et al., 1995; Kawano et al., 1995). As proposed by Serneri et al., "ET-1 appears to be an important mechanism for the long-lasting adaptations of venous wall tension to changes in blood volume." ETs raise venous and arterial resistance, systemic arterial pressure and MCFP when infused in vivo in chronically instrumented normal rats (Waite and Pang, 1992; Palacios et al., 1997a; 1997b). Exogenous ET-1 has been shown to increase MCFP in rats through activation of both ET_A and ET_B receptors (Waite and Pang, 1992; Palacios et al., 1997b). And importantly, Haynes and colleagues have found that venoconstriction (but not arterial constriction) to ET-1 is selectively enhanced in patients with essential hypertension (Haynes et al., 1994). In addition to its direct constrictor effects, ET-1 can facilitate sympathetic venoconstrictor tone in humans. In hypertensive but not normotensive human subjects, ET-1 potentiated sympathetically-mediated constriction of dorsal hand veins (Haynes et al., 1994).

V. *Hypotheses and specific aims*

Compelling evidence exists for a key role for ET-1 related mechanisms in animal models of experimental hypertension and essential hypertension in human subjects, particularly the salt-sensitive forms of hypertension. Taken together the findings presented also suggest venous tone as a target for ET-1 induced alterations in cardiovascular function. Therefore, the general aim of this research

project is to test the overall hypothesis that the endothelial cell-derived peptide endothelin-1 contributes to salt-sensitive hypertension by decreasing venous capacitance. The deoxycorticosterone acetate-salt model of experimental hypertension in the rat was chosen for study. The overall hypothesis will be addressed experimentally through a series of in vitro and in vivo studies outlined in the following specific aims.

Specific aim 1: **What are the mechanisms of endothelin-induced constriction in isolated mesenteric veins in vitro in the guinea pig?**

The venous system provides significant blood carrying capacity for the body with the splanchnic veins representing a major component of vascular capacitance (Monos et al., 1995). In order to validate agonists and antagonists used to characterize ET receptors mediating venoconstriction in vitro in the mesentery, an animal model of readily accessible mesenteric veins of all sizes was needed. For these purposes the guinea pig model was chosen over the rat. These studies examined ET receptor subtypes mediating venoconstriction in vitro to ET-1 and S6c in guinea pig mesentery using selective ET_A and ET_B receptor antagonists. They also explored the relative contribution of endothelial-cell derived vasodilator release to venous tone.

Specific aim 2: **Are mechanisms of endothelin-induced constriction in mesenteric veins in vitro altered in the DOCA-salt model of hypertension in the rat?**

Chronic hypertension is associated with structural and functional changes in VSM (Folkow, 1982). Whether such changes precede or are caused by hypertension is still controversial. There is little doubt, however, that vascular alterations make an important contribution to the maintenance of elevated arterial pressure in experimental and clinical hypertension. The hemodynaminc hallmark of chronic hypertension is elevated resistance to arterial blood flow through small arteries in most vascular beds, but another virtually invariable finding is decreased capacitance of the venous system (Folkow, 1982; Safar and London, 1985; Widgren et al., 1992). The cause of decreased venous capacitance in hypertension is unknown. Recent work suggests that one local factor which may have a role is ET-1 (Tabrizchi and Pang, 1992; Haynes et al., 1994; Rubanyi and Polokoff, 1994).

The major premise for the proposed studies in this thesis is that an alteration in vascular ET production or receptor dynamics contributes to increased venous tone in hypertension. In order to fully test the overall hypothesis, it is necessary to understand at the cellular level how ETs affect contractility of major capacitance vessels such as the mesenteric veins, and how these mechanisms may be altered in experimental models of hypertension in the rat. Convincing evidence exists suggesting a role for ETs in the pathophysiology of the DOCA-salt model in the rat

(Schiffrin, 1995a; Schiffrin et al., 1997a); therefore, this model of experimental hypertension was chosen for study. The first hypothesis to be tested was that ETs act at ET_A and ET_{B2} receptors to cause constriction and ET_{B1} receptors to cause vasodilation in mesenteric veins. These studies were performed in isolated preparations of rat mesentery maintained in vitro. Receptor classification was accomplished by using receptor selective agonists and antagonists. Finally, these studies addressed the hypothesis that in the DOCA-salt model of experimental hypertension in rats the receptor mechanisms used by ETs to directly regulate mesenteric veins are altered.

Specific aim 3: **Do acute bolus injections of endothelins reduce venous capacitance in vivo in the conscious rat, and is this influenced by salt intake and endothelial derived vasodilators?**

A relationship may exist between altered venous function and ET-1 in hypertension, particularly salt-dependent forms of hypertension. Several forms of experimental hypertension known to be salt-dependent also show ET-1 involvement (Schiffrin, 1999; Matsumura et al., 1999). Salt-intake may be an important factor in ET-induced forms of hypertension (Mortensen and Fink, 1992), particularly through altered venous function. Therefore, it was hypothesized that high salt intake would increase venomotor responses to ET-1. In order to evaluate this, studies were conducted examining the effects of differing salt intakes on acute

ET-induced changes in venomotor tone using measurements of MCFP in instrumented conscious rats.

The vascular endothelium is important in maintenance of arterial tone through the release of the vasodilators NO and prostanoids, however its role in modulating venous tone is not clear. NO production is impaired in several models of experimental hypertension including the DOCA-salt and Dahl salt-sensitive models which also show ET-1 involvement (Schiffrin, 1995b; Kassab et al., 1997; Shimokawa and Vanhoutte, 1997). Therefore, it was hypothesized that venoconstrictor effects of the selective ET_B receptor agonist S6c would be significantly increased by blockade of NOS and cyclooxygenase. To test this, studies were conducted to evaluate the effects of NOS and cyclooxygenase inhibition on S6c-induced changes to venomotor tone in intact, awake rats. Because ET-induced dilator release by the endothelium is mediated by the ET_B receptor, S6c was chosen over ET-1.

Specific aim 4: **Do endothelins reduce venous capacitance in vivo in the DOCA-salt model of experimental hypertension in the rat?**

The cause of decreased venous capacitance in hypertension is unknown. Changes in venous function may be especially important in the pathophysiology of those forms of hypertension known to be strongly influenced by alterations in salt intake and thus body fluid volumes (Simon, 1981; Simon, 1978; Simone et al., 1993;

Yamamoto et al., 1983). Probably the best characterized experimental model of salt and volume-dependent hypertension is the DOCA-salt model in the rat (Yamamoto et al., 1983). Much evidence supports the idea that abnormalities of the ET system are an important component of the pathophysiology of DOCA-salt hypertension (Schiffrin, 1995a; 1995b; Haynes et al., 1994). Furthermore, it is firmly established that the sympathetic nervous system plays a vital role in the development and maintenance of this model of hypertension (de Champlain, 1990; Kita et al., 1998; Lange et al., 2000).

Therefore it was hypothesized that the sustained hypertension in the DOCA-salt experimental model of hypertension in the rat was in part the result of elevated venous tone (MCFP) due to either direct ET-1 induced alterations in vascular function, or alterations in sympathetic control of venomotor tone by ET-1. The hypothesis was addressed using selective ET receptor antagonists, and the ganglion blocker hexamethonium to determine if increased venous tone in the DOCA-salt model of experimental hypertension in the rat is caused by endogenous ET.

The following chapters describe experiments conducted to evaluate the above specific aims. Methods for experiments conducted in this thesis work are found within each individual chapter. Redundancy in description of experimental methods has been eliminated by referring the reader to the appropriate chapter describing the methods in question. Chapter 2 addresses Specific aim 1 while

Chapter 3 addresses Specific aim 2. Specific aim 3 is addressed by Chapter 4 and Specific aim 4 is addressed by the remaining Chapters 5 and 6. In order to synthesize the findings in this thesis, a general discussion is provided in Chapter 7.

Chapter 2

Mechanisms of Endothelin-Induced Venoconstriction in Isolated Guinea Pig Mesentery

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INTRODUCTION

The endothelin family of peptides consists of three ET-isoforms (ET-1, ET-2 and ET-3) encoded by three distinct genes (Inoue et al., 1989a), and four highly homologous cardiotoxic peptides known as the sarafotoxins (S6a, S6b, S6c and S6d) which can be isolated from the venom of certain snakes (Landan et al., 1991). Isoforms of ETs are produced in many tissues and cell types, however, ETs are vasomodulatory peptides. Endothelial cells in veins or arteries exclusively produce ET-1 (Inoue et al., 1989a; Dohi et al., 1992; Rubanyi and Polokoff, 1994). ET-1 released by cultured endothelial cells occurs preferentially toward the basal side, implying that ETs act primarily on adjacent endothelial cells and VSM in an autocrine and paracrine manner (Wagner et al., 1992). Two receptors for ETs, the ET_A and ET_B receptors, have been cloned from bovine (Arai et al., 1990) and rat lung (Sakurai et al., 1990) cDNA libraries, respectively. Functional studies suggest that the ET_B receptor may be further subtyped as ET_{B1} and ET_{B2} receptors, however, no support for molecularly distinct ET_B receptors currently exists (Sudjarwo et al., 1993; Douglas et al., 1995).

ET_A and ET_B receptors are found in vascular tissue. The ET_A receptor is located on VSM where it mediates vasoconstriction with an agonist rank order potency of ET-1>ET-3 (Ihara et al., 1992; Moreland et al., 1992). The ET_{B1} receptor is localized to endothelial cells where it mediates release of vasodilator substances such as NO, PGI_2, EDHF (Fozard and Part, 1992; Nakashima and Vanhoutte,

1993). The ET_{B2} receptor is also found on VSM in certain vascular beds where it to mediates vasoconstriction (Sumner et al., 1992). ET_{B1} and ET_{B2} receptors have equal affinity for all endothelin isoforms (Sakurai et al., 1990). Sarafotoxin 6c is a highly selective ET_B receptor agonist (Williams et al., 1991).

The distribution of ET-receptor subtypes in vascular tissue varies considerably with animal species and between vascular beds. Vasoconstrictor responses to ET-1 in resistance arteries are largely mediated by ET_A receptors (Moreland et al., 1992; Sumner et al., 1992; Davenport et al., 1995). In contrast, in large calibre arteries and veins, contractions to ET-1 involve the ET_{B2} receptor (Sumner et al., 1992; Lodge et al., 1995). Vasoconstriction to ETs has been studied in many vascular beds both in vitro and in vivo from several animal species, including humans. Although the majority of studies have been performed in arteries, some studies have examined the effects of ETs on veins. Studies in vitro consistently show that maximal responses and potency for ETs in veins are greater than those of corresponding arteries (Cocks et al., 1989; Riezebos et al., 1994; Rubanyi and Polokoff, 1994). Venous systems such as the mesenteric veins serve a large capacitance function. ET-induced alterations in their tone could result in significant changes in blood volume distribution, cardiac output and blood pressure (Waite and Pang, 1990; Monos et al., 1995). Therefore, examination of ET receptors mediating venoconstriction in the mesentery will provide a better understanding of ETs potential contribution to physiologic control of body fluid distribution and blood pressure. In the present study, ET agonists and receptor specific antagonists were

used to characterize ET-receptor subtypes mediating venoconstriction in guinea pig mesenteric veins in vitro. Further, the contribution of ET-evoked vasodilator release to venous tone was also explored.

METHODS

Tissue preparation. Male guinea pigs (Michigan Department of Public Health, Lansing, MI) 325-350 g were anesthetized lightly via halothane inhalation, stunned and bled from the neck. The ileum and associated mesenteric vessels (including the root of the mesentery) were removed and placed in oxygenated (95% O_2/5% CO_2) Krebs' solution of the following composition (millimolar): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, NaH_2PO4 1.2 and glucose 11. A segment of ileum (5 cm) with associated mesenteric vessels was removed and pinned flat in a silicone elastomer-lined petri dish (Sylgard®, Dow Corning, Midland, MI). A section of mesentery containing vessels close to the mesenteric border was cut out using fine scissors and forceps. The preparation was transferred to a smaller silicone elastomer-lined recording bath (5-6 ml volume) and pinned flat. Veins approximately 250-300 μm in diameter (mean diameter 281 ±17 μm) were isolated by clearing surrounding arterioles and other tissue. Vessels were placed under no preset pressure. The chamber was then mounted on the stage of an inverted microscope (Olympus CK-2; Leco Corp., St. Joseph, MI) and superfused with warm (36°C) Krebs' solution at a flow rate of 7 ml/min. All preparations were allowed a 15 minute equilabration period during which time all vessels relaxed to a stable resting diameter.

Video monitoring of vessel diameter. The methods used to monitor the diameter of mesenteric vessels were identical to those described by others (Neild, 1989; Galligan et al., 1995). The output of a black and white video camera (Hitachi model KP-111; Leco, St. Joseph, MI) attached to the microscope was fed to a PCVision Plus frame-grabber board (Image Technology, Woburn, MA) mounted in a personal computer. The video images were analyzed using computer software (Diamtrak©, Adelaide, Australia). The digitized signal was converted to an analog output (model DAC-02, Keithley Megabyte, Tauton, MA) and fed to a chart recorder (EZ Graph; Gould Inc., Cleveland, OH) for an online record of vessel diameter. The sampling rate was 10 Hz, and changes in vessel diameter of 1.8 μm could be resolved.

Experimental protocols. Preparations were superfused with Kreb's solution and allowed a 15 minute equilibration period prior to addition of drug. Agonist concentration-response curves were generated using cumulative application of increasing agonist concentrations. Previous studies revealed no difference in concentration-dependent contractile responses to ET applied cumulatively versus single dose application. ET-1, ET-3 and S6c dissociate from receptor sites very slowly resulting in prolonged washout periods, therefore individual preparations were tested with one agonist only, with each concentration-response reaching a maximum (5-6 minutes) prior to addition of the next concentration of agonist (Waggoner et al., 1992). Contributions from dilators released by ET-1 and S6c

were studied by pretreatment of preparations with the cyclooxygenase inhibitor, indomethacin (1 μM) and the nitric oxide synthase inhibitor NLA (100 μM). In a separate experiment the effects of indomethacin (1 or 10 μM) or NLA (100 μM) on ET-1 concentration-responses were examined. Indomethacin and/or NLA were applied for 20 minutes prior to agonist application. The relative contributions of ET_A and ET_B receptors to constrictor responses was studied by comparing curves for ET-1 and S6c in the presence and absence of the ET_A receptor selective antagonists PD 156707 or BQ-610 and/or the ET_B receptor selective antagonist BQ-788. All ET antagonist experiments were conducted in the presence of indomethacin (1 μM) and NLA (100 μM) in order to study ET-1 and S6c evoked constrictor responses in the absence of dilator release.

Analysis of responses. For the comparison of agonist responses, a complete concentration-response curve for an agonist was obtained for a given preparation. Contractions obtained from unpressurized vessels were expressed as a percentage constriction (% reduction in resting vessel diameter), where the resting vessel diameter (vessel diameter after initial equilibration period) was taken to be baseline. Concentration-response curves were fit by a four parameter logistic concentration-response equation given as $Y=[(A_1-A_2)/[1 + (X-X_0)^P]] + A_2$. The derived parameters EC_{50} (X_0; concentration generating half-maximal response) and maximum response or E_{max} (A_2) were expressed as the mean± S.E.M. and *n* values refer to the number of preparations from which the data were obtained. Minimum

response (A_1; threshold response) and slope factor (P) were not significantly different across any experiment and therefore not reported. Statistical difference between means was determined by the Student's two-tailed, unpaired *t*-test. $P<0.05$ was considered statistically significant. Analyses was conducted on a PC using ORIGIN® software (Microcal® software, Northampton, MA).

Drugs. ET-1, ET-3, S6c, BQ-788 and BQ-610 were purchased from Peninsula Laboratories (Belmont, CA.). PD 156707 was a generous gift from Parke-Davis Pharmaceuticals Research (Ann Arbor, MI). All other drugs were obtained from Sigma (St. Louis, MO). Stock solutions of NLA were prepared in 1 part hydrochloric acid (1N): 9 parts deionized water, while BQ-788 and BQ-610 were prepared in 1 part acetic acid (1N): 1 part deionized water. PD 156707 was prepared in deionized water. Indomethacin was prepared in dimethylsulphoxide. Prazosin was prepared in 1 part methanol (100 %): 1 part deionized water. All other drugs were prepared as stock solutions in deionized water.

RESULTS

Concentration-responses to ET-1, ET-3 and S6c. ET-1 (0.01-10 nM), ET-3 (0.01-10 nM) and S6c (0.01-10 nM) produced sustained concentration-dependent contractions (decreases in vessel diameter) in guinea pig mesenteric veins. In order to establish that contractions to ETs occurred through direct activation of ET receptors on VSM, preparations were pretreated for 20 minutes with either tetrodotoxin (300 nM), guanethidine (10 μM) or prazosin (1 μM) prior to application of agonists. Agonist responses in the presence of tetrodotoxin, guanethidine or prazosin were unaffected when compared to control responses (data not shown). ET-1 concentration-response curves were left shifted compared to curves for ET-3 establishing a rank order potency of ET-1 > ET-3. The maximum contraction (E_{max}) caused by ET-1 was not different than ET-3. The E_{max} produced by ET-1 was greater than S6c, however, the two agonists were equipotent (Figure 2.1 and Table 2.1).

Effects of Indomethacin and NLA on dilator contribution to venous tone. In order to evaluate the contributions of vasodilator substances to ET-1 and S6c induced venous tone in guinea pig mesentery, preparations were pretreated with indomethacin (1 μM) and NLA (100 μM) for 20 minutes prior to agonist addition. Indomethacin and NLA produced no change in resting vessel diameter. Contractions to ET-1 and S6c were enhanced in the presence of indomethacin and

NLA when compared to control responses (Figure 2.2 and Table 2.1). In a separate experiment the effects of indomethacin or NLA on ET-1 concentration-responses were examined. Indomethacin at 1 or 10 μM produced no differences in ET-1 responses when compared to control values, however, NLA at 100 μM shifted ET-1 responses leftward and increased maximal responses when compared to control values (Figure 2.3 and Table 2.1). All of the remaining experiments were done in the presence of indomethacin (1 μM) and NLA (100 μM).

Effects of antagonists on ET-1 and S6c responses. Cumulative concentration-response curves were obtained for ET-1 and S6c in the absence and presence of the ET_A receptor selective antagonists PD 156707 (1 μM, 100 nM, 10 nM) and BQ-610 (100 nM), and the ET_B receptor selective antagonist BQ-788 (100 nM). Pretreatment of preparations with antagonists for 20 minutes prior to agonist application produced no change in resting vessel diameter. S6c concentration-response curves in the presence of BQ-788 were shifted rightward when compared to control S6c responses, while BQ-610 did not change S6c responses (Figure 2.4 and Table 2.2). PD 156707 at 1 μM or 100 nM produced rightward shifts in S6c concentration-responses (data not shown), while PD 156707 at 10 nM did not change S6c responses when compared to control S6c values (Figure 2.4 and Table 2.2). ET-1 concentration-responses in the presence of BQ-610 showed a rightward shift and a decrease in the maximal response when compared to control ET-1 responses (Figure 2.5 and Table 2.2). ET-1 responses in the presence of BQ-788

were not different when compared to control ET-1 responses (Figure 2.5 and Table 2.2). PD 156707 (10 nM) decreased maximal ET-1 responses when compared to control ET-1 responses (Figure 2.5 and Table 2.2). The combined application of BQ-788 and BQ-610 produced a rightward shift in ET-1 responses when compared to control ET-1 responses (Figure 2.6 and Table 2.2) or to ET-1 responses in the presence of BQ-610 alone (Table 2.2). Combined application of PD 156707 (10 nM) and BQ-788 also produced a rightward shift in ET-1 responses when compared to control ET-1 responses (Figure 2.6 and Table 2.2) or to ET-1 responses in the presence of PD 156707 (10 nM) alone (Table 2.2).

Treatment	*n*	pD_2	E_{max}
ET-1 (control)	6	9.17±0.07†	63.2±8.1‡
ET-3 (control)	5	8.65±0.11	41.3±4.5
ET-1 +NLA 100μM Indo 1μM	5	9.47±0.23	82.2±4.9‡
ET-1 (control)	5	8.88±0.05	71.2±5.2
ET-1 + Indo 1μM	4	8.83±0.04	61.0±9.0
ET-1 + Indo 10μM	5	8.94±0.08	69.5±4.0
ET-1 (control)	8	9.16±0.07	57.6±2.6
ET-1 +NLA 100μM	6	9.27±0.03	78.1±2.1*
S6c (control)	6	9.17±0.13	31.9±5.8
S6c +NLA 100μM Indo 1μM	6	9.67±0.20	52.1±2.9*

Table 2.1: Concentration-responses to ET-1, ET-3 and S6c in the presence and absence (control) of indomethacin and NLA in guinea pig mesenteric veins. Data are expressed as mean ± sem; *n* refers to the number of preparations; statistical comparisons were made by Students unpaired *t*-test with a significance level of $P<0.05$; pD_2 is the negative logarithm of the molar concentration of agonist producing half-maximal contraction (% constriction); E_{max} is the maximum contraction based on data fitted to the logistic equation; * significant compared to control value; † significant compared to corresponding ET-3 value. ‡ significant compared to corresponding S6c value.

Treatment	*n*	pD_2	E_{max}
ET-1 (control)	12	9.01±0.08	64.4±3.6
+ BQ-788	4	9.12±0.04	70.0±3.3
+ BQ-610	7	8.82±0.14	53.2±4.3
ET-1 (control)	5	9.06±0.13	48.7±5.0
+ BQ-788/BQ-610	5	7.99±0.15 * †	50.3±3.3
ET-1 (control)	6	9.07±0.30	65.7±3.1
+ PD156707	6	9.14±0.15	50.4±4.1 *
ET-1 (control)	5	9.47±0.47	65.6±6.2
+ BQ-788/PD 156707	5	8.20±0.20 * ‡	56.7±4.8
S6c (control)	7	9.36±0.11	51.6±2.7
+ BQ-788	4	7.08±0.12 *	35.1±13.1
+ BQ-610	4	9.59±0.08	55.0±4.7
S6c (control)	4	9.44±0.35	51.0±10.9
+ PD156707	4	9.14±0.24	46.5±8.8

Table 2.2: Effects of BQ-610 (100 nM), PD 156707 (10 nM) and BQ-788 (100 nM) on ET-1 and S6c concentration-responses in guinea pig mesenteric veins. Data are expressed as mean ± sem; *n* refers to the number of preparations; statistical comparisons were made by Students unpaired *t*-test with a significance level of $P<0.05$; pD_2 is the negative logarithm of the molar concentration of agonist producing half-maximal contraction (% constriction); E_{max} is the maximum contraction based on data fitted to the logistic equation; * significant compared to control value; † significant compared to ET-1 + BQ-610; ‡ significant compared to ET-1 + PD 156707; All preparations contain 1μM indomethacin and 100μM NLA.

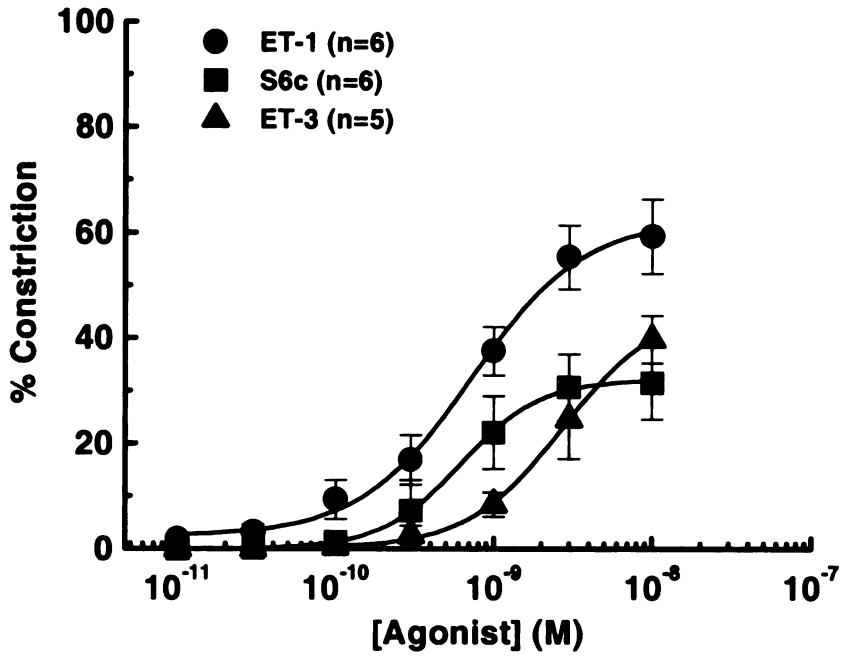

Figure 2.1: Cumulative agonist concentration-response curves in guinea pig mesenteric veins. Agonist contractile-responses are expressed as a % constriction of the resting vessel diameter (resting vessel diameter represents baseline). Data are the mean± s.e.m. from *n* preparations.

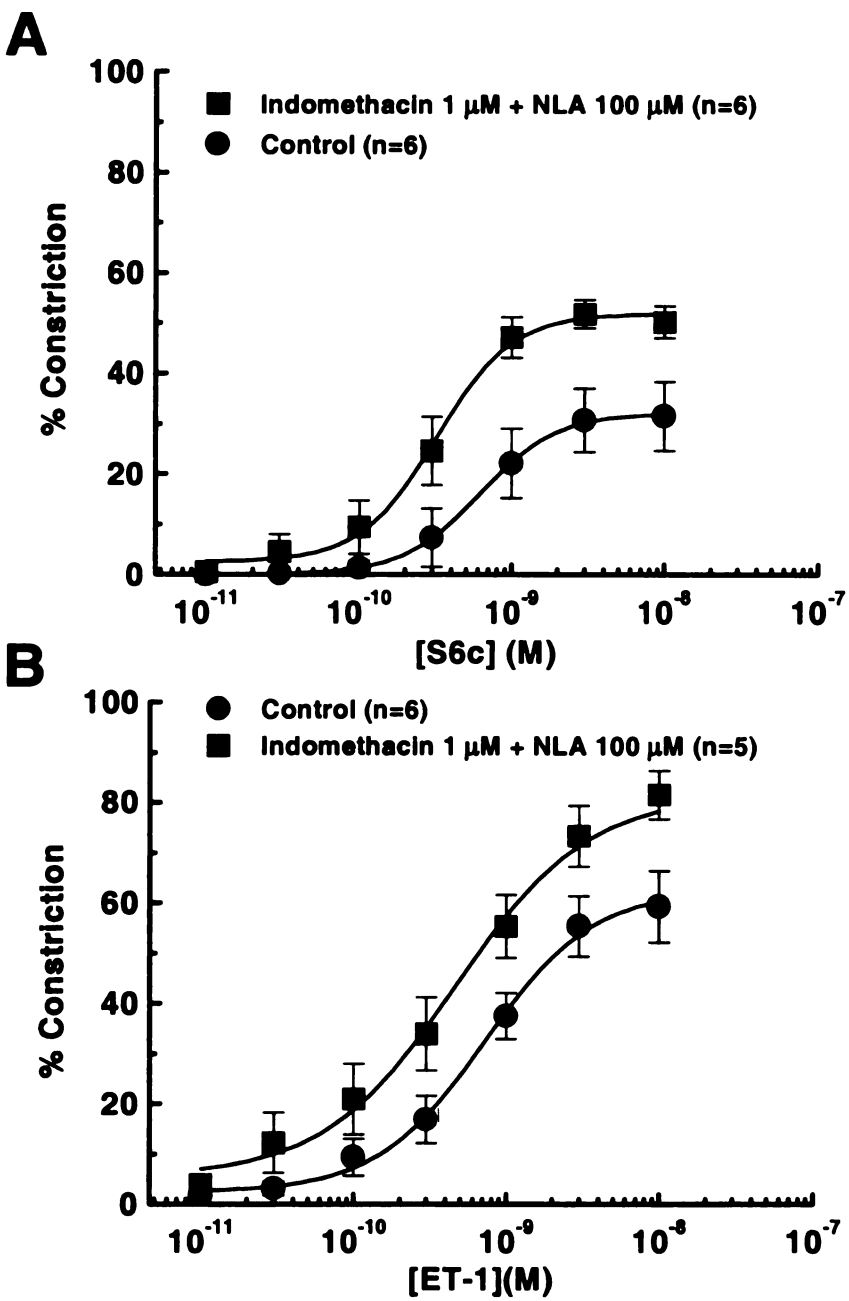

Figure 2.2: Effects of indomethacin and NLA on ET-1 and S6c concentration responses in guinea pig mesenteric veins. Indomethacin (1 μM) and NLA (100 μM) were applied for 20 minutes prior to application of A) S6c or B) ET-1. Agonist contractile-responses are expressed as a % constriction of the resting vessel diameter (resting vessel diameter represents baseline). Data are the mean± s.e.m. from *n* preparations.

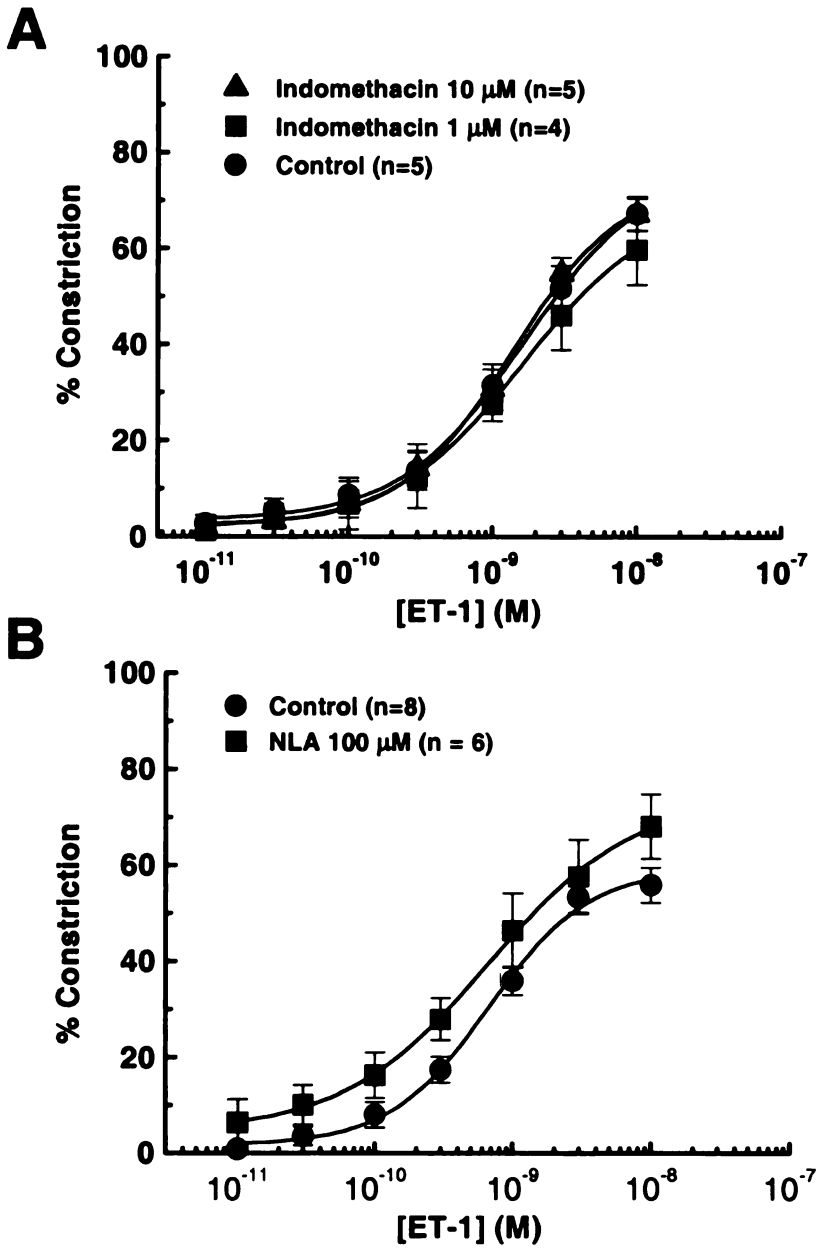

Figure 2.3: Effects of indomethacin or NLA on ET-1 concentration responses in guinea pig mesenteric veins. A) Indomethacin (1 μM or 10 μM) or B) NLA (100 μM) were applied for 20 minutes prior to application of ET-1. ET-1 contractile-responses are expressed as a % constriction of the resting vessel diameter (resting vessel diameter represents baseline). Data are the mean± s.e.m. from *n* preparations.

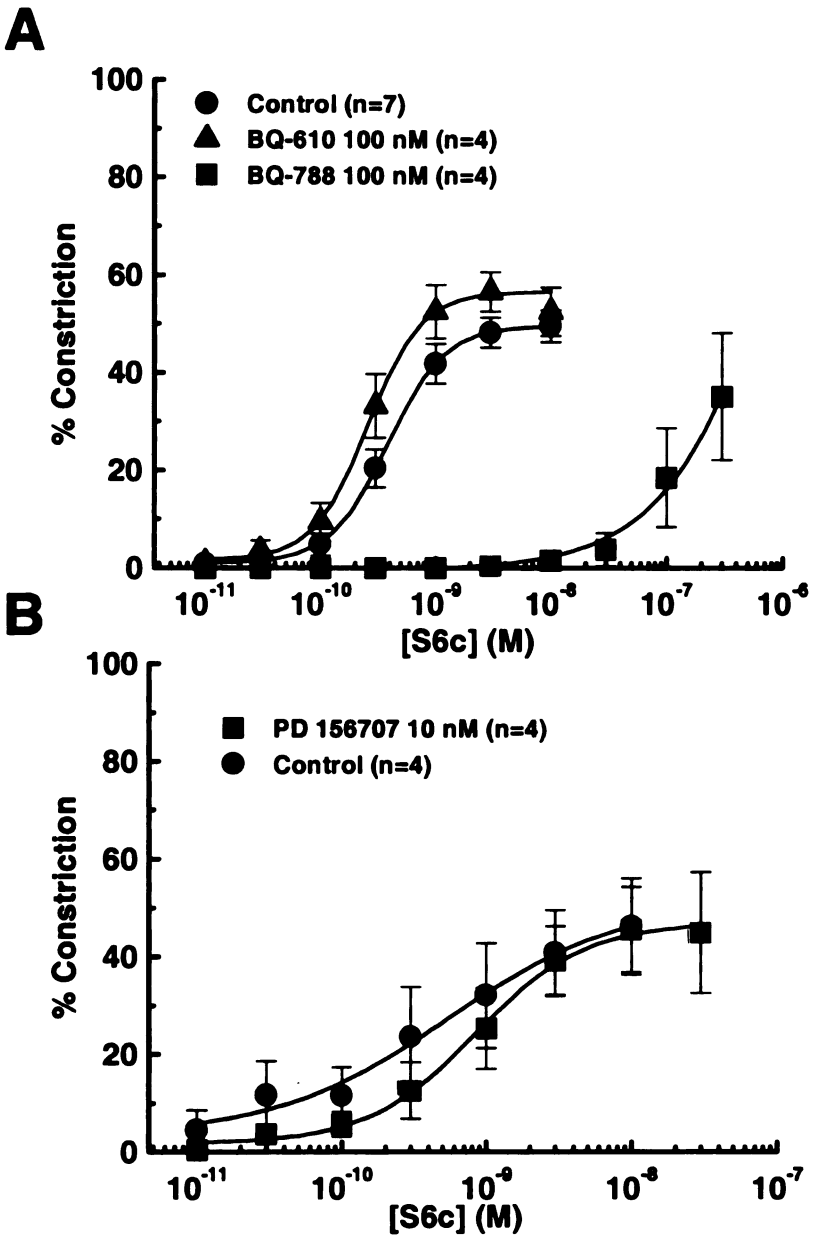

Figure 2.4: Effects of the ET_A selective antagonists BQ-610 and PD 156707 and the ET_B selective antagonist BQ-788 on S6c concentration-responses in guinea pig mesenteric veins. A) BQ-610 (100 nM) or BQ-788 (100 nM) or B) PD 156707 (10 nM) were applied in the presence of indomethacin and NLA for 20 minutes prior to S6c application. S6c contractile-responses are expressed as a % constriction of the resting vessel diameter (resting vessel diameter represents baseline). Data are the mean± s.e.m. from *n* preparations.

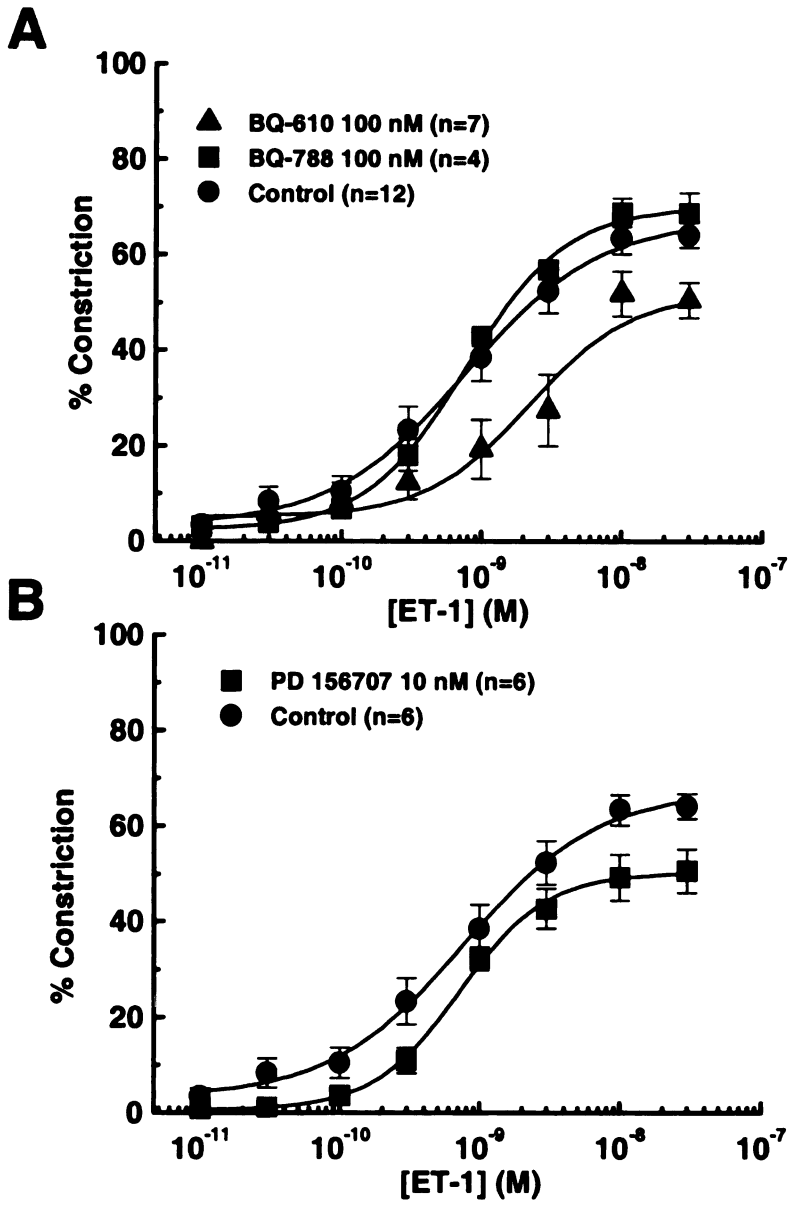

Figure 2.5: Effects of the ET_A selective antagonists BQ-610 and PD 156707 and the ET_B selective antagonist BQ-788 on ET-1 concentration-responses in guinea pig mesenteric veins. A) BQ-610 (100 nM) or BQ-788 (100 nM) or B) PD 156707 (10 nM) were applied in the presence of indomethacin and NLA for 20 minutes prior to ET-1 application. ET-1 contractile-responses are expressed as a % constriction of the resting vessel diameter (resting vessel diameter represents baseline). Data are the mean± s.e.m. from *n* preparations.

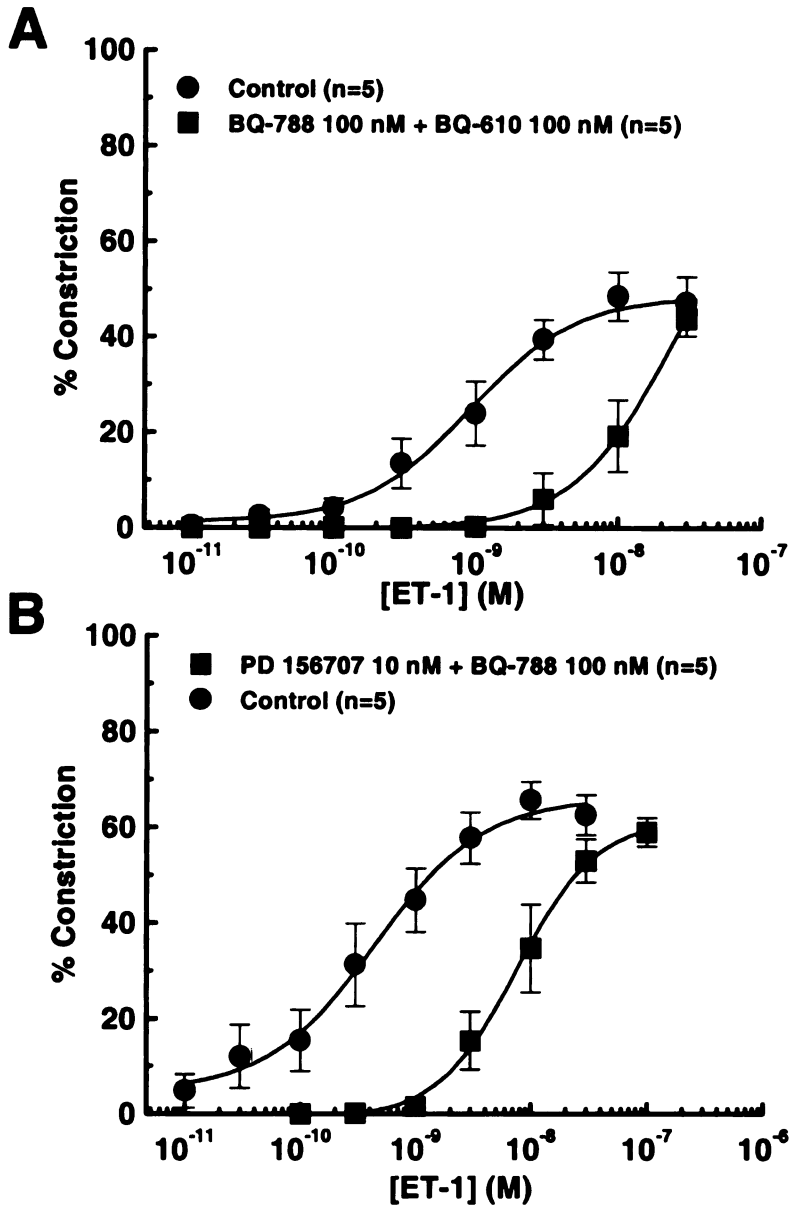

Figure 2.6: Effects of combined application of selective ET_A and ET_B antagonists on ET-1 concentration-responses in guinea pig mesenteric veins. A) BQ-788 (100 nM) and BQ-610 (100 nM) or B) BQ-788 (100 nM) and PD 156707 (10 nM) were applied in the presence of indomethacin and NLA for 20 minutes prior to ET-1 application. ET-1 contractile-responses are expressed as a % constriction of the resting vessel diameter (resting vessel diameter represents baseline). Data are the mean± s.e.m. from *n* preparations.

DISCUSSION

In the present study pharmacologic characterization of ET-receptor subtypes using ET-1, ET-3, S6c and ET-receptor selective antagonists revealed that guinea pig mesenteric veins possess ET_A and ET_B receptors coupled to contractile mechanisms. My data also show that activation of ET_{B1} receptors by ET-1 and S6c results in vasodilator release that reduces venoconstriction.

ET-1 and S6c induce the release of the NOS derived vasodilator product NO and the cyclooxygenase derived vasodilator product PGI_2 by binding to ET_{B1} receptors on the endothelial cell membrane (Sakurai et al., 1992; Douglas et al., 1995). In the present study, pretreatment of preparations with indomethacin which inhibits cycolooxygenase and NLA, an inhibitor of NOS, produced no changes to resting vessel diameter suggesting that dilator release was evoked by ETs and not the result of basal release from the vessel. My study did not examine the cell type expressing ET_{B1} receptors but endothelial cells lining the venous lumen are the most likely source of the vasodilators (Sakurai et al., 1992; Douglas et al., 1995). PGI_2 is the major product released by vascular cyclooxygenase, but its contribution to endothelium-dependent relaxation is minor (Shimokawa and Vanhoutte, 1997). However, others have shown that the cyclooxygenase inhibitor acetylsalicylic acid and not the NOS inhibitor L-NMMA potentiated ET-1 induced venoconstriction in human dorsal hand veins in vivo (Webb and Haynes, 1993). My findings in guinea pig mesenteric veins do not support a role for cyclooxygenase derived dilators, but instead show that NO is the major dilator released in response to ET-1. Inhibition

of NOS and cyclooxygenase removes ET-1 and S6c evoked dilator contributions to net vessel tone leaving ET-induced VSM constrictor responses unopposed. The present study shows that dilator release provides a significant contribution to S6c-induced changes in venous tone in guinea pig mesenteric veins. Comparison of ET-1 E_{max} values in the presence and absence of indomethacin and NLA with corresponding S6c values shows that ET-1 E_{max} values are greater than S6c responses, however, both ET-1 and S6c evoked dilator release account for approximately a 20 % reduction in venous tone (Table 2.1). Thus, dilator release by both agonists are comparable. This finding is supported by an equal affinity for all ET isoforms at the ET_{B1} receptor (Sakurai et al., 1990).

Pharmacological characterization of ET-receptor subtypes mediating venoconstriction in guinea pig mesentery was carried out using ET-receptor specific agonists and ET- receptor selective antagonists. Concentration-response curves for the mixed ET agonists ET-1 and ET-3 show a rank order agonist potency of ET-1>ET-3, thereby establishing the presence of ET_A receptors in guinea pig mesenteric veins. Sustained concentration-dependent contractions caused by S6c also establishes the presence of ET_{B2} constrictor receptors in guinea pig mesenteric veins. Application of indomethacin and NLA in all experiments conducted with ET antagonists allowed for characterization of ET-induced venoconstrictor mechanisms in the absence of ET-evoked dilator contributions. The ET_A selective antagonist BQ-610 at 100 nM did not affect responses to S6c suggesting that at this concentration BQ-610 did not block ET_{B2} receptors. Likewise, the ET_A selective

antagonist PD 156707 at 10 nM also showed no affect on S6c responses, while higher concentrations of the antagonist shifted S6c responses rightward suggesting that higher concentrations of PD 156707 (100 nM or 1 μM) were blocking ET_{B2} receptors. Application of BQ-610 (100 nM) or PD 156707 (10 nM) inhibited ET-1 responses, providing further support for the presence of ET_A receptors in guinea pig mesenteric veins. The ET_B selective antagonist BQ-788 (100 nM) shifted S6c responses rightward but had no effect on ET-1 responses. These findings suggest that while ET_{B2} constrictor receptors are present on the VSM, ET-1 may not activate them, or that an interaction occurs between the ET_A and ET_{B2} receptors resulting in masking of the ET_{B2} mediated contractile effect. An interaction between ET_A and ET_{B2} receptors is supported in the present study by the finding that combined application of BQ-788 (100 nM) and BQ-610 (100 nM) or BQ-788 (100 nM) and PD 156707 (10 nM) produces a rightward shift in ET-1 responses which is greater than BQ-610 (100 nM) alone or PD 156707 (10 nM) alone, respectively. Findings in other in vitro studies have also suggested the existence of an interaction between the ET_A and ET_{B2} receptors including vascular (Fukuroda et al., 1994b; Mickley et al., 1997) and non-vascular preparations (Fukuroda et al., 1996). It has been suggested that ET_A receptors are the major subtype mediating vasoconstriction in the arterial or high pressure side of the cardiovascular system while ET_{B2} receptors exert a significant constrictor role in low pressure systems such as the venous circulation (Moreland et al., 1994; Davenport et al., 1995). The results of the present study, while clearly demonstrating the presence of ET_{B2} constrictor

receptors, also show that responses mediated by these receptors are only revealed after ET_{B2} receptor blockade, in addition to effective ET_A receptor antagonism. The mechanism behind the proposed interaction between ET_A and ET_{B2} receptors is not understood but may involve interactions at the receptor level or through second messengers (Fukuroda et al., 1996). Biochemical studies examining receptor and second messenger interactions should provide valuable insight into the mechanism behind the proposed receptor "crosstalk".

In conclusion, guinea pig mesenteric veins express endothelial and VSM ET_B receptors as well as VSM ET_A receptors. Activation of either ET_A or ET_{B2} receptors on the VSM results in venoconstriction, however, the endogenous ET peptide ET-1 does not appear to activate the ET_{B2} receptor due to a proposed receptor "crosstalk" mechanism resulting in the masking of the ET_{B2} receptor mediated responses by ET_A receptor activation. Finally, ET-1 acting at ET_{B1} receptors results in dilator release which is largely NO mediated and provides a minor effect on net venous tone.

Chapter 3

Endothelin Receptor Function in Mesenteric Veins from Deoxycorticosterone Acetate-salt Hypertensive Rats

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INTRODUCTION

Hypertensive humans and experimental animals have decreased systemic venous capacitance due to reduced compliance of extrathoracic veins, particularly those in the splanchnic bed (Safar and London, 1987). This facilitates return of blood to the heart in the presence of decreased ventricular compliance and elevated end diastolic pressures. Limited evidence also implicates changes in venous function in the development of hypertension (Martin et al., 1998), although this idea is controversial. Proposed mechanisms for diminished venous compliance in hypertension include structural remodeling of the vascular wall (Mark, 1984), and neurogenic (Willems et al., 1982; Martin et al., 1998) or humoral (Simon, 1978) activation of venous smooth muscle. Recent evidence from human studies (Haynes et al., 1994; Serneri et al., 1995) indicates ET-1 may be an important determinant of venous smooth muscle activity. ET-1 causes venoconstriction by activating both ET_A and ET_B subtypes of ET-1 receptors on venous smooth muscle, whereas stimulation of ET_B receptors on endothelial cells causes release of vasodilators that oppose venoconstriction (Chapter 2). To test the hypothesis that ET-1 can decrease venous compliance in hypertension, I have studied DOCA-salt rats. There is abundant evidence that ET-1 has a major role in the pathogenesis of hypertension in this experimental model (Schiffrin, 1995a; 1998). Recently, I showed that venomotor tone is increased in vivo in hypertensive DOCA-salt rats compared to normotensive controls, and that the increase is reversed by the ET_A

selective receptor antagonist ABT-627 (Chapter 5). The goal of the in vitro study reported here was to test the hypothesis that increased ET-1 mediated venoconstriction in DOCA-salt rats is caused by changes in venous smooth muscle responsiveness to ET. Since changes in arterial reactivity to ET-1 have been well-characterized in DOCA-salt hypertensive rats (Nguyen et al., 1992) some comparative experiments also were performed on small mesenteric arteries.

METHODS

Animal Protocols. Upon arrival at my facility, male Sprague-Dawley rats weighing 175-225 g (Charles River, Portage, MI) were maintained according to standards approved by the Michigan State University All-University Committee on Animal Care and Use. Rats were housed in clear plastic boxes in groups of three with free access to standard pelleted rat chow (Harlan/Teklad 8640 Rodent Diet) and tap water. All rats were housed in a light and temperature controlled room and maintained in strict accordance with Michigan State University and National Institutes of Health animal care guidelines. Rats were acclimatized to their environment for 2 days prior to surgical manipulation.

Induction of DOCA-salt hypertension has been described by others (Ormsbee et al., 1973). Briefly, rats were nephrectomized unilaterally under anesthesia provided by an intraperitoneal (i.p.) injection of pentobarbital sodium (45 mg/kg; Abbott Laboratories, Abbott Park, IL). Bronchiolar secretions were controlled by administration of atropine sulfate i.p. (0.04 mg/kg; Sigma, St. Louis, MO). Silicone rubber patches (Dow Corning, Ferndale, MI) impregnated with deoxycorticosterone acetate (DOCA; Sigma, St. Louis, MO) were implanted subcutaneously (s.c.) in rats providing DOCA at 150 mg/kg. Postoperative analgesia was provided by a single injection of butorphanol tartrate s.c. (0.5 mg/kg; Abbott Laboratories, Abbott Park, IL). All DOCA-implanted rats were placed on salt water containing 1 % NaCl and 0.2 % KCl. Normotensive control rats were

nephrectomized unilaterally and placed on tap water. All rats were placed on standard pelleted rat chow. Systolic blood pressure was measured using the tail-cuff method after three weeks on the above protocol. After four weeks, rats were euthanized by an overdose of pentobarbital followed by exsanguination, and mesenteric vessels were extracted.

In vitro Preparation of Mesenteric Vessels. Following euthanasia, the small bowel was removed and placed in oxygenated (95 % O_2, 5 % CO_2) Krebs' physiological saline solution of the following composition (millimolar): NaCl 117; KCl 4.7; $CaCl_2$ 2.5; $MgCl_2$ 1.2; $NaHCO_3$ 25; glucose 11. A segment of ileum (5 cm) with associated mesenteric vessels was removed and pinned flat in a silicone elastomer-lined petri dish (Sylgard®, Dow Corning, Midland, MI). A section of mesentery containing vessels close to the mesenteric border was cut out using fine scissors and forceps. The preparation was transferred to a smaller silicone elastomer-lined recording bath (5-6 ml volume) and pinned flat. Second or third order mesenteric veins (mean diameter 281 ± 17.1 μm, n=121) or mesenteric arteries (mean diameter 162 ± 5.2 μm, n=57) were isolated for study by clearing surrounding tissue. Vessels were placed under no preset pressure. Previous studies have shown that venous pressures in vivo are not different in sham and DOCA-salt rats (Chapter 5). The chamber was then mounted on the stage of an inverted microscope (Olympus CK-2; Leco, St. Joseph, MI) and superfused with warm (37 °C) Krebs' solution at a flow rate of 7 ml/min. All preparations were allowed a 20 minute equilibration period

during which time all vessels relaxed to a stable resting diameter.

Video Monitoring of Vessel Diameter. See Chapter 2 page 49.

Experimental Protocols. All drugs were added in known concentrations to the superfusing Krebs' solution. Concentration-response curves were obtained following cumulative application of either ET-1 (Peninsula Laboratories, Belmont, CA) or S6c (Peninsula Laboratories, Belmont, CA). Each agonist concentration was applied for 5-6 minutes to allow sufficient time to reach a steady-state response. ET-1 and S6c dissociate from receptor sites very slowly resulting in prolonged washout periods (Waggoner et al., 1992), therefore only one concentration-response curve could be obtained in each preparation. Contributions from endothelium-derived vasodilator substances released by ET-1 and S6c were eliminated by pretreatment of preparations with the cyclooxygenase inhibitor, indomethacin (1 μM; Sigma, St. Louis, MO) and the NOS inhibitor NLA (100 μM; Sigma, St. Louis, MO) for 20 minutes prior to agonist application. The relative contribution of ET_A and ET_B receptors to constrictor responses was studied by comparing curves for ET-1 and S6c in the presence and absence of the ET_A receptor selective antagonist BQ-610 (100 nM; Peninsula Laboratories, Belmont, CA) and/or the ET_B receptor selective antagonist BQ-788 (100 nM; Peninsula Laboratories, Belmont, CA). Antagonists were applied for 20 minutes prior to the application of agonist. All ET antagonist experiments were conducted in the

presence of indomethacin (1 μM) and NLA (100 μM).

Wire Myograph Studies in Mesenteric Arteries. In order to validate observations made in small arteries using computer-assisted video microscopy in which vessels were placed under no predetermined pressure, a separate experiment was conducted with arteries placed under passive tension using methods similar to others (Ohlstein et al., 1998). Using a stereomicroscope with a calibrating eyepiece, small mesenteric arteries from sham normotensive (mean diameter 242 ± 22.0 μm, n=3) and DOCA-salt hypertensive rats (mean diameter 222 ± 27.0 μm, n=3) were suspended between an isometric force transducer and a micrometer-attached support in a dual chamber myograph (Instrumentation and Model Facility, University of Vermont, Burlington, VT). This allowed experimentation on arteries from sham normotensive and DOCA-salt hypertensive rats at the same time. The jacketed dual chamber was filled with oxygenated (95 % O_2, 5 % CO_2) physiological saline, kept warm (37 °C) by a heating water bath. Mounted vessels were equilibrated for 30 minutes with frequent buffer changes. Arteries were placed under passive tension (previous experiments determined that 0.4 g resulted in optimal force generation) and equilibrated for 30 minutes. Vessels were initially challenged with a maximal concentration of phenylephrine (10 μM; Sigma, St. Louis, MO) and subsequent responses were normalized to this reference contraction. Vessels were then washed and endothelial integrity assessed by the ability of acetylcholine (1 μM) to produce relaxation of vessels precontracted with

phenylephrine (1 μM). Vessels were washed and a cumulative concentration-response curve to ET-1 was generated. These studies were performed by Dr. Stephanie Watts, Department of Pharmacology and Toxicology, Michigan State University.

Analysis of Responses. A complete concentration-response curve for an agonist was obtained in a given preparation. Contractions obtained from unpressurized vessels were expressed as a percentage constriction (% reduction in resting vessel diameter), where the resting vessel diameter (vessel diameter after initial equilibration period) was taken to be baseline. Contractions obtained for small mesenteric arteries placed under passive tension were expressed as a percentage of the reference contraction (phenylephrine 10 μM). Concentration-response curves were fit by a four parameter logistic concentration-response equation given as:

$$Y=\{(E_{min}-E_{max})/[1 + (X-EC_{50})^{P}]\} + E_{max}.$$

The derived parameters, EC_{50} (concentration generating half-maximal response) and maximum response or $E_{max,}$ were expressed as the mean ± S.E.M. and *n* values refer to the number of preparations from which the data were obtained. Statistical difference between means was determined by Student's two-tailed, unpaired *t*-test. S6c produced negligible responses in arterial preparations and these responses could not be fitted to the logistic function. For these data, the observed E_{max} was taken directly from individual plots. Point by point comparisons were made for S6c

concentration-response curves generated from arteries and analyzed by ANOVA. $P<0.05$ was considered statistically significant. Analyses were conducted on a PC using ORIGIN® (Microcal Software, Northampton, MA) and Instat® (GraphPad Software, San Diego, CA) software.

RESULTS

ET-1 induced contractions of arteries and veins. Average systolic blood pressures recorded for DOCA-salt rats were higher than average systolic pressures recorded for sham-operated rats (DOCA-salt: 193 ± 3 mmHg, n=37 versus sham: 122 ± 1 mmHg, n=38; $P<0.05$). ET-1 produced sustained concentration-dependent contractions in mesenteric veins (0.01-30 nM) and arteries (0.01-100 nM) from DOCA-salt hypertensive and sham normotensive rats. In control solutions, ET-1 was a more potent agonist and caused greater maximum contractions in veins compared to arteries in preparations from sham normotensive and DOCA-salt hypertensive rats. (Figure 3.1 and Table 3.1). In veins, the ET-1 concentration-response curves were very similar in preparations from sham normotensive and DOCA-salt hypertensive rats; however, there was a rightward shift in the ET-1 concentration-response curve with a decreased maximum contraction in arteries from DOCA-salt hypertensive rats compared to those obtained from sham normotensive rats (Figure 3.1 and Table 3.1). Combined application of indomethacin/NLA did not cause a significant change in the ET-1 concentration-response curve in sham normotensive veins although there was a small increase in pD_2 values in the presence of the inhibitors (Figure 3.1 and Table 3.1). Similarly, the ET-1 concentration-response curve in veins from DOCA-salt hypertensive rats was not altered by indomethacin/NLA treatment (Figure 3.1 and Table 3.1). However, indomethacin/NLA did cause a leftward shift in the ET-1 concentration-

response curve in arteries from sham normotensive rats as indicated by a significant increase in the ET-1 pD_2, but not the E_{max}, in the presence of the inhibitors (Figure 3.1 and Table 3.1). Indomethacin/NLA also increased the E_{max} value for ET-1 in arteries from DOCA-salt hypertensive rats without changing the pD_2 value (Figure 3.1 and Table 3.1).

S6c induced contractions of arteries and veins. In control solution, the concentration-response curves for S6c were very similar in veins from sham normotensive and DOCA-salt hypertensive rats (Figure 3.2 and Table 3.1). In the presence of indomethacin/NLA, the S6c concentration-response curve obtained in mesenteric veins from sham normotensive, but not from DOCA-salt hypertensive rats, was shifted to the left with an increased pD_2, (Figure 3.2 and Table 3.1). S6c caused small contractions in arteries from both sham normotensive and DOCA-salt hypertensive rats and these responses were not significantly altered in the presence of indomethacin/NLA (Figure 3.2 and Table 3.1).

Myograph studies in mesenteric arteries. The data described above indicate that mesenteric veins are more sensitive than unpressurized arteries to the contracile actions of ET-1 and S6c. However, it is known that pressure can affect arterial sensitivity to contractile agonists. To address this issue, studies of the contractile effects of ET-1 on small mesenteric arteries were done in a wire myograph, in order to place the wall of the artery under tension. Maximal

contractions obtained to phenylephrine (10 μM) in these studies were not significantly different in arteries from DOCA-salt hypertensive compared to sham normotensive rats. Mesenteric arteries obtained from DOCA-salt hypertensive rats and sham normotensive rats produced sustained contractions in response to ET-1 (10 pM-300 nM) (Figure 3.3). Contractile responses in arteries from sham rats were enhanced compared to arteries from DOCA-salt rats (E_{max}-Sham artery; 136.5±6.2, n=3 vs DOCA-salt artery; 69.6±14.0, n=3 and pD_2-Sham artery; 8.49±0.1, n=3 vs DOCA-salt artery; 8.19±0.1, n=3; P<0.05, Figure 3.3). Results of ET-1 concentration-response curves obtained from unpressurized arteries (Table 3.1) were similar to those obtained for arteries placed under passive tension (see above), most notably showing a similar reduction in maximal contractions in DOCA-salt arteries compared to sham arteries, respectively (Figure 3.3).

Effects of BQ-788 and BQ-610 on S6c Responses. In order to study receptors mediating constriction, all antagonist experiments were conducted in the presence of indomethacin (1 μM) and NLA (100 μM). Concentration-response curves were obtained in mesenteric veins for S6c in the absence (control) and presence of the ET_A receptor selective antagonist BQ-610 (100 nM) or the ET_B receptor selective antagonist BQ-788 (100 nM). As S6c produced little or no contractions in arteries, ET-antagonists were not tested against S6c in arteries. Antagonist pretreatment produced no change in resting vessel diameter. S6c concentration-response curves in veins from both DOCA-salt and sham rats were

shifted rightward in the presence of BQ-788 when compared to control S6c responses (Figure 3.4 and Table 3.2). Application of BQ-610 to either DOCA-salt or sham veins did not change S6c responses (Figure 3.4 and Table 3.2). There were no differences between DOCA-salt versus sham S6c contractile response curves in the presence of BQ-788 or BQ-610 (Table 3.2).

Effects of BQ-788 and BQ-610 on ET-1 Responses. Concentration-response curves in mesenteric veins were obtained for ET-1 in the absence (control) and presence of BQ-610 (100 nM) and/or BQ-788 (100 nM). Maximum constrictor responses to ET-1 in mesenteric veins from sham normotensive rats were reduced in the presence of BQ-610 when compared to control values, while *p*D2 values did not differ between BQ-610 treated and control preparations (Figure 3.5 and Table 3.2). Similar findings were noted in mesenteric veins from DOCA-salt hypertensive rats with responses to ET-1 being reduced in the presence of BQ-610 when compared to control values (Figure 3.5 and Table 3.2). Comparison of ET-1 responses in the presence of BQ-610 in sham versus DOCA-salt venous preparations showed no differences (Table 3.2). Application of BQ-788 to either sham or DOCA-salt venous preparations did not change ET-1 contractile responses when compared to control values (Figure 3.5, Table 3.2). The combined application of BQ-788 and BQ-610 produced a rightward shift in ET-1 responses in sham vein preparations compared to control responses (Figure 3.5 and Table 3.2). Combined ET-antagonist application produced a rightward shift in ET-1 responses in DOCA-

salt vein preparations compared to control responses (Figure 3.5 and Table 3.2), or to BQ-610 responses alone (Table 3.2). Maximal ET-1 responses in DOCA-salt venous preparations in the presence of BQ-788 and BQ-610 were increased compared to responses in corresponding sham vein preparations, while there were no differences in pD_2 values (Table 3.2). ET-1 contractile-responses in arteries were tested against the ET_A selective antagonist BQ-610 (100 nM) in sham preparations only. Contractile responses in sham arteries to ET-1 were reduced and shifted rightward in the presence of BQ-610 when compared to control responses (Control; E_{max}=57.1±4.4, pD_2=8.9±0.3, n=7 vs BQ-610; E_{max}=23.0±6.5, pD_2=7.6±0.2, n=5, P<0.05).

	pD_2		E_{max}	
	Vein	**Artery**	**Vein**	**Artery**
Sham				
ET-1	9.4 ± 0.2 (5)	8.3 ± 0.1‡ (9)	61.8 ± 5.5 (5)	45.2 ± 4.6‡ (9)
ET1+NLA/Indo	9.6 ± 0.2 (11)	8.9±0.3*‡ (7)	64.2 ± 3.4 (11)	57.1 ± 4.4 (7)
DOCA-Salt				
ET-1	9.6 ± 0.4 (5)	8.4 ± 0.1‡ (6)	63.1 ± 4.6 (5)	27.5 ± 6.6†‡ (6)
ET1+NLA/Indo	9.7 ± 0.2 (12)	8.1±0.1†‡ (6)	65.4 ± 4.3 (12)	47.8 ± 5.8* (6)
SHAM				
S6c	9.3 ± 0.1 (6)	nd (6)	38.2 ± 3.6 (6)	5.1 ±4.7 (6)
S6c+ NLA/Indo	9.6±0.1* (10)	nd (5)	40.5 ± 2.6 (10)	19.9 ± 4.2 (5)
DOCA-Salt				
S6c	9.4 ± 0.1 (6)	nd (8)	40.0 ± 9.3 (6)	4.8 ± 3.5 (8)
S6c+ NLA/Indo	9.4 ± 0.1 (11)	nd (5)	38.5 ± 4.3 (11)	8.1 ± 4.5 (5)

Table 3.1: E_{max} and pD_2 values obtained from ET-1 and S6c concentration-response curves in the absence and presence of indomethacin and NLA in mesenteric blood vessels from DOCA-salt hypertensive rats and sham normotensive rats. Data are mean ± S.E.M.; Numbers in parenthesis are *n* values. NLA/Indo refers to data obtained from preparations pretreated with NLA (100 μM) and indomethacin (1 μM). Statistical comparisons were made by Students unpaired *t*-test with a significance level of $P<0.05$; pD_2 is the negative logarithm of the molar concentration of agonist producing half-maximal contraction (expressed as % Constriction); E_{max}- the maximum contraction based on data fitted to the logistic equation; nd-not determined; * significantly different from value obtained in the absence of NLA/Indo; † significantly different from corresponding sham value; ‡ significantly different from corresponding vein value; E_{max} values for S6c concentration-responses in arterial preparations taken directly from individual plots.

	pD_2	E_{max}
Sham		
ET-1 (control)	9.7±0.4 (6)	59.9±5.3 (6)
+ BQ-788	9.6±0.2 (5)	56.4±9.1 (5)
+ BQ-610	8.8±0.4 (6)	41.2±5.0* (6)
+ BQ-788/BQ-610	8.5±0.3* (5)	48.0±6.2 (5)
S6c (control)	9.4±0.1 (5)	38.4±4.0 (5)
+ BQ-788	7.6±0.1* (5)	nd (5)
+ BQ-610	9.1±0.1 (5)	41.1±4.0 (5)
DOCA-salt		
ET-1 (control)	9.4±0.2 (7)	71.4±4.1 (7)
+ BQ-788	9.4±0.2 (7)	63.3±4.1 (7)
+ BQ-610	9.1±0.2 (6)	46.7±7.0* (6)
+ BQ-788/BQ-610	8.5±0.1*† (7)	69.6±5.7†‡ (7)
S6c (control)	9.5±0.1 (5)	39.0±7.9 (5)
+ BQ-788	7.6±0.1* (4)	nd (4)
+ BQ-610	9.3±0.1 (5)	34.1±6.5 (5)

Table 3.2: Effects of BQ-610 (100 nM) and BQ-788 (100 nM) on ET-1 and S6c concentration-responses in mesenteric veins from DOCA-salt hypertensive rats and sham normotensive rats. Data are mean ± S.E.M.; Numbers in parenthesis are *n* values. All preparations were pretreated with NLA (100 μM) and indomethacin (1 μM). Statistical comparisons were made by Students unpaired *t*-test with a significance level of $P<0.05$; pD_2 is the negative logarithm of the molar concentration of agonist producing half-maximal contraction (expressed as % Constriction); E_{max}- the maximum contraction based on data fitted to the logistic equation; nd-not determined; * significantly different from control value; † significantly different from ET-1 +BQ-610; ‡ significantly different from sham ET-1 +BQ-788/BQ-610.

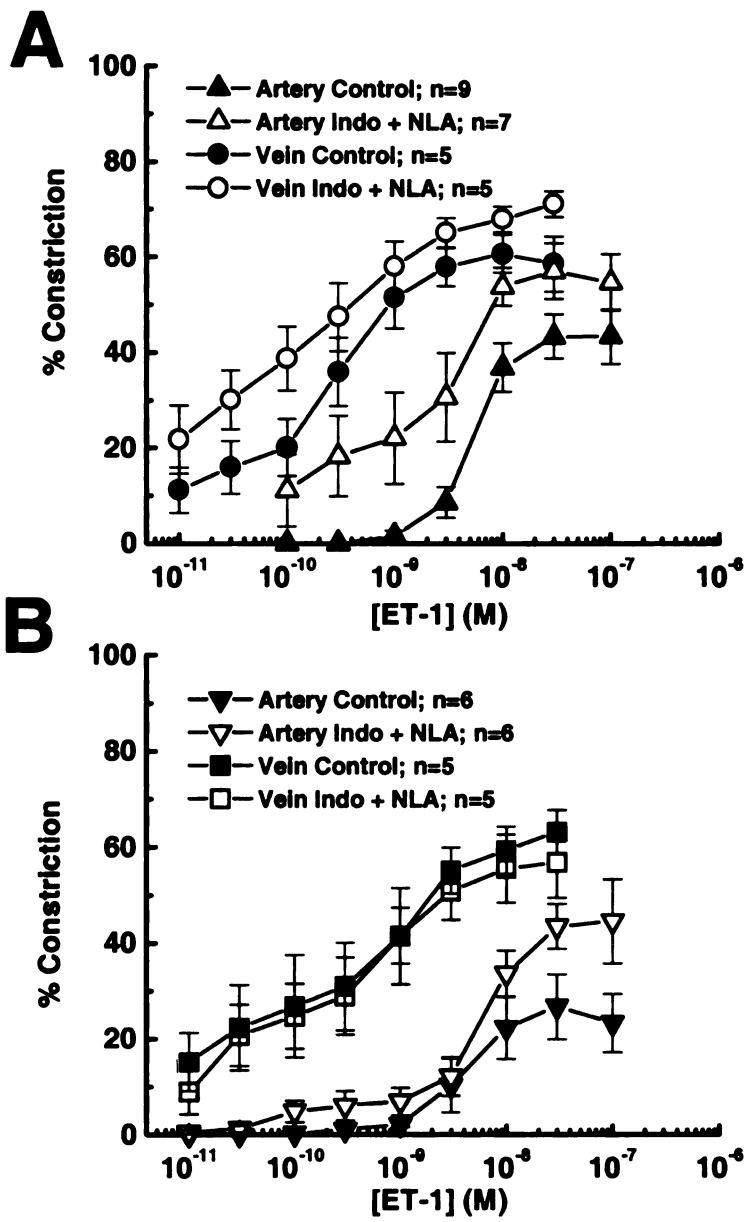

Figure 3.1: ET-1 concentration-response curves in the absence (control) and presence of indomethacin (Indo, 1 μM) and NLA (100 μM) in blood vessels from (A) sham-operated normotensive rats and (B) DOCA-salt hypertensive rats. Indomethacin and NLA were applied for 20 minutes prior to application of ET-1. Agonist contractile-responses are expressed as % constriction. Data are the mean ± S.E.M., *n* refers to the number of preparations.

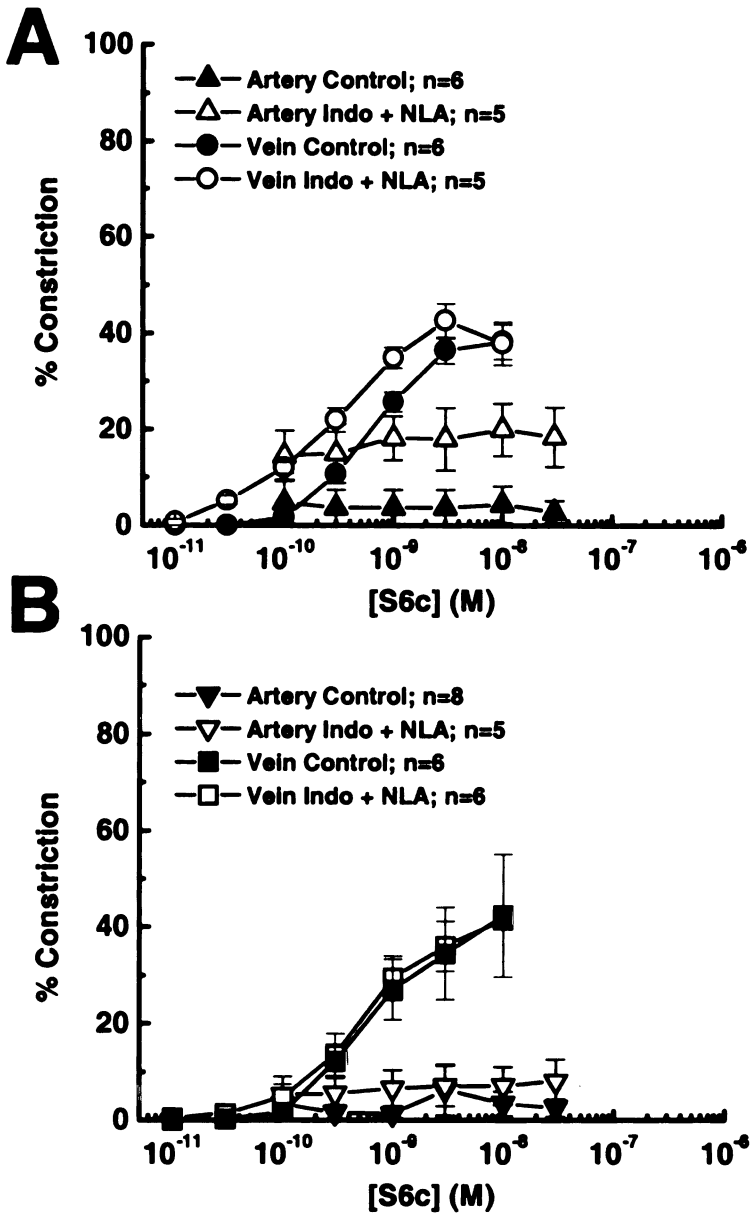

Figure 3.2: S6c concentration-response curves obtained in the absence (control) and presence of indomethacin (Indo, 1 μM) and NLA (100 μM) in blood vessels from (A) sham-operated normotensive rats and (B) DOCA-salt hypertensive rats. Indomethacin and NLA were applied for 20 minutes prior to application of S6c. Agonist contractile-responses are expressed as % constriction. Data are the mean ± S.E.M., *n* refers to the number of preparations.

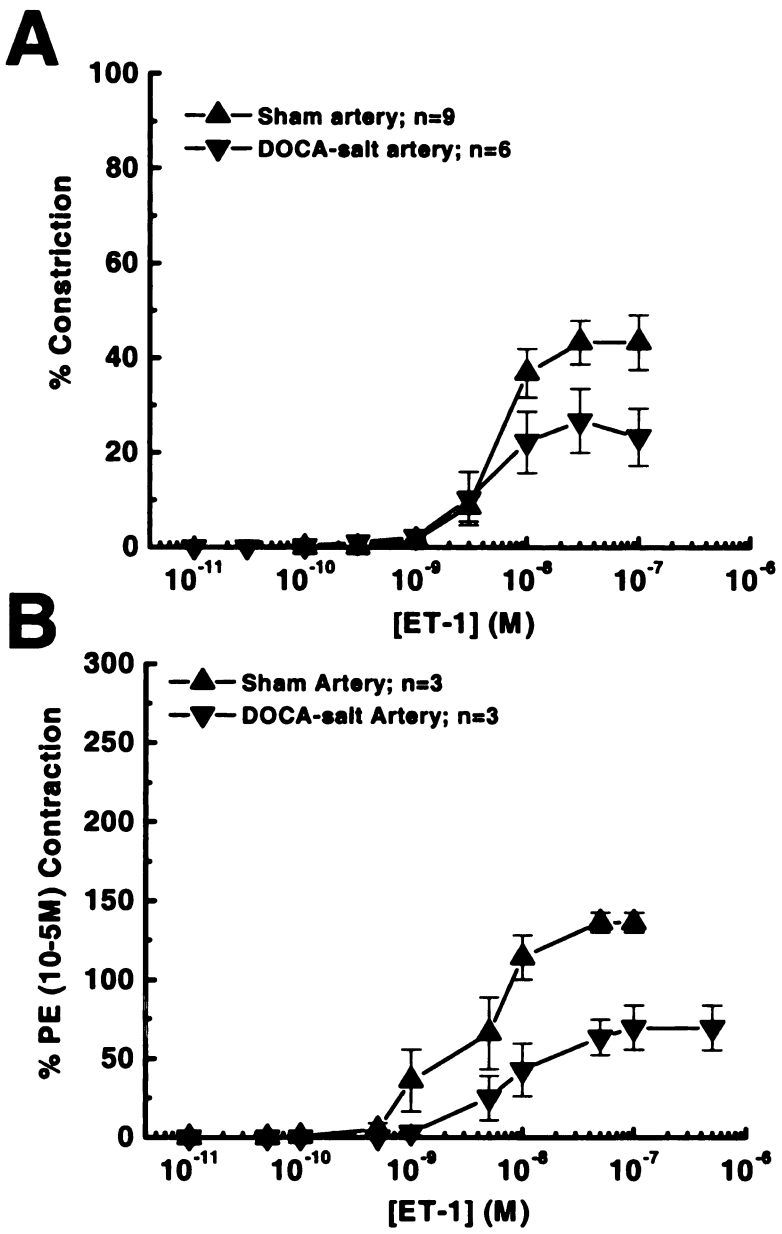

Figure 3.3: Comparison of contractile responses to ET-1 in mesenteric arteries from sham-operated normotensive rats versus DOCA-salt hypertensive rats in (A) unpressurized arteries or in (B) arteries placed under passive tension using a wire myograph. Agonist contractile-responses are expressed as (A) % constriction or (B) % phenylephrine contraction (10 μM). Data are the mean ± S.E.M., *n* refers to the number of preparations.

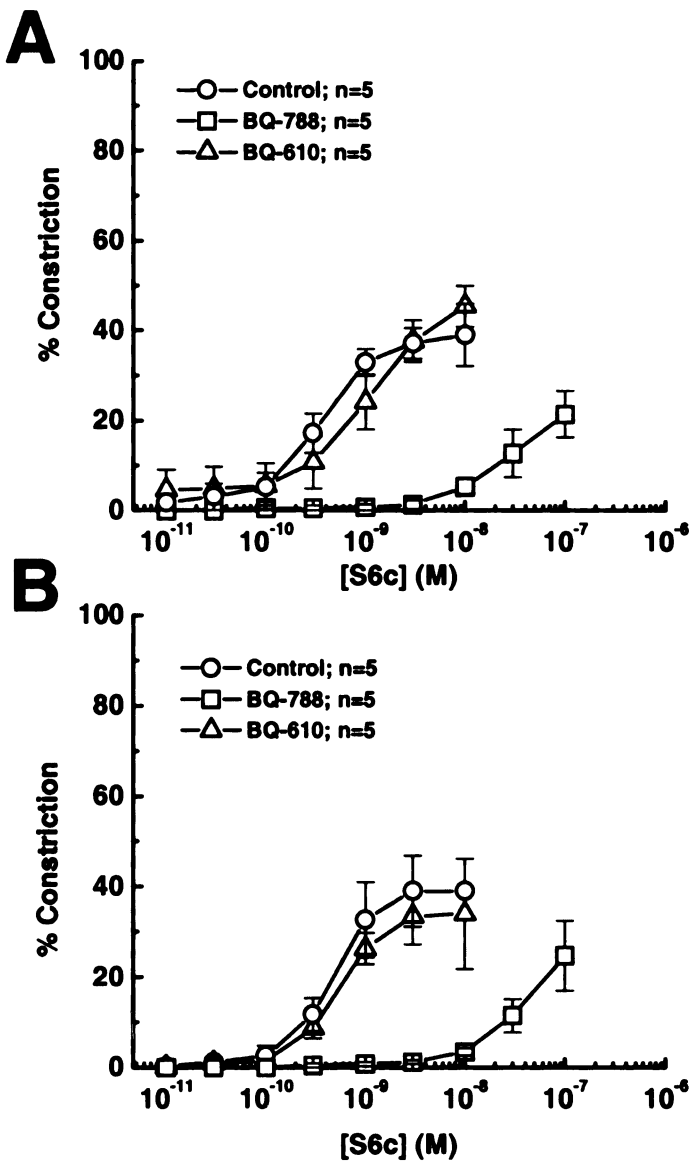

Figure 3.4: Effects of the selective ET_B receptor antagonist BQ-788 and the selective ET_A receptor antagonist BQ-610 on S6c concentration-response curves in mesenteric veins from (A) sham-operated normotensive rats and (B) DOCA-salt hypertensive rats. BQ-788 (100 nM) or BQ-610 (100 nM) were applied in the presence of indomethacin and NLA for 20 minutes prior to S6c application. S6c contractile-responses are expressed as % constriction. Data are the mean ± S.E.M., *n* refers to the number of preparations.

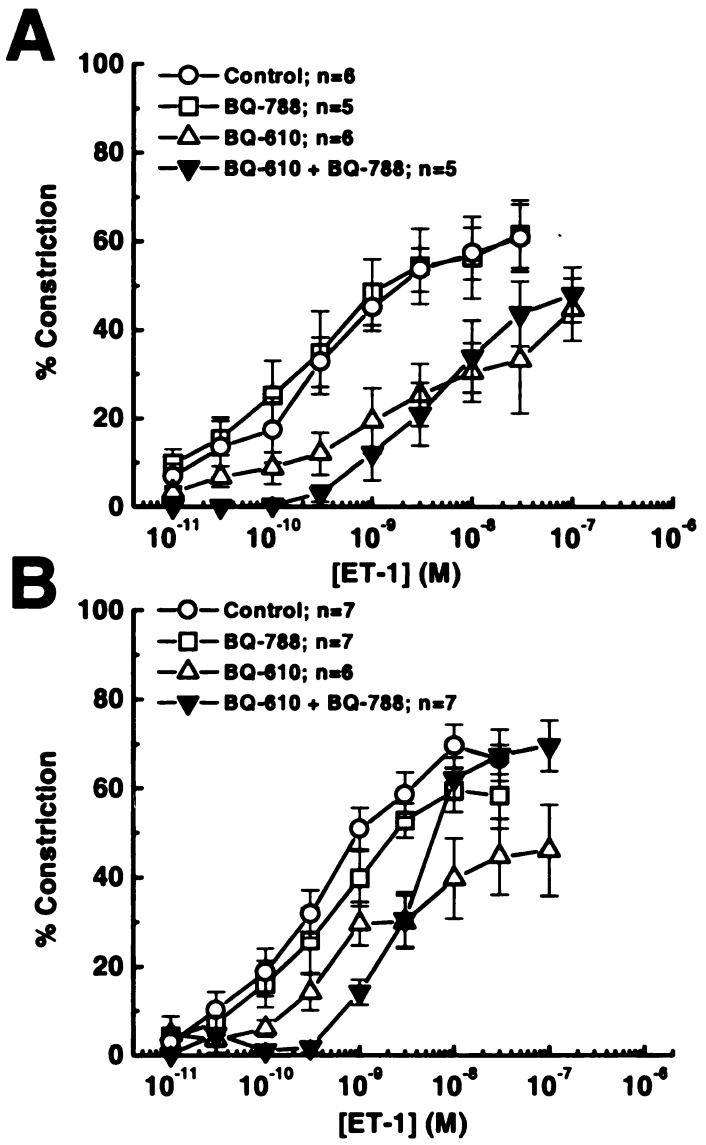

Figure 3.5: Effects of the selective ET_B receptor antagonist BQ-788 and the selective ET_A receptor antagonist BQ-610 alone or in combination on ET-1 concentration-response curves in mesenteric veins from (A) sham-operated normotensive rats and (B) DOCA-salt hypertensive rats. BQ-788 (100 nM) and/or BQ-610 (100 nM) were applied in the presence of indomethacin and NLA for 20 minutes prior to ET-1 application. ET-1 contractile-responses are expressed as a % constriction. Data are the mean ± S.E.M.,*n* refers to the number of preparations.

DISCUSSION

Systemic venous capacitance is reduced in hypertensive humans and experimental animals (Safar and London, 1987). Recent evidence indicates ET-1 is an important determinant of venous smooth muscle tone in humans (Serneri et al., 1995). Furthermore, constriction of dorsal hand veins to ET-1 was significantly greater in humans with essential hypertension than in normotensive controls (Haynes et al., 1994). This suggests that increased venous reactivity to ET-1 could contribute to reduced venous compliance observed in hypertensive patients. To date, the DOCA-salt model of experimental hypertension provides the most convincing evidence for a role of ET-1 in hypertension (Schiffrin, 1995a; 1998). I recently provided evidence that ET-1 exerts a larger effect on venomotor tone in vivo in hypertensive DOCA-salt versus sham normotensive rats (Chapter 5). While several in vitro studies have characterized ET-induced changes in arterial function in this model (Deng and Schiffrin, 1992; Nguyen et al., 1992; Giulumian et al., 1998), I am unaware of studies examining venous function in vitro. Therefore, in the present study, I compared ET-receptor mediated constrictor responses in vitro in veins from DOCA-salt hypertensive and sham normotensive rats. The important new findings of my work are that venous reactivity to ET-1 is well-maintained in DOCA-salt hypertension, through a combination of ET_A and ET_B mediated constrictor mechanisms unopposed by endothelial dilators.

Changes in ET-1 sensivitity in DOCA-salt hypertension. Contractile responses to ET-1 in mesenteric veins from DOCA-salt hypertensive rats were not different from those of veins of normotensive rats. In contrast, contractile responses to ET-1 in mesenteric arteries from DOCA-salt hypertensive rats were reduced compared to arteries from sham normotensive rats. Others have reported similar results using arteries in the DOCA-salt (Hagen and Webb, 1984; Nguyen et al., 1992; Giulumian et al., 1998) and Dahl salt-sensitive (d'Uscio et al., 1997) models of experimental hypertension in the rat. The reduction in contractile responses in arteries from DOCA-salt hypertensive rats may be caused by increased vascular ET-1 production and subsequent ET receptor-downregulation. Because ETs are secreted abluminally to act on vascular smooth muscle in a paracrine/autorcrine manner (Wagner, et al., 1992), plasma ET-1 levels do not provide a reliable indication of the status of ET production in the vasculature (Davenport et al., 1990). Other studies using the DOCA-salt model in the rat, however, have demonstrated increased preproET-1 gene expression (Lariviere et al., 1993a; Day et al., 1995) and immunoreactive ET-1 content (Lariviere et al., 1993b) in aorta and mesenteric arteries. It was also shown that endothelium denuded aorta and mesenteric arteries obtained from DOCA-salt hypertensive rats demonstrate reduced ET-1 binding compared to sham normotensive rats, suggesting a reduction in vascular smooth muscle ET-receptor density (Nguyen et al., 1992). Synthesis of ET-1 by venous endothelial cells per se has not been investigated in DOCA-salt hypertension. But my findings that venoconstrictor

responses to exogenous ET-1 are well maintained (unlike in arteries) indicate that venous capacitance changes in DOCA-salt rats (Yamamoto et al., 1983; Chapter 5) could be caused by increased endogenous ET-1 mediated venoconstriction.

Effects of endothelial dilators on arterial and venous reactivity. The present study shows sham arteries had enhanced contractions in the presence of indomethacin and NLA compared to untreated controls, while arteries from DOCA-salt rats were unaffected by indomethacin and NLA. Support for these findings comes from studies of endothelium-dependent relaxation in rat aorta which have demonstrated reduced formation of NO in most models of experimental hypertension, although other factors also may contribute importantly to endothelium mediated relaxation in resistance vessels (Shimokawa and Vanhoutte, 1997). Comparison of contractile responses in the absence and presence of indomethacin and NLA in mesenteric veins from sham or DOCA-salt rats revealed no difference. These findings suggest minimal ET-mediated NO or eicosanoid release in mesenteric veins.

Mechanism for enhanced ET-1 sensitivity in veins compared to arteries. Previous studies have demonstrated enhanced reactivity to ET-1 in veins compared to arteries (Cocks et al., 1989; Riezebos et al., 1994). In the present study, results of ET-1 concentration-responses in mesenteric veins from DOCA-salt hypertensive and sham normotensive rats also reveal enhanced reactivity compared to ET-1

responses in corresponding arteries. Enhanced production of ET-1-evoked endothelial-derived vasodilators in DOCA-salt arteries versus veins could account for a reduction in arterial contractions compared to contractile responses in veins. This explanation seems unlikely since even in the presence of indomethacin and NLA veins remained more sensitive to the contractile effects of ET-1 than were arteries. Because it is known that pressure can affect arterial sensitivity to contracile agonists, a separate experiment was conducted comparing ET-1 contractions in unpressurized versus pressurized arteries. In this experiment, concentration-responses to ET-1 in pressurized arteries from DOCA-salt versus sham rats were similar to findings in unpressurized arteries from DOCA-salt versus sham rats. Therefore, differences in ET-1 reactivity between veins and arteries in the present study were not due to a lack of pressurization of arteries. The distribution of ET-receptor subtypes in vascular tissue can vary with animal species and between vascular beds, however, vascoconstrictor responses to ET-1 in resistance arteries are largely mediated by ET_A receptors (Nguyen et al., 1992; Moreland et al., 1994; Davenport et al., 1995). In contrast, contractions to ET-1 in veins involve the vascular smooth muscle ET_B receptor (Sumner et al., 1992; Riezebos et al., 1994; Haynes et al., 1995; Lodge et al., 1995). My findings of sustained contractions to S6c in mesenteric veins from both sham normotensive and DOCA-salt hypertensive rats indicate the presence of contractile ET_B receptors in rat mesenteric veins. The selective ET_A receptor antagonist BQ-610 did not change S6c responses in either DOCA-salt or sham mesenteric veins suggesting

that BQ-610 did not block ET_B receptors. However, BQ-610 blocked contractions caused by ET-1 in veins from both sham and DOCA-salt rats, demonstrating that ET_A receptors also are present in rat mesenteric veins. Application of ET-1 to arteries from sham normotensive and DOCA-salt hypertensive rats produced sustained contractions while S6c produced negligible responses. These results suggest the presence of ET_A receptors, but not contractile ET_B receptors, in rat mesenteric arteries. These findings are in agreement with other studies (Sumner et al., 1992; Moreland et al., 1994; Davenport et al., 1995), and are further supported in the present study by the significant blockade of ET-1 responses in sham normotensive arteries by BQ-610. The selective ET_B receptor antagonist BQ-788 (100 nM) caused similar rightward shifts in S6c concentration-response curves in both sham and DOCA-salt mesenteric veins when compared to control values. Surprisingly, however, ET-1 responses were not affected by BQ 788 in either sham or DOCA-salt vein preparations. This result demonstrates that the presence of ET_B receptors on venous smooth muscle does not account for the increased sensitivity of veins to ET-1 when compared to arteries.

Interactions between ET_A and ET_B receptors. An interaction may occur between vascular smooth muscle ET_A and ET_B receptors on mesenteric veins resulting in masking of the ET_B-mediated contractile effect. The existence of such an interaction between these two constrictor receptors has been proposed based on other in vitro vascular studies of arteries (Furkuroda et al., 1994b; Mickley et al.,

1997) and veins (Chapter 2). The mechanism behind the proposed interaction between the vascular smooth muscle ET_A and ET_B receptor remains unclear, but may involve receptor mediated events or interactions at the second messenger level (Furkuroda et al., 1996). Importantly, if ET_A receptor activation does suppress smooth muscle ET_B receptor-mediated contraction, the effect is less pronounced in DOCA-salt rats, since addition of BQ-788 to BQ-610 shifted the dose-response curve further right than BQ-610 alone in veins from DOCA-salt rats, but not sham rats, particularly at the lower end of the concentration-response curve. These findings may indicate a greater role for constrictor ET_B receptors in mesenteric veins of DOCA-salt hypertensive rats versus sham normotensive rats. A potential implication is that in hypertensive animals combined ET_A/ET_B receptor blockade could produce larger increments in vascular capacitance than ET_A antagonism alone. This has relevance for development of this class of drugs as clinical tools for treating hypertension and heart failure (Rubanyi and Polokoff, 1994; Schiffrin, 1995).

Summary and conclusions. In conclusion, the results of this study show that activation of either ET_A or ET_B receptors on the vascular smooth muscle causes constriction in rat mesenteric veins that is similar in DOCA-salt and sham normotensive rats. Endothelial dilators do not oppose ET-1 venoconstriction in sham or DOCA-salt rats. Although, ET-1 appears to contract mesenteric veins mainly via activation of ET_A receptors, combined ET_A/ET_B receptor blockade is more

effective than ET_A receptor antagonism at inhibiting venous contractile responses to ET-1, perhaps due to an undefined interaction between the receptors or their signaling pathways. In contrast, contractions in mesenteric arteries are mediated exclusively by ET_A receptors, and are reduced in DOCA-salt rats compared to sham normotensive rats. These results suggest that net venoconstrictor activity produced by ET-1 in vivo is significantly larger in DOCA-salt hypertensive versus normotensive rats due to increased ET-1 formation (Schiffrin, 1995a) rather than enhanced venous smooth muscle reactivity.

Chapter 4

Factors Affecting Endothelin-Induced Venous Tone in Conscious Rats

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INTRODUCTION

ET-1 is known to cause a transient depressor response followed by a sustained pressor response in vivo (Yanagisawa et al., 1988a). The initial vasodilation is due to the actions of ET-1 at endothelial-cell ET_B receptors resulting in release of NO and prostacyclin (Rubanyi and Polokoff, 1994). The long-lasting pressor response is due to the activation of VSM ET_A receptors and ET_B receptors (Karaki et al., 1993). Recently, I have shown that ETs produce potent venoconstriction in guinea pig mesenteric veins in vitro through activation of VSM ET_A and ET_B receptors (Chapter 2). Results of in vivo studies consistently show that i.v. infusion of ET-1 in rats produces a prolonged increase in mean arterial blood pressure (MABP), increases in both venous and arterial resistance, and at high doses, an increase in MCFP (Waite and Pang, 1990; 1992; Palacios et al., 1997a; 1997b). MCFP is representative of whole body integrated venomotor tone, and provides the driving force for venous blood return to the heart (Guyton, 1955). Elevated venous tone has been observed in human hypertension (Schobel et al., 1993) and in several models of experimental hypertension (Ricksten et al., 1981; Chapter 5). An increase in venous tone may be compensatory, maintaining cardiac filling in the face of reduced cardiac compliance (Safar and London, 1987). Alternatively, increased venous tone could result in an increase in the pressure for venous return and a subsequent increase in cardiac output (Martin et al., 1998) thereby contributing to hypertension development. While the cause of elevated

venomotor tone observed in hypertension is unclear, it is known that compliance of the venous system is under the direct influence of the sympathetic nervous system and vasomodulatory substances such as the endothelial cell-derived factors, ET-1 and NO (Safar and London, 1987; Chapter 2; Chapter 5).

Changes in venous function may be especially important in the pathophysiology of those forms of hypertension known to be strongly salt-sensitive (Simon, 1978; 1981; Yamamoto et al., 1983; Simone et al., 1993). Several forms of experimental hypertension known to be salt-dependent also show ET-1 involvement in their pathogenesis, including the deoxycorticosterone acetate-salt (Schiffrin, 1999; Chapter 5), Dahl (Kassab et al., 1997) and reduced renal mass (Potter et al., 1997) models. Finally, in instrumented conscious rats, a high salt intake was necessary to produce sustained hypertension during chronic infusion with ET-1 (Mortensen and Fink, 1992). These findings suggest ETs may be a critical factor in salt-sensitive hypertension through control of venomotor tone. For example, high salt intake could increase venomotor responses to ET-1. Therefore, one aim of the present study was to evaluate the effects of differing salt intakes on acute endothelin-induced changes in venomotor tone using repeated measurements of MCFP in intact, conscious animals. Because ET-induced venoconstriction is dependent on activation of VSM ET_A and ET_B receptors, experiments were conducted with the selective ET_B receptor agonist S6c and ET-1, which is a mixed ET receptor agonist.

The vascular endothelium serves an important role in the control of arterial

tone, particularly through the release of the vasodilators, NO and prostanoids. Endothelium-dependent relaxations to acetylcholine are reduced in arteries from several models of experimental hypertension in which reductions in NO production predominates (Shimokawa and Vanhoutte, 1997). Alterations in arterial endothelial function have therefore been implicated in the pathophysiology of hypertension. The role of the endothelium in modulating venous tone is unclear but impaired release of endothelial dilators could contribute to decreased vascular capacitance by increasing venomotor tone (De Mey and Vanhoutte, 1982). In conscious, unrestrained rats, inhibition of NOS with N^G-nitro-L-arginine methyl ester (L-NAME) dose-dependently increased MABP but not MCFP, both in the absence and presence of a ganglion blocker (Wang et al., 1995). I am unaware of in vivo studies examining the effects of blockade of endothelial dilators on ET-peptide induced changes to venomotor tone. Therefore, a second aim of this study was to evaluate the effects of NOS and cyclooxygenase inhibition on ET-peptide changes to venomotor tone in intact, awake rats. Because ET-induced dilator release by the endothelium is mediated by the ET_B receptor, the selective ET_B receptor agonist S6c was chosen over ET-1 for these experiments. I hypothesized that venoconstrictor effects of S6c would be significantly increased by blockade of NOS and cyclooxygenase.

METHODS

Animals. Male Sprague-Dawley rats weighing 350-375 g (Charles River, Portage, MI) were maintained according to standards approved by the Michigan State University All-University Committee on Animal Care and Use. Rats were housed in clear plastic boxes in groups of three with free access to standard pelleted rat chow (Harlan/Teklad 8640 Rodent Diet) and tap water. All rats were housed in a light and temperature controlled room and maintained in strict accordance with Michigan State University and National Institutes of Health animal care guidelines. Rats were acclimatized to their environment for 2 days prior to surgical manipulation.

Catheterization. At the time of catheterization the average body weight of the rats was 406 ± 10 g. Catheters were permanently placed in each rat for measurement of arterial and central venous pressures, for drug administration, and to produce brief circulatory arrest. Anesthesia was produced using an i.p. injection of sodium pentobarbital (50 mg/kg), and atropine (0.2 mg/kg, i.p.) was given to decrease bronchial secretions. Silicone rubber tipped catheters were inserted through the internal iliac artery and vein coming to rest in the abdominal aorta and thoracic vena cava, respectively. A silicone rubber tipped balloon catheter (fabricated in the lab from 0.047" OD silicone tubing) was advanced via the right jugular vein into the right atrium. It was secured at a location where rapid inflation

with 0.15-0.25 ml saline caused a smooth decline in arterial pressure to ~25 mmHg and a simultaneous rise in central venous pressure to 6-8 mmHg within 4-5 seconds of circulatory arrest. The ends of all catheters were tunneled subcutaneously to the head, where they exited the rat inside a stainless steel spring secured to the skull with jeweler's screws and dental acrylic. Injections of enrofloxacin (5 mg/kg, i.v.) and butorphanol tartate (2 mg/kg, i.v.) were given for bacterial prophylaxis and post-operative pain relief, respectively. 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 daily to all rats for a three day period after surgery. Vascular catheters were filled with heparin solutions when not in use and flushed daily. In order to prevent adhesions to the atrial wall, balloon catheters were partially inflated for 3-5 seconds daily.

Hemodynamic measurements. Arterial pressure was determined by connecting the arterial catheter to a low volume displacement pressure transducer (TXD-300, Micro-Med, Louisville, KY) linked to a digital pressure monitor (BPA-200 Blood Pressure Analyzer, Micro-Med) that derived systolic, mean and diastolic pressures and heart rate every 0.5 seconds (sampling rate = 1000 Hz). All values were averaged minute-by-minute and saved using a computerized data acquisition system (DMSI-200/4 System Integrator, Micro-Med), except during measurement

of MCFP. The venous catheter was connected to a separate pressure transducer and digital monitor which allowed similar continuous recordings of central venous pressure (CVP). The transducer used to measure CVP was zeroed at mid-chest level of rats in a typical crouched posture, and was calibrated daily against a column of water.

Estimation of MCFP was performed according to established methods for the rat (Yamamoto et al., 1980; Waite and Pang, 1990). Briefly, the atrial balloon catheter was rapidly inflated with 0.15-0.25 ml saline for no longer than 5 seconds. This caused an immediate decline in arterial pressure, and simultaneous rise in CVP, both of which plateaued after 3-5 seconds of balloon inflation. Pressure averaging rate was increased to once per second during balloon inflation. This method however does not allow full equalization of pressure throughout the circulation due to "trapping" of blood on the low compliance arterial side. To correct for this, MCFP was computed from venous plateau pressure (VPP) and arterial plateau pressure (APP) using the formula:

$$MCFP = VPP + (APP - VPP)/60$$

Assuming a ratio of venous to arterial compliance of 60 in the rat (Palacios et al., 1997a), this method has been shown previously to produce values of MCFP nearly identical to those obtained by completely equalizing VPP and APP by pumping blood from the arterial to the venous side of the circulation (Yamamoto et al., 1980). Nevertheless, the correction needed for blood trapping is always very small, averaging ~ 0.4 mmHg (Samar and Coleman, 1979).

In order to allow time for the circulation to restabilize, each measurement of MCFP was taken at least 10 minutes after the previous one. The rats exhibited no visible adverse reactions to balloon inflation and deflation.

Experimental protocols. Experimentation began at least 4 days after surgery for catheter and balloon implantation. Catheters were flushed and attached to pressure transducers. Rats were then allowed to sit undisturbed for 10-20 minutes, with all hemodynamic measures being averaged and recorded in one minute time bins. The first control measurement of MCFP was obtained (labeled -10), followed 10 minutes later by a second control measurement (labeled 0). Then drug or drug vehicle (0.9% NaCl) was injected intravenously. Measurements of MCFP were repeated every 15 minutes for one hour (labeled 15, 30, 45 and 60). Values reported for MABP, HR and CVP are the average of the values obtained during the last three minutes before balloon inflation.

The effects of low salt (sodium deficient test diet, TD 170950, Harlan/Teklad, distilled water and furosemide at 5 mg/kg i.v. daily for 2 days to ensure sodium depletion), normal salt (normal rat chow and tap water) and high salt (normal rat chow and drinking water containing 1% NaCl / 0.2% KCl) intake on responses to ET receptor agonists were investigated using the above protocol. Some rats were tested on all three levels of salt intake, while others were randomly assigned to a salt intake group. Rats were placed on each salt intake protocol for 4 days prior to initiation of experiments. Rats then received either drug vehicle or the selective

ET_B receptor agonist S6c (500 pmol) or ET-1 (500 pmol) as a bolus i.v. injection (0.1 ml/100 g body weight). Only one protocol was run per day and the order was randomized. The contribution of endothelial dilator release to MCFP changes in response to S6c was evaluated in rats on normal salt intake by pretreating rats with either the cyclooxygenase inhibitor indomethacin (2.5 mg/kg) or the NOS inhibitor L-NAME (10 mg/kg) as a bolus i.v. injection (0.1 ml/100 g body weight) thirty minutes prior to S6c injection.

Drugs. Stock solutions of indomethacin (2.5 mg/ml; Sigma, St. Louis, MO) were prepared in sodium carbonate (0.01 M; Sigma, St. Louis, MO) while stock solutions of L-NAME (10 mg/ml; Sigma, St. Louis, MO), furosemide (5 mg/ml; Sigma, St. Louis, MO) and heparin saline (100 U/ml) were prepared in physiological saline (0.9% NaCl; Butler, Columbus, OH). ET-1 (Peninsula Laboratories, Belmont, CA) and S6c (Peninsula Laboratories, Belmont, CA) were prepared in physiological saline.

Statistical analyses. Comparisons between control period values were made using a mixed design ANOVA; where significant overall between groups differences were found, comparisons at individual times were achieved by testing simple main effects. Changes in hemodynamic variables within groups over time were evaluated using the method of contrasts, where values at each time point after drug injection were compared to the average of the two control period values.

For all tests the significance level was set at $P < 0.05$.

RESULTS

No consistent differences in resting MABP, HR, CVP or MCFP were found at the different levels of salt intake. The effects of salt intake on hemodynamic responses to administration of ET-1 are shown in Figure 4.1. ET-1 produced a significant increase in MABP in all groups which was more sustained in the high salt group than in the normal and low salt groups. Peak effects did not differ between groups. A brief reduction in HR was also noted in all three groups following i.v. administration of ET-1. Small increases in MCFP occurred following administration of ET-1, however these changes were not significant.

Figure 4.2 shows hemodyamic changes following administration of the selective ET_B receptor agonist S6c to rats on different salt intakes. MABP was significantly increased by S6c in all three groups, and then gradually returned to control period values. S6c also produced a brief bradycardia in all three treatment groups. While all groups showed a similar increase in MCFP following S6c administration, statistical significance was reached only in rats in the high salt group.

Hemodynamic responses to 0.9% NaCl, indomethacin (2.5 mg/kg) and L-NAME (10 mg/kg) in rats on normal salt intake are shown in Figure 4.3. No significant differences in hemodynamic variables were detected between groups during the control period with the exception of a higher CVP in the L-NAME group. No significant changes were noted in MABP, HR, CVP or MCFP over the 70 minute

protocol following injections of 0.9% NaCl (control group). Hemodynamic variables after administration of indomethacin (2.5 mg/kg) were not different from control period values. Administration of L-NAME (10 mg/kg) to rats resulted in a sustained, significant increase in MABP and a decrease in HR compared to control period values. L-NAME administration reduced MCFP, however statistical significance was not achieved.

Hemodynamic responses to the selective ET_B receptor agonist S6c (500 pmol) in rats on normal salt intake and following pre-treatment with 0.9% NaCl, indomethacin and L-NAME are shown in Figure 4.4. MABP and HR values were significantly different in L-NAME pre-treated rats when compared to indomethacin and 0.9% NaCl pretreated rats. Surprisingly, administration of S6c resulted in a sustained, significant reduction in MABP in the L-NAME group while MABP significantly and similarly increased in the indomethacin and 0.9% NaCl treated groups. Again, bolus injection of S6c did not significantly increase MCFP in rats on normal salt intake, even after pre-treatment with indomethacin or L-NAME.

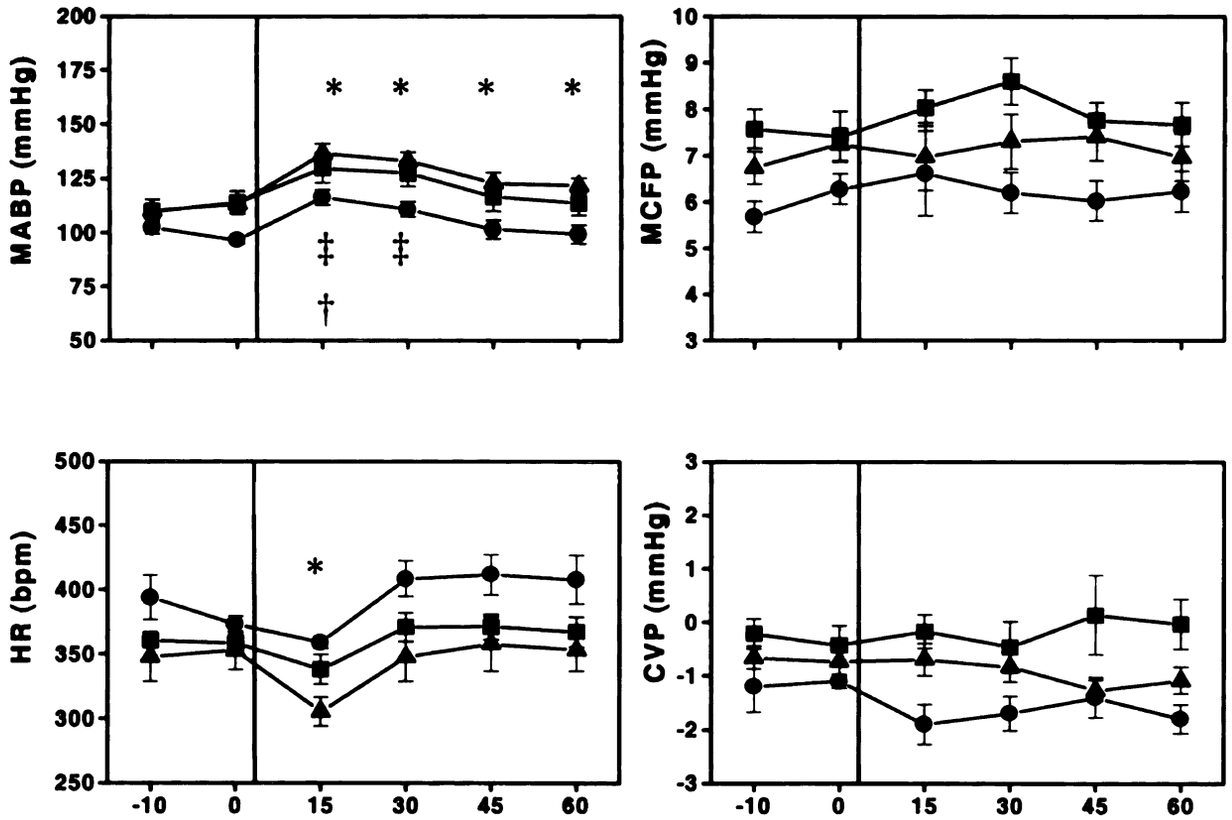

Figure 4.1: The response of hemodynamic variables to ET-1 (500 pmol) given intravenously immediately after the 0 time point to rats on low (●; n=6), normal (■; n=10) and high (▲; n=10) salt diets. Treatment administration indicated on figure by vertical line. Mean arterial blood pressure=MABP. Heart rate=HR. Central venous pressure=CVP. Mean circulatory filling pressure=MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Symbols (*= high salt; †= normal salt; ‡= low salt) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

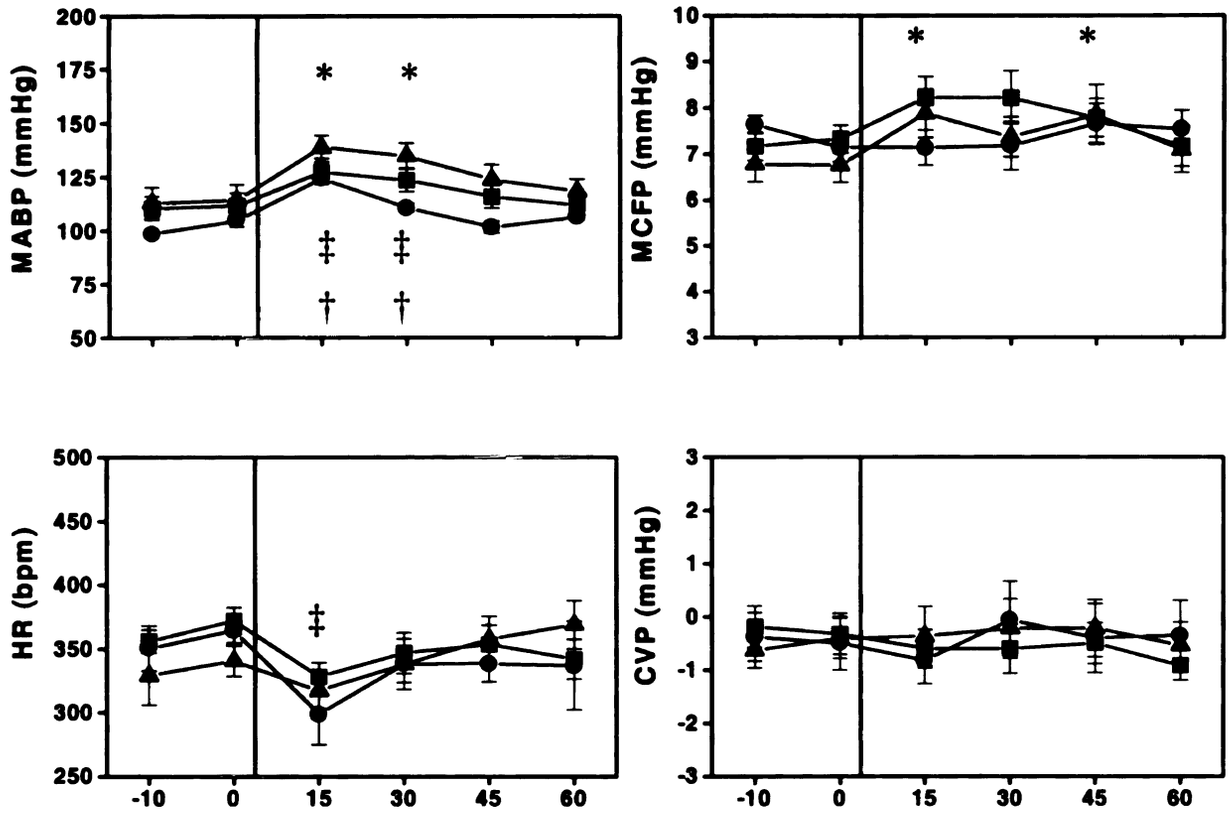

Figure 4.2: The response of hemodynamic variables to S6c (500 pmol) given intravenously immediately after the 0 time point to rats on low (●; n=5), normal (■; n=10) and high (▲; n=10) salt diets. Treatment administration indicated on figure by vertical line. Mean arterial blood pressure=MABP. Heart rate=HR. Central venous pressure=CVP. Mean circulatory filling pressure=MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Symbols (*= high salt; †= normal salt; ‡= low salt) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

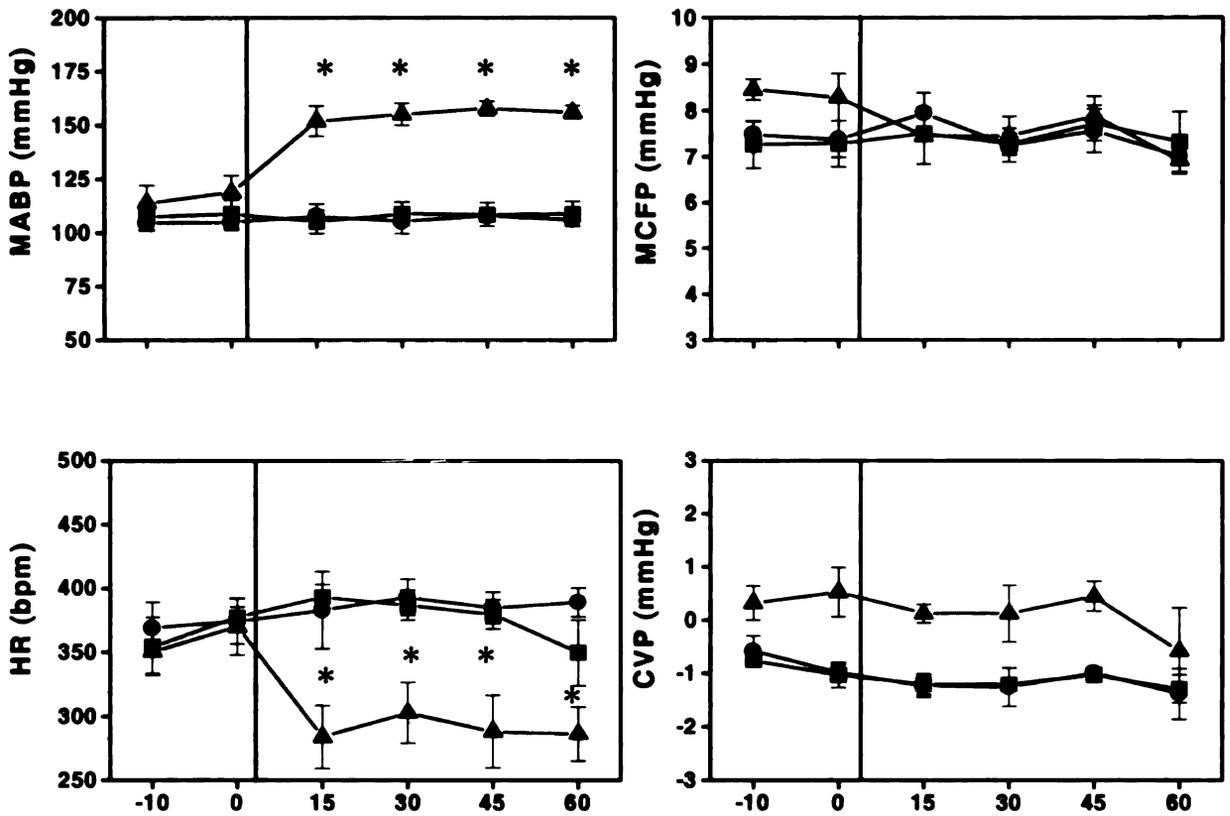

Figure 4.3: The response of hemodynamic variables to 0.9% NaCl (●; n=5), 2.5 mg/kg indomethacin (■; n=5) or 10 mg/kg L-NAME (▲; n=5) given intravenously immediately after the 0 time point. Treatment administration indicated on figure by vertical line. Mean arterial blood pressure=MABP. Heart rate=HR. Central venous pressure=CVP. Mean circulatory filling pressure=MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Symbols (*= L-NAME; †= indomethacin; ‡= 0.9% NaCl) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

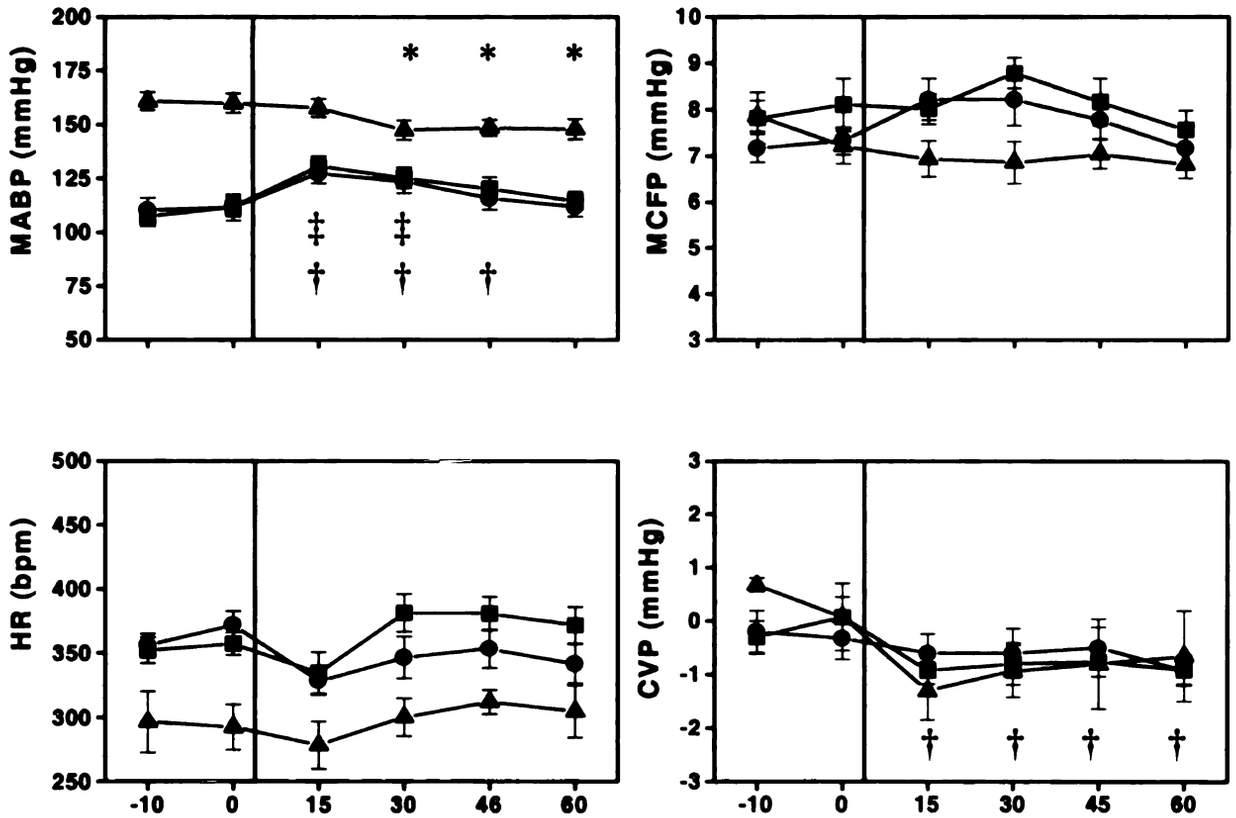

Figure 4.4: Hemodynamic responses to the selective ET_B receptor agonist S6c (500 pmol) given intravenously immediately after the 0 time point to rats pre-treated with 0.9% NaCl (●; n=10), 2.5 mg/kg indomethacin (■; n=10) or 10 mg/kg L-NAME (▲; n=5). Treatment administration indicated on figure by vertical line. Mean arterial blood pressure=MABP. Heart rate=HR. Central venous pressure=CVP. Mean circulatory filling pressure=MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Symbols (*= L-NAME; †= indomethacin; ‡= 0.9% NaCl) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

DISCUSSION

I observed only a small increase in MCFP in rats following i.v. administration of ET-1 or S6c, but MABP increased markedly and significantly. These findings suggest that the doses of ET-1 and S6c administered to rats in the present study was more efficacious in elevating MABP than MCFP. Other in vivo studies using instrumented awake rats on normal salt intake also have reported small increases in MCFP accompanying significant increases in MABP after injection of ET peptides (Waite and Pang, 1990; 1992). These findings are surprising given that in vitro studies consistently show that veins are more sensitive to the vasoconstrictor effects of ET-1 than corresponding arteries (Riezebos et al., 1994; Chapter 3). Venomotor tone is determined by neurotransmitters released from sympathetic nerve terminals and circulating and locally produced vasoactive factors (Monos et al., 1995). It is generally accepted however that sympathetic nerves have the greatest quantitative importance in minute-to-minute regulation of venomotor activity (Shoukas and Bohlen, 1990; Monos et al., 1995). Therefore, the disparity of hemodynamic changes in MABP compared to MCFP following injections of ET-1 or S6c may be explained by relatively stronger baroreflex mediated decreases in sympathetic tone to the veins compared to the arteries. Support for this comes from studies in conscious rats showing that sympathetic reflexes have a greater effect on MCFP than MABP changes elicited by vasoactive drugs (Waite et al., 1988; Waite and Pang, 1992).

Altered venous function has been implicated in the pathogenesis of human hypertension (Martin et al., 1998), and may be particularly important in salt-sensitive forms of hypertension (Simon, 1978; 1981; Simone et al., 1993). In addition, several experimental models of salt-sensitive hypertension demonstrating increased venous tone also show ET-1 involvement (Sventek et al., 1996; Chapter 5). Athough increased salt intake does not appear to have a consistent effect on vascular ET-1 formation (Michel et al., 1993; Ishimitsu et al., 1996; Oh et al., 1997), chronic hypertension produced by exogenous ET-1 administration is salt-dependent (Mortensen and Fink, 1992). This observation suggests that high salt intake could potentiate ET-induced changes in venomotor tone. Therefore, the first goal of this experiment was to test the effects of varying salt intakes on acute ET-induced changes to MCFP (an index of venomotor tone). I hypothesized that high salt intake would increase venomotor responses to ET-1. However, my results showed no change in MCFP after acute administration of ET-1 or S6c in rats on normal, high or low salt intake. Interestingly, the same was true for MABP and HR responses. Thus, salt intake does not appear to affect acute arterial or venous responses to ET peptides. This indicates that any contribution of endogenous ET-1 to control of venomotor activity secondary to changes in salt ingestion, at least in normotensive rats, is not due to alterations in vascular reactivity.

Endothelial factors such as NO and prostanoids may affect systemic venous tone (Rubanyi and Polokoff, 1994; Shimokawa and Vanhoutte, 1997). A deficiency in the production or action of these substances could contribute to decreased

vascular capacitance in hypertension. Furthermore, the only modest changes in MCFP observed after acute administration of low doses of ET peptides (Waite and Pang, 1992; Palacios et al., 1997a, 1997b) could result from direct venoconstrictor effects being opposed by the release of endothelial venodilators. This phenomenon was demonstrated in vitro in guinea pig mesenteric veins exposed to S6c (Chapter 2). Constriction of hand veins in vivo by S6c in humans was potentiated by cyclooxygenase inhibition with aspirin or inhibition of NO synthesis with L-NMMA (Strachan et al., 1995). However, constriction of hand veins to ET-1 was potentiated by aspirin but not by L-NMMA (Webb and Haynes, 1993), indicating cyclooxygenase products may be more important in controlling venomotor tone than NO. ET_B receptor activation opposed the effects of ET-1 on MCFP (Palacios et al., 1998), but inhibition of NO synthesis alone caused only modest changes in MCFP in conscious rats (Glick et al., 1993; Wang et al., 1995). Therefore, experiments were performed here to test the hypothesis that endothelium-derived NO or prostanoids importantly modify the response to MCFP to acute administration of S6c.

Confirming the results of others (Wang et al., 1995), L-NAME administration to intact conscious rats caused large increases in MABP and decreases in HR, but no significant change in MCFP. The failure of MCFP to increase could have been due in part to reflex withdrawal of sympathetic activity to veins, but previous studies (Wang et al., 1995) do not support this idea. Blockade of cyclooxygenase with indomethacin also did not affect MCFP, or any other hemodynamic variable. This

result shows for the first time that endogenous prostanoids do not regulate MCFP in conscious rats. Endothelial dilators apparently do not make a large contribution to control of venous capacitance in the intact rat under normal circumstances, possibly because shear stress is relatively low on venous endothelial cells (Monos et al., 1995). Furthermore, since neither L-NAME nor indomethacin potentiated MCFP responses to acute injection of S6c, it is unlikely that venomotor effects of ET peptides are importantly modulated by endothelial NO or prostanoids. These results are consistent with my in vitro findings in mesenteric veins from sham and DOCA-salt rats (Chapter 3). The data do not then support the idea that increased ET-1 mediated venomotor tone and resultant decreases in vascular capacitance in hypertension could be caused by impaired endothelial dilator release. More chronic studies are required to test that idea fully. Interestingly, administration of S6c to indomethacin and 0.9% NaCl pretreated rats produced an increase in MABP while the L-NAME pretreated group showed a paradoxical decrease in MABP. These findings are difficult to interpret since S6c and L-NAME given individually both increased MABP.

In conclusion, neither basal MCFP nor integrated venomotor responses to acute injection of ET-1 or S6c into conscious rats were significantly altered by short-term changes in salt intake, or blockade of NO synthase or cyclooxygenase. The data do not support the hypothesis that acute alterations in reactivity of capacitance vessels to endothelial cell factors contributes to the etiology of salt-sensitive hypertension.

Chapter 5

Mechanisms of Increased Venous Smooth Muscle Tone in Deoxycorticosterone Acetate-salt Hypertension

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INTRODUCTION

The effective driving force for venous return to the heart, MCFP, is usually increased in hypertension (Martin et al., 1998). This important physiological change is necessary to maintain normal cardiac output in the face of reduced diastolic compliance of the ventricles (Safar and London, 1987). Blood volume (BV) and compliance of the venous system are the major determinants of MCFP (Yamamoto et al., 1980). In established hypertension BV is either normal or reduced (Safar and London, 1987). Thus, elevated MCFP in hypertensives is caused by structural changes in the venous wall and/or increased venous smooth muscle contractile activity (i.e. venomotor tone). Reduced compliance of the extrathoracic venous system has been documented in humans (Schobel et al., 1993) and rats (Ricksten et al., 1981) with established hypertension.

The mechanisms responsible for adjustments of venous capacitance function in hypertension have not been completely defined. Current evidence indicates a small but significant role for structural modifications (Yamamoto et al., 1980, Mark, 1984), and an important contribution of sympathetically mediated venoconstriction in humans and experimental animals (Willems et al., 1982; Mark, 1984; Martin et al., 1998). Older studies also implicate an unknown humoral mechanism operating in some models of experimental hypertension (Simon, 1978).

The endothelium-derived peptide, ET-1, is a potent venoconstrictor in most vascular beds (Cocks et al., 1989). Changes in endogenous ET-1 formation

consistent with a role for the peptide in venomotor adjustments to volume stimuli have been measured in humans (Serneri et al., 1995). Interestingly, although ET-1 formation typically is not increased in hypertension (Schiffrin, 1998), forearm venoconstriction in response to exogenous ET-1 is significantly greater in patients with essential hypertension (Haynes et al., 1994). Venomotor tone induced by sympathetic nervous system activation also is potentiated by ET-1 in essential hypertensive, but not normotensive patients (Haynes et al., 1994). I recently studied contractile responses in vitro to ET-1 in mesenteric veins from rats with DOCA-salt hypertension (Chapter 3). This is an experimental model in which ET-1 formation in vascular tisssue is increased, and ET-1 is known to have a significant pathophysiological role (Schiffrin, 1998). Venoconstrictor responses to ET-1 were mediated through both ET_A and ET_B subtypes of endothelin receptor. Unlike in arteries, however, where contractile responses to ET-1 are decreased (Nguyen et al., 1992), presumably due to receptor downregulation, contraction of veins to ET-1 were well-maintained in DOCA-salt rats. The majority of the response was mediated through ET_A receptors. The present study was designed to extend those observations by exploring the contribution of endogenous ET-1 to venous function in vivo in rats with DOCA-salt hypertension. Venomotor tone was assessed using repeated measurements of MCFP in conscious, undisturbed animals.

METHODS

Animals. Experiments were performed in male Sprague-Dawley rats (Charles River Laboratories, Madison, WI) weighing 175-225 grams at the beginning of the study. All protocols were approved by the Michigan State University Committee on Animal Use and Care. Until the time of catheterization rats were housed 2-3 per cage in a temperature and humidity controlled room under a 12 hour on / 12 hour off light cycle. Free access was allowed to standard laboratory rat chow (Harlan/Teklad 8640 Rodent Diet). Housing was in strict accordance with National Institute of Health and Michigan State University care guidelines.

DOCA-salt hypertension. DOCA-salt hypertension was induced by the methods of others (Ormsbee and Ryan, 1973). Briefly, rats were unilaterally nephrectomized under anesthesia with sodium pentobarbitol (45 mg/kg, i.p.; Abbott Laboratories, Abbott Park, IL). Bronchiolar secretions were controlled by administration of atropine sulfate (0.04 mg/kg, i.p.; Sigma, St. Louis, MO). Silicone rubber patches (Dow Corning, Ferndale, MI) impregnated with deoxycorticosterone acetate (DOCA ;Sigma, St. Louis, MO) were implanted s.c. in rats providing DOCA at 150 mg/kg. Postoperative analgesia was provided by a single injection of butorphanol tartrate s.c. (0.5 mg/kg; Abbott Laboratories, Abbott Park, IL). All DOCA-implanted rats were placed on salt water containing 1 % NaCl and 0.2 % KCl. Normotensive control rats were unilaterally nephrectomized and provided tap

water. All rats were fed standard pelleted rat chow. Rats were maintained on the above protocols for 3 weeks prior to surgical catheterization.

Catheterization. See Chapter 4 page 99.

Hemodynamic measurements. See Chapter 4 page 100.

Volume measurements. Plasma volume (PV) was estimated using the 10-minute distribution volume of Evan's Blue dye. Hematocrit (Hct) was measured in duplicate from an arterial blood sample. Blood volume (BV) was computed using the formula:

$$BV = PV / [1 - Hct(0.8)/100]$$

The value of 0.8 corrects for differences in arterial and whole body hematocrit.

Experimental protocols. Experimentation began 2 days after surgery for catheter and balloon implantation. Catheters were flushed and attached to pressure transducers. Rats were then allowed to sit undisturbed for 10-20 minutes, with all hemodynamic measures being averaged and recorded in one minute time bins. The initial protocol in all rats was a time control. The first control measurement (labeled -10) of MCFP was obtained, followed 10 minutes later by a

second control measurement (labeled 0). Then drug vehicle (saline or a mixture of 70% water, 10% ethanol and 20% propylene glycol) was injected intravenously. Measurements of MCFP were repeated every 15 minutes for one hour (labeled 15, 30, 45 and 60). Values reported for MABP, HR and CVP are the average of the values obtained during the last three minutes before balloon inflation.

In most rats the effects of selective ET_A receptor antagonism and ganglion blockade were investigated using the same protocol in experiments conducted on subsequent consecutive days. Initially, in place of drug vehicle injections rats received either the ET_A receptor antagonist ABT-627, 1 mg/kg, i.v. or the ganglion blocker hexamethonium, 30 mg/kg, i.v. Only one protocol was run per day and the order was randomized. Finally, the ability of ET_A receptor antagonism to modify hemodynamic responses to ganglion blockade was examined by pretreating rats with ABT-627, 1 mg/kg, i.v. one hour before administering hexamethonium, 30 mg/kg, iv.

In five sham rats, immediately after completion of the studies described in the last paragraph, a 1% NaCl / 0.2% KCl solution was substituted for their drinking water; they continued to consume normal rat chow. After 3-4 days hemodynamic responses to ABT-627, 1 mg/kg, i.v. were recorded using the same protocol as previously indicated. Rats were then subjected to 3-4 days of sodium depletion. Distilled water was the sole drinking solution provided; the only food available was a modified diet containing negligible quantities of sodium (Sodium deficient test diet, TD 170950, Harlan/Teklad); and furosemide, 5 mg/kg, i.v. was administered

daily. A final determination of hemodynamic responses to ABT-627 then was obtained.

Volume measurements were made at least 24 hours after the completion of the initial hemodynamic protocols.

Statistical analyses. Comparisons between sham and DOCA-salt rats were made using a mixed design ANOVA; where significant overall between groups differences were found, comparisons at individual times were achieved by testing simple main effects. Changes in variables within groups over time were evaluated using the method of contrasts, where values at each time point after drug injection were compared to the average of the two control values. Comparisons between rats on high salt versus low salt intakes were performed using a two-factor ANOVA with both factors being repeated measures. Comparisons of maximum changes to drug treatment were performed using t-tests for paired or independent samples where appropriate. For all tests the significance level was set at $P< 0.05$.

RESULTS

A total of 19 rats (sham=9, DOCA-salt=10) were included in this study. At the time of catheterization body weight in sham rats was 412±12 g and in DOCA-salt rats was 336±8 g. All rats were tested two days after catheterization in a time control protocol; additional experiments were conducted in rats not experiencing catheter or balloon failure. Balloon rupture occurred in several rats early in the study (especially in the DOCA-salt group). Prestretching the balloons for 24-48 hrs prior to implantation virtually eliminated this problem. Time control data are shown in Figure 5.1. In both sham and DOCA-salt rats all hemodynamic variables were stable over the course of the 80 minute protocol with the exception of CVP, which tended to decline gradually. This was significant only in the sham group. In DOCA-salt rats HR rose, but this achieved statistical significance only at the last time point. Compared to sham rats, DOCA-salt animals had significantly higher MAP and MCFP, whereas HR and CVP were similar. To evaluate the stability of these same measures over a period of days, control period values (-10 and 0) were averaged in 11 rats in which data could be collected over 3 consecutive days (days 2-4 after catheterization surgery). The results are shown in Figure 5.2. In sham rats MAP and HR declined significantly over the 3 days; in DOCA-salt rats those variables were stable, but MCFP rose gradually and significantly by the fourth day after catheterization.

Hemodynamic responses to administration of the ET_A receptor antagonist

ABT-627 are shown in Figure 5.3. Peak changes were defined as those occurring one hour after drug administration, since in most rats the largest change in hemodynamic variables occurred at that time. MAP decreased significantly in sham and DOCA-salt rats after ABT-627 injection, but the peak fall was significantly larger in the DOCA-salt group (-24±3 versus -8±2 mmHg). Significant increases in HR occurred in both groups; the magnitudes did not differ. CVP declined modestly but not significantly in both groups. In DOCA-salt rats ABT-627 caused a gradual but significant decrease in MCFP (peak change = -1.1±0.2 mmHg) whereas in sham rats no change occurred (peak change= +0.1±0.2 mmHg). One hour after ABT-627 MCFP was not significantly different in sham and DOCA-salt rats.

Hemodynamic responses to ganglion blockade with hexamethonium are shown in Figure 5.4. Peak changes were defined as those occuring 15 minutes (labeled 15) after drug injection, since in all but one rat the largest change in hemodynamic variables occurred at this time. MAP decreased significantly in sham and DOCA-salt rats after hexamethonium, but the peak fall was significantly larger in the DOCA-salt group (-64±6 versus -28±3 mmHg). Significant decreases in HR occurred in both groups, but the peak response in DOCA-salt rats was significantly larger (-55±14 versus -20±8 beats/min). Modest decreases in CVP were observed in both groups of rats, but these reached statistical significance only in sham animals. MCFP fell in all rats in response to hexamethonium, but the peak decrement was significantly larger in the DOCA-salt group (-3.0±0.3 versus -1.8±0.1 mmHg).

Figures 5.5 and 5.6 illustrate the hemodynamic changes in response to hexamethonium one hour after administration of ABT-627, 1 mg/kg, i.v. compared to the responses of the same rats to hexamethonium alone. The values of all hemodynamic variables were the same in pretreated and non-pretreated rats before injection of hexamethonium in sham and DOCA-salt rats. The effects of hexamethonium were not significantly altered by one-hour pretreatment with ABT-627 in either sham or DOCA-salt rats.

Figure 5.7 shows hemodynamic changes produced by ABT-627, 1 mg/kg, i.v. in sham rats maintained on high and low salt intakes for 3-4 days. During the control period, MAP was slightly but significantly higher when rats were on high salt intake , but no other variable differed between the two conditions. Injection of ABT-627 caused a small, delayed decline in MAP in rats during high salt intake but not during salt restriction. Tachycardia occurred to a similar degree under both levels of salt intake. Neither CVP nor MCFP was affected by ABT-627 under high or low salt intake conditions.

Blood volume averaged 61.9±2.5 ml/kg in five sham rats and 69.3±2.2 ml/kg in four DOCA-salt rats. This difference did not achieve statistical significance ($p=0.07$).

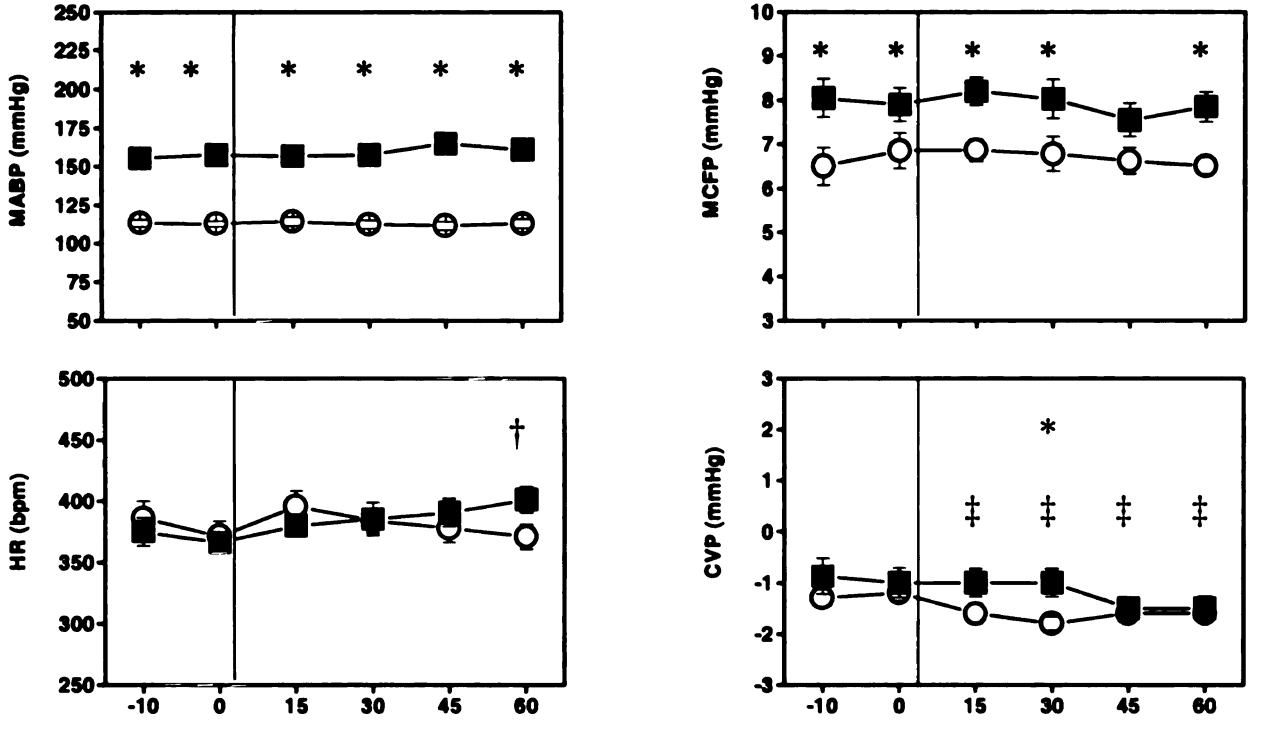

Figure 5.1: The response of hemodynamic variables to time (protocol) in minutes with drug vehicle (saline or mixture of 70% water, 10 % ethanol and 20% propylene glycol) given i.v. immediately after the 0 time point in sham-operated (○; n=9) and deoxycorticosterone acetate-salt treated rats (■; n=10). Vertical line = treatment administration. Mean arterial blood pressure= MABP. Heart rate= HR. Central venous pressure= CVP. Mean circulatory filling pressure= MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Asterisks (*) indicate a significant difference between sham and DOCA-salt values at a specific measurement time. Crosses (†= DOCA-salt; ‡= sham) indicate significant difference between 15-60 minute time point value compared to the average of the control time point values. $P<0.05$ considered statistically significant.

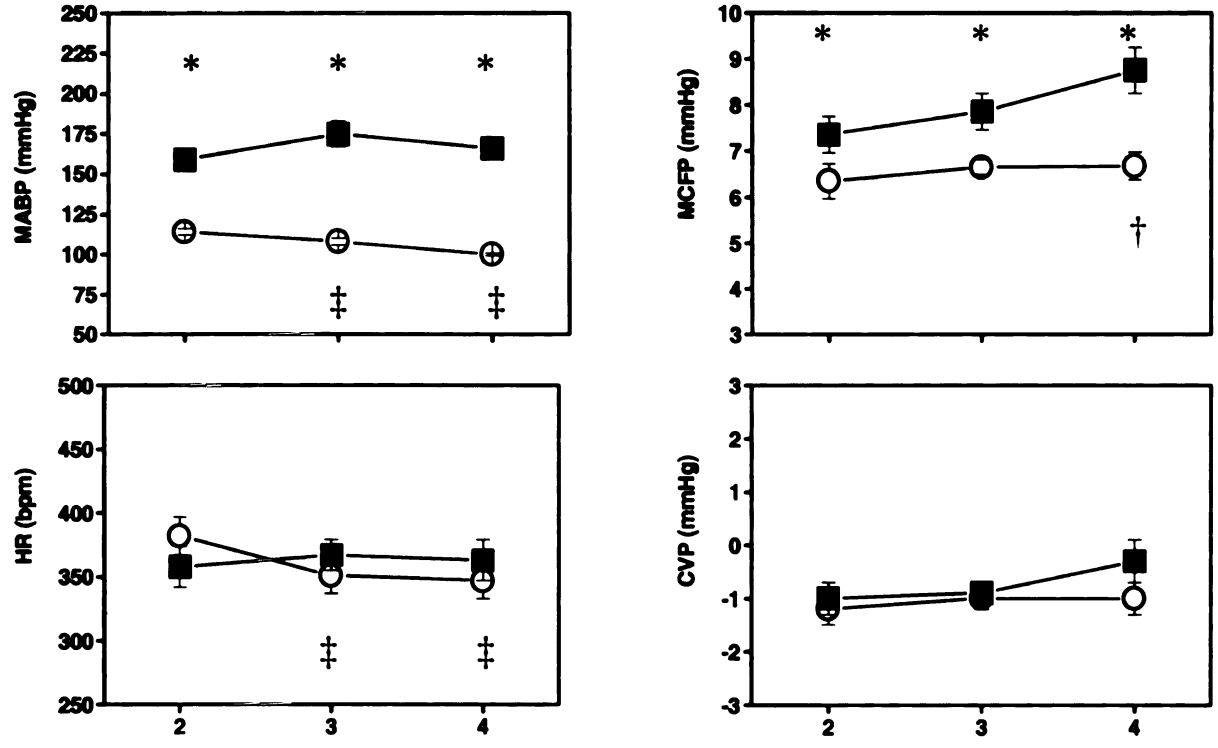

Figure 5.2: The response of hemodynamic variables to time (days after catheterization) with drug vehicle (saline or mixture of 70% water, 10 % ethanol and 20% propylene glycol) given i.v. immediately after the 0 time point in sham-operated (○; n=6) and deoxycorticosterone acetate-salt treated rats (■; n=5). Mean arterial blood pressure= MABP. Heart rate= HR. Central venous pressure= CVP. Mean circulatory filling pressure= MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Asterisks (*) indicate a significant difference between sham and DOCA-salt values at a specific measurement time. Crosses (†= DOCA-salt; ‡= sham) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

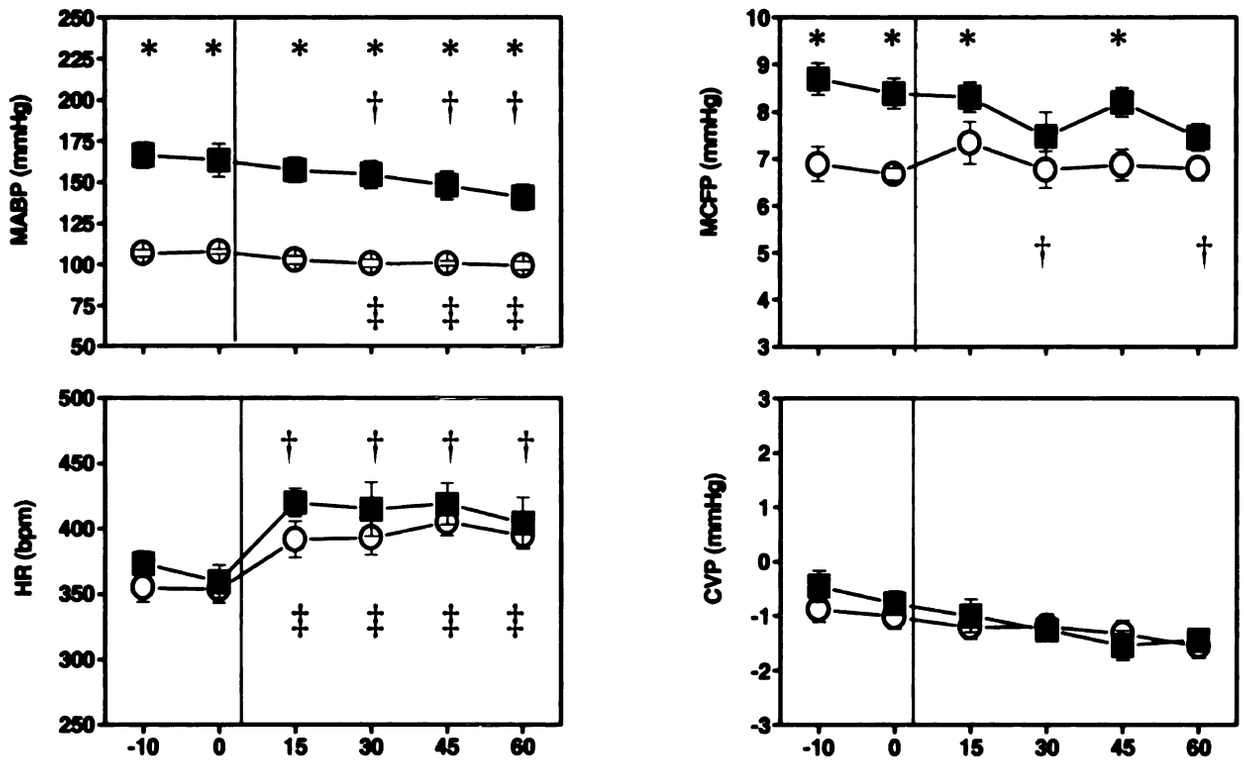

Figure 5.3: The response of hemodynamic variables to selective endothelin subtype A receptor blockade with ABT-627 (1 mg/kg; i.v.) given immediately after the 0 time point in sham-operated (○; n=9) and deoxycorticosterone acetate-salt treated rats (■; n=8). Vertical line = treatment administration. Mean arterial blood pressure= MABP. Heart rate= HR. Central venous pressure= CVP. Mean circulatory filling pressure= MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Asterisks (*) indicate a significant difference between sham and DOCA-salt values at a specific measurement time. Crosses (†= DOCA-salt; ‡= sham) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

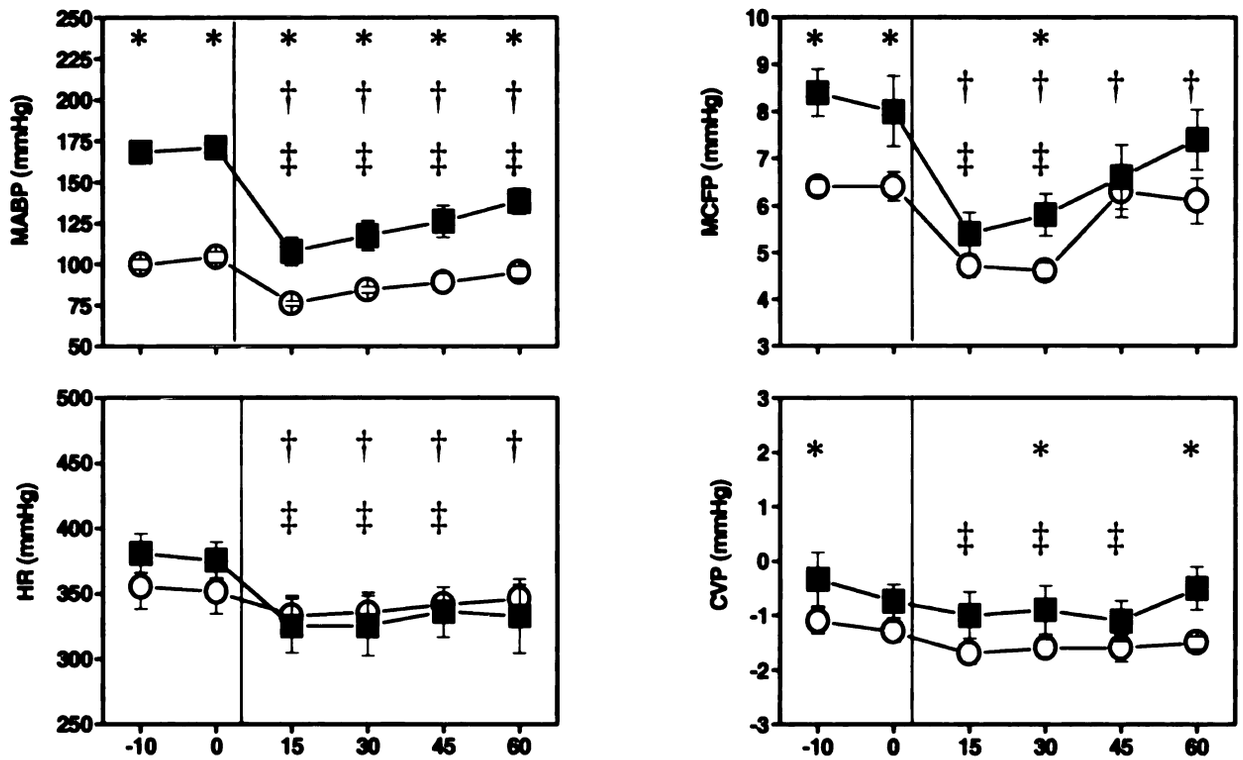

Figure 5.4: The response of hemodynamic variables to ganglion blockade with intravenous hexamethonium (30 mg/kg; i.v.) given immediately after the 0 time point in sham-operated (○; n=9) and deoxycorticosterone acetate-salt treated rats (■; n=6). Treatment administration indicated on figure by vertical line. Mean arterial blood pressure= MABP. Heart rate= HR. Central venous pressure= CVP. Mean circulatory filling pressure= MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Asterisks (*) indicate a significant difference between sham and DOCA-salt values at a specific measurement time. Crosses (†= DOCA-salt; ‡= sham) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

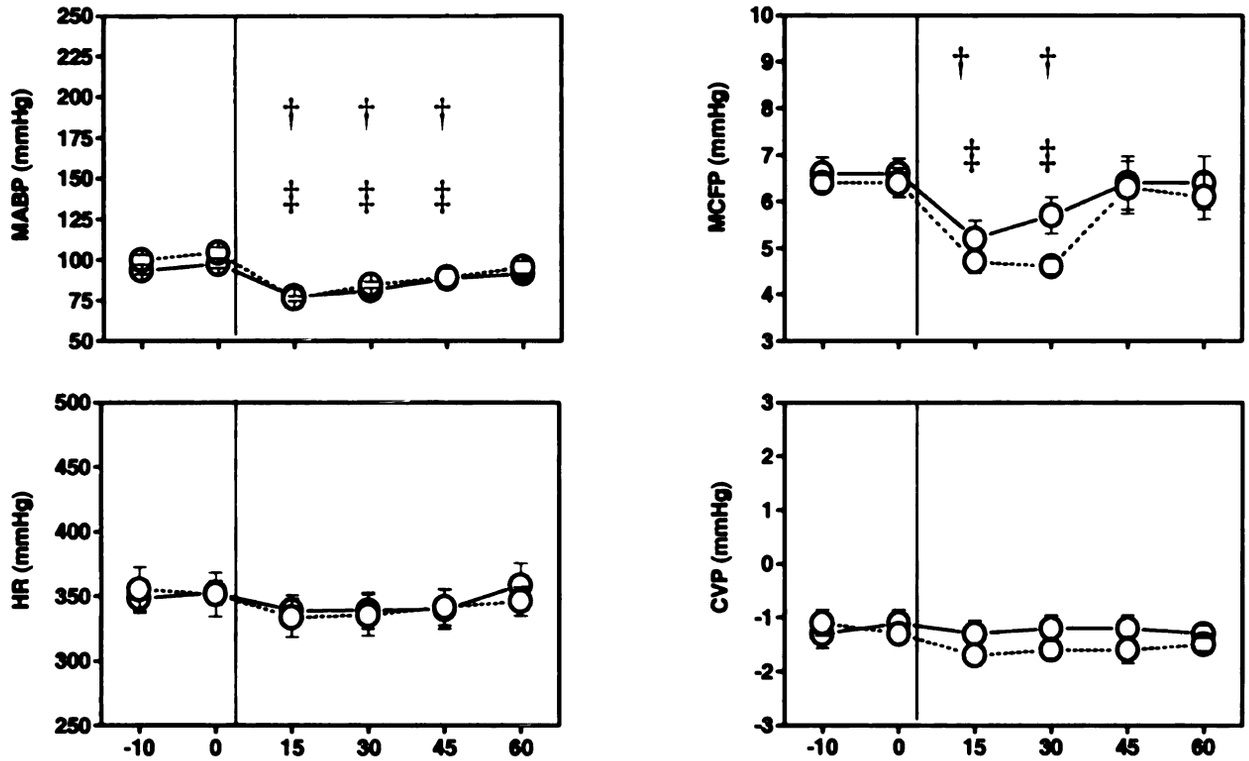

Figure 5.5: The response of hemodynamic variables in sham rats to ganglion blockade with hexamethonium (30 mg/kg; i.v.) given immediately after the 0 time point either alone (hexamethonium, -O-; n=6) or one hour after pretreatment with the selective ET_A receptor antagonist ABT-627 (O; 1 mg/kg; iv, n=6). Vertical line=treatment administration indicated on figure by vertical line. Mean arterial blood pressure=MABP. Heart rate=HR. Central venous pressure=CVP. Mean circulatory filling pressure=MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n=number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Asterisks (*) indicate a significant difference between ABT-627 pretreated and non-pretreated values at a specific measurement time. Crosses (†= ABT-627 pretreated; ‡= non-pretreated) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

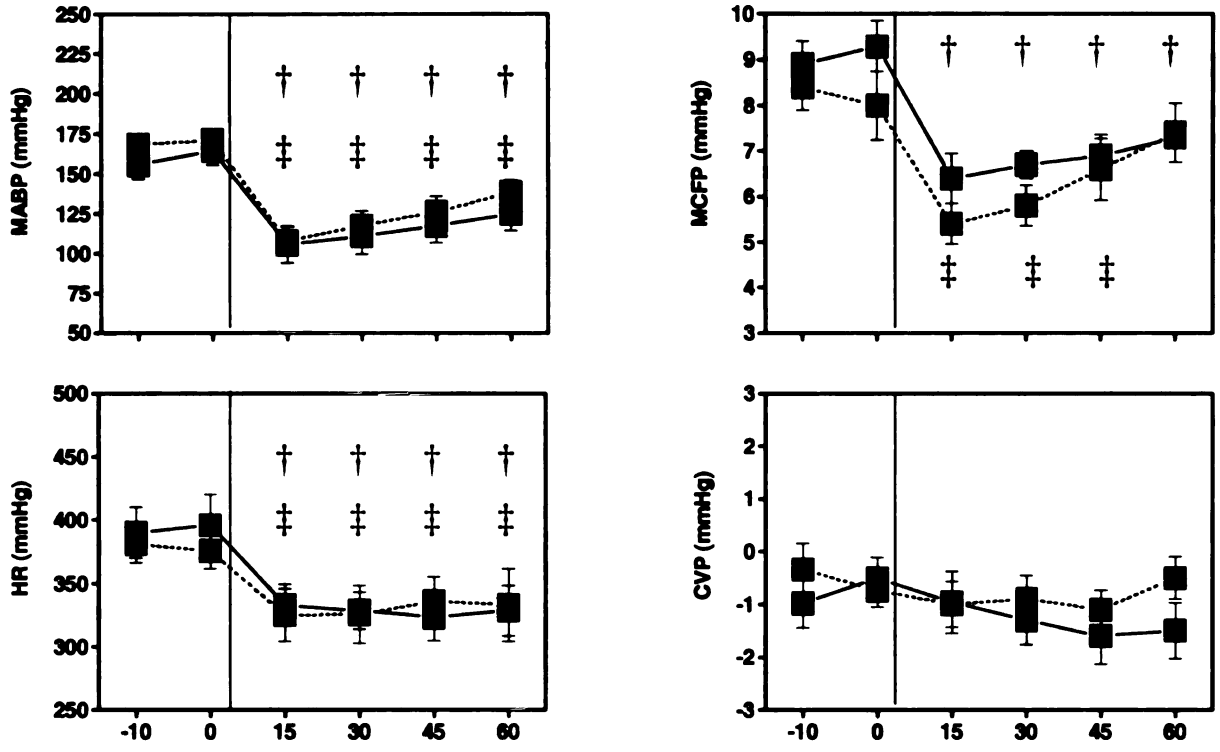

Figure 5.6: The response of hemodynamic variables in DOCA-salt rats to ganglion blockade with hexamethonium (30 mg/kg; i.v.) given immediately after the 0 time point either alone (hexamethonium, -■-; n=5) or one hour after pretreatment with the selective ET_A receptor antagonist ABT-627 (■; 1 mg/kg; iv, n=5). Vertical line=treatment administration indicated on figure by vertical line. Mean arterial blood pressure=MABP. Heart rate=HR. Central venous pressure=CVP. Mean circulatory filling pressure=MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n=number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Asterisks (*) indicate a significant difference between ABT-627 pretreated and non-pretreated values at a specific measurement time. Crosses (†= ABT-627 pretreated; ‡= non-pretreated) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

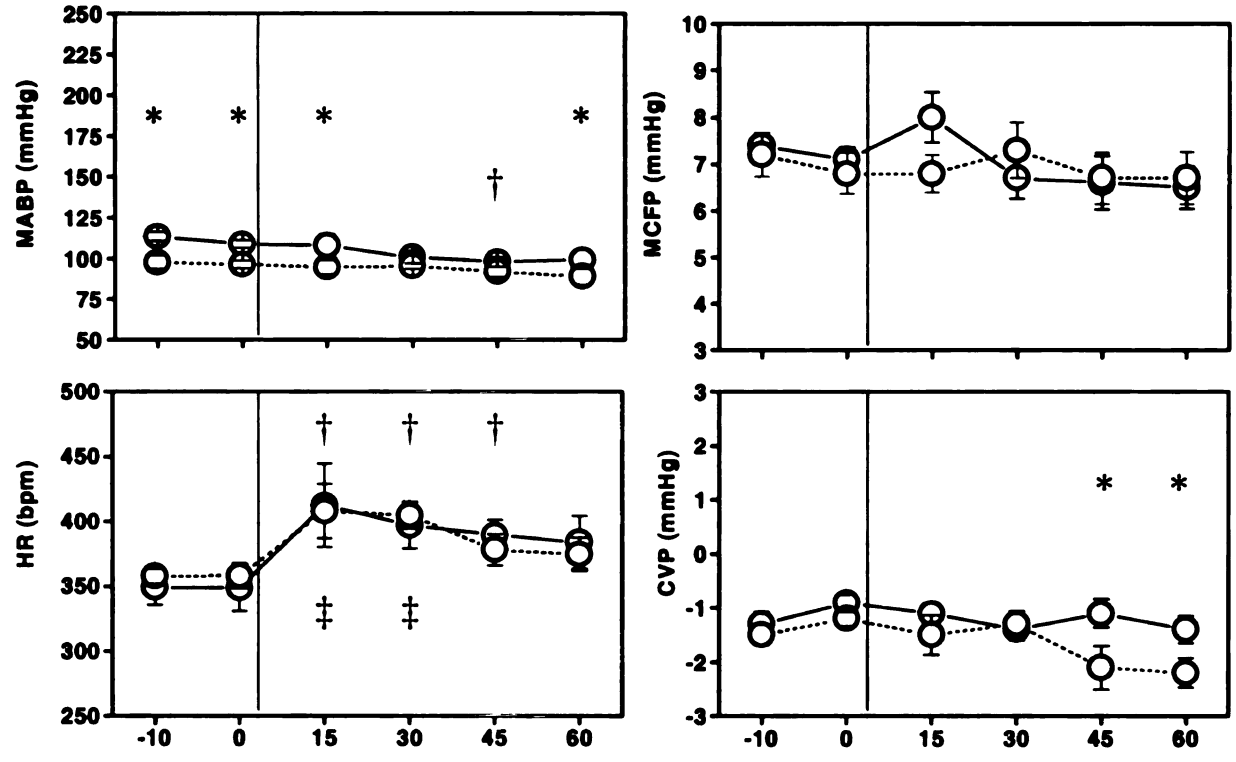

Figure 5.7: The response of hemodynamic variables in sham rats on high salt intake (○; n=5) or low salt intake (-○-; n=5) to the selective ET_A receptor antagonist ABT-627 (1 mg/kg; i.v.) given immediately after the 0 time point. Vertical line = treatment administration. Mean arterial blood pressure= MABP. Heart rate= HR. Central venous pressure= CVP. Mean circulatory filling pressure= MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Asterisks (*) indicate a significant difference between high salt intake and low salt intake values at a specific measurement time. Crosses (†= high salt; ‡= low salt) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

DISCUSSION

There are three new findings in this study. First, MCFP is increased in rats after 3-4 weeks of DOCA-salt hypertension. Second, administration of a selective antagonist of the ET_A subtype of endothelin receptor causes a larger acute fall in MCFP in DOCA-salt hypertensive rats than in normotensive rats. Third, acute ganglion blockade with hexamethonium also causes a larger fall in MCFP in DOCA-salt hypertensive rats versus normotensive rats.

Two separate studies in dogs with chronic mineralocorticoid-salt hypertension revealed increased MCFP when compared to normotensive controls (Pan and Young, 1982; Ogilvie and Zborowska-Sluis, 1987). Only one other report exists concerning MCFP in DOCA-salt rats. Yamamoto performed one-time-only measurements of MCFP in conscious rats 1, 2, 5 and 8 weeks after intitiating DOCA-salt treatment (Yamamoto et al., 1983). Although MCFP was numerically higher in the hypertensive rats compared to normotensive controls, the differences did not reach statistical significance. However, hemodynamic measurements were made only 3 hours after general anesthesia and surgery for catheter and balloon insertion. In contrast, values for MCFP reported here were obtained 2-5 days after surgery. A significantly higher MCFP was found in DOCA-salt rats compared to sham at 2 days, and this difference became even larger on subsequent days (Figure 5.2). I speculate that the discrepancy can be explained by suppression of sympathetic activity and relative dehydration associated with anesthesia and

surgery, especially in DOCA-salt rats. Supportive of this idea is the fact that saline drinking in the DOCA-salt rats studied here typically did not recover to pre-surgery amounts until at least 4 days of recovery. I conclude that MCFP is increased in DOCA-salt hypertension of 3-4 week duration.

Increased blood volume, constriction of venous smooth muscle and structural changes in the wall of venules and veins are the most likely causes of increased MCFP. Evidence of structural abnormalities in veins exists for hypertensive humans (Mark, 1984; Safar and London, 1987) and some experimental models of hypertension (e.g. spontaneously hypertensive rats, Ricksten et al., 1981). No data are available in the DOCA-salt hypertensive rat. Most studies of individuals with established hypertension, including DOCA-salt rats, reveal blood volumes (indexed to body weight) to be normal or even reduced compared to normotensive controls (Safar and London, 1987; Yamamoto et al., 1983). I could measure blood volume in only a sample of the rats studied here, and found the DOCA-salt group had moderately higher blood volumes than the sham group under the conditions of my study. I am uncertain why my results differ from the majority of published results, but conclude that elevated blood volume may have contributed to higher MCFP in my DOCA-salt hypertensive rats.

One goal of my experiments was to test the hypothesis that ET-1 produces greater venoconstriction in conscious, intact DOCA-salt rats than in normotensive sham animals. Venoconstriction in response to ET-1 can result from stimulation of either ET_A or ET_B receptors (Chapter 2). I have demonstrated previously that small

veins isolated from the mesentery of DOCA-salt rats exhibit normal contractile responses to ET_A and ET_B receptor activation in vitro (Chapter 3), even in the face of chronic increases in endogenous ET-1 formation (Schiffrin, 1998). Since ET_A receptor stimulation causes a larger venoconstrictor response than ET_B receptor activation, in the current study I investigated changes in MCFP produced by the selective ET_A receptor antagonist ABT-627, the active enantiomer of the better known A-127722 (Opgenorth et al., 1996). I chose a dose of 1 mg/kg, i.v. based on evidence that this dose produces plasma concentrations of the drug causing significant blockade of ET_A receptors with minimal effects on ET_B mediated responses in rats (Opgenorth et al., 1996). ABT-627 has already been shown to significantly decrease arterial pressure in DOCA-salt rats (Matsumura et al., 1999). That result was confirmed in the current study. In addition, the drug caused significant tachycardia in both sham and DOCA-salt rats; the mechanism is unknown. Most importantly, ABT-627 produced a significant decrease in MCFP in DOCA-salt but not sham rats. In general, the time course of the response paralleled the decline in arterial pressure. These results are consistent with the idea that DOCA-salt hypertension is associated with enhanced venoconstriction produced by endogenous ET-1 acting on ET_A receptors. This could be due to greater endothelial formation of ET-1 in DOCA-salt rats. No data exist, however, on venous endothelial cell ET formation in the DOCA-salt model, and reports on blood levels of ET-1 in DOCA-salt rats are conflicting (Schiffrin, 1995a; 1999).

It is possible that DOCA-salt rats showed greater decreases in MCFP to

ABT-627 than sham rats merely because the former were on a high salt intake. For example, Mortensen reported that pressor responses to chronic infusion of ET-1 in rats are markedly potentiated by high salt intake (Mortensen and Fink, 1992). My current results do not support that explanation: normotensive sham rats did not exhibit a decrease in MCFP in response to ABT-627 administration after 3-4 days of either sodium loading or sodium depletion.

The sympathetic nervous system has a major impact on venomotor tone and thus systemic vascular compliance and MCFP (Karim and Hainsworth, 1976; Greenway, 1983; Shoukas and Bohlen, 1990). There is much evidence that sympathetic nervous system activity and sympathetic support of arterial pressure (de Champlain, 1990) are increased in DOCA-salt hypertension. Therefore I compared the contribution of the sympathetic nervous system to control of MCFP in DOCA-salt and sham rats using acute ganglion blockade with hexamethonium. As many have reported previously (de Champlain, 1990), I found that acute ganglion blockade caused falls in AP and HR in both sham and DOCA-salt rats, and that the magnitude of these effects were significantly larger in DOCA-salt rats. CVP tended to decline in both groups as well. The decline in MCFP produced by hexamethonium was significantly larger in DOCA-salt than in sham rats; in fact, at all but one time point (30 minutes) after hexamethonium injection MCFP no longer differed significantly in sham and DOCA-salt rats (although remaining consistently higher in the DOCA-salt group). While not ruling out a contribution by other factors, this result suggests that increased sympathetically mediated venomotor tone plays

a major part in the elevated MCFP of DOCA-salt rats. Similar conclusions have been reached concerning the increased MCFP measured in SHR (Martin et al., 1998).

In some vascular beds exogenous ET-1 augments sympathetic neurotransmission. One such bed is forearm veins in humans with essential hypertension (Haynes et al., 1994). So I sought to determine if there was an interaction between endogenous ET-1 and sympathetic control of MCFP in DOCA-salt rats. I reasoned that if endogenous ET-1 potentiated sympathetic venomotor tone through activation of ET_A receptors, then block of ET_A receptors with ABT-627 would impair subsequent MCFP responses to ganglion blockade. Such an effect could not be demonstrated, however, in either sham or DOCA-salt rats. I conclude that endogenous ET-1 does not significantly influence sympathetic control of venomotor tone in sham or DOCA-salt rats solely through an action on ET_A receptors.

In summary, I have demonstrated that conscious DOCA-salt hypertensive rats have increased MCFP relative to that of normotensive rats. Possible mechanisms include higher blood volume, augmented ET-1 induced direct venoconstriction mediated by ET_A receptors and increased sympathetic venomotor tone. Whether this is a secondary adaptation or contributes to the development of hypertension remains to be determined.

Chapter 6

Effect of an ET_B-selective and a Mixed $ET_{A/B}$ Endothelin Receptor Antagonist on Venomotor Tone in Deoxycorticosterone Acetate-Salt Hypertension

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INTRODUCTION

The effective pressure for venous blood return to the heart, MCFP, is usually increased in hypertension (Martin et al., 1998). Venomotor tone and blood volume provide the determinants for MCFP (Yamamoto et al., 1980), however, blood volume is usually normal or reduced in established hypertension (Safar and London, 1987; Chapter 5). Therefore, MCFP represents whole body integrated venomotor tone which is dependent on sympathetic tone and the effects of circulating and locally produced hormones (Waite and Pang, 1990; 1992; Mono et al., 1995; Chapter 1; Chapter 4). The endothelial cell-derived peptide, ET-1, may play a key role in elevating venous tone in human hypertension (Haynes et al., 1994; Cardillo et al., 1999) and in models of experimental hypertension (Potter et al., 1997; Chapter 5). However, the use of ET receptor antagonists to treat hypertension remains uncertain, in part, due to controversy regarding the use of balanced $ET_{A/B}$ versus selective ET_A receptor antagonists as the preferred agents (Rubanyi and Polokoff, 1994).

Evidence shows that endothelins are important in the pathogenesis of salt-sensitive forms of hypertension including the DOCA-salt (Lariviere et al., 1993a; 1993b; Matsumura et al., 1999; Lange et al., 2000), reduced renal mass (Potter et al., 1997) and Dahl (Kassab et al., 1997) salt-sensitive models of experimental hypertension. I recently showed that intact conscious DOCA-salt hypertensive rats have increased MCFP compared to normotensive rats (Chapter 5). In the same

study using a selective ET_A receptor antagonist (ABT-627) and ganglion blockade (hexamethonium), I showed that elevated venomotor tone in DOCA-salt hypertensive rats was due to ET-1 acting at ET_A receptors and increased sympathetic tone to the veins. However, my results clearly showed that ET-1 did not significantly influence sympathetic contributions to venomotor tone through an action on ET_A receptors. I have also recently shown in vitro using mesenteric veins from DOCA-salt hypertensive and sham normotensive rats that although ET-1 appeared to contract mesenteric veins mainly via activation of ET_A receptors, combined ET_A/ET_B receptor blockade was more effective than ET_A receptor antagonism at inhibiting venous contractile responses to ET-1, perhaps due to an undefined interaction between the receptors or their signaling pathways (Chapter 3). In another study, intrarenal arterial infusion of ET-1 inhibited NE release induced by renal sympathetic nerve stimulation in anesthetized dogs (Suzuki et al., 1992). This neuroinhibitory effect of ET-1 was mediated through activation of ET_B receptor mechanisms (Matsuo et al., 1997). Taken together, these findings suggest that ET-1 acting at ET_B receptors may provide an important contribution to venomotor tone via direct actions on the VSM and through modulation of sympathetic tone to the veins. To investigate this hypothesis I examined the effects of a selective ET_B receptor antagonist and a mixed $ET_{A/B}$ receptor antagonist on endogenous ET-1 contributions to elevated venomotor tone in DOCA-salt hypertension. Venomotor tone was assessed using repeated measurements of MCFP in intact conscious, undisturbed animals.

METHODS

Animals. Male Sprague-Dawley rats weighing 175-225 g (Charles River, Portage, MI) were maintained according to standards approved by the Michigan State University All-University Committee on Animal Care and Use. Standards were in strict accordance with Michigan State University and National Institutes of Health animal care guidelines. All rats were kept in a light and temperature controlled room and housed in clear plastic boxes in groups of three with free access to standard pelleted rat chow (Harlan/Teklad 8640 Rodent Diet) and tap water. Rats were acclimatized to their environment for 2 days prior to surgical manipulation.

DOCA-salt hypertension. See Chapter 5 page 118.

Catheterization. See Chapter 4 page 99.

Hemodynamic measurements. See Chapter 4 page 100.

Volume measurements. See Chapter 5 page 119.

Experimental protocols. Experiments began at least 4 days after catheter and balloon implantation, as previous work in my lab indicated that saline intake in DOCA rats typically did not recover to pre-surgery amounts until 4 days (Chapter 5). I also showed in the same study that the hemodynamic variables MABP, HR, CVP and MCFP remained stable over the 80 minute experimental protocol with the exception of CVP which tended to decline gradually in the sham rats and HR which rose significantly in the last time point (labeled 60) in DOCA-salt rats. Therefore, a time control protocol was not done in the present study. Catheters were flushed and attached to pressure transducers. Rats were then allowed to sit 10-20 minutes undisturbed, with all hemodynamic measures being averaged and recorded in one minute time bins. The first control measurement (labeled -10) of MCFP was obtained, followed 10 minutes later by a second control measurement (labeled 0). Then drug or drug vehicle (saline or a mixture of 70% water, 10% ethanol and 20% propylene glycol) was injected iv. Measurements of MCFP were repeated every 15 minutes for one hour (labeled 15, 30, 45 and 60). Values reported for MABP, HR and CVP are the average of the values obtained during the last three minutes before balloon inflation.

In most rats I studied the effects of ET receptor antagonism and ganglion blockade using the same protocol. Initially, in place of drug vehicle injections rats received either the selective ET_B receptor antagonist A-192621 (12 mg/kg i.v.; Abbott Laboratories, Abbott Park, IL), the mixed $ET_{A/B}$ receptor antagonist A-182086 (12 mg/kg, i.v.; Abbott Laboratories, Abbott Park, IL) or the ganglion blocker

hexamethonium (30 mg/kg, i.v.). Also, the ability of ET receptor antagonism to modify hemodynamic responses to ganglion blockade was examined by pretreating rats with ET receptor antagonists, i.v. one hour before administering hexamethonium (30 mg/kg, i.v.; Sigma, St. Louis, MO). Only one protocol was run per day and the order was randomized with at least 48 hours between different ET antagonist treatments.

Volume measurements were made at least 24 hours after the completion of the initial hemodynamic protocols. Blood volume measurements were made on 5 sham rats and 9 DOCA-salt rats.

Statistical analyses. Comparisons between control period values were made using a two-way ANOVA; where significant overall between groups differences were found, comparsions at individual times were achieved by testing simple main effects. Changes in hemodynamic variables within groups over time were evaluated using the methods of contrasts, where values at each time point after drug injection were compared to the average of the two control period values. For all tests the significance level was set at $P< 0.05$.

RESULTS

A total of 28 rats (sham=12 , DOCA-salt=16) were studied. At the time of surgical catheterization sham rats weighed 369 ± 36 g while DOCA-salt rats weighed 353 ± 40 g. Hemodynamic responses following administration of the ET_B receptor antagonist A-192621 and the $ET_{A/B}$ receptor antagonist A-182086 to sham rats are shown in Figure 6.1. Hemodynamic responses in DOCA-salt rats to ET antagonists are shown in Figure 6.2. No consistent differences in MABP, HR, CVP or MCFP during the control period (-10 and 0 time points) were noted on the different days of antagonist administration in either the sham or DOCA-salt groups. MABP and MCFP were higher in DOCA-salt than in sham rats ($P<0.05$).

Following administration of A-182086 a significant reduction in MABP was noted in sham rats which persisted for the entire treatment period (Figure 6.1). The ET_B antagonist A-192621 produced a small but significant increase in MABP in sham rats which was also sustained over the length of treatment period. Injections of A-182086 in sham rats produced a significant increase in HR while neither HR, CVP nor MCFP was affected by administration of A-192621.

A-182086 caused a significant decline in MABP in DOCA-salt rats which persisted for the entire treatment period (Figure 6.2). A-182086 injections produced an increase in HR and a gradual decline in MCFP in DOCA-salt rats which achieved statistical significance by one hour after drug injection. A-192621 produced a small but significant increase in MABP, but no other variable changed.

Hemodynamic responses to ganglion blockade with hexamethonium in sham and DOCA-salt rats are shown in Figure 6.3. Compared to the sham group, DOCA-salt rats had significantly higher control period MCFP and MABP values ($P<0.05$). Following hexamethonium injection, MABP declined in both sham and DOCA-salt rats. MCFP decreased rapidly in both sham and DOCA-salt rats in response to hexamethonium, but the fall persisted over the entire treatment time period in DOCA-salt rats while the decline in sham rats occurred only at 15 minutes after drug injection.

Figure 6.4 illustrates hemodynamic responses to hexamethonium one hour after i.v. injection of A-192621 or A-182086 in sham rats. These responses are compared to the responses of the same sham rats a few days earlier to hexamethonium alone. In sham rats, a consistent difference was noted in control period MABP values with: A-192621 pretreatment > no pretreatment > A-182086 pretreatment. Control period HR values in sham rats were also consistently higher when pretreated with A-182086 compared to pretreatment with A-192621 or no pretreatment. Following injection of hexamethonium in the sham group, all rats experienced a reduction in MABP. Rats pretreated with either ET antagonist also showed a reduction in HR after hexamethonium. All sham rats showed a similar peak reduction in MCFP following hexamathonium which persisted throughout the treatment period in rats pretreated with either ET antagonist only.

Hemodynamic responses to hexamethonium one hour after iv injection of A-192621 or A-182086 in DOCA-salt rats is shown in Figure 6.5. These responses

are also compared to the responses of the same DOCA-salt rats a few days earlier to hexamethonium alone. In DOCA-salt rats, a consistent difference was also noted in control period MABP values with A-192621 pretreatment > no pretreatment > A-182086 pretreatment. Control period HR values in the DOCA-salt group were also consistently higher when rats were pretreated with A-182086 compared to no pretreatment or pretreatment with A-192621. A small but significant reduction in control period MCFP values were noted in the DOCA-salt group when rats were pretreated with either ET antagonist compared to no pretreatment. Following injections of hexamethonium, all DOCA-salt rats showed a significant reduction in MABP which persisted throughout the treatment period while only DOCA-salt rats pretreated with A-182086 experienced a decline in HR after hexamethonium. MCFP declined significantly in all DOCA-salt rats after hexamethonium and remained significantly reduced throughout the treatment time period. Peak effects however, were significantly greater after pretreatment with either A-192621 (decline of 3.7 ± 0.54 mmHg; n=9) or A-182086 (decline of 3.8 ± 0.3 mmHg; n=10) compared to hexamethonium alone (decline of 2.6 ± 0.3 mmHg; n=12) ($P<0.05$). Blood volume averaged 60.5 ± 6.0 ml/kg in sham rats (n=5) and 56.8 ± 5.2 ml/kg in DOCA-salt rats (n=9), and the differences were not statistically significant ($P>0.05$).

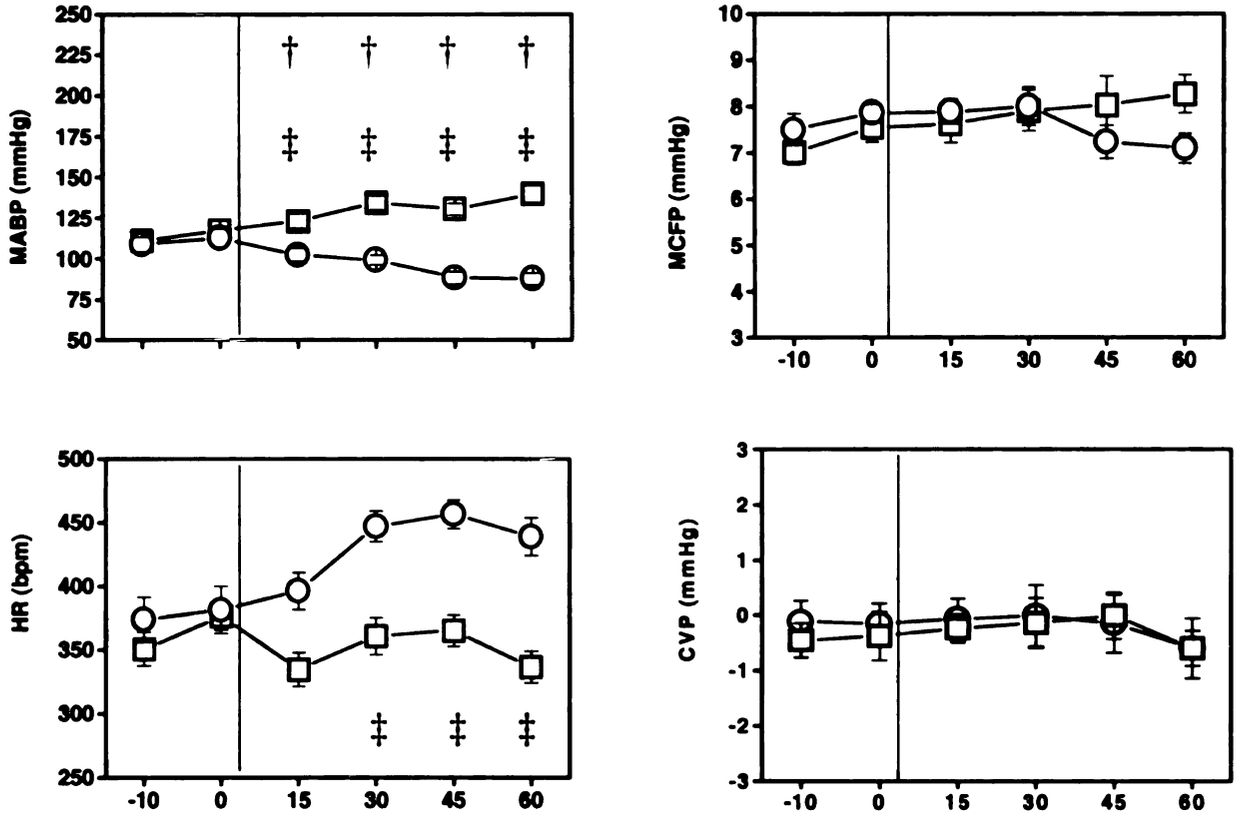

Figure 6.1: Response of hemodynamic variables in sham rats to the selective ET_B receptor antagonist A-192621 (□; 12 mg/kg, n=12) and the mixed $ET_{A/B}$ receptor antagonist A-182086 (○; 12 mg/kg, n=11) given intravenously immediately after the 0 time point. Treatment administration indicated on figure by vertical line. Mean arterial blood pressure=MABP. Heart rate=HR. Central venous pressure=CVP. Mean circulatory filling pressure=MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Symbols (†= A-192621; ‡=A-182086) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

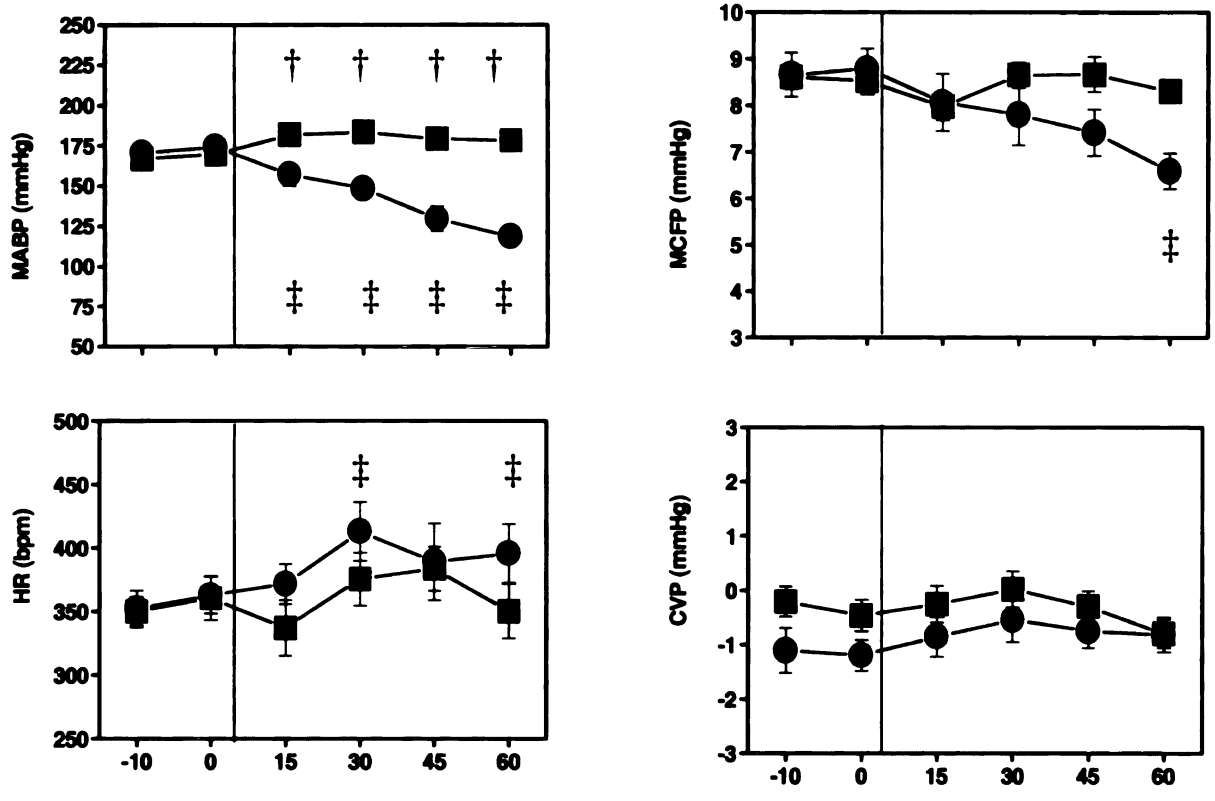

Figure 6.2: Response of hemodynamic variables in DOCA-salt rats to the selective ET_B receptor antagonist A-192621 (■; 12 mg/kg, n=12) and the mixed $ET_{A/B}$ receptor antagonist A-182086 (●; 12 mg/kg, n=11) given intravenously immediately after the 0 time point. Treatment administration indicated on figure by vertical line. Mean arterial blood pressure=MABP. Heart rate=HR. Central venous pressure=CVP. Mean circulatory filling pressure=MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Symbols (†= A-192621; ‡=A-182086) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

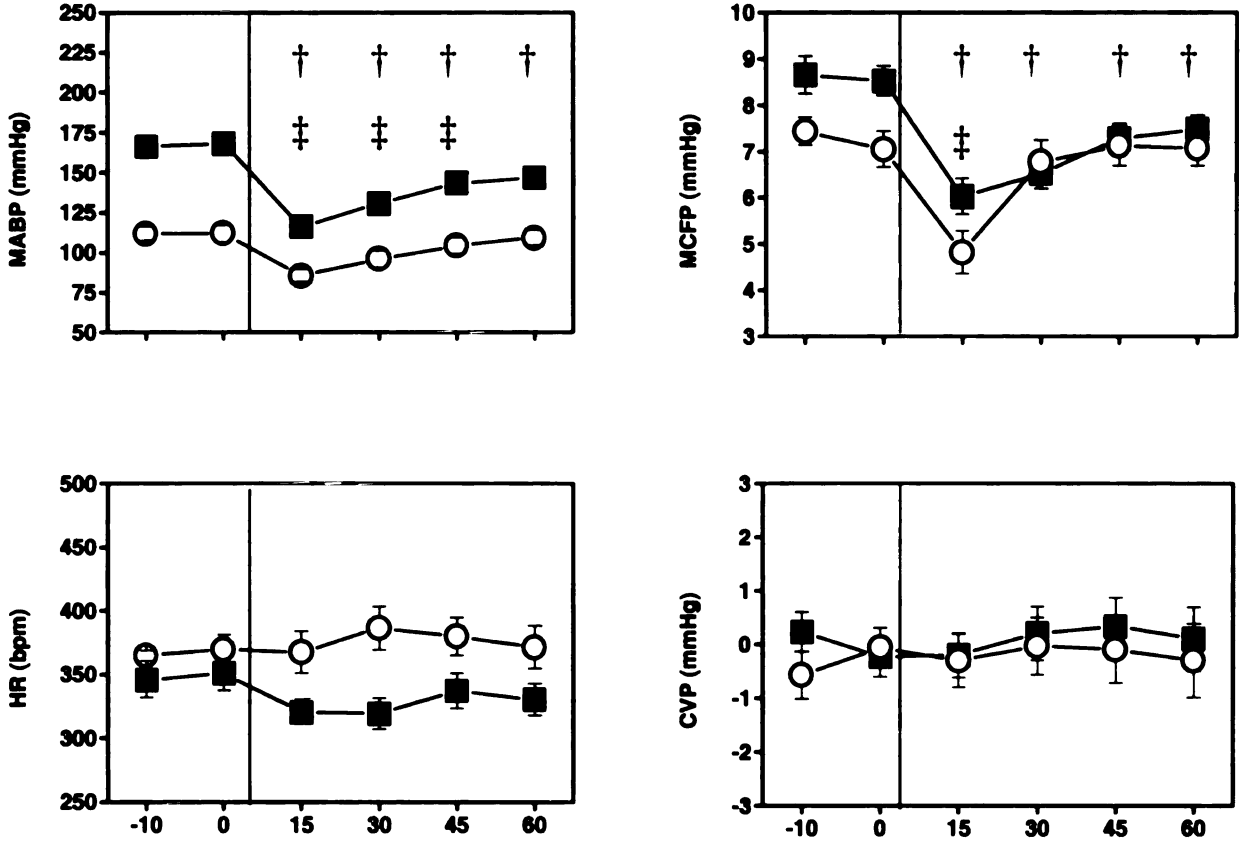

Figure 6.3: Response of hemodynamic variables to ganglion blockade with intravenous hexamethonium (30 mg/kg) given immediately after the 0 time point in sham rats (○; n=12) and DOCA-salt rats (■; n=12). Treatment administration indicated on figure by vertical line. Mean arterial blood pressure=MABP. Heart rate=HR. Central venous pressure=CVP. Mean circulatory filling pressure=MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Symbols (†= DOCA-salt; ‡=sham) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

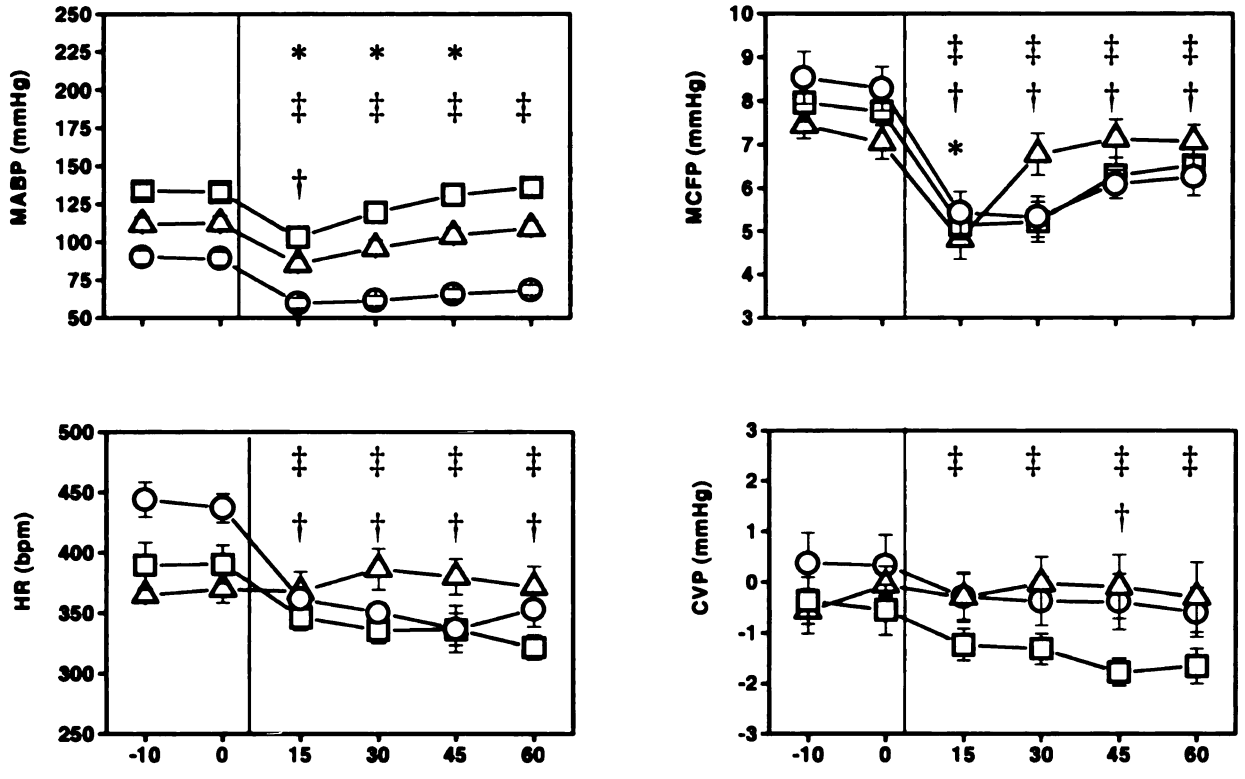

Figure 6.4: Response of hemodynamic variables in sham rats to ganglion blockade with intravenous hexamethonium (30 mg/kg) given immediately after the 0 time point either alone (hexamethonium, Δ; n=12) or one hour after pretreatment with the selective ET_B receptor antagonist A-192621 ((□; 12 mg/kg, n=11) or the mixed $ET_{A/B}$ receptor antagonist A-182086 (○; 12 mg/kg, n=10). Treatment administration indicated on figure by vertical line. Mean arterial blood pressure=MABP. Heart rate=HR. Central venous pressure=CVP. Mean circulatory filling pressure=MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Symbols (†=A-192621; ‡=A-182086; ✻=hexamethonium) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

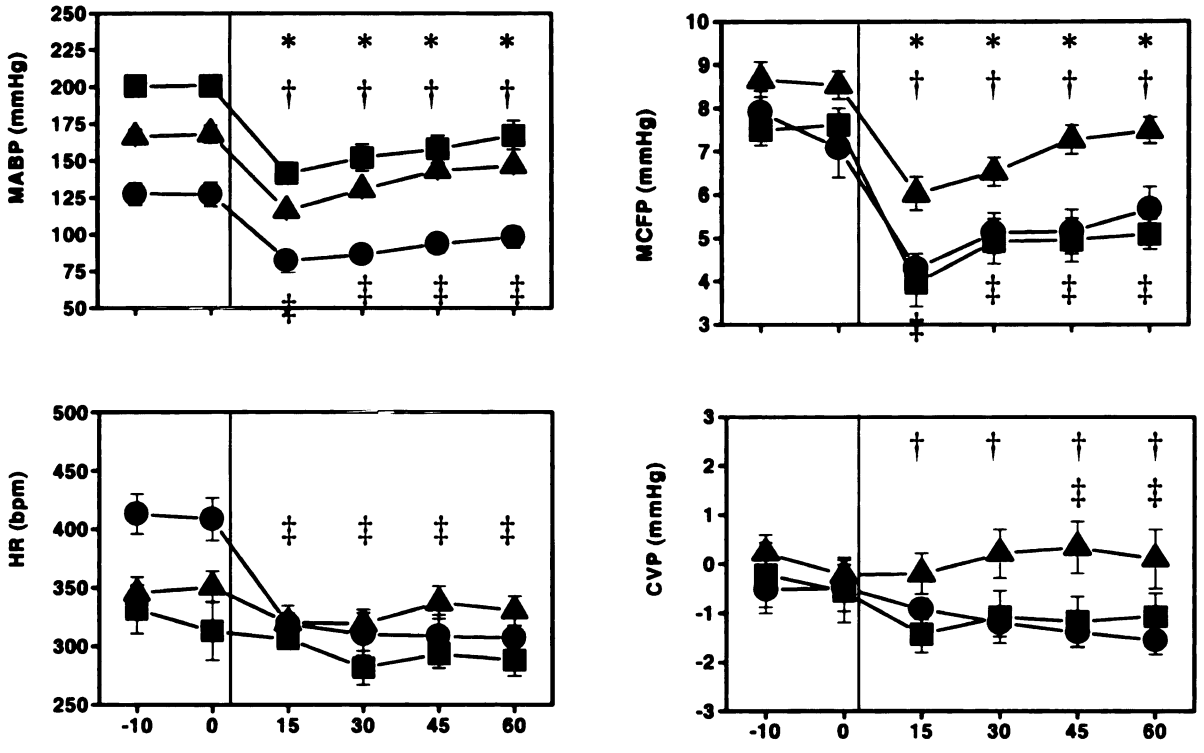

Figure 6.5: Response of hemodynamic variables in DOCA-salt rats to ganglion blockade with intravenous hexamethonium (30 mg/kg) given immediately after the 0 time point either alone (hexamethonium, ▲; n=12) or one hour after pretreatment with the selective ET_B receptor antagonist A-192621 (■; 12 mg/kg, n=9) or the mixed $ET_{A/B}$ receptor antagonist A-182086 (●; 12 mg/kg, n=10). Treatment administration indicated on figure by vertical line. Mean arterial blood pressure=MABP. Heart rate=HR. Central venous pressure=CVP. Mean circulatory filling pressure=MCFP. Control period (-10, 0) measurements separated by 10 minutes. Treatment period measurements (15-60) separated by 15 minutes. n= number of rats per treatment group. mmHg=millimeters mercury. bpm=beats per minute. Symbols (†=A-192621; ‡=A-182086; *=hexamethonium) indicate significant difference between 15-60 minute time point value compared to the average of the control period time point values. $P<0.05$ considered statistically significant.

DISCUSSION

I have previously shown that venomotor tone is increased in DOCA-salt hypertension through the independent actions of both endogenous ET-1 acting on venous smooth muscle ET_A receptors and sympathetically mediated venoconstrictor activity (Chapter 5). Results in the present study with acute ganglion blockade again illustrate the importance of the latter mechanism in DOCA-salt rats. Because both ET_A and ET_B receptors are important in venoconstriction, I hypothesized that ET-1 acting at ET_B receptors may provide an important contribution to venomotor tone in DOCA-salt hypertension via direct actions on the VSM or through modulation of sympathetic tone to the veins. To investigate this hypothesis I examined the effects of a selective ET_B receptor antagonist, A-192621, and a mixed $ET_{A/B}$ receptor antagonist, A-182086, on endogenous ET-1 contributions to elevated venomotor tone in DOCA-salt hypertension using repeated measurements of MCFP in conscious, undisturbed animals. Because there is evidence that ET-1 can modulate sympathetic neurotransmission (Suzuki et al., 1992; Matsuo et al., 1997), I also tested the actions of the antagonists on sympathetically mediated venoconstriction. In the present study I report the following new findings: the selective ET_B receptor antagonist A-192621did not affect MCFP in either sham or DOCA-salt rats, the mixed $ET_{A/B}$ receptor antagonist A-182086 reduced MCFP in DOCA-salt rats but not in sham rats, and the fall in MCFP after hexamethonium injection in DOCA-salt rats, but not in sham rats, was significantly greater in rats

pretreated with either ET antagonist compared to no pretreatment. In the present study I also confirmed previous findings showing that MCFP and MABP are increased in DOCA-salt hypertension compared to sham rats and that blood volume (an important determinant of MCFP) is not significantly different between the two groups (Chapter 5).

The endothelin system is activated in low renin, salt-sensitive forms of hypertension such as the DOCA-salt (Schiffrin, 1999), reduced renal mass (Potter et al., 1997) and one-kidney, one-clip models (Sventek et al., 1996), and in general in more severe forms of hypertension (Schiffrin, 1995a; 1999). However, ET-1 has a prominent role in the DOCA-salt model of experimental hypertension (Schiffrin, 1995a). Studies using the DOCA-salt model in the rat have demonstrated increased preproET-1 gene expression (Lariviere et al., 1993b; Day et al., 1995) and immunoreactive ET-1 content (Lariviere et al., 1993a) in aorta and mesenteric arteries. In arteries, ET-induced vasoconstriction is mediated by ET_A receptors (Sumner et al., 1992, Moreland et al., 1994). In contrast, in veins I and others have shown that ET-1 mediated constriction involves VSM ET_A and ET_B receptors (Moreland et al., 1992; 1994; Chapter 2; Chapter 5). I have shown that elevated venomotor tone in vivo in DOCA-salt rats is, in part, due to ET-1 activation of ET_A receptors (Chapter 5). I have also shown in vitro in mesenteric veins from DOCA-salt hypertensive rats that contractions induced by ET-1 and the selective ET_B receptor agonist S6c are maintained while contractile responses in corresponding arteries are reduced (Chapter 3). Therefore, increased ET-1 production observed

in DOCA-salt hypertension could result in elevated venous tone by the actions of ET-1 at ET_A and ET_B receptors.

In the present study the ET_B receptor antagonist A-192621 produced no change in MCFP in either DOCA-salt or sham rats but did produce a small but significant increase in MABP in both groups following iv administration. These results suggest that ET-1 does not act through ET_B receptors to elevate venous tone in DOCA-salt hypertension. Although this finding is consistent with findings in vitro showing that ET-1 induced contractions in mesenteric veins from DOCA-salt hypertensive rats are not affected by selective ET_B receptor blockade (Chapter 3), these results could also be explained by baroreflex mediated alterations in venous tone and subsequent blunting of any direct effects of A-192621 on the veins. Studies in conscious rats have shown that sympathetic reflexes elicited by vasoactive drugs have significant effects on MCFP (Waite and Pang, 1988; Waite and Pang, 1992). If the effects of ET_B receptor blockade on venomotor tone in the present study were opposed by baroreflex mediated decreases in sympathetic tone to the veins as a result of increased MABP, then it would be expected that administration of the ganglion blocker hexamethonium after pretreatment with A-192621 should produce a smaller fall in MCFP than with hexamethonium alone, owing to diminished sympathetic input to the veins. This was not the case, however, as the peak fall in MCFP in DOCA-salt rats, but not sham rats, pretreated with A-192621 was significantly greater following hexamethonium injection than the fall in non-pretreated rats following hexamethonium. These findings therefore

indicate that pretreatment of DOCA-salt rats with A-192621 increased rather than decreased sympathetic input to the veins. ETs can inhibit NE release from sympathetic nerve terminals in blood vessels while facilitating the actions of NE on postjunctional adrenergic VSM sites (Rubanyi and Polokoff, 1994). In rat tail arteries in vitro, electrically evoked release of NE was inhibited by low concentrations of ET-1 (1-30 nM), an effect which was blocked by the selective ET_B receptor antagonist BQ-788 (Moreland et al., 1992). In a similar study using rat tail artery, contractions caused by electric field stimulation were enhanced by pretreatment with BQ-788 but not the selective ET_A receptor antagonist BQ-123 (Garcia-Villalon et al., 1999). Therefore, in this study A-192621 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.

Another possibility to explain the failure of ET_B blockade to affect MCFP is that venoconstrictor affects of ET_B receptor activation of venous smooth muscle is opposed by release of endothelial cell dilators. I have shown in vitro though that ET-1 mediated release of endothelial-derived venodilators does not provide a significant contribution to the net constrictor effect of ETs in mesenteric veins (Chapter 2). I have confirmed these results in vivo by showing that acute bolus injections of S6c (500 pmol) did not significantly affect MCFP in conscious rats, even after treatment with indomethacin or L-NAME (Chapter 4). Therefore, it is not

likely that A-192621 in the present study failed to affect MCFP in sham or DOCA-salt rats due to opposing effects of blockade of endothelial ET_B mediated dilator release. On the other hand, the vascular endothelium does control arterial tone and the release of the nitric oxide and dilator prostanoids (De Mey and Vanhoutte, 1982; Chapter 3). A-192621 may increase MABP in part, by blocking endothelial ET-mediated dilator contributions to arterial tone.

A final potential reason ET_B blockade did not affect MCFP is decreased clearance of ET-1 by blockade of ET_B receptors. It has been postulated that the ET_B receptor also functions as an ET-1 clearance receptor (Fukuroda et al., 1994). Thus, blockade of the ET_B receptor by A-192621 would serve to elevate tissue ET-1 levels, thereby producing further venoconstriction at the ET_A receptor. I have no evidence from the current study to support or refute this possibility.

Blockade of both the ET_A and ET_B receptors following administration of the mixed ET antagonist A-182086 lowered MABP modestly in sham rats and dramatically in DOCA-salt rats. More importantly, mixed ET antagonism reduced MCFP in DOCA-salt rats, but not sham rats. This finding supports the idea that ET-induced increases in venomotor tone in DOCA-salt hypertension are due mainly to activation of ET_A receptors, since ET_B blockade alone did not affect MCFP. The results also are consistent with earlier observations in DOCA-salt rats given a selective ET_A receptor antagonist (Chapter 5). The responses of MCFP to selective ET_A receptor antagonism versus mixed ET_A/ET_B receptor blockade were not directly compared in the present experiments. Thus, it is not possible to determine if mixed

receptor antagonists offer any advantages as a way of reducing venomotor tone. It is worth noting, however, that sympathetic venoconstriction (indexed by the response of MCFP to ganglion blockade) was increased by both the ET_B blocker and the mixed receptor antagonist, an effect not observed with the ET_A selective antagonist (Chapter 5). Although in vitro data indicate combined ET_A/ET_B receptor blockade is more effective at inhibiting venomotor responses to ET-1 (Chapter 2). Facilitation of NE release by ET_B receptor blockade may negate that advantage in vivo.

In conclusion, the present study confirms previous findings of elevated MCFP in DOCA-salt hypertensive rats compared to normotensive rats (Chapter 5), but is the first to show that venomotor tone is affected by actions of endogenous ET-1 acting at ET_B receptors to inhibit sympathetic input to the veins, as well as direct actions of ET-1 on VSM ET_A receptors. I also showed that mixed ET_A/ET_B receptor antagonism was effective in lowering MCFP and MABP in DOCA-salt hypertensive rats. These findings may have ramifications regarding choice of balanced ET_A/ET_B versus ET_A receptor antagonists for therapeutic intervention in several cardiovascular diseases including congestive heart failure and hypertension.

Chapter 7

GENERAL DISCUSSION AND CONCLUSIONS

Cardiovascular disease is the leading cause of death in Westernized countries (Whelton et al., 1994). Among the many risk factors associated with the development of cardiovascular disease, hypertension is the most important modifiable factor (Burt et al., 1995). Hypertension may occur secondary to other diseases or conditions, but the vast majority of hypertensive individuals suffer from primary or essential hypertension. Essential hypertension is a worldwide epidemic which largely affects adults but can occur in all ages. Geographic patterns of essential hypertension primarily reflect economic and social development suggesting an environmental contribution to its development. There is little doubt however that essential hypertension also possesses a genetic component. Therefore, essential hypertension is a multifactorial condition with interactions between genetic and environment susceptibility. While the success of treating hypertension is indisputable, what has become evident is that not all essential hypertensives are treatable with the same classes of drugs suggesting that differing hypertensive phenotypes exist (Lilly, 1993). Essential hypertension is best described then as a syndrome of multiple etiologies resulting in subsets or subpopulations of hypertensives varying in their pathophysiology.

Elevated venous tone has been observed in essential hypertension (Schobel et al., 1993) and in models of experimental hypertension (Ricksten et al. 1981;

Chapter 5; Chapter 6). Increases in venous tone in hypertension may be necessary to maintain cardiac filling in the face of reduced compliance of the heart (Safar and London, 1987), however, increased venous tone could result in an increase in the pressure for venous return and CO, thereby contributing to hypertension development (Martin et al., 1998). Therefore, altered venous function has been implicated in the etiology of hypertension (Safar and London, 1985; Edmunds et al., 1989).

Subsets of essential hypertensive humans show a salt-related progression of disease (Tobian, 1991; Taubes, 2000). Hence, the term "salt-sensitive hypertension". Salt-dependent models of experimental hypertension such as the 1K1C (Sventek et al., 1996), reduced renal mass (Potter et al., 1997), DOCA-salt (Schiffrin et al., 1996) and Dahl (Kassab et al., 1997) models show ET-1 involvement as well as alterations in venous function (Yamamoto et al., 1981; 1983; Edmunds et al., 1989) in their development. Of these models, the DOCA-salt model provides the most evidence for a role of ETs in hypertension development (Schiffrin, 1995a; 1999).

In view of these observations, the aim of this research project was to test the overall hypothesis that the endothelial cell-derived peptide ET-1 contributes to the development of salt-sensitive hypertension by decreasing venous capacitance in the DOCA-salt model of experimental hypertension in the rat. To test my hypothesis a series of specific aims was devised to evaluate the effects of ETs on veins in DOCA-salt hypertensive rats. Studies conducted involved both in vitro and

in vivo approaches.

Endothelin-1 mediated increases in venomotor tone contribute to DOCA-salt hypertension in the rat: the model

For purposes of synthesizing my findings into a coherent discussion, a model proposing how ETs contribute to DOCA-salt hypertension through their actions on veins is provided in Figure 7.1 of the general discussion and conclusions. Derivation of the model from key results of experiments conducted in the thesis is discussed below. Based on findings from studies conducted in this thesis work it is proposed that increased activation of the endothelin system in DOCA-salt hypertension in the rat produces two significant effects on venomotor tone which contribute to hypertension development. First, direct actions of ET-1 on VSM ET_A receptors increases venomotor tone in DOCA-salt hypertension in the rat. Second, the increase in venomotor tone observed in DOCA-salt hypertension in the rat that is due to increased sympathetic input to the veins is modulated by neuroinhibitory actions of ET-1 via ET_B receptors. The role of both ET_A and ET_B receptors has important implications regarding the use of balanced ET_A/ET_B versus selective ET_A receptor antagonism in several cardiovascular diseases including heart failure and hypertension.

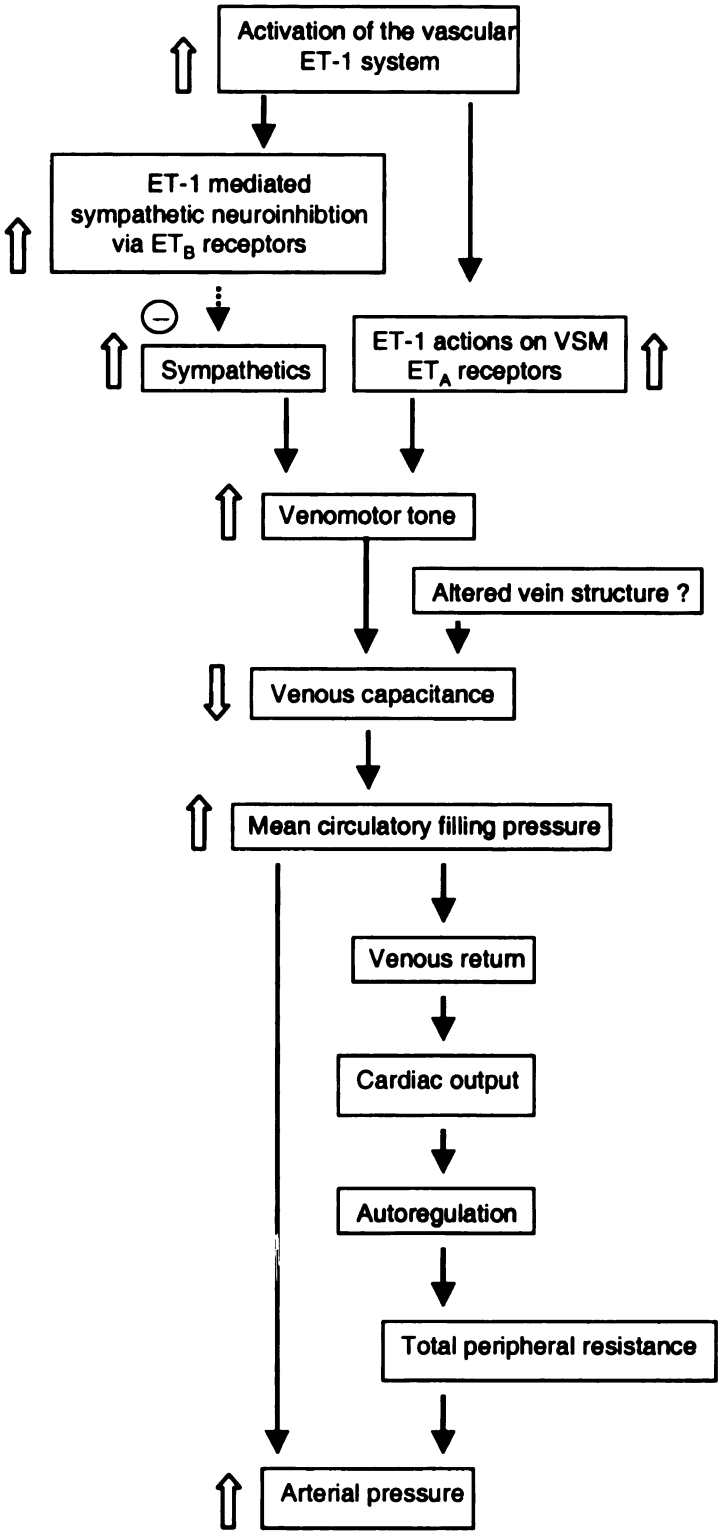

Figure 7.1: Endothelin-1 mediated increases in venomotor tone contribute to DOCA-salt hypertension in the rat: the model.

Reduction in venous capacitance elevates MCFP in DOCA-salt hypertension in the rat

In order to accept the overall hypothesis I must first show that MCFP is increased in DOCA-salt hypertensive rats compared to sham-operated normotensive rats. Results of in vivo studies conducted in chronically instrumented awake rats in Chapter 5 (Figure 5.1) and Chapter 6 (Figure 6.3) showed that MCFP is in fact significantly increased in DOCA-salt hypertensive rats. Evidence exists which supports the idea that some forms of experimental hypertension are initiated by elevated CO (Coleman and Guyton, 1969; Ferrario et al., 1970; Ledingham et al., 1970) which may be due to early increases in MCFP (Manning et al., 1979). Hemodynamic studies performed in young mild or borderline human essential hypertensives have also described a high CO in the presence of a normal TPR in the initial stages of hypertension development (Lund-Johansen, 1994). This idea is illustrated in Figure 7.2 of the general discussion and conclusions, which shows how increases in MCFP could result in elevated arterial blood pressure. My studies did not examine the temporal development of increased MCFP in DOCA-salt rats relative to the establishment of hypertension. Therefore I can not conclude from my studies that the observed increase in MCFP is an initiating event in DOCA-salt hypertension but rather only that MCFP is elevated in DOCA-salt rats with established hypertension of 3-4 weeks duration. Studies conducted early in the development of DOCA-salt hypertension ie. 1 or 2 weeks after surgical placement of DOCA implants may have allowed for the determination of early increases in

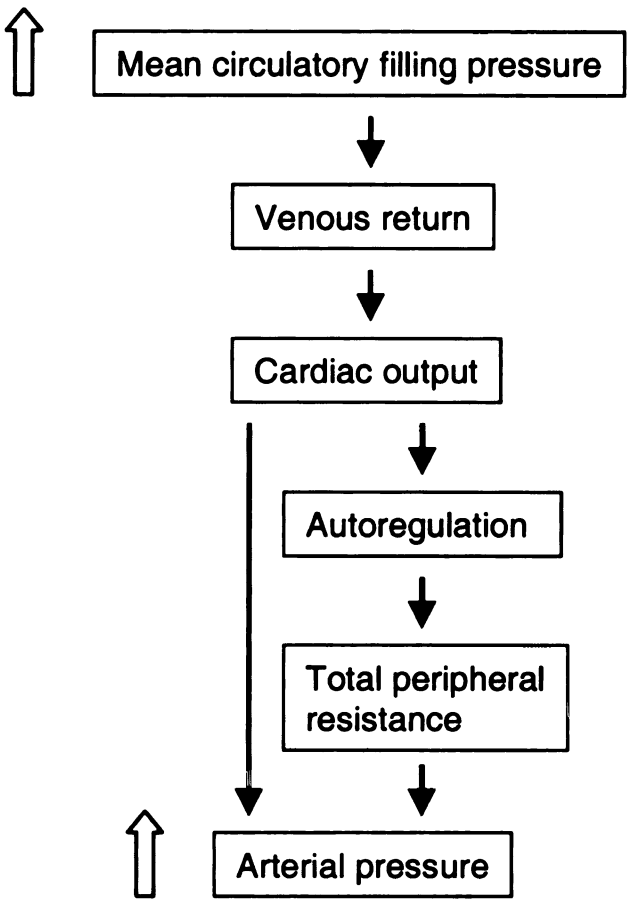

Figure 7.2: Relationship between mean circulatory filling pressure and mean arterial blood pressure in DOCA-salt hypertension in the rat.

MCFP compared to MABP. Yamamoto examined pressure-volume relationships in instrumented DOCA-salt rats at 1, 2, 5 and 8 weeks after DOCA-salt treatment and found no significant increase in MCFP compared to sham rats; however, one-time-only hemodynamic measurements were made only 3 hours after general anesthesia and surgery for catheter and balloon placement (Yamamoto et al., 1983). MCFP values reported here were obtained 2-5 days after surgery. A significantly higher MCFP was found in DOCA-salt rats compared to sham rats at 2 days, and this difference became even larger on subsequent days (Chapter 5, Figure 5.2). Suppression of sympathetic activity and relative dehydration associated with anesthesia and surgery, particularly in DOCA-salt rats may explain differing results between my study and the Yamamoto study. The fact that saline intake in the DOCA-salt rats did not recover to pre-surgery amounts until at least 4 days of recovery provides support for this idea (Chapter 5). In another study in which saline infusion was used to produce hypertension in reduced renal mass dogs (Manning et al., 1979), it was shown that early increases in arterial pressure were due to increased MCFP and CO while TPR was decreased. The findings of that study suggest that early elevations in MCFP are key to initiating increases in systemic blood pressure in salt-dependent hypertension.

In established hypertension both human and animal studies show that the contribution made by CO to chronic elevation in blood pressure is reduced while TPR contributions increase (Lund-Johansen, 1994). Although another virtually invariable finding in established hypertension is decreased capacitance of the

venous system (Safar and London, 1985; 1987). Venous capacitance and blood volume are the main determinants of MCFP. In established hypertension, blood volume is either normal or reduced (Safar and London, 1987). In vivo studies conducted in chapters 5 and 6 of the thesis revealed no significant differences in blood volume (indexed to body weight) between DOCA-salt hypertensive and sham normotensive rats. Therefore I concluded that elevated MCFP observed in established DOCA-salt hypertension was due to decreased venous capacitance (General discussion and conclusions, Figure 7.3).

Endogenous ET-1 increases venomotor tone via direct actions on the vascular smooth muscle in DOCA-salt hypertension in the rat

Multiple factors regulate vascular capacitance including the structure of veins, blood volume and venous smooth muscle activity (venomotor tone). Because veins have limits of distensibility, increases in venous blood volume can have significant effects on venous capacitance, cardiac filling pressure (preload) and blood pressure. This point has already been addressed with regards to findings in my studies. Structural abnormalities in veins of hypertensive humans (Mark, 1984) and in some animal models of experimental hypertension such as the SHR rat (Ricksten et al., 1981) have been documented. The mitogenic actions of ET-1 on VSMC's comprising the vascular media are associated with hypertrophic remodeling of resistance arteries in DOCA-salt rats (Li et al., 1994; Schiffrin et al., 1996) however no studies have examined ET-1 induced changes in venous wall

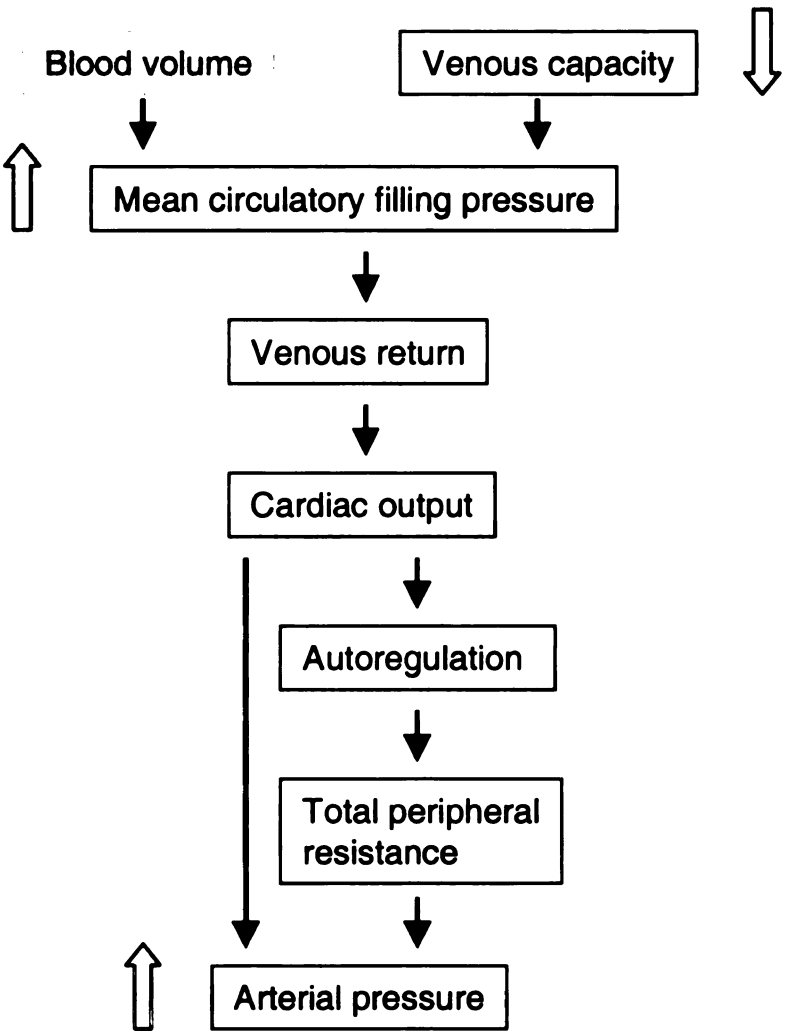

Figure 7.3: Relationship between venous capacitance, blood volume and mean circulatory filling pressure in DOCA-salt hypertension in the rat. The shaded area indicates no significant effects.

structure in DOCA-salt rats. Although it is possible that structural changes in the veins of DOCA-salt hypertensive rats may have contributed to the increased MCFP observed by reductions in wall distensibility and lumen size, I did not examine vascular structure in my studies.

Venomotor tone is determined by the level of sympathetic input to the veins and the effects of circulating and locally produced hormones. A main goal of this thesis was to show that the endothelial cell-derived peptide ET-1 contributes to DOCA-salt hypertension by decreasing venous capacitance through increases in venomotor tone. I hypothesized that the sustained hypertension in the DOCA-salt experimental model of hypertension in the rat was in part the result of elevated venous tone (MCFP) due to direct ET-1 induced alterations in vascular function. The rationale for the proposed hypothesis was based on in vitro evidence showing that ET-1 contracts capacitance vessels such as the mesenteric veins in DOCA-salt hypertensive rats and sham normotensive rats through activation of ET_A receptors (Chapter 3). In Chapter 5 (Figure 5.3), i.v. administration of the selective ET_A receptor antagonist ABT-627 produced a significant reduction in MCFP in DOCA-salt but not sham normotensive rats. While it can be concluded that ET-1 contributes to DOCA-salt hypertension in part by acting at ET_A receptors, it can not be concluded that the contribution was due to direct ET-1 actions on the VSM. ET-1 may have produced its effects on MCFP in DOCA-salt rats through modulating sympathetic activity to the veins, therefore the effects of selective ET_A receptor antagonism were evaluated in conjunction with ganglion blockade using

hexamethonium. Acute injections of hexamethonium alone caused falls in MCFP, MABP and HR in both sham and DOCA-salt rats, and the magnitude of these changes was significantly greater in DOCA-salt rats (Chapter 5, Figure 5.4). Hemodynamic responses to hexamethonium given one hour after pretreatment of sham and DOCA-salt rats with ABT-627 were not altered compared to non-pretreated rats (Chapter 5, Figures 5.5 and 5.6). Based on these findings, I concluded that the increased venomotor tone observed in DOCA-salt hypertension was due in part to increased sympathetically mediated venomotor tone and in part to enhanced ET-1 induced venoconstriction mediated by VSM ET_A receptors (General discussion and conclusions, Figure 7.4).

Increased sympathetic activity is an early feature of human hypertension and in several models of experimental hypertension including renovacular, steroid-induced and genetic hypertension in the rat, and is necessary for full expression of hypertension (Wyss, 1998; Fouad-Tarazi and Izzo, 1998). Recent work by Dr. James Galligan, Michigan State University, clearly shows that sympathetic input to the veins in DOCA-salt hypertension is increased. This statement is based on in vitro findings of reduced sensitivity to the contractile effects of exogenous NE in veins from DOCA-salt hypertensive rats compared to sham normotensive rats. Also, contractions caused by electrical stimulation of sympathetic nerves innervating DOCA-salt veins were increased while NE-content of DOCA-salt veins was decreased compared to sham normotensive veins. Contractions caused by electrical stimulation were blocked by the α1-adrenergic antagonist prazosin while

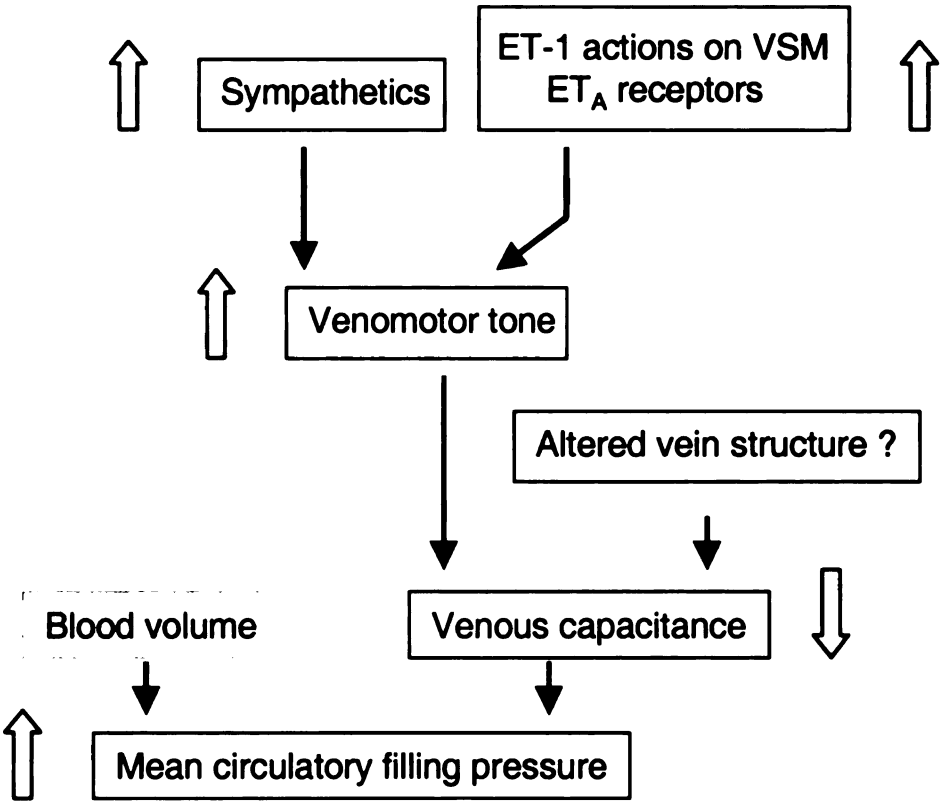

Figure 7.4: Increased sympathetic input to veins and direct actions of endogenous ET-1 acting at VSM ET_A receptors increases venomotor tone in DOCA-salt hypertension in the rat.

phenylephrine, but not clonidine, mimicked NE responses . Taken together, the data suggest that in DOCA-salt veins α1-adrenergic receptors are down-regulated due to enhanced NE release which would cause increased venomotor tone and contribute to hypertension development.

If an interaction between endogenous ET-1 and sympathetic control of MCFP in DOCA-salt rats existed, such that ET-1 potentiated sympathetic venomotor tone through activation of ET_A receptors, then blockade of ET_A receptors with ABT-627 should have blunted subsequent MCFP responses to ganglion blockade. This was not seen in either sham or DOCA-salt rats, therefore, endogenous ET-1 did not significantly influence sympathetic control of venomotor tone in sham or DOCA-salt rats via actions on ET_A receptors.

Venoconstriction to ET-1 in vitro is maintained in DOCA-salt hypertension in the rat

Experiments described in Chapter 5 showed that the increase in venomotor tone observed in DOCA-salt hypertensive rats was due in part to direct actions of endogenous ET-1 on the VSM, and that the increase observed was reversed by selective ET_A receptor antagonism. Increased venoconstriction caused by ET-1 acting at VSM ET_A receptors in DOCA-salt hypertension may be the result of enhanced reactivity of VSM ET_A receptors to ET-1, or increased production of ET-1 acting at ET_A receptors. ET-1 contractile responses in veins show enhanced reactivity when compared to contractions in corresponding arteries in vitro (Cocks

et al., 1989; Riezebos et al., 1994) and in vivo (Haynes et al., 1991). Also, constriction of dorsal hand veins to ET-1 are significantly greater in essential hypertensives compared to normotensives (Haynes et al., 1994). Therefore, in Chapter 3, I tested the hypothesis that increased ET-1 mediated venoconstriction in DOCA-salt rats was caused by changes in venous smooth muscle responsiveness to ETs. In order to examine contractile-mechanisms in the absence of ET-evoked dilator release I studied vessels in the presence of indomethacin and NLA. I showed that contractile responses to ET-1 in mesenteric veins in vitro from DOCA-salt hypertensive rats were not different from veins of normotensive rats (Chapter 3, Figure 3.1 and Table 3.1). I also showed that contractile responses to ET-1 in mesenteric arteries in vitro from DOCA-salt hypertensive rats were reduced compared to arteries from sham normotensive rats (Chapter 3, Figure 3.1 and Table 3.1). Taken together, these findings did not suggest that venous smooth muscle reactivity to ET-1 was enhanced in veins from DOCA-salt hypertensive rats compared to veins from sham normotensive rats. Finally, I showed that reactivity to ET-1 in mesenteric veins in vitro from sham normotensive and DOCA-salt hypertensive rats was enhanced compared to ET-1 responses in corresponding arteries (Chapter 3, Figure 3.1 and Table 3.1). These differences were not due to a lack of pressurization of arteries (Chapter 3, Figure 3.3). Therefore, based on results of experiments conducted in Chapter 3 in the presence of indomethacin and NLA, it was concluded that the increased venoconstrictor activity produced by the direct actions of endogenous ET-1 on the VSM in DOCA-salt rats in vivo was not

due to enhanced venous smooth muscle reactivity to ETs.

The reduction in contractile responses in arteries from DOCA-salt hypertensive rats (Chapter 3, Figure 3.1 and Table 3.1) may be the result of increased vascular production of ET-1 and subsequent ET receptor-downregulation. Increased preproET-1 gene expresssion (Lariviere et al., 1993a; Day et al., 1995) and immunoreactive ET-1 content (Lariviere et al., 1993b) have been demonstrated in aorta and mesenteric arteries of DOCA-salt hypertensive rats. Activation of the ET system and consequent down-regulation of ET receptors in arteries from DOCA-salt hypertensive rats is suggested by in vitro functional studies which show reduced contractions to ET-1 compared to responses in arteries from sham normotensive rats (Hagen and Webb, 1984; Nguyen et al., 1992; Giulumian et al., 1998). Other support comes from in vitro binding studies which show reduced density of ET-1 VSM receptors in endothelium denuded aorta and mesenteric arteries obtained from DOCA-salt hypertensive rats compared to arteries from sham normotensive rats (Nguyen et al., 1992). Although endothelial cells of venous origin have been shown to produce ET-1 in vitro (Inoue et al., 1988a; 1988b), no studies have examined ET-1 production in the veins of DOCA-salt hypertensive rats. Taken together, these findings strongly suggest, but do not allow us to conclude, that the venoconstrictor activity produced by ET-1 in vivo is significantly larger in DOCA-salt hypertensive versus sham normotensive rats due to increased ET-1 formation and activation of VSM ET_A receptors (General discussion and conclusions, Figure 7.5). Studies examining ET production in veins

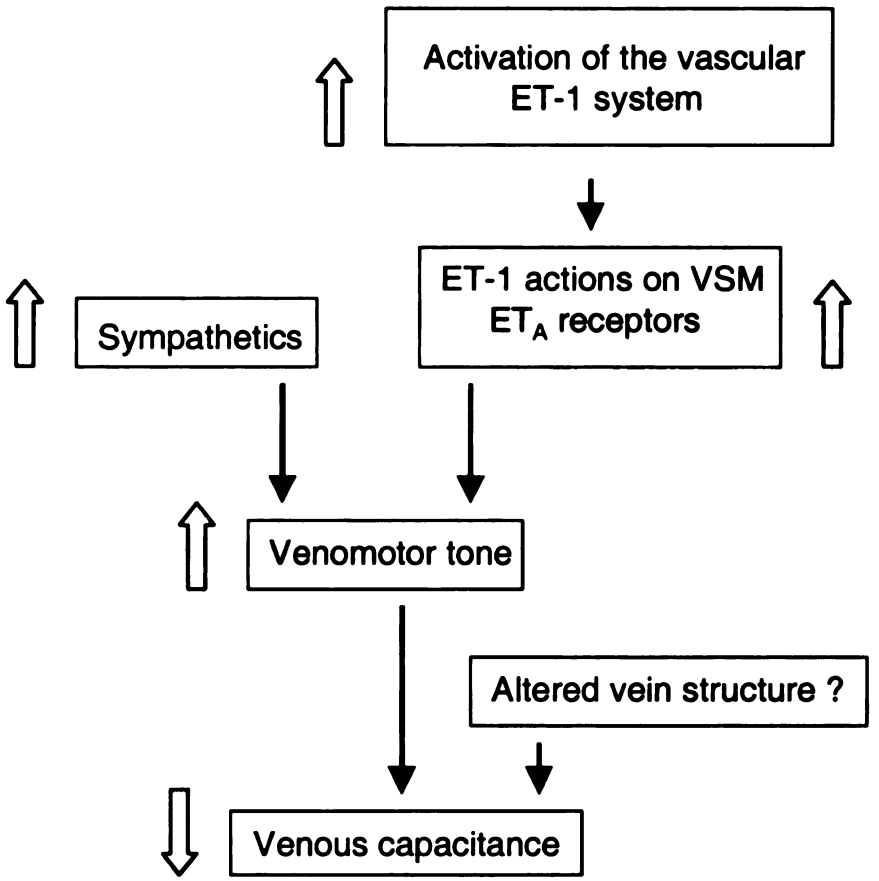

Figure 7.5: Enhanced activation of the vascular ET system contributes to increased venomotor tone in DOCA-salt hypertension in the rat by actions of ET-1 on VSM ET_A receptors.

of DOCA-salt hypertensive rats and sham normotensive rats will provide critical information regarding activation of the ET system in veins in hypertension and alterations in receptor dynamics. Conclusions may then be drawn concerning the source of ET-1 in ET-mediated venoconstriction in DOCA-salt hypertension ie. veins versus arteries.

ET-1 causes contractions in arteries via actions on VSM ET_A receptors (Moreland et al., 1992; Sumner et al., 1992; Cardell et al., 1993). Furthermore, in Chapter 5, I showed that the increase in venomotor tone observed in DOCA-salt hypertensive rats was reversed by a selective ET_A receptor antagonist. However, ET-1 can cause venoconstriction by activating both ET_A and ET_B VSM receptors (Moreland et al., 1992; Sumner et al., 1992). Therefore, I also tested the hypothesis that ET mediated venoconstriction in mesenteric veins from DOCA-salt rats is mediated by VSM ET_A and ET_B receptors. I showed that the ET_B selective agonist S6c produced contractions in sham and DOCA-salt veins but not arteries suggesting the presence of VSM ET_B receptors in mesenteric veins but not arteries in the rat (Chapter 3, Figure 3.2). However, contractions to ET-1, a mixed ET_A and ET_B receptor agonist, were blocked by selective ET_A receptor antagonism (BQ-610) but not with a selective ET_B receptor antagonist (BQ-788) in mesenteric veins from either sham or DOCA-salt rats (Chapter 3, Figure 3.5 and Table 3.2). Results of experiments conducted in Chapter 2 in guinea pig mesenteric veins revealed similar findings with ET-1 contractions being mediated by ET_A receptors (Chapter 2, Figure 2.5 and Table 2.2) even though S6c produced contractions in these vessels

(Chapter 2, Figure 2.4 and Table 2.2). Although in vitro experiments conducted in Chapters 2 and 3 clearly demonstrate the presence of VSM ET_A and ET_B receptors in mesenteric veins from DOCA-salt hypertensive rats and sham normotensive rats, their findings support the earlier conclusions that the direct actions of the endogenous ET peptide, ET-1, on the VSM to increase venomotor tone are mediated by ET_A receptors.

Interestingly, combined ET_A/ET_B receptor blockade in experiments conducted in Chapter 3 was more effective than ET_A receptor antagonism at inhibiting venous contractions to ET-1 in veins from sham normotensive and DOCA-salt hypertensive rats (Chapter 3, Figure 3.5 and Table 3.2). These findings suggest that an interaction may occur between VSM ET_A and ET_B receptors on mesenteric veins resulting in masking of the ET_B-mediated contractile effect. The existence of an interaction between the VSM ET_A and ET_B receptors has been demonstrated in other in vitro vascular studies of arteries (Fukuroda et al., 1994b; Mickley et al., 1997) and in guinea pig mesenteric veins studied in Chapter 2 (Chapter 2.1, Figure 2.6 and Table 2.2). Importantly, the proposed masking of VSM ET_B receptors by ET_A receptor activation in mesenteric veins was less pronounced in DOCA-salt hypertensive rats, as combined ET_A/ET_B receptor antagonism shifted ET-1 dose-response curves further right than ET_A receptor antagonism alone in veins from DOCA-salt rats, but not sham rats (Chapter 3, Figure 3.5 and Table 3.2). One possible interpretation of these findings may be that increased ET-1 production in DOCA-salt hypertension causes ET_A receptor downregulation in veins and

unmasking of the VSM ET_B receptor. Once unmasked, the contractile ET_B receptor may be responsible for the maintained venoconstriction to ET-1 in DOCA-salt hypertension. As such, it would be anticipated that selective ET_B receptor antagonism should have produced a shift in concentration responses to ET-1 in mesenteric veins from DOCA-salt hypertensive rats. This was not the case in experiments conducted in Chapter 3. Although the mechanisms behind the proposed interaction between the two ET contractile receptors are unclear, a potential implication is that in hypertensive animals combined ET_A/ET_B receptor blockade could produce larger increments in vascular capacitance that ET_A antagonism alone.

ET-evoked endothelial-derived dilator release from mesenteric veins in the rat do not make important contributions to net venous tone

In vivo and in vitro work conducted in this thesis have clearly shown that the increased venomotor tone observed in DOCA-salt hypertension is due in part to direct actions of ET-1 on VSM ET_A receptors. However, venomotor tone may also be augmented in DOCA-salt hypertension by alterations in ET-evoked endothelial-derived dilator contributions to net venous tone. The vascular endothelium serves an important role in the control of arterial tone, particularly through the release of NO and dilator prostanoids. The role of the endothelium in modulating venous tone is unclear. In Chapter 3, concentration-responses to ET-1 in sham arteries were enhanced in the presence of indomethacin and NLA compared to untreated

controls, while arteries from DOCA-salt hypertensive rats were unaffected by indomethacin and NLA, except at the highest concentrations of ET-1 (Chapter 3, Figure 3.1 and Table 3.1). Comparison of ET-1 contractile responses in the absence and presence of indomethacin and NLA in mesenteric veins from sham normotensive rats or DOCA-salt hypertensive rats revealed no differences. These findings suggest that ET-1 evoked dilator release by the endothelium of mesenteric arteries in the rat makes important contributions to net arterial tone while minimal ET-mediated dilator release occurs in mesenteric veins in the rat. In order to substantiate these findings in in vivo, the effects of NOS and cyclooxygenase inhibition on ET-peptide changes to venomotor tone in awake normal rats were tested in experiments conducted in Chapter 4. Because ET-induced dilator release by the endothelium is mediated by the ET_B receptor, the selective ET_B receptor agonist S6c was chosen over ET-1 for these experiments. Administration of L-NAME or indomethacin alone produced no changes in MCFP in awake rats (Chapter 4, Figure 4.3). It was possible that MCFP failed to increase due in part to reflex withdrawal of sympathetic activity to the veins. However, in other studies, administration of L-NAME to conscious rats also produced no effects on MCFP in the presence or absence of ganglion blockade (Wang et al., 1995), suggesting this is not the case. It was also found in Chapter 4 that neither L-NAME or indomethacin potentiated MCFP responses to acute injections of S6c (Chapter 4, Figure 4.4). Therefore, it was concluded that increased ET-mediated venomotor tone in DOCA-salt hypertension was not due to impaired endothelial dilator release.

Support for this conclusion may be gained by evaluation of hemodynamic changes following administration of L-NAME and indomethacin to DOCA-salt hypertensive rats, where it is anticipated that no significant changes would be observed in MCFP compared to no treatment.

High salt alone does not modify acute changes in venomotor tone to ET-peptides in the rat

It is well established that some forms of hypertension are salt-dependent (Taubian, 1991). Mortensen showed that pressor responses to chronic infusion of ET-1 in rats are markedly potentiated by high salt intake (Mortensen and Fink, 1992). Therefore, it is possible in experiments conducted in Chapter 5 that DOCA-salt rats showed greater falls in MCFP to selective ET_A receptor antagonism compared to levels of MCFP decline in sham rats because the DOCA-salt rats were provided a high salt intake. In Chapter 5, sham rats did not exhibit a decrease in MCFP in response to selective ET_A receptor antagonism after 3-4 days of either sodium depletion or sodium loading (Chapter 5, Figure 5.7). Further, in Chapter 4 it was shown that neither basal MCFP nor integrated venomotor responses to acute injection of ET-1 or S6c in awake rats were significantly affected by short-term changes in salt intake (Chapter 4, Figure 4.1 and Figure 4.2). In fact, the same was true for MABP and HR (Chapter 4, Figure 4.1 and Figure 4.2). Therefore, it was concluded that salt intake did not appear to affect acute vascular responses (arterial or venous) to ET peptides in normal rats. The role of salt in ET-1

contributions to DOCA-salt hypertension was not evaluated in my studies as it was not the goal of the thesis to explore the relationship between salt and ET-1 in DOCA-salt hypertension. However, others have shown that increased salt intake does not appear to consistently alter vascular formation of ET-1 (Michel et al., 1993; Ishimitsu et al., 1996; Oh et al., 1997). The relationship between salt-sensitivity and ET-1 in DOCA-salt hypertension remains unclear, as do the mechanisms responsible for ET-1 induction in this model of experimental hypertension. Schiffrin has provided evidence that activation of the vascular ET system may be mediated by increased vasopressin in DOCA-salt hypertension (Intengan et al., 1998; 1999).

Endogenous ET-1 modulates the enhanced sympathetic activity observed in DOCA-salt hypertension in the rat via ET_B receptors

Experiments conducted in Chapter 5 and Chapter 3 showed that the direct contractile actions of ET-1 on DOCA-salt hypertensive and sham normotensive veins were the result of ET_A receptor activation. However, results of experiments conducted in Chapter 3 also suggest that the VSM ET_B receptor may be important in ET-mediated venoconstriction, particularly in DOCA-salt hypertension. Other studies have shown that ET-1 inhibits NE release induced by sympathetic nerve stimulation in renal arteries (Suzuki et al., 1992), and that the neuroinhibition observed is mediated through ET_B receptor mechanisms (Matsuo et al., 1997). Therefore, it was hypothesized that ET-1 acting at ET_B receptors provides important contributions to venomotor tone via direct actions on the VSM and through

modulation of sympathetic tone to the veins. In Chapter 6, it was shown that the mixed ET_A/ET_B receptor antagonist A-182086, but not the ET_B selective antagonist A-192621, significantly reduced MCFP in DOCA-salt, but not sham rats (Chapter 6, Figure 6.1 and 6.2). These results suggest that endogenous ET-1 does not act through ET_B receptors to elevate venous tone in DOCA-salt hypertension. Alternatively, ET_B blockade may not have affected MCFP because of decreased clearance of ET-1 by blockade of ET_B receptors. It has been postulated that the ET_B receptor also functions as a clearance receptor (Fukuroda et al., 1994). As such, its blockade would serve to elevate tissue ET-1 levels, thereby producing further venoconstriction at the ET_A receptor. I do not have other evidence to support or refute this possibility. More likely, these results are explained by baroreflex mediated alterations in venous tone and subsequent blunting of any direct effects of selective ET_B receptor antagonism on the veins. This idea is supported by the small but significant increase in MABP in both sham and DOCA-salt groups following administration of A–192621 (Chapter 6, Figure 6.1 and Figure 6.2), and other studies in conscious rats showing that sympathetic reflexes elicited by vasoactive drugs have significant effects on MCFP (Waite et al., 1988; Waite and Pang, 1992). If the effects of ET_B receptor blockade on venomotor tone were opposed by baroreflex mediated decreases in sympathetic tone to the veins as a result of increased MABP, then it would be expected that ganglion blockade after pretreatment with A-192621 should produce smaller reductions in MCFP than with hexamethonium alone, owing to diminished sympathetic input to the veins. In fact,

it was found that the peak fall in MCFP in DOCA-salt rats, but not sham rats, pretreated with A-192621 was significantly greater following hexamethonium injection than the fall in non-pretreated rats following hexamethonium (Chapter 6, Figure 6.4 and Figure 6.5). These findings indicated that pretreatment of DOCA-salt rats with a selective ET_B receptor antagonist increased rather than decreased sympathetic input to the veins. Therefore, it was concluded that ET-1 acts via the ET_B receptor to inhibit sympathetic input to the veins in DOCA-salt hypertension (General discussion and conclusions, Figure 7.6). These findings are in agreement with studies mentioned earlier (Suzuki et al., 1992; Matsuo et al., 1997), however the results reported here are the first obtained in DOCA-salt hypertension.

In Chapter 6 (Figure 6.5), it was also shown that pretreatment of DOCA-salt rats, but not sham rats, with a mixed ET_A/ET_B receptor antagonist (A-182086) produced a peak fall in MCFP following hexamethonium administration that was significantly greater than MCFP declines with hexamethonium alone. Because of neuromodulation of sympathetic input to the veins by the actions of ET-1 at ET_B receptors, I could not conclude whether ET-1 increased venomotor tone via direct actions on the VSM ET_B receptor. In order to properly address this question it is necessary to remove sympathetic contributions to venomotor tone. Future experiments conducted in baroreflex enervated (sinoatrial denervated) DOCA-salt rats, and in the presence of adrenergic receptor antagonism would allow for a more complete understanding of ET receptor subtypes mediating direct venoconstriction to ET-1 in vivo. Specifically, pretreatment of sinoatrial denervated DOCA-salt

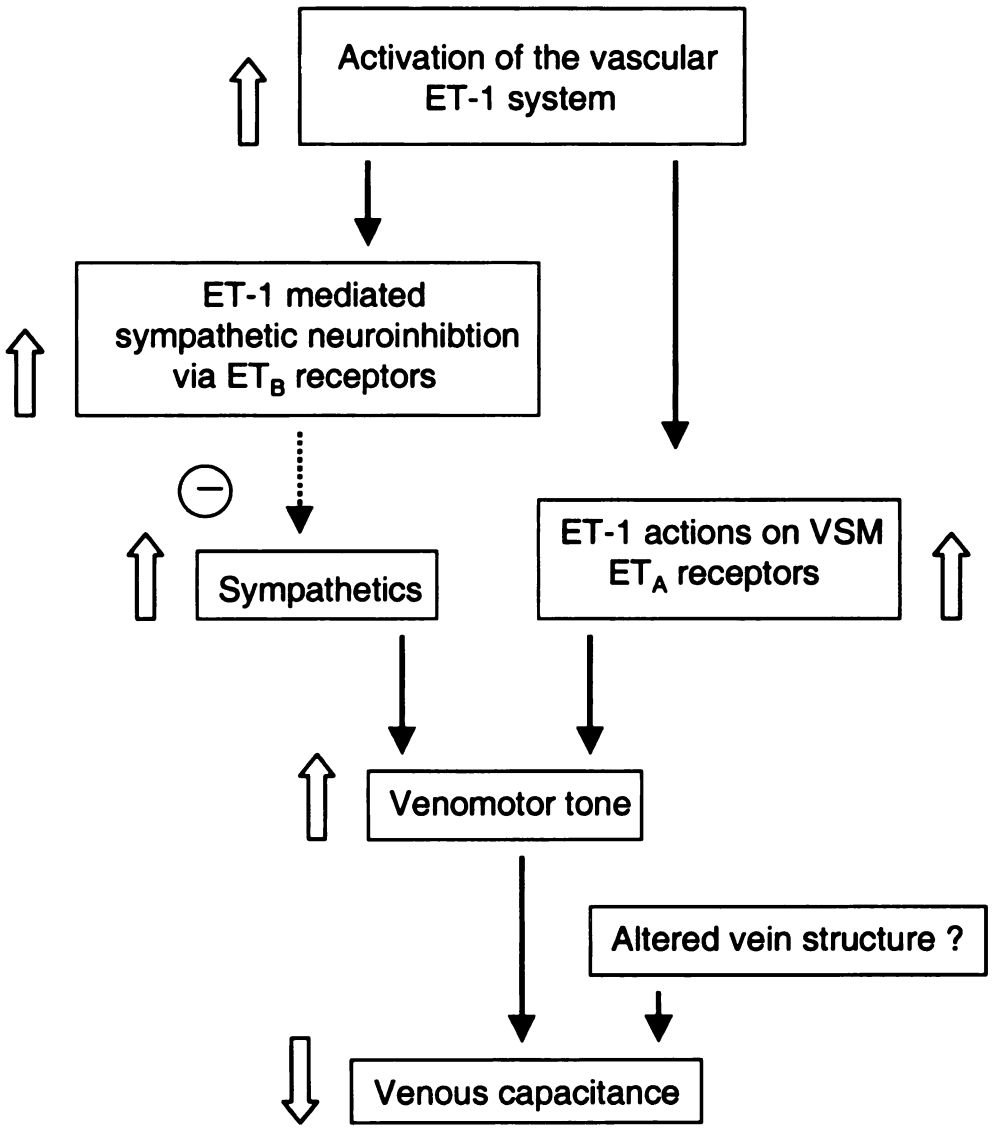

Figure 7.6: ET-1 acts via the ET_B receptor to inhibit sympathetic input to the veins in DOCA-salt hypertension in the rat.

hypertensive rats with adrenergic receptor blockers, and selective a ET_A receptor antagonist, would in theory leave the VSM ET_B receptor for study. Conclusions regarding superiority of mixed ET_A/ET_B receptor antagonism versus selective ET_A receptor antagonism in lowering MCFP in DOCA-salt rats were also not possible due to differing sets of rats studied in experiments conducted in Chapter 5 and 6. These comparisons would provide valuable information regarding therapeutic implication with the use of mixed ET receptor antagonists versus selective ET_A receptor antagonists in intact hypertensive animals.

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