ALTERED VASCULAR STRUCTURE AND FUNCTION IN THE SPONTANEOUSLY HYPERTENSIVE RAT: ROLE OF THE SYMPATHETIC NERVOUS SYSTEM AND THE RENIN-ANGIOTENSIN SYSTEM

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DECLARATION

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ABSTRACT

The principal aims of the studies presented in this thesis were to examine the role of the renin-angiotensin and sympathetic nervous systems in hypertension development in the spontaneously hypertensive rat and, in particular, their influence on certain biochemical, structural and functional properties of the cardiovascular system in relation to blood pressure changes in the model. This was achieved through pharmacological intervention which targeted specific areas responsible for the normal functioning of these systems.

Chronic captopril treatment was found to be effective in not only preventing hypertension development but also suppressing left ventricular hypertrophy, enhanced vascular actin and 3-methylhistidine (a marker of vascular actin levels) and enhanced vascular reactivity to noradrenaline, which is characteristic of the perfused mesenteric vascular bed in the SHR. The suppression in vascular reactivity and 3MH concentrations was greater than that predicted from captopril's blood pressure-lowering influence, indicating that the drug may exert additional effects on vascular biochemical and functional parameters aside from its influence on blood pressure. Moreover, the profound suppression in vascular reactivity achieved after just one week of captopril treatment in the adult SHR suggests an effect related to vascular neuroeffector function in this model. The ability of losartan to block the angiotensin II-mediated facilitation of responses to nerve-stimulation in the perfused mesenteric vascular bed from the SHR supports this premise and suggests that this occurs through actions of angiotensin II mediated at the AT_1 receptor.

Withdrawal from chronic captopril treatment was generally associated with a reversal in the enhanced expression of mesenteric vascular actin (and 3MH) and mesenteric vascular reactivity, while the redevelopment of hypertension remained suppressed. Other parameters, such as aortic angiotensin converting enzyme (ACE) activity, left ventricular hypertrophy and larger vessel contractile protein levels were more effectively correlated with systolic blood pressure after captopril withdrawal. These results suggest

that some of the measured vascular biochemical and functional indices are not tightly coupled to the prevailing systolic blood pressure in this model.

In comparison to captopril, chronic vasodilatation (hydralazine treatment) in the SHR exerted less influence on cardiovascular biochemical, structural and functional measures (despite affording equal protection against hypertension development during therapy), indicating that the potent influence of ACE-inhibition on cardiovascular biochemical, structural and functional alterations occurs in part independently of its effects on blood pressure in this model. After withdrawal, systolic blood pressure rapidly reverted to the hypertensive levels of untreated adult SHR. The likelihood that the influence of captopril treatment on these parameters may play a role in the suppression of hypertension redevelopment following drug withdrawal was not conclusively defined in these studies.

The defining of a specific role for angiotensin II in the ontogeny of hypertension development in the SHR was explored with the use of the angiotensin II type I (AT_1) receptor antagonist losartan. Losartan elicited a dose-dependent prevention in hypertension development with normotension achieved at a dosage of 30 mg/kg/day. The suppression in vascular reactivity was not dose-dependently linked to the fall in blood pressure. After withdrawal from losartan treatment (30 mg/kg/day), systolic blood pressure rose rapidly to control levels, while vascular reactivity remained attenuated. These results suggest that while angiotensin II and the AT_1 receptor are involved in the control of hypertension development in the SHR, actions of the ACE-inhibitor not mediated by the AT_1 receptor may have influenced the long-term resetting of blood pressure after drug withdrawal. Furthermore, there was a dissociation between vascular reactivity and blood pressure after treatment withdrawal, indicating this index of vascular function may be a poor predictor of the prevailing blood pressure during and after antihypertensive treatment. The lack of effect of losartan on vascular 3MH concentrations and cardiac hypertrophy also indicate that these features may be achieved independently of the AT_1 receptor. This may potentially occur at the level of the ACE or at another AT receptor subtype.

Chronic sympatholytic therapy with α-adrenoceptor antagonists (doxazosin (α_1) and phenoxybenzamine (α_1 and α_2)) and ganglion blockade (mecamylamine) was ineffective in preventing hypertension development in the SHR, despite pharmacological evidence for α_1-adrenoceptor blockade in vivo. The functional tolerance to α-adrenoceptor blockade was associated with an enhanced action of the renin-angiotensin system, as shown by the greater hypotension exhibited in response to *in vivo* administration of losartan in phenoxybenzamine and doxazosin-treated SHR. The recruitment of the renin-angiotensin system under these circumstances underpins the central role of angiotensin II in maintaining the phenotype in the SHR. Collectively, the results presented in this thesis highlight the integral role of the renin-angiotensin system in developing and maintaining the cardiovascular amplifying mechanisms associated with the expression of hypertension in this model.

ABBREVIATIONS

The following abbreviations have been used throughout this thesis.

%C	percentage crosslinking monomer concentration
%T	percentage total monomer concentration
3EtH	3-ethylhistidine
3MH	3-methylhistidine
5HT	serotonin
ACE	angiotensin converting enzyme
AII	angiotensin II
ANOVA	analysis of variance
$AT_{()}$	angiotensin II receptor
ATP	adenosine triphosphate
AVP	arginine vasopressin
$BaCl_2$	barium chloride
Bis	N,N'-methylene-bis-acrylamide
BSA	bovine serum albumin
BW	body weight
CAP(REL)	captopril, (captopril-released)
cm, mm, nm	length units (centimetres, millimetres, nanometres)
CNS	central nervous system
cpm	counts per minute
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DNA	deoxyribonucleic acid
DOCA	deoxycorticosterone acetate
DOX	doxazosin
DuP 753	losartan
EDRF	endothelial-derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
EIM	enzyme incubation mixture

fig.	figure
g	gravity units
GABA	gamma amino butyric acid
GHD	genetic hypertension development
HCl	hydrochloric acid
HHL	hippuryl-histidyl-leucine
HPLC	high performance liquid chromatography
HW	heart weight
HYD(REL)	hydralazine, (hydralazine-released)
Hz	frequency units
i.v.	intravenous
IGF	insulin-like growth factor
IML	intermediolateral column
IP_3	inositol triphosphate
KCl	potassium chloride
kg, g, mg, ug	weight units (kilogram, gram, milligram, microgram)
l, ml, ul	volume units (litre, millilitre, microlitre)
LOS(REL)	losartan, (losartan-released)
LVH	left ventricular hypertrophy
M, mM, uM, nM	concentration units (molar, millimolar, micromolar, nanomolar)
mA	current units (milliamps)
MAP	mean arterial pressure
MEC	mecamylamine
MHC	myosin heavy chain
mmHg	millimetres of mercury
MOPS	3-[morpholino] propanesulfonic acid
mRNA	messenger RNA
n	number of observations
NA	noradrenaline
NaCl	sodium chloride
NaOH	sodium hydroxide

NGF	nerve growth factor
NM	nonmuscle
NTS	nucleus tractus soltarius
°C	degrees Celsius
P	probability level
p.o.	per os
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PMSF	phenylmethylsulfonylfluoride
PMVB	perfused mesenteric vascular bed
POB	phenoxybenzamine
PRA	plasma renin activity
RAS	renin angiotensin system
RFLP	restriction fragment length polymorphism
s, ms, mins	time units (seconds, milliseconds, minutes)
SBP	systolic blood pressure
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SHR	spontaneously hypertensive rat
SM	smooth muscle
SNS	sympathetic nervous system
TEMED	N,N,N',N'-tetramethylenediamine
TGF-ß	transforming growth factor beta
TLC	thin layer chromatography
TPR	total peripheral resistance
TXA_2	thromboxane
U	International units
vs.	versus
VSM(C)	vascular smooth muscle (cell)
WKY	Wistar-Kyoto rat

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1.1 Hypertension-definition, prevalence, and epidemiology

Hypertension is a disorder with a prevalence sufficiently high in acculturated societies to warrant it being designated a serious public health concern. As a long term condition it occurred in 1.2 million persons, or 7.1% of the Australian population over the period 1985-1989. 2.2 million persons reported experiencing diseases of the circulatory system and hypertension occurred in 54.4% of those sufferers (Social Health Atlas of Australia, vol 1, 1992, p 68-71).

Hypertensive subjects occupy the upper tail of a continuous unimodal distribution of blood pressure, with the division between "normotension" and "hypertension" being arbitrary. Within this upper tail, further classification is based upon levels of both diastolic and systolic blood pressure. These include definitions of mild, moderate, severe and isolated systolic hypertension based on measured blood pressure values (WHO/ISH Guidelines for the Management of Mild Hypertension, June 1993).

Cardiovascular risk has a strong, direct association with levels of both systolic and diastolic blood pressure (Horan and Lenfant, 1990). Hypertension is a powerful independent contributor to cardiovascular mortality and morbidity, on average conferring a threefold increase in risk at all ages and in both sexes (Kannel and Higgins, 1990). Clear treatment benefits in intervention trials occur when either or both diastolic and systolic blood pressure levels are reduced (Kannel et al, 1980).

Hypertension is a contributor to and a risk factor for a wide range of other disorders. Target organ damage can occur as a result of the high blood pressure (eg., heart disease, renal insufficiency, aneurysm, retinopathy), or as a reaction to high blood pressure (eg., left ventricular hypertrophy). Hypertension is also linked with atherosclerotic vascular disease, including ischaemic heart disease and cerebrovascular and peripheral vascular disease.

Hypertension and its sequelae remain the leading cause of death in Australia, accounting for 47.1% of all deaths in the period 1985-1989 (Social Health Atlas of

Australia, vol 1, 1992, p 224-229). The main causes of death in this period were from ischaemic heart disease and stroke. Coronary heart disease is the most prevalent lethal sequel of hypertension, occurring at a rate two to three times that exhibited by normotensives (Kannel and Higgins, 1990).

1.2 Hypertension-aetiology, pathogenesis and risk factors

In general, hypertensive subjects can be subdivided into two groups, based on aetiology. In approximately 5-10% of cases, hypertension is secondary to distinct abnormalities eg., renal or endocrine conditions. These include chronic glomerulonephritis, pyelonephritis and analgesic nephropathy or phaeochromocytoma (endocrine). In many of these cases, once a correct diagnosis is obtained, the condition can be remedied pharmacologically or surgically. In the remaining 90-95% of cases, the underlying cause is unknown, and the patient is classified as having primary, or essential hypertension. Primary hypertension is a multifactorial disorder comprising polygenetic, environmental, and physiologically-based cardiovascular reinforcing factors that vary in their expression across each individual (Folkow, 1978). Thus, there are likely to be many variations within the classification of essential hypertension itself and finding a common basis to categorise essential hypertensives becomes difficult. A number of classifications have been adopted, according to plasma renin activity, salt-sensitivity (or non-sensitivity), and haemodynamic characteristics (vasoconstrictive or volume-dependent).

Blood pressure is regulated by multiple control mechanisms, abnormalities in any one of which can result in transient or sustained increases in blood pressure. These include signals of vascular, cardiac, endocrine/autocrine/paracrine, neural and renal origin, aberrations in one or more of these being associated either with essential hypertension or animal models of genetic hypertension (Dzau et al, 1989).

1.2.1. Genetic factors

Genetic influences on blood pressure, in which blood pressure measures correlate highly among related individuals, have been established along various sibships (Hunt et al, 1989) and with identical twins (Feinleib et al, 1978), with a small contribution from a shared family environment (Williams et al, 1988). Potential confounding factors to these statistics include not only the shared environment, but also shared genetically determined behaviour patterns to exposure to environmental influences such as obesity and alcohol and salt consumption, which may adversely affect blood pressure (Beilin, 1988).

Potential candidates for genetic aberrations in essential hypertension have been partly elucidated with the advent of innovative molecular biological technology. Molecular biological markers are increasingly being recognised as possible valid predictors of hypertension. Putative gene abnormalities have been put forward for components of the renin-angiotensin system (RAS), sodium, potassium and calcium transport systems, the sympathetic nervous system, central regulatory systems and lipoprotein metabolism, abnormalities in many of which show a positive relationship with the prevalence of hypertension in families (Williams et al, 1989). Methods of positively associating these aberrations with hypertension, at the molecular level, include linkage analysis of restriction fragment length polymorphisms (RFLPs) that may cosegregate with the desired phenotype (high blood pressure) or associated diseases. Such candidate genes in man are to date, sparse, but a link between the gene encoding angiotensin-converting enzyme and myocardial infarction has been reported (Cambien et al, 1993). Preliminary studies are yet to find a link between RFLPs in the renin gene and essential hypertension in humans, but have found such a link in Dahl salt-sensitive rats (Rapp et al, 1989). Their limitations arise from the difficulty in accurately defining hypertension-related phenotypes. As blood pressure is unimodally distributed, it cannot be used as a phenotype. However, a phenotype such as sodium sensitivity, which shows a strong bimodal distribution when linked to the expression of hypertension, has the potential to be a valuable candidate for linkage analysis, provided a major gene effect for this trait can be shown (Williams et al, 1989). This example also highlights the interplay between the predisposition of genetic variables and environmental influences in the expression of high blood pressure.

There exists the potential for a battery of biochemical tests, measuring a range of metabolic variables in individuals, to be utilised in a clinical setting as predictors of hypertension. Individual gene effects are believed primarily responsible for some of these biochemical abnormalities, including urinary kallikrein excretion, intracellular sodium levels, sodium-lithium countertransport and sodium-potassium cotransport (Williams et al, 1989).

1.2.2 Environmental factors

Environmental factors operate on the background of monogenetic and polygenetic influences to alter the development and maintenance of hypertension. Some of these are discussed below.

The role of sodium in hypertension has been clearly defined in a subset of hypertensives which display marked sensitivity to excessive salt intake. Essential hypertensives are more likely to display an increase in blood pressure to salt ingestion than normotensives (Horan and Lenfant, 1990).

In contrast, potassium and calcium consumption show an inverse relationship with blood pressure, increases in either being associated with a reduction in blood pressure. Their effects also follow a definable, hereditable relationship in subsets of the population that also exhibit salt sensitivity (Kotchen et al, 1991).

Obesity is also linked with hypertension. A high proportion of individuals in Western societies are obese, and this is consistently shown to influence blood pressure distribution and prevalence rates (Beilin, 1988). An Australian study found that up to 60% of hypertension in men aged less than forty-five years could be attributed to obesity and that calorie restriction in obese hypertensives can lower blood pressure (McMahon et al, 1984).

Alcohol consumption has been shown to raise blood pressure in both normotensive and hypertensive humans in direct proportion to the number of drinks consumed (Puddey et al, 1985, 1987).

Other factors which may influence blood pressure and hypertension development include psychological stress (Zimmerman and Frohlich, 1990), cigarette smoking (Kannel and Higgins, 1990) and physical inactivity (Jennings et al, 1986).

1.3 Vascular structural and functional factors in essential hypertension

There is doubt as to whether cardiovascular structural alterations, in humans, are involved directly in the aetiology of hypertension. This doubt has arisen primarily through difficulties in successfully distinguishing between those structural changes that are adaptive to the higher blood pressure and those changes which have a component that is environmentally and/or genetically determined, and precedes the onset of hypertension. As these changes occur *pari passu* with the rise in blood pressure, which itself can modulate vascular structure, establishing the sequence of events remains a challenging problem.

1.3.1 Vascular media

Alterations in structure in hypertension were first described by Bright in 1827, and excerpts from his diagnoses have been a favoured introduction in papers on this topic (Packer, 1990). Bright's patients, dying with dropsy, invariably displayed a marked derangement of the kidneys upon autopsy. This finding was much later extended to blood vessels, in particular large arteries and arterioles, where hypertrophy of the vessel media was linked to chronic hypertension, independently of the origin of the disorder, and was considered an adaptive response of the vessel wall to the high blood pressure. Folkow (Folkow et al, 1958) was the principal cardiovascular physiologist who attempted to define the results of these changes in relation to changes in vessel function. His postulates may be summarised as follows:

Folkow's postulates:

(i): the increased medial mass confers increased constrictor properties on the vessel

(ii): the hypertrophying media encroaches upon and diminishes the luminal area of the vessel.

This theory encompasses both an active and a passive component to the vascular medial hypertrophy, the capacity for exaggerated contraction and the potential for a significantly reduced lumen, even at a state of maximal relaxation.

Recently, the view that an increased media:lumen ratio of a blood vessel implied the presence of medial hypertrophy has been disputed (Mulvany, 1991, Heagerty et al, 1993). The argument centres on the capacity for a proposed "remodelling of the blood vessel" during the development of hypertension, in which the media:lumen ratio increases without the addition of new material to the vessel. The testing of this hypothesis is difficult, as a direct comparison of vessels between normotensive and hypertensive individuals must take into account the differing vascular architecture between the groups, making vessel selection fraught with inaccuracy (Mulvany, 1991).

In humans, the haemodynamics of essential hypertension are associated with an abnormally high peripheral vascular resistance distributed throughout all tissues (Lund-Johansen, 1980, London et al, 1984), and occurring even in maximally dilated vascular beds (Mulvany and Aalkjaer, 1990). The pressor responses to the infusion of agonists is increased in hypertensives when compared to normotensives (Doyle and Fraser, 1961), but the sensitivity to agonists such as noradrenaline appears normal (Folkow et al, 1958, Sivertsson, 1970) or even reduced (Aalkjaer et al, 1987). The increased total peripheral resistance, under fully relaxed conditions, is strong evidence for altered structure. Whether this alteration results from an increase in the overall vessel media:lumen ratio, (due to remodelling and/or growth), or is a result of vascular bed rarefaction, has yet to be thoroughly investigated in humans. However, histological

evidence from studying the former has demonstrated an increased vessel wall:lumen ratio in small arteries in essential hypertensives (Short, 1966)

It is not clear whether altered vascular function, in relation to changes in medial growth, plays a major role in the maintenance of essential hypertension. Most studies have found no change or even a reduced sensitivity to a range of vasoactive agents in blood vessels from hypertensives, while vascular reactivity, or the maximal pressor response, is increased (Aalkjaer et al, 1986). This can be solely attributed to the increased media:lumen ratio of these vessels. As it is assumed that the vasculature is not constantly called upon to adopt a perpetual state of maximal contraction in order to maintain the high total peripheral resistance, it would seem that the altered vascular structure (increased media:lumen ratio) is the more important determinant of the haemodynamics of essential hypertension, and so will be the focus of further discussion.

1.3.2 Endothelium

Over the last decade, the endothelium has been found to display a wide range of functions aside from its previously considered limiting role as simply a barrier between blood and the blood vessel. It is now also considered as an important regulator of growth and contraction of vascular smooth muscle (Bohr et al, 1991).

Structural changes in the vascular intima occur in hypertension, with morphological alterations occurring in the endothelium (Luscher and Vanhoutte, 1986). It is not clear what role, if any, these changes have in regard to vessel function. Physical forces such as shear stress, pressure and stretch can modulate endothelial responses (Luscher and Vanhoutte, 1986), suggesting a primary role for high blood pressure in observed endothelial abnormalities. Although overt endothelial denudation does not occur in hypertension, the altered function and morphological changes may lead to an increased risk of developing atherosclerosis and thrombus formation (Dzau, 1990).

Since the finding that acetylcholine and related muscarinic agonists relaxed blood vessels only in the presence of an intact endothelium, the search for a mediator of the endothelial-to-smooth muscle communication led to the discovery of endothelium-

derived relaxing factor (EDRF). This was subsequently found to be nitric oxide (Moncada et al, 1991). The identification of this molecule, and the pathway through which it is generated in the endothelium, has spurred research into the further capabilities of this tissue in regulating not only vascular function, but also vessel structure in the long term, through vascular smooth muscle cell growth. It is now known that the endothelium can produce a myriad of both relaxing and contracting factors in response to a wide range of stimuli (Furchgott and Vanhoutte, 1989), thus acting as a modulator of vasoactivity. These opposing forces are believed to operate in a finely tuned balance in normal blood vessels to maintain a relatively constant vascular tone *in vivo*. Mediators of vessel contraction generated by the endothelium include the arachidonic acid metabolites thromboxane A_2 (TXA_2) and prostaglandin H_2, superoxide anions, endothelins and angiotensin II (Luscher, 1990).

Aberrations in the function of the endothelium have been noted in vessels from hypertensive rats (Luscher and Vanhoutte, 1986, Diederich et al, 1990, Dyer et al, 1992) , and this has now been extended to essential hypertension in humans (Panza et al, 1990). These abnormalities may act to shift the balance of vascular tone in favour of contraction, thus leading to an increased peripheral resistance (Bohr et al, 1991). The focus of endothelial function in hypertension has been on the spontaneously hypertensive rat (SHR).

The endothelium is also likely to play an important role in the longer term growth and hypertrophy of the vasculature (characteristic of hypertensives). It is not only a source of various growth factors, but can influence the migration, attachment and subsequent function of platelets and leucocytes (Clozel et al, 1993), bodies also rich in a range of potent vasoactive agents and growth-promoting molecules (Dzau, 1990, Owens, 1989). These include platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), transforming growth factor ß (TGF-ß), angiotensin II and cytokines (Luscher and Vanhoutte, 1986, Owens, 1989, Bobik and Campbell, 1993, Gomez et al, 1993). Nitric oxide seems to play a key role in modulating all of the above effects; i.e., vasoconstriction, leucocyte adhesion, platelet aggregation and responses of the vascular smooth muscle cell to growth factors (Clozel

et al, 1993). Thus it seems that proper vessel function relies on the many physiological actions of nitric oxide, both in the short and long term.

1.3.3 Adventitia and sympathetic nervous system

The adventitial layer has an important focus with respect to studies on blood vessels of hypertensive subjects, due to the presence of sympathetic innervation within this layer of connective tissue.

The sympathetic nerve supply is pivotal to the overall cardiovascular homeostasis of the organism, controlling tone in peripheral resistance vessels to maintain adequate blood pressure and tissue perfusion. It does this directly through the release of the vasoactive catecholamine noradrenaline at the neuroeffector junction, and through the effects of blood borne adrenaline, and to a lesser extent noradrenaline released from the adrenal medulla. Indirectly, it can influence vascular tone through distinct neurohumoral pathways such as the endocrine renin-angiotensin-aldosterone axis.

In the longer term, the sympathetic nervous system may play a role in the development of cardiac and vascular medial hypertrophy in essential hypertension, both directly through the furnishing of growth-promoting factors to the tissue, and indirectly through its influence on blood pressure in the peripheral vasculature.

In some essential hypertensive humans and in the SHR, the contribution of the sympathetic nervous system to the development, and then maintenance, of established hypertension seems to have a biphasic nature. In the early phases of the disorder, individuals commonly have what is termed "hyperkinetic borderline hypertension", characterised by an elevated heart rate, cardiac output, and left ventricular ejection rate with normal total peripheral resistance (TPR) (Pfeffer and Frohlich, 1973, Julius, 1988). A strong neurogenic component to this hyperkinetic state has been found (Julius, 1987), with increases in plasma noradrenaline (an index of sympathetic tone) commonly observed (Esler, 1987). There is also, however, blunted vagal tone to the heart, which also contributes to the abnormally enhanced cardiac parameters already described (Egan, 1987). This pattern of involvement is also reflected in the development of hypertension

in the SHR (Pfeffer and Frohlich, 1973), where detailed investigations into developmental aspects of hypertension have uncovered a similar transitional pattern of haemodynamics, and an important role for the sympathetic nervous system in the pathogenesis of hypertension in this strain. This role will be discussed later.

As hypertension becomes established, sympathetic tone and cardiac output are normalised, and it is the elevation in TPR that maintains essential hypertension, in part due to the changes in cardiac and vascular structure that have taken place (Julius, 1988). Why the hyperkinetic factors are normalised is not clear, but blunted cardiac ß-adrenoceptor sensitivity (Ely et al, 1985), left ventricular hypertrophy leading to reduced diastolic filling and stroke volume (Shapiro and McKenna, 1984), and the ability of the hypertrophied vessels to maintain high resistance with less active tone are all candidates. Even though plasma noradrenaline is elevated in established hypertensives, it is believed to be a decreased clearance, rather than any consistently high sympathetic drive, that maintains high plasma noradrenaline spillover (Esler et al, 1981). There is still evidence for enhanced sympathetic activity in patients with accelerated, or malignant hypertension (Matsukawa et al, 1993).

Evidence for altered levels of sympathetic innervation in humans is scarce, despite the clear evidence for hypernoradrenergic innervation in the SHR (Head, 1989). One study, however, suggested a greater innervation in human subcutaneous small arteries from essential hypertensives, as judged from the greater leftward shift in the exogenous noradrenaline dose-response curves of the vessels in the presence of cocaine, when compared to normotensives (Aalkjaer et al, 1989).

The adventitial layer also serves an important role in the centrally-mediated regulation of blood pressure, through aortic and carotid baroreceptors. These detect changes in the deformation imposed on the vessel wall through changes in mean arterial pressure and convey appropriate signals through primary afferent nerves to the brain stem, activating the central nervous system (CNS) to ultimately control blood pressure levels.

The question of aberrant baroreceptor function in hypertension is yet to be fully answered, but there is evidence pointing to a resetting of baroreceptor activity to a higher threshold in hypertensive humans (Mancia et al, 1978). The ability to define a

pathogenetic role for arterial baroreceptors in hypertension is technically much more difficult (Brown, 1981); however, methods using an *in vitro* aortic arch preparation from SHR show that baroreceptors alter their rate of discharge when the vessel distensibility in which they reside changes (Brown, 1980). This is achieved through a resetting of the strain sensitivity of the baroreceptors in proportion to the increased deformation they encounter in the vessel wall. In hypertension, the strain sensitivity is enhanced, but is inadequate to compensate for the reduction in distensibility of the vessel, resulting in an imbalance in the relationship between pressure and discharge and a subsequent resetting of blood pressure to higher levels (Brown, 1981). The appearance of this dysfunction with increasing age has been put forward as an attractive candidate for a contributing factor to the development of hypertension, at least in the SHR.

1.4 Cardiac structure and function in essential hypertension

Aside from well documented changes in vascular structure in essential hypertension in humans, the heart also undergoes changes in both structure and function in hypertension. An understanding of the relationship between altered cardiac parameters and high blood pressure is as important as the knowledge of the interplay of those factors affecting vascular structure and function in hypertension, as target organ damage to the heart is often the end point of a sustained elevation in blood pressure, and is often fatal.

Hypertensives are more likely than normotensives to display left ventricular hypertrophy and heart failure (hypertensive heart disease), and to develop atherosclerotic coronary arteries leading to ischaemic heart disease. The potential sequelae include angina pectoris, cardiac arrhythmias, myocardial infarction and sudden death.

The heart influences blood pressure through cardiac output, a function of cardiac ventricular stroke volume and heart rate. These parameters are affected by a wide variety of stimuli. Heart rate is primarily modulated by neurogenic factors arising from sympathetic and parasympathetic innervation. Stroke volume is determined by ventricular preload, myocardial contractility, and ventricular afterload, factors which can

be controlled individually, or in combination with a number of intrinsic, neurogenic and haemodynamic modifiers.

Ventricular preload is altered by an increased venous return by way of the recruitment of the Starling principle, which defines the length-tension relationship of ventricular muscle fibres to an altered end-diastolic volume.

The sympathetic nervous system, through noradrenaline release at cardiac adrenergic nerves, and circulating adrenaline released from the adrenal medulla, has positive chronotropic and inotropic effects on the heart, increasing heart rate and myocardial contractility in the face of an altered haemodynamic burden.

Ventricular afterload is altered by factors which affect intraventricular systolic pressure. These are principally haemodynamic in origin, including such factors as systemic vascular resistance, mass and viscosity of blood and resistance at the outflow tract of the left ventricle.

As previously mentioned, altered cardiac structure and function related to high blood pressure is an important risk factor for the development of further cardiovascular disease, with left ventricular hypertrophy being associated with a six to eight-fold increase in the risk of developing acute myocardial infarction, congestive heart failure, cardiac arrhythmias and sudden death (Messerli and Ketelhut, 1993).

Left ventricular hypertrophy (LVH) is commonly associated with elevated blood pressure (Levy et al, 1988b), and is characterised by an increase in the muscle mass of the left ventricle. This can also occur in response to an increased volume load, but the pattern of hypertrophy development is different between the two stimuli (Messerli and Ketelhut, 1993). The elevated peripheral resistance in essential hypertension manifests its actions on the heart at the level of the afterload, placing greater stress on the ventricular myocardium during systole. The ventricle reacts by parallel replication of sarcomeres arranged to give a concentric hypertrophy, offsetting the greater stress level by reducing the radius and wall tension of the ventricle. The septum and posterior wall of the left ventricle thicken at the expense of chamber volume. Initially the adaptation allows the maintenance of a high systolic pressure or augmented cardiac output without placing undue stress on the ventricular myocardium, in the face of a sustained higher blood pressure. In the late phase of hypertensive heart disease, LVH may become

eccentric, a feature often associated with conditions characterised by volume overload. This type of LVH is a precursor to congestive heart failure. Severe LVH impairs myocardial contractility and coronary vascular reserve, which can lead to congestive heart failure and ischaemic heart disease (Hollander, 1976, Messerli and Ketelhut, 1993). Mild degrees of LVH do not appear to interfere with left ventricular function (Nichols et al, 1980).

While most evidence seems to suggest that LVH is a reaction to high blood pressure in humans, there is evidence, taken from studies using the SHR, to suggest that this is not the sole determinant of LVH, where there is a strong genetic determinant to the hypertension (Sen et al, 1974). Trophic influences, including those generated by the SNS and RAS, have been implicated in the abnormal growth of the myocardium of the SHR (Leenen and Harmsen, 1991).

1.5 *The spontaneously hypertensive rat*

The Spontaneously Hypertensive Rat (SHR) is an animal model of genetic hypertension that displays a number of features akin to essential hypertension in humans. Amongst these is a polygenetic causation of the phenotype, an increased severity of the condition in males, and a wide number of perturbations in systems responsible for blood pressure homeostasis in the animal.

The SHR was developed in 1963 by selective and consistent brother-sister matings of the progeny from a female Wistar rat displaying a slightly elevated systolic blood pressure (130-140 mmHg), mated with a male Wistar rat also displaying an elevated systolic blood pressure of 145-175 mmHg (Okamoto, 1969). Twenty generations on, in 1969, 100% of the progeny would proceed to develop spontaneous hypertension.

The evolution of systolic blood pressure in this model encompasses a defined "prehypertensive" phase from birth to four weeks of age, where measured blood pressure does not exceed that of its normotensive progenitor. During the ensuing weeks, to twenty weeks of age, systolic blood pressure rises steeply to approximately

200 mmHg, where it remains for the duration of the animal's life. In comparison, the normotensive Wistar-Kyoto (WKY) rat, a designated control strain for the SHR, displays a stable value of approximately 140 mmHg systolic blood pressure over its entire lifespan. Thus, there are three arbitrarily defined phases of hypertension expression in the SHR; prehypertensive (0-4 weeks), developing hypertensive (4-20 weeks) and established hypertension (20 weeks of age onwards).

1.5.1 Prehypertensive SHR

The young SHR, although its blood pressure is similar to that of the WKY rat, already displays evidence of aberrant cardiovascular structure, in particular the presence of increased vascular medial thickening in a range of large and small vessels, and left ventricular thickening (Gray, 1984, Eccleston-Joyner and Gray, 1988, Morton et al, 1990). Vascular resistance is also greater in four week-old SHR when compared to WKY rats (Adams et al, 1989). This suggests that part of the changes in cardiovascular structure associated with the development of hypertension in this strain occur before the full expression of the disorder, indicating a possible role in its pathogenesis. Adams et al (1989) proposed that an "immaturity" in heart and blood vessel function at the younger ages, together with a greater vascular density, are the reasons blood pressure remains low at this age despite the observed changes in cardiovascular structure and function.

1.5.2 Developing SHR

The transition from young to adult SHR is accompanied by the development of hypertension and it is during this phase that changes in the level of expression of physiological regulatory systems of cardiovascular tone and growth occur with the elevation in blood pressure. In the initial stages of developing hypertension the SHR possesses a "hyperkinetic circulation", characterised by a normal total peripheral resistance but elevated cardiac output (Pfeffer and Frohlich, 1973). As the animal matures the underlying haemodynamics alter to produce hypertension resulting from

sustained elevated total peripheral resistance with normal cardiac output. During this phase, cardiovascular structural change accelerates with the rise in blood pressure. This includes left ventricular hypertrophy, increases in vascular medial hypertrophy and amplification of the resistance properties of the vasculature (Adams et al, 1989, Leenen et al, 1994).

1.5.3 Established SHR

Established hypertension in the SHR is associated with aberrations in cardiovascular structure and function. Further vascular medial hypertrophy does occur during this period, but this is confined to smaller vessels, whereas in larger vessels medial hypertrophy is established by the age of sixteen weeks (Leenen et al, 1994). Left ventricular hypertrophy is established by the age of twenty weeks (Adams et al, 1989). If left unchecked, the rats suffer target organ damage at many sites. Hypertensive lesions include cerebral and subarachnoid haemorrhage, myocardial infarction, nephrosclerosis, periarteritis nodosa and arterionecrosis. Malignant hypertension is also common (Okamoto, 1969). Additional substrains of the SHR have also been developed which show a selective disposition to stroke, stroke-resistance, arteriolipidosis, thrombogenesis and myocardial ischaemia in the adult animal (Yamori, 1984).

1.6 Pathogenetic mechanisms in the SHR-the sympathetic nervous system

Early studies placed a strong emphasis on disorders of the neurogenic control of cardiovascular structure and function as the initiators of hypertension in the young animal (Yamori, 1984), with permanent vascular structural changes maintaining the high peripheral resistance observed in the adult SHR through Folkow's postulates.

There is an overwhelming body of evidence now supporting a neurogenic component of hypertension initiation and development in the SHR. The term "neurogenic" encompasses the interaction of multiple physiological systems, most of

which come under either direct or indirect control of the CNS. The CNS regulates blood pressure directly through the autonomic control of cardiovascular function and indirectly through the control of fluid, endocrine and electrolyte balance within the circulation via the hypothalamo-pituitary axis. Only aspects of the CNS as it relates to direct sympathetic nervous system (SNS) control will be discussed.

The autonomic nervous system comprises a sympathetic and parasympathetic division, with only sympathetically-mediated control of vascular tone controlling total peripheral resistance. Both divisions regulate cardiac chronotropism and inotropism.

There is considerable evidence implicating enhanced sympathetic activity in the development and maintenance of hypertension in the SHR (Brody et al, 1984), suggestive of an enhanced bulbospinal regulation of sympathetic tone. This encompasses vascular afferent baroreceptors, those regions in the CNS that process information relevant to required changes in blood pressure, sympathetic pre- and postganglionic fibres, ganglia and the neuroeffector junction. Despite the number of sites in this complex network where perturbations have been described, their role in hypertension development and/or maintenance in the SHR are not certain. Some of these are summarised below.

1.6.1 Baroreceptors

No inherent impairment of baroreceptor function in the SHR has been described, but function of these mechanoreceptors can be diminished by changes in the structure and mechanics of the blood vessels in which they reside (Andresen et al, 1980). As the SHR matures, the enhanced deposition of vascular collagen and extracellular matrix can diminish baroreceptor sensitivity, causing baroreceptor splinting and impaired function (Andresen et al, 1980). They may play a more important role in maintenance rather than development of hypertension in this scenario.

1.6.2 Central Neural Pathways

Altered central pathways regulating sympathoadrenal activity have been studied, and accumulating evidence suggests that defects in specific central neural function may play a role in hypertension maintenance in the SHR.

Primary afferent fibres from vascular baroreceptors terminate in the nucleus tractus solitarius (NTS) of the dorsomedial medulla. Information is processed in the NTS, the caudal ventrolateral medulla and rostral ventral medulla. This information is then relayed to the intermediolateral cell column (IML) of the spinal cord before being channelled as sympathetic nerve output (Chalmers et al, 1992).

Evidence supports a role for the impaired function of GABAergic fibres essential for the modulation of sympathetic output by glutaminergic neurons in the aforementioned regions of the CNS from adult SHR (Chalmers et al, 1992). Others note enhanced levels of markers of noradrenergic neurons in the NTS, locus coerulus and IML in young SHR (Nakamura and Nakamura, 1978). Their role, if any, in hypertension development in the SHR has not been investigated.

1.6.3 Sympathetic Ganglia

The sympathetic ganglia are another site where modulation of nerve traffic occurs. Deficiencies in the inhibitory neurotransmitter dopamine and hyperexcitable membrane properties have been noted in this tissue from the adult SHR (Magee and Schofield, 1992). Functionally, adult (but not young) SHR show an altered rate of active ganglionic transmission (Magee and Schofield, 1992). These results however do not support an active role for sympathetic ganglia in the development of hypertension in the SHR.

1.6.4 Neuroeffector Junction

There is a large body of evidence indicating that in peripheral blood vessels from the SHR the density of sympathetic innervation is increased from an early age (Head, 1989). This may be linked with increased levels of nerve growth factor in blood vessels from developing SHR (Ueyama et al, 1992, Zettler and Rush, 1993), produced

by the vascular smooth muscle (Creedon and Tuttle, 1991). Irrespective of its cause, the presence of hypernoradrenergic innervation of the vasculature has been shown to amplify its contractile responses to nerve stimulation (Cassis et al, 1988), and offers a potential end point amplification of the general increase in sympathetic activity observed in this model (Iruchijima, 1973). The development of hypernoradrenergic innervation takes place in the prehypertensive and early developing phases of hypertension in this strain. However, the shift from a high output to high resistance haemodynamic pattern occurs later in the developing phase of hypertension, well after the innervation has peaked. A reason why TPR does not peak with the innervation is possibly because of the vascular "immaturity" hypothesis stated by Adams et al (1989) in the young animal.

There are longer term effects of the SNS on blood vessel structure that suggest a pathogenetic role of the nerves in this setting. Catecholamines have been shown to have growth promoting effects on vascular smooth muscle cells VSMCs (Blaes and Boissel, 1983, Bobik and Campbell, 1993), and, as discussed later, selected antihypertensives that target the SNS also abolish cardiac and vascular medial hypertrophy in the SHR.

1.7 Pathogenetic mechanisms in the SHR-The renin-angiotensin system

1.7.1 Endocrine RAS

It is uncertain as to whether the endocrine RAS is involved in the development of hypertension in the SHR (Samani et al, 1989). When compared to WKY rats, levels of plasma renin activity (the rate-limiting factor in RAS activity) have been observed to be increased, decreased or unchanged with age in the SHR (Samani et al, 1989). Plasma angiotensin converting enzyme (ACE) activity is consistently lower in the SHR at all ages (Okunishi et al, 1991). In contrast to the ambiguous direct evidence, the results of pharmacological blockade of the RAS in developing SHR suggest a significant role for it in the development of hypertension.

1.7.2 Tissue RAS

Molecular biological techniques have detected the presence of components of what are believed to be functional renin-angiotensin systems in a range of tissues, including blood vessels (Dzau, 1993). This includes the presence of renin, ACE, angiotensinogen and angiotensin II localised to specific layers in the blood vessel which may play an important role in local vascular regulation (Dzau, 1993). Moreover, in the young and developing SHR, these tissue markers for the RAS have been found to be consistently enhanced in vascular tissue when compared to the WKY rat. These include increased levels of renin peptide (Siniako and Mirkin, 1974) and mRNA (Samani et al, 1989) in the kidney and blood vessels (Naruse and Inagami, 1982), as well as increased ACE activity in blood vessels from developing SHR (Okunushi et al, 1991). Angiotensin II concentrations have also been found to be higher in vascular tissue from young SHR than WKY rats (Matsushima et al, 1988), although, as with renin, the relative contribution of these peptides from plasma uptake is still to be fully elucidated. Furthermore, the prolonged suppression of blood pressure after a single dose of ACE-inhibitor correlates better with vascular ACE activity than plasma ACE activity (Unger et al, 1985). These findings support a role for the enhanced expression of a local autocrine and/or paracrine RAS in the development of hypertension in the SHR.

The implications of the local production of AII in blood vessels has a particular reference to the SHR, given the vascular structural and functional changes it undergoes in the transition to the hypertensive state. AII has both short and long term effects. As a powerful vasoconstrictor it can regulate vascular tone in peripheral vascular beds directly and through its facilitation of sympathetic nerve activity it can amplify the response to sympathetic outflow at the neuroeffector junction, as has been shown in rat mesenteric arteries from locally-generated AII (Malik and Nasjletti, 1976). The SHR exhibits enhanced responses to nerve stimulation from the presynaptic actions of AII (Cline, 1985). Given the SHR's increased vascular innervation, the potential for AII to greatly exaggerate vasoactivity through these multifunctional actions must be considered. AII can also modulate endothelial prostacyclin production (Jaiswal et al, 1993).

Local vessel AII may also act in a way that supports the development of hypernoradrenergic innervation in the vasculature of the young SHR, in what is proposed as a positive trophic feedback loop involving the VSMC and the sympathetic nerve (Head 1989). Within this cycle, the increased density of sympathetic innervation can act to augment VSMC proliferation through the provision of known growth-promoting factors such as noradrenaline, and possibly other candidates such as the cotransmitter neuropeptide Y or other products. Locally sequestered AII can augment sympathetic outflow at the neuroeffector junction as described earlier, which can further supplement VSMC growth. As the VSMC can synthesise NGF, the sympathetic nerve is controlling the provision of its own growth factor, thus completing the cycle. The discovery of AII's direct growth-promoting effects on the VSMC (discussed below) may also aid the furnishing of NGF to the nerve.

The presence of a tissue RAS in the SHR also applies to the CNS, where all components of the enzymatic cascade have been described to occur in different regions in the brain (Ruiz et al, 1990). Furthermore, altered levels of tissue RAS markers have been described in the CNS of the SHR compared with WKY rats, although their role in hypertension development is unclear (reviewed by Paul et al, 1993). The role of AII in the CNS appears to involve regulation of dipsogenesis, autonomic function and modulation of vasopressin release. However, inhibition of the brain RAS through intracerebroventricular administration of drugs has been shown to have variable effects on blood pressure (Stecklings et al, 1992). This may be due to the ages of the animals when studied, the agents used or to methodological differences (Stecklings et al, 1992). The definition of the precise role of the central RAS awaits further studies.

1.8 Angiotensin II as a growth-promoting molecule

In addition to AII's local role in modulating vasoactivity, the expression of a paracrine/autocrine-acting AII may have profound effects on the longer term changes in vascular structure and function through its role as a growth-promoting factor of the vascular smooth muscle cell (VSMC).

There is a diversity of VSMC phenotypes throughout the vascular tree which is believed to reflect the size of the vessel and its physiological role. The modulation of the VSMC phenotype during development is contingent upon overall cellular organisation, the density of innervation and intrinsic properties of the cell (Bobik and Campbell, 1993).

The hypertrophic response of the VSMCs typical to the SHR varies according to the vessel type, with larger conduit vessels (aorta) displaying true cellular hypertrophy associated with an increase in DNA content and total protein (Owens, 1989). In contrast, smaller resistance VSMCs display hyperplastic or mitogenic growth, a proliferative response producing more VSMCs with a similar phenotype to the progenitor cell (Owens, 1989). The mechanism of the selective nature of the response between vessel types is not clear, but may be related to the exposure of local neurohumoral factors across each vessel.

Cultured VSMCs from the SHR have a tendency to proliferate to a higher cell number, grow to a greater density and have a greater specific growth rate at higher saturation densities when compared to cells from the WKY rat (Hadrava et al, 1989). They are also more resistant to the growth-inhibiting effects of TGF-β_1 than cultured WKY rat VSMCs (Saltis and Bobik, 1992). These responses occur to a range of growth factors (Bobik and Campbell, 1993) and also to other growth-promoting molecules, such as AII and catecholamines (Owens, 1989), and involve interactions with specific cell receptors. Intracellular signalling is mediated by altered tyrosine kinase activity. Associated with the response to AII is activation of phospholipase C with the subsequent formation of diacylglycerol and protein kinase C activation. Proto-oncogene expression and mRNA for growth factors such as PDGF and TGF-β_1 are subsequently increased, with consequent DNA replication and cell growth (Gibbons et al, 1992, Saltis and Bobik, 1992, Itoh et al, 1993). AII stimulates proto-oncogene expression through this pathway (Baudouin-Legros et al, 1989).

The SHR VSMC displays an enhanced level of proto-oncogene expression after AII administration in culture (Paquet et al, 1990). In cultured aortic VSMCs, the response to AII tends to be hypertrophic and not hyperplastic (Geisterfer et al, 1988, Turla et al, 1991), with selective increases in contractile proteins (Turla et al, 1991).

The expression of TGF-β_1 in rat aortic VSMCs can also be increased by experimentally induced hypertension in previously normal cells (Sarzani et al, 1989). TGF-β_1 has been found to be the modulator of the cell's growth response to AII: when present, the VSMC will undergo hypertrophic growth in response to AII-stimulated PDGF secretion; when absent, mitogenic growth to PDGF predominates (Saltis and Bobik, 1992).

In contrast to the aorta, VSMCs isolated from small mesenteric arteries undergo AII-induced dose-dependent increases in cell numbers (Lyall et al, 1988). This growth reflects the *in vivo* situation, indicating that the selective trait(s) that dictate the response may be determined early in the cell's life. It must also be cautioned that the conditions of culture can alter the phenotype, specifically from a contractile to synthetic phenotype (Chamley-Campbell et al, 1981). The modulating influence of the endothelium and nerves are also lost in culture.

In intact aortic segments AII has been shown to selectively induce hypertrophy of the VSMC, and *in vivo* AII can elicit vascular medial hypertrophy independently of changes in blood pressure, although the exact type of VSMC growth response was not determined (Griffin et al, 1991).

1.9 ***Aspects of angiotensin II-mediated growth; cellular biochemical changes in contractile protein***

Preliminary studies have documented the selective nature of the effects of AII on the vascular smooth muscle, particularly directed towards the production of contractile protein (Turla et al, 1991). AII not only induced increases in actin and myosin, but specifically directed the induction of specific isoforms, especially of α-actin mRNA in cultured VSMCs from the rat (Turla et al, 1991) and α-actin gene expression in human cultured VSMCs (Andrawis et al, 1993). However, the potential link between the elevated tissue RAS in the SHR and altered changes in contractile protein, increased vascular contractility and elevated blood pressure has not yet been fully explored.

1.9.1 Actin in vascular smooth muscle

Actin forms a major cytoskeletal component of all eukaryotic cells and the contractile machinery of muscle cells. In smooth muscle there are three different isoforms which vary in their isoelectric electrophoretic properties. These consist of α - smooth muscle (SM)-specific, β-nonmuscle (NM) and γ-smooth and nonmuscle isoforms (Fatagati and Murphy, 1984), and their expression is regulated in an age, cell and tissue-specific manner (Owens et al, 1986). The expression of the α-SM actin has been correlated with the changing phenotype of the VSMC. In particular, as the cell moves from a "synthetic" to mature "contractile" phenotype its expression increases at the expense of the β-NM type (Fatagati and Murphy, 1984). This is seen in cultured aortic VSMCs, where in cells from newborn rats the major actin is the β-NM type (Owens et al, 1986), associated with a cell rapidly dividing and producing and secreting extracellular matrix and connective tissue components (Chamley-Campbell et al, 1981). With age, the α-SM isoform makes up to 70% of the total VSMC actin (Morano, 1992), correlating with the expression of the contractile phenotype and a reduced turnover of the variant.

In comparison to other types of smooth muscle, VSMCs contain double the actin content per cell wet weight, the extra actin being due solely to the increase in the α-isoform (Cohen and Murphy, 1979). The correlation between levels of this variant and the tonic activity of arterial muscle has led to speculation that actin variants may be responsible for altered contractile apparatus function (Fatagati and Murphy, 1984), although this has yet to be proven.

1.9.2 Myosin in vascular smooth muscle

The myosin heavy chains (MHCs) of VSMCs also exist as three distinct isoforms: two smooth muscle (SM1 and SM2 based on their electrophoretic mobilities) and a single nonmuscle (NM) variant (Sartore et al, 1994). Developmental expression studies reveal that, as with actin, the relative ratio of SM-specific MHC:NM increases with age in rat arterial VSMCs (Eddinger and Murphy, 1991). For SM-specific isoforms of rat aortic VSMCs, SM2 is gradually replaced by increasing amounts of SM1, but not to the same extent as that seen with actin (Eddinger and Murphy, 1991).

Altered contractile apparatus function with changing myosin isoform distribution in VSMCs has been reviewed (Sartore et al, 1994).

In human VSMCs, the switching of both actin and myosin isoforms occurs early in foetal development, so that the pattern of distribution changes very little when compared with adult VSMCs (Glukhova et al, 1990).

1.9.3 Actin and Myosin in vascular smooth muscle from the SHR

Despite the clear evidence of altered VSMC morphology in blood vessels from the SHR, relatively little is known regarding cellular contractile protein biochemistry in this model.

Sporadic papers on this topic report increased levels of total aortic actomyosin (actin plus myosin) with age in SHR versus WKY rats; however, relative to both total cellular protein and wet tissue weight these differences were normalised in adult rats (Seidel, 1979, Matsumura et al, 1991). An increase in aortic actomyosin concentration has been reported up until five weeks of age in the SHR (Seidel and Murphy, 1979b).

In mesenteric resistance vessels, the total actin and myosin contents were also elevated in adult SHR (Brayden et al, 1983). When normalised to DNA content, however, there were no differences, consistent with the tendency for these vessels to undergo hyperplastic VSMC growth. On a wet tissue weight basis there were significantly elevated levels of both actin and myosin in these vessels from the SHR (Brayden et al, 1983). These reports suggest variations occur between blood vessel types with regard to the relative amounts of actin and myosin they contain. Collectively, they suggest that the proportion of resistance vessel composed of contractile protein is greater in the SHR.

In terms of altered isoform expression in blood vessels from the adult SHR, there is no evidence to suggest significant myosin isoform switching occurs when compared to the WKY rat (Matsumura et al, 1991, Morano et al, 1986). There is a report that suggests dramatic increases in α-actin mRNA concentrations in the aorta from the SHR as early as five weeks of age, with an even greater difference occurring at seventeen weeks of age (Le Jemtel et al, 1993). Levels of the protein were not

measured, so it remains to be seen whether increased levels of this variant were actually expressed in blood vessels, particularly resistance vessels, from this strain.

1.10 3-Methylhistidine-a marker for vascular contractile proteins

3-Methylhistidine (3MH) has been developed as a novel biochemical marker for contractile protein in blood vessels and applied as such in comparisons between hypertensive and normotensive rats (Jonsson et al, 1991).

3MH is a postranslationally methylated histidine residue occurring exclusively in actin and myosin in adult skeletal, cardiac and smooth muscle. The exception is cardiac myosin, which does not contain 3MH (Kuehl and Adelstein, 1970).

There is one residue of 3MH per actin or myosin molecule. However, with actin being the predominant cellular contractile protein in the VSMC (stoichiometric ratio of actin:myosin equivalent to 35.5:1 (Cohen and Murphy, 1979), the relative contribution of actin to the total tissue 3MH concentration has been calculated to be more than 90% (Appendix I). Therefore, actin is the major source of 3MH in the vessel.

The primary structure of all actins contains a unique 3MH residue at position 73 in the molecule (Raghavan et al, 1989). The biological function, if any, that 3MH confers on the protein is as yet undetermined, but site-specific mutagenesis of the histidine residue does not alter a variety of properties of the molecule (Johnson and Perry, 1970, Solomon and Rubenstein, 1987). A single study has proposed a complex series of interactions by which 3MH may be involved in the phosphorylation of myosin (Fazekas et al, 1988).

As 3MH is not reutilised by the muscle cell following contractile protein catabolism, its urinary excretion rate has been widely used as an index of muscle protein breakdown, particularly from skeletal muscle (Tomas and Ballard, 1987). The use of 3MH as a direct index of tissue contractile protein levels was developed by Jonsson et al (1991), who documented selective increases in cellular 3MH in a variety of blood vessels, but not in nonvascular tissues, from the SHR when compared to the

WKY rat. 3MH concentrations have been shown to be markedly reduced after chronic ACE-inhibition (Jonsson et al, 1991).

1.11 Chronic antihypertensive therapies in the SHR

The use of chronic antihypertensive therapies in the developing SHR has revealed much in regard to the pathogenetic mechanisms of the disease, particularly the role of the SNS and RAS.

There are many clinically useful antihypertensive drugs that can control uncomplicated essential hypertension, but not all of these agents have been found to be effective in controlling genetic hypertension development (GHD) nor the associated vascular structural and functional abnormalities in the SHR.

1.11.1 Beta-blockers

In general, β-blockers vary from poor to moderate in their ability to control GHD in the SHR (Giudicelli et al, 1981). Systolic blood pressure (SBP)-lowering has been achieved with propranolol (Giudicelli et al, 1980, Owens, 1987), atenolol (Giudicelli et al, 1981), metoprolol (Christensen et al, 1989) and timolol (Goldberg and Triggle, 1978), but not with pindolol (Giudicelli et al, 1981), acebutolol (Giudicelli et al, 1981) or nadolol (Lee et al, 1992). Of those that have an effect, most required oral dosages of upwards of 100 mg/kg/day. This class of antihypertensive has little effect on vascular structure irrespective of its blood pressure-lowering efficacy (Christensen et al, 1989, Lee et al, 1992).

1.11.2 Alpha-blockers

In the few studies that have been performed, α_1-blockade with prazosin or its derivatives terazosin and doxazosin has consistently been ineffectual in controlling GHD in the SHR (Giudicelli et al, 1981, Sanchez et al, 1989a, Jonsson et al, 1992, Young et

al, 1993). Further studies using doxazosin and the irreversible nonspecific α-blocker phenoxybenzamine have also revealed a lack of effect, which will be discussed in Chapter 6. Sanchez et al (1989b) found modest reductions in blood pressure with prazosin in the SHR, but this occurred at the beginning of the phase of established hypertension. The role of α_2-adrenoceptors in hypertension in the SHR awaits further clarification, but preliminary studies have shown that chronic yohimbine treatment has no effect on GHD (Sanchez et al, 1989a, Young et al, 1993), although Sanchez et al (1989b) found a modest antihypertensive effect of the drug in older SHR. No vascular structural studies were undertaken.

1.11.3 Diuretics

There are few studies utilising diuretic monotherapy in this setting. Hydrochlorothiazide or clopamide alone afforded only minimal control of GHD in the SHR (Giudicelli et al, 1981), while indapamide was ineffective at a low dose and had no effect on mesenteric small artery structure, despite diminishing the enhanced contractile responses of these vessels to potassium chloride (Qui Zhou et al, 1993).

1.11.4 Nonspecific vasodilators

The nonspecific vasorelaxants hydralazine and minoxidil are effective at controlling GHD in the SHR (Giudicelli et al, 1980, Freslon and Giudicelli, 1983, Owens, 1987, Christensen et al, 1989, Lee et al, 1992,), although their effects on the development of left ventricular hypertrophy, resistance vessel medial hypertrophy and active and passive vascular functional parameters (compliance and contractility) are much less than those observed for other antihypertensives (Freslon and Giudicelli, 1983, Christensen et al, 1989). While there is a good correlation between the extent of larger vessel structural change and blood pressure, this correlation is lost for the smaller vessels, with effects on structure becoming more drug class-dependent than pressure-dependent (Christensen et al, 1989). Therapy with these drugs is often associated with a baroreflex-mediated increase in cardiac sympathetic activity resulting in tachycardia

(Giudicelli et al, 1981, Smeda and Lee, 1991), which may underlie the lack of influence of these agents on cardiac hypertrophy.

1.11.5 Angiotensin converting enzyme inhibitors

The effect of ACE-inhibitors on GHD in the SHR has already been alluded to briefly in earlier sections of this introduction. Generally, they are one of the most effective group of drugs known in this regard.

A range of ACE-inhibitors have been tried within this setting, and all have similar effects, including captopril (Giudicelli et al, 1980, Owens, 1987, Christensen et al, 1989), enalapril (Adams et al, 1990, King et al, 1992) and perindopril (Harrap et al, 1986, Christensen et al, 1988, Harrap et al, 1990, Black et al, 1993, Thybo et al, 1994). Oral dosages range from as little as 0.8 mg/kg/day (perindopril) to 100 mg/kg/day (captopril). Associated with the prevention in GHD is a marked effect on cardiac hypertrophy and vascular structure in large arteries and smaller resistance vessels. This includes a reduction in aortic medial hypertrophy and polyploid VSMC frequency (Owens, 1987), left ventricular hypertrophy (Cadilhac and Giudicelli, 1986, Christensen et al, 1988), and reduced medial thickness and increased media:lumen ratios in resistance vessels (Freslon and Giudicelli, 1983, Cadilhac and Giudicelli, 1986, Christensen et al, 1989, Harrap et al, 1990, Thybo et al, 1994). Moreover, the reduced medial hypertrophy occurs to a degree over and above that expected from the blood pressure-lowering effects alone, in comparison with other classes of equipotent antihypertensives (Owens, 1987, Christensen et al, 1989).

These results, along with the evidence that: (i) AII is a growth-promoting factor of the VSMC; (ii) the SHR VSMC is more responsive to growth-promoting molecules; and (iii) an increased vascular tissue RAS is evident in the SHR, lend firm support to the suggestion that AII, or possibly another product of the ACE, may play a pivotal role in the development of aberrant vascular structure in the SHR through effects independent of blood pressure.

Often associated with the normalisation in structural parameters is a change in the active and passive functional properties of the vessel and/or vascular bed, including

an increased compliance and reduced contractility of vessels (Freslon and Giudicelli, 1983), and reduced vascular resistance "amplifying" properties (Adams et al, 1990). The reductions in vessel contractility that accompanied chronic ACE-inhibition are assumed to be brought about by the reduced medial thickness, but there may also be a component arising from a direct effect of ACE-inhibition on VSMC responses to vasoactive substances (Major et al, 1993).

1.11.6 Calcium channel antagonists

Calcium channel blockers have been shown to be effective in opposing GHD in the SHR (Christensen et al, 1989, Godfraind et al, 1991, Kawashima et al, 1991), although nicardipine is less effective in young than in older SHR (Giudicelli et al, 1981, Godfraind et al, 1991). The modulation of vascular structure in one study was equivalent to that of the vasodilator hydralazine, but not as effective as ACE-inhibition and with no change in the active properties of the vessels studied (Christensen et al, 1989). Chronic nisoldipine treatment in 8 week-old SHR reduced cardiac and aortic hypertrophy and vascular contraction to potassium chloride (Godfraind et al, 1991).

1.11.7 Angiotensin II receptor antagonists

The development of selective ligands for the angiotensin II receptor has aided an elucidation of receptor subtypes for the peptide. The initial studies reported two receptors, designated AT_1 and AT_2 (Siegl, 1993). Functionally, most of the biochemical and physiological effects of angiotensin II are attributed to the AT_1 receptor (reviewed by Timmermans et al, 1993).

Since the arrival of the new orally available nonpeptide AII (AT_1 receptor subtype-specific) receptor antagonist losartan, a number of chronic studies have been undertaken in the SHR and, generally, they have found a full prevention of GHD or a normalisation of high blood pressure at doses upwards of 30 mg/kg/day p.o. (Oddie et al, 1992, Oddie et al, 1993). Associated with the lowering of SBP is a reduction in left ventricular hypertrophy and vascular amplifying properties of the SHR perfused

hindlimb preparation (Oddie et al, 1993), as well as reductions in media:lumen ratios from mesenteric resistance vessels (Morton et al, 1992).

1.11.8 Other antihypertensive treatments

Impairment of the SNS in the SHR can oppose GHD, provided the sympathetic nerves are destroyed. Chemical sympathectomy with 6-hydroxydopamine has been used effectively in the neonatal SHR to prevent GHD (Siniako et al, 1980), but there is a suggestion that the agent can also destroy central sympathetic structures (Clark et al, 1972). Antibodies to nerve growth factor often result in only partial sympathectomy (Folkow et al, 1972), and, even when combined with other agents like guanethidine, still do not normalise blood pressure in the SHR (Lee et al, 1987). On the other hand, cardiovascular structural properties of the SHR are attenuated or completely normalised by treatment, including medial mass, luminal diameters and cardiac hypertrophy (Lee et al, 1991a, Korner et al, 1993). Combined with adrenalectomy (Lee et al, 1991a) or α-blockade with prazosin (Korner et al, 1993), blood pressure and vascular structural aberrations can be reduced further in this model, suggesting that adrenal catecholamines contribute to some of the resistance these parameters display to antihypertensive strategies. The SHR seems resistant to other sympathoinhibitory agents alone, such as guanethidine, whereas WKY rats undergo complete neuronal loss (Johnson and Macia, 1979).

As mentioned earlier, hypernoradrenergic innervation of resistance vessels is already evident from as early as two weeks of age in the SHR, i.e., well before the development of high blood pressure and vascular medial changes (Scott and Pang, 1983). Together, these findings suggest that the hyperinnervation may have a strong causal role in the hypertension and vascular medial hypertrophic changes.

1.12 Withdrawal from antihypertensive treatment in the SHR

There is a disparity of effects on blood pressure after withdrawal from chronic antihypertensive treatments in the SHR. Most treatments lose their blood pressure-lowering influence once the drug is removed, with the exception of the ACE-inhibitors and low doses of the AII antagonist losartan (Giudicelli et al, 1980, Christensen et al, 1988, Christensen et al, 1989, Adams et al, 1990, Morton et al, 1992). Researchers have found that with as little as one month on the ACE-inhibitor captopril, prolonged reductions in SBP can be observed long after drug cessation (Harrap et al, 1990). In contrast, when the same treatment is instigated in adult (established) SHR and then withdrawn, the hypertension is restored relatively quickly (Harrap et al, 1990), emphasising the necessity for treatment in the developing phase. The permanency of captopril's effects can be taken a step further. The progeny of rats discontinued from early chronic captopril treatment also possess significantly lower blood pressures despite the likelihood of never having been exposed to the drug (Wu and Berecek, 1993) Obviously a wealth of research into the effects of ACE-inhibitors remains to be performed.

The prolonged suppression of the redevelopment of hypertension is believed to be due to the effects of treatment on cardiac and vascular medial hypertrophy, although some researchers disagree that vascular structure is an important determinant of the continuing effect (Christensen et al, 1988, Christensen et al, 1989, Mulvany, 1990, Thybo et al, 1994). There is far from a consensus view of functional changes in the vasculature that accompany a reduction in blood pressure from antihypertensive treatment. Parameters measured are passive (compliance) and active (contraction) vessel properties, including sensitivity and contractility of isolated vessels and perfused vascular beds to vasoactive agents and nerve stimulation.

Generally, when vessels have significant differences in structural parameters following chronic drug treatments these are accompanied by alterations in vessel function, at least for those treated with ACE-inhibitors. These changes have been described earlier. For non ACE-inhibitors, there are reports of altered active properties in response to hydralazine treatment, either alone (Freslon and Giudicelli, 1983, Jespersen et al, 1985) or in combination with a diuretic (Soltis and Bohr, 1987), diuretics alone (Qui Zhou et al, 1993), and calcium channel blockers (Nyborg and

Mulvany, 1985, Godfraind et al, 1991). Others however note no differences in vessel responses from calcium channel blockade (Christensen et al, 1989), β-blockade (Goldberg and Triggle, 1978, Christensen et al, 1989) or hydralazine treatment (Christensen et al, 1989).

After drug withdrawal even the effects of the ACE-inhibitors are contentious, with some authors reporting continued suppression of active (Adams et al, 1990) and passive (Freslon and Giudicelli, 1983) functional properties, while others note a loss of passive properties (Cadilhac and Giudicelli, 1986) after drug cessation. Others see the emergence of suppressed contractility when during treatment there was none (Christensen et al, 1989). Reasons for the discrepancies may lie in a number of areas related to study design, including: (i) the ACE-inhibitor used; (ii) the timing of treatment initiation and its duration; (iii) the period of drug withdrawal; and (iv) the vascular bed or vessels used for comparison.

Irrespective of the underlying mechanism(s) of action of the ACE-inhibitors, the prolonged suppression in hypertension redevelopment in this model has given researchers valuable insight into the role of the RAS in the pathogenesis of the disorder and, in particular, the multifunctional role of AII on cardiovascular design and function. It remains to be seen whether changes in RAS activity after treatment can prolong life in this model.

1.13 ***Summary***

In summary, the SHR is an animal model of genetic hypertension displaying a large number of alterations in vascular structure and function that are believed to play an important role in the development and maintenance of high blood pressure. A predominant feature of the cardiovascular system from the SHR is the presence of cardiac and vascular medial hypertrophy, the latter conferring properties on the vessels that may be responsible for the altered total peripheral resistance in this strain.

The precise factors responsible for the development of cardiovascular structural hypertrophy are still uncertain, but there is accumulating evidence pointing to the

involvement of neurohumoral systems expressed at the level of the vasculature in mediating VSM growth, vascular medial hypertrophy and the enhanced vascular contractility observed in this model.

The renin-angiotensin system (RAS) and sympathetic nervous system (SNS) are integrative pathways that can potentially produce long term effects on vascular structure and function in the SHR. There is strong evidence linking the expression of local vascular RAS components and the density of sympathetic innervation to these alterations in vessel properties, with the prospect that VSMC growth and proliferation may further augment the development of hypernoradrenergic innervation in a positive feedback cycle.

The effectiveness of pharmacological interference with RAS and SNS function also lends support to their involvement in vascular change, with ACE-inhibitors and certain sympatholytic treatments being able to prevent the development of hypertension and cardiovascular structural aberrations when administered during the developing phase of hypertension in this model. The ACE-inhibitors have the seemingly unique ability to prolong the suppression in hypertension redevelopment after drug withdrawal, although the exact mechanisms by which they operate to suppress blood pressure are not known with certainty.

There are currently anomalies in the efficacy of different classes of sympatholytics, with α-adrenoceptor antagonists and ganglion blockers yet to be tested or to yield consistent effects on hypertension development in the SHR. A full understanding of the precise mechanisms of the involvement of the SNS in these disorders of blood pressure and cardiovascular design awaits further studies with the available pharmacological tools.

1.14 Hypotheses and aims

The principal aims of this study are to examine aspects of cardiovascular structure and function during and after antihypertensive treatments in the developing SHR. Specifically, they focus on the RAS; its influence on these parameters and its

relationship with the SNS in mediating changes in cardiovascular structural, functional and biochemical parameters during the development of hypertension in the SHR.

Hypothesis 1: The RAS (and particularly the ACE) contributes to the long term development of hypertension and associated cardiovascular structural and functional changes in the SHR.

Aim: To investigate the effects of chronic angiotensin-converting enzyme (ACE)-inhibition and its withdrawal on blood pressure, vascular reactivity, cardiac hypertrophy and vascular contractile protein content in blood vessels from the SHR.

Hypothesis 2: Angiotensin II the predominant factor involved in the changes in cardiovascular structure and function observed in this model.

Aim: To compare the effects of chronic ACE-inhibition with that of other antihypertensive therapies (including acute ACE-inhibition), eg., the nonspecific vasodilator hydralazine and the angiotensin II (AII) receptor antagonist losartan. This permits a dissection of the effects of duration of treatment, blood pressure, inhibition of the ACE and blockade of the AII receptor on the aforementioned parameters of the SHR cardiovascular system.

Hypothesis 3: That the RAS is involved in the functional tolerance exhibited to chronic treatment with specific sympatholytic agents in the SHR

Aim: To investigate the effects of chronic α-adrenoceptor blockade and sympathetic ganglion blockade on the development of hypertension, cardiac structure, vascular contractile protein, and RAS activity in the SHR.

CHAPTER 2

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CHAPTER 2

METHODS

2.1 Animals

All of the studies in this thesis which involved the use of animals were conducted with the written approval of both the University of Adelaide Animal Ethics Committee and the CSIRO Animal Care and Ethics Committee.

2.1.1 Source and maintenance

Male spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats were obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) breeding colony (Glenthorne laboratories, O'Halloran Hill, South Australia). Breeding stocks of the SHR were developed from SHRs supplied by the Flinders Medical Centre (November 1981), and recent analysis revealed no genetic contamination (Appendix II). The rats were transferred postweaning (4-5 weeks of age) to the animal holding facility at the CSIRO Division of Human Nutrition, Kintore Avenue, Adelaide, prior to the chronic drug trials.

Rats were housed in wire-bottomed cages with 6-8 rats per cage and exposed to a 12 hour light/dark cycle, 22-24°C ambient temperature and food and water *ad libitum.* All rats received standard rat chow (Milling Industries, Australia) except for chronically phenoxybenzamine (POB)-treated SHR and their controls, which received the drug in a specially prepared synthetic diet (Appendix III). All rats received drinking water that was purified using reverse osmosis.

2.1.2 *Drug administration*

All antihypertensive drugs except POB displayed sufficient water solubility to allow them to be administered orally in the rat's drinking water. Body weight and fluid intakes were regularly monitored and were used to calculate the final concentration of drug to be made up in solution to give the desired dosage. Drugs in solution were prepared and replaced every 2-3 days. Diet containing POB was made up in a single batch, with the drug concentration based on an average body weight and expected food intake for that weight. Diet was stored frozen at -20°C, with the necessary quantity per cage thawed and fed every 2-3 days.

2.1.3 *Indirect blood pressure evaluation*

During the blood pressure developmental studies, drug-treated and untreated (control) rats had their systolic blood pressure measured using indirect tail-cuff plethysmography (IITC Life Sciences, California). Rats were placed in ventilated cylindrical perspex restraining tubes which were, in turn, placed in a large warming cabinet maintained at approximately 30°C. After an initial period of warming (20 mins) the tail cuff systolic blood pressure readings were systematically recorded, taking five to six measurements and disregarding exceedingly high or low readings. These occurred when the animals were insufficiently warm or were obviously agitated.

2.1.4 *Animal sacrifice and tissue collection*

Animals were sacrificed by stunning with a blow to the head followed by decapitation. At the completion of *in vivo* experimentation, anaesthetised rats were killed by a lethal intravenous injection of potassium chloride. Upon death, tissues were excised, cleaned and cleared of surrounding connective and adipose tissue, rinsed in ice-cold saline, blotted dry, weighed, wrapped in aluminium foil and snap-frozen in liquid nitrogen. Tissues were stored at -80°C until required for biochemical analyses. Blood

was collected into heparinised tubes, centrifuged at 2500*g* for 10 mins and the supernatant recovered and stored at -20°C until use.

All tissues were subsequently homogenised in ice-cold phosphate-buffered saline (PBS; 20mM sodium phosphate, 130 mM sodium chloride, pH 7.4) and stored at 4°C when not in immediate use.

2.2 *In vivo experiments*

In selected experiments, rats were anaesthetised for the direct intra-arterial monitoring of mean arterial blood pressure and its response to intravenous infusion of vasoactive agents.

Anaesthesia was induced by an intraperitoneal injection of a combination of methohexitone (Breital, Lilly, 10 mg/ml) and pentobarbitone (Nembutal, Boehringer, 60 mg/ml) in a ratio of 4:3, 0.3-0.5 mls per 100g body weight. After a midline incision down the neck the left carotid artery and jugular vein were exteriorised and cannulated for the direct recording of mean arterial blood pressure and administration of vasoactive agents respectively. Drug administration occurred in a volume of saline not exceeding 0.1 ml and was subsequently flushed with 0.2 mls heparinised saline (25U/ml). Cannula tubing was surgical grade (polyethylene, 0.4 mm internal diameter, 0.8 mm external diameter), and was connected to a Cobe CDX III pressure transducer (Cobe, Lakewood Co). Measurements were recorded on a Grass model 7D Polygraph.

2.3 *In vitro experiments*

A series of experiments involving the use of the perfused mesenteric vascular bed (PMVB) preparation was also performed. This entailed a midline laparotomy to expose the gastrointestinal tract and mesentery. The superior mesenteric artery was isolated and a cannula introduced into its lumen via its junction with the aorta (cannulated using tubing identical to that described for the *in vivo* experiments). The

gastrointestinal tract was severed near the stomach proximally and caecum distally and the abdominal aorta was divided on either side of the superior mesenteric artery. This allowed the complete mesenteric vasculature, intact with gastrointestinal tract, to be removed from the animal and mounted in a perfused organ bath system as previously described (Longhurst et al, 1986). The gastrointestinal tract was flushed before mounting. The preparation was maintained at 37°C in a water-jacketed organ bath and perfused with Kreb's solution consisting of the following: sodium chloride 113 mM, potassium chloride 4.8 mM, potassium dihydrogen phosphate 1.2 mM, magnesium sulphate 1.2 mM, sodium hydrogen carbonate 25 mM, calcium chloride 2.5 mM and glucose 11.2 mM. The solution was gassed with 95% carbon dioxide and 5% oxygen throughout the experiments and was perfused at a flow rate of 4 ml/min through the vascular bed via the superior mesenteric artery. Vasoactive agents were administered via an injection port proximal to the connection with the cannula. Changes in perfusion pressure were monitored using a Statham P23 AC pressure transducer and recorded on a Grass model 7D polygraph.

2.4 Biochemical analyses

2.4.1 Protein assay

The protein content of tissue homogenates was measured using a modification of the spectrophotometric method of Lowry et al (1951), using the Folins-Ciocalteu's phenol reagent. Blanks (100 ul water), protein standards (bovine serum albumin (BSA); 100 ul) or tissue homogenates (1-10 mg in 100 ul) were added to 2M sodium hydroxide (NaOH; 100 ul) and incubated in a 60°C water bath for 2 hours. Water was then added (800 ul), the samples diluted in 0.1 M NaOH (1/2-1/5) and an aliquot (20 ul) assayed using an automated preprogrammed bioanalyser (COBAS Bio, Roche), with absorbance set at 540 nM. The standard curve was linear over the range 2-20 ug protein. Intra and inter-assay coefficients of variation were both 2%.

2.4.2 3-Methylhistidine assay

The 3-methylhistidine (3MH) content of vascular and nonvascular tissues involved acid hydrolysis of the tissue homogenate, followed by ion exchange chromatographic purification, derivatisation with fluorescamine and analysis using high performance liquid chromatography (HPLC) with fluorometric detection.

Aliquots of tissue homogenate containing 10 mg tissue were made up in concentrated hydrochloric acid (HCl) to give a final concentration of 10 mg tissue per ml of 6M HCl. Samples also contained 3-ethylhistidine (3EtH; 20 nmoles) as an internal standard. Samples were placed in 5 or 10 ml culture tubes, flushed with nitrogen and sealed with caps containing teflon inners. Samples were then hydrolysed at 110°C for 20 hours.

Following hydrolysis, samples were purified by ion exchange chromatography, in a modification of the procedure of Tomas et al (1984). Samples were diluted to 10% v/v HCl and loaded on a strongly acidic cation exchange resin column (Dowex AG50X8 200-400, Sigma; 1 ml) that had been previously prepared and washed (Appendix IV). Columns were sequentially washed with a citrate-phosphate buffer (0.15 M, pH 4.5, 6 mls) and glass-distilled water. Free 3MH and 3EtH were eluted off the columns using a sodium phosphate buffer (0.4 M, pH 9.0) in 1 ml fractions, with the second and third ml pooled fractions representing approximately 85% of total eluted 3MH.

For convenience in another set of experiments the ion exchange purification was omitted. Post-hydrolysis, the HCl was removed from samples by drying under vacuum for 4 hours. Samples were reconstituted in water (1ml) with vigorous vortexing, left overnight at 4°C, spun at 10,000*g* in a microfuge for 1 min the following day and the supernatant, containing free 3MH and 3EtH, collected and stored at -20°C until use.

The 3MH content of the eluates was determined using a modification of the procedure of Wassner et al (1980), in which conditions favour the selective retention of histidine derivatives of flourescamine at the expense of other amino acid derivatives.

The eluate (100 ul) was derivatised in 0.2 M borate, pH 12.2 (250 ul) after the addition of fluorescamine (1.6 mg/ml in acetonitrile, 250 ul). The mixture was vortexed and left for 5 mins before concentrated perchloric acid (35 ul) was added and the

mixture again vortexed, before incubating in a hot water bath (80°C, 1 hour). 3-[N-morpholino] propanesulfonic acid (MOPS; 0.5 M in 3M NaOH, 100 ul) was added and the mixture vortexed prior to reverse phase HPLC with fluorometric detection. The excitation and emission wavelengths were 365 nm and 460 nm respectively. Isocratic elution was performed using a mobile phase of 30% acetonitrile in 10 mM sodium phosphate, pH 7.4, at a flow rate of 1ml/min. Peaks were recorded and the signal analysed using an automated data acquisition and on-line management system consisting of a K25-D HPLC pump (Kortec, Australia), 30 cm X 4.6 mm HPLC column packed with C_{18} S10 ODS2 Spherisorb (Adelab, Australia), LC 1600 Autosampler, LC 1250 Fluoro Detector, DP 800 Data Interface and Data Management Program (all ICI Instruments, Australia). 3MH was linear in the range of 0-100 picomoles (Fig. 2.1). Retention times for 3MH and 3EtH were 7.0 and 9.5 mins respectively (Fig. 2.2). The intra and inter-assay coefficients of variation were 2.6% and 4.75% respectively. 3MH was detectable to approximately 20 picomoles.

2.4.3 Synthesis and purification of 3-ethylhistidine (3EtH)

The internal standard was not commercially available. 3EtH was kindly synthesised from histidine by Dr D. Ward, Department of Chemistry, University of Adelaide, utilising the protocol of Noordam et al (1978).

Chromatographic analysis of the synthesised compound, using both thin layer chromatography (TLC) and HPLC revealed an impurity that co-chromatographed with 3MH (Appendix V), which was estimated to be approximately 20% of the synthetic yield. This necessitated further purification of the mixture, which was performed using TLC as described below.

Crude 3EtH mix was purified by dissolving 10 mg in 1 ml of 85% methanol and gradually applying 20 ul aliquots along the centre section of a single silica gel TLC plate (20X20 cm, 0.2 mm thick, 60 F_{254}, Merck). A stream of warm air was used to facilitate evaporation. Margins were left on either side of the applied 3EtH line where marker lanes of 3EtH (20 ug) were spotted. The TLC plate was developed for 3 hours in a standard solvent tank using a solvent system of chloroform/methanol/ammonium

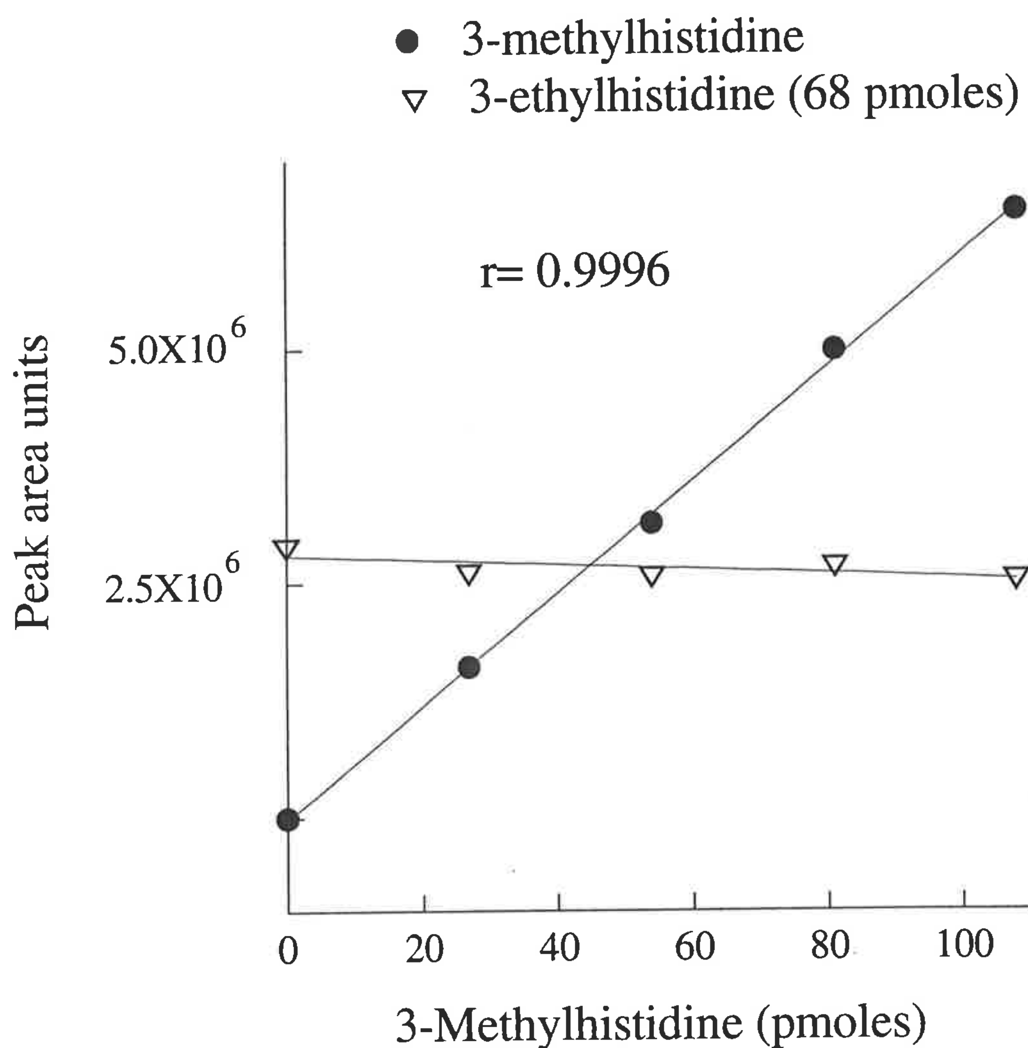
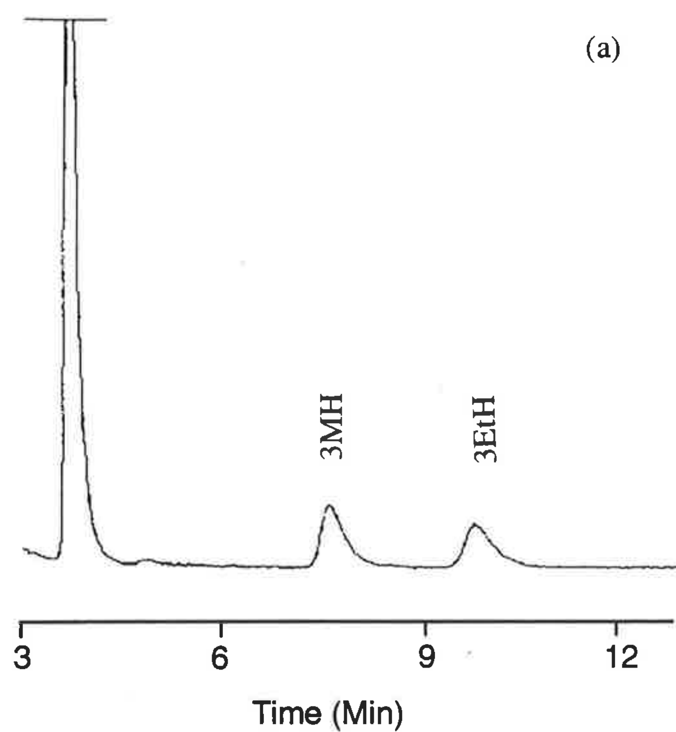


Fig 2.1. Standard curve of 3-methylhistidine (3MH) and 3-ethylhistidine (3EtH) for 5 standards. Based on total amount loaded onto HPLC column. Each standard contained 68 pmoles 3EtH.



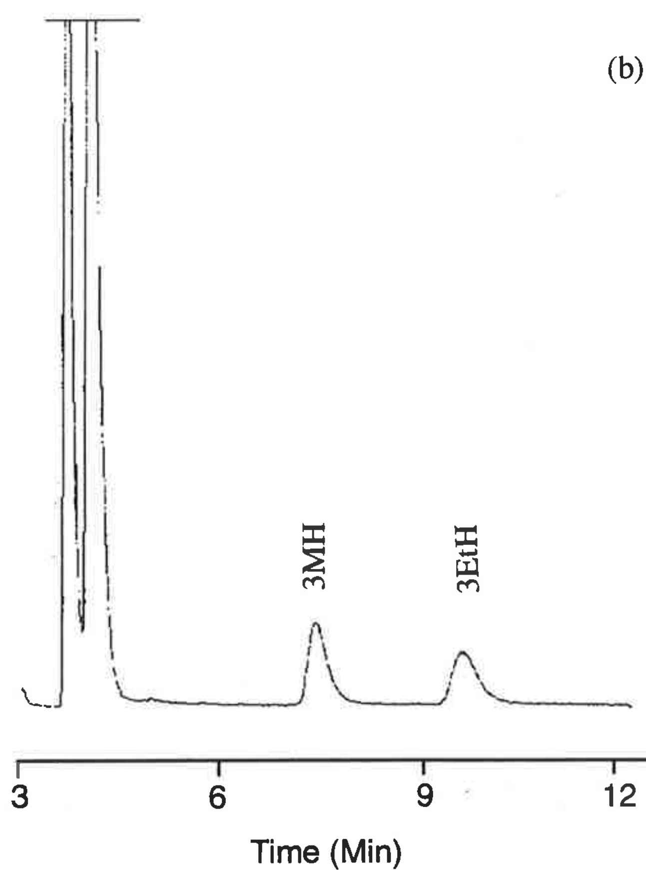


Fig 2.2. HPLC chromatograms representing (a) standards and (b) tissue samples containing 3-methylhistidine (3MH) and 3-ethylhistidine (3EtH). Amino acids are derivatised with Fluorescamine prior to reverse phase HPLC with a mobile phase of 30% acetonitrile, 10mM sodium phosphate, pH 7.4.

hydroxide (4:4:1). After removal and evaporation of ammonia the marker lanes were removed and sprayed with ninhydrin (0.2% in 95% ethanol). The marker lanes were allowed to develop for at least 1 hour at room temperature to allow visualisation of the amino acids (Appendix V). A wide section of the centre plate corresponding to the location of the pure 3EtH was outlined, the silica removed from the plastic backing and transferred to a 5 ml tube for extraction of pure 3EtH.

3EtH was extracted off the silica in 1M acetic acid/50% w/v methanol and left overnight on a tilting platform to optimise extraction. The extract was centrifuged (10,000*g* for 10 mins) and the supernatant removed. This was repeated 3 times and the supernatants pooled and dried under vacuum. The recovered fraction was reconstituted in dilute HCl (0.05M, 1 ml) and then subjected to HPLC analysis to determine purity and recovery. The relevant calculations are given in Appendix V. The 3EtH was sufficiently pure to be subsequently used as an internal standard in the HPLC determination of tissue 3MH concentrations.

2.4.4 Angiotensin converting enzyme assay

The radioenzymatic assay for angiotensin converting enzyme activity (ACE) activity was adapted from the method of Rohrbach et al (1978). Plasma (1/2 dilution; 10 ul) or tissue homogenate (10 ul) was incubated at 37°C with an enzyme incubation mixture (EIM; 40 ul) consisting of sodium borate (100 mM, pH 8.75), NaCl (300 mM) and the ACE substrate, hippuryl-histidyl-leucine (HHL; 5mM). The HHL consisted of a mixture of [^{14}C]-HHL (specific activity = 3 mCi/mmol, NEN) and unlabelled HHL in a ratio of 1:75. The reaction was stopped with HCl (1M; 50 ul) and the [^{14}C]-hippuric acid extracted into ethyl acetate (300 ul). The mixture was vortexed vigorously and briefly centrifuged (10 000*g*, 10s). The supernatant was recovered (200 ul) and added to scintillation fluid (Ready Value, Beckmann, 5 mls). The reaction was linear for 120 mins (Fig 2.3). Activity was expressed as nmol HHL hydrolysed/min/ml plasma or /mg protein for tissue samples. Blanks were constructed by addition of 1M HCl (50 ul) prior to adding EIM. The inter and intra-assay coefficients of variation were 7% and 6% respectively.

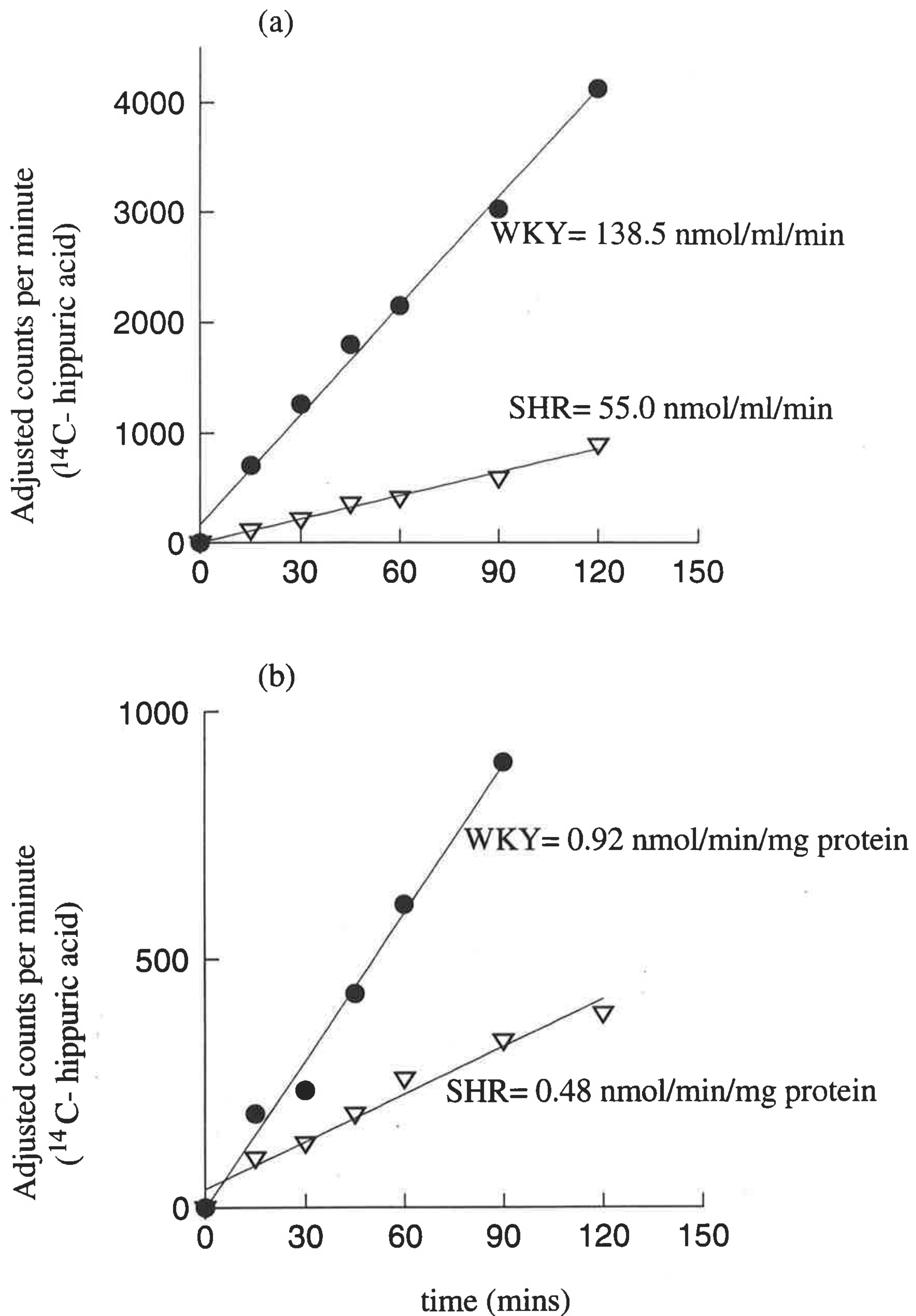


Fig 2.3. Linearity of ACE activity with time in SHR (▽) and WKY (●) rat plasma (a) and mesenteric vascular tissue (b). Activity was measured as the formation of ^{14}C-hippuric acid from the substrate ^{14}C-hippuryl-histidyl-leucine.

2.4.5 Actin determination: tissue preparation and polyacrylamide gel electrophoresis

Prior to gel electrophoresis, samples of aortic and mesenteric vascular tissue previously homogenised in phosphate-buffered saline (PBS) were centrifuged (10,000*g*, 5 mins) and the supernatant recovered and stored at -20°C until use. The tissue residue was reconstituted in an equivalent volume of cell lysis buffer consisting of urea (9M), sodium dodecyl sulfate (SDS, 5% w/v), ß-mercaptoethanol (5% v/v) and phenylmethylsulfonyl fluoride (PMSF, 5 mM). Samples were stored overnight at 4°C prior to being vortexed and then centrifuged (10,000*g*, 5 mins).

Samples of supernatant from both the PBS and lysis buffer phases of vascular tissue were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous buffer system technique employed by Laemmli (1970), employing standard total gel monomer (%T=12) and crosslinking (%C=1.07) concentrations. The complete set of conditions for electrophoresis, including all reagents and preparative procedures are outlined in Appendix VI. Gels were run using a Protean II Slab Cell System (BioRad Industries, California), with run times between 4-5 hours under constant current conditions (25 mA/stack gel, 35 mA/resolving gel). Gel temperature was maintained at 15-20°C by circulation of tap water through the cooling core.

After electrophoresis, gels were removed, fixed and stained overnight using Coomassie Brilliant Blue stain (R-250, 0.25 g/l in 7% acetic acid, 2% methanol). Gels were destained in 7% acetic acid for 7-8 hours prior to photography and quantitation of actin bands. Determination of actin levels were performed using laser densitometry (LKB Ultrascan XL, LKB Bromma), with bands scanned at 650 nm. The measurement of actin was linear up to at least 15 ug protein, with the inter and intra-assay coefficients of variation equal to 2.5% and 8% respectively. Total actin extracted was measured as a combination of values determined from both the PBS and lysis buffer phase supernatant actin content.

2.4.6 Statistical analyses

Values were expressed as the means ± standard errors of the means (SEM) of the relevant number of observations (n) within each control and treatment group. Statistical analyses of the data was as follows: normality of distribution was determined using a Bartlett's homogeneity of variances test. All parametric data was subjected to One-way ANOVA testing and nonparametric data was subjected to Kruskall-Wallis ANOVA testing to determine the significance of variation in group means ($P<0.05$ level). If significantly different, subsequent tests consisted of a Dunnett's multiple comparison test (parametric data), Dunn's multiple comparison test (nonparametric) and a Student's t-test for grouped data where only the means of measures from the SHR versus WKY rat were analysed. All blood pressure, vascular responses and biochemical determinations were analysed in this fashion using a compiled pharmacological statistics program (Pharcal v 6.62p, courtesy of G.A. Crabb, University of Adelaide) and Biostatistics program (Instat v 2.0, Graphpad). Correlation analysis was tested for significance ($P<0.05$) using a parametric (Pearson's) test.

Data tabulation was performed using Lotus 123 and 123 Release v 3.4 spreadsheet programs (Lotus inc.), and results were represented graphically using Sigmaplot v 5.01 scientific graphics software (Jandel Scientific) and Harvard Graphics for Windows (v 2.0). All programs were licensed to the CSIRO Division of Human Nutrition or the Department of Clinical and Experimental Pharmacology, University of Adelaide.

CHAPTER 3

The Effects of Chronic Captopril Treatment and its Withdrawal on Blood Pressure and Tissue 3-Methylhistidine in the Developing Spontaneously Hypertensive Rat (SHR)

3.1 Introduction ***51***

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3.1 Introduction

The identification of alterations in vascular structure in the SHR has been achieved using a variety of techniques. Measurements of vascular structure by histological means include selective staining of medial mass, nucleic acid, elastin and collagen within cross-sections of blood vessels (Levy et al, 1988a, Michel et al, 1990), allowing, amongst other things, a calculation of the media:lumen ratio. However, under standard and perfused fixing conditions medial contraction may occur, thus creating an artefact when this index is used to determine the presence of medial hypertrophy (Owens et al, 1988). The conditions of perfusion fixation have also been criticised for comparing hypertensive and normotensive vessels fixed under identical perfusion pressures, when obviously *in vivo* pressures are markedly different. A way of bypassing the problem of vascular smooth muscle cell (VSMC) activation is to perfuse under conditions of maximal vasodilatation, but this does not give an indication as to the functional state of the media *in vivo.* A preferred measure of vascular wall hypertrophy is the medial cross sectional area, which should be constant irrespective of changes in activation and pressure within the vessel (Greensmith and Duling, 1984).

Comparisons of cross sections from anatomically similar vessels may not mean that they are functionally similar, especially if the vasculature of hypertensive rats is altered in its fundamental design. This may occur in cases of vascular rarefaction, where the loss of density of blood vessels may place unusual haemodynamic restraints on certain vascular beds. Rarefaction of vessels has been documented in the SHR (Hutchins and Darnell, 1974).

With the questioning of techniques used in calculating alterations in medial mass in blood vessels, the estimation of the media-to-lumen ratio and its application to vessels from the SHR has also been examined. Researchers suggest that an increase in the ratio may in fact not involve an increase in medial mass at all, but instead reflect a change in the size of the vessel to one of smaller external diameter and lumen (Heagerty et al, 1993). In this case, the existing media arranges around a smaller lumen, causing an increase in the ratio without medial growth (Fig. 3.1). This has been loosely termed "remodelling" which may be a somewhat inaccurate term, as there is the

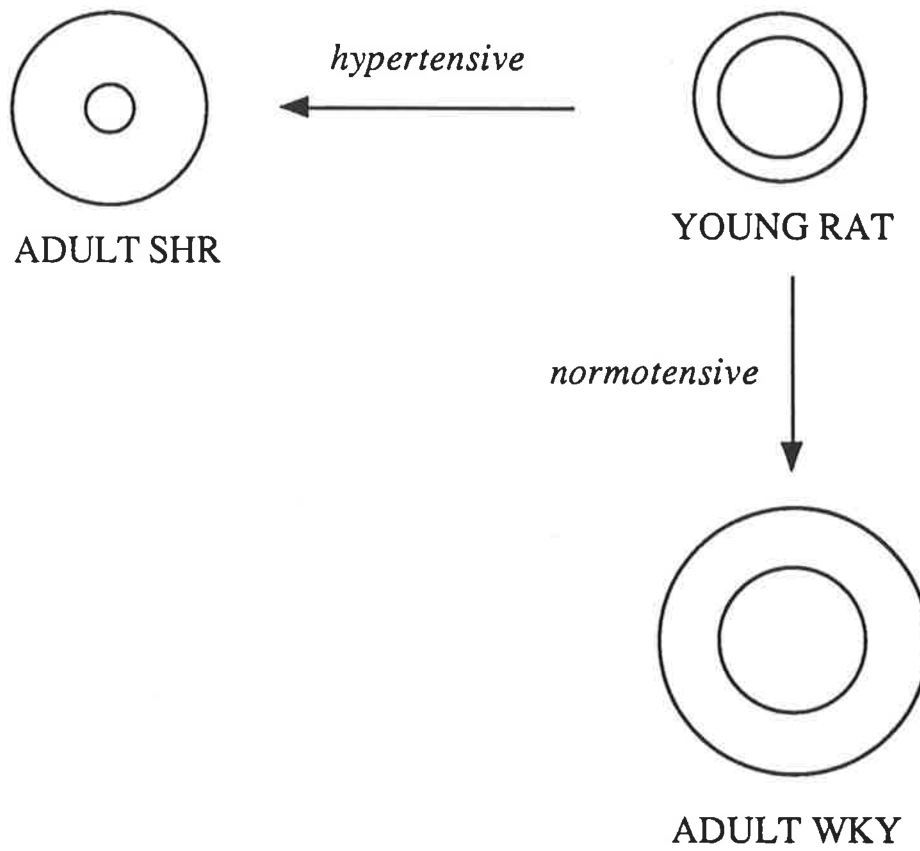

Fig. 3.1. Depiction of the proposed alterations in vascular medial structure occurring in the development of the hypertensive (SHR) and normotensive (WKY) rat. With vascular "remodelling", there exists equal medial mass arranged differently about the vascular lumen, resulting in an increase in the media:lumen ratio in the SHR.

implication of a dynamic alteration in the design of a vessel originally designated normotensive to that of one distinguished as hypertensive. The term may be more applicable to vessels from hypertensive rats that have undergone changes in structure from select antihypertensive treatments, where modifications in overall vessel structure have occurred to render the vessels similar to those from normotensive rats. Schiffrin (1994) has shown that the types of structural changes are different depending on the model of hypertension being investigated. For example, in DOCA-salt rats, small mesenteric arteries exhibit an increased volume of the media per unit length of vessel and a reduced lumen, implying medial growth projecting inwards into the lumen. In 17 week-old SHR, there is an increased medial width associated with an unchanged media volume, suggesting a distribution of media around a smaller vessel. In gluteal vascular biopsies from essential hypertensives, the findings are similar to those in the SHR (Schiffrin, 1994). The different growth responses may reflect the type of hypertension (volume or vasoconstriction-dependent) or the genetic basis of the VSMC response.

Irrespective of the underlying changes in vascular morphology in hypertension, an important postulate underlying Folkow's hypothesis is still met in vessels from hypertensive humans and animal models, i.e. narrowing of the vascular lumen and an increase in vascular resistance.

The SHR is a model which has been widely used to study the effects of chronic antihypertensive treatments on genetic hypertension development (GHD) and associated cardiovascular structural modifications. Of those, the ACE-inhibitors have been extensively studied and found to be one of the most effective therapies in controlling GHD and cardiovascular structural parameters in the developing SHR. Generally, the ACE-inhibitors are found to have an effect on structure over and above that expected from their blood-pressure lowering effects alone (see *1.11.5*). This may underlie a strong growth-promoting influence of the RAS on the SHR vasculature.

As mentioned previously (*1.12*), a feature of chronic ACE-inhibition in this animal is the prolonged suppression in hypertension redevelopment after drug withdrawal. Morphometric analysis of blood vessels post-withdrawal has yielded results suggesting that, although structure was a poor predictor of the attendant blood pressure during treatment with the ACE-inhibitor, there was a good correlation between

media:lumen ratios and blood pressure after a period of withdrawal from the antihypertensive (Christensen et al, 1989). Others (Freslon and Giudicelli, 1983) have found a continued, albeit diminished, effect on vascular structure after ACE-inhibition was discontinued. Left ventricular hypertrophy was also matched to blood pressure after withdrawal from chronic enalapril treatment (Adams et al, 1990).

The dissociation between this index of vascular structure and blood pressure during treatment may underlie a more complex relationship between these parameters in the SHR, seemingly dependent on other influences of the RAS. Another factor may be the effects of the RAS on vascular function, independent of its effects on structure (Purdy and Weber, 1988). Thus, the full extent of the ACE-inhibitor's actions on blood pressure may not be completely attributable to the altered media:lumen ratio. This aspect of RAS action will be discussed in Chapters 4 and 5.

A marker of contractile protein may be an index of vascular alterations more closely related to vascular function, and perhaps then also blood pressure, in this model. As described previously (*1.9.1*), there exists a proportionately greater level of actin in vascular smooth muscle (VSM) than other types of smooth muscle. This is associated with an increased force-generating capacity in blood vessels (Cohen and Murphy, 1979). As calculated in Appendix I, 3-methylhistidine (3MH) is expected to be a marker of contractile protein levels (predominantly actin) in the vasculature. With the evidence suggesting that AII regulates vascular actin expression, the possibility that 3MH levels may more closely follow systolic blood pressure changes with ACE-inhibition than the media:lumen ratio was explored in the following study.

In this chapter, the effects of chronic captopril treatment and its withdrawal on blood pressure and vascular and nonvascular 3MH concentrations were examined in developing SHR and WKY rats.

3.2 Methods

3.2.1 Animals, drug treatments and tissue collection

Male SHR and WKY rats were maintained as described previously (*2.1.1*). Drug treatments and blood pressure measurements were performed by S. Dyer, with some of this data appearing in a previous publication (Dyer et al, 1992). At 4-5 weeks of age, animals were randomly assigned to either untreated (control), captopril-treated or captopril-withdrawn ("released") groups. Captopril (100 mg/kg/day p.o.) was administered as previously described (*2.1.2*). Captopril-treated groups received the drug from 5 to 17 weeks of age; captopril-released groups received captopril from 5 to 13 weeks of age, whereupon drug treatment was withdrawn. Systolic blood pressure was measured fortnightly using an indirect tail-cuff procedure (*2.1.3*). At 17 weeks of age all animals were sacrificed and tissues removed and prepared for 3MH analysis as previously described (*2.1.4*). The mesenteric vascular bed was cleaned of surrounding adipose and connective tissue, leaving the superior mesenteric artery and its branches intact.

3.2.2 3-Methylhistidine (3MH) analysis

3MH was assayed as described previously (*2.4.2*) using ion exchange chromatography and tritiated histidine as the internal standard, which was used prior to the development of 3EtH as a more effective internal standard. Prior to tissue hydrolysis, l-(2,5)-^{3}H-histidine (15000 cpm/ml, 100 ul, NEN) was added to samples. After ion exchange chromatographic purification and fraction pooling, an aliquot of eluate (100 ul) was added to water (400 ul) and scintillation fluid (Beckmann Ready Value, 4mls) and the tritium counted (Rackbeta 1218, LKB). In this manner the 3MH content of nonvascular tissues (vas deferens, left ventricle and skeletal muscle) was compared with vascular tissue (mesenteric vascular bed). 3MH was expressed on a umol/g wet tissue weight basis.

3.3 Results

3.3.1 Blood pressure

The age-dependent development of hypertension was observed in untreated SHRs, where at 17 weeks of age systolic blood pressure (SBP) plateaus at approximately 200 mmHg (Fig. 3.2a). In contrast, the WKY rat displayed a relatively stable SBP profile, SBP rising only 30 mmHg from 5 to 17 weeks of age (Fig. 3.2b). Administration of captopril from 5 to 17 weeks of age prevented the development of hypertension in the SHR (Fig. 3.2a), and also elicited a modest overall decrease in systolic blood pressure (SBP) in the WKY rat (Fig. 3.2b). After 4 weeks of drug withdrawal from 13 weeks of age, SBP was unchanged in captopril-released versus captopril-treated WKY rats, and there was a modest increase in SBP of captopril-released SHRs to 140 mmHg, equivalent to the untreated (control) WKY at 17 weeks of age, and substantially lower than the untreated SHR at the same age.

3.3.2 Tissue 3-Methylhistidine content

When compared to tissues from control WKY rats, the concentrations of 3MH were elevated in the mesenteric vascular bed, but not in the left ventricle, skeletal muscle or vas deferens from the control SHRs (Fig. 3.3). Chronic captopril treatment for 8 weeks was associated with a 50% decrease in 3MH concentrations occurring solely in the vascular tissue studied, with no alterations from drug treatment in any of the other tissues analysed. Chronic captopril treatment did not alter vascular 3MH levels in the WKY rat.

Release from captopril treatment in the SHR was associated with a reversal of 3MH content to levels of the untreated SHR. Withdrawal from treatment did not significantly alter the levels of 3MH in any of the other tissues studied, and was without effect on mesenteric vascular 3MH concentrations in captopril-released WKY rats.

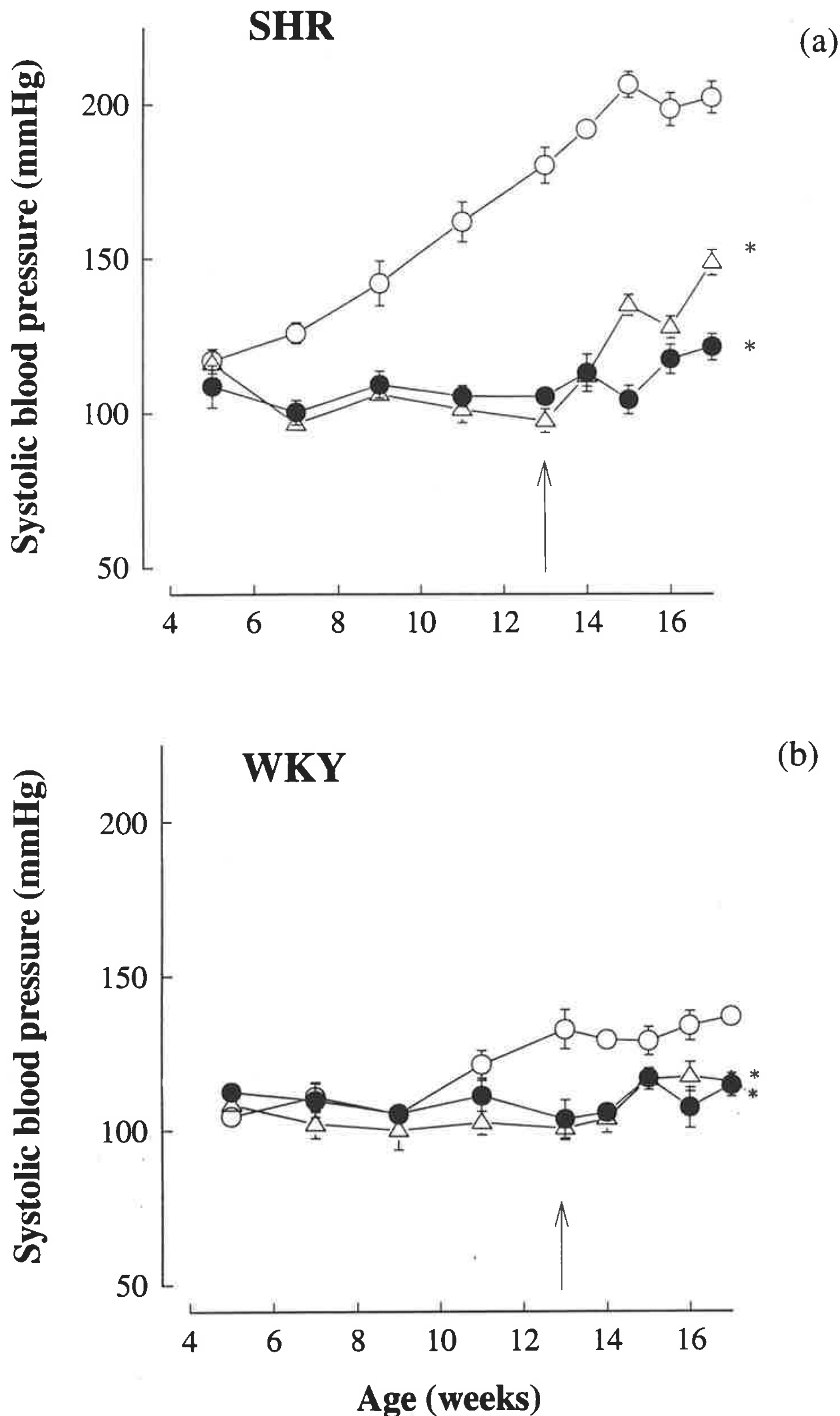

Fig 3.2. Changes in systolic blood pressure with age in untreated (control; ○), chronically captopril-treated (●) and captopril-withdrawn "released" (△) SHR (a) and WKY rats (b). Rats were orally administered captopril (100 mg/kg/day in drinking water) from 5 weeks of age. Systolic blood pressure was measured using an indirect tail cuff technique. Arrow denotes time of drug withdrawal in captopril-released SHR.

* significant ($P<0.05$) difference vs. control at 17 weeks (n=6).

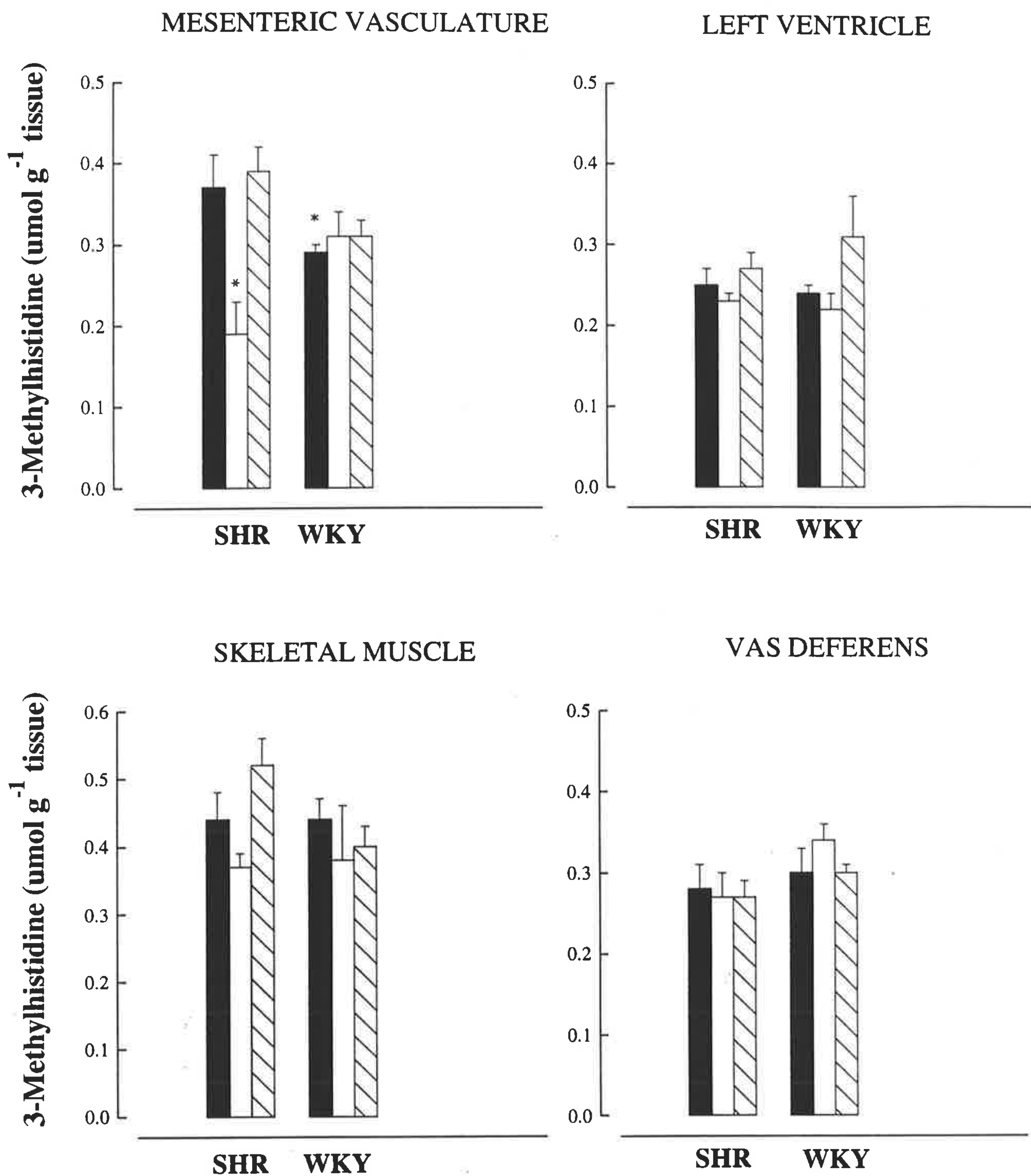

Fig. 3.3. 3-methylhistidine (3MH) concentrations from mesenteric vascular, left ventricular, skeletal muscle and vas deferens tissue from untreated (control ■),chronically captopril-treated (□) and captopril-withdrawn "released" (▨) SHR and WKY rats. Rats were orally administered captopril (100 mg/kg/day in drinking water) from 5 to 17 weeks of age; captopril-released SHR from 5 to 13 weeks of age. Rats were sacrificed at 17 weeks of age.
* significant ($P<0.05$) difference vs. control SHR (n=3-6).

3.4 Discussion

In the present study, 3MH content has been used as a marker of contractile protein in a range of nonvascular tissue and in the mesenteric vascular bed in the rat. An investigation into the relationship between 3MH concentrations and prevailing levels of SBP has been undertaken.

The enhanced 3MH content in the mesenteric vasculature from the SHR is similar to results previously published (Jonsson et al, 1991). Furthermore, chronic captopril treatment has been previously shown to elicit substantial reductions in vascular 3MH levels in this strain. Its use within this context supports the morphometric studies showing increased media:lumen ratios and medial mass in blood vessels from the SHR (Freslon and Giudicelli, 1983, Owens et al, 1986). It also supports the marked effect of ACE-inhibition on vascular medial hypertrophy in the SHR.

Interestingly, the withdrawal from captopril treatment resulted in a reversal of the vascular 3MH concentration to levels observed in control SHR, despite a continued suppression in the redevelopment of hypertension. There was a small increase in SBP in captopril-released SHR and the possibility that this is the stimulus for a complete reversal of the 3MH content cannot be excluded. It could also be argued that, if the changes in contractile protein occur after 13 weeks of age, then this treatment strategy missed the window for treatment to decrease 3MH levels. In this case 3MH content would not be reversed in the captopril-released SHR; it would never have decreased initially. However, an earlier study in which vascular 3MH concentrations in 12 week-old control and captopril-treated rats (treatment from 4-12 weeks of age) were measured showed that reductions had occurred by 12 weeks of age (Jonsson et al, 1991). Irrespective of the relationship between SBP and 3MH content during treatment, it appears that 3MH levels do not follow blood pressure in a simplistic fashion after treatment withdrawal.

Chronic captopril treatment elicited a significant overall lowering of SBP in WKY rats. Furthermore, SBP of captopril-released WKY rats remained at levels of captopril-treated rats after 4 weeks of drug withdrawal. These results suggest that the renin-angiotensin system (RAS) may regulate the development of "normal" blood

pressure to some extent. However, captopril-treated WKY rats did not exhibit a lowering of vascular 3MH concentrations.

Despite the observation that the adult SHR displays a readily observable degree of left ventricular hypertrophy (Chapter 5), there was no difference in the 3MH content in this tissue between the SHR and WKY rats. This is a consistent finding (Jonsson et al, 1991) and may be related to the absence of 3MH in cardiac myosin (Kuehl and Adelstein, 1970) and the large increases in connective tissue and extracellular matrix that accompany hypertrophic change in cardiac muscle (Sen et al, 1974). This may effectively dilute increases in 3MH content on a per weight or protein basis.

The mechanism by which captopril exerts a long-lasting influence on hypertension redevelopment remains unclear. Although prolonged tissue ACE-inhibition after cessation of ACE-inhibitor administration has been described, the period was only for approximately 1 week after the last dose in peripheral tissues (Unger et al, 1985). This is not long enough to account for the time course of suppression observed, although CNS levels have not yet been measured within this context and may be suppressed for longer periods.

Aside from the effects of drug treatment on vascular structure, ACE-inhibitors may be acting in other ways to control blood pressure long-term, including prolonged suppression of vascular neuroeffector function and/or normalisation of central nervous system pathways (bulbospinal and/or hypothalamo-pituitary) modulating blood pressure. An attractive hypothesis may relate to a facilitation of baroreceptor function in the SHR, which shows an impaired relationship to blood pressure control in this model (Andresen et al, 1980). This may be a consequence of the described ability of AII to dampen baroreceptor sensitivity directly (AII causes rightward shift in aortic arch pressure/nerve activity curves; Munch and Longhurst, 1989) or indirectly (AII is implicated in structural modifications of blood vessels which reduce distensibility and impair baroreceptor sensitivity; da Silva et al, 1994). The indirectly-acting component may be long lasting and involve changes in connective tissue expression, predominantly in large vessels. Chronic ACE-inhibition has been shown to inhibit the elevated deposition of vascular and left ventricular collagen in blood vessels from the SHR (Oshima et al, 1983, Levy et al, 1991), an integral structural protein which has a very slow turnover

(Keeley et al, 1991). Complete resetting of baroreceptor sensitivity has been observed after acute (7 day) and chronic blockade of RAS function (Howe, 1989, da Silva et al, 1994).

In this regard, vascular contractile protein by itself may only give limited information regarding the changes in vessel structure that occur after antihypertensive therapy. Other biochemical indices that may more closely follow SBP after drug withdrawal include markers of collagen and elastin, components that influence vessel elasticity, and thus resistance, in the cardiovascular system.

Based on the present results, it is anticipated that, if contractile protein levels were reflecting medial hypertrophy in the SHR, and this was an important determinant of the hypertension, then 3MH values would correlate with the prevailing blood pressure. The findings of the present study do not support a simple relationship between these parameters, possibly due to the following reasons.

3MH may not be indicative of a change in cellular actin levels, rather reflecting alterations in the disposition of actin isoforms in the vasculature. In this case, the relative content of 3MH in each isoform of actin may need to be considered to determine why the 3MH content is dramatically reduced after chronic captopril treatment.

Studies have documented the highly conserved amino acid sequence of actin throughout various tissues and animal species (Raghavan et al, 1989). However, there are no studies to date that have addressed actin isoform 3MH expression from any muscle type, possibly due to technical limitations in purification, resolution and extraction of the variants. As isoform expression changes with age in VSMCs (see *1.9.1*), the possibility exists that, if α-actin contains more 3MH than the ß or τ isoform, then isoform switching may be the cause of the reduced 3MH levels observed in chronically ACE-inhibited SHR and not a diminishing content of total actin.

Measurements of mesenteric resistance vessel actin concentrations suggest that actin levels are enhanced in the SHR relative to tissue wet weight, indicating selective increases in contractile protein content in these vessels (Brayden et al, 1983). This, together with the likelihood that α-actin isoform sequences are highly conserved, would suggest that isoform switching is not likely to be responsible for altered 3MH levels.

An alternative scenario is shown in Fig. 3.4, in which medial mass and contractile protein (and 3MH) are restored to levels exhibited in the untreated SHR after a period of withdrawal from chronic treatment with an ACE-inhibitor. However, due to the overt structural changes and a loss of developmental plasticity of the vessel in the adult animal, the adverse vascular "remodelling" cannot occur, and instead medial growth slowly projects inward to the lumen. In this situation the media:lumen ratio (and vascular 3MH content) would progressively increase, and blood pressure would slowly rise after drug withdrawal. This scenario obviates the need for vascular morphological and morphometric indices to be used in conjunction with biochemical markers, in order to form a more conclusive appraisal of vascular alterations from antihypertensive therapies.

In summary, chronic captopril treatment elicits a long lasting reduction in SBP after drug withdrawal that is dissociated from mesenteric vascular 3MH content in the SHR.

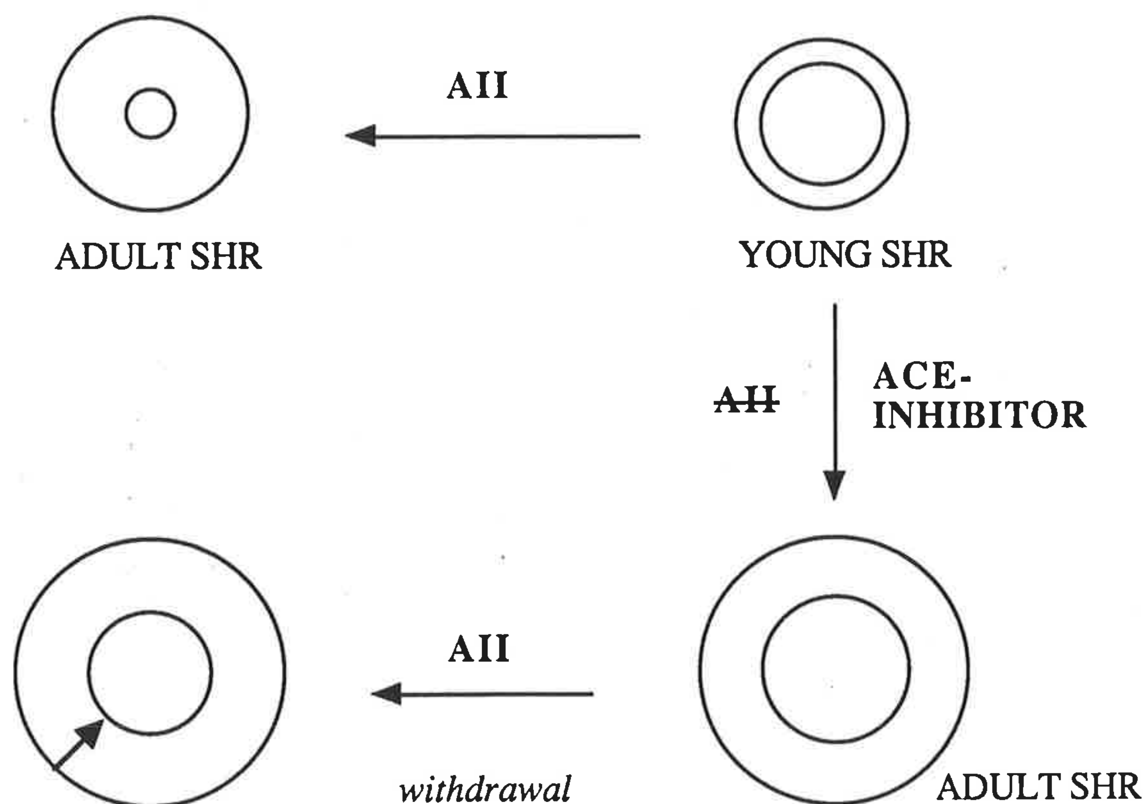

Fig 3.4. Depiction of hypothetical vascular morphological changes in SHR chronically treated with an ACE-inhibitor, which prevents the increase in media:lumen ratio. After withdrawal, medial growth projects inwards, but the media:lumen ratio is still less than that of the adult SHR, in keeping with the suppression in SBP after drug withdrawal.

CHAPTER 4

The Effects of Chronic Captopril Treatment and its Withdrawal on Blood Pressure, Vascular Reactivity and 3-Methylhistidine in the Developing SHR

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4.1 Introduction

The previous chapter identified changes in an index of vascular contractile protein in the SHR following chronic captopril treatment and its relationship with systolic blood pressure (SBP). The association between SBP, 3MH levels and responses of the isolated perfused mesenteric vascular bed (PMVB) have been studied in this chapter using an experimental design of drug treatments in the SHR identical to that in Chapter 3.

Assessment of certain aspects of vascular function, such as reactivity, agonist sensitivity and contractility are ideally performed *in vitro*. These techniques have been designed to characterise vascular functional parameters and their response to antihypertensive drugs in the SHR. Indeed, in this model, aberrant function is a well defined feature of those vascular beds expressing concomitant structural changes such as vascular medial hypertrophy/hyperplasia. These include enhanced reactivity of the isolated perfused kidney, hindlimb and mesenteric vasculature, defined as the altered maximal change in perfusion pressure to an agonist (Folkow et al, 1971, Triggle and Laher, 1985). The enhanced response occurs to a variety of agonists in the whole vascular preparations and is suggestive of altered vascular structure or a more effective coupling between receptor stimulation and vascular smooth muscle cell (VSMC) contraction. This may be related to a number of different factors, such contractile apparatus function or physical aspects of vessel design.

In vivo studies show discrepant results with regard to vascular responses to agonists, with authors reporting no change, decreased and increased systemic vascular responsiveness (as measured by changes in total peripheral resistance) to agonists in the SHR, even in cases where reflex blood pressure control pathways are blocked (Touw et al, 1980, Walsh, 1983, Leenen et al, 1994). Compensatory reflexes, differing responses of vascular beds to agonists, selective uptake of various agonists (and differences in innervation density) can all lead to altered responses to vasoactive agents. There are also age differences with respect to vascular responsiveness as has been shown *in vitro* (Adams et al, 1989) and *in vivo* (Leenen et al, 1994).

Studies utilising aortic or arterial segments often show differing contraction profiles to agonists when compared to whole vascular preparations. Responses are attenuated on a force per unit weight of tissue basis to noradrenaline (Dyer et al, 1992, Arvola et al, 1993) and potassium chloride (Arvola et al, 1993). Often, responses are greater or identical in SHR vessels until normalised to vessel weight, whereupon the differences are diminished. Whole vascular preparations are considered more pertinent to vascular function *in vivo* in that responses are modulated by the reaction to such physical forces as shear stress arising from changes in perfusion pressure.

Chronic captopril treatment had a marked influence on 3MH expression in the mesenteric vasculature of the SHR. If this reflects expression of vascular actin, then there is a likelihood that vascular reactivity may be similarly affected. The diminished capacity of the contractile apparatus would be expected to result in an attenuation of responses to a wider range of vasoactive agents, including nonspecific agents such as potassium ions and calcium ionophores.

Impairment of vascular contraction in response to α-adrenoceptor agonists after long-term ACE-inhibition has been noted previously in mesenteric vessels (Freslon and Giudicelli, 1983), and the perfused hindlimb vascular bed (Adams et al, 1990). Moreover, there was also a suppression in the redevelopment of enhanced vasoactivity after drug withdrawal, with vascular reactivity following the subsequent change in blood pressure (Adams et al, 1990).

This chapter describes the measurement of vascular reactivity in the isolated perfused mesenteric vascular bed of SHR and WKY rats under three conditions: (i) untreated, (ii) treated long-term with captopril and (iii) withdrawn from chronic treatment with captopril. Furthermore, corresponding 3MH measurements allow an investigation into the relationship between actin content, vascular reactivity and blood pressure in SHR and WKY rats and the effects of chronic captopril treatment and its withdrawal on these parameters.

4.2 Methods

4.2.1 Animals and drug treatments

Male 4 week-old SHR and WKY rats were maintained and administered captopril as previously described (*2.1.1, 2.1.2*), except that the treatment period was from 5 to 17 weeks of age in captopril-treated SHR (SCAP) and WKY rats (WCAP), and from 5 to 13 weeks of age in captopril-withdrawn ("released") SHR (SCAPREL) and WKY rats (WCAPREL). Monthly systolic blood pressure measurements were performed as described previously by S. Dyer (*3.2.1*), with some of this blood pressure data appearing in a previous publication (Dyer et al, 1994).

4.2.2 The perfused mesenteric vascular bed (PMVB)

At the end of all treatments rats were killed by stunning and decapitation. The PMVB was prepared as previously described (*2.3*). The responses to intraluminally injected noradrenaline (0.010-20 ug in ascorbic saline (9 g/l NaCl, 0.1 g/l ascorbic acid)) and potassium chloride (1-32 mg) were then measured. Responses to nerve stimulation in this preparation were also measured. This entailed electrical stimulation of the perivascular sympathetic nerves which supply the blood vessels of the mesenteric preparation. Frequency-response curves were obtained using a Grass model S9 stimulator producing pulses of 1 ms duration from 2 platinum electrodes at supramaximal voltage. The electrodes were positioned proximally (base of superior mesenteric artery) and distally (into the tissue fascia) to the cannula entry. Stimulation occurred over the frequency range 2-64 Hz for 15s duration every 4 mins. Injection volume for drugs did not exceed 0.1 ml. Responses were measured as changes in perfusion pressure. Dose and frequency-response curves were constructed in a noncumulative manner.

4.2.3 Tissue preparation

After responses of the PMVB were completed, the preparations were removed and the complete vascular bed dissected free from the intestinal tract and any attached adipose tissue, so that only blood vessels remained. These included the superior mesenteric arteries down to the smaller third order branching vessels. The vasculature was then rapidly frozen in liquid nitrogen and stored at -80°C prior to biochemical determinations.

4.2.4 3-Methylhistidine analysis

3-methylhistidine measurements were performed as previously described (*3.2.2*), except that only mesenteric vascular tissue was assayed.

4.3 Results

4.3.1 Blood pressure

As observed previously, chronic captopril treatment completely retarded the development of hypertension in the SHR (Fig. 4.1 (a)), and prevented any increase in SBP with age in the WKY rat (Fig. 4.1 (b)). Withdrawal from treatment at 13 weeks of age resulted in a sharp rise in SBP in the SHR to 150 mmHg and a return of SBP in the WKY rat to levels of the WCON group at 17 weeks of age. At the end of the treatment/release period, however, the SCAPREL group exhibited significantly lower SBP when compared to the SCON group.

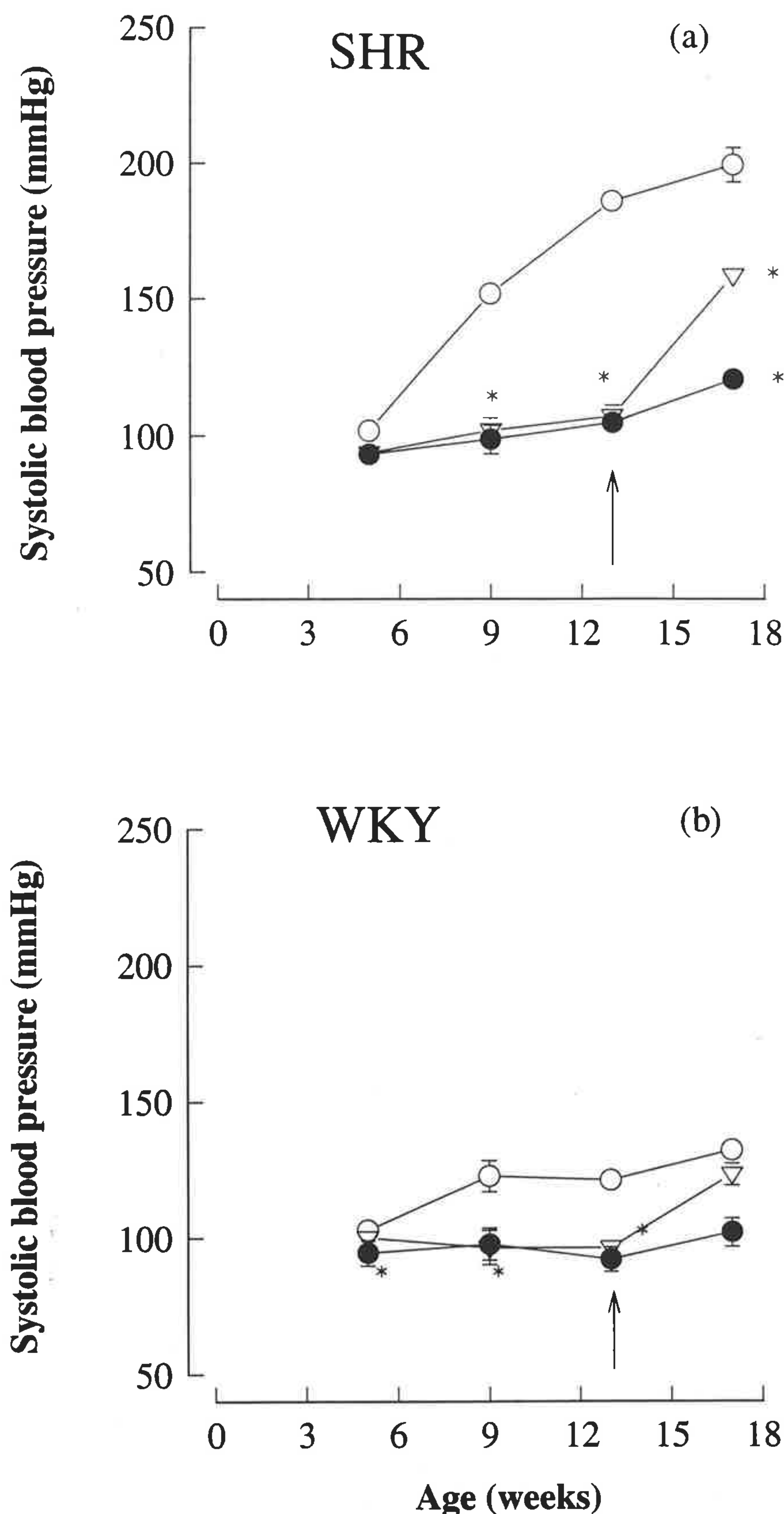

Fig 4.1. Changes in systolic blood pressure with age in untreated (control; ○), chronic captopril-treated (●) and captopril-withdrawn ("released" ; ▽) SHR (a) and WKY (b) rats. Blood pressure was measured using an indirect tail-cuff technique. Captopril (100 mg/kg/day) was administered in drinking water. Arrow denotes time of release in captopril-released SHR.
*significant ($P<0.05$) difference vs. control SHR (n=6-7 per group).

4.3.2 Vascular reactivity

Nerve stimulation

The PMVB of the untreated SHR (SCON) showed an enhanced response to nerve stimulation when compared to the control WKY (WCON) preparation (Fig. 4.2 (a)), significantly so at smaller frequencies (2-8 Hz) and at the highest frequency (64 Hz). Chronic captopril treatment exerted little influence on the frequency response curve of the WKY rat, as did 4 weeks withdrawal from captopril (Fig. 4.2 (c)). The SCAP group showed no significant attenuation in its frequency-response curve when compared to the SCON group (Fig. 4.2 (b)). Interestingly, after captopril withdrawal, vascular reactivity appeared greater than that displayed in the SCON group, although this was not significantly different.

Potassium chloride

Responses to potassium chloride were not different between the SCON and WCON groups, in terms of both slope and maximal responses, both generating approximately 200 mmHg (Fig. 4.3 (a)). In both the SCAP and WCAP groups chronic captopril treatment suppressed vascular responses to potassium chloride, in a single instance by 50% (Fig. 4.3 (b), 16 mg KCl). However large variations precluded statistical significance in this set of responses. Reactivity was mostly restored in the SCAPREL group and partially in the WCAPREL group (Fig. 4.3 (b) and (c)).

Noradrenaline

The untreated SHR (SCON) PMVB displayed greater vascular reactivity than the WCON group preparations over the upper dose range of noradrenaline (Fig. 4.4 (a)), although there were no statistically significant differences over the full dose range. Chronic captopril treatment imparted dramatic and significant reductions in vascular reactivity in the SCON group, resulting in reactivity of approximately 30% of the

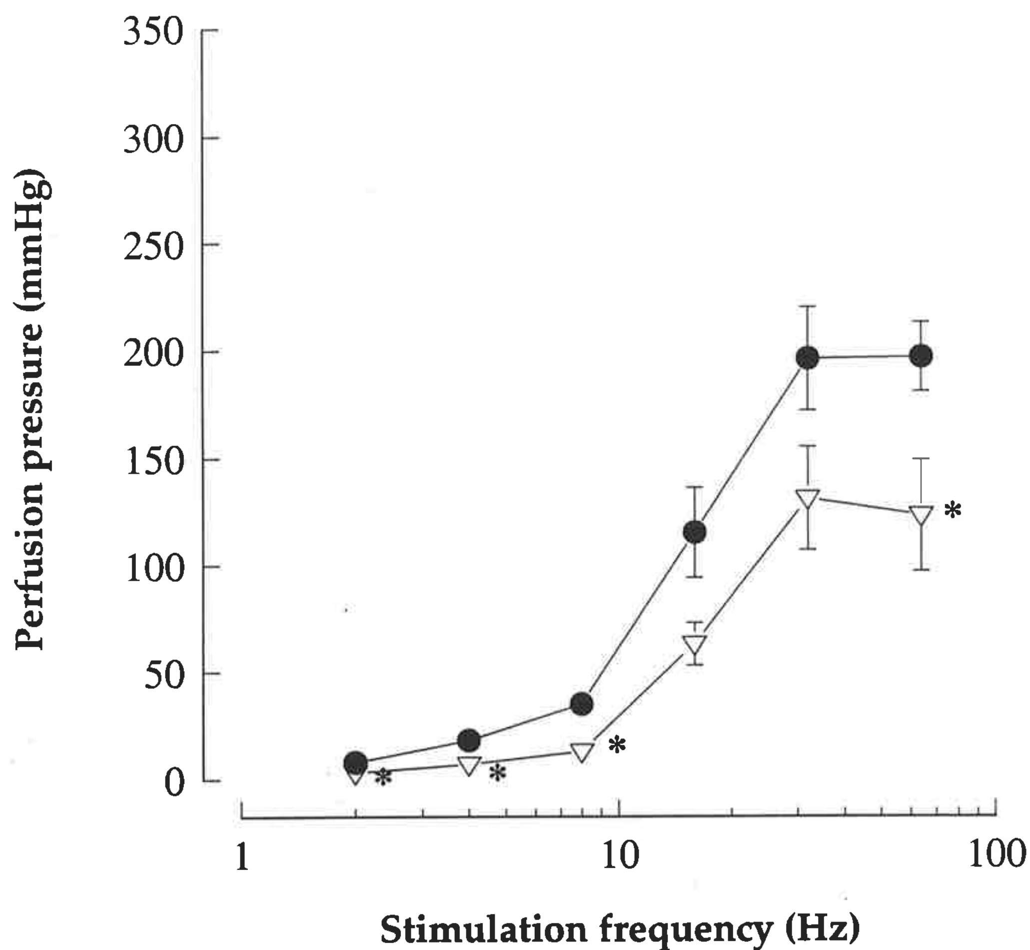

Fig 4.2 (a). Changes in perfusion pressure of the isolated perfused mesenteric vascular bed to perivascular sympathetic nerve stimulation (2-64 Hz) in untreated (control) SHR (●) and WKY (▽) rats (n=5-7 per group).

*significant (P<0.05) difference vs. control SHR.

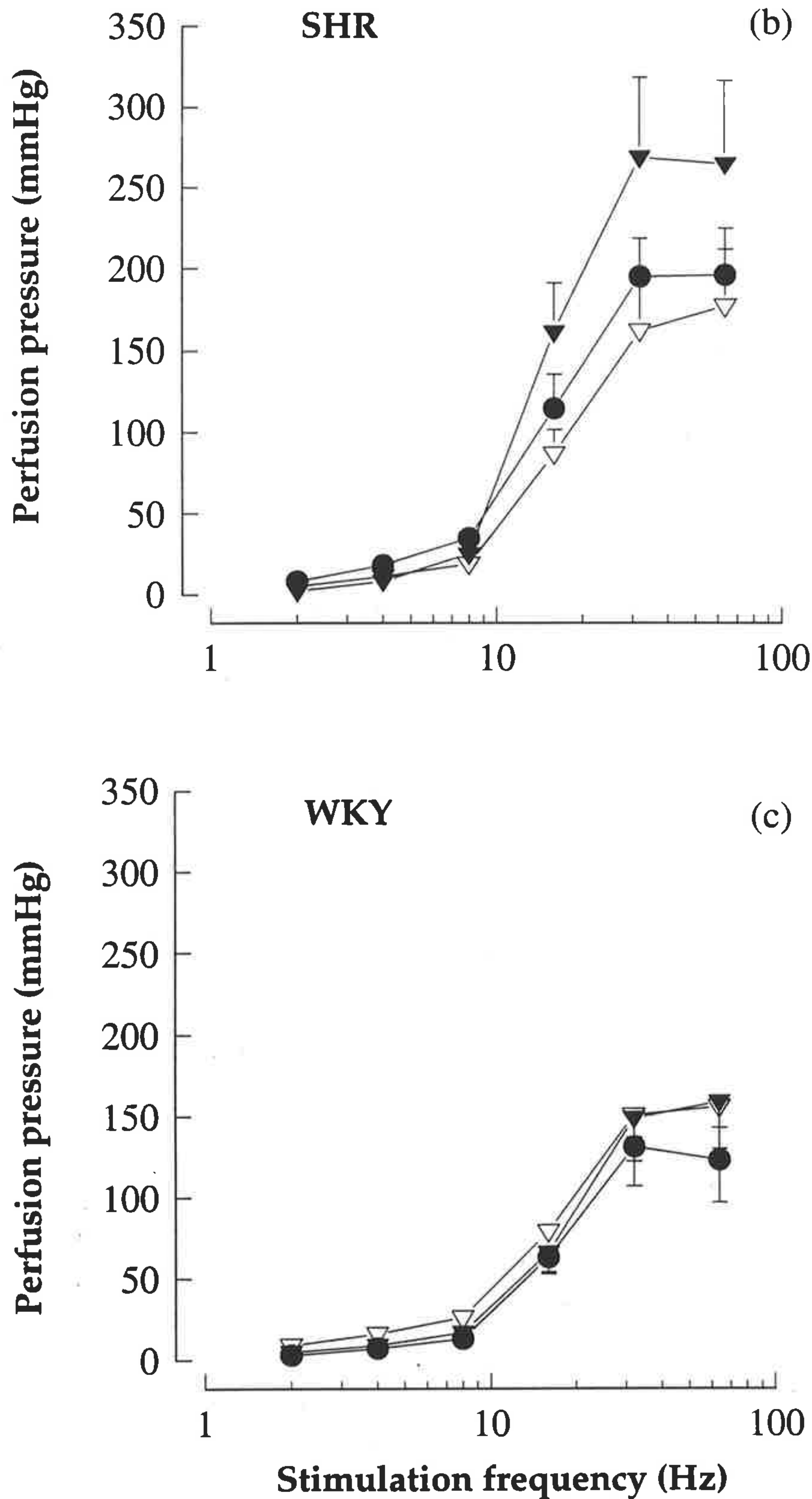

Fig 4.2. Changes in perfusion pressure of the isolated perfused mesenteric vascular bed to perivascular sympathetic nerve stimulation (2-64 Hz) in control (untreated; ●), chronic captopril-treated (▽) and captopril-withdrawn ("released"; ▼) SHR (b) and WKY (c) rats. N=6-7 per group.

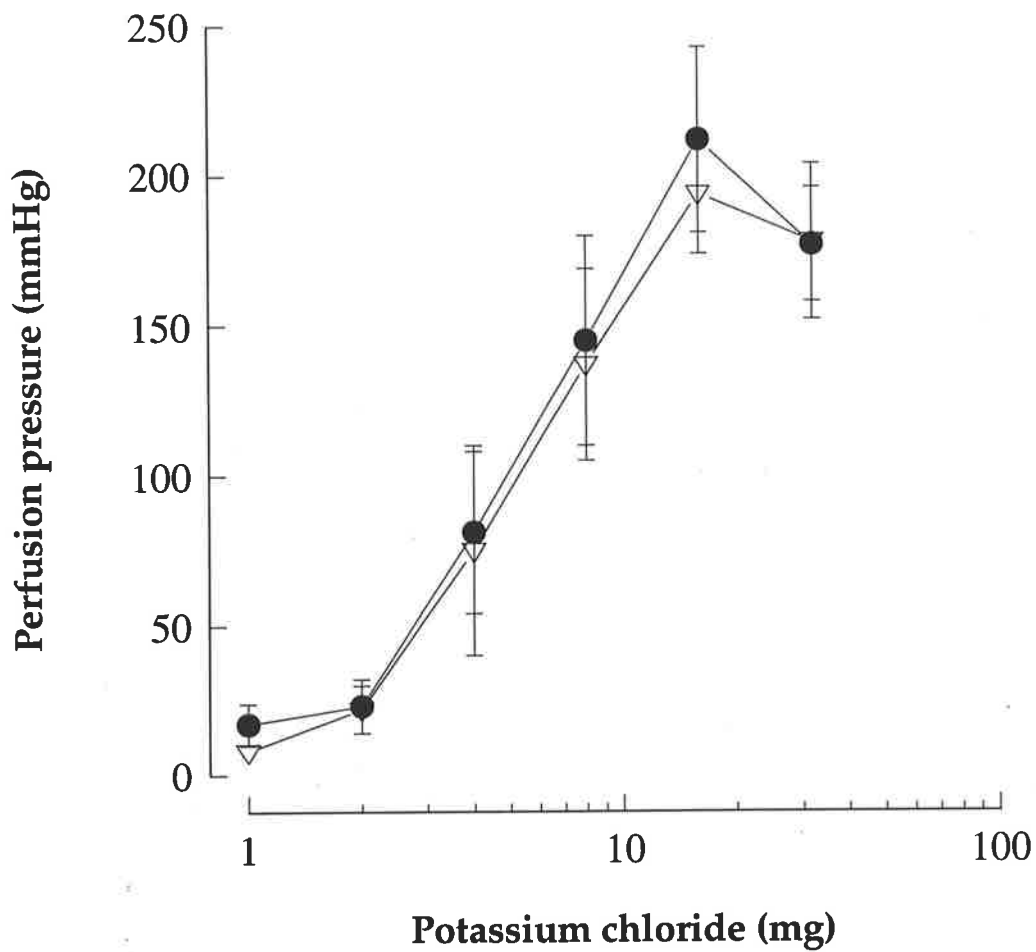

Fig 4.3 (a). Changes in perfusion pressure of the isolated perfused mesenteric vascular bed to potassium chloride infusion (1-32 mg) in untreated (control) SHR (●) and WKY (▽) rats. N=5-7 per group.

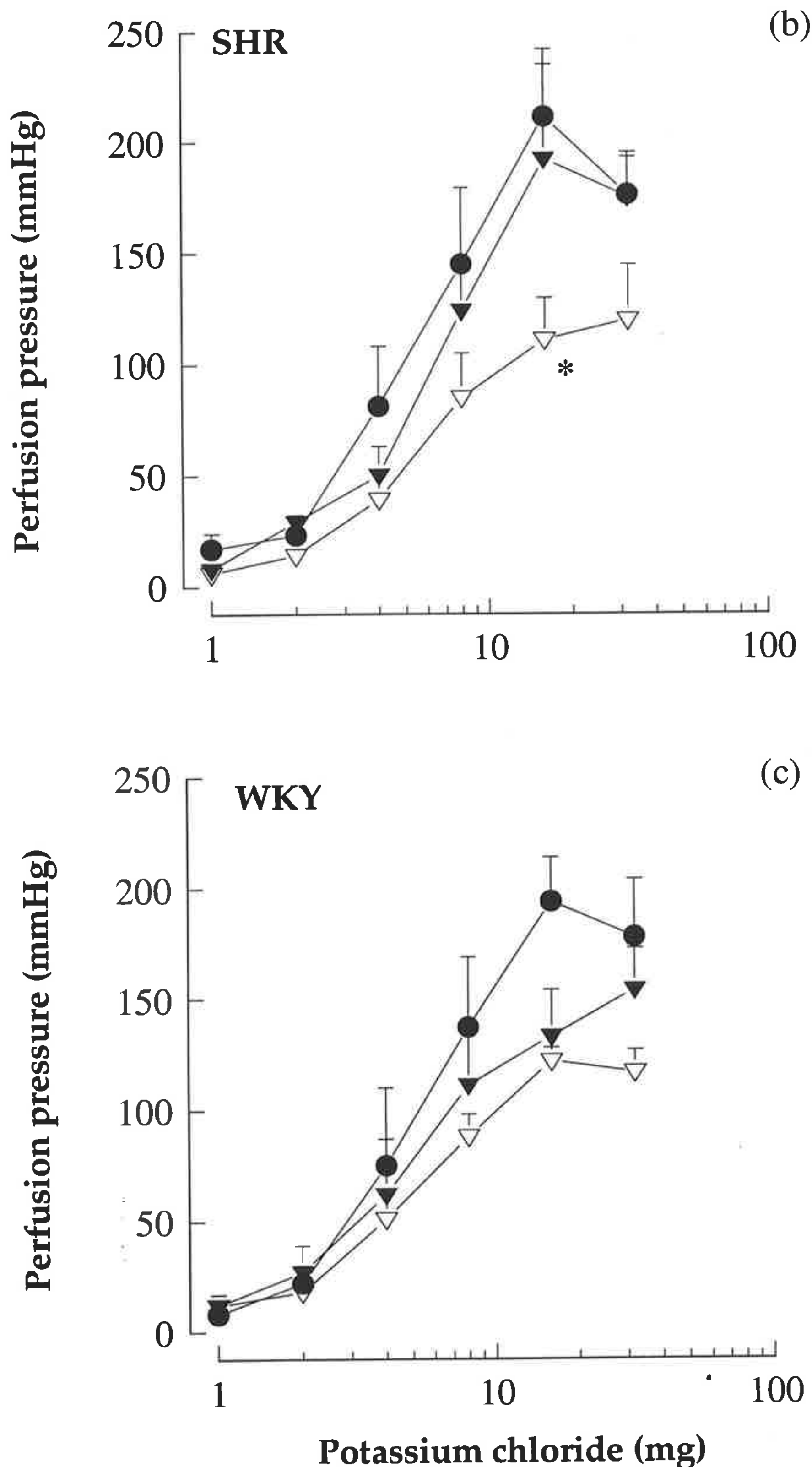

Fig 4.3. Changes in perfusion pressure of the isolated perfused mesenteric vascular bed to potassium chloride infusion (1-32 mg) in untreated (control; ●), captopril-treated (▽) and captopril-withdrawn ("released"; ▼) SHR (b) and WKY (c) rats. N=5-7 per group.

* significant (P<0.05) difference vs. control SHR.

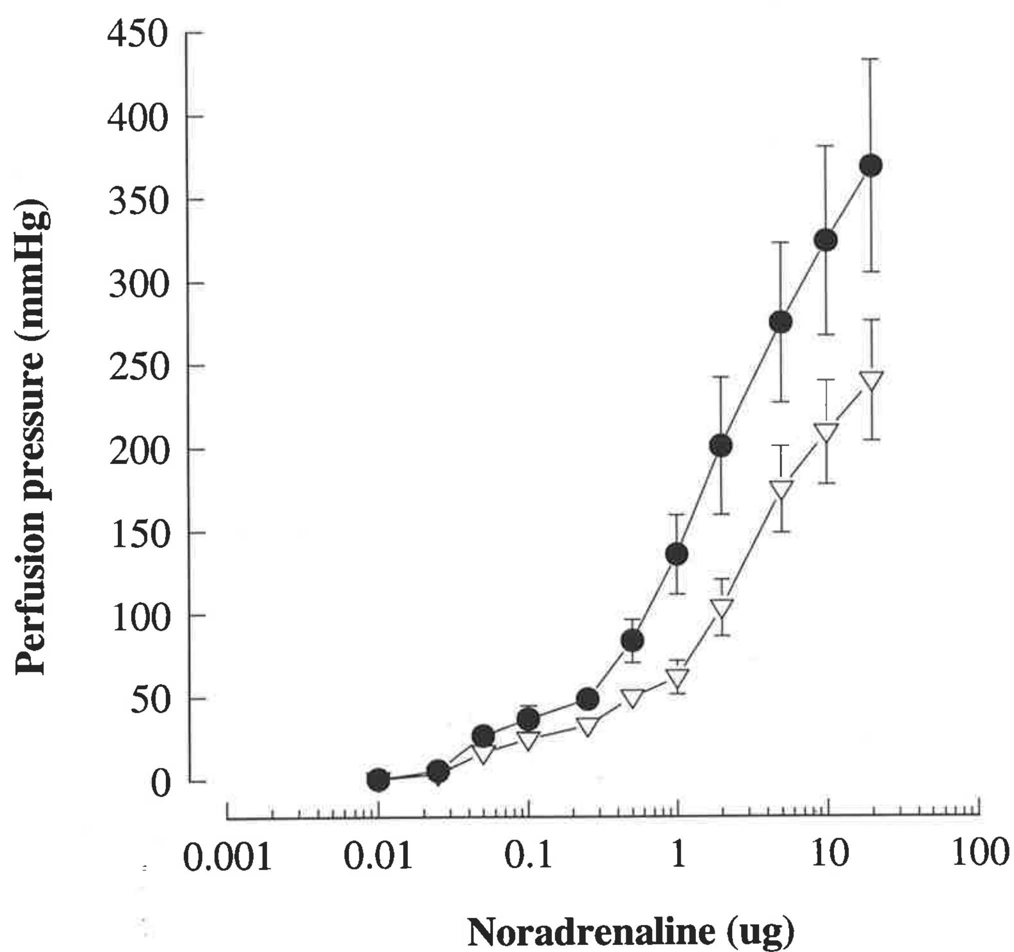

Fig 4.4 (a). Changes in perfusion pressures of the isolated perfused mesenteric vascular bed to exogenous noradrenaline infusion (0.01 to 20 ug) in untreated (control) SHR (●) and WKY (▽) rats (n=5-7 per group).

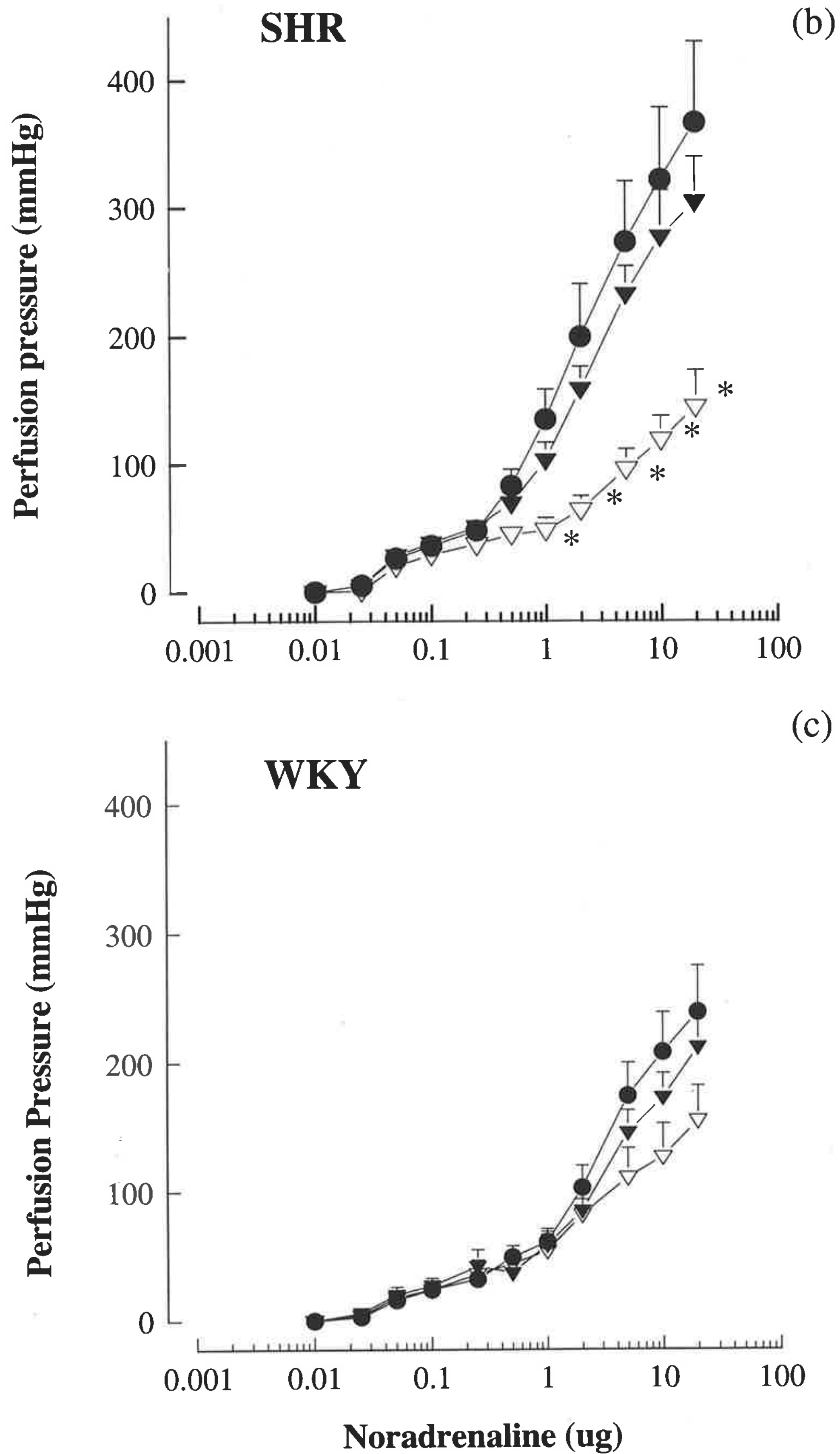

Fig 4.4. Changes in perfusion pressure of the isolated perfused mesenteric vascular bed to exogenous noradrenaline infusion (0.01 to 20 ug) in untreated (control; ●), captopril-treated (▽) and captopril-withdrawn ("released"; ▼) SHR (b) and WKY (c) rats. N=5-7 per group. * significant ($P<0.05$) difference vs. control SHR.

SCON group in the upper dose range (Fig. 4.4 (b)). Captopril treatment had a modest effect on noradrenaline responses in the WKY rat (Fig. 4.4 (c)). Interestingly, withdrawal from captopril treatment caused a return in the reactivity to NA in the SCAPREL group to levels that were modestly attenuated in comparison to untreated (control) SHR (maximal 305 mmHg vs. 370 mmHg, 20 ug NA).

4.3.3 Vascular 3-methylhistidine content

The 3MH content of vascular tissue from the mesenteric bed in all treatment/release groups and their controls is outlined in Figs. 4.5 (a) and (b). There was no difference between SHR and WKY rats with respect to 3MH levels in this tissue. However, chronic ACE-inhibition elicited large falls in mesenteric 3MH content in both SHR and WKY rats (significantly so in the SHR). Withdrawal from treatment was associated with a rebound in 3MH content in the SCAPREL group, but not in the WCAPREL group.

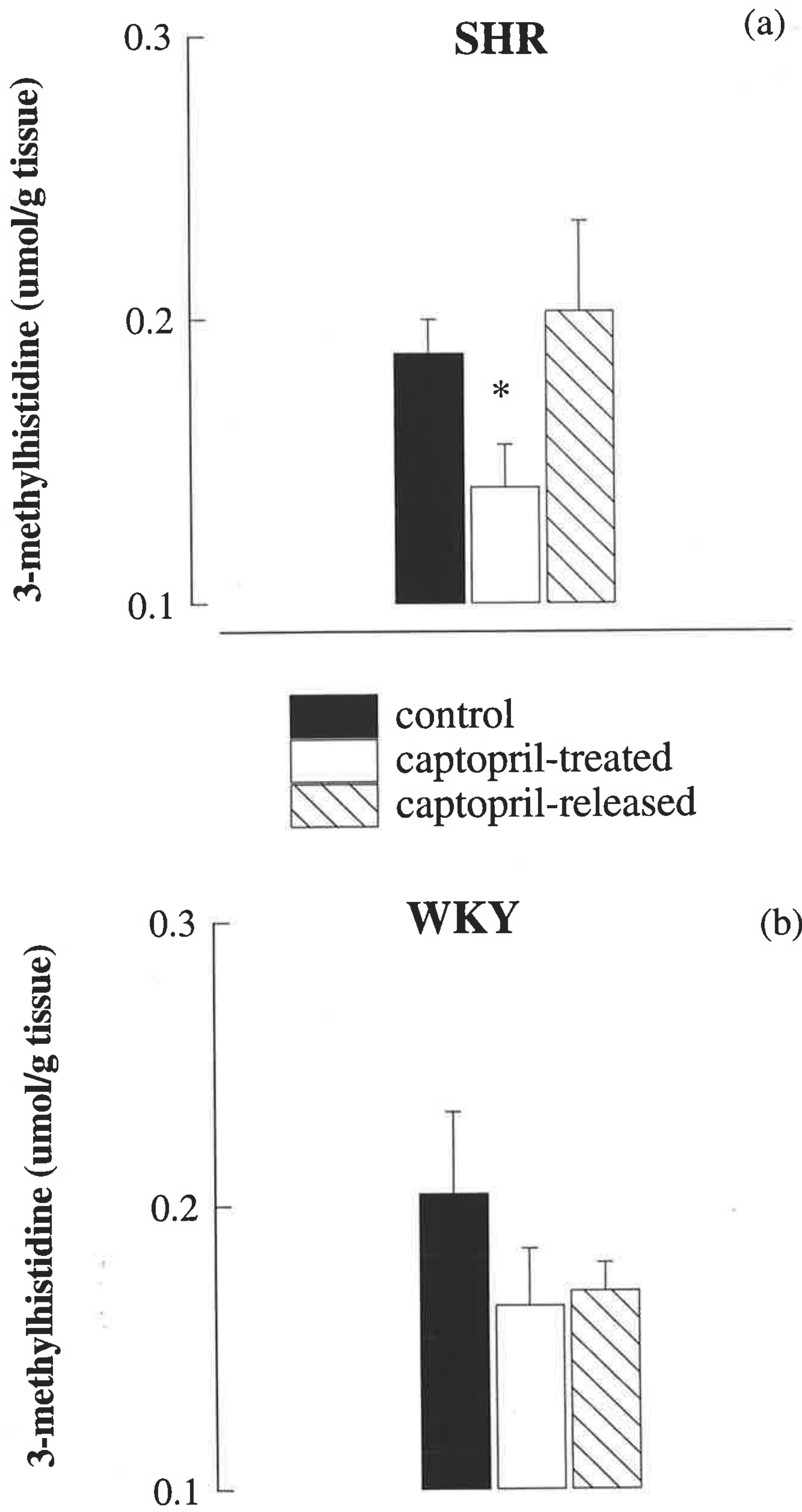

Fig 4.5. 3-methylhistidine (3MH) concentrations of mesenteric vascular tissue from untreated (control), chronic captopril -treated and captopril-withdrawn ("released") SHR (a) and WKY (b) rats (n=5-7 per group).
* significant ($P<0.05$) difference vs. control SHR.

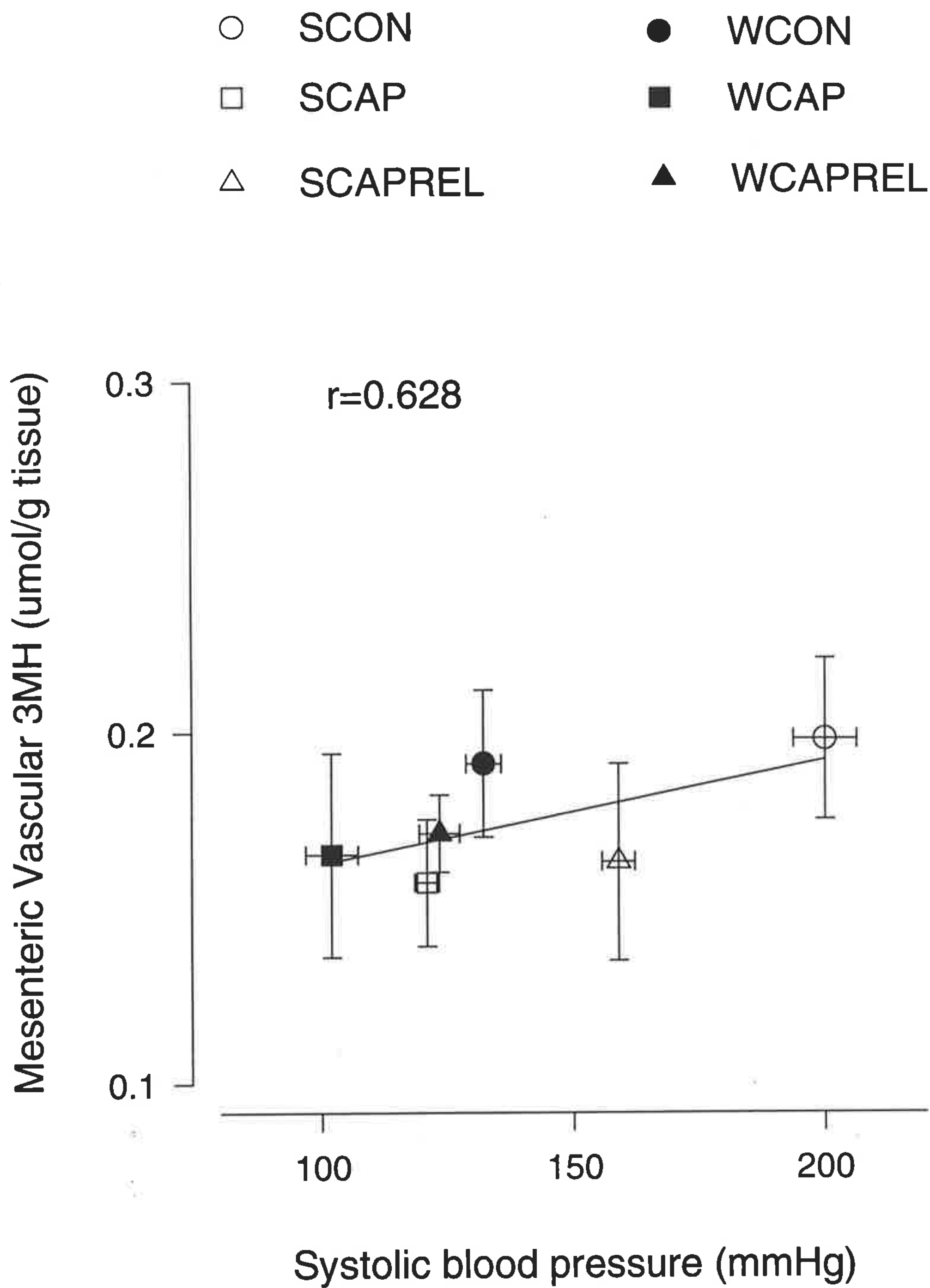

Fig 4.6. Correlation analysis of systolic blood pressure and mesenteric vascular 3MH from all treatment/release groups and their controls (n=5-7 per group). S=SHR; W=WKY; CON=control; CAP=captopril; CAPREL=captopril released.

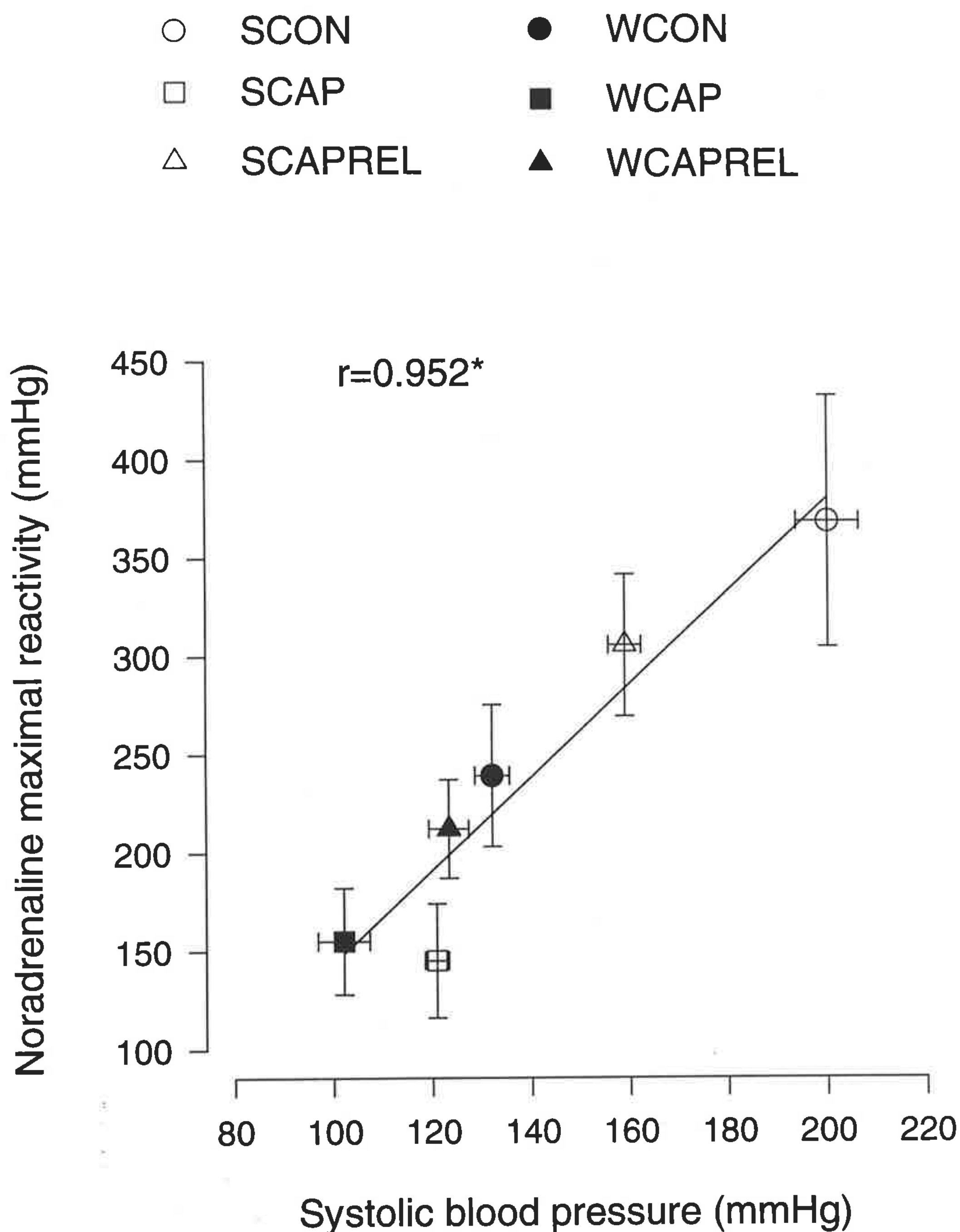

Fig 4.7. Correlation analysis of systolic blood pressure and maximal noradrenaline reactivity of the perfused mesenteric vascular bed from all treatment/release groups and their controls (n=5-7 per group). See Fig. 4.6 for legend explanations.

* significant (P<0.05) correlation.

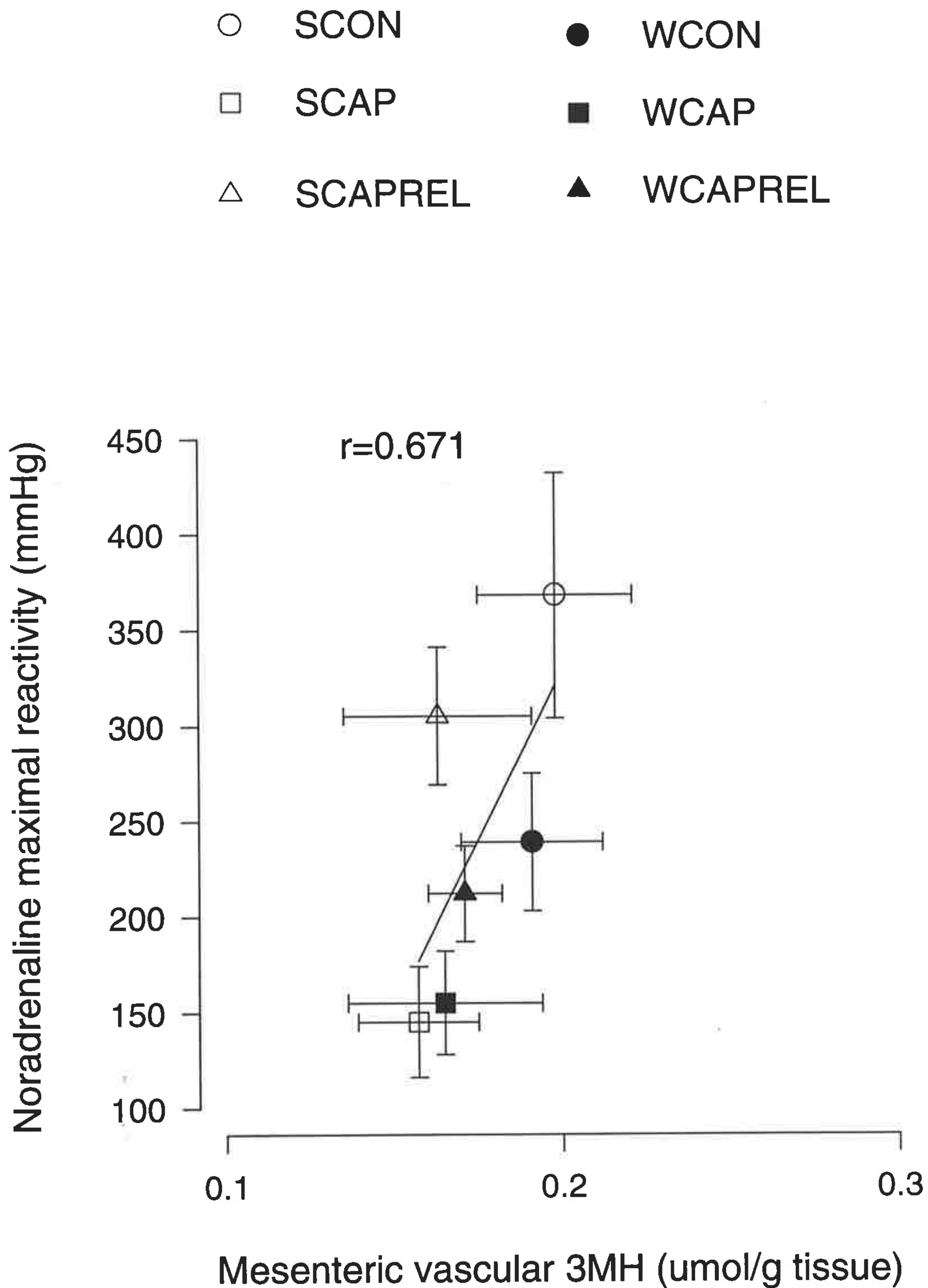

Fig 4.8. Correlation analysis of mesenteric vascular 3MH and maximal noradrenaline reactivity of the perfused mesenteric vascular bed from all treatment/release groups and their controls (n=5-7 per group). See Fig. 4.6 for legend explanations.

4.4 Discussion

The studies described in this chapter investigated the relationship between changes in systolic blood pressure (SBP), altered vascular 3MH concentrations and function (vascular reactivity), and the effects of chronic ACE-inhibition and its withdrawal on these indices in SHR and WKY rats.

4.4.1 Blood Pressure

The SBP profiles from all treatment groups and controls parallelled those seen earlier (Chapter 3), except that the partial redevelopment of elevated blood pressure after drug withdrawal occurred more quickly than was observed previously. A lifespan follow-up of SHR treated from 6 to 10 weeks of age with the ACE inhibitor perindopril showed a continued lower blood pressure until up to 80 weeks of age (Harrap et al, 1994), suggesting a sustained effect of ACE-inhibition on SBP after withdrawal.

4.4.2 Vascular Reactivity

Nerve stimulation

Responses to nerve stimulation were enhanced in control SHR when compared with control WKY rat preparations, indicating that the SHR preparation is capable of a greater response to any given nerve stimulus. This result has been shown previously (Longhurst et al, 1986). There was no significant difference in vascular responsiveness to nerve stimulation in SCON, SCAP and SCAPREL groups, although there was a tendency for responses of the SCAPREL group to be greater than that for the SCON group. The reason for this is unclear. Maximal nerve-stimulated reactivity in the SCON group was, however, only approximately 50% of the responses observed to exogenous noradrenaline (NA) administration. As this tissue may never be exposed to physiological pressures above 200 mmHg, the level of neuroeffector coupling producing

the responses may be sufficient for its function *in vivo*. However, differences in reactivity to exogenous noradrenaline were observed between SCAP and SCON groups at perfusion pressures as low as 50 mmHg. Why differences should become apparent at this perfusion pressure in NA (but not nerve) mediated responses is unclear. The discrepancy may be explained by an attempted increase in sympathetic neural activity, which may occur to offset the actions of ACE-inhibition related to AII's influence at the nerve (sympathofacilitation; Lewis and Coote, 1993) and the muscle (amplification of α-adrenoceptor agonist response; Purdy and Weber, 1988). For example, a downregulation of the presynaptic α_2-adrenoceptor-mediated inhibition of noradrenaline release from the nerve terminal may be occurring. Chronic enalapril treatment of developing SHRs has been shown to be ineffective in altering the density of postsynaptic renal α_2-adrenoceptors, but presynaptic postganglionic α_2-adrenoceptor changes were not evaluated (Gong et al, 1994).

Potassium chloride

In contrast to the differences in responsiveness to NA in the PMVB from SHR and WKY rats, there were no differences in response to KCl. Responses to potassium ions have been used as indicators of alterations in vascular structure, but a contributing factor may also be membrane properties (i.e., permeability, potassium and calcium channel affinity and number) that may influence the movement of calcium ions into the cell cytosol. Nevertheless, the similarity in reactivity between strains may suggest that, if there were alterations in structure or membrane ion permeability in SHR mesenteric vessels, they were of little consequence in terms of the potassium-induced maximal pressor responses. Previous studies utilising potassium chloride induced contractions of the isolated PMVB have disclosed enhanced reactivity of SHR preparations when compared with those of the WKY rat (Longhurst et al, 1986, Inoue et al, 1990, Criscione et al, 1990, Major et al, 1993). This is believed to be either an indirect effect of structural modifications or an effect of altered cell membrane ion permeability, a feature well documented in vessels from the SHR (Sada et al, 1989). Smeda et al (1988) reported no differences in vascular reactivity of the isolated perfused kidney

preparation to potassium chloride between 21 week-old SHR and WKY rats, despite observing increased responses to other agonists. Similarly, others have found that $BaCl_2$ and angiotensin II responses are equivalent between strains (Collis and Vanhoutte, 1977).

Chronic captopril treatment reduced the contraction profile of both SHR and WKY preparations to KCl, indicating that the ACE-inhibitor may have reduced plasmalemmal or sarcoplasmic potassium and/or calcium movement, and/or vascular structural parameters. This feature of ACE-inhibition has been observed after chronic administration (months), but not after 7 day or "in the bath" quinapril treatment (Sada et al, 1989, Major et al, 1993). The time course of the ACE-inhibitor's effects warrants further investigation, and will be discussed in the next chapter.

Vascular responses to KCl returned to control levels after withdrawal from captopril treatment. It is possible that those factors modifying the responses to K^+ ions are rapidly lost after treatment cessation. In a similar study Traub and Webb (1993) addressed the question of altered vascular ion channel function in the SHR after withdrawal from the ACE inhibitor ramipril. They found that the responses of aortic strips to calcium channel activation remained suppressed along with hypertension redevelopment 7 weeks after cessation of ramipril treatment from 6 to 10 weeks of age (Traub and Webb, 1993). Unfortunately these responses were not measured relative to tissue weight or length, and it is possible that differences may have been diminished if responses were adjusted. These responses were also measured in a large conduit (and not resistance) vessel, where calcium requirements may be different.

Noradrenaline

The SHR has a greater vascular reactive capacity to NA than the WKY rat, a feature well documented in the mesenteric vasculature (Longhurst et al, 1986, Inoue et al, 1990, Major et al, 1993). This may be an influence of vascular structural changes, but as KCl responses and mesenteric vascular 3MH were not altered when compared to the WKY rat, it suggests that the enhancement may not solely be due to this aspect of vessel design. The increased responsiveness to specific receptor agonists (eg., at α-

adrenoceptors) but not cations (eg., K^+, Ba^{++}) has been observed previously (Collis and Vanhoutte, 1977, Smeda et al, 1988). Moreover, Inoue et al (1990) reported a selective increase in α-adrenoceptor-mediated vasoconstriction in younger SHR. In adult animals, the increased reactivity occurs to many different agonists (α-adrenergic, nerve stimulation, KCl, 5HT, phorbol ester and endothelin), suggestive of a common pathway downstream from the receptors mediating the exaggerated responses (Medgett et al, 1984, Triggle and Laher, 1985, Longhurst et al, 1986, Criscione et al, 1990, Major et al, 1993). This could lie in areas related to calcium mobilisation and utilisation, contractile protein content and function, cellular and extracellular organisation and gross vessel structure.

Captopril treatment caused a marked suppression in NA responsiveness in the PMVB of the SHR, resulting in a suppression to levels beyond that of the control WKY rat preparation. This attenuation in vascular reactivity has been observed previously (Freslon and Giudicelli, 1983, Adams et al, 1990). It is not exclusive to captopril treatment because chronic enalapril treatment also has a similar effect (Adams et al, 1990), indicating that the effect is due to the impairment of ACE activity. Together with the decreased KCl responses and lower mesenteric vascular 3MH levels, these findings support a contribution of altered actin content or disposition towards captopril's effects on vascular function. The correlation analysis between maximal NA reactivity and SBP indicates that, for a given reduction in SBP, captopril-treatment has caused a greater fall in vascular reactivity than would be predicted from the regression line (Fig. 4.7). This suggests that captopril is exerting effects not simply related to the change in SBP. The additional effect may be related to the direct influence of captopril on vascular neuroeffector function (discussed in Chapter 5).

Withdrawal from captopril treatment in the SHR resulted in a return of the vascular reactive profile to NA to levels slightly less than that exhibited by the PMVB preparations of untreated SHR and similar to the KCl responses, suggesting a discrete reversibility of this ACE-dependent process. This pattern is similar to that observed in an earlier study using enalapril, where vascular reactivity to methoxamine in the perfused hindlimb preparation closely matched the change in SBP after withdrawal from chronic treatment (Adams et al, 1990). Correlation analysis of maximal vascular

reactivity to NA and SBP supports the findings of Adams et al, (1990), the value lying close to the regression line (Fig. 4.7).

4.4.3 Vascular 3-methylhistidine content

The lack of disparity in mesenteric 3MH values between strains contrasts to the findings of the previous study. In this regard, it appears that vascular bed 3MH differences between the SHR and WKY rat are not consistently observed, and further work is required to clarify 3MH expression and actin concentration within resistance vessels.

The most consistent finding thus far is the effect of chronic captopril treatment on vascular 3MH content. Similarly to the results in Chapter 3, 3MH levels were reduced in the SHR after chronic ACE-inhibition in the present study. Levels in the WKY rat were also lower, and SBP and vascular reactivity were also affected in the normotensive strain. This indicates that ACE activity is an important component for the development of "normal" blood pressure, vascular actin and function. Chronic ACE-inhibition has been shown to retard the development of high blood pressure and increased left ventricular mass in the WKY rat (Harrap et al, 1986, King et al, 1992), and the reduction in 3MH is consistent with the ability of ACE-inhibitors to reduce cellular actin levels in cultured WKY rat VSMCs (Uehara et al, 1993).

The fall in 3MH concentrations in the SHR after chronic captopril treatment was associated with a decrease in vascular reactivity and the prevention of hypertension development. This suggests that the SHR is exquisitely sensitive to the impairment of ACE activity with regard to the expression of vascular contractile protein. As therapy was not compared with a nonRAS-acting antihypertensive that is effective in this model, elucidating an effect distinct from blood pressure changes is not possible. A comparison of these parameters after chronic treatment with the vasodilator hydralazine has been performed in Chapter 5.

The sensitivity of vascular actin to ACE-inhibition can be explained by the regulatory role of AII on the expression of the protein, particularly the α-isoform. AII stimulates α-actin mRNA production in cultured rat and human VSMCs (Turla et al,

1991, Andrawis et al, 1993). In the SHR, increases in large vessel α-actin mRNA are present in 17 week-old SHR (Le Jemtel et al, 1993), but the prevalence or disposition of protein in resistance vessels is not known with certainty. However, it is plausible to suggest that the effects of captopril treatment on vascular 3MH are manifest through the suppression of AII's influence on cell growth and proliferation, and perhaps a more selective effect on actin synthesis and/or turnover. The angiotensin II (AT_1) receptor antagonist losartan also decreases markers of VSMC hypertrophy (Lyall et al, 1992), supporting the concept that this is an AII-dependent process.

Similar to results observed in Chapter 3, mesenteric vascular 3MH content increased to control levels after withdrawal from captopril treatment, indicating a distinct reversibility of this ACE-dependent process. This consistent finding supports previous evidence of a dissociation between the level or disposition of actin and SBP after withdrawal from chronic captopril treatment.

4.4.4 3-Methylhistidine, vascular reactivity and blood pressure

The present study has investigated the relationship between SBP development with respect to markers of vascular function and contractile protein under a number of conditions: (i) genetic hypertension and normotension, (ii) chronic antihypertensive treatment in hypertension and normotension, and (iii) long-term withdrawal from chronic antihypertensive treatment in hypertension and normotension.

Vascular reactivity to NA correlates extremely well with the prevailing SBP in both SHR and WKY rats (Fig 4.7). This analysis also highlights the sensitivity of the SHR vasculature to chronic ACE-inhibition, because reactivity fell in greater proportion to that expected from its blood pressure lowering effects. Responses of captopril-treated WKY rats lay close to the regression line, emphasising the exaggerated decrease in vascular reactivity of the PMVB after ACE-inhibition in the SHR. It also highlights how the complex relationship between two such variables can be unmasked after specific pharmacological intervention. While maximal vascular reactivity to NA correlates well with SBP after cessation from captopril therapy, the overall dose-response profile is closely associated with that of the untreated (control) SHR,

suggestive that vascular reactivity has been restored to levels exhibited by untreated SHR.

The relationship between 3MH and vascular reactivity is similar, indicating that vascular actin does not follow changes in vascular reactivity as simplistically as predicted (Fig 4.8). There are many other parameters that determine the contractility of peripheral vascular beds, acting at various organisational levels within the vessel (subcellular, cellular, extracellular, organ). For example, gross structural changes (eg., remodelling) could render a vessel more reactive without altering levels of contractile protein.

Similarly, the relationship between SBP and 3MH remains obscure, indicating that the factors regulating vascular actin expression are not related to the prevailing SBP in a straightforward manner. Once again, changes in vessel remodelling could increase vessel resistance and thus TPR without necessarily altering contractile protein content.

It is likely that a combination of biochemical, functional and structural features determine the role of the vascular bed in the control of TPR in the adult SHR, and that these are determined in the developing phase of hypertension in this model. The results highlight an effect of chronic captopril treatment on indices of vascular function and actin that may play a pivotal role in the drug's mechanism of action.

CHAPTER 5

The Effects of Acute and Chronic Antihypertensive Treatments and their Withdrawal on Blood Pressure and Cardiovascular Structural, Functional and Biochemical Properties in the Developing SHR

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5.1 Introduction

Angiotensin II can modify vascular neuroeffector function by facilitating NA release, impairing its reuptake from the nerve terminal *in vitro* (Starke, 1977) and augmenting catecholamine release from the adrenal medulla (MaClean and Ungar, 1986). It is plausible to suggest that enhanced local RAS expression may make a contribution to the increased sympathetic nerve activity at the vascular neuroeffector junction in the SHR.

AII can sensitise cardiac and VSMCs to the effects of vasoactive agents, typically occurring at subhaemodynamic concentrations of AII and not related to the effects of impairing catecholamine reuptake (Ziogas and Story, 1987, Purdy and Weber, 1988). The responses to α_1-adrenoceptor stimulation are particularly enhanced, although there are also reports to suggest that α_2-adrenoceptors can mediate vasoconstrictor responses to a greater extent in the SHR, and that AII can increase postjunctional α_2-adrenoceptor-mediated reactivity to agonists *in vitro* (Medgett et al, 1984, Ikeoka and Faber, 1993). There is accumulating data to suggest that ACE-inhibitors may owe a part of their sympathoinhibitory action to a suppression of responses mediated by postsynaptic α-adrenoceptors, as AII directly facilitates the response to exogenous noradrenaline in vascular tissue (Purdy and Weber, 1988), and the effectiveness of α-adrenoceptor antagonists has been shown to be dependent on the level of activation of the RAS (Marwood et al, 1992).

The role of AII-mediated facilitation *in vivo* is not as clearly defined, as the pressor response to nerve stimulation or exogenous noradrenaline may be modulated by the extent of change in vascular tone and altered vasodilator prostaglandin release (Grant and McGrath, 1988, Ogiku et al, 1993). Others also note the influence of enhanced bradykinin and prostaglandin levels after ACE-inhibition that may act to offset the agent's ability to enhance noradrenaline release from the sympathetic nerve (Schwieler et al, 1993). The interpretation of the *in vivo* results are clearly complicated by a number of haemodynamic and neurohumoral modifiers.

Another factor impeding the elucidation of the mechanism of action of the ACE-inhibitors in various settings is the existence of other substrates of the converting enzyme. The other commonly known substrate of ACE (kininase II) is bradykinin, which exhibits a wide range of effects on cardiovascular function, and possibly also structure, in the SHR. This nonapeptide when generated acts locally by stimulating vascular nitric oxide and prostacyclin release to elicit vasodilatation (Busse et al, 1993).

ACE-inhibitors have been shown to lead to increased levels of circulating and tissue bradykinin in experimental animal models, but their effects in human studies have been less conclusive, possibly related to difficulties in the assay and interpretation of plasma and urinary kinin levels (Sunman and Sever, 1993). The efficacy of ACE-inhibitors can be reduced by administration of a bradykinin receptor antagonist and the actions of the RAS are not completely abolished by the administration of an angiotensin receptor antagonist (Pontieri et al, 1990, Hajj-ali and Zimmerman, 1991). Bradykinin infusion protects against myocardial ischaemia after coronary ligation (Linz and Scholkens, 1992) and contributes to the antihypertensive effect of ramipril in experimental rat renal hypertension, but not in the SHR (Bao et al, 1992, Gohlke et al, 1994). Deficiencies in the SHR kallikrein-kinin system have been cited as reasons for this lack of effect (Bao et al, 1992). However, bradykinin does contribute to the beneficial myocardial effects of ACE-inhibition in this model (Gohlke et al, 1994). Aside from bradykinin, there are a multitude of peptides and hormones that are acted on by the ACE, including substance P and K, enkephalin, neurotensin and gonadotrophin leutinising-releasing hormone (Sunman and Sever, 1993, Johnston, 1994). This may be a consequence of having two potentially active sites per molecule, with each site possessing a different conformation and having varying pH and chloride dependencies (Johnston, 1994).

The development of the orally available, nonpeptide angiotensin II receptor antagonists has also allowed further developments in elucidating the mechanisms of action of ACE-inhibitors, as well as assisting in the classification of AII receptor subtypes. The poor bioavailability and residual agonistic activity of peptide analogues spurred on the development of the nonpeptide antagonists, assisting in the identification and classification of two subtypes, designated AT_1 and AT_2 (reviewed by Timmermans

et al, 1993). Most, if not all, physiological actions have been ascribed to the AT_1 receptor (Timmermans et al, 1993), and the presence of losartan-sensitive AT_1 subtypes ($AT_{1A,1B,1C}$) and possibly a third distinct receptor has also been recently revealed (Timmermans et al, 1993, Mendelsohn et al, 1994). Moreover, there may exist a differential expression of AT_1 receptor subtypes in the SHR, with reports of increased AT_{1B} mRNA content in the left ventricle (Iwai et al, 1992) and increased AT_{1A} and AT_{1B} mRNA levels in the hypothalamus and brainstem of the SHR (Raizada et al, 1993). Recent studies have ascribed *potential* functions to the AT_2 receptor, namely in cerebral blood flow autoregulation, renal and vascular development and renal water handling (Keiser et al, 1992, Ardaillou, 1994, Rosenfeld, 1994, Saavedra, 1994). The selective AT_1 blocker losartan (DuP 753) has been trialled in acute and long-term studies in the SHR and has been found to lower blood pressure acutely (Wong et al, 1990), and after prolonged oral administration and its withdrawal in both the developing and established model (Morton et al, 1992, Oddie et al, 1993).

Other antihypertensives that are efficacious in the SHR include the nonspecific vasodilators, such as hydralazine and minoxidil, which control SBP and genetic hypertension development as long as treatment is maintained (*1.11.4*). They are seen to have only a limited degree of influence on the development of cardiovascular structural parameters, even when effectively used to control blood pressure (Christensen et al, 1989). Their use in these settings allows an investigation of the modulating effect of blood pressure on vascular structural and functional parameters in the developing SHR.

In this chapter, previous findings with captopril have been extended and compared with the outcomes of a number of different antihypertensive strategies. Structural (cardiac hypertrophy), biochemical (vascular contractile protein) and functional (blood pressure and vascular reactivity) parameters have been investigated after chronic treatment with the ACE-inhibitor captopril, the vasodilator hydralazine and the AT_1 receptor antagonist losartan in the SHR. The aim was to further characterise the relationship between blood pressure and vascular contractile protein and function during and after these treatments, in relation to changes in blood pressure, angiotensin converting enzyme activity and angiotensin receptor antagonism. A part of the present study was to also investigate the effects of chronic and acute ACE-inhibition on

responses to vasoactive agents in the perfused mesenteric vascular preparation of the SHR, in an attempt to dissociate the functional versus structural effects of the ACE inhibitor and possible sites of action of the peptide.

5.2 Methods

5.2.1 Animals and drug treatments

Male 5 week-old SHR and Wistar-Kyoto (WKY) rats were obtained from the CSIRO Glenthorne breeding colonies and immediately designated to control (SCON, WCON), captopril (SCAP), hydralazine (SHYD) or losartan (SLOS) treatment groups (10-12 rats/group). Drugs were administered orally by dissolution in the drinking water at concentrations that would allow for doses of 100 mg/kg/day (captopril), 25 mg/kg/day (hydralazine) and 10 mg/kg/day (losartan). Drug solutions were prepared and replaced every 2-3 days, and their concentrations were adjusted to allow for variations in water intake. All treatments continued for 8 weeks, whereupon subgroups of drug-treated SHR were discontinued from therapy ("released") for 5 weeks (SCAPREL, SHYDREL, SLOSREL; n=5-6 per group). The remainder continued with the drug treatments for a further 5 weeks, whereupon all animals were sacrificed. An acute captopril-treated group also accompanied the chronic studies. This consisted of male SHR treated at 100 mg/kg/day from 17 to 18 weeks of age (SCAP.AC; n=5). Systolic blood pressure measurements were made at least fortnightly on all animals as previously described (*2.1.3*).

5.2.2 The perfused mesenteric vascular bed (PMVB)

Animals were sacrificed by stunning and decapitation. The perfused mesenteric vascular bed was prepared as previously described (*2.3*). The responses to injected noradrenaline (0.05 ug to 20 ug) were measured and recorded as described previously (*2.3*).

5.2.3 Tissue preparation and 3-methylhistidine analysis

After decapitation, the heart, vas deferens and aorta were removed and prepared as previously described (*2.1.4*). After the responses of each mesenteric vascular bed were elicited, the preparations were removed and the vascular bed prepared as previously described (*4.2.3*).

3-methylhistidine measurements were performed as previously described (*2.4.2*) using 3-ethylhistidine (20 nmoles/sample) as the internal standard.

5.2.4 Aortic angiotensin converting enzyme (ACE) activity

The ACE activity of aortic tissue homogenates was measured as described previously (*2.4.4*). Tissues were homogenised in potassium phosphate buffer (50 mM, pH 8.75 at 100 mg/ml).

5.3 Results

5.3.1 Blood pressure

The SHR developed hypertension over a period of 12 weeks (mean SBP, 200 mmHg vs. WKY rat, 130 mmHg, Figs. 5.1-5.3). These levels were maintained until sacrifice. Chronic treatment with captopril was associated with a reduction in SBP to levels similar to those observed in the WKY rat (Fig. 5.1). After drug withdrawal, blood pressure slowly rose, but plateaued well below that of control SHR (150 mmHg) after 4 weeks. Acute captopril treatment from 17 weeks of age rapidly lowered SBP of adult hypertensive SHR to normotensive levels within the week of treatment (Fig. 5.1).

Hydralazine-treated SHR exhibited a similar fall in SBP to that of captopril-treated SHR (130 mmHg at 17 weeks of age), but upon drug withdrawal at 13 weeks of age, SBP rose rapidly to that of control SHR values within two weeks (Fig. 5.2).

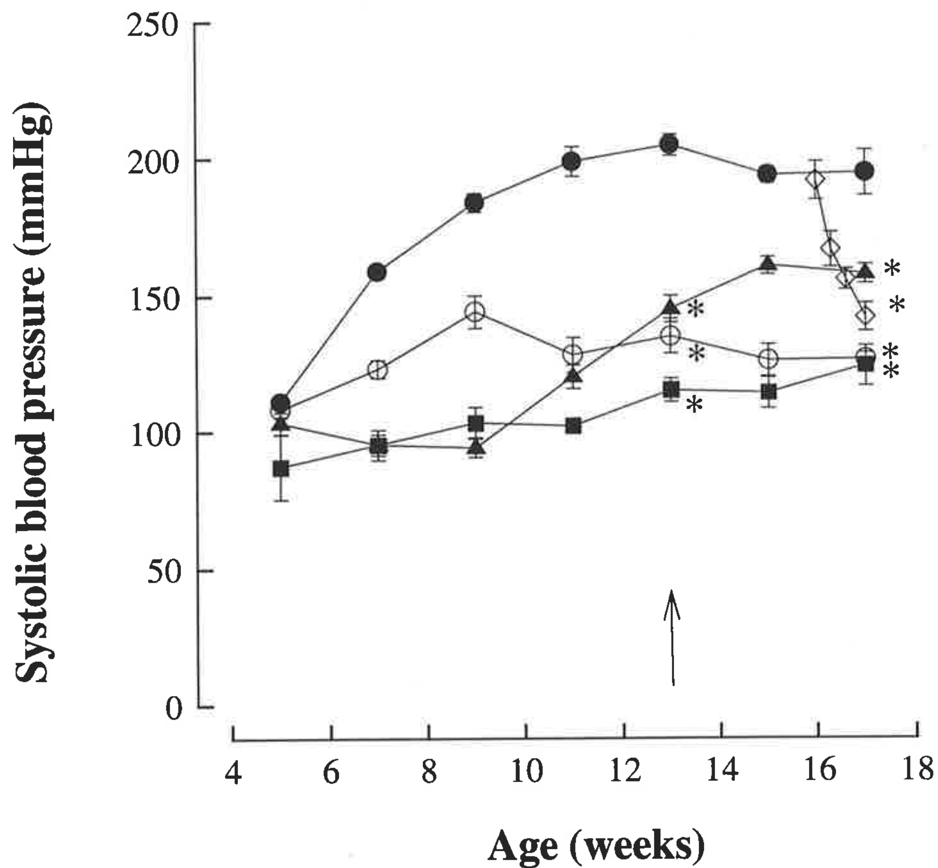

Fig. 5.1. Systolic blood pressure measurements in untreated (control) SHR (●) and WKY (○) rats, chronic (■) and acute (◇) captopril -treated and captopril-released (▲) SHR. Arrow denotes time of release. Rats were administered captopril in the drinking water (100 mg/kg/day). Systolic blood pressure was measured using an indirect tail-cuff technique.

* significant ($P<0.05$) difference vs. control SHR at 13 and 17 weeks of age (n=6 per group).

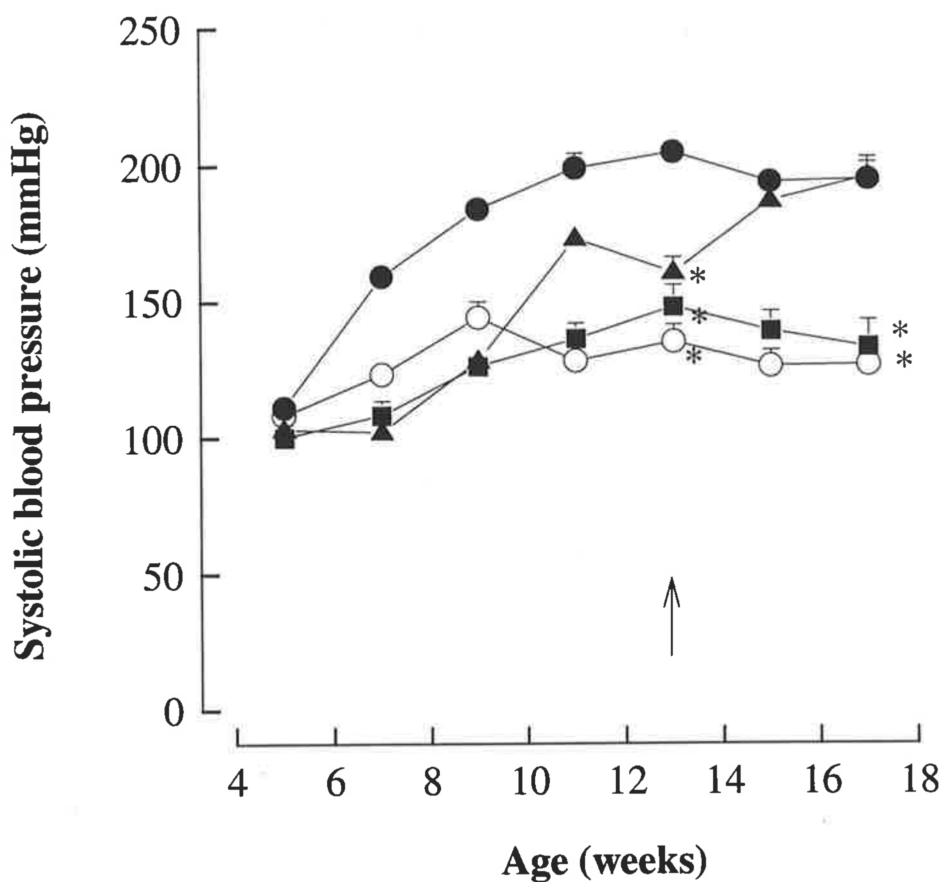

Fig. 5.2. Systolic blood pressure measurements from untreated (control) SHR (●) and WKY (○) rats, chronic hydralazine-treated (■) and hydralazine-released (▲) SHR. Arrow denotes time of release. Rats were administered hydralazine in the drinking water (25 mg/kg/day). Systolic blood pressure was measured using an indirect tail-cuff technique.
*significant ($P<0.05$) difference vs. control SHR at 13 and 17 weeks of age (n=6 per group).

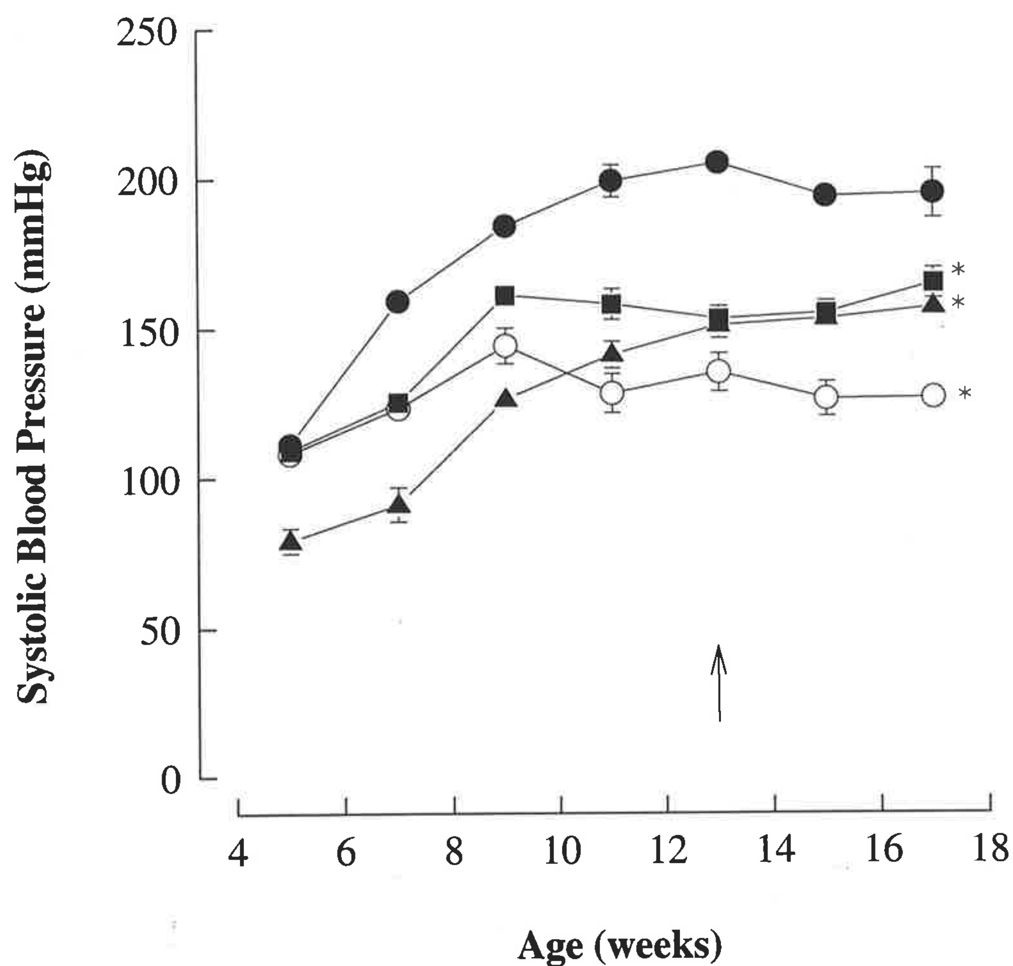

Fig. 5.3. Systolic blood pressure measurements of untreated (control) SHR (●) and WKY (○) rats, chronic losartan-treated (■) and losartan-released (▲) SHR. Arrow denotes time of release. Losartan was administered in the drinking water (10 mg/kg/day). Systolic blood pressure was measured using an indirect tail-cuff technique.

* significant ($P<0.05$) difference vs. control SHR at 13 and 17 weeks of age (n=6 per group).

Chronic losartan treatment, while not as effective as the other antihypertensives in lowering SBP (at a dose of 10 mg/kg/day), maintained SBP at a level significantly lower than the SCON group over the duration of treatment (Fig. 5.3; 160 mmHg at 17 weeks of age). Upon withdrawal of losartan, SBP levels did not rise.

5.3.2 Vascular reactivity to noradrenaline

Responses of the perfused mesenteric vascular bed (PMVB) to NA were markedly enhanced in 17 week-old SHR when compared to age-matched WKY rats over injected doses of upwards of 250 ng NA, with the SHR capable of contracting at approximately twice the maximal capacity of the WKY rat (290 mmHg vs WKY, 150 mmHg; Figs. 5.4-5.6).

Chronic captopril treatment resulted in a dramatic attenuation of vascular reactivity, normalising its contraction profile to that seen in preparations from the WKY rats, over the dose range of NA employed (Fig. 5.4). In contrast, once released from captopril treatment, vascular reactivity returned to levels similar to that of untreated (control) SHR. Acute captopril treatment was as effective as chronic captopril treatment in normalising the PMVB constriction profile to NA (Fig. 5.4).

Chronic hydralazine treatment reduced the response of the PMVB to NA such that the dose response curve was intermediate between that of control SHR and WKY rats, with a significant reduction in reactivity over nearly all doses used (Fig. 5.5). Withdrawal from hydralazine resulted in a dose response curve virtually indistinguishable from control SHR (Fig. 5.5).

The SLOS group exhibited a complete normalisation of vascular reactivity similar to captopril-treated SHR (Fig. 5.6). After drug withdrawal, vascular responses to NA were not fully restored, with mid-range (but not maximal) constriction remaining significantly diminished over the range 0.5 ug NA to 5.0 ug NA (Fig. 5.6).

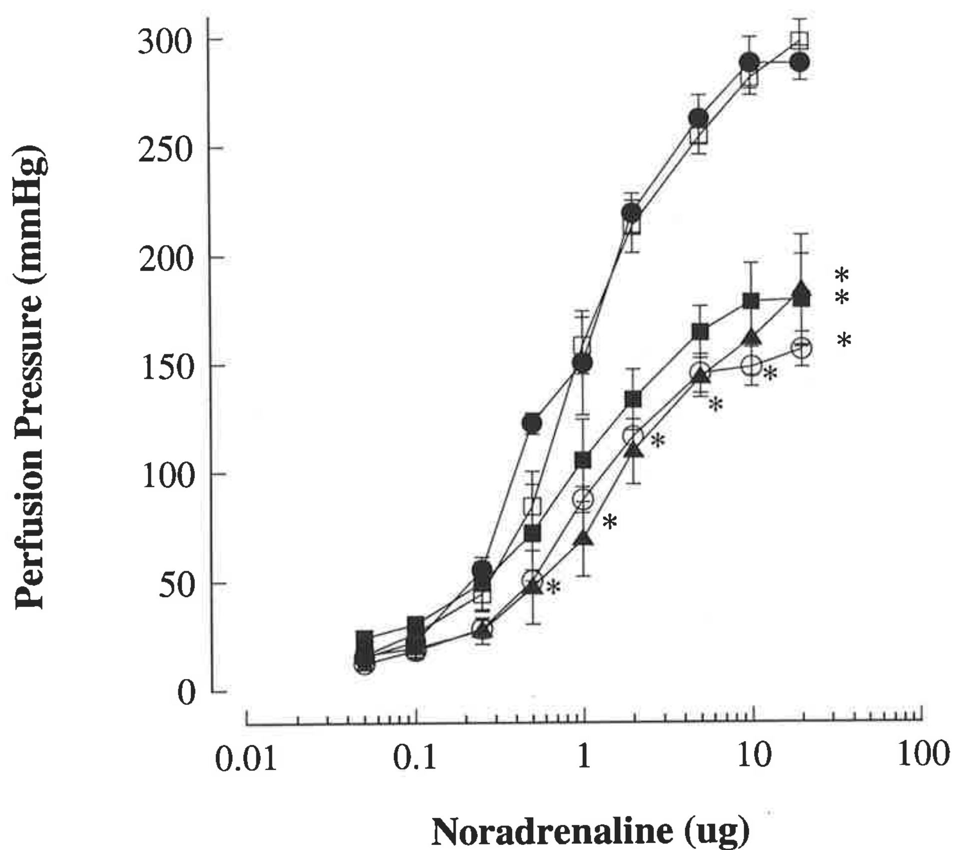

Fig. 5.4. Changes in perfusion pressure of the isolated perfused mesenteric vascular bed to exogenous noradrenaline infusion (0.05 to 20 ug) in untreated (control) SHR (●) and WKY (○) rats, chronic (■) and acute (▲) captopril-treated and captopril-released (□) SHR. N=6 per group.

* significant (P<0.05) difference vs. control SHR.

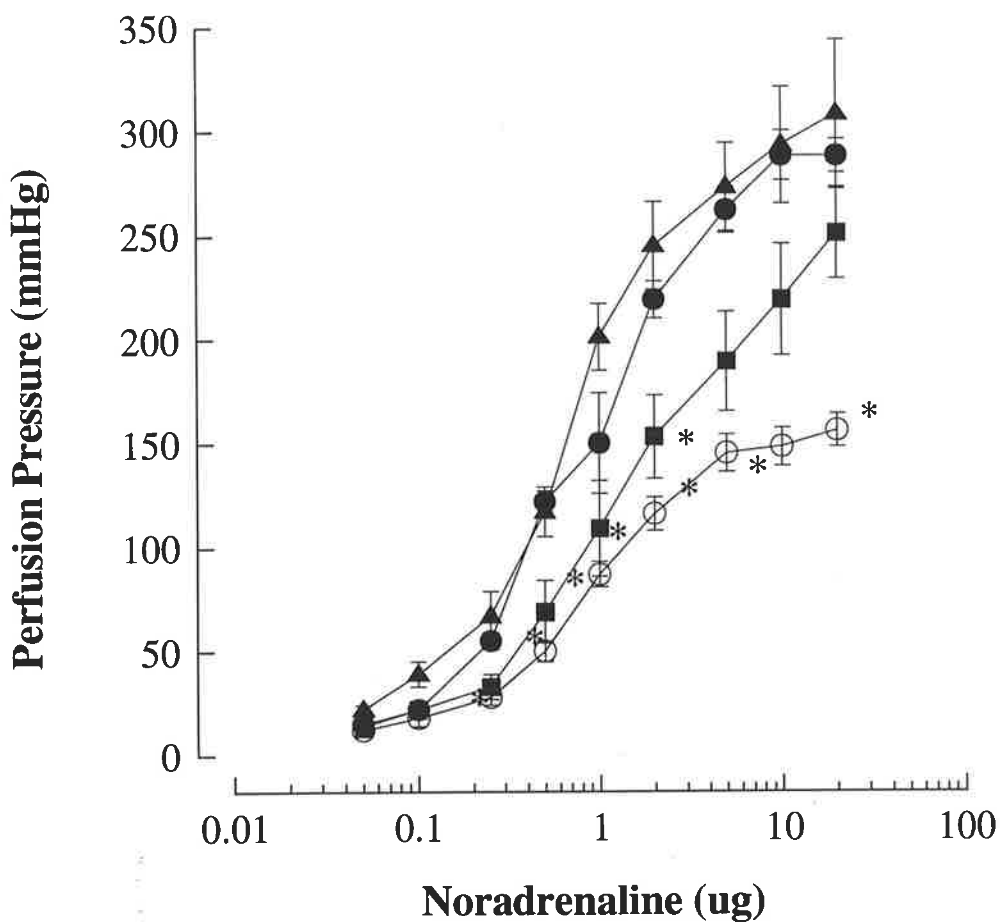

Fig 5.5. Changes in perfusion pressure of the isolated perfused mesenteric vascular bed to exogenous noradrenaline infusion (0.05 to 20 ug) in untreated (control) SHR (●) and WKY (○) rats, chronic hydralazine-treated (■) and hydralazine -released (▲) SHR. N=6 per group.

* significant (P<0.05) difference vs. control SHR

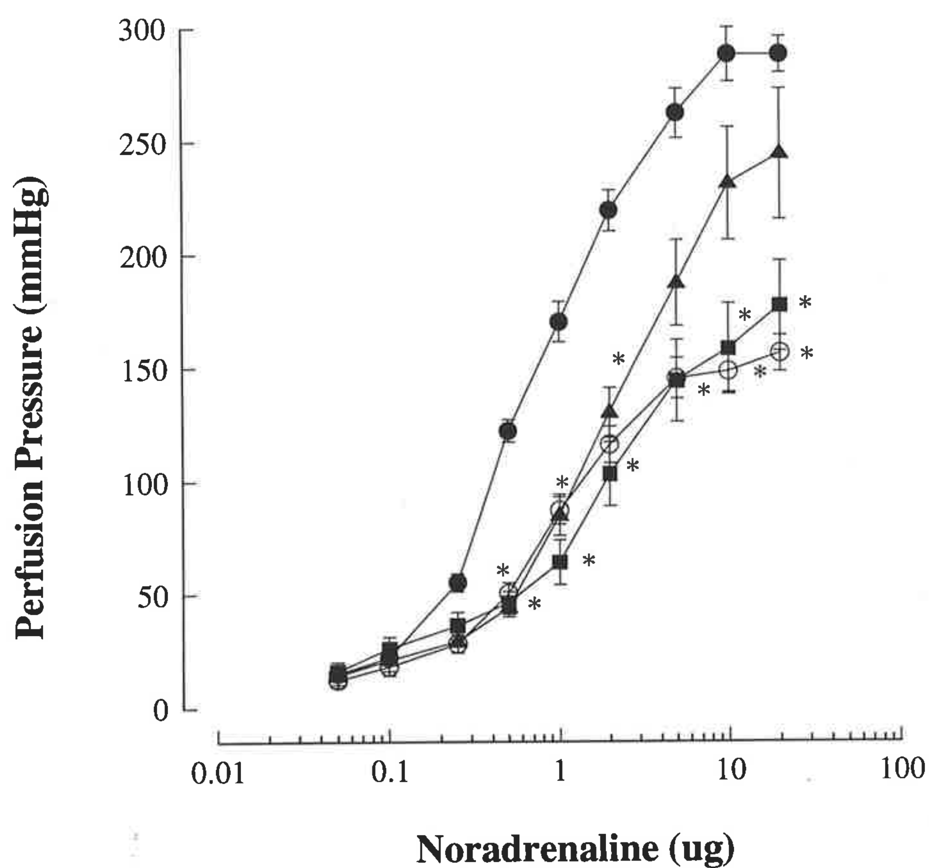

Fig. 5.6. Changes in perfusion pressure of the isolated perfused mesenteric vascular bed to exogenous noradrenaline infusion (0.025 to 20 ug) in untreated (control) SHR (●) and WKY (○) rats, chronic losartan-treated (■) and released (▲) SHR. N= 6 per group.

* significant (P<0.05) difference vs. control SHR.

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5.3.3 Heart weight to body weight ratios

Heart weight to body weight (HW:BW) ratios for all groups are shown in Fig. 5.7. The SHR exhibited a significantly higher ratio than the WKY rat, indicative of the presence of left ventricular hypertrophy in this strain.

Chronic captopril treatment elicited a marked and significant reduction in the HW:BW ratios in the SHR. Acute captopril treatment reduced heart weight to a lesser degree than that achieved with chronic therapy, but this was still significant. After withdrawal from captopril HW:BW ratios had increased, but were still significantly lower than those observed in control SHR.

Chronic hydralazine treatment afforded no reduction in HW:BW ratios despite good blood pressure control in this strain. Release from treatment also had no effect on HW:BW ratios.

Losartan, although not as effective as captopril in lowering blood pressure at a dose of 10 mg/kg/day, produced a significant reduction in the HW:BW ratio in the SLOS group. The SLOSREL group maintained a significantly lower HW:BW ratio compared to control SHR.

5.3.4 Tissue 3-methylhistidine content

Vas deferens:

As previously observed, differences in vas deferens (nonvascular) 3MH concentrations rarely exist between strains and after drug treatments. This pattern was similarly observed in the present study (Fig. 5.8), with no significant changes between control WKY rats and SHR.

Aorta:

3MH values for this tissue are displayed in Fig. 5.9. The WKY rat showed lower levels of 3MH when compared to the SHR, but this difference failed to reach

significance. Comparing across treatment groups, chronic (but not acute) captopril treatment caused a marked and significant reduction in aortic 3MH levels. Release from captopril treatment was associated with an increase in the 3MH concentration of the aorta that was intermediate between that seen in the aortae from SCAP and SCON groups.

Chronic hydralazine treatment also significantly lowered aortic 3MH concentrations, with a reversal of 3MH concentrations after release.

Losartan treatment also reduced aortic 3MH content, but this was not significant. After withdrawal from losartan treatment, 3MH concentrations remained low, increasing only slightly after 4 weeks off the drug.

Mesenteric vascular bed:

As in Chapter 4, mesenteric vascular 3MH levels were not altered in control SHR when compared with control WKY rats (Fig. 5.10). In general, 3MH values in this tissue were resistant to changes from all drug therapies and their withdrawal, with only chronic captopril and hydralazine treatment eliciting a modest reduction in mesenteric vascular 3MH concentrations.

5.3.5 Aortic angiotensin converting enzyme (ACE) activity

The ACE activity of aortic tissue homogenates is shown in Fig. 5.11. The SHR exhibited approximately double the activity when compared to the WKY rat in this vessel. Chronic and acute captopril treatment normalised ACE activity in the SHR, whereas levels in hydralazine-treated SHR were unaltered.

While chronic losartan treatment caused a reduction in aortic ACE activity, this failed to attain significance. After withdrawal from antihypertensive therapy (captopril, hydralazine or losartan), ACE activity was no different from untreated (control) SHR.

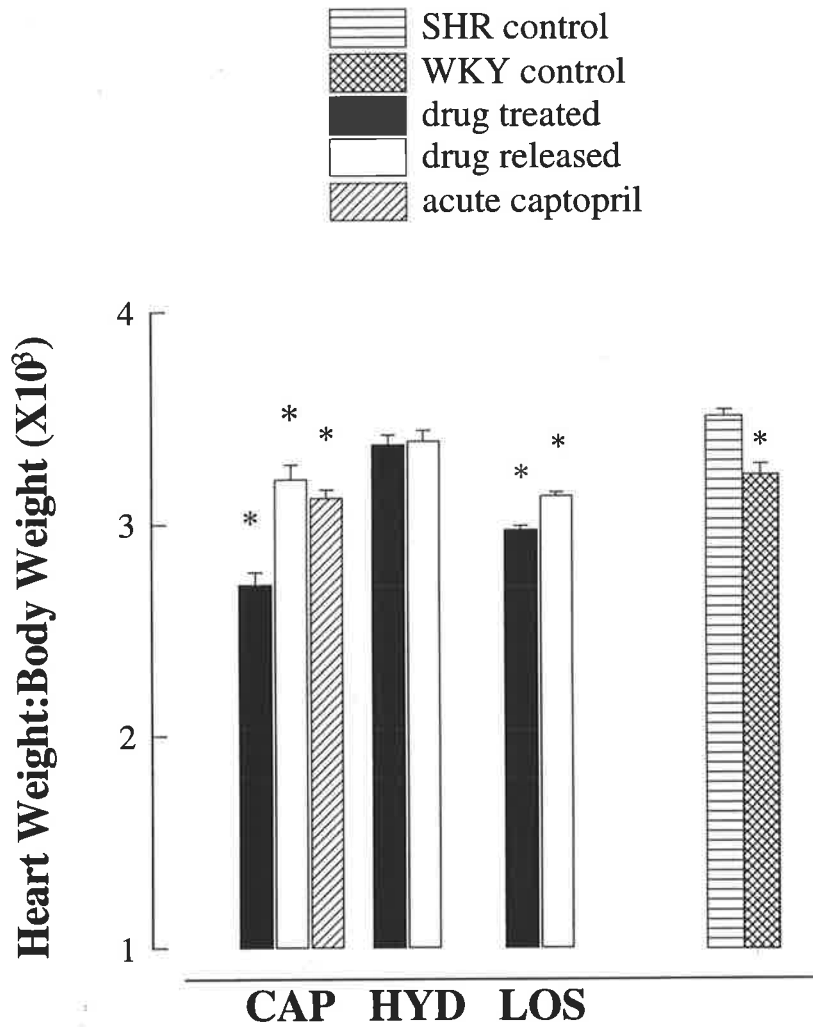

Fig 5.7. Heart weight: body weight ratios for all treatment and release groups and their controls. CAP=captopril, HYD=hydralazine, LOS=losartan. N=6 per group.
* significant ($P<0.05$) vs. control SHR

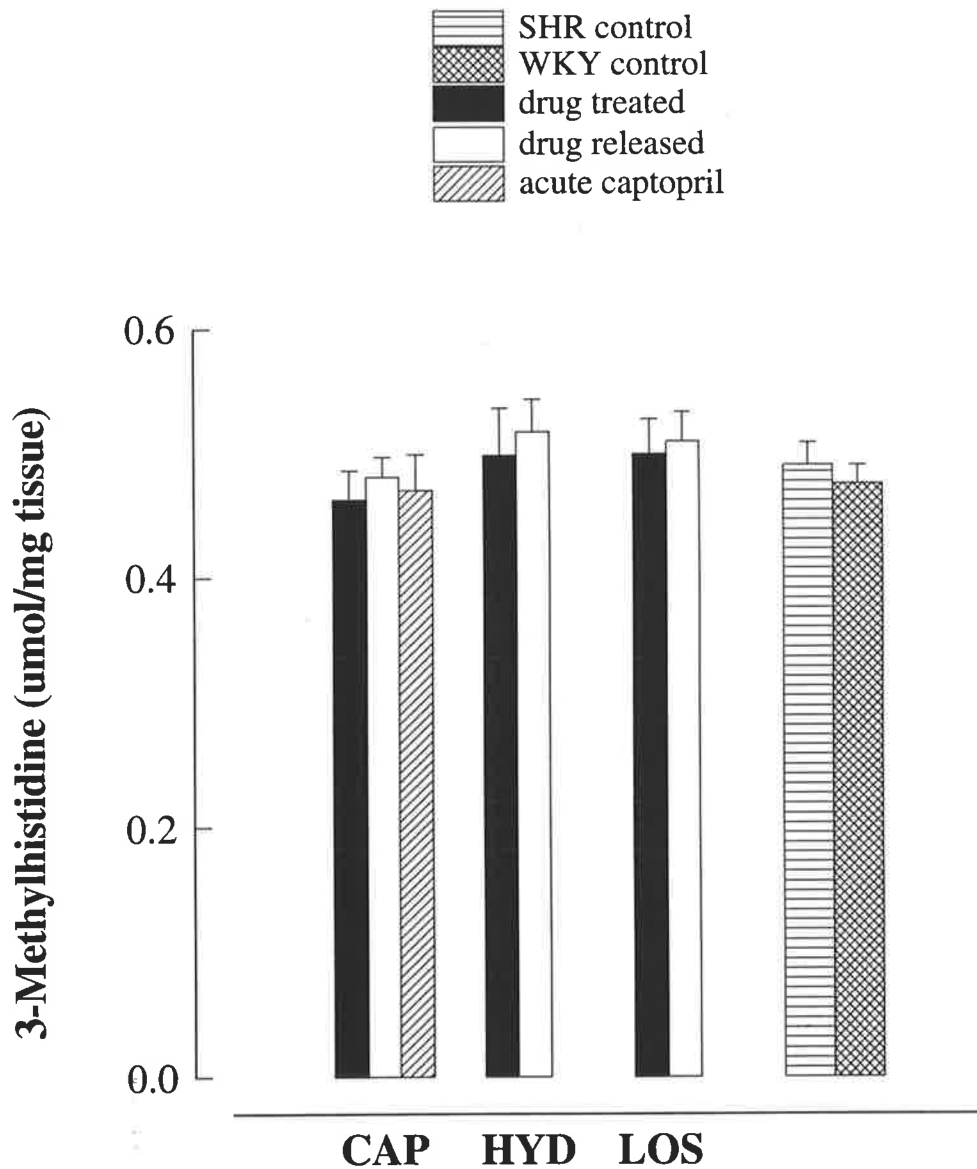

Fig. 5.8. Vas deferens 3-methylhistidine content from all treatment and release groups and their controls (n=6 per group). See Fig. 5.7 for legend details.

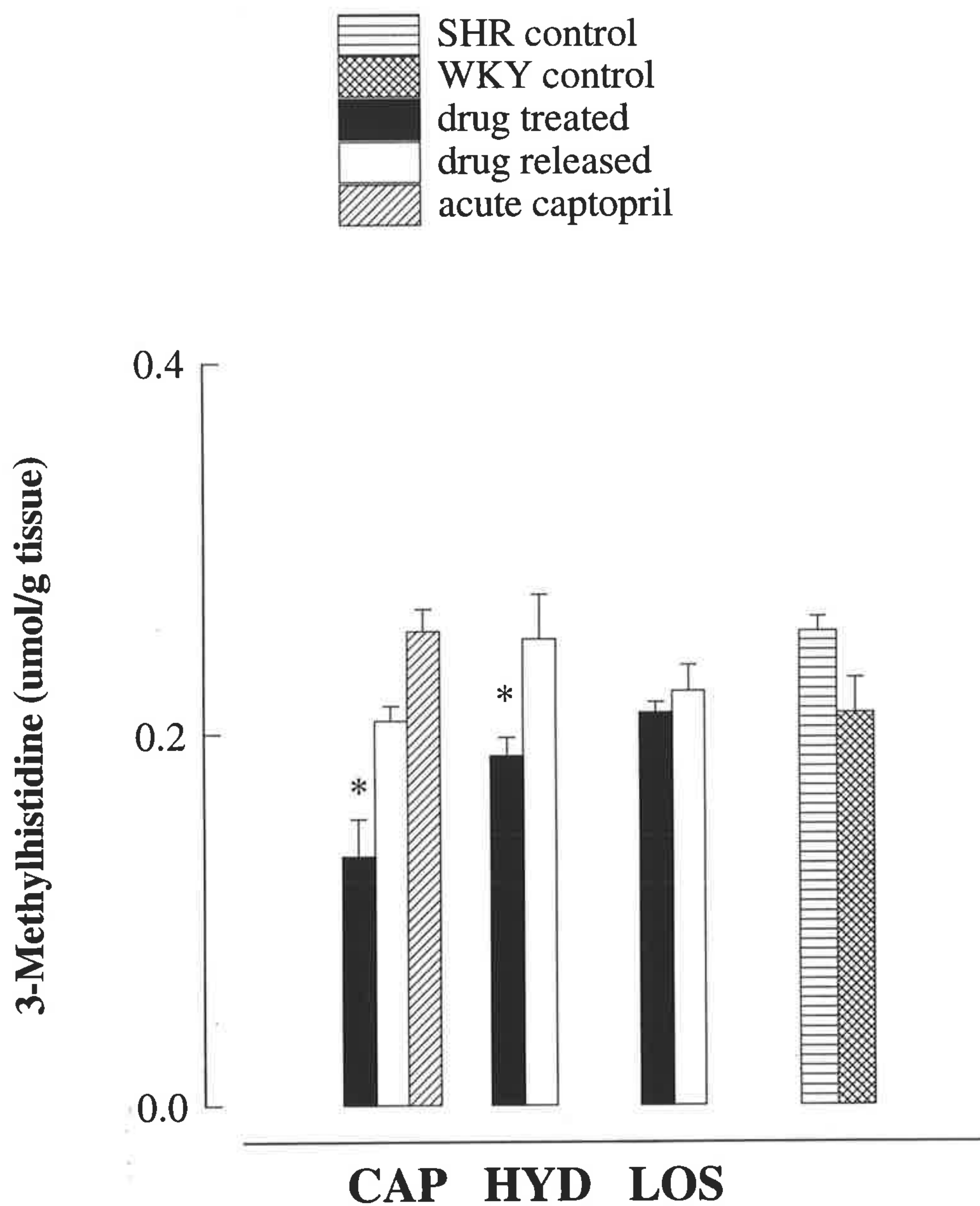

Fig. 5.9. Aortic 3-methylhistidine concentrations from all treatment and release groups and their controls. See Fig. 5.7 for legend details. N= 6 per group.
*significant (P<0.05) difference vs. control SHR.

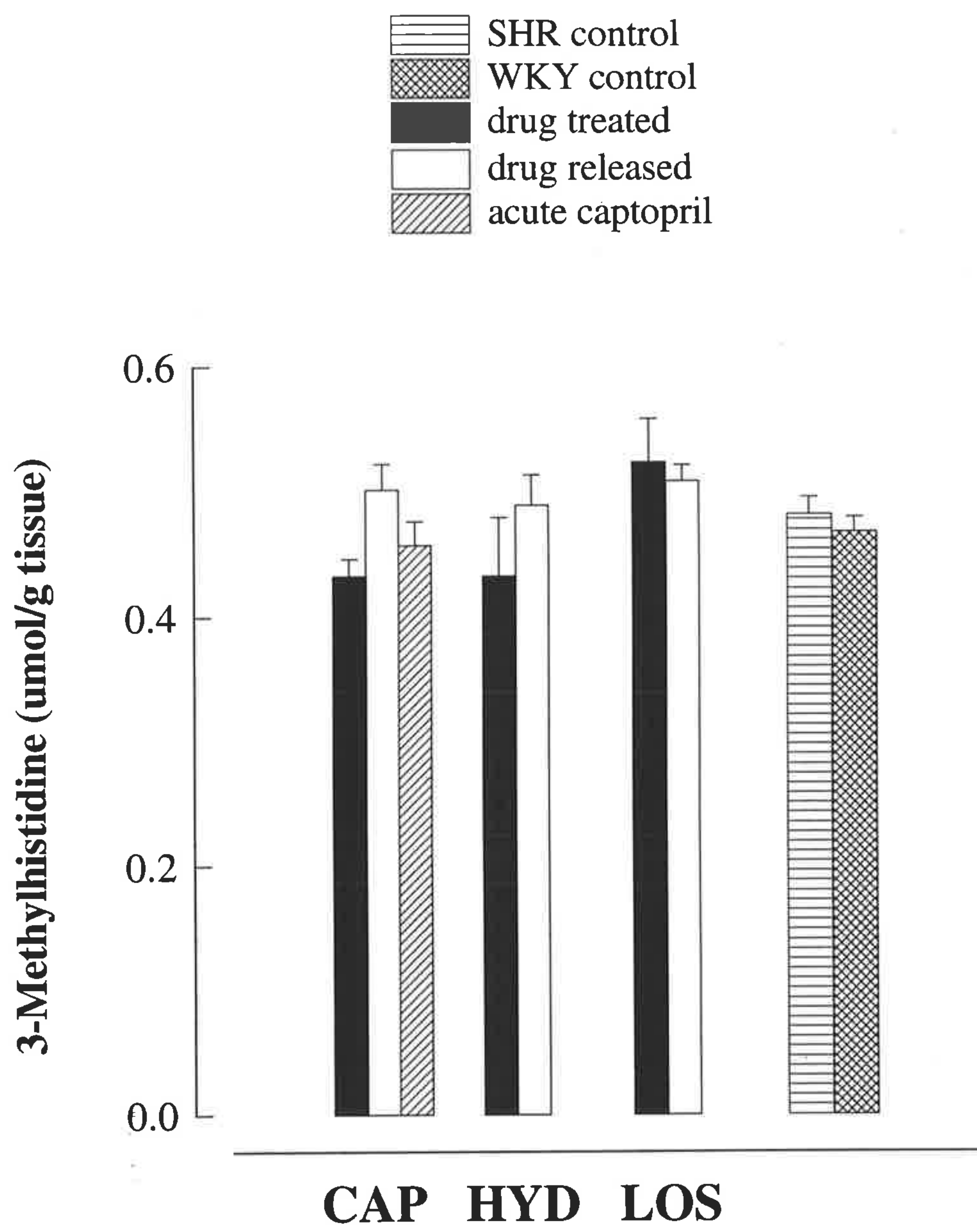

Fig 5.10. Mesenteric vascular 3-methylhistidine content of all drug treatment and release groups and their controls. See Fig. 5.7. for legend details. N= 6 per group.

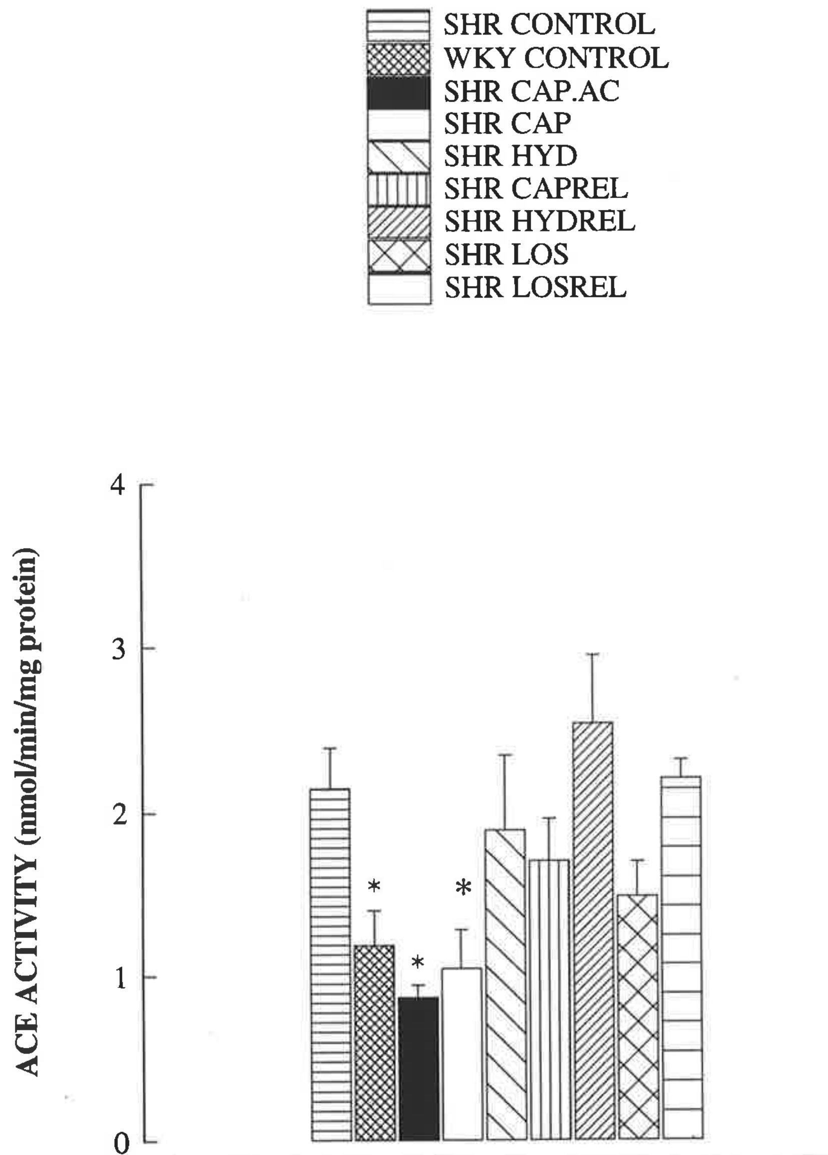

Fig 5.11. ACE activity from aortic tissue of all treatment groups and controls (n=5-11). CAP=captopril, HYD= hydralazine, LOS=losartan, REL=release, CAP.AC= acute captopril.
*significant (P<0.05) difference vs. control SHR.

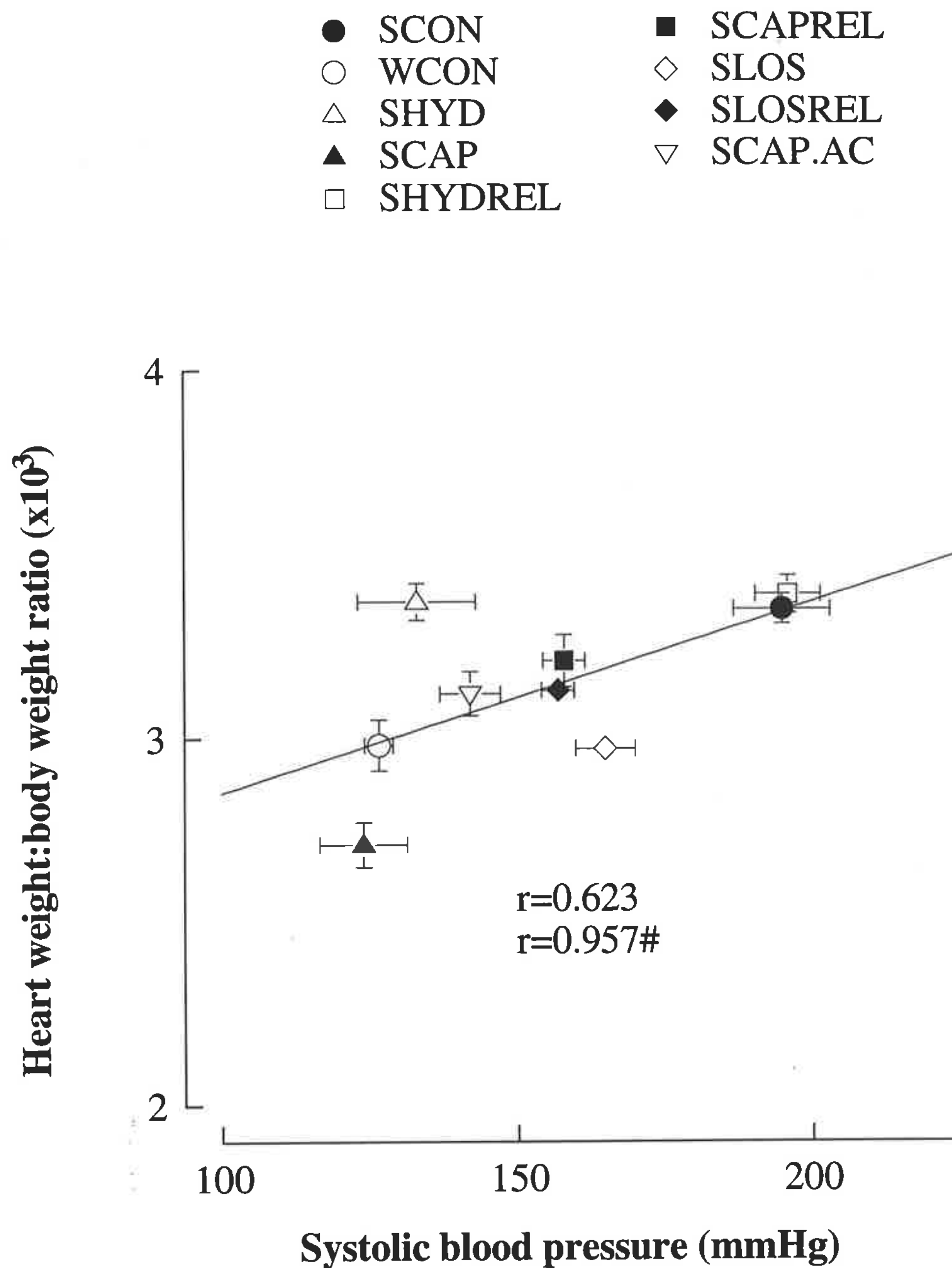

Fig. 5.12. Correlation analysis of systolic blood pressure vs. heart weight to body weight ratios in all treatment groups and controls. W=WKY rat, S=SHR, CON=control, HYD=hydralazine, CAP=captopril, LOS=losartan, REL=release. N=6 per group.
significant (P<0.01) correlation (excluding SCAP, SCAP.AC, SHYD and SLOS groups).

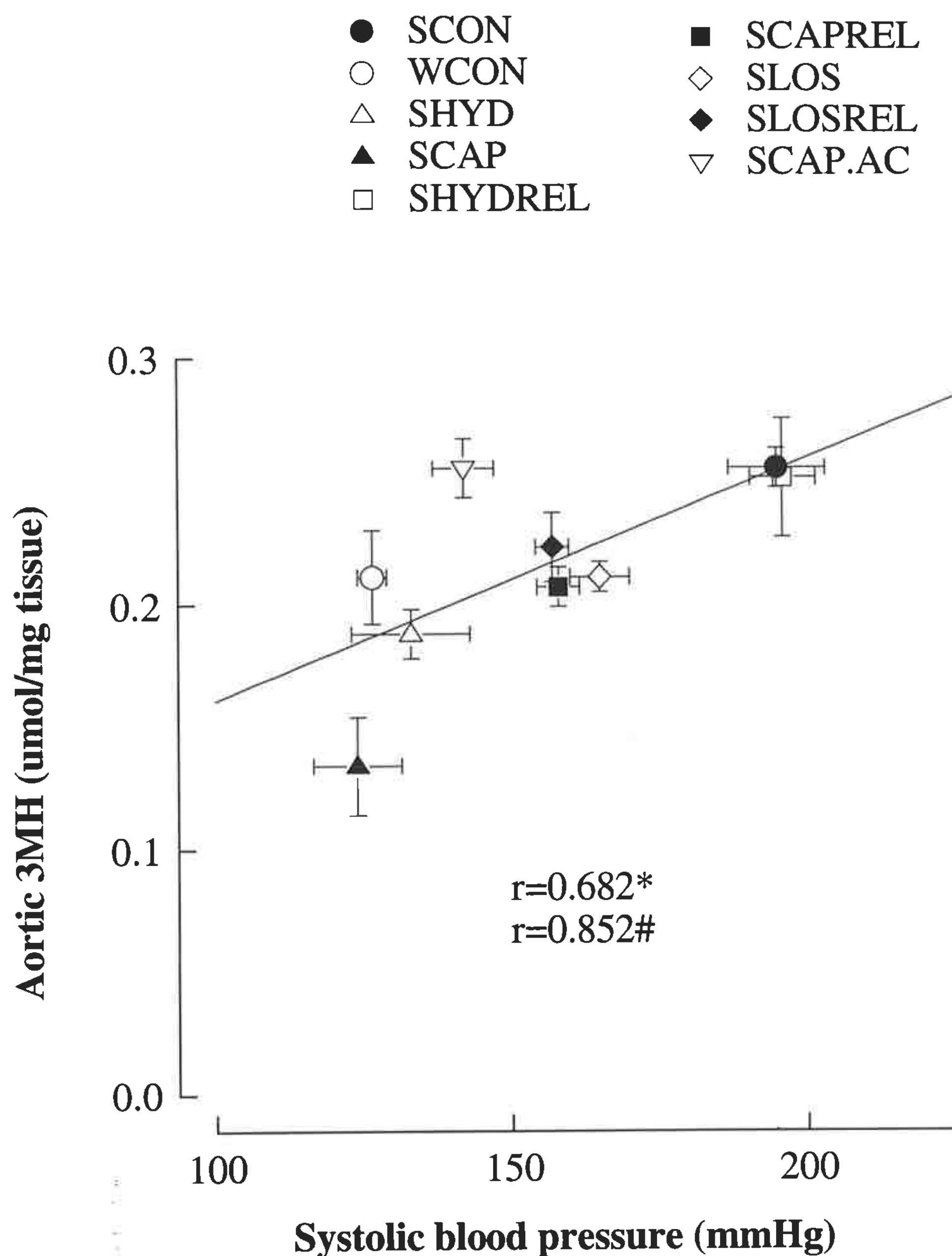

Fig. 5.13. Correlation analysis of systolic blood pressure vs. aortic 3MH in all treatment groups and controls. W=WKY rat, S=SHR, CON=control, HYD=hydralazine, CAP=captopril, LOS= losartan, REL=release. N=6 per group.

significant (P<0.05) correlation (excluding scap, scap.acute groups).

* significant (P<0.05) correlation (all groups).

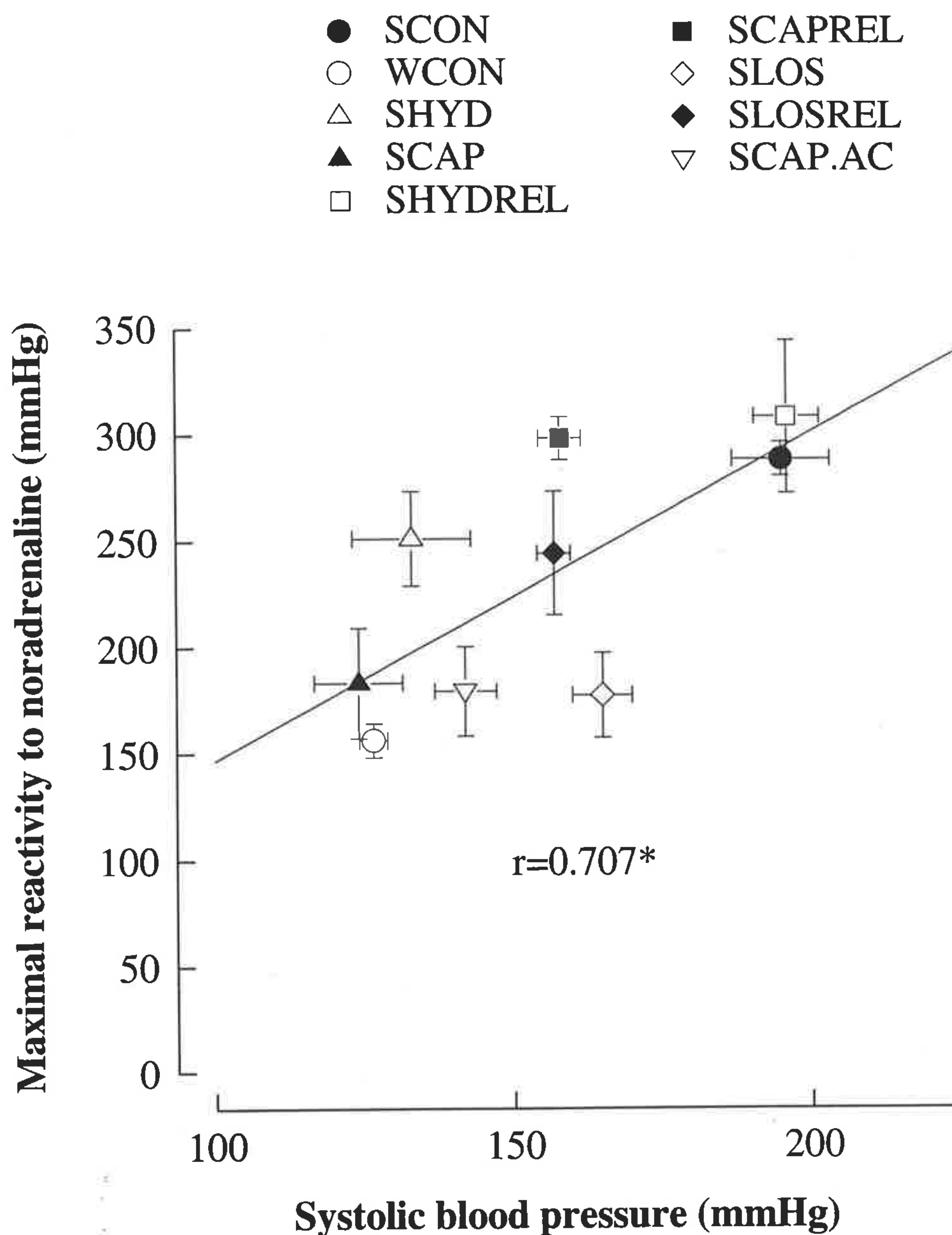

Fig. 5.14. Correlation analysis of systolic blood pressure vs. maximal noradrenaline responses of the mesenteric vascular bed in all treatment groups and controls. W=WKY rat, S=SHR, CON=control, HYD=hydralazine, CAP=captopril, LOS=losartan, REL=release. N=6 per group.
* significant ($P<0.05$) correlation (all groups).

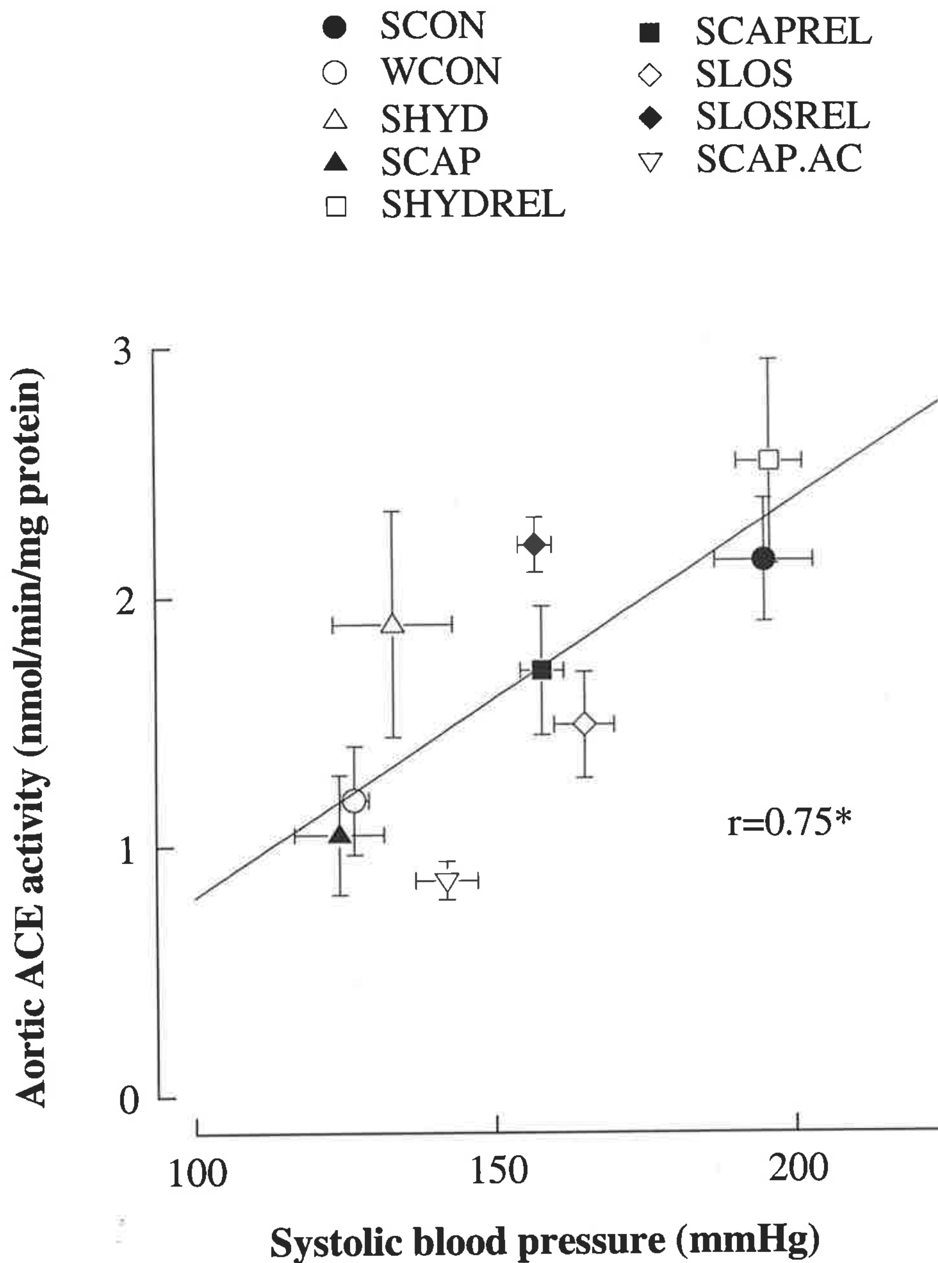

Fig. 5.15. Correlation analysis of systolic blood pressure vs. aortic ACE activity in all treatment groups and controls. W=WKY rat, S=SHR, CON=control, HYD=hydralazine, CAP=captopril, LOS=losartan, REL=release.
* significant (P<0.05) correlation (all groups).

5.4 Discussion

In this study, the blood pressure development of SHR has been monitored under conditions of acute and chronic treatment with selected antihypertensives, with subsequent measurements of vascular contractile protein and vascular reactivity. Blood pressure development has then been followed during a period of drug cessation, and a comparison made between these findings and those with the SHR maintained on antihypertensives.

5.4.1 Acute and chronic captopril treatment and withdrawal

Chronic captopril treatment prevented the age-dependent rise in blood pressure in the SHR. After withdrawal, blood pressure initially rose but remained lower than in untreated SHR after 5 weeks. This pattern of blood pressure development is a consistent finding (Giudicelli et al, 1980, Freslon and Giudicelli, 1983). Acute captopril treatment (7 days) lowered blood pressure in SHR with established hypertension to levels achieved with chronic treatment at an equivalent dose.

Both acute and chronic captopril regimens were equally effective in suppressing vascular reactivity to NA to levels observed in the WKY rat. The ability of ACE-inhibitors to modify vascular function in the SHR under conditions of chronic administration is not a novel finding (Freslon and Giudicelli, 1983, Adams et al, 1990). This is associated with, and generally believed to be attributable to, the reductions in vascular medial hypertrophy that accompany such treatments. It was initially considered that one week of treatment with the ACE-inhibitor was unlikely to be long enough for regression of structural changes sufficient to account for the observed reduction in responses after acute captopril treatment. However, two weeks of losartan treatment has been shown to decrease large and small artery weight and medial thickness in 24 week-old SHR (Soltis, 1993), so the possibility that medial structure was overtly affected after one week of ACE-inhibition could not be excluded. Alternatively, there may be a component of the attenuation in reactivity to acute captopril treatment mediated by the inhibition of the AII-driven facilitation of VSMC contraction. There is evidence from

other studies that ACE inhibition alters the function of post-junctional α-adrenoceptors in the normotensive rat (Marwood et al, 1992, Tabrizchi and Triggle, 1992), possibly through inhibiting an interaction between AII and plasmalemmal and sarcoplasmic reticular calcium channels (Purdy and Weber, 1988, Sada et al, 1989, Traub and Webb, 1993). There is accumulating evidence to suggest that VSMC calcium regulation is altered in the SHR (Erne and Hermsmeyer, 1989, Shibata et al, 1990). Long-term inhibition of ACE has been shown to reverse aberrant vascular plasmalemmal calcium permeability in the SHR (Sada et al, 1989). Others, however suggest extracellular calcium mobilisation is not enhanced by AII in either normotensive or hypertensive VSMCs, instead providing evidence linking the AII augmented NA response with an activation of protein kinase C (Henrion et al, 1992, Soloviev and Bershtein, 1992). VSMCs of the SHR exhibit accentuated protein kinase C activation to AII than do WKY rat VSMCs (Soloviev and Bershtein, 1992, Neusser et al, 1993). The suppression mediated through α-adrenoceptors has been observed in a previous study after one week of ACE-inhibition with quinapril, although reactivity to the α_1-agonist phenylephrine was not normalised to WKY rat levels (Major et al, 1993).

Chronic captopril treatment reduced cardiac hypertrophy and aortic 3MH content to levels below that of the normotensive WKY rat, making ACE-inhibition the most efficacious of therapies in terms of their effects on cardiac structure and vascular contractile protein in this study. These results support an essential relationship between the RAS and the long-term development of cardiovascular hypertrophy in this model, and not simply the influence of blood pressure *per se* on this feature. Angiotensin II, or possibly another substrate of the ACE, is important in the development of the normal myocardium and vasculature in the rat, as previously shown with chronic ACE-inhibition in normotensive rats (Owens, 1987, Jonsson et al, 1991, Keeley et al, 1992). Previous studies with ACE-inhibitors show consistent beneficial effects on myocardial and vascular hypertrophy after chronic treatment of young SHR (Freslon and Giudicelli, 1983, Lee et al, 1991b).

Acute treatment with captopril in the older SHR did not alter vascular 3MH levels, but did elicit a significant reduction in cardiac hypertrophy, indicating that regression of abnormal cardiac structure is possible after acute administration of the

ACE-inhibitor in the adult animal. These results suggest that a component of the cardiac hypertrophy is reversible in the adult SHR. This may be more dependent on the decrease in AII concentrations rather than the fall in blood pressure, as acute hydralazine treatment did not decrease heart weight despite normalising SBP, while AT_1-blockade with losartan reduced both parameters (Soltis, 1993). As aortic 3MH content was not reduced by acute treatment in the present study, vascular contractile protein seems less labile.

The effects of withdrawal from chronic captopril treatment on vascular reactivity showed that NA responses were restored to control levels after 5 weeks off the drug, despite the persistence of significantly lower blood pressure. Previous studies in chronically ACE-inhibited SHR have yielded variable results with regard to vascular functional parameters both during treatment and after withdrawal. However, these studies used *in vitro* segments of mesenteric resistance vessels, and not the whole vascular bed (Freslon and Giudicelli, 1983, Cadilhac and Giudicelli, 1986, Christensen et al, 1988). This lack of consistency merits studies of whole vascular preparations, rather than segments of vascular tissue. One previous study used the perfused *in vitro* hindquarter vascular bed under conditions of chronic treatment with enalapril in young SHR, and found a suppression of the enhanced vascular resistance that was observed 16 weeks after treatment withdrawal and which correlated well with the prevailing SBP (Adams et al, 1990). The correlation between SBP and maximal NA reactivity (Fig. 5.14) in the present chapter was less than that observed previously after withdrawal from captopril treatment (Fig. 4.7), but the dose-response curves to NA in captopril-released and control SHR were generally closely associated in both studies.

5.4.2 Hydralazine Treatment and Withdrawal

Chronic hydralazine treatment effectively controlled blood pressure during chronic therapy, but not after withdrawal. Unlike chronic captopril treatment, blood pressure rose rapidly after hydralazine treatment as has been described in other studies (Giudicelli et al, 1980, Freslon and Giudicelli, 1983, Christensen et al, 1988). Reactivity of the perfused mesenteric vascular bed, while suppressed to some extent

during treatment, was not normalised and, following drug withdrawal, returned to control levels. This result is consistent with a previous study (Freslon and Giudicelli, 1983). It is possible that hydralazine may reverse only the pressure-dependent stimulus to structural and functional change in this vascular bed, without affecting the pressure-independent stimulus, which is likely to involve the RAS. Another study has shown that chronic vasodilatation with hydralazine is associated with modest increases in luminal diameters of small arteries without alteration of medial mass (Smeda and Lee, 1991). This may underlie the slightly lower mesenteric reactive profiles to NA observed in this group.

The precise mechanism by which hydralazine lowers blood pressure is unknown. However, there is some evidence supporting a role in the promotion of potassium channel opening and inhibition of calcium channels (Gurney, 1994). This may also contribute to the observed attenuation in NA responses of the mesenteric vasculature.

The finding that cardiac hypertrophy was not prevented by chronic hydralazine treatment, despite effective blood pressure control, is consistent with the results of chronic trials in both young and older SHR (Tsoporis et al, 1991, 1993) indicating that its maintenance proceeds independently of blood pressure under these conditions. The present results are similar to those of others (Freslon and Giudicelli, 1983, Owens, 1987, Tsoporis et al, 1991 and 1993). The discarding of SBP as a determinant of LV hypertrophy is clouded by reflex increases in sympathetic activity of the heart that occur following hydralazine administration (Tsoporis et al, 1991 and 1993), underscoring the trophic actions of catecholamines on the myocardium (Leenen and Harmsen, 1991, Long et al, 1991), or the consequence of the adaptation to an increased workload. This may mask any true effect a change in blood pressure is having on myocardial growth. An increase in sympathetic drive to the heart can occur as a consequence of many other antihypertensive treatments (Leenen and Harmsen, 1991), and the noted lack of this effect by ACE-inhibitors may partly underlie the dramatic reduction in cardiac mass observed in response to this class of drugs. Tsoporis et al (1993) also note an increase in plasma and blood volume after minoxidil treatment of the SHR, with a subsequent increase in the progression of cardiac hypertrophy despite low blood pressure.

Chronic hydralazine treatment significantly reduced 3MH levels in the aorta, although to a lesser extent than captopril, but to levels below that of the WKY rat. This indicates that there may be a component of aortic contractile protein change occurring in response to the blood pressure, similar to the more general structural alterations in larger vessels after blood pressure reduction (Lee and Smeda, 1985). This was restored (along with the other parameters) to control levels following drug withdrawal and is consistent with the view that hydralazine only alters the pressure-dependent stimulus to cardiovascular structural and functional change, which is reversible following the cessation of treatment.

5.4.3 Losartan Treatment and Withdrawal

Losartan prevented hypertension development during chronic administration, but was less effective at a dosage of 10 mg/kg/day than the other antihypertensives. Previous studies using oral doses of losartan between 10 and 15 mg/kg/day have found that it exerts a modest effect on blood pressure in the SHR and SHR-SP strain (Morton et al, 1992, Fornes et al, 1993), and is less effective than captopril (100 mg/kg/day). These findings indicate that a significant part of the antihypertensive effect of ACE-inhibitors in this model is mediated by suppression of AII formation, acting at the AT_1 receptor. The AII involvement was supported by a study showing that the chronic antihypertensive effect of perindopril, both during treatment and after withdrawal, was abolished by coadministration of angiotensin II (Harrap et al, 1990).

The suppression of vascular reactivity, however, was not dependent on the fall in blood pressure, as this dosage normalised NA-induced pressor responses in the perfused mesenteric vasculature, clearly shown in the deviation from the regression line in Fig. 5.14. This suggests that angiotensin II, and not other factors (other ACE products, blood pressure), is an important component mediating changes in vascular reactivity to NA observed with those drugs modulating RAS function. Specifically, this is mediated through the AT_1 receptor. The attenuation in responses is qualitatively similar to that observed with captopril treatment, and is likely to occur through a similar mechanism.

Losartan caused a reduction in the development of cardiac hypertrophy that was greater than predicted from the blood pressure reduction alone and was similar to captopril in this regard (Fig. 5.12). Losartan was less effective than captopril, possibly due to actions of ACE-inhibition aside from prevention of AII generation, such as bradykinin accumulation, that may result in inhibition of myocardial growth (Linz and Scholkens, 1992). The added influence of the higher blood pressure may have also influenced the extent of LV hypertrophy. After withdrawal, losartan maintained a significantly lower HW:BW ratio, consistent with the subsequent changes in blood pressure following cessation of drug treatment.

After withdrawal from losartan, the reduction in SBP was maintained. This result has been similarly observed after withdrawal from 10 mg/kg/day losartan in another study (Morton et al, 1992). Vascular reactivity to NA was still reduced at lower (but not higher) NA doses. In this regard, losartan has a similar (but not identical) pharmacological profile to captopril after long-term treatment, indicating a distinct reversibility in vascular function following drug withdrawal. This is mediated through the inhibition of AII's actions on the AT_1 receptor in the SHR.

5.4.4 Correlation analyses

Correlation analyses was performed on a number or relevant variables in relation to the prevailing SBP in each of the treatment, withdrawal and control groups. This included HW:BW ratios (Fig. 5.12), aortic 3MH (Fig. 5.13), maximal vascular reactivity to NA (Fig. 5.14) and aortic ACE activity (Fig. 5.15). In most cases it was also desirable to investigate the relationship between certain variables in the absence of treatment, i.e. in untreated or drug-withdrawn groups. This excluded certain drug treatment groups in the analysis of the regression line.

In the absence of treatment (control and drug-withdrawn groups) LV hypertrophy (Fig. 5.12) and aortic 3MH (Fig. 5.13) were well correlated with SBP. These changes are likely to occur in response to the change in SBP.

Maximal vascular reactivity to NA (Fig. 5.14) was well correlated with SBP after withdrawal from both chronic losartan and hydralazine (but not captopril)

treatment, in contrast to the results of Chapter 4. This reflects the overall proximity of the dose-response curve of the SCAPREL group to that of the control SHR group.

Interestingly, aortic ACE activity correlated closely with SBP in the SCAPREL group. However, large sample variability precluded statistical significance. Although measurements of ACE activity were not performed in other vascular tissue, it seems unlikely that such a prolonged inhibition could occur after 5 weeks off the drug. Another study found that 2 weeks after cessation of chronic spirapril treatment, ACE activity was rapidly restored in plasma and mesenteric arteries. However, kidney, brain and aortic ACE activity remained low along with SBP (Okunishi et al, 1991). Prolonged tissue ACE-inhibition (especially in the CNS) may play an important role in determining SBP levels after chronic ACE-inhibition, and warrants further attention.

5.4.5 Blood pressure, vascular reactivity, cardiac hypertrophy and vascular 3MH

The foregoing results have described a number of relevant findings. Firstly, that chronic captopril treatment is not necessary to achieve a complete normalisation in vascular reactivity to NA, but is necessary to modify cardiovascular biochemical (actin) and structural (LV hypertrophy) features. This may be a feature related to the inhibition of the AII-mediated direct facilitation of VSMC contraction.

Chronic treatment with hydralazine is effective in preventing hypertension development and can influence larger vessel actin levels/disposition without exerting an effect on LV hypertrophy. This suggests that blood pressure partly modifies some aspects of vascular biochemical changes in hypertension without affecting cardiac mechanisms that generate hypertrophy, or alternatively instigating mechanisms that offset any preventative effects on LV mass from the lower SBP. However, all of these parameters undergo reversal after treatment withdrawal.

Aortic vascular actin and cardiac hypertrophy are sensitive to the effects of chronic ACE-inhibition and are also modified by chronic losartan treatment, indicative that the effects of captopril are, in part, due to a reduction in the formation or actions of AII acting at the AT_1 receptor.

The prolonged resetting of SBP after withdrawal from chronic ACE inhibition is partly mediated by its influence on AII levels in the SHR, also acting at the AT_1 receptor. This is because chronic losartan treatment also caused a prolonged suppression in blood pressure. However, the dissociation between vascular reactivity and SBP after captopril treatment suggests that other mechanisms may operate to maintain the partly suppressed SBP in this model after withdrawal from therapy. The use of losartan and hydralazine in the present study has confirmed the integral role of AII in generating and maintaining the aberrant vascular function, cardiac structure and aortic contractile protein. The peptide performs this through a combination of effects which are partly independent of changes in blood pressure and are mediated by the AT_1 receptor.

CHAPTER 6

The Effects of Chronic α-Adrenoceptor and Ganglion Blockade on Blood Pressure and the Renin-Angiotensin System in the Developing SHR

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6.1 Introduction

The importance of increased activity of the sympathetic nervous system (SNS) and its impact on the genesis and maintenance of hypertension in this strain was recognised by Yamori (1984). Aspects of SNS function in the SHR have been described elsewhere (see *1.6*).

Pharmacological (or other) treatments targeting the SNS in the developing SHR have had mixed success in preventing hypertension development. Surgical (ablation), immunological (antibodies to NGF) and chemical (6-hydroxydopamine) sympathectomy, either alone or in combination, are effective at preventing genetic hypertension development (GHD) and the accompanying cardiovascular structural changes, suggesting the involvement of the SNS in the disorder (see *1.11.8*). However, less permanent therapies are generally ineffective in controlling blood pressure. These include guanethidine (Johnson and Macia, 1979), reserpine (Kiprov and Dimitrov, 1977), α-methyldopa (Sen et al, 1974) and α- and ß-adrenoceptor antagonists (see *1.11.1-2*). Aside from the α- and ß-blockers, most sympatholytic treatments today find little clinical use in the treatment of essential hypertension, being replaced with more effective antihypertensives. Only modest falls in blood pressure are observed in the SHR during treatment with α-blockers (Watanabe et al, 1980, Chichester and Rodgers, 1987, Sanchez et al, 1989b, Takeda et al, 1991, Nishimura et al, 1993) and ß-blockers (Ieki et al, 1989, Lee et al, 1992), even with very high doses (Owens, 1987, Christensen et al, 1989). The development of tolerance to α-adrenoceptor antagonists occurs even when objective *in vivo* evidence for receptor blockade is present (Jonsson et al, 1992, Young et al, 1993).

The involvement of the RAS has been tentatively explored in previous studies after treatment with α-blockers, these reports supporting a role for the RAS in maintaining blood pressure during acute α-adrenoceptor blockade (Paller and Linas, 1984, Winn et al, 1985). This may be mediated through augmented ß-adrenoceptor-mediated renin release in the kidney or possibly at local tissue sites, or the lack of effect may simply be a response to increased ß-adrenoceptor stimulation in the heart. These antagonists also specifically impair vasopressin (AVP) release in the CNS (Paller

and Linas, 1984, Winn et al, 1985). Further evidence suggests that AVP release is mediated by both angiotensin II (AT_1) and α_1-adrenoceptors in rats (Qadri et al, 1993). The possibility that the RAS is involved in the tolerance exhibited to chronic α-adrenoceptor blockade and other sympatholytics was explored in the present study. The effects of chronic ganglion and α_1-adrenoceptor blockade on genetic hypertension development and *in vivo* responses to inhibition of the RAS have been investigated in order to elucidate the role of the RAS in the tolerance phenomenon. The expression of circulating and tissue ACE activity, left ventricular hypertrophy and tissue 3MH expression has also been evaluated.

6.2 Methods

6.2.1 Animals and drug treatments

The following experiments were conducted as two separate studies, using different drugs but similar protocols. In series one, five week-old male SHR were obtained from the CSIRO Division of Human Nutrition Animal Services Section and allocated to either control (CON) or chronic doxazosin (DOX) treatment groups (n=6/group). Untreated WKY rats served as their controls. Series two experiments involved the chronic treatment of five week-old SHR with either mecamylamine (MEC) or phenoxybenzamine (POB), with appropriate untreated SHRs serving as controls. Doxazosin and mecamylamine were administered orally in the drinking water at concentrations that would allow for doses of 10 mg/kg/day. These were prepared fresh every 2-3 days, and drug concentrations were adjusted to allow for variations in water intake. Phenoxybenzamine has poor water solubility, and was therefore incorporated into a synthetic diet at a concentration that would allow for a dosage of 10 mg/kg/day. Details of the diet are given in Appendix III. All chronic treatments continued for 13 weeks, with fortnightly or monthly systolic blood pressure measurements obtained through indirect tail-cuff plethysmography as previously described (*2.1.3*).

6.2.2 *In Vivo Experiments*

Control and chronic drug-treated SHR were anaesthetised and prepared for *in vivo* experimentation as previously described (2.2). Drugs were dissolved in physiological saline (9% NaCl) and administered via a catheter implanted in the left jugular vein in a volume not exceeding 0.1 ml. This was subsequently flushed with 0.2 mls heparinised saline (25 U/ml). In series one experiments, changes in mean arterial pressure (MAP) responses to potassium losartan (0.05 to 16.75 mg/kg) were measured. In series two experiments, changes in MAP responses to tyramine (0.25 mg/kg), noradrenaline (0.025 to 5 ug/kg), potassium losartan (0.05 to 50 mg/kg cumulative) and the competitive α-adrenoceptor antagonist terazosin (0.25 mg/kg) were measured.

6.2.3 *Angiotensin converting enzyme (ACE) activity*

After the *in vivo* experiments were completed, blood was collected by backflow from the carotid arterial catheter, the animals sacrificed and tissues and plasma treated as described previously (*2.1.4*). Angiotensin converting enzyme activity was measured as described in *2.4.4*. Tissues were homogenised in potassium phosphate buffer (50 mM, pH 8.75) at 100 mg/ml.

6.2.4 *Heart weight to body weight ratios and tissue 3-methylhistidine analysis*

The heart weight:body weight ratios were measured after opening the heart and removing all blood from the left ventricle. The 3-methylhistidine concentrations from vas deferens, aortic and mesenteric vascular tissue were assayed as previously described (*2.4.2*), using 3-ethylhistidine as the internal standard.

6.3 Results

6.3.1 Blood pressure development

The indirect tail-cuff measurements of systolic blood pressure levels of control and chronic DOX-treated SHR are presented in Fig. 6.1. As expected, the SHR developed hypertension over the period of 5 to 16 weeks of age, with systolic blood pressure (SBP) attaining a maximal level at approximately 200 mmHg. The WKY rat displayed a significantly lower, stable SBP over the same period, with measurements never exceeding 130 mmHg. Chronic administration of doxazosin failed to alter genetic hypertension development in the SHR.

The systolic blood pressure measurements of MEC and POB-treated SHR are given in Fig. 6.2. Chronic mecamylamine (MEC) or phenoxybenzamine (POB) treatment failed to significantly alter the development of hypertension in the SHR, although SBP values were intermittently lower during the course of treatment. However, this did not alter the final outcome with respect to hypertension in these groups.

6.3.2 The influence of losartan on blood pressure in SHR treated chronically with sympatholytics

The influence of losartan on directly measured MAP responses in anaesthetised control SHR and WKY rats, and chronic DOX-treated SHR are summarised in Fig. 6.3. The SHR exhibited a greater fall in MAP to losartan than untreated WKY rats at upwards of a 3.0 mg/kg cumulative dosage (WKY: 18.75 mmHg ± 3.5 vs. SHR: 35.7 mmHg ± 6.3 mmHg). The main finding is that DOX-treated SHR showed a significantly greater fall in blood pressure to losartan than untreated SHR at doses upwards of 10 mg/kg (Fig 6.3; SHR: 35.7 ± 6.3 mmHg vs. SHR-DOX: mean 53.7 ± 4.9 mmHg).

The MAP responses to losartan in POB and MEC-treated SHR are displayed in Fig. 6.4. As with doxazosin treatment, POB-treated SHR showed greater falls in blood

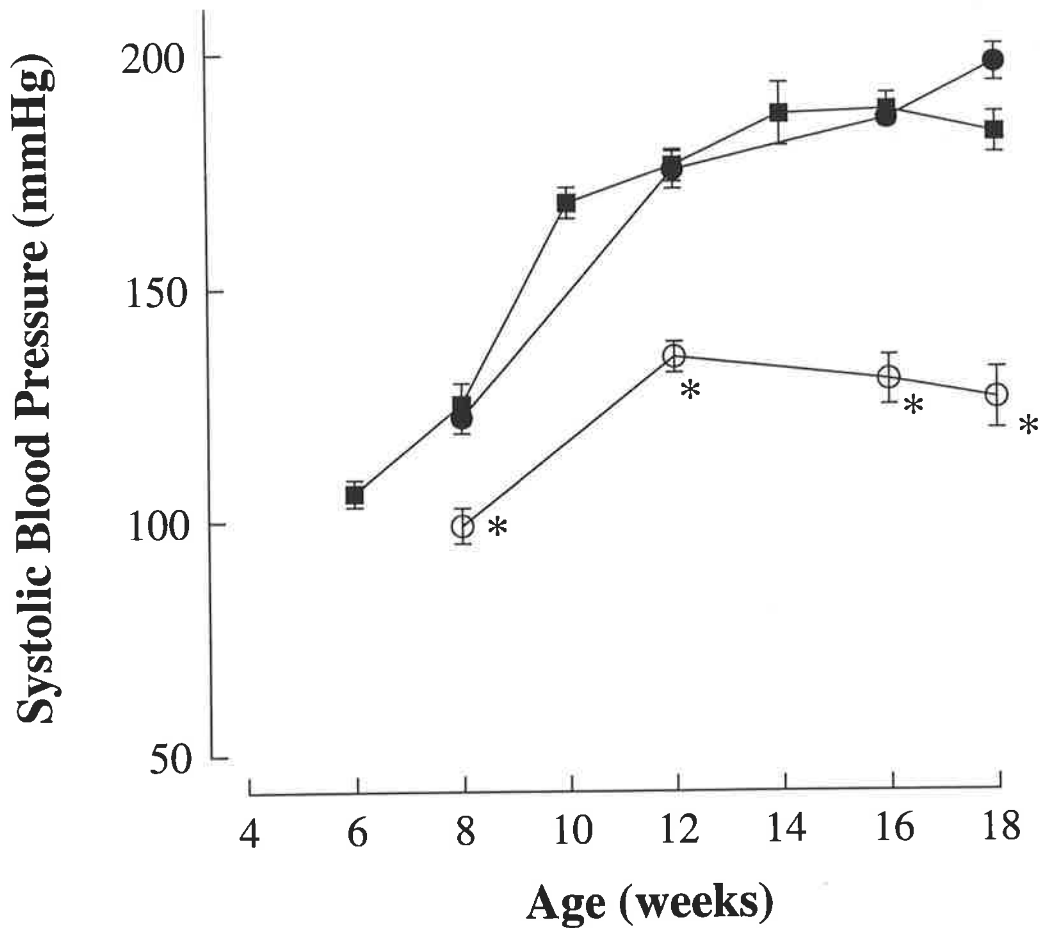


Fig 6.1. Changes in systolic blood pressure with age in untreated (control) SHR (●) and WKY (○) rats, and chronically doxazosin (■)-treated SHR (n=6 per group). Rats were administered doxazosin (10 mg/kg/day) in drinking water. Systolic blood pressure was measured using an indirect tail-cuff technique.

* significant (P<0.05) difference vs. control SHR

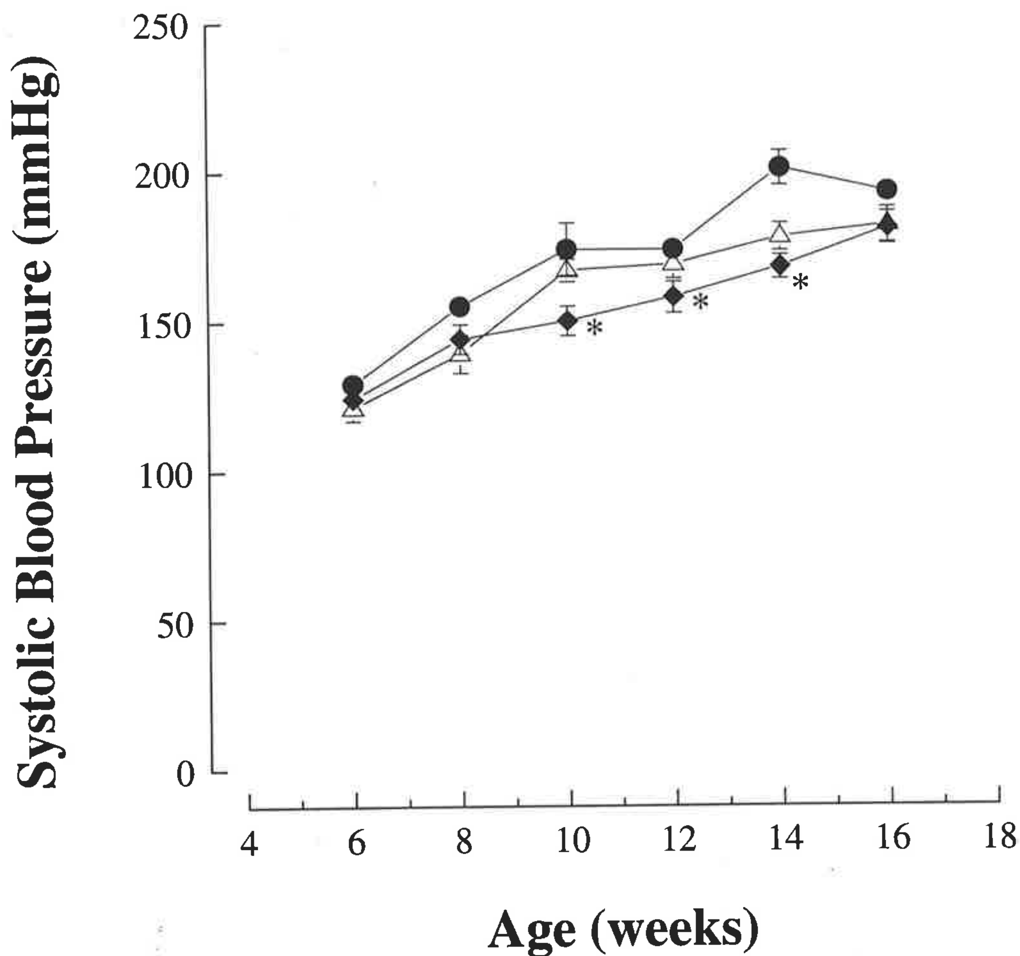


Fig 6.2 Changes in systolic blood pressure with age in untreated (control; ●), phenoxybenzamine (△) and mecamylamine (◆)-treated SHR. Rats were administered mecamylamine in drinking water (10 mg/kg/day) and phenoxybenzamine in a synthetic diet mix (10 mg/kg/day). Systolic blood pressure was measured using an indirect tail-cuff technique.
* significant ($P<0.05$) difference vs. control SHR (n=6 per group).

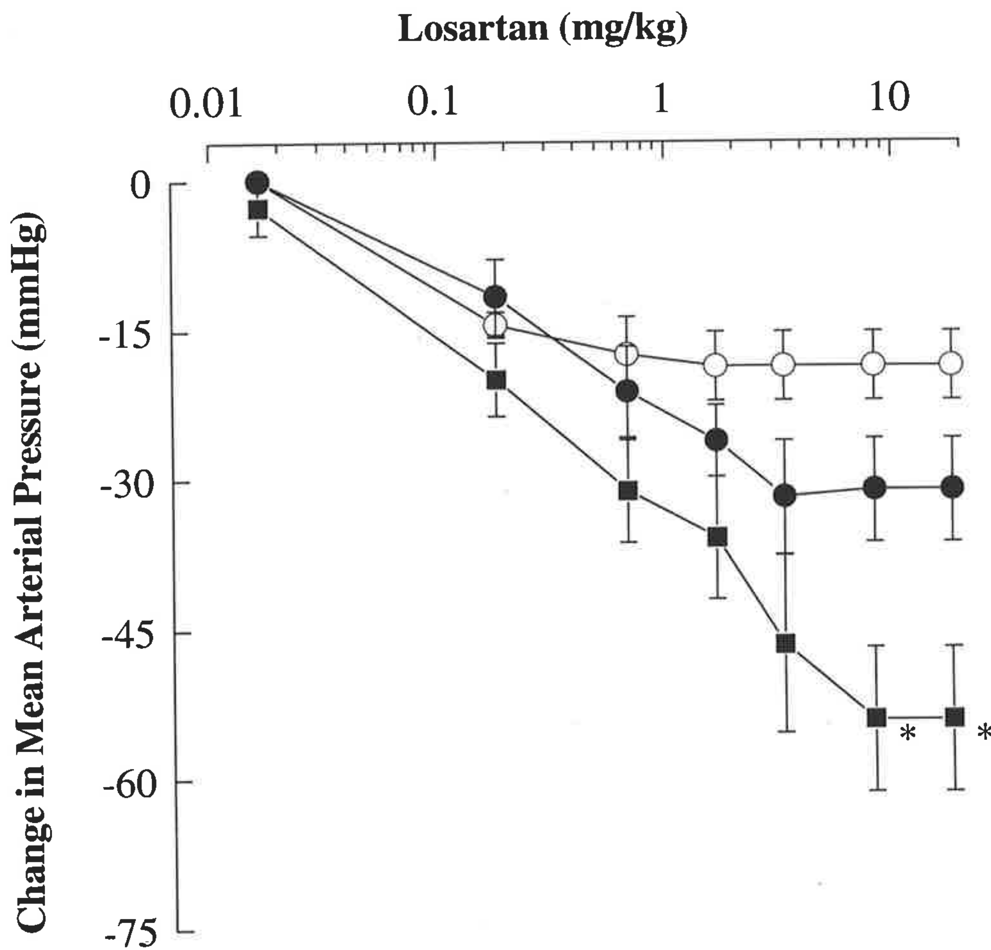


Fig 6.3. In vivo mean arterial blood pressure responses to *i.v.* losartan (0.0165 to 18 mg/kg) in control SHR (●) and WKY rats (○) and chronically doxazosin-treated SHR (■).
N= 6 per group.
* significant ($P<0.05$) difference vs. control SHR.

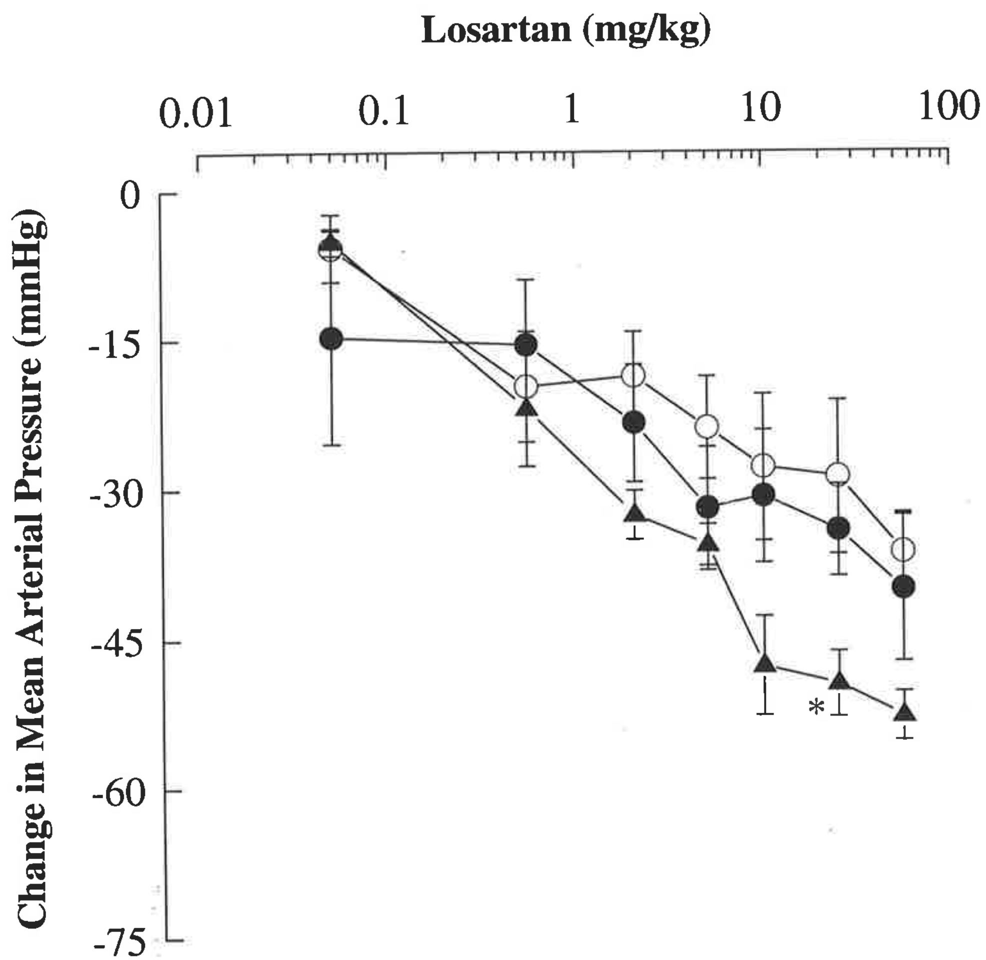


Fig 6.4. In vivo mean arterial blood pressure responses to *i.v.* losartan (0.05 to 55 mg/kg) in control (●), chronically phenoxybenzamine (▲) and mecamylamine (○)-treated SHR. N=6 per group.

* significant (P<0.05) difference vs. control SHR.

pressure to losartan doses upwards of 10 mg/kg. There was no difference in the responses to losartan from mecamylamine-treated SHR when compared with control SHR.

6.3.3 In vivo responses to noradrenaline, tyramine and terazosin

In vivo MAP responses to intravenously administered terazosin and tyramine in anaesthetised control, MEC and POB-treated SHR are summarised in Figs. 6.5 (a) and (b). POB-treated SHR exhibited dampened responses to terazosin and tyramine *in vivo.* MEC-treated SHR also exhibited a modest but nonsignificant attenuation in response to tyramine.

Noradrenaline responses are shown in Fig. 6.6. Noradrenaline elicited increments of up to 100 mmHg at higher doses (1.0 to 5.0 ug/kg) in control SHR. In contrast, the maximal pressor responses of chronic POB-treated SHR were approximately 60% of control SHR at similar doses, indicating a significant degree of α-blockade had been achieved. Interestingly, responses of chronically MEC-treated SHR were also suppressed (to 80% that of control SHR) at the higher doses. *In vivo* noradrenaline responses in chronic DOX-treated SHR were not performed as a previous study in our laboratory found a significant degree of α-blockade under similar treatment conditions (Young et al, 1993).

6.3.4 Angiotensin converting enzyme (ACE) activity

The ACE activities from plasma and tissues are outlined in Figs. 6.7 and 6.8. The WKY rat displayed significantly greater ACE activity in all tissues studied except the aorta. None of the tried sympatholytic treatments modulated ACE activity, although MEC-treatment did have a nonsignificant lowering effect on aortic ACE levels. The reason for this is unclear.

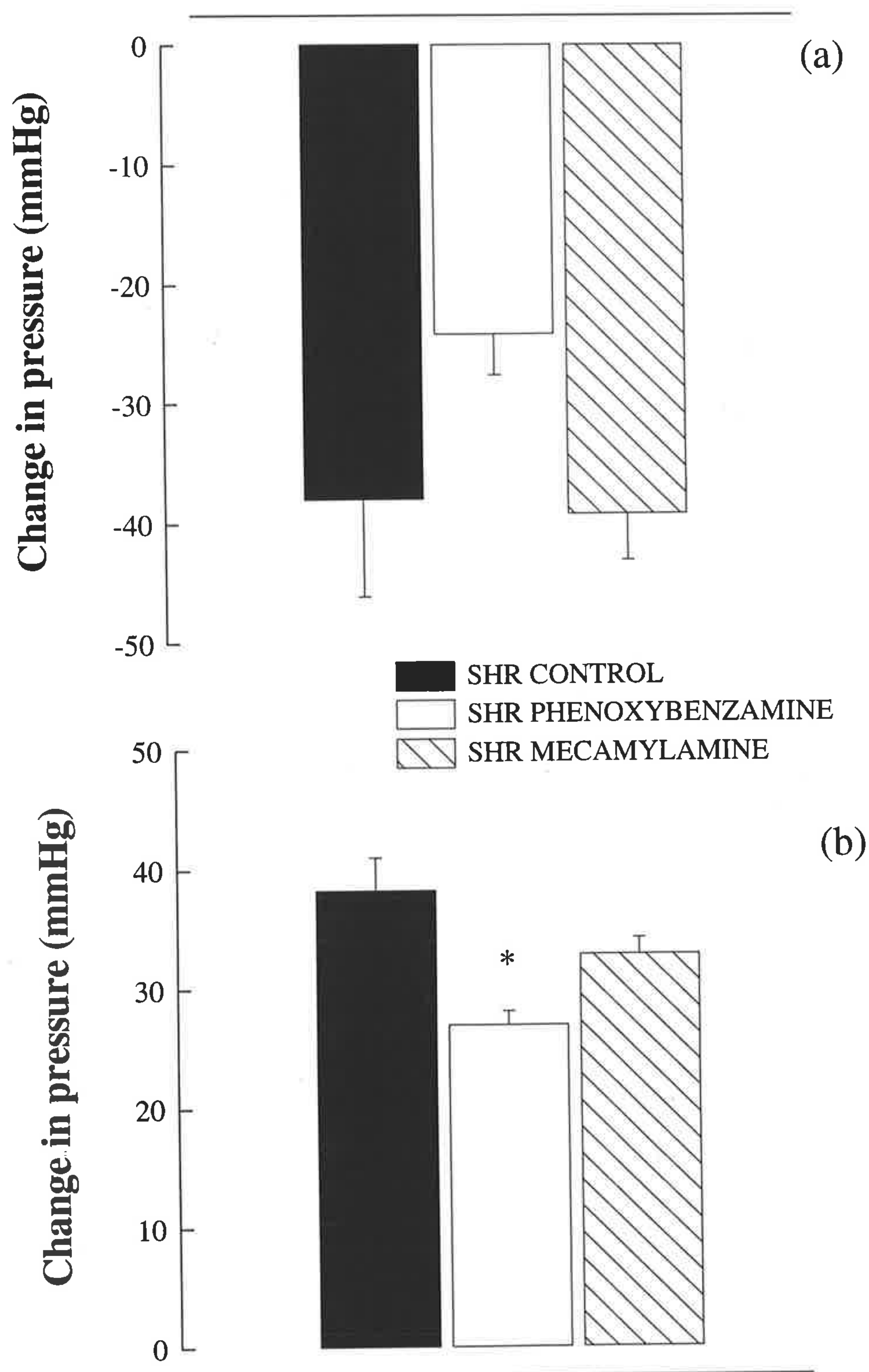


Fig 6.5. Mean arterial blood pressure responses to (a): *i.v.* terazosin (0.25 mg/kg) (b): *i.v.* tyramine (0.25 mg/kg) in control, phenoxybenzamine and mecamylamine-treated SHR. N=6 per group.
* significant ($P<0.05$) difference vs. control SHR.

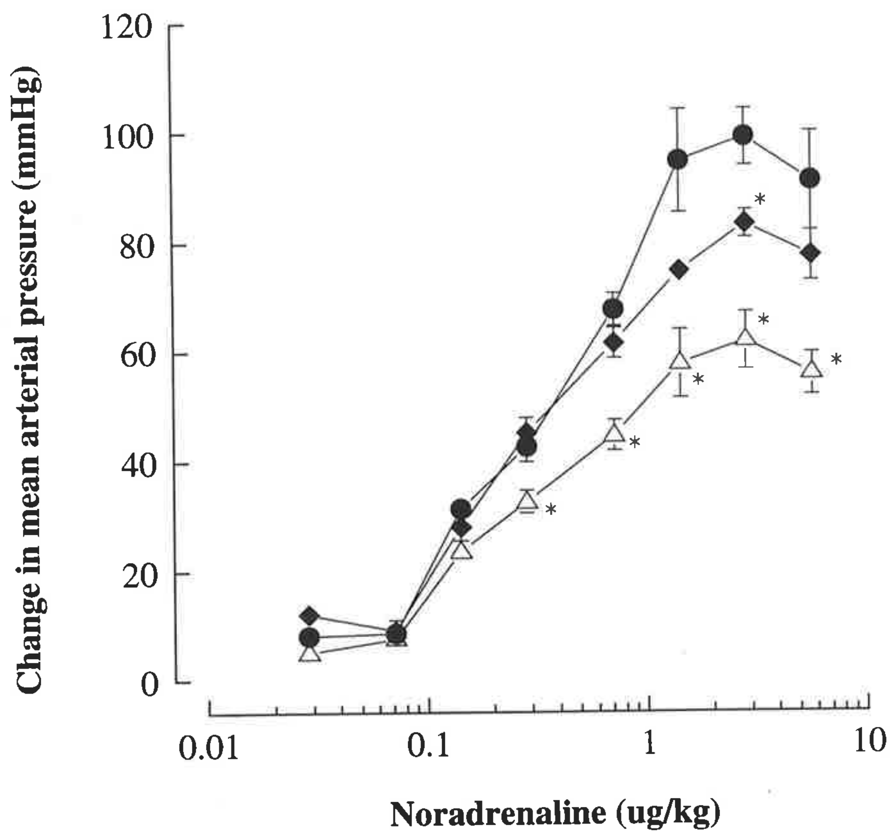


Fig 6.6. *In vivo* mean arterial blood pressure responses to noradrenaline (0.025 to 5 ug/kg) in untreated (control; ●), chronically phenoxybenzamine (△) and mecamylamine - treated SHR. N= 6 per group.

* significant (P<0.05) difference vs. control SHR.

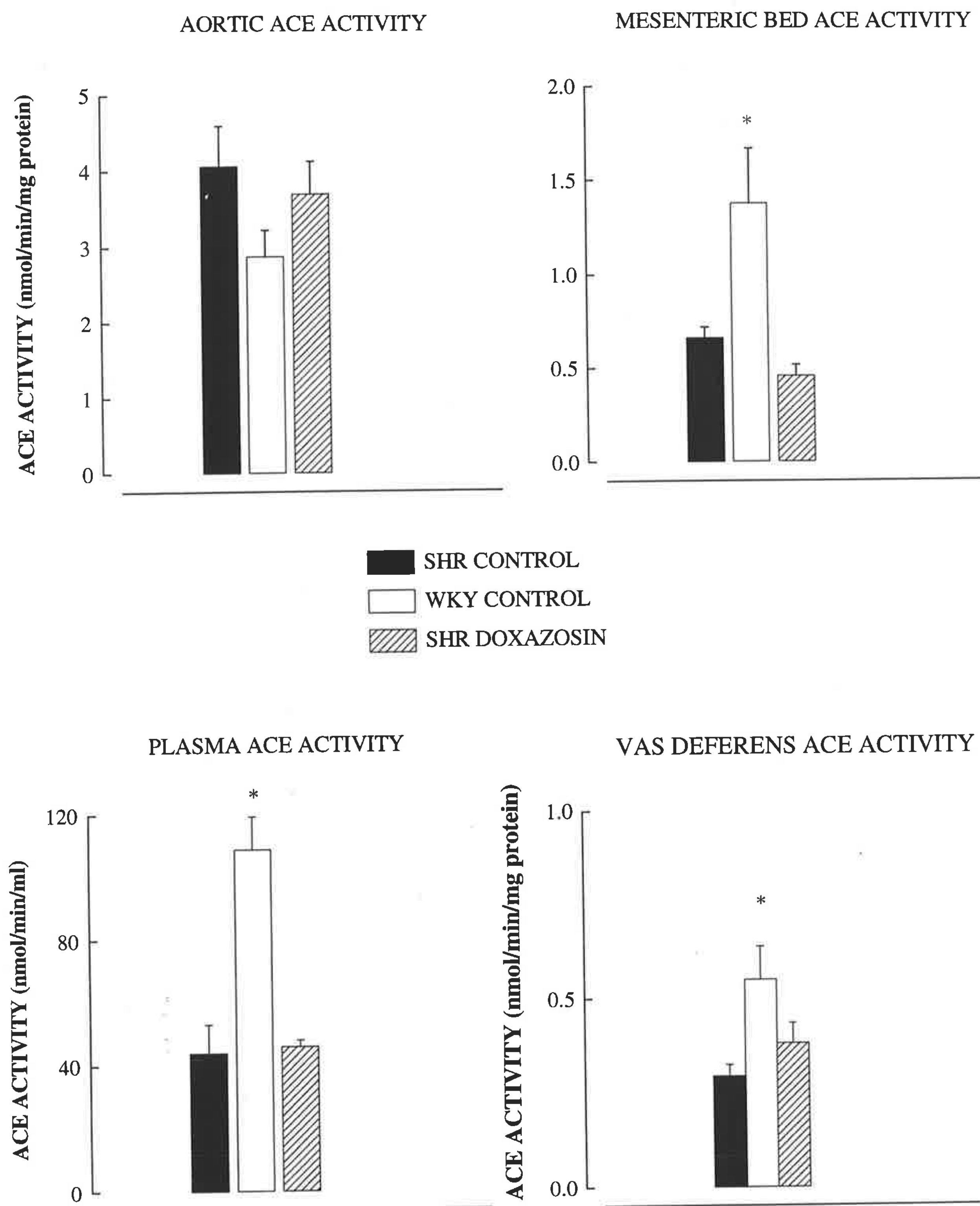


Fig 6.7. ACE activity in plasma and various tissues from untreated (control) SHR and WKY rats, and chronically doxazosin-treated SHR. Activity is measured as nmoles ^{14}C-hippuric acid formed from hydrolysis of substrate (Hippuryl-histidyl-leucine [glycine-1-^{14}C]).

* significant ($P<0.05$) difference vs. control SHR (n=4-6 per group).

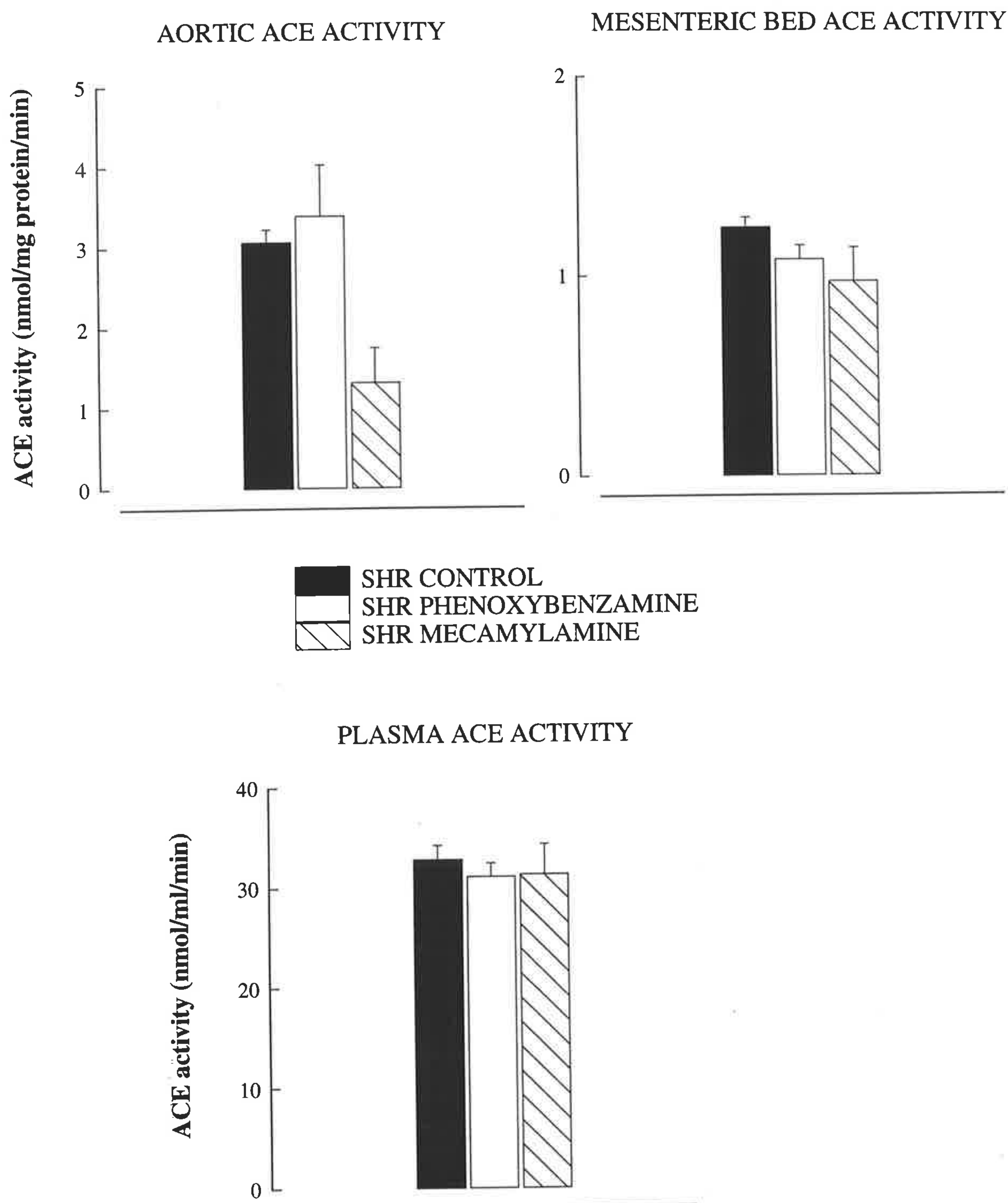


Fig 6.8. ACE activity from plasma and vascular tissue from untreated (control), chronically phenoxybenzamine and mecamylamine-treated SHR (n=4-6 per group). Substrate is Hippuryl-L-histidyl-L-leucine [Glycine-1-^{14}C].

6.3.5 Heart weight:body weight ratios and vascular 3-methylhistidine (3MH) content

Chronic drug treatments had no effect on HW:BW ratios, an index of hypertrophy (Fig. 6.9). The SHR displayed a small increase in this ratio when compared to the WKY rat, but this was not significant. Tissue 3MH levels were unaltered in the mesenteric vasculature, aorta or vas deferens (Figs. 6.10, 6.11). The WKY rat displayed significantly lower levels of 3MH in the aorta and mesenteric vasculature when compared with the SHR.

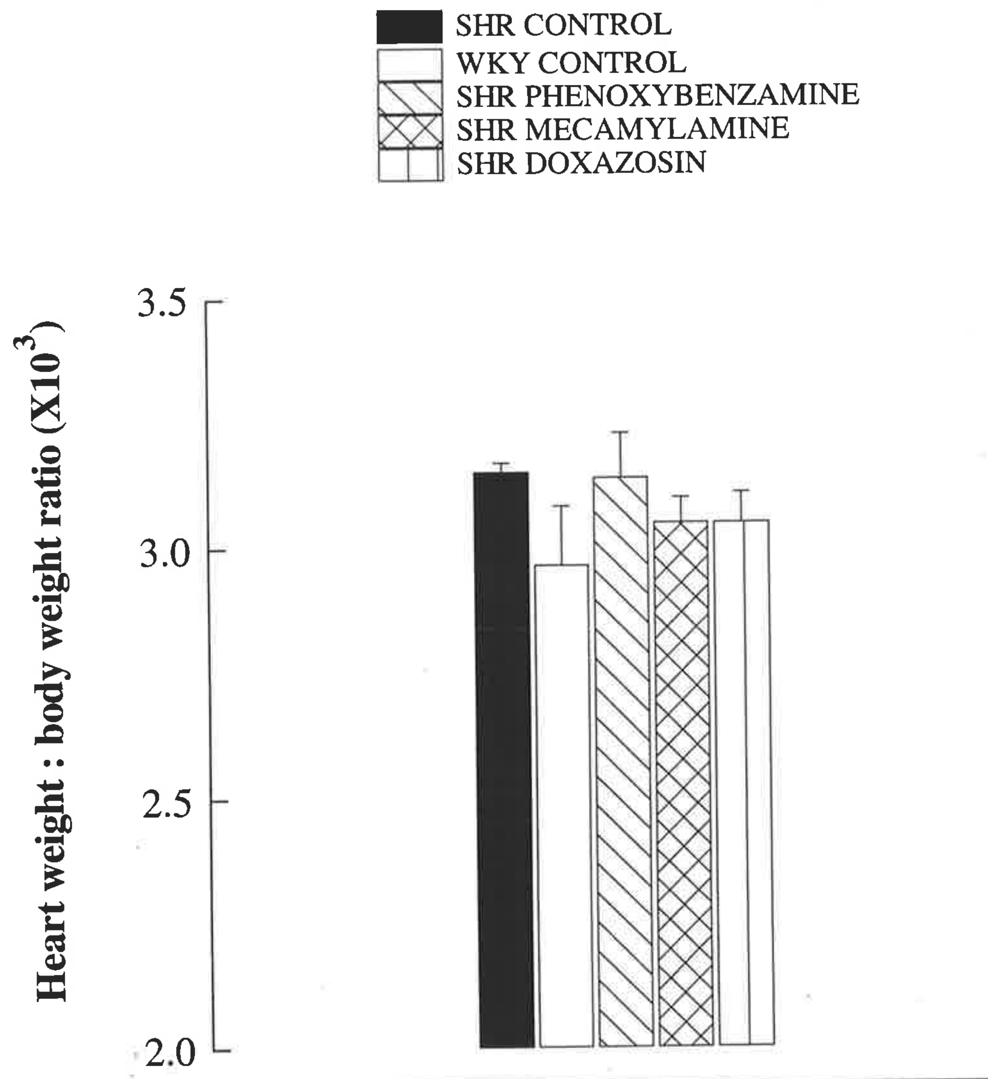
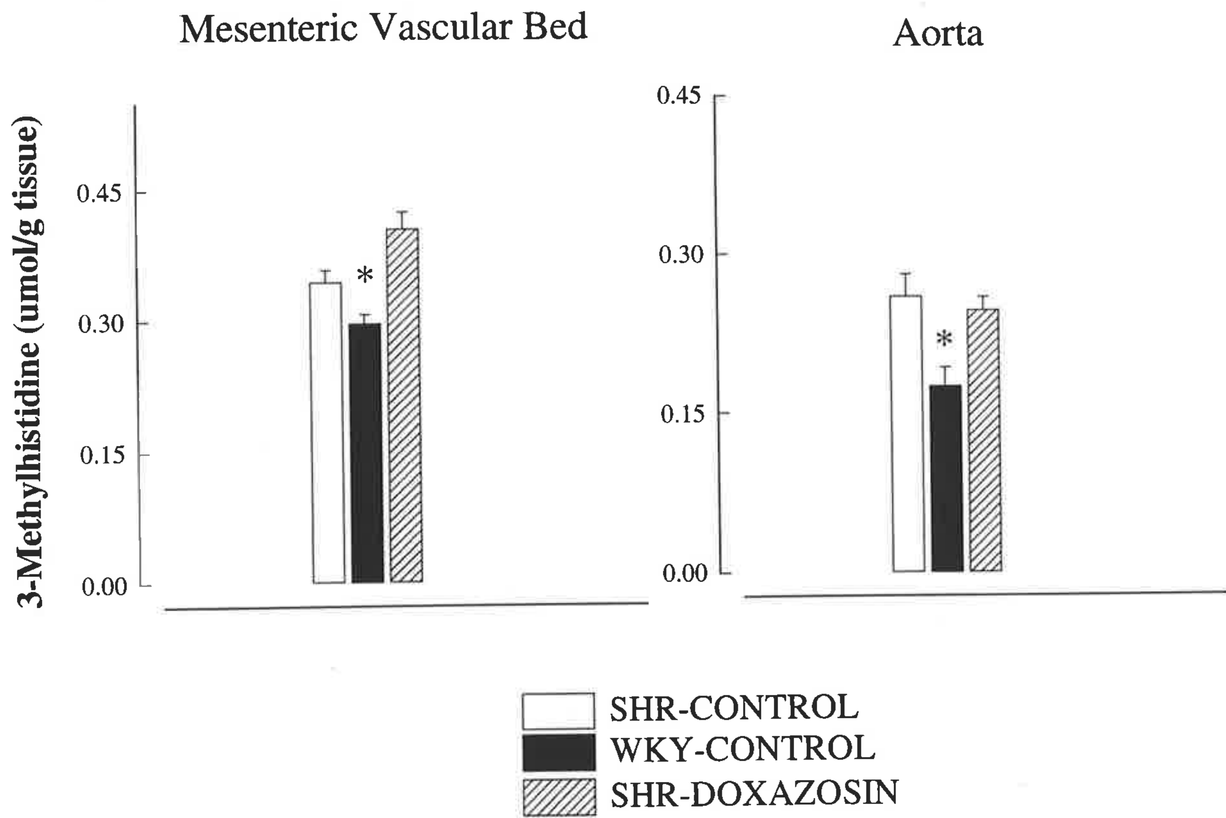


Fig 6.9. Heart weight : body weight ratios in untreated (control) SHR and WKY rats, chronically phenoxybenzamine, mecamylamine and doxazosin-treated SHR. All drug treatments were from 5 to 18 weeks of age. N=6 per group.



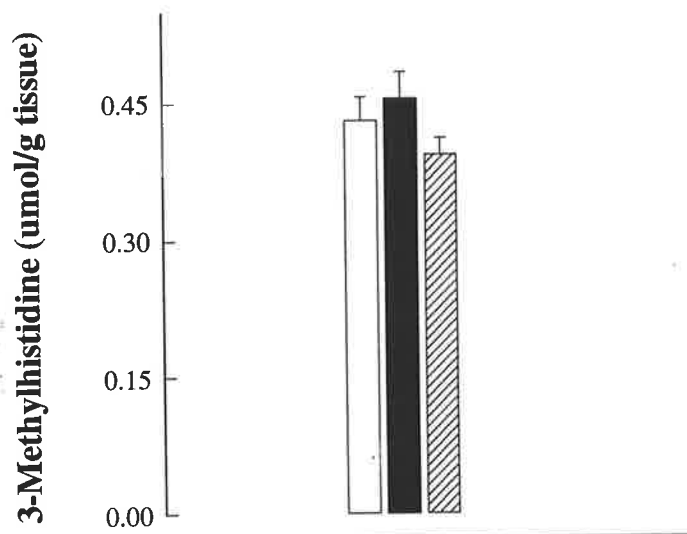


Fig 6.10. 3-Methylhistidine content of various tissues from untreated (control) SHR and WKY rats, and chronically doxazosin-treated SHR. * significant (P<0.05) difference vs. control SHR (n=6 per group).

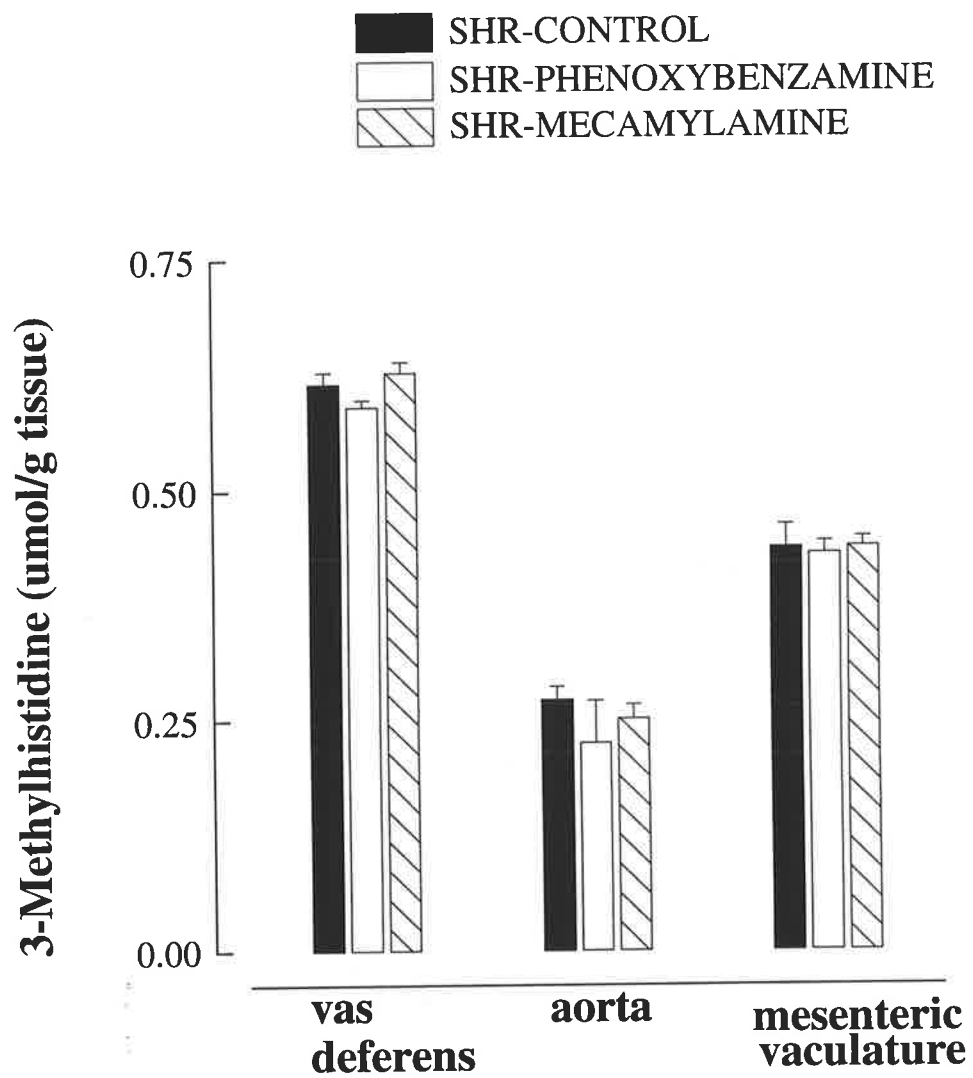


Fig 6.11. 3-Methylhistidine concentrations of various tissues from untreated (control), chronically phenoxybenzamine and mecamylamine-treated SHR (n=6 per group).

6.4 Discussion

The foregoing results highlight some interesting features of sympatholytic therapy in this strain, namely a lack of capacity to control genetic hypertension development. With regard to α-adrenoceptor antagonists, this is a consistent finding (Giudicelli et al, 1980, Jonsson et al, 1992, Young et al, 1993). These results present a paradox with respect to the evidence which suggests that: (i) quinazoline derivatives are effective antihypertensives when acutely administered orally in this model (Kyncl, 1986) and (ii) the sympathetic nervous system contributes to the prevailing blood pressure difference between adult SHR and WKY rats (Leenen and Klement, 1989, Jablonskis et al, 1992). The observation that a significant degree of α-blockade to phenoxybenzamine was present *in vivo* is indirect evidence that pharmacokinetic tolerance to this agent did not occur. This was similarly observed for doxazosin (Young et al, 1993) and terazosin (Jonsson et al, 1992). Direct measurements of serum doxazosin levels after prolonged oral treatment in the SHR have suggested that levels adequate for profound systemic α-blockade were still present (Chichester and Rodgers, 1987).

The time course for the antihypertensive action of a single dose of terazosin shows a maximal effect (70 mmHg fall in blood pressure) one to five hours following oral administration in the SHR, with a return to predrug levels after approximately 24 hours (Kyncl, 1986). When SBP was measured daily during acute doxazosin treatment in adult SHR (dosed by gavage every 12 hours for 5 days), tolerance developed to high doses within 4 days (200 mg/kg/day), with a maximal antihypertensive effect after one day. In contrast, 20 mg/kg/day doxazosin-treated SHR did not develop tolerance (Chichester and Rodgers, 1987). After chronic oral (drug in drinking water) dosing from 15 to 23 weeks of age (16 mg/kg/day), tolerance was also observed (Chichester and Rodgers, 1987).

Phenoxybenzamine, in a similar fashion to competitive α-blockers, has only a modest effect on SBP in the adult SHR using oral doses from 1 to 100 mg/kg/day (Watanabe et al, 1980). Heart rate was not altered by phenoxybenzamine treatment and the inclusion of a ß-blocker (propranolol) did not lower SBP, suggesting that changes in

cardiac output were not compensating for the vascular systemic effects of α-blockade (Watanabe et al, 1980). The rationale for utilising a nonselective antagonist such as phenoxybenzamine arises from the suggestion that postsynaptic α_2-adrenoceptors may be involved in the control of vascular tone in the SHR (Medgett et al, 1984), and possibly underlie the lack of effect of α_1-antagonists. However, others have shown a lack of effect of yohimbine, alone or in combination with an α_1-blocker, in this strain (Sanchez et al, 1989b, Young et al, 1993). The results with phenoxybenzamine support these previous findings. With regard to an α_2-adrenoceptor-mediated mechanism of tolerance, these findings may be difficult to resolve, as an equally feasible argument suggests that blockade of presynaptic α_2-adrenoceptors would facilitate NA release and impair subsequent α_1-blockade by phenoxybenzamine at VSMC α_1- and α_2-adrenoceptors (Winn et al, 1985).

In this study, ACE activity was not altered by chronic α-blockade. However, as ACE levels were generally elevated in WKY rat tissues and plasma when compared to the SHR, then the interpretation of potential differences in enzyme activities from the drug treatments is difficult. The SHR exhibited higher levels of aortic ACE activity. The increased ACE activity in WKY rats extends to not only the plasma, but also lung and kidney in developing and adult animals (Grima et al, 1990, Okunishi et al, 1991). The increased activity in aortic tissue from the SHR is also a consistent finding (Okunishi et al, 1991). Gene expression for ACE has been investigated in vascular tissue from the SHR, where findings show a large increase in mRNA in mesenteric arteries (but not the aorta) when compared with WKY rats (Fernandez-Alfonso et al, 1994). In contrast, ACE activity in the present study is elevated in the aorta and reduced in the mesenteric arteries. While the differential regulation of ACE message and activity is in itself interesting, other markers of RAS activity should be considered for use with the SHR.

Although not measured in this study, Watanabe et al (1980) found evidence of increased plasma renin activity (PRA) in the SHR after prolonged phenoxybenzamine treatment. It is possible that increased circulating catecholamines may have augmented β_1-mediated renin release in this instance, as concomitant propranolol treatment has been

shown to abolish the increase in PRA to well below control levels (Watanabe et al, 1980).

Angiotensin II may play a critical role in the mechanism by which tolerance is displayed to α-blockers, in an extension of its role as an amplifier of responses to noradrenaline as discussed in Chapter 5. The AII-mediated amplification of vasoconstriction to α-agonists appears to be greater in cases of reduced α-receptor reserve, as has been shown with the use of another irreversible α-blocker, benetrexamine (Purdy and Weber, 1988). In the case of the SHR, where autocrine/paracrine vessel RAS expression is believed to be integral in the local control of vascular tone and VSMC growth, the effects of blocking α-receptors may be offset by the increased facilitatory actions of AII on the remaining vascular adrenoceptors, with an increased uptake of plasma renin supplementing the cascade. This could be tested in a simple study design, whereby the effects of a combination of high dose α-antagonist/low dose ACE-inhibitor or AII antagonist on GHD could be compared to the α-blocker alone.

The reason that inhibition of the RAS, but not the SNS, is so successful in preventing hypertension development in the SHR is unclear. Three pressor systems are responsible for the maintenance of blood pressure in the rat; i.e., the RAS, SNS and arginine vasopressin (AVP) system. Under conditions of acute α-blockade, both the SNS and AVP system are inhibited, as the release of AVP has been shown to be in part mediated by α-adrenoceptors (Paller and Linas, 1984, Qadri et al, 1993). The RAS is clearly able to compensate for α-blockade in this situation (Winn et al, 1985). In fact, the degree of its recruitment was similar despite differences in the blood pressure fall generated by phentolamine versus phenoxybenzamine, suggestive that it was the level of α-blockade rather than the change in blood pressure that was the stimulus for RAS activation (Winn et al, 1985). This suggests a link between the two systems independent of blood pressure changes. Effective blood pressure control via RAS impairment may reflect the inhibition of the AII-mediated augmentation of sympathetic activity at multiple sites in the SNS (Lewis and Coote, 1993, Schwieler et al, 1993), causing inhibition of various pressor systems dependent on angiotensin II for their

overexpression in this model. In contrast, α and ß-adrenoceptor blockade may not alter, or possibly even facilitate, activity of the tissue RAS (Purdy and Weber, 1988).

Ganglion blockade was also ineffective in preventing GHD in the SHR, but as this was not accompanied by an augmented RAS, it is unclear as to how tolerance developed to mecamylamine in this setting. The dosage employed was approximately one hundred and forty times that needed to produce measurable blood pressure falls in man (Taylor, 1987). Pharmacological blockade of the ganglion was not demonstrated directly, so the possibility that pharmacokinetic tolerance had developed cannot be excluded in the present study. Mecamylamine and other nonquaternary agents are almost completely absorbed after oral administration, and the development of tolerance to their actions is less than that of other ganglion-blocking drugs (Bowman et al, 1968). It is possible that increased AVP levels may have supported blood pressure during ganglion blockade, as α-adrenoceptor function was not significantly impaired from mecamylamine treatment. Jablonskis and Howe (1993) note that AVP, while not contributing to the maintenance of hypertension in the SHR, supplements blood pressure during acute ganglion blockade in this model. This is in contrast to acute α-blockade (Winn et al, 1985).

The possibility that an upregulated RAS may have additive effects on the aberrant medial hypertrophy/remodelling of the vasculature in the SHR was explored with the 3MH analysis of blood vessels from chronically α-blocked SHR. While there were significant differences with respect to aortic and mesenteric vascular tissue between SHR and WKY rats, this was not extended further in the SHR after prolonged α-blockade, although 3MH was slightly elevated after doxazosin treatment. Possible reasons centre on the degree and site (plasma or local) of RAS upregulation, or that vascular changes in contractile proteins and actin may already be maximal in this strain. Heart weight to body weight ratios indicate that α-blockade did not modify the development of LV hypertrophy. Others have found a cardiomyocyte growth-promoting capacity of the SNS and catecholamines, potentially linked to α_1-adrenoceptors both *in vivo* (Korner et al, 1993) and *in vitro* (Ikeda et al, 1991). These findings are not supported by the results of the present study, or those utilising solely chronic monotherapy with α-blockers in the SHR (Nishimura et al, 1993).

In summary, the tolerance displayed to chronic α-adrenoceptor antagonism in the developing SHR is accompanied by an increased activity of the RAS, which contributes to the high blood pressure in the face of substantial α-adrenoceptor blockade in this model. This phenomena highlights the interaction and recruitment of neurohumoral systems, in particular the RAS, that are utilised to maintain expression of the phenotype in the SHR.

CHAPTER 7

Aspects of Contractile Protein and 3-Methylhistidine in the SHR and the Effects of Losartan on these Parameters in Relation to Blood Pressure and Cardiovascular Structure and Function

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7.1 Introduction

As observed in the Chapter 5, losartan attenuated the development of hypertension and normalised vascular reactivity in the SHR. However, there were some differences with respect to captopril treatment. Firstly, vascular reactivity was attenuated to levels of the WKY rat, but the reduction in blood pressure was not. Secondly, after drug withdrawal, there was no transient increase in blood pressure and vascular reactivity was not completely reversed. These discrepant findings may have been related to the degree of blood pressure reduction achieved with losartan. That is, the dose of captopril equipotent with 10 mg/kg/day losartan with regard to blood pressure may have had similar effects on vascular reactivity, or alternatively, a higher dose of losartan may have had effects on these parameters equal to those seen with 100 mg/kg/day captopril. With this in mind, the effects of chronic high-dose (30 mg/kg/day) losartan treatment and its withdrawal on blood pressure, vascular reactivity, cardiac hypertrophy and vascular 3MH were examined in the present study.

Losartan can be used to determine whether the effects of ACE-inhibition are solely attributable to the prevention of AII formation, or whether there is a component mediated through another product of ACE, specific to the drug used or occurring at the AT_2 receptor. Amongst these is a suppression of the sympathofacilitation attributed to AII (Malik and Nasjletti, 1976), which was investigated using the nerve-stimulated perfused mesenteric vascular bed (PMVB). The acute effects of AII with or without losartan allowed a specification of the receptor subtype (AT_1 or AT_2) subserving the actions of AII on the perivascular sympathetic nerves or the vascular smooth muscle.

Despite agreement that blood vessels from the SHR exhibit alterations in structure, the exact type of changes occurring in the vasculature is not known with certainty. Central to this issue is the question of altered medial mass in resistance vessels from this strain, with some researchers suggesting vessels remodel their existing media about a smaller lumen, causing an increased media:lumen ratio without extensive VSMC hypertrophic and/or hyperplastic change (Mulvany, 1993). In larger vessels it is generally well accepted that hypertrophy of the VSMC is responsible for the increased media:lumen ratio (Owens, 1987, Lee and Smeda, 1985). The larger vessels may

possess less developmental plasticity as an explanation for the observed differences, which may be a consequence of the variable degree of sympathetic innervation and prevailing blood pressure across vessel types.

The changes in resistance vessel contractile protein within this context suggest that total actin and myosin content may be unaltered relative to vessel weight, but comparisons of these proteins between strains have been limited, especially for actin, which makes up the bulk of contractile protein in the VSMC (Cohen and Murphy, 1979). However, a previous report has observed an increased resistance vessel actin concentration relative to tissue weight in the SHR, indicating selective increases in the contractile protein content in these vessels (Brayden et al, 1983).

The use of 3MH as an indicator of vascular actin levels in previous chapters has revealed ambiguous findings representative of mesenteric blood vessels from SHR and WKY rats, necessitating further elucidation of the actin protein in samples of these vessels. The effects on actin in response to chronic captopril treatment and its withdrawal have also been compared with 3MH measurements presented in earlier studies. In addition, a dissection of the mesenteric vascular bed was performed in an attempt to further characterise the level at which changes in 3MH may occur in this vascular tissue from the SHR. Specifically, a comparison of the expression of this marker between the superior mesenteric artery (conducting vessel) versus mesenteric arterioles and finer branches (resistance vessels) of SHR and WKY rats was performed.

7.2 Methods

7.2.1 Animals and drug treatments

Male 5 week-old SHR were obtained and maintained as described previously (*2.1.1*). Rats were immediately allocated to control (SCON; untreated), chronically losartan-treated (SLOS; 30 mg/kg/day p.o) and withdrawn (SLOSREL; "released") groups (n=5/group). Losartan was administered in the drinking water from 5 to 13 weeks of age, whereupon a subgroup of treated SHR were withdrawn from losartan

("released") for 5 weeks. The remainder of the SHR continued treatment until 18 weeks of age, whereupon all animals were sacrificed. Fortnightly measurements of systolic blood pressure were performed as described previously (*2.1.3*).

For the 3MH dissection study, adult (20-24 week-old) SHR and WKY rats were sacrificed and the mesenteric vascular bed isolated and cleaned of adhering connective and adipose tissue. All first order branches were severed from the superior mesenteric artery. The artery (conducting vessel) and all branches (resistance vessels) were processed separately for 3MH values. For the acute organ bath studies, perfused mesenteric vascular preparations from untreated (control; 17-18 weeks of age) SHR and WKY rats from a previous study (see *5.2.1*) were utilised.

For the actin measurements, aortic and mesenteric vascular tissue was used from untreated SHR and WKY rats sourced from previous studies. Aortic samples from captopril-treated and captopril-withdrawn "released" SHR were derived from animals used in Chapter 5. However, mesenteric vascular tissue from this study was not used as preliminary sample electrophoresis indicated significant protein degradation, as shown by the absence of actin banding and enhanced staining in low molecular weight sections relative to other samples.

7.2.2 Perfused mesenteric vascular bed (PMVB)

Chronic losartan treatment

After sacrifice, the perfused mesenteric vascular bed (PMVB) was prepared as previously described (*4.2.2*). Perfusion pressure responses to intraluminally applied noradrenaline and serotonin (both 0.025 to 20 ug in ascorbic saline) were obtained. Potassium chloride (1.0-24 mg) responses were also measured.

Acute effects of AII

After sacrifice, the PMVB from untreated SHR and WKY rats was prepared as described previously (*4.2.2*). Responses to nerve stimulation (2-32 Hz) alone, in the presence of a subthreshold concentration of AII (10 nM) and in the presence of both AII (10 nM) and losartan (1 uM) were obtained. AII, and then losartan were infused 10 mins prior to and during nerve stimulation.

7.2.3 3-Methylhistidine analysis

Mesenteric resistance (branches) and conducting vessels (superior mesenteric arteries) were assayed for 3MH as previously described (*2.4.2*), using 3-ethylhistidine as the internal standard. Heart weight to body weight ratios were measured as previously described (*6.2.4*).

7.2.4 Tissue preparation and electrophoresis of actin

The preparation of vascular tissue for separation and quantitation of actin has been described previously (*2.4.5*). A full listing of preparative procedures and reagents is described in Appendix VI.

7.3 Results

7.3.1 Blood pressure

The pattern of systolic blood pressure (SBP) development in untreated (control) and chronically losartan-treated (SLOS) and released (SLOSREL) SHR is shown in Fig. 7.1. At the higher dose of 30 mg/kg/day, hypertension development was prevented up until treatment cessation, when SBP levelled off under 150 mmHg. Interestingly, withdrawal of losartan resulted in a rapid return of SBP to hypertensive levels (Fig. 7.1).

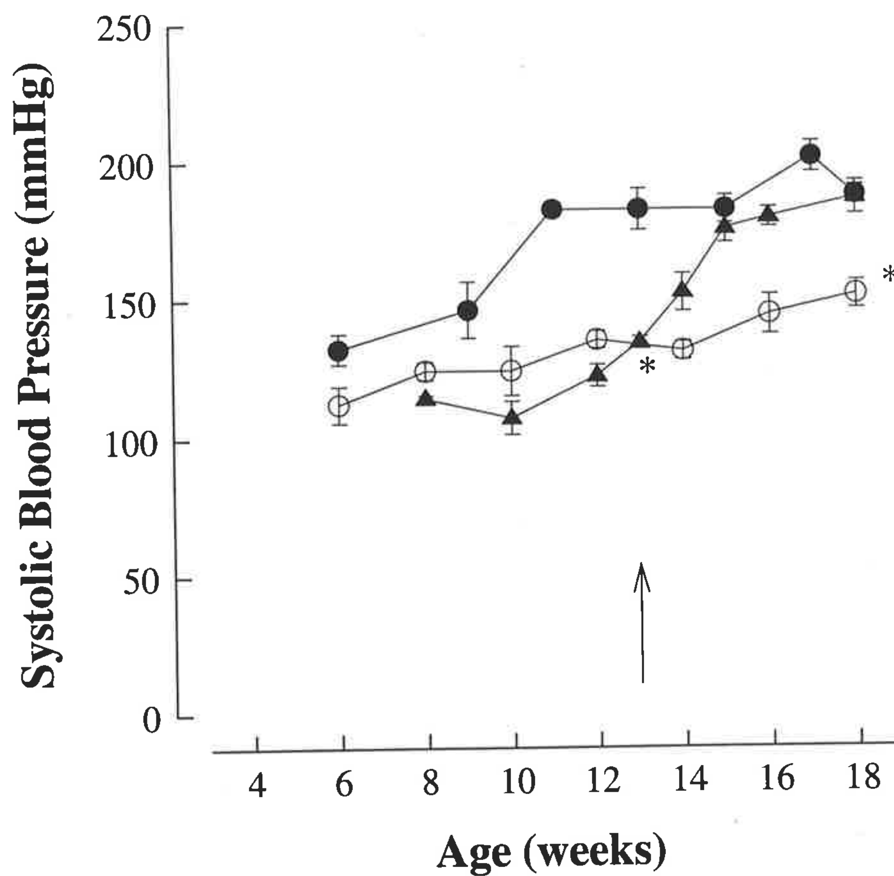


Fig 7.1. Changes in systolic blood pressure with age in untreated (SCON; ●), chronically losartan-treated (SLOS; ○) and losartan-withdrawn "released" (SLOSREL; ▲) SHR (n=5-6 per group). Arrow denotes time of release from losartan. Losartan was administered in the drinking water (30 mg/kg/day). Systolic blood pressure was measured using an indirect tail-cuff technique.

* significant ($P<0.05$) difference vs. SCON at 13 and 18 weeks of age.

7.3.2 Vascular reactivity

Noradrenaline

The perfusion pressure responses to noradrenaline (NA) in SCON, SLOS and SLOSREL groups are graphed in Fig. 7.2 (a). Chronic losartan treatment caused a significant suppression in the NA dose-response profile in the PMVB, a feature that was maintained after 5 weeks of drug withdrawal despite the reversion of blood pressure to the hypertensive state within that time (SLOSREL group).

Serotonin

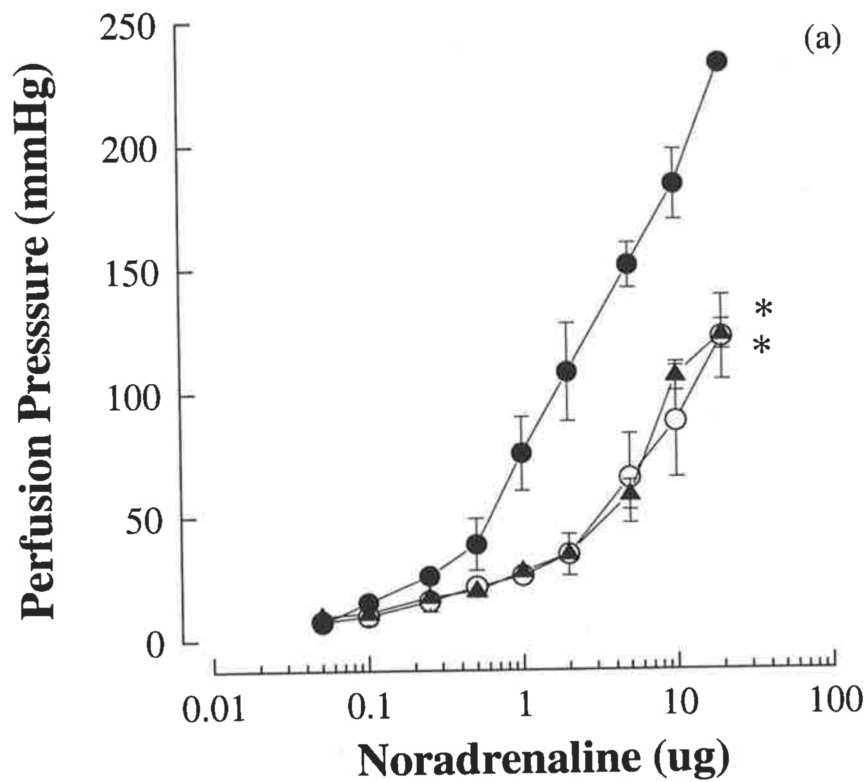
The perfusion pressure responses to serotonin (5HT) in SCON, SLOS and SLOSREL groups are shown in Fig. 7.2 (b). Chronic losartan treatment elicited a profound (over 50%) suppression in the 5HT dose-response curve. This attenuation was, like the noradrenaline responses, maintained after treatment withdrawal (SLOSREL group).

Potassium chloride (KCl) responses of the PMVB

The perfusion pressure responses to KCl in SCON, SLOS and SLOSREL groups are shown in Fig. 7.3. There were no significant differences in vascular reactivity to KCl in any of the groups studied, with responses showing a high degree of variability about the mean.

Angiotensin II-mediated sympathofacilitation; effects of losartan

The perfusion pressure responses to nerve stimulation in untreated (control) SHR and WKY rats are graphed in Figs. 7.4 (a,b). The SHR showed a significantly enhanced response to nerve stimulation over the upper frequency range when compared to the WKY rat (240 mmHg versus 160 mmHg respectively, 32 Hz). The effects of AII



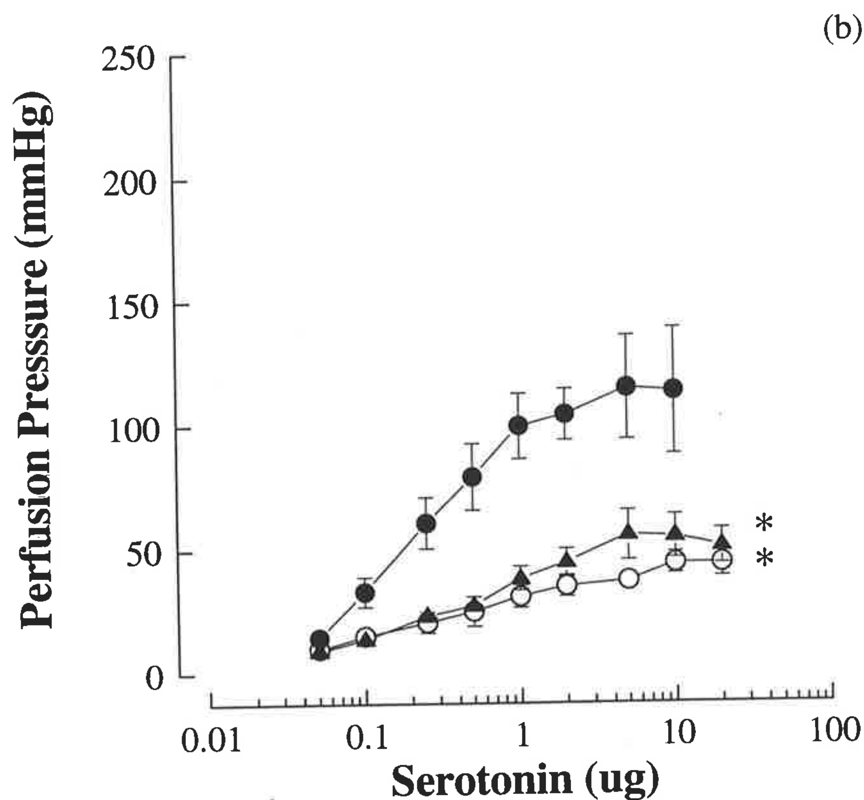


Fig 7.2. Changes in perfusion pressure of the isolated perfused mesenteric vascular bed to (a) noradrenaline and (b) serotonin in untreated (SCON: ●), chronically losartan-treated (SLOS; ○) and losartan-released (SLOSREL; ▲) SHR, (n=5 per group).

* significant (P<0.05) difference vs. SCON over upper dose range.

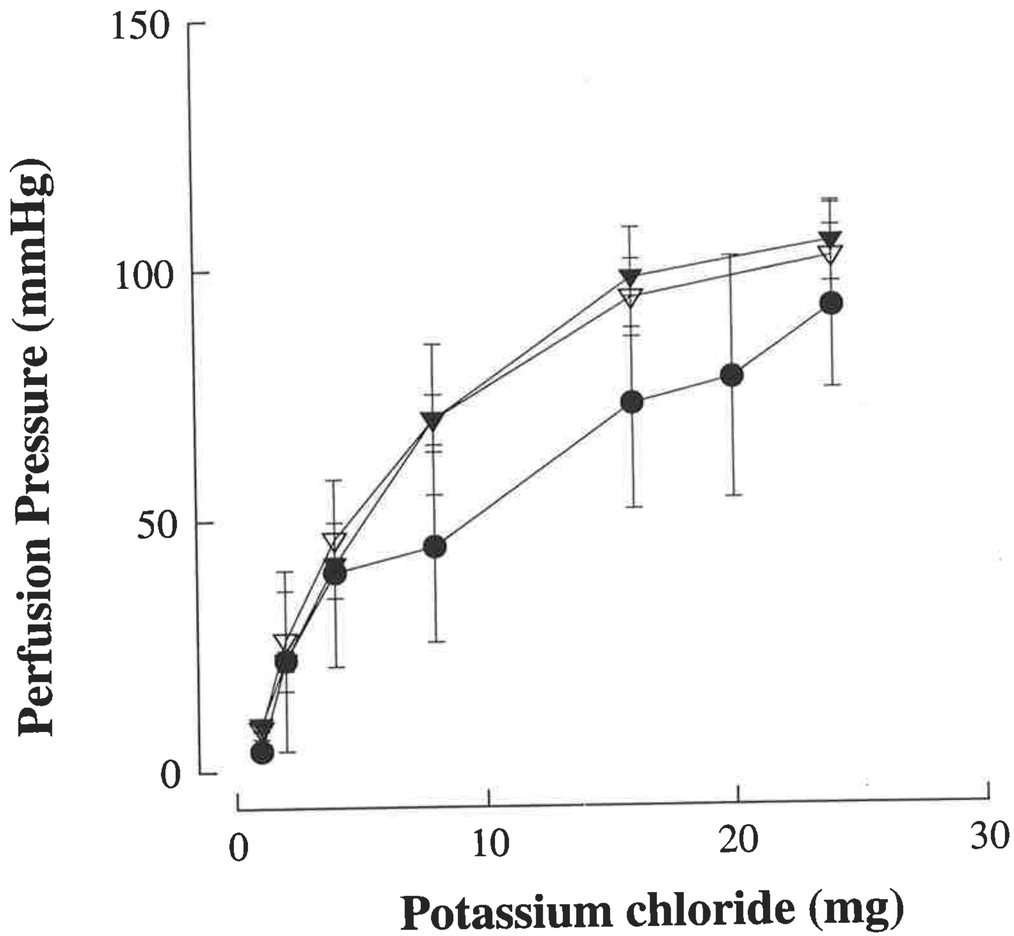


Fig. 7.3. Changes in perfusion pressure of the isolated perfused mesenteric vascular bed to potassium chloride from untreated (SCON; ●), chronically losartan-treated (SLOS; ▽) and losartan-released (SLOSREL; ▼) SHR, n=5 per group.

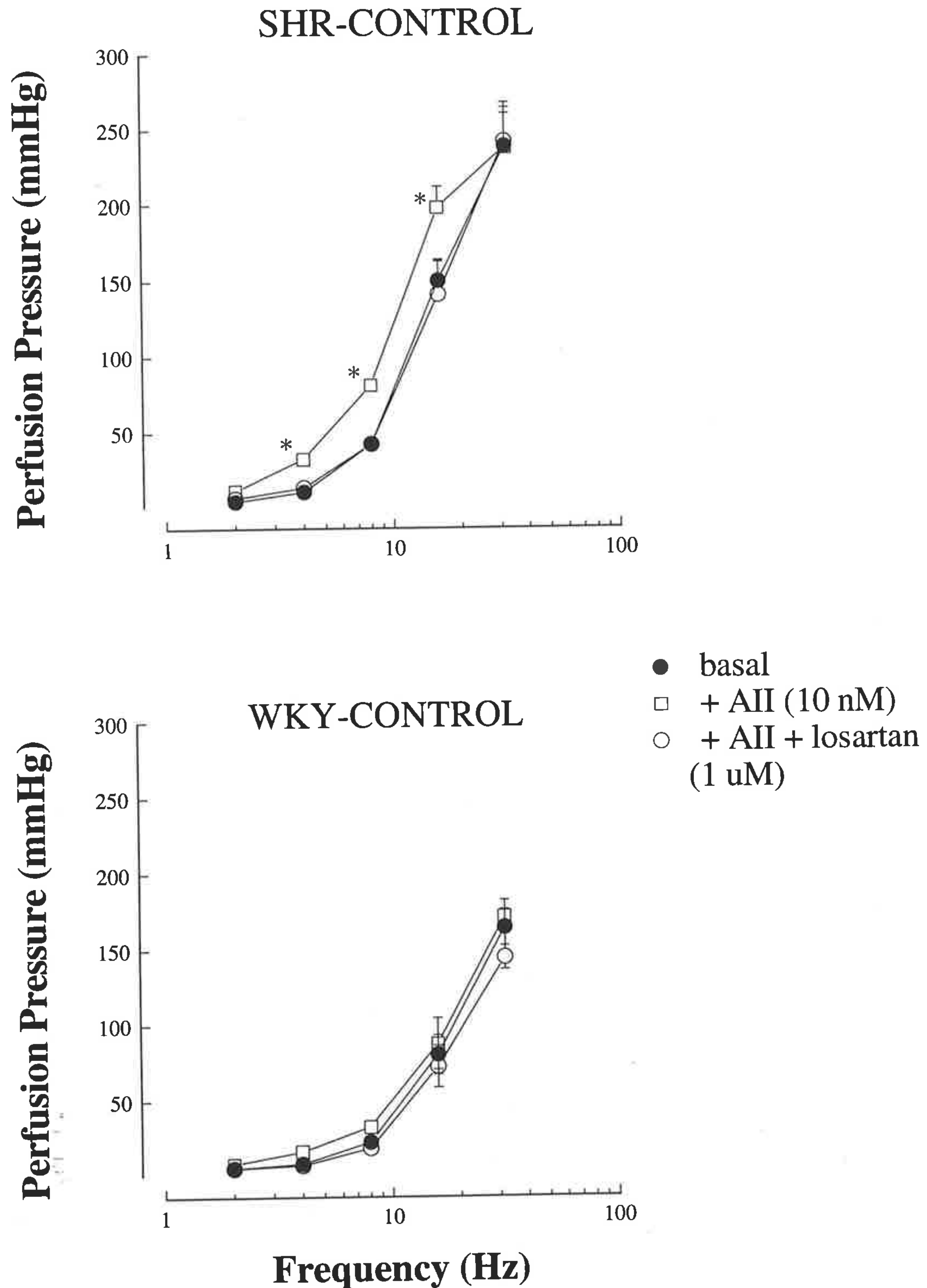


Fig 7.4. Changes in perfusion pressure to perivascular sympathetic nerve stimulation (2-32 Hz) in the isolated perfused mesenteric vascular bed in untreated (control; ●) SHR and WKY rats, alone and in the presence of angiotensin II (□ ; 10 nM) and AII (10 nM) + losartan (○ ; 1 uM). N=6 per group.

* significant (P<0.05) difference vs. basal stimulation.

and losartan on responses to nerve stimulation in the SHR and WKY rat show that, over mid-range frequencies of stimulation, the PMVB of the SHR showed an enhanced response to nerve stimulation in the presence of 10 nM AII, which was reversed once losartan (1 uM) was infused (Fig. 7.4 (a)). By comparison, the PMVB of the WKY rat did not exhibit any of the sympathofacilitatory actions of AII (Fig. 7.4 (b)), and losartan had no additional effects.

7.3.3 Vascular 3-methylhistidine content and heart weight to body weight ratios

The 3-methylhistidine (3MH) content of the superior mesenteric artery and its branches of untreated (control) adult SHR and WKY rats are depicted in Fig. 7.5. In both vessel preparations, the SHR exhibited a greater concentration of 3MH. This attained significance in the mesenteric resistance vessels.

The effects of chronic losartan treatment and its withdrawal on heart weight to body weight ratios and vascular and nonvascular tissue 3MH concentrations are graphed in Fig. 7.6. Neither losartan treatment nor release altered the HW:BW ratio or vas deferens 3MH content. Chronic losartan treatment effectively lowered aortic (but not mesenteric vascular) 3MH concentrations, which were reversed after a 5 week withdrawal period. Mesenteric 3MH concentrations were significantly higher after the withdrawal period.

7.3.4 Vascular actin levels

The actin content of aortic tissue from SHR and WKY rats is displayed in Figs. 7.7 (a,b). On both a wet tissue weight and protein basis actin concentrations were elevated in untreated SHR. Chronic captopril treatment resulted in a decrease in aortic actin content to WKY levels. Withdrawal from captopril treatment resulted in an increase in aortic actin levels that were not significantly different from untreated SHR, indicating a distinct reversibility in contractile protein after release from chronic ACE-inhibition.

The actin content of mesenteric vascular tissue from untreated SHR and WKY rats is shown in Fig. 7.7 (a,b). On a wet tissue weight (but not protein) basis the SHR exhibited significantly greater levels of actin in comparison to the WKY rat in this tissue.

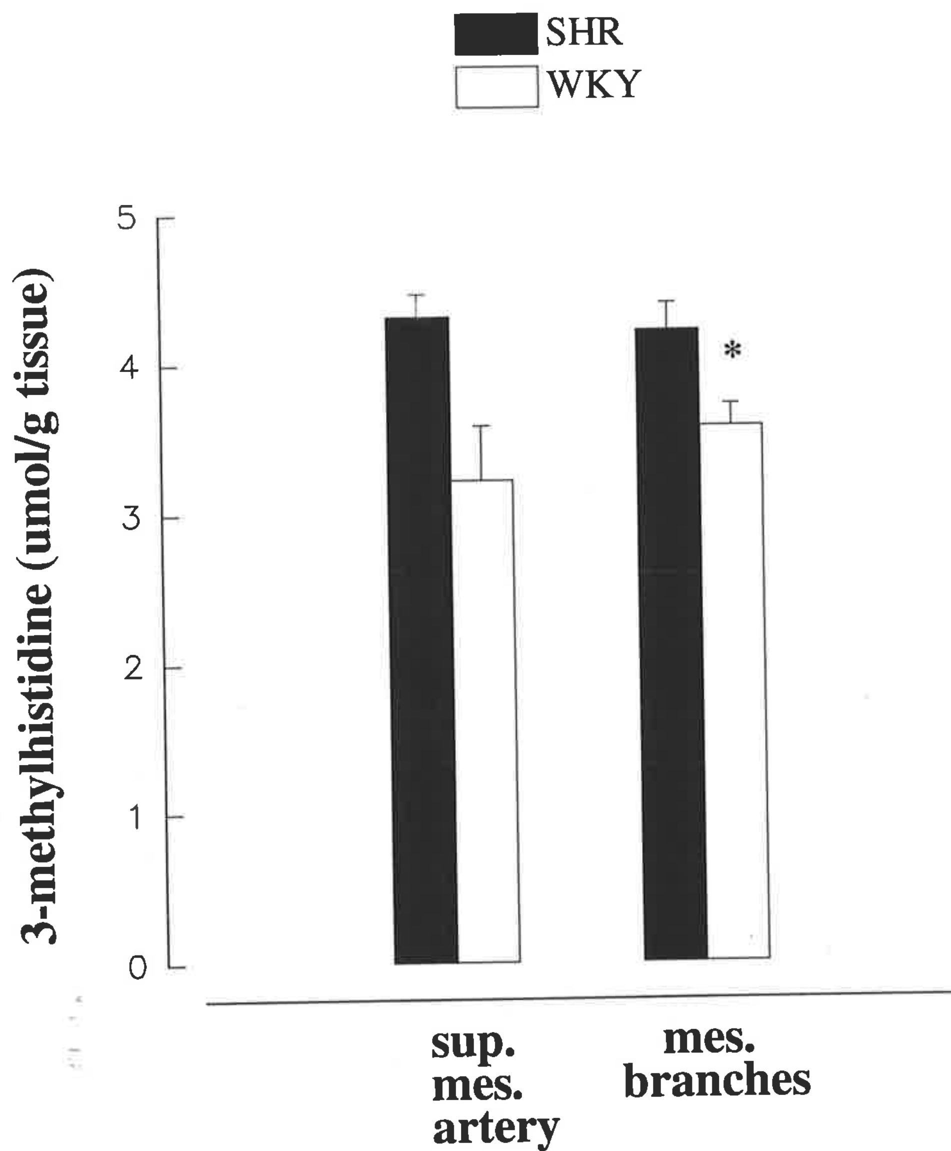


Fig 7.5. 3-Methylhistidine concentrations from mesenteric resistance vessels (branches) and the superior mesenteric artery from adult SHR and WKY rats (n=3-8 per group).
* significant (P<0.05) difference vs. SHR control

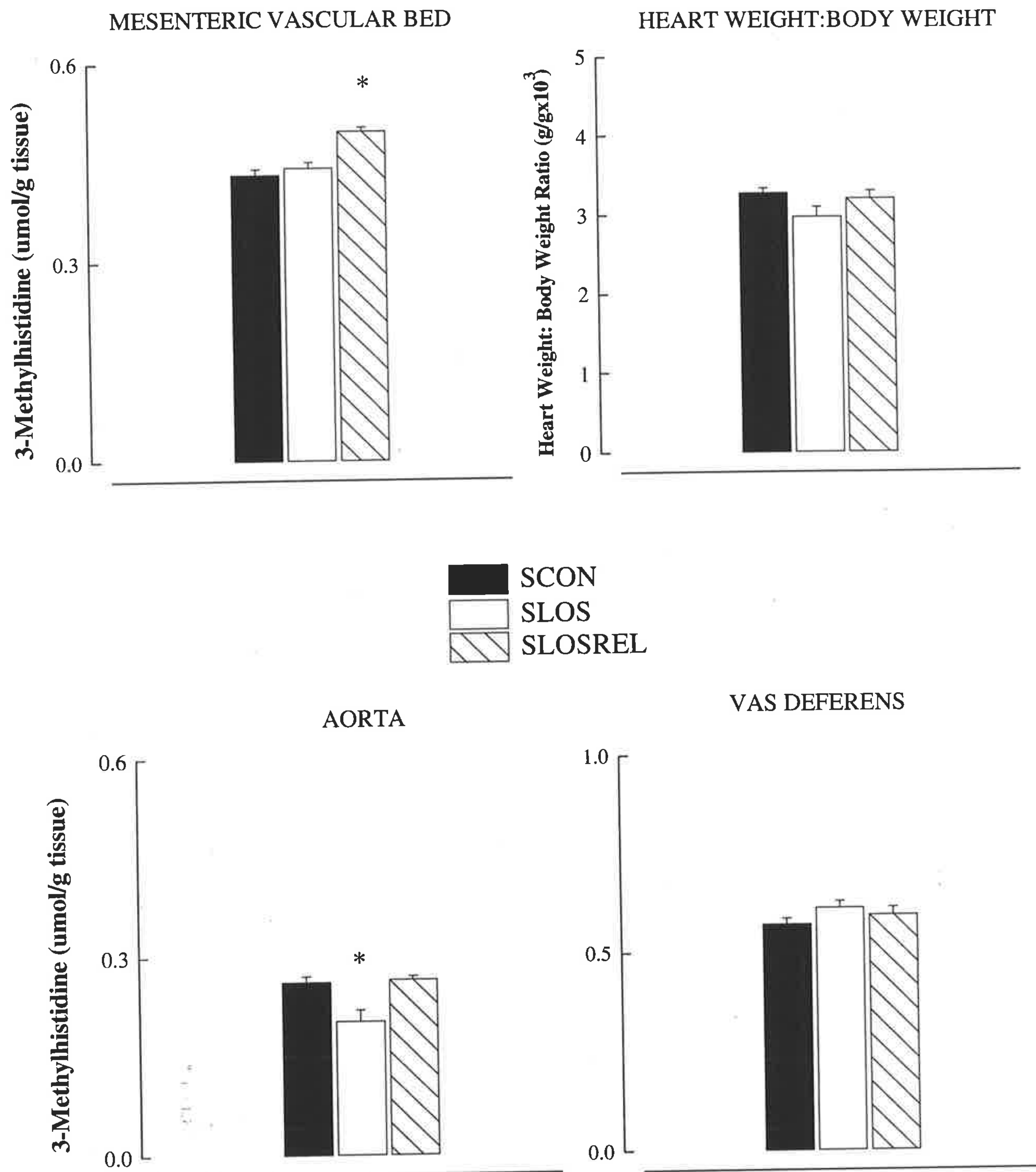
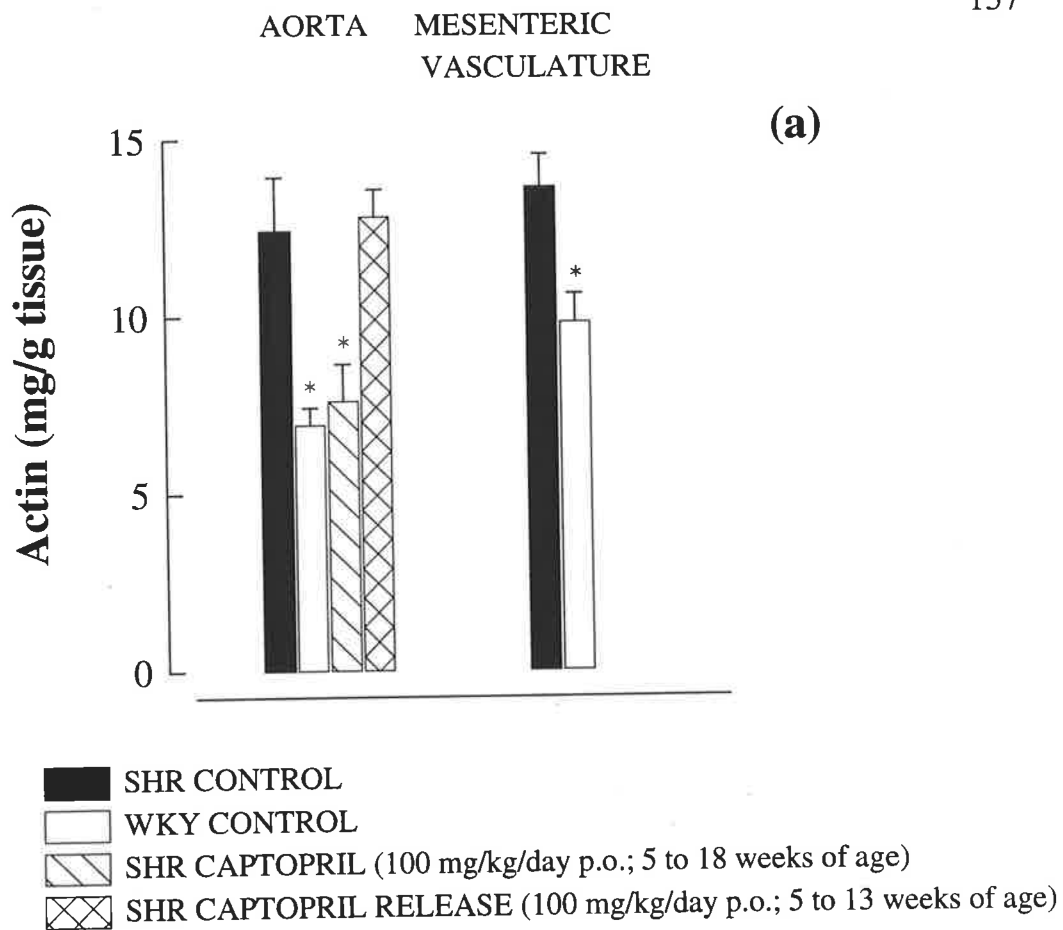
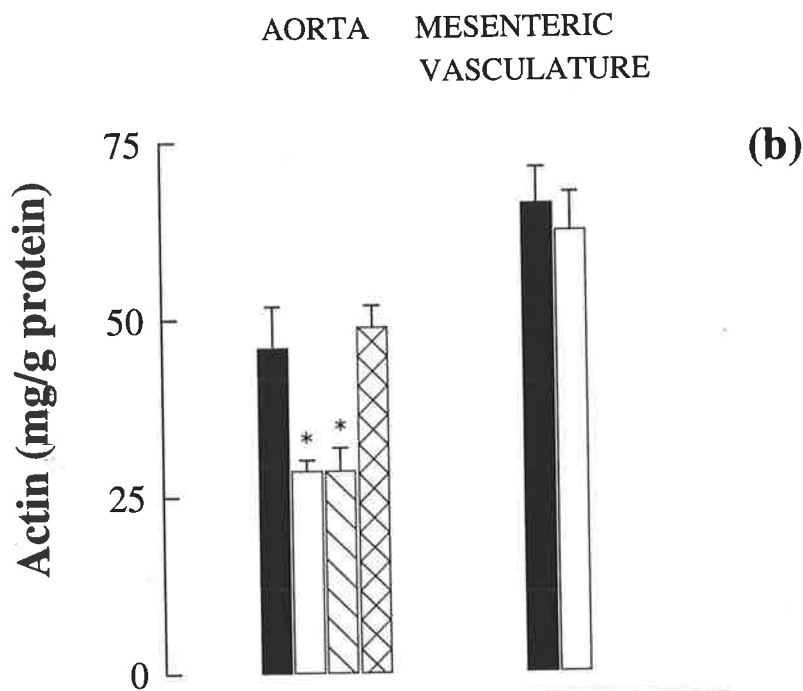


Fig 7.6. 3-Methylhistidine concentrations and heart weight to body weight ratios from untreated (SCON), chronically losartan-treated (SLOS; 30 mg/kg /day p.o. from 5 to 18 weeks of age) and losartan-withdrawn "released" (SLOSREL; 30 mg/kg/day p.o. from 5 to 13 weeks of age) SHR.
* significant ($P<0.05$) difference vs. SCON.





Figs. 7.7 (a,b). Actin concentrations in aortic and mesenteric vascular tissue per unit wet tissue weight (a) and per unit protein (b) in untreated SHR and WKY rats, chronic captopril-treated and captopril-withdrawn "released" SHR. N= 4-11.
* significant (P<0.05) difference vs. control SHR.

7.4 Discussion

The efficacy of chronic losartan treatment (30 mg/kg/day) in preventing hypertension development in the SHR supports a role for angiotensin II and the AT_1-receptor in the expression of aberrant cardiovascular function in this model. The reversal in SBP after treatment withdrawal from losartan is in contrast to that observed after captopril treatment, where blood pressure remains suppressed after drug withdrawal. The mechanism(s) through which this discrepancy becomes apparent are as yet unknown, but suggest that actions of the ACE-inhibitor not mediated by AT_1 receptors have influenced the long-term resetting of blood pressure after cessation of drug treatment. This feature of withdrawal from high dose losartan treatment has been observed previously, where 60 mg/kg/day p.o. losartan from 4 to 14 weeks of age, followed by withdrawal, resulted in a rapid rebound hypertension within 3 weeks (Oddie et al, 1992). After 18 weeks of withdrawal, there was a modest decrease in SBP relative to untreated SHR in this study. Reversion to hypertension after withdrawal from a similar dosage of losartan in adult SHR was also met with a rapid rebound in SBP (Oddie et al, 1993). However, hypertension redevelopment occurs rapidly after chronic ACE-inhibition is withdrawn in older animals (Harrap et al, 1990).

Losartan treatment was accompanied by a suppression in vascular reactivity to noradrenaline (NA) similar to that seen with captopril treatment. In addition, responses to serotonin (5HT) were also depressed, a feature observed after 1 week of quinapril treatment in the SHR (Major et al, 1993). These findings with regard to vascular reactivity are in agreement with the results of captopril treatment and the low dose losartan study (10 mg/kg/day; Chapter 5). After losartan withdrawal, the continued suppression of vascular reactivity confounds the relationship between this vascular functional index and blood pressure and contrasts with the findings of captopril-release. The possibility that prolonged tissue levels of losartan may be influencing vascular neuroeffector function in this study cannot be excluded. While other studies have shown losartan's effects on vascular reactivity were similar during treatment, there was a somewhat greater degree of return to enhanced vascular responses to α-adrenoceptor stimulation after treatment cessation, although this occurred after an extended period off

the drug (18 weeks; Oddie et al, 1993). A longer withdrawal period may be necessary to uncover changes in reactivity after losartan withdrawal. Irrespective of this, it appears that the suppressed vascular reactivity was of little consequence in terms of the control of blood pressure after treatment.

The lack of effect of losartan on mesenteric vascular 3MH and the heart weight to body weight ratio indicates that perhaps cardiovascular structural/biochemical alterations do not occur solely through an AT_1-mediated pathway. Why a lower dose of losartan (Chapter 5) is more effective in reducing cardiac hypertrophy than the dose used in this study is unclear. A modest yet significant decrease in mesenteric vascular cross-sectional area has been observed previously after chronic losartan treatment (Oddie et al, 1993), but the changes were generally greater for the equivalent degree of blood pressure-lowering achieved with an ACE-inhibitor. Aortic 3MH was significantly reduced, but the marker in this tissue generally follows the pattern of blood pressure more than the heart or resistance vessels. The lack of effect of losartan on KCl-mediated vascular responses may indirectly support an inability to modulate vascular structural change. In this regard it is tempting to speculate that the efficacy of ACE-inhibitors in suppressing the development of aberrant cardiovascular structural and biochemical changes is dependent on their capacity to reduce endogenous bradykinin breakdown. From this, the potential importance of endothelial function and nitric oxide's VSMC antiproliferative actions (Nakaki et al, 1990) becomes apparent. A component of the improvement in vascular function observed during treatment with ACE-inhibitors may also occur by virtue of increased release of vasodilators promoted by bradykinin accumulation (EDRF and prostacyclin) or reduced prostanoid vasoconstrictor (TXA_2) effectors derived mainly from the endothelium, but also from the VSMC (Clozel et al, 1993, Jaiswal et al, 1993, Vanhoutte et al, 1993). As losartan caused a profound suppression in vascular reactivity to 5HT, a response also observed after ACE-inhibition (Major et al, 1993) and in part mediated by the endothelium (Dohi and Luscher, 1991), part of the response may be occurring as a result of beneficial effects on the endothelium through AT_1 receptor blockade. Further work is required to clarify the potential role of AII within this context.

A role for the AT_2 (or other as yet uncharacterised AT) receptor cannot be excluded from the present study. The development of balanced AT_1/AT_2 receptor antagonists will assist in the further elucidation of AT receptor subtypes subserving the actions of AII in the SHR, which will also further the understanding of the mechanism of action of ACE-inhibitors in this model.

A part of losartan's effects is likely to include a suppression of the AII-mediated increase in sympathetic function, as losartan effectively abolished the enhancement by AII to nerve stimulation, a feature shown to be mediated by the prejunctional AT_1 receptor subtype (Wong et al, 1992). This property of AII is selective for the SHR, the WKY rat PMVB displaying no augmentation in the frequency-response curve to a subthreshold dose of AII. It is also possible that the increase occurred due to a selective facilitation of α-adrenoceptor function by AII; responses to exogenous NA were not performed to confirm this.

The observation that actin levels were elevated on a wet tissue weight basis in both aortic and mesenteric vascular tissue from the SHR supports evidence of increases in 3MH concentrations in these tissues presented in Chapters 3 and 6. The marked influence of chronic captopril treatment on aortic actin is also reflected by a parallel reduction in 3MH content observed in previous chapters. The effects of captopril treatment were profound in this tissue and suggest that ACE mediates changes in the medial thickness characteristic of this vessel. Increases in medial thickness are predominantly through hypertrophic change in the aorta (Lee and Smeda, 1985, Owens, 1987). The proportion of the aorta composed of actin also increased sufficiently to uncover differences on a protein basis.

In contrast, while there were significant differences in mesenteric vascular actin per unit wet tissue weight between strains, this was not reflected by a difference in actin when compared to total vessel protein content. When using 3MH concentrations as an index of actin, this has been a consistent finding by the author but is in contrast to others (Jonsson et al, 1991). The reasons for this are unclear, but improved assay conditions, including use of an internal standard (3EtH), may be of significance.

While actin (and 3MH) values give no indication as to the type of structural change occurring in resistance vessels from the SHR, they are commensurate with the

exaggerated vascular reactivity that occurs in the mesenteric vasculature in this model. The increase in actin on a wet tissue weight basis may be an indicator of the selective hyperplastic growth as opposed to a vessel like the aorta, where actin content relative to both indices may be more indicative of hypertrophic change. Previous biochemical measurements in SHR mesenteric arteries have revealed selective increases per wet tissue weight in actin and DNA, with no change in actin relative to DNA content (Brayden et al, 1983). While it is tempting to suggest this is indicative of hyperplastic growth, increases in cell ploidy during VSMC hypertrophy are also recognised (Owens, 1989).

In summary, chronic losartan treatment exerted effects on blood pressure and vascular reactivity similar to those of the ACE-inhibitor captopril in the developing SHR, suggesting the involvement of the AT_1 receptor in mediating the effects of AII. However, there were differences with respect to losartan's influence on cardiac and mesenteric vascular contractile protein that suggests the ACE-inhibitors possess additional effects beyond those related to inhibition of AII and/or expressed at the AT_1 receptor. Furthermore, losartan had dose-dependent effects on blood pressure that were manifest after treatment was discontinued when compared to low-dose losartan and captopril treatment (Chapter 5). These results highlight important differences in action between the ACE-inhibitor and AT_1 receptor antagonist that may explain features of blood pressure development after cessation of therapy with these agents.

Vascular actin content is elevated in aortic tissue on both a wet tissue weight and protein basis, and on a wet tissue weight basis only in the mesenteric vasculature. The difference in actin levels occurs in both the superior mesenteric artery and the finer resistance vessels of this vascular bed as reflected by differences in 3MH in this tissue. Chronic captopril treatment resulted in a marked decrease in aortic actin content that was reversed following drug withdrawal, indicating a distinct reversibility of this ACE-dependent process.

CHAPTER 8

GENERAL DISCUSSION

The study of genetic animal models of hypertension has greatly facilitated our understanding of the interaction between neurohumoral factors and the development and maintenance of the disorder. The SHR is a significant contributor to such knowledge, which is derived from two broad lines of investigation.

The first relates to studies in the pathophysiology of hypertension development. Researchers have now quite clearly determined the sites of expression where aberrations may be involved in the loss of control of "normal" blood pressure levels. Increased markers of sympathetic vascular nerve density and function (Iruchijima, 1973, Head, 1989), increased markers of tissue autocrine/paracrine renin-angiotensin systems (Samani et al, 1989) and the prevalence of cardiac and vascular structural change in the adult SHR (Folkow, 1978) are the predominant indicators.

The second relates to the selective impairment of neurohumoral function. This has been achieved primarily through pharmacological treatment, and therefore has been dependent on the available pharmacological tools. Initial studies utilising sympathectomy supported a role for the sympathetic nervous system (SNS) in the development of hypertension and cardiovascular structural changes (Bevan and Tsuru, 1979, Scott and Pang, 1983). With the advent of inhibitors of the renin-angiotensin system, support for its role in the disorder was seen to be strong (Cadilhac and Giudicelli, 1986). Moreover, the findings that a prolonged suppression in hypertension redevelopment occurred following treatment with the ACE-inhibitors guaranteed a place for the further development of these drugs (Giudicelli et al, 1980).

However successful the foregoing therapies were, there still existed a number of unresolved questions regarding the specific role of these pathophysiological markers in hypertension development. Central to these issues was the inability to determine a cause and effect scenario. Was altered innervation, tissue RAS expression and cardiovascular structural change a cause or result of the hypertension? Equally plausible was the possibility that they were neither, but instead simply traits associated with the

breeding of the SHR. Despite initial considerations that vessel structural changes were adaptive (Folkow et al, 1958), there were examples where successful antihypertensive therapies were ineffective in preventing the full development of cardiac and vascular medial hypertrophy/remodelling (Smeda et al, 1988). Likewise, the development of these alterations was shown to partly precede the onset of hypertension (Gray, 1984). These findings are not conclusive for there is evidence suggesting cardiovascular structural change can occur through both pressure-dependent and independent stimuli (Lee and Smeda, 1985). Nevertheless, their presence suggested they may play a role in the pathogenesis of the disorder. The suggestion that the narrowing of the vascular lumen was an important determinant of the increased resistance to flow (Korner et al, 1989) focused research on mechanisms through which aberrant growth/design of the vasculature occurred. Tissue culture and molecular biology have complimented the pharmacological evidence suggesting that, amongst a myriad of other factors, the augmented expression of the SNS and RAS is integral.

The aims of the studies in this thesis were to examine specifically which components of the RAS (and, to a lesser extent, the SNS) were important in the development of hypertension and what effects they have on enhanced vascular reactivity and cardiovascular structure, using pharmacological treatments and functional and biochemical markers of the vasculature from the SHR. The determination of mechanisms by which the ACE-inhibitors maintain a prolonged reduction in blood pressure was also investigated using these indices.

In Chapter 3 of this thesis the antihypertensive effect of chronic oral captopril treatment was demonstrated in the SHR. The prevention of genetic hypertension development was accompanied by a marked reduction in the enhanced vascular 3MH content observed in this strain, suggestive that captopril treatment had altered the disposition or content of contractile protein (actin) in the blood vessels analysed. Withdrawal from captopril treatment resulted in an attenuation of the redevelopment of high blood pressure, but not vascular 3MH, suggesting that this marker of cellular actin was a poor predictor of the prevailing blood pressure following treatment. These results indicated that either the ACE-inhibitors act at sites other than at the level of vascular

contractile protein to suppress hypertension redevelopment, or that 3MH is not simplistically related to blood pressure in the manner envisaged.

In Chapter 4 these studies were repeated, but accompanied by an index of vascular function, namely reactivity of an isolated vascular preparation (the perfused mesenteric vascular bed). Once again, findings consistent with those of previous chronic studies with captopril were seen with regard to blood pressure and chronic captopril treatment reduced the enhanced levels of 3MH in the mesenteric vascular bed. This pattern of effects with the ACE-inhibitor was reflected by the profound decrease in vascular reactivity to noradrenaline (NA). These results were in keeping with the suggestion that captopril exerts an influence on vascular contractile protein which contributes to the profound suppression in vascular function. From the correlation analysis it was also shown that for any given fall in blood pressure due to captopril treatment, vascular reactivity to NA decreased beyond that predicted from the regression analysis, indicating that captopril may exert additional effects on vascular contractility not related to its influence on contractile protein. The reasonable correlation between vascular reactivity and SBP continued in the absence of treatment, as has been observed previously after withdrawal from chronic enalapril therapy (Adams et al, 1990). This is associated with a progressive return in the increased media to lumen ratio of resistance vessels that also correlates well with SBP after treatment with an ACE inhibitor (Christensen et al, 1989). Collectively, the evidence presented in this thesis and by others supports the view that these agents exert their effects on blood pressure by increasing luminal diameter and preventing medial hypertrophy/hyperplasia/vascular remodelling, i.e., conditions which lead to accentuated vasoconstriction and increased resistance to blood flow. The reduction in 3MH is an indication of the sharp drop in vascular actin content, suggestive of an overt change in the contractile machinery of the vessels. However, 3MH does not give an indication as to the gross structural changes occurring in blood vessels from the SHR.

Inconsistencies in the difference in mesenteric vascular 3MH concentrations between SHR and WKY rats necessitated the quantitation of actin levels in this tissue between strains (Chapter 7). These results suggested that the SHR exhibits greater actin concentrations in aortic and mesenteric blood vessels dependent on the indices against

which the marker is compared. The differences (as expressed per unit wet tissue weight or total protein) may be indicative of the contribution of actin to total tissue protein and the type of VSMC growth that occurs in these vessels. Furthermore, the consistent influence of chronic captopril treatment on aortic 3MH levels paralleled the change in aortic actin content also.

In Chapter 5 the comparison between chronic captopril treatment and the effects of therapy with the nonspecific vasodilator hydralazine was used to differentiate the effects of ACE-inhibition related to the fall in blood pressure on structural, biochemical and functional indices in the cardiovascular system of the SHR. The findings suggest that altered blood pressure does contribute to some of the changes in larger vessel contractile protein content, but enhanced vascular reactivity and cardiac hypertrophy are some of the features that continue to develop seemingly unabated, despite successful blood pressure control during treatment. The rapid return of high blood pressure after withdrawal then is not surprising, given hydralazine's minimal impact on cardiovascular structural and functional parameters.

The use of the recently developed nonpeptide angiotensin type 1 (AT_1) receptor antagonist losartan allowed the elucidation of the mechanism(s) of action of captopril in this setting. In this regard, losartan was shown to be similar to captopril in its effects on vascular reactivity, aortic 3MH and cardiac hypertrophy, indicating that captopril in part exerts its effects via preventing the formation of AII acting at the AT_1 receptor. An interesting feature of high dose losartan treatment (Chapter 7) was the rapid return of hypertension displayed in SHR withdrawn from long-term treatment, while vascular reactivity to NA remained seemingly depressed. This is in contrast to withdrawal from captopril and low dose losartan treatment. The reason for the discrepancies in blood pressure and vascular reactivity are unclear, but indicate that captopril's (and possibly other ACE-inhibitor's) influence on blood pressure after drug withdrawal may be related to an as yet unrecognised feature of these antihypertensives. This may involve bradykinin or other products of the ACE, or may be a feature related to the relatively uncharacterised functions of the AT_2 receptor (or other AT receptor subtype) in this model. These results also question the validity of using vascular reactivity as a predictive index of systolic blood pressure, even in the absence of treatment. To this

end, while vascular reactivity is positively associated with SBP between strains, this relationship does not always hold during antihypertensive treatments and after drug withdrawal. This may underlie the complexity of vascular structural change in relation to Folkow's postulates. The permanency of vascular remodelling after drug treatments needs to be addressed, particularly as altered luminal diameter may be a greater determinant (and therefore be more predictive) of blood pressure than accentuated vasoconstriction.

Acute (1 week at 100 mg/kg/day) captopril treatment caused normotension in the adult SHR. This was associated with modest changes in aortic actin and cardiac hypertrophy and a complete normalisation of vascular reactivity to NA. As distinct structural alterations were only considered possible after chronic treatment, it was concluded that a significant proportion of the responses to NA in the vasculature of the SHR were enhanced due to a direct facilitatory effect of AII on the vascular smooth muscle cell, acting at the AT_1 receptor. This feature of acute treatment highlights the multifunctional role of AII in the SHR. The peptide can act through both pressure-dependent and independent pathways to mediate changes in cardiovascular structure and function.

In these studies, and especially in Chapter 5, the relationship between a number of cardiovascular parameters and blood pressure was explored, both during and after treatment with various antihypertensives. In general, the correlation analyses suggested that RAS impairment produced reductions in a number of parameters (heart weight to body weight ratios, vascular reactivity, aortic 3MH) that were greater than that predicted from their effects on SBP. The complexity of these relationships became apparent only after acute captopril and other chronic drug treatments. Otherwise, the parameters were often well correlated in the absence of any drug treatment. These findings serve to focus on the multiple loci of actions of AII in the SHR and emphasise the difficulty in using markers of cardiovascular structure and function to predict both the prevailing blood pressure and subsequent mechanisms of action of the appropriate antihypertensives.

In Chapter 6, the focus shifted to the potential interaction between the SNS and the RAS in the control of hypertension development and maintenance in the SHR. The

findings indicate that a proportion of the tolerance exhibited to chronic α-adrenoceptor blockade is mediated by the RAS in this model. The traditional relationship between the two systems has been well described. Sympathetic renal efferent activity is one stimulus for juxtaglomerular cell renin secretion, and AII has sympathofacilitatory properties at the vascular neuroeffector junction. However, the active contribution of the RAS in offsetting some of the effects of chronic α-adrenoceptor blockade in this model is the first such documentation of this interaction to date. A study designed to investigate aspects of SNS function has ultimately focused on the dominant role the RAS exerts in maintaining hypertension in the SHR.

The mechanism by which the RAS is recruited in this situation is not defined in the present study, but the efficacy of nonspecific vasodilators in this model suggests that it may not be due to any initial reduction in blood pressure the α-blocker may induce. The contribution of ß-adrenoceptor-mediated renin release from the kidneys (Watanabe et al, 1980) and peripheral blood vessels (Nakamura and Nakamura, 1978) are areas that require further investigation within this context.

The precise role of the SNS in the development of both hypertension and cardiovascular structural and functional abnormalities in the SHR is still unclear. Chronic treatment of normotensive rats with nerve growth factor enhances the vascular density of sympathetic innervation and medial thickness but does not alter blood pressure (Zettler et al, 1991), whereas normotensive rats made transgenic for the single mouse renin gene (TGR(mREN2)27) display many characteristics of the SHR, including aspects of hypertension which are similar in their degree and development, including an increased severity of condition in males, elevated tissue RAS markers (Hilgers et al, 1992) and vascular structural abnormalities (Mulvany, 1993). Hypertension development can be averted through treatment with captopril or losartan, but losartan's effects are lost after withdrawal (Bader et al, 1992). These animals express high adrenal renin content but not elevated sympathetic nerve activity (Tokita et al, 1994), making them different in some respects to the SHR. It may be in these differences that the true contribution of the SNS lies; however, the weight of evidence suggest the RAS exerts a more powerful role in genetic and transgenic rat models of hypertension.

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APPENDICES

Appendix I

Actin to myosin ratio in vascular smooth muscle cells and the proportion of 3-methylhistidine (3MH) from each contractile protein in vascular smooth muscle cells.

1. Stoichiometry of 3MH: MYOSIN

    * 1 mole of 3MH per mole myosin (MW= 200 kDa; Johnson and Perry, 1970)
    * 13 mg myosin per g cell weight (Cohen and Murphy, 1979)

    * therefore 0.013/200000= 65 nmoles myosin per cell

2. Stoichiometry of 3MH: ACTIN

    * 1 mole of 3MH per mole actin (MW= 42 kDa; Johnson and Perry, 1970)
    * 42 mg actin per g cell weight (Cohen and Murphy, 1979)

    * therefore 0.042/42000= 1000 nmoles actin per cell

3. Proportion of 3MH from actin per cell:

    * 1000/1065 X 100= 94%

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Appendix II

Genetic marker profiles of SHR, SHRSP and WKY rats from CSIRO Glenthorne Breeding Colonies, September 1994.

	SHR	SHRSP	WKY
Acon-1	*b*	*b*	*b*
Ahd-2	*b*	*b*	*b*
Ahd-C	*b*	*b*	*b*
Akp-1	*a*	*b*	*b*
Alp-1	*a*	*b*	*b*
Br-1	*b*	*b*	*a*
Es-2	*a*	*a*	*d*
Es-4	*a*	*a*	*b*
Es-10	*a*	*a*	*b*
Fh	*b*	*b*	*b*
Pep-3	*a*	*a*	*b*
Hbb	*a*	*a*	*a*
Pk	*a*	*a*	*a*

Nomenclature for allelic profiles according to Adams et al. "Laboratory protocols for detecting biochemical markers" in "Genetic Monitoring of Inbred Strains of Rat", Hedrich H. (ed), Gustav Fischer Verlag, Stuttgart (1990).

CONCLUSIONS

1. There is no evidence of genetic contamination in any strain. All animals representing a particular strain were identical at the 13 genetic markers tested.

2. The strain profiles obtained are consistent with those found in previous screens and those published in the literature.

Appendix III

Components of synthetic diet mix

Component	% (weight)		Component	% (weight)
Casein	12.5		Starch	35.2
Sucrose	32.5		Methyl cellulose	4.5
Butylated hydroxyanisole	0.01		Gelatin	5.0
Choline chloride	0.2		Olive oil	5.0
Amino acid mixture	0.352			
L-methionine	0.28		L-Phenylalanine	0.0618
L-Tryptophan	0.0103			
Water soluble vitamins	0.10			
Menadione	0.0112		Folic acid	0.0005
Niacin	0.02075		Thiamine HCl	0.0048
Pyridoxine HCl	0.0048		Inositol	0.0239
p-amino Benzoic acid	0.0239		Ca Pantothenate	0.01435
Biotin	0.0001		Vitamin B12 (1/100)	0.032
Riboflavin	0.0048			
Mineral mixture	4.0			
$CaCO_3$	0.741		$Ca_3(PO4)_2$	0.528
KH_2PO_4	1.095		KCl	0.423
NaCl	0.489		$MgSO_4.7H_2O$	0.637
$FePO_4.H_2O$	0.058		$MnSO_4.H_2O$	0.00109
$CuSO_4.5H_2O$	0.00132		KI	0.000016
NaF	0.00186		$Al_2(SO_4)3K_2SO_4.24H_2O$	0.000390
$ZnSO_4.7H_2O$	0.024			
Fat soluble vitamin mix	0.0046765			
Cholesterol	0.00375		α-Tocopherol	0.00050
Vitamin D	0.0000015		Retinol	0.000425
Phenoxybenzamine	0.010425			

Phenoxybenzamine calculations based on a dosage of 10 mg/kg/day, an average rat weight of 250 gms and an average nightly consumption of 25 gms diet.

Appendix IV

Ion exchange chromatography; equipment and resin preparation.

Equipment:

* syringe column (Terumo, 2 ml)
* filter paper plugs (Whatman #44)
* syringe needle (Terumo, 21G, 38 mm)
* one way stopcock (Vygon)

Preparation:

Two plugs of filter paper are inserted at the base of the syringe column, which in turn is fixed to the stopcock and the syringe needle. A 1 ml bed of resin is loaded onto the column. After a single sample run the used resin is replaced.

Resin Preparation:

* 100 mls crude resin added to 200 mls water (final volume=300 mls)
* supernatant (S/N) aspirated and 300 mls HCl (5M) added and mixed
* aspirate S/N and wash with water until pH≈5.0-6.0 (≈15 litres)
* add 300 mls NaOH (2M)+EDTA (1%w/v); mix and leave overnight
* aspirate S/N and wash with water until pH≈7.0 (10 litres)
* wash with 100 mls HCl (5M)
* wash with water (2 litres), leaving 100 mls S/N above resin
* transfer to labelled 250 ml glass bottle

Appendix V

Identification of 3EtH and calculations of recovery of 3EtH from TLC purification

1. Assumed recovery ≈ 5mg pure 3EtH from 10 mg crude mix
   (≈ 20% impurity, therefore ≈ 8 mg pure 3EtH loaded)

2. 5 mg 3EtH in 1 ml pool= 5 mg/ml (≈25 mM)

3. To attain 10 uM for HPLC analysis perform 1/25 and 1/100 dilutions

4. HPLC analysis revealed recovery ratio of 3EtH:

$$\frac{\text{peak height "10 uM" pure}}{\text{peak height "10 uM" crude}} = 1.875$$

5. Back calculate to determine actual concentration of pure 3EtH:

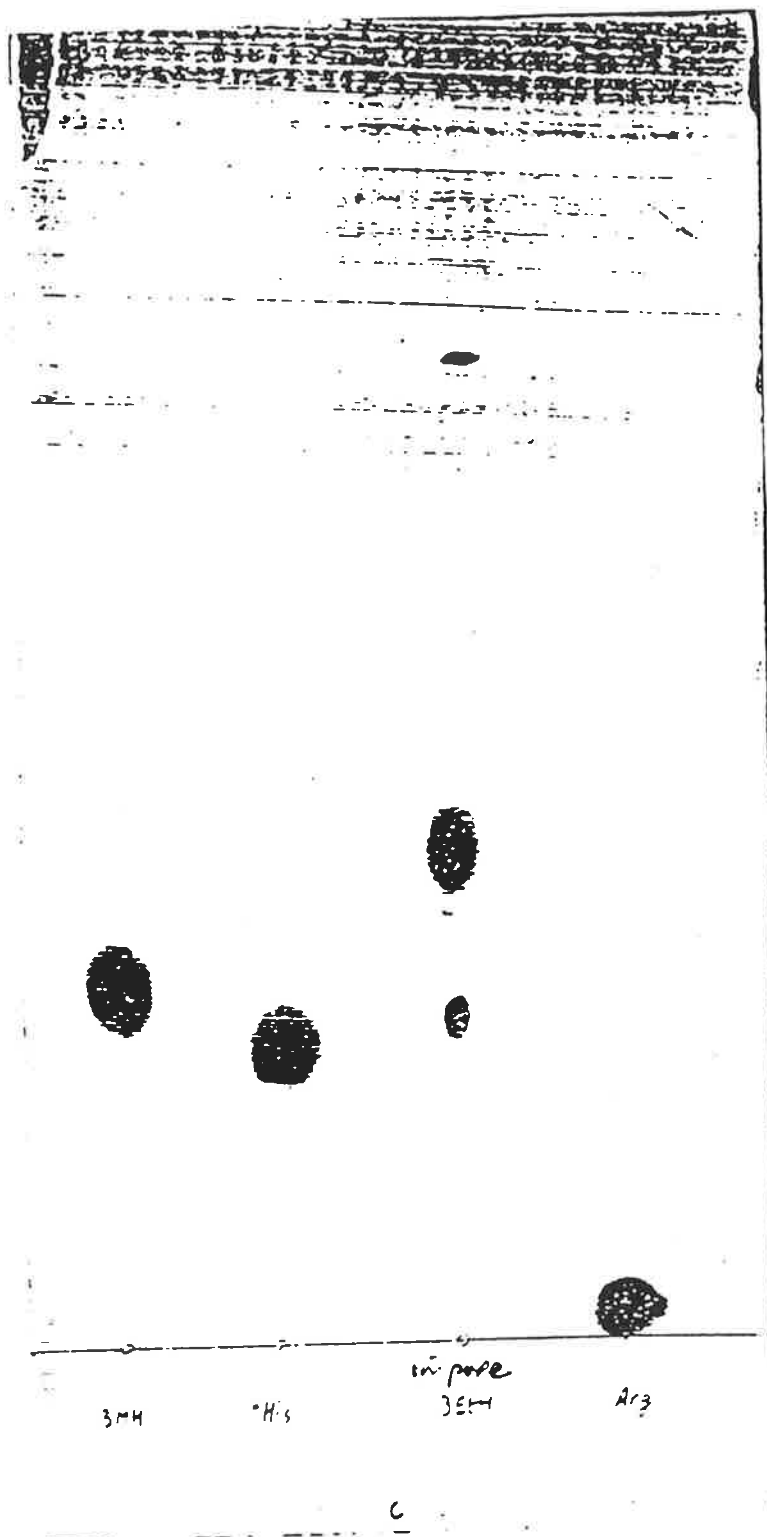
   1.875 * 8 uM * 25 * 100 = 37.5 umoles

6. Total 3EtH loaded:

   8.0/183 (MW 3EtH) = 43.7158 umoles

7. Recovery:

   37.5/43.7158 = 86%



TLC separation profile of various amino acids, including 3EtH. From left to right: lane 1, 3-methylhistidine (3MH); lane 2, histidine; lane 3, crude 3-ethylhistidine (3EtH) mix; lane 4, arginine. Note the presence of at least two distinct ninhydrin-staining impurities, and the co-chromatographing of the major impurity with 3MH. For all samples, 20 ug (in 85% methanol) were spotted, resolved and stained as previously specified (*2.4.3*).

Appendix VI

Reagents and Gel Preparation for SDS-PAGE Slab Gels based on the Laemmli (1970) Buffer System.

1. Stock Solutions

1a. Acrylamide/Bis (30% T, 2.67% C)

supplied as a preweighed mix of acrylamide/bis; total monomer: crosslinking ratio equivalent to 37.5:1 (BioRad, Ca). Store at 4°C.

1b. 1.5 M Tris-HCl, pH 8.8 (store at 4°C)

1c. 0.5 M Tris-HCl, pH 6.8 (store at 4°C)

1d. 10% w/v SDS

1e. Sample Buffer (SDS reducing buffer)

Distilled water	4.0 mls
0.5 M Tris-HCl, ph 6.8	1.0 ml
Glycerol	0.8 ml
10% w/v SDS	1.6 mls
ß-mercaptoethanol	0.4 ml
0.05% w/v Bromophenol blue	0.2 ml

1f. 5X Electrode (Running) Buffer, pH 8.3

Tris base	15g/l
Glycine	72 g/l
SDS	5g/l

Dilute 300 mls to 1.5 litres with distilled water for 1 gel run. 1.2 Litres of this is diluted 1/2 with water for the lower (anode) chamber; the remaining 300 mls is used undiluted in the upper (cathode) chamber. Store stock at 4°C.

2. Separating (resolving) gel preparation- 0.375 M Tris, pH 8.8

For two T=12% gels:

Distilled water	33.5 mls
1.5 M Tris-HCl, pH 8.8	25.0 mls
10% SDS	1.0 ml
Acrylamide/Bis (30%)	40.0 mls (degas before use)
Ammonium persulphate (10%)	0.5 mls (make fresh before use)
TEMED	50.0 **ul**

3. Stacking gel preparation- 4.0% gel, 0.125 M Tris, pH 6.8

For two gels of 1.5 mm thickness:

Distilled water	12.2 mls
0.5 M Tris-HCl, pH 6.8	5.0 mls
10% SDS	200.0 **ul**
Acrylamide/Bis (30%)	2.6 mls (degas)
Ammonium persulphate (10%)	100.0 **ul** (fresh)
TEMED	20.0 **ul**

4. Gel preparation and Running Conditions

To prepare the monomer solutions, all reagents are combined except the ammonium persulphate (APS) and TEMED. This is degassed under vacuum for 10 mins. Polymerisation is initiated after addition and mixing of the APS and TEMED. The time to complete polymerisation varies depending on the amount of APS and TEMED and the temperature of the solution.

The set up and casting of the gels is described in the Protean II Slab Cell System Instruction Manual (BioRad, Ca), Sections 4 and 5.

Gels are run under constant current conditions with either a Model 3000xi or 500/200 Power Supply (BioRad, Ca). Cooling is advised.

5. Gel Characteristics

$$\%T = \frac{\text{grams acrylamide} + \text{grams bis-acrylamide}}{\text{total volume}} \times 100 \text{ (total monomer)}$$

$$\%C = \frac{\text{grams bis-acrylamide}}{\text{grams acrylamide} + \text{grams bis-acrylamide}} \times 100 \text{ (total crosslinker)}$$

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