GENE EXPRESSION OF NERVE GROWTH FACTOR IN THE DEVELOPING SPONTANEOUSLY HYPERTENSIVE RAT

A Thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of

Doctor of Philosophy

in

The Department of Clinical and Experimental Pharmacology, University of Adelaide

by

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February 1992

DECLARATION

I declare this thesis to be based on original data obtained while I was enrolled as a Ph.D. candidate in the Department of Clinical and Experimental Pharmacology at the University of Adelaide. To the best of my knowledge this thesis contains no material which has been previously accepted for the award of any degree or diploma in any university, nor any material previously published by any person, except where due reference is cited in the text. The author consents to the thesis being made available for photocopying and loan if applicable and if accepted for the award of the degree.

Data from this thesis have been presented to a Meeting of the Australian Society of Clinical and Experimental Pharmacologists in Sydney (1989), a Meeting of the International Union of Pharmacologists in Bonn (1990) and a Meeting of the International Society of Neurochemistry in Sydney (1991).

Patrick HJ Falckh

February 1992

LIST OF PUBLICATIONS IN SUPPORT OF THIS THESIS

Falckh PHJ, Irvine R, Rush R, Bridges D and Head RJ, 1989, Gene Expression of Nerve Growth Factor in the Mouse Salivary Gland, *Clin. Exp. Pharmacol. Physiol.*, (Suppl. 16);

Falckh PHJ, Rush R, Bridges D and Head RJ, 1990, Expression of Nerve Growth Factor (NGF) and NGF-mRNA, Blood Vessels, Vol. 27(1):36;

Falckh PHJ, Harkin LA and Head RJ, Expression of NGF in the Heart and Kidney in the Developing Spontaneously Hypertensive Rat, *Clin. Exp. Pharmacol. Physiol.*, (in press);

Falckh PHJ, Harkin LA and Head RJ, Resistance Vessel Gene Expression of Nerve Growth Factor is Elevated in Young Spontaneously Hypertensive Rats, *Hypertension*, (submitted 1991).

ACKNOWLEDGMENTS

There are so many individuals to whom I would like to express my sincere gratitude for their help and support during my Ph.D. candidature that the list is far too long to include here, however, there are several who I feel I must mention.

Professor Richard John Head, not only provided me the opportunity to conduct my studies at the CSIRO, Division of Human Nutrition, but arranged a scholarship with CSIRO, and provided the impetus of the project. Thanks to Mark Mano for his friendship, discussions, support and technical advice over the last 5 years and to many of the staff at CSIRO who provided technical help and friendship especially Roger King, Colin Chandler, Yvette Schaeffer and Ian Pannent. David Courage for his uncanny ability to "pull-a-rat-out-of-a-hat", his wasted weekends and forbearance of the extensive breeding program I required over the years. Kath Illes and Pat Nayda for their advice, friendship and the annoyance I must have caused with interruptions to have my thesis, and drafts, printed.

Diana Bridges for her technical expertise in conducting the NGF determinations and Professor Robert (Bob) Rush for allowing the use of his laboratory for this work.

My most sincerest thanks to Dr Mavis Abbey, CSIRO, Division of Human Nutrition, for her expert discussions and assistance in understanding molecular biology, her willingness to guide a confused mind and the "lost days" of reading my thesis.

To many members of the Department for their friendship, stimulating discussions and elbow-bending especially Rod Irvine, Gordon Crabb and Julie Jonsson.

To Sotiria Bexis who has kept me sane (?) throughout my postgrad years with her unwavering friendship and loyalty, for the hours and days I "bent her ears", for the discussions we have shared and to whom the completion of this thesis is attributed for never letting me quit; I owe my greatest thanks and appreciation.

Finally to my wife, Heather, and her family for their encouragement and support during the difficult months of writing the thesis; especially for my bad moods.

This thesis is dedicated to my mother, who unfortunately did not see me complete my University studies, but whom I love and miss greatly.

ABSTRACT

Hypernoradrenergic innervation of the vasculature is a recognised as a key characteristic in the spontaneously hypertensive rat (SHR), a genetic animal model of hypertension. Nerve growth factor (NGF), a protein essential for the development and maintenance of sympathetic innervation, has been shown to be elevated in the young SHR when compared to the normotensive control, the Wistar-Kyoto (WKY) rat. NGF concentrations have been shown to correlate with gene expression for the protein and with the degree of sympathetic innervation in many species. The NGFmRNA levels in cardiovascular tissue of neonatal, and developing, SHR and WKY rats were investigated to determine if there is a link between the gene expression of NGF and the pattern of abnormal sympathetic innervation in this model.

The reliability of the cDNA probe used for the determination of NGFmRNA concentrations was initially verified by investigating NGFmRNA concentrations in the submaxillary salivary gland of the mouse after pharmacological manipulation.

Mesenteric and caudal arteries of the SHR were found to contain significantly elevated levels of NGFmRNA (> 5 fold) consistent with the hypernoradrenergic state reported for these blood vessels. The kidney of the SHR also displayed an elevated NGFmRNA production, consistent with the elevated innervation reported for this tissue. Cardiac and aortic tissues, which do not exhibit hypernoradrenergic innervation in the SHR, displayed low levels of NGFmRNA which were generally similar to levels seen in cardiac and aortic tissues from WKY rats. In the hypernoradrenergically

innervated tissues the NGFmRNA levels were elevated as early as 2 days of age and the elevated level sustained for 6 weeks. The latter period corresponds to the normal time course for sympathetic innervation of the vasculature.

It is proposed that the elevated degree of innervation seen in resistance vessels and organs (for example in the kidney) is due, in part, to a sustained elevation of NGFmRNA and that the elevated NGFmRNA is present at birth. The findings also provide a rational basis for the elevated noradrenaline (NA) content of vessels in the SHR, the larger releasable pool of NA in the SHR and the elevated levels of NGF peptide in vessels from the SHR. The results provide sufficient stimulus for examination of a role of NGF in the initiation of hypertension in the SHR.

ABBREVIATIONS USED IN THIS THESIS

σ	wall stress
^{32}P	^{32}Phosphorus
6-OHDA	6-hydroxydopamine
Ad	adrenalin
A_{750}	absorbance at 750nm
ABS	Australian Bureau of Statistics
BC	back cross
BC_2	second generation back cross
bp	base pairs
BP	blood pressure
BSA	bovine serum albumin
cDNA	complementary DNA
cm	centimetre
CNS	central nervous system
cpm	counts per minute
CsCl	cesium chloride
°C	degrees celsius
DβH	dopamine β-hydroxylase
dATP	2′-deoxyadenosine triphosphate
DBP	diastolic blood pressure
dCTP	2′-deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate

dGTP	2′-deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
DOCA	deoxycorticosterone acetate
DR	Dahl salt-resistant strain
DS	Dahl salt-sensitive strain
dTTP	2′-deoxythymidine triphosphate
EC_{50}	concentration required to produce 50 percent of the maximum response
ECD	electrochemical detection
EDTA	ethylenediametetra acetic acid
EtBr	ethidium bromide
F_1	first generation progeny
F_2	second generation progeny
g	grams
g	gravity units
GH	Genetically hypertensive strain
H/BW	heart to body weight ratio
HPLC	high performance liquid chromatography
hrs	hours
kb	kilo base pairs
LH	Lyon hypertensive strain
LL	Lyon low blood pressure strain
LN	Lyon normotensive strain
M	moles per litre
mg	milligrams

MHS	Milan hypertensive strain
min	minute
ml	millilitre
mm	millimetres
mM	millimoles per litre
mmHg	millimetres of mercury
MNS	Milan normotensive strain
MOPS	3-[N-morpholino] propane sulfonic acid
NA	noradrenaline
NaOH	sodium hydroxide
ng	nanogram
NGF	nerve growth factor
NGFmRNA	NGF messenger RNA
NGF-R-mRNA	NGF receptor messenger RNA
NGF-Rt	NGF receptor
NHF	National Heart Foundation
nm	nanometre
P	transmural pressure
PCA	perchloric acid
pmol	picomoles
r	radius
RNA	ribonucleic acid
rpm	revolutions per minute
SBH	Sabra hypertensive strain
SBN	Sabra normotensive strain
SBP	systolic blood pressure

SDW	sterile distilled water
sec	seconds
SEM	standard error of the mean
SHR	Spontaneously Hypertensive Rat
SHRSP	SHR-stroke prone strain
SMC	smooth muscle cells
SNA	sympathetic nervous activity
SNS	sympathetic nervous system
Soln 4	homogenising solution
Soln D	denaturing solution
SSC	standard sodium/citrate buffer
TAE	tris-acetate/EDTA buffer
µg	microgram
µl	microlitre
µM	micromoles per litre
V	volts
VSM	vascular smooth muscle
w	wall thickness
WKY	Wistar Kyoto Rat
x	multiply

CONTENTS

CHAPTER I

GENERAL INTRODUCTION

I.1 Hypertension

I.1.1 *Definition*

Hypertension is a disease state typified by an elevation in blood pressure (BP), either systolic, diastolic or both, that is considered to be detrimental to the well being of an individual (Doyle et al, 1980). In studies as early as 1959, it was demonstrated that individuals that had diastolic blood pressures (DBP) between 88 and 92 mmHg had an increased mortality (150%) compared with other individuals of the same age with DBP of 70 mmHg; elevation of diastolic pressures over 103 mmHg resulted in a 250% increase in mortality and morbidity (Smirk et al, 1959). Elevations in systolic blood pressure (SBP), between 125 and 165 mmHg, in 30-39 year old individuals, resulted in a 10% increase in mortality. Increases in an individual's age also elevated the probability of death due to hypertension by increasing associated risks to other diseases; eg heart disease, cerebral haemorrhage and nephritis.

In an Australian survey conducted in 1989 of over 9000 individuals, between the ages of 20-69 years, 16.7% of men, and 12.7% of women were defined by DBP (DBP $\geq$ 95 mmHg) to be hypertensive. Of these figures 10.9% of men and 4.6% of

women had DBP greater than 95 mmHg. The percentage of individuals classified as hypertensive, if extended to include those with SBP greater than 160 mmHg, rose to 18.3% and 14.3% respectively for men and women (National Heart Foundation of Australia (NHF), Risk Factor Prevalence Study No. 3, 1989).

Cardiovascular disease is still the most prevalent cause of death in Australia, responsible for 46% (55,080) of the total deaths in 1988; coronary heart disease and stroke accounting for 57.3% and 22.6% of this figure respectively (Australian Bureau of Statistics (ABS), 1988a)(Figure I.1). The percentage of deaths in females was 7.1% greater but this figure may be due to the lower mortality rate of females in general and thus give rise to a greater number of individuals that actually die from cardiovascular disease.

Whilst these figures are disheartening in themselves, the incidence of deaths from cardiovascular anomalies has decreased by 58% and 65% for males and females respectively, from 1967 to 1988 (NHF, 1988; ABS, 1988a,b). This decrease in mortality rates can be attributed to greater public awareness of health risks due to diet, stress and lifestyle, and in an appreciation of an annual expenditure of pharmaceuticals for the treatment of cardiovascular disease in excess of $A330 million (ABS, 1991). This figure alone represents 26% of the national expenditure on pharmaceuticals (prescribed drugs only) (ABS, 1991) to which must be added indirect costs of the disease which is believed to be in excess of $A800 million (NHF).

The relatively high cost of treating individuals suffering from cardiovascular disease makes it imperative to discern the pathogenesis of the disease. Hypertension itself is categorised into two areas; those individuals with elevated blood pressures from (a) known causes identified by alterations in biochemical and physiological function, eg pheochromocytoma, primary aldosteronism, renal artery disease or

All Deaths : Percentage Distribution by Cause

(Australia, 1989)

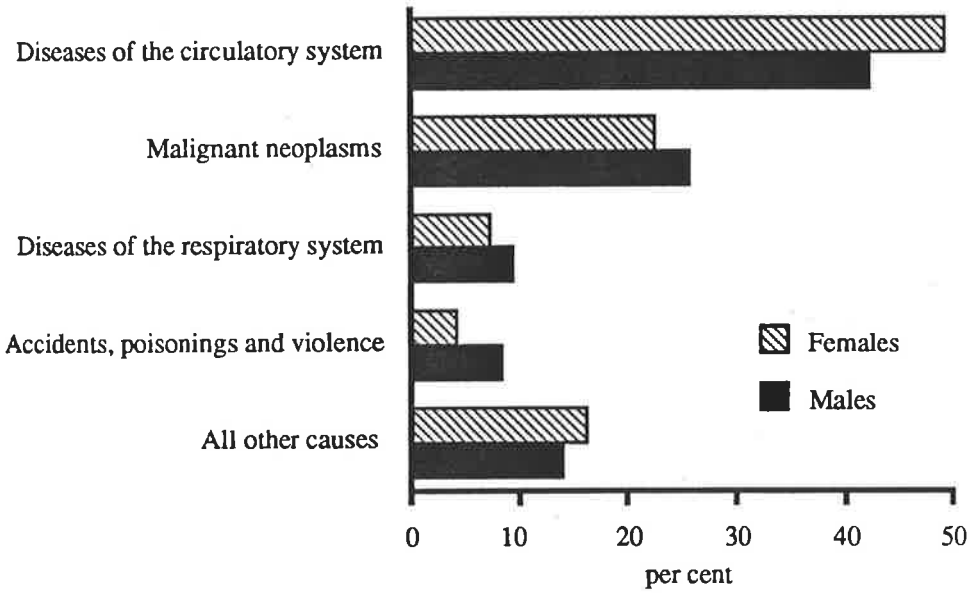


Figure I.1 : Percentage of deaths in Australia, by cause, in 1989. The largest cause of death is due to circulatory, or cardiovascular, disease (from the "Year Book Australia", ABS, Canberra, No 74)

nephritis (secondary hypertension)(Westfall, 1986) and (b) unknown aetiology (primary or essential hypertension). The numbers of patients suffering from secondary hypertension are relatively minor (5%) compared to those suffering from essential hypertension (95%).

I.1.2 *Aetiology of Essential Hypertension*

Although the causes of essential hypertension remain unknown several underlying mechanisms for elevating BP are considered as a basis for its aetiology. It has been suggested that these mechanisms can be classified into two distinct categories; environmental and genetic (Mendelowitz, 1979). Folkow (1982) segregated the factors involved in primary hypertension into ten categories;

1) *Hereditary predisposition* : polygenetic transference in both human and various hypertensive rat strains;
2) *Structural adaptations* : adaptive changes of contractile tissues that include the systemic arteries, precapillary vessels and the left ventricle;
3) *Neurogenic excitatory influences* : autonomic nervous mechanisms having extrinsic cardiovascular control which may help in the initiation of primary hypertension;
4) *Effector cells* : accentuated myogenic activity and muscle sensitivity, and reactivity;
5) *Nature of raised resistance* : ascribed to either an elevated equilibrium of unrestricted resistance or to increased activity of vascular effector cells;
6) *Environmental influences* : dietary excesses such as salt (Dahl, 1962) and deficiencies such as calcium (Resnick et al, 1986), as well as psycho-

emotional influences may aggravate and help precipitate the disease;

7) *Hemodynamic balance* : normality of cardiac output, blood volume and viscosity imply an elevated systemic resistance;

8) *Hormonal excitatory influences* : these may help initiate primary hypertension but are not a direct consequence of blood-borne constrictor influences in the establishment phase;

9) *Barostat resetting* : reflex adjustments and maintenance of BP set at a higher level;

10) *Renal involvement* : a major potential site for the initiation of essential hypertension.

Although each point specified by Folkow has its specific importance in initiating sustained elevated BP, either singularly or in conjunction with one or more of the other mechanisms, the points covered from a)-e) can be more simply categorised into;

i) Genetic Predisposition of Primary Hypertension;

ii) Sympathetic Innervation and Hypertension;

iii) Structural changes of cardiovascular tissues.

I.1.3 *Genetic Hypertension*

There are several hypotheses regarding the genetic predisposition to primary hypertension but it can be generally stated that the phenomenon is polygenic. Nojima (1990) proposed four different patterns for the genetic inducement of hypertension (see Figure I.2); from a single gene to interactions of multiple genes.

Patterns for inheritable hypertension

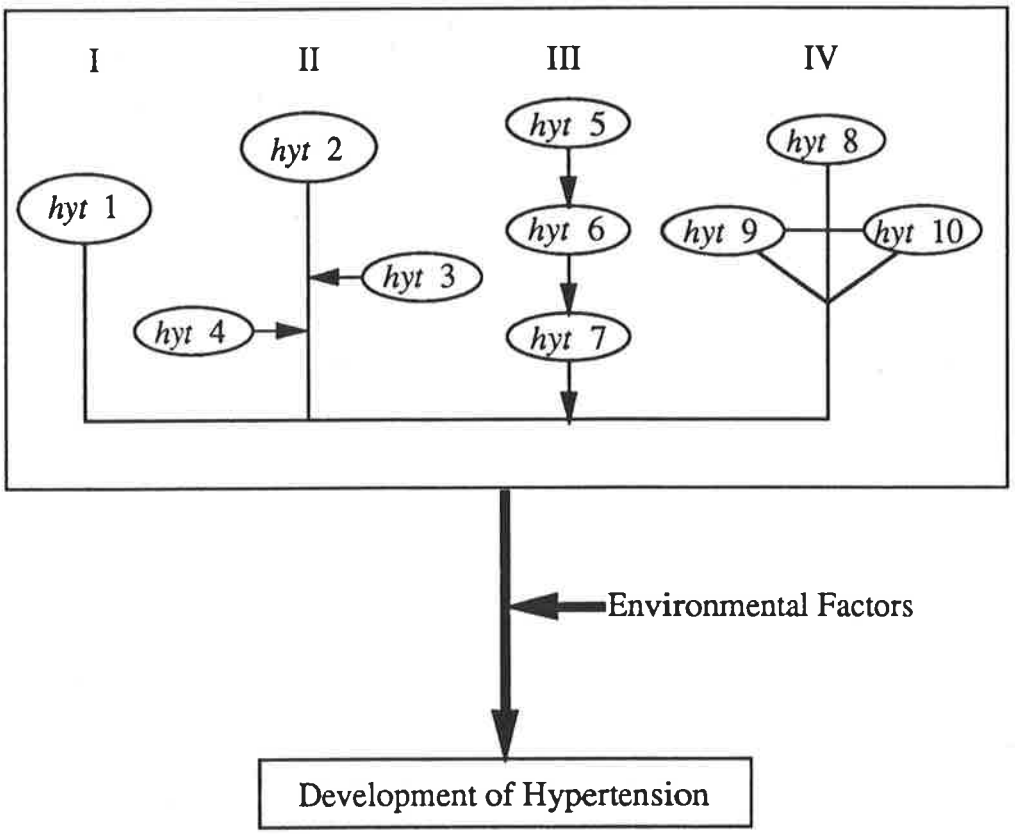


Figure I.2 The above diagram depicts four different types of patterns for the genetic predispositions for hypertension (*hyt* genes) and how they induce hypertension with the aid of environmental factors. The heredity of hypertension is suggested to result from; I : a single causative gene; II : a major, and two minor interacting genes; III : a cascade of three genes; IV : three genes interacting with each other (adapted from "Molecular Genetics in Hypertension", N. Nojima, 1990).

Hypersensitivity to endogenous and exogenous pressor agents (eg. noradrenaline) has been demonstrated in normotensive siblings from hypertensive families (Doyle and Fraser, 1961). Normotensive offspring with a family history of hypertension have also demonstrated a BP lowering effect with diuretics (Ferrier et al, 1983), a 36% lower urinary excretion of kallikerein and high sodium-lithium counter-transport (Berry et al, 1989). Williams and colleagues (1989) have examined a myriad of heritable traits that are associated with primary hypertension and have proposed a model where major genes, polygenes and environmental factors may interact, contributing to the multifactorial susceptibility to hypertension (Figure I.3). Studies on the occurrence of essential hypertension in twins has found that monozygotic twins have a higher susceptibility of inheriting elevated BP from hypertensive parents than do dizygotic twins (Annest et al, 1979; Williams et al, 1988) and that BPs of monozygotic twins tended to be similar (Smirk, 1976).

Needless to say there is considerable evidence for the acceptance of inherited hypertension that is polygenic and that the development of essential hypertension may be due to a multiplicity of different factors.

I.1.4 *Sympathetic Nervous System*

The sympathetic nervous system (SNS) innervates various tissues, and organs, and regulates a variety of mechanisms that directly influence BP. Whether enhanced activity of the SNS, either by measuring changes in cardiovascular reflex activity, or quantitating its principal neurotransmitters (catecholamines and their metabolites) in urine or plasma, contributes to essential hypertension is suspected but remains indeterminate and controversial.

What Causes Hypertension ?

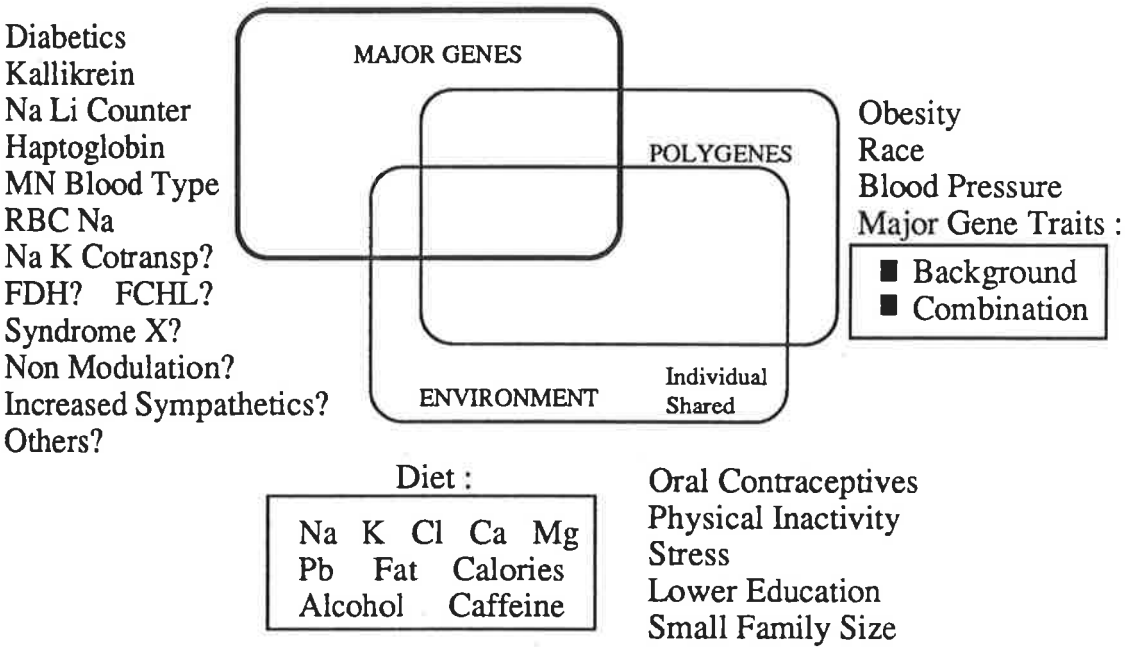


Figure I.3 A model showing overlapping contributions from major genes, poly genes and enviromental factors to the multifactorial susceptility to hypertension. [Na Li Counter = sodium-lithium countertransport; MN = blood group antigens; RBC Na = erythrocyte sodium; Na K Cotransp = sodium-potassium cotransport; FDH = familial dyslipidaemic hypertension: FCHL familial combined hyperlipidaemia (taken from "Current knowledge regarding the genetics of human hypertension", Williams et al, 1989).

The most documented evidence of elevated plasma levels of catecholamines, in particular noradrenaline (NA) and adrenalin (Ad), in relation to hypertension are studies conducted on patients suffering from phaeochromocytoma. In fact, measurement of catecholamines (both urinary or plasma) is not only a diagnostic tool but in some cases permits the time localisation of the tumour (ie. adrenal or extra adrenal). While spillover of NA and Ad into plasma may be a convenient measure of sympathetic nerve activity (SNA) the sensitivity of assays and the limit of detectability have been a problem due to the relatively small levels of catecholamines found in human plasma.

Analysis of plasma NA, generally considered to be a measure of sympathetic tone in essential hypertension, has not been conclusive in delineating hypertensives from normotensives. The fact that circulating catecholamine levels are dependent on a complex system of release (positive and negative feedback systems), uptake, re-uptake, receptor binding, spillover and metabolic degradation (Vanhoutte et al, 1981) brings into question the reliability of circulating catecholamines as a index of SNA (Tuck, 1986), regardless of the fact that overflow studies have demonstrated that the rate of SNS firing is proportional to NA released (Brown and Gillespie, 1957). Similar but more complex considerations have led to abandoning the measurement of serum dopamine β-hydroxylase (DβH) derived from adrenergic vesicles as an index of SNA.

Comparative studies of plasma catecholamines have suggested a general trend in hypertensive patients exhibiting elevated levels of NA (Engelman et al, 1970; de Quattro and Chan, 1972; de Champlain et al, 1977; Goldstein, 1983a,b; Tuck, 1986; Böhm et al, 1987; Esler et al, 1988; Iimura, 1989) however statistical analysis of the data suggests that approximately 40% of hypertensives have a positive correlation between circulating NA and persistent elevated BP (Goldstein, 1983a; Tuck, 1986).

Measurement of total catecholamines (NA, Ad and dopamine) while contributing to a total level of circulating catecholamines does not statistically influence the association between hypertension SNA. Increases in plasma and urinary dopamine has been associated with patients that demonstrate a salt-sensitive idiopathic hypertension (Gill et al, 1988).

Age of patients showed an influence on the levels of catecholamines in as much as young hypertensives tended to show a better correlation between the disease state and their plasma NA levels and that the basal level of plasma catecholamines, for both normotensive and hypertensive subjects, increased with age (Goldstein, 1983a; Tuck, 1986). It has been proposed that within the hypertensive population there are subcategories that are defined by; (1) those with elevated circulating levels of NA (hypernoradrenergic) and (2) those patients with circulating levels of NA indeterminate from normotensive patients (Tuck, 1986).

An increase in NA overflow can also be affected by the proposed positive-feedback modulation of activation of presynaptic β_2-adrenoceptors. The mechanism for this feed-back system is based on co-released Ad, derived from the adrenal gland and incorporated into sympathetic nerve endings, activating the presynaptic β-receptor and thereby increase NA release (Majewski et al, 1981). Ad infusion in humans has produced conflicting results in that increases in plasma NA have been observed with infused Ad in selected studies (Nezu et al, 1985; Chang et al, 1989; Gordon et al, 1989) whilst not in others (Brown, 1985). While there is adequate evidence to agree with the argument that Ad increases the release of NA, at least with infused Ad, studies with radiolabelled Ad suggest that, at least in humans, that Ad is taken up but not released concomitantly with NA (Gordon et al, 1989). The β-adrenoceptor feed-back theory fails to accommodate the findings that there is a blunted β-receptor

responsiveness and a hyperresponsiveness to pressor agents in patients with essential hypertension (de Champlain et al, 1989).

Many hypertensive patients demonstrate a reduced baroreceptor sensitivity (Takeshita et al, 1975; Goldstein, 1983b; Tuck, 1986) and as mentioned earlier an enhanced vascular response to NA (Doyle and Fraser, 1961; Goldstein, 1983b; Esler et al, 1988); these patients have all displayed a relationship between enhanced BP and elevated circulating NA. Of some interest, and in support of inheritable hypertension, young normotensive subjects with parents exhibiting hypertension, also demonstrated an increased sensitivity to NA (Doyle and Fraser, 1961). Results collectively suggest that in human hypertensives there exists the unusual relationship of enhanced transmitter release with enhanced activity of vascular smooth muscle (VSM) cells.

Direct recordings of sympathetic nerve activity in human skin and muscle nerves have demonstrated differences in the pattern and role of activity in tissues, as well as the same organ in different individuals (Wallin and Fagius, 1986). A positive correlation between plasma levels of NA (forearm) and the frequency of bursts in muscle sympathetic activity has been demonstrated in normotensive, hypertensive and cardiac failure patients (Wallin, 1989) despite the fact that microneurographic studies have failed to show a significant difference between normotensive and patients suffering from essential hypertension (Wallin, 1989).

Why some hypertensive patients exhibit elevated plasma NA while others appear to have normotensive levels remains a mystery, however, there does exist a possible explanation. NA is metabolised by two major enzymes; (1) monoamine oxidase (MAO) resident in neuronal mitochondria and, to a lesser extent, in effector cells, and (2) catechol-O-methyltransferase (COMT) residing in effector and liver cells. The re-uptake of NA into neuronal cells is the most important transport system for the

removal of the transmitter from both plasma and neuronally released NA. It is also well established that the major neuronal (deaminated) metabolite of NA is dihydoxy-phenylglycol (DOPEG). If an increase in spillover is due, in some cases, not to an increase in SNA but to an increase in the density of nerves, then the re-uptake and metabolism of the transmitter will also be enhanced. The increased spillover, in conjunction with an increased uptake, would mean that there would be an increase in neuronal metabolites present in plasma and urine. This explanation has been put forward, based on the findings, that plasma DOPEG was elevated in those hypertensive patients that did not display elevated plasma NA content (Ludwig et al, 1991). Patients administered desipramine, to block the neuronal uptake of NA, displayed a decreased plasma level of DOPEG although hypertensive patients retained an elevated level compared to normotensive patients. No difference was found between the plasma NA levels of hypertensive and normotensive patients after treatment with desipramine.

A large proportion of all antihypertensive drugs developed in the last four decades for the treatment of elevated BP interfere with the function of the autonomic nervous system either at central or peripheral levels, and in some case at both. Drugs effecting the peripheral autonomic system can be readily segregated into 5 classes (Doyle et al, 1980) :

1) ganglion blocking drugs (eg. hexamethonium)

2) adrenergic neurone blocking drugs (eg. guanethidine)

3) monoamine oxidase inhibitors (eg. pargyline)

4) α-adrenergic blocking agents (eg. phentolamine and more recently selective agents such as prazosin)

5) β-adrenoceptor blocking drugs (eg. propranolol and more

recently selective agents such as metoprolol).

To this list should be added drugs that are inhibitors of the synthesis of NA (eg. α-methyltyrosine (Aldomet)) and drugs that modify CNS baroceptor outflow (eg. clonidine)(Westfall DP, 1990). The fact that such a range of drugs can effectively interact with the SNS and result in a reduction in BP implicates a role of the SNS in contributing to the maintenance of hypertension. The critical question then arises for a possible additional role of the SNS in the initiation of hypertension. Given that there is a subgroup of hypertensive patients that exhibit a consistent elevation in plasma catecholamines in conjunction with a sustained elevation in BP, and that some patients exhibit elevated NA metabolites in the absence of elevated plasma NA, suggests that the sympathetic nervous system may play a role not only in the maintenance but in the pathogenesis of hypertension.

I.1.5 *Structural Changes*

It goes without question that primary essential hypertension is multifactorial in nature and that changes in the structure of conduit and contractile blood vessels, as well as structural aberrations in the heart, are the pathological characteristics of an elevation in BP. The question as to whether cardiovascular changes result in elevated BP or whether an increased BP cause adaptive cardiovascular changes that consequently result in sustained elevations in BP is the unresolved "chicken or the egg" phenomenon. This outlook is mainly reflective of the fact that structural alterations of blood vessels occur in close association with elevation of BP (Lee and Smeda, 1985) and it is extremely difficult to dissociate cause and effect in a system as closely integrated and interrelated as the cardiovascular system.

It is of some importance to discern whether structural alterations occur prior to, or after, the development of hypertension, as this will determine whether the therapy is treating the cause or a symptom of the disease. Primary changes can be classified as alterations to the cardiovascular structure of vessels, and organs, prior to the development of the disease while structural changes that are adaptive in response to elevated BP are then denoted as secondary changes (Lee and Smeda, 1985; Folkow, 1990). Early prevention of hypertension by antihypertensive therapy should therefore prevent any secondary structural modifications to blood vessels.

Structural adaptive change of cardiovascular tissue is a normal and essentially a local response to changes in tissue activity and load subjected to the tissue (Folkow, 1982; 1990). Increased vascular resistance has been implicated in patients suffering from hypertension and it has been implied that this increase is due to structural changes of arterioles (Folkow, 1958). The hemodynamic behaviour of peripheral resistance, *in vivo*, is dependent on four factors; (1) smooth muscle activity, (2) the vessels' geometric design, (3) the distensibility of the vessel wall, and (4) the pressure distending the system (Folkow, 1982). Thus the higher the pressure, the higher must be the wall thickness-to-radius ratio (w/r); this is in accordance with Laplace's law:

$$\sigma = P \times r/w$$

wall stress (σ) is equal to the product of the radius (r) and the transmural pressure (P), divided by the wall thickness (w). While changes in conduit vessels will ultimately alter the dynamics of the system the greatest changes to the pressure of the system would be generated by changes in the activity and structure of the VSM in resistance vessels. Changes in wall thickness, and an attenuation of the distensibility of the vessel wall, will result in an elevation of the transmural pressure to which must be added additional elevations of pressure due to a decrease of the luminal radius

resulting from encroachment of the lumen volume by vessel cells (Folkow et al, 1958). The wall-to-lumen ratio hypothesis of Folkow and his colleagues is based on perfusion studies and not on actual morphological, or morphometrical, studies. The difficulties in obtaining human tissues to study are self evident and thus little data from human studies is available.

While most of the evidence for structural changes are derived from animal model studies cardiovascular changes have been observed in patients suffering from primary hypertension. Medial hypertrophy of coronary vessels, and of coronary tissue in general, correlates strongly with the increase in aortic rigidity seen in patients with sustained essential hypertension, that is considered to be a consequence of increased after-load (Safar et al, 1987). The increased load is also related to increases in vascular resistance afforded by changes in total vascular resistance (Folkow, 1982). It is the attenuation of the distensibility of arteries that is considered to be a cause for the dysfunction of the baroreflex in established hypertension (Goldstein, 1983b).

I.2 Animal Models of Hypertension

The study of hypertension, in humans, presents a multitude of problems from ethical and legalistic considerations which often limit the scope of experimentation. It is therefore imperative that other models be available. *In vitro* experiments on tissue cultures produced from tissues obtained from patients during surgery is far too limiting, and does not allow a comprehensive examination of the causative effects in hypertension. Likewise, blood vessels removed from humans are often diseased and the results generated are often of limited value.

Animal models on the other hand allow for appropriate control experiments, a

greater flexibility in the type of studies available and the number of animals used, as well as providing a means to follow the progression of cardiovascular changes from initiation to the established stage. Hypertension can be induced chemically by exposing the animals to compounds and drugs that alter some physiological system more or less in a similar fashion to that seen in humans, eg. deoxycorticosterone acetate (DOCA)-salt hypertension (de Champlain et al, 1968), or by surgical intervention, as in the Goldblatt renal hypertension (Leenen and Meyer, 1984).

Animal models also allow the development of strains that are genetically predisposed to inherit hypertension. Over the last 40 years a number of different hypertensive rat strains have been developed (see Table I.1) and are used as an aid in determining the aetiology of hypertension in humans. Each animal model has its own unique attractiveness from the stand point of different facets of hypertension, eg. renal, volume expansion and hypernoradrenergic hypertension, however, the spontaneously hypertensive rat (SHR) and Dahl salt-sensitive rat (Tobian, 1984) strains have been the most extensively adopted and investigated over the last 25 years. From the standpoint of human essential hypertension the SHR has been exhaustively studied and like human essential hypertension there is no unifying hypothesis regarding (i) the relationship of the disease to the gene or (ii) the initiation of the disease.

I.2.1 *Spontaneously Hypertensive Rat*

The development of the SHR was commenced in 1959 by selectively inbreeding brother-sister pairs of American Wistar rats at the Animal Centre Laboratory of the Kyoto University, Japan, and was reported as a spontaneously hypertensive rat strain in 1963 (Okamoto and Aoki, 1963); the strain was established

Hypertensive rat strains

(The initial location of breeding is indicated in parentheses)

DR	Dahl salt-resistant strain
DS	Dahl salt-sensitive strain (Bookhaven)
GH	Genetically hypertensive strain (Dunedin)
LH	Lyon hypertensive strain
LN	Lyon normotensive strain
LL	Lyon low blood pressure strain (Lyon)
MHS	Milan hypertensive strain
MNS	Milan normotensive strain (Milan)
SBH	Sabra hypertensive strain
SBN	Sabra normotensive strain (Jerusalem)
SHR	Spontaneously hypertensive rat strain (Kyoto)
WKY	Wistar-Kyoto strain (Kyoto)
SHRSP	SHR-stroke prone strain (Kyoto)

Table I.1 : Different rat strains developed throughout the world for investigations into the pathogenesis of hypertension (taken from "Handbook of Hypertension, Vol. 4: Experimental and genetic Models of Hypertension", edited by W de Jong, 1984).

in 1969 when it had reached its 20th generation (Okamoto, 1969). The normotensive Wistar, from which the SHR was derived, constitutes the major control in experiments with the SHR and is referred to as the Wistar-Kyoto rat (WKY). An important characteristic of the SHR is that 100% of the adult population will be hypertensive by 18 weeks of age in the absence of any pharmacological or surgical intervention (Okamoto, 1969).

The heritability of the disease state in the SHR has been attributed to 3 major genes (Yamori, 1984). The SHR will develop secondary complications to elevated BP, as is the case in sub-populations of human hypertensives, such as myocardial and vascular lesions, as well as nephrosclerosis, and elevated peripheral vascular resistance in the absence of any changes in cardiac output (Okamoto, 1972). The hypertensive state of this animal model is effected by dietary calcium (Stern et al, 1984) and sodium (Pratt et al, 1989), psychoemotional factors (Yamori et al, 1969), and is not refractory to antihypertensive therapy.

The development of BP in the SHR has been proposed to occur in three stages; the prehypertensive stage (the first 40-50 days of age), the developmental or early hypertensive stage (ending at about 4 months of age) and an established or advanced hypertensive stage (extending from 4 months of age onwards) (Aoki et al, 1963; Okamoto, 1972). The BPs of SHR and WKY rats are reported to be significantly different at the end of each of these periods; 147 mmHg vs 131 mmHg, 185 mmHg vs 138 mmHg and 188 mmHg vs 133 mmHg respectively for each stage (Gray, 1984). BPs of neonatal SHR have been found to be similar to those measured in neonatal WKY pups (Lee et al, 1988). In contrast to this is observations that neonatal BP is elevated at 1-3 days of age (Bruno et al, 1979; Gray, 1984). Although these investigators used somewhat different techniques to study neonatal BP the evidence

indicates that, rather than an increase in BP at 6-7 weeks of age, an elevated pressure may exist at birth which gradually increases over the early period of development (Gray, 1984).

I.2.1.1 *Hypernoradrenergic Innervation*

Several studies have proposed that the higher the density of adrenergic varicosities within the medial and adventitial layers of a blood vessel the more neurotransmitter will be available to reach the smooth muscle cells (Bevan and Su, 1973; Su and Lee, 1976). Just as the concentration of NA present in plasma and urine samples from humans are considered to be a reflection of the activity of the sympathetic nervous system, the concentration of NA found in sympathetically innervated tissues is considered to reflect the degree of innervation present in that tissue. Studies have been conducted using a variety of techniques, from *in situ* histofluorescence to radioenzymic assays and high performance liquid chromatography (HPLC), all of which have demonstrated conclusively that the SHR have an over-developed peripheral SNS compared to the normotensive WKY (coined "hyper-noradrenergic innervation"; see review Head, 1989). The mesenteric vascular bed (Berkowitz and Spector, 1976; Berkowitz et al, 1980; Head et al, 1984,1985; Donohue et al, 1988; Kawamura et al, 1989), caudal (Cassis et al, 1985; Donohue et al, 1988), choroidal (Haebara et al, 1968) and renal (Head and Berkowitz, 1979) arteries all have demonstrated increased levels of NA relative to levels in control (WKY) tissues. Analysis of nerve bundles (Featherston et al, 1984; Cassis et al, 1985) and density (Lee, 1985) have also been shown to be increased in the SHR. The increased content and axonal cross-sections of sympathetic nerves innervating blood vessels has lead to

the concept of hypernoradrenergic innervation in the SHR (Cassis et al, 1985). An increased innervation has been suggested to be present as early as 2 day after birth in the SHR (Donohue et al, 1988) and it has been demonstrated that the innervation is consistently elevated during most stages of development (Donohue et al, 1988). The densities of other nervous tissue, specifically cholinergic nerve fibres, vasoactive intestinal polypeptide containing and substance-P containing nerve fibres, in the mesenteric vasculature, are not found to be significantly different between the SHR and WKY strains at 7 weeks or at 6 months of age (Kawamura et al, 1989).

In contrast SHR cardiac tissue does not reflect the increase in sympathetic innervation that is seen in blood vessels. Moreover there is some contention as to the actual level of innervation in the SHR heart as investigators have shown NA concentrations to be either decreased (Howe et al, 1979; Berkowitz et al, 1980) or indistinguishable (Donohue et al, 1988) from WKY. The apparent lack of an increase in cardiac innervation is significant in the light that elevation of BP in the SHR is not associated with an increase in cardiac output (Smith and Hutchins, 1979; Trippodo and Frohlich, 1981) but with an increase in peripheral resistance, at least in adult animals.

The activity of renal nerves (Judy et al, 1976; 1979) and splanchnic nerves (Iriuchijima, 1973) has been shown to be significantly higher in the SHR than in Wistar rats. It would follow that enhanced SNA would result in a greater discharge of transmitter over a given time and that this would be amplified by the hyper-noradrenergic innervation seen in the SHR. Ganglion blockade with hexamethonium effectively reduces SNA and arterial BP of SHR rats to levels similar to those seen in ganglion blocked WKY rats (Judy et al, 1976). Moreover Yarowsky and Weinreich (1985) have shown that synaptic transmission through SHR ganglia is heightened compared with transmission through WKY ganglia. Cholinergic-noradrenergic

neurotransmission in superior cervical ganglia has also been demonstrated to be hyperreactive in young but not adult SHR rat (Debinski and Kuchel, 1989). Due to the hypernoradrenergic innervation demonstrated in the SHR it is not surprising that researchers have shown an increase in NA release upon nerve stimulation in vascular tissues from SHR rats (Zsotér et al, 1982; Westfall et al, 1984, 1986).

Responses to graded nerve stimulation of SHR blood vessels from the mesenteric vascular bed have been demonstrated to be shifted to the left when compared to the same vessel of the WKY (Nilsson and Folkow, 1982; Yamamoto and Cline, 1988) although the contractile responses of the smooth muscle to NA appears to be similar (Whall et al, 1980; Cassis et al, 1985). Perivascular stimulation of the sympathetic nerves of mesenteric and caudal arteries, in the presence of cocaine, an inhibitor of catecholamine neuronal uptake, shifts both the SHR and WKY frequency response curves to the left although the shift in the SHR curve was significantly greater (Cassis et al, 1985). The greater shift in the SHR frequency response curve supports the suggestion of a greater release of NA from the SHR (normally modulated by an enhanced NA uptake) and thus an increased degree of innervation (Whall et al, 1980; Mulvany, 1989). The "cocaine-shift" to exogenous NA in mesenteric ring preparations seen by Mulvany (1989, 1990) was also explained as an index of increased innervation.

Chemical denervation of mesenteric arteries with 6-hydroxydopamine (6-OHDA) also increases the sensitivity of vessels from SHR animals by 2 fold compared to sympathetically denervated WKY rats (Whall et al, 1980). Chemical sympathectomy of neonatal SHR, with either 6-OHDA or antibodies to nerve growth factor (anti-NGF), can markedly reduce the development of hypertension (Yamori et al, 1972; Provoost et al, 1978; Bevan and Tsuru, 1981; Nyborg et al, 1986; Lee et al,

1987) and reduce vascular hyperplasia (Bevan and Tsuru, 1981; Lee et al, 1987).

The indifferent contractile responses observed by Whall et al (1980) are consistent with those reported by Mulvany et al (1978) who demonstrated a similar ED_{50} value for NA in both SHR and WKY resistance vessels. In contrast, other investigators have demonstrated that the responses of VSM preparations to exogenous NA from the SHR are greater than those seen from the WKY (Lais and Brody, 1975, 1978; Longhurst et al, 1986; Debinski and Kuchel, 1989).

Nerve growth factor (NGF), a protein considered to be essential for the development, and maintenance, of sympathetic and sensory nerves has been found to be significantly elevated in mesenteric arteries (≈59%) and aortae (≈43%) of 20 day old SHR compared to age-matched WKY rat pups (Donohue et al, 1989). Moreover when adult tissues were analysed NGF content did not differ significantly between the two strains. The fact that antibodies to NGF can not only denervate the sympathetically innervated tissues but abolish the hyperplastic changes in those tissues suggests some relationship between innervation, VSM growth and the development of hypertension. Consistent with this is the observation that administration of NGF not only enhances sympathetic innervation but also increases VSM growth (Zettler et al, 1991). It has yet to be determined how the increased NGF concentration seen in young SHR resistance vessels relates to the gene and what is the specific period of increased NGF production which enforces enhanced sympathetic growth. It is evident from chronic NGF studies that increased growth per se (eg. in the adult rat) does not lead to the hypertensive state although sympathetic innervation is elevated (Zettler et al, 1991).

I.2.1.2 *Structural Changes*

Primary and secondary adaptive cardiovascular changes are also a major factor in initiation and maintenance of hypertension in the SHR. Several studies have demonstrated morphological alterations of both vascular and cardiac vessels in both young and adult SHR.

Cardiac hypertrophy, specifically ventricular hypertrophy, has been considered to be an adaptive change to sustained elevated BP. Heart-to-body weight (H/BW) ratios of adult SHR are consistently greater than that seen in adult WKY (Mulvany et al, 1978, 1985; Cutilletta and Oparil, 1980; Owens, 1985; Nyborg et al, 1986) indicating cardiac hypertrophy present in adult animals. Whether cardiac mass is increased in neonatal SHR is still to be resolved as several researchers have noted that H/BW ratios are also greater in neonatal SHR pups (Gray, 1984; Nyborg et al, 1986) whilst others (Owens and Schwartz, 1982) have not. The fact that neonatal SHR pups may exhibit cardiac hypertrophy may reflect species differences and not an adaptive change to elevated and sustained BP; this dilemma has yet to be solved.

Medial hypertrophy of resistance vessels of the mesenteric bed (Mulvany et al. 1978, 1985; Lee et al, 1983, 1987, 1988; Owens et al, 1988), as well as the aorta (Limas, 1980; Owens and Schwartz, 1982; Owens, 1985), has also been demonstrated to exhibit hypertrophic expansion in adult SHR. As well as hypertrophic changes in mesenteric vessels, increases in the number of smooth muscle cells (hyperplasia) have also been reported (Lee et al 1983,1987). Neonatal hypertrophy of mesenteric vessels has not been shown (Lee et al, 1988) suggesting that any changes in the SHR mesenteric vasculature occur at some point after birth.

With the observation that hypertrophy and hyperplasia of resistance blood

vessels exists for the SHR it is not surprising to note that the wall thickness-to-lumen radius ratio is greater in the SHR compared to age matched WKY (Mulvany et al, 1978, 1985; Limas, 1980; Owens and Schwartz, 1982; Owens, 1985; Lee et al, 1983, 1987, 1988; Owens et al, 1988; Mulvany, 1987, 1990). The encroachment of the lumen volume by cells of the media resulting from a thickening of the vessel wall has been reported (Mulvany et al, 1978; Mulvany, 1987, 1990; Owens et al; 1988) as predicted earlier by Folkow et al (1958).

While SHR rats are more resistant to chemical denervation with 6-OHDA than WKY rats (Nyborg et al, 1986) 6-OHDA has been shown to attenuate the development of hypertension (Yamori et al, 1972; Provoost et al, 1978; Bevan and Tsuru, 1981; Nyborg et al, 1986). Complete sympathectomy of neonatal SHR, with a combined therapy of anti-NGF and guanethidine, prevented the hyperplastic but not the hypertrophic changes in mesenteric smooth muscle cells (Lee et al, 1987); the hypertension in these animals was completely abolished. In contrast, Nyborg et al (1986) failed to observe any changes in morphology following sympathectomy of SHR rats with 6-OHDA of mesenteric vessels; the failure to notice any changes may be due to the incomplete denervation seen by Nyborg with 6-OHDA (1986). SHR rats immuno-sympathectomized with antiserum to NGF prevented the development of hypertension but failed to prevent the development of left ventricular hypertrophy (Cutilletta and Oparil, 1980).

Nonvascular smooth muscle mass, Müller's muscle, has also been reported to be increased in the SHR (Smith et al, 1988) suggesting that other factors may also be responsible for increased smooth muscle mass in hypertension. Cultured smooth muscle cells from SHR replicate, *in vitro*, at a greater rate than cells from normotensive rats (Yamori et al, 1981; Suithichaiyakul et al, 1990) and it has been

suggested that a β-adrenergic neurohumoral mechanism may accelerate smooth muscle cell growth independent of BP (Yamori et al, 1981). Cultured SMC from the SHR are reported to be more prolific in the presence of a number of factors: angiotensin II (Baudouin-Legros et al, 1989; Lyall et al, 1990), epidermal and insulin-like growth factors (Scott-Burden et al, 1989) and fetal calf serum (Clegg et al, 1986; Baudouin-Legros et al, 1989).

I.3 Nerve Growth Factor

In 1913, Ramon y Cajal (Levi-Montalcini and Angeletti, 1968; Chamley, 1973) suggested the existence of a neurotrophic agent but the first observation of such an agent was not witnessed until Bueker (1948) noted hyperplasia and hypertrophy in sensory ganglia after explanting a mouse tumour, sarcoma 180, into the hind limb of a chick embryo. The existence of a specific promoting agent was later confirmed when mouse tumours, sarcomas 180 and 37, grafted into chick embryos resulted in increased neurite outgrowth from sensory (250%) and sympathetic (600%) ganglia (Levi-Montalcini and Hamburger, 1951; Cohen et al, 1954). Cohen (1954) coined the term "Nerve Growth Promoting Factor" to describe the nucleoprotein responsible for the growth and differentiation of nerve cells which was later shortened to Nerve Growth Factor (NGF)(Levi-Montalcini and Angeletti, 1968).

Since 1956, NGF has been extensively characterised and identified as a protein rather than a nucleoprotein molecule (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980; Levi-Montalcini, 1987). In determining the biochemical characteristics of purified NGF Varon et al (1967) noted that the molecule had a molecular weight of approximately 140 kilo Daltons (kDa). Sucrose sedimentation of

this compound resulted in a sediment coefficient of 7.1 and the NGF complex designated as 7S. A secondary complex, designated 2.5S, was also found that represented an active fraction with a molecular weight of 30 kDa. The 7S complex dissociates into 3 smaller complexes of approximately 30 kDa when subjected to a pH outside a range of 5-8. These complexes are designated the α, β and γ subunits and the stoichiometry of the 7S complex is $\alpha_2\beta\gamma_2$ (Thoenen and Barde, 1980). The acidic α-subunit has not been found to demonstrate any known physiological activity to date while the γ-subunit is known to be an arginine-specific esteropeptidase (Server and Shooter, 1977). The β-subunit is the only subunit capable of producing the NGF biological response which is stimulation of neurite outgrowths (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980; Levi-Montalcini, 1987) and physiological active NGF is generally known as β-NGF. The arrangement of the α and γ-subunits affords protection of β-NGF and the proNGF complex must have the subunits cleaved to form the active molecule (Figure I.4)(Levi-Montalcini and Calissano, 1983). Multiple forms of NGF, in association with its subunits, have been found (Smith et al, 1968) suggesting that active NGF may be formed by differential cleavage of the α and γ-subunits.

Active NGF from snake venom is a protein with a molecular weight of approximately 20 kDa and exists in multiple aggregate states with similar α and γ-subunits as those found in mouse NGF (Angeletti et al, 1967; Levi-Montalcini and Angeletti, 1968). In contrast, NGF synthesised by cultured mouse fibroblasts has the ability to elicit the NGF-biological response and is of the same molecular weight as submaxillary gland NGF (140-160 kDa)(Pantazis et al, 1877). The α and γ-subunits however are not found in fibroblastic cell media and may constitute a different form of NGF (Pantazis et al, 1977; Pantazis, 1983).

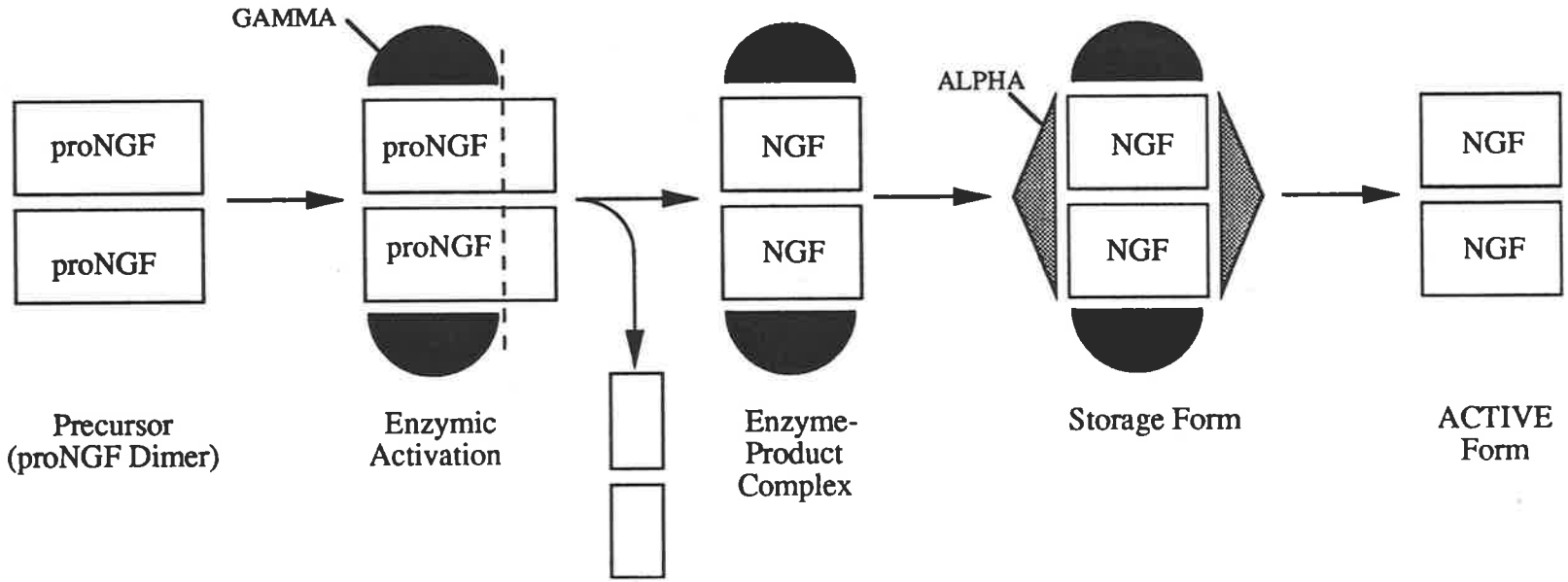


Figure I.4 The manufacture of NGF requires a number of "processing"steps. The intial form of the molecule is a precursor chain called proNGF. The chains form a dimer which are cleaved by an enzyme known as the gamma subunit. The two gamma subunits remain associated with the NGF dimer and two additional proteins known as alpha subunits then bind to the complex. The association of both the alpha and gamma subunits, with the NGF dimer, appears to protect the complex from further degradation by other enzymes. The subunits are cleaved to produce the active NGF dimer (adapted from "The Nerve Growth Factor" by Levi-Montalacini and Calissano, *Sci. Am.*, 1979).

In contrast to Pantazis's claim of a different NGF from fibroblasts Greene and Shooter (1980) have demonstrated that the stability of 7S NGF is dependent on zinc ions (Thoenen and Barde, 1980) and that this may account for the lack of α and γ-subunits in fibroblast cell media.

NGF elicits its effect by initially binding to a surface receptor, is internalised and then transported retrogradely to the perikaryon. The embryonic NGF-responsive targets of chicks have been shown to possess 2 classes of receptors on their membrane surface; one a low affinity site with a Kd of 1.2nM and the other a high affinity site with a Kd of 0.02nM (Frazier et al, 1974a,b; Riopelle et al, 1980). Receptors from cultured neural crest cells were characterised as having a much lower affinity site with a Kd of 3.2nM suggesting that these cells may not be terminally differentiated (Bernd,1987, 1989). It is also noted that dorsal root ganglia (DRG) of chick embryos are only minimally responsive to NGF prior to embryonic day 7 (Bernd, 1987). Thus the sensory and sympathetic ganglia that derive from neural crest cells must undergo differentiation and it is suggested by Bernd that the appearance of the receptors is in-association with the cells acquiring phenotypic characteristics. On the other hand, embryonic catechol-aminergic cells from embryonic ganglia possess both high and low affinity receptors (Bernd, 1989) but are unable to alter the activity of tyrosine hydroxylase, a function characteristic of PC12 cells (Gizang-Ginsberg and Ziff, 1990) and seen in neonatal rats (Goedert et al, 1978). In contrast placode-derived neurons, taken from chick embryos between embryonic day 6 and day 12, present conflicting data as some researchers have found these neurons to be either devoid of NGF receptors (Straznicky and Rush, 1985; Lindsay and Rohrer, 1985) or are shown to express NGF receptor mRNA (NGF-R-mRNA)(Ernfors et al, 1988; Hallböök et al, 1990). A truncated form of the NGF receptor (NGF-Rt) has been found in the media

from cultured Schwann cells as well as being found in amniotic fluid and neonatal rat urine and plasma (DiStefano and Johnson, 1988). During development the level of NGF-Rt found in rat bodily fluids has been found to decrease but upon lesioning adult peripheral nerves these levels have been shown to increase (DiStefano and Johnson, 1988). High affinity receptors have recently been found on Ewing's sarcoma and Wilm's tumour cell (Thomson et al, 1989) and these cells present further models in which to study the effects of NGF. The bivalent nature of NGF to bind to its receptor is unusual and indicates that NGF is internalised after binding to its receptor (Hendry and Iversen, 1973; Dumas et al, 1979); this bivalent binding and internalisation is also displayed by insulin (De Meyts et al, 1973).

The evidence for retrograde axonal transport of NGF is supported by the results obtained when radioactive NGF (^{125}I-NGF) was injected unilaterally into the anterior chamber of the eye and radioactivity was found to accumulate in the superior cervical ganglia of the injected side (Hendry et al, 1974 a,b; Stöckel and Thoenen, 1975; Greene and Shooter, 1980).

Levi-Montalcini and Hamburger (1951) extended the sarcoma findings of Bueker (1948) and noted that; (1) sensory and sympathetic ganglia produced a greater degree of outgrowth in the presence of the sarcoma 180 than in the presence of the limb itself, (2) the outgrowth was observed to affect only sympathetic and sensory neurons whilst parasympathetic, motor and autonomic preganglionic neurons were not affected, (3) the degree of outgrowth tended to be enhanced if the neurons had extensions into the tumor and (4) the neurites entering the tumors did not produce any specialised synapses. Thus, it was concluded that the normal development of neurons can be attributed to peripheral target tissue production of NGF, or some trophic factor, and not to the type of synapse formed. It also indicated that NGF was a specific

trophic factor that effects sensory and sympathetic innervation. Injecting exogenous NGF into neonatal rat pups results in profuse peripheral sprouting of sympathetic nerves and an overdevelopment of sympathetic innervation (Levi-Montalcini and Calissano, 1983; Zettler et al, 1991). Further evidence of NGF's importance for the development of sympathetic neurons are experiments where neonatal rats were injected with anti-NGF that resulted in animals developing to maturity essentially lacking a sympathetic nervous system (Levi-Montalcini and Angeletti, 1968; Bjerre et al, 1975; Goedert et al, 1978). The administration of anti-NGF to neonatal rat pups results in a dramatic, and irreversible, reduction in NA-synthesising enzymes, specifically tyrosine hydroxylase and dopamine β-hydroxylase (Goedert et al, 1978), but has only transient (decreased) effect in adult rats. Injecting anti-NGF into neonatal rat pups has been shown to induce sprouting of sensory axons in dorsal root ganglia (Hulsebosch et al, 1987; Urschel and Hulsebosch, 1990). The interpretation of this was that the cell bodies respond in a manner conducive to a state of denervation and initiates sprouting, and that Schwann cell-neuronal interactions are altered by the inactivation of NGF. The changes seen in sensory neurons occur only in the central processes of sensory fibres and not in peripheral processes (Urschel and Hulsebosch, 1990). The other possibility is that some central sensory neurons are dependent on another growth factor, either brain derived neurotrophic factor (BDNF)(Barde, 1989; Wetmore et al, 1990) or NT3 (Ernfors et al, 1990), and the removal of NGF-dependent neurons allows additional collateral sprouting of BDNF-dependent neurons. Both BDNF and NT3 are structurally and functionally related to NGF and it appears that these factors belong to a growing family of neurotrophic factors that may cooperate to support the development and maintenance of the sympathetic nervous system. An increase in specific sensory nerve fibres (D-hair afferents) has been observed in conjunction with

a depletion of high threshold mechanoreceptors after anti-NGF treatment (Ritter et al. 1991). Thus a subpopulation of sensory nerves are refractory to NGF, or specific NGF-antibodies, and proliferate with the removal of NGF-dependent nerves. Evidence of other growth factors accounting for sensory nerve survival has been shown when cells from DRG cultures survived in the presence of anti-NGF when incubated with fluid collected from either portion of a severed sciatic nerve (Lundborg et al, 1982).

Tissue culture techniques have clearly demonstrated that specific tissues tend to attract neurite growths and that the growth is toward tissues that express and produce NGF (Chamley et al, 1973; Chamley and Dowel, 1975; Furukawa et al, 1984; Southwell eta al, 1985; Sefton et al, 1987). Cultured sympathetic ganglia will preferentially extend neurite outgrowths towards vas deferens, atria (Chamley et al, 1973; Chamley and Dowel, 1975; Furukawa et al, 1984) and caudal artery (Southwell et al, 1985) than toward kidney, uterus, lung or aorta. Neurite outgrowth will occur toward the other tissues but the growth appears to be random and at a much later time. It was proposed (Chamley et al; 1973) that neurite growth may be related to the normal potential of the tissue to be sympathetically innervated and the NGF produced by the effector organs was responsible. In accordance with Chamley's proposal both the caudal artery (Cassis et al, 1985; Westfall et al, 1986; Donohue et al, 1988) and vas deferens (Head et al, 1985) have been shown to be highly sympathetically innervated tissues compared to the lung, kidney and aorta.

The richest source of NGF is found in the male submaxillary gland (Cohen et al, 1960; Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980; Levi-Montalcini, 1987) while relatively high concentrations have also been demonstrated in snake venom (Cohen and Levi-Montalcini, 1956; Cohen, 1959) and the prostate glands of the guinea-pig, rabbit and bull (Harper et al, 1979; Harper and Thoenen, 1980;

MacGrogan et al, 1991). Aortae, and highly sympathetically innervated mesenteric arteries, have been found to contain significantly greater levels of NGF in young (20-day-old) SHR when compared to age matched WKY. NGF content was noted to decreased with age in aortic tissue and no significant difference was found between adult (6-month-old) SHR and WKY (Donohue et al, 1989). In contrast, WKY mesenteric artery NGF increased to a level greater than, but not significantly different from, SHR at 6-months of age. These findings illustrated that a specific period of elevated NGF may be necessary for a hypernoradrenegic state to exist.

NGF content is relatively small in most tissues and determination of NGF concentrations has generally proven to be unsuccessful. Alternative methods, namely the measurement of messenger RNA for NGF (NGF-mRNA), has proven to be a more useful tool in determining NGF content and with the advent of the sequencing of the mouse NGF β-chain (Angeletti et al, 1973) and NGF-mRNA (Scott et al, 1983) has led to the successful production of complementary DNA (cDNA) and RNA (cRNA) probes used for the detection of NGF-mRNA in RNA extracts and tissues. Using radiolabelled probes (cDNA and cRNA) it has been possible to demonstrate that NGF is synthesised by Schwann cells ensheathing sympathetic fibres (Bandtlow et al, 1987), astrocytes and glioma cells (Yamakuni et al, 1987; Dal Taso et al, 1987) and target organs themselves (Bandtlow et al, 1987). NGF-mRNA has been detected in a variety of different sympathetically innervated tissues and has been demonstrated to correlate with the density of innervation (Shelton and Reichardt, 1984; Heumann et al, 1984) and NGF (Korsching and Thoenen, 1983; Heumann and Thoenen, 1986) found in these tissues. Analysis of the timing and site of NGF synthesis in mouse embryos shows a close time sequence in the appearance of NGF-mRNA and NGF in the target field and that NGF synthesis during development is governed by the level of NGF-

mRNA (Davies et al, 1987). More recently NGF-mRNA content has been shown to be elevated (5-fold) in mesenteric arteries from 10-day-old SHRs when compared to age-matched WKY rat pups (Curto et al, 1988) which is in accordance with the sympathetic innervation found in these animals ((Donohue et al, 1988). The gene sequence of human β-NGF has been shown to be highly homologous (90%) to mature mouse β-NGF (Ullrich et al, 1983) and shows some homology (10-23%) with the insulin gene family (insulin, relaxin and insulin-like growth factors)(Ullrich et al, 1983). More recently a recombinant human NGF has been produced that is comparable with 2.5S NGF (Iwane et al, 1990)

NGF production has been shown to be under the influence of a number of hormones. Thyroxine (T_4)(Aloe and Levi-Montalcini, 1980; Walker et al 1981), corticosterone (Walker et al, 1981) and testosterone (Ishii and Shooter, 1975; Walker et al, 1981) treatment have all proven to effectively elevate mouse salivary gland NGF well above control levels after only a brief (5 day) treatment; >400%, >150% and >700% respectively. In contrast, testosterone attenuates the level of NGF-mRNA seen in Sertoli cells suggesting that NGF regulates testicular function in an androgen-modulated fashion (Persson et al, 1990); ie. a testosterone-NGF feed-back system regulates the function of the testes. NGF synthesis is also elevated in astrocytes by interleukin-1 (Spranger et al, 1990) and β-adrenergic stimulation (Dal Taso et al, 1987, 1988; Schwartz and Mishler, 1990). Experiments in cultured mouse fibroblasts (L-M cells) has demonstrated that NGF is also elevated by catecholamine however in contrast to astrocyte NGF production the L-M cells do not require adrenergic receptor activation (Furukawa et al, 1986 a,b). The neurite promoting effect of NGF on PC12 cells has been demonstrated to cause a rise in cytosolic Ca^{++} (Pandiella-Alonso et al, 1986; Nikodijevic and Guroff, 1991) and activation of protein kinase C (Doherty et al,

1988; Kondratyev et al, 1990; Damon et al, 1990), tyrosine hydroxylase (Gizang-Ginsberg and Ziff, 1990) and S6 kinase (Matsuda and Guroff, 1987) in PC12 that involves activation of *c-fos* (Gizang-Ginsberg and Ziff, 1990). The neuronal differentiation of PC12 cells to produce neurites, on the other hand, have been shown to occur independently of protein kinase A (Ginty et al, 1990) and protein kinase C (Damon et al, 1990). In contrast to NGF activation of *c-fos* in PC12 cells increases in NGF caused by lesions of the sciatic nerve are mediated by *c-fos* (Hengerer et al, 1990). In newborn rats exogenous NGF increase choline transferase activity, choline uptake and acetylcholine content in rat brain (Kewitz et al, 1990; Williams and Rylett, 1990).

The effect of NGF on neurite outgrowths from PC12 cells has been shown to be synergistic in the presence of fibroblast growth factor (FGF), a factor derived from the pituitary (Togari et al, 1985; Rydel and Greene, 1987). FGF, either acidic (aFGF) or basic (bFGF), has been shown to effect neurite outgrowth in PC12 cells independent of NGF effects and some of the changes induced by FGF are similar to those seen after NGF administration (Togari et al, 1985; Rydel and Greene, 1987).

I.4 Introduction Summary

Detailed clinical studies have characterised many features of human essential hypertension. Among these features are a genetic predisposition to the disease and evidence for an overt role of the SNS. These two features are also present in the animal model of hypertension, the SHR, where there is an absolute genetic predisposition to the disease as well as an extensively documented abnormality in the development of the SNS. This abnormality comprises of an enhanced sympathetic innervation of resistance blood vessels that is not seen in cardiac tissue of the SHR. Fundamental consequences of this abnormal innervation include abnormal organ bath responses of isolated vascular preparations from the SHR. It is also known that the altered innervation that occurs in the SHR does so during the normal time course of sympathetic innervation of the blood vessels and that the neurotrophic factor NGF, which is responsible for the regulation of vascular sympathetic innervation, is produced in larger quantities in the blood vessels of 20 day old SHR that is attenuated in the adult animal. What is unknown, and is of considerable importance, is whether there exists a link between the gene expression of NGF and the hypernoradrenergic innervation seen in the SHR.

I.5 Aims

The principal aim of this study follows from the discussion immediately above, viz;

Is the gene expression of NGF enhanced in blood vessels that display hypernoradrenergic innervation in the SHR ?

To answer this question the following objectives were undertaken:

1) The development of suitable techniques to isolate mRNA from extremely small segments of blood vessels;

2) To establish that the cDNA probe for NGF was suitable for detecting changes in the NGFmRNA. Manipulation of the gene expression of NGF in male mouse salivary gland by androgens was developed as a test system;

3) To determine the status of NGFmRNA in cardiac and aortic tissue, tissues that do not exhibit hypernoradrenergic innervation in the SHR;

4) To determine whether NGFmRNA was elevated in those tissues in the SHR that display hypernoradrenergic innervation ie. mesenteric and caudal arteries and renal tissue;

5) To characterise the NGFmRNA in blood vessels from SHR and WKY rats during the developmental period of innervation;

6) To investigate the NA content in vascular tissue of male and female F1 progeny from a SHR/WKY cross and characterise the inheritability of the hypernoradrenergic state.

CHAPTER II

GENERAL METHODS

***Introductory Note** : The methods described in this chapter are common to many experiments and are described here to avoid repetition.*

II.1 Glassware and Solutions

All Schott bottles, polypropylene tubes and vials, pipette tips, glass rods and spatula were sterilised by steam autoclaving for 25 min. Sterile distilled water (SDW) was used in all solutions; glass distilled reverse-osmosis water was treated with 0.1% diethyl pyrocarbonate (DEPC) and sterilised by autoclaving (Blumberg DD, 1987). Flasks and open beakers were rinsed in 0.1% DEPC, covered with foil and baked for at least 2 hours at 180°C. All solutions used were either sterilised by filtration or autoclaved (Maniatis et al, 1989). Where possible all inorganic reagents were of molecular biology grade.

II.2 Gel Electrophoresis

Nucleic acids were fractionated according to size (in base pairs) on 1% agarose gels by electrophoresis. A 1% agarose gel gives optimal separation of DNA in the

range of 7.0-0.5 kilobase pairs (kb)(Ogden and Adams, 1987). Agarose electrophoresis of RNA gives better separation of different sized molecules than sedimentation through sucrose gradients (Ogden and Adams, 1987). Nucleic acid samples were run in conjunction with size markers; either RNA markers (7.4-0.3 kb fragments of linearized plasmids, Boehringer Mannheim) for RNA samples or phage DNA (SPP-1 bacteriophage digested with *EcoR*I to give 8.51-0.36 kb fragments) for DNA samples. Specific markers are necessary for the type of nucleic acid electrophoresed as the migration of RNA through agarose gels is faster than the migration of DNA through the same medium (Wicks, 1986).

II.2.1 *Equipment*

BioRad electrophoresis equipment was used throughout, either the minisub (64x101 mm gel tray; 200 ml buffer) or large (151x203 mm gel tray; 1000 ml buffer) horizontal electrophoretic chamber powered by a Biorad 200/2.0 power supply. Electrophoresis buffer was circulated with the aid of a peristaltic pump. The gel trays were immersed in 5% laural sulphate (SDS) when not in use and were rinsed several times prior to use with SDW.

II.2.2 *RNA Electrophoresis*

All RNA electrophoretic fractionations were conducted with denaturing agarose gels containing formaldehyde (Lebrach et al, 1977). The formaldehyde concentration was reduced from 2.2M to 0.66M as the lower concentration of formaldehyde was sufficient to totally denature the RNA (Davies et al, 1986; Fourney et al; 1988).

II.2.2.1 *Buffers*

The stock Gel Running buffer was 10xMOPS containing 0.2M MOPS (3-[N-morpholino] propane sulfonic acid), 30mM sodium acetate and 10mM EDTA. The buffer was adjusted to pH 7.0 with NaOH. The solution was prepared by dissolving 42.0 g of MOPS and 4.1 g sodium acetate in 800 ml SDW with 4.0 ml 0.5M EDTA (pH 8.0), the pH was adjusted to 7.0 with a small quantity of 2M NaOH and the final volume made to 1 litre with SDW. The solution turned a faint yellow colour during sterilisation but this does not effect the solution (Maniatis et al, 1989).

The Sample buffer contained 1.0 ml deionised formamide, 350 µl formaldehyde and 200 µl 10xMOPS. The buffer was stored as 150 µl aliquots at -20°C.

The Gel Loading buffer contained bromophenol blue for the purpose of tracking the migration of the samples. In a sterile 15 ml tube 1.0 ml glycerol and 200µl 2.5% bromophenol blue were mixed with 800 µl SDW. The buffer was stored at -20°C in 1.5 ml sterile microfuge tubes as 100 µl aliquots.

II.2.2.2 *Gel Preparation*

Small Gel

In a sterile 100 ml conical flask 0.336 g molecular biology grade agarose was combined with 28.5 ml SDW and 3.3 ml 10xMOPS. The agarose was dissolved by heating the solution in a microwave oven. The solution was cooled to approximately 55°C and 1.8 ml formaldehyde was added with constant mixing. The gel was cast in a perspex tray sealed at the open ends with autoclave tape. Sample wells were formed with an 8 tooth comb. The gel was allowed to set for at least 30 min at which time

the comb and end tapes were removed. The gel (including tray) was placed within the minisub electrophoretic chamber and submerged in 1xRunning Buffer. The gel was run for 5 min at 60V prior to loading the samples into the wells.

Large Gel

The large gels were 4 times the volume of the small gel. Agarose (1.35 g) was melted in 114 ml SDW and 13.2 ml 10xMOPS, cooled to approximately 55°C and 7.2 ml formaldehyde added. The gel tray was sealed with autoclave tape and the gel was cast using a 16 tooth comb of the same dimensions and spacing as the small comb. The gel was allowed to set for at least 30 min before the removal of the tape and comb. The gel was submerged in 1xRunning buffer and run at 60V for 5 min prior to loading the samples.

II.2.2.3 *Sample Preparations*

RNA (4.5 µl containing up to 10µg of RNA) was mixed with 15.5 µl Sample buffer in a sterile microfuge tube. The tube was heated in a water bath at 65°C for 10 min then chilled on ice for 15 min to linearise the RNA. Gel Loading buffer (2 µl) was added, mixed and the sample loaded onto the gel. Both the large and small gels were run at either 30V or 60V until the bromophenol blue had migrated to within 1 cm from the end of the gel.

II.2.2.4 *Visualisation*

Nucleic acids were visualised under ultraviolet (UV) illumination after

fluorescent staining with ethidium bromide (EtBr) (Sharpe et al, 1973). However, the fluorescence of RNA is poor as EtBr has a low affinity for single stranded nucleic acid. EtBr was used to stain the RNA samples in two different procedures. One method consisted of staining the RNA after electrophoresis by soaking the gel in Etbr (0.5 µg/ml) for 15 min and destaining in SDW for 40 min. The banding on the gel was then visible under UV illumination (model 3-3002, Fotodyne Inc., WI, USA) and was photographed using Polaroid 668 film (45 sec at *f*4.5). The second method involved inclusion of 1µl EtBr (10µg/ml) in the sample prior to electrophoresis. EtBr was added to the samples after heat shocking the RNA and just prior to loading the sample onto the gel. This allowed visualisation of the electrophoresed RNA without further treatment and kept the concentration of the toxic bromide to a minimum (Fourney et al, 1988). Addition of 1 µl EtBr (10 µg/ml) to the samples prior to heat shocking resulted in more intense staining of the bands under UV illumination than addition of EtBr after shocking (Figure II.1). EtBr impedes the migration of RNA through agarose gels whether it is present in the gel or the sample. The degree of impedance is independent on whether the EtBr is added prior to or after, heat shocking or included in the gel itself.

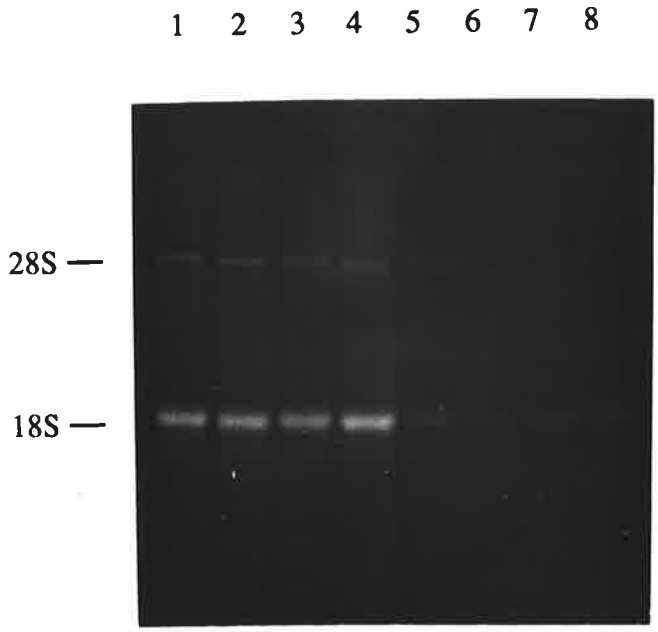


Figure II.1 Rat liver RNA extracts (5µg/lane) fractionated on a 1% agarose gel and visualised under UV illumination. Ethidium bromide (1µl of 1.0mg/ml) was added to each sample either before (lanes 1-4) or after (lanes 5-6) heat shocking. Addition of the stain prior to shocking the sample clearly intensifies the illumination of the RNA.

II.2.3 *DNA Electrophoresis*

The electrophoresis of DNA samples was performed in nondenaturing agarose gels run in the presence of a tris-acetate buffer. The buffering ability of tris-acetate is low and it was necessary to recirculate the buffer (Loening, 1967).

II.2.3.1 *Buffers*

The stock Gel Running buffer was 5xTAE (tris-acetate/EDTA) containing 40mM Tris-acetate and 1mM EDTA and was prepared by dissolving 24.2 g Tris base in 800 ml SDW to which 5.71 ml glacial acetic acid and 10 ml 0.5M EDTA (pH 8.0) were added; the volume was made to 1 litre with SDW (Ogden and Adams, 1987). The Gel Loading buffer was the same as for RNA electrophoresis (see II.2.2.1).

II.2.3.2 *Gel Preparation*

Small Gel

In a sterile 100 ml conical flask a 1% agarose gel was made by dissolving 336 mg molecular biology grade agarose with 26.9 ml SDW and 6.8 ml 5xTAE and heating in a microwave oven. The solution was cooled to approximately 55°C and the gel was cast in a perspex tray sealed at the open ends with autoclave tape. Sample wells were formed with an 8 tooth comb. The gel was allowed to set for at least 30 min at which time the comb and end tapes were removed. The gel (including tray) was placed within the minisub electrophoretic chamber and submerged in 1xTAE buffer. The gel was run for 5 min at 60V prior to loading the samples into the wells.

Large Preparative Gel

Agarose (1.42 g) was dissolved in 114 ml SDW and 28.5 ml 5xTAE in a microwave oven, cooled to approximately 55°C and cast in a tray sealed with autoclave tape. Sample wells were formed with a preparative comb consisted of 3 teeth; a centre tooth 100x1 mm and two outer teeth both 5x1 mm. The gel was allowed to set for at least 30 min before the removal of the tape and comb. The gel was submerged in 1xRunning Buffer and run at 60V for 5 min prior to loading the samples.

II.2.3.3 *Sample Preparation*

A volume of sample, or marker DNA (20 µl), was transferred to a sterile microfuge tube and 5 volumes of loading buffer was added. The samples were mixed and then loaded into the wells of the gel. Both the large and small gels were run at 60V until the bromophenol blue had migrated at least 50% of the length of the gel.

II.2.3.4 *Visualisation*

The gel was carefully removed from the tray and immersed in SDW containing 1.0 µg/ml EtBr for 30 min. The gel was destained for 10 min in SDW to remove background fluorescence caused by unbound EtBr in the gel. The DNA was visualised under UV illumination and photographed using Polaroid 668 film (45 sec at *f*4.5).

II.3 Northern Transfers

RNA was transferred from small gels to nitrocellulose filters using a Millipore Vacuum transfer apparatus (MilliBlot-V-Jr) while RNA from large gels was transferred using the Biorad Vacuum blotter (model 785); the vacuum was applied by a Bio-Rad Vacuum Station. The apparatus was washed twice with 10xMOPS buffer before preparing for transfer.

A nitrocellulose filter (Schleicher and Schuell, Germany) the same size as the gel was pre-wet in SDW before soaking the filter for 20 min in 20xSSC (3M NaCl, 0.3M tri sodium citrate; pH 7.0). A filter paper, 1cm larger than the nitrocellulose membrane, was soaked in 20xSSC and laid on the porous vacuum plate. The nitrocellulose membrane was overlaid with a rubber gasket which was smaller (by 1 cm longitudinally and laterally) than both the nitrocellulose membrane and agarose gel; this allowed a seal to form between the gel and the membrane. The gel was placed face up over the hole in the gasket. A small vacuum was applied to the apparatus to initiate the seal and the upper reservoir of the transfer system filled with 10xSSC.

A vacuum was applied to the apparatus and the RNA was transferred to the nitrocellulose membrane within 30 min. The membrane was washed briefly in 10XSSC then allowed to air-dry overnight before baking at 80°C *in vacuo* for 2 hours. The membrane was stored in a sterile blotting paper envelope at -20°C until required.

II.4 Slot Blots

MilliBlot-S (slots) and Milliblot-B (blots) manifolds were purchased from Biorad and the vacuum was applied by a water pump. Both manifolds were used in

different experiments but the preparation and application of the samples are exactly the same (Maniatis et al, 1989). The apparatus was washed with 0.1M NaOH and rinsed with several passages of SDW prior to applying the RNA samples to nitrocellulose filters.

II.4.1 *Sample Preparation*

Denaturing buffer was prepared by thoroughly mixing 4 ml deionised formamide, 1.4 ml formaldehyde (37%) and 400 µl 20xSSC in a sterile 15 ml polypropylene tube. RNA samples were thawed at room temperature and 6µg of RNA (not more than 4.5 µl) was transferred to a sterile microfuge tube. The volume was adjusted to 30µl with SDW and serially diluted (1:1) with SDW to give a dilution series of each sample from 2.0-0.25µg/ml. Denaturing buffer (3.9 volumes) was added to each tube and vortexed. The tubes were incubated for 15 min at 65°C, then cooled on ice for at least 15 min before loading the samples onto the nitrocellulose filter.

II.4.2 *Sample Application*

A nitrocellulose filter (Schleicher and Schuell, Germany) was soaked for 5 min in SDW then immersed in 20xSSC for 30 min at room temperature. Two sheets of filter paper were placed on the porous lower section of the manifold and saturated with 20xSSC. The nitrocellulose filter was placed on top of the absorbent paper, ensuring that no air was trapped between the layers and the top of the manifold clamped in place. The vacuum line was connected to the manifold and a gentle vacuum was applied for 15 sec to remove excess SSC. The vacuum was released and

the slots in the manifold were filled with 10xSSC. The vacuum was reapplied until all the fluid had been removed from the wells at which time the vacuum was ceased.

The samples (117 µl) were loaded into the slot wells and a gentle vacuum applied. After all the samples had passed through the filter the wells were washed twice with 500 µl of 10xSSC. The vacuum was applied for several minutes to dry the nitrocellulose filter.

The filter was removed from the manifold and placed in a sterile blotting paper folder and allowed to air dry overnight. The filter was then baked in a vacuum oven for 2 hours at 80°C. After cooling filters were stored at -20°C until required for hydridization with a radiolabelled probe.

II.5 The cDNA Probe

The cDNA probe used for the quantitation and identification of NGFmRNA was a 917 base pair cDNA sequence (Scott et al, 1984) obtained originally from Dr Rutter (University of California). The quantity of the cDNA probe required amplification by insertion into bacteria and harvesting the cells after an overnight culture.

II.5.1 *NGFmRNA Probe Production*

The cDNA probe for NGFmRNA was received with the sequence inserted into the *PstI* site of an artificial plasmid; pBR322. The pBR322 plasmid is a 4363 base pair (bp) sequence which has several restriction enzyme sites (Figure II.2) which allow the incorporation of an extraneous sequence.

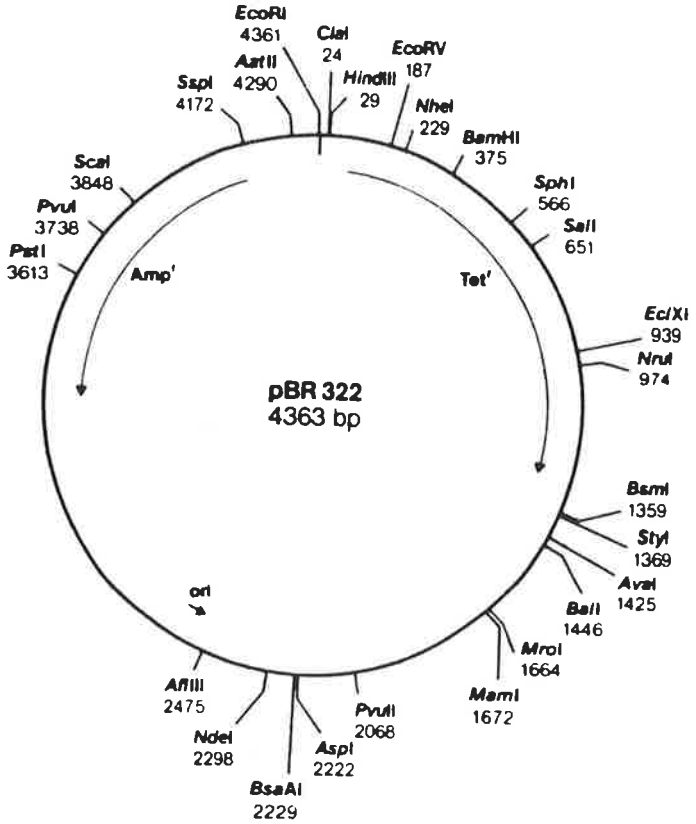


Figure II.2 The above diagram depicts the restriction map of the pBR322 plasmid; the restriction enzyme sites are shown. The plasmid carries the genes for ampicillin and tetracycline resistance.

The plasmid was transfected into *E Coli*, streaked onto agar plates containing Ampicillin and allowed to grow overnight. Several 500 ml flasks containing 100 ml sterile growth medium, (Circlegrow, BIO 101, La Jolla, USA) were inoculated with several surviving colonies from the agar plates. The flasks were incubated at 37°C overnight, with aeration. The transfection, growth of Ampicillin resistant bacteria and the overnight flask cultures were conducted in the laboratories of the Department of Biochemistry, University of Adelaide.

The cells from the overnight cultures were transferred to sterile 500 ml centrifuge bottles and the cells pelleted. The supernatant was discarded and the plasmid containing the cDNA insert was isolated from the cells using a commercial kit, Circleprep (BIO 101, La Jolla, USA).

The cells were lysed under alkaline conditions and the linear cellular DNA, and RNA, were removed in three successive alkaline denaturations; 80% of linear DNA is removed with each denaturing step with minimal loss of circular plasmid DNA. The RNA and ssDNA were eliminated by precipitation in the presence of LiCl. The supernatant containing only plasmid DNA divided into 6 equal aliquots. 'Glassmilk' (silica beads) was added to each aliquot which selectively bound the DNA (Marko et al, 1982). Protein, polysaccharides and any remaining RNA was removed by several washes. The plasmid DNA was eluted by resuspending the pellet in a small volume of SDW (300 µl) and incubating for 5 min in a water bath maintained at 55°C. The suspension was centrifuged and the supernatant containing plasmid DNA was transferred to a sterile 2.0 ml Sarsdent microfuge tube. From a total culture volume of 500 ml, 5 g of cells were harvested; this equated to approximately 500 µg of plasmid DNA. The insert sequence represented approximately 16% of the DNA harvested and thus it was estimated that the maximum quantity of probe available was 80 µg.

To reduce nonspecific binding and increase the hybridisation of the probe it was necessary to cut the insert from the plasmid and purify the 917 base pair sequence. The insert was cut from the plasmid by incubating a 220 µl aliquot of plasmid (50 µg) with 50 µl of the restriction enzyme *PstI* (500 units) (Pharmacia, Sweden) and 30 µl of tris-phosphate buffer (100mM tris-acetate, 100mM magnesium acetate and 500mM potassium acetate) for 2 hrs at 37°C. The digestion mix was electrophoresed on a 1% agarose gel to separate the insert from the plasmid (see II.2.3).

The gel was stained with 1.0 µg/ml EtBr for 30 min, destained with SDW for 10 min and the bands visualised under UV illumination. Four distinct bands were visualised representing; (1) undigested plasmid at 5.3 kb, (2) the plasmid with the insert deleted at 4.2 kb, (3) the cDNA insert at 0.92 kb and (4) a 0.5 kb fragment (Figure II.3). The band corresponding to the cDNA insert was cut from the gel with a sterile scalpel and sectioned into pieces approximately 1 cm long. The small sections were transferred to a pre-weighed sterile 15 ml polypropylene tube and the volume calculated; by weight. The DNA was purified from the agarose by utilising a commercial kit; GeneClean (BIO 101,La Jolla, USA). The agarose was dissolved in 2.5 volumes NaI and the DNA was then adsorbed to 'Glassmilk'. The pellet was washed five times, the DNA eluted with 150µl SDW and the probe stored at -20°C. The concentration of the purified cDNA was determined using bizbenzamide and fluorescent spectrophotometry (see II.8). The standard curve was performed using 31.25-1000 ng/ml salmon sperm DNA. The sperm DNA was considered to be an appropriate standard for determining the probe concentration due to the smaller molecular weight of salmon sperm when compared to other DNA sources (eg. calf thymus DNA).

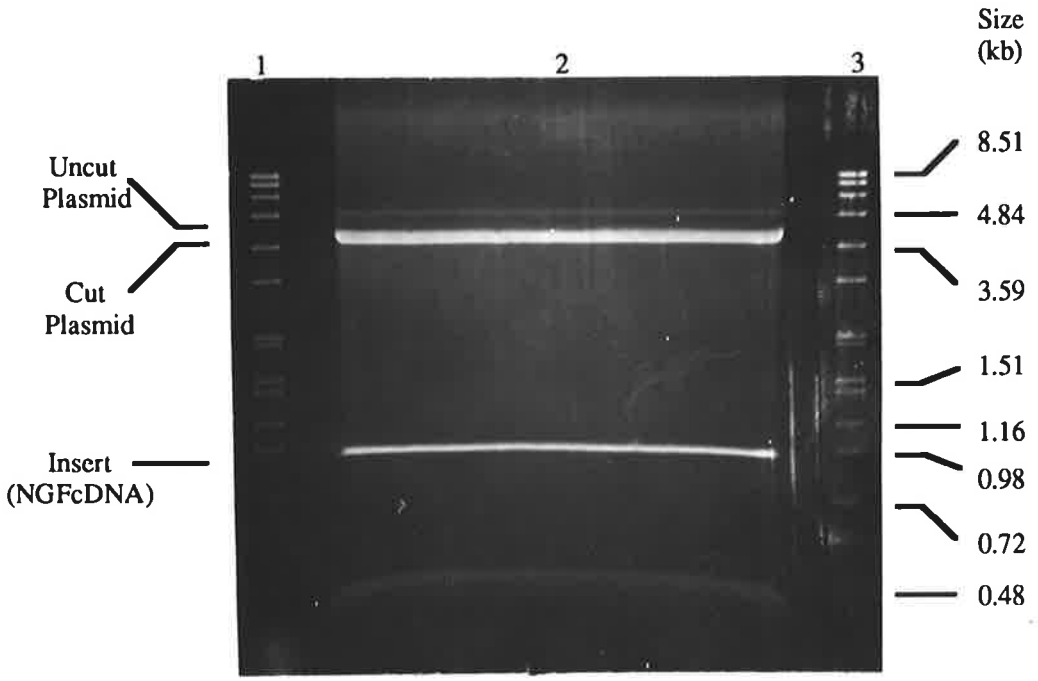


Figure II.3 Plasmid DNA (50µg) was digested with Pst I restriction enzyme and fractionated on a 1% agarose gel (lane 2). The gel was stained with ethidium bromide and the fragments visualised under UV illumination. Uncut plasmid (faint band) and the plasmid with the insert removed are seen at 5.3kb and 4.2kb respectively. The band at 0.92kb represents the NGFcDNA probe. Size markers (SPP-1 bacteriophage digested with *EcoRI* to give 8.51-0.36 kb fragments) are to either side of the plasmid lane.

The concentration of the probe was determined to be 25 ng/µl; ie 3.75 µg of cDNA was purified from 50 µg of plasmid. As the insert DNA represented only 16% of the total plasmid DNA then the maximum return of cDNA would have been 8 µg. Additionally, the recovery of DNA utilising GeneClean averages about 60% which suggests that the maximal recoverable DNA is 4.6 µg.

A small quantity of the purified DNA probe (250 ng) was electrophoresed on a 1% agarose gel. A single discrete band was observed at approximately 900 bp, the position corresponding to that expected for the probe (Figure II.4). The summary of the probe production, isolation and purification is illustrated in Figure II.5.

II.6 Radiolabelling of the cDNA Probe

The most widely used radiolabel in molecular biology is radioactive phosphorus (^{32}P) which is available at high specific activity and gives maximum sensitivity in filter hybridisations.

II.6.1 *Nick Translation*

A nick translation kit was purchased from Boehringer Mannheim (Mannheim, Germany) and the reaction carried out as specified by the manufacturers instructions and as described by Rigby et al (1977). In a sterile 1.5 ml microfuge tube 0.125 µg DNA, 20µM dATP, dGTP, dTTP, 100 µCi [α-^{32}P]dCTP (3000 Ci/mol, Bresatec, Australia), $MgCl_2$, DNA-polymerase I and DNase I were incubated in a Tris buffer for 70 min at 15°C. The reaction was stopped by the addition of 2 µl EDTA (0.2M, pH 8.0).

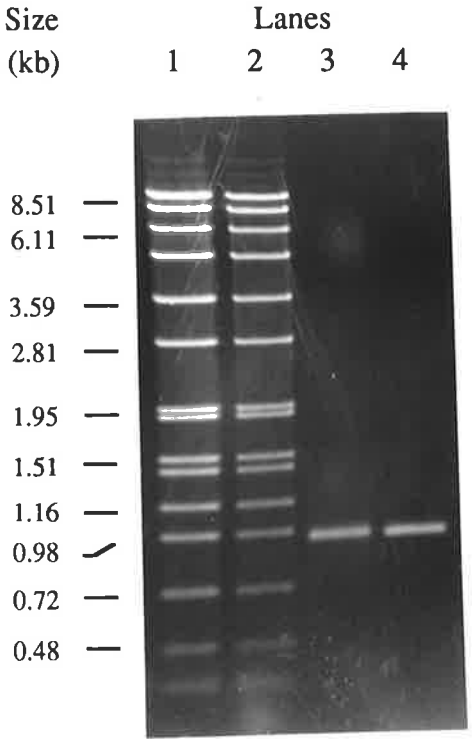


Figure II.4 Purified NGFcDNA (0.5µg/lane; lanes 3 & 4) was run on a 1% agarose gel in conjunction with phage DNA size markers and stained with ethidium bromide. A discrete band at approximately 0.92kb is seen without degradation.

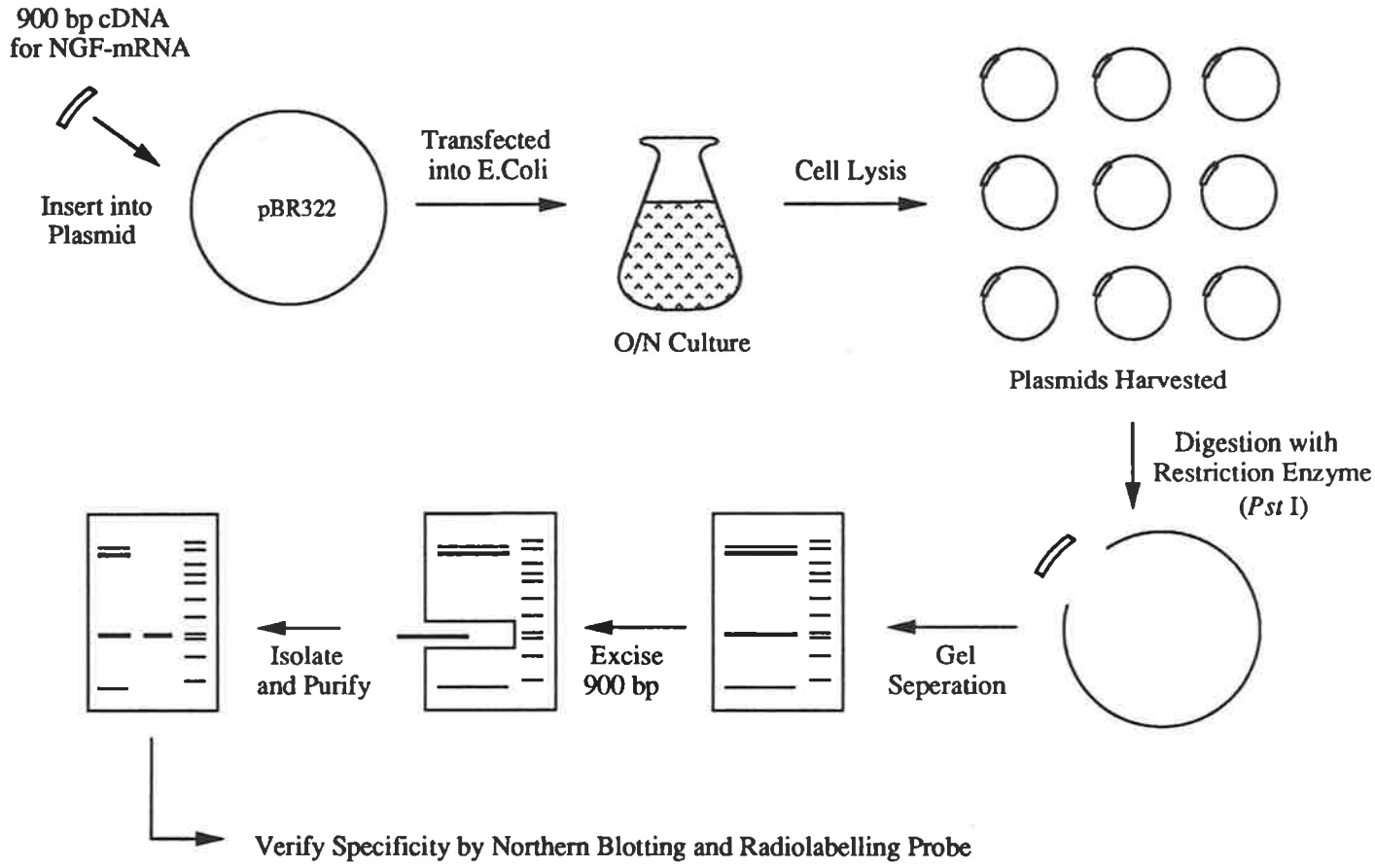


Figure II.5 Diagramatical view of the amplification and isolation of the cDNA probe.

To the reaction mixture 45 µl of phenol:chloroform (1:1) was added, the tube vortexed briefly, then centrifuged at room temperature in a benchtop microfuge for 30 sec at 3000 rpm. The aqueous phase was transferred to a sterile microfuge tube and the organic phase was back extracted with 20 µl of chromatography buffer (10mM Tris-HCl, 0.1mM EDTA and 5mM β-mercaptoethanol). After centrifugation the aqueous phase was removed and pooled with the initial aqueous aliquot.

The non-incorporated deoxyribonucleotide triphosphates (dNTP) (both labelled and unlabelled) and non-incorporated ^{32}P were removed by chromatography through Sephadex G-50. A sterile Pasteur pipette containing a small wad of glass wool was packed (to within 2 cm of the top) with Sephadex G-50 (Pharmacia, Sweden) that had been suspended in chromatography buffer and autoclaved. The pooled aqueous phases were loaded on the G-50 column with 300 µl of buffer and the fraction collected discarded. The column was then loaded with 100 µl aliquots of buffer and the eluent collected in sterile 1.5 ml microfuge tubes; 14 fractions were collected. A 5 µl aliquot of each fraction was transferred to a 5 ml Beckmann scintillation vial with 4.5 ml of scintillant and the radioactive content determined on a β counter (LKB Wallac, 1218 Rackbeta).

The general profile of the fractions eluted from the column demonstrated a skewed distribution of radioactivity. The commencement of a second peak of radioactivity signalled the elution of the non-incorporated radioligand and all subsequent fractions were discarded. The separation of the non-incorporated radioligand from radiolabelled cDNA has been previously demonstrated on a 8x300 mm Sephadex G-50 column. The labelled DNA is impeded less than the non-incorporated radionucleotide and is eluted from the column first, preceding the non-incorporated fraction by approximately 5 cm; an area intermediate between the two

radioactive fractions was virtually devoid of radioactivity. The larger column allowed better separation of the two radioactive peaks however the time period for elution was approximately 30 min, the fractions were larger and the label significantly diluted; for this reason the shorter column was preferred.

The first 8 fractions containing radioactivity in excess of $1x10^4$ cpm/µl were pooled. The other fractions were discarded due to possible inclusion of non-incorporated radionucleotides (due to the relatively short column). The radioactivity of the pooled fractions was counted and the specific activity of the DNA determined. The labelled probe was utilised for hybridisation experiments the day it was prepared. The specific activity of the samples, and the test DNA included in the kit, corresponded to 70±4% (n=6) incorporation of the label into the cDNA sequences and a specific activity of at least $1x10^8$ cpm/µg DNA.

II.7 Hybridisation of Nitrocellulose Filters

The protocol for the hybridisation of both the slot blot and Northern transfer membranes was a compilation of the methods described by several authors (Thomas et al, 1980; Shelton and Reichardt, 1984; Dal Taso et al, 1987; Ouafik et al, 1989). The major difference in the following protocol to those mentioned above is that the percentage of deionised formamide was reduced from 50% to 40%.

II.7.1 *Prehybridisation*

The prehybridisation solution was prepared in a sterile conical flask and contained the following; 40% deionised formamide, 3.2x Denhardt's solution (1x =

0.02% each of Ficoll 400, polyvinyl pyrrolidine and bovine serum albumin (BSA)), 4xSSC, 40mM sodium phosphate (pH 6.5), 10% dextran sulphate and 250 µg/ml denatured salmon sperm DNA. The baked filter was placed into a plastic bag and the appropriate amount of prehybridisation solution was added; 0.1 ml/cm^2 filter. The air bubbles were removed and the bag heat sealed. The bag was checked to ensure there was no leakage of the solution and that the solution completely covered both sides of the filter.

The sealed bag was placed into a plastic container and the container half filled with water. A glass plate (edges smoothed) was placed over the bag to ensure that the membrane was kept flat (Figure II.6). The container was sealed and placed in a shaking water bath maintained at 42°C for at least 6 hours. The water in the container ensures that the temperature of the membrane, and the prehybridisation solution, are the same as the water bath. The glass plate ensures that the membrane is kept flat and in association with the shaking of the bath helps to circulate the prehybridisation solution around the filter.

II.7.2 *Hybridisation*

After hybridisation a corner of the bag was cut and approximately half the contents were drained into a sterile 15 ml tube; the bag was placed at an angle to avoid loss of the remaining solution. Radioactive probe calculated (1x10^6 cpm/ml of prehydridisation solution) was placed in the tube and vortexed briefly. The tube was placed into boiling water for 5 min then placed on ice for at least 5 min before returning the contents of the tube to the bag. The bag was heat sealed, ensuring no air bubbles were trapped, and the contents mixed by inverting the bag several times.

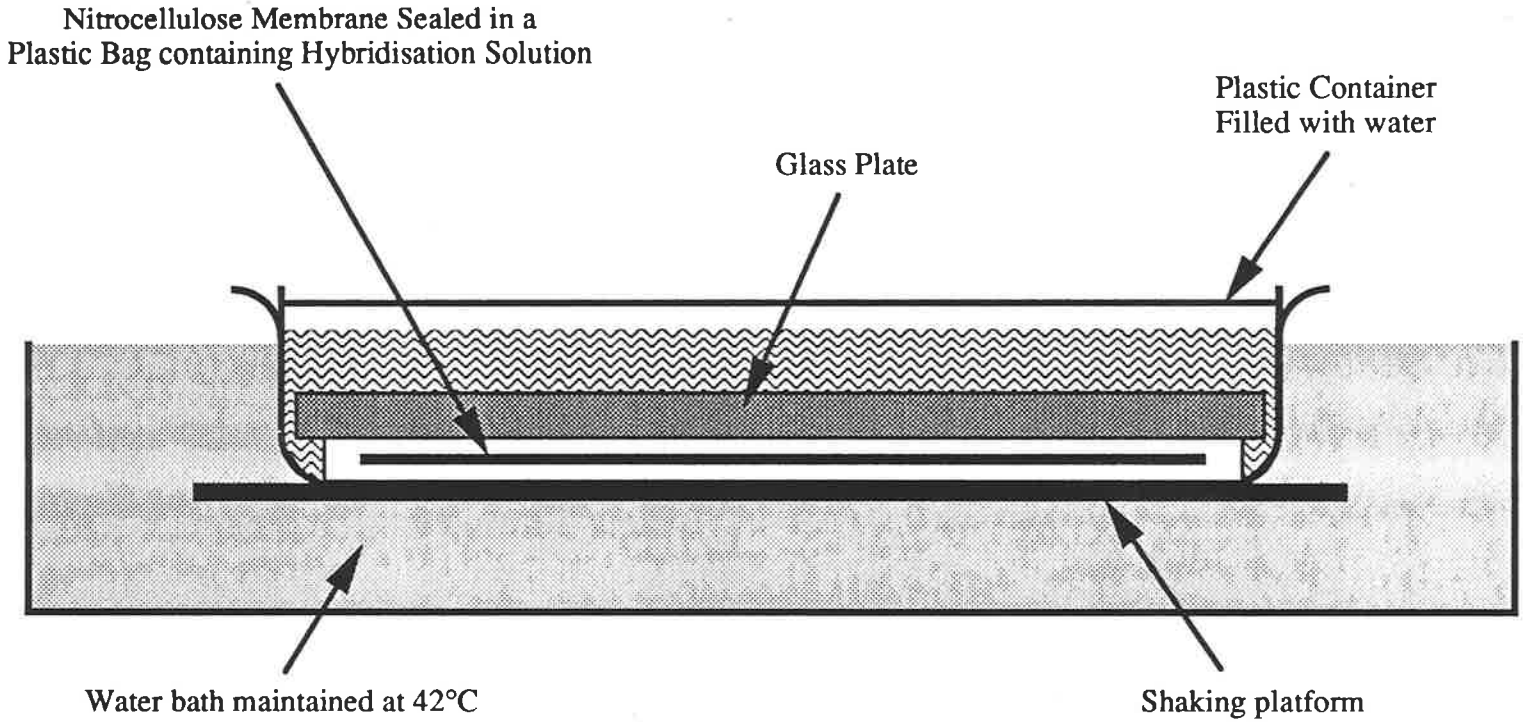


Figure II.6 Diagramatical view of the pre- and hybridisation baths. The nitrocellulose membrane is sealed in a plastic bag with 0.1 ml/cm² prehybridisation solution. The glass plate ensures that the membrane is kept flat. The shaking platform and the glass plate helps to move the solution in the bag so that all the membrane is subject to the solution. The water in the container is maintained at the same temperature as the bath while the container itself ensures containment of all radioactivity.

The bag was returned to the plastic container and positioned as described for prehybridisation. The container was returned to the water bath and left to hybridise at 42°C for at least 16 hrs; ie overnight.

II.7.3 *Stringency Washes*

Upon the completion of hybridisation the bag was removed from the water bath and the contents of the bag drained into a radioactive waste container. The filter was given an initial wash in the bag with 15 ml 2xSSC containing 0.1% SDS (lauryl sulphate). The solution was moved around the bag to remove any remaining radioactive hybridisation solution and then discarded. The filter was transferred to a 750 ml plastic container and washed as follows; 2 times for 30 min with 500 ml 2xSSC containing 0.1% SDS at room temperature then once for 45 min with 750 ml 0.2xSSC containing 0.1% SDS at 55°C.

The membrane was removed from the last wash and placed in a sterile blotting paper folder and allowed to air dry for 15 min. The membrane was then sealed between two sheets of Gladwrap® and the membrane autoradiographed.

II.8 Autoradiography

The membrane, sealed in Gladwrap®, was sandwiched between two sheets of X-ray film (Curix RP1, Agfa-Gevaert Ltd, Australia) and sealed in an x-ray cassette (Dupont) with intensifying screens (Cronex, Xtra Life). The cassette was wrapped in plastic and stored in a perspex box at -70°C. The exposure period for the autoradiograph varied between 4 hrs and 7 days depending on the probe and the tissue

sample.

The film was manually developed for 3 min (Agfa-Gevaert, rapid developer), fixed for 4 min (Kodak rapid fixer) and the radiograph was analysed by densitometry (LKB Laser Densitometer, Pharmacia/LKB, Bromma, Sweden) incorporating area integration.

II.9 DNA Determinations

The DNA content of tissue samples and RNA extracts were determined by the method described by Labarca and Paigen (1980). In a sterile microfuge tube 25µl of RNA extract, tissue homogenate or standard DNA was mixed with 1975 µl of DNA assay buffer (50mM $NaPO_4$, 2M NaCl; pH 7.4) containing 0.2 µg/ml bisbenzamide. The fluorescence of each solution was determined using a Perkin-Elmer LS-5 Luminescence Spectrometer with the excitation and emission wavelengths set at 360 nm and 460 nm respectively. The DNA content was determined by comparison with standards comprising of calf thymus DNA. The DNA concentrations of the standards was proportional to the fluorescence in the range 15.6 ng/ml to 2 µg/ml.

II.10 Protein Determinations

RNA extracts were analysed for protein content using an automated version of the methodology described by Lowry et al (1951) and adapted by Clifton et al (1988). The automated system comprised of an auto-analyser (Roche Diagnostica, Cobas Bio) that dilute 20 µl of either RNA extract or protein standard with SDW (1:1) in a cuvette maintained at 37°C. To each cuvette was added 250 µl of 2% Na_2CO_3 in 0.1M NaOH

: 1% Na/K tartrate : 1% $CuSO_4$ (100:1:1) and 25µl of 50% Folin's reagent and the absorbance of the mixture determined at 750 nm (A_{750}). Each sample was assayed in duplicate and the protein concentration determined by comparison to standards of bovine serum albumin (BSA). The A_{750} was proportional to the protein concentration in the 100 ng/ml to 1250 ng/ml range. The coefficient of variance of the Cobas was determined at the commencement of each day, using sodium chromate, and was always less than 1.5%.

CHAPTER III

ISOLATION OF RNA USING GUANIDINIUM-PHENOL-CHLOROFORM METHODOLOGIES

III.1 Introduction

The most important factor in RNA extraction is the ability to produce consistent yields with respect to quality and quantity. Impurities in the RNA preparation produce major difficulties in the quantification of the RNA and its use in hybridisation studies. Techniques for the extraction of RNA have been evolving since the late 1950's and can be broadly grouped into three categories; (1) those that use SDS to disrupt the cells in conjunction with proteinase K to partly digest the denatured proteins, (2) those that make use of phenol to denature proteins and (3) those that make use of guanidinium salts that can denature proteins and inhibit ribonuleases.

The two former methods are not as highly favoured as those which use the chaototrophic guanidinium salts. The two most prominent methods for extracting RNA are homogenisation of tissue in guanidinium thiocyanate and either, (a) layer the homogenate on a cesium chloride (CsCl) cushion followed by centrifugation (Glisin et al (1974)) or (b) rehomogenisation in guanidinium HCl after initially precipitating the RNA with ethanol (Chirgwin and Przybyla, 1979). The major drawbacks of these methods are the need for an ultra-centrifuge and long run times (24-48 hrs), the

relative expense of the CsCl and the fact that guanidinium HCl is not as strong a ribonuclease inhibitor as the thiocyanate salt. Thus the processing of a large number of samples can be an expensive project (both in time and reagents) and the RNA product susceptible to degradation by RNase. A procedure that is quick and relatively inexpensive, and is able to produce undegraded RNA in substantial quantities, was described by Chomczynski and Sacchi (1987). This method involves both guanidinium isothiocyanate and phenol and is able to extract undegraded RNA from as little as 100 mg tissue samples. A minor adaption in the homogenisation of the tissue samples allows extraction of RNA from tissue samples as small as 10 mg.

III.2 Isolation of RNA

III.2.1 *The Chomczynski-Sacchi Method*

This protocol incorporates a phenol:chloroform extraction of a guanidinium isothiocyanate lysate and has been modified slightly for the extraction of intact RNA from small tissues; approximately 10 mg. The volume for the initial homogenisation was altered such that tissues smaller than 50 mg were homogenised in 750 µl of homogenising solution while tissues greater than 100 mg were homogenised in a ratio of 1 ml homogenising solution per 100 mg of tissue (v/w). All other procedures were as described by Chomczynski and Sacchi (1987). All tissue samples were blotted dry, weighed, frozen in liquid nitrogen (after wrapping in foil) and stored at -70°C until required.

III.2.1.1 *Reagents*

The 4.0M guanidinium isothiocyanate stock solution was prepared by dissolving 500 g guanidinium thiocyanate with 648 ml SDW and 25 ml 1.0M sodium citrate (pH 7.0) directly in the manufacturers bottle and was stored at 4°C until required. The denaturing solution (Soln D) used for homogenising tissue was prepared from this stock by adding 720 µl β-mercaptoethanol/100 ml of stock solution and was stored at room temperature. Fresh Soln D was prepared on a fortnightly basis.

Water-saturated phenol was prepared as described by Maniatis et al (1989). Phenol was melted in a water bath and 100 ml aliquots were transferred to 250 ml sterile Schott bottles and stored under nitrogen at -20°C until required. The solidified phenol was allowed to thaw to room temperature then melted in a water bath. 8-Hydroxyquinoline was added to a final concentration of 0.1% and the phenol was extracted twice with 1.5 volumes of 1.0M Tris (pH 8.0) and once with 1.5 volumes 0.1M Tris (pH 8.0); the pH of the aqueous phase was >7.6 at this stage (Maniatis et al, 1989). The equilibrated phenol solution was stored at 4°C.

Sarcosyl (10%) was prepared by dissolving 10 g sarcosyl in sufficient SDW to make 100 mls and then filter sterilised.

III.2.1.2 *Small Scale Homogenisations*

Tissue samples (between 8-50 mg) stored at -70°C were transferred directly into liquid nitrogen. The samples were placed into 6 ml polypropylene tubes that were maintained in liquid nitrogen and the tissue was pulverised with the aid of a stainless steel pestle; the pestle's temperature was lowered by immersing in liquid nitrogen for

15 min prior to use. Care was taken to ensure that tissue did not adhere to the pestle due to electrostatic forces generated in the steel at the low temperature. Adhering tissue was removed with the aid of a sterile spatula.

Excess liquid nitrogen was "boiled off" and the tube was placed in a ethanol-ice bath. The tissue was homogenised in 800µl of Soln D using an Ultra-Turrax with a 6 mm probe (T25, Janke and Kunkel) for 60 sec at 24000 rpm then placed on ice.

III.2.1.3 *Large Scale Homogenisation*

This protocol was used for tissue samples greater than 100 mg, namely hearts and kidneys. Tissue samples stored at -70°C were placed directly into liquid nitrogen. The tissue was placed in a stainless steel mortar that was immersed in liquid nitrogen and crushed with a stainless steel pestle that also had its temperature lowered by immersing in liquid nitrogen. The crushed sample was transferred to a sterile 15 ml polypropylene tube maintained in liquid nitrogen. The liquid nitrogen was "boiled off" and the tube placed in an ethanol-ice bath. The tissue was homogenised in Soln D (1 ml/100 mg) using an Ultra-Turrax for 60 sec at 24000 rpm.

III.2.1.4 *RNA Extraction*

A 25 µl aliquot of each sample was transferred to a sterile 1.5 ml microfuge tube, frozen in liquid nitrogen and stored at -70°C until required for determination of total DNA and protein.

The remaining homogenate was transferred to either a sterile 2.0 ml microfuge tube or 30 ml polypropylene tube to which the following was added sequentially, with

brief vortexing between additions; 0.05 volume 10% sarcosyl, 0.1 volume 2M sodium acetate (pH 4), 1.0 volume phenol and 0.2 volume chloroform:isoamyl alcohol (49:1). The tube was left on ice for 15 min before centrifugation for 20 min at 12000 *g* (4°C). The aqueous phase was transferred to a sterile polypropylene tube (2 or 30 ml) with 1 volume of ice-cold isopropanol, vortexed, and left at -70°C for at least 30 min to precipitate the RNA. The samples were centrifuged at 4°C for 15 min at 12000 *g* and the supernatant discarded. The pellet was resuspended in 0.3 volume Soln D by vortexing and repeated passage by pipetting. One volume of isopropanol was added to the suspension, vortexed, and the tube returned to the -70°C freezer for 30 min. The RNA was pelleted by centrifugation at 4°C for 10 min at 12000 *g* and the supernatant discarded. The pellet was washed with 1.5 ml 75% ethanol by vortexing. The RNA was again pelleted at 12000 *g* at 4°C for 10 min and the supernatant again discarded. Extreme care was taken with the removal of the supernatant as the pellet was quite soft and easily dislodged. The pellet was dried *in vacuo* for 15 min and dissolved in 0.2 ml SDW per 100 mg of starting tissue. The sample was placed in a water bath, maintained at 65°C, for 10 min to help dissolve the RNA pellet. The sample was stored at -20°C until required.

III.2.1.5 *Purity*

The concentration of the sample was determined by absorbance at 260 nm using a Beckmann DU-65 spectrophotometer and the purity of the samples were determined by the 260/280 nm ratios.

Rat liver was used for test samples and all RNA extracts had ratio levels greater than 1.7; RNA samples having a ratio of 2 are considered to be pure. Protein

and DNA contamination were investigated using Lowry and fluorescence protocols respectively (see II.9 and II.10 for protocols) and neither protein or DNA were detected. Yields of RNA for liver were 4.76±0.10 µg RNA/mg of tissue.

Fractionation of the test samples on a 1% agarose gel showed distinct bands for the 28s and 18s ribosomal RNA, as well as several other bands; no degradation of the samples (or very little) was seen at completion of the extraction or after storage of the samples at -20°C for up to 3 months (Figure III.1).

III.2.2 *The Combined Phenol Method*

The acid guanidinium extraction described above is comparable to other methods for the isolation of intact, undegraded RNA in considerable quantities. The question arose as to whether this method could be simplified such that the several additions after the homogenisation phase could be either reduced or eliminated completely by altering the original homogenisation solution. A protocol that reduces the number of steps in the isolation of quality RNA could possibly enhance RNA recovery and reliability while on the other hand could fail to remove all contaminants; eg. ribonuclease, protein and DNA. Thus the aim of this experiment was to incorporate the features of Chomczynski's method into a method which would give faster isolation of RNA without compromise in quality or quantity.

Phenol extraction has been extensively used to extract RNA from mammalian tissues (Kirby, 1968; Wallace, 1987) but the basic methods generally resulted in degradation of the RNA extracted (Kirby, 1968).

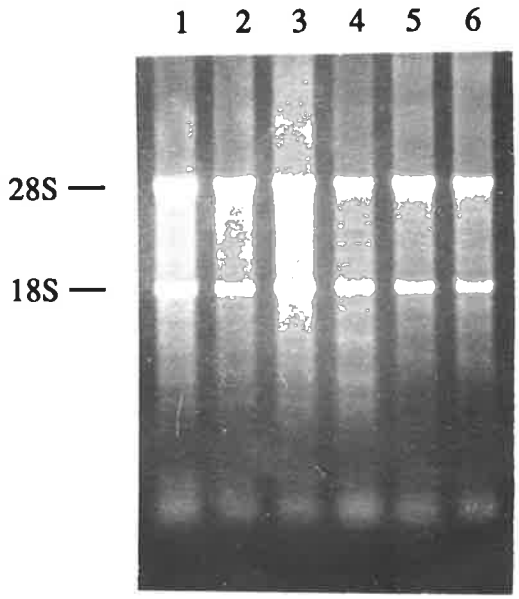


Figure III.1 Rat liver RNA samples (5µg/lane) were fractionated on a 1% agarose gel and visualised under UV illumination. The samples had been stored at -70°C for 3 months. Little degradation is seen in the samples after storage.

The degradation of RNA samples can be reduced by the inclusion of a number of different inhibitors of ribonuclease in the phenol; eg. inclusion of 8-hydroxy-quinoline to phenol improves the RNA yield, decreases ribonuclease activity and protein contamination (Kirby, 1968). Inclusion of guanidinium thiocyanate, a potent inhibitor of ribonuclease, as well as 8-hydroxyquinoline, with phenol should result in an efficient solution to extract intact, undegraded RNA, in a single homogenisation step.

A major consideration of the constituents of the extraction buffer was to ensure that the DNA was selectively retained in the organic phase, leaving the RNA in the aqueous phase. At a pH in the range of 5-6 DNA is selectively inhibited from the aqueous phase while at a pH greater than 8 DNA is included (Wallace, 1987). A specific salt concentration was also required to efficiently retain optimal RNA yields where the ionic strength was less than 0.2M ; the maximum solubility of RNA occurs at 0.15M Na^+ (Wallace, 1987).

III.2.2.1 *Homogenisation Solution*

The homogenisation solution (Soln 4) consisted of 2.0M guanidinium thiocyanate, 25mM Na_3 citrate, 60mM Na acetate, 0.05% isoamyl alcohol, 0.5% sarcosyl, 50mM β-mercaptoethanol and 49% phenol; the pH was 6.4. The phenol was prepared as described above (see III.2.1.1).

III.2.2.2 *RNA Extraction*

Liver tissue samples (50 and 100 mg) and small segments of mesenteric artery

(50 mg) stored at -70°C were placed directly into liquid nitrogen. The samples were placed in the well of a stainless steel mortar immersed in liquid nitrogen. The sample was pulverised with a stainless steel pestle that had its temperature also lowered in liquid nitrogen. The crushed samples were transferred to sterile 15 ml polypropylene tubes maintained in liquid nitrogen.

Excess liquid nitrogen was "boiled off" and the tube was placed in a ethanol-ice bath. The liver tissues were homogenised in Soln 4 (2 ml/100 mg tissue) while mesenteric artery tissues were homogenised in 2 or 4 ml of Soln 4 per 100 mg of tissue using an Ultra-Turrax for 60 sec at 24000 rpm. One-tenth volume of chloroform was added, the tube vortexed and left on ice for 15 min. The homogenate was centrifuged at 12000 *g* at 4°C for 20 min to separate the phases. The aqueous phase was transferred to a sterile 15 ml polypropylene tube and one volume of ice-cold isopropanol was added to precipitate the RNA. The tube was vortexed and placed in a -70°C freezer for 30 min to assist precipitation of the RNA. The RNA was pelleted by centrifugation at 12000 *g* for 15 min at 4°C and the supernatant discarded. The pellet was washed twice with several volumes of ice-cold 70% ethanol with the RNA pelleted between washes by centrifugation at 12000 *g* for 10 min at 4°C. The pellet was dissolved in SDW and the concentration, and purity, determined by absorbance at 260 nm and 280 nm.

III.2.2.3 *Purity*

The liver samples had purities (260/280 nm ratio) better than 1.7 (1.75±0.02) (n=10) with yields of 3.42±0.17 mg RNA/g of tissue. The samples were subjected to analysis for DNA and protein contamination by the methods described earlier (see II.9

and II.10). Within the parameters and sensitivities of the assays used no protein was detectable in any of the samples. DNA was detectable only in minute quantities and only when the entire total RNA extracted was assayed; ie less than 20 ng/100 mg tissue. The amount of DNA contamination in the RNA extracts was only 0.2% of the total nucleic acid extracted and was considered to be negligible.

The RNA from the liver samples were fractionated by electrophoresis on a mini gel (see section II.2.2) to observe if the samples were degraded. The gel showed two distinct bands denoting the RNA species at 28s and 18s with no degradation of the sample (Figure III.2).

The RNA extracted from the mesenteric arteries proved to be far from successful. The yield of RNA from the mesenteric arteries was 0.847±0.099 mg/g tissue (n=9) with an average purity of 1.63±0.05. A varying degree of DNA was found to be present in the samples although generally at the limit of detection; ie 20-30 ng per 100mg of tissue. Protein was not detectable in any of the samples. The DNA was able to be eliminated with successive washes with 70% ethanol but substantial amounts of RNA were lost each wash resulting in decreased RNA yield. The RNA was fractionated on a 1% agarose gel as described above (II.2.2) but some degree of degradation was seen in all mesenteric artery samples tested. The data indicates that the extraction of RNA from liver samples, by the incorporation of both phenol and guanidinium thiocyanate in the homogenisation solution, is possible and that the RNA is undegraded and essentially devoid of contaminants. Mesenteric artery tissue tended to be resilient to the combined phenol-guanidinium salt method and the degrading of the RNA mark this method to be not as efficient as the aforementioned method.

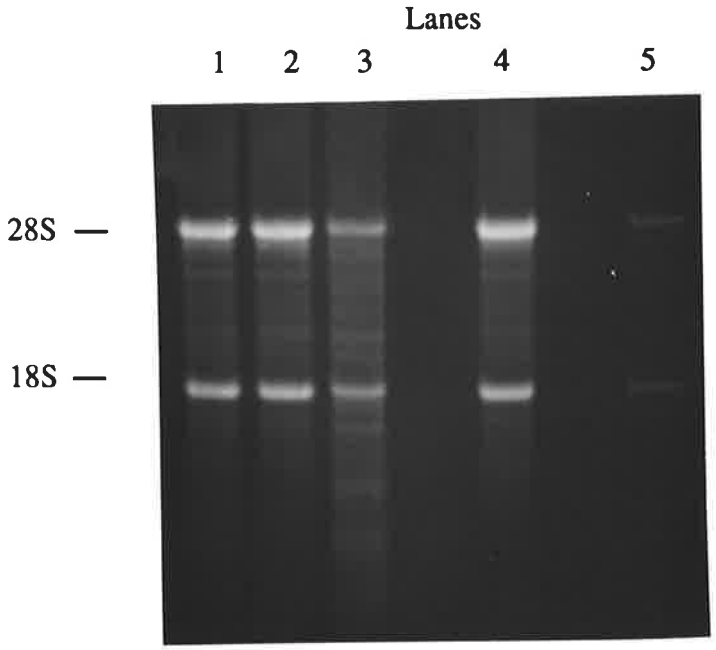


Figure III.2 RNA extracted by the modified extraction method were fractionated on a 1% agarose gel and visualised with ethidium bromide under UV illumination. Rat liver (lanes 1,2 & 3; 5,5 & 2.5µg respectively) and mouse salivary gland (lanes 4 & 5; 5 & 1µg respectively) RNA appeared undegraded with several bands observed between the expected 28s and 18s bands.

CHAPTER IV

CHANGES IN NGFmRNA AND NGF BY ANDROGEN MANIPULATION

Introductory Note : This experiment was conducted to verify that the cDNA probe was not altered after transfection and that it was capable of binding to the messenger RNA for proNGF and thus be effective in detecting changes in vascular NGFmRNA in latter studies.

IV.1 Introduction

The production of NGF has been demonstrated to be controlled by a myriad of compounds ranging from catecholamines (Furukawa, 1986) to hormones (Ishii and Shooter, 1975; Wion et al, 1985; Lakshmanan, 1986; Siminoski et al, 1987). The production of NGF has also been shown to follow an increase in mRNA levels (Heumann and Thoenen, 1986) and that the quantity of NGF correlates to the specific protein mRNA (Korsching and Thoenen, 1983; Heumann et al, 1984; Shelton and Reichardt, 1984). Chemically induced changes in NGF content in cell cultures have been shown to be preceded by an associated change in the messenger RNA for NGF (NGFmRNA) (Korsching and Thoenen, 1983; Heumann et al, 1984), hence it can be

stated that changes in NGFmRNA precede a subsequent change in NGF production.

The submaxillary gland of the male mouse has been shown to be an organ containing an exceptionally high concentration of NGF (Murphy et al, 1977). The rate of synthesis of NGF in this organ has been shown to be under the control of the androgen testosterone (Ishii and Shooter, 1975). Castration of adult male mice attenuated the production of NGF, in the submaxillary gland, but supplementation of testosterone to the castrated mice returned the level of NGF to normal (Ishii and Shooter, 1975). Similarly, injecting female mice with testosterone also elevated their level of NGF found in the submaxillary gland by several fold.

The probe used to quantitate NGFmRNA in the cardiovascular studies was obtained from Dr Rutter (University of California, USA) and was a 917 base pair (bp) sequence homologous for the mRNA for proNGF (Scott et al, 1983). The probe was received with the cDNA sequence inserted into the *PstI* site of an artificial plasmid, pBR322. It was necessary to amplify the probe for the following experiments and the plasmid was transfected into the bacteria *E.Coli*, grown in culture and purified (see II.5.1).

Electrophoresis of the purified probe demonstrated that the size of the sequence was maintained after transfection and excision from the plasmid with the restriction enzyme *PstI*. The probe was to be tested to confirm that the cDNA sequence had not been altered during the amplification process and that it was specific for the mRNA for proNGF. Changes in NGFmRNA, and NGF, by manipulating the level of circulating testosterone, was used to provide a model to test the cDNA NGFmRNA probe which was to be used in cardiovascular experiments.

IV.2 Methods

IV.2.1 *Animals and Tissue Collection*

Adult (60 day old) male, and female, Swiss Webster mice were obtained from the breeding colony of the University of Adelaide (Australia). The male mice were randomly allocated to one of four groups; A: control mice; B: castrated mice supplemented with testosterone; C: castrated mice treated with vehicle and D: castrated mice. The female mice provided a group (E) that had low levels of NGF present in the salivary gland and constituted a control for the castrated male mice.

The mice to be castrated (groups B,C and D) were anaesthetised with ether and a sagittal incision made in the scrotum. The vas deferens was tied off by a ligature and the testes were removed. The scrotum was sutured with cat gut and the animals allowed to recover for 2 days before any further treatments. Testosterone propionate (4-androsten-17β-ol-3-one; Sigma) was dissolved in a small quantity of ethanol and then suspended in peanut oil to give a final concentration of 2 mg/ml. Groups B and C were injected subcutaneously with 0.1 ml of either 2 mg/ml testosterone or the vehicle (peanut oil), respectively. All animals were fed standard chow and water ad libitum, and were housed in the animal colony at the University of Adelaide.

After 21 days of treatment the mice were sacrificed by cervical dislocation and the two lobes of the submaxillary gland were removed. The two lobes were separated, rinsed in ice-cold saline, blotted dry on wetted filter paper, weighed and frozen in liquid nitrogen. The tissues were stored at -70°C until required; one lobe was used to analyse the NGF content while the other was used to quantify the level of NGFmRNA.

IV.2.2 *RNA Analysis*

The RNA was extracted using the modified guanidinium thiocyanate-phenol-chloroform methodology of Chomzynski and Sacchi (1987) (see III.2.2) and redissolved in SDW. The purity and yields of RNA were determine by absorbance at 260 and 280 nm before the samples were stored at -70°C until required for Northern blot and slot blot analysis.

IV.2.2.1 *Northern Transfer*

Aliquots of the total RNA (5 and 10 µg) extracted from the tissue samples were fractionated on a 1% agarose formaldehyde-denaturing gel, visualised under UV illumination in the presence of EtBr and then transferred to nitrocellulose filters (Schleicher and Schuell, Germany) by vacuum (see II.2). The filters were transferred to sterile folders, allowed to air dry overnight then baked for 2 hrs at 80°C *in vacuo* (see section II.3). The membrane was stored at -70°C until required.

IV.2.2.2 *Slot Blots*

Serial dilutions of total RNA from each sample were prepared in sterile microfuge tubes in 2xSSC containing 50% formamide and 6.3% formaldehyde and applied to nitrocellulose membranes (see II.4).

IV.2.2.3 *Hybridisation*

The nitrocellulose membranes (Northern transfers and dot blots) were prehybridised for 6 hrs, then hybridised with the ^{32}P nick translated probe (see II.6.1) for a further 16 hrs (42°C) in sealed bags (see II.7). The membranes were air-dried for 15 min then placed in plastic wraps for autoradiographing. The membranes were placed in x-ray film cassettes (Dupont) with intensifying screens (Cronex, Xtra Life) and x-ray film (Curix RPI, Agfa-Gevaert, Australia) and stored at -70°C for several days. The autoradiographs were developed after 3-5 days and the signals quantitated using a densitometer (LKB Laser Ultrascan Densitometer, Bromma, Sweden). The area under the curve was analysed by computer using an integrating program that was incorporated in the operations program for the densitometer.

IV.2.3 *NGF Analysis*

The quantitation of NGF in each lobe of the submaxillary gland was determined using the methodology described by Gasser et al (1986) and modified by Abrahamson et al (1987). These measurements were conducted in the laboratories of Professor Bob Rush at Flinders Medical Centre and the procedure carried out in collaboration with Ms Dianne Bridges.

Samples were homogenised in 10M phosphate-buffered saline and 100 µl aliquots of each sample were assayed; in duplicate. Sheep antibodies to mouse NGF, which were affinity-purified, were adsorbed to the base of a polystyrene microtitre plate (Greiner, West Germany) in carbonate-bicarbonate buffer (pH 9.8) and used as the capture antibody. Tissue homogenates, or standard NGF, were incubated overnight

in the wells of the plates in a shaking water bath at room temperature and then thoroughly washed with buffer (50mM Tris-HCl, 0.2M NaCl, 10mM $CaCl_2$, 0.1% Triton X-100, 1% gelatine and 0.05% NaN_3; pH7.0). Rabbit antibodies to mouse NGF and alkaline-phosphatase-conjugated anti-rabbit antibodies (Sigma, St Louis) were incubated sequentially at 37°C for 2 hrs in a shaking water bath.

Colour was developed using para-nitrophenol phosphate (Sigma, St Louis) as the substrate and the plate was read on a ELISA reader (Dynatech Instruments Inc., Torrance) at 410 nm. The assay had a sensitivity of 2 pg/100 µl and the values for the NGF in each tissue sample were adjusted for recovery; recovery of pure NGF added to tissue homogenates was 48%.

IV.2.4 *Statistical Analysis*

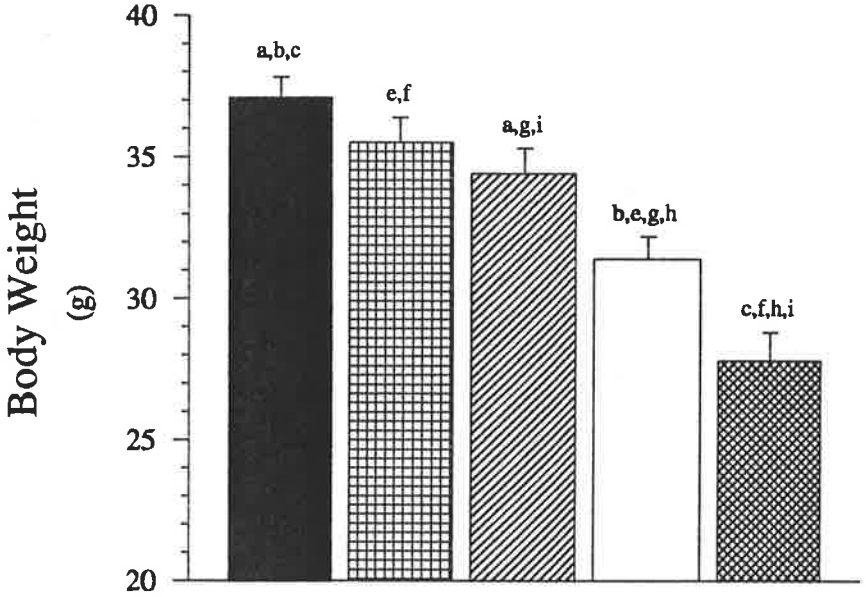
All data were subjected to statistical analysis using the Student's t-test with a significance difference of $p<0.05$. NGF and NGFmRNA content was additionally subjected to analysis using Kruskall-Wallis rank analysis ($p<0.05$). All data are expressed as the mean ± standard error of the mean (SEM).

IV.3 Results

IV.3.1 *Body and Tissue Weights*

The female mice were significantly smaller, by weight, than all male groups. Castration of the male mice however, resulted in a reduction in body weight which was restored, in part, by supplementation with testosterone (Figure IV.1a). The control

(a)



(b)

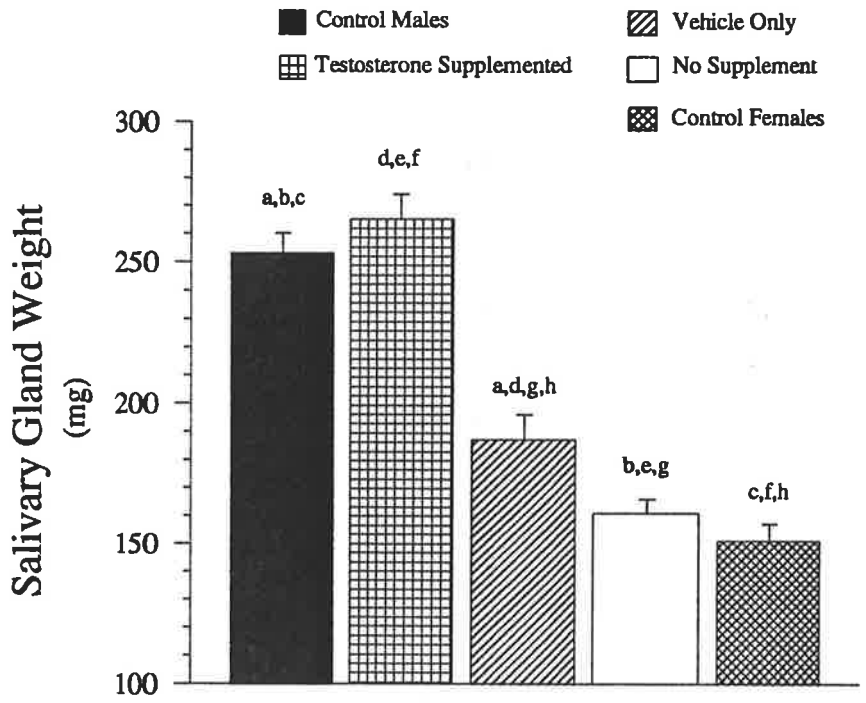


Figure IV.1 Body (a) and submaxillary salivary gland (b) weights for control male and female mice, and all treatmented groups (n=9). Significant differences between columns are noted by having the same character (Student's *t*-test; $p<0.05$).

male mice were heavier than all castrated mice but this was only statistically significant when compared to mice not supplemented with testosterone ($p<0.05$)(Figure IV.1a). The castrated mice injected with the vehicle, peanut oil, were significantly heavier ($p<0.05$) than the castrated mice that were not given any treatment, but were lighter than the mice supplemented with testosterone; though not significantly so (Figure IV.1a).

The weights of the submaxillary gland demonstrated a similar pattern as that seen for the body weights. The salivary glands of the control male mice, and the testosterone supplemented mice, were significantly heavier than all other groups ($p<0.05$); there was no significant difference between the control male and the supplemented mice although the latter weighed approximately 5% more (Figure IV.1b). As expected, based upon body weights, the female salivary glands weighed the least of the 5 groups however the weights of the glands were not significantly different from the castrated male mice which did not receive any treatment.

The figures pertaining to body and salivary weight, as well as RNA yields are listed in the appendices (Appendix II)

IV.3.2 *Extraction and NGFmRNA Content*

The extraction of total RNA, from all the submaxillary salivary glands, resulted in an optical density ratio (260/280 nm) of 1.68 ± 0.01 ($n=51$). The samples were devoid of protein, as determined by the protein assay of Lowry (1951), and DNA was undetectable using the fluorescence methodologies (Labarca and Paigen, 1980). RNA aliquots fractionated on a 1% agarose gel, stained with EtBr, showed no degradation of the samples.

The untreated castrated male mice demonstrated a significantly lower concentration of total RNA than all other groups ($p<0.05$)(Figure IV.2). The female mice had a greater amount of total RNA than the castrated male mice treated with peanut oil but this was not significant. Control male mice and testosterone supplemented mice showed a significantly greater level of total RNA than all other groups but were not significantly different from each other (Figure IV.2).

The Northern transfer of 5 and 10 µg of total RNA from control male mice, analysed with the ^{32}P nick-translated probe, demonstrated a single hybridisation band that corresponded to 1.35 kb (Figure IV.3). Absence of smearing of the hybridisation bands demonstrated that the samples had not degraded during storage.

Dot blot analysis of the RNA samples indicated that the concentration of NGFmRNA was significantly greater in the testosterone supplemented and control male mice than all other groups ($p<0.05$)(Figure IV.4a). The intensity of the blots of the supplemented mice were greater than the control male mice (Figure IV.4a) possibly indicating that the concentration of exogenous testosterone administered may have been greater than the endogenous levels normally present. The NGFmRNA values (and NGF concentrations) of the different tissues are listed in the appendices (Appendix III).

IV.3.3 *NGF Determinations*

Both the control males and the testosterone supplemented castrated males had significantly greater amounts of NGF than all other groups ($p<0.005$)(Figure IV.4b). The testosterone supplemented males also had significantly greater NGF than the control males; almost 2 fold difference between the two groups (Figure IV.4b).

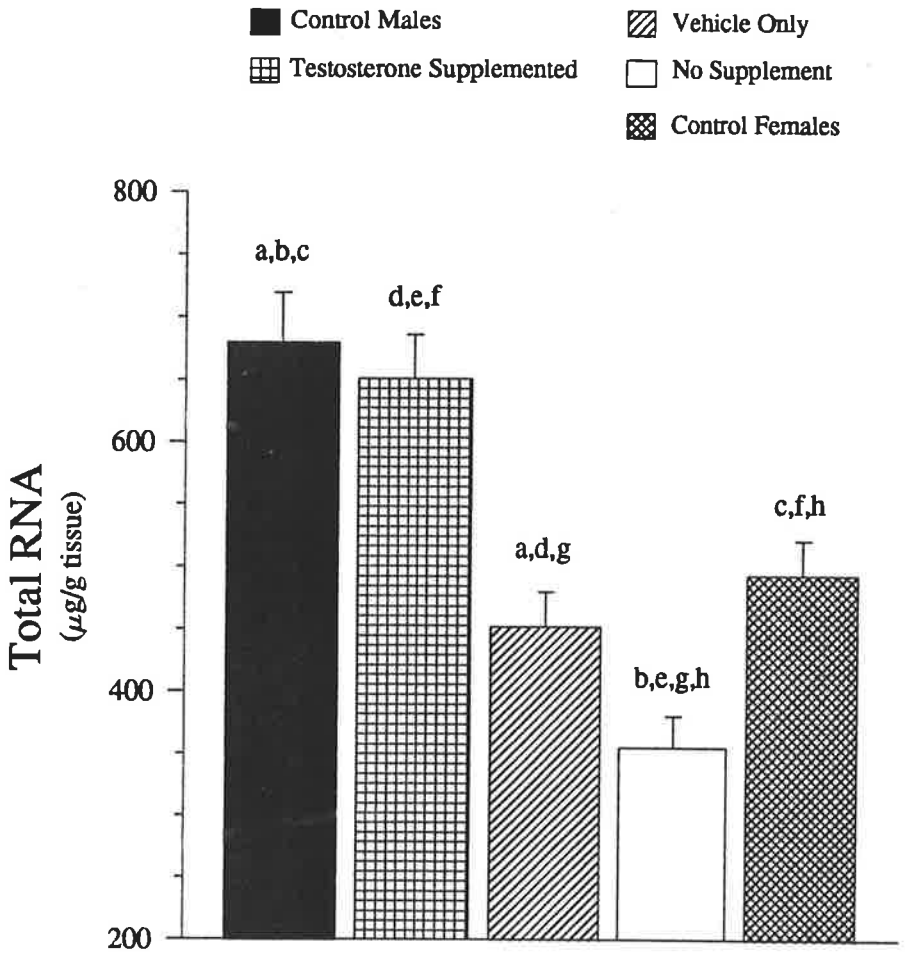


Figure IV.2 Total RNA extracted from each mouse group using the combined phenol-guanidinium-chloroform technique (n=9). Significant differences between columns are noted by having the same character (Student's *t*-test; $p<0.05$).

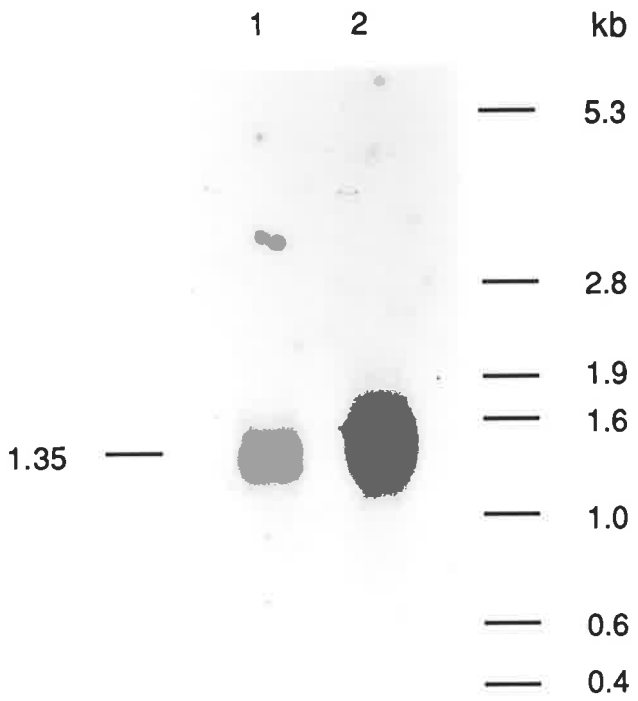
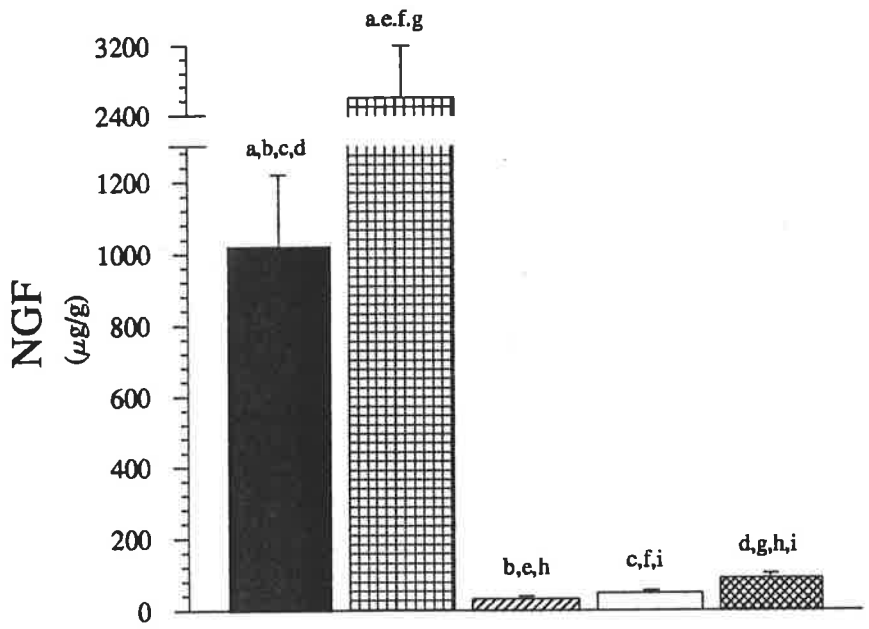


Figure IV.3 Northern transfer analysis of submaxillary gland RNA from male control mice (5 and 10µg total RNA). The nitrocellulose membrane was probed with 0.5µg of the purified 917bp NGFcDNA, nick translated with ^{32}P; only a single band is observed corresponding to 1,350bp. RNA size markers (fragments of linearized plasmids) in base pairs are noted on the right.

(a)



(b)

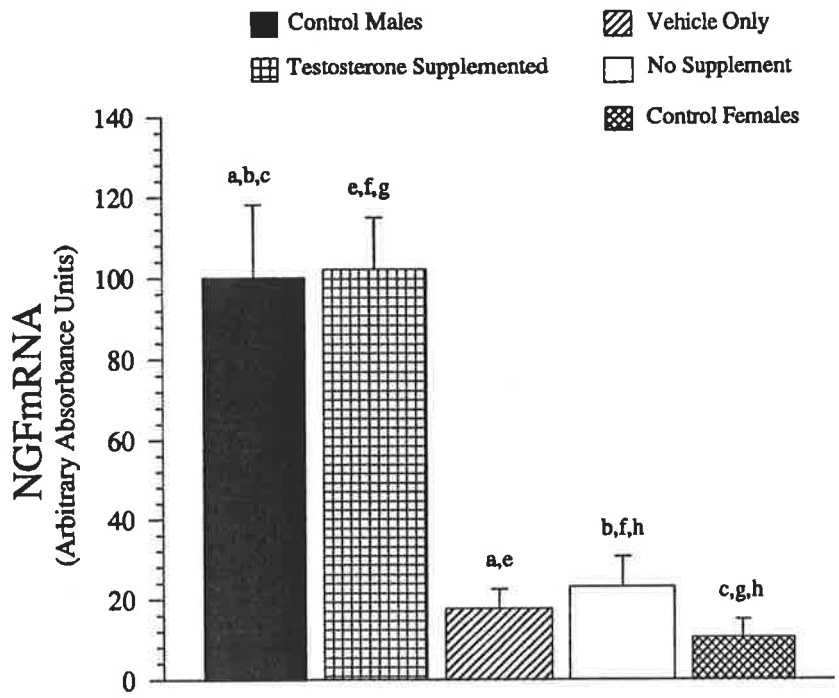


Figure IV.4 (a) Dot blot analysis of RNA samples of all groups (n=9). The absorbance of all treatment groups are relative to the control (male). (b) NGF concentrations in a single lobe of all groups (n=8). Significant differences between columns are noted by having the same character (Student's *t*-test; $p<0.05$).

The female mice demonstrated significantly greater amounts of NGF than the two other castrated male groups ($p<0.05$) while there was no significance between the two latter groups in regard to NGF content (Figure IV.4b).

IV.4 Discussion

Developing mice demonstrate a fluctuating serum level of testosterone (Lucas and Eleftheriou, 1979; Bartke et al, 1973; Jean-Faucher et al, 1978) and while there is a positive correlation for age and plasma testosterone between 1 and 40 days there exists a negative correlation from day 40 onwards (Jean-Faucher et al, 1978). Aggression (Lakshmanan, 1986b) and induced hyperthyroidism (Lakshmanan, 1986a) in mice has been shown to elevate serum NGF while the hyperthyroidism of neonatal mice will impair the developmental rise in salivary gland NGF (Lakshmanan, 1986a).

The submandibular salivary glands of male mice contain a relatively high concentration of nerve growth factor that appears to be under the control of the androgen testosterone (Ishii and Shooter, 1975; Lakshmanan, 1986b). Castration of male mice, while depriving the animal of its source of testosterone, decreased the NGF content in the salivary gland. Treating castrated mice with supplemental injections of testosterone will return the glandular NGF content to control levels while treating female mice with exogenous testosterone results in an elevation of salivary NGF content (Ishii and Shooter, 1975).

The results from the experiment reported in this chapter are in agreement with the findings of Ishii and Shooter (1975) in that the submandibular NGF was greatly reduced in castrated animals while supplementation of testosterone elevated the level of NGF. In point of fact the supplementation, with alternate day injections, of 200µg

testosterone raised the glandular NGF content in excess of the levels found in control animals. This elevation may reflect that the plasma concentration of testosterone may have reached higher plasma levels than expected and that testosterone may influence NGF production; directly or via some feedback system effected by the level of testosterone. It could also be argued that testosterone may have some effect on NGF production, other than via NGFmRNA, for while NGF content in the glands was elevated, by almost 2 fold, there was not a significant difference between the control and testosterone supplemented groups total RNA (652±42 µg/g and 670±31µg/g respectively), nor was there a significant difference in NGFmRNA content (100.0±18.1 and 102±12.8 respectively). Total RNA and salivary gland weight showed a strong correlation (r=0.72) that was not seen with NGF content despite the fact that NGF has been shown to correlate with the product of its gene expression, NGFmRNA, in other tissues which have not been pharmacologically manipulated. Gonadectomy in rats results in the onset of a negative-feedback regulation of pituitary gonadotrophin synthesis (Pakarinen and Huhtaniemi, 1989) and this may inturn effect other factors that may result in overproduction of NGF.

The vehicle, peanut oil, had a direct influence on the animals body and gland weight and total RNA content of the gland. Increases in the body and gland weight could possibly be explained by the incorporation of fatty acids within tissues, derived from the oil. The increased total RNA content seen in control (vehicle only) treated mice, compared to castrated mice (approximately a 25% increase), cannot be so easily reconciled as there is no such increase observed when comparing control males with testosterone supplemented mice; this phenomena remains unexplained.

The single band seen in the Northern transfer demonstrated that the probe bound to a specific RNA species and the band was identical with the mRNA species

specific for the precursor to NGF (Scott et al, 1983). The lack of smearing of the tracking of the RNA and the single band indicates that the method used for extracting the RNA was capable of extracting undegraded RNA from submaxillary gland tissue and that the binding of the probe was specific.

The overall objectives of the experiment were achieved in that; (1) undegraded RNA was extracted from the mouse salivary gland using the modified extraction procedure described earlier (III.2.2), (2) the probe amplified by culture was specific for the NGFmRNA species as described by Scott et al (1983) and (3) changes in the gene expression for NGF are, in general, reflective of changes seen in the concentration of the protein itself (Davies et al, 1987). The probe's ability to detect mRNA for the precursor for NGF presents a useful tool for further investigations into the gene expression of NGF. Moreover the results of the experiment provides confidence that the cDNA probe used for subsequent studies with tissues expressing minimal quantities of NGFmRNA is in fact reliable.

CHAPTER V

EXPRESSION OF NGF IN THE HEART AND KIDNEY IN THE DEVELOPING SHR

V.1 Introduction

In general, there is widespread agreement that the sympathetic innervation of selected vascular and non–vascular tissues is enhanced in the SHR (Head, 1989). It has been suggested that this enhanced innervation occurs during the normal time course of sympathetic innervation of tissues (Donohue et al, 1988), and it is believed that the abnormal innervation in the vasculature of the SHR is related to an elevation in the neurotrophic peptide NGF (Donohue et al, 1989; Hamada et al; 1990).

Not all tissues in the SHR display the characteristics of the enhanced innervation. For example, the NA content of ventricles from hearts of SHR rats have been shown to be either similar to (Louis et al, 1969; Nakamura et al, 1971) or smaller than (Howe et al, 1979), those seen in ventricles from normotensive control WKY. In contrast the NA content of kidneys and the mesenteric vasculature is greater in SHR rats, a feature consistent with an enhanced sympathetic innervation in these tissues (Head et al, 1985; Donohue et al, 1988). It has been shown that the prevailing density of innervation of tissues innervated by the sympathetic nervous system correlates with the tissue expression of NGFmRNA (Korsching and Thoenen, 1983;

Shelton and Reichardt, 1984; Heumann et al, 1984). It follows that a comparison of NGFmRNA expression in tissue that does not display an enhanced innervation (heart) with those that do (kidney and mesenteric vasculature) in the SHR, will help to define the relationship between gene expression of NGF and the hypernoradrenergic innervation of tissues in the SHR.

V.II Methods

V.II.1 *Animals and Tissue Removal*

Male SHR and WKY rats, and male Swiss Webster mice, were supplied from the CSIRO Glenthorne barrier facilities. The BPs of adult male SHR and WKY rats from this colony were analysed and were found to be 183±2 and 131±2 mmHg respectively (n=10). The rats were sacrificed by initially stunning the animals then decapitation. Hearts and kidneys were removed from 2, 10 and 43 day old rats (30, 18 and 12 rats respectively) while mesenteric veins were removed using a dissecting microscope from only the 10 and 43 day old rats. Male mice were sacrificed by cervical dislocation and the submaxillary salivary gland removed. All tissues were blotted on moistened filter paper, weighed, frozen in liquid nitrogen and stored prior to analysis in sterile tubes at -70°C. The male mouse submaxillary gland was used as a NGFmRNA standard due to the relatively high concentration of NGF found in this tissue.

V.II.2 *RNA Isolation and Application to Nitrocellulose membranes*

The RNA from male mouse submaxillary salivary glands, rat hearts and kidneys was isolated using the method described by Chomczynski and Sacchi (1987) (III.2.1; subsections 3 and 4). For extraction purposes, 5 hearts were pooled for each extraction for 2 day-old pups, 2 for 10 day-old pups, whilst individual hearts were extracted for 43 day-old rats; the same criteria applied for kidneys. Four mesenteric veins were pooled for extraction of total RNA, thus giving a pooled n value (n=3). RNA from mesenteric veins was also isolated by the same method.

RNA from hearts, kidneys, mesenteric veins and male mouse submaxillary glands were heated in the presence of formamide and formaldehyde and electrophoresed on a 1% agarose gel containing formaldehyde (II.2.2). The fractionated RNA was transferred to nitrocellulose membranes by vacuum (II.3) and annealed by baking for 2 hrs *in vacuo* (80°C). For slot blot analysis, several dilutions of total RNA from rat hearts, kidneys and mesenteric veins were prepared in sterile microfuge tubes in 10xSSC containing formaldehyde and applied to nitrocellulose membranes under vacuum (II.4). The membranes were placed in sterile folders, air dried and then baked at 80°C for 2 hrs *in vacuo*.

Both the Northern and slot blot filters were then analysed using the radiolabelled probe and the hybridisation conditions described below.

V.II.3 *Hybridisation of Filters*

The 917 base pair cDNA sequence previously demonstrated to be specific for the mRNA for the precursor of NGF (Chapter IV; Falckh et al, 1990) was used to

probe the Northern and slot blots membranes. The membranes were placed in separate sealed plastic bags with 0.1 ml/cm^2 prehybridisation solution and incubated for at least 6 hrs in a water bath maintained at 42°C (II.7.1). The cDNA sequence was nick translated with ^{32}P (II.6.1) to give a specific activity of 1x10^9 cpm/µg of DNA then heated shocked prior to its addition to the prehybridisation solution. The membranes were hybridised for a further 16 hrs (II.7.2) then washed twice in 2xSSC containing 0.1% SDS and once in 0.2xSSC containing 0.1% SDS (II.7.3).

V.II.4 *Autoradiography*

The filters were allowed to air-dry for 15 min then wrapped in plastic and placed in X-ray cassettes with intensifying screens and x-ray film (II.8). The cassettes were placed in perspex boxes and left for several days in a -70°C freezer. The autoradiographs were developed, fixed and analysed using a laser densitometer incorporating a computer integration program for area under the curve (II.8). The samples were normalised to the signal generated by 100 ng of male mouse submaxillary gland RNA for each membrane.

V.II.5 *Statistical Significance*

Data are shown as the mean (± SEM) for the number of measurements shown in the results section. Statistical significance was determined by using Student's t–test and analysis of variance with a p value less than 0.05.

V.III Results

The body weights of WKY pups were significantly greater ($p<0.01$) than those of SHR pups for all but the youngest animals (Figure V.1a). WKY kidney weights followed a similar trend, being significantly heavier than the SHR at 10 and 43 days-of-age, however SHR kidneys were greater than WKY kidneys at 2 days-of-age (Figure V.1b). Heart weights ranged between the two species (Figure V.2a) although heart-body weight ratios were always significantly greater for SHR than for WKY animals (Figure V.2b). Body and tissue weights are listed in the appendices (Appendix IV).

The yields of RNA for each tissue are shown in Table V.2. While younger animals showed some differences in the amount of RNA extracted from both hearts and kidneys no significant difference was found for either tissue at 6 weeks of age (Table V.2). Aliquots of total RNA from kidney, heart and mesenteric veins showed no evidence of RNA degradation after separation on agarose gels. Extracts of kidney, heart and mesenteric vein showed single bands after hybridisation with Northern blot analysis. The position of the hybridisation bands for the rat tissues were similar to those for male mouse submaxillary glands at approximately 1350 base pairs (Figure V.3).

Slot blot analysis indicated that the concentration of NGFmRNA in mesenteric veins from 43 day old SHR's was dramatically greater than the concentrations present in mesenteric veins from WKY rats (Figure V.4). In hearts from SHR and WKY rats there was an age dependent decline in the concentration of NGFmRNA with the values at 2 days of age being very much greater than those present in hearts from 43 day old animals (Figure V.5a).

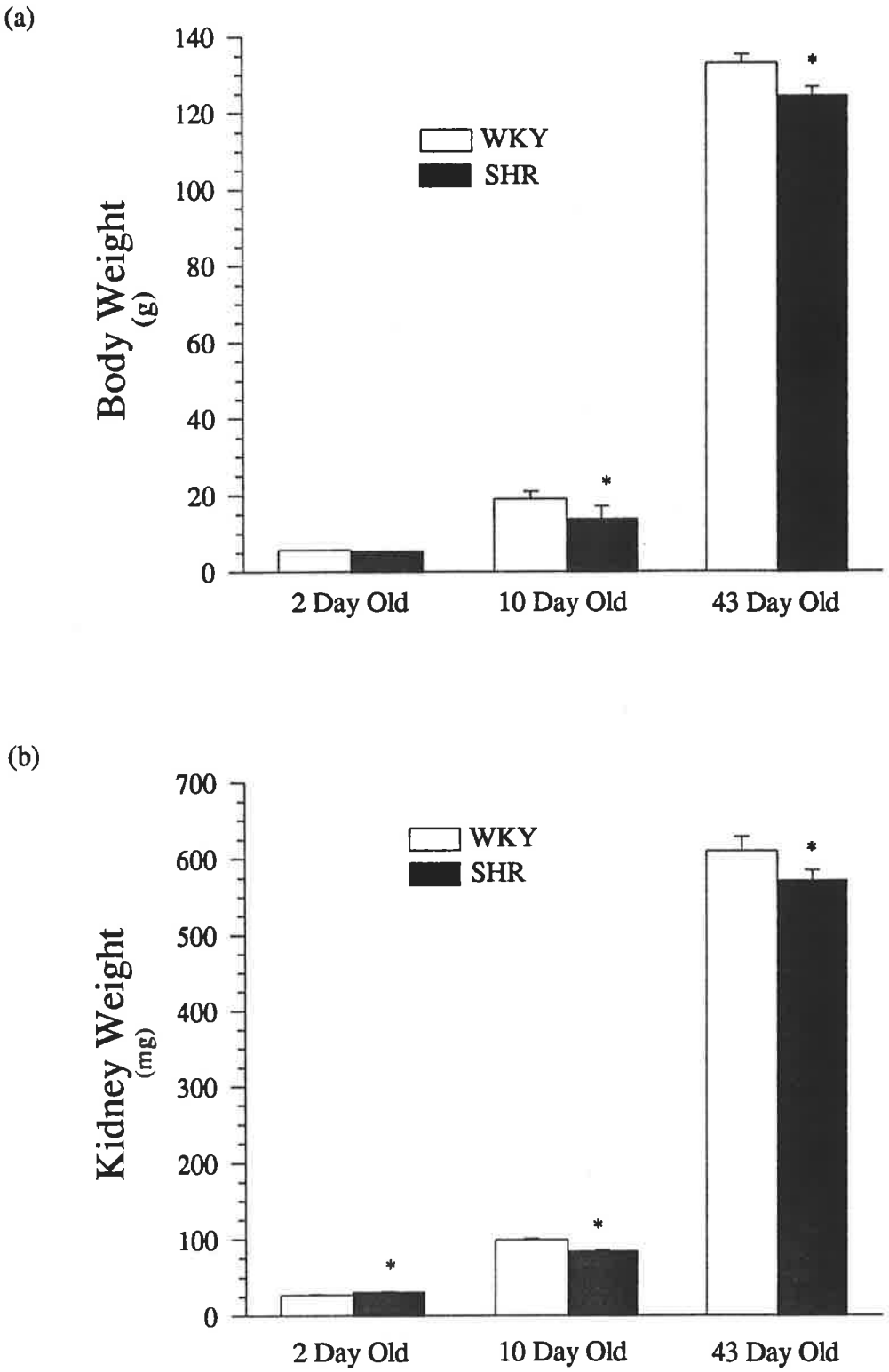


Figure V.1 (a) Body and (b) kidney weights of WKY and SHR pups at 2 (n=30), 10 (n=18) and 43 (n=12) days of age (* denotes significant differences between SHR and WKY, for each age group; Student's *t*-test, $p<0.05$).

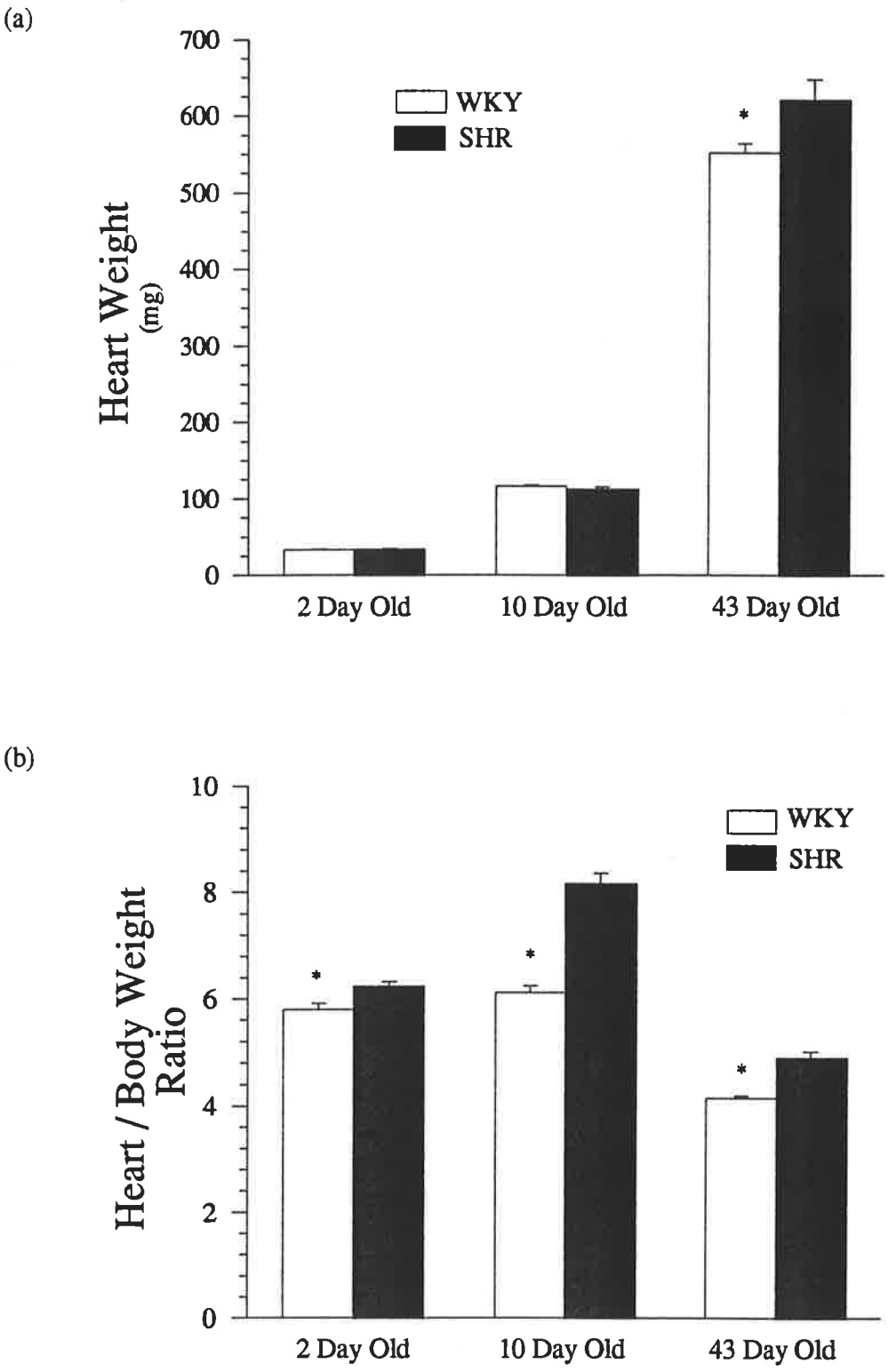


Figure V.2 (a) Heart weights and (b) heart-to-body weights of WKY and SHR rat pups at 2 (n=30), 10 (n=18) and 43 (n=12) days of age (* denotes significant differences between SHR and WKY, for each age group; Student's *t*-test, $p<0.05$).

Tissue	Species	RNA (µg/mg tissue)		
		2 Day Old	10 Day Old	43 Day Old
Kidney	WKY	3.44±0.21*	4.76±0.16	2.70±0.08
	SHR	2.02±0.26	9.91±0.43*	2.99±0.27
Heart	WKY	4.42±0.16*	3.05±0.10*	1.29±0.04
	SHR	3.14±0.52	1.26±0.08	1.23±0.09

Table V.2

RNA yields (µg/mg tissue) of hearts and kidneys from WKY and SHR rat pups at 2, 10 and 43 days of age (* denotes significant differences between SHR and WKY yields; $p<0.05$, Student's *t*-test).

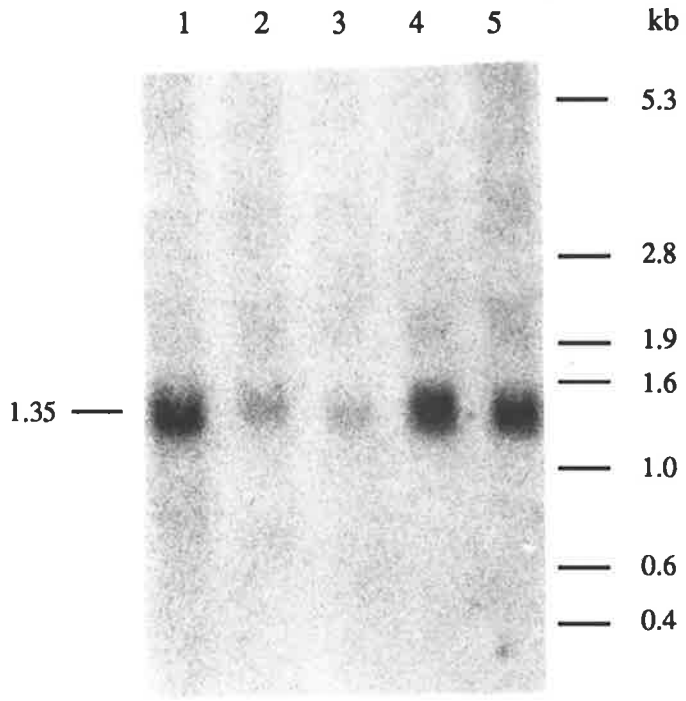


Figure V.3 Northern transfer analysis of RNA fractionated on a 1% agarose gel of kidneys (lanes 4 & 5; 10µg/lane) and hearts (lanes 2 & 3; 5µg/lane) from 43 day old SHR (lanes 2 & 4) and WKY (lanes 3 & 5) rat pups. Mouse salivary gland RNA (2µg;lane 1) was used as a positive control for the hybridisation of the NGFcDNA. RNA size markers (fragments of linearized plasmids) in base pairs are noted on the right. A single band is seen for all tissues corresponding to 1350bp.

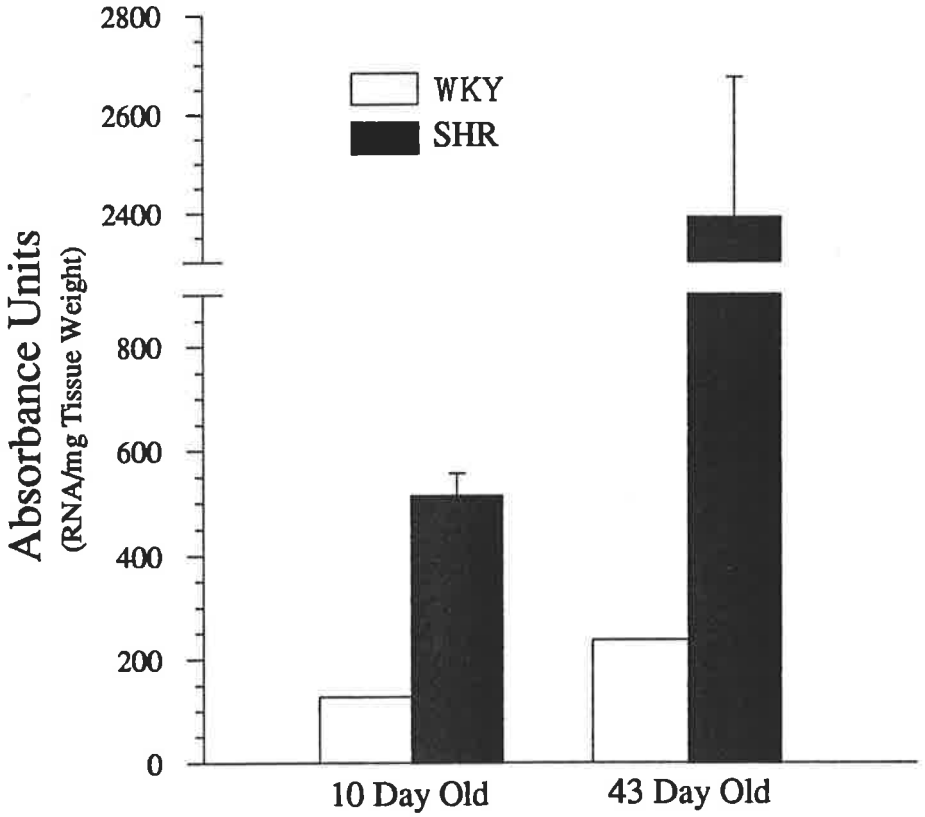
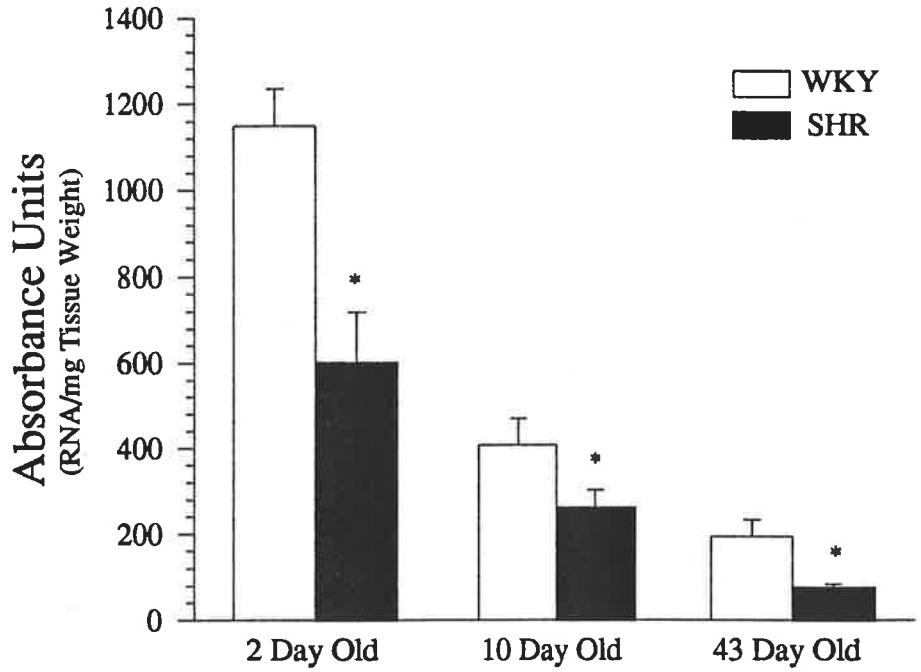


Figure V.4 Slot blot analysis of NGFmRNA in mesenteric veins from WKY and SHR rats. The absorbance of slots were standardised to 100 ng of RNA from male mouse submaxillary salivary glands.

(a)



(b)

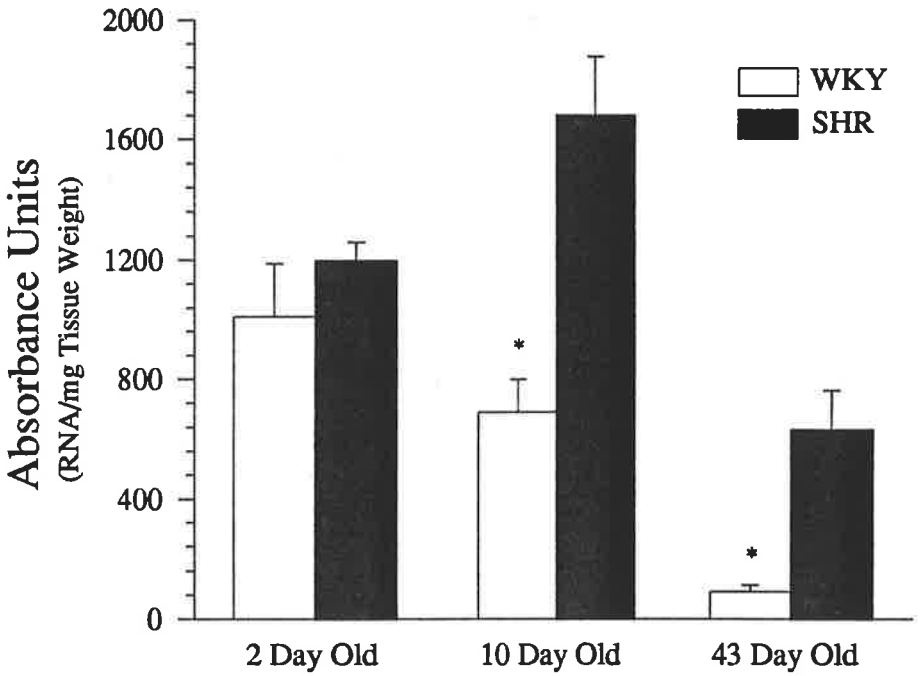


Figure V.5 Slot blot analysis of NGFmRNA of (a) hearts and (b) kidneys from WKY and SHR rat pups at 2 (n=3), 10 (n=4) and 43 (n=6) days of age (* significant differences between age groups, Kruskal-Wallis one way ANOVA by ranks; $p<0.05$).

At all ages studied (ie. 2, 10 and 43 days), the content of NGFmRNA in hearts from WKY rats was greater than those present in cardiac tissue from SHR's (Figure V.5a). The NGFmRNA in kidneys from WKY rats demonstrated an age dependent decline (Figure V.5b) similar to that seen in hearts from WKY. In contrast the NGFmRNA level in kidneys of 10 day old SHR pups was approximately 2 fold greater than that seen in 10 day old WKY and this difference increased to 6 fold at 6 weeks of age (Figure V.5b). SHR pups displayed a decrease in NGFmRNA of 62.5% from day 10 to day 43 while WKY NGFmRNA decreased 87% in the same period. Thus the SHR displayed a sustained, and elevated, increase in NGFmRNA consistent with the increased innervation seen in adult SHR. NGFmRNA levels are listed in the appendices (Appendix V).

V.IV Discussion

The predominant finding in the present study was the identification of a larger content of NGFmRNA in mesenteric veins and kidneys but not in cardiac tissues from SHR rats when compared to the levels prevailing in corresponding tissues from WKY rats. The significance of this finding relates to the pattern of abnormal sympathetic innervation in the SHR. There is compelling evidence to suggest that there is a greater sympathetic innervation of selected cardiovascular tissues in the SHR when compared with the innervation of corresponding tissues in the normotensive WKY rat (Head 1989). This enhanced innervation is characterised by an increased NA concentration which has been reported for the SHR mesenteric vein (Head and Berkowitz, 1979) and the kidney (Head et al, 1985). Evidence has been presented indicating that the enhanced sympathetic innervation in the SHR occurs in selected vascular beds as early

as 2 days of age and remains elevated into adult life (Donohue et al, 1988).

Based upon the observation that NGF was elevated in the SHR mesenteric vascular bed in 20 day old rats it was suggested that an early and enhanced elevation of NGF during the normal time course of sympathetic innervation is responsible for the hypernoradrenergic innervation seen in the SHR (Donohue et al, 1989). The results of the present study extend this hypothesis to suggest that the enhanced sympathetic innervation in the kidney and mesenteric vein is associated with an increased content of NGFmRNA.

There is no evidence for hypernoradrenergic innervation of the SHR heart. The NA concentrations in cardiac tissue from SHR and WKY rats have been reported to be indistinguishable from each other (Louis et al, 1969; Nakamura et al, 1971; Donohue et al, 1988) or slightly less in SHR rats (Howe et al, 1979; Berkowitz et al, 1980; Cassis et al, 1988). The results of the present study in which the NGFmRNA levels in hearts from WKY and SHR rats were compared is consistent with an absence of hyper-noradrenergic innervation in the SHR heart. In particular, the NGFmRNA content of cardiac tissue from WKY rats, at all ages studied, was greater than the levels found in hearts from SHR rats. Heart-to-body weight ratios were significantly greater in the SHR pups as early as 2 days of age. These data are consistent with the findings of Gray (1984) and Nyborg et al (1986) and may reflect some species differences between the SHR and WKY strains.

The results of the present study demonstrate that in those tissues of the SHR where there is evidence for an enhanced sympathetic innervation there is a greater content of NGFmRNA. In contrast in cardiac tissue where there is no evidence of hypernoradrenergic innervation the content of NGFmRNA in hearts from SHR rats is less than that seen in hearts from WKY rats. This study provides the first evidence that

NGFmRNA content parallels the altered sympathetic innervation in the SHR. In addition, the results strengthen the hypothesis that an early and overt production of NGF due to an enhanced vascular expression of NGFmRNA plays a pivotal role in the development of hypertension in the SHR.

CHAPTER VI

EXPRESSION OF NGF IN RESISTANCE VESSELS IN THE DEVELOPING SHR

VI.1 Introduction

There is general agreement that there is a greater sympathetic innervation of blood vessels in the SHR when compared to the innervation of blood vessels in the normotensive genetic control WKY rat (Head, 1989). A great deal of this evidence is based upon histofluorescence, morphometric analysis and chemical analysis of mesenteric arteries and caudal arteries (Berkowitz and Spector, 1976; Berkowitz et al, 1980; Cassis et al, 1985; Donohue et al, 1988). In association with the enhanced innervation of the SHR vasculature is a significant elevation of the blood pressure compared to the WKY (Okamoto, 1969; Limas et al, 1980) as well as structural changes of the vessels (Lee et al, 1983a,b). Vascular changes have been observed in neonatal pups (Gray, 1984; Lee et al 1985) suggesting that abnormalities may occur either during embryogenesis or early in postnatal development.

The enhanced innervation of the vasculature occurs for most vessels during the normal time course of sympathetic innervation and for selected vessels can be observed within days of birth (Donohue et al, 1988). The development of the sympathetic innervation, as well as that of sensory nerves, has been demonstrated to be

under the influence of a neurotrophic peptide, NGF (Levi-Montalcini and Calissano, 1979) and it is during the developmental phase of innervation that NGF plays a functional role in the vasculature (Levi-Montalcini and Calissano, 1979; Lee et al, 1987). The possibility that this neurotrophic regulation may be abnormal in the SHR was suggested by Donohue et al (1989), after they detected a larger content of NGF in mesenteric arteries from 20 day old SHR's when compared to the concentrations of the peptide found in mesenteric vessels from WKY rats. Subsequently, it was demonstrated that NGF concentrations were elevated in iliac nerves from 6 week old SHR's (Hamada et al, 1990) compared to age-matched WKY rats. The level of NGF has been correlated with the density of sympathetic innervation in different tissues and in several species (Korsching and Thoenen, 1983) as well as correlating with the gene expression for the peptide (Shelton and Reichardt, 1984; Heumann et al, 1984).

As reports suggest that abnormalities during the postnatal period may contribute to the development of the disease state in the SHR the pattern of NGFmRNA was determined in blood vessels of the SHR that have been shown to display enhanced levels of NGF, and a concomitant increased sympathetic innervation. NGFmRNA levels in blood vessels from age-matched WKY rats were also investigated and whether the NGFmRNA pattern was different between the two strains.

VI.2 Methods

VI.2.1 *Animals and Tissue Removal*

Adult male Swiss Webster mice, male SHR and WKY rat pups at 2, 10 and 43 days of age were obtained from the small animal colony at CSIRO Glenthorne (South Australia). Animals were weighed and sacrificed within hours of receipt of the pups

from the colony. Tissues (aortae, mesenteric arteries and caudal arteries) were removed from the rat pups using a dissection microscope, cleaned of all adhering extraneous tissue, were blotted on moistened filter paper and weighed. The tissues were wrapped in foil, frozen in liquid nitrogen and stored at –70°C until required. Submaxillary salivary glands were dissected from euthanised mice and treated as described above; the NGFmRNA from these glands was used as a standard.

VI.2.2 *RNA Isolation*

Due to the small weights of vascular tissue it was necessary to pool tissues prior to extracting total RNA. Mesenteric arteries from 10, 4 and 2 rat pups were pooled for the 2, 10 and 43 day old animals respectively; aortae were treated in a similar manner. Caudal arteries could only be obtained from 43 day old animals and extracts were prepared from a pool of 2 arteries; individual glands were processed from mice. Total RNA was extracted from tissues using the modified guanidinium isothiocyanate–phenol chloroform procedure of Chomczynski and Sacchi (1987) described in Section II.2.1. The RNA was dried *in vacuo*, resuspended in SDW and the RNA content determined by measuring the absorbance at 260 nm.

VI.2.3 *Northern Blot*

Extracts of RNA from pooled mesenteric and caudal arteries as well as male mouse submaxillary gland RNA were subjected to Northern blot analysis (II.3). Aliquots of RNA extracts, and RNA size markers, were fractionated on a 1% agarose gel containing formaldehyde for 8 hrs at 60 volts in sterile 1xMOPS buffer (II.2.2).

The RNA was transferred by vacuum to a nitrocellulose membrane then baked for 2 hrs at 80°C *in vacuo*. The marker lanes of gels were separated from the rest of the gel and stained with EtBr to permit visualisation of the RNA size markers. The blots were analysed using a ^{32}P radiolabelled probe which has been previously demonstrated to be able to detect NGFmRNA (Chapter IV; Falckh et al, 1990).

VI.2.4 *Slot Blots*

Serial dilutions of total RNA from rat blood vessels (2 to 0.25 µg/100 µl) and male mouse salivary gland (200 to 100 ng/100 µl) were prepared in sterile microfuge tubes and applied to nitrocellulose membranes as described previously (II.4). The membranes were placed in sterile folders, air dried then baked for 2 hrs at 80°C *in vacuo*. The filters were then analysed for NGFmRNA using the hybridisation conditions described below.

VI.2.5 *Hybridisation*

The nitrocellulose membranes (either from Northern transfers or slot blots) were incubated for 6 hrs at 42°C in sealed plastic bags containing 0.1ml/cm^2 pre–hybridisation solution (II.7.1). After prehybridisation, sufficient ^{32}P nick-translated cDNA probe (II.6.1) was added to each bag to achieve a ^{32}P radioactive concentration of $1x10^6$ cpm/ml. The membranes were incubated for an additional 16 hrs at 42°C then washed several times in different stringent SSC solutions (II.7.3). The membranes were air-dried, covered in plastic wraps and placed on x–ray film in x-ray film cassettes with intensifying screens at -70°C (II.8). The films were developed (3

to 5 days) and the signals quantitated using a laser densitometer and the area under the curves analysed using an integration program. Using the integration program the samples were normalised to the signal generated by 100 ng of male mouse submaxillary gland RNA for each membrane.

VI.2.6 *Statistical Significance*

Values shown are the means (± SEM) for the number of measurements shown in the results. Statistical significance was determined using students t-test and analysis of variance with a p value less than 0.05.

VI.3 Results

The mesenteric tissue weight of the SHR was significantly lower at the intermediate age but significantly higher in the older pups (Figure VI.1a). The mesenteric weight/body weight ratio of WKY pups was significantly greater at 2 days of age but significantly lower at 43 days of age than their age matched SHR (Figure VI.1b). No statistical differences were observed for mesenteric tissue/body weight at the intermediate weight. Aortic tissue weights followed the same trend as seen for the mesenteric arteries being significantly smaller for SHR pups at 10 and 43 days of age (Figure VI.2). No statistical difference was found for the body or tissue weights between the 2 day old pups nor was there a statistical difference between the caudal tissue weights of these species.

The yield of RNA (µg/mg tissue) for each tissue are shown in Table VI.2.

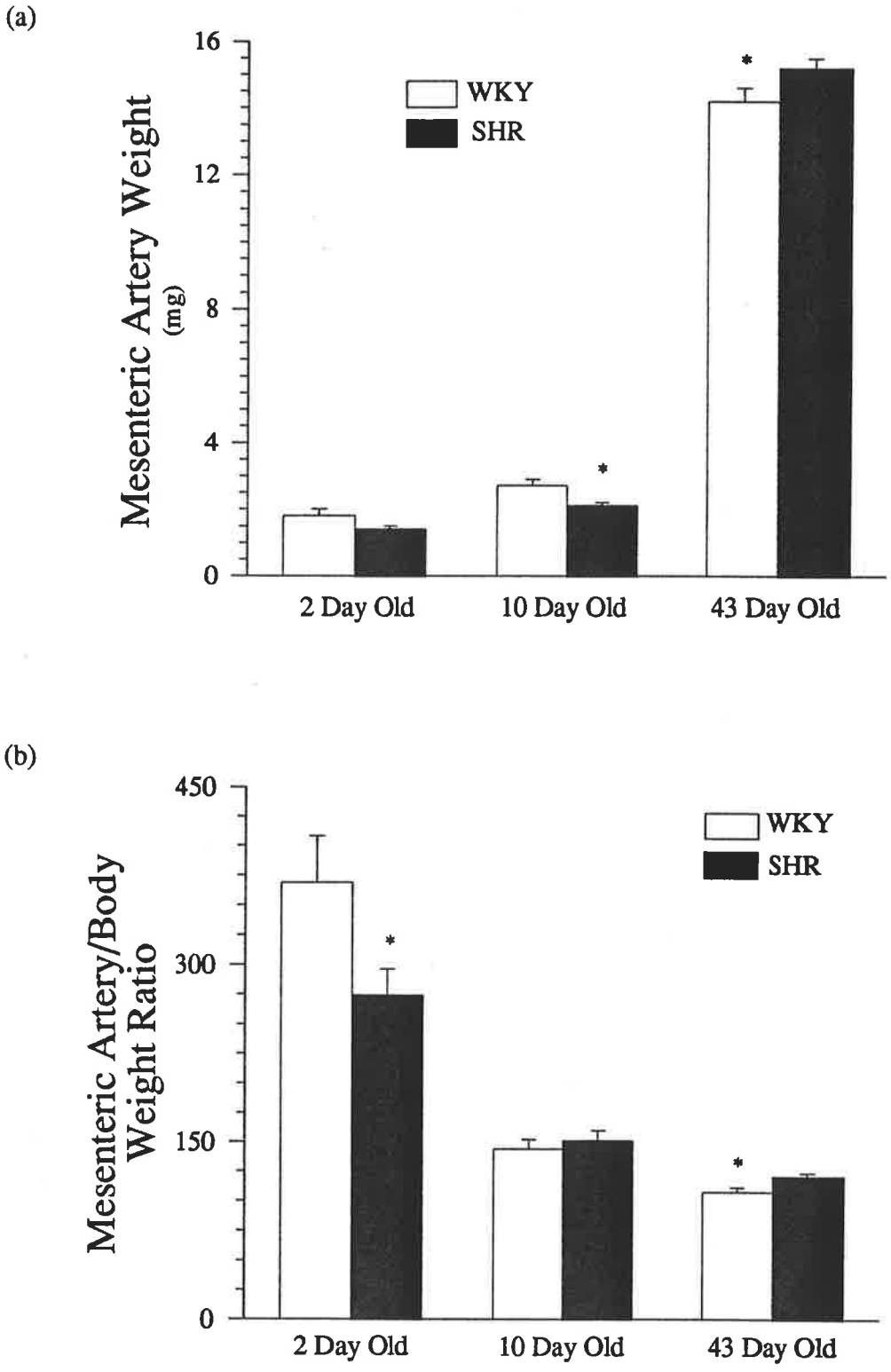


Figure VI.1 (a) Mesenteric artery tissue weights and (b) mesenteric artery-to-body weights of WKY and SHR rat pups at 2 (n=30), 10 (n=18) and 43 (n=12) days of age (* denotes significant differences between SHR and WKY, for each age group; Student's *t*-test, $p<0.05$).

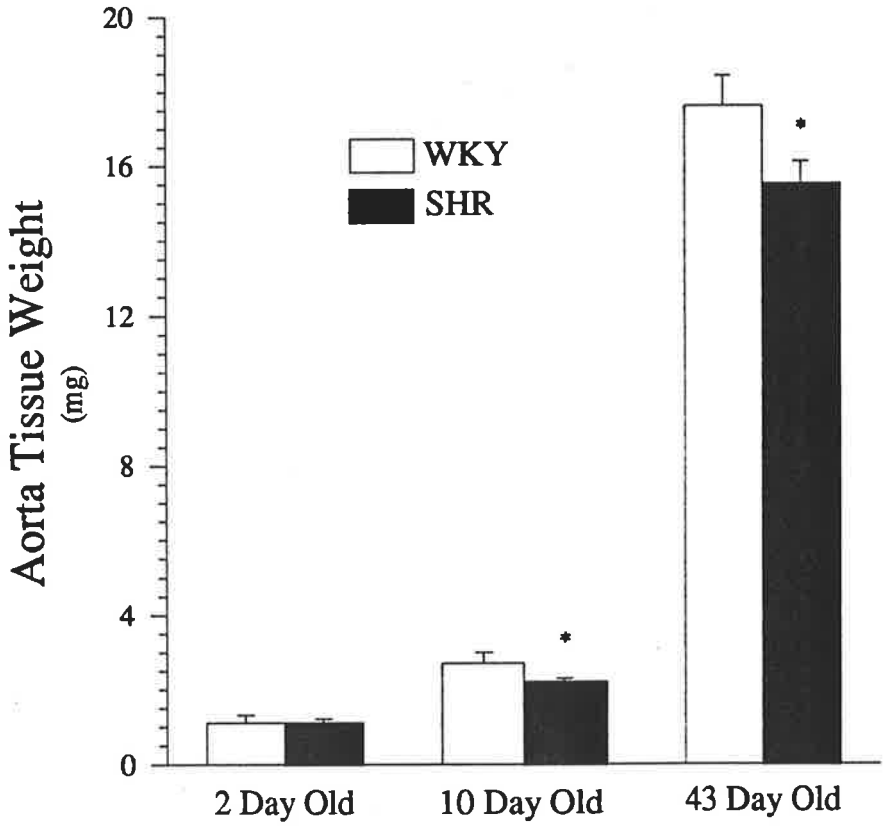


Figure VI.2 Aortic tissue weights of WKY and SHR rat pups at 2 (n=30), 10 (n=18) and 43 (n=12) days of age (* denotes significant differences between SHR and WKY, for each age group; Student's *t*-test, $p<0.05$).

Tissue	Species	RNA (µg/mg tissue)		
		2 Day Old (n=3)	10 Day Old (n=6)	43 Day Old (n=6)
Aorta	WKY	4.09±1.53	2.05±0.14	0.73±0.14
	SHR	4.87±0.39	1.76±0.12	1.06±0.09*
Mesenteric Artery	WKY	2.18±0.30	2.49±0.20	1.08±0.06
	SHR	4.21±0.07*	2.41±0.27	1.57±0.14*
Caudal Artery	WKY	-	-	1.10±0.04
	SHR	-	-	1.87±0.16*

Table VI.2

RNA yields (µg/mg tissue) of aorta, mesenteric and caudal arteries from WKY and SHR rat pups at 2, 10 and 43 days of age (* denotes significant differences between SHR and WKY yields; $p<0.05$, Student's *t*-test).

RNA extracted from SHR aortae were significantly greater than from WKY at only 43 days of age and both species showed a age-dependent decrease in total aortic RNA.

Total RNA derived from mesenteric arteries from SHR pups also demonstrated an age-dependent decrease in RNA and was significantly greater than RNA obtained from 2 and 43 day old WKY pups. No statistical difference was found for mesenteric artery RNA between 10 day old SHR and WKY pups (Table VI.2), nor was there a statistical difference between the RNA yields obtained for 2 and 10 day old WKY pups.

Aliquots of extracts of total RNA from mesenteric and caudal arteries separated on agarose gels and stained with EtBr showed no evidence of RNA degradation. Northern blot analysis of NGFmRNA in extracts of mesenteric and caudal arteries showed hybridisation of single bands at approximately 1.35 kb (Figure VI.3). The hybridisation bands for the rat blood vessels were similar to the single bands obtained from male mouse submaxillary glands which also migrated to the 1.35 kb region (Figure VI.3).

Slot blot analysis indicated that the concentration of NGFmRNA for mesenteric (at all ages; Figure VI.4a) and caudal arteries (43 day olds; Figure VI.4b) were greater in the blood vessels from the SHR rats when compared to the levels present in arteries from WKY rats. The greater concentration of NGFmRNA in these blood vessels from SHR rats was apparent regardless of whether the hybridisation signal was related to total RNA (data not shown) or alternatively to tissue weight. In the mesenteric artery the expression of NGFmRNA increased with age in vessels from SHR and WKY rats. In contrast, NGFmRNA in SHR aorta was significantly greater at the two younger groups but significantly lower at 43 days of age compared to age-matched WKY (Figure VI.5). The values for body and tissue weight, and NGFmRNA levels, are listed in the appendices (Appendices VII and VIII respectively).

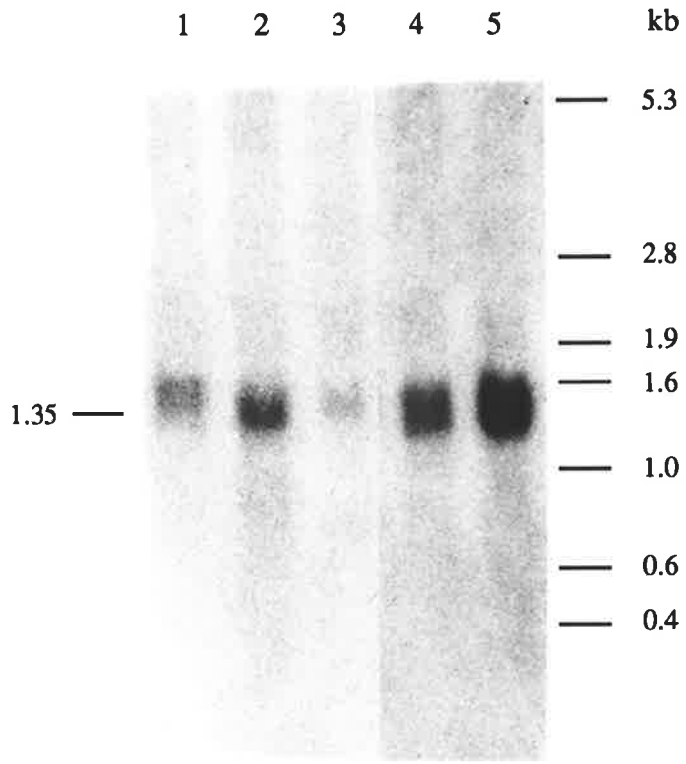


Figure VI.3 Northern transfer analysis of RNA fractionated on a 1% agarose gel of caudal (lanes 1 & 2) and mesenteric (lanes 4 & 5) arteries from 43 day old SHR (lanes 2 & 5) and WKY (lanes 1 & 4) rat pups. Mouse salivary gland RNA (lane 3) was used as a positive control for the hybridisation of the NGFcDNA. RNA size markers (fragments of linearized plasmids) in base pairs are noted on the right. A single band is seen for all tissues corresponding to 1350bp.

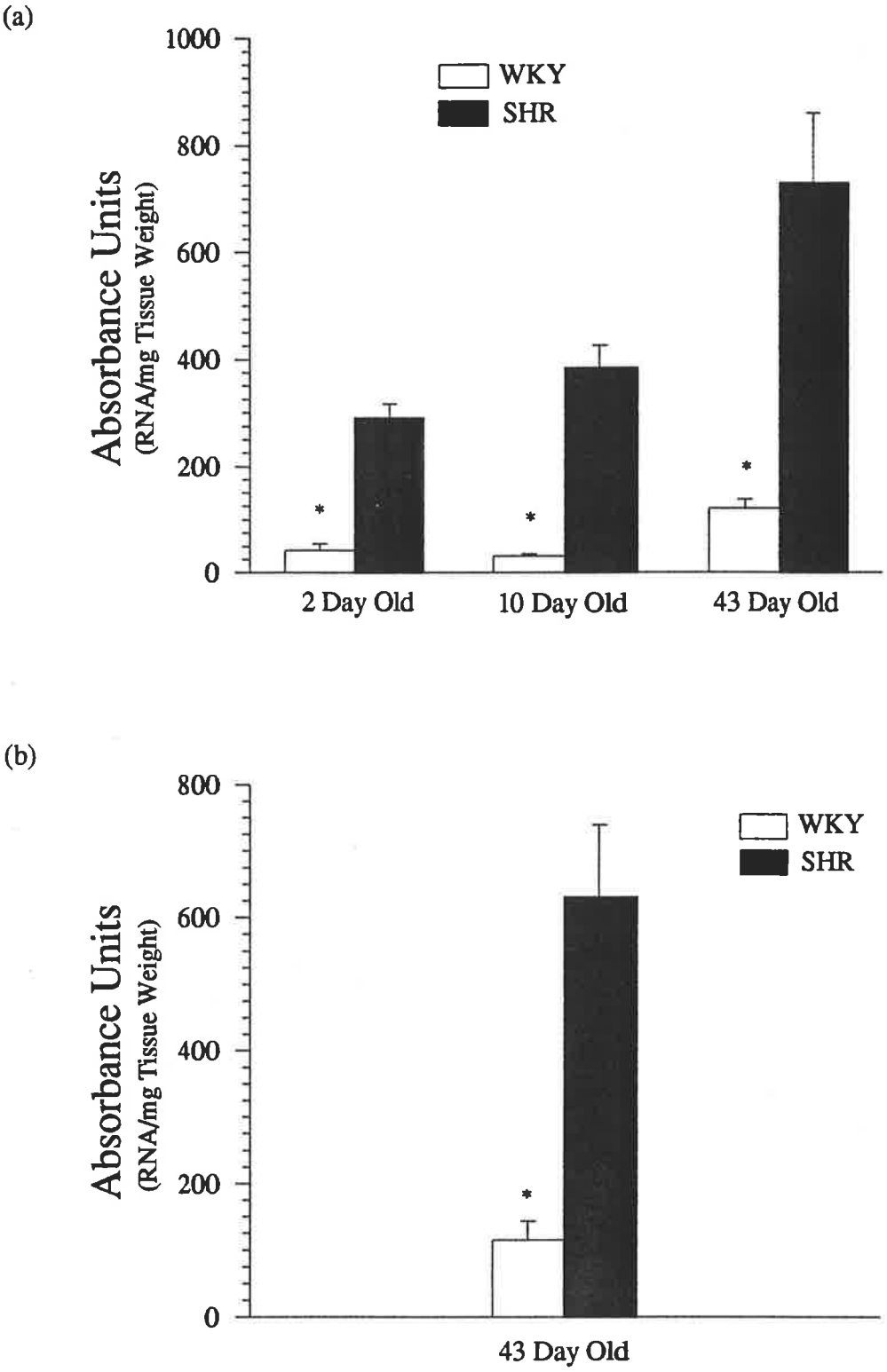


Figure VI.4 Slot blot analysis of NGFmRNA of (a) mesenteric and (b) caudal arteries from WKY and SHR rat pups at 2 (n=3), 10 (n=4) and 43 (n=6) days of age (* denotes significant differences between age groups, Kruskal-Wallis one way ANOVA by ranks; $p<0.05$). Slots were standardised to 100 ng of mouse salivary gland RNA.

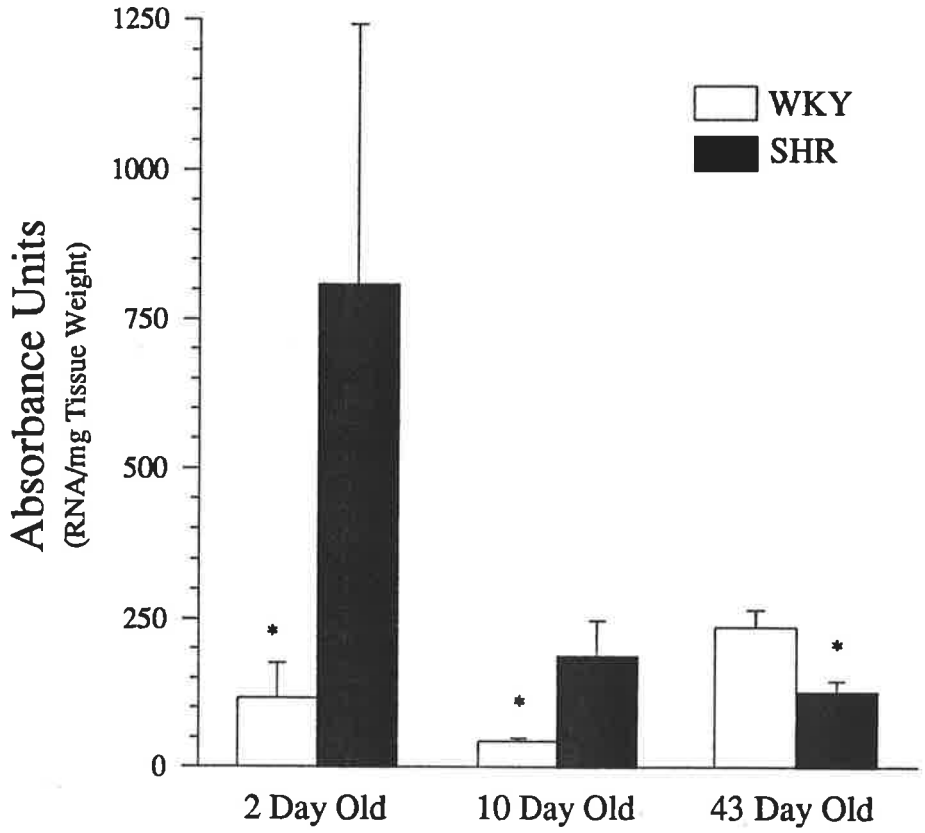


Figure VI.5 Slot blot analysis of NGFmRNA in aortae from WKY and SHR rats at 2 (n=3), 10 (n=4) and 43 (n=6) days of age (* denotes significant differences between age groups, Kruskal-Wallis one way ANOVA by ranks; $p<0.05$). The absorbance of slots were standardised to 100 ng of RNA from male mouse submaxillary salivary glands.

VI.4 Discussion

In the present study it has been demonstrated that NGFmRNA expression in extracts of mesenteric and caudal arteries, as well as aorta, can be determined by dot blot analysis. This was based on the observation that extracts of RNA from the blood vessels were not degraded and that Northern blot analysis displayed single hybridisation bands, at 1,350 bp, identical to that seen with RNA extracts from the male mouse submaxillary gland (Scott et al, 1983; Falckh et al, 1990). The findings in the present study provide evidence that NGFmRNA levels are substantially elevated in the mesenteric vasculature from SHR rats as early as 2 days of age and persist for at least 6 weeks. Elevated NGFmRNA, of the same magnitude as seen for mesenteric arteries, was also observed in the caudal arteries of SHR pups at 6 weeks of age. Aorta, a tissue not known for being highly sympathetically innervated, did show elevated NGFmRNA prior to 2 weeks of age. A dramatic decrease in NGFmRNA content was observed from birth such that at 6 weeks of age WKY rats displayed a greater level of NGFmRNA than SHRs of the same age. The large decrease in NGFmRNA, and presumably an equivalent decrease in NGF production, in the aorta between 2 and 10 days may be responsible for the paucity of sympathetic innervation seen in this tissue. It has been demonstrated repeatedly that the NA content of mesenteric and caudal arteries is elevated in the SHR and this increase is a consequence of a greater sympathetic innervation of the vasculature of the SHR rat (Head, 1989; Berkowitz and Spector, 1976; Berkowitz et al, 1980; Cassis et al, 1985; Donohue et al, 1988). It was suggested that an early appearance of an enhanced sympathetic innervation in the SHR may be a significant contributing factor to the development of hypertension in the SHR (Donohue et al, 1989). The enhanced sympathetic innervation (as reflected by NA

content) demonstrated in the SHR as early as 2 days of age (Donohue et al, 1988) is consistent with the elevated level of NGFmRNA found in this study. In line with the enhanced innervation and NGFmRNA demonstrated in neonatal SHR is a significant increase in blood pressure compared to WKY pups (Donohue et al, 1988).

The significant decrease in mesenteric vascular tissue weight at 10 days of age would appear, at first hand, to be a result commensurate with the attenuation in body weight. However it was observed that the branching of secondary vessels from the superior mesenteric artery (SMA) of the SHR was less than that seen in the WKY and this maybe attributable to the decrease in vascular tissue. After day 10 the mesenteric vascular tissue weight dramatically increased such that 43 day-old SHR vasculature was significantly greater than age-matched WKY. Changes in vascular morphology at this age are clearly evident with an increase in medial smooth muscle content (Lee et al, 1983a,b; Lee, 1985; Mulvany, 1985, 1989) and it is most probably this hyperplastic change that accounts for the significant changes in tissue weight. In association with the vascular changes at 43 days of age the NA content of mesenteric arteries from SHR and WKY rats reach their highest values indicating that the process of sympathetic innervation is complete at that age (Donohue et al, 1988). At an intermediate point of peripheral sympathetic development (viz; 20 days of age) the NGF content of mesenteric arteries from SHR's was found to be greater than that present in mesenteric arteries from WKY rats (Donohue et al, 1989). The present study demonstrates that the levels of NGFmRNA present in mesenteric arteries from SHR rats are also elevated, when compared to the values seen in arteries from WKY rats. This increase occurs during the normal time course of sympathetic innervation of the mesenteric vascular bed and differences were 7-fold at 2 days of age, 12-fold at 10 days of age and 6-fold at 43 days of age.

VSM cells from the SHR show a greater response to growth factors (Hamet et al, 1988) and proliferate more *in vitro* (Michel et al, 1990) than do VSM cells from WKY rats. In conjunction with the findings that catecholamines can stimulate NGF in fibroblasts (Furukawa et al, 1989) and SMC (Yamori et al, 1981) a hypernoradrenergic state, as seen in the SHR, would lend itself to a self promoting production of NGF and growth of vascular SMC. The interaction of VSM and innervation, initially suggested by Head (1989), is proposed to be responsible for the increase in both VSM and innervation, given that the system has an initiation point. The fact that NGFmRNA is elevated in 2 day old SHR rat pups seems to be a factor relevant to the hypernoradrenergic state, and elevated blood pressure, of adult rats. It has been established that the sympathetic innervation of the caudal artery in the SHR is also greater than that seen in caudal arteries from WKY rats (Cassis et al, 1985). Consistent with this finding is the observation that the concentrations of NGFmRNA present in caudal arteries from SHR rats were approximately 5-fold greater than those present in arteries from WKY rats.

It is conceivable that an early over production of NGF (Donohue et al, 1989), due to an enhanced vascular expression of NGFmRNA (Head, 1989), plays a pivotal role in development of the pathophysiology of hypertension in the SHR model. This conclusion is viewed as important for several reasons. Firstly, the results of the present study now provide a link between an enhanced level of NGFmRNA, an enhanced concentration of NGF and the appearance of vascular hypernoradrenergic innervation in the SHR. Secondly, recent clinical studies have described an enhanced production of the principal presynaptic metabolite, dihydroxyphenylglycol (DOPEG), in human essential hypertensives which can be interpreted as reflecting an increased noradrenergic innervation of vascular tissue (Ludwig et al, 1991). It follows that the results of our

present study now provide the stimulus for the analysis of the role of nerve growth factor and particularly its gene expression in the development of human essential hypertension.

The fact that morphometric changes are absent in neonatal SHR when compared to age-matched WKY pups (Lee et al, 1988) and that these young animals do not demonstrate elevated BPs at birth makes it difficult to determine how the increased NGFmRNA production comes about. In contrast to the findings of Lee et al (1988) are those that have found significant changes in neonatal SHR BP (Gray, 1984) suggesting that some changes may occur prior to parturition. The questions that arise from this are; (1) whether polymorphism of the NGF gene exists in the SHR which may result in overproduction of NGF, (2) does the mother produce some hormonal factor at, or near parturition, that results in an early overproduction of NGFmRNA, or (3) whether some particular cell type that is able to produce NGF within the vasculature, other than VSM, is sufficiently elevated in number to account for the elevated NGFmRNA seen in 2 day-old SHR rat pups ? These questions have yet to be answered and present several avenues for further investigations into how NGFmRNA is elevated in some tissues that are genetically committed to an increased innervation.

It is interesting to note that fibroblasts, known for their ability to produce NGF (Furukawa et al, 1986a,b; Furukawa et al, 1989), as well as other growth factors, exist in a closer anatomical relationship to adrenergic varicosities than do VSM cells (Soares-da-Silva and Azevedo, 1985). It is therefore not inconceivable to consider that a small increase in fibroblastic cell number would result in a greater production of NGF and ultimately lead to an increase in innervation. An increased overflow of NA commensurate with an increased innervation would then be self-reinforcing by further elevating NGF production by fibroblast cells. The inclusion of fibroblast in the

feedback system described by Head (1989) would result in a self-reciprocating system (Figure VI.6).

The number of fibroblast cells found in the adventitia from small mesenteric vessels has been found to be significantly elevated in 10-12 week-old SHR rats compared to WKY rats (2.17±0.18 and 1.64±0.22 respectively) as have the number of fibroblasts found in the large mesenteric artery (16.24±0.8 and 8.99±0.9 respectively)(Lee et al, 1983b). In contrast no statistical difference was found in adventitial fibroblast cell number in the superior mesenteric artery of SHR and WKY rats. This is consistent with findings that the smaller branches of the mesenteric vascular bed display hypernoradrenergic innervation and not the superior mesenteric artery itself (Head, 1991). 'Fibroblast layers' (undifferentiated cell layers) in large mesenteric arteries from neonatal SHRs were larger but not significantly different to neonatal WKY pups (5.59±0.26 and 4.91±0.34 respectively) (Lee et al, 1988) while fibroblast cell number followed the same trend seen in 10-12 week old rats (1,228±34 and 988±51 respectively). No investigation of fibroblast cell number in small mesenteric arteries or vessels has been done to date and may possibly reflect changes seen in older animals (10-12 week-old; Lee et al, 1983b). If the number of fibroblast cells found in the small mesenteric vessels are of the same magnitude as that seen in older (10-12 week-old) rats then the involvement of this cell type in the overproduction of NGF is not only feasible but possible. *In situ* hybridisation of the neonatal mesenteric vasculature with radiolabelled cRNA or cDNA NGFmRNA probes of both the SHR and WKY rats would demonstrate which cells are responsible for the production of NGF.

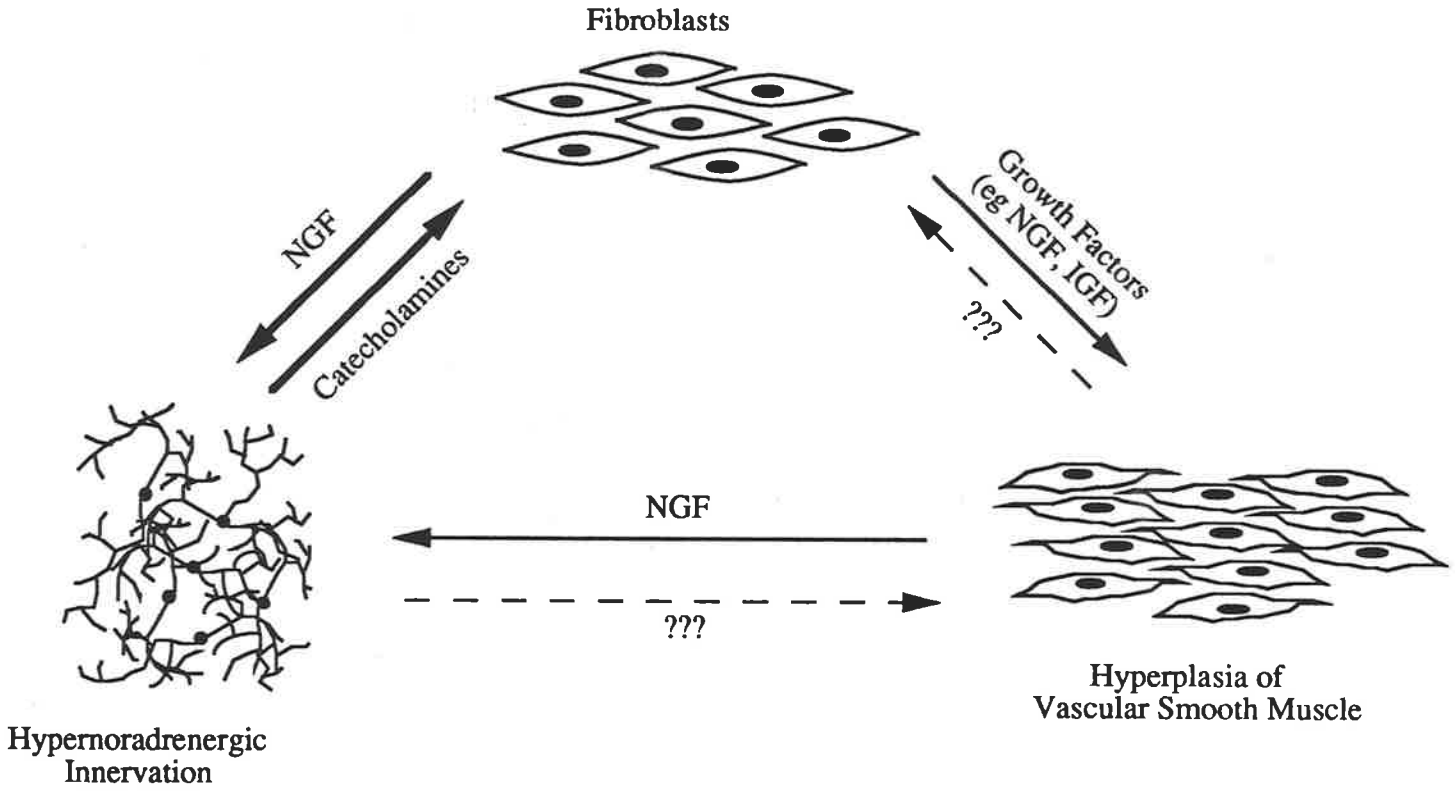


Figure VI.6 The postulated intervention of fibroblasts, stimulated by NA, initiating NGF production. VSM cells also produce NGF in attracting neurite outgrowths. Growth of VSM is suggested to be stimulated by growth factors from fibroblasts and whatever contribution from the increased innervation eventuating in the mesenteric vasculature. A small increase in SHR fibroblast number would result in an elevated NGF production and sympathetic neurite outgrowth.

CHAPTER VII

TISSUE NORADRENALINE CONTENT IN MALE AND FEMALE F1 HYBRID RATS

VII.1 Introduction

The hypertensive disease state seen in the SHR is associated with an increased degree of sympathetic innervation, increased heart weight, enhanced sensitivity of VSM and an increased peripheral resistance when compared to the normotensive, and genetic control, WKY. Significant changes in vascular wall structure have also been associated with the disease state in the SHR and includes an increase in media/luminal cross-sectional area and a decrease in the luminal cross-sectional area that leads to the increased resistance and elevated BP. The sympathetic nerve activity in the SHR is also markedly increased and pharmacological intervention of the SNS in the SHR leads to a greater decrease in BP than that seen in the WKY. In the previous chapters it has been shown that the SHR also has a higher NGFmRNA level in vasculature that display hypernoradrenergic innervation but not in the heart or aorta, tissues which do not display enhanced innervation.

Although the aforementioned differences between the SHR and WKY have been extensively investigated there is still some question as to whether these differences are a cause or a consequence of hypertension and whether they are species

differences that exists between the SHR and WKY that are unrelated to the development of hypertension. Investigations of the inheritability of specific traits from SHR and WKY rats has been studied in F_2-generation of hypertensive/normotensive rats (Tanase et al, 1970; Judy et al, 1979; Mulvany and Korsgaard, 1983; Mulvany, 1987,1988) and has shed some light on whether the differences between SHR and WKY cosegregate with hypertension. The increased calcium sensitivity seen in the SHR has been shown not to be correlated with BP in the F_2 hybrids (Mulvany, 1987, 1988) while there is a positive correlation between BP and SNA, and the development of hypertension (Judy et al, 1979).

In all studies of F_2 generations, and in some instances studies of more distantly outbred generations, there is a significant shift in the mean BP of hybrid rats. The BP profile of F_1, and successive backcross rats, displays an attenuation of the mean BP and an increase in the standard deviation of each progressive cross (Tanase et al, 1970; Judy et al, 1979; Mulvany and Korsgaard, 1983; Mulvany, 1987,1988). In contrast to the decreasing mean BP seen with successive hybrids of backcross, and outbred F_1/WKY rats, a backcross of the F_1-generation with the SHR will produce a BC_2 hybrid with an increased mean BP with an associated increase in the variation of the population (Tanase et al, 1970).

While various aspects of hypertension have been studied in the hybrid hypertensive/normotensive rats there appears to be a paucity of investigations into how the hypernoradrenegic innervation seen in the SHR is affected by outbreeding with the WKY strain. The outbreeding of the SHR, with the WKY, would be expected to alter (decrease) the degree of sympathetic innervation seen in the F_1 population, consistent with the reported decrease in BP found by previous investigators. On the basis that NA is a means to measure the degree of innervation in cardiovascular tissue it was

decided to analyse the NA content of the F_1 hybrid from a SHR sire/WKY dame cross. Analysis of NA content and BP were to be conducted in both the male and female progeny of the SHR/WKY cross. The purpose of this study was that if clear cut changes in vascular NA content were seen with the backcross population then subsequent experiments would be conducted to measure changes in the NGFmRNA content, in the vasculature, of the backcross.

VII.2 Methods

VII.2.1 *Animals*

The animals were bred at the barrier colony of the CSIRO, Division of Human Nutrition, Adelaide, Australia. The F_1 hybrid rats were developed as follows: 3 hypertensive male inbred SHRs were mated to 6 normotensive female inbred WKY rats that were randomly selected from the breeding stock of the colony. The F_1 hybrids were weened at four weeks and transferred to the CSIRO facility situated on the University of Adelaide campus. The male and female hybrids were housed separately and were fed standard chow and water ad libitum. At the end of the breeding program the SHR and WKY parents, as well as 3 litter mates of the SHR sires, were also transferred and housed at the University facility.

VII.2.2 *BP Analysis*

The systolic blood pressure (SBP) in conscious unanaesthetised rats were measured by the tail-cuff plethysmographic method (Yong et al, 1992). The rats were

weighed then placed in a perspex restrainer and an inflatable cuff placed on the proximal end of the tail. The inflation of the cuff was automated (IITC Inc., USA; Pulse Amplifier and Cuff Pump) and incorporated a doppler photocell to measure blood flow. The rats were placed in a flow cabinet that was maintained at 32°C.

The animals were habituated to the inflation and deflation of the cuff during the first week. Weekly BP readings were conducted on male and female F_1 rats for 18 weeks at which time the rats were sacrificed and the required tissues removed. At least 6 BP measurements were made on each occasion and the average of the last 4 readings taken as the SBP estimate for that week. The BP of the SHR and WKY rats were habituated as above and the SBP estimate of these rats were determined on two successive days after which the animals were sacrificed and the tissues removed.

VII.2.3 *Tissue Collection*

The rats were sacrificed by initially stunning, followed by decapitation. The mesenteric artery, caudal artery and heart were removed, placed in ice-chilled ascorbic-saline and cleaned of adhering extraneous tissue. The tissues were blotted dry, weighed, individually wrapped in foil and frozen in liquid nitrogen. The frozen tissues were stored at -70°C until required. The vas deferens were removed from all male rats and treated as described above.

VII.2.4 *Catecholamine Analysis*

Tissues were homogenised in a motor driven Wheaton glass/glass homogeniser with 0.4M perchloric acid (PCA) containing 50 ng/ml of 3,4-dihydroxybenzylamine

(DHBA) as an internal standard. Individual mesenteric arteries, caudal arteries, hearts and vas deferens (pairs) were homogenised in 0.4M PCA. The homogenate was transferred to a 6 ml tube and the homogeniser rinsed with a small quantity of 0.4M PCA that was added to the initial homogenate; the tube was maintained on ice.

The tubes were centrifuged in a bench centrifuge (MSE) for 1 min at 3000rpm. An aliquot of the homogenate (500 µl) was transferred to a 750 µl auto-analyser cup, the pH adjusted to 2 with 45-85 µl of 2M Tris. The samples were analysed using a modification of the HPLC-ECD (high performance liquid chromatography in conjunction with electrochemical detection (LKB, Bromma, Model 2143)) technique described by Stitzel et al (1983). The modified technique incorporated an automated enrichment of the injected sample on an alumina column prior to determination by the standard HPLC-ECD method.

The catecholamines were injected (Kortec K65 auto-injector) onto the alumina cartridge and bound to the alumina in the presence of a 0.5M phosphate buffer (pH 8.0) containing 100 mg/ml EDTA; mobile phases were delivered by a Kortec K25D pump. An automated valve switched the mobile phase to a Tris/citric acid buffer (6.06 g Trizma base, 10.6 g citric acid, 100 mg EDTA, 50 mg octane sulphonic acid and 40 ml methanol per litre; pH 3.6) that eluted the catecholamines onto a C_{18} reverse phase column (5µm Bondapak silica). The catecholamine content in the samples were determined by comparison with standards comprising of NA and DHBA.

VII.2.5 *Statistical Analysis*

All data was subjected to statistical analysis using the Student's *t*-test with a significance difference of at least $p<0.05$.

VII.3 Results

The SBP values of the SHR sires and the WKY dames were 182±3 and 131±2 mmHg, respectively. The F_1 male SBP showed a steady increase from week 5 and the group achieved a SBP not statistically different from the SHR sires' (181±4 mmHg). In contrast, the female F_1 population achieved a SBP (146±4 mmHg) significantly higher ($p<0.05$) than that found in the WKY dames but significantly lower than both the male rat groups (Figure VII.1). The SBP of the female F_1 rats appeared to plateau at 13 weeks, a week earlier than that seen in the male F_1s.

The body and heart weight of F1 males was significantly greater than F_1 females (Table VII.1), however, the heart/body weight ratios were not significantly different between the two F_1 sexes (3.47±0.07 and 3.56±0.04 respectively). The SHR sires also demonstrated greater body and heart weights than the WKY dames. The weight of the mesenteric vasculature of the F_1 males was significantly greater than the F_1 females ($p<0.05$) and the weights of the SHR and WKY parents followed the same trend (Table VII.1). The mean F_1 male mesenteric vascular weight was greater than, but not statistically different from, the SHR sires. In contrast female F_1 mesenteric vasculature was significantly smaller ($p<0.05$) than both the SHR and WKY parents' vasculature (Table VII.1). The weight of the vas deferens of the SHR and F_1 rats were not significantly different.

Heart NA content (μg/g) was not significantly different between any of the groups (Table VII.2). The NA content of F_1 caudal arteries were not significantly different from each other nor were they significantly different from the NA content found in either parent group (Table VII.2).

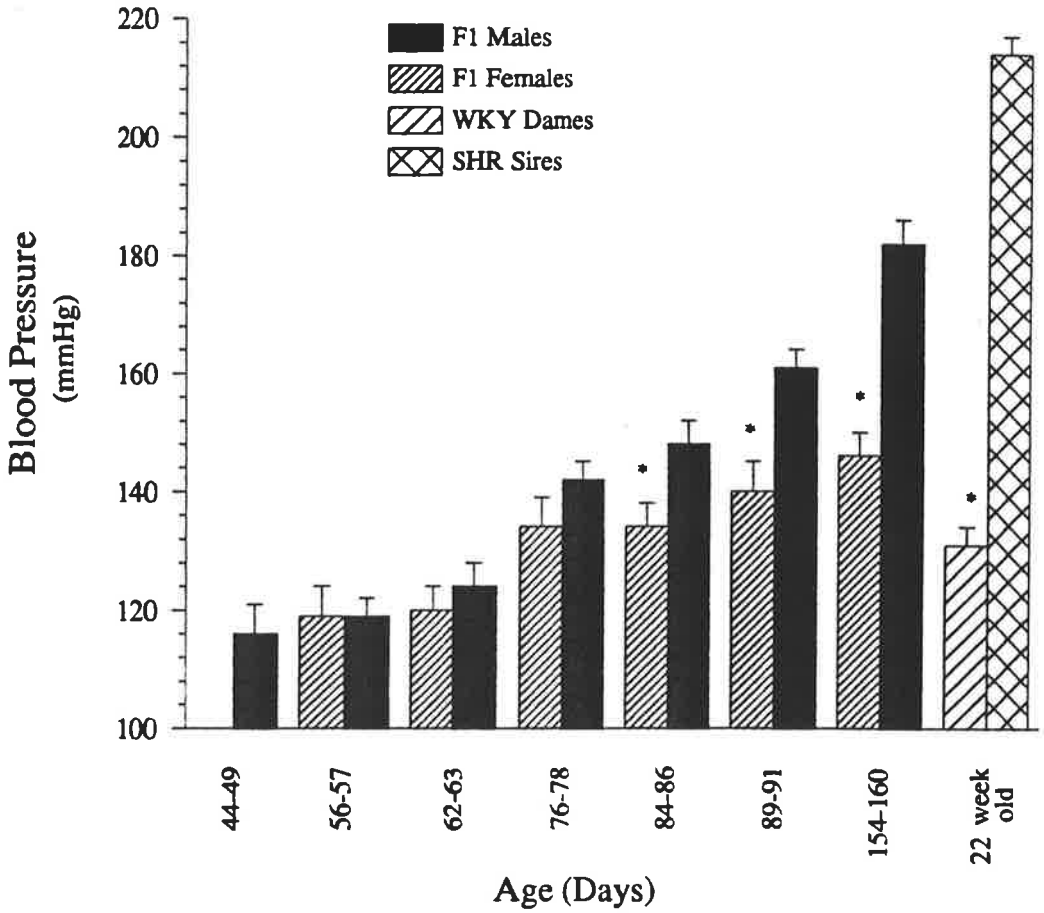


Figure VII.1 Blood pressure (BP) values (mean±SEM) of F_1 males (n=25) and females (n=20) from 4 to 22 weeks. The BP of the SHR and WKY parents is also shown (* denotes significant differences between F_1 male and female, or SHR males and WKY females).

Tissue Type	Animal Strain			
	SHR ♂	WKY ♀	F_1 ♂	F_1 ♀
Body Weight (g)	-	-	436±5	266±2*
Heart/Body Weight Ratios	-	-	0.347±0.007	0.356±0.004
Heart (g)	1.49±0.04	1.09±0.04*	1.50±0.03	0.96±0.01*
Caudal Artery (mg)	25.30±1.30†	17.60±0.70*,†	33.28±0.80	21.30±0.40*
Mesenteric Artery (mg)	31.10±1.50†	18.90±0.80*,†	34.60±2.80	14.70±0.03*
Mesenteric Artery/Body Weight Ratio	-	-	8.97±0.74	5.53±0.21*
Vas Deferens (mg)	211.70±9.10	-	200.30±3.20	-

Table VII.1 Body and tissue weights of SHR (♂, n=6), WKY (♀, n=6) and F_1 (♂, n=25 & ♀, n=20) hybrid rats. The heart-to body weight and mesenteric artery-to-body weight ratios is also listed (* denotes significant differences between SHR and WKY, or F_1 males and F_1 females; † denotes significant differences between SHR and F_1 males, or WKY and F_1 females; Student's *t*-test, $p<0.05$).

Tissue Type	NA Concentration (µg/g)			
	SHR ♂	WKY ♀	F_1 ♂	F_1 ♀
Heart	0.60±0.04	0.56±0.02	0.55±0.04	0.51±0.02†
Caudal Artery	7.74±0.31	7.87±0.34	7.67±0.17	7.20±0.26
Mesenteric Artery	6.51±0.91	-	6.51±0.20	3.82±0.23*,†
Vas Deferens	8.18±0.38	-	7.09±0.18	-

Table VII.2 NA concentrations (µg/g tissue weight) of tissues from SHR (♂, n=6), WKY (♀, n=6) and F_1 (♂, n=20 & ♀, n=16) hybrid rats (* denotes significant differences between F_1 males and F1 females; † denotes significant differences between SHR and F_1 females, or SHR and F_1 males; Student's *t*-test, $p<0.05$).

Mesenteric vascular NA content was not significantly different between SHR and F_1 males although the NA content of the F_1 females was significantly lower than both male groups. In contrast, the SHR vas deferens contained a significantly greater amount of NA than that seen in the F_1.

VII.4 Discussion

The BP of WKY females was significantly lower than SHR males and was consistent with the BP generally seen between SHR and WKY. In two studies (Tanase et al, 1970; Judy et al, 1979) the F_1 male population displayed a lower mean SBP than that seen for the SHR sires and in the former study this trend was also observed for F_1 females. In both studies the BP of the F_1 population was greater than that of female WKY. The F_1 male population of the present study was not consistent with these findings as the SBP of this group was not smaller than that found in the SHR sires. The SBP of F_1 females however were as expected and were intermediate to that of adult SHR and WKY rats and statistically different from both parent groups. A consequence of increased BP is the observation that cardiac tissue undergoes hypertrophy and there is an increase in the heart-to-body weight ratio (see I.2.1.2). The heart-to-body weight ratios in F_1 females was statistically indistinguishable from that of F_1 males and it appears that the 11% increase in BP, in the F_1 females, was sufficient to cause cardiac hypertrophy in these animals, demonstrating that cardiac hypertrophy is a consequence, and not a cause, of elevated BP.

In several studies it has been demonstrated that male SHR and WKY rats do not display a difference in the concentration of NA content found in cardiac tissue (Louis et al, 1969; Nakamura et al, 1971) or at least that the content in SHR

myocardium is smaller than that found in the WKY (Howe et al, 1979). The data of the present study is consistent with these findings in that the NA concentration in SHR male hearts was found to be indistinguishable from that observed in female WKY hearts. Moreover, there was no difference between the NA content of hearts from F1 males and females, and WKY females. Thus an increase in BP, and a consequential increase in cardiac mass, is not associated with a change in cardiac sympathetic innervation.

The SHR caudal artery content of NA was not different to that found in the female WKY caudal artery tissues. This was not initially expected as it has been previously demonstrated that the caudal artery of the male SHR has approximately twice the NA content as found in the same artery of the male WKY (Cassis et al, 1985; Donohue et al, 1987). The NA concentration of the caudal artery of all animals was similar to that found in the SHR of previous studies (Cassis et al, 1985; Donohue et al, 1987). The inability of this study to detect a difference between male SHR and female WKY caudal artery NA content remains puzzling. It should be noted that the NA content of female SHR rats is also greater than the NA content of female WKY rats (Yong et al, 1992).

The results of experiments on the mesenteric vasculature raises several questions. The weight of the male mesenteric vasculature (F_1 and SHR) was significantly greater than that seen in the female mesenteric vasculature (F_1 and WKY) and this difference was consistent when considering the mesenteric weight/body weight which was again significantly greater in the F_1 males (8.97±0.74) than in the F_1 females (5.53±0.21). The NA concentration in F_1 female mesenteric vasculature was smaller than that found in either of the male populations. In contrast, there was no difference between SHR and F_1 male NA concentrations in the mesenteric vasculature.

The NA concentrations in WKY female mesenteric arteries were found to be greater, but not statistically different, than that of the SHR males. This result is not consistent with the findings in the F_1 female population and remains unexplained (other than a possible experimental error). The NA concentrations in male mesenteric artery tissues is consistent with the concentrations found by Head et al (1985) but several fold smaller than that found by Donohue et al (1988).

The failure to attenuate the BP in F_1 males, as well as the unaltered mesenteric sympathetic innervation, demonstrates that the disease state of the male SHR was not significantly altered by outbreeding with the normotensive WKY; in this series of experiments. The hypertrophy seen in the F_1 male hearts was consistent with elevated BP in SHRs and the only evidence for an altered sympathetic innervation in the F_1 male population was that the NA content was significantly decreased in the F_1 population.

The F_1 female data does little for the understanding of the inheritability for the disease state other than to raise more questions as to how is the sympathetic innervation in females different from that seen in the male? The increase in the BP of F_1 females, in conjunction with the hypertrophic changes of the heart, were consistent with the inheritability of hypertension despite the fact that Ely and Turner (1990) have suggested that hypertension in the SHR is linked to the Y chromosome. The Y chromosome linkage was proposed when it was demonstrating that the BP of male F_1s from a SHR and WKY cross was greater when the sire was a SHR rather than a WKY; BP of females were not investigated in their study. The inability to find a difference between the mesenteric and caudal artery NA content in F_1 male and female tissues also raises some question as to the degree, and involvement, of hypernoradrenergic innervation in the initiation of the hypertensive state. Previously

the inheritability of the disease state in the SHR has been attributed to 3 major genes (Yamori, 1984) and the data from the current study suggests that the inheritance of the disease state is not a simple linear function.

The paucity of investigations into the sympathetic innervation of female rats, and of female rats in general, makes interpretation of the data of the current study difficult. It is conceivable that sex differences will result in a completely different pattern of sympathetic innervation and it is necessary to further investigate sex differences within strains as well as across strains, before the inheritability of the disease state of hypertension can be fully realised.

Unfortunately, the failure to isolate the enhanced innervation and the development of hypertension with the backcross breeding indicated that it was not worth pursuing the expression of NGF in this type of experiment. That is not to say that the next step in this line of investigation should be linkage analysis.

CHAPTER VIII

GENERAL CONCLUSIONS

As indicated in Chapter I the principle aim of this study was to determine if the gene expression of NGF (NGFmRNA) is enhanced in blood vessels that display hypernoradrenergic innervation in the SHR. The study investigated NGFmRNA concentrations in both the SHR and WKY rat strains for a period of 6 weeks, commencing at 2 days of age. The use of a specific cDNA probe for NGF, together with suitable techniques for isolating mRNA, indicated that NGFmRNA, in SHR pups, was elevated in the mesenteric and caudal artery, as well as the mesenteric vein, when compared to aged-matched WKY rat pups. These elevated levels were observed as early as 2 days of age and demonstrated an age-dependent increase that persisted to 6 weeks of age. The expression of NGF was also found to be elevated in the SHR kidney from 2 days of age but the profile of the expression of NGF over the 6 week period was somewhat different and did not show an age-dependent increase. The SHR kidney did however maintain an elevated level of NGFmRNA when compared to normotensive controls. In contrast to the aforementioned tissues NGFmRNA concentrations were not elevated in the SHR heart or aorta.

The significance of these findings are threefold. Firstly the pattern of expression of NGF, at least in the initial 6 weeks of development, parallels the

intensity of innervation in the SHR when compared to that seen in the WKY. For example, the mesenteric artery, caudal artery, mesenteric vein and kidney all have demonstrated larger NA content in these tissues from the SHR when compared to levels found in the WKY (Table VIII.1). In contrast, NA content in the SHR heart has been shown to be either the same as, or less than, that found in age matched WKY heart. Moreover, the SHR heart does not show an elevated NGFmRNA concentration but rather a decreased NGFmRNA level when compared to that seen in the WKY (Table VIII.1). Secondly, the work of Donohue et al (1989) shows an increased concentration of the NGF peptide in the mesenteric artery of young (20 day old) SHR pups. In the present study it was shown that there was an increase in NGFmRNA suggesting that the increase in peptide seen by Donohue was in fact due to an enhance expression of the messenger RNA for the peptide. Thus, in those tissues found to display an elevated level of NGFmRNA it would be expected to find an elevated level of NGF that would result in those tissues having an increased sympathetic innervation (Table VIII.1). Thirdly, tissues that display hypernoradrenergic innervation had NGFmRNA concentrations that were maintained at an elevated level for several weeks. In contrast, NGFmRNA concentrations in aortae from 10 day old SHR and WKY pups were attenuated by 77% and 63% respectively from those seen at 2 days of age. This dramatic decrease in NGFmRNA level was also seen in the heart; 57% and 65% respectively for SHR and WKY pups. These data suggest that a sustained, elevated NGFmRNA, and not an increase per se, is required in tissues to display hypernoradrenergic innervation.

The strength of the findings lie in the ability of the cDNA probe to detect specific messenger RNA for NGF. The sequence obtained was that of Scott et al (1983) that has been used to detect NGFmRNA levels of the pro molecule of NGF in

Tissue	SHR compared to WKY				
	NA content	NGF peptide	NGFmRNA		
			2 DO	10 DO	43 DO
Mesenteric Artery	↑↑	↑↑	↑↑	↑↑↑	↑↑
Mesenteric Vein	↑	-	-	-	↑↑↑↑
Caudal Artery	↑↑	-	-	-	↑↑
Aorta	↑		↑↑	↑	↓
Kidney	↑↑	-	↔	↑↑	↑↑↑
Heart	↔ or ↓	-	↓	↓	↓

Table VIII.1 The above table displays differences between tissue content of noradrenaline (NA), nerve growth factor (NGF) and messenger RNA for NGF (NGFmRNA). The arrows represent increased (↑), decreased (↓) or equivalent (↔) levels between SHR and WKY rats.

a variety of different tissues, both from tissue cultures and the intact animal. In the present study the reliability of the probe was examined by pharmacologically manipulating the submaxillary salivary gland from the male mouse. As discussed in Chapter 4, the message for NGF was found to be larger in the male salivary gland than that seen in the female submaxillary salivary gland, as was the concentration of the peptide. Depleting the male mouse of its source of testosterone (by castration) resulted in a decrease in total body weight, weight of the submaxillary salivary gland, total RNA, NGFmRNA and NGF. Supplementation of the androgen with exogenous testosterone was sufficient to elevate all the aforementioned changes to that of control (untreated) male mice. These data provided convincing evidence that the cDNA probe was reliable in detecting NGFmRNA levels, even in tissues that had significantly diminished levels of NGF and NGFmRNA.

The NGFmRNA study presented contains two possible shortfalls. The first is that while the relationship between NGFmRNA, NGF production and the development of hypertension is indicated, the fact is not proven. A thorough examination of this relationship would involve linkage analysis where the loci of the gene for NGF is clearly shown to cosegregate with hypertension. The possibility thereby remains that the greater production of NGF and NGFmRNA seen in the SHR is simply a feature of the SHR that is unrelated to the development of hypertension. Unfortunately the F_1 hybrid experiment did not demonstrate a decrease in the degree of hypernoradrenergic innervation as expected (except in the vas deferens) and further outbreeding of rats (F_2, F_3 etc.) may be required to give a better insight into the relationship between hypertension and hypernoradrenergic innervation. Further investigations into the NA concentrations of tissues from female rats is also a necessity. The second shortfall regards the lack of a suitable 'house-keeping' gene. Generally β-actin is used as a

control when investigating changes in mRNA, in both cell culture and intact tissues, as the concentration of messenger RNA for β-actin is considered to remain constant. Perhaps, and unfortunately, the only exception to this rule seems to be the cardiovasculature. Actin is present in blood vessels and the heart as an integral component of contractile protein. Unfortunately, contractile protein content has been found to change with vascular hypertrophy and hyperplasia that accompanies hypertension and the tissues examined in the present study are subjected to these changes (Siedel, 1979; Owens and Thompson, 1986). Furthermore, the concentration of β-actin mRNA has been shown to alter with anti-hypertensive therapy (R King, private communication).

While providing answers to some questions the study has presented additional questions, not the least of which are three. The first question that arises is, which cell type is responsible for the enhanced production of NGF in the SHR? (Figure VIII.1). SHR NGFmRNA is elevated to a greater degree in mesenteric vascular tissue than can be accounted for by any differences in VSM cell mass between the two species. Thus VSM cells may not be the only source of NGF in the mesenteric vasculature. It is suggested that the other source of NGF may be fibroblasts and that an increase in fibroblast cell number accounts for the unusually high level of NGFmRNA seen in this tissue. As fibroblasts reside in a closer relationship to sympathetic nerve varicosities than do VSM cells (Soares-da-Silva and Azevedo, 1985) then they are more apt to respond to released NA; this hypothesis is included as a feed-back system in Chapter VI (Figure VI.6). More recently, cultured VSM cells have been shown to be induced to synthesise greater concentrations of NGF in the presence of angiotensin II and phenylephrine (Creedon and Tuttle, 1991). Whether cultured SMC are in fact responding as SMC or have reverted to fibroblasts is a controversial point. This,

however, then presents the second question as to, what are the possible promoters that effect an increase in NGF synthesis? NGF synthesis has been shown to be influenced by a variety of compounds such as T_4 (Aloe and Levi-Montalcini, 1980; Walker et al 1981), corticosterone (Walker et al, 1981), testosterone (Ishii and Shooter, 1975; Walker et al, 1981), interleukin-1 (Spranger et al, 1990), β-adrenergic stimulation (Dal Taso et al, 1987, 1988; Schwartz and Mishler, 1990) and catecholamines that do not require receptor interactions (Furukawa et al, 1986 a,b). Whether these compounds act in concert, are required to act in a complicated sequence or which compound may be the initiator of a possible sequence has yet to be answered but presents a myriad of choices for further investigations. The third question arising from the present study regards the observation that the branching of secondary arteries from the superior mesenteric artery in the SHR was to a lesser degree than that seen in the WKY. As the SHR was a smaller animal this in itself would possibly constitute a smaller vasculature but the ratio of mesenteric tissue weight to body weight was only a third of that seen in the WKY at 2 days of age. Aortic tissue weight was not different between the two species at this age and thus the 'smaller animal = smaller tissue weight' hypothesis may not apply. A decreased branching in resistance vasculature would itself constitute an increase in blood pressure given that cardiac output was not different between the two rat strains. The degree of branching, and its contribution to elevating blood pressure, has yet to be studied but may reflect a further difference between SHR and WKY rats and may relate to the initiation of hypertension in the SHR.

The above highlight some of the future directions of research which are presented in Figure VIII.1. A priority investigation is the localisation of NGF in tissue and the determination of the cell types responsible for the production of NGF.

Future Directions

Investigations into :

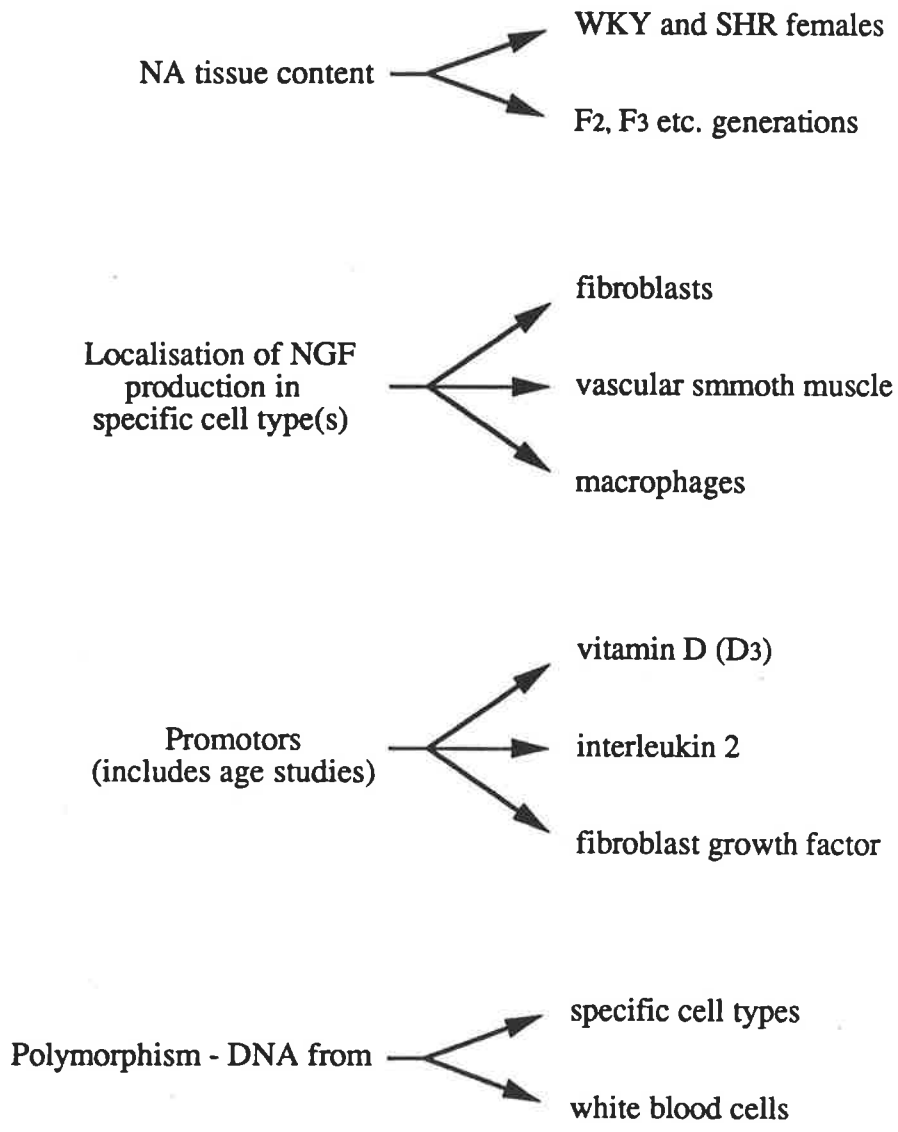


Figure VIII.1 The above diagram indicates some of the directions for future research to gain better insights into the relationship between hypernoradrenergic innervation and hypertension.

The new techniques of *in situ* hybridisation (with cDNA as well as cRNA probes), on intact tissues, offers the potential to localise the cell types that generate NGFmRNA and should be applied to tissues in the SHR. Promotor studies would present their own difficulties in that cultured cells allow a controlled environment but the question of cells dedifferentiating to another form arises (Stadler et al, 1989). Intact tissue would be ideal systems to validate promotors but as blood vessels contain a large number of cell types (eg. endothelial, VSM, fibroblasts, macrophages etc) the interpretation on how the promotors initiate increased synthesis becomes difficult.

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APPENDICES

APPENDIX I

The chemicals and reagents used in the experiments for this thesis are listed below. The chemicals are listed as either standard reagents (Univar) or were 'molecular biology grade'.

Ajax Chemical Pty. Ltd.

Standard Reagents

absolute ethanol
chloroform
disodium hydrogen orthophosphate
formaldehyde
glacial acetic acid
glycerol
isopropanol
sodium dihydrogen orthophosphate
sodium chloride
trisodium citrate

Biorad Laboratories

Standard Reagent

mixed bed resin - AG 501-X8 (D) : 20-50 mesh

Boehringer Mannheim

Molecular Biology Grade

Nick translation kit
salmon sperm DNA
RNA molecular size markers (7.4-0.3 kb fragments)

Bresatec Ltd

Molecular Biology Grade

DNA molecular size markers (8.51-0.36 kb fragment)
[α-^{32}P]dCTP 3000 Ci/mol

Merck

Standard Reagent

Folin's Reagent

Pharmacia

Molecular Biology Grade

*Pst*I restriction enzyme

Standard Reagent

dextran sulphate

Sigma Chemical Company, St Louis, MO.

Molecular Biology Grade

3-[N-morpholino] propane sulfonic acid (MOPS)

agarose

aminomethanamidie thiocyanate salt
(guanidinium thiocyanate)

diethyl pyrocarbonate

ethidium bromide (10 mg/ml)

ethylenediametetra acetic acid (EDTA)

Ficoll 400

formamide

magnesium chloride

lauryl sulphate

phenol

polyvinylpyrroldone

sodium acetate

sodium/potassium tartrate

Trizma Base

Trizma HCl

Standard Reagent

8-hydroxyquinolone

bovine serum albumin

bromophenol blue (sodium salt)

copper sulphate

sodium carbonate

sodium hydroxide

Treatments		N	Body Weight (g)	Salivary Gland Weight (mg)	Total RNA (µg/g tissue)
Control Males		12	$37.1\pm0.7^{a,b,c}$	$253\pm7^{a,b,c}$	$680\pm39^{a,b,c}$
Castrated	Testosterone Supplemented	10	$35.5\pm0.9^{e,f}$	$265\pm9^{d,e,f}$	$651\pm35^{d,e,f}$
	Vehicle Only	9	$34.4\pm0.9^{a,g,i}$	$187\pm9^{a,d,g,h}$	$452\pm28^{a,d,g}$
	No Supplement	10	$31.4\pm0.8^{b,e,g,h}$	$161\pm5^{b,e,g}$	$355\pm25^{b,e,g,h}$
Control Females		10	$27.8\pm1.0^{c,f,h,i}$	$151\pm6^{c,f,h}$	$493\pm28^{c,f,h}$

Table IV.1 The above table lists the body and submaxillary salivary weights of the 5 different groups described in Chapter IV. The yield of total RNA (µg RNA / g tissue wet weight) is also listed. Statistical differences between groups are denoted by the same superscript character (Student's t-test; $p<0.05$).

APPENDIX III

Treatment		NGF† (µg/g)	NGFmRNA† (arbitrary units)
Control Males		$1020\pm200^{a,b,c,d}$	$100.0\pm18.1^{a,b,c}$
Castrated	Testosterone Supplmented	$2600\pm600^{a,e,f,g}$	$102.1\pm12.8^{e,f,g}$
	Vehicle Only	$31\pm8^{b,e,h}$	$17.6\pm4.7^{a,e}$
	No Supplement	$47\pm9^{c,f,i}$	$23.0\pm7.5^{b,f,h}$
Control Females		$89\pm14^{d,g,h,i}$	$10.4\pm4.5^{c,g,h}$

Concentrations of NGF and NGFmRNA are listed for each group. The NGFmRNA concentration is noted as arbitrary (absorbance) units and are normalised for the absorbance of 1 µg of control male mouse RNA. Statistical differences between groups are denoted by the same superscript character (Student's t-test; $p<0.05$).

APPENDIX IV

Tissue	Species	Weights/Age Groups		
		2 Day Old	10 Day Old	43 Day Old
Total Body Weight (g)	WKY	5.66±0.11	19.03±0.33†	133.06±2.18†
	SHR	5.36±0.13	13.83±0.32	124.30±2.50
Kidney (mg)	WKY	27.5±0.6	99.1±1.8†	610.0±18.5#
	SHR	31.0±0.8#	84.1±1.8	571.0±13.1
Heart (mg)	WKY	33.2±0.9	116.0±1.9	552.0±12.0
	SHR	33.6±1.0	112.5±3.2	621.0±27.2†
Heart/Body Weight Ratio	WKY	5.80±0.11	6.12±0.13	4.15±0.04
	SHR	6.24±0.08#	8.16±0.20†	4.90±0.12#

Body and tissue weights of WKY and SHR rat pups at different ages. The Significant differences between WKY and SHR weights are noted (Student's *t*-test; #=$p<0.05$; †=$p<0.01$.

APPENDIX V

Tissue	Species	NGFmRNA (arbitrary units)		
		2 Day Old	10 Day Old	43 Day Old
Kidney	WKY	1009±179	689±109	91±20
	SHR	1198±60	1681±195*	631±131*
Heart	WKY	1149±85*	407±62*	194±38*
	SHR	602±117	262±40	76±9
Mesenteric Vein	WKY	-	127	237
	SHR	-	514±42	2390±284

NGFmRNA content (arbitrary units) of hearts, kidneys and mesenteric veins from WKY and SHR rat pups at 2, 10 and 43 days of age (* denotes significant differences between SHR and WKY yields; $p<0.05$, Student's *t*-test and Kruskall-Wallis). No statistics were done on the mesenteric veins as the n=1 for WKY tissue. All values have been standardised to 1 µg of mouse salivary gland RNA.

APPENDIX VI

Tissue	Species	Weights/Age Groups		
		2 Day Old (n=30)	10 Day Old (n=18)	43 Day Old (n=12)
Total Body Weight (g)	WKY	5.66±0.11	19.03±0.33†	133.06±2.18†
	SHR	5.36±0.13	13.83±0.32	124.30±2.50
Aorta (mg)	WKY	1.1±0.2	2.7±0.3#	17.6±0.8#
	SHR	1.1±0.1	2.2±0.1	15.5±0.6
Mesenteric Artery (mg)	WKY	1.8±0.2	2.7±0.2#	14.2±0.4
	SHR	1.4±0.1	2.1±0.1	15.2±0.3#
Mesenteric artery/body weight ratio (x1000)	WKY	369±39†	144±8	107±4
	SHR	274±22	151±9	120±3†
Caudal Artery (mg)	WKY	-	-	11.2±0.5
	SHR	-	-	10.9±0.5

Body and tissue weights of WKY and SHR rat pups at different ages. The fact that no difference was found for tissues at 2 day of age is possibly due to the difficulty in cleaning tissue; the tissue is extremely soft and fragile at this age. Increases in the mesenteric vasculature possibly represents hyperplastic changes (significant differences between WKY and SHR weights; Student's *t*-test, #=$p<0.05$; †=$p<0.01$).

APPENDIX VII

Tissue	Species	NGFmRNA (arbitrary units)		
		2 Day Old (n=3)	10 Day Old (n=6)	43 Day Old (n=6)
Aorta	WKY	116±60	43±6	237±29
	SHR	808±434	187±61	127±18
Mesenteric Artery	WKY	42±13	31±4	121±17
	SHR	291±25	385±41	731±130
Caudal Artery	WKY	-	-	115±28
	SHR	-	-	630±109

NGFmRNA content (arbitrary units) of aorta, mesenteric and caudal arteries from WKY and SHR rat pups at 2, 10 and 43 days of age. Significant differences between SHR and WKY were found at all ages, for all tissues; $p<0.05$, Student's *t*-test and Kruskall-Wallis). All values have been standardised to 1 µg of mouse salivary gland RNA.

Thesis Corrections and Explanations

1) The kidney weight for 10 day old WKY rat pups was significantly greater than those found in the 10 day old SHR (99.1 ± 1.8 and 84.1 ± 1.8 respectively) yet the total RNA extracted, per mg of wet tissue weight, was significantly greater for the SHR (4.76 ± 0.16 and 9.91 ± 0.43 μg/mg respectively). Allowing for the same densiometric analysis of 2 μg RNA, from both the SHR and WKY rat pups, then the SHR produced at least twice as RNA as the WKY; ergo twice the amount of NGFmRNA and subsequently twice the amount of protein (assuming a linear relationship).

2) Asterisks were placed consistently over all groups that were significantly lower.

3) The statistical tests used were the analysis of variance of the ranked data using the Kruskall–Wallis ANOVA followed by a Student's *t*–test; both with $p<0.05$. These tests were performed in accordance with directions from Dr Peter Baghurst, Senior Research Scientist (Statistician), CSIRO, Division of Human Nutrition, Adelaide South Australia; specifically as the *n* number was small in a several cases and the variance in ranks can be used on small numbers.

4) (i) pg 129: line 12: – extensive data from numerous laboratories are available that clearly demonstrate that the adult male SHR has an increased heart/body weight ratio (H/BW), without an increase in cardiac NA tissue content, when compared to age–matched WKY; yet there is a paucity of data on females from either WKY or SHR rats. The data of this study show no statistical difference between H/BW ratios for F1 males, SHR males and F1 females that suggests that the F1 females also displayed hypertrophy of the heart in a manner similar to that seen in the normally developing SHR.

(ii) The H/BW presented on pg 94 are from 43 days old (≈7 wks) SHRs whilst those on pg 127 are from 22 wks old F1 rats; it is not possible to compare the two data due to the substantial difference in the age of the two groups and the different stages in the development of hypertension.

5) (i) pg 130: line 15: the sentence should read:
"...that the caudal artery NA content..."

(ii) pg 131: line 12: the sentence should read:
"...was that the NA content in the vas deferens was significantly decreased in the F1 population."