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CHRONIC REGULATION OF ARTERIAL BLOOD PRESSURE BY ATRIAL NATRIURETIC PEPTIDE: ROLE OF ENDOTHELIAL FACTORS, SYMPATHETIC NERVOUS SYSTEM AND RENIN-ANGIOTENSIN SYSTEM

by

Luis Gabriel Melo

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Physiology
University of Toronto

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ABSTRACT

CHRONIC REGULATION OF ARTERIAL BLOOD PRESSURE BY ATRIAL NATRIURETIC PEPTIDE: ROLE OF ENDOTHELIAL FACTORS, SYMPATHETIC NERVOUS SYSTEM AND RENIN-ANGIOTENSIN SYSTEM

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CANADA

Atrial Natriuretic Peptide (ANP) exerts a chronic hypotensive effect that is mediated by relaxation of the resistance vasculature. Two functionally opposite mouse models of chronic ANP activity; an ANP-over expressing hypotensive transgenic mouse (TTR-ANP) characterized by reduced peripheral vascular resistance (TPR) and an hypertensive ANP gene knockout mouse (-/-), were used to evaluate the relative contribution of endothelial factors and tonic sympathetic nervous activity to the chronic vasodilatory effect of ANP. The general hypothesis that was tested is that the chronic ANP-dependent vasodilation is mediated by potentiation of vasodilatory nitric oxide (NO) and C-type natriuretic peptide (CNP) and inhibition of vasoconstrictor endothelin-1 (ET-1) from the vascular endothelium (VE) of the resistance vasculature, and by attenuation of tonic vascular sympathetic tone. The consequences of life-long deficiency in ANP activity on renal and hormonal adaptations to increased dietary salt intake were also evaluated in order to test the hypothesis that chronic deficiency in ANP activity leads to salt sensitivity of arterial blood pressure (ABP). Characterization of systemic hemodynamics in -/- and +/- using the thermal dilution technique indicated that the hypertension in -/- mice is specifically due to an elevation of basal TPR, thus confirming that the chronic hypotensive effect of ANP is mediated primarily by vasodilation of the resistance vasculature. Steady-state concentrations of ET-1, CNP and endothelial cell nitric oxide synthase (ecNOS) and ecNOS activity, measured in whole organ homogenates from TTR-ANP, -/- and the genetically-matched control (NT,
+/+) mice as an index of local synthesis from resistance vessel endothelium, and responsiveness of ABP and HR to acute inhibition of endogenous ET-1, CNP and NO in +/- and +/+ did not differ significantly between the mutant and wild-type control mice. This indicates that the chronic vasodilatory effect of ANP is not mediated by alterations in the activity of these endothelial vasoregulatory modulators. The hypotensive response to acute autonomic ganglionic blockade (AGB), taken as as an indirect index of tonic sympathetic tone, was quantitatively smaller in TTR-ANP and greater in +/- mice relative to their respective wild-type controls. These differences were paralleled by directional differences in total plasma catecholamine concentration, and were not attributable to altered peripheral adrenergic receptor responsiveness. These findings suggest that the chronic hypotensive effect of ANP is, at least partially, dependent on attenuation of cardiovascular sympathetic tone. Prolonged maintenance (3-4 weeks)on high dietary salt (HS, 8% NaCl) exacerbated the hypertension in +/- mice while having no effect on +/+ . The hypertensive effect of salt in the +/- mice was not accompanied by salt retention, despite an inherent reduced capacity for renal excretion of salt. Instead, it was associated with failure to downregulate plasma renin activity (PRA) and total plasma catecholamine concentration. Chronic treatment with the angiotensin II (ANG II) receptor antagonist Losartan during HS intake abolished the salt-induced increases in ABP and plasma catecholamine concentration, indicating the dependency of the hypertensive effect of salt on ANG II activity. In conclusion, the results of this thesis show that ANP exerts a chronic vasodilatory effect that is mediated by attenuation of vascular sympathetic tone. In the absence of endogenous ANP activity, an ANGII-dependent salt-sensitive component of hypertension develops in +/- mice, which is due to the removal of antagonism by ANP on renin synthesis.
DEDICATION

This thesis is dedicated to the memory of my beloved brother Carlos Alberto Melo (1947-1994), who, in life provided me with much enthusiasm and encouragement. He would be very proud of this achievement.
ACKNOWLEDGEMENTS

First and foremost, I wish to acknowledge that this thesis is as much mine as it is my wife’s and my daughters’, because this achievement was only made possible by their numerous sacrifices, patience and love over the years. To them, my deepest felt thank you for all the support.

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<tbody>
<tr>
<td>ABP</td>
<td>arterial blood pressure</td>
</tr>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AGB</td>
<td>autonomic ganglionic blockade</td>
</tr>
<tr>
<td>AHA</td>
<td>anterior hypothalamic area</td>
</tr>
<tr>
<td>ALDO</td>
<td>aldosterone</td>
</tr>
<tr>
<td>ANG</td>
<td>angiotensinogen</td>
</tr>
<tr>
<td>ANG II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>ANP-C</td>
<td>ANP C &quot;(clearance)&quot; receptor</td>
</tr>
<tr>
<td>AP III</td>
<td>atriopeptin III, ANP_{103-126}</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
</tr>
<tr>
<td>BNP</td>
<td>brain natriuretic peptide</td>
</tr>
<tr>
<td>BV</td>
<td>blood volume</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic 3',5'-adenosine monophosphate</td>
</tr>
<tr>
<td>C-ANP</td>
<td>ANP_{4-24}</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic 3',5'- guanosine monophosphate</td>
</tr>
<tr>
<td>CHF</td>
<td>congestive heart failure</td>
</tr>
<tr>
<td>CNP_{424}</td>
<td>C-type natriuretic peptide</td>
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</table>
CO cardiac output
DAG diacylglycerol
ECFV extracellular fluid volume
eNOS endothelial cell nitric oxide synthase
EPI epinephrine
ET-1 endothelin-1
G G- protein
GC-A particulate guanylate cyclase-linked receptor subtype A
GC-B particulate guanylate cyclase-linked receptor subtype B
GFR glomerular filtration rate
HR heart rate
icv intracerebroventricular
IP3 inositol 1'-3'-5'-trisphosphate
L-NAME Nω-nitro-arginine methyl ester
L-NMMA Nω-monomethyl-L-arginine
MAP mean arterial pressure
mRNA messenger ribonucleic acid
NADPH nicotinamide adenine dinucleotide phosphate
NE norepinephrine
NO nitric oxide
NT non-transgenic control mouse
NTS nucleus tractus solitarius
OVLT organum vasculosum of the lamina terminalis
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>pGC</td>
<td>particulate guanylate cyclase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphoinositol biphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>proANP</td>
<td>pro-atrial natriuretic peptide</td>
</tr>
<tr>
<td>RAS</td>
<td>renin-angiotensin system</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RSNA</td>
<td>renal sympathetic nerve activity</td>
</tr>
<tr>
<td>RVR</td>
<td>resistance to venous return</td>
</tr>
<tr>
<td>SAG</td>
<td>sympathetic autonomic ganglion</td>
</tr>
<tr>
<td>SFO</td>
<td>subfornical organ</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylate cyclase</td>
</tr>
<tr>
<td>SHR</td>
<td>spontaneously hypertensive rat</td>
</tr>
<tr>
<td>SNA</td>
<td>sympathetic nerve activity</td>
</tr>
<tr>
<td>SNS</td>
<td>sympathetic nervous system</td>
</tr>
<tr>
<td>SV</td>
<td>stroke volume</td>
</tr>
<tr>
<td>THB₁</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>TPR</td>
<td>total peripheral resistance</td>
</tr>
<tr>
<td>TTR-ANP</td>
<td>transthyretin-ANP transgenic mouse</td>
</tr>
<tr>
<td>VE</td>
<td>vascular endothelium</td>
</tr>
<tr>
<td>VSM</td>
<td>vascular smooth muscle</td>
</tr>
</tbody>
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INTRODUCTION

A. PREAMBLE

Atrial Natriuretic Peptide (ANP), a peptide hormone produced and secreted by the mammalian heart (1), elicits an array of cardiovascular responses that lead to the lowering of arterial blood pressure (ABP) (2). Acutely, ANP exerts a transient hypotensive effect that is mediated primarily by a reduction in cardiac output (CO) (2, 3, 4, 5, 6, 7). This occurs independently of the natriuretic and diuretic actions of the hormone (6, 7, 8), and is attributed to a decrease in ventricular preload (2-7) and inhibition of compensatory autonomic baroreflex responses (9, 10).

In addition to its well-defined acute hypotensive effect, there is evidence that ANP may also participate in chronic regulation of ABP. Prolonged (3-7 days) infusions of ANP into conscious rats (11, 12), dogs (13) and sheep (14, 15), producing plasma levels of the hormone within the physiological-to-pathophysiological range, cause sustained hypotension. As in the acute situation, the chronic hypotensive effect of ANP occurs, apparently, in the absence of detectable changes in renal function (11-15). However, in contrast to the acute situation, the long-term hypotensive action of ANP is mediated by a decrease in total peripheral resistance (TPR) (11-15). These findings were recently corroborated in genetic mouse models expressing alterations in endogenous ANP activity. Transgenic mice overexpressing a transthyretin-ANP fusion gene (TTR-ANP) are markedly hypotensive compared to non-transgenic (NT) control mice, in association with lifelong 8-10 fold elevation in plasma ANP (16, 17). The hypotension in the TTR-ANP mice is due to a reduction in TPR, consequent to generalized vasodilation in regional vascular beds (18). In contrast, "knockout mice", in which synthesis of ANP (-/-) (19) or its guanylate cyclase-a (-/- GC-a) receptor (20) are prevented by targeted homozygous disruption of the native genes, develop hypertension relative to their wild-type (+/+ ) siblings. The marked cardiac hypertrophy and elevated baseline diastolic blood
pressure observed in these knockout models suggest that the hypertension associated with lack of ANP activity is mediated by an elevation in TPR. However, a direct assessment of the hemodynamic characteristics of these models is necessary for confirmation of this hypothesis.

The mechanism by which ANP reduces TPR chronically is not known. ANP, at high concentration, directly relaxes vascular smooth muscle in some large arteries, via GC-A- mediated stimulation of cGMP (21, 22, 23, 24). However, the resistance vasculature, with the possible exclusion of the renal vascular bed (25), has a scarcity of GC-A receptors (26, 27) and is insensitive to direct relaxation by ANP (26, 27, 28, 29, 30, 31). These findings suggest that the chronic ANP-dependent dilation of resistance vessels is mediated by intermediary vasoeffecter mechanisms whose single or combined actions reduce peripheral vascular resistance. In this respect, ANP has been shown to modulate the activity of several vasoregulatory systems, including the vascular endothelium (VE) (for review see ref. 32) and the autonomic nervous system (for review see ref. 33). In addition, ANP exerts physiological antagonism of the renin-angiotensin aldosterone hormonal system (RAS) (for review see ref. 34). Specifically, ANP acutely inhibits production of locally-acting vasoconstrictor peptide endothelin-1 (ET-1) (35, 36, 37, 38) and stimulates synthesis of vasodilator C-type natriuretic peptide (CNP) (39) and possibly nitric oxide (NO) (40, 41) from VE, and modulates the target cardiovascular effects of these vasoactive agents (42). In addition, ANP inhibits sympathetic nervous activity (SNA), both centrally (43, 44) and peripherally (45, 46), and inhibits renin (47) and aldosterone (48) synthesis and antagonizes the target actions of ANG II (49, 50).

It may be inferred from these findings, that such interactions with the VE, SNA and RAS, if tonically active, may contribute to the chronic vasodilatory effect of ANP. Thus, ANP could reduce peripheral vascular resistance by potentiating the vasodilatory moieties of NO and CNP from VE concurrently with attenuation of the vasoconstriction associated with ET-1 and SNA activities and
by modulating the target effects of RAS. This thesis investigates the contribution of these vasoregulatory systems to the chronic hypotensive effect of ANP, based on the general hypothesis that ANP-dependent relaxation of the resistance vasculature is mediated by simultaneous tonic potentiation of NO and CNP and inhibition of ET-1 activities from the VE of the resistance vasculature, and attenuation of vascular sympathetic tone. This hypothesis was tested in two genetic mouse models expressing life-long alterations in endogenous ANP activity: the hypotensive ANP-overexpressing (TTR-ANP) transgenic mouse, and the hypertensive ANP gene knockout (−/−) mouse characterized by lack of ANP activity.

B. BIOCHEMICAL ASPECTS OF ANP

1. Biosynthesis, structure of the prepro-ANP gene, pro-ANP and ANP

ANP is the most abundant of a family of structurally and functionally related peptides (for review see refs. 51, 52) that are involved in homeostatic regulation of ABP (2) and fluid and electrolyte balance (2, 53, 54). Under normal hemodynamic conditions, ANP is predominantly synthesized by the myocytes of the cardiac atria (55) (Figure 1.1). However, in pathophysiological conditions characterized by hemodynamic overload, such as in congestive heart failure (CHF) (56), the ventricles contribute significantly to the circulating levels of the hormone. ANP is also synthesized in lesser amounts in peripheral tissues and central nervous structures, including the kidney, lung, aortic arch, vascular endothelium, autonomic ganglia, adrenal medulla, brain, pituitary and the hypothalamus (57, 58).
Figure 1.1. Transmission electron micrograph (x6000) of a secretory myocyte in the right atrium. ANP is stored within the dense perinuclear granules.
In all mammalian species studied to date, ANP was found to be the product of a single gene that has been highly conserved through evolution (59, 60, 61, 62). The gene consists of three exons separated by two introns (61, 62) (Fig. 1.2A). The first exon encodes the leader sequence and the first 16 residues of pro-ANP. The second exon encodes the intervening peptide sequence which contains the biologically active peptide. The third exon contains the carboxyl terminal tyrosine. Transcription and translation of the gene produces the 152-amino acid preproANP (63, 64) from which a 24 aminoacid hydrophobic leader sequence is removed (65). The 128- amino acid peptide undergoes further posttranslational processing during which a carboxyl end Arginine-Arginine sequence is removed (66). The final 126- aminoacid proANP is the primary storage form found in the perinuclear granules of secretory atrial myocytes (67, 68). The biologically active 28- aminoacid peptide (ANP) is cleaved from the carboxyl end of proANP during secretion, by atrioactivase (69), a serine protease that cleaves between Arginine$_{98}$ and Serine$_{99}$.

In consonance with the high degree of conservation of the ANP gene, there is a high interspecies homology of the aminoacid sequences of proANP and ANP, with the ANP sequence of rodents differing from that of humans and dogs by one aminoacid at position 110 (65, 66, 70). The primary structure of the active peptide contains an intrachain disulfide bond between cysteine residues at 105 and 121 positions, forming a 17 aminoacid ring (71) (Fig. 1.2B) that is essential for biological activity (72).

2. Regulation of proANP gene expression and post-translational processing

Regulation of ANP biosynthesis occurs primarily at the transcriptional level (73). In cardiac atria, the ANP gene is expressed constitutively at high levels (74). In the adult ventricles, the proANP gene is quiescent, but its expression can be induced by neurohumoral and hemodynamic signals (56, 73). The mechanisms involved in regulation of proANP gene expression, however, have
not been fully clarified. Several regulatory sequences (cis- elements) have been mapped in the 5' flanking region of the proANP gene (74, 75, 76, 77), but their roles in in vivo regulation of proANP gene transcription, in most cases remain undefined, as much of the evidence was obtained in cultured neonatal ventricular myocytes, whose phenotype differs from that of secretory atrial myocytes.

**Figure 1.2.** A) Structure of the pro-ANP gene and biosynthetic pathway of ANP. B) Primary structure of mature ANP (1-28).

Nevertheless, several chronic in vivo interventions are associated with changes in proANP gene expression. In rats, ANP mRNA levels decrease in parallel with low dietary sodium intake (78) and with water deprivation (79), suggesting that the transcriptional activity of the proANP gene is adjusted, possibly adaptively, to the salt and hydration state of the body. Glucocorticoids (80, 81), thyroid hormone (82, 83) and mineralocorticoids (84) have all been found to increase ANP mRNA
in vivo. In the case of glucocorticoid and thyroid hormone, the elevation in proANP mRNA is, at least partially, due to a direct increase in transcription rate associated with activation of glucocorticoid (85) and thyroid hormone receptor responsive cis elements (86). Interestingly, the effect of thyroid hormone on proANP mRNA is dependent on volume status, with no effect present in hydrated rats (83) and maximal effect seen in dehydrated rats (83). The effect of mineralocorticoid on ANP mRNA, on the other hand, is indirect, and appears to be due to stretch-induced transcriptional activation in the atrial myocytes, consequent to blood volume expansion (84, 87).

More recently, early gene components of the activator protein -1 complex (AP-1), c-jun and c-fos, were found to activate the hANP promoter coupled to the chloramphenicol acetyltransferase (CAT) reporter gene in cultured neonatal rat ventricular myocytes (88). This occurs via a TRE (12-o-tetradecanoylphorbol 13-acetate) responsive element (88) located upstream (~200 bp) from the transcription initiation site (CAP) (89). Although the physiological significance of this interaction has not been established, it is possible that activation of this enhancer cis-element by these early gene transcriptional factors may, in part, underlie the increase in proANP gene transcription that accompanies cardiac hypertrophy such as in severe CHF (90) and chronic hypertension (91). Basal and phenylephrine-induced proANP gene expression were also found to be dependent on activation of several serum responsive elements (SRE) localized along the 5' flanking sequence (92). Stretch-induced stimulation of proANP gene transcription appears to be mediated by mitogen-activated protein kinase (MAPK kinase)-dependent phosphorylation of serum response factor (SRF) and subsequent activation of these SRE elements (93).

Tissue- or cell-specific expression of the ANP gene is also controlled by the interaction of nuclear regulatory proteins with cis-elements. A sequence of the 5' flanking region of the proANP gene localized ~3500 nucleotides from the CAP site was required for specific expression of proANP-
CAT chimaeric genes in neonatal ventricular myocytes as in adult atrial myocytes (93), whereas no expression was seen in non-myocyte cells (94). The absence of proANP gene expression in non-myocardial cells is partly due to activation of repressor cis-elements. In the human proANP gene, two such elements sharing homology with an E-box motif, were localized proximal (≈222 bp) and distal (≈2593 bp) to CAP, in the 5' flanking region (95). However, the mechanism of activation/deactivation of these repressor elements is not known.

Following translation, proANP undergoes a series of posttranslational modifications, in preparation for release of the bioactive hormone. The principal maturing events are the formation of the intrachain disulfide bond between cysteine residues 105 and 121 to form the 17-aminoacid ring that is required for bioactivity (72), phosphorylation of some aminoacid residues in the peptide sequence, the significance of which is not known (96), and proteolytic cleavage of the 28 aminoacid bioactive peptide from the carboxyl end of proANP by atrioactivase (69). The time and site(s) where cleavage of proANP takes place have not been established. Recent evidence suggests that proANP cleavage can occur cosecretionally (97) via activation of intragranular serine protease (i.e. atrioactivase) by secretory stimuli. However, this was observed in cultured neonatal atrial myocytes supplemented with dexamethasone, and only after 4 days, during which time only proANP was released, thus raising doubts about the physiological significance of this mechanism. It is possible that proANP is cleaved after exocytosis by proteases in the interstitial space (98), or alternatively, it could be picked up and processed by adjacent non-myocyte (i.e. endothelial, mesenchymal) cells (99).

3. Mechanisms of secretion and clearance of ANP

Evidence from in vivo and from in vitro studies in a variety of experimental models, suggests that at least two types of stimuli are involved in modulation of ANP secretion from cardiac atria (Fig. 1.3). Myocyte stretch is widely accepted as the primary stimulus for ANP release. ANP secretion is
increased severalfold in isolated heart preparations following distension of the atria (100, 101, 102) and *in vivo* in association with experimental procedures that increase intraatrial pressure, such as acute volume loading (103, 104) and head-out water immersion (105), as well as in certain cardiovascular diseases characterized by vascular volume congestion (106) and in response to central hypervolemia caused by postural changes and pregnancy (107, 108). The common feature in all these situations is distension of the atrial wall. The secretory response to stretch is not affected by prior bilateral cervical vagotomy, nor by adrenergic receptor inhibition (109, 110), suggesting that stretch-induced release of ANP occurs independently of autonomic neural activity. The increase in ANP release following volume-induced atrial stretch correlates well with wall stress during the V-wave of the atrial cycle, suggesting that the effect of stretch on ANP release occurs mainly during atrial diastole (111). However, an increase in systolic atrial wall tension may also contribute to stimulation of ANP release, particularly in response to chronotropic stimulation (112, 113).

In addition to stretch, ANP release can be modulated by a variety of hormones, most notably pressor hormones, endothelium-derived factors and autonomic neurotransmitters (Fig. 1.3). There are, however, some discrepancies in the findings of different groups, and observations in *in vitro* preparations have not always been replicated *in vivo*. In many cases, these discrepancies cannot be ascribed to specific differences in the methodology, or any particular characteristics of the preparations, making it difficult to achieve a reasonable consensus with physiological validity. ANG II, vasopressin (AVP) and catecholamines stimulate ANP release, both *in vitro* (114, 115, 116, 117) and *in vivo* (118, 119, 120, 121). However, in most *in vivo* studies, the observed stimulation of ANP release could not be dissociated from the increases in mean arterial pressure and atrial wall stress accompanying the administration of these hormones (118, 120, 121). This raises doubt whether the agonist effect that these hormones have in some *in vitro* preparations is due to direct stimulation of
Figure 1.3. Physical and humoral factors that regulate ANP secretion, and the preferential intracellular signal transduction pathways utilized by these factors. Solid lines indicate stimulation. Broken lines indicate inhibition.
the secretory myocytes, or whether it occurs secondarily to changes in atrial function. ET-1 potently stimulates ANP secretion both in vivo (122, 123) and in vitro (124, 125). The effect of ET-1 on ANP secretion in vivo can occur independently of ensuing changes in MAP (126), suggesting the participation of a direct receptor-mediated mechanism coupling ET-1 to ANP secretion. Similarly, several arachidonic acid metabolites, possibly of endothelial and/or myocardial origin, have also been reported to stimulate ANP secretion to various degrees (127, 128) independently of hemodynamic changes (127). Endothelium derived relaxing factor/NO in contrast, exerts primarily an inhibitory effect on basal (129) and agonist-mediated (130) ANP release (Fig. 1.3).

A newly emerging hypothesis is that stretch and hormones may interact synergistically to produce maximal rates of ANP secretion. AVP (131), ET-1 (132) and to a lesser degree ANG II (132, 133) and epinephrine (134) enhances release of ANP in response to volume expansion in conscious rats. This effect is abolished in each case by specific pharmacological receptor blockade (131-133), and persists even after the hemodynamics return to baseline conditions, suggesting that the synergy between these hormones and stretch in potentiating ANP release is due to superimposition of the direct secretagogue action of these substances upon the stretch stimulus.

The putative cellular mechanism(s) linking stimulus to secretion of ANP from the myocytes are shown in Figure 1.3. Most evidence suggests a preferential activation of the phosphoinositide-protein kinase C (PIP2-PKC), as most of the identified stimuli for ANP secretion, both stretch (135) and humoral (136, 137, 138), are functionally linked to PIP2 hydrolysis and PKC activation in cardiac tissue. Under certain conditions, the adenylate cyclase-cAMP pathway (AC-cAMP) may also contribute to ANP secretion. Dibutyryl-cAMP, a membrane permeable analogue of cAMP, and forskolin, a diterpene activator of adenylate cyclase, potentiate release of ANP from isolated paced rat atria in a manner analogous to isoprenaline, a β-adrenergic agonist that activates the AC-cAMP
pathway (139), suggesting a role of this pathway in mediating β-adrenergic-dependent stimulation of ANP release. Less is known about the role of cGMP in ANP secretion. In the isolated rat heart, neither 8-bromo-cGMP, an analogue of cGMP, nor nitroprusside, a stimulator of guanylate cyclase, have a direct effect on ANP secretion (140). However, these agents delay phorbol ester- induced ANP secretion (140). Recently, ANP was reported to inhibit its own secretion in response to volume loading in rats, specifically via activation of its guanylate cyclase-linked (GC-A) receptors (141), suggesting that ANP may operate in a negative feedback loop to regulate its own secretion.

Many of the stimuli for ANP secretion, including stretch (142) and hormones (143, 144) are associated with a rise in intracellular calcium concentration, suggesting that this ion may play a physiological role in modulating ANP secretion from the heart. In isolated rat hearts, basal ANP secretion is potentiated by perfusion with calcium ionophore A23187 (145) and with calcium-channel agonist Bay K8644 (140, 146). The mechanism appears to be dependent both on influx of extracellular calcium as well as on calcium release from intracellular stores, since ANP secretion is inhibited by sarcolemma and sarcoplasmic reticulum-specific calcium channel blockers verapamil (147) and ryanodine (148) respectively. Similarly, stretch-induced ANP release from spontaneously-beating isolated hearts is attenuated by reductions in intracellular calcium concentration with channel blockers (149). The precise role of calcium in the mechanism of ANP secretion has not been defined. A likely role for calcium is in modulating the events leading to fusion of ANP-containing secretory granules with the sarcolemma and exocytosis. In support of this, recently two non-calmodulin, calcium-dependent proteins, annexins V and VI were isolated from rat atrial myocytes (150). These proteins bind specifically to ANP secretory granules at physiological calcium concentrations, and are thought to regulate exocytosis of ANP.
Soon after release, ANP is removed from the circulation by several organs and tissues, of which the most important are the kidney, lung, intestine and vasculature (151). At least two mechanisms are involved in clearance of ANP, endocytosis of receptor-ligand complexes and enzymatic degradation. Receptor-mediated endocytosis involves binding of ANP to its C- (clearance) type receptor (152) and internalization of the receptor-ligand complexes. These complexes then undergo intralysosomal degradation by peptidases (153). The clearance receptors, because of their abundance in most ANP-binding tissues (99% of all receptors in kidney and endothelial cells), thus, may operate as a physiological buffer of plasma ANP concentration (154). The second major mechanism of ANP degradation occurs by enzymatic hydrolysis of the Cysteine 105-Phenylalanine 106 bond by neutral endopeptidase (NEP, EC 3.4.24.11) (155), a widely distributed transmembrane, zinc-dependent metalloprotease that is particularly abundant in the brush border of the kidney proximal tubular epithelia (156). The hydrolysis of this bond disrupts the disulfide-linked ring of ANP and results in loss of bioactivity. It is not known how much each of the two mechanisms of degradation contribute to the catabolism of ANP. The relative contribution of each mechanism may be in part determined by the specific biochemical and morphological characteristics of the tissues where metabolism of ANP is taking place.

4. Cellular mechanisms of action of ANP

Upon release, ANP exerts its biological actions by interacting with specific, high affinity membrane-bound glycoprotein receptors in target tissues (157, for review see ref. 27). These receptors have been localized by autoradiography and radioligand binding in a variety of mammalian tissues, including the endothelium, vascular smooth muscle, heart, lung, adrenal gland, kidney, pituitary and various regions of the brain (27). At least two structurally and functionally distinct receptor subtypes have been identified to date by cross-linking and photoaffinity labelling (27, 157,
on the basis of their ability to stimulate cGMP synthesis. The R₁ subtype is coupled to particulate guanylate cyclase (pGC) and is thought to mediate most of the biological actions of ANP (158, 159). Two isoforms of this receptor, GC-A and GC-B, have been distinguished based on their selectivity for ANP or ANP-related peptides, brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). The GC-A receptor binds ANP with high affinity, whereas GC-B binds BNP and CNP with greater affinity than ANP (160, 161). The second receptor subtype (R₂, C receptor) binds all members of the natriuretic peptide family with equivalent affinity (27). This receptor is not functionally linked to pGC, and until recently, it was thought to subserve solely the role of clearance and metabolism of natriuretic peptides from the circulation (152, 153).

The GC-coupled receptors function as homodimers of a single transmembrane protein of approximately 130 Kda (162). These receptors have an heterologous extracellular ligand binding domain, a membrane spanning domain, an intracellular catalytic domain that co-purifies with guanylate cyclase (163), and a cytosolic kinase-like domain (164). The kinase-like domain appears to function as a repressor of basal pGC activity (164, 165). The binding of ANP to the receptor releases this inhibition and stimulates pGC activity (166). This requires prior phosphorylation of the ligand-binding and kinase domains (167) and ATP as a cofactor (168). Activation of pGC converts GTP to cGMP. The newly formed cGMP stimulates intracellular protein kinases. The phosphorylation of target proteins by these cGMP-dependent kinases leads to the manifestation of some of the biological effects of ANP (27, 161, 169). The ANP-stimulated activity of pGC becomes desensitized over time, possibly due to dephosphorylation of the kinase-like domain (170), and is also prone to desensitization by protein kinase C (PKC) (171) and phorbol esters (172).

The R₂ receptor (more commonly designated as C-receptor) consists of an extracellular binding domain sharing some homology with GC-receptors, a transmembrane domain, and a very
short (~37 amino acids) cytosolic domain (152, 173). This receptor subtype is preponderant in most ANP-binding tissues. In some tissues, such as the kidney and the vascular endothelium, it constitutes more than 95% of the total receptor population (174). There is evidence that the C-receptor may be negatively coupled to adenylate cyclase (AC) via an inhibitory G (G_i) protein, and that this may represent an alternative signal transduction mechanism for some of the biological actions of ANP (175, 176). C-ANP (ANP_{4-24}), a truncated analogue of ANP specific for the C-receptor (152) reduces AC activity dose-dependently, in absence of any detectable changes in cGMP, in several rat tissues, including aortic smooth muscle, adrenal cortex, renal glomeruli, collecting duct and anterior pituitary (177). The inhibition of AC activity requires addition of GTP and is sensitive to pertussis toxin, indicating the involvement of a G_i protein (178). Some of the effects of ANP that have been ascribed to activation of the C-receptor include inhibition of growth and proliferation of vascular smooth muscle (179) and inhibition of aldosterone synthesis from the adrenal glomerulosa (180). In some tissues, the ANP-dependent inhibition of AC activity leads indirectly to stimulation of phospholipase C (PLC) activity and phosphoinositide (PIP_2) hydrolysis (181). However, the significance of this interaction in mediating ANP-dependent effects is unknown. On the other hand, GC-A activation by ANP inhibits ANG II and ET-1-induced stimulation of PLC and mobilization of intracellular calcium in vascular smooth muscle (for review see ref. 27).

The magnitude of the biological response to ANP is modulated by changes in receptor affinity and density (27, 169, 173). Prolonged exposure to ANP, even at physiological concentrations, almost invariably leads to receptor downregulation (27, 169, 182, 183). Although this process of homologous downregulation affects both GC-A and C-receptors, the latter is more susceptible. Incubation of aortic endothelial cells (184, 185) and vascular smooth muscle cells (186, 187) with ANP leads to a time-dependent decrease in C-receptor mRNA levels. This is inversely related to the
magnitude of ANP-induced cGMP accumulation, and is mimicked by membrane-permeable analogues of cGMP (185) and by the cGMP phosphodiesterase inhibitor, zaprinast (187). These findings are compatible with the interpretation that homologous ANP receptor downregulation is primarily due to GC-A/cGMP-dependent decrease in C-receptor synthesis. The extent to which receptor internalization and catabolism may contribute to downregulation has not been elucidated. Receptor density also changes in response to manoeuvres that alter plasma ANP concentration, such as salt and water loading (188, 189). Ballerman et al (188) showed that renal glomerular ANP receptor density change in inverse proportion to dietary salt content in rats fed on high or low salt diets for 2 weeks. Similarly, dehydration increases glomerular ANP receptors (189). The effect of dietary salt on glomerular ANP receptors is selectively due to changes in GC-A receptor number, whereas the effect of water loading appears to be primarily due to changes in C-receptor density (189). ANP receptor density in the vascular endothelium is also decreased by salt loading (190), and this is associated with downregulation of C-receptors (190).

In addition to homologous downregulation, ANP receptors in the vasculature undergo heterologous downregulation after prolonged exposure to elevated concentrations of ANG II in vitro (191). This appears to be selectively due to reduction of C-receptors, since the ANG II-mediated inhibition of ANP binding is not accompanied by a decrease in GC-A responsiveness (192). These findings were recently confirmed in vivo in rats infused with ANG II (193).

Some pathophysiological changes of the cardiovascular system are accompanied by ANP receptor downregulation. In general, these conditions are characterized by elevated plasma ANP levels, such as congestive heart failure (CHF) (194) and some types of experimental hypertension (195, 196, 197). In CHF, plasma ANP increases proportionally to the severity of the disease (194). In most studies, this was found to be accompanied by a decrease in ANP receptors (198, 199, 200).
The C-receptor subtype appears to be predominantly downregulated (201). Although the significance of this has not been established, the decrease in C-receptor binding and its associated clearance function may serve as a compensatory mechanism to increase GC-A receptor activity in the kidney, vasculature and the nervous system. Spontaneously hypertensive rats (SHR), a genetic model with some resemblance to human essential hypertension, display a generalized decrease in ANP receptor density in brain structures involved in fluid and electrolyte and blood pressure (ABP) regulation, namely the circumventricular organs and in the area postrema (AP) and nucleus tractus solitarius (NTS), in the kidney and in the mesenteric and brain vessels (202, for review see ref. 27). This appears to be due, at least in part, to reduced GC-A receptor activity, since ANP-mediated cGMP production is reduced in this model (198). Paradoxically, C-receptor mediated inhibition of adenylate cyclase was accentuated in SHR (203) despite no change in the abundance of this receptor subtype. The role that these alterations in ANP receptor function may play in development of hypertension is not known.

C. PHYSIOLOGICAL ACTIONS OF ANP

1. Renal effects of ANP

Although the physiological significance of ANP is not fully established, this hormone exerts several effects on renal and cardiovascular function that contribute towards maintenance of circulatory homeostasis and blood pressure (for review see ref. 2) (Figure 1.4). In the whole animal, ANP causes a potent and short lasting diuresis and natriuresis (1) by a mechanism that is partly hemodynamic (204) and partly due to direct inhibition of renal sodium reabsorption (205). The hemodynamic component of ANP-dependent natriuresis/diuresis is manifested by rises in glomerular filtration rate (206) and vasa recta blood flow (207), the former leading to increased delivery of fluid
to distal tubular segments, the latter by causing changes in transcapillary Starling forces (208) and medullary solute gradients (209) that are unfavourable to solute and water reabsorption. The direct tubular effect of ANP is predominantly localized in the inner medullary collecting duct (210). In this segment of the nephron, ANP inhibits sodium and water reabsorption via activation of CG-A receptors (211). The mechanism involves inhibition of an apical amiloride-sensitive cation channel (212) and inhibition of basolateral Na⁺/K⁺ATPase pumps (213), and, possibly, stimulation of a basolateral secretory pathway mediated by Na⁺/K⁺/2Cl⁻ (214).

The renal action of ANP may also result, in part, from its antagonism to other major salt and water-regulating systems. ANP inhibits renin (47, 215) and aldosterone (48, 216) synthesis, and antagonizes the renal (49) and vascular (50, 217) actions of ANG II. In addition, ANP inhibits AVP-dependent reabsorption of water in the cortical collecting duct (218), and reduces renal sympathetic nerve activity (219). Although it has been difficult to establish the extent to which these interactions, single or cumulative, contribute to the net renal effect of ANP, there are at least two experimental situations in which this antagonism has been verified. First, in deoxycortisone acetate induced hypertension, ANP is released consequent to expansion of the extracellular fluid volume (ECFV). The ensuing elevation in plasma ANP concentration is temporally (220) and causally (221) implicated in mediating the subsequent escape from the salt-retaining effect of mineralocorticoid. Secondly, ANGII-induced sensitization of ABP to salt becomes attenuated over time, in parallel with an increase in plasma ANP concentration (222). The rise in plasma ANP leads to an increase in the sensitivity of the pressure natriuresis relationship, possibly due to inhibition of ANG II-dependent sodium reabsorption in the proximal tubule (223).

The magnitude of the renal response to ANP is largely determined by the degree of filling of the extracellular fluid volume compartment (224). In general, the kidney shows refractoriness to
Figure 1.4. Coordinated renal and cardiovascular effects of ANP in maintenance of circulatory homeostasis. (See text for details).
ANP in volume depleted animals (224, 225), and becomes increasingly responsive to ANP with expansion of the ECFV (224, 225, 226). Acute extracellular volume expansion with saline in rats led to significant increases in renal salt and water excretion, in parallel with a several fold increase in plasma ANP concentration (225). The renal response to hypervolemia is attenuated by atrial appendectomy (226) and by anti-ANP monoclonal antibodies (227), thereby implying an obligatory role of ANP in mediating volume-induced diuresis and natriuresis.

Despite compelling evidence in support of a physiological role of ANP in renal regulation of fluid and electrolyte balance, there have been reports of experimental, physiological and pathophysiological situations in which increases in circulating ANP levels were found to be dissociated from diuresis and natriuresis. For example, volume loading or left atrial distension with a balloon is followed by the expected rise in plasma ANP concentration and natriuresis and diuresis (228). The renal responses are abolished by cardiac denervation, even though plasma ANP concentration remain elevated. Although this finding was taken as indication that volume-induced natriuresis occurs independent of ANP, the same study failed to demonstrate natriuresis/diuresis in the absence of a rise in plasma ANP. Recently, Kishimoto et al (229) showed that "knockout" mice lacking the GC-A receptor fail to respond to acute ECFV expansion with an increase in renal sodium and water excretion. This finding provides unequivocal confirmation that the GC-A receptor is involved in mediating the renal response to hypervolemia. On the other hand, ANP knockout mice respond adequately to ECFV expansion (230), suggesting that another member of the natriuretic peptide family, possibly BNP, with affinity for the GC-A receptor, or another natriuretic hormone, partly compensate for the absence of ANP activity. Other arguments against a role of ANP in day-to-day maintenance of sodium and water are derived from isolated observations that changes in dietary sodium intake were not followed by directional changes in plasma ANP concentrations (231); from
the lack of correspondence between the diurnal variations in plasma ANP and sodium excretion (232); and the absence of natriuresis during physical exercise, despite a rise in plasma ANP (233). With respect to the first two arguments, it can be said that a lack of relationship does not necessarily imply lack of causality. In this regard, it was recently shown that mice lacking the ability to synthesize ANP, show a predisposition towards development of salt sensitive hypertension when maintained on high dietary salt intake (19), thus suggesting a role of ANP in chronic maintenance of salt and fluid balance. The third argument is a poor example, since exercise stimulates a whole array of antinatriuretic mechanisms that are aimed at conserving fluid and electrolytes (234). Congestive heart failure is characterized by high plasma ANP concentration (106, 194). Despite this, there is fluid and salt retention, and generalized peripheral vasoconstriction (235). This, however, appears to be mainly due to simultaneous activation of antinatriuretic mechanisms that tend to override the renal effects of ANP. Indeed there is evidence that ANP compensatorily maintains adequate renal function against the prevalent background of antinatriuresis in CHF. In rats with experimentally-induced CHF, renal function was significantly depressed after chronic treatment with the GC-A/GC-B receptor antagonist HS-142-1 (236), whereas no effect of the treatment was seen in sham operated rats. Similarly, sodium excretion was markedly reduced following a bolus injection of anti-ANP monoclonal antibody in anesthetized rats with CHF, independent of changes in GFR or ABP (237). Thus, salt and water retention in CHF would be even higher if elevated levels of ANP were not present.

In summary, most evidence indicates that ANP plays a physiological role in regulating extracellular fluid volume, via its direct effects on renal hemodynamic and tubular function, and possibly, in part, by modulating the activity of the other major salt-regulating mechanisms. Such requirement of ANP activity for fluid and electrolyte balance may have implications for long-term regulation of arterial blood pressure (ABP), given the dependency of chronic ABP on maintenance
of ECF volume constancy by the kidneys (for review see ref. 238). Thus, pathophysiological situations in which ANP activity is reduced or impaired, may be accompanied by salt retention and expansion of the ECF volume (239). The ensuing increase in ABP could then partially compensate for the reduced ANP activity by pressure natriuresis (238). In the long term, body sodium balance would be achieved, albeit, at the higher ABP. Reduced renal sensitivity to ANP is indeed observed in some sodium-retaining disorders (240), and mice expressing one half of the normal GC-A receptor copy number develop salt-sensitive increases in ABP (229).

2. Cardiovascular effects of ANP

Constancy of mean arterial blood pressure within the appropriate physiological level is necessary for optimal functioning of the cardiovascular system. Long-term departure from this operational level severely compromises the structural and functional integrity of the system (for review see ref. 238, 241). Homeostasis of ABP is dependent upon the activity of several interacting regulatory mechanisms that function to correct, in a compensatory fashion, changes in cardiac output (CO), total peripheral resistance (TPR) and blood volume (BV) (238, 241). Moment-to-moment changes in CO and TPR are continuously buffered by pressure/stretch sensitive mechanoreceptors, and to a lesser extent, chemoreceptors strategically located in the large arteries, heart and lungs. Activation of these receptors then initiates appropriate adjustments in cardiac performance and arteriolar tone, by altering the activity of autonomic nervous outflow to the heart and vasculature (238, 241). This reflex regulation of ABP is further complemented by the action of locally-active intrinsic myogenic mechanisms, vasoactive metabolites, endothelium-derived factors, as well as circulating hormones that collectively contribute to local regulation of vascular resistance and distribution of blood flow. The short-term mechanism of regulation of ABP by baroreceptors is, however, ineffective in the presence of prolonged changes in ABP, because the sensitivity of these
mechanoreceptors is quickly reset to the prevailing level of arterial pressure. Long term constancy of ABP is dependent upon the activity of renal, hormonal, neural and local mechanisms involved in tonic regulation of extracellular fluid volume, vascular resistance and cardiac function (238).

ANP has been reported to exert a wide range of cardiovascular and hemodynamic effects that converge to elicit a pronounced decrease in ABP acutely (2-6) (Fig. 1.4) and chronically (11-15). In either case, the hypotensive effect of ANP is, apparently, not dependent on the renal actions of the hormone (11-15), since it is preserved in acutely nephrectomized rats (242). In addition, rats chronically infused with exogenous ANP over several days, develop hypotension in absence of detectable changes in renal function (243). The nature of the mechanism(s) underlying the hypotensive activity of ANP, however, has not been unequivocally defined, and although much of the dissension can be attributed to methodological differences among studies (244), there is also some controversy surrounding the physiological significance of the observed cardiovascular effects of ANP, because some of these effects were elicited with pharmacological doses of ANP. The most convincing evidence for a physiological role of ANP in cardiovascular regulation is seen in genetic mouse models expressing life-long alterations in ANP activity. Mice harboring a functional deletion of the genes for ANP (19) and its GC-A receptor (20) develop chronic hypertension, whereas mice overexpressing the transthyretin-ANP transgene (16) are hypotensive with respect to the genetically-matched wild-type mice. These findings indicate that ANP exerts a tonic hypotensive effect, and that in its absence (i.e ANP and GC-A knockout mice), the consequent elevation of ABP is not compensated by other cardiovascular regulatory mechanisms.

The nature of the hemodynamic change(s) underlying the blood pressure-lowering activity of ANP differs between the acute and chronic states. Acutely, the hypotensive effect of ANP is mediated primarily by a reduction in cardiac output (CO), brought about by a combination of direct effects
of the peptide on vascular function and interactions with autonomic and hormonal cardiovascular regulatory mechanisms (for review see ref. 2, 245). In contrast, the chronic hypotensive effect of ANP is mediated by a sustained decrease in total peripheral resistance (TPR) by an unidentified mechanism(s).

D. HYPOTENSIVE ACTIONS OF ANP

1. Acute

a) Effect of ANP on cardiac output

Acute administration of ANP reduces ABP in a dose-dependent manner in all species studied (2-8, 245-249) (Fig. 1.5). Bolus injection or short-duration infusion of ANP into rats, dogs, sheep, rabbits and humans was reported to cause a reduction in CO between 10%-40% (2-8, 245-249, 250, 251, 252).

(i) role of intravascular volume

The mechanism by which ANP reduces CO is not well characterized. In the species studied, ANP does not appear to affect cardiac contractility. ANP did not have a direct negative inotropic effect in the heart in situ (252) or in isolated ventricular and atrial muscle strips (252, 253, 254). However, in isolated rat hearts, atriopeptin II (APII) caused coronary vasoconstriction (255). In chronically instrumented dogs, infusion of atriopeptin III (APIII), although reducing left ventricular end-diastolic diameter, had no effect on left ventricular function (7, 256). Similarly, intracoronary injection of ANP was without effect (257). Cardiac performance did not differ between anesthetized rats receiving an intravenous infusion of ANP and control rats coinjected with phenylephrine to restore ABP to pre-ANP injection level (258), thus indicating that ANP-mediated changes in ABP and CO were not mediated by a negative inotropic effect of the hormone.
The primary cause for ANP-induced reduction of CO is a decrease in ventricular preload. This is due to a reduction in cardiac filling pressures (Fig. 1.5), and has been seen in rats (247, 248, 252, 259), rabbits (260) dogs (7, 250, 257, 261)), sheep (6) and human (251, 262). Theoretically, a decrease in cardiac filling pressure could be induced by a series of interacting factors, including an improvement in diastolic function and a decrease in venous return due to an increase in venous capacitance. Regarding the first possibility, if ANP improved ventricular end diastolic function, it is expected that this would be accompanied by an increase in left ventricular diastolic volume. This is unlikely, since ANP was found to have the opposite effect (6, 7, 261, 262). With respect to the second possibility, ANP was found to reduce venous filling by decreasing blood volume (7, 8, 242, 263-266) and by increasing resistance to venous return (7, 267, 268) (Fig. 1.5).

Administration of ANP leads to a decrease in plasma volume, and concomitant increase in hematocrit (7, 8, 264-266). In conscious rats, Sugimoto et al (8) showed that blood volume decreases immediately following a bolus injection of ANP. The effect is rapidly attenuated, achieving a minimum after 15 minutes. Thereafter blood volume returned gradually to control values. The ANP-induced reduction of blood volume is also present in nephrectomized animals (7, 243, 260, 263, 265, 269) suggesting that the renal effects of the hormone are not essential for its effects on blood volume. The mechanism by which ANP decreases plasma volume is not completely understood. The shift of plasma fluid between the intravascular and extravascular space is determined by capillary hydrostatic pressure, plasma oncotic pressure, and to a lesser degree by capillary permeability (capillary hydraulic conductivity). To this point there is no direct evidence that ANP affects capillary hydrostatic pressure; however, Sarelius et al (270) showed that local application of pharmacological concentrations of ANP to isolated cheek pouch arterioles of anesthetized golden hamsters selectively dilates terminal arterioles that are involved in the distribution of blood flow to the capillary beds. On
ANP

- stimulates baroreceptors
- stimulates cardiopulmonary receptors
- stimulates parasympathetic outflow to S-A node

inhibits sympathetic outflow

- reduces intravascular volume
- venodilation (?)
- reduced TPR (?)

- increased RVR

reduced preload

- decreased cardiac filling pressure

- decreased SV

- decreased CO

- HR

HYPOTENSION

Figure 1.5. Acute cardiovascular effects of ANP. (See text for details)
the other hand, Sugimoto et al (8) showed that ANP significantly elevates the capillary filtration coefficient in anesthetized rats undergoing volume expansion with isotonic saline. A similar conclusion was reached by Huxley et al (271) who showed that perfusion of frog mesenteric capillaries with $10^{-5}$ M ANP causes a four fold reversible increase in hydraulic conductivity, resulting in an increase in transcapillary flux of water independently of changes in hydrostatic pressure or capillary surface area. In addition, ANP increases albumin permeability coefficients in isolated capillaries (272) and augments transcapillary protein escape, both in normovolemic animals (273, 274) and during acute volume loading (275). These observations suggest that ANP reduces intravascular volume, at least partially, by increasing capillary filtration of plasma fluid. Interestingly, ANP was found to increase the permeability of the endothelium to thrombin (276). This interaction of ANP with capillary endothelial cells may be the mechanism mediating the ANP-induced increase in capillary filtration, given the primary role of endothelium in regulation of transcapillary fluid movement (265, 277). In addition to effects on capillary permeability, there is some evidence that ANP may also reduce whole body capillary absorption, possibly by decreasing the precapillary to postcapillary resistance ratio (265, 268, 270, 278). ANP further reduces intravascular volume by inhibiting spontaneous pumping activity in lymphatic vessels (279, 280, 281), thereby reducing lymph flow (281).

The other major determinant of venous capacitance is venous tone. Most evidence indicates that ANP has no direct effect on venous tone in vitro (23, 24, 282, 283) or in vivo (7, 103, 284-286). However, ANP has been shown to increase resistance to venous return (RVR) in rats and dogs (7, 259). In rats, ANP infusion significantly reduces ABP in association with decreases in central venous pressure and cardiac output (259). The reduction in cardiac output occurs in the absence of changes in mean circulatory filling pressure, total peripheral resistance, or splanchnic and skeletal muscle.
blood flow, and was not attributed to the diuretic effects of the hormone. However, the calculated resistance to venous return is significantly increased during ANP infusion, suggesting that this effect may contribute to the decrease in cardiac output. The mechanism by which ANP may lead to an increase in resistance to venous return is not known. There is some evidence that it may be brought about by sympathetic-mediated increases in postcapillary resistance subsequent to the initial volume-dependent decrease in CO and hypotension (259).

(ii) role of cardiovascular autonomic reflexes

The acute hypotensive effect of ANP, unlike that of other agents (i.e. sodium nitroprusside), is not accompanied by reflex tachycardia (3, 7, 10, 259, 264, 287-289), and, in some cases, is associated with bradycardia, suggesting that interactions with elements of cardiovascular autonomic reflex regulation may contribute, at least in part, to the reduction in cardiac output that precedes the fall in blood pressure. There is evidence that ANP exerts generalized cardiosuppressive effects by modulating cardiovascular autonomic reflex responses, both peripherally and centrally (3, 9, 260, 290, 291). Such effects have most frequently been observed in response to bolus injections of exogenous peptide and are transient in nature. In an early study, Ackermann et al (3) showed that injection of ANP-rich atrial extract into anesthetized rats caused a drop of ≈ 20 mm Hg in mean arterial pressure (MAP), that was associated with a decrease in CO and stroke volume. The response appeared to be due, in part, to a depression of heart rate (HR) and decrease in cardiac performance. The decreases in MAP and HR were partially overcome by prior vagotomy, and it was suggested that the hypotensive effect of ANP was partly due to stimulation of vagal afferents originating in cardiopulmonary mechano- and chemoreceptors (likely those receptors mediating the von Bezold-Jarisch reflex). This finding was subsequently confirmed with synthetic ANP (290, 292). More recently, Atchison and Ackermann showed that ANP decreases heart rate in cardiac-denervated rats
by enhancing parasympathetic activity, independent of the underlying level of sympathetic tone (293). The negative chronotropic effect of ANP is not due to activation of postsynaptic receptors in pacemaker cells (294), and appears to be mediated via interaction with presynaptic α-adrenergic receptors located on parasympathetic efferent nerves (295).

ANP may also alter ABP indirectly by interfering with the activation of the aortic and carotid baroreceptors (9, 10, 85, 219, 290, 296). In vagotomized rats with intact arterial baroreceptor innervation, ANP inhibits the expected hypotension-driven reflex tachycardia (219). The suppressive effect of ANP is greatly attenuated by denervation of the aortic baroreceptors (219, 290). In humans, ANP blunts reflex tachycardia in response to an increase in external pressure to the neck, and potentiates the reflex bradycardia associated with neck suction (289). These findings suggest that ANP inhibits the compensatory arterial baroreflex response to hypotension, possibly by stimulating baroreceptor activation, despite hemodynamic conditions that would favour their unloading. The effect of ANP on arterial baroreceptors is thought to be mediated primarily by circulating peptide, however, the expression of the ANP gene in the aortic arch (297) suggests a potential paracrine role of locally-produced ANP on baroreceptor function.

b) Effect of ANP on peripheral vascular resistance

Another major site of acute regulation of mean arterial pressure is the segment of the vasculature that is involved in control of peripheral resistance, namely, the small diameter arteries and arterioles in the different regional vascular beds. Frequently, the hypotensive response to bolus administration of ANP is accompanied by a transient decrease in total peripheral resistance (TPR) (2, 3, 245, 247, 298), suggesting that the acute hypotensive effect of this peptide may be caused, in part, by relaxation of the resistance vasculature.
(i) direct vascular effects of ANP

ANP causes relaxation of several preconstricted vascular preparations in vitro (2, 23, 282, 299, 300), but there is pronounced regional heterogeneity in the magnitude of this response. In general, large arteries are the most susceptible to direct relaxation by ANP. Faison et al (23) showed that segments of the thoracic and abdominal aortae and the renal artery were relaxed by ANP. The more distal arteries are less sensitive to ANP (282, 299, 300). In vitro it has been difficult to show ANP-dependent relaxation of the small resistance arterioles, even when maximally preconstricted (23, 299). In organ bath preparations, high concentrations of ANP fails to relax resistance arteries from the coronary (301), mesenteric (22, 31), skeletal (28-30) and cerebral (31) vascular beds. Only the renal vasculature is relaxed by ANP (22, 25), suggesting that ANP may function as a renal-specific vasodilator. Thus, on the basis of this evidence, it is unlikely that the acute hypotensive effect of ANP is caused by direct relaxation of the resistance vasculature.

In responsive blood vessels, the direct relaxant effect of ANP occurs independently of the endothelium (24, 299, 302) and appears to be mediated primarily by interaction with GC-linked receptors (27, 299, 303-306) (Fig. 1.6). The concomitant rise in cGMP inhibits the translocation of extracellular calcium into the smooth muscle cell (307) and the mobilization of calcium from the sarcoplasmic reticulum (308), and stimulates the activity of the sarcolemmal Ca\(^{2+}\)-ATPase (309). The resultant reduction in intracellular calcium concentration leads to dephosphorylation of myosin light chain and relaxation. The inhibitory effect of ANP on VSM calcium fluxes has been suggested to be associated with inhibition of phosphoinositide hydrolysis (310), and this possibly explains, in part, the greater efficacy of ANP in relaxing vessel segments precontracted by vasoconstrictor agents such as ANG II and norepinephrine (50). The ANP-mediated increases in cGMP in VSM also lead to stimulation of the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport (311) and Na\(^+\)/H\(^+\) antiport (312) systems in rabbit and rat
aorta explants resulting in intracellular accumulation of sodium. The role that these changes in ionic currents may play in mediating the relaxant effect of ANP, however, is controversial. Nevertheless, the vasodilatory action of ANP on the aorta is attenuated in low sodium buffer (313), and the in vivo hypotensive effect of ANP is potentiated in animals maintained on high dietary salt intake (314).

The physiological significance of such direct, cGMP-mediated vasodilation by ANP, however, is questionable. First, the vasculature expresses an overwhelming preponderance (>90%) of ANP "C" (R2, clearance) receptors (174, 315), that are not involved in mediating ANP-dependent relaxation. Secondly, expression of GC-A receptors in VSM is mostly confined to large arteries such as the aorta (for review see ref. 27), and is absent in resistance vessels (26, 27), with the exception of the renal vasculature (22, 25, 316). Third, the cGMP-mediated vasodilatory activity of ANP requires, in most cases, pharmacological concentrations of exogenous peptide (for review see ref. 299). In addition, there are several instances in which ANP induced vasodilation occured.

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**Figure 1.6.** Intracellular signal transduction mechanisms mediating vasodilatory and antimitogenic effects of ANP in a prototypical vascular smooth muscle cell from a large artery. Similar mechanisms of transduction for ANP have been found in other responsive cell types. (See text for details)
independently of GC-A activity, suggesting the possible existence of additional mechanisms of ANP-mediated vasodilation. For example, a structural analogue of ANP (ANP<sub>103-125</sub>) potently relaxes rabbit aortic rings without stimulating guanylate cyclase (317). Also, the GC inhibitor, LY83583, although preventing the cGMP response to ANP, does not reduce its relaxant effect in aortic rings (312). Furthermore, inhibition of the GC receptors with the antagonist HS-142-1 fails to inhibit ANP-dependent vasodilation <i>in vivo</i> (318) and in rabbit aortic rings (312) despite suppressing cGMP production. These observations, however, do not negate a role of GC-A receptors in mediating ANP-dependent vasodilation. Mice harboring a deletion of the GC-A receptor gene are chronically hypertensive with respect to their wild-type siblings (20, 320). The rise in MAP is associated with significant increases in systolic and diastolic blood pressures, and is accompanied by marked cardiac hypertrophy (321), suggesting that the hypertension in these mice is, at least partially, determined by elevated peripheral resistance. Also, mice expressing different copy numbers of the GC-gene, ranging from one-half normal (1 copy) to twice normal (4 copies), display parallel changes in ABP; the mice with one copy being hypertensive, and the mice with multiple copies (3 and 4 copies) being hypotensive relative to the normal (2 copies) mice (229). These findings provide concrete evidence that ANP exerts a tonic hypotensive effect that is partly dependent on GC-A receptor activity. However, given the scarcity of these receptors in resistance vessel smooth muscle (26, 27), and the relative insensitivity of these vessels to direct relaxation by ANP (28-31, for review see ref. 299), it is likely that the GC-A-dependent effect of ANP on vascular resistance is mediated by indirect mechanisms, possibly via interactions with other vasoregulatory systems, such as the endothelium, the sympathetic nervous system, and vasoactive hormonal systems such as the renin-angiotensin-aldosterone (RAS) axis. Also, there is the possibility that GC-A-mediated decreases in vascular resistance may result from compensatory autoregulatory adjustments in regional resistance and blood
flow, secondary to ANP-induced decrease in venous return and cardiac output.

(ii) indirect vascular effects of ANP

As previously mentioned, when administered as a bolus, ANP, depending on the species, may lead to a transient fall in calculated peripheral resistance (2, 3, 244, 245, 247, 298). In anesthetized rats, injection of ANP leads to dose-dependent decrease in MAP that is accompanied by significant decreases in renal, mesenteric and muscle vascular resistances measured with implanted doppler flowmeters (244), or with radioactive microspheres (322, 323). Similar reduction in calculated TPR was observed in conscious dogs (250, 317). The reduction in MAP is not followed by compensatory reflex tachycardia, suggesting that the instantaneous decrease in peripheral vascular tone that follows injection of a large hypotensive dose of ANP may be due to inhibition of cardiovascular sympathetic tone (244, 250, 323, 324). However, this sympathetic-mediated decrease in TPR is, likely, pharmacological in nature, since the doses administered in these studies, in most cases produced rises in plasma ANP concentrations that were above the pathophysiological range. When ANP is infused, on the other hand, the ensuing hypotension is frequently accompanied by a significant increase in TPR (4-6, 248, 249, 252, 259, 268, 289, 325) and is sustained by the decrease in CO. This is indicative that over time, the sympathoinhibitory effect of ANP, eventually, is overridden, possibly by baroreflex-mediated increase in sympathetic outflow. Alternatively, this increase in TPR may reflect local compensatory autoregulatory adjustments to the decrease in CO. For example, infusion of ANP into conscious dogs (326, 327), rats (328, 329) and rabbits (287) subjected to autonomic ganglionic blockade, reduced ABP dose-dependently in association with a concomitant fall in CO, and a rise in TPR. The increase in vascular resistance likely represents an autoregulatory response to the decrease in CO, rather than a direct constrictor effect of ANP.
2. Chronic cardiovascular effects of ANP

a) Role of peripheral vascular resistance

When ANP is infused over a period of several days, the accompanying hypotension is maintained by a gradual hemodynamic shift towards a fall in peripheral vascular resistance (TPR) from the initial (acute) decrease in CO (11-15). The fall in TPR sustains the chronic hypotensive effect of ANP for the remainder of the treatment, as CO gradually returns to the preinfusion level (11-14). In conscious normotensive rats (11, 12) dogs (13) and sheep (14), a 5-day infusion of ANP, producing sustained plasma ANP concentrations in the pathophysiological range, leads to a significant fall of ~15% in MAP on days 1 and 5. The initial decrease in MAP is associated with a fall in CO concomitant with an increase in TPR. By day 5, TPR and CO had returned to preinfusion values, indicating that TPR had decreased and CO had increased from their respective levels on day 1. In a recent study, Charles et al (15) showed that chronic infusion of ANP in sheep at a rate that barely increased the basal plasma concentration of the hormone, causes a significant decrease in calculated total peripheral resistance and MAP within 24 hours of beginning the infusion, in the absence of any changes in CO. A similar hemodynamic profile is observed in genetic mouse models harboring lifelong alterations in endogenous ANP activity (16-20). Transgenic mice overexpressing a transthyretin-ANP fusion gene constitutively in the liver (TTR-ANP) have ~20-25% lower ABP than their genetically-matched wild-type counterparts (NT), in association with chronic 8-10 fold elevation in plasma ANP concentration (16, 17). The marked hypotension in the TTR-ANP mice is characterized by 21% lower TPR and accompanying 27% decrease in total heart weight compared to the NT, whereas CO, stroke volume and heart rate does not differ significantly between genotypes (18). The lower TPR in the TTR-ANP mice is associated with 19%-35% reductions in vascular resistances in most regional vascular beds, with the exception of the coronary and splanchnic circulations (18), as assessed with
the reference microsphere technique. In contrast, "knockout" mice harboring deletions of the ANP (19) or GC-A receptor (20) genes are markedly hypertensive throughout life, with respect to their wild type siblings. Although the systemic hemodynamics in these knockout mouse models of ANP activity have not been characterized, indirect evidence suggests that the hypertension is caused, at least in part, by elevated peripheral resistance. For example, the marked cardiac hypertrophy and elevated basal diastolic pressure that are observed in these knockout mice likely develop in response to an increase in peripheral vascular resistance and ventricular afterload (19, 20).

E. INDIRECT MEDIATION OF CHRONIC HYPOTENSIVE ANP EFFECTS

It is evident from the preceding discussion that the chronic hypotensive effect of ANP is mediated by a reduction in peripheral vascular resistance.

The mechanism by means of which ANP chronically reduces TPR is not known. Although the early phase of the hemodynamic transition to lower TPR may, possibly, be due to whole-body autoregulation in response to the acute fall in CO (11, 13, 14), it is unlikely that this mechanism is capable of maintaining the sustained fall in TPR seen during prolonged ANP infusions (11-14) and in the TTR-ANP mice (18), since the autoregulatory mechanism(s) would be expected to progressively increase TPR as CO returns to basal level over time, restoring normal ABP. On the other hand, as previously described (28-31, 299), the resistance vasculature, with the possible exception of the renal vascular bed (25, 27) has a scarcity of GC-A receptors and is insensitive to direct relaxation by ANP (26, 27). This suggests that the chronic ANP-dependent dilation of the resistance vasculature may be mediated by interactions with intermediary vasoeffectector mechanisms whose single or combined actions reduce peripheral vascular resistance. In this regard, ANP has been shown to modulate the activity of several vasoregulatory systems, including the vascular endothelium.
(VE) (32) and the autonomic nervous system (33). Specifically, when administered acutely, ANP inhibits the synthesis of locally-acting vasoconstrictor peptide ET-1 (36-38) and potentiates the synthesis and release of locally-acting vasodilators CNP (39) and possibly NO (40, 41) from the vascular endothelium (VE). In addition, ANP modulates the target cardiovascular effects of these vasoactive agents (42, 184, 330), and exerts a generalized sympatholytic effect by inhibiting central (43, 44) and peripheral (45, 46) sympathetic nervous activity.

The extent to which these effects of ANP may be chronically active is not known. However, some preliminary evidence suggests that ANP can tonically alter the activity of these vasoregulatory systems. In dogs with congestive heart failure, plasma ET-1 concentration is significantly increased following antagonism of GC-A receptor function with HS-142-1, suggesting that the underlying elevated plasma ANP concentration in these animals is exerting a tonic inhibitory effect on endothelin synthesis (331). Also, CNP-dependent vasodilation is enhanced in CHF (332), possibly due to reduced clearance, consequent to ANP-induced downregulation of endothelial C-receptors (184).

In general, the chronic hypotensive effect of ANP is accentuated in situations characterized by elevated basal sympathetic tone, such as in the spontaneously hypertensive rat model (SHR) (121, 333, 334). Also, in normal Sprague-Dawley rats, ANP significantly attenuates the hypertension caused by chronic infusion of norepinephrine (NE) (335), suggesting that the hypotensive action of ANP is partly mediated by interactions with noradrenergic mechanisms of blood pressure regulation. Also, HR decreases (12) or remains unchanged (13, 15) during chronic infusion of ANP, and does not differ between TTR-ANP and NT mice (16-18), despite the chronic decrease in ABP. Given the tonic influences that the VE (32, 336) and SNS (337) exert on vascular tone, it is conceivable that interactions of ANP with these vasoregulatory systems, if tonically active, may represent an effector mechanism by means of which this hormone exerts its chronic vasodilatory effect. Thus, ANP could
decrease peripheral vascular resistance chronically by concurrently regulating the synthetic activity of the VE in the resistance vasculature, such that the vasodilatory moieties NO and CNP are potentiated and the vasoconstrictor ET-1 is reduced, as well as by attenuating the activities of SNS.

An additional component of chronic regulation of ABP by ANP may involve interactions of this hormone with ECF volume-regulating mechanisms. There is evidence that ANP opposes the antidiuretic and antisuuretic actions of other renal regulatory mechanisms. It acutely inhibits renal sympathetic nerve activity (219, 290, 338), vasopressin-dependent increase in water permeability in the distal tubule (339), renin (47, 215), angiotensin converting enzyme (ACE) (340) and aldosterone (48) activities, and antagonizes ANG-II-dependent sodium reabsorption from the proximal tubule (49). It may be speculated that such ANP-mediated antagonism of these mechanisms, if tonically active, could contribute to the chronic hypotensive effect of this hormone. This antagonism would be expected to assume particular physiological significance in long-term regulation of ABP in conditions in which the activity of these salt-retainilng mechanisms needs to be maximally suppressed, such as during dietary salt loading (341); or when these mechanisms are pathologically activated by circulatory insufficiency, such as in CHF (235). The extent to which these interactions occur chronically remains unclear.

1. Endothelial factors

Vascular endothelium, a layer of epithelial-like cells lining the lumen of all blood vessels and the heart (342) is involved in the homeostatic regulation of nearly all aspects of cardiovascular function (32, 336, 343-346). Owing to its anatomical localization, the VE is optimally suited for its physiological role as a mechano- and chemical sensor and transducer of local hemodynamic or chemical changes, eliciting appropriate compensatory adjustments in vascular and/or cardiac function to restore homeostasis. The modulatory actions of VE on cardiovascular function are mediated
primarily via production and release of a variety of vasoactive substances that are loosely classified into two broad categories as relaxing factors, of which NO, CNP, prostaglandins, carbon monoxide and endothelium-derived hyperpolarizing factor (EDHF) are the most prominent, and contracting factors, such as ET-1 and thromboxanes (32, 336). These locally-acting substances alter the contractility of the underlying vascular smooth muscle (VSM) and bring about adjustments in vascular tone, in response to acute and chronic changes in blood flow, shear stress, transmural pressure and oxygen tension (PO₂) (336). In addition to their role in these minute-to-minute autoregulatory adjustments to local hemodynamic and chemical changes, endothelial factors such as NO and ET-1, and, likely, CNP contribute to maintenance of long-term constancy of ABP by exerting tonic effects on the resistance vasculature (32, 347, 348).

The endothelial cell membrane has a wide variety of receptors for several neurotransmitters and hormones that can influence the synthesis and release of endothelial factors (32, 349, 350). ANP could potentially exert its chronic vasorelaxant effect by modulating the synthesis and/or the target actions of locally-acting vasoactive endothelial factors (Fig. 1.7). Vascular endothelium (VE) is the preferential site of interaction with ANP in the vasculature (27, 174, 315), and endothelial cells from various regions of the vascular tree, including resistance vessels, express both C and GC-A receptors and bind ANP with high affinity (for review see ref. 27). In vitro, ANP inhibits the synthesis of the potent vasoconstrictor peptide ET-1 (36-38) and stimulates synthesis of vasodilator CNP (39) and possibly NO (40, 41) from cultured endothelial cells. Although these ANP-endothelium interactions remain largely untested in vivo, they provide a sound rationale for a working hypothesis that ANP may elicit its vasodilatory action by modulating the synthetic activity of vascular endothelium. Conceptually, the vasodilatory activities associated with CNP and NO are potentiated concurrently with inhibition of the vasoconstrictive activity of ET-1, to produce a net vasodilatory influence from
the endothelium.

a) Nitric oxide (NO)

Nitric oxide is a very labile free radical gas produced from oxidation of the terminal guanidino nitrogen of L-arginine by a group of enzymes that are collectively referred to as nitric oxide synthases (NOS) (351). At least three isoforms of NOS have been identified on the basis of their molecular structure and calcium dependency for activity. These enzymes are ubiquitously distributed in the cardiovascular system and in other tissues (for review see ref. 352). In the vasculature, NO is continuously synthesized and released from the endothelium by the constitutive endothelial nitric oxide synthase isoform (ecNOS) and this largely accounts for the cardiovascular actions of NO under normal conditions (353). The activity of ecNOS is upregulated by agonists that increase intracellular calcium, such as ANG II and catecholamines (32, 349). NO is also produced in high amounts by cardiac myocytes and VSM after stimulation of an inducible NOS isoform (iNOS) by cytokines and/or bacterial lipopolysaccharide (351, 354), but this is thought to have a greater relevance in pathophysiological situations such as in septic shock (355).

(i) biochemistry of NO production and action

All three NOS enzyme isoforms share a relatively high degree (50-55%) of structural homology within species, and their genetic sequences are highly conserved across species (352, 356). However the NOS enzymes differ significantly in their mechanisms of regulation of gene expression, catalytic activity and tissue distribution and abundance (354, 357, 358), with ecNOS mostly confined to vascular endothelium (358, 359) and to a lesser extent in cardiac myocytes (360), VSM (41) and renal tubular epithelium (361) and in some populations of neural cells (357). On the other hand, iNOS and nNOS have a more ubiquitous pattern of distribution in a variety of neural and non-neural cell types, including VSM, myocardium, skeletal muscle, enteric nervous system, renal tubular and
Endothelial cell

Vascular smooth muscle cell

Figure 1.7. Interactions of ANP with vasoactive secretory pathways in vascular endothelium and action in vascular smooth muscle.
juxtaglomerular cells and in macrophages (354, 356).

The NOS enzymes are cytochrome P-450-like hemeproteins, 135-155 KDa with two distinct catalytic activities; an oxidase domain located at the amino (NH₂) terminus and a reductase domain at the carboxyl (COOH) terminus (354). All NO synthases function as homodimers (354-356) and contain binding sites for calmodulin (CaM), flavin adenine mono- and dinucleotide (FAM, FAD), nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (THB₄) and cytochrome P-450. CaM, FAM, FAD, NADPH and THB₄ function as cofactors and prosthetic group respectively and are required for homodimerization and catalytic activity (for review see ref. 354). In addition, ecNOS undergoes co-translational myristoylation of an NH₂ terminal glycine residue (362) and post-translational palmytoylation of two cysteine residues (363). These modifications target and stabilize ecNOS in specialized glycosphingolipid:cholesterol-rich invaginations in the cell membrane called caveolae (358, 364). These structures function as signal transduction domains for ecNOS and other regulatory proteins (358). In the caveolae, ecNOS associates with caveolin (358, 364), a "scaffolding" integral membrane protein that provides structural support for proteins and lipids within the membrane. The activity of ecNOS is inhibited by this association with caveolin (358, 366, 367). The reversal of this inhibition is dependent on a rise in intracellular calcium (358, 368) which binds to calmodulin and interacts with the CaM domain in ecNOS, thereby promoting dissociation of the ecNOS-caveolin heteromeric complexes (358). The overall activity of ecNOS is regulated by this dynamic cycle of association/dissociation with caveolin and by depalmytoylation and translocation of the enzyme to the cytosol which also decreases ecNOS activity (358, 366). The activity of nNOS also shows dependence on Ca-CaM, but no such association with caveolin has been described, and the enzyme is localized in the cytosol. The inducible NOS (iNOS), on the other hand, has a much lower calcium threshold for catalytic activity, because it binds CaM tightly (354, 358, 370).
Both ecNOS and nNOS display basal constitutive activity (45, 354, 370-373), and agonist-mediated upregulation is largely dependent on activation of a pre-formed enzyme pool (370-373), whereas induction of iNOS activity requires de novo protein synthesis and takes several hours (374). There is evidence that both ecNOS and nNOS gene expression can also be induced in response to certain physiological stimuli, such as, for example, a chronic elevation in hemodynamic shear stress (357, 373, 376). The ecNOS and nNOS genes contain several consensus sequences in the 5'-flanking promoter region that may be involved in transcriptional regulation of gene expression. Most notable, is the identification of AP-1/ AP-2 (activator protein-1/2), CRE (cAMP-responsive element) and NF-1 (nuclear factor-1) cis-acting sequences that may mediate gene expression in response to phorbol esters/PKC, cAMP and TNF-1 (for review see ref. 357). For example, cAMP significantly decreases steady-state mRNA levels of ecNOS in endothelial cells and in cardiac myocytes (377), and cGMP increases both ecNOS and iNOS mRNA in cultured endothelial cells (41) and VSM (378) respectively, in part by stimulating gene transcription and by increasing mRNA stability. Also, staurosporine, an inhibitor of PKC, significantly increases ecNOS mRNA in bovine aortic endothelial cells and this effect is mimicked by prolonged (24 hr) incubation with phorbol ester (379), which downregulates PKC activity, thus indicating that sustained activation of PKC by agents such as vasoconstrictors (i.e. ANG II, ET-1) may inhibit ecNOS gene expression. The ecNOS gene also contains a shear-stress responsive element which is likely involved in mediating transcriptional upregulation of this gene in response to chronic increases in shear stress (357, 375). Mature ecNOS, nNOS and iNOS proteins have consensus sequences that are susceptible to posttranslational phosphorylation by protein kinase C and A (PKC, PKA) and calmodulin-activated protein kinase (CaMK) (379-381). The functional role of phosphorylation in regulation of NOS catalytic activity, however, is not fully established. All three isoforms have been isolated from cell culture as
phosphoproteins (for review see ref. 358). The activity of ecNOS is reversibly decreased after brief incubation with agonists of PKC and PKA in cultured endothelial cells (379-381) and cardiac myocytes (377-382), in association with phosphorylation of serine residues in the enzyme (358, 364, 381). The phosphorylation of ecNOS is accompanied by translocation from the cell membrane to the cytosol (358, 381), suggesting that this may be one mechanism of regulation of catalytic activity. In cultured bovine aortic endothelial cells, an increase in flow-induced shear stress leads to a potent biphasic increase in NO production that parallels an increase in phosphorylation of tyrosine and serine residues in ecNOS (383). The effect of shear stress is attenuated by inhibition of tyrosine kinase activity (383) and potentiated by inhibition of tyrosine phosphatase (384), suggesting a role of tyrosine kinase-mediated phosphorylation of ecNOS in mediating the response to increased shear stress. The possible physiological significance of protein kinase-mediated phosphorylation of ecNOS is that it may serve as a mechanism of “cross-talk” by which other signal transduction pathways regulate the activity of the enzyme. The role of posttranslational phosphorylation in the activity of the other NOS isoforms have not been as intensely studied as ecNOS. A recent study suggests that iNOS activity is increased by tyrosine phosphorylation (385), and the activity of nNOS is not affected by PKA or PKC (358).

The basic mechanism of catalysis that leads to formation of NO is similar for the three isoforms. The reaction requires molecular oxygen, L-arginine as substrates, NADPH as an electron donor and Ca-CaM, THB₄ and flavin nucleotides as cofactors and electron acceptors respectively. This reaction is competitively inhibited by guanidino nitrogen-modified analogues of L-arginine, such as N⁰-monomethyl-L-arginine (L-NMMA) or N⁰-nitro-L-arginine methyl ester (L-NAME) (354, 370). NO is formed by a 5 electron oxidation of the terminal guanidino nitrogen of L-arginine by two successive monooxygenation reactions (354, 386-389). The first monooxygenation step converts L-
arginine to $\text{N}^\omega$-hydroxyarginine. The second step involves oxidation of hydroxyarginine to form NO and citrulline (Figure 1.8). NO exerts its diverse autocrine and paracrine biological effects by activating soluble guanylate cyclase (sGC) which produces cGMP from GTP in the cytosol. cGMP serves as the intracellular mediator of NO effects by participating in phosphorylation of target proteins (390) (Figure 1.8). There is evidence that some of the biological effects of NO may be mediated by cADP-ribose, a cyclic nucleotide second messenger derived from NAD$^+$ (391).

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**Figure 1.8.** Molecular mechanism of nitric oxide synthesis and action in target tissues.
(ii) role of NO in cardiovascular regulation

The participation of NO in determination of ABP is well established. Both acute (385) and chronic (393, 394) inhibition of nitric oxide synthesis with guanidino substituted analogues of L-arginine (i.e. L-NMMA, L-NAME) result in a sustained increase in arterial blood pressure that is reversed by L-arginine. Furthermore, mice harboring a deletion of the ecNOS gene are hypertensive compared to their normal littermates (395, 396). Finally, NO synthesis is impaired in some cardiovascular diseases such as in hypertension (397, 398) and congestive heart failure (399).

In the microvasculature, NO release from the endothelium plays a fundamental role in regulation of basal arteriolar tone (for review see ref. 348, 400, 401). In anesthetized rabbits (392), cats (401) rats (402) and dogs (394, 403), and in chronically instrumented conscious rats (404), inhibition of NO synthesis with L-NMMA or L-NAME causes dose-dependent increases in ABP in association with vasoconstriction and reduction of blood flow in most vascular beds. These effects are reversed by L-arginine, thereby implicating a role of endothelium-derived NO in tonic modulation of arteriolar resistance. The mechanism by which NO decreases vascular tone locally appears to involve direct relaxation of arteriolar smooth muscle (405, 406), buffering of vasoconstrictor moieties, such as \( \text{ANG II} \) and ET-1 (407-409), and presynaptic inhibition of sympathetic neurotransmission in the perivascular nerves (410-414). New evidence is emerging that some of the perivascular nerve fibers express the neuronal type NOS (nNOS) and synthesize and release NO as a vasodilatory neurotransmitter (415-418). These so-called nitrovasodilator nerves are particularly abundant in cerebral arteries, and have also been localized peripherally in skeletal muscle and skin vascular beds (for review see ref. 416), where, together with noradrenergic vasoconstrictor nerves, they may exert reciprocal control of vascular tone.
The constitutive nature of ecNOS activity implies that this enzyme is optimally suited to respond to local hemodynamic and chemical changes. Flow-induced dilation of the coronary and skeletal muscle vascular beds in rats is prevented by inhibition of NO synthesis (400, 419, 420), and pressure-induced myogenic arteriolar constriction is opposed by NO in some of the species studied (421, 422). Coronary vasodilation in isolated guinea pig hearts perfused with hypoxic saline was associated with a rapid overflow of cGMP into the effluent, later followed by accumulation of adenosine (423). The hypoxic vasodilatory response and accompanying rise in cGMP spillover is greatly attenuated by NO inhibition with L-NMMA, suggesting that coronary hypoxic vasodilation is partially mediated by an instantaneous increase in NO synthesis. Collectively, these findings indicate that locally-derived NO plays a crucial role in mediating autoregulatory adjustments in blood flow in response to acute changes in hemodynamic shear stress, transmural pressure and hypoxia.

In addition to its direct effects on vascular tone, NO may also affect ABP via its modulatory actions on cardiac function (for review see ref. 346) and on neural cardiovascular regulatory elements. Cardiac endothelium influences cardiac contractility indirectly by regulating coronary vascular tone (419) and, consequently, coronary flow, upon which metabolic activity is dependent, and directly by modulating the activity of myofibrillar contractile proteins (346, 424-426). Stimulation of endothelial cell-cardiac myocyte co-cultures with bradykinin, a known agonist of NO release, significantly reduces myocyte contraction in a fashion that is inhibited by L-NMMA, indicating that NO mediates the negative inotropic effect of bradykinin (427). Also, exogenous NO attenuates contraction significantly in isolated, electrically-stimulated guinea pig ventricular myocytes (427). These results suggest that NO released from cardiac endothelium may function as a paracrine relaxing factor of contractile activity of the myocardium. This has been suggested to function as an extrinsic mechanism of regulation of cardiac performance serving as an accessory to intrinsic cardiac
autoregulation. (for review see ref. 346).

In the central nervous system, nNOS is localized in close proximity to cardiovascular regulatory areas in the medulla (416) and NO is released in these areas upon stimulation of glutamate (N-methyl-D-aspartate, NMDA-sensitive) receptors that are known to be involved in modulation of peripheral autonomic outflow (428). This suggests that locally-derived NO may function as a neurotransmitter or neuromodulator of central cardiovascular regulatory activity. The hypertensive response to systemic inhibition of NO synthesis with analogues of L-arginine is usually accompanied by a paradoxical increase or no change in heart rate and an increase in renal sympathetic nerve activity (RSNA) (429), suggesting that NO may exert its hypotensive effect partly by inhibiting peripheral sympathetic activity. The rise in RSNA in response to inhibition of NO is significantly attenuated by cervical transection of the spinal cord and potentiated by baroreceptor deafferentation, indicating that NO may potentiate baroreceptor sensitivity and suppress central sympathetic outflow. Recent work indeed provides evidence for a central vasodepressor effect of NO. Injection of a nitric oxide donor or L-arginine into the rostral ventrolateral medulla in cats (430) and in rats (431) greatly reduces RSNA, HR and ABP, an effect that mimics baroreflex activation. Conversely, intracisternal injection of N\textsuperscript{\textalpha}-methyl-L-arginine or microinjection of L-NMMA into the lateral ventral medulla or the nucleus tractus solitarius (the principal central sensory nucleus for most cardiovascular afferent fibers) significantly increases RSNA, HR and ABP in anesthetized rats (432) and in rabbits (433). Furthermore, L-NAME reduces single unit discharge rate of of a population of neurons in the nucleus tractus solitarius of Fischer-344 rats in a manner that is rapidly reversed by L-arginine or nitroglycerin (434). These results imply that NO locally produced in the medulla oblongata by nNOS may participate in reflex and tonic modulation of cardiovascular sympathetic outflow. On the other hand, nNOS knockout mice, apart from displaying excessive aggressive behaviour, do not show any
obvious defect in cardiovascular regulation (435). These mice do not develop hypertension, and this cannot be attributed to supercompensation by the other NO synthases (i.e. ecNOS, iNOS). Thus, the physiological significance of nNOS-derived NO in central ABP regulation remains to be fully established.

Chronic inhibition of NO synthesis also leads to a sustained and reversible increase in ABP (393, 404, 436). It is likely, on the basis of the hemodynamic characteristics of ecNOS mice (395, 396), and the constitutive nature of this enzyme, that the hypertension associated with chronic NO inhibition is mostly due to inhibition of basal endothelial NO production. However the potential contribution of nNOS inhibition to this effect cannot be entirely discounted. In rats maintained on normal or low salt diets, L-NAME administered to the drinking water for up to six weeks significantly raises blood pressure by the same relative amount in both groups (437). The hypertension is associated with a marked decrease in whole brain NOS activity (mostly derived from nNOS) and significant increases in plasma catecholamines and renin activity. Furthermore, acute inhibition of ANG II or α-adrenergic receptors reduces ABP in the L-NAME hypertensive rats but not in the normotensive controls. These results suggest that the hypertension caused by chronic inhibition of NO synthesis is partially mediated by abnormal activation of the SNS and RAS, possibly due to inhibition of nNOS. In the kidney, nNOS is expressed in the juxtaglomerular cells (438), and has been implicated in mediating the known inhibitory effect of NO on renin secretion (439). This regulatory action of nNOS on renin activity may be of particular significance in the renal adaptation to high dietary salt. In rats, chronic NO inhibition reduces renal sodium excretion, and sodium balance is achieved only at the expense of elevated ABP (440). When animals are maintained on high salt diet, there is a marked upregulation of ecNOS in the renal cortex and medulla and upregulation of nNOS specifically in the renal medulla. This upregulation of NOS activity is essential for the renal adaptation
to the high salt intake (441). Presumably, the elevation in ecNOS increases renal blood flow, whereas the increase in nNOS may lead to greater inhibition of renin and the antinatriuretic activity associated with it. It may be speculated, based on these findings, that sensitization of ABP to salt could, in some cases, be associated with decreased NOS activity in the kidney. In this regard, sensitivity of ABP to salt in Dahl rats was markedly attenuated by chronic dietary supplementation of L-arginine (442). Thus NO may also contribute to chronic regulation of ABP, ultimately by regulating ECFV volume.

(iii) interactions of ANP with NO in cardiovascular regulation

A clear-cut effect of ANP on acute NO synthesis has not been reported in endothelium. Agonist-induced release of NO from endothelial cells is accompanied by a rise in intracellular cGMP which is thought to be due to autocrine activation of soluble guanylate cyclase by NO (443). 8-bromo cGMP inhibits agonist-induced release of NO (444), and it has been suggested that NO exerts inhibition of its own release by stimulating cGMP formation (445). However, more recent work showed that pharmacological doses of ANP (0.1 μM) or sodium nitroprusside (10 μM) have no effect on basal or agonist-dependent NO release from bovine aortic cells, despite raising cGMP several fold (446). This suggests that acute ecNOS activity is not affected by ANP or other cGMP-stimulating agents in endothelial cells.

On the other hand, ANP may chronically affect the expression and/or activity of ecNOS via its multiple effects on endothelial intracellular signal transduction pathways (for review see ref. 27). VE has an abundance of receptors that bind ANP with high affinity (174, 315). The preponderance (> 90%) of endothelial ANP receptor sites are of the clearance (C) subtype; however, the abundance of GC-A receptors in these cells is sufficient to elicit robust stimulation of cGMP (174, 315, 347). The C- receptor subtype is negatively coupled to adenylate cyclase by an inhibitory G (Gi) protein
and activation of these receptors in endothelial cells decreases cAMP production (177). Since cAMP-dependent PKA can reduce ecNOS gene expression (382) and lead to reversible subcellular translocation and inactivation of the enzyme (381), it is expected that the inhibitory effect of ANP on adenylate cyclase activity in endothelium, if tonically active in vivo, will indirectly lead to an increase in ecNOS activity by attenuating the inhibitory effect of cAMP on gene expression and subcellular enzyme translocation. In addition, cGMP increases ecNOS gene expression in cultured endothelial cells (41), and ANP potentiates cytokine-induced iNOS expression in VSM (378) and in cardiac myocytes (345) by a cGMP-dependent pathway, suggesting that chronic activation of GC-A receptors in endothelium may also potentiate ecNOS expression. Thus, ANP may exert its chronic vasodilatory effect, in part, by stimulating ecNOS activity in VE, via its dual effects on cAMP and cGMP production. This potential mechanism would be self-perpetuated by the vasodilation and the associated increases in blood flow and shear stress, which would exert positive feedback on ecNOS gene expression and enzyme activity, resulting in potentiation of NO-dependent relaxation of the resistance vasculature.

b) C-type natriuretic peptide (CNP)

C-type natriuretic peptide (CNP) is a 22-aminoacid peptide that is structurally and functionally related to ANP and brain natriuretic peptide (BNP) (for review see ref. 448). The peptide was originally isolated in the central nervous system (449), but is now known to be synthesized in several peripheral tissues, including the heart (450, 451) vasculature (452), kidney (453) adrenal (454), immune organs (455) and gonads (456). The pattern of distribution of CNP in most tissues is mirrored by a similar distribution of the guanylate cyclase B (GC-B) receptor subtype, which binds this peptide much more selectively than either ANP or BNP (448, 452). The proximity of these receptors to the sites of CNP synthesis and the low concentration of the peptide in the circulation
suggest that CNP may exert its biological effects in a paracrine and/or autocrine fashion.

In the cardiovascular system, CNP is a potent vasodilator (457) and inhibitor of vascular wall hypertrophy (458). At comparable doses, this peptide is more effective in stimulating cGMP in VSM (459) and relaxing preconstricted blood vessels than ANP (460), in part because the GC-B are the predominant guanylate cyclase-linked receptors expressed in VSM (461). Vascular endothelium synthesizes and secretes CNP constitutively at physiologically effective concentrations (452, 462), and ANP potently stimulates CNP synthesis in cultured endothelial cells (39). In view of these observations, it is expected that the influence of ANP on endothelial synthesis of CNP, if tonically active, could operate as an intermediary vasoeffector mechanism mediating, at least in part, the chronic effect of the hormone on peripheral vascular resistance.

(i) biochemistry of CNP production and action

CNP is synthesized from a gene that is structurally and functionally independent from the genes of the other natriuretic peptides (for review see ref. 448). The gene is highly conserved across species and consists of two exons separated by one intron. Translation of the mRNA produces a 126 aminoacid pre-pro peptide which is posttranslationally cleaved by an unspecified protease to produce a 103 aminoacid pro-CNP (463). The pro-CNP undergoes further enzymatic processing, from which a 53 aminoacid fragment (CNP-53) is cleaved from the carboxyl end. The mature, biologically active molecule is a 22 aminoacid peptide(CNP) with the 17-aminoacid ring characteristic of all members of the natriuretic peptide family. However CNP is truncated at the carboxyl end (464). The peptide has been detected in plasma of some species (448). In endothelial cells, the peptide is found primarily as CNP-53, whereas the released form is predominantly CNP-22 (39, 44). There is no evidence for intracellular storage of CNP, and its secretion appears to be constitutive (448).

The stimuli for CNP production and release from the endothelium are not well characterized.
In endothelial cells, basal CNP secretion is greatly enhanced when these cells are co-cultured with VSM cells (465), compared to endothelial cells alone, suggesting that maximal CNP production requires chemical and/or physical contact with the underlying VSM. The increase in basal CNP production in these endothelial cell-VSM co-cultures is associated with an increase in TGF-β secretion from VSM and is inhibited by anti-TGF-β monoclonal antibody (466), indicating a role of this cytokine in mediating the effect of VSM on CNP synthesis and release from endothelial cells. Interestingly, cytokines potently stimulate CNP release in endothelial cell cultures (467).

The biological effects of CNP are mediated primarily by the GC-B receptors, which display high selectivity for this peptide (160, 161). The receptor is a particulate guanylate cyclase that is structurally and functionally similar to GC-A (27). The GC-B receptor is widely expressed in most tissues (27, 468), in contrast to the GC-A isoform which shows a more restricted pattern of distribution. As with GC-A, activation of the GC-B leads to generation of cGMP, the intracellular second messenger mediating CNP signal transduction (27, 469). CNP also interacts with equal affinity to other natriuretic peptides with the C-type receptors, but it is not known whether these receptors mediate any biological effects of CNP, other than its clearance (153, 330).

(ii) role of CNP in cardiovascular regulation

The physiological role of CNP in determination of ABP is not completely defined, partly due to the unavailability of suitable natural or genetic models of under- or overproduction of the peptide, and the lack of a specific GC-B receptor blocker. Acute intravenous infusion of CNP in anesthetized dogs causes a greater decrease in ABP than an equimolar dose of ANP (457, 470), in association with reductions in CO and right and left atrial pressures, indicating a decrease in ventricular preload. In contrast to ANP, infusion of CNP does not stimulate natriuresis, indicating a selective cardiovascular action of CNP. The hypotensive response to CNP infusion, however, is accompanied
by reflex sympathetic peripheral vasoconstriction, and this could have obscured any direct effect that systemic CNP may have exerted on vascular resistance (357). The hemodynamic effects of CNP described in these experiments, however, were accomplished by pharmacological elevations in the plasma concentration of the peptide, thus raising some doubt about their physiological significance. More recently, Charles et al (471) and Hunt et al (472) showed that intravenous infusion of CNP in sheep and humans respectively, to raise the plasma concentration of the peptide to supraphysiological levels, has no effect on basal hemodynamics, supporting the idea that CNP exerts primarily local effects on cardiovascular regulation. Nevertheless, systemic cardiovascular responses to CNP may occur in pathophysiological conditions in which the synthesis and plasma concentration of the peptide are greatly increased such as in atherosclerosis and septic shock (for review see ref. 473).

CNP displays regional heterogeneity in its ability to relax isolated vascular preparations (i.e. vessel strips, segments, etc) in vitro. In large blood vessels, CNP has a less potent relaxant effect on arteries than does ANP (470). However, unlike ANP, CNP relaxes several vein preparations, such as the saphenous, femoral and renal veins (460), suggesting that this peptide may function as a selective venodilator in vivo. In the microvasculature CNP is a more potent dilator of coronary (474-476), cerebral (477, 478) and renal afferent (479) arterioles than ANP, however, the effect of CNP on other vascular beds has not yet been described. CNP-dependent dilation can occur in the absence of endothelium (460, 474-476), suggesting that the mechanism of relaxation is, at least in part, mediated by direct effects on VSM. In isolated porcine coronary arteries, the dilatory response to CNP is attenuated by potassium channel antagonists glibenclamide and charybdotoxin, and in enzymatically-dispersed coronary VSM cells, CNP stimulates potassium channel activation and hyperpolarization in a dose-dependent manner (474), indicating that direct relaxation of VSM by CNP is caused by hyperpolarization of the cell membrane. The hyperpolarizing effect of CNP on VSM is
accompanied by a rise in intracellular cGMP concentration (476, 480), indicating that this effect is likely mediated by activation of GC-B receptors.

In addition to its direct effects of VSM tone, CNP may also cause relaxation by interacting and modifying the activity of other locally-acting vasoregulatory mechanisms. In cultured endothelial cells, CNP potently inhibits basal and agonist-mediated release of ET-1 (481) by stimulating cGMP accumulation. This suggests that CNP may exert GC-B-mediated tonic autocrine inhibition of ET-1 synthesis and release from endothelium and, thereby, reduce the vasoconstrictor activity associated with this peptide. In humans, CNP significantly reduces forearm vasoconstriction when co-infused with ANG I, but has no effect on the constrictive effect of ANG II (482), indicating that it may function as an endogenous antagonist of angiotensin converting enzyme. Also, in some tissues, CNP inhibits norepinephrine release prejunctionally from sympathetic nerve terminals (483), and when administered centrally, CNP reduces ABP in normotensive sheep (484), suggesting that sympatholysis may contribute to the vasodilatory action of this peptide.

(iii) interactions of ANP with CNP in cardiovascular regulation

In cultured bovine aortic endothelial cells, ANP (as well as BNP) increases the accumulation of CNP in the culture medium, whereas other vasoactive peptides ET-1 and ANG II have no effect (39). The effect of ANP is associated with an increase in CNP mRNA expression and protein translation, and is significantly attenuated by LY 83583 and KT 5823, inhibitors of guanylate cyclase activation and cGMP-dependent protein kinase respectively, whereas the C-receptor agonist C-ANP (ANP_{4-23}) has no effect on CNP synthesis (39). This implies a role for GC-A receptors in mediating the effects of ANP on CNP synthesis. The potential physiological significance of these findings is that CNP may serve as an endothelium-derived mediator/effect of ANP action on vascular tone. However, an interaction of ANP with endothelial cell production of CNP has not yet been examined
in vivo.

Another mechanism by means of which ANP may enhance the biological activity of CNP is by inhibiting its clearance and metabolism. The endothelial C-receptors, that are involved in the clearance of CNP (as well as ANP and BNP) (330) undergo homologous down regulation in response to stimulation with ANP (184, 185), an effect that is mediated by activation of GC-A and cGMP (185). Conceivably, then, ANP-mediated stimulation of GC-A in the vascular endothelium could have dual beneficial effects on CNP activity, by stimulating its rate of synthesis and by reducing its clearance, thereby prolonging the biological effect of this peptide on vascular function. Such interaction could subserve a major compensatory role in cardiovascular diseases such as CHF. Elevated plasma ANF levels are characteristic of CHF (194), and this leads to homologous receptor downregulation that is almost exclusively due to a decrease in C-receptor density (27, 200), whereas the activity of GC-A is unaltered or may even be increased in certain sites (27). This would be expected to potentiate the vascular actions of CNP, by reducing its clearance. The increase in CNP activity could play a significant role in preventing excessive proliferation of the vascular wall, and in counteracting the high constrictor tone imposed by the abnormally elevated activity of the SNS and RAAS that is characteristic of CHF (235).

c) Endothelin

Endothelin-1 (ET-1) is one of a family of three functionally-related isopeptides (ET-1, ET-2, ET-3) that are structurally related to sarafotoxin, a cardiotoxin first isolated from the burrowing asp Actractuspis engaddensis (32, 348, 485). The three isopeptides are coded by different genes (486), and are expressed and synthesized in various tissues and cell types, including lung, kidney, heart, liver, neural tissue and muscle (487-490). However, endothelial cells from every region of the vasculature produce exclusively ET-1 (32, 348, 488), and this isopeptide likely elicits most, if not all, of the
cardiovascular actions of endothelin (348). ET-1 is released abluminally (491) in a constitutive manner (348, 492). The close proximity of specific membrane receptors to the sites of synthesis suggests that ET-1 exerts primarily local paracrine and autocrine effects on cardiovascular function, and the constitutive nature of its release suggests that ET-1 may play a role in determination of basal vascular tone. Although the peptide is detectable in plasma, due to spillover from the abluminal side, in normal physiological conditions, the measured concentrations are below the threshold for receptor activation (348, 493).

ET-1 exerts a wide range of effects on cardiovascular and renal function that ultimately affect ABP. When acutely administered ET-1 elicits a very potent hypertensive response, caused, in part, by generalized vasoconstriction and elevation of TPR, (348, 494, 495) and, in part, by interacting with other cardiovascular regulatory mechanisms and modulating cardiac function (341, 494, 496). Chronically, ET-1 exerts a potent mitogenic effect on the heart (497) and the vasculature (498), and this may influence chronic hemodynamic function and ABP.

(i) biochemistry of ET-1 production and action

Endothelin-1 is derived from a complex gene consisting of 5 exons and 4 introns (341, 499). Each of the five exons encodes a segment of prepro-ET-1, however, the biologically active peptide is completely encoded within exon 2 (499). The gene contains several regulatory elements in the 5' and 3' untranslated- and intron regions that may be involved in regulation of gene expression. Of particular significance are multiple consensus binding sequences dispersed throughout the gene for nuclear factor-1 (NF-1) (500), and several AP-1/Jun binding sequences in the 5' and 3' untranslated regions that may be involved in mediating rapid induction of ET-1 gene expression by phorbol esters, protooncogene protein products (i.e C-jun, C-fos) and endogenous activators of PKC such as ANG II and AVP (32, 348, 499). In addition, several stress-responsive elements within the 5' untranslated
region induce gene expression in response to hemodynamic shear stress and other stresses such as hypoxia and injury (499). The gene also has two highly conserved AUUUA sequences in the 3' untranslated region that may be involved in mediating mRNA degradation (499, 501). The expression of the gene is inhibited by cGMP and cGMP-raising agents such as NO and ANP, presumably by inhibiting PKC activity (35, 38, 502). The gene is constitutively expressed and there is no storage of peptide, indicating an increase in the rate of secretion above basal levels requires de novo induction of gene expression which is predominantly regulated at the level of transcription, translation or mRNA stability (32, 348, 485). Transcription and processing of the gene gives rise to ~2.3 Kb long mRNA which undergoes translation to produce a 203- aminoacid prepro-ET-1 (Fig. 1.9). The propeptide undergoes several steps of posttranslational processing to produce a 38- aminoacid propeptide (big-ET-1). Big ET-1 undergoes a final proteolytic cleavage at Trp\(^{21}\)-Val\(^{22}\) by a specific neutral metalloprotease, endothelin converting enzyme (ECE), to produce the mature, biologically-active 21 aminoacid ET-1 (348). ET-1 has an hairpin configuration held in place by two disulfide bonds between Cys\(^{1}\)-Cys\(^{15}\) and Cys\(^{3}\)-Cys\(^{11}\) (Fig. 1.9). The hairpin region is highly polar, and the hydrophobic Trp\(^{21}\) at the carboxyl end is required for bioactivity (348).

The diverse biological actions of ET-1 are mediated by specific high affinity membrane receptors that have a high degree of interspecies homology (348, 503). The structure of the endothelin receptors is similar to that of adrenergic receptors, consisting of 7 amphipathic transmembrane domains connected by extracellular and cytosolic loops, a long 75- aminoacid extracellular amino terminus with glycosylated aminoacid residues and a cytosolic carboxyl terminus containing a site for posttranslational palmitoylation, which anchors the receptor to the membrane (341, 504, 505). At least two isoforms of these receptors, ET\(_{A}\) and ET\(_{B}\) have been identified and found to be widely distributed in the vasculature and in several organs (504-506). ET\(_{A}\) is more
Figure 1.9. Gene structure, proteolytic processing and structure of mature ET-1. (From Gray et al. In: Molecular Biology and Pharmacology of Endothelins, edited by G. A. Gray and D. J. Webb, Austin, R. G. Landes Company, 1995.)
selective for ET-1 and ET-2 than for ET-3 (507), whereas ETb binds all three endothelin isopeptides with equal affinity (508). ETa is the predominant receptor mediating ET-1-dependent vasoconstriction and cardiac effects (348, 507), however, recent studies indicate that ETb also mediates vasoconstriction in some vascular beds (508, 509). ETb is abundantly expressed in vascular endothelium and is thought to mediate autocrine effects of ET-1 such as stimulation of NO production (510).

The endothelin receptors are coupled by a G-protein to the phosphoinositide second messenger pathway, for intracellular signal transduction (348, 495, 511-513) (Fig. 1.10). After binding to its receptor, ET-1 activates phospholipase C which hydrolyses phosphatidyl inositol (PIP2), a membrane phospholipid, to produce a rapid increase in inositol trisphosphate (IP3) and a sustained accumulation of diacylglycerol (DAG). These two by-products of PIP2 hydrolysis function as second messengers of ET-1-mediated cellular effects. IP3 stimulates calcium release from intracellular storage depots, and DAG activates protein kinase C (PKC) which sensitizes the contractile proteins to calcium (pharmacomechanical coupling) in VSM, and mediates long-term effects of ET-1 on gene expression and mitogenesis (348). In addition, ET-1 also stimulates phospholipase A (PLA₂) (511, 514) and phospholipase D (PLD) (515), which hydrolyze arachidonic acid and phosphatidic acid respectively, providing substrate for formation of eicosanoids (i.e. prostaglandins) and DAG (348, 495). ET-1 binding also stimulates calcium influx via selective voltage-gated (VOC) L-type calcium channels (485, 512) and possibly via non-selective receptor-operated cation channels (ROCC) (348, 512). When ET-1 activates its receptors in VSM there is a biphasic rise in intracellular calcium (Ca₂⁺) concentration, a transient increase that is likely due to release of calcium from intracellular stores (485, 514), followed by a prolonged rise in calcium that is inhibited by pertussis toxin (485, 516), suggesting that the influx of extracellular Ca is mediated
by G protein-dependent receptor gating of L-type VOC calcium channels and, possibly ROCC, thereby causing sustained contraction (348, 485, 495, 517). The mechanism by which ET-1 stimulates opening of VOC calcium channels is not well understood, but is thought to be caused, in part, by membrane depolarization, caused by the opening of chloride channels and efflux of chloride (348), and an increase in intracellular free sodium due to stimulation of the Na⁺/H⁺ antiport by PKC (348).

Figure 1.10. Intracellular signalling pathways used by the endothelin peptide family. (From Gray et al. In Molecular biology and pharmacology of endothelins, edited by G. A. Gray and D. J. Webb, Austin, R. G. Landes Company, 1995.)
(ii) role of ET-1 in cardiovascular regulation

Acute intravenous infusion of ET-1 in different species produces a biphasic change in ABP; a transient, short-lasting hypotensive response associated with peripheral vasodilation, followed by a potent and long-lasting increase in ABP that is associated with vasoconstriction (348, 485, 518-525). The initial vasodilatory effect is likely due to ET-1-mediated autocrine stimulation of NO, since it can be prevented by L-NAME (526, 527), whereas the pressor effect is largely due to direct stimulation of VSM contraction (485, 523-525, 528). ET-1 constricts vessel segments isolated from every region of the vascular tree, with greater potency than any other endogenous vasoconstrictor (348, 494). In large blood vessels of the systemic circulation, ET-1 is 3-10 more potent constrictor of veins than arteries (348, 529). However, this pattern of sensitivity is reversed in the pulmonary circulation (530) and in the microcirculation, with arterioles from mesenteric (531), splanchnic (532), renal (533), coronary (534), cerebral (535), cutaneous (536) and muscle (528, 537, 538) beds being more sensitive to ET-1 induced constriction than postcapillary venules. In addition to direct effects on vascular reactivity, ET-1 stimulates cardiovascular sympathetic tone centrally (539, 540) and peripherally (541, 542), and reduces the sensitivity of the baroreflex (543, 544). ET-1 also stimulates the release of some vasoconstrictor substances, such as AVP (545, 546) and norepinephrine (546) and Ang II (547-549), and amplifies their contractile effects on VSM even at threshold concentrations (348, 494, 547). These effects may partially account for the vasoconstrictor action of ET-1 (550).

In the heart, ET-1 is produced in the endothelium (551), as well as in myocytes (552). Endothelin binding sites are distributed throughout the myocardium, both in contractile myocytes (553) and in the atrioventricular node (554), suggesting that locally-released peptide may modulate cardiac contraction. Administration of ET-1 to isolated perfused hearts induces positive inotropic
and chronotropic (557, 558) effects and a prolongation of the action potential (559). These effects appear to be due to direct action of peptide on the myocardium. In addition, ET-1 affects cardiac function indirectly via coronary vasoconstriction and reduction of blood flow (534, 560). The role that these actions of ET-1 may play in normal regulation of cardiac function, however, remains to be established. When injected or infused into the coronary circulation in vivo, ET-1 induces bradycardia consequent to reduction in coronary blood flow (561), and, depending on the dose, it may even reduce cardiac output (562) which is in contrast to the putative cardiotonic action of ET-1 in vitro (555, 556). These findings indicate that the overall acute hemodynamic effect of exogenous ET-1 in vivo is determined by a complex interaction of competing direct and indirect effects of the peptide on cardiac, vascular, autonomic and endocrine function.

The role of ET-1 in chronic regulation of ABP is not fully established. Chronic infusion of ET-1 leads to sustained hypertension in rats (563, 564), and the circulating level of ET-1 is elevated several fold in several cardiovascular diseases, including chronic CHF, renal failure, atherosclerosis and some types of hypertension (348, 565-568). However it has not yet been unequivocally established whether the increase in plasma ET-1 concentration underlies the etiology of these diseases. One case of secondary hypertension caused by malignant hemangioendothelioma was found to be associated with elevated plasma ET-1 concentration. In this case, ABP returned to normotensive levels following surgical removal of the tumour and normalization of ET-1 levels (569), suggesting a causative role of this peptide in causing the rise in blood pressure. More recently, Douglas et al (570) showed that chronic infusion of ±SB 209670, a high-affinity non-peptide antagonist of ET<sub>AB</sub> receptors into spontaneously hypertensive rats (SHR) and renin-dependent hypertensive rats and their respective normotensive controls, significantly decreased ABP in both hypertensive rat models in association with a decrease in TPR, but had no effect on the normotensive


controls. On the other hand, inhibition of ET-1 synthesis with phosphoramidon, a nonselective inhibitor of endothelin converting enzyme (571), or specific inhibition of ET\textsubscript{A} receptor activity with BQ-123 (572) significantly decreases ABP both in SHR rats and in normotensive control rats, indicating that ET-1 contributes to maintenance of basal vascular tone in both types of animals. In contrast to these findings, mice heterozygous for a deletion of the ET-1 gene (homozygous genotype is lethal), paradoxically develop hypertension in association with \textasciitilde50\% decrease in plasma ET-1 levels (573). These results would seem to suggest that ET-1 exerts a chronic vasodilatory effect. However, the consequence of the reduced plasma ET-1 levels on endothelin receptor sensitivity and vascular reactivity were not assessed. It is possible that the chronic decrease in ET-1 synthesis in the heterozygous ET-1 knockout mice may lead to receptor upregulation, and that the increase in receptor density mediates an hypertensive response even with a reduced level of ligand. The physiological significance of ET-1 in long-term regulation of blood pressure thus remains unresolved. On the basis of available evidence, a conceivable hypothesis is that ET-1 exerts tonic constricting and mitogenic effects on the vasculature, and that under normal physiological conditions, these effects are counterbalanced by endothelium-derived vasodilatory/antimitogenic moieties such as NO in order to prevent excessive basal tone. In some types of cardiovascular disease, this balance is disturbed in favour of ET-1, due to decreased generation of these vasodilatory substances and/or in some cases, increased production of ET-1. The end result of this imbalance would be an amplification of the vascular effects of ET-1, leading to sustained hypertension.

(iii) Interactions of ANP with ET-1 in cardiovascular regulation

Endothelin-1 potently stimulates ANP synthesis (497, 574, 575) and release \textit{in vivo} (122, 123) and ANP inhibits the release of ET-1 (35-38, 331) and its target actions on the cardiovascular and renal systems (42, 576). The interaction between these two regulatory peptides may function both
as an acute negative feedback mechanism of regulation of cardiovascular and renal function, and as a tonic modulator of basal tone. For example, plasma ET-1 concentration was markedly elevated after inhibition of GC-A receptor activity in dogs (331) suggesting that ANP exerts a tonic inhibitory effect on ET-1 release. In cultured endothelial cells, ANP reduces basal (38) and agonist-mediated release of ET-1 (35-37). The cellular mechanism(s) mediating ANP-dependent inhibition of ET-1 secretion in vitro remain unclear. It appears that depending on the conditions, either ANP receptor type may be involved. In bovine aortic endothelial cells, both ANP and the C-receptor ligand ANP_4-23 inhibit translation of prepro-ET-1 and release of ET-1 into the culture media (36). These effects are not affected by inhibition of guanylate cyclase with LY 83583, but are reversed by 8-bromo cAMP, suggesting that inhibition of basal ET-1 release by ANP is mediated by the C-receptor (36). In contrast, inhibition of ET-1 release from human umbilical endothelial cells by ANP is mimicked by 8-bromo cGMP and by other cGMP-stimulating agents, such as nitroprusside and nitroglycerin (38), thereby implying a role of the GC-A receptor in mediating the effects of ANP, at least in these cell cultures. Inhibition of agonist-mediated ET-1 release by ANP is also associated with intracellular accumulation of cGMP (35, 37), suggesting a primary role of GC-A receptors in mediating the inhibitory effect of ANP.

ANP antagonizes the vascular effects of ET-1 in some situations. When co-infused with ET-1 in conscious rats, ANP reduces the pressor effect of the latter (577). In nephrectomized rats, ET-3 infusion produces a hypertensive effect and an increase in hematocrit (578). Pretreatment of these animals with an ANP antiserum exacerbates the hypertensive response to ET-3 and inhibits the increase in hematocrit (578), suggesting that release of ANP consequent to stimulation by ET-1 attenuates the pressor effect of the latter, and mediates the change in hematocrit. In isolated perfused rat kidney, ANP completely abolishes ET-1-induced increase in vascular resistance (579). In denuded
porcine (580) and rabbit (42) aortic rings, ANP reduces the contraction efficacy of ET-1, particularly at low subnanomolar concentrations, resulting in a rightward shift of the dose-response curve to ET-1. ANP has no effect on the potency of ET-1 contraction when the rings are incubated in the absence of calcium, or pretreated with the L-type channel blocker nifedipine (580). These findings indicate that ANP antagonizes the direct constricting effect of ET-1 in VSM, at least in part, by inhibiting ET-1-induced calcium mobilization. The mechanism involved in mediating ANP-induced decrease in intracellular calcium is not known, but is likely to involve activation of the GC-A receptors and cGMP accumulation (27, 307, 308). The extent to which this interaction occurs in the resistance vasculature, with the exception of the renal vasculature (579), is not known. However, since GC-A receptors are scarcely expressed in the resistance vasculature (26, 27), it is unlikely that ANP significantly inhibits ET-1-mediated changes in calcium flux in the resistance vessel VSM, suggesting that any inhibitory effect of ANP on ET-1-dependent increase in peripheral vascular resistance may be mediated via inhibition of ET-1 synthesis/release.

2. Sympathetic nervous system (SNS)

ANP exerts acute inhibition of all levels of sympathetic neurotransmission (for review see refs. 33, 581), and there is evidence that the full expression of acute ANP-mediated hypotension requires an intact sympathetic nervous system (287, 326, 328, 329). The extent to which the sympatholytic activity of ANP may contribute to the chronic hypotensive effect of the hormone is not known. Some indirect evidence suggests a chronic interaction of ANP with sympathetic nervous activity. For example, the hypotensive response to chronic ANP infusion is exacerbated in the spontaneously hypertensive rat (333, 334), a model of genetic hypertension that is characterized by elevated sympathetic tone. This may be interpreted as an indication that the chronic hypotensive effect of
ANP is brought about by attenuation of basal sympathetic tone, and that the magnitude of this effect is determined by the underlying level of sympathetic tone. However, a direct association between the level of endogenous ANP activity, arterial blood pressure and sympathetic activity has not been described.

a) Biochemistry of sympathetic neurotransmission and mechanism of action

The sympathetic nervous system is intimately involved in regulation of all aspects of cardiovascular function and blood pressure homeostasis (for review see ref. 248). The catecholamines norepinephrine (NE) and epinephrine (EPI) are the primary postganglionic and adrenomedullary neurotransmitters respectively, mediating the sympathetic actions on the cardiovascular system (582). However there is evidence that other vasoactive substances are co-localized with norepinephrine in synaptic vesicles, and are released as cotransmitters during sympathetic nerve stimulation (582-584). Among these sympathetic co-transmitters are neuropeptide Y (NPY) and adenosine 5'-triphosphate (ATP) (582) which appear to function in synergy with catecholamines in local regulation of vascular tone and blood flow (583, 584).

In adrenergic nerves and in the adrenal medulla, catecholamines are synthesized from tyrosine which is taken up from the blood by the nerve cells (583). The tyrosine is first hydroxylated to 3,4-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase in the cell body and/or in the nerve terminals, then converted to dopamine by DOPA decarboxylase. The final step in catecholamine synthesis is the hydroxylation of dopamine to norepinephrine by dopamine-β-hydroxylase (583, 585). The catecholamines are then packaged into vesicles and stored at the nerve terminals (also called varicosities at neuromuscular junctions). Depolarization of the synaptic terminal leads to calcium influx and exocytosis of the vesicular contents into the synaptic cleft. The contents of several vesicles (≈100-200) are released synchronously and produce a large endplate potential in the post synaptic...
cell (584). The release of catecholamines and co-transmitters is prejunctionally modulated by NE, which exerts an autoinhibitory effect via $\alpha$-2 adrenergic receptors (584). Termination of catecholamine action occurs via endocytotic reuptake into the presynaptic terminal, and subsequent enzymatic degradation by monoamine oxidase and catechol-O-methyltransferase (585-586).

At the neuroeffector junction, the released catecholamines mediate their target actions by interacting with specific postjunctional membrane-bound adrenergic receptors. Co-released ATP acts on purinergic “P2” receptors and NPY acts via its own receptors, of which there are several subtypes (583). There are several pharmacologically and physiologically distinct adrenoreceptors (for review see ref. 587) that are loosely categorized under two subtypes, $\alpha$ and $\beta$, on the basis of their ligand specificity and the intracellular signalling pathways mediating their actions. These subtypes are further subdivided into $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$, $\beta_3$. Recent evidence indicates that there are several isoforms of each one of these subtypes (587). All adrenergic receptors share a similar structure consisting of seven hydrophobic membrane-spanning domains (like the ET receptors) coupled to the intracellular signal transduction cascades by G proteins (587). The $\alpha_1$ receptor is coupled to phospholipase C; however, there is evidence that the receptor can also activate phospholipase A as well as receptor-operated calcium channels (587). The $\alpha_1$ receptor is the predominant subtype found in VSM from most vascular beds, with the exception of muscle, and is primarily involved in mediating the vasoconstrictor activity of catecholamines (587). The $\alpha$-2 subtype is negatively coupled to adenylate cyclase, and primarily subserves inhibitory actions, such as autoinhibition of norepinephrine release at sympathetic nerve terminals, and central inhibition of sympathetic outflow from the medulla (587). $\beta$-adrenoreceptors stimulate adenylate cyclase and mediate the positive inotropic and chronotropic effects of norepinephrine in the heart ($\beta_1$ subtype). In the microvasculature, the $\beta$-adrenoreceptors are greatly outnumbered by $\alpha_1$ receptors, and contribute little to basal vascular sympathetic tone, with
the exception of the skeletal muscle vasculature, where they elicit profound vasodilation, particularly in response to exercise (587).

b) Role of the SNS in chronic regulation of arterial blood pressure

In addition to its primary role in acute feedback reflex regulation of ABP (287), there is evidence that the SNS contributes prominently to determination of chronic ABP (for review see ref. 588). When peripheral sympathetic neurotransmission is interrupted by procedures such as chemical sympathectomy (589, 590) or ganglionic blockade (591), there is a significant decrease in blood pressure in association with reductions in cardiac function and peripheral vascular resistance. Furthermore, hypertension can be induced by chronic sympathetic nerve stimulation (592, 593), and efferent sympathetic activity is elevated in some forms of hypertension (594, 595) indicating that the central nervous system provides a tonic level of sympathetic activity which is required for maintaining adequate cardiac output and peripheral vascular tone (588, 596), and that aberrations in the level of sympathetic activity lead to chronic alterations in ABP.

Although there is no doubt that sustained changes in sympathetic nerve activity lead to chronic alterations in ABP, there is much disagreement whether the SNS subserves a physiologically significant role as a homeostatic regulatory mechanism of long-term ABP. The arguments against such a role are that: 1) Baroreceptor denervation, although increasing the short-term lability of ABP, does not chronically increase ABP (245, 588, 597). 2) Sympathetic overactivity by itself is not sufficient to raise ABP, because some pathophysiological states, such as CHF and cirrhosis, although characterized by elevated sympathetic activity, are not accompanied by hypertension (595). 3) Sympathetic-mediated increase in peripheral vascular resistance and ABP will not be permanent because, in the absence of any excretory dysfunction, the kidney will counterbalance this by increasing salt excretion by pressure natriuresis (245, 246). These arguments may be countered on the basis that:
1) Baroreceptors, although providing the primary short-term error signal to the central autonomic nervous system, quickly reset to prevailing pressure, thus, are not adaptable to long-term changes in ABP. In addition, the possibility of other mediators serving as error signals (i.e. ANG II, endothelial factors) cannot be discounted (for review see ref. 596). Furthermore, baroreceptors are an afferent system and should not be taken as synonymous with sympathetic nerve activity which is efferent in nature and modulated by other influences, in addition to moment-to-moment regulation by baroreflexes. 2) Pathophysiological situations such as CHF and cirrhosis are accompanied by some hormonal changes that may oppose and mask the hypertensive effect of elevated sympathetic tone (e.g. increase in ANP release) (595). 3) Increased sympathetic activity has profound direct and indirect effects on renal function, causing antinatriuresis and opposing pressure natriuresis (598), thereby leading to salt and water retention.

Regardless of whether the SNS operates as a “genuine” homeostatic mechanism of chronic regulation of ABP, sustained sympathetic hyperactivity will elicit chronic hypertension due to multiple effects on: vascular and cardiac growth, VSM membrane electrical properties and renal function. 1) Elevated sympathetic tone has trophic effects on cardiac myocytes and on VSM, causing selective increases in contractile protein gene transcription and hypertrophy (599, 600). In the resistance vasculature this leads to remodelling of the vessel wall, resulting in increased wall thickness and reduced lumen diameter (594, 601), which increases vascular resistance and contributes to perpetuation of hypertension. 2) In addition to stimulating vascular hypertrophy, sustained sympathetic hyperactivity increases the passive permeability of VSM membrane to sodium, leading to decreased resting membrane potential. This has been shown to result in potentiation of the vasoconstrictor response to norepinephrine in SHR (602). 3) Renal sympathetic nerve stimulation, even at frequencies below the threshold required to elicit renal vasoconstriction, results in significant
antinatriuresis that is partly due to direct effects on tubular transport, and stimulation of RAAS (598).

c) Interactions of ANP with SNS in cardiovascular regulation

When administered acutely, ANP exerts widespread inhibition of sympathetic nervous activity by interacting with all levels of sympathetic function centrally and peripherally (for review see ref. 33, 581). Centrally, ANP reduces sympathetic outflow from cardiovascular regulatory areas in the brain stem (43, 44, 338, 603). Peripherally, ANP inhibits ganglion transmission (604), spontaneous and evoked norepinephrine synthesis and release from post-ganglionic sympathetic nerve fibers (45, 46), and adrenal medulla (605), and it antagonizes postsynaptic α-1 receptor activity (606). On the other hand, catecholamines stimulate ANP release in vitro (116, 117), indicating that this interaction may operate as a negative feedback mechanism of blood pressure regulation. It is not well established whether these interactions are tonically active and modulate the long-term hypotensive effect of ANP.

(i) central effects of ANP on sympathetic outflow

In addition to peripheral effects on the vasculature and cardiovascular autonomic reflex afferent mechanisms, ANP may also interact with central cardiovascular regulatory areas. ANP immunoreactivity and binding sites are found in brain structures closely involved in the regulation of ABP, particularly in hypothalamic nuclei, median eminence, septal areas, the anteroventral region of the third ventricle (AV3V), and to a lesser extent in the circumventricular organs (607-609). This suggests that locally-derived peptide could potentially exert paracrine neuromodulatory effects on cardiovascular regulatory sites. Injection of ANP into the nucleus tractus solitarius of anesthetized rats decreases MAP and HR significantly, in association with an increase in the firing rate of a discrete population of neurons that are also stimulated by activation of arterial baroreceptors and chemoreceptors (43, 44, 603, 610, 611). Similarly, injection of ANP into the fourth ventricle of conscious, unrestrained spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto
(WKY) rats reduces MAP in both strains (43, 612). The effect of ANP is abolished by selective antagonism of α-2 adrenergic receptors with yohimbine or by prior chemical sympathectomy with 6-hydroxydopamine. Also, injection of ANP into the third ventricle of anesthetized rats leads to a decrease in peripheral sympathetic nerve activity (613). Thus, ANP may operate as a neuromodulator of baro- and chemoreceptor responses within the dorsal medulla, by interacting with central catecholaminergic neurotransmission and sympathetic outflow. These neuromodulatory effects may partly account for the acute hypotensive action of the ANP. Furthermore, ANP may also modulate the central effects of other pressor hormones. Intracerebroventricular (icv) injection of ANP in anesthetized rats with sinoaortic denervation decreases MAP, HR and renal sympathetic nerve activity (RSNA), and attenuates the stimulatory effect of icv injection of ANG II on these parameters (614). The depressor effect of icv injection of ANP on MAP, HR and RSNA is abolished by prior blockade of the ANG II receptors or by local inhibition of converting enzyme, suggesting that the central depressor effect of ANP in this region of the brain is mediated by inhibition of local neuromodulatory effects of ANG II on sympathetic nerve activity. The hypotensive effect of ANP may, also, in some situations, be enhanced by the action of this hormone on AVP activity. ANP inhibits basal and ANG II-stimulated (615) release of AVP from the hypothalamus. The ensuing decrease in plasma AVP concentration could result in reduced pressor activity associated with this peptide, thereby contributing indirectly to the hypotensive effect of ANP.

The extent to which chronic changes in locally-derived ANP activity in central cardiovascular regulatory areas will affect long-term ABP is not well characterized. Microinjection of an ANP monoclonal antibody into the caudal nucleus tractus solitarius raises ABP in SHR rats (616), a genetic hypertensive model characterized by elevated sympathetic tone (617), suggesting that endogenous ANP, likely produced locally, mediates a tonic central depressor effect on ABP. Overall, brain ANP
concentration and receptor density are decreased in the SHR (27, 202). However, in this model, the concentration of ANP is significantly elevated in the anterior hypothalamic area (AHA) (618), a region that subserves a tonic inhibitory influence of central cardiovascular sympathetic outflow, via $\alpha$-2 adrenergic stimulation of neurons in the nucleus tractus solitarius. The elevated ANP concentration in the AHA area is associated with significant local inhibition of norepinephrine release (610), suggesting that abnormally high ANP activity in this area can lead to hypertension by reducing tonic excitatory $\alpha$-2 adrenergic input to the NTS.

(ii) peripheral effects of ANP on sympathetic nerve function

In the periphery, ANP-like immunoreactivity (27, 33, 619) and binding sites (620) have been localized in preganglionic cholinergic neurons within autonomic ganglia. In isolated superior cervical ganglia of rat in vitro, ANP potently inhibits cholinergic-mediated synthesis of norepinephrine in association with a rise in cGMP formation (621). In humans, the sympathoexcitatory response to the cold pressor test, a stimulus for increased central sympathetic outflow, and to edrophonium, a reversible acetylcholinesterase inhibitor devoid of central activity, is significantly inhibited by ANP, as assessed by a decrease in peripheral muscle sympathetic nerve activity (MSNA) (622). These observations suggest that locally-derived ANP and its GC-A receptors may function as a “self-contained” tonic inhibitory unit of sympathetic ganglionic neurotransmission.

In addition to blockade of ganglionic function, ANP modulates sympathetic neurotransmission pre- and postjunctionally in the adrenal medulla (605, 623) and at the neuroeffector junction in the vasculature (44, 45, 50, 335, 606, 624, 625). Infusion of ANP inhibits release of epinephrine from the adrenal medulla in conscious dogs (605), in association with a decrease in ABP, indicating that under physiological conditions, ANP exerts an inhibitory effect on sympathoadrenal activity that may contribute to its hypotensive effect. In isolated perfused rat mesenteric arteries, ANP inhibits
norepinephrine release in a dose-dependent fashion, in response to nerve stimulation, suggesting that ANP inhibits noradrenergic neurotransmission at the neuroeffector junction, possibly via a prejunctional mechanism (45). A similar conclusion was reached in vivo by Haass et al (625). Using a pithed rat model, this group showed that injection of ANP significantly reduces the pressor response to norepinephrine. When co-administered as a bolus with α-1 or α-2 receptor agonists phenylephrine and clonidine respectively, ANP antagonizes the pressor effect of clonidine but not that of phenylephrine. However when co-infused with one of these agonists, ANP decreases the pressor response to both clonidine and phenylephrine (625). These results indicate that ANP exhibits a sequential pattern of inhibition of adrenoreceptor activity at the neuroeffector junction; an immediate inhibition of pre-junctional α-2 pressor action followed by sustained inhibition of post-junctional α-1 pressor effects.

3. Renin-angiotensin system (RAS)

Angiotensin II, the principal biologically active product of the RAS cascade plays a major role in acute (238) and long-term (238, 605, 607) regulation of ABP via its multiple actions on renal salt excretion, vascular tone and cardiac function and growth (626-628). ANG II is produced both systemically and locally in the vasculature and in several other tissues, including the brain, heart and the adrenal glands (627). Circulating ANG II is the primary hormonal effector of RAS and accounts for most of the effects on renal function, vascular tone and cardiac function (626, 627). Locally-produced ANG II is thought to exert autocrine and paracrine effects on cellular growth, cardiac function, neuromodulation, and possibly vascular tone (626, 628, 629).
a) Biochemistry of ANG II production and mode of action

The octapeptide ANG II is produced from a larger α-2 globulin precursor, angiotensinogen, synthesized in the liver, by a cascade of enzymatic reactions catalyzed by two main enzymes, renin and angiotensin converting enzyme (ACE) (Figure 1.11). In the first step of the cascade, a decapeptide, ANG I, is cleaved from the amino terminal of angiotensinogen by renin. ANG I is then cleaved to ANG II by ACE, a membrane-bound glycoprotein that is abundantly expressed in endothelial cell membranes of pulmonary capillaries, and to a lesser extent in other vascular beds (626, 627). The rate limiting step in this enzymatic cascade is the availability of angiotensinogen substrate for renin. The cascade is set in action by the release of renin from the juxtaglomerular cells, in response to hemodynamic, neurogenic or humoral signals that are activated, for example, by lower effective circulating volume, such as in hemorrhage, or in response to extracellular sodium depletion (626, 627). Systemic ANG II is synthesized in this fashion from blood-borne angiotensinogen. Extrarenal sites of ANG II synthesis have all the components of RAS and synthesize ANG II from locally-derived angiotensinogen (627).

The target actions of ANG II are mediated by membrane-bound receptors that are structurally similar to adrenergic and endothelin receptors, consisting of seven membrane-spanning domains, coupled to a G regulatory protein (630-632). At least two high affinity receptor subtypes, AT₁ and AT₂, have been identified (631, 632). The AT₁ receptor has a widespread pattern of distribution in many tissues (632) and is thought to mediate, almost exclusively, the vascular, cardiac, neuromodulatory and renal and adrenal effects of ANG II (631). This receptor subtype is specifically blocked by the non-peptide antagonist DuP-753 (losartan) (632). The AT₂ displays a more restricted pattern of distribution, being found in the adrenal gland, brain, heart and in-reproductive organs (632).
The physiological role of the AT$_2$ receptor remains relatively obscure. On the basis of studies with PD 123177, a specific antagonist of AT$_2$ (632), it is suggested that this receptor may be partially involved in mediating the neuromodulatory and growth-promoting effects of ANG II (632). The signal transduction pathways activated by AT$_1$ are similar to those employed by endothelin and other growth factors and cytokines and involve stimulation of the PLC/PKC, PLA$_2$, and PLD intracellular cascades, calcium mobilization, tyrosine kinase-mediated protein phosphorylation, and stimulation of mitogen-activated protein (MAP) kinases (630, 632).
b) Role of ANG II in cardiovascular regulation

ANG II exerts a multiplicity of cardiovascular, renal and neural effects that ultimately influence ABP acutely and chronically (626, 628, 631, 632). In the heart, ANG II exerts a positive inotropic effect that is due, in part, to direct actions of the peptide on the myocardium, and in part, due to stimulation of sympathetic activity (628, 629, 633, 634). ANG II also exerts a direct positive chronotropic effect \textit{in vitro} (629); however, \textit{in vivo}, this effect is not consistently observed, because it is confounded by effects of ANG II on vagal tone and baroreflex-mediated decreases in HR (628). Most evidence suggests that the positive inotropic and chronotropic effects of ANG II are mediated by enhancement of the slow inward calcium current, and consequent influx of extracellular calcium, resulting in an increase in the height and duration of the plateau phase of the cardiac action potential and an increase in peak tension (628). In the vasculature, ANG II potently constricts resistance vessels via direct effects on VSM, and indirectly by potentiating vascular sympathetic tone (626, 627). Acutely, RAS plays a major role in re-establishing normal circulatory homeostasis in response to emergency situations such as hemorrhage and orthostatic hypotension (627).

ANG II also contributes significantly to chronic ABP. Several hypertensive diseases are characterized by chronically elevated ANG II levels (635), and these are generally ameliorated by treatment with ACE or AT\textsubscript{i} receptor antagonists (i.e. losartan), thereby establishing a causal relationship between ANG II plasma concentration and chronic ABP. Perhaps a more definitive implication of RAS in determination of chronic ABP is seen in genetic mouse models expressing lifelong alterations in the different components of the RAS. In mice carrying 0 to 4 copies of the angiotensinogen gene, ABP increases almost linearly with gene copy number and plasma angiotensinogen concentration (636). Mice harboring homozygous or heterozygous disruptions of the AT\textsubscript{1A} receptor gene have \textasciitilde24 mm Hg and \textasciitilde12 mm Hg lower ABP respectively than their wild
type counterparts, showing also a linear relationship between the endogenous level of ANG II activity and ABP (637). It is interesting to note that exogenous ANG II fails to raise ABP in the AT₁A knockout mice, thus showing the dependency on this receptor of the vasoconstrictor activity of ANG II (637).

Chronically, an elevation in ANG II can lead to hypertension by a combination of effects. First, high ANG II levels exert a persistent direct vasoconstrictor effect, leading to an elevation of basal vascular tone. This would be compounded by the facilitatory effects that ANG II exerts on sympathetic neurotransmission. Second, the elevated ANG II and sympathetic tone have trophic effects on the vasculature and the heart, and, in time, the structural remodelling of the resistance vasculature may exacerbate the increase in vascular resistance (628, 635). Cardiac hypertrophy, which develops in response to direct effects of ANG II, and as an adaptive response to elevated ventricular afterload (628), may, in time, become pathological (628, 635). Third, chronically elevated ANG II causes hypertension by stimulating sodium and water retention, via its direct effect on proximal sodium reabsorption and stimulation of aldosterone (341, 605, 627, 638). This salt-retaining effect of ANG II is counteracted, in part by pressure natriuresis (238, 239, 638) and by ANP (48, 49, 220, 221), such that salt balance is chronically maintained (638), albeit at a higher blood pressure. On the other hand, the vascular effects of ANG II are not compensated (635). Thus a chronic increase in ANG II activity leads to hypertension that is characterized by elevated TPR and ECFV expansion.

c) Interactions of ANP with ANG II in cardiovascular regulation

In rats with renovascular hypertension, chronic infusion of ANP has an hypotensive effect only when ABP is sensitive to ANG II blockade (639), and a 5-day infusion of ANP reduces ABP significantly in 2 kidney/1 clip (Goldblatt hypertension), renin-dependent hypertensive Wistar rats, but not in normotensive control rats (640). In addition, infusion of ANP concurrently with ANGII
for 3 days, in normal and salt loaded rats significantly attenuates the pressor effect of the latter independently of salt intake (641). These findings suggest that under certain conditions, the hypotensive action of ANP is dependent on the functional antagonism of ANG II. Also, ANP appears to be temporally and causally implicated in mediating renal escape from aldosterone action following prolonged exogenous administration of this mineralocorticoid (220, 221, 642). In addition, in experimental ANG II-dependent salt sensitive hypertension the time-dependent inhibition of aldosterone is mirrored by an increase in ANP release (226, 638). On the other hand, chronic administration of a hypotensive dose of ANP for 2-7 days does not significantly affect plasma renin activity (PRA), aldosterone or AVP concentrations in conscious rats (11), dogs (13) or sheep (14, 15), neither are the plasma levels of these hormones significantly altered in the TTR-ANP mice (16, 17). These observations were interpreted as indicative that ANP does not chronically inhibit the activity of salt conserving mechanisms. This interpretation, however, may be challenged on the basis that the failure of PRA to increase, in the face of a chronic reduction in ABP and renal perfusion pressure, may be due to a tonic inhibitory effect of ANP on renin (47, 215) and/or ACE activity (340). The reduced renal perfusion pressure per se is a potent stimulus for sodium retention (238). Thus the ability of these animals to maintain salt balance against this antinatriuretic background could be due to increased renal excretory capacity, brought about by persistent effects of ANP on salt excretion by collecting duct and on RAS activity. Since the basal level of renal excretory activity is determined by ECFV, which itself is determined by extracellular sodium content (238), it is expected that the renal effects of ANP and its antagonism of renal salt-conserving mechanisms will play an essential role in the renal adaptation to high dietary salt intake, when these antinatriuretic mechanisms normally are minimally active. On this basis, conditions (pathological or hereditary) characterized by decreased or deficient ANP biological activity are expected to be accompanied by an inherent
propensity for sensitization of ABP to high salt intake; with the increase in ABP, thus, becoming the supporting mechanism for renal salt excretion. The ANP-mediated antagonism of renal salt-retaining mechanisms may also have functional implications for regulation of salt excretion in pathological conditions in which these salt-retaining mechanisms are inappropriately activated, such as in CHF (595) and in mineralocorticoid-dependent hypertensive states (595, 638).

It is also possible that the vasodilatory action of ANP may result, in part, from antagonism of vasoconstrictor activity of ANG II. ANP apparently antagonizes the central (614, 643-645) and peripheral (50, 217, 646-649) pressor effects of Ang II. In man (639, 640) and in dogs (649), for example, the acute pressor effect of ANG II is significantly attenuated by ANP. However, it is not discernible from these studies whether the observed effect of ANP on the pressor response to ANG II is due to specific antagonism of the latter, or whether it simply reflects the overall response to opposite and independent vasoeffecter mechanisms. Other evidence from animal models does suggest a dependency on inhibition of ANG II pressor activity for expression of ANP-induced hypotension. In anesthetized rats, the acute hypotensive response to ANP infusion is almost fully abolished by prior inhibition of ACE with captopril (650), and sheep chronically infused with an hypotensive dose of ANP had an attenuated response to an acute pressor dose of exogenous ANG II (15). Interestingly, the antagonism of ANG II pressor activity by ANP is accentuated in high renin-dependent hypertensive states (639, 640), suggesting that in such cases, the suppressive action of ANP on ANG II-mediated cardiovascular effects may effectively function as a counterregulatory mechanism contributing significantly to homeostatic regulation of blood pressure.
4. Summary

The hypotensive response to ANP cannot be attributed to one single hemodynamic effect of this hormone. Rather, it results from the integration of coordinated direct effects of the peptide on the vasculature, and interactions with other mechanisms involved in regulation of ABP, including the autonomic nervous system, vasoconstrictor hormones, and possibly the vascular endothelium. The antagonism of vasoconstrictor activity by ANP may operate as a negative feedback mechanism that could contribute significantly to homeostatic regulation of ABP. The fact that vasoconstrictor substances, including norepinephrine (116, 117), ANG II (118) and endothelin (122, 331) invariably stimulate ANP release, provide further support for such negative feedback modulation of ABP by ANP. This putative mechanism of blood pressure regulation may play a significant role in blood pressure control acutely, particularly in pathophysiological situations characterized by elevated vasoconstrictive activity (595). In the long-term, ANP may exert its vasodilation-dependent hypotensive effect primarily by modulating the activity of tonic vasoregulatory mechanisms mediated by vasoactive endothelial factors and the sympathetic nervous system, such that a net vasodilatory effect prevails. When maximal renal excretory activity of ANP is required, such as during elevated dietary salt intake, the chronic hypotensive effect of ANP may be partly dependent on its physiological antagonism of RAS activity. Consequently, pathophysiological conditions in which such antagonism is absent or suppressed are expected to lead to sensitization of ABP to high salt.
F. RATIONALE FOR THE STUDY AND DEVELOPMENT OF HYPOTHESES

When I began the Ph.D. program in 1994, it was known that ANP exhibited acute and chronic hypotensive effects, and that these effects were mediated by differing underlying hemodynamic changes. The acute hypotensive effect of ANP was brought about by a reduction in cardiac output (3-7), whereas the chronic hypotensive effect was mediated by vasodilation of the resistance vasculature (11-15, 18). The chronic vasodilatory effect of ANP seemed paradoxical, in light of the evidence that the resistance vasculature, with the possible exclusion of the renal vascular bed (24, 25), displayed generalized insensitivity towards ANP-induced relaxation (28-32), in part due to the scarcity of GC-A receptors in resistance VSM membranes (26, 27). This suggested that chronic ANP-dependent dilation of the resistance vasculature could be mediated by intermediary vasoeffectector mechanisms whose single or combined activity leads to a reduction in peripheral vascular resistance.

The existent and emerging evidence at the time was that ANP could modulate the activity of several tonic vasoregulatory systems, including the vascular endothelium (32), the autonomic nervous system (33), and under certain conditions, the RAAS (34). More specifically, ANP was known to stimulate production of locally-acting vasodilators CNP and NO and inhibit ET-1 synthesis from VE (35-41), and to modulate the target cardiovascular responses of these vasoactive substances (42, 578-581). Furthermore, ANP was known to exert widespread acute inhibition of central (43) and peripheral (44, 45) mechanisms of sympathetic neurotransmission, and to antagonize the renal and cardiovascular actions of RAAS (49-50).

The extent to which these interactions of ANP might occur chronically were not characterized, although some indirect evidence suggested that the actions of ANP on VE, SNS and the RAS were chronically active in vivo (330-335, 618, 639, 640). Thus, ANP could decrease peripheral vascular resistance chronically by tonically reducing sympathetic vascular tone, and by
regulating the synthetic activity of the VE, such that the vasodilatory moieties associated with NO and CNP are potentiated and the vasoconstriction associated with ET-1 is reduced. An additional component of chronic ANP-dependent hypotension could be determined by interaction of the peptide with the activity of RAAS. Such interaction would be maximally active during maintenance on high dietary salt, when endogenous ANP activity and renal and cardiovascular actions of the peptide are potentiated (231, 234, 314).

This thesis therefore investigated the relative contribution of tonic vasoregulatory influences from the vascular endothelium and sympathetic nervous system to the chronic hypotensive effect of ANP, based on the general hypothesis that ANP-dependent vasodilation is mediated by potentiation of vasodilatory NO and CNP and inhibition of vasoconstrictor ET-1 from VE of the resistance vasculature, and by attenuation of vascular sympathetic tone and/or the RAAS. In addition, I evaluated the consequence of life-long deficiency in endogenous ANP activity on the renal and hormonal adaptations to increased dietary salt intake. The objective was to determine whether chronic deficiency in ANP activity compromises the renal and hormonal adaptations to high salt intake and leads to salt sensitivity of ABP. The following specific hypotheses were tested:

1. **Specific hypotheses**

1. Since ANP exerts a chronic hypotensive effect that is mediated by reduced peripheral resistance, chronic endogenous ANP deficiency will lead to hypertension in association with elevated TPR.

2. ANP upregulates basal tissue content and activity of ecNOS and CNP and tonically inhibits ET-1 synthesis in resistance vessel endothelium.

3. ANP chronically attenuates basal vascular sympathetic tone.

4. The combined effects of ANP on upregulation of ecNOS and CNP activities, inhibition of ET-1 synthesis and attenuation of sympathetic vascular tone lead to reduction of peripheral vascular resistance.
5. Chronic ANP deficiency leads to sensitization of arterial blood pressure during elevated dietary salt intake due to failure to downregulate plasma renin activity.

2. Choice of experimental models

The traditional approaches for assessing the physiological functions of a hormone involve both ablation of the endogenous source as well as studying natural pathological models of over-or underproduction of the hormone. However, it is not technically feasible to remove all ANP-producing cells from the heart and other sources, neither are there any identifiable natural disease models. These technical difficulties have been overcome with the development of genetic models of ANP over- and underproduction. The hypotheses proposed in this thesis were tested in two complementary, functionally-opposite genetic mouse models expressing life-long alterations in endogenous ANP activity; an hypotensive ANP-overexpressing (TTR-ANP) transgenic mouse, and an hypertensive ANP gene knockout (-/-) mouse characterized by lack of ANP activity. The physical and hemodynamic characteristics of the two models are summarized in Table 1.1.

a) Experimental models

(i) transgenic mouse

The generation of ANP transgenic mice was previously described in detail (16) and is briefly outlined here. The mouse ANP structural gene containing all three exons as well as approximately 1.75 Kb of the 3' flanking region was fused to the transthyretin (TTR) promoter to target ectopic constitutive expression of ANP to the liver. The fusion gene (TTR-ANP) was linearized (HindIII) and microinjected into the male pronucleus of fertilized C3HeB/FeJ embryos. Embryos at the two cell stage were implanted into oviducts of pseudopregnant females and allowed to develop to term. The resulting animals were screened for the transgene by Southern blot analysis of tail tissue. Immunoreactive plasma ANP level was 8-10 fold higher and the animals were significantly hypotensive relative to their control (NT) littermates (Table 1.1).
(ii) ANP gene knockout mouse

The ANP gene knockout is fully described in (19). Briefly, a targeting construct which deletes 11 base pairs from exon 2 of mouse preproANP and replaces it with the neomycin resistance gene, was introduced into embryonic stem cells from mouse strain 129 by electroporation. Targeted cells were injected into recipient blastocysts and returned to pseudopregnant foster mothers. The resulting chimaeric mice harboring the mutation were mated to mice of strain C57BL/6J (B6). Matings between the resulting 129 x B6 heterozygotes produced homozygous mutant (-/-), heterozygous (+/-) and wild type (+/+) F3 offspring in roughly Mendelian ratios. Plasma and atrial ANP were undetectable by radioimmunoassay, and ANP-specific granules were absent in the mutant (-/-) mice.

<table>
<thead>
<tr>
<th></th>
<th>ABP, mm Hg</th>
<th>HR, bpm</th>
<th>Hct, %</th>
<th>HW/BW, g/g</th>
<th>KW/BW, g/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTR (n = 9)</td>
<td>77 ± 2*</td>
<td>395 ± 17</td>
<td>45.2 ± 1</td>
<td>0.5 ± 0.02*</td>
<td>0.85 ± 0.03</td>
</tr>
<tr>
<td>NT (n = 9)</td>
<td>104 ± 3</td>
<td>390 ± 11</td>
<td>45.2 ± 1</td>
<td>0.7 ± 0.02</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td>-/- (n = 9)</td>
<td>119 ± 2#</td>
<td>459 ± 15</td>
<td>48.2 ± 1</td>
<td>0.6 ± 0.04#</td>
<td>1.31 ± 0.07</td>
</tr>
<tr>
<td>+/- (n = 9)</td>
<td>91 ± 3</td>
<td>484 ± 13</td>
<td>47.7 ± 0.4</td>
<td>0.5 ± 0.02</td>
<td>1.31 ± 0.03</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

TTR, NT, -/-, +/-, transgenic, non transgenic, knockout, and wild type respectively.


*, # Statistical difference (P<0.05, unpaired t-test) between TTR, NT (*) or -/-, +/- (#)
3. Experimental approach

a) Animals

F₂ homozygous ANP knockout, TTR-ANP and the corresponding wildtype littermates were used in all experiments. The knockout mice and their controls were obtained from our resident colony. The TTR-ANP and their controls were kindly supplied by Drs. Loren Field (Krannert Inst. of Cardiology, Ind.) and by Dr. Mark L. Stainhelper (U. of Texas, San Antonio). The genotypes of the animals were identified by Southern blot analysis of DNA extracted from tail tissue.

b) Experiments

(i) systemic hemodynamics in +/- and +/+ mice (Chapter 2)

Although the systemic hemodynamics in the TTR-ANP mouse have previously been characterized (18), this has not been done in the ANP knockout mice and their wild type controls. In this study, I used the thermodilution method to characterize basal systemic hemodynamics in +/- and +/+ mice, with the objective of identifying the hemodynamic alteration(s) underlying the elevation of ABP in the +/- mice. This study tested hypothesis # 1.

(ii) role of endogenous vasoactive endothelial factors in chronic regulation of arterial blood pressure by ANP (Chapter 3)

In this study, I measured the steady state concentrations of ET-1, CNP and ecNOS in representative whole-organ homogenates from TTR-ANP, NT, +/- and +/+ mice as an index of local synthesis from resistance vessel endothelium and tested in +/- and +/+ mice whether the responsiveness of ABP and HR to endogenous ET-1, CNP and NO is affected by the level of endogenous ANP activity. This study investigated hypotheses # 2 and 4.
(iii) role of cardiovascular sympathetic tone in chronic regulation of arterial blood pressure by ANP (Chapter 4)

ABP and HR were measured in TTR-ANP, NT, -/- and +/+ mice under basal conditions and following autonomic ganglionic blockade (AGB), in order to determine whether chronic differences in endogenous ANP activity alter the underlying cardiovascular sympathetic tone. In addition, I measured pressor and chronotropic responses to norepinephrine infusion during autonomic ganglion blockade in order to determine if cardiovascular responsiveness to peripheral adrenergic receptor stimulation is affected by the chronic level of ANP activity. I also measured total catecholamine (epinephrine + norepinephrine) in plasma from the different genotypes as complementary indirect index of sympathetic activity. This study tested hypotheses # 3 and 4.

(iv) role of ANP in cardiorenal adaptation to chronically elevated dietary salt intake (Chapter 5)

In this study, ABP and plasma renin activity (PRA) were measured in -/- and +/+ mice maintained either on low (0.008% NaCl) or high (8% NaCl) salt diet for 3-4 weeks, in order to assess whether the -/- mice develop salt-sensitive hypertension in association with absence of ANP-dependent physiological antagonism of RAS. A second group of +/+ and -/- animals were chronically treated with AT-1 antagonist losartan for the duration of the dietary regimen to determine if salt-induced changes in ABP are functionally linked to ANG II receptor activation. A third group of -/- and +/+ mice, also maintained on 8% NaCl, were used to assess renal and collecting duct function during acute ECF volume expansion, in order to determine whether lack of ANP limits renal excretory capacity for salt. This study evaluates hypothesis # 5.
CHAPTER 2

SYSTEMIC HEMODYNAMICS IN ANP KNOCKOUT MICE

INTRODUCTION

Recent findings in genetic mouse models expressing alterations in endogenous activity of ANP suggest that this hormone, in addition to its well-defined acute hypotensive effect (3, 4) also participates in chronic regulation of blood pressure. Overexpression of a transthyretin-ANP fusion gene (TTR-ANP) in mice leads to a marked reduction in arterial blood pressure (ABP), in association with the lifelong elevation of plasma ANP concentration (16). In contrast, disruption of the native genes for ANP (4) or its guanylate cyclase-A (GC-A) receptor (20) by gene targeting results in hypertension, consequent to elimination of ANP activity.

The chronic hypotensive effect of ANP in the TTR-ANP mice is mediated by a reduction in total peripheral resistance (TPR), due to vasodilation of several regional vascular beds (11, 12, 18). The hemodynamic correlates underlying the expression of hypertension in the ANP “knockout” mice, however, are not known. On the basis of the hemodynamic characteristics of the TTR-ANP mice (18), it may be expected that the hypertension of -/- mice is, at least partially, determined by an elevation in TPR. This is indeed implied by the observed increases in heart weight/body weight ratios (19) and baseline diastolic pressures (Melo and Sonnenberg, unpublished observations) in these mice, relative to their normotensive wild-type littermates. Cardiac hypertrophy and high diastolic pressure are hallmarks of chronic hypertension (651, 652), the former developing as a physiological adaptive response to a chronic increase in aortic afterload (651), the latter as a manifestation of elevated TPR (652).

In the present study, the thermodilution method was employed in order to characterize basal systemic hemodynamics in -/- and +/- mice, with the purpose of identifying the hemodynamic alteration(s) underlying the elevation of ABP in -/- mice.
METHODS

Animals. F₂ homozygous mutant (/-) and wild-type (+/+ ) mice of both sexes, 17-22 weeks old and weighing 31-34 grams were used in this study. The animals were obtained from our resident colony, which was founded with pathogen-free heterozygous (+/-) breeding pairs. The genotypes were identified by Southern blot analysis of EcoR I-digested genomic DNA from the tail (19) soon after weaning. The animals were housed according to sex in groups of two to four per cage and kept at ambient 23 °C and 40% humidity in a room with a 12:12-hour light-dark schedule, and maintained on normal rodent chow (0.4% NaCl, Ralston Purina no. 5001).

Surgical preparation. On the day of the experiment the animals were anesthetized with Inactin (150 µg/g body weight ip) and kept at a body temperature near 38 °C with a heat lamp. After tracheostomy, a jugular vein and femoral artery were cannulated with catheters (300-400 µm diameter) fashioned from pulled-out PE-50 polyethylene tubing, for intravenous infusion and measurement of mean arterial pressure (ABP, mm Hg) and heart rate (HR, beats.min⁻¹) respectively. A thermistor F#1 (Columbus Instruments, Columbus, OH) was inserted in the right common carotid artery and advanced to the junction at the aortic arch for measurement of cardiac output (mL.min⁻¹) by thermodilution. All catheters were held firmly in place with cotton ligatures and kept patent by prior flushing with heparinized (20 U/ml) isotonic saline. Upon completion of surgery, 0.12 ml of isotonic saline containing 2.25% bovine serum albumin (BSA) and 1% glucose were infused over 15 minutes and followed by constant infusion of the same solution at 0.12 ml/hr for the duration of the experiment, except for brief interruptions for measurement of cardiac output, during which time the solution was delivered as a 25 µl bolus. The experiment was begun after an additional 15 min equilibration period.
**Measurement of blood pressure and heart rate.** ABP and HR were monitored continuously during the experiment using a small volume displacement pressure transducer (model RP 1500, Narco Systems, Toronto, Ontario) connected to a MacLab/4e data acquisition system. HR was calculated instantaneously from the pressure pulses. Measurements of ABP, HR and CO were taken simultaneously.

**Measurement of cardiac output.** The experimental set-up for measurement of cardiac output by thermodilution is shown in Figure 2.1. Twenty five microliters of 0.9%NaCl:2.25% BSA:1% glucose at room temperature (≈24 °C) were rapidly delivered (≈1 sec) into the jugular vein by a microinjector pump (model 500, Columbus Instruments). Injectate and blood temperatures were recorded individually by a Cardiomax II-R CO computer (Columbus Instruments). CO was calculated instantaneously from the thermodilution curve of the injected saline by the Cardiomax II computer using the equation

\[
CO = \frac{(T_{\text{blood}} - T_{\text{inj}}) \times (\text{vol.})}{\int T \, dt}
\]

where:

- \( T_{\text{blood}} \) = temperature of blood prior to injection (°C).
- \( T_{\text{inj}} \) = temperature of injectate (°C).
- \( \text{vol.} \) = volume of injected bolus (ml).
- \( \int T \, dt \) = area under the thermodilution curve (°C/min).

The thermodilution curve was recorded with an IBM-compatible computer (486 DX2-66) equipped with the Easyest LX (Kithley Asyst, Taunton, MA) data acquisition system. Its area was calculated by a factory-installed algorithm that is based on the two-sample points method of Williams et al (653). The averages of four successive CO measurements taken 1 minute apart are reported in this study. Stroke volume (SV, μl.beat⁻¹) and total peripheral resistance (TPR, mm Hg.min.ml⁻¹) were derived
from the equation \( ABP = (HR \times SV \times TPR) \). The omission of right atrial pressure from the equation was considered to have been a negligible error. At termination of the experiment, the position of the thermocouple microprobe was verified by autopsy.

**Statistical analysis.** All results are presented as means ± SE. The unpaired t-test was used to compare differences between mutant and wild-type mice. A \( P \) value of <0.05 was considered to indicate statistically significant difference.

**Cardiac output measurements using the indirect thermodilution technique**

![Diagram showing the setup for cardiac output measurement using the thermodilution technique.](image)

**Figure 2.1.** Set-up for measurement of cardiac output using the thermodilution technique.
RESULTS

Representative thermodilution curves for four successive measurements of CO in one -/- mouse are shown in Fig. 2.2. The individual curves were superimposable, and the calculated CO values in each animal were within 10% of one another, and were highly comparable to those obtained by others using an aortic electromagnetic flow probe (654). Recirculation was negligible, as suggested by the smoothness of the descending limb of the curves, indicating that the injected bolus of cold (room temperature) saline equilibrated with blood temperature within one circulatory passage. Comparable characteristics were observed in the thermodilution curves of control +/+ mice.

Figure 2.2. Thermodilution curves for four successive CO measurements taken ~ 1 minute apart in a -/- mouse.
Basal ABP and HR of -/- and +/- mice are shown in Fig. 2.3. ABP was significantly elevated in the -/- mice (132 ± 4) compared to the +/- control (95 ± 2) (Fig. 2.3A) (P<0.0001). HR tended to be lower in -/- mice (407 ± 22) than in +/- mice (462 ± 21) (Fig. 2.3B) but this did not reach statistical significance. The baseline systemic hemodynamics for -/- and +/- are shown in Fig. 2.4. There was a tendency towards lower cardiac output in -/- mice (7.3 ± 0.5) compared to the +/- mice (8.3 ± 0.6) as a result of lower basal HR, but this did not reach statistical significance (Fig. 2.4A). Stroke volume (Fig. 2.4B) did not differ significantly between genotypes. Total peripheral resistance was significantly elevated in the -/- mice (18.4 ± 0.7) compared to the +/- mice (12.3 ± 1.0) (Fig. 2.4C) (P<0.0003).

**DISCUSSION**

Chronic increases in ANP activity, either by administration of exogenous hormone (11-15), or by *in vivo* overexpression of an ANP transgene in mice (18) lead to hypotension, due to a reduction in TPR. The current study shows that mice rendered genetically incapable of synthesizing ANP develop hypertension, in association with an elevation of TPR. These findings thus provide complementary evidence that ANP exerts a chronic hypotensive effect, which is primarily mediated by vasodilation of the resistance vasculature.

The mechanism by which ANP chronically reduces TPR is not known. The insensitivity of the resistance vasculature to ANP (28-31), suggests that the relaxant effect of this hormone in the microvasculature may be mediated by an indirect vasoeffector mechanism. In this regard, ANP has been shown to acutely potentiate synthesis of locally-acting vasodilatory NO (40-41) and CNP (39) and to inhibit release of vasoconstrictor ET-1 (35-38) from VE. Such effects of ANP on the synthetic activity of VE, if chronically active, would result in a net vasodilatory influence which could account, at least partially, for the tonic relaxant effect of this hormone in the resistance vasculature. In
Figure 2.3. Baseline arterial blood pressure (ABP) (A) and heart rate (HR) (B) in -/- (n = 7) and +/+ (n = 10) mice. ABP differed significantly between genotypes (*, p<0.0001).
Figure 2.4: Baseline cardiac output (CO) (A), stroke volume (SV) (B) and total peripheral resistance (TPR) (C) in +/+ (n = 7) and +/− (n = 4) mice. TPR differed significantly between genotypes (p > 0.0003).

A. CO (ml/min)
B. SV (ul/beat)
C. TPR (mm Hg/ml/min)
addition, there is evidence that the autonomic nervous system may also be involved as an intermediary effector of ANP-dependent vasodilation, inasmuch as the ability of ANP to reduce ABP, is partly determined by its widespread inhibition of sympathetic nervous activity. (33, 43-46). For example, the acute hypotensive effect of ANP is greatly attenuated by autonomic ganglionic blockade (326-329), and is exacerbated by conditions characterized by high sympathetic tone (333, 334).

In conclusion, the present study shows that knockout mice lacking endogenous ANP activity develop high resistance hypertension. These findings, in conjunction with previous observations that ANP exerts a chronic, low-resistance hypotensive effect, thus, provide complementary evidence that ANP participates in the long-term regulation of arterial blood pressure, primarily by affecting the tone of the resistance vasculature.

**Perspectives**

Previous work has shown that chronic increases in endogenous ANP activity lead to a sustained, resistance-dependent reduction in ABP (11-15, 18), in the absence of any major changes in renal function (15, 17). The present study extends these observations and shows that chronic deficiency in ANP activity leads to high resistance hypertension. These complementary findings suggest that the long-term hypotensive effect of ANP is predominated by its indirect action on vascular resistance.

It remains controversial whether deficiencies in endogenous ANP activity contribute to the pathology of hypertensive disease. A decrease in ANP secretion is seen in the prehypertensive stages of several hypertensive rat models with close resemblance to variants of human essential hypertension (655, 656), suggesting that a defect in ANP synthesis/secretion may be an initiating factor in the development of hypertension. Polymorphisms of the ANP gene have recently been reported to occur with significantly greater frequency in humans with essential hypertension (657), and mutations in the
GC-A receptor gene cosegregate with ABP in Dahl salt-sensitive rats (658). The common theme between these and the current findings is that genetic defects in ANP activity increase the predisposition for development of hypertension.
CHAPTER 3

CHRONIC REGULATION OF ARTERIAL BLOOD PRESSURE BY ANP: ROLE OF ENDOGENOUS VASOACTIVE ENDOTHELIAL FACTORS

INTRODUCTION

ANP reduces ABP chronically, consequent to generalized vasodilation of vascular beds (11-15). The resistance vasculature has, however, been reported to be insensitive to direct relaxation by ANP (28-31), suggesting that the chronic ANP-dependent dilation of resistance vessels is mediated by intermediary vasoeffector mechanisms whose single or cumulative action(s) reduce peripheral vascular resistance. In this regard, the vascular endothelium (VE), which is known to play a major role in local regulation of vascular tone (32, 336), may act as a modulator of chronic ANP-dependent vascular effects, especially in light of recent evidence that ANP may affect synthesis and secretion of locally-acting vasoactive substances from VE (35-41, 331) as well as their effects on target tissues (42, 184, 185, 330, 576, 577, 579, 580). ANP inhibits production of vasoconstrictor endothelin-1 (ET-1) (34-36, 331) and stimulates synthesis of vasodilators C-type Natriuretic Peptide (CNP) (39) and possibly nitric oxide (NO) (40, 41) from vascular endothelial cells. Furthermore, ANP attenuates the pressor effect of ET-1 (42, 576-580) and may potentiate the vasodilatory action of CNP by downregulating endothelial “C”-type receptors (184, 185, 330) involved in clearance of natriuretic peptides.

These findings imply that such ANP-endothelium interactions, if operative in vivo, may represent a novel mechanism by which ANP exerts its chronic hypotensive effect. Thus, ANP could reduce peripheral vascular resistance chronically by regulating the synthetic activity of VE in the resistance vasculature, such that the vasoconstrictor moiety associated with ET-1 is reduced and vasodilatory CNP and NO are potentiated, and/or by modulating target responses to these substances.
In the present study, I measured steady-state concentrations of ET-1, CNP and endothelial constitutive nitric oxide synthase (ecNOS) in several tissues of ANP-overexpressing transgenic mice (TTR-ANP) and pro-ANP gene knockout mice (-/-) and their respective wild type controls (NT, +/-) in order to determine whether the chronic effect of ANP on blood pressure is attributable to differences in the synthesis of these paracrine vasoactive factors by resistance vessel endothelium. In addition, I measured changes in arterial blood pressure (ABP) and heart rate (HR) in +/- and +/- mice following antagonism of ET\(_{A/B}\) receptors, inhibition of nitric oxide synthase (NOS) activity or immunoneutralization of guanylate cyclase-B (CG-B) receptors in order to test whether the responsiveness of these target cardiovascular parameters to endogenous ET, NO and CNP is affected by the level of ANP activity.

METHODS

**Animals.** Male TTR-ANP mice and their respective NT littermates and +/- and +/- mice of both sexes, 8-13 months old and weighing 27-42 grams were used in this study. The TTR-ANP mice were kindly provided by Dr. L.J. Field, Krannert Institute of Cardiology (Indianapolis, IN). The +/- and +/- animals were obtained from a resident colony. The animals were housed according to sex and genotype in groups of 2-4 per cage and kept at ambient 23 °C and 40% humidity in a room with a 12 hour light-dark cycle.

**Materials.** All materials for the ET-1 and CNP radioimmunoassays (RIA), including porcine ET-1 and CNP(1-22) standards, [\(^{125}\)I]-CNP(1-22), rabbit polyclonal anti-ET-1 and anti-CNP sera, goat-anti-rabbit-IgG serum and normal rabbit serum were from Peninsula Laboratories (Belmont, CA), with the exception of [\(^{125}\)I]-ET-1 (NEN Dupont, Markham, Ontario). Acrylamide, bis-acrylamide, Dowex-AG50W-X8 (H\(^+\)) resin, Poly-Prep chromatography columns and protein assay kit (Bradford) were from Bio Rad (Mississauga, Ontario). The ecNOS monoclonal antibody was
from Transduction Labs (Lexington, KY). [³H]-L-arginine, Hybond-C nitrocellulose membranes and enhanced chemiluminescence (ECL) kit were purchased from Amersham (Oakville, Ontario). ±SB 209670 was a generous gift of SmithKline Beecham (King of Prussia, PA). 3G12 monoclonal antibodies were kindly donated by Genentech (San Francisco, CA). CNP-22 was from Phoenix Pharmaceuticals (Mountain View, CA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

**Tissue preparation.** ET-1, CNP and ecNOS immunoreactivities were measured in whole organ homogenates of kidney, heart, lung, brain and hindquarter skeletal muscle. The tissues were frozen in liquid nitrogen immediately upon dissection from anesthetized mice (Inactin, 100 µg/g body weight) and stored intact at -70 °C until assayed. The tissues were washed with two rinses of cold (~ 4 °C) phosphate buffered saline (PBS, pH 7.4), cut into small pieces and homogenized (~ 5:1, ml:g tissue) on ice in protein lysis buffer (50 mM Tris.HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 2µM leupeptin, 2 µM pepstatin A, 0.1% β-mercaptoethanol) with 2-4 successive bursts (11,000 rpm, 10 sec) as required, from a Polytron (PT 3300, Brinkmann Instruments, Littau, Switzerland). The homogenates were mixed with glycerol (10% vol/vol) and centrifuged at 10,000 g for 15 min in a refrigerated (4 °C) microfuge, to remove cellular debris.

**Tissue concentration of ET-1 and CNP.** ET-1 and CNP immunoreactivities were measured by RIA according to published methods (130). For the ET-1 and CNP RIA, 100 µl of the cleared supernatants were incubated (1:1:1) with the respective rabbit polyclonal anti-ET-1 or anti-CNP sera and [¹²⁵I]-ET-1 or [¹²⁵I]-CNP tracers. The sensitivity of the ET-1 and CNP assays were 5.8 pg/100 µl and 3.8 pg/100 µl respectively. Cross-reactivity of the ET-1 antiserum with big ET-1, ET-2 and ET-3 and CNP was 35%, 7% and 7% and 0% respectively. CNP antiserum crossreactivity with CNP(1-53) and ET-1 was 100% and 0% respectively. All values for ET-1 and CNP were normalized
for total protein concentration in the sample, as determined by the Bradford method.

**Tissue concentration of ecNOS.** ecNOS immunoreactivity in the tissue homogenates was measured by Western blot (659). The homogenates were treated with 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) for 20 min at 4 °C for solubilization of membrane bound protein. One hundred micrograms of total protein extract were electrophoresed according to the method of Laemmli, in 6% sodium dodecyl sulfate-polyacrylamide gels (Mini-Protean II, BioRad) under reducing and denaturing conditions, and transferred to Hybond-C nitrocellulose membranes by electroblotting. The membranes were blocked overnight (12-15 hr) at 4 °C with 4% BSA (fraction V, Sigma) in Tris buffered saline:0.1% Tween 20 (TBS-T, pH 7.5) and incubated with 1:2000 dilution of mouse anti-ecNOS monoclonal antibody for 1 hour. After three washes (15 min each) with TBS-T, the membranes were incubated with horseradish peroxidase anti-mouse IgG (1:5000) secondary antibody for 2 hours. The ecNOS signal was detected by ECL and quantified with NIH Image 1.52 (National Institutes of Health, Bethesda, MD).

**Total NOS enzyme activity.** NOS enzyme activity in the tissue homogenates was determined by measuring the rate of formation of [3H]-L-citrulline from [3H]-L-arginine (372). The tissues were homogenized as described above in lysis buffer (25 mM Tris.HCl (pH 7.4), 1mM EDTA, 1 mM EGTA). Fifty microliters of supernatant were incubated at 37 °C for 1 hour with reaction buffer (50 mM Tris.HCl (pH 7.4), 2.5 mM NADPH, 10μM tetrahydropterin, 10 μM flavin adenine dinucleotide, 10 μM flavin adenine mononucleotide, 50 U calmodulin, 2 μM [3H]-L-arginine, 10 μM L-arginine, 5 mM L-valine). Corresponding blank reactions were prepared by combining each sample with reaction buffer supplemented with 300 μM each of N^G^-nitro-L-arginine methyl ester (L-NAME) and N^G^-monomethyl-L-arginine (L-NMMA). The reaction was terminated by adding 400 μl of ice-cold stop buffer (50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), pH 5.5, 5 mM
EDTA). The total volume was applied to Poly-Prep chromatography columns containing 1 ml of Dowex AG 50W-X8 (Na⁺) pre-equilibrated with 2 ml of stop buffer. The [³H]-L-citrulline was eluted with 2 ml of distilled water. The radioactivity was quantified in 10 ml of scintillant in a β-liquid scintillation counter. Each blank was subtracted from its corresponding non-inhibited sample. Under these assay conditions, NOS activity displayed time-dependent linearity up to 60 minutes (Fig. 3.1). The results are presented as counts per minute (CPM).min⁻¹.mg protein⁻¹.

![Graph](image)

**Figure 3.1.** Nitric oxide synthase activity as a function of duration of incubation
**In vivo studies.** Mice (-/-, +/-) were anesthetized with Inactin (150 μg/g body wt ip) and kept at a body temperature near 38 °C with a heat lamp. After tracheostomy, a jugular vein and carotid artery were cannulated with catheters (300-400 μm diameter) fashioned from PE-50 tubing for intravenous infusion and measurement of mean blood pressure (ABP) and heart rate (HR) respectively. Upon completion of surgery, 0.12 ml of isotonic saline containing 2.25% bovine serum albumin and 1% glucose were infused over 15 min as a priming dose, followed by constant infusion of the same solution at 0.12ml/hr for the duration of the experiment. The experiment was begun after an additional 30 min equilibration period. BP and HR were monitored continuously and recorded at 10 min intervals with a small displacement pressure transducer (model RP 1500, Narco Systems) connected to a MacLab/4e data acquisition system. Each experiment consisted of a 30 min control period, followed by 30 min infusion of L-NAME (0.12 mg.kg⁻¹.min⁻¹) or ±SB 209670 (100 μg.kg⁻¹.min⁻¹) or 3G12 (20 μg.kg⁻¹.min⁻¹ ) to inhibit NOS (398), ET₄₅ (652) or GC-B (653) receptors respectively. At the end of the experiment, the inhibitors were coinfused with L-arginine (1.2 mg.kg⁻¹.min⁻¹) or ET-1 (100 ng.kg⁻¹.min⁻¹) or CNP (100 ng.kg⁻¹.min⁻¹) respectively, to assess the effectiveness and selectivity of inhibition.

**Statistical analysis.** All results are presented as means ± SE. Unpaired t-test was used to compare differences between mutant (TTR-ANP, -/-) mice and their respective wild-type controls (NT, +/-), with respect to ET-1, CNP and ecNOS concentration and NOS activity. One-way ANOVA followed by the Bonferroni multiple comparison test was employed to compare ABP and HR responses in vivo to the different treatments within and between genotypes. A P value of <0.05 was considered to indicate statistically significant difference.
RESULTS

The concentration of immunoreactive ET-1 in tissues of TTR-ANP, NT, -/- and +/- mice is shown in Table 3.1. Variable amounts of ET-1 were detected in the different tissues, but the distribution and abundance patterns were similar in all genotypes, with lung and skeletal muscle expressing the highest and lowest concentrations, respectively. No statistically significant differences in tissue ET-1 concentration were observed between mutant (TTR-ANP, -/-) mice and the corresponding control (NT, +/-) mice. However, there was a prominent strain-related difference in kidney ET-1 concentration between animals of the TTR-ANP, NT background (C3HeB/FeJ) and those of the -/-, +/- background (C57BL/6J).

The tissue concentrations of immunoreactive CNP are shown in Table 3.2. As for ET-1, the abundance patterns of CNP in the tissues were similar between the various genotypes. As expected, the brain contained the highest concentration of CNP. Lowest concentration was detected in skeletal muscle. There were also strain-related differences in kidney CNP concentration, with animals of the -/-, +/- strain having lower concentrations than those of the TTR-ANP, NT strain. No significant differences in tissue CNP concentrations were found, however, between mutant mice and their respective control mice.

Table 3.3 shows the concentration of immunoreactive ecNOS in the different tissues from the various genotypes. As for ET-1 and CNP, the relative tissue abundance of ecNOS was similar in all genotypes and did not differ statistically between mutant and control mice. The heart had the highest ecNOS concentration per unit of total homogenate protein, and comparable amounts were found in all other tissues. Likewise, total nitric oxide synthase (NOS) enzyme activity in the tissue homogenates (Table 3.4) was similarly distributed in all genotypes. NOS activity was several fold higher in brain and muscle than in the other tissues, reflecting the contribution of neuronal NOS, which was detected only in these two tissues (Melo and Sonnenberg, unpublished observations).
### TABLE 3.1. Tissue concentration of ET-1 (RIA) in TTR, NT, -/-, and +/- mice

<table>
<thead>
<tr>
<th></th>
<th>Kidney</th>
<th>Heart</th>
<th>Lung</th>
<th>Muscle</th>
<th>Brain</th>
</tr>
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<tbody>
<tr>
<td><strong>TTR (n = 8)</strong></td>
<td>41 ± 5</td>
<td>5.9 ± 0.5</td>
<td>99 ± 10</td>
<td>3.6 ± 1</td>
<td>40 ± 10</td>
</tr>
<tr>
<td><strong>NT (n = 8)</strong></td>
<td>39 ± 5</td>
<td>6.5 ± 2</td>
<td>93 ± 13</td>
<td>3.6 ± 0.3</td>
<td>38 ± 8</td>
</tr>
<tr>
<td><strong>-/- (n = 8)</strong></td>
<td>15 ± 2</td>
<td>4.7 ± 1</td>
<td>67 ± 15</td>
<td>4.4 ± 0.4</td>
<td>24 ± 3</td>
</tr>
<tr>
<td><strong>+/+ (n = 8)</strong></td>
<td>15 ± 5</td>
<td>3.7 ± 1</td>
<td>61 ± 10</td>
<td>4.6 ± 0.5</td>
<td>29 ± 4</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

ET-1, endothelin-1.

TTR, NT, -/-, +/-, transgenic, non transgenic, knockout, and wild type respectively.
TABLE 3.2. Tissue concentration of CNP (RIA) in TTR, NT, -/-, and +/- mice.

<table>
<thead>
<tr>
<th></th>
<th>Kidney</th>
<th>Heart</th>
<th>Lung</th>
<th>Muscle</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTR (n = 8)</td>
<td>38 ± 7</td>
<td>3.6 ± 0.4</td>
<td>11 ± 2</td>
<td>2.4 ± 0.2</td>
<td>43 ± 8</td>
</tr>
<tr>
<td>NT (n = 8)</td>
<td>39 ± 6</td>
<td>3.9 ± 0.6</td>
<td>9 ± 2</td>
<td>2.7 ± 0.3</td>
<td>44 ± 4</td>
</tr>
<tr>
<td>-/- (n = 7)</td>
<td>12 ± 2</td>
<td>3.9 ± 0.5</td>
<td>7 ± 2</td>
<td>2.7 ± 0.3</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>+/- (n = 8)</td>
<td>12 ± 2</td>
<td>3.2 ± 0.5</td>
<td>6 ± 1</td>
<td>3.1 ± 0.3</td>
<td>36 ± 3</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

CNP, C-type natriuretic peptide.

TTR, NT, -/-, +/-, transgenic, non transgenic, knockout, and wild type respectively.
TABLE 3.3. Tissue concentration of ecNOS (Western blot) in TTR, NT, -/-, and +/- mice.

<table>
<thead>
<tr>
<th></th>
<th>Kidney</th>
<th>Heart</th>
<th>Lung</th>
<th>Muscle</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TTR (n = 8)</strong></td>
<td>107 ± 18</td>
<td>214 ± 26</td>
<td>145 ± 19</td>
<td>106 ± 17</td>
<td>127 ± 14</td>
</tr>
<tr>
<td><strong>NT (n = 8)</strong></td>
<td>101 ± 19</td>
<td>214 ± 30</td>
<td>145 ± 20</td>
<td>118 ± 14</td>
<td>118 ± 9</td>
</tr>
<tr>
<td><strong>-/- (n = 8)</strong></td>
<td>115 ± 13</td>
<td>229 ± 31</td>
<td>174 ± 22</td>
<td>151 ± 11</td>
<td>112 ± 8</td>
</tr>
<tr>
<td><strong>+/- (n = 8)</strong></td>
<td>125 ± 18</td>
<td>226 ± 28</td>
<td>172 ± 24</td>
<td>143 ± 10</td>
<td>128 ±10</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

tcNOS, endothelial constitutive nitric oxide synthase.

TTR, NT, -/-, +/-, transgenic, non transgenic, knockout, and wild type respectively.
The cardiovascular effects of antagonism of ET$_{A}$ receptors with ±SB 209670 in -/- and +/- mice are shown Fig. 3.2. Basal ABP (Fig. 3.2A) differed significantly between genotypes. Basal HR (Fig. 1B) tended to be lower in -/- (Fig. 3.2B) than in +/- mice, but this difference did not reach statistical significance. The antagonist reduced ABP significantly (Fig. 3.2A) and decreased HR slightly (Fig. 3.2B) in both genotypes. The effects of the antagonist on ABP and HR were not reversed by co-infusion (10 minutes) with a dose of ET-1 previously titrated to produce a rise of 30-35 mm Hg in ABP. The relative changes (%) in ABP and HR following ±SB209670 administration were quantitatively similar in both genotypes (Table 3.5).

The effect of immunoneutralization of GC-B receptor activity with 3G12 monoclonal antibody on ABP and HR is shown in Fig. 3.3. Basal ABP (Fig. 3.3A) and HR (Fig. 3.3B) for the two genotypes was similar to those of the previous group (Fig. 3.2). The antibody slightly, but not significantly increased ABP (Fig. 3.3A) and HR (Fig. 3.3B)) by comparable relative (%) amounts (Table 3.5) and reduced the hypotensive effect of CNP (10 min) infused at a dose producing 20-25 mm Hg decrease in ABP (Fig. 3.3A).

Figure 3.4 shows the effect of inhibition of endogenous NOS activity with L-NAME on ABP and HR in -/- and +/- mice. As in the previous two groups, basal ABP was significantly higher in -/- mice than in +/- mice (p<0.0001) (Fig. 3.4A) and HR was slightly, but not significantly lower in -/- mice than in +/- mice (Fig. 3.4B). L-NAME increased ABP significantly in both genotypes (p<0.0001) (Fig. 3.4A). HR did not change in -/-, but increased slightly in +/- . The effects of L-NAME on ABP and HR were not prevented by co-infusion (10 minutes) with a 10-fold molar excess of L-arginine. Comparable changes (%) in ABP and HR were found between genotypes (Table 3.5).
TABLE 3.4. Total NOS enzyme activity in tissues of TTR, NT, -/-, and +/- mice.

<table>
<thead>
<tr>
<th></th>
<th>[³H]-L-citrulline, CPM.min⁻¹.mg protein⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kidney</td>
</tr>
<tr>
<td><strong>TTR (n = 6)</strong></td>
<td>578 ± 113</td>
</tr>
<tr>
<td><strong>NT (n = 6)</strong></td>
<td>507 ± 22</td>
</tr>
<tr>
<td><strong>-/- (n = 6)</strong></td>
<td>849 ± 100</td>
</tr>
<tr>
<td><strong>+/+ (n = 8)</strong></td>
<td>1141 ± 213</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

ecNOS, endothelial constitutive nitric oxide synthase.

TTR, NT, -/-, +/-, transgenic, non transgenic, knockout, and wild type respectively.

NOS, nitric oxide synthase, CPM, counts per minute.
**TABLE 3.5.** Changes (%) in ABP and HR in response to infusion (30 min) of ±SB 209670, L-NAME, or 3G12 in -/- and +/- mice.

<table>
<thead>
<tr>
<th></th>
<th>ABP (% change)</th>
<th>HR (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SB209670  L-NAME  3G12</td>
<td>SB209670  L-NAME  3G12</td>
</tr>
<tr>
<td>-/-</td>
<td>-27 ± 4  53 ± 5  10 ± 6</td>
<td>-6 ± 8  -1 ± 5  4 ± 1</td>
</tr>
<tr>
<td>+/-</td>
<td>-25 ± 2  50 ± 6  8 ± 3</td>
<td>-4 ± 4  7 ± 5  4 ± 1</td>
</tr>
</tbody>
</table>

Values are mean ± SE

-/-, +/-, knockout, wild type mice

ABP, arterial blood pressure, HR, heart rate

**DISCUSSION**

A contributory role of ANP to chronic regulation of ABP is implied by observations that lifelong genetic alterations in the level of endogenous ANP activity uncover a tonic hypotensive effect of this hormone (16, 19, 20) which is associated with vasodilation of the resistance vasculature (18). The rationale for the present study is based on the premise that the chronic hypotensive effect of ANP may be mediated by an intermediary vasoeffectector mechanism, inasmuch as previous observations had shown that the resistance vasculature per se is, at least acutely, insensitive to ANP (27-32). Our aim was to examine whether the chronic vascular effects of ANP are attributable to differences in activity of locally-acting vasoactive substances ET-1, CNP and NO. I measured concentrations of ET-1, CNP and eNOS in whole organ homogenates from TTR-ANP, NT, -/- and +/- mice as an index of local synthesis from resistance vessel endothelium (32, 359, 451, 487, 488, 662), and tested whether the
Figure 3.2  Average arterial blood pressure (ABP), (A) and heart rate (HR), (B) during baseline conditions (solid bar) and after infusion (30 min) of ET antagonist ±SB 209670 (hatched bar) in -/- (n = 8) and +/- (n = 8) mice. Baseline ABP differed significantly between genotypes (*, p<0.0002). Significant differences were also found between baseline ABP and ±SB 209670 in both genotypes (#, -/- = p<0.005; +/- = p<0.0001), as well as between genotypes after ±SB 209670 (**, p<0.03) and between baseline ABP and ±SB209670 + ET-1 (+, p<0.05).
Figure 3.3  Average ABP (A) and HR (B) during baseline conditions (solid bar) and after immunoneutralization of GC-B receptors (iv infusion, 30 min) with 3G12 (hatched bar) in +/- (n = 5) and +/-/+ (n = 5) mice. Baseline ABP differed significantly between genotypes (*, p<0.05). No significant differences in ABP or HR were found between baseline conditions and 3G12.
Figure 3.4  Average ABP (A) and HR (B) during baseline conditions (solid bar) and after inhibition of NOS (iv infusion, 30 min) with L-NAME (hatched bar) in -/- (n = 6) and +/- (n = 6) mice. Baseline ABP differed significantly between genotypes (*, p<0.0001). Significant differences were found between baseline ABP and L-NAME and L-NAME + L-ARG (#, +, -/- = p<0.0001; +/+ = p<0.0001) in both genotypes as well as between genotypes after L-NAME (**, p<0.005)
responsiveness of ABP and HR to endogenous ET-1, CNP and NO were affected by the level of ANP activity. Our results show that between mice of identical genetic background, neither the synthesis of ET-1, CNP or NO from the resistance vasculature nor their effects on cardiovascular regulation were altered by the chronic level of ANP bioactivity, thus indicating that long term vascular effects of ANP are not determined by differences in activity of these endothelial modulators.

The lack of a detectable effect of ANP on the synthetic activity of VE is surprising. Endothelial cells have an abundance of both C and GC-A ANP binding sites (27, 174, 447). Activation of the preponderant C-receptor subtype, leads to inhibition of basal ET-1 synthesis (36) and may potentiate basal ecNOS activity via inhibition of adenylate cyclase (40, 381), whereas GC-A-dependent elevation in cGMP production inhibits agonist-mediated ET-1 synthesis (35, 37) and stimulates ecNOS (41, 380) and CNP synthesis (39). I hypothesized that the chronic vascular effects of ANP could be effected by these receptor-mediated interactions with the endothelium. Thus, tonic activation of resistance vessel endothelium by ANP in TTR-ANP would lead to hypotension, concomitant with potentiation of CNP and ecNOS and inhibition of ET-1, whereas the absence of such modulation in the ANP knockout (-/-) mice would result in hypertension. This is clearly not supported by our findings. A possible cause for the lack of differences in synthetic activity of VE between TTR-ANP and NT, is that endothelial cell responsiveness to ANP is attenuated in the TTR-ANP mice, due to homologous receptor downregulation (184). Conversely, absence of ANP may result in an increase in the number of its endothelial receptors, and the consequent increase in basal activity of these receptors may account for the lack of differences in synthetic activity of VE between -/- and +/-.
Alternatively, ANP may exert its effects on peripheral vascular resistance by modulating the responsiveness of target cardiovascular effects of endothelial factors. A functional interaction between ET-1 and ANP, for example, is suggested by the almost identical distribution of receptors for both peptides in many target tissues (659). ANP attenuates vascular reactivity to ET-1 by reducing intracellular calcium concentration (580) and possibly, by decreasing the number of ET\(_A\) receptors in vascular smooth muscle (VSM) (177, 664). ANP may also lead to upregulation of soluble guanylate cyclase in VSM (665) via its inhibitory action on adenylate cyclase (177), and prolong the vascular effects of CNP by downregulating C-receptors (184). Our in vivo findings indicate that endogenous ET-1 and NO, and to a lesser extent CNP participate significantly in cardiovascular regulation in +/- and +/+ mice, but their relative contributions to maintenance of basal arterial blood pressure and heart rate are not influenced by the chronic level of ANP activity. These observations suggest that ANP interactions with the target cardiovascular effects of ET-1, NO and CNP are ineffective in the chronic state, or else, that they may be overcome by counteracting influences on cardiovascular function.

A direct effect of ANP on the resistance vasculature, however unlikely, cannot be totally discounted. On the basis of the similarities in cardiovascular phenotype between the GC-A and the ANP knockout models, it is tempting to speculate that the chronic hypotensive effect of ANP may be due to direct GC-A-mediated relaxation of the resistance vasculature. However, the scarcity of GC-A receptors and relative insensitivity to ANP-mediated cGMP production in resistance vessel smooth muscle (26, 27), would preclude a role of this pathway in ANP-induced dilation of the resistance vasculature. Indeed, the physiological significance of cGMP-mediated ANP vasodilation is doubtful, as it appears to be a pharmacological characteristic of large arteries (299, 317). Nevertheless, the GC-A knockout model firmly establishes the role of this receptor in chronic
regulation of blood pressure (327). To this extent, the ANP and CG-A models share similarities in cardiovascular function, at least with respect to actions of ANP that are mediated by the GC-A receptor.

In conclusion, the results of the present study show that over a wide range of chronic ANP activity, neither the synthesis of ET-1, CNP and NO from the resistance vasculature, nor their actions on the cardiovascular system are affected, thus indicating that the chronic effect of ANP on vascular resistance is not mediated by the endothelium.
CHAPTER 4.

CHRONIC REGULATION OF ARTERIAL BLOOD PRESSURE BY ANP: ROLE OF CARDIOVASCULAR SYMPATHETIC TONE

INTRODUCTION

As discussed in the preceding chapter, the chronic effects of ANP on vascular resistance and ABP are not attributable to differences in synthesis or activity of locally-acting vasoactive endothelial factors NO, CNP or ET-1, inasmuch as the concentration of these substances in the resistance vasculature and their target cardiovascular effects are not altered by chronic differences in endogenous ANP activity. This finding prompted me to test an alternate hypothesis that the chronic hypotensive effect of ANP may be mediated via attenuation of cardiovascular sympathetic tone. This hypothesis was formulated on the basis of well-documented evidence that ANP acutely exerts widespread sympathectomy at all levels of sympathetic nervous function (33, 43-46, 337, 581, 603, 605, 606), and from observations that the hypotensive effect of ANP is significantly reduced by autonomic ganglionic blockade (287, 326, 328, 329), and is exacerbated by chronically elevated sympathetic tone (333-335). These findings suggest a dependency on inhibition of sympathetic tone for expression of the hypotensive effect of ANP.

It could be inferred from these findings that the sympathectomy activity of ANP, if tonically active, could contribute to the chronic vasodilatory effect of this hormone in the resistance vasculature. Thus, in the absence of this antagonism, the -/- mice would be expected to develop hypertension, in association with an elevation of cardiovascular sympathetic tone, whereas the chronically elevated plasma ANP activity in the TTR-ANP would lead to attenuation of sympathetic tone and manifestation of the hypotensive phenotype characteristic of this model. The present study was designed to determine if tonic cardiovascular sympathetic tone is altered by chronic differences
in endogenous ANP activity. I measured total plasma catecholamine concentration and changes in ABP and HR following autonomic ganglionic blockade in TTR-ANP, +/- and control mice as indirect indices of sympathetic nerve activity. In addition, I measured pressor and chronotropic responses to exogenous norepinephrine infusion during ganglion blockade in all genotypes and compared norepinephrine receptor binding in representative tissues of +/- and +/+ in order to determine whether peripheral cardiovascular responsiveness to adrenergic stimulation is affected by the level of endogenous ANP activity.

METHODS

Animals. TTR-ANP, +/- and their respective controls, 8-12 months old, and weighing 24-38 grams were used in this study. The physical and genetic characteristics of the different strains and the housing conditions have been described previously in the thesis (Chapters 1 and 2). The +/- and +/+ mice were obtained from our resident colony. The TTR-ANP and NT mice were kindly supplied by Dr. Mark E. Steinhelper (U. of Texas, San Antonio, TX).

Materials. All materials for the catecholamine radioenzymatic assay were provided with the kit (Amersham, Oakville, Ontario), with the exception of toluene and isoamyl alcohol which were purchased from Sigma Chemical Co. (St. Louis, MO) and Liquifluor which was purchased from Canberra Packard (Mississauga, Ontario). Pentolinium, hexamethonium, norepinephrine and bacitracin were also from Sigma. PMSF, leupeptin and pepstatin were from ICN (Montreal, Quebec). Scintiverse scintillation cocktail was obtained from Fisher Scientific (Nepean, Ontario) and l-[2,5,6-^3^H]- Norepinephrine was purchased from NEN Dupont (Markham, Ontario). All other chemicals were from VWR (Mississauga, Ontario).

Surgical preparation, cardiac output, blood pressure and heart rate measurement. The surgical preparation for intravenous infusion and measurement of ABP and HR was described in
Chapter 2 (pp. 88-89). CO, ABP and HR were recorded as previously described as in Chapter 2.

**Autonomic ganglion blockade (AGB).** Total autonomic ganglion blockade was achieved by intravenous infusion of 0.5 mg.min⁻¹.kg⁻¹ hexamethonium:0.05 mg.min⁻¹.kg⁻¹ pentolinium for 30 minutes. The effectiveness of blockade was assessed at the end of the experiment by absent or diminished reflex changes in ABP and HR subsequent to bilateral carotid artery ligation.

**Experimental protocol for in vivo studies.** All in vivo experiments consisted of a 30 min control period, followed by 30 min autonomic ganglionic blockade and a further 30 min infusion of norepinephrine (±arterenol, 1μg.min⁻¹.Kg⁻¹) under continuous ganglionic blockade (Fig. 4.1). ABP and HR were monitored continuously and recorded every 10 min for the duration of the experiment. The end point of each period is reported.

<table>
<thead>
<tr>
<th>ABP AND HR MEASUREMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibration</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>-30</td>
</tr>
</tbody>
</table>

**Figure 4.1.** Experimental protocol for in vivo autonomic ganglionic blockade experiments

**Blood sample collection.** Blood for catecholamine analysis was collected by exsanguination of freshly decapitated animals into chilled tubes containing a final concentration of 1.8 mg EGTA:1.2 mg glutathione per ml of blood. The tubes were inverted several times to mix the blood with the preservatives and centrifuged at 3000 revolutions/min (rpm) for 15 minutes at 3°C. The cleared plasma samples were stored at -70 °C until assayed.
**Total plasma catecholamines.** Total plasma catecholamine (norepinephrine and epinephrine) concentration was measured by a modified radioenzymatic method of Peuler and Johnson (666) with a commercially available kit (Amersham, Oakville, Ont.). The sensitivity of the assay is in the range of 2-5 pg per 50 μl of sample. The assay is based on the catalytic transfer of a [3H]-methyl group from tritiated S-adenosyl-L-methionine ([3H]-SAM) to the hydroxyl group in the 3-position of epinephrine and norepinephrine by catechol-O-methyltransferase (COMT), to produce [3 H]-metanephrine and normetanephrine respectively. These derivatives are then converted to [3H]-vanillin by periodate oxidation. The radioactivity associated with [3H] vanillin is proportional to the unknown catecholamine (norepinephrine and epinephrine) concentration in the sample being analyzed.

The plasma samples were assayed according to the instructions provided by the manufacturer. Briefly, 50 μl of plasma were mixed in duplicate with 40 μl of a reaction mixture consisting of Tris-EGTA-MgCl₂ buffer pH 8.5, [3H]-SAM and COMT in disposable glass tubes. A norepinephrine + epinephrine standard was added to one of the plasma samples to a final concentration of 400 pg/ml in a final volume of 100 μl, and an equivalent volume of stabilizing buffer was added to the duplicate sample. All samples were incubated at 37 °C for 1 hr. At the end of the incubation the contents of each tube were mixed vigorously with 50 μl of of a 4 mM mixture of metanephrine and normetanephrine for termination of the methylation reaction. Each sample was mixed with 2 ml of toluene/isoamyl alcohol (3:2 v/v) for catecholamine extraction, centrifuged at 1000 rpm and frozen for 15 sec in a dry ice:ethanol bath. The upper organic phase was decanted into a second set of tubes and mixed vigorously with 100 μl of 0.1M acetic acid. The mixture was frozen in the dry-ice:ethanol bath as previously, and the organic phase was aspirated. The acetic acid residue was dried under a stream of air for 2 hr and mixed vigorously with 1 ml of 0.05M ammonium hydroxide. Periodate oxidation of the samples was initiated by addition of 50 μl of 4% (w/v) of sodium metaperiodate and
terminated after 5 minutes by addition of 50 μl of 10% (v/v) glycerol and 1 ml of 0.1 M acetic acid. Each sample was mixed vigorously for 20 sec with 10 ml of toluene/Liquifluor (1000:50, v/v) and frozen in dry ice-ethanol. The upper organic phase was decanted in separate scintillation vials containing 2 ml of 0.1M acetic acid and counted in a liquid scintillation counter for 2 minutes. Calculation of sample catecholamine concentration was performed according to the instructions of the manufacturer, using the formula

\[
\frac{\text{CPM sample} - \text{CPM blank}}{\text{CPM (sample +st) - CPM sample}} \times \frac{\text{pg standard}}{\text{sample vol. (ml)}}
\]

where:

CPM = counts per minute

st = standard (400 pg/sample)

**Tissue norepinephrine binding.** Membranes for norepinephrine receptor binding were prepared from tissues (kidney, heart, brain, liver and skeletal muscle) of -/- and +/- mice. Tissues were harvested from anesthetized (sodium pentobarbital, 45 mg/kg) mice, flash-frozen in liquid nitrogen and stored at -80 °C until processed. Frozen tissues were thawed and rinsed in 0.9% NaCl. The tissues were then minced and homogenized in a Teflon/glass homogenizer with 10 volumes of cold homogenization buffer containing 10 mM MgCl₂, 10 mM Hepes pH 7.5, 10 mM NaCl, 5 mM PMSF, 5 μg/ml leupeptin and 5 μl/ml pepstatin. The suspension was centrifuged at 4,000g for 10 minutes at 4 °C. The pellet was discarded, and the membrane in the supernatant was pelleted by centrifugation at 16,000g for 15 minutes at 4 °C. The membrane pellet was then resuspended in 10 mM NaHCO₃ at pH 8, containing 5 mM PMSF, 5 μg/ml leupeptin and 0.1% bacitracin and stored at -70 °C. Protein concentration was determined by a modified Lowry method using bovine serum albumin (BSA) as a standard.
Norepinephrine binding assays were performed at 20 °C in a binding buffer containing 50 mM Hepes pH 7.5, 5 mM MgCl₂, 10 mM NaCl, 1 mM PMSF, 2 mg/ml leupeptin, 0.1% BSA and 0.1% bacitracin. The binding reaction was initiated by the addition of 100,000 cpm l-[2,5,6-³H]-Norepinephrine, in the presence or absence of competing 100 μM unlabelled norepinephrine, to 100 μg of membrane in a final volume of 300 μl. Duplicate samples were incubated for 30 minutes and then centrifuged at 16,000g for 10 minutes to separate free from bound ligand. The membrane pellets were washed three times with 100 μl of wash buffer (10 mM Hepes, 10% sucrose, pH 7.5). The washed pellet was resuspended in 200 μl of wash buffer and added to 5 ml of ScintiVerse and counted on a Beckman LS 1801 scintillation counter.

Statistical analysis. All results are presented as means ± SEM. Unpaired t-test was employed to compare differences in plasma catecholamine between mutant (TTR-ANP, -/-) and their controls (NT, +/-), as well as differences in tissue norepinephrine binding and in systemic hemodynamic changes to AGB between -/- and +/- mice. One-way ANOVA coupled with the Bonferroni multiple comparison test was used to compare ABP and HR responses to the different treatments within each group and between control and mutant mice of each genotype. A P value of <0.05 was considered to indicate statistically significant difference.

RESULTS

The effectiveness of AGB with hexamethonium:pentolinium in -/- and +/- mice is shown in Fig. 4.2. The blocker mixture effectively abolished reflex increases in ABP and reduced increases in HR following bilateral carotid artery ligation, indicating physiologically effective blockade. Comparable characteristics were observed in the TTR-ANP and NT mice.

The effect of AGB on ABP and HR of TTR-ANP and NT mice is shown in Fig. 4.3. Basal ABP was significantly lower in TTR-ANP than in NT (p<0.0001) (Fig. 4.3A). Basal HR also tended
Figure 4.2. Reflex changes in ABP and HR in response to bilateral carotid artery ligation in a -/- (A) and a +/- (B) mouse, following autonomic ganglionic blockade.
to be lower in TTR-ANP, but this difference was not significant (Fig. 4.3B). Ganglionic blockade significantly decreased ABP (p<0.05) (Fig. 4.3A) and tended to reduce HR (Fig. 4.3B) in both genotypes. However, the relative decrease in basal ABP following AGB was significantly greater in the NT mice compared to the TTR-ANP mice (Table 4.1). In contrast, the relative fall in HR was greater in the TTR-ANP mice than in the NT mice (Table 4.1). Norepinephrine infusion during AGB elicited similar pressor (Fig. 4.3A) and chronotropic (Fig. 4.3B) responses in both genotypes.

**TABLE 4.1.** Changes (%) in ABP and HR after autonomic ganglionic blockade.

<table>
<thead>
<tr>
<th></th>
<th>ABP (% change)</th>
<th>HR (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AGB</td>
<td>AGB + NE*</td>
</tr>
<tr>
<td>TTR-ANP (n = 6)</td>
<td>-14 ± 3*</td>
<td>58 ± 4†</td>
</tr>
<tr>
<td>NT (n = 6)</td>
<td>-34 ± 1</td>
<td>86 ± 7</td>
</tr>
<tr>
<td>-/- (n = 7)</td>
<td>-48 ± 2*</td>
<td>114 ± 9*</td>
</tr>
<tr>
<td>+/- (n = 7)</td>
<td>-21 ± 2</td>
<td>73 ± 6</td>
</tr>
</tbody>
</table>

* relative to AGB alone

Values are means ± SE

*, †, ‡ Statistical difference between mutant (TTR-ANP, -/-) and controls (NT, +/-) (* P < 0.0001, † P < 0.007, ‡ P < 0.05)
Figure 4.3. Average ABP (A) and HR (B) during baseline conditions (solid bar) and after autonomic ganglionic blockade (HEX, hatched bar), and in response to intravenous norepinephrine infusion (HEX + NE, speckled bar) in TTR-ANP (n = 6) and NT (n = 6) mice. Baseline ABP differed significantly between genotypes (*, p<0.0001). Significant differences were also found between baseline ABP and HEX (#, TTR-ANP, p<0.05; NT, p<0.001) and HEX +NE (+, TTR-ANP, p<0.0001, NT, p<0.05), as well as between HEX and HEX + NE (**, p<0.0001). Differences in HR were found between baseline and HEX+ NE (+, p<0.001) and between HEX and HEX + NE (**, p<0.0001).
Figure 4.4 shows the effect of AGB in -/- and +/+ mice. Basal ABP was significantly elevated in -/- mice (p<0.05) (Fig. 4.4A), whereas HR did not differ significantly between genotypes (Fig. 4.4B). AGB reduced ABP (Fig. 4.4A) and HR (Fig. 4.4B) in both genotypes. However, the relative fall in ABP was twice as great in the -/- mice compared to the +/+ mice (Table 4.1). This was associated with a significant relative decrease in TPR (Fig. 4.5C), whereas the fall in CO (Fig. 4.5A) and SV (Fig. 4.5B) did not differ between -/- and +/+ mice. The pressor (Fig. 4.4A) and chronotropic (4.4B) responses to norepinephrine during AGB did not differ significantly between the two genotypes.

Total plasma catecholamine concentration in the four genotypes is shown in table 4.2. Plasma catecholamine concentration was lower in the TTR-ANP mice, but this difference did not reach statistical significance. In contrast, -/- mice had significantly higher plasma catecholamine concentration compared to the +/+ mice.

**TABLE 4.2.** Total plasma catecholamine concentration in TTR-ANP, NT, -/- and +/+ mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Catecholamine concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTR-ANP (n = 4)</td>
<td>5848 ± 514</td>
</tr>
<tr>
<td>NT (n = 4)</td>
<td>7475 ± 1124</td>
</tr>
<tr>
<td>-/- (n = 4)</td>
<td>13,803 ± 2125*</td>
</tr>
<tr>
<td>+/+ (n = 4)</td>
<td>8109 ± 852</td>
</tr>
</tbody>
</table>

Values are means ± SE

* Statistical difference between -/- and +/+ (P = 0.047)
Figure 4.4. Average ABP (A) and HR (B) during baseline conditions (solid bar) and after autonomic ganglionic blockade (HEX, hatched bar), and in response to intravenous norepinephrine infusion (HEX + NE, speckled bar) in -/- (n = 7) and +/- (n = 7) mice. Baseline ABP differed significantly between genotypes (*, p<0.05). Significant differences were also found between baseline ABP and HEX (#, -/-, p<0.0001; +/-, p<0.05) and between HEX and HEX + NE (**, p<0.0001). Significant differences in HR were found between baseline and HEX (#, p<0.05) and HEX + NE (+, p<0.05), as well as between HEX and HEX + NE (**, p<0.0001).
Figure 4.5. Changes in systemic hemodynamics following autonomic ganglionic blockade in -/- (n = 7) and +/+ (n = 10) mice. Significant differences in the % change in TPR were found between -/- and +/+ mice (*, p<0.02).
Table 4.3 shows norepinephrine receptor binding in representative tissues of -/- and +/- mice. Kidney and skeletal muscle had the highest and lowest binding respectively, and variable amounts of receptor binding were found in cardiac ventricle, brain and liver. No statistically significant differences in norepinephrine binding were found between tissues of -/- and +/- mice.

**TABLE 4.3.** Adrenergic receptor binding in tissues of -/- and +/- mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Kidney</th>
<th>Ventricle</th>
<th>Brain</th>
<th>Liver</th>
<th>Skeletalmuscle</th>
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<tbody>
<tr>
<td>-/-</td>
<td>1732 ± 405</td>
<td>1244 ± 190</td>
<td>1105 ± 108</td>
<td>1007 ± 178</td>
<td>584 ± 155</td>
</tr>
<tr>
<td>(n = 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/-</td>
<td>2025 ± 329</td>
<td>1207 ± 230</td>
<td>1140 ± 179</td>
<td>993 ± 163</td>
<td>535 ± 208</td>
</tr>
<tr>
<td>(n = 4)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Values are means ± SE
DISCUSSION

The present study shows that tonic cardiovascular sympathetic tone, as assessed by the magnitude of the changes in ABP and HR to autonomic ganglionic blockade, is attenuated in transgenic mice with chronically elevated plasma ANP, and is increased in knockout mice with absent ANP activity. These differences in cardiovascular sympathetic tone are paralleled by chronic hypotension in the transgenic mice and by hypertension in the +/- mice, and are accompanied by directional changes in plasma catecholamine concentration. Furthermore, the magnitude of the fall in ABP and HR to AGB is not determined by the basal ABP and HR (Fig. 4.6), indicating that the observed differences in cardiovascular sympathetic tone underlie genotype-specific differences in sympathetic nerve activity. These findings indicate that the chronic hypotensive effect of ANP is dependent on attenuation of cardiovascular sympathetic tone.

The differences in ABP between the mutant mice and their genetically-matched controls are fully abolished by AGB, indicating that the underlying differences in sympathetic tone per se, account for the differences in cardiovascular phenotype. Furthermore, the exaggerated hypotensive response of +/- mice to AGB is determined by a decrease in TPR, in the absence of apparent changes in cardiac performance. These findings imply that the chronic hypotensive effect of ANP requires attenuation of sympathetic tone to the resistance vasculature. The requirement for such an intermediary effector mechanism of ANP-dependent relaxation of the resistance vasculature is further suggested by the apparent scarcity of GC-A receptors in resistance vessels, and their relative insensitivity to ANP-dependent cGMP synthesis (26, 27), thus precluding a direct role of this pathway in mediating the effect of ANP on vascular resistance.

The nature of the neuromodulatory action of ANP on sympathetic nervous activity has not been fully elucidated. When administered acutely, ANP exerts a pervasive sympatholytic effect which
Figure 4.6. Relationship between baseline ABP (A, B) and HR (C, D) and the final ABP and HR during ganglionic blockade (ABP = A, HR = C) and norepinephrine infusion (ABP = B, HR = D).
is mediated primarily by GC-A receptor activation (27, 33). Centrally, ANP reduces sympathetic outflow from cardiovascular regulatory areas in the brain stem (41, 42, 338, 581, 603) and inhibits autonomic ganglion transmission (604). Peripherally, ANP inhibits spontaneous and evoked norepinephrine synthesis and release from post-ganglionic sympathetic nerve fibers (45, 46) and adrenal medulla (605), and it interferes with the functional expression of post-synaptic α-1 adrenergic receptors (606). The extent to which these interactions may occur chronically is not known. The present study clearly shows that the genotype-dependent differences in sympathetic tone are not due to changes in responsiveness of target cardiovascular responses to peripheral adrenergic receptor stimulation, given that norepinephrine infusion elicits comparable absolute pressor and positive chronotropic responses in ganglion-blocked mutant and wild type control mice. In concordance with this, whole-organ adrenergic receptor binding is not altered by the level of endogenous ANP activity, and although the binding data do not totally discount the possibility of regional differences in vascular adrenergic receptor density or subtype between genotypes, the absence of differences in norepinephrine binding in the whole-organ membrane preparations is in good agreement with the observed in vivo responses to adrenergic receptor stimulation. These findings suggest that the chronic effect of ANP on sympathetic nerve activity is mediated by pre-junctional mechanism(s). It could be argued that the widespread co-localization of ANP and GC-A in the sympathetic nervous system (27, 619, 620), may function as a tonically active neuromodulatory unit (33, 619, 621), participating in local inhibition of sympathetic nervous activity. In this regard, the low vascular resistance in ANP-overexpressing transgenic mice (18) could be due to tonically elevated ANP-dependent modulation of sympathetic nerve function, whereas the lack of such neuromodulation in +/- mice could account for the high peripheral resistance seen in these animals. This may also explain the hypertension of GC-A knockout mice (20), since the sympatholytic effects of ANP are mediated by the GC-A receptor
Thus, the -/- and GC-A knockout models would be expected to share a common mechanism of hypertension, characterized by elevated vascular sympathetic tone.

Alternatively, the differences in cardiovascular sympathetic tone between the mutant and control mice may develop secondarily to abnormalities in other ANP-dependent actions. ANP, for example, reduces renin release (47) and antagonizes the central pressor effect of ANG II, in part, by attenuating ANG II-dependent increases in sympathetic outflow (60). Furthermore, chronic increase in ANG II activity produces an hypertensive phenotype that is determined, at least in part, by an increase in sympathetic outflow (597, 638, 641). The development of ANG II-dependent hypertension is effectively abrogated by ANP (641), suggesting that under normal conditions, the antagonism of ANG II pressor activity by ANP may subserve a physiologically significant role in chronic regulation of ABP. In light of this evidence, it is conceivable that the observed ANP genotype-dependent differences in cardiovascular sympathetic tone may be determined, in part, by the tonic level of ANG II activity. The absence of such antagonism in the -/- mice would be expected to lead to an increase in basal ANG II activity. The resultant potentiation of sympathetic activity would then account, wholly or in part, for the observed increase in cardiovascular sympathetic tone and the underlying hypertensive phenotype. This mechanism would be self-sustaining, as the increase in renal sympathetic nerve activity would maintain plasma renin activity elevated (588, 597, 598).

The extent to which structural alterations in the resistance vasculature may contribute to the ANP-dependent differences in basal blood pressure is not known. Chronic increases in ABP are accompanied by hypertrophy and remodelling of the tunica media of resistance vessels (601, 667). These changes could increase vascular resistance directly by reducing lumen diameter (663), and indirectly by exacerbating the pressor effects of vasoconstrictors (668). Furthermore, chronically elevated sympathetic nerve activity has been shown to have trophic effects on the resistance
vasculature (599, 600). It may be speculated on the basis of these findings that the hypertension of -/- mice could potentially be caused by hypertrophy of the resistance vasculature, whereas the hypotension in the TTR-ANP may be due, in part, to reduced resistance vessel wall thickness and increased lumen diameter. However, the results of the present and previous (Chapter 3) study, while not excluding the possibility of structural changes in the resistance vasculature, do not show a greater pressor response to NE or ET-1 infusion in -/- mice compared to the +/- mice. This would have been expected if significant hypertrophy of the vasculature had occurred.

In conclusion, the present study shows that chronic differences in endogenous ANP activity in mice, resulting in life-long resistance-dependent alterations in ABP are accompanied by directional changes in underlying cardiovascular sympathetic tone; this being attenuated in hypotensive transgenic mice overexpressing ANP and elevated in hypertensive ANP-gene deleted mice. These findings indicate that ANP exerts a chronic vasodilatory effect leading to hypotension that is dependent on attenuation of sympathetic tone.
CHAPTER 5

SALT-SENSITIVITY OF ABP IN ANP KNOCKOUT MICE: ROLES OF ANGIOTENSIN II, ALDOSTERONE, SYMPATHETIC ACTIVITY AND COLLECTING DUCT FUNCTION

INTRODUCTION

An unresolved aspect of the physiological mechanism of chronic regulation of ABP by ANP, is the extent, if any, to which the effects of this hormone on regulation of renal salt excretion and ECFV may contribute to its hypotensive effects. ANP elicits pronounced natriuresis and diuresis acutely, directly by inhibiting sodium reabsorption in the medullary collecting duct (205), and indirectly, by antagonizing the actions of major hormonal and neural salt-conserving mechanisms such as the RAS (47-49, 215, 340) and the sympathetic nervous system (33, 581). Theoretically, it may be predicted that such effects, if tonically active, could contribute to the chronic hypotensive effect of ANP, given the fundamental dependency of chronic ABP on maintenance of ECFV by the kidney (238). Thus, a chronic increase in ANP activity would be expected to lead to hypotension in association with persistent natriuresis and reduction of ECFV, whereas chronic ANP deficiency would lead to hypertension in association with salt retention and expansion of the ECFV.

If such assumptions are correct, then the hypotension in TTR-ANP mice should be accompanied by reduced ECFV, and this should be further aggravated by low dietary salt intake, whereas high salt intake should tend to normalize ECFV and ABP. Surprisingly, ECFV is increased in the TTR-ANP mice, apparently due to higher plasma aldosterone concentration (17), whereas, neither plasma catecholamines (16, 17) nor plasma renin activity (PRA) (17) differ from the levels in the non-transgenic control mice. Furthermore, the TTR-ANP mice maintain salt balance on either dietary regimen, and there is no differential effect of salt on basal ABP between these mice and their NT controls (17). A priori, these findings appear to suggest that the chronic hypotensive action of
ANP occurs independently of its renal effects. However, it must be considered that the TTR-ANP mice maintain salt balance despite the reduced renal perfusion pressure. This may be due to an increase in the sensitivity of the renal pressure natriuresis mechanism (223), since the reduced perfusion pressure per se would tend to counteract the natriuretic effect of ANP. Furthermore, the failure of PRA and plasma catecholamines to increase, despite the underlying hypotension, may indeed be due to tonic inhibition by ANP.

With respect to the second assumption that ANP deficiency leads to salt retention and expansion of the ECFV, there is evidence that ANP activity is necessary for maintenance of salt balance during normal and high (for review see refs. 224, 234) salt intake. Plasma ANP concentration increases in parallel with salt intake (231, 669), and high dietary salt content potentiates the vasorelaxant effect of ANP in the renal vasculature (314), suggesting that ANP may be involved in maintaining constancy of ABP during increased dietary salt intake. On the basis of this, it is expected that a decrease in endogenous ANP activity predisposes to development of sensitivity of ABP to high dietary salt. Previous studies showed that ABP increased further in conscious -/- mice kept for 2 weeks on 2% salt (19), but not in anesthetized -/- mice after 1 week on 8% salt (230). Notwithstanding the methodological differences between these two studies, such as the state of alertness of the animals and the method of ABP measurement, the results suggest, nevertheless, that -/- mice may develop time-dependent sensitivity of ABP to high salt intake.

In principle, the sensitization of ABP to elevated salt intake in the -/- mice could be due to failure to adequately regulate the level of activity of salt-conserving mechanisms, such as the RAS and the sympathetic nervous system, and/or due to increased salt reabsorption from the inner medullary collecting duct (IMCD), since the physiological antagonism that ANP normally exerts on these systems and its natriuretic action in this tubular segment is absent.
The studies presented in this chapter were undertaken with the following objectives in mind:

1) In order to resolve the inconsistencies in the observed effects of salt on ABP of -/- mice, I measured ABP during, and after recovery from anesthesia in adult +/- and -/- mice kept on low (LS, 0.008% NaCl) or high (HS, 8% NaCl) salt diets for 3-4 weeks (study 1). PRA was measured at termination of the experiment, in order to determine whether potential differences in sensitivity to salt between -/- and +/- mice are attributed to abnormalities in regulation of PRA. In addition, given that increases in local production of NO and ET-1 are considered essential for the chronic renal adaptation to high salt intake (439, 670-673), I measured concentration of ecNOS and ET-1 in kidney extracts from the different groups of animals, to determine whether the sensitization of ABP to salt in the -/- mice is associated with failure to adequately upregulate renal synthesis of NO and ET-1. 2) In order to further characterize the role of ANG II in sensitization of ABP to salt in -/-, two groups each of -/- and +/- mice were maintained on 8% salt for 4 weeks (study 2). One group of each genotype was treated with daily injections of the AT-1 receptor antagonist losartan for the duration of the dietary regimen, and the two remaining groups served as controls and received an equivalent volume of vehicle as placebo. ABP and HR were measured at the end of the dietary regimen. Daily food and water intake and urinary excretion of fluid and electrolytes were measured for the first and last week of the dietary regimen to determine whether -/- mice develop time-dependent retention of salt relative to the +/- mice, and whether this is corrected by AT-1 receptor inhibition. At termination of the experiment, total plasma catecholamine and aldosterone concentrations were measured to determine their role in development of salt-sensitivity in -/- mice.

3) In order to evaluate whether chronic lack of ANP limits the excretory capacity for salt, renal and collecting duct function was assessed in -/- and +/- mice maintained on 8% salt for 3-4 weeks (study 3).
METHODS

Animals. The production of ANP knockout mice and the housing conditions were described in Chapter 1. F2 homozygous mutant (-/-) and wild-type (+/+) mice of both sexes, 20–24 weeks old and weighing 20–35 g, were used in all studies.

Dietary regimen. For study 1, two groups each of +/+ and -/- mice were maintained on a powdered Purina diet containing either low salt (0.008% NaCl, n = 10 +/+; n = 9 -/-) or high salt (8% NaCl, n = 9 +/+; n = 10 -/-) for 3–4 weeks before beginning the study. Food and distilled drinking water were available ad libitum. Except for the sodium content, the LS and HS diets were of identical composition. For study 2, two additional groups each of +/+ and -/- mice were maintained on the HS diet for 4 weeks. One group each of +/+ (n = 7) and -/- (n = 5) mice received daily injections of ANG II receptor antagonist losartan (DuP 753, 20 mg/Kg BW, courtesy of Merck-Dupont) for the duration of the dietary regimen. The antagonist was dissolved in distilled deionized water (10 mg/ml) and kept at 4 °C shielded from light in an aluminum foil-wrapped dark bottle. The remaining two groups (+/+, n = 6; -/-, n = 6) received equivalent daily injections of vehicle. All animals were housed in individual metabolic cages during the first and last weeks of the dietary regimen. Daily food and water intake and urinary excretion of fluid and electrolytes were measured during these periods. For assessment of renal and collecting duct function (study 3), two additional groups of -/- (n = 10, BW = 27 ± 1g) and +/+ (n = 10, BW = 24 ± 1g) were maintained on HS for 3 weeks. A third group of outbred mice (Crl-CD-1[CR]BR, n = 10, BW = 28 ± 1g) from Charles River Laboratories was used for comparative purposes. The outbred mice were kept on standard rodent chow (0.4% NaCl) prior to the experiment.

Surgical preparation for blood pressure measurement. For study 1, on the day of the experiment, the animals were anesthetized with 0.03–0.04 ml intramuscular injection of a 2:1 mixture
of ketamine (100 mg/ml) and xylazine (20 mg/ml) (Sigma Chemicals, St. Louis, MO). A catheter fashioned from pulled-out PE-50 polyethylene tubing was tunneled subcutaneously to exit at the nape of the neck. The catheter was flushed with heparinized (20 U/ml) saline, and secured in place with silk sutures. The beveled tip of the catheter (300–400 μm diameter) was then inserted into the previously dissected right common carotid artery and advanced to the junction at the aortic arch and firmly tied in position with silk sutures for measurement of blood pressure. For studies 2 and 3 the surgical preparation for ABP measurement was as described in Chapter 2.

**Blood pressure measurements.** In all experiments, ABP was monitored continuously during the experiment using a small volume displacement pressure transducer (model RP 1500, Narco Systems) connected to a MacLab/4e data acquisition system. Measurements of blood pressure were taken at 30 minute intervals. In study 1, two measurements were obtained while the mice were still under anesthesia. Following these measurements, the mice were returned to their individual cages and allowed to recover under an open bottomed box (12.5 cm x 10 cm x 5 cm) with a slot in the top for passage of the catheter. On average, recovery was complete within three hours following induction of anesthesia. After recovery, an additional four measurements of blood pressure at 30 minutes apart were taken from the conscious mice. In all other studies, ABP was measured in Inactin-anesthetized mice. In study 2, four measurements of ABP and HR were taken after the initial 30 min equilibration period.

**Kidney and nephron function study.** Animals were prepared for assessment of kidney and nephron function during acute plasma volume expansion as described previously (674). Briefly, after anesthesia with Inactin, tracheostomy and cannulation of carotid artery and jugular vein were performed for ABP measurement and intravenous infusion respectively. The bladder was catheterized, and the left kidney prepared for collecting duct microcatheterization similarly to the method
developed for rats (675). After completion of surgery, the outbred group (CD-1) received an intravenous priming dose (0.24 ml) of 2.25% BSA: 1% glucose in 0.9% NaCl followed by intravenous infusion (4 μl/min) of the same solution. The other two groups (+/+ HS, -/- HS) received a priming dose (1 ml) of isotonic saline, followed by constant infusion at 21 μl/min for intravascular volume expansion. The infusate contained sufficient 3H-inulin to allow assay of its concentration in samples of tubular fluid (674). After a 40 min equilibration period, three consecutive 20 min collections of bladder urine were taken. Arterial blood samples (5 μl) were obtained at 20 min intervals. ABP was monitored with the MacLab recording system as above. During this time, 6 pairs of fluid samples from deep (near the border between inner and outer medulla) and shallow (near the papillary orifice) sites were taken from different collecting duct systems (676). The concentrations of 3H-inulin and electrolytes in the urine and tubular fluid were used to calculate renal excretions as well as deliveries to, and transport along, the inner medullary collecting duct in each animal. 3H-inulin was measured by liquid scintillation counting. Concentration of sodium and potassium in plasma and urine were measured by flame photometry. Plasma, urinary and tubular fluid chloride concentrations were measured by electrometric titration (676), and tubular fluid sodium and potassium concentrations were measured by helium glow photometry.

**Blood sample collection.** In study 1, the mice were re-anesthetized with an intraperitoneal injection of sodium pentobarbital at termination of the experiment, and quickly exsanguinated. Blood was collected in chilled tubes containing 1 mg/ml EDTA and spun at 3000 rpm in a centrifuge for 10 minutes at 4°C. Plasma samples were stored at -70°C until assayed for plasma renin activity. In study 2, blood for catecholamine and aldosterone analysis was collected in chilled tubes containing a final concentration of 1.8 mg EGTA:1.2 mg glutathione per ml of blood. The samples were processed as above.
Plasma renin activity. PRA was measured in unextracted plasma with the RIANEN Angiotensin I [$^{125}$I] radioimmunoassay kit (Du Pont NEN, Boston, MA), according to the instructions provided by the manufacturer. All reactions were prepared in an ice bath. Briefly, 500 $\mu$l of plasma were mixed with 5 $\mu$l of dimercaprol, 5 $\mu$l of 8-hydroxyquinoline and 500 $\mu$l of maleate buffer. This mixture was then split equally into two separate aliquots. One aliquot was incubated at 37 °C for 1 hour. The other aliquot was kept on the ice bath (4 °C) for the same length of time. At the end of incubation, the 37 °C samples were transferred to the ice bath and paired with the corresponding 4 °C samples. For the radioimmunoassay, 100 $\mu$l of each sample were incubated with 100 $\mu$l of [$^{125}$I] Ang I tracer (= 12,000 cpm) and 100 $\mu$l of Ang I antiserum (rabbit) for 2 hours at room temperature (= 23 °C). The immunocomplexes were then precipitated by incubation with 500 $\mu$l of normal rabbit serum (secondary antibody) for 30 minutes at room temperature. The precipitates were centrifuged at 3,000 rpm and 4 °C for 20 minutes. The supernatants were removed by aspiration, and the radioactivity (cpm) of the pellets counted. The concentration of Ang I (ng/ml) was obtained from a standard curve of Ang I in the range of 0.1-10 ng/ml. Only concentrations falling in the linear range of the curve (0.25-6.0 ng/ml) were used, and samples beyond this range were diluted with maleate buffer and reassayed. Plasma renin activity (ng Ang I/ml/hour) was calculated by subtracting the concentration of Ang I in the 4 °C sample from the concentration of Ang I in the corresponding 37 °C sample after application of a correction factor for sample dilution.

Plasma aldosterone concentration. Plasma aldosterone was measured in unextracted plasma samples collected at the end of experiment from the animals in study 2. Aldosterone was measured with the Coat-A-Count Aldosterone radioimmunoassay kit (Diagnostics Products Corp., Los Angeles, CA) according to the procedures provided by the manufacturer. All reactions were prepared in an ice bath (= 4 °C). Briefly, 200 $\mu$l of plasma were incubated in duplicate with 1 ml of $^{125}$I.
aldosterone in aldosterone antibody-coated polypropylene tubes. The mixture was incubated at 37 °C for 3 hours. At the end of the incubation, the tubes were decanted thoroughly and the bound radioactivity counted for 2 minutes in a gamma counter. The concentration of aldosterone in the plasma samples was obtained from a standard curve prepared with human serum-based calibrators having aldosterone concentrations in the range of 25-1200 pg/ml. Under these conditions, the sensitivity of the assay was 19 pg/ml, the 50% displacement was at 340 pg/ml and the intrassay coefficient of variation was 5.4%.

**Total plasma catecholamine concentration.** Total plasma catecholamine concentration was measured in the plasma from the animals in study 2 by the modified radioenzymatic method of Peuler and Johnson (666) as described in Chapter 4.

**Tissue concentration of ET-1 and ecNOS.** ET-1 and ecNOS immunoreactivity was measured by radioimmunoassay (RIA) and Western blot respectively (659), in supernatants cleared at 10,000 G from whole organ homogenates of kidney and heart from the animals in study 1 as described previously in Chapter 3.

**Statistical analysis.** All results are presented as means ± SEM. In study 1 the data were analyzed by two-way analysis of variance (ANOVA) to test for separate and combined effects of genotypes and diet on ABP, PRA and ecNOS and ET-1 concentrations. One-way ANOVA followed by Bonferroni multiple comparison test was used to compare differences in ABP between groups at each timed measurement, and between individual measurements in each experimental period (anesthesia, conscious) within the groups. Inasmuch as no differences were found in the individual measurements of ABP within the groups, the average ABP during (-60, -30 min) and following recovery (30, 60, 90, 120 min) from anesthesia was calculated and the genotype and diet-related differences in means were further compared by one-way ANOVA. Statistical differences between
groups in the combined effects of genotype and diet on PRA, ecNOS and ET-1 concentrations were isolated from the 2-way ANOVA by a multiple comparison test. In study 2, the separate and combined effects of genotype and treatment (losartan vs vehicle) on ABP and HR and on plasma aldosterone, total catecholamine concentrations, food and water intake and urinary excretion of fluid and electrolytes were also analyzed by 2-way ANOVA. Statistical differences between groups were isolated with a multiple comparison test as for study 1. Differences in food and water intake and urinary excretion of fluid and electrolytes between weeks 1 and 4 of the metabolic study were analyzed by t-test for each group. In study 3, one-way ANOVA followed by Fisher’s probable least significant difference (PLSD) test was employed to identify statistical differences between the three groups. A P value of < 0.05 was considered to indicate statistically significant difference.

RESULTS

The time course patterns of ABP during and following recovery from anesthesia are shown in Fig. 5.1. ABP was uniformly and significantly higher (P < 0.05) in -/- mice maintained on high salt compared to -/- mice on low salt and +/- mice on either diet (P < 0.001, genotypes; P < 0.001, diets, 2-way ANOVA). The average ABP (mm Hg) during the conscious period (Fig. 5.2A) did not differ significantly between +/- mice on either diet (HS = 113 ± 9, LS = 110 ± 5). However, -/- mice on high salt diet had significantly elevated ABP compared to -/- mice kept on low salt diet (HS = 135 ± 3, LS = 115 ± 2) (P < 0.01) and +/- mice on either diet (P < 0.01) (Fig. 5.2A). The ABP of -/- mice on low salt diet did not significantly differ from that of +/- mice on either diet. Anesthesia lowered ABP slightly, but not significantly in all groups (HS -/- = 134 ± 6, +/- = 97 ± 7; LS -/- = 106 ± 5, +/- = 100 ± 6), and did not affect the genotype- and diet-related differences in ABP observed in the conscious state (Fig. 5.2B).
Figure 5.1. Patterns of mean arterial pressure (ABP) during (-60, -30) and after recovery (30, 60, 90, 120 min) from anesthesia in -/- and +/- mice previously maintained on high (HS, *, -/-; o, +/-)- or low (LS, *, -/-; a, +/-)- salt diets for 3-4 weeks. Significant differences were found at every interval between -/- mice on HS diet and +/- mice on LS diet (*) and between -/- on HS and +/- mice on either diet (+).

Plasma renin activity (ng Ang I/ml/hr) for all groups is given in Fig. 5.3. On the low salt diet, the +/- and -/- mice had comparable and appropriately elevated PRA values (+/+ = 21.1 ± 2.8; -/- = 19.1 ± 3.7). On the high salt diet, PRA decreased significantly (P < 0.05) in +/- mice to 4.9 ± 1.9, as expected, but not in -/- mice (17.7 ± 2.9). Indeed, there was no difference in PRA of the -/- mice over the 1000-fold difference in dietary salt content.
Figure 5.2. Average ABP in conscious state (A) and during anesthesia (B) in -/- and +/- mice fed on HS (solid bars) or LS (hatched bars) diets for 3-4 weeks. Differences were significant between -/- mice fed on HS and LS (*) and between -/- mice on HS diet and +/- mice on either diet (+). P< 0.05 by one-way ANOVA.
Figure 5.3. Plasma renin activity in -/- and +/+ mice fed on HS (n = 5 -/-; n = 7 +/+ ) and LS (n = 8 -/-; n = 10 +/+ ) for 3-4 weeks. Significant differences were found between +/+ mice fed on HS and LS diets (+ P < 0.5 by 2-way ANOVA and between -/- and +/+ mice fed on HS diet (*P < 0.003 by unpaired t-test.

The concentrations of ET-1 and ecNOS in kidneys and hearts of +/+ and -/- mice are shown in Table 5.1. There were no statistically significant differences in ET-1 and ecNOS concentrations between +/+ and -/- mice on either diet. However ET-1 and ecNOS concentrations were significantly increased (P < 0.05) in kidneys of both genotypes fed on HS diet.
TABLE 5.1. Concentration of endothelin-1 (ET-1, RIA) and endothelial nitric oxide synthase (ecNOS, Western blot) in kidneys and hearts of +/+ and -/- mice. Values are mean ± SEM. NS, not significant, +/+, -/-, normal, absent ANF respectively.

<table>
<thead>
<tr>
<th></th>
<th>ET, pg/mg protein</th>
<th>ecNOS, density/100μg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kidney</td>
<td>heart</td>
</tr>
<tr>
<td>Low-salt diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+ (n = 6)</td>
<td>21.4 ± 1.8*</td>
<td>8.2 ± 1.1</td>
</tr>
<tr>
<td>-/- (n = 6)</td>
<td>19.3 ± 1.9*</td>
<td>9.8 ± 2.1</td>
</tr>
<tr>
<td>High-salt diet</td>
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<tr>
<td>+/+ (n = 6)</td>
<td>31.9 ± 4.1</td>
<td>8.7 ± 2.3</td>
</tr>
<tr>
<td>-/- (n = 6)</td>
<td>31.3 ± 4.7</td>
<td>8.0 ± 0.5</td>
</tr>
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<td>2-Way ANOVA</td>
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</tr>
<tr>
<td>P, genotypes</td>
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<tr>
<td>P, diets</td>
<td>0.0048</td>
<td>NS</td>
</tr>
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</table>

*Statistical difference between diets (* = P < 0.05 by unpaired t-test)
Figure 5.4 shows the effect of chronic treatment with the AT-1 antagonist losartan on ABP and HR of -/- and +/+ mice maintained on 8% NaCl for 4 weeks. Basal ABP (Fig. 5.4A) and HR (Fig. 5.4B) were significantly elevated in control -/- mice compared to control +/+ mice (ABP, -/- = 139 ± 5, +/+ = 82 ± 1; HR, -/- = 504 ± 20, +/+ = 425 ± 25, P < 0.05). The antagonist had no effect on ABP and HR in +/+ mice, but reduced ABP and HR significantly (P < 0.05) in the -/- mice to the levels in the +/+ mice.

Total plasma catecholamine concentration (pg/ml) in control and Losartan-treated -/- and +/+ mice is given in Fig. 5.5. The control -/- mice had a significant 10-fold elevation in basal plasma catecholamine concentration compared to the control +/+ mice (-/- = 8633 ± 1769, +/+ = 827 ± 133, P < 0.05). Chronic treatment with losartan lowered plasma catecholamine significantly (P < 0.05) in the -/- mice (1860 ± 328), and tended to increase plasma catecholamine concentration in the +/+ mice.

Plasma aldosterone concentration (pg/ml) in the same control and Losartan-treated mice is shown in Fig. 5.6. Plasma aldosterone did not differ significantly between control -/- and +/+ mice (-/- = 137 ± 23, +/+ = 156 ± 37). Losartan treatment did not significantly alter plasma aldosterone in either genotype, however, there was a tendency for lower aldosterone concentration in -/- mice compared to control -/- mice.

Table 5.2 summarizes the cumulative food and water intake and urinary excretion of fluid and electrolytes for the first and fourth weeks of the dietary regimen. Food and water intake was significantly higher in +/+ mice compared to the -/- mice during the first week, resulting in higher absolute urinary excretion of water and electrolytes. However the relative urinary excretion of fluid and electrolytes, as represented by the ratio of urinary output to dietary intake, did not differ significantly between genotypes. These genotype-related differences in food and water intake and
Figure 5.4. Average ABP (A) and HR (B) in -/- and +/- mice fed on HS and treated daily with Losartan (n = 5 -/-; n = 7 +/-) or vehicle (Control, n = 6 -/-, n = 6 +/-) for 4 weeks. ABP and HR differed significantly between -/- control and Losartan-treated mice (*, P < 0.05), as well as between -/-control mice and +/- control (#, P < 0.05) and Losartan-treated (+, P < 0.05) mice.
Figure 5.5. Total plasma catecholamine concentration in -/- and +/+ mice maintained on HS and treated daily with Losartan (n = 4) or vehicle (Control, n = 5) for 4 weeks. Plasma catecholamine differed significantly between control and Losartan-treated -/- mice (*) and between -/- control mice and control (#) and Losartan-treated (+) +/+ mice. P < 0.05 by 2-way ANOVA coupled to Bonferroni multiple comparison test.

Figure 5.6. Plasma aldosterone concentration in -/- and +/+ mice on HS for 4 weeks and treated daily with Losartan (n = 5) or vehicle (Control, n = 6). No significant differences in plasma aldosterone were found between genotypes or treatments.
Table 5.2. Cumulative dietary intake of food and water and urinary output of water, sodium, chloride and potassium for the first and last weeks in control and Losartan-treated -/- and +/- mice maintained on a dietary regimen of HS (8% NaCl) diet for four weeks

<table>
<thead>
<tr>
<th></th>
<th>Food (g)</th>
<th>Water, m/7 days</th>
<th>Na, μmol/7 days</th>
<th>Cl, μmol/7 days</th>
<th>K, μmol/7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In</td>
<td>In</td>
<td>Out (%)</td>
<td>In</td>
<td>Out (%)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>16±3</td>
<td>80±8</td>
<td>59±9</td>
<td>72±4</td>
<td>21700±3992</td>
</tr>
<tr>
<td>-/- (n = 6)</td>
<td>19±5</td>
<td>116±16</td>
<td>81±18</td>
<td>67±8</td>
<td>25725±6207</td>
</tr>
<tr>
<td>Week 4</td>
<td>24±3</td>
<td>108±8</td>
<td>82±7</td>
<td>76±3</td>
<td>32344±3412</td>
</tr>
<tr>
<td>+/- (n = 6)</td>
<td>25±2</td>
<td>126±9</td>
<td>95±9</td>
<td>75±5</td>
<td>33595±3163</td>
</tr>
<tr>
<td>Losartan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>16±3</td>
<td>90±10</td>
<td>70±10</td>
<td>78±4</td>
<td>21850±3804</td>
</tr>
<tr>
<td>-/- (n = 5)</td>
<td>17±4</td>
<td>76±17</td>
<td>76±17</td>
<td>73±4</td>
<td>22801±5700</td>
</tr>
<tr>
<td>Week 4</td>
<td>23±4</td>
<td>112±11</td>
<td>96±7</td>
<td>86±3</td>
<td>31340±4713</td>
</tr>
<tr>
<td>+/- (n = 7)</td>
<td>17±2</td>
<td>98±8</td>
<td>80±8</td>
<td>81±3</td>
<td>22466±2128</td>
</tr>
</tbody>
</table>

2-WAY ANOVA

| P, genotypes | Week 1 | 0.02 | 0.01 | 0.009 | NS | 0.02 | 0.02 | NS | 0.02 | 0.01 | NS | 0.02 | 0.04 | NS |
|              | Week 4 | NS   | NS   | NS    | NS | NS   | NS   | NS | NS   | NS   | NS | NS   | NS   | NS |
| P, treatment | Week 1 | NS   | NS   | NS    | 0.04| NS   | NS   | NS | NS   | NS   | NS | NS   | NS   | NS |
|              | Week 4 | NS   | NS   | NS    | NS | NS   | NS   | NS | NS   | NS   | NS | NS   | NS   | NS |
| P, Interaction | Week 1 | NS   | NS   | NS    | NS | NS   | NS   | NS | NS   | NS   | NS | NS   | NS   | NS |
|              | Week 4 | NS   | NS   | NS    | NS | NS   | NS   | NS | NS   | NS   | NS | NS   | NS   | NS |

NS = not significant.
urinary excretion of fluid and electrolytes were absent by the last week of the dietary regimen. There were no statistical differences (paired t-test) between weeks 1 and 4 in the intake and urinary parameters in any of the groups. Losartan treatment did not significantly affect food and water intake or urinary excretion of fluid and electrolytes on either genotype through the duration of the metabolic study.

Table 5.3 shows the ABP and plasma electrolytes and total solute concentrations for -/- and +/+ mice maintained on HS for 4 weeks and for outbred CD-1 mice kept on normal (0.4% NaCl) rodent chow (study 3). As expected, ABP was significantly elevated in -/- mice compared to their genetically-matched +/+ controls and to the outbred mice. There were no significant differences in plasma Na⁺, Cl⁻, K⁺ or total plasma solute concentration between -/- and +/+ mice. However, plasma Cl⁻ and K⁺ concentrations were significantly lower and higher respectively in the CD-1 mice compared to the -/- and +/+ mice, likely reflecting differences in dietary content of these ions, as well as the larger saline infusion that the two latter groups received during the acute experiment.

**Table 5.3.** ABP and plasma solute concentrations in normal and in salt loaded -/- and +/+ mice.

<table>
<thead>
<tr>
<th></th>
<th>ABP (mm Hg)</th>
<th>PNa (mM)</th>
<th>PCl (mM)</th>
<th>PK (mM)</th>
<th>POsm (mOsm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 10)</td>
<td>86 ± 4</td>
<td>150 ± 1</td>
<td>115 ± 1</td>
<td>6.0 ± 0.5</td>
<td>304 ± 6</td>
</tr>
<tr>
<td>-/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 10)</td>
<td>139 ± 4*†</td>
<td>149 ± 2</td>
<td>125 ± 2*</td>
<td>4.4 ± 0.2*</td>
<td>315 ± 5</td>
</tr>
<tr>
<td>+/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 10)</td>
<td>101 ± 2*</td>
<td>147 ± 2</td>
<td>126 ± 1*</td>
<td>4.3 ± 0.2*</td>
<td>315 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SE

* - statistically different from CD-1 (P < 0.05)
† - statistical difference between -/- and +/+ (P < 0.05)
Glomerular filtration rates (GFR) and fluid and electrolyte excretion for the three groups of mice are shown in Table 5.4. Although there were no differences in GFR between groups, fluid and sodium chloride excretion were elevated in both -/- and +/+ mice, in association with the HS intake and acute saline infusion. The -/- mice, however, showed a deficit in salt and water excretion relative to the +/+ mice.

Table 5.4. Glomerular filtration and urinary fluid and electrolyte excretion in normal and in salt-loaded -/- and +/+ mice.

<table>
<thead>
<tr>
<th></th>
<th>GFR (ml/min)</th>
<th>V (µl/min)</th>
<th>U₉₅V (nmol/min)</th>
<th>U₉₅V (nmol/min)</th>
<th>U₂₅V (nmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD-1</strong> (n = 10)</td>
<td>0.81 ± 0.07</td>
<td>6.6 ± 0.8</td>
<td>580 ± 140</td>
<td>660 ± 140</td>
<td>1180 ± 180</td>
</tr>
<tr>
<td><strong>-/-</strong> (n = 10)</td>
<td>0.84 ± 0.06</td>
<td>34.3 ± 3.7* †</td>
<td>8650 ± 1090* †</td>
<td>10060 ± 1350* †</td>
<td>1130 ± 240</td>
</tr>
<tr>
<td><strong>+/+</strong> (n = 10)</td>
<td>0.81 ± 0.04</td>
<td>63 ± 3.8*</td>
<td>13980 ± 1070*</td>
<td>16020 ± 1080 *</td>
<td>1640 ± 230</td>
</tr>
</tbody>
</table>

Values are means ± SE.

* - statistically different from CD-1 (P < 0.05)

† - statistical difference between -/- and +/+ (P < 0.05)
The fractional remainders of filtered loads of fluid and electrolytes at the beginning of the inner medullary collecting duct (IMCD) for the three groups are shown in Table 5.5. The increase in water, sodium and chloride deliveries in the -/- and +/- mice relative the the CD-1 mice shows that upstream tubular reabsorption was equally inhibited in the -/- and +/- mice as a result of high salt intake and saline infusion. The increase in fractional potassium delivery in the -/- and +/- mice did not reach statistical significance.

Table 5.5. Deliveries of water and electrolytes to the medullary collecting duct as a fraction of filtered loads in normal and in salt-loaded -/- and +/- mice.

<table>
<thead>
<tr>
<th></th>
<th>Water (%)</th>
<th>Sodium (%)</th>
<th>Chloride (%)</th>
<th>Potassium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-1 (n = 10)</td>
<td>7.3 ± 1.2</td>
<td>6.3 ± 1.2</td>
<td>9.7 ± 1.7</td>
<td>51 ± 7</td>
</tr>
<tr>
<td>+/- (n = 10)</td>
<td>17.0 ± 2.9*</td>
<td>22.5 ± 4.2*</td>
<td>27.1 ± 4.1*</td>
<td>76±26</td>
</tr>
<tr>
<td>+/- (n = 10)</td>
<td>13.8 ± 1.7*</td>
<td>17.5 ± 1.7*</td>
<td>23.9 ± 2.4*</td>
<td>69+12</td>
</tr>
</tbody>
</table>

Values are means ± SE

*- statistically different from CD-1 (P < 0.05)
The fractional reabsorption of delivered fluid and electrolytes along the inner medullary collecting duct is shown in Table 5.6. As expected, sodium reabsorption was significantly decreased in both +/- and +/- mice in comparison to the normal mice, due to the elevated salt intake and the acute saline load. Chloride reabsorption was also decreased in both +/- and +/- mice but this reached statistical significance only in the latter. However, both sodium and chloride reabsorptions were significantly higher in the +/- mice, compared to the +/- mice. The difference in water reabsorption between the two genotypes was not statistically significant (p = 0.06), and there were no differences in potassium reabsorption among the three groups.

Table 5.6. Fractional reabsorption of delivered water and electrolytes in the medullary collecting duct of normal and of salt-loaded +/- and +/- mice.

<table>
<thead>
<tr>
<th></th>
<th>Water (%)</th>
<th>Sodium (%)</th>
<th>Chloride (%)</th>
<th>Potassium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-1</td>
<td>57 ± 3</td>
<td>84 ± 2</td>
<td>67 ± 4</td>
<td>15 ± 10</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/-</td>
<td>59 ± 5</td>
<td>64 ± 5*†</td>
<td>55 ± 4†</td>
<td>24 ± 13</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/-</td>
<td>44 ± 7</td>
<td>45 ± 7*</td>
<td>38 ± 8*</td>
<td>7 ± 9</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE

* - statistically different from CD-1 (P < 0.05)

† - statistical difference between +/- and +/- (P < 0.05)
DISCUSSION

The principal finding of this study is that mice rendered genetically incapable of synthesizing ANP develop sensitivity of arterial blood pressure to prolonged high (8% NaCl) dietary salt intake. Taken together with previous observations that one week of feeding on the same diet failed to exacerbate hypertension in this model (230), the present results indicate that development of salt-sensitive hypertension in ANP knockout mice is time-dependent, with at least one week of latency.

Despite the inherent reduced capacity for renal excretion of salt by the -/- mice (due to the absence of excretory effects of ANP on the IMCD) (Tables 5.4 and 5.6), the mechanism sustaining the hypertensive effect of salt in these mice is not mediated by salt retention and expansion of the ECFV, since the relative cumulative renal excretion of fluid and electrolytes at the end of four weeks on the HS diet, does not differ from the relative renal excretions after just one week on the dietary regimen. This indicates that the -/- mice maintain salt balance even on this very high salt intake. Furthermore, the genotype-dependent difference in salt sensitivity of ABP is not attributable to differences in renal excretion of fluid and electrolytes, since these parameters are comparable between the -/- and +/- mice. Nevertheless, the results do not exclude the possibility that differences in renal excretion between the genotypes during the unmonitored intermediate weeks (weeks 2 and 3) of the dietary regimen, may contribute towards the appearance of the salt-sensitive phenotype in the -/- mice. In the long term, however, the increased renal perfusion pressure in the -/- mice seem to adequately compensate for the reduced renal excretory capacity, and maintain salt balance by the pressure natriuresis mechanism.

Increased dietary salt intake is normally accompanied by homeostatic deactivation of antinatriuretic mechanisms, and in particular, the RAAS and sympathetic nervous activity (238, 596-598). The present study provides evidence that the hypertensive effect of salt in the -/- mice is
associated with failure to downregulate plasma renin activity in response to the excessive salt intake (Fig. 5.3). This is suggested by the observation that +/- mice respond to the high salt diet with an appropriate decrease in PRA and remain normotensive for the duration of the dietary regimen. Furthermore, chronic blockade of ANG II receptor activity with the antagonist Losartan fully abrogates the hypertension of -/- mice fed on HS, while having a negligible hypotensive effect on similarly maintained +/- mice, thus showing the dependency of salt-sensitivity of ABP in -/- mice on the underlying high basal ANG II activity. There is evidence that the chronic hypotensive effect of ANP in animals fed on high salt diet is partly mediated by suppressing the activity of RAS (34). ANP inhibits renin (47) and aldosterone secretion (48) and opposes ANG II-mediated vascular (22, 50) and renal effects (49), thus, suggesting a functional role of ANP as a physiological antagonist of the RAS. Considering that plasma ANP increases adaptively with elevated salt intake (231, 669), the failure of RAS to respond to changes in dietary salt content in the -/- mice may be caused, in part, by the absence of such antagonism.

The nature of the derangement in PRA in the -/- mice is not known. Dietary salt loading is accompanied by a compensatory increase in delivery of NaCl to the distal tubule; an adaptation that is normally potentiated by ANP (676). Since the inhibitory effect of ANP on renin release is mediated by increasing NaCl delivery to the macula densa (215), the initiating defect in renin release in the -/- mice could, in theory, be due to the inability to increase distal NaCl delivery in face of increased dietary intake. The consequence of this would be inappropriate activation of the macula densa. The antinatriuresis that would be triggered by the resultant increase in ANG II production (638) could then operate as a positive feedback signal to the macula densa, thereby overriding any direct inhibitory effect of ANG II on the juxtaglomerular cells, and sustain the chronic activation of renin. The present results, however, do not lend support to this hypothesis, since fractional sodium delivery
to the IMCD does not differ between the -/- and +/+ mice.

The derangement in PRA in the -/- mice could, in principle, be implicated in sensitization of ABP to high salt through, at least, three non-mutually exclusive mechanisms. First, the persistent direct antinatriuretic effects of chronically-elevated ANG II concentration may sensitize ABP to salt by causing excessive salt retention and expansion of the extracellular fluid volume (ECFV) (638). Exogenous ANG II at subpressor concentrations raises ABP when administered in combination with high dietary salt intake (222). This salt-sensitive hypertension is triggered by a sequential mechanism which is initiated by an increase in cardiac output, consequent to sodium retention and expansion of the ECFV, and later maintained by a sustained increase in total peripheral resistance (TPR) (222). The elevation in ABP and renal perfusion pressure then increases salt excretion by the pressure natriuresis mechanism, thereby bringing salt balance and normalization of ECFV volume (222). Interestingly, the temporal increase in ECFV volume in ANG II-induced salt sensitive hypertension is accompanied by a parallel increase in release of ANP, suggesting that the escape from the salt-retaining effects of ANG II may, in part, be mediated by ANP (222). Indeed, ANP increases the sensitivity of the pressure-natriuresis relationship partly by inhibiting ANG II-stimulated proximal tubular sodium reabsorption (49, 223). Thus, the absence of this ANP-mediated counteraction of ANG II effects in the -/- mice could result in a state of antinatriuresis. The maintenance of salt balance in -/- animals in this background of antinatriuresis could, thereby be achieved by raising ABP and renal perfusion pressure, which would overcome the direct stimulatory effect of ANG II on proximal tubular sodium reabsorption. The fact that fractional deliveries of sodium and water to the IMCD in the -/- mice do not differ from those of +/+ mice (Table 5.5) does indeed support this possibility. Furthermore, the fact that the Losartan-treated -/- mice maintain balance despite the marked decrease in ABP (Table 5.2), suggests that primary role of the elevation of ABP in the untreated -/- mice is to overcome the
salt retaining effects of ANG II and the reduced sensitivity of the renal pressure natriuresis mechanism. If this were not the case, then the decline in ABP in the Losartan-treated -/- mice would have been expected to lead to relative salt retention and reduced renal excretion.

In addition to the direct salt-retaining effects of ANG II, a potential contribution of aldosterone to salt sensitivity in the -/- mice needs to be evaluated. Although the high dietary salt intake is expected to exert a direct suppressive effect on aldosterone synthesis (334) and to reduce the sensitivity of the adrenal glomerulosa to ANG II (334, 677), the tonic elevation in ANG II could potentially increase adrenal output of aldosterone (677). The attendant secondary aldosteronism would then compound the direct antinatriuretic effects of ANG II. Furthermore, ANP is known to reduce aldosterone secretion both by directly inhibiting its synthetic pathway and by preventing the agonist effect of ANG II (48), and the elevation in ANP release that occurs coincident with expansion of the ECFV has been shown to be temporally (220, 642) and causally (221) implicated in mediating the escape from aldosterone action. Since these counterregulatory effects of ANP are effectively absent in the -/- mice, it would have been predicted that sensitization of ABP to salt in these mice could, at least in part, result from failure to appropriately overcome the antinatriuretic effect of aldosterone. However, the results of the current study do not show any difference in basal plasma aldosterone concentration between the -/- and +/+ mice maintained on HS diet, thus indicating that the absence of ANP-dependent antagonism of aldosterone synthesis in -/- mice during increased dietary salt intake, is adequately compensated for by other regulatory mechanism(s).

A third possibility by means of which the derangement in PRA could lead to sensitization of ABP to salt is via the excitatory effect that the elevated ANG II may exert on sympathetic nerve activity (597, 614). ANG II stimulates SNA at all levels of sympathetic nerve function (678). Centrally, both locally-derived and systemic ANG II stimulate sympathetic outflow, the former by
stimulating nuclei in the anterior hypothalamus, the latter by stimulating receptors in the blood-brain barrier devoid area postrema in the circumventricular region of the brain (678). These regions send excitatory multisynaptic fibers to the rostral ventrolateral medulla, the medullary region where peripheral sympathetic outflow originates (678). Peripherally, ANG II stimulates sympathetic ganglionic transmission and catecholamine release from the adrenal and from sympathetic nerve terminals (632). Furthermore, chronic increases in ANG II, either experimentally (596, 597) or pathologically (595-597) lead to sustained increases in SNA and sensitization of ABP to dietary salt (589), and these effects are prevented by blockade of RAS (597), indicating a role of the sympathetic nervous system as an intermediary effector of the pressor activity of chronically elevated ANG II.

In analogy with these situations, it was anticipated that the increased level of ANG II in the salt-fed -/- mice would provide continuous stimulatory input to the sympathetic nervous system, and that the resultant increase in SNA would, at least in part, account for the pressor effect of salt. The present study shows that the salt-fed -/- mice had an eightfold elevation of total plasma catecholamine concentration as well as higher basal HR, in comparison to the similarly maintained +/- control mice. Interestingly, the differences in plasma catecholamine concentration and HR between the -/- and +/- mice were abolished by chronic ANG II receptor blockade, showing the dependency of these differences on ANG II activity. Notwithstanding the fact that these differences provide only indirect evidence for increased SNA in the -/- mice, it seems unlikely, nevertheless, that the attenuating effect of chronic losartan treatment on plasma catecholamine and HR, and the accompanying decrease in ABP, specifically in the -/- mice, is merely a coincidental characteristic of these mice, unrelated to the underlying level of ANG II and SNA activity. In this regard, it is noted that the magnitude of the antihypertensive effect of losartan is directly proportional to the level of ANG II activity, which, itself,
is directly related to the level of SNA, due to the synergistic effect that these two vasoregulatory and salt-conserving mechanisms exert upon each other (632). Thus, on the basis of these findings, I suggest that the sensitization of ABP to dietary salt in the +/- mice is due to failure to adequately downregulate PRA and consequent tonic potentiation of SNA. The resultant increase in cardiovascular sympathetic tone supports the hypertensive effect of salt. The mechanism is non-adaptive and synergistic, and the increase in sympathetic tone maintains PRA elevated. The elevated ANG II in turn, sustains the increase in sympathetic tone.

A surprising finding of this study, is that the initial difference in basal ABP between +/- and +/+ mice is abolished after prolonged feeding on the low salt diet (Fig. 5.2). This equalization of ABP between the two genotypes may, in part, be due to development of refractironess to ANP in the +/+ mice, because prolonged exposure to low dietary salt intake decreases synthesis and release of ANP (679) and upregulates endothelial ANP-C receptors (190). Thus, the combined effects of reduced synthesis and accelerated removal of ANP could render the salt-deficient +/+ mice in a state of physiological equivalence to the +/- mice on the same dietary regimen, and may explain the elimination of the genotype-dependent difference in ABP by the low salt diet.

It has previously been shown that an increase in local production of nitric oxide (NO) (440, 670-672) and ET-1 (673) are essential for the chronic renal adaptation to high dietary salt intake. This is likely related to the ability of these factors to promote natriuresis and diuresis by their dual actions on the renal vasculature (442, 680, 681) and tubular function (682-684). On the basis of these premises, I suggest that the genotype-independent increase in content of eNOS and ET-1, specifically in the kidneys of both +/+ and +/- mice fed on HS diet (Table 5.1), may be an adaptive counterregulatory adjustment, unrelated to endogenous ANP activity, that is aimed at improving kidney function and counteracting the pressor effect of salt.
In conclusion, this study shows that ANP knockout mice develop sensitivity of ABP to increased dietary salt in a time-dependent fashion, in association with failure to downregulate plasma renin activity. I postulate that the sensitization of ABP to salt in the ANP knockout mice may partly be due to potentiation of cardiovascular sympathetic tone by ANG II, and that the ensuing increase in ABP is essential for overcoming the antinatriuretic potential of ANG II and SNA, and maintain salt balance.

On the basis of the evidence presented here and the documented roles of ANP on regulation of renal salt excretion, ECFV and chronic ABP, I propose the following mechanism to describe the role of ANP in mediating chronic cardiovascular and renal adaptations to high dietary salt intake (Figure 5.7): During high salt (HS) intake, there is a progressive accumulation of sodium and expansion of the extracellular fluid volume (ECFV) and a rise in ABP. The resultant expansion of the intravascular volume leads to an increase in right atrial pressure (RAP) which potently stimulates release of ANP. In parallel with this, the expansion of the ECFV also leads to decreases in renal sympathetic nervous activity (SNA) and angiotensin II (ANG II). This is greatly enhanced by ANP which directly inhibits sympathetic neurotransmission as well as renin synthesis from the juxtaglomerular cells of the renal afferent arteriole. The inhibition of these antinatriuretic mechanisms leads to increased distal delivery of water and electrolytes. In the inner medullary collecting duct, ANP inhibits sodium reabsorption, and the ensuing natriuresis and diuresis reduces ECFV and normalizes ABP. When endogenous ANP activity is reduced or absent, the homeostatic downregulation of antinatriuretic mechanisms is impaired, in part, due to the lack of antagonism by ANP, and the kidneys have an inherent reduced capacity for excretion of salt due to the absence of ANP natriuretic effect. High salt intake leads to salt retention and expansion of the ECFV. The consequent rise in ABP becomes essential for counteracting the antinatriuretic effects of elevated ANG II and long-term maintenance of salt balance.
Figure 5.7. Putative role of ANP in long-term maintenance of ABP during prolonged high dietary salt intake. Genetic defects in ANP synthesis and/or target organ hyporesponsiveness lead to sensitization of ABP to dietary salt due to absence of ANP-dependent antagonism of SNA and ANG II and failure of these pressure and salt-regulating systems to be compensatorily downregulated (see details in the text)
Perspectives

Previous work from our laboratory has shown that mice overexpressing an ANP-transgene have lifelong hypotension (17). These animals are capable of maintaining salt balance on a very low salt diet (0.008%) without evidence of salt depletion, suggesting independent actions of ANP on blood pressure and renal function. A similar conclusion may be reached based on results in the ANP-deficient model (230), since relative hypertension was observed after one week of high salt feeding (8%) without evidence of sodium accumulation. However, as the present study indicates, with time the lack of ANP action, apparently via inability to properly regulate PRA, results in development of an additional component of salt-sensitive hypertension.

Whether or not deficiencies in endogenous ANP activity play a contributory role in hypertensive diseases, and salt-sensitive variants of hypertension in particular, remains controversial. The present study provides evidence that chronic lack of ANP impairs the ability of regulatory system(s) to maintain constancy of ABP in face of increased salt intake. Similar dependency on ANP has been observed in other salt-sensitive animal models (685, 686), and human populations (687). It is likely that sensitization of ABP to dietary salt develops as a consequence of physiologically inappropriate functional alterations in salt- and pressure-regulating mechanisms, amongst which a deficiency in ANP synthesis may play a contributory role.
CHAPTER 6
GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

1. Synthesis of major findings

The initial study in this thesis characterized the basal systemic hemodynamics of the -/- mice and their wild type counterparts (Chapter 2). Using the thermodilution technique for measurement of CO, I identified that the predominant hemodynamic alteration underlying the hypertension in -/- mice is an elevation in basal TPR, in the absence of any significant differences in CO, SV or HR. This chronic alteration in TPR and the consequent increase in aortic afterload, likely is one of the causative factors in the development of cardiac hypertrophy (651) that characterizes this model. In conjunction with previous observations in the TTR-ANP mice (18), the current results support a physiological role of ANP in chronic regulation of ABP that is primarily mediated by relaxation of the resistance vasculature.

The mechanism(s) by means of which ANP exerts its chronic vasodilatory effect in the resistance vasculature is not known. It is unlikely that this is due to direct relaxation of vascular smooth muscle, given the insensitivity of the microvasculature to ANP (28-31). On the other hand, ANP has been shown to interact with and modulate the activity of several tonic vasoregulatory mechanisms, including endothelial factors and the sympathetic nervous system. The initial hypothesis that I tested in this thesis was that the chronic vasodilatory effect of ANP is mediated by interactions of this hormone with the synthetic activity of VE in the resistance vasculature, such that locally acting vasodilatory moieties associated with NO and CNP are potentiated, whereas the vasoconstrictor ET-1 is reduced. This was based on evidence that, at least acutely, ANP was shown to inhibit production of ET-1 (34-36, 331) and to stimulate synthesis of CNP (39) and possibly NO (40, 41). In addition, ANP was found to attenuate the target pressor effect of ET-1 (42, 576-580) and, possibly, potentiate the vasodilatory action of CNP. The premise was that such interactions, if tonically active in vivo,
would operate as an intermediary effector mechanism of ANP-dependent relaxation of the resistance vasculature. Measurement of whole organ homogenate concentrations of ET-1, CNP and ecNOS as an index of synthesis of these vasoactive substances from resistance vessel VE does not reveal any significant differences between the mutant mice (−/−, TTR-ANP) and the respective genetically-matched wild type (+/+, NT) mice (Chapter 3), indicating that the synthesis of ET-1, CNP and NO is not altered by the chronic level of endogenous ANP activity. Furthermore, the cardiovascular responsiveness of ABP and HR to acute pharmacological inhibition of endothelin receptors, nitric oxide synthase activity or immunoneutralization of CNP-specific GC-B receptors, taken as an index of the tonic cardiovascular activity of ET-1, CNP and ecNOS also does not differ between −/− and +/+ mice. Thus, in their totality, these results do not support the hypothesis that the chronic vasodilatory effect of ANP is mediated by potentiation of NO and CNP and/or inhibition of ET-1 from the VE the resistance vasculature, or by alterations in target cardiovascular responsiveness to these endothelial factors.

The lack of a chronic effect of ANP in the synthesis of these endothelial factors, nevertheless, is surprising, given the abundance of ANP binding sites in VE (27, 174, 447) and the ability of ANP to alter the activity of intracellular signalling pathways that indirectly affect the synthetic pathways involved in formation of these factors (27, 158, 174). It may be inferred from the current results, that the acute effects that ANP was previously found to exert on ET-1, CNP and NO synthesis and activity are either ineffective in the chronic state, or else, that they may be overcome by counteracting mechanisms. These findings, however, do not exclude the possibility that ANP may affect the activity of other endothelial vasoactive systems, such as the cyclooxygenase- arachidonic acid pathway leading to synthesis of vasodilatory eicosanoids (32) and/or the heme oxygenase-heme pathway leading to the formation of vasodilatory carbon monoxide (688).
On the basis of these negative findings, I tested the alternative hypothesis that the chronic vasodilatory effect of ANP is dependent upon attenuation of cardiovascular sympathetic tone. The premise of this hypothesis was that the widespread sympathoinhibitory action of ANP (33, 43-46, 338, 581, 603, 605, 606), if chronically active, would lead to attenuation of tonic vascular sympathetic tone, resulting in lower peripheral resistance and hypotension. This hypothesis is partly supported by evidence that the hypotensive response to exogenous ANP is exacerbated in conditions characterized by chronically elevated sympathetic tone (333, 334), and is greatly attenuated by AGB (287, 326, 328, 329), thus suggesting that the hypotensive effect of ANP is, at least partly dependent on inhibition of sympathetic tone.

Measuring the magnitude of the hypotensive response to acute AGB as an indirect index of tonic sympathetic activity, I determined that cardiovascular sympathetic tone is attenuated in TTR-ANP mice and is elevated in the +/- mice relative to their wild type controls (Chapter 4). Furthermore, these differences in sympathetic tone are accompanied by directional changes in total plasma catecholamine concentration, and the differences in basal ABP between mutant and control mice are abolished by AGB, indicating that the underlying sympathetic tone per se accounts for the differences in cardiovascular phenotype. These findings show that the chronic hypotensive effect of ANP is, at least partially, dependent on attenuation of cardiovascular sympathetic tone. To the best of my knowledge, these findings demonstrate for the first time, a direct reciprocal relationship between the chronic level of endogenous ANP activity and the level of sympathetic activity, thus implicating a functional role of the SNS in mediating the chronic vasodilatory effect of ANP.

The nature and localization of the neuromodulatory action of ANP on sympathetic nervous activity is not fully characterized. Acutely, ANP exerts an inhibitory effect at all levels of sympathetic nerve function, including reduction of sympathetic outflow from cardiovascular regulatory nuclei in
the medulla (41, 42, 331, 603), and inhibition of ganglionic neurotransmission (604) and catecholamine synthesis from post-ganglionic nerve fibers (45, 46). In addition, ANP has been shown to interfere with post-junctional α-1 adrenergic receptor function (606, 625). My results do not show any significant differences between mutant and wild-type mice in the responsiveness of ABP or HR to peripheral adrenergic receptor stimulation with norepinephrine, and in whole-organ adrenergic receptor binding, suggesting that the genotype-dependent differences in sympathetic tone are likely mediated by prejunctional effects of ANP. In principle, any of the identified neuromodulatory effects of ANP, either singly or in combination, could account for the observed differences in sympathetic tone between the genotypes. For example, the absence of a tonic inhibitory effect of ANP on tyrosine hydroxylase activity in postganglionic nerve terminals (46) and in the adrenal medulla (605) could partially account for the elevated plasma catecholamine levels in the -/- mice. It could also be argued that the co-localization of ANP and its GC-A receptors in autonomic ganglia (27, 619, 620) and in central sympathetic nuclei could function as a neuromodulatory unit (33, 619, 621) of sympathetic outflow. In this regard, Floras et al (622) has recently shown that, at least in humans, ANP-dependent sympatholysis is preferentially mediated by inhibition of autonomic ganglionic neurotransmission. Jin et al (689) showed that in a substrain of SHR rats with an inherent predisposition to sensitization of ABP to HS intake, ganglionic content of ANP failed to increase in response to HS intake. The sensitization of ABP to salt was associated with high basal sympathetic tone (690) and was abolished by chronic infusion of ANP (686), suggesting a dependency of ganglionic function on ANP activity for maintenance of normal sympathetic tone. Interestingly, these salt-sensitive SHR rats have reduced catecholamine synthesis in the anterior hypothalamic area, a region that subserves α-2-mediated inhibition of central sympathetic outflow (618). The inhibition of catecholamine synthesis in this nucleus is associated with increased local ANP synthesis (618). Thus, abnormalities in ANP synthesis
in sympathoregulatory areas can lead to elevated sympathetic tone and hypertension.

The possibility that the sympatholytic action of ANP may be dependent on interactions of the peptide with other regulatory systems must also be considered. In particular, ANP has been shown to antagonize the central pressor effect of ANG II, in part, by attenuating ANG II-dependent increases in sympathetic outflow (614, 643, 644). Indeed, it has been suggested that the central hypotensive effect of ANP is fully determined by inhibition of central ANG II activity (614). Thus, ANP may be exerting its sympatholytic activity indirectly partly by antagonizing the central sympathoexcitatory actions of ANG II. The potential role of such ANP-ANG II-mediated interactions in modulation of sympathetic nerve activity would assume particular relevance in pathophysiological conditions characterized by reduced synthesis or hyporesponsiveness to ANP, such as in congestive heart failure and in some forms of hypertension. In such cases, the physiological antagonism that ANP normally exerts on renin synthesis (47) and ANG II activity (22, 48, 50) is attenuated (239) and this could partly account for the elevated sympathetic tone characteristic of these diseases. In analogy with these conditions, it is possible that the absence of ANP-dependent antagonism of RAS in the -/- mice may, at least in part, lead to the observed increase in vascular sympathetic tone and hypertension.

It is not known whether structural changes in the resistance vasculature contribute to the ANP-dependent differences in ABP. Chronic increases in ABP are often accompanied by adaptive hypertrophy and remodelling of the tunica media of resistance vessels (594, 601, 667). The ensuing expansion the media could encroach into the lumen and raise vascular resistance (667). The resultant increase in smooth muscle mass may further elevate resistance by exacerbating the pressor response to vasoconstrictors. (667, 691). In addition to this, chronically elevated sympathetic nerve activity per se has direct trophic effects on the resistance vasculature (599, 600), and ANP has been shown
to inhibit proliferation of vascular smooth muscle *in vitro* (692), and to reduce hyperplasia of the media in response to balloon injury *in vivo* (693). These observations raise the possibility that the combined effects of elevated sympathetic tone and lack of ANP antimitogenic activity in the -/- mice may lead to hypertrophy of the resistance vasculature. The preliminary evidence presented in this thesis is that the acute pressor responsiveness of -/- mice to two potent vasoconstrictors, ET-1 and norepinephrine does not differ from that of +/- mice. A greater pressor response of -/- mice to ET-1 and norepinephrine would have been expected if significant vascular hypertrophy had occurred. It is entirely possible that media proliferation indeed occurs in the -/- mice, but that the subsequent remodelling of the vessel wall leads to a redistribution of wall materials, such that there is no permanent increase in wall thickness or reduction of luminal diameter.

The third aspect investigated in this thesis was the issue of whether the antagonism of salt conserving mechanisms (i.e., RAS, SNA) (47-49, 215, 238, 340) by ANP and/or the tubular natriuretic activity of this hormone is/are essential for maintenance of salt balance and constancy of ABP, during HS intake. Plasma ANP concentration increases in response to chronic HS intake (231, 669), and there is evidence that the obligatory increase in renal salt excretion that occurs during HS intake is partly dependent on the increased renal excretory response to ANP (215, 224, 314). In principle, this could be due to potentiation of the natriuretic activity of ANP in the IMCD (205) and/or due to ANP-mediated inhibition of RAS. On this basis, I predicted that a decrease in endogenous ANP activity leads to sensitization of ABP to chronic HS intake, associated, in part, with inappropriately high activity of RAS, and with reduced renal excretory capacity for salt. This premise was tested in -/- and +/- mice maintained on HS (8% NaCl) diet for 3-4 weeks. My findings show that, relative to their wild type controls, -/- develop salt sensitivity of ABP after prolonged (>1 week) feeding on HS, in association with failure to downregulate PRA (Chapter 5). This confirms
that under normal conditions, ANP-mediated antagonism of RAS activity is essential for the chronic adaptation to HS intake. Furthermore, the -/- mice show increased capacity for reabsorption of sodium in the IMCD, thereby confirming this region of the collecting duct as the preferential nephron site for the natriuretic action ANP. However, the -/- mice, despite their inherent reduced capacity for renal salt excretion, maintain salt balance. The increase in ABP and renal perfusion pressure appears to overcome the antinatriuretic background associated with elevated ANG II, and maintain salt excretion by pressure natriuresis (238). Thus, the salt-induced increase in ABP in -/- mice is a necessary adaptation for maintenance of salt balance.

The salt-fed -/- mice are also characterized by an inappropriately elevated sympathetic tone, as evidenced by the eightfold elevation of total plasma catecholamine concentration and higher HR compared to the similarly fed +/- mice. This increase in basal sympathetic tone is prevented by chronic ANG II receptor blockade, thereby showing its dependency on the elevated PRA (Chapter 5). This suggests that the sensitization of ABP to dietary salt in -/- mice is due to tonic potentiation of SNA by ANG II, the increase in cardiovascular sympathetic tone being largely responsible for supporting the hypertensive effect of salt. Chronic ANGII-mediated increases in SNA have previously been documented (696). For example, long-term ANP infusion increases splanchnic and muscle sympathetic nerve activity in rats and rabbits (696, 697), and in CHF, a pathological state of effective arterial blood volume depletion characterized by elevated PRA and SNA (235), losartan decreases ABP and prevents a compensatory increase in plasma catecholamine concentration (698), suggesting that in this disease, ANG II is involved in maintaining the elevated sympathetic tone. A characteristic of this putative mechanism of interaction is that it is non-adaptive and “self-sustaining” due to the synergistic interaction between ANG II and the SNA. A similar dysfunction has been shown in Dahl salt-sensitive rats. These rats also fail to suppress PRA (694) and SNA (695) when fed on HS, but
the extent to which the interdependency between ANG II and SNA account for this has not been tested in this model. These similarities, nevertheless, suggest some common features between salt-sensitive hypertensive models.

2. Methodological considerations

This thesis successfully employs for the first time, a commercially available thermodilution method to characterize systemic hemodynamics in mice. Successive measurements within and between experiments were highly reproducible and within an acceptable margin of error (< 10%). Our measurements of CO compare very favourably with those obtained by others, using aortic electromagnetic flow probes (654), thus validating the use of the thermodilution for hemodynamic measurements in mice.

The measurement of whole organ homogenate concentration of ET-1, CNP and ecNOS as an index of endothelial synthetic activity is based on the assumption that endothelial cells are the exclusive site of production of these factors. Although this holds true for many tissues (32), a possible caveat with this assumption is that it excludes the potential contribution of other cell types in the homogenate and or receptor-bound peptide (ET-1, CNP) to the total homogenate concentration. This may in some cases lead to an overestimation of the synthetic capacity of VE. For example, in the kidney, ET-1 and CNP are also produced by tubular epithelial and mesangial cells, albeit at much lower concentrations (423, 488, 489). Also, ET-1 is known to bind very tightly to its receptors (34). The analytical methods used in the thesis to measure these substances (RIA, Western blot) do not discriminate between these possibilities. The validity of these measurements would be further enhanced by characterizing cell-specific expression of these factors in the different organs. Nevertheless, these measurements are in good agreement with the in vivo measurements of basal cardiovascular responsiveness to these factors, that is, the lack of differences in tissue concentration of ET-1, CNP and ecNOS between the different genotypes was paralleled by lack of differences in
basal cardiovascular responsiveness to ET-1, CNP or NO.

Both the magnitude of the hypotensive response to autonomic ganglionic blockade and plasma catecholamine concentration provide only indirect indexes of sympathetic activity. The basic assumption underlying the use of AGB as an index of sympathetic tone is that the fall in ABP following blockade is proportional to the level of sympathetic activity (581, 588, 594, 597, 695). A potential shortcoming of this assumption is that it does not take into account possible compensatory adjustments in other vasoregulatory systems, such as an increase in ANG II or other vasoconstrictors, to the AGB-induced hypotension. Despite this caveat, I believe, nevertheless, that AGB provides a good indirect index of “overall” sympathetic activity. The interpretation of results obtained with this method should be restricted to this level of indirectness, and must not be overextended to describe any particular mechanism of action, unless complemented with other appropriate tests.

In general plasma catecholamine concentration correlates well with the underlying level of sympathetic nerve activity (594, 700). However, plasma catecholamine concentration is a function of both neurotransmitter release and reuptake, as well as blood flow and the volume of distribution (701). This problem can be partially circumvented by measuring catecholamine spillover, which allows more precise evaluation of changes in sympathetic activity in specific vascular beds. It would have been preferable to supplement the results in this thesis with a direct assessment of sympathetic nerve activity. However, at this time we are still limited by the relative novelty of mice as experimental models and by the unavailability of expertise and tools to perform such measurements. At the moment, there are no flawless methods for quantifying sympathetic activity. Recently developed microneurographic methods allow direct recording of multifiber nerve activity from efferent sympathetic nerves in humans and some animals (588); however, the limitation of the method is that it provides a measure of sympathetic nerve activity only to the region/vascular bed being supplied by that particular nerve.
3. **Summary**

1. ANP knockout mice are chronically hypertensive relative to their wild type siblings in association with an elevation of basal peripheral vascular resistance *(Chapter 2).*

2. Tissue content of ET-1, CNP and ecNOS and total NOS activity does not differ between mutant (-/-, TTR-ANP) and their genetically-matched wild type (+/+, NT) mice *(Chapter 3).*

3. Responsiveness of ABP and HR to acute pharmacological antagonism of ET<sub>A/B</sub> receptor, or immunoneutralization of CNP-specific GC-B receptors, or competitive inhibition of nitric oxide synthase *in vivo,* does not differ between -/- and +/+ mice *(Chapter 3).*

4. The hypotensive response to acute autonomic ganglionic blockade was attenuated in TTR-ANP mice and elevated in -/ mice relative to the respective wild type mice *(Chapter 4).*

5. The differences in magnitude of the hypotensive response to acute autonomic ganglionic blockade between mutant (-/-, TTR-ANP) and wild type (+/+, NT) are paralleled by directional differences in total plasma catecholamine concentration *(Chapter 4).*

6. Responsiveness of ABP and HR to acute peripheral adrenergic receptor stimulation and tissue adrenergic receptor binding do not differ between mutant and wild type mice *(Chapter 4).*

7. ANP knockout mice develop time-dependent sensitivity of ABP to high dietary salt intake with a latency of at least one week *(Chapter 5).*

8. The hypertensive effect of salt in -/- mice is accompanied by elevated plasma renin activity compared to similarly maintained wild type mice. *(Chapter 5).*

9. ANP knockout mice maintained on HS diet have elevated total plasma catecholamine concentration compared to wild type mice *(Chapter 5).*

10. The elevation in total plasma catecholamine in -/- mice on HS is abolished by chronic inhibition of ANG II receptor activation *(Chapter 5).*
4. Conclusions

Based on the results presented in this thesis, the following conclusions are made:

1. ANP exerts a chronic hypotensive effect that is mediated by reduction of peripheral vascular resistance.

2. The chronic hypotensive effect of ANP is not determined by differences in synthesis of locally-acting endothelium-derived vasoactive factors ET-1, CNP or NO in the resistance vasculature, or in the tonic responsiveness of ABP and HR to these factors.

3. The chronic hypotensive effect of ANP is mediated by tonic attenuation of vascular sympathetic tone.

4. ANP knockout mice develop salt-sensitive hypertension due to failure to downregulate plasma renin activity.

5. The failure of plasma renin activity to decrease in ANP knockout mice in response to high dietary salt intake is due to lack of antagonism of renin synthesis by ANP.

6. ANG II-mediated potentiation of cardiovascular sympathetic tone supports the hypertensive effect of salt in +/- mice.

5. Overall significance of the findings

The results of this thesis confirm that ANP plays a significant role in long term regulation of arterial blood pressure. When ANP activity is reduced, an uncompensated chronic rise in ABP occurs. This thesis provides evidence that the hypertensive phenotype associated with deficient ANP activity is due to failure of other homeostatic pressure regulating mechanisms, most notably the sympathetic nervous system and the renin angiotensin system to adequately compensate for the rise in ABP. The striking similarity of the abnormalities in ABP regulation between +/- mice and cardiovascular
diseases such CHF and some salt-sensitive variants of hypertension, suggest that a deficiency in ANP synthesis and/or resistance to the actions of ANP may play a pathophysiological role in the development of these diseases.

6. Future Directions

Several topics of investigation are left open with the results of this thesis. In my opinion, the most pressing future experiments should be aimed at further characterizing the site(s) and mechanism of ANP-mediated chronic sympathoinhibition. As previously discussed, ANP exerts generalized inhibition of sympathetic neurotransmission, and it is likely that the overall chronic sympatholytic effect of ANP results from neuromodulatory actions of the peptide at multiple sites in the sympathetic nervous system. This could include regulation of central sympathetic outflow from the RVLM, inhibition of ganglionic neurotransmission and inhibition of catecholamine synthesis in sympathetic nerve terminals and in the adrenal medulla.

The reciprocal relationship between plasma ANP concentration and sympathetic tone seen in the TTR-ANP and -/- mice must be confirmed, to exclude the unlikely possibility that this relationship is merely a phenomenological characteristic of these genetic mouse models. A reasonable and uncomplicated approach to this would be to compare the changes in sympathetic tone in TTR-ANP and -/- mice after chronic GC-A receptor inhibition or implantation of an ANP-containing "slow release" pellet respectively, with NT and +/- mice similarly treated with a placebo.

The developmental time frame of the sympathetic dysregulation in -/- mice needs to be characterized. It is not known whether this dysfunction is present from birth, or whether it develops later with aging. A logical approach to this would be to assess changes in ABP and sympathetic activity from birth and throughout life in chronically instrumented animals. However there are some technical restrictions to this approach, particularly when the animals are young and the surgical
procedure for chronic instrumentation is complicated by the small size of the animals.

Although this thesis provides evidence for a role of elevated ANG II activity in potentiating cardiovascular sympathetic tone in -/- mice during HS intake, it did not investigate whether ANG II plays any role in establishing basal sympathetic tone in -/- mice during normal salt intake. This possibility should be investigated in the future. An approach similar to that used in Chapter 5 using Losartan to chronically inhibit ANG II activity would provide adequate preliminary evidence. Briefly, changes in ABP, HR and plasma catecholamine concentration would be compared between untreated -/- mice and -/- mice chronically treated with Losartan, in order to determine the contribution of ANG II to basal sympathetic tone in these mice. Similarly treated +/- mice would serve as controls.

A thorough morphometric assessment of the resistance vasculature in these genetic mouse models also merits investigation, in order to determine whether structural changes and remodelling in this segment of the vasculature contribute significantly to the chronic alterations in peripheral vascular resistance that are associated with differences in endogenous ANP activity. This is particularly relevant, in light of the documented antigrowth activity of ANP on vascular smooth muscle (688, 689).

A possible role of adrenomedullin (ADM), a vasoactive and natriuretic peptide structurally related to calcitonin gene-related peptide (CGRP) (702, for review see ref. 703), in mediating the tonic vasodilatory effect of ANP must also be investigated. This is particularly relevant in light of recent evidence that this peptide has a vasodilatory profile similar to that of ANP (704, 705, 706), and that ANP stimulates the release of this peptide from endothelium (707), suggesting that ADM may subserve a role as an intermediary effector of the chronic vasodilatory action of ANP. Furthermore, ADM inhibits ANP gene expression (708) and secretion from the heart (709), suggesting that these two vasoactive peptide systems may act in a negative feedback fashion to
contribute to long-term blood pressure homeostasis.

7. A model of chronic regulation of arterial blood pressure (ABP) by ANP

Based on the current results and on prior work reviewed in this thesis, the following model is proposed to describe the mechanism of chronic ANP-dependent vasodilation (Figure 6.1). Some of the concepts are based on experimental evidence presented in this thesis and/or elsewhere. Other concepts are speculative at this time and will require experimental confirmation.

According to this model, the chronic hypotensive effect of ANP is determined by tonic inhibitory influences of the peptide on multiple sites in the SNS and in the RAAS cascade. Centrally, locally-derived ANP potentiates sympathoinhibitory neurotransmission from the nucleus tractus solitarius (NTS) to the caudal ventrolateral medulla (CVLM); the site of origin of peripheral sympathetic outflow. Paradoxically, ANP also inhibits $\alpha$-2-mediated sympathoinhibitory influences from the anterior hypothalamic area (AHA) to the NTS by reducing NE synthesis, and an increase in ANP synthesis in this region has been identified as a causative factor in sensitization of ABP to salt in SHR. In the area postrema of the circumventricular organ, a region devoid of blood-brain barrier, systemic ANP antagonizes the central sympathoexcitatory activity of circulating ANG II. Peripherally, the co-localization of ANP and its GC-A receptors in sympathetic autonomic ganglia (SAG) function as a neuromodulatory unit and inhibit cholinergic ganglionic transmission. At the neuroeffector junction, ANP inhibits tyrosine hydroxylase in sympathetic nerve terminals and reduces NE release. In the kidney, ANP inhibits renin synthesis and in the lung capillaries and in tissues expressing RAS, ANP inhibits angiotensin converting enzyme (ACE) activity, thereby decreasing the vasoconstrictor potential of ANG II. The attenuation of this functional antagonism of SNA and RAS in conditions characterized by reduced ANP activity leads to hypertension in association with elevated vascular tone.
Figure 6.1. Proposed model of chronic regulation of arterial blood pressure by ANP (see text for details). Solid lines represent pathways leading to sympathoexcitation. Broken lines represent sympatheinhibitory pathways.
REFERENCES


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