CARDIOVASCULAR EFFECTS OF ACTIVATED COAGULATION FXII, 'NEW PRESSOR PROTEIN' (NPP), ARE ASSOCIATED WITH POTENT ADRENAL MEDULLARY CATECHOLAMINE RELEASE

by

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ABSTRACT

Cardiovascular effects of activated coagulation FXII, 'new pressor protein' (NPP), are associated with profound adrenal medullary catecholamine release.

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"New Pressor Protein" (NPP), related to coagulation βFXIIa, raises systolic blood pressure (SBP >DBP) and heart rate (HR) after injection into bioassay rats ganglion blocked with Ansolysen (+A). The ACE inhibitor captopril (+C), but not the angiotensin II receptor antagonist losartan, greatly potentiates NPP effects while total adrenal medullectomy virtually obliterates them. Massive increases in plasma adrenaline and noradrenaline also follow NPP and are captopril-potentiated. Without ganglion blockade (–A), BP and plasma adrenaline responses to NPP are different, reduced and less potentiated by captopril. Comparisons with angiotensin and sodium nitroprusside effects suggest that NPP acts agonistically and probably indirectly via a peptidergic pathway for adrenal medullary catecholamine release – a pathway that is probably enhanced in +A+C rats. We propose that NPP somehow triggers this peptidergic pathway and represents a potentially new axis for BP regulation by connecting the blood coagulation and 'fight-flight' sympatho-adrenal systems.

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All my love, Mimi.

"All things excellent are as difficult as they are rare."

-Spinoza, Ethics, Pt.V

LIST OF ABBREVIATIONS

acetylcholine	Ach
adrenaline	A
angiotensin I	Ang I
angiotensin II	Ang II
angiotensin converting enzyme inhibitor	ACEI
bilateral adrenalectomy	2AX
bilateral adrenal medullectomy	2MDX
cardiac output	CO
centimeter	cm
central nervous system	CNS
corn trypsin inhibitor	CTI
degrees celcius	°C
diastolic blood pressure	DBP
dopamine	DA
grams	g
heart rate	HR
hydrochloric acid	HCl
intraperitoneal	i.p.
intravenous	i.v.
kilogram	kg
liter	L
mean arterial pressure	MAP
microgram	µg
microliter	µL
milligram	mg
millileter	mL
millimeters of Mercury	mmHg
minutes	min
moles	mol

nanogram	ng
New Pressor Protein	NPP
noradrenaline	NA
normal saline	0.9% NaCl
not statistically significant	n.s.
picograms	pg
pituitary adenylate cyclase-activating polypeptide	PACAP
polyethylene tubing	PE
radioimmunoassay	RIA
renin-angiostensin-aldosterone system	RAA system
sodium chloride	NaCl
sodium nitroprusside	SNP
soy bean trypsin inhibitor	SBTI
standard error of the mean	SEM
stroke volume	SV
subcutaneous	s.c.
systolic blood pressure	SBP
total peripheral resistance	TPR
unit	U
vasoactive intestinal peptide	VIP

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1. INTRODUCTION

1.1 PREAMBLE

This thesis investigates the role of adrenal medullary catecholamines in mediating the pressor effect of activated coagulation factor XII (FXII), "new pressor protein" (NPP) in bioassay rats. Given that the field is relatively new (<10 years) and still in its infancy, there is little literature to review. This Introduction will examine in some detail what is currently understood regarding the biochemistry and physiology of NPP in order to set the stage for the current set of experiments.

1.2 OBSERVATIONS LEADING TO DISCOVERY OF 'NEW PRESSOR PROTEIN' (NPP)

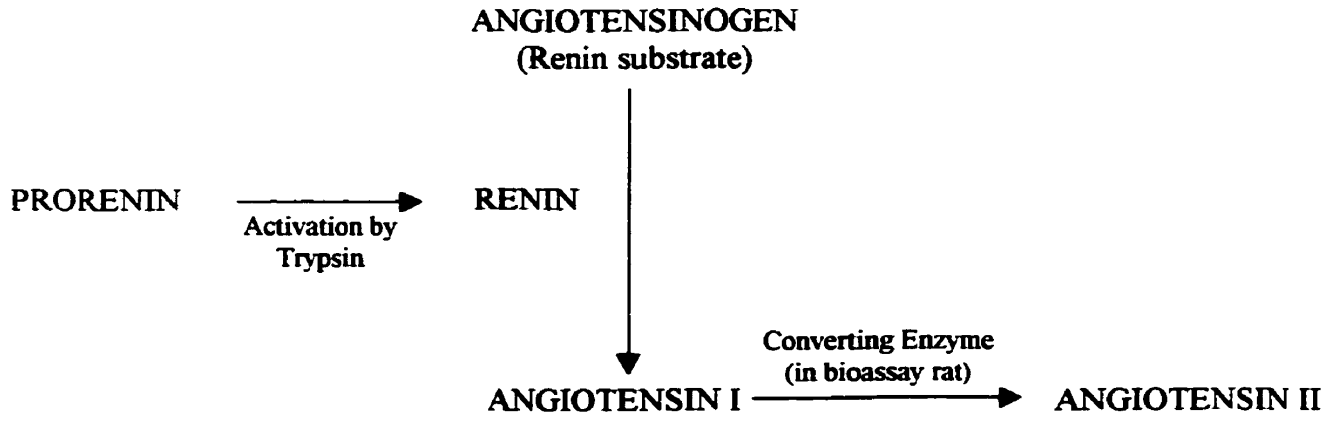
During the investigation of *in vitro* activation of prorenin to renin in human and animal plasmas (Ioannou *et al.* 1991; Osmond *et al.* 1991; Cooper *et al.* 1977), it was customary to use a radioimmunoassay (RIA) technique designed to measure the generation of angiotensin I (Ang I) from renin that was formed via prorenin (Ioannou *et al.* 1991; Osmond *et al.* 1991; Cooper *et al.* 1977; Oparil, 1976). The preferred method of such activation involved the use of trypsin but, at the time, not all laboratories were necessarily using the same trypsin concentration and reaction conditions. This inevitably lead to divergent reports of prorenin and renin, both quantitatively and functionally (Hagemann *et al.* 1992; Johannessen *et al.* 1989; Barrett and Eggena, 1988).

To further complicate the matter, a debate began to form around the issue of what precisely was being called prorenin. Reports of "pseudo" prorenin began to surface in the literature which described the inability of specific antibodies to bind to plasma prorenin and that this "pseudo" prorenin generated "renin" that was not recognized by antibodies directed against "true" renin

derived from normal plasma (Kim *et al.* 1991a; Kim *et al.* 1991b). This controversy regarding "pseudo" prorenin and "true" prorenin prompted Osmond and Cotter (1992) to activate plasma prorenin and test it for renin activity using a bioassay method based mainly on Pickens *et al.* (1965), with some features adapted from Boucher *et al.* (1964). Chervu *et al.* (1972) had reported that there was a high degree of correlation between the bioassay of the renin-angiotensin-aldosterone (RAA) system and RIA results. This allowed for reliable comparisons to be made between bioactivity and relevant immunogenicity.

The bioassay model modified from Pickens *et al.* (1965) was used primarily because it was considered a "sensitive" method for the detection of angiotensin responses (Page and Taylor, 1947) and was thus considered to be an appropriate choice for detecting prorenin activation to renin. This is because bioassay of renin is expressed via the activity of angiotensin II (Ang II, Fig. 1.2.1).

Fig. 1.2.1: Mechanism of Ang II formation *in vivo* by the activation product of prorenin *in vitro*.



In preparation for bioassay, the rat is anesthetized with Inactin (100 mg/kg) and bilaterally vagotomized to isolate the heart. The rat is treated with atropine (2.4 mg/kg) to maintain patency of the airway and to prevent anesthetic depression of heart rate. A ganglion blocking agent (pentolinium, 19.2 mg/kg, Methods), which is a nicotinic acetylcholine (Ach) receptor antagonist, is also given to increase the animal's sensitivity (Page and Taylor, 1947). Page and Taylor (1947) noted that such ganglioplegics greatly augmented the responses to relatively small doses of angiotensin, and subsequent work employed this principle in rats, cats and dogs. Blood pressure is monitored via a carotid artery cannula and heart rate is derived by computer software (see Methods).

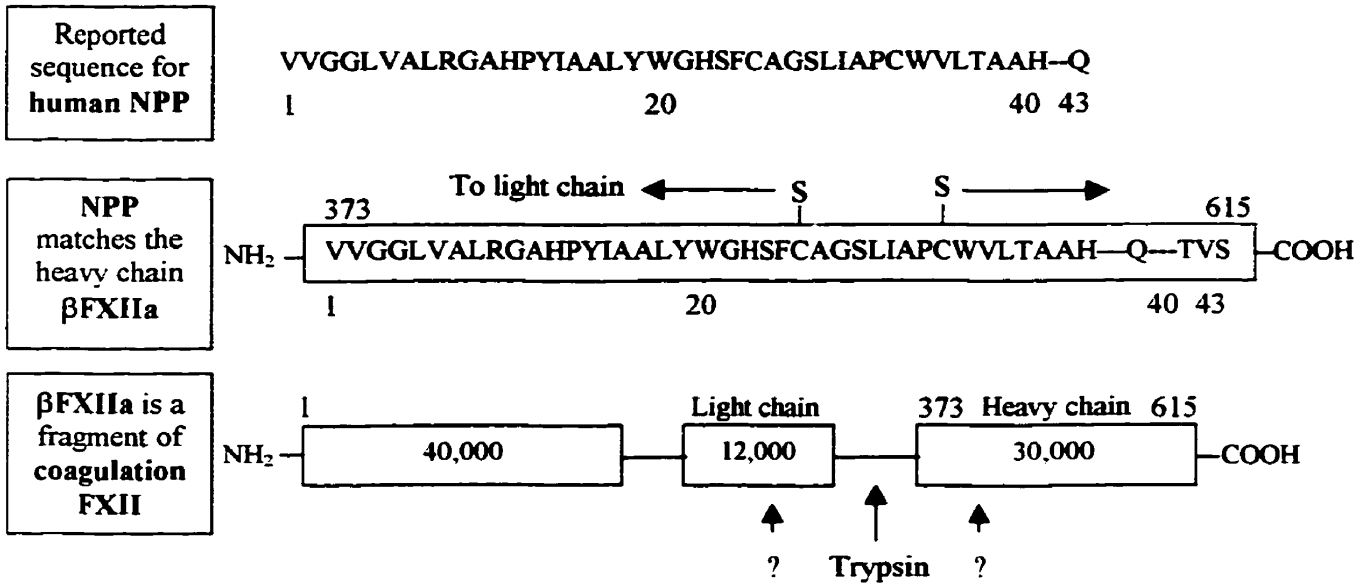
It was expected that intravenous (i.v.) injection of the trypsin-activated product of plasma prorenin into a bioassay rat would produce a pressor response similar to that of Ang II. This was based on the assumption that prorenin activation produced Ang I in the test tube, which was then converted *in vivo* to Ang II via the angiotensin converting enzyme (ACE, Fig. 1.2.1). The blood pressure responses to renin and Ang II are quite characteristic in shape and duration, making it possible to identify renin or angiotensin as the causative agent. In addition, the use of ACE inhibitors (ACEI), such as captopril or enalapril, that block the renin system helps to differentiate responses in blood pressure due to renin-angiotensin from those due to some other agonist.

It was no surprise then, that i.v. injection of the activation product in small plasma volumes (10-20 μL) produced a very modest increase in both blood pressure and heart rate in bioassay rats (Osmond and Cotter, 1992). Given that these responses were thought to be due to the action of renin produced from prorenin and acting through Ang II, the ACEIs captopril and enalapril were

given to the bioassay rat to confirm the involvement of renin. But instead of abolishing the pressor effect, blockade with ACEIs resulted in an unexpected, major potentiation of the pressor response. The increase in systolic blood pressure (SBP, >30 mmHg) was much more pronounced than the increase in diastolic blood pressure (DBP, <5-10 mmHg). Heart rate (HR) was also potentiated after ACE inhibition (Cotter, 1995; Osmond and Cotter, 1993). Trypsin-activated rat plasma also evoked increases in SBP which were similar to those observed using human plasma (Osmond *et al.* 1997a, 1997b, 1997c; Osmond and Cotter, 1992, 1996; Osmond and Cotter, 1993). This suggested a lack of species specificity which, reportedly, is not the case for human renin in the rat (Skinner, 1967).

Since no explanation for these observations was available in the literature, this pressor activity was tentatively called "new pressor protein", or NPP (Osmond *et al.* 1998; Osmond *et al.* 1997a, 1997b, 1997c; Osmond and Cotter, 1992, 1996; Cotter and Osmond, 1993a; Osmond and Cotter, 1993). The pressor activity of NPP was shown to be heat labile, enzymically active, with a relative molecular mass of >30 kDa, and isoelectric point(s) 4.7-4.9. Highly purified material was sequenced and the N-terminal sequence (19 residues) was found to be homologous with the β factor XIIa fragment of blood coagulation factor XII (FXII, Mavrogiannis, 1998).

Fig. 1.2.2: Amino acid sequence of human plasma NPP and its confirmed homology to coagulation factor XII (βFXIIa)



1.3 CONFIRMATION OF NPP'S LINK TO COAGULATION FXII

The apparent link to coagulation FXII was investigated by treating the plasma preparation prior to injection with corn trypsin inhibitor (CTI), reportedly specific against FXII *in vitro* (Kirby and McDevitt, 1983). Such CTI treatment abolished the NPP effect on blood pressure and heart rate, suggesting that direct inhibition occurred (Osmond and Cotter, 1996). It is not known whether CTI administered *in vivo* has the same effect, since it would presumably also inhibit the rat's own FXII, which may itself be activated upon injection of active β-fragment.

This apparent link to coagulation FXII was also confirmed by testing specific coagulation factor deficiency plasmas for pressor activity. Of the coagulation factor deficiency plasmas tested, i.e., prekallikrein, factors I, XI, XII and kininogen, only FXII deficient plasma was found to profoundly lack NPP activity (Mavrogiannis, 1998). Furthermore, adding highly purified

coagulation FXII, αFXIIa or βFXIIa fragment to factor XII deficiency plasmas fully restored NPP pressor activity, but only after activation with trypsin (Mavrogiannis *et al.* 1997). This requirement of trypsin suggested that none of the factors are structurally identical with NPP at the outset, but that all can be activated to NPP. In addition, when injected directly by vein, only βFXIIa was found to be pressor (Mavrogiannis *et al.* 1997).

1.4 THE EVIDENCE SUPPORTING CATECHOLAMINE INVOLVEMENT IN THE NPP PRESSOR EFFECT

The preferential action of NPP on SBP and HR (with only small changes in DBP) suggested to us a specific effect on the heart. Indeed, NPP was found to increase cardiac output (CO) by increasing both stroke volume (SV) and HR (Mavrogiannis, 1998). There was minimal change in total peripheral resistance (TPR), also arguing in favor of an action mainly on the heart and not on the peripheral vasculature.

1.4.1 Effect of Acute Bilateral Adrenalectomy (2AX) on the NPP Pressor Response

The action of NPP to increase SBP and HR strongly suggested that the pressor effect might be mediated by catecholamines released from the adrenal gland. Thus, the pressor responses of NPP and Ang II (for comparison) were tested in bioassay rats after acute bilateral adrenalectomy (2AX).

Peripheral administration of Ang II increases mean arterial pressure (MAP) by a direct vasoconstrictor action and indirectly by its actions on the sympathetic nervous system. Although Ang II is well known to facilitate sympathetic transmission by potentiating neurotransmitter

release (Starke, 1977), as well as inhibiting noradrenaline re-uptake (Khairallah, 1972), it can also stimulate the release of catecholamines from the adrenal medulla (Butler *et al.* 1994; Feldberg and Lewis, 1964). The ability to stimulate catecholamine release was confirmed indirectly by observing a reduction (approx. 20%) in the pressor response to Ang II after 2AX (Mavrogiannis, 1998; Osmond *et al.* 1998).

The NPP pressor response (SBP, DBP, and HR) was >90% reduced within 10 min of 2AX compared to control (Mavrogiannis, 1998; Osmond *et al.* 1998). These results suggested that secretions from the adrenal gland were involved in the pressor response. The rapid onset of the effect of adrenal ablation on reducing the NPP response prompted Mavrogiannis (1998) to hypothesize that medullary catecholamines were the likely candidates given that their effects manifest themselves rapidly and their half-life is relatively short (i.e. 1-2 min, Discussion). Such strong evidence implicating adrenal catecholamines in the action of NPP prompted further studies to investigate the links between catecholamine action and the NPP pressor effect.

Since adrenal medullary catecholamines, specifically adrenaline and noradrenaline, express their effects through adrenergic receptor activation, Mavrogiannis (1998) also suggested that if catecholamines were involved in the pressor effect, these effects would be due to activation of perhaps both α- and β- adrenergic receptors. In order to test this hypothesis, the NPP pressor effect was investigated after treatment of the bioassay rat with adrenergic receptor antagonists. Thus, in order to facilitate the forthcoming discussion, a brief review of catecholamine action by adrenergic receptor activation follows.

1.4.2 Consideration of Adrenergic Receptor Types: Distribution and Function Relative to Observed Effects of NPP

There are generally two types of adrenergic receptors, the alpha (α) and beta (β) receptors. These receptors have seven transmembrane domains, are G protein-linked and multiple subtypes of each exist. Adrenaline and noradrenaline secreted from the adrenal medulla have different effects on α and β receptors. For instance, noradrenaline stimulates mainly α receptors but can also stimulate β receptors to a somewhat lesser degree. On the other hand, adrenaline can stimulate both α and β receptors with equal efficacy. The types of receptors on effector organs determine the relative effects of adrenaline and noradrenaline. Thus, if the receptors in a particular organ are β-adrenergic, adrenaline will be more effective at stimulating these receptors than noradrenaline (Ganong, 1997).

Adrenergic receptors in the heart are primarily β_1. When these receptors are stimulated by noradrenaline released from noradrenergic terminals, they increase HR, contractility, excitability and conduction velocity (Lees, 1981). β_2 receptors are also present in the heart (Stene-Larsen *et al.* 1986), but β_1 receptors usually predominate. Adrenaline's affinity for the β_2 receptor is 200-fold greater than noradrenaline, so circulating adrenaline released from the adrenal medulla can also facilitate the effects of noradrenaline in the heart (Stene-Larsen *et al.* 1986). In the coronary arteries, activation of β_1 receptors results in vasodilation; in most other arteries, vasodilation occurs as a result of β_2 activation.

Adrenergic receptors in the vasculature are primarily α_1 and α_2. The general effects of α receptor activation is constriction of arteries and veins in such vascular beds as the skin and

mucosa, cerebral arteries, the pulmonary circulation, abdominal viscera, salivary glands and the renal circulation. Adrenaline and noradrenaline have similar affinities for the α_1 receptor, but of the two, noradrenaline is more effective at the α_2 receptor (Lees, 1981).

If catecholamines are indeed involved in mediating the NPP pressor response, the increase in HR following NPP injection might be due to effects of adrenaline activating pre-junctional β_2 receptors in the heart to facilitate noradrenaline release from sympathetic varicosities, or noradrenaline directly activating β_1 receptors. Similarly, the increase in SBP might also be due to the effect on SV and HR, that when combined also increase CO. Thus, the NPP effect was tested in bioassay rats that were adrenergically blocked with the non-selective β-antagonist, propranolol, alone and in combination with, the α-antagonist phentolamine (Mavrogiannis, 1998).

1.4.3 Possible Effects of β-Adrenergic Blockade on NPP Responses

Under conditions of β-adrenergic blockade with propranolol, NPP caused a major potentiation of both SBP and DBP, while the characteristic increase in HR was virtually eliminated (Mavrogiannis, 1998). The loss of the HR effect supports the view that the observed chronotropy was catecholamine dependent. Thus, with the β_1 and β_2 receptors blocked, unopposed activation of the α_1 receptors in the periphery might have resulted in vasoconstriction, increased TPR and hence DBP, thereby contributing to the rise in SBP (Mavrogiannis, 1998). Since the known effect of noradrenaline on peripheral blood vessels is vasoconstriction and the effect of β-blockade on the NPP response was potentiation of both SBP

and DBP, complete activation of α_1 receptors by circulating adrenaline and noradrenaline is a possibility since these catecholamines have similar affinities for the α_1 receptor.

Activation of peripheral α_2 receptors may have also occurred since noradrenaline has a greater affinity than adrenaline for this receptor. In addition, it is well established that circulating adrenaline can facilitate noradrenaline release from sympathetic nerve endings via stimulatory pre-junctional β_2 receptors in the heart, as well as in the adrenal medulla (Floras, 1992; Foucart *et al.* 1988). Thus, while the primary effect of adrenaline on β receptors in the periphery is to decrease TPR via vasodilatation, if adrenaline is present in high enough concentrations, it might in fact facilitate noradrenaline's α_1 effect in the peripheral vasculature, as well as its β_1 effect in the heart (Buhler *et al.* 1982) thereby leading to the increase in both SBP, DBP and hence TPR.

1.4.4 Possible Effects of Combined α-and β-Adrenergic Blockade on NPP Responses

In order to assess and confirm the possible involvement of α receptor activation in the NPP effect, rats were treated with phentolamine (α-adrenergic antagonist) in addition to propranolol. This combination of α- and β-adrenergic blockade substantially prevented any increase in SBP, DBP or HR (Mavrogiannis, 1998). These results support the earlier suggestion that activation of α-receptors in the periphery might be responsible for the potentiation of both SBP and DBP in propranolol-treated rats. Thus, α-blockade with phentolamine probably prevented the increase in SBP while complete β-adrenergic blockade with propranolol likely acted to inhibit the increase in HR.

1.5 THE CONCEPT OF NPP AS A SYSTEM

The relationship of NPP to the FXII enzyme, the rapid onset of the pressor effect (within 20 seconds of injection), its long duration (10-15 min) and potentiation by captopril, as well as confirmation of adrenergic receptor activation, all argue in favor of recruitment of multiple blood pressure regulating mechanisms. Evidence reported in this thesis suggests that ganglion blockade with pentolinium might also influence the pressor effect (Results). Since NPP is a heat labile protein and an enzyme, it is unlikely to have a direct agonistic effect of increasing blood pressure when injected into the bioassay rat (Mavrogiannis, 1998; Osmond *et al.* 1998). More likely it acts indirectly, by initiating one, or more, enzymatic reactions (cascade?) that produces a protein/peptide end product which exerts an agonistic effect to increase blood pressure, presumably by inducing the release of adrenal catecholamines.

Studies with protease inhibitors would appear to support the hypothesis of a cascade of events after injecting NPP into the rat. Treatment of NPP *in vitro* with the non-selective protease inhibitor soy bean trypsin inhibitor (SBTI) did not inhibit its activity when injected into bioassay rats (Cotter, 1995). However, when the rats themselves were infused with SBTI, just before NPP, its pressor response was completely blocked (Cotter, 1995). Similar treatment of plasma NPP with another non-selective protease inhibitor, aprotinin, did not block the subsequent NPP effect, but it did inhibit the response when given *in vivo*. Thus, neither SBTI nor aprotinin blocked the NPP pressor activity directly (i.e. *in vitro*), but rather did so indirectly (*in vivo*), presumably by inhibiting some other product formed by NPP once introduced into the bioassay rat (Cotter, 1995).

These four lines of evidence...

1) the relationship to FXII and enzymatic activity;
2) adrenal catecholamine involvement and adrenergic receptor activation;
3) potentiation by Captopril; and
4) possible influence of ganglion blockade

...suggest a complex system of events, initiated by i.v. injection of NPP. Therefore, it is pertinent to provide additional material relevant to the experiments and hypotheses of this thesis, i.e.:

1) ACE inhibition by captopril and the possible mechanism of its potentiating effect on NPP;
2) Adrenal medullary physiology as it relates to catecholamine storage and release, and;
3) Ganglion blockade by pentolinium and the possible role it plays in altering adrenal gland function and catecholamine release.

1.5.1 ACE Inhibition by Captopril and the NPP Pressor Effect

NPP effects on blood pressure and HR are greatly potentiated after treatment with the ACE inhibitor captopril. Given the widespread use of captopril as an anti-hypertensive agent, it is very important to determine the mechanism by which this potentiation occurs. If NPP does indeed generate a peptide end product having the ability to stimulate adrenal medullary catecholamine release, the question is whether the actions of this peptide(s) are influenced (i.e. or even augmented) by captopril, given that the degradative effects of ACE are inhibited (see below).

ACE is a member of the kininase II family of proteases, and is responsible for the bioregulation of many other peptides, including: bradykinin, vasoactive intestinal polypeptide (VIP), the enkephalins, neurotensin, substance P, substance K, luteinizing hormone-releasing hormone, the

hemoregulatory peptide AcSDKP, α/β-neoendorphins, β-endorphin and the dynorphins, and possibly others (Reiger *et al.* 1993; Handa *et al.* 1991; Erdös, 1990; Skidgel and Erdös, 1987; Theile *et al.* 1985; Ulrich and Hersh, 1985;). There is long standing evidence to suggest that some of these peptides can stimulate adrenal medullary catecholamine release (Chowdhury *et al.* 1994; Guo and Wakade, 1994; Malhotra and Wakade, 1986, 1987; Lang and Pearson, 1968; Staszewska-Barczak and Vane, 1965, 1967; Feldberg and Lewis, 1964, 1965).

Thus, if NPP exerts its effects by generating a peptide(s) that is capable of causing catecholamine release, and if that peptide(s) is also regulated by ACE, the potentiation by captopril might be a result of prolonging the half-life of such a peptide. It is unknown which peptide(s) or combination of peptides (known or unknown) is generated in sufficiently high concentration to stimulate adrenal medullary catecholamine release. Since this thesis is centered on implicating catecholamine release as the critical mediator of the NPP effect, a brief discussion of the key factors controlling catecholamine secretion follows.

1.5.2 Possible Basis for NPP's Selective Effect on Adrenaline vs. Noradrenaline Release

Based on the above-mentioned results of adrenergic receptor antagonist studies, Mavrogiannis (1998) hypothesized that a higher concentration of adrenaline, rather than noradrenaline, might be responsible for the observed cardiotonic effects of NPP.

The proportions of adrenaline and noradrenaline in the adrenal medulla vary from species to species. In the human adrenal, 90% of cells are of the adrenaline-secreting type and 10% are of the noradrenaline-secreting type (Ganong, 1997). In the rat adrenal, the adrenaline to

noradrenaline ratio varies from 2.5 to 5 in favor of adrenaline (Tomlinson *et al.* 1987; Tischler *et al.* 1987; Wakade and Wakade, 1983; Eranko and Raisanen, 1957). Generally, the ratio of adrenaline to noradrenaline secretion in the rat is reported to be about 4:1 (Parker *et al.* 1993; Verhofstad *et al.* 1985), but there is some evidence to suggest that this ratio can be substantially increased (Vollmer *et al.* 1997; Vollmer *et al.* 1992; Feuerstein and Gutman, 1971).

Adrenaline and noradrenaline enter the circulation mainly by release from the medulla, but noradrenaline also originates from sympathetic adrenergic neurons and spillover from neuronal release. The exact source of circulating dopamine is probably from the medulla, although the specific cell type is unknown. Normal resting levels of catecholamines in the peripheral plasma vary greatly depending on the time of day, stress level of the individual, and the route from which blood samples are obtained (Callingham and Barrand, 1979).

Adrenaline and noradrenaline are secreted from two distinct populations of adrenal medullary chromaffin cells (Hillarp and Hokfelt, 1953). In addition to adrenaline and noradrenaline secretory vesicles, chromaffin cells also contain dopamine, and other neuropeptides (Discussion). For the most part, chromaffin cells are stimulated to secrete their contents into the bloodstream upon stimulation by preganglionic nerve fibers that innervate the medulla via the splanchnic branch of the autonomic nervous system (Parker *et al.* 1993). It is generally accepted that excitation of splanchnic nerve terminals causes release of Ach that activates nicotinic (and muscarinic) cholinergic receptors on the chromaffin cells to evoke the secretion of catecholamines (Wakade and Wakade, 1983; Wilson and Kirshner 1976, 1977; Feldberg *et al.* 1934; Dale, 1914). Exocytosis is primarily dependent on intracellular calcium (Baker and Rink,

1975; Douglas, 1968), but other second messengers eg. cAMP, cGMP, protein kinase C and inositol phosphate have also been known to be involved in mediating release of catecholamine stores (Malhotra *et* al. 1989).

The adrenal medulla is generally described as a modified ganglion that is controlled primarily by a preganglionic sympathetic nerve supply via the splanchnic branch of the autonomic nervous system (Ganong, 1997). However, a more complex picture of the innervation of the adrenal gland is emerging. While it is true that the medulla receives primarily a preganglionic sympathetic innervation, there is ample evidence to suggest that the medulla also receives a postganglionic sympathetic innervation, a parasympathetic innervation and also has an afferent innervation (Parker *et al.* 1993; Coupland *et al.* 1989; Afework, 1988; Kesse *et al.* 1988; Mohammed *et* al. 1988; Coupland, 1965). With this apparently complex innervation, it seems likely that control of catecholamine secretion would also be complex and indeed recent reports indicates this to be the case (Discussion).

If catecholamines are secreted primarily as a result of sympathetic nerve activity, it follows that under conditions of sympathetic blockade, the adrenal medulla would be essentially "cut off" and unable to respond to sympathetic stimulation. Since there is little evidence to suggest that the adrenal medulla can be stimulated by the periphery (i.e. the circulation), it is unlikely that the product(s) of NPP injection which are present in the circulation act directly on medullary chromaffin cells to stimulate catecholamine release. The fact that our rats are ganglion blocked with the nicotinic Ach antagonist pentolinium (see below), raises the question as to what control mechanism might be operative for release of medullary catecholamines. Is there another route

by which the adrenal medulla can be stimulated? What role does ganglion blockade have on the NPP pressor response? Since these questions have arisen as a result of prior experiments, it becomes relevant to briefly discuss the issue of ganglion blockade as it relates to catecholamine release in our rats.

1.5.3 Ganglion Blockade and Adrenal Function As It Relates to Observed NPP Effects

It is well established that the adrenal gland is controlled primarily by the sympathetic nervous system (Parker *et al.* 1993) and that catecholamine release is triggered by the binding of Ach, released from preganglionic sympathetic nerve terminals, to act on nicotinic and/or muscarinic receptors on chromaffin cells. In the presence of ganglion blockade with pentolinium, the question arises as to how the isolated adrenal is capable of releasing catecholamines.

Pentolinium (a.k.a. Ansolysen) is a bisquaternary, symmetrical ammonium compound that blocks impulse transmission from the preganglionic neuron to the postganglionic neuron in autonomic ganglia (Klowden *et al.* 1978). The specific action of the drug is to block Ach stimulation of nicotinic receptors on the postganglionic neuron in the sympathetic system as well as stimulation of muscarinic receptors in the parasympathetic systems simultaneously, although the effect on the sympathetic system is far greater. The liberation of Ach at the synapse is not interfered with, nor are the rates of Ach synthesis or hydrolysis. Furthermore, when both the sympathetic and parasympathetic ganglia are blocked, there is often variation in sensitivity from one ganglion to another and in some cases, even among different cells of the same ganglion (Klowden *et al.* 1978).

Ganglion blockade was used in our bioassay rats as a tool to render the animal more sensitive to agonists (Page and Taylor, 1947). In the late 1950s, ganglionic blocking agents were administered in humans to reduce blood pressure during surgery. The first dose usually produced a significant fall in blood pressure but the pressure often returned quite rapidly to its initial level and subsequent doses had little or no effect (Mantegazza *et al.* 1958). At that time there were published reports regarding the potentiation of adrenaline and noradrenaline effects in the cat that appeared to occur in the presence of pentolinium and hexamethonium (another ganglion blocking agent) which persisted after vagotomy and spinal cord section at a high level (Bartorelli *et al.* 1954).

Mantegazza *et al.* (1958) tested the effects of adrenaline and noradrenaline on certain peripheral effector cells in the cat (venous outflow from the hind limb and contractions of the nicitiating membrane) before and after intravenous or close-arterial injection of hexamethonium and pentolinium. They observed increased responses to adrenaline and noradrenaline after the ganglion blocking agents were administered, regardless of the route of administration, and attributed this to a "sensitization" of the effector cells to the catecholamines and to sympathetic stimulation. The authors speculated that the increasing dosage of ganglionic blocking drugs produced decreasing blood pressure effects in humans, not because the magnitude of the blockade was diminished, but because this effect was partially masked by the peripheral sensitization, i.e. up-regulation of adrenergic receptors, of the blood vessels to catecholamines (Mantegazza *et al.* 1958; Shimamoto *et al.* 1955).

Preliminary experiments in unblocked rats also indicated to us that ganglion blockade might potentiate the NPP effect, as did captopril. Since we know that the adrenal medulla receives most, if not all, of its 'commands' from the central nervous system (CNS) and not from the periphery (i.e. the circulation), under conditions of sympathetic blockade, the adrenal medulla should not be capable of releasing catecholamines. However, the adrenergic receptor antagonist studies in our ganglion blocked rats clearly indicate that catecholamines are involved in the NPP effect and perhaps also argues in favor of other mechanisms or pathways controlling adrenal medullary function.

Since the adrenal medulla has always been considered an integral part of the sympathetic nervous system, this is little evidence to suggest that other non-nervous pathways also stimulate the adrenal medulla. Therefore, might the adrenal medulla be stimulated through a nervous pathway that bypasses the routes blocked by pentolinium? We know that under conditions of ganglion blockade, preganglionic transmission is blocked, but the same is not true for postganglionic transmission (Klowden *et al.* 1978). There are many other neuropeptides/neuromodulators present in the postganglionic nerve terminal, as well as in the adrenal medulla itself (Discussion) that could play a role in mediating the pressor effect of NPP in the absence of the nicotinic-Ach pathway. Whether NPP influences any of these peptides to the extent that they can cause catecholamine release is yet to be determined.

The adrenal ablation studies, the effect of adrenergic receptor antagonists, and the effect of NPP on SBP, CO, SV and HR all support involvement of adrenal medullary catecholamines in mediating the observed pressor response. These strong cardiotonic effects also argue in favor of

high circulating levels of adrenaline and noradrenaline. The evidence supporting continued adrenal function despite sympathetic system inhibition and the role that ganglion blockade plays in modifying the pressor response of NPP adds further complexity to understanding the mechanism of this extremely potent blood pressure-raising system. This thesis will attempt to provide some insight into these issues, specifically by confirming the involvement of the adrenal medulla, quantifying the extent of catecholamine release after NPP and investigating the role of ganglion blockade in these responses.

RATIONALE FOR THE PRESENT STUDY

The primary focus of this thesis is the factor(s) involved in causing the blood pressure, cardiotonic and catecholamine releasing effects seen after NPP injection in bioassay rats. Since detection and measurement of NPP is only available to us by the use of bioassay animals (rats), all of the experiments in this thesis will deal with data obtained using this preparation.

Adrenal Gland Involvement and Catecholamine Release

Since 2AX abolished >90% of the pressor effect of NPP, it is important to confirm the involvement of the adrenal medulla and to understand the possible mechanisms responsible for the effect. Thus, one of the key experiments will be to test the effect of NPP in bioassay rats that have had their adrenal medullae removed, but are left with the cortex intact. In addition, since combined treatment with α- and β-adrenergic antagonists also produced a significant reduction in the pressor effect of NPP, it is important to measure circulating plasma catecholamine levels (adrenaline, noradrenaline and dopamine) at the peak SBP response to further characterize the response to NPP. Such experiments will provide further support to the suggestions by Mavrogiannis (1998) regarding the role of catecholamines in the pressor effect.

Issues Surrounding Ganglion Blockade

Our established bioassay rat model is ganglion blockade with pentolinium (i.e. ansolysen, 19.2 mg/kg) and captopril pre-treatment (0.25 mg/kg). Thus, the initial observations that lead to the characterization of NPP were made in this +A (ganglion blocked, ansolysen treated) and +C (captopril treated) rat. Since it has become apparent that the adrenal gland might very well be a

key factor in mediating the pressor effect of NPP, we initiated a specific investigation into mechanisms involved in adrenal medullary catecholamine release.

As discussed in the Introduction, it is necessary to investigate the NPP pressor effect (and catecholamine release) in unblocked bioassay rats (i.e. no ansolysen, -A) and compare these responses to what is observed under "normal" conditions. Since it is now well established that the NPP pressor effect is potentiated after captopril treatment, it is also critical to investigate the NPP effect in unblocked rats, both in the absence and presence of captopril (i.e. –A-C and –A+C). These experiments should illuminate the role ganglion blockade has on the NPP effect, not only by itself, but also in combination with captopril.

Early experiments of NPP injection in unblocked bioassay rats before captopril (i.e. -A-C) resulted in a surprising biphasic response (Fig. 3.5.1, Results) that began with a drop in pressure, followed by a secondary pressor phase. In light of this different character of the NPP response in the absence of ganglion blockade and captopril, we were prompted to compare the blood pressure and catecholamine responses of NPP to a known depressor agent, sodium nitroprusside (SNP). SNP was chosen because it acts by direct vasodilatation and therefore, was not expected to trigger adrenal medullary catecholamine release directly.

<u>Other Anti-Hypertensive Drug Effects on NPP Pressor Response</u>

The potentiation of the NPP pressor effect after treatment with ACEIs begs the question whether this effect is specific to this class of drug. As already discussed, ACEIs inhibit the conversion of Ang I into Ang II. In addition to this effect, ACEIs also prolong the half-lives of certain classes

of peptides that are normally degraded by the ACE enzyme. Thus, we decided to begin the investigation of possible roles of peptides and receptor interaction as possible causes of the potentiation of NPP. We decided to test one of the first angiotensin AT_1 receptor antagonists, losartan, on the pressor effect of NPP. Losartan functions by blocking the effects of Ang II at the receptor level. Thus, it appeared relevant to investigate the effect of losartan alone, and in combination with captopril, on the NPP-mediated pressor and catecholamine effects.

HYPOTHESES

1. *The previously demonstrated effect of the adrenal gland in mediating the cardiovascular effects of injected NPP is due primarily to the medulla, not the cortex.*

2. *If the cardiovascular effects of NPP are mediated by the adrenal medulla, they are associated substantially with the release of catecholamines reflected by high plasma concentrations in concert with pressor and heart rate responses.*

3. *Since the ACE inhibitor captopril enhances the cardiovascular effects of NPP, its administration will also produce enhanced release of catecholamines.*

4. *Since the cardiovascular effects of NPP are observed in ganglion blocked bioassay rats (pentolinium) which should block the main nicotinic nerve pathway to the adrenal medulla, another pathway must be present to mediate such NPP effects, especially if catecholamine release is enhanced.*

5. *Any potentiating effect of captopril on cardiovascular responses and catecholamine release will not be duplicated by the anti-hypertensive drug losartan (angiotensin II AT_1 receptor antagonist) because the two act through different pathways, thereby implicating the ACE mechanism.*

6. *If captopril exerts its effect through ACE inhibition and this effect is not duplicated by losartan, then combining the latter with captopril will produce no further potentiation.*

7. *The cardiovascular and catecholamine effects of NPP are the product of some unknown agonistic action and are not simply a consequence of blood pressure changes such as are produced by the reference drug sodium nitroprusside (SNP).*

OBJECTIVES

1. *To implicate the adrenal medulla specifically in mediating the pressor and cardiotonic effects of NPP.*

2. *To determine the magnitude and nature of adrenal medullary catecholamine responses to NPP by measuring plasma catecholamines before, during and after NPP injection.*

3. *To determine whether captopril potentiates the pressor effect of NPP by also potentiating the release of adrenal medullary catecholamines.*

4. *To investigate the possible role of ganglion blockade on the NPP-mediated pressure and catecholamine effect.*

5. *To investigate the effect of losartan on the NPP pressor response and catecholamine release.*

6. *To determine the effect of combined losartan and captopril treatment on the NPP-mediated pressor effect.*

7. *To investigate the effect of combined losartan and captopril treatment on adrenal medullary catecholamine responses.*

8. *To compare the effect of sodium nitroprusside (SNP) in unblocked rats on blood pressure and catecholamine responses to that of NPP.*

2. METHODS

2.1 HUMAN PLASMA

Human plasma, considered normal but not suitable for transfusion purposes, was provided by the Canadian Blood Services (formerly the Canadian Red Cross Society, Toronto Center). These bags of frozen plasmas (≅250 mL) from male and female donors were derived from ≅450 mL units of blood containing 63 mL of CP2D anticoagulant made up as follows (g/63 mL water): sodium citrate, 1.66; monobasic sodium phosphate, 0.14; citric acid, 0.206; dextrose, 3.22. The plasma bags (containing most of this 63 mL anticoagulant) were thawed in cold tap water and aliquots were used immediately or frozen at -20°C in capped polystyrene tubes for later use.

2.1.1 Preparation of NPP from Human Plasma

Plasmas were activated with trypsin as described previously for prorenin (Ioannou *et al.* 1991; Hare *et al.* 1989; Ioannou *et al.* 1989; Cooper *et al.* 1977). Essentially trypsin (type III, bovine, T-8253; Sigma Chemical Co., St Louis, Missouri, USA) was prepared as a stock solution in 0.002 N HCl and added to plasma at 3-10% v/v to achieve the required final trypsin concentration of 1 mg/mL for human plasmas (Ioannou *et al.* 1989) with minimal plasma dilution. After it had been mixed for a few seconds with a Vortex shaker (Sybron Thermolyne; Thermolye Corp., Dubuque, Iowa, USA), human plasma was incubated at 23°C for 10 min. Such "activated" plasma was divided into 1 mL aliquots, placed in small polystyrene tubes which were covered with parafilm and immediately frozen at -20°C. On the day of each experiment, one tube was thawed to provide NPP (20 μL human plasma equivalent) for intravenous (i.v.) injection in bioassay rats.

2.2 ANIMALS

All animals were cared for and used in accordance with the principles and guidelines of the Canadian Council on Animal Care. Male Wistar rats, 300-400g, (Canadian Biobreeding Laboratories) were fasted overnight before surgery and prepared for bioassay essentially according to Pickens *et al.* 1965, with some modifications (see below).

2.2.1. Anesthesia

Rats were anesthetized with Inactin (sodium ethyl-[1-methyl-propyl]-malonyl-thio-urea, Promonta Hamburg, Germany) at a dose of 100 mg/kg intraperitoneally (i.p.). Solutions (50 mg/mL) were prepared in normal saline (0.9% NaCl in distilled water) just prior to use.

2.2.2. Surgery

Prior to the start of surgery, atropine sulphate (0.5 mg/mL saline, Ormond Veterinary Supply Ltd., Ancaster, Ontario, DIN 153656) was injected subcutaneously (s.c.) at a dose of 2.4 mg/kg to help maintain a patent airway by controlling for bronchial secretions. The ganglion blocking agent ansolysen (4.8 mg/mL, pentolinium tartrate, 1,1'-pentamethylenebis[1-methylpyrrolidinium hydrogen tartrate], P-3520, Sigma Chemical Co., St. Louis, USA) was given s.c. at a dose of 19.2 mg/kg in 20% polyvinylpyrrolidone solution (Sigma, PVP-40T), to provide for gradual release of the drug during the bioassay period.

A tracheotomy was performed to secure the airway using a short polyethylene (PE 240) cannula (Clay Adams, Becton Dickinson) and both vagi were severed. The right carotid artery was dissected free of the carotid sheath and cannulated with PE-50 tubing filled with heparinized

saline (20 units/mL Hepalean, Organon Teknika Inc., Toronto, Canada). This cannula was used for blood pressure measurement, for blood sampling and blood replacement. The incision was covered with surgical gauze to minimize loss of heat and fluid.

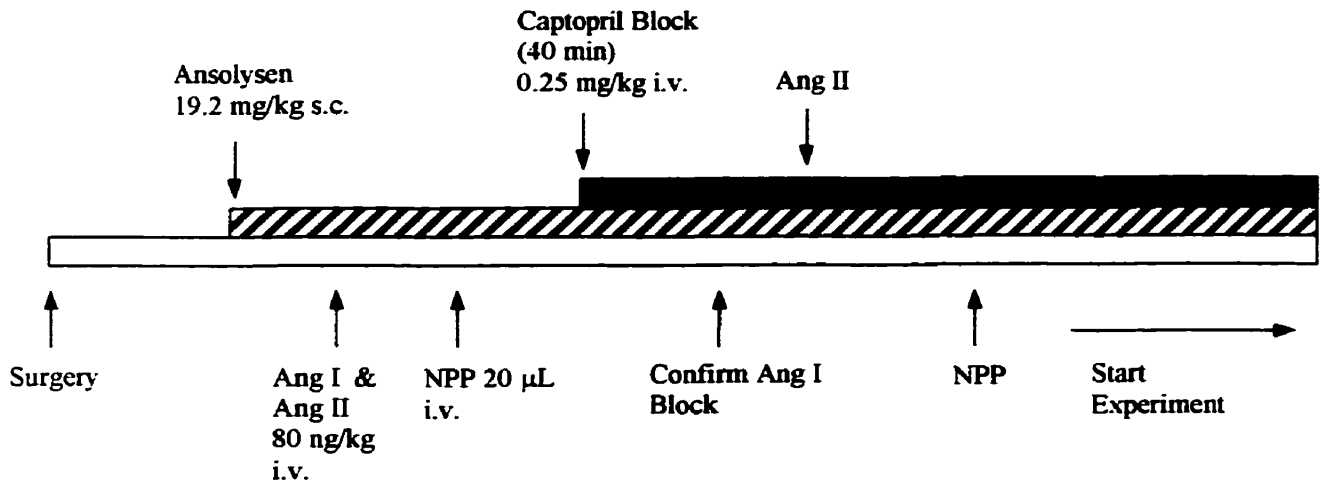
In experiments where the carotid artery cannula was used for blood sampling, the left femoral artery was cannulated for measuring blood pressure using PE-20 tubing filled with heparinized saline. Blood pressure measurements were made using a Statham Dc pressure transducer (Hato Rey, Puerto Rico) connected to a MacLab/8 data acquisition system (AD Instruments, Castle Hill, Australia) and an Apple Power Macintosh 7200/1200 PC Compatible computer driven by MacLab Chart v.3.5.6 software. The recording system was calibrated each day against a mercury sphygmomanometer (Tycos, Taylor Instrument Co., Rochester, NY, USA).

Injection of agonists was via a PE-20 cannula, filled with heparinized saline, fitted with a 27-gauge needle (Becton Dickinson and Co., Sparks, Maryland, USA). After a cut-down to expose the area, the needle was inserted freely at the junction of the superficial epigastric vein and the superficial circumflex iliac vein to allow for continued venous return. In some instances, a PE-20 cannula was inserted into the jugular vein. The incision was covered with surgical gauze to prevent loss of heat and fluid. A rectal thermometer was used to monitor core body temperature, which was maintained at 37°C by warming the rat with 60-watt incandescent bulbs that were placed 25-30 cm above the tail area.

2.3 TYPICAL RAT BIOASSAY PROTOCOL

Following administration of the ganglion blocking agent ansolysen and after an initial stabilization period, the pressor responses to injections of angiotensin I (Ang I) and angiotensin II (Ang II) were determined in each rat in every experiment. Ang I and Ang II were each administered at a dose of 80 ng/kg in a 20 μL injection volume. The pressor responses to Ang I and Ang II are quite characteristic and are used to establish adequate responsiveness of the bioassay rat. The angiotensin I converting enzyme inhibitor (ACEI) captopril (see below) was given (i.v.) at a dose of 0.25 mg/kg, after which basal blood pressure usually stabilized at about 80/40 mmHg, systolic/diastolic (SBP/DBP). This dose of captopril abolished the pressor response to injected Ang I, thereby verifying complete renin system blockade (Fig. 2.3.1).

Figure 2.3.1
Typical bioassay protocol.



2.3.1 Reproducibility of Blood Pressure Responses

Bioassay rats were excluded from our experiments if they did not meet the established criteria of minimal trauma and blood loss during surgery, stability of basal blood pressure and good responsiveness to angiotensins I and II. The reproducibility of blood pressure responses to repeated injections of the same dose of Ang II was investigated in 4 rats treated with both ansolysen and captopril. Test doses of Ang II (80 ng/kg i.v.) were given every 5-10 min and SBP, DBP and HR were recorded.

2.4 PREPARATION AND STORAGE OF AGONISTS

Ang I (Sigma A-9650) and Ang II (Sigma A-9525) were dissolved in distilled water to prepare stock solutions of 2.0 mg/mL and were stored frozen in small aliquots at -20°C. Aliquots were thawed and diluted for i.v. injection with 0.9% NaCl in distilled water just before use. Captopril (Sigma, C-4042) was prepared fresh daily, dissolved in 0.9% NaCl in distilled water and given i.v. at 0.25 mg/kg in two injections, 20 min apart.

The angiotensin AT_1 receptor antagonist losartan (Merck Sharp & Dohme Research Laboraties, Rahway NJ, USA) was dissolved in distilled water and fresh stock solutions of 50 mg/mL were prepared shortly before each experiment. The stock was diluted with 0.9% NaCl in distilled water for i.v. injection at a dose of 10 mg/kg.

Sodium nitroprusside (SNP, $Na_2[Fe(CN)_5NO]$-$2H_20$, Product #10257, BDH Chemicals Ltd., Poole, England) was made fresh each day and dissolved in distilled water to prepare stock solutions of 1 mg/mL. The stock was diluted for i.v. injection with 0.9% NaCl in distilled water,

given i.v. at a dose of 5 μg/kg and all solutions were protected from light by wrapping the tube in aluminum foil.

2.5 ACUTE BILATERAL ADRENAL MEDULLECTOMY (2MDX)

Following the test doses of Ang I, Ang II and NPP injections before and after captopril blockade, the rat was disconnected from the recording system in order to remove the adrenal medullae. With the carotid artery and jugular vein cannulae tied firmly in place, the rat was positioned on its side, taking care to maintain a patent airway. Flank incisions were made to expose the adrenal glands and the medullae were removed by enucleation so as to spare the adrenal cortex (Borkowski & Quinn, 1983). The muscle layer was closed using 3.0 silk suture (Ethicon Inc.,) and the skin was stapled using wound clips (Autoclip 9mm, Becton Dikinson and Co., Sparks, MD, USA). The rat was reconnected to the blood pressure recording system, allowed to stabilize for about 10 min and the pressor response to 20 μL of NPP was assessed.

2.6 MEASUREMENT OF PLASMA CATECHOLAMINES IN RESPONSE TO HUMAN NPP IN RATS

Blood for catecholamine analysis was obtained from rats prepared as already described, in the absence, or presence, of ganglion blockade and captopril (see below). Blood pressure was monitored in the recipient bioassay rat via a femoral artery cannula. Blood (1 mL) from a control donor rat was infused 10-15 min before each blood sample was removed in order to maintain blood volume (see below). Depending on the experiment, 3-4 blood samples were taken for catecholamine analysis (see below). Blood, 1 mL, was withdrawn via the carotid artery cannula and transferred to a chilled polystyrene tube containing 1.2 mg glutathione (Lot #

13644446-85, Boehringer Mannhiem GmbH, West Germany) in 0.5 mL heparinized saline (10 U/mL, Hepalean).

2.6.1 Preparation of Donor Rats

Male Wistar rats weighing 350-400g were prepared essentially as described above. Rats were anesthetized with Inactin (100 mg/kg i.p.), both vagi were severed, and the carotid artery was cannulated for blood withdrawal. Unlike the bioassay rats, donor rats were not treated with ansolysen or captopril. Following removal of 1 mL blood for donation to the bioassay rats, the donor rat received 1 mL of normal saline i.v. (0.9% NaCl in distilled water) as fluid replacement.

2.6.2 Determination of Plasma Catecholamines

Plasma was prepared by centrifugation of anti-coagulated blood (section 2.6) at 2000x*g* for 15 min. It was separated and stored at -40°C until the time of assay. Plasma catecholamines were determined courtesy of Dr. Frans Boomsma (Internal Medicine I, Dijkizgt Hospital, Rotterdam, The Netherlands) by high performance liquid chromatography (HPLC) with fluorimetric detection (Van der Hoorn *et al.* 1989). The fluorimetric method is advantageous because it requires smaller plasma volumes (300 µL) and is very sensitive for detecting picogram (pg) levels of catecholamines.

2.7. EXPERIMENTAL GROUPS

Four experimental groups of animals were established to investigate the effects of ganglion blockade and captopril on the pressor response to NPP and adrenal medullary catecholamine release.

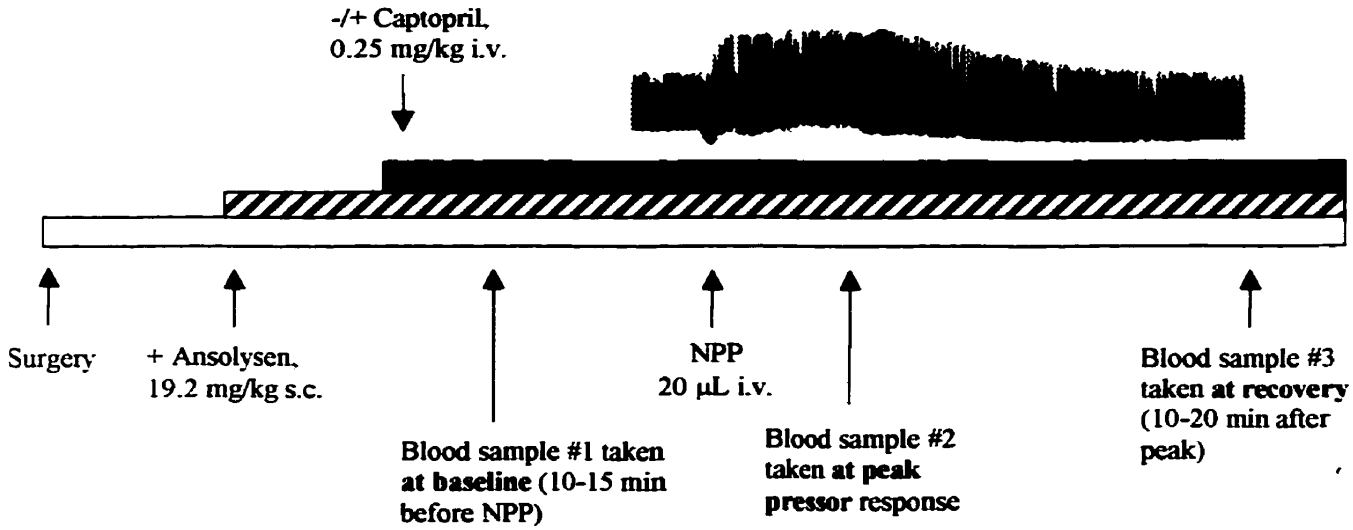
2.7.1 Presence of Ganglion Blockade -/+ Captopril

Given the potentiation of the NPP pressor response following captopril treatment, plasma catecholamines were also assessed in rats before, and after, treatment with the ACE inhibitor (Fig.2.7.1). Three blood samples were taken in these rats; at baseline, at peak SBP response to NPP, and at recovery (10-20 min after peak). Before each sampling, 1 mL of blood was infused from a control donor rat as described above. The two groups of rats were given the following designation:

+A-C (ansolysen treated, without captopril treatment)

+A+C (ansolysen treated with captopril treatment)

Fig 2.7.1
Protocol for catecholamine experiments in ganglion blocked rats, before and after captopril. Note that blood donations were given 10-15 min before baseline and recovery samples were taken and 15 min before NPP injection.



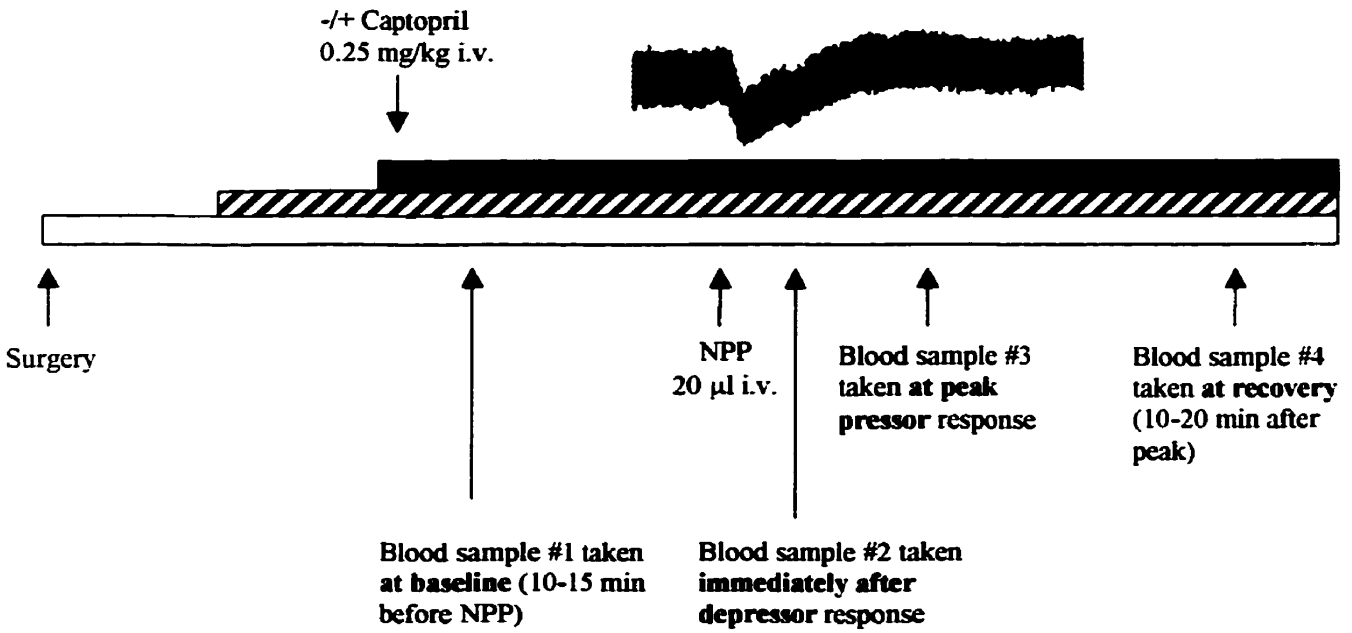
2.7.2 Absence of Ganglion Blockade -/+ Captopril

The effect of ganglion blockade on the pressor and catecholamine effects of NPP was investigated in bioassay rats. Plasma catecholamines were measured in rats without ganglion blockade, in the absence or presence of captopril, before, during, and after injection of NPP (Fig. 2.7.2). Note that because the blood pressure response pattern was different (cf. Fig 2.7.1 & 2.7.2), the blood samples had to be taken at different times. Thus, four blood samples (instead of three) were taken in these two groups of rats (see Results). Before each sampling, 1 mL of blood was infused from a control donor rat as described above. These two groups of rats were given the following designation:

-A-C (no ansolysen, no captopril)

-A+C (no ansolysen with captopril treatment)

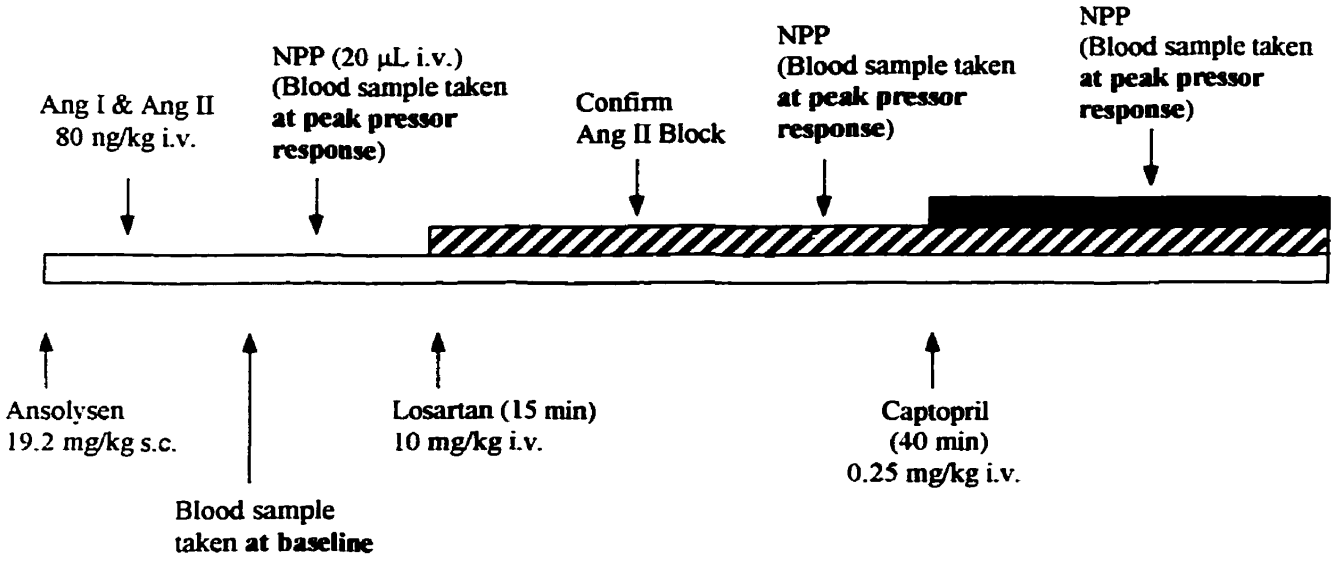
Fig 2.7.2
Protocol for catecholamine experiments in unblocked rats, before and after captopril. Note that blood donations were given 10-15 min before baseline and recovery samples were taken and 15 min before NPP injection.



2.8 EFFECT OF ANGIOTENSIN AT_1 RECEPTOR ANTAGONIST ON CARDIOVASCULAR FUNCTIONS OF NPP

The NPP pressor response was determined in ganglion blocked bioassay rats before treatment with losartan (angiotensin AT_1 receptor antagonist, 10 mg/kg i.v.), after losartan, and after losartan + captopril (Fig. 2.8.1). Blood (1 mL) was taken at peak SBP response to NPP before, and after, losartan and after losartan + captopril. As described in section 2.7, blood from a control donor rat was infused prior to each blood sampling. Blood was treated as previously described, and plasma adrenaline, noradrenaline and dopamine were measured by HPLC with fluorimetric detection.

Figure 2.8.1
Experimental protocol with losartan. Blood was donated 10-15 min before each sample was taken.



2.9 EFFECT OF SODIUM NITROPRUSSIDE ON PLASMA CATECHOLAMINES

The effect of the direct vasodilator sodium nitroprusside (SNP) on blood pressure, heart rate and plasma catecholamine responses was assessed in rats that were not ganglion blocked, before and after captopril treatment. SNP was administered in 0.9% NaCl in distilled water at a dose of 5 μg/kg. Blood for catecholamine analysis was taken at similar time points as those described for NPP (section 2.7.2, Fig. 2.7.2).

2.10 STATISTICAL ANALYSES

Numerical data were analyzed using the Analytical MacLab Program (InStat for Macintosh, GraphPad Software v.1.12). Pressure data are expressed in terms of increments of SBP and DBP (means ±SEM), and catecholamine data are expressed in terms of picograms (pg) per mL plasma (means ±SEM). Statistical comparison of blood pressure and heart rate data were made using Student's *t* test for paired or unpaired data, as appropriate. Statistical comparisons of plasma catecholamine data were made using the repeated measures analysis of variance with correction for multiple *t* tests (Bonferroni) where appropriate.

3. RESULTS

3.1 REPRODUCIBILITY OF INJECTION TECHNIQUE

The stability of our bioassay rats and reproducibility of their responses to angiotensin II (Ang II, as a representative agonist) was tested in 4 rats that were ganglion blocked with ansolysen and captopril treated (+A+C). A succession of 10 injections, 5-10 min apart, gave very reproducible systolic blood pressure (SBP) and diastolic blood pressure (DBP) as well as heart rate (HR) responses (Fig. 3.1.1). The increments in DBP responses were consistently greater than SBP responses ($p<0.001$ for injection #1 and $p<0.0001$ for both injections #5 and #10) and there was no significant change in HR (Fig. 3.3.1). These results confirm that rats meeting our acceptance criteria for bioassay are substantially stable and give reproducible responses to Ang II. The group data for NPP, with relatively small standard errors of the mean, suggest that NPP responses are similarly reproducible (Fig. 3.2.3)

3.2 EFFECT OF CAPTOPRIL ON THE NPP PRESSOR RESPONSE

Effects of NPP injection in ganglion blocked rats before captopril (+A-C) and after (+A+C) are shown in representative blood pressure traces (Fig. 3.2.1a & 3.2.1b, respectively). As previously described (Methods), basal blood pressure typically stabilized around 80/40 mmHg (SBP/DBP, Fig.3.2.2, left panel). Baseline HR was significantly increased ($p<0.05$) after Captopril treatment (Fig. 3.2.2, right panel).

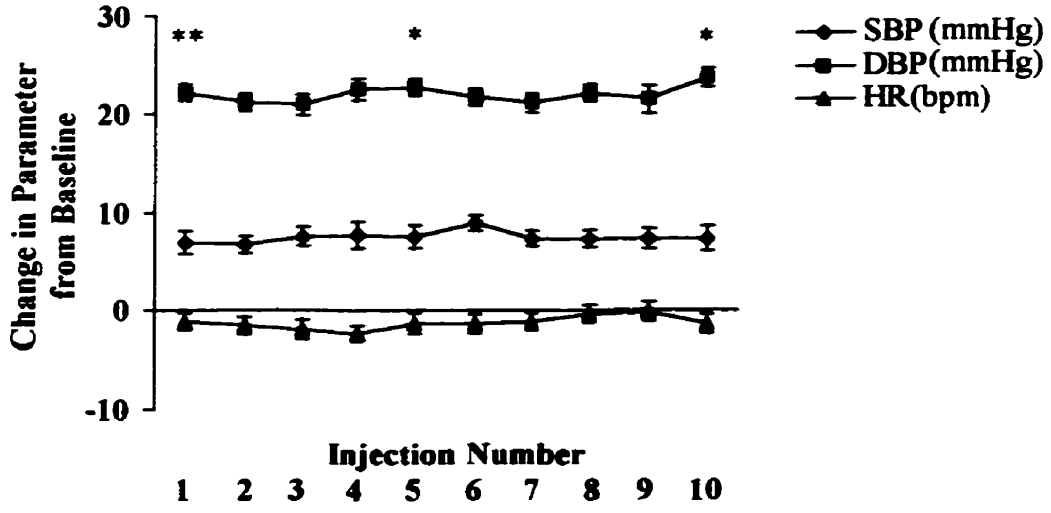


Figure 3.1.1. Reproducibility of Ang II responses in +A+C bioassay rats (n=4).

A typical injection of Ang II in our bioassay rat model always increases diastolic blood pressure (DBP) more than systolic blood pressure (SBP), which is consistent with effects mediated in the peripheral vasculature. There was no significant change in HR responses.

Each point represents the mean of four rats.
**p<0.001 DBP vs. SBP, *p<0.0001 DBP vs. SBP

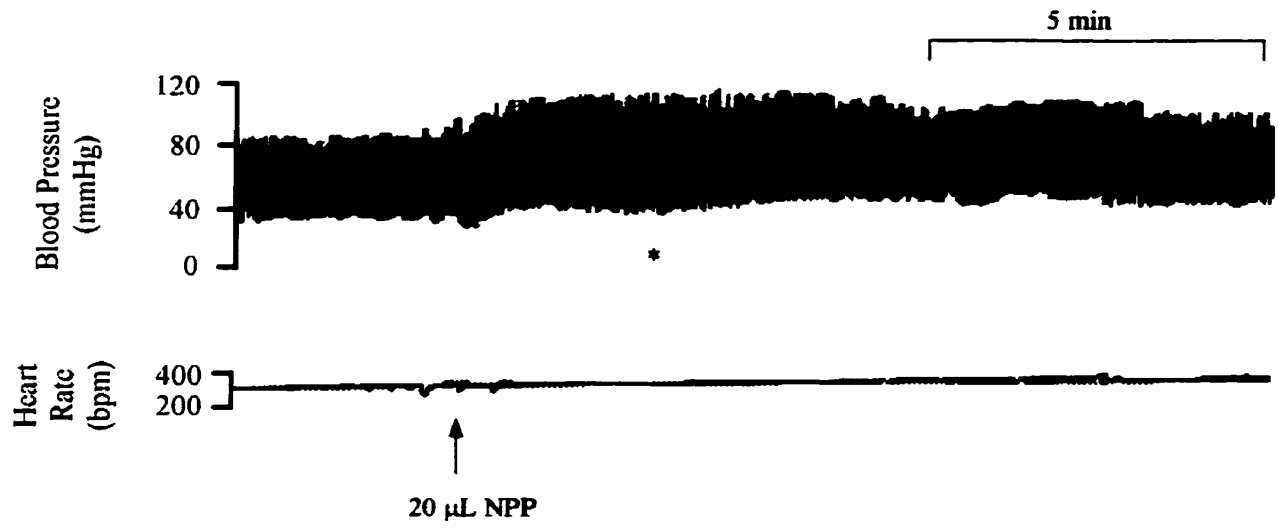


Figure 3.2.1a. NPP response in ganglion blocked rats before captopril (+A-C)
Representative blood pressure response to NPP injection. Note the modest increase in SBP with a smaller change in DBP. *Indicates where blood was taken for catecholamine analysis, at peak pressor response. Blood samples were also taken 15 min before injection (baseline control) and 15-20 min after the peak response (recovery control).

Figure. 3.2.1b. NPP response in ganglion blocked rats after captopril (+A+C)
Representative blood pressure response to NPP. Note the greater increase in SBP relative to DBP and the greater increase in HR compared to the +A-C rat. *Indicates where blood was taken for catecholamine analysis. Blood samples were also taken 15 min before injection (baseline control) and 15-20 min after the peak response (recovery control).

In ganglion blocked rats before captopril (n=8, Fig. 3.2.3), NPP produced a slight increase in SBP (11 ± 3 mmHg) accompanied by a smaller increase in DBP (3 ± 2 mmHg) and a small increase in HR (about 4 ± 1 bpm). After captopril (n=8), NPP caused a significantly greater SBP response (30 ± 3 mmHg, $p<0.01$, Fig. 3.2.3) compared to control (before captopril) and there was no change in DBP. Captopril treatment also potentiated the increase in HR after NPP (83 ± 11 bpm, $p<0.001$, Fig. 3.2.3, right panel).

3.3 EFFECT OF ACUTE BILATERAL ADRENAL MEDULLECTOMY (2MDX)

The NPP pressor effect has been shown to be reduced >90% within 10 min of acute bilateral adrenalectomy (Mavrogiannis, 1998; Osmond *et al.* 1997). With rats serving as their own control, the NPP pressor response was assessed before and after acute bilateral adrenal medullectomy (2MDX). Medullary ablation resulted in a significant reduction of the NPP effect (n=6, Fig.3.3.1). 2MDX caused virtually complete inhibition of the SBP component of the NPP pressor response ($p<0.01$), while there was no significant change in DBP. The characteristic increase in HR that usually followed NPP was also reduced by 2MDX (76 ± 12 bpm before vs. 19 ± 6 bpm after 2MDX, Fig. 3.3.1, $p<0.01$, right panel).

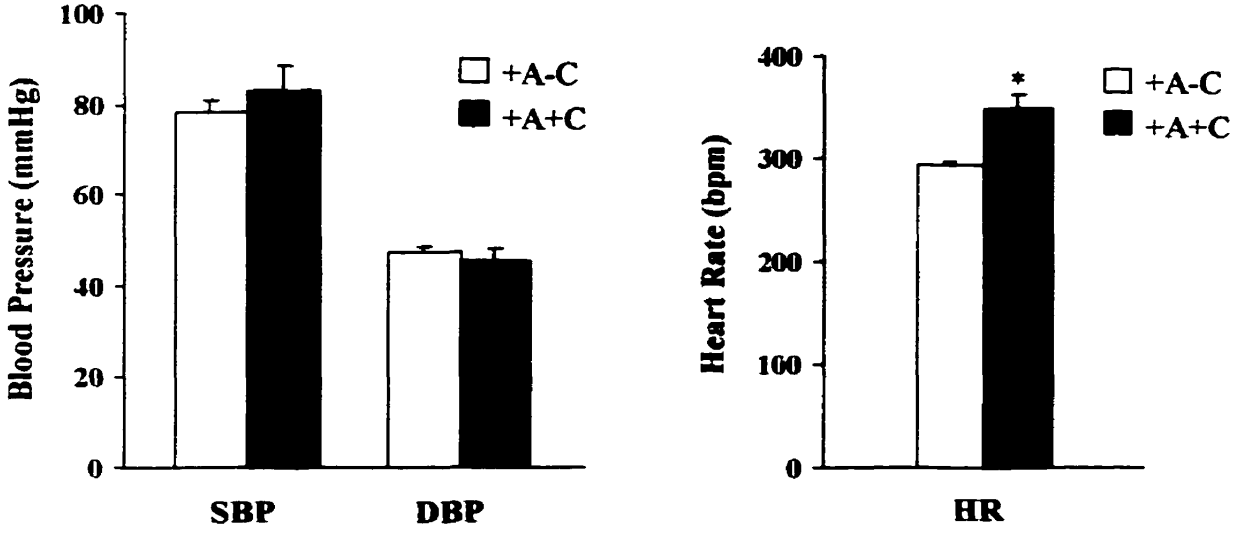


Figure 3.2.2. Baseline values in +A-C and +A+C rats (n=8): SBP, DBP and HR.

Left Panel: Baseline SBP and DBP were similar in +A-C and +A+C rats. In both groups blood pressure typically stabilized around 80/40 mmHg (SBP/DBP).

Right Panel: Baseline HR in +A+C rats significantly higher than was the case for +A-C rats (*$p<0.05$).

Data presented in Fig.3.2.2 (means ± SEM)
Left panel: From left to right, 78±3; 83±5; 47±1; 45±3 mmHg.
Right panel: From left to right, 293±3; 348±13 bpm.

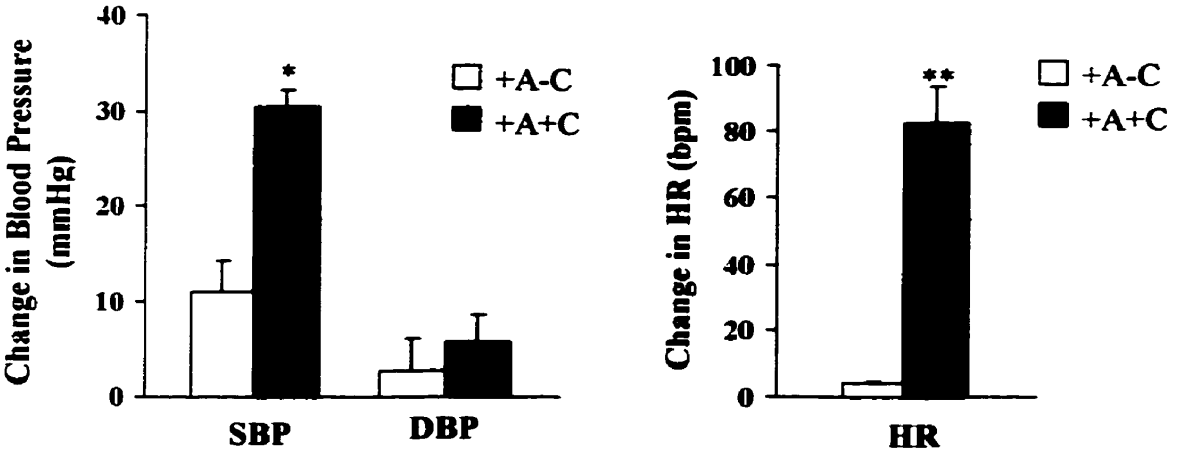


Figure 3.2.3. Effect of NPP in +A-C and +A+C rats (n=8): Changes in SBP, DBP and HR.

Left Panel: In +A-C rats, NPP caused a small increase in SBP and an even smaller increase DBP. In +A+C rats, only the SBP effect was potentiated (*$p<0.01$).

Right Panel: In +A-C rats, NPP caused a trivial increase in HR. This effect was greatly potentiated after Captopril treatment (**$p<0.001$, +A+C rats).

Data presented in Fig.3.2.3 (means ± SEM)
Left panel: From left to right, 11±3; 30±3; 3±2; 6±3 mmHg.
Right panel: From left to right, 4±0.8; 83±11 bpm.

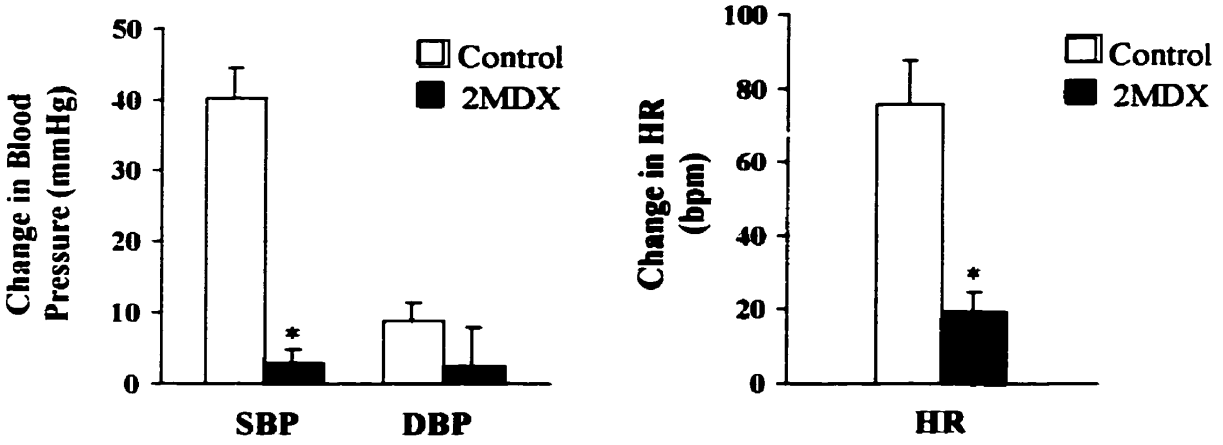


Figure 3.3.1. Effect of 2MDX in +A+C rats (n=6): Changes in NPP response.

Left Panel: In intact rats, NPP increased SBP much more potently than DBP. After 2MDX, NPP injection produced a much lower increase in SBP (>90%, *p<0.01) and there was no change in DBP.

Right Panel: In control rats, NPP produced a prominent increase in HR which was reduced >70% (*p<0.01) after 2MDX.

Data presented in Fig.3.3.1 (means ± SEM)
Left panel: From left to right, 40±4; 3±2; 9±3; 2±5 mmHg.
Right panel: From left to right, 76±12; 19±6 bpm.

3.4 EFFECT OF CAPTOPRIL ON CATECHOLAMINE RESPONSES TO HUMAN NPP

Plasma catecholamine levels (adrenaline, A; noradrenaline, NA; dopamine, DA) were measured in ganglion blocked rats before and after captopril. Three blood samples were obtained for analysis (Fig. 2.7.1, Methods): 10-15 min before NPP (baseline), at peak pressor response to NPP, and 15-20 min after the peak response (recovery).

In ganglion blocked rats before captopril (n=8, **+A-C**), the baseline ratio of A:NA was approximately 3:1 and A:DA was aproximately 6:1 (Table 3.4.1). At the peak SBP pressor response to NPP, A and NA levels rose significantly ($p<0.01$ and $p<0.05$, respectively), while there was no significant change in DA levels. Furthermore, during the peak pressor response the ratio of A:NA increased from 3:1 to 17:1 and the A:DA ratio increased accordingly (Table 3.4.1). At recovery, catecholamines returned to baseline levels and the A:NA ratio was about 3:1.

In ganglion blocked rats after captopril (n=8, **+A+C**), baseline A levels were significantly higher than before captopril ($p<0.05$, Table 3.4.1 vs. 3.4.2) and there was no significant difference in NA and DA levels between the two groups. The ratio of A:NA at baseline was approximately 4:1 and not statistically different from the **+A-C** group (Table 3.4.3). At peak SBP response to NPP, all catecholamine levels and ratios increased significantly ($p<0.001$, $p<0.01$ and $p<0.05$, A, NA and DA, respectively), and were higher than **+A-C** rats (for statistics see Table 3.4.3). At recovery, all catecholamines returned to near baseline levels and the A:NA and A:DA ratios were about 5:1. The recovery levels of A were significantly elevated compared to **+A-C** rats (Table 3.4.3).

Condition	*Adrenaline**	*Noradrenaline*	*Dopamine*	*Ratio# A:NA*	*Ratio A:DA*
Baseline	45 ± 18	14 ± 7	31 ± 12	3:1	6:1
Peak Pressor	671 ± 137***	47 ± 10**	27 ± 7	17:1	34:1
Recovery	31± 0.5	27 ± 16	28 ± 9	3:1	3:1

Table 3.4.1. Effect of NPP injection on plasma catecholamines in ganglion blocked rats before captopril (+A-C, n=8).

Plasma catecholamine levels at baseline, at peak SBP response to NPP and at recovery. Note the 15-fold increase in adrenaline level and the 3-fold increase in noradrenaline at peak response to NPP injection.

*Values are pg/mL plasma ± SEM
***$p<0.01$ vs. baseline
**$p<0.05$ vs. baseline

#Ratios are calculated from individual raw data.

Condition	*Adrenaline**	*Noradrenaline*	*Dopamine*	*Ratio§ A:NA*	*Ratio A:DA*
Baseline	118 ± 26	30 ± 7	36 ± 10	4:1	4:1
Peak Pressor	7884 ± 1196#	488 ± 128***	84 ± 21**	18:1	180:1
Recovery	270 ± 87	34 ± 7	56 ± 24	5:1	5:1

Table 3.4.2. Effect of NPP injection on plasma catecholamines in ganglion blocked rats after captopril (+A+C, n=8).

Plasma catecholamine levels at baseline, at peak SBP response to NPP and at recovery. Note the 67-fold increase in adrenaline, the 16-fold increase in noradrenaline, and the 2-fold increase in dopamine levels at peak NPP pressor response.

*Values are pg/mL plasma ± SEM

#p<0.001 vs. baseline
***p<0.01 vs. baseline
**p<0.05 vs. baseline

§ Ratios are calculated from individual raw data

Condition	*Adrenaline*	*Noradrenaline*	*Dopamine*	*Ratio A:NA*	*Ratio A:DA*
Baseline Values	$p<0.05$	n.s.	n.s.	n.s	n.s
Peak Pressor Responses	$p<0.001$	$p<0.01$	$p<0.05$	$p<0.001$	$p<0.001$
Baseline Values	$p<0.05$	n.s.	n.s.	n.s.	n.s.

Table 3.4.3. Statistical comparisons of the NPP effect on plasma catecholamines in +A-C vs. +A+C groups.

In +A+C rats, baseline plasma adrenaline levels were significantly elevated compared to the +A-C group. Captopril treatment also potentiated the NPP effect on release of adrenal medullary catecholamines, as indicated by circulating plasma levels.

3.5 EFFECT OF GANGLION BLOCKADE ON THE NPP PRESSOR RESPONSE

Effects of NPP in non-ganglion blocked rats before captopril (-A-C) and after (-A+C) are shown in Fig. 3.5.1a and 3.5.1b. Baseline blood pressure in these rats usually stabilized around 120/80 mmHg (SBP/DBP). The NPP response was markedly different compared to rats that were ganglion blocked (cf. Fig. 3.2.1 & Fig. 3.5.1). In the absence of either drug (ansolysen or captopril), NPP injection led to a biphasic response that was initially depressor and was followed by a secondary pressor phase. There was no statistical difference in the blood pressure response of NPP in -A+C rats vs. –A-C rats.

Baseline SBP, DBP and HR in unblocked rats before (n=9) and after (n=6) captopril are shown in Fig. 3.5.2. There was no difference in the two groups. However, when compared to ganglion blocked rats, all parameters were consistently higher ($p<0.01$).

In unblocked rats before captopril, NPP caused an initial drop in both SBP and DBP ($p<0.05$ vs. baseline, Fig. 3.5.3, first two bars, left panel). The depressor response was accompanied by a very small compensatory increase in HR (Fig. 3.5.3, first bar, right panel). After the depressor response, blood pressure rebounded and increased slightly from baseline (Fig 3.5.3, last two bars, left panel). The rebound was accompanied by a small decrease in HR (Fig. 3.5.3, last bar, right panel) that was not statistically different from the change observed after the depressor response.

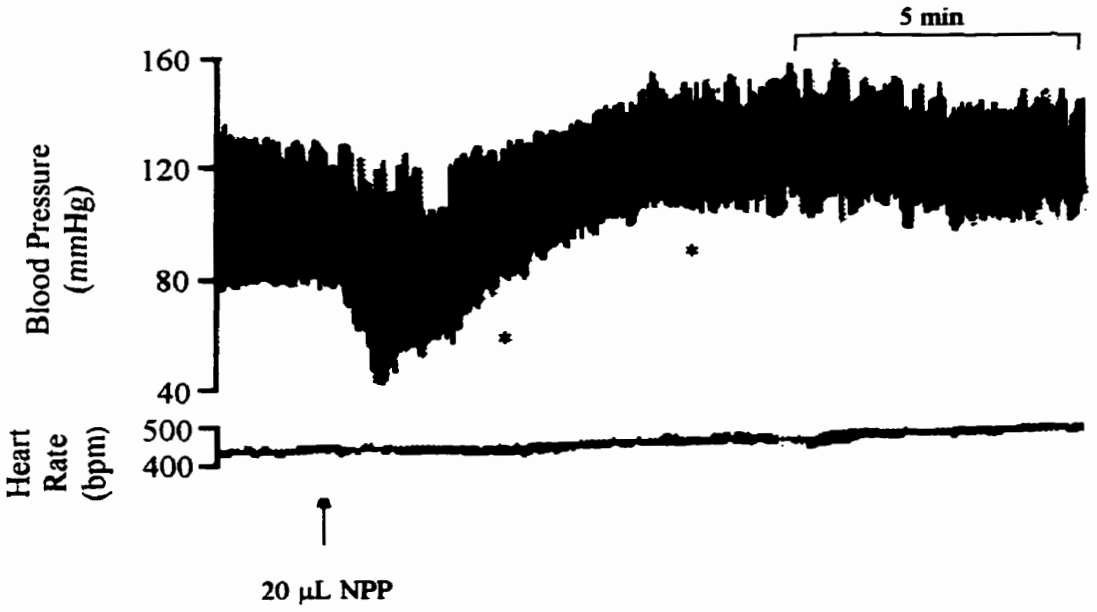


Figure 3.5.1a. NPP response in unblocked rats before captopril (-A-C).
Representative blood pressure response to NPP injection. *Indicates points where blood was taken for catecholamine analysis. Samples were also taken 15 min before NPP (baseline control) and 15-20 min after the peak response (recovery control).

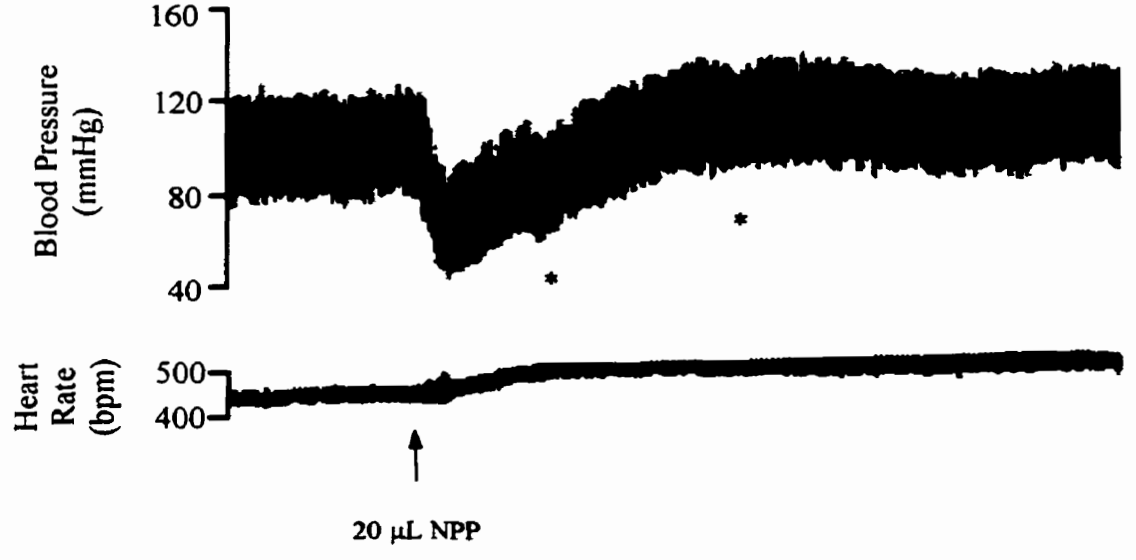


Figure 3.5.1b. NPP response in unblocked rats after captopril (-A+C).
Representative blood pressure response to NPP injection. *Indicates points where blood was taken for catecholamine analysis. Samples were also taken 15 min before NPP (baseline control) and 15-20 min after peak (recovery control).

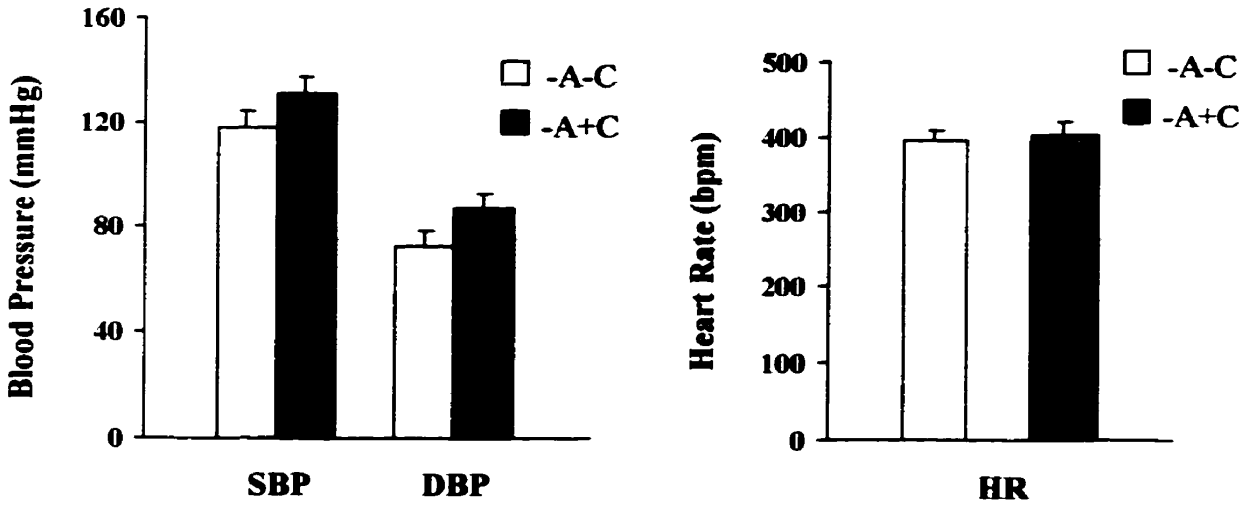


Figure 3.5.2. Baseline values in -A-C (n=9) and –A+C (n=6) rats: SBP, DBP and HR.

Left Panel: Baseline SBP and DBP were similar in the two groups. Blood pressure typically stabilized around 120/80 mmHg.

Right Panel: There was no difference in baseline HR in the two groups of rats.

Data presented in Fig.3.5.2 (means ± SEM)
Left panel: From left to right, 118±7; 131±6; 72±5; 87±6 mmHg.
Right panel: From left to right, 394±14; 402±17 bpm.

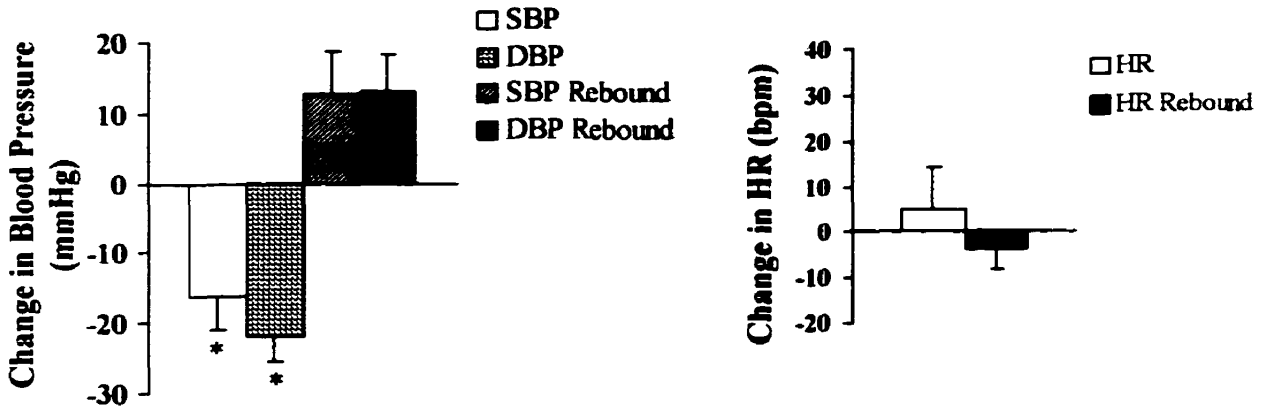


Figure 3.5.3. Effect of NPP in –A-C rats (n=9): Changes in SBP, DBP and HR.

Left Panel: In –A-C rats, NPP caused an initial drop in blood pressure ($p<0.05$, both SBP and DBP), which was followed by a rebound in pressure that slightly exceeded baseline (n.s.).

Right Panel: During the depressor response HR increased slightly from baseline, and when pressure rebounded, HR decreased slightly from baseline.

Data represented in Fig.3.5.3 (means ± SEM)
Left panel: From left to right, -16±5; -22±3; 13±6; 13±5 mmHg.
Right Panel: From left to right, 5±9; -4±4 bpm.

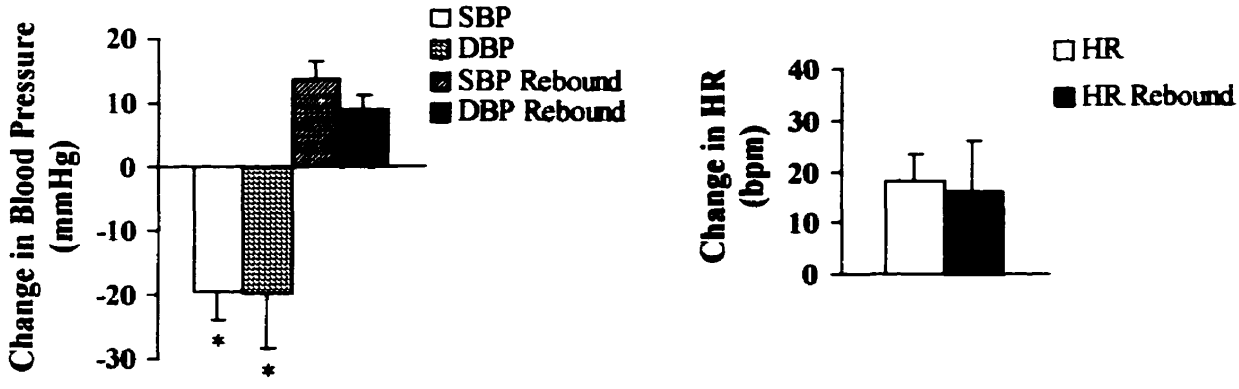


Figure 3.5.4. Effect of NPP in –A+C rats (n=6): Changes in SBP, DBP and HR.

Left Panel: In -A+C rats, NPP caused a depressor response similar to the –A-C group (Fig. 3.5.3). There was no difference in the magnitude of blood pressure change between the two groups of rats. *$p<0.05$ vs. baseline

Right Panel: During the biphasic response, HR increased from baseline. There was no difference in HR responses between –A-C and –A+C rats.

Data presented in Fig.3.5.4 (means ± SEM)
Left panel: From left to right, -19±4; -20±9; 14±3; 9±2 mmHg.
Right panel: From left to right, 18±5; 16±10 bpm.

In unblocked rats after captopril, NPP caused a similar reduction in blood pressure ($p<0.05$, SBP and DBP) compared to before captopril (Fig. 3.5.4, first two bars, left panel). After the depressor response, blood pressure rebounded slightly above baseline (Fig. 3.5.4, last two bars, left panel) and was accompanied by an increase in HR (Fig. 3.5.4, last bar, right panel). There was no difference between the magnitude of pressure reduction (both SBP and DBP) or HR in the two groups.

3.6 EFFECT OF GANGLION BLOCKADE ON CATECHOLAMINE RESPONSES TO NPP

Plasma catecholamines were measured in unblocked rats before and after captopril (Tables 3.6.1 & 3.6.2, respectively). Four blood samples for analysis were obtained (Fig. 2.7.2, Methods): 10-15 min before NPP (baseline), immediately after the depressor response, at the secondary peak pressor response and 15-20 min after the peak response (recovery).

In unblocked rats before captopril ($n=9$, Table 3.6.1), the ratios of A:NA and A:DA at baseline were approximately 1:2 and 3:1, respectively. Baseline levels of NA (121 ± 35 pg/mL) were significantly higher ($p<0.05$) than those observed in the +A-C group (14 ± 7 pg/mL, Table 3.4.1) and the +A+C group (30 ± 7 pg/ml, Table 3.4.2). Immediately after the depressor response, levels of A increased significantly from baseline ($p<0.05$), while NA and DA levels did not change. The A:NA and A:DA ratios increased to approximately 7:1 and 70:1, respectively. At the secondary pressor response, levels of A were slightly higher than baseline (n.s.) and there was no change in NA or DA. All catecholamines returned to near baseline levels with A:NA and A:DA ratios of about 1:2 and 10:1, respectively.

In unblocked rats after captopril (n=6, Table 3.6.2), the ratio of A:NA and A:DA at baseline were approximately 1:3 and 2:1, respectively. NA levels (307 ± 124 pg/mL) were also significantly higher ($p<0.05$) than those observed in the +A-C group (14 ± 7 pg/mL, Table 3.4.1) and the +A+C group (30 ± 7 pg/mL, Table 3.4.2). Immediately after the depressor response, levels of A increased significantly from baseline ($p<0.001$) and were also much higher compared to the –A-C group ($p<0.01$, Table 3.6.3). The A:NA and A:DA ratios increased to approximately 14:1 and 140:1, respectively, while levels of NA and DA did not change. At the secondary pressor phase, levels of A exceeded baseline ($p<0.01$); A levels were significantly higher compared to –A-C rats (for statistical comparison see Table 3.6.3) and there was no change in NA or DA levels. All catecholamine levels returned to near baseline with A:NA and A:DA ratios of about 1:2 and 10:1 respectively.

Condition	*Adrenaline**	*Noradrenaline*	*Dopamine*	*Ratio# A:NA*	*Ratio A:DA*
Baseline	59 ± 9	121 ± 35	17 ± 2	1:2	3:1
Depressor	1359 ± 6**	196 ± 57	19 ± 4	7:1	70:1
Peak Pressor	141 ± 28	155 ± 42	14 ± 1	1:1	10:1
Recovery	107 ± 25	203 ± 25	12 ± 2	1:2	10:1

Table 3.6.1: Effects of NPP injection on plasma catecholamines in unblocked rats before Captopril (-A-C, n=9).

Note the increase in noradrenaline at baseline compared to either +A-C or +A+C rats (see Tables 3.4.1 and 3.4.2). Also note the 23-fold increase in A levels immediately following the initial depressor response.

*Values are pg/mL plasma ± SEM
**$p<0.05$ vs. baseline

Ratios are calculated from individual raw data

Condition	*Adrenaline•*	*Noradrenaline*	*Dopamine*	*Ratio# A:NA*	*Ratio A:DA*
Baseline	112 ± 35	307 ± 124	47 ± 27	1:3	2:1
Depressor	4158 ± 424***	312 ± 25	23 ± 3	14:1	140:1
Peak Pressor	431 ± 65**	206 ± 26	14 ± 2	2:1	20:1
Recovery	92 ± 23	204 ± 56	13 ± 3	1:2	10:1

Table 3.6.2: Effects of NPP injection on plasma catecholamines in unblocked rats after Captopril (-A+C, n=6)

Note the increase in noradrenaline at baseline compared to +A-C or +A+C rats (see Tables 3.4.1 and 3.4.2). Also note the 37-fold increase in levels of adrenaline immediately following the initial depressor response.

Values are pg/mL plasma ± SEM
***p<0.001 vs. baseline
**p<0.01 vs. baseline

#Ratios are calculated from individual raw data

Condition	*Adrenaline*	*Noradrenaline*	*Dopamine*	*Ratio A:NA*	*Ratio A:DA*
Baseline Values	n.s.	n.s.	n.s.	n.s.	n.s.
Depressor Responses	$p<0.01$	n.s.	n.s.	$p<0.01$	$p<0.01$
Peak Pressor Responses	$p<0.01$	n.s.	n.s.	$p<0.05$	$p<0.05$
Recovery Values	n.s.	n.s.	n.s.	n.s.	n.s.

Table 3.6.3. Statistical comparisons of the NPP effect on plasma catecholamines in –A-C vs. –A+C rats.

Captopril had no significant effect on baseline catecholamines, despite a suggestively higher level of noradrenaline in –A+C rats compared to –A-C rats. Captopril also appears to have potentiated the effect of NPP on plasma catecholamines released immediately following the depressor response.

3.7 EFFECT OF SODIUM NITROPRUSSIDE (SNP) ON PRESSURE AND CATECHOLAMINE RESPONSES

A dose response curve (Fig. 3.7.1) was established in order to determine the optimum dose of sodium nitroprusside (SNP) to administer to unblocked bioassay rats before and after captopril. The goal was to mimic a similar reduction in blood pressure seen previously with NPP under the same conditions in order to compare and contrast the effect of a direct vasodilator (i.e. SNP) to our observed NPP effect. This comparison was done with respect to SBP, DBP and HR changes as well as to changes in plasma catecholamine levels. SNP produced a rapid drop in blood pressure (both SBP and DBP) with a concomitant increase in HR. A dose of 5 μg/kg was chosen because it produced a reduction of SBP of about 20-25 mmHg, similar to that observed with NPP. The blood pressure response to SNP was of shorter duration (2-3 min) compared to the NPP response (5-7 min).

Representative blood pressure responses to SNP in unblocked rats before and after captopril (n=6, both groups) are shown in Fig. 3.7.2a and 3.7.2b, respectively. SNP produced rapid vasodilatation indicated by the fall in both SBP and DBP. In comparison, HR increased slightly. There were no statistical differences in the BP or HR responses to SNP before and after captopril.

Baseline SBP, DBP and HR in the two experimental groups are shown in Fig. 3.7.3. There were no statistical differences in any of the measures in the two groups, nor was there any difference with other rats treated under the same conditions described earlier (cf. Fig. 3.5.2)

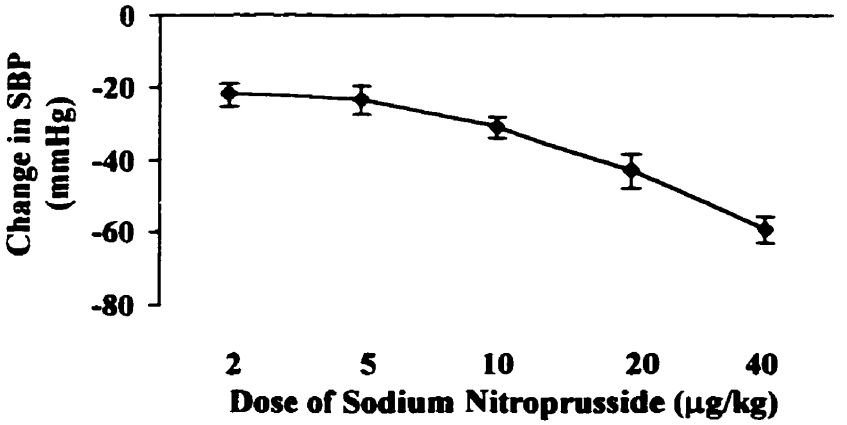


Figure 3.7.1. SNP dose response curve in –A-C rats (n=5).

A dose of 5 μg/kg SNP lowered SBP on average by about 20-25 mmHg. This dose was chosen because it produced a similar magnitude drop in pressure in –A-C rats as compared to the drop observed with NPP. The overall response to SNP was shorter (2-3 min) compared to NPP (5-7 min).

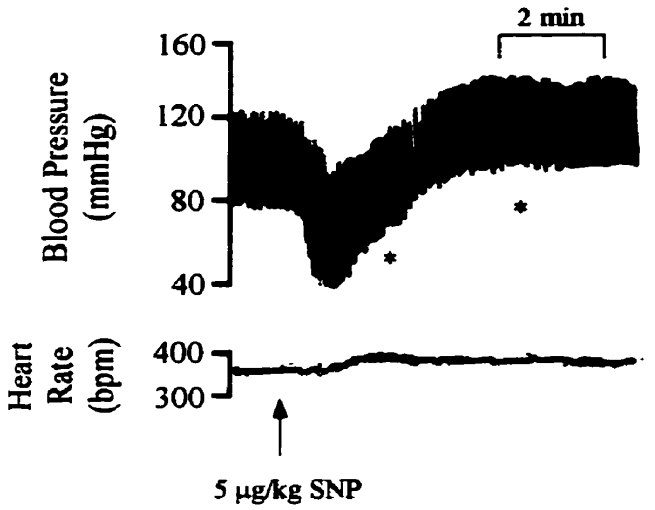


Figure 3.7.2a. SNP response in unblocked rats before captopril (-A-C).
Representative blood pressure response to SNP (5 µg/kg). *Indicates points when blood was taken for catecholamine analysis. Blood was also taken 15 min before SNP (baseline control) and 15 min after the peak response, i.e. rebound to baseline (recovery control).

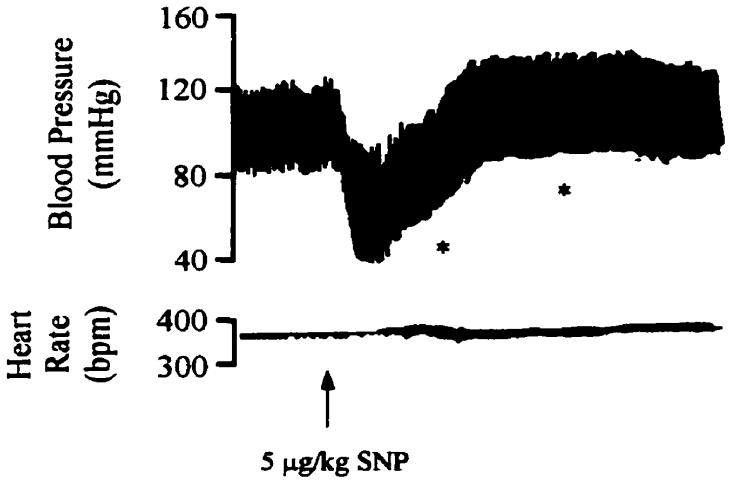


Figure 3.7.2b. SNP response in unblocked rats after captopril (-A+C).
Representative blood pressure response to SNP (5 µg/kg). *Indicates points when blood was taken for catecholamine analysis. Blood was also taken 15 min before SNP (baseline control) and 15 min after the peak response (recovery control).

In unblocked rats before captopril (n=6), SNP caused an initial depressor response, with similar drops in both SBP and DBP (Fig. 3.7.4, first two bars, left panel) and a slight increase in HR (Fig. 3.7.4, first bar, right panel). Immediately after the depressor response, there was a secondary pressor phase similar to that observed with NPP (Fig. 3.7.4, last two bars, left panel) with a small increase in HR (Fig. 3.7.4, last bar, right panel). The changes in HR during the biphasic response were not statistically different from each other. After captopril (n=6, Fig. 3.7.5), SNP also produced a reduction in SBP and DBP, with an increase in HR that was not statistically different compared to unblocked rats before captopril. During the secondary pressor response, SBP, DBP and HR all increased above baseline, but these changes were not statistically different compared to unblocked rats before captopril, nor were they statistically different compared to NPP (cf. Fig. 3.7.5 & 3.5.4).

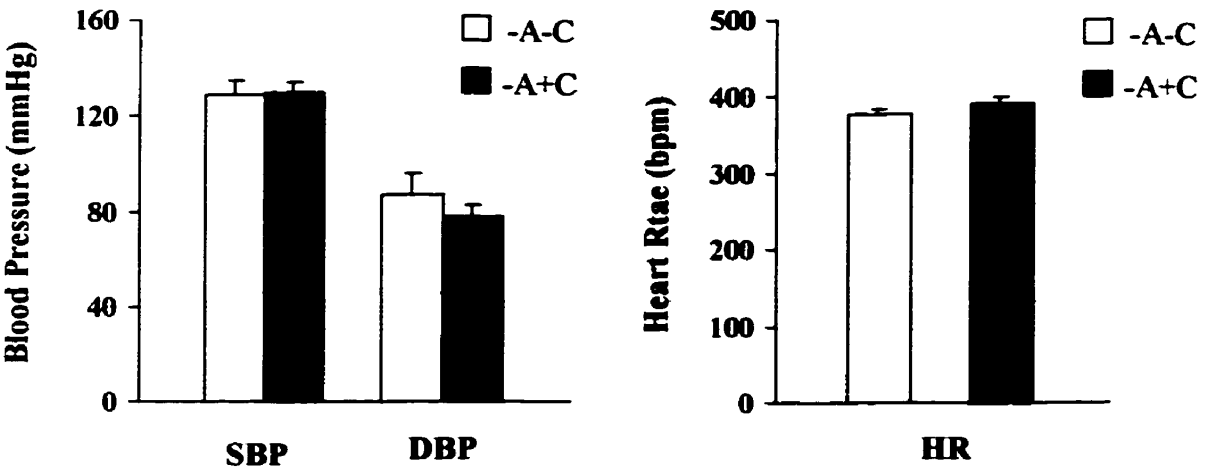


Figure 3.7.3. SNP Experiments: Baseline values in -A-C and –A+C rats (n=6) - SBP, DBP and HR.

Left Panel: Baseline SBP and DBP were similar in the two groups, nor were they different from other rats treated similarly (cf. Fig. 3.5.2, left panel).

Right Panel: There was no difference in baseline HR in the two groups, nor was there any difference from other rats treated similarly (cf. Fig 3.5.2, right panel).

Data presented in Fig. 3.7.3 (means ± SEM)
Left panel: From left to right, 123±6; 123.0±5; 87±9; 77±6 mmHg.
Right panel: From left to right, 377±8; 391±9 bpm.

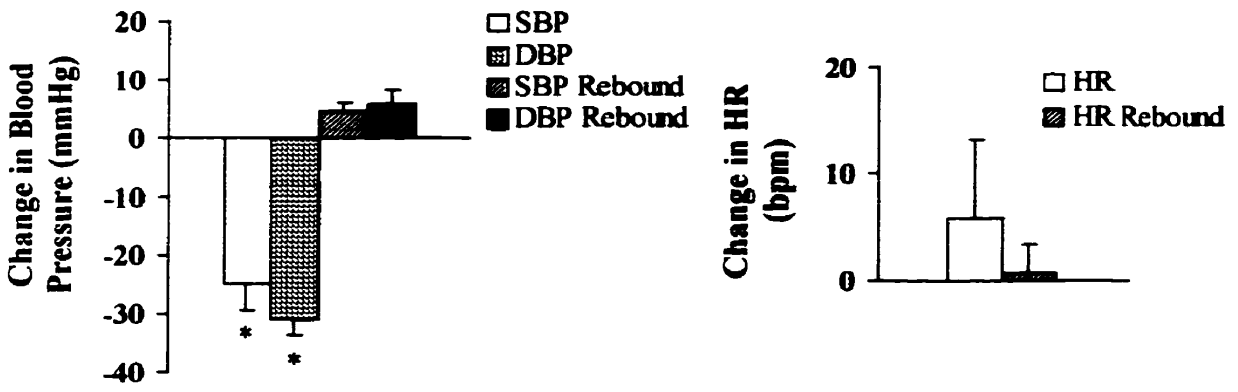


Figure 3.7.4: Effect of SNP (5 μg/kg) in –A-C rats (n=6). Changes in SBP, DBP and HR.

Left Panel: In –A-C rats, SNP caused a biphasic response similar to NPP (Fig. 3.5.3). There was no difference in the magnitude of the drop in pressure between –A-C rats treated with SNP and those treated with NPP (cf. Fig. 3.5.3).
*$p<0.05$ vs. baseline

Right Panel: During the initial depressor phase, HR increased to compensate. There was no difference in the magnitude of the changes in HR between these rats and those treated with NPP (cf. Fig. 3.5.3).

Data presented in Fig. 3.7.4 (means ± SEM)
Left panel: From left to right, -25±5; -31±3; 4±1; 6±2 mmHg.
Right panel: From left to right, 6±7; 0.7±3 bpm.

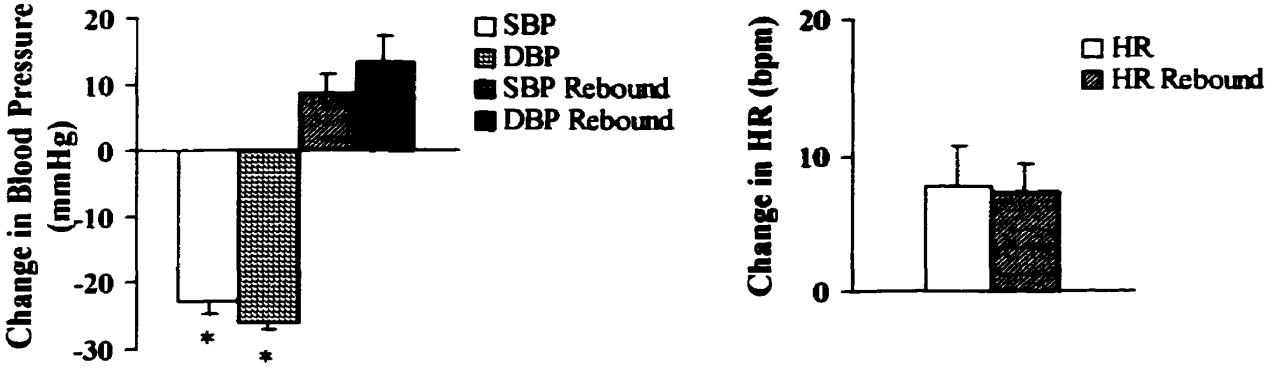


Figure 3.7.5: Effect of SNP (5 μg/kg) in –A+C rats (n=6). Changes in SBP, DBP and HR.

Left Panel: In –A+C rats, SNP caused a biphasic response similar to that seen in –A-C rats (Fig. 3.7.4). There was no difference in the magnitude of the drop in pressure with these rats and the –A-C group, nor was there any difference with the rats that were given NPP (cf. Fig. 3.5.3). *$p<0.05$ vs. baseline

Right Panel: HR increased during the biphasic response. There was no statistical difference in the magnitude of the change in HR in the two groups of rats treated with SNP or NPP (cf. Fig. 3.5.3).

Data presented in Fig.3.7.5 (means ± SEM)
Left panel: From left to right, -23±2; -26±1; 9±3; 13±4 mmHg.
Right panel: From left to right, 8±3; 7.3±2 bpm

3.8 EFFECT OF SNP ON PLASMA CATECHOLAMINE RESPONSES

Plasma catecholamines were measured in unblocked rats before and after captopril (Tables 3.8.1 & 3.8.2, respectively). As discussed in Methods, four blood samples for analysis were obtained: 10-15 min before SNP (baseline), immediately after the depressor response, at the peak pressor phase and 15-20 min after peak (recovery).

In unblocked rats before captopril (n=6, Table 3.8.1), the ratios of A:NA and A:DA at baseline were approximately 1:3 and 5:1, respectively. Baseline levels of all catecholamines measured were similar to those observed for other rats treated similarly (cf. Table 3.8.1 & 3.6.1). Generally, there were no significant changes in any of the catecholamines measured during the SNP response. When compared to the effect of NPP on plasma catecholamines, significant differences appear in the levels of A. Immediately after the depressor response to NPP the effect of medullary release of A was significantly higher ($p<0.05$, Table 3.8.3) compared to that of SNP at the same time point (1359 ± 6 pg/mL vs. 52 ± 20 pg/mL, respectively). Similar trends were observed at the peak pressor response to SNP; A levels were also much lower ($p<0.05$) compared to those seen after NPP in –**A-C** rats (Table 3.8.3) and the ratios of A:NA and A:DA were approximately 1:3 and 3:1, respectively. Although NA levels tended to increase after SNP, the changes were not significant in this group of rats. Catecholamine levels returned to baseline after SNP and the ratios of A:NA and A:DA were approximately 1:5 and 3:1, respectively.

In unblocked rats after captopril (n=6, Table 3.8.2), the ratios of A:NA and A:DA at baseline were approximately 1:2 and 10:1, respectively. Baseline levels of all catecholamines were similar to those observed for other rats treated similarly (cf. Table 3.8.2 & 3.6.2), however when

compared to the unblocked rats before captopril (Table 3.8.1), all catecholamines were slightly elevated, although not statistically significant. As observed with –A-C rats, SNP in –A+C rats did not significantly alter plasma catecholamines levels. When compared to the effect of NPP on plasma catecholamines, significant differences appear in the levels of A. Immediately after the depressor response to NPP the effect on medullary release of A was significantly higher ($p<0.0001$, Table 3.8.3) compared to that of SNP at the same time point (4158 ± 424 pg/mL vs. 161 ± 55 pg/mL, respectively). Similar trends were observed at the peak pressor response to SNP; A levels were also much lower ($p<0.0001$) compared to those seen after NPP in –A+C rats (Table 3.8.3) and the ratios of A:NA and A:DA were approximately 1:2 and 10:1, respectively. Although NA levels tended to increase after SNP, the changes were not statistically significant. At recovery, all catecholamine levels returned to near baseline values and the ratios of A:NA and A:DA remained at approximately 1:2 and 10:1, respectively.

Condition	*Adrenaline**	*Noradrenaline*	*Dopamine*	*Ratio** A:NA*	*Ratio A:DA*
Baseline	46 ± 10	160 ± 45	14 ± 4	1:3	5:1
Depressor	52 ± 20	131 ± 30	11± 2	1:2	5:1
Peak Pressor	58 ± 21	161 ± 40	16 ± 2	1:3	3:1
Recovery	35 ± 6	150 ± 19	9 ± 1	1:5	3:1

Table 3.8.1. Effect of SNP on plasma catecholamines in unblocked rats before captopril (–A-C, n=6).

There were no significant changes in any of the catecholamines measured during the SNP response in this group of rats.

* Values are pg/mL plasma ± SEM
** Ratios are calculated from individual raw data

Condition	*Adrenaline**	*Noradrenaline*	*Dopamine*	*Ratio** A:NA*	*Ratio A:DA*
Baseline	110 ± 49	190 ± 31	19 ± 6	1:2	10:1
Depressor	161 ± 55	268 ± 51	30 ± 12	1:2	10:1
Peak Pressor	138 ± 52	256 ± 36	24 ± 5	1:2	10:1
Recovery	152 ± 66	258 ± 59	19 ± 5	1:2	10:1

Table 3.8.2. Effect of SNP on plasma catecholamines in ganglion blocked rats after captopril (–A+C, n=6).

There were no significant changes in any of the catecholamines measured during the SNP response in this group of rats.

* Values are pg/mL plasma ± SEM
** Ratios are calculated from individual raw data

Condition	*Adrenaline*	*Noradrenaline*	*Dopamine*	*Ratio A:NA*	*Ratio A:DA*
Baseline	n.s.	n.s.	n.s.	n.s.	n.s.
Values	n.s.	n.s.	n.s.	n.s.	n.s.
Depressor	$p<0.05$*	n.s.	n.s.	$p<0.05$*	$p<0.05$*
Responses	$p<0.0001$**	n.s.	n.s.	$p<0.001$**	$p<0.001$**
Peak Pressor	$p<0.05$*	n.s.	n.s.	n.s.	n.s.
Responses	$p<0.0001$**	n.s.	n.s.	$p<0.05$**	$p<0.05$**
Recovery	$p<0.05$*	n.s.	n.s.	n.s.	n.s.
Values	n.s.	n.s.	n.s.	n.s.	n.s.

Table 3.8.3. Statistical comparisons of NPP vs. SNP effects on plasma catecholamines in –A-C rats (*) and –A+C rats (**).

In unblocked bioassay rats before or after captopril, SNP had no significant effect on plasma catecholamines (Table 3.8.1 & 3.8.2). NPP, however, significantly increased plasma adrenaline levels when compared to SNP. Thus, NPP appears to have a specific effect on adrenal medullary *adrenaline* release.

3.9 EFFECT OF ANGIOTENSIN AT_1 RECEPTOR BLOCKADE ON CARDIOVASCULAR FUNCTIONS OF NPP

Representative blood pressure responses to NPP before and after losartan are shown in Fig. 3.9.1a and 3.9.1b, and to NPP after losartan + captopril are shown in Fig. 3.9.1c. These rats were also treated with the ganglion blocking agent ansolysen, and as was the case with other ganglion blocked rats, baseline blood pressure typically stabilized around 80/40 mmHg (SBP/DBP, not shown).

In ganglion blocked rats before losartan or captopril (n=6, Fig. 3.9.2), NPP caused a typical small increase in SBP (cf. +A-C rats, Fig. 3.2.3), very little change in DBP and a moderate increase in HR. There was no statistical difference between these NPP responses and those obtained in other +A-C rats. Losartan alone did not potentiate the pressor response to NPP (Fig.3.9.2). However, losartan combined with captopril resulted in a major potentiation of the NPP pressor effect (Fig. 3.9.2). The increases in SBP, DBP and HR were significantly higher than those observed before and after losartan treatment alone ($p<0.001$, $p<0.01$, and $p<0.0001$, SBP, DBP and HR respectively). The NPP effect on SBP and DBP in losartan- and captopril-treated rats was significantly higher ($p<0.05$ for both) compared to captopril alone (Fig. 3.2.3).

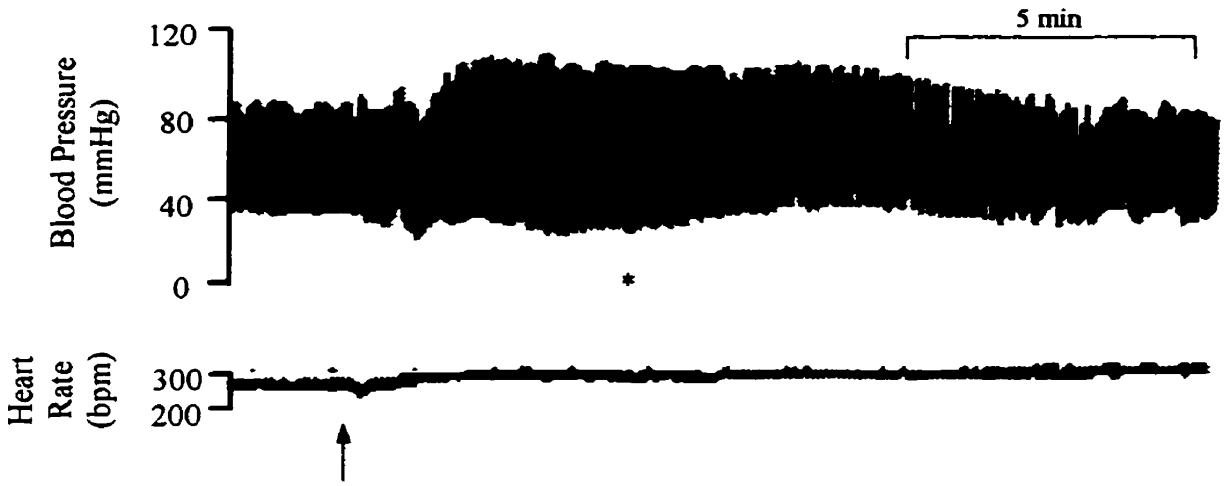


Figure 3.9.1a. NPP response in ganglion blocked rats before losartan or captopril. Representative blood pressure response to NPP. *Indicates where blood was taken for catecholamine analysis. Blood was also taken 15 min before NPP (baseline control).

Figure 3.9.1b. NPP response in ganglion blocked rats after losartan. Representative blood pressure response to NPP. *Indicates where blood was taken for catecholamine analysis. Blood was also taken 15 min before NPP (baseline control).

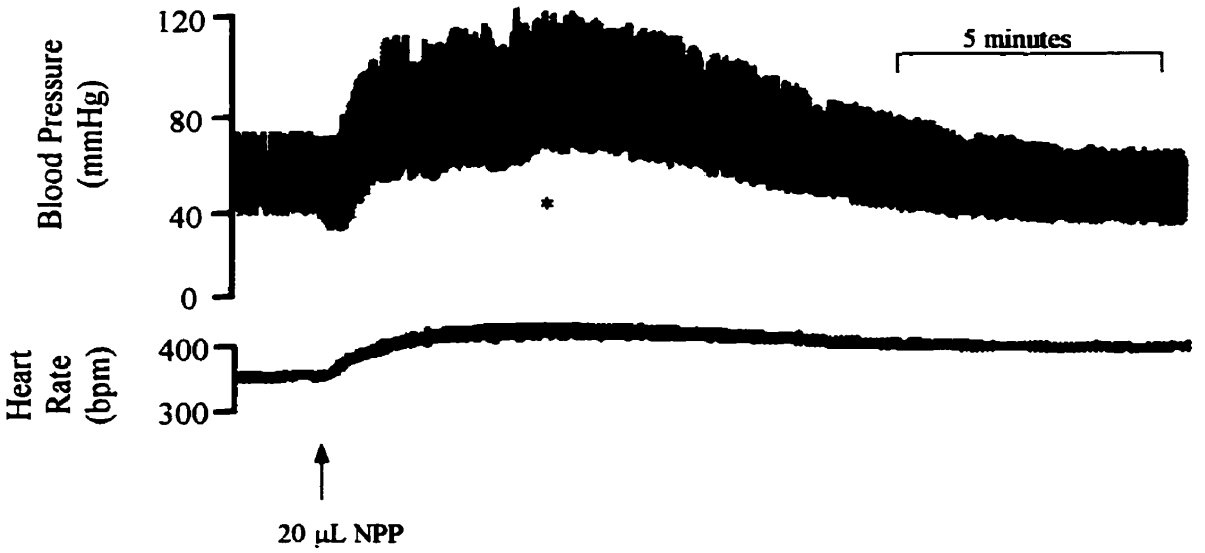


Figure 3.9.1c. NPP response in ganglion blocked rats after loartan and captopril. Representative blood pressure response to NPP. *Indicates where blood was taken for catecholamine analysis. Blood was also taken 15 min before NPP (baseline control).

Note the substantial increase in SBP and DBP as well as HR.

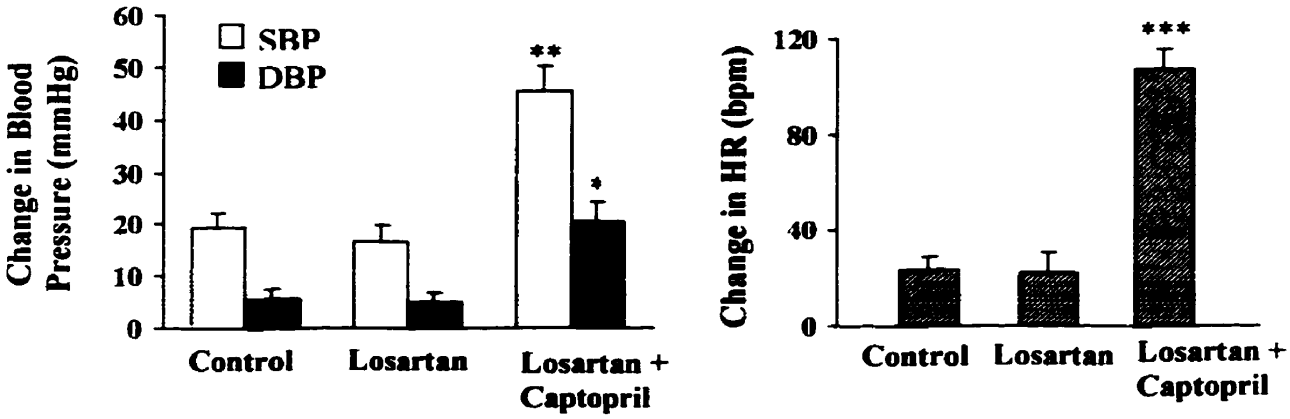


Figure 3.9.2. Effect of NPP in losartan- and captopril-treated rats (n=6). Changes in SBP, DBP and HR.

Left Panel: Losartan treatment had no effect on the NPP pressor response. In combination with captopril however, there was significant potentiation of SBP (**$p<0.001$) and DBP (*$p<0.01$). The potentiation of the SBP and DBP response was also significantly greater than what was observed in rats treated with captopril alone ($p<0.05$, cf. Fig. 3.2.3).

Right Panel: Losartan treatment also had no measurable effect on the HR response to NPP however, in combination with captopril, there was significant potentiation (***$p<0.0001$).

Data presented in Fig. 3.9.2 (means ±SEM)
Top panel: From left to right, 20±3; 5±2; 17±3; 5±2; 45±5; 20±4 mmHg.
Bottom panel: From left to right, 23±6, 22±8; 107±8 bpm.

3.10 EFFECT OF LOSARTAN ON CATECHOLAMINE RESPONSES TO HUMAN NPP

Plasma catecholamines were measured at peak pressor response to NPP in ganglion blocked rats before and after losartan, as well as after combined treatment with losartan and captopril (Table 3.10.1). Blood samples were taken at baseline, at peak SBP response to NPP *before* losartan, at peak SBP response to NPP *after* losartan and at peak SBP response to NPP *after* losartan + captopril.

The baseline ratios of A:NA and A:DA before treatment with either drug were approximately 3:1. At the peak SBP response to NPP (before losartan or captopril), all catecholamine levels increased significantly ($p<0.05$ for A, NA and DA). The ratios of A:NA and A:DA also rose significantly to about 15:1 and 30:1, respectively. NPP after losartan resulted in catecholamine levels that were significantly higher than baseline ($p<0.01$ for A; $p<0.05$ for NA and DA), but not different than before losartan. Similarly, the ratios of A:NA and A:DA were about 16:1 and 40:1. Thus, losartan treatment had no significant effect on the levels of catecholamines released in response to NPP.

In rats treated with both losartan and captopril, NPP caused a major potentiation of all catecholamines released ($p<0.01$ for A; $p<0.05$ for NA and DA, compared to losartan alone). The ratios of A:NA and A:DA also increased to approximately 21:1 and 105:1, respectively.. There were no statistically significant differences in the amounts of catecholamines released between losartan + captopril treated rats versus captopril-alone treated rats (cf. Table 3.4.2 & 3.10.1).

Condition	*Adrenaline**	*Noradrenaline*	*Dopamine*	*Ratio# A:NA*	*Ratio A:DA*
Baseline	77.35 ± 29.75	25 ± 9	23 ± 7	3:1	3:1
NPP Pre-Losartan	1837 ± 709***	93 ± 33***	33 ± 8***	15:1	30:1
NPP Post-Losartan	1770 ± 380**	115 ± 29***	33± 9***	16:1	40:1
NPP Post-Losartan + Captopril	7366 ± 1381***	383 ± 120***	73 ± 13***	21:1	105:1

Table 3.10.1: Losartan experiments. Plasma catecholamine levels in ganglion blocked rats (n=6) in response to NPP.

Losartan alone does not potentiate catecholamine release. Captopril on top of losartan, however, does potentiate adrenal medullary catechoalmine release. Note the 4-fold increase of adrenaline after treatment with both losartan and captopril.

*Values are pg/mL plasma ± SEM

***$p<0.05$ vs. baseline
**$p<0.01$ vs. baseline

Ratios are calculated from individual raw data

4. DISCUSSION

4.1 PREAMBLE

This thesis investigates the role of the adrenal medulla and catecholamine release in mediating the pressor and cardiotonic effects of an active fragment of coagulation factor XII, 'new pressor protein', or NPP. As indicated in the Introduction and Results sections, NPP appears to be strongly cardiotonic and it also appears to express its pressor potency via adrenergic receptor activation. Thus, the specific objective of this study was to implicate adrenal medullary catecholamines as the critical mediators of the pressor effect. Therefore, this discussion will review and integrate the data presented and attempt to further interpret the mechanism(s) of action of NPP.

4.2 REPRODUCIBILITY OF BLOOD PRESSURE RESPONSES

Bioassay procedures were used in the present studies because rats respond as well to human plasma NPP as they do to rat plasma NPP (Mavrogiannis, 1998; Osmond *et al.* 1998). At the present moment, the only method we have for detecting and quantitating NPP is by rat bioassay. The adequacy of our bioassay rat preparation is illustrated by the reproducibility of systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) responses to successive injections of angiotensin II (Ang II) (Fig. 3.1.1). Similar reproducibility has been observed throughout our studies with NPP (Mavrogiannis, 1998; Osmond *et al.* 1998; Mavrogiannis *et al.* 1997; Osmond *et al.* 1997a, 1997b, 1997c; Cotter, 1995;Osmond and Cotter, 1992, 1993, 1996) and is reflected in the acceptable standard errors presented here, especially in ganglion blocked (+Ansolysen) and captopril treated (+C) rats (Fig. 3.2.3).

The data show that DBP increments after Ang II (80 ng/kg i.v.) are very consistently greater than the increments of SBP (Fig. 3.1.1). This confirms an earlier report (Osmond *et al.* 1998) and further suggests that under our standard bioassay procedure of ganglion blockade with ansolysen in the presence of captopril, Ang II seems to exert a particularly strong action on peripheral resistance relative to any effect on the heart. After Ang II, both the relatively small increase in SBP compared to DBP and the minimal stimulation of HR contrast sharply with the responses to NPP (cf. Fig. 3.3.1 & Fig. 3.2.3).

4.3 ADRENAL MEDULLECTOMY: EFFECT ON THE NPP PRESSOR RESPONSE

Adrenal medullary gland involvement in the NPP effect was first suspected because the increase in SBP was always greater than the increase in DBP and was always accompanied by an increase in HR (Osmond and Cotter, 1992). Such effects argued strongly for the involvement of catechoiamines secreted from the adrenal medulla. Indeed, within 10 min of acute bilateral adrenalectomy (2AX), the pressor response to NPP was greatly reduced (>90%) (Mavrogiannis, 1998; Osmond *et al.* 1998).

We have demonstrated that acute bilateral adrenal medullectomy (2MDX, Fig. 3.3.1) replicates the effect of total 2AX, suggesting that the primary site mediating the NPP pressor effect is the medulla, not the cortex. Although it is well known that the adrenal cortex is involved in regulating blood pressure by the cardiovascular effects of glucocorticoids (Baxter, 1976; Bondy, 1974; Liddle, 1974) and by fluid and electrolyte regulation via the actions of mineralocorticoids (Leaf and Liddle, 1975), there are reasons for excluding the cortex as having a major role in the NPP effect. The half-life of these corticosteroids is approximately 20-60 min

(mineralocorticoids vs. glucocorticoids, Ballard, 1979) and it is unlikely that removal of the adrenal cortex would result in the immediate effect we observe on the cardiovascular system. On the other hand, the half-lives of adrenal medullary catecholamines in the circulation are approximately 1-2 min (Best and Halter, 1982) so any effects of medullary ablation would probably dissipate quickly.

The results of the 2MDX experiment (Fig. 3.3.1) confirmed hypothesis #1 (pg. 23) that secretions from the medullae are necessary to manifest the NPP pressor effect. The SBP component of NPP was reduced >90% after 2MDX, and the HR response was reduced >70%. This confirmation of adrenal medullary involvement in the NPP effect prompted us to measure plasma catecholamines in order to quantify the pressure data.

4.4 BASELINE PRESSURES AND PLASMA CATECHOLAMINES

Blood pressure and plasma catecholamine levels were measured after the standard drug treatments were given to bioassay rats. Although we were primarily interested in the levels of catecholamines at the peak pressor response to NPP, it soon became apparent that ganglion blockade (and perhaps even captopril) had a significant effect on basal levels of circulating catecholamines. This discussion will focus primarily on changes in adrenaline and noradrenaline since dopamine levels did not change significantly under baseline conditions.

4.4.1 Unblocked Rats Before and After Captopril

Under Inactin anesthesia, basal blood pressure (SBP and DBP) remains relatively stable for at least 3 hours (Buelke-Sam *et al.* 1978). Baseline pressure in unblocked rats was similar (approx.

120/80, SBP/DBP, Fig.3.5.2) whether captopril was present, or not, confirming earlier suggestions by Mavrogiannis (1998) that under Inactin anesthesia, the sympathetic nervous system assumes a greater role in maintaining blood pressure once the renin-angiotensin system is inhibited. This was partially evident in terms of suggestively higher levels of both circulating adrenaline and noradrenaline (albeit not statistically significant) in unblocked rats after renin system blockade with captopril (Table 3.6.1 vs. 3.6.2).

The levels reported here, particularly the higher noradrenaline concentrations, are within ranges reported by Buhler *et al.* (1978) for conscious Wistar Fullinsdorf (SPF) rats, but are somewhat lower than values reported by Picotti *et al.* (1982) for Wistar SPF and Wistar Kyoto normotensive rats. The ratio of A:NA reported by these authors was also similar to our observed ratios (1:2-1:3, Tables 3.6.1 & 3.6.2). The higher concentration of noradrenaline may have been the result of normal sympathetic drive resulting in increased spillover from adrenergic nerve terminals. Picotti *et al.* (1982) reported that normotensive rats of different strains and origins exhibited different resting plasma catecholamine levels, despite similar blood pressure values. Apparently, there is appreciable variability in catecholamine levels among rat populations.

4.4.2 Ganglion Blocked Rats Before and After Captopril

The use of ganglion blocking agents in our rat model creates a "sensitive" method for the detection of Ang II responses in a biological system (Page and Taylor, 1947; Pickens *et al.* 1965). Confirmation of ganglionic blockade was seen when the animal's blood pressure dropped and stabilized around 80/40 mmHg (Fig. 3.2.2). Ganglion blocking agents usually cause decreased autonomic responses and inhibition of catecholamine release (Picotti *et al.*

1979), a possible explanation for the rather low levels of circulating catecholamines in these rats (Table 3.4.1 cf. unblocked rats Table 3.6.1). Such a decrease in basal secretion might theoretically up-regulate peripheral adrenergic receptors, thereby attributing to the increased sensitivity of the animal.

Assuming that Inactin anesthesia causes the sympathetic system to assume greater control in maintaining blood pressure, it was no surprise that renin system blockade with captopril did not depress blood pressure any further compared to ganglion blockade alone (Fig. 3.2.2). The elevated baseline HR seen in ganglion blocked rats after captopril ($p<0.05$, Fig. 3.2.2) might be explained by the significant increase in adrenaline concentration compared to those rats with an intact renin system ($p<0.05$, Tables 3.4.1 & 3.4.2). The catecholamine ratios in ganglion blocked rats after captopril shifted in favor of adrenaline (3:1 to 4:1), which might reflect an attempt by the animal to maintain blood pressure, particularly after both sympathetic and renin system blockade (Mavrogiannis, 1998).

4.5 PEAK BLOOD PRESSURE AND CATECHOLAMINE RESPONSES TO NPP

Blood pressure and catecholamine levels were measured at the peak pressor response to NPP, which usually occurred about 2-3 min after injection in ganglion blocked rats. Due to the rather surprising effect in unblocked rats, catecholamine levels were measured immediately after the depressor response (1-2 min after injection) and at the peak response (4-5 min after injection). Each group will be discussed separately at first, followed by a brief synthesis.

4.5.1 Captopril Potentiation in Ganglion Blocked Rats

In ganglion blocked rats before captopril, NPP initiated a rise in SBP and HR (Fig. 3.2.3) that was accompanied by a rather significant increase in circulating adrenaline (15-fold) and noradrenaline (3-fold, $p<0.01$ and $p<0.05$ vs. baseline, respectively, Table 3.4.1). These results confirmed hypothesis #2 (pg. 23) that plasma catecholamines increase after NPP. They also support the earlier suggestion by Mavrogiannis (1998) that high concentrations of adrenaline and noradrenaline might stimulate α- and β-adrenergic receptors, thereby accounting for the hypothesized specific action on the heart (i.e. increased SBP, CO and SV), rather than in the periphery (i.e. DBP and TPR). Of particular interest was the significant increase in the ratio of circulating catecholamines. NPP appeared to stimulate preferential secretion of adrenaline, which was reflected by a significant shift in the adrenaline to noradrenaline ratio (17:1 vs. 3:1 at baseline, $p<0.01$, Table 3.4.1).

The NPP pressor effect was greatly potentiated in ganglion blocked rats after captopril (Fig. 3.2.3). However, the effect of captopril was not limited to blood pressure; plasma adrenaline and noradrenaline also increased significantly at the peak response to NPP (Table3.4.2). The increase in catecholamines was substantially higher after captopril ($p<0.001$ adrenaline; $p<0.01$ noradrenaline), but the ratio of adrenaline to noradrenaline (18:1) was not significantly different from the ratio observed in rats before captopril treatment. Thus, despite the fact that the ratio of release was similar, captopril resulted in a higher absolute amount of adrenaline over noradrenaline (cf. Tables 3.4.1 & 3.4.2). These results confirmed hypothesis #3 (pg. 23) that catecholamine release is potentiated by captopril.

The overwhelming increase in adrenaline secretion over noradrenaline suggests that the hypothesized peptide end product generated by NPP might have the ability to preferentially stimulate secretion by adrenaline-storing chromaffin cells. Whether this peptide(s) might also be influenced by ACE is both important (Introduction) and likely, but this is not the focus of discussion here.

There are reports in the literature which demonstrate that selective activation of noradrenaline- and adrenaline-secreting chromaffin cells in the rat adrenal can occur (Vollmer *et al.* 1997; Vollmer *et al.* 1992). Vollmer *et al.* (1992) reported that induction of hypoglycemia in Sprague-Dawley rats produced a 70% decrease in adrenal medullary adrenaline content 3 hours after insulin administration, with no change in noradrenaline content. Similarly, plasma adrenaline concentration increased significantly after insulin treatment, with a smaller increase in noradrenaline. In contrast, exposure to a 4°C environment resulted in a marked decrease in adrenal noradrenaline content with an increase in plasma levels. In response to this type of stressor, there was no change in adrenal content or plasma levels of adrenaline.

As indicated earlier (Introduction), the ability to stimulate adrenal medullary catecholamine release under conditions of sympathetic blockade is both intriguing and perplexing. Intriguing, because the adrenal gland has always been considered to be under the strict control of the CNS and sympathetic nervous system (Parker *et al.* 1993); perplexing, because ganglion blockade was always seen as a tool, not an active agent for exerting a direct effect on the NPP pressor response. In order to settle the question of ganglion blockade, we decided to explore what role,

if any, ganglion blockade with pentolinium exerts on the pressor and catecholamine response to NPP.

4.5.2 Pressure and Catecholamine Responses to NPP in Unblocked Rats

As discussed earlier (section 4.3), the basal blood pressure and catecholamine levels in unblocked rats were usually higher than in ganglion blocked rats. Mavrogiannis (1998) demonstrated that the starting blood pressure was not a major determinant in the manifestation of the NPP pressor effect, but that both ganglion blockade and captopril were necessary to observe the response.

In unblocked rats before captopril, NPP caused an initial depressor response that was followed rather quickly by a secondary pressor phase. While it is still unknown what caused this depressor response, we do know that immediately after the fall in pressure plasma adrenaline levels increased significantly ($p<0.05$ vs. baseline, Table 3.6.1). It is known that low doses of adrenaline can reduce vascular resistance and cause a drop in blood pressure (Freyschuss *et al.* 1986). It would be particularly interesting to explore whether NPP administration to rats causes an initially low level of adrenaline secretion that might account for the drop in pressure. This could be tested by measuring plasma catecholamine levels immediately after NPP injection, just before the fall in pressure.

Activation of β_1 receptors in the heart by noradrenaline from adrenergic nerves can increase chronotropy, conduction velocity and excitability. Circulating adrenaline can also facilitate this effect, since the affinity of the pre-junctional β_2-adrenergic receptor for adrenaline is relatively

the same as it is for noradrenaline (McPherson *et al.* 1985; Lees, 1981). However, despite the large amount of circulating adrenaline present after the depressor response, it did not appear to have much of an effect on HR, since the increase in chronotropy was negligible (Fig. 3.5.3). There was also no significant change in noradrenaline levels which probably accounted for the shift in ratio from baseline 1:2 (A:NA) to 7:1 (Table 3.6.1)

Since HR did not increase to any great extent, but blood pressure reversed and overshot baseline, this suggests that the high levels of circulating adrenaline may have been responsible for the rebound in pressure. The affinity of adrenaline for the α_1-adrenergic receptor is equal, if not greater than noradrenaline (Lees, 1981), so it is conceivable that massive vasoconstriction via α_1-adrenergic receptor activation was responsible for the recovery in blood pressure. Blood pressure reached a peak that was about 10 mmHg above baseline (Fig. 3.5.1 & 3.5.3) and plasma catecholamine levels were slightly higher than baseline, although not to the level of statistical significance.

Captopril had no significant effect on the biphasic response of NPP in unblocked rats (Fig. 3.5.4). The initial depressor response was accompanied by an increase in HR that tended to be greater compared to unblocked rats before captopril, but not to the level of statistical significance (cf. Fig. 3.5.3 & Fig 3.5.4). Despite the rather similar effect on blood pressure, plasma adrenaline levels were markedly elevated immediately after the depressor response compared to unblocked rats before captopril ($p<0.01$, Table 3.6.2). If captopril potentiated both the blood pressure and catecholamine effect of NPP in ganglion blocked rats, it is interesting why captopril only potentiated the catecholamine effect of NPP in unblocked rats. Indeed, despite the fact that

the secondary pressor response was identical in magnitude in unblocked rats before captopril, the levels of circulating adrenaline were significantly increased compared to baseline in unblocked rats after captopril ($p<0.001$, Table 3.6.1 vs. 3.6.2). This relatively large increase in adrenaline secretion might explain why HR tended to be higher, but it does not help explain the obvious dichotomy in pressure and catecholamine responses.

What role might ganglion blockade play? As discussed in the Introduction, it was theorized early on that ganglion blocking agents "sensitized" peripheral effector cells (i.e. adrenergic receptor sites) to circulating catecholamines and sympathetic stimulation (Mantegazza *et al.* 1958; Shimamoto *et al.* 1955; Bartorelli *et al.* 1954). Reports of potentiation of the pressor action of i.v. adrenaline, noradrenaline, angiotensin and renin after ganglionic blockade offered little insight into the mechanism at the time (Page and Taylor, 1947). With the increasing knowledge of adrenergic receptors and their regulation, the potentiation of adrenaline and noradrenaline after ganglionic blockade in the earlier studies was probably due to an increase in the sensitivity (i.e. up-regulation) of α- and β-adrenergic receptors for their agonists. Thus, the presence of ganglion blockade in our animals undoubtedly influences the effect of NPP on blood pressure and catecholamine release, but it is also not necessarily required.

There were significant increases in circulating catecholamines in the presence of blockade (+A, Tables 3.4.1 & 3.4.2) as well as in the absence of blockade (-A, Tables 3.6.1 & 3.6.2). This strongly suggests that the ability of NPP to stimulate catecholamine release is independent of ganglion blockade. The same might be said of captopril as well. Indeed, captopril does appear to heavily influence the NPP pressor and catecholamine effect, but it is not absolutely required.

We have demonstrated that NPP provokes substantial adrenaline release in the absence of captopril (-C, Tables 3.4.1 & 3.6.1) as well as in its presence (+C, Table 3.4.2 & 3.6.2). Thus, with the combination of both agents (+A+C), NPP exerts its maximal effect.

In view of the unexpected depressor responses to NPP seen in –A rats, we tested the responses of such rats to the vasodilator sodium nitroprusside (SNP, Appendix A), a nitric oxide (NO) donor known to produce sustained hypotension under constant infusion. Since we were interested in mimicking the depressor effect of NPP, a bolus dose of SNP of 5 μg/kg was administered i.v. to unblocked rats before and after captopril. The effect of SNP on blood pressure, heart rate and catecholamine release was compared to that of NPP.

4.6 PRESSURE AND CATECHOLAMINE RESPONSES TO SODIUM NITROPRUSSIDE (SNP)

As discussed briefly in Appendix A, SNP is a nitric oxide (NO) donor that has a direct effect on peripheral vascular beds to induce hypotension. The restoration of blood pressure immediately after the hypotensive event is usually the result of increased sympathetic activity reflected by increased splanchnic nerve activity (Struthers and Dollery, 1985) and circulating catecholamines (Grossman *et al.*1982). Thus, in our bioassay rat, it was expected that with the increase in sympathetic nerve activity following the hypotension, this might be reflected in higher levels of circulating noradrenaline which may be the result of spillover from neuronal release.

4.6.1 Baseline Blood Pressure and Catecholamine Levels

Basal blood pressure stabilized around 120/80 mmHg (SBP/DBP) and plasma catecholamines were similar to those observed in other unblocked rats (Table 3.6.1 & 3.8.1), with noradrenaline

levels higher than adrenaline, thus reflecting normal sympathetic drive (section 4.3). After captopril, baseline blood pressure remained around 120/80 mmHg and there was a similar elevation in adrenaline and noradrenaline after renin system blockade seen in earlier rats (section 4.3.1 & Table 3.8.2).

4.6.2 Pressure and Catechoalmine Responses to SNP in Unblocked Rats Before Captopril

A single dose of 5 μg/kg SNP in unblocked rats before and after captopril produced a remarkably similar blood pressure response to that obtained for NPP in similar rats (cf. Fig. 3.7.2a & 3.5.1), although of shorter duration (1-3 min rather than 5-7 min). The depressor response following SNP in unblocked rats before captopril was a little more pronounced than that observed with NPP (Fig. 3.7.4, not statistically significant) and was also accompanied by a small increase in HR. It is well established that constant infusion of NO and NO donors can inhibit sympathetic neurotransmission and baroreceptor activity (Liu *et al.* 1996; Bucher *et al.* 1992) and recent evidence suggests that NO donors can also increase HR directly (Hogan *et al.* 1999; Musialek *et al.* 1997). Almost immediately after the infusion, blood pressure usually reverses quickly, often increasing significantly above baseline. However, these effects were not observed in our rats. It may be that a relatively low, bolus dose of SNP does not exert the same potent effects as constant infusion does. Also, given that the half-life is approximately 1-3 min, SNP is probably cleared quite rapidly after its introduction into the circulation and this might also account for the rapid increase in blood pressure.

After the depressor response, catecholamine levels were contrasted with those observed for NPP under the same conditions (Table 3.8.1). In response to the vasodilatation, noradrenaline levels

remained relatively constant. If the rebound in pressure were due to a reflex increase in sympathetic activity, then release of noradrenaline from adrenergic terminals would activate post-synaptic α-adrenergic receptors to constrict vessels, increase DBP and TPR, thus correcting the deficit in pressure. Spillover into the circulation need not have occurred if sufficient sympathetic activity corrected the deficit in pressure and this seems to have been the case in our rats.

In contrast to the effect of NPP on adrenaline release, after SNP, adrenaline levels stayed relatively constant (Table 3.8.1). The ratio of adrenaline to noradrenaline during the SNP response shifted in favor of noradrenaline, as expected. At the pressor phase of the response to SNP, adrenaline and noradrenaline levels resembled those observed at baseline. These data confirmed hypothesis #7 (pg. 23) that in comparison to SNP, the effect of NPP in –A rats is not simply a consequence of blood pressure changes.

4.6.3 Pressure and Catecholamine Responses in Unblocked Rats After Captopril

Since SNP is not known to interact with the angiotensin converting enzyme (ACE), captopril was not expected to potentiate the blood pressure response to SNP nor to catecholamine release. Indeed, there was no statistically significant difference in the magnitude of the depressor response or the secondary pressor phase before or after captopril (Fig. 3.7.5) compared to NPP under the same conditions. After the depressor response, the ratio of adrenaline to noradrenaline shifted in favor of the latter, with no significant difference compared to the –A-C group. As in unblocked rats before captopril, at the secondary pressor phase, adrenaline and noradrenaline values were similar to baseline values. Thus, in these captopril-treated rats, there appeared to be

no evidence of any potentiation of catecholamine release after SNP, contrary to what occurred after NPP (cf. Table 3.6.2 & 3.8.2).

The similarities of NPP and SNP end at their effects on blood pressure. We have shown that in unblocked rats, NPP caused an initial depressor response that was followed by massive release of adrenaline as opposed to noradrenaline. In contrast, SNP also produced a similar blood pressure effect, but the catecholamine profile was much different. The data obtained from the SNP and NPP experiments in unblocked rats support the view that NPP preferentially stimulates adrenaline secretion and that the presence of ganglion blockade in increases the sensitivity of the animal to circulating catecholamines. These data also confirm that in our bioassay rat model, under conditions of no ganglion blockade (-A), the response to a direct vasodilator appears to be direct sympathetic stimulation of peripheral vessels without adrenal medullary involvement. This contrasts to the significant levels of circulating adrenaline seen after NPP in similarly unblocked rats.

How is a sympathetically blocked adrenal gland still capable of secreting such large amounts of catecholamines as we observe in the ganglion blocked (+A) condition? The following discussion is aimed at providing a possible explanation for the level of catecholamine release we observe in ganglion blocked rats.

4.7 ALTERNATE ROUTES TO ADRENAL GLAND STIMULATION AND CATECHOLAMINE RELEASE

A key question that remains is how the adrenal gland can stimulate catecholamine secretion when it is sympathetically blocked. In the Introduction, we alluded to the possibility that there

might be other pathways capable of stimulating adrenal medullary catecholamine release in the presence of ganglion blockade. Thus, in light of the magnitude of catecholamine release observed in ganglion blocked rats relative to those obtained in unblocked rats, it is pertinent to discuss briefly what is known regarding other pathways of catecholamine release. Whether or not they play a role in our system remains to be determined.

4.7.1 Evidence for Other Peptides in the Adrenal Medulla

Several polypeptides that exist in the adrenal glands of different animal species could act as neurotransmitters and/or neuromodulators in this synaptic region. Ultrastructural and immunohistochemical studies of axon terminals and nerve fibers in the medulla indicate that, in addition to the classical cholinergic-containing terminals, there is extensive evidence of peptide-containing terminals as well. Such neuropeptides include enkephalins, VIP, substance P (SP), pituitary adenylate cyclase-activating polypeptide (PACAP) and neuropeptide Y (Moller and Sundler, 1996; Frodin *et al.* 1995; Pelto-Huikko *et al.* 1985; Varndell *et al.* 1984; Allen *et al.* 1983; Linnoila *et al.* 1980; Lundberg *et al.* 1979; Schultzberg *et al.* 1978).

Receptors on chromaffin cells that mediate catecholamine release are numerous and include: cholinergic agonists (nicotinic and muscarinic), VIP, opioid peptides, SP, bradykinin, and PACAP. It is well established that exocytotic release of adrenal medullary catecholamines is dependent on intracellular calcium (Baker and Rink, 1975; Douglas, 1968). However, there is some evidence to suggest that other second messengers, eg. cAMP, cGMP, protein kinase C (PKC) and inostiol triphosphate (IP_3) can also stimulate secretion of catecholamine stores (Malhotra *et* al. 1989). All of the peptides mentioned above are capable of stimulating

catecholamine release to one degree or another, using a variety of second messengers, particularly in the absence of acetylcholine (Ach, see below).

4.7.2 Catecholamine Secretion in the Presence of Cholinergic Antagonists

Wakade and coworkers demonstrated that the stimulatory effect of exogenous Ach on the isolated perfused adrenal gland (Wakade, 1981) was fully blocked by nicotinic and muscarinic receptor antagonists, but the secretion of catecholamines evoked by stimulation of splanchnic nerves persists to a significant degree even in the presence of cholinergic antagonists (Malhotra and Wakade, 1986). They showed that increasing the frequency of stimulation of the splanchnic nerve from 0.5 to 10 Hz (300 pulses) enhanced the secretion of catecholamines from the isolated perfused adrenal gland. After blockade of nicotinic and muscarinic receptors with mecamylamine and atropine, the neurally-evoked secretion was reduced 40%. On the other hand, exogenously applied Ach-evoked secretion was reduced 93% in the presence of mecamylamine and atropine (Malhotra and Wakade, 1986). Given the evidence of the presence of other peptides in the nerve terminals innervating the adrenal medulla, the authors hypothesized that the splanchnic nerves might release an excitatory transmitter in addition to Ach (Malhotra and Wakade, 1987).

One such candidate is vasoactive intestinal polypeptide (VIP) because of its presence in the splanchnic nerves of the adrenal gland (Hokfelt *et al.* 1981; DiGuilio *et al.* 1979; Schulzberg *et al.* 1978) and the fact that VIP exerts a significant stimulatory effect on the rat adrenal medulla (Malhotra and Wakade, 1987). The effect of such a non-cholinergic transmitter was especially prominent when splanchnic nerves were stimulated at low (0.5 Hz) rather than high frequencies

(10 Hz) (Malhotra and Wakade, 1987), suggesting that it acts independently from Ach and thus is released independently. These observations suggest that under biological conditions of low neuronal activity (e.g. ganglion blockade, as in our model), the adrenal gland could be capable of being stimulated by other neurotransmitters released from the splanchnic nerves that innervate it.

We have shown that in ganglion blocked rats after Captopril, NPP caused massive release of adrenal medullary catecholamines, at a ratio of about 18:1 adrenaline to noradrenaline. Is there precedence in the literature for such massive release of catecholamines in such ratios and does it relate to our model? Although the purpose of this thesis is not to determine what peptide(s) is/are involved in triggering the release of catecholamines, there is evidence in the literature to suggest that there might be a possible candidate, as follows.

4.7.3 Alteration in Catecholamine Release Ratios and Possible Peptidergic Mediators of the NPP Effect

To reiterate, the typical release ratio of adrenaline to noradrenaline from the adrenal medulla is 4:1 in favor of adrenaline (Parker *et al.* 1993; Verhofstad *et al.* 1985), but this ratio can be substantially increased (Vollmer *et al.* 1997; Vollmer *et al.* 1992; Feuerstein and Gutman, 1971). The increase in the ratio of circulating adrenaline (compared to noradrenaline) at the peak SBP response to NPP is very intriguing and further supports the view that the proposed peptide end product might be preferentially stimulating adrenaline release over noradrenaline.

In addition to VIP, it has recently come to light that pituitary adenylate cyclase-activating polypeptide (PACAP) is present in splanchnic nerve terminals innervating the adrenal medulla (Arimura, 1992; Waymire *et al.* 1992). The adrenal medulla also contains endogenous PACAP

and in fact, has the second highest concentration of PACAP among the peripheral tissues (Arimura, 1992; Watanabe *et al.* 1992; Arimura *et al.* 1991;). PACAP binding sites are also present in the adrenal medulla (Shivers *et al.* 1991) and stimulation of adenylate cylcase in PC12 cells strongly suggests that the adrenal chromaffin cells are one of the major target cells for PACAP (Arimura, 1992). VIP and PACAP also share approximately 60% structural homology which might also argue in favor of PACAP being influenced by ACE, as is the case for VIP (Duggan and Ye, 1996; Woie *et al.* 1987).

In the isolated perfused adrenal gland of the rat, Guo and Wakade (1994) showed that perfusion with 10μM Ach for 4 min caused a significant increase in the secretion of adrenaline and noradrenaline, at a ratio of about 4:1, similar to what is normally observed in the rat adrenal. Perfusion with PACAP (as low as 0.1μM) also produced a significant increase in the secretion of catecholamines; the release ratio of adrenaline to noradrenaline also increased to approx. 7:1. Finally, perfusion with VIP (10μM) stimulated catecholamine release as well and the ratio of adrenaline to noradrenaline was closer to 10:1.

These authors also reported that the ratio of catecholamines released at low levels of nerve stimulation (0.5-3 Hz) was similar to that observed with the peptides and that at higher frequencies of stimulation (10-30 Hz) the ratio shifted to 5:2, closer to what was observed with Ach (Guo and Wakade, 1994). They concluded that release of PACAP (or VIP) under conditions of low neuronal activity stimulates adrenaline-storing cells, possibly to support a low level of metabolic function.

In contrast, when the splanchnic nerve activity increases, as during stress, release of Ach stimulates the secretion of noradrenaline to meet the higher metabolic needs created by the stressful condition (Guo and Wakade, 1994). PACAP was also shown to be capable of stimulating catecholamine release in the rat using an *in vivo* microdialysis technique that allows for local application of the drug (Watanabe *et al.* 1995).

When injected i.v. into the cat, PACAP is shown to be pressor (Champion *et al.* 1996; Minkes *et al.* 1992), whereas VIP is known to be a depressor agent (Duckles and Said, 1987; Brayden and Bevan, 1986). The pressor activity of PACAP was reduced in cats that were bilaterally adrenalectomized. In animals treated with phentolamine (α-adrenergic antagonist), the pressor effect was also reduced (Minkes *et al.* 1992). In the same study, the authors reported that in three cats pretreated with the ganglion blocking agent hexamethonium, the responses to PACAP were enhanced. There is evidence from preliminary experiments in our lab to indicate that in our bioassay rat model, PACAP is indeed pressor and can duplicate the NPP response (unpublished observations). The relatively high dose (μg range) we administered systemically might be required to mimic concentrations normally seen at the nerve terminal. There is also evidence to suggest that in response to intravenously administered PACAP, plasma adrenaline levels increase significantly compared to noradrenaline (unpublished observations).

If a peptide(s) is involved in stimulating catecholamine release, and if that peptide shares a relationship to the angiotensin converting enzyme (ACE), it is plausible that the potentiation of the NPP pressor response after captopril may be due to a sustained presence of that peptide after ACE inhibition. If ganglion blockade unmasks an alternate route to adrenal gland stimulation,

and if captopril prolongs the half-life of a peptide that can stimulate catecholamine release, this may help to explain how the NPP pressor effect is at is greatest potency when the two drugs are administered. Thus, the preceding discussion illustrates the degree of complexity that is emerging as we attempt to understand the mechanism(s) underlying the NPP pressor effect. Whether or not NPP generates a peptide(s) has yet to be determined; as candidate peptides emerge, their ability to stimulate catecholamine release and the degree to which their action is potentiated by ACEIs will be subject to close scrutiny.

4.8 DOES LOSARTAN POTENTIATE THE NPP PRESSOR AND CATECHOLAMINE EFFECT?

We have shown time and time again that the NPP effect is greatly potentiated by captopril and that ganglion blockade has a significant effect on catecholamine release, which has yet to be fully understood. Given the value of ACE inhibitors in clinical practice and their widespread use, we needed to verify that the effect of captopril was specific to ACEIs and not to anti-hypertensive drugs in general. Thus, we decided to investigate the effect of a new class of anti-hypertensive drug, the angiotensin AT_1 receptor antagonist (losartan, Appendix B), on the pressor and catecholamine response to NPP. The NPP pressor response and subsequent catecholamine release was measured in ganglion blocked rats before and after losartan (10 mg/kg i.v.), and after losartan + captopril.

4.8.1 Baseline Blood Pressure and Catecholamine Responses

Baseline blood pressure in ganglion blocked rats before and after losartan or captopril typically stabilized at about 80/40 mmHg (SBP/DBP, Fig. 3.9.1), illustrating the relative importance of the sympathetic system in assuming greater control in the maintenance of blood pressure relative to

the renin system (Mavrogiannis, 1998). This was also reflected in slightly higher baseline catecholamine levels of adrenaline over noradrenaline and in the ratio of A:NA of about 3:1 (Table 3.10.1).

4.8.2 NPP Pressor and Catecholamine Responses Before and After Losartan

Angiotensin AT_1 receptor antagonism with losartan did not potentiate the NPP pressor response or catecholamine release (Fig. 3.9.1a, 3.9.1b, 3.9.2 & Table 3.10.1). The increase in SBP before and after osartan was within the 10-20 mmHg range seen for NPP before captopril (Fig. 3.2.3 &3.9.2, Mavrogiannis, 1998). Thus, the data support hypothesis #5 (pg. 23) that the pressor effect of NPP is not enhanced by angiotensin AT_1 receptor antagonists.

It is well known that angiotensin II (Ang II) can stimulate adrenal medullary catecholamine release (Livett and Marley, 1993) via AT_1 receptors on chromaffin cells. In the rat adrenal, AT_2 receptors are also present, but catecholamine release is mainly mediated by AT_1 (Wong *et al.* 1990). Under ACE inhibition, it is conceivable that local generation of Ang II in the adrenal might still exist and contribute to catecholamine release. However, in the absence or presence of losartan, stimulation of catecholamine release after NPP was unchanged (Table 3.10.1), suggesting that catecholamine release in our rats is independent of AT_1 receptor effects. The characteristic increase in the ratio of A:NA after NPP was similar to the ratio observed in other ganglion blocked rats (Table 3.4.1) and did not change before or after losartan (15:1 vs. 16:1, respectively). These data also support hypothesis #5 (pg. 23) that angiotensin AT_1 receptor antagonism with losartan does not alter adrenal medullary catecholamine release.

4.8.3 NPP Pressor and Catecholamine Responses After Losartan and Captopril

Combined treatment of ganglion blocked rats with losartan and captopril resulted in major potentiation of the NPP pressor and catecholamine effect (Fig. 3.9.1c and Table 3.10.1). A rather surprising result was the potentiation of not only the SBP component of NPP but also of the DBP component, an effect that is not normally seen after captopril alone (cf. Fig. 3.2.3 & 3.9.2). The effect on HR was also greatly potentiated after losartan and captopril. The data presented here refute hypothesis #6 (pg. 23) that captopril on top of losartan does not potentiate the NPP pressor effect. In fact, we show that the DBP pressor response is potentiated after combined treatment of captopril and losartan, for reasons that are at the moment, unclear. However, it is possible that alternate, local generation of Ang II, which escapes inhibition by ACE and the action of losartan, might be contributing to the increase observed. Whether this occurs remains to be determined.

We were curious to observe whether the increase in the DBP response was reflected by either increased circulating noradrenaline levels or a shift in the A:NA ratio. This did not appear to be the case. Although the levels of noradrenaline were significantly increased after NPP in losartan and captopril treated rats ($p<0.05$ vs. baseline, Table 3.10.1), the levels were not different from captopril treated rats alone (Table 3.4.2). Much the same was observed for adrenaline; the catecholamine levels (and ratios) were significantly increased after combination treatment ($p<0.05$ vs. baseline), but not when compared to captopril treatment alone and this was reflected in the increase in the A:NA ratio of approximately 21:1.

Interestingly, dopamine levels were also significantly increased after losartan alone and in combination with captopril, but because the levels were much lower compared to either adrenaline or noradrenaline it is doubtful that they contributed much to pressure. However, the data do not adequately explain the potentiation of DBP observed with combined treatment of the two drugs. Perhaps the combined treatments of ganglion blockade, ACE inhibition and AT_1 receptor antagonism in our bioassay rats increased basal catecholamine release from the medulla to maintain blood pressure. However, since we did not measure baseline plasma catecholamine levels after combined treatment of captopril and losartan, it is not clear whether catecholamine levels were indeed altered. Further studies will be required to elucidate the mechanism underlying the effect of NPP on DBP in losartan- and captopril-treated rats.

4.9 POSSIBLE PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL SIGNIFICANCE OF NPP

We have demonstrated that an activated fragment of coagulation FXII (i.e.βFXIIa) when injected into a bioassay rat, increases SBP, HR, cardiac output (CO) and stroke volume (SV) and does so by stimulating massive release of adrenal medullary catecholamines, particularly adrenaline. That these responses are potentiated in the presence of ganglion blockade itself is not necessarily of therapeutic interest since these drugs are no longer being used to lower blood pressure in humans. However, we have shown that even under these conditions, the adrenal medulla is still capable of being stimulated through other, as yet undetermined, pathways. Furthermore, in bioassay rats that are not ganglion blocked, NPP can still stimulate adrenal medullary catecholamine release, suggesting a significant effect even in the absence of blockade.

We have also demonstrated that the NPP pressor response and subsequent catecholamine release are greatly potentiated after treatment with one class of anti-hypertensive drug, the angiotensin converting enzyme (ACE) inhibitors, captopril and enalapril. Since these drugs lower blood pressure in many hypertensive patients, it is possible that NPP is not activated under normal circumstances *in vivo* (see below) or that NPP effects are necessarily present. However, for individuals whose blood pressure might not be lowered while taking ACE inhibitors, NPP could be present and stimulating catecholamine release contributing to the elevation in blood pressure. This hypothesis deserves investigation.

NPP is a fragment of coagulation FXII, a key enzyme in the intrinsic coagulation pathway. Contact activation of plasma involves the interaction of negatively charged surfaces with plasma proteins and initiation of blood coagulation upon the activation of FXII (Kaplan *et al.* 1981). NPP can theoretically be released into the blood in situations where the coagulation system is activated. Such situations can occur when blood vessels are exposed to particularly high pressures, as in hypertension. Indeed, the main complications of hypertension, i.e. stroke and myocardial infarction, are thrombotic in nature rather than haemorrhagic (MacMahon *et al.* 1990). In fact, it has recently been suggested that hypertension does confer a 'hypercoagulable state' (Lip and Li-Saw-Hee, 1998). Thus, during such episodes of thrombotic events, the possibility exists that NPP is released into the blood and can stimulate adrenal medullary catecholamine release, further aggravating an already jeopardized condition. This proposed link between a coagulation factor involved in thrombosis and the release of catecholamines to increase blood pressure seems to be of particular interest and clinical importance.

5. *SUMMARY*

- Bilateral adrenal medullectomy caused >90% inhibition of the pressor and cardiotonic effect of NPP in bioassay rats (supporting hypothesis #1 and fulfilling objective #1).

- Plasma catecholamines (adrenaline and noradrenaline) increase significantly after NPP in ganglion blocked rats; the ratio of A:NA increases from about 4:1 to 17:1 (supporting hypothesis #2 and fulfilling objective #2).

- Plasma catecholamines and the ratio of release are greatly potentiated after NPP in ganglion blocked and captopril treated rats (supporting hypothesis #3 and fulfilling objective #3).

- Plasma catecholamines and the ratio of release increase significantly after NPP in unblocked rats, despite an opposite effect on blood pressure, suggesting that ganglion blockade heavily influences the action of NPP as well (supporting hypothesis #4, partially objective #4).

- Sodium nitroprusside produces a similar drop in pressure as NPP does in unblocked rats, but does not stimulate catecholamine release to the extent that NPP does in the same situation (supporting hypothesis #7 and fulfilling objective #8).

- Angiotensin AT_1 receptor antagonism with losartan does not alter the NPP pressor effect or catecholamine release (supporting hypothesis #5 and fulfilling objective #5).

- Combined treatment with losartan and captopril significantly potentiated the SBP, DBP and HR effect of NPP as well as catecholamine release (not supporting hypothesis #6, but fulfilling objectives #6 and #7).

6. CONCLUSIONS

Activated coagulation FXII, "new pressor protein" (NPP) raises systolic blood pressure (SBP) and heart rate (HR) when injected intravenously (i.v.) in ganglion blocked bioassay rats. That the effect is specific to SBP, and has negligible effect on diastolic blood pressure (DBP), suggests a specific action on the heart and not on the peripheral vasculature. These effects are greatly potentiated after angiotensin converting enzyme inhibition (ACEI) with captopril.

Acute bilateral adrenal medullectomy (2MDX) confirmed earlier work on adrenalectomy (2AX), that secretions from the adrenal medulla mediate the NPP pressor response. Plasma catecholamines increase significantly when measured at the peak pressor response to NPP in rats before captopril treatment. The ratio of catecholamines also increases significantly in favor of adrenaline. These levels are greatly potentiated after captopril treatment, in accordance with the increase in BP and HR.

NPP stimulates massive release of adrenal medullary catecholamines, particularly adrenaline, despite the fact that the adrenal gland is ganglion blocked. The adrenal medulla is controlled primarily by preganglionic sympathetic nerves via the splanchnic branch of the autonomic nervous system. As such, the primary stimulus for catecholamine secretion is Ach released from sympathetic nerve terminals and synapsing on their nicotinic (and muscarinic) receptors on chromaffin cells. However, recent evidence suggests that the medulla also receives a postganglionic nerve supply, with many other neurotransmitters that are capable of stimulating catecholamine release. Thus, the possibility exists that NPP can act through other pathways to stimulate catecholamine release.

In the absence of ganglion blockade (-A), NPP causes an initial depressor response that is followed immediately by a significant increase in circulating adrenaline, presumably to assist in reversing the pressure. The presence of captopril (+C) in these unblocked rats did not have a significant effect on the blood pressure response to NPP, but adrenaline secretion was greatly potentiated. The data presented suggest that the NPP pressor and catecholamine effects do not necessarily depend on the presence of either ganglion blockade or captopril, since significant increases in plasma catecholamine levels persist in the –A-C condition. However, given that NPP's potent effects on blood pressure and catecholamine release are at their greatest when both drugs are present suggests a complex interplay between ganglion blockade and ACE inhibition that will need further elucidation.

Sodium nitroprusside (SNP) caused direct vasodilatation that resulted in an initial hypotensive event in unblocked bioassay rats similar to what was observed with NPP. Despite similarities in the blood pressure responses, the effect on adrenal medullary catecholamine release was quite different, arguing in favor of a specific agonistic effect of NPP on the adrenal gland that is not present with SNP.

Since the NPP pressor response is potentiated by ACE inhibition, we tested the effects of another class of anti-hypertensive drug, the angiotensin AT_1 receptor antagonist, losartan on the NPP pressor response. Losartan alone did not potentiate the NPP-mediated pressure or catecholamine effect. However, losartan treatment combined with captopril did potentiate the blood pressure (both SBP and DBP) and catecholamine effect. The mechanism of such potentiation of DBP remains to be elucidated.

7. LIMITATIONS OF THE STUDY

The physiological effects of adrenaline, noradrenaline and dopamine differ in various vascular beds. They depend on both the quantities and proportions (ratios) of the catecholamine species. The critical quantities and ratios for producing a given effect (heart rate, cardiac output and peripheral vasoconstriction) are not readily knowable under the varying conditions of ganglion blockade (+A) and captopril (+C). This study determined plasma concentrations of adrenaline, noradrenaline and dopamine, but was not able to ascertain exactly what levels and proportions can fully account for the observed cardiovascular effects. Thus, high levels of plasma adrenaline are found in association with strong pressor effects (Figs. 3.2.1a & 3.2.1b, Tables 3.4.1 & 3.4.2) as well as depressor effects (Figs. 3.5.1a & 3.5.1b, Tables 3.6.1 & 3.6.2). This means that while a strong association between catecholamine levels and cardiovascular effects has been established herein, a distinct link between cause and effect cannot be known exactly. Some other adrenal medullary secretagogue may also be involved, e.g. neuropeptide Y (Varndell *et al.* 1984; Allen *et al.* 1983), working in co-operation with catecholamines. Alternatively, given plasma levels and proportions of catecholamines may correlate with the observed blood pressure changes, up or down. Resolution of this issue will have to await the measurement of other adrenal medullary secretagogues.

The studies show marked changes in catecholamine ratios, especially with reference to adrenaline:noradrenaline (A:NA). This happened both at the baselines of the various groups (+A vs. –A, +C vs. –C) and during the pressor and depressor responses to NPP (e.g. Tables 3.4.1, 3.4.2, 3.6.1 & 3.6.2). Such changes could be significant but we cannot, at this time, assess such differences in ratios relative to the cardiovascular effects noted.

All catecholamine changes reported are evaluated relative to baseline (control) levels. It is not known how these so-called baseline values compare with some 'true' baseline to be found in a normal animal perfectly at rest.

We used impure trypsin-activated plasma preparations of human NPP in our bioassay rats based on earlier findings from our laboratory that these fully reproduce the effects of highly purified human NPP preparations (Mavrogiannis, 1998). Moreover, the human and rat NPP preparations appear to be interchangeable in their physiological effects (Mavrogiannis, 1998). The clinical significance of our work rests on the assumption that NPP is formed in the human body under thrombotic conditions. Such conditions are common but the endogenous formation of NPP remains to be verified before all the present experimental observations are assumed to be applicable to clinical situations.

Our bioassay technique is presently the only method we have for measuring and evaluating NPP. It was essential to use it in the present study in which system interactions were being evaluated. Future studies will require isolated organs, tissues, cells and molecular techniques in order to further the foundational knowledge that is being developed.

8. *FUTURE DIRECTIONS*

Studies that are on going in our laboratory that are directly related to the findings of this thesis are aimed at determining the end product of NPP injection that causes such massive release of adrenal medullary catecholamines, particularly adrenaline.

That NPP can stimulate adrenal medullary catecholamine release in bioassay rats with, for all intents and purposes, isolated adrenal glands is quite perplexing. As discussed briefly, there are many peptides that can stimulate catecholamine release both *in vivo* and *in vitro*, and a few have the ability to alter the release ratio of adrenaline. Of such peptides, preliminary experiments in our lab suggest that pituitary adenylate cyclase-activating polypeptide (PACAP) can duplicate the NPP pressor effect in ganglion blocked, captopril treated bioassay rats (unpublished observations). These exciting results suggest that PACAP may be involved downstream of NPP injection, perhaps by stimulating the adrenal medulla to release catecholamines. Preliminary data also suggests that intravenously administered PACAP in our ganglion blocked and captopril treated rat stimulates adrenal medullary catecholamine release, since plasma catecholamines are significantly increased at the peak pressor response.

We have shown that the NPP-mediated pressure and catecholamine effects are potentiated by ACE inhibition with Captopril. Thus, it was not a surprise that Losartan (an angiotensin AT_1 receptor antagonist) did not potentiate the effect, since Mavrogiannis (1998) ruled out angiotensin II depletion as a possible mechanism in the NPP pressor effect. However, the potentiaton of both SBP and DBP after combined treatment of losartan and captopril is perplexing and needs to be investigated further.

9. APPENDIX A

EFFECT OF SODIUM NITROPRUSSIDE (SNP) IN –A-C AND –A+C RATS

Rationale for Choosing SNP

Sodium nitroprusside (SNP) is a strong hypotensive agent when infused intravenously (i.v.) and has been shown to exert a direct action on peripheral vascular beds (Kyněl, 1971; Johnson, 1929; Thiens, 1926). SNP is a nitric oxide (NO) donor and like other NO donors, it reduces arterial blood pressure by decreasing vascular resistance and inhibits the vascular and chronotropic response to sympathetic stimulation (Choate and Paterson, 1999; Elvan *et al.* 1997). SNP has a rapid onset and short duration of effect (1-3 min, Cohn and Burke, 1979). It is degraded in the blood (by hemoglobin) into cyanide, which is in part detoxified by the liver and kidney to thiocyanate (Smith and Kruszyna, 1974, 1975).

We therefore chose SNP to compare its blood pressure and catecholamine effects against the known effects of NPP in –A-C rats. Our objective was to determine if the effect of NPP on catecholamine release was primarily a reflex response to the drop in pressure, or if NPP injection produced some type of agonistic effect on the adrenal medulla.

Most reports of SNP use in rats involve i.v. drug infusion studies at doses between 10-100 μg/kg/min (for 20-60 min). This produces a sustained hypotension with typical decreases in blood pressure of about 40 mmHg (Vollmer *et al.* 1989; Eveguoz *et al.* 1987; Bush and Vollmer, 1984; Gustafson, 1984; Knight *et al.* 1983; Hoffman *et al.* 1982; Gmeiner *et al.* 1975). Preliminary experiments (data not shown) indicated to us that bolus injection of doses

greater than 40 μg/kg produced a fall in blood pressure >45 mmHg. Thus, a dose-response curve was constructed in order to determine the optimal dose of SNP that would produce a decrease in blood pressure similar to that observed with NPP in –A-C rats. The dose chosen was 5 μg/kg (see Fig. 3.7.1).

10. APPENDIX B

ANGIOTENSIN AT_1 RECEPTOR ANTAGONIST LOSARTAN

The effects of angiotensin II (Ang II) are mediated predominantly by the AT_1 receptor that is a seven-transmembrane domain protein coupled by a G-protein to phospholipase C. When bound to its receptor, Ang II increases the cytosolic free calcium level in the cell to bring about its biological effects (Ganong, 1997). Ang II is a potent vasoconstrictor; it stimulates aldosterone secretion from the adrenal cortex; it facilitates noradrenaline release by a direct action on postganglionic sympathetic neurons (Starke, 1977); it acts on the brain to increase blood pressure and water intake (Ganong, 1997) and can also stimulate adrenal medullary catecholamine release (Butler *et al.* 1994; Straszewska-Barczak and Vane, 1967; Lewis and Reit, 1966; Feldberg and Lewis, 1964, 1965).

Both the AT_1 and AT_2 receptor subtypes have been identified on rat adrenal chromaffin cells (Marley *et al.* 1989; Healy *et al.* 1985; Quirion *et al.* 1983). In the rat adernal, 70% of the receptors are of the AT_2 subtype, and 30% are the AT_1 (Balla et al. 1991; Chang and Lotti, 1990; Chiu et al. 1989). However, despite the higher density of AT_2 receptors, adrenal catecholamine secretion induced by Ang II in the rat is due to activation of AT_1 receptors (Wong et al. 1990). The function of AT_2 is known in other tissues, for example, it exerts a role in angiogenesis in smooth muscle and in thirst and behaviour (Messerli *et al.* 1996) but its role in the rat adrenal is unclear.

Losartan is a nonpeptide, AT_1-selective receptor antagonist and a relatively new drug that reduces blood pressure by inhibiting the effects of Ang II at the receptor level (Smith and Timmermans, 1994). We were interested in observing the effect, if any, of this very different anti-hypertensive agent on the pressor effect of NPP, given what we know about the effect of ACE inhibitors. Thus, we tested NPP in ganglion blocked bioassay rats before losartan treatment, after losartan and after captopril + losartan.

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