Endocrine and Paracrine Aspects of Vascular Control

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The effects of natriuretic peptides on human capacitance in health and chronic heart failure

by

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SUMMARY

The effects of natriuretic peptides on vascular control were investigated. In the major part of this thesis emphasis is placed on the effects of natriuretic peptides, in particular ANP, on regulation of regional vascular volume and venous tone in healthy volunteers (Chapter 3) and patients with chronic heart failure (Chapter 4). The second part of this thesis investigates the effects and mechanisms of action of ANP, BNP and CNP on large artery function in an ovine hind limb model (Chapter 5). Finally, the actions of the latest members of the natriuretic peptide family, namely DNP and NNP are investigated in vitro using rings of rabbit aorta in organ bath experiments (Chapter 6).

The important new findings of this thesis are;

1. ANP regulates regional vascular volume and venous tone over a wide range of physiological and pathophysiological plasma levels without affecting compliance.
2. Most importantly, basal ANP plasma levels contribute significantly to regulation of resting vascular tone.
3. The rank order of potency of NP in the forearm capacitance vasculature of patients with chronic heart failure is ANP>> BNP>CNP/Urodilatin.
4. Venous ANP responsiveness is preserved in patients with chronic heart failure despite marked impairment in the resistance vasculature. This preservation may be due to preserved venous endothelial function.
5. ANP acting locally modifies pulse wave velocity via the NPR_A receptor. Neither CNP (acting via the NPR_B receptor) nor cANF (acting via NPR_C) elicit any immediate vasoactive effects.

6. Novel natriuretic peptide (NNP), a newly isolated NP from the venom of the green mamba snake (dendroaspis angusepticus) has arterial vasorelaxant properties similar to those of ANP and DNP.

ACKNOWLEDGEMENTS

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The organ bath studies organised, designed and supervised by myself were carried out in the WHRI by Sophie Reshamwalla with support and co-supervision of Derek Lang.

Most importantly I would like to thank Professor Michael P. Frenneaux for introducing me to the interesting field of venous physiology and for *infecting me* with scientific curiosity. His unrestricted support has sustained the work that forms this thesis.

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ABBREVIATIONS

CHF	= congestive heart failure
cHF	= chronic heart failure
SNS	= sympathetic nervous system
RAAS	= renin angiotensin aldosterone system
ESC	= European Society of Cardiology
NP(s)	= natriuretic peptide(s)
NPS	= natriuretic peptide system
LV	= left ventricular
P/V	= pressure/volume
NO	= nitric oxide
PVR(s)	= pressure/volume relation(s)
RPY	= radionuclide plethysmography
DVI	= diastolic ventricular interaction
UVV	= unstressed vascular volume
NA	= noradrenaline
NE	= norepinephrine
ACE	= angiotensin converting enzyme
Ang II	= angiotensin II
CVP	= central venous pressure
LVEDP	= left ventricular end diastolic pressure
RVEDP	= right ventricular end diastolic pressure
PCWP	= pulmonary capillary wedge pressure
RV	= right ventricular
RCT	= randomised controlled trial
ANP	= atrial natriuretic peptide
BNP	= brain natriuretic peptide
CNP	= c-type natriuretic peptide
DNP	= dendroaspis natriuretic peptide
NNP	= novel natriuretic peptide
SNP	= sodium nitroprusside
ER	= endoplasmatic reticulum
aa	= amino acids
N	= nucleus
G	= Golgi apparatus
SG	= secretory granules
LANP	= long acting natriuretic peptide
ANF	= atrial natriuretic factor
α-TNF	= tumour necrosis factor α
GC	= guanylate cyclase
NPR	= natriuretic peptide receptor
AV	= arterio-venous
eNOS	= endothelial nitric oxide synthase
VSMC(s)	= vascular smooth muscle cell(s)
NEP	= neutral endopeptidase
BP	= blood pressure
CO	= cardiac output
TPR	= total peripheral resistance

Asp13	= aspartic acid in position 13
Phe 26	= phenylalanine in position 26
Tic	= tetrahydroquinoline-3-carboxylate
NPE	= non-pigmented epithelial cells
d-MSH	= delta-Melanocyte-stimulating hormone
SVR	= systemic vascular resistance
PAP	= pulmonary artery pressure
SVI	= stroke volume index
CI	= cardiac index
HR	= heart rate
PCWP	= pulmonary artery wedge pressure
VP	= vasopeptidase
VPI	= vasopeptidase inhibition
OMA	= Omapatrilat
ET	= exercise tolerance
ETT	= exercise tolerance test
ADM	= adrenomedullin
EBPS	= equilibrium blood pool scintigraphy
ROI	= region of interest
SFOV	= small field of view
FBF	= forearm blood flow
VOP	= venous occlusion plethysmography
SGP	= strain gauge plethysmography
FVV	= forearm vascular volume
ECG	= electrocardiography
IVV	= intestinal vascular volume
ANOVA	= analysis of variance
SEM	= Sandard error of the mean
LNMMA	= N^G-monomethyl-L-arginine
PWV	= Pulse wave velocity
MAP	= Mean arterial pressure
ISH	= Isolated systolic hypertension
GSK	= Glaxo Smith Kline
PE	= Phenylepinephrine
ODQ	= 1H-[1,2,4] oxaddiazol94,3-alpha quinoxaline-1
L-NAME	= N^G-Nitro-D-Arginine-Methyl Ester
EDHF	= Endothelium derived hyperpolarising factor

"The very essence of cardiovascular practice is the early detection of heart failure"

Sir Thomas Lewis, 1933

CHAPTER 1 – Literature Review

1.1 Heart Failure

1.1.1. Introduction

Chronic heart failure (cHF) is the potential end stage of any cardiac disease and is a complex syndrome rather than a single disease entity. Being a diverse syndrome arising from multiple underlying aetiologies it has defied a complete and accurate yet simple definition (1-4). Indeed there are no cut off values of cardiac or ventricular dysfunction or changes in flow, pressure, diameter or volume that can be used reliably to identify patients with heart failure (5). It is probably fair to say that the intense search for an ideal definition has, at times, been somewhat counterproductive as many proposals have not only confused the word "definition" of heart failure with "diagnosis" of heart failure but also disregarded the importance of an integrative approach towards the syndrome.

Nevertheless the hallmarks of heart failure described and defined by Sir William Osler (6) and modified and "modernised" by Eugene Braunwald (3) can still serve as useful description of the syndrome: "a pathophysiological state in which an abnormality of the cardiac function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirements of the metabolising tissues".

It should however be emphasised that today's perception of heart failure is constantly evolving and quite different from that of only half a century ago.

Over recent decades it has become increasingly clear that the syndrome of heart failure is associated with a characteristic pattern of cardiac and extra-cardiac responses. The latter including renal, biochemical, neural, vascular and hormonal changes – especially: stimulation of vasopressin, the sympathetic nervous system (SNS), the renin-angiotensin-aldosterone system (RAAS), increased release of

inflammatory markers and cytokines, increased oxidative stress and endothelial dysfunction.

In particular up-regulation of the natriuretic peptide (NP) system (NPS) has emerged as a hallmark of several cardiovascular disorders, most evident in CHF.

The diagnosis of heart failure continues to rely on clinical judgement based on a history, physical examination and appropriate investigations. Clinically heart failure is characterised by breathlessness, effort intolerance, fluid retention and poor survival. It is clear from the aforementioned complexities that any attempt to reduce heart failure to a sole problem of reduced myocardial contractility would be inadequate.

As cardiac output (CO) is a complex and dynamic relationship between preload, myocardial (myocyte) contractility, and afterload, it becomes clear that arterial compliance, venous capacitance, ventricular-interdependence, ventricular-arterial and veno-ventricular coupling all have their role to play in the complex adaptive and maladaptive processes taking place following an injury sufficient enough to lead to heart failure, not to speak about primary myocardial changes itself.

Whilst our understanding of resistance and conduit vascular function has expanded enormously over the past two decades, our understanding and appreciation of venous physiology and pathophysiology in relation to congestive heart failure (CHF) remained rudimentary.

In this thesis the major emphasis is placed on the neurohormonal (in particular enocrine and paracrine), regulation of venous capacitance and tone (Chapter 3 + 4). Especially under consideration is the body's endogenous response to the deleterious activation of the SNS and RAAS – paticularly the role of the natriuretic peptide system (NPS). In the second part (Chapter 5 + 6) the role of natriuretic peptides (NPs) in the functional regulation of conduit arteries in vivo and in vitro is investigated.

1.1.2. Epidemiology

The importance of heart failure as a public health problem predominantly relates to the prevalence of the syndrome, in particular that of more severe stages. The prevalence is determined by the incidence and the survival following the onset of heart failure. Whilst much is known about the epidemiology of heart failure in North America and Europe, regional differences (not only between the US and Europe but also between and within European Member States) exist in risk factors, prevalence, incidence, prognosis and hospitalisation rates. Estimates of the prevalence of symptomatic heart failure in the general European population range from 0.4%-2% (7-9) and it is believed that asymptomatic left ventricular (LV) systolic dysfunction has a similar prevalence (10;11). Each of these two groups probably comprise in Europe approximately 10 million people at any given time (5). Heart failure related health cost expenditures within the UK are estimated to be £ 751 million accounting for roughly 1.83% of the total NHS budget (12). The average yearly incidence in Western countries varies between 1-4/1000 (13) and is highly age dependent. In the 45-55 years age group the prevalence of CHF is considerably less than 1% (14-16). This increases to 2-5% in the 65-75 age group (14;16-18) and approaches 10% in the over 80 years old (14-16;19). Men are more frequently affected than women of the same age with a male/female ratio of roughly 1.5/1. The mean age of affected individuals is now in the mid seventies in Europe and North America. Heart failure prevalence has risen dramatically due to both population aging and the fact that thrombolysis has improved survival post myocardial infarction, but survivors often have significant LV dysfunction. A simulation model by Bonneux and colleagues (20) predicts a transition from acute to chronic cardiovascular disease, resulting in a dramatic increase in age adjusted prevalence rates of ischaemic heart disease in the

Netherlands by 2010, that is largely attributable to CHF. "In absolute terms prevalence rates are predicted to increase 70%; adjusting for the increasing age of the population the net increase will be around 20%" (21).

1.1.3. Prognosis

The prognosis of heart failure is poor (14). Half of patients carrying a diagnosis of heart failure will die within 4 years and in patients with severe heart failure more than 50% will die within 1 year (14;16;22).

1.1.4. Aetiology and Pathophysiology

As noted above CHF can principally be the consequence of any cardiac disease. It is believed that in roughly 80-90% of patients the symptoms are caused by ventricular dysfunction, in possibly more than 60% due to systolic dysfunction with a reduced ejection fraction (EF<40%) (23-25). Systolic dysfunction results from a loss of intrinsic inotropy (contractility), either due to loss of viability or due to alterations in signal transduction mechanisms responsible for regulating inotropy. Diastolic dysfunction refers to the diastolic properties of the ventricle and occurs when the ventricle becomes less compliant (i.e. "stiffer"), which impairs ventricular filling. Both systolic and diastolic dysfunction result in a higher ventricular end-diastolic pressure which serves as a compensatory mechanism by utilising the Frank-Starling mechanism to augment stroke volume. In certain types of heart failure (e.g. dilated cardiomyopathy) this leads to ventricular enlargement as preload pressure increases in order to maintain normal stroke volumes. The leading cause of CHF in Western countries is coronary artery disease (CAD; ≈ 54-70%), which in 35-52% of these patients is associated with arterial hypertension (13). Hypertension as the sole cause

of CHF accounts possibly for 9-20% of cases, whilst idiopathic dilated cardiomyopathy is believed to account for 18-28% of cases. Rarer causes are congenital heart disease, degenerative valvular abnormalities, myocarditis, endocarditis, alcoholic cardiomyopathies and other causes including myocardial storage diseases (7;13-15;26-28).

Following an initial myocardial injury (tissue necrosis, volume overload, pressure overload) compensatory mechanisms become established to maintain stroke volume. These mechanisms lead to macroscopic (ventricular enlargement and change of ventricular geometry) and microscopic (myocyte hypertrophy and interstitial fibrosis) remodeling (29). Ventricular enlargement is a self-perpetuating process as stretch induced apoptosis leads to further loss of viable myocardium (30). As a consequence of reduced organ perfusion and in an attempt to compensate for the reduced CO a complex neuroendocrine activation (activation of the SNS, RAAS, increased release of vasopressin, nitric oxide (NO), cytokines and endothelins) sets in (31-34) . The consequences of long-term activation of these "short-term compensatory mechanisms" however include venous and arterial vasoconstriction (31;35), fluid retention (31;36), and facilitation of life-threatening arrhythmias, finally leading to increased symptoms and death (31).

1.1.5. Classification

Patients were traditionally classified on the basis of their symptoms according to the revised classification of the New York Heart Association (NYHA). This classification stages the exercise tolerance (ET) of patients into 4 groups (NYHA I-IV).

NYHA I: cardiac diseases without physical limitation. Daily activities due not cause inadequate exhaustion, dyspnoea, arrhythmias or angina.

NYHAII: cardiac disease leading to mild limitation of ET. No rest symptoms but normal daily activity causes exhaustion, arrhythmias, dyspnoe or angina pectoris.

NYHA III: cardiac disease with marked limitation in ET. No symptoms at rest but minimal daily activities can cause exhaustion, arrhythmias, dyspnoea, or angina pectoris.

NYHAIV: cardiac disease leading to symptoms at any level of exercise and at rest.

As heart failure is (at least in theory) a largely preventable disease, primarily through the control of blood pressure and other cardiovascular risk factors, the new guidelines of the American College of Cardiology and the American Heart Association (37) have expanded the classification of heart failure now encompassing patients at high risk for the development of heart failure, but without any evidence of structural disease. This new approach to the classification of heart failure emphasises its evolution and progression, and defined four stages: A-D.

Stage A: high risk without structural heart disease and no symptoms.

Stage B: structural heart disease, no symptoms.

Stage C: structural heart disease, previous or current symptoms.

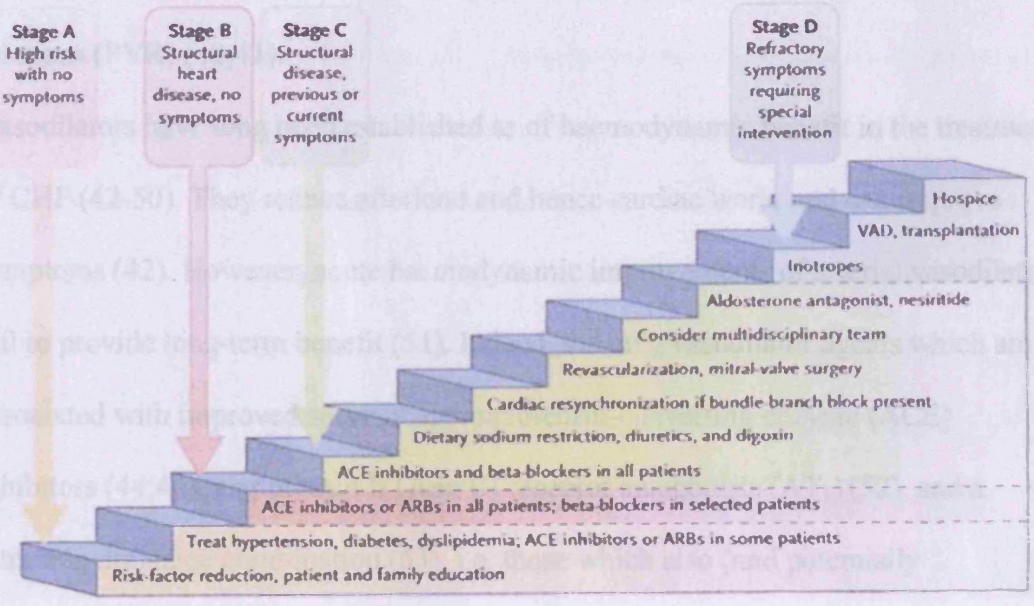
Stage D: refractory sumptoms requiring special interventions.

This new staged classification and treatment options for systolic heart failure are summarised in Figure 1.1.5.

It remains to be seen if this newer classification will be universally accepted, clinically implemented and used in future heart failure trials. The usefulness of this

approach is likely to lie in the extension, and combination with, rather than the replacement of the traditional NYHA classification.

Figure 1.1.5.



Patients with stage C heart failure have evidence of structural heart disease and a history of previous or ongoing symptoms, which can be classified as NYHA class I, II, III, or IV. (From Medical Progress - Heart Failure. In NEJM 2003;348:2007-18. With kind permission of the Massachusetts Medical Society).

1.2. Venous Physiology

1.2.1. Introduction

Many cardiovascular drugs act predominantly on the peripheral circulation and their effects on resistance vessels can be relatively easily assessed by relating changes in pressure to changes in flow (27;38;39). The venous circulation however serves a capacitance function and is better described in terms of pressure/volume (P/V) relations (PVR) (40;41).

Vasodilators have long been established as of haemodynamic benefit in the treatment of CHF (42-50). They reduce afterload and hence cardiac work, and can improve symptoms (42). However, acute haemodynamic improvements of arterial vasodilators fail to provide long-term benefit (51). Indeed, the only vasodilator agents which are associated with improved survival are angiotensin-converting enzyme (ACE) inhibitors (44;46), angiotensin II (Ang II) receptor antagonists (AT_1) (52), and a nitrate-hydralazine combination (53), i.e. those which also (and potentially predominantly) act on the venous beds (54). This observation illustrates the importance of the venous circulation, and the need to gain a greater understanding of the role this relatively neglected part of the vasculature plays in the pathophysiology of CHF.

1.2.2. Terminology

Because confusion can arise over the terminology used to describe the physical characteristics of veins, I include (Table 1.2.2.1.) a short description of the terms used in this thesis.

Capacitance	The term capacitance remains poorly defined and is not synonymous with venous volume as a smaller amount of volume resides within the heart and the arterial tree (Figure 1). In general terms it relates the total contained volume of the vasculature to a given transmural pressure over at least the physiological range of transmural pressures. In this thesis I will use, for simplicity, the term *venous capacitance* synonymously with venous volume.
Compliance	Is a general term describing the change in dimension following a change in stress. Translated into venous physiology this means that compliance is the ratio of the change in volume (ΔV) resulting from a change in transmural distending pressure (ΔP), or $\Delta V/\Delta P$. It is the slope of the PVR at a given point of the "curve", see Figure 2. Because venous compliance is very high at low pressures, the slope of the venous PVR over the physiological pressure range (10 to 40mmHg) is nearly linear.
Unstressed volume	The volume of blood in a vessel at zero transmural pressure is defined as unstressed volume. It is a virtual volume established by extrapolating the linear portion of the PVR to zero transmural pressure.

1.2.3. Importance of venous physiology

The veins and venules return the blood from the microcirculation to the heart. However they are far more than simple conduits. Indeed by regulating central blood volume and therefore preload, changes in venous tone regulate stroke volume via the Frank-Starling mechanism. Almost 80% of blood volume lies in these vessels, representing a large capacitance reservoir (41) (see Figure 1.2.3.1.).

Changes in regional venous volume can be mediated via 3 mechanisms. First, passively along the P/V axis (as illustrated by "A" in Figure 1.2.3.2.), for example if venous outflow is obstructed temporarily by pressures between 10-40 mmHg. Secondly, by a change in compliance (when the veins are not operating on the flat portion of the P/V curve – see Figure 1.2.3.3.), in which case the slope of the P/V relation is changed, as illustrated by "B". Thirdly, actively due to primary changes in venous tone, as illustrated by "C" (the latter results in a parallel shift of the P?V relation).

Veins are thinner-walled than arteries (see Figure 1.2.3.1.) and can therefore expand greatly. This explains why veins are much more compliant than arteries at low pressures (Figure 1.2.3.3. and Figure 1.2.3.4.). Despite this, there is sufficient smooth muscle in the walls of all but the smallest venules to actively modulate venous tone. It therefore follows that even small changes in venous tone are capable of translocating relatively large amounts of blood to and from the central compartment.

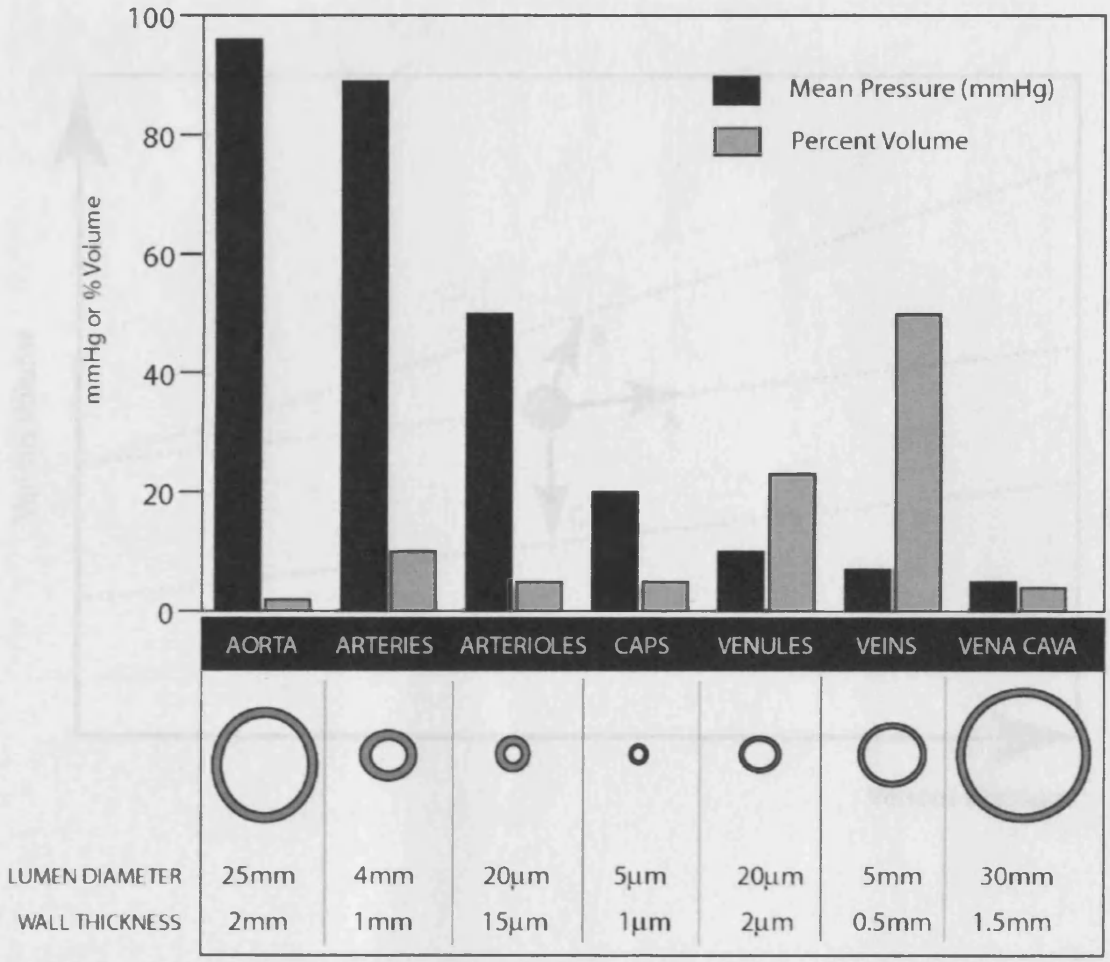


Figure 1.2.3.1. illustrates the blood volume distribution, pressures within each vascular compartment, corresponding lumen diameters and wall thickness. Modified from Vascular anatomy. In: Aarson PI and Ward JPT, ed. *The Cardiovascular System at a Glance.* Oxford: Blackwell Science Ltd, 1999: Page 10. With kind permission.

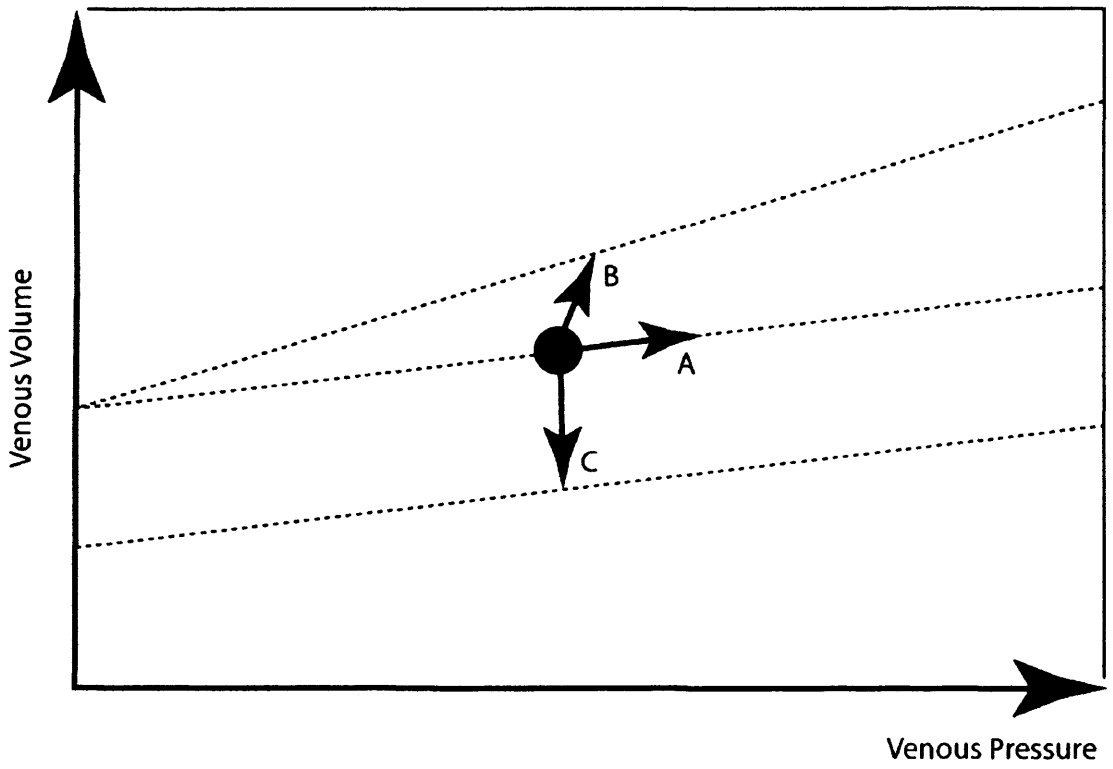


Figure 1.2.3.2. shows a venous PVR. Venous volume, depicted by the black dot can change in 3 ways. "A" illustrates an increase in venous volume along the slope of the P/V relation (dotted line), e.g. when venous outflow is obstructed temporarily over a physiological pressure range. "B" illustrates an increase in venous volume following a change (here increase) in venous compliance, demonstrated by a changing slope of the PVR. "C" illustrates a decrease in venous volume, in absence of a compliance change. Parallel-shifts reflect changes in venous tone (up-ward shift = decrease in tone, down-ward shift=increase in tone).

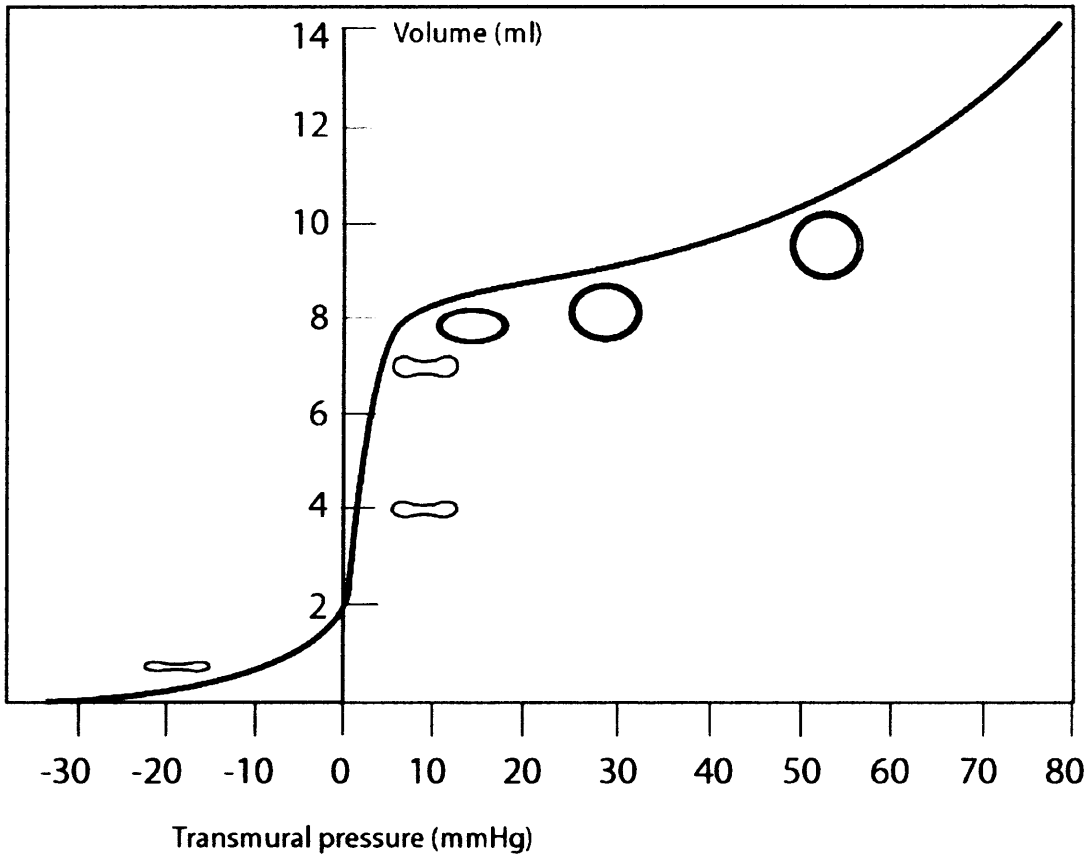


Figure 1.2.3.3. illustrates the venous transmural PVR over a wide range of pressures (note venous [transmural] pressure may be negative). The slope of the curve is referred to as compliance. Modified from The venous system. In: Aaronson PI and Ward JPT, ed. *The Cardiovascular System at a Glance.* Oxford: Blackwell Science Ltd, 1999: Page 46. With kind permission.

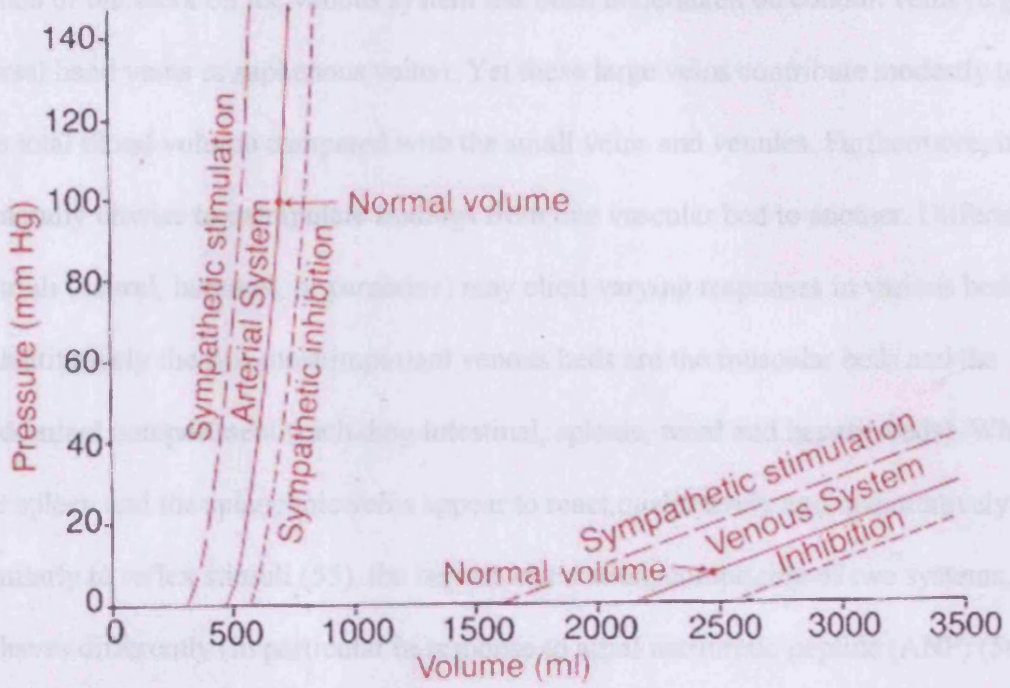


Figure 1.2.3.4. demonstrates parallel shifting arterial an venous pressure-volume relationships. The figure illustrates the marked differences in compliance and capacitance between the arterial and venous circulation. Taken from Physiology and Mechanisms of Disease. Guyton AC, Hall JE. 6th Edition, WB Saunders Company, Philadelphia, 1997, p.120. With kind permission.

1.2.4. Varying responses of individual venous beds

Much of the work on the venous system has been undertaken on conduit veins (e.g. dorsal hand veins or saphenous veins). Yet these large veins contribute modestly to the total blood volume compared with the small veins and venules. Furthermore, it is generally unwise to extrapolate findings from one vascular bed to another. Different stimuli (neural, humoral, or paracrine) may elicit varying responses in various beds. Quantitatively the two most important venous beds are the muscular beds and the abdominal compartment (including intestinal, splenic, renal and hepatic beds). Whilst the spleen and the splanchnic veins appear to react qualitatively and quantitatively similarly to reflex stimuli (55), the hepatic circulation, comprising of two systems, behaves differently (in particular in response to atrial natriuretic peptide (ANP) (56)) and meaningful assessment requires an invasive approach (57-59). Capacitance vessels in skeletal muscle may also behave differently and appears to vary between species. For example, some studies found little effects of arterial and cardiopulmonary receptors on limb venous capacitance (60) (61) despite marked changes in intestinal capacitance (62-66) whilst others found that the carotid sinus reflex had marked effects on skeletal muscle capacitance (67), indeed in one study there was a greater effect on skeletal muscle versus intestinal capacitance vessels (68). Part of these differences may depend on the species studied. Nevertheless part of the constricting effect on small muscular veins may be via a humoral effect, due to circulating catecholamines, following intense excitation of the vasomotor centre (68;69;69). In contrast, in larger veins such as the femoral veins and the vena cava, active changes can be elicited easily by the carotid sinus mechanism (70-72). From the response of the vessels to electric stimulation of the sympathetic nerves, as an index of changes in sympathetic outflow, it appears that a decrease in carotid sinus pressure causes the

same increase in nerve traffic to the resistance and capacitance vessels of the splanchnic region but a greater increase in traffic to the limb resistance vessels (68;72;72-74;74). The density of adrenergic innervation of individual blood vessels varies widely, partly reflecting their degree of participation in centrally controlled responses. The cutaneous veins are densely innervated (75) and respond to a variety of stimuli (e.g. cold pressor, limb exercise or deep breathing which can be inhibited with alpha blockade, consistent with a neurally mediated mechanism). However, cutaneous veins show not only no consistent immediate response to changes in arterial baroreceptor activity but changes in sympathetic outflow to cutaneous veins are often opposite to those of other capacitance vessels, e.g. stimulation of carotid chemoreceptors and muscle metabo- receptors cause reflex constriction in the splanchnic circulation whilst dilating cutaneous veins (76). Furthermore, whilst thermoregulatory mechanisms are believed to predominate over other inputs in the control of venomotor tone at ambient temperatures, during prolonged exercise in the heat a cutaneous vasoconstrictor drive superimposes its effect upon a high vasodilator drive and thus limits the absolute volume contained in the skin (77-81). The total amount of blood contained at a given time within the skin circulation at ambient temperatures is believed to be relatively small (≈3%). However, Fortney at al (82) could show that the degree of cutaneous vasoconstriction during exercise in the heat is greater after an acute reduction in blood volume than in normovolaemic conditions, implying that skin veins are indeed part of a functioning efferent arm of the blood pressure regulating mechanism. Tripathi and colleagues (83) provided information that this venoconstriction could be due to low pressure baroreceptor unloading. With a constant input from peripheral receptors of vascular beds with varying demands, metabolic states and function, the interaction between the pressor and

depressor areas will be modulated both by the metabolic conditions in the brain and by the activity of higher centres such as the cortex, hypothalamus, and limbic system, thus fulfilling the role of a central integration of all information in order to optimise the overall performance of the whole body.

It is clear from the foregoing that an understanding of the regulatory mechanisms involved in the control of venous tone is an important aspect of cardiovascular physiology. Yet our understanding of normal venous physiology in man is poor. Difficulties in developing valid and reliable techniques for assessing venous tone and compliance in the human capacitance bed have hampered attempts to gain a greater understanding of its precise role in human health and disease. Established techniques are available (84-89), but as discussed below, all have limitations when applied to the assessment of the capacitance bed. The introduction of "radionuclide plethysmography" (RPY) into the research arena more than 20 years ago (90-92) was able to overcome some of the limitations of conventional plethysmographic techniques and has contributed to a better understanding of human venous physiology (93-96) and exercise physiology in health (97) and disease (98-102).

1.2.4.1. A mechanical analogue of the venous capacitance system, or how changes in venous tone modulate cardiac preload

Gow suggested that a useful mechanical analogue when considering the reservoir function of the venous capacitance bed is that of a spring-loaded plunger in a syringe (Figure 1.2.4.1.) (103). The adjusting screw alters the position of the plunger and thus the capacity of the reservoir, analogous to alterations in venous tone. The compliance of the veins is represented by the stiffness of the spring, which may change when the position of the plunger (or tone) changes. In fact, changes in venous capacitance, and

hence unstressed volume, tend to be dominated by changes in tone. In contrast to the arterial bed, such changes in tone are not usuaaly accompanied by significant changes in venous compliance (103-105).

It is evident from the plunger model that an increase in tone in the venous capacitance bed will reduce volume in the peripheral "reservoir", displacing this volume into the central compartment, and consequently increasing central blood volume and right ventricular preload (volume). Initial support for such a theory, that changes in capacitance of the venous reservoir, mediated by changes in venous tone, determine central blood volume, came from Robinson's work (106). He found that the cold pressor test, which it was assumed would decrease the volume of the capacitance veins, significantly increased the cross-sectional area of the conducting veins. More conclusive evidence arose from the studies of Smiseth and colleagues (107). They investigated the effects of the contrasting vasoactive agents angiotensin and nitroprusside on splanchnic blood volume, pericardial pressure, and the left ventricular pressure/diameter relationship in healthy dogs. Nitroprusside caused an increase in splanchnic blood volume, which was associated with a fall in pericardial pressure, left ventricular end diastolic pressure (LVEDP) and LV diameter. Angiotensin had exactly the opposite effect. Furthermore, changes in pericardial pressure with the two drugs were inversely correlated with changes in splanchnic blood volume. Thus tone in the venous capacitance bed regulates central blood volume and right ventricular preload, by causing shifts of blood volume between the venous capacitance bed and the heart. Further evidence of this is provided by studies in (chronic) heart failure.

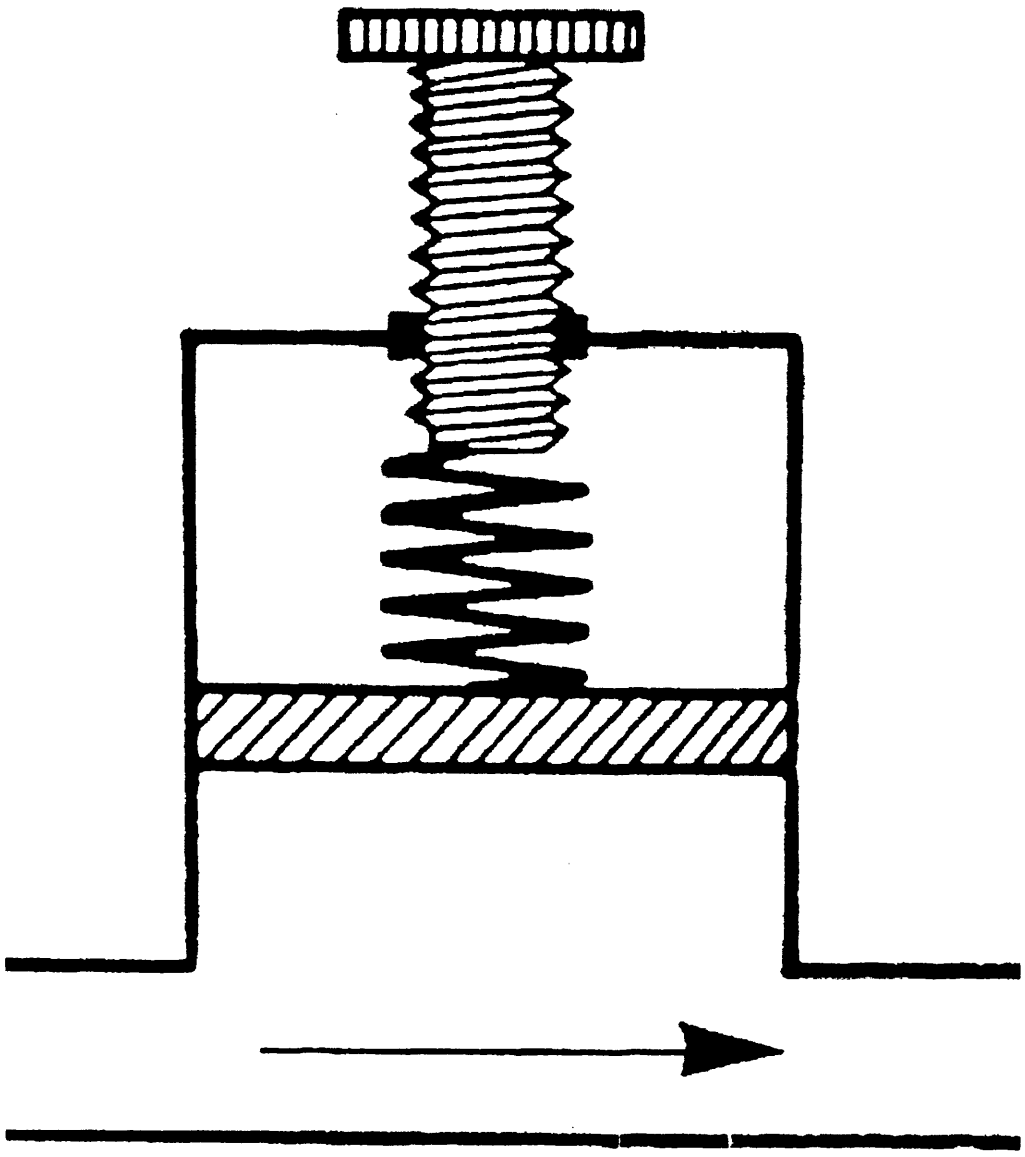


Figure 1.2.4.1. A mechanical analogue of the venous capacitance system. The adjusting screw alters the position of the plunger and thus the capacity of the reservoir, analogues to alterations in venous tone. The compliance of the veins is represented by the stiffness of the spring.

Modified from Gow BS. In: Bohr DF, Somlyo AP, Sparks HV Jr, eds. Handbook of Physiology. Volume II. American Physiological Society; 1980:353-408.

1.2.5. How changes in preload may affect cardiac output in health and heart failure

As noted above the importance of preload in regulating CO is described by the Frank-Starling relationship (Figure 1.2.5.1.). Frank, in 1895 (108) and later Starling, in 1914 (109), described the relationship between the volume of blood in the ventricles at the moment they begin to contract (end-diastolic volume) and the forced contraction developed by the ventricle. As left ventricular end-diastolic volume (LVEDV) increases, stroke work increases progressively, resulting in an increased CO. Starling's original curve constructed from an isolated heart preparation, described both an ascending and descending limb. According to this model, when the descending limb is reached, further increments in EDV would cause a decrease in stroke work. Conversely, reduction in preload, i.e. LVEDV, would result in an increase in cardiac function. However, the degree of LV stretch required to elicit a "descending limb" is thought to be considerably greater than that seen in human physiology or pathophysiology. However in clinical practice LVEDP (or its indirect measure pulmonary capillary wedge pressure (PCWP)) is often used as an index of LV preload. Using this measure of "preload" a descending limb of the Starling curve is frequently observed in heart failure. This is because increases in LVEDP may be accompanied by proportionately greater increases in pericardial pressure and right ventricular (RV) end diastolic pressure (RVEDP) resulting in constraint to LV filling by the pericardium (pericardial constraint) and the RV (diastolic ventricular interaction (DVI)), a reduction in LV and diastolic volume, and a fall in stroke volume.

The Frank-Starling relationship has been thought analogous to the length-tension relationship of skeletal muscle. Clearly, if an increase in preload is causes an increase

in stroke work, this must imply that an increase in sarcomere length causes an increase in tension. Indeed many authorities define preload as end-diastolic sarcomere length rather than end-diastolic volume (110). In skeletal muscle the explanation for this lies in the sliding filament theory, in which increased force generation occurs with increased sarcomere stretch due to stretching of the thin actin filaments to a configuration that allows maximal cross-bridge interaction with thick myosin filaments (111). However, there are clear differences in the behaviour between skeletal and cardiac muscle and the favoured explanation for the length-tension relationship in cardiac muscle is length-dependent activation. As sarcomere length increases, there is sensitisation of troponin-C to calcium, resulting in an increase in force development (112). Recently attention has focused on the role of titin, a giant protein spanning half the sarcomere, in the Frank-Starling phenomenon (113;114). Titin is now believed to be responsible for passive and restoring forces in cardiac myofilaments during sarcomere elongation and compression, respectively(115-117). In addition titin has been implicated in the length dependent activation that occurs in the stretched sarcomere, during the transition from diastole to systole (115). The Frank-Starling relationship thus illustrates clearly how ventricular preload is of vital importance in determining CO. It is the venous capacitance system which in turn regulates ventricular preload, and therefore critically influences cardiac function.

It is well established that CHF is associated with increased central blood volume (118). Similarly the efficacy of nitrate therapy to reduce central blood volume is clinically evident and scientifically proven (119-121). However, according to the Frank-Starling relationship, an increase in central blood volume and consequently ventricular preload should lead to an improvement in stroke work, whereas any treatment which reduces central blood volume, would be expected to result in a fall in

CO. The explanation for this apparent paradox, which underpins the importance of venous regulation of ventricular preload in the pathophysiology of CHF, lies in the phenomenon of direct DVI, as described above

Cardiac performance is exquisitely sensitive to the state of cardiac filling such that stroke volume may change as much as 50% in response to a change in filling pressure of as little as 1 cm H_2O if buffering reflexes are blocked or exhausted (40).

The importance of active control of the capacitance beds is especially relevant during exercise and in disease states when cardiac reserve is limited. For example, in healthy subjects upright exercise is associated with a 23% reduction in abdominal blood volume (liver 18%, kidney 24%, spleen 46%) and a 30% reduction of leg blood volume. This translocation of blood volume to the central compartment is closely correlated to oxygen consumption, contributing (via the Frank-Starling mechanism) to the increase in CO during exercise (97). Whilst impaired venoconstriction contributes to exercise hypotension in some patients with vasovagal syncope (98) and hypertrophic cardiomyopathy (99), an exaggerated response may potentially contribute to increased left ventricular and diastolic pressure on exercise in heart failure.

When acute heart failure is induced in a canine model, there is profound baroreflex mediated venoconstriction (due to hypotension) accounting for roughly 80% of the increase in LVEDP, the left ventricular dysfunction itself only accounting for 20 % of this increase in LVEDP (89;89;122;123). In CHF a rise in LVEDP (due to venoconstriction) may result in a fall rather than a rise in stroke volume (124). This is due to the aforementioned phenomenon of DVI (89;92;124-126) in which filling of the LV is impeded by external constraint from the right ventricle (RV) and pericardium (Figure 1.2.5.2.). The implication is that whilst in most physiological

situations, venoconstriction may be a compensatory mechanism to maintain stroke volume, in pathophysiological situations in which RVEDP and LVEDP are elevated, venoconstriction could be deleterious due to a reduction in stroke volume. In general, this phenomenon appears to be apparent when LVEDP is greater than approximately 15mmHg. This implies not only that ventricular interaction and venous capacitance modulate left ventricular preload (89) but also that there is an optimal LVEDP to maximize use of the Frank-Starling mechanism (Figure 1.2.5.1.).

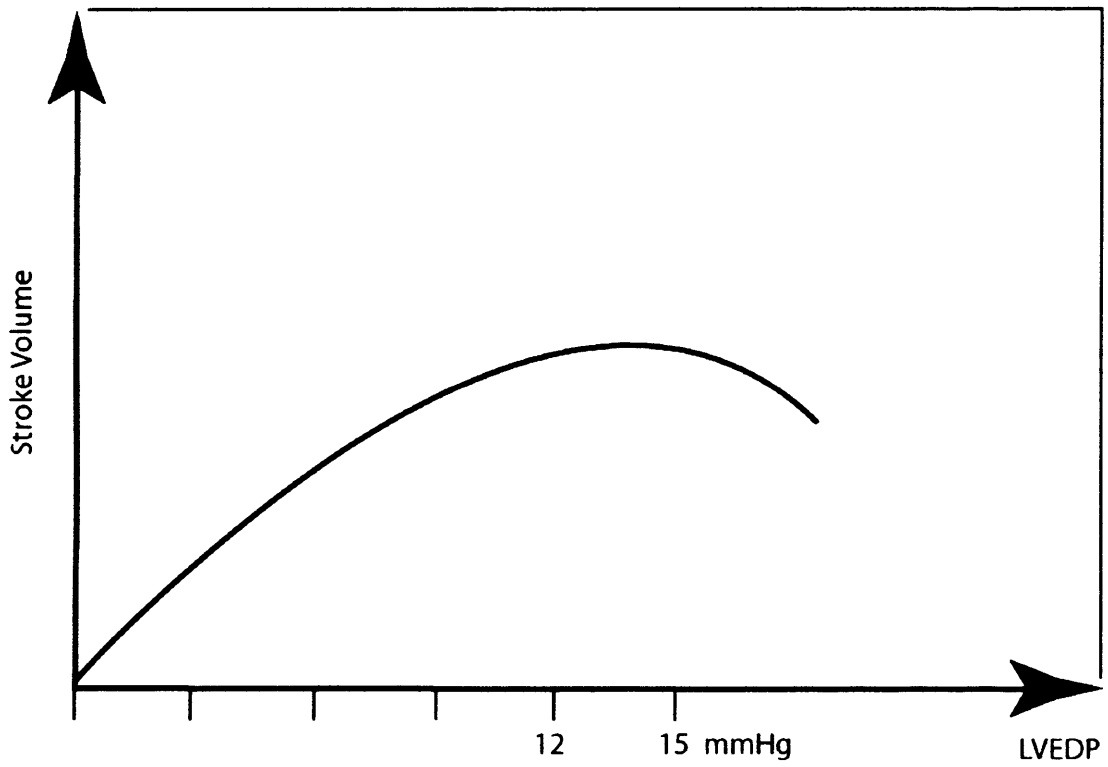


Figure 1.2.5.1. Illustrates the Frank-Starling or ventricular function curve with a descending limb, implying that there may be an optimal left ventricular end-diastolic pressure (between 12-15mmHg) to maximize use of the Frank-Starling mechanism.

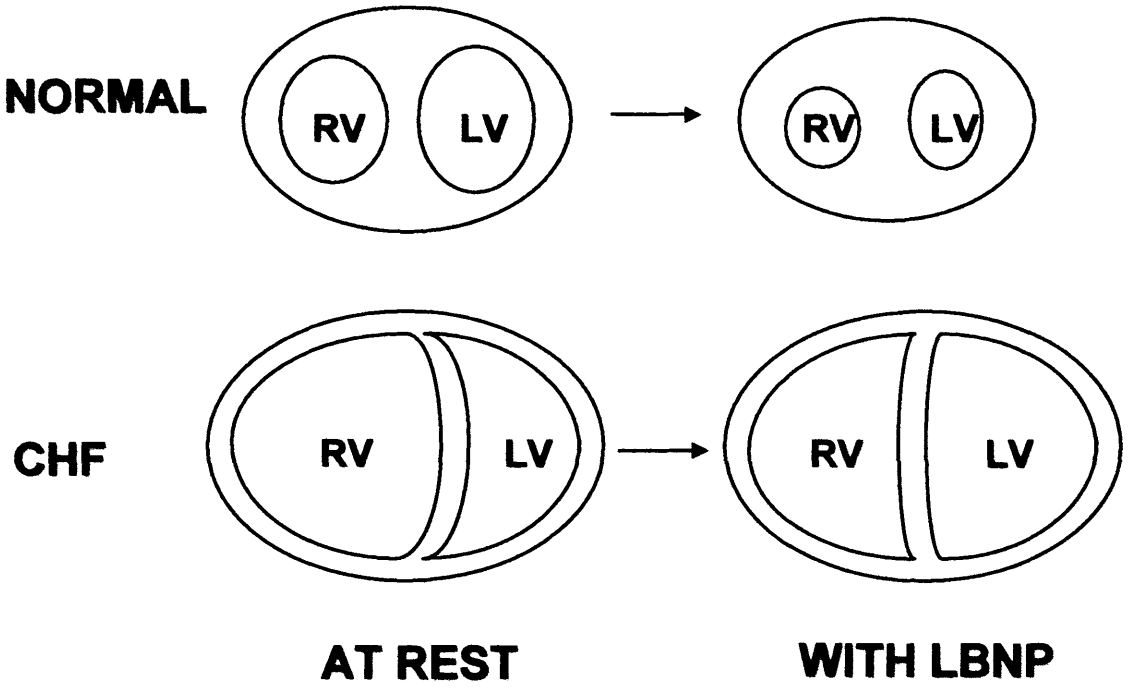


Figure 1.2.5.2. Illustrates the phenomenon of DVI. In health lower negative body pressure (LNBP) reduces LV and RV volume. In CHF patients with DVI, LNBP reduces RV volume but increases LV volume.

1.2.6. Venous tone in chronic heart failure

The concept of increased venous tone in CHF was first described over 40 years ago by J. Edwin Wood, who assessed venous volume and compliance in the forearm using fluid displacement plethysmography (127). He found that venous occlusion resulted in a much smaller increment in venous volume in patients with overt heart failure than in normal subjects or patients with compensated heart failure i.e. venous compliance was reduced in decompensated heart failure. He concluded that this was the first clear demonstration of peripheral venoconstriction in human CHF. In fact it is also consistent with a shift upwards to the steep portion of the PVR. A multitude of studies have since confirmed decreased limb vein distensibility in CHF, which can be reversed by either alpha-adrenergic blocking drugs or with appropriate anti-failure therapy (75;128;129). Elegant demonstrations of the increased tone in the venous capacitance bed, and its association with increased ventricular preload, have been provided by studies using canine heart failure models. Using a rapid ventricular pacing model and classic indicator-dilution methods, Ogilvie and Zborovska-Sluis demonstrated that the development of heart failure resulted in a profound parallel downward shift of the splanchnic venous PVR consistent with venoconstriction (130). They documented almost a 50% decrease in unstressed venous volume, with no associated change in compliance. Furthermore, central blood volume as a proportion of total blood volume increased markedly from 9% to 16%, indicating a shift of blood volume into the central compartment. These findings were replicated in a study of microembolisation-induced experimental acute ischaemic heart failure (122). Induction of heart failure was associated with a decrease in the splanchnic unstressed vascular volume (UVV). Furthermore, the venodilator drug with the greatest effect on UVV had the greatest effect on LVEDP and vice-versa. These observations suggest

that changes in LVEDP during development of heart failure, and during the administration of vasodilator agents, are mediated by the displacement of blood from the capacitance veins to the central compartment and from the central compartment to the capacitance veins respectively. They are compatible with the hypothesis that changes in venous capacitance modulate ventricular preload (89;92;104).

1.2.6.1. Causes of increased venous tone in chronic heart failure

The changes that occur in the regional circulations with CHF depend on a number of variables: (A) the stimulus for heart failure; (B) the severity of heart disease; (C) the stage of development of heart failure; and (D) the stress placed on the cardiovascular system. The development and progression of cHF are heralded by the activation of circulating neurohormonal systems that modulate vascular tone. In addition, more recent evidence has pointed to the importance of local changes in the peripheral vasculature, both structural and functional, which contribute to increased vascular tone. Table 1.2.6.1. summarises the causes of increased vascular tone in CHF. Although a considerable body of evidence exists to support the contribution of these factors to increased arterial tone, the importance of each in underlying the increase in venous tone which concerns this thesis is less certain.

Table 1.2.6.1. Causes of increased vascular tone in chronic heart failure

(A) Neurohormonal activation

1) Autonomic dysfunction

Activation of the SNS

Increased α-adrenergic receptor responsiveness

2) RAAS activation

3) Vasopressin

4) Natriuretic peptide tolerance/resistance

(B) Local Factors

1) Structural alterations (loss of elastin)

Interstitial oedema

Increased sodium content of vascular wall

2) Endothelins

3) Decreased NO bioavailability

4) Endothelium derived hyperpolarising factor(s) – reduced activity?

5) Dysfunctional Prostacyclins?

A) Neurohormonal activation

Activation of the sympathetic nervous system

It is well established that SNS activity is increased in CHF (131). This is characterised by both increased adrenergic nerve outflow and an increase in circulating catecholamines (132;133). The latter arises due to increased release of noradrenaline

(NA) from nerve endings and its consequent "spillover" into plasma (134), reduced uptake of NA by nerve endings (135), and a reduction in renal excretion of NA (134). Furthermore, during comparable levels of exercise, much greater elevations in circulating NA occur in CHF patients than in normal subjects (136), presumably reflecting greater activation of the SNS during exercise in these patients. Wood first suggested that increased adrenergic activity was a cause of increased venous tone in CHF (137). He found that ganglionic blockade caused an increase in forearm venous volume in CHF patients, albeit slight. Zelis later demonstrated a more impressive increase in limb venous volume after administration of the α-antagonist phentolamine, indicating that circulating catecholamines have an additional effect to that mediated by direct adrenergic nerve activity (138). However, Zelis also noted that even after α-blockade venous volume remained considerably lower in CHF patients than in normal subjects. He therefore concluded that sympathetic activation is not the only cause of venoconstriction in heart failure.

Increased α-adrenoceptor responsiveness

There is conflicting evidence as to whether CHF is also characterised by an increase in α-adrenoceptor responsiveness. The only evidence that this may contribute to increased tone in veins comes from Forster's demonstration of enhanced responsiveness to NA and phenylepinephrine in saphenous vein rings of dogs with rapid pacing-induced CHF (139). No human study has yet attempted to corroborate this finding.

Activation of the renin-angiotensin system

Activation of the renal renin-angiotensin system occurs initially due to renal sympathetic nerve stimulation (140), and as a consequence of diuretic therapy (141). Later, renal hypoperfusion directly stimulates renin release. In patients with mild

symptoms, plasma renin activity is normal at rest but rises on exertion (142). As symptoms become progressively more severe and renal perfusion pressure falls, plasma renin activity becomes elevated at rest, and the magnitude of the elevation parallels the degree of haemodynamic and functional impairment (143). In addition to the renal system, renin, angiotensin and ACE have been identified in blood vessels, resulting in local production of Ang II (144;145). Ang II is a potent direct vasoconstrictor, but also facilitates the vasoconstrictor actions of the SNS, as well as the release of vasopressin (146). In addition, Ang II may contribute to endothelial dysfunction by stimulating free radical production via Ang II-dependent NADPH-oxidase (147). In animal models of heart failure, Ang II has been shown to cause constriction of capacitance veins with a corresponding shift of blood volume into the central compartments (148). In patients with cHF, ACE inhibition results in a fall in Ang II levels (at least initially) and an associated reduction in venous tone (149;150). Thus it seems highly likely that activation of the RAAS contributes to venoconstriction in CHF.

Vasopressin

Vasopressin has a direct vasoconstrictor effect, but may also mediate vasodilation by causing baroreceptor mediated withdrawal of sympathetic activity, and by increasing the synthesis and release of NO and vasodilator prostaglandins (151). High plasma vasopressin concentrations have been documented in some patients with heart failure, its release stimulated by a reduction in CO and ineffective peripheral perfusion (152;153). In those patients in whom vasopressin levels are elevated, use of vasopressin antagonists has suggested that it contributes to arterial vasoconstriction (154). However, evidence that vasopressin may contribute to venoconstriction in CHF

is sparse. Indeed it appears to be a far less potent constrictor of veins than arteries (148), and therefore is unlikely to play a significant role in increasing venous tone.

Natriuretic peptides

NPs are ontogenetically ancient hormones with vasorelaxant properties. Plasma levels are generally increased in patients with heart failure, and correlate with atrial filling pressures and disease severity (155-157). In addition to having a direct vasodilator effect, ANP inhibits biosynthesis, release and turnover of NA (158;159), suppresses renin formation (157), opposes the systemic vasoconstrictor actions of Ang II, and inhibits both the release and the vasoconstrictor effect of vasopressin (160).
Whilst the vasorelaxant effects of NPs are well documented in the resistance vasculature, controversy exists about their effects in the venous system. A number of human studies assessing the effects of NPs on human conduit veins such as the dorsal hand veins (161;162) or the saphenous veins (163) have suggested that NPs have very little if any effects on these veins. However, ANP infusion consistently reduces central venous pressure (CVP), even in nephrectomised animals (164) and in patients with heart failure in the absence of significant diuresis or changes in haematocrit (165-167). This raises the possibility that ANP regulates regional venous volume by modifying venous tone in the small veins and venules. Further support for a venodilating role of ANP comes from a study by Roy et al who found that low dose infusion of ANP decreased left ventricular end-systolic and end-diastolic volumes in the absence of any change in, total peripheral resistance (TPR) or forearm vascular resistance. These authors concluded that short-term increases in plasma ANP within the physiologic range may primarily affect the venous vascular bed either by decreasing vascular volume or by venodilation (168). To date only a single study,

using an in-vivo trout model, has provided direct evidence that NPs have venodilating effects in capacitance vessels (169). The effects of NPs on regulation of regional venous volume and tone in health and CHF will be the main focus of this thesis.

(B) Local Factors

Structural

In CHF, abnormalities in vascular structure may contribute to increased vascular resistance at rest and impair the vasodilator response to exercise or hormonal stimuli (151). Whereas there is no direct histopathological evidence of altered vascular structure in humans with heart failure, physiological evidence suggests that (potentially reversible (170)) functional vascular remodeling does occur (171). Zelis found that calf venous volume was reduced in CHF patients compared to healthy controls, even after maximal venodilation induced by intraarterial injection of sodium nitrite (138). He concluded that there are mechanical factors limiting metabolic vasodilation. These latter mechanical factors have been called the heart failure "stiffness component" and are related to sodium retention and plasma volume expansion, which results in an increased vascular sodium content (172) and to interstitial oedema (35). In support of a role for salt and water retention in increasing vascular tone, it has been shown that administration of diuretics increases the arterial vasodilator response of reactive hyperemia (173).

Functional

Over the last 3 decades considerable interest has focused on the role of the endothelium in regulating vascular function. It has become apparent that the endothelium itself is the source and side of action of a multitude of vasoactive signalling molecules and peptides. The bioavailability of some of these agents is markedly altered in CHF. The role of NO and the endothelium in regulation of venous

tone (96) and exercise limitation in cHF (174) were investigated by my colleagues and predecessors at the WHRI; Dr Dan Blackman and Dr Angus Nightingale respectively. The role of endothelial dysfunction in regulation of vascular control has consequently not been the focus of the present work and is reviewed in detail in above referenced work and elsewhere (175-177).

1.3. The natriuretic peptide system

1.3.1. Introduction

Up-regulation of the NPS, most evidently in CHF and hypertension, is not only a hallmark of many cardiovascular disease states but also a useful diagnostic tool (178;179), prognosticator (180), and potentially a guide to optimise therapy (181;182). The early recognition of the NPS as a potential target for therapeutic intervention was an important driving force behind the concentrated research efforts in this field. This enthusiasm has recently been damped by the somewhat disappointing results of OVETURE (183), which was at the time the largest ever randomised controlled trial (RCT) undertaken in CHF.

1.3.2. Natriuretic peptide research – a historic perspective

Historical descriptions of a phenomenon related to the endocrine heart were made more than 2000 years ago. Flavius, a Roman historian called the workers diving to build the maritime harbour of Caesarea "urinatores". Due to intra-thoracic pressure changes during immersion, the increased diuresis forced these divers to urinate frequently.

Thus, the later discovery of the so-called Henry-Gauer-reflex (184) was anticipated much earlier. The Henry-Gauer hypothesis postulates that changes in left atrial pressure induce changes in the release of arginine vasopressin, which subsequently modulates the renal output of fluid (185;186). However, results of subsequent studies indicate that this hypothesis is too simplistic in explaining the complexity of extracelular fluid volume control (187-189). The identification of atrial granules, using electron microscopy (190) (see plate 1.3.2.1) , possibly marks the real start of natriuretic peptide research. This publication appeared simultaneously but

independently from the physiological studies by Henry and Gauer (184) demonstrating that diuresis is induced by atrial distension, a phenomenon long known and described earlier by Karel Frederick Wenkebach in his work about cardiac arrhythmias and their clinical significance (191). Initially progress was slow, but following de Bold's seminal observation (192) that infusion of atrial but not ventricular extracts into rats caused copious natriuresis, diuresis and hypotension, research gathered momentum and was advanced rapidly by the application of the emerging molecular biological methodologies. The bioactive substances causing this natriuresis were soon purified and their amino acid sequence identified by several researchers. In 1983 Flynn first purified ANP from rat atria to homogeneity and produced the first amino acid sequence of the molecule (193). One year later Kanagawa and Matsuo reported the complete amino acid sequence of α-human atrial natriuretic polypeptide (194), Figure 1.3.2.1. The structure of the prohormone revealed that the ANP peptide comprised of the c-terminal 28 amino acids of the precursor. It is sometimes written ANP (1-28) and often written ANP (99-126). In 1984 the m-RNA's of the cardiac peptides of several species were analysed (195;196;196;197;197-199) and the gene structure of human ANP was also identified and sequenced (200;201;201;202;202).

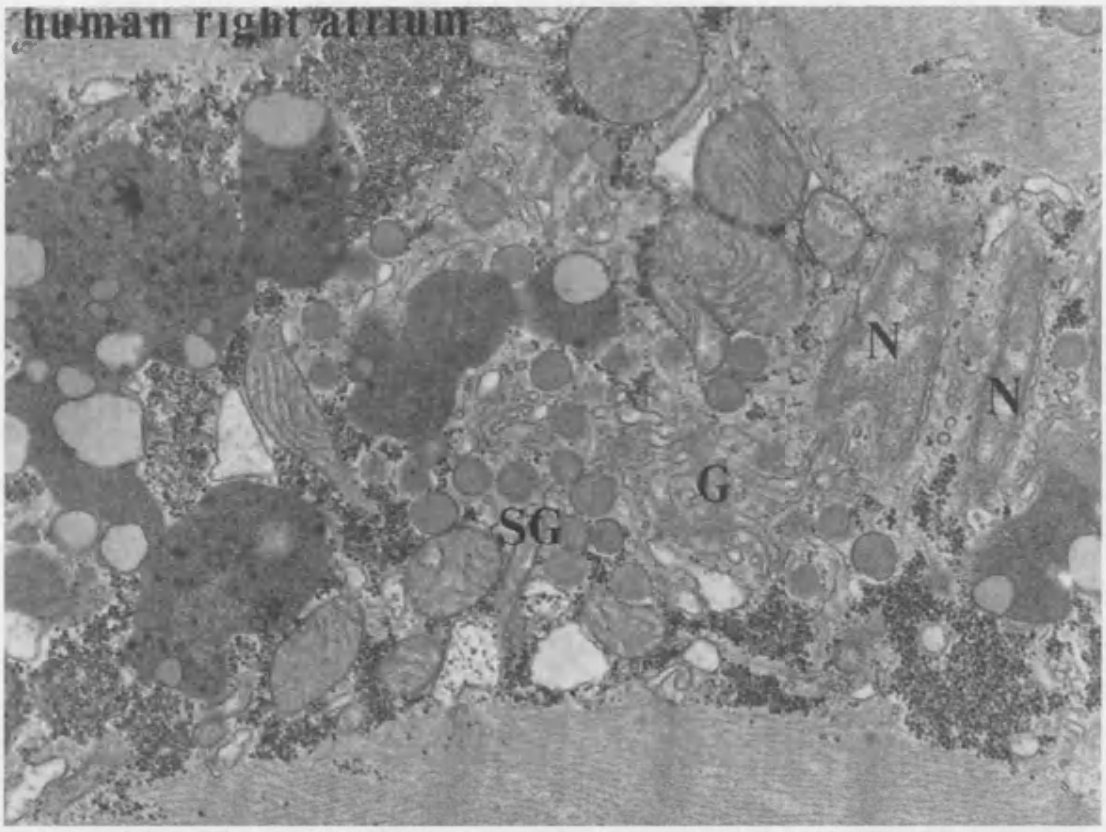


Plate 1.3.2.1. Electromicroscopic ultrastructure of human atrial myoendocrine cells: the perinuclear region containing the Golgi apparatus (G) is seen. The Nucleus (N) is round and lobated, further myofibrils, lysosomes, and mitochondria are present. The secretory granules (SG) are slightly variable in density and size (Reproduced from Ref (203) with kind permission from Springer).

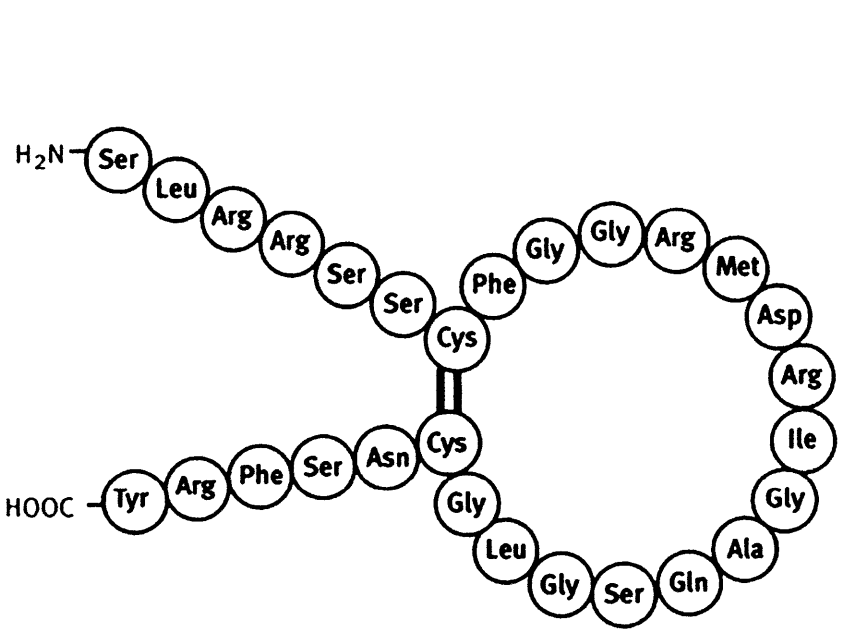


Figure 1.3.2.1. The aminoacid sequence and ringstructure of ANP (99-126) is shown.

1.3.3. Biochemistry and Molecular Biology of Natriuretic Peptides

The natriuretic peptide family consists of several "mature" NPs that share a common structural motif, consisting of a 17 amino acid loop formed by an intracellular disulphide linkage, Figure 1.3.3.1. Furthermore, some (few) authors also consider the amino- terminal cleavage products, arising during processing from the pre-prohormones via prohormones to the final mature hormones as essential parts of the NP-system (204) (Table 1.3.3.1.). In humans 4 "mature" NPs, ANP, Urodilatin, brain natriuretic peptide (BNP) and c-type natriuretic peptide (CNP) have been isolated. A further natriuretic peptide, dendroaspis natriuretic peptide (DNP), has been isolated from the venom of the green mamba (205) and "DNP-like immuno-reactivity" has recently been shown in patients with CHF (206). The significance of these findings is currently debated (207) and is the subject of ongoing research (208;209).

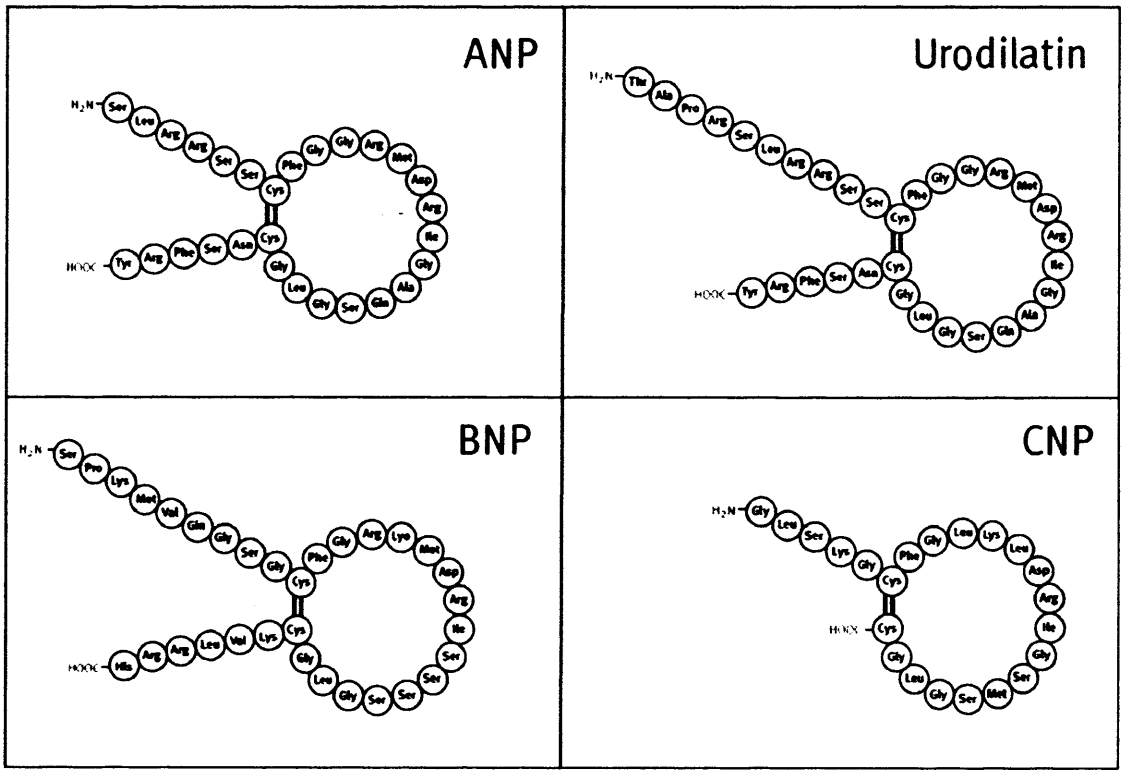


Figure 1.3.3.1. Demonstrates the aminoacid and ringstructure of all four human NPs.

Table 1.3.3.1. "ANP-Family"

Mature ANP's	amino-terminal cleavage products	synthetic "ANP's"
ANP_{99-126}	LANP (i.e. pro-ANP_{1-30})[210]	cANF[211]
ANP_{95-126}	Vessel dilator (i.e. pro-ANP_{31-67})[212]	Anaritide®[213] (i.e. $ANP_{102-126}$)
	Kaliuretic peptide (i.e. pro-ANP_{79-98})[210]	Mini-ANP[214]

1.3.3.1. Processing of natriuretic peptides: from gene to peptide

The structure of the ANP gene, in humans located on the long arm of chromosome 1 (p36.2), is schematically shown in Figure 1.3.3.1.1. The BNP gene is also located on chromosome 1 in close proximity to the ANP gene. The ANP and BNP genes both comprise 3 exons separated by 2 introns. The CNP gene is located on chromosome 2 and is comprised of 2 exons and 1 intron.

Exon 1 encodes the signal sequence which is cleaved from the pre-prohormone (151aa) in the endoplasmatic reticulum (ER) to form a pro-hormone of 126 amino acid residues, which is the storage form of ANP, and the first 16 amino acid residues of the pro-hormone. Exon 2 encodes for the majority of the remainder of the pro-hormone (i.e. aa 17-125 in humans), whereas Exon 3 encodes only for amino acid 126 (Tyrosine) in humans, and the 3 C-terminal amino acids in rat, mouse, rabbit and cow. As noteded above, in humans, ANP is produced from a 151 amino acid pre-pro-hormone, which after cleavage of a 25 amino acid hydrophobic signal sequence is stored in atrial granules, predominantly as pro-hormone 1-126. Figure 1.3.3.1.1. shows in schematic form the gene structure and post-translational processing from pre-pro ANP to pro-ANP, ANP and Urodilatin. The proteolytic cleavage of pro-ANP 1-126 occurs normally at Arg98-Ser99 in the C-terminal region during secretion to

yield mature ANP 99-126. This cleavage step is performed by corin, a cardiac serine protease and this protease may therefore be seen as the "pro-ANP-converting enzyme" (215;216;216).

Corin does not convert pro-CNP into CNP, the latter conversion step is highly dependent on the presence and activity of the endoprotease furin, and furin processed CNP has been shown to be biologically active (217).

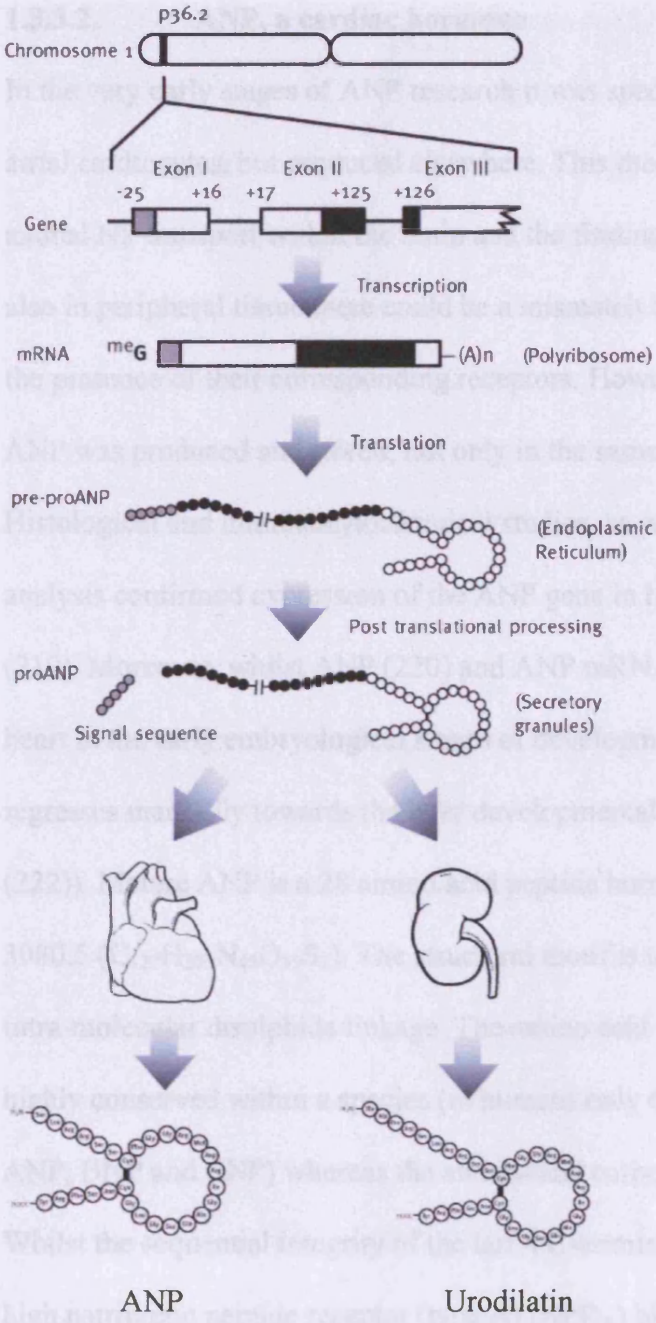


Figure 1.3.3.1.1. The ANP gene comprises three exons and two introns. Exon 2 codes for the majority of the pro-hormone (i.e. amino acids 17-125 in humans). Note the different posttranslational processing of the cardiac and renal peptides from the same gene.

1.3.3.2. ANP, a cardiac hormone

In the very early stages of ANP research it was speculated that ANP may be stored in atrial cardiocytes, but produced elsewhere. This theory was based on the finding of axonal NP transport within the brain and the finding that especially in the brain but also in peripheral tissue there could be a mismatch between the presence of NPs and the presence of their corresponding receptors. However, it soon became clear that ANP was produced and stored, not only in the same region but also in identical cells. Histological and immunocytochemical studies, as well as in-situ hybridisation analysis confirmed expression of the ANP gene in human (218) and ovine fetal heart (219). Moreover, whilst ANP (220) and ANP mRNA (221) are present throughout the heart in the early embryological stages of development, the ventricular representation regresses markedly towards the later developmental phases (221) (for review see Ref. (222)). Mature ANP is a 28 amino acid peptide hormone with a molecular weight of 3080.5 ($C_{127}H_{203}N_{45}O_{39}S_3$). The structural motif is a 17 amino acid loop, formed by an intra-molecular disulphide linkage. The amino acid sequence of the ring of the NPs is highly conserved within a species (in humans only 6 aa within the ring differ between ANP, BNP and CNP) whereas the amino- and carboxy-terminal tail vary markedly. Whilst the sequential integrity of the last 4 C-terminal amino acids is important for high natriuretic peptide receptor (type A) (NPR_A) binding affinity, the additional presence of Aspartic acid in position 13 and Phenylalanine in position 26 appears to be crucial for activation of guanylate cyclase (GC) and cGMP production following receptor binding (223;224). It is noteworthy that there are several reduced-size "mini-ANP" available that vary in their affinity to the NPR_A-receptor as well as their ability to generate cGMP. Furthermore, due to variation in post-translational processing, several different "amino-terminal ANP's" exist (204). The fate and biological activity

of these cleavage products of the amino-terminal tail, occasionally called "long acting ANP" (LANP, i.e. proANP 1-30), "vessel dilator" (i.e. proANP 31-67), and "kaliuretic peptide" (i.e. proANP 79-98) is highly controversial. Whilst they were previously believed to have little or no biological effects, some evidence suggest that they have some natiuretic, kaluretic and vasoactive potency (210), but that the mode of action is different from mature ANP (225). For example, kaliuretic peptide and LANP are believed to exert their biological action via inhibition of a Na^+-ATPase (226) by increasing prostaglandin E_2 (227).

1.3.3.2.1. ANP secretion

Following processing from pre-prohormone to prohormone (the storage form), cleavage and secretion of mature ANP is predominantly in response to increased transmural atrial pressure or stretch (228), which in an intact physiological organism is mainly a consequence of volume expansion. However, several other stimuli are capable of inducing ANP release (for review see Ref. (229)). These include vasopressin-, adreno-, endothelin-, angiotensin II-, enkephaline- and morphine receptor stimulation. Very recent evidence also suggests that endothelin, tumour necrosis factor α (α-TNF) and other cytokines may play a regulatory role in its production and release (230;231;231;232;232;233;233).

1.3.3.3. Urodilatin

Urodilatin is the name given to a naturally occurring paracrine acting ANP homologue first reported by Schulz-Kappe in 1988 (234). The amino acid sequence reveals that it is an N-terminal extension of ANP (see Figure 1.3.3.1.). It comprises residues 95-126 of pro-ANP, and therefore it is very likely that both Urodilatin and

ANP are derived from a common precursor ANP 1-126. Urodilatin is believed to be a kidney derived peptide and immunohistochemical studies identified high concentrations in the cortical tubules and around collecting ducts (235-237) contrasting with the predominant cortical location of renal ANP binding sites (238). Whilst the overall biological actions of Urodilatin closely resemble those of ANP (239) it is believed that Urodilatin is more important than ANP in regulating renal sodium excretion (240-242). Like ANP, Urodilatin is cleared by the C-type receptor, but in contrast is not degraded by neutral endopeptidase 24.11. (243).

1.3.3.4. BNP

B-type or BNP was first isolated from pig brain as either a 26 or a 32 aminocid peptide (244;245). Human BNP was isolated in 1989 and found to comprise of 32 residues, consisting of amino acids 77-108 of the BNP precursor peptide. Whilst these peptides, derived from the C-terminus of the pro-BNP molecule, show great similarity to the structure of ANP it is noteworthy, that in contrast to ANP, the amino-acid sequence of BNP exhibits marked inter-species variation. The same is true for the pro-hormones. Furthermore, the post-translational processing of BNP appears to differ from ANP, in that conversion of BNP precursors occurs intra-cellularly rather than during secretion. BNP binds to the same receptors as ANP (see Figure 1.3.3.1.) and is believed to exert its biological actions, namely natriuresis, diuresis and vasorelaxation via the GC coupled NPR_A-receptor. BNP is predominantly synthesised in the ventricles and when tissue weight is taken into account, the total content of BNP mRNA is approximately 3-fold greater in the ventricles than in the atria. In marked contrast ANP mRNA content in the ventricle is only 7% of that in the atria (246). Interestingly, whilst 60% of circulating BNP is ventricular in origin the mature BNP

tissue concentration in the ventricle is only 1% of that in the atria. This observation of a large amount of production and secretion but a small amount of storage of BNP in the ventricles (in health) is consistent with the previous finding that ventricular cardiocytes secrete ANP more rapidly after its synthesis via the constitutive pathway than atrial myocytes (247). Due to the greater augmentation of gene expression, the more sustained production and secretion process, and the longer plasma half-life, BNP has certain practical advantages as a diagnostic tool over ANP. In disease states that cause hypertrophy, myocardial necrosis or heart failure there is greater induction and more rapid turnover of BNP mRNA and a much greater increase in circulating levels over basal levels compared to ANP (248). For these reasons it has been suggested that BNP may act as an evolutionarily younger ANP support peptide against ventricular P/V overload, compensating for an insufficient ANP-NPR_A system. However, this theory is not completely supported by evidence from knockout mouse models where interruption of the ANP-NPR_A system was not consistently accompanied by up-regulation of BNP production. BNP appears slightly less potent in generating cGMP in human arterial and venous tissue than ANP (249), has similar potency in endothelin pre-constricted conductance and resistance coronary arteries (250), but has been shown to have less powerful local vasorelaxant effects in the resistance vasculature of the human forearm in health (251;252;252), whilst it was found to be roughly equipotent to ANP in the forearm resistance vasculature in CHF patients (252). Hunt et al studied the biological effects of ANP and BNP infusions in normal subjects (253). Equimolar infusions caused a similar rise in ANP and BNP levels. Whilst the increase in cGMP was 4 fold higher with ANP than with BNP, natriuresis, contraction in plasma volume and inhibition of plasma aldosterone were comparable. Interestingly, whilst the pressor response to Ang II was unaffected by

ANP or BNP, ANP but not BNP significantly inhibited the plasma aldosterone response to Ang II (253). In a series of experiments the same group further investigated the interactions between several NPs in health (254;255) and disease (256),(257), (258). They found similar interactions between ANP-BNP and BNP-ANP infusions with regard to metabolic clearance rate and disappearance rate from plasma (in health) suggesting that additive effects in regard to cGMP stimulation and blood pressure lowering effects resulted from dissociation of pre-bound hormones, presumably from biological or clearance receptors. Intriguingly, no additive effect on the RAAS and SNS (heart rate (HR) and plasma catecholamines) was seen at the infused doses (leading to low pathophysiological concentrations) (254;255). In a study in mildly hypertensive subjects, both peptides (given intravenously) suppressed the RAAS to a similar degree (258), whilst in another study intra-renal BNP infusion did not induce changes in renal blood flow, secretion of active renin, or creatinine extraction (259).

Interestingly, a very recent study using a canine model of pacing induced heart failure showed that BNP infusion improved central hemodynamics whilst there was resistance to the effects of ANP. The authors speculated that these findings would support the existence of a so far undetected BNP-selective NP receptor (260). Support for such a theory derives from an earlier study by Goy et al (261). Using a NPR_A knockout mouse model these authors found that testis and adrenal gland retained statistically significant, high affinity responses to BNP, which could not be accounted for by $NPR_{B/C}$, suggesting the presence of a novel receptor in these tissues that prefers BNP over ANP. However, at pharmacological doses the overall hemodynamic profile of ANP and BNP, given to patients with CHF, appear to be comparable.

1.3.3.5. CNP

CNP, originally isolated from porcine brain (262), exists in two mature forms, as 22 amino acid form CNP-22 (see Figure 1.3.3.1.), highly homologous to ANP but lacking the C-terminal tail, and as CNP-53, which N-terminal sequence is extended by 31 amino acids (263). Unlike ANP and BNP, both of which originate from the heart, humoral CNP is believed to be predominantly of endothelial origin. However, highest tissue levels in humans are found throughout the brain (10 times higher than concentrations of ANP or BNP), consequently CNP is believed to be a major non-conventional neuro-transmitter. Its actions include inhibition of vasopressin and ACTH secretion as well as modulating central regulation of blood pressure (264). Until recently there was no entirely convincing evidence for CNP-synthesis within the heart. Kalra et al (265) found that in patients with CHF the central arterio-venous (AV)-gradient for CNP is raised in a NYHA class dependent fashion, indicating a cardiac origin and up-regulation of CNP in this condition. Very recently, Horito et al could demonstrate that CNP is indeed synthesised in and released from cardiac fibroblasts suggesting that CNP has a suppressive effect on fibroblast proliferation and extracellular matrix production (266). This assumption would be in keeping with the earlier finding by Doyle et al which demonstrated that NPR_B receptors in adult rat ventricle are predominantly confined to the nonmyocyte population, i.e. cardiac fibroblasts (267). Furthermore, findings that specific CNP receptors (NPR_B) and CNP gene transcripts exists within the vascular wall, the kidney (268), and within the central and peripheral nervous system strongly indicate a possible regulatory role for CNP in cardiovascular homeostasis (for review see Ref. (269)). However, in contrast to ANP and BNP, CNP actions appear to be predominantly autocrine/paracrine rather than endocrine. CNP is vasorelaxant in dogs (270) and humans (271) and when tested

in isolated canine vessels (272), was relatively more potent as a venodilator than ANP. Furthermore, in canine arterial vessel preparations there were differential responses to ANP and CNP. CNP was more active in saphenous rings and less active in renal artery rings (272). Similar findings were reported in the pulmonary circulation of newborn lambs were ANP caused greater relaxation of pulmonary arteries than veins, and CNP was more potent in relaxing pulmonary veins than arteries (273). CNP also possesses coronary vasodilator properties. The mechanisms of this vasodilatation appear complex and include at least the particulate GC system (in dog coronaries) (274) as well as smooth muscle membrane hyperpolarization through potassium channel activation (in pig coronaries) (275). In isolated canine femoral veins the soluble and particulate GC system as well as activation of large-conduction, calcium-activated, potassium channels contribute in mediating vasodilatation (276). In human preparations CNP has both veno- and arterial dilator actions (161;277;278). In-vitro experiments, using human vascular tissue, showed CNP to augmented cGMP production weakly (less than ANP) and equally in human saphenous veins, gastroepiploic and internal mammary arteries (279). Intra-arterial infusions of CNP in humans also induced much less arterial dilatation than does ANP (278). Importantly, in contrast to ANP (280) the forearm arterial vasodilatation following local CNP infusion appears less dependent on endothelial NO synthase (eNOS) but at least in large part dependent on hyperpolarisation (271).

Other cardiovascular actions of CNP include inhibition of Ang II stimulated endothelin-1 (ET-1) release in porcine endothelial cells (281), where CNP was found to be more potent than either ANP or BNP. In addition CNP was found to inhibit vascular ACE activity (282). CNP-22 inhibits vascular smooth muscle cell (VSMC) growth in tissue culture (283) and inhibits intimal thickening after vascular injury

(284) raising the possibility that CNP may have an important anti-mitogenic role in the prevention of atheroma. Taken together these findings support the existence of a vascular NPS (285) in which CNP participates as an endothelium derived autocrine/paracrine regulator of vascular tone and remodelling.

1.3.4. Natriuretic peptide receptors, intracellular signalling and metabolism

Circulating ANP is rapidly removed from the circulation by two mechanisms. First, by binding to the abundantly expressed NPR_C receptor (also called the "clearance receptor"), second, via enzymatic degradation through a zinc dependent metalloprotease termed neutral-endopeptidase (NEP 24.11). This enzyme was previously known as "enkephalinase", because of its role in morphine and enkephaline metabolism. NEP 24.11 is an non-specific enzyme that is also involved in the breakdown of a variety of vasoactive peptides including the vasoconstrictors Ang II and endothelin-1, as well as several vasodilators including NPs, bradykinin, substance-P and adrenomedullin (ADM) (286). In sheep the contribution of each ANP eliminating factor is roughly equal (287). In humans the significance of both NP-eliminating systems is not well defined but the contribution of NEP is possibly less than that of clearance via NPR_C binding.

The plasma half-life time of mature ANP is 150-200 seconds and the various amino-terminal pro-ANP-cleavage products are believed to have a half life of several minutes. In contrast to CNP, where plasma levels appear to be consistently higher in venous than arterial blood, ANP has consistently higher arterial than venous levels (at a ratio of around 2:1) in all vascular beds so far studied. ANP is believed to exert its biological effects predominantly via binding to the NPR_A-type or $NPR1_A$. Of all the known NPs, ANP exhibits the highest affinity for this receptor, found on the luminal

surface of endothelial cells and the endothelial surface of VSMCs. The affinity for NPR_A binding is ANP>>BNP>>>CNP (Figure 1.3.4.1.). The same trend is demonstrated with respect to cyclic GMP production following ligand-receptor binding. Whilst ANP appears to be the natural ligand for the NPR_A, CNP is the natural ligand for the NPR_B. So far no natural/primary BNP receptor has been found. Whilst the acute haemodynamic effects of BNP are weaker, its secretion process is more sustainable than that of ANP. The structure of NPR_A and NPR_B bear close resemblance whilst NPR_C differs (Figure 1.3.4.1.). NPR_{A+B} are membrane bound GC coupled receptors with a molecular mass of 130-180 kDa (288-291). The structure and roles of these GC-receptors in blood pressure regulation, cardiac and renal physiology have recently been reviewed in detail (292). NPR_C, a homodimer of 64-66 kDa (293), is lacking a GC domain but contains a 39 amino acid intracellular tail that contains a G-protein activating sequence. It appears to mediate signal transduction (for review see Ref. (294)) either through inhibition of adenyl cyclase (possibly via Gi-2) (295-300) or activation of phospholipase C (possibly via the βγ-subunit of Gi1 and Gi2) (294;301). Following receptor-ligand (ANP/BNP/CNP to NPR_{A+B}) binding there is activation of the GC subunit. This in turn appears to be regulated by the attached protein kinase homology region, because if deleted, ligand binding and GC activity are uncoupled from each other (302). Increased intracellular cGMP levels alter the conductivity of several ion channels and ultimately result in VSMC relaxation. Furthermore, ANP is believed to affect metabolism of phosphatidyl-inositol biphosphate to IP_3 and diacylglycerol, a recognised important signal transduction pathway for hormones mobilizing intracellular calcium, in distinct partially opposing ways. Resink et al initially observed this process (303) and Hirata (304) demonstrated that a truncated ANP analogue (amino acids 103-123), produced

the same effect, thereby dissociating the action from the $NPR_{A/B}$ receptor. This ANP-NPR_C mediated stimulation of phospholipase C was observed in quiescent cells. Conversely, hormone-stimulated phospholipase C activity was inhibited by either ANP or other stimulants of GC activity (305). Whilst stimulation of phospholipase C activity by ANP has been observed only in vascular tissue (303) an inhibitory effect has been observed in vascular tissue and the kidney (306;307). Although the ultimate significance of ANP actions on phospholipase C activity are still not completely known it is clear from the aforementioned that the proportional presentation of $NPR_{A/B}$ and NPR_C within a vascular bed will affect the biological response to ANP exposure and may therefore differ in health and disease.

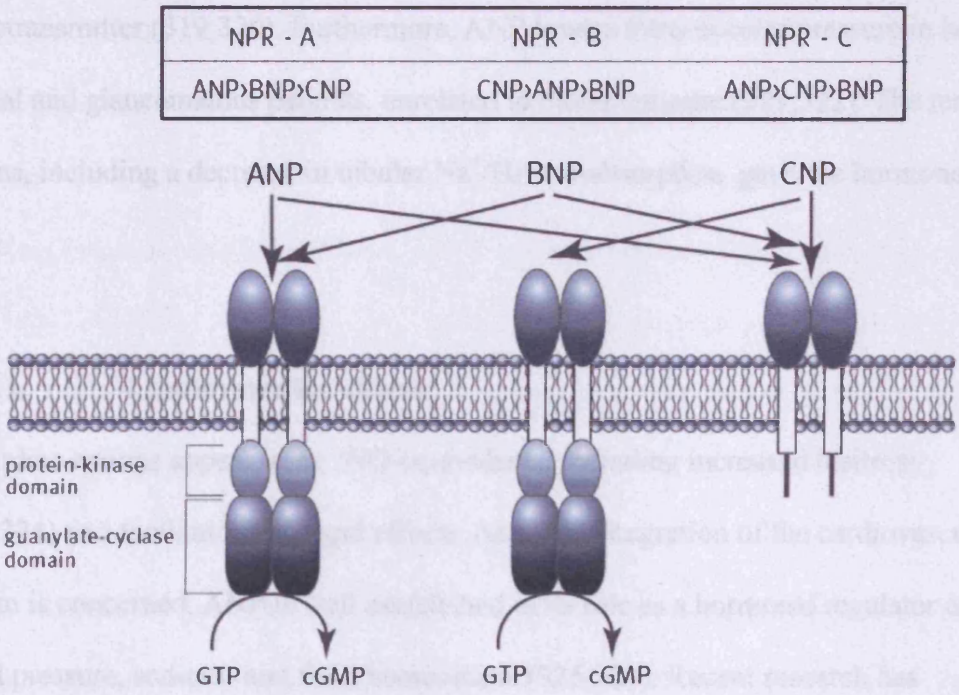


Figure 1.3.4.1. Note all 3 receptors have an extracellular ligand-binding domain, as well as a transmembrane segment. NPR_A and NPR_B also contain a protein kinase homology region, which appears to exert some regulatory function on the GC domain.

1.3.5. Physiological aspects in health (ANP)

ANP is an almost ubiquitous hormone which has been detected in mammals, many reptiles, some single cell organisms (308) and in several chlorophyll containing plants, where it enhances solute flow (309). NP and ANP in particular appear to have a regulatory and integrating role in organo- and embryogenesis (222), i.e. they seem to play an active role in differentiation and regulation of multiple organ systems, including the skeletal- (stimulating osteoclasts and increasing bone-turn-over (310;311)), immune defence (312;313;313) (important role in host defence (314;315;315), histamine release (316), activation of natural killer cells (317)), somatostatin secretion from antrum mucosa (318), reproductive- (testosterone production), and the central nervous system where they act as a non-conventional neurotransmitter (319;320). Furthermore, ANP lowers intra-occular pressure in both normal and glaucomatous patients, unrelated to blood pressure (321;322). The renal actions, including a decrease in tubular Na^+/H_2O re-absorption, gave the hormone its name.

1.3.5.1. Cardiovascular effects

Its cardiac actions appear to be "NO-equivalent", including increased lusitropy (323;324) and facilitation of vagal effects. As far as integration of the cardiovascular system is concerned, ANP is well established in its role as a hormonal regulator of blood pressure, sodium- and fluid homeostasis (325-327). Recent research has highlighted its anti-proliferative potency (328-333), a property shared by BNP and CNP (334-336), emphasising a potentially important role in cardiovascular remodelling. In CHF the NPS appears to be the body's endogenous defence against the deleterious effects of a chronically activated RAAS (337-340). The vasorelaxant

effects of ANP, (BNP and CNP) on resistance vessels are well documented in vitro (341) and in vivo (161;251;342;343) and it was initially believed that this effect explained the rapid fall of blood pressure (BP) following intravenous ANP infusion (see Figure 1.3.5.1.). However, several animal studies (344;345) showed that the fall in BP and CO was almost invariably associated with a fall in CVP and in fact occurred despite an increase in TPR. Groban (346) demonstrated in humans that low dose systemic ANP infusion reduced CVP without affecting BP and Roy et al showed that low dose short term ANP infusion reduced left ventricular end-systolic and end-diastolic volume in the absence of evidence for arterial vasodilatation (no decrease in diastolic pressure, TPR, or forearm vascular resistance) (168). It is now generally accepted that the fall in CO/BP following systemic infusion of ANP is a consequence of reduced preload and the mechanisms involved may include a) volume contraction as a consequence of diuresis and increased capillary filtration (167;167;258;347) and b) direct venodilation (325). Whilst the former is well documented evidence for the latter is sparse and ambivalent. Indeed ANP has no effect on dorsal hand veins (161;348), and little (349), or no (163) venodilating potency on saphenous veins. However, Olson et al [166], using an in-vivo trout model, showed that ANP actively regulates venous capacitance (comprised predominantly of the volume of blood contained within the small veins and venules).

The development of genetically modified animals that either have augmented NPR populations or peptide production, or knockout of either the peptide production or the NPR_A or NPR_C have markedly improved our understanding of the role of NPs in cardiovascular control. Discussion of the rapidly expanding literature in this field has been done elsewhere (350;351) and is beyond the scope of this thesis. However, the most pertinent findings of this research has been summarised in Table 1.3.5.1.

Finally, genetic targeting approaches to understanding hypertension have highlighted that molecular variants of the ANP gene may represent an independent risk factor for cerebrovascular accidents in animal models (352) and in humans (353) (for review see Refs (354;355)) . Interestingly, some of these genetic variations exert their biological effects clearly without altering the amino-acid sequence of the mature ANP(99-126).

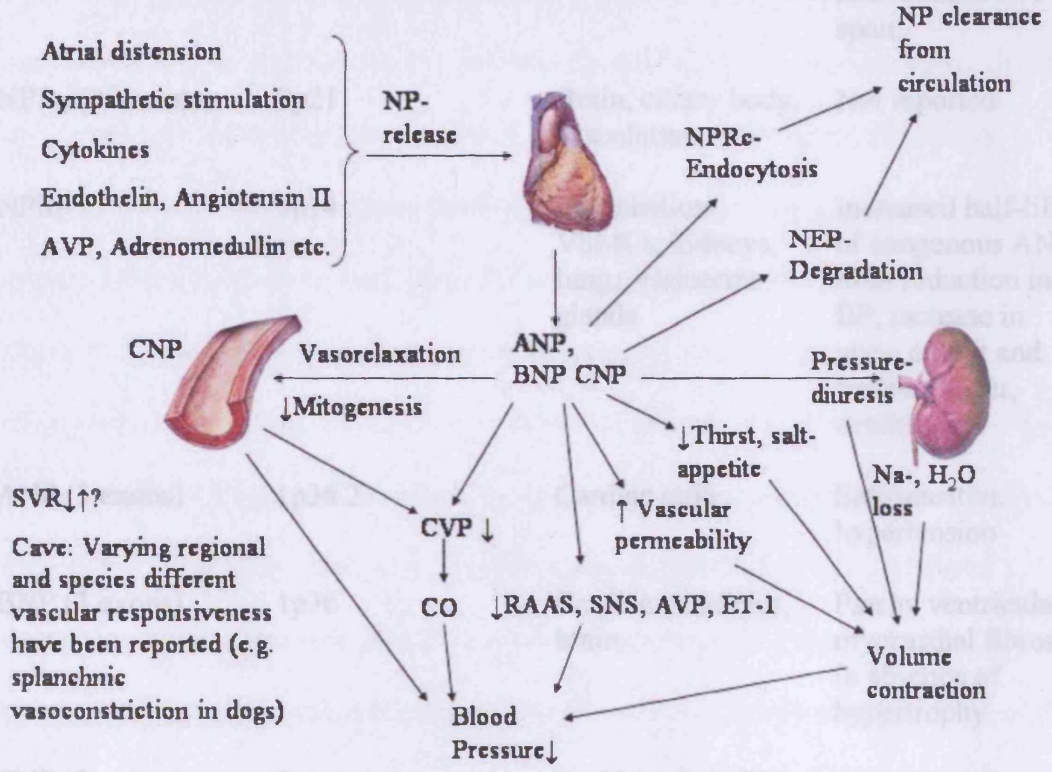


Figure 1.3.5.1. Aspects of cardiovascular natriuretic peptide physiology

Table 1.3.5.1. Phenotype of genetically modified mice.

Gene	Human chromosomal location	Principal tissue distribution	Phenotype of knockout mice
NPR_A(22 exons)	1q21	Vasculature, kidneys, adrenal glands	Hypertension, cardiac-hypertrophy + dilatation, reduced testosterone levels and reduced live span
NPR_B (22 exons)	9p21	Brain, ciliary body, vasculature	Not reported
NPR_C	5p14	Endothelium, VSMCs, kidneys, lungs, endocrine glands	Increased half-life of exogenous ANP, mild reduction in BP, increase in urine output and bone turnover, deafness
ANP (3 exons)	1p36.2	Cardiac atria	Salt-sensitive hypertension
BNP (3 exons)	1p36	Cardiac ventricles, brain	Patchy ventricular myocardial fibrosis in absence of hypertrophy
CNP (2 exons)	2	Brain, endothelium, ovary, uterus, testes	Not reported

1.3.5.2. The need for in vivo studies using ANP receptor antagonism

Whilst in-vitro studies and tissue culture experiments were indispensable in identifying cellular and sub-cellular mechanisms in response to ANP, the application of this information to and comparison with in vivo studies was often surprisingly limited, in fact often contradictory. This is for several reasons; Firstly, in vitro studies using vascular tissue were, for experimental and practical reasons, largely confined to the use of conduit veins and arteries, yet it is clear that arterial resistance (afterload) and venous capacitance (preload) are largely regulated by the small pre- and post-capillary arterioles and venules respectively. Secondly, in vitro studies are unaffected by (do not take account of) the activation of complex counter-regulatory neuro-homonal mechanisms such as the baro-reflex and the renin-angiotensin-aldosteron system, which needs to be considered for an integrated approach, especially as there is ample evidence to suggest that the vasoactive and BP regulating actions of NPs are often through inhibiting the secretion or action of other vasoactive hormones (356-358). Thirdly, it has become evident that the process of culturing vascular smooth muscle cells itself may result in a significant phenotypic shift involving altered expression of the natriuretic peptide receptors. Suga et al (359) demonstrated that although cultured rat aortic smooth muscle cells express high levels of NPR_C receptor binding sites and mRNA, receptor expression is reduced markedly in aortic media tissue derived from the same animals. in contrast, although cultured cells express low levels of NPR_A and NPR_B receptors, cells derived from aortic media tissue contains significantly higher levels of these receptors (359).

1.3.5.2.1. A71915 - ANP receptor antagonism

Receptor antagonists have traditionally provided powerful tools for understanding the physiological role of ligands. It is informative to recall arguments concerning the true functions of the RAAS. It was only when effective chemical inhibitors of the RAAS were discovered, e.g. ACE-inhibitors, AT_1-receptor antagonists and Aldosterone receptor antagonists, that the true nature of the RAAS was appreciated.

Von Geldern et al (360) described a series of ANP analogues capable of interfering with ANP-induced cyclic GMP accumulation in bovine tansformed aortic cells. These agents were prepared using solid phase techniques on an automated synthesiser.

Delporte et al (223) used cultured human neuroblastoma cells (NB-OK-1), which exclusively express NPR_A but not NPR_B or NPR_C, to evaluate the potency of seven of this agents to suppress cGMP production in more details. ANP analogues with a shortened Cys7-Cys18 bridge, Asp 13 and a hydrophobic tetrahydroquinoline-3-carboxylate (Tic) residue at position 16 expressed antagonistic activity, while Ala16 provoked lower antagonistic potency and Phe16 induced receptor activation. A71915 ([Cys2-Cys13]H-Arg-Cys-Cha-Gly-Gly-Arg-Met-Asp-Arg-Ile-D-Tic-Arg-Cys-NH_2; Mwt. 1634.0) emerged as the most potent antagonist with a binding inhibition of; pKi = 9.18, and a inhibition of ANP-induced cyclic GMP of; $pA_2 = 9.48$. The binding affinity of A71915 to NPR_A was only 22 lower than that of ANP, and the antagonist itself (at a concentration of up to 10μM) had no intrinsic potency to increase cGMP. It also appears that selectivity towards the NPR_A as opposed to the NPR_C, is facilitated by the presence of the bulky, hydrophobic Tic residue at position 16 within the Cys^7-Cys^{18} bridge (223).

Further evidence for NPR_A selectivity for A71915 derives from a study by Crook and Chang (361). They examined the effects of NPs (ANP, BNP, CNP) on cGMP

production and ^{125}I-labelled-ANP binding to cultured NPE-cells (non-pigmented ciliary epithelial cells, believed to be devote of NPR_A- but expressing NPR_B- and NPR_C), derived from human ciliary body epithelium, the site of aqueous humour production in the eye (ANP [and in rabbits more potent CNP] lowers intraoccular pressure in both normal and glaucomatous patients, unrelated to blood pressure (321),(362),(322)). In their experiments the fungal product HS-142-1, a non-protein antagonist of both NPR_A and NPR_B inhibited CNP induced cGMP production (via NPR_B blockade; NPR_B is the major GC containing NPR-subtype in choroids plexus, pituitary and brain as well as ciliary epithelium (363)). In contrast A71915 did not inhibit CNP- or ANP-stimulated cGMP production, which was 10 times greater following CNP exposure than ANP exposure (all three NPs bound to binding sites on NPE cells with high affinity, which is characteristic of NPR_C but not NPR_A).

Equally important evidence for functional antagonism derives from further studies. Brunner and Woelkert (364) using rat hearts showed that the reduction in coronary perfusion pressure (-46% decrease) achieved following ANP (10nmol/l) infusion was diminished by half (-23%decrease; 50% inhibition) by co-infusion of ANP (10nmol/l) and A71915 (0.1μmol/l). Furthermore, they investigated the physiological role of endogenous ANP in blood pressure regulation using a conscious mouse model. Intraperitoneal injection of A71915 (50 nmol) raised the blood pressure from 120±3mmHg to a mean of 144±5mmHg within 10-20minutes ($p<0.05$), followed by a decline to baseline within 30 minutes (five measurements). Injection of saline (0.2ml) had no effect (121±1mmHg throughout the test). Finally Ni et al (365) using anaesthesised Sprague-Dawley rats showed that intrarenal infusion of A71915 (5pmol/min) effectively inhibited the natriuresis induced by intravenous infusion of ANP (1pmol/min). In contrast A71915 did not inhibit reflex mediated (delta-

Melanocyte-stimulating-hormone [d-MSH] signalled) natriuresis following acute unilateral nephrectomy.

1.3.6. Therapeutic principles and rationale

The NP-system is the main endogenous defence system to counter-regulate the deleterious chronic activation of vasopressin, the RAAS and the SNS, found in several cardiovascular diseases (340). However, it appears that in advanced disease states the beneficial effects of the NPs are masked by the opposing actions of increased sympathetic drive, increased circulating and local cytokines, and vasoconstricting factors. Thus reversing this imbalance in favour of the NP-system, reducing vascular tone, inhibiting cardiovascular remodeling, and decreasing neurohumoral activation has been considered a promising therapeutic approach in the treatment of essential hypertension and CHF.

Enhancement of the NP-system can principally be achieved via 3 mechanisms:

1) Administration of NPs, peptide analogues or mimetics.
2) Increasing synthesis and release of endogenous NPs.
3) Prolonging life-time and preventing breakdown of endogenous NPs.

Preventing the breakdown of endogenous NPs appeared to be the most promising approach and can generally be achieved either by agents targeted to block the clearance receptor or by blocking enzymatic degradation of endogenous NPs via blockade of NEP 24.11.

1.3.6.1. NP-administration:

1.3.6.1.1. ANP administration

A large number of studies have assessed the efficacy (366-368) and mechanisms of action (251) (161;198;346;347;367;369-373) of ANP infusion in health and CHF. These studies varied widely in design (acute versus cHF, continuous versus repeated infusion etc.) and patient selection. Importantly, some studies used shorter ANP analogues ("mini-ANP") with a reduced biological profile. Not surprisingly, theses studies have produced variable results, in particular regarding renal responsiveness following ANP administration. Whilst some investigators found little (166;374;375) or no (368) effect on sodium and water excretion in patients with CHF, others observed marked responsiveness (157). The causes of these discrepancies may be attributed to variations in study design. Since Ang II enhances cGMP degradation (376) it has been suggested (377) that concomitant ACE-inhibition or AT_1-receptor blockade may actually enhance the biological effects of NPs, potentially explaining preserved renal responsiveness in some of the more recent studies (366). Interestingly, ACE-inhibition appears to blunt renal ANP actions in healthy controls (378) whilst it was shown to have little effect on ANP induced natriuresis, diuresis and creatinine clearance in CHF patients (375). In a more recent study, AT_1-receptor blockade, using Irbersartan, has been shown to increase ANP levels (15.7% over a 30 day treatment period) despite a drop in BP and a decrease in atrial and ventricular diameter (379). However, a unifying feature of all the studies using ANP infusion is the almost invariable reduction in cardiac filling pressures. Elevating NPs either by infusion (167;366), or by augmenting endogenous NPs (367) has been shown to increase stroke volume (167) and cardiac index (CI) (366) in patients with heart failure despite a fall in CVP (167;366;367) a finding apparently in conflict with the Starling

mechanism. This is almost certainly due to relief of constraint by the stretched pericardium (pericardial constraint) and the volume/pressure loaded right ventricle (DVI) resulting in an increase in true LV preload despite a fall in LVEDP as previously shown by Atherton et al (124) and discussed in chapter 1.2.7. The fact that ANP infusion is able to reduce cardiac filling pressures, too early to be solely accounted for by a fluid shift into the interstitial space (ANP has been shown to reduce cadiac preload in nephrectomised rats to a similar degree as in sham operated animals (164)), and in the absence of significant changes in diuresis and pulmonary and peripheral vascular resistance, has re-emphasised the potential role of ANP on venous tone.

1.3.6.1.2. Urodilatin

The rationale behind the use of the renal ANP homolog Urodilatin instead of ANP is multifold. It has been suggested that urodilatin, rather than ANP, regulates renal sodium excretion (240;242), and Urodilatin excretion has been found to be increased in CHF (380). Some studies also found Urodilatin to cause a less marked hypotensive effect when compared to ANP at equimolar doses (381). Furthermore, there is some evidence that exogenously infused Urodilatin interacts with the same receptors as ANP but is, presumably due to its amino-terminal extension, almost inert to enzymatic degradation by neutral endopeptidase (243). A recent study by Bonatti et al (249) assessing the potency of ANP, BNP, CNP and Urodilatin to stimulate cGMP production in rings of saphenous veins and internal mammary arteries showed that whilst Urodilatin had a slightly weaker overall effect, the ratio of its veno-arterial potency was higher than ANP and BNP. This may imply a better tolerability in patients with low output failure. Riegger et al (382) offered several arguments for the

therapeutic role of Urodilatin in CHF. Further studies by Kentsch (383) and Elsner (384) also underscored a potential use of Urodilatin in CHF. In their studies Urodialtin improved CI and stroke volume. Urodilatin was infused intravenously for 10 hours at a dose of 15ng/kg per minute in 12 patients suffering from CHF (classified NYHA II/III). In this randomised double-blind, placebo-controlled trial (384), Urodilatin was shown to significantly decrease systolic blood pressure and CVP. However, diastolic blood pressure and HR were unaffected. No relevant side effects were observed, showing hat Urodilatin was well tolerated in this prolonged infusion. So far, clinical phase I and II studies using Urodilatin in the treatment of CHF, renal failure and bronchial asthma have been performed (for review of these studies see ref (385-387). The renal Urodilatin system, its implications and indications have been reviewed elsewhere (388).

1.3.6.1.3. BNP

Intravenous infusion of Nesiritide, a human recombinant B-type natriuretic peptide, has been investigated in more than 1700 patients with acute decompensated heart failure (389), and as such is the clinically most advanced NP in the drug development process. Indeed, it is the first new parental agent (Natrecor™) to be approved by the FDA for treating heart failure in more than a decade (390). Nesiritide causes rapid, dose-dependent vasodilatation that is sustained for the duration of treatment and it appears to have balanced arterial and venous effects as evidenced by decreases in systemic vascular resistance (SVR), systemic arterial pressure, and mean pulmonary arterial pressure (PAP) (391;392). Vasodilatation occurs without a change in HR and is associated with increased stroke volume index (SVI) and CI (393). A recent landmark study investigated the clinical use of Nesiritide in patients with decompensated CHF (394). The study enrolled patients in either an efficacy trial

(n=127, double blind parallel group design) or a comparative trial (n=305, open label parallel group, 7 day follow up). Nesiritide infusion significantly reduced pulmonary capillary ewdge pressure (PCWP), dyspnoea and fatigue and improved global clinical status in the efficacy trial at 6 hours. The improvements in global clinical status, dyspnoea and fatigue were sustained with Nesiritide treatment for up to 7 days in the comparative arm and were similar to those seen with standard intravenous therapy. A healthcare cost analysis in 261 patients with CHF from an open-label randomised study comparing Nesiritide versus standard care suggested that treatment of decompensated CHF using Nesiritide instead of dobutamine may reduce readmission rate, healthcare costs and mortality (391). The latter is most likely attributable to the better safety profile of Nesiritide which in contrast to the pro-arrhythmic and chronotropic effects of dobutamine actually reduces ventricular ectopy or has a neutral effect (395;396).

Similar to the use of ANP, reports about the renal effects of BNP in heart failure are also variable. Whilst Marcus et al (397) and Yoshimura and co-workers (398) found that Nesiritide or BNP respectively increased natriuresis and diuresis, Abraham reported that the magnitude of this response seemed to be blunted in 6 out of 10 patients (399).

Despite impressive haemodynamic and neurohormonal responses, that are generally very similar for the use of varying NPs, some caveats remain; the short biological half-life, the need for parenteral administration and the high production costs are likely to limit the widespread use of NPs in the foreseeable future, at least in public healthcare systems such as the NHS.

1.3.6.2. Vasopeptidase inhibition

Vasopeptidase (VP) - inhibitors are a class of drugs including Omapatrilat (OMA), Samapatrilat and Fasidotrilat, that simultaneously inhibit both ACE and neutral endopeptidase 24.11 (NEP 24.11) (for review see Ref. (400-402). NEP is a widely distributed ectodermal enzyme, present not only in endothelial cells, but also in cardiac myocytes, fibroblasts, smooth muscle cells, adrenal glands, brain-, lung-, and renal tissue. Because NEP is involved in the breakdown of both, vasodilators as well as vasoconstrictors, the balance of effects of NEP inhibition on TPR will depend on whether the predominant substrate degraded consists of vasoconstrictors or vasodilators (400). This may vary between vascular beds and may be more dependent on tissue levels than on circulating plasma levels of these vasoactive substances. For example, in the human forearm, NEP inhibition using Candoxatrilat caused resistance vessel constriction (400). Furthermore, the vascular responsiveness to NEP-inhibition may differ in the pulmonary and peripheral circulation independent or over and above the degradation ratio between vasoconstrictors and vasodilators. In CHF NEP-inhibition reduces PCWP without significantly affecting afterload (400). Inhibition of breakdown of endogenous vasoconstrictor peptides (i.e. Ang II and ET-1) can, at least partly, offset the benefitial effect of NP augmentation. Therefore, combined inhibition of ACE activity to reduce Ang II and of NEP to enhance endogenous NP should theoretically provide additional benefits compared to mono therapy. Some promising small-animal experiments showed improved haemodynamics following VP-inhibition (VPI) over and above that of selective ACE-inhibition or NEP-inhibition (403-406). Similar findings have recently been reported in large-animal models of experimental CHF. Chen et al compared VPI with OMA and acute ACE-inhibition with Fosinoprilat in a canine model of pacing induced mild heart failure (407). Using

intrarenal administration of a NP receptor blocker they demonstrated that the beneficial renal effects of OMA were mediated via augmentation of endogenous NP. The same group recently extended these findings, comparing the effects of OMA, with and without a diuretic, with those of ACE-inhibition plus a diuretic (ACI-I + D) in the same animal model of CHF. Again OMA, with or without a diuretic, resulted in a more favorable cardiorenal and humoral response than ACI-I + D. OMA given alone did not cause activation of the RAAS (408) (for review see Ref (409)). Administration of NEP inhibitors has also been shown to reduce bradykinin breakdown (410;411). Since bradykinin is also a substrate for ACE, inhibition of both ACE and NEP is likely to lead to an even greater enhancement of endogenous bradykinin activity (412). Whilst bradykinin clearly mediates some of the beneficial vascular effects of ACE- and NEP- inhibition, these benefical effects need to be weighed against the potential for an increased side-effect profile (including angioedema) arising from dual blockade of bradykinin breakdown during VPI (for review see Ref. (413)). However, angioedema seems to be a lesser problem in the treatment of CHF compared to hypertension.

Early human studies in advanced CHF were promising. In a study by McClean and co-workers in 48 patients 3 months treatment with OMA improved cardiac function and in turn clinical status (414). In a study comparing the effects of 40mg OMA against 20mg Lisinopril on ET in 573 patients with NYHA II-IV treatment with OMA lead to a better clinical status and lower incidence of combined mortality and morbidity (admission and discontinuation of study treatment for worsening heart failure), whilst there was no significant difference in ET, the pre-specified primary endpoint. Both agents increased ET to a similar degree (415). A further multicentre study, enrolling 369 patients with symptomatic heart failure evaluated the

haemodynamic and neurohormonal effects, safety and tolerability of increasing doses of OMA after a single oral dose and after 12 weeks of once-daily oral therapy (416). Higher doses were associated with greater increases in vasodilatation and NPs, in addition to ACE inhibition. Furthermore, higher doses (20mg and 40mg) showed greater falls from baseline in PCWP and systolic BP than 2.5mg, whilst the incidence of adverse experiences and patient withdrawal were similar in all groups. First presentation of the only adequately powered mortality/morbidity trial (OVERTURE) at the American College of Cardiology in March 2002 received little enthusiasm (417). The results indicated a non-significant reduction of 6% in the primary combined endpoint of all-cause death and cardiovascular hospitalisations with OMA versus ACE inhibition alone, making the drug equivalent, but not superior to ACE inhibitor treatment alone. Following a re-evaluation, using criteria for non-inferiority, based on the Studies of Left Ventricular Dysfunction (SOLVD) treatment trial (46), the final publication of OVERTURE, however suggested a significant (HR of 0.89, p=0.012) incremental benefit on cardiovascular events with OMA of around 10% (183). It has been argued that in patients with advanced CHF (and a systolic BP < 110 mmHg) the BP lowering effect of OMA may partly outweigh the beneficial neurohormonal effects. Consequently it has been suggested that VPI may be most beneficial in early heart failure when NPs are activated in the absence of significant RAAS activation (402). It remains to be investigated whether VPI can delay disease progression from (relatively) asymptomatic to symptomatic forms of heart failure.

1.3.6.3. Combined NP administration and NEP- / VP- inhibition

Very recent work from the Christchurch Cardioendocrine Research Group and the Mayo Clinic has attempted to further augment the NP system in animal models of heart failure by combining inhibition of endogenous NP breakdown and exogenous

NP administration, a concept first investigated by Seymour and colleagues (418;419). Rademaker et al (420) evaluated the combination of a 3 hour infusion of ADM and an endopeptidase inhibitor (SCH32615) in an ovine model of pacing induced CHF. ADM induced directionally similar but greater changes in all hemodynamic variables compared to SCH32615. Coadministration of ADM and SCH32615 produced hemodynamic effects greater than those achieved during ADM infusion alone. The authors concluded that cotreatment with ADM and an endopeptidase inhibitor has beneficial renal and hemodynamic effects in heart failure beyond those of either agent separately. Chen and coworkers extended their previous studies, using subcutaneous administration of BNP (421), by comparing this treatment with VPI (using OMA) and with a combination of both treatments in a canine model of pacing induced heart failure (422). Similar to the study by Rademaker, combination therapy yielded greater renal, humoral and hemodynamic (increase in CO and reduction in filling pressures) effects compared to either treatment alone. Whilst these studies are encouraging it remains to be seen whether oral VP-inhibition, combined with subcutaneous BNP administration, is able to provide longer term benefits in patients with CHF.

1.3.7. Problems and Pitfalls: natriuretic peptide resistance

There is ample evidence of reduced vascular natriuretic peptide responsiveness (NP resistance) in CHF (199;423). The mechanisms underlying this NP-resistance are potentially complex and are not completely understood. Theoretically, all of the following may contribute;

1.3.7.1. NPR_{A+B} -receptor down-regulation

Decreased ANP binding in a rat model of CHF was reported by Tsunoda (424) in 1988 and later suggested in humans following the observation that the good correlation between the arterio-venous decrease in ANP-levels and the AV-increase in cGMP levels seen across the lower limb of patients with mild CHF, was lost in patients with advanced disease stages (425). This, albeit indirect evidence, was partially in keeping with an earlier study by Hirooka that showed a decreased FBF response in CHF patients compared to normal controls following intra-arterial ANP infusion (199). However, the reduced hemodynamic response in this study was not paralleled by a reduced cGMP production, indeed absolute cGMP levels were higher in the CHF group.

1.3.7.2. Fetal gene activation / endogenous β-ANP

One should principally differentiate between the response to endogenous NPs and exogenous NPs. Wei et al previously showed that β-ANP, an anti-parallel dimer of α-ANP, with diminished cGMP generating potency, is the principal form of circulating ANP in patients with severe CHF (426). The fact that following ANP administration patients with advanced heart failure consistently exhibit a marked reduction in CVP and PCWP may be seen as supportive of the concept that NP-resistance in CHF is more likely the consequence of an impaired endogenous ligand rather than receptor down-regulation, at least in the capacitance vasculature and pulmonary circulation.

1.3.7.3. Clearance receptor (NPR_C) up-regulation

An additional, potentially alternative concept to explain NP-resistance is that of NPR_C up-regulation. Matsukawa et al showed that the NPR_C locally modulates the physiological effects of vascular NP responses (427). A preliminary report suggested increased NPR_C gene expression in human failing hearts (428). This was supported by the finding of up-regulated NPR_C expression in platelets of CHF patients (429). However, the latter line of evidence is particular controversial because platelets, in contrast to VSMC and endothelial cells, only express NPR_C and because previous investigations have contrastingly reported ANP platelet receptor down-regulation (430).

1.3.7.4. Receptor desensitisation

NPR_{A+B} are densely phosphorylated transmembranous GC coupled receptors (431;432), Figure 1.3.7.4. Ligand binding leads to dephosphorylation which in turn appears to lead to desensitisation of the receptor towards the ligand (433;434). This mechanism per se could explain reduced vascular responsiveness despite sustained or even up-regulated ligand / receptor availability. However the ultimate significance of this process has not been determined since rephosphorylation of GC-A has not been accomplished.

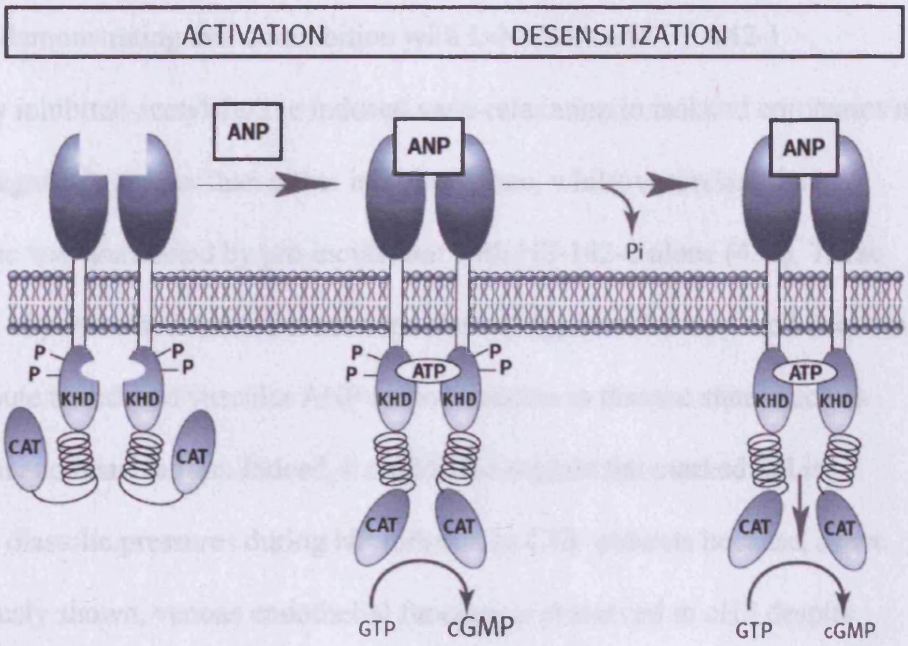


Figure 1.3.7.4. The receptor exists in a phosphorylated state in absence of ligand; when the ligand is bound, the receptor becomes dephosphorylated. Dephosphorylation correlates with desensitization of the receptor to its ligand. KHD, kinase homology domain; CAT, catalytic domain.

1.3.7.5. Endothelial dysfunction - soluble and particulate GC-crosstalk

There is accumulating evidence of intense cross-talk between both GC pathways (soluble and particulate) especially in disease states such as CHF. Hussain et al found a cooperative interaction between NO and ANP (435). In their experiments aortas from eNOS knockout mice were less sensitive to ANP than aortas from wild type animals. The potency of ANP in wild type animals was also decreased after pre-treatment with inhibitors of either eNOS- or sGC. Wenneberg et al, using the non-

peptide ANP receptor antagonist HS-142-1, revealed an interaction between NO and the NPS by demonstrating that coinhibition with L-NMMA and HS-142-1 significantly inhibited acetylcholine induced vaso-relaxation in isolated coronaries of dogs to a magnitude greater than either inhibitor alone, whilst vasorelaxation to acetylcholine was unaffected by pre-incubation with HS-142-1 alone (436). These findings are of particular interest because endothelial dysfunction may well (explain and) contribute to reduced vascular ANP responsiveness in disease states such as hypertension and heart failure. Indeed, it could also explain the marked fall in intracardiac diastolic pressures during NP infusion in CHF patients because, as we have previously shown, venous endothelial function is preserved in cHF despite marked impairment of arterial endothelial function (437). Furthermore, in disease states signalling pathway abnormalities downstream from NP-NPR interaction and activation of either soluble or particulate GC may also play a role in diminished NP responsiveness (438).

1.3.7.6. Neutral endopeptidase up-regulation (NEP24.11)

Alternativelly, increased NEP-activity in CHF could also contribute to reduced NP-responsiveness. Indeed, a very recent study found increased NEP activity in kidneys of several different models of severe heart failure in the rat (439). For a review on the mechanisms of reduced renal ANP responsiveness in heart failure see reference (440).

1.3.7.7. Area of uncertainty; cGMP second messenger of vascular ANP actions ?

Whilst it is generally accepted that the renal actions of ANP, comprising of pressure diuresis and increased Na^+ excretion, are cGMP mediated, there is some doubt that

the same is true for vascular ANP responsiveness. Von Geldern et al (360) showed in a murine in-vivo model that ANP receptor blockade using A74186 during ANP co-infusion prevented the increase in cGMP increase which was paralleled by prevention of natriuresis and diuresis whilst vascular responsiveness remained unaffected; in other words A74186 did not antagonize the hypotensive or vasorelaxant effect of ANP. These authors concluded that, although it may mediate the renal responses to ANP, cGMP is not responsible for the vascular and hemodynamic effects that result from the action of the hormone. Using a multitude of ANP analogues the same authors also found that aminoacids Aspartic acid (in position 13) and Phenylalanine (in position 26) were important for cGMP production following ligand-receptor binding (360;441), whilst they were not essential for ANP-NPR_A receptor binding (360). Furthermore, a study by Elsner and co-workers (442), using the lipohilic cGMP analogue 8-Br-cyclic GMP in a pacing- induced conscious dog heart failure model, provided evidence that the renal effects of ANP can be attenuated in CHF whilst vascular effects remained preserved. This was seen as in agreement with the hypothesis that an intracellular defect beyond cGMP might be involved in the phenomenon of NP tolerance/resistance in CHF. However, it could also be seen as indirect evidence that the renal and vascular ANP effects are mediated via differing pathways. Furthermore, as outlined above there is strong evidence of cross-talk between the soluble and particulate GC pathways and this might be of particular importance in disease states where one or both of these pathways becomes insufficient. It remains to be shown if this cross-talk is a clinically relevant compensatory or maladaptive mechanism.

1.3.7.8. ACE-escape

Finally, the phenomena of aldosterone escape (443) and Ang II reactivation (444), sometimes summarised as "ACE-escape" can theoretically contribute to the decreased renal and vascular response to ANP in CHF. This is likely due to their ability to augment the SNS and interfere and oppose the actions of NPs on several levels. The presence of this phenomenon may be seen as evidence of a shifted neurohormonal balance following ACE-inhibitor treatment.

Furthermore, escape from the sodium-retaining effects of aldosterone is associated with significant increases in the circulatory levels of ANP (445), and failure of "renal escape" in patients with hydropic diseases such as cirrhosis (446), but potentially also CHF, may be due to the renal resistance to ANP with insufficient increase of urinary cGMP excretion. (for summary of mechanisms see reference (440)).

1.3.8. Conclusion and Perspective

NP-research has come a long way since de Bold's seminal observations in the early 80's. Whilst NP administration in advanced heart failure is effective but expensive, and restricted to continuous intravenous use, the first outcome study using VP inhibitor in CHF was somewhat inconclusive. Because the NPs oppose RAAS and SNS actions, it remains important to further pursue their therapeutic potential in cardiovascular disease. The current emphasis in NP-research is in the field of molecular biology and genetics. However, in parallel it appears mandatory to gain a greater understanding of NP physiology and pathophysiology. In particular to improve understanding of venous ANP effects and to investigate the mechanisms underlying reduced NP responsiveness in CHF.

1.4. Original Hypotheses

The starting point of the present research work was that we generally thought it counterintuitive to believe that a hormone released in response to atrial stretch should lack veno-dilatory actions.

Consequently we first hypothesised that, despite the absence of significant effects on conduit veins, ANP may modulate regional venous volume by modifying venous tone in the small veins and venules (i.e. in the capacitance vasculature).

Furthermore, given the fact that the ANP gene knockout mouse (ANP -/-) (447) is hypertensive (20-30mmHg higher BP levels than wild-type animals (448)) I reasoned that basal ANP plasma levels are likely to contribute to resting venous (vascular tone).

Also, recent data suggests that ANP vascular effects may be in part endothelium dependent (364;435;436). Endothelial dysfunction may therefore, at least partly, be responsible for the phenomenon of ANP tolerance/resistance in arteries of CHF patients. However, we recently reported preserved endothelial function in the capacitance vasculature of CHF patients (437). The possibility therefore existed that venous ANP responsiveness was preserved in CHF and potentially due to preserved or even up-regulated activity of endothelial NOS activity.

Consequently we hypothesised:

- ANP regulates regional vascular volume by modifying venous tone in the small veins and venules.

- Physiological, including basal-, ANP plasma levels exert venodilating actions.

- Whilst there is "arterial tolerance" to the effects of ANP, venous responsiveness to ANP is preserved in CHF.

- The endothelial NO/cGMP pathway contributes to ANP induced venodilation in health and/or CHF.

- ANP locally modifies arterial distensibility, as assessed by PWV, via ANP-NPR_A interaction.

- Novel natriuretic peptide (NNP) relaxes rings of rabbit aorta via either NNP-$NPR_{A/B/C}$ interaction.

The background and rationale of each of these hypotheses is outlined in more details in the relevant chapters.

CHAPTER 2 – METHODOLOGY

2.1. History, Applicability and Basic Principle of Radionuclide plethysmography

Imaging of the peripheral blood pool, using labelled albumin (449) or red blood cells (450), was first used to assess changes in venous volume and tissue fluid accumulation in animal experiments in the early 60's by Ablad et al and Baker et al. In 1981 Rutlen (90) and Clements (91) independently described the application of this technique, termed radionuclide plethysmography (RPY), to study the human peripheral circulation. The technique, which despite its name actually does not involve a plethysmographic element, combines equilibrium blood pool scintigraphy (EBPS) with a standard occlusive technique. In brief: red cells are labelled using a modified in-vivo labelling technique, based on the method described by Callahan (451). Using this technique (details described below) at least 95% of injected isotope is bound to red cells, and therefore confined to the intravascular space. It follows that radioactive counts are proportional to intravascular volume. Since the vast majority of blood in the peripheral circulation is contained within the veins (41), and since veins are much more distensible than arteries, changes in counts largely reflect changes in venous volume. Following radiolabelling P/V relations (PVR) are constructed by obstructing venous outflow in a stepwise manner for 1 minute. During each 1-minute interval a dynamic image (split into 6 intervals of 10 seconds) of the region of interest (ROI) is continuously acquired. The technique allows the assessment of changes in venous tone with either local or systemic drug infusion, or physiological stimuli, such as lower body negative pressure, carotid stimulation or exercise. A parallel shift of the PVR implies a change in venous tone. A change in slope indicates altered compliance. The technique was further validated and refined by Manyari, Wang, Tyberg and co-workers who modified its use and expanded its application to assess splanchnic

capacitance in animal experiments (92;122;123;161;452) and humans (94) in health and disease. This group also demonstrated the suitability and applicability of the technique to investigate venous reflex control in health and disease states associated with abnormal vasomotor response (453). Our group later expanded this application to investigate venous reflex responses in hypertrophic cardiomyopathy (99), post myocardial infarction (101), and during dynamic leg exercise (100-102;345;346). We have also adapted the technique to assess venous endothelial function in health (95;96) and CHF (437) and more recently improved the technique by expressing changes in one arm to changes in the contralateral arm, as is convention for plethysmographic techniques, to take account of potential systemic changes during pharmacological intervention and prolonged experiments.

Whilst some of the earlier studies assessed peripheral counts by means of simple scintillation probes, mobile small field of view (SFOV) gamma cameras now permit simultaneous imaging of the ROI (see below).

2.2. Practical Methodology

2.2.1. Red cell labeling

Red cells are labelled with ^{99m}Technetium (^{99m}Tc) using a modified in-vivo labelling technique (451). The content of one vial of Amerscan™ Stannous agent (Amersham, UK) is dissolved in 6 ml of normal saline to form stannous medronate complex. Immediately after solution, 0.03ml/kg/body weight is withdrawn from the vial and injected directly into a vein of the non-dominant arm. In the presence of stannous ion ^{99m}Tc is reduced in the cells and becomes bound to the β-chains of the globin. Twenty minutes after injection of the stannous solution 3 ml of blood are withdrawn from an antecubital vein into a 10 ml syringe containing 750 MBq of ^{99m}Tc pertechnetate

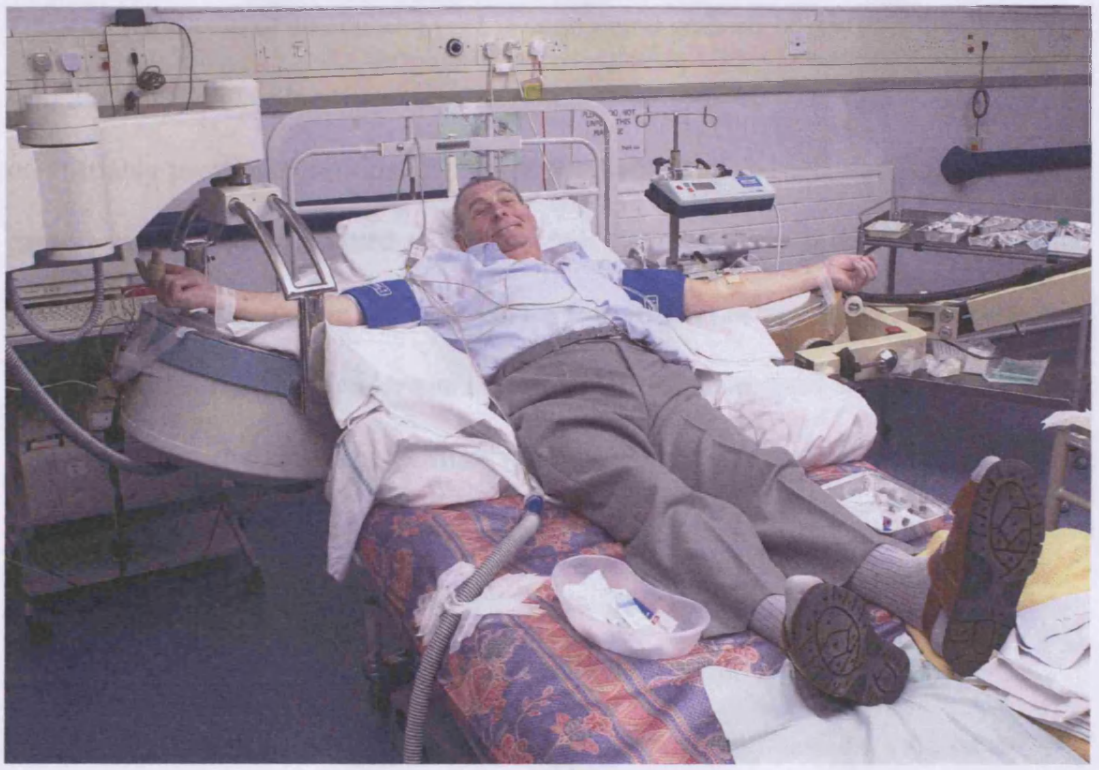
diluted in 2.5 ml normal saline. The syringe is placed into a lead cylinder and gently agitated for 10 minutes to prevent clotting and facilitate binding. The now (ex-vivo) labelled blood is re-injected 10 minutes later via a separate butterfly. To further minimize the amount of free circulating ^{99m}Tc and to allow complete in-vivo binding to occur, scintigrams are not recorded for a further 15 minutes. Using this technique at least 95% of the injected radionuclide is bound to red cells, and therefore confined to the intravascular space.

2.3. General Protocol

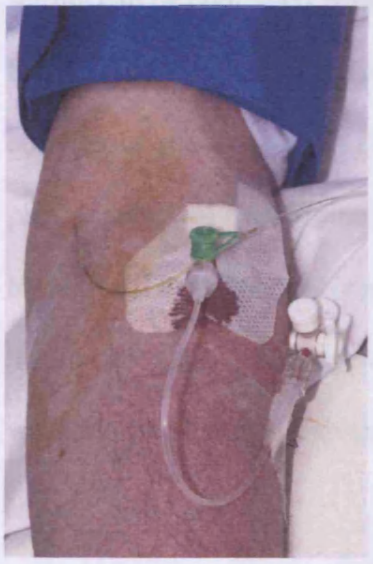
2.3.1. Forearm studies:

In studies using intra-arterial drug infusion antecubital veins of both arms are cannulated with 18 gauge indwelling cannulas for baseline blood sampling. After red cell labelling (see above), a 27-gauge unmounted steel needle sealed with dental wax to an epidural catheter is inserted into the brachial artery of the non-dominant arm under sterile conditions. The infused arm is then positioned on the face of a SFOV camera equipped with an integrated computer system. During intra-arterial infusion studies the non-infused arm may be studied using a second camera to take account of systemic vascular changes over time (see plate 2.3.1.1). Twenty to 30 minutes after infusion of saline is commenced, two baseline venous PVR are recorded (see below). This can be combined with assessment of forearm blood flow (FBF) and forearm vascular resistance (see below). Thereafter the infusion of the study drug commences at the chosen concentration and an infusion speed of $\leq$1 ml/min. Venous dose-response curves can than be created by repeating PVR at incremental drug doses. Venous-effluent blood samples from both arms can be taken for assessment of plasma concentration and for calculation of e.g. second messenger "spillover" at each dose.

A)



B)



C)

Plate 2.3.1.1. A, B, C illustrate a typical setting of a forearm study. Both forearms rest on the surface of a gamma camera collimator. The radial artery and a cubital vein in the intervention arm are cannulated.

2.3.2. Splanchnic/intestinal studies:

A dorsal hand vein or antecubital vein is cannulated with an 18 gauge indwelling cannula and adequate baseline blood sampling is performed. The subject is then comfortably positioned on a bed in supine position. After red cell labelling both gamma cameras are positioned as follows: one gamma camera is positioned above the subjects abdomen to record (changes of) venous volume in the intestinal vascular bed (see plate 2.3.2.1.). The bladder and the iliac bifurcation are used as landmarks, and lead-strips applied to the skin facilitate monitoring of a constant region. The liver, spleen and kidneys are excluded from the ROI. A second camera may be positioned to monitor the count rate derived from the spleen. This setting can than be combined with either systemic drug infusion or physiological stimuli as outlined above. Constant dynamic monitoring allows assessment of regional vascular volume at "baseline transmural pressure" (= Capacity). In order to assess capacitance, this setting can be combined with breathing continuous positive pressure in order to create PVRs. Subjects are first taught to relax while breathing with various levels of continuous airway pressure (CPAP). This procedure can then be combined with pharmacological interventions. Subjects are instructed not to move, and care is taken to maintain a constant subject – camera position throughout the experiment. Changing the level of CPAP requires between 5-10 seconds. This technique has a coefficient of variation for changes in splanchnic vascular volume (SVV) of 2-3% (94)

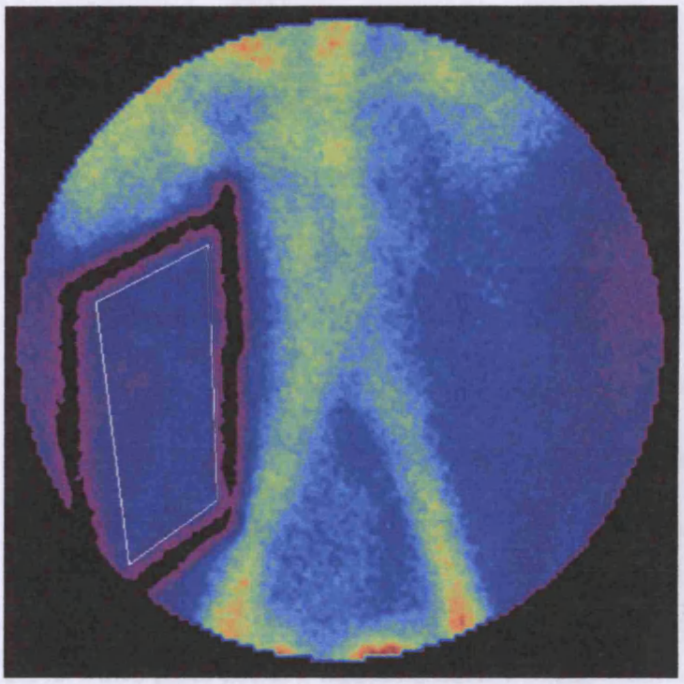


Plate 2.3.2.1. Shows an abdominal scintigram. A single splanchnic ROI is drawn clear of large conduit vessels (multiple ROIs can be drawn). The liver, spleen, bladder and bifurcation serve as landmarks. Lead strips attached to the skin facilitate monitoring of a constant region.

2.4. Data Analysis

Following red cell labelling images are aquired as a dynamic study of 10-second frames, either continuously or as a series of 1-minute studies. Regional PVR are constructed by increasing venous transmural pressure in a stepwise fashion, either by inflation of upper arm cuffs (arm-studies) or by incremental CPAP (splanchnic-studies). Following any intervention (e.g. cuff inflation or alteration of CPAP) the first 30-second data-set is ignored (upper panel in plate 2.4.1.), to allow regional volume to stabilise and only the next 30 second data-set (lower panel in plate 2.4.1.) is used for analysis. The images are summed and a ROI is defined on the image (plate 2.3.2.1 and plate 2.4.2.), obtained during normal saline infusion (baseline). All images in the study are viewed with this ROI to confirm that no patient movement has occurred during the acquisition. The counts from this ROI for each of the appropriate 30-second intervals are corrected for physical decay and the count obtained with no intervention (baseline) is arbitrarily taken to reflect 100% volume. All subsequent readings are expressed as percentage change of this baseline value. These counts, or scintigraphic vascular volumes in %-units are plotted on the y-axis against cuff/CPAP pressure on the x-axis to form venous P/V plots (see Figure 2.4.1.). Linear regression is performed for each plot, and a linear model is accepted if the R^2 value is ≥ 0.9. The slope of each plot is a measure of venous compliance. Therefore a parallel shift in P/V relation not only reflects a change in venous capacitance, but in the absence of a change in compliance is simultaneously indicative of a change in venous tone. A parallel up-ward shift reflects a reduction in venous tone whilst a parallel downward shift reflects an increase in venous tone. This is exemplified in Figure 1.2.3.2. Relative capacitance changes following intervention can be grouped and are compared to baseline and expressed as "% units" (Figure 2.4.1.).

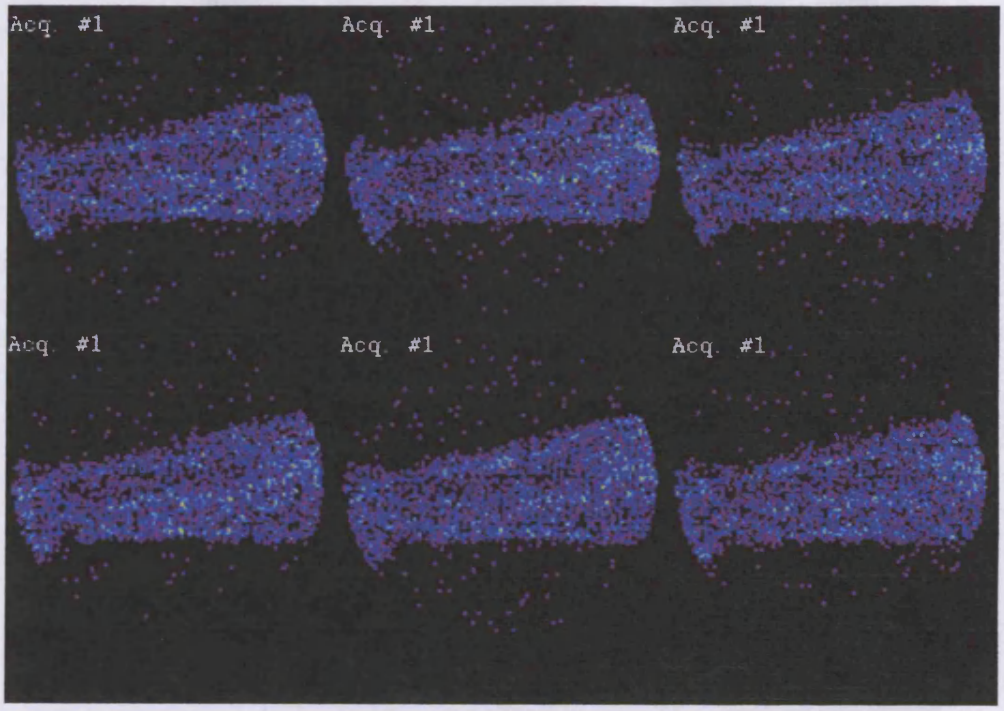


Plate 2.4.1. Shows a 1-minute dynamic recording split into 6 intervals of 10 seconds. The lower panel (the last 30 seconds after pressure has equalized) is used for analysis.

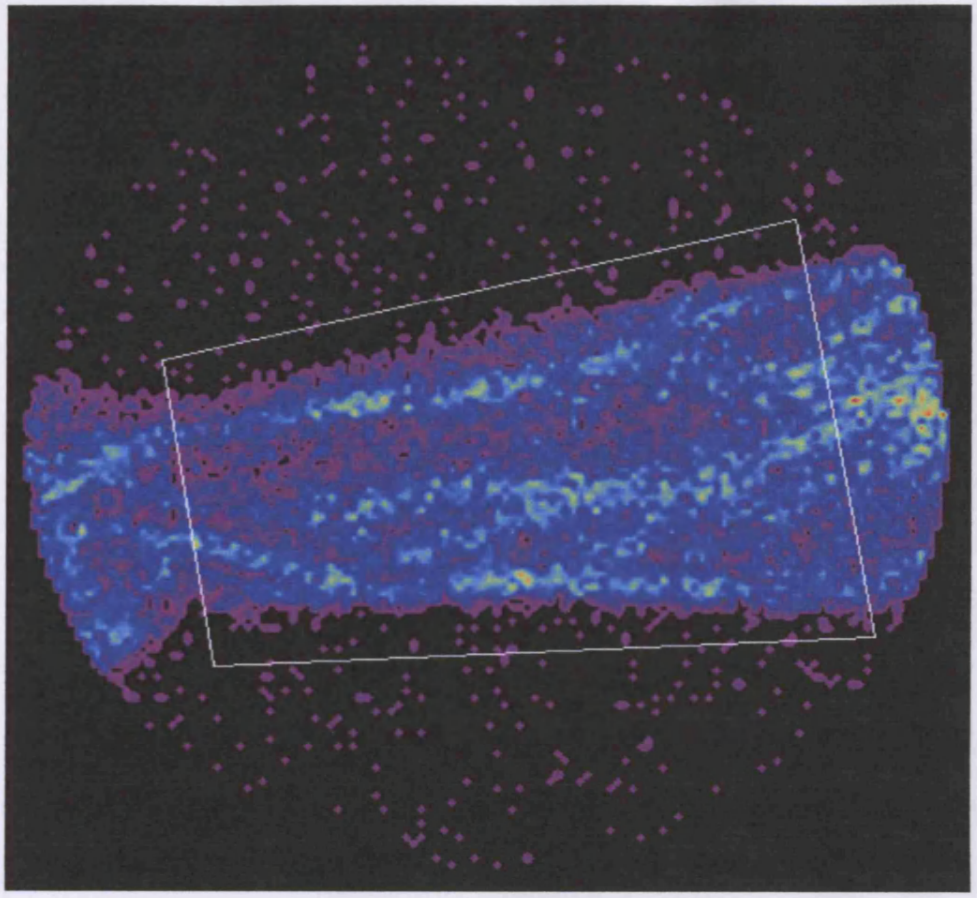


Plate 2.4.2. Demonstrates a ROI drawn over a forearm. It is suggested that the region should be drawn just clear of the edge of the arm (to allow for small expansion and movements) but inside the edge of the camera field.

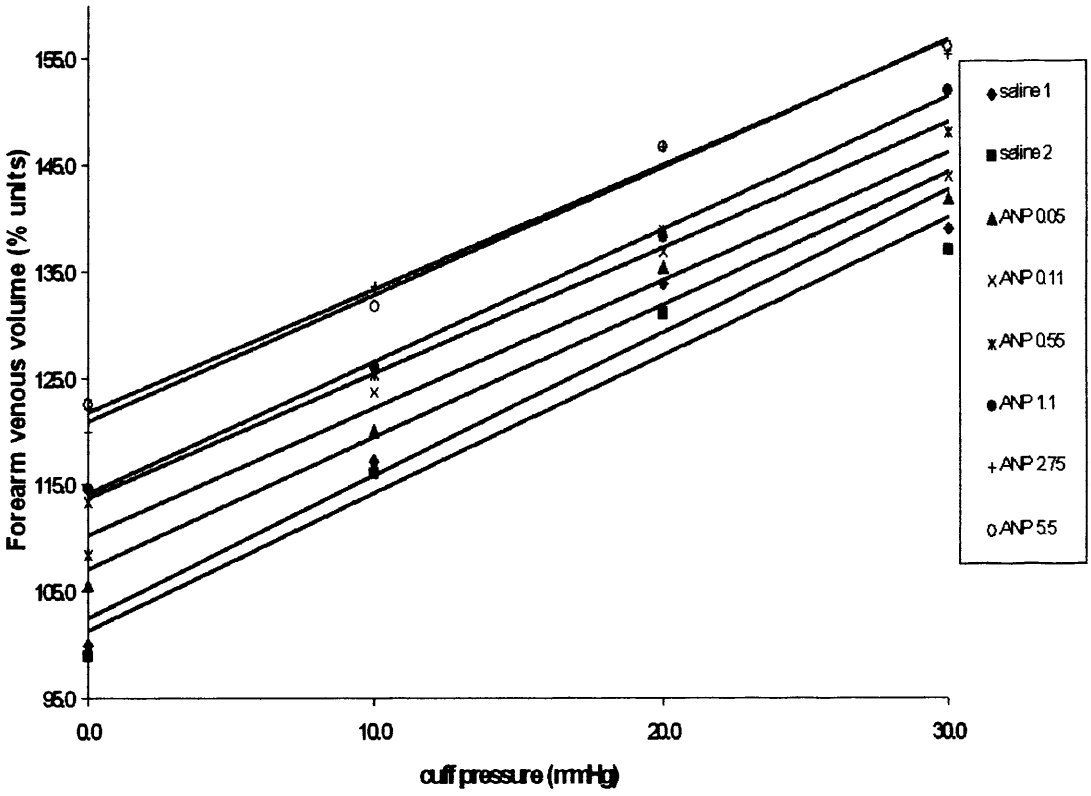


Figure 2.4.1. Illustrates grouped PVRs of a typical dose-response study. A dose dependent parallel up-ward shift, indicating increase in venous volume and decrease in venous tone, following intra-arterial ANP administration, is shown.

2.5. Validation

2.5.1. Variability

In his original description of RPY in 1981, Rutlen (90) obtained absolute counts in the calf, under resting conditions, over a 30-minute period (n=14). Count rate varied very little under resting conditions. Manyari evaluated variability between two control measurements of forearm venous volume using the standard error of estimate (*Syx*), computed as the square root of the residual mean square as applied to consecutive quantitative radionuclide studies. Variability was 3.11%, which compared favourably with variability using strain gauge plethysmography (SGP) of 3.24% (454).

2.5.2. Validation against fluid displacement plethysmography

To determine if changes in intravascular volume obtained by imaging the radiolabelled blood pool correlated with changes recorded with a standard plethysmographic technique, Rutlen also simultaneously recorded changes following cuff occlusion (15 and 30mmHg), before and after GTN, in 9 healthy volunteers. Changes obtained in one arm as assessed by RPY were correlated in a linear fashion ($r=0.71$ to $r=0.98$) with simultaneously obtained changes in the contralateral arm, as assessed by water displacement plethysmography before and after these interventions (90).

2.5.3. Validation against strain gauge plethysmography

Manyari assessed changes in regional forearm volume in response to sublingual GTN and oral Nifedipine in 16 patients with a history of recurrent chest pains who had otherwise a low probability of coronary artery disease and no history or evidence of heart failure, hypertension, diabetes mellitus or peripheral vascular disease (454). He

constructed PVR and assessed changes in one arm using RPY and SGP in the contralateral arm. Results using the different methods were closely correlated (r=0.91 to r=0.99). Clements correlated %-changes in count rate and changes in forearm volume simultaneously in the same arm in 8 subjects. He also demonstrated a close correlation with *r*-values between 0.95-0.99 (91).

2.6. Issues regarding red cell binding

RPY uses radioactive counts within a ROI to represent venous volume. As binding to red cells is not 100% it is likely that a small amount of activity derives from tissue, i.e. is extravascular. Poor labelling efficiency will therefore impact on the accuracy of the technique and has the theoretical potential to lead to systematic overestimation of intravascular volume via extravasate of unbound ^{99m}Tc. Furthermore, if binding varies between different subjects results would not be comparable. Therefore it is essential that red cell binding is as close to 100% as possible with minimal variation between individuals.

Principally there are 3 methods of labeling (455). In vivo technique: stannous (such as Amerscan Stannous Agent from Amersham International) is injected intravenously followed by ^{99m}Tc pertechnetate injection after approximately 20 minutes. This is the simplest technique. However this technique has not only the lowest binding efficacy but also a high inter-individual variation. In previous validation studies from our unit (96) binding efficacy using this technique was 79.8 ± 16.7% (SEM, n=20). In vitro technique: a sample of blood is taken and red cells are separated and incubated first with stannous and than with ^{99m}Tc pertechnetate. The cells are washed with saline before and after each step to eliminate unbound material. The cells are re-injected into the patient with little or no free pertechnetate, approaching a labelling efficacy of

100%. This technique however is technically difficult and requires a higher level of logistic organisation. Modified in vivo technique: this technique (described above) is easier to perform and results in high labelling efficacy and little interindividual variability. In previous departmental validation studies, performed in healthy volunteers, we achieved a mean binding efficacy of 96.8 ± 0.6% (varying from 96-98%, n=20) (96). In my own validation studies, performed in patients with CHF, the binding efficacy was 94.6±1.7% (see Table 2.6.1.).

To establish the reliability of binding throughout lengthy studies I also assessed unbinding from erythrocytes over a 3-hour period (see Table 2.6.2. and Plates 2.6.1. and 2.6.2.). I found no significant unbinding over this period (% labelling efficiency was determined by the ratio: $A_c * 100/A_c+A_s$, where A_c is the activity in the labelled cell suspension (middle panel in Plate 2.6.1.) and A_s is the activity remaining in the plasma supernatant – lower panel in Plate 2.6.1.).

Whilst correction for physical decay is easily performed by a computer program on the basis of the tracer half-life (6 hours for technetium) and the study length, biological decay (biological decay refers to changes in the count rate due to loss of the isotope from the erythrocyte or change in haematocrit) may have to be taken into account depending on the study setting and nature of the intervention. For example when performing exercise studies it is good practice to correct for changes in radioactive counts/ml as this may change significantly due to release of erythrocytes from the spleen (97) that may escape effective labelling. We previously observed a 10-12% fall in radioactive counts/ml blood in healthy individuals during maximal exercise.

Table 2.6.1. Red cell labelling efficacy in patients with cHF (ANP study)

Subject number	activity in erythrocyte cell suspension (c/s)	activity in plasma supernatant (c/s)	labelling efficacy (%)
1	2754.5	116.75	95.76
2	912.2	28.4	96.89
3	1747	71	95.96
4	849.75	47.75	94.38
5	1227	56.5	95.4
6	1072	98.75	90.79
7	546.25	116.5	78.62
8	1120	18.4	98.31
9	1321	22.4	98.3
10	1450.5	33.8	97.67
11	1622	24.8	98.47
mean			94.59
SD			5.75
SEM			1.73

Table 2.6.2. 3-hour "*Unbinding-studies*" Red cell labelling efficacy in patients with cHF (ANP study)

Subject number	baseline LE %	1-hour LE %	2-hour LE %	3-hour LE %
1	95.76	95.62	95.6	95.59
2	96.89	96.92	96.61	96.7
3	95.96	96.1	95.8	95.85
4	94.38	95.4	95.5	95.3
5	95.4	95.9	95.7	95.5
6	90.79	92.0	92.7	92.5
7	78.62	82.3	83.8	83.2
8	98.31	97.1	97.3	95.9
9	98.3	97.9	97.6	97.2
10	97.67	97.43	97.4	96.7
mean	94.21	94.67	94.8	94.44
SD	5.91	4.64	4.12	4.15
SEM	1.87	1.47	1.30	1.31

A)

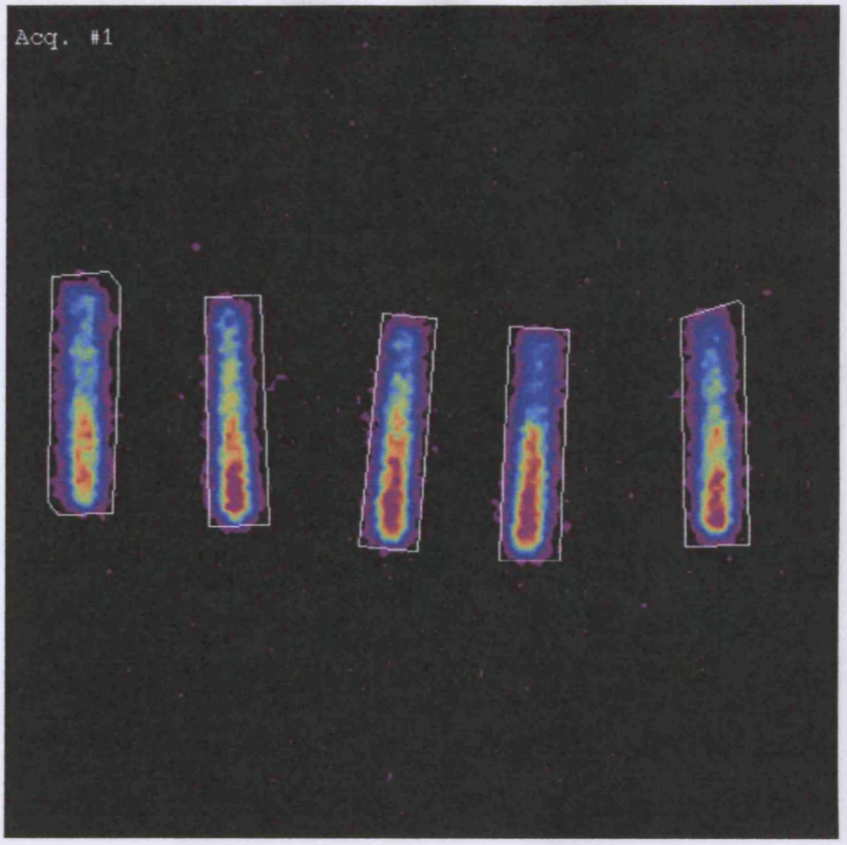


Plate 2.6.1.a: shows blood samples taken over a 3-hour period (left to right; Baseline, 30 minutes, 1-hour, 2-hours, 3-hours).

B)

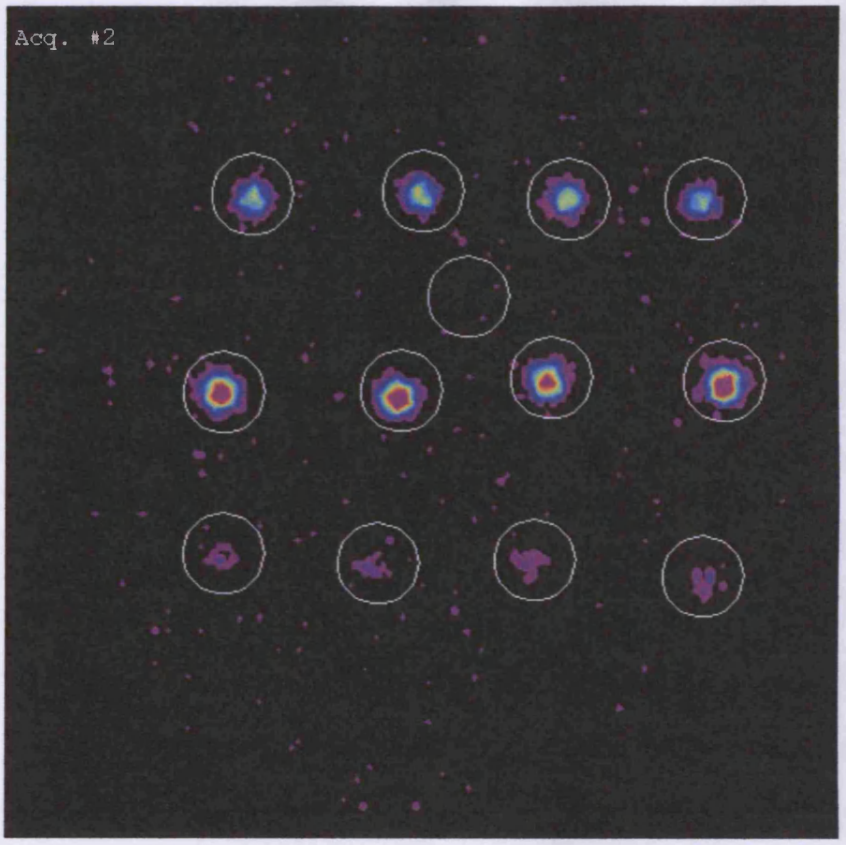


Plate 2.6.1.b. The upper panel shows whole blood-activity (at 1, 2, 3, and 4 hours) the middle panel concentrated erythrocyte-activity, and the lower panel the activity in plasma. The single ROI between upper and middle panel serves to correct for background radiation.

2.7. Effects of arterial inflow on venous volume

A crucial question is whether changes in regional blood volume are influenced by changes in arterial inflow. The vast majority of vasoactive drugs affect both arterial and venous tone and the possibility exists that changes in venous volume are at least in part consequence of the changes in arterial inflow. Whilst an increase in flow might be predicted to cause a passive increase in venous volume (along the same PVR) it would not be expected to alter venous tone. To investigate this issue, our group (i.e. my predecessor Dr D. Blackman) investigated the effect of brachial artery infusion of Hydralazine on both FBF and the venous PVR. Hydralazine is a selective arterial vasodilator, the peak effect of which is delayed for 30 (to 45) minutes after administration (49;161;456). Five healthy subjects had FBF measured by SGP for 30 minutes following an intra-arterial infusion of 800μg Hydralazine in 8ml at 1 ml/min. Hydralazine caused progressive arterial vasodilatation, with a peak increase in FBF of 262±102% at 30 minutes, but there was no change in the forearm venous PVR (95). Our findings are in keeping with previous studies. Wathan et al (457) compared the effects of Hydralazine (10 mg i.v.) and GTN (0.6mg s.l.) using radiolabelled albumin and a collimated scintillation probe to assess calf venous volume. Both drugs reduced vascular resistance. Whereas GTN increased calf venous volume, Hydralazine did not change it. Wang et al (122) compared the effects of systemic Hydralazine, Enalaprilat and GTN on the intestinal venous PVR in a canine acute heart failure model. Enalaprilat and GTN both induced an upward shift of the PVR (=venodilation) and markedly reduced LVEDP, whereas Hydralazine had no effect on the PVR and minimal effect on LVEDP. Manyari et al (163;454;458) compared the effects of systemic Nifedipine and GTN administration. Both agents reduced BP. GTN caused an upward shift in the PVR (=venodilation), but Nifedipine had no effect. Further

strong, albeit indirect evidence is derived from the observation that leg blood volume remains virtually unchanged during a work load increase between 50% to 100% of maximal VO_2 (97) whilst there is a remarkable increase in blood flow (459). Given the fact that RPY is able to detect blood volume changes as little as 5ml (452), these data support the view that changes in the venous PVR are largely independent of changes in arterial inflow. Measurement of venous tone is therefore valid even when arterial inflow is also markedly altered.

2.8. Limitations of Radionuclide plethysmography

Beside the obvious disadvantages of the radiation exposure (roughly 6.4 mSV per study), the financial aspects (expensive equipment), the considerable logistic efforts, regulatory, ethical and health and safety aspects involved when performing studies using radioactive substances, the major disadvantage is the inability to express the changes in volume in absolute (volume) units. Thus changes are expressed as a percentage of control measurements. Simultaneous measurement of volume changes using radionuclide and SGP has the theoretical potential to overcome the latter limitation. Whilst invasive assessment of venous pressure is not necessary as there is a close relationship between cuff pressure and invasively assessed conduit vein pressure (460), the radionuclide technique shares the inheritent limitation of all venous occlusion plethysmographic techniques, that the pressure considered to plot venous PVR is an "upstream" pressure and not that of the small veins and venules. However the pressure drop between the small veins and venules and the "upstream" veins is in most situations minimal. Finally, RPY does not provide a measure of capillary filtration. Therefore increased tissue attenuation as a consequence of increased

capillary filtration can lead, at least theoretically, to underestimation of the vasodilating potency of the studied agent.

2.9. Comparison to Available Methods to Assess Venous Function and Capacitance

As outlined above measurement of changes in venous capacitance is essential to describe venous actions of cardiovascular drugs and key to assessing the importance of the venous system in cardiovascular haemostasis. Because the vast majority of blood is contained in microscopic vessels, hidden in tissue, blood volume and distending pressure are notoriously difficult to assess.

2.9.1. Established techniques

2.9.1.1. Dorsal hand vein technique

The principal method used for in vivo evaluation of human venous tone is the dorsal hand vein technique described by Aellig (86-88;163;163;163;345). The diameter of a single superficial hand vein is recorded by measuring the linear displacement of a lightweight probe resting on the skin over the summit of the vein, when the pressure in a congesting cuff placed around the upper arm is lowered from 45 mmHg to 0 mmHg. Changes in vein diameter following various interventions can be measured in order to determine the effects of these interventions on venous tone. However, this technique does not quantify intravascular volume, nor does it permit construction of a complete PVR. Thus it is not possible to distinguish whether an intervention is affecting tone alone, or whether it is causing alterations in compliance, passively altering volume, or having a combination of these effects. More importantly this technique measures diameter in a superficial conduit vein, and results from such

studies should not be extrapolated to the small veins and venules (and vice versa) in which a much larger proportion of the blood volume lies.

2.9.1.2. Plethysmographic techniques for volume measurement

As outlined and illustrated in a detailed review (see reference (461)) conventional venous occlusion plethysmography (VOP) (water displacement or strain gauge) can be used to assess changes in "limb venous volume" (84;85;345;462;463) and when modifying the protocol accordingly to assess capillary filtration (369) or permeability (464) and microvascular monitoring (369;465). Measuring changes of the total volume of tissue, by placing the tissue (conventionally the forearm) in a chamber (plethysmograph) or measuring circumferential changes of a cross-sectional area of a limb (strain gauge) is the classical approach. Whilst this technique can provide a highly reproducible and accurate measure of tissue volume (462), several assumptions have to be made before concluding that the change in total tissue volume is representative of a change in vascular volume.

2.9.1.2.1. Strain gauge plethysmography (SGP)

SGP measures changes in circumference of a cross-sectional area in a limb. It is based on the (usually reliable) assumption that circumferential changes reflect limb volume changes. It does not measure intravascular volume alone and assumes that the rate of venous pressure rise in the assessed cross-section equals the rate of capacity pressure rise, not taking account of varying viscoelastic properties of different veins leading to different filling pressures. Measurement of the entire PVR is not possible, nor is it possible to be sure that the preparation has returned to the same control volume after experimental stimuli (40). Whilst the technique is well validated and reproducible it is

highly sensitive to limb movements which can lead to error over a prolonged period. When assessing vascular compliance using strain gauges, venous outflow is either obstructed abruptly or stepwise and the progressive change in volume (producing a lengthening of the tube and thus a thinning of the mercury column consequently leading to an increase in electrical resistance) is derived from the different sections (slopes) of the curve reflecting circumferential extension. After an early (seconds) rapid phase of circumferential increase, reflecting the rate of arterial inflow, the slope flattens out. This slope reflects a change in forearm volume, believed to be mainly caused by venous expansion. This is followed by a final slow steady state increase believed to represent interstitial fluid volume accumulation (leakage, lymph, secretion, viscoelastic creep). During this latter phase intravascular volume may actually start to decrease due to a change in transmural pressure. The difference between the asymptotic volume and steady state rate of change and the moment by moment changes in volume tend to follow a single exponential pattern between about 30 seconds and less than 5 minutes after a sudden change in venous pressure (40). In other words the difference in volume of the studied limb before and after inflation of the occlusion cuff consequently reflects the blood pooled in the limb at the occlusion pressure applied. It does not however represent the total blood volume of this segment, but only the volume increase from basal volume after the occlusion (87). Furthermore the small continued increase in forearm volume following prolonged venous occlusion is sometimes used to assess capillary filtration (347;369). Finally, there are some caveats to be considered when applying these assumptions; although vascular capacitance refers to a static, time independent relation, veins and capacitance vessels in particular also elicit some stress relaxation (466;467) and delayed compliance (468), the time course of which extends to minutes (467-469).

The technique of VOP itself however is at its best when limited to relatively short time intervals because repeated and/or prolonged obstruction of venous outflow (via inflation of upper arm cuff) itself can cause a paradoxical decrease in forearm (vascular) volume for reasons outlined below.

2.9.2. Comparing Radionuclide plethysmography and Strain Gauge Plethysmography

Whilst PVR and acute changes in venous volume are highly correlated and reproducible (91;369;454) using both techniques, detailed analysis shows that they are not identical. Clements et al (91) performed a series of simple but elegant experiments comparing both techniques. They recorded simultaneous changes in forearm volume and forearm counts during sequential 1-minute acquisitions, in which they inflated an upper arm cuff ("collecting cuff") in a stepwise manner, from 0 to 80 mmHg, followed by deflation back to 0 mmHg. One-minute equilibration was allowed after each pressure change at each 20-mmHg step before measurements were taken. A close correlation ($r=0.94$ to $r=0.99$) between % changes in count rate and forearm volume changes was present in each subject ($n=6$). However, whereas forearm count rates had returned to baseline following final deflation, forearm circumferential volume remained slightly elevated, probably due to tissue fluid accumulation. To investigate this finding further they obstructed venous outflow for 18 minutes in a single subject. After 2 minutes of continuous cuff pressure, forearm circumferential volume continued to increase but forearm count rate decreased. Following cuff deflation the count rate returned to baseline within 1 minute whilst arm volume remained elevated 5 minutes after cuff deflation. In a second experiment they occluded arterial inflow using a 2nd cuff inflated to 300 mmHg proximal to the

collecting cuff. Whilst in this setting inflation of the collecting cuff had no effect on count rate it produced a sustained upward deflection in the strain gauge measurement, most likely due to forearm distortion. Other groups reported a similar inflation artifact (470). These findings are in keeping with earlier work by Zelis and coworkes (471) showing that "in normal human subjects short term venous congestion and subsequent edema fluid accumulation can result in qualitatively similar changes in limb vascular dynamics as are shown in heart failure patients".

Whilst SGP is the gold standard for assessing FBF, in our view the technique is sub-optimal to assess venous capacitance when performing prolonged studies, dose response studies with cumulative drug infusion, or assessing capacitance effects of drugs with marked effects on vascular conductivity, such as natriuretic and other vasoactive peptides, which have well documented effects on capillary filtration (198;343;347;369). Indeed, VOP previously showed only spurious changes in venous vascular volume and tone in health (343) and CHF (423), whilst our own studies (see chapter 3 and 4) using RPY demonstrate that ANP has marked effects on venous volume and tone in health (472) and CHF (280;473). Also the issue of increased vascular permeability must be considered, especially in disease states with established increased capillary permeability such as type-1 diabetes (474), CHF or sepsis (464). More importantly conventional VOP does not provide a measure of unstressed volume or change in unstressed volume. In contrast RPY provides a direct measure of intravascular unstressed volume and relative changes in unstressed volume.

2.10. Intravenous pressure versus cuff pressure

In this thesis cuff pressure is used as a surrogate for the average pressure in the veins of the forearm. The use of cuff occlusion pressure has previously been validated

against directly measured conduit vein pressure and been found to be closely correlated (460). Furthermore, Brown et al. (475) argue that rapid cuff inflation can cause non-uniform filling of forearm conduit veins (which in their studies was exaggerated by increasing flow !), preventing reliable assessment of distensibility when relating forearm volume to pressure in a single vein.

2.11. RPY Conclusion and Perspective

Despite some theoretical limitations, SGP is regarded as the gold standard for assessment of changes in limb volume and has been used extensively to study human limb veins. Importantly, when modifying protocols accordingly, the technique is able to assess the effect of vasoactive drugs on capillary permeability. Whilst VOP serves a similar purpose as RPY (without the need of complex technology) when assessing limb hemodynamics and the effects of local drug infusions, the latter technique has the potential to assess (simultaneously) further vascular beds, such as the splanchnic and pulmonary circulation, inaccessible to VOP. Furthermore, RPY allows precise assessment of right and left ventricular function at baseline and during/following physiological (e.g. exercise or lower body negative pressure) and pharmacological stimuli. By combining the advantages of EBPS and conventional occlusive techniques, RPY has the potential to be used in the complex, acute hemodynamic assessment of cardiovascular drugs, a prerequisite for optimising and tailoring medical treatment in conditions such cHF, pulmonary and essential hypertension. Finally, as a research tool, RPY has the advantage of providing a direct measure of changes in unstressed intravascular volume and may be the more suitable technique for prolonged studies, and when assessing changes in vascular volume in disease states with known increased capillary filtration such as diabetes and heart failure,

albeit at the cost of not providing a measure of the degree of capillary filtration. The latter weakness could be overcome by combining both techniques.

2.12. Animal work

2.12.1. General and historic consideration of the sheep as an experimental animal

The sheep's (ovis aries) place in research, albeit comparatively small, has a long tradition. In 1667, Jean Dennis, physician to Luis XIV of France transfused a boy aged 15 years with blood taken from the carotid artery of a lamb. The boy felt "a great heat along his arm", probably the first description of a transfusion incompatibility (476). The first recorded transfusion experiments in England, demonstrated to the Members of the Royal Society also in 1667, again used sheep blood (477). In Paris in 1790, a man whose name became a symbol of the French Revolution, Dr Guillotine, perfected his decapitating machine on sheep (478). For reasons that are largely historical the standard haemoglobin used for blood agar plates in bacteriology and the standard red cells for many immunological tests are from sheep. The large volume of sheep endocrine organs available in abattoirs has permitted the isolation of many of the sheep hormones in pure form. The sheep is also an animal ideally suited to surgical modification for experimentation and instrumentation. Its size is convenient and somewhat similar to that of man. This was the reason that many medical device manufacturers have in the past often used the sheep as their research animal of choice. For example early research into the development of an artificial heart was performed in sheep as the thorax of a sheep is large enough to accommodate an artificial heart suitable for implantation in a human (479). For each species of animal there are aspects of its physiology which are similar to general mammalian physiology and

aspects that are different. It is these similarities and differences which mainly determine if a particular species can be used in experiments as a model for other species. Whilst ovis aries and homo sapiens for example differ markedly in their digestive system, for most cardiovascular parameters, the sheep is similar to man if allowance is made for the smaller weight of the average sheep. Published values for most cardiovascular parameters are summarized in Table 2.12.1.1.

Besides the availability and the surgical suitability the sheep was the animal of choice for the present work as there is no other mammal, apart from homo sapiens, in which the cardiovascular effects of NPs have been equally well characterised, in both health (287;480-485) and heart failure (420;486-497). This work has been performed by the Christchurch Cardioendocrine Research Group in New Zealand .

Table 2.12.1.1. Selected ovine cardiovascular parameters

Parameter	Range of published values
Heart rate (beats/min)	66 - 129
Intrinsic heart rate	120
Maximum hear rate	260-280
Midwall aortic stress (dynes x $10^5/cm^2$)	6.9
Stroke volume (ml)	44-95
Cardiac output (l/min)	3.2-5.5
Cardiac output (ml/min/kg)	115-143
BP systolic (mmHg)	115
BP diastolic (mmHg)	83-111
Peripheral resistance (dynes x m^2/cm^{-5})	1840-2726
Arterial baroreceptor sensitivity (msec/mmHg)	29-45
Arteriovenous oxygen difference (ml/100ml)	5.5

2.12.2. Specific Methodology

The specific methodology of the ovine hind-limb model is described in Chapter 5.2.

2.13. Organ bath experiments

For isometric tension recordings 2 to 3mm rings of male New Zealand rabbit aorta were suspended in organ baths. The response to cummulative concentrations of NNP, DNP and ANP was obtained in presence and absence of inhibiting agents in order to determine the mechanism of this vasorelaxation. The detailed methodology is described in Chapter 6.2.

Chapter 3 – The effects of ANP on venous capacitance in man

3.1. Background / Introduction

ANP plays an important role in cardiovascular control, being a major determinant of sodium homeostasis, plasma volume and BP. Although intraarterial infusion of ANP produces vasorelaxation of resistance vessels (161;251) this may not be the underlying mechanism of the fall in BP that accompanies systemic ANP infusion. Animal studies (344;345) have shown that the fall in BP and CO are almost invariably associated with a fall in CVP and, in fact, an increase in TPR. Indeed, Groban et al (346) demonstrated in humans that low dose systemic infusion of ANP reduced CVP without affecting BP. It is now generally accepted that the fall in CO/BP following systemic infusion of ANP is a consequence of reduced preload (498). Possible mechanisms include: volume contraction as a result of diuresis; increased capillary filtration (369); and direct venodilatation, even though no venorelaxant effects of ANP were observed in dorsal hand veins (161) and saphenous veins (163). Given that atrial stretch is the principal stimulus for ANP release (228), the apparent lack of a direct effect on venous tone in man seems counterintuitive. In addition, the contribution of basal levels of ANP to human resting vascular function is unknown but acceptance of ANP as a physiologically important vasoactive hormone in health clearly depends on its contribution being significant.

We hypothesised that, despite its lack of action on conduit veins, ANP may act as a venodilator on the small veins and venules that constitute the majority of the capacitance vasculature. To study the effect of ANP on vascular function in general and venous tone in particular, 4 experiments were performed. In experiment 1 we evaluated the effect of incremental doses of intraarterial ANP on forearm vascular volume (FVV) and venous tone. In experiment 2 we assessed the contribution of

basal ANP plasma levels to FVV and venous tone, by intraarterial infusion of the NPR_A-selective ANP-receptor antagonist A71915. In experiment 3 we further explored the specificity and potency of A71915 by measuring FBF during intraarterial co-infusion of incremental doses of this receptor antagonist and either ANP or sodium nitroprusside (SNP). In experiment 4 we determined whether or not the potential effects of basal ANP on vascular volume were specific to the forearm, by assessing the effects of intravenous (i.v.) infusion of A71915 on regional vascular volume in the physiologically important intestinal bed.

3.2. Methods

3.2.1. Subjects

Four groups of seven healthy volunteers with no history of or evidence for cardiovascular disease or other cardiovascular risk factors were studied. Baseline characteristics are shown in Table 3.2. None were taking any medication. All had normal clinical examination, electrocardiograms (ECGs) and ventricular function as assessed by radionuclide ventriculography. All gave written consent. The study was approved by the local research ethics committee. Heart rhythm/rate and BP were recorded continuously and samples for determination of hematocrit and total protein were taken at the beginning and the end of each study.

TABLE 3.2. Baseline Characteristics of Subject Group

	Group 1	Group 2	Group 3	Group 4
Age, y (range)	34 (26-48)	50 (27-66)	32 (27-36)	58 (35-79)
Sex, M/F, n	7/0	4/3	7/0	5/2
Body mass index, kg/m^2	24.1±0.6	25.9±1.3	24.0±0.4	26.8±1.5
Forearm volume, mls	1751±72	1580±105	1671±82	1572±98
LV ejection fraction, %	57±3	51±4	59±3	50±4
Serum Na^+, mmol/l	140.3±0.6	140.2±0.8	140.4±0.6	140.1±0.7
Urinary Na^+, mmol/l	105±13	124±25	112±16	123±17
Serum Creatinine, µmol/l	96±10	83±4	94±8	104±9
Serum Urea, mmol/l	5.4±0.9	5.1±0.2	5.3±0.7	5.8±0.4
Heart rate, min^{-1}	62±6	63±8	61±5	65±7
Blood pressure, mmHg				
Systolic	128±8	131±9	127±7	130±8
Diastolic	70±6	71±7	69±7	72±7

Continuous data are presented as mean±SEM.

3.2.2. Assessment of venous function

Changes in forearm and intestinal vascular volume (IVV) were assessed by EBPS. Combining EBPS with a standard occlusion technique ("RPY"), as described in details above (Chapter 2), we also determined forearm venous compliance from which venous tone was derived.

Briefly, a dynamic image of the forearm was continuously acquired and a ROI was defined (Plate 2.4.2). The count in the ROI, obtained with no occluding pressure, was taken as baseline or UVV. Forearm pressure volume relationships were then constructed by inflating upper arm cuffs for 1 minute to pressures of 10, 20 and 30 mmHg. Scintigraphic vascular volumes were plotted against cuff pressure to form venous PVR. Linear regression was performed for each PVR, and a linear model was accepted if $R^2 \geq 0.9$. The slope of each PVR is a measure of venous compliance. A

parallel upward shift in PVR indicates a decrease in venous tone whilst a parallel downward shift indicates an increase. In order to allow presentation of grouped data, results are presented as percentages.

3.2.2.1. Experiment 1: effect of intrabrachial ANP on UVV and venous tone in the forearm

An antecubital vein in each arm was cannulated with an 18 gauge cannula. After red cell labelling with ^{99m}Technetium, a 27 gauge steel needle, sealed with dental wax to a 16 gauge epidural catheter, was inserted into the brachial artery of the left arm of 7 volunteers (Group 1) under sterile conditions and kept patent by continuous infusion of saline at a rate of 1.0ml/min. The left arm was positioned on the face of a gamma camera (Elscint Apex 215M). The right arm was also placed on a gamma camera (ADAC-Transcam) and thus served as a control for the left arm. Thirty minutes after needle insertion, two baseline venous PVR were recorded. Thereafter incremental ANP (Clinalfa) infusions at concentrations of 0.05µg/ml, 0.1µg/ml, 0.5µg/ml, 1µg/ml, 2.75µg/ml and 5µg/ml commenced, at 1ml/minute (in pilot studies doses of 10µg/ml had shown to exert systemic effects). At each dose one PVR was performed after a 5-minute run-in period. Venous sampling from the infused arm was attempted at baseline and before the end of each infusion step for measurement of a) plasma ANP concentration (in all subjects) and b) plasmanorepinephrine (499) (in 4 subjects). For the former 10 ml of blood was collected in chilled polypropylene tubes containing 400µl of propylalcohol-conserved aprotinin. Plasma was immediately separated and frozen at -70°C. 37 out of a possible 49 samples could be obtained without a visible degree of hemolysis. Natriuretic peptide analysis was performed in two assay runs and measured by radioimmunoassay as described elsewhere (500). Results from a

further 4 samples had to be excluded because duplication of results was poor, even on repeat testing.

3.2.2.2. Experiment 2: effect of intrabrachial A71915 on UVV and venous tone in the forearm

The setup for this experiment was identical to that of experiment 1. Thirty minutes after needle insertion, two baseline venous PVR were recorded in a further 7 volunteers (Group 2). Thereafter incremental A71915 (Clinalfa) infusions at concentrations of 0.5μg/ml and 1μg/ml commenced at 1 ml/minute. At each dose one PVR was recorded after a 20-minute run-in period. As the binding affinity of A71915 to the GC-coupled ANP receptor (NPR_A) is 22 times lower than that of ANP (223), the infused concentrations of A71915 were chosen to produce approximately 50:1 and 100:1 ratios of A71915 and basal ANP in the venous effluent. The lengths of run-in periods in experiments 1 and 2 were chosen to allow a steady state to be reached, based on pilot data.

3.2.2.3. Experiment 3: effect of intrabrachial A71915 on ANP/SNP induced changes in FBF

In this experiment FBF was recorded simultaneously in both arms by strain-gauge VOP as described previously (501). The left brachial artery of 7 volunteers (Group 3) was cannulated as before and kept patent with a 15-minute infusion of saline. Thereafter ANP was infused at 0.1μg/min followed by coinfusion with A71915 at 0.5μg/min, 1μg/min, 2μg/min and 5μg/min to achieve approximate ANP:A71915 ratios of 1:5, 1:10, 1:20 and 1:50 in the brachial artery. In order to assess whether the inhibitory effect of A71915 on ANP induced vasodilatation was specific or non-

specific, more than a week later 5 out of the 7 volunteers underwent a second study to assess the effects of A71915 (5μg/min) on SNP (1μg/min) (David Bull Laboratories) induced changes in FBF. In all FBF studies measurements were made during the last 90 seconds of each 6-minute infusion period and the ratio in FBF between arms was expressed as "%-change" (ΔFBF%) from baseline (FBF during saline infusion).

3.2.2.4. Experiment 4: effect of intravenous A71915 on regional IVV

Measurement of regional IVV was performed in all 7 volunteers (Group 2) from experiment 2, over 90 minutes after completion of their forearm study, plus an additional 7 volunteers (Group 4) who acted as "time controls" but did not receive A71915. Volunteers were positioned supine on a bed. The gamma camera (ADAC-Transcam) was positioned approximately 2 cm above each volunteer's anterior abdominal wall. The bladder, the lower edge of the liver and the bifurcation were used as landmarks. Lead strips attached to the skin facilitated the monitoring of a constant ROI (Plate 2.3.2.1.). Volunteers were instructed to breathe regularly and not to move. A constant patient-camera position was maintained throughout. At baseline 5ml of saline were injected intravenously followed by an infusion at 1ml/minute, during which a 200 second scintigram was recorded. The 7 subjects of Group 2 were then injected with 5μg A71915 in 5ml saline followed by an infusion of A71915 at 1μg in 1ml/minute, during which another 200 second scintigram was recorded. The 7 time control volunteers underwent the same protocol but received only saline. Since the count from a region is directly proportional to the quantity of blood in that region, changes in count rate (corrected for physical decay) are proportional to changes in regional vascular volume.

3.3. Statistics

Data are expressed as mean +/- SEM. In experiment 1 and 2 the effects of ANP and A71915 on FVV in the infused arm were assessed by 2-way analysis of variance (ANOVA) with post-hoc comparison to baseline. A value of $p<0.05$ was considered significant. Original data (counts/sec) were analysed when assessing drug effects. Conversion to percentage was then undertaken to allow presentation of grouped data. When additionally presenting "corrected data" in experiment 2, changes in the infused arm (venoconstriction) were first corrected for systemically occurring venoconstriction and converted into percentage before assessed by 2-way ANOVA as above. In experiments 3 changes in FBF were assessed either by 2-way ANOVA (ANP dose-ranging studies)) or the non-parametric Wilcoxon test (SNP) as appropriate. In experiment 4 regional IVV was analysed by unpaired t test.
A non-parametric Mann-Whitney test was used to compare changes in plasma norepinephrine.

3.4. Results

3.4.1. Subject characteristics

Grouped baseline characteristics are shown in Table 3.2. There were no significant changes in BP, HR, haematocrit or plasma proteins in any of the groups in any experiment.

3.4.2. Experiment 1: effect of intrabrachial ANP on UVV and venous tone in forearm

Intraarterial infusion of incremental ANP concentrations caused a dose dependent parallel upward shift in the PVR in all 7 subjects, indicating venodilation in the infused arm (Figure 3.4.2.1. and Figure 2.4.1.). ANP infusion of 0.05μg/min caused a

modest but non-significant 6% ($p=0.1$) increase in UVV. ANP infusions of 0.1μg/min, 0.5μg/min, 1μg/min, 2.75μg/min and 5μg/min caused significant increases in UVV of 8% ($p<0.05$), 13% ($p<0.001$), 14% ($p<0.001$), 18% ($p<0.001$), and 20% ($p<0.001$) respectively, compared to baseline in the same arm (Figure 3.4.2.2.).

UVV decreased gradually in the non-infused arm during the study, by 12 ± 6% (p=ns versus baseline) during the initial infusion rate and by 43 ± 8% ($p=0.01$ versus baseline) at the final/peak infusion step.

Baseline plasma ANP level was 14.6 ± 8.4 pg/ml. To further investigate the relation between venous ANP and venodilatation, we grouped the effect on UVV according to ANP levels in the venous effluent from the infused arm (Figure 3.4.2.3.). High physiological/low pathophysiological ANP levels (25-100pg/ml) were associated with a non-significant 11 ± 4% ($p=0.1$) increase in UVV. Infusions achieving pathophysiological (101-400pg/ml) and high pathophysiological (401-600pg/ml) concentrations caused a significant 18 ± 6% ($p<0.05$) and 19 ± 5% ($p<0.05$) increase in UVV respectively. Pharmacological levels (>600pg/ml) caused a significant 16 ± 3% increase in UVV ($p<0.01$).

Changes in plasma norepinephrine in the infused and non-infused arm

Plasma norepinephrine levels rose during the study by 269±99pg/ml in the non-infused arm (from 327±70pg/ml at baseline to 596±123pg/ml at maximum dose; $p=0.07$) compared to 58±87pg/ml (from 362±20pg/ml at baseline to 421±101pg/ml at peak dose; $p=0.47$) in the infused arm; $\Delta p=0.34$.

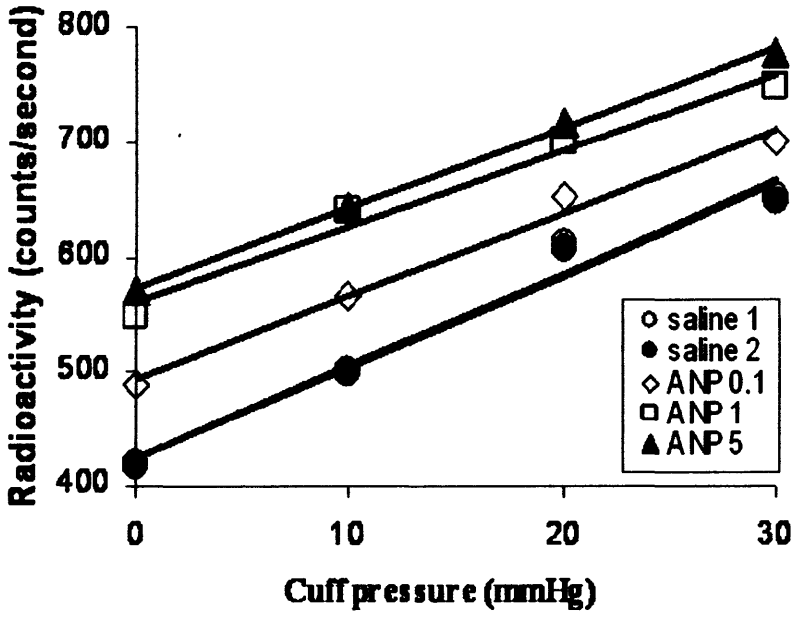


Figure 3.4.2.1. Typical PVR of the infused arm following ANP. The parallel up-ward shifts in the PVR following 0.1μg/min, 1μg/min, and 5μg/min ANP reflect a reduction in venous tone. The slope of each line reflects compliance. Linear regression equations; ○ $y=8.0X + 424$; $R^2=0.973$, ● $y=7.9X + 422$; $R^2=0.973$, ◇ $y=7.2X + 492$; $R^2=0.986$, □ $y=6.5X + 560$; $R^2=0.976$, ▲ $y=6.9X + 572$; $R^2=0.998$.

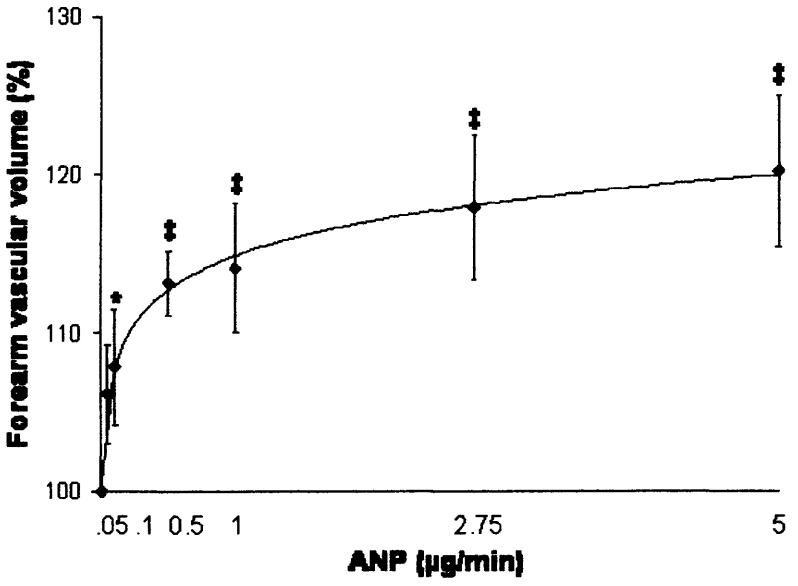


Figure 3.4.2.2. ANP dose response curve; Increase in vascular volume according to infused ANP concentration. *p<0.05, ‡p<0.001, 2-way ANOVA, vs baseline.

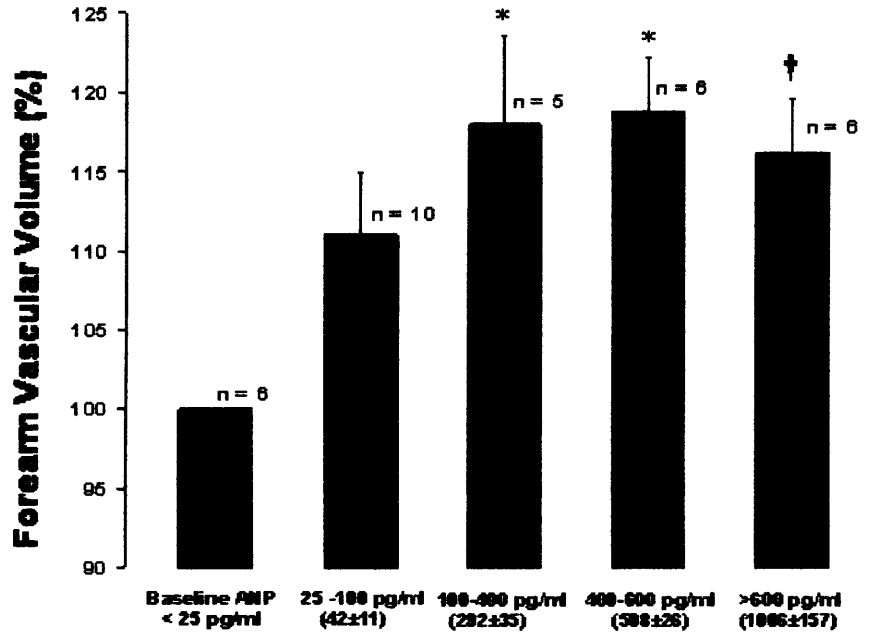


Figure 3.4.2.3. Increase in vascular volume according to plasma levels (n=33 samples). Venous plasma levels similar to those seen in severe heart failure (i.e. between 100 and 400pg/ml) achieve near-maximal venodilatation. *$P<0.05$, †$P<0.01$ vs baseline.

3.4.3. Experiment 2: effect of intrabrachial A71915 on UVV and venous tone in forearm

Infusion of A71915 caused a dose dependent parallel downward shift of PVR (Figure 3.4.3.) in all but one volunteer, reflecting increased venous tone. Infusion of 0.5μg/min and 1μg/min caused 7.7±2.3% ($p=0.01$) and 16.1±3% ($p<0.01$) reductions in UVV in the infused arm respectively.

UVV decreased gradually in the non-infused arm during the study, by 3.3±2.4% (p=ns) at 0.5μg/min and by 7.0±3.2% ($p=0.08$) at 1μg/min.

When correcting for the reduction in vascular volume observed in the contralateral arm, the net effect of A71915 infusion was 4.4±1.5% ($p=0.01$) and 9.6±1.1 % ($p<0.001$) respectively.

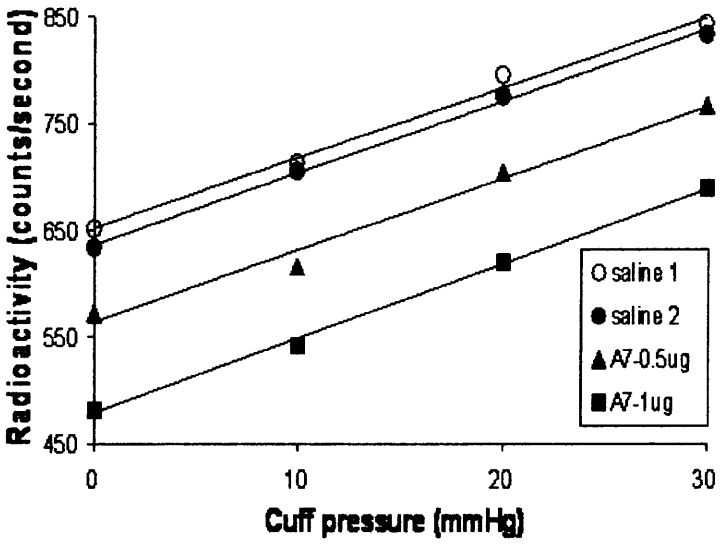


Figure 3.4.3. Typical PVR of the infused arm following A71915. Note the marked reduction in counts/sec after 0.5μg/min (≅12%) and after 1μg/min (≅ 26%) are not yet corrected for systemic venoconstriction. Furthermore, the dose dependent parallel down-ward shift reflects venoconstriction. Linear regression equations; ○ y=6.5X + 651; R^2=0.990, ● y=6.7X + 635; R^2=0.997, ▲ y=6.6X + 569 R^2=0.985, ■ y=6.9X + 478; R^2=0.998.

3.4.4. Experiment 3: effect of intrabrachial A71915 on FBF

The results of the dose-ranging studies are shown in Figure 3.4.4.1. At an infusion ratio of 50:1 A71915 almost completely abolished the effects of ANP on FBF. SNP increased FBF by 111±29 % ($p<0.05$). A71915 did not attenuate the SNP-induced increase in FBF.

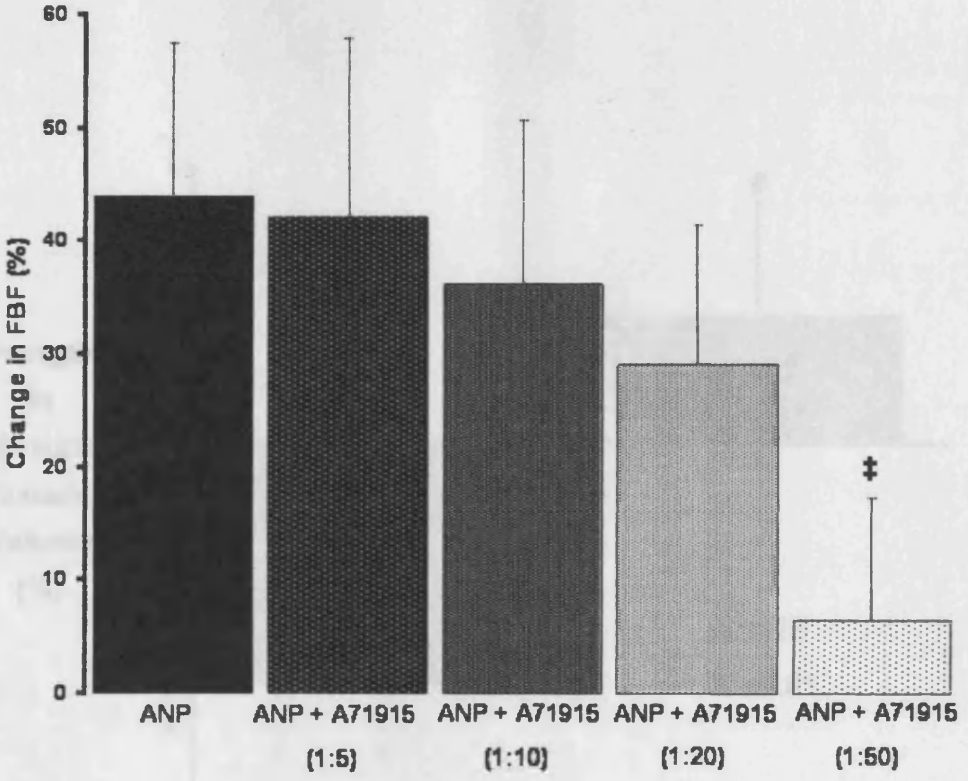


Figure 3.4.4.1. Effect of ANP and ANP/A71915 coinfusion on FBF. ANP (0.1μg/min) vs ANP/A71915 **(1:50)** coinfusion; ‡$p<0.01$, 2-way ANOVA.

3.4.5. Experiment 4: effect of intravenous A71915 on IVV

Intravenous bolus injection of 5μg A71915 followed by intravenous infusion of 1μg/min for 200 seconds reduced regional IVV in all subjects of Group 2. The mean reduction was 2.6±0.5%; p=0.01. At the same time regional IVV in the 7 time control volunteers (Group 4) increased by 1.4±1.2%; p=0.3. The difference between both groups was significant; p<0.05. The results are also illustrated in Figure 3.4.5.1.

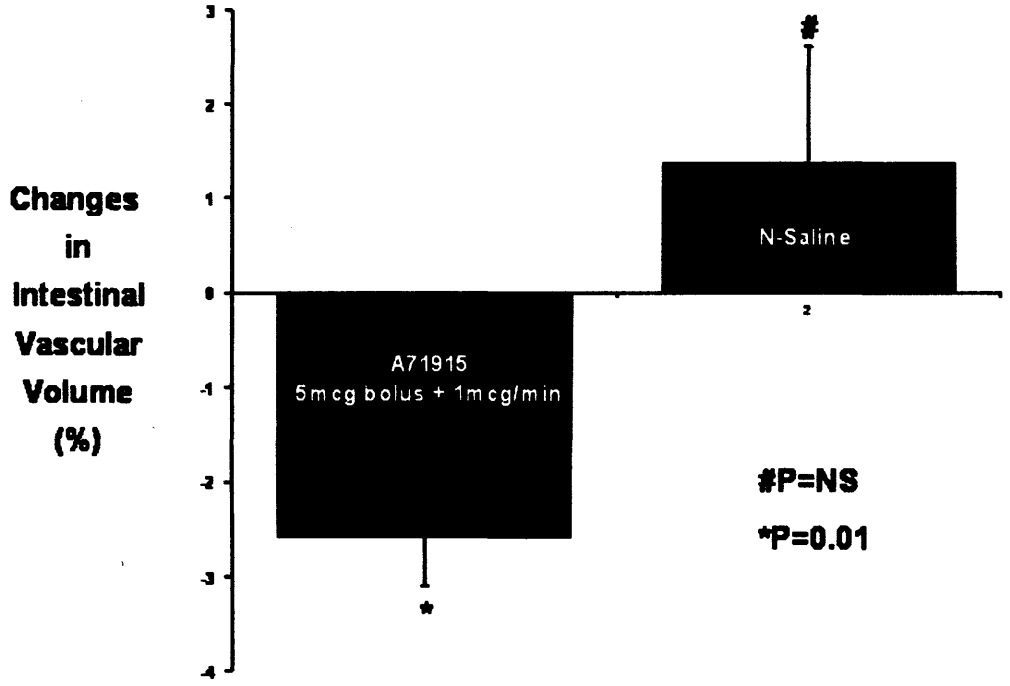


Figure 3.4.5.1. The effects of A71915 and N-Saline on intestinal capacity (IVV) are illustrated. Recumbency tended to increase IVV. This trend was reversed following A71915 administration.

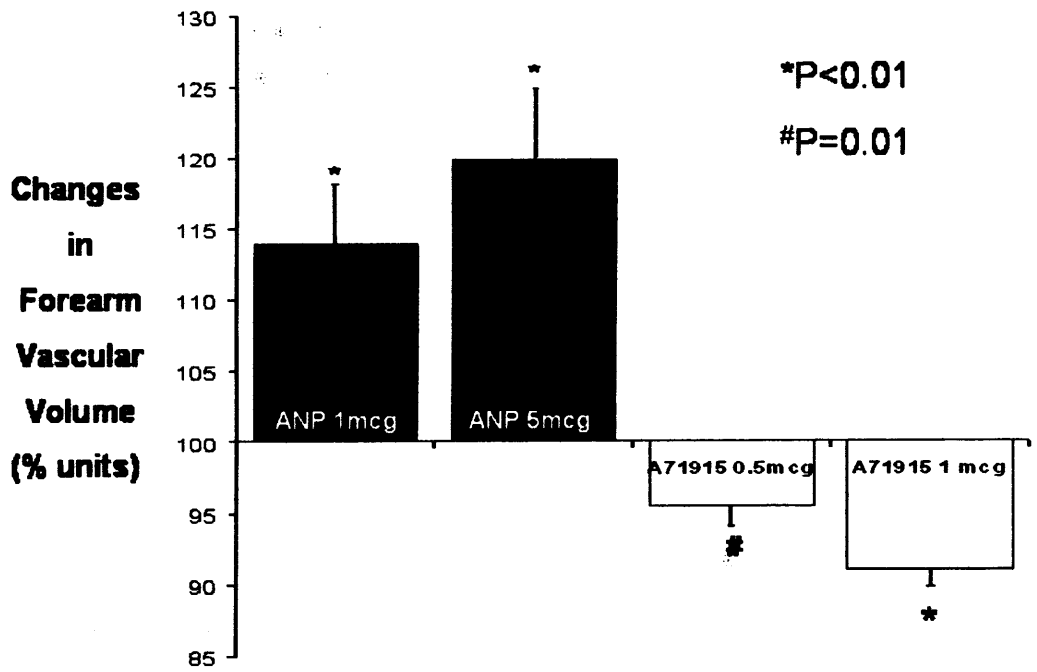


Figure 3.4.5.2. The contrasting effects of ANP and A71915 on FVV in vivo are illustrated

3.5. Discussion

In this study we provide direct evidence that ANP is a local regulator of FVV and IVV in health. This is in keeping with a previous study demonstrating that systemic ANP infusion increases intestinal blood content (56) but contrasts with other reports that ANP has minimal or no activity on conduit veins (161;163). Venodilator activity was seen across a wide range of physiological and pathophysiological plasma levels with a near maximum venodilating effect at plasma levels similar to those seen in severe heart failure (between 100-400pg/ml). Importantly, we show for the first time that basal ANP levels influence vascular volume and resting venous tone in both

forearm and intestinal beds. The forearm studies exclude ANP-mediated central sympathetic withdrawal as the mechanism.

3.5.1. Previous studies assessing the effects of ANP on venous function

Several studies showed that ANP consistently reduced CVP (344;345), even at very low doses, without measurable changes in BP (346). This was in keeping with a primary venodilating ANP effect. However, other studies failed to detect a direct venodilating action. Holtz et al observed that systemic infusion of ANP had little or no effect on venous tone in anaesthetised dogs (anaesthetics in general and pentobarbital inparticular have been shown to alter ANP responsiveness (502;503)), despite potent effects on arterioles (504). Studies in human dorsal hand veins (161) and saphenous veins (163) also failed to demonstrate any significant effect. Doorenbos et al, using a strain gauge technique, investigated the effect of brachial artery infusion of ANP on venous compliance (343). ANP infusion alone had no effect, but antagonised the effects of Ang II on venous compliance during coinfusion. Ando et al used a water displacement plethysmography technique to compare the effects of brachial artery infusion of SNP and a single dose of ANP on the forearm vascular bed (369). Both agents shifted the forearm PVR upwards, but ANP had less marked effects than SNP and simultaneously increased capillary filtration. Because this technique measures total limb volume (i.e. tissue plus blood volume) it is difficult to be certain about the relative contributions of venodilation and increased tissue fluid.

3.5.2. How can these discrepancies be explained?

It has become increasingly clear that conduit veins in general and dorsal hand veins in particular behave differently from the physiologically important small veins and venules. Furthermore, much of the in vitro work assessing the vascular effects of ANP has been carried out on mammary arteries and saphenous vein grafts harvested from patients undergoing bypass operations (163). The vascular response in these specimens may not reflect the in-vivo response in healthy people. In addition, the absolute number and proportional representation of NPR subtypes (NPR_{A+B} versus NPR_C) varies from tissue to tissue (505), and such differences may well account for the different response between the small veins and venules and large conduit veins. Moreover, some human studies employed doses of ANP that resulted in plasma concentrations far above those seen in health or disease (370). Because of the consequent fall in BP, baroreflex mediated changes may supervene, causing reflex-mediated constriction to over-ride any local venodilator effects.

3.5.3. Alternative explanations for increased vascular volume?

ANP has well documented effects on sympathetic nerve activity (198). Vatta et al demonstrated that ANP plays a role in the modulation of norepinephrine (NE) metabolism in the rat hypothalamus and adrenal medulla, affecting storage, release and uptake of NE (159). They concluded that ANP acts as an inhibitor of noradrenergic neurotransmission. Also, as Ang II is known to potentiate peripheral sympathetic activity (506) (the mechanism in veins is believed to be pre-synaptic), the well documented effects of NPs on reducing Ang II levels (370) could indirectly reduce NE release. However, withdrawal of sympathetic activity was not the mechanism of venodilation in the present work, either at a central or a peripheral

level, since we observed venoconstriction in the contralateral arm, and venodilation in the infused arm occurred despite an increase in plasma NE in the venous effluent from this arm.

3.5.4. Effects of ANP-receptor antagonism

Acceptance of vasoactive compounds as physiologically significant depends on the demonstration of changes in vascular tone in response to blockade of basal levels. This has traditionally been achieved using receptor antagonism. A71915, an antagonistic ANP-analogue, has emerged as the most potent inhibitor of ANP-stimulated, NPR_A-mediated, cyclic guanylate monophosphate production (223). Brunner and Woelkert used A71915 to show that basal ANP contributes to regulation of coronary and TPR in rodents (364). To our knowledge the present work is the first to assess the effects of ANP-receptor antagonism in a human (in vivo) study. We provide direct evidence that blocking the effects of basal ANP levels increases venous tone, thereby decreasing vascular volume. Furthermore, we provide evidence that A71915 inhibits ANP-induced increases in FBF in a dose dependent, NPR_A mediated fashion.

3.5.5. Study limitations

Observations in the contralateral arm merit comment. During infusion of A71915 vascular volume decreased in both arms, though significantly more in the infused arm. Because of this unidirectional behaviour, changes in vascular volume and venous tone in the infused arm were corrected for changes in the contralateral arm. In contrast, infusion of ANP increased vascular volume in the infused arm whilst there were opposing effects in the contralateral arm. Expressing volume changes in the infused

arm in relation to the changes in the contralateral arm, as is convention in assessing FBF using VOP, would therefore have been potentially misleading and was ultimately not performed in the ANP dose/response study as data for the contralateral arm was not recorded in the first two study subjects. The contralateral arm venoconstriction, initially surprising, is a consistent phenomenon during our studies. Potential mechanisms include: increased sympathetic outflow caused by discomfort arising during the lengthy study; and reflex-mediated vasoconstriction via baroreceptors or cardiopulmonary receptors, in response to subtle changes in BP or central blood volume due to systemic drug effects and/or prolonged semi-recumbence. Our observation of an increase in plasma NE levels is consistent with any of these mechanisms. Furthermore, it has previously been shown that prolonged venous congestion per se significantly reduces venous vascular volume in the forearm of healthy volunteers (507). Consequently our measurements of the venodilating ANP action in the infused arm are likely to underestimate the true magnitude of its effect as ANP actions were opposed by the effects of increased sympathetic stimulation.

It is clear that the findings of this study cannot simply be extrapolated to the syndrome of cHF as there is evidence of hyporesponsiveness or "resistance" to ANP in situations associated with chronic elevation of ANP levels (199). Furthermore, it is important to note that the observations cannot be explained simply on the basis of increased arterial inflow. We and others have previously shown that limb PVR are unaffected by large increases in flow (for detailed discussion see Chapter 3). Finally, in experiment 1, the documented increase in count rate does not represent increased extravasate as a consequence of increased capillary permeability, since 95-98.5% of the technetium was bound to erythrocytes and vascular volume returned below baseline within 30 minutes following the cessation of ANP infusion. The

measurement therefore reflects changes in vascular volume. Indeed, increased capillary permeability would tend to blunt the magnitude of the measured change in venous tone using this technique, by increasing tissue attenuation.

3.6. *Conclusion*

These studies provide direct evidence that ANP is a regulator of regional vascular volume and venous tone in healthy subjects. Importantly, we show that basal ANP levels contribute significantly to resting venous tone, and that plasma levels similar to those seen in severe heart failure exert a powerful venodilator effect.

CHAPTER 4 – THE EFFECTS OF NATRIURETIC PEPTIDES ON VENOUS CAPACITANCE IN cHF

4.1. Introduction

Animal and human studies have suggested a variable reduction in hormonal, renal and resistance-vessel responsiveness to NPs in heart failure (HF) (157;166;199;425;438;508-511). Despite minimal effects on plasma volume and resistance-vessel tone, infusion of NPs in patients with cHF has consistently been shown to reduce CVP (366;367). This raises the possibility that the venodilator effects of NPs may be preserved in HF.

Although earlier data suggested that NPs exert their effects solely via a non-endothelium-dependent, particulate GC pathway (512), recent evidence suggests that the vaso-relaxant effects may, at least in part, be mediated via an endothelium-dependent pathway involving NO and soluble GC (435;436). Endothelial dysfunction, a characteristic feature in the arterial vessels of patients with CHF (177), may therefore theoretically contribute to the phenomenon of NP hypo-responsiveness ("NP resistance"). We previously demonstrated preserved venous endothelial function in cHF patients despite the presence of marked arterial endothelial dysfunction (437). We hypothesised that cHF patients may have preserved venous NP responsiveness despite diminished resistance-vessel responsiveness and that this may be explained by preservation of venous endothelium-dependent NO release.

This studies had four aims: first, to compare the effects of the currently known human NPs (ANP^{99-126}, ANP^{95-126} [Urodilatin], BNP, CNP) on the forearm capacitance vasculature in 53 cHF patients on optimal therapy; second, to examine the contribution of basal NP plasma levels to regulation of regional vascular volume (VV) and venous tone by intra-arterial infusion of the NPR_A-selective ANP-receptor

blocker A71915; third, to test the hypothesis that venous responsiveness to exogenous ANP was preserved but resistance-vessel responsiveness attenuated in cHF patients versus controls; and fourth, to assess the contribution of NO to NP-induced changes in venous and resistance vessels by co-infusion with the NO- synthase inhibitor, N^G-monomethyl-L-arginine (LNMMA).

4.2. Methods and Data Analysis

Subjects: fifty-three consecutive patients with clinical features of cHF, recruited from the Heart Failure Clinic at the University Hospital of Wales, were enrolled to receive intra-arterial ANP, BNP, CNP, Urodilatin, or A71915. All patients satisfied the European Society of Cardiology (ESC) criteria for the diagnosis of CHF (5) and had impaired left ventricular systolic function (EF<45%) as assessed by radionuclide ventriculography. Patients with hypertensive heart disease were excluded. All were on diuretics, a maximally tolerated dose of an ACE inhibitor or an AT_1-antagonist, and unless contraindicated or previously not tolerated, on maximally tolerated doses of β-blockers. Their symptomatic status had remained unchanged on stable medical therapy for at least 2 months. Long-acting nitrates (n=5) were stopped 24 hours before the study and all medications were withheld on the morning of the study. Eleven healthy volunteers with no history of or evidence for cardiovascular disease or other cardiovascular risk factors were also studied as a control group. None was taking any medication. All had normal clinical examination, ECG's and ventricular function (EF>45%) as assessed by radionuclide ventriculography. Volunteer and patient characteristics are shown in Table 4.2.1. and Table 4.2.2. All participants gave written informed consent. The study was approved by the local research ethics committee.

Table 4.2.1. Baseline Characteristics

	Controls	cHF Patients				
	ANP	ANP	URODILATIN	BNP	CNP	A71915
Age, y (range)	57 (34-69)	63 (42-79)	67 (53-77)	63 (38-79)	65 (54-76)	59(40-83)
Sex, M/F, n	10/1	18/0	8/0	8/0	7/1	9/2
NYHA class		2.3±0.1	2.4±0.2	2.4±0.2	2.4±0.2	2.2±0.1
Etiology, (IHD/DCM), n		12/6	7/1	4/4	5/3	6/5
Body mass index, kg/m^2	25.1±1.2†	29.2±0.7	29.4±1.0	28.1±1.6	31.7±1.7	27.9.0±2.4
Forearm volume, mls	1709±77	1694±53	1690±94	1646±31	1929±203	1702±148
FBF, ml/100ml FV	2.14±0.37	2.18±0.18	2.01±0.17	2.10±0.27	2.63±0.36	2.36±0.33
LV ejection fraction, %	50±1‡	29±3	24±4	25±4	38±5	32±5
BP - Systolic, mmHg	119±4	110±4	117±8	107±6	104±5	122±9
BP - Diastolic, mmHg	64±3	65±3	54±3	61±3	60±4	73±7
Heart rate, min^{-1}	65±4	66±5	67±5	65±4	65±5	65±6
Diuretic / + Spironolactone		18/5	8/2	8/4	8/0	11/6
ACE/AT_2		17/1	8/0	6/2	8/0	9/2
β-Blocker		10	4	6	5	6

Continuous data are presented as mean±SEM. Symbols †$p \leq 0.01$, ‡$p \leq 0.001$ depict comparison between both ANP groups.

Table 4.2.2. Humoral, Urinary, and Hemodynamic Parameters at Baseline and End of Study

	Controls	cHF Patients				
	ANP	ANP	URODILATIN	BNP	CNP	A71915
Baseline						
Serum Na^+, mmol/l	139.4±0.5	139.3±0.6	137.1±1.2	137.3±0.8	139.2±0.8	137.4±1.2
Urinary Na^+, mmol/l	77±14	59±13	81±19	61±17	89±31	88±15
Serum Creatinine, µmol/l	88±4	118±7	112.9±12	90.1±4	107.8±9	89.6±10
Serum Urea, mmol/l	6.3±0.5	8.8±1.2	9.7±2	6.4±0.7	8.4±0.8	7.1±1.2
Serum Albumin, g/l	44.3±0.5	43.9±0.7	44±0.7	44±0.8	42.7±0.9	46.6±0.8
Hemoglobin, g/dl	14.2±0.3	14.4±0.3	14.1±0.3	14.6±0.6	13.9±0.5	14.3±0.7
Hct	0.41±0.01	0.42±0.01	0.41±0.01	0.43±0.02	0.40±0.01	0.42±0.02
Heart rate, min^{-1}	65±7	66±8	68±8	66±6	66±6	67±8
Mean BP, mmHg	83±3	80±3	75±3	76±4	75±4	90±5
End of Study						
Serum Na^+, mmol/l	138.2±0.5	138.6±0.6	137.0±1.2	137.4±1.0	139.7±0.8	136.2±0.8
Urinary Na^+, mmol/l	96±21	92±16‡	105±28	66±12	154.5±17	76.2±19.2
Serum Creatinine, µmol/l	82±4*	97±8‡	102.5±12†	83.3±3*	101.4±8	76.5±8.9†
Serum Urea, mmol/l	6.1±0.5*	7.8±1.2†	9.6±2*	6.3±0.7*	7.9±0.6	6.9±1.0*
Serum Albumin, g/l	42.1±0.8*	39.1±1.2‡	40±0.9*	39.5±1.0†	38.8±1.4	42.2±0.8†
Hemoglobin, g/dl	13.8±0.4*	13.6±0.3‡	13.4±0.3‡	13.8±0.6†	13.5±0.6	13.2±0.6†
Hct	0.40±0.01	0.40±0.01‡	0.39±0.01‡	0.41±0.01*	0.38±0.02	0.39±0.02†
Heart rate, min^{-1}	66±7	67±6	67±8	65±6	65±6	65±8
Mean BP, mmHg	82±4	81±4	74±6	76±6	75±5	90±5

Continuous data are presented as mean±SEM. Symbols *$p \leq 0.05$, $^{\dagger}p \leq 0.01$, $^{\ddagger}p \leq 0.001$, depict comparison to baseline.

4.2.1. Assessment of Arterial Resistance-Vessel Effects

The forearm resistance vasculature was assessed using conventional VOP as described previously (501). We interpreted changes in FBF to have been due to changes in to changes in arterial (arteriolar) resistance. FBF was calculated according to the formula of Whitney (85). Changes in FBF (ml/100ml/min) were assessed in the infused and non-infused arms and changes in the former were expressed as a ratio of those in the latter (513). Changes in flow ratios following NP infusions are expressed as percentage changes from the baseline (i.e., normal saline infusion) that preceded the administration of each drug. FBF results are expressed as mean values with 95% CIs, unless stated otherwise. With respect to the dose-response curves (Figures 4.2.1.1a+b), the area under the curve (AUC) was calculated using the trapezoid rule (514).

4.2.2. Assessment of Venous Function

Changes in FVV, compliance and venous tone were assessed by RPY, as described previously (437;515;516) and outlined in the Methodology section of this thesis.

4.2.3. Protocol

All studies commenced between 8.30 and 9.30am and lasted between 2.5 to 3.5 hours. Patients were asked to abstain from caffeine-containing drinks for at least 12 hours beforehand. Forearm volume (FV) was assessed by water displacement. Antecubital veins of both arms were cannulated with 18-gauge cannulae and baseline blood sampling was performed. After red-cell labelling, a 27-gauge unmounted steel needle, connected to an epidural catheter and sealed with dental wax, was inserted into the brachial artery of the non-dominant arm under sterile conditions. Each arm was then

positioned on the face of a gamma camera. Thirty minutes after infusion of saline was commenced, FBF was assessed. This was followed by recording of two baseline venous pressure-volume relations (PVR). Thereafter, incremental NP (ANP* [* = all Clinalfa, Läufelfingen/Switzerland], Urodilatin [Cardiopep, Hannover/Germany], BNP*, CNP*; 0.05μg/min, 0.5μg/min, 1.0μg/min) or A71915* (0.5μg/min and 1μg/min in 7 subjects and 20μg/min in 4 further subjects) infusions were commenced at a volume infusion rate of 1 ml/min. Following a 5-minute run-in period (10 minutes for A71915), FBF was assessed, followed by a 2-minute gap and measurement of one PVR. This was repeated during co-infusion of NP (1μg/ml) + LNMMA* (12mg/ml), following a 15-minute run-in period.

4.2.3.1. Plasma ANP Levels and cGMP Spillover

Venous-effluent blood samples were taken from the infused arm at the end of each infusion period for assessment of plasma NP and cGMP. NP and cGMP measurements were performed by blinded operators using standard competitive radioimmunoassay (Peninsula Laboratories, Bachem Ltd UK) and enzyme immunoassay kits (Cayman Chemical, Michigan/USA), respectively.

In both ANP groups the difference between plasma cGMP concentration at the baseline and after the maximum dose of ANP was multiplied by the increase in FBF from the baseline level induced by the corresponding maximum dose, and the resulting values were taken as representative of the change in the amount of forearm cGMP spillover.

4.2.3.2. Statistics

Continuous data are expressed as mean ± SEM. The baseline characteristics of the cHF groups (ANP, BNP, CNP, Urodilatin) were compared using chi-square tests and one-way analysis of variance (ANOVA) for categorical variables and continuous data respectively. Baseline characteristics between both ANP groups were compared using the unpaired *t*-test. Changes in FBF and FVV results are expressed as mean values with 95% CIs. The effects of NP on FBF and FVV (within-group comparisons) were assessed by 2-way analysis of variance (ANOVA) with post-hoc comparison to baseline. Between-cHF-group comparisons were performed using repeated measures ANOVA. The comparison of the ANP dose-response curves (Figure 1a+b) between cHF patients and controls was performed using the trapezoid rule (514), as is conventional when presenting FBF data. Correlation between baseline plasma ANP levels and venous responsiveness was assessed by Spearman rank correlation. A p-value less than 0.05 was considered statistically significant. In order to allow presentation of grouped data, results are presented as percentages.

4.3. Results

4.3.1. Subject Characteristics:

The patient groups were well matched, without any significant differences in their baseline characteristics, including humoral, urinary and hemodynamic parameters. The ANP control group had significantly higher EF and lower body mass index than the ANP cHF group. Importantly, there was no significant difference in mean FV (see Table 4.2.1.) or resting forearm counts between patients and controls (ANP-cHF; 691±82 counts/s, controls; 793±60, p=0.3). Baseline characteristics are summarised

in Tables 4.2.1. and Table 4.2.2. The renal and humoral changes are summarised in Table 4.2.2.

4.3.2. ANP Levels

Baseline venous plasma ANP levels in the control and cHF group were 21.7 ± 4.8pg/ml and 100.5 ± 8.1 respectively, $p<0.0001$. Intra-arterial ANP infusions of 0.05μg/min, 0.5μg/min, and 1.0μg/min raised ANP concentrations in the venous effluent to 196 ± 62pg/ml, 785 ± 256pg/ml, 1113 ± 244pg/ml ($p<0.01$ for all) in the control group and to 208 ± 33pg/ml, 234 ± 38pg/ml, 280 ± 32pg/ml ($p<0.05$ for all) in the cHF group. The increase in venous ANP concentration at 0.5μg/min and 1μg/min was significantly higher in controls than patients ($p<0.01$, for both).

Baseline cGMP levels in the control and cHF group were 0.73 ± 0.15pg/ml and 8.98 ± 1.0pg/ml respectively ($p<0.0001$). The cGMP spillover was 20.0 ± 9.2 pmol/min per 100ml FV in the control group and 11. 6 ± 4.5 pmol/min per 100ml FV in patients (p=NS).

4.3.3. The Effects of NP on FBF in Health and cHF

The effects of ANP on the FBF ratio in patients with cHF and healthy controls are shown in Figure 4.3.3. A) and Table 4.3.

Intra-arterial infusion of incremental ANP concentrations caused a dose-dependent increase in FBF in both controls and patients. ANP infusions of 0.05μg/min, 0.5μg/min, and 1μg/min increased FBF in the infused arm of controls from 2.14±0.37ml/100ml FV to 3.22±0.48ml/100ml FV (95% CI, -0.1 to 2.27), to 3.65±0.44ml/100ml FV (95% CI, 0.32 to 2.70; $p<0.05$), to 4.50±0.75ml/100ml FV

(95% CI, 1.17 to 3.55; $p<0.001$), respectively. In patients, FBF increased in the infused arm from 2.18±0.18ml/100ml FV to 2.86±0.78ml/100ml FV (95% CI, -0.13 to 1.09), to 3.02±0.54 (95% CI, 0.12 to 1.3, $p<0.05$), to 3.27±0.47 (95% CI, 0.48 to 1.7, $p<0.001$), respectively. Changes in FBF were significantly blunted in patients when compared with controls (AUC; $p<0.01$).

The effects of Urodilatin, BNP and CNP on the FBF ratio in patients are summarised in Table 4.3.

4.3.4. Changes in Forearm Venous Tone

For each subject, two baseline PVR were generated during infusion of normal saline and 1 during infusion of each dose of NP and NP/LNMMA co-infusion. Linear regression was performed for each PVR, and R^2 values were 0.88 to 1 (mean 0.96±0.03). Therefore a linear model was adopted. We then determined whether the slopes of the lines in each data set were different (i.e., to determine whether the lines were parallel or not) using a standard method for comparing 2 independent regressions (517). Although PVR varied in slope (compliance) between individuals, within individuals there was very little change in slope at different stages of the study. In other words, shifts in the plots induced by infusion of the active agents were parallel and were due to changes in venous tone rather than compliance.

The baseline slope of the PVR was 7.3±1.2 counts/s/mmHg in the ANP-cHF group and 7.5±0.8 counts/s/mmHg in the controls (p=NS). Furthermore these parameters were similar at baseline in the patient groups randomised to each of the NPs.

4.3.5. The Effects of NP on FVV in Health and in cHF

ANP caused a significant and similar dose-dependent increase in FVV in controls and in patients. The results are summarised in Figure 4.3.3. and Table 4.3.

The effects of Urodilatin, BNP, and CNP on FVV in patients and controls are shown in Table 4.3.

The overall effects of ANP on FVV in cHF patients was significantly greater than those of Urodilatin, BNP and CNP, $p<0.05$ (for all, repeated measures ANOVA).

There was no significant correlation between baseline ANP plasma level and maximal venous responsiveness to i.a. ANP infusion (r_s=-0.19; p=NS).

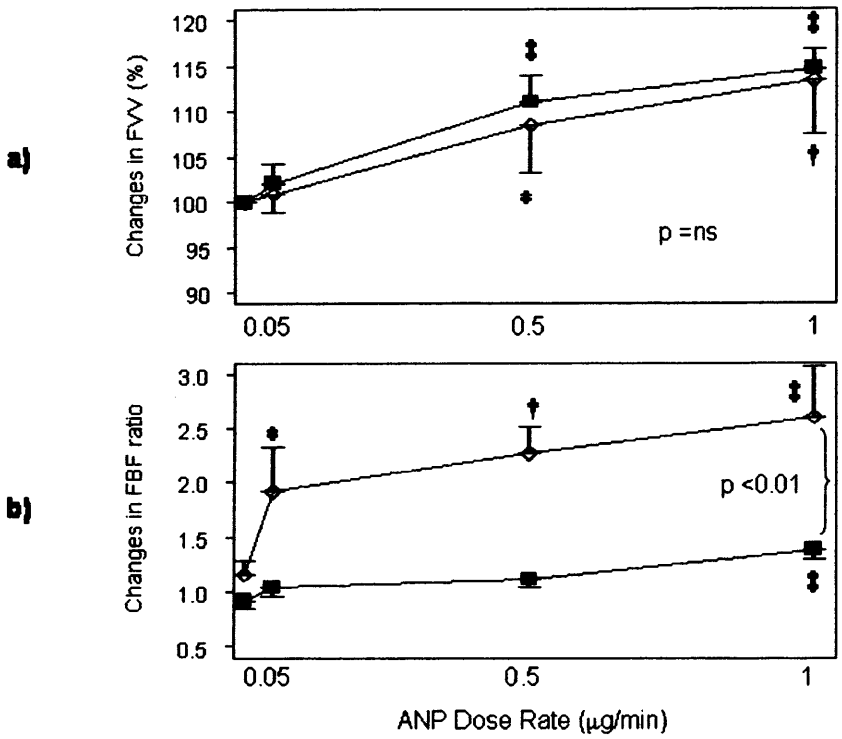


Figure 4.3.3. Changes in FVV (Figure 4.3.3.a) and FBF (Figure 4.3.3.b) following incremental cumulative dose rates of ANP are shown. Symbols depict *$p<0.05$, †$p<0.01$, ‡$p<0.001$ and refer to within group comparison using 2-way ANOVA. P-values within the figures (p=ns in Fig 1a, and $p<0.01$ in Fig 1b) refer to AUC comparison between both groups (patients = ■, controls = ◊).

4.3.6. The Effects of A71915 on FBF and FVV in Patients with cHF

Intra-arterial infusion of A71915 at 0.5µg/min and 1µg/min [n=7] had no significant effect on FBF or FVV. Infusion of 20µg/min [n=4]) reduced FVV by 4.4±1.2%; $p<0.05$ without significant changes in FBF.

Table 4.3.3.1. Changes in forearm vascular volume and blood flow

Group	Dose		
	0.05μg/min	0.5μg/min	1μg/min
ANP-Controls			
Change in FBF ratio	+0.756* (0.03 to 1.49)	+1.111† (0.38 to 1.84)	+1.438‡ (0.71 to 2.17)
Change in FVV in %	+0.930 (-7.26 to 9.12)	+8.476* (0.28 to 16.67)	+13.424† (5.23 to 21.62)
ANP-cHF			
Change in FBF ratio	+0.122 (-0.08 to 0.32)	+0.191 (-0.01 to 0.39)	+0.467‡ (0.27 to 0.67)
Change in FVV in %	+2.149 (-2.25 to 6.55)	+11.057‡ (6.66 to 15.45)	+14.669‡ (10.3 to 19.1)
Urodilatin-cHF			
Change in FBF ratio	+0.117 (-0.19 to 0.42)	+0.397* (0.09 to 0.70)	+0.695‡ (0.39 to 1.00)
Change in FVV in %	-1.938 (-4.76 to 0.9)	+1.438 (-1.39 to 4.26)	+6.49‡ (3.66 to 9.31)
BNP-cHF			
Change in FBF ratio	+0.114 (-0.24 to 0.46)	+0.03 (-0.32 to 0.38)	+0.648† (0.30 to 1.00)
Change in FVV in %	+1.120 (-4.43 to 6.67)	+4.829 (-0.72 to 10.38)	+7.281* (1.73 to 12.83)
CNP-cHF			
Change in FBF ratio	0.020 (-0.44 to 0.48)	-0.137 (-0.59 to 0.32)	+0.238 (-0.22 to 0.70)
Change in FVV in %	-1.521 (-7.00 to 3.96)	+1.106 (-4.43 to 6.54)	+7.381* (1.90 to 12.87)

Symbols: * $p<0.05$, † $p<0.01$, ‡ $p<0.001$, depict within group comparison (2 way-ANOVA). FBF data are mean absolute changes in the ratio of infused versus non-infused arm, (95% confidence interval). Changes in FVV are corrected for systemic changes and expressed in %.

Table 4.3.7. The effects of LNMMA on NP induced changes in FBF and FVV

	Group				
	ANP-Controls	**ANP-cHF**	**Urodilatin-cHF**	**BNP-cHF**	**CNP-cHF**
Mean Change in FBF					
NP (1.0μg/min) versus NP/LNMMA Co-infusion	-1.37 (-2.48 to –0.26)*	-0.64 (-1.31 to 0.03)	-0.81 (-1.20 to –0.43)‡	-0.73 (-1.19 to –0.26)†	-0.56 (-1.07 to –0.05)*
Mean change in FVV					
NP (1.0μg/min) versus NP/LNMMA Co-infusion	-6.32 (-4.11 to –8.53)‡	-8.00 (-4.10 to -11.92)‡	-7.46 (-4.24 to 10.69)‡	-6.86 (2.70 to 11.03)†	-8.97 (-4.84 to –13.09)†

Symbols *, †, ‡ depict within group comparison (2 way-ANOVA). *p<0.05, †p<0.01, ‡p<0.001. FBF is expressed as ml/100ml FV. Change in FBF reflects mean absolute change, (95% CI). FVV (forearm vascular volume) is expressed in %.

4.3.7. The Effects of Endothelium-Derived NO on NP-Induced Changes in FBF and FVV in Health and cHF

eNOS-blockade reduced ANP-induced FBF changes in controls but not in patients ($p<0.05$), whilst similar reductions in FVV were seen in both ANP groups (both $p<0.001$) The effects of LNMMA/NP co-infusion on all NP-induced changes in FBF and FVV are summarised in Table 4.3.7.

4.4. Discussion

Many studies have detailed the central hemodynamic, arterial, renal and adrenal effects of exogenous (157;166;199;438;509-511) and endogenous (367;416;508) NPs in animal models of heart failure (438;508) and in human heart failure (157;166;199;509-511), but little is known about the effects of NPs on the capacitance vasculature in this condition. In the present study we assessed the effects of all 4 currently known human NPs on the forearm vasculature in well matched groups of optimally treated cHF patients. Furthermore, we compared the effects of ANP on the forearm capacitance vasculature in patients with optimally treated cHF with those in normal controls and evaluated the contribution of endothelium-derived NO on NP-induced vasodilation in the forearm resistance and capacitance vasculature. Finally, we assessed for the first time the effects of basal NPs on forearm vascular function in patients with cHF.

The principal findings were as follows: first, we demonstrated that in cHF ANP is a significantly more potent venodilator of forearm capacitance vessels than other NPs, the rank order of potency in the forearm capacitance bed being ANP>> BNP > CNP/ Urodilatin. Second, basal NPs contribute to regulation of regional VV and venous tone in cHF, but the contribution appears to be less than that previously shown by us

in younger normal volunteers (516). Third, we demonstrated that venous responsiveness to exogenous ANP is preserved in patients with cHF, despite marked attenuation of its effects in the forearm resistance vasculature. Fourth, eNOS blockade reduced ANP-induced-induced FBF changes in controls but not in patients, whilst similar reductions in FVV were seen in both groups.

4.4.1. Mechanism of the Attenuated Forearm Resistance-Vessel Response to ANP in cHF

Our observation of an attenuated forearm resistance-vessel response to intra-arterial ANP infusion is consistent with the majority of the literature, which documents attenuated arterial, renal and endocrine responses to ANP in patients with cHF (157;166;199;509-511). Hirooka et al (199), and Nakamura et al (278;510), reported reduced forearm resistance-vessel responses to intra-arterial infusion of ANP and BNP (but not CNP), in HF patients versus healthy controls. Interestingly, in these studies, responses (i.e changes in FBF) to BNP and CNP were less than those to ANP (on an equimolar basis) in healthy controls, but roughly equipotent in patients with CHF. Conversely, Kubo et al reported no significant difference between the forearm resistance-vessel responses to maximal doses of ANP in HF patients and controls (518). However, the maximal dose of ANP employed in this study was considerably higher than that used in other studies (199;510;519). There was a clear reduction in responsiveness at lower doses in HF patients versus controls, indicating a rightward shift in the dose-response relation.

The mechanisms responsible for reduced NP responsiveness in patients with HF are likely to be complex and may differ between the hormonal, renal, and vascular responses to these peptides. All of the following have been implied to contribute (for

review see ref. (520)); NPR_{A+B} receptor down-regulation, NPR_C (clearance receptor) and NEP up-regulation, receptor desensitisation, fetal NP-gene activation, down-stream signalling abnormalities beyond cGMP generation, and increased activity of functional antagonists of the NP system, such as increased sympathetic nerve activity (especially renal sympathetic activity) and activation of the RAAS. The present study provides evidence that endothelial dysfunction might contribute to arterial NP resistance. There was considerable impairment of the NO-mediated component of the vasodilator effect of ANP in cHF patients versus controls, but the non-NO-mediated component was preserved. Furthermore, our finding that identical ANP infusions caused a significantly greater increase in venous effluent plasma ANP levels in controls compared to cHF patients would be in keeping with the notion of up-regulation of NPR_C-receptor density in this condition.

4.4.2. Venous Effects of Natriuretic Peptides in cHF

Studies in conduit veins (e.g., saphenous vein and dorsal hand vein) have suggested that NPs have minimal or absent vasorelaxant effect on veins (161;521). In contrast we have previously demonstrated that intra-brachial arterial ANP infusion causes a significant increase in forearm venous capacitance in healthy controls across a venous plasma concentration range spanning from physiological values to those seen in severe HF (516). These observations suggest that in contrast to the lack of effect seen in conduit veins, ANP dilates small veins and venules, which contain most of the venous (and total vascular) volume. In the present study brachial artery ANP infusion resulted in a similar increase in FVV in cHF patients versus healthy controls, in marked contrast with the attenuated effects in the forearm resistance beds. To the best of our knowledge, there is only one other published study of the venous effects of

ANP in HF (423). That study differed from ours in three important respects. First, the ANP was given as a single systemic bolus dose that produced hypotension. The authors commented that baroreflex-mediated adjustments to the hypotension may have overcome any local venodilator effects of ANP. Second, our patients were symptomatic (NYHA II/III) but on optimal contemporary medical therapy. In contrast that study (423) was undertaken in the pre-ACE inhibitor era. Third, they employed strain-gauge VOP which measures total limb volume, and this may be influenced by changes in tissue fluid content when capillary permeability is altered by a vasoactive agent; in contrast radionuclide-plethysmography provides a more direct measure of VV and is therefore a more reliable technique when assessing vasodilators which alter vascular permeability (515).

Our observations raise the fascinating question of why the venous effects of ANP are preserved in patients with cHF when the resistance vessel responses are markedly attenuated. There is growing evidence for an interdependence between both GC-systems (i.e. soluble and particulate), in particular in situations with altered NO-bio-availability (435;436). We recently demonstrated that carbachol-induced, NO-mediated venodilation was preserved in the forearm of patients with cHF, whilst endothelial function in arterial vessels was markedly impaired (437). Emmick and Cohen previously demonstrated that, in older animals, vascular ANP responsiveness declined in arteries whilst it remained preserved in veins (522). Taken together there seems to be an impairment in arterial, but a preservation in venous ANP responsiveness with aging and with cHF. The same appears to be true for vascular NO-responsiveness and it is therefore conceivable that a NO-dependent component of ANP induced vasodilation accounts for these observations.

4.4.3. Effects of Basal ANP Levels

A71915 is an ANP analogue capable of interfering with ANP-induced cyclic GMP accumulation. The binding affinity of A71915 to NPR_A is only 22 times lower than that of ANP (223). A71915 has previously been shown to antagonise the cGMP dependent renal (365) and vascular responses (364) of ANP (the renal and vascular tissue is rich on NPR_A receptors) without inhibiting ANP (and CNP) induced cGMP production in non-pigmented ciliary epithelial cells (tissue believed to be rich in NPR_B and NPR_C receptors but devoid of NPR_A receptors) (361).

Infusion of A71915 at a rate identical to that which had previously shown to induce venoconstriction in healthy controls (516) had no effect. Given that basal ANP plasma levels in our patients were 10-fold higher than in the normal controls of the previous (and the present) study, the lack of effect can be explained by a rightward shift of the dose-response curve, in keeping with a competitive antagonism between endogenous ANP and exogenous A71915. Indeed, infusion of a 20-fold higher concentration was able to elicit a significant, albeit in relative terms still reduced (4.4% in present study versus 9.6% in previous study (516)) reduction in FVV. The latter might be explained by the observation that β-ANP, an antiparallel dimer of α-ANP with reduced biological action, is the principal form of circulating ANP in patients with cHF (426).

These observations may also help to explain the finding that acute administration of exogenous NPs have marked beneficial hemodynamic effects (394) whilst potentiating endogenous NP by preventing their breakdown has been somewhat disappointing.

4.4.4. Relative Potency of the Different NPs

With respect to the effects of ANP and BNP on forearm resistance vasculature, our study largely confirms previous findings discussed above (199;278;509;510).

We found no significant vasodilatory effect of CNP in the resistance vasculature, but some venodilation at pharmacological levels. This is at least partly in keeping with an earlier finding by Wei et al (272) demonstrating that CNP is a relaxing factor in isolated canine saphenous vein rings. It is noted that a previous study by Nakamura et al found no significant increase in FBF at similar doses to those used in our study but a significant, albeit small and similar increase in FBF in patients and controls at a dose equalling roughly twice our peak infusion rate (278). Our study suggests that CNP has little or no vasodilatory potency in the forearm resistance vasculature of patients with cHF, at least at physiological and pathophysiological concentrations. However, there remains an important caveat, as CNP is an abluminally secreted paracrine hormone measurements of plasma levels may be a poor indicator for more important tissue concentrations. Consequently studies utilising specific NPR_B-receptor blockers have to be awaited to ultimately confirm or rule out a potentially vasodilating action of CNP.

4.4.5. Clinical and Pathophysiological Implications

Systemic infusion of NP (167;366) (or elevation of endogenous NP by inhibiting their breakdown) (367;416) has been shown to increase stroke volume (SV) (167) and CI (366) in patients with HF, despite a fall in CVP (366;367), a finding apparently in conflict with the Frank-Starling mechanism. This is almost certainly due a decrease in the volume of the right ventricle and a decrease in pericardial pressure, which increases effective LV distending pressure despite a decrease in LVEDP (i.e., it

reduces DVI) (124). ANP infusion reduces cardiac filling pressures too rapidly to be solely accounted for by a fluid shift into the interstitial space and in HF patients there is no significant diuresis or change in haematocrit (157;166;167). These observations re-emphasise the potentially important venous effects of ANP, despite studies in conduit veins suggesting little or no venodilator effects. Extending our previous findings in healthy controls (516), the present study suggests that ANP also has important venodilator activity in cHF, but only modest effects on the resistance vasculature. This is in keeping with an earlier study by Serizawa et al (523) who found that ANP reduced cardiac filling pressures in the absence of changes in SVR. The relatively selective venodilator action of NP in HF results in marked reductions in CVP and increases SV without a major fall in SVR, avoiding serious hypotension. This is a favourable haemodynamic profile in the treatment of patients with decompensated HF.

4.4.6. Study limitations

One caveat is that, using the radionuclide technique, changes in VV during brachial artery infusion are expressed as a percentage of baseline using the radionuclide technique. If FVV were reduced in cHF patients versus controls, a similar percentage increase in FVV might nevertheless represent a smaller absolute increase in volume. However, forearm radioactive counts at baseline and total forearm volume were similar in patients and controls, making it likely that baseline FVV was similar in the two groups. This is perhaps not surprising; although SVR is increased in untreated HF, it is normal or even reduced in patients with well-treated (but symptomatic) HF such as those we studied (524). Although small, statistically non-significant clinical differences (i.e. cardiovascular risk profile) may have had some bearing on the

vascular responsiveness to the various NPs, the degree of atherosclerotic burden in the forearm circulation is generally believed to be relatively minor. Furthermore, this was not a randomised study. Patients were consecutively enrolled to receive ANP, BNP, CNP, Urodilatin, or A71915 in this order. However, patient characteristics between the NP groups were well matched and the study setting and protocol remained unchanged throughout. Finally, this study was intentionally performed in patients who were on contemporary medical therapy for cHF, although they were nevertheless symptomatic. Our findings may not therefore apply to untreated HF.

4.5. Conclusion

Venous ANP responsiveness is preserved in patients with cHF despite markedly diminished responsiveness of the arterial resistance vasculature. This difference may partially be due to arterial endothelial dysfunction.

CHAPTER 5 – THE ROLE OF NATRIURETIC PEPTIDES IN THE FUNCTIONAL REGULATION OF CONDUIT ARTERY STIFFNESS

5.1. Introduction

Aortic pulse wave velocity (PWV), a measure of distensibility is an important, independent determinant of cardiovascular risk (525;526). Structural components within the arterial wall (mainly collagen and elastin), together with transmural pressure and smooth muscle tone are all important determinants of arterial distensibility (527;528). Vascular smooth muscle tone is dependent on locally derived signalling molecules, such as NO (529) and endothelin-1,(530) but may also be influenced by paracrine and endocrine acting NPs. Although ANP is a potent vasorelaxant on smaller conduit and resistance vessels in vivo and in vitro, there is considerable heterogeneity in responsiveness dependent on size and site of the vessels (163;251;521;531;532). The in vivo-effects of ANP on large artery vasomotor control, however, have not been investigated. Therefore we aimed to establish whether NPs, acting locally, modify regional distensibility in large muscular arteries. In order to investigate the potency and mechanisms of action we performed 4 studies. First, we performed a comparative dose-response study assessing the effects of ANP, BNP, and CNP on PWV in a previously validated ovine hind limb model.(529) Second, to determine the contribution of endogenous ANP to basal arterial distensibility we studied the effects of the natriuretic peptide receptor A (NPR_A-) selective ANP receptor-antagonist A71915 (223) on resting PWV. Third, we assessed the effects of A71915 on ANP induced changes in PWV. Fourth, to establish the role of the NPR_C receptor in regulation of regional iliac artery distensibility we studied the

effects of cANF, an ANP analogue that selectively binds to the NPR_C receptor(211;533), on PWV.

5.2. Methods

Studies were conducted in 18 adult (n=number of sheep), crossbred Suffolk sheep aged between 12 and 18 months, weighing between 37 and 60 kg, at the University of New South Wales, Sydney, Australia. The studies were approved by the University's Animal Care and Ethics Committee. Anaesthesia was induced by intramuscular injection of Zoletil 100® (10mg/kg) (Virbac, Peakhurst NSW, Australia), and maintained by inhalation of 2-3% isofluorane, administered via a Boyle's re-breathing apparatus with an oxygen flow rate of 2 L min^{-1} (see Plate 5.2.1.) Animals were studied in the supine position, breathing spontaneously (see Plate 5.2.2).

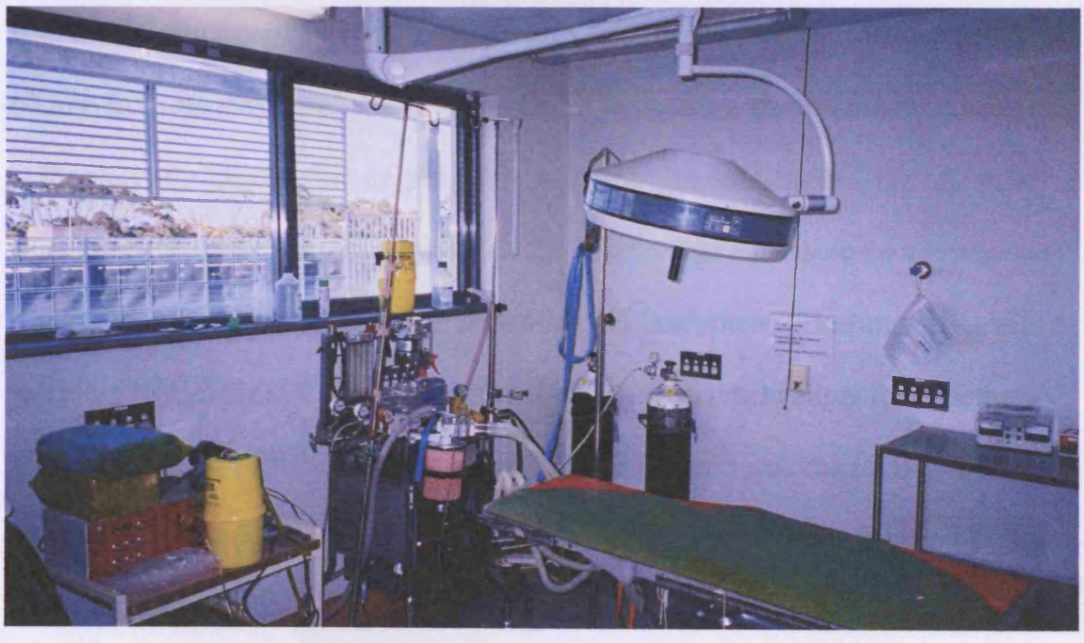


Plate 5.2.1. Shows the animal-theatre; a Harvard respirator (red) to the left, the Boyle's re-breathing apparatus and the OP- table to the right.

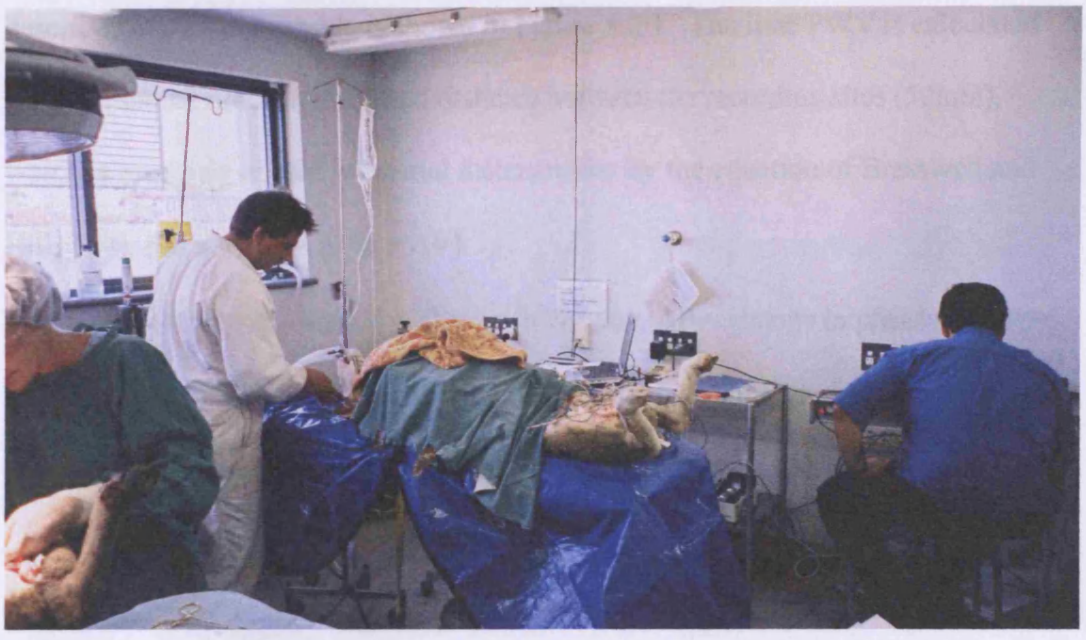


Plate 5.2.2. Illustrates (supine) intra-operative positioning.

5.2.1. Haemodynamic measurements

Pressure measurements were made using a 6F end-hole catheter (Gaeltec, Skye, UK) with a 0.46 mm internal lumen, and dual high fidelity pressure sensors located 10 and 60 mm from the distal end. Calibration of both sensors was performed simultaneously at the start of each experiment using a mercury sphygmomanometer. The analogue signal from the pressure control unit was fed directly into a portable microcomputer using a PC Lab analogue-to-digital converter (AD Instruments, Hastings, UK) with a sampling rate of 1kHz. Data were recorded over 20 seconds to allow for variations within the respiratory cycle. Mean arterial pressure (MAP) was calculated from integration of the distal pressure waveform using CHART software (Version 4). Data were then exported and re-sampled at 10kHz for further analysis with custom-written MATLAB analysis programme (Math Works, Cambridge, UK). This allows identification of the foot of each of the simultaneously recorded pressure waveforms and calculates the transit time from the foot-to-foot delay, as previously

described.(529) An example is shown in Figure 5.2.1. The iliac PWV is calculated from the transit time and the fixed distance between the recording sites (50mm), which is inversely related to arterial distensibility by the equation of Bramwell and Hill,(534) $PWV=\sqrt{[V \bullet \Delta P/\rho \bullet \Delta V]}$

Where: V= artery volume, AV = change in volume, AP = change in pressure and ρ= blood density (assumed to be constant during he study). HR is derived over the measurement period from a simultaneously recorded electrocardiogram.

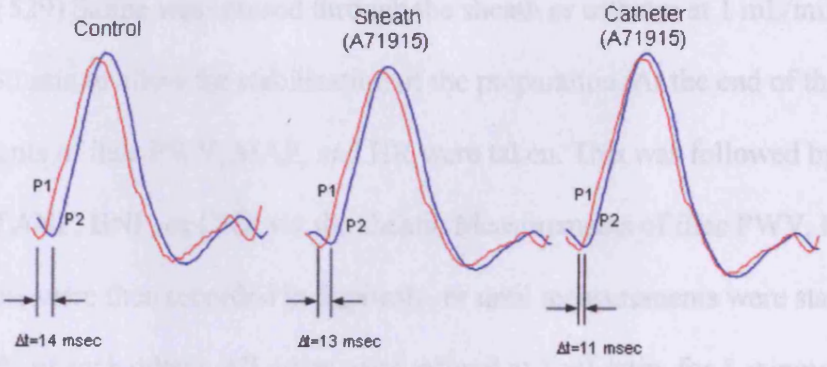


Figure 5.2.1. This figure shows the recording of an intra-arterial pressure wave form sampled at 1000Hz before and after infusion of the NPR_A selective receptor blocker A71915. For illustration purposes the foot of each wave form is highlighted.

5.2.2. Drugs

Human α-ANP, BNP and CNP (Clinalfa, Läufelfingen/Switzerland), A71915, c-ANF (Bachem, UK) were all prepared in 0.9% saline in an aseptic manner on the day of the study. The NP doses and infusion periods were based on our previous experience in the human forearm, (516) and were titrated to produce local and not systemic effects.

5.2.3. Protocol / Surgical preparation

The femoral artery was identified by palpation and a 30 mm segment of each artery was exposed by limited dissection into which a 7F sheath was inserted (see Plates 5.2.3.1 a, b, c). The arterial catheter was then positioned in the common iliac artery with the tip below the level of the aortic trifurcation (see Plate 5.2.3.2), as previously described.(529) Saline was infused through the sheath or catheter at 1 mL/min for a period of 30 min to allow for stabilization of the preparation. At the end of this period, measurements of iliac PWV, MAP, and HR were taken. This was followed by infusion of ANP, BNP, or CNP via the sheath. Measurements of iliac PWV, MAP, HR and flow were then recorded in duplicate, or until measurements were stable (within 10% of each other). All drugs were infused at 1 mL/min, for 5 minutes in doses as outlined below. Repeated pressure waveforms were recorded for 20 seconds during the last minute of each infusion period. Potentially confounding changes in flow were controlled for by drug infusion via the catheter and the sheath. Infusion of drugs through the catheter expose the arterial segment under-study to the drug, while infusion via the sheath do not, since the drug is delivered distal to the pressure sensors (see Figure 5.2.3.).

With the exemption of study 3 no two drugs were infused in the same limb. Where animals were studied twice hemodynamics had returned to baseline and a minimal

washout period of 40 minutes was interposed before the contralateral limb was studied.

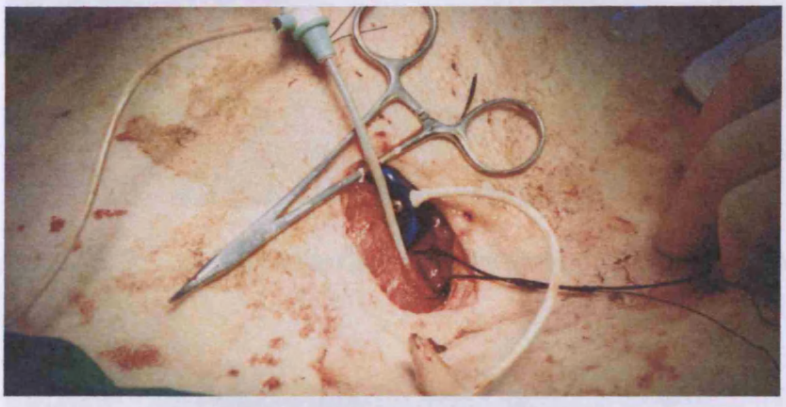


Plate 5.2.3.1. a) illustrates a "minimal invasive" inguinal cut-down. The sheet is inserted via seldinger technique under direct inspection.

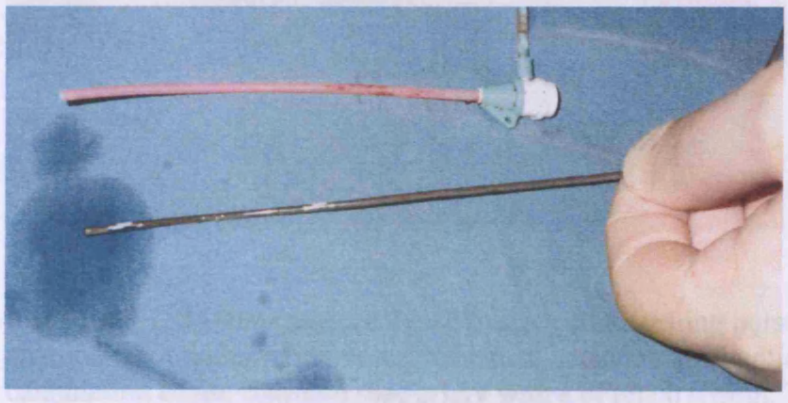


Plate 5.2.3.1. b) shows the two high fidelity pressure sensors, mounted at the distal end of the 5 French Gaeltec catheter. The pressure sensors are spaced 50 mmm apart.

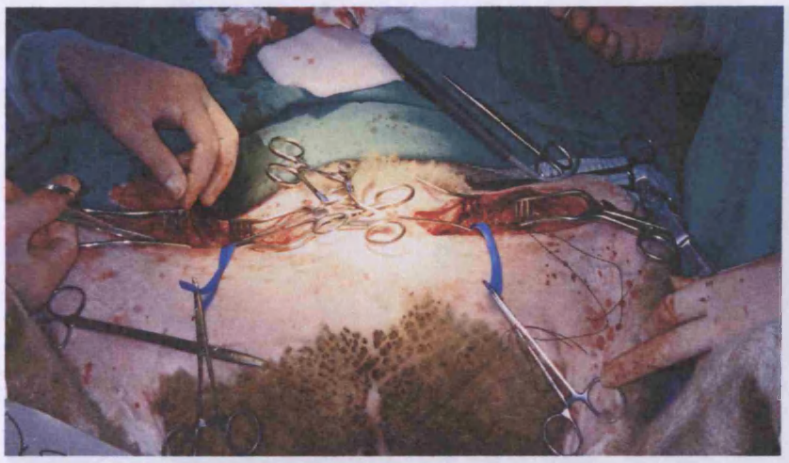


Plate 5.2.3.1. c) Illustrates the operative setting of a bilateral inguinal cut-down.

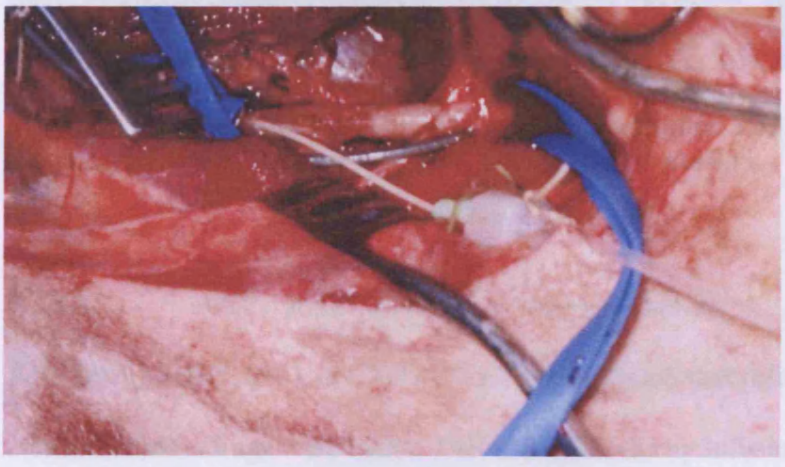


Plate 5.2.3.1. d) demonstrates the alternative study setting pursued in animals where anatomical variants, small vessel caliber, or technical difficulties prevented cannulation of the common iliac artery with a 6French sheeth. The catheter is directly inserted via an arteriotomy and advanced proximally whilst a 2nd vigon tube is inserted distally in downstream direction.

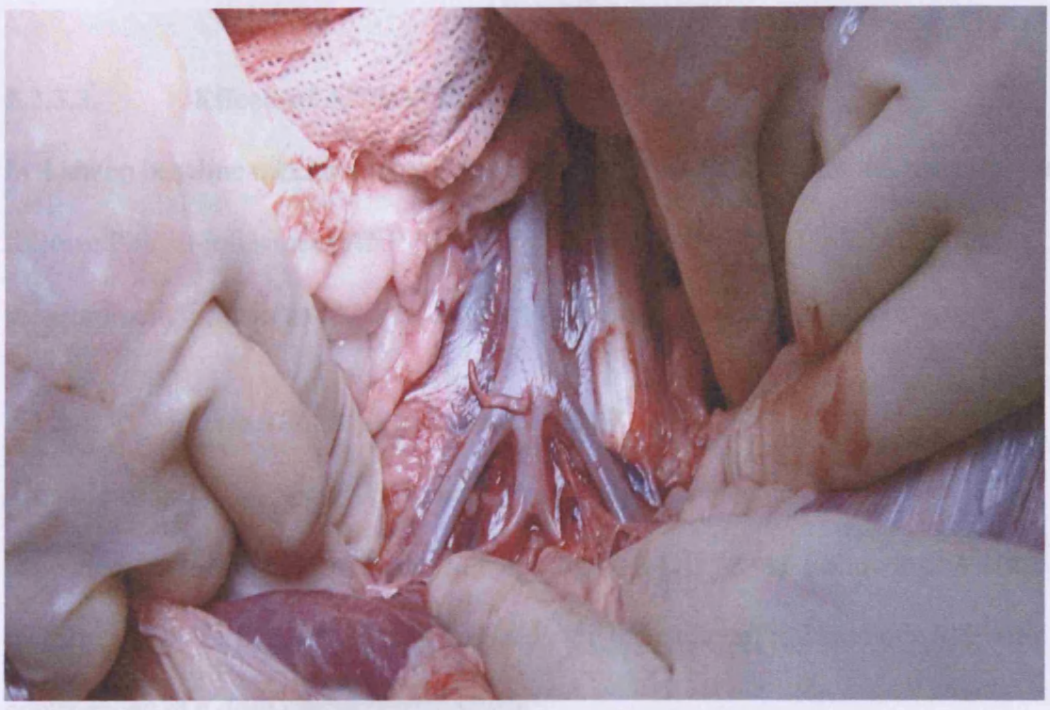


Plate 5.2.3.2. Illustrates the ovine pelvic arterial anatomy. The sheep has a very strong mediosarcal artery, essentially forming an aortic trifurcation.

5.2.3.1. ANP / BNP / CNP dose response studies

ANP (n=6), BNP (n=6), or CNP (n=6) (0.3nMol/min) was infused via the sheath and 2 baseline recordings were taken. This was followed by infusion of equimolar doses of ANP (~0.03nMol/min [≈0.1μg/min], ~0.15nMol/min [≈0.5μg/min], ~0.3nMol/min [≈1μg/min]), BNP (~0.03nMol/min [≈0.11μg/min], ~0.15nMol/min [≈0.56μg/min], 0.3nMol/min [≈1.1μg/min]) or CNP (~0.15nMol/min and ~0.3nMol/min) via the catheter, in an incremental cumulative fashion.

5.2.3.2. Effects of A71915 on resting PWV

Following baseline measurement during normal saline via the catheter the NPR_A-selective ANP receptor antagonist A71915 was infused at an infusion rate of 6.1nMol/min (n=7) and measurements of HR, MAP and PWV were repeated.

5.2.3.3. Effects of ANP/A71915 co-infusion

In 4 sheep baseline measurements during normal saline infusion via the catheter were followed by co-infusion of ANP (0.15nMol/min)/A71915 (6.1nMol/min) a further measurement, infusion of ANP (0.15nMol/min) alone, and a final measurement.

5.2.3.4. Effect of cANF on regional PWV

The role of the NPR_C receptor in mediating NP induced changes in regional PWV were studied by infusing the NPR_C selective agent cANF (n=4). Following baseline measurements during normal saline infusion via the catheter, infusion of cANF was commenced at a dose rate of 0.3nMol/min.

5.3. Statistics

Data are expressed as mean±SEM. Data were analysed by 2-way ANOVA with post-hoc comparison to baseline. A value of $p<0.05$ was considered significant.

5.4. Results

Changes in HR and MAP are summarised in Table 5.4. Importantly, in the ANP/BNP/CNP dose response studies, NP infusion distal to the common iliac artery (via the sheath) did not have any (reflex) effects on (proximal) PWV.

5.4.1. The effects of ANP / BNP / CNP on iliac PWV

ANP and to a lesser degree BNP dose dependently decreased regional PWV. CNP, despite having a marked effect on BP, caused little change in PWV. The results, using 0.3nMol sheath infusion as baseline, are summarised in Table 5.4. Additionally, the

ANP and BNP dose response curves (using normal saline infusion as baseline) are shown in Figure 5.4.1.a.and Figure 5.4.1.b. respectively.

5.4.2. The effect of A71915 on iliac PWV

The effects of A71915 on iliac PWV are shown in Figure 5.4.2. A71915 increased iliac PWV from 2.97±0.13m/s to 3.06±0.13m/s; $P<0.01$.

5.4.3. The effect of ANP/A71915 co-infusion on iliac PWV

Iliac PWV did not change significantly during ANP/A71915 co-infusion (PWV increased from 3.32±0.2m/s during N-saline infusion to 3.36±0.2m/s during co-infusion; p=NS). ANP infusion alone reduced PWV to 3.23±0.1ms; $p<0.01$.

5.4.4. The effects of cANF on iliac PWV

cANF infusion had no significant effect on resting PWV (see Figure 5.4.3.).

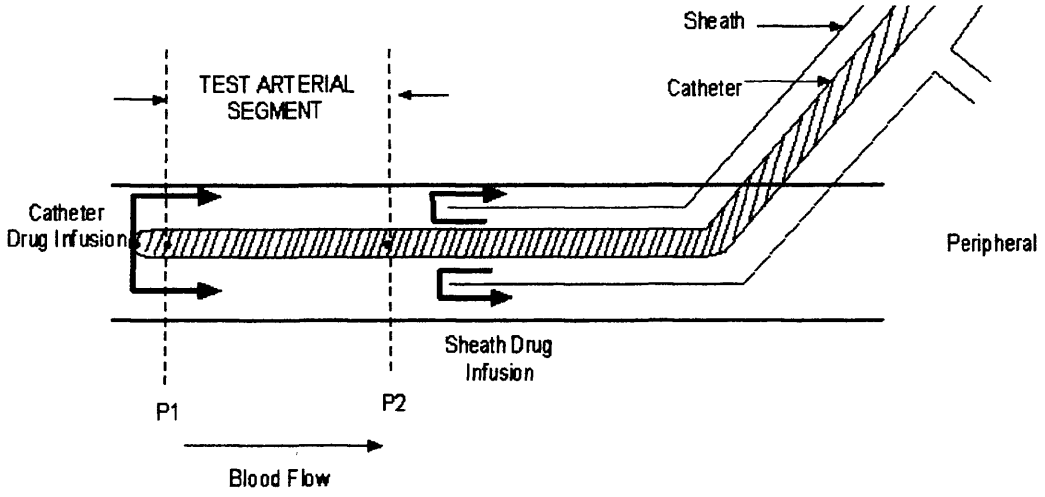


Figure 5.2.3. Schema showing infusions via the catheter (proximal) and sheath (distal).
P1 = pressure sensor 1; P2 = pressure sensor 2.

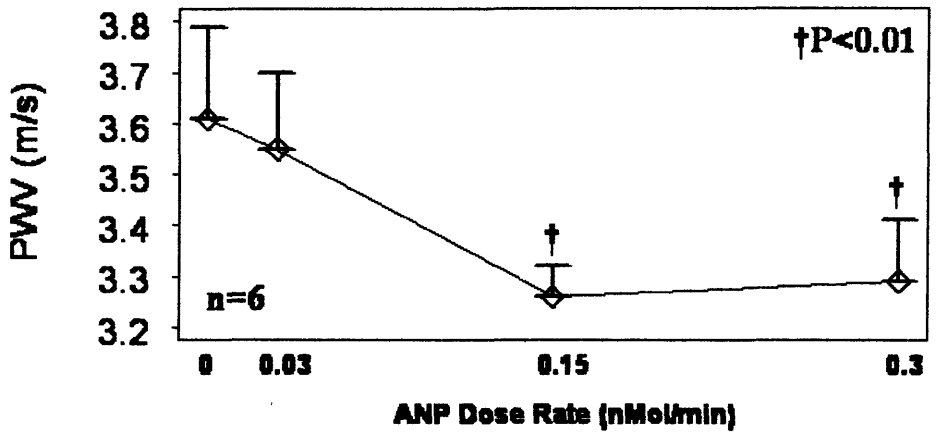


Figure 5.4.1.a. The effect of intra-arterial ANP infusion on regional PWV is illustrated. Values represent means±SEM. Symbol † depicts within group comparison (2 way-ANOVA).

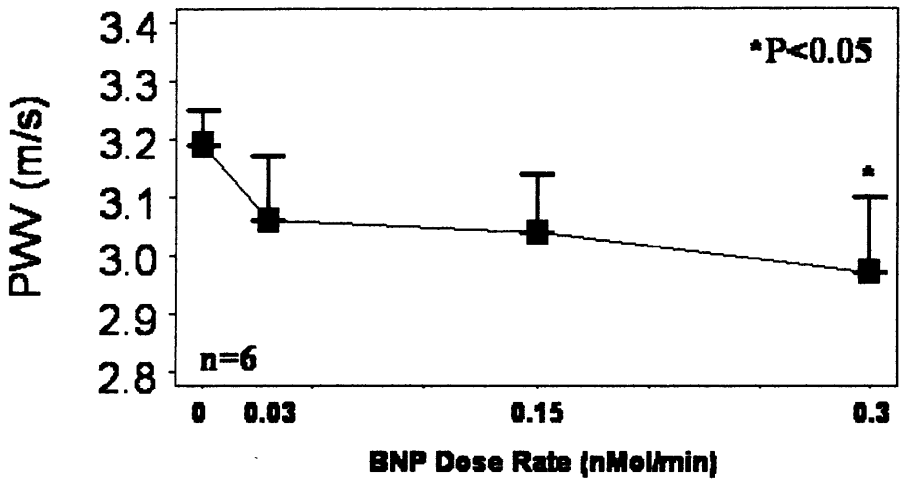


Figure 5.4.1.b. BNP dose-response curve.
The effect of intra-arterial BNP infusion on regional PWV is illustrated. Values represent means±SEM. Symbol * depicts within group comparison (2 way-ANOVA).

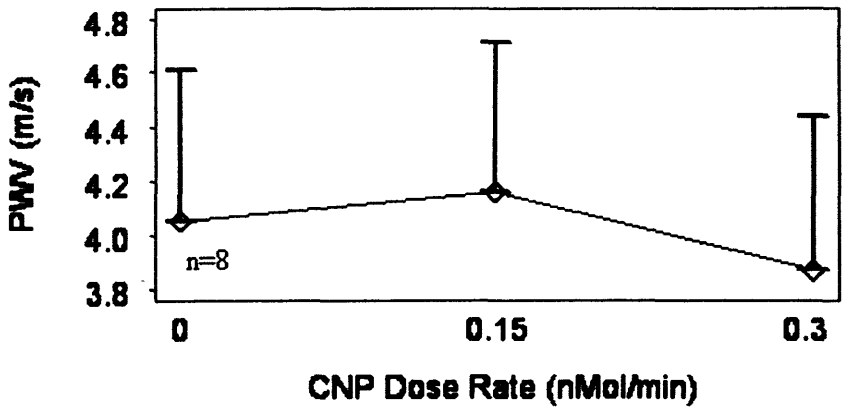


Figure 5.4.1.c. CNP dose-response curve.
The effect of intra-arterial CNP infusion on regional PWV is illustrated. Values represent means±SEM

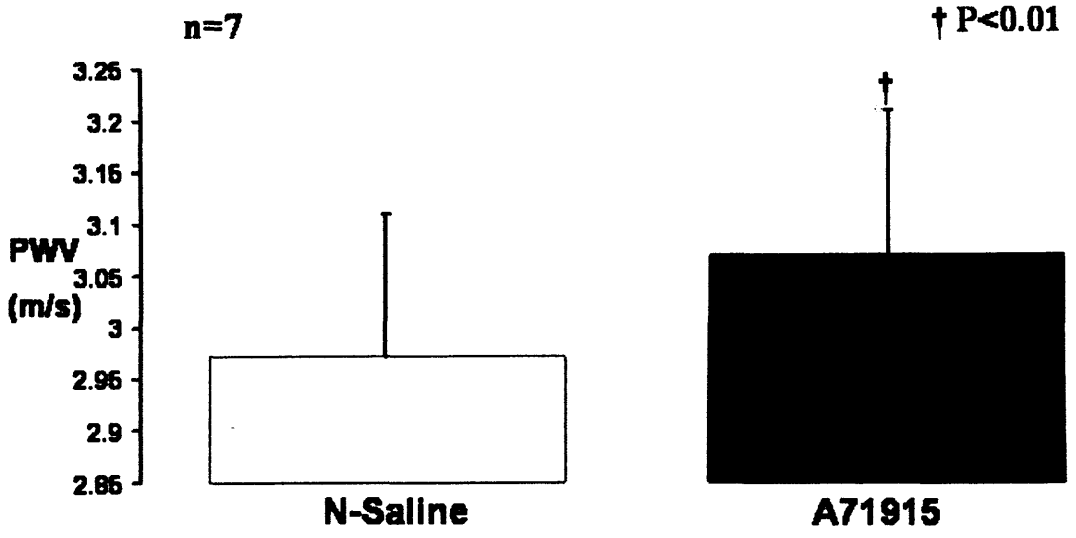


Figure 5.4.2. The effects of A71915 (NPR_A-receptor blockade) on iliac PWV. The effect of inhibiting basal ANP plasma level on resting regional PWV is illustrated. Values represent means±SEM. Symbol † depicts within group comparison (student's t-test).

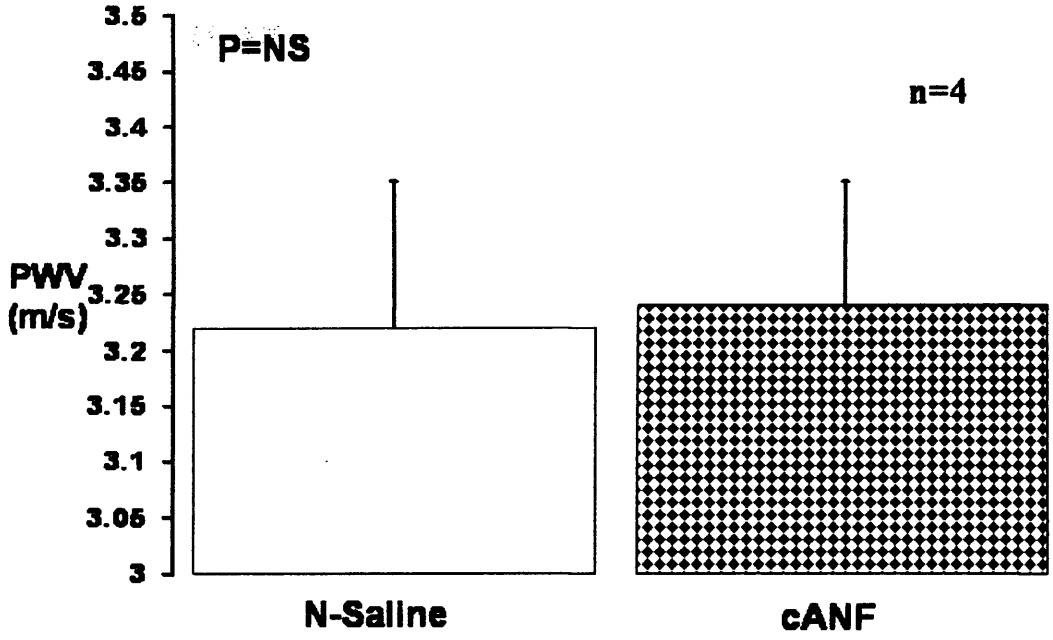


Figure 5.4.3. Effect of cANF on PWV. The effect of cANF-NPR_C-interaction on regional PWV is illustrated. Values represent means±SEM.

Table 5.4. Effects of ANP, BNP, CNP on Hemodynamics

N=18 sheep		ANP			BNP			CNP		
	Sheath	0.15	0.3	Sheath	0.15	0.3	Sheath	0.15	0.3
Iliac PWV, m/s	3.6±0.2	3.3±0.1†	3.3±0.1‡	3.2±0.1	3.1±0.1	3.0±0.1*	4.0±0.6	4.2±0.6	3.9±0.6
MAP, mmHg	84±5	82±7	82±6	89±2	88±3	86±4	90±7	88±7	81±6†
HR, bpm	141±6	142±10	142±8	151±7	152±5	152±6	101±4	101±5	97±3

Continuous data are presented as mean±SEM. Symbols depict *P≤0.05, †P≤0.01, ‡P≤0.001.

5.5. Discussion

Large artery stiffness, the inverse parameter distensibility, and aortic PWV are powerful, independent predictors of cardiovascular risk.(525;526) Whilst age-related arterial stiffening was thought to be mainly the consequence of structural changes within the arterial wall, we (529;530) and others (535) recently demonstrated a significant degree of functional regulation.

In the present study we investigated the role of NPs in the functional regulation of large artery distensibility.

The principal findings were as follows: first, we demonstrate that ANP and to a lesser degree BNP regulate regional large artery distensibility. Second, the effect of ANP was completely antagonised by the NPR_A-selective receptor blocker A71915 suggesting that this effect was solely mediated via the NPR_A-receptor. Third, A71915 infusion increased resting PWV suggesting that basal ANP plasma levels contribute to resting large artery distensibility. Fourth, neither CNP nor the NPR_C-selective receptor agonist cANF altered regional PWV suggesting that the NPR_B and NPR_C receptors do not acutely modify large artery distensibility *in vivo* (keeping in mind the caveat referred to in Chapter 4.4.4 on page 157).

5.5.1. The effect of ANP and BNP on large conduit arteries

ANP and BNP exert well documented vasorelaxant effects in the resistance vasculature *in vivo* (251;521). Furthermore, in organ bath experiments various NPs have been shown to stimulate cyclic guanosine monophosphate production and to dilate rings of smaller conduit arteries, including human internal mammary (249;521;531;536). However, compelling evidence of a biological effect of ANP on *in vivo* large artery function, including resting distensibility is lacking. In the present

study we provide direct evidence that ANP modulates regional large artery distensibility. The study design rules out central sympathetic or general systemic effects. The results therefore reflect local ANP actions.

5.5.2. CNP – a vasoactive NP?

The NPS consists at least of ANP, BNP, both predominantly of myocardial origin, and CNP, largely of endothelial cell origin.(325) ANP and BNP are believed to exert their vasorelaxant effects mainly through binding to NPR_A, a membrane bound GC-coupled receptor that signals via the 2nd messenger cGMP. CNP in contrast is the natural ligand for the NPR_B receptor. All 3 peptides have high binding affinity to the NPR_C receptor,(537) the latter being devoid of a GC-domain. The NPR_C-receptor (previously believed to mainly act as a clearance receptor) has now been recognised to play a crucial role in mediating the anti-proliferative action of the NP. Activation of NPR_C inhibits adenylate cyclase, increases phospholipase C activity and has very recently been shown to mediate CNP induced endothelium derived hyperpolarising factor (EDHF) dependent vasorelaxation in mesenteric resistance arteries (533). In contrast to the latter study neither cANF-NPR_C- nor CNP-$NPR_{B/C}$-interaction elicited any immediate regional arterial effects in the present study. The latter is not surprising given that the amount of EDHF dependent vasorelaxation is believed to decrease with increasing vessel diameter (538;539). Furthermore, it has long been noticed that the effects of NP are size and site dependent (163;531). The potential vasodilatory role of CNP remains controversial. Whilst some in-vitro (533;540) and in-vivo studies (271;278), (the latter following local drug administration), have reported vasodilatory effects, at least at pharmacological concentrations, others have failed to demonstrate important hemodynamic effects at pathophysiological plasma concentrations

(483;484;541;542). Our study suggests exogenously (intra-arterial) administered CNP has little local vasodilatory potency, at least in large muscular conduit arteries. However, as CNP is an abluminally secreted paracrine acting hormone plasmalevels are possibly a poor surrogate for more important tissue concentrationsin. Therefore, definitive assessment of a potential vasoactive role of CNP will have to await the results of studies using a selective NPR_B-receptor antagonist (which has become commercially available in 2004).

5.5.3. Effects of Basal ANP plasma levels on large artery distensibility

The effects of NP on vascular function have traditionally been investigated by assessing changes in blood pressure, central hemodynamics, vascular tone, blood flow and resistance in response to either exposure to exogenous ANP (167;251;373) or in response to inhibition of breakdown of endogenous NPs (367). Most studies have employed doses of ANP that result in elevation of plasma peptide concentrations to values far above normal (370;371). Richards et al demonstrated that even very low dose infusions caused significant hemodynamic effects (543). However, acceptance of ANP as a physiologically significant vasoactive hormone depends on the demonstration of changes in vascular tone in response to inhibition of the effects of basal plasma levels. Studies by Brunner and Woelkert (364), using the ANP-analogue A71915, provided evidence that ANP-antagonism contributes to regulation of basal coronary and TPR, at least in rodents. Using the same receptor antagonist we previously demonstrated that basal ANP plasma levels contribute to regulation of regional vascular volume and venous tone in healthy volunteers. Furthermore, we demonstrated that A71915 dose dependently antagonised ANP but not sodium nitroprusside induced changes in FBF (516). Here we extend these findings to large

conduit arteries. Indeed the present study, to the best of our knowledge, is the first to show that basal ANP plasma levels contribute to resting large artery distensibility *in vivo*.

5.5.4. Clinical considerations

Decreased arterial distensibility (increased stiffening) causes increased PWV so that wave reflection affects the systolic rather than the diastolic part of the wave, creating a secondary rise in pressure in late systole thereby increasing afterload without adequately augmenting coronary perfusion pressure (544). The sequelae are isolated systolic hypertension (ISH), left ventricular hypertrophy and ultimately heart failure. Wave reflection itself arises from a myriad of sites at which there is a change in vascular impedance, including the aorto-iliac bifurcation. Besides the largely age dependent and at present untreatable structural abnormalities (fatigue and fracture of elastin), it is now well recognised that functional changes, most importantly smooth muscle tone, will also affect PWV. Interestingly patients with ISH exhibit lower baseline ANP plasma levels compared to age matched patients with essential hypertension, but exhibit a higher renal ANP sensitivity (545). If the same were true for their vasculature then NP based treatment regimes may prove particularly useful in this subgroup of hypertensive patients. Indeed, NPs have the potential to counteract both, pathological remodelling and increased vascular tone. Here we show that ANP-NPR_A-interaction acutely modulates regional PWV thereby providing a potential mechanism by which NP based treatment regimes may exert beneficial effects in ISH.

5.5.5. Limitations

The present study used an ovine hind limb as model of large arteries in humans. Therefore, general concerns of transferring data obtained from animal research to humans will apply to this study. However, whilst ovis aries and homo sapiens for example differ markedly in their digestive system, for most published cardiovascular parameters, the sheep is similar to man if allowance is made for the smaller weight of the average sheep. Besides these cardiovascular similarities and its surgical suitability, the sheep was the animal of choice for the present work as there is possibly no other mammal (apart from homo sapiens) in which the cardiovascular effects of NPs have been equally well characterised (480;484). In addition, inhibition of basal NO production with LNMMA, has a similar qualitative effects on arterial distensibility in ovine (529) and human iliac artery (own unpublished data) *in vivo*.

Furthermore, a non-homologues NP system was used in the present study. Given the different degree of species variation between the amino-acid sequence of human and ovine NP (minor for ANP and CNP but relatively large for BNP) the absolute vascular responses observed (in particular in the BNP study) have to be interpreted with caution. It is also self evident that the use of general anaesthesia may have influenced our results to some degree. However, due to the need to make very high fidelity recordings, it was not possible to use conscious animals.

Although PWV is a measure of distensibility, factors which influence distensibility include vessel diameter, wall thickness and wall stiffness, possibly due to altered relative loading of elastin and collagen fibres within the arterial wall, accompanying changes in smooth muscle tone. Therefore, we are unable to identify which of these parameters are responsible for the observed changes in PWV in the present study. However, it would seem unlikely that alterations in vascular resistance in the hind

limb is responsible for the changes in PWV since infusion of NPs via the sheath had no effect.

5.6. Conclusions

ANP, and to a lesser degree BNP, play a role in regulating regional large artery distensibility via the NPR_A receptor. Neither CNP nor cANF altered PWV, suggesting that NPR_B and NPR_C do not acutely influence distensibility *in vivo*.

CHAPTER 6 – THE EFFECTS OF NOVEL NATRIURETIC PEPTIDES (NNP) ON ISOLATED RINGS OF RABBIT AORTA: COMPARISON TO ANP AND DNP

6.1. Background

Unravelling of the NPS is a rapidly evolving process but far from complete. Recently dendroaspis natriuretic peptide (DNP) a 38 amino acid peptide [Sequence: Glu-Val-Lys-Tyr-Asp-Pro-Cys-Phe-Gly-His-Lys-Ile-Asp-Arg-Ile-Asn-His-Val-Ser-Asn-Leu-Gly-Cys-Pro-Ser-Leu-Arg-Asp-Pro-Arg-Pro-Asn-Ala-Pro-Ser-Thr-Ser-Ala-; discuphide bridge Cys7-Cys23] with a Molecular Weight of 4190.7, has been isolated from the venom of the green mamba snake (*Dendroaspis angusticeps*) (205). Apart from the 17 amino acid ring structure shared by all NPs, DNP shares eleven amino acid residues with CNP, twelve residues with ANP and fourteen with BNP but has a 15 amino acid carboxy terminal tail whereas ANP and BNP have only a 6 amino acid extension and CNP is completely devote of a c-terminal tail. Furthermore, DNP was found to contain 4 proline residues, three of which are clustered in the C-terminal region of the molecule. This characteristic is unique to DNP, as neither ANP, BNP nor CNP contain proline residues. Interestingly, DNP immuno-like reactivity has been found in the plasma of patients with CHF (206) but the significance of this finding is controversially debated (207). Meanwhile DNP has been shown to relax human arteries and veins in vitro (208), to be a potent natriuretic and diuretic peptide with tubular actions linked to cGMP (546), and when administered in dogs, with (547) and without heart failure (548), exhibited cardiac unloading and lusitropic properties. Even more recently, a further novel natriuretic peptide, here preliminarily termed NNP, has been isolated in the research laboratory of Glaxo Smith Kline (GSK) from

the same snake venom as DNP. There is so far no published literature about this peptide. NNP has a Molecular Weight of 3690 and its cDNA sequence revealed classic characteristics for NPs. A first cell based (PC12) assay showed that its ability to produce cGMP is about the same as rat BNP(1-45) but less than that of ANP. In contrast its potency in primary cultured rat aortic smooth muscle cells is superior to that of ANP. Furthermore, NNP also exhibits some similarity to CNP but as ANP, BNP, and DNP has a c-terminal tail, which appears to be of the same length as that reported for DNP. In an in-vitro relaxation experiment using rat mesenteric artery NNP was roughly equipotent to BNP(1-45) (Liang Lee [GSK]; personal communication).

The aim of this work was to assess the vasorelaxant potency of NNP on conduit arteries, using DNP and ANP as comparative agents. Rabbit aortic rings were suspended in organ bath and the response to cumulative concentrations of NNP, DNP and ANP was obtained following phenylephrine induced pre-constriction and consecutive equilibration. Additionally, inhibiting agents were used to determine the mechanism of vasorelaxation.

6.2. Methods

Male, New Zealand white rabbits (2-2.5kg) were euthanased by intravenous injection of sodium pentabarbitone. The thoracic aorta was then removed into fresh Krebs buffer composed of (mmol/L) NaCl 138, KCl 5.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, Glucose 15, $NaHCO_3$ 24, and $CaCl_2$ 1.5, and gassed with 95% O_2 and 5% CO_2. The aorta was then carefully cleared of all blood, fat, muscle and connective tissue.

6.2.1. Isometric Tension Recordings

For isometric tension recording, 2- to 3-mm-wide aortic rings were mounted on a force transducer in tissue baths containing fresh Krebs buffer with a resting tension set at 2 g (see Figure 6.2.1.). After a 1 hour equilibration period, contractions were produced by addition of a submaximal concentration of (PE, 1μmol/L) and the responses allowed to reach a plateau. The aortic rings were then exposed to 1μmol/L acetylcholine to determine integrity of the endothelium. Tissues were then repeatedly exposed to PE (1μmol/L) until a stable reproducible level of constriction was obtained. Experiments were then carried out as outlined below and all data are expressed as percentage relaxation of the appropriate PE-induced constriction (with the constriction produced by 1μmol/L PE considered as 0% baseline tension) (549). Contractions were recorded and analysed using MacLab 8 hardware.

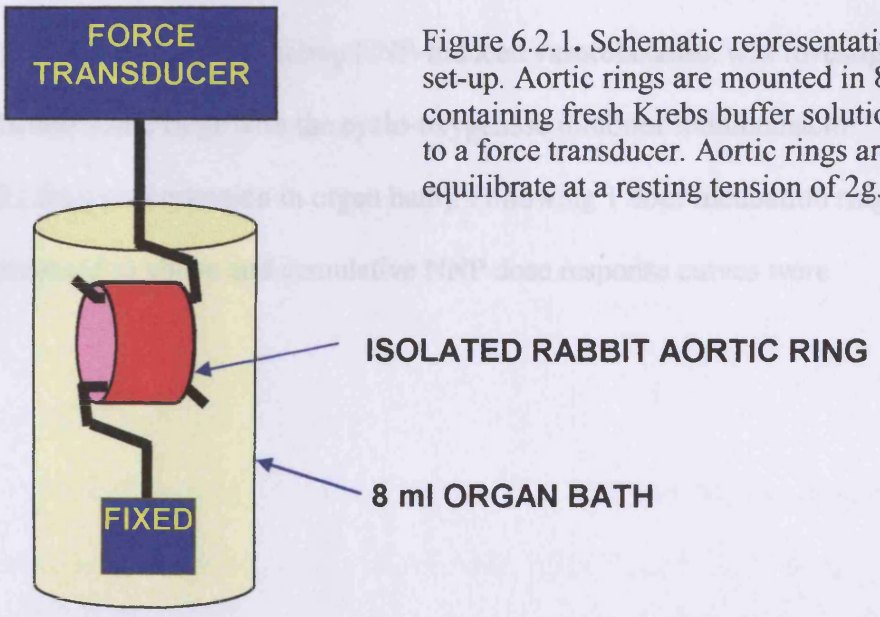


Figure 6.2.1. Schematic representation of organ bath set-up. Aortic rings are mounted in 8ml tissue baths containing fresh Krebs buffer solution and attached to a force transducer. Aortic rings are allowed to equilibrate at a resting tension of 2g.

6.2.1.1. Effects of ANP, DNP and NNP on vascular smooth muscle tone

The effects of ANP, DNP and NNP on PE (1 μmol/L – final concentration in organ bath) pre-constricted rings of rabbit aortic tissue was measured by generating concentration-response curves. Relaxation to all three peptides was determined over a concentration range of 0.1nmol/L to 0.1μmol/L (final concentration in organ bath). The relaxation response was measured during the 5-minute interval after each cumulative addition of the natriuretic peptide in question.

6.2.1.2. The Role of Nitric Oxide in NNP induced vasorelaxation

To determine a potential role of NO in NNP mediated vasorelaxation, 0.3mmol/L of L-nitro arginine methyl ester (L-NAME), a NO synthase inhibitor, was added to the organ baths one hour before the pre-contraction with PE and subsequent NNP administration.

6.2.1.3. The Role of Prostaglandins in NNP-induced vasorelaxation

The role of prostaglandins in mediating NNP-induced vasorelaxation was investigated by pre-incubation aortic rings with the cyclo-oxygenase inhibitor indomethacin (0.01mmol/L; final concentration in organ bath). Following 1 hour incubation rings were pre-contracted as above and cumulative NNP dose response curves were performed.

6.2.1.4. The Role of Soluble Guanylate Cyclase (sGC) in NNP-induced vasorelaxation

To assess the potential role of soluble GC (s-GC) in NNP induced vaorelaxation, aortic rings were incubated for 1 hour with the s-GC-inhibitor ODQ (1H-[1,2,4]oxadiazol 9,4,3-alpha) quinoxaline-1, 10μmol/L; final concentration in organ bath).

6.2.1.5. The Role of NPR_A in NNP induced vasorelaxation

To assess whether NNP induced vasorelaxation is mediated via NNP-NPR_A interaction, we performed NNP dose-response studies in the presence and absence of the NPR_A-selective receptor antagonist A71915 (1μmol/L, 3μmol/L, 10μmol/L). A prior pilot dose-response study using A71915 had revealed no intrinsic vasodilating property of A71915 over the applied dose range.

6.2.1.6. The Role of NPR_C in NNP induced vasorelaxation

To assess whether NNP induced vasorelaxation is mediated via NNP-NPR_C interaction, we performed NNP dose-response studies in presence and absence of the NPR_C-selective receptor antagonist cANF (1μmol/L).

6.2.2. Drugs and Chemicals

NNP was donated by Glaxo Smith Kline (London, UK). DNP was purchased from Phoenix Pharmaceuticals (Belmont CA, USA). ANP, A71915, and cANF were purchased from Bachem LTD (St Helens, UK). ODQ, L-NAME, and Indomethacin and Phenylepinephrine were purchased from Sigma.

6.2.3. Statistical analysis

Data are expressed as mean ± standard error of the mean (S.E.M.). In all experiments, *n* equals the number of vascular rings. All comparisons were performed by one-way ANOVA followed by Bonferroni's post hoc test as appropriate. Concentrations causing 50% of the maximal response (EC_{50}) were calculated using GraphPad Prism Software. Significance is defined as $p<0.05$.

6.3. Results

6.3.1. Comparative ANP, DNP, NNP dose response study

ANP, DNP, and NNP produced similar concentration-dependent relaxation of PE-pre-constricted rabbit aortic tissues (see Fig 6.3.1 and Table 6.3.1).

Table 6.3.1. Effects of ANP, DNP and NNP on vascular smooth muscle tone

Natriuretic peptide	% Aortic Relaxation ± S.E.M.	-Log EC_{50} ± S.E.M. (mol/l)
ANP	91.46 ± 1.50	7.957 ± 0.09
DNP	94.78 ± 0.45	8.105 ± 0.04
NNP	92.04 ± 0.61	8.157 ± 0.04

Maximal relaxations to 0.1μmol/l natriuretic peptide (final concentration in organ bath). -Log EC_{50} values are also shown Data are expressed as means ± Standard error of the mean (S.E.M.).

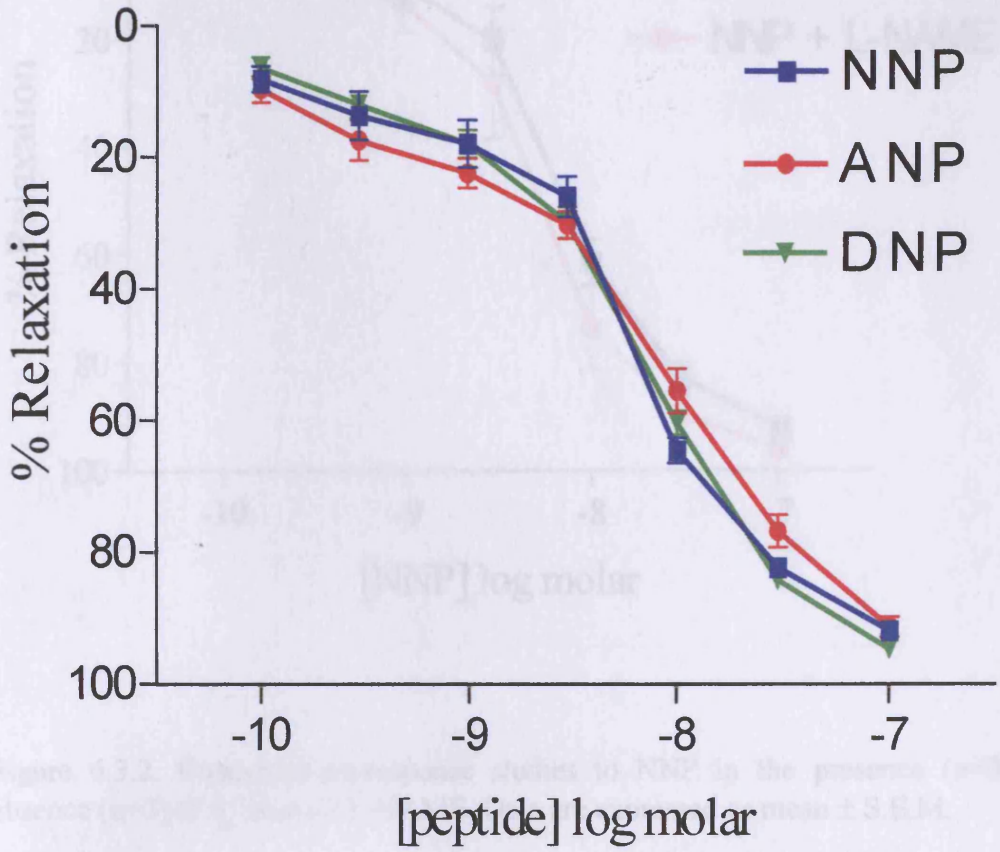


Figure 6.3.1. Concentration response studies to NPs ANP (n=8), DNP (n=13) and NNP (n=16) (0.1nmol/l-0.1μmol/l) on 1μmol/l PE-induced preconstricted rabbit aortic tissue. Data are expressed as mean ± S.E.M.

6.3.2. The Effects of Nitric Oxide in NNP induced vasorelaxation

Figure 6.3.2. Illustrates the role of endothelial NO synthase on NNP (0.1nmol/l-0.1μmol/l) induced vasorelaxation. Pre-incubation with L-NAME (0.3mmol/L) had no effect on NNP induced vasorelaxation.

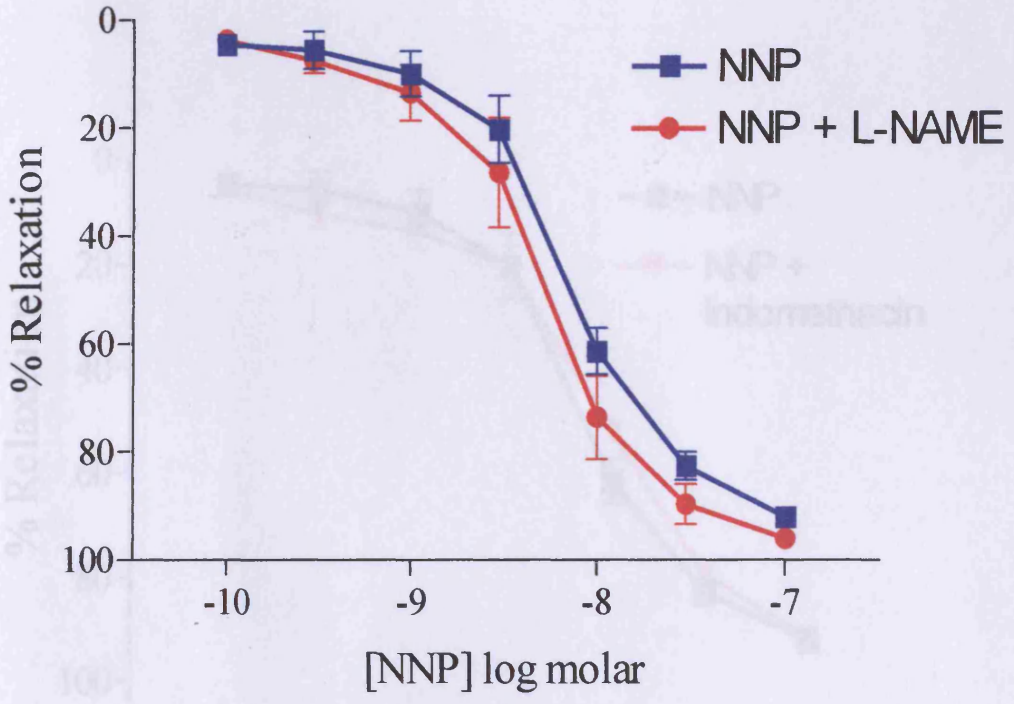


Figure 6.3.2. Concentration-response studies to NNP in the presence (n=3) and absence (n=3) of 0.3mmol/l L-NAME. Data are expressed as mean ± S.E.M.

6.3.3. The Effects of Prostaglandins in NNP-induced vasorelaxation

Figure 6.3.3. Illustrates the effect of cyclo-oxgenase inhibition on NNP (0.1nmol/l-0.1μmol/l) induced vasorelaxation. Pre-incubation with Indomethacin (1μmol/L) had no effect on the subsequent relaxation response to NNP.

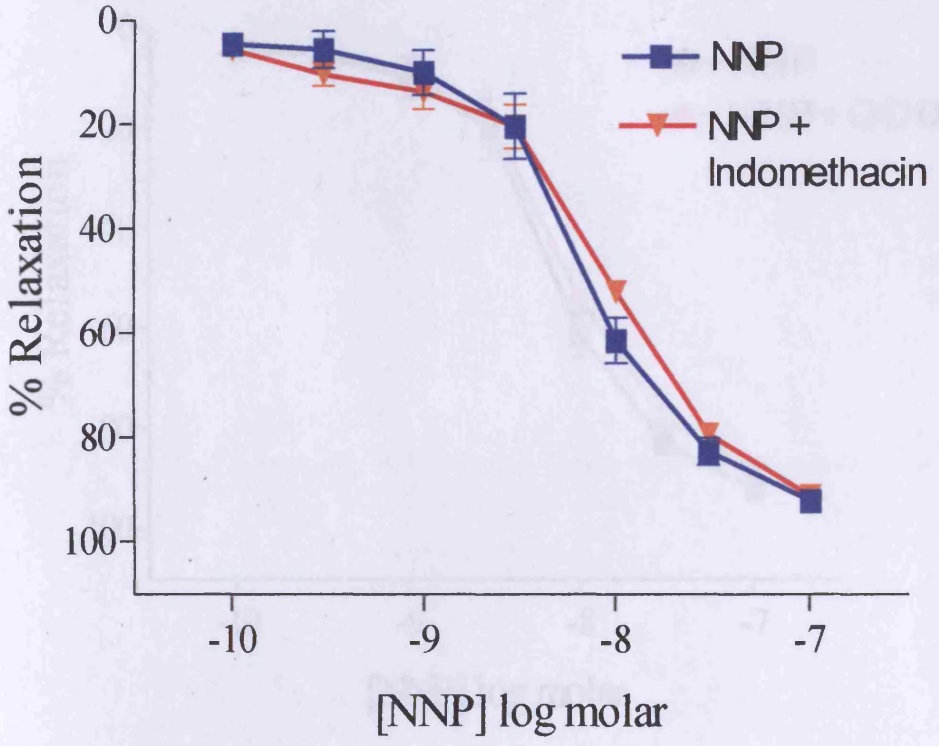


Figure 6.3.3. Concentration-response studies to NNP (0.1nmol/l-0.1μmol/l) in the presence (n=3) and absence (n=3) of 1μmol/l Indomethacin. Data are expressed as mean ± S.E.M.

6.3.4. The Effects of sGC in NNP-induced vasorelaxation

Figure 6.3.4. Illustrates the role of the sGC-cGMP pathway in mediating NNP induced vasorelaxation. Pre-incubation with 10μmol/l ODQ had no effect on NNP (0.1nmol/l-0.1μmol/l) induced vasorelaxation.

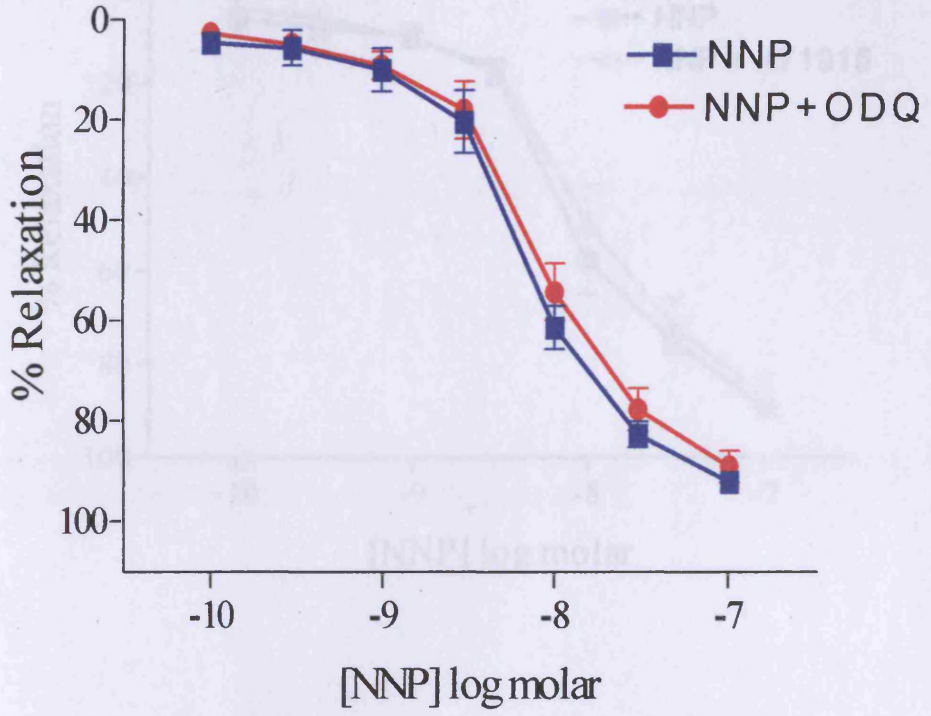


Figure 6.3.4 Concentration-response studies to NNP (0.1nmol/l-0.1μmol/l) in the presence (n=3) and absence (n=3) of 10μmol/l ODQ. Data are expressed as mean ± S.E.M.

6.3.5. The Effects of NPR_A-NNP interaction on vascular smooth muscle tone

Figures 6.3.5.a.-c. Illustrate the effects of selective NPR_A-receptor blockade on NNP induced vasorelaxation. Pre-incubation with A71915 (1μmol/L, 3μmol/L and 10μmol/L) had no effect on NNP induced vasorelaxation.

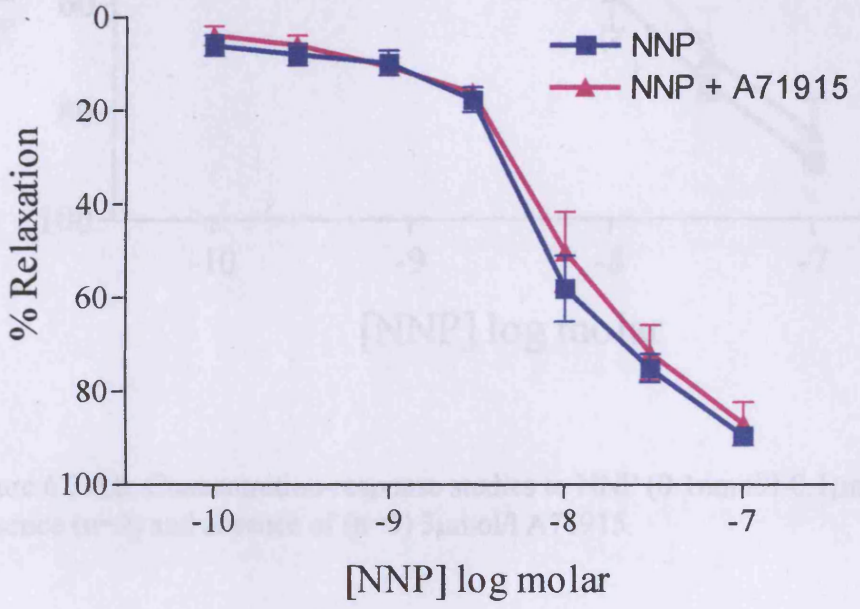


Figure 6.3.5.a. Concentration-response studies to NNP (0.1nmol/l-0.1μmol/l) in the presence (n=4) and absence of (n=5) 1μmol/l A71915.

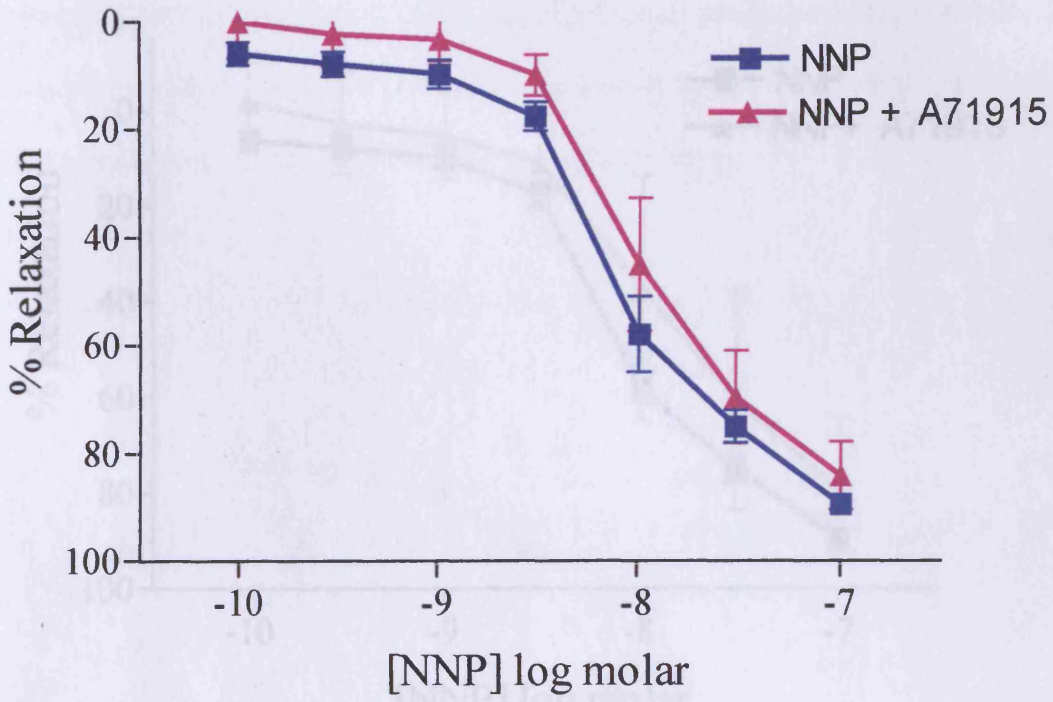


Figure 6.3.5.b. Concentration-response studies to NNP (0.1nmol/l-0.1μmol/l) in the presence (n=2) and absence of (n=5) 3μmol/l A71915.

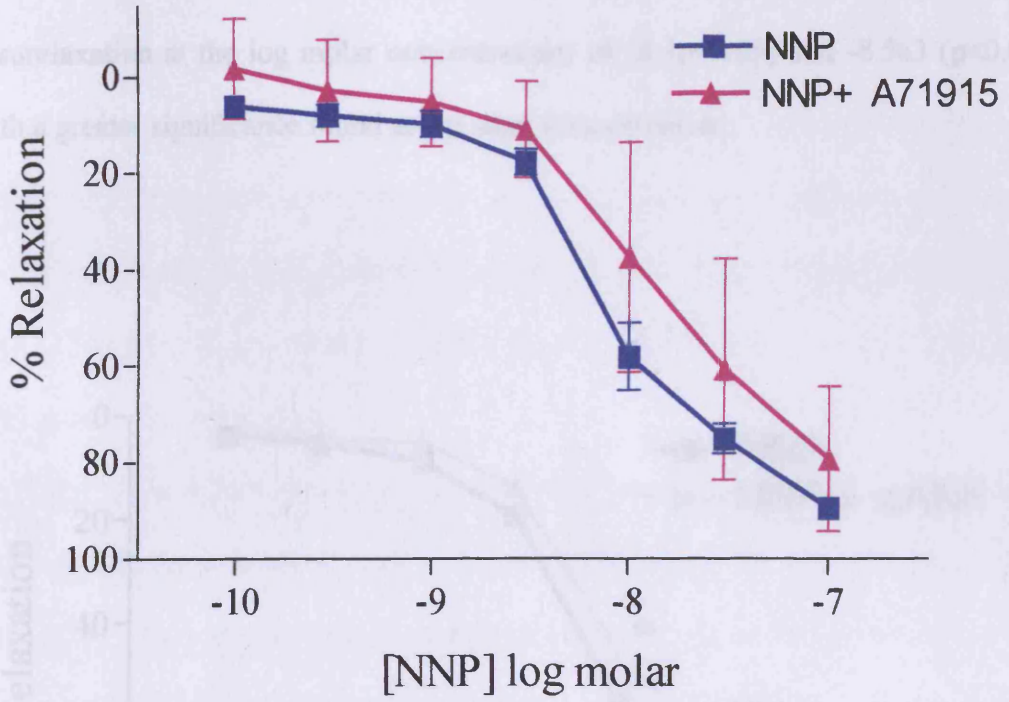


Figure 6.3.5.c. Concentration-response studies to NNP (0.1nmol/l-0.1μmol/l) in the presence (n=2) and absence of (n=5) 10μmol/l A7191515.

6.3.6. The Effects of NPR_C-NNP interaction on vascular smooth muscle tone

Figure 6.3.6. Illustrates the effects NPR_C-inhibition on NNP induced vasorelaxation. Pre-incubation with cANF caused a significant inhibition of NNP-induced vasorelaxation at the log molar concentrations of -8 ($p<0.05$) and -8.563 ($p<0.001$) with a greater significance found at the latter concentration.

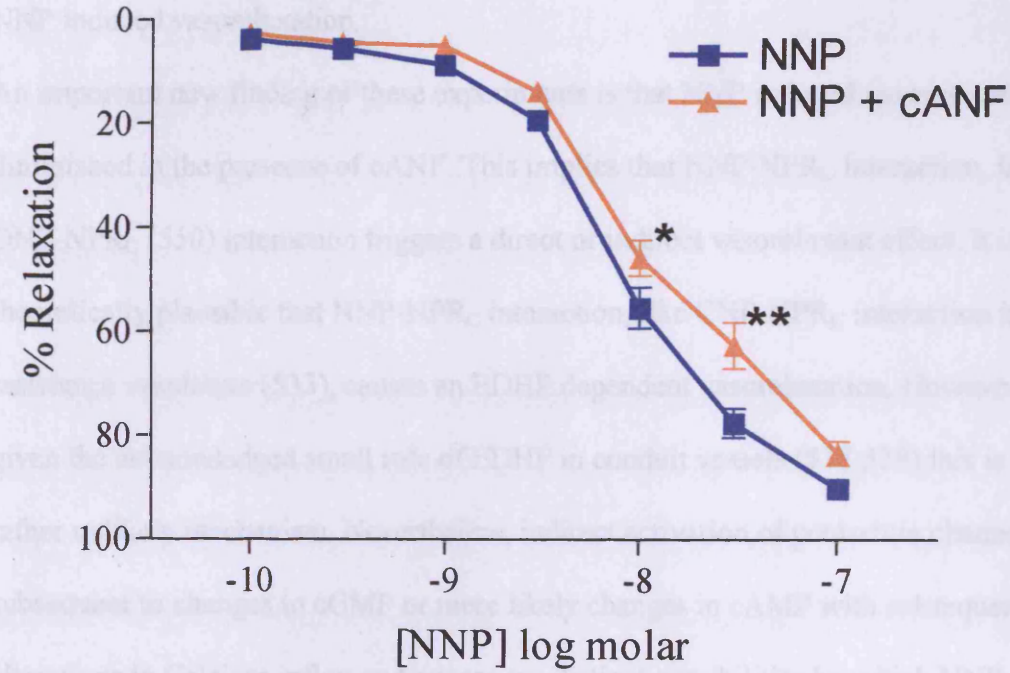


Figure 6.3.6. Concentration-response studies to NNP (0.1nmol/l-0.1μmol/l) in the presence (n=4) and absence (n=4) of 1μmol/l cANF. Symbols depict *$p<0.05$), **$p<0.001$).

6.4. Discussion

We tested the ability of NNP to decrease PE induced isometric tension in rabbit aortic rings. Our studies revealed that NNP has vasorelaxant potency very similar to those of DNP and ANP.

In line with previous studies (550) assessing the vascular reactivity of tissue from healthy animals to various NP's our studies provide evidence that the vasorelaxant properties of NNP are independent of cyclo-oxygenase. Furthermore, as is the case for CNP (274;276) neither, eNOS nor soluble guanylate-cyclase appear to be involved in NNP induced vasorelaxation.

An important new finding of these experiments is that NNP induced vasorelaxation is diminished in the presence of cANF. This implies that NNP-NPR_C interaction, like DNP-NPR_C (550) interaction triggers a direct or indirect vasorelaxant effect. It is theoretically plausible that NNP-NPR_C interaction, like CNP-NPR_C interaction in the resistance vasulature (533), causes an EDHF dependent vasorelaxation. However, given the acknowledged small role of EDHF in conduit vessels (538;539) this is a rather unlikely mechanism. Nevertheless, indirect activation of potassium channels subsequent to changes to cGMP or more likely changes in cAMP with subsequent alterations in Calcium influx and release are distinct possibilities by which NNP could exert a NPR_C mediated effect. Finally, NNP-NPR_C binding could induce a conformational change in the 39 amino acid intracellular tail of the receptor thereby activating its G-protein containing sequence with subsequent signal transduction either via inhibition of adenylate cyclase (potentially via G_{i2}) or alternatively via activation of phospholipase C (potentially via the $\beta\gamma$-subunit of G_{i1} and G_{i2}).

As with other NPs NNP induced vasorelaxation does not appear to depend on the soluble GC system, as evidence by a lack of effect of ODQ.

The results of study 6.3.5. using the NPR_A-selective receptor antagonist A71915 deserve comment. Pre-incubation of aortic rings with A71915 at a concentration of 1μmol/l failed to affect NNP induced vasorelaxation. Pre-incubation of aortic rings with A71915 at a concentration of 3μmol/l and 10μmol/l tended to shift the relaxation curves up-ward (i.e. reduced vasorelaxation) without reaching statistical significance. Because of the need to perform dose finding studies, the limited amount of NNP and A71915 available at the time of experimentation, and the decision to test the interactions of NNP with several possible vasorelaxant systems (as outlined above) the studies are likely to be underpowered. It is therefore concluded that whilst the NPR_A receptor is unlikely to be the natural receptor for NNP, NNP induced NPR_A mediated effects can not be ruled out on the basis of these experiments and repetition of these experiments with higher numbers is needed.

DNP has structural features, receptor characteristics, second messenger activity, and some biological effects (i.e. vasorelaxation) in common with ANP and BNP. However, despite some studies showing DNP-like immuno reactivity in human plasma (206;551) the significance of this finding and consequently the question if DNP is a member of the NPS in man remains controversial (207), even 11 years after its discovery (205).

It has been pointed out that albeit intriguing, the finding of DNP-like immunoreactive staining (206) and DNP-like immuno-reactivity in human plasma (206;551) "*do not establish DNP as a member of the mammalian cardiac natriuretic peptide family or even as an endogenous peptide in human beings*" (207). Because of the limitations associated with the techniques applied in aforementioned studies it will be mandatory to identify the peptide in human plasma and tissue using high-performance

chromatography linked to immunoassay, followed by purification and analysis to establish the human aminoacid sequence. Furthermore, the gene must be identified in the Green Mamba so that appropriate probes for the cloning of the human homologue can be generated (207).

As far as NNP is concerned the same criteria will have to apply as for DNP. The publication of the aminoacid sequence is eagerly awaited in order to confirm that NNP and DNP are indeed different in their amino acid sequence and not simply different slicing products of an identical NNP/DNP pre-hormone. Time will tell if these peptides are true additions to the (human) natriuretic peptide family or just primitive evolutionary precursors to ANP and BNP, or even snake BNP itself given, the enormous species variation and the lack of an identified snake BNP.

CHAPTER 7 – DISCUSSION AND CONCLUSIONS

7.1. Discussion

Over the last two decades several well conducted large randomised controlled clinical trials, in patients with heart failure on the basis of systolic left ventricular impairment, have unequivocally demonstrated reductions in overall mortality for the use of ACE – inhibitors (46;552-554) or alternatively AT_1-antagonists (52;555), β-blockers (556-561), and spironolactone (562). However, epidemiological surveys, including the Framingham Study, have not documented any meaningful reduction in overall death rates (563). Instead it appears that the aforementioned therapies delay the development of heart failure following myocardial injury (45) and possibly slow down disease progression after heart failure has set in, thereby prolonging a life with heart failure (563).

Heart failure is also the reason for at least 20% of all hospital admissions among persons older than 65, and over the past decade the rate of hospitalisations has increased by 159% (564). Besides the pharmacological interventions listed above, coronary artery bypass grafting in ischemic heart disease (565;566), mitral valve repair (567), left ventricular geometry restoration (568), implantation of left ventricular assist devices, implantable cardioverter-defibrillators (569;570) and/or Bi-ventricular pacemakers (571), have all been proven to be beneficial in (more or less) selected patients. Finally, the use of multidisciplinary teams (572) has been demonstrated to be cost effective and to reduce the rate of hospitalisation (573).

In line with these findings and because of the large burden that heart failure presents not only to the affected individual but also to the society, modern RCTs are now also assessing the effects of the studied intervention on rate of hospital admissions and other markers of morbidity and economic factors (574).

From a pathophysiological point of view heart failure remains imperfectly understood. A variety of different model approaches have placed different weight on different aspects of the clinical syndrome. The present thesis is performed at a time when the neurohormonal model of heart failure is popular. Indeed, current therapies of heart failure are largely based on inhibition of neuroendocrine responses, and the diagnostic (178;180) and prognostic (575-577) value of an abundant number of circulating peptides and proteins (578) (be it of myocardial (578) or vascular in origin (579)) have been proven beyond doubt.

However, it is acknowledged that the recent failure of several therapeutic concepts, grounded in the neurohormonal concept have sobered the "scientific heart failure community". The findings and/or failures of OVERTURE (183;580), ENABLE (581), and RENEWAL (= RENAISSANCE+ RECOVER) (582) have raised the questions if we have either reached the "asymptote of efficacy" (580) and/or, if some of the concepts that appeared so promising in animal models of heart failure are simply "unsuccessful, to be specific" (583) in the clinical scenario.

Several "opinion leaders" recently commented on these issues and discussed a possible scenario of "the foreseeable future" (584) for some of the agents that failed to deliver on earlier promises (582;585-587).

From a clinician's and more importantly a patient's point of view the existing armamentarium of heart failure medication, complemented by non-pharmacological and dietary interventions, already seems to be a sizable challenge to completely comply with. With this in mind and recognising that Nesiritide (a recombinant BNP), a drug that only confers marginal symptomatic benefit over existing much cheaper therapeutic alternatives, is the only true new drug that has been licensed for the

treatment for heart failure by the FDA over the last two decades, it is possibly fair to assume that (immediate) further improvements in morbidity and/or mortality in the management of heart failure, is most likely to be achieved only by a truly individualised treatment approach. Such an individualised approach will have to take account of the degree and pattern of neurohormonal activation. Encouragingly a recent, albeit small randomised study, showed that pharmacotherapy guided by plasma N-BNP reduced the number of cardiovascular events as compared to trial-based therapy dictated by clinical acumen (181).

Heart failure has all too long remained a "low profile diagnosis" despite a prognosis worse than most cancers (588;589). The introductory Chapter 1.1. briefly describes the pandemic dimensions, pathophysiology and classification of this disease.

Chapter 1.2. outlines the physiological importance of the venous capacitance bed in man, and describes the adverse pathohysiological consequences of increased venous tone in heart failure.

The dearth of knowledge concerning these small veins and venules and the mechanism of venoconstriction in heart failure is in no small part the consequence of absence of simple techniques that allow their study. The methodology chapter (chapter 2) describes the basic principle, applicability and validity of RPY, a technique that combines EBPS and conventional VOP, thereby allowing detailed assessment of the small veins and venules (i.e. the capacitance circulation).

For reasons outlined in chapter 2.8. RPY will remain limited in its application. However, in view of the unique information that this technique can provide it is hoped that RPY will find its niche and indeed a wider application than at present (to the best of our knowledge Cardiff is currently worldwide the only institute applying RPY in human research). Combining the advantages of EBPS and conventional occlusive

techniques has the potential to be used in the complex assessment of cardiovascular drugs, physiologic, and mechanistic interventions (e.g. cardiac resynchronisation therapy) in order to optimise individual treatments in patients with e.g. heart failure (or essential and pulmonary hypertension). The use of multiple gamma cameras and mobile "nuclear vests" will permit investigation of the effects of variable interventions on right and left ventricular volumes (and function) and dynamic blood volume shifts (or regional re-distributions) in health, disease states, and importantly following mechanistic interventions, and during exercise.

Several such studies are already underway and it is hoped that the work of this thesis provides a platform and a rationale for a wealth of further studies of the venous capacitance bed, in particular in heart failure but also in pulmonary and essential hypertension.

Venodilatation, has been shown to confer some of the symptomatic benefits derived from diuretic (590;591) and morphine (592;593) therapy and may partly account for the superior outcome data of ACE-inhibitors when compared to pure arterial dilators (54). In view of the fact that only vasoactive agents with a balanced veno-/arterio-dilating profile have so far been shown to improve outcome in heart failure the marked imbalance of scientific focus on the arterial circulation and the relative neglect of the venous circulation, in particular the small veins and venules, seems somewhat unjustified.

The interpretation of studies assessing venous physiology and pathophysiology will depend on the conceptual framework that is deployed. The methodology and concepts used in the present thesis are based on Tyberg's (and co-workers, for review see (89;104)) extended P/V model of the circulation, and ultimately based on Levy's

(594) concepts. The applied methodology is, from a theoretical and practical point of view, presented in chapter 1.2 and 2 respectively.

The principal objective of the work presented in this thesis was to investigate the role of ANP in the regulation of regional vascular function, in particular regional volume and tone, in both health and heart failure.

We generally thought it counterintuitive to believe that a hormone released in response to atrial stretch should not have an effect on venous tone. A starting point was that, despite the uniform observation of a reduced CVP following ANP administration, and the specific finding that low dose ANP reduced CVP without a fall of SVR, the majority of studies in the mid eighties and early nineties concluded that ANP had very little, if any effect on venous tone. These negatives studies can generally be divided into two groups; The first group are studies assessing the effects of ANP on conduit veins, either using the "Aellig-dorsal hand vein technique" (86) in vivo, or assessing other conduit veins, such as the saphenous vein, in vitro using organ chamber experiments. Besides the general limitations that need to be considered when applying these techniques (for discussion see chapters 2.9.1. and 3.5.1.-4.) A(NP) indeed appears to have little effect on conduit veins. However, in analogy to the arterial circulation where it has long been recognised that there are marked differences in the control of conduit and resistance arteries, it has become clear that conduit veins in general and the dorsal hand vein in particular behave differently from the physiologically more important small veins and venules. Some of the reasons for such differences have been discussed in chapter 3.5.2. The second group of studies finding a lack of A(NP) actions on venous tone are grounded in *Guytonian Physiology* and specifically in the concept of *increased resistance to venous return* (595;596).

In contrast to the approach presented in the current thesis, which as stated above is based on Tyberg's modified P/V model of the circulation, the *Guytonian approach* to the veins is based on pressure-flow relationships and the concept postulates that the driving force behind venous return is the difference between mean circulatory filling pressure (MCFP) and RA pressure. Based on this understanding, the venous- return-right-atrial-pressure representation was used to argue that venous return increases because right atrial pressure ("back-pressure") decreases, given a constant MCFP (596). Levy (594) questioned the assertion that venous return increases because of a decrease in right atrial pressure, repeated Guyton's experiment, and interpreted the results exactly oppositely – that right atrial pressure decreased because CO increased. It is possibly worthwhile mentioning that Guyton was one of the referees of Levy's work and his comments were judged to merit publication (and make interesting reading) (597). In his comment he found himself "*in complete agreement with Levy on all but two minor points, neither of which is important conceptually and both of which justify discussion only from a semantic view*" (597).

However, deductions about venous tone from standard hemodynamic studies may be misleading since filling pressures may be reduced by improved left ventricular function (54;75) (improvement in left ventricular function following (A)NP administration is undisputed). Recognising this, M. Packer (598) pointed out that the concept of venous return may be semantically (if not also fundamentally) problematic when analyzing the hemodynamics of CHF.

Further, we suggest that there are significant problems with Gauer's approach (599), as used by Holtz (504) and Trippodo (600) and their associates. Before and after the intervention, they defined pressure-volume relationships by quickly and substantially changing total blood volume while measuring mean circulatory filling pressure

(MCFP). Recently, Tyberg's group (601) has studied the effects of acute volume infusion (40 ml/kg) and hemorrhage (sufficient to reduce MAP by 50-60%) and found that volume infusion increased capacitance (i.e., the volume contained at a given pressure) by ~60% and hemorrhage decreased it by ~15%. As venous capacitance is so sensitive to changes in blood volume, we contend that the results of previous experiments are difficult to interpret and need to be re-evaluated.

Further, we contend that our method has fundamental and substantial advantages in that it disturbs the total circulation so minimally. In un-sedated, comfortable patients, we define the vascular pressure-volume relationship of the forearm simply by inflating a cuff to ~30 mm Hg, in marked contrast to the experimental studies of anesthetized (anaesthesias itself alters the response to ANP (502;503)), surgically operated animals in which MCFP was measured by stopping the heart repeatedly for many seconds.

Finally, although in his recent review (92) Tyberg has acknowledged that both the Guyton and Levy models are both internally consistent and that it is difficult to prove one at the expense of the other, we feel we have very good reasons to prefer the Levy model. First and foremost, *capacitance* is a pressure-volume property and to investigate it by studying pressure-*flow* relationships (i.e., right atrial pressure vs. venous return) is profoundly indirect, at the very best. Second, venous resistance, which forms such an important part of the Guyton-Permutt approach, is only an extremely small fraction of the total SVR and assumes significance only in Guyton's model — and that, primarily, in the unsteady state when the peripheral capacitance is being discharged (most clinical situations of interest are steady-state, in which capacitors completely disappear from the circuit). Third, our approach is entirely consistent with classical pre-Guyton venous physiology (602-604), in fact, Guyton

provided a figure (Figure 1.2.3.4.) showing parallel shifting arterial and venous pressure-volume relationships in several of his textbooks (e.g., Guyton AC, Hall JE: Human Physiology and Mechanisms of Disease. 6th Edition, WB Saunders Company, Philadelphia, 1997, p. 120). Because venous capacitance is a measure of the volume that the veins contain at a given pressure (40) it is in our view the most intuitive way to describe venous capacitance in terms of PVRs.

The studies by Ando et al (369), Olson et al (169) and Peters et al (56) hinted that ANP affected venous compliance and/or capacitance in the small veins and venules. The study by Peters (56) (a randomized, vehicle controlled, double blind study evaluating how ANP altered the regional distribution of blood in the capacitance vessels of eight healthy volunteers) found that a significant decrease in cardiac radioactivity (and fall in CVP) was paralleled by a significant increase in intestinal activity (56). This study in our view is strong evidence that ANP regionally "pools", indeed translocates central blood reserves into intestinal (and skeletal) capacitance vessels.

Applying our P/V model we demonstrate in chapter 3 that ANP increases regional vascular volume over a wide range of physiological, pathophysiological, and pharmacological plasma levels without significantly affecting compliance.

The finding of regionally increased blood volume in absence of compliance changes therefore allows us to conclude that changes in regional volume were the consequence of venodilatation. Interestingly the studies confirmed that plasma levels similar to those in severe heart failure, achieve a near maximal venodilating effect and that further increases fail to elicit an additional increase in venodilatation, possibly (partly) due to systemic effects and overriding baro-reflex-mediated venoconstriction. Most importantly, we demonstrate for the first time that basal natriuretic peptide levels

modulate vascular tone. It is worthwhile pointing out (and in my own view this is indeed the greatest merit of the present work) that the studies summarised in chapter 3 are the first investigating NPR blockade (NPR_A-receptor blockade to be specific) in humans.

In chapter 4 we present the effects of all four currently known human NPs on regional vascular function in symptomatic patients with cHF on maximal medical therapy. In particular we tested the hypothesis that venous but not arterial ANP responsiveness is preserved. We demonstrate that ANP despite lesser effects in the resistance vasculature has significantly more potent effects on the capacitance vasculature compared to BNP, CNP and Urodilatin. This finding is not only supportive of the original hypothesis but also renders indirect support to our previous validation studies demonstrating that changes in venous volume due not merely reflect changes in arterial inflow (95). Indeed, in the present studies ANP causes the least increase in FBF but the highest increase in FVV. The finding of preserved venous ANP responsiveness in presence of impaired arterial responsiveness together with the indirect observation that the older patients in chapter 4 had an almost identical venous responsiveness to the younger patients in chapter 3 despite clearly decreased arterial responsiveness, is in keeping with an often overlooked study by Emmick and Cohen which examined the possibility that vascular relaxation to atrial NPs may be affected by (animal) age (522). These investigators found that in vitro relaxation to atrial peptide 25 (AP-25) and atriopeptin (AP-21) in serotonin contracted aorta, carotid artery and mesenteric artery was greatest in young animals (rats 1-2 months of age) with a roughly 20% reduction in vascular responsiveness in older animals (rats 18-19 months of age). Unlike the arterial preparations examined, portal veins from rats of all ages relaxed similarly to AP-25. Interestingly, AP-25 given intravenously lowered

blood pressure to a similar extent in animals of all ages. Thus the greater arterial in vitro sensitivity of younger animals did not result in a greater reduction in BP in these younger rats (522). This observation is in keeping with the aforementioned studies by Groban (346) and Serizawa (523) showing that ANP could lower CVP without affecting SVR. In other words the effect of atrial peptides on blood pressure may not be associated with an arterial event but instead may be the consequence of its venous effects with subsequent reduction in cardiac preload.

Decreased ANP binding, first observed in a rat model of heart failure (see also chapter 1.3.7.1.NPR_{A+B}-receptor downregulation) was reported by Tsunoda (424) and later suggested in humans (425). It is conceivable that modest down-regulation of NPR_A-receptors occurs as ANP levels increase with age and with certain diseases, including heart failure and hypertension. Indeed ANP induced NPR_A-receptor down regulation has been observed in aortic VSMCs (605). To a certain degree the venous circulation may be protected from such a down-regulatory process due to the abundance of NPR_C-, or clearance receptors.

Our studies, albeit not specifically designed to investigate receptor density, nevertheless provided nevertheless some interesting findings. Baseline cGMP levels in heart failure patients were 10-fold higher than in controls, potentially arguing against $NPR_{A/B}$ receptor down-regulation. Most intriguingly, and somewhat in support of the assumption of a protected venous circulation (protected against ANP induced NPR_A receptor down-regulation), we found that identical infusion rates raised venous ANP plasma levels significantly more in controls than in cHF patients. Given that these ANP concentrations were infused directly into the brachial artery this can only mean that there was a higher clearance activity across the forearm bed of patients than controls, which raises the distinct possibility of NPR_C-receptor up-regulation.

Chapter 5 focusses on conduit arteries. The ovine hind limb model was chosen mainly for 3 reasons. First, the sheep is similar to man for most cardiovascular parameters and the cardiovascular effects of NPs have been extensively studied in ovis aries in both, health (287;480-484) and heart failure (486-488;490;491;493;495). Second, the aforementioned surgical suitability (and of course the possibility of access to sheep) and pre-existing sound validation (529;530) of this model.

Third, and most importantly the ovine hind limb model was chosen because of its unique potential to investigate the mechanisms of NP tolerance/resistance (for discussion see chapter 1.3.7) in the future (see future work).

Besides the fact that the in-vivo effects of NPs on regional large artery function had not been studied adequately a further important aspect of this work (which because cANF is not licensed for human use could only be studied in an animal model) was to gain some knowledge about the role of the NPR_C-receptor in regulating vasomotor tone. Whilst this receptor was long believed to play a somewhat dormant role as a clearance receptor, some recent work had demonstrated that cANF-NPR_C interaction could elicit vasomotor changes in resistance arteries.

The data presented in chapter 5.4 in my view provide compelling evidence that ANP modulates large artery distensibility predominantly, if not exclusively, via the NPR_A. Neither CNP, acting via the NPR_B, nor cANF, a NPR_C-selective agonist, altered PWV suggesting that NPR_B and NPR_C do not acutely influence distensibility in vivo.

In chapter 6 we present first results assessing the vasorelaxant properties of a potentially new member of the natriuretic peptide family. Glaxo-Smith-Kline provided us with a small amount of a peptide preliminarily termed NNP. This peptide was isolated from the venom of the green mamba snake (Dendroaspis angusticeps).

According to personal communication (Liang Lee – GSK) the peptide's cDNA sequence revealed classic characteristics for NPs. Interestingly, its carboxy-terminal tail is of the same length as that of DNP but its molecular weight differs (3690 for NNP versus 4190.7 for DNP). Consequently there are two possibilities; Either NNP is indeed a new member of the (serpent) natriuretic peptide family, or NNP and DNP are both derived from an identical precursor ("pre-NNP/DNP") and are only spliced at a different amino-terminal side (analogy to ANP and Urodilatin). Only the publication of the full amino-acid sequence and/or identification of the serpent NNP / DNP gene will clarify this issue and reveal the full significance of the NNP discovery. Whatever the outcome, the data in chapter 6.3. demonstrate that this peptide is roughly equipotent to ANP and DNP in its vasorelaxant properties. The mechanisms of this vasorelaxation will need further study.

7.2. Future work

The most important question arising from a countless number of studies investigating the effects of NPs in heart failure is;

What are the mechanisms of natriuretic peptide resistance and/or tolerance?

The answer to this question will help to explain why, NP-based, treatment strategies have so far failed to provide a tangible success, and may indeed open up new therapeutic avenues.

A further question directly arising from our previous work (437) and chapter 4 is;

What are the reasons for the apparent preservation of natriuretic peptide responsivness in the venous circulation?

Starting from the current understanding of *NP-resistance*, reviewed in chapter 1.3.7., I recently extended our established ovine hind limb model into a pacing induced heart

failure model based on the earlier work of Fitzpatrick and Rademaker (486-488;490;491;493;495). Ethical approval has meanwhile been granted for a large animal study to investigate the mechanisms of natriuretic peptide resistance. Following successful completion of the feasibility pilot studies (all 8 animals survived a left lateral thoracotomy and 6-8 days epicardial pacing [4 animals] I now hope to embark in the main study. All studies will be conducted using the above mentioned model. PWV will be conducted by the foot to foot methodology as described in chapter 5. Additionally iliac artery blood flow will be measured with electromagnetic flow probes in response to local administration of NPs, selective and unselective NPR antagonists, as well as endothelium dependent and independent vasodilating agents. Commercially available radio-immuno assays will be used for NP analysis, including determination of β-ANP sub-fraction. Segments of iliac artery will be harvested (post mortem) from sham operated and paced sheep for characterisation of gene expression of biologically active natriuretic peptide receptors. Gene expression of NPR_{A+B} will be characterised by reverse transcriptase polymerase chain reaction method (279). NPR_C density will be assessed using an immuno- fluorescence method.
In the long term this model could be used to study pharmacological interventions, e.g. with an clearance receptor antagonist.
The study of "signalling abnormalities" down-stream from cGMP-generation will be facilitated by the current arrival and development of agents selectively inhibiting varies sub-fractions of G-proteins.

"Only those who risk going too far can possibly find out how far one can go"

T.S. Eliot

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PUBLICATIONS RELATING TO THIS WORK

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