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BASIS FOR A SYMPATHOLYTIC APPROACH IN THE TREATMENT OF HUMAN HEART FAILURE

by

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THESIS

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For my father
Kailash Chander Aggarwal

DECLARATION

The work presented in this thesis is derived from experiments performed by me in the clinical and pharmacological laboratories of the Alfred and Baker Medical Unit, Alfred Hospital and the Baker Medical Research Institute, Melbourne, Australia. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

[redacted]

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LIST OF PUBLICATIONS

Sections of the work described in the thesis have been published or submitted for publication:

PUBLISHED PAPERS

Aggarwal A, Esler MD, Socratous F, Kaye DM. Evidence for functional presynaptic α_2.adrenoceptors and their down-regulation in human heart failure. J Am Coll Card 2001;37:1246-51.

Aggarwal A, Esler MD, Lambert GW, Hastings J, Johnston L, Kaye DM. Noradrenaline turnover is increased in suprabulbar subcortical brain regions and is related to whole body sympathetic activity in human heart failure. Circulation (In Press).

SUBMITTED PAPER

Aggarwal A, Esler MD, Morris MJ, Lambert GW, Kaye DM. The Effects of low dose Clonidine on Cardiac and Renal Sympathetic Activity and Brain Monoamine Turnover in Human Heart Failure.

SYNOPSIS

Congestive heart failure (CHF) is a progressive disorder, and one that is a major cause of morbidity and mortality in the community. In the last few decades, a neurohormonal hypothesis of disease progression in heart failure has emerged, based upon the findings of several studies that the renin-angiotensin-aldosterone axis, the sympathetic nervous system system, and the hypothalamic-neurohypophyseal system are activated. In addition, there is increased release of endothelin from the vascular bed. The activation of these systems initially serves to maintain arterial pressure and thereby coronary and cerebral perfusion pressures. However, it is now clear that their heightened activity contributes to worsening heart failure and the occurrence of sudden cardiac death. This understanding has resulted in the successful implementation of several strategies to counteract neurohormonal derangement. Our group has previously made several contributions to elucidate the role played by the sympathetic nervous system in heart failure progression, and the aim of this thesis was to further advance these concepts. In so doing, the intent was to further develop strategies for slowing the progression of heart failure and to provide new therapeutic alternatives.

Whilst it is established that the sympathetic nervous system is activated in heart failure, with resultant elevated cardiac and renal spillover of noradrenaline and increased levels of circulating noradrenaline, the processes by which this occurs are not yet fully elucidated. Some of the work presented in this thesis expands on potential mechanisms. In chapter 3, the role of the peripheral α_2-adrenoceptor functionality was addressed. In patients with CHF, my studies showed that these are down-regulated. Specifically, these receptors were seen to be effective in reducing

noradrenaline spillover across the forearm in the healthy subjects, but this sympathoinhibitory function appeared to be lost in heart failure. In chapter 5, my studies were directed at further elucidating the efferent signals responsible for the heightened sympathetic drive in heart failure. Studies utilising bilateral jugulovenous sampling showed a significant elevation of noradrenergic turnover in the suprabulbar subcortical regions of the brain, with central noradrenergic activity positively correlated with peripheral sympathetic activity.

Anti-adrenergic therapy with β-blockers such as carvedilol has become a cornerstone of modern heart failure therapy. In chapter 4, it is argued that such an approach is somewhat limited as it cannot counteract the potentially harmful effects of vasoactive cotransmitters such as neuropeptide Y that are co-released with noradrenaline. A major component of heart failure symptomatology is consequent upon heightened renal sympathetic tone and this is also not antagonised by β-blockade alone. It is therefore also argued that a central sympatholytic approach may be beneficial in this regard. This study was conducted in the aftermath of the prematurely terminated "MOXCON" heart failure trial, which examined the effects of the powerful sympatholytic agent moxonidine. A novel approach to sympathetic attenuation was undertaken in this chapter. Given that the cardiac adrenergic drive is disproportionately activated in heart failure, it was hypothesised that this drive may be preferentially sensitive to a sympatholytic agent such as clonidine, at doses that do not cause significant systemic effects resulting from significant systemic sympathetic withdrawal. It was shown that the heart is more sensitive to the sympathoinhibitory effects of clonidine than is systemic sympathetic drive, and that renal sympathetic tone in heart failure can also be reduced with clonidine. Interestingly, the anticipated

reduction in cardiac neuropeptide Y release was not observed, with baseline cardiac neuropeptide Y levels being lower than previously observed in heart failure patients. The patients described in this study were on carvedilol and, on the basis of pulmonary pressures, were in a state of more optimal control of their heart failure.

In chapter 6, I aimed to evaluate the role of β-adrenoceptor kinase (β-ARK) in the favorable action of Carvedilol on myocardial systolic function. The study was predicated on previous work that shows that β-adrenoceptor kinase (β-ARK) levels are increased in heart failure. In chapter 6, it was hypothesised that in heart failure patients, the expression of β-ARK is increased in peripheral lymhocytes, and that with therapy with carvedilol, β-ARK expression may decrease. No significant change was observed in lymphocytic β-ARK expression in this group of heart failure patients after the initiation of carvedilol.

SUMMARY

Studies presented in this thesis make significant new contributions to the understanding of the role played by the sympathetic nervous system in congestive heart failure. The chief observations are that in heart failure, the heightened sympathetic drive arises from the suprabulbar subcortical regions of the brain; that peripheral sympathoinhibitory α_2-adrenoceptor functionality is down-regulated; that sympatholytic treatment at low doses can target the heart whilst also effective in attenuating renal sympathetic tone, and that this therapeutic approach is probably synergistic with anti β-adrenergic therapy.

ADDENDUM

PUBLISHED PAPERS

Aggarwal A, Esler MD, Morris MJ, Lambert G, Kaye DM. Regional sympathetic effects of low dose clonidine in heart failure. Hypertension (In Press).

2.5.2a Non-compartmental approach of radioisotope dilution methodology

A particular criticism of the radioisotope dilution methodology has been its assumption that plasma noradrenaline kinetics is non-compartmental. This approach assumes that all newly released noradrenaline enters into and leaves from an accessible compartment. However, it is known that only 10-20% of the NA released into the neuroeffector junction "spills over" into the circulation. Both the neuroeffector junction into which NA is released and the post-ganglionic neurone into which it is taken back are sites which are inaccessible to sampling. Therefore, it is argued that because NA has multiple sites for production and removal that are remote from the sampling site (ie the plasma pool), the radio-isotope dilution methodology, using a non-compartmental model, is an oversimplification of noradrenaline kinetics. This contention is strengthened somewhat by the observation that the disappearance of [^{3}H] NA from plasma in humans is at least bi-exponential, indicating there are at least two compartments into which NA is distributed (1).

To address these theoretical considerations, Linares et al (2) have proposed a compartmental approach to the study of noradrenaline kinetics in man. In this two-compartment model, compartment 1 represents the vascular compartment, which is therefore accessible for sampling. Compartment 2 represents a "lumped" compartment that is extravascular and inaccessible for sampling, with contributions from the heart, kidneys and gut that are available for neuronal reuptake. The principals of this methodology are to again employ an intravenous infusion of [^{3}H]-NA with multiple arterial blood samples to provide a two-compartment modeling analysis of NA kinetics.

The NA kinetic parameters estimated by this model include the rates of appearance of noradrenaline into compartment 1 and 2, the metabolic clearance rate from compartment 1, the NA spillover fraction from compartment 2 to 1, and the volume of distribution of NA in compartment 1. It has been proposed that a non-compartmental approach to the estimate of NA appearance into the circulation, as offered by radio-isotope dilution methodology, underestimates the rate of NA release into the extravascular space [denoted as NA_2], as estimated by a two-compartment model(3).

Therefore, it is argued that a two-compartment approach gives a better measure of global sympathetic activity.

However, despite these limitations of the non-compartmental approach inherent in radio-isotope dilution methodology, this technique has been well-validated. Unlike the two-compartment model, where a correlation between NA_2 and actual measures of sympathetic nerve activity (such as can be obtained from nerve traffic recordings) has not been as yet shown, the relationship between regional NA spillover into the circulation and regional sympathetic nerve activity is well-established. In addition, total overflow of NA to plasma as measured by radio-isotope dilution methodology correlates well with the sum of regional NA spillover into the central pool, when measured individually (4).

3.5.6A Further discussion

In considering the results of this component of the thesis, it is of relevance to consider other factors that may also exert influence on the role of potentially regulatory pre-synaptic receptors. Of some relevance to this study is the concept of "accentuated antagonism", which has been variously applied to issues related to the functional status of pre-synaptic receptors at given levels of nerve firing, and separately to the effects of sympatho-vagal interaction. In relation to the functional role of pre-synaptic alpha-2 adrenoceptors, Grossman et al demonstrated in two separate experiments (5,6) that intravenous and regional yohimbine, an α_2-adrenoceptor antagonist, resulted in an increase in forearm NA release for a given amount of sympathetic nerve traffic in the human limb at rest. This result may relate to the abolition of the 'auto-inhibitory' effect of local release catecholamines at the alpha-2 adrenoceptor or possibly as a result of changes in the expression or functional coupling of the alpha-2 adrenoceptor. Hence, in the present study it is also conceivable that some of the differences in response to clonidine were mediated by different levels of nerve traffic. The extent to which this is likely however, is somewhat limited, by virtue of the fact that no difference in baseline forearm spillover of noradrenaline was detected and in other studies in well-controlled patients, the increase in muscle sympathetic nerve activity is relatively modest (7).

In relation to the possible effects of sympathovagal interaction, Levy et al (8) observed that, in the left ventricle of the dog, the effects of vagal stimulation at low levels of sympathetic drive were not particularly strong. However, the vagal effects were enhanced during sympathetic activation. This phenomenon of increasing vagal effect with increasing sympathetic drive has been termed "accentuated antagonism". The relevance of this observation to the periphery however, is less clear as most observations on this phenomenon have related to myocardial control.

Of direct relevance to the findings of this study is an earlier study by Parker et al(9). In this earlier study, phentolamine, a nonselective α-adrenergic antagonist, was given as an intracoronary infusion to patients with heart failure and to a control group with normal left ventricular systolic function. Phentolamine caused a significant increase in left ventricular inotropic and lusitropic performance in the failing heart, and that this was accompanied by an increase in the venous-arterial gradient for NA across the failing heart. The conclusion from this study was that the positive functional effects were mediated by increased release of cardiac NA secondary to blockade of presynaptic α_2-adrenergic receptors, and that these receptors were functional in the failing heart. The two studies would therefore appear to be contradictory in their findings.

The limitations of the current study are

- a relatively small sample size,
-measuring NA kinetics in the forearm rather than in the heart, with the possibility that regional differences in receptor regulation may exist, and
-relatively wide confidence intervals for NA spillover and appearance rate in the patient group, compared to the control group.

The limitations of the earlier phentolamine study were that

- coronary blood flow was not measured. It is apparent from previous discussion that regional blood flow is a major determinant of regional NA spillover. The effects of phentolamine on coronary blood flow are controversial. Gould et al (10) showed that *intravenous* phentolamine increased coronary blood flow in patients with a recent myocardial infarct. In a later study in *unstressed humans*, Hodgson et al (11) found no change in coronary vascular resistance with intracoronary phentolamine

- radiotracer methodology was not applied. The advantage of this methodology is that it recognises that noradrenaline flux is bidirectional, with extraction and release occurring concurrently. It therefore allows for and measures changes in tissue extraction of NA. Extraction of NA can be altered by changes in blood flow, as may be effected by phentolamine.

In addition, the possibilty of inconsistent sample from versus non-muscular beds in the forearm has to be acknowledged. A future study amalgamating the methodologies of these two studies may be worthy of consideration. Such a study may consist of the administration of intracoronary phentolamine, with measures of coronary blood flow and cardiac spillover of NA as measured by radiotracer kinetics.

3.6.B Conclusions

The propranolol arm of the study was incorporated into the clonidine study as reported in the first section of this chapter. It was found that the addition of this intervention resulted in a greatly prolonged study time and therefore in patient discomfort. This led to the decision to discontinue this facet of the study into presynaptic receptor function in heart failure. A future study may be undertaken to specifically study the effects of intra-arterial propranolol upon forearm NA release. Therefore the findings presented here really represent a work in progress.

4.5.1 Further discussion

Whilst clonidine has complex neurohormonal effects, in CHF (as discussed in Chapter 3), it appears to act predominantly via stimulation of sympatho-inhibitory α_2-adrenergic and/or imidazoline receptors in the central nervous system. In addition, it appears to have effects on regional blood flow, presumably by a post-junctional effect on α_1-adrenoceptors.

In a previous study, Azevedo et al (12) demonstrated that intravenous clonidine resulted in impressive sympatholysis in heart failure patients, both in terms of cardiac and global effects. In this study, two doses of clonidine at 50 and 100μg were administered at 20 minute-intervals. No change in left or right heart filling pressures were noted with the two doses of clonidine. A significant reduction in left ventricular inotropic and lusitropic responses was demonstrated with clonidine.

In the present study, an attempt was made to extend these observations. The effects of clonidine on the filling pressures of the heart were not measured in this study in part because the prior study did not demonstrate any changes, but also because such a measurement would have significantly added to the complexity and the duration of an already complex study. In addition, the present group of patients were a well treated group with normal resting mean pulmonary capillary wedge pressures. There are several novel aspects to the present study.

Firstly, the effects of clonidine on renal sympathetic activity in CHF are largely unstudied. The findings suggest that clonidine has a renal sympatholytic effect, but that this is accompanied by a reduction in renal blood flow. Intuitively, a reduction in renal blood flow would not be a desirable outcome in CHF. A more comprehensive assessment of the renal effects of intravenous clonidine should include measurement of the glomerular filtration rate. As is the case with the undesirable cardiac effects of acute clonidine administration as described by Azevedo et al, and for that matter, with acute β-blocker administration, there may be undesirable renal function effects with acute clonidine administration. These, as is the case with chronic β-blocker

administration, may be different from those effected by long- term administration. In order to address some of these issues, we are currently engaged in a study involving chronic oral dosing with clonidine and its effects on renal sympathetic function and glomerular filtration rate.

A particular second novel aspect of the present study that may have implications for the future therapy of CHF is that this is the first study to examine the effects of a central sympatholytic approach in a group of patients already on carvedilol. The findings of this study indicate that this combined therapeutic approach is safe and may be synergistic.

Another future study that is suggested by the findings of the present study would be to investigate the effects of clonidine on neuropeptideY release by the heart in CHF. Such a study would have to be performed on patients before they are stabilised on carvedilol.

5.5. Further discussion

With respect to central noradrenergic responses to SNP, cortical NA turnover appeared to increase in CHF, with the p value just failing to reach significance ($p=0.05$). It is difficult to be sure of the robustness of this finding. Given the difficulties in controlling haemodynamic responses to SNP in this study, it seems more prudent to not make any definitive conclusions from this arm of the study.

5.6 Further conclusions

Clearly from the present human series of studies, the presence or absence of any primary central abnormalities that lead to heightened central sympathetic outflow cannot be determined. It is also plausible that abnormal afferent inputs appear to regulate this augmentation of sympathetic outflow in heart failure. From this study, and those previously performed by our group, it is becoming increasingly evident that a high level of spatial heterogeneity in terms of sympathetic outflow and the relevant controlling mechanisms exists both under normal conditions and in the presence of heart failure.

6.6 Further conclusions

This study is presented here as a work in progress. It is being included for presentation because it does establish a methodology for studying β-ARK levels in peripheral monocytes of CHF patients. The findings, as reported here, are that carvedilol did not effect a significant difference in β-ARK levels, and that it was not possible to conclude that changes in β-ARK levels in individual patients were predictive of change in left ventricular systolic function.

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Chapter 1

Heart Failure:

General Introduction and Overview

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1.1 General overview

Congestive heart failure (CHF) is a major health problem in the developed world, being responsible for major morbidity and mortality in the community. It is a condition that principally affects the elderly, and with the progressive aging of the population, it seems highly likely that its prevalence will continue to increase. The increase in prevalence has occurred despite a reduction in age-standardised mortality associated with cardiovascular diseases such as myocardial infarction and stroke (Bonneux et al, 1994). Factors responsible for this may, in addition to the ageing of the population, include decreased mortality rates following myocardial infarction, and more frequent use of investigations such as echocardiography to detect left ventricular systolic dysfunction (McGovern et al., 1996).

The clinical syndrome of CHF has an estimated prevalence of approximately 1% in the general population, and perhaps as high as 10% in those older than 75 years (Dargie et al, 1994). Age-adjusted hospital admissions for heart failure continue to rise by about 10% per annum, particularly in patients older than 65 years (Cleland et al, 1998). As a result, the cost of management of heart failure is high with the estimate across Europe being about £530 million per million adult population, with similar estimates in the USA (Cleland et al, 1998). In addition, the annual mortality of new-onset heart failure in the community still exceeds 25% (Cowie et al, 2000), exceeding mortality rates associated with many cancers.

During the past half-century, the causes of heart failure have changed considerably. In addition, major advances have been made in the understanding of the pathophysiology of CHF. These advances have led to major shifts in therapy, with newer strategies beginning to demonstrate reductions in both the need for hospitalisations and in mortality.

1.2 Definition of heart failure

A major stumbling block to the acquisition of accurate epidemiological data has been the lack of a uniform definition for heart failure. It is a clinical syndrome that develops as a consequence of cardiac disease, and is recognised clinically by a constellation of symptoms and signs produced by complex circulatory and neurohormonal responses to cardiac dysfunction. However, the sensitivity and specificity of clinical symptoms and signs is low (Cowie et al, 1997). While echo cardiography and radionuclide ventriculography provide measures of the systolic function of the heart (and in the case of the former also diastolic function), they do not determine the presence or absence of the clinical syndrome of heart failure. For example, the existence of both asymptomatic left ventricular systolic dysfunction (SOLVD, 1992) and heart failure resulting from pure diastolic dysfunction are established (Grossman, 1991).

Recognising these limitations, it is still possible to have a working definition of CHF that identifies a syndrome that is progressive and debilitating. Most diagnostic difficulties arise in individual cases where the syndrome is mild. In the guidelines issued by The Task Force on Heart Failure of the European Society of Cardiology, both symptoms and objective evidence of cardiac systolic dysfunction need to be present to satisfy a diagnosis of heart failure (European Society of Cardiology, 1995). The World Health Organisation has issued guidelines for assessment of possible heart failure patients (Table 1), with the recommendation that patients suspected of having CHF based on these criteria have further investigations (WHO, 1997).

Table 1: Modified World Health Organization criteria for assessment of possible chronic heart failure

Symptoms: Dyspnoea, chronic fatigue, oedema, and exercise intolerance.

Signs: Third or fourth heart sounds, heart murmur, cardiomegaly, pulmonary crackles, raised jugular venous pressure, and dependent oedema.

Causative factors: Angina, previous myocardial infarction, hypertension, valvular heart disease/rheumatic fever, and cardiomyopathy.

Patients were considered to have possible CHF if they had:

> 2 symptoms,
> 2 signs,
> 1 symptom and > 1 sign, or
> 1 symptom and > 1 causative factor.

Reproduced from (WHO, 1997)

1.3 Aetiology, Incidence and Prevalence of heart failure

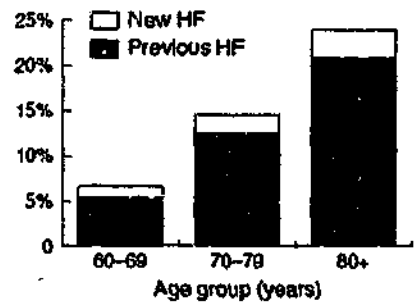
Heart failure is the common end-result of many different disease processes that impair cardiac function. Hypertensive and valvular heart diseases were the most frequent causes of heart failure in Western nations in 1950. Currently, ischaemic heart disease, hypertensive heart disease, and idiopathic dilated cardiomyopathy are dominant.

Epidemiological information regarding the incidence of heart failure has been obtained from two basic approaches: re-examining individuals within a cohort at intervals to identify those who have developed heart failure; or by a population-based surveillance system in which subjects developing heart failure for the first time are identified. The Framingham Heart Study (Ho et al., 1993) and the Study of Men Born in 1913 in Sweden (Eriksson et al., 1989) are good examples of the first approach. The Finnish Study, the Rochester study and the United States Two Counties Study (Cowie et al., 1997) are examples of the second approach. In these studies, the crude incidence (unadjusted for age) in the general population ranges from 1.0 to 5.0 cases

per 1000 population per annum, with a steep increase with advancing age. The incidence rate for those aged over 75 years is reported to be as high as 40 cases per 1000 population per annum in some studies (Cowie et al., 1997).

Prevalence estimates vary widely from study to study, reflecting the differences in methodology rather than true differences between populations. The crude prevalence (unadjusted for age) ranges from 3 to 20 individuals per 1000, with a prevalence of 30 to 130 individuals per 1000 for those aged over 65 years (Cowie et al., 1997). The epidemiology of CHF in Australia has in the past been assumed to be similar to that in the US and Europe, and it is only recently that local national data has emerged (Krum et al., 2001). In the recently published Cardiac Awareness Survey and Evaluation (CASE) Study, a general practice based study focusing on patients older than 60 years, a higher prevalence rate than above of 13.2% was reported (Krum et al., 2001). Prevalence of CHF in the CASE study increased dramatically with age, with more than 20% of patients aged 80 years or older diagnosed with CHF (Figure 1.1).

Figure 1.1 Percentage of patients with chronic heart failure in each group



Adapted from [Krum, 2001 #218]

1.4 Pathophysiology of Heart Failure

1.4.1 Neurohormonal activation

In recent times, major gains have been made in the understanding of the pathophysiology of CHF, such that it is now regarded as a syndrome characterised by neurohormonal derangement rather than as a purely haemodynamic disorder. Studies in the early 1960s demonstrated the presence of increased concentrations of circulating norepinephrine in heart failure (Gaffney et al., 1962). A large number of investigations on other neurohormonal changes in CCF followed, revealing activation of the renin-angiotensin-aldosterone system, the hypothalamic-neurohypophyseal system with release of vasopressin, the release of endothelin from the vascular bed and of the natriuretic peptides from the heart (Packer et al., 1992; Chatterjee et al., 1996; Eichhorn et al., 1996b). The activation of these systems, which results in vasoconstriction and sodium-retention, initially is beneficial by maintaining arterial pressure and blood volume. However, it has become clear that persistent activation is maladaptive in chronic heart failure. A prominent clinical example of this is the patient who is initially well compensated after extensive myocardial infarction, only to return 6 to 9 months later in decompensated CHF, without clinical or angiographic evidence of further infarction. In the prevention arm of the SOLVD study, 30% of the initially asymptomatic patients with left ventricular systolic dysfunction in the placebo arm developed CHF over 37 months (SOLVD, 1992).

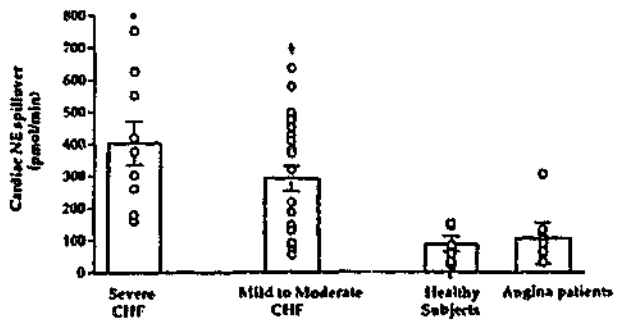
The initial insult to pump function may be from myocardial infarction, inflammation, haemodynamic overload from hypertensive or valvular disease or from genetic causes. Regardless of the nature of the insult, similar compensatory mechanisms are activated to support the failing heart. The signals responsible for entraining some of these mechanisms are yet to be clearly defined. The major concern of this thesis is the

role of the sympathetic nervous system in the progression of congestive heart failure. It is now established that SNS stimulation is commonly present in patients with heart failure to the detriment of the failing myocardium (Cohn et al., 1984; Hasking et al., 1986), predisposing even in mild failure to lethal ventricular arrhythmias (Meredith et al., 1991). In addition, increased cardiac adrenergic drive precedes generalized sympathetic activation (Rundqvist et al., 1997). As presented in Figure 1.2, Rundqvist et al found that cardiac NA spillover in patients with mild-to-moderate CHF was increased threefold versus that in healthy subjects, whereas total body and renal NA spillover and muscle sympathetic activity did not differ from those in healthy subjects.

There is an emerging understanding of the detrimental biological effects that excess catecholamine exposure has on the myocyte (Mann et al, 1992). In the studies presented, there is an emphasis on the reflexes responsible for the heightened adrenergic drive that exists in CHF, and on their possible manipulations. A particular focus is on further understanding the role of the central nervous system and whether centrally acting sympatholytic drugs could potentially have therapeutic value in heart failure.

Figure 1.2 Cardiac Adrenergic Drive

Adapted from [Rundqvist, 1997 #158], where NE is noradrenaline



1.4.2 Myocardial biology of heart failure

In the last 10 years there has been a heightened focus on research into the pathobiology of the cardiac myocyte. Specifically, the aim of this work has been to explain the progressive nature of CHF, and consequently to lead to new therapeutic insights. For some time, it has been postulated that exposure to high levels of catecholamines might be toxic to myocytes. Mann et al (Mann et al., 1992) advanced this view by demonstrating that noradrenaline (NA) exerts a direct toxic effect on cardiac myocytes in vitro, and that this effect is mediated by β-adrenergic receptor stimulation and involves increase in cAMP and calcium influx (Colucci et al., 1996).

Apoptosis, or programmed cell death, is a central feature of normal tissue development in the fetus and of cell replacement in certain adult tissues, eg. the thymus. Histologically, cells undergoing apoptosis show several characteristic features, including blebbing of the cell membrane, a reduction in cell volume, and condensation of nuclear chromatin (Steller et al., 1995). As apoptosis is most often associated with cells that are progressing through the cell cycle, it has been generally believed that this process does not occur in terminally differentiated adult cells such as cardiac myocytes. However, there has been a recent report of apoptosis occuring in the heart in the setting of dilated cardiomyopathy (Narula et al., 1996). Therefore, it is possible that reduced myocardial function in the setting of a cardiomypathy is in part due to apoptosis (Colucci et al., 1996).

Relatively little is known about the stimuli that initiate the program of apoptosis. However, Communal et al (Communal et al., 1998) report that noradrenaline, acting via the β-adrenergic pathway, stimulates apoptosis in adult rat cardiac myocytes in vitro. The authors therefore hypothesise that the high adrenergic tone that exists in CHF stimulates apoptosis of cardiac myocytes and that this contributes to the

progression of heart failure. In further experiments on adult rat ventricular myocytes, Communal found that stimulation of β_1-adrenoceptors increases apoptosis via a cAMP-dependent mechanism, whereas stimulation of β_2-adrenoceptors inhibits apoptosis via a G_i-coupled pathway (Communal et al., 1998).

1.5 Treatment and Prognosis of heart failure

In 1971, McKee et al noted that the clinical course and prognosis of CHF were "surprisingly grim and not much better than those for cancer in general"(McKee et al., 1971). Death in CHF principally results from either worsening pump failure or from an arrhythmia. In the Framingham Heart Study subjects, a median survival of 1.7 years in men and 3.2 years in women was observed, with no significant temporal change in the prognosis of CHF during the 40 years of observation (Ho et al., 1993). This is in contrast to the declining trends for coronary heart disease mortality (Goldman et al., 1984).

Previously, heart failure had been seen as the mechanical failure of the heart to maintain systemic perfusion commensurate with the demands of metabolising organs. Accordingly, early pharmacological approaches were designed to increase the performance of weakened heart muscle. Between 1986 and 1993, multiple clinical trials were conducted with positive inotropic and vasodilator agents to improve systolic performance. However, with the exception of one trial (V-HeFT), examining the benefits of treatment with vasodilators hydralazine and isosorbide dinitrate, these all resulted in worsening of the natural history of CHF (Cohn et al., 1986).

An understanding of the neuroendocrine activation in CHF has led to a major shift in our approach to treatment, from merely treating symptoms with diuretics and positively inotropic agents, to attempting to curtail the disadvantageous effects of

these neurohormonal derangements with agents such as β-adrenergic antagonists (Packer et al., 1996), angiotensin converting-enzyme inhibitors (CONSENSUS, 1987) and aldosterone antagonists (Pitt et al., 1999). Commensurately, there have been major advances made in the understanding of the biology of the myocyte. Myocardial failure, from being considered as an irreversible and relentlessly progressive process, can now be regarded as a dynamic pathophysiological entity with opportunities to manipulate it to achieve more favorable outcomes (Eichhorn et al., 1996) . This change is reflected in a current therapeutic approach that has made important in-roads into reducing both morbidity and mortality resulting from CCF. With respect to the latter, it has been estimated that there has been a 46% reduction in mortality in the last 10 years in heart failure clinical trials (Bristow et al., 2000).

1.5.1 Rationale for β-blocker therapy in heart failure

Long-term infusions of noradrenaline (NA) in animals produces a cardiomyopathy, characterised by cardial hypertrophy, necrosis, and fibrosis (Schenk et al., 1966). In phaeochromocytoma, a state of excess NA production, a cardiomyopathy is well described in humans (Imperato-McGinley et al., 1987). In this context, increased sympathetic activity in human heart failure has been closely related to mortality and need for cardiac transplantation (Cohn et al., 1984; Kaye et al., 1994). Combining all these observations, it seems logical to attempt sympathoinhibitory treatment in CHF in order to favorably alter prognosis.

The failing human heart is adrenergically activated and this has been ascribed a key role in maintaining cardiac performance over the short term by increasing contractility. Traditionally, therefore, adrenergic blockers have been considered to be contraindicated in heart failure because of their acute negative inotropic effect. As

shown in Table 2, there are 3 adrenergic receptors (β_1, β_2, and α_1) expressed in human cardiac myocytes that are coupled to a positive inotropic response and cell growth (Bristow, 2000). β_3-adrenoceptors are also present and functional in the human heart; and these receptors may be present as a counterregulatory receptor coupled to the "inhibitory" G protein G_i (Gauthier, Tavernier, Charpentier, Langin, & Le Marec, 1996).

In nonfailing human left or right ventricles, the β_1/ β_2 ratio is 70 to 80/30 to 20, but in failing human ventricles, 35% to 40% of the total number of β-receptors are β_2 because of selective down-regulation in the β_1 subtype (Bristow et al., 1982). Because α_1-receptors are upregulated in the failing heart, the cardiac myocyte adrenergic receptor profile changes from predominately (>70% of the total adrenergic receptor population) β_1 to more of a mixed 2:1:1 ratio in end-stage heart failure.

Noradrenaline (NA) is mildly β_1–receptor selective, having an affinity to this receptor that is 10- to 30- fold compared with the binding affinity to β_2-receptors, and its cytotoxicity appears to be mediated through β-rather than α-adrenergic receptors (Communal et al., 1998). In addition to changes in receptor number in the failing heart, there is also a down-regulation in adrenergic receptor functionality. In the end-stage failing heart, β-adrenergic signal transduction is reduced by 40-50% (Bristow, 2000).

Therefore, the continuously increased adrenergic drive that exists in the failing heart leads to the delivery of adverse biological signals to the cardiac myocyte, and this forms the basis for the use of antiadrenergic agents in the treatment of CHF.

Table 2. Biological Responses Mediated by Adrenergic Receptors in the Human Heart

Biological Response	Adrenergic Receptor Mediation
Cardiac myocyte growth	$ß_1$, $ß_2$, α_1
Positive inotropic response	$ß_1$, $ß_2$, α_1 (minimal)
Positive chronotropic response	$ß_1$, $ß_2$
Myocyte toxicity	$ß_1$, $ß_2$ (?<$ß_1$)
Myocyte apoptosis	$ß_1$

Reproduced from (Bristow, 2000)

1.5.2 Clinical Trials with β-blockade

Propranolol is the prototype non-selective β- blocker that was introduced into clinical practice in 1968 as an anti-anginal agent. In the 1970s, pharmaceutical companies developed "cardic-selective", or second-generation, β-blockers that were relatively β_1 – selective. Examples of this class are metoprolol and bisoprolol. In the 1980s, another drug development effort aimed at improving the tretament of hypertension led to the creation of β-blockers with vasodilating activity. The prototype of this "third-generation" class of compounds is labetolol, possessing both β- and α_1 – blocking capabilities. A more recent development in this class is carvedilol.

Since the recognition of the elevated circulating plasma noradrenaline levels in CHF, there have been attempts to trial β-adrenergic blockers in CHF, but these studies had until recently involved relatively small number of patients (Currie et al., 1984,

Massie, 1998; Doughty et al., 1994). The first placebo-controlled multicenter trial with a β-blocking agent was the Metoprolol in Dilated Cardiomyopathy (MDC) trial (Waagstein et al., 1993). Although this trial failed to demonstrate a significant mortality benefit, there were promising trends and this led to the Metoprolol CR/XL Randomised Intervention Trial in Congestive Heart Failure (MERIT-HF) (MERIT-HF, 1999). This trial and the subsequent CIBIS-II trial, with the drug bisoprolol, showed a >30% mortality benefit (CIBIS, 1999).

Carvedilol, a non-selective β-blocker with α-blocking activity and antioxidant properties (Yue et al., 1992), is currently approved for treatment of chronic heart failure in the USA and most other countries. Its use stems from the findings of the U.S. Carvedilol study, involving 1094 patients in total (Packer et al., 1996). Over an average of 68 months of follow-up, a substantial decrease in mortality of 65% was demonstrated in the treatment arm. The striking mortality benefit was derived from both reductions in sudden death and in death from worsening heart failure, and was associated with an improvement in the systolic function of the heart. The findings of this trial are largely responsible for the prominent role that β-blocker therapy now plays in the management of CHF.

Potential mechanisms that may explain the improvements in cardiac contractility and survival with β-blocker therapy are discussed in more detail below.

1.6 Measures of sympathetic nervous system activity and their prognosic value

The sympathetic nervous system (SNS) plays an integral part in the acute maintenance of cardiovascular homeostasis in disease states such as heart failure and hypertension. Over time, several methodologies have been developed to measure adrenergic activity.

1.6.1 Microneurography

Hagbarth and Vallbo (Hagbarth et al., 1969) devised clinical electrophysiological methods for studying nerve firing in subcutaneous sympathetic nerves and this technique was further applied by Wallin et al (Wallin et al., 1981). Subsequently, Leimbach et al (Leimbach et al., 1986) obtained direct evidence from intraneural recordings (from the peroneal nerve) for increased central sympathetic outflow in patients with heart failure. They were able to show a significant correlation between plasma NA levels and sympathetic nerve firing rates. However, microneurography is time consuming and may be uncomfortable for the patient, and therefore is relatively limited in its clinical application. Secondly, the nerves to internal organs are not accessible for such testing, and therefore the sympathetic activity of organs such as the heart can not be assessed.

1.6.2 Biochemical measures

Noradrenaline (NA) is both the principal neurotransmitter of the mammalian sympathetic nervous system and a major transmitter within the central nervous system. Since the identification of its role by von Euler in 1946 (Von Euler, 1946), there have been a variety of techniques developed to measure NA and, thereby, sympathetic activity. By 1954, urinary NA measurements had been used as a measure of sympathetic nervous activity. Subsequently, further clinical methods were devised, including measures of tissue NA concentration and turnover and the concentration of NA in the synaptic space (Esler et al., 1988). These methods had major limitations to widespread clinical use.

The development of a sensitive plasma assay for NA by Engleman et al (Engleman K, 1968) represented a major advance in this biochemical approach. The observation that plasma NA is increased in heart failure was the first step in the formation of the current consensus that sympathetic nervous overactivity plays a crucial part in the pathophysiology of the disorder. Cohn et al found that whereas patients with resting plasma NA of less than 400 pg/ml had greater than 60% four-year survival, NA levels of greater than 800 pg/ml were associated with less than a 20% two-year survival rate (Cohn et al., 1984).

In terms of the origin of plasma NA in the healthy human, it is now understood that the sympathetic nerves of the kidneys and skeletal muscles each contribute ~25% of the total. The sympathetic innervations of the heart, skin, gastro-intestinal tract, and liver each are responsible for <10% of the total plasma NA appearance rate (Esler, 1990).

Nevertheless, plasma NA is an inadequate guide to either total or regional sympathetic activity for two reasons. The plasma concentration of NA is determined by the combined influences of the release from sympathetic neurons and of the rate of removal from plasma. Indeed, in CHF the elevation in peripheral plasma NA has been shown to represent the effect of increased rates of NA spillover from nerves and of reduced clearance from plasma, by virtue of reduced cardiac output. A second difficulty is the inability to estimate sympathetic activity in internal organs such as the heart and kidneys. This is of importance since sympathetic nervous responses are not generalised but organ specific.

These limitations of plasma NA have led to the development of a radioisotope dilution methodology (Esler, 1979) that has allowed for measurements of specific organ and total body sympathetic activity. Regional NA spillover is a legitimate

method of studying regional sympathetic nervous activity, as the rate at which the NA released from sympathetic nerves "spills into" the venous drainage of individual organs is usually proportional to their rate of sympathetic nerve firing. In implementing this methodology, it has become clear that the high circulating plasma NA in heart failure derives from excessively heightened cardiorenal sympathetic activity (Hasking et al., 1986).

1.6.3 Cardiac noradrenaline concentration

A central paradox in the study of cardiac sympathetic activity in CHF has been the repeated observations of increased cardiac NA spillover in the presence of depleted cardiac NA stores (Chidsey CA, 1963), (Petch et al., 1979; Meredith et al., 1993). These observations combined led, for some time, to the mistaken view that a functional cardiac sympathetic denervation existed in CHF.

The rate at which NA escapes from the neuroeffector junction and appears in coronary sinus plasma is dependent on several factors, including the rate of neuronal release of NA and the capacity for neuronal reuptake. If neuronal reuptake is diminished, cardiac NA spillover would increase without any concomitant increase in cardiac sympathetic nerve activity. Conflicting evidence is available to support both normal (Hasking et al., 1986; Meredith et al., 1993) and reduced (Rose et al 1985; Kaye et al., 1994) neuronal uptake of NA by the cardiac nerves. In the most comprehensive study to date, Eisenhofer et al (Eisenhofer et al., 1996) demonstrated that increased neuronal release of NA and decreased efficiency of NA reuptake both contribute to increased cardiac adrenergic drive in CHF, and these processes combined are also responsible for depleted cardiac NA stores observed in CHF (Brunner-La Rocca et al., 2001). Further evidence for the contention that NA reuptake

is reduced in CHF, is the observation that patients have significantly reduced myocardial retention of I-123 Metaiodobenzylguanidine, a compound that appears to share common uptake and storage mechanisms with NA (Henderson et al., 1988).

1.7 Sympathetic nervous system

The SNS can be compartmentaized into a central organisation within the central nervous system (CNS) and a peripheral component. Control of sympathetic efferent nervous activity is achieved through interrelated neuronal cell groups located in the brainstem and hypothalamus. Evidence exists that the release of central nervous system monoaminergic neuronal transmitters (adrenaline, NA and serotonin) is increased in human heart failure, and that the apparent degree of activation of aminergic brain neurons is linked to the magnitude of peripheral (Lambert et al., 1995a) and cardiac (Kaye et al., 1994) sympathetic nervous stimulation present.

1.7.1 Peripheral organisation

The SNS is organised at a spinal and peripheral level such that cell bodies within the thoracolumbar sections of the spinal cord provide preganglionic efferent innervation to postsynaptic sympathetic neurons that reside in ganglia dispersed in three arrangements: paravertebral, prevertebral and previsceral ganglia (Hamill, 1996). Paravertebral ganglia are paired structures which are located bilaterally along the vertebral column and extend from the rostrally situated cervical ganglia to ganglia located in the sacral region. There are 3 cervical ganglia (the superior, middle and inferior cervical ganglia), 11 thoracic ganglia, 4 lumbar ganglia, and 4 or 5 sacral ganglia. The inferior cervical ganglion is usually fused with the first thoracic ganglion, and this structure is known as the stellate ganglion.

The outflow from the spinal cord to peripheral ganglia is segmentally organised in a rostral-caudal pattern. Therefore, the superior cervical ganglia (SCG) receives innervation from spinal segments T1 to T3; stellate ganglia, T1-T6, and so on. Similarly, the distribution of postsynaptic fibers also follows a regional pattern with

the head, face and neck receiving innervation from the cervical ganglia (T1-T4), the upper limb and thorax from the stellate and upper thoracic ganglia (T1-T8) and so on. With the aid of transneuronal tracing techniques using, for example, pseudorabies virus, it has become possible to trace connections between these peripheral ganglia and central autonomic organisation. For example, following injections in either the SCG or stellate ganglia, the following brain areas are labeled: ventromedial and rostral ventrolateral medulla, caudal raphe nuclei, A5 noradrenergic cell group, and the paraventricular nucleus of the hypothalamus (Strack et al., 1989).

The postganglionic fibers in the SNS originate from projections from these central nuclei to the spinal preganglionic neurons and travel quite lengthy paths to arrive at target organs. The target organs of sympathetic neurons include smooth muscle and cardiac muscle, and parenchymal organs such as the kidney. The fibers to the heart originate from the superior, middle and inferior (or stellate) ganglia.

At the autonomic neuroeffector junction, the interface between the postganglionic fiber and the target organ, the postganglionic fibre becomes beaded with varicosities. These varicosities are packed with mitochondria and vesicles containing various transmitters, as discussed below. The autonomic neuroeffector junction is not a well defined synaptic structure, and the lack of a restrictive synaptic arrangement permits the released neurotransmitter to diffuse various distances, thereby expanding the overall effect of sympathetic activation. The complex nature of this junction is discussed in more detail below. The main neuronal phenotype in peripheral sympathetic ganglia is the noradrenergic neuron, with 80-95% of ganglion cells staining positively for tyrosine hydroxylase, the enzyme catalyzing the rate-limiting step in catecholamine biosynthesis (Hamill, 1996).

1.7.2 Central autonomic organization

Central autonomic regulation is achieved through interrelated neuronal cell groups located in the brainstem. The most commonly regarded centrally occurring

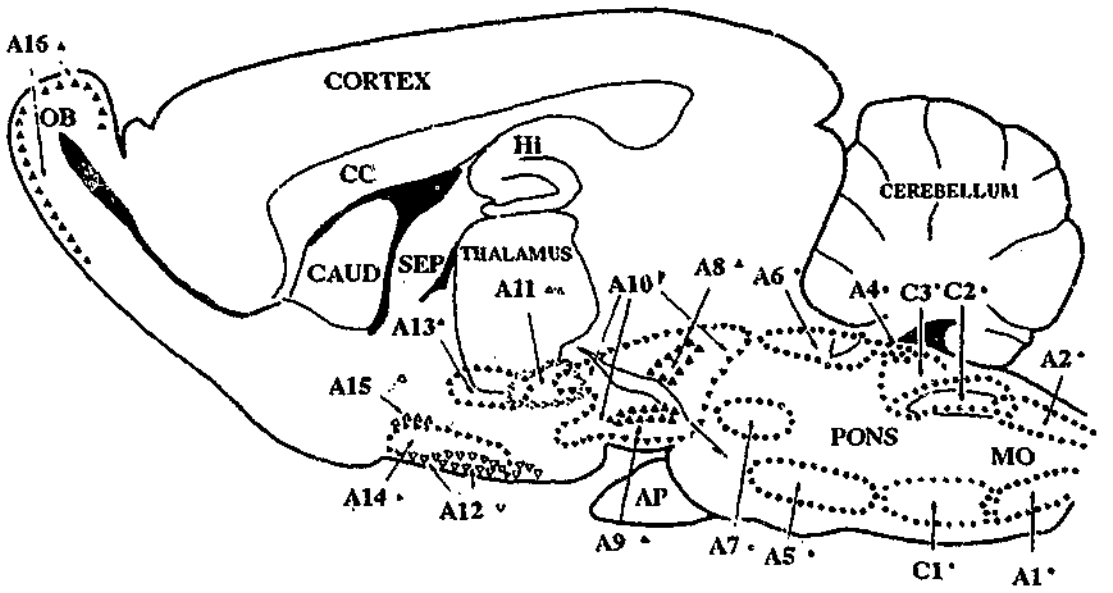
neurotransmitters can be broadly divided into four groups: amines, peptides, amino acids and others. Emphasis here will be placed on those neurons using the monoamine noradrenaline as their neurotransmitter.

Most studies concerning the mapping of monoamine neurotransmitter systems have been carried out using the rat brain. There exist 15 catecholaminergic neuronal groups in the reticular formation, comprising approximately 50,000 neurons. Of these, around 80 % utilise dopamine as their neurotransmitter and 20 % use noradrenaline (Hokfelt et al., 1984). A schematic overview of the distribution of the catecholaminergic neuronal groups in the rat brain is shown in Figure 1.3 (Hokfelt et al., 1984).

The cell bodies of the noradrenergic groups, designated A1-7, are confined to medullary and pontine parts of the brainstem (Figure 1.3) but exhibit complex ascending and descending projections in addition to local destinations in the brainstem. The more caudally located cell groups (A1-A5) are found in the ventrolateral portion of the pons (A5) the medulla oblongata (A1) and the nucleus tractus solitarii (A2). The majority of brain noradrenaline is located in the pontine locus coeruleus (A6), so named because of its propensity to stain blue. This consists of a group of cells in the dorsolateral tegmentum of the pons (Foote et al., 1983), accounting for approximately 50% of the brain NA. This centre has been ascribed a central role in cardiovascular control, with a suggested direct functional connection with the peripheral sympathetic nervous system (Elam et al., 1985). In rats, Elam et al (Elam et al.,1984) showed that an acute reduction in blood pressure elicited a marked increase in locus coeruleus neuronal activity. In humans, previous investigators have demonstrated a relationship between subcortical noradrenergic neuronal activity, as measured by central noradrenaline turnover, and renal sympathetic activation (Ferrier et al., 1993) in human hypertension. These findings are supplemented by the results of

animal studies in which stimulation of noradrenergic pressor regions of the hypothalamus and amygdala have been shown to increase renal firing and renal vascular resistance (Koepke et al, 1991).

Figure 1.3 Localisation of catecholamine cell groups in the rat brain



——————— DOPAMINE ———————— NOR—— ADR – NOR

The approximate distribution of dopamine (triangles), noradrenaline (NOR, as indicated by dots) and adrenaline (ADR, represented by asterisks) cell groups are shown. Each catecholamine has its own domain in the brain with dopamine cell bodies (A8 - A16) in the rostral parts of the brain (mesencephalon, hypothalamus and olfactory bulb) and noradrenaline cells in the pontine (A5 and A6) and lower medullary (A1 and A2) levels. The adrenaline cells lie between the rostral and caudal noradrenaline complexes. The catecholamine cells may be found along the entire brain axis. There is a tendency to a dorsal and a ventral catecholamine complex at all levels from the spinal cord to the rostral hypothalamus. Abbreviations used are: AP, anterior pituitary; Caud, caudate nucleus; CC; crus cerebri; HI, hippocampal complex; MO, medulla oblongata; OB, olfactory bulb; and Sep, septum. Figure reproduced from Hokfelt *et al* (Hokfelt et al., 1984).

1.8 Baroreflexes

It has been recognised for over 100 years that stimulation of reflexogenic areas within the heart and lungs can lead to dramatic changes in heart rate and blood pressure. Bainbridge demonstrated that the rapid intravenous infusion of fluids with resultant baroreceptor loading, led to a reflex tachycardia (Bainbridge, 1915). A mechanism for this response was subsequently described by investigators who obtained tachycardia in response to discrete distensions of the junctional regions between the atria and the pulmonary veins or the venae cavae (Linden et al., 1982).

Contrastingly, baroreceptor unloading with manoeuvres such as head-up tilt and lower body negative pressure in normal subjects, has been observed to increase forearm vascular resistance and heart rate, and these changes are accompanied by activation of the SNS and the renin-angiotensin systems (Zoller et al., 1972). These complex reflexes are mediated through afferents running in the vagus nerves to the above-mentioned cardiovascular centres in the brain (Hainsworth, 1991). However, the nature of the afferent signals and the subsequent reflex responses remains enigmatic. Whilst the diversity of responses to discrete stimulation of these areas is now recognised, there remains a tendency to group them together as "cardiopulmonary" baroreflexes.

In health, arterial baroreceptors exert a tonic inhibitory influence on the sympathoadrenal drive to the heart and peripheral circulation as well as a tonic excitatory influence on vagal outflow to the heart (Eckberg et al., 1971). From animal models of CHF (Higgins et al., 1972) and in human CHF, as discussed below, the evidence indicates that these baroreflexes are blunted. The mechanisms responsible have not as yet been determined. There may be structural abnormalities in the baroreceptors, central neural abnormalities, or impaired neuroeffector mechanisms.

1.8.1 Abnormal baroreflexes in heart failure

Even in patients without evidence of heart failure, there is already evidence of neurohormonal excitation once left ventricular systolic dysfunction is present (Francis et al., 1990; Rundqvist et al., 1997). In addition, recordings of muscle sympathetic nerve activity (Leimbach et al., 1986) and measures of cardiac NA release (Kaye et al., 1994) in human CHF show progressive increases as the level of cardiac filling pressure increases. However, the mechanism for the establishment of the neurohormonal excitatory state that begins even before heart failure develops has not been established. A hypothesis that has been advanced is that abnormalities of arterial and cardiopulmonary baroreflexes exist, leading to reduced tonic inhibitory influences on the sympathetic nervous system centres in the brain (Thames et al., 1993).

It has been long recognised that patients with chronic CHF have abnormal autonomic neuronal reflex responses to changes in cardiopulmonary loading conditions. Upright tilt has been associated with forearm vasodilation in CHF, as has lower body negative pressure (Ferguson et al., 1984), (Levine et al., 1983), in contrast to the vasoconstriction observed in healthy subjects (Zoller et al., 1972). It is also clear that patients with CHF have abnormal responses to increases in cardiac filling pressures, with the demonstration that mean pulmonary arterial pressure is an independent predictor of cardiac sympathetic activity (Kaye et al., 1994). This relation is paradoxical, since in the healthy individual, increases in cardiopulmonary pressures have sympathoinhibitory effects manifest as reduction in peripheral vascular resistance (Shi et al., 1993; Ferguson et al., 1992). Baroreflex responsiveness may be most impaired in patients with the greatest v ricular dysfunction (Hirsch et al., 1987), although it appears that that even in mild CHF, the baroreceptor inhibitory influence on heart rate and muscle sympathetic nerve activity is already impaired (Grassi et al., 1995). These abnormalities in baroreflex responses have been demonstrated to reverse as early as 2 weeks after cardiac transplantation (Ellenbogen et al.,1989). When baroreflex sensitivity was assessed with intravenous bolus

injections of phenylephrine in patients before and after orthotopic heart transplantation, a markedly diminished arterial baroreflex slope (msec/mmHg) was noted in the CHF patients and this was reversed to a normal response after transplantation.

1.8.2 Manipulations of the reflexes responsible for high sympathetic tone

The afferent signals responsible for the demonstrated increased central monoamine turnover in human CHF (Lambert et al., 1995a) have yet to be fully elucidated. Evidence to date suggests that there may be crucial afferents from the cardiopulmonary baroreceptors that are activated in situations of high pressure and/or volume overload that exists in CHF. Peripheral muscle sympathetic nervous activity has been most closely related to left ventricular filling pressure (Leimbach et al., 1986). Distension of the left atrium and pulmonary veins has been demonstrated to result in a positive chronotropic response, with the suggestion of an afferent pathway lying in the vagus and mediation of the efferent response by cardiac sympathetic nerves (Furnival et al., 1971; Kurz et al., 1990). Similarly, cardiac sympathetic activation in severe heart failure bears a close relationship with pulmonary artery pressures (Kaye et al., 1994).

In recent years, there have been several studies that have tried to manipulate this postulated cardiopulmonary baroreceptor – afferent, locus coeruleus - efferent reflex in human heart failure. In CHF patients with high pulmonary pressures, sodium nitroprusside administration led to directionally opposite changes in whole-body (increase) and cardiac (reduction) sympathetic nervous activity (Kaye et al., 1998). This finding suggests that there is a positive feedback loop between pulmonary artery filling pressure and cardiac sympathetic tone, and that when these receptors are acutely off-loaded, cardiac sympathetic drive is reduced. In another investigation, lower body negative pressure (LBNP) was used in an effort to unload cardiopulmonary baroreceptors, in the absence of measurable changes in systemic arterial blood pressure (Azevedo et al., 2000; Floras, 2001). Non-hypotensive LBNP

caused a significant reduction in cardiac noradrenaline spillover in the CHF group, but no change in the normal left ventricular function group.

1.8.3 Further manipulations of baroreflexes

Our group has previously demonstrated a positive correlation between pulmonary artery pressures and cardiac noradrenaline spillover rate (NASR) (Kaye et al., 1994) in patients with CHF; further, our group has shown that a positive correlation also exists between monoamine turnover in the brain and cardiac NASR (Lambert et al., 1995a). These two observations combined suggest the existence of a reflex link, consisting of afferent neural traffic from the cardiopulmonary receptors and sympathetic efferent outflow from the brain. Adding further weight to this, investigators have reported that muscle sympathetic nervous activity was most closely related to left ventricular filling pressure (Leimbach et al., 1986).

As mentioned above, the demonstration of an apparent reflex link between cardiac filling pressures and cardiac NA release (Kaye et al., 1998; Azevedo et al., 2000), leads one to speculate on the relationship between cardiac filling pressure and central monoamine turnover. In Chapter 4, I describe the effects of the administration of intravenous SNP upon central monoamine turnover, in both healthy volunteers and CHF patients.

1.9 Localisation of the central origin of heightened sympathetic drive

With the aid of combined cerebral venous blood pool scanning and bilateral internal jugular venous cannulation, it is feasible to broadly localise the heightened central sympathetic drive to the cortex or the suprabulbar subcortex. As discussed above, there is a substantial body of literature derived from animal studies that indicates that the important centres for sympathetic cardiovascular control are located in the

brainstem. In human hypertension, investigators have demonstrated that there is increased spillover of NA and its metabolites from the subcortical regions (Ferrier et al., 1993).

Hitherto, attempts have not been made to better localise the heightened central sympathetic drive in human or experimental heart failure. This is the subject of the study presented in Chapter 4. The original aim of this study was to use SPECT (single photon emission computerised tomography)- I-123 MIBG (metaiodobenzyluanidine) scanning of the central nervous system to localise these centres. I-123 MIBG is an analogue of the adrenergic blocking agent guanethedine that has been used to image catecholamine-containing tumors such as phaeochromocytomas. It shares the same uptake mechanism and storage in the sympathetic nerve endings as NA but is not metabolised. Accordingly, I-123 MIBG imaging could be used as a measure of sympathetic activity in an organ-specific level. This technique has been used to assess myocardial adrenergic activity in patients with CHF (Henderson et al., 1988; Imamura et al., 1995). The original aim was to correlate the invasively derived measures of CNS sympathetic activity with SPECT images, thereby establishing a non-invasive method of assessment of central sympathetic drive in CHF.

Unfortunately, in a test case, in which a patient with CHF underwent SPECT scanning of the CNS, there was no uptake of I-MIBG, presumably due to the actions of the blood-brain barrier. Therefore, this aspect of the study reported in Chapter 4 was abandoned.

From animal studies, it is apparent that central monoaminergic systems are important in the regulation of cardiovascular reflexes, with pressor noradrenergic neurons influencing sympathetic outflow. However, there are limited methodologies available to study central nervous system control of human sympathetic nervous outflow. As

mentioned above, direct imaging using MIBG does not appear to hold much promise. Another approach has been to study catecholamine turnover in the brain by measuring the spillover of NA, its precursor, dihydroxyphenylalanine (DOPA), and its lipophilic metabolites into the internal jugular vein, the so-called central noradrenaline turnover. Historically, the first support of such an approach were reports of an elevated NA concentration in the cerebrospinal fluid of some patients with essential hypertension (Eide et al., 1979).

A previous report has demonstrated a significant relationship between the internal jugular venous spillover of NA and the estimated CNS turnover of this neurotransmitter (Lambert et al., 1998). However, this methodology has two major limitations. Firstly, the existence of a blood-brain barrier to the efflux of NA from the brain may lead to an underestimate of the degree of cerebral noradrenergic neuronal activation (Lambert et al., 1998). Secondly, the origin of the NA spillover in internal jugular venous blood has been a point of some controversy. There is well-developed sympathetic innervation of the cerebral arterial blood vessels, and this could be a potential contaminating source. However, in a study of patients with pure autonomic failure, normal trans-cerebral NA overflows were demonstrated, despite a marked reduction in peripheral NA spillover to plasma, thereby supporting an origin from the brain (Lambert et al., 1998). In addition, ganglion blockade with trimehaphan does not diminish NA overflow from the CNS (Esler et al., 1995).

A brief outline of the noradrenergic synthetic pathway is given in Figure 1.4 and the pathways of catecholamine metabolism are presented in Figure 1.5. The relative amounts of the NA metabolites largely depends on the site being sampled. For instance, NA accumulated by extraneuronal cells via Uptake 2 may be metabolised by COMT to form normetanephrine or, after formation of its deaminated intermediates,

MHPG or VMA. Intraneuronal NA in the axonal cytoplasm is subject to local deamination by MAO and reduction by aldehyde reductase with subsequent production of DHPG. DHPG is lipophilic and readily crosses the neuronal cell membrane and spills over into plasma. DHPG may be taken up into extraneuronal cells and be transformed to MHPG via the actions of COMT. MHPG in plasma, therefore, may be derived from two sources: extraneuronal uptake and metabolism of NA by MAO and COMT, and extraneuronal removal and O-methylation of DHPG. In primates, free MHPG is the predominant NA metabolite released from the brain into the peripheral circulation. However, in the heart, DHPG is the predominant metabolite. This may result from the fact that, in the heart, the synaptic cleft width is narrow and that, therefore, NA dissipation after release is much more dependent on neuronal reuptake and subsequent intraneuronal metabolism (Esler et al., 1990).

There has been a long-held interest in possible neurochemical methodologies for the clinical study of brain NA turnover, with measures of MHPG in urine, CSF and plasma. Initially, it was believed that MHPG was a terminal metabolite and that as much as 60% of urinary MHPG was derived from the brain. Subsequently, it has been established that MHPG in the periphery is further metabolised, and that consequently, the brain's contribution to the total body MHPG plasma pool is less than 20%. In a study examining the regional origins of MHPG in plasma, Lambert et al concluded that the contribution of the brain to plasma MHPG must be less than 10% (Lambert et al., 1995b). Therefore, plasma or urine measures of metabolites of NA are not accurate indicators of brain noradrenergic activity.

Fortunately, the two major NA metabolites in the brain – MHPG and DHPG- are lipophilic and readily cross the blood-brain barrier into the venous drainage of the brain, creating positive jugular-arterial plasma concentration differences. Following

the demonstration by Maas et al (Maas et al., 1977) that CNS neuronal activity of stump-tailed monkeys could be studied by direct internal jugular vein blood sampling, this technique has been applied to humans and at present remains the "gold standard" for measuring central noradrenergic activity. This methodology is applied in studies presented in Chapters 4 and 5.

Figure 1.4 Biosynthesis of the catecholamines

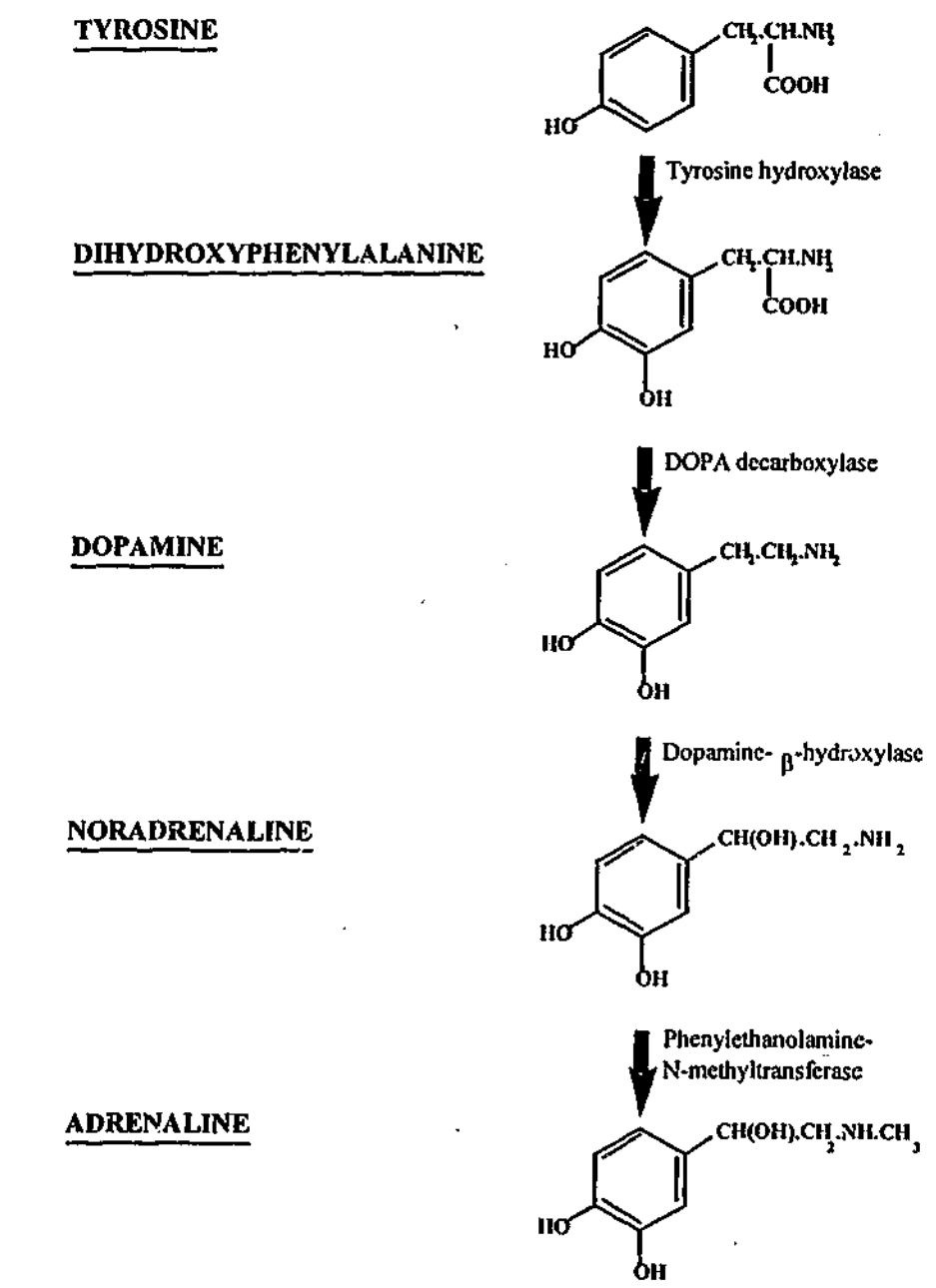
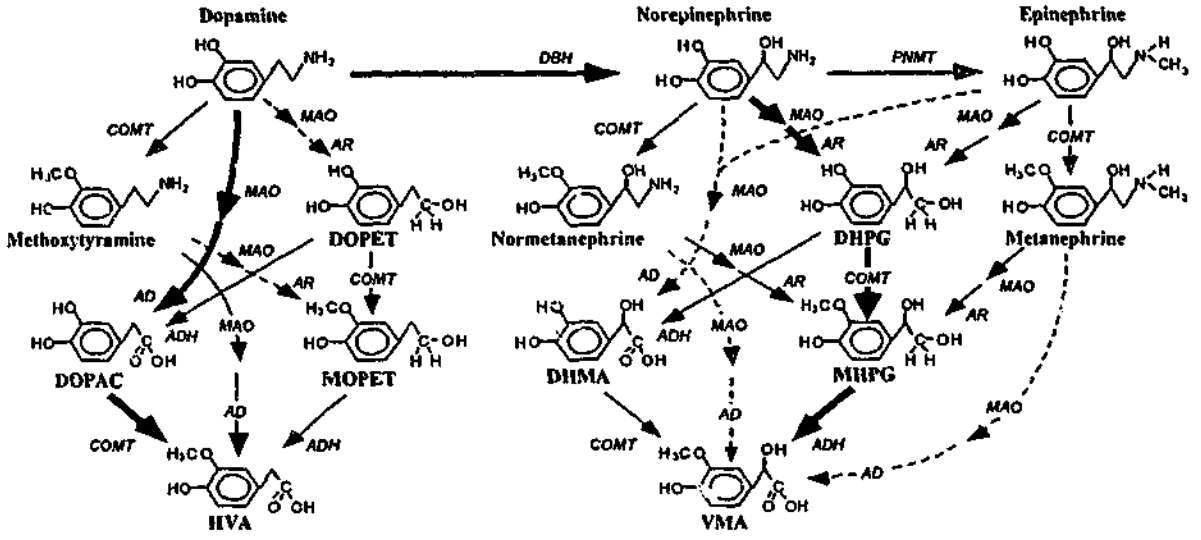


Figure 1.5 The pathways of catecholamine metabolism



This scheme outlining the general metabolic pathways involved in the inactivation of the catecholamines was compiled by Dr Graeme Eisenhofer and reproduced with his permission. The most active pathways are shown by the most solid arrows and the least active by dotted arrows. The enzyme responsible for each step is shown at the head of each arrow. The pathways of sulpho and glucuronide conjugation are not shown. Note that norepinephrine and epinephrine are synonyms for noradrenaline and adrenaline respectively.

Abbreviations used are: DBH, dopamine-β-hydroxylase; PNMT, phenylethanolamine-N-methyltransferase; COMT, catechol-O-methyltransferase; MAO, monoamine oxidase; AR, aldehyde reductase; AD, aldehyde dehydrogenase; ADH, alcohol dehydrogenase; DOPET, dihydroxyphenylethanol; DHPG, 3,4-dihydroxyphenylglycol; DOPAC, 3,4-dihydroxyphenylacetic acid; MOPET, methoxyhydroxyphenylethanol; DHMA, dihydroxymandelic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; HVA, homovanillic acid; VMA, vanillylmandelic acid.

1.9.1 Manipulations of central monoamine turnover

Thus far, there are a limited number of animal and human studies in which central monomaine turnover has been manipulated, either by pharmacological means or by manipulating baroreflexes. Maas and colleagues demonstrated that clonidine resulted in a reduction in MHPG jugular overflow in monkeys (Maas et al., 1977). Clonidine is a centrally acting inhibitor of the sympathetic nervous system, known to inhibit the firing rate of locus coeruleus neurons (Svensson et al., 1975). This centrally sympatholytic action of clonidine has since then been reproduced in healthy human subjects (Lambert et al., 1998; Esler et al., 1995).

These observations, when combined with the acknowledged limited therapeutic approach of peripheral adrenergic blockade, encourage the investigation of centrally acting sympatholytic therapy for heart failure. In Chapter 5, the effects of clonidine on central monoamine turnover in patients with CHF are described.

1.10 Presynaptic modulation of noradrenaline release

Up to 75% - 80% of the released NA is disposed of by reuptake into the nerve terminal of the parent adrenergic neuron and stored in vesicles for release. The remainder "spills over" into the venous circulation or extraneuronal metabolism. Whilst possible determinants of this rate of NA spillover include factors such as regional blood flow and the adequacy of neuronal NA uptake, generally the overflow of NA serves as an indirect measure of sympathetic neural activity in a particular organ.

A substantial body of work exists to support the view that postganglionic fibres have functionally important pre-synaptic receptors that modify neurotransmitter release, thereby creating a mismatch between sympathetic nerve firing rate and rate of NA release. Alterations in these mechanisms of "neuromodulation" could then be another means, in addition to abnormal baroreflexes, by which high circulating NA levels exist in CHF. The existence of functionally important peripheral, presynaptic α_2- and

β_2- adrenoceptors has been postulated for some time. There is a large body of evidence from, animal in vitro and in vivo studies, which suggests that noradrenaline release following nerve stimulation is regulated by a presynaptic α_2-adrenoceptor-mediated negative feedback mechanism (Westfall et al., 1977). The role of presynaptic α_2- and β_2- adrenoceptors in modulating NA release was examined in isolated cat spleen prelabeled with tritiated NA (Dixon et al., 1979). Perfusion of the splenic bed with NA reduced release of [^{3}H] NA. Isoprenaline, a β_2- adrenoceptor agonist, however, increased labelled NA release.

In humans, the physiological role of pre-synaptic adrenoceptors has been somewhat more difficult to clarify. It became possible to approach this issue with the advent of the techniques of forearm venous occlusive plethysmography, intra-arterial administration of sympathoactive agents (Robertson et al.,1986), and of radiotracer kinetics. These methods will be dealt with in more detail in Chapter 2.

1.10.1 α-adrenoceptors

The α-adrenergic component of the sympathetic nervous system plays a major role in the pathophysiology, clinical manifestations, and natural history of human heart failure (Leier et al., 1990). Noradrenaline is the predominant endogenous agonist of α-adrenoceptors. NA is stored within vesicles in nerve terminals and is released into the junctional cleft when the action potential of the nerve is sufficient to increase the concentration of intraneuronal calcium at the nerve terminal, thereby prompting vesicular exocytosis.

On the basis of ligand binding studies and pharmacological properties, the α-adrenergic receptors are divided into α_1 – and α_2 – adrenoceptors. In addition, on the basis of multiple cellular responses to adrenergic stimulation, there appear to be a number of α_1 – and α_2 – adrenoceptor subtypes (Minneman, 1988).

The predominant α-receptors in the CNS are central α_2 – adrenergic postsynaptic receptors. Stimulation of these receptors leads to a fall in blood pressure and bradycardia. In the periphery, the noradrenaline released from the nerve terminal

diffuses across the junctional cleft to activate the α_1–and α_2–adrenoceptors located within the nerve junction on the effector organ, eg myocardium. Most of the postsynaptic α-adrenergic receptors located within the neuroeffector junction are of the α_1-type, while presynaptically, the α_2-type predominates (Brown, 1989). It has been theorised that when these receptors are stimulated by the NA in the synaptic cleft, further NA release from the nerve terminal is inhibited. Therefore, NA has been postulated to have its own inhibitory or "negative" feedback loop to control its own release (Dixon et al., 1979). The importance of this mechanism in man remains controversial.

1.10.2 Role of the peripheral α_2 - adrenoceptors

Clonidine, an α_2–adrenoceptor agonist, and yohimbine (Grossman et al.,1991c), an α_2–adrenoceptor antagonist, when administered intravenously in humans, have demonstrable effects on blood pressure, heart rate, and plasma catecholamines (Robertson et al., 1986). Clonidine is sympatholytic, whereas yohimbine raises plasma NA and blood pressure. However, systemic administration allows the drug to act both centrally and peripherally, and therefore the function of peripheral α_2–adrenoceptors in isolation cannot be studied in this way. Evidence from microneurographic studies have suggested that clonidine has a combined central and peripheral antihypertensive action in man (Mark et al., 1985). By measuring the forearm NA spillover response to intra-brachial artery clonidine, it is possible to make conclusions about the functional importance of the presynaptic α_2–adrenoceptors. This study is described in Chapter 3.

1.10.3 Peripheral presynaptic β_2-adrenergic receptors – The "Adrenaline Hypothesis"

In heart failure, the plasma concentration of adrenaline is significantly elevated and may bear some relationship to subsequent mortality (Swedberg et al., 1990).

According to the "adrenaline hypothesis" of hypertension pathogenesis, sympathetic nerve terminals take up circulating adrenaline by neuronal uptake (uptake –1), and with sympathetic stimulation, adrenaline is co-released with NA. The co-released adrenaline is then postulated to bind to presynaptic β_2-adrenergic receptors, thereby augmenting NA release during sympathetic stimulation (Majewski et al., 1981; Majewski et al., 1983).

Tests of this hypothesis have, to date, led to conflicting results in humans. Intra-arterial adrenaline infusion, in the brachial artery, has been demonstrated to both augment (Floras et al., 1988) and not change forearm NA spillover (Stein et al., 1997). In a more recent examination of the hypothesis, Goldstein et al (Goldstein et al., 1999) found that loading of forearm sympathetic terminals with adrenaline in the healthy human did not augment subsequent neurogenic vasoconstriction or forearm NA spillover in response to sympathetic stimulation by lower body negative pressure and intravenous nitroprusside. However, intracoronary infusion of salbutamol, a β_2–receptor agonist, has been demonstrated to enhance cardiac NA spillover, in a group of patients with normal left ventricular function (Newton et al., 1999). Other investigators have found that therapy with carvedilol caused significant decreases in systemic and cardiac NA spillover, and that these changes are not observed in patients treated with metoprolol. Additionally, there was no effect of either agent on sympathetic efferent neuronal discharge to skeletal muscle. These findings led the authors to conclude that carvedilol caused its sympathoinhibitory effect by blocking peripheral, prejunctional β_2-adrenergic receptors (Azevedo et al., 2001). In Chapter 3, the effects of brachial intra-arterial propranolol on forearm NE spillover in CHF and in healthy volunteers are presented.

1.11 Potential mechanisms for beneficial actions of β-adrenergic blockade

The striking generalisation about the long – term effects of β-blockade on myocardial function is that they are diametrically opposite to the short-term negative inotropic effects (Eichhorn et al., 1996). Long-term β-blockade in heart failure results in significant hemodynamic improvement, with increased left ventricular stroke work index, left ventricular ejection fraction and decreased pulmonary capillary wedge pressure (Swedberg et al., 1980). The mechanisms for the hemodynamic improvement and for the mortality benefit are not yet fully elucidated.

Eichorn et al (Eichhorn et al., 1990), in a nonrandomised study of the effects of 3 months of treatment with bucindolol in patients with CHF, demonstrated an increase in ejection fraction despite an decrease in preload and an increase in afterload, suggesting improvement in myocardial contractility. The cellular basis for this improved inotropy remains unclear.

There appear to be significant differences between the acute and long-term haemodynamic effects of anti-adrenergic therapy. After one day of therapy with metoprolol, left ventricular ejection fraction is depressed compared with baseline, but returns to "normal" by one month of treatment (Hall et al., 1995). This is in keeping with the depression in myocardial contractility after acute administration of β-blocking agents in CHF, presumably due to the effect of withdrawal of β-adrenergic support to the failing heart. Any improvement in ejection fraction takes up to three months after initiation of therapy (Packer et al., 1996). The delay in improvement in myocardial contractility, therefore, appears to be within the time frame necessary for an as yet undetermined secondary biological effect to take place.

It has been established for some time that there is a selective down-regulation of the myocardial β_1–adrenergic receptor number and function in the failing human heart

(Bristow et al., 1982), and this would appear to result from chronic agonist overexposure (Hadcock et al., 1988). This down-regulation leads to the reduced positive inotropic effects of β-adrenergic receptor agonists in the failing heart. Whilst upregulation and restoration of β-adrenergic signal transduction is described with metoprolol (Heilbrunn et al., 1989), thereby providing a mechanism for improved myocardial contractility, these molecular changes have not been seen with carvedilol (Heilbrunn et al., 1989; Yoshikawa et al., 1996; Gilbert et al., 1996). In a study comparing the effects of metoprolol and carvedilol on cardiac adrenergic drive, only carvedilol was found to selectively lower coronary sinus noradrenaline levels (Gilbert et al., 1996; Azevedo et al., 2001). However, Kaye et al found that carvedilol did not reduce cardiac NA spillover in a group of CHF patients, and concluded that a reduction in cardiac sympathetic drive was not a mechanism by which it resulted in improved myocardial function (Kaye et al., 2001).

1.11.1 Receptor uncoupling

In addition to reduced expression of β_1–adrenergic receptors in CHF, there also appears to be reduced receptor coupling to secondary intracellular signalling pathways. Studies have shown that a major mechanism leading to this uncoupling involves phosphorylation of the beta-adrenoceptor by the specific β-adrenergic receptor kinase (βARK) (Ungerer et al., 1993; Ungerer et al., 1994). It has been demonstrated that there is increased expression of βARK in the failing human heart (Ungerer et al., 1993), with the signal for this being the agonist-occupied β-adrenergic receptor. It is possible, therefore, that therapy with anti-adrenergic agents may resensitize the receptor system, perhaps by reducing βARK expression thereby resulting in improved cardiac function (Manning et al., 2000). Given that a high

expression of βARK has been shown in human peripheral lymphocytes, a study was conducted in which βARK levels were measured in peripheral lymphocytes of CHF patients, before and after the initiation of carvedilol therapy. The results of this are presented in Chapter 6.

1.12 Possibility of sympatholytic therapy for heart failure

Peripheral β-adrenergic blockade, whilst resulting in major gains in prognosis, is somewhat limited in its objectives. Firstly, it cannot antagonise the effects of potentially harmful, vasoactive cotransmitters that are released with NA, such as neuropeptide Y (Maisel et al., 1989; Kaye et al., 1994). Secondly, the heightened renal sympathetic tone that is a hallmark of CHF (Hasking et al., 1986; Middlekauff et al., 2000) is unabated with carvedilol (Dupont, 1990). With the understanding that there is increased central monamine turnover in CHF (Lambert et al., 1995a), the possibility of attenuating sympathetic drive directly arises. This idea is certainly not new, having first been proposed by Kelly et al in 1953 (Kelly RT, 1953).

Clonidine, an imidazoline and α_2-adrenoceptor agonist, is a potent sympatholytic agent that has been demonstrated to inhibit sympathetic outflow (Isaac, 1980). In states of heightened sympathetic drive, such as CHF and hepatic cirrhosis, parenteral clonidine has been demonstrated to have potent sympathoinhibitory action. In a study of CHF patients, intravenous clonidine resulted in a 50% reduction in cardiac and global NA spillover (Azevedo et al., 1999). Similarly, when given to patients with alcoholic cirrhosis, clonidine resulted in significant decreases in NA spillover from the body as a whole, the kidneys and the gut and liver (Esler et al., 1992).

Despite this experience with sympatholytic treatment, the only large scale to trial to date of sympathoinhibition in CHF- "MOXCON"- was terminated prematurely

because of excess mortality. Whilst a detailed report of the trial results has yet to be published, a preliminary study demonstrated a powerful sympatholytic effect of the drug (Swedberg et al., 2000). It remains unclear as to why this potentially favorable pharmacological effect did not translate into clinical benefit for CHF patients. One possible explanation is that the uptitration of moxonidine was too aggressive and the acute sympathetic withdrawal led to poor outcomes.

In Chapter 5, I present a study in which low doses of clonidine were used intravenously in patients with CHF. The effects on global, cardiac and renal NA spillover were measured. In addition, I studied the effect of clonidine on central monoamine turnover.

1.12.1 Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino-acid peptide that is co-localised and co-released with NA in many central and peripheral neurons (Tatemoto et al., 1982). Plasma NPY correlates better with plasma NA than with plasma adrenaline, indicating its origin from sympathetic nerve terminals. Under resting conditions, the venous plasma concentrations of NPY are found to be 200-250 times smaller than the corresponding plasma concentration of NA (Haass, 1998). The acute sympathetic activation achieved by a cold-pressor test appears insufficient to significantly increase the plasma concentrations of NPY, although the plasma concentration of NA more than doubles (Haass, 1998). Similarly, dynamic exercise increases plasma NA levels but does not significantly alter the NPY concentration (Maisel et al., 1989). This lack of responsiveness to acute stimulation has led authors to speculate that plasma NPY might be an even more reliable prognostic indicator than plasma NA.

The perfused guinea pig heart has been chosen to further characterize cardiac NPY release in comparison to NA release. In this model, several different stimuli resulted in a concomitant overflow of both NPY and NA into the coronary sinus. The molar ratio of the concentrations of NPY and NA in the coronary sinus was approximately 1:500 (Haass, 1998). Circulating levels of NPY are elevated in human CHF (Maisel et al., 1989; Morris et al., 1986). Furthermore, the cardiac release of NPY is significantly increased in patients with CHF (Kaye et al., 1994).

NPY is an even more potent vasoconstrictor than NA, by activation of its specific Y1 receptors, and additionally potentiates α-adrenergic receptor-mediated vasoconstriction (Daly et al., 1987). In addition to being a potent vasoconstrictor, NPY has been reported to exert both negative cardiac inotropic and chronotropic actions (Zukowska-Grojec et al., 1987; Gray et al., 1986). Whether these latter effects are due to baroreflex activation in response to the peripheral vasoconstriction, to coronary vasoconstriction, or to a direct effect on the heart is not yet clear. Further evidence of the pleiotropic functions of NPY is the finding that it stimulates both vascular smooth muscle proliferation and angiogenesis (Zukowska-Grojec, 1998).

Whilst the prolonged release of NPY in CHF may contribute to the deterioration of ventricular function, the current therapeutic approach of adrenergic blockade cannot counter this. Therefore, strategies to attenuate sympathetic drive directly should be investigated. In Chapter 5, I present the effects of intravenous clonidine on cardiac spillover of NA and NPY.

1.12.2 Imidazoline receptors

Evidence from studies in animals indicates that clonidine lowers blood pressure predominantly by stimulation of α_2–adrenoceptors in the brainstem (Kobinger et al., 1967). Activation of these receptors in the ponto-medullary region is hypothesized to increase the firing of central inhibitory neurones, thereby leading to a reduction in sympathetic activity. The concept of imidazoline receptors (IR) arose from the observation that the central hypotensive actions of clonidine did not relate solely to the drug's actions as an α_2–adrenergic agonist but also to its structure as an imidazoline (Reis et al., 1995). Definite evidence for a new receptor came from the animal studies of Bousquet et al (Bousquet et al., 1984) who studied the effect of the microinjection of clonidine and α-methylnoradrenaline into the rostral ventro-lateral medulla (RVLM). In contrast to clonidine (a pure α_2-adrenoceptor agonist), α-methylnoradrenaline failed to lower blood pressure, leading the authors to conclude that there were imidazoline binding sites in the RVLM. Subsequently, these IR have been detected with radioligand binding assays in the brainstem of several species including human (Chan et al., 1996).

Although it seems likely that central α_2-adrenoceptors and IR are related functionally, they appear to mediate different effects – IR appear to be involved in sympathoinhibitory actions, whereas α_2-adrenoceptors, in addition to these actions, are responsible for adverse events such as sedation and dry mouth. These adverse side-effects of traditional centrally-acting antihypertensives such as clonidine have led to the development of agents with a higher affinity for IR than for the α_2-adrenoceptors. The possibility that the central sympatholytic action of clonidine may be due to activation of these IR is considered in the study presented in Chapter 3.

Chapter 2

General Description of Methods

In this Chapter, the methods that are that are common to more than one subsequent experimental Chapter are described. Any methods that are study-unique are detailed in the relevant Chapter.

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2.1 Subject selection

Much of the work documented in this thesis was carried out in healthy human volunteers aged between 18 and 75 years. These subjects were recruited by local advertisement from the general community and were reimbursed a nominal amount for their time and efforts. All subjects underwent a comprehensive clinical and physical examination to screen for any previously undiagnosed medical conditions prior to their acceptance in any of the experimental protocols. Exclusion criteria for all studies included a history of major illness, cardiovascular disease, current drug medication and previous psychiatric therapy.

The congestive heart failure patients were recruited from the medical outpatient clinics of the Alfred Hospital and many of these subjects were undergoing assessment for suitability as candidates for cardiac transplantation. All patients were moderately to severely symptomatic with breathlessness at rest, or on minimal exertion (New York Heart Association symptom class III to IV). Left ventricular ejection fractions assessed by radionuclide ventriculography was less than 40%. All patients were treated with standard anti-failure medication. In view of the severity of their heart failure medication was continued throughout the research testing.

2.2 Institutional review and informed consent

The protocols reported in this thesis conformed to the relevant guidelines of the National Health and Medical Research Council of Australia and were approved by the Alfred Hospital Human Research Ethics Committee. All patients and healthy volunteers gave written informed consent prior to their participation in the experimental procedures.

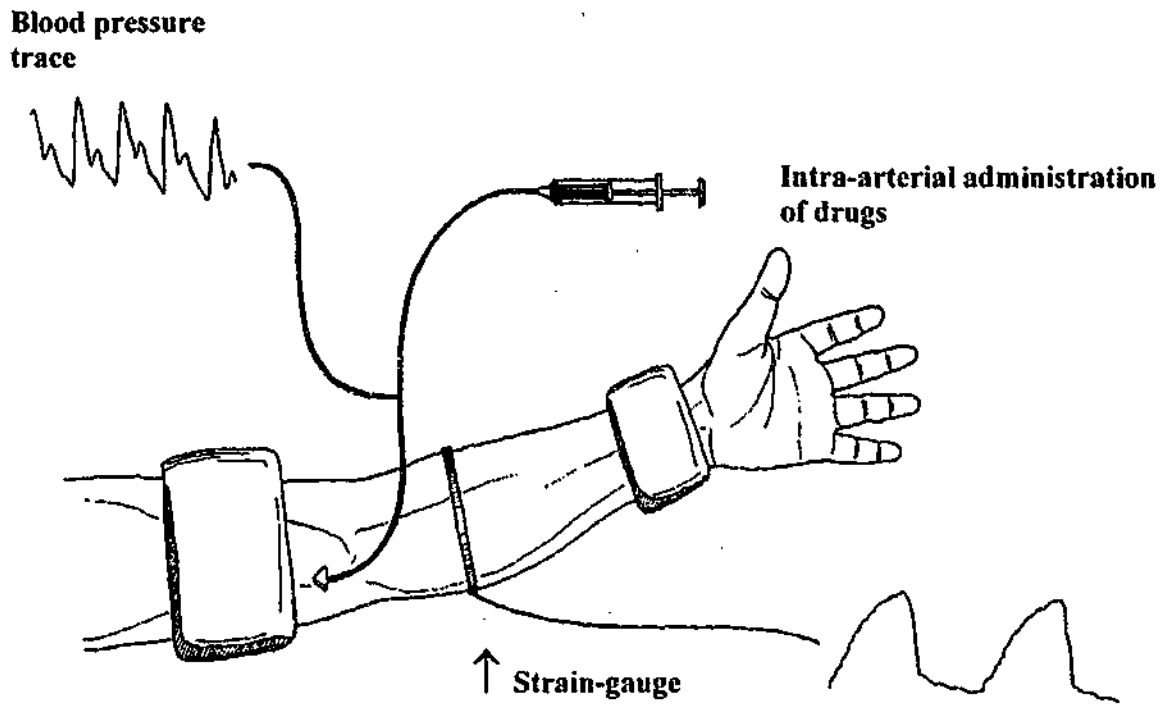
2.3 Regional blood flow determination and sampling

2.3.1 Forearm

In Chapter 3, forearm blood flow was obtained using the mean of 3-5 measurements of forearm venous occlusion plethysmography utilising an alloy filled (gallium and indium) double strand strain gauge (Medasonic, Mountain View, California). The experimental set-up is as depicted in Fig. 2.1. The underlying principle of venous occlusion plethysmography is straightforward: If venous return from the arm is obstructed and arterial inflow continues unimpeded, the forearm swells at a rate proportional to the rate of arterial inflow (Benjamin et al., 1995). Venous occlusion pressure was set at 40 mmHg while blood flow to the wrist and hand was excluded by application of a blood pressure cuff to the wrist raised to supra-systolic blood pressure. Venous occlusion plethysmography measures total forearm blood flow, of which, under resting conditions, blood flow through skeletal muscle is the bulk (Cooper, 1955). The hand has to be excluded from the circulation, as the blood flow in the hand is predominantly through skin (Benjamin et al., 1995). The flow is expressed as milliliters per 100 ml forearm/minute.

Arterial and venous blood samples were obtained simultaneously with the assumption that arterio-venous transit of blood across skeletal muscle is rapid at rest (Mottram et al., 1973). It was assumed that venous neurochemical concentrations were largely derived from skeletal muscle with negligible contributions from skin or adipose tissue.

Figure 2.1 A schematic representation of forearm strain gauge venous occlusive plethysmography.



2.3.2 Internal jugular vein

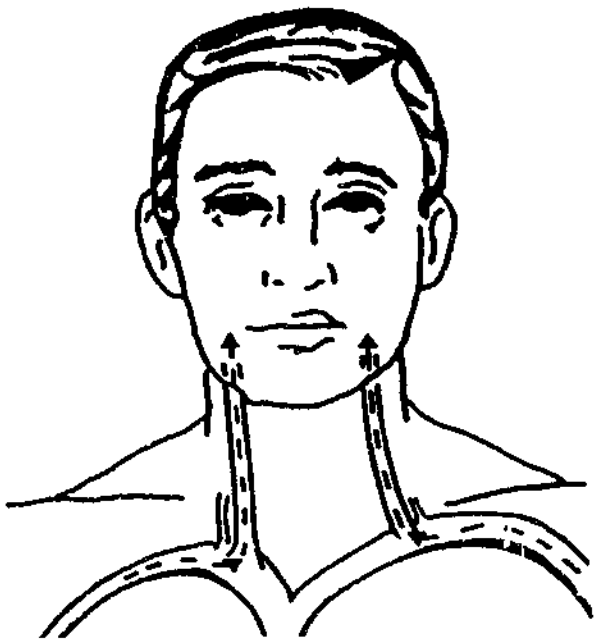
2.3.2.1 Catheter placement

Caffeinated beverages, alcohol and tobacco smoking were prohibited for the twelve hours preceding the catheter study. Subjects had a standardised light breakfast, typically comprising fruit juice and toast, at 08:00 on the morning of the procedure. While meals with high-monoamine content have been shown to drastically influence certain plasma neurotransmitter metabolite concentrations (Davidson et al., 1987), a light breakfast, low in monoamine content, does not appreciably influence plasma neurochemical concentrations (Lambert et al., 1993).

Blood samples were obtained from central venous and arterial catheters percutaneously inserted under strict aseptic conditions in the cardiac catheterisation laboratory of the Alfred and Baker Medical Unit. Subjects were studied in the supine position on a manoeuvrable laboratory table (Model 1212, NVC Australia Pty. Ltd., Australia) equipped with fixed anteroposterior fluoroscopic screening facilities (Model DC 12MB-1, Toshiba Industries, Osaka, Japan).

Following skin preparation and adequate local anaesthesia (1-2 mls of 2% lignocaine, Delta West Pty. Ltd., Bently, WA, Aust), a model C-PMS-301-RA 3.0F, 5 cm arterial catheter (Cook Australia, Eight Mile Plains, Queensland, Aust) was inserted percutaneously into a brachial artery. The arterial catheter was connected to a continuous infusion of 5% dextrose containing 2 U/ml heparin (David Bell Laboratories, Mulgrave, Vic, Aust) via an intraflow adaptor and pressure bag, which ensured delivery of 3 mls/hr at a bag pressure of 300 mmHg, if the system was not flushed manually. Intraarterial pressure was recorded continuously via a Spacelabs Inc. model 90603 2-channel pressure monitor (Redmond, WA, USA). Bilateral internal jugular vein catheterisation was performed under direct fluoroscopic vision

via an 8.5F percutaneous introducing sheath (Arrow International Inc., Reading, Pennsylvannia, USA) inserted into a median antecubital vein and a 7F type CCS-7U coronary sinus thermodilution catheter (Webster Laboratories, CA, USA). The catheter was positioned in the internal jugular vein beyond the mandibular angle, upstream to points of entry of veins draining the face and neck to minimise contamination of the cerebral venous effluent (Fig 2.2). The catheter's position was verified with 2 ml radiopaque contrast medium (Omnipaque, Winthrop Pharmaceuticals, NY, USA) and its patency ensured with heparinised dextrose as outlined above. These catheters were used for simultaneous bilateral internal jugular vein blood sampling and for the determination of internal jugular vein blood flows.



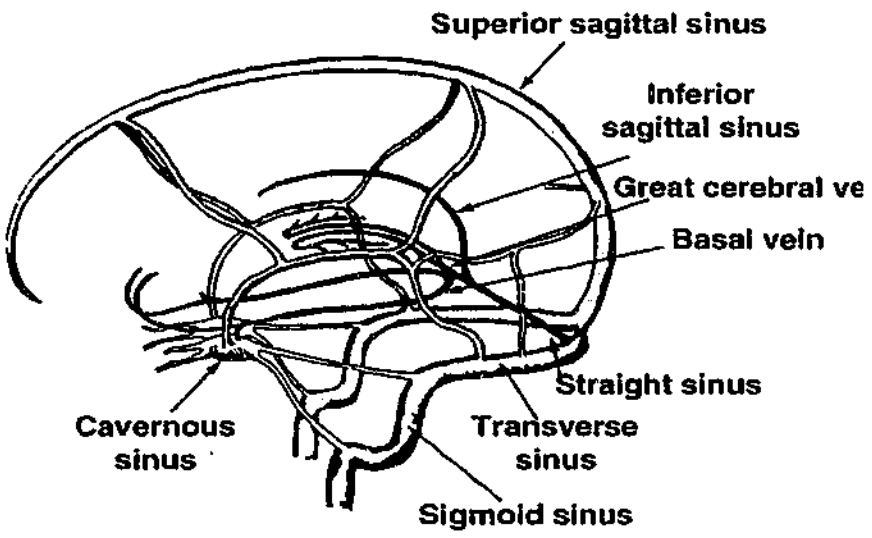


Figure 2.2 Diagrams representing positioning of internal jugular venous catheters and anatomy of cerebral venous blood flow.

Left: Schematic presentation of cannulation of the internal jugular veins via an antecubital approach.

Right: Diagramatic representation of the venous sinuses of the dura mater and their associations with the cerebral veins, deeply placed vessels are shown in black. The superior sagittal sinus begins marginally posterior to the foramen caecum and runs backwards across the falx cerebri and predominantly drains the cerebral hemispheres. As the sinus nears the internal occipital protuberance it deviates to one side or the other and forms the transverse sinus which in turn becomes an internal jugular vein. In the majority of cases the superior sagittal sinus tracks to the right and forms the right transverse sinus, as illustrated above. Although this vessel contains the bulk of cortical venous effluent it also contains blood derived from some subcortical areas via the cavernous sinus. Subcortical brain regions drain predominantly into the inferior sagittal and straight sinuses which in turn from the transverse sinus generally on the opposite side to that derived from the superior sagittal sinus. The inferior sagittal sinus also receives some cortical venous effluent from the falx cerebri. Reproduced with kind permission from Gray's Anatomy

2.3.2.2 Internal jugular vein blood flow

Internal jugular vein blood flow was measured according to the continuous infusion thermodilution method described by Ganz et al (Ganz et al., 1971b), and generally applied to the assessment of coronary sinus blood flow. The coronary sinus catheter was interfaced with a Webster CF-300 Flowmeter connected to a chart recorder (Gould 2800, 8 channel model). The technique involves the injection of an indicator fluid miscible with blood (5% dextrose in water), at a lower temperature (room temperature), into the bloodstream at a constant rate (35 ml/min) and recording of the resultant change in temperature a short distance downstream. Mixing of the blood and the colder infusate results in an equilibrium temperature that depends on the relative flow rates of blood and the infusate, thus blood flow can be calculated from the following equation:

$$\text{Blood Flow} = F_I \times 1.08 \times K \{ [(T_b - T_i) / (T_b - T_m)] - 1 \}$$

where F is the constant infusion rate of the indicator, T_b, T_i and T_m are the blood, indicator and equilibrium temperatures (^oC) respectively and K is the correction factor for heat flow between the indicator thermistor and the surrounding external catheter (supplied with each catheter).

The fundamental and practical limitations of this method, when applied to the assessment of coronary sinus blood flow, have been well documented (Ganz et al., 1971b). In applying this technique for our purposes it was critical that the tip of the catheter be positioned sufficiently high in the jugular vein so as to avoid the downstream contamination from facial veins entering the internal jugular vein lower in the neck, and that the catheter's thermistor was not in contact with the vessel wall (Fig 2.2).

2.3.3 Coronary sinus

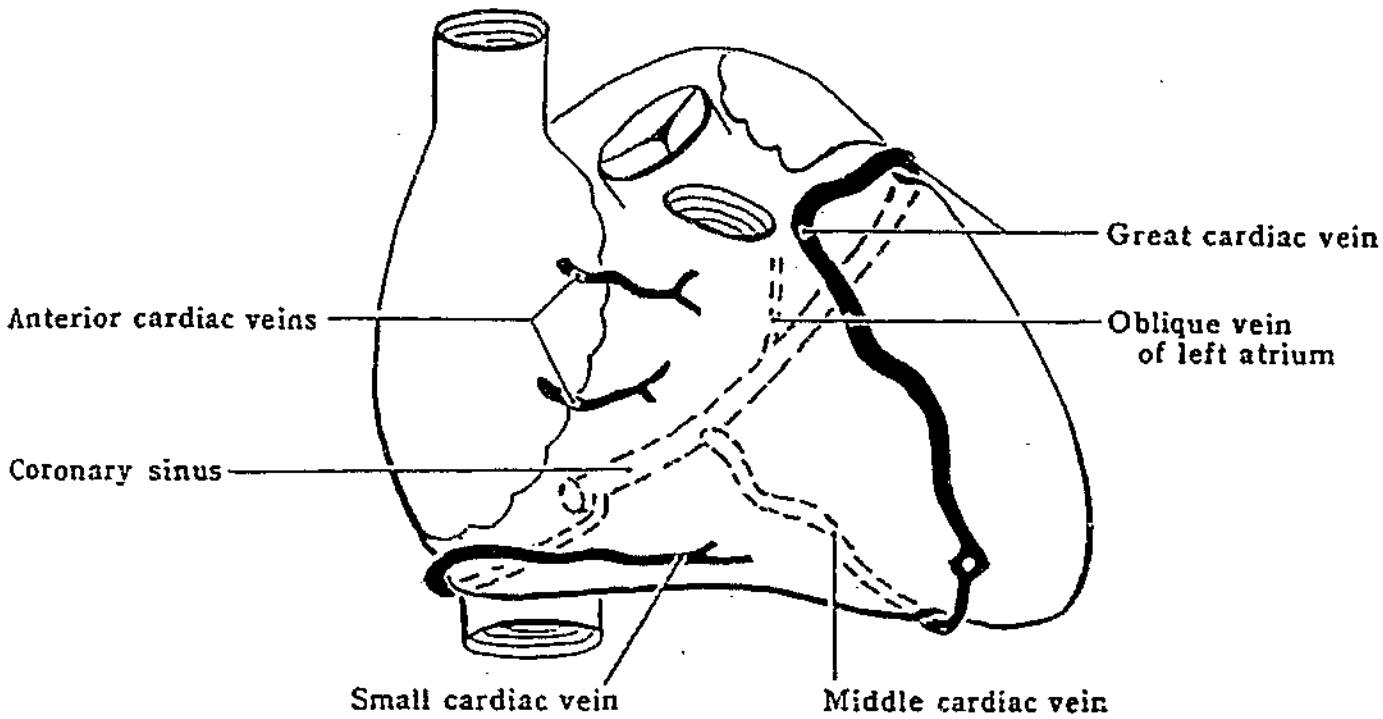
For the determination of cardiac sympathetic activity and cardiac neurochemical overflows, left ventricular myocardial blood flow was estimated by measurement of the coronary sinus blood flow, by the thermodilution technique as outlined in the previous section (Ganz et al., 1971b). A schematic diagram of the venous drainage of the heart is presented in Fig 2.3. With fluoroscopic control, a coronary sinus thermodilution catheter was advanced into the coronary sinus, the tip lying 2 to 3 cm proximal to the ostium of the sinus, being confirmed by the injection of 2ml radiographic contrast media. After confirmation of appropriate positioning of the catheter within the coronary sinus, blood flow determination was performed using a continuous infusion of an indicator solution (at room temperature) consisting of 5% dextrose, delivered at a constant rate of 35 ml/min by infusion pump into the coronary sinus.

A number of factors require consideration when interpreting data obtained using this method. Foremost amongst these is an understanding of the venous anatomy of the circulation of the heart. The coronary sinus usually receives venous drainage from all regions of the heart except those portions drained by the anterior cardiac veins and the venae cordis minimae, which both enter the right atrium directly. In approximately 10% of cases though the middle cardiac vein enters the right atrium directly. As such, measurement of the coronary sinus blood flow, and collection of venous samples provides an incomplete indication of myocardial blood flow and biochemical processes in the heart as a whole, and largely samples the left ventricle only. Furthermore, unlike positron emission tomography, the technique displays no capacity to provide measurements of regional myocardial blood flow and is unable to quantify phasic blood flow. The frequency response of thermodilution determination of coronary sinus blood flow is sufficient to detect flow changes which occur in 2 to 3 seconds (Ganz et al., 1971b).

Analagous to measuring internal jugular vein blood flow, other technical factors require special attention so as to minimise the chance of error during the application

of this method. These include the influences of incomplete indicator mixing in the blood stream, and the potential effect of local infusion on true blood flow. Other sources of artefact also include incorrect positioning of the catheter within the lumen of the coronary sinus such that the outflow port is partially obstructed by the vessel wall (Ganz et al., 1971b), instability of the catheter within the vessel and prolonged infusions which carry the possibility of error due to infusate recirculation.

Figure 2.3. Coronary venous anatomy of the heart



2.3.4 Renal vein flow

Renal plasma flow was estimated by measuring the rate of plasma clearance of para-aminohippuric acid (PAH) using the technique of Schurr (Schurr, 1980). Approximately 90 % of PAH is cleared in a single circulation through the kidney. Effective renal plasma flow was derived by dividing the clearance rate for PAH by the renal extraction of PAH (obtained by determining the arterial and renal venous PAH concentrations simultaneously), as given by the following equation:

$$\text{Renal Plasma Flow} = \frac{\text{Total plasma clearance PAH}}{\text{Renal fractional extraction PAH}}$$

2.3.5 Cardiac output measurement

Cardiac output was also measured by the thermodilution technique (Ganz et al., 1971a). A balloon-tipped Swan-Ganz (7F, 110 cm, Baxter-Edwards) thermodilution catheter was interfaced with a cardiac output computer (American Edwards model 9520 A). The technique involved the rapid injection of boluses (10 mls) of room temperature 5% dextrose solution into the right atrium and following mixing of the injectate with blood on passage through the right ventricle, the measurement of the resultant change in blood temperature in the pulmonary artery. Although the principle relating change in blood temperature to blood flow is the same as described above for coronary sinus flow, its computation is different due to the method of delivery of the indicator. Following a bolus injection, cardiac output is inversely proportional to the area under the resultant temperature – time curve. The values for cardiac output are the means of at least 3 determinations differing by less than 10%.

2.3.6 Blood sampling

In all studies, 10 ml blood samples for plasma neurochemical evaluation were obtained simultaneously from the arterial and venous catheters and immediately placed in chilled tubes containing an anticoagulant/antioxidant mixture of 0.30 M ethyleneglycol-bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid and 0.25 M reduced glutathione in 200 µl of water. At the completion of the catheter study and within 15-75 minutes of sampling the blood samples were centrifuged (800 g for 30 min at 4° C) and the plasma stored at -80° C until assayed. Blood and plasma flows were interconverted using the subject's haematocrit.

2.4 Cerebral venous blood pool scan

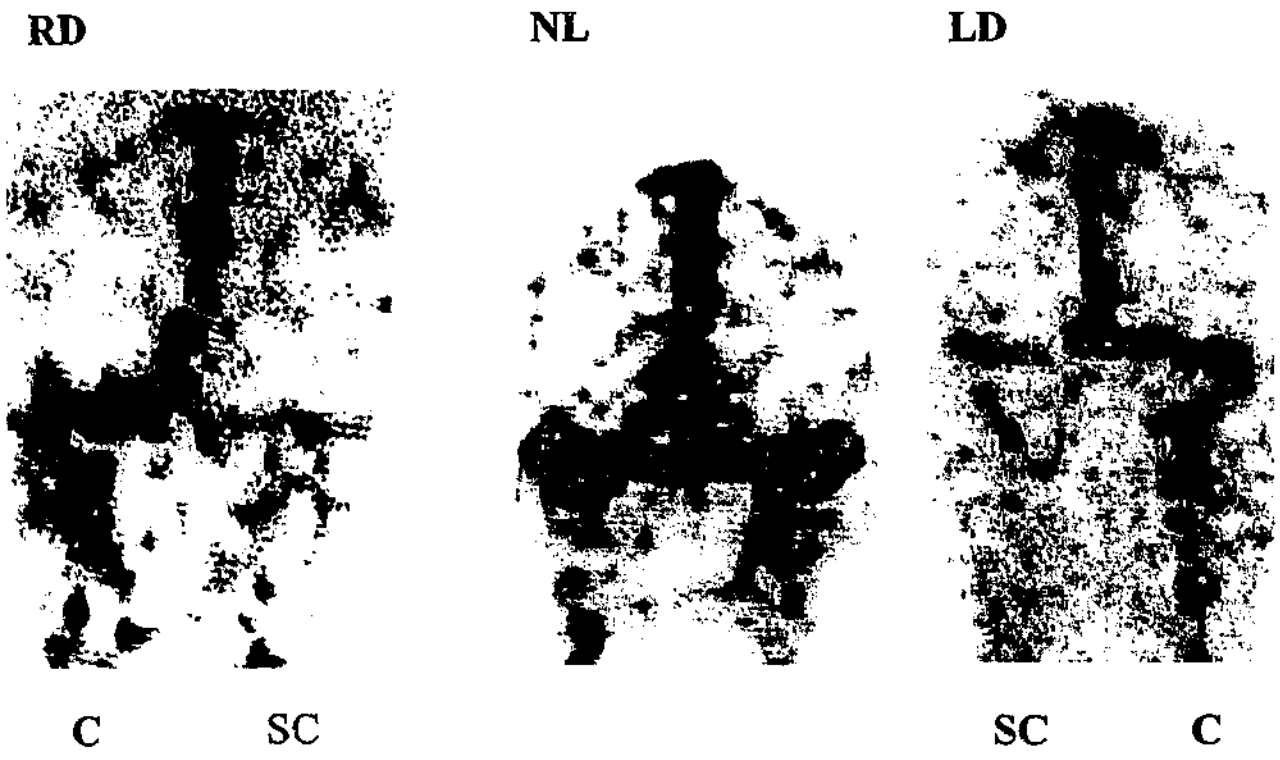
The cerebral venous sinus drainage of the human brain displays a degree of asymmetry with the route of superior sagittal sinus drainage (carrying the bulk of cortical venous return) more commonly being via the right internal jugular vein than the left (Gray, 1980), as presented in Fig 2.2. Cerebral blood flow scans were performed in the Division of Nuclear Medicine of the Alfred Hospital. Blood cells were labelled using the modified *in vivo* technique first described by Callahan et al (Callahan et al., 1982). In brief, 1 mg of stannous pyrophosphate was intravenously administered prior to an intravenous line being inserted into an antecubital vein. Ten minutes after the administration of the pyrophosphate 2-3 ml of blood was withdrawn from the intravenous line and incubated with 8 mCi of Technetium 99m for 15-20 minutes. The scintigraphic imaging procedure was then performed in two parts, a dynamic flow study followed by a single photon emission computed tomographic (SPECT) study of the cerebral blood pool. In the dynamic flow study the technetium labelled red cells were injected as a rapid bolus into the antecubital vein and images of the occipital region were taken immediately. Data was acquired in digital format on a large field of view gamma camera with a cut away head (General Electric 400AC) fitted with a high resolution collimator. Dynamic data was obtained for 32 sec at a frame rate of 2 sec per frame using a 64 x 64 matrix.

Ten minutes later, using the same gamma camera, the SPECT study was performed. Data was acquired as a 64 frame, 128 x 128 matrix series of 30 second images. This data was then prefiltered using a count density dependent Butterworth filter (typical critical frequency 0.3/cm, power factor 8.0) and reconstructed using a linear attenuation correction algorithm. Data was reformatted into transverse, coronal and sagittal images, each of approximately 1cm slice thickness. The plane of the transaxial images was based on a line drawn from the nuchal crest to the orbit. Images were photographed on transparencies for storage.

Data analysis consisted of visual inspection of the digital images of the dynamic flow and SPECT studies. In the dynamic flow study, the images were classified according to whether most of the sagittal sinus flow went to the left or to the right transverse sinus. Cases in which the direction of flow could not be determined were classified as indeterminant. SPECT analysis consisted of visual comparison of the degree of asymmetry between the radioactivity in the left and right transverse-sigmoid sinus systems. Almost invariably, the side nominated from the dynamic study as being the dominant route of sagittal sinus drainage was also the side with the greatest blood volume as determined by the degree of tracer activity. In those cases where the flow study was difficult to interpret, the SPECT study was also difficult to interpret. A representative scan is presented in Fig 2.4.

Retrospective analysis of cerebral blood flow scans of healthy volunteers performed in the past revealed that in 49% of cases, the route of superior sagittal sinus drainage was via the right internal jugular vein, and in approximately 25 % of cases the route of drainage was via the left internal jugular vein. In the remaining cases the cerebral sinus drainage displayed a non lateralizing pattern. In approximately 90 % of cases the internal jugular vein identified as the continuation of the superior sagittal sinus by the technetium blood flow scan agreed with thermodilution derived measurements of the internal jugular vein with the highest blood flow.

Figure 2.4 Cerebral venous blood pool scan



Patterns of cerebral venous drainage demonstrated by dynamic and static SPECT cerebral sinus tecnesium-99 scans representing a coronal slice through the junctions of the sagittal and transverse sinuses. Examples of internal jugular vein flow types, which allows selective sampling of cortical and subcortical-derived blood, and their representative venous scans are shown.
RD, right dominant (superior sagittal sinus drains to right internal jugular vein); LD, left dominant (sagittal sinus drainage to left internal jugular); NL, nonlateralizing (sagittal sinus drainage to both internal jugular veins); C, cortical drainage; SC, subcortical drainage.

2.5 Assessment of total and regional sympathetic nervous activity

In the previous chapter, several of the techniques, including the biochemical methods, used to measure sympathetic nervous activity in humans were discussed in the light of their relative strengths and limitations. In the following section, the radiotracer kinetic method for determining total and regional noradrenaline spillover to plasma is described in detail.

2.5.1 Total body noradrenaline spillover to plasma

The concept of measuring the rate of NA release to plasma, as an index of sympathetic nerve firing, began with the observation that NA appeared in the venous effluent of cat spleen during electrical stimulation of its nerve supply (Peart, 1949). The existence of a proportionality between nerve firing and transmitter release to plasma has been well validated (Yamaguchi et al., 1975; Blombery et al., 1983; Bradley et al., 1984; Kahan et al., 1984) and has provided the foundation for the use of measurements of NA spillover to plasma as an index of sympathetic nerve firing rates in experimental physiology and clinical medicine.

As mentioned in the previous chapter, only a small percentage (~10-20%) of neuronally released NA actually "escapes" from the synaptic cleft and diffuses into the plasma, with most of it being recaptured by neuronal uptake. It is on this basis of this "fraction" of NA appearing in plasma, that neuronal release rate of NA and hence sympathetic activity is inferred. Although in general, the rate at which NA released into the interstitial space spills over to plasma is proportional to the rate of sympathetic nerve firing, other factors may possibly influence this. These factors include:

- the adequacy of neuronal reuptake (Cousineau et al., 1984)
- capillary permeability and the surface area of the microcirculation available for exchange (Cousineau et al., 1984)
- regional organ blood flow (Esler et al., 1988)
- and presynaptic modulation.

2.5.2 Radiotracer kinetic determination of whole body noradrenaline spillover

In the studies presented, the noradrenaline appearance, or spillover, rate to plasma was determined by the principle of isotope dilution during an intravenous infusion of a tracer dose of tritiated noradrenaline. Levo-[7-^{3}H]-noradrenaline was infused for approximately 60 minutes prior to blood sampling to ensure that a plateau concentration of tritium was achieved and plasma noradrenaline specific activity could be determined (Esler, 1979). At steady-state, the total body noradrenaline spillover to plasma and the total body clearance rate of noradrenaline were determined according to the following formulas:

$$\text{Total Spillover Rate} = \frac{[^{3}\text{H}]\ \text{Noradrenaline Infusion Rate (dpm/min)}}{\text{Plasma NA Specific Radioactivity (dpm/pmol)}}$$

and

$$\text{Total Body Clearance} = \frac{[^{3}\text{H}]\ \text{Noradrenaline Infusion Rate (dpm/min)}}{\text{Arterial } [^{3}\text{H}]\ \text{NA concentration (dpm/ml)}}$$

Where NA=noradrenaline, dpm=disintegrations per minute of tritiated NA and [^{3}H]NA=tritiated NA.

Analogous to noradrenaline, adrenaline secretion rates can also be determined by the principle of isotope dilution, and calculated according to the above equations, during an infusion of radiolabelled adrenaline.

Assumptions that underly this method are as follows:

- Both tritium labelled and endogenous NA rates of entry to and removal from the plasma are equal. This hold only if the concentration of tracer and unlabelled NA is at steady-state (Esler, 1979).
- Recirculation of the tracer, by release from nerve varicosities after uptake, is negligible in comparison with the rate of infusion of tracer.
- The infusion of tritium labelled NA has no haemodynamic effects that might secondarily alter sympathetic nervous activity or NA clearance. The use of high specific activity NA infused at 0.8μCi/min has negligible influence on the plasma concentration (increase of 1-2ng/ml only) and is well below the threshold concentration for haemodynamic and metabolic effects (Esler, 1979; Silverberg et al., 1978) and,
- With respect to sampling from the plasma, infused tritium labelled NA is evenly mixed in a "central" plasma pool. NA kinetics are, infact, more complex than the simple "entry and exit from a common plasma pool" concept. Regional differences in the plasma concentration exist, dependent upon local processes of NA release and removal (Esler et al., 1988). Furthermore, NA is released in plasma from the pulmonary and systemic circulation in series. Although no ideal sampling site exists in such circumstances, arterial blood was sampled to avoid difficulties with mixed venous sampling and to include the contribution of pulmonary release and extraction of NA.

2.5.3 Regional noradrenaline spillover to plasma

The sympathetic outflow to individual organs is not uniform and regional sympathetic outflows, such as to the heart and kidneys, are capable of responding in a differentiated fashion to a variety of stimuli both physiological and pathophysiological (Hasking et al., 1986, Esler et al., 1988). Assessment of regional and organ-specific sympathetic activity therefore provides more accurate information regarding the sympathetic nervous function in health and disease.

The spillover of noradrenaline from an individual organ to plasma can be calculated according to the Fick Principle from the product of the venoarterial difference in plasma noradrenaline and the plasma flow with an adjustment allowing for the neuronal uptake of noradrenaline. Since NA flux is generally bidirectional, net overflow calculations tend to underestimate regional NA outward flux, and therefore an adjustment needs to be made for NA uptake.The neuronal uptake of noradrenaline is estimated from the fractional extraction of tritium labelled noradrenaline across an organ during a constant-rate infusion of radiolabelled noradrenaline (Esler, 1979; Esler et al., 1988; Esler et al; 1990). At steady state:

$$\text{Regional Noradrenaline Spillover} = [(NA_{ven} - NA_{art}) + (NA_{art} \times NA_{ex})] \times \text{Plasma Flow}$$

where NA_{ven} and NA_{art} are the venous and arterial noradrenaline concentrations respectively and NA_{ex} is the fractional extraction of tritiated noradrenaline in a single passage through an organ.

2.5.4 Preparation and administration of tritium-labelled catecholamines

2.5.4.1 Supply

Levo-[7-^{3}H]-noradrenaline (specific activity of 11-25 Ci/mmol) and levo-[N-methyl-^{3}H]-adrenaline (specific activity 69-78) were obtained from New England Nuclear

(Boston, MA, USA) packaged in 0.2 M acetic acid: ethanol (9:1), under argon and shipped in dry ice.

Levo-[7-^{3}H]-noradrenaline was prepared enzymatically from [7-^{3}H]-dopamine using partially purified bovine dopamine-β-hydroxylase and purified chromatographically. The radiochemical purity of the noradrenaline, as determined by the manufacturer using HPLC on a cation exchange column using a mobile phase of 25 mM potassium phosphate (pH 4.3), was typically between 99.4 and 99.9%. Tritium nuclear magnetic resonance analysis revealed the ^{3}H label to be exclusively at the C-7 position.

Levo-[N-methyl-^{3}H]-adrenaline was prepared enzymatically from cold levo-noradrenaline and S-[methyl-^{3}H]-adenosyl-L-methionine. The radiochemical purity of the adrenaline, as determined by the manufacturer using HPLC on a Zorbax SCX column using a mobile phase of 100 mM potassium phosphate (pH 4.3) was typically between 97.7 and 99.9 % with less than 0.1 % of the total radioactivity co-chromatographing with S-[methyl-^{3}H]-adenosyl-L-methionine.

2.5.4.2 Preparation

The stock tritiated adrenaline and noradrenaline were prepared identically but separately for later use in catheter studies. Each ^{3}H-catecholamine was diluted in 0.2 M acetic acid in normal saline containing 10 mM ascorbic acid under a laminar airflow hood in the drug preparation laboratory of the Alfred Hospital. The diluted catechols were then sterilised by filtration through a Millex-GS 0.22 μm filter unit (Model SLGS025OS, Millipore Products, Bedford, MA, USA) and divided into 3-6 ml aliquots containing approximately 50 μCi/ml. The first and last sample prepared of each batch were cultured in the microbiology department of the Alfred Hospital to confirm sterility.

2.5.4.3 Administration

Immediately prior to the commencement of a study, a single vial of ^{3}H-noradrenaline (± a vial of ^{3}H-adrenaline) was thawed and diluted 1:12.5 in normal saline, resulting

in a final concentration of approximately 4 μCi/ml. After a bolus injection of 16 μCi, the tritium labelled catecholamines were infused intravenously via a peripheral vein at 0.8 μCi/min. This rate has been demonstrated to yield readily measurable concentrations of tritium in plasma and alter total plasma noradrenaline concentrations by only 0.01% (Esler, 1979).

2.5.5 Arterial and venous plasma sampling

Ten mls of paired arterial and venous blood samples were collected during the course of the studies presented. This blood was transferred to tubes containing EGTA and reduced glutathione. All samples were placed on ice immediately upon collection. Plasma was separated by centrifugation at 4°C upon completion of the study and was subsequently stored at -70°C until assayed.

2.6 HPLC determination of catecholamines

The neurochemical analysis of the plasma samples obtained was achieved using high performance liquid chromatography (HPLC) coupled with electrochemical detection.

2.6.1 General chromatographic system

The chromatographic system, common to all assays, consisted of a Model 510 Solvent Delivery System (Waters Millipore, MA, USA), Model 5100A coulometric detector, Model 5021 conditioning cell, Model 5011 analytical cell (Environmental Sciences Associates, MA, USA), Shimadzu Model C-R4A Chromatopac integrater (Shimadzu Corporation, Kyoto, Japan), Model AS-100 HRLC automatic sampling system (Bio-Rad Laboratories, Richmond, CA, USA) and a 25 cm Altex Ultrasphere column (ODS 4.6 mm x 25 cm, 5μ particle size: Beckman Instruments, Inc. CA, USA).

2.6.2 Plasma catechol determination

The catechols, DOPA, dopamine, noradrenaline, adrenaline, DOPAC and DHPG, were extracted from plasma with alumina adsorption, separated by HPLC and the

amounts quantified by electrochemical detection according to previously described and validated methods (Eisenhofer et al., 1986), (Medvedev, Esler, Angus, Cox, & Eisenhofer, 1990).

2.6.2.1 Reagents and HPLC conditions

The catechol standards and internal standard, dihydroxybenzylamine (DHBA), were all obtained from the Sigma Chemical Company. The mobile phase, delivered at a flow rate of 1.2 ml/min, consisted of: 0.1 M sodium dihydrogen phosphate, 0.05 mM EDTA, 0.16 mM octanesulfonic acid (Sigma Chemical Company), and 0.5% HPLC grade acetonitrile. The pH was set at 3.4 with concentrated phosphoric acid. The mobile phase was degassed by vacuum filtration through a 0.2 µm Millipore membrane prior to use. The operating potentials of -0.35 V for the guard cell and +0.35 and -0.30 V for detectors 1 and 2 respectively were chosen after generating hydrodynamic voltnammograms for all compounds and determining the highest signal-to-noise ratio. All measurements were made using the reducing potential applied at detector 2 and compounds in plasma extracts were identified by their retention behaviour compared to that of authentic standard solutions.

2.6.2.2 Plasma extraction of catecholamines

Plasma samples were thawed at room temperature and placed on ice until required. Two ng of DHBA, 400 µl of 1 M TrisHCl / EDTA (pH 8.6) and approximately 20 mg of acid washed alumina was added to 1 ml of plasma. The samples were briefly vortexed and then gently shaken on a rotary mixer for 30 minutes. The plasma-Tris effluent was discarded and the alumina particles were washed with 1 ml of freshly prepared 0.2M $NaHCO_3$ followed by 2 ml deionised water, each time the samples were vortexed, centrifuged and the effluent discarded. One hundred µl of a solution containing 5-20 % 0.2 M phosphoric acid in 0.2 M acetic acid was added to the alumina and the catechols were eluted by vortexing for 2 minutes. After centrifugation the eluant was transferred into microsample vials and the acid elution

step repeated with a further 50 µl of acid. The acid eluates were pooled and the total volume injected onto the HPLC for analysis. The outlet of the analytical cell was connected to a Pharmacia PSV-100 switching valve (Uppsala, Sweden) which directed the effluent to either the mobile phase reservoir or to scintillation vials loaded within a carousel on a Pharmacia FRAC-100 fraction collector. Fractions of the eluant leaving the electrochemical cell were collected for measurement of tritium labelled noradrenaline and adrenaline by liquid scintillation spectroscopy Model 1900CA Liquid Scintillation Analyzer, Packard Instrument Company, Downer's Grove, Il, USA). All compounds of interest in the plasma extracts were separated from each other and the solvent front. Plasma concentrations of ^{3}H-labelled and endogenous catecholamines were corrected according to the recovery of the internal standard. Compounds whose recovery differed to that of DHBA were adjusted according to the recovery of external standards.

2.6.3 Reagents and HPLC conditions for plasma MHPG determination

The internal standard was 3-ethoxy-4-hydroxyphenylglycol (EHPG). MHPG was obtained from the Sigma Chemical Company and sequanal grade ethyl acetate was purchased from Pierce (Rockford, Ill, USA). The mobile phase, delivered at a flow rate of 1.0 ml/min, consisted of 0.1M sodium dihydrogen phosphate, 0.1mM EDTA and 3% acetonitrile. The pH was set at 3.4 with concentrated phosphoric acid. The mobile phase was degassed by vacuum filtration through a 0.2 µm Millipore membrane prior to use. The operating potentials were chosen after generating a hydrodynamic voltnammogram for MHPG and determining the highest signal-to-noise ratio. The conditioning cell electrode was set at + 0.3 V and the first and second electrodes of the analytical cell were set at +0.15 and -0.55 V respectively. Measurements were made using the final electrode in the series. MHPG and EHPG in plasma extracts were identified by their retention behaviour compared to that of putative standards.

2.6.3.1 Plasma extraction of MHPG

MHPG was extracted from plasma using an adaptation of the technique used by Eisenhofer et al (Eisenhofer et al., 1988). Twenty ng of EHPG was added to 0.5 ml of plasma and the samples were deproteinated using an AMICON micropartition system utilising YMT ultrafiltration membranes (AMICON Scientific Australia, Vic, Aust) by fixed angle centrifugation at 2000g for 45 min at 4 ^{o}C. Membranes were then rinsed with 150 µl distilled water and centrifuged for a further 45 min. MHPG was extracted from the ultrafiltered plasma by vigorous shaking for 30 sec with 2.5 ml sequanal grade ethyl acetate. This step was repeated and the acetate aliquots pooled. Samples were then dried under gaseous nitrogen (ICI, Vic, Aust) and reconstituted in 120 µl mobile phase and quantified by HPLC with coulometric detection.

2.7 Statistical analysis

The statistical methods used to analyse data varied slightly between chapters according to the type of data and the comparisons made. In general, all results, unless otherwise specified, are express as mean ± standard error of the mean. The null hypothesis was rejected at $p < 0.05$. Between group comparisons were performed using Student's t-test or one-way analysis of variance for multiple groups. Statistical techniques peculiar to specific chapters are described in full in the Methods section of the relevant chapter.

Chapter 3.

Pole of Presynaptic α_2 – and β_2 - adrenoceptors in Modulating Noradrenaline Release in the Forearm

PART A

Peripheral α_2- adrenoceptors

PART B

Peripheral β_2- adrenoceptros

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PART A

Peripheral α_2- adrenoceptors

3.1A Introduction

Sympathetic nervous activation is a hallmark of congestive heart failure (CHF) that is a powerful predictor of prognosis (Cohn et al., 1984; Meredith et al., 1991; Kaye et al., 1995). Therapy with β-adrenergic blockade has been shown to enhance systolic function and to improve prognosis (Packer et al., 1996; CIBIS, 1999). However, such an approach does not entirely negate the detrimental effects of the exaggerated sympathetic outflow observed in CHF. The effects of vasoactive sympathetic co-transmitters, such as neuropeptide Y (Maisel et al., 1989) are not attenuated; further, the high renal sympathetic tone in CHF is left unchecked, with limited evidence indicating that carvedilol has a neutral effect on glomeular filtration rate and renal blood flow (Dupont, 1992).

A logical step in therapy of CHF might be to investigate the value of reducing sympathoneural neurotransmitter release. Increased activity of central noradrenergic neurons, as reflected indirectly by the increased rates of central monoamine turnover, has been demonstrated in CHF patients (Lambert et al., 1995a, Kaye et al., 1994). This observation has renewed interest in the possibility of central sympathetic inhibition. While it is known that there are sympathoinhibitory α_2–adrenoceptors in the brainstem (Chalmers et al., 1991), they are also known to exist presynaptically on peripheral sympathetic nerves (Leier et al., 1990). The issue of peripheral receptor functionality is of clinical interest given that centrally-acting sympatholytic drugs such as clonidine tend to possess untoward side-effects, such as sedation and depression. If it were evident that peripheral α_2–adrenoceptors are physiologically active in CHF, then future drug therapy could be targeted directly at them. In so doing, direct sympathetic attenuation could be achieved without the side-effects resulting from central α_2–adrenoceptor activation.

The evidence to date of peripheral α_2–adrenoceptor functionality is somewhat conflicting. Kiowski et al (Kiowski et al., 1985) infused clonidine into the brachial artery of healthy volunteers, and in doing so, were able to study the effects of local α_2–adrenoceptor stimulation on forearm NA release, as measured by a venous-arterial gradient. The investigators were unable to demonstrate a reduction in forearm NA "spillover". However, this study preceded the technique of radioisotope dilution, and it is possible that a high NA extraction across the forearm led to an underestimate of local NA release. In a more recent study, Grossman et al (Grossman et al., 1991a) demonstrated, by administering locally active agents, that α_2–adrenoceptors do modulate release of NA from vascular sympathetic nerve endings in healthy humans.

In states of high adrenergic drive, such as congestive heart failure, with overexposure of adrenoceptors to catecholamines, alterations in receptor number and/or function do occur. The premier example of this is the down-regulation that is found in the β-adrenoceptor system (Bristow et al., 1982). However, there have been a few studies to date, comparing the effects of local α_2–adrenoceptor stimulation on NA release, in CHF and healthy volunteers (Kubo et al., 1989). When phentolamine, a nonselective α-adrenergic antagonist, was administered as an intracoronary infusion into patients with normal left ventricular function and those with CHF, only those patients with CHF demonstrated an increase in coronary sinus NA concentration (Parker et al., 1995). This finding led the authors to conclude that pre-synaptic α_2–adrenoceptors exert an inhibitory effect on NA release from cardiac nerves in patients with CHF. However, a major limitation of the study was that only the venous–arterial gradient was employed to measure myocardial NA release. Coronary sinus blood flow and radioisotope dilution, when factored in with a venous–arterial gradient, allow measurement of cardiac NA spillover, a more accurate index of the activity of cardiac sympathetic nerves.

In a different approach to this issue, Weiss et al have demonstrated that there is a decrease in platelet α_2–adrenoceptors in CHF patients, having established that human platelets have α_2–adrenoceptors similar to those present on noradrenergic nerve

terminals (Weiss et al., 1983). However, in the absence of sympathetic innervation of platelets, the physiological importance of this finding is uncertain.

3.2A. Aim of study

The aim of this study was to investigate the role of peripheral presynaptic α_2–adrenergic receptors in modulating NA release in health and in congestive heart failure.

3.3A Methods

3.3.1A Patient characteristics

The study group comprised a consecutive series of 10 patients with heart failure (9males, 1 female, age 58.1 ± 5.8 years) and 15 healthy volunteers (all male, 45.9 ± 8.5 years), age ± SD. The patients with CHF were all in New York Heart Association functional class II or III for heart failure severity. Their left ventricular ejection fraction (LVEF) was 23.8 ± 4.4% (mean ± SD). Of these patients, six had an ischaemic and four had a dilated cardiomyopathy. No patient had diabetes or a diagnosed peripheral neuropathy. All patients continued taking their normal medications. No patient was taking a β-blocker, α_2–adrenergic agonist or antidepressant medication. Medications consisted of an angiotensin-converting enzyme inhibitor, digoxin and diuretics. Both the patients' clinical condition and their medications had been stable for at least one month. The healthy control subjects were recruited by advertisement in the general community. The study was performed with the approval of the Alfred Hospital Ethics Review Committee and all the subjects gave written informed consent.

3.3.2A Experimental procedures

All experiments were performed in the morning after a light breakfast. All subjects had refrained from smoking and consuming caffeinated beverages over the 12 hours before the procedure. Forearm volume was measured by water displacement. Under a

local anaesthetic, the brachial artery of the nondominant arm was cannualted (3F, 5cm, Cook, Brisbane, Australia) for arterial pressure monitoring, blood sampling and drug administration. In the same arm, the antecubital vein was cannulated for deep venous (skeletal muscle drainage) blood sampling (5F Hoffman sheath, Cook). This cannula was advanced retrogradely into the forearm so that its tip could no longer be palpated, or for a distance of 10 cm. Forearm blood flow (FBF) was measured by strain-gauge venous occlusion plethysmography (Benjamin et al., 1995), as described in Chapter 2 (Figure 2.1). For each determination, four to five measurements were performed, and the results were averaged. Hand blood flow was excluded by the use of a cuff at the wrist that was inflated to suprasystolic levels.

3.3.3A Study protocol

The noradrenaline (NA) isotope-dilution technique, as described in chapter 2, was employed to provide a biochemical index of global and forearm sympathetic nerve activity. Once steady-state plasma concentrations of tritiated NA had been achieved, baseline FBF was measured. Arterial and deep venous samples were obtained for calculation of forearm and global NA spillover. Then clonidine (Boehringer-Ingleheim, Ingeheim, Germany) was infused into the brachial artery at 0.05 μg/100 ml forearm/ 5 min. The FBF measurements and blood sampling were again undertaken. Immediately after this, clonidine was administered intra-arterially at 0.48μg/100 ml forearm/ 5 min. All measurements were then repeated.

After an interval of > 30 min, intravenous clonidine was successively administered at two doses, 1 and 2 μg/kg, each infused over 10 min. At the end of each infusion, FBF and blood sampling for catecholamine spillover was again performed.

3.3.4A Noradrenaline spillover measurements

Calculations of global NA spillover were performed according to the methods described in Chapter 2. Forearm spillover (FSO) of NA was obtained as follows:

$$\text{FSO of NA} = [\ (C_V - C_A) + C_A \times (NA_E)\ ] \times PF$$

Where C_V is the plasma NA concentration in the deep forearm vein, C_A is the arterial plasma NA concentration, and NA_E is the fractional extraction of radio-labeled NA across the forearm.

3.3.5A Plasma appearance rate of noradrenaline

As discussed in more detail below, regional NA spillover can be influenced by blood flow. Recently, the plasma appearance rate (PAR) of NA has been proposed as a flow-independent measure of regional sympathetic activity (Chang et al., 1994; Rongen et al., 2000):

$$PAR = \frac{\text{FSO of NA}}{1 - [NA_E]}$$

3.3.6A Statistical analysis

Data are presented as mean value ± SEM, unless otherwise stated. Statistical analysis was performed using statistical software (SigmaStat, version 2.03, Chicago, Illinois). Within – group analysis of variance (ANOVA) and the Tukey Test was employed in post-hoc analysis. Group differences were obtained using two-way repeated measures ANOVA with the Bonferroni multiple comparison test. A p value of <0.05 was considered statistically significant.

3.4A Results

The baseline comparisons between the two groups are given in Table 3.1. There was no significant difference in baseline FBF and FSO of NA. However, the patient group had a much higher total body spillover of NA than the healthy control subjects.

Table 3.1 Baseline Characterisics

Parameter	Control	Heart Failure	p Value
FBF, ml/min	21.3 ± 3.8	24.7 ± 7.3	NS
FSO, pmol/min	12.1 ± 1.4	8.97 ± 2.65	NS
TBS, nmol/min	1.86 ± 0.33	5.26 ± 1.39	0.01

Where FBF is forearm blood flow, FSO is forearm spillover of NA, TBS is total body spillover of NA and NS is nonsignificant.

3.4.1A Effects of intra-arterial clonidine on forearm blood flow

Intra-arterial clonidine at the two doses did not result in any significant changes in global NA spillover (control 1.86 ± 0.33 to 1.87 ± 0.27 and 1.86 ± 0.28 nmol/min, and CHF 5.26 ± 1.39 to 4.44 ± 1.1, 4.33 ± 1.2 nmol/min).

I/A clonidine caused a decrease in FBF in both groups (Fig. 3.1A and 3.1B). At the low dose, there was a small nonsignificant fall in FBF: 21.3 ± 3.8 to 19.4 ± 2.7 ml/min in the control group and 24.7 ± 7.35 to 23.2 ± 6.4 ml/min in the patient group. With the high dose, the changes in FBF were 21.3 ± 3.8 to 15.4 ± 2.0 ml/min (p=0.01) and 24.7 ± 7.35 to 14.8 ± 3.5 ml/min (p=0.015), respectively. At the higher dose, this represented a decrease in FBF in the control and patient groups of 28% and 40%, respectively (p=NS for between group comparison).

Figure 3.1A Line graph representing the change in forearm blood flow (FBF) in response to I/A clonidine in healthy volunteers, n=15.

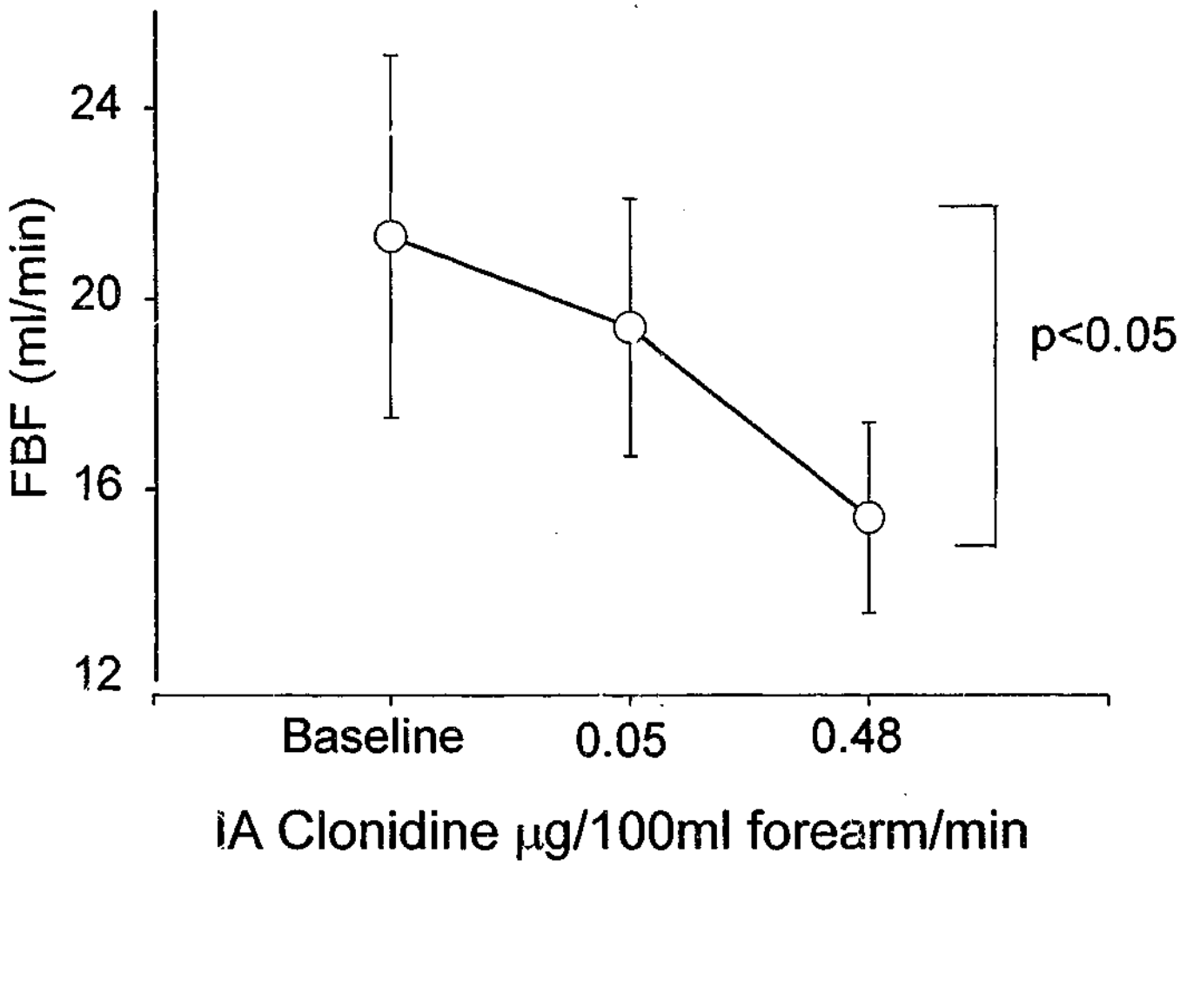
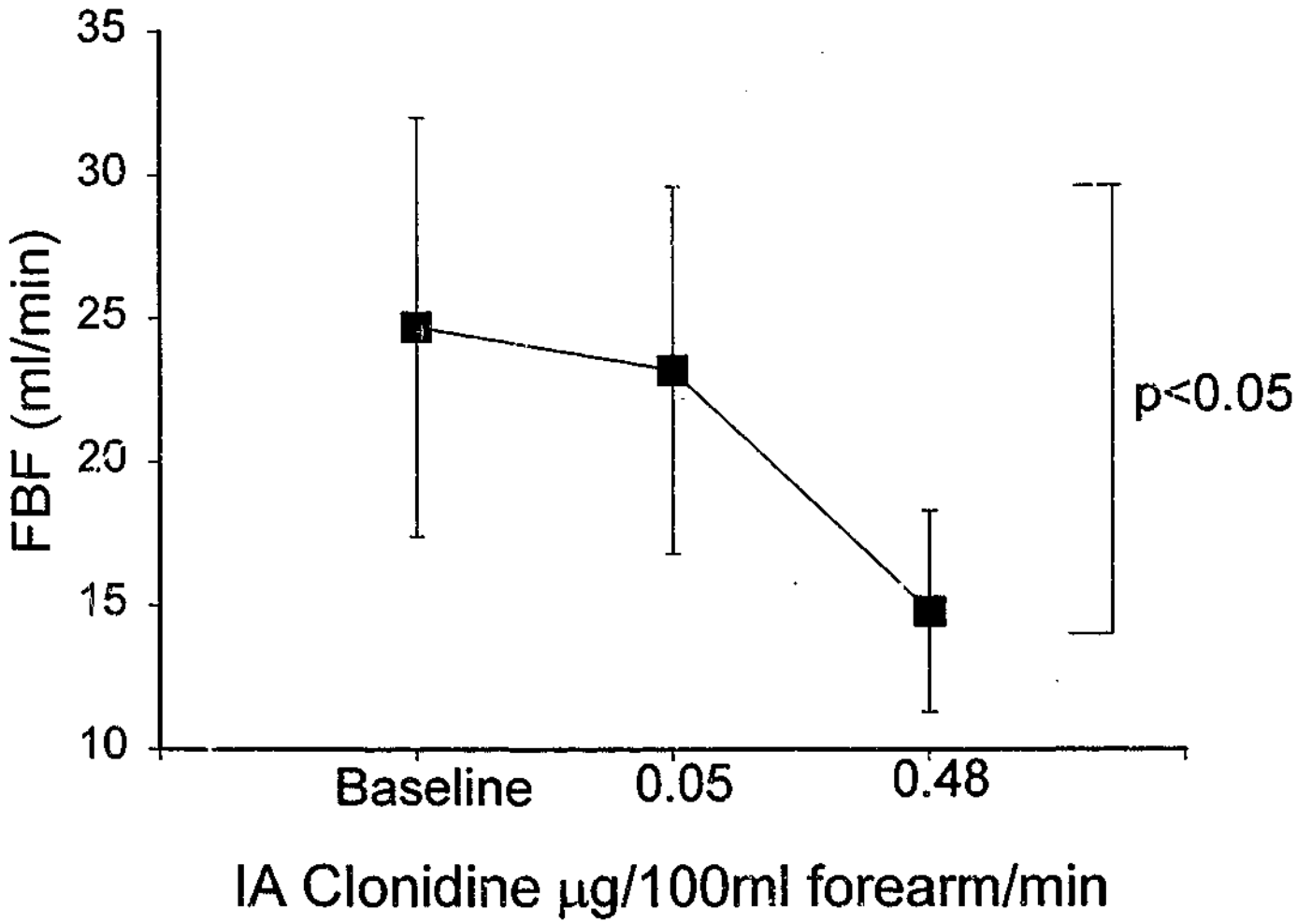


Figure 3.1B Line graph representing the change in forearm blood flow (FBF) in response to I/A clonidine in CHF patients, n=10.



3.4.2A Effects of intra-arterial clonidine on forearm noradrenaline spillover

With low dose I/A clonidine, FSO of NA in the control group decreased from 12.1 ± 1.4 to 8.96 ± 1.18 pmol/min (p=0.03). In the patient group, a nonsignificant increase in FSO was seen from baseline of 8.97 ± 2.65 to 14.3 ± 6.23 pmol/min. High dose I/A clonidine resulted in a change from 12.1 ± 1.4 to 6.19 ± 1.01 pmol/min (p< 0.001) and 8.97 ± 2.65 to 11.88 ± 5.72 pmol/min (p=NS), respectively (Fig 3.2A and 3.2B). The difference in the response to I/A clonidine between the two groups (decrease by 49% and increase by 32%) was significant (p=0.004).

3.4.2.1A Plasma appearance rate of noradrenaline

Since regional NA spillover is to some extent dependent on blood flow, the plasma appearance rate (PAR) of NA in the forearm was also calculated. In the control group, PAR (pmol/min) decreased from 30.3 ± 7.4 to 18.1 ± 3.4 (p<0.05) and to 14.7 ± 3.6 (p<0.001) with the two doses of i/A clonidine. In the patient group, the change in PAR was from 9.1 ± 2.4 to 13.9 ± 7.2 (p=NS) and to 13.5 ± 3.3 (p= NS). Again, as with FSO, the difference in response to I/A clonidine between the groups was significant (p=0.005).

Figure 3.2A Line graph represents the forearm spillover (FSO) of noradrenaline (NA) in response to intra-arterial (I/A) clonidine, in healthy volunteers, n=15.

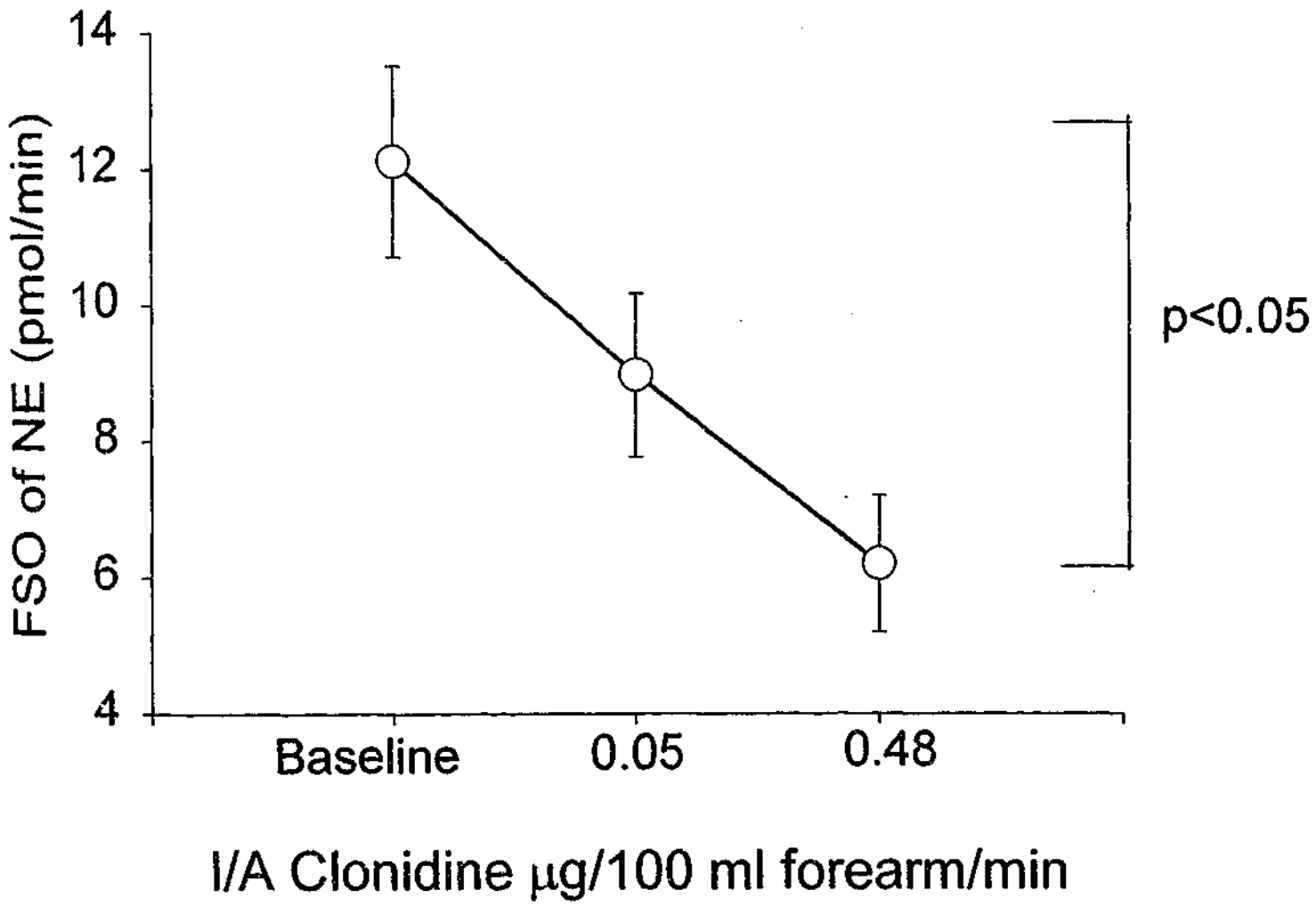
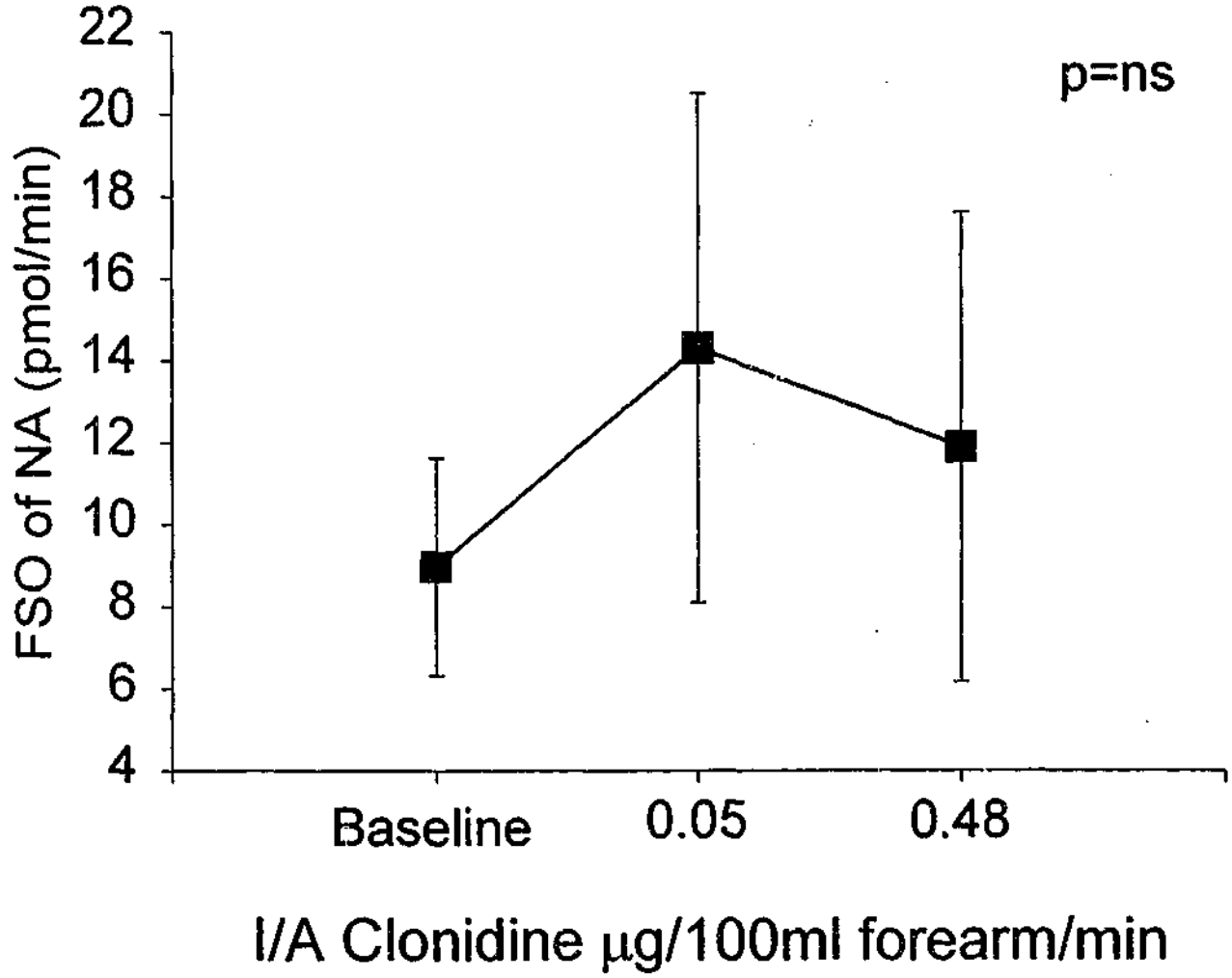


Figure 3.2B Line graph represents the forearm spillover (FSO) of noradrenaline (NA) in response to intra-arterial (I/A) clonidine, in CHF patients, n=10.



3.4.3A Effects of intravenous clonidine

IV clonidine resulted in a reduction in the systolic blood pressure: in the control group, from 134 ± 5.8 to 127 ± 4.4 and 114.3 ± 4.0 mmHg, p= 0.02 and p<0.001, respectively and in the patient group, from 125.8 ± 9.8 to 116.4 ± 9.2 and 100.2 ± 6.2 mmHg, p=NS and p=0.01, respectively. There was no significant change in FBF in either group with IV administration.

In the control subjects, IV clonidine resulted in a decrease in FSO (12.1 ± 1.4 to 6.87 ± 0.95 and 3.12 ± 0.70 pmol/min, p<0.001). In patients with CHF, IV clonidine elicited a change in FSO from 8.97 ± 2.65 to 10.2 ± 4.1 at low dose and 5.43 ± 3.25 pmol/min at the higher dose (p=0.06).

When examining the effects of IV clonidine on global NA spillover, the following results were obtained: in the healthy subjects, 1.86 ± 0.33 to 1.62 ± 0.28 and 1.01± 0.18 nmol/min, p=NS and p<0.001; in the patients, 5.26 ± 1.39 to 3.49 ± 0.84 and 2.48 ± 0.74 nmol/min, p=NS and p=0.004.

3.5A Discussion

3.5.1A Effects of intra-arterial clonidine on regional blood flow and noradrenaline release

The novel finding of this study is that NA spillover in the healthy forearm is substantially reduced by intra-brachial artery clonidine, but that this effect is lost in CHF patients. This indicates a down-regulation of the peripheral α_2–adrenoceptor functionality in heart failure.

As previous investigators have found, I/A clonidine does result in a reduction in FBF (Kiowski et al., 1985), and in our study, this effect was preserved in CHF (Kubo et al., 1989). Most of the postsynaptic α–adrenoceptors located within the nerve junction are

of the α_1–type (Leier et al., 1990). Clonidine has a high, though not absolute, selectivity for the α_2–adrenoceptor (Kiowski et al., 1985), so that the demonstrated reduction in FBF in patients with CHF may be due to vasoconstriction caused by activation of the postsynaptic α_1-adrenoceptor on vascular smooth muscle. Alternatively, in heart failure, down–regulation of neuronal presynaptic α_2–adrenoceptors may occur in the absence of down–regulation of the vascular α_2–adrenoceptors, which remain capable of eliciting unimpaired vasoconstriction. In support of this hypothesis, Hein et al have shown functional differences within the α_2-adrenergic subtypes (α_{2A}, α_{2B}, α_{2C}) in mice, raising the possibility that they may be differentially expressed and regulated (Hein et al., 1999).

3.5.2A Regional noradrenaline spillover and its dependence on flow

In this study and others (Kiowski et al., 1985), intra-arterial clonidine resulted in a significant reduction in FBF. The reduction in forearm NA spillover that was demonstrated in the control group in this study could have resulted from true inhibition of NA release, or purely from a reduction in forearm blood flow.

Forearm NA spillover is a measure of the outward "flux" of NA from the forearm to the plasma. Whilst regional calculations of NA spillover have been found to correlate strongly with directly recorded sympathoneural activity in rat kidneys (Deka-Starosta et al., 1989), and in limbs of humans (Grossman et al., 1991b), there are several factors that can potentially affect this relationship. Remembering that only a small fraction of the released NA enters plasma, with the majority returning to the nerve varicosity via the NA transporter, the extent of neuronal reuptake is influenced by factors including synaptic cleft width, and organ blood flow.

The potential flow dependency of NA spillover can easily be explained as follows. In steady state there is a concentration gradient between the sites of release in the tissue and the blood that depends on diffusion equilibrium between the NA that is being secreted in the tissues and the NA that is in the blood. When regional blood flow is low, the transit time of the NA that is in the blood is longer and, therefore, more NA can diffuse back into the tissue and can be removed by neuronal and extraneuronal processes. Conversely, when the blood flow is high, the transit time of the blood is short and, as there is a high concentration of NA at the sites of release in the tissue favoring diffusion into the blood, both more of the NA originating from the tissue and entering the organ with the blood will be washed out of the organ.

Flow dependence of NA spillover is typically not observed in the kidney (Esler et al., 1988), but forearm NA spillover has been demonstrated to be dependent on flow (Grossman et al., 1991b; Rongen et al., 2000). For example, neither sodium nitroprusside nor methoxamine affect neuronal NA release directly, yet, when infused into the brachial artery, both substances change forearm blood flow and forearm NA spillover (Grossman et al., 1991b).

Although it is appreciated that regional spillover of NA is altered by changes in blood flow, decreasing with declining flow, we believe that this study provides evidence for a true peripheral sympatholytic effect of clonidine in the healthy human, as a result of activation of peripheral α_2-adrenoceptors. The arguments for this conclusion are as follows. Firstly, on existing evidence, a reduction in regional NA release of 50% with I/A clonidine is too profound to have resulted alone from a flow reduction of 28% (Esler et al., 1990; Esler et al., 1979). Most importantly, no reduction in regional NA release was seen with local α_2-adrenoceptor stimulation in the heart failure group, despite a 40% decrease in FBF, a reduction that was similar in magnitude to that

observed in the control group. Finally, the plasma appearance rate, which has been proposed to be a largely flow independent measure, also showed a dose-dependent reduction in forearm NA spillover in the control group.

3.5.3A Plasma appearance rate of noradrenaline

In a recent validation of this method (Rongen et al., 2000) initially proposed by Chang et al (Chang et al., 1994), the effects of two interventions known to exert contrasting actions on neuronal forearm NA release and forearm blood flow were compared. For calculation of forearm NA release, both the usual kinetic equation and the modified formula, were used. Locally administered SNP increased forearm blood flow, reduced forearm NA extraction, and increased forearm NA spillover. However, when the PAR calculation was used, there was no significant change from baseline. Conversely, when lower body negative pressure was applied, a manoeuvre known to increase sympathetic drive to the forearm, forearm blood flow fell. The forearm NA spillover rate did not increase in response to this intervention, but the increased sympathetic drive to the forearm was detected as a rise in the forearm PAR of NA. These findings led the authors to conclude that the noradrenaline appearance rate provides the better approximation of changes in forearm neuronal noradrenaline release in response to stimuli which alter local blood flow.

3.5.4A Effects of systemic clonidine on regional noradrenaline spillover

The well-recognised sympatho-inhibitory actions of intravenous clonidine (Isaac, 1980; Azevedo et al, 1999) have been explained pharmacologically in the context of two possible sites of action: in the periphery on sympathetic nerves, and the brain centres controlling sympathetic outflow. In this study, control subjects demonstrated a

dramatic reduction in regional (74%) and global (46%) spillover. In the CHF group, IV clonidine resulted in a 54% reduction in global NA spillover; however, only a trend to reduction in regional NA spillover was seen. An explanation for these disparate effects of IV clonidine on forearm spillover of NA in CHF could be that the major determinant of forearm NA spillover is local rather central α_2–adrenoceptor activity, and that these autoinhibitory peripheral receptors are down-regulated, with preservation of the central α_2–adrenoceptors. Alternatively, it is also possible that, in CHF, there is a global down-regulation of the α_2–adrenoceptor, and the inhibitory effects on whole body NA spillover are mediated through central imidazoline receptors (Guyenet, 1997).

3.5.5A Clinical implications

Moxonidine, an imidazoline ligand acting on the CNS receptors to decrease sympathetic activation, has been demonstrated to result in a dramatic reduction in global NA release in patients with heart failure (Swedberg et al., 2000). It should be noted that the "MOXCON" study, designed to examine the effect of moxonidine in heart failure, was recently terminated because of excess mortality in the treatment group. Given that moxonidine has a powerful sympatholytic effect, it is unclear why this favorable effect should be associated with excess mortality in heart failure. A detailed report of the study is yet to be published.

3.6A Conclusions

This study aimed to clarify the importance of presynaptic α_2-adrenoceptors in the regulation of NA release in health and in heart failure. When compared with control subjects, a substantial down-regulation of peripheral α_2-adrenoceptor functionality

was demonstrated in CHF. This data, therefore, mitigates against a likely clinical role for agents that specifically target these receptors. As a further conclusion, the findings suggest that the lack of sympathoinhibitory activity demonstrated by these receptors in heart failure may possibly contribute to the higher NA release in CHF, due to a failure of the auto-inhibitory feedback mechanism in the synaptic cleft (Dixon et al., 1979).

PART B

Peripheral β_2-adrenoceptros

3.1B Introduction

Controlled clinical trials have shown that β-blockers produce consistent benefits in patients with CHF (CIBIS, 1994; Packer et al., 1996) and have become an integral part of modern heart failure treatment. However, β-blockers differ significantly in their pharmacological properties. Metoprolol and bisoprolol selectively inhibit β_1–receptors; propranolol blocks both β_1-and β_2-receptors; and carvedilol blocks α_1-,β_1- and β_2–receptors.

Although β-adrenergic blockade has become a central component of heart failure treatment, it is not yet clear whether one type of β-blocker offers a particular clinical advantage. One point of difference may be the extent to which cardiac sympathetic activity is inhibited. Stimulation of prejunctional β_2–adrenergic receptors on adrenergic nerve terminals has been demonstrated to augment NA release (Majewski, 1983; Chang et al., 1994; Newton et al., 1999), a finding that lends support to the "adrenaline hypothesis" as discussed in Chapter 1. In healthy hearts, intracoronary salbutamol, a β_2–adrenoceptor agonist, has been observed to increase noradrenaline release from cardiac sympathetic nerves (Newton et al., 1999), as evidenced by the observations that atenolol suppressed the salbutamol inotropic response (thereby demonstrating that this response was mediated in part by β_1-receptors), but not the increase in cardiac NA spillover. The authors contend that this result provides in vivo evidence, in humans, for the role of sympathoexcitatory presynaptic cardiac β_2-receptors.

Recently, the significance of these receptors in the setting of human heart failure has been the subject of further study (Newton et al., 1996; Azevedo et al., 2001). Newton et al administered intravenous propranolol and metoprolol to a group of heart failure patients. At similar haemodynamic end-points, metoprolol increased and propranolol decreased cardiac NA spillover. A possible explanation for this finding is that facilitative β_2-adrenoceptors were antagonised by propranolol. Azevedo et al (2001) randomised CHF patients to receive either metoprolol or carvedilol, with subjects undergoing microneurography in addition to measures of cardiac and whole body NA spillover. Unlike metoprolol, carvedilol reduced cardiac and global sympathetic activity. Microneurographic measures of sympathetic nerve traffic to skeletal muscle did not change in either group, leading the authors to conclude that carvedilol caused its sympathoinhibitory effect by blocking peripheral presynaptic β_2-adrenoceptors. However, Kaye et al (Kaye et al., 2001) did not find that carvedilol reduced cardiac NA spillover. To avoid any systemic actions, we infused propranolol into the brachial artery of both healthy and heart failure subjects and studied forearm adrenaline and noradrenaline kinetics.

3.2B Aims of study

As an adjunct to the forearm clonidine study described above, we sought to study adrenaline kinetics in the forearm, and then by injecting intra-brachial artery propranolol, we aimed to study the role of the peripheral β_2- adrenoceptors in health and in CHF.

3.3B Methods

Propranolol was administered into the brachial artery of seven healthy and four CHF subjects, as an addition to the forearm clonidine study described in Part 1 of this chapter. In this subset of patients, both tritiated noradrenaline and adrenaline were infused into a peripheral vein. After the phase of intra-arterial clonidine, thirty minutes were allowed to lapse. At the end of this time, baseline FBF and arterio-venous sampling for regional spillover calculations were obtained.

Then propranolol was infused intra-arterially at 0.1μg/kg, over 15 minutes. After this infusion, FBF and blood samples were again undertaken. Forearm spillover of NA was calculated as previously described and forearm adrenaline (Adr) spillover was similarly calculated:

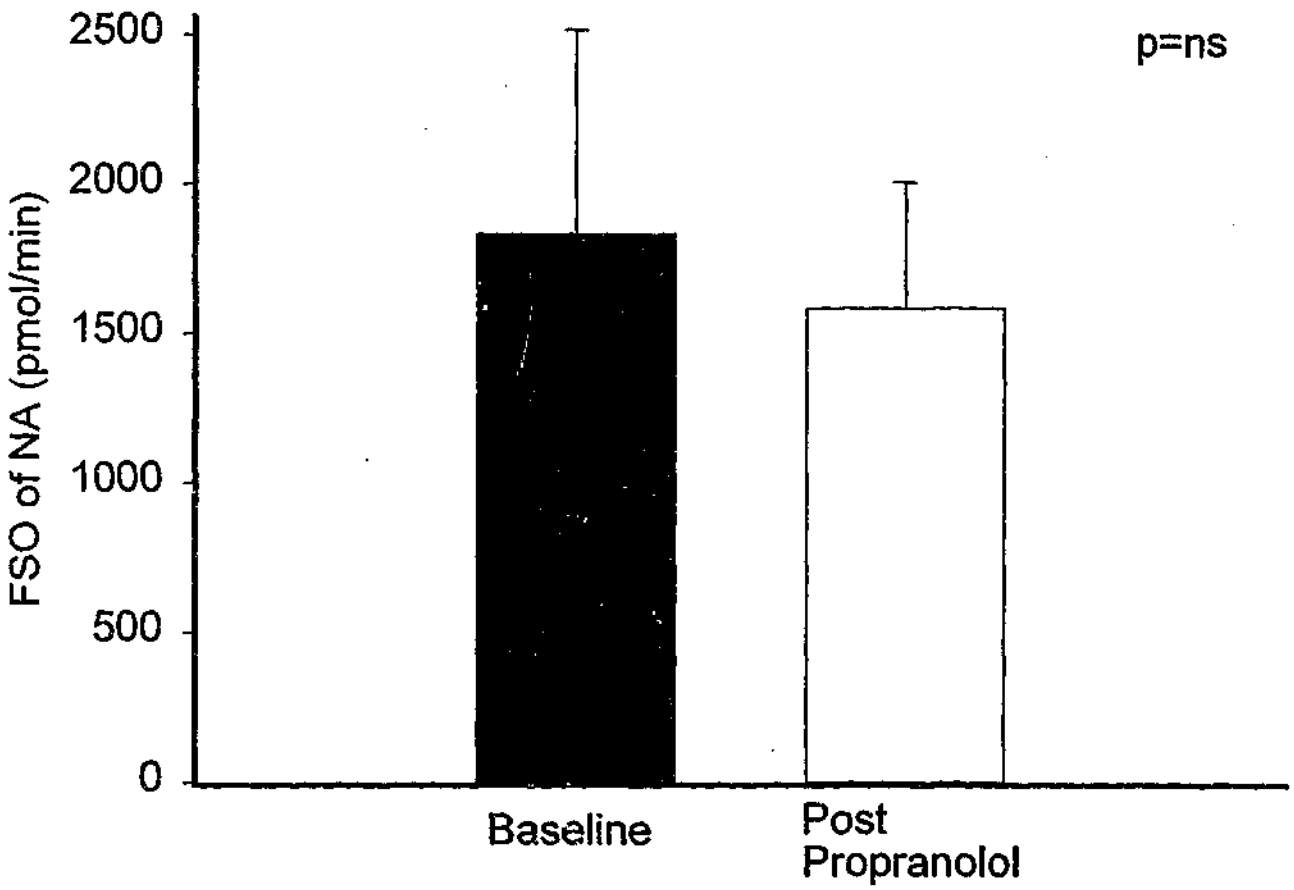
$$\text{FSO of Adr} = [\ (C_V - C_A) + C_A \times (Adr_E)\ ] \times PF$$

Where C_V is the plasma Adr concentration in the deep forearm vein, C_A is the arterial plasma Adr concentration, and Adr_E is the fractional extraction of radio-labeled Adr across the forearm.

3.4B Results

Intra-arterial propranolol did not result in any significant change in forearm blood flow: in CHF, 31.6±8.2 to 34.3±12.1 mls/min; in controls, 20.1±4.3 to 20.0±3.4 mls/min (p=NS). The values obtained for adrenaline in the arteriovenous samples were very low (<30 pg/ml). Forearm NA spillover in the control subjects fell slightly from 10.0±3.7 to 8.65±2.31 nmol/min (p=NS), as presented in Figure 3.3; and in CHF, from 14.9±9.4 to 12.5±8.2 nmol/min (p=NS).

Figure 3.3. Bar graph depicting the forearm spillover (FSO) of noradrenaline (NA) in response to intra-arterial propranolol, in healthy volunteers, n=7.



3.5B Study limitations

The study is obviously limited by the small sample size. It was found that the addition of the propranolol intervention meant the study duration was very prolonged, and therefore was not appropriate for all subjects.

3.6B Conclusions

The sample size of seven healthy subjects is reasonable on which to base conclusions. The results indicate that in health, forearm presynaptic β_2-adrenoceptors do not appear to augment noradrenaline release. However, in the study presented, the sample size for the heart failure is too small to form any conclusions. It is possible that peripheral β_2-adrenoceptor functionality is altered in CHF and it would be reasonable to study a larger sample size with the intervention of intra-arterial propranolol alone.

Chapter 4

The Effects of low dose Clonidine on Cardiac and Renal Sympathetic Activity and Brain Noradrenergic Turnover in Human Heart Failure

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4.1 Introduction

Congestive heart failure (CHF) is characterised by heightened sympathetic activity, particularly in the heart and kidneys (Hasking et al., 1986). Neurohormonal activation is initially beneficial, helping to maintain systemic blood pressure and perfusion to vital organs, but maladaptive in the long-term, contributing to worsening of CHF and sudden cardiac death (Kaye et al., 1995; Brunner-La Rocca et al., 2001). Beta-adrenergic antagonists have become a cornerstone of modern heart failure management, resulting in major gains in prognosis (Waagstein et al., 1993; Packer et al., 1996). However, β-adrenoceptor antagonism cannot attenuate the effects of vasoactive cotransmitters such as neuropeptide Y that is coreleased with noradrenaline (NA) (Zukowska-Grojec, 1998; Haass, 1998). In addition, the high renal sympathetic renal tone observed in CHF that contributes to sodium retention and circulatory overload, is unabated (Middlekauff et al., 2000; Dupont, 1990). These limitations, when coupled with the observation that there is increased central monoamine turnover in CHF (Kaye et al, 1994; Lambert et al., 1995), suggest that therapy to attenuate sympathetic drive directly should be investigated.

Clonidine is a potent sympatholytic drug with central and peripheral effects (Svensson et al., 1975; Isaac, 1980). In CHF, it appears to act predominantly via stimulation of sympatho-inhibitory α_2-adrenergic and/or imidazoline receptors in the central nervous system (Aggarwal et al., 2001). Clonidine, in acute doses, has been demonstrated to attenuate the systemic sympathetic activation that occurs during exercise in CHF (Lang et al., 1997). In studies of more long-term dosing, systemic sympathetic suppression and favourable haemodynamic effects have been observed (Manolis et al., 1995; Grassi et al., 2001). Moreover, Azevedo et al (1999) have shown that acute administration of clonidine to CHF patients resulted in a significant reduction in

cardiac noradrenaline spillover, a measure of cardiac sympathetic activity. However, the only controlled clinical trial to date employing a strategy of attenuating sympathetic drive in CHF – "MOXCON" – was terminated early because of excess mortality in the treatment arm. This outcome was surprising in light of evidence that moxonidine has a powerful sympatholytic effect in heart failure (Swedberg et al., 2000). Whilst a detailed report of this trial is yet to be published, a possible explanation for this apparent contradiction may be that the doses of moxonidine used were excessive, leading to haemodynamic decompensation.

4.2 Aims

In this study, we aimed to test the hypothesis that clonidine may selectively attenuate cardiac sympathetic drive at low doses, on the basis that cardiac activation is greatest and earliest (Rundqvist et al., 1997), in the absence of significant systemic effects. In addition, we studied the effects of clonidine on sympathetic nerve cotransmitter release, by measuring transcardiac neuropeptide Y levels. We also tested whether clonidine reduced renal sympathetic activity and central monoamine turnover in heart failure. The latter component of the study was based on the fact that the high sympathetic tone in human (Lambert et al., 1995a) and experimental heart failure (Elam et al., 1985) appears to be generated by increased activity in noradrenergic neurons projecting from the brainstem to suprabulbar subcortical brain regions.

4.3 Methods

4.3.1 Patient characteristics

Ten patients with chronic congestive heart failure (mean age, 57 years; range 49 to 67 years) participated in this study, which was performed with written informed consent and the approval of the Alfred Hospital Ethics Review Committee. All patients had a protracted history of symptomatic CHF consequent upon either coronary artery disease and previous myocardial infarction (n=4) or an idiopathic dilated cardiomyopathy (n=6). All had left ventricular ejection fractions measured by radionucleotide ventriculography of <40% (mean LVEF 25.4 ± 8.0%, mean ± SD), and were in NYHA Class II – III for heart failure symptoms. Due to the severity of their illness, all patients continued taking their normal medications. Medications consisted of an angiotensin-converting enzyme inhibitor (n=9), carvedilol (n=9), anti-arrhthymic therapy (amiodarone n=2, sotalol n=1), aldactone (n=6), digoxin (n=3) and frusemide (n=9). Both the patients' clinical condition and their medications had been stable for at least one month.

4.3.2 Experimental procedures

All patients were studied at rest in the supine position 2 hours after eating a standardized light breakfast. Tea, coffee, and alcohol were withheld for a minimum of 12 hours before the study. Under local anaesthesia, the radial artery was cannulated (3F, 5cm, Cook) for arterial pressure monitoring and blood sampling. A venous introducer sheath was placed in the right antecubital fossa (n=7) or, when this was not possible, the right internal jugular vein (n=3).

After a priming bolus of 12μCi of 1-[ring-2,5,6-^{3}H]- noradrenaline (New England Nuclear, specific activity 40 to 50 μ Ci/mmol) and 120mg of p-aminohippurate (PAH,

Clinalfa) via a peripheral vein, infusions were commenced that maintained plateau plasma concentrations during the study. Tritiated NA was infused at 0.7μCi/m^2/min and PAH at 5mg/m^2/min. To ensure steady-state levels of the infusates, a period of at least 45 minutes was allowed to elapse after commencement of the infusions before baseline blood sampling was undertaken.

A pulmonary artery thermodilution catheter (7F, Arrow, Arrow International) was advanced to the pulmonary circulation for the measurement of right heart pressures, pulmonary capillary wedge pressure and cardiac output. The right renal vein (8F Cournand, n=9), right internal jugular vein (n=7) and the coronary sinus (n=9, Webster CCS 7/8U 90A, Webster Laboratories) were then catheterised sequentially with fluoroscopic monitoring. At each site venous blood samples were taken for measurement of catecholamines. Radial artery samples were obtained simultaneously. After baseline sampling at each site, the catheter was left in position in the coronary sinus. After the last dose of intravenous clonidine, as described below, the right internal jugular vein was recannulated. Finally, the Cournand catheter was reintroduced into the right renal vein.

4.3.3 Blood flow determination

Cardiac output, coronary sinus and right internal jugular venous blood flow were measured by thermodilution. Blood and plasma flows were interconverted using the subjects' hematocrit. Paired arterial and venous samples were collected for the estimation of renal plasma flow from clearance of PAH. Briefly, standard curves relating optical density to the concentration of PAH were constructed with the subject's plasma. The concentrations of PAH in duplicate experimental samples were

determined from the curves and used to calculate total body clearance, which was corrected for fractional extraction to derive plasma flow.

4.3.4 Study protocol

Clonidine (Boehringer-Ingelheim) was given via a peripheral vein at the following doses: 0.1μg/kg, 0.25μg/kg, and 1μg/kg. Each dose was given as an infusion over 15 minutes. There was then a 20 minute delay before the next dose. Blood sampling and flow measurements were taken at the end of this 20 minute period. This protocol was derived from the observation that the haemodynamic effects of a single intravenous bolus of clonidine peak at 15 to 20 minutes and return to baseline by 45 minutes (Giles et al., 1981). Upon completion of the final coronary sinus sampling, the catheters were repositioned to allow right internal jugular and renal vein sampling.

4.3.5 Biochemical assays

Noradrenaline, dihydroxyphenylalanine and dihydroxyphenylglycol concentrations were measured with high performance liquid chromatography (HPLC). Fractions from the HPLC effluent containing tritium-labeled NA were assayed by liquid scintillation spectroscopy. Plasma concentrations of 3-methoxy-4-hydroxyphenylglycol (MHPG) were determined using a technique adapted from Eisenhofer et al (Eisenhofer et al., 1986). In brief, MHPG was extracted from ultrafiltered plasma samples by the addition of ethyl acetate, followed by drying under nitrogen. MHPG was then reconstituted and underwent liquid chromatography with electrochemical detection. Plasma neuropeptide Y levels were determined by direct immunoassay using antiserum raised in rabbits immunized with synthetic neuropeptide Y as previously described (Morris et al., 1986).

4.3.6 Calculations for global and regional sympathetic activity

The overall, cardiac, renal and central nervous system spillover rates of norepinephrine were determined by the principle of isotope dilution, as given below:

$$\text{Total Spillover Rate NA} = \frac{[^3\text{H}]\ \text{NA Infusion Rate (dpm/min)}}{\text{Plasma NA Specific Radioactivity (dpm/pmol)}}$$

Where NA=norepinephrine, dpm=disintegrations per minute of tritiated NA and [^{3}H]NA=tritiated NA.

$$\text{Regional NA Spillover} = [(NA_{ven} - NA_{art}) + (NA_{art} \times {}^{r3}\text{H}]\ NA_{ex})] \times \text{Plasma Flow}$$

where NA_{ven} and NA_{art} are the venous and arterial noradrenaline concentrations respectively and [^{3}H] NA_{ex} is the fractional extraction of tritiated noradrenaline in a single passage through an organ.

The net cardiac overflow of neuropeptide Y was calculated as the product of coronary sinus plasma flow and the venoarterial concentration gradient for NPY (Kaye et al., 1994). Regional releases of dihydroxyphenylglycol (DHPG) and MHPG were computed by using blood rather than plasma flow as these lipophilic compounds are evenly distributed between plasma and red blood cells (Eisenhofer et al., 1988).

Central nervous noradrenergic activity was assessed by measuring the rate of spillover into the right internal jugular vein of NA, its precursor dihydroxyphenylalanine (DOPA), and principal centrally occurring metabolites DHPG and MHPG (Lambert et al., 1995a).

As regional NA spillover is to some extent dependent on organ blood flow, we also calculated, where appropriate, the plasma appearance rate (PAR) (Chang et al., 1994; Rongen et al., 2000) of NA:

$$PAR = \frac{\text{Regional spillover of NA}}{1 - [^3H]\,NA_{ex}}$$

4.3.7 Statistical methods

Data are presented as mean value ± SEM, unless otherwise stated. Statistical analysis was performed using statistical software (SigmaStat, version 2.03, Chicago, Illinois). Within – group analysis of variance (ANOVA) and the Tukey Test was employed in post-hoc analysis. A p value of <0.05 was considered statistically significant.

4.4 Results

4.4.1 Haemodynamic profiles

Baseline heart rate (HR) and mean arterial blood pressure (MAP) were 77±4 beats per minute and 89±4.5 mmHg respectively. Mean pulmonary capillary wedge pressure was 14.8±2.4 mmHg. The two lower doses of clonidine did not result in any significant change in HR and MAP. After the final dose, that is at a cumulative dose of clonidine of 1.35μg/kg, HR was 72±3 bpm (p=NS) and MAP was 77±3.3mmHg (p=0.001).

4.4.2 Systemic and cardiac sympathetic response to clonidine

As shown in Table 4.1, the first two doses of clonidine did not cause any change to the baseline total body NA spillover of 4.0±0.6 nmol/min. After the final dose, a

reduction to 3.1±0.5 nmol/min was observed ($p<0.01$). This represented a 23% reduction in global sympathetic activity. No significant change in NA clearance was observed with the administration of clonidine.

Across the heart, extraction of tritium labelled NA did not change significantly during the course of the study. When compared with baseline, a significant reduction in arterial and coronary sinus plasma NA levels was observed only at the highest dose of clonidine, (arterial) 3.6±0.6 to 2.6±0.4($p<0.001$) and (coronary sinus) 5.1±0.9 to 3.6±0.7($p<0.01$) pmol/ml respectively. As such, a dose of 1μg/kg clonidine produced a significant reduction in cardiac NA spillover, from 326±73 to 160±40 pmol/min, $p<0.001$. This represented a 50% reduction in cardiac sympathetic activity (Figure 4.1). When the plasma appearance rate of NA from the heart was calculated, a reduction of similar magnitude was observed at the highest dose of clonidine, from 791±212 to 370±107 pmol/min ($p=0.002$).

4.4.3 Cardiac neuropeptide Y release

Data is available from nine patients for neuropeptide Y dynamics across the heart and is also presented in Table 1. At rest, a net cardiac extraction of NPY was demonstrated. Clonidine did not result in any significant change in cardiac NPY release.

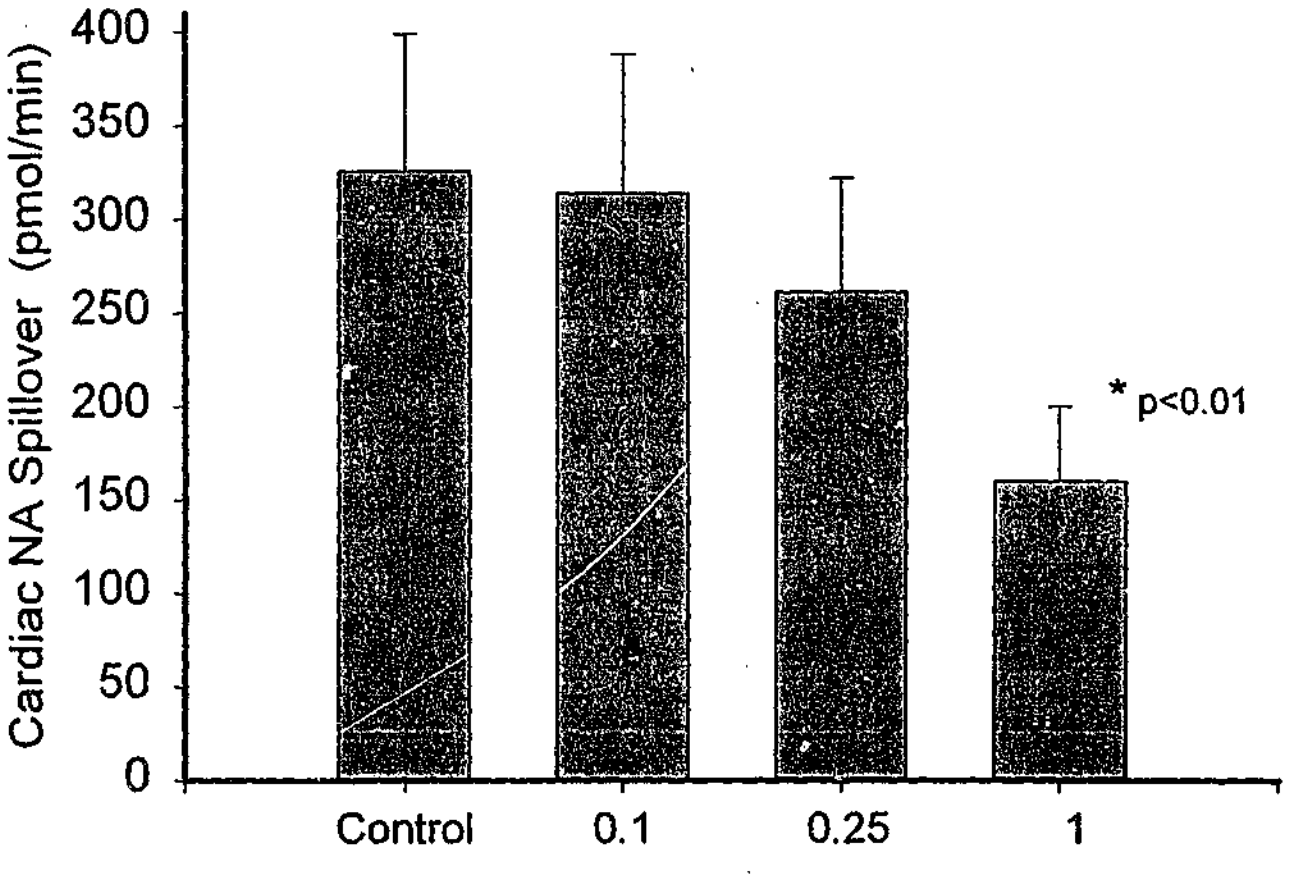
Table 4. 1 Neurochemical and flow responses to clonidine

	Control	0.1μg/kg	0.25μg/kg	1μg/kg
NA_{art} (pmol/ml)	3.6±0.6	2.8±0.6	3.2±0.5	2.6±0.4*
NA_{CS} (pmol/ml)	5.1±0.9	4.1±0.8	4.5±0.8	3.6±0.7**
CardiacV-A (pmol/ml)	1.5±0.3	1.3±0.3	1.2±0.3	1.0±0.3
$Cardiac_{NA}Ext$ (%)	55±6	61±5	61±5	54±7
CSPF (ml/min)	98±11	94.5±13	84±11	72±7*
$Cardiac_{NA}SR$ (pmol/min)	326±73	314±74	261±61	160±40*
$TB_{NA}Cl$ (L/min)	1.3±0.2	1.3±0.2	1.3±0.2	1.4±0.2
$TB_{NA}SR$ (nmol/min)	4.0±0.6	3.8±0.7	4.0±0.8	3.1±0.5**
$Cardiac_{NA}$:TB_{NA} SR	0.08±0.0	0.08±0.01	0.06±0.01	0.05±0.01***
$Cardiac_{NPY}SR$ (pg/min)	-998±616	-105±600	409±241	915±782

*p<0.001 versus control. **p<0.01 versus control. ***p<0.05 versus control.

NA_{art} = arterial plasma noradrenaline; NA_{cs} = coronary sinus plasma noradrenaline; Cardiac V-A = coronary sinus-arterial concentration of noradrenaline; $Cardiac_{NA}Ext$ = cardiac extraction of noradrenaline; CSPF =coronary sinus plasma flow; $Cardiac_{NA}SR$ =cardiac spillover of noradrenaline; $TB_{NA}Cl$ = total body clearance of noradrenaline; $TB_{NA}SR$ = total body spillover of noradrenaline, $Cardiac_{NA}$:TB_{NA} SR = Ratio of cardiac to total body noradrenaline spillover, and $Cardiac_{NPY}SR$ = cardiac spillover of neuropeptide Y.

Figure 4.1 Bar graph representing cardiac noradrenaline spillover in response to intravenous clonidine, n=9

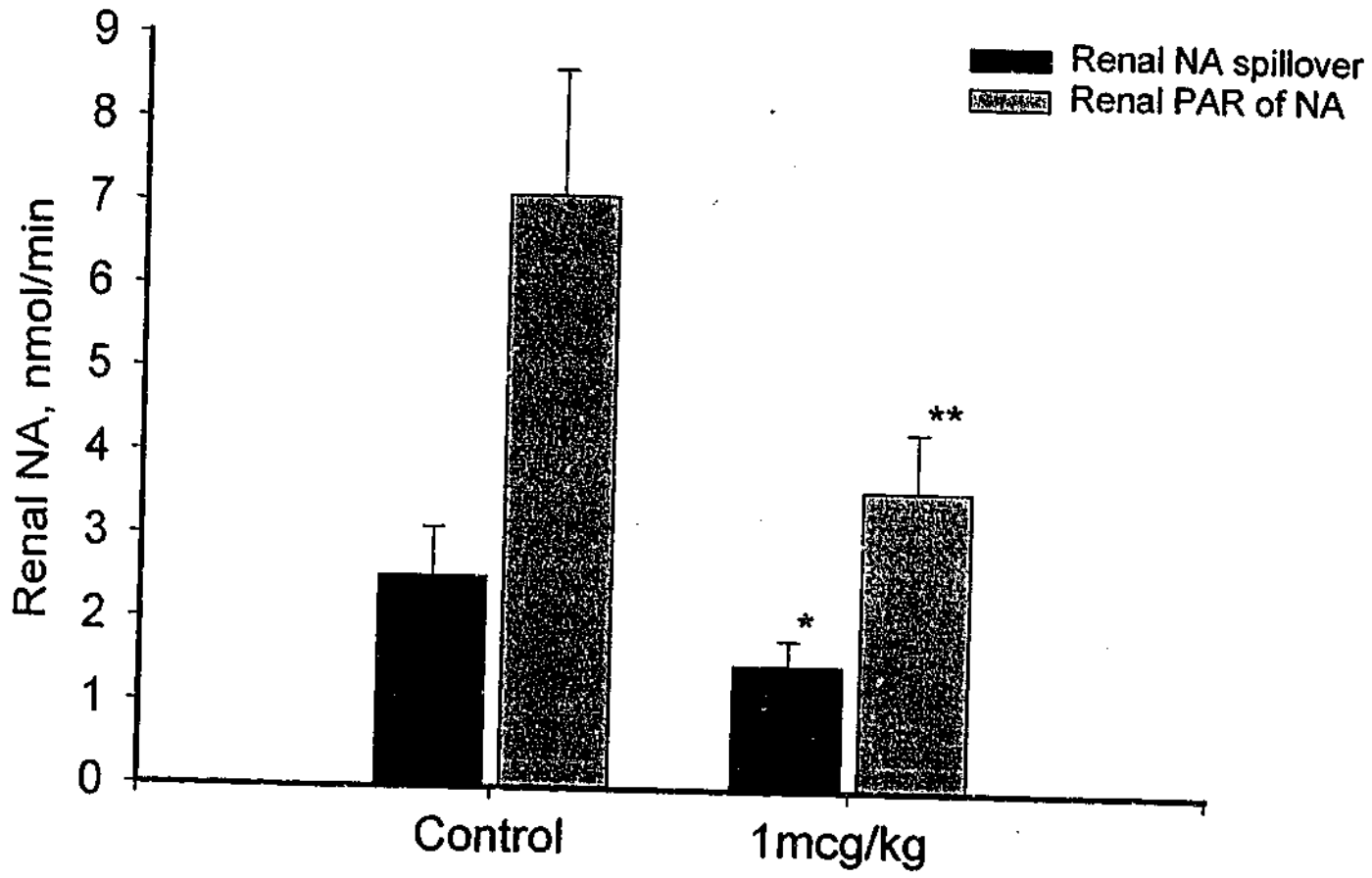


4.4.4 Renal noradrenaline release

Renal NA spillover data is available from nine patients and is presented in Figure 4.2. Baseline renal plasma flow was 739±111 ml/min. After the final dose of clonidine, there was a significant fall in flow to 494±59 ml/min (p=0.01). Baseline renal NA spillover was 2.53±0.6 nmol/min and this fell to 1.5±0.3 nmol/min (p=0.01). This represented a reduction by a mean of 26% and 32% respectively in renal plasma flow and renal NA spillover.

As regional NA spillover is to some extent dependent on blood flow, we also calculated the plasma appearance rate (PAR) of NA (Rongen et al., 2000). Renal PAR of NA decreased from a baseline of 7.1±1.5 to 3.6±0.7 nmol/min in response to the highest clonidine dose (p=0.009).

Figure 4.2 Bar graph representing renal noradrenaline spillover and plasma appearance rate in CHF before and after clonidine, n=9

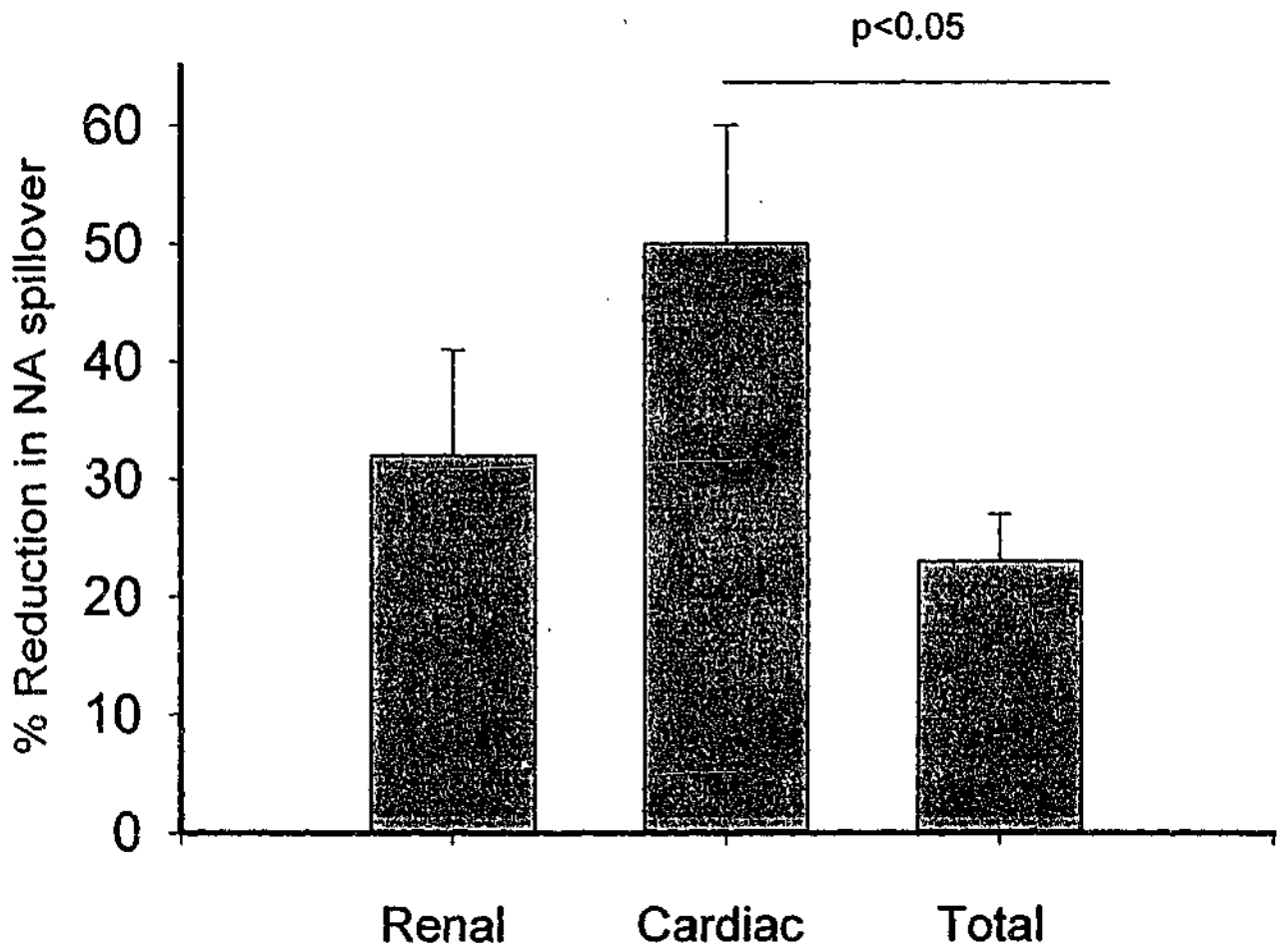


* p=0.01, **p=0.009

4.4.5 Regional versus total sympathetic outflow sensitivities to clonidine

When comparing the regional sympathetic responses to clonidine, the heart was significantly more sensitive than the global sympathetic outflow (Figure 4.3). There was a 32±9%, 50±10%, and 23±4% reduction in renal, cardiac and total body sympathetic outflow achieved with the final dose of clonidine. To examine this regional sensitivity in another way, when cardiac noradrenaline spillover is expressed as a percentage of total noradrenaline spillover at baseline and at the three doses of clonidine, the values are: 8±1%, 8±1%, 6±1% and 5±1% ($p<0.05$, between baseline and final dose results).

Figure 4.3 Bar graph representing % reduction from baseline in noradrenaline spillover after intravenous clonidine, n=9



4.4.6 Effects of clonidine on brain noradrenaline turnover

The right internal jugular vein (RIJV) was cannulated in 7 patients via the antecubital approach. Baseline RIJV blood flow was 405±41 ml/min, and this reduced to 320±44 ml/min after the highest dose of clonidine ($p<0.05$). Clonidine did not result in any significant reduction in the release of noradrenaline and its lipophilic metabolites (MHPG and DHPG) from the brain into the right internal jugular vein (1048±557 to 981±267 pmol/min). When comparing the rate of release of dihydroxyphenylalanine (DOPA), the precursor of noradrenaline, a significant reduction was seen (366±67 to 101±78 pmol/min, $p<0.05$) after clonidine.

4.5 Discussion

Activation of the sympathetic nervous system is an important pathophysiological feature in heart failure, with its links to mortality (Kaye et al., 1995), arrhythmogenesis (Meredith et al., 1991), and progression of heart failure (Kaye et al., 1995; Brunner-La Rocca et al., 2001) clearly established. Further evidence of the detrimental effects of this neurohormonal excitation is that β-adrenergic blockade markedly improves prognosis in CHF (Packer et al., 1996). Therefore, sympathetic deactivation has become a desirable therapeutic goal in patients with cardiac systolic dysfunction.

The strategy of β-adrenergic receptor blockade is limited in its objective to adrenoceptor antagonism. A logical progression in therapy of CHF would be to investigate a sympatholytic approach, utilising pharmacological central suppression of sympathetic outflow. The sympathetic excitation observed in CHF is not uniform but regional, with the major focus on the heart and the kidneys (Hasking et al., 1986). A further refinement of the sympatholytic approach may then be to selectively target

these organs. Some supportive evidence to this contention already exists in alcoholic cirrhosis, another disease model of sympathetic overexcitation. In this condition, Esler et al have previously demonstrated that sympathetic outflow to the hepatomesenteric circulation was more sensitive to pharmacological suppression with clonidine than was the systemic sympathetic outflow (Esler et al., 1992).

In this study we have confirmed the finding of Azevedo et al (1999) that clonidine suppresses cardiac sympathetic drive in CHF. We have extended this observation by using lower doses of clonidine, and have shown that the heart is disproportionately more sensitive to the sympatholytic effects of this drug than is global sympathetic activity. It is known that cardiac sympathetic activation is a more powerful prognostic indicator than generalized sympathetic tone, as indicated by plasma noradrenaline (Kaye et al., 1995). Therefore, an implication from this finding is that low doses of sympatholytic drugs may possibly produce prognostic benefit, by selectively suppressing cardiac sympathetic drive, in the absence of significant systemic side-effects associated with pronounced sympathetic withdrawal such as hypotension.

One of the potential advantages of central sympathetic inhibition over anti-adrenergic therapy is that release of vasoactive sympathetic cotransmitters, such as neuropeptide Y, could also be suppressed. Previous investigators have shown that transcardiac (Kaye et al., 1995) and arterial plasma NPY (Maisel et al., 1989) levels are increased in CHF. These findings were in an era before the routine use of β-adrenergic blockers. Interestingly, in the current study, we were not able to demonstrate net cardiac release of NPY at rest or any change after clonidine, possibly due to carvedilol use in the current group of patients (8 out of 9 patients sampled) substantially reducing sympathetic activity. In our earlier report (Kaye et al., 1995), the mean cardiac and total NA spillover values were 394±46 pmol/min and 5.5±0.4 nmol/min respectively

(compared with 326±73 and 4.0±0.6 in the present study). Further, the mean pulmonary capillary wedge pressure in the previous group was 21.5±1.3 mmHg, whereas in the current group this was 14.8±2.4 mmHg, again reflecting the better control of CHF in this group. It is possible that relatively minor reductions in cardiac sympathetic nerve activity have resulted in major decreases in cardiac NPY release, given that NPY sympathetic cotransmission only occurs at high rates of sympathetic nerve firing (Morris et al., 1986; Haass, 1998).

Another possible advantage of the sympatholytic approach is that the high renal sympathetic tone that exists in CHF (Hasking et al., 1986) could be attenuated. In rats, increased activity of the efferent renal sympathetic nerves exerts a potent antidiuretic and antinatriuretic influence (DiBona et al., 1982). By extrapolation, in human CHF, the exaggerated renal sympathetic activity could be a major contributing factor to the salt and water retention which is characteristic of the disease. Attenuation of this sympathetic drive could offer symptomatic and prognostic benefit. Carvedilol appears to have neutral effects on surrogate markers of renal sympathetic tone such as renal blood flow and glomerular filtration rate (Dupont, 1990; Dupont, 1992).

In the present study, we demonstrate a substantial reduction in renal norepinephrine spillover with clonidine. Whilst clonidine did produce a significant reduction in renal plasma flow (26%), we believe that the decrease in NE spillover resulted from a true reduction in sympathetic discharge, rather than from a reduction in flow. In support of this are two observations. Firstly, we have previously shown that renal norepinephrine spillover is unchanged with blood flow reductions of less than 30% (Esler et al; 1988). Secondly, the plasma appearance rate of NE, proposed as a flow-independent measure of regional sympathetic activity (Chang et al., 1994; Rongen et al., 2000), was also substantially reduced by clonidine. In a study of Sprague-Dawley rats with

experimental CHF, intravenous clonidine has been demonstrated to substantially reduce renal sympathetic nervous activity (Feng et al., 1992). However, to our knowledge, the effects of clonidine on renal sympathetic tone in human CHF have not been previously investigated.

A final aspect of the current study was to investigate the effects of clonidine on central monoamine turnover, a measure of brain noradrenergic activity. We have previously established an association between the degree of activation of central monoaminergic neurons and the level of sympathetic nervous tone in the heart (Lambert et al., 1995) in CHF. The cell bodies of the noradrenergic groups, designated A1-7, are confined to medullary and pontine parts of the brainstem but exhibit complex ascending and descending projections in addition to local destinations in the brainstem, with the majority of brain norepinephrine located in the pontine locus coeruleus (A6) (Hokfelt, 1984).

Clonidine is a centrally acting suppressant of sympathetic nervous activity which is known to inhibit the firing rate of locus coeruleus neurons (Foote et al., 1983) and to decrease the concentration of MHPG in rat brain (Braestrup, 1974). Maas et al (1977) have demonstrated a reduction in 'HPG jugular overflow from the brain of stump-tailed monkeys after clonidine adi. nistration, and this finding was subsequently reproduced in healthy human subjects (Lambert et al., 1998).

In the group of heart failure patients presented in this report, the values for central monoamine turnover were substantially lower than in our earlier reports (Lambert et al., 1995a; Kaye et al., 1994). A possible explanation for this is that the mean pulmonary capillary wedge pressure in the present study is much lower. A trend has been previously observed for mean pulmonary artery pressure and central monoamine turnover to be related in human heart failure (Lambert et al., 1995a), and from animal

studies, it is known that pulmonary afferents do project to the locus coeruleus (Ela... et al., 1984). In the present study, we were unable to demonstrate a significant decrease in the right internal jugular venous spillover of noradrenaline and its lipophilic metabolites after clonidine administration. There are two possible explanations for this finding. Firstly, we did not use radionuclide cerebral venous scanning to lateralise the venous drainage of the brain in this study. As would be inferred from studies of central sympathetic organization in rats (Hokfelt, 1984), and from studies in human hypertension (Ferrier et al., 1993), central monoamine turnover is significantly higher in the jugular vein that receives drainage from the subcortical ares of the brain. In the majority of humans, this area drains to the left internal jugular vein (Ferrier et al., 1993). Therefore, in this small group of seven patients, we may have failed to see a clonidine effect due to insufficient sampling of subcortical venous drainage. A second possible explanation is that the central noradrenergic centres in CHF are resistant to the relatively modest doses of clonidine used in the current study. When previously given to healthy humans, a dose of 150-225μg of clonidine was used to effect a substantial reduction in central monoamine turnover (Lambert et al., 1998).

The novel findings of this study are, first, that cardiac sympathetic activity is more sensitive to central sympatholytic therapy than is global sympathetic tone and, second, that renal sympathetic tone is significantly reduced by clonidine. These findings have implications for the future therapy of heart failure. Further clinical trial evaluation of the efficacy of this drug class, with study design using low doses of sympatholytic agents such as rilmenidine and moxonidine, is needed. The probability that sympatholytic therapy may be synergistic with β-anti-adrenergic therapy in the treatment of heart failure means that in the conduct of such a trial, concurrent β-

adrenoceptor blocker therapy would be acceptable, allowing the ethical dilemmas associated with a β-blocker free trial to be avoided.

Chapter 5

Noradrenaline turnover is increased in suprabulbar subcortical brain regions and is related to whole body sympathetic activity in human heart failure

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5.1 Introduction

High cardiac sympathetic tone, as measured by cardiac noradrenaline spillover, has been correlated with worsening heart failure (Kaye et al., 1995), need for cardiac transplantation (Kaye et al., 1995) and the occurrence of arrhythmic death (Meredith et al., 1991). Whilst the role of the sympathetic nervous system in the pathophysiology of heart failure has been established, the nature of the afferent signals and the sites of origin of the efferent signals from the central nervous system responsible for the heightened sympathetic tone have not been fully elucidated.

Noradrenaline-releasing neurons in the brainstem and forebrain have an important regulatory influence over the sympathetic nervous outflow. Electrophysiological and anatomical evidence exists of a connection between pressor noradrenergic hypothalamic and brain stem centres and sympathetic preganglionic neurones in the thoracolumbar cord (Chalmers et al., 1991). Noradrenaline-containing cell bodies are assorted into nuclei designated as A1 to A7 and located in the medulla and the pons (Hokfelt et al., 1984). The main source of noradrenaline in the brain is the locus coeruleus (A6), an area of cells near the wall of the fourth ventricle in the dorsal pons, accounting for approximately 50% of cerebral noradrenaline (Foote et al., 1983). From animal studies, evidence exists that the firing rate of the locus coeruleus is regulated by cardiac volume receptors (Elam et al., 1984).

Our group has previously demonstrated a positive correlation between pulmonary artery pressures and cardiac noradrenaline spillover rate (NASR) (Kaye et al., 1994) in patients with congestive heart failure (CHF). Further, we have shown that a positive correlation exists between brain monoamine turnover, a measure of central noradrenergic neuronal activity, and cardiac NASR (Lambert et al., 1995a). These two findings combined suggest the existence of a reflex link, consisting of afferent

neural traffic from the cardiopulmonary receptors and sympathetic efferent outflow from the brain. Adding further weight to this, investigators have reported that muscle sympathetic nervous activity was most closely related to left ventricular filling pressure (Leimbach et al., 1986). We have also previously observed a nonsignificant trend for mean pulmonary artery pressure in CHF patients to be related to cerebral monoamine turnover (Lambert et al., 1995a). The afferent limb of this reflex has been recently investigated in human heart failure (Kaye et al., 1998; Azevedo et al., 2000; Floras, 2001). When cardiac filling pressure was lowered in CHF patients, a directionally opposite change in whole-body (increase) and cardiac (reduction) sympathetic nervous activity was demonstrated.

5.2 Aims

In this present study, our aims were to further investigate this reflex by firstly attempting to broadly localise the sites in the CNS that are responsible for the efferent signals; in hypertensive patients, there is evidence of increased subcortical monoamine turnover (Ferrier et al., 1993), but this relationship has not been previously examined in CHF. Secondly, we wished to investigate the relationship between central monoamine turnover and global sympathetic activity. Finally, we studied the effect of sodium nitroprusside (SNP) on brain monoamine turnover in CHF and in healthy volunteers.

5.3 Methods

5.3.1 Subject characteristics

Results from studies performed on 13 CHF patients (age 53±5.7 years) and 12 healthy volunteers (age 55.1±8.2 years) form the basis for this report. The heart failure group were in New York Heart Association functional class II or III with markedly impaired

left ventricular function (LVEF 23.5±5.9%), as assessed by radionuclide ventriculography. All patients were on standard anti-failure therapy which consisted of digoxin (n=8), diuretics (13), and an angiotensin-converting enzyme inhibitor (n=13). Eight of the CHF patients were also on carvedilol. In all cases, the patient's heart failure was considered to be of such severity that discontinuation of medications was not appropriate. The healthy control subjects were recruited by advertisement in the general community. The study was performed with the approval of the Alfred Hospital Ethics Review Committee and all the subjects gave written informed consent.

5.3.2 Catheterization protocol

All studies were performed in the morning after a light breakfast. After a priming bolus of 12μCi of 1-[ring-2,5,6-^{3}H]- noradrenaline (New England Nuclear, specific activity 40 to 50 μ Ci/mmol) via a peripheral vein, an infusion was commenced at 0.7μCi/m^{2}/min to maintain plateau plasma concentrations during the study. To ensure steady-state levels of the infusate, a period of at least 45 minutes was allowed to elapse after commencement of the infusion before baseline blood sampling was undertaken. Under local anesthesia, the radial artery was cannulated (3F, 5cm, Cook) for arterial pressure monitoring and blood sampling. Venous introducer sheaths were placed in the antecubital fossae bilaterally, where possible. In the patients with heart failure, a pulmonary artery thermodilution catheter (7F, Arrow, Arrow International) was advanced under fluoroscopic control to the pulmonary circulation, for the measurement of right-sided heart pressures, pulmonary capillary wedge pressure and cardiac output. Subsequently in these patients and the healthy control subjects, coronary sinus thermodilution catheters (Webster CCS 7/8U 90A, Webster Laboratories) were introduced bilaterally, where possible, via the antecubital venous

sheaths. These were manipulated into both the right and left internal jugular veins (RIJV and LIJV) above the confluence of the facial veins, for measurement of jugulo-venous blood flow by thermodilution (Lambert et al., 1995a; Lambert et al, 1995b) and for jugular venous blood sampling for the determination of plasma catecholamines in the venous effluent from the brain. The tip of the catheter was placed beyond the mandibular angle, upstream to the point of entry of any venous tributaries from the tissues of the face to minimize contamination of the cerebral venous effluent.

5.3.3 Study protocol

After the completion of baseline haemodynamic measurements and internal jugular venous blood sampling, an intravenous infusion of SNP was commenced. SNP was administered through a dedicated peripheral vein at an initial rate of 0.5μg/kg body weight per minute, if the baseline systolic blood pressure was ≥ 100 mmHg. The infusion rate was increased at 5-min intervals, titrated to achieve a stable reduction in arterial systolic blood pressure of 20mmHg. Once the desired reduction in blood pressure had been achieved, internal jugular venous blood sampling was repeated. In the heart failure patients, the pulmonary artery thermodilution catheter was then repositioned for repeat hemodynamic measurements.

5.3.4 Cerebral venous blood flow lateralisation

Using a technetium-99 cerebral venous sinus scan to delineate the pattern of venous drainage in individual subjects, subcortical and cortical neurotransmitter turnover can be distinguished, as described in Chapter 2. The usual pattern is for the right IJV to have the superior sagittal sinus as its major tributary and the cerebral cortex as its predominant field of drainage. This we designate the "dominant" or "cortical" IJV. In this situation, the suprabulbar subcortical venous drainage from regions such as the hypothalamus and amygdala is into the left IJV and we designate this the "non-

dominant" or "subcortical" IJV (See Fig.2.4). Venous drainage from the medulla oblongata is primarily into the veins of the spinal cord and dural venous sinuses, not the internal jugular veins (Gray, 1980). Sometimes the venous sinus drainage pattern is reversed, with cortical venous drainage being into the left IJV. Another normal variant is that the drainage is non-lateralising, with ready admixture of blood occurring at the confluence of the sagittal and straight sinuses (Esler et al., 1995; Ferrier et al., 1993).

5.3.5 Neurochemical measures of sympathetic activity

The central nervous system and overall release of noradrenaline was determined by the principle of isotope dilution during an infusion of tritiated NA as previously described (Esler et al., 1990). Venoarterial plasma concentration differences combined with internal jugular vein flow were used, in accordance with the Fick principle, to determine metabolite spillover from the brain. The central nervous system noradrenergic turnover was estimated by summing the IJV spillover of NE, MHPG and DHPG (Ferrier et al., 1993; Kaye et al., 1994; Lambert et al, 1995a and 1995b).

5.3.6 Biochemical assays

Noradrenaline, dihydroxyphenylalanine (DOPA) and DHPG concentrations were measured with high performance liquid chromatography (HPLC). Fractions from the HPLC effluent containing tritium-labelled NA were assayed by liquid scintillation spectroscopy. Plasma concentrations of MHPG were determined as previously described (Eisenhofer et al., 1986).

5.3.7 Statistical analysis

Data are presented as mean value ± SEM, unless otherwise stated. Statistical analysis was performed using Student's t-test (SigmaStat, version 2.03, Chicago, Illinois).

Relations between continuous variables were examined using the least squares method of linear regression. A p value of <0.05 was considered statistically significant.

5.4 Results

In 11 out of the 13 CHF patients, both internal jugular veins were successfully entered via the antecubital approach. In 2 patients, only the RIJV or the LIJV was cannulated respectively. Both internal jugular veins were entered in 11 of the 12 healthy volunteers; in one subject, only the RIJV could be cannulated. All subjects underwent radionuclide cerebral venous sinus scanning; in the control group, 7/12 had right dominance, 3/12 had symmetrical drainage, and 2/12 had a left dominant system. In the CHF group, 7/13 had right dominance, 4/13 had symmetrical drainage, and 2/13 had left dominance. Due to systemic hypotension (systolic blood pressure <100mmHg), 2/13 CHF patients were not given SNP. SNP was administered to 10/12 control subjects.

5.4.1 Haemodynamic and global sympathetic responses to SNP

This data is presented in Table 5.1. In the patient group, baseline systolic BP and pulmonary capillary wedge pressure were 117.7±4.4 and 22.7±1.8 mmHg, and after SNP, these pressures reduced to 90.2±3.5 and 13.2 ± 1.3 mmHg respectively, p<0.001 for both measures. In the control group, systolic BP fell from 138.2±4.0 to 102.4±2.8 mmHg, p<0.001.Baseline total body spillover of NA was 2.2±0.4 nmol/min in the control group and 3.5±0.4 nmol/min in the CHF group, p=NS. After SNP, a rise to 4.3±0.6 and to 5.8±0.4 nmol/min was observed respectively, p<0.01 for both groups.

Table 5.1 Haemodynamic and global sympathetic responses to sodium nitroprusside

	Healthy Subjects, n=12	CHF patients, n=13
Baseline systolic BP, mmHg	138.2±4.0	117.7±4.4
Post-SNP systolic BP	102.4±2.8*	90.2±3.5*
Baseline mean PCWP, mmHg	N/A	22.7±1.8
Post-SNP mean PCWP	N/A	13.2±1.3*
Baseline TBS, nmol/min	2.2±0.4	3.5±0.4
Post-SNP TBS	4.3±0.6**	5.8±0.4**

Where PCWP is the pulmonary capillary wedge pressure, TBS is total body spillover of noradrenaline, N/A is not available and SNP is sodium nitroprusside. Statistical comparisons are made within the group, between baseline and post SNP values.

*denotes $p<0.001$, **denotes $p<0.01$.

5.4.2 Central noradrenergic activity at rest

In 8/13 CHF and 8/12 control subjects, data is available that allows lateralisation of central noradrenergic activity at rest. In the remainder, this data was not available either because of inaccessibilty of the internal jugular vein and/or symmetrical cerebral venous blood flow.

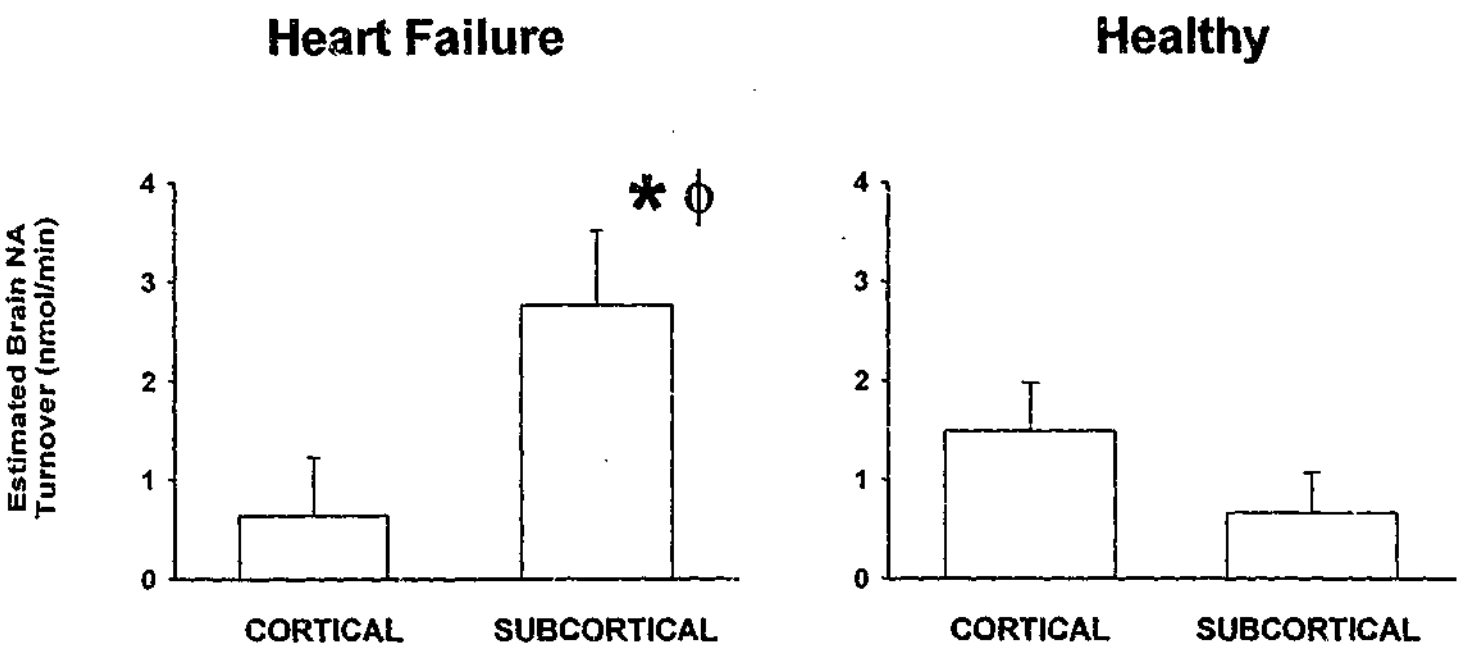
Internal jugular blood flows were not significantly different between the two groups and were as follows: in CHF, cortical 405±80 and subcortical 318±115 ml/min; in the healthy group, cortical 436±60 and subcortical 284±40 ml/min.

As presented in Fig. 5.1, suprabulbar subcortical noradrenergic turnover was greater in CHF than in the control group, 2.77±0.75 versus 0.66±0.40 nmol/min ($p<0.05$). When examining the relationship between cortical and subcortical noradrenergic activity, there was evidence of a significant increase in subcortical noradrenergic activity in CHF, 0.64±0.59 (cortical) versus 2.77±0.75 (subcortical) nmol/min ($p<0.05$). This relationship did not exist in the control group, 1.37±0.54 (cortical) versus 0.66±0.40 (subcortical) nmol/min (p=ns).

5.4.3 Relationship between central and peripheral sympathetic activity

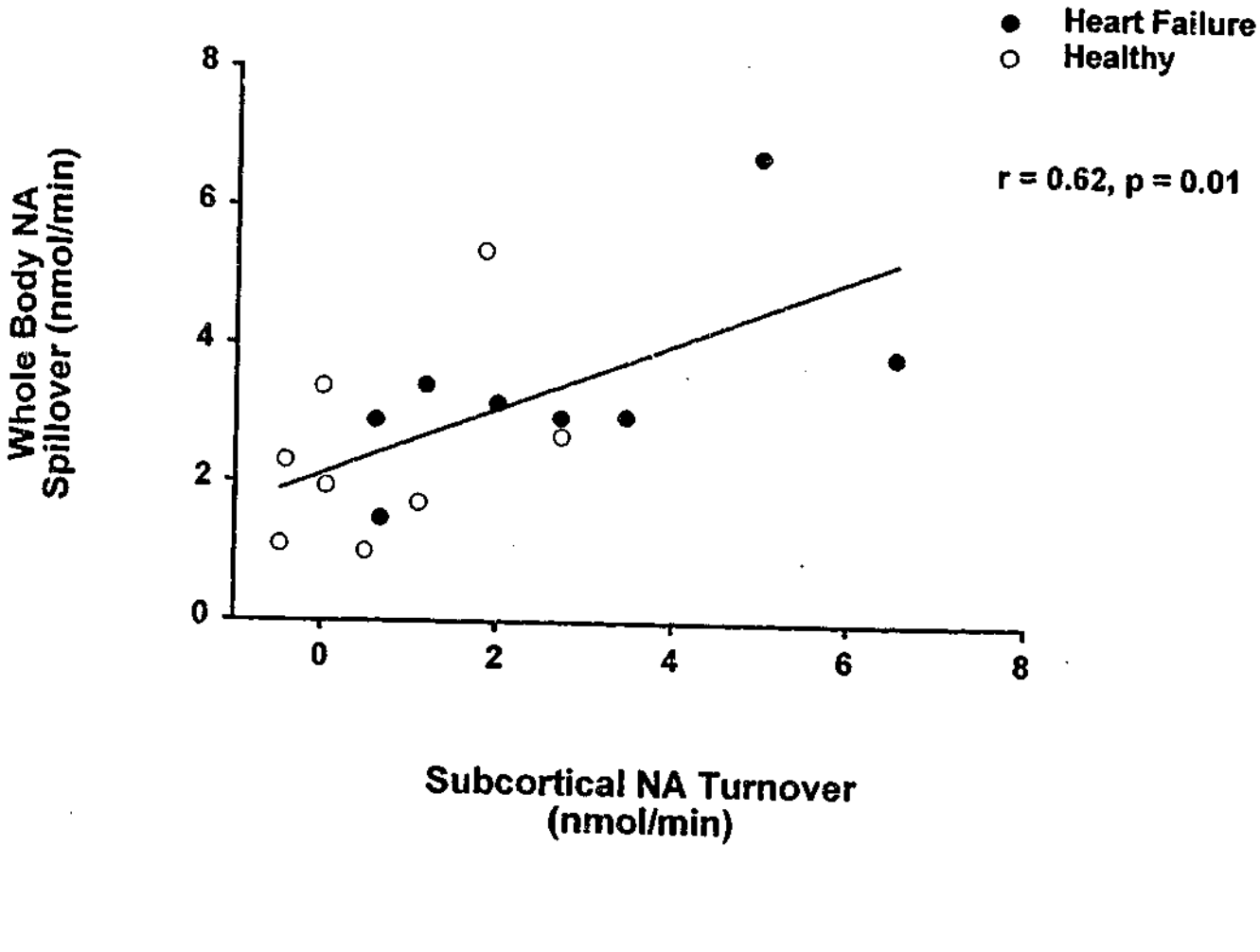
As presented in Fig.5.2, when data from the subcortical vein in all 16 subjects was analysed, significant positive correlations were observed between total body NE spillover and subcortical noradrenergic turnover ($y=2.12+0.47x$; $r=0.62$, $p=0.01$). No significant relationship was observed to exist between cortical venous noradrenergic turnover and global NA spillover.

Figure 5.1 Bar graphs representing brain noradrenergic turnover in health and heart failure.



*for comparison between subcortical and cortical in heart failure, $p<0.05$.
ϕfor comparison between subcortical in CHF and healthy volunteers, $p<0.05$.

Figure 5. 2 Correlation between subcortical internal jugular venous noradrenergic turnover and total body noradrenaline spillover



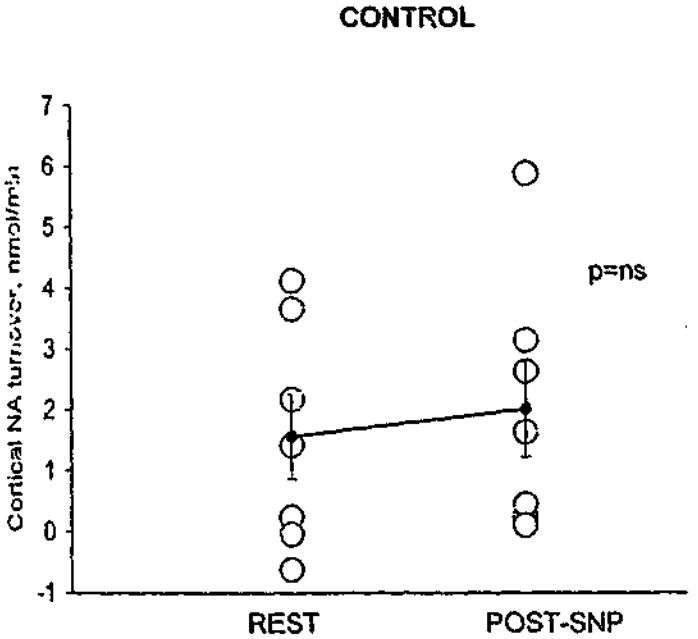
5.4.4 Haemodynamic correlates of sympathetic activity

Data is available in 8 patients to study the relationship between cardiac filling pressures and central noradrenergic activity. No significant correlation was observed between pulmonary capillary wedge pressure and noradrenaline turnover, either from the cortex or subcortex. We also calculated a global central nervous system noradrenergic turnover, by summing together NA turnover from both the cortex and subcortex. This allowed incorporation of data from a further 4 CHF patients in whom there was symmetrical cerebral venous drainage. Again, no correlation was observed between this "global" CNS noradrenergic turnover and left sided cardiac filling pressures. Similarly, no significant correlation was demonstrated between the wedge pressure and total body NA spillover.

5.4.5 Central noradrenergic response to SNP

As noted above, SNP elicited a significant increase in global sympathetic activity, as measured by total body NA spillover. We examined the effects of SNP on cortical, subcortical and total (cortical + subcortical) noradrenergic turnover in both groups. We were unable to demonstrate any significant change in these parameters after SNP in either group. This data is presented in Figures 5.3a and 5.3b. In the control group, cortical NA turnover was 1.56±0.7, increasing to 2.01±0.80nmol/min after SNP (p=ns); subcortical NA turnover in this group was 0.95±0.50, increasing to 1.39±0.6 nmol/min after SNP (p=ns). In CHF, cortical NA turnover was 0.33±0.57, increasing to 2.12±0.49 nmol/min after SNP (p=0.05); subcotical NA turnover in this group was 2.90±0.80, decreasing to 2.14±1.12nmol/min after SNP (p=ns).

Figure 5.3a. Cortical and Subcortical noradrenergic turnover in control subjects, before and after intravenous sodium nitroprusside.



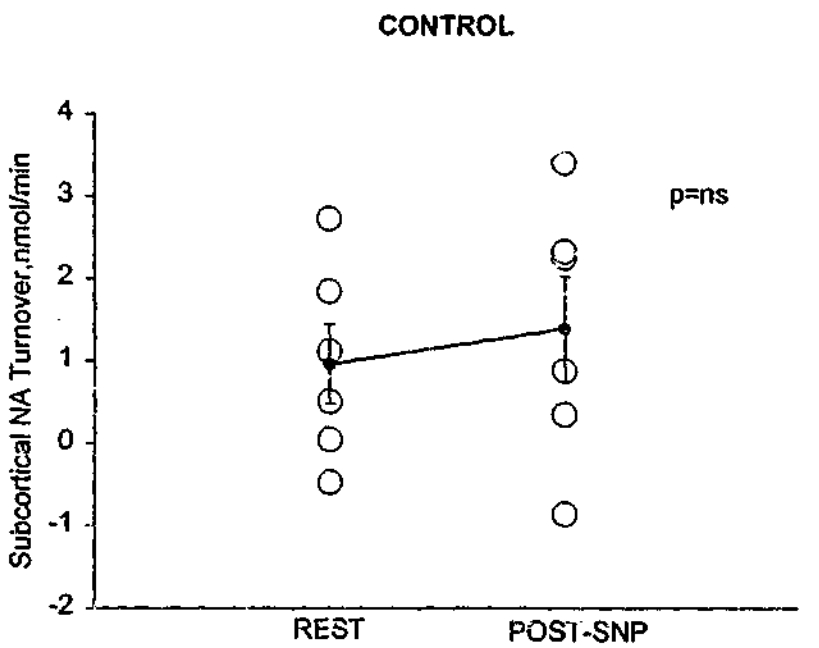
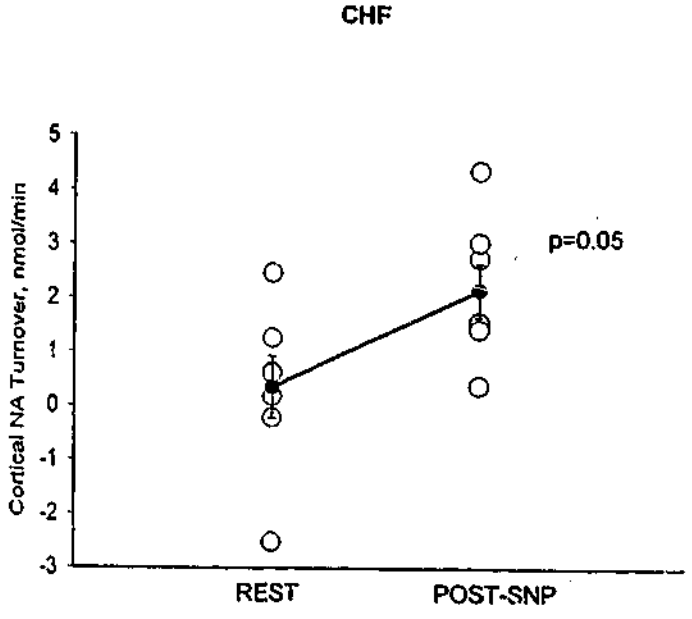
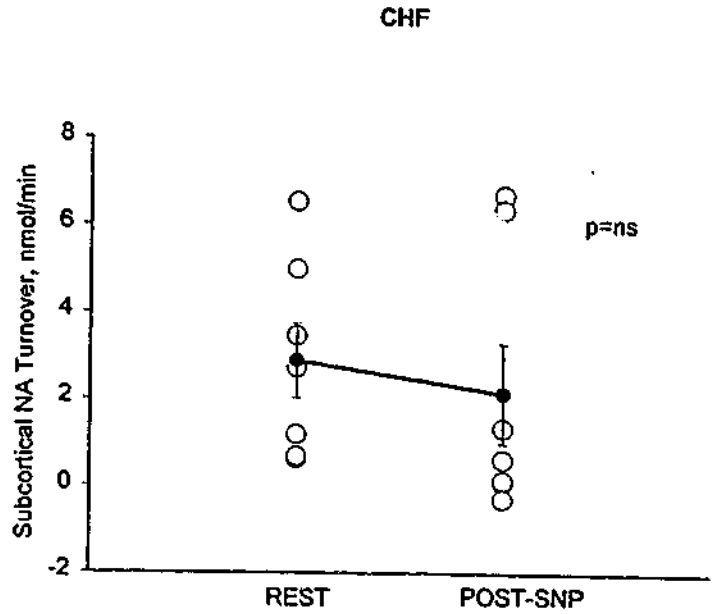


Figure 5.3b. Cortical and Subcortical noradrenergic turnover in heart failure subjects, before and after intravenous sodium nitroprusside.





5.5 Discussion

Sympathetic activation, whilst initially compensatory, is eventually pathogenic in heart failure, such that sympathetic deactivation has become a major aim of modern heart failure therapy (Packer et al., 1996). Despite the central importance of this neurohormonal activation, an understanding of the mechanisms responsible remains elusive. Abnormalities in afferent control, local prejunctional modulation, and/or central processing may be involved. Identifying these mechanisms may allow early therapeutic intervention and ensuing clinical benefit.

The aims of the study presented in this chapter were to better localise the origins of efferent signals arising from the central nervous system in heart failure, to examine any relationship between these and global sympathetic activity, and finally to manipulate this central activity by altering baroreceptor loading.

Employing techniques such as the retrograde transynaptic transport of live pseudorabies virus, a substantial body of evidence now exists from animal studies that a number of brain regions in the hypothalamus and the brainstem innervate all levels of sympathetic nervous outflow (Strack et al., 1989). In humans, techniques to examine central sympathetic function are limited to measuring the overflow from the brain of noradrenaline and its metabolites. Following the demonstration by Maas et al (Maas et al., 1977) that CNS neuronal activity of stump-tailed monkeys could be studied by direct internal jugular vein blood sampling for MHPG (the major central nervous system metabolite of NA), this technique has been applied to humans and at present remains the "gold standard" for measuring central noradrenergic activity.

Whilst it is known in human hypertension that subcortical overflow of noradrenaline and its metabolites is higher than that from the cortex, and that this is positively correlated with total body sympathetic activity (Ferrier et al., 1993), these relationships have not been previously examined in human heart failure.

In the present study, NA turnover was increased in the suprabulbar subcortical areas in CHF when compared to a healthy, age-matched control group. Furthermore, subcortical NA turnover was significantly greater than in the cortex in CHF, but this

differentiation did not exist in the control subjects. In addition, there was a strong positive correlation between suprabulbar subcortical NE turnover and global sympathetic activity.

Another aim of this study was to examine the relationship between cardiac filling pressures and brain noradrenergic turnover. We did not demonstrate a correlation between cardiac filling pressures and overflow of monoamines from the cortex or subcortex in CHF. This was somewhat surprising given that in our earlier report, a trend for cardiac filling pressures to positively correlate with central monoamine turnover was noted (Lambert et al., 1995a). In addition, it is known from rat studies that cardiopulmonary volume afferents project to the noradrenergic nuclei of the locus coeruleus, and the firing rate of locus coeruleus neurons is changed by alterations in cardiopulmonary pressures (Elam et al., 1984). An alternative explanation of the sympathetic nervous stimulation present in CHF may perhaps be that a state of desensitization of low-pressure baroreflexes exists, forming a basis for withdrawal of tonic inhibition of peripheral sympathetic activity (Thames et al., 1993), mediated centrally by activation of brainstem noradrenergic neurons projecting rostrally to suprabulbar subcortical areas. Another possible explanation for the lack of correlation observed in this group of patients between pulmonary pressures and central noradrenergic turnover is that, when compared with previous study groups (Lambert et al., 1995a) this group consisted of patients who were in a state of more optimally controlled heart failure, as reflected by the observed lower total body spillover of noradrenaline.

It is recognized that patients with chronic CHF have abnormal autonomic neuronal reflex responses to changes in cardiopulmonary loading conditions. Patients with severe CHF develop forearm vasodilation in response to lower body negative pressure (LBNP) (Ferguson et al, 1983), where such reductions in cardiopulmonary filling pressures should cause sympathoexcitation. It also appears that patients with CHF have abnormal responses to increases in cardiac filling pressures, with the observation that a positive correlation exists between these and measures of sympathetic activity

(Kaye et al., 1994). Reducing cardiac filling pressures with sodium nitroprusside (Kaye et al., 1998) or LBNP (Azevedo et al., 2000; Floras, 2001) lowers cardiac sympathetic activity. These observations led to the hypothesis in the current study that lowering cardiac filling pressures in CHF may lower brain noradrenergic turnover.

To date, limited data is available regarding the effects of systolic blood pressure reduction upon central noradrenergic turnover in healthy subjects. We have previously observed an increase in central monoamine turnover from unilateral jugular venous sampling in 4 healthy subjects after the administration of trimetaphan, a ganglion blocker (Lambert et al., 1998). We feel that the most likely explanation for the lack of response to SNP in healthy subjects in this current study is that we found it technically difficult to maintain a steady hypotensive state with SNP during the period of bilateral jugular venous sampling.

In the CHF patients, we also did not observe any significant change in central noradrenergic activity after SNP. Whilst again there was a difficulty in maintaining steady state hypotension, another explanation is that there was possibly competing afferent information to the CNS from cardiopulmonary and peripheral arterial baroreceptors. A future study employing nonhypotensive lower body negative pressure may be useful to overcome these limitations. Application of LBNP will offload cardiopulmonary baroreceptors, without influencing peripheral arterial baroreceptor afferent input (Azevedo et al., 2000).

5.6 Conclusions

This study examines the central origins and regulatory mechanisms underlying the heightened sympathetic drive in human heart failure. The novel findings are that neuronal noradrenaline turnover in suprabulbar subcortical regions of the brain is increased, and is positively correlated with the level of whole body noradrenaline spillover in human CHF. The findings support the hypothesis that activation of noradrenergic neurons projecting rostrally from the brainstem mediates the sympathetic nervous stimulation present in CHF.

Chapter 6
Effects of carvedilol therapy on peripheral lymphocytic β-adrenoceptor kinase expression in human heart failure

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6.1 Introduction

Carvedilol, a non-selective α_1, β_1, and β_2 - adrenoceptor antagonist has been shown in placebo – controlled clinical trials to markedly reduce mortality in heart failure (Packer et al., 1996; Packer et al, 2001). A possible explanation for this may be attenuation of cardiac sympathetic drive (Metra et al., 2000) via blockade of β-adrenoceptors, given that cardiac noradrenaline spillover has been demonstrated to be powerful prognostic indicator (Kaye et al., 1995). Another common finding in the published trials is that carvedilol results in improvement of left ventricular ejection fraction (LVEF), with gains of up to 50% from baseline having been reported (Metra et al.,1994; Olsen et al., 1995; Bristow et al., 1996). The mechanisms for this have not yet been fully elucidated.

Whilst it has been clearly established that there is a down-regulation of cardiac β_1 – adrenergic receptors in heart failure (Bristow et al., 1982), the improvement in left ventricular systolic performance repeatedly observed with carvedilol does not appear to arise secondary to an upregulation of these receptors (Yoshikawa et al., 1996). Improvements in LVEF are not seen immediately upon commencement of therapy, but take several months (Eichhorn et al., 1996; Packer et al., 1996), thereby allowing for the possibility that the biological function of the cardiac myocyte is in some way improved.

6.1 1 Mechanisms responsible for progressive cardiac myocyte dysfunction

In the failing heart, although activation of the adrenergic and renin-angiotensin systems is quite effective for short-term compensation, there are long-term adverse consequences of chronic activation of these systems that may override any initial benefit. With the conditions of increased heart rate and enhanced contractile state

resulting from activation of these neurohormonal systems, the failing dilated ventricle has a much greater metabolic need than smaller ventricles operating at a lower heart rate. Katz has called the failing heart an "energy-starved heart" and has suggested that this state of energy deprivation may damage subcellular processes in the long term and contribute to progressive cardiac myocytic dysfunction (Katz, 1988).

Another adverse biological effect of activation of these systems is direct cardiac toxicity (Communal et al., 1998), and this appears to be primarily mediated by β-adrenergic mechanisms for both noradrenaline and angiotensin II (Mann et al., 1992; Henegar et al., 1995), with the observation that in the failing heart, angiotensin II appears to be a powerful facilitator of noradrenaline release (Malik et al., 1976).

The β-adrenergic receptor mediates the stimulatory effects of noradrenaline on the adenylyl cyclase system and thereby regulates intracellular cyclic AMP levels (Lefkowitz et al.,1983). Whilst one of the mechanisms underlying the process of reduced myocyte contractile function is down-regulation in β-adrenoceptor density (Bristow et al., 1982), another is β-adrenoceptor "homologous desensitisation". This term refers to the observation that exposure of cells containing β-adrenergic receptors to β-adrenergic agonists results in rapid and reversible loss of the receptor-mediated response to subsequent stimulation (Hausdorff et al., 1990). It is now accepted that receptor phosphorylation is a key step in the process of receptor desensitisation (Hausdorff et al., 1990).

6.1.2 β-adrenergic receptor kinase

A selective kinase, termed β-adrenergic receptor kinase (β-ARK) has been identified which phosphorylates the agonist-occupied form of the receptor. β-ARK is one of a family of enzymes that phosphorylates β-adrenoceptors (β-AR) and other G protein-

coupled receptors after they have been stimulated, thus leading to their desensitisation (Pitcher et al.,1998). This process causes inhibition of receptor functionality by up to 70% (Ungerer et al., 1994). β-ARK is thought to effect desensitisation by acting in concert with an inhibitor protein called β-arrestin (Pitcher et al., 1998). Phosphorylation of the agonist-occupied β-receptors by the specific β-ARK is followed by binding of the inhibitor protein β-arrestin to the phosphorylated receptor (Ungerer et al., 1994). Two isoforms have been identified both for β-ARK and β-arrestin. Ungerer et al have demonstrated that in the failing human heart, quantitation by reverse-transcription polymerase chain reactions shows that there are no changes of the mRNA levels for β-arrestin-1 and β-arrestin-2, a slight (<50%) increase of the mRNA for β-ARK-2, and a threefold increase for β-ARK-1 mRNA (Ungerer et al., 1994). A hypothesis has emerged that increased sympathetic nervous system activity associated with heart failure might be the initial stimulus for β-AR signaling alterations, including desensitisation (Iaccarino et al., 1998). In a test of this hypothesis, investigators have demonstrated in mice, β-AR stimulation significantly increases the expression of β-ARK-1, whereas β-blockade decreases expression (Iaccarino et al., 1998).

In the mouse heart, a 3-fold transgenic overexpression of β-ARK reproduces the marked biochemical and physiological desensitisation to β-adrenergic stimulation observed in heart failure (Koch et al., 1995). The blunted cardiac response to isoprenaline and elevated cardiac β-ARK activity seen in these mice are both reversed when these animals are mated with mice overexpressing the β-ARK inhibitor peptide in the heart (Akhter et al., 1999). With such experimental data showing the beneficial effects of the β-ARK inhibitory peptide on reversing β-AR desensitisation, the

possibility arises that β-blocker therapy with β-ARK inhibition may be complementary therapeutic modalities (Lefkowitz et al., 2000).

6.1.3 β - adrenergic receptor kinase levels in peripheral lymphocytes

Previous investigators have demonstrated that the β-receptor density in human atria correlates with that observed in peripheral lymphocytes (Brodde et al., 1986), and that, commensurate with altered cardiac receptor density, there is decreased lymphocytic β-adrenergic receptor density in patients with heart failure (Colucci et al., 1981). As a corollary to this, a high expression of β-ARK in circulating lymphocytes has been identified (Chuang et al, 1992).

6.2 Aims of study

Understanding that therapy with carvedilol improves left ventricular ejection fraction, and that this improvement occurs in the absence of an upregulation in cardiac β_1-adrenoceptor density, the hypothesis is that carvedilol reduces β-ARK levels in heart failure.

6.3 Methods

Seven patients with moderate to severe CHF were recruited into the study. At baseline, no patient was on β-anti-adrenergic therapy. All patients underwent assessment of left ventricular systolic function either by radionuclide ventriculography or echocardiography. Twenty-five ml of blood was then taken from the patients' antecubital vein for extraction of mononuclear cells as described below. All patients were then commenced on carvedilol, and their therapy was uptitrated over the next 6 weeks. At the end of three months from the time of commencement of the

study, left ventricular systolic function was reassessed and peripheral blood was again taken for extraction of mononuclear cells.

6.3.1 Isolation of mononuclear cells from human blood

The basic method for separating lymphocytes from other blood cell types involves mixing the blood with some erythrocyte aggregating agent, thereby causing the erythrocytes to clump and sediment to the bottom of the tube (Boyum, 1964). A solution of Ficoll-Paque (Pharmacia Biotech) was placed in a centrifuge tube. Blood was layered on top, and the two-phased system was centrifuged for 40 minutes at 1800rpm. The erythrocytes and granulocytes sedimented to the bottom of the tube, and the purified lymphocytes were then collected from the interface between the two phases. For the purposes of this study, the mononuclear cells were transferred into an Eppendorf tube and snap frozen in liquid nitrogen and stored at -70°C until used.

6.3.2 Protein immunoblotting

Protein extracts were prepared from the extracted peripheral blood mononuclear cells and homogenised in a radio-immunoprecipitation assay lysis buffer [1% Triton, 150mM NaCl, 10mM Tris (pH 7.5), 1mM EDTA, 250µM EGTA (pH 9.0), 0,5% Nonidet P-40, 40mM PMSF]. Fifty µg of the mononuclear cell extract was solubilised in SDS-loading buffer at 95°C for 5 minutes prior to separation on a 10% SDS-polyacrylamide gel. Subsequently, the extracts were transferred to a nitrocellulose membrane using a transfer buffer (48mM Tris, 39mM Glycine, 0.037% SDS, 20% Methanol). Western analysis was performed by blocking the nitrocellulose membrane in 5% nonfat milk powder in TBS buffer (10Mm Tris, 0.15 M NaCl, 0.05% Tween 20) for one hour at room temperature. The membrane was incubated with anti-GRK

2/3 (β-ARK 1/2) monoclonal antibody (1:2000, Upstate Biotechnology) in blocking buffer (5% nonfat milk powder in TBS), followed by HRP-conjugated anti-mouse IgG antibody (Amersham). The 80-kDa β-ARK protein was visualised by chemilluminescence (Renaissance; NEN) and autoradiography using Kodak XOMat AR film. A representative radiograph of a Western analysis is presented in Fig 6.1.

6.4 Results

The mean LVEF at the commmencement of the study was 31.4±11.0% (mean±SD) and was 36.6 ±9.2% after therapy with carvedilol (p=NS). When comparing the β-ARK/β-tubulin optical density before and after carvedilol therapy, no significant difference was demonstrated, 1.57±0.49 to 2.58±1.5 (OD±SEM), as depicted in Fig 6.2. Five of the seven patients had no change in their LVEF with carvedilol therapy. The remaining two patients had an increase in LVEF (31 to 46% and 15 to 36%). When examining the change in OD in these two patients alone, both demonstrated an increase in OD with therapy (0.58 to 1.05 and 0.98 to 1.25, subjects 4 and 5 in Fig 6.2).

Fig. 6.1 Representative Western Blot of two patients before and after carvedilol

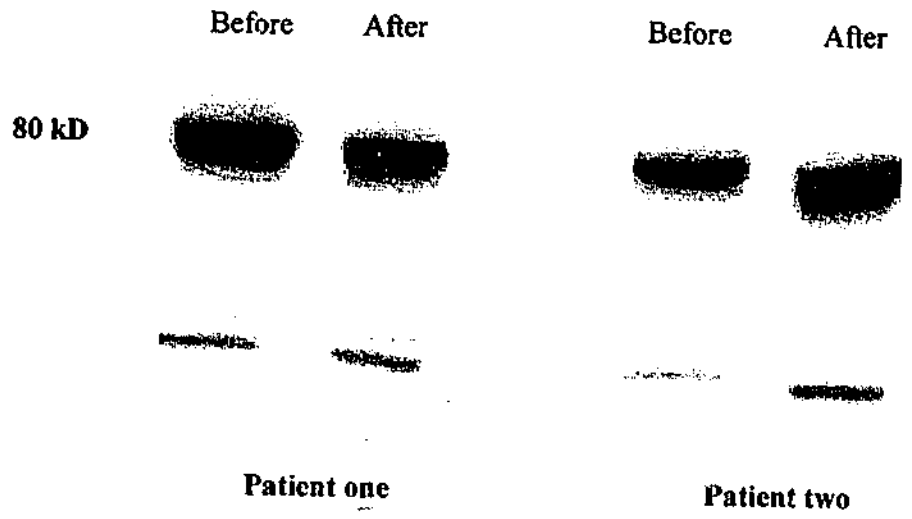
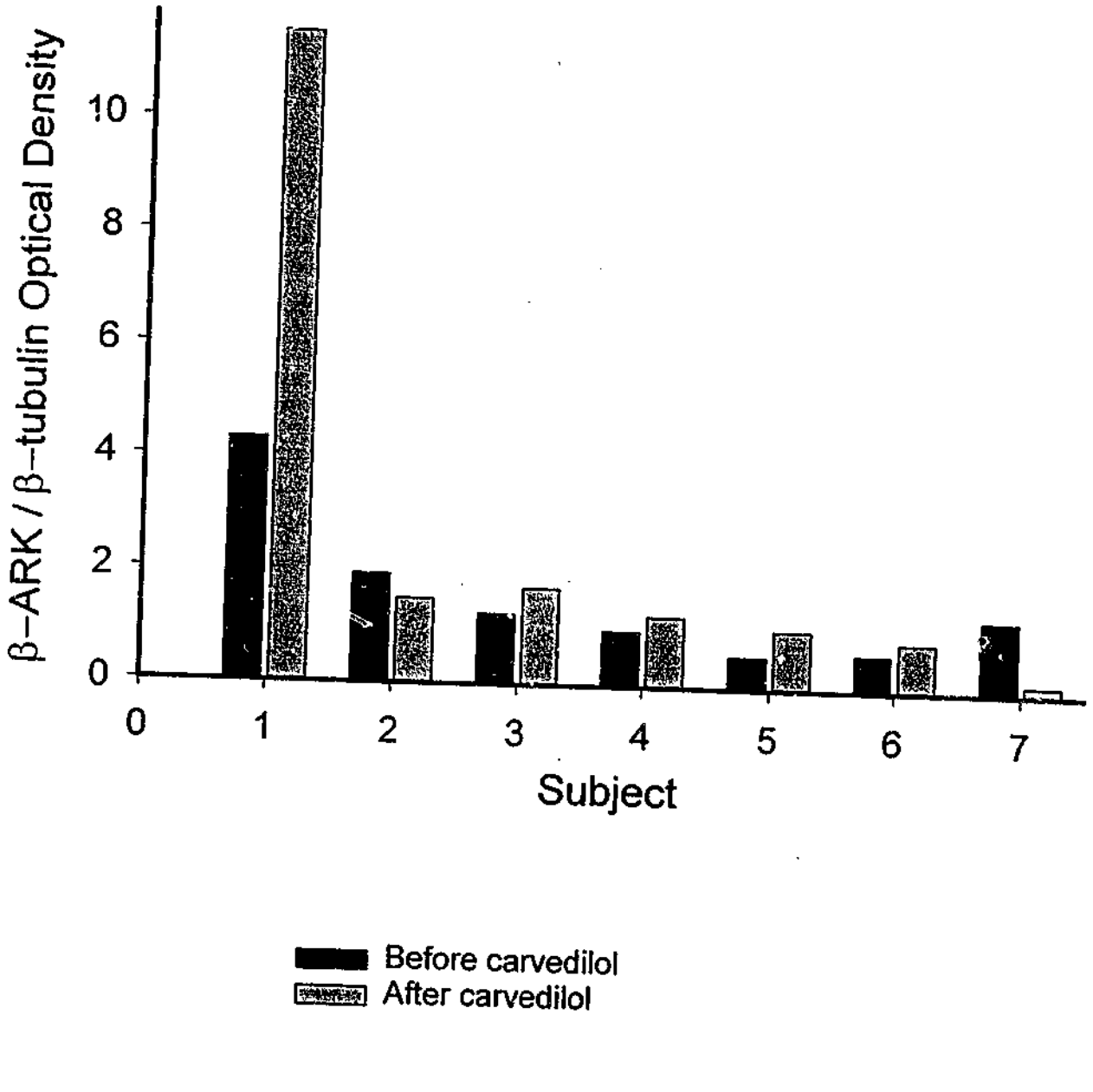


Fig 6.2 The optical density of β-ARK / β- Tubulin in heart failure subjects, before and after carvedilol.



6.5 Discussion

The aim of this study was to investigate whether there was a change in the expression of β-adrenoceptor kinase on peripheral mononuclear cells with carvedilol therapy in heart failure patients, and to correlate this with changes in left ventricular ejection fraction. In so doing, one would then be able to identify a mechanism by which β-blocker therapy improves myocardial contractile function in CHF.

In the study presented in this chapter, of the seven patients recruited, five had no demonstrable change in left ventricular ejection fraction after at least three months of carvedilol therapy. No significant change in β-ARK levels was demonstrated by Western analysis in these patients or in the two patients in whom an improvement in left ventricular systolic function was recorded.

6.6 Conclusions

It is not possible to make any definitive conclusions on the basis of the findings of this study. The sample size is too small and one of the problems this created was that the majority of patients had no demonstrable change in left ventricular systolic function with anti-adrenergic therapy. The technique employed to examine β-ARK levels in circulating lymphocytes was Western analysis, and a potential for error is controlling for the quantity of protein that is loaded. Whilst β-tubulin was used to adjust for this, this technique may not have eliminated this confounding factor entirely. It would be reasonable to expand this study by recruiting more patients and by incorporating analysis of mRNA expression for β-ARK by competitive PCR (Wang et al., 1989).

A possible explanation for the observed lack of effect of carvedilol could be that any change in β-ARK levels that may be instigated by therapy may be regionalised, and therefore lymphocyte findings might be difficult to interpret in the context of cardiac

effects of carvedilol. Another explanation for the lack of effect in β-ARK expression may be that other cellular proteins are altered by anti-adrenergic therapy in CHF, and that these changes result in improvements in myocardial contractile function. Possibilities include alterations in myosin heavy chain isoforms (Mercadier et al., 1990) and in the expression of proinflammatory cytokines such as TNF-α (Murray et al., 2000).

Chapter 7.

CONCLUSIONS

The central aims of this thesis were to further understand the central and peripheral processes that are responsible for the release of excess noradrenaline from sympathetic nerve terminals in human heart failure, and then secondly to investigate the therapeutic option of direct attenuation of the observed heightened sympathetic drive. The possibilities of peripheral presynaptic neuromodulation, and of the presence of a reflex arc involving cardiopulmonary afferents and central noradrenergic efferent fibres, were considered. In addition, an attempt was made to better localise the centres in the brain responsible for the efferent signals.

It was shown that presynaptic α_2-adrenoceptor functionality was down-regulated in heart failure, suggesting this as another mechanism by which circulating noradrenaline levels are increased in heart failure. Additionally, this finding suggests that attempting sympathetic attenuation with peripherally acting α_2-adrenoceptor agonists would not be a successful therapeutic modality in heart failure.

An initial aim of this work was to develop a non-invasive method of localising and studying central noradrenergic neurons in the brain. Whilst direct imaging with I-123 MIBG (metaiodobenzyluanidine), was not possible, it was shown (with the aid of cerebral venous blood pool scanning and bilateral internal jugular venous sampling for noradrenaline and its lipophilic metabolites) that central noradrenergic activity in heart failure arises from suprabulbar subcortical projections in the brain, and is positively correlated with the level pperipheral sympathetic activity in human heart failure. These findings support the hypothesis that activation of noradrenergic neurons projecting rostrally from the brainstem mediates the sympathetic nervous stimulation present in heart failure.

When attempting to elucidate further the afferent signals responsible for the activation of these suprabulbar subcortical regions, no relationship was found to exist between

left-sided cardiac filling pressures and noradrenergic turnover in these regions. The implication from this finding is that the sympathetic nervous stimulation present in CHF may perhaps result from a state of desensitization of low-pressure baroreflexes, thereby forming a basis for withdrawal of tonic inhibition of peripheral sympathetic activity, mediated centrally by activation of brainstem noradrenergic neurons projecting rostrally to suprabulbar subcortical areas.

Given that heart failure continues to increase in prevalence in our community, with attendant high morbidity and mortality, additional therapeutic approaches need to continue to be investigated. Whilst anti-adrenergic therapy and angiotensin-converting enzyme inhibition have made impressive inroads, some of the findings presented in this work suggest that the option of sympatholytic therapy should also be seriously considered. Evidence is presented that the cardiac adrenergic drive, just as it is disproportionately activated in heart failure, is disproportionately sensitive to sympatholytic therapy with low doses of clonidine. Further evidence to support the option of sympatholytic therapy in heart failure is the finding that renal sympathetic tone is also reduced with this approach, unlike with beta-adrenergic blockade. In addition evidence is presented that suggests that β-anti-adrenergic therapy may be synergistic with centrally acting sympatholytic therapy.

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