

B-type natriuretic peptide (BNP) in myocardial ischaemia-reperfusion injury

Thesis submitted by

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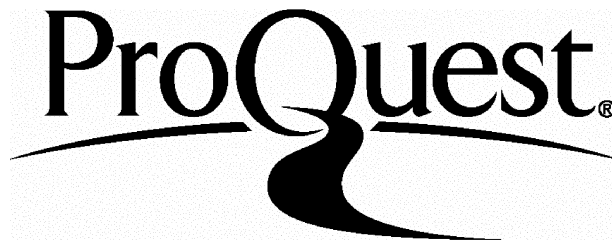
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Abstract

Type-B natriuretic peptide (BNP) is an important hormone abundantly present as a pro-peptide in cardiomyocytes. Its release is triggered by exercise, hypoxia and myocardial ischaemia, in addition to chronic haemodynamic cardiac overloading states. BNP's main endocrine actions of vasodilation and natriuresis are mediated by a particulate receptor guanylyl cyclase, natriuretic peptide receptor-A (NPR-A), with subsequent elevation of intracellular cGMP. The role and mechanisms of action of BNP in cardiac ischaemia are not known. We hypothesised that BNP mediates cardioprotection during acute ischaemia-reperfusion, via guanylyl cyclase and cGMP elevation, and examined the role of K_{ATP} channel opening in the protective mechanism. The role of nitric oxide (NO) in BNP's signal transduction was also evaluated.

Pharmacological studies were carried out in the Langendorff perfused rat heart. Endogenous BNP release was assessed by radio-immunoassay of coronary effluent samples following global normothermic ischemia. Peak concentrations in the first min of reperfusion were markedly elevated following 2 min, 5 min and 20 min of ischaemia. In rat hearts subjected to 35 min regional ischaemia, exogenous BNP limited infarct size in a concentration-dependent manner. The protective action of BNP was sensitive to inhibition by glibenclamide and 5-hydroxydecanoate, blockers of the mitochondrial K_{ATP} channel, but not by HMR1098, a blocker of the sarcolemmal K_{ATP} channel.

Radio-immunoassayed cGMP in cardiac tissue showed a proportionate rise when hearts were subjected to graded durations of ischaemia and when perfused with BNP. Hearts perfused with varying concentrations of 8-bromo-cGMP, a cell-permeable cGMP analogue, were protected against infarction at the lower concentration.

L-NAME a blocker of nitric oxide synthase (NOS), and ODQ a blocker of soluble guanylyl cyclase (sGC), both abolished cardioprotection when co-perfused with BNP, suggesting that NO and its activation of sGC play key roles in the protective effect of BNP. To evaluate the source of NOS, studies were undertaken using Western Blot techniques to probe the involvement of endothelial isoform of NOS (eNOS) known to be partially responsible for BNP's vasodilation. However, we found no evidence for acute phosphorylation of eNOS at serine 1177, following BNP or acute ischaemia.

Finally, together, our findings indicate a previously-unrecognised cardioprotective action of exogenous BNP via opening of the putative mitochondrial K_{ATP} channel with the involvement of basal nitric oxide in the NO/sGC, and cGMP release in the signal transduction. The work contained in this thesis thus confirms a cytoprotective role for BNP in myocardial ischaemia-reperfusion injury and requires further studies in other species and in transgenic models.

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List of Abbreviations

The following abbreviations are used in this thesis

5-HD	5 hydroxy decanoate
8-Br-cGMP	8-Bromoguanosine 3':5'- cyclicmonophosphate sodium salt
A ₁	Adenosine type 1 receptor
A ₃	Adenosine type 3 receptor
ACE	Angiotensin converting enzyme
ACS	Acute coronary syndromes
AEBSF-HCl	4-(2-Aminoethyl)-benzene sulfonyl fluoride hydrochloride
Akt	serine/threonine kinase or protein kinase B
AMI	Acute myocardial infarction
ANP	Atrial or type-A natriuretic peptide
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BNP	B-type or brain natriuretic peptide
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CABG	Coronary artery by-pass grafting
cAK	cyclic adenosine monophosphate dependent protein kinase
CaM	Calmodulin
cAMP	cyclic adenosine monophosphate
CFTR	Cystic fibrosis trans-membrane conductance regulator
cGK	cyclic guanosine-5'-monophosphate dependent protein kinase

cGMP	cyclic guanosine-5'-monophosphate
CHF	Congestive heart failure
CHD	Coronary heart disease
CNP	type-C natriuretic peptide
cDNA	complementary deoxyribonucleic acid
DAG	Diacyl glycerol
DMSO	Dimethyl sulfoxide
DNP	Dendroaspis natriuretic peptide
DPHPC	Department of public health and primary care
DTT	Dithiothreitol
eNOS	endothelial nitric oxide synthase
EDP	End diastolic pressure
EDTA	Ethylene diamine tetra acetic acid
ERK	Extracellular signal related kinase
ET-1	Endothelin 1
G-protein	Guanine nucleotide binding regulatory protein
GC	Guanylyl cyclase
GTP	Guanosine triphosphate
IL	Interleukin
I/R (%)	Infarct-risk ratio (%)
iNOS	inducible nitric oxide synthase
IP ₃	Inositol 3,4,5 triphosphate
IPC	Ischaemic preconditioning
JAK	Janus kinase
JNK	c-jun N-terminal kinase
KATP	Adenosine triphosphate-sensitive potassium channel

KLD	Kinase-like domain
LV	Left ventricle
L-NAME	N ω -Nitro-L-arginine methyl ester hydrochloride
MAP	Mitogen activated kinase
MPTP	Mitochondrial permeability transition pore
mK _{ATP}	mitochondrial adenosine triphosphate-sensitive potassium channel
MPG	N-2-(mercaptopropionyl) glycine
mRNA	messenger ribonucleic acid
N-ANP	amino-terminal Atrial or type-A natriuretic peptide
N-BNP	amino-terminal B-type or brain natriuretic peptide
NADH	Nicotinamide adenine dinucleotide phosphate
NEP	Neutral endopeptidase 24.11
nNOS	neuronal nitric oxide synthase
NO	Nitric oxide
NP	Natriuretic peptide
NPR-A	Natriuretic peptide-A receptor
NPR-B	Natriuretic peptide-B receptor
NPR-C	Natriuretic peptide-C receptor
NSTEMI	Non-ST-elevation myocardial infarction
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
PCI	Percutaneous coronary intervention
PDE	Phosphodiesterase
pGC	particulate guanylyl cyclase
phospho-eNOS	phosphorylated endothelial nitric oxide synthase
PI3 kinase	Phosphatidylinositol-3 kinase
PKA	cyclic adenosine monophosphate dependent protein kinase

PKC	Protein kinase C
PKG	cyclic guanosine-5'-monophosphate dependent protein kinase
PLC	Phospholipase C
PTCA	percutaneous transluminal coronary angioplasty
R-A-A	Renin-angiotensin-aldosterone axis
ROS	Reactive oxygen species
Ras	small guanine-nucleotide binding protein
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
sGC	soluble guanylyl cyclase
sKATP	sarcolemmal adenosine triphosphate-sensitive potassium channel
STAT	Signal transducer and activator of transcription
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylenediamine
TNF- α	Tumour necrosis factor- α
Tris	Trizma base
TTC	Triphenyltetrazolium chloride
TyrK	Tyrosine kinase
UA	Unstable angina

Publications arising from this thesis

Abstracts

- Cardioprotective action of B-type natriuretic peptide is NO-dependent.

SP D'Souza, DM Yellon & GF Baxter. *Br J Pharmacol* 2003; 138 (Suppl): 21P

- Type-B natriuretic peptide limits infarct size via K_{ATP} channel opening in the langendorff perfused rat heart.

SP D'Souza, DM Yellon & GF Baxter. *Circulation* . Nov 2002, 106(19), II-192.

- Type-B natriuretic peptide limits infarct size via K_{ATP} channel opening.

SP D'Souza, DM Yellon & GF Baxter. *J Mol Cell Cardiol* June 2002, 34(6): A21.

- Type-B natriuretic peptide limits infarct size in rat isolated heart.

SP D'Souza, DM Yellon & GF Baxter. *Br J Pharmacol* March 2002, 135, 325P.

Full papers

- B-type natriuretic peptide limits infarct size in rat isolated hearts via K_{ATP} channel opening.

D'Souza SP, Yellon DM, Martin C, Schulz R, Heusch G, Onody A, Ferdinandy P, Baxter GF. *Am J Physiol Heart Circ Physiol* 284(5):H1592-H1600, 2003.

- B-type natriuretic peptide: A good omen in myocardial ischaemia?

SP D'Souza and GF Baxter. *Heart* (2003, in press).

Chapter One

Introduction

1.1 General Introduction

An estimated 2.6 million of the United Kingdom population have coronary heart disease (CHD). About 86,000 cases of acute myocardial infarction (AMI) per year occur in men and woman under the age of 65. However, despite recent improvement, the mortality from CHD in the UK is still amongst the highest in the world, with around 125,000 deaths in the year 2000. UK statistics reveal that one in four men and one in six women die from the disease (DPHPC, 2002) and by current trends, the World Health Organisation predicts ischaemic heart disease will be the leading cause of disease burden worldwide in 2020 (Lopez et al, 1998). Thus, limitation of the pathophysiological process in ischaemic heart disease remains a priority for the managing physician. With the world-wide use of thrombolytic agents and the more affluent societies resorting to primary angioplasty, damage from myocardial infarction has been limited. But, the need for treatments to maintain viability of the myocardium remains paramount. An appreciation of the molecular triggers, cell signalling pathways and target proteins associated with protection, indicate the ability of the myocardium to adapt to ischaemia and thereby protect itself from otherwise lethal ischaemic insult. More than any time in the past, it is conceivable that specific pharmacological treatments can be developed to protect the ischaemic and reperfused myocardium through direct targeting of endogenous cytoprotective pathways (Yellon et al, 2000).

Acute ischaemia is associated with the release of endogenous mediators, the therapeutic potential of which is not exploited. One such mediator is a peptide called

B-type natriuretic peptide (BNP) which has long been known to play an important role in the control of cardiovascular homeostasis of mammalian and non-mammalian vertebrates, but its relevance in coronary ischaemia remain obscure. The potential protective role of BNP in myocardial ischaemia is addressed in this thesis.

1.2 Natriuretic Peptide System

1.2.1 Overview and historical background

The natriuretic peptide (NP) system is made up of a family of structurally similar, but genetically distinct polypeptide hormones (see figure 1.1). The peptides constituting this system tend to be quite highly conserved across species. They are not restricted to mammals – fish express them abundantly and they are important for fluid regulation in invertebrates, micro organisms and even plants (Loretz et al, 2000; Takei 2001). Four endogenous NPs have been isolated to date. These include atrial, or type-A natriuretic peptide (ANP), BNP, type-C natriuretic peptide (CNP) and the more recent addition of *Dendroaspis* natriuretic peptide (DNP). The history of the research on natriuretic peptides can be traced back to 1956, when Kisch noted membrane bound granules in the atrial (but not ventricular) myocytes of guinea pig heart (Kisch, 1956). In a major advancement, the function of these granules and the heart as an endocrine organ only began to be understood a quarter of a century later in 1981, when de Bold discovered the potent natriuretic and vasodepressor activity of ANP in atrial (not ventricular) extracts (de Bold et al, 1981). Subsequently studies aimed at discovering the other family members, resulted in the isolation of two other factors -BNP in 1988 (Sudoh et al, 1988) and CNP in 1990 (Sudoh et al, 1990) both from porcine brain. Hence, BNP

was originally termed 'brain natriuretic peptide'. This peptide was then isolated from porcine heart and blood (Aburaya et al, 1989b) and subsequently using porcine cDNA as a screening probe, human and canine gene homologues of porcine BNP were isolated and sequenced (Seilhammer et al, 1989; Sudoh et al, 1989). BNP was also isolated from the rat species, elucidated by cDNA sequencing. The amino acid sequences of BNPs vary from species to species, unlike those of mammalian ANPs. DNP was originally isolated from the venom of the green mamba snake (*Dendroaspis angusticeps*; Schweitz et al, 1992) and subsequently the human homologue was isolated (Schirger et al, 1999; Best et al, 2002).

A striking feature of the NP system is its diversity of action on all *in vivo* tissues concerned with regulation of sodium and blood pressure homeostasis under basal conditions and in pathophysiological conditions such as congestive heart failure (Stein et al, 1998). Important actions to increase haematocrit, diminish sympathetic neuronal activity, and reduce vascular cell growth (Espiner et al, 1994) are opposed by those of the renin-angiotensin-aldosterone (RAA) system, making it likely for the two systems to counterbalance each other in the regulation of sodium and blood pressure homeostasis.

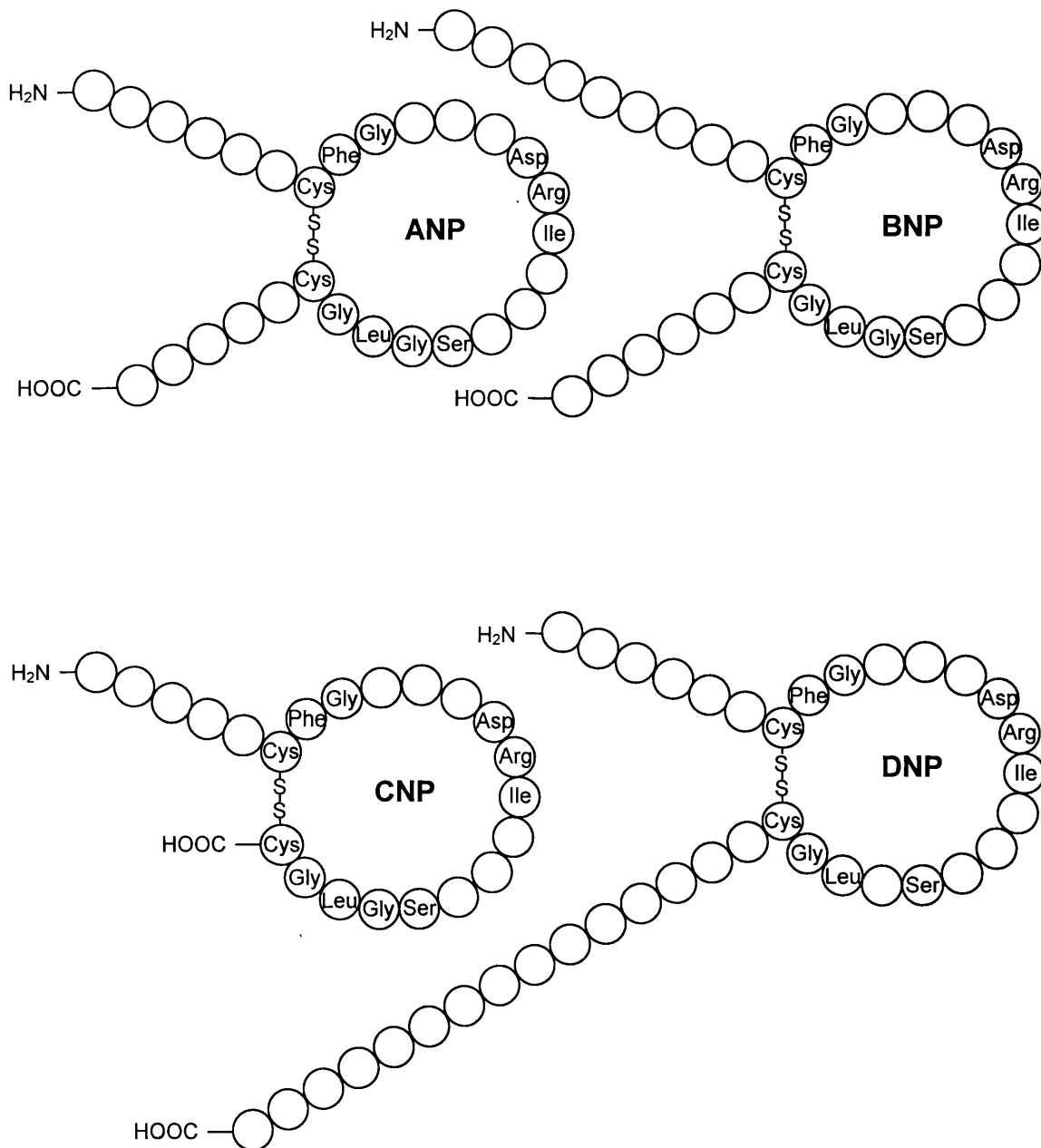


Figure 1.1 Structure of natriuretic peptides

Mature forms of the human natriuretic peptides belonging to the atrial natriuretic peptide (ANP-28), B-type natriuretic peptide (BNP-32), C-type natriuretic peptide (CNP-22) and *dandrosapis* natriuretic peptide (DNP-35) families. Every member has a disulphide bond between two cysteine residues, producing a 17-amino acid ring structure responsible for biological action. Residues homologous to all four peptides are shown.

1.2.2 Biological structure, tissue distribution and action

The NP family consists of structurally homologous peptides having at their core a 17-amino acid disulphide ring structure as shown in figure 1.1, essential for receptor recognition and biological function (Inagami et al, 1987). All the NPs are initially synthesised as precursors that are post-translationally modified. Biological activity of the peptides generally resides in the mature forms of the hormones, particularly the carboxy-terminal residues, although prohormones such as proANP and proBNP also may have bioactivity (Kangawa et al, 1985). The amino (N)-terminal fragments are generally inactive. However, it has been reported that further degradation of N-ANP, may lead to several smaller fragments some of which may also have biological activity (Vesely et al, 1994). Less is known about the processing of BNP; it is believed to be more constitutively expressed. The N-terminal fragments have no specific clearance receptor and as a result have a longer half-life than the primary peptide.

All these peptides are cleared by the natriuretic peptide-C receptor (NPR-C) - a clearance receptor, and binding of this ligand leads to internalisation of the receptor-ligand complex to the cell and degradation of the ligand (Levin et al, 1998). An ectoenzyme neutral endopeptidase 24.11 (NEP) also takes part in the inactivation of natriuretic peptides. Both NPR-C and NEP are widely expressed in the kidneys, lungs and the vascular wall (Shima et al, 1988).

1.2.2.1 *Atrial natriuretic peptide (ANP)*

ANP is a 28-amino acid polypeptide secreted largely from human atria in a prompt response to stretch (Espiner et al, 1995). Cloning of the human ANP, cDNA revealed that it encodes a 151 amino acid prohormone (Oikawa et al, 1984). Proteolytic

processing removes the signal peptide, and in some species the carboxy-terminal di-Arg, to form a 126 amino acid prohormone (proANP) that is stored in dense granules in the atrial myocytes. Recent studies indicate that during the secretory process, corin, a cardiac serine protease, cleaves proANP to ANP in a highly sequence-specific manner and may represent a proANP-converting enzyme (Yan et al, 2000) to form proANP₁₋₉₈ (N-terminal ANP), and the biologically active hormone, carboxy-terminal peptide (ANP₉₉₋₁₂₆) (Vuolteenaho et al 1985; Ruskoaho 1992). The ANP precursor peptide gene resides on human chromosome 1 and comprises three exons and two introns. Although expressed under physiological conditions primarily in the atrium, the induction of left ventricular-ANP gene expression is seen in most of the clinical disorders as well as experimental models with pressure or volume overload, and the increase occurs within the first day of experimental overload (Ruskoaho 1992). Much lower levels of ANP mRNA are present in normal adult ventricle (1% of atrial ANP mRNA), although gene expression here may increase markedly in pathological states, such as congestive heart failure (CHF) and patients with left ventricular hypertrophy (Ruskoaho 1992). *In vivo*, plasma ANP increases rapidly in response to pressure as well as volume loading, (Lang et al, 1985; Ruskoaho 1992), and also in response to physical exercise (Vuolteenaho et al 1985), but has a short half-life of one to three minutes (Ruskoaho 1992; Espiner et al, 1995).

In isolated perfused hearts, increased atrial pressure very rapidly releases ANP to the perfusate (Ruskoaho et al, 1986). Non-cardiac sources include the brain, eye, lung, thymus, vascular tissue, kidney gastrointestinal tract and adrenal medulla where gene expression levels are low. Besides the major cardiovascular effects, i.e. vasodilation, diuresis and natriuresis, ANP reportedly has a direct inotropic effect, mediated via its receptor natriuretic peptide receptor-A (NPR-A) and the second messenger cGMP (see

below), leading to a decrease of intracellular pH and subsequently decreased Ca^{+2} sensitivity (Tajima et al, 1998).

1.2.2.2 Type-B natriuretic peptide (BNP)

BNP is a 32-amino acid polypeptide secreted predominantly by cardiac atria and ventricles. Human BNP is synthesised as a preproBNP of 132 amino acids that is processed to a 108 amino acid precursor protein (proBNP₁₋₁₀₈) which is cleaved into a biologically active 32 amino acid carboxy-terminal fragment and a 76 amino acid amino-terminal fragment (Sudoh et al, 1988; Suzuki et al, 2001; Espiner et al, 1995). , Unlike ANP, which is stored as the 126-amino acid prohormone, the most abundant form of BNP in the rat atria, is composed of 45 amino acids (Aburaya et al, 1989a; Nakao et al, 1990) and 32 amino acids in human atria (Hino et al, 1990; Kambayashi et al 1990b; Espiner et al, 1995). BNP coexists with ANP in secretory granules of human cardiomyocytes (Nakamura et al, 1991). The BNP precursor peptide gene resides upstream of the ANP precursor peptide gene on human chromosome 1 and comprises two exons and an intron. BNP gene expression, in atria and ventricles is induced within one hour in response to stretch from increase venous volume overload (Nakagawa et al, 1995; Hama et al, 1995) and/or an increase in pressure (Boomsma et al, 2001) and has a plasma half-life of about twenty one minutes (Espiner et al, 1995).

Though released from the atria and ventricles, the ratio of ventricular-to-atrial BNP mRNA is higher than that observed for ANP. Thus, when allowance is made for organ weight, these findings are consistent with the view that the ventricle is the main source of BNP secretion in contrast to ANP, where secretion in health occurs from the atrium (Minamino et al, 1988; Ogawa et al, 1991; Yandle et al, 1994; Espiner et al, 1995). Extracardiac BNP synthesis has been reported in human brain and amnion tissue, and

bovine adrenal medulla (Espiner et al, 1995). With chronic overload, BNP mRNA levels have been suggested to remain constantly increased (de Bold et al, 1996). The rapid induction of BNP gene expression in response to overload has been used as a marker of elevated loading (Tokola et al, 2001). Short-lived transcripts are a characteristic of many genes that are rapidly induced (Sachs, 1993). For this reason, BNP has been proposed to be a primary response gene to modulate the mitogenic effects of other hormones.

Human BNP is more potent than rat BNP in the relaxation of porcine coronary artery strips (IC_{50} of 0.02 v 1.10 nM respectively), indicating that the biological actions of BNP are species-specific (Kambayashi et al, 1990a). The haemodynamic effects of BNP are largely similar to ANP. Like ANP, BNP binds to NPR-A, a trans-membrane bound particulate guanylyl cyclase, which via 3',5' -cyclic guanosine monophosphate (cGMP), mediates natriuresis, vasodilation, renin inhibition, lowering plasma volume and blood pressure, antimitogenesis, and lusitropic properties (Koller et al, 1992; Espiner et al, 1995; Silberbach et al 2001; see figure 1.2). The regulation, metabolism and actions of BNP in comparison to ANP and CNP are summarized in table 1.1.

1.2.2.3 *Type-C natriuretic peptide (CNP)*

From a 103 amino acid CNP pro-peptide, CNP produces 22 and 53 amino acid fragments with the former contained within the latter. The 22 amino acid fragment is the mature and more active form, and is expressed in the nervous system and endothelial cells (Espiner et al, 1995; Suzuki et al, 2001). While, ANP and BNP have five and six amino acid residues attached to the carboxy-terminal tail respectively, CNP completely lacks this tail.

Heart tissue contains little CNP and, only small amounts if any are found in plasma (Yandle et al, 1994). The situation is reversed in the brain and nervous tissue where CNP seems to be the most abundant form of natriuretic peptide in most species (Minamino et al, 1993a; Yandle et al, 1994). Several cytokines, including transforming growth factor beta, interleukin-1 alpha, tumour necrosis factor alpha (TNF- α) and endotoxin, stimulate CNP mRNA suggesting a regulatory role for CNP in vascular remodelling. CNP is more potent than ANP in eliciting smooth muscle relaxation but is a less potent inducer of diuresis and natriuresis (Sudoh et al, 1990; Clavell et al, 1993). Perhaps reflecting its functional and evolutionary distinction from ANP and BNP, the CNP gene is located on human chromosome 2 and has three exons (Ogawa et al, 1992). Its gene transcripts are also found in the vascular endothelium, consistent with the peptide's putative/paracrine role in the regulation of vascular tone and cell growth (Komatsu et al, 1992; Furuya et al 1993) via the guanylyl cyclase-linked natriuretic peptide-B receptor (NPR-B).

1.2.2.4 *Dendroaspis natriuretic peptide (DNP)*

DNP, the newest member of the natriuretic family has 38 amino acids and a 17-aminoacid disulphide ring structure resembling ANP, BNP and CNP (Schweitz et al 1992). It has been suggested to exist, in the plasma and atrial myocardium of normal humans and is elevated in CHF (Schirger et al, 1999).

Like ANP and BNP, DNP also acts via NPR-A in the activation of the particulate guanylyl cyclase mediating a vasorelaxing action in human vasculature (Schweitz et al, 1992; Best et al 2002). Its action appears to be mediated via the inward rectifying potassium channels, prostaglandin production and involves elevation of cGMP.

	ANP	BNP	CNP
Major site of synthesis	Cardiac atria	Cardiac ventricles	Brain Vascular endothelium Kidney tubules
Major stimulus to synthesis	Atrial transmural pressure	Ventricular wall tension	? Cytokines ? Other natriuretic peptides
Major plasma forms	ANP ₉₉₋₁₂₆	BNP ₇₇₋₁₀₈ (BNP-32) and proBNP	CNP-22
Plasma half-life	3 min	21 min	2.6 min
Bioreceptor type	NPR-A	NPR-A	NPR-B
C-receptor affinity	High	Lower than ANP/CNP	High
Affinity for endopeptidase	High	Lower than ANP/CNP	High
Major actions	Natriuresis Vasodepression Inhibition of RAA Antimitogenesis	Natriuresis Vasodepression Inhibition of aldosterone ? antimitogenesis	? Vasodepression Antimitogenesis

Table 1. 1 Human natriuretic peptides compared

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; NPR-A, natriuretic peptide receptor subtype A; NPR-B, natriuretic peptide receptor subtype B; RAA, rennin-angiotensin-aldosterone axis (modified after Espiner et al, 1995).

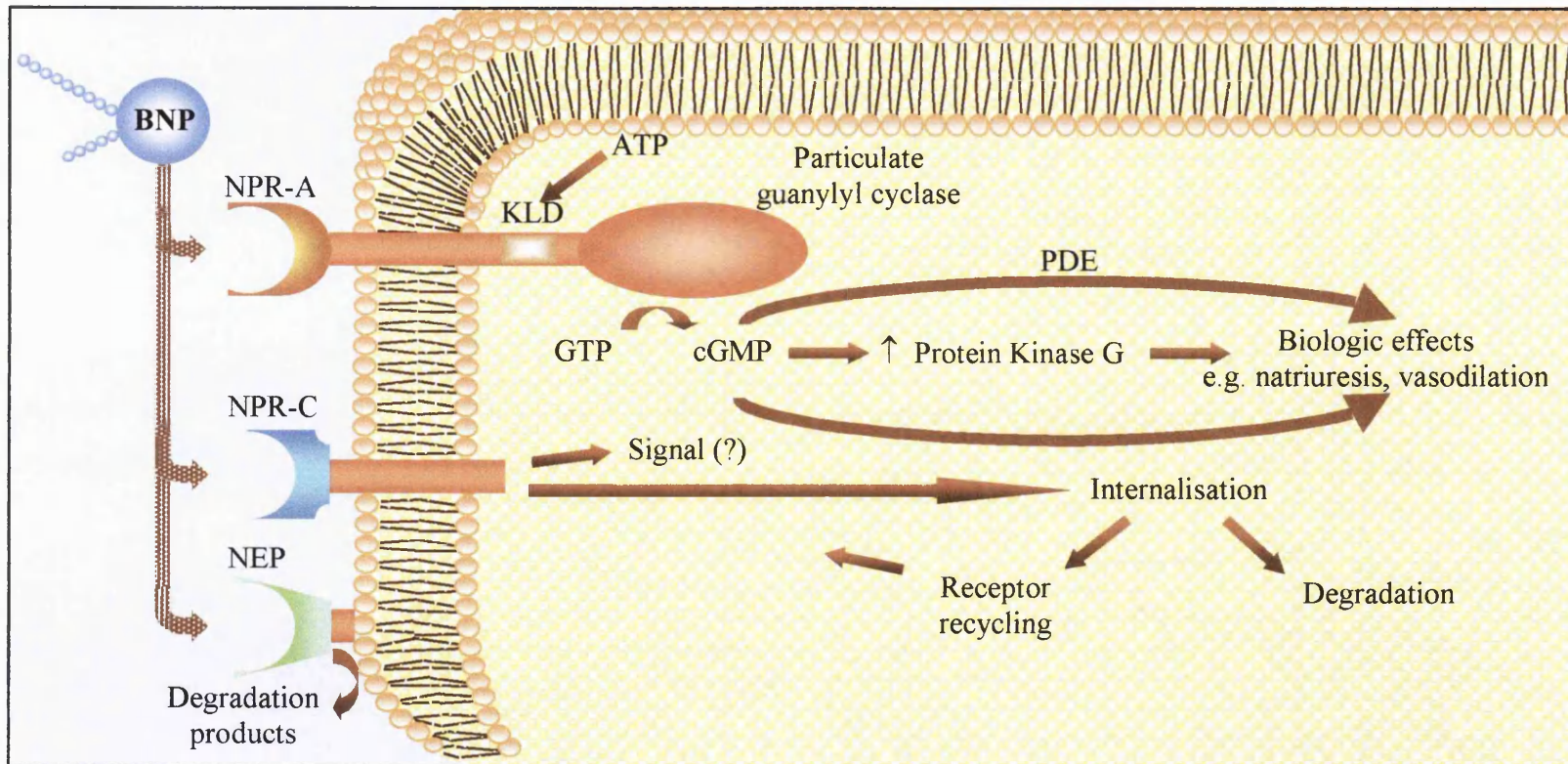


Figure 1.2 BNP signal transduction.

Type-B natriuretic peptide (BNP) binds to the extracellular domain of natriuretic peptide receptor A (NPR-A) and, in an ATP-dependent fashion, stimulates the intrinsic guanylyl cyclase activity of the receptor. Cyclic guanosine monophosphate (cGMP) exerts its biologic effects indirectly through cGMP-dependent protein kinase G (or cGK) or one or more phosphodiesterases (PDEs), or by direct action on the effectors such as amiloride-sensitive sodium channels in the kidney. ATP dependence requires the kinase-like domain (KLD) of the receptor. BNP also binds to natriuretic peptide receptor C (NPR-C), after which it is internalised and degraded. The C receptor may also have independent signalling functions. Finally, BNP may be degraded by extracellular neutral endopeptidases (NEPs) in the kidney and vasculature. GTP denotes guanosine triphosphate (adapted from Levin et al, 1998).

1.3 Regulation of BNP release and synthesis

In normal healthy adults, plasma concentrations of BNP are in the range of 0.3-10 pmol/L and vary with sodium intake and increase with age (Richards et al, 1993). Myocardial stretch resulting from an increased end-diastolic pressure of the left ventricle and/or an increase in wall stress has been postulated to act as a major physiological stimulus to BNP release and increased the BNP gene transcription (Tervonen et al, 1998). Plasma BNP concentration is a sensitive and specific marker of the altered left ventricular structure and function in a patient population at risk for cardiovascular disease (Yamamoto et al, 1996a).

Pathologically, highest levels of BNP (100- to 200-fold) are seen following congestive heart failure, suggesting a compensatory role for BNP (Yasue et al, 1994; Luchner et al, 1998), acute myocardial infarction (AMI) and cor-pulmonale (Nicholls, 1994). Hama et al also reported rapid induction of ventricular BNP gene expression in rats with AMI compared with ANP gene expression in acute ventricular overload (Hama et al, 1995). The rapid induction of BNP gene expression in response to overload has been widely used as a marker of elevated loading (Tokola et al, 2001). Non-cardiac oedematous states, such as nephrosis and cirrhosis where central blood volume may be increased, potentiate BNP release (Wong et al, 1994). Inflammatory conditions, such as endotoxaemia are known to trigger the release of BNP (Tomaru et al, 2002) as does angiotensin II and endothelin.

The mammalian myocytes are able to shift between the two pathways of protein secretion: constitutive pathway when the peptides are secreted as fast as they are synthesized and the regulated pathway when newly synthesized peptides are stored in membrane-enveloped granules before their secretion (Kelly et al, 1985). After birth,

while the atrial cells continue to store the peptides and to secrete them intermittently, the ventricular cells containing the mRNA for these peptides lack the secretory granules, indicating the secretion of natriuretic peptides through the constitutive pathway (Bloch et al, 1986). Hence, the immediate release of BNP is from the stored/cleaved form, followed later by gene transcription and new protein synthesis. A full-length human BNP promoter in the molecular regulation of the BNP gene expression following conditions outlined above, exerts greater activity in the ventricular than in atrial myocytes and is inactive in fibroblasts. The BNP gene promoter has both positive and negative regulatory elements (LaPointe et al, 1996).

1.4 The BNP receptors

1.4.1 Natriuretic peptide receptor-A

The diverse biological effects of BNP are mediated by binding of the hormone to a specific membrane-associated guanylyl cyclase (GC) receptor or natriuretic peptide receptor-A (NPR-A) also referred to as particulate guanylyl cyclase-pGC (distinct from the soluble or cytoplasmic form -sGC, stimulated in vascular smooth muscle cells by the actions of endothelium-derived relaxing factor nitric oxide) - (Espiner et al, 1995). NPR-A is widely expressed in the cardiovascular system with localisation in both the atria and the ventricles (Gutkowska et al, 1989). Other sites expressing the receptor include the aorta and generalised vasculature (Espiner et al, 1995; Kone et al, 2001), lungs, kidney, plasma, skin and sympathetic cholinergic fibres. Both ANP and BNP can effectively stimulate NPR-A; BNP, however is approximately 10-fold less potent. CNP, in contrast, does not significantly increase intracellular cGMP in cells

expressing NPR-A. For NPR-B expressing cells, only CNP can effectively stimulate the receptor's enzymatic activity. Neither ANP nor BNP increase cGMP levels in these cells except at very high non-physiological concentrations (Koller et al, 1992).

Studies by Bennett et al (1991) on extracellular domains of the receptors show a correlation between the ability of NPs to stimulate guanylyl cyclase activity and the affinities of these hormones for the binding domains of the receptors, as shown in table 1.2. Both ANP and BNP can bind NPR-A with relative high affinity, but ANP is 4-70 times more potent than BNP. This result corresponds well with a 10-fold difference in EC_{50} s of these hormones to stimulate NPR-A cyclase activity (Koller et al, 1992; Yandle et al, 1994).

NPR-A contains an extracellular ligand-binding domain linked by a transmembrane domain to an intracellular domain.

Receptor	<u>Dissociation constants</u>		
	ANP	BNP	CNP
NPR-A	1.9 pmol	7.3 pmol	> 500 nmol
NPR-C	2.6 pmol	13 pmol	10.8 pmol

Table 1. 2 Affinity of natriuretic peptide receptors

Affinities of ANP, BNP and CNP for the natriuretic peptide receptor of the subtype A (NPR-A) and the clearance receptor natriuretic peptide receptor of the subtype C (NPR-C). The affinities are represented by their dissociation constants (after Bennett et al, 1991).

1.4.1.1 Extracellular domain

The ligand-binding extracellular domain contains several consensus sites for N-linked glycosylation as well as a number of conserved cysteine residues postulated to mediate intramolecular folding as well as intermolecular dimer- or oligomerisation.

1.4.1.2 Intracellular domain

The intracellular domain has a kinase homology domain which has proved to be central in regulating guanylyl cyclase activity and in modulating receptor sensitivity. Furthermore, critical ATP binding sites reside within this domain. It serves to repress the activation of the C-terminal guanylyl cyclase catalytic domain. The NPR-A receptor is phosphorylated in the basal state, and hormone binding is responsible for dephosphorylation and desensitisation (Potter et al, 1992 and 1994). Both, peptide binding and ATP are required for activation of the receptor and dephosphorylation of the kinase homology domain is part of the desensitisation phenomenon resulting in the generation of second messenger cyclic guanosine monophosphate (cGMP) (Silberbach et al, 2001).

1.4.2 Natriuretic peptide receptor-B

Like NPR-A, natriuretic peptide receptor-B (NPR-B) is also a specific transmembrane-associated guanylyl cyclase receptor with mRNA expression predominantly in neuronally derived tissue such as the brain, pituitary, adrenal medulla and to a minor extent in the cardiac atria and other sites (Koller et al, 1992; Espiner et al, 1995; Silberbach et al, 2001). It has an extracellular domain partly identical to NPR-A, an intracellular domain consisting of a kinase homology domain and a C-terminal guanylyl cyclase catalytic domain, activated on peptide-binding to its extracellular

domain. In NPR-B expressing cells, only CNP can effectively stimulate the receptor's enzymatic activity, synthesising the second messenger cGMP to coordinate body fluid homeostasis. Neither ANP nor BNP can increase cGMP activity in these cells except at very high, nonphysiological concentrations (Koler et al, 1992).

1.4.3 Natriuretic peptide receptor-C

BNP along with the other NPs also acts on another cell-surface receptor called natriuretic peptide receptor-C (NPR-C or clearance receptor). Molecular cloning has shown NPR-C to contain a very short 37-amino-acid cytoplasmic tail that bears no homology to the intracellular domain of any other known receptors. Its extracellular domain, however, is approximately 30% identical to NPR-A and NPR-B. It appears to be widely expressed in the kidneys, adrenals, brain, lungs and the vascular wall (Shima et al, 1988; Porter et al, 1990). Overall, NPR-C seems to be the most abundant receptor in most tissues and comprises more than 90% of the total NPR complement in important target tissues such as the kidney, at least in rats (Maack et al, 1993).

All four human NPs and a diversity of natriuretic hormones and analogues bind NPR-C with high affinity. NPR-C is not specific for BNP and tends to bind ANP and CNP as well and with higher affinity (Yandle et al, 1994). The dissociation constants are shown in table 1.2. Amongst species, ANP and CNP bind similarly to both rat and human C receptor, while the binding affinity of human BNP to the human C receptor is ten fold lower than the binding of human BNP to the rat C receptor. This explains the differences in half-life of Nps, particularly BNP, across species (Espiner et al, 1995).

The physiological role for NPR-C is not clear. The NPR-C along with neutral endopeptidase (see below) is thought to contribute in a combinatorial manner to the

clearance of the NPs. It is postulated that this protein functions as a clearance receptor to remove large amounts of ANP (and BNP) from the circulation (Maack et al, 1993). Its abundance in vascular tissue may serve to absorb excess ANP and protect against sudden onset of dangerously low blood pressure. It has also been suggested that NPR-C may mediate biological effects of ANP through second messengers other than cGMP, via coupling to G-proteins as well as adenylyl cyclase/cAMP system (Murthy et al, 2000; Anand-Srivastava et al, 1991).

1.4.4 Neutral Endopeptidase

Plasma levels of NPs are determined by secretion, uptake by specific receptors (chiefly NPR-C), degradation by enzymes (neutral endopeptidase EC 24.11), and to a minor extent by renal elimination.

Neutral endopeptidase (NEP) is a membrane-bound ectodermal enzyme widely distributed in many tissues including the vascular endothelium and the kidneys (Erdo et al, 1989; Soleilhac et al, 1992). Although its exact location in relation to NP receptors is unknown, it is possible that the enzyme is juxtaposed to the NPR-A in the cell membrane (Okamoto et al, 1994) making it uniquely placed to regulate the bioactive concentration of the receptor bound natriuretic hormone. Of the NP hormones, CNP is the preferred substrate, while hBNP-32 has the lowest affinity for the enzyme (Kenny et al, 1993). In rats, NPR-C and neutral endopeptidase contribute nearly equally, and together, the two pathways account for approximately 70% total-body clearance of ^{125}I -ANP (Yandle et al, 1994).

This enzyme is non-specific in its action and also hydrolyses other vasodepressor peptides such as bradykinin, angiotensin II and endothelin.

1.5 Post-receptor signalling

BNP, like several other endogenous and exogenous compounds produces cellular responses via cGMP (Espiner et al, 1995). The biochemical mechanisms underlying those responses depend on further targeting and degradation of cGMP. A cGMP-dependent protein kinase - cGK (or PKG) represents the principal intracellular mediator of cGMP signals (reviews Vaandrager et al, 1996; Lohmann et al, 1997; Silberbach et al, 2001). BNP induced elevation of intracellular cGMP, induces a binding-dependent activation of cGK leading to the catalytic transfer of the phosphate from ATP to a serine or threonine residue of the target protein. This phosphorylated protein then mediates the translation of the extracellular stimulus into a specific biological function. Two different genes for cGK have been identified in mammals. One is located on human chromosome 10 and encodes cGK-I, which is cytosolic, widely expressed in mammalian tissues and has two isoforms I α and I β . cGK-I α is detected mainly in the cardiac myocytes, vascular system, lung, cerebellum, kidney and adrenal glands, whereas cGK-I β is only present in the uterus (Keilbach et al, 1992; Tamura et al, 1996). cGK-II is a membrane bound homodimer on human chromosome 4 and absent from the cardiovascular system, but expressed in brain, intestine, lung, kidney and bone (Lohmann et al, 1997). cGK-I, acts as a soluble intracellular modulator of Ca²⁺, while cGK-II regulates fluid homeostasis at the cell membrane. cGKs activate cyclic nucleotide-gated ion channels, which regulate the influx of Na⁺ and Ca²⁺ into cells (Ludwig et al, 1999).

Activity of cGMP-regulated phosphodiesterases (PDE) is crucial for cellular signalling because metabolism of cyclic nucleotides modulates their intracellular concentrations and affects subsequent cellular and behavioural responses (Beavo, 1995; Essayan, 2001; Mehats et al, 2002). There is clear evidence that PDEs allow crosstalk and integration between different pathways (figure 1.3): PDE1 located in heart, lungs, brain and smooth muscle is activated by binding of Ca^{2+} -calmodulin to cause a decrease in cyclic nucleotide (cGMP or cAMP) concentration. NPs activate PDE2 distributed in heart, lungs, liver, adrenals and platelets, via an increase in cGMP levels (cGMP-stimulated PDE2), and the activation of this PDE2 in turn decreases cAMP signalling and inhibits adrenocorticotrophin-induced mineralocorticoid production. Nitric oxide is a potent secretagogue for renin secretion in the juxtaglomerular cells. It activates a soluble guanylyl cyclase, causing an increase in cGMP concentration in these cells. This is followed by the inactivation of PDE3 (cGMP-inhibited PDE3) also present in the heart, lung liver, platelets and immunocytes, followed by an increase in cAMP, activation of cAMP-dependent protein kinase – cAK (or PKA) and stimulation of renin secretion.

In vascular smooth muscle relaxation, different mechanisms operate synergistically to lower intracellular Ca^{2+} , using cGMP as a second messenger. These include, reducing Ca^{2+} influx, increasing Ca^{2+} efflux, promoting Ca^{2+} sequestration in sarcoplasmic reticulum and by decreasing Ca^{2+} mobilisation. The Ca^{2+} -pumping ATPase and the $\text{Na}^+ -\text{K}^+$ ATPase in the plasma membrane are activated by cGMP through PKG (Yoshida et al, 1992, Tamaoki et al, 1997). In the intestine, again PKG appears to be the principal molecular target of cGMP in the signal sequence leading to CFTR activation, promoting chloride efflux.

cAK contains nucleotide-binding sites homologous to those of cGK implying possible activation by cGMP. However, cGMP and cAMP signalling cascades elicit various important physiological effects independently and cross-regulation cannot compensate for loss of the other nucleotide (Lucas et al, 2000).

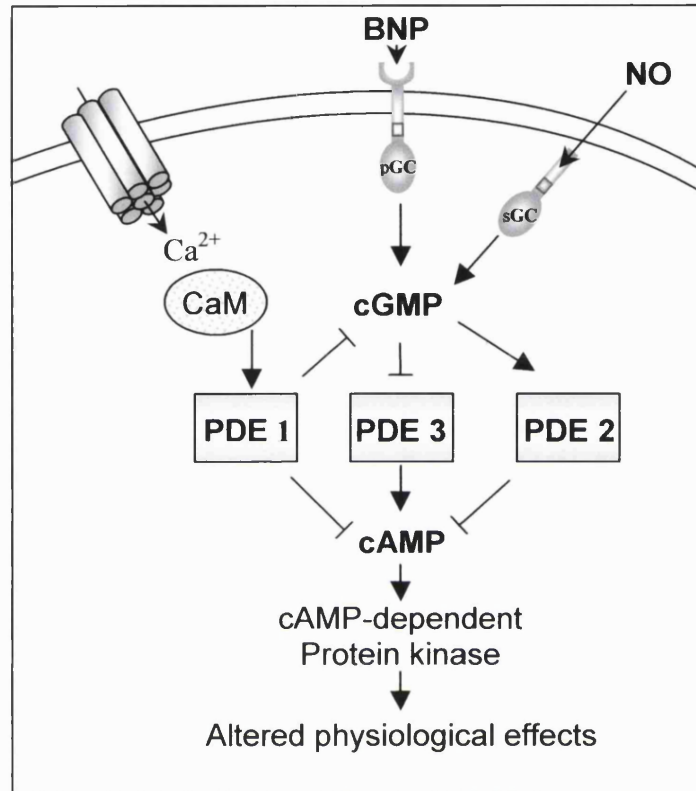


Figure 1. 1 PDEs and crosstalk between cGMP and cAMP

Following an increase in cytosolic Ca^{2+} concentration and binding to calmodulin (CaM), PDE1s are activated causing a decrease in cAMP and cGMP concentration. Stimulation of the particulate or soluble guanylyl cyclase (pGC or sGC) by BNP or nitric oxide (NO) respectively causes an increase in cGMP concentration that affects cAMP depending on the phosphodiesterase (PDE) isoform present. In some cells, cGMP activates the PDE2s, thereby decreasing cAMP concentration. In other cells, cGMP inhibits the PDE3s causing an increase in cAMP concentration (adapted from Beavo, 1995; Mehats et al, 2002).

1.6 BNP and cardiac ischaemia

When isolated rat ventricles are perfused using the Langendorff method, both ANP and BNP are released into the perfusate. Toth et al, have shown that hypoxia after removal of the atria in the isolated rat heart preparation, significantly enhanced both ANP and BNP release, demonstrating that these peptides are ventricular hormones stimulated by hypoxia (Toth et al, 1994). Thus during myocardial ischaemia, the increase in natriuretic peptide release may be linked to the decrease in the cellular energy state.

1.6.1 BNP expression in acute myocardial infarction

Plasma levels of ANP and BNP are markedly elevated in heart failure (Mukoyama et al, 1991) and are powerful predictors of ventricular dysfunction and mortality (Lainchbury et al, 1997). Both plasma ANP and BNP, measured in patients in the early phase of acute myocardial infarction (AMI), showed plasma BNP level to increase rapidly (within hours from the onset of AMI) and markedly (greater than 100 times the normal level) as compared to ANP (Mukoyama et al, 1991; Morita et al, 1993). The ANP level correlated with the pulmonary capillary wedge pressure, whereas the BNP level did not, but correlated inversely with the cardiac index. Foy et al, reported similar findings in patients post-AMI, with significant correlation with left ventricular ejection fraction measured at day 5 and month 3 after the episode (Foy et al, 1995). These observations suggested the possibility of BNP synthesis rapidly in the infarcted ventricle.

The BNP peptide (in plasma and ventricles) and ventricular BNP mRNA levels were determined in an experimental rat model of AMI produced by *in vivo* coronary artery

ligation (Hama et al, 1995). BNP concentration in the left ventricle increased about 2-fold as early as 12 hours post-AMI and 5-fold day 1 post-AMI compared to controls, whereas left ventricular ANP concentration which was also measured remained unchanged within one day. BNP tissue concentration was increased in the infarcted and non-infarcted regions as well as the right ventricle. BNP mRNA expression in the left ventricle was augmented 3-fold as early as 4 hours post-AMI (in contrast to ANP mRNA expression, which remained unchanged). Hence, the comparative and rapid BNP gene expression against acute ventricular overload post-AMI, suggests a pathophysiological role of BNP distinct from ANP in AMI. The study also demonstrated that in acute heart failure, BNP was secreted from the ventricle via *de novo* synthesis against ventricular overload, whereas ANP was secreted from the storage sites in the atrium. Thus, BNP can be a more sensitive marker of ventricular function than ANP.

In an ovine experimental model of *in vivo* coronary artery ligation (Cameron et al, 2000), ANP mRNA was documented within the infarct from 7 days post-AMI but no BNP gene expression, though a transient increase could not be excluded. The peri-infarct region showed predominant BNP gene expression rather than ANP expression, especially over the first 18 hours. This may be a response to both mechanical stress as well as haemodynamic overload.

1.6.2 BNP expression in unstable angina

The N-terminal proBNP reflects *de novo* synthesis of BNP rather than the stored form and can better reflect the natriuretic pathway activation. Both BNP and N-BNP concentrations are raised following AMI (Richards et al, 1998). Talwar et al,

compared circulating concentrations of N-BNP and cardiotrophin 1 (a cytokine of the interleukin 6 – IL6 family) in patients with stable and unstable angina. Blood was drawn within 48 hours of chest pain and in case of stable angina, prior to exercise testing and compared to controls. Both circulating N-BNP and cardiotrophin 1 were raised in unstable angina in comparison to controls and stable angina, while cardiotrophin 1 alone was raised in stable angina. The mechanism of the peptide released remained unclear and was postulated to be secondary to increase regional wall stretch secondary to myocardial ischaemia. Mechanical stretch can activate the Janus kinase/transcription 3 (JAK/STAT) pathway and may stimulate N-BNP secretion and augment the mRNA expression of IL6 and cardiotrophin 1. Alternatively, cardiotrophin 1 may directly regulate myocardial transcription of BNP (Talwar et al, 2000). The study suggested that N-BNP and cardiotrophin 1 may have a therapeutic potential in the protection of cardiac cells from ischaemic injury.

1.6.3 BNP expression following coronary angioplasty

Percutaneous transluminal coronary angioplasty (PTCA) is widely used to re-establish coronary artery flow, in the treatment of ischaemic heart disease. Inflation of the PTCA balloon during the procedure induces transient myocardial ischaemia. Tateishi et al (2000), designed a study to examine plasma concentrations of BNP over a period of 4 days in patients undergoing PTCA in order to assess the response to the transient myocardial ischaemia. Patients undergoing diagnostic angiographies served as the control group. Blood sampling for radioimmunoassay of plasma BNP was obtained on the morning (after an overnight fast), immediately before and after the procedure. Plasma BNP was increased significantly 24 h after PTCA, with no significant rise in

the plasma creatine phosphokinase. Coronary angiography per se did not cause an increase of plasma BNP 24 h after the procedure. No significant changes were present in the haemodynamic parameters (Swan-Ganz catheter pressures, cardiac index, heart rate and arterial blood pressure), obtained immediately before and 24 h after PTCA. Further, no significant difference was noted in BNP levels in either of the subgroups subjected to PTCA of the left anterior descending artery, circumflex artery or the right coronary artery, indicating BNP release from both ischaemic and non-ischaemic myocardium. Severity of ischaemia being multifactorial, no correlation was found between the magnitude of BNP increase and the duration of myocardial ischaemia produced by PTCA balloon inflation (Tateishi et al, 2000). The authors conclude that plasma BNP may potentially play a role as an index of recent myocardial ischaemia in patients with angina pectoris. In other studies, BNP has been shown to increase acutely and much more prominently than ANP following the first balloon inflation during PTCA (Kyriakides et al, 2000).

1.7 Mechanisms of cardioprotection – Ischaemic preconditioning (IPC)

Innate myocardial adaptive processes render the heart more resistant to ischaemia-reperfusion injury. One such mechanism is the phenomenon of myocardial ischaemic preconditioning (IPC). This is a phenomenon in which single or multiple brief periods of ischaemia have been shown to protect the heart against a more prolonged ischaemic insult, the result of which is a marked reduction of myocardial infarct size, severity of stunning, or incidence of cardiac arrhythmias. IPC was first demonstrated in 1986, when Murry et al, made the novel discovery that brief periods of ischaemia and

reperfusion afforded cardioprotection to a prolonged ischaemic insult in the canine coronary occlusion model (Murry et al, 1986). Since then, extensive studies have demonstrated this cardioprotection in all animals studied and have shown that IPC-like effects exist in cultured cells exposed to hypoxia or metabolic inhibition. There appear to be two phases of protection following preconditioning, the first phase occurs within minutes and is extremely pronounced but transient (classical IPC). A delayed phase (also known as the 'second window of protection') takes hours to become apparent and can last for days. The mechanisms involved in these phases are different but linked (Yellon et al, 1998; Cohen et al, 2000; Baxter, 2002a).

Preconditioning studies in humans date back to 1993, when Yellon et al, in a setting of coronary artery bypass grafting (CABG), subjected patients to intermittent application of an aortic cross clamp to produce global ischaemia – a stimulus for IPC. ATP levels used as the experimental end point, were better preserved in patients subjected to a subsequent 10 min ischaemic episode (Yellon et al, 1993). In similar experiments, Jenkins et al, showed attenuated troponin T levels in CABG patients subjected to IPC during revascularisation (Jenkins et al, 1997). IPC has been shown to induce protection in isolated human atrial trabeculae (Speechly-Dick et al, 1995; Morris et al, 1997). Isolated human ventricular myocytes also demonstrated the preconditioning phenomenon (Arstall et al, 1998).

In a clinical context, the administration of an injury modifying manoeuvre i.e. IPC, must occur either before the onset of ischaemia or after the onset of ischaemia but before therapeutic reperfusion (review - Yellon and Baxter, 2000). Hence, pre-infarct angina could be a clinical correlate of the experimental IPC phenomenon. In fact, Andreotti et al, showed that thrombolytic therapy given to patients with acute myocardial infarction preceded by unstable angina, resulted in more rapid reperfusion

and smaller infarcts compared with those sans pre-infarction angina (Andreotti et al, 1996). Patients with prodromal angina 24 h prior to infarction had noticeably lower hospital mortality rates and associated with a better 5 year survival rate (Ischiara et al, 1997).

Although the mechanisms governing IPC are far from completely understood, great progress has been made in identifying a number of endogenous autocrine and paracrine mediators released from the myocytes and vascular endothelium during the IPC stimulus. These triggers, via a receptor mediated mechanism, activate a complex intracellular signalling pathway involving a number of protein kinases. The signalling cascade in turn modifies potential end effector or target proteins.

1.7.1 Molecular triggers of IPC

The ischaemic heart undergoing classical IPC has been shown to release a number of endogenous substances possibly protecting the heart (Fryer et al, 2002). The presence of these triggers in the myocardium is considered a prerequisite to protection prior to the index ischaemic episode.

1.7.1.1 Adenosine

Adenosine was the first endogenous ligand to be identified as a trigger of the cardioprotective action of IPC. Following extensive research, it is evident that infarct limiting effects of both endogenous adenosine and exogenously applied analogues work via the A₁ and/or the A₃ receptor subtypes in isolated cardiac myocytes (Wang et al, 1997), animal studies (Liu et al, 1991; Tracey et al, 1997) and human cardiac tissue (Walker et al, 1995; Carr et al, 1997).

1.7.1.2 *Bradykinin*

Another important trigger in the IPC protection mechanism is bradykinin. Its release from the ischaemic myocardium has been well documented (Schulz et al, 2001; Baxter et al, 2002b). Further, it has been reported that Hoe 140 (a selective B₂ receptor antagonist) abolished the protective effect of IPC in rabbit heart studies *in vivo* (Goto et al, 1995; Wall et al, 1994).

1.7.1.3 *Opioids*

Endogenous opioid peptides have also been implicated in the triggering mechanism of IPC. These act through the δ -receptor subtype activating the signalling cascade of preconditioning (Schultz et al, 1997; Aitchison et al, 2000). Selectivity of the δ -receptor subtype has been demonstrated using selective δ -receptor agonists in the isolated rat heart, with infarct size as an end point (Schultz et al, 1998; Aitchison et al, 2000).

1.7.1.4 *Nitric Oxide*

Graded reductions in the coronary flow, in a canine model of ischaemia result in the release of the end-products of NO viz. nitrates and nitrites (Node et al, 1995). Vegh et al demonstrated that a NO inhibitor partially attenuated the anti-arrhythmic effect of IPC in the canine model (Vegh et al, 1992). Further, Horimoto et al, showed that L-arginine a precursor of NO, preconditioned the rabbit heart using infarct size as the end point. This response was mediated through a cGMP-dependent mechanism, but interestingly, independent of the K_{ATP} channels using glibenclamide as the K_{ATP} blocker (Horimoto et al, 2000). However, several investigators showed conflicting results on the influence of NO on lethal ischaemic injury in various species, tissues and severity of injury (Weyrich et al, 1992; Engleman et al, 1996; Maulik et al, 1996;

Takano et al, 1998). This is likely to be possible, because massive NO formation may be detrimental (from tyrosine nitration, dityrosine formation, thiol oxidation and lipid peroxidation), whereas lower concentrations of NO may be protective (secondary to high super oxide dismutase expression). Unfortunately, no further studies are available to date implicating a role for cGMP and the mitochondrial K_{ATP} channels.

1.7.1.5 Other triggers

Catecholamines are also released following cardiac ischaemia. A role for both the α - and the β - adrenoreceptor is well established in some models (Hu et al, 1995; Sharma et al, 1997; Lochner et al, 1999). Oxygen free radicals released in ischaemia have infarct limiting IPC effects in the rabbit heart (Baines et al, 1997) and can also trigger preconditioning in post-CABG patients (Wu et al, 2001).

Linkage to G-proteins is central to the many diverse triggers of IPC. Hence, adenosine, bradykinin, opioid and catecholamines, all act on G-protein coupled receptors (transmembrane domain receptors). Some G-proteins couple to phospholipase C, subsequently believed to activate protein kinase C (PKC).

1.7.2 Signalling cascades

The intracellular transduction mechanisms involved in IPC are grouped under two main receptor categories. The G-protein coupled receptors and tyrosine kinase receptors.

1.7.2.1 G-protein coupled receptors

As described above, many of the triggers mediate their preconditioning effect via the G-protein. It consists of three polypeptide sub units - α , β and γ in two alternate

conformations according to GTP binding. $G\alpha$ exists in an active GTP bound form and an inactive GDP bound form. Mammalian α -subunits are further divided into four subfamilies – an adenylyl cyclase stimulating form - G_s , an adenylyl cyclase inhibiting form - G_i , a phospholipase C (PLC) activating form – G_q and an ion channel regulating subfamily – G_o , present mainly in brain tissue (Nagao et al, 1998).

Adenosine and opioid receptors are linked via G_i proteins; the α -subunit inhibits the adenylyl cyclase activity and hence the production of the second messenger cAMP. Also, $G_i\alpha$ subunits are able to open voltage independent Ca^{2+} channels, activate phosphatidylinositol-3 kinase (PI3 kinase) (Ge et al, 1998), and lead to generation of inositol 3,4,5 triphosphate (IP_3), capable of mobilising intracellular calcium stores. Bradykinin and noradrenaline signal via the G_s protein activating cAMP-dependent protein kinase (PKA), which is associated with activation of the endothelial isoform of nitric oxide synthase (eNOS), phosphorylating the enzyme at ser 1177 (Butt et al, 2000). $G\beta$ and $G\gamma$ have multifarious signalling properties, including activation of PI3 kinase, PLC, ion currents (I_k and I_{ca}), tyrosine kinases and G-protein coupled receptor kinases (review – Kehrl, 1998). PLC and its product diacyl glycerol (DAG) have been demonstrated to activate protein kinase C (PKC) and precondition the heart (Ytrehus et al, 1994; Downey et al, 1995).

PI3 kinase, like PKA, phosphorylates protein eNOS through the action of Akt (cellular Akt/protein kinase B; Dimmeler et al, 1999).

1.7.2.2 Tyrosine kinase coupled receptors

Experimental work indicates that tyrosine kinase is downstream of PKC in the IPC signal transduction (Baines et al, 1998). It phosphorylates tyrosine residues on target proteins. The activation of the receptor/tyrosine complex is thought to cause direct

activation of the Ras/mitogen activated protein (MAP) kinase pathway, possibly independent of PKC (although there still is activation of PLC and PKC) (Bogoyevitch et al, 1994). Hence, G-protein receptor and tyrosine receptor linked pathways appear to converge and integrate with the activation of MAP kinases. The three major MAP kinases include – extracellular signal regulated kinases (p42/p44 ERK), p38MAP kinase and the stress activated C-jun N-terminal kinase (JNK/SAPK).

1.7.3 End-effector mechanism of IPC

The growing consensus is that the distal target or effector of protection is the K_{ATP} channel.

1.7.3.1 Sarcolemmal K_{ATP} channel

Initially research concentrated on the sarcolemmal K_{ATP} channel, with Gross et al, implicating its role in IPC in the canine model. Blocking the channel with glibenclamide in dog hearts prevented preconditioning, while the drug had no effect on the non-preconditioned heart. Although glibenclamide closes both mitochondrial and sarcolemmal K_{ATP} channels, it was initially assumed that the sarcolemmal K_{ATP} was the end-effector of the IPC protection attributed to shortening of action potential (Gross et al, 1992). However, other K_{ATP} channel openers such as, cromokalim were protective, independent of any action potential shortening (Grover et al, 1995). Further, isolated non-beating cardiac myocytes could be preconditioned and were amenable to protection induced by K_{ATP} channel openers in the absence of a ventricular action potential (Armstrong et al, 1995), throwing into doubt the protective role of the sarcolemmal K_{ATP} channel itself. Subsequently, it was proposed that cardiomyocytes contain two different types of K_{ATP} channels, sarcolemmal and mitochondrial, with each having a distinct pharmacological profile.

1.7.3.2 Mitochondrial K_{ATP} channel

Although the mitochondrial K_{ATP} (mK_{ATP}) channel has not yet been cloned and its existence not universally accepted, based on pharmacological profiles, several investigators have now shown that it is not the surface but the mK_{ATP} channel that is involved in protection. Garlid et al, first showed that a selective mK_{ATP} opener protected the *in vitro* rat heart, this was blocked with 5-hydroxy decanoate (5-HD) an inhibitor of mK_{ATP} (Garlid et al, 1997). Subsequently, convincing evidence in different models showed that it was indeed the mK_{ATP} channel that was involved in protection (Liu et al, 1998; Sato et al, 2000). The exact mechanism behind the opening of the mK_{ATP} and the observed protection is not fully understood. mK_{ATP} opening causes depolarisation of the inner mitochondrial membrane, matrix swelling, enhanced respiration and reduced calcium overload (Gross et al, 1999).

1.7.3.2.1 mK_{ATP} as an IPC trigger?

Recent experiments have re-examined the role of mK_{ATP} as an end-effector of protection. In examining the critical opening of the mK_{ATP} channel, Downey's group perfused the isolated rabbit heart, preconditioned with diazoxide, with either 5-HD (mK_{ATP} blocker), chelerythrine (PKC inhibitor), genistein (tyrosine kinase inhibitor) or free radical scavengers, to either bracket diazoxide or precede the index ischaemia. They conclude that mK_{ATP} channel opening triggers protection but is not the final step in IPC mediated protection (Pain et al, 2000). Similar observations were noted by Ashraff's group, in the rat heart (Wang et al, 2001). These data implicate the opening of the mK_{ATP} channel as a possible trigger of IPC, although it could also play a distal role.

1.7.3.2.2 *mK_{ATP} and downstream kinases*

In further experiments, Ashraf's group showed that the PKC blocker chelerythrine could block protection from a pulse of diazoxide in the isolated rat heart (Wang, Ashraf et al, 2001). While, Pain et al, could not show a similar result in the rabbit heart, they were able to block diazoxide's protective action with a tyrosine kinase blocker genistein, indicating there was at least one tyrosine kinase downstream from mK_{ATP} opening in the rabbit model (Pain et al, 2000). Hence a role for mK_{ATP} opening as an upstream link in the signal transduction chain remains a possibility.

1.7.3.2.3 *mK_{ATP} and reactive oxygen species*

Experiments performed by Downey's group showed that the free radical scavenger N-2-(mercaptopropionyl) glycine (MPG), abolished protection in the diazoxide preconditioned rabbit hearts (Pain et al, 2000). Steenbergen's group, also found that diazoxide's protection could be blocked by a free radical scavenger, N-acetylcysteine (Forbes et al, 2001). Further, Yao et al, found that pharmacological preconditioning of chick cardiomyocytes with the muscarinic agonist acetylcholine, caused the cells to produce small bursts of free radicals. The probe used, 2',7'-dichlorofluorescein diacetate fluoresced when oxidised by free radicals. This burst was blocked with myxothiazol indicating that the increased reactive oxygen species (ROS) production was the result of electron transport within the mitochondria. Also this burst could be blocked using 5-hydroxydecanoate (Yao et al, 2001).

Krenz et al tried to block the ROS signal from diazoxide with the PKC blocker chelerythrine used by Wang et al (described above) (Krenz et al, 2002). Chelerythrene did not block the diazoxide-induced increase in fluorescence, indicating PKC to be downstream of K_{ATP} and presumably of ROS production (Oldenberg et al, 2002).

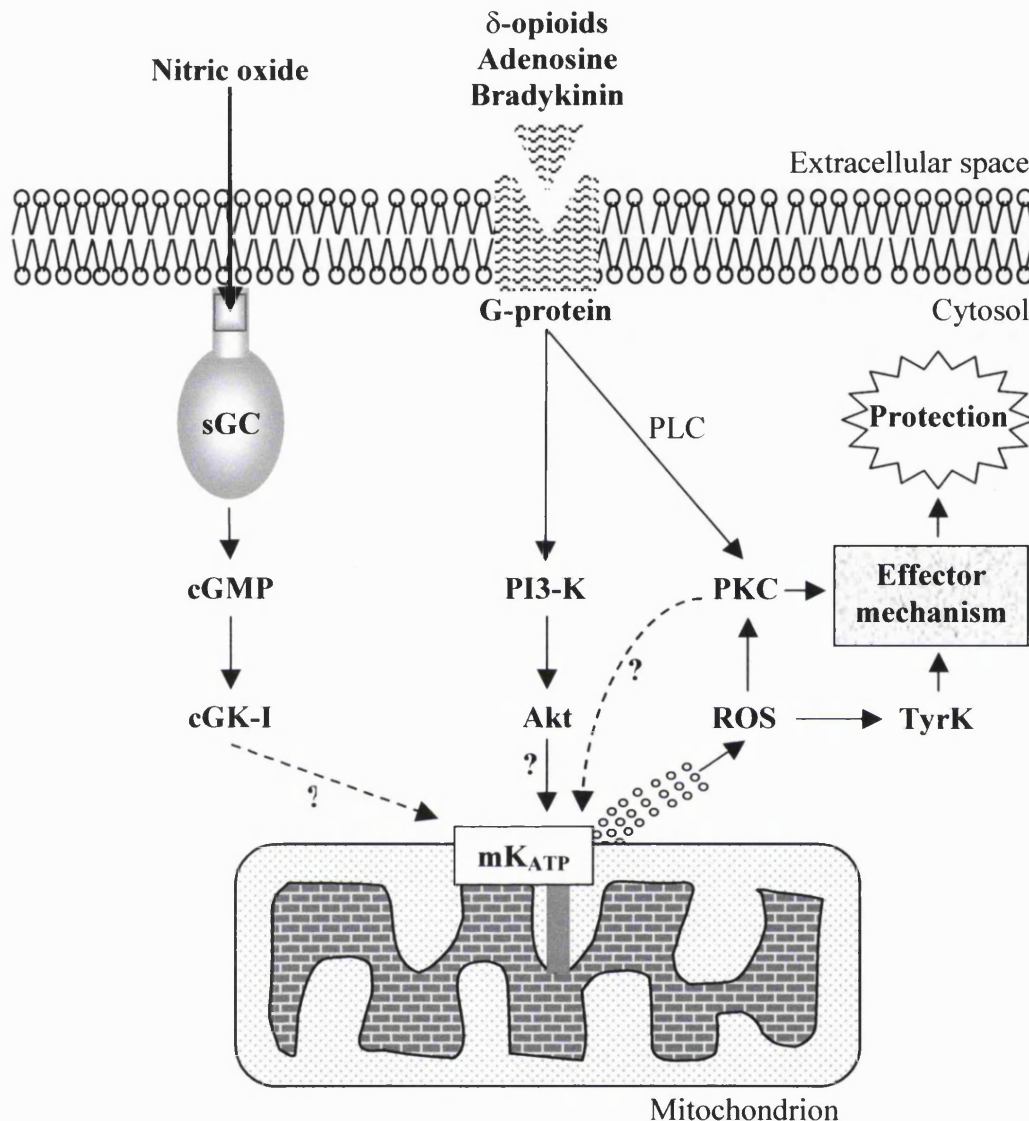


Figure 1. 1 Signalling pathway for cardiac ischaemic preconditioning

Schematic representation of the receptor-mediated protection is shown. G-protein-linked agonists bind to a 7 transmembrane-domain surface receptor. Receptor activation leads to the activation and binding of protein kinase C (PKC) via phospholipase C (PLC). This may in turn phosphorylate and open the mitochondrial K_{ATP} (mK_{ATP}) channel. G-protein activation can also activate phosphatidylinositol 3 kinase (PI3-k) and open the mK_{ATP} channel possibly through cellular Akt. cGMP, derived from nitric oxide activated soluble guanylyl cyclase (sGC), influences opening of the mK_{ATP} channel possibly mediated through its cGMP-dependent kinase of the subtype-I (cGK-I). The linked release of reactive oxygen species (ROS) from the mitochondrion, possibly in context with other mediators, leads to activation of PKC and other tyrosine kinases (TyrK). The purpose of all these steps is to activate the end-effector, which then mediates protection.

1.8 cGMP, BNP and cardioprotection

Several endogenous mediators released in the very early stages of myocardial ischaemia are known to be protective e.g. adenosine, prostacyclin, plasma kinins, NO (Parratt, 1993) and BNP as described above. However, no studies to date have examined the protective effects of BNP in ischaemia-reperfusion or the molecular mechanism of any such protection.

Further, Parratt et al, hypothesised that in response to brief ischaemic stimulus, endothelial cells in the heart release protective mediators which diffuse to cardiac myocytes to increase resistance to a subsequent ischaemic stress. These substances include NO, generated through initial bradykinin release, and prostacyclin. The evidence included measurement of mediator release and the pharmacological attenuation of the antiarrhythmic effects of preconditioning by blockade of bradykinin receptors and inhibition of NO production (Parratt et al, 1999). A proposed mechanism for this antiarrhythmic effect of IPC is elevation of cardiac myocyte cGMP levels (Parratt et al, 1997), a hypothesis first put forward by Opie in 1982 (Opie, 1982).

Another mechanism, by which myocardial cGMP levels can be increased, albeit by a different mechanism, is by elevating plasma levels of BNP. At present, a functional role for the rapid release of BNP in response to brief periods of myocardial ischaemia is not known. Many of the documented actions of BNP are attributable at least in part to cGMP elevation and activation of cGK-I. To date no published data exists to invoke the link between the direct action of cGMP and the mK_{ATP} channel which is known to play a key role in cardioprotection.

In the coronary conductance and resistance arteries, human BNP exerts a direct vasodilatory effect (Okumura et al, 1995). Under physiological conditions and after precontraction of coronary arteries with endothelin-1 (ET-1), Zellner et al, studied the vasodilatory response to BNP in the swine model *in vivo*. They found that BNP exerts coronary vasodilator effects, predominantly in epicardial conductance vessels. An accentuated vasodilatory response to BNP occurred in ET-1 precontracted arteries. The BNP-induced vasodilation was largely blocked by L-NAME, suggesting the contributory role of endothelium-derived NO (Zellner et al, 1999). In common, the second messenger for BNP and NO is cGMP: BNP acts on the extracellular domain of the membrane-bound NPR-A receptor to activate its intracellular guanylyl cyclase domain, resulting in the catalysis of cGMP from GTP, while NO appears to activate the intracellular cytosolic sGC to invoke the second messenger (Murad, 1999).

How elevated cGMP levels could be cardioprotective is illustrated in figure 1.5. The proposal is that, there is a 'cross-talk' between the endothelial cells and cardiac myocytes through the liberation of NO. BNP may activate cGMP via NPR-A in the endothelial cell, which phosphorylates the endothelial nitric oxide synthase (eNOS) by its kinase dependent protein - cGK-I. This statement though attractive, is only speculative. The NO can diffuse rapidly across quite long distances, stimulates guanylyl cyclase in cardiac myocytes and elevates cGMP. BNP can directly elevate intracellular cGMP in cardiomyocytes via its NPR-A receptor, and combined with the sGC-derived cGMP, opens the mK_{ATP} channel via cGK-I leading to cardioprotection. Pharmacological blockers of the transduction pathway help to decipher the mechanism. Three other known possible effects of cGMP in cardioprotection include – (i) stimulation of a cGMP sensitive phosphodiesterase enzyme which leads to an increase breakdown of cAMP (Lohman et al, 1991, Hove-Masden et al, 1996, Mery et al,

1993), (ii) inhibition of calcium entry through L-type calcium channels and (Lohman et al, 1991) (iii) NO is a known to depress myocardial contractility, reducing myocardial oxygen a factor suggested to play a an important role in protecting the ischaemic myocardium (Parratt & Vegh, 1996). A role for inhibition of the mitochondrial permeability transition pore (Takuma et al, 2001), and opening of K_{ATP} channels (Kubo et al, 1994) has also been postulated.

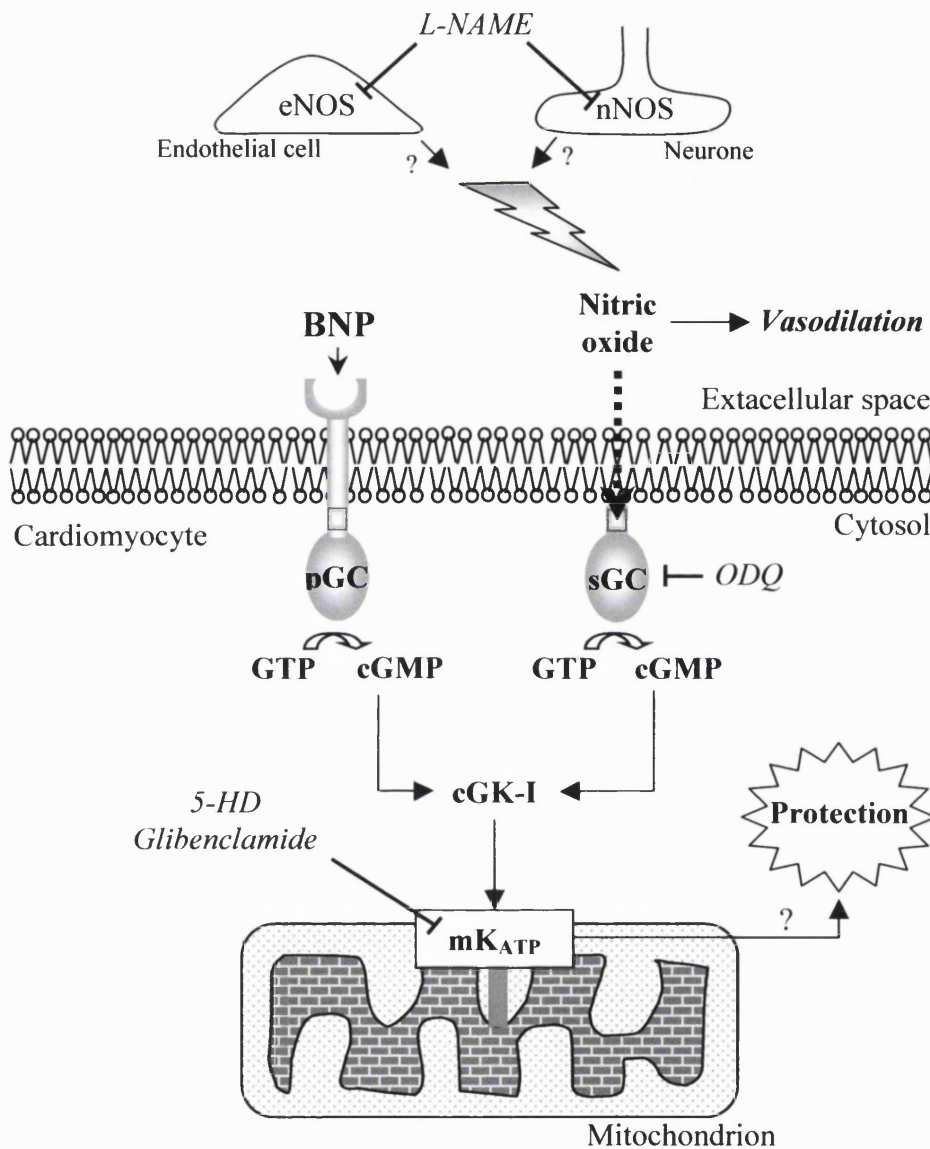


Figure 1.1 BNP and NO signalling in cardioprotection

Schematic representation of the possible signalling cascade in BNP mediated cardioprotection. A basal level of nitric oxide (NO) derived from either the endothelial cell (eNOS) or the neurone (nNOS) appears necessary, and then 'talks' to the cardiac myocyte, stimulates its soluble guanylyl cyclase (sGC) receptor and elevates cGMP. In the cardiomyocyte itself, BNP interacts via its particulate guanylyl cyclase (pGC) receptor to produce cGMP which via its dependent kinase (cGK-I) opens the K_{ATP} channel in the mitochondrion, possibly mediating protection. Pharmacological blockers *N* ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME), 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), 5-hydroxydecanoate (5-HD) and glibenclamide, have been used to elucidate the pathway thus far.

1.9 Aims of the thesis

Previous work, has implicated a role for NO-cGMP signalling in cardioprotection but this has never been resolved with the K_{ATP} hypothesis. Here, we propose that BNP-cGMP offers an alternative humoral mediator, signalling through cGMP, leading to cardioprotection through a preconditioning-like mechanism i.e. K_{ATP} channel opening. The primary hypothesis was that BNP exerts an anti-ischaemic action in myocardium and that this cardioprotective role of BNP is mediated via cGMP by opening of the mitochondrial K_{ATP} channel.

This thesis focuses primarily on the effects of exogenously administered BNP on the *in vitro* Langendorff perfused rat heart in ischaemia-reperfusion using infarct size as an endpoint and index of cardioprotection.

The study examined the release of mature BNP₁₋₃₂, following various durations of regional ischaemia in the isolated perfused rat heart. Myocardial infarction studies were carried out in the isolated perfused rat heart and the anti-ischaemic concentration-response relationship to exogenous BNP was examined. Infarct size, a robust reflector of cardiac injury was used as an end-point.

A subsequent study defined the involvement of K_{ATP} channel opening using selective pharmacological blockers of sarcolemmal and mitochondrial K_{ATP} channels in BNP's cardioprotective action.

The study proceeds to examine the role of cGMP in modulating ischaemia-reperfusion injury in the isolated perfused heart. Local tissue concentration was also studied following exposure to ischaemia and perfusion with various concentrations of exogenous BNP.

The implications of nitric oxide and eNOS phosphorylation in the BNP's cardiac signalling were also studied using a combination of nitric oxide synthase inhibitors in Langendorff perfusion and Western blot techniques.

Chapter Two

General Methods

2.1 Introduction

Experimental studies have been of tremendous importance for the understanding of the physiology, biochemistry and molecular biology of ischaemic heart disease. Experimental models in use for the study of ischaemic heart involve studies on the integrated organism, experiments in isolated hearts or multicellular preparations. As ethical implications preclude the use of the human heart model in most scientific research, fragments of knowledge put together from different types of experimental models create the background for successful design for potential treatment. Regional ischaemia, infarct size limitation, and heart function in the isolated perfused heart has been extensively studied and is the basis of the experimental approach used here.

Experiments described in this thesis have been performed in the laboratory of The Hatter Institute and Centre for Cardiology, University College London Hospitals and Medical School, London, UK. The studies were conducted in accordance with The Home Office Guidance on the operation of Animals (Scientific Procedures) Act, 1986, published by the Stationary Office, London (project licence no. 70/16554; personal investigator licence no.1811).

2.2 Choice of animal model

The rat is an established experimental species, and the isolated heart preparation has been extensively characterised. The ease of breeding and maintenance of these

animals is quite cost-effective compared to other animal models. Sprague-Dawley rats were used in our experiments and were purchased from Charles River, Bicester, Oxon. The influence of gender upon ischaemia/reperfusion injury in the rat heart has not been shown to influence infarct size (Li et al, 1995), but male animals were consistently used. On arrival these animals were housed in groups of three to four to a cage in the animal house and fed on standard pellet chow. They were allowed to acclimatise for a minimum of four days prior to use, during which time a 12 hour light-dark cycle was maintained at 19-22°C, and 55 ± 10% humidity.

2.3 Isolated heart studies

2.3.1 The Langendorff heart preparation

2.3.1.1 Principle

Most of the experiments in this study have employed the isolated perfused heart technique according to Oscar Langendorff (1853-1908). The underlying principle is to force blood, or any other oxygenated fluid appropriate to maintain cardiac activity, towards the heart through a cannula inserted into the ascending aorta. Retrograde perfusion closes the aortic valves – just as the in situ heart during diastole – and the perfusate is displaced through the coronary arteries into the coronary sinus and opened right atrium. The cardiac cavities remain basically empty throughout the experiment and do not perform pressure-volume work.

The Langendorff preparation is appropriate for all warm-blooded animals with a coronary vascular system. Even human hearts have been kept alive for several hours during heart surgery using this principle (Doring et al, 1988). The preparation can be operated with either a constant pressure or constant flow. From a physiological

standpoint, perfusion at constant pressure should be given preference, because whenever possible mean perfusion pressure is maintained constant in the intact circulation.

2.3.1.2 *Measuring parameters*

Mechanical parameters of the working myocardium (contractile force, volume, and ventricular diameter), mean coronary flow, bioelectrical parameters (electrogram, monophasic injury potentials) and cardiac rhythm can be measured and recorded.

The intact function of the working myocardium and coronary vessels can be examined using various simple tests regarding the myocardium, the smooth musculature and the endothelium of the coronary vessels. Due to technical ease, a variety of parameters can be measured, providing information on the state of the myocardium and coronary vessels (Doring, 1990). Further these experiments can be easily repeated and is economically feasible. The heart tends to remain devoid of central neuronal stimulation/depression which makes it ideal for studying the direct effects of treatment(s).

2.3.2 **Chemicals and drugs**

Chemicals used for Krebs-Henseleit buffer (NaCl 118, NaHCO₃ 25, glucose 11, KCl 4.7, MgSO₄.7H₂O 1.2, KH₂PO₄ 1.21, CaCl₂.2H₂O 1.8 mM) and the 1 µm filters were purchased from BDH Laboratory Supplies (Merck Eurolab, UK). Carbogen (95% O₂/5%CO₂) for perfusing the buffer was obtained from our local hospital supplies. Rat BNP₁₋₃₂, glibenclamide, sodium 5-hydroxydecanoate (5-HD), N^o-Nitro-L-arginine methyl ester hydrochloride (L-NAME), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and 8-Bromoguanosine 3':5'-cyclicmonophosphate sodium salt (8-Br-cGMP)

were purchased from Sigma (Poole, UK). HMR 1098 was a gift from Aventis Pharma. Triphenyltetrazolium chloride (TTC) and formaldehyde, were also purchased from Sigma (Poole, UK). All other reagents were of analytical standard.

2.3.3 Perfusion equipment

The Langendorff apparatus used in these studies was based on the same principle as originally pioneered by Oscar Langendorff in 1895. A schematic representation of the same and the set-up in our laboratory are shown in figure 2.1 and figure 2.2 respectively. The buffer pH was monitored using a blood gas system (AVL995 analyser, AVL Medical Instruments, Stonebridge, UK). A high rate flow inducer from Watson Marlow (Cornwall, UK) was used to fill the reservoirs with Krebs-Henseleit buffer. A saline-filled latex balloon was attached to a pressure transducer (Oxnard, CA), coupled to a pen-recorder multitrace2 machine (Lectromed, Letchworth, UK). A thermostat regulated circulator (Techne, Cambridge, UK) maintained the heart at body temperature as measured by a digital thermometer (Digitron Instrumentation Ltd., Hertfordshire, UK).

2.3.4 Anaesthesia

All animals were anaesthetised with pentobarbitone sodium (Sagatal - 50 mgK⁻¹) obtained from Rhone Merieux (Tallaght, Dublin, Eire). This was administered intraperitoneally (ip) and concomitantly with heparin (1 IU g⁻¹) obtained from Leo Laboratories Ltd (Bucks, UK) to prophylactically prevent coronary thrombosis in the

coronary vasculature or ventricular chambers. Barbiturates produce negative inotropic action on the myocardium and affect the coronary arteries, but these effects are completely abolished 10 minutes after discontinuing the anaesthetic (Doring et al, 1988). Consciousness was usually lost in five to ten minutes and the animal was then weighed and transferred to the operating board. Depth of anaesthesia was confirmed by the loss of the pedal withdrawal reflex before thoracotomy was performed.

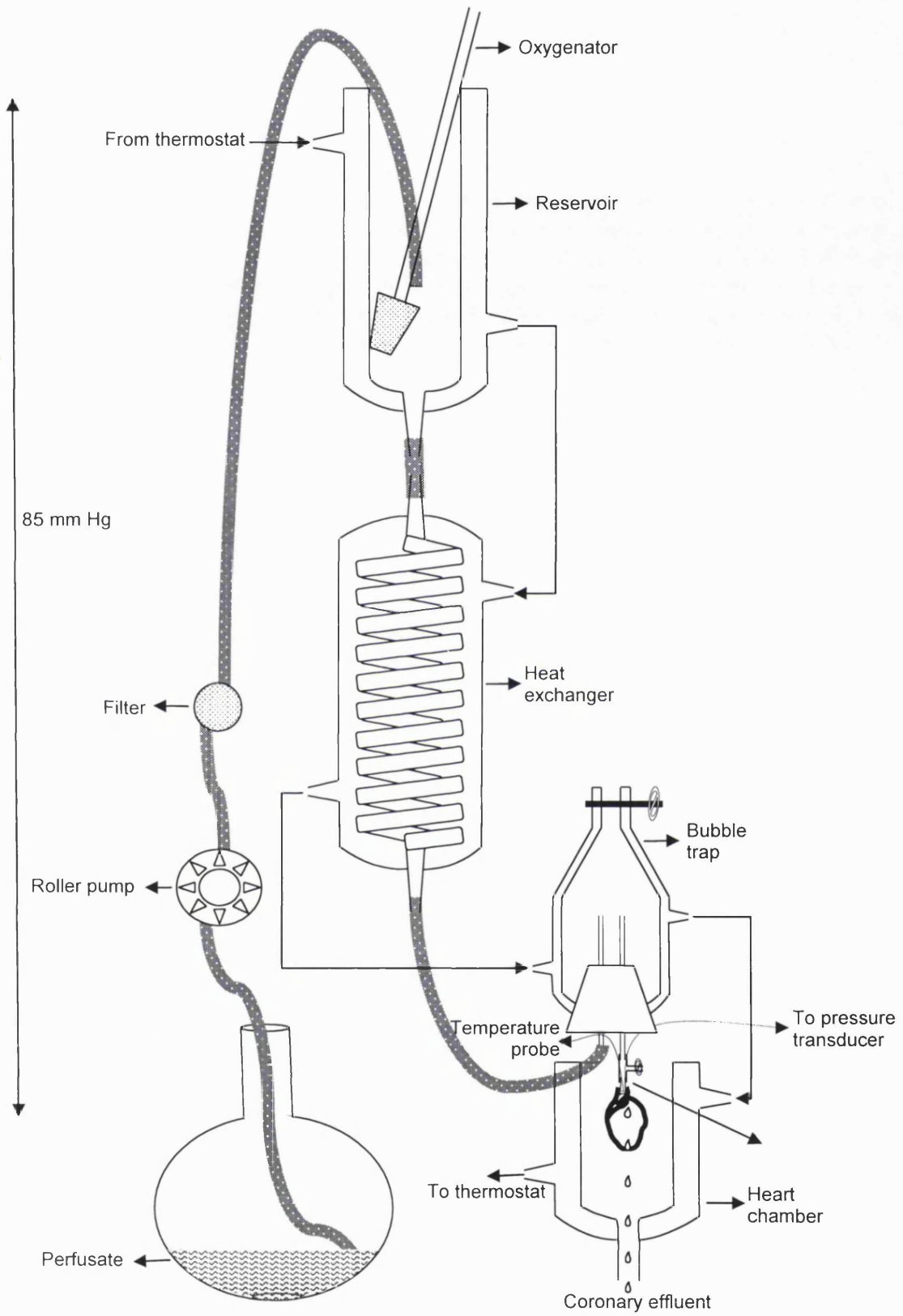


Figure 2. 1 The Langendorff apparatus

Schematic representation of the Langendorff perfusion equipment used in isolated rat heart studies.

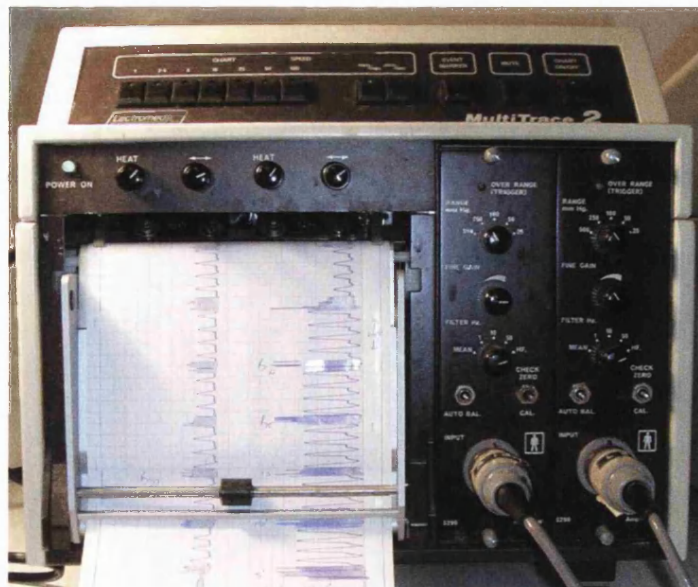
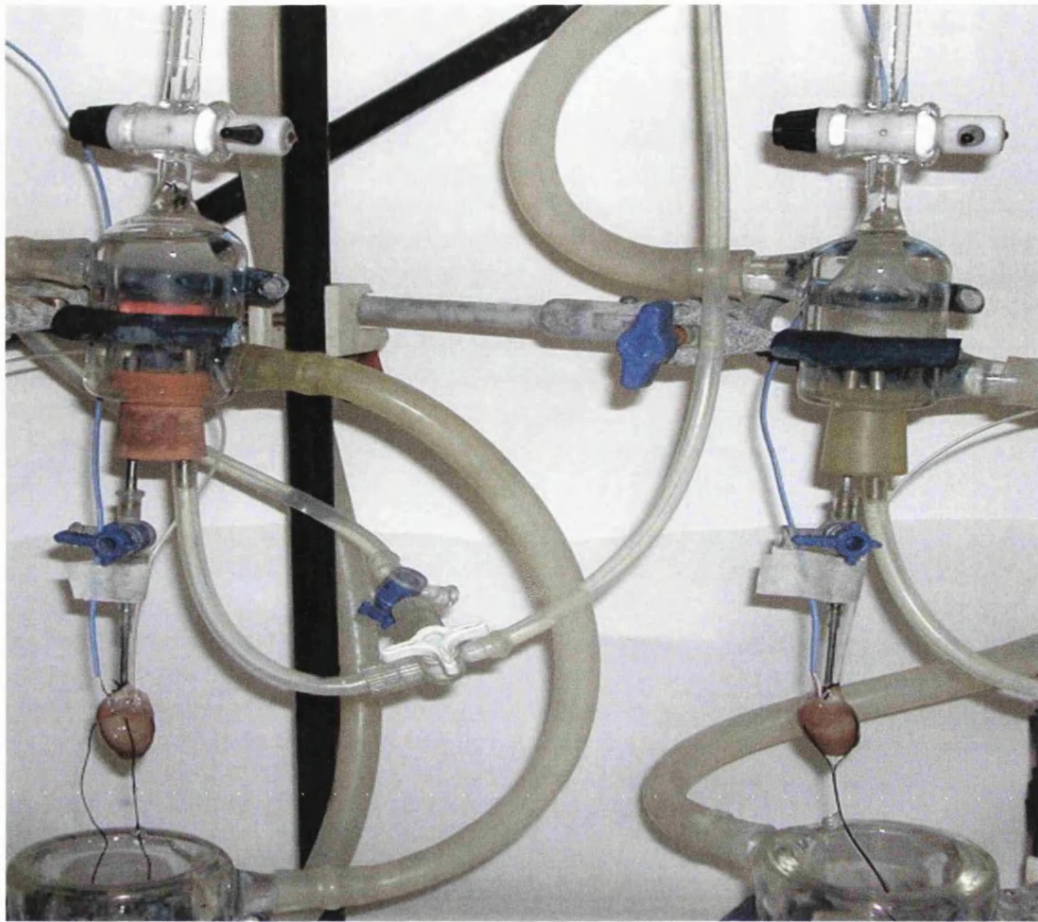


Figure 2. 2 The laboratory equipment set-up

2.3.5 Isolated heart preparation

After confirming satisfactory depth of anaesthesia, the animal was subjected to parasternal incisions to reflect the anterior chest and optimise the field of view. The heart was removed by transecting the ascending aorta and venous connections, and immediately transferred to an ice-cold buffer solution to arrest contraction. The heart was then held by the aorta and mounted onto the Langendorff apparatus. After considerable practice, it was possible to adequately cannulate the aorta without damaging the aortic valves or coronary ostia, and restore perfusion within 3 min of heart isolation. This is essential to reduce the potential risk of ischaemic preconditioning due to perfusion delay (Minhaz et al, 1995; Awan et al, 1999).

Perfusion pressure was fixed and maintained constant at 85 mmHg throughout the duration of the experiment. A 4/0 silk suture was positioned around the left coronary artery and threaded through a plastic snare to induce reversible regional ischaemia. The left atrial appendage was excised and a saline-filled latex balloon was inserted into the left ventricle to connect to a pressure transducer. Baseline end diastolic pressure (EDP) was set at 1-10 mmHg. The temperature was monitored continuously via a probe into the pulmonary conus and maintained at 36-37°C via a thermostat controlled water bath providing the water jacket to the apparatus. Raising or lowering the heart chamber with or without a lamp heat source aided with temperature control. A bubble trap in the system prevents the probability of an air embolus in the perfusate to the heart

2.3.6 Regional Ischaemia

All hearts were allowed to stabilise for a minimum of 15 min, before being subjected to regional ischaemia. The snare permitted reversible occlusion of the coronary artery. Coronary occlusion was induced for 35 min by clamping the snare onto the epicardium. This was verified by about 50% reduction of the coronary flow as well as the rate-pressure product. Reperfusion was achieved by releasing the snare and verified with a sharp rise of the coronary flow and onset of arrhythmias. The pre- and post-ischaemic hearts are shown in figure 2.3. When regional ischaemia has been employed, it becomes necessary to delineate the field of the occluded artery. This field is referred to as 'the region at risk' or the 'risk zone' as it is at risk of infarction. At the end of 120 min reperfusion the left main coronary artery was re-occluded and the risk zone delineated by injecting via a side port about 1-2 ml of Evan's blue dye (0.25%) into the aorta. Figure 2.4 shows the risk zone delineated by Evan's blue.



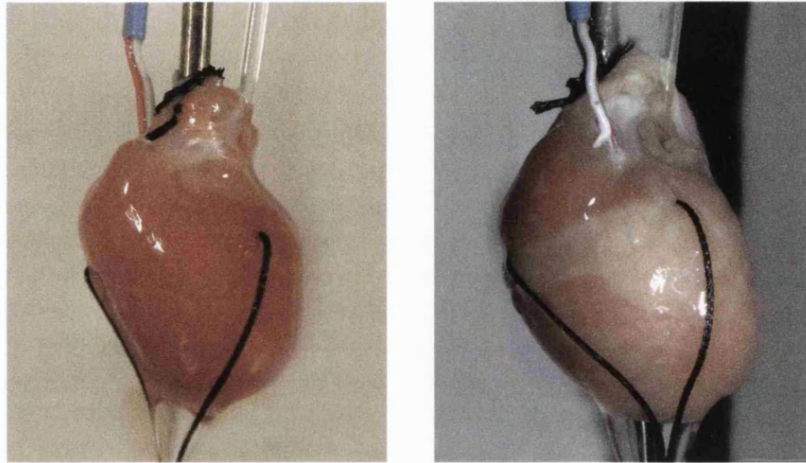


Figure 2.3 Pre-ischaemic and post-ischaemic hearts

On the left, the pre-ischaemic Langendorff perfused rat heart in stabilisation phase. Shown are the temperature probe (red and white electrode) in the pulmonary conus, cannula arising from an inflated balloon in the left ventricle and a ligature around the left coronary artery ligature in situ. The post-ischaemic heart on the right is shown in the reperfusion phase. The pale area in the heart surface is the infarcted risk zone following a 35 min regional ischaemia.

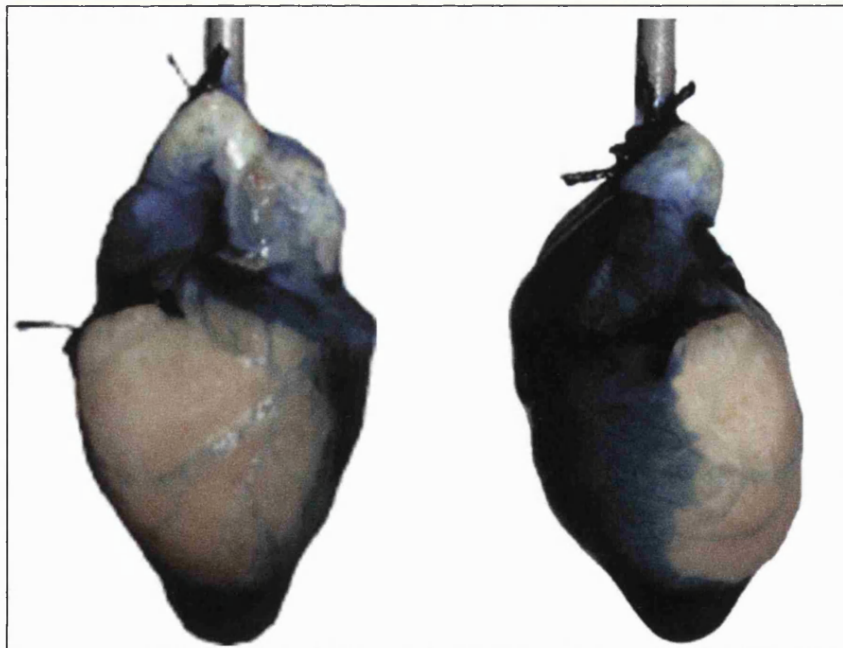


Figure 2.4 Delineating the risk zone

Langendorff perfused rat hearts stained with Evan's blue following a two hour reperfusion. The left coronary artery has been re-ligated prior to injection of the dye through the aortic cannula. The dye delineates the risk zone as it appears on the epicardial surface as seen here in the frontal and lateral views.

2.3.7 Parameters measured

2.3.7.1 *Coronary flow rate*

The Langendorff hearts were perfused at a constant pressure, hence coronary flow was linked to the vascular resistance of the coronary arterial beds, as determined by heart contraction and arteriolar vascular smooth muscle tone. Coronary flow was measured very simply by collecting the coronary effusate over a duration of 1 min in a graduated measuring tube and expressed as rate of millilitres per minute (ml/min). Coronary flow was monitored at regular intervals during the experiment. In a preliminary assessment of the preparation, coronary flow was shown to diminish with time in control hearts not subjected to ischaemia as shown in figure 2.5.

2.3.7.2 *Temperature*

Temperature control is important in the perfused heart in view of the large surface to volume ratio resulting in significant heat loss. Hypothermia has been shown to attenuate infarction resulting from ischaemia-reperfusion injury (Hale et al, 1997). Hence, temperature during the experiments was rigorously maintained at 36-37 °C and documented at every stage of the protocol. The method of measurement is described in the isolated heart preparation above (section 2.3.5).

2.3.7.3 *Rate pressure product*

The left ventricle contracts against the inflated latex balloon, which serves as a pre-load. The contractions are pressure transduced on to the pen recorder as described in the perfusion equipment above (section 2.3.3). The rate-pressure product is a good and reliable index of cardiac workload and function. It is calculated as the product of the ventricular heart rate and the developed pressure at a given time and is expressed as

millimetres of mercury per minute (mmHg/min). It was determined at various time points of ischaemia-reperfusion.

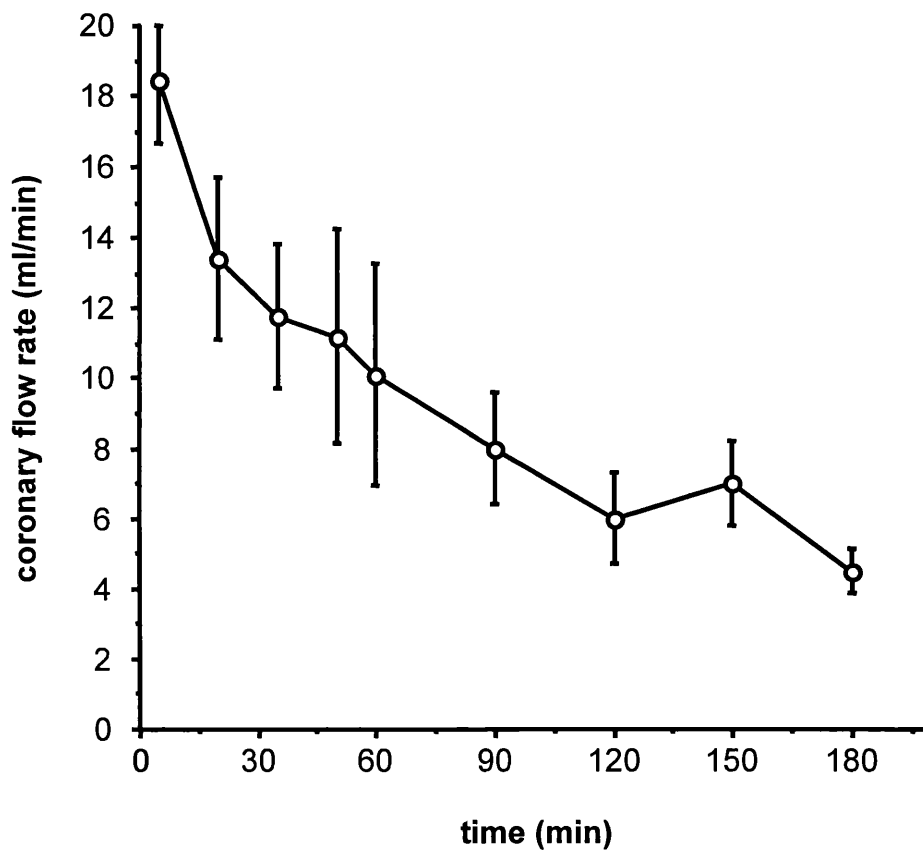


Figure 2.5 Characterisation of the instrumented heart

Coronary flow rate (ml/min) in the isolated rat heart instrumented to be Langendorff perfused. These hearts were perfused with Krebs-Henseleit buffer over the three hour time interval and have not been made ischaemic. Flows are expressed as mean \pm s.e.m.; $n = 6$.

2.3.8 Infarct size evaluation

At the end of the 120 min reperfusion period, the hearts were infused with Evan's blue, to delineate the risk zone as described above (section 2.3.6) and stored in the freezer at -20°C for one-four hours. Hearts were then sliced into 2 mm sections from apex to base for infarct determination. Triphenyltetrazolium staining is a widely used and validated method available for infarct detection in the whole heart (Ito et al, 1997; Schwarz et al, 2000). Triphenyltetrazolium chloride (TTC) is reduced by NADH and cofactors in viable tissue, producing a formazan pigment, which is distinct from white necrotic tissue. The 2 mm heart slices were incubated with 1% TTC in phosphate buffer (pH 7.4) at body temperature for 10-15 min. in a water bath. The viable tissue turned deep red. The slices were then fixed in 10% formalin for 24 hours. This also tends to increase the contrast between coloured living tissue and the pale infarcted tissue.

The formalin fixed and stained heart slices were mounted on a glass plate. A cover glass plate was then placed over the tissue. Two mm thick shims in the corners hold the glass plates away from each other. The risk area, infarcted and non-infarcted areas were demarcated as shown in figure 2.4 and traced on to an acetate sheet by an operator blinded to the study. Infarct area and the risk zone area were determined by computerised planimetry (Planimetry+ V1.0 for windows) and volumes calculated by multiplying area and thickness (i.e. 2 mm).

2.3.9 Perfusion protocol

Control hearts were perfused using a well established standard protocol – 15 min stabilisation, followed by 35 min of left coronary regional ischaemia and 120 min reperfusion. Rat BNP₋₃₂ and 8-Br-cGMP in various concentrations further described in chapters 4 and 6, were perfused from 10 min prior to ischaemia up to 30 min into reperfusion. L-NAME and ODQ detailed in chapter 7, were similarly perfused with or without BNP from 10 min pre-ischaemia up to 30 min into reperfusion. Mitochondrial and sarcolemmal K_{ATP} channel blockers were also similarly perfused with and without BNP as detailed in chapter 5.



Figure 2. 6 Control heart slices

Heart slices following ischaemia-reperfusion injury. The infarcted area (white), the risk zone area (deep red) demarcated by triphenyltetrazolium and the non-infarcted area (blue) are shown.

2.4 Myocardial BNP assay

To examine release of endogenous BNP in response to ischaemia, samples of coronary effluent were collected from perfused hearts and immediately frozen. Following stabilisation during which baseline samples were collected, hearts were rendered globally ischaemic for 2, 5 or 20 min by cessation of perfusate flow while maintaining temperature in the normothermic range by a jacketed chamber. Following global ischaemia, flow was re-instituted and coronary effluent was sampled at 30 seconds, 2 min and 5 min of reflow. Control hearts were perfused without ischaemia. The ventricular tissue was immediately frozen in liquid N₂. These samples were then packed on dry ice and transported to Institut für Pathophysiologie, Universitätsklinikum Essen, Germany for further analysis by Prof Gerd Heusch and his team.

Coronary effluent and ventricular tissue samples were analysed for BNP₁₋₃₂ immunoreactivity by radioimmunoassay using a commercially available kit (RIK 9085 BNP-32 (rat), Peninsula Laboratories Inc., San Carlos, CA, USA). Tissue samples were homogenised and extracted with trifluoroacetic acid. The peptide was purified on C18 columns, freeze-dried and redissolved in "RIA Buffer". To samples of coronary effluent, "RIA Buffer" concentrate was added, and aliquots were used for the assay. Based upon the competition of labelled ¹²⁵I-BNP and unlabeled BNP binding to a limited amount of specific antibodies, a standard curve was constructed from which the concentration of BNP in the samples was determined (Dwenger, 1984; Vollmar et al, 1995).

2.5 Myocardial cGMP assay

Following varying intervals of ischaemia (see Chapter 5), hearts were frozen with a Wollenberger clamp prechilled in liquid nitrogen and stored at -81°C . Frozen ventricular tissue, was then deproteinised by ten volumes of 6% trichloroacetic acid. Using mortar and pestle, the homogenate was obtained, then centrifuged (10,000 g for 10 min) at 4°C , and the supernatant was then extracted seven times in water-saturated diethylether. The remaining water phase was evaporated in nitrogen flow and these samples were then packed on dry ice for transportation. This initial extraction process was performed by Annamaria Onody, before the samples were transferred to the Department of Biochemistry, University of Szeged, Hungary for further assay of cyclic GMP by Dr Peter Ferdinandy and his team. The assay has been fully validated in their laboratory. Radioimmunoassay kits were purchased from Amersham Biosciences (UK) (Szilvassy et al, 1994; Csont et al, 1999).

2.6 Western blot analysis

Western blotting is useful in detecting a protein of interest that may be mixed with others (such as in a cell lysate) or verifying the identity of a protein on a gel. In general, a mixture of proteins is resolved using a denaturing acrylamide gel and then transferred from gel onto a nitrocellulose membrane. The eNOS and phospho-eNOS proteins of interest are probed by incubating the membrane with specific antibodies. The membrane next is incubated with a secondary antibody which recognizes any bound primary antibody, and allows eventual identification. This technique is limited by the success of the gel separation, the transfer step and specificity of the antibodies.

2.6.1 Chemicals and drugs

Chemicals used for Western blotting viz. sodium dodecyl sulfate (SDS), Trizma base (Tris), ammonium persulfate (APS), TEMED, bromophenol blue, Ponceau solution, reagents for suspension/sample buffers and Kodak film were purchased from Sigma (Poole, UK). Methanol and glycine were purchased from BDH Laboratories (Merck Eurolab, Dorset, UK) and Acrylamide (protogel) from National diagnostics (Hull, UK). The anti-eNOS/NOS III (rabbit polyclonal IgG) and horseradish peroxidase (HRP)-linked anti-rabbit IgG (H&L) were purchased from Cell Signalling technology (Hertfordshire, UK). The phospho-eNOS (Ser 1177) antibody and goat anti-rabbit IgG HRP conjugate were from Upstate Cell Signalling Solutions (Milton Keynes, UK). Nitrocellulose membrane and ECL plus detection reagents were obtained from Amersham Biosciences (Buckinghamshire, UK).

2.6.2 Tissue preparation

Langendorff perfused rat hearts were stabilised for 15 min, before being perfused with varying concentrations of BNP for 10 min as further described in chapter 7. Control hearts received no further treatment. The coronary flow was terminated, both atria were excised and the ventricles were immediately freeze-crushed with a Wollenberger clamp prechilled in liquid nitrogen and stored at -81°C . In the sham controls, hearts were excised below the atria in the animal itself (i.e. unperfused) and immediately dry-blotted to remove blood and freeze-clamped.

2.6.3 Protein extraction

Approximately 50 mg of frozen ventricular tissue was used for each protein extraction sample. Using an IKA Labortechnik T25 basic homogeniser, tissue was then homogenised on ice in 250 ml suspension buffer (NaCl 100, Tris 10 (pH 7.6), EDTA 1 (pH 8.0), sodium pyrophosphate 2, sodium fluoride 2, β -glycerophosphate 2, AEBSF {4-(2-Aminoethyl)-benzene sulfonyl fluoride hydrochloride} 0.5 mM, and 1 μ g/ml each of aprotinin, leupeptin, trypsin inhibitor and protease inhibitor in a distilled water base. Samples were then centrifuged, and the supernatant divided in duplicate samples of 2 μ l each for protein quantification and the remainder of the sample for electrophoresis. The purified protein was then further diluted in 2x sample buffer (Tris 100 - pH6.8), DTT 200 mM, SDS 2%, bromophenol blue 0.2% and glycerol 20%) and subsequently boiled for 10 min at 100°C, and stored at -20°C for later analysis.

2.6.4 Protein quantification

Protein concentrations were estimated using a Bicinchoninic acid based (BCA™) protein assay reagent system (Pierce, Rockford, USA). The assay relies on reduction of ionised copper, and the formation of a purple coloured BCA-copper complex measured by optical densitometry at 562 nm.

Duplicate samples of bovine serum albumin (BSA) in suspension buffer (contents as described in chapter 7), were quantified to generate a standard curve derived from the results of the optical density using a photospectrometer (Janway model 6405 UV/Vis, Dunmow, UK). The protein in the samples was then quantified using this method, and the protein content compared against the standard curve to provide an estimate of the protein concentration (μ g/ μ l) to enable equal loading of the polyacrylamide gel.

2.6.5 Polyacrylamide gel electrophoresis

2.6.5.1 *Gel preparation*

For eNOS and phospho-eNOS electrophoresis, 16% acrylamide gels were made using 36 ml deionised water, 16 ml acrylamide, 18 ml running gel base (1.5 M Tris and 0.4% SDS in deionised water, pH 8.8), 80 μ l TEMED and 400 μ l 10% ammonium sulphate). This running gel was introduced between two glass plates separated by spacers and sealed with vaseline. Once set, protein loading wells were made in the stacking gel prepared using 14 ml deionised water, 6 ml stacking gel base (0.5 M Tris, 0.4% SDS in deionised water, pH 6.8), 4 ml 30% acrylamide, 40 μ l 8% bromophenol blue, 48 μ l TEMED and 240 μ l 10% APS).

2.6.5.2 *Electrophoresis*

A total of 100 μ g of protein for each sample was loaded into the gel. A molecular weight rainbow marker (10 μ l) and human endothelial cells lysate (100 μ g protein) to provide control banding for eNOS and phospho-eNOS (molecular weight 140) were also loaded. The gel was allowed to run at 125 V constant for 4 hours.

2.6.5.3 *Protein Transfer*

Following electrophoresis, the gel was then apposed to a same size of nitrocellulose Hybond ECL membrane, and both were sandwiched between sheets of filter paper, ironing out air-bubbles. The gel was then mounted in a transfer tank containing transfer buffer - 200 ml methanol, 700 ml deionised water and 100 ml 10x transfer buffer (glycine, Tris and deionised water). Transfer proceeded at 140 mA constant overnight (12-16 hours).

The next morning, the membranes were removed and equal protein loading confirmed with Ponceau stain.

2.6.6 Immunoblotting

The protein loaded membranes were then washed in washing buffer (50 ml 10xTBS (Tris-buffered saline), 450 ml deionised water and 0.5 ml Tween-20) and then incubated on a rocking platform in blocking buffer (washing buffer + 5% non-fat milk) for 3 hours.

2.6.6.1 *Primary antibody*

The membranes were subsequently, washed in washing buffer and one incubated in primary antibody in a concentration of 1:500 for eNOS and the other incubated in primary antibody concentration of 1:250 for phospho-eNOS for 2 hours.

2.6.6.2 *Secondary antibody*

The primary antibody was washed off the membranes with washing buffer, and membranes then incubated in secondary antibody in a concentration of 1:5000 for eNOS and the other incubated in secondary antibody in a concentration of 1:2500 for phospho-eNOS for 1 hour. Subsequently the membranes were again washed in washing buffer and proteins detected by using enhanced chemiluminescence ECL plus detection reagents.

Bands on the membranes were visualised by autoradiography onto Kodak film. The membranes were finally stained with Ponceau stain and laminated.

2.6.7 Quantification

Autoradiography films and Ponceau stained membranes were scanned on a flat-bed picture scanner and the digital imaged saved to disc. Images may be calibrated against absolute densitometer values if one of the lanes has so been measured. Results are

expressed as a percentage proportion of wild-type control expression (\pm s.e.m.), and corrected for Ponceau determined protein loading.

2.7 Statistical analysis

Data were expressed as means \pm s.e.m. Statistical computations were undertaken using the StatView programme (version 4). Differences between group means for infarct/risk ratios, risk zone volumes, BNP tissue concentrations and cGMP tissue concentrations were analysed using one-way analysis of variance (ANOVA). When treatment constituted a significant source of difference overall ($P < 0.05$), individual comparisons between groups were undertaken with the Fisher's protected least significant difference post-hoc test. LV function parameters, coronary flow rate and BNP coronary effluent concentrations were examined with respect to treatment and time using repeated measures ANOVA with Bonferroni's post-hoc test (Fisher's post-hoc test is not available for use with repeated measures ANOVA in the StatView programme). Differences in group means were accepted as being significant when $P < 0.05$.

Chapter Three

BNP Release in Response to Ischaemia

3.1 Introduction

Since its initial discovery in 1988 from the porcine brain, BNP has established itself as a cardiac hormone being released both from the atrial and the ventricular cardiomyocytes, with the ventricles being the predominant source (Minamino et al, 1988; Ogawa et al, 1991; Espiner et al, 1995;), contributing significantly to its circulating levels.

Plasma BNP elevation in patients with heart failure, ventricular hypertrophy and ventricular overload is well known (Yassue et al, 1994; Hasegawa et al, 1993; Tokola et al, 2001). Stretching the ventricular tissue of an isolated Langendorff perfused rat heart, after removal of the atria, causes considerable elevation in the BNP secretion (Kinnunen et al, 1993). Hama et al, have reported that in acute heart failure, BNP is secreted rapidly from the ventricles via de novo synthesis against ventricular overload, whereas atrial natriuretic peptide (ANP) is secreted from storage in the atrium. Thus, BNP can be a more sensitive marker of ventricular function than ANP (Hama et al, 1995).

Previous experimental and clinical evidence suggests that brief episodes of ischaemia or hypoxia, insufficient to cause alterations in end-diastolic pressure or irreversible tissue injury, can evoke rapid release of BNP from cardiac tissue. Hypoxic perfusion of isolated hearts led to a rapid increase of BNP immunoreactivity in the coronary effluent (Toth et al, 1994). Further, recent studies in patients with acute myocardial infarction (AMI) and unstable angina have documented significant elevation of plasma BNP levels following the event (Richards et al, 1998; Talwar et al, 2000). Plasma

BNP was also found to be increased by transient myocardial ischaemia induced in patients undergoing a percutaneous transluminal coronary angioplasty procedure. However the quantitative relationship between ischaemia duration and BNP release has not been previously assessed.

The present study was designed to examine the effect of ischaemia on the ventricular BNP release, by exposing the Langendorff perfused rat heart, to graded episodes of global normothermic ischaemia.

3.2 Materials and methods

Male Sprague-Dawley rats (300 – 400 g) were used for these studies.

3.2.1 Isolated heart preparation

Rats were anaesthetised with pentobarbitone sodium (50 mg/Kg) intra-peritoneally. Heparin (1 IU/g) was administered concomitantly to prophylactically prevent thrombus formation in the coronary vasculature or ventricular chambers. Hearts were then excised and retrogradely perfused through the aorta on the Langendorff apparatus. Perfusate contained Krebs-Henseleit buffer (see chapter 2). Perfusion pressure was kept constant at 85 mmHg. By closing a port, the perfusate to the heart was stopped and global ischaemia was induced for the specified ischaemic time interval. The heart was jacketed by an external water-bath to maintain a physiological temperature (37°C) during this time. Reperfusion was achieved by opening the port to release perfusate once again to the heart. Baseline coronary effluent was collected at 5 min and 10 min

stabilisation and then again sampled at 30 seconds, 2 min and 5 min of reflow. These samples were then stored at -81°C for BNP assay.

At the end of each experiment, both the atria were excised above the ventricles, and the ventricular tissue was immediately freeze-clamped with a Wollenberger clamp prechilled in liquid nitrogen. These tissue samples and the coronary effluent samples were stored at -81°C until shipped to Essen for assay.

3.2.2 Ischaemia protocols

The experimental protocol is illustrated in figure 3.1. Hearts were randomly assigned to one of the following groups.

Group 1, Control: Following a stabilisation period of 10 min, hearts continued to be perfused for a further 5 min without an intervening ischaemic interval.

Group 2, Ischaemia 2 min: Hearts in this group were stabilised for 10 min before being subjected to 2 min of global ischaemia, followed by 5 min reperfusion.

Group 3, Ischaemia 5 min: In this group hearts were stabilised for 10 min before being subjected to 5 min of global ischaemia, followed by 5 min reperfusion.

Group 4, Ischaemia 20 min: Following the initial stabilisation of 10 min, hearts in this group were subjected to a more severe global ischaemia of 20 min, followed by 5 min reperfusion.

At the end of each experiment, hearts were excised below the atria and immediately freeze-clamped in liquid nitrogen and stored at -81°C for later BNP assay.

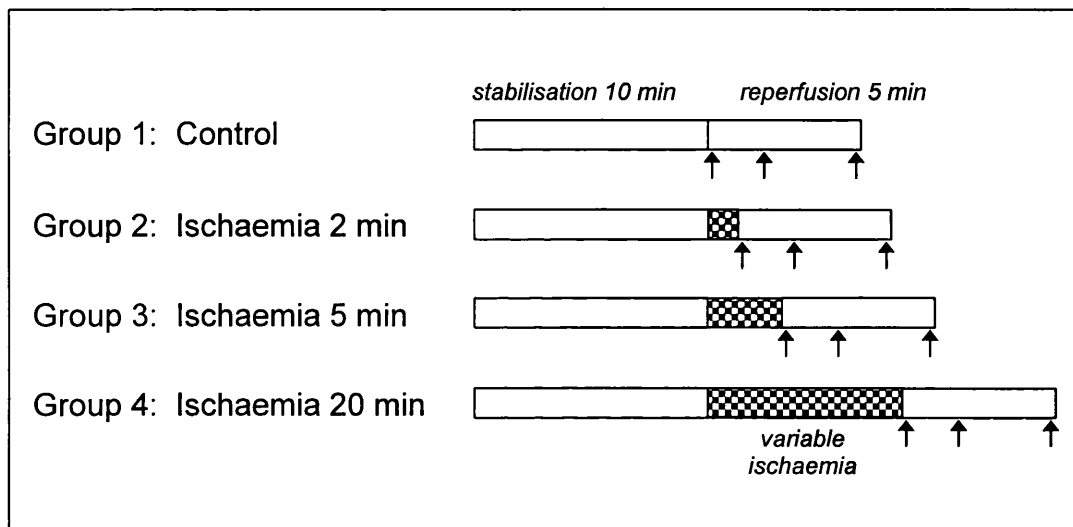


Figure 3. 1 Ischaemia protocol

Hearts in all four groups were initially stabilised for 10 min, during which time coronary effluents were sampled at 5 min intervals. In group 1, control hearts were perfused without ischaemia. In the other groups, hearts were rendered globally ischaemic for either 2, 5 or 20 min. All hearts were then perfused for 5 min, during which time, coronary effluents were sampled at 30 sec, 2 min and 5 min (arrowed). At the end of all the experiments ventricles were freeze-clamped for BNP assay.

3.2.3 Endogenous BNP assay

BNP concentration in the ventricular tissue and coronary effluents was determined as per the technique described in chapter two.

3.2.4 Statistical analysis

Data are expressed as means \pm s.e.m. BNP tissue concentrations were estimated from a linear extrapolation of the standard curve, and the corrected results derived from the reassured exponential standard curve. BNP tissue concentrations were analysed using one-way ANOVA and Fisher's protected least significant difference post-hoc test. BNP coronary effluent concentrations were evaluated using repeated-measures ANOVA with Bonferroni's post-hoc test. Statistical significance between group means was defined as $p < 0.05$.

3.3 Results

3.3.1 Technical exclusions

A total of 17 animals were used in this study. Two hearts were excluded; one due to poor contractility in the stabilisation phase, and one for failure to reperfuse. Therefore, data for 15 successfully completed experiments is reported.

3.3.2 BNP concentration in coronary effluent

A low basal release of BNP₁₋₃₂ was found in the coronary effluent in the range of 1.1 – 9.7 pmol/L, in the stabilisation phase as shown in figure 3.2. In the control group where hearts were not subjected to ischaemia, this basal efflux of BNP remained stable throughout the perfusion. Peak post-ischaemic BNP₁₋₃₂ concentrations in the coronary effluent were related to the duration of preceding ischaemia: ischaemia 2 min, 11.0 ± 2.4 pmol/L; ischaemia 5 min, 20.1 ± 2.2 pmol/L; ischaemia 20 min, 41.5 ± 3.3 pmol/L (all $P < 0.05$ v control values) – see figure 3.2. Global ischaemia of 2 min and 5 min, were not associated with changes in end diastolic pressure above baseline. Hearts subjected to to global ischaemia of 20 min, displayed an increase in end diastolic pressure of approximately 10 mm Hg above baseline values.

3.3.3 BNP concentration in ventricular myocardium

BNP₁₋₃₂ levels in the ventricular myocardium obtained at the end of each experiment are shown in table 3.1. The concentration (pmol/g wet weight) was 1.58 ± 0.16 in control hearts, and 1.77 ± 0.11 , 3.69 ± 0.65 ($P < 0.05$ v control values) and 1.82 ± 0.14 respectively in hearts subjected to 2, 5 and 20 min global ischaemia with 5 min reperfusion (n = 3-4 per group).

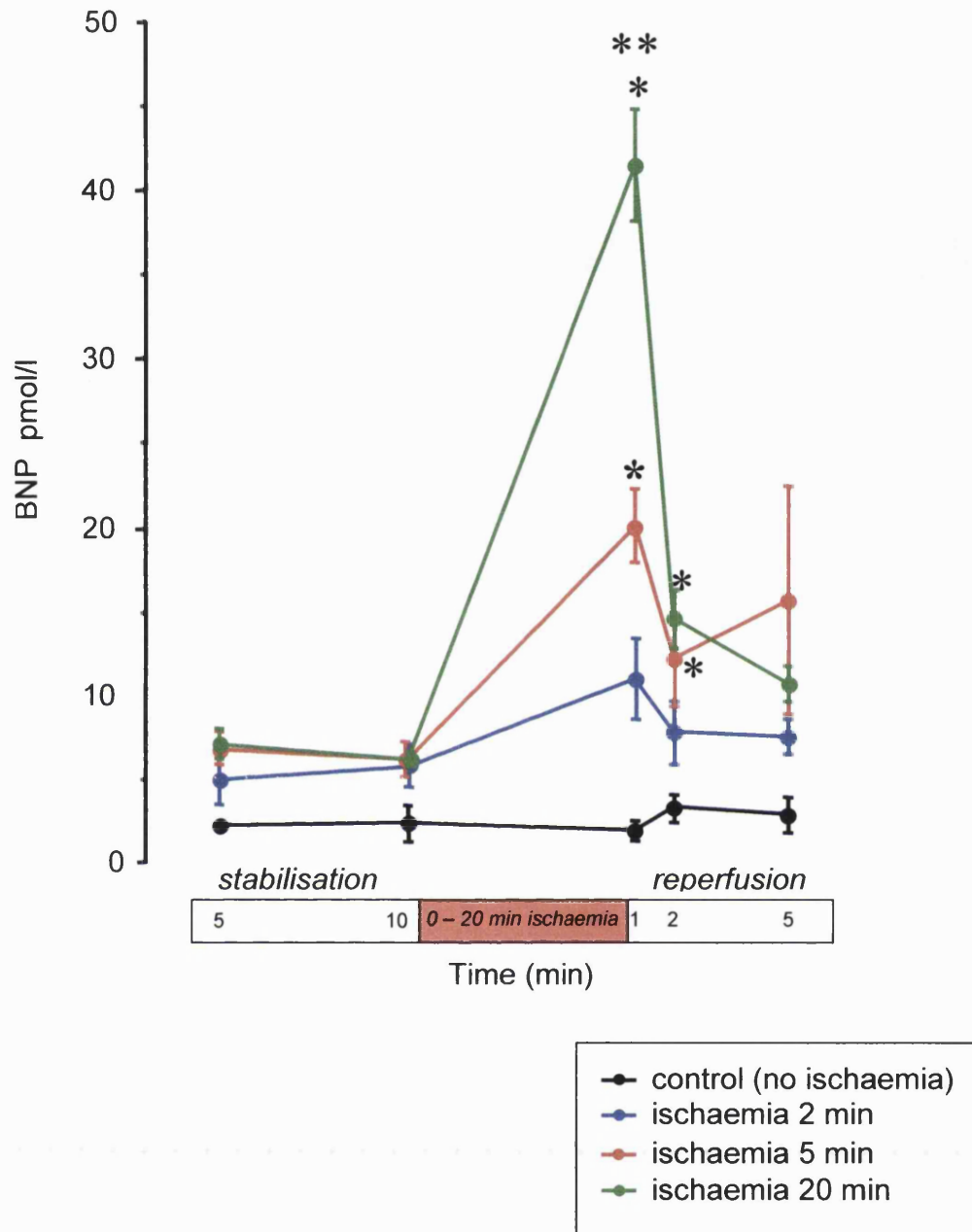


Figure 3. 2 BNP concentration in coronary effluent

BNP concentrations as measured from the coronary effluents in hearts subjected to different time intervals of regional coronary ischaemia. BNP release was directly proportional to the duration of ischaemia. * $P < 0.05$ and ** $P < 0.005$ v control and ischaemia 2 min groups (2-way ANOVA). $n = 3-4$ per group.

<i>Group</i>	<i>BNP levels (pmol/g wet weight)</i>
Control	1.58 ± 0.16
Ischaemia 2 min	1.77 ± 0.11*
Ischaemia 5 min	3.69 ± 0.65*
Ischaemia 20 min	1.82 ± 0.14

Table 3.1 BNP concentration in ventricular myocardium

Tabular representation of the BNP concentration in the ventricular tissue obtained at the end of each experiment. Values are mean ± s.e.m (one-way ANOVA). * Indicate $P < 0.05$ v control values. $n = 3 - 4/\text{group}$

3.4 Discussion

Several previous studies have shown that plasma BNP levels are increased in various types of heart disease, such as heart failure, acute myocardial infarction and unstable angina. In vivo, myocardial ischaemia is a strong stimulus for BNP release. Further, BNP has recently, been shown to provide independent and incremental prognostic information in patients with non-ST elevation acute coronary syndromes (Sabatine et al, 2002). Data in this study characterises the release of BNP into the coronary effluent following global ischaemia in the isolated perfused rat heart. The study demonstrates that BNP release occurs immediately following even brief, non-injurious ischaemic episodes, correlating in a graded fashion with the ischaemia severity and the levels then returning to the baseline trend following a 5 min reperfusion. With the rapid release of the peptide into the coronary effluent the ventricular myocardium stores tend to get depleted reflected by the low concentration of BNP in the ventricular

myocardium following the 20 min ischaemic episode. The increased tissue levels of BNP₁₋₃₂ after 2 min and 5min ischaemic stimuli, likely reflect the cleavage of the stored pro-peptide in response to ischaemia, prior to release.

The mechanism of release of BNP in myocardial ischaemia is unclear. Myocardial ischaemia in unstable angina may increase regional ventricular wall stretch owing to local depression of myocardial contraction. Mechanical stretch can activate the JAK/STAT pathway and may stimulate N-BNP secretion (Nakao et al, 1991). Alternatively, cytokines released in myocardial ischaemia may directly promote myocardial transcription of BNP (Kuwahara et al, 1998). However, since end-diastolic pressure did not change substantially with 2 or 5 min ischaemia, mechanical factors may not be the primary stimulus for release.

In summary, this study characterises the post-ischaemic endogenous release of BNP₁₋₃₂ in a graded manner with ischaemia severity. While other endogenous mediators released in myocardial ischaemia e.g. adenosine, opioids, bradykinin, nitric oxide, etc. have been shown to play a role in ischaemic preconditioning of the heart, the functional role of BNP in ischaemia-reperfusion injury is uncertain. However, these data show that even brief episodes of ischaemia, normally associated with preconditioning are sufficient to elicit the release of BNP.

Chapter Four

Exogenous BNP and Cardioprotection

4.1 Introduction

Our previous study described in chapter three confirms the graded release of cardiac BNP in response to severity of ischaemia, with no substantial change of end-diastolic pressure. However, a functional role for this endogenous BNP release in protection against myocardial infarction has not been studied.

The recognition in recent years that several neurohormonal mediators are released from the myocardium during brief periods of ischaemia underpins the current mechanistic model of ischaemic preconditioning (Murry et al, 1986; Gross et al, 1995; Yellon et al, 1998; Cohen et al, 2000; Schulz et al, 2001; Baxter et al, 2002b). As described in chapter one, autocrine and paracrine mediators acting on G-protein coupled receptors, notably adenosine, bradykinin, opioids peptides and catecholamines, participate in the activation of a multiple-stage signal transduction pathway. This involves opening of the ATP sensitive potassium (K_{ATP}) channels either as a downstream or proximal event, essential for conferring resistance to a subsequent episode of ischaemia. NPR-A does not couple through G-proteins, but through elevation of cGMP, NPR-A activation can modulate K_{ATP} channel activity (Kubo et al, 1994). Thus NPR-A activation by BNP could represent an alternative signal for this pro-survival mechanism. To test the hypothesis that BNP is cytoprotective during ischaemia-reperfusion, isolated rat hearts were perfused with graded concentrations of rat BNP₁₋₃₂ in a concentration-response study to determine the ability of exogenous BNP to limit irreversible myocardial injury.

4.2 Materials and methods

Male Sprague-Dawley rats (300 – 400 g) were used for these studies. Hearts were isolated, Langendorff perfused and infarct size evaluated as described in chapter two.

4.2.1 Infarction protocol

The experimental protocol is illustrated in figure 4.1. The initial concentration of BNP (BNP 10^{-8} mol/L) was calculated as 10 times the EC_{50} value - the concentration required to produce 50% relaxation of the precontracted, isolated rat aorta (Kabayashi et al, 1990a). This stock solution was diluted further to make up sequential 10-fold dilutions of BNP. Hearts were randomly assigned to one of the following six groups.

Control: In the control group, hearts were stabilised for 15-20 min before being subjected to 35 min of regional ischaemia, followed by 120 min reperfusion

BNP 10^{-8} : BNP 10^{-8} mol/L was added to Krebs-Henseleit buffer and commenced 10 min into stabilisation and continued for a further 10 min prior to ischaemia and then continued until 30 min reperfusion.

BNP 10^{-9} : In this group, BNP 10^{-9} mol/L was added to Krebs-Henseleit buffer and perfusion started 10 min prior to ischaemia and continued until 30 min reperfusion.

BNP 10^{-10} : BNP 10^{-10} mol/L treatment as described above

BNP 10⁻¹¹: BNP 10⁻¹¹ mol/L treatment as described above

BNP 10⁻¹²: BNP 10⁻¹² mol/L treatment as described above

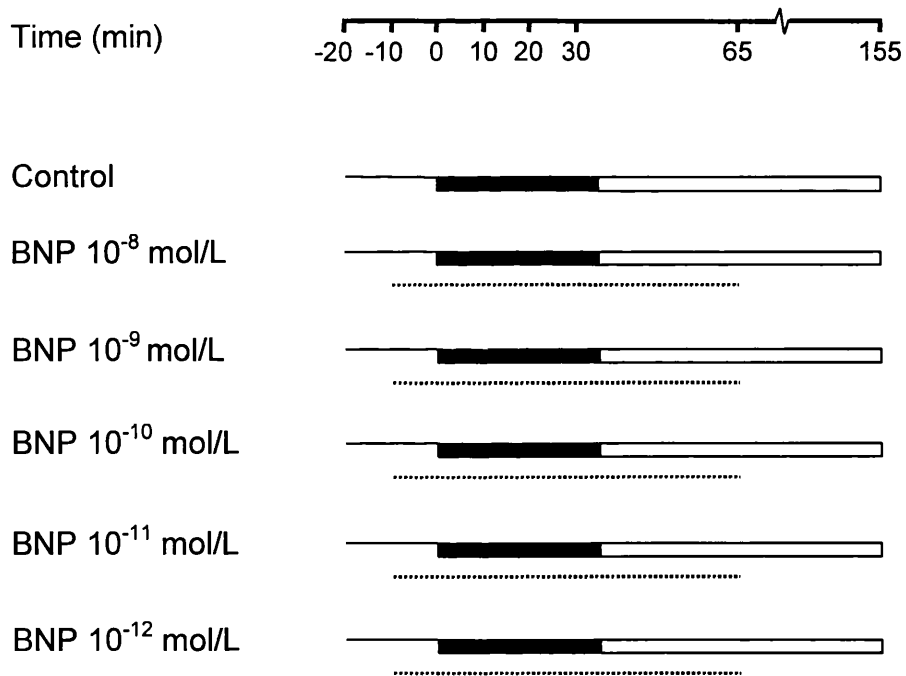


Figure 4. 1 Experimental protocol

Protocol used in the BNP concentration-response study is shown. Dotted lines represent the time course of perfusion with different BNP concentrations as indicated.

4.2.2 Statistical analysis

Data were analysed as described in chapter two.

4.3 Results

4.3.1 Technical exclusions

A total of 65 animals were used in this study. Five hearts were excluded; one was damaged during instrumentation, one had failure of tetrazolium stain, two had persistent bradyarrhythmias in the stabilisation phase, and one failed to reperfuse. Therefore, data for 60 successfully completed experiments is reported.

4.3.2 Infarct size and risk zone

The risk zone volumes, body weights and heart weights were similar among all the groups and are shown in table 1.1. Control infarct/risk zone ratio was $44.8 \pm 4.4\%$ without BNP treatment. Treatment with BNP limited infarct size in a concentration-dependent manner (figures 4.2, 4.3 and 4.4). Significant limitation of infarction was observed with BNP 10^{-10} , 10^{-9} and 10^{-8} mol/L. The highest concentration of BNP studied (10^{-8} mol/L) resulted in the smallest infarct size ($20.1 \pm 5.2\%$, $P < 0.01$ v control).

Group	n	Body weight (g)	Wet heart weight (g)	Risk zone volume (mm ³)	Infarct size (%)
Control	11	357±10	1.8±0.07	352±26	44.8±4.4
BNP 10 ⁻⁸ mol/L	8	338±17	1.5±0.07*	325±29	20.1±5.2**
BNP 10 ⁻⁹ mol/L	9	342±14	1.6±0.08	318±27	26.5±5.8**
BNP 10 ⁻¹⁰ mol/L	8	378±10	1.8±0.07	335±33	30.5±3.7*
BNP 10 ⁻¹¹ mol/L	12	351±14	1.7±0.11	323±19	37.4±5.3
BNP 10 ⁻¹² mol/L	12	361±18	1.7±0.05	334±16	39.7±2.4

Table 4. 1 Summary of body weight, wet heart weights, risk zone volume and infarct size

Figures are mean ± s.e.m. Infarct size is the ratio of infarct area to the risk zone area.

*P < 0.05 v control and **P < 0.01 v control values (one-way ANOVA).

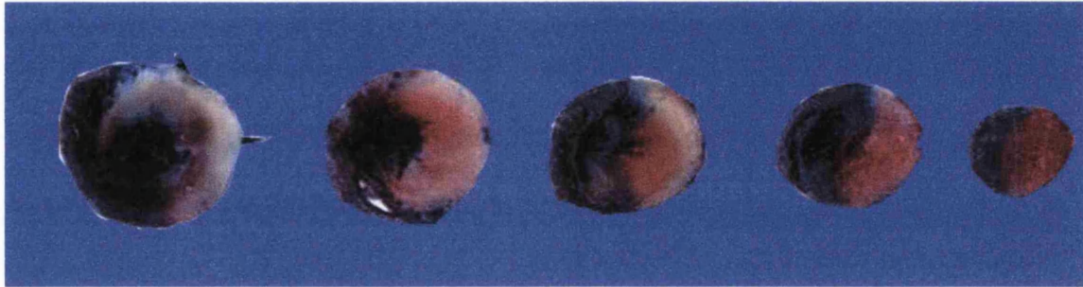


Figure 4.2 Control heart slices

Heart slices in the control group, following ischaemia-reperfusion injury. Infarct/risk zone ratio averaged $44.8 \pm 4.4\%$.

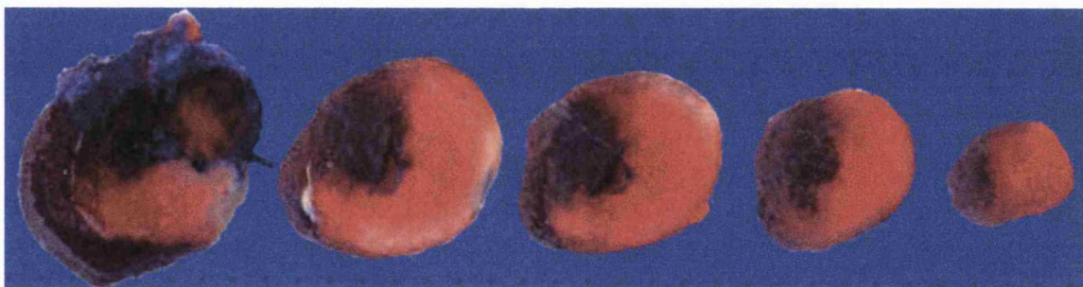


Figure 4.3 BNP treated heart slices

BNP perfused rat heart showing significant protection against ischaemia-reperfusion injury (v Control) with average infarct-to-risk ratio of $20.1 \pm 5.2\%$ in the BNP 10^{-8} mol/L treatment group.

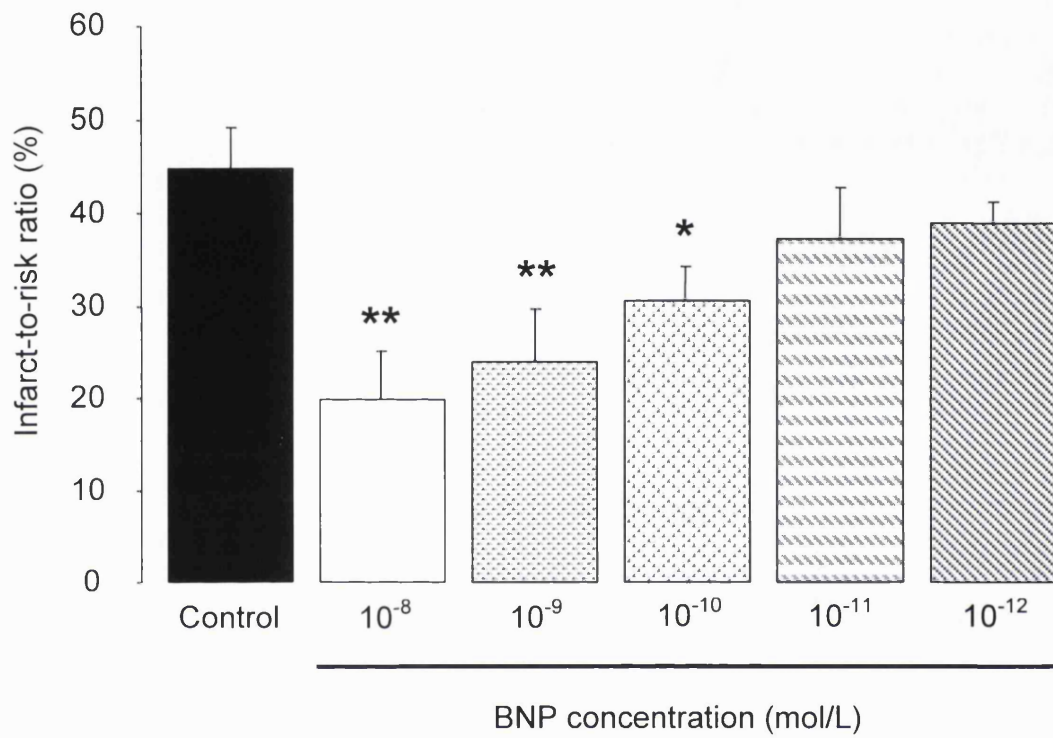


Figure 4. 4 Infarct to risk ratio

Infarct-to-risk ratios for the hearts perfused with BNP 10^{-12} to 10^{-8} mol/l. $n = 8-12$ experiments in each group. Bars represent mean \pm s.e.m. * $P < 0.05$; ** $P < 0.01$ v control (1-way ANOVA).

4.3.3 Coronary flow and LV function

Pre-ischaemic global coronary flow averaged 12.5 ml/min among the six experimental groups. After coronary occlusion, there was a decrease in global coronary flow rate of approximately 35% and a recovery to pre-ischaemic values immediately after reperfusion with a gradual “run-down” during the remaining 120 min perfusion (figure 4.5 A). Global coronary flow rate measured at intervals throughout the protocol did not differ substantially among the experimental groups.

There were no detectable differences between the groups in any of the parameters of LV function measured (spontaneous heart rate, developed pressure or the double product – figure 4.5 B). Developed pressure and double product declined immediately after the onset of coronary occlusion to the same extent in all the experimental groups. A tabular representation of the coronary flow and the double product amongst the experimental groups is presented in tables 4.2 and 4.3.

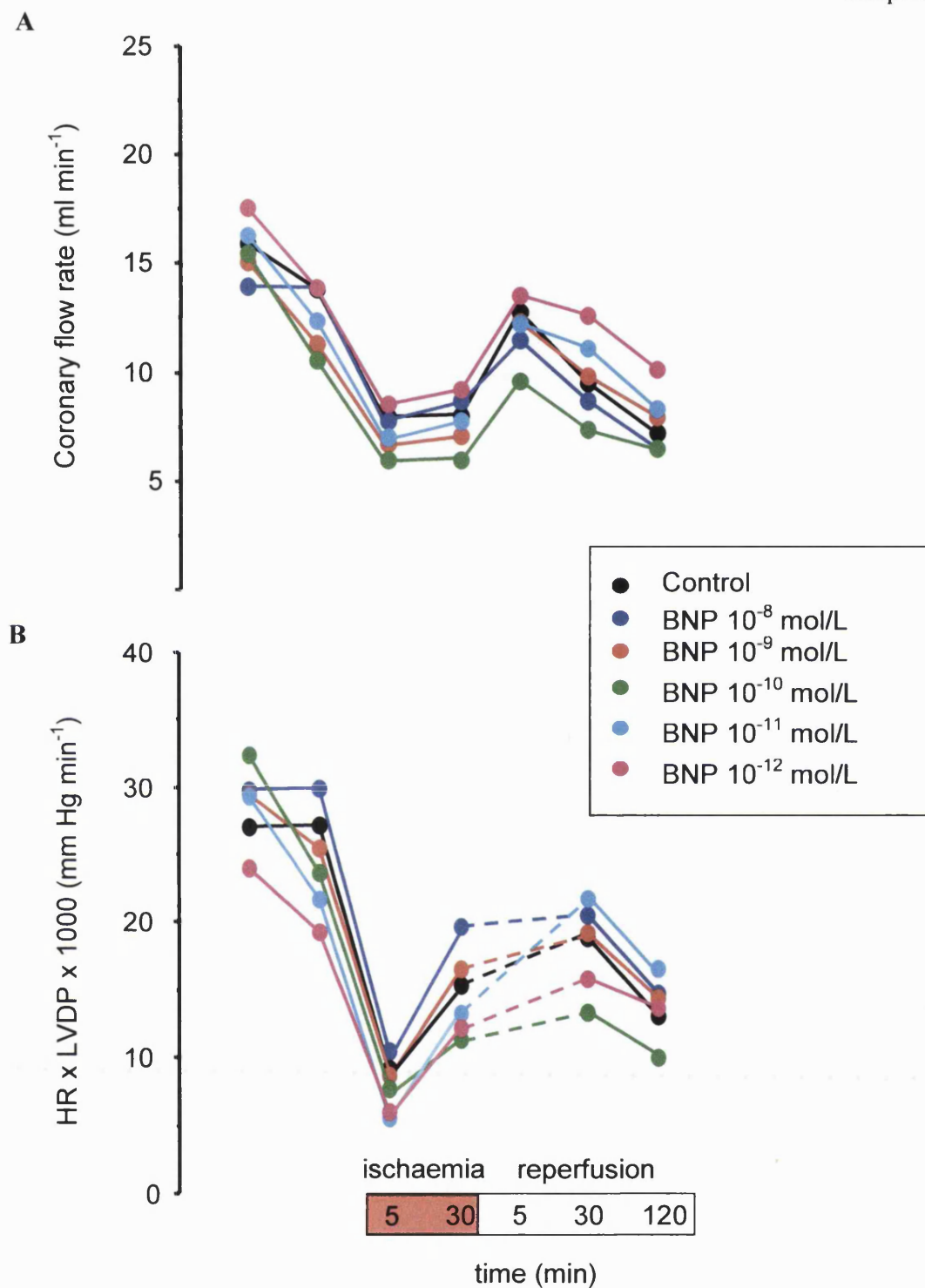


Figure 4.5 Cardiodynamics

Global coronary flow rate and the rate-pressure or double product for the BNP concentration response study. Symbols indicate mean values. Standard error bars have been removed for clarity.

Group	Basal	Pre-ischaemia	<u>Ischaemia</u>		<u>Reperfusion</u>		
			5 min	30 min	5 min	30 min	120 min
Control	15.9±1.0	13.8±0.9	7.8±0.6	8.0±0.4	12.3±0.8	9.5±0.9	7.2±0.8
BNP 10 ⁻⁸ mol/L	14.0±1.4	13.8±1.1	7.7±0.9	8.7±1.1	13.1±1.8	8.7±1.4	6.5±0.9
BNP 10 ⁻⁹ mol/L	15.1±1.2	11.2±1.0	6.6±0.7	7.1±0.7	11.3±1.1	9.8±1.1	7.9±1.2
BNP 10 ⁻¹⁰ mol/L	15.5±0.7	10.6±0.8*	6.0±0.6	5.8±0.5	9.0±1.0	7.1±0.6	6.5±0.1
BNP 10 ⁻¹¹ mol/L	16.3±0.9	12.4±0.9	7.0±0.7	7.8±0.8	12.3±1.1	11.1±1.2	8.3±1.1
BNP 10 ⁻¹² mol/L	17.6±0.8	13.9±1.1	8.4±0.9	9.2±1.0	13.8±1.1	12.5±0.9	10.0±0.6

Table 4. 2 Summary of coronary flow (ml/min)

Figures are mean ± s.e.m. *P < 0.05 v control v control values (repeated-measures ANOVA).

Group	Basal	Pre- <i>ischaemia</i>	<u><i>Ischaemia</i></u>		<u><i>Reperfusion</i></u>	
			5 min	30 min	30 min	120 min
Control	27.1±3.8	27.2±4.1	8.8±1.4	15.4±1.8	18.8±2.1	13.0±2.0
BNP 10 ⁻⁸ mol/L	29.8±3.0	29.9±3.2	10.4±1.3	19.7±1.6	20.5±2.7	14.8±2.8
BNP 10 ⁻⁹ mol/L	29.5±2.3	25.4±2.5	8.8±1.7	16.5±2.6	19.2±2.2	14.4±2.3
BNP 10 ⁻¹⁰ mol/L	32.3±5.0	23.7±5.0	7.7±1.8	11.3±2.1	12.3±2.9	10.4±1.2*
BNP 10 ⁻¹¹ mol/L	29.4±2.4	21.8±2.6	5.6±0.9	13.3±1.2	22.4±2.5	16.4±2.5
BNP 10 ⁻¹² mol/L	24.1±3.0	19.3±2.9	6.0±1.1	12.2±1.4	15.7±1.6	13.6±1.3

Table 4.3 Summary of rate pressure product (mm Hg min⁻¹ x 10³)

Figures are mean ± s.e.m.; *P < 0.05 v control values (repeated-measures ANOVA).

4.4 Discussion

The present study provides insight into the cardioprotective role of exogenously administered BNP, in myocardial ischaemia-reperfusion injury in the isolated rat heart model. An acute infusion of exogenous BNP protects the ischaemic myocardial injury, leading to a concentration-dependent limitation of infarct size. Although recent studies have reported that ANP (Csont et al, 1998) and the related non-cardiac peptide urodilatin (Takagi et al, 2000) limit infarct size *in vivo*, this is the first study to show a previously undefined cardioprotective effect of BNP. However, the mechanism of the primary cytoprotective effect of BNP in myocardial ischaemia-reperfusion remains to be elucidated.

The cell-surface receptor mediating the biological actions of BNP is a particulate guanylyl cyclase-A (NPR-A) receptor, which as discussed in chapter one is abundantly expressed in cardiac tissue (Boomsma et al, 2001; Silberbach et al, 2001). However, unlike receptors for other endogenous mediators released in myocardial ischaemia viz. adenosine, bradykinin and opioids, the NPR-A is not a G-protein-coupled receptor. This receptor contains an intracellular guanylyl cyclase catalytic domain, which mediates most of the known biological actions of the natriuretic peptide through the conversion of guanosine mono phosphate (GTP) to cyclic-guanosine monophosphate (cGMP) (Padilla et al, 2001; Kambayashi et al, 1990a; Lucas et al, 2000; Chinkers et al, 1991).

BNP is a vasodilator in several vascular beds including coronary epicardial conductance arteries and coronary microvessels (Zellner et al, 1999; Brunner et al, 2001). However, a surprising finding in our study was the absence of any gross

alterations in global coronary flow rate secondary to BNP-mediated coronary vasodilation. It is conceivable that an effect might be observed at higher concentrations. At present, effects of higher concentrations could not be studied due to larger molar quantity of BNP required. A further explanation relates to the coronary resistance and conductance arteries being maximally vasodilated in this constant pressure preparation. Indeed, in a previous study examining the coronary vasodilator mechanisms of ANP in a constant flow preparation in which endothelin was applied to raise coronary vascular tone, ANP reduced coronary perfusion pressure and this effect was sensitive to inhibition by nitro-L-arginine (Zellner et al, 1999). A soluble guanylyl cyclase derived nitric oxide (NO)-cGMP signalling complex may thus be implicated in natriuretic peptide mediated effects.

BNP at the concentrations used in this study did not significantly affect the functional determinants of the left ventricle reflected from the heart rate, developed pressure and the double product, when compared to the control group. Further, there were no significant differences amongst the BNP treatment groups. The infarct-limiting effect of BNP, thus looks likely to be independent of the altered functional modalities of the heart.

The relationship between BNP mediated cardioprotection and the role of the mitochondrial K_{ATP} channel, the putative effector of ischaemic preconditioning is not defined. Further study to elucidate this relationship is important in further defining BNP's cytoprotective effect and is described in the next chapter.

In conclusion, these studies are the first to demonstrate a cardioprotective effect of exogenously administered BNP against ischaemia-reperfusion injury. The BNP protection accorded was concentration-dependent. The molecular mechanism of this previously undefined action of BNP is unknown.

Chapter Five

Role of K_{ATP} channels in BNP-induced cardioprotection

5.1 Introduction

Our study thus far has shown, BNP to be released post-ischaemically from the ventricular tissue and proportionate to the duration of cardiac ischaemia as described in chapter three. Further, in chapter four the study implicates BNP for the first time to be cardioprotective in a concentration-dependent manner.

Many of the endogenous mediators like opioids, bradykinin and adenosine, described in chapter one, are released in response to cardiac ischaemia. These substances have been shown to trigger ischaemic preconditioning, signalling via a trans-membrane G-protein and activating intracellular kinases eventually opening the mitochondrial K_{ATP} channel, either as a downstream or a proximal event, shown to be cardioprotective to a subsequent episode of ischaemia. While endogenous BNP has been shown to be cardioprotective, its molecular mechanistic remains unresolved. BNP, in contrast to other endogenous mediators binds to its own membrane bound particulate guanylyl cyclase receptor (natriuretic peptide receptor-A; NPR-A) in exerting its known biological effects of vasodilation and natriuresis (Yamamoto et al, 1997a). NPR-A can modulate K_{ATP} channel activity, through elevation of cGMP (Kubo et al, 1994). There is a general consensus that the K_{ATP} channel opening in ischaemia limits the development of injury. In relation to IPC, the K_{ATP} channel opening appears to play a central role, either as a distal mediator (Grover et al, 2000) or, as has been suggested more recently, as a proximal (trigger) event (Wang et al, 2001). Opening of both the sarcolemmal and mitochondrial K_{ATP} (sK_{ATP} and mK_{ATP}) channels has been shown to

be enhanced following activation of intracellular mediators or kinases (Gross et al, 1999). While sK_{ATP} channel opening is responsible for shortening of the myocyte action potential, membrane hyperpolarisation, decreased calcium entry and preservation of ATP, mK_{ATP} channel opening causes membrane depolarisation, matrix swelling, enhanced respiration and reduced calcium overload (Gross et al, 1999). Subsequently, persuasive evidence in different models has shown that it was likely to be the mK_{ATP} channel that is involved in protection (Garlid et al, 1997; Liu et al, 1998; Sato et al, 2000).

To test the hypothesis that the cytoprotective action of BNP during ischaemia-reperfusion injury is mediated through opening of the K_{ATP} channels, various pharmacological blockers of the sK_{ATP} and mK_{ATP} channels have been used in this study to define the role of K_{ATP} channel involvement in the infarct limiting effect of exogenous BNP. Inhibitors of the K_{ATP} channel used here include sodium 5-hydroxydecanoate (5-HD) 10^{-4} mol/L - a selective mitochondrial K_{ATP} blocker (Grover, 1994; Wang et al, 2001), glibenclamide 10^{-6} mol/L - a non-selective blocker of sarcolemmal and mitochondrial K_{ATP} (Workman et al, 2000; Ferdinandy et al, 1995; Mocanu et al, 2001; Legtenberg et al, 2001) and HMR1098 10^{-5} mol/L a selective blocker of the sK_{ATP} channel (Kita et al, 2000; Manning Fox et al, 2002).

5.2 Materials and methods

Hearts were excised, Langendorff perfused and infarct size evaluated as described in chapter two.

5.2.1 Infarction protocol

The experimental protocol is illustrated in figure 5.1. A concentration of BNP at 10^{-8} mol/L was selected based on preceding experiments described in chapter four. Hearts were randomly assigned to one of the following eight groups.

1. A control group in which hearts were stabilised for 15-20 min before being subjected to 35 min of regional ischaemia, followed by 120 min reperfusion. Six control hearts were perfused with DMSO 0.016% (the vehicle solvent for glibenclamide). Since there was no effect on infarct size of DMSO, these hearts were combined for statistical evaluation with non-DMSO treated control hearts to comprise group 1.
2. BNP 10^{-8} mol/L treatment group, with BNP added to the Krebs-Henseleit buffer and perfusion started 10 min prior to ischaemia and continued 30 min into reperfusion.
3. 5-HD (10^{-4} mol/L), was perfused 10 min prior to ischaemia and continued until 30 min reperfusion.

4. 5-HD 10^{-4} mol/L + BNP 10^{-8} mol/L, co-perfused as described above.

5. Glibenclamide (10^{-6} mol/L), perfused 10 min prior to ischaemia and continued until 30 min into reperfusion. Glibenclamide was dissolved in DMSO (final concentration not more than 0.016%).

6. Glibenclamide 10^{-6} mol/L + BNP 10^{-8} mol/L, co-perfused as described above.

7. HMR1098 (10^{-5} mol/L), perfused 10 min prior to ischaemia and continued until 30 min into reperfusion.

8. HMR1098 10^{-5} mol/L + BNP 10^{-8} mol/L, co-perfused as described above.

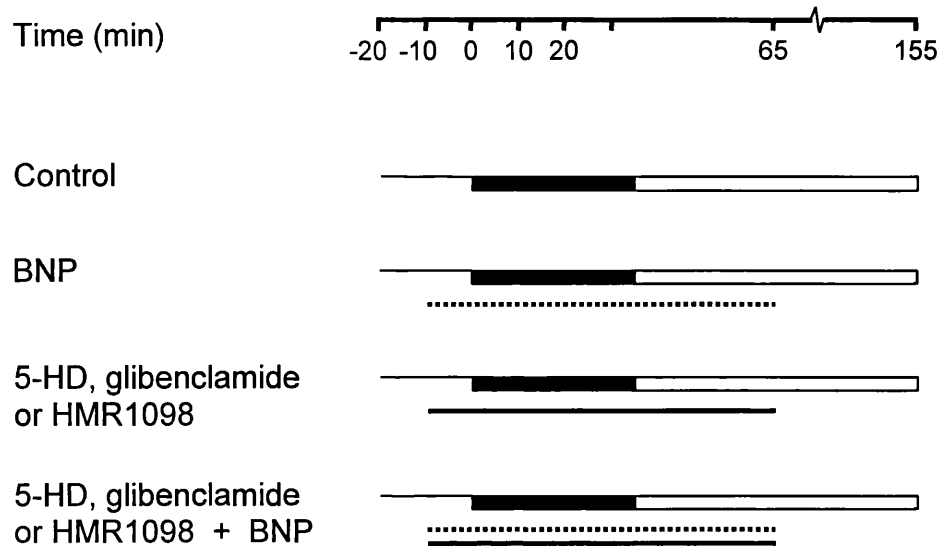


Figure 5.1 Experimental protocol

Protocol used in the K_{ATP} blockade study is shown. Dotted lines represent the time course of perfusion with BNP. Solid lines represent K_{ATP} channel blocker perfusion.

5.2.2 Statistical analysis

Data are expressed as means \pm s.e.m. Infarct-to-risk ratios and risk zone volumes were analysed using one-way ANOVA and Fisher's protected least significant difference post-hoc test. Left ventricular function parameters and coronary flow rate were evaluated using repeated-measures ANOVA. Statistical significance between group means was defined as $p < 0.05$.

5.3 Results

5.3.1 Technical exclusions

A total of 55 animals was used in this study. Five hearts were excluded; two failed to reperfuse, one was damaged by instrumentation which prevented precise LV function assessment, one had failure of tetrazolium stain and one had persistent bradyarrhythmias throughout reperfusion. Therefore, data for 50 successfully completed experiments are reported.

5.3.2 Infarct size and risk zone

The risk zone volumes, body weights and heart weights were similar among all the groups and are shown in table 5.1 and infarct sizes represented in figure 5.2. Control infarct-to-risk zone ratio was $47.1 \pm 2.8\%$ without BNP treatment. BNP 10^{-8} mol/L treatment resulted in significant limitation of infarct size ($21.3 \pm 2.8\%$, $P < 0.01$ v control). Co-perfusion of BNP with either 5-HD or glibenclamide resulted in abolition of the protective effect of BNP (infarct-to-risk ratio $52.3 \pm 5.7\%$ and $52.0 \pm 5.7\%$ respectively, P not significant v control). However, infarct size limitation with BNP was not abolished by HMR1098 (infarct-to-risk ratio $14.7 \pm 2.7\%$, $P < 0.01$ v control, P not significant v BNP). None of the K_{ATP} channel blockers per se influenced infarct size.

Group	n	Body weight (g)	Wet heart weight (g)	Risk zone volume (mm ³)	Infarct size (%)
Control	10	361±8	1.7±0.04	384±32	47.1±2.8
BNP	5	358±3	1.7±0.07	339±15	21.3±2.8**
5-HD	6	333±3*	1.5±0.03**	339±29	40.5±4.1
5-HD + BNP	6	352±6	1.6±0.05	312±35	52.3±5.7
Glibenclamide	4	373±9	1.7±0.07	367±36	41.0±7.1
Glibenclamide + BNP	6	370±6	1.7±0.04	370±25	52.0±5.7
HMR1098	5	374±13	1.7±0.04	375±39	48.6±2.3
HMR1098 + BNP	8	379±11	1.7±0.06	320±23	14.7±2.7**

Table 5.1 Summary of body weight, wet heart weights, risk zone volume and infarct size

Figures are mean ± s.e.m. Infarct size is the ratio of infarct area to the risk zone area.

*P < 0.05 v control and **P < 0.01 v control values (one-way ANOVA).

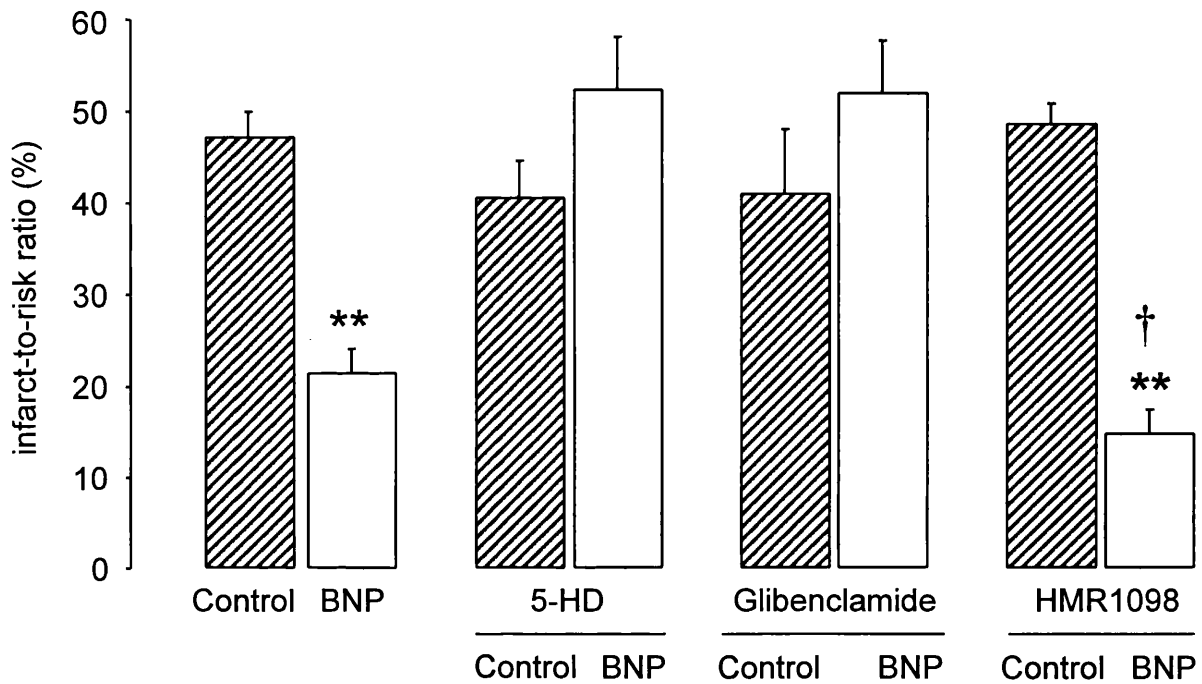


Figure 5. 2 Infarct to risk ratio

Infarct-to-risk zone ratios for hearts perfused with K_{ATP} blockers with and without BNP 10^{-8} mol/l. $n = 5-10$ experiments in each group. Bars represent mean \pm s.e.m.

** $P < 0.01$ v control; † $P < 0.01$ v HMR1098 control (1-way ANOVA).

5.3.3 Coronary flow and LV function

Pre-ischaemic global coronary flow averaged 15 ml/min among the eight experimental groups. After coronary occlusion, there was a decrease in global coronary flow rate of approximately 35% and a recovery to pre-ischaemic values immediately after reperfusion with a gradual “run-down” during the remaining 120 min perfusion. Global coronary flow rate measured at intervals throughout the protocol did not differ substantially among the experimental groups.

There were no detectable differences between the groups in any of the parameters of LV function measured (spontaneous heart rate, developed pressure or the double product). Developed pressure and double product declined immediately after the onset of coronary occlusion to the same extent in all the experimental groups. Tabular presentation of the coronary flow and the double product amongst the experimental groups is given in tables 5.2 and 5.3.

Group	Basal	Pre-ischaeamia	<u>Ischaemia</u>		<u>Reperfusion</u>		
			5 min	30 min	5 min	30 min	120 min
Control	19.4±1.0	17.9±1.3	8.3±0.5	9.6±0.6	18.3±1.2	18.4±1.1	14.4±1.1
BNP	18.2±1.1	16.2±1.5	8.0±0.3	8.9±0.7	15.6±0.7	15.0±0.4*	12.6±1.1
5-HD	17.0±1.1	12.7±1.1**	7.2±0.6	7.8±0.6	14.7±0.9*	12.7±1.1**	10.3±1.2**
5-HD + BNP	19.0±1.3	15.2±1.2	8.0±0.5	9.8±0.8	17.2±1.2	14.2±1.6**	12.0±1.7
Glibenclamide	18.5±1.0	14.7±1.4	9.2±1.1	10.7±1.7	17.1±1.2	13.7±1.5**	12.7±1.6
Glibenclamide + BNP	19.3±0.4	12.3±0.4**	7.1±0.2	7.9±0.3	11.8±0.6**	11.0±0.7**	13.1±1.1
HMR1098	19.0±0.8	15.2±1.2	8.2±0.7	9.2±0.5	13.6±0.5**	12.8±0.5**	9.7±0.4**
HMR1098 + BNP	17.5±1.0	15.2±1.1	7.2±0.6	9.4±0.9	14.6±1.3*	12.7±1.3**	9.4±0.7**

Table 5. 2 Summary of coronary flow (ml/min)

Figures are mean ± s.e.m. Infarct size is the ratio of infarct area to the risk zone area.

*P < 0.05 v control and **P < 0.01 v control values (repeated-measures ANOVA).

Group	Basal	Pre- <i>ischaemia</i>	<u>Ischaemia</u>		<u>Reperfusion</u>	
			5 min	30 min	30 min	120 min
Control	27.9±2.7	33.1±2.3	8.5±1.4	20.0±1.9	29.7±3.8	20.8±2.7
BNP	34.4±3.7	32.0±3.6	6.5±1.1	19.9±4.4	29.3±6.3	20.1±3.7
5-HD	33.0±3.9	22.5±5.8*	6.5±2.2	11.6±3.3*	20.2±5.2	16.5±3.6
5-HD + BNP	32.7±4.6	34.5±5.1	10.6±2.3	26.7±2.8	29.5±5.7	21.3±5.1
Glibenclamide	31.1±7.6	27.1±3.5	11.4±2.0	26.7±4.3	26.5±5.2	18.5±3.0
Glibenclamide + BNP	35.9±3.5	27.6±1.9	7.7±1.4	20.8±2.5	21.3±2.2	22.3±2.4
HMR1098	28.9±3.2	28.8±5.2	6.6±1.9	18.5±3.1	22.5±4.5	12.1±1.6
HMR1098 + BNP	26.9±3.1	28.9±3.9	7.7±1.2	20.1±3.0	19.6±2.5*	15.8±2.9*

Table 5.3 Summary of rate pressure product (mm Hg min⁻¹ x 10³)

Figures are mean ± s.e.m. Infarct size is the ratio of infarct area to the risk zone area.

*P < 0.05 v control values (repeated-measures ANOVA).

5.4 Discussion

The present study implicates the involvement of the K_{ATP} channels in the BNP mediated cytoprotection in myocardial ischaemia-reperfusion injury. The pharmacological selectivities of the widely used K_{ATP} channel blockers, applied at conventional inhibitory concentrations (Workman et al 2000; Ferdinandy et al, 1995; Mocanu et al, 2001; Legtenberg et al, 2001; Kita et al, 2000; Manning Fox et al, 2002), may indicate involvement of the mK_{ATP} channel, rather than the sK_{ATP} channel. As in the BNP concentration-response study described in chapter 4, hearts treated with BNP and/or the K_{ATP} blockers in this study, did not exhibit an increase in the coronary flow indicative of a vasodilatory response compared to control hearts. Again, this may be explained on the basis of the low concentration of BNP used or the coronary vasculature being maximally dilated in the constant-pressure experimental set-up.

BNP is known to mediate its biological actions by binding to its membrane-bound particulate guanylyl cyclase-A receptor, NPR-A abundantly expressed in cardiac tissue (Boomsma et al, 2001; Silberbach et al, 2001). Unlike other endogenous mediators in the likes of opioids and bradykinin, this is not a G-protein receptor, and an active moiety of the receptor is a guanylyl cyclase domain. This catalytic domain mediates BNP's activity through conversion of GTP to cGMP (Padilla et al, 2001; Kambayashi et al, 1990a; Lucas et al, 2000; Chinkers et al, 1991). cGMP has been postulated to a mechanism of injury limitation in the ischaemic myocardium (Opie, 1982), but the distal molecular mechanisms resulting in enhanced tolerance to ischaemic injury associated with cGMP elevation are unclear and the proposed mechanisms are enumerated in chapter one. The cGMP/cGK-I pathway could link NPR-A receptor

activation and the cytoprotective K_{ATP} channel opening, representing an alternative signal cascade to the widely studied G-protein receptor coupled-PKC pathway.

In the myocardium, sK_{ATP} channels were originally postulated to participate in salvage from irreversible ischaemia-reperfusion injury. Opening of these sK_{ATP} channels produce an increase in the outward potassium current leading to shortening of the action potential duration which would in turn reduce the Ca^{2+} influx through voltage-dependent Ca^{2+} channels and increase the time during which $Na^+ - Ca^{2+}$ exchanger would operate to extrude Ca^{2+} from the cell. Since 1998, attention has been focused on mK_{ATP} channels in both ischaemic preconditioning and pharmacological preconditioning studies (Schulz et al, 2001). Much of the evidence implicating a role of the mK_{ATP} channels is reliant on the reputed selectivity of pharmacological agents such as 5-HD (a blocker of mK_{ATP}) and HMR1098 (a selective blocker of sK_{ATP}). With the caution that pharmacological specificity and selectivity may be subjected to revision, this study provides pharmacological evidence for the involvement of a K_{ATP} channel subtype, possibly a mK_{ATP} channel, in the infarct limiting action of BNP. Further studies in appropriate isolated and mitochondrial preparations using biophysical and electrophysiological approaches may be able to probe the mK_{ATP} channel opening.

In summary the present study shows BNP's cytoprotective effect in myocardial ischaemia-reperfusion injury is limited by glibenclamide and 5-HD, but not by HMR1098. This is consistent with, but does not constitute proof of a mechanism involving opening of the putative mK_{ATP} channel. The relationship between cGMP levels in response to BNP release/activation and its role in myocardial ischaemia-reperfusion injury is studied further in the next chapter.

Chapter Six

cGMP activation and cardioprotection

6.1 Introduction

Endogenous and exogenous compounds, including autacoids, hormones, neurotransmitters and toxins produce cellular responses via cGMP. The biochemical mechanisms underlying those responses include synthesis, targeting and degradation of cGMP. Intracellular cGMP ($[cGMP]_i$) regulates cellular physiology by activating protein kinases, directly gating specific ion channels, or altering intracellular cyclic nucleotide concentrations through regulation of phosphodiesterases (PDEs) (Lucas et al, 2000). In nitric oxide induced ischaemic preconditioning, an elevated cardiac myocyte cGMP has been proposed as the mechanism for the antiarrhythmic effect observed (Parratt et al, 1995 and 1999), a hypothesis first put forward by Opie in 1982 (Opie, 1982).

Synthesis of cGMP occurs in response to guanylyl cyclase mediated catalytic conversion of GTP to cGMP (Lucas et al, 2000). The guanylyl cyclase (GC) family comprise both membrane-bound or particulate (pGC) and intracellular heme-containing soluble (sGC) isoforms which are activated on ligand binding to diverse signals. The natriuretic peptide receptors, GC-A (also termed NPR-A) and GC-B (or NPR-B) were the first pGCs cloned from mammalian tissues (Chinkers et al, 1989; Schulz et al, 1989) and are distributed in the heart and other tissues as described in chapter one. A GC-C is distributed in the intestinal mucosa and activated by heat-stable enterotoxins, guanylin and uroguanylin ligands whereas other pGC sequences (GC-D to GC-G) of olfactory, retinal or intestinal distribution have no known ligand receptors. The α - and β -subunits of sGC, present in the heart and other tissues are

activated by NO, protoporphyrin IX and fluxes of divalent cations like Mg^{2+} and Ca^{2+} (Lucas et al, 2000).

The principal signalling mechanism, by which natriuretic peptides induce their physiological effects involves activation of GC-coupled receptors and accumulation of $[cGMP]_i$. ANP and BNP have selective affinity for GC-A, compared to CNP which has a greater affinity for GC-B. BNP is 10-fold less potent than ANP in stimulating GC-A, but increases guanylyl cyclase activity and accumulation of $[cGMP]_i$ in cells and tissues in a pattern that mimics that of ANP (Koller, 1992). In studies thus far, we have shown that BNP is released in the isolated Langendorff perfused rat heart following ischaemia and limits infarct size in a concentration-dependent manner via opening of the mitochondrial K_{ATP} channels. However, the role of cGMP as the second messenger of BNP in this cytoprotective process is unknown.

To test the hypothesis that cGMP is involved in BNP's cardioprotection, the present study examines the cardiac cGMP concentration in response to ischaemia and graded perfusions of exogenous BNP and further examined the ability of exogenous cGMP to limit irreversible myocardial injury. To this end we have used the well-described cell-permeable analogue 8-bromoguanosine 3',5'-cyclic monophosphate sodium salt monohydrate (8-Br-cGMP; Boss, 1989), commencing at previously used doses (Kawada et al, 1997; Frolidi et al, 2001).

6.2 Materials and methods

Heart tissue was harvested for cGMP assay as described in chapter two. For 8-Br-cGMP infarct studies, hearts were excised, Langendorff perfused and infarct size evaluated as again described in chapter two.

6.2.1 Determination of myocardial cGMP concentration

The protocol for hearts perfused for cGMP analysis following ischaemia and BNP perfusion is shown in figure 6.1.

6.2.1.1 *Ventricular cGMP following ischaemia*

In the first set of experiments (figure 6.1A), hearts were randomised to one of the following four experimental groups

1. Group 1; hearts were initially stabilised for 10 min and continued to be perfused with Krebs-Henseleit buffer for a further 5 without ischaemia.
2. Group 2; hearts were stabilised for 10 min and then subjected to 2 min global ischaemia.
3. Group 3; hearts in this group were stabilised for 10 min as above, then subjected to 5 min global ischaemia.
4. Group 4; hearts in this group were stabilised for 10 min and then 20 min of global ischaemia.

6.2.1.2 Ventricular cGMP following BNP

In the second set of experiments (figure 6.1B), hearts were randomised to one of the following two experimental groups

5. Group 5; control hearts in this group were stabilised for 10 min and continued to be perfused for further 10 min with Krebs-Henseleit buffer without BNP.

6. Group 6; hearts were stabilised as above for 10 min, then perfused with either concentration of BNP 10^{-12} , 10^{-11} , 10^{-9} or 10^{-8} mol/L for the next 10 min. (BNP perfused hearts).

At the end of each experiment, hearts were excised below the atria and immediately freeze-clamped with a Wallenberger clamp pre-chilled in liquid nitrogen and stored at -81°C . Frozen ventricular tissue, was then deproteinised by ten volumes of 6% trichloroacetic acid. The homogenate was centrifuged (10,000 rpm for 10 min) at 4°C , and the supernatant was then extracted seven times in water-saturated diethylether. The remaining water phase was evaporated in nitrogen flow and these samples were then packed on dry ice and transported to The Department of Biochemistry, University of Szeged, Hungary for further assay of cyclic GMP, as described in chapter two.

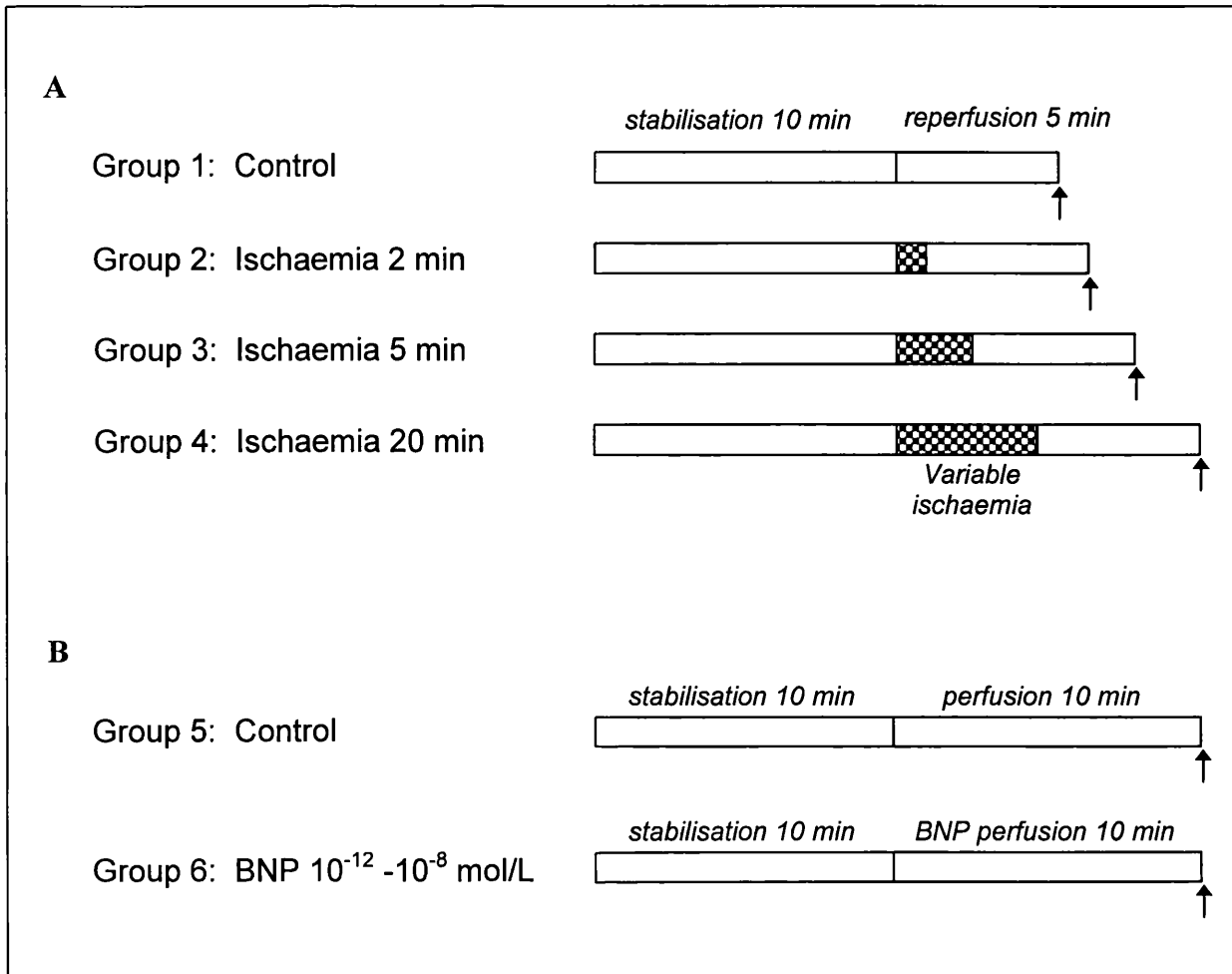


Figure 6. 1 Experimental protocol for heart-tissue sampling for cGMP

In A, hearts in all four groups were initially stabilised for 10 min. In group 1, control hearts were perfused without ischaemia. In groups 2-4, hearts were rendered globally ischaemic for 2, 5 and 20 min respectively. All hearts were then reperfused for 5 min.

In B, control hearts in group 5 were stabilised for 10 min and continued to be perfused for further 10 min with Krebs-Henseleit buffer without BNP. In group 6, hearts were stabilised for 10 min, then perfused with either concentration of BNP 10^{-12} , 10^{-11} , 10^{-9} or 10^{-8} mol/L for the next 10 min.

At the end of all the experiments (arrowed), ventricles were immediately freeze clamped and stored at -80°C for cGMP analysis.

6.2.2 Examination of infarct-limitation by cGMP

8-Br-cGMP was used in a concentration-dependent fashion in this study and the experimental protocol is illustrated in figure 6.2 A. Concentrations of 8-Br-cGMP $5 \times 10^{-5} - 10^{-8}$ mol/L were prepared using deionised water as a solvent. Hearts were randomly assigned to one of the following six experimental groups

1. Control; in this group, hearts were stabilised for 15-20 min before being subjected to 35 min of regional ischaemia, followed by 120 min reperfusion
2. 8-Br-cGMP 5×10^{-5} mol/L was added to Krebs-Henseleit buffer and commenced 10 min into stabilisation and continued for a further 10 min prior to ischaemia and then continued until 30 min reperfusion.
3. 8-Br-cGMP 10^{-5} mol/L was added to Krebs-Henseleit buffer and perfusion started 10 min prior to ischaemia and continued until 30 min reperfusion.
4. 8-Br-cGMP 10^{-6} mol/L treatment as described above
5. 8-Br-cGMP 10^{-7} mol/L treatment as described above
6. 8-Br-cGMP 10^{-8} mol/L treatment as described above

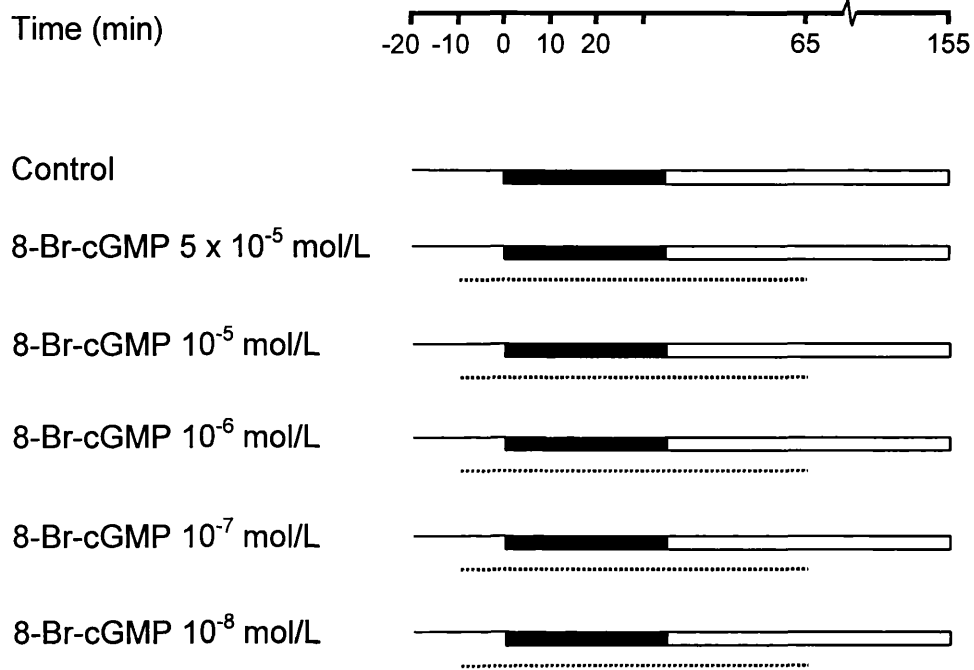


Figure 6. 2 Infarction protocol

Protocol used in the 8-Br-cGMP concentration-response study is shown. Dotted lines represent the time course of perfusion with different 8-Br-cGMP concentrations as indicated.

6.2.3 Statistical analysis

Data are expressed as means \pm s.e.m. cGMP levels, infarct-to-risk ratios and risk zone volumes were analysed using one-way ANOVA and Fisher's protected least significant difference post-hoc test. For cGMP assay if a difference was established, each group was compared to the solvent-treated group using a modified t-test corrected for simultaneous multiple comparisons according to Bonferroni method. Left ventricular function parameters and coronary flow rate were evaluated using repeated-measures ANOVA. Statistical significance between group means was defined as $p < 0.05$.

6.3 Results

6.3.1 Technical exclusions

A total of 106 animals were used in this study. Three hearts were excluded; one was damaged by instrumentation which prevented precise LV function assessment, one had failure of tetrazolium stain and one had persistent bradyarrhythmias throughout reperfusion. Therefore, data for 103 successfully completed experiments is reported.

6.3.2 Effect of ischaemia on myocardial cGMP concentration

A low basal concentration of cGMP with a mean value of 2.99 ± 0.51 pmol g⁻¹ wet weight (n = 3) was observed in the ventricular myocardium of control hearts not subjected to ischaemia. This concentration increased progressively in a graded fashion related to the duration of preceding ischaemia: 2 min ischaemia 7.43 ± 2.11 pmol g⁻¹ wet weight; 5 min ischaemia 7.68 ± 2.61 pmol g⁻¹ wet weight (both n = 4 and P < 0.05 v control values) and 20 min ischaemia 10.58 ± 1.71 pmol g⁻¹ wet weight (n = 4, P < 0.01 v control). The percentage increase of released cGMP following ischaemia, relative to the basal cGMP value in the control hearts is represented in figure 6.3.

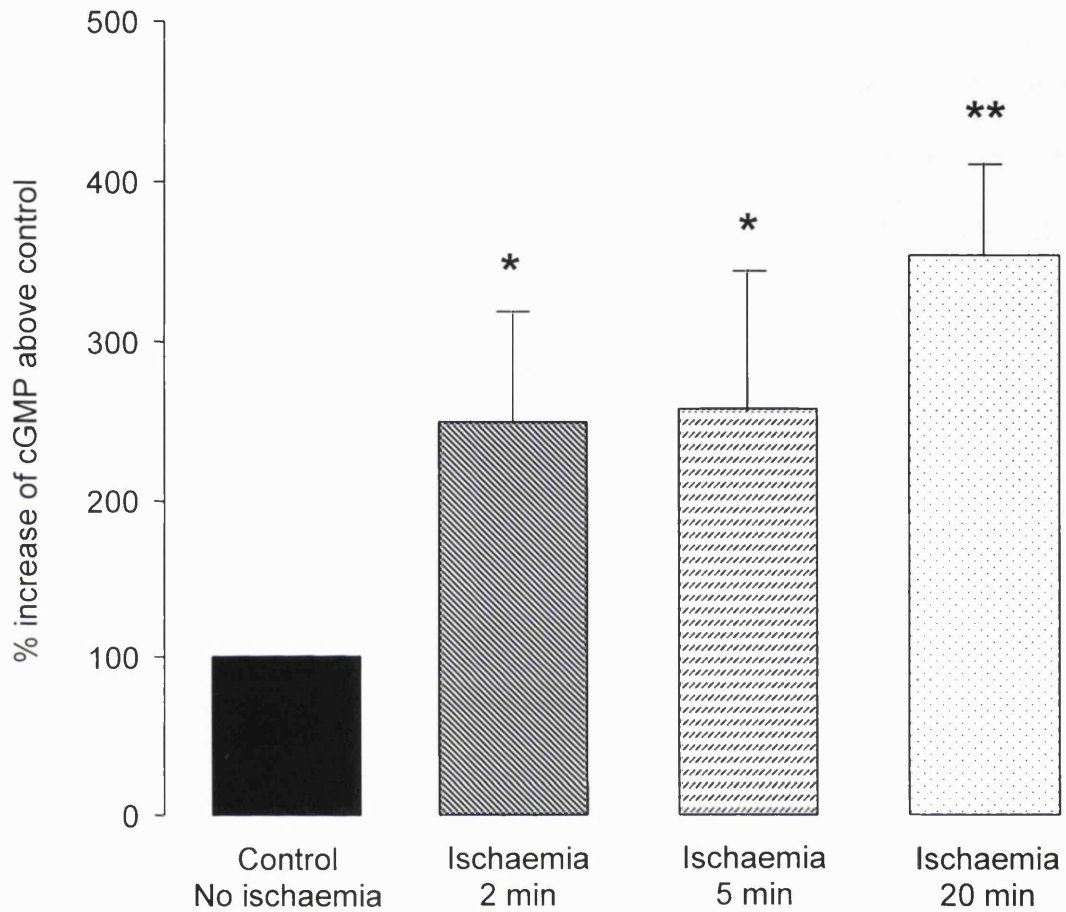


Figure 6.3 Myocardial cGMP concentration in ischaemia

Percentage (%) increase of ventricular cGMP above control values, following graded durations of global ischaemia in the isolated rat heart. Bars indicate mean \pm SE; $n = 3-4$ experiments per group, * $P < 0.05$ v control and ** $P < 0.01$ v control values (one-way ANOVA)

6.3.3 Effect of BNP on myocardial cGMP concentration

The basal level of cGMP concentration in the ventricular myocardial tissue was in the range of 9.2 – 13.1 pmol g⁻¹ wet weight in the control hearts (n = 9) perfused with Krebs-Henseleit buffer without BNP. Perfusion with BNP for 10 min caused a concentration-dependent increase of cGMP concentration in the ventricular myocardium. Significant increases in tissue cGMP concentration were observed following perfusion with BNP 10⁻⁸ mol/L (cGMP 28.6 ± 1.5 pmol g⁻¹ wet weight, n = 8, P < 0.01 v control values) and BNP 10⁻⁹ mol/L (cGMP 20.3 ± 5.2 pmol g⁻¹ wet weight, n = 5, P < 0.05 v control). Lower concentrations of BNP did not cause statistically significant elevation of cGMP concentration. The percentage increase of released cGMP following BNP perfusion, relative to the basal cGMP value in the control hearts is in illustrated in figure 6.4.

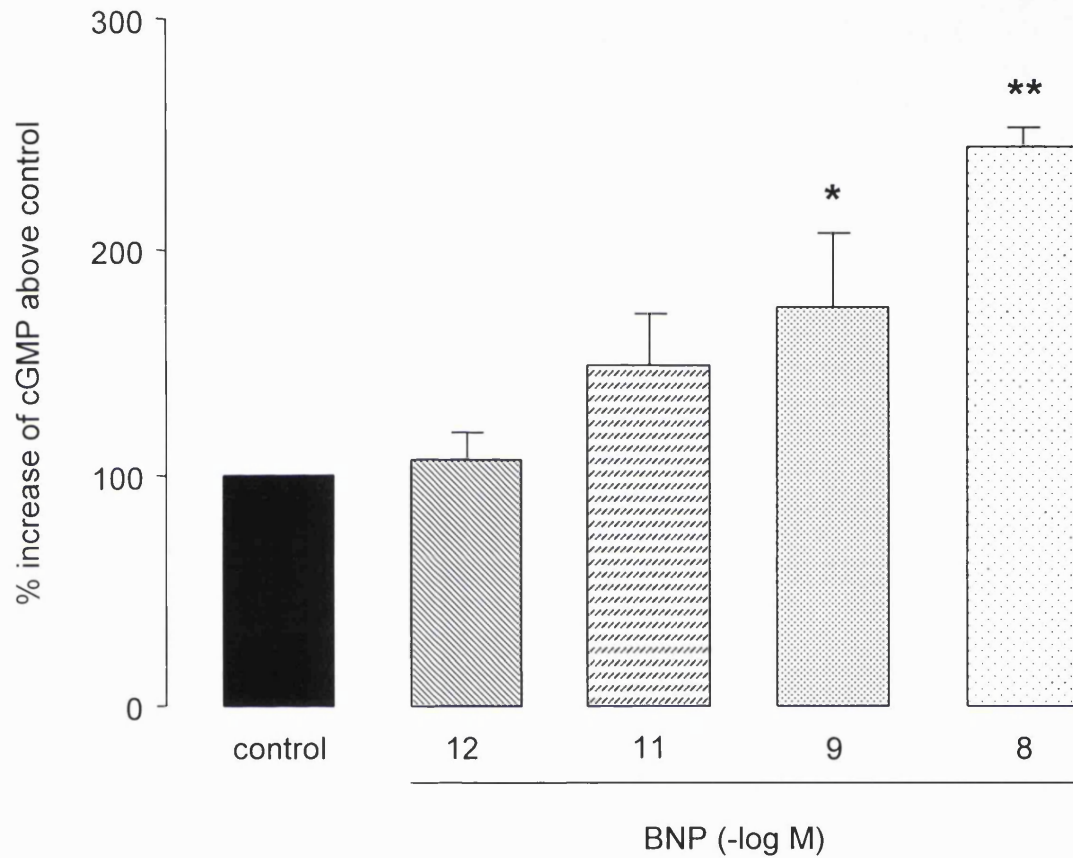


Figure 6. 4 BNP activation of cGMP

Percentage (%) increase of cGMP from ventricular heart tissue above control values, following a 10 min perfusion of the rat heart with BNP 10^{-12} – 10^{-8} mol/L concentrations. Bars indicate mean \pm SE, n = 5-9 experiments per group, *P < 0.05 v control and **P < 0.01 v control values (one-way ANOVA)

6.3.4 8-Br-cGMP infarct size and risk zone

The risk zone volumes, body weights and heart weights were similar among all the groups and are shown in table 6.1 and infarct sizes represented in figure 6.5. Control infarct-to-risk zone ratio was $38.7 \pm 3.6\%$ without 8-Br-cGMP treatment. Treatment with 8-Br-cGMP concentrations of 10^{-7} and 10^{-8} mol/L resulted in significant limitation of infarct size ($23.1 \pm 6.3\%$ and $20.8 \pm 4.1\%$ respectively, both $P < 0.05$ v control). However, perfusion with 8-Br-cGMP in the concentrations of 5×10^{-5} , 10^{-5} and 10^{-6} mol/L were not found to be protective (infarct sizes – 37.4 ± 7.9 , 32.0 ± 5.6 and 31.6 ± 6.3 respectively).

Group	n	Body weight (g)	Wet heart weight (g)	Risk zone volume (mm ³)	Infarct size (%)
Control	13	372±9	1.6±0.05	320±25	38.7±3.6
Br cGMP 10 ⁻⁸ mol/L	7	331±3	1.5±0.03	329±27	20.8±4.1*
Br cGMP 10 ⁻⁷ mol/L	7	356±8	1.5±0.04	371±34	23.1±6.3*
Br cGMP 10 ⁻⁶ mol/L	9	382±8	1.7±0.05	314±25	31.6±6.3
Br cGMP 10 ⁻⁵ mol/L	10	414±5*	1.9±0.04*	402±14*	32.0±5.6
Br cGMP 5 x 10 ⁻⁵ mol/L	6	338±3*	1.6±0.07	323±44	37.4±7.9

Table 6. 1 Summary of body weight, wet heart weights, risk zone volume and infarct size

Figures are mean ± s.e.m. Infarct size is the ratio of infarct area to the risk zone area, n = number of experiments per group, *P < 0.05 v control values.

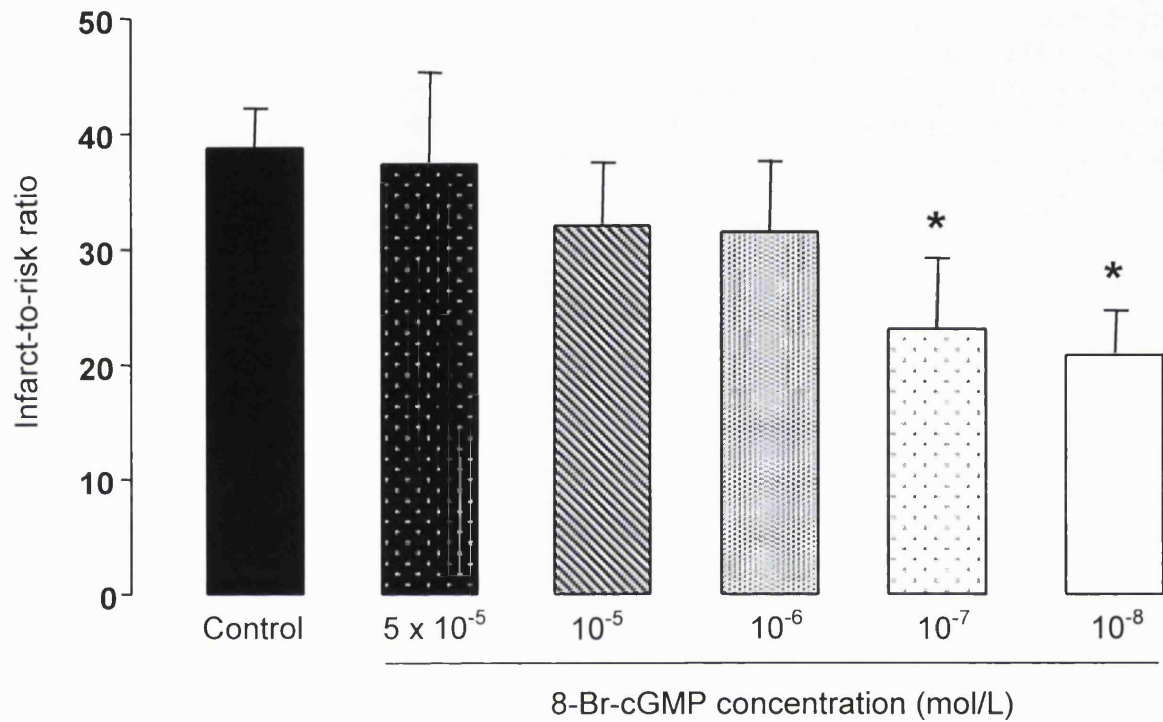


Figure 6. 5 Infarct-to-risk ratio in the 8-Br-cGMP concentration response study

Infarct-to-risk zone ratios for hearts perfused with 8-Br-cGMP 10^{-8} - 5×10^{-5} mol/L concentrations. Bars represent mean \pm SE, n = 6 - 13 experiments in each group, *P < 0.05 v control (one-way ANOVA).

6.3.5 Coronary flow and LV function

Pre-ischaemic global coronary flow averaged 15 ml/min among the six experimental groups in the 8-Br-cGMP concentration response study. After coronary occlusion, there was a decrease in global coronary flow rate of approximately 35% and a recovery to pre-ischaemic values immediately after reperfusion with a gradual “run-down” during the remaining 120 min perfusion. Global coronary flow rate measured at intervals throughout the protocol did not differ substantially among the experimental groups.

There were no detectable differences between the groups in any of the parameters of LV function measured (spontaneous heart rate, developed pressure or the double product). Developed pressure and double product declined immediately after the onset of coronary occlusion to the same extent in all the experimental groups. A tabular representation of the coronary flow and the double product amongst the experimental groups is presented in tables 6.2 and 6.3.

Group	Basal	Pre-ischaemia	<u>Ischaemia</u>		<u>Reperfusion</u>		
			5 min	30 min	5 min	30 min	120 min
Control	16.0±0.9	16.9±1.0	9.8±0.6	10.7±0.6	16.2±0.9	14.8±0.7	11.0±0.6
Br cGMP 10 ⁻⁸ mol/L	17.6±1.3	12.9±1.3*	6.8±0.5*	7.1±0.5**	14.8±0.7	14.1±1.2	11.5±1.0
Br cGMP 10 ⁻⁷ mol/L	17.0±1.2	12.3±0.8*	7.8±0.9	8.6±0.4	13.5±0.9	13.1±0.7	10.8±1.0
Br cGMP 10 ⁻⁶ mol/L	19.6±1.2*	15.2±1.3	8.8±1.2	12.2±1.1	16.2±1.4	15.8±1.0	12.0±1.7
Br cGMP 10 ⁻⁵ mol/L	20.0±1.2**	17.2±1.9	10.1±0.8	11.3±1.1	16.7±1.3	15.7±1.2	11.7±0.6
Br cGMP 5 x 10 ⁻⁵ mol/L	14.8±1.0	12.3±0.8	10.5±1.7	12.4±1.2	18.9±1.9	16.8±1.6	12.3±1.0

Table 6.2 Summary of coronary flow (ml/min)

Figures are mean ± s.e.m. *P < 0.05 v control values; **P < 0.01 v control values (repeated-measures ANOVA)

Group	Basal	Pre-ischaemia	<u>Ischaemia</u>		<u>Reperfusion</u>	
			5 min	30 min	30 min	120 min
Control	23.3±2.9	24.5±2.5	8.9±0.8	16.9±2.4	22.6±2.5	15.6±2.1
Br cGMP 10 ⁻⁸ mol/L	27.3±4.6	20.6±3.2*	5.1±0.8*	11.2±2.0	23.2±3.1	15.2±2.7
Br cGMP 10 ⁻⁷ mol/L	33.5±2.6*	25.3±4.3*	6.6±1.2	13.3±1.6	24.6±2.3	18.8±1.1
Br cGMP 10 ⁻⁶ mol/L	23.7±3.2	20.3±3.3	6.1±1.6	14.8±2.4	17.8±3.3	13.1±2.3
Br cGMP 10 ⁻⁵ mol/L	31.8±2.0*	29.1±3.3	7.8±1.2	16.1±1.3	26.8±3.5	19.6±2.9
Br cGMP 5 x 10 ⁻⁵ mol/L	26.9±3.2	25.3±4.3	10.6±1.2	18.7±2.4	27.0±1.8	16.0±1.6

Table 6.3 Summary of rate pressure product (mm Hg min⁻¹ x 10³)

Figures are mean ± s.e.m. *P < 0.05 v control values (repeated-measures ANOVA)

6.4 Discussion

This present study provides three principal findings. First, cGMP level in the ventricular myocardium is elevated and directly related to the severity of the duration of preceding cardiac ischaemia. Second, the mechanism of protection afforded by BNP is associated with the elevation of cGMP in a concentration-dependent manner. Third, exogenous administration of a synthetic analogue of cGMP is protective against myocardial ischaemia-reperfusion injury at low concentrations of 10^{-7} and 10^{-8} mol/L concentrations, while higher concentrations were non-protective.

The biological actions of BNP are mediated by binding to its particulate guanylyl cyclase cell-surface receptor NPR-A, abundantly expressed in cardiac tissue (Boomsma et al, 2001; Silberbach et al 2001). The catalytic domain of this receptor is responsible for the conversion of GTP to cGMP to mediate the action of the natriuretic peptide (Padilla et al, 2001; Kambayashi et al, 1990a; Lucas et al, 2000; Chinkers et al, 1991). While as the study shows, ischaemia itself can trigger the elevation of tissue concentration of cGMP, a significant increase in ventricular cGMP concentration occurs following a 10 min perfusion with BNP 10^{-9} or 10^{-8} mol/L, probably consistent with NPR-A activation. However, in the absence of studies with NPR-A antagonists, we cannot conclude definitively that the BNP-induced elevation of cGMP is receptor-mediated. cGMP elevation has been proposed to be a mechanism of injury limitation in the ischaemic myocardium (Opie, 1982), but the distal molecular mechanisms resulting in enhanced tolerance to ischaemic injury associated with cGMP elevation are unclear. Proposed mechanisms include inhibition of L-type calcium channel opening (Lohmann et al, 1991), decreased intracellular concentrations of cAMP through a feedback mechanism and stimulation of cAMP phosphodiesterase (Lohmann

et al, 1991; Hove-Masden et al, 1996; Mery PF, et al, 1993), inhibition of the MPTP - mitochondrial permeability transition pore (Takuma et al, 2001) and opening of the K_{ATP} channels (Kubo et al, 1994). Further, our study implies that although $[cGMP]_i$ may be triggered by either ischaemia or BNP, consolidating the link in signal transduction of BNP's cardioprotection, cGMP itself is only partially responsible for the direct limitation of myocardial ischaemia-reperfusion injury. The unexpected finding that higher concentrations of 8-Br-cGMP (similar to those frequently applied in isolated cell and tissue pharmacology) were not protective is intriguing and unexplained at present. Previous work has reported that high concentrations of cGMP are associated with cell injury. For example, cytotoxic effects of a NO donor in a pheochromocytoma line were augmented by a cell-permeable cGMP analogue (Nakamura et al, 1997). Tepperman et al, extended these observations to rat intestinal epithelial cells, showing that dibutyl cGMP at millimolar concentrations reduced cell viability in culture (Tepperman et al, 1998). The cytotoxic effect of high concentrations of $[cGMP]_i$ may be related to generation of reactive oxygen species, since superoxide dismutase attenuated the injurious effects of the cGMP analogue in intestinal epithelial cells. Further, 8-Br-cGMP, the cGMP analogue used in this study although a lipophilic membrane-permeant, relatively resistant to hydrolysis by phosphodiesterases and potent activators of cGK (or PKG), can at high concentrations also activate cAMP-dependent protein kinase cAK (or PKA), known to counteract cGMP (see review by Smolenski et al, 1998)

While not constituting proof of mechanism, the association between concentration-dependent elevation of cGMP by BNP and infarct limitation observed, leads us to hypothesise a role for cGK. cGKs are composed of N-terminal, regulatory and catalytic domains. Two different isoenzymes of cGK have been isolated in mammals, cGK-I and cGK-II. cGK-I is soluble located on chromosome 10 and has two isoforms

cGK-I α and cGK-I β . cGK-I α is abundant in cardiac myocytes and the vascular system and is responsible for dampening myocardial contractility, inhibiting cAMP-stimulated Ca²⁺ influx across the L-type Ca²⁺ channel, reduces the conductance of the major cardiac gap junction and inhibits platelet activation (Lucas et al, 2000; Smolenski et al, 1998; Lohmann et al, 1997). cGK-I β is detected only in the uterus. The membrane-bound cGK-II, located on chromosome 4, is present mainly in the intestine, kidney and brain, but is absent in the cardiovascular system. It phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR) to secrete Cl⁻ and via natriuresis and diuresis regulates fluid homeostasis at the cell membrane (Lucas et al, 2000; Smolenski et al, 1998; Lohmann et al, 1997; Vaandrager et al, 1996). Quantitative analysis of cGK activation in intact cells has been very low due to a relatively low expression in most cell types and due to the biochemical and kinetic properties. Further, specific inhibitors and antagonists which are invaluable tools for evaluating pharmacological functions of cGKs do not exist. Two major groups available – the H-series (KT5823, H8, H89) interfere at the level of the ATP binding site of the catalytic domain; and the Rp-stereoisomers of cGMP phosphorothioates prevent activation of cGK at the cGMP binding site of the regulatory domain (Smolenski et al, 1998). Approaches using cultured myocytes may be feasible but may not accurately model endogenous protective mechanisms in intact tissue where there is interaction with several cell-types and humoral mediators. Injection/transfection of cGKs and analysis of cGK-deficient systems, together with molecular approaches may be increasingly used in the future to evaluate contributions of cGKs to the physiological and pharmacological effects of cGMP-elevating agents in the intact organism. Thus, the cGMP/cGK-I pathway could link NPR-A receptor activation and the cytoprotective K_{ATP} channel implicated in BNP's cardioprotection in our recent study.

Vasodilatory properties of BNP appear to be largely blocked by L-NAME, suggesting the contributory role of endothelium-derived relaxing factor – NO (Zellner et al, 1999). In common, the second messenger for BNP and NO is the cGMP: BNP acts on the extracellular domain of the membrane-bound NPR-A receptor to activate its intracellular guanylyl cyclase domain, resulting in the catalysis of cGMP from GTP, while NO appears to activate the intracellular cytosolic form of guanylyl cyclase (sGC) to invoke the second messenger (Murad, 1999). The precise mechanism by which BNP could lead to vascular NO release is unknown. At this stage of the study, we speculate that BNP, may act on its NPR-A receptor on the endothelium, triggering the release of endothelial cGMP which via cGK, stimulates NO production which then cross-talks with the cardiomyocytes via the sGC to invoke the cardiocyte cGMP and activate a NO-cGMP signal transduction pathway (see figure 1.5).

In conclusion, this study implicates the release of cGMP in cardiac ischaemia as well as following exogenous BNP infusion, with a possible protective role for cGMP in BNP's cytoprotection.

Chapter Seven

Nitric oxide in BNP cardioprotection

7.1 Introduction

The BNP study thus far has shown the release of endogenous BNP and its second messenger cGMP to be positively expressed by the myocardial ventricular tissue in response to cardiac ischaemia. Further, we have shown that exogenously administered BNP, protects the isolated perfused rat heart from ischaemia-reperfusion injury via opening of the mitochondrial K_{ATP} channels and that this protection is partially mediated via BNP's second messenger cGMP, however the intracellular transduction mechanism is not known. As discussed in chapter one, most of BNP's traditionally known biological actions are presumed to be mediated via its transmembrane NPR-A receptor (or pGC), which invokes cGMP via phosphorylation of ATP and catalytic conversion of GTP.

BNP is known to influence vascular tone. In the swine model, Zellner et al, studied the vasodilatory response to BNP under physiological conditions and after pre-constriction with endothelin-1 (Zellner et al, 1999). They showed that BNP, exerted coronary vasodilatory effects, predominantly in epicardial conductance vessels, further accentuated in the pre-constricted vessels. Pretreatment with the nitric oxide (NO) inhibitor N^{ω} -nitro-L-arginine methyl ester (L-NAME), attenuated the coronary vasodilatory effect of BNP in the resistance arteries without influencing epicardial artery tone. Hence, NO has been implicated in the vasodilatory action of BNP. Further, in experiments conducted in humans, exogenous BNP infusion induced a dose-dependent dilatation of the brachial artery, measuring the forearm blood flow by venous occlusion plethysmography (van der Zander et al, 2002). There was no change

in systemic haemodynamics, and vasodilation appeared to be significantly blocked by co-perfusion with both NO and K^+Ca^{2+} inhibitors (l-NG-monomethyl arginine and tetraethylammonium respectively), again implicating a role for NO in BNP's vasodilatory action. Brunner et al, have performed studies in the rat and mouse models, to demonstrate that natriuretic peptides relax coronary and peripheral resistance vessels partly through activation of the NO/cGMP pathway implicating a link between soluble and particulate GC in resistance vessel function (Brunner et al, 2001). Their studies, along with others' (Moritoki et al, 1992), also indicated that natriuretic peptides have a potent endothelium- dependent coronary vasorelaxation and that nonendothelial derived nitric oxide was unlikely to contribute to the relaxation response.

NO is an endogenously formed radical acting as a signalling messenger. It is formed from NO synthases (NOSs), which convert L-arginine to citrulline and NO, a process requiring O_2 and NADPH derived electrons. NOSs have a widespread distribution in the body serving various physiological functions of blood pressure regulation, local vasomotion and sexual function, neuro-and immuno-protective roles (see review by Gewaltig et al, 2002). In the myocardium, NO is synthesised by one of the major three isoforms of NOS viz. NOS-I or neuronal NOS (nNOS), NOS-II or inducible NOS (iNOS) and NOS-III or endothelial NOS (eNOS) of approximate molecular weights of 130, 135 and 160 KD respectively. The constitutively expressed isoforms, eNOS and nNOS, require Ca^{2+} for calmodulin binding and enzymatic activity. The iNOS calmodulin binding domain has such a high affinity for calmodulin, that Ca^{2+} is not required for catalytic activity. Therefore, regulation of iNOS activity appears primarily dependent upon transcriptional and translational modulation (Abu-Soud et al, 1994). Many agonists like bradykinin, angiotensin II and endothelin-1 which trigger ischaemic preconditioning possess a binding domain for eNOS (Jin et al, 1998; Diaz et

al, 1997; Bugge et al, 1996; Marrero et al, 1999). In endothelial cells this enzyme is bound to cell membrane associated caveolae. NO activates the enzyme soluble guanylyl cyclase (sGC) to produce the second messenger cGMP. Activation of this enzyme is dependent on binding of NO to the heme moiety of sGC to form the nitrosyl-heme adduct of sGC. As a consequence, the heme-iron is shifted out of the plane of the porphyrine ring configuration which initiates the binding of GTP and formation of cGMP (overview by Ignarro et al, 1999). NO synthesised by eNOS promotes the relaxation of vascular smooth muscle cells and its activity is linked to phosphorylation to three or more sites in the enzyme: serine 1179 (ser 1177 in human eNOS sequence, Gallis et al, 1999; Fulton et al, 1999; Dimmeler et al, 1999); threonine 497 (threonine 495 in the human sequence, Harris et al, 2001; Michell et al, 2001, Fleming et al, 2001) and serine 116 (Gallis et al, 1999). Of these sites, regulation of eNOS serine 1179 has been most extensively characterised; protein kinase Akt, the AMP-activated protein kinase and the cAMP-dependent protein kinase among others have been shown to modify eNOS at this residue, potentiating eNOS enzyme activity. The phosphorylation of threonine 497 appears to be modulated by the agonist bradykinin (Harris et al, 2001; Fleming et al, 2001), though the intracellular pathways involved in this response remain less well understood. Phosphorylation of eNOS at serine 116 has been observed in intact endothelial cells subjected to haemodynamic shear stress (Gallis et al, 1999), but the resulting consequence is not known.

In view of the evidence that the vasodilator actions of BNP are at least partially dependent on NO generation, in this two-part study we hypothesise a role for NO in BNP mediated cardioprotection and that this cytoprotection is dependent on a NO/sGC transduction system. To study this effect, L-NAME, the water soluble non-selective NOS-inhibitor (Rees et al, 1990; Crabos et al, 1997) and 1H-[1,2,4}oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) a selective sGC inhibitor (Garthwaite et al, 1995) were

used in these experiments. In the second arm to the study, expression of eNOS and phosphorylation of eNOS (phospho-eNOS) at serine 1177 were studied in ischaemic and BNP perfused hearts.

7.2 Materials and methods

Rat hearts were isolated, Langendorff perfused and infarct size evaluated as described in chapter two. Western blot assay was performed as per the technique also described in chapter two.

7.2.1 Infarction protocol for NO/sGC blockade study

The experimental protocol is illustrated in figure 7.1. A concentration of BNP at 10^{-8} mol/L was selected based on preceding experiments described in chapter four. Hearts were randomly assigned to one of the following six groups.

1. Control; in this group, hearts were stabilised for 15-20 min before being subjected to 35 min of regional ischaemia, followed by 120 min reperfusion. Three control hearts were perfused with DMSO 0.016% (the vehicle solvent for ODQ). Since there was no effect on infarct size of DMSO, these hearts were combined for statistical evaluation with non-DMSO treated control hearts to comprise group 1.
2. BNP; in this group BNP 10^{-8} mol/L was added to Krebs-Henseleit buffer and commenced 10 min into stabilisation and continued for a further 10 min prior to ischaemia and then continued until 30 min reperfusion.

3. L-NAME; here, L-NAME 10^{-4} mol/L was added to Krebs-Henseleit buffer and perfusion started 10 min prior to ischaemia and continued until 30 min reperfusion.
4. L-NAME / BNP; BNP 10^{-8} mol/L treatment was co-perfused with L-NAME 10^{-4} mol/L as above, 10 min prior to ischaemia and continued for 30 min into reperfusion.
5. ODQ 10^{-5} mol/L treatment as described above. ODQ was dissolved in dimethylsulfoxide (DMSO - final concentration not more than 0.016%).
6. ODQ/BNP; BNP 10^{-8} mol/L treatment was co-perfused with ODQ 10^{-5} mol/L as above, 10 min prior to ischaemia and continued until 30 min into reperfusion.

With the exception of ODQ, agents were dissolved in aqueous buffer immediately before use.

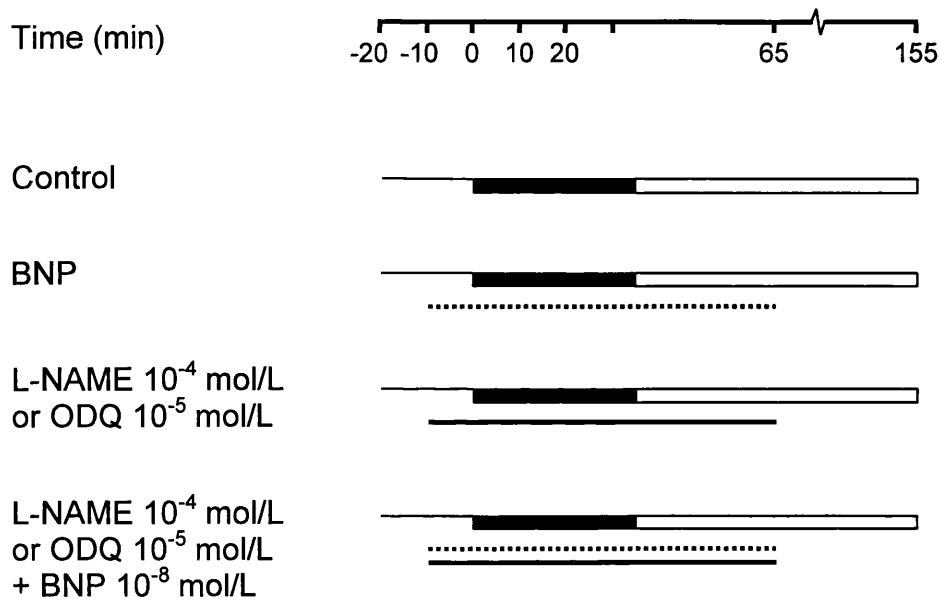


Figure 7.1 Experimental protocol for infarct studies

Protocol used in the nitric oxide/soluble guanylyl cyclase blockade study is shown. Dotted lines represent the time course of perfusion with BNP. Solid lines represent L-NAME or ODQ perfusion.

7.2.2 Western Blot assay for eNOS and phospho-eNOS

The experimental protocol used in the Western Blot assay is shown in figure 7.2. In the first set of experiments (figure 7.2 A), hearts were not subjected to ischaemia, while in the second set of experiments (figure 7.2 B), an ischaemic stimulus was used. Hearts were randomly assigned to one of the following six groups.

1. Group 1; in this control group, hearts received no treatment and were perfused with Krebs-Henseleit buffer for 25 min.
2. Group 2; hearts in this group, were stabilised for 15 min before perfusion with either of BNP 10^{-8} , 10^{-10} or 10^{-12} mol/L for 10 min. Concentrations of BNP were similar to those used in the previous studies.
3. Group 3; this group was used as sham controls, where hearts were not perfused, but excised and immediately blot-dried to remove blood.
4. Group 4; control hearts were stabilised as group 1, above without treatment or ischaemia.
5. Group 5; hearts were stabilised for 15 min, and then subjected to 15 min global ischaemia.
6. Group 6; following 15 min of stabilisation, hearts were perfused with BNP 10^{-8} mol/L for 10 min and then 15min of global ischaemia.

At the end of each experiment, hearts were immediately excised below the atria and freeze-clamped with liquid nitrogen and stored at -81°C for Western Blot assay to probe for eNOS and phospho-eNOS.

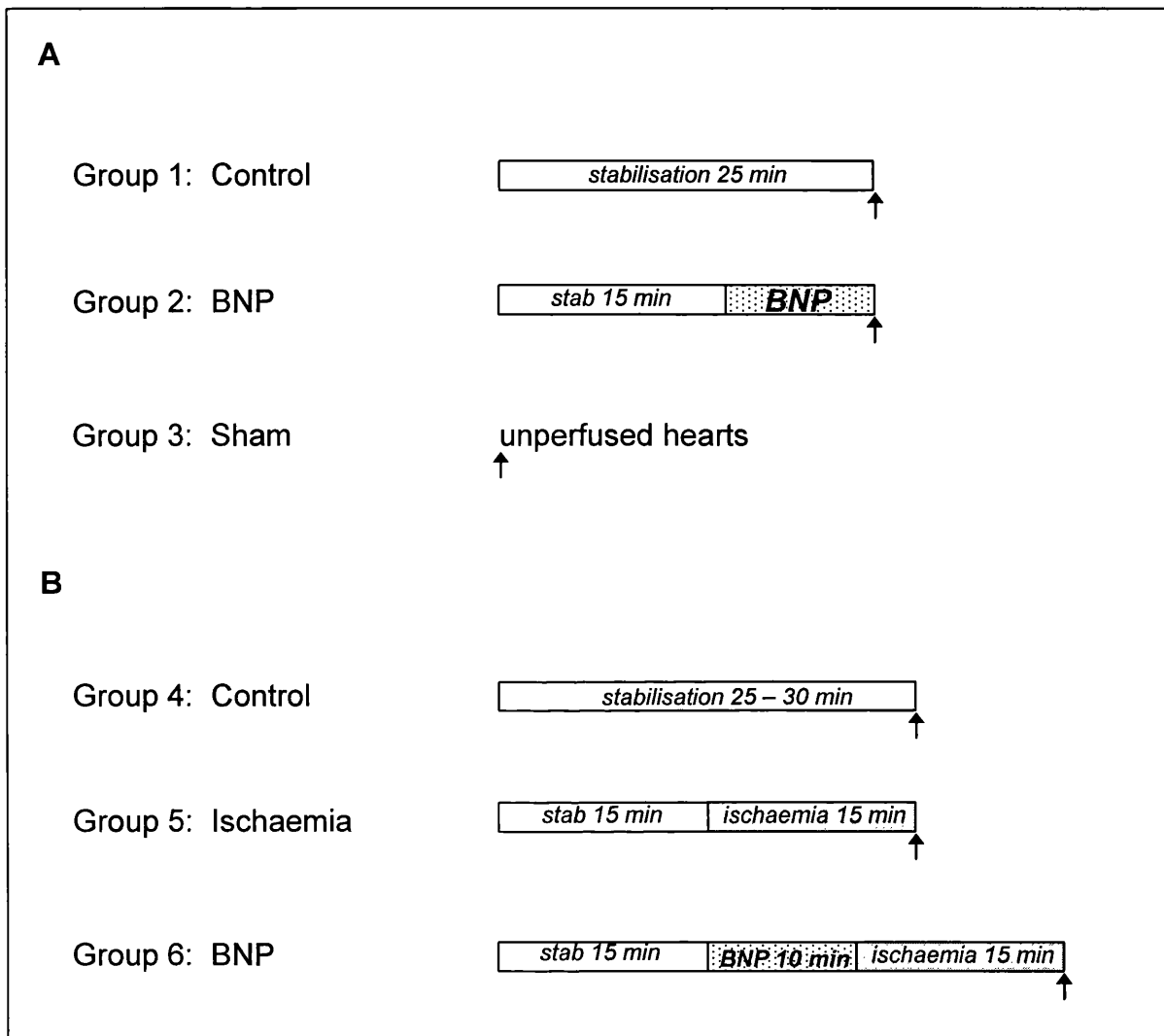


Figure 7.2 Protocol for tissue harvested for Western Blot studies

Experimental protocol for myocardial ventricular tissue sampling - In set A, hearts were not subjected to ischaemia, and BNP treated hearts received either BNP 10^{-8} , 10^{-10} or 10^{-12} mol/L concentration, sham control hearts were dry-blotted and not perfused. In set B, hearts were subjected to a 15 min global ischaemia; the BNP group hearts were perfused with BNP 10^{-8} mol/L concentration prior to ischaemia.

At the end of each experiment (indicated by arrow) hearts were immediately freeze-clamped and stored at -81°C for Western blot assay

7.2.3 Statistical analysis

Infarct-to-risk ratios and risk zone volumes and densitometry analysis of protein bands were determined as described in chapter two.

7.3 Results

7.3.1 Technical exclusions

A total of 47 animals were used in the NO/sGC blockade study. One heart was excluded due to persistent bradyarrhythmias in the stabilisation phase. Therefore, data for 46 successfully completed experiments is reported. In the Western blot assay, 27 animals were used.

7.3.2 NO/sGC blockade study

7.3.2.1 *Infarct size and risk zone*

The risk zone volumes, body weights and heart weights were similar among all the groups and are shown in table 7.1 and infarct sizes represented in figure 7.3. Control infarct-to-risk zone ratio was $45.0 \pm 5.5\%$ without BNP treatment. Treatment with BNP 10^{-8} mol/L resulted in significant limitation of infarct size ($22.8 \pm 3.1\%$, $n = 10$, $P < 0.01$ v control). Co-perfusion of BNP with either L-NAME or ODQ resulted in abolition of the protective effect of BNP (infarct-to-risk ratio $38.0 \pm 3.7\%$ and $41.1 \pm$

5.3% respectively, n = 6-10, P not significant v control). Neither L-NAME nor ODQ per se influenced infarct size.

Group	n	Body weight (g)	Wet heart weight (g)	Risk zone volume (mm ³)	Infarct size (%)
Control	8	361±8	1.6±0.05	388±43	45.0±5.5
BNP 10 ⁻⁸ mol/L	10	358±10	1.6±0.05	362±9	22.8±3.0**
L-NAME 10 ⁻⁴ mol/L	6	367±7	1.7±0.04	397±37	40.7±5.6
BNP/L-NAME	10	328±4**	1.4±0.05	377±15	38.0±3.6
ODQ 10 ⁻⁵ mol/L	6	380±4	1.6±0.07	435±33	44.1±2.7
BNP/ODQ	6	357±8	1.6±0.05	378±34	41.1±5.3

Table 7.1 Summary of body weight, wet heart weight, risk zone volume and infarct size

Figures are mean ± s.e.m. Infarct size is the ratio of infarct area to the risk zone area, n = number of experiments per group, **P < 0.01 v control values.

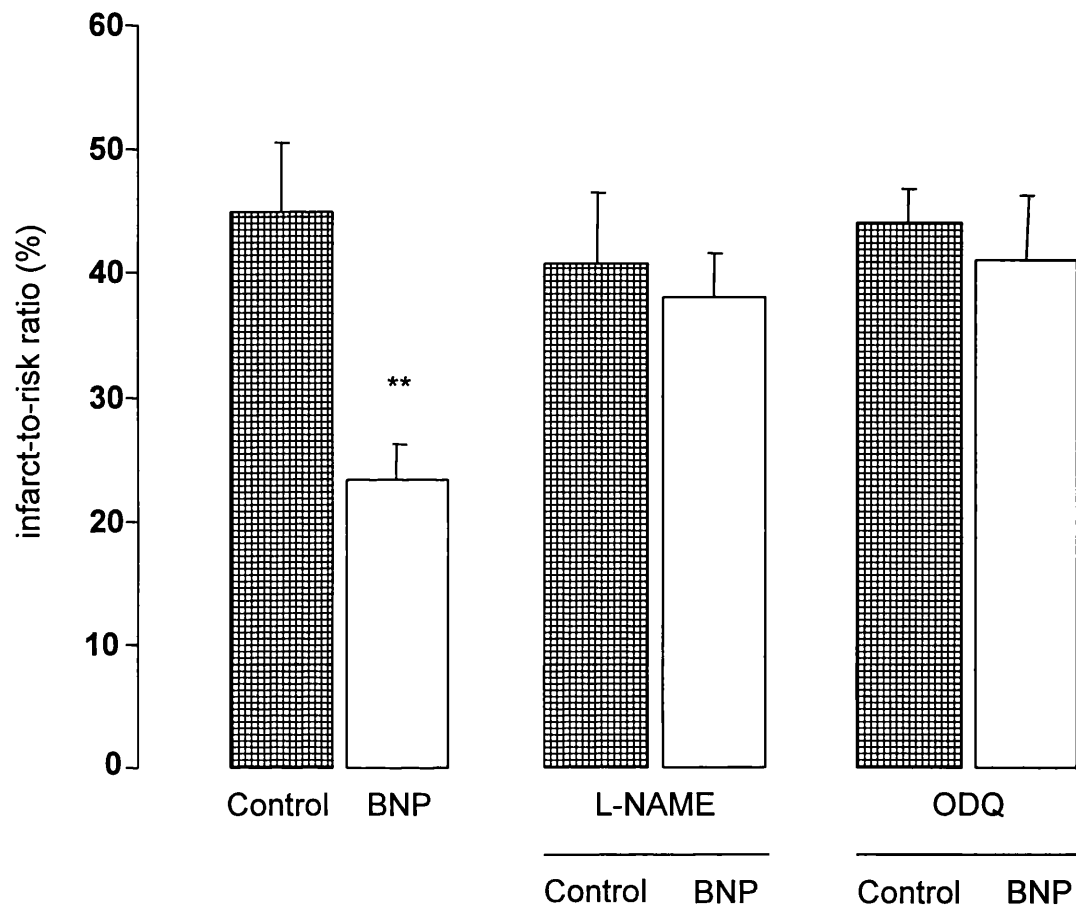


Figure 7.3 Infarct-to-risk ratio

Infarct-to-risk zone ratios for hearts perfused with L-NAME 10^{-4} mol/L and ODQ 10^{-5} mol/L with and without BNP 10^{-8} mol/L. Bars represent mean \pm SE. $n = 6 - 10$ experiments in each group. * $P < 0.01$ v control (one-way ANOVA)

7.3.2.2 *Coronary flow and LV function*

Pre-ischaemic global coronary flow averaged 14.4 ml/min among the six experimental groups. After coronary occlusion, there was a decrease in global coronary flow rate of approximately 35% and a recovery to pre-ischaemic values immediately after reperfusion with a gradual “run-down” during the remaining 120 min perfusion (figure 7.4 A). Global coronary flow rate, generally appeared to be significantly lower in the L-NAME , BNP/L-NAME and BNP/ODQ treated groups, however this did not appear to affect the double product indicative of cardiac function which was comparable amongst the groups.

There were no detectable differences between the groups in the parameters of LV function measured (spontaneous heart rate, developed pressure or the double product – figure 7.4 B). Developed pressure and double product declined immediately after the onset of coronary occlusion to the same extent in all the experimental groups. A tabular representation of the coronary flow and the double product amongst the experimental groups is presented in tables 7.2 and 7.3.

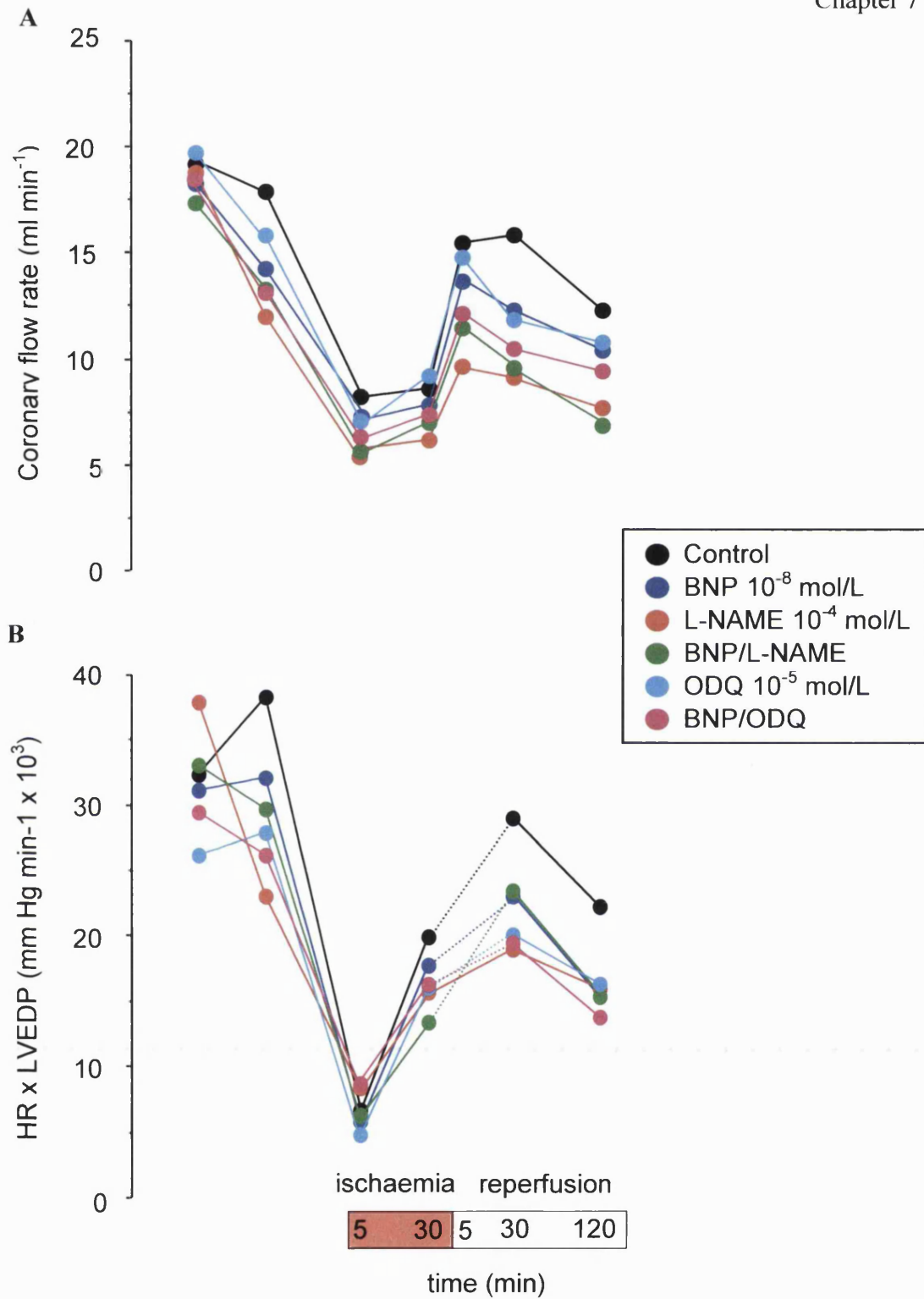


Figure 7.4 Cardiodynamics in the NO/sGC inhibitor study

Global coronary flow rate and the rate-pressure product for the NO/sGC inhibitor study. Symbols indicate mean values. Standard error bars have been removed for clarity.

Group	Basal	Pre- <i>ischaemia</i>	<u>Ischaemia</u>		<u>Reperfusion</u>		
			5 min	30 min	5 min	30 min	120 min
Control	19.0±0.6	17.8±0.8	8.2±0.5	8.6±0.3	15.5±0.8	15.8±0.8	12.3±0.4
BNP 10 ⁻⁸ mol/L	18.5±0.8	14.3±0.9**	7.2±0.4	7.8±0.6	13.7±0.8	12.3±0.7	10.4±0.7*
L-NAME 10 ⁻⁴ mol/L	18.8±0.9	12.0±1.0**	5.8±0.6**	6.2±0.8*	9.7±1.2**	9.2±1.1**	7.6±0.8**
BNP/L-NAME	17.4±0.9	13.3±0.6**	5.6±0.4**	7.0±0.4	11.5±0.6**	9.6±0.5**	6.9±0.3**
ODQ 10 ⁻⁵ mol/L	19.5±0.3	15.8±0.7	7.1±0.7	9.2±0.8	14.8±0.5	11.8±0.9	10.8±0.8
BNP/ODQ	18.7±1.0	13.2±0.8**	6.3±0.7*	7.4±0.8	12.2±0.5**	10.5±0.8**	9.4±0.9**

Table 7.2 Summary of coronary flow (ml/min)

Figures are mean ± s.e.m. *P < 0.05 v control values; **P < 0.01 v control values (repeated-measures ANOVA).

Group	Basal	Pre-ischaemia	<u>Ischaemia</u>		<u>Reperfusion</u>	
			5 min	30 min	30 min	120 min
Control	32.3±3.5	38.2±2.9	6.5±1.5	19.9±4.1	29.0±4.3	22.1±3.4
BNP 10 ⁻⁸ mol/L	31.2±3.5	32.1±4.3	6.2±0.8	17.8±2.5	23.4±3.1	15.3±2.4
L-NAME 10 ⁻⁴ mol/L	37.9±5.4	23.1±2.8*	8.8±2.2	15.7±3.9	18.9±5.2	15.9±3.8
BNP/L-NAME	33.1±3.0	29.7±3.6	6.2±1.2	13.4±2.1	23.5±4.7	15.4±3.1
ODQ 10 ⁻⁵ mol/L	26.2±3.7	27.8±6.1	4.9±0.8	16.1±2.2	19.7±2.6	16.3±2.7
BNP/ODQ	29.5±4.1	26.2±4.0	8.8±2.2	16.3±2.5	19.4±3.1	13.8±2.5

Table 7.3 Summary of rate pressure product (mm Hg min⁻¹ x 10³)

Figures are mean ± s.e.m. *P < 0.05 v control values (repeated-measures ANOVA).

7.3.3 eNOS and phospho-eNOS expression

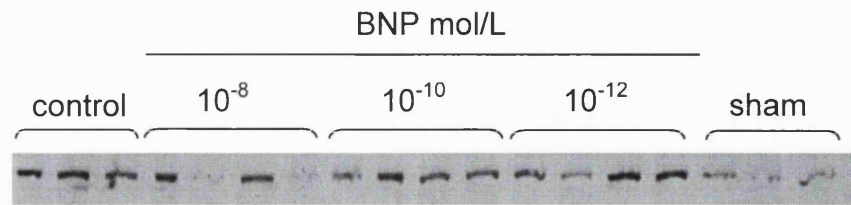
7.3.3.1 *Expression of eNOS and phospho-eNOS following BNP treatment*

Expression of eNOS and phospho-eNOS were investigated, following treatment with graded concentrations of BNP and the immunoblots and optical densitometries are shown in figures 7.5 and figure 7.6. There was no alteration of either eNOS or phospho-eNOS levels in the treated hearts when compared to control or sham hearts. In fact, phospho-eNOS was down regulated in hearts treated with BNP 10^{-10} mol/L concentration. In particular, treatment with BNP 10^{-8} mol/L, which has shown maximum protection in limiting infarct size (chapter 4), did not induce eNOS or phospho-eNOS.

7.3.3.2 *Expression of eNOS and phospho-eNOS following ischaemia*

Immunoblots and optical densitometries for the ischaemic hearts are shown in figure 7.7 and figure 7.8. eNOS expression in BNP treated hearts following ischaemia, was not significantly different from control hearts. phospho-eNOS was significantly down regulated in the BNP treated hearts compared to control.

A: Immunoblot



B: Optical densitometry

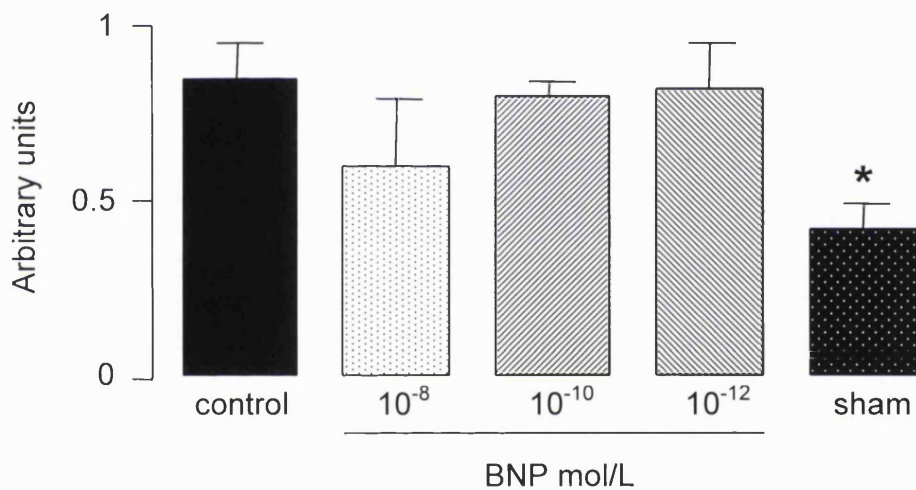
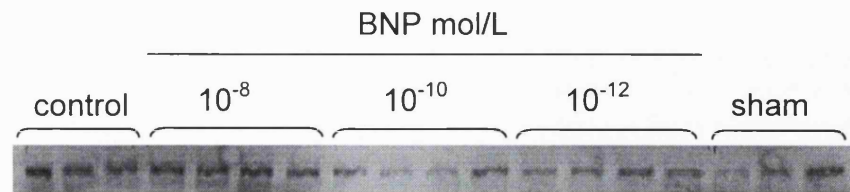


Figure 7.5 Western blot detecting eNOS expression following BNP

Panel A: Lanes 1-3 represent control hearts perfused with Krebs-Henseleit buffer, lanes 4-7 represent heart tissue perfused with BNP 10⁻⁸ mol/L, lanes 8-11 are hearts subjected to BNP 10⁻¹⁰ mol/L, lanes 12-15 are hearts subjected to BNP 10⁻¹² mol/L and lanes 16-18 are non-perfused sham hearts. Equal loading was ensured by Ponceau staining of membrane.

Panel B: Expression of eNOS in arbitrary units. *P < 0.05 v control values (one-way ANOVA).

A: Immunoblot



B: Optical densitometry

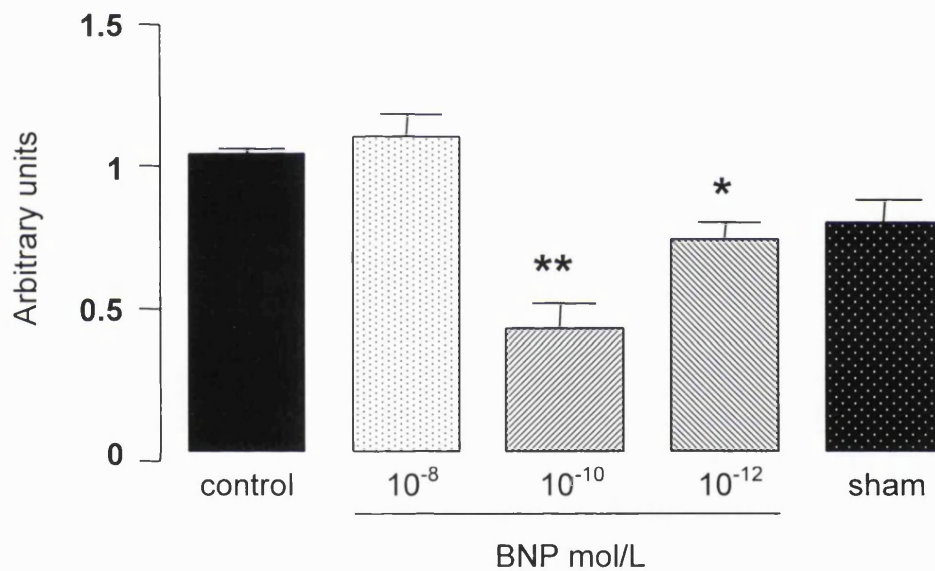


Figure 7.6 Western blot detecting phospho-eNOS expression following BNP

Panel A: Lanes 1-3 represent control hearts perfused with Krebs-Henseleit buffer, lanes 4-7 represent heart tissue perfused with BNP 10⁻⁸ mol/L, lanes 8-11 are hearts subjected to BNP 10⁻¹⁰ mol/L, lanes 12-15 are hearts subjected to BNP 10⁻¹² mol/L and lanes 16-18 are non-perfused sham hearts. Equal loading was ensured by Ponceau staining of membrane.

Panel B: Expression of phospho-eNOS in arbitrary units. *P < 0.05 v control and **P < 0.01 v control values (one-way ANOVA).

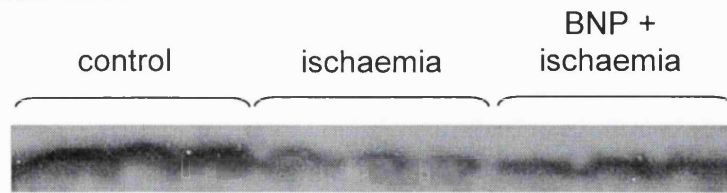
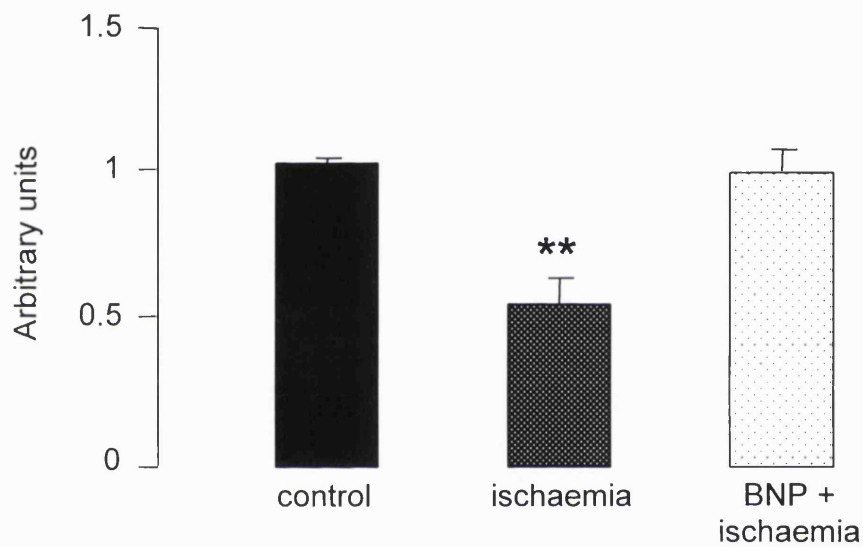
A: Immunoblot**B: Optical densitometry**

Figure 7.7 Western blot detecting eNOS expression following ischaemia

Panel A: Lanes 1-3 represent control hearts perfused with Krebs-Henseleit buffer without ischaemia, lanes 4-6 represent heart tissue subjected to 15 min ischaemia and lanes 7-9 are hearts subjected to BNP 10^{-8} mol/L followed by 15 min ischaemia. Equal loading was ensured by Ponceau staining of membrane.

Panel B: Expression of eNOS in arbitrary units. ** $P < 0.01$ v control values (one-way ANOVA).

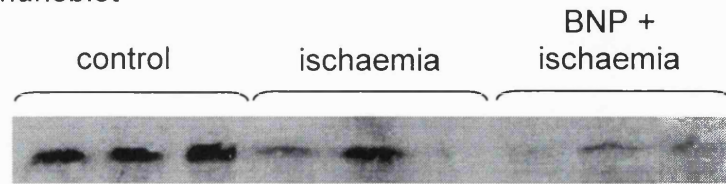
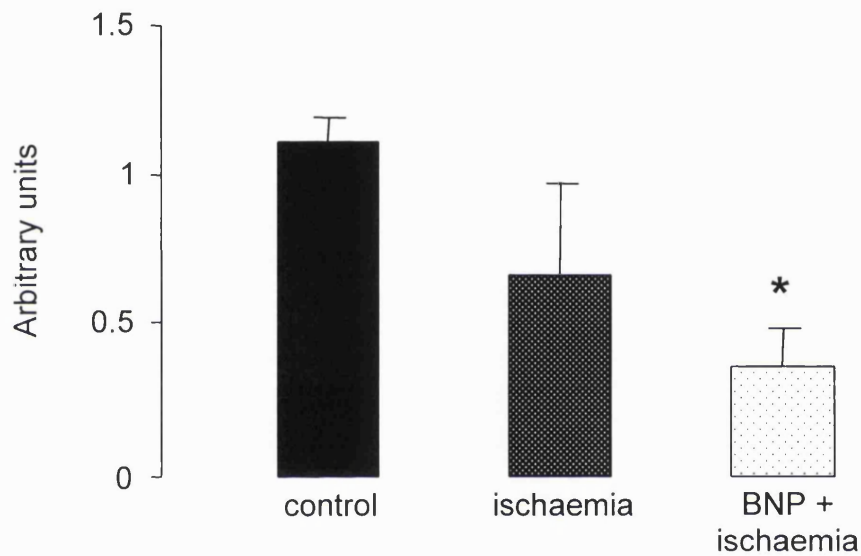
A: Immunoblot**B: Optical densitometry**

Figure 7.8 Western blot detecting phospho-eNOS expression following ischaemia

Panel A: Lanes 1-3 represent control hearts perfused with Krebs-Henseleit buffer without ischaemia, lanes 4-6 represent heart tissue subjected to 15 min ischaemia and lanes 7-9 are hearts subjected to BNP 10^{-8} mol/L followed by 15 min ischaemia. Equal loading was ensured by Ponceau staining of membrane.

Panel B: Expression of phospho-eNOS in arbitrary units. * $P < 0.05$ v control values (one-way ANOVA).

7.4 Discussion

This study confirms that BNP limitation of infarct size in myocardial ischaemia-reperfusion injury is abolished by the NOS inhibitor L-NAME and the soluble GC inhibitor ODQ, implicating a role for NO in BNP mediated cardioprotection via its sGC receptor. However in the preliminary investigation undertaken here, we could find no evidence that eNOS is acutely phosphorylated following application of BNP, either under normal aerobic conditions or during acute ischaemia.

BNP and NO are known vasodilators in several vascular beds including coronary epicardial conductance arteries and coronary microvessels (Zellner et al, 1999; Brunner et al, 2001, Gewaltig et al, 2002). Again, however, a surprising finding in the infarct study was the absence of any gross alterations in global coronary flow rate secondary to BNP-mediated coronary vasodilation. It is conceivable that an effect might be observed at a higher concentration of BNP. At present, effects of higher concentrations could not be studied due to larger molar quantity of BNP required. A further explanation relates to the coronary resistance and conductance arteries being substantially vasodilated in this constant pressure preparation. A lower coronary flow in the L-NAME, BNP/L-NAME and BNP/ODQ treated groups compared to control, might be expected. Cardiodynamic parameters were comparable amongst the groups.

While this study does not implicate the eNOS isoenzyme as the source of endothelial release of NO, other studies reported that natriuretic peptides augment iNOS in rat vascular smooth muscle cells (Marumo et al, 1995). iNOS is up-regulated in several tissues including cardiomyocytes and microvascular endothelium by lipopolysaccharide and cytokine stimulation. BNP has been shown to augment NO

synthesis in cardiac myocytes under cytokine-stimulated conditions due to enhanced induction of iNOS mRNA and protein levels (Yamamoto et al, 1997). The contributory role of iNOS to NO production in myocardial ischaemia-reperfusion injury needs to be defined. Further, in recent studies in a human-derived cell model, preconditioning-mediated neuroprotection, showed an increased in the expression of nNOS (Andoh et al, 2002). Again the role of iNOS in BNP mediated cardioprotection will need to be explored. However, it is unlikely that significant upregulation of iNOS occurs during the time course of the present experiments.

Thus the present experiments provide persuasive pharmacological evidence for a role of the NO/sGC signalling pathway in BNP's cardioprotection but no direct biochemical evidence for phosphorylation of eNOS above basal values by BNP. This is surprising and difficult to resolve. It is possible that BNP does not directly lead to activation of eNOS in this preparation but a basal production of NO is required for the protective action of BNP to occur (see figure 1.5)

Owing to its radical and lipophilic properties, NO does not adhere to its archetypal signalling process, but diffuses randomly away from its point of synthesis to interact with various intracellular molecules (Hobbs et al, 1996). Nevertheless, the best characterised target site for NO is iron, bound within certain proteins as heme or iron-sulfur complexes. Of primary physiological significance is the interaction of NO with the heme component of sGC forming a ferrous-nitrosyl-heme complex, and after shifting out of the heme iron, stimulates the enzyme conversion of GTP to cGMP; as such sGC is often termed the 'NO receptor'. It is this mechanism which is responsible for the overwhelming majority of biological effects mediated by NO (Hobbs et al, 1996; Koesling, 1999).

sGC is expressed in the cytoplasm of almost all mammalian cells including cardiomyocytes (Lucas et al, 2000), and mediates a wide range of important physiological functions, such as inhibition of platelet aggregation, relaxation of smooth of smooth muscle, vasodilation, neuronal signal transduction and immunomodulation. The enzyme is a heterodimer composed of α - and β -subunits. The C-terminal regions of both subunits have a high degree of sequence identity with cloned adenylyl and pGCs, suggestive of a catalytic domain (Lucas et al, 2000). The N-terminal regions of the subunits are responsible for binding of the prosthetic heme group of the enzyme, required for stimulatory effect of NO (Koesling, 1999). Protoporphyrin IX and carbon monoxide can also activate sGC, but are less potent than NO. The NO-sGC complex has a high NO dissociation rate which is increased about 40-fold in the presence of Mg^{2+} as a substrate for catalytic conversion of GTP to cGMP (Hobbs et al, 1996). The half-life of NO-sGC complex is about 5 s and is fast enough to allow rapid deactivation of the enzyme in biological systems, although a NO scavenger is a prerequisite (Koesling, 1999). ODQ, the potent and selective inhibitor of sGC provides an important tool to discriminate between cGMP-dependent and cGMP-independent signalling (Garthwaite et al, 1995).

The NO-sGC-cGMP transduction system could thus be implicated upstream in BNP's cardioprotection, converging with a signalling pathway associated directly via the BNP-pGC-cGMP signalling system, activating cGK-I and the mitochondrial K_{ATP} channel. Studies measuring NO levels in the cardiac tissue following BNP treatment or the use of eNOS knockout animals may help in further consolidating the role of NO in BNP's signalling. The role of the other isoforms of NOS (iNOS and nNOS) in BNP's cardioprotection merit further studies.

In summary, exogenous BNP is able to limit infarct size in myocardial ischemia-reperfusion injury in the isolated rat heart model and pharmacological evidence suggests that this cytoprotective effect is likely to be dependent on activation of the NO/sGC pathway.

Chapter Eight

Conclusion

8.1 Summary of BNP's mechanism of action in cardioprotection

BNP an endogenous natriuretic peptide has, since its first isolation in 1988, established itself as a predominant cardiac hormone with various autocrine and paracrine actions. The traditional role of BNP in natriuresis and vasodilation is mediated via its cell-surface particulate guanylyl cyclase, NPR-A receptor. The receptor activation requires ATP binding, to convert GTP to cGMP responsible for the biological effects of BNP. Experimental and clinical evidence, suggested BNP release from cardiac tissue following brief episodes of ischaemia or hypoxia. However, its role *per se* in myocardial-ischaemia reperfusion injury was unknown.

Work in this thesis has confirmed that BNP is released from the ventricular myocardium, in response to various durations of an ischaemic insult in the isolated, Langendorff perfused rat heart. To explore the possibility of a cardioprotective affect on BNP in myocardial ischaemia-reperfusion injury, rat hearts were treated with graded concentrations of BNP and subjected to regional ischaemia. This is the first study to show that exogenous BNP is markedly protective against myocardial ischaemia-reperfusion injury, leading to a concentration-dependent limitation of infarct size.

In elucidating, BNP's cytoprotective mechanism of action, the possible involvement of the mitochondrial K_{ATP} was implicated using various pharmacological inhibitors of the K_{ATP} channel. The study then proceeded to evaluate the myocardial ventricular response, to graded durations of ischaemia and treatment with graded concentrations of

BNP. Myocardial ischaemia produced a substantial elevation in the tissue cGMP concentration which was directly proportional to the duration of the ischaemic stimulus. As expected, cGMP levels in the ventricular myocardium also increased significantly following BNP treatment, and this increase also correlated positively as the BNP treatment concentration was raised. In view of BNP and ischaemia-triggered release of ventricular cGMP, the role of cGMP itself on ischaemia-reperfusion injury was next studied. Treatment of hearts with graded concentrations of a synthetic cGMP analogue, revealed protection from the ischaemia-reperfusion injury at lower concentrations, and absence of protection at the higher concentrations of cGMP used in the study.

Previous studies showed BNP-mediated vasodilation to be dependent on NO release from the endothelium. Hence, in the next set of experiments the role of NO/sGC was studied in BNP's signal transduction. Hearts were perfused with pharmacological blockers of NOS and sGC in either the presence or absence of BNP. Inhibiting, both the NOS and sGC independently abolished BNP-induced limitation of infarct size in the ischaemia-reperfusion injury, implicating a role for the activation of NO/sGC in BNP cytoprotection. However, attempting to identify the isoform of NOS responsible for the BNP triggered NO release in cardioprotection, Western blot analysis, failed to reveal phosphorylation of eNOS at Ser 1177, a common site of phosphorylation for other IPC triggers, suggesting nNOS as a possible source of NO. Endothelial release of NO is known to cross-talk to cardiac myocytes to engage the cardiomyocyte sGC receptor and subsequently elevate intracellular cGMP concentration, which along with the known cGMP derived from BNP's direct action on the cell-surface NPR-A receptor invokes cGK-I to act downstream on the mitochondrial K_{ATP} channel well known to mediate ischaemic preconditioning. This leads us to hypothesise that cGK is central to the cardioprotective effects of BNP

In summary, these studies are the first to demonstrate a cardioprotective effect of exogenous BNP against ischaemia-reperfusion injury by a mechanism involving the K_{ATP} mitochondrial channel opening and induction of the NO/sGC upstream of NPR-A activation.

8.2 Study limitations and the future

Normal physiological circulating concentrations of endogenous BNP are in the picomolar range. The concentrations of exogenous BNP used in our study, as derived from previous experiments, were supra-physiological. However, the cytoprotective effect obtained with these high pharmacological concentrations did not appear to affect the general function – coronary flow and rate-pressure-product of the perfused hearts when compared to untreated control hearts throughout the duration of the experiment. Further *in vivo* monitoring will be required to determine if these concentrations are pathological in the general context in the intact rat model as well as other species. While it is likely that BNP released from the ischaemic heart is derived from the ventricular cardiomyocyte injury, specific release from and actions upon the myocardial vascular endothelium, smooth muscle, fibroblasts and interstitium will almost certainly be contributory in myocardial ischaemia. The interaction of various intrinsic neurohormonal mechanisms and cross-talk will make isolated cardiomyocyte and/or coronary vascular studies non-reflective of the intact body mechanism.

Coronary flow in the isolated perfused hearts was assessed on a constant-pressure Langendorff apparatus which was pre-established in our laboratory and its use validated. As stated in the methods, this is more physiological and simulates the intact circulation. Failure of this constant-pressure setup to reproduce increased coronary

flow even with established vasodilators like nitrates, suggest that the constant-flow Langendorff equipment may be better suited to study BNP induced coronary vasodilation.

Time constraints did not permit the use eNOS knock-out animals and further Western Blot evaluation of the other NOS isoenzymes in the study. The lack of easily available NPR-A knock-out animals, limits the evaluation of BNP in the ischaemic heart. NPR-A receptor inhibitors are available but financial constraints, the non-selective nature and debate about their effectiveness *in vitro* studies precluded their use in this study. cGK-I inhibitors again were not easily available, prohibitively costly and the high molar concentration of these substances required for isolated heart perfusion prevented their use. cGK-I-knockout mice would greatly facilitate the examination of our hypothesis that cGK is central to the cardioprotective effects of BNP.

Studies evaluating BNP's cardioprotection may be undertaken using cultured cardiac myocytes but again may not accurately model endogenous protective mechanisms in intact tissue where there is interaction between several cell lines and humoral mediators. The role of K_{ATP} channels may be further studied in isolated cells or mitochondria using cellular imaging techniques like electron microscopy and confocal fluorescence microscopy. NO in relation to BNP cytoprotection may be probed further using eNOS knock-out mice. Alternative approaches using mutant mouse strains with targeted deletion of either pro-BNP or NPR-A genes may also be possible in future studies although interpretation of findings from these animals, in the absence of complementary pharmacological data, may be clouded by uncertainties of altered expression of other gene products and high level of biological redundancy. BNP action is species specific and as such further studies in human isolated atrial trabeculae and *in vivo* work in other species is required to fully elucidate the cytoprotective potential of BNP in myocardial ischaemia-reperfusion injury.

8.3 Clinical implications

BNP is a potent natriuretic, diuretic vasorelaxant peptide. It coordinates fluid and electrolyte homeostasis through its activity in the central nervous system and peripheral tissues. BNP promotes vascular relaxation and lowers blood pressure, particularly in states of hypovolaemia. It inhibits sympathetic tone, the RAA system, and synthesis of vasoconstrictor molecules such as catecholamines, angiotensin II, aldosterone and endothelin-I. Its renal effects include increasing glomerular filtration rate and enhancing sodium excretion (Koller et al, 1992). It also inhibits vascular smooth muscle cell proliferation and potentiates the generation of cGMP (Schirger et al, 2000) and inhibits apoptosis and prolongs survival in cultured neural PC12 cells (Fiscus et al, 2001).

Neurohormonal activation following cardiovascular disease is now well known. This mechanism is well illustrated by the beneficial effects of ACE inhibition and β -blockade in hypertension, ischaemic heart disease, heart failure and renal disease. However, despite success of these therapies, mortality and morbidity from myocardial infarction is a global predicament. In addition, blood pressure is not adequately controlled in a large proportion of hypertensive patients and heart failure and renal disease continue to present a therapeutic challenge. The limitations of current therapies serve to stimulate research and development of new therapies.

8.3.1 BNP in health

BNP levels tend to rise with age, presumably secondary to LV stiffening over time and stimulating BNP production. Women have somewhat higher BNP levels than men of the same age group. In a recent study, 23 healthy male subjects aged 23-27 years were subjected to treadmill exercise according to the Bruce protocol, which was fully

completed. Even after correction for haematocrit, a significant increase in plasma BNP was found immediately after the test, which returned to normal within one hour (Huang et al, 2002).

8.3.2 BNP in diagnosis and management of CHF

BNP levels in patients diagnosed with CHF are much higher compared to those in the non-CHF group and those with lung disease. Levels of BNP have been shown to be elevated in patients with asymptomatic and symptomatic left ventricular dysfunction and correlate with the New York Heart Association class, as well as with prognosis (Lerman et al, 1993; Cowie et al, 1997; Koglin et al 2001). This is recognised by their inclusion in the European guidelines for the diagnosis of CHF (Remme et al, 2001). In the recent Breathing Not Properly multinational study (Maisel et al, 2002), patients presenting to emergency departments with acute dyspnoea had their blood BNP measured with a bedside assay. BNP levels by themselves were more accurate than any historical or physical findings or laboratory values in identifying CHF as the cause of dyspnoea. The diagnostic accuracy of BNP at a cut-off of 100 pg/ml was 83.4%, with a sensitivity of 90% and specificity of 76% for differentiating CHF from other causes of dyspnoea. The negative predictive value of BNP at levels less than 50 pg/ml was 96%. In conjunction with other clinical information, BNP levels should lead to a more accurate initial diagnosis of CHF.

In prognosis and risk stratification, BNP levels have been shown to be a powerful tool in heart failure. Harrison et al (2002) followed up 325 patients for six months after an index visit to the emergency department for dyspnoea. Higher BNP levels were associated with a progressively worse prognosis. The relative risk of 6-month CHF death in patients with BNP levels greater than 230 pg/ml was 24. In patients

undergoing haemodynamic monitoring, changes in wedge pressures were strongly correlated with dropping BNP levels and clinical improvement and in future may serve as a substitute for invasive Swan-Ganz catheterisation (Kazanegra et al, 2001). Further, in modulating and optimising effectiveness of therapy for CHF - BNP or N-BNP have been shown to be very effective in an outpatient setting (Richardson et al, 1999; Troughton et al, 2000).

While increasing levels of BNP represent a compensatory response to LV strain, importantly, there is no suggestion that BNP itself causes deterioration. A recombinant form of human BNP - nesiritide (natrecor®) manufactured from *E. coli* using recombinant DNA technology is now available and has recently been used to evaluate the role of BNP in CHF therapy. In clinical studies, in patients with acutely decompensated CHF, nesiritide reduced pulmonary capillary wedge pressure, right atrial pressure (preload) and systematic vascular resistance (afterload) while increasing cardiac output without affecting heart rate and improved symptoms (Mills et al, 1999; Colucci et al, 2000). In the PRECEDENT study, nesiritide used in the recommended dose of 2 µg/Kg bolus followed by an infusion of 0.01 µg/Kg/min when compared to standard dose dobutamine therapy was not proarrhythmic, but in fact reduced ventricular ectopy or has a neutral effect obviating a need for intense electrocardiographic monitoring (Burger et al, 2002). Although nesiritide was associated with a higher incidence of hypotension than dobutamine, this effect of the drug was easily treated with dose reduction or discontinuation.

8.3.3 RAA and NP systems

The RAA and NP systems play important roles in the regulation of fluid and electrolyte homeostasis, affecting blood pressure and volume status. The physiological relationship between these two systems is antagonistic in nature. The RAA system is activated by volume contraction and decreases in renal perfusion, whereas the NP system is primarily activated by volume expansion. The RAA system raises blood pressure through vasoconstriction mediated by angiotensin II and sodium and water retention mediated via production and release of aldosterone. In contrast, the NP system lowers blood pressure, through vasodilation, diuresis, natriuresis, and shifts of intravascular fluid to extravascular spaces. NPs antagonise a number of biological effects of angiotensin II including vascular cell growth, hypertrophy (Stein et al, 1998; Itoh et al, 1991) and vasoconstriction (Kleinert et al, 1984).

Under normal circumstances, the renal-body fluid feedback mechanism and the RAA system play a major role in the long-term control of fluid and electrolyte homeostasis, whereas the NP system plays a minor role (Lohmeier et al, 1995). Therefore only small changes in volume pressure occur leading to minimal activation of the NP system. However, in conditions with large and persistent volume expansion (i.e. CHF), significant and long-term activation of the NP system occurs. This results in sustained pharmacological effects such as natriuresis and suppression of pressor hormones and amines secretions by NPs. In the absence of this long-term effect, reduced arterial pressure in compensated heart failure would lead to protracted salt and water retention and persistent increase in body fluid volume. Therefore while playing a minor role in normal conditions; the NP system plays an important role in long-term fluid and electrolyte homeostasis in pathophysiological conditions such as CHF and hypertension (Lohmeier et al, 1995). The success of ACE inhibition for the treatment of many conditions has led to the development of strategies that build on this success.

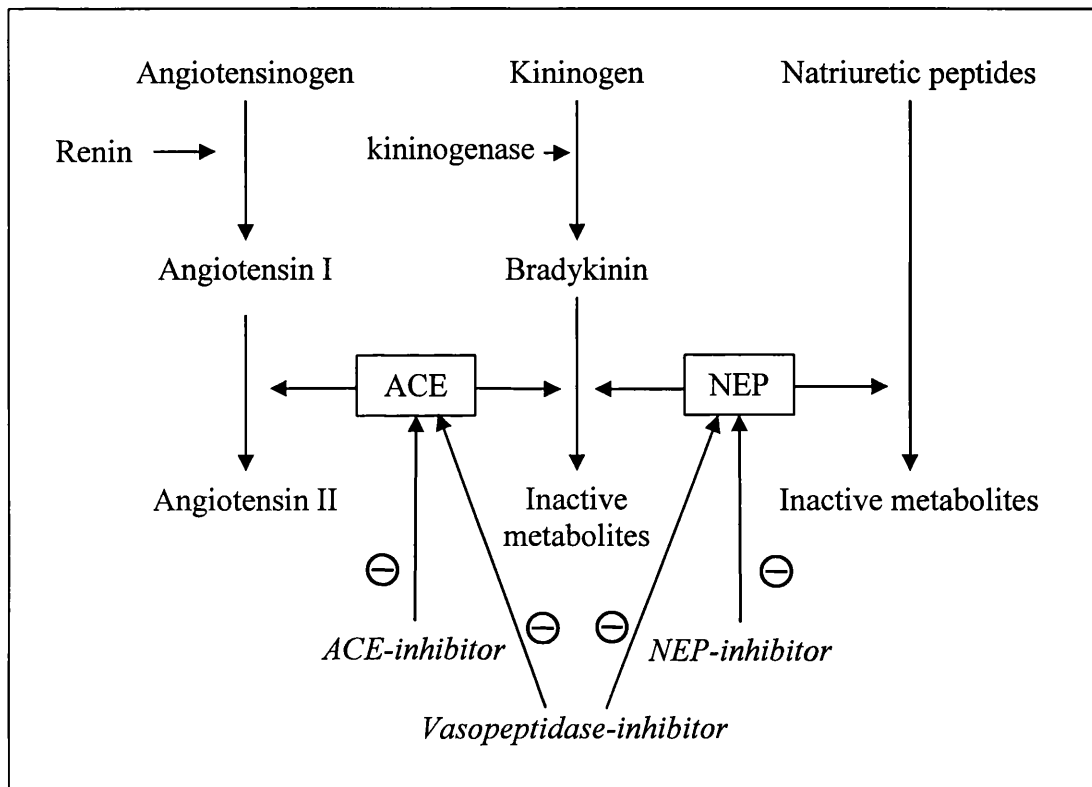


Figure 8.1 Natriuretic peptide and renin-angiotensin-aldosterone systems

ACE, angiotensin-converting enzyme; NEP, neutral endopeptidase; ⊖, inhibition process. In addition, NEP also hydrolyses other vasodepressor peptides including angiotensin II and endothelin, omitted for clarity.

8.3.3.1 *NP system inhibition*

Since NPs have to be administered as a continuous infusion because of their short half-lives (BNP 18-21 min; ANP 3 min), they are not practical for use in the clinical management of hypertension and heart failure. Measures targeting potentiation of NP effects by inhibiting NEP, the enzyme responsible for degradation of these peptides, have been at the centre of drug development.

- *In CHF* – In patients with CHF, NEP inhibition reduced cardiac filling pressures and decreased indices of the renin axis (Munzel et al, 1992). Preliminary experimental evidence that NEP inhibition may directly protect endothelial function and reduce atheromatous changes in the vascular wall (Schirger et al, 2000), suggest that this type of treatment could contribute to improving cardiovascular prognosis.
- *In hypertension* - NP levels are elevated in patients with hypertension (Santucci et al, 1990; Nishikimi et al, 1997) and increasing levels correlate with severity of hypertension and LV and left atrial size (Testaert et al, 1989; Richard et al, 1994) likely representing a compensatory mechanism. Preclinical and clinical studies in animals and humans show that exogenous ANP infusion consistently lowers blood pressure (Tonolo et al, 1989; Charles et al, 1993). Several NEP inhibitors have been developed (e.g. candoxatril), however clinical studies of these agents failed to show sustained benefit in blood pressure reduction. The cause of this failure could be possibly due to increased angiotensin II levels since NEP can also degrade angiotensin II (Richards et al, 1992; Ando et al, 1995). Therefore, blood pressure response with NEP inhibition alone may depend on the net effects on vasodilator and vasoconstrictor systems.

8.3.3.2 Combined RAA and NP systems inhibition

Focus of drug development has now shifted to dual inhibition of both NP degradation and activation of the RAA system a strategy known as vasopeptidase inhibition. Vasopeptidase inhibitors inhibit both NEP and ACE while blocking the degradation of bradykinin – more powerfully than an ACE inhibitor alone (see figure 8.1). They increase the availability of NP that have vasodilatory and other vascular effects and inhibit the production of angiotensin II. Hence, the profile renders the drug very effective at reducing blood pressure, theoretically very attractive for management of heart failure, but at an increase risk of provoking side effects such as cough and angioedema. One such inhibitor well researched is omapatrilat. Early clinical trials with omapatrilat provided additional support for the potential incremental efficacy of vasopeptidase inhibition, although concerns about drug related adverse effects (including angioedema) also emerged (Rouleau et al, 2000).

- *In CHF* - In the OVERTURE study (Packer et al, 2002), a comparison was made of omapatrilat (40 mg once daily) and an ACE inhibitor enalapril (10 mg twice daily) randomly assigned to 5770 patients with New York Heart Association class II-IV CHF for a mean of 14.5 months. Omapatrilat reduced the morbidity and mortality in CHF but interestingly was not more effective than ACE inhibition alone in reducing the risk of a primary clinical event – the combined risk of death or hospitalisation for heart failure requiring intravenous treatment. However, post hoc analyses based on a broader definition of heart failure or focussed on all cardiovascular events suggested the possibility of between-group differences in favour of omapatrilat warranting further study. Angioedema occurred rarely in both groups, but was more common in the omapatrilat treated group (0.8% v 0.5%), possibly and fortunately because patients with CHF seem to be resistant to this potential side effect. Hypotension and dizziness

were more common in patients treated with omapatrilat, however despite these effects, worsening renal function were less frequent in the omapatrilat group.

- *In hypertension* - In the OCTAVE study – omapatrilat cardiovascular treatment assessment v enalapril in hypertension, 25, 000 hypertensive patients were randomly assigned to either of the drug (Armstrong et al, 2002). The primary aim of the study was to assess the safety of omapatrilat (up to 80 mg) and enalapril (up to 40 mg), especially in relation to angioedema. Omapatrilat had a superior anti-hypertensive effect, requiring less up-titration in dose and add-on therapy while maintaining a 2-3 mmHg greater reduction in systolic blood pressure compared to enalapril treatment. Overall, 2.17% omapatrilat treated patients developed angioedema, compared to 0.68% in the enalapril treated group. These rates were 2-3 times higher in the African-American patients and smokers. Half the patients required steroids or epinephrine for treatment. Most events occurred on the first day of dosing and only two patients required intubation and none died. Because of these concerns, omapatrilat has not been approved for treatment of hypertension.

8.3.4 BNP in coronary syndromes

With BNP known to be a strong independent diagnostic and prognostic marker in CHF, it has subsequently found to predict outcomes in patients with AMI (Hall et al, 1995; Omland et al, 1996). These findings were extended across the spectrum of patients with acute coronary syndromes (ACS), including those with unstable angina (UA), in whom elevated levels of BNP predicted a twofold to threefold higher risk of death by ten months (de Lemos et al, 2001). Recently, in 1,676 patients with non-ST-elevation ACS randomised to early invasive v conservative management, BNP levels at baseline (>80 pg/ml) were at higher risk of death at seven days and six months

(Morrow et al, 2003). The association between BNP levels and mortality at six months was independent of cardiac troponin-I and CHF. Thus clinical use may be made of circulating BNP levels for risk assessment and triage in patients with suspected ACS including non-ST-elevation myocardial infarction (NSTEMI).

Work in this thesis has shown for the first time, that exogenous BNP is cardioprotective in limiting infarction in myocardial ischaemia-reperfusion injury without impairing cardiac haemodynamic function. The intracellular cytoprotective action appears complex with the possible involvement of NO, a cross-talk between particulate and soluble guanylyl cyclases, cGMP and its dependent kinases and mK_{ATP} cascade. The mechanism ultimately reduces intracellular Ca^{2+} associated with myocardial cell death. It is likely that this cytoprotection accorded by BNP is well complemented by its traditionally known biological actions viz. vasodilation, natriuresis, antagonism of the RAA system (including vascular cell growth and hypertrophy), anti-sympathetic action and inhibition of platelet aggregation.

Based on present clinical correlates it may seem reasonable to speculate a further extension of exogenous BNP's cytoprotective role to include prophylaxis of coronary micro-embolism especially during a percutaneous coronary intervention (PCI) procedure in our cardiac catheterisation laboratories.

At the time of writing, the therapeutic application of nesiritide has been licensed in the United States to treat patients with acute decompensated CHF who have dyspnoea at rest or with minimal activity. It appears to be effective and well-tolerated, with the most common side effect reported to be dose-related hypotension which was usually mild. It is conceivable, that in the foreseeable future well-defined clinical trials will build on this important pharmacological action of BNP and extend the therapeutic efficacy to treat patients with ACS including myocardial infarction.

Another attractive target is the pharmacological inhibition of neutral endopeptidase (NEP) responsible for the degradation of BNP, thus increasing BNP's biological activity. NEP inhibitors currently used therapeutically in the management of patients with heart failure and further experimental and clinical trials may give them an extended role in protection against myocardial ischaemia-reperfusion injury.

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CARDIOPROTECTIVE ACTION OF B-TYPE NATRIURETIC PEPTIDE IS NO-DEPENDENT

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B-type natriuretic peptide (BNP) is one of a family of cardiac and vascular-derived peptides. BNP exerts a number of systemic actions through activation of a particulate guanylyl cyclase cell-surface receptor, NPR-A, and the subsequent elevation of intracellular cyclic GMP. We have recently reported that exogenous BNP confers protection in a concentration-dependent manner against acute ischaemia-reperfusion injury (D'Souza et al., 2002a). The mechanisms of this cytoprotective action are not fully elucidated although there is evidence to suggest a pivotal role of K_{ATP} channel opening (D'Souza et al., 2002b). While NPR-A is a particulate guanylyl cyclase, actions of BNP in some vascular beds have been attributed to generation of NO (Zellner et al., 1999). This study was undertaken to identify if the NO/soluble guanylyl cyclase pathway plays a role in the cytoprotective action of BNP in myocardium.

Male Sprague Dawley rats (200-300 g) were deeply anaesthetised with pentobarbitone sodium (60 mg/kg). Hearts were excised and Langendorff-perfused at constant pressure (100 cm H₂O) with Krebs-Henseleit buffer. After instrumentation and stabilisation, hearts were subjected to 35 min left coronary artery occlusion and 120 min reperfusion. Infarct size was determined as a percentage of the ischaemic risk zone by computerised planimetry of sections, double stained with Evans' blue and triphenyltetrazolium chloride. Control hearts received no treatment. BNP treated hearts were perfused with BNP 10⁻⁸ M, beginning 10 min before the onset of coronary occlusion until 30 min reperfusion. To assess the involvement of NO synthase activation, L-NAME 10⁻⁴ M was co-perfused with BNP. To assess the involvement of soluble guanylyl cyclase, the inhibitor compound 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) 10⁻⁵ M, which does not inhibit particulate guanylyl cyclase, was co-perfused with BNP. Infarct size data (I/R) and ischaemic risk zone data (R) are presented in table 1.

Table 1. Infarct size data

Treatment	n	R cm ³	I/R %
Control	8	0.39±0.04	45.0±5.5
BNP	11	0.37±0.01	23.4±2.8*
L-NAME+BNP	10	0.38±0.02	38.0±3.7
L-NAME	6	0.40±0.04	40.8±5.7
ODQ+BNP	6	0.38±0.03	41.1±5.3
ODQ	6	0.43±0.03	44.1±2.7

Data are mean ± s.e. * P < 0.01 versus control (1-way ANOVA)

BNP treatment significantly limited infarction during ischaemia-reperfusion. This cytoprotective effect was abolished by the non-specific NO-synthase inhibitor L-NAME or by the particulate guanylyl cyclase inhibitor ODQ. The differences in infarct size were independent of variations in ischaemic risk zone size since this was similar in all treatment groups. There were no substantial differences in coronary flow rate or left ventricular contractility between the groups (data not presented).

We conclude that the ability of BNP to limit myocardial infarction is dependent on activation of the NO/soluble guanylyl cyclase pathway.

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TYPE-B NATRIURETIC PEPTIDE LIMITS INFARCT SIZE VIA KATP CHANNEL OPENING IN THE LANGENDORFF PERFUSED RAT HEART

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Increasing evidence suggests that type-B natriuretic peptide (BNP) exerts important autocrine/paracrine actions in the heart. Acute ischemia triggers the rapid release of BNP from ventricular myocardium. We have recently shown exogenously-administered BNP to limit infarct size in rat heart in a concentration-dependent manner. The mechanism of cytoprotection is unknown. Since all the recognized actions of BNP are exerted at least in part through cGMP signaling, we hypothesised that the infarct-limiting effect of BNP is mediated by opening of KATP channels, a downstream target of cGMP-dependent protein kinase. To test this hypothesis, Langendorff-perfused rat hearts were subjected to 35 min of regional ischemia and 120 min reperfusion. KATP channel blockers used were the mitochondrial and sarcolemmal KATP blocker glibenclamide (1 mM), the selective mitochondrial KATP blocker 5-hydroxydecanoate (5-HD; 100 mM) and the selective sarcolemmal KATP blocker HMR1098 (10 mM). Controls received no further treatment. Either rat BNP-32 (10 nM), a KATP blocker or a combination of the two agents was perfused from 10 min pre-ischemia up to 30 min reperfusion. Infarct size was determined by triphenyltetrazolium staining and expressed as a percentage of the ischemic risk zone (I/R%). Data are shown in table 1. BNP 10 nM significantly limited infarct size. This protective effect was abolished by glibenclamide and 5-HD but not by HMR1098, suggesting participation of mitochondrial but not sarcolemmal KATP channel opening. These data are the first to suggest that BNP is protective via opening of mitochondrial KATP channels. However, details of the signaling cascade underlying this newly-described action of BNP remain to be determined.

	Control	BNP	Glibenclamide		5-HD		HMR1098	
			control	BNP	control	BNP	control	BNP
I/R%	47.1±2.8	21.3±2.8*	41±7.1	52.0±5.7	40.5±4.1	52.3±5.7	48.6±2.2	14.7±2.7*

mean±s.e.m.; n=5-10 per group; *P<0.01 v control

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**TYPE-B NATRIURETIC PEPTIDE LIMITS
INFARCT SIZE VIA K_{ATP} CHANNEL OPENING**

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The Hatter Institute, UCL Hospitals and Medical School, London, UK. & *The Royal Veterinary College, London, UK.

Type-B natriuretic peptide (BNP) is released rapidly from the heart in acute ischaemia. We have recently shown exogenous BNP to limit myocardial infarct size in a concentration-dependent manner but the mechanism of protection is unknown. We hypothesise that this action of BNP may involve K_{ATP} channel opening.

Langendorff-perfused rat hearts were subjected to 35 min regional ischaemia and 120 min reperfusion. Controls received no further treatment. Either BNP (10 nM), the putative mitochondrial K_{ATP} channel blocker 5-hydroxydecanoate (5HD; 100 µM) or a combination of both was perfused 10 min before ischaemia until 30 min of reperfusion. Infarct size was determined using triphenyltetrazolium stain and expressed as a percentage of the ischaemic risk zone (I/R) shown in table 1.

	control	BNP	5HD	5HD+BNP
I/R	46.4±5.8	21.3±6.3*	40.6±4	50.1±5.3

*mean±s.e.m.; n=6-7 per group; *P<0.01 v control*

BNP significantly limited infarct size. This protective action was abolished by 5HD. These data are the first to suggest that BNP may confer protection through opening of the mitochondrial K_{ATP} channel. The intracellular signalling mechanism is as yet unknown.

TYPE-B NATRIURETIC PEPTIDE LIMITS INFARCT SIZE IN RAT ISOLATED HEART

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Chronic haemodynamic overload leading to cardiac dilatation triggers the release of atrial natriuretic peptide (ANP) and type-B natriuretic peptide (BNP). ANP and BNP are abundant in cardiomyocytes, in the form of pro-peptides stored in secretory granules. Both peptides bind to natriuretic peptide receptor-A (NPR-A), and subsequent elevation of intracellular cGMP concentration mediates the endocrine actions of ANP and BNP, including vasodilatation and natriuresis. Acute ischaemia or hypoxia can evoke the rapid release of natriuretic peptides from cardiac tissue (Toth et al., 1994; Hama et al., 1995) but the local role of the peptides during ischaemia has not been defined. ANP was shown to be antiarrhythmic in dog heart subjected to acute coronary artery occlusion and reperfusion (Rastegar et al., 2000). We hypothesised that BNP, the predominant natriuretic peptide in ventricular myocardium, exerts an autocrine cytoprotective action during ischaemia, independent of systemic haemodynamic unloading.

Male Sprague Dawley rats (275-400 g) were anaesthetised with pentobarbitone sodium (50 mg kg⁻¹, i.p.) and heparinised (300 i.u.). All hearts were excised and Langendorff perfused with Krebs-Henseleit buffer at 37 °C and subjected to 35 min left coronary artery occlusion and 120 min reperfusion. Control hearts received no further treatment. BNP was perfused at various concentrations commencing 10 min before ischaemia and continued until 30 min reperfusion. Infarct size was determined using triphenyltetrazolium stain and expressed as a percentage of the ischaemic risk zone. Infarct size data are shown in figure 1.

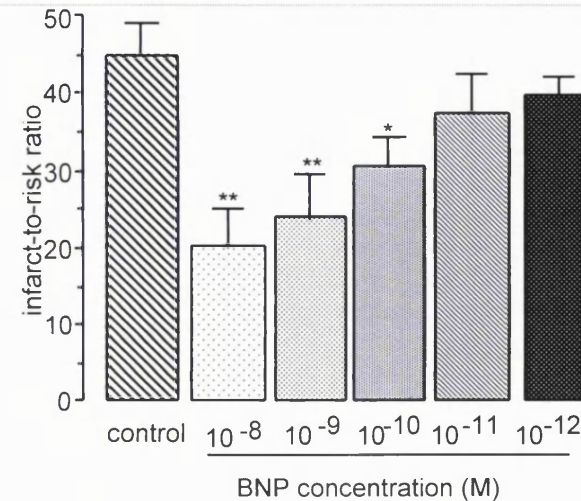


Figure 1. Infarct size. Bars represent mean ± s.e.m. *P < 0.05; ** P < 0.01 v control (1-way ANOVA). n = 8-12 per group

BNP limited infarct size in a concentration-dependent manner, with significant protection being seen at 10⁻⁸, 10⁻⁹ and 10⁻¹⁰ M. The risk zone volume averaged 0.35 cm³ for all the groups. There were no detectable differences in coronary flow rates at these concentrations, suggesting that the cytoprotective action of BNP was not mediated by coronary vasodilatation in this model. We conclude that BNP protects against acute ischaemia-reperfusion injury and may represent an innate cardioprotective response. The molecular mechanism of this previously undefined action of BNP is unknown.

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B-type natriuretic peptide limits infarct size in rat isolated hearts via K_{ATP} channel opening

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D'Souza, Savio P., Derek M. Yellon, Claus Martin, Rainer Schulz, Gerd Heusch, Annamaria Onody, Peter Ferdinandy, and Gary F. Baxter. B-type natriuretic peptide limits infarct size in rat isolated hearts via K_{ATP} channel opening. *Am J Physiol Heart Circ Physiol* 284: H1592–H1600, 2003. First published January 9, 2003; 10.1152/ajpheart.00902.2002.—B-type natriuretic peptide (BNP) has been reported to be released from the myocardium during ischemia. We hypothesized that BNP mediates cardioprotection during ischemia-reperfusion and examined whether exogenous BNP limits myocardial infarction and the potential role of ATP-sensitive potassium (K_{ATP}) channel opening. Langendorff-perfused rat hearts underwent 35 min of left coronary artery occlusion and 120 min of reperfusion. The control infarct-to-risk ratio was $44.8 \pm 4.4\%$ (means \pm SE). BNP perfused 10 min before ischemia limited infarct size in a concentration-dependent manner, with maximal protection observed at 10^{-8} M (infarct-to-risk ratio: $20.1 \pm 5.2\%$, $P < 0.01$ vs. control), associated with a 2.5-fold elevation of myocardial cGMP above the control value. To examine the role of K_{ATP} channel opening, glibenclamide (10^{-6} M), 5-hydroxydecanoate (5-HD; 10^{-4} M), or HMR-1098 (10^{-5} M) was co-perfused with BNP (10^{-8} M). Protection afforded by BNP was abolished by glibenclamide or 5-HD but not by HMR-1098, suggesting the involvement of putative mitochondrial but not sarcolemmal K_{ATP} channel opening. We conclude that natriuretic peptide/cGMP/ K_{ATP} channel signaling may constitute an important injury-limiting mechanism in myocardium.

cGMP; ischemia-reperfusion; infarct size; preconditioning

NATRIURETIC PEPTIDES ARE RELEASED from many tissues in response to physiological and pathological stimuli. A-type (atrial) natriuretic peptide (ANP) and B-type (brain or ventricular) natriuretic peptide (BNP) are the predominant natriuretic peptides in mammalian myocardium, stored within secretory granules as propeptides (2, 6, 24). Release of propeptides and the cleaved products in response to dilatation of the cardiac chambers in conditions such as heart failure has been well described. Under such conditions, the classical endocrine actions of ANP and BNP include vasodilatation of some peripheral vascular beds and natriuresis (38, 39).

These actions are mediated by elevation of intracellular cGMP after peptide binding to natriuretic peptide receptor type A (NPR-A), a membrane-bound particulate guanylyl cyclase (2, 6, 15, 29).

BNP is the principal natriuretic peptide in ventricular myocardium. Experimental and clinical evidence suggests that brief episodes of ischemia or hypoxia, insufficient to cause alterations in end-diastolic pressure or irreversible tissue injury, can evoke a rapid release of BNP from cardiac tissue. Hypoxic perfusion of isolated hearts led to a rapid increase of BNP immunoreactivity in coronary effluent (35). In patients undergoing percutaneous transluminal coronary angioplasty, coronary sinus BNP concentration increased rapidly after balloon deflation (33), and circulating plasma concentrations of BNP were elevated 4.5-fold in patients after episodes of unstable angina (32). A functional role for the rapid release of BNP in response to brief periods of myocardial ischemia is not known. The recognition in recent years that several neurohormonal mediators are released from myocardium during brief periods of ischemia underpins the current mechanistic model of ischemic preconditioning (1, 8, 28). Autocrine and paracrine mediators acting on G protein-coupled receptors, notably adenosine, bradykinin, opioid peptides, and catecholamines, participate in the activation of a multiple-stage signal transduction pathway. This involves opening of ATP-sensitive potassium (K_{ATP}) channels as either a downstream or proximal event essential for conferring resistance to a subsequent episode of ischemia. NPR-A does not couple through G proteins but, through elevation of cGMP, NPR-A activation could modulate K_{ATP} channel activity (16). Thus the signal pathway activated by BNP might represent an alternative prosurvival mechanism. To test the hypothesis that BNP is cytoprotective during ischemia-reperfusion through the opening of K_{ATP} channels, we examined the ability of exogenous BNP to limit irreversible myocardial injury, defining the involvement of K_{ATP} channel opening using pharmacological blockers of K_{ATP} channels.

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MATERIALS AND METHODS

Male Sprague-Dawley rats (300–400 g) were used for these studies. Animals were treated in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. Rat BNP_{1–32} (hereinafter referred to as BNP), glibenclamide, sodium 5-hydroxydecanoate (5-HD), 8-bromo-cGMP (8-Br-cGMP), and triphenyltetrazolium chloride were from Sigma (Poole, UK). HMR-1098 was a gift of Aventis Pharma. All other reagents were of analytic standard.

Isolated Heart Preparation

Rats were anesthetized with pentobarbital sodium (50 mg/kg ip). Heparin (1 IU/g) was administered concomitantly. Excised hearts were perfused retrogradely through the aorta at 11.3 kPa with Krebs-Henseleit buffer [containing (in mmol/l) 118 NaCl, 25 NaHCO₃, 11 glucose, 4.7 KCl, 1.2 MgSO₄·7H₂O, 1.2 KH₂PO₄, and 1.8 CaCl₂·2H₂O; aerated with carbogen, pH 7.3–7.5, at 37°C]. Coronary flow rate (CFR) was determined by timed collection of the coronary effluent. A saline-filled latex balloon connected to a pressure transducer was inserted into the left ventricle (LV), and baseline end-diastolic pressure was set at 5–10 mmHg. Heart rate, LV end-diastolic pressure, and developed pressures were recorded continuously.

Infarct Size Evaluation

A 4-0 silk suture was positioned around the left main coronary artery and threaded through a plastic snare to permit reversible occlusion of the coronary artery. Coronary occlusion was induced for 35 min by clamping the snare onto the heart. Reperfusion was achieved by releasing the snare. At the end of 120-min reperfusion, the coronary artery was reoccluded, and the risk zone was delineated with Evans' blue. Hearts were sectioned (2 mm) and incubated in 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4, 37°C) for 15 min to define white necrotic tissue when fixed in 10% formalin for 24 h. Images of the sections were drawn by an operator blinded to the experimental treatment. Risk zone

areas and infarct-to-risk ratios were determined by computerized planimetry (Planimetry+ version 1.0 for Windows).

Infarction Protocols

The experimental protocols for the three separate infarction studies are illustrated in Fig. 1.

Study 1: BNP concentration-response study. In the control group, hearts were stabilized for 15–20 min and then subjected to 35 min of regional ischemia, followed by 120 min of reperfusion. BNP (10^{-12} – 10^{-8} mol/l) was added to the Krebs-Henseleit buffer, and perfusion was started 10 min before ischemia and continued until 30-min reperfusion.

Study 2: K_{ATP} channel blockade study. BNP (10^{-8} mol/l) was selected after the experiments described above. Hearts were randomized to one of the following experimental groups (Fig. 1): 1) Control group, as described above. Six control hearts were perfused with 0.016% DMSO (the vehicle for glibenclamide). Because there was no effect on infarct size of DMSO, these hearts were combined for statistical evaluation with non-DMSO-treated control hearts to comprise *group 1*. 2) BNP (10^{-8} mol/l) treatment, as described above. 3) 5-HD (10^{-4} mol/l), a blocker of mitochondrial K_{ATP} channels (9, 36), was perfused 10 min before ischemia and continued until 30-min reperfusion. 4) 5-HD (10^{-4} mol/l) + BNP (10^{-8} mol/l), coperfused as described above. 5) Glibenclamide (10^{-6} mol/l), a nonselective blocker of sarcolemmal K_{ATP} and mitochondrial K_{ATP} channels (7, 17, 23, 37), was perfused 10 min before ischemia and continued until 30-min reperfusion. Glibenclamide was dissolved in DMSO (final concentration not more than 0.016%). 6) Glibenclamide (10^{-6} mol/l) + BNP (10^{-8} mol/l), coperfused as described above. 7) HMR-1098 (10^{-5} mol/l), a selective blocker of sarcolemmal K_{ATP} channels (14, 21), was perfused 10 min before ischemia and continued until 30-min reperfusion. 8) HMR-1098 (10^{-5} mol/l) + BNP (10^{-8} mol/l), coperfused as described above.

Study 3: effects of 8-Br-cGMP. In the third infarct study, 8-Br-cGMP, a cell-permeable analog of cGMP, was perfused at 10^{-8} – 10^{-5} mol/l, commencing 10 min before ischemia and continued until 30-min reperfusion. A control group was

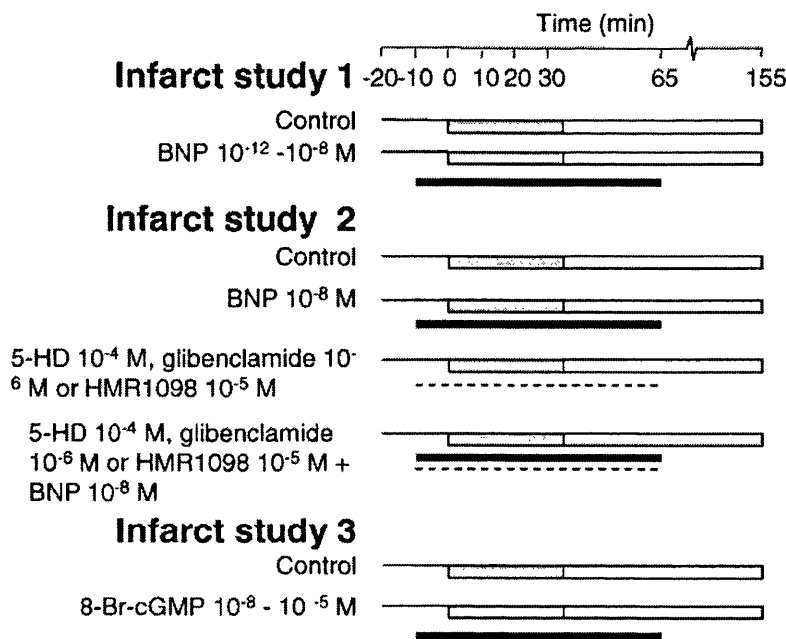


Fig. 1. Experimental protocols for infarct studies. Solid lines, time course of B-type natriuretic peptide (BNP) perfusion or 8-bromo-cGMP (8-Br-cGMP) perfusion; dotted lines, ATP-sensitive potassium (K_{ATP}) channel blocker perfusion; 5-HD, 5-hydroxydecanoate.

subjected to coronary artery occlusion and reperfusion only, as described above.

Myocardial cGMP Concentration

After stabilization, hearts were perfused for 10 min with BNP (10^{-12} – 10^{-8} mol/l) as described above. Ventricular myocardium was then rapidly freeze-clamped to the temperature of liquid nitrogen. After extraction with trichloroacetic acid, the tissue cGMP concentration was determined by radioimmunoassay as described previously (5).

Endogenous BNP Release After Ischemia

After stabilization, during which baseline samples were collected, hearts were rendered globally ischemic for 2, 5, or 20 min. Flow was reinstated, and the coronary effluent was sampled during reflow. Control hearts were perfused without ischemia. The ventricular tissue was immediately frozen in liquid N_2 . Coronary effluent and ventricular tissue samples were analysed for BNP immunoreactivity by radioimmunoassay using a commercially available kit [RIK 9085 BNP-32 (rat), Peninsula Laboratories; San Carlos, CA]. Tissue was extracted with trifluoroacetic acid. The peptide was purified on C18 columns, freeze-dried, and redissolved in "RIA buffer." RIA buffer concentrate was added to the samples of coronary effluent, and aliquots were used for the assay. On the basis of the competition of ^{125}I -labeled BNP and unlabeled BNP binding to a limited amount of specific antibodies, a standard curve was constructed from which the concentration of BNP in the samples was determined.

Statistical Analysis

Data are expressed as means \pm SE. Infarct-to-risk ratios, risk zone volumes, and BNP tissue concentrations were analyzed using one-way ANOVA and Fisher's protected least-significant-difference post hoc test. LV function parameters, CFR, and BNP coronary effluent concentrations were evaluated using repeated-measures ANOVA with Bonferroni's post hoc test. Statistical significance between group means was defined as $P < 0.05$.

RESULTS

Technical Exclusions

A total of 217 animals was used. For the concentration-response infarct experiments, 65 hearts were used, of which 5 hearts were excluded: one was damaged by instrumentation, one had failure of the tetrazolium stain, and three had persistent bradyarrhythmia in the stabilization phase. In the second series of experiments, 55 hearts were used, of which 5 hearts were excluded: two failed to reperfuse, one had an instrumentation error that prevented precise LV function assessment, one had failure of the tetrazolium stain, and one had persistent bradyarrhythmia throughout reperfusion. In the third infarct series, 46 hearts were successfully perfused without exclusion. Therefore, we report the data for 156 successfully completed infarct experiments. An additional 36 hearts were used to examine tissue cGMP concentration, and

15 hearts were used to study the release of endogenous BNP.

Infarct Study 1: BNP Concentration-Response Study

The risk zone volumes were similar among all the groups (Table 1). The control infarct-to-risk zone ratio was $44.8 \pm 4.4\%$ without BNP treatment, consistent with previous results (20). Treatment with BNP limited infarct size in a concentration-dependent manner (Fig. 2). Significant limitation of infarction was observed with BNP (10^{-10} , 10^{-9} , and 10^{-8} mol/l). The highest BNP concentration studied (10^{-8} mol/l) resulted in the smallest infarct size ($20.1 \pm 5.2\%$, $P < 0.01$ vs. control).

Preischemic global coronary flow rate averaged 12.5 ml/min among the six experimental groups. After coronary artery occlusion, there was a decrease in the global CFR of $\sim 35\%$ and a recovery to preischemic values immediately after reperfusion with gradual "rundown" during the remaining 120-min of perfusion (Fig. 3A). The global CFR measured at intervals throughout the protocol did not differ substantially among the experimental groups. There were no detectable differences among the groups in any of the parameters of LV function measured (spontaneous heart rate, developed pressure, or the rate-pressure product; data not presented). Developed pressure and rate-pressure product declined immediately after the onset of coronary occlusion to the same extent in all experimental groups.

Table 1. Risk zone volume in the BNP concentration-response study (study 1), the K_{ATP} channel blocker study (study 2), and the 8-Br-cGMP study (study 3)

Treatment Group	n	Risk Volume, mm ³
<i>Study 1</i>		
Control	11	353 \pm 26
BNP (10^{-8} mol/l)	8	311 \pm 26
BNP (10^{-9} mol/l)	9	309 \pm 26
BNP (10^{-10} mol/l)	8	336 \pm 33
BNP (10^{-11} mol/l)	12	323 \pm 19
BNP (10^{-12} mol/l)	12	337 \pm 15
<i>Study 2</i>		
Control	10	384 \pm 32
BNP (10^{-8} mol/l)	5	345 \pm 13
5-HD (10^{-4} mol/l)	6	339 \pm 29
5-HD + BNP	6	312 \pm 35
Glibenclamide (10^{-6} mol/l)	4	367 \pm 36
Glibenclamide + BNP	6	370 \pm 26
HMR-1098 (10^{-6} mol/l)	5	351 \pm 40
HMR-1098 + BNP	8	348 \pm 34
<i>Study 3</i>		
Control	13	320 \pm 25
8-Br-cGMP (10^{-5} mol/l)	10	402 \pm 14
8-Br-cGMP (10^{-6} mol/l)	9	315 \pm 22
8-Br-cGMP (10^{-7} mol/l)	7	372 \pm 34
8-Br-cGMP (10^{-8} mol/l)	7	329 \pm 27

Values are means \pm SE; n, number of experiments. BNP, B-type (brain or ventricular) natriuretic peptide; K_{ATP} channel, ATP-sensitive K^+ channel; 5-HD, 5-hydroxydecanoate; 8-Br-cGMP, 8-bromo-cGMP. No statistically significant differences between groups were detected (one-way ANOVA).

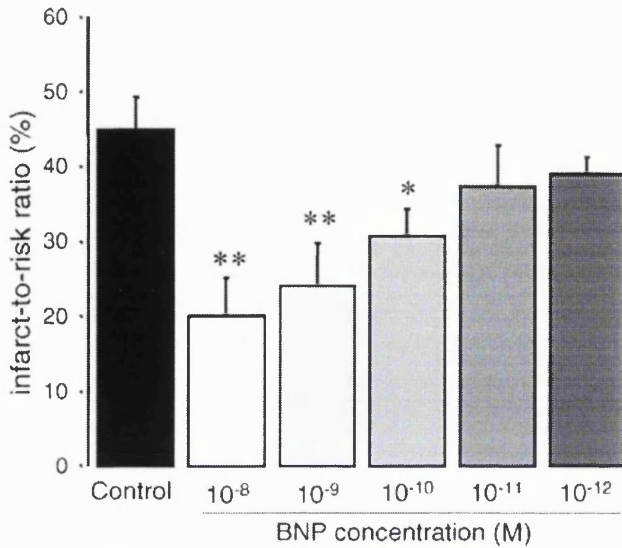


Fig. 2. Infarct-to-risk zone ratios for hearts perfused with BNP (10^{-12} – 10^{-8} mol/l). Bars represent means \pm SE; $n = 8$ – 12 experiments in each group. * $P < 0.05$ and ** $P < 0.01$ vs. control (one-way ANOVA).

Infarct Study 2: K_{ATP} Channel Blockade Study

In the second series of infarct experiments, BNP was applied at a concentration of 10^{-8} mol/l and K_{ATP} channel blockers were coperfused with BNP.

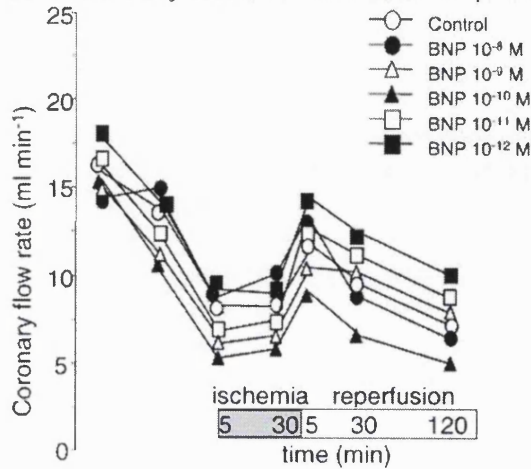
The risk zone volume did not differ among the groups (Table 1). The control infarct-to-risk ratio was $47.1 \pm 2.8\%$ (Fig. 4). BNP (10^{-8} mol/l) treatment resulted in a significant limitation of infarct size ($21.3 \pm 2.8\%$, $P < 0.01$ vs. control). Coperfusion of BNP with either 5-HD or glibenclamide resulted in abolition of the protective effect of BNP (infarct-to-risk ratio: $52.3 \pm 5.7\%$ and $41.0 \pm 7.1\%$, respectively, $P =$ not significant vs. control). However, the infarct size limitation with BNP was not abolished by HMR-1098 (infarct-to-risk ratio: $14.7 \pm 2.7\%$, $P < 0.01$ vs. control and $P =$ not significant vs. BNP). None of the K_{ATP} channel blockers per se influenced infarct size.

As in study 1, the global CFR and LV function were not altered by BNP perfusion or any of the K_{ATP} channel blockers (Fig. 3B).

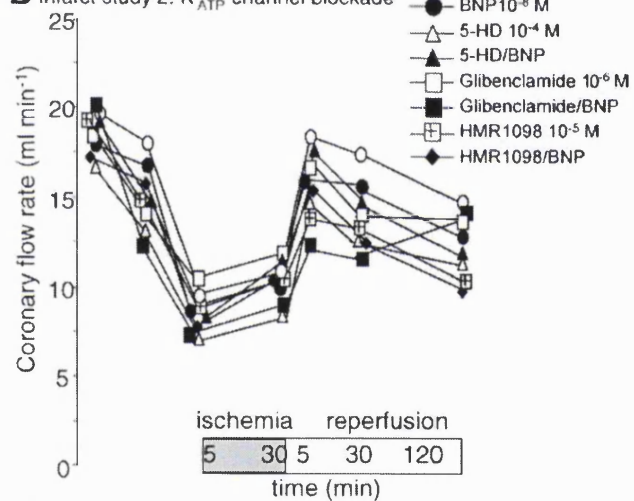
Infarct Study 3: 8-Br-cGMP Study

In the third series of experiments, 8-Br-cGMP was applied across a range of concentrations to examine the role of receptor-independent elevation of intracellular cGMP in myocardial responses to ischemia-reperfusion. Risk zone volume was similar among the experimental groups, and the control infarct-to-risk ratio was $38.7 \pm 3.6\%$. We observed a paradoxical inverse concentration response with increasing concen-

A Infarct study 1: BNP concentration - response



B Infarct study 2: K_{ATP} channel blockade



C Infarct study 3: 8-Br-cGMP

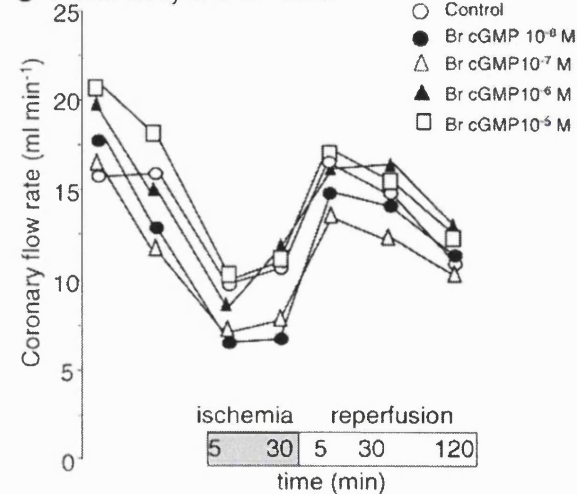


Fig. 3. Global coronary flow rate data for the BNP concentration-response study (A); K_{ATP} channel blockade study (B); and 8-Br-cGMP study (C). Symbols indicate mean values; SE bars have been removed for clarity.

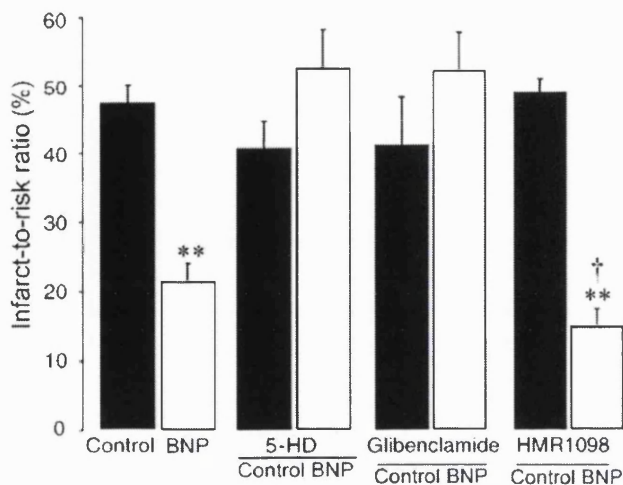


Fig. 4. Infarct-to-risk zone ratios for hearts perfused with K_{ATP} channel blockers with and without BNP (10^{-8} mol/l). Bars represent means \pm SE; $n = 4-10$. ** $P < 0.01$ vs. control; † $P < 0.01$ vs. HMR-1098 control (one-way ANOVA).

trations of 8-Br-cGMP (Fig. 5). Statistically significant limitation of infarction was observed with the lowest concentrations examined (10^{-7} mol/l: $23.1 \pm 6.3\%$, $P < 0.05$ vs. control; 10^{-8} mol/l: $20.8 \pm 4.2\%$, $P < 0.05$ vs. control). However, treatment with higher concentrations did not result in a significant limitation of infarction.

Perfusion with 8-Br-cGMP at 10^{-5} and 10^{-6} mol/l resulted in a nonsignificant increase in the preischemic CFR (Fig. 3C; 16.0 ± 1.0 ml/min in the control group vs. 19.5 ± 1.1 and 20.3 ± 1.1 ml/min in the 10^{-5} and 10^{-6} mol/l 8-Br-cGMP-treated groups, respectively, $P =$ not significant). These influences of 8-Br-cGMP were not observed with the lower, infarct-limiting concentrations of the agent. No consistent patterns of

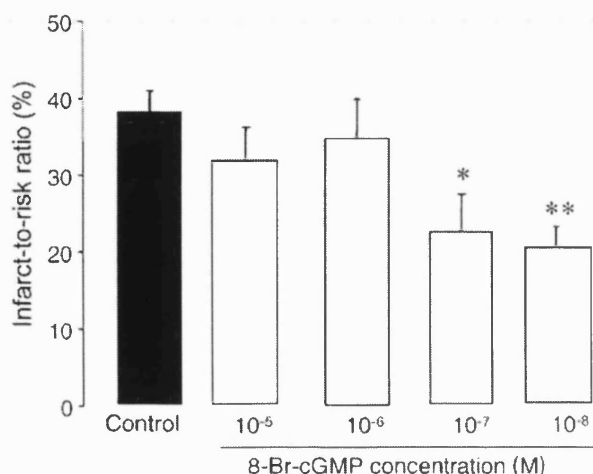


Fig. 5. Infarct-to-risk zone ratios for hearts perfused with 8-Br-cGMP. Bars represent means \pm SE; $n = 7-13$. * $P < 0.05$ and ** $P < 0.01$ vs. control (one-way ANOVA).

8-Br-cGMP treatment on LV contractility were observed during the perfusion protocol.

Effect of BNP on Myocardial cGMP Concentration

Perfusion with BNP for 10 min caused a concentration-dependent increase of the cGMP concentration in ventricular myocardium (Fig. 6). In control tissue, at the time point corresponding to the onset of myocardial ischemia, the cGMP concentration was 11.6 ± 0.5 pmol/g wet wt ($n = 9$). Significant increases in the tissue cGMP concentration were observed after perfusion with 10^{-9} mol/l BNP (cGMP: 20.3 ± 5.2 pmol/g wet wt, $n = 5$, $P < 0.05$) and 10^{-8} mol/l BNP (cGMP: 28.6 ± 1.5 pmol/g wet wt, $n = 8$, $P < 0.01$). Lower concentrations of BNP did not cause statistically significant elevation of the myocardial cGMP concentration.

BNP Release and Ventricular Myocardial BNP Concentrations

We observed a low basal release of BNP into the coronary effluent in the range of 1.1–9.7 pmol/l (see Fig. 7). In the control group (no ischemia), this efflux of BNP remained stable throughout 35 min of perfusion. Peak postischemic BNP concentrations in the coronary effluent were related to the duration of preceding ischemia: 2-min ischemia, 11.0 ± 2.4 pmol/l; 5-min ischemia, 20.1 ± 2.2 pmol/l; and 20-min ischemia, 41.5 ± 3.3 pmol/l (all $P < 0.05$ vs. control values; Fig. 7). Global ischemia for 2 and 5 min was not associated with changes in end-diastolic pressure above baseline. Hearts subjected to 20-min ischemia displayed an increase in end-diastolic pressure of ~ 10 mmHg above baseline values. Myocardial BNP concentration was 1.58 ± 0.16 pmol/g wet wt in control hearts and 1.77 ± 0.16 , 3.69 ± 0.65 ($P < 0.05$ vs. control), and 1.82 ± 0.14 pmol/g wet wt, respectively, in hearts subjected to 2-, 5-, or 20-min global ischemia with 5-min reperfusion ($n = 3-4$ hearts/group).

DISCUSSION

The present investigation provides two principal new findings. First, acute infusion of exogenous BNP

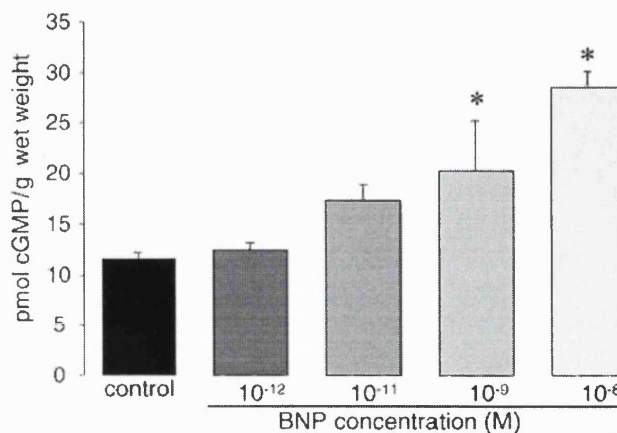


Fig. 6. Ventricular cGMP concentration after 10-min perfusion with BNP. Bars represent means \pm SE; $n = 5-10$. * $P < 0.01$ vs. control.

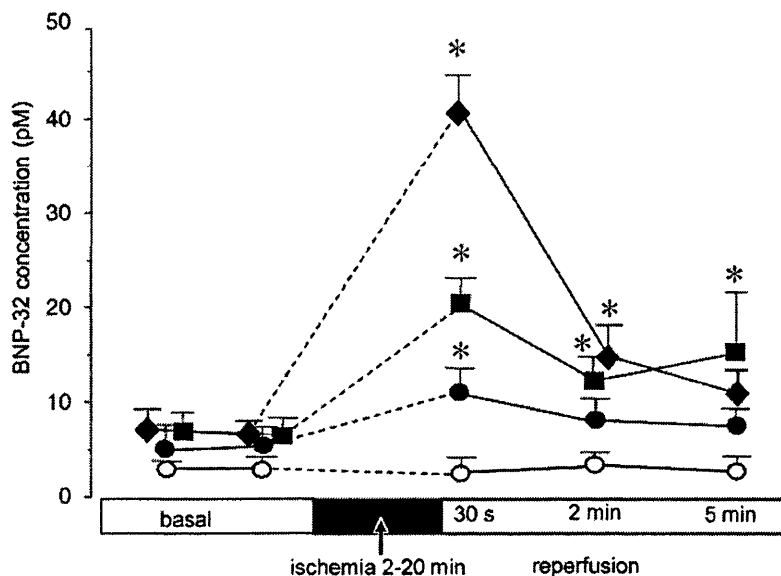


Fig. 7. Release of BNP into coronary effluent during control perfusion (○; $n = 3$), after 2-min ischemia (●; $n = 4$), 5-min ischemia (■; $n = 4$), or 20-min ischemia (◆; $n = 4$). * $P < 0.05$ vs. the corresponding control value (repeated-measures ANOVA).

is markedly protective against myocardial ischemia-reperfusion injury, leading to concentration-dependent infarct size limitation. Second, the mechanism of protection afforded by BNP is associated with elevation of cGMP and appears to involve K_{ATP} channel opening. The pharmacological selectivities of the widely used K_{ATP} channel blockers, applied at conventional inhibitory concentrations, may indicate involvement of the mitochondrial K_{ATP} channel rather than the sarcolemmal K_{ATP} channel. Although recent studies have reported that ANP (30) and the related noncardiac peptide urodilatin (27) limit infarct size in vivo, this is the first study to show the cardioprotective effect of BNP and to provide evidence of a primary mechanism of action of a natriuretic peptide on myocardium mediated by K_{ATP} channel activation.

The cell surface receptor mediating the biological actions of BNP is a particulate guanylyl cyclase A receptor, NPR-A, abundantly expressed in cardiac tissue (2, 29). Unlike receptors for adenosine, bradykinin, and opioids, the NPR-A receptor is not G protein coupled. This receptor contains an intracellular guanylyl cyclase catalytic domain that mediates most of the biological action of the natriuretic peptide through the conversion of GTP to cGMP (4, 13, 20). We observed significant increases in the ventricular cGMP concentration after 10-min perfusion with BNP (10^{-9} or 10^{-8} mol/l). Although this observation is consistent with NPR-A activation, we are unable to conclude at present that the infarct-limiting action of BNP in ischemic myocardium is a receptor-mediated action. Unfortunately, reliable NPR-A inhibitors such as HS-142-1 and A71915 are not available in sufficient quantity to undertake perfused heart or in vivo studies. Approaches using cultured cardiac myocytes may be feasible but may not accurately model endogenous protective mechanisms in intact tissue where there are interactions among several cell types and humoral

mediators. Alternative approaches using mutant mouse strains with targeted deletion of either pro-BNP or NPR-A genes may also be possible in future studies. However, interpretation of findings from such animals, in the absence of complementary pharmacological data, may be clouded by the uncertainties of altered expression of other gene products, high levels of redundancy in signaling pathways, and the spontaneous development of cardiac pathology in these animals. For example, Izumi et al. (12) recently reported that mice with targeted deletion of the NPR-A gene sustained infarcts that were 20% smaller than wild-type control animals. However, the mutant animals had a substantial degree of LV hypertrophy. Moreover, interpretation of this study is predicated on the observation that natriuretic peptides upregulated vascular adhesion molecule expression in vitro, an effect that has not been demonstrated in humans or in intact animal models.

Our observation that the synthetic cGMP analog 8-Br-cGMP evoked, at low concentrations, an infarct-limiting effect similar to that observed with BNP is consistent with the notion that elevation of intracellular cGMP may indeed be a mechanism that is central to the cardioprotective action of BNP. cGMP elevation has been proposed to be a mechanism of injury limitation in ischemic myocardium (26), but the distal molecular mechanisms resulting in enhanced tolerance to ischemic injury associated with cGMP elevation are unclear. Proposed mechanisms include inhibition of L-type calcium channel opening (18), decreased intracellular concentrations of cAMP through a feedback mechanism and stimulation of cAMP phosphodiesterase (11, 18, 22), inhibition of the mitochondrial permeability transition pore (31), and opening of K_{ATP} channels (16). However, the unexpected finding that higher concentrations of 8-Br-cGMP (similar to those frequently applied in isolated cell and tissue pharmacol-

ogy) were not protective is intriguing and unexplained at present. Previous work has reported that high concentrations of cGMP are associated with cell injury. For example, Nakamura et al. (25) reported that the cytotoxic effects of a NO donor in a pheochromocytoma line were augmented by a cell-permeable cGMP analog. A role of cGMP and cGMP-dependent protein kinase (cGK) in mediating apoptosis of pancreatic β -cells has been reported (19). Tepperman et al. (34) extended these observations to rat intestinal epithelial cells, showing that dibutyl cGMP at millimolar concentrations reduced cell viability in culture. This cytotoxic effect of high intracellular concentrations of cGMP may be related to the generation of reactive oxygen species, because superoxide dismutase attenuated the injurious effects of the cGMP analog in intestinal epithelial cells. Thus our apparently paradoxical observation that increasing concentrations of 8-Br-cGMP were not associated with infarct limitation, whereas low concentrations were protective, may reflect an ambivalent effect of cGMP, both pro-survival and pro-injury effects being mediated by this second messenger depending on concentration and pathophysiological context.

In myocardium, sarcolemmal K_{ATP} channels were originally postulated to participate in salvage from irreversible ischemia-reperfusion injury, because their opening would produce an increase in the outward potassium current leading to shortening of action potential duration, which would in turn reduce the Ca^{2+} influx through voltage-dependent Ca^{2+} channels and increase the time during which the Na^+/Ca^{2+} exchanger would operate to extrude Ca^{2+} from the cell. Since 1998, attention has focused on mitochondrial K_{ATP} channels in both ischemic preconditioning and pharmacological preconditioning studies (28). Much of the evidence implicating a role of mitochondrial K_{ATP} channels is reliant on the reputed selectivity of pharmacological agents such as 5-HD (a blocker of mitochondrial K_{ATP} channels) and HMR-1098 (a blocker of sarcolemmal K_{ATP} channels). With the caution that pharmacological specificity and selectivity may be subject to revision, our study provides pharmacological evidence for involvement of a K_{ATP} channel subtype, possibly a mitochondrial K_{ATP} channel, in the infarct-limiting action of BNP. Further studies in appropriate isolated cell and mitochondrial preparations using biophysical approaches are now being planned to probe the specific involvement of mitochondrial K_{ATP} channel opening.

While not constituting proof of mechanism, the association between concentration-dependent elevation of cGMP by BNP and infarct limitation that we observed leads us to hypothesize a role for cGK-I (protein kinase G). The cGMP/cGK-I pathway could promote K_{ATP} channel opening, representing an alternative signal cascade to the widely studied G protein receptor-coupled-protein kinase C pathway. Indeed, recent studies support the concept that K_{ATP} channel activation may be promoted by cGK in a variety of cell types, including ventricular myocytes (10). The contribution of cGK signaling to mitochondrial K_{ATP} channel activation

and infarct limitation after BNP treatment will be the subject of further studies, using mice with targeted deletion of cardiac cGK-I.

BNP is a vasodilator in several vascular beds including coronary epicardial conductance arteries and coronary microvessels (3, 40). A surprising finding in our studies was the absence of any gross alterations in the global CFR secondary to BNP-mediated coronary vasodilatation. It is likely that a more consistent and marked effect would be observed at higher concentrations than those used in our studies. The highest concentration of BNP we used (10^{-8} mol/l) is at the threshold for vasorelaxation in rat aortic rings (13). At present, we must conclude that the protective effect of BNP on ischemic myocardium is apparently independent of coronary vasodilatation or collateral vessel recruitment, because the rat heart is devoid of native coronary collateral vessels. It is of interest that in a previous study examining the coronary vasodilator mechanisms of ANP in a constant flow preparation, ANP reduced coronary perfusion pressure, an effect sensitive to inhibition by N^{ω} -nitro-L-arginine methyl ester (40). The possibility that BNP-associated cardioprotection may be related, at least in part, to NO generation and activation of soluble guanylyl cyclase is currently being investigated in our laboratory.

We observed that postischemic release of endogenous BNP increased in a graded fashion with ischemia severity. Moreover, the increase of tissue BNP after 2- and 5-min ischemia likely reflects cleavage of the stored propeptide in response to ischemia; after a 20-min ischemic stimulus, tissue levels of BNP were reduced as a consequence of massive release of the peptide. The immediate stimulus to BNP release could be either ischemia per se or local tissue deformation as a result of ischemia. At present, we are unable to comment on this except to say that we observed graded release of BNP after ischemia that was not associated with substantial changes in end-diastolic pressure. It is impossible to directly relate the concentrations of BNP in coronary effluent to the concentrations required to protect against infarction. Although the coronary effluent concentrations were two to three orders of magnitude less than the protective concentrations infused, local interstitial concentration during ischemia, when there is no flow and thus no washout, would be considerably higher than that detected in the coronary effluent during reflow. Thus changes in the coronary effluent concentrations reflect changes in the interstitial concentrations but cannot predict the interstitial concentrations.

In conclusion, this study is the first to demonstrate a cardioprotective effect of exogenous BNP against ischemia-reperfusion injury. The abrogation of this protective effect by glibenclamide and 5-HD, but not by HMR-1098, is consistent with, but does not constitute proof of, a mechanism involving opening of the putative mitochondrial K_{ATP} channel. Although we postulate that elevation of cGMP with activation of cGK-I is a plausible mechanism of K_{ATP} channel opening, the signaling pathway underlying this newly defined ac-

tion of BNP requires further elucidation, as does the involvement of NPR-A activation. Further studies in vivo and in other species are indicated to elucidate fully the cytoprotective potential of BNP and cGMP signaling in myocardial ischemia-reperfusion, especially the therapeutic application of recombinant BNP and inhibitors of neutral endopeptidase, the major enzymatic route for BNP degradation.

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EDITORIAL

B Type natriuretic peptide: a good omen in myocardial ischaemia?

S P D'Souza, G F Baxter

Heart 2003;89:1-3

Although BNP is now firmly established as a diagnostic and prognostic marker of ventricular dysfunction, its autocrine and paracrine actions within the heart have received less attention

The natriuretic peptide family consists of a number of structurally homologous but genetically distinct polypeptide mediators. The peptides are highly conserved across mammalian and invertebrate species and, in humans, are released from various tissues in response to physiological and pathological stimuli. All the peptides have at their core a 17-amino acid disulfide ring essential for receptor recognition and biological function. [AQ:1]

The first description of secretory granules in the cardiac atria was given in 1956, to be followed by the physiological characterisation of an atrial natriuretic factor in the work of de Bold and colleagues in 1981.¹ Several further natriuretic peptides have subsequently been identified. These include B type natriuretic peptide (BNP) predominantly from ventricular myocardium (although originally isolated from pig brain)² and C type natriuretic peptide (CNP) expressed in the nervous system and endothelial cells.³ A homologue of the natriuretic factor, DNP, in the venom of the green mamba *Dendroaspis angusticeps*, is found in plasma and atrial myocardium of normal humans.⁴ Urodilatin is a renal derived natriuretic peptide.⁵ ANP and BNP, the predominant natriuretic peptides in mammalian cardiomyocytes, are stored within secretory granules as pro-peptides, pro-ANP and pro-BNP, that are post-translationally modified by peptide bond cleavage to form the mature circulating peptides and amino (N)-terminal residues.⁶

Biological activity of the peptides generally resides in the mature carboxy-terminal residues, although pro-ANP and pro-BNP may exhibit some activity. The (N)-terminal residues are generally inactive. Relatively little is known about the processing of BNP in the circulation. The peptides are eventually cleared by the natriuretic peptide-C receptor (NPR-C)—a clearance receptor. Binding of natriuretic peptides to NPR-C leads to internalisation and degradation.⁷ Neutral endopeptidase 24.11 (NEP) also takes part in the inactivation of natriuretic peptides and there is a minor contribution from renal excretion. Both NPR-C and NEP are widely expressed in the kidneys, lungs, and the vascular wall.

A variety of physiological and pathological stimuli evoke the release of ANP and BNP. Such stimuli include exercise, hypoxia, ischaemia,

increased wall stress and dilatation of the cardiac chambers, and sepsis. The classical endocrine actions of ANP and BNP, namely vasodilatation and natriuresis, are mediated by interaction with a natriuretic peptide receptor (NPR)-A which is a particulate guanylyl cyclase (pGC). This cyclase subserves the same biochemical function as soluble guanylyl cyclase (sGC)—the principal intracellular receptor for NO—namely, the conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP). NPR-A is widely expressed in cardiovascular tissues, and its activation by ANP and BNP leads to elevation of intracellular cGMP and activation of the cGMP dependent protein kinase (PKG-I).⁷ CNP engages another receptor subtype, NPR-B, also a pGC receptor, while DNP acts via NPR-A, mediating a predominant vasorelaxing action.⁸

BNP RELEASE IN CARDIAC DYSFUNCTION AND MYOCARDIAL ISCHAEMIA

BNP is released following left ventricular overloading and wall stress, underpinning its diagnostic and prognostic value in patients with heart failure.⁹ Other cardiac pathologies including hypertensive heart disease, atrial fibrillation, and valvar heart disease are also associated with elevation of circulating BNP.¹⁰ Experimental and clinical myocardial infarction is clearly associated with gradual and sustained elevation of tissue and circulating BNP. BNP is deemed to be a strong independent predictor of prognosis in patients with heart failure and following myocardial infarction.^{11, 12} However, the rapid release of BNP following very brief periods of ischaemia, insufficient to provoke irreversible tissue injury or pronounced changes in end diastolic volume, is of considerable interest. Elevated concentrations of BNP in humans are detected following unstable angina¹³ and immediately after short balloon inflations during coronary angioplasty.¹⁴ In the rat heart, experimental ischaemia of as little as two minutes leads to an immediate increase of BNP outflow, which correlates with the duration of ischaemia.¹⁵ Such observations are a stimulus to identifying the functional role of BNP in the ischaemic myocardium.

Abbreviations: ANP, atrial natriuretic peptide; BNP, B type or brain natriuretic peptide; CNP, C type natriuretic peptide; DNP, *Dendroaspis* natriuretic peptide; K_{ATP} , ATP dependent potassium channel; NEP 24.11, neutral endopeptidase; NO, nitric oxide; NPR, natriuretic peptide receptor; pGC, particulate guanylyl cyclase; PKG-I, cGMP-dependent protein kinase subtype I; sGC, soluble guanylyl cyclase

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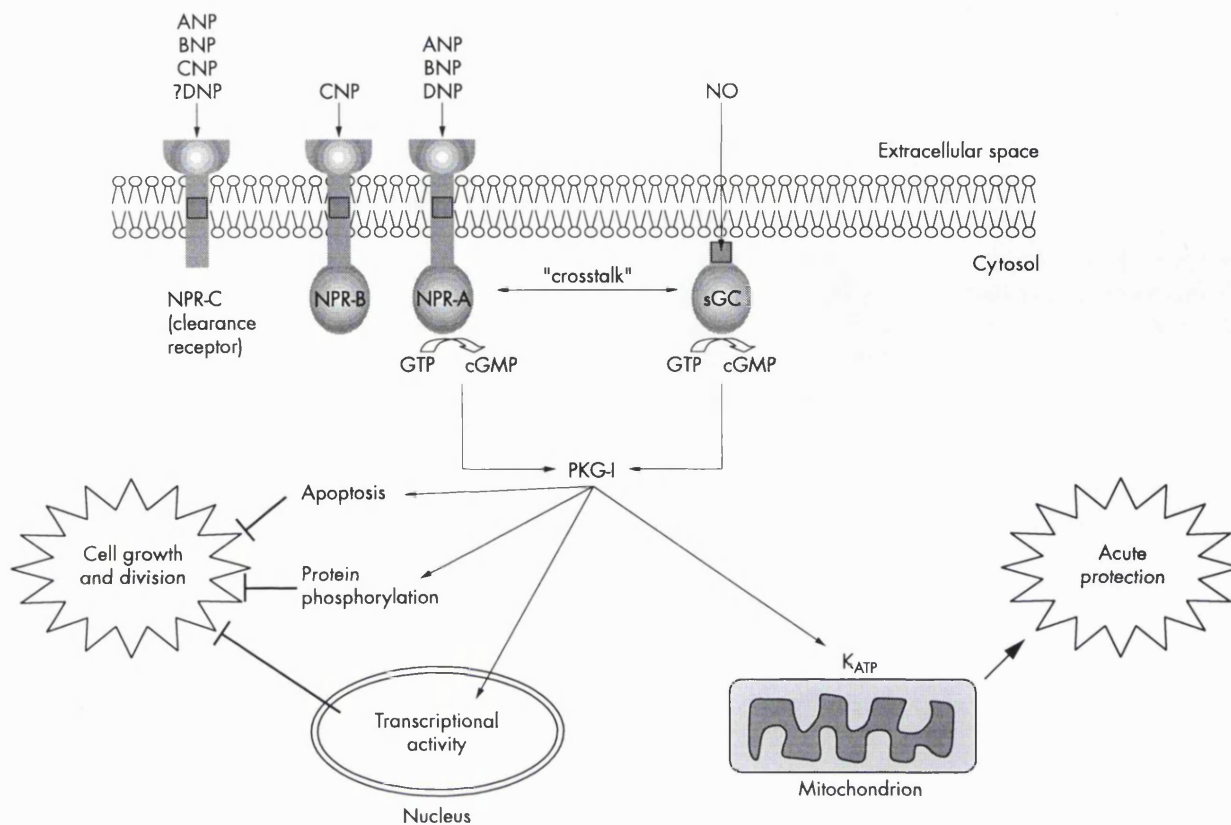


Figure 1 Mechanism of acute protective effect of BNP.

BNP MODIFIES ACUTE ISCHAEMIC INJURY

Myocardial ischaemia triggers the release of a number of endogenous cytoprotective mediators. These include adenosine, opioid peptides, and bradykinin¹⁶. However, the role of BNP in modifying acute myocardial ischaemic injury has only recently been identified. In a rat heart model of acute myocardial infarction, exogenously administered BNP limited infarct size in a concentration dependent manner.¹⁵ This action was independent of the peptide's endocrine actions that lead to systemic haemodynamic unloading, or to recruitment of native coronary collateral vessels, pointing to a primary cytoprotective action. The mechanism of this acute protective effect of BNP appears to be associated with an increase of the second messenger cGMP and involves opening of ATP sensitive potassium (K_{ATP}) channels (fig 1). Pharmacological evidence suggests that this channel may be a mitochondrial K_{ATP} channel, rather than the sarcolemmal K_{ATP} channel. There is further evidence that the nitric oxide/soluble guanylyl cyclase (NO/sGC) system may also play a role in the anti-ischaemic actions of BNP that we have described.¹⁷ There is recent evidence for a "cross-talk" between the BNP/NPR-A pathway and the NO/sGC pathway in vascular cells and this may also be relevant to the myocardium. [AQ:2]

The mitochondrial K_{ATP} channel has been prominent recently in relation to protection of the ischaemic myocardium by preconditioning and other protective strategies. Many endogenous protective mediators are thought to produce their anti-ischaemic actions through mitochondrial K_{ATP} channel opening, so why should BNP be regarded differently from, say, adenosine or bradykinin? One reason is the novel molecular signalling pathway employed by BNP. We postulate that BNP/NPR-A induced elevation of cGMP with activation of cGMP-dependent protein kinase-I (cGK-I or protein kinase-G-I) is a plausible mechanism of K_{ATP} channel opening via the cGMP/cGK-I pathway, and may therefore represent an alterna-

tive signalling cascade resulting in tissue protection and salvage. Most of the endogenous mediators of cardioprotection studied so far act on G-protein-coupled receptors. In several disease states, including cardiac hypertrophy and cardiac failure, there may be downregulation of G-protein-coupled receptor responses. Under these conditions, the BNP/NPR-A signalling pathway could act as a very effective reserve salvage pathway.

BNP MAY INFLUENCE POSTINFARCTION REMODELLING

The sustained elevation of cardiac BNP expression following myocardial infarction is likely to be a reflection of important counter-regulatory actions of natriuretic peptides on cell growth and proliferation. These actions may include inhibition of fibroblast activity and an antihypertrophic effect on cardiac myocytes.

Following infarction, ANP and BNP exert potent actions on cardiac fibroblasts. Both peptides inhibit collagen synthesis by cardiac fibroblasts in response to hypoxia *in vitro* and they inhibit fibroblast proliferation in response to angiotensin II stimulation.¹⁸ Recently, multifocal cardiac fibrosis has been described in mice with deletion of the *bnp* gene.¹⁹ NPR-A^{-/-} knockout mice also develop hypertension with an exaggerated cardiac hypertrophy and fibrosis.²⁰ Limited information is available on the role of natriuretic peptides in regulating cardiomyocyte growth. In cultured cardiac myocytes, natriuretic peptides inhibit hypertrophy and in vascular smooth muscle cells, BNP and CNP inhibit growth and proliferation. The mechanism of growth inhibition is far from clear but may involve an inhibitory action on the mitogen activated protein kinase cascade.⁶ Another action of natriuretic peptides that may be relevant to tissue remodelling is the ability to stimulate apoptosis. However, this issue is presently unresolved. Although cyclic nucleotides such as cGMP, the principal

second messenger in the BNP/NPR-A signalling pathway, are known to regulate the processes of cell proliferation, differentiation, and apoptosis, there are currently no clear patterns to predict how cGMP could modify apoptosis in ischaemic myocardium.

CONCLUSION

In conclusion, BNP is now firmly established as a diagnostic and prognostic marker of ventricular dysfunction, but its autocrine and paracrine actions within the heart have received less attention. Experimental evidence suggests that BNP is a pleiotropic peptide and its salutary effects in acute ischaemia and following infarction go beyond its classical natriuretic and cardiac unloading effects. Therapeutically, this range of actions could be of great benefit. Conceivably, the therapeutic role for recombinant human BNP (nesiritide), currently indicated in the USA for acute decompensated heart failure,²¹ may be extended to limit damage from unstable angina and acute myocardial infarction.

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Author Queries [AQ:]

1. Author: a short introduction has been added. Please amend if necessary.
2. Author: there was no caption for fig 1. Please amend current caption as necessary.
3. Author: any updates for refs 15 and 17

