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CHARACTERISATION OF THE ANTI-INFLAMMATORY AND ANTI-PROLIFERATIVE EFFECTS OF NATRIURETIC PEPTIDES IN RODENTS

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DECLARATION OF OWNERSHIP

I, Catherine Maria Panayiotou, declare that this thesis is the result of my own work. All help and advice has been acknowledged and primary and secondary sources of information have been properly attributed.

ABSTRACT

Natriuretic peptides are a family of vasoactive hormones that play important roles in cardiovascular homeostasis. These peptides exert biological effects primarily via activation of guanylate cyclase (GC)-coupled natriuretic peptide receptors (NPR) that generate the intracellular messenger cyclic guanosine 3',5'-monophosphate (cGMP). Activation of the cytosolic GC by NO is well-established to mediate cGMP-dependent, anti-atherogenic effects; however, an analogous cytoprotective role for natriuretic peptides has yet to be fully elucidated. Since many cardiovascular disorders (e.g. atherosclerosis, septic shock) are now accepted as inflammation-based diseases, identification of potential roles for natriuretic peptides in regulating vascular inflammation might assist in the prevention and treatment of cardiovascular pathology.

The studies described in this thesis investigated the hypothesis that natriuretic peptides (i.e. atrial natriuretic peptide [ANP], C-type natriuretic peptide [CNP]) affect pro-inflammatory protein expression (i.e. inducible NO synthase, iNOS) and cell proliferation via activation of GC-linked NPR.

Herein, it is demonstrated that in NPR-A knockout mice, iNOS expression and NO production in response to intravenous administration of bacterial lipopolysaccharide is significantly reduced compared to wild-type controls; this difference is mirrored in the *ex vivo* functional reactivity of vessels from such animals. However, neither ANP nor CNP were able to alter iNOS expression or NO production *in vitro* in RAW264.7 murine macrophages or primary rat aortic smooth muscle cells. CNP, but not ANP, transiently enhanced phosphorylation of extracellular signal-regulated kinase (ERK)1/2. CNP-induced ERK 1/2 phosphorylation was blocked by the selective ERK 1/2 inhibitor PD98059, the G_i-protein inhibitor Pertussis toxin, and the selective NPR-C antagonist M372049. Accordingly, CNP inhibited vascular smooth muscle proliferation in a PD98059- and M372049-reversible manner.

These observations suggest that part of the anti-atherogenic profile of CNP is mediated via NPR-C, G_i-dependent ERK 1/2 phosphorylation and inhibition of vascular smooth muscle proliferation. Moreover, my findings identify a potential pro-inflammatory role for ANP/NPR-A-dependent signalling *in vivo*.

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PUBLICATIONS

Work described in this thesis has given rise to the following manuscripts:

1. Inmaculada C. Villar, **Catherine M. Panayiotou**, Adil Sheraz, Melanie Madhani, Ramona S. Scotland, Muriel Nobles, Barbara Kemp-Harper, Amrita Ahluwalia & Adrian J. Hobbs (2007). Definitive role for natriuretic peptide receptor-C in mediating the vasorelaxant activity of endothelium-derived C-type natriuretic peptide. *Cardiovasc. Res.*, **74**:515-525

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1^{o}	– Primary
8-pCPT-cGMP	– 8-(4-chlorophenylthio)-cGMP
aa	– Amino acids
ACh	– Acetylcholine
Ang II	– Angiotensin II
ANP	– Atrial natriuretic peptide
AP-1	– Activating protein-1
ASK	– Apoptosis signal-regulating kinase
ATF-2	– Activated transcription factor-2
ATP	– Adenosine 5'-triphosphate
BH_4	– Tetrahydrobiopterin
BMI	– Body mass index
BNP	– Brain natriuretic peptide
BrdU	– 5-bromo-2'-deoxyuridine
C/EBP	– CCAAT/enhancer-binding protein
CaM	– Calmodulin
cAMP	– Cyclic adenosine 3',5'-monophosphate
cGK	– cGMP-dependent protein kinase
cGMP	– Cyclic guanosine 3',5'-monophosphate
CNG	– Cyclic nucleotide-gated
CNP	– C-type natriuretic peptide
COX-1	– Cyclooxygenase-1
COX-2	– Cyclooxygenase-2
CREB	– cAMP response element binding protein
CXCR4	– Chemokine receptor 4
DAPI	– 4',6-diamidino-2-phenylindole
DLK	– Dual leucine zipper-bearing kinase
DMEM	– Dulbecco's modified eagle medium
DMSO	– Dimethyl sulphoxide
DNA	– Deoxyribonucleic acid
DNP	– Dendroaspis natriuretic peptide
dNTP	– Deoxynucleotide triphosphate
ECL	– Enhanced chemiluminescence
EDHF	– Endothelium-derived hyperpolarising factor
EDRF	– Endothelium-derived relaxing factor
EDTA	– Ethylenediamine-tetraacetic acid
EGF	– Epidermal growth factor
EGTA	– Ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
eIF-4E	– Eukaryotic initiation factor 4E
eNOS	– Endothelial nitric oxide synthase
ERK	– Extracellular signal-regulated kinases
FAD	– Flavin adenine dinucleotide
FCS	– Foetal calf serum
FDA	– Food and drug administration
FGF	– Fibroblast growth factor
FITC	– Fluorescein isothiocyanate

FMN	– Flavin mononucleotide
g	– Relative centrifugal force
GC-A	– Guanylate cyclase-A
GC-B	– Guanylate cyclase-B
GIRK	– G-protein gated inward rectifying K^+ channels
GM-CSF	– Granulocyte-macrophage colony-stimulating factor
gp 96	– Glycoprotein 96
G-proteins	– Regulatory guanine nucleotide binding proteins
GTN	– Glyceryl trinitrate
GTP	– Guanosine 5'-triphosphate
h	– Hours
HEPES	– N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMG	– High mobility group
HPB	– (3E)-4-(2-hydroxyphenyl)but-3-en-2-one
Hsp	– Heat shock protein
Hsp 1	– Heat shock factor transcription factor 1
Hsp27	– Heat shock protein 27
HUVEC	– Human umbilical vein endothelial cells
ICAM-1	– Intercellular adhesion molecule-1
ICU	– Intensive care unit
IFN-γ	– Interferon-γ
IKK	– Inhibitors of κB kinase
IL-1R	– IL-1 receptor
IL-1β	– Interleukin-1β
iNOS	– Inducible nitric oxide synthase
IP_3	– Inositol triphosphate
IRAK	– IL-1 receptor associated protein kinase
IRF	– Interferon regulatory factor
IκB	– Inhibitory κB subunit
JAK	– Janus tyrosine kinase
JNK/SAPK	– C-Jun N-terminal kinases/stress activated protein kinases
K_{ATP}	– ATP sensitive potassium
KHD	– Kinase homology domain
KO	– Knockout
LBP	– LPS-binding protein
LDL	– Low density lipoprotein
L-NAME	– N^G-nitro-L-arginine methyl ester
L-NMA	– N^G-methyl-L-arginine
LPS	– Lipopolysaccharide
LRR	– Leucine-rich repeats
LSP1	– Lymphocyte specific protein-1
Mal	– MyD88-adaptor-like
MAPK	– Mitogen-activated protein kinase
MCP-1	– Monocyte chemotactic protein-1
M-CSF	– Macrophage colony stimulating factor
MD	– Myeloid differentiation protein
MEK	– MAPK/ERK kinase
MEKK	– MAPK/ERK kinase kinase
min	– Minutes
MKK	– MAPK kinase

MKP-1	– MAPK phosphatase-1
MLK	– Mixed lineage kinase
Mnk	– MAP kinase-interacting kinase
mRNA	– Messenger ribonucleic acid
Msk	– Mitogen and stress activated protein kinases
MyD88	– Myeloid differentiation factor-88
NADH	– Nicotinamide adenine dinucleotide
NADPH	– Nicotinamide adenine dinucleotide phosphate
NF-κB	– Nuclear factor-κB
NIK	– NF-κB inducing kinase
NLK	– Nemo-like kinase
nNOS	– Neuronal nitric oxide synthase
NO	– Nitric oxide
NO_2^-	– Nitrite
NO_3^-	– Nitrate
NOD	– Nucleotide oligomerisation domain
NOHA	– N^G-Hydroxy-L-arginine
NOS	– Nitric oxide synthase
NO_x	– Total [NO_2^-] and [NO_3^-]
NPR	– Natriuretic peptide receptor
NT-proBNP	– Amino-terminal pro-brain natriuretic peptide
O_2^-	– Superoxide anion
ODQ	– 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
$ONOO^-$	– Peroxynitrite
PAF	– Platelet-activating factor
PBS	– Phosphate buffered saline
PD98059	– 2′-amino-3′-methoxyflavone
PDE	– Phosphodiesterase
PDGF	– Platelet derived growth factor
PDTC	– Pyrrolidine dithiocarbamate
pGC	– Particulate guanylate cyclase
PGE-2	– Prostaglandin E_2
PI3K	– Phosphoinositide-3-kinase
PKC	– Protein kinase C
PKG	– Protein kinase G
PLC	– Phospholipase C
PMN	– Polymorphonuclear neutrophils
PTB	– Phosphotyrosine-binding
RAoSMC	– Rat aortic smooth muscle cells
RNA	– Ribonucleic acid
ROS	– Reactive oxygen species
RP105	– Radioprotective 105
RPMI	– Roswell park memorial institute
RSK	– Ribosomal S6 kinase
RTK	– Receptor tyrosine kinases
RT-PCR	– Reverse transcriptase polymerase chain reaction
SB203580	– 4-(4-Fluoreophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole
SDS-PAGE	– Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sGC	– Soluble guanylate cyclase

SH2	– Src-homology 2
SIRS	– Systemic inflammatory response syndrome
SPER-NO	– Spermine NONOate
SRC-1	– Steroid receptor coactivator-1
SRE	– Serum response element
STAT	– Signal transducers and activators of transcription
TAE	– Tris-acetate Na_2EDTA
TAK	– TGF-β-activated protein kinase
TAO	– Thousand and one amino acid kinase
TCF	– Ternary complex factor
TEMED	– N,N,N',N'-tetramethylethylenediamine
TF	– Thrombotic protein tissue factor
TGF	– Transforming growth factor
TIR	– Toll/IL1R
TIRAP	– TIR domain-containing adaptor protein
TLR	– Toll-like receptor
TNF-α	– Tumour necrosis factor-α
Tpl-2	– Tumour progression locus-2
TRAF6	– TNF receptor-activated factor 6
TRIKA-2	– TRAF6-regulated IKK activator 2
Tris-HCl	– Tris(hydroxymethyl)aminomethane hydrochloride
Tween 20	– Polyoxyethylene-sorbitan monolaurate
VCAM-1	– Vascular-cell adhesion molecule-1
VEGF	– Vascular endothelial growth factor
VPIs	– Vasopeptide inhibitors
WT	– Wild type
Z-VAD-FMK	– Benzyloxycarbonyl-val-ala-asp-fluromethylketone

CHAPTER 1
INTRODUCTION

1. Introduction

The defining clinical features of an inflammatory response were first described by a Roman physician, Celsus – Aulus Cornelius, in *De re medicina* circa 30 AD, but it was only in the late 20th century that the involvement of nitric oxide (NO) was first characterised (Ignarro *et al.*, 1986; Palmer *et al.*, 1987; Ignarro *et al.*, 1987). In *De re medicina*, the cardinal signs of inflammation were described as calour (warmth), dolour (pain), tumour (swelling) and rubour (redness and hyperaemia). Following the establishment of an infection in the body the immune system acts to fight and eradicate it in a multifaceted process involving many mediators. Blood flow around the infected area is increased as a result of vasodilatation, making the tissues red and warm. Capillaries in the area become more permeable allowing cell and fluid infiltration, and thus causing oedematous swelling. As a result of the swelling and the release of various chemical mediators, the infected area becomes painful as tissue expansion causes mechanical pressure on nerve cells. NO contributes to each of these cardinal signs of inflammation by causing vasodilatation, increasing vascular permeability and regulating nociception (Moncada and Higgs, 1993 ; Hobbs *et al.*, 1999). Thus, NO has a key role to play in the development of an inflammatory response. More recently, the involvement of natriuretic peptides in the development of an inflammatory response has been revealed (Hama *et al.*, 1994; Kiemer and Vollmar, 1997; Casco *et al.*, 2002; Scotland *et al.*, 2005a) suggesting that these complementary cardiovascular mediators might act in concert to govern vascular inflammation. Moreover, this implies that both soluble (sGC) and particulate (pGC) isoforms of guanylate cyclase are capable of modulating an inflammatory response.

1.1. Nitric oxide

1.1.1. History

In 1818, Prout observed large amounts of nitrite in the urine of a febrile patient (Prout, 1818) but these findings were not pursued until 1981 when Tannenbaum and Green made similar observations and concluded, following work with germ-free rats, that the increased nitrite (and nitrate) excretion exceeded the dietary intake and was due to the host and not its microbial flora (Green *et al.*, 1981a; Green *et al.*, 1981b).

Following these observations Stuehr & Marletta identified activated macrophages as the predominant nitrogen oxide producers (Stuehr and Marletta, 1985). But the true pioneering work came from the groups of Murad (Arnold *et al.*, 1977; Murad *et al.*, 1978), Furchgott (Furchgott and Zawadzki, 1980), Ignarro (Ignarro *et al.*, 1987) and Moncada (Palmer *et al.*, 1987) who worked independently to conclude that NO played an important physiological role as a signalling molecule. In the late 1970s and early 1980s Furchgott reported that relaxation of smooth muscle in blood vessels by acetylcholine (ACh) required an intact endothelium and concluded that the endothelial cells produced a compound in response to ACh, named endothelium-derived relaxing factor (EDRF), which acted on smooth muscle cells to bring about relaxation (Furchgott and Zawadzki, 1980). At the same time, Murad demonstrated that NO activated sGC, the enzyme responsible for producing the secondary messenger cyclic guanosine 3',5'-monophosphate (cGMP), and that the active metabolite of glyceryl trinitrate (GTN) and related organic nitrates is NO (Murad *et al.*, 1978; Howlett, 1998). Following these observations it was independently concluded by Furchgott and Ignarro at a conference (Mayo Clinic in Rochester, Minn) in 1986 that EDRF is NO (Howlett, 1998). The first published evidence came from Moncada and Ignarro in 1987 showing that NO produced by endothelial cells had the same biological activity and chemical properties as EDRF (Palmer *et al.*, 1987; Ignarro *et al.*, 1987).

Since its discovery as a signalling molecule, NO has received immense attention from the scientific community. It was described as "Molecule of the year" in 1992 by Science Magazine (Culotta and Koshland, 1992) and Robert Furchgott, Louis Ignarro and Ferid Murad were awarded the Nobel prize in physiology or medicine in 1998 for their breakthrough work (Howlett, 1998). NO has now been implicated in numerous physiological processes including smooth muscle contractility, platelet reactivity, central and peripheral neurotransmission and the immune response (Hobbs *et al.*, 1999). Regulation of this unique signalling molecule has been shown to be invaluable in highly diverse pathologies including cancer, impotence and inflammatory diseases like atherosclerosis, sepsis and circulatory shock, asthma and colitis (Moncada and Higgs, 1993; Hobbs *et al.*, 1999).

1.1.2. Nitric oxide synthases (NOS)

NO is a stable, hydrophobic, diatomic radical gas, which is both lipid and water soluble. It is produced by the catalytic conversion of L-arginine to L-citrulline by one of three nitric oxide synthase (NOS) isoforms (Bredt and Snyder, 1990; Pollock *et al.*, 1991; Stuehr *et al.*, 1991a). Several co-factors have been implicated in the reaction including nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin (CaM) and O_2 (Knowles and Moncada, 1994). The catalytic reaction involves two steps. L-arginine is initially hydroxylated to N^G-hydroxy-L-arginine (NOHA; Stuehr *et al.*, 1991b) followed by NOHA oxidation to equimolar quantities of NO and L-citrulline (Lirk *et al.*, 2002).

Three isoforms of NOS have been identified: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). They all exist as homodimers in their active form, share 50 – 60 % sequence homology and show 80 – 90 % conservation among species (Forstermann and Kleinert, 1995).

The enzymes are composed of a C-terminal reductase domain containing the NADPH, FAD and FMN binding sites and a N-terminal oxygenase domain containing a haem binding domain and binding sites for BH_4 and L-arginine (Stuehr, 1999). The two domains are joined by a calmodulin (CaM) binding site, which permits the transfer of electrons from the flavins to the haem iron. The haem iron can then bind and activate O_2 and ultimately synthesise NO (Stuehr, 1997). The constitutive isoforms eNOS and nNOS regulate NO synthesis by CaM binding giving a rapid on/off switch, while the inducible isoform iNOS binds CaM irreversibly (Cho *et al.*, 1992). Therefore eNOS and nNOS, the 'low output' enzymes, are regulated by intracellular Ca^{2+} fluxes while iNOS, the 'high output' enzyme, is Ca^{2+}-independent and is controlled primarily by enzyme expression (Forstermann and Kleinert, 1995). NO cannot be formed from monomeric subunits as the transfer of electrons occurs between the reductase and oxygenase regions of the opposing monomers in the dimerised active enzyme (Siddhanta *et al.*, 1998).

1.1.2.1. Neuronal nitric oxide synthase (nNOS/NOS I)

Neuronal NOS is a 160 kDa protein first cloned and purified from rat cerebellum (Bredt and Snyder, 1990). It is expressed mostly in the central and peripheral nervous system but it has also been identified in human skeletal muscle (Stamler and Meissner, 2001). NO synthesised by nNOS acts primarily as a neurotransmitter (Bredt, 1996). In the central nervous system NO has been implicated in the formation of memory via long-term potentiation, since in nNOS knockout (KO) mice or following inhibition of NO, learning behaviour is impaired (Chapman *et al.*, 1992). Neuronal NOS-derived NO has also been shown to have a physiological role in vision (Venturini *et al.*, 1991), feeding behaviour (Morley and Flood, 1991), pain (Moore *et al.*, 1991) and olfaction (Breer and Shepherd, 1993), and pathologically, has been implicated in cerebral ischaemia and epilepsy (Dalkara *et al.*, 1994). In the periphery there are numerous organ systems that use NO as a neurotransmitter. For example, in the gastrointestinal tract relaxation and dilatation of the stomach (Schuurkes and Meulemans, 1994), in addition to control of the sigmoid colon muscle and sphincter muscle are modulated by NO (Burleigh, 1992). Furthermore, NO is responsible for penile erection, as its release from nerve endings causes vasorelaxation of the blood vessels in the corpus cavernosum bringing a flow of blood to the blood sinuses resulting in an erection (Ignarro *et al.*, 1990; Moncada and Higgs, 1993).

1.1.2.2. Endothelial nitric oxide synthase (eNOS/NOS III)

Endothelial NOS was first purified from native bovine aortic endothelial cells (Pollock *et al.*, 1991) and is a 135 kDa protein (Pollock *et al.*, 1991). Like nNOS its activity is regulated by intracellular free Ca^{2+} and it is constitutively expressed. Endothelial NOS can also be activated by phosphorylation (in the absence of increased Ca^{2+}) by shear-stress via regulatory guanine nucleotide binding proteins (G-proteins; Kuchan *et al.*, 1994). Activated G-proteins stimulate effector molecules including phosphoinositide-3-kinase (PI3K). PI3K subsequently phosphorylates and activates the serine/threonine protein kinase Akt/protein kinase B, which can then phosphorylate eNOS (Fulton *et al.*, 1999). Endothelial NOS is mostly found in the vasculature, being expressed by vascular endothelial cells of both arteries and veins,

but this isozyme has also been detected in both rodent and human macrophages (Hecker *et al.*, 1992; Reiling *et al.*, 1994). NO produced by eNOS in the vasculature has been shown to have anti-thrombotic and anti-atherogenic properties as well as being an inhibitor of platelet adhesion and preventing initiation of the coagulation pathway by inhibiting the expression of thrombotic protein tissue factor (TF; Naseem, 2005). In addition, NO has a role in vascular remodelling and angiogenesis and inhibits smooth muscle cell proliferation via a protein kinase G (PKG)-dependent mechanism (Assender *et al.*, 1992) as well as cGMP-independently via inhibition of ornithine decarboxylase (Ignarro *et al.*, 2001). It has been shown that NO bioavailability is reduced in cardiovascular disease and that normal function of eNOS is required for a fully functional and compliant endothelium (Naseem, 2005).

Perhaps the most important role for NO is in controlling systemic blood pressure. Under physiological conditions, endothelial cells lining blood vessels release NO synthesised by eNOS, which diffuses into the underlying smooth muscle cells causing them to relax and thus increasing vessel diameter. Therefore, aberrant NO bioactivity has been implicated in hypertension, characterised by endothelial dysfunction and impairment of eNOS expression and activity (Naseem, 2005). Endothelial NOS KO mice also suffer from hypertension, decreased heart rate and increased plasma renin concentrations (Shesely *et al.*, 1996). Thus, NO plays a key anti-atherogenic role in maintaining the integrity of blood vessels. In the immune system, and particularly in macrophages, low level NO from eNOS has been shown to be essential for obtaining maximal levels of iNOS expression and activity in cells stimulated with bacterial lipopolysaccharide (LPS; Connelly *et al.*, 2003) suggesting that NO can also expedite host defence. The importance of eNOS as a regulator of iNOS has also been demonstrated *in vivo* using eNOS KO mice, where iNOS expression and activity are reduced, vascular reactivity to the NO donor spermine NONOate (SPER-NO) is enhanced, and hypotension is reversed following LPS treatment (compared to wild type (WT) animals; Connelly *et al.*, 2005; Vo *et al.*, 2005). PI3K and Akt/Protein kinase B-dependent enzyme phosphorylation have been shown to be involved in eNOS activation in response to LPS/inflammatory stimuli (Connelly *et al.*, 2005), collectively giving eNOS a role in the pathogenesis of sepsis and other inflammatory disorders.

1.1.2.3. Inducible nitric oxide synthase (iNOS/NOS II)

In the late 1970s and early 1980s it was postulated that mammals were able to produce high levels of urinary nitrate, 4 fold higher than ingested in humans (Green *et al.*, 1981a) and 9 fold higher than ingested in *Escherichia coli* LPS treated rats (Wagner *et al.*, 1983). In 1985 it was shown that macrophages were responsible for most of this nitrate production (Stuehr and Marletta, 1985) and later iNOS was identified as the NOS isoform responsible for NO production in macrophages (Stuehr *et al.*, 1991a). Unlike eNOS and nNOS, iNOS is inducible (Geller and Billiar, 1998) and does not require Ca^{2+} and CaM for activation (Cho *et al.*, 1992). It is a 130 kDa protein (Stuehr *et al.*, 1991a) expressed in numerous cell types including chondrocytes, kupffer cells, hepatocytes, neutrophils, pulmonary epithelium and vascular smooth muscle cells in response to inflammatory cytokines and bacterial metabolites (Lirk *et al.*, 2002).

NO provides a primary defence mechanism against tumour cells and intracellular and extracellular microorganisms in macrophages (Moncada and Higgs, 1993). The 'high output' NO from iNOS is cytotoxic and cytostatic and is thus important in combating bacterial infections. During the course of an inflammatory response, stimulated macrophages produce high levels of NO that surpass physiological concentrations and this diffuses to target cells (e.g. bacteria, fungi or tumour cells) where it causes deoxyribonucleic acid (DNA) damage, low density lipoprotein (LDL) oxidation, isoprostane formation, tyrosine nitration and inhibits mitochondrial respiration (Guzik *et al.*, 2003). NO binds with iron-sulphur centres in key enzymes involved in the target's respiratory chain and DNA synthesis pathway. NO target enzymes include aconitase, which is part of the Krebs cycle, reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase, which belongs to complex I of the mitochondrial respiratory pathway, succinate-NADPH dehydrogenase part of mitochondrial complex II and ribonucleoside-diphosphate reductase, which is essential in DNA synthesis (Moncada and Higgs, 1993). The high NO levels are usually paralleled with the production of superoxide anion (O_2^-). NO and O_2^- combine to give peroxynitrite ($ONOO^-$), which complements the cytotoxic effects of NO (Linares *et al.*, 2001). Animal models and human clinical data have

demonstrated the importance of iNOS and 'high output' NO in the development of septic shock, a heightened inflammatory response, as will be discussed in greater detail in section 1.3.3.

The most prominent mediators involved in iNOS expression are interferon (IFN)-γ, interleukin (IL)-1β, tumour necrosis factor (TNF)-α and bacterial LPS (Lirk *et al.*, 2002). The activation of iNOS expression varies profoundly from one cell type to another. Macrophages activated with LPS alone give a high level of iNOS (Kiemer and Vollmar, 1998; Connelly *et al.*, 2001; Connelly *et al.*, 2003) while combinations of cytokines and LPS are required in vascular smooth muscle cells, for example, to give maximal iNOS expression (MacNaul and Hutchinson, 1993; Matsumura *et al.*, 2001). Vascular smooth muscle cells derived from different species require differential combinations of LPS and pro-inflammatory cytokines to induce iNOS expression; indeed, it is important to note that induction of iNOS in human cells *in vitro* is notoriously difficult (MacNaul and Hutchinson, 1993). This gives an indication that different pathways are involved in iNOS regulation, either individually or synergistically.

The nuclear factor-κB (NF-κB) signal transduction pathway has been extensively studied and shown to be of major importance in iNOS expression. iNOS expression is blocked by a number of NF-κB inhibitors including salicylates (Wu, 2003), (3E)-4-(2-hydroxyphenyl)but-3-en-2-one (HPB; Tsao *et al.*, 2005), pyrrolidine dithiocarbamate (PDTC; Cuzzocrea *et al.*, 2002) and dexamethasone (Matsumura *et al.*, 2001). Moreover, promoter-reporter studies have shown that NF-κB binding sites on the iNOS promoter are key to its expression (Xie *et al.*, 1994; Taylor *et al.*, 1998; Marks-Konczalik *et al.*, 1998; Ganster *et al.*, 2001). NO has been shown to have a biphasic effect on NF-κB in murine macrophages such that it can both downregulate and upregulate iNOS expression and a number of other pro-inflammatory proteins (Connelly *et al.*, 2001) perhaps explaining why eNOS has been reported to have both pro- and anti-inflammatory actions. In addition to NF-κB, alternative pathways are involved in the regulation of iNOS expression including janus tyrosine kinase (JAK)-signal transducers and activators of transcription

(STAT; Kleinert *et al.*, 1998; Xuan *et al.*, 2001), and mitogen-activated protein kinases (MAPK; Guha and Mackman, 2001).

1.2. Mitogen-activated protein kinases (MAPK)

The MAPK network targets are involved in controlling numerous physiological and pathophysiological processes including proliferation, differentiation, development, inflammatory responses and cell death (Pearson *et al.*, 2001). Three main mammalian MAPK signalling pathways have been identified; extracellular signal-regulated kinases (ERK), p38 pathway and c-Jun N-terminal kinases/stress activated protein kinases (JNK/SARK; although other MAPKs exist including ERK3, ERK5, ERK7, nemo-like kinase (NLK) and MOK, but these are less well characterised; Pearson *et al.*, 2001). These pathways involve the stimulation of extracellular receptors, which then transmit the signal to intracellular targets via a cascade of consecutive cytoplasmic protein kinases. Up to six tiers of protein kinases have been identified and these assist in the amplification and specificity of the signalling pathway via phosphorylation cascades (Seger and Krebs, 1995).

The distinguishing feature of MAPK is that direct phosphorylation of two sites, one threonine and one tyrosine, in the kinase activation loop is required to bring about their activation. MAPK are activated through the consecutive phosphorylation of kinases higher up in the hierarchy. The first kinase in the phosphorylation sequence belongs to the MEK (MAPK/ERK kinase) kinase (MEKK) family, which are subject to numerous regulators including G-protein coupled receptors (GPCRs) and multiple protein kinases (e.g. protein kinase C (PKC) and Akt; Pearson *et al.*, 2001). MEKK activates MEK downstream by phosphorylating two conserved adjacent serine/threonine residues in the activation loop (Zheng and Guan, 1994). MEK in turn activates MAPK (Crews *et al.*, 1992) by phosphorylating both serine/threonine and tyrosine residues with aliphatic or aromatic side chains (Kosako *et al.*, 1992). In the case of ERK 1/2, tyrosine residues are phosphorylated prior to threonine, and tyrosine phosphorylation must accumulate at a threshold before threonine phosphorylation is enabled (Pearson *et al.*, 2001). Once activated, MAPK can phosphorylate targets and thus affect biological processes. The various different members of each step in the MAPK cascade are shown in Figure 1.

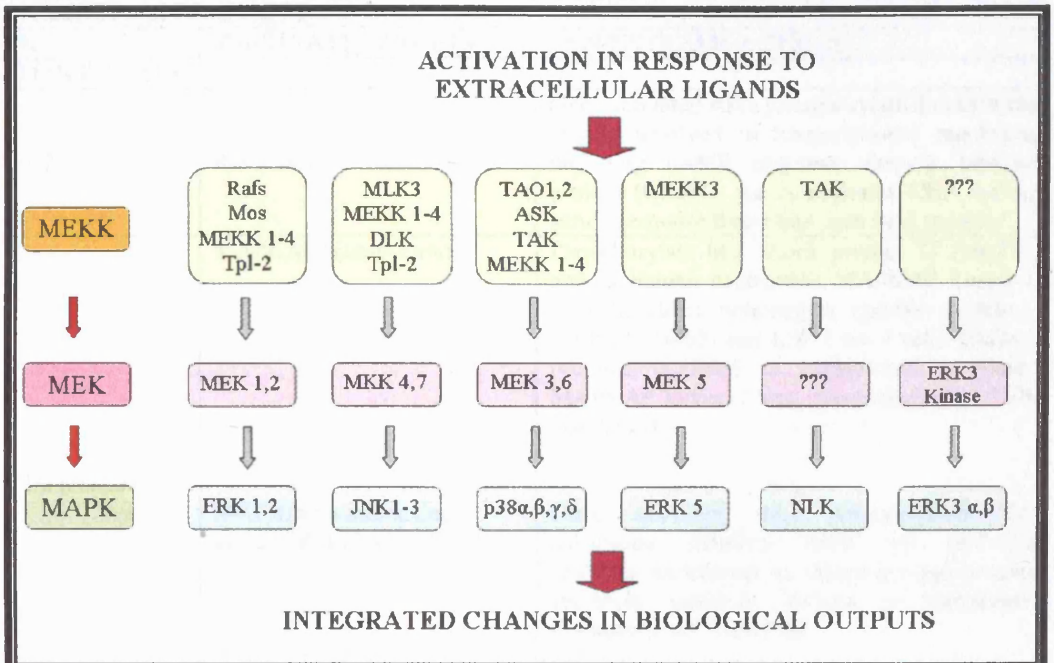


Figure 1 – MAPK cascades (Abbreviations: ASK – apoptosis signal-regulating kinase, DLK – dual leucine zipper-bearing kinase, ERK – extracellular signal-regulated kinase, JNK – c-Jun N-terminal kinase, MAPK – mitogen-activated protein kinase, MEK – MAPK/ERK kinase, MEKK – MAPK/ERK kinase kinase, MKK – MAPK kinase, MLK – mixed lineage kinase, NLK – nemo-like kinase, TAK – transforming growth factor (TGF)-β-activated protein kinase, TAO – thousand and one amino acid kinase, Tpl-2 – tumour progression locus-2).

1.2.1. Extracellular signal-regulated kinases (ERK 1/2)

ERK 1 and ERK 2 (ERK 1/2) are 43 and 41 kDa proteins respectively and show 85 % homology. They are ubiquitously expressed but their respective distributions vary with individual cell types. The two phosphorylation accepting residues are separated by a single glutamate residue in the enzyme's active loop (Payne *et al.*, 1991). As shown in Figure 1, ERK 1/2 are activated by MEK 1 and 2 and MEK 1/2 are phosphorylated by Raf isoforms including A-Raf, B-Raf and Raf-1 (Avruch *et al.*, 1994; Daum *et al.*, 1994) or Mos (Pearson *et al.*, 2001). The downstream activation of ERK 1/2 is brought about by activation of either RTKs and Ras or GPCRs at the cell membrane. Alternatively the ERK 1/2 pathway can be activated through heterotrimeric GPCRs. ERK 1/2 have multiple effector targets both cytoplasmic and nuclear and these are summarised in Table 1. A number of gene disruption experiments have been carried out involving numerous members of each of the three main MAPK signalling pathways. The phenotypes of such animals are summarised in Table 2.

MAPK SUBSTRATE TARGETS		
MAPK	SUBSTRATE TARGETS	DOWNSTREAM ACTIONS
PROTEIN KINASES		
ERK 1/2	Rsk 1, 2, 3	Once activated Rsks phosphorylate downstream targets involved in transcriptional regulation including cAMP response element binding protein (CREB), the co-activator CBP, c-Fos, serum response factor and oestrogen receptor.
ERK 1/2 and p38	MAPKAP kinase-2 and 3	Phosphorylate heat shock protein 27 (hsp27), and in human neutrophils MAPKAP kinase-2 phosphorylates lymphocyte specific protein-1 (LSP 1). Hsp27 and LSP 1 are F-actin binding proteins involved in cytoskeletal structure. MAPKAP kinase-2 also phosphorylates CREB and ATF-1.
	MAP kinase-interacting kinase (Mnk) 1 and Mnk 2	Once activated they phosphorylate the eukaryotic initiation factor 4E (eIF-4E) enabling recruitment of ribosomes and protein synthesis initiation factors to messenger ribonucleic acid (mRNA).
	Mitogen and stress activated protein kinases (Msk)	Phosphorylates CREB
MEMBRANE AND CYTOPLASMIC SUBSTRATES		
ERK 1/2 and p38	Phospholipase A_2	
ERK 2	Heat shock factor transcription factor 1 (hsp 1) Topoisomerase II-b Ral-GDS ZNF-7 (zinc finger protein)	
NUCLEAR SUBSTRATES Nuclear Substrates are regulated indirectly via other protein kinases or MAPKs can translocate to the nucleus and phosphorylate transcription factors themselves		
ERK 1/2	Ribosomal S6 kinase (Rsk) 2	Phosphorylates histone H3
	Mnks	Phosphorylates histone H3 and high mobility group (HMG) protein 14, thus controlling DNA packaging into chromatin
	Transcription factors of the activating protein-1 (AP-1) family.	c-Jun (N-terminal phosphorylation), c-Fos, activating transcription factor (ATF-2)
ERK 2	Steroid receptor coactivator-1 (SRC-1)	SRC-1 has histone acetyltransferase activity. SRC-1 also interacts with CREB to enhance oestrogen and progesterone receptor-mediated gene activation and HAT protein p300/CBP-associated factor. SRC-1 is involved in chromatin remodelling and gene expression
JNK/SARK	c-Jun (C-terminal phosphorylation)	
ERK, p38 and JNK/SARK	Ternary complex factor (TCF) family of transcription factors	Ets domain proteins including Elk-1, which mediates transcription from serum response elements (SREs). SAP-1 and SAP-2 (ternary complex factors)

Table 1 – MAPK substrate targets (Pearson *et al.*, 2001).

ERK 1/2 is partly involved in cytokine-induced iNOS expression (without modulating NF-κB) in vascular smooth muscle cells (Doi *et al.*, 2000) and cyclooxygenase-2 (COX-2) expression in human tracheal smooth muscle cells (Lin *et al.*, 2004). In human airway epithelial cells, ERK is the major kinase regulating prostaglandin E_2 (PGE-2) synthesis, and inhibition of ERK phosphorylation with 2′-amino-3′-methoxyflavone (PD98059) almost completely abrogates PGE-2 synthesis in response to LPS, IL-1β or TNF-α (Petrovic *et al.*, 2006). Furthermore, it has been demonstrated that ERK 1/2 signalling is important in the regulation of baseline permeability and cGMP-induced hyperpermeability in endothelial cells (Varma *et al.*, 2002) and in platelet-activating factor (PAF)-induced microvascular hyperpermeability *in vivo* (Yu et al., 2005), a process that is vital in the inflammatory reaction that characterises vascular disease and wound healing.

1.2.2. p38

The p38 MAPK pathway is less well defined and unlike ERK 1/2, p38 is activated by a variety of environmental stresses and pro-inflammatory signals. These include cytokines, hormones, osmotic shock and heat shock but they can also be activated by GPCRs (Pearson *et al.*, 2001). This 38 kDa protein exists as four analogous family members, p38α, β, γ and δ (Pearson *et al.*, 2001). All p38 isoforms have the sequence TGY in their active loop thus having a single glycine separating the two threonine/tyrosine phosphoaccepting residues. As shown in Figure 1, the MEK family members that phosphorylate p38 are MEK 3 and MEK 6 and these are phosphorylated further upstream by TAO 1 and 2, ASK, TAK and MEKK 1, 2, 3 and 4. The downstream effectors of p38 are summarised in Table 1, while Table 2 shows the phenotypic results of gene disruption.

p38, like ERK 1/2 has been implicated in iNOS and COX-2 gene expression. In glial cells p38α and β have been shown to modulate iNOS expression via transcriptional control. Activated transcription factor (ATF-2), CCAAT/enhancer-binding protein (C/EBP) and NF-κB increase expression, while cyclic adenosine 3′,5′-monophosphate (cAMP) response element binding protein (CREB) decreases expression (Bhat *et al.*, 2002). In murine astrocytes the p38 pathway is partly responsible for inhibiting iNOS expression (Da Silva *et al.*, 1997) while in

human tracheal smooth muscle cells, p38 is involved in regulation of COX-2 (Lin *et al.*, 2004). Akin to ERK 1/2, p38 is important in mediating hyperpermeability in endothelial cells and in PAF-induced microvascular hyperpermeability *in vivo* (Yu et al., 2005).

1.2.3. c-Jun N-terminal kinases/stress activated protein kinases (JNK/SAPK)

JNK/SAPK are 46 and 54 kDa proteins encoded on three genes producing 10 or more alternative splice variants (Pearson *et al.*, 2001). The phosphoacceptor residues are separated by a single proline in the active loop and they are activated by cytokines, certain ligands for GPCRs (e.g. growth factors), agents that interfere with DNA and protein synthesis, serum, transforming agents as well as various other stresses (Pearson *et al.*, 2001). These MAPK are activated by MKK 4 (phosphorylates tyrosine) or MKK 7 (phosphorylates threonine), which are MEK family members and MEKs are in turn phosphorylated by MEKK family members including MLK 3, MEKK 1, 2, 3 and 4, DLK and Tpl-2 (Figure 1). The phenotypes of MEKK and MAPK KO mice are shown in Table 2. Principal downstream effectors of the JNK/SAPK pathway are shown in Table 1.

JNK/SAPK signalling has also been implicated in the regulation of iNOS. In murine macrophages, LPS-induced iNOS expression is blocked by the overexpression of a JNK/SAPK dominant negative mutant or by the broad caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluromethylketone (Z-VAD-FMK; Chakravortty *et al.*, 2001). This inhibitory effect is also seen with a dominant negative mutant of MKK 4, the upstream MEK for JNK/SAPK (Chan and Riches, 2001). In insulin-producing cells it has been hypothesised that activation of ATF-2 by JNK 1 could result in ATF-2 synergising with NF-κB and AP-1 to enhance iNOS transcription (Welsh, 1996). In rat glomerular mesangial cells IL-1β-induced iNOS stimulation requires both p38 and JNK/SAPK activation (Guan *et al.*, 1999). Furthermore JNK/SAPK has been shown to be overexpressed and activated in atherosclerotic lesions. It has therefore been proposed that JNK/SAPK could play a key role in mediating cell differentiation and apoptosis during atherosclerosis development with the possible involvement of the p53 transcription factor (Metzler *et al.*, 2000). Finally, the JNK/SAPK cascade has been implicated in the development of cardiac

hypertrophy and could therefore be pursued as a drug target for treatment of this condition (Choukroun *et al.*, 1999).

MAPKK/MAPK	PHENOTYPE
MEK 1	Defective placental vascularisation
MKK 4	Defective liver development
MKK 7	Embryonic lethality of unknown cause
MEK 3	Defective IL-12 production
ERK 1	Defective T-cell development (positive selection)
JNK 1	Defective T-cell differentiation to Th2 cells
JNK 2	Defective T-cell differentiation to Th1 cells
JNK 1 or JNK 2	Defective T-cell proliferation and IL-1 production
	Defective activation induced death of thymocytes
JNK 1 and JNK 2	IL-2 production
	Neural tube disclosure
JNK 3	Resistance to excitotoxic neuronal cell death
p38α	Placental defect (trophoblast cells)
	Insufficient production of erythropoietin

Table 2 – Phenotypes of MEK and MAPK KO mice (adapted from Chang and Karin, 2001).

1.3. LPS and septic shock

1.3.1. LPS and its signalling

LPS is a complex glycolipid macromolecule found on the outer membrane of all gram-negative bacteria such as *Escherichia coli* and *Salmonella*, and is one of the most potent microbial initiators of inflammation (Schaechter *et al.*, 1999; Parslow *et al.*, 2001). Each LPS molecule consists of three regions, a core carbohydrate domain consisting of a variable and an invariable portion linked on one side by a hydrophilic polysaccharide moiety (O-Antigen), and on the other side by hydrophobic phospholipids (Lipid A), which anchors LPS to the bacterial membrane (Raetz, 1990). The repeating sugar subunits in the O-Antigen region are highly variable and

species-specific giving rise to the different antigenic specificities among Gram-negative bacteria (Schaechter *et al.*, 1999). To the contrary, the core polysaccharide and Lipid A moieties are essentially invariable thus acting as targets for binding by human serum proteins (Parslow *et al.*, 2001). The common LPS structure is shown in Figure 2.

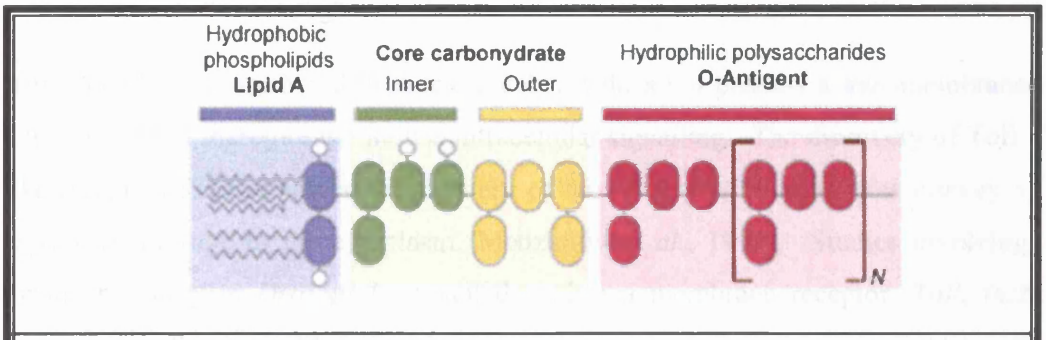


Figure 2 – Structural organisation of LPS.

LPS activates monocytes and macrophages to produce pro-inflammatory cytokines including IL-1, IL-6 and TNF-α (Cohen, 2002) and humans have developed a complex system to recognise minute amounts of LPS ($<10^{-12}$ M) to combat infection (Guha and Mackman, 2001; Parslow *et al.*, 2001). LPS binds to LPS-binding protein (LBP), first isolated from acute phase rabbit serum (Tobias *et al.*, 1986). LBP is a 60 kDa serum glycoprotein that binds Lipid A of LPS with high affinity and is normally present in serum at <0.5 μg/ml, but levels rise 100-fold to 50 μg/ml 24 h after induction of an acute phase response (Schumann *et al.*, 1990). LBP-LPS complexes enhance LPS-induced TNF production 250-fold, while levels are unaltered in the presence of LPS alone (Schumann *et al.*, 1990). The LBP-LPS complexes bind to CD14, a 55 kDa differentiation antigen of monocytes, as determined using CD14 monoclonal antibodies, which abrogate the binding of these complexes to cells (Wright *et al.*, 1990). CD14 is found anchored to myeloid cell membrane (especially at the final stage of monocyte/macrophage maturation and weakly on neutrophils) via a glycosyl phosphatidyinositol tail or as a soluble form circulating free in plasma at a concentration of 2 – 6 μg/ml (Guha and Mackman, 2001), which enables cells such as endothelial and smooth muscle cells to respond to LPS even though they do not possess membrane bound CD14 (Guha and Mackman, 2001). CD14/LPS complexes reduce the concentration of LPS required to activate macrophages compared to LPS alone (Fujihara *et al.*, 2003) and mice lacking CD14

are highly resistant to endotoxic shock and almost no secreted TNF-α is detectable in the serum of these animals (Haziot *et al.*, 1996). Isolated peripheral mononuclear cells from CD14-deficient mice also do not secrete detectable levels of TNF-α and IL-6 (Haziot *et al.*, 1996). Interestingly, increased levels of circulating soluble CD14 (3.23 vs 2.48 μg/ml vs control) are associated with high mortality in patients with gram-negative septic shock (Landmann *et al.*, 1996).

Although CD14 is anchored to myeloid cells, it does not possess a transmembrane domain and can therefore not induce intracellular signalling. The discovery of Toll-like receptors (TLRs) solved the mystery of how CD14/LPS complexes convey a signalling message to the cytoplasm (Medzhitov *et al.*, 1997). Studies involving innate immunity in *Drosophila* unveiled a plasma membrane receptor, *Toll*, that recognises pathogens and activates an innate immune response via activation of *Dorsal*, *Dif* and *Relish*, transcription factors of the Rel family that are homologous to mammalian NF-κB (Ghosh *et al.*, 1998). Mutations in *Toll* render *Drosophila* susceptible to fungal infections as they are unable to produce drosomycin, an antifungal peptide (Lemaitre *et al.*, 1996). In *Drosophila*, *Toll* is a plasma membrane receptor that possesses a cytoplasmic domain homologous to the mammalian IL-1 receptor (IL-1R) (Medzhitov *et al.*, 1997). The *Dorsal* system in *Drosophila*, which is important in the formation of dorsoventral patterning in the embryo, is paralleled in mammalian species by NF-κB (Wasserman, 1993). *Dorsal* and NF-κB are retained in the cytoplasm by the inhibitor proteins cactus and inhibitory κB subunit (IκB) respectively. Upon receptor stimulation (Toll/IL-1R) *dorsal* and NF-κB are released from cactus and IκB and are free to translocate to the nucleus and interact with target promoters bringing about controlled gene expression (Wasserman, 1993; Lemaitre *et al.*, 1996).

Sequence homology between *Toll* and human IL-1R was used in a blast search of human cDNA libraries. A cDNA clone with an open reading frame of 2523 bp encoding a 841 amino acid (aa) chain was found and was termed TLR (Medzhitov *et al.*, 1997). Five TLRs were originally cloned (Medzhitov *et al.*, 1997) but there are now known to be at least 10 members in the family (Takeda *et al.*, 2003; Pandey and Agrawa, 2006) and they are characterised structurally by leucine-rich repeats (LRR) in the extracellular domain and a *Toll*/IL-1R (TIR) domain in the intracellular

region (Rock *et al.*, 1998; Akira *et al.*, 2001; Takeda *et al.*, 2003). TLR-4 was the first mammalian TLR to be identified. mRNA encoding this protein has been found in monocytes, macrophages, T-cells, small intestinal epithelial cells and B-cells and activation of the receptor induces expression of inflammatory cytokines (Medzhitov *et al.*, 1997). In 1998, TLR-4 was established as the receptor for LPS (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999; Hoshino *et al.*, 1999). C3H/HeJ and C57BL/10ScCr mice were known to be hyposensitive to LPS and the group of Poltorak *et al* showed that both had mutations in the *Tlr4* gene. C3H/HeJ mice have a missense mutation in the third exon, which replaces a highly conserved proline with histidine at position 712, while C57BL/10ScCr mice are homozygous for a null mutation of *Tlr4* (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). Subsequently, the involvement of TLR-4 in LPS recognition was further confirmed using TLR-2 and TLR-4 deficient mice. In WT and TLR-2 deficient mice, shock was induced by day 5 following LPS challenge and only one fifth survived on day 6. In contrast all the TLR-4 deficient mice were alive on day 6 showing that TLR-4 but not TLR-2 is involved in LPS signalling and septic shock (Takeuchi *et al.*, 1999). Furthermore WT and TLR-2 deficient macrophages produced IL-6, TNF-α and NO (i.e. nitrite) in response to LPS while TLR-4 deficient macrophages were unable to produce these inflammatory mediators (Takeuchi *et al.*, 1999). Although TLR-4 is the principal receptor for LPS, it has been shown that LPS can be recognised and internalised independently of CD14 and TLR-4. Studies using affinity chromatography, peptide mass fingerprinting and fluorescence resonance energy transfer have shown the involvement of heat shock proteins (hsp) 70 and 90, chemokine receptor 4 (CXCR4) and growth differentiation factor 5 (Triantafilou *et al.*, 2001). Furthermore, nucleotide oligomerisation domain (Nod) 1 and 2 have been identified as intracellular recognition molecules for LPS, which can directly activate NF-κB (Inohara *et al.*, 2002).

In addition to LBP, CD14 and TLR-4, the LPS binding complex requires a further protein for signalling, myeloid differentiation protein-2 (MD-2). Radioprotective 105 (RP105) is a lymphocyte specific receptor that has LRR similar to *Drosophila Toll* and TLR-4; it was found that a secretory molecule called MD-1 physically associates with RP105 and is essential for B-cell activation (Miyake *et al.*, 1998). The amino acid sequence for MD-1 was used in a computer blast of an expressed

sequence tag database and a human cDNA clone with an open reading frame of 160 aa termed MD-2 was identified (Shimazu *et al.*, 1999). It was shown that MD-2 associates with TLR-4, as expression of MD-2 confers responsiveness to LPS in cells co-expressing TLR-4 and it co-localises with TLR-4 on the cell surface (Shimazu *et al.*, 1999). Furthermore, MD-2 enhances TLR-4 dependent activation of NF-κB by 2 – 3 fold (Shimazu *et al.*, 1999). Studies using MD-2 KO mice have shown that MD-2 is indispensable for LPS responses both *in vitro* and *in vivo*. Cells isolated from MD-$2^{-/-}$ mice are hyporesponsive to LPS and are unable to produce inflammatory cytokines. MD-$2^{-/-}$ mice have similar phenotypes to the TLR-$4^{-/-}$ mice and are hyporesponsive to LPS and survive endotoxic shock (Nagai *et al.*, 2002). In addition it was shown that MD-2 contributes to the correct intracellular distribution of TLR-4. In MD-$2^{-/-}$ cells TLR-4 predominantly resides in the golgi apparatus associated with the chaperone protein glycoprotein 96 (gp 96) and is unable to translocate to the cell membrane and thus recognise LPS (Nagai *et al.*, 2002). Cross-linking studies have shown that LPS comes into close proximity to TLR-4 only when it is bound to CD14 and its interaction with TLR-4 involves MD-2 (Da Silva Correia *et al.*, 2001).

All the components of the LPS recognition and signalling pathway discussed thus far are involved extracellularly. The LPS intracellular signal transduction pathway was first elucidated by its similarities (once again) to the *Drosophila Toll* and IL-1R signalling cascades. In *Drosophila* an adaptor protein known as *Tube* binds to the intracellular portion of the *Toll* receptor and brings about the activation of *Dorsal*, the NF-κB homologue, via the *Pelle* pathway (Akira *et al.*, 2001). In the mammalian system the adaptor protein myeloid differentiation factor-88 (MyD88), a homologue of *Tube*, binds intracellularly to IL-1R. MyD88 links IL-1R to IL-1 receptor-associated kinase (IRAK), a serine-threonine kinase that is a homologue of *Drosophila Pelle* (Muzio *et al.*, 1997; Wesche *et al.*, 1997; Medzhitov *et al.*, 1998). The same MyD88 adaptor protein has been found to bind to the intracellular region of TLR-4 as all receptors possess a common conserved Toll/IL1R (TIR) domain and TLR-4 immunoprecipitates with MyD88 (Medzhitov *et al.*, 1998). MyD88-deficient mice are resistant to high doses of LPS and increases in serum levels of IL-6, TNF-α and IL-1β levels are not detected in these animals. To the contrary, WT animals have elevated levels of inflammatory cytokines and almost all these animals die

within 96 h after LPS injection (Kawai *et al.*, 1999). Similarly, macrophages isolated from MyD88 KO mice fail to produce any inflammatory cytokines or mediators in response to LPS (Kawai *et al.*, 1999).

Upon ligand binding to TLR-4, IRAK is phosphorylated and dissociates from the receptor complex and interacts with another adaptor protein TNF receptor-activated factor 6 (TRAF6) (Akira *et al.*, 2001; Fujihara *et al.*, 2003). TRAF6 activates both the MAPK pathway resulting in AP-1 activation and the inhibitors of κB kinase (IKK) complex allowing NF-κB activation (Fujihara *et al.*, 2003). Evidence for the involvement of both IRAK and TRAF6 in LPS signalling was provided mainly from genetic manipulation studies. In the human promonocytic THP-1 cell line it was shown that LPS activates endogenous IRAK as measured by its *in vitro* kinase activity toward myelin basic protein and that LPS triggers the association of IRAK with MyD88 (Li *et al.*, 2000). Furthermore, IRAK-deficient mice are resistant to the lethal effects of LPS, and macrophages isolated from IRAK deficient mice exhibit impaired TNF-α production in response to LPS (Swantek *et al.*, 2000). In TRAF6-deficient macrophages induction of iNOS is impaired in response to high doses of LPS and NF-κB activation is impaired in embryonic fibroblasts and splenocytes isolated from the same animals (Lomaga *et al.*, 1999). Finally, dominant negative TRAF6 mutations significantly impair TLR-4-induced NF-κB activity (Muzio *et al.*, 1998).

NF-κB has been shown to be activated by LPS through TGF-β-activated kinase (TAK-1) and TRAF6-regulated IKK activator 2 (TRIKA-2) by activating IKK (Wang *et al.*, 2001). However, IKK phosphorylation and hence NF-κB activation can also be brought about by numerous MEKs as well as NF-κB inducing kinase (NIK; Guha and Mackman, 2001).

In addition to NF-κB activation, LPS can induce IFN-α/β expression in murine macrophages in an autocrine/paracrine, MyD88-independent fashion. The transcription factor signal transducer and activator of transcription 1α (Stat1α) is activated by phosphorylation and together with interferon regulatory factor (IRF) 1 regulate the expression of iNOS (Jacobs and Ignarro, 2001; Kleinert *et al.*, 2003). IFN-α/β expression is also important for the expression of other LPS-inducible genes

including IFN-inducible protein-10 and monocyte chemoattractant protein 5 (Fujihara *et al.*, 2003). Studies using IRF-3-deficient mice have shown the importance of this regulatory factor in the stimulation of IFN-β as the mice become resistant to LPS-induced endotoxic shock (Sakaguchi *et al.*, 2003). Independent studies have demonstrated the involvement of TIR domain-containing adaptor protein (TIRAP; Horng *et al.*, 2001) and MyD88-adaptor-like (Mal; Fitzgerald *et al.*, 2001) in the MyD88-independent activation of TLR-4 by LPS and the induction of IFN-β as well as NF-κB. Furthermore, LPS activates a number of other MyD88-independent signalling molecules including PKC (Paul *et al.*, 1997), Src-type tyrosine kinases, small G proteins, PI3K and the serine/threonine protein kinase Akt/protein kinase B, all of which can activate NF-κB as well as members of the MAPK pathways (Akira *et al.*, 2001).

1.3.2. LPS and the MAPK pathway

The activation of MEK/ERK 1/2 by LPS has been shown to be both Ras and c-Raf dependent (Reimann *et al.*, 1994; van der Bruggen *et al.*, 1999) and independent (Buscher *et al.*, 1995). Dominant negative repressors of Ras and c-Raf inhibit LPS induction of the TNF-α promoter in RAW264.7 macrophages (Guha and Mackman, 2001) and treatment of BAC-1.2F5 macrophages with LPS causes phosphorylation and activation of Raf-1 followed by stimulation of MEK-1 and MAPK activity with subsequent phosphorylation of the transcription factor, Elk-1 (Reimann *et al.*, 1994). Inhibition of MEK by the specific inhibitor U0126 causes failure of macrophages to release cytokines such as IL-1β, IL-8 and TNF-α as well as prostaglandin E_2 (Scherle *et al.*, 1998). Treatment of human monocytes with PD98059 (MEK inhibitor) inhibits LPS induction of TNF-α (van der Bruggen *et al.*, 1999; Guha and Mackman, 2001) and in murine macrophages, PD98059 decreases LPS-induced iNOS mRNA and expression (Kim *et al.*, 2004).

The activation of p38 has also been implicated in LPS signalling. Targeted disruption of *Mkk3*, the gene encoding MEK3, upstream of p38, causes a selective defect in the response of fibroblasts to TNF-α, including reduced p38 activation and cytokine expression (Wysk *et al.*, 1999). Inhibition of p38 using the pathway specific inhibitor 4-(4-Fluoreophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-

imidazole (SB203580) results in reduced cytokine expression (IL-1β and IL-6) as well as a decrease in LPS-induced iNOS expression (Kim *et al.*, 2004).

The third pathway in the MAPK network, the JNK pathway is also involved in LPS signalling (Hambleton *et al.*, 1996). Treatment of RAW264.7 cells, murine bone marrow-derived macrophages and the human monocyte cell line THP-1 with LPS results in rapid activation of the two isoforms of JNK, p46 and p54 (Hambleton *et al.*, 1996). JNK is regulated by the upstream MEK 4 and MEK 7 (Yang *et al.*, 1997; Tournier *et al.*, 1999) and treatment of THP-1 cells with the monoclonal antibody 60b, which blocks LPS-LBP binding to CD14, results in inhibition of JNK activation indicating the importance of JNK in downstream gene expression initiated by LPS (Hambleton *et al.*, 1996).

The pathways involved in LPS signalling, as detailed above, are summarised in Figure 3.

1.3.3. Septic (endotoxic) shock

Septic shock can be defined as a progressive failure of the circulation to provide blood to vital organs. Symptoms of septic shock include severe hypotension and inadequate tissue oxygenation, resulting in multiple organ failure (Thiemermann, 1997; Wolkow, 1998). The most common cause of septic shock is the contamination of blood with gram-negative bacteria (bacteraemia) and thus the presence of LPS, although gram-positive bacteria, viruses and parasites can also cause sepsis (Thiemermann, 1997). Septic shock and multiple organ failure are an important cause of morbidity and mortality affecting around 1 % of all hospitalised patients (Wolkow, 1998) and 30 – 40 % of patients admitted to intensive care units (ICU; Pittet *et al.*, 1995). It is known that the development of multiple organ failure involves numerous pathways including inflammatory, immune, microvascular, hormonal, bioenergetic and metabolic systems (Vincent *et al.*, 2006).

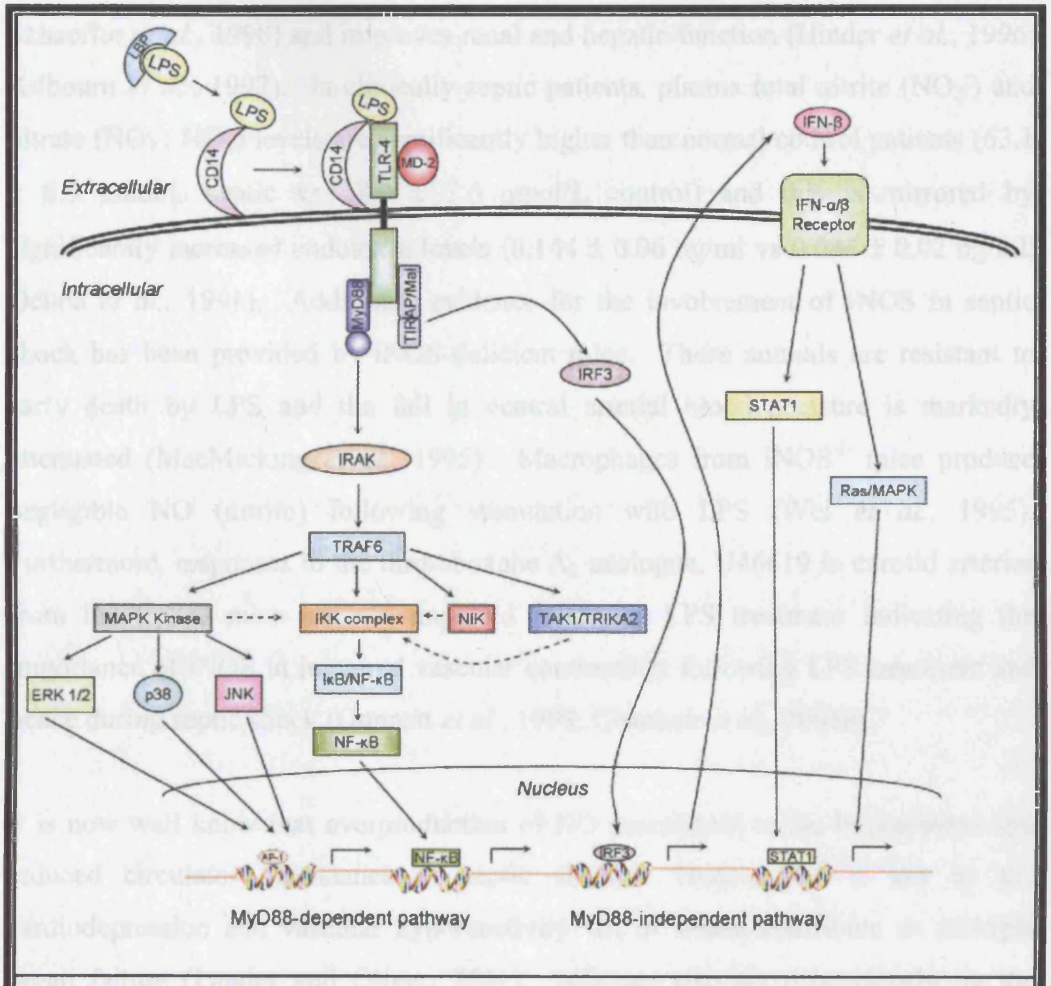


Figure 3 – LPS-triggered signalling cascades (Abbreviations: ERK – extracellular signal-regulated kinases, IFN – interferon, IKK – inhibitors of κB kinase, IRAK – IL-1 receptor associated protein kinase, IRF3 – interferon regulatory factor 3, IκB – inhibitory κB subunit, JNK – C-Jun N-terminal kinases, LBP – LPS-binding protein, LPS – lipopolysaccharide, Mal – MyD88-adaptor-like, MAPK – mitogen-activated protein kinase, MD-2 – myeloid differentiation protein 2, MyD88 – myeloid differentiation factor-88, NF-κB – nuclear factor-κB, NIK – NF-κB inducing kinase, STAT – signal transducers and activators of transcription, TAK – TGF-β-activated protein kinase, TIRAP – TIR domain-containing adaptor protein, TLR-4 – toll-like receptor 4, TRAF6 – TNF receptor-activated factor 6, TRIKA2 – TRAF6-regulated IKK activator 2).

Animal models and human clinical data have shown a definitive role for NO in the development of septic shock (Ochoa *et al.*, 1991; Nava *et al.*, 1992; Evans *et al.*, 1993; Liu *et al.*, 1993). Treatment of rats *in vivo* with LPS causes an increase in iNOS mRNA levels in lung, liver, spleen, skeletal muscle and kidney and this is attenuated in the presence of dexamethasone (Liu *et al.*, 1993). Administration of the NOS inhibitor N^G-methyl-L-arginine (L-NMA) to animal models of sepsis restores blood pressure (Kilbourn *et al.*, 1990; Kilbourn *et al.*, 1992; Kilbourn *et al.*, 1994; Kilbourn *et al.*, 1997), hyporesponsiveness to vasoconstrictors (Julou-

Schaeffer *et al.*, 1990) and improves renal and hepatic function (Hinder *et al.*, 1996; Kilbourn *et al.*, 1997). In clinically septic patients, plasma total nitrite (NO_2^-) and nitrate (NO_3^-; NO_x) levels are significantly higher than normal control patients (63.1 ± 6.5 μmol/L septic vs 28.9 ± 3.6 μmol/L control) and this is mirrored by significantly increased endotoxin levels (0.144 ± 0.06 ng/ml vs 0.044 ± 0.02 ng/ml; Ochoa *et al.*, 1991). Additional evidence for the involvement of iNOS in septic shock has been provided by iNOS-deficient mice. These animals are resistant to early death by LPS and the fall in central arterial blood pressure is markedly attenuated (MacMicking *et al.*, 1995). Macrophages from $iNOS^{-/-}$ mice produce negligible NO (nitrite) following stimulation with LPS (Wei *et al.*, 1995). Furthermore, responses to the thromboxane A_2 analogue, U46619 in carotid arteries from iNOS KO mice are not impaired following LPS treatment indicating the importance of iNOS in impaired vascular contractility following LPS treatment and hence during septic shock (Gunnett *et al.*, 1998; Chauhan *et al.*, 2003a).

It is now well know that overproduction of NO contributes to the hypotension and reduced circulatory resistance in septic shock. Hence, NO is key to the cardiodepression and vascular hyporeactivity, all of which contribute to multiple organ failure (Landry and Oliver, 2001). NO can also act deleteriously by the formation of peroxynitrite resulting in DNA damage and impairment of mitochondrial respiration (Hauser *et al.*, 2005; Levy *et al.*, 2005). On the other hand, NO can be protective as it can inhibit leukocyte and platelet aggregation as well as scavenge oxygen radicals (Hauser *et al.*, 2005).

Since the high levels of NO produced by iNOS can be so deleterious in septic shock, clinical studies were conducted in an attempt to find a suitable drug for the treatment or improvement of this systemic condition. Results from a phase II randomised, double-blind, placebo-controlled study using L-NMA promoted the resolution of shock (Wei *et al.*, 1995), reduced the elevated plasma nitrate concentrations, increased vascular tone and reduced both cardiac index and oxygen delivery as well as successfully maintaining a target mean arterial blood pressure of ≥ 70 mm Hg and reducing the requirement for, or withdrawal of, conventional inotropic vasoconstrictor agents (Watson *et al.*, 2004). Despite the promising results from this

study, a subsequent phase III clinical trial was terminated prematurely as there was increased mortality in the treatment group (Lopez *et al.*, 2004). However, the trials met criticism in an accompanying editorial stressing the importance of specific versus non-specific NOS inhibitors as complete NO inhibition by inhibiting all NOS isoforms could result in blockade of physiological NO functions (Phillip and Parrillo, 2004).

1.4. Guanylate cyclase (GC) and cGMP

Cyclic GMP is a second messenger produced by the enzymatic catalysis of guanosine 5'-triphosphate (GTP) by the guanylate cyclase (GC) enzymes and is degraded by hydrolysing phosphodiesterases (PDE). It was first identified in urine in 1963 (Ashman *et al.*, 1963) and was later designated as a second messenger in 1970 (Kuo and Greengard, 1970). cGMP can bring about its effects by interacting with cyclic nucleotide-gated (CNG) cation channels, cGMP-regulated PDEs and PKG. There are two forms of guanylate cyclase, soluble (sGC) and particulate (pGC). sGC is principally activated by NO, while pGC is activated by the family of natriuretic peptides (Feil *et al.*, 2003; although there are a number of membrane bound GCs, which are orphan receptors and whose ligands have yet to be identified; Kuhn, 2003). The soluble isoform exists as a heterodimer composed of an α and β subunit and has a covalently bound prosthetic haem group. Two isoforms of each subunit have been identified, α_1/α_2 and β_1/β_2 (Russwurm and Koesling, 2002). The particulate isoform is made up of an extracellular ligand binding domain (for natriuretic peptide binding), a short transmembrane region and an intracellular GC catalytic domain and acts as the natriuretic peptide receptor (Kuhn, 2003). The ability of NO and natriuretic peptides to stimulate GCs underlies their analogous effects in the vasculature.

1.5. Natriuretic peptides

1.5.1. Introduction

Natriuretic peptides are a family of highly conserved but widely distributed cardiac hormones, which play important roles in cardiovascular homeostasis (Baxter, 2004).

Three members of this family, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) have been well-characterised and extensively reviewed in the literature (Ahluwalia *et al.*, 2004; Baxter, 2004; Kuhn, 2004; Potter, 2004; Vanderheyden *et al.*, 2004); other members have been documented but are less well characterised and their function and physiological importance have yet to be fully elucidated (Baxter, 2004). ANP and BNP act in an endocrine manner to regulate fluid and electrolyte balance, blood pressure and cardiac morphology, while CNP is thought to have a complementary role acting locally in the vascular endothelium in a paracrine fashion to control vascular smooth muscle tone and proliferation (Ahluwalia *et al.*, 2004).

1.5.2. History of natriuretic peptides

The presence of endocrine-like secretory granules in the atria was first described by Kisch and Jamieson in 1956 (Kisch, 1956). However, it was not until the late 1970s that the role of the heart as an endocrine organ and its connection to the kidney was elucidated when de Bold and his co-workers detected a change in atrial granularity in accordance with changes in body fluid balance (de Bold, 1979). This was later attributed to an atrial hormone now know as ANP. In 1981 de Bold infused anaesthetised rats with an atrial myocardial homogenate (de Bold *et al.*, 1981). An increase in sodium, potassium and chloride excretion as well as an increased urine volume were observed (de Bold *et al.*, 1981), confirming the link between natriuresis, ANP and atrial granularity (de Bold, 1982). Following these observations two more members of the natriuretic peptide family with potent natriuretic, diuretic and vasodilating activity (Levin *et al.*, 1998), as well as anti-proliferative and anti-migratory effects on vascular smooth muscle cells (Casco *et al.*, 2002) were identified. In 1988 Sudoh *et al* identified a peptide in porcine brain that had similar biological and structural properties as ANP and hence was called BNP (Sudoh *et al.*, 1988; Sudoh *et al.*, 1989). CNP was isolated two years later, again from porcine brain by Sudoh *et al* (Sudoh *et al.*, 1990) but it was later revealed that the endothelium is the main site for CNP synthesis (Suga *et al.*, 1992b). Additional members of the natriuretic peptide family exist with structural, biological and distributional similarities to ANP, BNP and CNP. Dendroaspis natriuretic peptide (DNP) was isolated from the venom of the green mamba, *Dendroaspis angusticeps*

(Schweitz *et al.*, 1992), urodilatin was isolated from human urine (Schulz-Knappe *et al.*, 1988) while guanylin and uroguanylin are both intestinal peptides produced by the gut epithelium (Kuhn, 2004).

1.5.3. Natriuretic peptide synthesis, processing and expression

Each natriuretic peptide is encoded by a separate gene in humans and all are produced as unique prohormones that undergo post-translational modification to yield the active peptides. Peptide synthesis is shown in Figure 4 and synthesis for each peptide is described below.

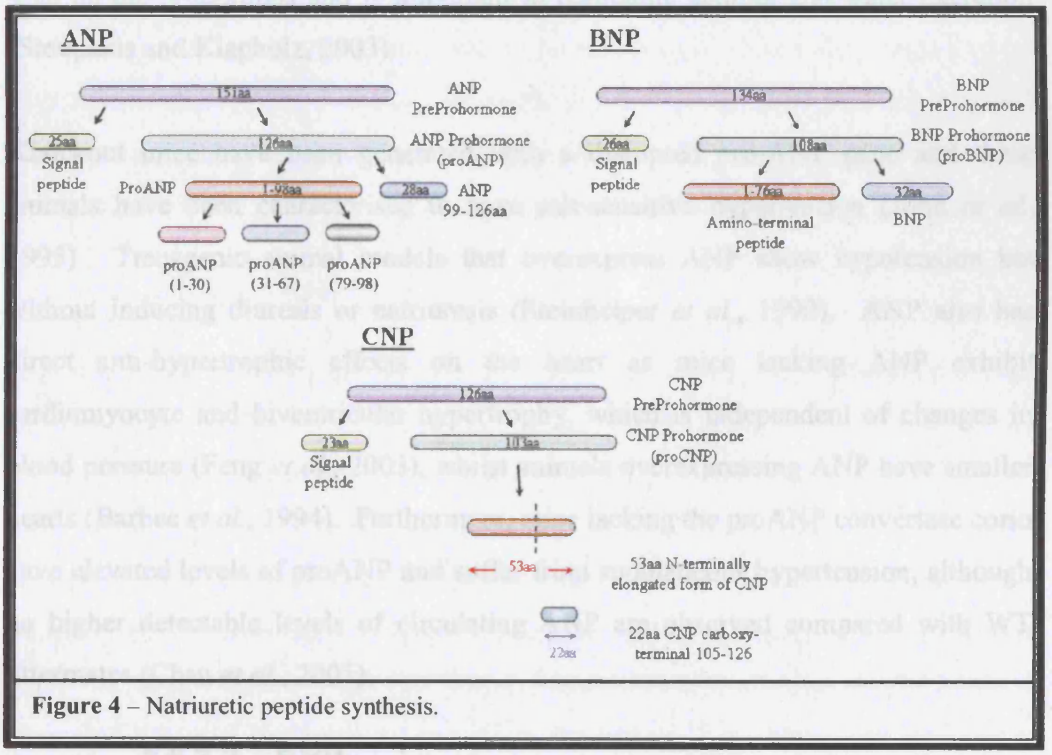


Figure 4 – Natriuretic peptide synthesis.

1.5.3.1. ANP

ANP is primarily produced in the atria of the heart. It is encoded in humans as a 151 aa preprohormone by the *Nppa* gene (Baxter, 2004) on chromosome 1 (Baxter, 2004; Vanderheyden *et al.*, 2004) and the mRNA transcript is approximately 1 kb in size (Vanderheyden *et al.*, 2004; the ANP gene in mice is located on chromosome 4; Tamura *et al.*, 1996). The *Nppa* gene consists of 3 exons separated by 2 introns (Vesely, 2002). The preprohormone undergoes cleavage of its 25 aa signal peptide

in the endoplasmic reticulum giving the 126 aa prohormone (proANP), which is stored in atrial myocyte granules (Vuolteenaho *et al.*, 1985) and released into the circulation in response to stretch, thyroid hormones, glucocorticoids, mineralocorticoids, calcium and vasoconstrictive peptides such as endothelin-1, norepinephrine and angiotensin II (Ang II; Vesely, 2002). Cleavage of this prohormone by corin, a cardiac serine protease (Yan *et al.*, 2000) produces an amino-terminal fragment of 98 aa and a carboxy-terminal fragment of 28 aa (ANP), which is the biologically active peptide (Vesely, 1995). In addition, alternative processing of the 126 aa prohormone in the kidney produces a 32 aa peptide, urodilatin (Schulz-Knappe *et al.*, 1988). It is identical to ANP but with an additional 4 aa on the N-terminus and is important in regulating sodium and water excretion (Stoupakis and Klapholz, 2003).

Knockout mice have been generated with a disrupted pro-ANP gene and these animals have been characterised to have salt-sensitive hypertension (John *et al.*, 1995). Transgenic animal models that overexpress ANP show hypotension but without inducing diuresis or natriuresis (Steinhelper *et al.*, 1990). ANP also has direct anti-hypertrophic effects on the heart as mice lacking ANP exhibit cardiomyocyte and biventricular hypertrophy, which is independent of changes in blood pressure (Feng *et al.*, 2003), whilst animals overexpressing ANP have smaller hearts (Barbee *et al.*, 1994). Furthermore, mice lacking the proANP convertase corin have elevated levels of proANP and suffer from spontaneous hypertension, although no higher detectable levels of circulating ANP are observed compared with WT littermates (Chan *et al.*, 2005).

1.5.3.2. BNP

BNP was first identified in porcine brain (Sudoh *et al.*, 1988) but in humans it is produced predominantly by the cardiac ventricles (Yasue *et al.*, 1994). PreproBNP is encoded by the gene, *Nppb*, in tandem with *Nppa* (approximately 8 kb upstream) on the distal short arm of chromosome 1 in humans (Tamura *et al.*, 1996). *Nppb* consists of 3 exons and 2 introns (Ogawa *et al.*, 1994a). Its preprohormone consists of 134 aa that undergoes endoprotease cleavage to give a 108 aa prohormone (proBNP; Baxter, 2004). In humans the prohormone is processed to create the 32 aa

carboxy-terminal, biological active BNP (77 – 108), and an amino-terminal peptide (1 – 76) whose physiological role has yet to be identified (Sudoh *et al.*, 1989; Hunt *et al.*, 1995). The 108 aa prohormone as well as the two cleavage products, BNP and amino-terminal proBNP (NT-proBNP), circulate in the plasma (Vanderheyden *et al.*, 2004). The porcine and rat BNP prohormone, in addition to the 32 aa BNP, can be processed to yield pBNP-26 and rBNP-45 respectively (Koller and Goeddel, 1992).

Targeted disruption of the *Nppb* gene has shown the importance of BNP as an antifibrotic factor and its role as a regulator of ventricular remodelling. These KO animals demonstrate cardiac fibrosis but have normal blood pressure and exhibit no left ventricular hypertrophy (Tamura *et al.*, 2000). In earlier transgenic mouse studies in which BNP was overexpressed, the mice were hypotensive suggesting the potential use of BNP as a long-term therapeutic agent (Ogawa *et al.*, 1994a). However Natrecor® (nesiritide; synthetic recombinant form of human BNP) can be detrimental to the patient's health (Topol, 2005) as will be discussed in section 1.6.

1.5.3.3. CNP

In humans CNP is highly prevalent in the central nervous system, anterior pituitary, hypothalamus, midbrain, kidney and especially in vascular tissues (Levin *et al.*, 1998; Stoupakis and Klapholz, 2003). CNP is encoded by the *Nppc* gene on chromosome 2 in humans and chromosome 1 in mice (Ogawa *et al.*, 1992) and is thus physically separate from the ANP and BNP coding regions. The human gene consists of 2 exons separated by an intron and produces a 126 aa residue preprohormone (Ogawa *et al.*, 1992) that is processed at the N-terminus by 23 aa to give proCNP (23-126; Stoupakis and Klapholz, 2003). The carboxy-terminal end is then metabolised by the endoprotease furin (Wu *et al.*, 2003) to give the 22 aa active form of CNP and 53 aa, N-terminally elongated form of CNP (Minamino *et al.*, 1990), with the 22 aa peptide being more potent than its 53 aa counterpart (Tawaragi *et al.*, 1991; Stoupakis and Klapholz, 2003).

Nppc gene disruption has revealed CNP's importance in endochondral ossification and its role as a local positive regulator of this process. *Nppc* KO mice exhibit dwarfism with the length of femurs, tibiae and vertebrae being 50 – 80 % of their

WT littermates, in addition to striking narrowing of the growth plate (Komatsu *et al.*, 2002). Less than 50 % of CNP KOs are able to survive during postnatal development, although targeted expression of CNP in the growth plate chondrocytes improves their survival rate (Chusho *et al.*, 2001).

1.5.4. Natriuretic peptide structure and homology

All natriuretic peptides possess a common 17 aa ring structure formed by an intramolecular disulfide bond between 2 conserved cysteine residues (Silberbach and Roberts, 2001). The ring component of the natriuretic peptides consists of 11 invariant and 6 variable amino acids. ANP and BNP are very similar and both have amino and carboxyl-termini attached to the ring structure while CNP is lacking the C-terminal tail (Ogawa *et al.*, 1994b). This ring structure is imperative for receptor recognition and physiological functions (Inagami *et al.*, 1987). The common ring structure and homology of ANP, BNP and CNP are shown in Figure 5.

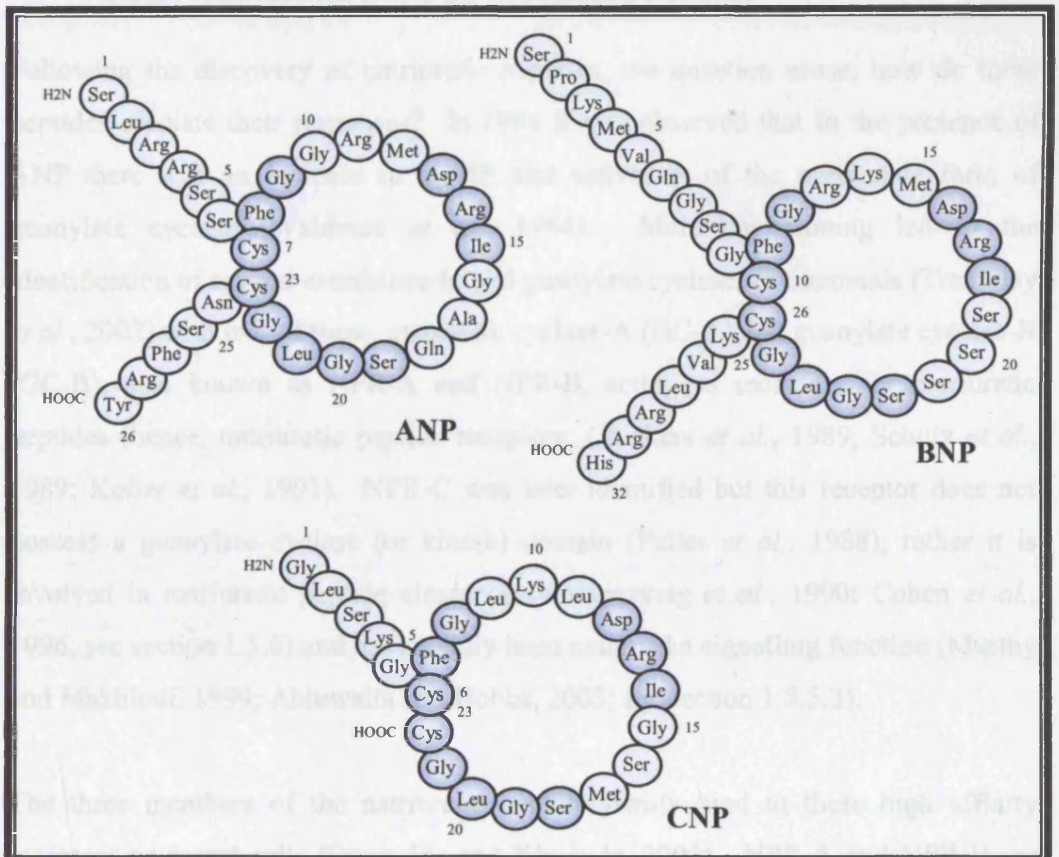


Figure 5 – Amino acid sequence and primary structure of mammalian natriuretic peptides. Invariable amino acids are shaded darker blue (adapted from Silberbach and Roberts, 2001).

ANP and CNP are highly conserved among species with CNP being considered the ancestral form of all natriuretic peptides. All three peptides are found in tetrapods but CNP is the only form found in elasmobranches (Inagami *et al.*, 1987) and this lead to the speculation that CNP was the original natriuretic peptide giving rise to ANP and BNP by gene duplication (Silberbach and Roberts, 2001) through evolutionary development. Human and porcine ANP are identical while there is only 1 aa substitution between the human and rat forms (Koller and Goeddel, 1992). Similarly porcine and rat CNP-53 are identical, with human CNP-53 having 2 aa substitutions; however, CNP-22 is conserved across all mammals (Koller and Goeddel, 1992). In contrast, BNP is not as well conserved. At the amino acid level, human, rat and pig BNP are only 50 % homologous while the mature peptides vary in size from 26 aa in pigs to 32 aa in humans and 45 aa in rats (Koller and Goeddel, 1992).

1.5.5. Natriuretic peptide receptors (NPRs)

Following the discovery of natriuretic peptides, the question arose, how do these peptides mediate their responses? In 1984 it was observed that in the presence of ANP there was an increase in cGMP and activation of the particulate form of guanylate cyclase (Waldman *et al.*, 1984). Molecular cloning led to the identification of several membrane-bound guanylate cyclases in mammals (Tremblay *et al.*, 2002) with two of these, guanylate cyclase-A (GC-A) and guanylate cyclase-B (GC-B), also known as NPR-A and NPR-B, acting as receptors for natriuretic peptides (hence, natriuretic peptide receptors; Chinkers *et al.*, 1989; Schulz *et al.*, 1989; Koller *et al.*, 1991). NPR-C was later identified but this receptor does not possess a guanylate cyclase (or kinase) domain (Fuller *et al.*, 1988); rather it is involved in natriuretic peptide clearance (Nussenzveig *et al.*, 1990; Cohen *et al.*, 1996; see section 1.5.6) and has recently been assigned a signalling function (Murthy and Makhlouf, 1999; Ahluwalia and Hobbs, 2005; see section 1.5.5.3).

The three members of the natriuretic peptide family bind to these high affinity receptors on target cells (Stoupakis and Klapholz, 2003). NPR-A and NPR-B are guanylate cyclase linked receptors and consist of an extracellular 440 aa binding domain, a single transmembrane spanning region, a 280 aa kinase homology domain

(KHD; which shows approximately 30 % homology to protein kinases), and a 250 aa guanylate cyclase domain (55 % homologous to other mammalian members of the receptor guanylate cyclase family). There exists a 44 % homology between the extracellular regions, 63 % homology between the intracellular kinase-like domain and 88 % homology between the guanylate cyclase domains (Koller and Goeddel, 1992). Like NPR-A and NPR-B, NPR-C has an extracellular domain and a single membrane spanning region but it only has a 37 aa intracellular domain that bears no homology to the NPR-A and NPR-B intracellular domains. The intracellular domain of NPR-C is devoid of both guanylate cyclase and kinase activities but it selectively activates G proteins of the $G_{i/o}$ family via a G-protein-activating domain (Murthy and Makhlouf, 1999), which links it to adenylyl cyclase (Pagano and Anand-Srivastava, 2001), phospholipase-Cβ (PLC-β; Murthy *et al.*, 2000) and G-protein gated inwardly rectifying K^+ channels (GIRKs; Chauhan *et al.*, 2003b). The structures of the natriuretic peptide receptors are shown in Figure 6.

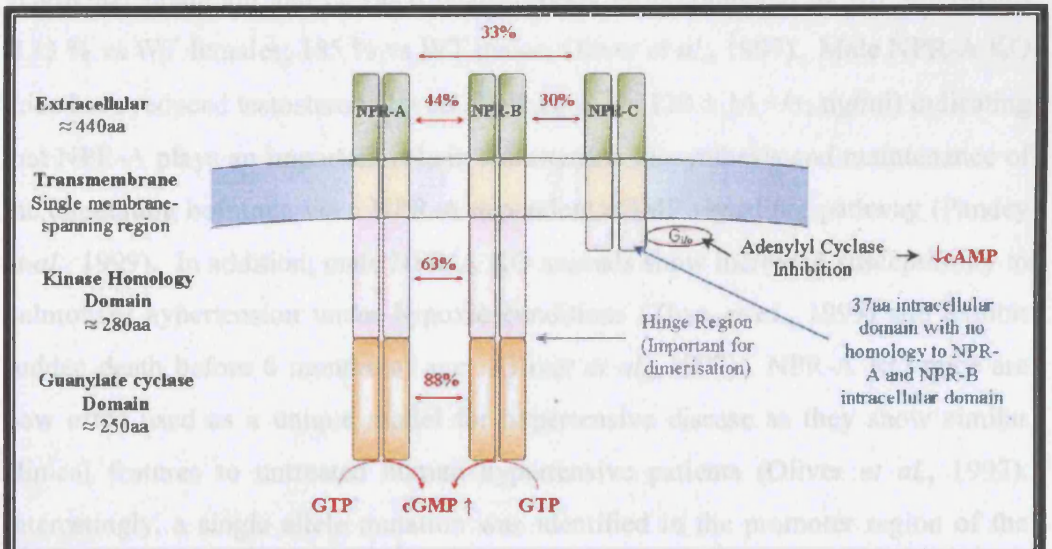


Figure 6 – Natriuretic peptide receptors NPR-A, NPR-B and NPR-C, showing homology between the three structures.

1.5.5.1. NPR-A

NPR-A is encoded by a 16 kb gene on human chromosome 1, consists of 22 exons giving rise to a 1061 aa, 135 kDa peptide (Kone, 2001) and the crystal structure of the glycosylated, unliganded, dimerised extracellular domain of rat NPR-A has been solved (van den Akker *et al.*, 2000; Ogawa *et al.*, 2004). The mature NPR-A peptide

is found in abundance in the vasculature, kidneys and adrenal glands (Levin *et al.*, 1998; Kone, 2001) and stimulation by natriuretic peptides, especially ANP or BNP, brings about vasorelaxation, natriuresis, a decrease in aldosterone synthesis (Kone, 2001) and stimulation of testosterone production (Pandey *et al.*, 1999). NPR-A is most abundant in large blood vessels, which is consistent with the vasorelaxation effects brought about by receptor activation (Levin *et al.*, 1998). Although NPR-A binds all three natriuretic peptides, it does so with different affinities and kinetics; ANP≥BNP>>CNP (Suga *et al.*, 1992a). More recently, DNP has been shown to bind to NPR-A with higher affinity than ANP and BNP (Singh *et al.*, 2006a; Singh *et al.*, 2006b).

The functions of NPR-A have partly been elucidated using KO animals. NPR-A KO mice are hypertensive (with an average increase in blood pressure of 16 mmHg), have low plasma renin concentrations (one third normal), cardiac hypertrophy, ventricular dilatation and elevated heart-to-body ratio compared to WT littermates (133 % vs WT females; 185 % vs WT males; Oliver *et al.*, 1997). Male NPR-A KO mice have reduced testosterone levels (80 ± 10 -/- vs 120 ± 14 +/+, ng/ml) indicating that NPR-A plays an important role in testosterone biosynthesis and maintenance of the circulating hormone via a NPR-A dependent cGMP signalling pathway (Pandey *et al.*, 1999). In addition, male NPR-A KO animals show increased susceptibility to pulmonary hypertension under hypoxic conditions (Zhao *et al.*, 1999) and exhibit sudden death before 6 months of age (Oliver *et al.*, 1997). NPR-A KO mice are now often used as a unique model for hypertensive disease as they show similar clinical features to untreated human hypertensive patients (Oliver *et al.*, 1997). Interestingly, a single allele mutation was identified in the promoter region of the NPR-A gene, which causes a 70 % decrease in NPR-A expression in Japanese people. Out of eight people with this mutation, seven suffered from hypertension and one had congestive heart failure (Nakayama *et al.*, 2000).

1.5.5.2. NPR-B

NPR-B is encoded by a 16.5 kb region of human chromosome 9. Like NPR-A, this receptor consists of 22 exons producing a 1047 aa peptide of approximately 120 kDa (Kone, 2001) but the crystal structure has yet to be solved. NPR-B is highly

abundant in the brain, including the pituitary glands (Levin *et al.*, 1998; Kone, 2001). Binding and guanylate cyclase activation studies have shown that CNP is likely to be the only endogenous ligand for NPR-B as it selectively activates the receptor at physiological concentrations of 100 nM but has the ability to stimulate cGMP signalling at concentrations as low as 0.5 nM. Human CNP binding is 50 – 500 fold higher than the other human natriuretic peptides (Koller *et al.*, 1991) with the order of binding affinity being CNP>ANP≥BNP (Suga *et al.*, 1992a). In NPR-B KO mice there is dramatic impairment of endochondral ossification and attenuation of longitudinal vertebra or limb-bone growth leading to reduced body size and dwarfism. Using this model it has also been shown that NPR-B is important in the embryonic development of the female reproductive tract as female KO animals are infertile while males are fertile (Tamura *et al.*, 2004).

1.5.5.3. NPR-C

NPR-C is encoded on human chromosome 5 and is a 540 aa peptide (Kone, 2001). The mature peptide exists as a homodimer (Kone, 2001), each monomer having a size of approximately 60 kDa (Cohen *et al.*, 1996), and possessing its own membrane spanning domain (Levin *et al.*, 1998); monomers are linked by a disulfide bond (Kone, 2001). The crystal structure of the unbound and natriuretic peptide bound receptor extracellular domain have been solved (He *et al.*, 2001; He *et al.*, 2006). NPR-C is the most abundantly and widely expressed natriuretic peptide receptor and is found in major endocrine glands, lungs, kidneys and the vascular wall (Kone, 2001). In addition it is highly localised in cells and tissues that receive a large proportion of cardiac output; these include vascular endothelial cells, smooth muscle cells and the renal cortex (Cohen *et al.*, 1996). All natriuretic peptides bind to NPR-C with high affinity but it has highest affinity for ANP (ANP>CNP>BNP; Suga *et al.*, 1992a). NPR-C is known to act as a clearance receptor (Maack *et al.*, 1987; Matsukawa *et al.*, 1999; see section 1.5.6) but studies have also suggested that it has a signalling function through a G-protein-activating domain (Murthy and Makhlouf, 1999).

NPR-C KO mice have been developed and have assisted in clarifying the biological functions of NPR-C (Matsukawa *et al.*, 1999). These animals show prolonged half-

life of exogenous ANP (2.40 ± 0.08 -/- vs 1.44 ± 0.05 +/+, min) and are mildly hypotensive (110 ± 2.3 -/- vs 118.7 ± 1.9 +/+, mmHg), in accord with a clearance function for this NPR subtype. They also have increased daily urinary output (2.0 ± 0.1 -/- vs 1.1 ± 0.1 +/+, ml/day) and inability to concentrate urine indicating altered renal function, decreased intravascular volume, increased number of red blood cells (10.4 ± 0.1 -/- vs 9.7 ± 0.1 +/+, $10^6/mm^3$), haematocrit (53.6 ± 0.9 -/- vs 49.9 ± 1.0 +/+, %) and haemoglobin (16 ± 0.2 -/- vs 15.6 ± 0.2 +/+, g/dl) levels. However, plasma ANP and BNP levels are not higher in these animals (plasma ANP 88.5 ± 9.0 -/- vs 114.5 ± 5.9 +/+, pg/ml; plasma BNP 22.1 ± 1.9 -/- vs 21.6 ± 3.8 +/+, pg/ml) suggesting that there is probably a homeostatic decrease in cardiac secretion of ANP and BNP, and any pathological effects seen in the NPR-C KO animals are not due to elevated plasma levels of ANP and BNP (Matsukawa *et al.*, 1999). NPR-C KO mice all have increased basal bone turnover and skeletal abnormalities including hunched backs, dome-shaped skulls, elongated tails, femurs, tibiae, metatarsal and digital bones, increased body length, decreased body weight, smaller and constricted thoracic cages as well as delay in development of secondary ossification centres in long bones (Matsukawa *et al.*, 1999) suggesting a role akin to NPR-B in regulating bone homeostasis.

1.5.6. Natriuretic peptide clearance

Plasma natriuretic peptide levels are controlled by both the rate of synthesis and release as well as their removal from the circulation. Natriuretic peptides can be removed by two distinct methods, receptor mediated endocytosis involving NPR-C and enzymatic degradation by neutral endopeptidase (Vanderheyden *et al.*, 2004). Neutral endopeptidase is a non-specific, membrane-bound, zinc containing enzyme present on the surface of endothelial cells, smooth muscle cells, cardiac myocytes and fibroblasts as well as on brush border membranes in the proximal tubules of the kidneys (Valli *et al.*, 1999; Vanderheyden *et al.*, 2004). Natriuretic peptides are cleaved by this enzyme opening the ring structure and thus making them inactive (Valli *et al.*, 1999). Alternatively, natriuretic peptide plasma levels are controlled by NPR-C. NPR-C has a single tyrosine in the cytoplasmic domain (Tyr^{508}), which plays an important role in clathrin-coated pit receptor internalisation although it is not flanked by the degenerate sequence found on other receptors having the same

method of internalisation (Cohen *et al.*, 1996). Native NPR-C undergoes rapid constitutive endocytosis in mammalian cells, the rate is not altered by ligand binding and dimerised receptors are internalised twice as fast as monomers (Cohen *et al.*, 1996). Endocytosed receptor-ligand complexes are dissociated intracellularly in endosomes, the ligand (natriuretic peptide) is lysosomally hydrolysed and the native receptors are recycled back to the cell surface (Nussenzveig *et al.*, 1990; Cohen *et al.*, 1996). Under normal conditions neutral endopeptidase is likely to play a relatively minor role in metabolic clearance but in cardiovascular disease where there is increased levels of natriuretic peptides and a large fraction of NPR-C are occupied, neutral endopeptidase could play a greater role in natriuretic peptide clearance (Okolicany *et al.*, 1992; Maack, 2006), hence the effectiveness of neutral endopeptidase inhibitors in cardiovascular disease (but do little in healthy volunteers).

1.5.7. Natriuretic peptide signalling

Both ANP and BNP exert their biological effect by binding to NPR-A while CNP is the sole endogenous ligand for NPR-B (Koller *et al.*, 1991). Binding of all natriuretic peptides to their cognate receptors activates the guanylate cyclase moiety, which then brings about an increase in intracellular cGMP subsequently initiating the cGMP-dependent signalling cascade (Koller *et al.*, 1991; see section 1.4).

NPR-A and NPR-B exist as homodimers and in a highly phosphorylated basal state (phosphorylated on conserved serines and threonines) when ligand is not bound. Upon ligand binding the receptor is transformed from a "loose" to a "tight" conformation that facilitates activation (Silberbach and Roberts, 2001) and is concomitantly dephosphorylated and desensitised. The binding of natriuretic peptide to the extracellular domain brings about conformational changes in the KHD allowing adenosine 5'-triphosphate (ATP) binding and 'disinhibition' of the catalytic guanylate cyclase domain thus bringing about cGMP production (Silberbach and Roberts, 2001; Tremblay *et al.*, 2002). More recently it has been suggested that NPR-B can alternatively signal through G-protein coupling, as it possesses G-protein coupling domains (Borges *et al.*, 2001; Alfonzo *et al.*, 2006).

In the case of NPR-C, natriuretic peptide binding to the extracellular domain of the dimerised receptor allows G_i activator peptide sequences on the cytoplasmic domain to bind and activate G_i regulatory proteins, bringing about G_i-coupled signalling and inhibition of adenylyl cyclase activity (Pagano and Anand-Srivastava, 2001). NPR-C G_i-coupling can also result in inositol triphosphate (IP_3) and diacylglycerol (DAG) formation, which are involved in intracellular Ca^{2+} mobilisation and PKC activation (Anand-Srivastava, 2005).

1.6. Functions of natriuretic peptides in the cardiovascular system

Natriuretic peptides exert a wide range of physiological actions and are thought to be critical 'cardioprotective hormones'. Well characterised cardiovascular actions of ANP and BNP include stimulation of renal natriuresis and diuresis, suppression of the renin-angiotensin-aldosterone system, inhibition of vasopressin and catecholamines, vasodilatation and inhibition of growth of vascular smooth muscle and endothelial cells (Ahluwalia *et al.*, 2004; Woods, 2004). ANP and BNP are secreted from the heart in response to atrial stretch and pressure, but BNP has lower circulating levels than ANP, which become equal in certain pathophysiological conditions such as myocardial hypertrophy and congestive heart failure (Tremblay *et al.*, 2002). Plasma concentrations of natriuretic peptides under physiological and pathophysiological conditions are summarised in Table 3. ANP acts primarily on the kidney by increasing glomerular filtration rate, and salt and fluid excretion, in response to increases in blood pressure and/or volume. This effect is brought about through NPR-A and cGMP as it can be blocked by the NPR-A/NPR-B specific antagonist, HS-142-1 (Zhang *et al.*, 1994), and NPR-A KO mice develop salt-sensitive hypertension (John *et al.*, 1995).

Administration of ANP to normotensive rodents and humans brings about small decreases in blood pressure; these changes are exacerbated in models of hypertension. ANP alters blood pressure by reducing plasma volume via a shift of fluid from the intravascular to the interstitial compartment, decreasing central venous pressure and thus reducing cardiac output but maintaining total peripheral resistance (Wijeyaratne and Moult, 1993).

	ANP	BNP	CNP
Plasma half life (min)	~ 2	~ 20	~ 2.6
Plasma Concentration (pmol/ml)			
Normal	6.4 ± 0.9	0.9 ± 0.007	1.4 ± 0.6
In Congestive heart failure	87 ± 12*	87 ± 11*	1.4 ± 0.2
In myocardial infarction	33.4 ± 6.1*	60 ± 9.4*	N.D.
In pulmonary arterial hypertension	14.0*	15.3*	N.D.
In chronic renal failure	43 ± 11*	130 ± 37.4*	3.0 ± 0.4*
In subarachnoid haemorrhage	5.9 ± 1.0	15.1 ± 3.8*	2.0 – 2.6
In cirrhosis	27.98 ± 3.71*	16.0 ±1.91*	1.36 ± 0.18

Table 3 – Plasma concentrations of natriuretic peptides under physiological and pathophysiological conditions. Values represent the mean patient values from a representative study (Potter *et al.*, 2006), * indicates significant increases.

As will be discussed in greater detail in section 1.8.2.1, ANP acts in a vasoprotective manner, especially during the development of atherosclerosis (Kiemer *et al.*, 2005). The Framingham Heart Study offspring cohort investigated the relationship between plasma BNP and N-terminal proANP to body mass index (BMI) and found that obese and overweight individuals have considerably lower plasma natriuretic peptide levels than those with a normal BMI, which could contribute to their susceptibility to cardiovascular disease (Wang *et al.*, 2004). ANP and BNP have been shown to have potent lipolytic effects in isolated human adipocytes similar to the effects seen with β-adrenergic receptor agonists (e.g. isoproterenol), but ANP and BNP bring about their effects through an increase in cGMP (not linked to cGMP-inhibitable phosphodiesterase-3B and changes in cAMP production; Sengenes *et al.*, 2000). However, it is worth noting that epidemiological studies have suggested that NPR-C could be important in modulating abdominal adiposity, since individuals carrying an allele variant in the promoter region of NPR-C, resulting in reduced NPR-C expression in adipocytes, have a significantly lower prevalence of obesity and abdominal adiposity (Sarzani *et al.*, 2004). It has been suggested that this could be due to reduced clearance of natriuretic peptides, which increases their local concentrations favouring fat mobilisation (Maack, 2006). In addition, ANP levels are elevated following prolonged exercise suggesting that ANP could play a role in

lipid mobilisation from adipose tissue required by the huge energy demand during exercise (Moro *et al.*, 2004). Since obesity presents an underlying risk for cardiovascular disease, it is not surprising that exogenous application of ANP in the form of carperitide (recombinant human ANP), which is available for the treatment of acute and refractory heart failure, has beneficial vasoprotective effects (Kikuchi *et al.*, 2001) and increases lipid mobilisation (Lafontan *et al.*, 2005).

Carperitide has also been shown to protect against ischaemia-reperfusion injury. Intravenous administration of synthetic ANP limits infarct size in dogs (Takagi *et al.*, 2000) and preischaemic infusion reduces infarct size after ischaemia-reperfusion in isolated rat hearts (Okawa *et al.*, 2003). This cardioprotective effect of ANP is dependent on the NO-PKC pathway (Okawa *et al.*, 2003). Sangawa *et al* have also reported that perfusion of ANP at the beginning of reperfusion protects isolated rat hearts from ischaemia-reperfusion injury (Sangawa *et al.*, 2004). A clinical study involving intracoronary injection of ANP following a first attack of myocardial infarction and subsequent coronary angioplasty, demonstrated that ANP attenuates myocardial reperfusion injury, preserves left ventricular function and prevents late remodelling after angioplasty (Kuga *et al.*, 2003). Akin to ANP, BNP is released during ischaemia and can act in a cytoprotective manner (D'Souza and Baxter, 2003). In isolated rat hearts, acute infusion of exogenous BNP markedly protects against myocardial ischaemia-reperfusion injury in a concentration-dependent manner and involves elevation of cGMP and ATP sensitive potassium (K_{ATP}) channel opening (D'Souza *et al.*, 2003b). Furthermore, there is evidence to suggest that the NO/sGC system may play a role in the anti-ischaemic actions of BNP, since the infarct-limiting actions of this natriuretic peptide are abrogated by treatment with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) and the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; D'Souza *et al.*, 2003a).

Taken together, the reduced natriuretic peptide levels observed in individuals susceptible to cardiovascular disease (Wang *et al.*, 2004) and the elevated levels seen during heart failure, myocardial infarction and pulmonary hypertension (Potter *et al.*, 2006) suggest that a basal level of natriuretic peptides is required to prevent the development of cardiovascular disease, but once a cardiovascular insult has occurred then natriuretic peptide levels are increased to help in the resolution of disease.

However, it is worth noting that patients with elevated levels of BNP after an acute coronary syndrome have a higher likelihood of further adverse cardiovascular events (de Lemos *et al.*, 2001).

In accordance with the functions of natriuretic peptides, they have been implicated in a number of cardiovascular diseases, thus representing prime targets for therapeutic intervention (Tremblay *et al.*, 2002). Recently there has been great interest in the levels of BNP and NT-proBNP as diagnostic tools for cardiovascular risk (Talwar *et al.*, 2000; de Lemos *et al.*, 2001; Kragelund *et al.*, 2005). During heart failure BNP levels in the circulation are raised (Yandle *et al.*, 1993; Gehi *et al.*, 2005); patients with BNP levels >80 pg/ml after an acute coronary syndrome are at high risk of death, a new myocardial infarction and heart failure, while those with normal BNP levels appear to have a low long-term risk of death and heart failure (de Lemos *et al.*, 2001). NT-proBNP has also been established as a marker of long-term mortality in patients with stable coronary disease (Kragelund *et al.*, 2005) and it has been shown to have a biphasic pattern of plasma concentration following acute myocardial infarction. Measurements of NT-proBNP prior to hospital discharge, following acute myocardial infarction are more predictive of adverse outcome in comparison to measurements taken soon after admission (Talwar *et al.*, 2000). Therefore BNP and NT-proBNP levels following acute coronary events are useful tools in predicting long-term risk of death and future nonfatal cardiac events (de Lemos *et al.*, 2001).

In addition to the raised BNP levels, BNP clearance is upregulated in patients with heart failure and this limits the rise in the concentration of circulating BNP (Adams *et al.*, 2003). For this reason two classes of therapeutic agents have been developed, recombinant human BNP and neutral endopeptidase inhibitors. Human recombinant BNP (identical to the endogenously produced hormone; Adams *et al.*, 2003) has been marketed as Natrecor® (nesiritide) and following extensive clinical trials was approved for human use by the Food and Drug Administration (FDA) in 2001 for the treatment of acute decompensated congestive heart failure (Topol, 2005). Although nesiritide has been on the market for a few years now, there has been no long term study showing decrease in length of hospitalisation, re-admission rate, or mortality and there have been safety problems associated with its administration (Gehi *et al.*,

2005; Topol, 2005). Neutral endopeptidase inhibitors have been developed and are currently administered in conjunction with angiotensin-converting enzyme inhibitors (vasopeptide inhibitors (VPIs), marketed as omapatrilat, fasidotril, sampatrilat; Campbell, 2003) in patients with congestive heart failure (Lapointe and Rouleau, 2002).

CNP, in contrast to ANP and BNP acts in a paracrine fashion and does not circulate in high concentrations (Ahluwalia *et al.*, 2004). It possesses weak natriuretic properties but is a potent vasodilator and shows anti-mitogenic properties (Ahluwalia *et al.*, 2004). CNP increases cGMP levels more than ANP in cultured vascular smooth muscle cells and relaxes aortic rings (Tremblay *et al.*, 2002), and local infusion of CNP increases forearm blood flow NO-independently (Honing *et al.*, 2001). CNP has also been characterised as an endothelium-derived hyperpolarising factor (EDHF) via activation of NPR-C (Chauhan *et al.*, 2003b). CNP released from the endothelium activates NPR-C on the underlying smooth muscle cells bringing about relaxation via G_i-coupling to GIRKs on smooth muscle cells and subsequent hyperpolarisation (Chauhan *et al.*, 2003b). Responses to CNP and EDHF are not blocked by HS-142-1 and are attenuated by Pertussis toxin (PTx; Chauhan *et al.*, 2003b) giving a definitive role for G_i-coupling and signalling via NPR-C. Furthermore, CNP has been shown to be protective against ischaemia-reperfusion injury through vasorelaxation with the involvement of NPR-C activation and signalling (Hobbs *et al.*, 2004). Finally, a single nucleotide polymorphism (G2628A) in the 3'-untranslated region of the CNP gene has been reported to be associated with hypertension (Ono *et al.*, 2002).

More recently, the newest member of the natriuretic peptide family, DNP, has been shown, by immunoreactivity, to be present in human plasma and to be elevated in congestive heart failure (Schirger *et al.*, 1999). Akin to other natriuretic peptides it possesses natriuretic and diuretic properties (Lisy *et al.*, 1999), is anti-proliferative (Woodard *et al.*, 2002), causes vasorelaxation of isolated human arteries via cGMP (Best *et al.*, 2002) and has been proposed to protect against post-ischaemic myocardial dysfunction (Ha *et al.*, 2006). However, DNP has yet to be purified from human plasma and therefore its relevance to human physiology is uncertain (Richards *et al.*, 2002).

1.7. Functions of natriuretic peptides in the immune system

Natriuretic peptides play a vital role in the immune system. ANP has been shown to prime polymorphonuclear neutrophils (PMNs) to secrete superoxide anion, mobilise PMNs and increase oxygen radical production in the macrophage cell line J774 (Vollmar *et al.*, 1997). Furthermore, ANP at high concentrations suppresses, while at low concentrations stimulates, IgG phagocytosis (Vollmar *et al.*, 1997). In macrophages ANP affects pro-inflammatory protein expression including iNOS and COX-2. For example, in LPS activated murine macrophages ANP inhibits iNOS expression and NO production via GC coupled NPR-A (Kiemer and Vollmar, 1998) while CNP has no effect (Kiemer and Vollmar, 1997). In addition to iNOS, ANP affects COX-2 by reducing both mRNA and protein expression via inhibition of LPS-induced PGE-2 with the involvement of NPR-C and inhibition of cAMP production (Kiemer *et al.*, 2002b). Furthermore, ANP inhibits TNF-α production in IFN-γ-activated macrophages via p38 suppression (Tsukagoshi *et al.*, 2001) and inhibits pro-inflammatory transcription factors such as NF-κB and AP-1 in LPS-activated macrophages (Kiemer *et al.*, 2000; with the possible involvement of p38 MAPK). Thus, ANP can affect TNF-α effector functions (Vollmar, 2005) such as endothelial permeability (Tsukagoshi *et al.*, 2001). ANP has also been shown to be released endogenously by macrophages when activated (Vollmar and Schulz, 1994). In human umbilical vein endothelial cells (HUVEC), haem oxygenase-1 (HO-1) is induced by ANP via cGMP with the involvement of ERK and AP-1/JNK pathways (Kiemer *et al.*, 2003). In messengial cells ANP negatively regulates platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) -stimulated ERK activity via NPR-A and the involvement of cGMP (Pandey *et al.*, 2000). In addition to the innate immune system ANP also affects adaptive immunity. It regulates thymopoesis and T-cell maturation via NPR-A/cGMP signalling in dendritic cells, as well as controlling the balance between Th 1 and Th 2 responses (Vollmar, 2005).

CNP has also been shown to play a role in inflammation. Akin to ANP, CNP suppresses COX-2 but does not affect cyclooxygenase-1 (COX-1) in LPS-activated murine macrophages (Kiemer *et al.*, 2002b). Furthermore, CNP reduces cytokine or histamine-induced leukocyte rolling in the mouse mesenteric system possibly

through the activation of NPR-C (Scotland *et al.*, 2005b). In addition it has been postulated that CNP could play a role in protecting against development of atherosclerosis. CNP and NPR-B/NPR-C mRNA expression is high in low-severity disease while levels are reduced in high severity cases, indicating a potential role for CNP in protection against atherosclerosis (Casco *et al.*, 2002) as will be discussed further in section 1.8.2.3. Moreover, circulating BNP and CNP levels are elevated in patients with septic shock (BNP: 500 (239 – 1017) septic vs 319 (132 – 808) non-septic ICU patients pg/ml (Cuthbertson *et al.*, 2005); CNP: 13.2 ± 10.1 septic vs 1.4 ± 0.6 healthy volunteers, pmol/ml; Hama *et al.*, 1994). Finally CNP (and ANP) inhibit oxidised low density lipoprotein (LDL)-stimulated vascular smooth muscle cell migration and proliferation by inhibiting the MAPK signalling pathway (Prins *et al.*, 1996) thus affecting vascular remodelling, a major contributor to development of vascular inflammatory disease.

1.8. Role of natriuretic peptides in atherosclerosis

1.8.1. Introduction

Atherosclerosis, hardening of the arteries, is a multi-factorial pathological and inflammatory process resulting in cardiovascular disease including myocardial infarction and stroke, which are the primary causes of death in the western world (Shoenfeld *et al.*, 2001; Sherer and Shoenfeld, 2006).

Russell Ross originally proposed the "response to injury" hypothesis in which he described the first step in the development of atherosclerosis to be endothelial denudation followed by adherence and aggregation of platelets together with uncontrolled smooth muscle proliferation (Ross and Glomset, 1976a; Ross and Glomset, 1976b). However, this hypothesis has since been revised and Russell Ross published a historic paper in 1999 emphasising the importance of endothelial dysfunction, rather than complete loss, in the early development of atherosclerosis (Ross, 1999). Initiation of atherosclerosis is influenced by a number of 'risk factors' including smoking, hypertension, hyperlipidemia, hyperhomocysteinemia and diabetes mellitus, all of which cause endothelial dysfunction (Celermajer *et al.*, 1994).

It has been demonstrated that both cellular and humoral immunity play a key role in atherosclerotic plaque development (Ross, 1999; Sherer and Shoenfeld, 2006; Tedgui and Mallat, 2006). Atherosclerotic plaques are more likely to form in arterial sites where there is decreased shear stress, increased turbulence and in the setting of an unfavourable serum lipid profile, especially in the presence of LDL cholesterol (Ross, 1999; Crowther, 2005). Under physiological conditions circulating blood constantly induces shear-stress on the vessel wall and the endothelium. This shear stress induced by laminar flow is mechanotransduced into a biochemical signal resulting in gene expression, which is crucial for normal vascular function and is atheroprotective by inhibiting proliferation, thrombosis and inflammation of the vessel wall (Cunningham and Gotlieb, 2005). Normal laminar flow causes the expression of eNOS and the production of NO, which enhances vasorelaxation, inhibits platelet activation and aggregation, apoptosis and endothelial-dependent monocyte adhesion (Tsao *et al.*, 1996; Freedman *et al.*, 1999; Dimmeler *et al.*, 1999). Around curvatures, branch ostia and bifurcations, there is disruption to the blood flow and hence reduced shear stress, which is associated with reduced eNOS production, reduced vasodilatation and impaired endothelial cell repair. In addition there is an increase in reactive oxygen species (ROS), endothelial permeability, leukocyte adhesion, apoptosis, smooth muscle cell proliferation and collagen deposition (Cunningham and Gotlieb, 2005). It is at these sites that atherogenesis is favoured and most commonly observed. Reduction in shear stress causes upregulation of leukocyte adhesion molecules including L-selectin, integrins and platelet-endothelial-cell adhesion molecule-1 as well as upregulation of endothelial adhesion molecules such as E-selectin, P-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular-cell adhesion molecule-1 (VCAM-1; Ross, 1999). Expression of these molecules on the endothelial surface causes recruitment of monocytes and T-cells and the migration of leukocytes into the artery wall. It is the macrophages in the artery wall that take up oxidised-LDL to become foam cells. The presence of foam cells and T lymphocytes marks the beginning of the fatty-streak formation (Ross, 1999). The immune cells release a milieu of inflammatory mediators including growth factors and cytokines, which cause migration and proliferation of the underlying smooth muscle cells, which can then release their own mediators including IL-1β, TNF-α and β, IL-6, macrophage colony-stimulating factor (M-CSF), monocyte chemotactic protein-1 (MCP-1), IL-18 and CD-40L

resulting in a self-perpetuating inflammatory process within the plaque (Ross, 1999; Crowther, 2005; Sherer and Shoenfeld, 2006). The early development of an atherosclerotic plaque is shown in Figure 7. As the fatty streak grows it forms a fibrous cap (often associated with calcification) that isolates the lesion from the lumen and this can become progressively weaker resulting in plaque rupture, thrombosis and distal occlusion (Ross, 1999).

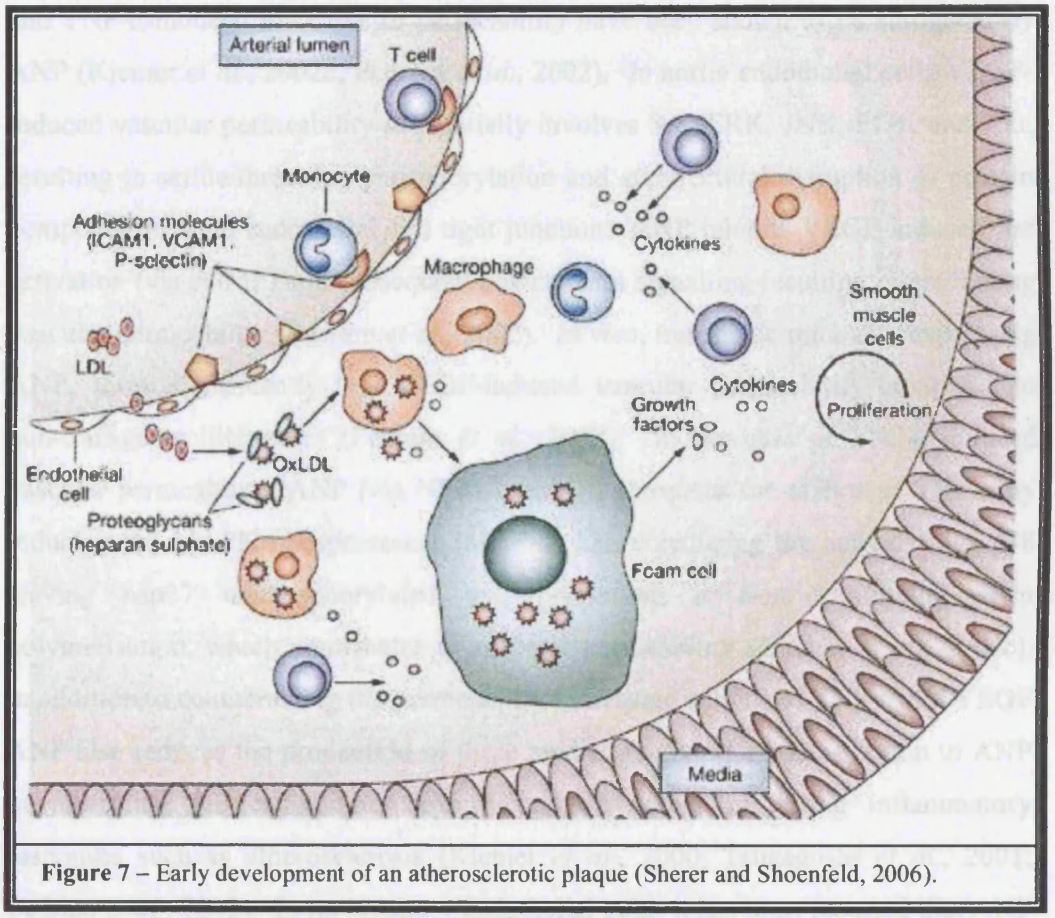


Figure 7 – Early development of an atherosclerotic plaque (Sherer and Shoenfeld, 2006).

1.8.2. Role of natriuretic peptides in the pathogenesis of atherosclerosis

Natriuretic peptides are known to contribute to the regulation of inflammatory responses, as discussed in section 1.7, and their role in the development of atherosclerosis might be of utmost clinical relevance (Kiemer *et al.*, 2005).

1.8.2.1. ANP

Vascular permeability, the increase in endothelial cell leakiness and formation of intercellular gaps in vascular endothelium, is considered to be one of the initial stages in the development of an atheromatous plaque (Kiemer *et al.*, 2005). Both vascular endothelial growth factor (VEGF) and TNF-α are able to induce increases in permeability involving fibre formation and actin polymerisation. These VEGF- and TNF-α-induced increases in permeability have been shown to be abrogated by ANP (Kiemer *et al.*, 2002c; Pedram *et al.*, 2002). In aortic endothelial cells VEGF-induced vascular permeability sequentially involves Src, ERK, JNK, PI3K and Akt, resulting in serine/threonine phosphorylation and architectural disruption of protein components of the endothelial cell tight junction. ANP inhibits VEGF-induced Src activation (via cGMP) and subsequent downstream signalling resulting in preventing vascular permeability (Pedram *et al.*, 2002). *In vivo*, transgenic mice overexpressing ANP, show significantly less VEGF-induced vascular permeability compared to non-transgenic littermates (Pedram *et al.*, 2002). In the case of TNF-α-induced vascular permeability, ANP (via NPR-A/cGMP) abrogates the effects of TNF-α by inducing the MAPK phosphatase-1 (MKP-1) hence reducing the activation of p38 leaving hsp27 unphosphorylated and preventing it from promoting actin polymerisation, which contributes to vascular permeability (Kiemer *et al.*, 2002c). In addition to counteracting the permeability-increasing actions of TNF-α and VEGF, ANP also reduces the production of these mediators giving a double action to ANP in protecting the endothelium from excessive activation during inflammatory responses such as atherosclerosis (Kiemer *et al.*, 2000; Tsukagoshi *et al.*, 2001; Pedram *et al.*, 2002). In rat coronary endothelial cells, it has been reported that ANP decreases endothelial permeability via NPR-C (Hempel *et al.*, 1998).

However, there are studies indicating that ANP can increase vascular permeability. Sarker and Fraser have shown that ANP increases endothelial permeability in rat pial venular capillaries (Sarker and Fraser, 2002), while Hölschermann *et al* have reported that increases in cGMP can both increase and decrease endothelial permeability in the same endothelial cell preparation depending on whether the cells are in a basal state where Ca^{2+} is not involved (cGMP reduces permeability), or

whether they have been stimulated and hence Ca^{2+} has already been released from endogenous stores (cGMP increases permeability; Hölschermann *et al.*, 1997).

ANP has also been reported to prevent leukocyte attraction and adhesion. As discussed above in section 1.8.1, MCP-1 is one of the mediators involved in leukocyte recruitment in atherosclerosis (Ross, 1999) and is transcriptionally activated by TNF-α (Young *et al.*, 2002). TNF-α-induced expression of MCP-1 is reduced by ANP involving p38 and MKP-1 as discussed above (Young *et al.*, 2002). Furthermore, ANP significantly reduces other TNF-α-induced cell surface proteins involved in leukocyte adhesion, such as E-selectin and ICAM-1 via transcriptional activation of IκB (Kiemer *et al.*, 2002d). Cell adhesion molecules are known to be expressed on endothelial cells under hypoxic conditions (Kokura *et al.*, 2002) and this has been shown to be attenuated by ANP (Mtairag *et al.*, 2002). Mtairag *et al* inhibited neutral endopeptidase, thus increasing the levels of ANP and demonstrated that ANP inhibits neutrophil-endothelial interactions under hypoxic conditions (Mtairag *et al.*, 2002). Furthermore, Kugiyama *et al* demonstrated that inhibition of neutral endopeptidase in cholesterol-fed rabbits suppressed atherosclerotic changes and atheromatous plaque formation (Kugiyama *et al.*, 1996). They also showed that relaxation of isolated aorta in response to ACh is significantly impaired in cholesterol and cholesterol plus neutral endopeptidase inhibitor fed rabbits, although the impairment was significantly less in the latter group. Vasorelaxation responses to ANP were also impaired in cholesterol fed rabbits, but were preserved in cholesterol plus neutral endopeptidase inhibitor fed animals (Kugiyama *et al.*, 1996).

In addition to vascular permeability and leukocyte adhesion, migration of endothelial cells as well as arterial medial smooth muscle cells, are key processes in the initiation, progression and intimal thickening during the development of an atherosclerotic lesion (Ross, 1999). Ikeda *et al* have shown that ANP inhibits both rat vascular endothelial and smooth muscle cell migration in a concentration- and cGMP-dependent fashion (Ikeda *et al.*, 1995; Ikeda *et al.*, 1997), once again supporting its anti-atherogenic properties. Furthermore, *ScaI* ANP gene polymorphism, leading to the extension of human ANP (the peptide of 28 aa is extended to 30 aa by 2 additional arginines), is significantly associated with multiple-vessel coronary atherosclerosis (Gruchala *et al.*, 2003).

Finally, a study employing immunocytochemistry and *in situ* hybridization has revealed the localisation of the three natriuretic peptides and their receptors in human coronary arteries with variable degrees of atherosclerosis (Casco *et al.*, 2002). During the formation of early atherosclerotic lesions, ANP is localised in the intima, at very low levels in the media and in the adventitia, particularly in the vasa vasora, while NPR-A, NPR-B and NPR-C are not detected. In intermediate plaques ANP is localised in the intimal level, in the lipid core regions and in the media. NPR-A mRNA is not present, but NPR-B and NPR-C mRNA is present in the intima of the plaques. In advanced atherosclerotic lesions however both peptide and mRNA are restricted to intimal myofibroblasts and microvessels of the intima and vasa vasorum, suggesting that atherosclerotic plaque progression entails the downregulation of locally expressed ANP (Casco *et al.*, 2002).

1.8.2.2. BNP

Although BNP is known to play a pivotal role in cardiovascular homeostasis and disease, a lot less is known about its involvement in the development of atherosclerosis. Casco *et al* have demonstrated the localisation of BNP at the different stages of plaque development. BNP is not present in early and intermediate atherosclerotic lesions, but it is detected in the intima and adventitia of advanced atherosclerotic lesions together with NPR-B and NPR-C (Casco *et al.*, 2002).

BNP has been shown to induce potent relaxation in normal aortae from rabbits preconstricted with phenylephrine, and relaxations are endothelium-independent as removal of the endothelium does not affect relaxation potency (Schirger *et al.*, 2000). However, BNP-induced relaxations are impaired in aortae from rabbits fed a high cholesterol diet possessing atherosclerotic lesions. In rabbits fed a high cholesterol diet but concomitantly given the neutral endopeptidase inhibitor candoxatril, BNP-induced relaxations are preserved when compared to aortae from control animals (Schirger *et al.*, 2000).

Akin to ANP, BNP inhibits rat endothelial and smooth muscle cell migration in a concentration- and cGMP-dependent manner (Ikeda *et al.*, 1995; Ikeda *et al.*, 1997). Furthermore, BNP significantly blunts the extent of thrombin-induced permeability

and enhances the rate of barrier restoration in pulmonary microvascular endothelial cells in a cGMP-independent manner (Klinger *et al.*, 2006). Although this study was conducted in pulmonary endothelial cells and is primarily applicable to lung injury, it could be extrapolated to other inflammatory diseases such as atherosclerosis where endothelial barrier dysfunction is key to progression of disease.

1.8.2.3. CNP

CNP, like ANP and BNP has been shown to possess anti-atherogenic properties. Immunological studies on human coronary atherosclerotic lesions have revealed that CNP is present in different cell types according to progression of atherosclerosis. Under normal physiological conditions, arterial segments show CNP-positive endothelial cells (Naruko *et al.*, 1996) and monocytes/macrophages (Ishizaka *et al.*, 1992). As the development of the atherosclerotic plaques progresses, the hypercellular atherosclerotic lesions have both CNP-positive smooth muscle cells and macrophages while CNP levels in the endothelial cells are decreased (Naruko *et al.*, 1996). Advanced atherosclerotic lesions show CNP expression in the plaque microvessels and adventitial vasa vasora suggesting a role for CNP in vascular remodelling and in progression/regression of the plaque of the diseased arteries (Casco *et al.*, 2002). Furthermore, advanced atherosclerotic lesions have CNP-positive macrophages but the majority of the smooth muscle cells within the fibrous cap and the surface endothelial cells are CNP-negative (Naruko *et al.*, 1996). Therefore, the severity of atherosclerotic lesions inversely correlates with CNP expression (Casco *et al.*, 2002). It is also interesting to note that oxidized LDL suppresses the secretion of CNP from endothelial cells (Sugiyama *et al.*, 1995), possibly allowing the growth of smooth muscle cells into the lesion. However, there is conflicting evidence as CNP inhibits oxidized LDL-stimulated smooth muscle cell migration and proliferation as discussed in section 1.7. CNP has also been shown to have potent inhibitory effects on foetal calf serum (FCS)-induced or PDGF-induced migration of rat vascular smooth muscle cells (Ikeda *et al.*, 1997).

CNP has also been implicated in the regulation of leukocyte adhesion, platelet activation and P-selectin expression (Scotland *et al.*, 2005a). In eNOS KO mice where basal leukocyte activation is high, CNP is able to suppress basal leukocyte

rolling and this is mimicked by the NPR-C selective agonist $cANF^{4-23}$, implying the involvement of NPR-C. CNP also suppresses leukocyte rolling induced by IL-1β or histamine, inhibits platelet-leukocyte interactions and prevents thrombin-induced platelet aggregation. Furthermore, CNP attenuates the expression of P-selectin in HUVEC, platelets and leukocytes (Scotland *et al.*, 2005a). It is also worth noting that relaxation to CNP in atherosclerotic aortae from cholesterol rabbits is impaired compared to aortae from normal dietary fed rabbits; however, incubation of the atherosclerotic aortae from cholesterol rabbits with a neutral endopeptidase inhibitor attenuates the impairment of the vasorelaxation response to CNP (Kugiyama *et al.*, 1996). Taken together, the above evidence suggests an anti-atherogenic role for CNP by regulating smooth muscle migration, cellular adhesion and P-selectin expression.

1.9. Role of natriuretic peptides in cellular growth

All natriuretic peptides have been shown to have anti-proliferative properties and this is particularly important in smooth muscle, endothelial and cardiomyocyte cells as it can be cardioprotective (Furuya *et al.*, 1991; Furuya *et al.*, 1993; Silberbach and Roberts, 2001). Since it has been suggested that excessive proliferation of smooth muscle cells in particular, is central to the development of restenosis and atherosclerosis (Ross, 1999), the molecular mechanisms underlying the anti-proliferative effects of natriuretic peptides have been investigated.

1.9.1. ANP

ANP exhibits anti-proliferative properties in a variety of cell types including renal mesangial cells (Appel, 1988), astrocytes (Levin and Frank, 1991), endothelial cells (Itoh *et al.*, 1992; Morishita *et al.*, 1994), cardiac myocytes and fibroblasts (Cao and Gardner, 1995; Calderone *et al.*, 1998), and vascular smooth muscle cells (Morishita *et al.*, 1994; Hutchinson *et al.*, 1997). Astrocyte growth inhibition by ANP involves inhibition of MAPK via NPR-C (Prins *et al.*, 1996), while in cardiac myocytes and fibroblasts the inhibitory effects are exerted through NPR-A and cGMP-dependent mechanisms (Cao and Gardner, 1995; Calderone *et al.*, 1998). Endothelial growth inhibition by ANP has been reported as cGMP-mediated (NPR-A; Itoh *et al.*, 1992) and cGMP-independent (NPR-C; Pedram *et al.*, 1997). In addition, ANP attenuates

the expression of VEGF, which is vital in the stimulation of normal angiogenesis (Pedram *et al.*, 1997). Interestingly however, a study by Hook *et al* in human endothelial cells showed that ANP increases growth, thus potentiating endothelial regeneration in a cGMP-dependent manner involving cGMP-dependent protein kinase stimulation and subsequent Akt and ERK 1/2 activation (Kook *et al.*, 2003). The discrepancies between the different studies could possibly be explained by the inconsistent concentrations of ANP used in the individual experimental setups. High ANP concentrations of 10^{-7} – 10^{-6} M result in growth inhibition (Itoh *et al.*, 1992; Pedram *et al.*, 1997) while low, physiological ANP concentrations of 10^{-11} M cause proliferation (Kook *et al.*, 2003). It could therefore be postulated that the actions of ANP differ between maintaining normal homeostasis and regulating pathophysiological conditions where ANP levels are elevated (Kiemer *et al.*, 2005). Like endothelial cell proliferation, control of smooth muscle growth and proliferation by ANP has also been reported, by independent studies, to involve both NPR-A- (cGMP-dependent) and NPR-C- (cGMP-independent) mediated mechanisms. Sharma *et al* have shown that growth inhibition by ANP in vascular smooth muscle cells is mediated via NPR-A, GC and PKG, perhaps involving the inhibition of ERK 1/2 phosphorylation (Sharma *et al.*, 2002) while Cahill *et al* have reported the involvement of NPR-C in growth inhibition by ANP in a cGMP- and cAMP-independent manner (Cahill and Hassid, 1994). However, ANP can also phosphorylate and activate the MAPK pathway (Tremblay *et al.*, 2002), which suggests it can act in a proliferative fashion under certain circumstances as seen in endothelial cells. It has been reported that ANP exerts proliferative actions on smooth muscle cells by enhancing the mitogenic effects of FGF-2, via a mechanism that may involve elevation of immediate early gene expression but not enhancement of FGF receptor protein levels (Dhaunsi and Hassid, 1996). However, in support of the anti-proliferative actions of ANP it is worth noting that NPR-A KO mice show marked cardiac hypertrophy (Knowles *et al.*, 2001) and increased susceptibility to hypoxia-induced pulmonary hypertension (Zhao *et al.*, 1999).

1.9.2. BNP

Unlike ANP and CNP, few studies have addressed the anti-proliferative properties of BNP. Levin *et al* have shown that akin to ANP, BNP inhibits rat astroglial

proliferation probably through NPR-C and cGMP-independent mechanisms (Levin and Frank, 1991). BNP also inhibits FCS-stimulated human aortic vascular smooth muscle cell proliferation, which is accompanied by an increase in cGMP (Schirger *et al.*, 2000) suggesting the involvement of NPR-A. Furthermore, BNP abrogates Ang II-stimulated increases in markers of hypertrophy in adult rat cardiomyocytes through cGMP-dependent protein kinases (Rosenkranz *et al.*, 2003). However, *in vivo* BNP appears to exert antifibrotic rather than antihypertrophic actions (Tamura *et al.*, 2000). Despite NPR-A KO mice exhibiting cardiac hypertrophy (Oliver *et al.*, 1997), BNP null animals suffer from fibrosis but are not hypertrophic (Tamura *et al.*, 2000).

1.9.3. CNP

The anti-atherogenic profile of CNP, like ANP and BNP is strengthened by its anti-proliferative properties. CNP has been reported to inhibit DNA synthesis in cardiac fibroblasts (Cao and Gardner, 1995) and inhibit vascular smooth muscle growth (Furuya *et al.*, 1991; Furuya *et al.*, 1993; Cahill and Hassid, 1994; Hutchinson *et al.*, 1997). However, controversy exists as to the receptor responsible for the anti-mitogenic activity and the underlying mechanisms involved in smooth muscle cell inhibition by CNP. Several publications have documented the involvement of NPR-B, and thus cGMP-dependent signal transduction pathways, in growth inhibition. Furuya *et al* have shown that CNP inhibits DNA synthesis and growth in vascular smooth muscle cells via cGMP-dependent mechanisms (Furuya *et al.*, 1991). The same group also reported that CNP inhibits intimal thickening following vascular injury with an elevation of cGMP levels, concluding that NPR-B is probably the receptor responsible for these inhibitory effects (Furuya *et al.*, 1993). These findings have been further substantiated by Hutchinson *et al* who showed increases in cGMP levels correlate with growth inhibition and that CNP decreases PDGF-stimulated MAPK activity (Hutchinson *et al.*, 1997). In contrast, Cahill *et al* have suggested that CNP inhibits growth in aortic smooth muscle cells via NPR-C and thus in a cGMP-independent manner, as cGMP levels are not altered (Cahill and Hassid, 1994). It is also worth noting that small cytoplasmic domain peptides of NPR-C have been shown to inhibit vasoactive peptide (e.g. Ang II, endothelin-1, arginine-

vasopressin)-induced phosphorylation of ERK 1/2 and growth inhibition in vascular cells (Hashim *et al.*, 2006).

Endothelial/smooth muscle cell co-culturing experiments have revealed that endothelial expression of CNP is at least in part regulated by TGF-β, and CNP released from endothelial cells inhibits growth of the underlying smooth muscle cells (Komatsu *et al.*, 1996). In addition, adenovirus-mediated gene transfer of CNP causes G1 growth inhibition of vascular smooth muscle cells (Doi *et al.*, 1997). However, CNP has an opposite effect on endothelial cells, in which it stimulates growth. Rabbit jugular vein grafts incubated *ex vivo* with adenovirus containing CNP, inducing overexpression of CNP, undergo accelerated re-endothelialisation (Ohno *et al.*, 2002), suggesting that CNP could play an important local role in angiogenesis.

To the contrary however, CNP has been shown to enhance the mitogenic effect of FGF-2 in aortic smooth muscle cells (Dhaunsi and Hassid, 1996), intimating that regulation of smooth muscle growth could be highly specific and tightly regulated depending on the surrounding environment.

1.10. Current study

In summary, there is compelling evidence in the literature to suggest a role for natriuretic peptides in regulating an inflammatory response, but data are conflicting. Both up and downregulation of iNOS expression and other pro-inflammatory proteins, by natriuretic peptides have been reported in isolated cell systems (Marumo *et al.*, 1995; Vollmar and Schulz, 1995a; Kiemer and Vollmar, 1998; Kiemer and Vollmar, 2001) and therefore it is important to determine a definitive role for natriuretic peptides during an inflammatory response. Many cardiovascular disorders are inflammatory diseases (i.e. atherosclerosis, septic shock) and since natriuretic peptides are thought to be cardioprotective (Ahluwalia *et al.*, 2004), it is imperative to determine their mechanisms of action, thus helping in establishing ways in which progression of such disorders might be prevented/regulated by natriuretic peptides and therapeutics that can modulate natriuretic peptide activity.

In the current study NPR-A KO mice, the RAW264.7 murine macrophage cell line and primary rat aortic smooth muscle cells were used to investigate:

1. *Ex vivo* pharmacological characterisation of the role of NPR-A in the pathogenesis of septic shock.
2. The effect of natriuretic peptides on pro-inflammatory protein expression (iNOS) via the particulate guanylate cyclase isoform.
3. The involvement of MAPK pathways in regulating pro-inflammatory protein expression.
4. The role of natriuretic peptides, and specific natriuretic peptide receptors, in MAPK signalling and downstream regulation of cell proliferation.

The underlying hypotheses for the current study are:

1. Natriuretic peptides (ANP and BNP) affect pro-inflammatory protein expression and vascular function *in vivo* under inflammatory conditions (Chapter 3 – *Ex vivo* pharmacological characterisation of the role of NPR-A in pathogenesis of septic shock).
2. Natriuretic peptides (ANP and CNP) affect pro-inflammatory protein expression *in vitro* (Chapter 4 – Regulation of pro-inflammatory protein expression (iNOS) by natriuretic peptides (ANP and CNP) *in vitro*)
3. Natriuretic peptides (ANP and CNP) modulate MAPK pathways and regulate cell proliferation via activation of GC-linked NPRs (Chapter 5 – Role of natriuretic peptides and their receptors in regulating MPAK signalling and cell proliferation).

CHAPTER 2
MATERIALS AND METHODS

2. Materials and Methods

2.1. Reagents

Salmonella typhimurium or *Escherichia coli* (serotype O111:B4) LPS were purchased from Sigma (Poole, Dorset, UK) and re-suspended in distilled water to a concentration of 10 mg/ml. Recombinant rat IFN-γ, IL-1β and TNF-α were purchased from PeproTech EC (London, UK). IFN-γ was reconstituted in 5 mM Tris HCl pH 8.0 and 100 mM NaCl to a concentration of 1 mg/ml, equivalent to 1 x 10^6 units specific activity. IL-1β and TNF-α were reconstituted in distilled water to a concentration of 100 μg/ml, equivalent to 1 x 10^5 and 4 x 10^5 specific activity units, respectively. Rat ANP was obtained from Sigma and re-suspended to a concentration of 1 mM using distilled water. CNP (human and porcine) was purchased from Calbiochem (Nottingham, UK) and re-suspended in distilled water to a concentration of 1 mM. PD98059 and SB203580 were purchased from Alexis Biochemicals (Distributed by Axxora (UK) Ltd, Nottingham, UK), reconstituted in dimethyl sulphoxide (DMSO) to concentrations of 10mM. 8-Bromoguanosine 3′, 5′-cyclic monophosphate (8-Bromo-cGMP) was purchased from Sigma and reconstituted to 100 mM in distilled water. Pertussis toxin *(Bordetella pertussis)* was purchased from Calbiochem and was reconstituted to a stock concentration of 100 μg/ml in distilled water. Angiotensin II was purchased from Sigma and reconstituted in distilled water to a concentration of 1 mM. U46619 was purchased from Biomol International (Exeter, UK) and re-suspended in ethanol to a concentration of 1 mM, ACh was obtained from Sigma and re-suspended in distilled water to a concentration of 10 mM, SPER-NO was bought from Calbiochem and was re-suspended in distilled water to a concentration of 10 mM just prior to use. All other reagents above were aliquoted and stored at -20^{0}C until required when they were diluted to appropriate concentrations in growth medium or distilled water. M372049, a selective NPR-C antagonist (Veale *et al.*, 2000; Figure 8) was a kind gift from AstraZeneca Pharmaceuticals (Wilmington, USA) and was reconstituted to 1 mg/ml in distilled water just prior to use. M372049 is a competitive antagonist with a K_i = 21 nM with respect to inhibition of I^{125}-ANP binding to recombinant CHO cells expressing human NPR-C or membranes (48000 g fraction) prepared from recombinant baculovirus-Sf21 cells expressing NPR-C (personal

communication, Dr C.A. Veale, AstraZeneca Pharmaceuticals). It has an approximate pA_2 value of 11.14 against CNP-induced relaxation of rat mesenteric small arteries *in vitro* (IC_{50} = 4 nM; Villar *et al.*, 2007). In rat isolated aorta, M372049 does not alter relaxant responses to ANP, CNP, ACh and SPER-NO which have been shown previously to be mediated via NPR-A and NPR-B (Madhani *et al.*, 2003), however, in rat isolated mesenteric resistance arteries M372049, at a concentration of 100 nM, completely blocks the CNP-induced relaxation (Villar *et al.*, 2007).

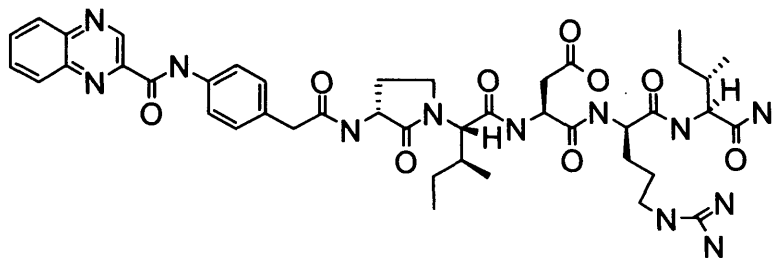


Figure 8 – Chemical structure of M372049.

2.2. Cell culture

2.2.1. RAW 264.7 – Murine macrophage cell line

RAW 264.7 murine macrophage cells (TIB-71 from American Type Culture Collection, Manassas, USA) were cultured in suspension in glass stirrer bottles using Roswell Park Memorial Institute (RPMI) 1640 medium with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all purchased from Life Technologies, Paisley, UK) and 10 % heat inactivated New Zealand FCS (TCS Biologicals Ltd, Buckingham, UK). Cells were grown at 37^{0}C in a humidified incubator with 5 % CO_2 in air. Routinely cell number and viability were quantified using trypan blue exclusion. Equal volumes of cell suspension and trypan blue (Life Technologies) were mixed and a small volume was transferred to a haemocytometer. Cells were counted under an inverted microscope (Axiovert25, Carl Zeiss MicroImaging Inc., Herfordshire, UK) with unstained, luminescent cells counted as

viable and blue stained cells counted as dead. Cells were only used for experimentation if viability was above 95 %.

2.2.2. Rat aortic smooth muscle cells (RAoSMC)

2.2.2.1. Isolation

Vascular aortic smooth muscle cells were prepared by enzymatic dissociation from rat aortae of male Sprague-Dawley rats (200 - 250 g). The rats were euthanised by cervical dislocation and thoracic aortae excised aseptically into phosphate buffered saline (PBS) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Loose fat and adventitia were removed with fine forceps and the cleaned aortae were preincubated in PBS containing 1 mg/ml collagenase Type I, 0.25 mg/ml soybean trypsin inhibitor, 1 mg/ml bovine albumin, 0.2 mM $CaCl_2$ (all purchased from Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (referred to as 'enzyme solution') for 30 min at 37^0C in a humidified incubator with 5 % CO_2 in air. Following incubation the aortae were opened longitudinally and the intima was removed by scraping the inner surface of the vessels using the blunt edge of a pair of scissors. The remaining tissue, comprising mostly media, was transferred into fresh enzyme solution, minced to approximately 1 mm^2 pieces and incubated as described above for 1 h vortexing every 10 min. Following incubation, enzyme solution was replenished at 30 min intervals, cells pelleted by centrifugation (200 g for 5 min), resuspended in Dulbecco's modified eagle medium (DMEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM L-glutamine and 10 % heat inactivated FCS, transferred to a tissue culture flask and incubated for 15 min. The medium containing cells that had not adhered was replenished with fresh medium and transferred to a clean tissue culture flask. This was incubated for a further 15 min and then discarded replacing it with fresh medium. This process of differential plating was repeated every 30 min until most of the aortae had been digested. Cells grown to confluency were detached using trypsin/ethylenediamine-tetraacetic acid (EDTA; 0.25 % trypsin/1 mM EDTA; Life Technologies) and seeded in secondary culture. Once a pure population of RAoSMC was obtained cells were used for experimentation up to passage 15.

2.2.2.2. Characterisation

2.2.2.2.1. Immunocytochemistry

Immunocytochemistry was used to ascertain that a homogeneous population of smooth muscle cells had been isolated. Cells were seeded onto sterilised glass coverslips in 6 well plates and grown in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10 % heat inactivated FCS at 37^{0}C in a humidified incubator with 5 % CO_2 in air for 24 h. Coverslips with an attached monolayer of cells were washed several times in PBS, fixed and permeabilised in 100 % ice cold methanol at <4^{0}C for 5 min, washed several times in PBS and blocked with PBS containing 5 % goat serum (Sigma, Poole, Dorset, UK) for 30 min. Cells were subsequently labelled with 1:1600 dilution of primary antibody, mouse monoclonal anti- smooth muscle α-actin (Sigma, Missouri, USA) in PBS by incubating for 2 h at room temperature. After 3 washes with PBS cells were incubated with secondary antibody, fluorescein isothiocyanate (FITC) -conjugated goat anti-mouse IgG (Chemicon), 1:500 dilution for 90 min at room temperature. Cells were washed 3 times in PBS followed by several washes in distilled water. Slides were mounted with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vectashield, Vector Laboratories, Burlingame, USA) and the bound antibody was visualised using a Leica TCS-SP1 confocal microscope (Leica, Milton Keynes, Bucks, UK; Figure 28, Figure 29, Figure 30).

2.2.2.2.2. Hill and valley morphology

Primary rat aortic smooth muscle cells (1° RAoSMC) were seeded into tissue culture vessels and allowed to grow in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10 % heat inactivated FCS at 37^{0}C in a humidified incubator with 5 % CO_2 in air. Medium was replaced every 2 – 3 days until overconfluency was achieved; at this time cells push each other to form areas of focal overgrowth. This was observed using a Leica DM IRB inverted microscope (Leica) and photographs were taken using a JVC KY-F55B Colour Video Camera (JVC Professional, London, UK) attached to the KS300 Imaging system release 3.0 (Imaging Associates Limited, Thame, UK; Figure 31).

2.2.3. Freezing and thawing cells

2.2.3.1. Freezing cells

Long term preservation of cells was achieved by storing in liquid nitrogen. Cells were trypsinised in the case of adherent cells or simply removed from culture and pelleted by centrifugation (RAoSMC: 200 g for 5 min; RAW264.7: 400 g for 5 min). Cells were resuspended in supplemented DMEM or RPMI1640 growth medium as described for the respective cell types and counted using trypan blue exclusion. 10 % DMSO was added to the cells just prior to freezing. Cells were frozen in cryogenic vials at a concentration of 0.5 – 1.0 x 10^6 cells/ml. Cryogenic vials containing cells were transferred to a Cryo 1^0C freezing container (Fisher Scientific, Leicestershire, UK) and placed at -80^0C for at least 24 h after which they were stored until required in the vapour phase of liquid nitrogen. Alternatively cryogenic vials were kept on ice in the fridge (4^0C) for 10 min, transferred to a polythene box and kept at -20^0C for 2 h and then moved to -80^0C overnight. Following overnight freezing vials were transferred to liquid nitrogen and stored as described above.

2.2.3.2. Thawing cells

Growth medium was placed into the culture flasks and equilibrated in a humidified incubator with 5 % CO_2 in air. Cells were removed from liquid nitrogen and thawed by constantly agitating in a 37^0C waterbath. Once half the content had thawed the vial was removed from the waterbath, the screwtop was opened slightly to reduce the overpressure and re-screwed tightly. Thawing was continued until all the content was thawed. Cells were resuspended by carefully pipetting and the content of the vial was transferred to the culture flasks. Alternatively, cells were resuspended in 50 ml medium, centrifuged at appropriate speeds as described previously for each cell type, the supernatant was discarded, the pellet was resuspended in 5 ml medium and transferred to culture flasks. Medium was replaced after 24 h and cells were subcultured once 80 – 90 % confluence was achieved. For adherent cell lines cells were subcultured as described in sections 2.2.2.1. For suspension cells (RAW264.7), cells were dislodged from the culture flask by scraping and transferred to a stirrer bottle containing RPMI 1640 growth medium.

2.3. Determination of iNOS activity and expression *in vitro*

RAW264.7 cells were seeded into 6 well plates at a concentration of 1 x 10^6 cells per well. RAoSMC were seeded into 6 well plates at a concentration of 0.5 x 10^6 cells per well or 10 cm dishes at a concentration of 0.5 x 10^6 cells per dish. Cells were allowed to grow and adhere at 37^0C in a humidified incubator with 5 % CO_2 in air overnight or until 80 – 90 % confluent. Growth medium was changed prior to experimentation. Activators (i.e. LPS, IFN-γ, IL-1β or TNF-α) were added at appropriate concentrations and cells were incubated (as above) for appropriate times. Alternatively, cells were preincubated with ANP or CNP for 5 min or MAPK inhibitors (PD98059 or SB203580; both at 30 μM) for 30 min at appropriate concentrations prior to activator addition. Medium was removed at each timepoint and an aliquot was stored at -20^0C for nitrite determination by chemiluminescence (see section 2.6). Cells were washed twice with cold PBS. 100 – 200 μl whole cell homogenisation buffer (10 ml of 50 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 150 mM NaCl, 1 % Triton X-100, 2 mM EDTA, 8 mM ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), protease inhibitor cocktail; Roche, East Sussex, UK) was added to each well and incubated on ice for 5 min. The cells were dislodged by scraping and the lysate was centrifuged at 16060 g for 5 min at 4^0C. The pellet was discarded and the supernatant (whole cell extract) stored at -20^0C for iNOS protein determination by Western blotting (see section 2.5).

2.4. Determination of MAPK phosphorylation *in vitro*

RAW264.7 cells or RAoSMC were seeded and allowed to reach confluency as described in section 2.3. Growth medium was changed prior to experimentation. Cells were treated with a cocktail of LPS (100 μg/ml), IFN-γ (200 U/ml), IL-1β (400 U/ml) and TNF-α (1000 U/ml) for 0 - 60 min in the presence and absence of natriuretic peptides (5 min pre-incubation). Alternatively, cells were treated with ANP (1 μM), CNP (1 nM – 10 μM) or 8-Bromo-cGMP (300 μM) alone. In certain experiments, cells were pre-incubated with PTx (100 ng/ml; 16 h), M372049 (10 μM) or PD98059 (30 μM; both 30 min) prior to the addition of CNP. At appropriate timepoints medium was removed and discarded and cells were washed twice with

cold PBS. 150 µl/well (6 well-plate) or 200 µl/dish (10 cm dishes) of phospho-homogenisation buffer (10 mM Tris-HCl, 50 mM NaCl, 30 mM sodium pyrophosphate, 2 mM EDTA, 50 mM NaF, 1 % Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 1 µg/ml benzamidine, antipain, leupeptin and aprotinin) was added and cells were dislodged into the buffer by scraping. Lysate was centrifuged at 16060 g for 5 min at 4^0C, the pellet was discarded and the supernatant (whole cell extract) was stored at 20^0C for total MAPK or phosphorylated MAPK protein determination by Western blot.

2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

2.5.1. Sample preparation for SDS-PAGE

Protein concentrations were determined using the bicinchoninic acid based BCA™ protein assay kit. Total protein was quantified by its reaction with Cu^{2+} reducing it to Cu^{1+} in an alkaline medium known as the biuret reaction. The colorimetric change is brought about by the reaction of bicinchoninic acid with Cu^{1+} and the protein concentrations are determined based on a standard curve produced from a series of dilutions of known concentrations using the common protein bovine serum albumin in whole cell homogenisation buffer or phospho-homogenisation buffer (standard concentrations: 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125 and 0 mg/ml). 25 µl sample or standard were mixed with 200 µl working reagent (made by mixing 50 parts Reagent A with 1 part Reagent B) in a 96-well plate and incubated for 30 min at 37^0C. Absorbance was measure at 570 nm (Victor^2™ 1420 multilabel counter, PerkinElmer Life Sciences, Boston, USA) and total protein was calculated from the standard curve. Samples were diluted using whole cell homogenisation buffer or phospho-homogenisation buffer to ensure equal loading of protein to the gel and allow comparative analysis. A 1:1 dilution was prepared of homogenised samples and sample buffer (20 mM Tris HCl, pH 6.8, 2 mM EDTA, 2 % SDS, 10 % 2-mercaptoethanol, 20 % glycerol, 0.025 % bromophenol blue in distilled water). The samples were heated at 100^0C in a heating block (Grant Instruments, Cambridge, UK) for 5 min, placed on ice for a further 5 min and centrifuged at 16060 g for 2 min at 4^0C. Samples were then loaded onto the gel for separation or stored at -20^0C for future analysis.

2.5.2. SDS-PAGE

Two glass plates were assembled using a casting frame and held in an upright position using a casting stand (Mini-Protean 3 cell and system, Biorad, Hertfordshire, UK). A separating gel containing 7.5 or 15 % acrylamide was prepared by mixing acrylamide/bis acrylamide (30 % solution, mix ration 37.5:1, Sigma), separating gel buffer (0.375 M Tris Base, 0.1 % SDS, distilled water, pH 8.8), 0.1 % ammonium persulphate (Sigma), 0.001 % N,N,N',N'-tetramethylethylenediamine (TEMED; Sigma) in distilled water, and poured into the glass assembly. The gel was overlaid with isopropanol to remove any bubbles and smooth out the surface; this was removed after the separating gel had set and polymerised. A stacking gel containing 4 % acrylamide was prepared by combining acrylamide/bis acrylamide, stacking gel buffer (0.125 M Tris Base, 0.1 % SDS, distilled water, pH 6.8), 0.1 % ammonium persulphate, 0.001 % TEMED in distilled water and poured on top of the separating gel. Combs were inserted and the gel was allowed to set. Combs were removed and the gels were assembled using the Protean III gel tank system, running buffer was added (50 mM Tris Base, 0.384 M glycine, 0.1 % SDS in distilled water) and 20 µl of diluted sample to ensure equal protein were loaded into each well. Samples were run alongside 10 µl rainbow high-range molecular weight markers (GE Healthcare, Bucks, UK) and 15 µl positive control at 100 – 150 V until the dye front reached the bottom of the gel.

2.5.3. Western blotting

2.5.3.1. Wet transfer

Transfer of proteins from gel to nitrocellulose was carried out using the Mini trans-blot electrophoresis transfer cell (Biorad). Transfer buffer (25 mM Tris Base, 192 mM glycine, 20 % methanol; made up to 1 litre with distilled water) was prepared just before required and stored at 4^0C until used. A sandwich of filter paper, nitrocellulose, SDS-PAGE gel and fibre pads was built up on the positive side of the gel holder cassette (see Figure 9).

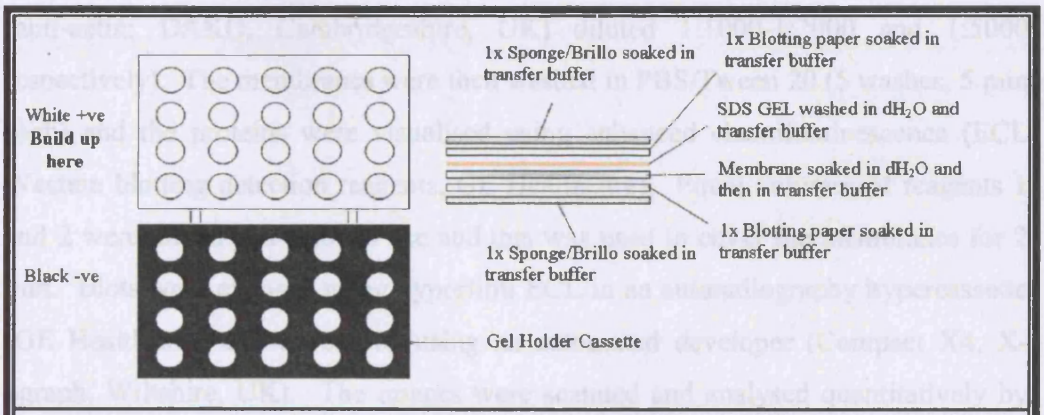


Figure 9 – Blotting setup using mini trans-blot electrophoretic transfer cell.

The gel holder cassettes were placed into the electrode module and the tank was filled with transfer buffer. The transfer was carried out at 100 V for 75 min.

2.5.3.2. Immunoblotting

Following transfer, nitrocellulose was stained with 1 % Ponceau S solution (Sigma) to visualise transferred proteins and then washed three times in distilled water. Membranes were incubated in 5 % dried skimmed milk (Marvel, Premier International Foods Ltd, Spalding, Lincs, UK) in PBS/Tween 20 (20 % 10x PBS without calcium and magnesium (Life Technologies), 0.001 % polyoxyethylene-sorbitan monolaurate (Tween 20) from Sigma in distilled water) for 60 min or overnight with constant shaking. The blot was washed 5 times in PBS/Tween 20 (5 min each wash with shaking) followed by incubation overnight at 4^0C with constant agitation with primary antibody diluted in 5 % dried skimmed milk in PBS/Tween 20 (polyclonal anti-iNOS (BD Transduction Laboratories, Cowley, Oxford, UK), polyclonal anti-p44/p42 MAP kinase, polyclonal anti phospho-p44/42 MAP kinase (Thr202/Tyr204), polyclonal anti-p38 MAP kinase, anti-phospho-p38 MAP kinase (Thr180/Tyr182; Cell Signaling Technology, distributed by New England Biolabs, Hertfordshire, UK), monoclonal anti-actin (Chemicon) diluted 1:2000 (anti-iNOS), 1:500 (anti-p44/42, anti-phospho-p44/42, anti-p38 and anti-phospho-p38), 1:20000 (anti-actin). Following incubation the blot was washed with 5 changes of PBS/Tween 20 (5 min each wash) and incubated for 60 min with secondary antibody diluted in 5 % dried skimmed milk in PBS/Tween 20 (peroxidase-conjugated goat anti-rabbit immunoglobulins (anti-iNOS, anti-p44/42, anti-phospho-p44/42, anti-p38 and anti-phospho-p38), peroxidase-conjugated goat anti-mouse immunoglobulins

(anti-actin; DAKO, Cambridgeshire, UK) diluted 1:1000-1:2000 and 1:5000 respectively). The membranes were then washed in PBS/Tween 20 (5 washes, 5 min each) and the proteins were visualised using enhanced chemiluminescence (ECL Western blotting detection reagents, GE Healthcare). Equal volumes of reagents 1 and 2 were mixed just prior to use and this was used to cover the membranes for 2 min. Blots were exposed using hyperfilm ECL in an autoradiography hypercassette (GE Healthcare) and developed using an automated developer (Compact X4, X-ograph, Wiltshire, UK). The images were scanned and analysed quantitatively by densitometry (AlphaImager® Imaging System, AlphaEase® FC software version 4; AlphaInnotech, San Leandro, California, USA).

2.6. Chemiluminescence

NO released by cells in culture or *in vivo* is immediately converted to its oxidative products nitrate and nitrite. Nitrate/nitrite accumulation in the samples was measured using chemiluminescence, which has been shown to provide greater accuracy and precision than colourimetric methods (Cox, 1980). The chemical reduction of nitrate and nitrite to NO is achieved using the strong reducing agent vanadium III chloride (0.1 M) in 1 M hydrochloric acid refluxing at 90^0C under nitrogen, while nitrite only reduction to NO involves the use of 25 % sodium iodide (6 % solution) and 75 % glacial acetic acid; NO produced by the reduction reaction can be subsequently used for nitrate/nitrite determination via its chemiluminescent reaction with ozone. When the NO is combined with ozone, metastable nitrogen dioxide (NO_2^*) is produced, which subsequently emits a photon as it returns to ground state, and the emitted light is detected by a photomultiplier (Figure 10). The integrated electrical signal produced by the photomultiplier is proportional to the content of nitrate/nitrite in the sample. Analysis was carried out using Sievers Nitric Oxide Analyser NOA™ 280 (Sievers Instruments, Inc, Boulder, CO80301 USA).

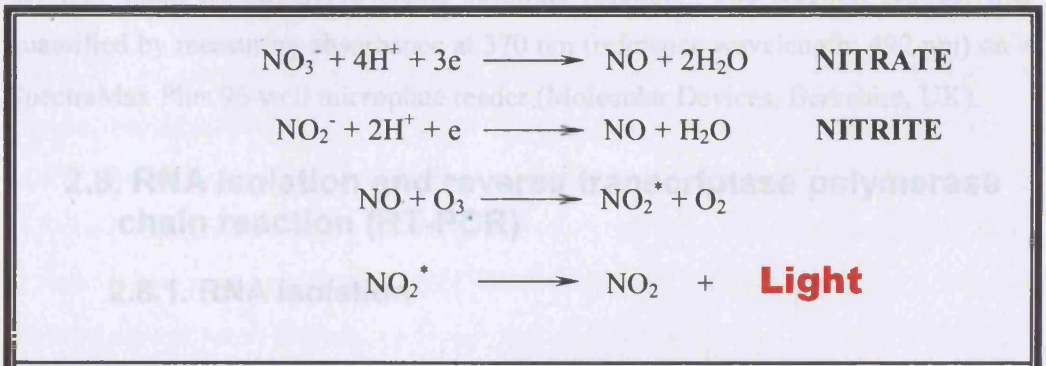


Figure 10 – Reactions underlying chemiluminescence analysis of nitrate/nitrite concentration.

A standard curve was produced using 10 µl of 100, 50, 25, 10, 1 and 0.1 µM sodium nitrate/nitrite standards according to the species to be analysed. 10 µl of each sample (cell free medium or plasma) was injected into the system and the concentrations of nitrate/nitrite were calculated from the standard curve.

2.7. Assessment of cell proliferation

RAoSMC proliferation was assessed by determining 5-bromo-2′-deoxyuridine (BrdU) incorporation using a commercially available assay (Roche Diagnostics, East Sussex, UK). Replication of genomic DNA is a prerequisite for cellular proliferation and therefore monitoring of DNA synthesis is an indirect parameter of cell proliferation. BrdU, a pyrimidine analogue, is incorporated into replicating cell DNA in place of thymidine and an anti-BrdU antibody is used coupled to a substrate reaction to quantify proliferating cells (Gratzner, 1982; Porstmann *et al.*, 1985). The assay was carried out according to the manufacturer's protocol. In brief, RAoSMC were plated into 96-well plates at a density of 25000 cells/well in triplicate in DMEM containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10 % heat inactivated FCS for 24 h in a humidified incubator with 5 % CO_2 in air. Cells were switched to DMEM as above but with 0.1 % FCS for 24 h. Cells were then pretreated with 10 µM M372049, 30 µM PD98059 or vehicle for 30 min and then exposed to 1 µM CNP or 100 nM Ang II for 24 h. At 20 h cells were labelled with BrdU for 4 h. Culture medium was then removed, cells were fixed and the DNA denatured using the FixDenat solution. Peroxidase-conjugated anti-BrdU antibody (anti-BrdU-POD) was added to detect the newly synthesised cellular DNA incorporated BrdU and the bound anti-BrdU immune complexes were

detected using tetramethylbenzidine substrate reaction. The reaction product was quantified by measuring absorbance at 370 nm (reference wavelength: 492 nm) on a SpectraMax Plus 96-well microplate reader (Molecular Devices, Berkshire, UK).

2.8. RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

2.8.1. RNA isolation

Total ribonucleic acid (RNA) was extracted from cells using the RNeasy mini kit (Qiagen, West Sussex, UK). Briefly, 4 – 5 x 10^6 cells were disrupted in buffer containing guanidine isothiocyanate and homogenised using QIAshredder homogenizers (Qiagen). Ethanol was added to the lysate enabling selective RNA binding to the RNeasy silica containing gel membrane. DNase was added (RNase-free DNase Kit, Qiagen) to eliminate any DNA contamination and spin columns were centrifuged to remove waste and impurities. Total RNA was eluted into RNase free water and stored at -80^{0}C.

RNA concentration and purity were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). Concentration was determined by measuring absorbance at 260 nm (A_{260}), while the ratio between 260 nm and 280 nm, and 260 nm and 230 nm gave an indication of RNA purity. Values of A_{260}/A_{280} ratio should be approximately 2.0 (Glasel, 1995; Manchester, 1995) and A_{260}/A_{230} ratio should be in the range of 1.8 – 2.2 for pure RNA according to the manufacturer's instructions.

RNA integrity was checked by running 0.5 μg and 1 μg RNA on a 1 % agarose gel. Agarose multi-purpose molecular grade (Bioline, London, UK) was dissolved by heating in 0.04 M Tris-acetate and 0.002 M Na_2EDTA in distilled water (TAE; National Diagnostics, Hessle Hull, UK). 0.5 μg/ml ethidium bromide (Sigma) was added to the gel before setting. DNA coloured loading buffer (Bioline) was added to RNA samples and gels were run at 50 – 100 V in TAE. Samples were run alongside DNA marker HyperLadder II (Bioline) and bands were visualised and photographed using the automated gel documentation AlphaImager® imaging system and AlphaEase® FC software, version 4 (AlphaInnotech).

2.8.2. RT-PCR

RT-PCR was carried out using Qiagen one-step RT-PCR kit (Qiagen). Briefly, 1x Qiagen one-step RT-PCR buffer (containing $MgCl_2$), deoxynucleotide triphosphate (dNTP) mix (containing 400 µM final concentration of each dNTP), 1x Qiagen Q solution, Qiagen one-step RT-PCR enzyme mix (containing Omniscript and Sensiscript reverse transcriptases and HotStarTaq DNA polymerase), gene specific forward and reverse primers (0.6 µM each; Figure 11), 1 µl template RNA and RNase free water to make up to 50 µl were mixed and RT-PCR was performed on a Primus 96 thermocycler (MWG Biotech, Milton Keynes, UK). Reverse transcription was carried out at 50^0C for 30 min followed by thermal cycling conditions as follows: 95^0C for 15 min then 40 cycles of denaturation at 94^0C, annealing at 55^0C for 1 min and extension at 72^0C for 1 min, followed by a final extension at 72^0C for 10 min.

NPR-A	Sense	5′-CATCCTGGACAACCTGC-3′
	Antisense	5′-TAGGTCCGAACCTTGCC-3′
NPR-B	Sense	5′-CATGGCAGGACAATCGAACC-3′
	Antisense	5′-TGCCTGCACCTTTGTGATATCG-3′
NPR-C	Sense	5′-GGCTCAATGAGGAGGATTACGTG-3′
	Antisense	5′-AATCTTCCCGCAGCTCTCGATG-3′

Figure 11 – Rat NPR-A, B and C gene specific primers.

PCR products were resolved on a 1 % agarose gel as described in 2.8.1. 5 µl of 5x DNA coloured loading buffer (Bioline) were added to 20 µl of each PCR product, the entire volume was loaded onto the gel and run at 50 – 100 V in TAE alongside HyperLadder 2 marker. Bands were visualised and photographed using the AlphaImager® imaging system as described in 2.8.1.

2.9. *Ex vivo* animal studies

All experiments were conducted according to the Animals (Scientific Procedures) Act 1986, United Kingdom and conform to the *Guide to the care and use of laboratory animals* (NIH publication no. 85-23, 1996).

2.9.1. Genotyping NPR-A knockout mice

Tail snips from WT (C57BL6; Harlan UK Limited, Bicester Oxon, UK) and NPR-A KO (Oliver Smithies, University of North Carolina, USA) mice were cut into approximately 4 mm lengths. Total DNA was extracted using the DNeasy tissue kit (Qiagen) as described by the manufacturer's protocol. Briefly, tail snips were homogenised and lysed with ATL buffer and proteinase K overnight at 55^0C with shaking. Lysate was then loaded onto a DNeasy mini spin column where the DNA is selectively bound to the silica-gel-membrane in the presence of chaotropic salts. The column was washed and DNA eluted into AE buffer containing 10 mM Tris HCl and 0.5 mM EDTA at pH 9.0.

PCR was carried out using Taq DNA polymerase kit (Roche Diagnostics). Briefly, 1x PCR buffer supplied with enzyme (10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), dNTP mix (200 µM final concentration of each dNTP), gene specific primers NPR-A WT forward, 3′-GCATGGTTCAGCTCTAAGAC-5′, NPR-A NEO (KO) reverse, 3′-CTAACCCTGTGAACTGTAAGC-5′, NPR-A reverse, 3′-CCTCAGTTATCTACATCTGC-5′, all at 0.8 µM, 1 unit Taq DNA polymerase, 2 µl template DNA and RNase free water to make up to 25 µl were mixed and PCR was performed on a Primus 96 thermocycler (MWG Biotech). Initial denaturation was carried out at 95^0C for 30 sec, followed by thermal cycling conditions as follows: 35 cycles of denaturation at 94^0C for 30 sec, annealing at 60^0C for 30 sec and extension at 72^0C for 1 min, followed by a final extension at 72^0C for 10 min.

PCR products were resolved on a 2 % agarose gel as described in 2.8.1.

2.9.2. Determination of iNOS activity and expression *in vivo*

Salmonella typhimurium LPS (12.5 mg/kg, i.v.) or vehicle (physiological saline, sodium chloride 0.9 % w/v, Ivex Pharmaceuticals, Antrim, Northern Ireland, UK) were administered to 9 – 10 week old male WT (C57BL6; 25 – 30 g) or NPR-A KO mice (25 – 30 g) via the tail vein. After 16 h animals were euthanised by cervical dislocation and tissues (heart, lung and aorta) and blood collected. Tissues were snap frozen in liquid nitrogen and stored at -80^0C. They were then homogenised using an ice cold mortar and pestle, transferred to whole cell homogenisation buffer

(composition as in section 2.3) and incubated on ice with vortexing every 10 min for a minimum of 30 min. Samples were centrifuged at 16060 g for 15 min at 4^0C and the supernatant was retained and stored at -80^0C for iNOS protein determination.

Blood samples were centrifuged at 16060 g for 5 min at room temperature and plasma was retained for nitrate/nitrite determination by chemiluminescence.

2.9.3. Functional reactivity of isolated thoracic aorta

Male NPR-A KO or WT mice were treated with LPS or vehicle as described in section 2.9.2. After 16 h the animals were euthanised by cervical dislocation and the thoracic aortae were carefully excised and cleaned of connective tissue. The aortae were cut into 3 rings approximately 4 mm in length and mounted in 10 ml organ baths containing Krebs bicarbonate buffer (NaCl 118.41 mM, $NaHCO_3$ 25 mM, KCl 4.75 mM, KH_2PO_4 1.19 mM, $MgSO_4.7H_2O$ 1.19 mM, D-glucose 11.1 mM and $CaCl_2.6H_2O$ 2.5 mM; pH 7.4) maintained at 37^0C and bubbled with 95 % O_2/ 5 % CO_2. The tension was set at 0.3 g and the rings were equilibrated for approximately 1 h; Krebs solution was replenished every 15 – 20 min and the tension was re-adjusted to 0.3 g if required. After equilibration, the rings were primed with KCl (4.8×10^{-2} M). The KCl was washed out and cumulative concentrations of U46619 (10^{-10} – 10^{-6} M) were added; approximately 80 % maximum (EC_{80}) U46619-induced response was used for experimentation. Endothelial integrity was assessed by establishing greater than 50 % relaxation using ACh (10^{-6} M; for non-LPS treated animals). If the contractions to U46619 were not maintained, or relaxation greater than 50 % of the U46619-induced tone to ACh was not observed, the tissues were discarded. Following washing, an EC_{80} concentration of U46619 was added to the organ bath and following attainment of a stable contraction, cumulative concentration-response curves to ACh (10^{-9} – 10^{-5} M), ANP (10^{-11} – 10^{-6} M) and SPER-NO (10^{-9} – 10^{-4} M) were obtained. Data were captured using PowerLab and Chart version 4 (AD Instruments, Oxfordshire, UK).

2.10. Data Analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Densitometric analyses were performed using AlphaEase® FC software version 4 (AlphaInnotech). Exposed films were illuminated with

transilluminating white light and an image was captured using a motorised lens camera (focal length: 8.0 ~ 48 mm, maximum aperture ratio: 1:1.2, 6x zoom) with flat field calibration to easily correct for non-uniformities in light gathering. The captured image was then analysed using AlphaEase® FC software version 4 utilising 256 gray scale values. The software captures an integrated density value which is the sum of the gray levels of all the pixels within the boundaries of each band and then subtracts a background value computed as a mean of 16 lowest intensity points within the assigned boundaries. The boundary size is kept constant for all bands. All data are plotted graphically as mean values, with vertical bars representing standard error of the mean (SEM). A Student's t-test was used to assess differences between two data groups (iNOS expression, plasma NO_x and survival in WT versus NPR-A KO mice). One-way ANOVA followed by Bonferroni's multiple comparison test was used to assess differences between individual experimental conditions when multiple comparisons were being made to a single control. For organ bath experiments relaxations are expressed as percent reversal of U46619-induced tone. Curves were fitted to all the data using nonlinear regression and the concentration of each drug, giving a half-maximal response (EC_{50}), were used to compare potency. Curves were analysed using two-way ANOVA. For all statistical analyses a probability (*P*) value, *, of <0.05 was considered significant.

CHAPTER 3

RESULTS 1

Ex vivo pharmacological characterisation of the role of NPR-A in the pathogenesis of septic shock

3. Results 1

3.1. Introduction

NO and natriuretic peptides have both been described to play crucial roles in inflammation (Moncada and Higgs, 1993; Hobbs *et al.*, 1999; Vollmar, 2005) and maintaining cardiovascular homeostasis (Ahluwalia *et al.*, 2004; Woods, 2004; Naseem, 2005). During an inflammatory response, various stimuli, including IFN-γ, IL-1β and TNF-α, cause an increase in NO levels, primarily due to an increase in iNOS expression and activity (Lirk *et al.*, 2002). NO can then act in a cGMP-dependent or independent fashion to bring about cytostatic and cytotoxic effects (Guzik *et al.*, 2003). However, the high NO levels generated during an inflammatory response can prove detrimental, exemplified by the development of septic shock (Titheradge, 1999). Natriuretic peptides parallel the effects of NO in the vasculature (Ahluwalia *et al.*, 2004), but there is conflicting evidence concerning the effects of natriuretic peptides in the immune system (Vollmar and Schulz, 1995b; Kiemer and Vollmar, 1997; Kiemer *et al.*, 2002a; Vollmar, 2005) and further investigations are warranted.

Studies described in this chapter investigate the hypothesis that natriuretic peptides (ANP and BNP) affect pro-inflammatory protein expression and vascular function *in vivo* under inflammatory conditions. This was evaluated by examining the effect of NPR-A gene deletion on *in vivo* LPS-induced pro-inflammatory protein expression (i.e. iNOS), NO production and vascular function.

3.2. Effect of NPR-A gene deletion on LPS-induced iNOS expression and activity *in vivo*

Genotyping by RT-PCR was carried out on tail snips from both WT and NPR-A KO mice to ensure that NPR-A was absent from the KO animals and that the WT mice contained the receptor being investigated. RT-PCR revealed that all NPR-A KO animals used did not possess NPR-A while all the WT mice had NPR-A present (Figure 12).

WT and NPR-A KO mice were treated with saline (volume matched to LPS) or 12.5 mg/kg *Salmonella typhimurium* LPS (both i.v.) for 16 h. Animals were killed by cervical dislocation and organs/blood removed for analysis. Inducible NOS protein expression was determined by Western blot in heart, lung and aorta and plasma NO_x was determined using chemiluminescence. Inducible NOS protein expression in response to LPS was reduced in the lung (Figure 14) and aorta (Figure 15) of NPR-A KO animals when compared to WT. Inducible NOS protein expression in the heart in response to LPS exhibited an inhibitory trend in NPR-A KO animals but did not reach statistical significance (Figure 13). NO_x levels in plasma were indistinguishable between saline treated NPR-A KO and WT animals, however levels were significantly lower in NPR-A KO mice in comparison to WT animals treated with LPS (Figure 16), indicating that iNOS activity is also reduced in NPR-A KO animals following administration of LPS.

The survival rates of WT and NPR-A KO mice treated with LPS were indistinguishable (Figure 17).

GENOTYPING OF WILD TYPE AND NPR-A KNOCKOUT MICE

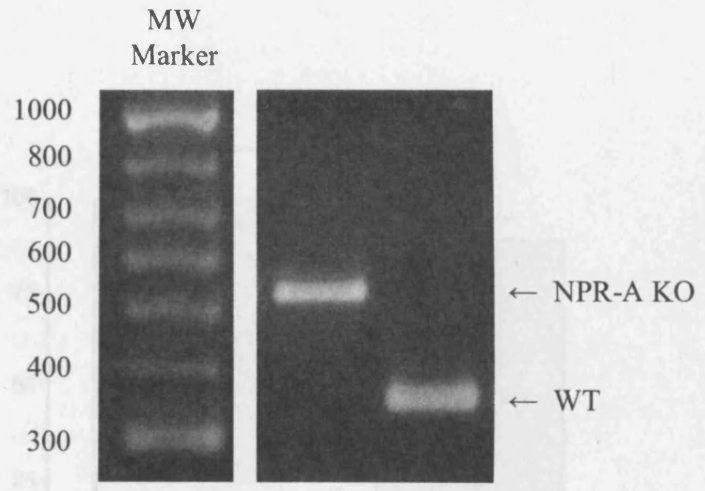


Figure 12 – Genotyping of WT and NPR-A KO mice. Products of expected size were obtained (550 bp NPR-A KO, 350 bp WT).

LPS-INDUCED iNOS EXPRESSION IN THE HEART OF WILD TYPE AND NPR-A KNOCKOUT MICE

(A)

(B)

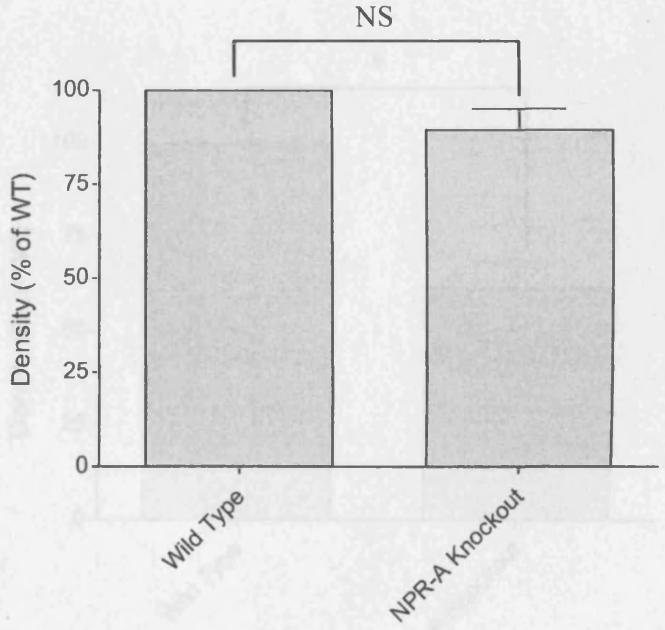


Figure 13 – Expression of iNOS protein in heart tissue from WT and NPR-A KO mice treated with LPS (12.5 mg/kg; i.v.) for 16 h. Protein expression was analysed by Western blot **(A)** and quantified by densitometry **(B)**. Data are represented as mean ± SEM, n = 5.

LPS-INDUCED iNOS EXPRESSION IN THE LUNG OF WILD TYPE AND NPR-A KNOCKOUT MICE

(A)

(B)

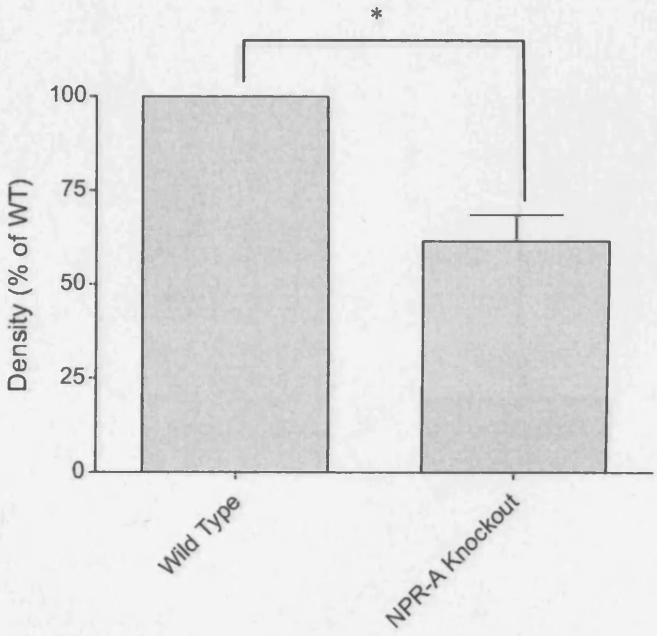


Figure 14 – Expression of iNOS protein in lung tissue from WT and NPR-A KO mice treated with LPS (12.5 mg/kg; i.v.) for 16 h. Protein expression was analysed by Western blot **(A)** and quantified by densitometry **(B)**. Data are represented as mean ± SEM, n = 5; *, $p<0.05$ versus control.

LPS-INDUCED iNOS EXPRESSION IN THE AORTA OF WILD TYPE AND NPR-A KNOCKOUT MICE

(A)

(B)

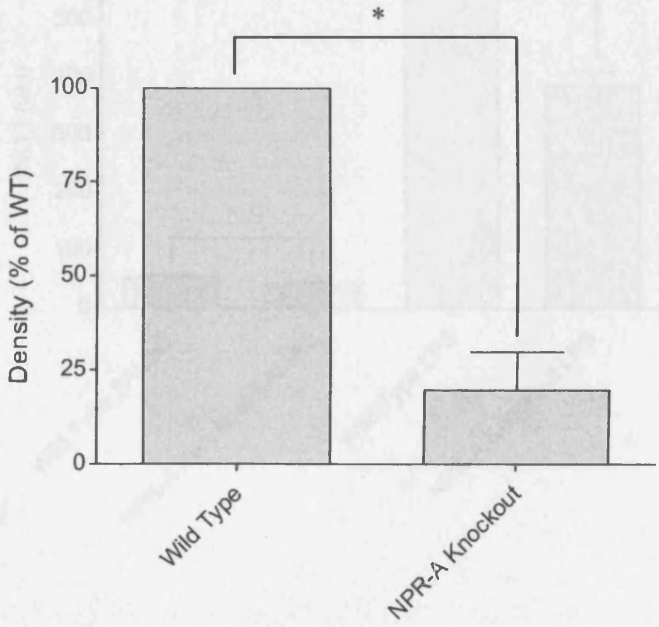


Figure 15 – Expression of iNOS protein in aortic tissue from WT and NPR-A KO mice treated with LPS (12.5 mg/kg; i.v.) for 16 h. Protein expression was analysed by Western blot **(A)** and quantified by densitometry **(B)**. Data are represented as mean ± SEM, n = 4; *, $p<0.05$ versus control.

LPS-INDUCED PLASMA NO_x IN WILD TYPE AND NPR-A KNOCKOUT MICE

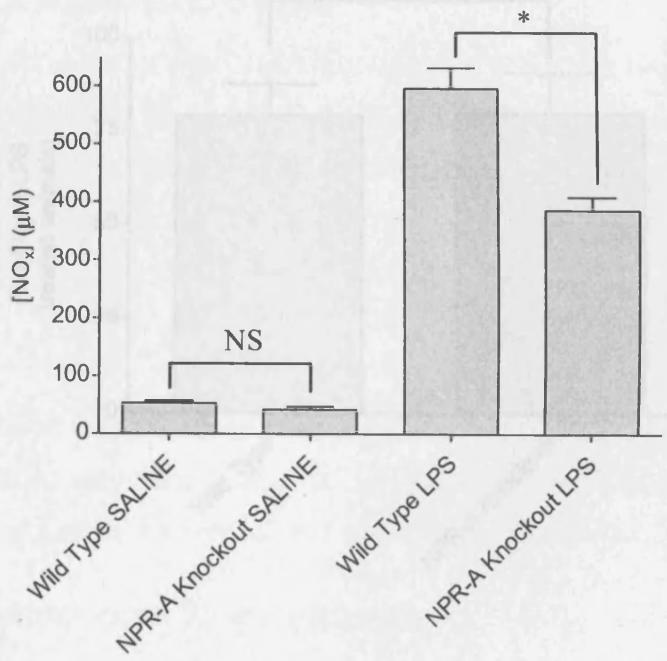


Figure 16 – Plasma NO_x levels from WT and NPR-A KO mice treated with LPS (12.5 mg/kg) or saline (both i.v.) for 16 h, determined by chemiluminescence. Data are represented as mean ± SEM, n = 7; *, $p<0.05$ versus control.

SURVIVAL RATES OF WILD TYPE AND NPR-A KNOCKOUT MICE FOLLOWING TREATMENT WITH LPS

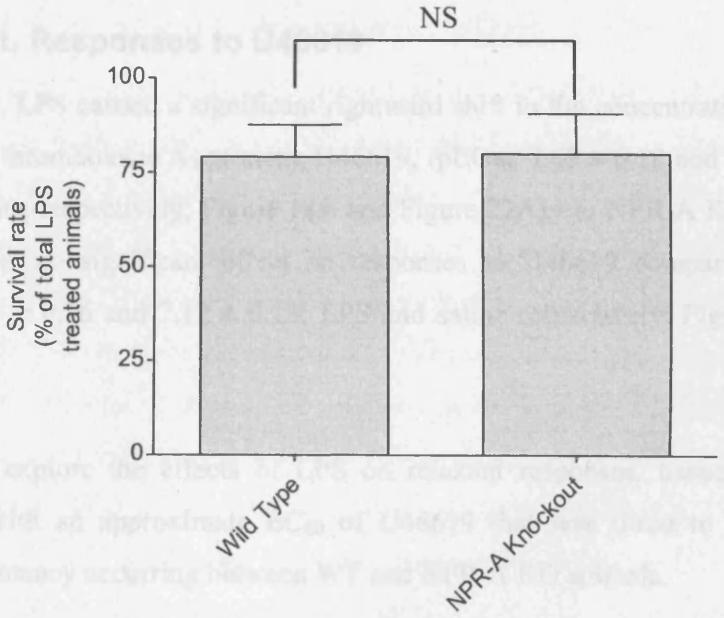


Figure 17 – Survival rates of WT and NPR-A KO mice treated with LPS (12.5 mg/kg; i.v.) for 16 h. Data are represented as mean ± SEM, n = 15.

3.3. Effect of NPR-A gene deletion on vascular function induced by LPS *in vivo*

Since treatment of NPR-A KO mice with LPS (12.5 mg/kg; i.v. for 16 h) revealed a decrease in iNOS expression and NO_x plasma levels in comparison to WT mice, functional pharmacological studies were conducted using thoracic aortae from saline-(volume matched to LPS) or LPS-(12.5 mg/kg), both i.v. for 16 h, treated NPR-A KO and WT mice.

3.3.1. Responses to U46619

In WT mice, LPS caused a significant rightward shift in the concentration-response curve to the thromboxane A_2 mimetic U46619, (pEC_{50}: 7.69 ± 0.12 and 7.94 ± 0.05; LPS and saline respectively; Figure 18A and Figure 22A). In NPR-A KO mice LPS treatment had no significant effect on responses to U46619 compared to saline (pEC_{50}: 6.41 ± 0.16 and 7.12 ± 0.18; LPS and saline respectively; Figure 18B and Figure 22B).

In order to explore the effects of LPS on relaxant responses, tissues were pre-contracted with an approximate EC_{80} of U46619 that was titred to account for changes in potency occurring between WT and NPR-A KO animals.

3.3.2. Responses to acetylcholine

Treatment of WT animals with LPS caused a significant rightward shift in the concentration-response curve to the endothelium-dependent relaxant ACh (pEC_{50}: 6.69 ± 0.21 and 7.00 ± 0.08; LPS and saline respectively; Figure 19A and Figure 23A) and a significant decrease in E_{max} (E_{max}: 25.46 ± 2.11 and 66.36 ± 2.22; LPS and saline respectively; Figure 23A). Vessels from LPS treated NPR-A KO mice also showed a trend towards reduced responsiveness to ACh (pEC_{50}: 6.70 ± 0.24 and 7.30 ± 0.28; LPS and saline respectively; Figure 19B and Figure 23B) but the concentration-response curves were not significantly different. Furthermore, E_{max} was indistinguishable in LPS and saline treated NPR-A KO mice (E_{max}: 77.31 ± 6.92 and 79.58 ± 6.93; LPS and saline respectively; Figure 23B).

3.3.3. Responses to ANP

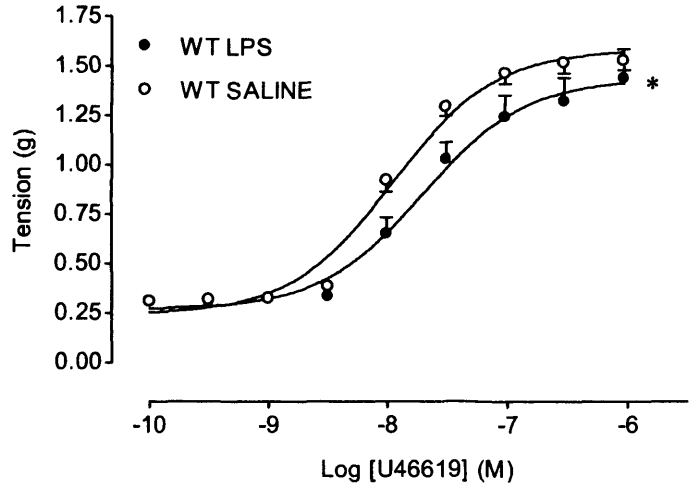
Aortae from WT mice treated with LPS exhibited a significant rightward shift in the concentration-response curve to ANP (pEC_{50}: 7.89 ± 0.20 and 8.13 ± 0.13; LPS and saline respectively; Figure 20A and Figure 23A) accompanied by a significant decrease in E_{max} (E_{max}: 53.61 ± 4.13 and 76.16 ± 3.58; LPS and saline respectively; Figure 23B). Aortae from NPR-A KO animals showed no vasorelaxation in response to ANP (Figure 20B).

3.3.4. Responses to spermine NONOate

Treatment of WT mice with LPS caused a significant rightward shift in the concentration response curve to the NO donor SPER-NO (pEC_{50}: 5.08 ± 0.16 and 5.86 ± 0.07; LPS and saline respectively; Figure 21A and Figure 23A). However, E_{max} was unchanged (E_{max}: 101.4 ± 12.13 and 94.40 ± 3.01; LPS and saline respectively; Figure 23A). This shift was not observed in LPS treated NPR-A KO animals as the curves were superimposable (pEC_{50}: 5.25 ± 0.12 and 5.42 ± 0.18; E_{max}: 95.73 ± 7.09 and 91.64 ± 8.83; LPS and saline respectively; Figure 21B and Figure 23B). SPER-NO was used as a NO donor in this set of experiments as it has been reported to have reproducible rates of dissociation, producing NO in a first order kinetic process at constant pH. It spontaneously generates NO without the need for a redox activation step and has a half-life of 39 min which ensures constant release of NO for the duration of the experiment (Morley and Keefer, 1993; Fitzhugh *et al.*, 2000).

EFFECT OF LPS *IN VIVO* ON RESPONSES TO U46619 IN WILD TYPE AND NPR-A KNOCKOUT MICE

(A)



(B)

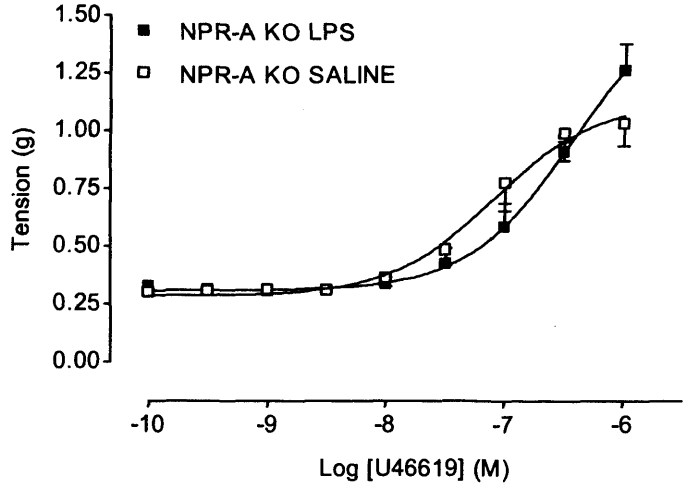
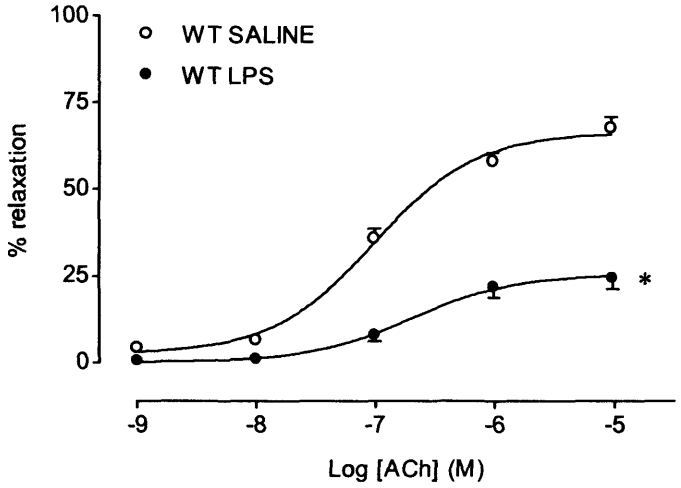


Figure 18 – Concentration-response curves to U46619 in aortic rings from WT **(A)** and NPR-A KO **(B)** mice treated with LPS (12.5 mg/kg) or saline (both i.v.) for 16 h. Contraction is expressed as mean ± SEM tension in g; *, $p<0.05$ versus saline treated animals, n = 7.

EFFECT OF LPS *IN VIVO* ON RESPONSES TO ACETYLCHOLINE IN WILD TYPE AND NPR-A KNOCKOUT MICE

(A)



(B)

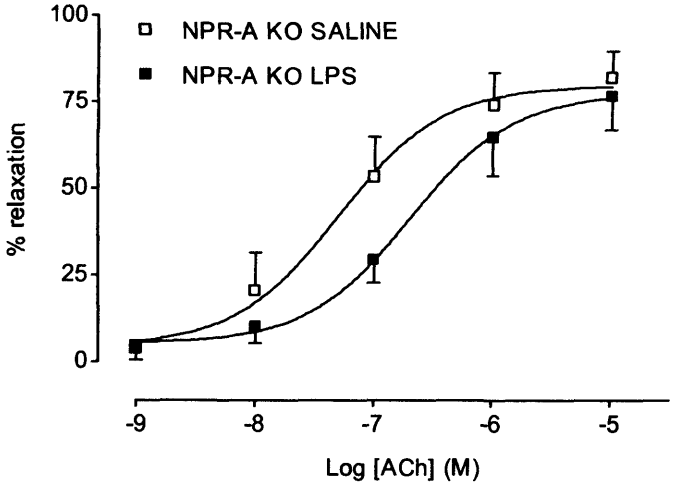
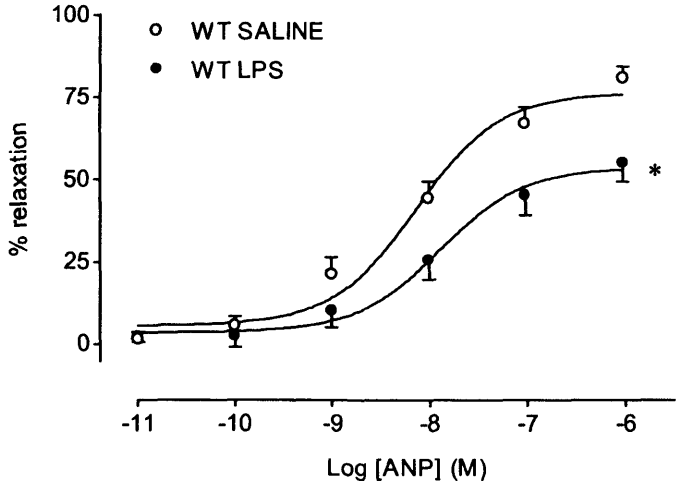


Figure 19 – Concentration-response curves to ACh in aortic rings from WT **(A)** and NPR-A KO **(B)** mice treated with LPS (12.5 mg/kg) or saline (both i.v.) for 16 h. Relaxation is expressed as mean ± SEM percentage reversal of U46619-induced tone; *, $p<0.05$ versus saline treated animals, n = 5.

EFFECT OF LPS *IN VIVO* ON RESPONSES TO ANP IN WILD TYPE AND NPR-A KNOCKOUT MICE

(A)



(B)

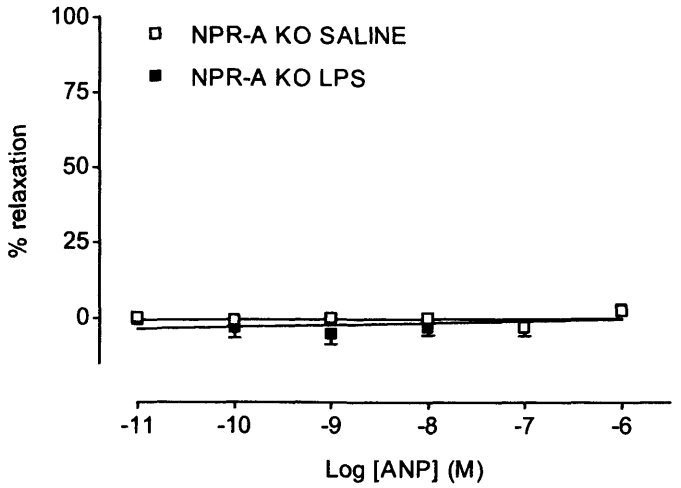
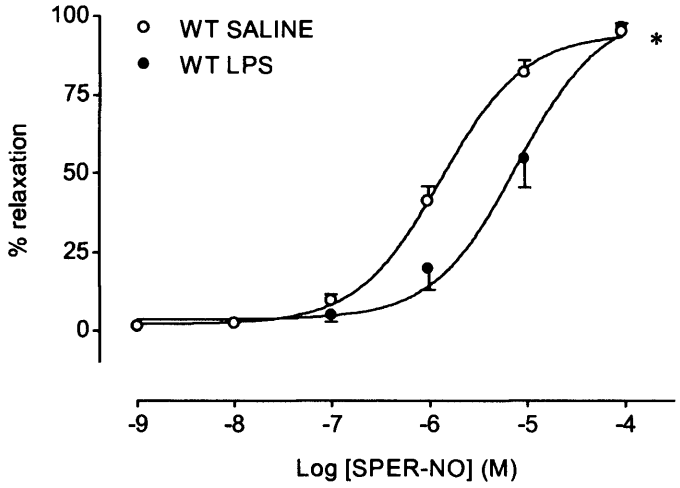


Figure 20 – Concentration-response curves to ANP in aortic rings from WT **(A)** and NPR-A KO **(B)** mice treated with LPS (12.5 mg/kg) or saline (both i.v.) for 16 h. Relaxation is expressed as mean ± SEM percentage reversal of U46619-induced tone; *, $p<0.05$ versus saline treated animals, n = 9.

EFFECT OF LPS *IN VIVO* ON RESPONSES TO SPERMINE NONOATE IN WILD TYPE AND NPR-A KNOCKOUT MICE

(A)



(B)

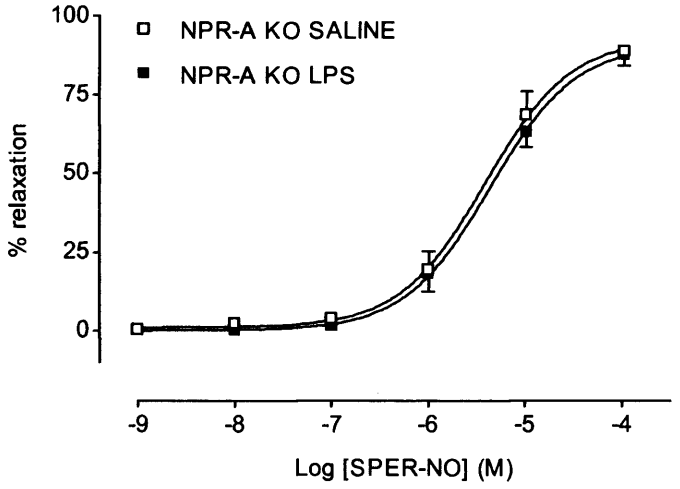


Figure 21 – Concentration-response curves to SPER-NO in aortic rings from WT **(A)** and NPR-A KO **(B)** mice treated with LPS (12.5 mg/kg) or saline (both i.v.) for 16 h. Relaxation is expressed as mean ± SEM percentage reversal of U46619-induced tone; *, $p<0.05$ versus saline treated animals, n = 3.

EFFECT OF LPS *IN VIVO* ON E_{max} AND POTENCY (EC_{50}) TO U46619 IN WILD TYPE AND NPR-A KNOCKOUT MICE

(A)

	WT LPS pEC_{50} -log [M]	WT SALINE pEC_{50} -log [M]	WT LPS E_{max} (% contraction)	WT SALINE E_{max} (% contraction)	FOLD SHIFT IN EC_{50} (POTENCY)
U46619	7.69 ± 0.12	7.94 ± 0.05	458.7 ± 20.39	520.2 ± 9.73	1.79

(B)

	NPR-A KO LPS pEC_{50} -log [M]	NPR-A KO SALINE pEC_{50} -log [M]	NPR-A KO LPS E_{max} (% contraction)	NPR-A KO SALINE E_{max} (% contraction)	FOLD SHIFT IN EC_{50} (POTENCY)
U46619	6.41 ± 0.16	7.12 ± 0.18	529.7 ± 60.64	363.8 ± 28.56	5.09

Figure 22 – E_{max} and pEC_{50} values determined from concentration-response curves to U46619 in aortic rings from WT **(A)** and NPR-A KO mice **(B)** treated with LPS (12.5 mg/kg) or saline (both i.v.) for 16 h. E_{max} is expressed as mean ± SEM percentage of basal tone and pEC_{50} is expressed as –log [M], mean ± SEM, n = 7.

EFFECT OF LPS *IN VIVO* ON E_{max} AND POTENCY (EC_{50}) TO ACETYLCHOLINE, ANP AND SPERMINE NONOATE IN WILD TYPE AND NPR-A KNOCKOUT MICE

(A)

	WT LPS pEC_{50} -log [M]	WT SALINE pEC_{50} -log [M]	WT LPS E_{max} (% relaxation)	WT SALINE E_{max} (% relaxation)	FOLD SHIFT IN EC_{50} (POTENCY)
ACh	6.69 ± 0.21	7.00 ± 0.08	25.46 ± 2.11	66.36 ± 2.22	2.07
ANP	7.89 ± 0.20	8.13 ± 0.13	53.61 ± 4.13	76.16 ± 3.58	1.77
SPER-NO	5.08 ± 0.16	5.86 ± 0.07	101.4 ± 12.13	94.40 ± 3.01	6.05

(B)

	NPR-A KO LPS pEC_{50} -log [M]	NPR-A KO SALINE pEC_{50} -log [M]	NPR-A KO LPS E_{max} (% relaxation)	NPR-A KO SALINE E_{max} (% relaxation)	FOLD SHIFT IN EC_{50} (POTENCY)
ACh	6.70 ± 0.24	7.30 ± 0.28	77.31 ± 6.92	79.58 ± 6.93	4.04
ANP	N/A	N/A	N/A	N/A	N/A
SPER-NO	5.25 ± 0.12	5.42 ± 0.18	95.73 ± 7.09	91.64 ± 8.83	1.46

Figure 23 – E_{max} and pEC_{50} values determined from concentration-response curves to ACh, ANP and SPER-NO in aortic rings from WT **(A)** and NPR-A KO mice **(B)** treated with LPS (12.5 mg/kg) or saline (both i.v.) for 16 h. E_{max} is expressed as mean ± SEM percentage reversal of U46619-induced tone and pEC_{50} is expressed as –log [M], mean ± SEM; n = 5 ACh, n = 9 ANP, n = 3 SPER-NO.

3.4. Summary of key results

Deletion of NPR-A *in vivo* results in NPR-A KO animals presenting with less vascular dysfunction following LPS treatment when compared to WT control animals. Responses to U46619, ACh, ANP and SPER-NO were suppressed to a greater extent in WT compared to NPR-A KO mice and this was paralleled by a decrease in iNOS expression and activity in lung and aortic tissue, suggesting that ANP or BNP (both signal through NPR-A) can act in a pro-inflammatory fashion.

CHAPTER 4

RESULTS 2

Regulation of pro-inflammatory protein expression (iNOS) by natriuretic peptides (ANP and CNP) *in vitro*

4. Results 2

4.1. Introduction

The findings presented in chapter 3 contrast with studies that suggest ANP acts in an anti-inflammatory fashion. Vollmar and Kiemer have shown that ANP inhibits NO production in macrophages by inhibiting iNOS expression via the pGC-coupled NPR-A with the involvement of cGMP (Vollmar and Schulz, 1995b; Kiemer and Vollmar, 1998; Kiemer and Vollmar, 2001). CNP however has been reported to have no effect on NO production and iNOS expression in macrophages (Kiemer and Vollmar, 1997). Yet, the role of natriuretic peptides in regulating iNOS expression remains unclear since ANP, BNP and CNP all upregulate iNOS induction by raising intracellular cGMP levels in vascular smooth muscle cells (Marumo *et al.*, 1995).

Thus, studies described in this chapter investigate the hypothesis that natriuretic peptides (ANP and CNP) affect pro-inflammatory protein expression *in vitro*. This was achieved by assessing the effects of ANP and CNP on pro-inflammatory protein expression and activity (iNOS) *in vitro*, in murine macrophages and vascular smooth muscle cells, in the presence of LPS and pro-inflammatory cytokines.

4.2. Optimisation of LPS-induced activation of RAW264.7 macrophages

In order to determine an effective, submaximal concentration of LPS with which to study the effect of natriuretic peptides on iNOS expression *in vitro*, RAW264.7 cells were activated with *Salmonella typhimurium* LPS (0 – 1000 ng/ml) and iNOS protein expression and activity determined. LPS produced a concentration dependent increase in iNOS expression at 24 and 48 h post activation. Maximum iNOS expression was seen at 24 h post activation using 1000 ng/ml LPS (Figure 24) and a suboptimal concentration of 100 ng/ml was selected for use in future experimentation.

LPS-INDUCED iNOS EXPRESSION AND ACTIVITY IN RAW264.7 MACROPHAGES

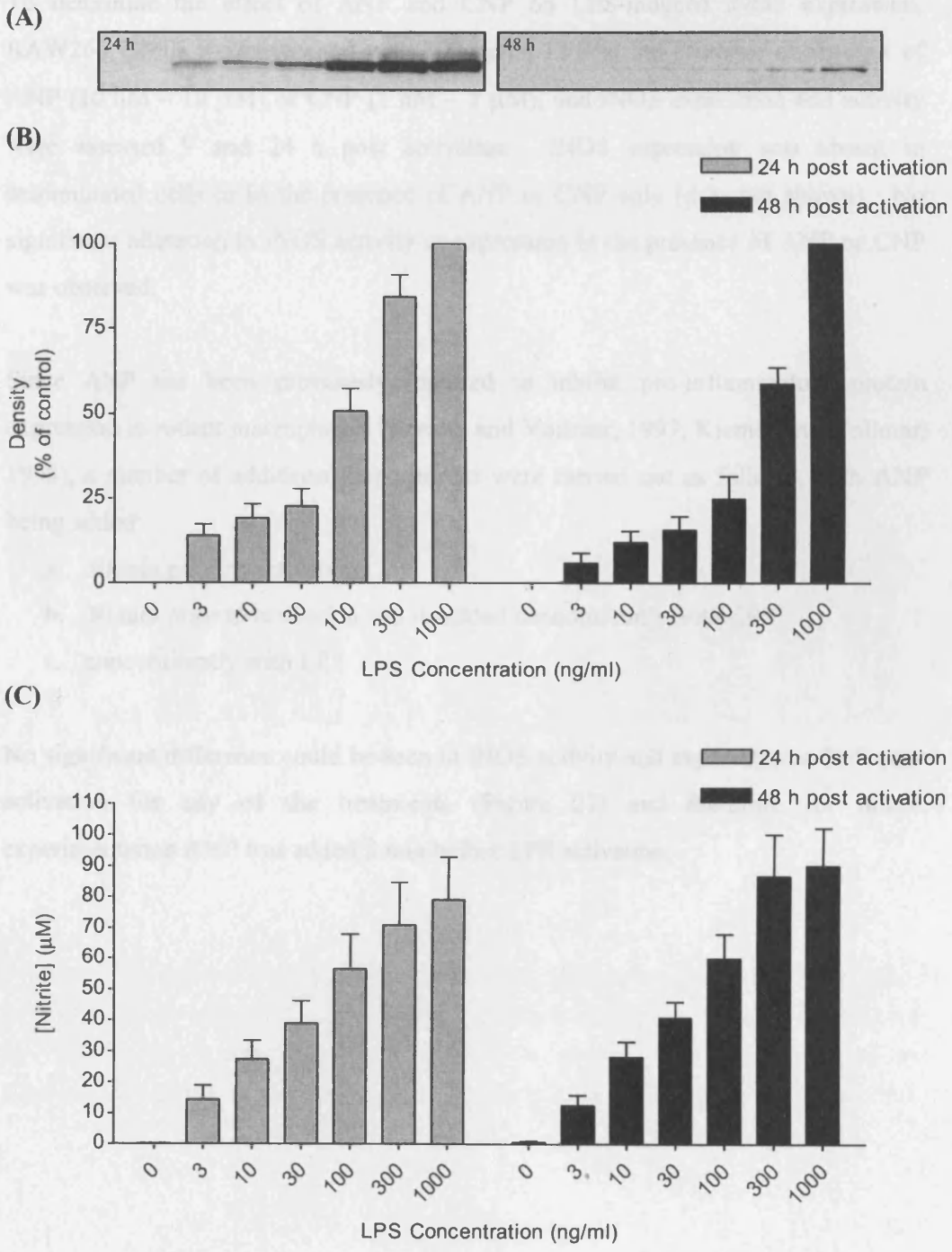


Figure 24 – Expression of iNOS protein in RAW264.7 macrophages activated with LPS (0 – 1000 ng/ml) for 24 or 48 h. Protein expression was analysed by Western blot **(A)** and quantified by densitometry **(B)**. Nitrite accumulation in the culture medium was determined by chemiluminescence **(C)**. Data are represented as mean ± SEM, n = 6.

4.3. Effect of ANP or CNP on LPS-induced iNOS in RAW264.7 macrophages

To determine the effect of ANP and CNP on LPS-induced iNOS expression, RAW264.7 cells were activated with 100 ng/ml LPS in the presence or absence of ANP (10 nM – 10 μM) or CNP (1 nM – 1 μM), and iNOS expression and activity were assessed 9 and 24 h post activation. iNOS expression was absent in unstimulated cells or in the presence of ANP or CNP only (data not shown). No significant alteration in iNOS activity or expression in the presence of ANP or CNP was observed.

Since ANP has been previously reported to inhibit pro-inflammatory protein expression in rodent macrophages (Kiemer and Vollmar, 1997; Kiemer and Vollmar, 1998), a number of additional experiments were carried out as follows, with ANP being added:

a. 30 min prior to activation
b. 30 min prior to activation and re-added concomitantly with LPS
c. concomitantly with LPS

No significant difference could be seen in iNOS activity and expression at 24 h post activation for any of the treatments (Figure 27) and therefore for future experimentation ANP was added 5 min before LPS activation.

EFFECT OF ANP ON LPS-INDUCED iNOS EXPRESSION AND ACTIVITY IN RAW264.7 MACROPHAGES

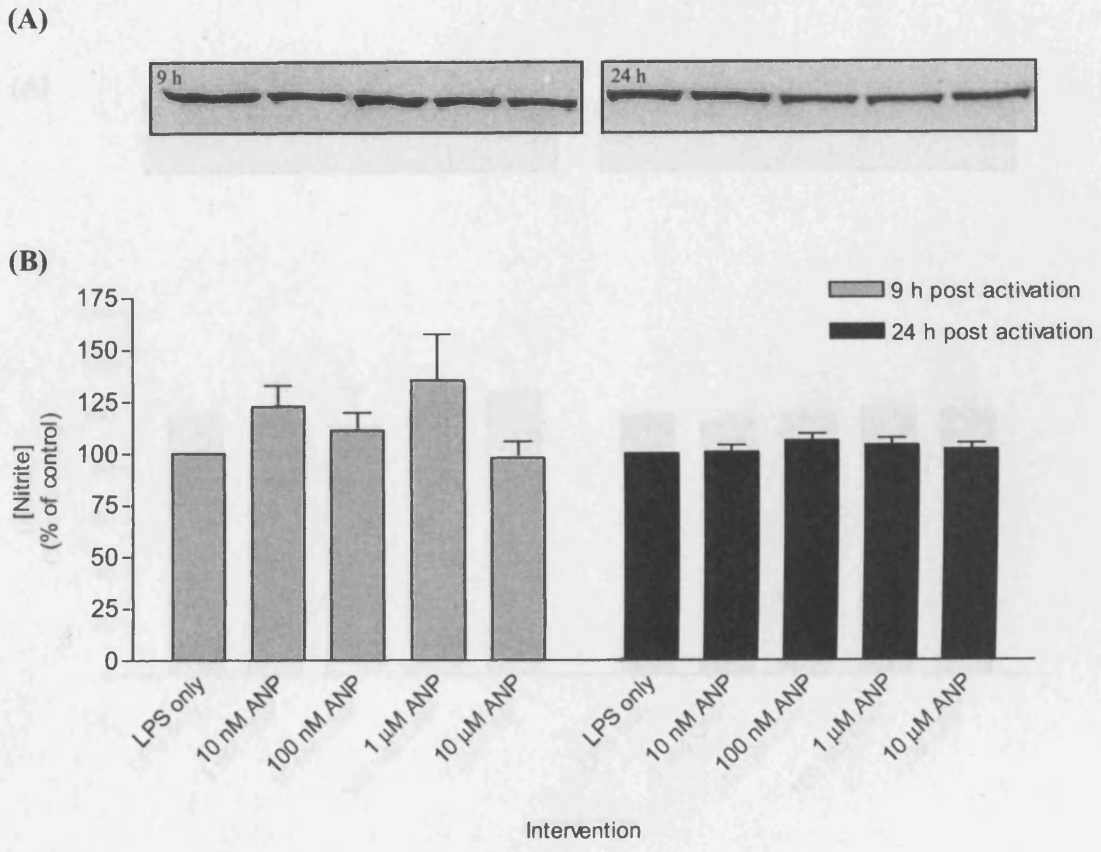


Figure 25 – Expression of iNOS protein in RAW264.7 macrophages activated with LPS (100 ng/ml) for 9 or 24 h in the presence of increasing concentrations of ANP. Protein expression was analysed by Western blot **(A)** and nitrite accumulation in the culture medium was determined by chemiluminescence **(B)**. Data are normalised to LPS (control) and represented as mean ± SEM, n = 3.

EFFECT OF CNP ON LPS-INDUCED iNOS EXPRESSION AND ACTIVITY IN RAW264.7 MACROPHAGES

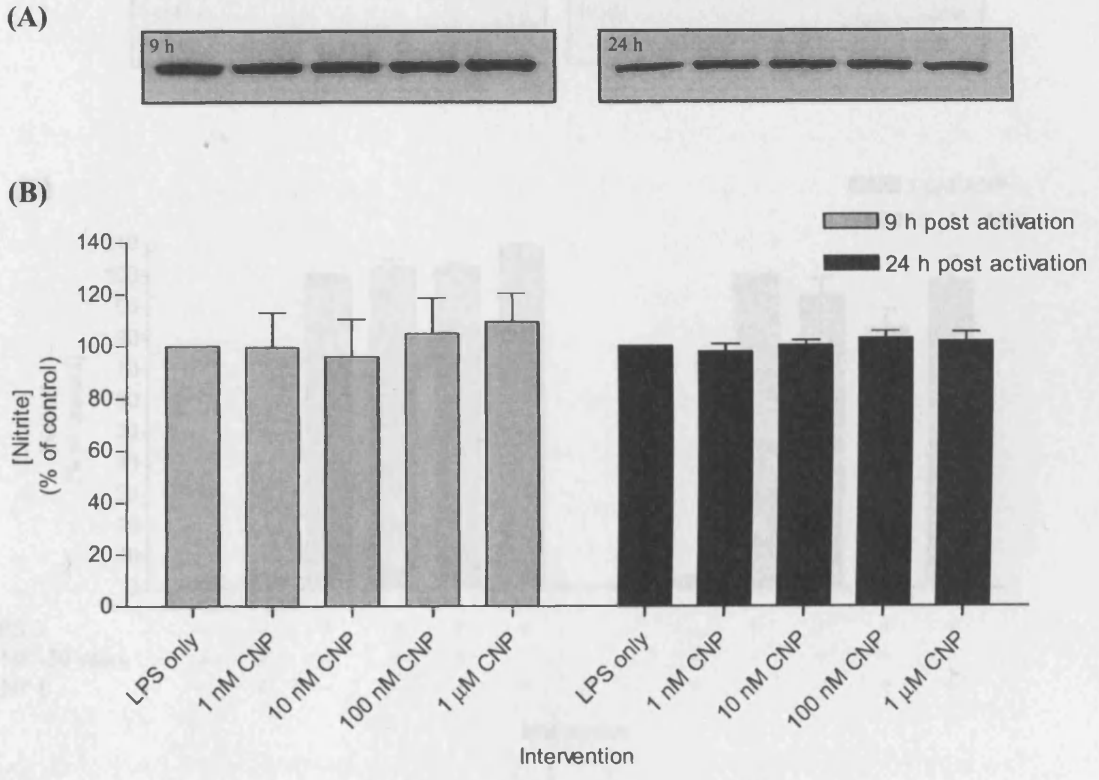


Figure 26 – Expression of iNOS protein in RAW264.7 macrophages activated with LPS (100 ng/ml) for 9 or 24 h in the presence of increasing concentrations of CNP. Protein expression was analysed by Western blot **(A)** and nitrite accumulation in the culture medium was determined by chemiluminescence **(B)**. Data are normalised to LPS (control) and represented as mean ± SEM, n = 6.

EFFECT OF TIME OF ADMINISTRATION OF ANP ON LPS-INDUCED iNOS EXPRESSION AND ACTIVITY IN RAW264.7 MACROPHAGES

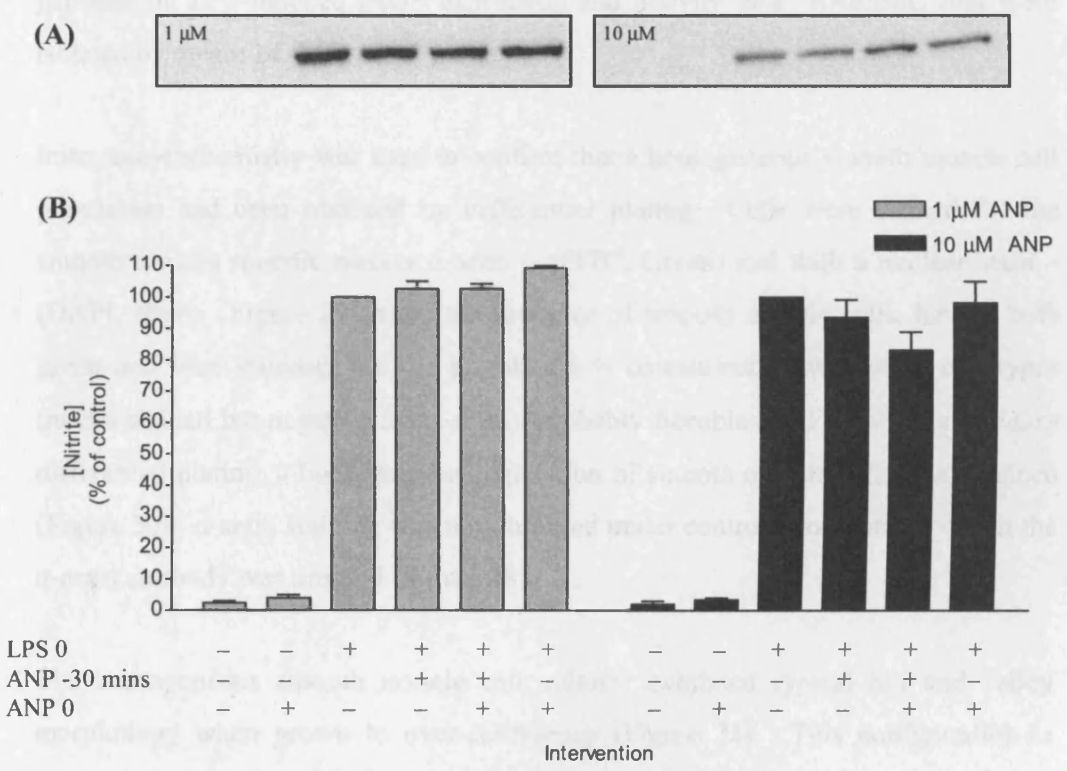


Figure 27 – Expression of iNOS protein in RAW264.7 macrophages activated with LPS (100 ng/ml) for 24 h in the presence of 1 or 10 µM ANP. ANP was added 30 min before activation, 30 min before activation and re-added at the same time as activation or concomitantly at the time of activation. Protein expression was analysed by Western blot **(A)** and nitrite accumulation in the culture medium was determined by chemiluminescence **(B)**. Data are normalised to LPS (control) and represented as mean ± SEM, n = 3.

4.4. Characterisation of primary rat aortic smooth muscle cells

4.4.1. Morphology

Since vascular smooth muscle cells also express iNOS during septic shock (Beasley *et al.*, 1991; Kusano *et al.*, 1999), I chose to examine the effect of natriuretic peptides on LPS-induced iNOS expression and activity in 1° RAoSMC that were isolated by means of differential plating.

Immunocytochemistry was used to confirm that a homogeneous smooth muscle cell population had been obtained by differential plating. Cells were stained for the smooth muscle specific marker α-actin – (FITC, Green) and with a nuclear stain – (DAPI, Blue). Figure 29 shows the presence of smooth muscle cells, having both green and blue staining, but the population is contaminated with other cell types (nuclei stained but negative for α-actin), probably fibroblasts. Following secondary differential plating, a homogeneous population of smooth muscle cells was obtained (Figure 30). α-actin staining was not observed under control conditions in which the α-actin antibody was omitted (Figure 28).

The homogeneous smooth muscle cell cultures exhibited typical hill and valley morphology when grown to over-confluency (Figure 31). This configuration is characterised by intertwined and overlapping cells with numerous multilayered and focal overgrowth areas (Chamley-Campbell *et al.*, 1979).

PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS WITH NUCLEAR STAINING

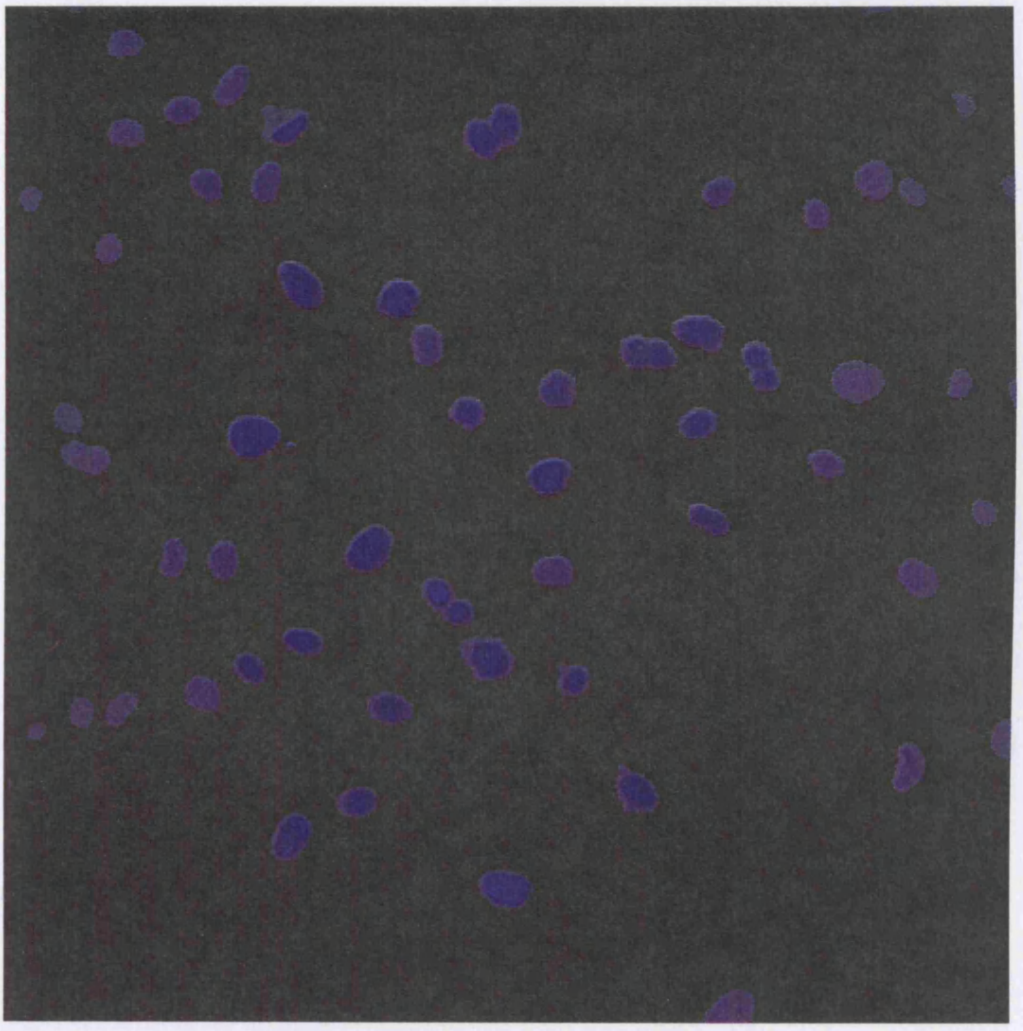


Figure 28 – Primary rat aortic smooth muscle cells stained with DAPI. The α-actin antibody was omitted for control purposes. Image generated using confocal microscopy; representative of several images.

NON-HOMOGENEOUS POPULATION OF PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

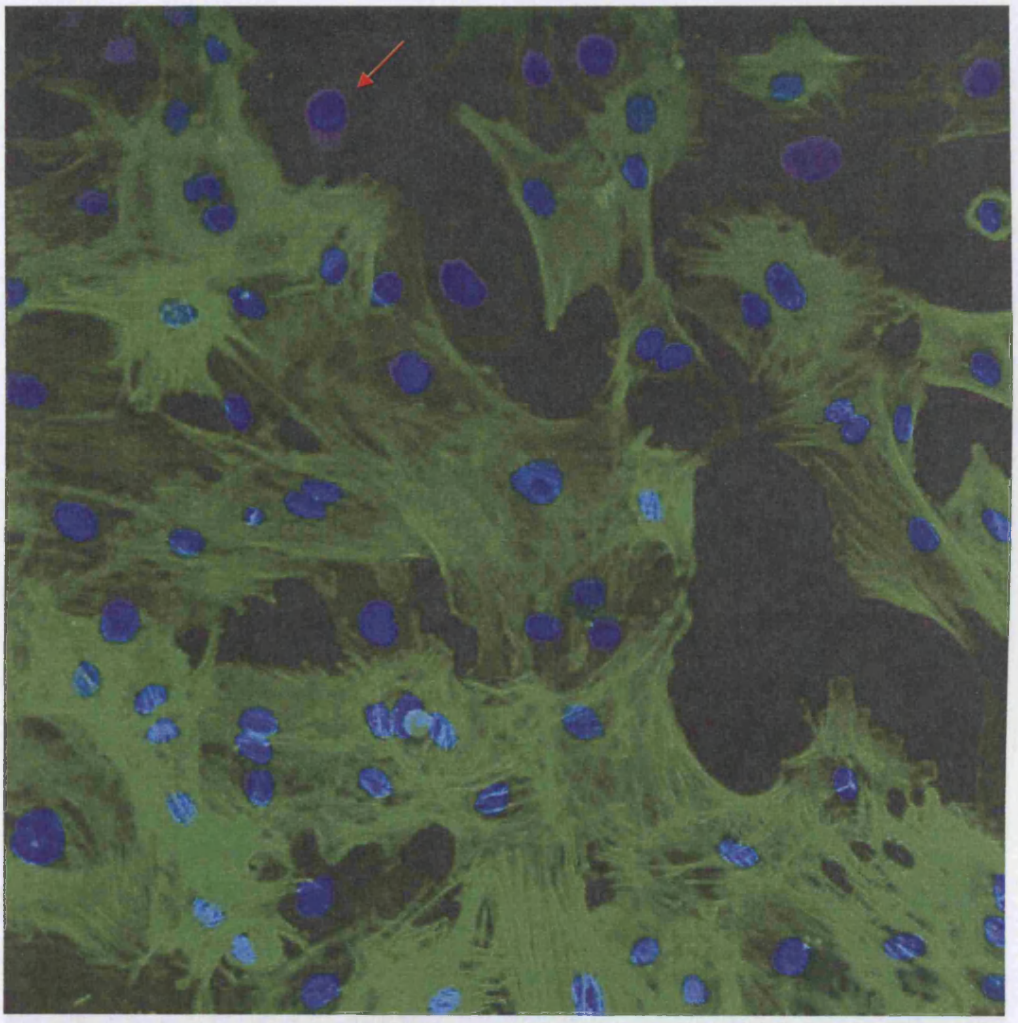


Figure 29 – Primary rat aortic smooth muscle cells stained with both DAPI (blue) and smooth muscle specific α-actin (green). Cells showing both green and blue staining are smooth muscle cells; cells showing only blue staining are other cell types, probably fibroblasts, contaminating the population (indicated by a red arrow). Image generated using confocal microscopy; representative of several images.

HOMOGENEOUS POPULATION OF PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

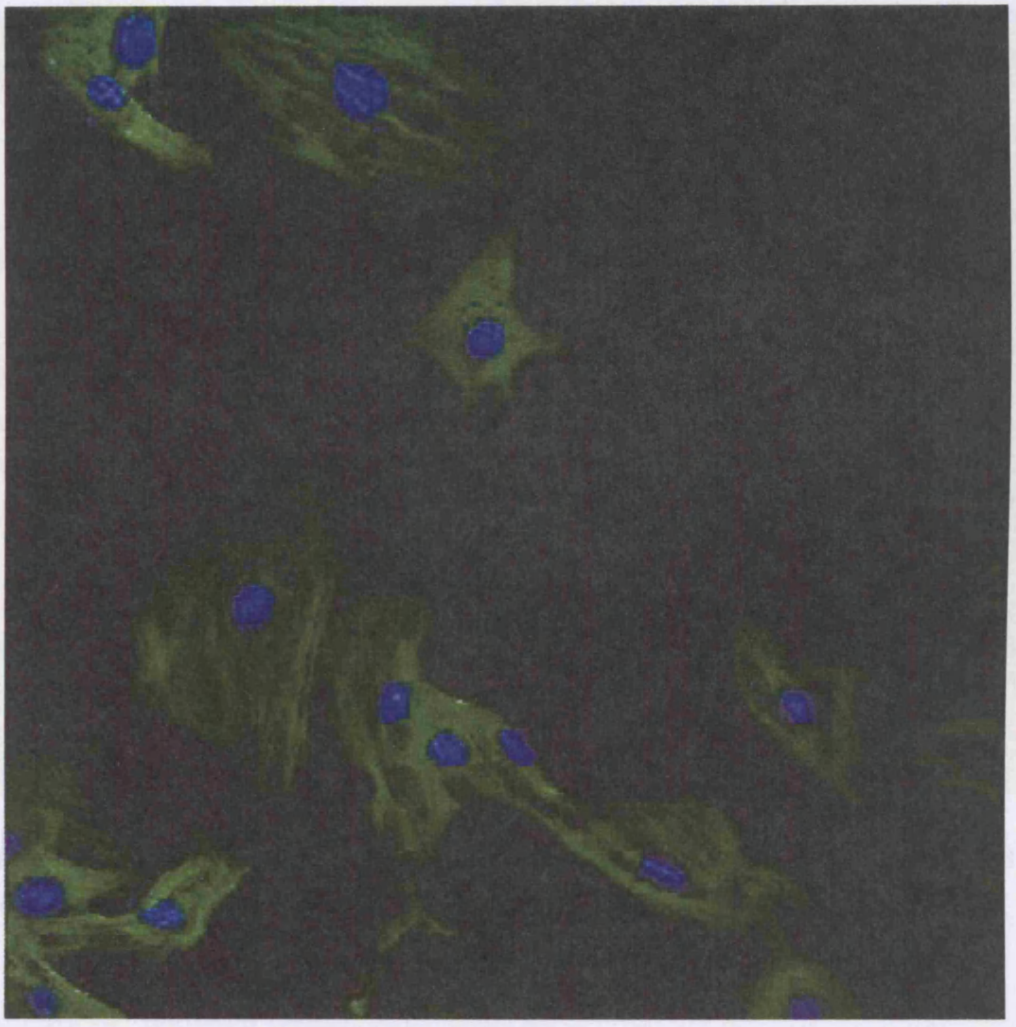


Figure 30 – Primary rat aortic smooth muscle cells stained with both DAPI (blue) and smooth muscle specific α-actin (green). Image depicts a homogeneous smooth muscle cell population with each cell positive for both nuclear and α-actin staining. Image generated using confocal microscopy; representative of several images.

PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS EXHIBITING TYPICAL HILL AND VALLEY MORPHOLOGY

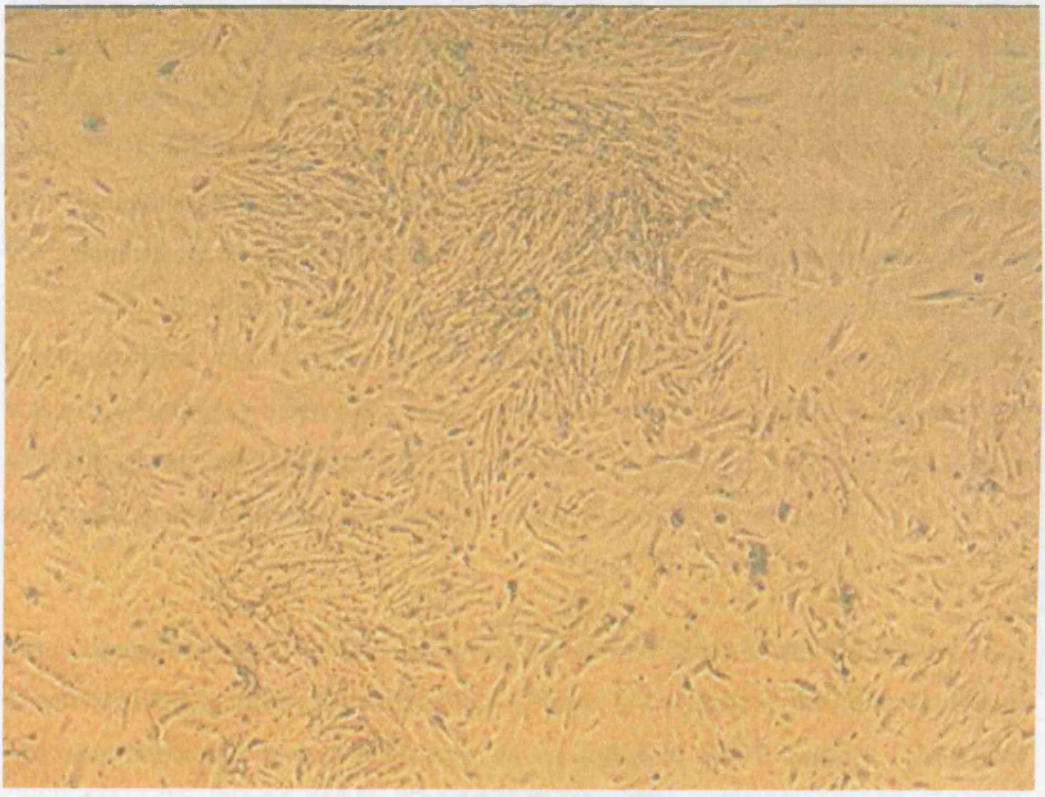


Figure 31 – Homogeneous primary rat aortic smooth muscle cell culture exhibiting typical hill and valley morphology; representative of several images.

4.4.2. Optimisation of primary rat aortic smooth muscle cell activation

Primary vascular smooth muscle cells do not express significant amounts of iNOS in response to LPS alone, but rather require a 'cocktail' of LPS and cytokines (MacNaul and Hutchinson, 1993; Matsumura *et al.*, 2001). Therefore, in order to determine an effective, submaximal concentration of LPS and cytokines with which to study the effect of natriuretic peptides on iNOS expression, 1° RAoSMC were activated with 100 μg/ml LPS (*Escherichia coli* or *Salmonella typhimurium*), 100 U/ml IFN-γ, 400 U/ml IL-1β and 1000 U/ml TNF-α in varying combinations (Figure 32). Inducible NOS expression and activity were determined by Western blot and chemiluminescence (nitrite) respectively. Inducible NOS expression and activity were most prominent in the presence of LPS and all three cytokines. Although cytokines alone, and cytokines plus *Escherichia Coli* LPS gave cell activation, this was not as strong as when *Salmonella typhimurium* LPS was added in the presence of IFN-γ, IL-1β and TNF-α (Figure 32). This combination of activators was used in additional studies to further optimise the activator cocktail to be used.

Three concentrations for each mediator/activator were selected and these were used to activate cells in different combinations (Figure 33). Nitrite in the culture medium was determined 24 h post activation as an index of iNOS activity. Maximal activation was seen with 100 μg/ml LPS, 200 U/ml IFN-γ, 400 U/ml IL-1β and 2000 U/ml TNF-α (Figure 33). This gave an approximately 50 % increase in nitrite in comparison to the initial combination of 100 μg/ml *Salmonella typhimurium* LPS, 100 U/ml IFN-γ, 400 U/ml IL-1β and 1000 U/ml TNF-α. For subsequent studies a suboptimal activator regime was used comprising of 100 μg/ml *Salmonella typhimurium* LPS, 200 U/ml IFN-γ, 400 U/ml IL-1β and 1000 U/ml TNF-α (Figure 33). This will be referred to as 'activator cocktail'.

ACTIVATION OF PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS WITH DIFFERENT LPS/CYTOKINE COCKTAILS

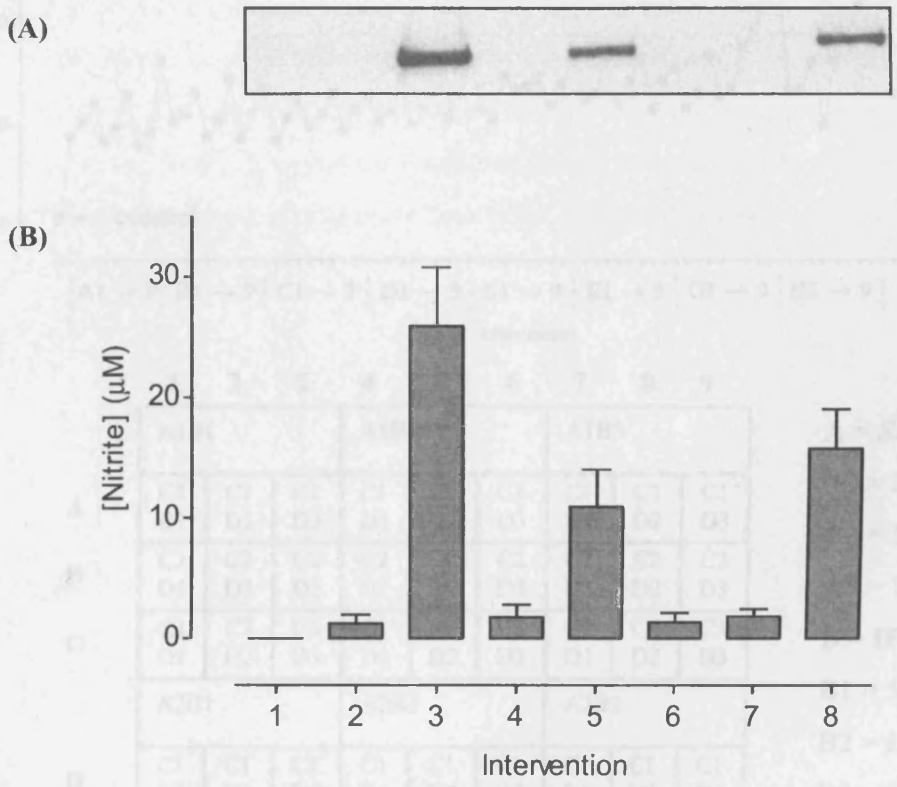
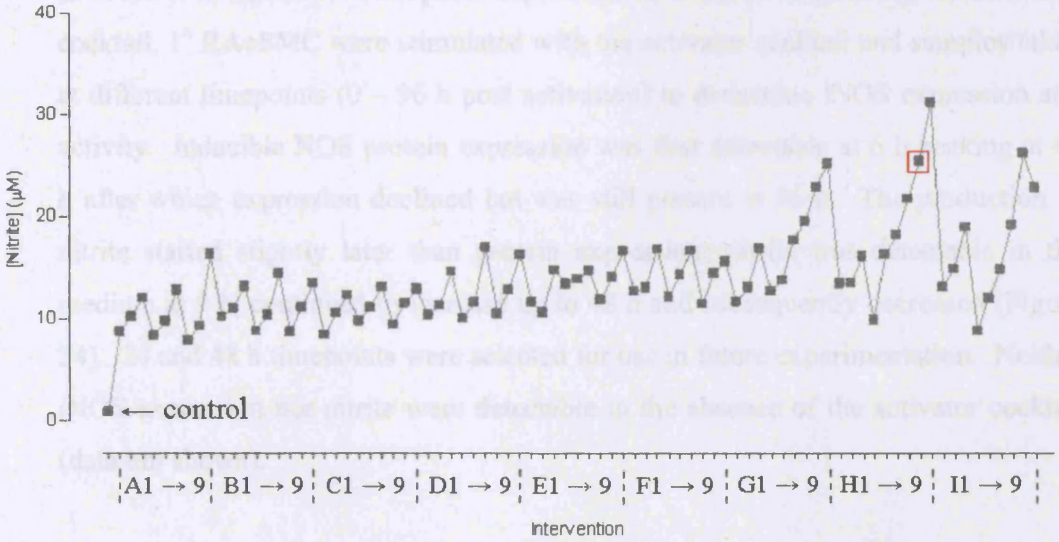


Figure 32 – Expression of iNOS protein in 1° RAoSMC treated with no activators (1), activated with 100 μg/ml *Salmonella typhimurium* LPS (2), 100 μg/ml *Salmonella typhimurium* LPS, 100 U/ml IFN-γ, 400 U/ml IL-1β and 1000 U/ml TNF-α (3), 1000 U/ml TNF-α and 400 U/ml IL-1β (4), 1000 U/ml TNF-α, 400 U/ml IL-1β and 100 U/ml IFN-γ (5), 1000 U/ml TNF-α and 100 U/ml IFN-γ (6), 100 μg/ml *Escherichia Coli* LPS (7), 100 μg/ml *Escherichia Coli* LPS, 100 U/ml IFN-γ, 400 U/ml IL-1β and 1000 U/ml TNF-α (8) for 24 h. Protein expression was analysed by Western blot **(A)** and nitrite accumulation in the culture medium was determined by chemiluminescence **(B)**. Data are represented as the mean ± SEM, n = 4.

RAT AORTIC SMOOTH MUSCLE CELL ACTIVATION WITH LPS (*SALMONELLA TYPHIMURIUM*) IN COMBINATION WITH VARYING CYTOKINE CONCENTRATIONS



	1	2	3	4	5	6	7	8	9
	A1B1			A1B2			A1B3		
A	C1 D1	C1 D2	C1 D3	C1 D1	C1 D2	C1 D3	C1 D1	C1 D2	C1 D3
B	C2 D1	C2 D2	C2 D3	C2 D1	C2 D2	C2 D3	C2 D1	C2 D2	C2 D3
C	C3 D1	C3 D2	C3 D3	C3 D1	C3 D2	C3 D3	C3 D1	C3 D2	C3 D3
	A2B1			A2B2			A2B3		
D	C1 D1	C1 D2	C1 D3	C1 D1	C1 D2	C1 D3	C1 D1	C1 D2	C1 D3
E	C2 D1	C2 D2	C2 D3	C2 D1	C2 D2	C2 D3	C2 D1	C2 D2	C2 D3
F	C3 D1	C3 D2	C3 D3	C3 D1	C3 D2	C3 D3	C3 D1	C3 D2	C3 D3
	A3B1			A3B2			A3B3		
G	C1 D1	C1 D2	C1 D3	C1 D1	C1 D2	C1 D3	C1 D1	C1 D2	C1 D3
H	C2 D1	C2 D2	C2 D3	C2 D1	C2 D2	C2 D3	C2 D1	C2 D2	C2 D3
I	C3 D1	C3 D2	C3 D3	C3 D1	C3 D2	C3 D3	C3 D1	C3 D2	C3 D3

A = *S.typhimurium* LPS
A1 = 25 μg/ml
A2 = 50 μg/ml
A3 = 100 μg/ml
B = IFN-γ
B1 = 50 U/ml
B2 = 100 U/ml
B3 = 200 U/ml
C = IL-1β
C1 = 200 U/ml
C2 = 400 U/ml
C3 = 600 U/ml
D = TNF-α
D1 = 500 U/ml
D2 = 1000 U/ml
D3 = 2000 U/ml

Figure 33 – Nitrite accumulation in the culture medium determined by chemiluminescence following activation of 1° RAoSMC with different combinations of *Salmonella typhimurium* LPS, IFN-γ, IL-1β and TNF-α at increasing concentrations. Suboptimal activator regime, 'activator cocktail', used in subsequent studies is indicated in red. Graph is representative of 2 separate experiments.

4.4.3. Timecourse of iNOS expression in primary rat aortic smooth muscle cells in response to activator cocktail

In order to determine the temporal expression of iNOS in response to the activator cocktail, 1° RAoSMC were stimulated with the activator cocktail and samples taken at different timepoints (0 – 96 h post activation) to determine iNOS expression and activity. Inducible NOS protein expression was first detectable at 6 h peaking at 48 h after which expression declined but was still present at 96 h. The production of nitrite started slightly later than protein expression; nitrite was detectable in the medium at 9 h, continued to increase up to 48 h and subsequently decreased (Figure 34). 24 and 48 h timepoints were selected for use in future experimentation. Neither iNOS expression nor nitrite were detectable in the absence of the activator cocktail (data not shown).

TIMECOURSE OF iNOS EXPRESSION AND ACTIVITY IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS IN RESPONSE TO ACTIVATOR COCKTAIL

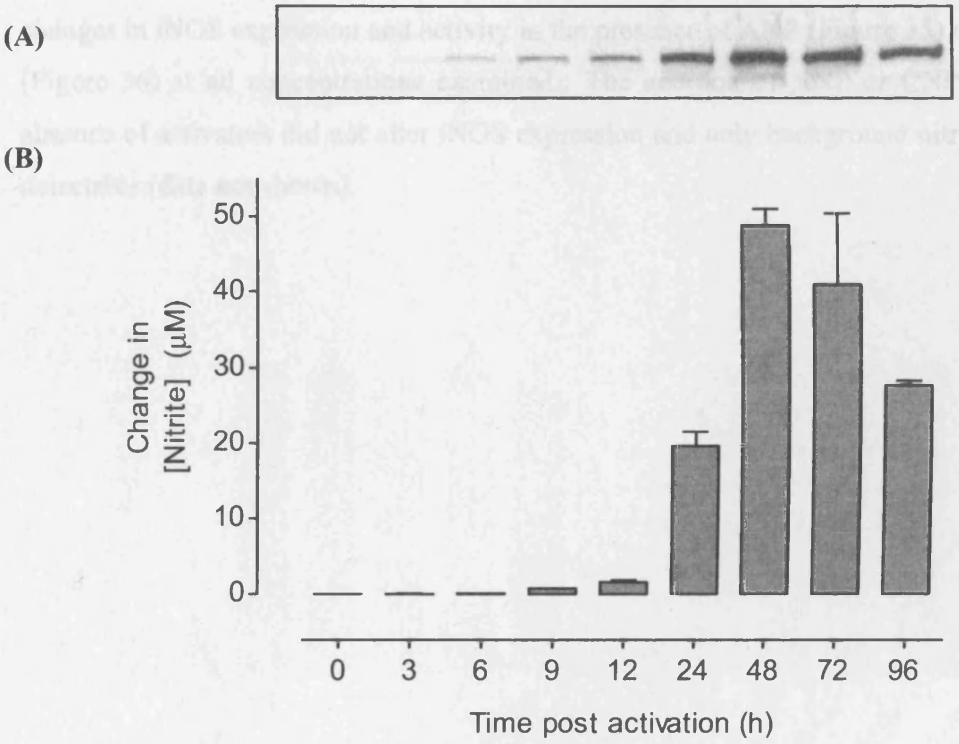


Figure 34 – Expression of iNOS protein in 1° RAoSMC activated with 100 μg/ml *Salmonella typhimurium* LPS, 200 U/ml IFN-γ, 400 U/ml IL-1β and 1000 U/ml TNF-α. Protein expression was analysed by Western blot **(A)** and accumulation of nitrite in the culture medium determined by chemiluminescence **(B)**. Data are represented as mean ± SEM, n = 4.

4.5. Effect of ANP and CNP on activator cocktail-induced iNOS expression in primary rat aortic smooth muscle cells

To determine the effect of ANP and CNP on iNOS expression in response to activator cocktail, 1° RAoSMC were treated with increasing concentrations of ANP or CNP (10 nM – 10 μM), 5 min prior to activation. There were no significant changes in iNOS expression and activity in the presence of ANP (Figure 35) or CNP (Figure 36) at all concentrations examined. The addition of ANP or CNP in the absence of activators did not alter iNOS expression and only background nitrite was detectable (data not shown).

EFFECT OF ANP ON ACTIVATOR COCKTAIL-INDUCED iNOS EXPRESSION AND ACTIVITY IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

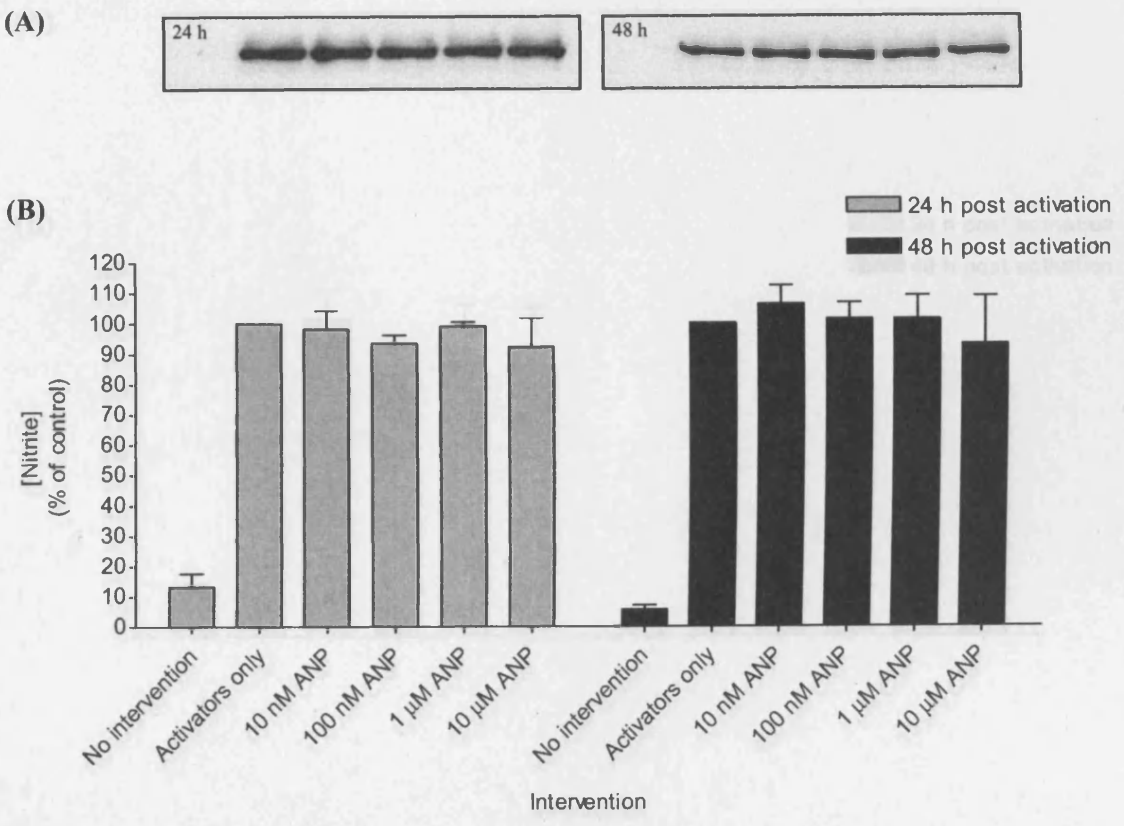


Figure 35 – Expression of iNOS protein in 1° RAoSMC activated with activator cocktail for 24 or 48 h in the presence of increasing concentrations of ANP. Protein expression was analysed by Western blot **(A)** and nitrite accumulation in the culture medium was determined by chemiluminescence **(B)**. Data are normalised to activators only (control) and represented as mean ± SEM, n = 4.

EFFECT OF CNP ON ACTIVATOR COCKTAIL-INDUCED iNOS EXPRESSION AND ACTIVITY IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

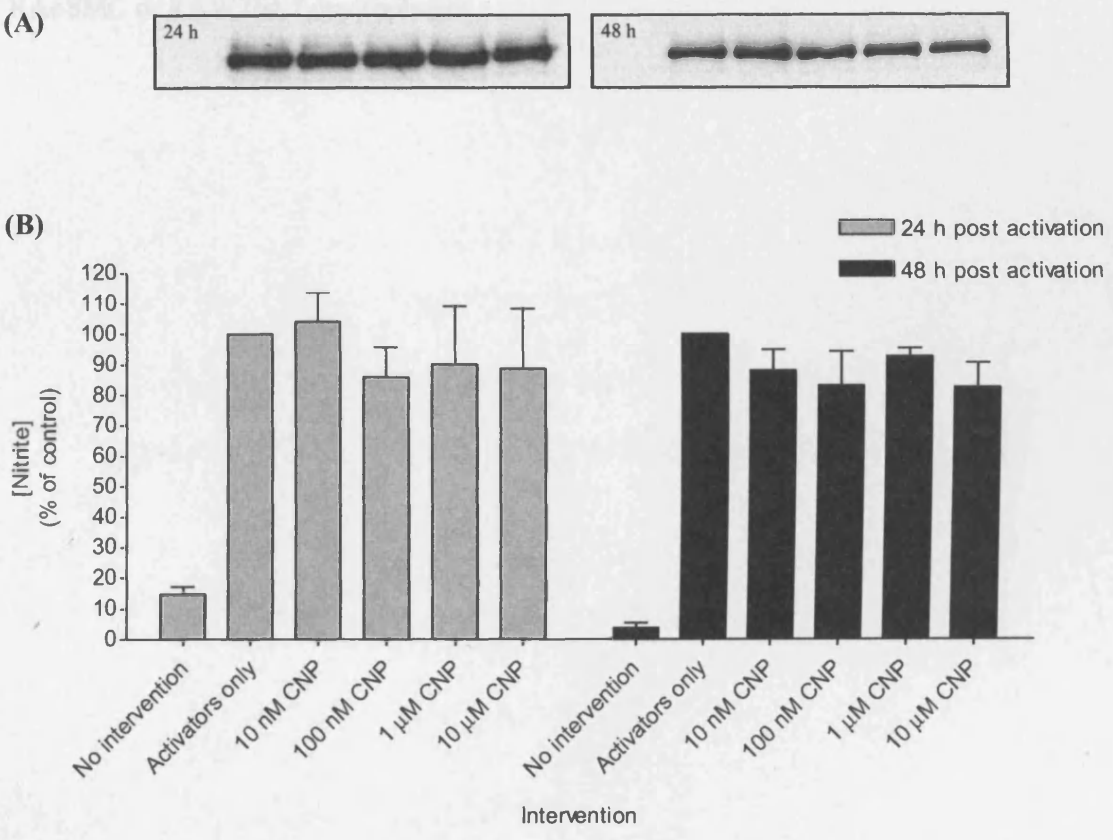


Figure 36 – Expression of iNOS protein in 1° RAoSMC activated with activator cocktail for 24 or 48 h in the presence of increasing concentrations of CNP. Protein expression was analysed by Western blot **(A)** and nitrite accumulation in the culture medium was determined by chemiluminescence **(B)**. Data are normalised to activators only (control) and represented as mean ± SEM, n = 4.

4.6. Summary of key results

Primary rat aortic smooth muscle cells were successfully isolated and characterised. They were shown to be positive for smooth muscle specific α-actin and exhibited a typical hill and valley morphology. A submaximal activator cocktail for the induction of iNOS in 1° RAoSMC was identified. However, no effect on iNOS expression or activity was seen in the presence of ANP or CNP in either 1° RAoSMC or RAW264.7 macrophages.

CHAPTER 5

RESULTS 3

Role of natriuretic peptides and their receptors in regulating MAPK signalling and cell proliferation

5. Results 3

5.1. Introduction

Previous results presented herein indicate that there is a lack of effect of ANP and CNP on iNOS expression *in vitro* in both macrophages and RAoSMC, which does not fit with preceding studies reporting ANP-mediated inhibition of iNOS expression (Kiemer and Vollmar, 1997; Kiemer and Vollmar, 1998). Moreover, my pharmacological data in NPR-A KO animals shown in chapter 3 suggest that ANP/BNP act in a pro-inflammatory fashion *in vivo*, and although there was a slight trend for ANP/CNP to increase iNOS expression in macrophages *in vitro*, there was essentially no effect.

In order to try and reconcile these differences and provide more definitive evidence supporting a pro- or anti-inflammatory role for natriuretic peptides, I investigated the relationship between ANP and CNP, MAPK and iNOS expression/activity. ANP and CNP have been shown to alter MAPK activity (Kiemer and Vollmar, 1997; Kiemer and Vollmar, 1998; Pandey *et al.*, 2000; Sharma *et al.*, 2002; Yasoda *et al.*, 2004), and this pathway is known to regulate iNOS expression (Da Silva *et al.*, 1997; Doi *et al.*, 2000; Chakravortty *et al.*, 2001; Bhat *et al.*, 2002) as well as cell proliferation (Pearson *et al.*, 2001). Furthermore, all natriuretic peptides have been shown to possess anti-proliferative properties, which appear to be important in cardiomyocytes and vascular smooth muscle (Furuya *et al.*, 1991; Furuya *et al.*, 1993; Silberbach and Roberts, 2001).

In this chapter I explored the hypothesis that natriuretic peptides (ANP and CNP) modulate MAPK pathways and regulate cell proliferation via activation of GC-linked NPRs. Initially, I investigated which 'arms' of the MAPK pathway are involved in iNOS expression and subsequently established the effects of ANP and CNP on MAPK (ERK 1/2 and p38) phosphorylation. Furthermore, I investigated which natriuretic peptide receptor is involved in the regulation of MAPK phosphorylation. Finally, I explored whether MAPK regulation by natriuretic peptides might influence cell proliferation.

5.2. Effect of MAPK phosphorylation inhibitors on activator cocktail-induced iNOS expression and activity in primary rat aortic smooth muscle cells

In an attempt to investigate which 'arms' of the MAPK pathway are involved in activator cocktail-induced iNOS expression, 1° RAoSMC were treated with the ERK 1/2 inhibitor PD98059 (30 μM), or the p38 inhibitor SB203580 (30 μM), for 30 min prior to the addition of activator cocktail (100 μg/ml *Salmonella typhimurium* LPS, 200 U/ml IFN-γ, 400 U/ml IL-1β and 1000 U/ml TNF-α). Nitrite accumulation in the culture medium and protein expression were determined 24 h post activation. PD98059 did not modulate iNOS but SB203580 brought about a significant inhibition of iNOS expression and activity (Figure 37). Therefore, the various components of the MAPK phosphorylation pathway were investigated further to determine if natriuretic peptides were able to modulate their activity.

EFFECT OF MAPK PHOSPHORYLATION INHIBITORS ON ACTIVATOR COCKTAIL-INDUCED iNOS EXPRESSION AND ACTIVITY IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

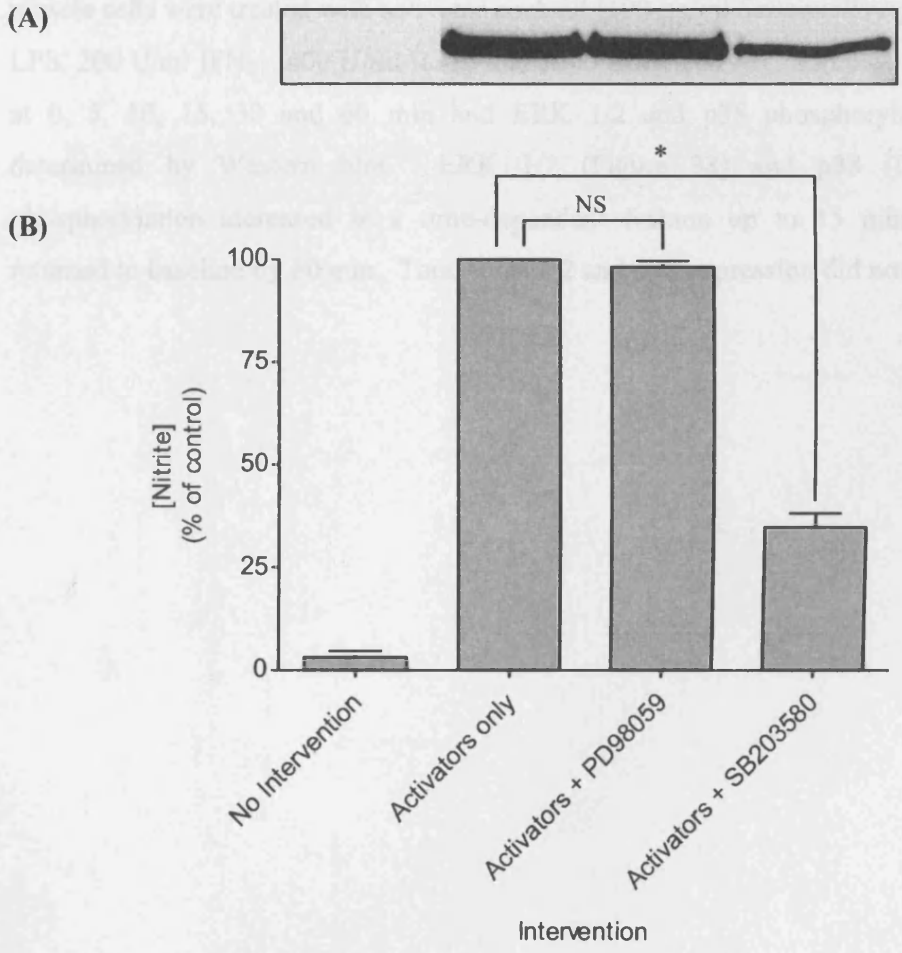


Figure 37 – Expression of iNOS protein in 1° RAoSMC activated with activator cocktail for 24 h in the presence of ERK 1/2 pathway inhibitor, PD98059 or p38 pathway inhibitor, SB203580 (both 30 μM). Protein expression was analysed by Western blot **(A)** and nitrite accumulation in the culture medium was determined by chemiluminescence **(B)**. Data are represented as mean ± SEM, n = 4; *, $p<0.05$ versus activators only.

5.3. Activation of MAPK pathway phosphorylation in primary rat aortic smooth muscle cells

To investigate further the potential effects of natriuretic peptides, primarily CNP, on MAPK regulation, I first examined which arms of the MAPK system were phosphorylated in response to the activator cocktail. Primary rat aortic smooth muscle cells were treated with activator cocktail (100 μg/ml *Salmonella typhimurium* LPS, 200 U/ml IFN-γ, 400 U/ml IL-1β and 1000 U/ml TNF-α). Samples were taken at 0, 5, 10, 15, 30 and 60 min and ERK 1/2 and p38 phosphorylation were determined by Western blot. ERK 1/2 (Figure 38) and p38 (Figure 39) phosphorylation increased in a time-dependent fashion up to 15 min and then returned to baseline by 60 min. Total ERK 1/2 and p38 expression did not change.

EFFECT OF ACTIVATOR COCKTAIL ON ERK 1/2 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

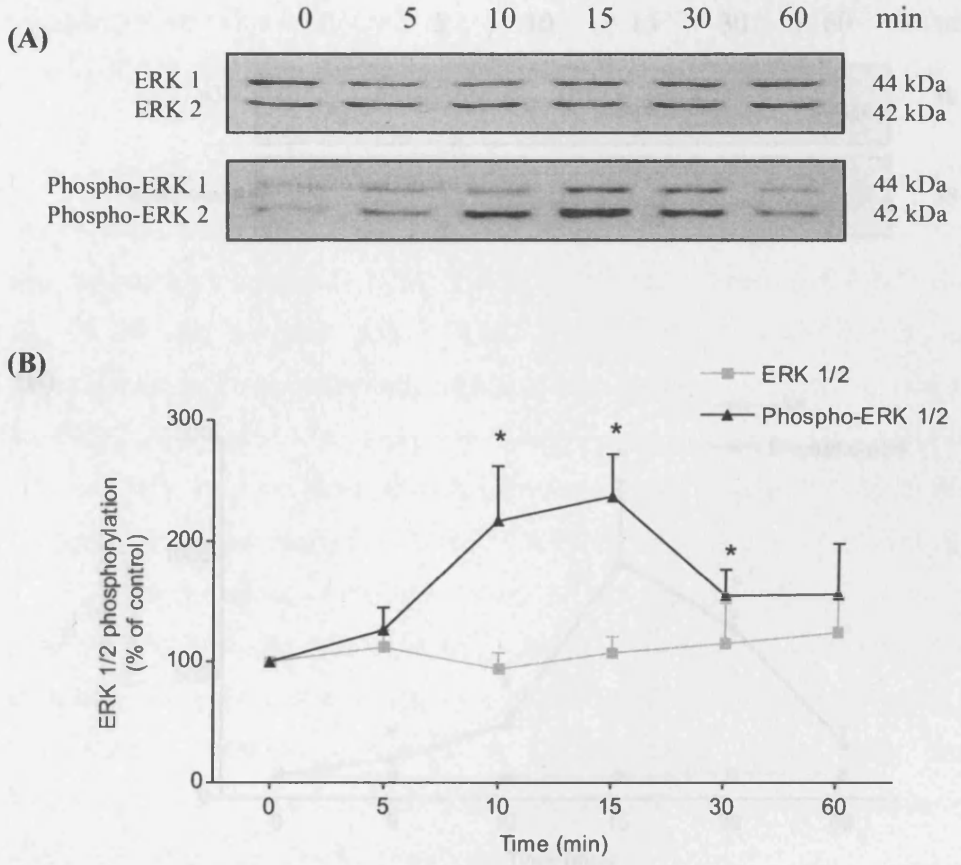


Figure 38 – Expression and phosphorylation of ERK 1/2 (p44/p42) protein in 1° RAoSMC treated with activator cocktail for 0 – 60 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (0 min) **(B)**. Data are represented as mean ± SEM, n = 4; *, $p<0.05$ versus control.

EFFECT OF ACTIVATOR COCKTAIL ON p38 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

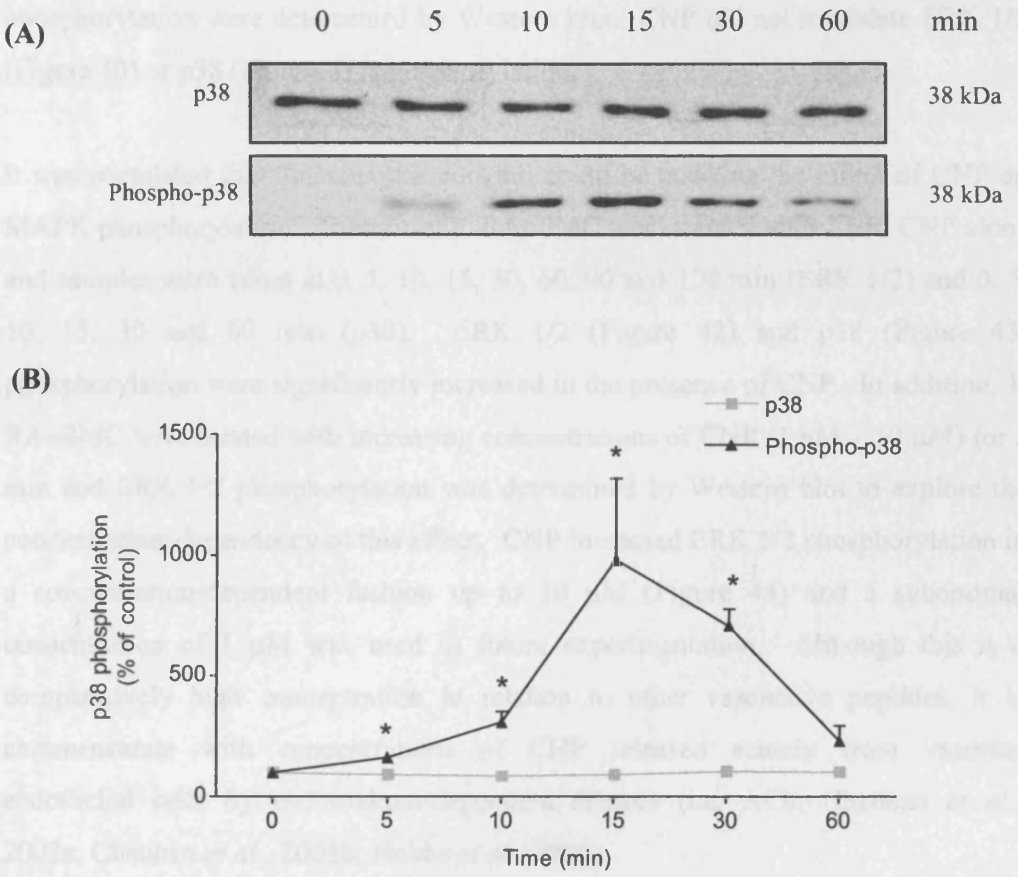


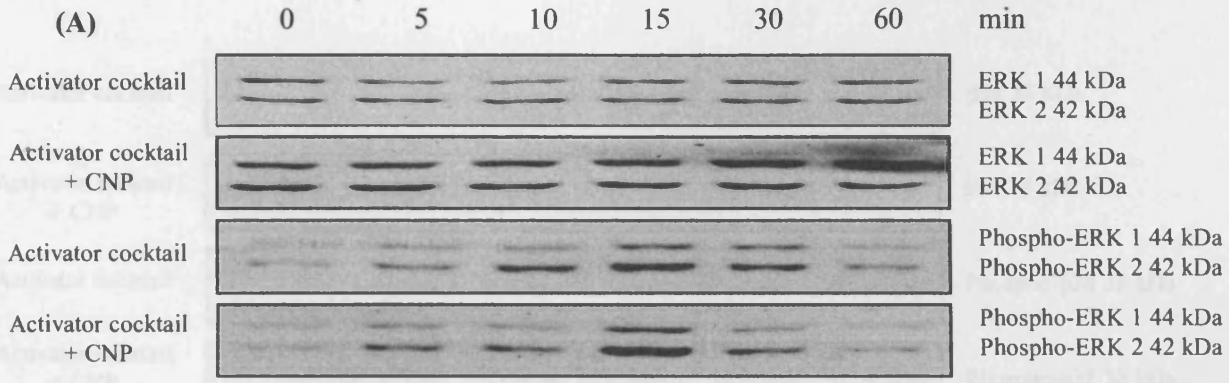
Figure 39 – Expression and phosphorylation of p38 protein in 1° RAoSMC treated with activator cocktail for 0 – 60 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (0 min) **(B)**. Data are represented as mean ± SEM, n = 3; *, $p<0.05$ versus control.

5.4. Effect of CNP on MAPK phosphorylation (ERK 1/2 and p38) in primary rat aortic smooth muscle cells

To determine the effect of CNP on ERK 1/2 and p38 phosphorylation, 1° RAoSMC were treated with 1 μM CNP for 5 min prior to the addition of activator cocktail. Samples were taken at 0, 5, 10, 15, 30 and 60 min and ERK 1/2 and p38 phosphorylation were determined by Western blot. CNP did not modulate ERK 1/2 (Figure 40) or p38 (Figure 41) phosphorylation.

It was postulated that the activator cocktail could be masking the effect of CNP on MAPK phosphorylation. Therefore, 1° RAoSMC were treated with 1 μM CNP alone and samples were taken at 0, 5, 10, 15, 30, 60, 90 and 120 min (ERK 1/2) and 0, 5, 10, 15, 30 and 60 min (p38). ERK 1/2 (Figure 42) and p38 (Figure 43) phosphorylation were significantly increased in the presence of CNP. In addition, 1° RAoSMC were treated with increasing concentrations of CNP (1 nM – 10 μM) for 5 min and ERK 1/2 phosphorylation was determined by Western blot to explore the concentration-dependency of this effect. CNP increased ERK 1/2 phosphorylation in a concentration-dependent fashion up to 10 μM (Figure 44) and a suboptimal concentration of 1 μM was used in future experimentation. Although this is a comparatively high concentration in relation to other vasoactive peptides, it is commensurate with concentrations of CNP released acutely from vascular endothelial cells by endothelium-dependent dilators (i.e. ACh; Chauhan *et al.*, 2003a; Chauhan *et al.*, 2003b; Hobbs *et al.*, 2004).

EFFECT OF CNP ON ERK1/2 PHOSPHORYLATION IN THE PRESENCE OF ACTIVATOR COCKTAIL IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS



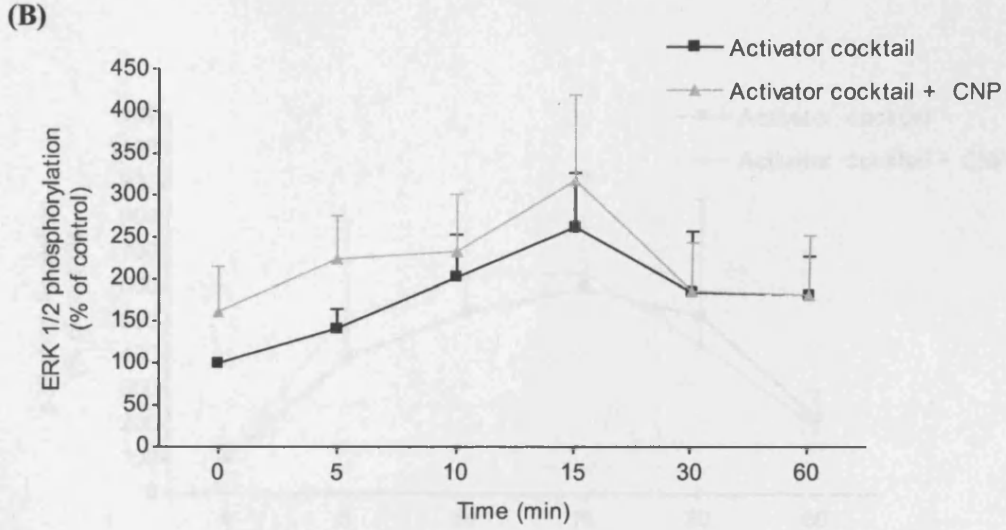


Figure 40 – Expression and phosphorylation of ERK 1/2 (p44/p42) protein in 1° RAoSMC treated with activator cocktail in the presence or absence of CNP (1 μM) for 0 – 60 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (0 min with activator cocktail only) **(B)**. Data are represented as mean ± SEM, n = 4.

EFFECT OF CNP ON p38 PHOSPHORYLATION IN THE PRESENCE OF ACTIVATOR COCKTAIL IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

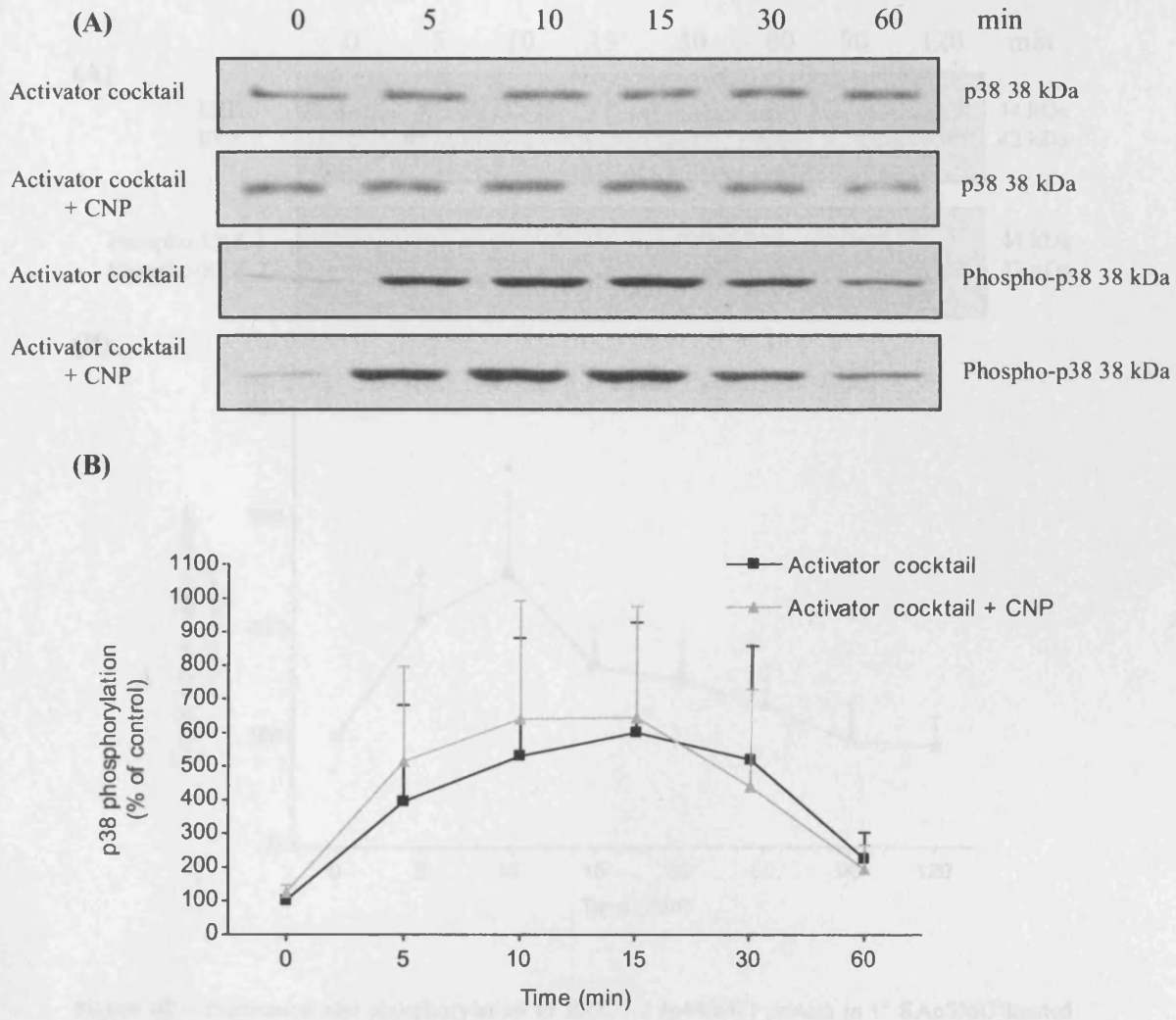


Figure 41 – Expression and phosphorylation of p38 protein in 1° RAoSMC treated with activator cocktail in the presence or absence of CNP (1 μM) for 0 – 60 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (0 min with activator cocktail only) **(B)**. Data are represented as mean ± SEM, n = 3.

EFFECT OF CNP ON ERK 1/2 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

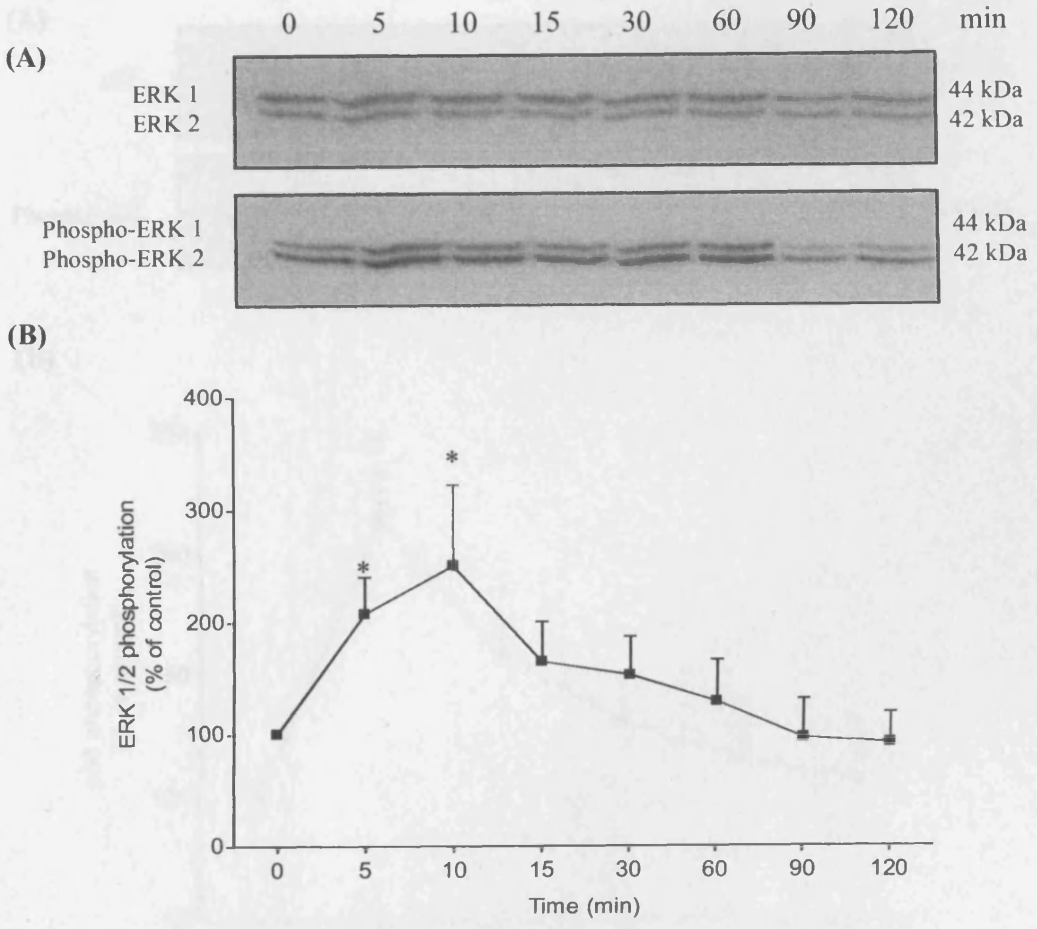


Figure 42 – Expression and phosphorylation of ERK 1/2 (p44/p42) protein in 1° RAoSMC treated with CNP (1 μM) for 0 – 120 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (0 min) **(B)**. Data are represented as the mean ± SEM, n =8; *, $p<0.05$ versus control.

EFFECT OF CNP ON p38 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

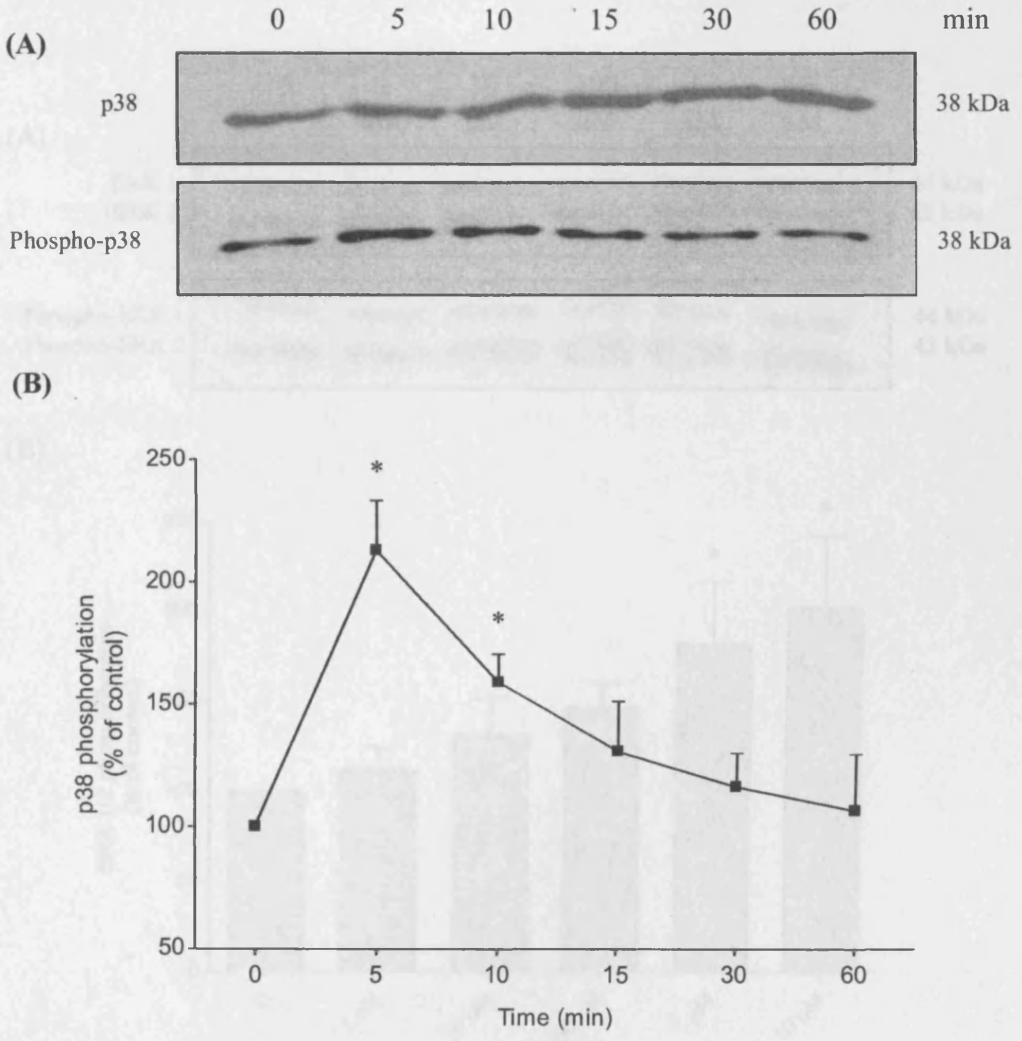


Figure 43 – Expression and phosphorylation of p38 protein in 1° RAoSMC treated with CNP (1 μM) for 0 – 60 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (0 min) **(B)**. Data are represented as the mean ± SEM, n =4; *, $p<0.05$ versus control.

CONCENTRATION-DEPENDENT EFFECT OF CNP ON ERK 1/2 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

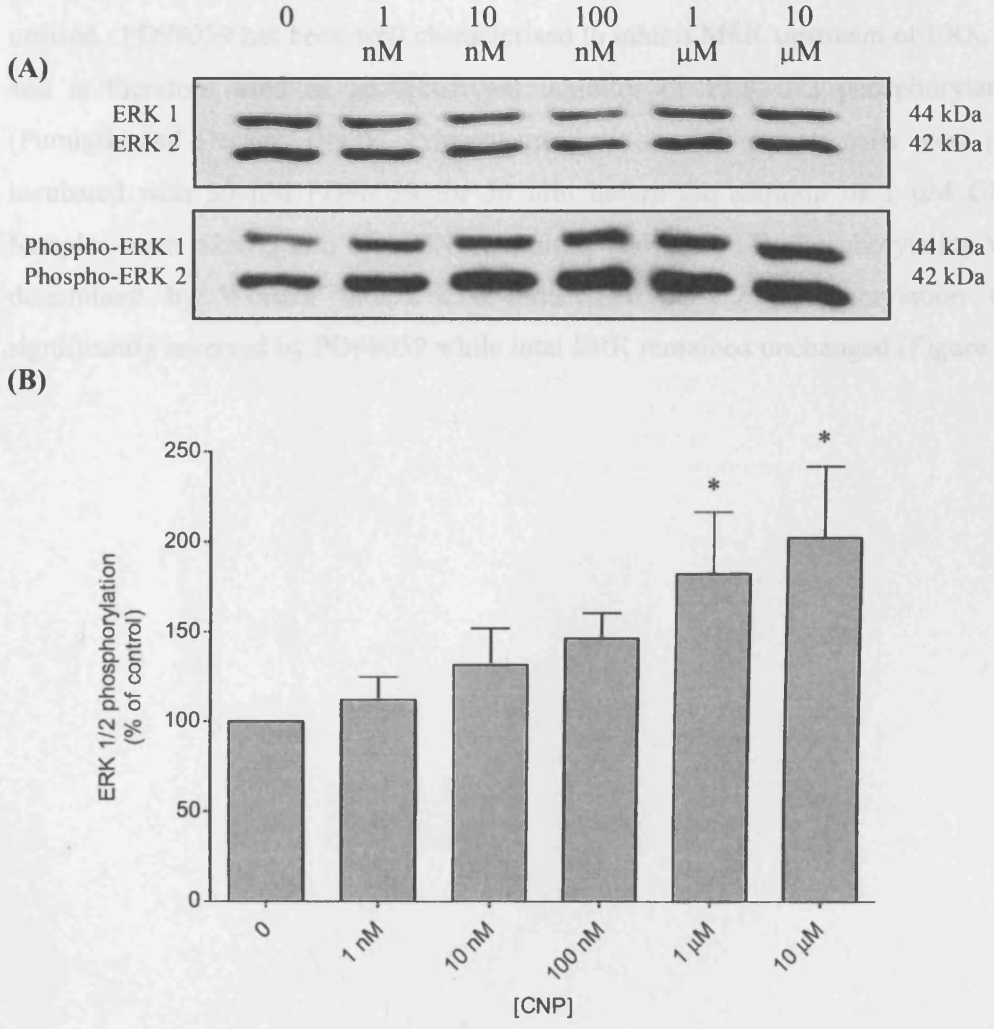


Figure 44 – Expression and phosphorylation of ERK 1/2 (p44/p42) protein in 1° RAoSMC in the presence of increasing concentrations of CNP (1 nM – 10 μM) for 5 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (without CNP) **(B)**. Data are represented as the mean ± SEM, n = 8; *, $p<0.05$ versus control.

5.5. Effect of PD98059 on CNP-induced ERK 1/2 phosphorylation in primary rat aortic smooth muscle cells

To confirm that activation of ERK 1/2 signalling is due to the actions of CNP, PD98059, a selective MAPK/ERK kinase (MEK), ERK 1/2 pathway inhibitor was utilised. PD98059 has been well characterised to inhibit MEK upstream of ERK 1/2 and is therefore used as an archetypal inhibitor of ERK 1/2 phosphorylation (Pumiglia and Decker, 1997). Primary rat aortic smooth muscle cells were pre-incubated with 30 μM PD98059 for 30 min before the addition of 1 μM CNP. Samples were taken 5 min after CNP treatment and ERK 1/2 phosphorylation was determined by Western blot. CNP-induced ERK 1/2 phosphorylation was significantly reversed by PD98059 while total ERK remained unchanged (Figure 45).

EFFECT OF PD98059 ON CNP-INDUCED ERK 1/2 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

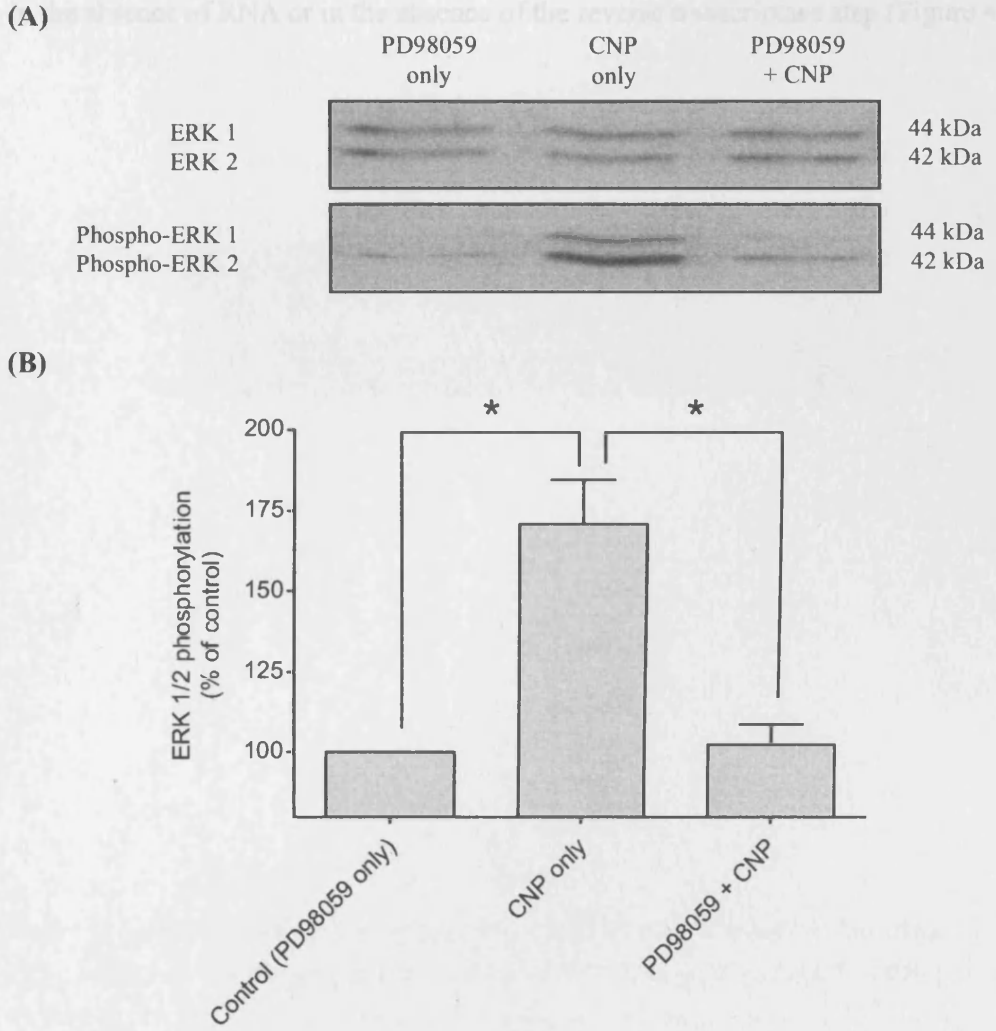


Figure 45 – Expression and phosphorylation of ERK 1/2 (p44/p42) protein in 1° RAoSMC in the presence of PD98059 (30 μM; 30 min prior to CNP) and CNP (1 μM) for 5 min. Protein expression and phosphorylation determined by Western blot (A) and quantified by densitometry normalised to control (PD98059 only) (B). Data are represented as the mean ± SEM, n = 6; *, $p<0.05$ versus control.

5.6. NPR-A, NPR-B and NPR-C mRNA is present in primary rat aortic smooth muscle cells

In an attempt to determine which receptor is responsible for CNP signalling in the regulation of the MAPK pathway, RT-PCR was carried out on 1° RAoSMC. mRNA for all three natriuretic peptide receptors was detected and no amplification occurred in the absence of RNA or in the absence of the reverse transcriptase step (Figure 46).

mRNA FOR NPR-A, NPR-B AND NPR-C IS PRESENT IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

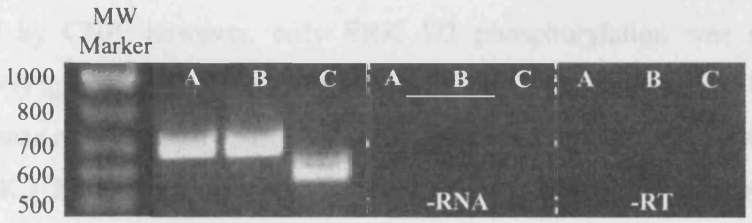


Figure 46 – mRNA expression for NPR-A, NPR-B and NPR-C in 1° RAoSMC determined by RT-PCR. Products of expected size were obtained for all NPRs (714 bp NPR-A, 720 bp NPR-B and 554 bp NPR-C). RNA or the RT steps were omitted for control reactions.

5.7. Effect of the NPR-C antagonist, M372049 on CNP-induced MAPK phosphorylation (ERK 1/2 and p38) in primary rat aortic smooth muscle cells

In an attempt to assign a role to NPR-C in MAPK phosphorylation induced by CNP, 1° RAoSMC were treated with 1 μM or 10 μM NPR-C antagonist M372049 for 30 min before the addition of CNP. Samples were taken 10 min after CNP (1 μM) addition and ERK 1/2 and p38 were determined by Western blot. M372049 alone did not alter the background phosphorylation of ERK 1/2 and p38 (Figure 48 and Figure 50 respectively). ERK 1/2 and p38 phosphorylation were significantly increased by CNP; however, only ERK 1/2 phosphorylation was attenuated by M372049 (Figure 47/Figure 48). The increase in p38 phosphorylation brought about by CNP was not altered by M372049 at either concentration (Figure 49/Figure 50). Total ERK 1/2 and p38 levels were unaltered in all experimental treatments.

EFFECT OF THE NPR-C ANTAGONIST, M372049 (1 μM AND 10 μM) ON CNP-INDUCED ERK 1/2 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

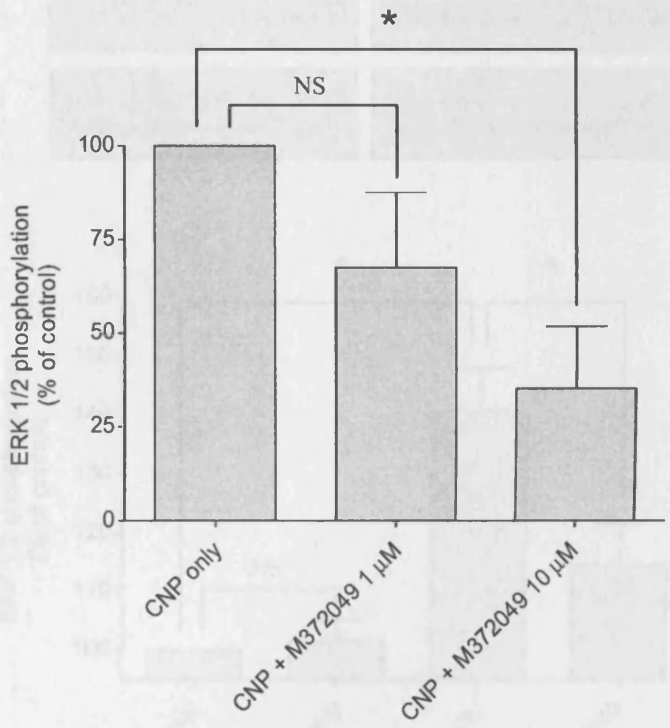
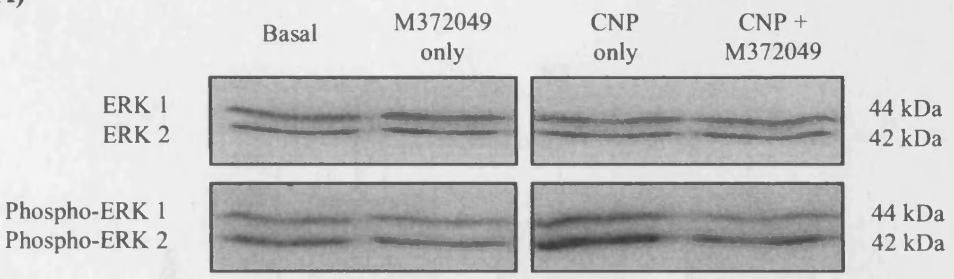


Figure 47 – Phosphorylation of ERK 1/2 (p44/p42) protein in 1° RAoSMC in the presence of M372049 (1 μM or 10 μM) and CNP (1 μM) for 10 min. Phosphorylation determined by Western blot and quantified by densitometry normalised to control (CNP only). Data are represented as the mean ± SEM, n = 5; *, $p<0.05$ versus control.

EFFECT OF THE NPR-C ANTAGONIST, M372049 (10 μM) ON CNP-INDUCED ERK 1/2 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

(A)



(B)

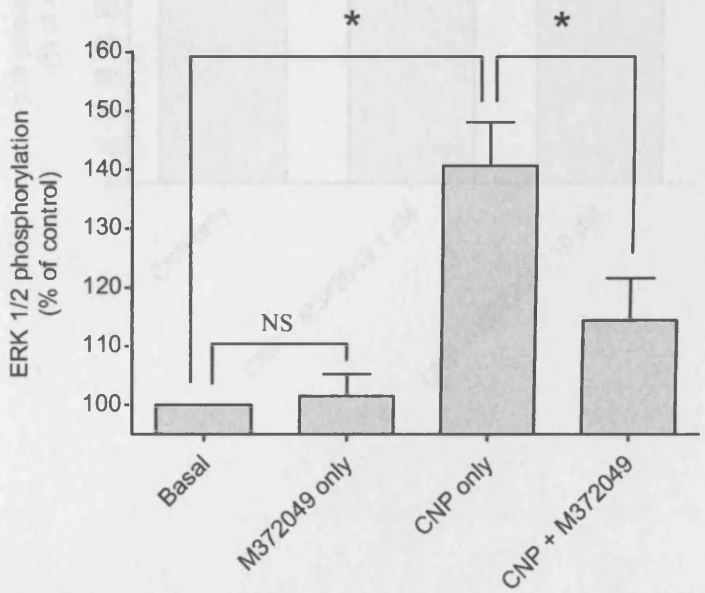


Figure 48 – Expression and phosphorylation of ERK 1/2 (p44/p42) protein in 1° RAoSMC in the presence of M372049 (10 μM) and CNP (1 μM) for 10 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (basal) **(B)**. Data are represented as the mean ± SEM, n = 5; *, $p<0.05$ versus control.

EFFECT OF THE NPR-C ANTAGONIST, M372049 (1 μM AND 10 μM) ON CNP-INDUCED p38 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

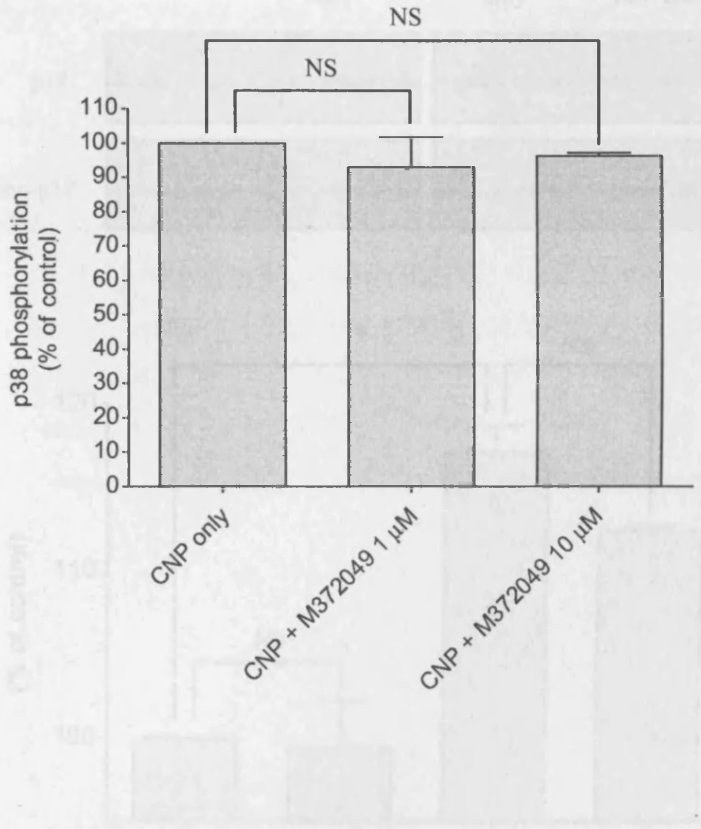


Figure 49 –Phosphorylation of p38 protein in 1° RAoSMC in the presence of M372049 (1 μM or 10 μM) and CNP (1 μM) for 10 min. Phosphorylation determined by Western blot and quantified by densitometry normalised to control (CNP only). Data are represented as the mean ± SEM, n = 4.

EFFECT OF THE NPR-C ANTAGONIST, M372049 (10 μM) ON CNP-INDUCED p38 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

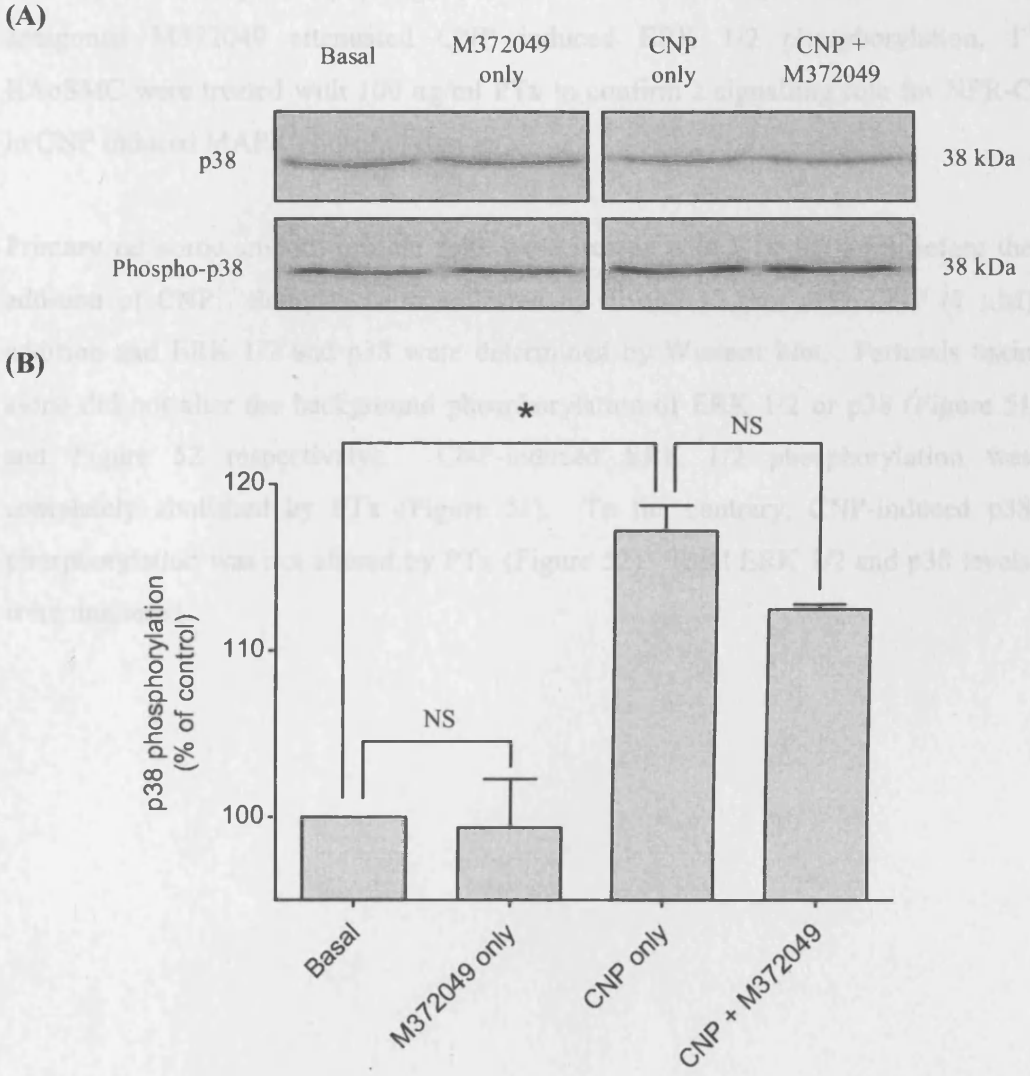


Figure 50 – Expression and phosphorylation of p38 protein in 1° RAoSMC in the presence of M372049 (10 μM) and CNP (1 μM) for 10 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (basal) **(B)**. Data are represented as the mean ± SEM, n = 3; *, $p<0.05$ versus control.

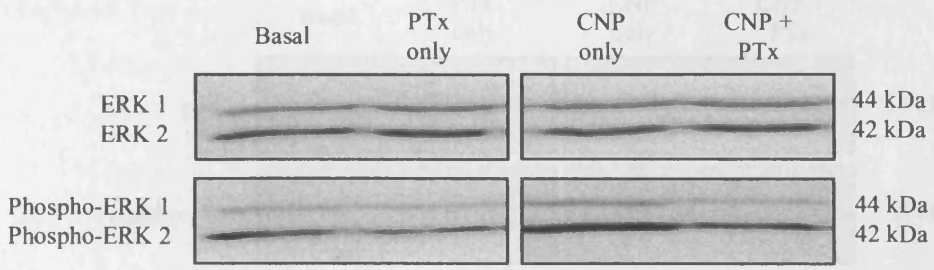
5.8. Effect of G_i-protein inhibitor, Pertussis toxin on CNP-induced MAPK phosphorylation (ERK 1/2 and p38) in primary rat aortic smooth muscle cells

NPR-C has previously been characterised to bring about smooth muscle relaxation via G_i-protein coupled signalling (Chauhan *et al.*, 2003b). Since the NPR-C antagonist M372049 attenuated CNP induced ERK 1/2 phosphorylation, 1^o RAoSMC were treated with 100 ng/ml PTx to confirm a signalling role for NPR-C in CNP induced MAPK phosphorylation.

Primary rat aortic smooth muscle cells were treated with PTx for 16 h before the addition of CNP. Samples were collected 5, 10 and 15 min after CNP (1 μM) addition and ERK 1/2 and p38 were determined by Western blot. Pertussis toxin alone did not alter the background phosphorylation of ERK 1/2 or p38 (Figure 51 and Figure 52 respectively). CNP-induced ERK 1/2 phosphorylation was completely abolished by PTx (Figure 51). To the contrary, CNP-induced p38 phosphorylation was not altered by PTx (Figure 52). Total ERK 1/2 and p38 levels were unaltered.

EFFECT OF PERTUSSIS TOXIN ON CNP-INDUCED ERK 1/2 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

(A)



(B)

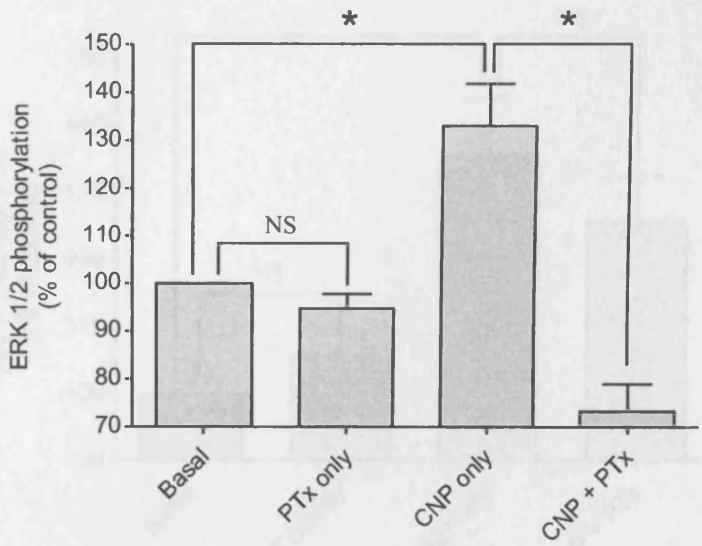
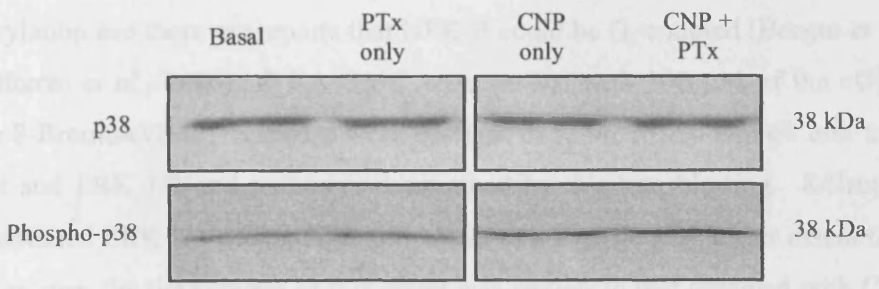


Figure 51 – Expression and phosphorylation of ERK 1/2 (p44/p42) protein in 1° RAoSMC in the presence of PTx (100 ng/ml) and CNP (1 μM) for 10 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (basal) **(B)**. Data are represented as the mean ± SEM, n = 8; *, $p<0.05$ versus control.

EFFECT OF PERTUSSIS TOXIN ON CNP-INDUCED p38 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

(A)



(B)

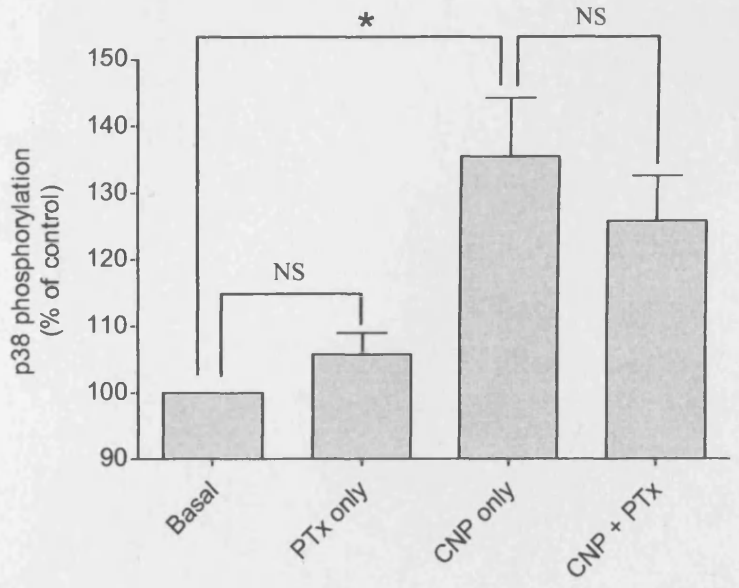


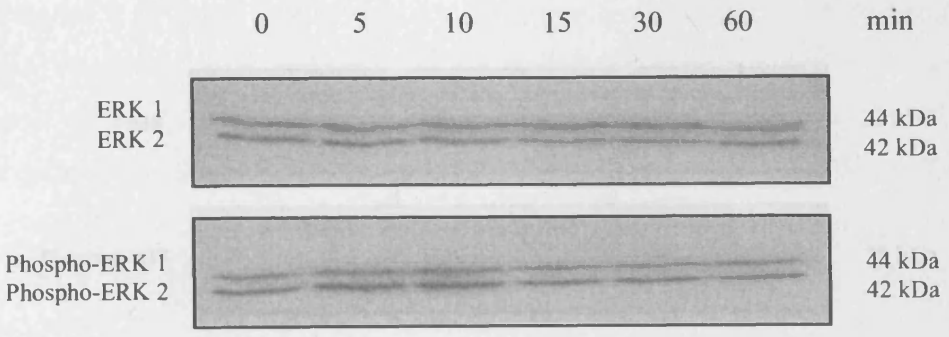
Figure 52 – Expression and phosphorylation of p38 protein in 1° RAoSMC in the presence of PTx (100 ng/ml) and CNP (1 μM) for 10 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (basal) **(B)**. Data are represented as the mean ± SEM, n = 7; *, $p<0.05$ versus control.

5.9. Effect of 8-Bromo-cGMP on MAPK phosphorylation (ERK 1/2 and p38) in primary rat aortic smooth muscle cells

CNP has been shown to signal through both NPR-B in a cGMP-dependent manner (Tremblay *et al.*, 2002) and NPR-C in a cGMP-independent fashion (Hutchinson *et al.*, 1997). Since M372049 did not entirely block CNP-induced ERK 1/2 phosphorylation and there are reports that NPR-B could be G_i-coupled (Borges *et al.*, 2001; Alfonzo *et al.*, 2006), 1° RAoSMC were treated with 300 μM of the cGMP analogue 8-Bromo-cGMP. Samples were taken at 0, 5, 10, 15, 30 and 60 min after treatment and ERK 1/2 and p38 were determined by Western blotting. 8-Bromo-cGMP increased ERK 1/2 phosphorylation, albeit to a significantly lesser extent than CNP. However, the time course of this effect was similar to that obtained with CNP (Figure 53). p38 phosphorylation was not altered by 8-Bromo-cGMP (Figure 54). Total ERK 1/2 and p38 remained unchanged.

EFFECT OF 8-BROMO-cGMP ON ERK 1/2 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

(A)



(B)

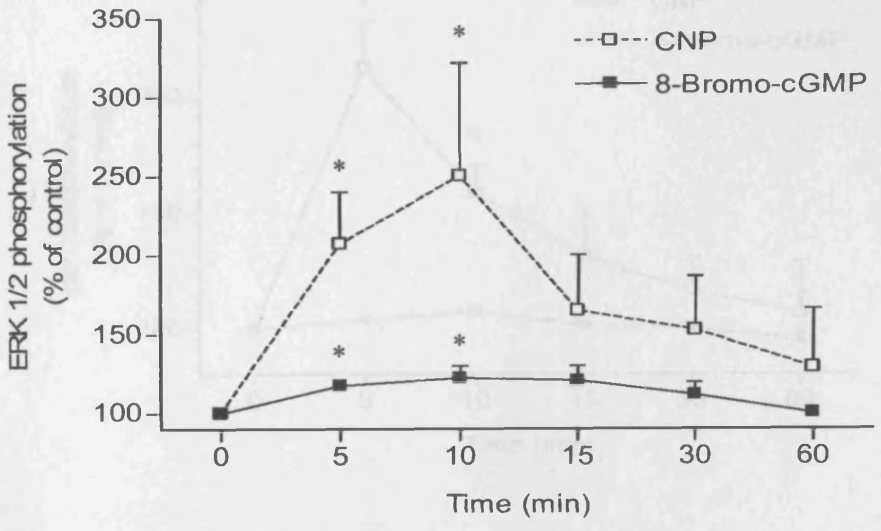
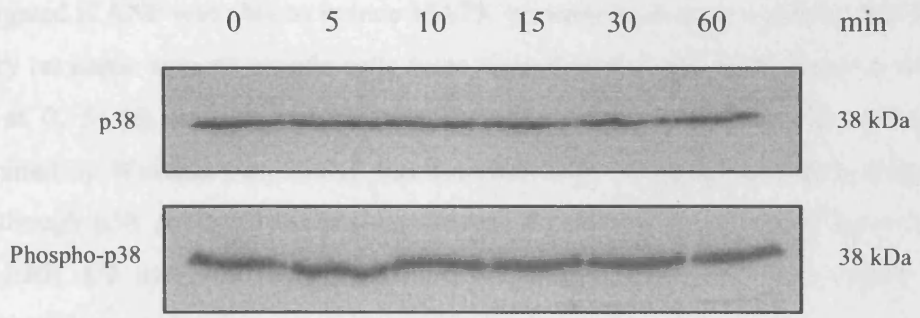


Figure 53 – Expression and phosphorylation of ERK 1/2 (p44/p42) protein in 1° RAoSMC treated with 8-Bromo-cGMP (300 μM) for 0 – 60 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (0 min) **(B)**. Data are represented as the mean ± SEM, n =3; *, $p<0.05$ versus control.

EFFECT OF 8-BROMO-cGMP ON p38 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

(A)



(B)

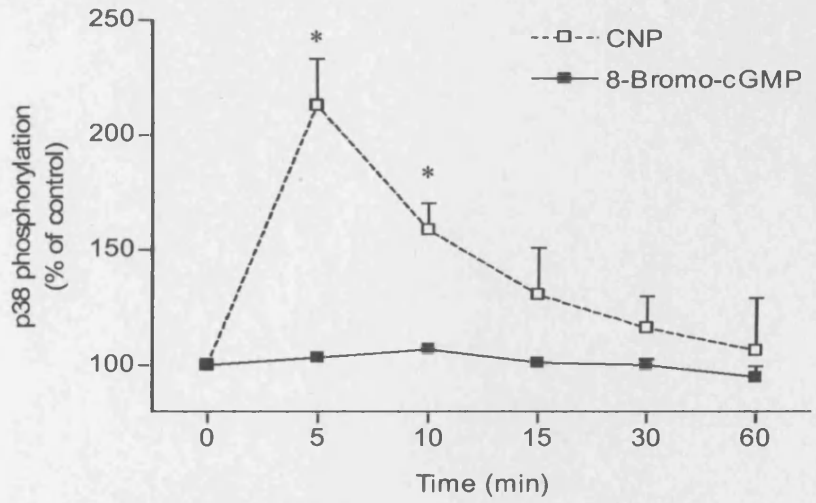


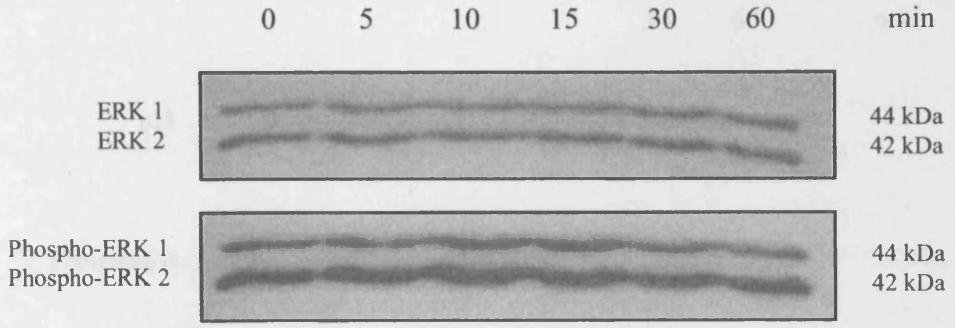
Figure 54 – Expression and phosphorylation of p38 protein in 1° RAoSMC treated with 8-Bromo-cGMP (300 μM) for 0 – 60 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (0 min) **(B)**. Data are represented as the mean ± SEM, n =3.

5.10. Effect of ANP on MAPK phosphorylation (ERK 1/2 and p38) in primary rat aortic smooth muscle cells

Since I had established that CNP elicited a time-dependent phosphorylation of ERK 1/2 and p38, and given that ANP also binds NPR-C (Koller and Goeddel, 1992), I investigated if ANP was able to induce MAPK phosphorylation in a similar fashion. Primary rat aortic smooth muscle cells were treated with 1 μM ANP, samples were taken at 0, 5, 10, 15, 30 and 60 min and ERK 1/2 and p38 phosphorylation determined by Western blot. ANP did not alter ERK 1/2 phosphorylation (Figure 55) although p38 phosphorylation was increased by ANP at 10 min (Figure 56). Total ERK 1/2 and p38 levels remained unchanged (Figure 55 and Figure 56 respectively).

EFFECT OF ANP ON ERK 1/2 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

(A)



(B)

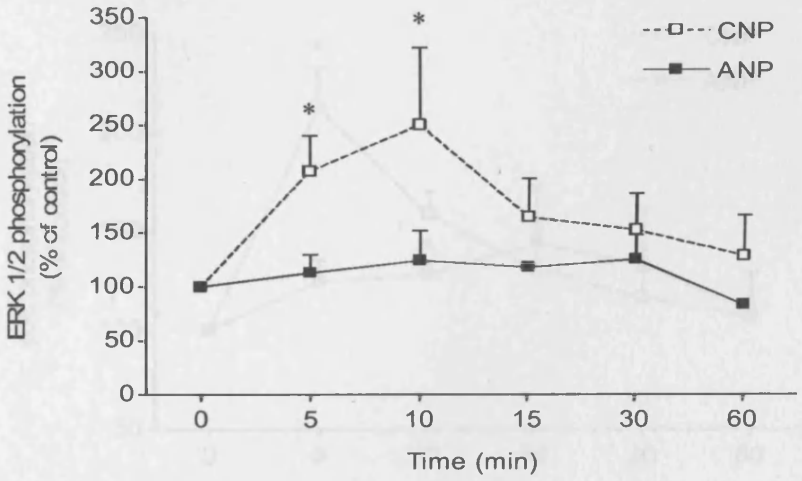
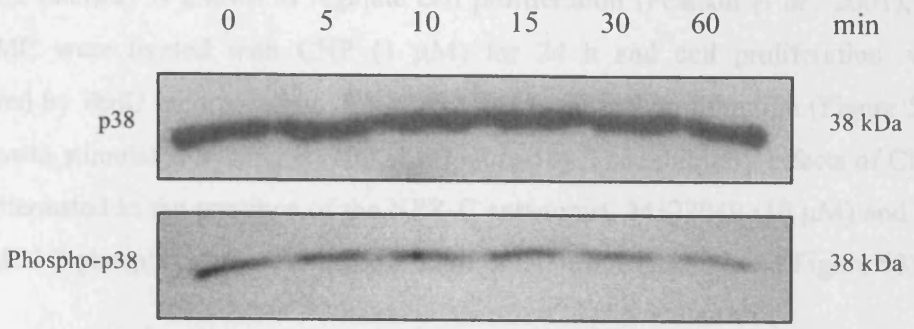


Figure 55 – Expression and phosphorylation of ERK 1/2 (p44/p42) protein in 1° RAoSMC treated with ANP (1 μM) for 0 – 60 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (0 min) **(B)**. Data are represented as the mean ± SEM, n =3.

EFFECT OF ANP ON p38 PHOSPHORYLATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

(A)



(B)

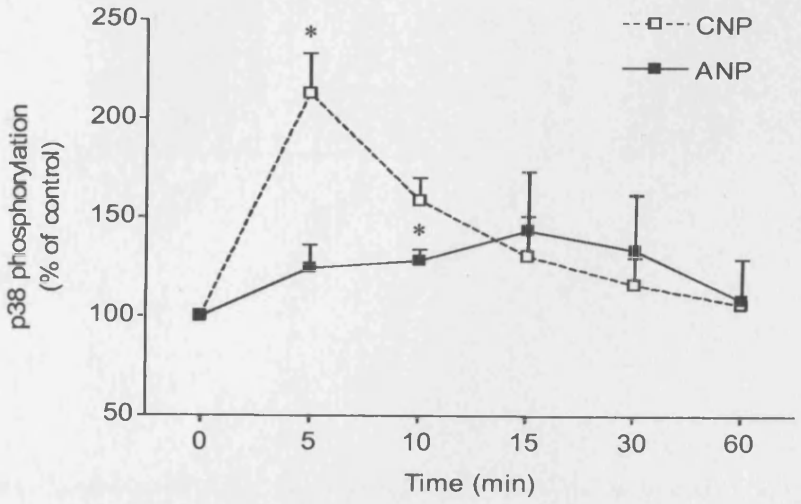


Figure 56 – Expression and phosphorylation of p38 protein in 1° RAoSMC treated with ANP (1 μM) for 0 – 60 min. Protein expression and phosphorylation determined by Western blot **(A)** and quantified by densitometry normalised to control (0 min) **(B)**. Data are represented as the mean ± SEM, n =3; *, $p<0.05$ versus control.

5.11. Effect of CNP on cell proliferation in primary rat aortic smooth muscle cells

In an attempt to establish a downstream target for the actions of CNP, and since the ERK 1/2 pathway is known to regulate cell proliferation (Pearson *et al.*, 2001), 1° RAoSMC were treated with CNP (1 μM) for 24 h and cell proliferation was measured by BrdU incorporation. CNP inhibited basal cell proliferation (Figure 57) and growth stimulated by Ang II (100 nM; Figure 58) The inhibitory effects of CNP were attenuated in the presence of the NPR-C antagonist, M372049 (10 μM) and by the ERK 1/2 phosphorylation inhibitor PD98059 (30 μM; Figure 57 and Figure 58)

EFFECT OF CNP ON BASAL CELL PROLIFERATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

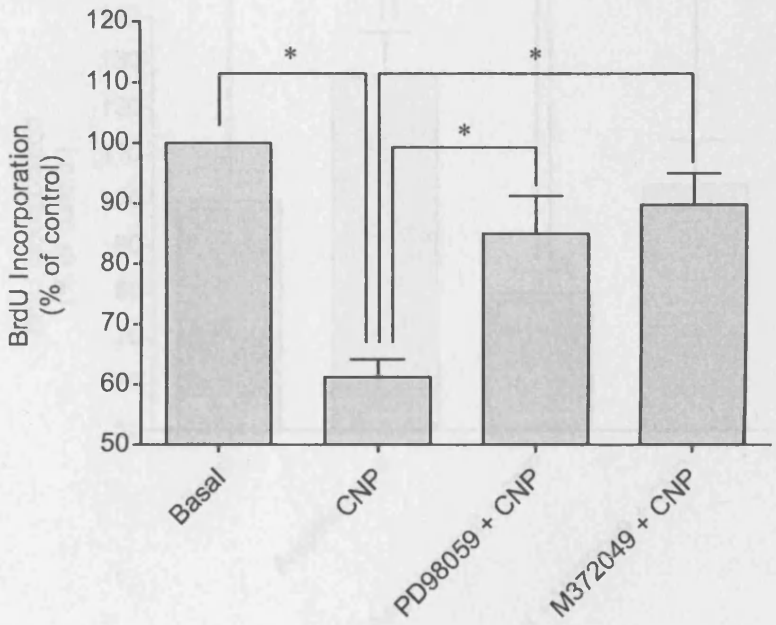


Figure 57 – BrdU incorporation in 1° RAoSMC under basal conditions in the absence and presence of CNP (10 μM), with or without pre-treatment with PD98059 (30 μM) or M372049 (10 μM). Data represented as the mean ± SEM absorbance, expressed as a percentage of basal cell growth, $n \geq 6$; *, $p<0.05$ versus control.

EFFECT OF CNP ON ANGIOTENSIN II-STIMULATED CELL PROLIFERATION IN PRIMARY RAT AORTIC SMOOTH MUSCLE CELLS

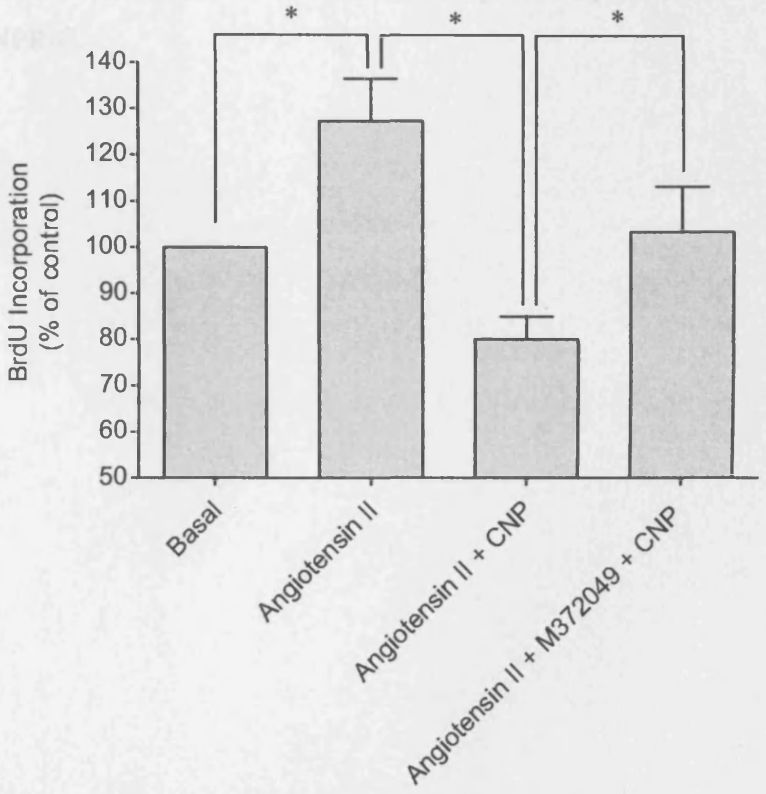


Figure 58 – BrdU incorporation in 1° RAoSMC following growth stimulation with Ang II (100 nM) in the absence and presence of CNP (10 μM), with or without pre-treatment with PD98059 (30 μM) or M372049 (10 μM). Data represented as the mean ± SEM absorbance, expressed as a percentage of basal cell growth, $n \geq 6$; *, $p<0.05$ versus control.

5.12. Summary of key results

In this chapter I have shown that CNP causes a strong ERK 1/2 and p38 phosphorylation; however, ANP was essentially unable to cause ERK 1/2 or p38 phosphorylation. CNP-induced ERK 1/2 phosphorylation occurs predominantly via G_i-coupled NPR-C since phosphorylation was blocked by the G_i-protein inhibitor PT_x and the NPR-C selective inhibitor M372049, and this is linked to growth inhibition in 1^o RAoSMC. CNP-induced p38 phosphorylation does not appear to involve NPR-C.

CHAPTER 6
DISCUSSION AND CONCLUSIONS

6. Discussion and Conclusions

6.1. Background and aim of current study

The involvement of both NO and natriuretic peptides in the control of vascular homeostasis has been described extensively (Moncada and Higgs, 1993; Levin *et al.*, 1998; Baxter, 2004; Ahluwalia *et al.*, 2004). The role of NO in the immune system has also been well-characterised. NO is produced in response to inflammatory stimuli including cytokines and LPS in numerous cell types and acts in both cGMP-dependent and independent manners to exert cytotoxic and cytostatic effects (Nathan and Hibbs, 1991; Nathan and Xie, 1994). This 'high output' NO production is generated predominantly by the inducible isoform of NOS. Unfortunately, excessive NO production during inflammatory episodes results in NO-mediated host damage. For example, septic shock occurs when there is extensive release of NO in an attempt to combat a systemic infection. These high NO levels cause dysfunction within the body, exemplified by a dramatic decrease in blood pressure followed by failure of vital organs often resulting in death (Titheradge, 1999). NO has therefore been termed a vital poison, as a balanced amount of NO production is essential for combating infections but mis-regulation leading to either excessive or deficient levels is deleterious.

Natriuretic peptides parallel the effects of NO in the vasculature via activation of the particulate isoform of guanylate cyclase; however, the role(s) of natriuretic peptides in the immune system remain unclear. Studies evaluating the effect of ANP on pro-inflammatory protein expression (e.g. iNOS), do not provide a consensus as to whether these peptides act in a pro- or anti-inflammatory manner (Vollmar and Schulz, 1995b; Kiemer and Vollmar, 1997; Kiemer *et al.*, 2002a; Vollmar, 2005). Thus, I hypothesised that natriuretic peptides (specifically ANP and CNP) affect pro-inflammatory protein expression (e.g. iNOS) via activation of the pGC isoform. The investigations described in this thesis were designed to test this hypothesis and to elucidate the mechanisms by which natriuretic peptides can regulate cell proliferation, with particular emphasis on the MAPK pathways.

6.2. Summary of key results

In NPR-A KO mice, iNOS expression and NO production in response to systemic administration of LPS are significantly reduced compared to WT controls; this is mirrored in the *ex vivo* functional reactivity of vessels from these animals in which aortae from WT mice display greater vascular dysfunction. Such observations intimate that ANP (and/or BNP – since it too signals via NPR-A) act in a pro-inflammatory manner, *in vivo*, contradicting previous findings (Vollmar and Schulz, 1995b; Kiemer and Vollmar, 1997; Vollmar, 2005). Since a reduction in iNOS was observed in NPR-A KO animals, the effects of ANP and CNP on iNOS expression and activity *in vitro*, in cell based systems, were investigated. Despite previous reports suggesting that ANP inhibits iNOS expression (Vollmar and Schulz, 1995b), neither ANP nor CNP were able to alter iNOS expression in murine macrophages or RAoSMC. However, CNP did increase MAPK phosphorylation (specifically ERK 1/2 and p38) in RAoSMC, pathways that have previously been implicated in the regulation of iNOS (Chen and Wang, 1999; Monier *et al.*, 2002; Kim *et al.*, 2004); indeed, I demonstrate that iNOS expression is dependent on p38 activation in 1° RAoSMC.

The MAPK pathway has also been implicated in growth regulation (Chen and Wang, 1999; Pearson *et al.*, 2001; Monier *et al.*, 2002), and the anti-proliferative properties of CNP have been well-characterised, especially in the vasculature (Furuya *et al.*, 1991; Furuya *et al.*, 1993; Cahill and Hassid, 1994; Hutchinson *et al.*, 1997). Although the anti-proliferative properties of CNP have been previously investigated, the receptor and signalling pathways involved in mediating this effect are not clearly defined. In an attempt to establish the transduction pathway(s) and natriuretic peptide receptor responsible for the anti-mitogenic actions of CNP, I employed 1° RAoSMC and a selective NPR-C antagonist, M372049. CNP caused inhibition of RAoSMC growth that was blocked by M372049 and the ERK 1/2 pathway inhibitor PD98059. CNP elicited a transient increase in ERK 1/2 phosphorylation, which was also blocked by PD98059, PTx and M372049. Interestingly however, ANP was unable to reproduce the ERK 1/2 phosphorylation induced by CNP, despite the ability of 8-Bromo-cGMP to elicit ERK 1/2 phosphorylation. These observations

imply that CNP is acting predominantly through NPR-C in a cGMP-independent manner to bring about its anti-proliferative actions via activation of ERK 1/2 phosphorylation.

6.3. Effect of ANP on iNOS expression and vascular reactivity *in vivo*

LPS has long been recognised as one of the prime inducers of septic shock (Thiemermann, 1997) and the associated iNOS expression and elevated NO levels (Liu *et al.*, 1993). This overproduction of NO contributes to the cardinal symptoms of septic shock, hypotension, reduced circulatory resistance, cardiodepression and vascular hyporeactivity, all of which result in multiple organ failure (Landry and Oliver, 2001). ANP, a key cardiovascular homeostatic hormone has also been implicated in inflammation and has been shown to inhibit iNOS expression in a number of cell types *in vitro*, suggesting an anti-inflammatory role (Vollmar and Schulz, 1995a; Pandey *et al.*, 2000; Tsukagoshi *et al.*, 2001; Kiemer *et al.*, 2003). Moreover, there is a considerable body of evidence intimating an important role for natriuretic peptides in the pathogenesis of sepsis *in vivo*, in both animals models and humans (Hinder *et al.*, 1997; Witthaut *et al.*, 2003; Stubbe *et al.*, 2004; Miyoshi *et al.*, 2006; Shor *et al.*, 2006; Vellaichamy *et al.*, 2007). For instance, Miyoshi *et al* reported that i.v. administration of LPS in rats causes an increase in IL-1β and fever, which is enhanced by administration of the NPR-A/B antagonist HS-142-1, suggesting that ANP acting outside the blood-brain barrier, inhibits IL-1β leading to an inhibition of second phase fever (Miyoshi *et al.*, 2006). Furthermore, in an experimental model of ovine sepsis, blockade of NPR-A/B by HS-142-1 increases cardiac filling pressures, maintains mean arterial pressure and increases pulmonary transvascular fluid flux independently of changes in permeability to protein, suggesting that ANP may play a protective role during sepsis (Hinder *et al.*, 1997; Stubbe *et al.*, 2004). A single *in vivo* study conducted by Vellaichamy *et al* in NPR-A KO mice reported that these mice have elevated basal levels of the pro-inflammatory cytokines TNF-α, IL-6 and TGF-β1, and exhibit greater NF-κB binding activity as compared to WT animals, suggesting that ablation of NPR-A/cGMP signalling is associated with a pro-inflammatory phenotype (Vellaichamy *et al.*, 2007).

LPS-induced endotoxaemia in rats causes a rise in plasma ANP and a reduction in plasma volume (Aiura *et al.*, 1995). The early rise of plasma ANP after LPS injection may be due to local release of ANP by macrophages of the spleen and regulated by PAF and not from a cardiac source (Qu *et al.*, 1998). Furthermore, the increase in plasma ANP following LPS is dampened by iNOS-derived NO. In the presence of the iNOS inhibitor, aminoguanidine, ANP plasma levels are significantly higher following LPS administration, suggesting that the NO pathway may activate an inhibitory control mechanism that attenuates ANP secretion during sepsis (Stabile *et al.*, 2007). An increase in cGMP levels in human sepsis has been reported, which is associated with a decline in peripheral resistance. The raised cGMP levels however cannot be attributed to sGC or pGC exclusively, suggesting that both natriuretic peptides and NO play a role (Schneider *et al.*, 1993).

Elevated plasma natriuretic peptide levels have also been demonstrated in the clinical arena. Plasma ANP and BNP levels in patients with septic shock are significantly higher than in control subjects (ANP: 82.7 ± 9.9 pg/ml septic vs 14.9 ± 1.2 pg/ml control; BNP: 12.4 ± 3.6 pg/ml septic vs 5.5 ± 0.7 pg/ml control; Witthaut *et al.*, 2003). The increased ANP levels are tightly correlated to increases in IL-6 suggesting that excessively increased pro-inflammatory cytokines contribute to the modulation of ANP levels in septic patients (Witthaut *et al.*, 2003). Natriuretic peptides, particularly ANP and BNP, are also known to be elevated in cardiovascular diseases such as heart failure, which is a common complication in sepsis (Potter *et al.*, 2006). Furthermore, it has been suggested that ANP, endothelin-1 and cGMP could be markers of severe and fatal myocardial depression early in the course of human septic shock (Hartemink *et al.*, 2001). Moreover, pro-ANP and N-terminal proANP have been described as useful markers of cardiac depression (Mazul-Sunko *et al.*, 2001) and myocardial dysfunction (Hoffmann *et al.*, 2005) caused by sepsis respectively. It is worth noting however, that it is hard to distinguish in these studies whether the elevated ANP levels are due to the sepsis or due to the associated cardiac condition.

Akin to ANP, BNP is elevated in sepsis (Witthaut *et al.*, 2003) and several studies have addressed the question whether BNP and proBNP are of prognostic value in patients with sepsis especially in terms of adverse cardiac events (Witthaut *et al.*,

2003; Brueckmann *et al.*, 2005; Ueda *et al.*, 2006). Some studies have shown a correlation between BNP or NT-proBNP and cardiac functional measures such as cardiac index (Witthaut *et al.*, 2003; Charpentier *et al.*, 2004; Brueckmann *et al.*, 2005), while others observed no correlation (Tung *et al.*, 2004; Bal *et al.*, 2006; Rudiger *et al.*, 2006). BNP has also been suggested as a potential marker for mortality in septic shock (Castillo *et al.*, 2004). Recently, a study by Maeder *et al* has demonstrated elevated BNP levels in patients with sepsis and normal left ventricular function (Maeder *et al.*, 2005). This is further supported by Shor *et al* who showed elevated BNP levels in septic patients who were not in a clinical state of congestive heart failure and had no echocardiographic evidence of systolic dysfunction (Shor *et al.*, 2006), suggesting that BNP is elevated in sepsis, without any indirect effects on the heart. Thus, there is good evidence that both ANP and BNP are elevated as a consequence of sepsis and may contribute to or help prevent pathogenesis of the disease.

Sepsis is characterised by vascular smooth muscle dysfunction resulting in impaired contractile function, hypotension, inadequate tissue perfusion and organ failure. This vascular dysfunction has been attributed to raised iNOS expression and activity, and the resulting increase in NO-mediated dilatation (Julou-Schaeffer *et al.*, 1990; Fleming *et al.*, 1991). Inhibition of iNOS activity in animal models of sepsis as well as humans reverses hypotension (Petros *et al.*, 1994). Endothelial dysfunction has also been shown to occur in sepsis and other inflammatory cardiovascular disease (Mombouli and Vanhoutte, 1999; Hingorani *et al.*, 2000). Utilisation of iNOS KO mice treated with LPS to induce sepsis demonstrated that the absence of iNOS protects against LPS-induced contractile hyporeactivity (Boyle, III *et al.*, 2000; Hollenberg *et al.*, 2000) as well as endothelial dysfunction (Chauhan *et al.*, 2003a). In a rat model of septic shock antisense oligonucleotides to iNOS mRNA inhibit iNOS activity and expression and prevent vascular hyporeactivity to norepinephrine (Hoque *et al.*, 1998). These studies indicate that iNOS induction plays an integral role in mediating endothelial dysfunction associated with sepsis.

Nitric oxide is known to control smooth muscle relaxation via NO-sensitive guanylate cyclase signalling and it has been suggested that the vascular hyporeactivity observed in sepsis could be due to guanylate cyclase desensitisation

(Hussain *et al.*, 2001; Madhani *et al.*, 2003; Friebe and Koesling, 2003; Madhani *et al.*, 2006; Mullershausen *et al.*, 2006). Short term exposure of rat thoracic aortae to NO causes desensitisation of NO-induced relaxation as well as heterologous desensitisation of ANP-induced relaxation suggesting a common underlying mechanism of negative feedback for regulation of NO/cGMP signal transduction, perhaps involving PDE-5 (Mullershausen *et al.*, 2006). Madhani *et al* have demonstrated that NPR-A/NPR-B-linked pGC pathways are modulated by NO/sGC/cGMP in mouse aorta and there is cross-desensitisation between NPR subtypes (Madhani *et al.*, 2003). This has also been shown in human vessels where the relaxant potency of SPER-NO and ANP is increased following inhibition of endogenously produced NO and decreased with prior exposure to GTN (Hussain *et al.*, 2001). Inhibition of endogenous generation of NO by L-NMA in healthy volunteers significantly enhances further the ANP-dependent forearm blood flow (Madhani *et al.*, 2006). Furthermore, aortae from eNOS KO mice are more sensitive to ANP compared to WT animals and responses to ANP in WT mice are increased in the presence of NOS or sGC inhibitors (Hussain *et al.*, 2001). *In vivo* administration of sodium nitroprusside and ANP to eNOS KO and NPR-A KO mice causes a significant enhancement in mean arterial blood pressure reduction (Madhani *et al.*, 2006), suggesting that loss of endothelium-derived NO results in upregulation of the sensitivity of both sGC and pGC *in vivo*, strengthening the evidence for NO/sGC and ANP/pGC cooperative interactions and intimating that at least in part, ANP-mediated relaxations are dependent on endothelial NO release. Conversely, mice with selective vascular smooth muscle NPR-A deletion have normal arterial blood pressure and increased vasorelaxing sensitivity to sodium nitroprusside and ACh compared to control animals showing that endothelial NO contributes to compensate for the missing vasodilating effect of ANP (Sabrane *et al.*, 2003). Taken together, these studies hint that NO/sGC and ANP/pGC crosstalk may be an important physiological homeostatic mechanism, which links the paracrine activity of NO and CNP with the endocrine functions of ANP and BNP in regulating vascular tone and blood pressure.

In the present study, I used a genetic approach (i.e. NPR-A KO mice) to explore the role of ANP/BNP in sepsis and to test the hypothesis that ANP acts in an anti-inflammatory manner by inhibiting iNOS expression and activity *in vivo*, as

previously reported *in vitro* (Vollmar and Schulz, 1995b; Vollmar, 2005). The study herein has demonstrated that treatment of NPR-A KO mice with LPS for 16 h results in inhibition of iNOS expression in lung and aortic tissue, reduced plasma NO_x and vascular dysfunction (in terms of both constrictor and dilator pathways), in comparison to WT animals. Relaxation responses to ACh and SPER-NO were impaired in aortae from WT mice consistent with high output NO production by iNOS and subsequent endothelial and smooth muscle hyporeactivity. However, this dysfunction was abrogated in NPR-A KO animals. It is worth noting that there is a difference in basal WT and NPR-A KO contractile and relaxant responses and hence differences in basal endothelial function. Therefore, it is important to compare fold shift in potency since the starting contraction/relaxation curves are not superimposable. NPR-A KO animals are less responsive to the vasoconstrictor U46619 and show more potent vasorelaxant responses. This could be explained by the crosstalk between pGC and sGC as discussed above; elimination of NPR-A and hence pGC-generated cGMP could result in a compensatory upregulation of (endothelial (eNOS) derived) NO/sGC signalling, hence dampening the contractile responses while enhancing NO-dependent vasorelaxation (ACh). In the presence of the NO donor SPER-NO however, NPR-A KO aortae do not respond more potently in comparison to WT and this could be due to the upregulation of basal EDRF release in NPR-A KO, which might offset any shift in the SPER-NO curve. In the presence of LPS however, there is less "high output" NO from iNOS in NPR-A KO animals resulting in reduced endothelial dysfunction and hence normalised responses to both contractile and relaxing agents when compared to saline treated NPR-A KO littermates. Taken together, this study suggests for the first time a pro-inflammatory role for ANP/BNP through NPR-A/pGC signalling in sepsis. It might be expected that CNP, acting through NPR-B could have a similar pro-inflammatory role; however, it is worth remembering that this natriuretic peptide is expressed and released locally (i.e. in a paracrine fashion) and is known to signal through both NPR-B (cGMP-dependent) and NPR-C (cGMP-independent). Furthermore, it could be speculated that NPR-A KO mice, akin to iNOS KO animals (MacLean *et al.*, 1998; McInnes *et al.*, 1998), might be more susceptible to infection since ANP/BNP appear to act in a pro-inflammatory manner to increase iNOS expression and help expedite host defence.

A pro-inflammatory role for ANP/BNP would fit with previous studies demonstrating a key pro-inflammatory role for eNOS-derived NO, that is cGMP-dependent (at least in part). Thus, since ANP/BNP act via NPR-A to increase cGMP, a similar effect in sepsis would be expected. Endothelial NOS expression and activity has been shown to modulate iNOS expression (Connelly *et al.*, 2003; Connelly *et al.*, 2005) and a decrease in eNOS has been suggested to reduce the vascular dysfunction associated with sepsis (Vo *et al.*, 2005). It has been shown that NO has a biphasic effect on NF-κB activity and can hence both upregulate and downregulate pro-inflammatory proteins (e.g. iNOS). Nanomolar concentrations of NO as might be produced by constitutive NOS were shown to augment macrophage activation and expression of iNOS while low micromolar concentration of NO, similar to those generated by iNOS inhibit gene expression (Connelly *et al.*, 2001). This suggests that eNOS could provide the source of "low output" NO to potentiate iNOS expression and facilitate the initial cellular response to inflammatory stimuli as appears to be mimicked by NPR-A activation.

Vo *et al* and Connelly *et al* have shown that treatment of WT mice with LPS enhanced plasma NO_x levels and these were reduced by 50 % in eNOS KO animals. This was accompanied by reduced iNOS expression and enhanced vascular reactivity to SPER-NO (Connelly *et al.*, 2005; Vo *et al.*, 2005), as has been demonstrated for NPR-A KO mice in the current study. Furthermore, eNOS KO animals exhibit reduced hypotension in response to LPS (Connelly *et al.*, 2005) and this is something that merits further investigation in NPR-A KO animals following sepsis. Perhaps it is not unreasonable to speculate that the NPR-A KO animals should have an improved haemodynamic profile and hence have reduced hypotension in response to LPS in comparison to WT animals as they have reduced iNOS and reduced high output NO, similar to that observed in eNOS KO mice. In further support of a pro-inflammatory role for eNOS, bone marrow-derived macrophages from eNOS KO mice exhibit reduced NF-κB activity, iNOS expression and NO production following exposure to LPS when compared to WT mice, suggesting constitutive eNOS is necessary to obtain maximal iNOS expression and activity (Connelly *et al.*, 2003). Furthermore, in HUVEC, LPS activates eNOS (and its phosphorylation) through PI3K and Akt/protein kinase B-dependent enzyme phosphorylation (Connelly *et al.*, 2005). Therefore, a sGC and pGC-mediated increase in cGMP in response to eNOS-

derived NO and ANP/BNP, appear to have a similar role in facilitating iNOS expression, at least during sepsis. The crosstalk between sGC and pGC could also act in a compensatory fashion under pathological conditions (Madhani *et al.*, 2003). For instance, in cardiovascular disease (e.g. hypertension and atherosclerosis) there is marked endothelial dysfunction associated with reduced NO levels, and this could be compensated by ANP/CNP-cGMP pathways. Conversely, during heart failure where there are elevated natriuretic peptide levels (Potter *et al.*, 2006), the downregulation of NO/sGC/cGMP signalling could help offset the systemic hypotension seen in such conditions (Madhani *et al.*, 2003).

The regulation of gene expression, particularly iNOS, by cGMP-dependent mechanisms has been previously documented. In human glomerular mesangial cells cGMP analogues amplify IL-1β/TNF-α-elicited iNOS induction and this is abrogated in the presence of the sGC inhibitor ODQ (Perez-Sala *et al.*, 2001). Furthermore, in rat glial cell cultures, cGMP analogues and zaprinast, an inhibitor of cGMP-selective phosphodiesterases, enhance LPS-stimulated iNOS expression and NO accumulation while KT5823, an inhibitor of PKG, has an inhibitory effect (Choi *et al.*, 1999; Choi *et al.*, 2002). The regulation of iNOS by sGC and cGMP has also been demonstrated in macrophages. In the presence of ODQ a reduction in iNOS expression in response to LPS is observed, while the sGC activator BAY 41-2272 increases iNOS expression (Connelly *et al.*, 2003). Adenoviral transfer of iNOS to the endothelium and adventitia of intact vessels impairs contractile responses to phenylephrine and this is reversed in the presence of ODQ, suggesting that sGC and cGMP are involved in regulating iNOS (Gunnett *et al.*, 2005). However, the mechanisms underlying the NO/sGC/cGMP regulation of iNOS are unclear. The NO donor S-nitroso-N-acetyl-D,L-pencillamine has been shown to activate NF-κB and induce TNF-α mRNA and protein expression in feline cardiac myocytes, and this is blocked by pre-treatment with ODQ or a PKG antagonist (Rp-8-Br-cGMPS; Kalra *et al.*, 2000). As mentioned above, LPS-induced iNOS expression in rat glial cell cultures is inhibited by a PKG inhibitor (Choi *et al.*, 1999; Choi *et al.*, 2002), suggesting the involvement of cGMP/PKG. Interestingly, PKG phosphorylates IκB at the critical Ser32 residue, which leads to its proteasomal degradation and activation of NF-κB (Traenckner *et al.*, 1995; Kalra *et al.*, 2000). Hence, cGMP generation by sGC and the involvement of PKG could be crucial in controlling iNOS expression. This can be extrapolated to

the current study, such that ANP/BNP activate NPR-A to increase cGMP production and iNOS expression via PKG and IκB degradation. Therefore, it would be interesting to investigate the cGMP levels present in NPR-A KO and WT mice following LPS as well as the phosphorylation status of IκB.

In the current study, only the cGMP-dependent pathways were investigated since cAMP-dependent systems are not affected by desensitisation; vascular responsiveness to the cAMP-dependent vasodilator epoprestenol and to the adenylyl cyclase activator, forskolin, are similar in WT and eNOS KO mice (Hussain *et al.*, 1999; Madhani *et al.*, 2006). However, confirmation of this in a septic model will be required in the future by looking at the relaxant responses to forskolin in NPR-A KO and WT mice following LPS treatment. Isolation of bone marrow-derived macrophages from NPR-A KO mice will help determine if PI3K/Akt, PKG and phosphorylated IκB are involved in the control of iNOS by ANP. Moreover, measurement of eNOS expression in heart, lung and aortic tissue from NPR-A KO and WT mice following treatment with LPS might help to determine if cooperative cGMP-dependent regulation of pro-inflammatory protein expression occurs in response to LPS. Furthermore, the plasma levels of ANP, BNP and CNP as well as cGMP and various pro-inflammatory cytokines e.g. IL-1, IL-6 and TNF-α should be determined in NPR-A KO and WT mice treated with LPS or saline, to further support the proposed pro-inflammatory role for ANP/BNP.

6.4. Effect of ANP and CNP on iNOS expression *in vitro*

Previous studies evaluating the effects of natriuretic peptides on pro-inflammatory protein expression have suggested an anti-inflammatory role for natriuretic peptides, and particularly ANP (as mentioned above). However, these results conflict with the *in vivo* studies described herein in NPR-A KO mice. Vollmar and Kiemer have shown that ANP inhibits NO production in macrophages by inhibiting iNOS expression via pGC-coupled NPR-A with the involvement of cGMP (Vollmar and Schulz, 1995b; Kiemer and Vollmar, 1998; Kiemer and Vollmar, 2001). This inhibition is seen in the macrophage cell lines RAW264.7 and J774, in addition to primary bone marrow-derived and peritoneal macrophages (Kiemer and Vollmar, 1997). In contrast, changes in iNOS expression and NO production were not

observed in Kupffer cells, although an increase in cGMP levels was apparent (Kiemer *et al.*, 2002a). CNP has been reported to have no effect on NO production and iNOS expression in macrophages (Kiemer and Vollmar, 1997). Yet, the role of natriuretic peptides in regulating iNOS expression remains unclear since Maruno *et al* have shown that ANP, BNP and CNP all upregulate iNOS induction by raising intracellular cGMP levels in vascular smooth muscle cells (Marumo *et al.*, 1995).

Since a pro-inflammatory role for ANP/BNP in the immune system was suggested from the *in vivo* NPR-A KO studies, the effect of natriuretic peptides on iNOS expression in RAW264.7 murine macrophages was investigated. Macrophages were treated with LPS to induce iNOS and the effects of ANP and CNP were investigated. No significant changes in iNOS expression were observed in the presence of ANP or CNP in contrast to previous findings. The discrepancy between the *in vitro* and *in vivo* systems could be explained by additional factors present *in vivo* not being available in a cell-based approach (e.g. release of additional pro-inflammatory cytokines/mediators by natriuretic peptides).

Since no effect of natriuretic peptides on pro-inflammatory protein expression was observed in murine macrophages, similar studies were conducted in 1° RAoSMC. Induction of iNOS was brought about by treating 1° RAoSMC with a combination of LPS, IFN-γ, IL-1β and TNF-α. There have been numerous reports documenting the activation of smooth muscle cells with single or dual-combinations of cytokines (Beasley *et al.*, 1991; Marumo *et al.*, 1995; Kusano *et al.*, 1999; Zhang *et al.*, 2001; Hsiao *et al.*, 2003) and induction of iNOS expression in cells *in vitro*, particularly human, has been notoriously difficult (MacNaul and Hutchinson, 1993). Inducible NOS activation (and nitrite accumulation in the culture medium as an index of NO production) in 1° RAoSMC used in the present study was best achieved using a combination of all four activators, LPS, IFN-γ, IL-1β and TNF-α. Treatment of cells with LPS alone, a combination of TNF-α and IL-1β or a combination of TNF-α and IFN-γ did not bring about significant increases in iNOS expression and no corresponding increase in nitrite was seen. The levels of nitrite production observed in 1° RAoSMC were approximately 2-fold lower in comparison to RAW264.7 macrophages. It has been suggested previously that NO formation in vascular smooth muscle cells in contrast to monocytes/macrophages, could be limited to some

extent by the availability of both the NOS substrate L-arginine and certain co-factors such as BH_4 (Hecker *et al.*, 1999), giving a possible explanation for the lower nitrite levels observed in 1° RAoSMC.

Previous studies in smooth muscle cells have investigated the effects of NO, cGMP and natriuretic peptides on iNOS expression. NO has been shown to inhibit cytokine-induced iNOS expression and NF-κB activity in RAoSMC (Katsuyama *et al.*, 1998; Hattori *et al.*, 2004). Yet, it has also been reported that NO has little or no effect on iNOS and NF- κB activity (Zhang *et al.*, 2001). Further studies have documented that cGMP-dependent vasodilators such as sodium nitroprusside significantly increase the IL-1β-stimulated expression of iNOS protein and activity (Boese *et al.*, 1996). Importantly however, iNOS was not induced using the same cytokine combinations in these studies, suggesting that there are multiple pathways controlling iNOS expression, which are all tightly and differentially regulated. A promoter study using A7r5 cells (rat aortic smooth muscle cells), has shown that the iNOS promoter is regulated differentially compared to macrophages and that different κB binding sites are involved in the two cell types (Spink *et al.*, 1995). The production of NO in vascular smooth muscle cells causes profound vasodilatation, which could be beneficial at the local area of inflammation to facilitate delivery of inflammatory cells and mediators to combat the infection. However, iNOS expression and NO production must be tightly controlled as inappropriate high levels of NO causes widespread vasodilatation resulting in hypotension and multiple organ failure as exemplified in sepsis. Therefore, the need for multiple cytokines to achieve iNOS induction in smooth muscle cells fits with a systemic protective mechanism. Two independent groups have shown that akin to NO, ANP, BNP and CNP augment the induction of iNOS via cGMP in vascular smooth muscle, however, these effects are independent of cGMP-dependent protein kinases and may involve cAMP-dependent kinases (Marumo *et al.*, 1995; Boese *et al.*, 1996; Iimura *et al.*, 1998).

In the current study, 1° RAoSMC were exposed to ANP or CNP prior to activation and the effect on iNOS expression and activity was determined. Once again, in contrast to previous findings (Marumo *et al.*, 1995; Boese *et al.*, 1996; Iimura *et al.*, 1998), no changes in iNOS were observed, despite the presence of mRNA for all

natriuretic peptide receptors, suggesting that each isoform is expressed in these cells. At present it is unclear why the natriuretic peptides were unable to alter iNOS expression/activity, although this may be due to the phenotype of the smooth muscle cells. Vascular smooth muscle cells switch from a contractile to a synthetic state *in vivo* in response to vascular injury, and NO, cGMP and PKG have been postulated to be involved in the switch (Komalavilas *et al.*, 1999). Therefore, whether the RAoSMC used in this study were contractile or synthetic could play a role in the activation of iNOS and thus the modulation of pro-inflammatory proteins by natriuretic peptides. Furthermore, *in vivo* there are other mediators produced by surrounding cells, especially endothelial cells, which could have an effect on the responsiveness of the underlying smooth muscle. In addition, the MAPK pathway has been shown to be important in the regulation of iNOS in vascular smooth muscle cells (Baydoun *et al.*, 1999; Doi *et al.*, 2000; Jiang *et al.*, 2004) and natriuretic peptides might modulate these pathways to alter iNOS expression. However, multiple pathways are involved in controlling iNOS expression and therefore a subtle effect on one pathway by natriuretic peptides could be 'masked' or 'compensated' for by an alternative modulatory mechanism.

6.5. iNOS expression and the MAPK pathway

The involvement of the MAPK pathway in the regulation of iNOS has been inferred previously using well characterised, highly specific MAPK inhibitors. PD98059 inhibits ERK 1/2 MAPK pathway by blocking the upstream activators (MEK 1/2) of ERK 1/2 (Dudley *et al.*, 1995) while SB203580 inhibits p38 MAPK pathway (Kumar *et al.*, 1997). In murine macrophages (RAW264.7), SB203580 but not PD98059 inhibits the LPS-induced stimulation of iNOS by inhibiting NF-κB DNA binding indicating that p38, but not ERK 1/2, is involved in LPS induced iNOS expression (Chen and Wang, 1999). Similar observations have been reported by Monier *et al* (Monier *et al.*, 2002). In contrast, Sharma *et al* have reported that both ERK 1/2 and p38 MAPK cascades are involved in endotoxin-induced NO production and iNOS expression, although PD98059 seems to inhibit iNOS to a lesser extent than SB203580 (Kim *et al.*, 2004), suggesting a predominant role for p38. In rat vascular smooth muscle cells transiently transfected with an iNOS-luciferase promoter construct, PD98059 inhibits luciferase activity in response to IL-1β and

TNF-α suggesting the involvement of the ERK pathway in iNOS regulation (Doi *et al.*, 2000). In addition, Baydoun *et al* observed that SB203580, but not PD98059, inhibits iNOS expression and activity, and L-arginine transport, in RAoSMC (Baydoun *et al.*, 1999). In contrast, p38 MAPK has been shown to be involved in Ang II regulated IL-1β induced iNOS in rat vascular smooth muscle cells (Jiang *et al.*, 2004).

Thus, although it is likely that ERK 1/2 and p38 MAPK both play a role in iNOS expression, the relative contribution of each pathway appears to be tissue specific. It is evident that the protein kinases that are involved in iNOS gene transcription and regulation vary from cell to cell (Baydoun *et al.*, 1999). Different cell types could express different combinations of the p38 isoforms. p38α and p38β isoforms are known to be sensitive to SB203580 and other pyridinyl imidazoles while p38γ and δ are thought to be insensitive to these inhibitors (Kumar *et al.*, 1997). Therefore, it is possible that p38α and β are the active isoforms in the RAoSMC system being studied and thus p38α and β could still be involved in iNOS regulation. It is also possible that there are variations in the iNOS promoter between different species (Geller and Billiar, 1998; Baydoun *et al.*, 1999) thus giving an explanation for the disparity in the results of the studies described above.

In the current study, 1° RAoSMC were exposed to PD98059 or SB203580 prior to stimulation with the activation cocktail. iNOS expression was inhibited by SB203580, but not by PD98059, implicating the involvement of the p38 MAPK signalling pathway in the regulation of iNOS expression in this cell type, but ruling out a contribution from the ERK 1/2 pathway. Thus, mediators inhibiting p38 but not ERK 1/2 should have a negative effect on iNOS. Conversely, p38 but not ERK 1/2 activators should facilitate iNOS expression. Therefore, I explored if ANP or CNP altered ERK 1/2 or p38 phosphorylation.

The pattern of MAPK phosphorylation following activation of 1° RAoSMC with LPS and cytokines was investigated in the current study. Both ERK 1/2 and p38 were phosphorylated in 1° RAoSMC in a transient fashion over a duration of 60 min following exposure to activator cocktail. Phosphorylation increased up to 15 min post activation and returned to baseline by 60 min. This indicates that both ERK 1/2

and p38 can be phosphorylated by the activator cocktail and are likely to be involved in pro-inflammatory protein regulation in this cell type, although it is clear that only p38 is key for the control of iNOS. The involvement of the ERK 1/2 pathway in pro-inflammatory protein expression has been described previously. Uric acid has a direct pro-inflammatory effect on vascular smooth muscle cells by stimulating MCP-1 production via activation of NFκB, ERK 1/2 and p38 (Kanellis *et al.*, 2003). In human airway smooth muscle cells, the release of granulocyte-macrophage colony-stimulating factor (GM-CSF), which is known to prime monocytes for a pro-inflammatory cytokine response (i.e. elevation in TNF-α, IL-1β and IL-6) is ERK 1/2 dependent (Hallsworth *et al.*, 2001). In macrophages, MEKK3, which lies upstream of all three MAPK pathways, is a crucial regulator of LPS-induced GM-CSF and IL-6 production (Kim *et al.*, 2007). The phosphorylation of MAPK cascades by LPS and inflammatory cytokines has also been described previously. In macrophages LPS can phosphorylate both ERK 1/2 and p38 (Kim *et al.*, 2004) while in RAoSMC ERK 1/2, p38 and JNK/SAPK are phosphorylated by LPS (Yamakawa *et al.*, 1999; MacKenzie *et al.*, 2003). Yamakawa *et al* reported ERK 1/2 and p38 phosphorylation increases time dependently in the presence of 100 μg/ml LPS with maximal phosphorylation occurring at 30 min post activation (Yamakawa *et al.*, 1999), observations that are similar to the ERK 1/2 and p38 phosphorylation patterns described in the present study.

6.6. Effects of ANP and CNP on MAPK pathways

Since phosphorylation of MAPK cascades by LPS and pro-inflammatory cytokines was observed in 1° RAoSMC, it was postulated that natriuretic peptides could modulate these protein kinases either through guanylate cyclase coupled NPRs or through G-protein coupled NPR-C. To test this thesis, 1° RAoSMC were treated with ANP and CNP prior to activator cocktail and ERK 1/2 and p38 phosphorylation monitored. CNP did not modulate ERK 1/2 or p38 phosphorylation to a great extent although there was a slight upward trend, especially in the case of p38 phosphorylation. However, in the absence of activator cocktail, CNP significantly enhanced ERK 1/2 and p38 phosphorylation. Conversely, ANP was essentially unable to alter ERK 1/2 or p38 phosphorylation. Therefore, the current study provides the first evidence that CNP can bring about MAPK phosphorylation

directly and independently of LPS and pro-inflammatory cytokines in vascular smooth muscle cells. An independent study by Ho *et al* using rat pinealocytes has shown that CNP and ANP are able to elevate the levels of phosphorylated MAPK, which is accompanied by an increase in cGMP accumulation with the possible involvement of PKG (Ho *et al.*, 1999). ANP has also been shown to inhibit Akt as well as ERK 1/2 phosphorylation in response to lysophosphatydic acid, a bioactive lipid mainly released from activated platelets, which participates in the development of atherosclerotic plaques by stimulating vascular smooth muscle cell proliferation and ROS production (Baldini *et al.*, 2005). Furthermore, NPR-A cardiomyocyte specific KO mice in an eNOS KO background show cardiac hypertrophy, which is accompanied by an increase in ERK 1/2 phosphorylation, suggesting that local ANP signalling prevents hypertensive cardiac hypertrophy (Bubikat *et al.*, 2005). More recently, a microarray study has confirmed the involvement of PKC-MAPK pathways in the prevention of cardiac hypertrophy (Ellmers *et al.*, 2007). CNP has also been shown to modulate the MAPK pathways. Overexpression of CNP in chondrocytes of a mouse model for achondroplasia rescues the disease phenotype by correcting the decreased extracellular matrix synthesis through inhibition of the ERK 1/2 pathway (Yasoda *et al.*, 2004). This was further substantiated by Krejci *et al* who demonstrated that CNP antagonises FGF-2-induced growth arrest of rat chondrosarcoma chondrocytes by the inhibition ERK 1/2 phosphorylation in a cGMP-dependent manner involving PKG (Krejci *et al.*, 2005).

6.7. MAPK, CNP and proliferation

The MAPK pathways are known to be involved in controlling numerous physiological and pathophysiological processes including proliferation (Pearson *et al.*, 2001). The anti-proliferative properties of CNP have been well-characterised, especially in the vasculature, but the receptor and the signalling pathways involved in mediating this effect are not clearly defined. CNP, which acts as a local mediator in the vasculature has been shown to act through NPR-B/cGMP (Marumo *et al.*, 1995) as well as in a cGMP independent manner (e.g. through NPR-C/G_i-coupling) as shown in the resistance arteries (Chauhan *et al.*, 2003b). Since CNP was able to elicit a transient increase in ERK 1/2 and p38 phosphorylation, and all three natriuretic peptide receptors have been found to be expressed in 1° RAoSMC, the

involvement of the MAPK pathways in the anti-proliferative actions of CNP, and the receptor responsible, were investigated.

There is compelling evidence for the involvement of GPCRs in cell growth in association with the MAPK signalling pathway (Gutkind, 1998a; Gutkind, 1998b; Gutkind, 2000; Waters *et al.*, 2004). Ligands as diverse as bombesin, endothelin-1, somatostatin, thromboxane A_2, prostaglandin F2α, interleukin-8, vasopressin, bradykinin, angiotensin, insulin, PDGF, epidermal growth factor (EGF) and many more have been shown to have mitogenic properties involving activation of GPCRs and the MAPK signalling pathway (Rozengurt, 1986; Gutkind, 2000). It is perhaps surprising that a number of these mitogens signal through the non-classical GPCR, involving activation of single transmembrane receptors coupled to either tyrosine kinases and the small G-protein Ras or heterotrimeric G-proteins (Patel, 2004). In the case of receptor tyrosine kinases (RTKs), upon ligand binding to an extracellular binding domain the receptor dimerises and catalytic activity increases enabling phosphorylation of tyrosine residues on the intracellular catalytic domain, by transferring phosphates from ATP, a process known as autophosphorylation (Mohammadi *et al.*, 1996). Once the receptor has been phosphorylated the phosphorylation sites act as docking sites for multiprotein complexes including proteins containing Src-homology 2 (SH2) or phosphotyrosine-binding (PTB) domains (Mohammadi *et al.*, 1996). These protein complexes can then bring about the activation of Ras, a monomeric GTP-binding switch G-protein that can itself activate Raf isoforms bringing about MAPK signalling. In the case of heterotrimeric G-proteins both free G_α and $G_{\beta\gamma}$ subunits initiate downstream MAPK activation (Hawes *et al.*, 1995). Recently, natriuretic peptide receptors have been characterised to possess G-protein binding domains (Murthy and Makhlouf, 1999; Murthy *et al.*, 2000; Borges *et al.*, 2001; Alfonzo *et al.*, 2006), and although they do not possess a seven transmembrane structure of classical GPCRs, they mimic single transmembrane GPCR signalling. NPR-C is linked to $G_{\alpha i}$, which can bring about inhibition of adenylyl cyclase upon ligand binding (Anand-Srivastava *et al.*, 1996; Pagano and Anand-Srivastava, 2001) as well as production of IP_3 and DAG resulting in PKC activation (Anand-Srivastava, 2005). It has also been reported that NPR-C is implicated in cell proliferation through $G_{\alpha i}$-protein dependency (Levin and Frank, 1991; Cahill and Hassid, 1994), but whether this involves MAPK is not known.

NPR-B has also been shown to possess G-protein binding domains but little work has been carried out in terms of signalling with the involvement of G-proteins from this receptor (Borges *et al.*, 2001; Alfonzo *et al.*, 2006).

The results presented herein demonstrate that CNP causes an inhibition of 1° RAoSMC growth (both basal and Ang II-stimulated), that is blocked by the selective NPR-C antagonist M372049 and the ERK 1/2 pathway inhibitor PD98059. The transient increase in ERK 1/2 phosphorylation elicited by CNP is also blocked by PD98059, the G_i-protein inhibitor PTx and M372049. Conversely, CNP-induced p38 phosphorylation was unaltered by M372049 and PTx. These observations imply that CNP is acting through NPR-C and G_i-protein coupling in a cGMP-independent manner to bring about its anti-proliferative actions via activation of ERK 1/2 phosphorylation.

Although previous studies have acknowledged the inhibitory effect of CNP on vascular smooth muscle growth, controversy has arisen when trying to establish a role for a NPR-B (cGMP-mediated) or NPR-C (cGMP-independent) effect. Furuya *et al* have reported that CNP inhibits intimal thickening following vascular injury accompanied by an increase in cGMP levels (Furuya *et al.*, 1993) and inhibits DNA synthesis and growth in vascular smooth muscle via cGMP-dependent mechanisms (Furuya *et al.*, 1991). This led to the conclusion that NPR-B is probably the receptor involved in these inhibitory effects (Furuya *et al.*, 1991; Furuya *et al.*, 1993). In accord, Hutchinson *et al* have shown that increases in cGMP levels correlate with growth inhibition and have further demonstrated that CNP inhibits PDGF-stimulated MAPK activity (Hutchinson *et al.*, 1997). MAPK inhibition by CNP has been reported by other groups in non-vascular cells via NPR-C (Prins *et al.*, 1996) and small cytoplasmic domain peptides of NPR-C are able to inhibit the proliferative responses of vasoactive peptides through G_i-protein and ERK 1/2/PI3K/Akt pathways in vascular smooth muscle cells (Hashim *et al.*, 2006). The involvement of NPR-C in growth inhibition is further substantiated by Cahill *et al* who have suggested that CNP inhibits growth in aortic smooth muscle cells via NPR-C in a cGMP-independent manner (Cahill and Hassid, 1994). Furthermore, growth inhibition via NPR-C and decreased adenylyl cyclase activity has been reported in gastric epithelial cells (Gower *et al.*, 2006).

Evidence from this study confirms the anti-proliferative properties of CNP in vascular smooth muscle cells but goes one step further in apportioning a definitive role for NPR-C-mediated ERK 1/2 phosphorylation in this phenomenon. The data reveal that the NPR-C antagonist M372049 is able to reverse the growth inhibition elicited by CNP; however, this reversal is not complete giving rise to the possibility that both NPR-B and NPR-C may coordinate the anti-mitogenic actions of CNP (see below). The results also provide insight into the intracellular pathways that mediate the anti-proliferative effects of CNP/NPR-C signalling. Exposure of vascular smooth muscle cells to CNP causes a rapid, transient increase in the phosphorylation of ERK 1/2, a key arm of the MAPK cascade that is well-established to regulate cell growth (Pearson *et al.*, 2001). The concentrations of CNP (1 μM and 10 μM) that elicit significant increases in ERK 1/2 phosphorylation (and inhibit smooth muscle proliferation) are commensurate with those released acutely from vascular endothelial cells by ACh (Chauhan *et al.*, 2003a; Chauhan *et al.*, 2003b; Hobbs *et al.*, 2004). Moreover, both NPR-C antagonism and the G_i-protein inhibitor PTx attenuated CNP induced enhancement in ERK 1/2 phosphorylation, suggesting that this CNP-triggered activation of the pathway is also NPR-C-dependent. The ERK 1/2 pathway inhibitor, PD98059 was employed to link these two facets of CNP bioactivity. In accord with the proposed thesis, PD98059 blocked both the ERK 1/2 phosphorylation and inhibition of vascular smooth muscle growth induced by CNP. Although a decrease in ERK 1/2 phosphorylation has been reported to result in growth inhibition (Prins *et al.*, 1996; Hutchinson *et al.*, 1997), it is also established that activation of ERK 1/2 can lead to $p21^{waf1/cip1}$ stimulation and cessation of the cell-cycle (Pumiglia and Decker, 1997; Olson *et al.*, 1998; Ray *et al.*, 1999; Figure 59). This is exemplified by the study of Bauer *et al* who reported that NO, a key regulator of vascular responsiveness and inflammation, can increase ERK 1/2 phosphorylation and bring about inhibition of cell proliferation in vascular smooth muscle cells (Bauer *et al.*, 2001). Since NO and CNP are both key endothelium-derived vasoactive mediators that possess complementary anti-atherogenic functions (Ahluwalia and Hobbs, 2005), it is perhaps not surprising that they evoke the same intracellular signalling systems to exert their anti-proliferative effects. It could be speculated that CNP might increase $p21^{waf1/cip1}$ in RAoSMC, akin to NO, and this might form part of future studies, which will endeavour to link $p21^{waf1/cip1}$ to CNP-induced growth inhibition via NPR-C. Moreover, the anti-mitogenic effects of NO

and CNP appear to be mediated in a cGMP-independent fashion, despite the ability of both mediators to activate guanylate cyclase enzymes that synthesise cGMP (Hobbs and Ignarro, 1996; Ahluwalia *et al.*, 2004), which is well-established to inhibit cell growth. It is likely, however, that these discrete pathways are cell specific and that in vascular smooth muscle cells, the cGMP-independent anti-mitogenic effects of NO and CNP predominate. Moreover, activation of the ERK 1/2 pathway by G_i-coupled NPR-C adds a new dimension to the signalling capacity of this receptor and suggests it can fulfil a role akin to other well-recognised G_i-coupled receptors that activate the MAPK cascade, such as sphingosine-1-phosphate and insulin-like growth factor (Zondag *et al.*, 1998; Hallak *et al.*, 2000; Figure 59).

Although NPR-C is probably the primary receptor responsible for the increase in ERK 1/2 phosphorylation and growth inhibition by CNP, NPR-B and cGMP could also be involved, as 8-Bromo-cGMP enhances ERK 1/2 phosphorylation in a similar fashion to CNP, albeit with a significantly reduced magnitude. This would fit with previous reports linking NPR-B activation, cGMP formation and inhibition of mitogenesis (Furuya *et al.*, 1991; Furuya *et al.*, 1993; Hutchinson *et al.*, 1997). The fact that PTx was able to completely abolish CNP-induced ERK 1/2 phosphorylation, while NPR-C blockade only achieved approximately 70 % inhibition, could be explained by the recent finding that NPR-B also possesses G-protein coupling domains (Alfonzo *et al.*, 2006). Treatment with PTx could potentially eliminate signalling from both NPR-B and NPR-C whereas M372049 would solely remove NPR-C-mediated effects. It is also worth noting, that in non-vascular cell types (e.g. adipocytes), it has been proposed that non-primate species (e.g. rodents, rabbits and dogs) express NPR-C as the predominant receptor with very low expression of NPR-A (Sengenes *et al.*, 2002). Moreover, vascular smooth muscle cells have previously been shown to express predominantly NPR-C, although NPR-A and NPR-B are also present (Hutchinson *et al.*, 1997). This could account for the proposed NPR-C-mediated effects on ERK 1/2 phosphorylation and growth presented in this study.

CNP is a potent vasorelaxant mediator in both conduit and resistance arteries (Madhani *et al.*, 2003; Ahluwalia *et al.*, 2004); ANP to the contrary, is a very poor relaxant in the mesenteric resistance vasculature (Villar *et al.*, 2007), unlike its strong relaxant effects in conduit vessels (Madhani *et al.*, 2003). The lack of

responsiveness to ANP in mesenteric resistance arteries is mirrored in the current study, since ANP was unable to induce ERK 1/2 phosphorylation, intimating that NPR-A does not couple to the MAPK cascade and that ANP cannot activate NPR-C to produce an analogous effect to CNP (despite the fact that both natriuretic peptides bind NPR-C (Maack *et al.*, 1987; Matsukawa *et al.*, 1999; Potter and Hunter, 2001)). This differential activity, paralleling the observed differences in vasorelaxant activity in resistance arteries, may be the result of the disparate binding characteristics of ANP and CNP at NPR-C (He *et al.*, 2006), and merits further attention.

In addition to the importance of NPR-C to the vasodilator properties of endothelium-derived CNP in the resistance vasculature, endothelial CNP contributes to the regulation of coronary blood flow and is a protective mechanism against ischaemia-reperfusion injury; these actions are also mediated via NPR-C (Hobbs *et al.*, 2004). Moreover, the importance of CNP in the vasculature, particularly during inflammatory disease, has been highlighted by the fact that it possesses significant anti-leukocyte and anti-platelet effects; again these effects have been attributed to NPR-C activation (Scotland *et al.*, 2005a). Taken together, these observations stress the importance of NPR-C signalling in bringing about the anti-atherogenic properties of CNP and data from the present study reveal that the anti-mitogenic properties of CNP are an additional cytoprotective property of this endothelium-derived peptide that can be attributed to NPR-C.

Future studies will be required to investigate further the signalling cascade that leads to CNP-induced ERK 1/2 phosphorylation and growth inhibition in an attempt to elucidate other members of this signalling pathway. It is known that signalling through G_i-coupled receptors can lead to Src tyrosine kinase activation (Milligan and Kostenis, 2006), which lies upstream of ERK 1/2. Furthermore, Src tyrosine kinase and ERK 1/2 can modulate eNOS activation (Fulton *et al.*, 2005), which could subsequently inhibit growth (Cooney *et al.*, 2006). Therefore, the effects of CNP and M372049 on Src tyrosine kinase and eNOS should be investigated. In addition, the effect of ANP and CNP on the third component of the MAPK family cascade, JNK/SAPK, might be investigated since this arm of the MAPK family is also known to play a key role in mediating cell differentiation and apoptosis during atherosclerotic plaque development (Metzler *et al.*, 2000).

6.8. Conclusions

In conclusion the results from the current study indicate for the first time that NPR-A KO mice are less susceptible to LPS-induced endothelial dysfunction. This is accompanied by reduced iNOS expression and plasma NO_x, suggesting that ANP/BNP, possess a pro-inflammatory role in sepsis. Despite these observations *in vivo*, ANP and CNP were unable to modulate iNOS expression and activity in murine macrophages and 1° RAoSMC. Furthermore, the p38 MAPK cascade inhibitor SB203580 inhibits LPS and cytokine-induced iNOS in 1° RAoSMC, but this was not mimicked by the ERK 1/2 inhibitor PD98059, giving a role for p38 but not ERK 1/2 signalling in the regulation of iNOS expression in these cells.

I have also demonstrated that CNP enhances ERK 1/2 phosphorylation and inhibits cell proliferation via NPR-C involving G_i-coupling. These data strengthen the evidence supporting NPR-C signal transduction as a principal mechanism conveying the anti-atherogenic actions of this peptide. Together with previous work (Cahill and Hassid, 1994; Chauhan *et al.*, 2003b; Hobbs *et al.*, 2004; Scotland *et al.*, 2005a), it is clear that CNP has an important role to play in maintaining vascular homeostasis at a local level. The NPR-C-dependent anti-proliferative properties of CNP highlighted in this study could prove a novel means of therapeutic intervention in the treatment of inflammatory cardiovascular diseases such as atherosclerosis, sepsis and coronary artery disease.

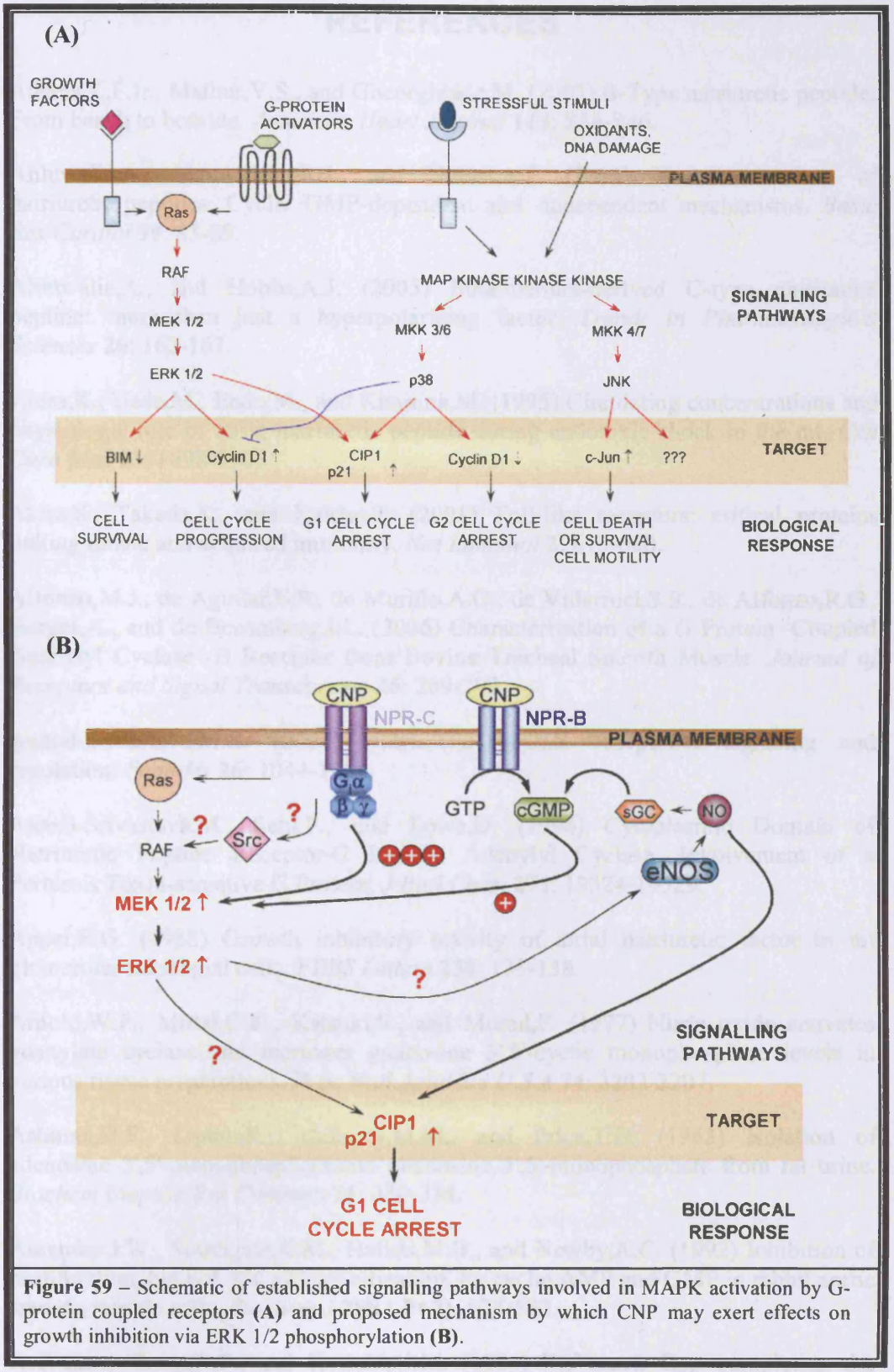


Figure 59 – Schematic of established signalling pathways involved in MAPK activation by G-protein coupled receptors **(A)** and proposed mechanism by which CNP may exert effects on growth inhibition via ERK 1/2 phosphorylation **(B)**.

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