RELAXIN: A NEW CARDIOVASCULAR HORMONE IN HUMANS? COMPARATIVE POTENCY AND MECHANISMS OF ACTION.

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AUTHOR'S DECLARATION

I declare that the work presented has been done, and the thesis composed, by myself and that the papers cited were all consulted by me personally, unless otherwise stated.

Dr Carol J. Fisher

PRESENTATIONS AND PUBLICATIONS

1. Fisher C, Johnston F, Hillier C and McMurray JJ. "Relaxin, a Newly Described Cardiac Hormone is More Potent than Atrial Natriuretic Factor in Human Resistance Arteries". Oral presentation at American Heart Association, November 2001.

2. Fisher C, Johnston F, Hillier C and McMurray JJ. "Relaxin, a Newly Described Cardiac Hormone is More Potent than Atrial Natriuretic Factor in Human Resistance Arteries". Poster presentation at Medical Research Society, Royal College of Physicians, London, January 2002.

3. Fisher C, Johnston F, Hillier C and McMurray JJ. "Relaxin, a Newly Described Cardiac Hormone is More Potent than Atrial Natriuretic Factor in Human Resistance Arteries". Oral and poster presentation at British Cardiac Society, May 2002.

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5. Fisher C, Blue L, Berry C, Morton JJ, Hillier C, McMurray J. "N-terminal B-type natriuretic peptide, but not the putative cardiac hormone relaxin, predicts adverse outcome in patients with chronic heart failure". Oral presentation at British Cardiac Society, April 2003.

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Publications

1. C. Fisher, M. Maclean, I. Morecroft, A. Seed, F. Johnston, C. Hillier, J. McMurray.
Is the pregnancy hormone relaxin also a vasodilator peptide secreted by the heart? Circulation 2002;106:292-295.

2. C. Fisher, S Al-Benna, A. Kirk, J.J. Morton, J. McMurray. Trans-cardiac and trans-pulmonary gradients in the putative new cardiovascular hormone relaxin. Heart, 2003 Jul;89(7):789-90.

3. C. Fisher, C. Berry, L. Blue, J.J. Morton, J. McMurray. N-Terminal pro B type natriuretic peptide, but not the new putative cardiac hormone relaxin, predicts prognosis in patients with chronic heart failure. Heart, 2003 Aug;89(8):879-91.

ABBREVIATIONS

ACh Acetylcholine

ACEI Angiotensin converting enzyme inhibitor

Ang II Angiotensin II

ANP atrial natriuretic peptide

AVP arginine vasopressin

BNP brain natriuretic peptide

Ca^{2+} calcium

CaI calcium ionophore

cAMP cyclic adenosine monophosphate

CCRC cumulative concentration response curve

cGMP cyclic guanosine monophosphate

CHF congestive heart failure

COA patient on ACEI

CNOA patient not on ACEI

CS coronary sinus

EDHF Endothelium – derived hyperpolarising factor

ERPF effective renal plasma flow

ESKD end stage kidney disease

ET-1 endothelin-1

FGF fibroblast growth factor

FSH follicle stimulating hormone

GFR glomerular filtration rate

GPCR G-protein couple receptor

GTP guanosine triphosphate

HMGCoA Hydroxymethyl-glutaryl-CoA reductase.

hRLX human relaxin

IMA Internal mammary artery

iNOS inducible nitric oxide synthase

LGR7 Leucine rich repeat containing guanine nucleotide binding receptor 7

LGR8 Leucine rich repeat containing guanine nucleotide binding receptor 8

LH luteinising hormone

L-NAME *N*ω-nitro-L-arginine methyl ester

L-NOARG *N*ω-nitro-L-arginine

LSV Long saphenous vein

LV left ventricle

LVEF left ventricular ejection fraction

MI myocardial infarction

MMP matrix metalloproteinases

mRNA messenger ribonucleic acid

NE Norepinephrine

NO Nitric oxide

NOS nitric oxide synthase

NT pro BNP N-Terminal pro-type brain natriuretic peptide

NYHA New York Heart Association

ODQ 1H-oxadiazolo-qui-noxaline-1-one

PA pulmonary artery

PCR Polymerase chain reaction

PCWP pulmonary capillary wedge pressure

PDE phosphodiesterase

PE Phenylephrine

PGI2 epoprostenol

PSS physiological saline solution

PVD peripheral vascular disease

RCE rat coronary endothelial cells

RIA radioimmunoassay

RLX Relaxin

RNA Ribonucleic acid

RT Reverse transcriptase

RXFP relaxin family peptide

SHR Spontaneously hypertensive rat

SRA Subcutaneous resistance artery

VEGF vascular endothelial growth factor

VSMC vascular smooth muscle cells

ABSTRACT

INTRODUCTION

The focus of this MD thesis has been relaxin, a member of the insulin family, which is a protein composed of two disulphide linked chains of approximately 6000 Daltons. Relaxin has been traditionally recognised as a hormone of parturition, though more recently it has been postulated that relaxin may be involved in cardiovascular regulation. We used concentrations similar to those found in the plasma in physiological (non-pregnant, pregnancy) and pathophysiological (chronic heart failure) states. Firstly, we characterised the effects of relaxin in small human resistance arteries *ex vivo* using wire myography obtained from gluteal biopsies taken from patients with coronary heart disease (CHD) and normal left ventricular systolic function. We also studied the same effects in larger calibre arteries (internal mammary) and veins (saphenous) using standard organ bath techniques. The effect of relaxin in veins has not previously been described. Internal mammary arteries and saphenous veins were obtained from patients undergoing coronary artery bypass surgery. Small pulmonary arteries were obtained from patients undergoing thoracotomy for bronchial carcinoma. In addition, we wished to determine if a transcardiac or transpulmonary gradient of relaxin could be measured to suggest either pulmonary or cardiac secretion or clearance of the hormone. Relaxin secretion in heart failure has previously been described. Lastly, we wished to determine whether an increased relaxin plasma concentration in patients with chronic heart failure (CHF), is of prognostic importance.

METHODS AND RESULTS

i)comparative potency of relaxin compared to other vasodilators: Small resistance arteries were obtained from biopsies taken from patients with CHD. Each set of vessels was preconstricted with noradrenaline. Thereafter, cumulative concentration response (relaxation) curves (CRCs) were constructed with known vasodilators

atrial natriuretic peptide (ANP), epoprostenol, substance P and relaxin (n=8). Relaxin was found to be a more potent vasodilator than ANP and equipotent to epoprostenol.

ii) mechanism of vasorelaxation: CRCs to relaxin (as above) were constructed to identify the importance of the endothelium – following the removal of the endothelium by the established method of intraluminal rubbing with a human hair. We found that relaxin is endothelium dependent.

iii) interaction of relaxin with nitric oxide and other possible mechanisms of vasodilation and importance of ACE inhibitor treatment: We identified the importance of the effect of ACE inhibitor treatment on the action of relaxin in human resistance arteries. Relaxin's vasodilatory action was significantly reduced in those patients on ACE inhibitors (n=28) compared with those patients not on ACE inhibitors (n=30). In patients treated with an ACE inhibitor, we found that manipulation of prostanoids is important. Indomethacin, (a cyclooxygenase inhibitor) (n=8) blocked relaxin's vasodilatory action. Manipulation of the cAMP second messenger system, with milrinone, (a cAMP phosphodiesterase inhibitor) (n=6) is also important as relaxin's vasodilatory action was enhanced. Manipulation of cyclic GMP second messenger system is also important. ODQ, (a guanylate cyclase inhibitor) (n=10) reduced relaxin's action while zaprinast, (a cGMP phosphodiesterase inhibitor) (n=7) enhanced relaxin's action. Manipulation of nitric oxide with L-NAME (n=8) and L-NOARG (n=10), nitric oxide synthase (NOS) inhibitors and EDHF with apamin and charybdotoxin (potassium channel blockers) (n=7) had a curious effect causing the opposite action to that expected, by enhancing relaxin's vasodilatory action. In patients not treated with an ACE inhibitor, we found that manipulation of nitric oxide with L-NAME (n=8) and L-NOARG (n=8), is important, as both reduced relaxin's vasodilatory action. Manipulating the cGMP second messenger system with ODQ (n=8) greatly reduced relaxin's action. but zaprinast (n=9) did not. Manipulation of EDHF with apamin and charybdotoxin (n=8) had no effect on relaxin's action. Manipulation of prostanoids with indomethacin (n=10) reduced relaxin's action but manipulation of cAMP with milrinone (n=8), had no effect.

iv)relaxin and small human pulmonary arteries: We determined, using wire myography, that relaxin is not a vasodilator of small pulmonary resistance arteries (n=5).

v)relaxin and large calibre vessels: We determined, using the organ bath technique, that relaxin is not a vasodilator of larger calibre arteries i.e. internal mammary arteries removed from patients during coronary artery bypass surgery (n=5).Relaxin is not a venodilator studying saphenous veins removed from patients during coronary artery bypass surgery (n=5).

vi)transmyocardial and transpulmonary gradient of relaxin: Plasma relaxin concentrations were measured using a validated assay. Samples were taken from patients undergoing CABG surgery, from the aorta, coronary sinus, pulmonary artery and pulmonary vein. We found that in 20 patients with normal left ventricular function that there was no transpulmonary gradient but there was a transcardiac gradient suggesting net cardiac extraction of relaxin.

vii)prognostic value of relaxin in patients with chronic heart failure: Relaxin was compared with N-terminal pro brain natriuretic peptide to determine whether relaxin is of prognostic importance. Plasma concentrations of the hormones were measured in 87 patients admitted with CHF. These patients were followed up for a year during which time hospitalisations due to CHF and death were recorded. While NT-proBNP was found to be a powerful and independent predictor of outcome in these patients, relaxin was not.

CONCLUSION.

In addition to its established role in pregnancy, relaxin has many other actions. In particular, its antihypertensive, antithrombotic and vasodilatory properties suggest that relaxin may have a central role in cardiovascular regulation.

CHAPTER 1:

INTRODUCTION.

1.1 THE DISCOVERY OF RELAXIN.

Relaxin, a peptide hormone, is a member of the insulin-like growth factor family with a molecular weight of 6000 daltons. It is made up of 53 amino acids with two disulfide-linked chains, A and B, with B bearing the receptor interaction site (Fig 1).

Structure of Relaxin

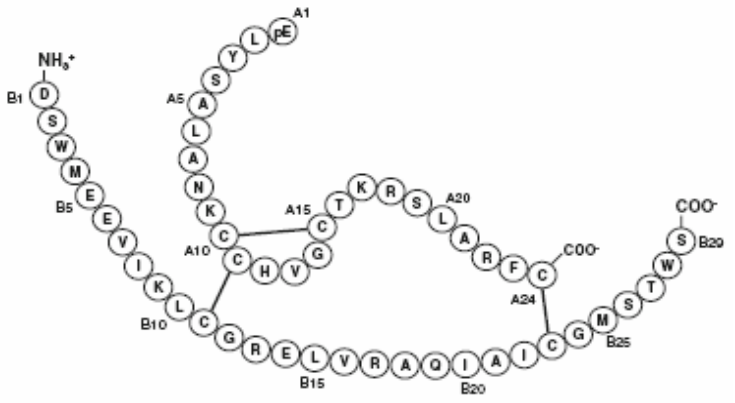


Fig. 1 Structure of native and manufactured human relaxin

(Adapted from Teichman et al, 2008).

Discovered in 1926 by Frederick Hisaw, it is best known as a hormone of parturition, responsible for structural remodeling of the birth canal. This effect is thought to reflect complex actions on reducing fibroblast collagen synthesis and enhancing collagen breakdown (Bani, 1997). Hisaw first reported on the relaxation of the interpubic ligament of female guinea pigs following injections of serum from pregnant guinea pigs or rabbits (Hisaw, 1926). Four years later, Hisaw and

his group (Fevold *et al*, 1930) obtained a crude aqueous extract from sow corpora lutea with chemical characteristics of a peptide and which retained the property to elongate the interpubic ligament. This substance was identified as a new hormone and was given the name "relaxin".

1.2 RELAXIN FAMILY PEPTIDES

The relaxin family peptides are a sub-group of the relaxin- insulin peptide family. All peptides within this family have a uniform two chain structure, with two inter-chain and one intra-chain disulphide bond. In the human, there are seven relaxin family peptides: the human gene 1 (H1-relaxin), human gene 2 (H2-relaxin, commonly referred to as relaxin and equivalent to other species' relaxin-1) and human gene 3 (H3-relaxin), and the insulin/relaxin-like peptides INSL3, INSL4, INSL5 and INSL6. Currently only four of the peptides have identified receptors, although there is a degree of cross-reactivity between peptides and receptors.

1.3 RELAXIN RECEPTORS.

It was with great excitement within the world of relaxinologists in 2002, the news was received that the relaxin receptors had now been identified. Hsu *et al*, described two G-protein coupled receptors LGR7 (Leucine-rich repeat containing guanine nucleotide binding receptor 7) and LGR8 that mediate the action of relaxin

through a cAMP dependent pathway (Hsu *et al*, 2002). They transfected cells with known G-protein coupled receptors with no known ligands. Treatment of these cells with porcine relaxin resulted in a dose dependent increase in cAMP production. LGR7 and LGR8 were not affected by treatment with insulin or insulin growth factor I or II (despite these proteins having a similar domain arrangement to prorelaxin, the precursor of relaxin). As relaxin belongs to the group of peptide hormones that includes insulin, the finding that relaxin receptors are G-protein coupled receptors rather than an orphan membrane-associated tyrosine kinase receptor resembling those that bind to insulin, is surprising.

The expression pattern of these receptors was also examined. LGR7 was expressed in the brain, kidney, testis, placenta, uterus, ovary, adrenals, prostate, skin and heart whereas LGR8 was mainly present in brain, kidney, muscle, testis, thyroid, uterus, peripheral blood cells and bone marrow. The wide distribution of these relaxin receptors is in keeping with relaxin's pleiotropic actions.

Meanwhile, a group of investigators at the Howard Florey Institute in Australia, discovered relaxin-3. (Bathgate *et al*, 2002). An intriguing finding of relaxin-3 was its nearly exclusive expression in the brainstem. Subsequent searches of all available genomes using the relaxin-3 sequence found that relaxin is not mammalian specific. Relaxin-3 sequences were found in fish species (zebrafish, fugufish and Tetraodon, a frog (Xenopus) and a chicken. Hsu et al demonstrated that relaxin-3 was capable of activating LGR7 relaxin receptor but not LGR8.

The discovery that the orphan receptor LGR7 is the relaxin receptor was largely attributable to the pursuit of an idea raised by the combination of the similarity of the structure of LGR7 to LGR8 and the similarity of the structure of relaxin to INSL3 (gene encoding insulin-like peptide) (Hsu *et al*, 2002). LGR7 and LGR8, which are 757 (Hsu *et al*, 2002) and 737 (Overbeek *et al*, 2001) amino acids in length, respectively, share about 60% amino acid sequence identity and contain 10 leucine-rich repeats in their large N-terminal extracellular domain. Two orphan G-protein-coupled receptors designated GPCR135 and GPCR142 were recently proposed as putative receptors for relaxin-3 (Liu *et al*, 2003a,b). Both receptors belong to the type I family of GPCRs. Unlike LGR7 and LGR8, GPCR135 and GPCR142 have short N-terminal extracellular domains, and they contain only 469 and 374 amino acid residues, respectively.

With the discovery of more relaxin receptors, it was decided by the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR), established in 1987, that the nomenclature for relaxin receptors should be examined (Table 1.1). Led by Professor Summers, Professor of Pharmacology, University of Monash, Australia, a review was published in 2006 with the revision of the nomenclature for the relaxin family peptides and their receptors (Bathgate *et al*, 2006).

Relaxin receptors have now been identified in many tissues including reproductive tissues, cardiovascular and renal systems and the brain. Table 1.2 provides a

summary of the distribution of human tissues where RXFP receptors 1 to 4 have been detected.

Table 1.1. Recommendations for the Nomenclature of Receptors for Relaxin Family Peptides

RECEPTOR	PREVIOUS NAME
RXFP 1	LGR7
RXFP 2	LGR8
RXFP 3	GPCR135
RXFP 4	GPCR142

RXFP = Relaxin Family Peptide Receptor

LGR = Leucine-Rich Repeat Containing Guanine Nucleotide Binding Receptor

GPCR = G-Protein-Coupled Receptor

Adapted from Bathgate *et al*, 2006

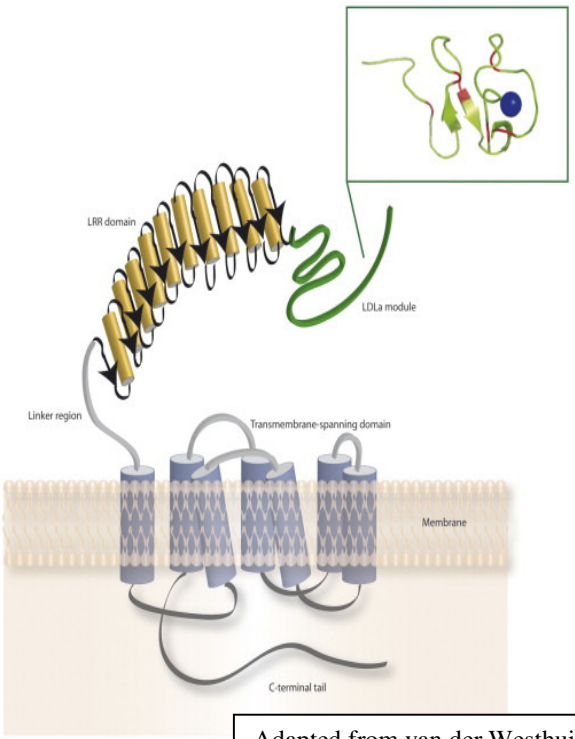
Table 1.2 Distribution of Receptors for Relaxin and Relaxin Related Peptides in Humans.

Tissue	RXFP 1	RXFP 2	RXFP 3	RXFP 4
Ovary	mRNA*			mRNA
Uterus	mRNA	mRNA		mRNA
Placenta	mRNA			mRNA
Breast	protein**			
Testis	mRNA	mRNA	mRNA	mRNA
Prostate	mRNA			mRNA
Brain	mRNA	mRNA	mRNA	mRNA
Pituitary			mRNA	
Kidney	mRNA	mRNA		mRNA
Heart	mRNA			mRNA
Lung	mRNA			
Liver	mRNA			
Muscle	mRNA	mRNA		mRNA
Thyroid	mRNA	mRNA		mRNA
Adrenal	mRNA		mRNA	mRNA
Skin	mRNA			

*mRNA by Northern blot analysis, **protein by immunohistochemical analysis
Adapted from Bathgate *et al*, 2006.

RXFP1 and RXFP2 are both classified as type C LGRs. **Figure 1.2** below is a schematic diagram of a type C LGR.

These receptors have a large and distinctive ectodomain encompassing an LDL class A (LDLa) module at the extreme N-terminus followed by 10 LRR (leucine rich repeats), a unique hinge region leading into the transmembrane domain (seven transmembrane helices) and an intracellular C-terminal tail. The LDLa module distinguishes RXFP1 and RXFP2 from other LGR receptors and other GPCRs.



Adapted from van der Westhuizen et al, 2008

Figure 1.2 Relaxin Receptor.

1.4 RELAXIN COMPARED WITH INSULIN.

Relaxin was compared to insulin as early as 1930. Both porcine relaxin and insulin have a molecular weight of 6000 daltons and consist of two chains, a smaller A chain and a larger B chain. With the elucidation of the first primary structure of relaxin and the positive identification of the crosslink pattern, a direct comparison of the structures of these two hormones became possible. Surprisingly only a small number of amino acids are identical in insulin and relaxin. Six of the eight identities are the cysteines from an insulin-like disulfide structure (Schwabe and McDonald, 1977), and two additional homologous residues are the structurally important glycines of the B chains (B11, B23).

Amino acid residues forming the hydrophobic core of insulin are hydrophobic in relaxin as well and the helical segments localized in the N-terminal and C-terminal region of the A chain and the mid-region of the B chain of relaxin contain amino acids that would also be consistent with helical conformations. The three glycine residues of the insulin B chain allow for β-turn-formation, and since the two glycines in the relaxin B chain are homologous, two of the three β-turns may be expected to occur in relaxin.

Although there is strong evidence that relaxin is structurally related to insulin there are also distinct differences in terms of the surface properties of these two hormones, i.e. all relaxins of known primary structure are basic proteins whereas

insulins are generally acidic proteins. In spite of overall structural similarities between insulin and relaxin the primary sequences vary sufficiently to eliminate all antigenic and biological cross-reactivity (Schwabe and Bullesbach, 1990).

1.5 RELAXIN IN OTHER ANIMALS.

Soon after its discovery in the ovaries of guinea pigs, investigators became curious to establish how widely distributed relaxin is in the animal kingdom. (Schwabe and Bullesbach, 1990). Using the guinea pig relaxation assay, it has been possible to find out that relaxin is not only a hormone of viviparity in mammals. There have been reports of relaxin in shark and whale ovaries as well as rooster and armadillo testes. Pregnant bats have also been found to rely on relaxin for propagation. Detection of relaxin in these unusual places has been confirmed at least in part by isolation and sequence analysis. In the dog, relaxin persists after pregnancy during lactation. Although the source of relaxin in the dog is not known, it has been suggested that both ovaries and the placenta might be a site of synthesis. Postpartum persistence of relaxin during lactation and the discovery of an immuno-reactive relaxin-like component in the milk of Labrador retrievers suggest the mammary gland as an additional source. (Steinetz *et al*, 1987).

Preliminary reports of relaxin in protozoa have been described by Schwabe *et al* (Schwabe *et al*, 1983) and immunoreactive molecules have also been detected in

prokaryotes (*Bacillus subtilis*) and even in lower plants (*Spirogyra*) by radioimmunoassay (RIA) with sheep anti-porcine relaxin antibodies. Whilst these studies are in need of confirmation, similar findings have been reported with insulin (LeRoith *et al*, 1980, 1981) which increases the likelihood that the genes for relaxin and insulin are present in unicellular life and in the plant kingdom.

A plethora of relaxin functions has been proposed on the basis of physiological studies in a large variety of animals. The single most characteristic property of relaxin appears to be the widening of the birth canal in mammals prior to parturition. However, the response of different components of the birth canal, vary to differing degrees in various species. For example, the symphysis pubis relaxes proportionately less in women than in mice, guinea pigs or bats. In humans, other parts of the pelvic girdle, such as the sacroiliac joint are also affected whereas in guinea pigs major remodeling occurs only in the symphysis pubis.

1.6 THE PRODUCTION OF RELAXIN IN HUMANS.

Relaxin is produced in the corpus luteum, placenta, prostate (in men) and the heart. In humans, three molecular forms, each encoded by separate genes, have been identified - H1, H2 and H3. H1 and H2 are found on chromosome 9 while H3 is found on chromosome 19. Only the H2 form circulates in plasma. In women, H2 is also expressed in the corpus luteum, endometrium, placenta and breast, while H1 is

expressed only in the placenta. In men, both H1 and H2 are expressed in the prostate gland. H3, which has only recently been identified, (Bathgate *et al*, 2002) is expressed in the brainstem. Relaxin mRNA H1 and H2 are expressed in human left ventricle, right atrium, the internal mammary artery and saphenous vein.

1.6.1 In Health.

Relaxin is produced in both pre- and post-menopausal women and also in men. The highest plasma concentrations are attained during pregnancy at around 1-2ng/ml, when relaxin is produced mainly by the corpus luteum and also the placenta. Relaxin can be found in peripheral blood in conception cycles by the time of missed menses and concentrations rapidly rise and peak by the middle of the first trimester of pregnancy. Serum levels then fall by approximately 20% and remain stable throughout pregnancy. (Weiss, 1991).

As previously stated, relaxin prepares the birth canal for delivery. Initially however, relaxin increases the secretion of prostacyclins which decrease myometrial contractility. Later, relaxin ripens the human cervix at term therefore inducing labour and plays a part in the changes in pelvic diameter as demonstrated radiographically.with pubic symphysis changes. Elevated first trimester relaxin levels have been shown to be associated with an increased risk of premature delivery (Weiss *et al*, 1993).

Another action of relaxin during pregnancy is on glucose metabolism. Relaxin increases the affinity of insulin to its own receptor in isolated human adipocytes in vitro from women at term gestation. Thus it is possible that circulating relaxin may have a protective effect from the diabetogenetic effects of pregnancy (Weiss, 1991).

In non-pregnant women, relaxin is produced by the corpus luteum. Levels during the menstrual cycle are highest after the luteinising hormone (LH) surge, around 30-150 pg/ml. Since this is the period of nidation, a role for relaxin during this phase has been postulated.

In men, relaxin is produced by the prostate gland. This is verified by taking semen samples that exclude testicular and seminal vesicle components, such as samples from men who have had a vasectomy or from men with congenital absence of the vas and seminal vesicles, where relaxin concentrations are undiminished. (Weiss, 1991). This observation has been confirmed by the finding that in split ejaculates the first part of the ejaculate is richer in relaxin. The first part of the split ejaculate is predominantly prostatic, whereas, the second part is predominantly from the seminal vesicles. (DeCooman *et al*, 1983).

It has been demonstrated that physiological concentrations of relaxin are effective in increasing sperm penetration into cervical mucus, which is the first barrier that sperm meets in traversing the female genital tract (Brenner *et al*, 1984). Relaxin

also significantly increases the motility of sperm in situations of decreased motility i.e. when sperm motility is apparently optimal, as in normal samples, addition of relaxin does not produce a further increase in motility. (Lessing *et al*, 1986). Relaxin secretion into seminal plasma by the male represents a novel physiological mechanism for hormone delivery.

1.6.2 In Disease.

Plasma relaxin concentrations are also increased in the pathophysiological states. The heart has been identified both as an additional source of relaxin and also as a target for this hormone. Specific binding sites for relaxin have been found in cardiac tissues, particularly the atria (Taylor *et al*, 1994), (Osheroff *et al*, 1992). Relaxin has been reported to increase natriuretic peptide secretion and to have positive chronotropic actions. (Toth *et al*, 1996) Of note, cardiac secretion and plasma concentrations of relaxin increase in heart failure and these increases are greatest in those patients with the most severe degrees of failure (15-20pg/ml) (Dschietzig *et al*, 2001). This suggests that the heart may be producing relaxin as a "compensatory" or protective response, consistent with its role as an endocrine organ. This will be discussed in more detail later.

Hocher *et al*, investigated the impact of relaxin on deaths in patients with end-stage kidney disease (ESKD). Patients with ESKD have a reduced life expectancy

mainly as a result of cardiovascular disease. 245 patients (123 male) on long term haemodialysis were followed up for 1140 days. Survival was compared by the Kaplan-Meier method and Cox regression analysis. Elevated serum relaxin concentrations (greater than median of 28.8 pg/ml) independently predicted all-cause and cardiovascular death in male but not female patients (Hocher *et al*, 2004).

1.7 SECRETION OF RELAXIN.

It is known that relaxin is produced in the corpus luteum. Taylor and Clark examined whether prostacyclin may act as a secretagogue for relaxin release from cultured porcine luteal cells (Taylor and Clark, 1987).

The release of relaxin from cultured porcine luteal cells (derived from pregnant sows) was detected by a reverse haemolytic plaque assay. In this assay, luteal cells are cocultured in monolayers with protein-A coupled ovine erythrocytes. In the presence of porcine relaxin antiserum and complement, a zone of haemolysis i.e. a plaque, develops around relaxin-releasing luteal cells. Treatment with a prostacyclin analogue, carba-prostacyclin, significantly accelerated the rate of plaque formation in a dose dependent manner. This analogue, chemically stable in aqueous medium, exerts potent PGI-2-like effects both *in vivo* and *in vitro*. Therefore, it appears that PGI-2 acts as a secretagogue for relaxin release from cultured porcine luteal cells.

Taylor and Clark also studied what may act as a local inhibitory mechanism to regulate relaxin secretion (Taylor and Clark, 1992). They examined basic fibroblast growth factor (FGF) which is involved in the development and function of the corpus luteum and has been shown to regulate ovarian steroidogenesis (Baird and Hsueh, 1986). Exposure of luteal cell-containing monolayers to basic FGF resulted in a significant reduction ($p<0.05$) in the rate of relaxin-induced plaque formation. In addition, PGE-2-stimulated secretion of relaxin by porcine luteal cells was diminished by basic FGF suggesting that there stimulatory and inhibitory agents acting to achieve fine control of relaxin secretion.

1.8 VASCULAR ACTIONS OF RELAXIN.

Before discussing the current literature on the vascular actions of relaxin, I will briefly describe the anatomy and physiology of blood vessels.

1.8.1 Anatomy and Function of Blood Vessels

Arteries have three layers: the intima, media and adventitia. The intima consists of the vascular endothelium, which is a single layer of cells and a thin layer of connective tissue. It is separated from the media by the internal elastic lamina made of elastin and fibrous tissue. The media consists of fibrous tissue, vascular smooth muscle and elastin. The media is separated from the adventitia by the

external elastic lamina. The adventitia consists of collagen and fibrous tissue that forms loose connective tissue.

The connective tissue of large arteries contains more elastin, whereas smaller arteries have more collagen. The elastic properties of healthy large arteries, such as the ascending aorta, help to cushion the stroke volume, decrease the work of ejection by the left ventricle and maintain pressure during diastole. The smaller arterioles and resistance arteries (the focus of my research) are able to regulate peripheral resistance by changing vascular smooth muscle tone to alter the lumen size.

1.8.1.1 Endothelial function

The healthy endothelium is an autocrine and paracrine organ that produces substances that decrease vascular smooth muscle tone and inhibit inflammation and thrombosis. These substances include nitric oxide, prostacyclin other endothelium dependent vasodilators such as endothelium-derived hyperpolarizing factor and plasminogen activators. In disease states or after injury by factors such as abnormal strain, temperature or risk factors for atherosclerosis, the endothelium produces substances that increase vascular tone, promote inflammation and enhance thrombosis. These substances include cytokines, growth factors, endothelins and plasminogen inhibitors.

The principle vasodilators produced by the endothelium include nitric oxide, prostacyclin and endothelium- derived hyperpolarising factor (EDHF). Of these nitric oxide has a central role in mediating many functions of the endothelium aside from vasodilation (Alexander and Dzau, 2000), (Kinlay, 2007).

1.8.1.2 Nitric oxide

Nitric oxide is generated in the endothelium from the amino acid L-arginine by nitric oxide synthase (NOS). Nitric oxide diffuses through the arterial wall and enters vascular smooth muscle cells in the media, where it increases the activity of guanylate cyclase and the concentration of cyclic guanosine monophosphate (cGMP). The increased level of cGMP relaxes vascular smooth muscle and leads to vasodilation.

1.8.1.3 Prostacyclin

Prostacyclin is another endothelial product that induces arterial dilation. It is produced from arachidonic acid by cyclooxygenase in response to shear stress or certain factors that also increase nitric oxide production. Prostacyclin activates adenylate cyclase to increase production of cyclic adenosine monophosphate (cAMP).

1.8.1.4 EDHF

A residual vasodilatory response to various stimuli after blocking nitric oxide and prostacyclin generation led to the discovery of EDHF. EDHF appears to be more important in the small arteries than large conduit arteries.

1.8.2 Systemic Arteries.

Relaxin may be a circulating endogenous agent capable of regulating vascular tone (Bani *et al*, 1998). In keeping with this hypothesis, relaxin has vascular effects. Though the focus of existing studies has been on the uteroplacental bed, which is not typical of the systemic or pulmonary circulations, two recent studies have examined the effect of this hormone in other vessels. Bani *et al* examined vascular smooth muscle cells cultured form bovine aorta. These cells were incubated with relaxin at concentrations ranging from 1nmol/L to 1μmol/L. The expression and activity of nitric oxide synthase, production of nitric oxide and the intracellular levels of cGMP and calcium (Ca^{2+}) were determined. The cell morphology and signal transduction mechanisms of these bovine aortic smooth muscle cells in response to relaxin were also studied. Relaxin was found to increase the expression and activity of inducible nitric oxide synthase (iNOS), nitric oxide production and intracellular concentrations of cyclic GMP. Concurrently, relaxin significantly decreased cytosolic Ca^{2+} concentrations and caused changes in cell shape and the

actin cytoskeleton that were consistent with cell relaxation (Bani *et al*, 1998). Danielson et al have shown that relaxin causes renal vasodilation and this will be discussed further in a later section (Danielson *et al*, 1999).

Relaxin has recently been described as a "potential new treatment for vasoconstrictive disorders". (Whelan J, 2000). However, its vasodilatory action was first recognised over forty years ago. Casten *et al* in an uncontrolled trial, found that, in patients with severe obliterative peripheral vascular disease (PVD), treated with intramuscular injections of porcine relaxin, there was consistent healing of ischaemic ulcerations (Casten *et al*, 1960). Patients also reported an improvement in symptoms of intermittent claudication and rest pain although there was a return of symptoms with cessation of the treatment. Interestingly, patients in this trial who also had coexistent severe coronary artery disease, reported a dramatic improvement with regard to their nitroglycerin requirements. One patient who, prior to treatment, had required an average of 100 nitroglycerin tablets per week, only needed 2-3 tablets during relaxin therapy.

A phase II clinical trial of patients with peripheral arterial disease who have recently undergone surgical revascularization of a lower extremity and who have at least one unhealed ischaemic or operative wound in that region, is currently underway. Treatment consists of a subcutaneous infusion of relaxin at 10, 25, or 100µg kg^{-1} day^{-1} or placebo. The study will evaluate the time to complete wound healing as well as a range of related parameters. As peripheral arterial disease is

often accompanied by renal disease, the study will also evaluate the effect of relaxin on renal function (Whelan, 2000). The results of this study have yet to be published.

It has been noted that Raynaud's phenomenon, related to vasoconstriction in the arteries in the fingers, disappears during pregnancy (when relaxin levels are at their highest). (Casten, 1958). This observation would therefore support relaxin's vasodilatory effect in humans.

Also in keeping with its vasodilatory action, relaxin has been found to decrease blood pressure and blunt responses to vasoconstrictors of mesenteric vessels in spontaneously hypertensive rats. Massicotte *et al* investigated the vascular reactivity to angiotensin II, arginine-vasopressin and norepinephrine in the perfused mesenteric artery and portal vein of control and relaxin-treated virgin spontaneously hypertensive rats. The latter received an intravenous infusion of 75ng/hr purified rat relaxin for 2 days, whereas the controls were given an equal infusion of saline. All of the animals were then killed and their tissues processed for in vitro study. In the perfused mesenteric artery, the concentration-response curves for arginine-vasopressin and norepinephrine were shifted to the right by a factor of about 2 ($p<0.05$ and $p<0.005$, respectively) after relaxin treatment. In the isolated portal vein, the response to angiotensin II was not affected; the effect of norepinephrine was slightly displaced to the right (increase in EC_{50}) and the maximum response remained unchanged. Relaxin, however, has no effect on Wiskar - Kyoto controls. (Massicotte *et al*, 1989).

1.8.3 Renal Arteries.

Relaxin is a potent renal vasodilator. (Danielson *et al*, 1999). After several days of treatment with either purified porcine relaxin or recombinant human relaxin, effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) increased by 40% in adult female rats. This was also observed in ovariectomized rats, suggesting that neither oestrogen nor progesterone is necessary for the renal response to relaxin. Further to this, the role of relaxin on the renal vasculature during pregnancy has been investigated. Striking changes are seen in the maternal cardiovascular system during pregnancy. Cardiac output, global arterial compliance, effective renal plasma flow (ERPF) and GFR rise from 30% to 80%. Vascular resistance and blood pressure decrease. These alterations begin immediately after conception, peak by the end of the first or the beginning of the second trimester and persist throughout gestation. Traditionally, oestrogen has been viewed as the uterine and systemic vasodilator of pregnancy but this hormone has little effect on the renal circulation. (Christy *et al*, 1974).

Novak et al used two approaches to eliminate relaxin or its biological activity from the circulation: ovariectomy and administration of relaxin neutralizing antibodies. (To maintain pregnancy in the ovariectomized rats, silastic tubes containing 17β oestradiol and progesterone were implanted). Neutralizing mAb against rat relaxin (MCA1) or control mAb against fluorescein (MCAF) was administered daily. They showed that the relaxin neutralizing antibody administered to pregnant Long-Evans

rats completely abolished the increase in GFR and ERPF associated with pregnancy. The same was seen in the ovariectomized, steroid replaced rats. Therefore, relaxin is in fact essential for renal vasodilation during pregnancy. (Novak *et al*, 2001).

Evidence of interaction between relaxin and other vasoactive peptides has been demonstrated in the renal arteries. Relaxin attenuates the vasoconstrictor effect of angiotensin II (Danielson *et al*, 1999).

1.8.4 Relaxin and Myogenic Reactivity.

Myogenic reactivity is defined as the response (either constriction or dilation) of an artery to a rapid change in intraluminal pressure. Novak *et al* investigated the myogenic responses of small renal and mesenteric arteries in relaxin-treated Long-Evans rats. They found that arteries from relaxin-treated rats constrict less than those from vehicle-treated rats in response to an increase in intraluminal pressure. (Novak *et al*, 2002).

In a previous study, Novak et al had demonstrated that circulating relaxin mediates myogenic reactivity of isolated small renal arteries in gravid rats. When relaxin neutralizing antibodies were administered or pregnant rats were ovariectomized, myogenic reactivity was restored to virgin levels (Novak *et al,* 2001). Thus Novak

et al, have demonstrated the influence of relaxin on myogenic reactivity in both the pregnant and non- pregnant state.

1.8.5 Penile Arteries .

Further evidence of relaxin's vasodilatory action has been demonstrated. Bigazzi *et al* showed that local injection of relaxin into the cavernous body of human volunteers increased blood flow in the deep penile artery. This was evaluated by echo Doppler apparatus (Bigazzi *et al*, 1995).

1.8.6 Uteroplacental Arteries.

It should be noted though that relaxin does not have a vasodilatory effect on all blood vessels. Peterssen *et al*, investigated the effect of synthetic human relaxin (hRLX-2) on isolated human myometrium and on uteroplacental arteries from term pregnant women. Relaxin did not dilate the arteries preconstricted with norepinephrine, endothelin or U46619. (Petersen *et al*, 1991).

This is in contrast with the findings of Longo *et al* who investigated the effects of recombinant human relaxin on rat uterine arteries and myometrial rings. In rats, relaxin did cause uterine artery relaxation. This was noted to be greater at mid

pregnancy compared to term. Relaxin was also found to inhibit spontaneous contractions at mid pregnancy but not at term. Relaxin had no effect on oxytocin- or indolactam-V-induced contractions. (Indolactam-V is a protein kinase C activator). (Longo *et al*, 2003).

1.8.7 Pulmonary Arteries.

During my MD thesis, I have investigated the potential role of relaxin in human pulmonary resistance arteries. Prior to this, the vasoactive role of relaxin in human pulmonary arteries had not been described. The methods and results shall be discussed in detail in later chapters (2 and 3).

1.8.8 Veins.

Prior to my work on human long saphenous veins as part of my MD thesis, the effect of relaxin on the human venous system had not been described. The methodology used will be described in Chapter 2 and the results will be described in Chapter 5.

1.9 RELAXIN AND THE HEART.

In recent years, evidence is accumulating that relaxin has a major influence on the cardiovascular system and may be involved in cardiovascular regulation. (Bigazzi *et al*, 2001). Relaxin has positive chronotropic actions and this may contribute to the increase in cardiac output seen in early pregnancy. During the first trimester, cardiac output increases by about 40%. During this time relaxin levels are at their highest peaking at week 10.

1.9.1 The Heart as a Source of Relaxin

The heart has been identified both as an additional source of relaxin and also as a target for this hormone (Taylor *et al*, 1994). Antibody-directed, complement-induced erythrocyte lysis (reverse haemolytic plaque assay) around atrial cardiocytes was used to determine whether this cell type possesses the capacity to secrete relaxin. After two hours of incubaton, 33 +/- 4% (n=3) of cardiocytes derived from the atria of neonatal rats secreted detectable amounts of immunoreactive relaxin (i.e. formed plaques) when cultured in monolayers. The observation that only one third of atrial cardiocytes secreted relaxin under basal conditions is consistent with a similar study which reported that only a subset of (27%) of rat atrial cardiocytes secreted atrial natriuretic peptide (ANP) under non-stimulated conditions. (Miller and Southerland, 1990).

Increased culture time of cardiocytes failed to increase the fraction of cardiocytes that secreted relaxin. However, the cumulative amount of relaxin secreted after 3 hours of incubation (plaque area) was 31% greater ($p<0.05$) than the amount of hormone present after 1 hour of incubation, providing evidence for sustained peptide secretion by cultured cardiocytes. The authors conclude that these data suggest that the source of the endogenous ligand for the specific and high-affinity relaxin receptors located in the rat atria is the atrial cardiocyte itself, following on from the work performed by Osheroff *et al* (Osheroff *et al*, 1992).

1.9.2 Relaxin and Atrial Binding Sites.

Osheroff *et al,* demonstrated that specific binding sites for relaxin can be found in cardiac tissues, particularly the atria. Nine different rat tissues, including the liver, spleen, thymus, kidney, adrenal gland, heart, lung, skin and testis were examined for the binding of the tracer ^{32}P- relaxin (phosphorylated relaxin). Of all of these tissues, specific binding was seen clearly in the heart atria. The ventricles by contrast did not show detectable binding under these experimental conditions. The specificity of binding was demonstrated by the binding displacement of 100pM ^{32}P- relaxin by 100nM unlabelled relaxin but not by 100nM insulin like growth factor I (IGF-I), insulin, angiotensin II and atrial natriuretic peptide (Osheroff *et al*, 1992).

These specific and high affinity relaxin receptors present in both male and female rat atria are regulated differently than the relaxin receptors in the uterus. It was found that the relaxin binding in the uterus was diminished by 53% overall following ovariectomy but was restored to 90% of normal levels when treated with oestrogen. Relaxin binding in the heart however was not affected by ovariectomy or oestrogen therapy. This is in keeping with the findings, previously described, demonstrating the lack of oestrogen requirement of relaxin for its renal vasodilatory action. (Danielson *et al*, 1999).

1.9.3 Inotropic and Chronotropic Effects of Relaxin.

The cardiac effects of human gene-2 relaxin (hRlx-2), in isolated rat atria, has been investigated (Kakouris *et al*, 1992). Using hearts from male Sprague-Dawley rats, atria were removed and mounted separately under a resting tension of 0.25g in 20ml organ baths containing Krebs' bicarbonate buffer. Right atria were allowed to beat spontaneously, whereas left atria were driven at 5 Hz with square-wave pulses of 2ms at 1.5 times the threshold voltage with a stimulator. Synthetic hRlx-2 (0.03-3 nmol/l) increased the heart rate of contraction in rat spontaneously beating right atria by a maximum of 131 (15) beats per minute from a baseline of 179 (25) beats per minute. The $EC_{50,}$ i.e. the concentration required to produce 50% of the maximal response, was 0.09 (SE 0.03) nmol/l. In the electrically driven left atria, synthetic hRlx-2 increased the force of contraction by a maximum of 0.19 (0.03) g

from a baseline of 0.15 (0.02) g (EC_{50} =0.31 (0.02) nmol/l). The authors noted that the EC_{50} value of 0.1-0.3 nmol/l for synthetic hRlx-2 in isolated rat atria is lower than that of endothelin, angiotension II or isoprenaline, making relaxin one of the most potent chronotropic and inotropic agents known.

More recently, Debrah *et al*, have shown that relaxin increases the cardiac output and reduces systemic arterial load in hypertensive rats (Debrah *et al*, 2005). Two models were used in this study: Long-Evans rats chronically administered with angiotensin II (AII) and spontaneously hypertensive rats (SHR). Debrah et al had noted and as I describe elsewhere, relaxin antagonizes the action of AII in rats. (Danielson et al,1999). Cardiac output and systemic arterial load were quantified by systemic vascular resistance (SVR) and AC_g (global arterial compliance). Rats were either administered relaxin acutely over a 6 hour period or chronically over 6 days.

In rats with AII-induced hypertension, acute RLX administration (up to 6 hours) significantly increased CO and AC_g (24.9+/-3.9 and 34.3+/-12.6% above baseline respectively) and significantly decreased SVR (17.2+/-3.5%) without changing mean arterial pressure (MAP). In contrast, acute RLX administration to SHR and normotensive rats for up to 6 hours failed to produce any significant change in CO, AC_g, SVR or MAP. However, chronic administration of RLX (1 to 7 days) to SHR yielded significant changes (24.0+/-8.1 and 22.3+/-6.6% increases in CO and AC_g, respectively, and a 13.3+/-5.3% decrease in SVR with no change in MAP.

Therefore, the time course to RLX treatment is dependent on the model of hypertension as rats with AII-induced hypertension responded more rapidly to RLX administration than SHR.

Debrah *et al* have since shown that relaxin is essential for the systemic vasodilation and increased global arterial compliance during early pregnancy in conscious rats (Debrah *et al*, 2006). They administered relaxin neutralizing antibodies daily beginning on day 8 of gestation to block the functional effects of circulating relaxin and used an antibody against fluorescein as a control. In the pregnant rats administered the relaxin neutralising antibody, there was no gestational increase in stroke volume, cardiac output and global arterial compliance or decrease in SVR, that would be expected and which was observed in the pregnant control rats. This suggests that relaxin mediates the transition of changes observed in the systemic circulation from the non-pregnant to the pregnant state in the rat model.

1.9.4 Relaxin and Heart Failure.

Relaxin production is increased in heart failure, higher levels correlating with the severity of heart failure. (Dschietzig *et al*, 2001). In this study, patients were classified as having severe heart failure if in functional class New York Heart Association (NYHA) class IV who required intensive care treatment for acute left heart decompensation (orthopnoea and signs of severe pulmonary congestion) with a pulmonary wedge pressure (PCWP) of >25mmHg and cardiac index (CI) < 2.5

l/min/m^2 . Acute ischaemia was excluded on the basis of electrocardiography as well as kinetics of enzymes (troponins, creatinine kinase) and myoglobin. The mean left ventricular ejection fraction (LVEF) was 19%. Patients were assigned to the moderate congestive heart failure (CHF) group if in NYHA class II and displayed a PCWP <20mmHg and a CI > 2.5 l/min/m^2 . The mean LVEF of these patients was 27%. The controls were patients who underwent cardiac catheterization for suspected coronary artery disease and in whom no structural cardiovascular disease was detected.

In all groups, catheters were positioned in the pulmonary artery (PA) (Swan Ganz), the coronary sinus (CS), and the left ventricle (LV) under fluoroscopic control. Blood sampling and haemodynamic measurements were then carried out. Whilst men and women with normal coronary arteries and normal left ventricular systolic function were noted to have plasma concentrations in the range 1-2 pg/ml, patients with mild to moderate heart failure, had plasma relaxin concentrations 5-10 pg/ml and those with severe heart failure concentrations of 15-20 pg/ml. Eleven out of the fourteen patients with severe CHF (i.e. 79%) showed higher relaxin plasma levels in the coronary sinus than in the left ventricle which indicates net coronary release of relaxin in the majority of patients suffering from severe heart failure.

The same group found that myocardial expression of the two relaxin genes, (H1 and H2) correlates with the severity of heart failure. For the RNA analysis, control samples were human total ventricular RNA from healthy individuals and a sample

from one donor heart that was not transplanted. Failing myocardium was from patients with dilated cardiomyopathy and ischaemic heart disease who underwent partial ventriculectomy or transplantation.

Dschietzig *et al* demonstrated pronounced up-regulation of GAPDH-normalized H1 mRNA (failing hearts, 14.4+/-2.4 vs. non-failing hearts 1.0+/-0.5 arbitary units; <0.05) and a moderate increase in H2 mRNA (2.6+/-0.4 vs. 1.0+/-0.2 units, <0.05) in left ventricular specimens of failing hearts compared with non-failing hearts. Results for right atrial H2 mRNA also showed a more pronounced elevation in failing hearts (3.8+/-1.0 vs. 1.0+/-0.1 units, <0.05). In contrast, expression of relaxin mRNA did not appear to differ in mammary arteries and saphenous veins from patients with normal heart function and patients with chronic heart failure.
Increased ventricular filling pressures results in up-regulation of relaxin expression. Elevation of left ventricular end diastolic pressure from 5 to 25mmHg up-regulates preprorelaxin (which processes the precursor of relaxin) mRNA in isolated rat hearts. In contrast, elevation of both right and left atrial pressures has no effect on the expression of relaxin mRNA.

In severe heart failure, a significant inverse correlation was found between relaxin and endothelin-1 (ET-1). ET-1 represents one of the most powerful mediators in CHF progression. Patients in this group (i.e. severe heart failure) with high relaxin levels had the lowest circulating levels of ET-1. It would seem, therefore, that

relaxin may be produced as a compensatory mediator in cardiovascular disease. In patients with moderate CHF and in controls, no such correlation was seen.

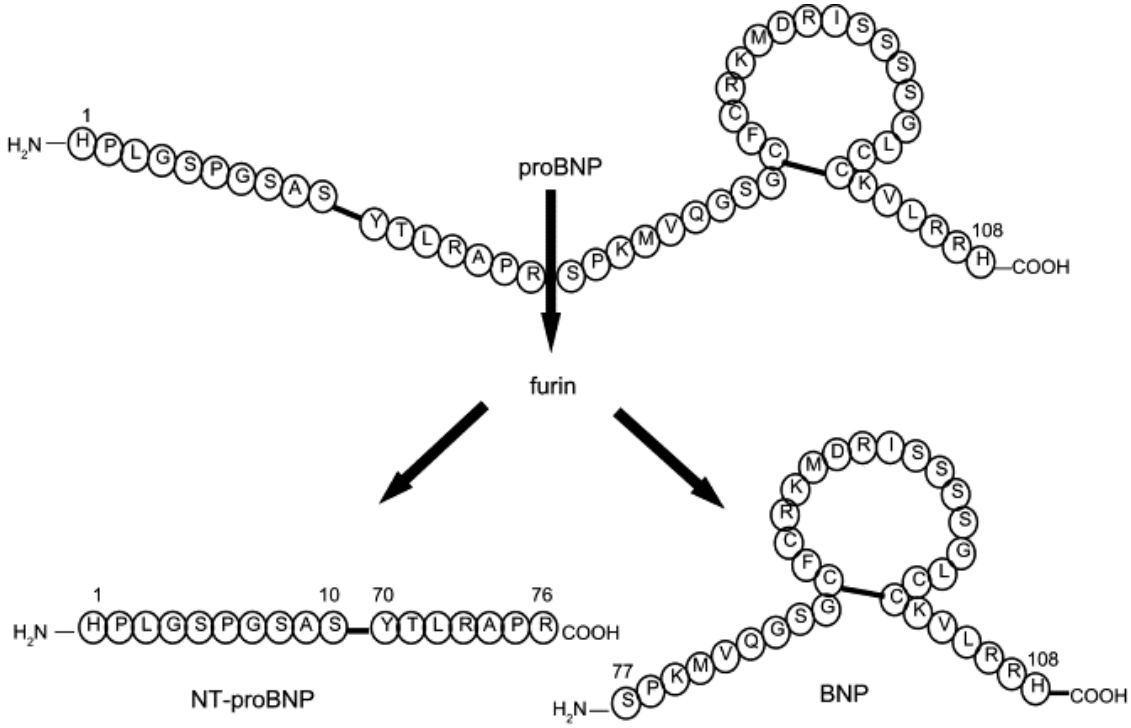
1.9.5 NT pro BNP and Heart Failure

Another peptide, N-terminal pro B type natriuretic peptide (NT pro BNP) has been shown to be elevated in patients with heart failure. As part of my MD thesis, I investigated whether the plasma concentration of relaxin predicts clinical outcome in patients with CHF and compared with this NT pro BNP (see chapter 6). I will therefore at this stage, describe the peptide NT pro BNP in more detail.

In the early 1980s, DeBold *et al* observed that extracts of atrial tissue infused into rats caused a massive diuresis (DeBold *et al*, 1981). From this work came the isolation of atrial natriuretic peptide (ANP), the first in the family of natriuretic peptides (DeBold, 1985). A decade later, the second member of the family was described, B-type natriuretic peptide (BNP). Since then, four different natriuretic peptides have been described: ANP, BNP, C-type natriuretic peptide (CNP) and D-type natriuretic peptide (DNP). They all contain a characteristic 17 amino-acid ring structure, formed by a disulfide bridge between two cysteine residues. The amino- and carboxy- terminal tail varies between the different peptides: ANP has a 28 amino acid polypeptide, BNP a 32 amino acid polypeptide, CNP a 53 amino acid polypeptide and DNP a 38 amino acid polypeptide. They all exist as pro-hormones with relatively high molecular weight, which are cleaved to active moieties before release into the circulation.

BNP is released in bursts as a 108 amino acid prohormone which is then cleaved to form biologically active BNP, a 32 amino acid molecule and the 76 amino acid molecule, NT-proBNP. Figure 1.2 summaries this process.

Figure 1.3 Structure and formation of BNP and NT-proBNP (adapted from Hall, 2004).



BNP, and more recently NT-proBNP have been recognised to be of particular importance in heart failure. The predominant source of BNP in humans is the ventricular myocardium (Hunt, 1997). In patients with LV systolic dysfunction

both BNP and NT-proBNP are elevated, however, increases in NT-proBNP are two-to ten-fold greater than increases in BNP. The explanation for this is unknown. Both BNP and NT-proBNP can be measured by radioimmunoassay (RI). The combination of its longer half-life and greater increases in heart failure, may make NT-proBNP a better marker of heart failure (Hunt, 1997). Recently, NT-proBNP has been shown to be elevated in patients with heart failure, post-MI LV systolic dysfunction and asymptomatic LV dysfunction (Hobbs *et al*, 2002), (Masson *et al*, 2002), (Hunt *et al*, 1997), (Richards *et al*, 1998) , (Groenning *et al*, 2002).

1.9.6 Relaxin and Cardiac Anaphylaxis

Cardiac anaphylaxis refers to the functional and metabolic changes in the heart caused by the anaphylactic release of histamine and vasoactive products of arachidonic acid cascade by mast cells and basophils. As in most type 1 hypersensitivity-based diseases, histamine plays a key role in the pathophysiology of cardiac anaphylaxis. In the heart, mast cell activation and histamine release are controlled by multiple endogenous mechanisms, including adrenergic neural control, histamine-dependent negative feedback operated through H2 receptors and the endogenous generation of nitric oxide and carbon monoxide.

A reliable model of cardiac anaphylaxis *ex vivo* can be reproduced using perfused hearts isolated from sensitized guinea pigs and subjected to challenge with the

specific antigen. A typical anaphylactic crisis is characterized by arrhythmias, sinus tachycardia and increase in the strength of contraction. These mechanical abnormalities are accompanied by a fast, short lasting decrease in the coronary flow followed by a sustained flow increase.

Masini *et al* demonstrated that *ex vivo* perfusion of hearts isolated from ovalbumin-sensitised guinea pigs with relaxin affords a marked protection against anaphylactic reaction induced by challenge with the specific antigen. They found that the protective effect of relaxin was exerted at low, nanomolar concentrations ($30ngml^{-1}$) and for short exposure times (30 minutes). A significant reduction of histamine release from resident mast cells (the main repository of cardiac histamine) was seen. No significant changes were observed in beat rate or contraction strength. No short-lasting decrease in coronary flow took place and the subsequent flow increase was significantly higher than in the untreated control hearts (Masini *et al*, 2002).

1.9.7 Relaxin and Prevention of Cardiac Ischaemia/Reperfusion Injury

Relaxin has been shown, as detailed above, to increase coronary blood flow and acts as a positive chronotropic and inotropic agent in the heart. Perna *et al*, tested the therapeutic potential of relaxin in a swine model of ischaemia/reperfusion-induced acute myocardial infarction which is used to test cardiotropic drugs.

Ischaemia for 30 minutes was obtained by transient ligation of the left anterior descending artery after the 2nd diagonal branch and was followed by reperfusion for a further 3 hours. Five minutes before reperfusion, lidocaine was administered to reduce the occurrence of lethal ventricular fibrillation. Relaxin was infused at reperfusion through the right atrial catheter. Measurements of blood and tissue markers of myocardial injury and inflammation were taken. In addition, functional evaluation of myocardial salvage by cardiac single- photon emission computed tomography (SPECT) was performed. By using the tracer, ^{201}Thallium chloride (which is a potassium competitor and therefore labels viable cardiac cells), the investigators were able to distinguish between normal myocardium, ischaemic myocardium and irreversibly damaged myocardium.

Administration of relaxin caused a statistically significant reduction of serum myoglobin, CK-MB and troponin T (markers of myocardial cell damage) which rose markedly in the control animals given the vehicle alone. Relaxin also reduced myeloperoxidase (MPO), a marker of inflammatory leukocyte infiltration and malondialdehyde (MDA), a marker of free radical-mediated cell damage. By SPECT analysis, in the relaxin treated swine, there was a striking reduction of the irreversibly injured myocardial tissue (Student's *t*-test: $p<0.05$). The relaxin-induced salvage improved contractile performance of the heart, indicated by a stable increase in cardiac index. The authors point out that the protection afforded by relaxin is exerted at plasma levels which are within the physiological range in humans. They feel that clinical trials with relaxin as adjunctive therapy to catheter-

based coronary angioplasty in patients with acute myocardial infarction may be warranted (Perna *et al,* 2005).

1.10 RELAXIN AND FLUID BALANCE.

During pregnancy, there is a decrease in plasma osmolality which begins early around gestational week 5 in women which returns to normal by 2 weeks post partum. This has also been demonstrated in the rat where the decrease in plasma osmolality is thought to be due to a decrease in both the threshold for thirst and for arginine vasopressin (AVP) release such that the relationship P_{osmol}/P_{AVP} shifts to the left. Thus a decrease of about 10 mosmol/kg occurs because more water is drunk and less excreted because the P_{AVP} is seemingly inappropriately high for the P_{osmol}. The decrease in P_{osmol} is dependent on the presence of the ovary and treatment of rats with various amounts of oestrogen and/or progesterone does have a small effect on the P_{osmol}/P_{AVP} relationship. However, treatment of rats or non-pregnant women with the appropriate amount of ovarian steroids does not produce the changes in the P_{osmol}/P_{AVP} relationship seen in pregnancy. (Barron *et al*, 1986; Lindheimer *et al*, 1989). Weisinger *et al*, noted that relaxin concentration increases in the rat and in the pregnant women at the appropriate stage of pregnancy and investigated this further. They found that in ovariectomized rats treated with intravenous synthetic human relaxin, P_{osmol} was significantly lower ($p<0.001$) than that in the two control groups (either no treatment or treatment with vehicle), but

the P_{AVP} was unchanged. This decrease in osmotic threshold for AVP release produced by intravenous relaxin in ovariectomized rats, which is observed in pregnant women and rats, was achieved at concentrations of relaxin found in pregnant rats. Weisinger *et al* were unable to demonstrate the exact mechanism of action.

Robertson *et al*, showed that relaxin stimulates vasopressin secretion from the posterior pituitary gland, triggering thirst and increasing water intake. (Robertson *et al*, 1991).

This key role in fluid balance played by relaxin during pregnancy has been further demonstrated by Novak *et al*. Neutralizing antibodies against rat relaxin (MCA1) or control antibodies against fluoroscein (MCAF) were administered to pregnant Long-Evans rats. In pregnancy there is an expected rise in effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) of between 30 and 80%. This did not occur in the rats that received the neutralizing MCA1 (ie the relaxin neutralizing antibody). The usual decreases in plasma osmolality and sodium concentration found during pregnancy were also inhibited by the MCA1 antibody. Relaxin therefore plays a central role in fluid homeostasis (Novak *et al,* 2001)

1.11 RELAXIN AND VASCULAR ENDOTHELIAL GROWTH FACTOR.

The actions of relaxin in pregnancy have been well documented. By inhibiting collagen synthesis and promoting collagen breakdown through increased synthesis of collagenase, relaxin facilitates enlargement of the uterus, abdomen and breasts and loosens the pelvic ligaments. It is also thought to mediate blood vessel dilation, both in pregnancy and during the menstrual cycle. (Unemori *et al*, 1999).

The importance of relaxin in early pregnancy centres on the induction of expression of an angiogenic agent, vascular endothelial growth factor (VEGF). This suggests that relaxin may be involved in the preparation of the endometrium for nidation. The evidence for the induction of VEGF by relaxin expression is supported clinically. In a recent trial investigating relaxin as a potential anti-fibrotic agent for the treatment of systemic sclerosis, (Seibold *et al*, 2000), the most commonly reported side effect of treatment, was menorrhagia. This in keeping with the hypothesis that relaxin mediates neovascularization of the endothelial lining.

Furthermore, relaxin is also responsible for stimulating ischaemic wound healing by increasing blood flow through vasodilation and angiogenesis. Rats were infused subcutaneously with recombinant human relaxin. Fluid and cells collected from the wounds were analysed for VEGF and basic fibroblast growth factor (bFGF);

another angiogenic agent. Expression of both factors was increased at wound sites in relaxin-treated rats compared with control rats (Arnold *et al*, 2000).

Another action of relaxin may also be responsible for the increase in menstrual blood flow mentioned above. In a study of platelet function, preincubation of human platelets with relaxin resulted in a significant, concentration dependent, inhibition of platelet aggregation. This was accompanied by an elevation of intraplatelet cGMP and a decrease in the rise of cytosolic calcium levels. Its effects appeared to be mediated through nitric oxide. This antiaggregatory property of relaxin suggests that it may play a role as an antithrombotic agent. (Bani *et al*, 1995).

Hypertension affects 10-15% of the adult population leading to structural remodeling of the left ventricular myocardium and eventually heart failure. Cardiac fibrosis is a hallmark of hypertensive heart disease and interferes with with normal structure and function of myocardium. Cardiac fibroblasts are activated and differentiate into myofibroblasts after cardiomyocyte death, inflammation enhanced workload, hypertrophy and stimulation by a number of hormones (e.g. angiotensin II), cytokines (e.g. interleukin-1) and growth factors (e.g. transforming factor- β [TGF-β].

Renal fibrosis is another major complication associated with the development and progression of hypertension. Collagens accumulate around renal resistance vessels,

glomeruli and interstitium which contributes to remodeling and progression of renal injury. Relaxin has been shown to reduce collagen synthesis, increase expression of matrix metalloproteinases (MMPs) to degrade collagen and antagonize the influence of pro-fibrotic factors.

Lekgabe *et al*, investigated the antifibrotic effects of relaxin on cardiac and renal fibrosis. (Lekgabe *et al*, 2005). They studied spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY). They found that over a 14 day period, H2 relaxin significantly reduced the elevated collagen content in the left ventricle and kidney, in particular types I, II and V collagen. In addition to this, H2 relaxin inhibited fibroblast proliferation and differentiation and induced a significant rise in MMP-2 expression. The authors conclude that since relaxin reverses cardiac and renal fibrosis that it may have therapeutic potential in hypertensive disease.

1.12 SUMMARY OF THE ACTIONS OF THE RELAXIN FAMILY PEPTIDES.

Knowledge of the relaxin family peptides and their receptors with their actions is developing at a rapid pace. **Figure 1.4** below summarises what is currently known of the tissue localisation of the relaxin family peptides, their receptors and their functions. Adapted from van der Westhuizen et al, 2008.

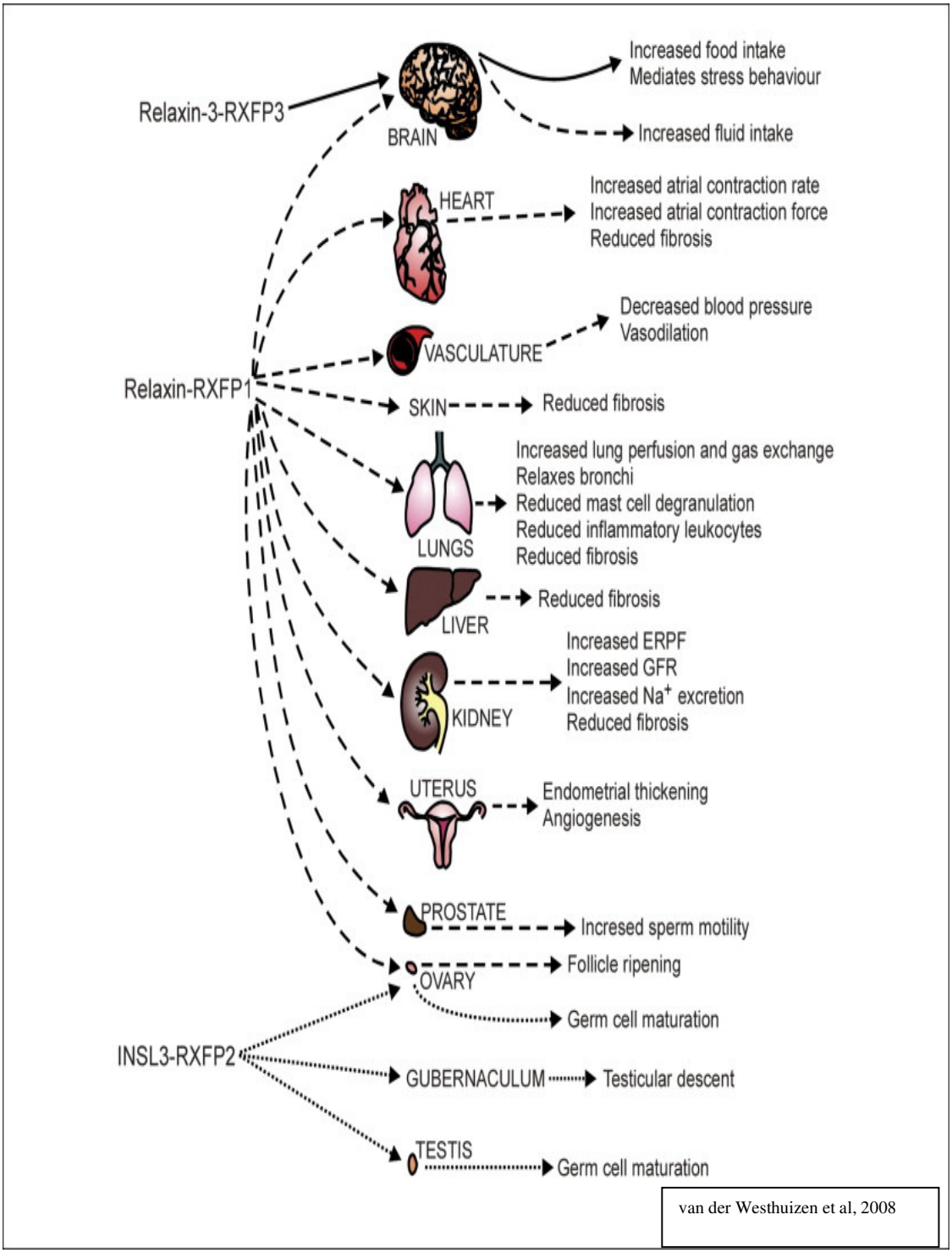


Figure 1.4 Relaxin family peptides, their receptors and their functions.

1.13 THE MECHANISM OF ACTION OF RELAXIN.

Although there has been a great deal of work demonstrating the mechanism of action of relaxin in animals, little is known of its mechanism of action in humans.

1.13.1 Relaxin and Nitric Oxide.

The mechanism of action of relaxin appears to involve the stimulation of the biosynthesis of nitric oxide (NO), a gaseous free radical and potent endogenous vasodilator. Under physiological conditions, the vasodilatory action of NO is thought to be primarily an endothelium-dependent process. Endothelial cells constitutively express a Ca^{2+} / calmodulin-dependent isoform of NOS (NOS III or eNOS) that continuously produces small amounts of NO, involved in a moment-to-moment regulation of the vascular tone. Endothelial cells also express the inducible, high- yield NO synthase isoform (NOS II or iNOS), which synthesizes greater amounts of NO than NOS III and can be up-regulated by different stimuli, especially inflammatory cytokines and mediators.

Relaxin generates nitric oxide in experimental animals. Bani *et al* examined vascular smooth muscle cells (VSMCs) cultured from bovine aorta. VSMCs were incubated with relaxin at concentrations ranging from 1nmol/l to 1 μmol/l. The choice of VSMCs of bovine origin was motivated by the fact that these cells retain a distinct muscular phenotype in *in vitro* culture, including the ability to produce nitric oxide (Mollace *et al*, 1991). Porcine relaxin has been found to be active in

cattle heifers *in vivo* (Musah *et al*, 1986). NOS activity was determined in cell homogenates by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. Nitric oxide production was determined by measuring the accumulation of nitrite, the stable end product of nitric oxide, in bovine aortic smooth muscle cell (BASMC) supernatants. Relaxin was found to increase the expression and activity of inducible nitric oxide synthase (iNOS), nitric oxide production and intracellular concentrations of cyclic GMP. The addition of the NOS inhibitor L-NMMA (*N*ω-monomethyl-L-arginine) abolished the effect of relaxin. This shows that relaxin directly activates the L-arginine-NO pathway in bovine arterial SMCs in culture. Relaxin was also noted to induce changes in cell shape and the actin cytoskeleton that are consistent with cell relaxation. (Bani *et al*, 1998).

Relaxin has also been shown to upregulate inducible nitric oxide synthase expression and nitric oxide generation *in vitro* in rat coronary endothelial (RCE) cells (Failli *et al*, 2001). RCE cells were isolated from Wistar rat hearts and incubated in the presence or absence of relaxin (60ng/ml) Evaluation of nitric oxide production was performed by measuring the accumulation of nitrite, a stable end product of nitric oxide, in the supernatant of the RCE cells. Expression of NO synthase isoenzymes II and III was analyzed by immunocytochemistry. The immunostained RCE cell cultures were examined. The control cells were almost negative for NOS II but showed a clear-cut cytoplasmic immunoreactivity for NOS III. A 24 hour incubation with relaxin caused a marked increase in the immunoreactivity for NOS II, whereas the immunoreactivity for NOS III was not

affected substantially. Since relaxin does not seem to influence NOS III isoform in RCE cells, the authors suggest that NOS II contributes the major amounts of biologically active NO in response to relaxin. The relaxin induced increase in coronary flow in experimental animals is also felt to be nitric oxide dependent (Bani Sacchi, 1995). More recently, Bani's group demonstrated that relaxin potentiates the expression of iNOS (or NOS II) by endothelial cells from human umbilical vein in *in vitro* culture (Quattrone et al, 2004). Relaxin treated cells showed an increased expression of NOS II, attaining a maximum with 1000ng/ml relaxin, which gave rise to increased NO generation, as shown by nitrite assay. This effect of relaxin appears to be mediated by activation of NOS II transcription factor NF-kappaB, since it was abolished by the NF-kappaB inhibitors curcumin-95 and dexamethasone. At variance with NOS II, the constitutive NOS III isoform appears unchanged upon relaxin treatment. Relaxin can therefore influence human umbilical vein endothelial cells (HUVEC) by up-regulating the expression of inducible NOS II mRNA and protein and in this respect, HUVEC behave similarly to bovine vascular smooth muscle cells and rat coronary endothelial cells.

Danielson *et al* found that the relaxin induced renal vasodilatation in both male and female Long Evans rats was likely to be nitric oxide dependent. Their study has been described previously in section 1.5.2 on renal arteries (Danielson *et al*, 1999). The renal vasodilation and hyperfiltration in the relaxin-treated rats observed on day 5 of the infusion was completely abolished by the infusion of *N*ω-nitro-L-

arginine methyl ester (L-NAME), a substrate competitive inhibitor of NO synthase, which was administered intravenously by infusion pump.

Novak *et al*, investigated the effect of relaxin, on the myogenic reactivity in rat renal arteries (section 1.5.3), and also tried to gain mechanistic insight. Thus myogenic reactivity was investigated following pretreatment of the small renal vessels with the NO synthesis inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) at 0.25mmol/l for 15 minutes. Relaxin treatment decreased myogenic reactivity but in the presence of L-NAME, the myogenic reactivity of relaxin-treated rat arteries was significantly increased (to the same response as vehicle-treated rats).

1.13.2 Relaxin and ANP

Relaxin has been reported to increase atrial natriuretic peptide (ANP) secretion, a peptide hormone involved in the regulation of blood pressure and fluid balance. ANP is synthesized and secreted predominantly by the atria of the adult mammalian heart. Toth *et al*, investigated the effect of relaxin on the isolated perfused spontaneously beating rat heart. A cyclic adenosine monophosphate (cAMP) dependent protein kinase inhibitor (H-89) was found to substantially reduce the ANP secretory effect of relaxin and a calcium/calmodulin dependent protein kinase inhibitor (KN-62) was found to decrease the positive chronotropic effect of relaxin. Both of these observations were statistically significant

($p<0.001$). The ANP secretory and chronotropic effects of relaxin were thought to involve activation of protein kinase C since administration of the protein kinase C inhibitor staurosporine at a concentration of 30nM completely blocked the effect of relaxin (10nM) on immunoreactive ANP (IR-ANP) secretion ($p<0.001$) and heart rate ($p<0.001$). The ANP secretory and chronotropic effects of relaxin in rats appear to be mediated by intracellular signal transduction pathways. (Toth *et al*, 1996).

1.13.3 Relaxin and NO-cGMP and cAMP Pathways.

Nitric oxide is generated in the vascular endothelium from L-arginine by a calcium-dependent NO synthase. Nitric oxide activates the soluble guanylate cyclase of the vascular smooth muscle, which increases intracellular cyclic guanosine monophosphate (cGMP) concentration, thus causing smooth muscle relaxation.

The vasodilatory effect of relaxin on the pregnant rat uterine artery has previously been described (section 1.5.5). Longo *et al*, investigated the mechanism of action of relaxin by using three inhibitors to examine the second-messenger systems involved. Therefore, relaxin effects in segments of uterine arteries were studied after preincubation for 30 minutes with, the NO synthetase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME 10^{-4} mol/l), the soluble guanylate cyclase

inhibitor, 1H-oxadiazolo-qui-noxaline-1-one (ODQ, 10^{-5} mol/l) or the adenylate cyclase inhibitor, 9-tetrahydro-2-furanyl-9-H-purin-6-amine (SQ-22,536,10^{-5} mol/l). L-NAME, ODQ and SQ-22,536 all decreased responses to relaxin in uterine artery rings in mid-pregnant rats. Blockade of the NO synthase with L-NAME or of soluble guanylate cyclase with ODQ caused a significant inhibition of the relaxant effect of relaxin. This suggests that the NO-cGMP pathway is one of the second messenger systems that mediates the vascular effects of relaxin in pregnancy in rats. Inhibition of the adenylate cyclase, the enzyme responsible for the production of cAMP, with SQ-22,536 decreased the response of the uterine arteries to relaxin, suggesting that cAMP is also involved in the vascular effects of relaxin in pregnancy (Longo *et al*, 2003).

The molecular mechanism of the anti-anaphylactic action of relaxin previously discussed (section 1.6.5) appeared to involve an up-regulation of the NO biosynthetic pathway. Upon perfusion with relaxin, the release of nitrite (the stable end product of NO metabolism) increased in the perfusates. In addition, relaxin treatment caused an increase in the expression of iNOS protein and in the activity of NOS. Tissue levels of cGMP were also increased by treatment with relaxin. (Masini *et al*, 2002).

It should be noted that the work described in section 1.9, above, has focused on the mechanism of action of relaxin in animals. As part of my MD thesis, I investigated the mechanism of action of relaxin in humans.

Aims and Hypothesis

The aim is to characterise the actions and potency of relaxin in human arteries and veins, of various calibre, *ex vivo* using concentrations similar to those found in the plasma in physiological (non-pregnant, pregnancy) and pathophysiological (chronic heart failure) states. I will study the mechanism of action of relaxin. In addition, I will study the heart's possible secretion and extraction of relaxin and determine whether relaxin is of prognostic importance in heart failure. The hypothesis is that relaxin is a circulating hormone that has measurable vascular effects at biological concentrations and that these actions are of potential benefit in cardiovascular disease.

CHAPTER 2:

METHODS.

SUMMARY

My thesis was funded by a British Heart Foundation project grant (Project number 2001/147). In this chapter there will be a detailed description of the methods used for each of the studies that comprise the M.D. thesis.

ETHICS

Ethics approval was obtained for all the studies performed in this thesis from the West Ethics Committee of the North Glasgow Hospitals University NHS Trust. Written informed consent was obtained from all the subjects.

2.1 METHODS FOR STUDY OF EFFECT OF RELAXIN ON SMALL HUMAN RESISTANCE ARTERIES FROM THE SYSTEMIC AND PULMONARY CIRCULATIONS.

2.1.1 PATIENTS

The University of Glasgow is located adjacent to the Western Infirmary, Glasgow which is a large tertiary referral healthcare centre. Patients attending the cardiology and cardiothoracic departments of the Western Infirmary were asked to participate in the study.

Gluteal biopsies taken from patients with coronary heart disease but normal left ventricular systolic function provided the source of the small resistance arteries (SRA) from the systemic circulation. All patients with renal failure (creatinine>200 μmol/l) and diabetes mellitus were excluded from the study.

Lung tissue obtained from patients undergoing pneumonectomy for cancer provided the source of the small resistance arteries from the pulmonary circulation.

The technique of gluteal biopsy has been used for many years by our research group as a source of SRA. (Hillier *et al*, 1999; Coats *et al*, 2001; Petrie *et al*, 2001).

Patients attended the Clinical Investigations Research Unit of the Department of Medicine and Therapeutics. Transport was provided. Clinical details such as smoking history, past medical history of myocardial infarction or hypertension and current medication were noted.

2.1.2 MATERIALS

Relaxin was gifted by Connetics Corporation, Palo Alto, USA. Many thanks to Dr Elaine Unemori for this gift.

Experiments were carried out in physiological salt solution (PSS) with the following composition (mM): NaCI 118.4, KCI 4.7, $MgSO_4H_2O$ 1.2, KH_2PO_4 1.2. Na HCO_3 24.9, $CaCI_2$ 2.5, glucose 11.1, EDTA 0.023 which gives a pH of 7.4 when gassed with a 5% CO_2 / 95% O_2 mixture.

A Mulvany-Halpern four-channel wire myograph (Danish Myotech, Aarhus, Denmark) was used.

2.1.3 SYSTEMIC RESISTANCE ARTERY STUDIES

2.1.3.1 Gluteal Biopsy Procedure and Artery Preparation

Small resistance arteries (SRA) are those blood vessels which contribute the greatest resistance to blood flow, and are therefore most involved in regulating blood flow and capillary pressure (Mulvany and Aalkjaer, 1990). These arteries can be readily obtained from gluteal biopsies in humans. Resistance arteries are those blood vessels which contribute the greatest resistance to blood flow, and are therefore most involved in regulating blood flow and capillary pressure (Mulvany and Aalkjaer 1990). SRA wire myography is an *in vitro* technique which allows resistance arteries with a diameter of 100 – 500μm to be studied under precise and standardised conditions. Use of this technique yields information on the contractile or relaxant properties, and morphology, of SRA under isometric tension (Mulvany and Aalkjaer 1990; Mulvany and Halpern 1977). In the present investigation, functional studies were undertaken with SRA because of the physiological importance of these blood vessels. Wire myography was employed for these studies because this is an established technique in our laboratory (Hillier *et al* 1999, 2001a; Padmanabhan *et al* 1999; Petrie *et al* 2001), in which several arteries may be studied at any one time, using one or more protocols.

The gluteal biopsy was performed by myself under local anaesthetic. 10mls of 1% lidocaine was injected into the upper, outer quadrant of the buttock using an aseptic technique. Usually the right buttock was used. However if a patient had had a

gluteal biopsy performed previously by another research fellow for their study, then the left buttock was used instead. An incision was made with a scalpel and a gluteal biopsy (around the size of a 10 pence coin) was taken. Three to four non-absorbable sutures were then used to close the skin and a dressing was placed on the skin over the sutures. Patients were asked to return to the research unit one week later to have the sutures removed by the research nurse. Again, transport was provided for this. There were no complications from this procedure.

The biopsy was placed in a universal container in cold 0.9% sodium chloride solution and sent by taxi to Caledonian University for dissection of the resistance arteries found within the gluteal biopsy. This was performed by Fiona Johnston, Research Technician under the guidance of Dr Chris Hillier. Isolating SRA within the biopsy requires careful dissection using surgical grade instruments with the aid of a high power microscope. The biopsy was placed in a Petri dish filled with ice cold Krebs buffer, which was regularly changed during the dissection process. Dissection of the SRA can take several hours.

Once dissection of the biopsy was complete, the SRA were placed in a universal container and stored at $4^{o}C$ in a refrigerator overnight. Storage of resistance arteries in this way has been previously demonstrated to have no effect on the vasoactive properties of these blood vessels (McIntyre *et al*, 1998).

A single biopsy may yield several blood vessels (average 2 – 4). Isolated SRA were divided into segments approximately 2mm long. When possible, four resistance arteries, were carefully mounted on two 40-µm-diameter stainless steel wires and mounted in the bath of a 4-channel myograph (Halpern & Mulvany,

Danish Myotech, Aarhus, Denmark), in which the wires are attached to a force transducer and micrometer, respectively. The bath contained physiological salt solution (PSS), which gives a pH of 7.4 when gassed with a 5% CO2/95% O2 mixture and pre-heated at 37°C. These conditions were maintained for the duration of the experiment. In addition, the physiological salt solution was regularly changed throughout the experiment.

2.1.3.2 Experimental Protocol (with intact endothelium)

After a rest period of 30 minutes, a normalisation procedure was followed for each artery to determine the normalised internal diameter (ID), L0, at which contraction is thought to be optimal, and the vessel was set to that diameter (Mulvany and Halpern 1977). In the present study, arteries with a normalised ID of 200 – 400μm were used. After a rest period of 30 minute each artery was stretched at 1 minute intervals to determine the passive exponential wall tension-internal circumference (L) relationship. From the Laplace equation, where P=T/r (P is the effective pressure, T is the wall tension and r is the internal radius), the equivalent circumference (L_{100}) for a transmural pressure of 100mmHg, was calculated for each artery by an iterative computer method. Each artery was then set to the normalised internal diameter, $L_1=0.9 \times L_{100}/\pi$, at which contraction is thought to be optimal (Mulvany and Aalkjaer 1990; Mulvany and Halpern 1977).

Following normalisation, the vessels were left for a further hour. They were then exposed to a high (123mM) concentration of potassium (KPSS, solution identical

to PSS except that sodium is replaced by potassium on an equimolar basis) for a series of 5 minute periods until repeatable maximal contractions were achieved, and then once to 10 µmol/L of norepinephrine (NE). After a plateau contraction had been attained with NE, 3 µmol/L of acetylcholine (ACh) was added to stimulate endothelium-dependent vasodilatation. Arteries that were unable to contract to either KPSS or norepinephrine or showed no relaxation to acetylcholine (and were therefore considered to have no functionally intact endothelium) were discarded. The arteries were then incubated for a further 30 minutes in Krebs solution prior to the commencement of the concentration-response curves (CRC) incorporated in the study protocol.

2.1.3.3 Comparative Potency of Relaxin Compared To Other Vasodilators.

Cumulative concentration response curves (CCRC) were then constructed in vessels pre-constricted with 10^{-5}M norepinephrine, using substance P (a powerful vasodilator, 10^{-14}M to 10^{-9}M), epoprostenol (a moderately potent vasodilator, 10^{-11}M to 10^{-8}M), atrial natriuretic peptide (ANP, a weak vasodilator, 10^{-11}M to 10^{-8}M), and relaxin (concentration range 10^{-13} -10^{-7}M).

Our research group has studied each of these comparators previously. (Hillier *et al*, 1999), (Petrie *et al*, 2000).

The concentration of relaxin used will cover the known physiological (pregnant, 10^{-9}M and non-pregnant state, 10^{-12}M) and pathophysiological (seen in chronic heart failure, 10^{-11}M) range of plasma concentrations.

2.1.3.4 Procedure for Removal of Endothelium and Experimental Protocol in De-endothelialised Vessels.

We were keen to establish if the action of relaxin in SRA was endothelium dependent. Thus, endothelium was mechanically removed from a further set of vessels by gently rubbing the luminal side of the arterial wall with a human hair (stored in ethanol and rinsed with PSS before use). Endothelial removal was confirmed by the lack of relaxation to ACh. A CCRC was then constructed with relaxin 10^{-13} to 10-^{7}M.

2.1.4 PULMONARY RESISTANCE ARTERY STUDIES.

2.1.4.1 Artery Preparation.

Lung tissue was placed in cold Krebs-buffer solution. Pulmonary resistance arteries (diameter < 300 μm, length approximately 2mm) were dissected and mounted in the myograph as described above and previously (Stirrat *et al*, 2001).

2.1.4.2 Experimental protocol.

Tension was applied to vessels to give transmural pressures equivalent to 12 – 16mmHg to simulate *in vivo* pressures. Vessels were allowed to equilibrate and endothelial integrity was tested, as described above. CCRC were then constructed with relaxin 10^{-15} to 10^{-7} M, after preconstricting each vessel with U46619 – a thromboxane A2 mimetic (norepinephrine does not induce sustained constriction of human pulmonary vessels).

2.1.5 ANALYSIS OF DATA

Responses (mean ± standard error of the mean [SEM]) are expressed as % relaxation from maximally preconstricted levels.

2.1.5.1 Comparisons Between Relaxin and Other Vasodilators

Statistical comparison of maximum responses (within the concentration range tested) was performed using unpaired Student's t test and Dunnett's post hoc test for multiple comparisons. Comparison of curves was by one way ANOVA for repeated measures (Hillier *et al*, 2001b, Jarajapu *et al*, 2001, Stirrat *et al*, 2001).

2.2 METHODS FOR THE STUDY OF THE MECHANISM OF ACTION OF RELAXIN IN SYSTEMIC RESISTANCE ARTERIES.

2.2.1 PATIENTS

Patients with coronary heart disease but normal left ventricular systolic function were studied. All patients with renal failure (creatinine>200 μmol/l) and diabetes mellitus were excluded from the study.

Patient characteristics such as smoking history and current medication were documented.

2.2.2 MATERIALS

Relaxin was gifted by Connetics Corporation, Palo Alto, USA.

Experiments were carried out in physiological salt solution (PSS) with the following composition (mM): NaCI 118.4, KCI 4.7, $MgSO_4II_2O$ 1.2, KH_2PO_4 1.2. Na HCO_3 24.9, $CaCI_2$ 2.5, glucose 11.1, EDTA 0.023 which gives a pH of 7.4 when gassed with a 5% CO_2 / 95% O_2 mixture.

A Mulvany-Halpern four-channel wire myograph (Danish Myotech, Aarhus, Denmark) was used.

2.2.3 GLUTEAL BIOPSY PROCEDURE AND ARTERY PREPARATION

Gluteal biopsies were obtained under local anaesthesia (1% lidocaine), as previously described. Resistance arteries (diameter < 300 μm, length approximately 2mm), were dissected and mounted in the myograph. A Mulvany-Halpern four-channel wire myograph (Danish Myotech, Aarhus, Denmark) was used. The bath was gassed and heated for the duration of the experiment.

2.2.4 EXPERIMENTAL PROTOCOL (WITH INTACT ENDOTHELIUM)

Experiments were carried out in physiological salt solution (PSS) with the following composition (mM): NaCI 118.4, KCI 4.7, $MgSO_4II_2O$ 1.2, KH_2PO_4 1.2. Na HCO_3 24.9, $CaCI_2$ 2.5, glucose 11.1, EDTA 0.023 which gives a pH of 7.4 when gassed with a 5% CO_2 / 95% O_2 mixture.

After 30 minutes rest, each artery was set to the normalised internal diameter at which contraction is thought to be optimal. The vessels were left for a further hour and then repeatedly exposed to a high potassium salt solution until reproducible maximal contractions were achieved. Vessels were preconstricted with 10^{-5} norepinephrine and 10^{-6} ACh was added to test for endothelial integrity since relaxin is endothelium dependent. (We have demonstrated this with a previous experiment detailed above, section 2.1.3.3).

Cumulative concentration response curves (CCRC) were constructed in vessels pre-constricted with 10^{-5}M norepinephrine and relaxin (concentration range 10^{-13} - 10^{-7}M).

2.2.4.1 Relaxin and the Prostacyclin Pathway

CCRCs to relaxin (as above) were constructed to identify the importance of prostacyclin – before the following incubation with the cyclooxygenase inhibitor indomethacin.(10^{-5} M).

2.2.4.2 Relaxin and EDHF

Next, we studied endothelium-derived hyperpolarising factor – before and following incubation with apamin and charybdotoxin, (blockers of Ca^{2+} activated K^+ channels).

2.2.4.3 Interaction of Relaxin with Nitric Oxide.

The bulk of the data from studies in experimental animals suggest that relaxin exerts at least some of its vasodilator effect via nitric oxide. (Bani, 1997), (Bani *et al*, 1998), (Masini *et al*, 1997). We therefore studied the vasorelaxant effect of relaxin in small human resistance arteries in the absence and presence of nitric

oxide synthase (NOS) inhibitors, L- NAME (*N*ω-nitro-L-arginine methyl ester) and L-NOARG (*N*ω-nitro-L-arginine).

2.2.4.4 Second Messengers Mediating Vasodilator Action of Relaxin.

It has been suggested that the vasodilator action of relaxin is mediated via the second messengers cyclic GMP and cyclic AMP (Bani, 1997). We studied the vasodilator action of relaxin in the presence of milrinone (a cAMP phosphodiesterase inhibitor), zaprinast (a cGMP phosphodiesterase inhibitor) and ODQ, a soluble guanylate cyclase inhibitor.

2.2.4.5 Relaxin and Patients taking Angiotensin Converting Enzyme (ACE) Inhibitors.

On gaining mechanistic insight, an unexpected finding was that prior treatment with an ACE inhibitor substantially attenuated the vasodilator effect of relaxin. Consequently, all subsequent analyses were carried out separately, according to whether vessels had been taken from patients treated with, or not treated with, an ACE inhibitor.

2.3 METHODS FOR STUDY OF EFFECT OF RELAXIN ON HUMAN INTERNAL MAMMARY ARTERIES AND SAPHENOUS VEINS.

2.3.1 PATIENTS

Coronary artery bypass graft (CABG) surgery is a common procedure at the Western Infirmary, with approximately 1000 operations performed each year. Usually distal segments of the left internal mammary artery (IMA) and saphenous vein (SV) are surplus to requirement and subsequently discarded. These segments can thus be used for scientific research.

Patients undergoing elective CABG for coronary artery disease were included in this study. These patients were invited to consent for the study prior to the CABG being performed. The study was fully approved by the West Ethics committee of the Western Infirmary, on behalf of the North Glasgow Hospitals NHS Trust.

Clinical details were recorded from case note examination. A history of current cigarette smoking, hypertension (defined as either current anti-hypertensive medication or blood pressure >140/90 mmHg, diabetes mellitus (insulin treated or non-insulin treated) and hypercholesterolaemia (plasma cholesterol >5.4) were noted. Information on other current medication was also documented at this point.

2.3.2 VESSEL PREPARATION

Distal segments of left internal mammary artery (IMA) and saphenous veins (SV), were harvested at the time of routine coronary artery revascularization surgery in the Cardiac Surgical Theatre Suites within the Western Infirmary, and surplus to requirement. The discarded distal end of the IMA (1–2 cm) and segments of SV (1–2 cm) were immediately taken to the laboratory in Krebs –HEPES buffer on ice. The blood vessels were then carefully dissected free from connective tissue under these conditions and divided into 4-5mm segments. The vessels were then incubated in Krebs buffer at pH 7.4 $\pm$ 2 and maintained in atmospheric conditions (PO_2 19 $\pm$ 4 kPa; PCO_2 3 $\pm$ 4 kPa) at 37^{o}C.

2.3.3 ORGAN BATH STUDIES

Some rings were studied immediately, others were stored in PSS overnight. The PSS (pH 7.490.1) had the following composition (in mM): 130 NaCl, 4.7 KCl, 14.9 NaHCO3 , 1.18 KH2 PO4 , 5.5 glucose, 1.17 MgSO4 .7H2 O, 1.6 CaCl2 .2H2 O, and 0.03 CaNa2 EDTA. Storage under these conditions had no effect on endothelial responses. The vessels were cleaned of connective tissue and cut into 2–3 mm long segments. Rings were suspended on wires in 10 ml organ chambers filled with physiological salt solution (PSS), maintained at 37°C, and aerated with a mixture of 95% O2 -5% CO2 . The rings were connected to force transducers, and changes in isometric tension were recorded.

The rings of human IMAs and SVs were equilibrated in the organ baths in PSS solution before the protocol was initiated. Optimal tension, defined as the tension at which maximum constriction to phenylephrine (PE) occurred, was determined.

In the series of experiments, cumulative dose-response curves to phenylephrine (10^{-8} -10^{-5} M ITA, 10^{-8} -10^{-5} M SV) were constructed. Baths were washed out and the tissues allowed to relax. They were then constricted to their individual EC50 values for phenylephrine and relaxation to carbachol (10^{-8} – 10^{-5} M) studied.

Following normalisation, the vessels were left for a further hour. Potassium chloride (KCl) was used as a receptor-independent vascular smooth muscle cell depolarizing agent. At 100mmol/L, maximal contraction is obtained. The noradrenaline analogue PE was used to constrict the rings via α-adrenoceptors. Carbachol, a stable analogue of acetylcholine, was used to relax the rings in an endothelium-dependent manner via muscarinic receptors, resulting in stimulated NO release. After a plateau contraction had been attained with PE, the vessels were incubated for a further 30 minutes in PSS solution prior to the commencement of the concentration-response curves (CRC) incorporated in the study protocol. Arteries that were unable to contract to either KPSS or phenylephrine were discarded.

Cumulative vasorelaxation curves to relaxin 10^{-13}M to 10^{-7}M were constructed in rings preconstricted with phenylephrine. Since relaxin is endothelium dependent, the calcium ionophore A23187 (CaI) was added to the bath following the CRC. A vasorelaxant response of the vessel to CaI (10^{-4}M to 10^{-2}M) confirmed that the

endothelium was intact (as CaI is also endothelium dependent). If there was no vasorelaxation response to CaI then we concluded that the endothelium was not functionally intact and the vessel was not included in the data.

2.4 METHODS FOR THE STUDY OF THE PROGNOSTIC EFFECT OF RELAXIN COMPARED WITH NT-BNP IN PATIENTS WITH HEART FAILURE.

2.4.1 PATIENTS

Patients taking part in a randomised controlled trial of specialist nurse intervention were studied (Blue *et al*, 2001). Patients admitted to hospital, as an emergency, with heart failure due to left ventricular systolic dysfunction were enrolled in a trial comparing conventional care to conventional care supplemented by specialist heart failure nurse intervention. Patients were followed for a mean of 12 months after randomisation. Deaths and hospital re-admissions were recorded. Re-admissions were adjudicated by a blinded end-point Committee. The primary end-point was death or readmission with heart failure. Patients consented to have venous blood collected for measurement of neurohumoral factors.

2.4.2 ASSAYS

2.4.2.1 NT-pro BNP Assay

NT-pro BNP was measured in blood samples using a validated and commercially available immunoassay (Roche Diagnostics, Germany).

The Roche Elecsys proBNP (Roche Diagnostics, East Sussex, England) Immunoassay was used to analyse NT-proBNP (proBNP). The Elecsys method used was an electrochemiluminescent immunoassay on an Elecsys 2010 autoanalyser. This has a within-assay and between-assay confidence variable of 2.7 and 3.2% respectively. The measuring range of the assay is 5 – 35,000 pg/ml. The analytical sensitivity of the assay is 5pg/ml. The diagnostic information quotes the cut-off for patients younger than 75 years to be 125 pg/ml and 450pg/ml for those 75 years and older. From work conducted on a healthy population, the following 95th percentile figures were established as normal ranges dichotomised for age and sex (Table 2.1). An elevated NT-proBNP was taken to be a value greater than the 95th percentile for each age and sex category.

Table 2.1 95th percentile according to age and sex of NT-proBNP for a healthy population

	NT-proBNP concentration pg/ml			
Age (years)	≤ 64	65-69	70-74	≥ 75
Female	213.4	314.2	338.5	355.3
Male	122.6	112.6	236	295.7

2.4.2.2 Relaxin Assay

Plasma relaxin was determined using an ELISA kit (Immundiagnostik, Bensheim, Germany). The polyclonal antibody was raised in rabbits. The kit has a detection limit of 0.40 pg/ml that was calculated from the mean optical density of the zero standard (measured in duplicate), plus 2 standard deviations. The intra-assay coefficient of variation is 9.6% (=18, at 15 pg/ml) and the interassay coefficient of variation is 10.2% (=12, at 15 pg/ml). The kit is highly selective for human relaxin with cross-reactivity measuring 100% for the H1 form and 100% for the H2 form. Cross-reactivity against insulin, insulin-like growth factors, luteinising hormone (LH), follicle stimulating hormone (FSH), and prolactin is less than <0.01%.

2.4.3 STATISTICAL ANALYSIS

Event rates were compared for patients with plasma concentrations of NT-pro BNP and relaxin above and below the group median. As NT-pro BNP concentrations above the median were associated with a significantly worse clinical outcome, multivariate analyses were carried out in order to determine whether or not NT-pro BNP was an independent predictor of outcome (death and death or hospital admission for CHF). Firstly, a univariate analysis was performed using all relevant baseline data (e.g. age, sex, NYHA Class, left ventricular function, heart rhythm, co-morbidity, history of prior CHF hospitalisation, creatinine etc) and variables significantly ($p<0.05$) associated with outcome were then examined in a stepwise multivariate analysis.

2.5 METHODS FOR THE STUDY OF THE TRANSPULMONARY AND TRANSCARDIAC GRADIENTS OF RELAXIN.

2.5.1 PATIENTS

20 consecutive patients undergoing elective coronary artery revascularization surgery for coronary artery disease, at the Cardiac Surgical Theatre Suites, within the Western Infirmary, were studied. These patients were invited to consent for the study prior to the CABG being performed. The study was fully approved by the West Ethics committee of the Western Infirmary, on behalf of the North Glasgow Hospitals NHS Trust.

Clinical details were recorded from case note examination. A history of current cigarette smoking, hypertension (defined as either current anti-hypertensive medication or blood pressure >140/90 mmHg, diabetes mellitus (insulin treated or non-insulin treated) and hypercholesterolaemia (plasma cholesterol >5.4) were noted. Information on other current medication was also documented at this point. Left ventricular systolic function (estimated ejection fraction) as determined by Simpson's biplane method on echocardiography was noted for each patient.

2.5.2 BLOOD SAMPLING

Immediately prior to institution of cardiopulmonary bypass, blood samples were taken by the operating Cardiothoracic Surgeon, in rapid succession, from the aorta, coronary sinus, pulmonary artery and pulmonary vein. I then placed the samples into chilled tubes and transported them directly to the lab for analysis.

2.5.3 ASSAYS

A validated relaxin immunoassay was used (Immundiagnostik, Bensheim, Germany) as detailed previously (section 2.4.2).

2.5.4 STATISTICAL ANALYSIS

Statistical analysis was performed using a Wilcoxon signed rank test.

CHAPTER 3:

THE COMPARATIVE VASODILATOR ACTION OF RELAXIN IN HUMAN RESISTANCE AND PULMONARY ARTERIES

3.1 Summary

It has been shown recently that relaxin is secreted by the heart. Cardiac mRNA expression and secretion of relaxin are increased in chronic heart failure. Circulating concentrations of relaxin are also markedly elevated in heart failure in proportion to clinical severity. (Dschietzig *et al*, 2001). As with other peptides, such as atrial natriuretic peptide and brain natriuretic peptide, cardiac relaxin secretion may be a compensatory response in heart failure and relaxin may be a circulating vasoactive hormone. This study examined the effects of relaxin in small resistance arteries from the systemic and pulmonary circulations.

3.2 PATIENTS

Thirteen patients with coronary heart disease but normal left ventricular systolic function were studied. Lung tissue was obtained from another 5 patients undergoing pneumonectomy for cancer.

The study had Ethics Committee approval and patients gave informed consent.

3.3 MATERIALS

Relaxin was gifted by Connetics Corporation, Palo Alto, USA. Experiments were carried out in physiological salt solution (PSS) with the following composition (mM): NaCI 118.4, KCI 4.7, $MgSO_4II_2O$ 1.2, KH_2PO_4 1.2. Na

HCO_3 24.9, $CaCI_2$ 2.5, glucose 11.1, EDTA 0.023 which gives a pH of 7.4 when gassed with a 5% CO_2/95% O_2 mixture. A Mulvany-Halpern four-channel wire myograph (Danish Myotech, Aarhus, Denmark) was used.

3.4 SYSTEMIC RESISTANCE ARTERY STUDIES

3.4.1 Gluteal biopsy procedure and artery preparation

Gluteal biopsies were obtained under local anaesthesia (1% lidocaine), as previously described (Methods 2.1.3). Resistance arteries (diameter < 300 μm, length approximately 2mm), were dissected and mounted in the myograph as previously described. The bath was gassed and heated for the duration of the experiment.

3.4.2 Subjects

Table 3.1 shows the characteristics of the patients studied.

Table 3.1. Characteristics of Patients Providing Small Systemic Resistance Arteries

Number of patients	13
Sex M/F	8/5
Age, y (range)	67 (55-78)
Previous MI	4
Previous CABG	6
Current smoker	3
Drug therapy -β blocker -Aspirin -HMG CoA reductase inhibitor -ACE inhibitor -Calcium channel blocker -Diuretic -Digoxin -Nitrate -Nicorandil	 11 13 13 6 2 4 3 5 2
Creatinine, μmol/L Mean LVEF % (range)	$105^{\pm}$ 5 55 (45-70)

M/F indicates male/female; MI indicates myocardial infarction; CABG, coronary artery bypass grafting; ACE, angiotensin-converting enzyme, HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A, LVEF, left ventricular ejection fraction.

3.5 STUDIES IN SMALL SYSTEMIC RESISTANCE ARTERIES WITH INTACT ENDOTHELIUM

3.5.1 Resistance artery diameter

The mean internal diameter (ID) of the systemic resistance arteries was 283 ± 21 μm. ACh caused an 88± 4% reduction in norepinephrine-induced tone, verifying the existence of an intact endothelium.

3.5.2 Response to vasodilators

Figure 3.1 below shows the vasodilator activity of substance P (n=8), epoprostenol (PGI 2) (n=6), ANP (n=6) and relaxin (n=8).

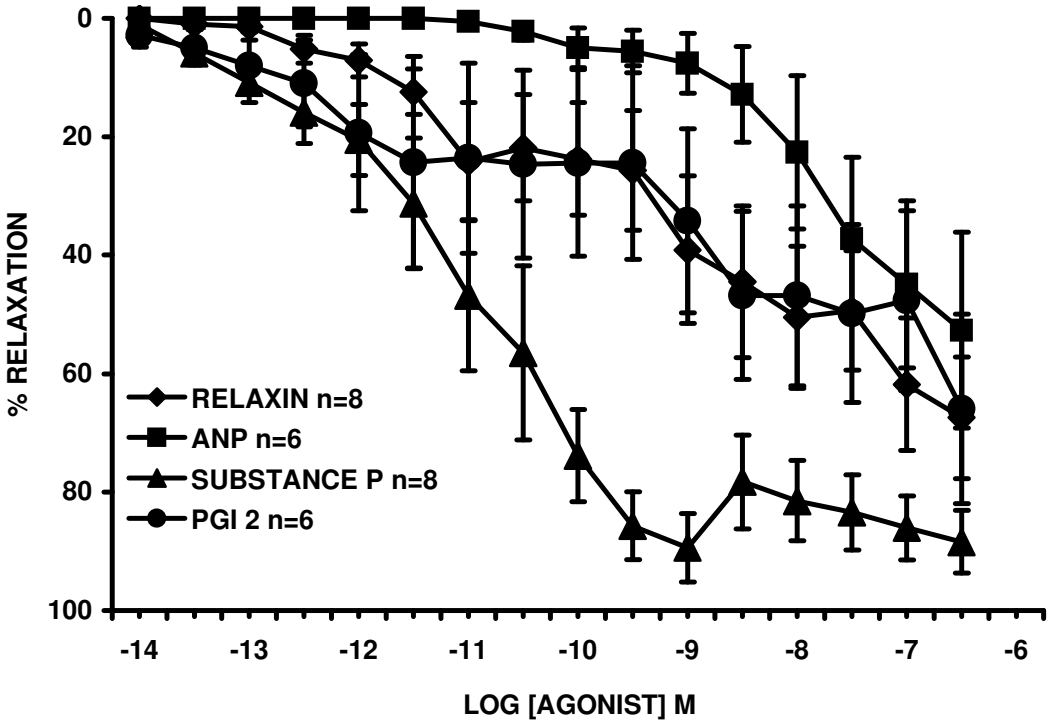
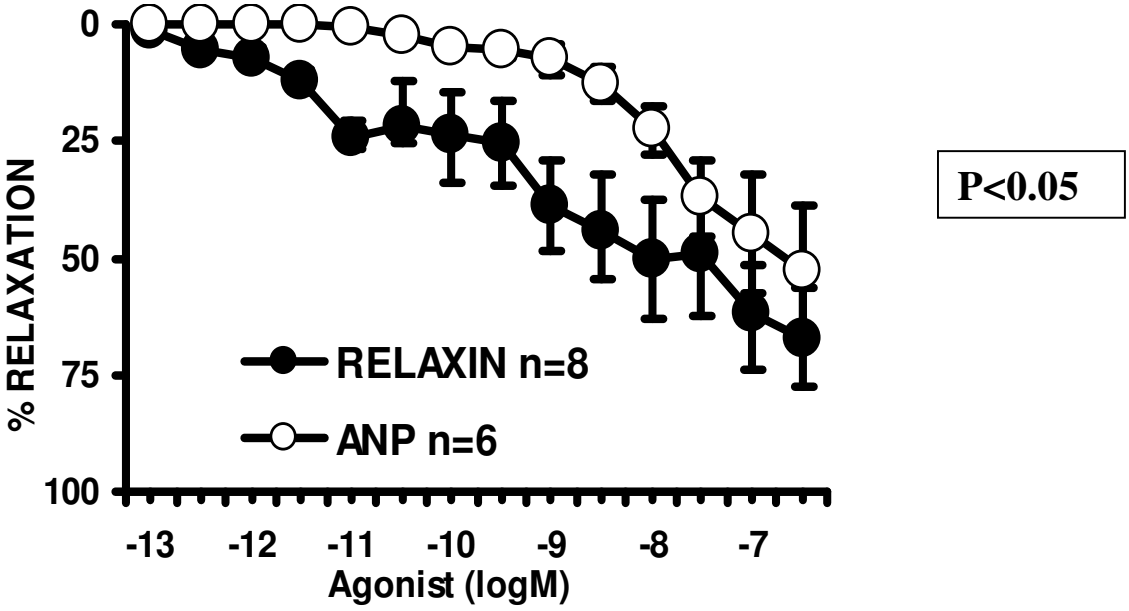


Figure 3.1. Cumulative concentration response curves for relaxin, ANP, substance P and epoprostenol in small human resistance arteries with intact endothelium.

Substance P, as noted previously, was a powerful vasodilator. Relaxin had comparable activity to epoprostenol (prostaglandin I_2). ANP was the weakest of the vasodilators studied. The maximal responses were 88($\pm$5) %, 66($\pm$16) %, 67($\pm$10) % and 52($\pm$16) %, respectively.

A 10% of maximal vasodilator response was achieved with $1x10^{-13}$M substance P, $2x10^{-13}$M epoprostenol, $1x10^{-12}$M relaxin and $3x10^{-10}$M ANP ($P<0.05$ for relaxin versus ANP).

Figure 3.2. Cumulative concentration response curve for relaxin compared with ANP in small human resistance arteries with intact endothelium.



3.5.3 Relaxin versus ANP

We found that relaxin is vasoactive at concentrations comparable to those found in chronic heart failure. Mean plasma relaxin concentrations in patients with severe chronic heart failure, average 2.5 to 3.34 x 10^{-11} mol/L while mean plasma ANP concentrations in patients with severe CHF, average 2.5 x 10^{-11} mol/L. At 10^{-11} mol/L relaxin caused 26% vasodilation of resistance arteries versus 0.68% with the same concentration of ANP.

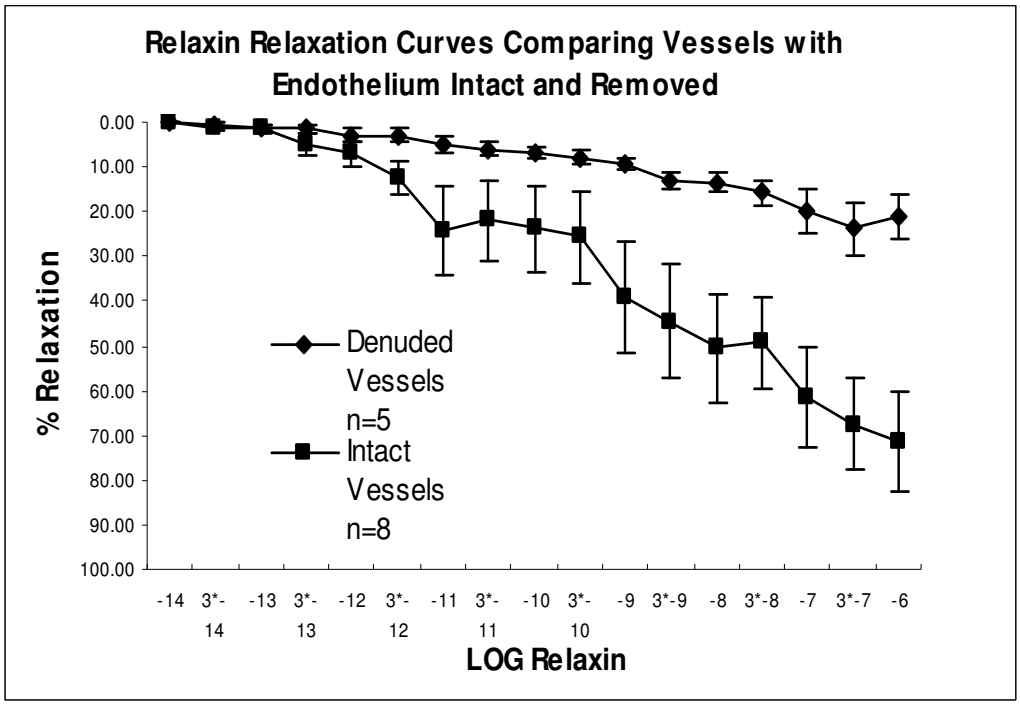
3.5.4 Procedure for removal of endothelium and experimental protocol in de-endothelialised vessels.

To gain further mechanistic insight into relaxin, endothelium was mechanically removed from a further set of vessels by gently rubbing the luminal side of the arterial wall with a human hair (stored in ethanol and rinsed with PSS before use). Endothelial removal was confirmed by the lack of relaxation to ACh. A CCRC was then constructed with relaxin 10^{-13} to 10-^{7}M.

3.5.5 Response to relaxin in systemic resistance arteries following removal of endothelium

Figure 3.3 shows that endothelial rubbing virtually abolished the action of relaxin ($n=8$, $p<0.05$).

Figure 3.3 Cumulative concentration response curve for relaxin in small human resistance arteries with intact endothelium and after removal of the endothelium.



3.6 PULMONARY RESISTANCE ARTERY STUDIES.

3.6.1 Artery preparation.

Lung tissue was placed in cold Krebs-buffer solution. Pulmonary resistance arteries (diameter < 300 μm, length approximately 2mm) were dissected and mounted in the myograph as described in Methods 2.1.4.

3.6.2 Experimental protocol.

Tension was applied to vessels to give transmural pressures equivalent to 12 – 16mmHg to simulate *in vivo* pressures. Vessels were allowed to equilibrate and endothelial integrity was tested, as described above. CCRC were then constructed with relaxin 10^{-15} to 10^{-7} M, after preconstricting each vessel with U46619 – a thromboxane A2 mimetic (norepinephrine does not induce sustained constriction of human pulmonary vessels).

Table 3.2 shows the characteristics of the patients studied.

Table 3.2. Characteristics of Patients Providing Small Pulmonary Resistance Arteries

Number of patients	5
Sex M/F	2/3
Age, y (range)	60 (52-66)
Previous MI	0
Previous CABG	0
Current smoker	1
Drug therapy -β blocker -Aspirin -HMG CoA reductase inhibitor -ACE inhibitor -Calcium channel blocker -Diuretic -Digoxin -Nitrate -Nicorandil	 1 0 0 2 1 1 0 0 0
Creatinine, μmol/L	$106^{\pm}$ 3

M/F indicates male/female; MI indicates myocardial infarction; CABG, coronary artery bypass grafting; ACE, angiotensin-converting enzyme, HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

3.6.3 Response to relaxin in small pulmonary resistance arteries.

The mean ID of the pulmonary vessels (n=5) was 208.8 ±10.6μm. ACh caused a 76±21% reduction in agonist-induced tone yet relaxin had no effect.

3.7 Summary of Chapter Results

In small human systemic resistance arteries, relaxin is a potent vasodilator.

The vasodilatory action of relaxin is more potent than that of ANP and equipotent to epoprostenol. Relaxin's vasodilatory action, however, is less potent than that of substance P. In these vessels, the vasodilatory action of relaxin is endothelium dependent.

In small human pulmonary resistance arteries, relaxin has no effect.

These findings have been published.

C Fisher, M Maclean, I Morecroft, A Seed, F Johnston, C Hillier and J McMurray. Is the Pregnancy Hormone Relaxin Also a Vasodilator Peptide Secreted by the Heart? *Circulation.* 2002;106:292-295.

CHAPTER 4:

THE MECHANISM OF ACTION OF RELAXIN

4.1 Summary

Having established that relaxin is a potent, endothelium dependent, vasodilator in human systemic resistance arteries, I then went on, in detail, to research its mechanism of action.

As described in Section 1.5, the endothelium is an autocrine and paracrine organ that produces substances that decrease vascular smooth muscle and inhibit inflammation and thrombosis. These substances include nitric oxide, prostacyclin and endothelium derived hyperpolarizing factor (EDHF). The focus of the experiments in this chapter, therefore, was to block each of the pathways involving these substances: nitric oxide, prostacyclin and EDHF, in turn.

Firstly, I investigated whether manipulation of nitric oxide and cyclic GMP would have an affect on relaxin's vasodilatory action. Secondly, I looked at whether manipulation of prostanoids and cAMP altered relaxin's action. Lastly, I determined whether manipulating EDHF would have an affect on relaxin's action.

4.2 PATIENTS

Patients with coronary heart disease with no history of heart failure were studied. Details of the patients studied are given in Tables 4.1 and 4.2. For reasons detailed below, groups were split into those patients taking ACE inhibitors and those patients not taking ACE inhibitors.

The study had Ethics Committee approval and patients gave informed consent.

Table 4.1. Characteristics of Patients Providing Small Systemic Resistance Arteries On an ACE Inhibitor

Number of patients	28
Sex M/F	22/6
Age, y (range)	61 (44-74)
Previous MI	11
Previous CABG	3
Current smoker	3
Drug therapy -β blocker -Aspirin -HMG CoA reductase inhibitor -ACE inhibitor -Calcium channel blocker -Diuretic -Digoxin -Nitrate -Nicorandil	 24 28 27 28 7 6 0 5 5
Creatinine, μmol/L	$109^{\pm}$ 4

M/F indicates male/female; MI indicates myocardial infarction; CABG, coronary artery bypass grafting; ACE, angiotensin-converting enzyme, HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

Table 4.2. Characteristics of Patients Providing Small Systemic Resistance Arteries Not On an ACE Inhibitor

Number of patients	30
Sex M/F	23/7
Age, y (range)	62 (41-80)
Previous MI	8
Previous CABG	3
Current smoker	4
Drug therapy -β blocker -Aspirin -HMG CoA reductase inhibitor -ACE inhibitor -Calcium channel blocker -Diuretic -Digoxin -Nitrate -Nicorandil	 25 30 26 0 16 6 0 9 6
Creatinine, μmol/L	$111^{\pm}$ 5

4.3 MATERIALS

Relaxin was gifted by Connetics Corporation, Palo Alto, USA. Experiments were carried out in physiological salt solution (PSS) with the following composition (mM): NaCI 118.4, KCI 4.7, $MgSO_4II_2O$ 1.2, KH_2PO_4 1.2. Na HCO_3 24.9, $CaCI_2$ 2.5, glucose 11.1, EDTA 0.023 which gives a pH of 7.4 when gassed with a 5% CO_2/95% O_2 mixture. A Mulvany-Halpern four-channel wire myograph (Danish Myotech, Aarhus, Denmark) was used.

4.4 GLUTEAL BIOPSY PROCEDURE AND ARTERY PREPARATION

Gluteal biopsies were obtained under local anaesthesia (1% lidocaine), as previously described (Methods 2.1.3). Resistance arteries (diameter < 300 μm, length approximately 2mm), were dissected and mounted in the myograph as previously described. The bath was gassed and heated for the duration of the experiment.

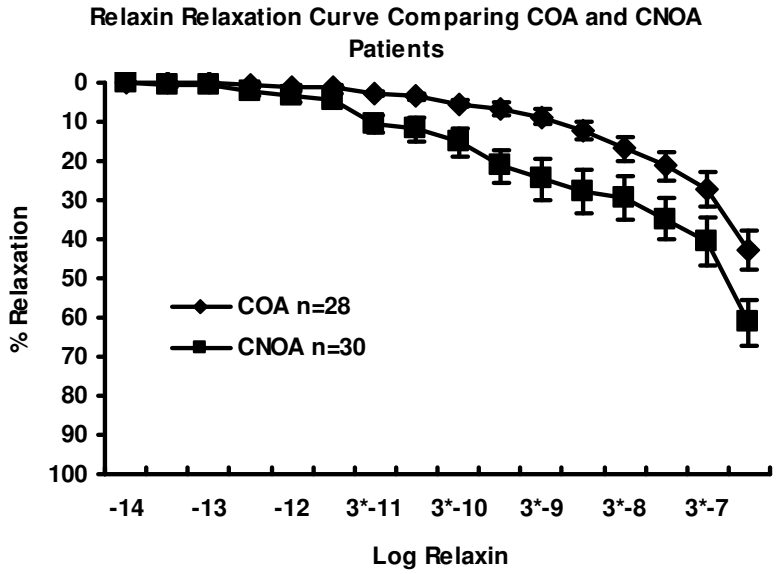
4.4.1 Resistance Artery Diameter

The mean internal diameter of the human small systemic resistance arteries studied was 290 ± 25 μ M. ACh caused a 90 ± 5% reduction in norepinephrine induced tone, verifying the existence of an intact endothelium.

4.5 EFFECT OF PRIOR TREATMENT WITH AN ACE INHIBITOR

An unexpected finding was that prior treatment with an ACE inhibitor substantially attenuated the vasodilator effect of relaxin ($p<0.0001$) (Figure 4.1)

Figure 4.1.



COA = patient on ACE inhibitor

CNOA = patient not on ACE inhibitor

Consequently, all subsequent analyses were carried out separately, according to whether vessels had been taken from patients treated with, or not treated with, an ACE inhibitor.

The results of these analyses will be presented separately.

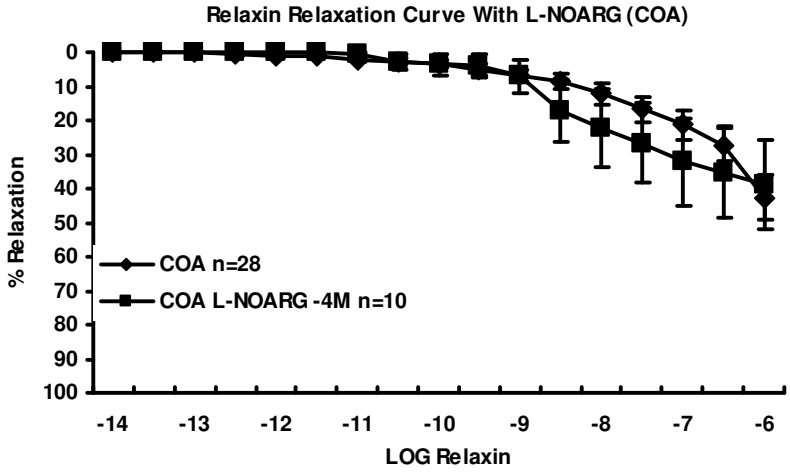
4.6 PATIENTS ON ACE INHIBITORS

4.6.1 Manipulation of Nitric Oxide and cyclic GMP

I) Inhibition of Nitric Oxide Synthase

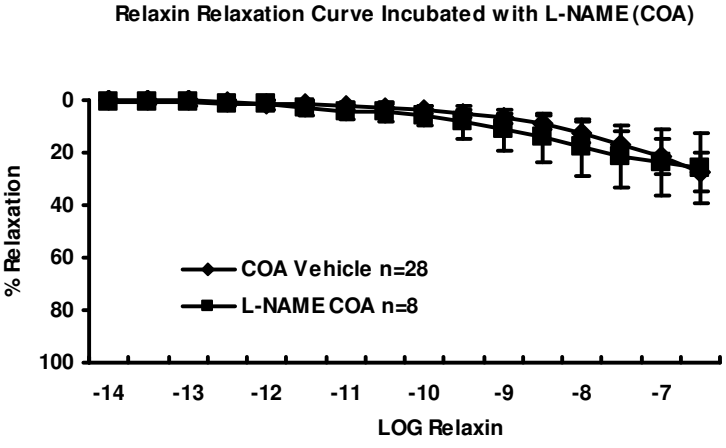
Both L-NOARG and L-NAME affected the vasodilator response to relaxin in arteries from patients treated with an ACE inhibitor. As each graph demonstrates, relaxin's vasodilator action was *enhanced* by blocking nitric oxide. This result was not expected and indeed was the opposite of what was anticipated (Figures 4.2 and 4.3).

Figure 4.2



p<0.05

Figure 4.3.

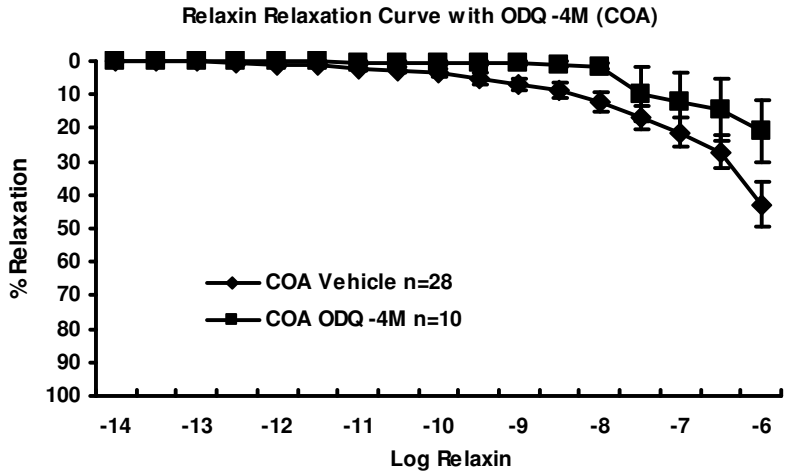


p<0.05

II) Inhibition of Soluble Guanylate Cyclase

Prior incubation with ODQ, the soluble guanylate cyclase inhibitor, reduced the vasodilator response to relaxin (Figure 4.4) in arteries taken from patients treated with an ACE inhibitor (Soluble guanylate cyclase converts GTP to cGMP)

Figure 4.4.

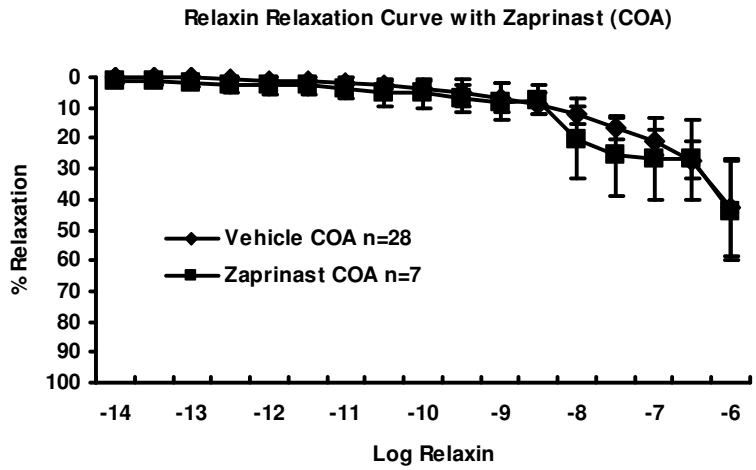


p=0.0009

III) Inhibition of cGMP Phosphodiesterase:

Prior incubation of arteries with zaprinast, which prevents the breakdown of cGMP, appeared to mildly enhance the vasodilator response to relaxin in patients treated with an ACE inhibitor (figure 4.5).

Figure 4.5.



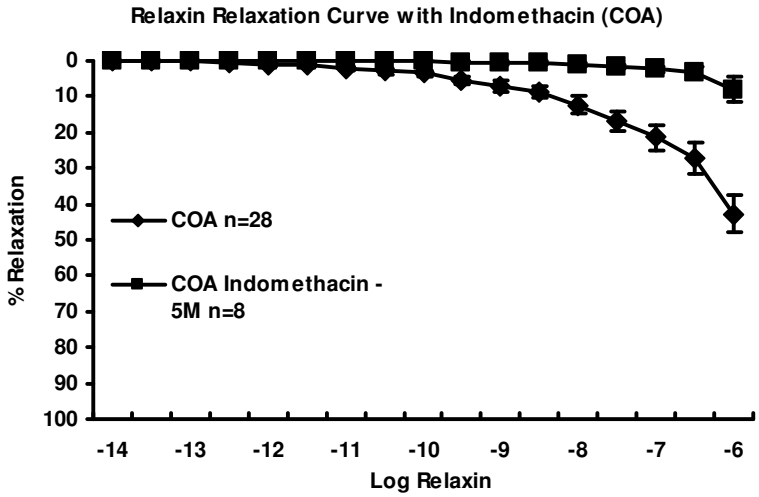
p<0.05

4.6.2 Manipulation of Prostanoids and cyclic AMP

I) Inhibition of Cyclo-oxygenase

Indomethacin, which inhibits cyclo-oxygenase, greatly reduced the vasodilator effect of relaxin in arteries obtained from patients taking an ACE inhibitor (figure 4.6).

Figure 4.6.

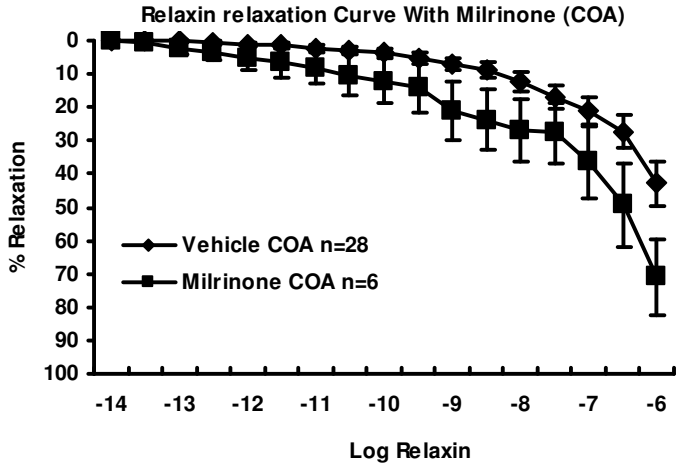


p=0.003

II) Inhibition of cAMP Phosphodiesterase

In arteries from patients treated with an ACE inhibitor, prior incubation with milrinone (which prevents the breakdown of cAMP) enhanced the vasodilator response to relaxin (Figure 4.7).

Figure 4.7.

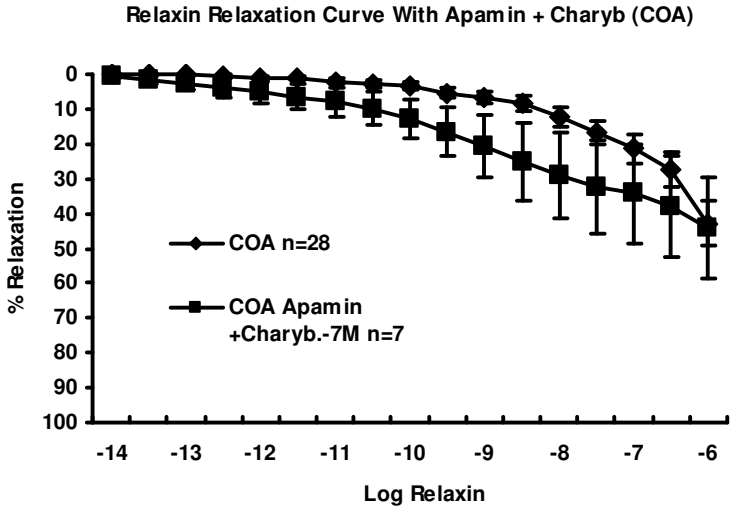


p<0.0001

4.6.3 Inhibition of Endothelium-Derived Hyperpolarising Factor

Prior incubation with the EDHF inhibitors, charybdotoxin and apamin, in arteries from patients treated with an ACE inhibitor (Figure 4.8), enhanced the vasodilator response of relaxin. (Charybdotoxin and apamin block calcium activated potassium channels).

Figure 4.8.



p<0.0001

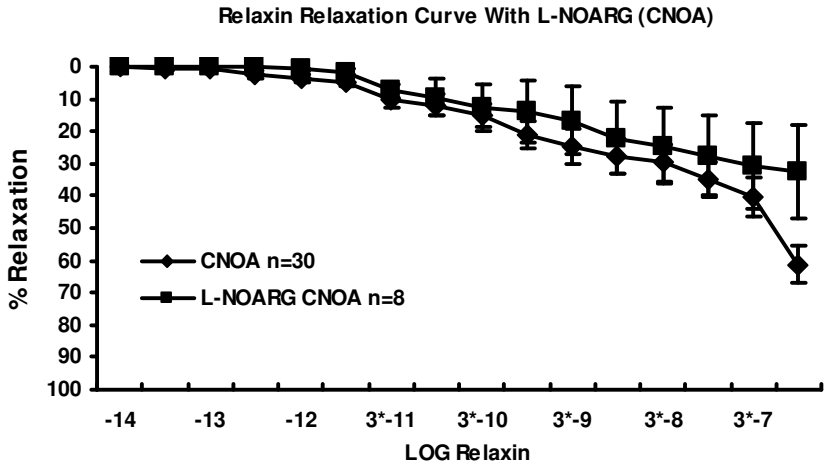
4.7 PATIENTS NOT ON ACE INHIBITORS

4.7.1 Manipulation of Nitric Oxide and cyclic GMP

I) Inhibition of Nitric Oxide Synthase

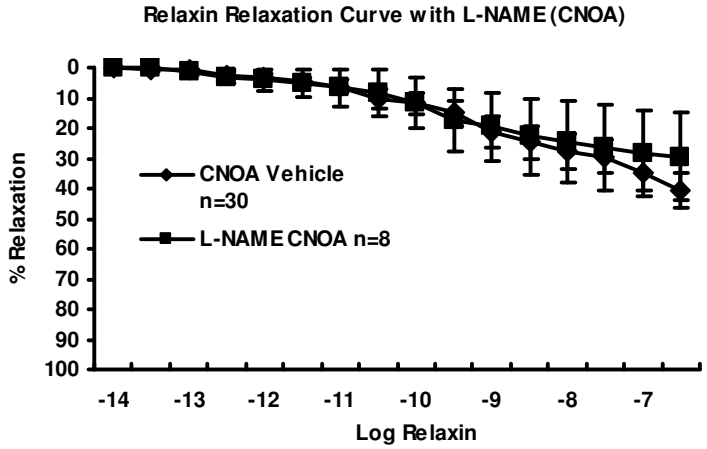
Inhibition of nitric oxide synthase with L-NOARG and L-NAME reduced the vasodilator response to relaxin in arteries taken from patients not taking an ACE inhibitor (figure 4.9 and figure 4.10).

Figure 4.9.



p=0.001

Figure 4.10.

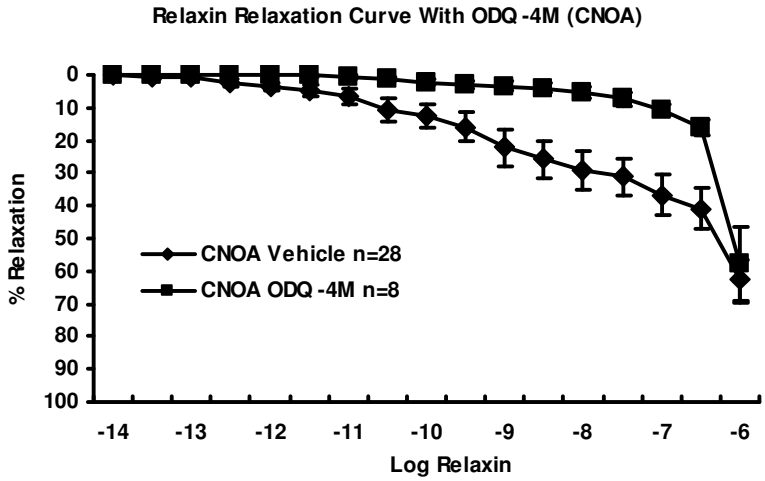


p<0.05

II) Inhibition of Soluble Guanylate Cyclase

Prior incubation with ODQ greatly reduced the vasodilator response to relaxin (Figure 4.11) in arteries taken from patients not treated with an ACE inhibitor. (Soluble guanylate cyclase converts GTP to cGMP).

Figure 4.11.

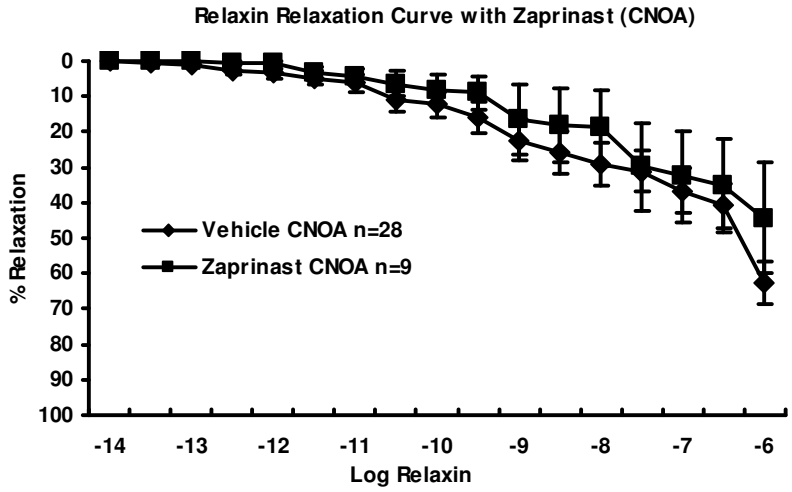


p=0.0001

III) Inhibition of cGMP Phosphodiesterase

Prior incubation of arteries with zaprinast (which prevents cGMP breakdown) reduced the vasodilator response to relaxin in patients not taking an ACE inhibitor (figure 4.12).

Figure 4.12.



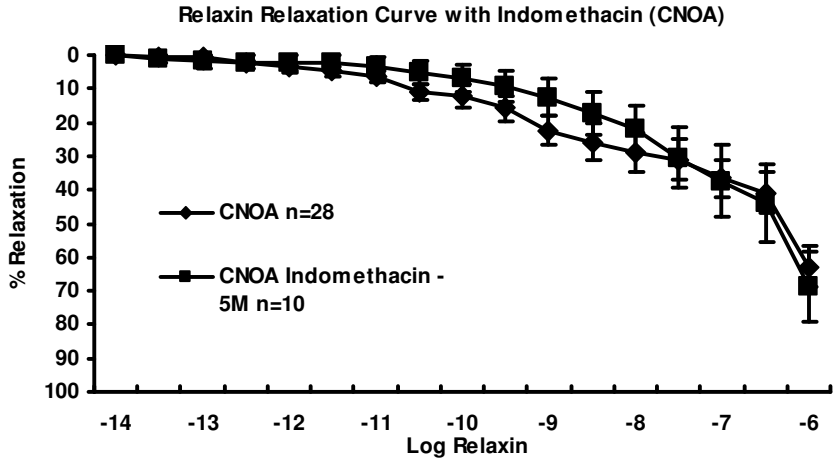
p=0.0004

4.7.2 Manipulation of Prostanoids and cyclic AMP

I) Inhibition of Cyclo-oxygenase

In arteries from patients not treated with an ACE inhibitor, prior incubation with indomethacin reduced the vasodilator response to relaxin (Figure 4.13).

Figure 4.13.

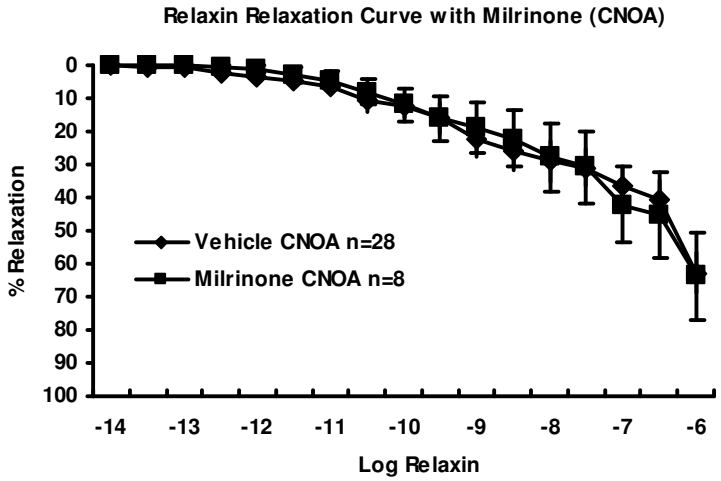


p<0.05

II) Inhibition of cAMP phosphodiesterase

In arteries from patients not treated with an ACE inhibitor, prior incubation with milrinone (which prevents cAMP breakdown) had no effect on the vasodilator response to relaxin (Figure 4.14).

Figure 4.14.

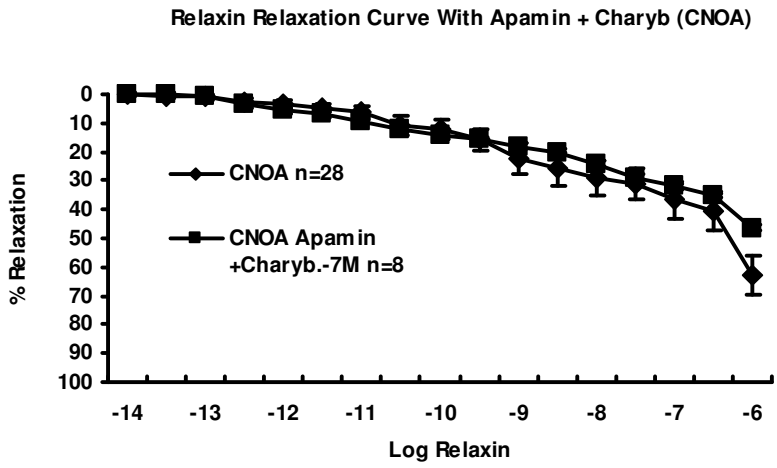


p=n.s.

4.7.3 Inhibition of Endothelium-Derived Hyperpolarising Factor

Prior incubation with the EDHF inhibitors, charybdotoxin and apamin, in arteries from patients not treated with an ACE inhibitor, had no effect on the vasodilator response of relaxin (Figure 4.15). (Charybdotoxin and apamin block calcium activated potassium channels).

Figure 4.15.



p=n.s.

4.8 Summary of Chapter Results

4.8.1 PATIENTS ON ACE INHIBITORS

1) Manipulation of prostanoids and cyclic AMP.

In patients treated with an ACE inhibitor, manipulation of prostanoids and cAMP is important. Blocking the prostanoid pathway with indomethacin blocked relaxin's vasodilatory action. By preventing the breakdown of cAMP with milrinone, relaxin's vasodilatory action was enhanced.

2) Manipulation of cyclic GMP

Manipulation of the cyclic GMP second messenger system may be important in patients treated with an ACE inhibitor. By inhibiting guanylate cyclase with ODQ and thus the conversion of GTP to cGMP, relaxin's vasodilatory effect was reduced. In keeping with this, preventing the breakdown of cyclic GMP with zaprinast, did enhance (although mildly) relaxin's vasodilatory effect. However, these findings were at supraphysiological levels of relaxin and so may not be clinically relevant.

3) Manipulation of nitric oxide and 4) Manipulation of EDHF

Manipulation of the nitric oxide pathway by blocking nitric oxide with L-NOARG and L-NAME and EDHF pathway by blocking potassium channels with apamin and charybdotoxin had curious effects, in patients taking an ACE inhibitor, by enhancing relaxin's vasodilatory action which is the opposite of what one might expect. The results for L-NOARG and L-NAME were at supraphysiological levels of relaxin and may not be clinically relevant.

4.8.2 PATIENTS NOT ON ACE INHIBITORS

1) Manipulation of nitric oxide

Manipulation of the nitric oxide pathway is important. In patients not treated with an ACE inhibitor, blocking nitric oxide synthesis with L-NAME and L-NOARG, resulted in reduced vasorelaxation with relaxin.

2) Manipulation of cGMP

Manipulation of the cGMP second messenger system by inhibiting guanylate cyclase (with ODQ) and thus the conversion of GTP to cGMP, is also important in patients not treated with an ACE inhibitor. ODQ greatly reduced relaxin's

vasodilatory action. However, preventing the breakdown of cyclic GMP with zaprinast did not enhance relaxin's vasodilatory effect and if anything reduced vasodilation.

3) Manipulation of prostanoids and cyclic AMP

Manipulation of prostanoids by blocking prostacyclin with indomethacin in patients not treated with an ACE inhibitor, reduced relaxin's vasodilatory action. However, preventing the breakdown of cAMP with milrinone had no effect on the vasodilatory action of relaxin in these patients.

4) Manipulation of EDHF

Manipulation of the EDHF pathway by blocking potassium channels with apamin and charybdotoxin respectively had no effect on relaxin's vasodilatory action in patients not treated with an ACE inhibitor.

CHAPTER 5:

RELAXIN IN HUMAN INTERNAL MAMMARY ARTERIES AND SAPHENOUS VEINS.

5.1 Summary

Having shown that in small human systemic resistance arteries, relaxin is a potent vasodilator, I was keen to establish whether the same were true in larger calibre arteries. Also, the action of relaxin in the venous system has not been investigated, previously. In this chapter, therefore, the results of this investigation into the action of relaxin in human internal mammary arteries (IMA) and saphenous veins (SV) are reported.

5.2 PATIENTS

IMA and SV from ten patients with coronary heart disease undergoing CABG were studied, as described in Methods 2.3.1

Table 5.1 below gives the characteristics of the patients involved in the study.

Table 5.1. Characteristics of Patients Providing Internal Mammary Arteries and Saphenous Veins

Number of patients	10
Sex M/F	10/0
Age, y (range)	68 (54-78)
Previous MI	4
Previous CABG	0
Current smoker	1
Drug therapy -β blocker -Aspirin -HMG CoA reductase inhibitor -ACE inhibitor -Calcium channel blocker -Diuretic -Digoxin -Nitrate -Nicorandil	 8 10 8 2 8 2 1 8 5
Creatinine, μmol/L	$120^{\pm}$ 8

M/F indicates male/female; MI indicates myocardial infarction; CABG, coronary artery bypass grafting; ACE, angiotensin-converting enzyme, HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

The study had Ethics Committee approval and patients gave informed consent.

5.3 MATERIALS

Relaxin was gifted by Connetics Corporation, Palo Alto, USA. Experiments were carried out in physiological salt solution (PSS) with the following composition (mM): NaCI 118.4, KCI 4.7, $MgSO_4II_2O$ 1.2, KH_2PO_4 1.2. Na HCO_3 24.9, $CaCI_2$ 2.5, glucose 11.1, EDTA 0.023 which gives a pH of 7.4 when gassed with a 5% CO_2/95% O_2 mixture.

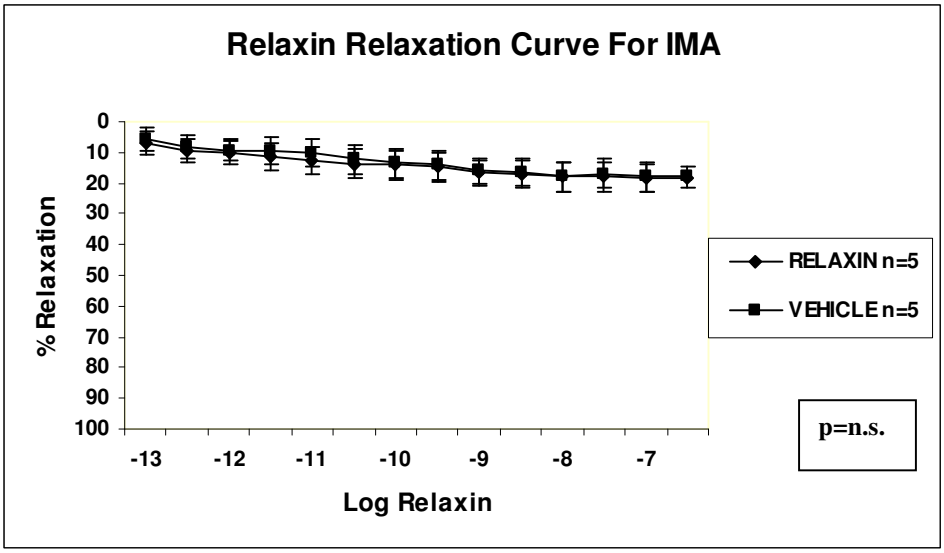
5.4 ORGAN BATH TECHNIQUE

Rings of IMA and SV were set up in organ baths as described in Methods 2.3.2. Cumulative response curves were then constructed to relaxin in vessels pre-constricted with phenylephrine. Since relaxin is endothelium dependent, an intact endothelium was verified, following the CCRC, if relaxation to the calcium ionophore (CaI) A23187 occurred. If no relaxation to CaI occurred then the vessel was not included in the data. IMA from 12 patients and SV from 14 patients were rejected because the endothelium was not intact.

5.5 RELAXIN CUMULATIVE CONCENTRATION RESPONSE CURVE IN HUMAN IMA

In human internal mammary arteries, no significant difference was found between relaxin and the control (vehicle) i.e. relaxin did not cause significant relaxation in these large calibre arteries (Figure 5.1).

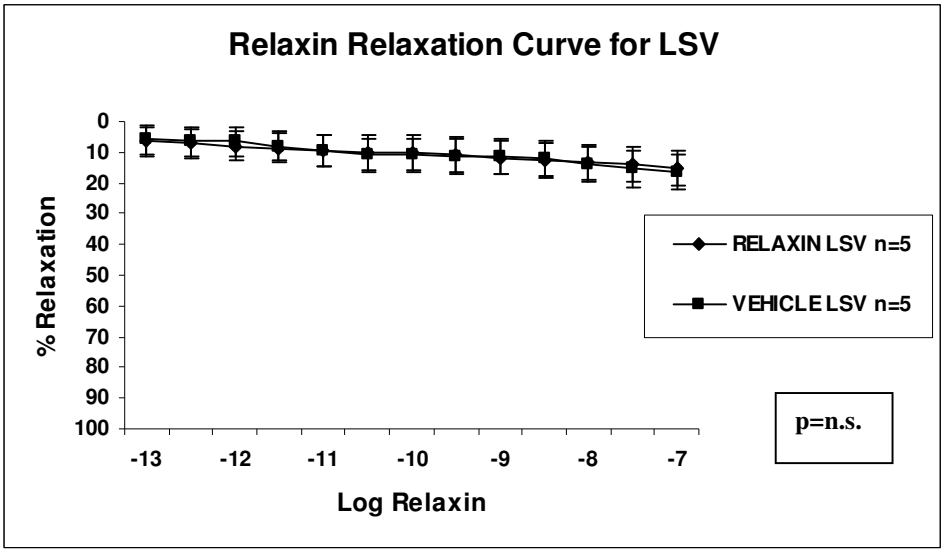
Figure 5.1.



5.6 RELAXIN CUMULATIVE CONCENTRATION RESPONSE CURVE IN HUMAN LSV

In human long saphenous veins, no significant difference was found between relaxin and the control (vehicle) i.e. relaxin did not cause significant relaxation in these large calibre veins (Figure 5.2).

Figure 5.2.



5.7 Summary of Chapter Results

Relaxin was not found to cause relaxation in human large calibre vessels i.e internal mammary arteries or long saphenous veins harvested at the time of coronary artery bypass grafting.

CHAPTER 6:

RELAXIN COMPARED WITH NT-BNP IN HEART FAILURE

6.1 Summary

Relaxin has been shown to be a powerful systemic arterial vasodilator released from the heart. (Fisher *et al*, 2002). One recent report has shown increased cardiac relaxin release and elevated plasma relaxin concentrations in heart failure. (Dschietzig *et al*, 2001). Consequently, it has been postulated that relaxin, like the natriuretic peptides, may be secreted as a compensatory neuroendocrine response in heart failure. ((Dschietzig *et al*, 2001), (Fisher *et al*, 2002). With other pathophysiologically important neurohumoral mediators there is a clear relationship to outcome in heart failure, whereby higher plasma concentrations, indicating greater neurohumoral activation, are associated with a worse prognosis.(Francis *et al*, 1984), (Cohn *et al*, 1984), (Swedberg *et al*, 1990). Consequently, we have examined the relationship between plasma relaxin concentration and clinical events in patients with chronic heart failure (CHF). As a "positive control", we also examined the prognostic importance of N-terminal pro B-type natriuretic peptide (NT pro BNP) in the same patients (Hunt *et al*, 1995).

6.2 PATIENTS

Patients taking part in a randomised controlled trial of specialist nurse intervention were studied. This trial has been described in detail elsewhere (Blue *et al*, 2001). Briefly, patients admitted to hospital, as an emergency, with heart failure due to left ventricular systolic dysfunction were enrolled in a trial comparing conventional care to conventional care supplemented by specialist heart failure nurse intervention. Patients were followed for a mean of 12 months after randomisation. Deaths and hospital re-admissions were recorded. Re-admissions were adjudicated by a blinded end-point Committee. The primary end-point was death or readmission with heart failure. Patients consented to have venous blood collected for measurement of neurohumoral factors.

Of the 165 patients randomised, plasma NT-pro BNP and relaxin concentrations were available in 87.

Details of these patients are given in Table 6.1 below.

Table 6.1. Characteristics of Patients Studied

Number of patients	87
Age (range)	75(51-93)
Sex (male/female)	51/36
Current angina	38
Previous MI	40
Diabetes	13
COPD	24
AF	25
Beta-blocker	9
ACE inhibitor	37
Diuretic	60
HMG CoA reductase inhibitor	2
Calcium channel blocker	20
Digoxin	17
Nitrate	12
Aspirin	43
Warfarin	9
Admission NYHA class II III IV	 21 28 38
Creatinine μmol/l	132+/-7.2

MI indicates myocardial infarction; COPD, chronic obstructive pulmonary disease; AF, atrial fibrillation; ACE, angiotensin-converting enzyme, HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A and NYHA, New York Heart Association.

6.3 NT-pro BNP CONCENTRATIONS DETECTED IN PATIENTS

The median (range) plasma NT-pro BNP concentration was marked elevated at 2994 (134-35,000) pg/ml compared to normal (< 334 pg/ml for females and <227 pg/ml for males).

Plasma NT-pro BNP was a predictor of both death or heart failure hospitalisation and death (Figures 6.1 and 6.2). Of patients with NT-pro BNP above the median concentration (n=43), 23 (53%) died and 30 (70%) died or were hospitalised with CHF. For those with NT- pro BNP concentrations below the median (n=44), these proportions were 5 (11%) and 12 (27%) ($p<0.0001$ for death, and $p<0.0001$ for death or CHF hospitalisation).

Figure 6.1 Time to death in patients with plasma NT pro BNP concentrations above and below the median

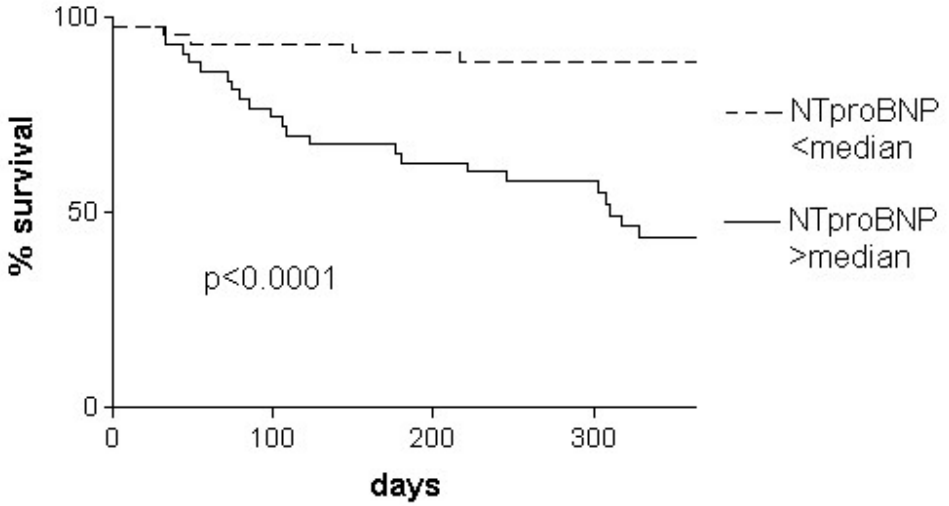
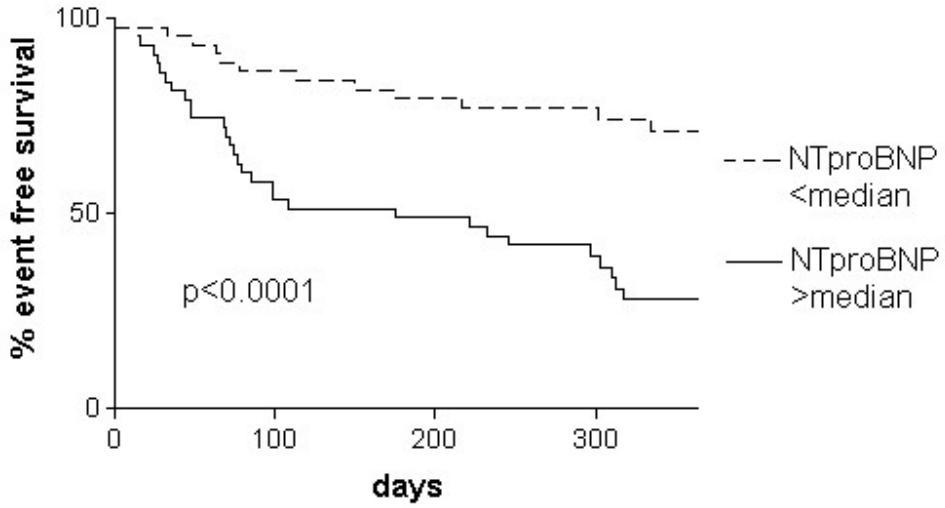


Figure 6.2. Time to death or hospital admission for heart failure in patients with plasma NT pro BNP concentrations above and below the median.



In the multivariate analysis, plasma NT pro BNP concentration was a significant, independent predictor of death or CHF hospitalization (odds ratio 4.15, p=0.003) and of death alone (odds ratio 2.22, p=0.03). Details of the univariate and multivariate analyses are given below.

Univariate analyses

An increased risk of death was conferred by having a plasma NT-proBNP concentration above the median value (OR 8.3±4.7, 95% CI 2.8,25.1; P<0.0001) or increasing plasma creatinine concentration (R^2=0.05, P=0.04). An increased risk of death was also conferred by having COPD (OR 2.9±1.5, 95% CI 1.1,7.8; P=0.031), a history or previous hospitalisation for CHF (OR 2.6±1.2, 95% CI 1.0, 6.5; P=0.043). A reduced risk of death was associated with the presence of atrial fibrillation (OR 0.2±0.1, 95% CI 0.05, 0.7; P=0.015).

An increased risk of death or CHF hospitalisation was associated with having a plasma NT-proBNP concentration above the median value (OR 8.8±4.4, 95% CI3.3,23.2; P<0.0001), increasing plasma creatinine concentration (R^2 = 0.06; P=0.023), the presence of valve disease (OR 3.6±2.3, 95% CI 1.0,12.5; P=0.041). A reduced risk of death was associated with the presence of atrial fibrillation (OR 0.4±0.2, 95% CI 0.1, 1.0; P=0.049).

Multivariate analyses

The results of the multivariate analyses are detailed below.

MULTIVARIATE ANALYSIS FOR *DEATH*, BASED ON FINDINGS FROM UNIVARIATE ANALYSES.

Outcome variable: death (DEATH), n=87

Covariate	n	Odds Ratio	P>|z| (95% Conf. Interval)
NT-proBNP			
per S.D. (= 6409)	87	2.220	0.029 (1.179 to 4.603)
CREATININE			
per S.D. (= 67.31)	87	1.110	0.745 (0.593 to 2.078)
AF			
0*	60	1	
1	25	0.240	0.053 (0.057 to 1.019)
COPD			
0*	61	1	
1	24	2.011	0.239 (0.628 to 6.437)
CHFADMISSION			
0*	47	1	
1	38	1.926	0.263 (0.612 to 6.060)

Plasma NT-proBNP was found to be an independent predictor of death (OR 2.2, 95% CI1.2,4.6; P=0.03).

MULTIVARIATE ANALYSIS FOR ***DEATH/CHF ADMISSION*** BASED ON FINDINGS FROM UNIVARIATE ANALYSES

Outcome variable: DEATH/CHF, n=87, using standard deviation for NT-proBNP and CREATININE

Covariate	n	Odds Ratio	P>|z| (95% Conf. Interval)
NT-proBNP			
per S.D. (= 6409)	87	4.152	0.003 (1.784 to 12.028)
CREATININE			
per S.D. (= 67.31)	87	1.061	0.873 (0.516 to 2.178)
AF			
0*	60	1	
1	25	0.426	0.163 (0.128 to 1.415)
VALVEDISEASE			
0*	70	1	
1	15	2.891	0.185 (0.601 to 13.902)

NT-proBNP was an independent predictor of death/CHF hospitalisation (OR 4.152 1.7 to 12.0; P=0.003).

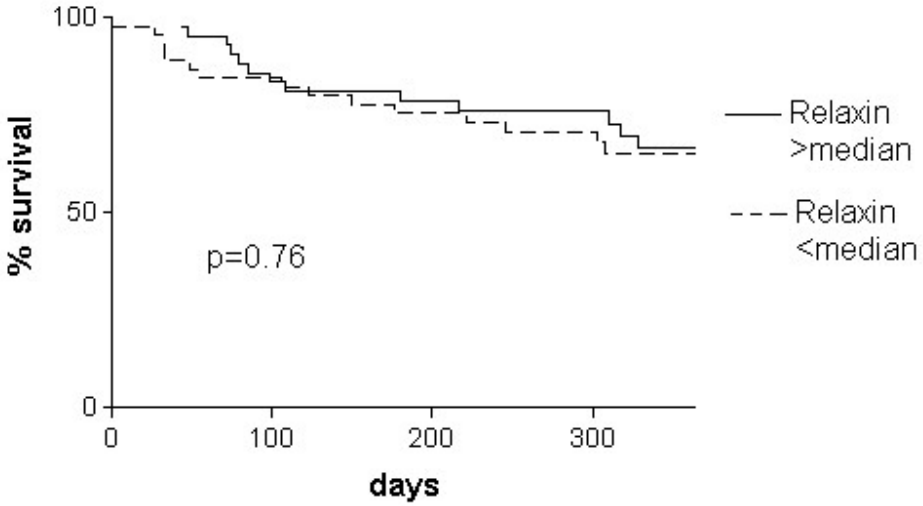
6.4 RELAXIN CONCENTRATIONS DETECTED IN PATIENTS.

The median (range) plasma relaxin concentration was also markedly elevated at 89 (11-644) pg/ml compared to normal (< 2 pg/ml). However, in contrast to NT pro BNP, there was no relationship between relaxin and outcome (Figures 6.3 and 6.4). Of those patients with a relaxin concentration above the median (n=42), 13 (31%) died and 20 (48%) died or were hospitalised. These proportions for patients with a plasma relaxin concentration below the median (n=45) were 15 (33%) and 22 (49%) (p=0.76 for death and p=0.84 for death or CHF hospitalisation). Plasma relaxin concentration was not a significant predictor of outcome in the univariate analysis.

There was no correlation between plasma concentrations of relaxin and NYHA functional class (as a marker of severity of heart failure).

There was no correlation between plasma concentrations of relaxin and those of NT-proBNP (Figure 6.5).

Figure 6.3. <u>Time to death in patients with plasma relaxin concentrations above and below the median.</u>



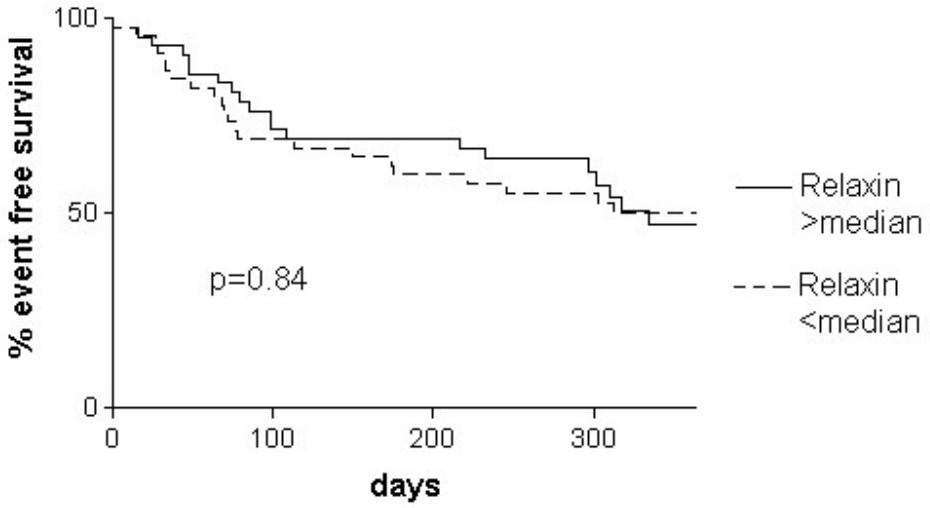


Figure 6.4. Time to death or hospital admission for heart failure in patients with plasma relaxin concentrations above and below the median.

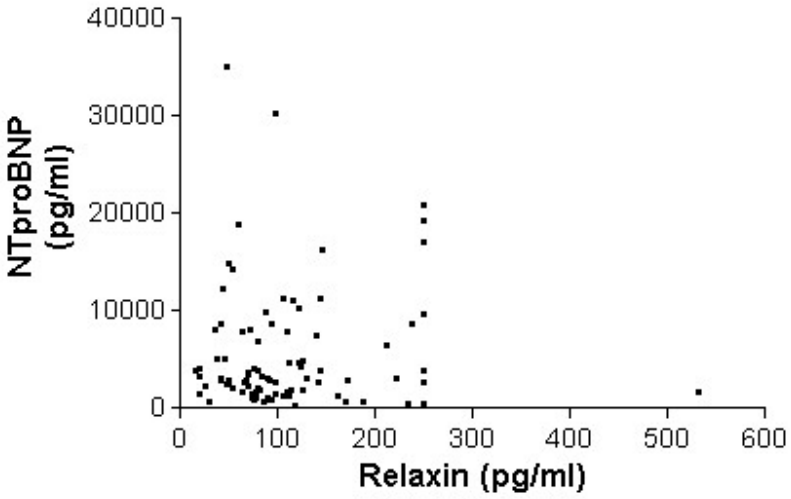


Figure 6.5. Correlation plot for NT pro BNP and relaxin concentrations.

6.5 Summary of Chapter Results.

NT-pro BNP is predictive of both death and death or readmission with worsening heart failure.

We have shown that NT-pro BNP is a powerful and independent predictor of outcome in chronic heart failure (CHF).

Although plasma levels of relaxin were elevated in patients with CHF, it is not predictive of outcome.

This work has been published.

C Fisher, C Berry, L Blue, J J Morton, J McMurray. N-terminal pro B type natriuretic peptide, but not the new putative cardiac hormone relaxin, predicts prognosis in patients with chronic heart failure. *Heart* 2003; 89:879-881.

CHAPTER 7:

TRANSPULMONARY AND TRANSCARDIAC GRADIENT OF RELAXIN

7.1 Summary

Relaxin has only lately been shown to have renal and haemodynamic actions. Relaxin may also be secreted by the heart, at least when it is failing (Dschietzig *et al*, 2001) Plasma relaxin concentrations are elevated in chronic heart failure (CHF) and myocardial expression of the H1 and H2 relaxin gene is increased in proportion to the severity of CHF. Whether the heart is a source of relaxin when cardiac contractility is preserved is unknown. The lungs are commonly involved in the clearance or secretion of vasoactive peptides but their role in relaxin metabolism is unknown. The aim of this study was to measure trans-cardiac and trans-pulmonary relaxin gradients in subjects with preserved left ventricular ejection fraction (LVEF). Patients undergoing coronary artery bypass grafting (CABG) were studied as both pulmonary and cardiac arterial inflow and venous effluent can be readily sampled.

Our study had Ethics approval.

7.2 PATIENTS

20 consecutive patients were studied. Immediately prior to institution of cardiopulmonary bypass, blood samples were taken into chilled tubes, in rapid succession, from the aorta, coronary sinus, pulmonary artery and pulmonary vein. A validated relaxin immunoassay was used (Immundiagnostik, Bensheim, Germany). Statistical analysis was performed using a Wilcoxon signed rank test.

Patient characteristics are shown in Table 7.1 below.

Table 7.1. Characteristics of Patients Studied.

male/female	(n=14/6)
mean age (range)	62 (44 – 74)
mean LVEF % (range)	55 (25 – 70)
medical history • hypertension • diabetes mellitus • prior MI • asthma	 4 3 7 2
current smoker	3
medication • beta-blocker • ACE inhibitor • CCB • long acting nitrate • nicorandil • HMG CoA reductase inhibitor • diuretic	

CCB	= calcium channel blocker
HMG CoA	= 3-hydroxy-3-methylglutaryl coenzyme A
LVEF	= left ventricular ejection fraction

7.3 RESULTS.

7.3.1 Transcardiac Gradient of Relaxin.

There was a decrement in the concentration of relaxin between aorta and coronary sinus in 16 of the 20 subjects studied, suggesting *cardiac extraction* of relaxin. The mean (SD) concentration fell from 38.1(61.3) to 32.8(57.8) pg/ml ($p<0.04$).
No characteristic differentiated the 16 patients with cardiac extraction of relaxin from the remaining four (Figure 7.1).

7.3.2 Transpulmonary Gradient of Relaxin.

There was no trans-pulmonary gradient in relaxin concentration. The mean (SD) concentration in the pulmonary artery was 42.0 (68.3) pg/ml versus 41.8(69.1) pg/ml in the pulmonary vein (p=n.s). (Figure 7.2).

Figure 1. Plasma relaxin concentrations (pg/ml) in aorta and coronary sinus

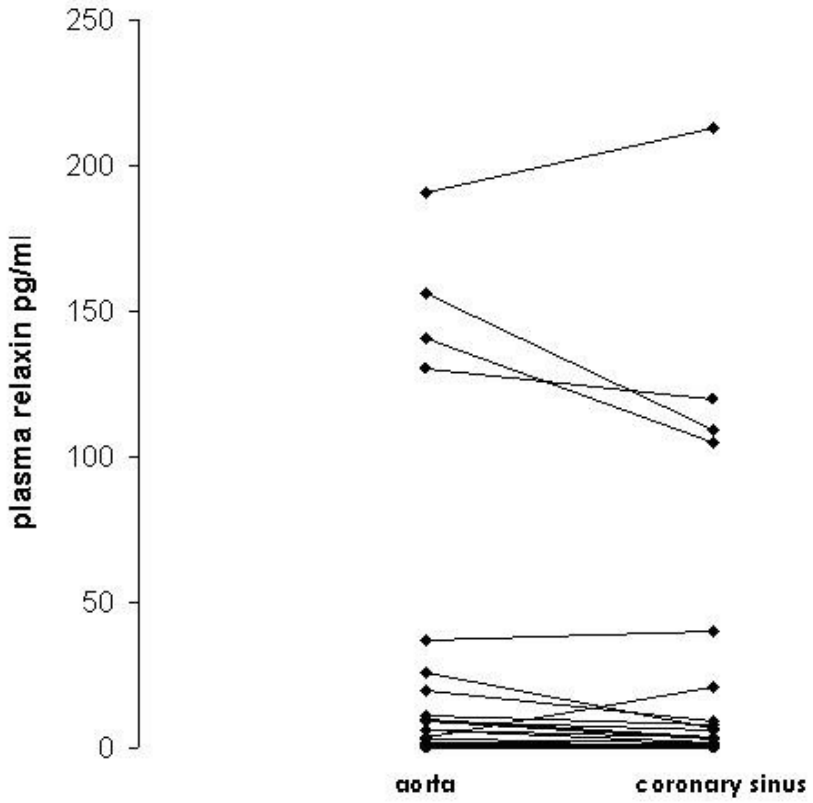
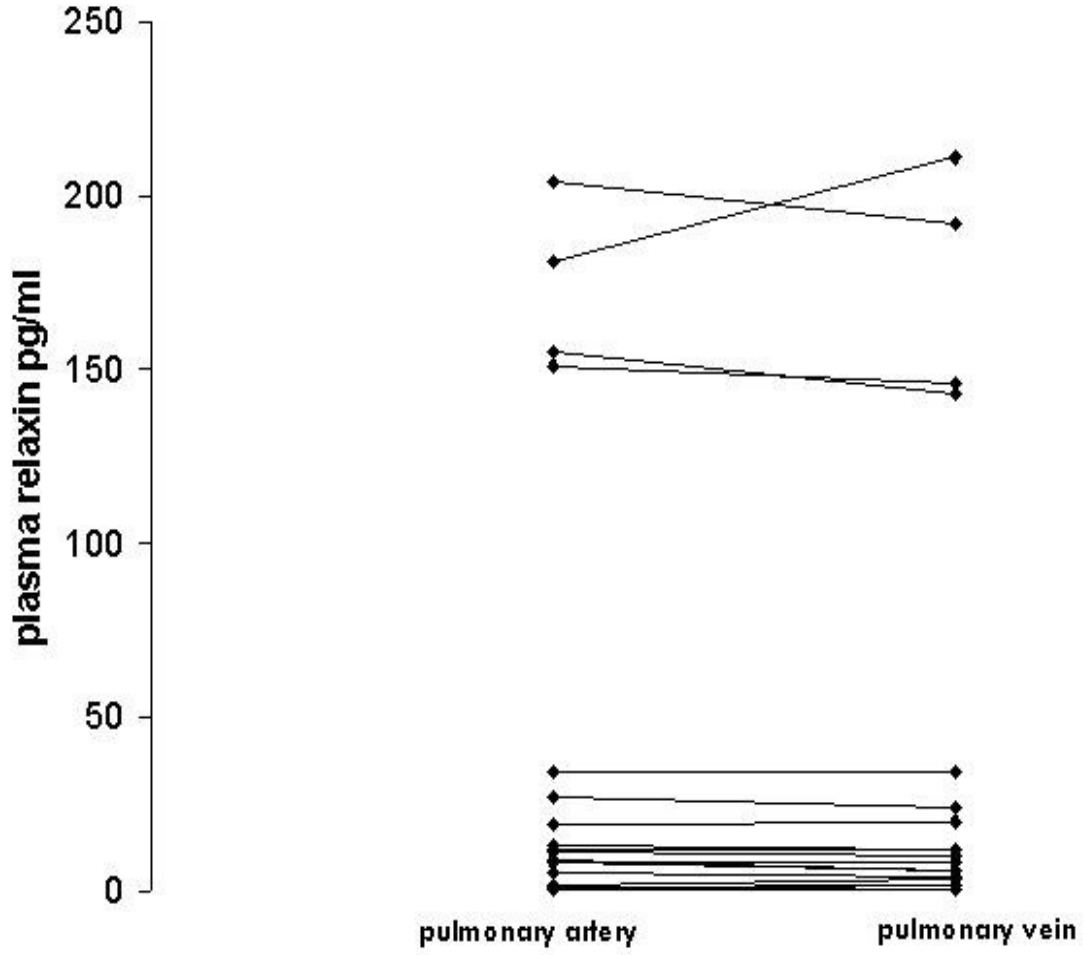


Figure 2 Plasma relaxin concentrations (pg/ml) in pulmonary artery and pulmonary vein



7.4 Summary of Chapter Results.

In patients with coronary disease but without CHF, there is net cardiac extraction of relaxin in contrast to reported secretion in CHF. In patients without CHF there is no transpulmonary gradient in relaxin.

This work has been published.

C Fisher, S Al-Benna, A Kirk, JJ Morton, JJV McMurray. Transcardiac and transpulmonary gradients in the putative new cardiovascular hormone relaxin. *Heart* 2003;89:789-790.

CHAPTER 8:

DISCUSSION

8.1 COMPARATIVE POTENCY OF RELAXIN

8.1.1 Relaxin as a Systemic Vasodilator

Our findings show that relaxin is a more potent arterial vasodilator than ANP, also secreted by the heart. It had been suggested that relaxin might exert its systemic vasodilator effects by stimulating the secretion of ANP (Toth *et al*, 1996). However, our results show that relaxin is a vasodilator in its own right.

Furthermore, relaxin is vasoactive at concentrations comparable to those found in CHF. Mean plasma relaxin concentrations in patients with severe CHF average 2.5 – 3.34 x 10^{-11} M while mean plasma ANP concentrations in patients with severe CHF, average 2.5 x 10^{-11} M. At 10^{-11} M, relaxin caused 26 % vasodilation of resistance arteries (*versus* 0.68 % with the same concentration of ANP).

The potency of relaxin is impressive. It is equipotent to epoprostenol, a substance regarded as a powerful vasodilator and used therapeutically in cardiovascular disease (Kieler-Jensen *et al*, 1995). Of course, epoprostenol is a particularly effective pulmonary vasodilator whereas relaxin seems to be devoid of this action.

8.1.2 Relaxin is Endothelium Dependent

We also found that relaxin induced vasodilation is endothelium dependent. Removal of the endothelium almost abolished its effect. This is in keeping with the observation that relaxin increases nitric oxide (NO) in cultured vascular smooth

muscle cells from bovine aortae (Bani *et al*, 1998). Similarly, NO inhibition abolished the increase in renal plasma flow induced by relaxin in rats (Danielson, 1999). Relaxin also increases coronary blood flow in experimental animals through NO (Bani, 1997).

8.1.3 Relaxin Does Not Vasodilate all Arteries.

Interestingly, relaxin does not seem to vasodilate all arteries. Human relaxin did not dilate human myometrial and uteroplacental arteries pre-contracted with norepinephrine, endothelin or U46619 (Petersen *et al*, 1991). Similarly, porcine relaxin has no effect on human umbilical arteries pre-contracted with potassium chloride or serotonin (Dombrowski *et al*, 1986). This suggests that relaxin does not act as a vasodilator in the placental-fetal circulation. We found that relaxin is inert in preconstricted human *pulmonary* resistance arteries, in contrast to systemic vessels. This may reflect differing relaxin receptor distribution in the circulation, as the nitric oxide vasodilator pathway was functionally intact in these pulmonary vessels (Bathgate *et al*, 2002). It should also be noted, however, that the sample of patients providing small pulmonary resistance arteries was small (n=5) and 2 out of 5 patients were taking ACE inhibitors which may have influenced the overall result.

8.1.4 Other Actions of Relaxin in Cardiovascular Disease

Relaxin could have other potentially favourable vascular and non-vascular actions in cardiovascular disease. For example, relaxin increases vascular endothelial growth factor, (Unemori *et al*, 1999) antagonises the vasoconstrictor action of other peptides such as angiotensin II (Massicotte *et al*, 1989) and inhibits collagen synthesis/increases collagen breakdown (Qin *et al*, 1997). The last action, key to the pelvic remodeling effect of relaxin, could also be important in cardiac and vascular remodeling. In addition, relaxin has also been reported to protect against experimental ischemia-reperfusion injury (Bani 1997).

Though the true vaso-regulatory role of relaxin can only be assessed using an antagonist, it does seem to be more potent than ANP, a hormone that circulates at a similar concentration and the inhibition of which leads to vasoconstriction and other potentially adverse cardiovascular effects (Drexler *et al*, 1990).

8.1.5 Summary

In summary, we have shown that relaxin, a hormone now known to be secreted by the heart, is a potent vasodilator of small systemic resistance arteries at pathophysiological concentrations and that this action is endothelium dependent. Relaxin is not, however, a pulmonary vasodilator. The pleiotropic actions of

relaxin suggest that its potential role in cardiovascular regulation merits further investigation.

8.2 THE MECHANISM OF ACTION OF RELAXIN

This series of experiments, in conjunction with our prior observations, suggests that the vasodilator action of relaxin is endothelium-dependent in small resistance arteries and involves both NO-dependent and NO-independent cyclic GMP and prostanoid-dependent cyclic AMP second messenger pathways (though the latter was only clearly revealed in vessels taken from patients treated with an ACE inhibitor).

8.2.1 In Patients Not Treated with an ACE inhibitor.

In patients not treated with an ACE inhibitor, the vasodilatory action of relaxin appears to be mediated via the nitric oxide pathway since both L-NAME and L-NOARG reduced relaxin's action. In keeping with this, by blocking the conversion of GTP to cGMP with the guanylate cyclase inhibitor, ODQ, the vasodilatory action of relaxin was reduced. However, preventing the breakdown

of cGMP, with the cGMP phosphodiesterase inhibitor, zaprinast did not augment relaxin's vasodilatory action.

The Bani group in recent years has provided increasing evidence that relaxin can act on several of its targets by increasing the expression and/or activity of nitric oxide synthase (NOS) isoenzymes, thereby promoting the generation of nitric oxide (NO). In 1994, Masini *et al*, showed that the relaxin attenuated calcium ionophore-induced granule exocytosis by isolated rat serosal mast cells, was mediated by nitric oxide (Masini *et al*, 1994).

This group then went on to show the existence of a relaxin- NO pathway causing increased levels of cGMP in rat and guinea pig hearts (Bani-Sacchi *et al*, 1995), in human and rabbit platelets (Bani *et al*, 1995a), in human breast cancer cells (Bani *et al*, 1995b) and in mouse small bowel (Bani *et al*, 2002). The NO synthase involved depends on the cell type under investigation. In the mouse uterus, relaxin causes up-regulation of NOS-III expression in epithelium, glands, endometrial stromal cells and myometrium while leaving inducible NOS (NOS II) expression unaffected (Bani *et al*, 1999). However, in rat coronary endothelial cells (Failli *et al*, 2002) and in bovine aortic smooth muscle cells (Bani *et al*, 1998), relaxin promoted expression and activity of the inducible NO synthase (NOS II) with negligible effects on NOS-III.

The protective effect of relaxin in cardiac anaphylaxis involves an up-regulation of the NO pathway (Ndisang *et al*, 2001), (Masini *et al*, 2002).

In this group of patients, not treated with an ACE inhibitor, the prostacyclin pathway also appears to be important since the cyclo-oxygenase inhibitor, indomethacin, reduced the vasodilatory action of relaxin. As previously described, prostacyclin activates adenylate cyclase to produce cAMP. When we used the cAMP phosphodiesterase inhibitor, milrinone, to prevent cAMP breakdown, however, the vasodilatory action of relaxin was not augmented. The EDHF pathway does not appear to be important in this group of patients.

8.2.2 In Patients Treated with an ACE inhibitor.

The most surprising finding of this study was, however, that ACE inhibitor treatment substantially blocked the vasodilator action of relaxin (and revealed a second prostanoid-cAMP dependent vasodilator mechanism of action of relaxin).
By discovering that the mechanism of action of relaxin in these patients is different to its action in those patients not treated with ACE inhibitors, we have shown that the prostacyclin pathway and the cAMP second messenger pathway are also important. Indomethacin blocked relaxin's action while milrinone, by preventing cAMP breakdown, enhanced the vasodilatory action of relaxin in this group of patients.

The cGMP second messenger pathway may also be important in these patients. By blocking conversion of GTP to cGMP, ODQ reduced relaxin's action while preventing cGMP breakdown with zaprinast, enhanced relaxin's action. These findings were at supraphysiological levels of relaxin, however, and so may not be clinically relevant.

Blocking the NO and EDHF pathways had a curious effect, though as rather than reducing the vasodilatory action of relaxin as expected, its vasodilatory effect was actually enhanced in patients treated with an ACE inhibitor. Again, it should be noted that blocking of the NO pathway causing enhancement of relaxin's vasodilatory action was found at high doses i.e. supraphysiological levels of relaxin and so may not be clinically relevant.

Why ACE inhibitor treatment should block the effect of relaxin is unknown and can only be speculated about. Of note, the other patient characteristics in the ACEI treated group and the non-ACEI treated group are very similar i.e. it does appear to be purely an ACE inhibitor effect. ACE inhibitors do, however, up-regulate the endothelium-vascular smooth muscle nitric oxide-cGMP pathway in human arteries. If it was already substantially activated in our patients taking an ACE inhibitor, there may be little remaining potential for relaxin to further stimulate this pathway. This circumstance may, however, have allowed

alternative vasodilator pathways to be revealed. This is indeed what our studies with indomethacin and milrinone seem to show. In vessels taken from patients treated with an ACE inhibitor, indomethacin blocked the vasodilator action of relaxin whereas milrinone enhanced it. This suggests that relaxin can also act through a prostanoid-cAMP pathway in small human resistance arteries.

The recent findings from PERTINENT, a substudy of the EUROPA study which demonstrated reduction in cardiovascular mortality and myocardial infarction in patients with stable coronary artery disease taking the ACEI perindopril, demonstrated up-regulation of the nitric oxide pathway via bradykinin (Ceconi et al, 2007). In this study, the effect of perindopril on endothelial function was determined by cultivating *in vitro* human umbilical vein endothelial cells (HUVECs). Incubation of HUVECs with serum taken at baseline from patients with CAD showed a significant down-regulation of endothelial nitric oxide synthase (eNOS) protein expression and activity (-26% and -30%, respectively; both $p<0.01$ compared with incubation with serum from the control group). At 1 year, the down-regulation of eNOS protein expression and activity was modulated by the treatment with perindopril: up-regulation by 19% and 27% for eNOS protein expression (p=ns) and ($p<0.05$), respectively, was observed. This modulation was at least in part mediated by the activation of bradykinin B2 receptors since the use of a specific B2 receptor antagonist, icatibant, conteracted the beneficial effect of perindopril. In addition, a correlation between increased plasma bradykinin levels and eNOS up-regulation, was noted.

Relaxin has previously been described to act via cAMP in animal models. Braddon first described relaxin-induced cAMP changes in the mouse symphysis in 1978 (Braddon, 1978). The causal relation between relaxin exposure, cAMP rise and inhibition of spontaneous contractile activity in the rat uterus was described back in 1980 (Sanborn *et al*, 1980). These observations were confirmed for human endometrial glandular cells (Chen *et al*, 1988) and human breast cells (Bigazzi *et al*, 1992). Cronin et al found that relaxin increases cAMP levels in cultured anterior pituitary cells (Cronin *et al*, 1987). Toth *et al*, investigated the effect of relaxin on the isolated perfused rat heart. A cAMP dependent protein kinase inhibitor (H-89) was found to substantially reduce the ANP secretory effect of relaxin (Toth *et al*, 1996).

When Hsu and colleagues discovered the relaxin receptors in 2002, LGR7 and LGR8 (now renamed RXFP1 and RXFP2 respectively), they showed that these G protein coupled receptors mediate the action of relaxin through an cAMP dependent pathway (Hsu *et al*, 2002).

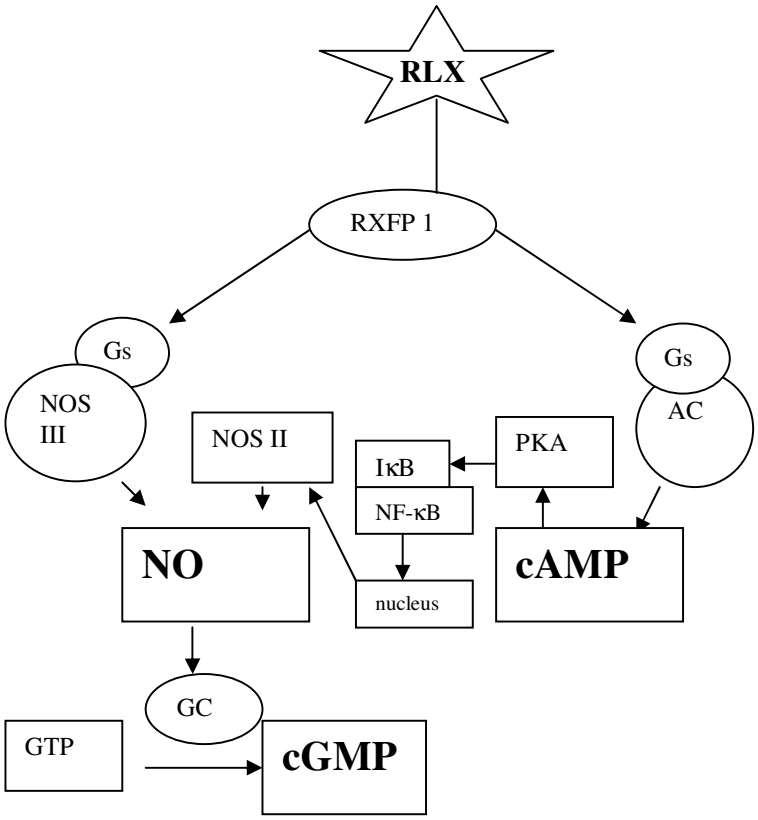
The Ivell group suggest that activation of the relaxin receptor leads to tyrosine phosphorylation, which, in turn, inhibits phosphodiesterase (PDE) activity and further upregulates cAMP levels. They examined human endometrial stromal cells and human macrophages (THP-1) in the human monocyte cell line to show that the relaxin receptor also initiates tyrosine kinase activation (Bartsch *et al*, 2001, Ivell, 2002, Bartsch *et al*, 2004, Ivell *et al*, 2005).

Previously we showed that endothelial denudation greatly reduced the vasodilation action of relaxin in human small resistance arteries. This observation was in keeping with a number of animal studies and a study in human vascular smooth muscle cells suggesting the action of relaxin involves nitric oxide-cGMP signalling. In spite of this, neither inhibition of nitric oxide synthase with L-NOARG, or with L-NAME, blocked the vasodilator effect of relaxin in this study, in patients treated with ACE inhibitors. Direct inhibition of soluble guanylate cyclase with ODQ did, however, reduce the vasodilator action of relaxin, raising the possibility that relaxin causes NO-independent activation of soluble guanylate cyclase in these patients. In keeping with this, the cGMP phosphodiesterase inhibitor zaprinast, which prevents the breakdown of cGMP, augmented the vasodilator effect of relaxin.

Other groups have demonstrated that relaxin's vasodilatory effect is mediated via more than one distinct pathway. Longo *et al*, studied uterine arteries from pregnant rats and found that relaxin's effect in these arteries was mediated by nitric oxide, soluble guanylate cyclase (which converts GTP to cGMP) and adenylate cyclase (which is responsible for the production of cAMP). They suggest that the NO-cGMP pathway is one of the second messenger systems involved in the vascular effects of relaxin in pregnancy but that cAMP is also involved (Longo *et al*, 2003).

Putative interactions between relaxin receptor signaling and the intrinsic NO pathway have been described (Nistri and Bani, 2003). See Figure 8.1 below.

Figure 8.1 Possible Interactions Between Relaxin Receptor and NO Pathway.



AC: adenylate cyclase, cAMP: cyclic adenosine monophosphate, cGMP: cyclic guanosine monophosphate, GC: guanylate cyclase, Gs: Gs proteins, GTP: guanosine triphosphate, IκB: inhibitor subunit of nuclear factor kappa-B, PKA:

protein kinase, NF-κB: nuclear factor kappa-B, NO: nitric oxide, NOS II: inducible NO synthase, NOS III: constitutive NO synthase, RLX: relaxin
(Adapted from Nistri and Bani, 2003).

Our studies have, however, by necessity been carried out in arteries from patients with coronary heart disease and it is possible that the balance between these vasodilator mechanisms could be different in healthy controls. Coats *et al*, showed that EDHF is the major contibutor to endothelium-dependent vasorelaxation in human subcutaneous resistance arteries in healthy volunteers (Coats *et al*, 2001). We have been unable to confirm this as a mechanism of action for relaxin, in resistance arteries taken from patients with coronary artery disease.

From a therapeutic perspective, however, our patient group is relevant and interesting. The therapeutic potential of relaxin as a vasodilator is perhaps less than previously considered, given that so many patients with cardiac disease have an indication for treatment with an ACE inhibitor.

Our results, however, open up another possible therapeutic avenue for relaxin. Type 5 phosphodiesterase inhibitors seem to have limited effectiveness as a treatment for erectile dysfunction in certain subsets of patients, for example diabetics, probably because of impaired endogenous NO production.

Theoretically, NO-independent activators of guanylate cyclase (of which relaxin seems to be one) may be more effective.

8.2.3 Summary

In summary, we have shown that the vasodilator peptide relaxin acts differently in human small resistance arteries in patients treated and not treated with ACE inhibitors. In ACEI-treated patients, relaxin appears to work via two distinct second messenger systems. One pathway involves vasodilator prostanoids and cAMP. The other pathway may involve NO-independent activation of guanylate cyclase and cGMP.

In non-ACEI-treated patients, relaxin works via an NO-dependent cGMP second messenger pathway. The other pathway involves vasodilator prostanoids but this does not appear to be as powerful as the cGMP pathway.

8.3 RELAXIN IN HUMAN INTERNAL MAMMARY ARTERIES AND SAPHENOUS VEINS

Although, in small human systemic resistance arteries, we showed that relaxin is a potent vasodilator (Fisher et al, 2002), we were unable to find that in human large calibre vessels i.e. internal mammary arteries and long saphenous veins that relaxin had any vasodilator effect compared to control.

8.3.1 Relaxin is Endothlium-Dependent

We know that relaxin is endothelium-dependent and it proved to be extremely difficult to obtain samples of IMA and LSV where the endothelium was indeed intact. Although for this set of experiments, only vessels where the endothelium had been found to be intact, were used in the data set, perhaps the endothelial function of the vessels was impaired and this had an impact with regard to relaxin's lack of action. Of course, relaxin may not act as a vasodilator of large calibre vessels and certainly Reid *et al*, reported that relaxin had no effect on endothelium intact rat aortae pre-constricted with noradrenaline (Reid *et al*, 2001). Of course, there may also be marked species differences in the action of relaxin as documented with other peptides. Interestingly, Hillier *et al,* investigated the action in humans, of the peptide, human urotensin II (hUII) which is a potent vasoconstrictor in some fish and mammals (e.g. cynomolgus monkey). They found

that hUII had no vasoconstrictor action in human arteries and veins of different sizes and vascular beds showing a marked species difference (Hillier *et al*, 2001b).

8.3.2 Relaxin and Arteries

It would seem, therefore, that relaxin does not vasodilate all arteries. Human relaxin did not dilate human myometrial and uteroplacental arteries pre-contracted with norepinephrine, endothelin or U46619 (Petersen *et al*, 1991). Similarly, porcine relaxin has no effect on human umbilical arteries pre-contracted with potassium chloride or serotonin (Dombrowski *et al*, 1986). This suggests that relaxin does not act as a vasodilator in the placental-fetal circulation. We found that relaxin is inert in preconstricted human *pulmonary* resistance arteries, in contrast to systemic vessels (Fisher *et al*, 2002). This may reflect differing relaxin receptor distribution in the circulation, as the nitric oxide vasodilator pathway was functionally intact in these pulmonary vessels (Bathgate *et al*, 2002).

8.3.3. Relaxin and Veins

There is very little reported in the literature regarding the action of relaxin on the venous system. Massicotte *et al*, found that there was a blunted response to vasoconstrictors, arginine-vasopressin and norepinephrine in the perfused mesenteric artery (with the concentration response curves shifted to the right by a

factor of two ($p<0.05$ and $p<0.005$ respectively) after treatment with relaxin. However, in the isolated portal vein, no statistical differences were observed in either the maximum response or sensitivity to angiotensin II or norepinephrine (Massicotte *et al*, 1989).

8.3.4 Relaxin and ACE Inhibitors

We reported that the vasodilatory effect of relaxin in patients treated with ACE inhibitors is significantly reduced. Of the ten patients whose IMA and LSV were harvested at the time of coronary artery bypass grafting, only 2 of them were on ACE inhibitors, so this is unlikely to be the cause of relaxin's lack of effect in IMA and LSV.

8.3.5 Relaxin and Beta-blockers

There has been very little reported in the literature on the interaction of betablockers with relaxin. One *in vivo* study reported that treatment with the β-adrenoreceptor antagonist propanolol significantly reduces the heart rate response to relaxin (Summerlee and Parry, 1993). In *in vitro* studies, propanolol has a small inhibitory action on the effects of relaxin in the heart (Kakouris *et al*, 1992). Of the 10 patients whose IMA and LSV were used in the study, 8 of them were on beta-blockers so this may have had an effect on relaxin in these vessels. Against this hypothesis however, is the fact that in our study on small resistance arteries where

relaxin was found to be a potent vasodilator, 11 out of 13 patients were on long term betablockers.

8.3.6 Summary

We have been unable to show that relaxin has a vasodilatory effect in human large calibre vessels, neither internal mammary arteries nor long saphenous veins.

8.4 RELAXIN COMPARED WITH NT pro BNP AS A PROGNOSTIC INDICATOR IN HEART FAILURE

Our study confirms that heart failure due to left ventricular systolic dysfunction is associated with marked elevation of the plasma concentration of the two new neurohumoral markers N-terminal pro BNP and relaxin.

8.4.1 NT pro BNP in Heart Failure

Prior to this study, there were few reports of plasma NT pro BNP levels in heart failure. Hobbs *et al*, investigated the performance of NT pro BNP in diagnosing heart failure in various randomly selected general and high risk community populations. They found that for NT pro BNP in the diagnosis of heart failure in the general population, a level of >36 pmol/l had a sensitivity of 100%, a specificity of 70%, a positive predictive value of 7% and a negative predictive value of 100% (Hobbs *et al*, 2002). Masson *et al* compared BNP with NT pro BNP in ambulatory patients with heart failure and found that the concentration of both peptides increased in a similar fashion according to the severity of NYHA class, left ventricular ejection fraction, aetiology and age. They concluded that NT pro BNP correlates equally to BNP with clinical variables in patients with CHF. Hunt *et al*, also compared BNP with NT pro BNP in patients with heart failure. They noted that plasma levels of NT pro BNP are raised in cardiac impairment, including NYHA class I i.e. asymptomatic patients, and rise with cardiac

decompensation. They found that the proportional and absolute increment above normal levels of NT pro BNP exceeds that for BNP suggesting that NT pro BNP may be a more discerning marker of early cardiac dysfunction than BNP (Hunt *et al*, 1997). Talwar *et al*, found that NT pro BNP is a strong, independent predictor of left ventricular dysfunction demonstrating a linear relationship between NT pro BNP and left ventricular wall motion index. NT pro BNP had a sensitivity of 95% and specificity of 55%. Of more clinical importance, they felt, was the high negative predictive value of 93% of NT pro BNP in the diagnosis of left ventricular dysfunction (Talwar *et al*,1999).

8.4.2 Relaxin in Heart Failure

At the time of this study, there was only one other report describing relaxin concentrations in heart failure (Dschietzig *et al*, 2001).

More recently, Kupari *et al*, investigated the role of relaxin in pressure overload-induced human heart failure. They studied patients undergoing cardiac catheterisation for isolated aortic stenosis. Blood was sampled from the aortic root and from the coronary sinus. The concentration gradients of relaxin from the aortic root to the coronary sinus indicated relaxin extraction by the heart in control patients in keeping with the findings from our study (Fisher *et al*, 2003a) described in Chapter 7. In patients with systolic heart failure the transcardiac gradient

indicated relaxin production. However, this did not translate into elevated systemic concentrations. They conclude that relaxin is not a major player in human heart failure (Kupari *et al*, 2005). Kruger *et al*, investigated circulating relaxin and its potential role in stable CHF at rest and after physical exercise. They were unable to find a difference in relaxin plasma levels at rest and after exercise between patients with CHF and controls (Kruger *et al*, 2004).

8.4.3 NT pro BNP as a Predictor of Outcome.

In this study, NT pro BNP was also predictive of both death and death or readmission with worsening CHF. Though the predictive value of other natriuretic peptides in CHF has been extensively reported (Hall, 1994), (Eriksson, 1995), (Dickstein, 1997), (Selvais, 2000), (McDonagh, 2001), (Bettenscourt, 2002), we know of only one other study (at the time of this study) describing the prognostic importance of NT pro BNP in CHF (Richards, 2001). In a sub-study of the Australia New Zealand carvedilol trial, Richards *et al* found that NT pro BNP was a strong predictor of all cause mortality as well as admission to hospital with CHF. In that study, NT pro BNP had more predictive power for these outcomes than left ventricular ejection fraction. We also found NT-pro BNP to be an independent predictor of adverse clinical outcome. Consequently, there are now two studies confirming that this very stable peptide (Hughes *et al*, 1999), (Downie *et al*, 1999), which is easy to assay (Hunt *et al*, 1997), (Hughes *et al*, 1999), (Downie *et al*, 1999), has prognostic as well as diagnostic value in CHF. Since publishing our

data (Fisher *et al*, 2003b), there have been numerous studies published showing the predictive value of NT pro BNP in heart failure prognosis (Hartmann *et al*, 2004), (Groenning *et al*, 2004), (Kirk *et al*, 2004), (Squire *et al*, 2004).

NT pro BNP also gives prognostic information after myocardial infarction and in acute coronary syndromes (Richards *et al*, 1998), (Omland *et al*, 2002). Richards *et al*, found that plasma NT pro BNP levels measured 2 to 4 days after myocardial infarction independently predicted left ventricular function and 2-year survival. They conclude that stratification of patients into low and high risk groups could be facilitated by NT pro BNP measurements.

Recently, NT pro BNP has been shown to be an independent prognostic marker in severe sepsis and septic shock (Varpula *et al*, 2007); infective endocarditis (Kahveci *et al*, 2007); after vascular surgery (Mahla *et al*, 2007) and in stable coronary artery disease (Omland *et al*, 2007).

8.4.4 Summary

Dschietzig *et al* have recently shown that cardiac relaxin production and plasma relaxin concentrations are increased in CHF, the latter finding confirmed in the present study (Dschietzig *et al*, 2001). As relaxin is a powerful vasodilator secreted by the heart (Fisher *et al*, 2002), our hypothesis was that like the

natriuretic peptides, plasma concentrations might be related to prognosis. We were not able to confirm this.

The timing of blood samples may be relevant. Blood samples were taken from CHF patients prior to discharge i.e. once stable. Perhaps if the sampling had been done on admission (when the patient was in acute heart failure) we may have been able to show relaxin concentrations related to prognosis.

Though plasma concentrations of most neurohumoral factors are predictive of outcome, not all are (*e.g.* arginine vasopressin) (Richards *et al*, 1998). We were unable to show any correlation between plasma NT pro BNP and relaxin concentrations suggesting different release mechanisms. The atria may be a more important source of relaxin than the ventricles, whereas the opposite is true for NT pro BNP (Dschietzig *et al*, 2001), (Taylor *et al*, 1994). As a biochemical marker of left ventricular dilatation and wall stress, it is perhaps not surprising, therefore, that NT pro BNP is a powerful prognostic factor (Richards *et al*, 2001). Furthermore, net cardiac relaxin release is not pronounced except in moderate to severe CHF and even then is not always observed. These reasons may explain why relaxin was not predictive of outcome. However, it remains possible that relaxin does have some weak predictive effect which was not revealed because of the modest size of our study.

In summary, even though relaxin has been shown to be a potent vasodilator released by the failing heart, it is not a powerful prognostic indicator in CHF.

8.5 TRANSCARDIAC AND TRANSPULMONARY GRADIENTS OF RELAXIN

Dschietzig *et al* showed higher coronary sinus than left ventricular relaxin concentrations in 11 of 14 patients with severe CHF, suggesting that the failing heart may be a source of circulating relaxin (Dschietzig *et al*, 2001). We found the opposite across the *non-failing* heart i.e. net *extraction* of relaxin. Kupari *et al*, have since confirmed this finding in patients without heart failure (Kupari *et al*, 2005). Further inspection of the data from Dschietzig *et al* shows no trans-cardiac gradient in patients with moderate CHF and an aorta-coronary sinus decrement in controls, in keeping with our findings (Dschietzig *et al*, 2001). This suggests that the contribution of the heart to circulating relaxin varies according to the presence or absence of CHF. Whether it is left ventricular systolic dysfunction, abnormal pulmonary or systemic haemodynamics, neurohumoral activation or other factors that leads to net cardiac secretion of relaxin in CHF is presently unknown.

The pattern of relaxin secretion/extraction is distinct from other hormones. A-type and B-type natriuretic peptide increase from aorta to coronary sinus in both the non-failing and failing heart (more markedly in the latter) (Northridge *et al*, 1992). Adrenomedullin is also secreted by both the failing and non-failing heart. In contrast, endothelin-1 is extracted by the failing heart, whereas there seems to be either no trans-cardiac gradient or higher coronary sinus concentrations in non-failing hearts (Stangl *et al*, 2000). There is no trans-cardiac gradient in plasma

aldosterone concentration in the non-failing heart but an increment in coronary sinus aldosterone concentration in CHF.

Interpretation of the cardiac extraction of relaxin by the normal heart, compared to its secretion by the failing heart, is difficult. The mechanisms of relaxin clearance from the circulation are unknown. Changes in the trans-cardiac concentration of other peptides seem to reflect changes in receptor density/affinity e.g. decreases in endothelin concentration across the failing heart are probably caused by the increase in myocardial ET_A and ET_B (Stangl *et al*, 2000). The receptors for relaxin have only recently been described and nothing is known about the effect of CHF on their expression (Dschietzig *et al*, 2001).

The transpulmonary gradient in relaxin concentration has not been described before. Neither net extraction nor secretion occurred, which is different than for other peptides. Pulmonary extraction of ANP (Northridge *et al*, 1992) and adrenomedullin has been described. For endothelin, some have described no transpulmonary concentration gradient, others pulmonary extraction and others still that both secretion and extraction, which balance each other out, occur (Stangl *et al*, 2000). Relaxin also differs from other peptides in having no effect in small pulmonary resistance arteries (whereas it is a potent vasodilator in comparable vessels from the systemic circulation) (Fisher *et al*, 2002).

In summary, in patients with coronary disease but without CHF, there is net cardiac extraction of relaxin in contrast to reported secretion in CHF. In patients without CHF there is no transpulmonary gradient in relaxin.

8.6 LIMITATIONS OF RESEARCH

8.6.1 Subjects studied

On determining the comparative potency of relaxin and its mechanism of action, patients with coronary artery disease generously provided buttock biopsies as a source of small resistance arteries. In addition to this, it would have been useful to have had similar samples from healthy volunteers to determine the comparative potency and mechanism of action of relaxin in these subjects.

Also, the patients that had buttock biopsies taken, had coronary artery disease but were not known to have heart failure and it would have been interesting to see if relaxin's comparative potency and mechanism of action differs in patients with heart failure.

8.6.2 *In Vivo* Studies

We had hoped as part of my MD thesis to perform *in vivo* studies of the action of relaxin in human resistance arteries with plasma concentrations reflecting the physiological and pathophysiological range. This would have involved 1) dorsal hand vein studies, using a modified Aellig technique, to document the local effect of relaxin in a human dorsal hand vein and 2) forearm venous occlusion plethysmography to study the local arterial effect of relaxin in the human brachial artery.

Unfortunately, Connectics Corporation who kindly gifted the relaxin for use in my *ex vivo* experiments, would not allow *in vivo* studies using relaxin gifted by them to be performed in humans.

8.7 FUTURE RESEARCH

The field of relaxin research is progressing rapidly. A single site open-label study of relaxin in patients with compensated CHF has recently been completed in Germany (Dschietzig et al, 2009). This was a safety and dose-finding study of intravenous relaxin given for 24 hours at doses ranging from 10 to 960 μg/kg/day. Pharmacodynamic dose-response parameters (serial haemodynamic measurements using pulmonary artery and radial artery catheters and serial renal chemistry

parameters) were evaluated to define relaxin doses for further study. The study enrolled 16 subjects with compensated CHF, NYHA class II-III due to ischaemic heart disease, hypertensive heart disease or dilated cardiomyopathy with keft ventricular ejection fraction <35%, pulmonary capillary wedge pressure (PCWP) $\geq$ 16mmHg and cardiac index $\leq$ 2.5 l/min/m^2. All 16 subjects completed dosing and the day 9 follow-up visit. Relaxin was safe and well-tolerated in all subjects. Doses of relaxin in the range of 10-100 μg/kg/day appeared to have a more pronounced effect than higher doses on right atrial pressure, pulmonary artery pressure, PCWP and NT pro-BNP while higher doses in the range of 240-960 μg/kg/day tended to have a greater effect on CI. Values for systemic vascular resistance decreased at all doses. The different dose responses observed may be explained by imbalances in baseline haemodynamic status of the dose groups or may be due to random variability in a small pilot study. However, the effect of relaxin may follow a U-shaped dose-response curve.

A high proportion of patients with acute heart failure have elevated blood pressure at the time of presentation, so called acute vascular failure (Teichman et al, 2008). Renal dysfunction is a common co-morbidity and major predictor of poor outcomes in patients with acute heart failure and appears to be particularly common in patients with acute vascular failure. Currently, no therapy has been demonstrated to improve symptoms or renal function in this group of patients. The current understanding of the haemodynamic and renovascular effects of relaxin, as well as the encouraging results from the evaluation of relaxin in patients with compensated heart failure, support the investigation of the use of relaxin as a therapeutic agent

for the treatment of patients with acute vascular failure. The RELAX-AHF study has been undertaken to evaluate the effects of relaxin therapy on symptoms, signs and outcomes in these patients and its results are eagerly awaited.

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