Vasoactive hormone studies in man using urotensin II and vasopressin

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MRCP

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

TABLES

FIGURES

ABSTRACT

Background Within this thesis we have examined the effects on vascular tone of two peptide hormones, urotensin II and vasopressin. Human urotensin II is a novel vasoactive peptide hormone with receptors located on human arterial tissue and ventricular myocytes. It is the most potent arterial vasoconstrictor yet discovered and has a sustained effect on blood vessels from a variety of species with potency 28 to 50 fold greater than endothelin-1. Vasopressin has however, been known to have arterial vasoconstrictor properties in man *in vivo* for many years. However, peptide antagonists for the V1 receptor, mediating vasoconstriction, and the V2 receptor, mediating vasodilatation, have often been of poor selectivity and subject to inter-species variation, thus clouding the true arterial pharmacodynamic effects of vasopressin. Two novel peptidic arginine vasopressin antagonists have recently been produced and offer potentially more receptor selectivity.

Objectives The aims of this thesis were: first to assess the effects of urotensin II on human resistance vessels and venous tone *in vivo*; second, to study the effects of systemic intravenous urotensin II on human haemodynamics such as blood pressure, pulse, vascular resistance and arterial stiffness; third, to explore the human *in vivo* pharmacodynamics of vasopressin in human resistance vessels in the skin microcirculation and forearm as preparation for V1 and V2 receptor antagonist studies; forth, to demonstrate that vasopressin induced vasodilatation is mediated by nitric oxide; and finally, fifth, to combine novel V1 and V2 receptor antagonists with vasopressin in the human skin and forearm to assess the efficacy of novel vasopressin peptide antagonists.

Methods Using the established method of bilateral venous occlusion plethysmography to measure forearm blood flow, combined with intra-arterial infusion of drugs into the brachial artery, we sought to determine the effects of urotensin II on human forearm blood flow. Other methods such as the Aellig venous displacement technique, to assess venous tone, and pulse wave analysis, to quantify arterial stiffness were also used during local and systemic urotensin II intravenous infusions respectively. Doppler flowmetry was used to assess skin microcirculation combined with intra-dermal peptide injection we assessed skin blood flow in response to vasopressin alone and in combination with a novel selective V1 antagonist. Venous occlusion plethysmography was again used to determine forearm blood flow responses to vasopressin alone and in combination with V1 and V2 antagonists.

Results During intra-arterial infusion of urotensin II we did not observe any significant changes in forearm blood flow, even in the presence of endothelial inhibitors such as aspirin and a 'nitric oxide clamp' nor was change observed in venous tone. Moreover, no alteration in systemic haemodynamics or arterial stiffness was seen during systemic intravenous infusion. We observed a significant fall in skin blood flow with intra-dermal injection of vasopressin, however, the V1 receptor antagonist did not alter skin vasoconstriction. Intra-arterial infusion of vasopressin caused a reproducible biphasic change in forearm blood flow, low doses causing vasoconstriction and high doses, nitric

oxide mediated vasodilatation. Vasodilatation was subject to tachyphylaxis during prolonged infusion of high dose vasopressin. Neither intra-arterial V1 or V2 antagonist, when co-infused with vasopressin, altered this biphasic vasoconstriction and vasodilatation.

Conclusion The majority of our findings for urotensin II were in contrast to our hypothesis. Until a selective urotensin II antagonist is developed the physiological role of urotensin II in human cardiovascular physiology will remain difficult to ascertain. Its role may alternatively lie in longer term regulation of vascular tone or in sodium and metabolic homeostasis. We defined the pharmacodynamics of vasopressin in humans *in vivo* to a greater depth than previous studies and confirmed, using a 'nitric oxide clamp', the dependence of vasopressin induced vasodilatation on locally derived nitric oxide. Both vasopressin antagonists have yet again been subject to considerable inter-species variation, as demonstrated by the efficacy of the V1 receptor antagonist in our rat biopressor assay. To date there are no truly selective peptidic vasopressin receptor antagonists available for human *in vivo* studies.

DECLARATION

This thesis represents research undertaken in the Clinical Pharmacology Unit and Research Centre at the Western General Hospital in Edinburgh. The substantial part of the work described has been my own and carried out during the period between May 2000 and July 2002 when I was a British Heart Foundation Junior Research Fellow. The majority of the data chapters have been published in peer reviewed journals (see bibliography). The thesis has not been accepted in any previous applications for a degree and all sources of information have been acknowledged.

Jonathan Theodore Affolter

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CHAPTER 1

INTRODUCTION: CHARACTERISTICS OF UROTENSIN II AND VASOPRESSIN

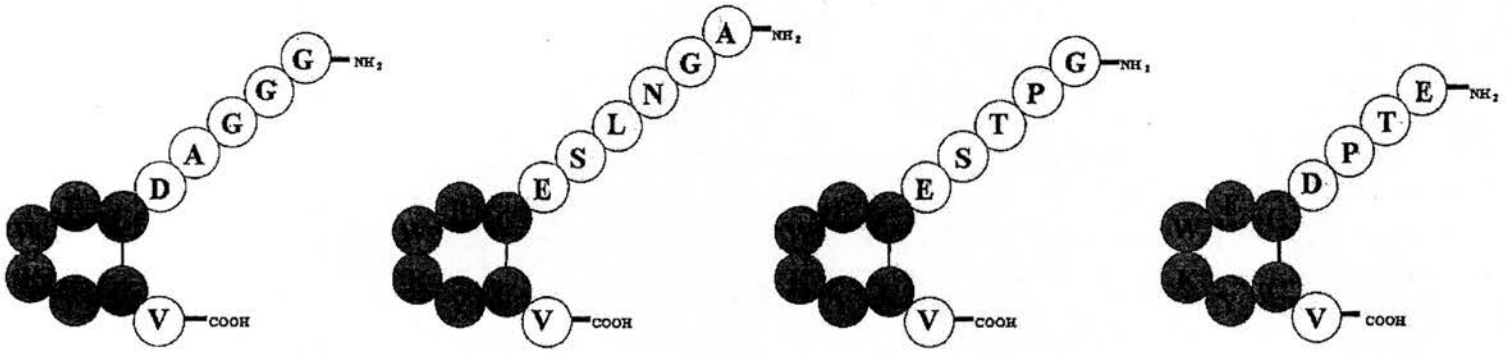
1.1 UROTENSIN II

1.1.1 Origins of Urotensin II

The "urotensins" are a group of peptides that were discovered within the tissue extracts of the urophysis, a caudal neurosecretory organ of the teleost fish. They were loosely classified into urotensin I to V, urotensin I and II being transcribed from different genes. Urotensin I is a longer peptide with 41 amino acids and is similar to corticotrophin releasing factor in man. Urotensin II was first characterised in 1967 from teleost fish [Bern *et al* 1967] and sequenced in 1980 [Pearson *et al* 1980]. It is a dodecapeptide, the cyclical moiety being the biologically active site. The concept that urotensin II was restricted to marine species was challenged only later when it was isolated from frogs [Conlon *et al* 1992], mice, rats and humans [Coulouarn *et al* 1998, Coulouarn *et al* 1999]. Although the number of amino acids that formed urotensin II differs amongst species, it is of potentially great evolutionary significance given that the biologically active cyclical portion of the peptide is entirely unchanged (Figure 1.1.1). This is despite the obvious ancestral divergence of man and the river lamprey (*Lampetra Fluviatilis*), the oldest know species to have urotensin II, occurring 550 million years ago [Waugh *et al* 1995]. In the context of the future finding that urotensin II caused profound vasomotor changes in mammalian studies, the potential reasons for careful evolutionary preservation of this peptide is of great interest.

Figure 1.1.1

The structure of urotensin II and variation amongst species [Coulouarn *et al* 1998].



Carp Urotensin II **Frog Urotensin II** **Porcine Urotensin II** **Human Urotensin II**

1.1.2 Characteristics of human urotensin II

Human urotensin II (hUII) is an 11 amino acid peptide (Figure 1.1.1). It is cleaved from a larger 130 amino acid prepro urotensin II precursor that itself is transcribed from a 688 base pair strand of DNA [Ames *et al* 1999]. Human prepro urotensin II mRNA was first located in the spinal cord, particularly in the motor neurones. Lesser concentrations have been found in other human tissues including kidney, spleen, small intestine, thymus, prostate, pituitary and adrenal glands, stomach, pancreas, liver and ovary [Coulouarn *et al* 1998]. Of all the organs, including the central nervous system, the kidney has the highest mRNA expression of prepro urotensin II [Nothacker *et al* 1999].

1.1.3 Discovery and characteristics of the urotensin II receptor

The receptor and ligand, were brought together by means of "reverse pharmacology". Using sequence homology, genomic DNA library screening and the polymerase chain reaction a rat "orphan receptor", called GPR-14, was identified [Marchese *et al* 1995] ("orphan receptor" refers to a receptor with an as yet undiscovered physiological and/or pharmacological ligand). It was shown to have features consistent with those of G-protein coupled receptors with 7 hydrophobic transmembrane domains and a third intracellular loop, indicative of a peptide binding receptor. Reverse pharmacology involves the expression of the orphan receptor on cultures cells allowing the trial of various potential ligands, a potentially protracted process [Stadel *et al* 1997]. Clues from the GPR-14 receptor such as its similarity to somatostatin receptors did suggest the ligand to be peptidic. By assessing the second messenger responses, including changes in intracellular Ca^{2+}, stimulatory ligands could be identified and in 1999 Lui *et al*

showed that frog urotensin II was an agonist ligand [Lui *et al* 1999]. Using similar techniques to Marchese *et al*, the human genome was probed for the GPR-14 equivalent gene and was located on chromosome 17 and the receptor had high affinity binding for both fish and human urotensin II [Ames *et al* 1999; Nothacker *et al* 1999]. Using immunohistochemistry on human tissues, the receptor distribution has been mapped to show extensive binding to cardiovascular tissues including coronary arteries, internal mammary arteries and ventricular cardiac myocytes [Ames *et al* 1999; Maguire *et al* 2000]. It seems likely that the kidney, with its high prepro urotensin II expression, is the source of circulating urotensin II and that urotensin II acts as an endocrine hormone.

1.1.4 *In vitro* animal evidence for urotensin II as a vasoactive peptide

The mammalian vasomotor effects of urotensin II first came to light when rat arterial strips from the most proximal and distal circulatory sites where exposed, *in vitro*, to fish urotensin II with subsequent vascular smooth muscle contraction. Moreover, the thoracic aorta was the most sensitive with progressive loss of effect through abdominal aorta, mesenteric and femoral arteries [Itoh *et al* 1987; Gibson 1987]. The pattern of effects corresponded to the distribution of receptors as shown by radio-labelled fish urotensin II [Itoh *et al* 1988]. To investigate the specificity of the urotensin II response investigators used agents that block α and β adrenergic receptors, cholinergic, histaminergic and serotonergic receptors and all failed to change the urotensin response [Gibson 1987; Itoh *et al* 1987]. The second messenger system was also scrutinised and it appears that the activity of phospolipase C [Gibson 1987], inositol phosphates

[Opgaard *et al* 2000] and Ca^{2+} [Gibson *et al* 1988] are essential for the vasoactive response. Human urotensin II has been used in similar *in vitro* studies on non-human primate vessels where it exceeded the potency of endothelin-1 and caused vasoconstriction in all arteries tested, although these were all conduit arteries [Ames *et al* 1999]. In addition, there was clearly a preferential effect on arterial over venous tissue, a selective feature not seen with endothelin-1 responses [Ames *et al* 1999].

However, despite the above evidence, some animal studies have demonstrated variation in response between species [Douglas *et al* 2000] as well as the anatomical variation in response and moreover, vasodilator [Katano *et al* 2000; Stirrat *et al* 2001] responses and potential interaction with nitric oxide have been uncovered [Bottril *et al* 2000; MacLean *et al* 2000]. Furthermore, the activity of human urotensin II extends beyond the vascular tree causing contraction of primate airway [Hay *et al* 2000], although receptor mapping has not yet been performed for these tissues.

1.1.5 *In vivo* animal responses to urotensin II

Animal *in vitro* studies have given mixed results and must be considered in light of the *in vivo* investigations. At low intravenous doses of human urotensin II in *Cynomologous* monkey cardiac output increased slightly without a change in mean arterial pressure. In contrast higher dose ranges caused a dose dependent increase in vascular resistance, bradycardia and decrease in cardiac output with haemodynamic and echocardiographic evidence of severe depression of myocardial contractility (Figure 1.1.2) [Ames *et al* 1999]. Despite these significant changes mean arterial pressure did not change. In some

cases the dose was sufficient to cause total cardiovascular collapse and death. Electrocardiographic (ECG) recordings during the studies did show marked ST segment depression (Figure 1.1.3) coinciding with reduced stroke volume and contractility as determined by echocardiography. Equivalent doses of endothelin-1 had no such effects and showed more simply a slow and sustained rise in mean arterial pressure [Ames *et al* 1999].

Figure 1.1.2

Cardiovascular effects of intravenous human urotensin II in non-human primates [Ames *et al* 1999].

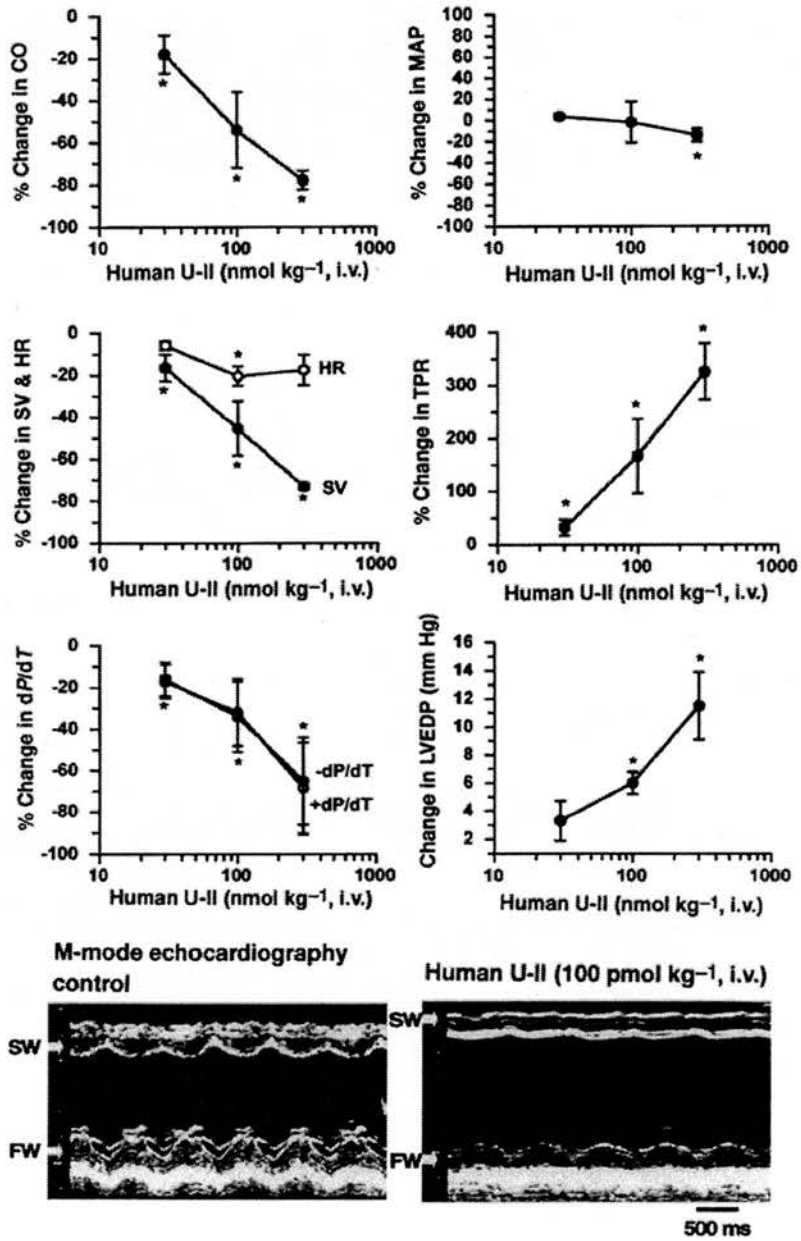
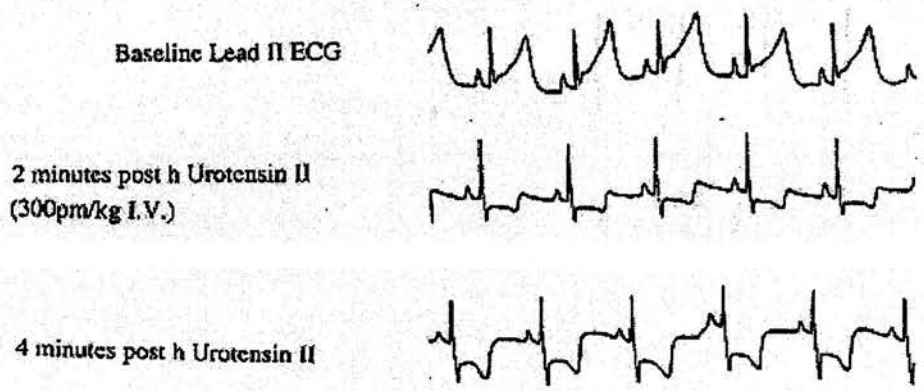


Figure 1.1.3

ECG changes in anaesthetised *cynomologous* monkey during human urotensin II [Personal communication from S.A. Douglas].



1.1.6 Potential role of urotensin in human cardiovascular regulation and pathophysiology

The systemic haemodynamic findings in the non-human primates were intriguing, as is the degree to which this peptide has been preserved through so many species over millions of years of evolution. Exactly what role urotensin II plays in the either homeostasis or in pathophysiology of man and animals is not yet clear. Some investigators have now shown that urotensin II plasma concentrations are increased in end stage cirrhosis [Heller J *et al* 2002] and heart failure [Richards *et al* 2002]. Furthermore, in patients with heart failure, the myocardial expression of urotensin II and its human receptor are both increased. This has led to speculation that urotensin II may be involved in the pathophysiological circulatory states found in both these diseases. Whether urotensin II influences vascular tone alone or also plasma volume is not yet clear but given that the kidney is the most likely source of circulating urotensin in man [Nothacker *et al* 1999], urotensin II may have a possible role in water and electrolyte homeostasis.

At the level of the endothelium, urotensin II receptors have been located in human coronary atheromatous plaque using immunostaining; particularly in lipid laden smooth muscle/macrophage rich areas of plaques [Ames *et al* 1999]. Watanabe has also demonstrated that urotensin II augments the proliferative effects of partly oxidised LDL-cholesterol on vascular smooth muscle cells [Watanabe *et al* 2001].

Few data have emerged on the basic physiological responses of urotensin II in man *in vivo* and how they might compare to a well established endocrine hormone. Nor unfortunately is there an antagonist to help clarify the true role of human urotensin II *in vitro* and *in vivo*.

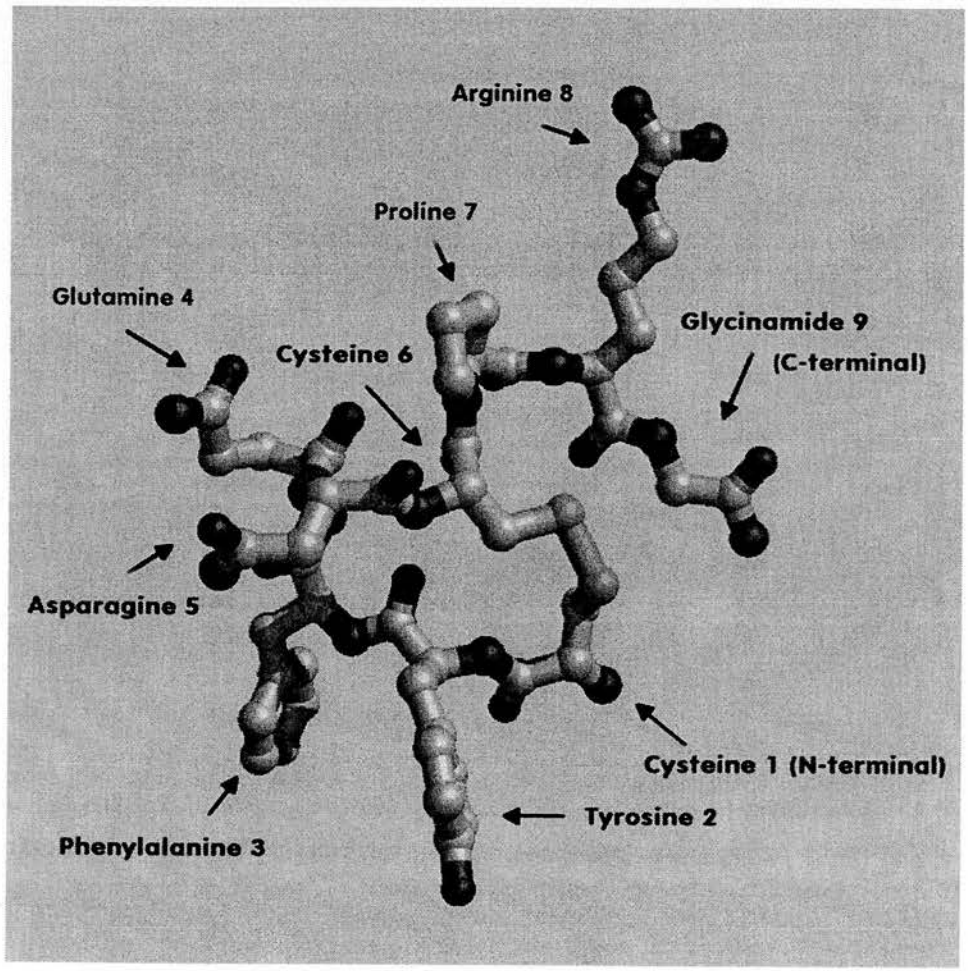
1.2 VASOPRESSIN

1.2.1 Origins of vasopressin

Arginine vasopressin (AVP), also known as anti-diuretic hormone, was first studied indirectly by Oliver and Shafer in 1895 when they discovered that pituitary gland extracts had potent pressor actions [Oliver and Shafer 1895]. Both vasopressin and oxytocin are secreted from the posterior pituitary gland where axons from the supraoptic and paraventricular nuclei of the hypothalamus terminate. Vasopressin is formed predominantly in the supraoptic nucleus and oxytocin in the paraventricular nucleus. Despite differing in only two amino acids (positions 3 and 8) vasopressin and oxytocin serve very different endocrine functions (see Figure 1.2.1 for structure of vasopressin). The main endocrine functions of vasopressin are to control body water and osmolarity and the contraction of vascular smooth muscle, whereas oxytocin is responsible for uterine contraction and milk letdown reflexes. While the cardiovascular and renal effects of vasopressin are well described, the central nervous system actions of vasopressin still raise many questions [Barberis and Tribollet 1996] where it acts as a neurotransmitter/neuromodulator of major body functions including blood pressure, body temperature, memory, anterior pituitary hormone secretion and brain development.

Figure 1.2.1

The structure of vasopressin (note the disulphide bond between cysteine residues creating a cyclical ring structure).



1.2.2 Characteristics of vasopressin

Vasopressin is a cyclical nonapeptide and like many hormonal peptides is cleaved from a larger molecule, prepropressophysin, which is produced in the cell bodies of the supraoptic neurones and then transported by carrier proteins, called neurophysins, to the posterior pituitary. During transit the translated prepropressophysin molecule is split and stored in histologically evident "Herring" granules of free vasopressin, neurophysin II and glycopeptide ready for release into adjacent capillaries. Beyond the stage of release, it is unknown if neurophysin II has a further role. Normal circulating concentrations of vasopressin is in the approximate range of 0.9 to 6.5 pmol/L increasing to as high as 187 pmol/L during extreme stimuli [Landry and Oliver 2001]. Stimuli that provoke the release of vasopressin are wide and numerous as listed in the table below.

Table 1.2.1

Factors influencing release of vasopressin

Stimuli provoking vasopressin	**Stimuli inhibiting vasopressin**
↑ Extracellular osmolarity	↓ Extracellular osmolarity
↓ Extracellular volume	↑ Extracellular volume
↓ Blood pressure	↑ Blood pressure
Angiotensin II	↓ Temperature
Pain/emotion	α- adrenergic agonists
Exercise	γ-aminobutyric acid (GABA)
Nausea and vomiting	Ethanol
Hypoglycaemia	Cortisol
Standing	Atrial naturetic peptide (ANP)
↑ Temperature	
Drugs: nicotine, opiates, barbiturates, sulphonylureas, vincristine	

1.2.3 Vasopressin receptors and their functions

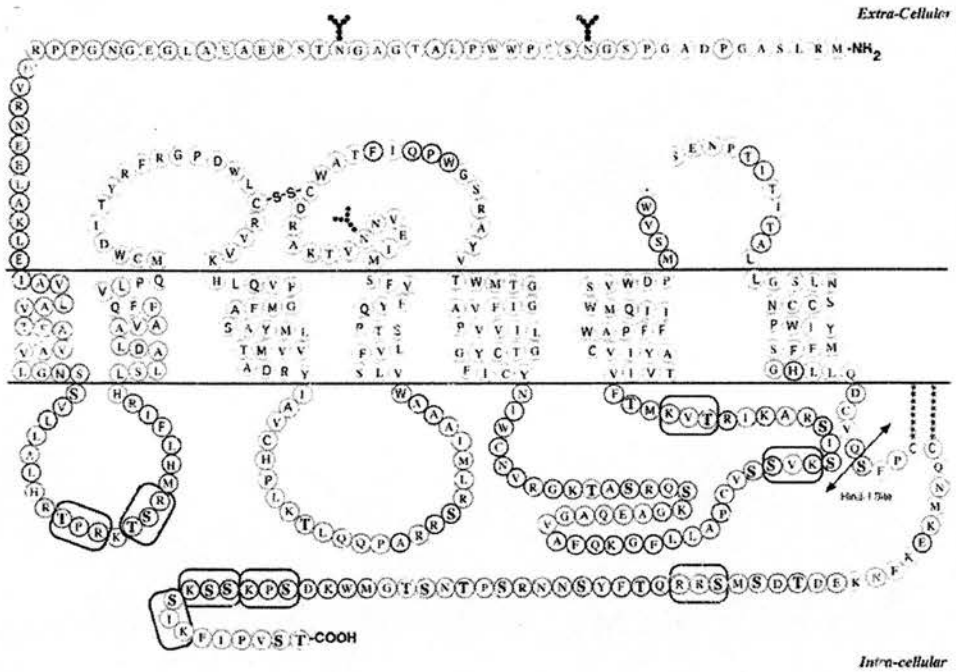
Receptors for vasopressin are distributed widely within the central nervous system and the rest of the body. There are 3 subtypes, the V1, V2 and V3 receptor forms and all are G protein coupled receptors (see Figure 1.2.2 for structure of V1 and V2 receptors). The majority of the central nervous system receptors are V1 but receptor subtypes exist in different quantities throughout the rest of the body. The principal effect of vasopressin on the periphery is to contract arterial vascular smooth muscle cells, via V1 receptors and to enhance water reabsorption in the medulla of the kidney via V2 receptors. The V3 receptor primarily mediates the release of adrenocorticotrophin from the anterior pituitary [Lui 1994], thus influencing steroidogenesis from the adrenal cortex. V1 receptors also exist on hepatocytes (causing glycogenolysis and gluconeogenesis), platelets, renal medullary collecting duct cells, spleen and the zona glomerulosa and zona fasiculata of the adrenal gland [Thiobonnier *et al* 2001]. On binding the V1 receptor, vasopressin activates phospholipase C allowing formation of inositol 1,4,5 triphosphate and diacylglycerol thus activating numerous protein kinases (Figure 1.2.3) [Thiobonnier 1992; Birley *et al* 1994; Granot *et al* 1993; Thiobonnier *et al* 2000]. In addition to vascular contraction, AVP mediates mitogenic stimuli, again via the V1 receptor, increasing cell protein synthesis and content with resultant hypertrophy and proliferation [Thiobonnier *et al* 2000]. The V2 receptor is principally located on the basolateral membrane of the collecting duct cells in the renal medulla, although it probably exists in the vasculature as well. In the kidney it enhances water reabsorption via increased transcription of aquaporin 2 protein, increases urea permeability into the collecting ducts, and stimulates sodium reabsorption at the cortical and outer medullary

collecting ducts [Bankir 2001]. In contrast to the V1 receptor, vasopressin binding to V2 receptors causes stimulation of adenylate cyclase increasing intracellular cAMP (Figure 1.2.4). Both receptors are internalised within the cell after vasopressin binding leading to receptor desensitisation [Lutz *et al* 1993; Fishman *et al* 1985]. Up to 90% of receptors are internalised after vasopressin binding, the half life of such a process being 5 and 13 minutes for the V1 and V2 receptors respectively [Thiobonnier *et al* 1992]. Receptors are recycled after vasopressin removal and return to the cell membrane in 20 minutes.

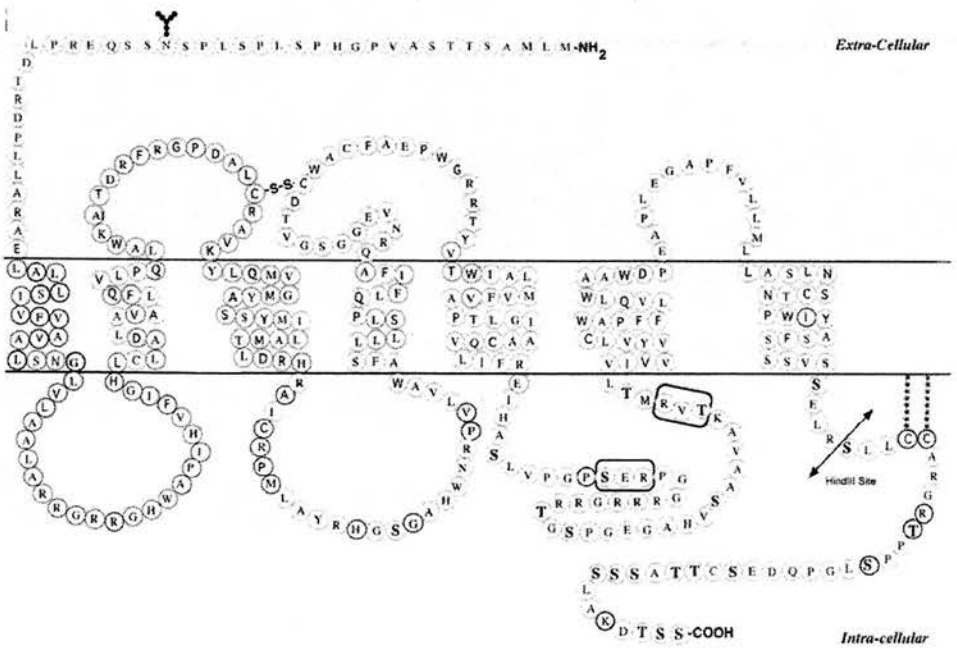
In its endocrine capacity, vasopressin is released under osmotic control to regulate permeability of the renal collecting ducts to water. Vasopressin is also under baroreflex control causing vasoconstriction in response to hypotension. The former mechanism is by far the more sensitive mode of control, with collecting duct permeability regulation occurring between 1 and 6 pmol/L (i.e. within the normal circulating vasopressin range), its vasoconstrictor effects are however, mediated at higher concentrations, up to 187 pmol/L. As part of the early response to hypotension, vasopressin plasma concentration is increased by up to 50-fold. The rapid rise in vasopressin helps maintain arterial pressure but, as has been shown in animal hypotension models, is not sustained and falls over several hours to a modestly elevated level [Morales *et al* 1999]. This most likely reflects vasopressin depletion from the neurohypophysis [Negro-Vilar *et al* 1979].

Figure 1.2.2

The structure of both vasopressin receptors [Thibonnier *et al* 2001].



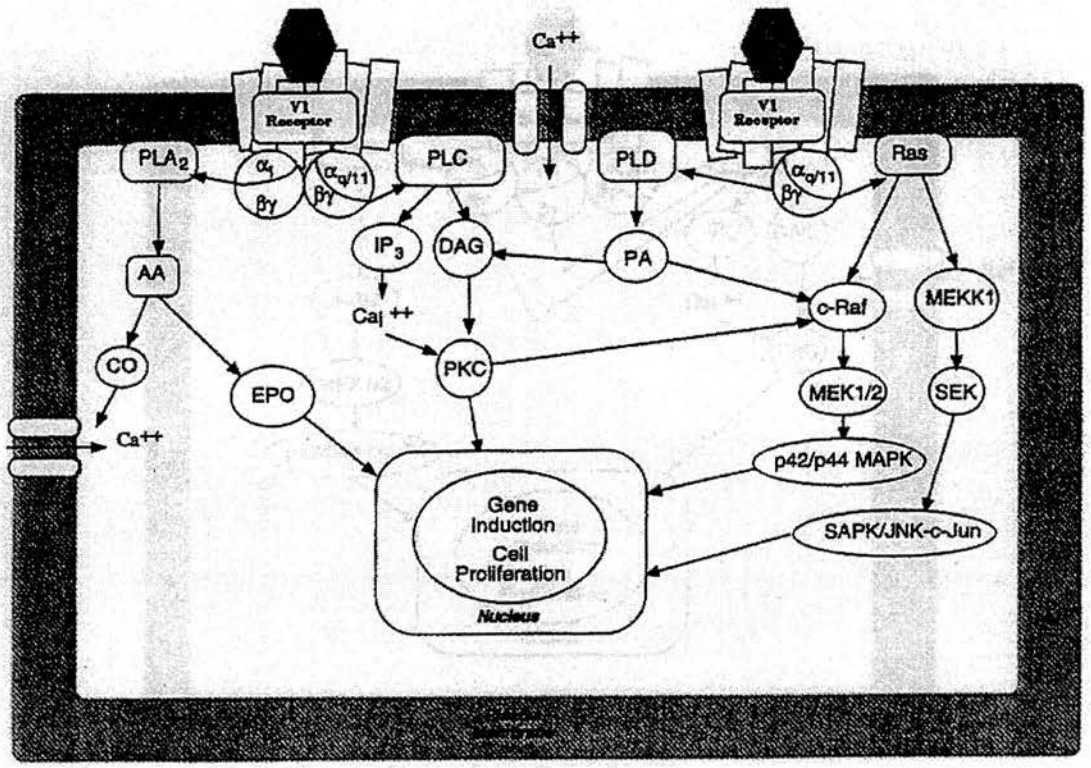
Human V_1 Vascular AVP Receptor



Human V_2 Renal AVP Receptor

Figure 1.2.3

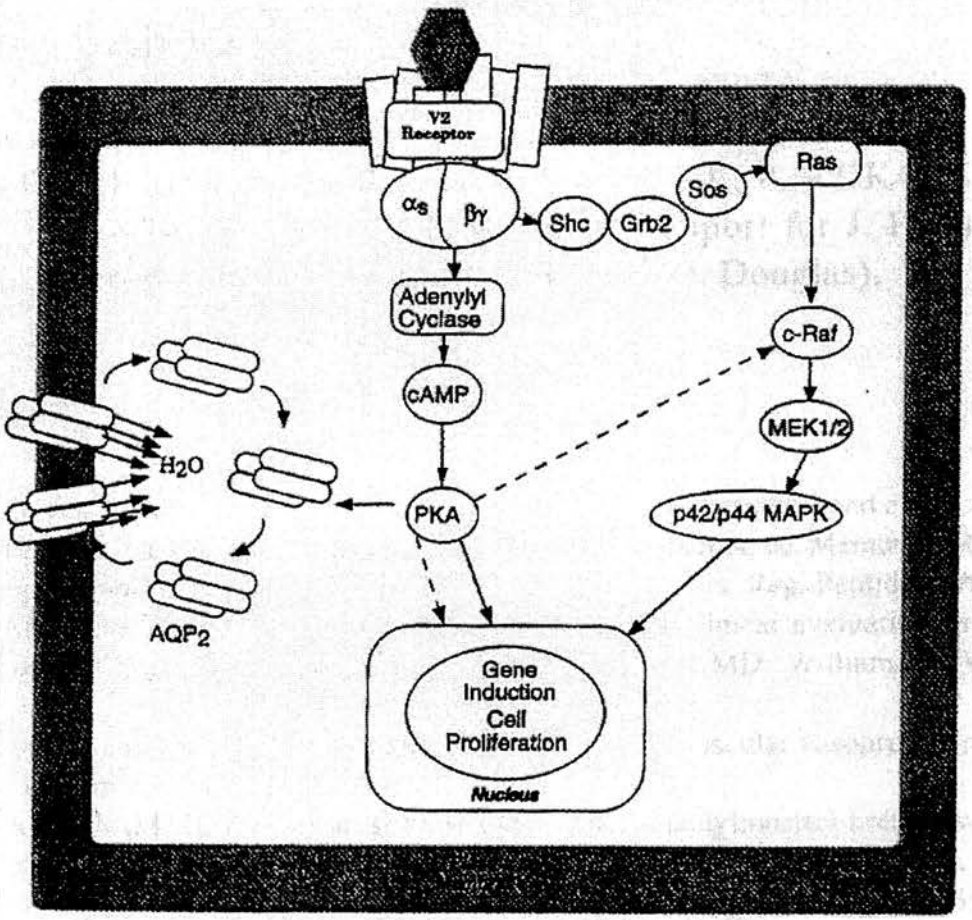
Detailed intracellular pathways coupled to the activation of the human V1 receptor [Thiobonnier *et al* 1998].



Abbreviations: α, alpha subunits; βγ, beta-gamma dimers of G-proteins; PLC, phospholipase C; PLD, phospholipase D; PLA_2, phospholipase A_2; IP_3, inositol-1,4,5 triphosphate; DAG, diacylglycerol; PKC, protein kinase C; AA, arachidonic acid; CO, cyclo-oxygenase; EPO, epoxygenase; PA, phosphatidic acid; ERK, extracellular signal-regulated kinase; MEK, mitogen activated ERK kinase; MAPK, mitogen activated protein kinase; SEK, SAPK/ERK kinase; cAMP, cyclic AMP; PKA, protein kinase A; APQ_2, aquaporin 2 protein.

Figure 1.2.4

Detailed intracellular pathways coupled to the activation of the human V2 receptor [Thiobnnier *et al* 1998].



Abbreviations: as for **Figure 1.2.3**

1.2.4 Localised human vascular studies

The direct vascular actions of parenteral vasopressin administration have been explored in clinical studies for many years [Kitchin 1957]. However, despite this, the direct vascular actions of vasopressin are controversial and the evidence conflicting. Some workers have found that intra-arterial vasopressin causes vasoconstriction only [Weber *et al* 1997] whilst others report isolated vasodilatation [Hirsch *et al* 1989]. This disparity is likely to reflect a biphasic vascular response with vasopressin infusion causing an initial vasoconstriction at low doses (1-10 pmol/min) followed by vasodilatation at higher doses (>10 pmol/min) [Kitchin 1957; Hirsch *et al* 1989; Tagawa *et al* 1993; Suzuki *et al* 1989]. This is consistent with a predominantly V1 receptor mediated effect with low concentrations and possibly "vascular V2 like" receptor effects at higher concentrations, and is borne out by the potentiation of vasopressin induced vasodilatation when administered in the presence of V1 receptor antagonism [Imaizumi *et al* 1992; Hirsch *et al* 1989]. Although the physical presence of extra-renal V2 receptors in arteries has yet to be demonstrated, there is strong pharmacological evidence for their existence. Intra-arterial infusion of desmopressin, a synthetic V2 receptor agonist, causes vasodilatation in the human forearm which can be abolished by selective V2 antagonism [Tagawa *et al* 1995]. In addition, patients with congenital diabetes insipidus due to a genetic defect in the V2 receptor do not respond to intra-arterial desmopressin and demonstrated only vasoconstriction in the face of increasing doses of vasopressin [Van Lieburg *et al* 1995].

1.2.5 Vasopressin induced arterial vasodilatation

The mechanism of vasopressin and desmopressin mediated vasodilatation has been poorly characterised. Previous studies have suggested that it is independent of prostaglandin production but is, in part, mediated by nitric oxide [Tagawa *et al* 1993; Okamura *et al* 1999]. However, the demonstration of the contribution of nitric oxide was achieved by the co-infusion of L-N^G-monomethyl arginine which causes basal vasoconstriction and makes interpretation of the subsequent blood flow responses unclear. Moreover, other groups have reported desmopressin induced vasodilatation is reduced by indomethacin but not by nitric oxide synthase inhibition [Aldasoro *et al* 1997; Medina *et al* 1999].

1.2.6 Endothelial, haemostatic and fibrinolytic effects of vasopressin receptor agonists

Vasopressin causes platelet aggregation through V1 receptor stimulation [Thibonnier *et al* 1999], and may release haemostatic (von Willebrand factor, vWf, and Factor VIII:C), fibrinolytic (tissue plasminogen activator, t-PA) and cytokine (interleukin-6, IL-6, and tumor necrosis factor, TNF) factors through V2 receptor stimulation using intra-arterial desmopressin [Newby *et al* 2000; Kaufmann *et al* 2000].

1.2.7 Differential responses to infused vasopressin in man

Distinct from its effects on total forearm blood flow, vasopressin also causes a dose-dependent reduction in skin blood flow when given intra-dermally [Hirsch *et al*, 1989; Weber *et al*, 1997] suggesting the presence of vascular bed specific differential V1 and

vascular V2 like receptor expression. Indeed intra-brachial vasopressin at high dose increases the composite measure of total forearm blood flow whilst both skin and radial artery blood flow decrease [Hayoz *et al.* 1997]. Despite the name, and given that *in vitro* [Ohlstein *et al.* 1986] and *in vivo* it causes vasoconstriction, when given systemically to healthy volunteers vasopressin causes only modest changes in heart rate but little change in mean arterial blood pressure [Aylward *et al.* 1986].

1.2.8 Potential role of vasopressin in disease states

Heart failure is characterised by increased vascular tone, water retention and subsequent oedema and dilutional hyponatraemia [Burrell *et al* 2000]. It has been suggested that a pathologically activated vasopressin axis may lead to many of the features we associate with heart failure although the exact role of vasopressin in heart failure remains controversial. It appears likely that during heart failure, hypo-perfusion leads to activation of the sympathetic nervous system, which in turn increases vasopressin release. This is supported by increased plasma vasopressin concentrations in patients with heart failure [Goldsmith *et al* 1983; Preibisz *et al* 1983; Schrier *et al* 1998]. This is particularly so in the case of patients with heart failure and hyponatraemia [Szatalowicz *et al* 1981], a feature of heart failure that confers a poor prognosis. Moreover, for unknown reasons, plasma vasopressin concentrations remain elevated despite adequate water loading in patients with heart failure [Goldsmith *et al.* 1986], it may be that non-osmotic stimuli are able to over-ride the osmotic control mechanisms. Although the rises in plasma vasopressin are modest in patients with heart failure, the levels in

subjects with haemodynamic instability and/or hyponatraemia are often 10 to 20-fold higher [Nicod *et al,* 1984].

Stimulation of the V2 receptor with desmopressin has been implicated in the endothelial release of haemostatic and fibrinolytic factors such as vWf factor VIII:C, t-PA and endothelial derived cytokines [Cash *et al* 1974; Mannucci *et al* 1975; Newby *et al* 2000]. The balance of such cytokines and haemostatic factors is altered by vascular injury [Sporn *et al* 1986] and inflammatory states, including atherosclerosis and a higher risk of coronary events is conferred by their imbalance [Nusinow *et al* 1986; Blann *et al* 1994; Haverkate *et al* 1995; Thompson *et al* 1995].

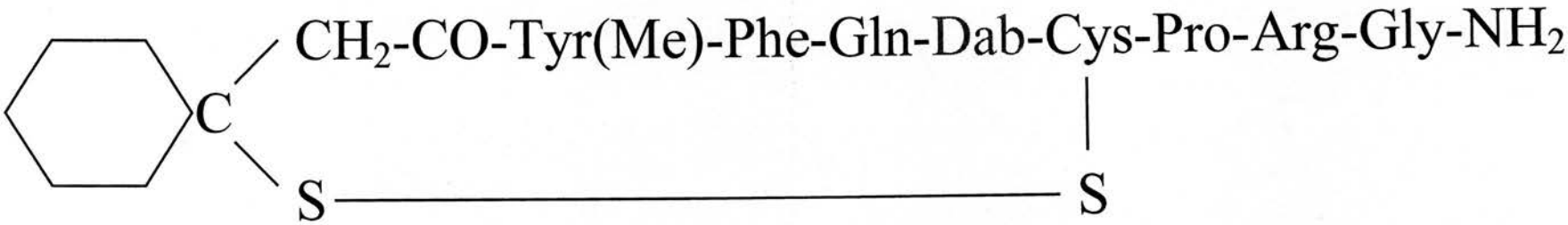
1.2.9 Peptidic vasopressin antagonists

Recently, many selective and non-selective oral non-peptidic vasopressin antagonists have been developed [Imaizumi *et al* 1992; Weber *et al* 1997]. However, for over 15 years, peptidic antagonists have been available for parenteral use and preliminary clinical studies in heart failure were conducted using the V1 receptor antagonist, $d(CH_2)_5Tyr(Me)AVP$ [Nicod *et al* 1985]. This antagonist does, however, have partial V2 agonist effects and cannot be viewed as a purely selective antagonist. In contrast, $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ and $d(CH_2)_5[D\text{-}Ile^2,Ile^4,Tyr\text{-}(NH_2)^9]AVP$ are more selective V1 and V2 receptor antagonists (Figure 1.2.5) [Chan *et al* 1996; Cotte *et al* 1998]. The effect of intra-arterial administration of these more selective peptidic antagonists has not been previously described in man.

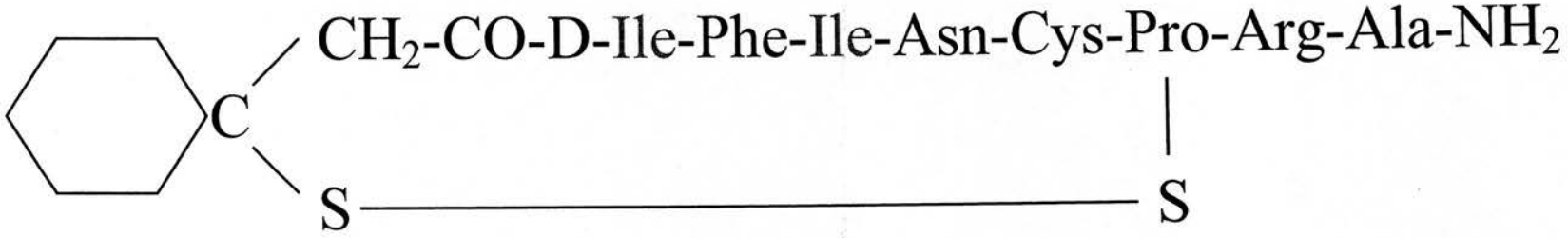
Figure 1.2.5

Structures of selective peptidic vasopressin antagonists

V1 antagonist [Chan *et al* 2000]



V2 antagonist [Cotte *et al* 1998]



1.3 HYPOTHESIS

The relative preservation of the amino acid sequence in urotensin II across many species suggests that it plays a highly significant role in animal physiology. The *in vivo* studies using human urotensin II in non-human primates demonstrated substantial haemodynamic changes [Ames *et al* 1999]. These changes were specific for the arterial side of the circulation. As yet the physiological role of urotensin II, especially the vasomotor response to urotensin II, in man is unknown. In contrast, vasopressin has been extensively studied in man and animals providing a positive control to which a novel peptide could be compared. However, some aspects of the underlying pharmacology of vasopressin responses are not fully understood and the use of selective vasopressin receptor antagonists may help clarify this. This will be of particular interest as vasopressin receptor antagonists are now in stage 3 clinical trials for patients with heart failure and end stage cirrhosis.

Validated methods such as venous occlusion plethysmography to measure forearm blood flow, pulse wave analysis to assess arterial stiffness, the Aellig technique for venous tone and laser Doppler assessment of skin blood flow provide excellent human *in vivo* methods to examine novel vasoactive peptides. The following hypothesis will be explored regarding urotensin II and vasopressin.

1. Urotensin II will cause arterial vasoconstriction in the human forearm and, when given in systemic doses, change haemodynamic parameters. Anticipated effects include an increase in blood pressure, peripheral vascular resistance and arterial stiffness.

2. Vasopressin will cause arterial vasoconstriction in the human skin microcirculation and forearm at low intra-arterial doses and cause nitric oxide mediated vasodilatation in the forearm at high intra-arterial doses.

3. Selective vasopressin V1 receptor antagonism will abolish vasoconstriction in the skin and forearm and enhance vasodilatation in the forearm. Selective V2 receptor antagonism will attenuate forearm vasodilatation.

1.4 AIMS

The aims of the thesis were:

(Chapter 3). To determine the intra- and inter-operator repeatability of saline injection volume and to assess repeatability of skin blood flow responses to intra-dermal injection of saline.

(Chapter 4). In healthy volunteers, to undertake the first human *in vivo* study addressing local responses to urotensin II in arterial vessels and dorsal hand veins.

(Chapter 5). In healthy volunteers, to determine the effects of systemic intravenous urotensin II on a range of systemic haemodynamic parameters including blood pressure and central arterial stiffness.

(Chapter 6). In healthy volunteers, to describe the forearm vascular actions of vasopressin to facilitate studies with vasopressin antagonists. Pharmacodynamics and reproducibility of responses to intra-brachial vasopressin and the contribution of nitric oxide to its vasomotor actions will be assessed.

(Chapter 7). In healthy volunteers, to use intra-dermal injection of peptides and laser Doppler flowmetry to assess a range of vasopressin doses in comparison to endothelin-1 and to co-administer vasopressin with a V1 antagonist.

(Chapter 8). In healthy volunteers, to study the forearm blood flow responses of intra-arterial V1 and V2 receptor antagonists before co-infusing vasopressin with the individual antagonists and saline placebo.

CHAPTER 2

METHODS

2.1 LOCALISED VERSUS SYSTEMIC HUMAN *IN VIVO* VASCULAR STUDIES

During systemic administration of vasoactive drugs in man, many organs can be affected directly and indirectly through the action of the drug as well as neurohormonal reflexes and changes in haemodynamics. Thus, multiple systemic effects can make the change in measurement a composite of local and systemic changes and obscuring the "true" direct pharmacological actions of the drug. However, local vascular bed study techniques permit more pure investigation of drug actions on the vasculature. Study methods such as intra-dermal drug injection combined with measurement of skin micro circulation, localised drug intravenous infusion combined with dorsal hand vein diameter measurement and intra-brachial drug infusion combined with bilateral venous occlusion plethysmography provide a method of assessing the *in vivo* effects of a drug on human vessels and endothelium [Benjamin *et al* 1995; Webb 1995]. By using this technique, the role of nitric oxide and endothelin-1 in the maintenance of basal vascular tone were first successfully demonstrated [Vallance *et al* 1989; Haynes *et al* 1994].

2.2 GENERAL

2.2.1 Ethical considerations

All subjects were recruited from a bank of healthy community volunteers held by the Clinical Research Centre at the Western General Hospital in Edinburgh. The studies were conducted with the approval of the local research ethics committee and the written

informed consent of each subject. Investigations conform to the principles of the Declaration of Helsinki 1989.

2.2.2 General requests made to volunteers prior to a study

Subjects were asked to fast from midnight before each study and to abstain from caffeine containing drinks, alcohol and smoking over the preceding 24 hours. Subjects also had to be on no prescribed medication, free from significant clinical illness and not be participating in other research.

2.2.3 Haemodynamic measurements

Blood pressure was recorded over the brachial artery in the non-infused arm at various intervals using a validated semi-automated ocillometric sphygmomanometer (HEM 705CP, Omron, Japan) [O'Brien *et al* 1996]. Non-invasive estimates of cardiac index were assessed using a validated transthoracic electrical bioimpedance technique [Northridge *et al* 1990] (NCCOM3, BoMed Irvine CA, USA). Mean arterial pressure was defined as the diastolic pressure plus a third of the pulse pressure. Systemic vascular resistance was defined as the mean arterial pressure divided by the cardiac output and then converted from Wood units to dynes.s.m^2/cm^5 on the basis that 1 Wood unit equals 80 dynes.s.m^2/cm^5 made.

2.3 FOREARM RESISTANCE VESSELS

2.3.1 Cannulation of the brachial artery

The brachial artery of the non-dominant arm was cannulated with a 27 gauge steel needle (Coopers Needle Works Ltd, Birmingham, UK). Discomfort was minimised using 1% lignocaine (Astra Pharmaceuticals Ltd., Hertfordshire, UK) as local anaesthesia. The cannula was attached to a 16 gauge epidural catheter (Portex Ltd, Hythe, UK) using sealing wax. Patency was maintained by infusion of saline (0.9%: Baxter Healthcare Ltd, Thetford, UK) via a IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). During all studies intra-arterial infusion rates were maintained at a constant 1 mL/min.

2.3.2 Blood flow measurement

Blood flow was measured in the infused and non-infused arms by venous occlusion plethysmography using mercury-in-silastic strain gauges applied to the widest part of the forearm (Figures 2.1 and 2.2) [Webb 1995]. During measurement periods, the hands were excluded from the circulation by rapid cuff inflation of the wrist cuffs to a pressure of 200 mmHg using E20 Rapid Cuff Inflators (D.E. Hokanson Inc, Washington, USA). Upper arm cuffs were inflated to 40 mmHg for 10 seconds in every 15 seconds to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge plethysmograph (D.E. Hokanson) was processed by a Mac Lab® analogue to digital converter and Chart™ *v*3.4.3 software (A.D. Instruments Ltd, Castle Hill, Australia) and recorded onto a Macintosh Classic II computer (Apple

Computers Inc, Cupertino, USA) (Figure 2.3). Calibration was achieved using an internal standard of the plethysmograph.

2.3.3 Data analysis

Plethysmographic data were extracted from Chart™ data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 98, Microsoft). Recordings from the first 60 seconds after wrist cuff inflation were not used due to the variability in blood flow that this incurs [Webb 1995]. The last five flow recordings in the 3 minute measurement period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point, thus using the non-infused arm as a contemporaneous control for the infused arm [Benjamin *et al* 1995; Webb 1995]. Percentage change in the infused arm blood flow were calculated as follows:

$$\% \text{ Change in forearm blood flow ratio} = 100 \times \{I_t/NI_t - I_b/NI_b\} / I_b/NI_b$$

Where I_b and NI_b are the infused and non-infused forearm blood flows at baseline respectively, and I_t and NI_t are the infused and non-infused forearm blood flows at a given time respectively.

Figure 2.1

Forearm venous occlusion plethysmography: overall set up.

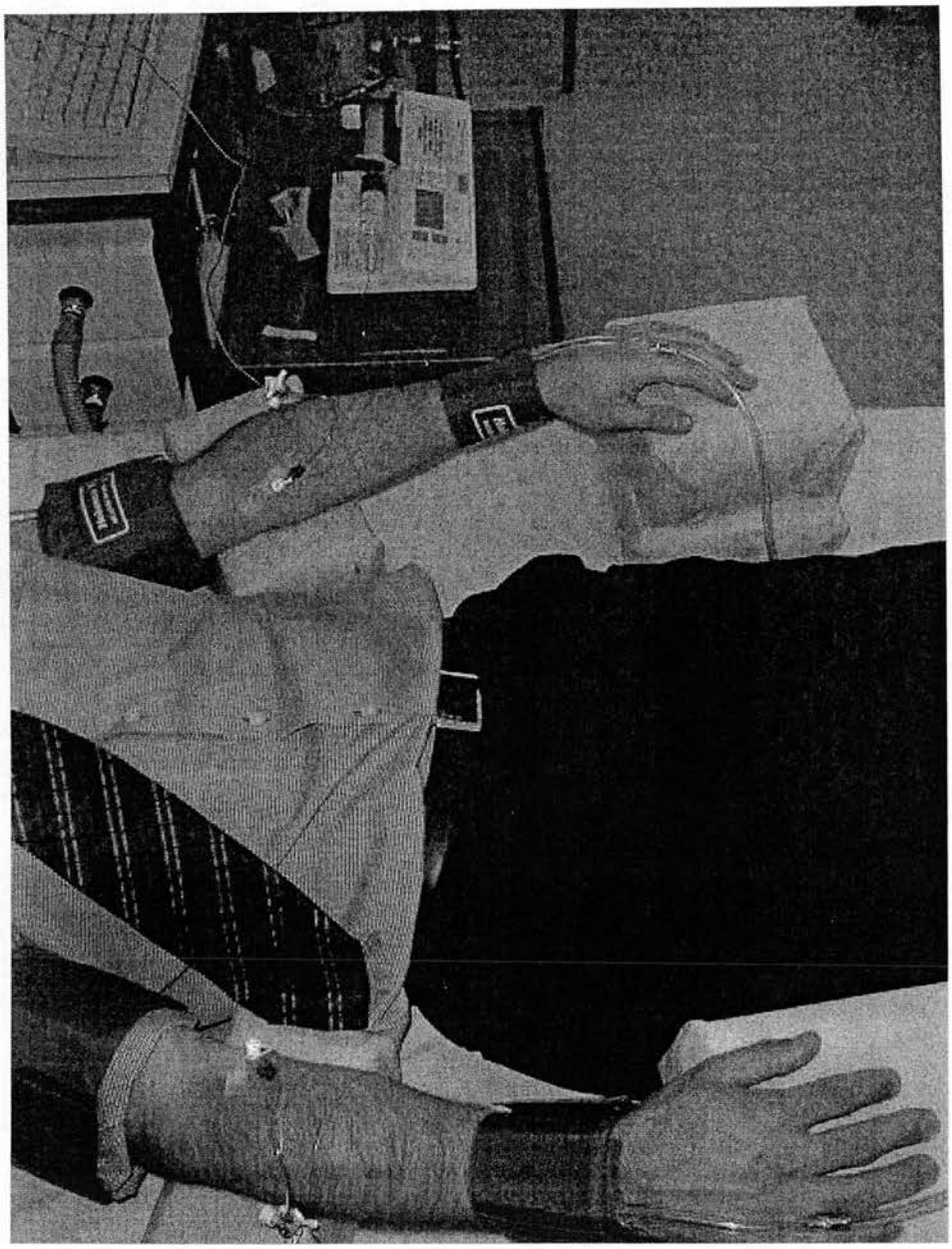


Figure 2.2

Forearm venous occlusion plethysmography: brachial artery cannulation.

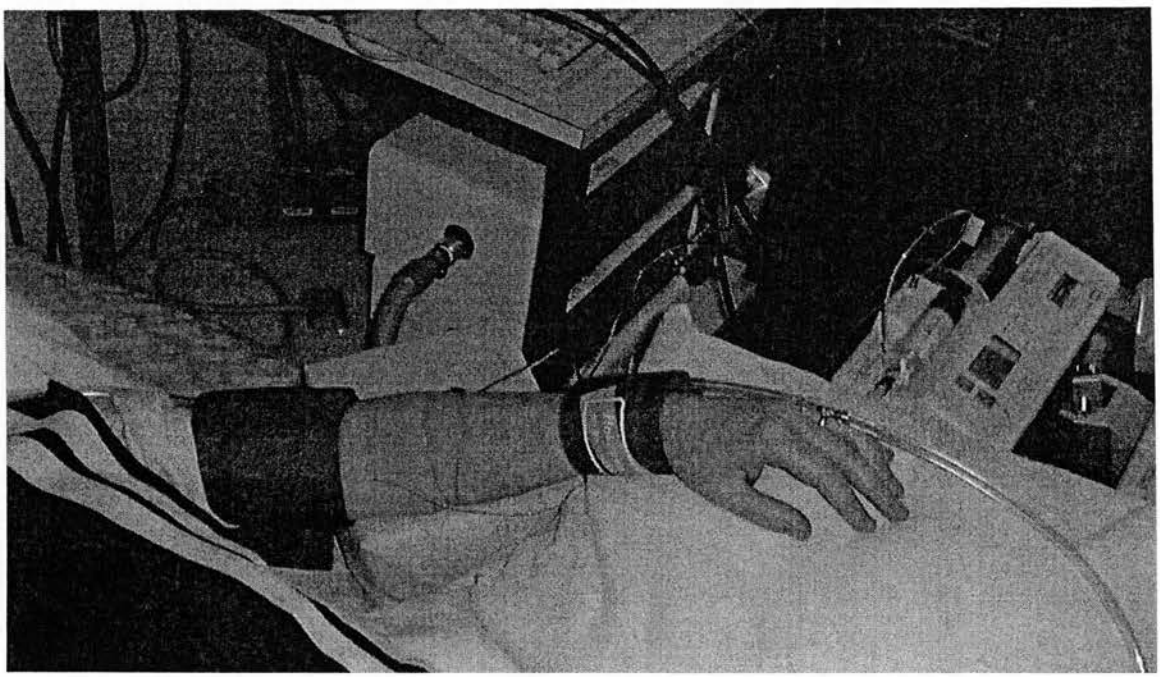
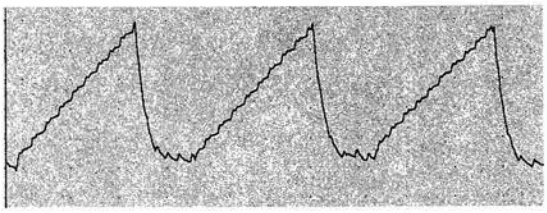


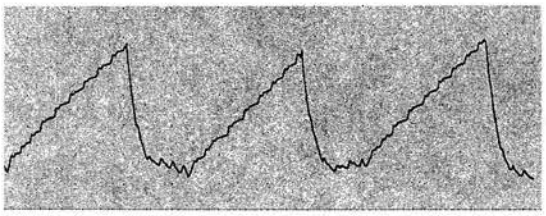
Figure 2.3

Forearm venous occlusion plethysmography: Typical Chart™ recording.

Intra-arterial saline infusion

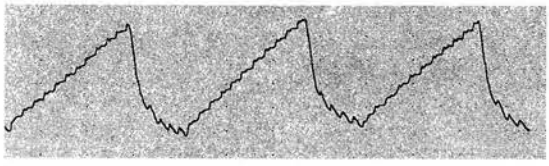


Non-infused arm

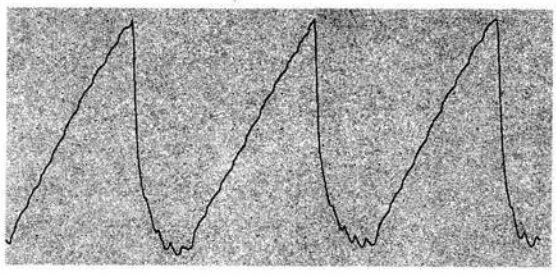


Infused arm

Intra-arterial vasopressin 300pmol/min



Non-infused arm



Infused arm

2.4 SKIN MICROCIRCULATION

2.4.1 Laser doppler flowmetry

An accurate estimate of blood flow in the skin microcirculation can be assessed using this validated method [Kubli *et al* 2000]. Measurement of skin blood flow depends on the principle of the Doppler shift of laser light backscattered from moving red blood cells in the skin microcirculation [Holloway *et al* 1977].

2.4.2 Drug administration

Drugs were delivered by intra-dermal injection using a graduated syringe with a 29.5 gauge needle (Becton-Dickinson, Dublin, Ireland). All drugs were administered in standardised 10 μL volume that causes minimal discomfort to the volunteers.

2.4.3 Measurement of skin blood flow

Skin blood flow was recorded from the volar aspects of both forearms. Sites were selected with care to avoid underlying arteries (as assessed by a pulsatile doppler signal) or veins (as assessed by a high baseline doppler signal). Usually four sites on each forearm could be selected and a probe holder attached to the skin with adhesive tape to ensure no probe movement during the study (Figure 2.4). Biological zero recordings were made at each site during inflation of a blood pressure cuff to 200 mmHg and after 5 minutes rest, baseline recordings were taken for the same sites. Following intra-dermal drug injections recordings were made at each site for 30 seconds. Analogue voltage output from a laser source (Laser Blood Flow Monitor MBF3D, Moor Instruments,

Devon, UK) and P2 Laser Probe (Moor Instruments, Devon, UK) was processed by a Mac Lab® analogue to digital converter and Chart™ v3.4.3 software (A.D. Instruments Ltd, Castle Hill, Australia) (Figure 2.5), and recorded onto a Macintosh Classic II computer (Apple Computers Inc, Cupertino, USA). Calibration was achieved using a flux standard solution of micro-spheres (Moor Instruments Ltd, Devon, UK).

2.4.4 Data analysis

The mean signal values for sequential 30 second time points were extracted from Chart™ files and applied to a spreadsheet (Excel 98; Microsoft) and a curve generated for each site. Area under the curve could then be determined and expressed in arbitrary perfusion units.

Figure 2.4

Skin micro-circulation: whole set up.

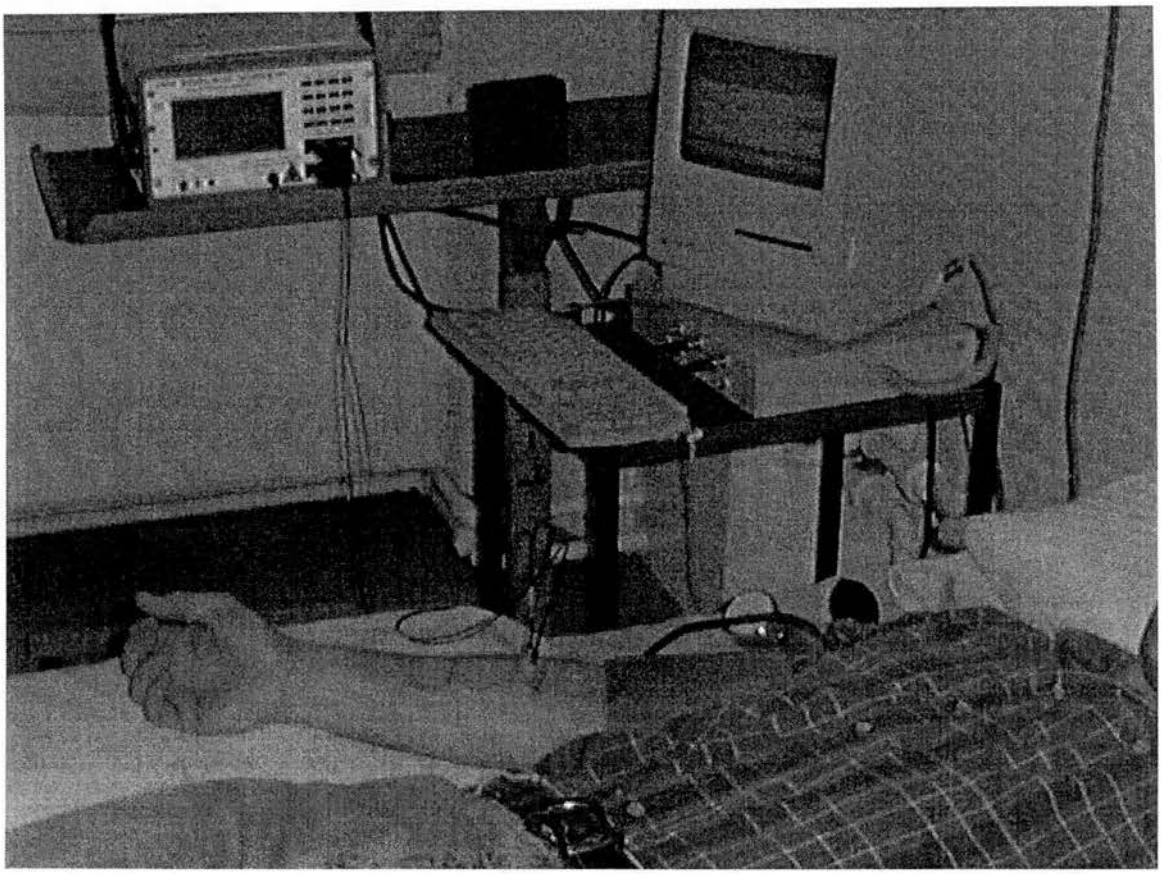
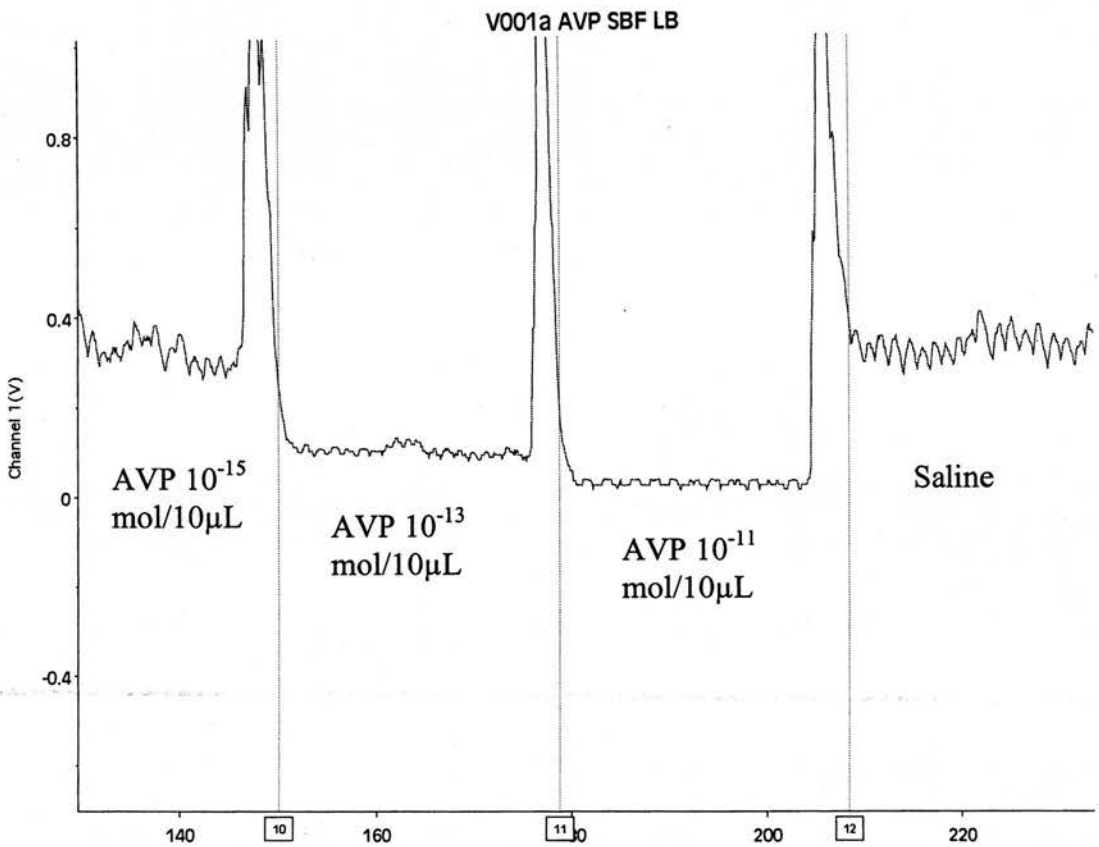


Figure 2.5

Skin micro-circulation: Typical Chart™ recording.



2.5 PULSE WAVE ANALYSIS

2.5.1 Principles of Pulse Wave Analysis

The arterial wave form that travels down the arterial tree, initiated at the start of systole, is reflected back principally by the resistance arterioles. While the wave form is known to alter as it progresses down the arterial tree, the profile of the wave form in the most proximal aorta influences the left ventricular afterload and coronary blood flow. Augmentation index is a measure of how much the reflected wave contributes to the initial systolic wave. Normally when arteries are compliant the reflected wave returns in diastole enhancing diastolic blood pressure and coronary flow. However, if the reflected wave returns from a more proximal site in the arterial tree (such as stiffened large arteries) and with greater velocity, it can arrive earlier in systole which augments systolic rather than diastolic blood pressure, increasing left ventricular afterload and reducing coronary flow [Nichols and O'Rourke 1998; Oliver and Webb 2003]. By measuring augmentation index a composite measure of central arterial stiffness can be obtained, whereas aortic pulse wave velocity examines the contribution of large arterial stiffness [Nichols and O'Rourke 1998; O'Rourke 2001].

2.5.2 Measurement of Augmentation index

Augmentation index was measured via applanation tonometry with a high fidelity micromanometer (SPC-301; Miller Instruments, Texas, USA) over the radial artery, where it lies anterior to the distal radius. Data were collected on to a portable computer

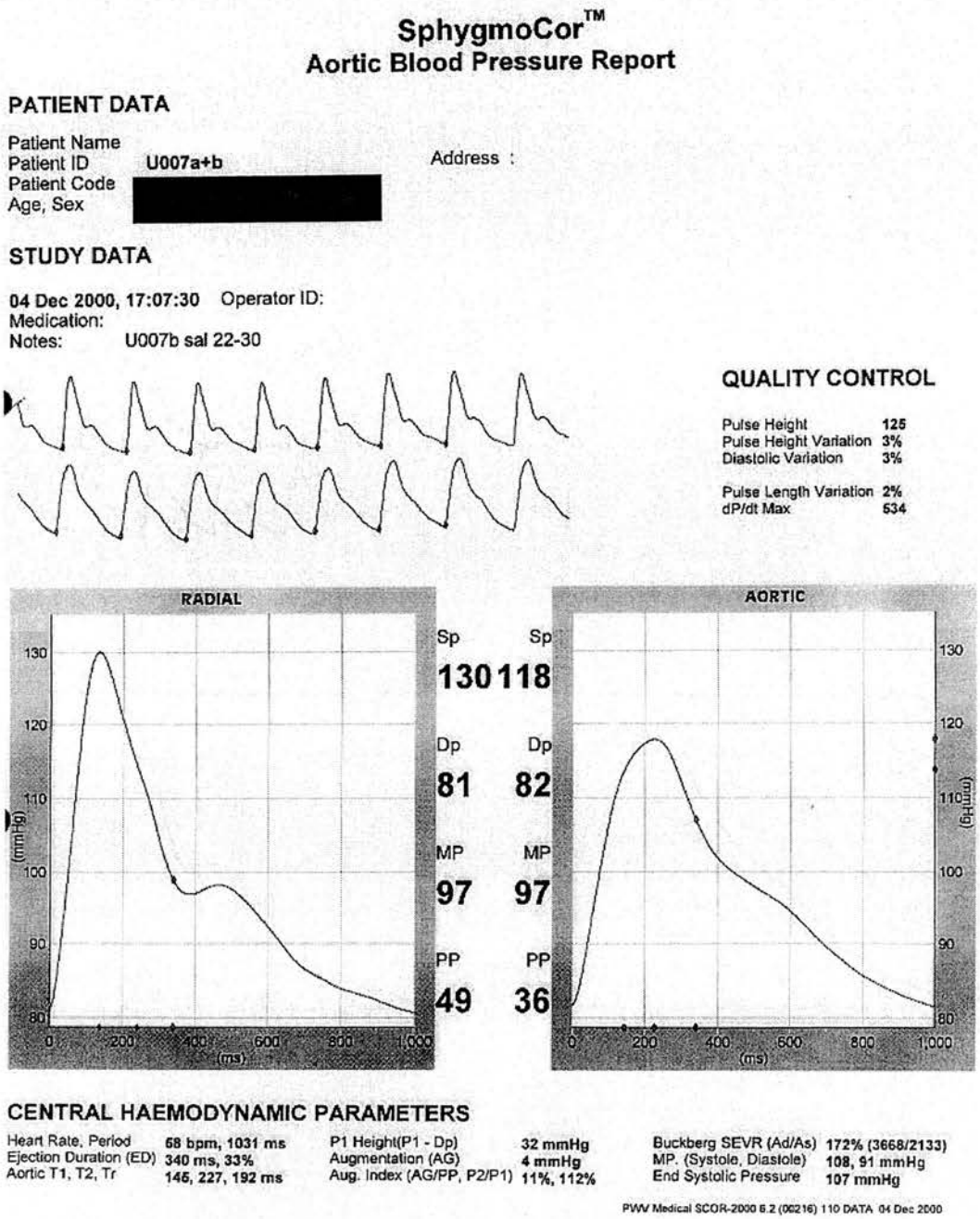
and allowed quality of the recordings was assessed to ensure freedom from artefacts. Recordings with systolic and diastolic variability in excess of 5% were excluded and the measurement repeated. After 20 sequential waveforms have been acquired the integrated software was used to generate an average peripheral and central waveform, calibrated against brachial blood pressure, which was subject to further analysis to determine augmentation index (Figure 2.6) (SphygmoCor 2000 version 6.2; PWV Medical PTY Ltd, Sydney, Australia). Augmentation index was defined as the distance between the first and second peaks of the central waveform, expressed as a percentage of the pulse pressure. All measurements were made in duplicate and mean values used in subsequent data analysis. Recordings with systolic and diastolic variability in excess of 5% were excluded and the measurement repeated.

2.5.3 Measurement of Aortic Pulse Wave Velocity

Pulse wave velocity (PWV) was determined using electrocardiographic monitoring combined with pulse wave analysis (SphygmoCor 2000 version 6.2) at the carotid artery (adjacent to the thyroid cartilage) and femoral artery (immediately below the inguinal ligament). The separation of the pulse waveforms was defined as the distance between the sternal notch and both the inguinal ligament and the thyroid cartilage. All measurements were made in duplicate and mean values used in subsequent data analysis.

Figure 2.6

Pulse wave analysis: typical result sheet.



SphygmoCor™
Aortic Blood Pressure Report

PATIENT DATA

Patient Name
Patient ID U007a+b
Patient Code
Age, Sex [redacted]
Address :

STUDY DATA

04 Dec 2000, 17:07:30 Operator ID:
Medication:
Notes: U007b sal 22-30

QUALITY CONTROL

Pulse Height	125
Pulse Height Variation	3%
Diastolic Variation	3%
Pulse Length Variation	2%
dP/dt Max	534

RADIAL

AORTIC

	Sp	Sp
	130	118
Dp	81	82
MP	97	97
PP	49	36

CENTRAL HAEMODYNAMIC PARAMETERS

Heart Rate, Period	58 bpm, 1031 ms	P1 Height(P1 - Dp)	32 mmHg	Buckberg SEVR (Ad/As)	172% (3668/2133)
Ejection Duration (ED)	340 ms, 33%	Augmentation (AG)	4 mmHg	MP. (Systole, Diastole)	108, 91 mmHg
Aortic T1, T2, Tr	145, 227, 192 ms	Aug. Index (AG/PP, P2/P1)	11%, 112%	End Systolic Pressure	107 mmHg

PWV Medical SCOR-2000 5.2 (00216) 110 DATA 04 Dec 2000

2.6 HAND VEIN STUDIES

2.6.1 Venous displacement technique and venous cannulation

Studies were carried out using a standard displacement technique [Aellig 1981; Webb and Haynes 1993]. During studies subjects were seated on a bed with hand and arm of the measured vein placed on an arm rest above the level of the heart. A 23 gauge butterfly® cannula (Abbott, Sligo, Ireland) was placed in a 2 cm length of non-branching dorsal hand vein in the direction of blood flow and attached to a 16 gauge epidual catheter (Potex Portex Ltd, Hythe, UK). Patency was maintained by infusion of saline (0.9%: Baxter Healthcare Ltd, Thetford, UK) via a IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK) at a rate of 0.25 mL/min. A tripod was placed 1.5 cm proximal to the cannulation site, ensuring it was not overlying any other veins. The tripod supported a linear variable differential transformer, the central bar of which rested on the apex of the chosen vein. The total infusion rates were kept constant at 0.25 mL/min throughout studies.

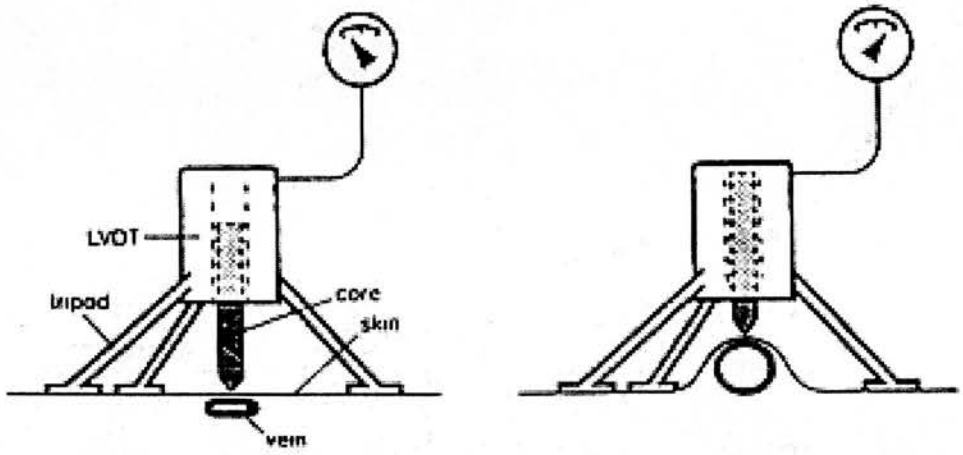
2.6.2 Measurement of venous tone

Measurements were made every 5 minutes during which a cuff is inflated intermittently to 30 mmHg using an E20 Rapid Cuff Inflator (D.E. Hokanson Inc, Washington, USA), thus occluding venous outflow in the studied arm. Analogue voltage, generated by displacement of the central rod in the tripod (Figure 2.7), was processed by a Mac Lab® analogue to digital converter and Chart™ *v*3.4.3 software (A.D. Instruments Ltd, Castle Hill, Australia) and recorded onto a Macintosh Classic II computer (Apple Computers

Inc, Cupertino, USA). The difference between the voltage recorded during inflation and deflation of the cuff is a measure of the diameter of the studied vein [Aellig 1981].

Figure 2.7

Aellig hand vein technique: tripod setup [Aellig 1981].



Deflated cuff

Inflated cuff (30mmHg)

2.7 BLOOD COLLECTION AND PEPTIDE ASSAY

2.7.1 Forearm venous sampling

Venous cannulae were inserted into large subcutaneous veins of the anticubital fossa in both arms as previously described [Plumpton *et al* 1995]. Blood was withdrawn simultaneously from each arm during the last 2 min of each infusion. Samples for urotensin II estimation were collected in tubes containing ethylene diamine tetra-acetic acid (EDTA) tubes (Monovette®, Sarstedt, Numbrecht, Germany). Samples were centrifuged at 3,000 *g* for 10 min at 4°C and the plasma stored at -80°C until subsequent analysis.

2.7.2 Urotensin II assay

Plasma human urotensin II (hUII) concentrations were determined by radioimmunoassay using rabbit anti-flounder urotensin II antibody and human urotensin II iodinated by the Iodogen method of Fraker and Speck [Fraker and Speck 1978]. The antibody had equal specificity for human and flounder UII, and there was no cross-reactivity in the assay with endothelin-1, angiotensin II or somatostatin-14 (Sigma Chemical Co, UK). Before assay, plasma samples were subject to reverse-phase chromatographic purification using Sep-Pak C18 cartridges (Millipore UK Ltd) with acetonitrile solvent. The assay protocol was based on that previously described for flounder urotensin II [Winter *et al* 1999]. Briefly, sample extract was incubated with antibody (38,400 dilution) and ^{125}I human urotensin II at 4°C for 24 hours. Following this, the complexes formed were precipitated by the addition of bovine γ-globulin (Sigma Chemical Co, Dorset, UK) and

polyethylene glycol (Sigma Chemical Co, Dorset, UK), and the bound fraction was counted for 10 min in a gamma counter (1275 minigamma, Wallac, Finland). A typical standard curve for the human radio-immunoassay is shown in Figure 4.1. Also shown is the parallelism of serial dilutions of human plasma extract with the standard curve established for synthetic human urotensin II, confirming the specificity of the assay and its suitability for measurement of plasma human urotensin II. Recovery of human urotensin II in plasma extracts was 63% and intra- and inter-assay coefficients of variation in our laboratory were 7.6% and 13.3% respectively; the sensitivity of the assay was 1 fmol/mL.

CHAPTER 3

VALIDATION OF LASER DOPPLER FLOWMETRY COUPLED WITH INTRA-DERMAL INJECTION

SJ Leslie, **JT Affolter**, MA Denvir, DJ Webb. Validation of laser Doppler flowmetry coupled with intra-dermal injection for investigating effects of vasoactive agents on the skin microcirculation. *Eur J Clin Pharm* 2003;**59**:99-102.

3.1 SUMMARY

We aimed to determine the repeatability and reproducibility of laser Doppler flowmetry coupled with intra-dermal saline delivery. Two operators each performed 100 injections. Delivery of saline was judged 'by eye' using a graduated syringe (Becton-Dickinson) by injecting onto a weighing boat. Saline volume of 10μL was assessed by weight were 1 g ≡ 1 mL saline. Skin blood flow following intra-dermal injection of saline was assessed in 18 healthy volunteers; 10 attended twice to assess between-day reproducibility, and 8 attended once to assess between-site repeatability. Results are expressed as mean value and 95% confidence interval for mean differences. There was no difference in mean injection weight between operators, both being 10.3 ± 0.1 mg (0.08, -0.23 to 0.39 mg: mean difference, 95% confidence, n=100, P=0.9). Intra-dermal delivery of saline was well tolerated with only mild discomfort experienced during the injection at some of the sites. Intra-dermal saline caused a 9-fold increase in skin blood flow (P<0.001). This response was rapid in onset with the maximal effect seen at 4 min and apparent duration of greater than 30 min. There was no difference in the magnitude of the response between the dominant and non-dominant arms, the area under the curve being 2.9 ± 0.4 perfusion units (PU) for both (-0.05, -0.8 to 0.73 PU: mean difference, 95% confidence, n=18, P=0.9). There was no statistical difference between study visits 1 and 2, area under the curve was 3.2 ± 0.6 and 2.0 ± 0.5 PU respectively (1.2, 0.03 to 2.43 PU: mean difference, 95% confidence, n=10, P=0.7). There was no difference in the magnitude of responses between different sites on the forearm (P=0.6). These

studies demonstrate that the technique of laser Doppler flowmetry coupled with intra-dermal injection is a safe, well-tolerated technique with good repeatability. A trend towards reduced between-day reproducibility emphasises the importance of vehicle control sites when investigating the effects of vasoactive compounds. This technique provides a reliable method for the intra-dermal delivery of the potential drug vehicle saline, despite the direct effect of injection of saline on blood flow.

3.2 INTRODUCTION

Laser Doppler flowmetry is a well validated technique [Nilsson *et al* 1980] used for the investigation of the effects of vasoactive substances on the skin microcirculation [Hovell *et al* 1987; Haynes *et al* 1991; Wenzel *et al* 1994). Saline is widely used as vehicle for drug administration in other vascular beds and it has been previously demonstrated that intra-dermal injection of saline, when used alone, causes an increase in laser Doppler flowmeter signal [Wenzel *et al* 1994]

Laser Doppler flowmetry coupled with intra-dermal injection has several potential advantages over other techniques for the initial study of drugs in humans in that it is minimally invasive and relatively safe because it uses very small doses of study compound with a mainly local activity. It also allows separate sites on the skin to be studied simultaneously and thus the investigation of a range of concentrations on the same study visit.

While it is possible to deliver some vasoactive substances such as acetylcholine and noradrenaline by skin iontophoresis, many peptides cannot be delivered by this means due to their large size, poor solubility or lack of electrical charge. In these situations, intra-dermal injection can be used. However, this technique has the theoretical disadvantage that it results in a small degree of skin trauma [Holloway 1980] and delivery of small volumes may increase errors and variability in responses. Physiological saline is commonly used in studies as a drug vehicle and there are a

variety of high precision syringes, which can be used for the intra-dermal injection. However, these are expensive and when used in human studies can, by necessity, be used only for a single subject. In the current studies we used standard clinical insulin syringes with a 29.5 SWG gauge needle to administer intra-dermal injections. The advantages of these syringes are that they are relatively inexpensive and easy to use. These syringes have been used by others in SBF studies [Wenzel *et al* 1994, 1996 and 1998] but have not been validated for use for intra-dermal injection in pharmacological studies. There are potential sources of error in the injection technique because the plunger is depressed by only 1 mm to deliver 10 μl and is judged 'by eye'. The repeatability of intra-dermal delivery in terms of volume delivered and the effect of intra-dermal saline injection on skin blood flow has not previously been reported.

The aim of these studies was to determine the intra- and inter-operator repeatability and reproducibility of saline administration in terms of injection volume and to determine repeatability of skin blood flow responses to intra-dermal injection.

3.3 METHODS

3.3.1 Subjects

Eighteen healthy men (22-45 years, all right handed) were studied. Studies were performed with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki of the World Medical Association. Written informed consent was obtained from each subject before entry to the study. None of the subjects were taking regular medication and all avoided any medication for 1 week prior to the study. All subjects abstained from alcohol for 24 hours and from food, tobacco and caffeine containing drinks for at least 12 hours before each study.

3.3.2 Injection volume

Graduated 29.5 SWG syringes (Becton-Dickinson, Dublin, Ireland) were used for saline delivery. Each 1 mm graduation on the 0.5 mL syringe represents 10 μL, thus to deliver this volume the syringe plunger was depressed by 1 mm. The repeatability of injection volume was assessed by injecting 10 μL saline judged by depressing the syringe plunger by one graduation, onto a weighing boat placed on a balance (Mettler Toledo® MT5). This balance has an accuracy and precision of 1 μg and therefore can measure changes of 1 nL assuming a specific gravity of saline (0.9%; Baxter Healthcare Ltd, Thetford, UK) of 1.00. A new syringe was used for each injection and care was taken, as in our clinical studies, to expel any air bubbles from the syringe before injecting. The balance was set to zero before each injection. Two operators each performed 100 injections.

3.3.3 Study design

Studies were performed in a quiet temperature controlled, draught free room (22-24°C). Each subject was allowed to rest for at least 20 min before the study protocol was started. Baseline recordings were made and then volunteers received intra-dermal injections of 10 μL 0.9% saline. Following intra-dermal injection, laser Doppler signal was recorded every 2 min until 10 min and every 5 min until 30 min. To assess between-day reproducibility, 10 subjects attended for 2 study visits. To assess between-site repeatability, 8 of the subjects received 4 intra-dermal injections of saline on the volar aspect of each forearm.

3.3.4 Data analysis

Increases in weight on the balance following saline injection were recorded manually and entered onto a spreadsheet (Excel v5.0; Microsoft). The accuracy was assessed by mean values, and reproducibility by assessing the spread of results. Results are expressed as mean ± SEM perfusion units (PU). Differences between results were compared using Student's *t*-test. Statistical significance was taken at the 5% level. Calculated area under the curve results were assessed using the method of Bland and Altman [Bland & Altman 1986]. Bland-Altman analysis allows the assessment of agreement and systematic bias. Coefficients of repeatability were determined for 95% confidence intervals. Statistical analysis was performed using Student's *t*-test and single factor ANOVA for between-site repeatability. Statistical significance was taken at the 5% level.

3.4 Results

3.4.1 Injection volume

Two operators performed 100 injections each. There was no difference in mean injection weight between operators, both being 10.3 ± 0.1 mg (figure 3.1, mean difference 0.08, 95% confidence intervals -0.23 to 0.39 mg, n=100, P=0.9).

3.4.2 Tolerability of intra-dermal saline

The technique was well tolerated by subjects with only mild discomfort experienced during the injection of saline at some of the sites. This discomfort was variable in intensity. In the majority of cases the trauma from intra-dermal injection did not leave any discernible mark on the skin by the end of the study although several injections did cause a small degree of bleeding along the track of the needle. This did not appear to affect the results in terms of repeatability.

3.4.3 Skin blood flow responses: effect of intra-dermal injection of saline

Saline caused a 9-fold increase in skin blood flow (0.03 ± 0.003 to 0.27 ± 0.02 PU, n=18, P<0.001). This effect was rapid in onset with maximal response seen at 4 min, see figure 3.2.

3.4.4 Between-arm repeatability

There was no difference in magnitude of response between dominant and non-dominant arms, area under the curve was 2.9 ± 0.4 PU for both (figure 3.2, mean difference -0.05,

95% confidence intervals -0.8 to 0.73 PU, n=18, P=0.9). Bland-Altman analysis was performed demonstrating no systemic bias and a co-efficient of repeatability of 3.54 PU (figure 3.5).

3.4.5 Within-subject same-day repeatability: Between-site repeatability

Area under the curve was constructed for the responses at 4 different sites on each forearm in 8 subjects. There was no difference in the magnitude of responses between sites on the forearm as assessed by area under the curve (figure 3.4, ANOVA, P=0.6).

3.4.6 Between-day reproducibility

There was a trend towards a difference between study visits 1 and 2, area under the curve was 3.2 ± 0.6 and 2.0 ± 0.5 PU respectively (1.2 mean difference, 95% confidence intervals 0.03 to 2.43 PU, n=10, P=0.7) although this did not reach statistical significance (figure 3.3). Bland-Altman analysis was performed demonstrating no systemic bias and a co-efficient of reproducibility of 3.48 PU (figure 3.6).

Figure 3.1

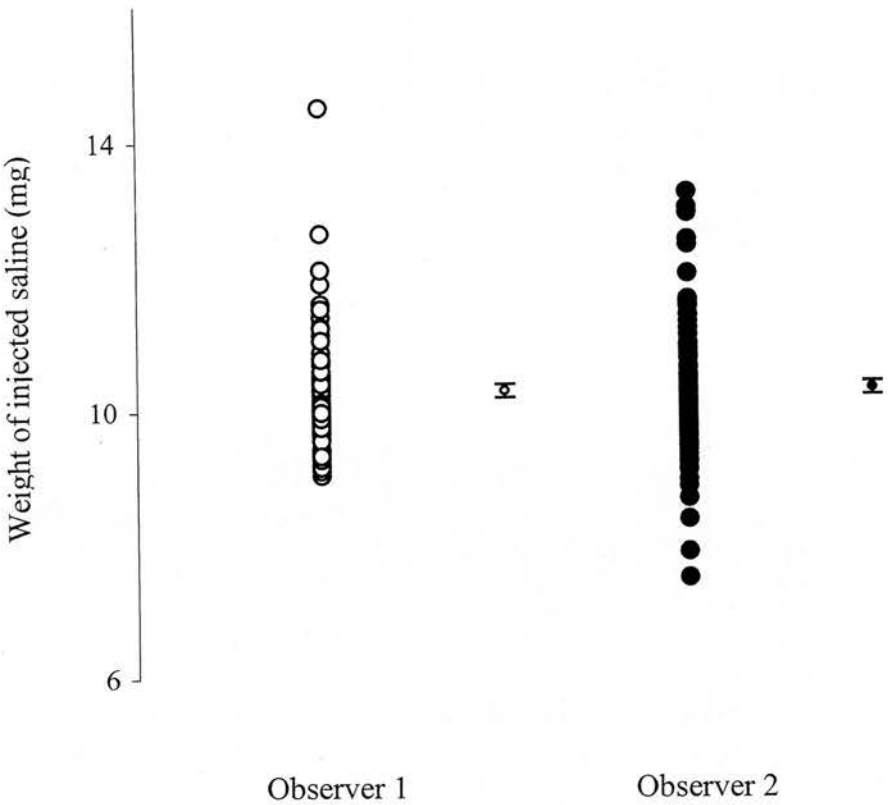
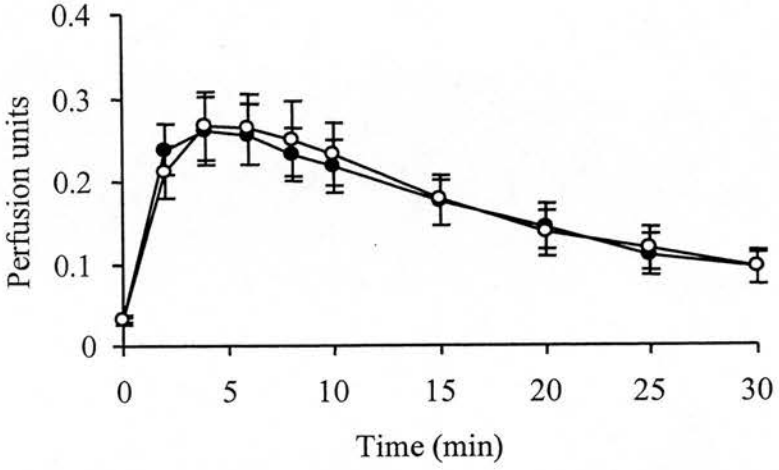


Figure 3.1

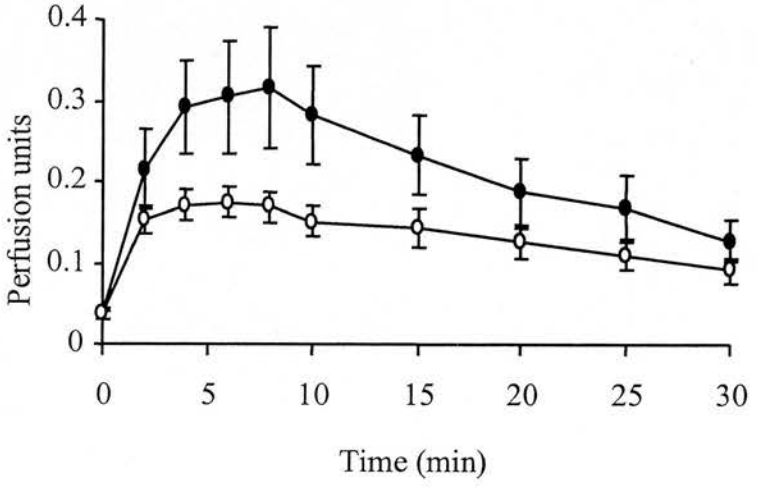
Weight of 100 injections by 2 observers, spread of points and mean ± SEM.

Figure 3.2



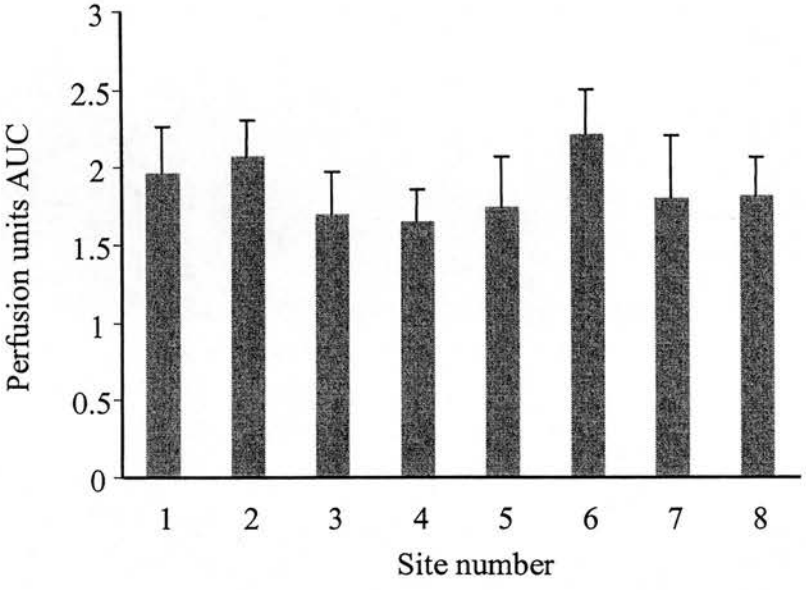
Effect of intra-dermal saline on SBF between dominant (○) and non-dominant (●) arm on the same study visit. Mean ± SEM.

Figure 3.3



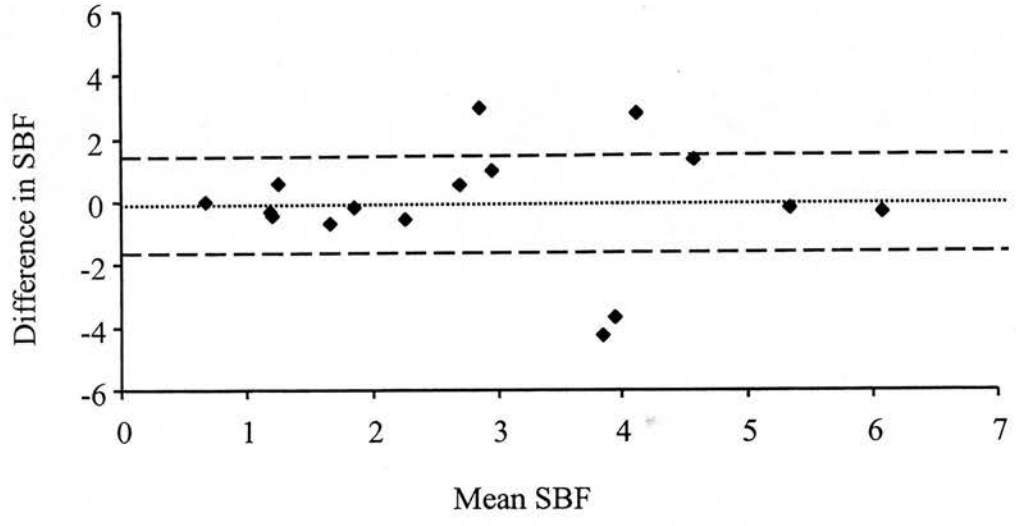
Effect of intra-dermal saline on SBF on the dominant arm on the different study visits 1 (●) and 2 (○). Mean ± SEM.

Figure 3.4



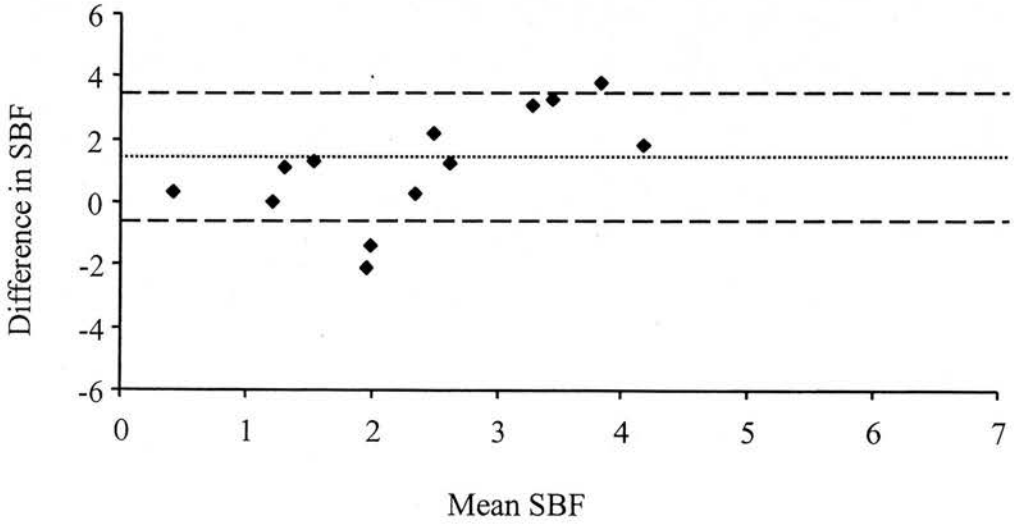
Simultaneous effect of intra-dermal saline injection on SBF between 8 sites on the same day. Sites 1-4 were chosen on the dominant arm (numbered proximal to distal on forearm), sites 5-8 were chosen on the non-dominant arm (numbered proximal to distal on forearm). Mean ± SEM.

Figure 3.5



Bland and Altman plot for between-arm repeatability

Figure 3.6



Bland and Altman plot for between-day reproducibility.

3.5 DISCUSSION

In this study we have demonstrated that saline delivery using the Becton-Dickinson syringe is accurate and repeatable with low intra-operator and inter-operator variability. In addition, we have demonstrated that intra-dermal injection of saline causes an increase in Doppler signal but that the magnitude of this increase is similar between different sites on the forearm. There is good within-day and between arm repeatability.

There was occasionally mild discomfort experienced by the subjects at the time of injection. However, there did not appear to be any pattern to explain the fact that some sites developed more discomfort than others. Although the non-uniform distribution of cutaneous nerves may explain this finding, this was not formally assessed and did not appear to affect the results in terms of repeatability.

While there is often greater interest in the systemic effects of vasoactive compounds, there are potential risks with administration of systemic doses of vasoactive compounds. The use of local techniques, such as intra-dermal administration with laser Doppler microcirculatory blood flow measurement has allowed the relatively safe observation of the *in vivo* effects of vasoactive compounds, without causing confounding compensatory systemic effects. While the skin blood flow is under different regulatory mechanisms and responses can differ from other vascular beds [Weber *et al* 1997], the effects of compounds in the skin have been generally reflected in other less accessible vascular beds [Wenzel *et al* 1994; Wenzel *et al* 1995]. Therefore the assessment of skin

microcirculation changes following intra-dermal injection can offer a safe, well tolerated, easy to use method to the initial investigation of vasoactive compounds.

Most vasoactive compounds will be prepared in saline for dose ranging studies. Some may be delivered to the skin by iontophoresis, however, this is not suitable for many compounds due to their large size and relative insolubility. Intra-dermal injection of study compounds is employed in these cases.

The Becton-Dickinson syringe is commonly used to delivery intra-dermal injections in clinical practice. Although it has the advantage that it is inexpensive and easy to use, its accuracy has not previously been described. Here, we found good agreement between operators as seen by mean values that were similar and close to the intended volume of 10 μL with most injections very close to this volume (figure 3.1). We conclude that this syringe can be used to deliver intra-dermal injections in a clinical research setting with sufficient accuracy and repeatability.

Intra-dermal saline causes an increase in laser Doppler signal. In this study, we have demonstrated that the technique of laser Doppler flowmetry coupled with intra-dermal injection is a repeatable method and that responses between subjects were similar. There was no difference in skin blood flow in response to intra-dermal saline injection between sites on the same arm or in the same subject on different study visits. This indicates the importance of employing saline placebo controls for all studies using intra-dermal injections. There was, however, a trend towards a small difference in skin blood

flow between study visits which emphasises the importance of a vehicle control site when investigating vasoactive compounds. The reasons for this between-day variability are not clear as ambient temperature was controlled, subjects were fasted and under similar conditions during each study visit. Although careful attempts were made to keep room temperature and conditions constant, the skin is more sensitive than other vascular beds to changes in ambient conditions and small temperature changes, draughts or emotional factors may be more important than in other vascular beds. Nevertheless, these apparent between day differences did not reach statistical significance and the within-subject and between-day coefficients of repeatability were similar. We conclude that despite small difference between days that vehicle control injections should be performed during each study visit.

In conclusion, the technique of intra-dermal injection coupled with laser Doppler flowmetry offers a safe, well-tolerated, repeatable technique for the potential investigation of vasoactive compounds using saline as a drug vehicle in human *in vivo*.

CHAPTER 4

HUMAN UROTENSIN II LOCAL VASCULAR STUDIES IN MAN

IB Wilkinson, **JT Affolter**, SL De Haas, MP Pellegrini, J Boyd, MJ Winter, RJ Balment and DJ Webb. High plasma levels of urotensin II do not alter local or systemic haemodynamics in man. *Cardiovascular Research* 2002;**53**:341-347.

4.1 SUMMARY

Human urotensin II is an endocrine hormone that acts as a potent arterial vasoconstrictor in *in vitro* and *in vivo* studies in animals. We examined, for the first time, the local and systemic haemodynamic response to urotensin II in man *in vivo*. Four healthy male volunteers took part in pilot studies and 11 in definitive studies. Forearm blood flow was measured in response to intra-arterial infusion of authentic, biologically active urotensin II (incremental rates of 0.001-300 pmol/min) and saline placebo using venous occlusion plethysmography. Blood pressure, heart rate, cardiac output and urotensin II plasma concentrations were also measured. Forearm studies were repeated in 5 subjects with inhibition of endothelial mediators using aspirin and a 'nitric oxide clamp'. Dorsal hand vein diameter was determined by a standard displacement technique in response to local administration of urotensin II (3-300 pmol/min) with and without nitric oxide synthase inhibition. There was no significant change in forearm blood flow during brachial infusion of saline or urotensin II (dose range, 0.001 to 300 pmol/min). A nitric oxide clamp did not unmask vasoactive effects of urotensin II infusions (100 and 300 pmol/min) significantly increased plasma concentrations from baseline (12 ± 3 pmol/mL) to 106 ± 15 and 307 ± 98 pmol/mL respectively. Despite high circulating urotensin II concentrations, no change was seen in systemic haemodynamics and ECGs were unchanged. Human urotensin II had no effect on hand vein diameter (n=6). In contrast to our hypothesised role of urotensin II, we found no vasoactive responses to urotensin II *in vivo*, consistent with recent *in vitro* studies in human blood vessels but in contrast to non-human primate studies *in vivo*. Our data do not support a key role for

urotensin II in the regulation of vascular tone and resting blood pressure in man. However, studies with urotensin II receptor antagonists are also needed before firm conclusions can be drawn.

4.2 INTRODUCTION

Human urotensin II is a recently discovered vasoactive peptide hormone that acts as a high affinity ligand for rat G-protein receptor 14 (GPR14) [Coulouarn *et al* 1997; Ames *et al* 1999; Nothacker *et al* 1999] and the more recently discovered human receptor [Nothacker *et al* 1999]. It is the most potent arterial vasoconstrictor yet discovered and has a sustained effect in blood vessels from a variety of species [Ames *et al* 1999; Maguire *et al* 2000; MacLean *et al* 2000].

Human urotensin II was first isolated in man from subgroups of motor neurones in the spinal cord [Coulouarn *et al* 1997]. Outwith the CNS, the kidney has the highest expression of prepro urotensin II mRNA and, therefore, appears the most likely source of circulating urotensin II receptor [Nothacker *et al* 1999]. Its receptor distribution has been mapped using immuno-histochemistry, confirming target binding sites for urotensin II in cardiovascular tissues including coronary arteries, internal mammary arteries and ventricular cardiomyocytes [Ames *et al* 1999; Maguire *et al* 2000]. Thus, it is likely that circulating urotensin II functions as an endocrine hormone. *In vitro* animal data and studies in rats and non-human primates *in vivo*, indicate that urotensin II is a potent vasoconstrictor and influences cardiac function [Ames *et al* 1999; Maguire *et al* 2000]. However, conflicting results have been obtained from human blood vessels *in vitro* [Maguire *et al* 2000; MacLean *et al* 2000; Stirrat *et al* 2001]. Although some studies suggest that urotensin II is 28 to 50 fold more potent than endothelin-1 [Ames *et al* 1999; Maguire *et al* 2000], others show urotensin II to be a vasodilator [Stirrat *et al*

2001]. In addition, there is some variability in response to urotensin II amongst species [Douglas *et al* 2000], depending on vessel location and type, and between individual preparations. This is highlighted by some of the *in vitro* studies having responding and non-responding vessels [MacLean *et al* 2000]. Interestingly, in the rat, the activity of urotensin II is most marked in the region of the proximal aorta, decreasing rapidly further down the arterial tree [Maguire *et al* 2000]. Venoconstriction has been found only in some studies in vessels from non-human primates and humans and, in contrast to the effects of endothelin-1 and norepinephrine, and where present, urotensin II is less potent in veins than arteries [Ames *et al* 1999; Maguire *et al* 2000].

To date, there have been no *in vivo* physiological studies of the actions of urotensin II in man. On the basis of animal *in vivo* and the positive human *in vitro* studies, we hypothesised that urotensin II would cause arteriolar vasoconstriction in the human forearm, but have little or no effect in veins. We also anticipated that systemic dosing would raise peripheral resistance and, hence, blood pressure. Our aim was to undertake the first human *in vivo* study with urotensin II, addressing local responses in human arterial vessels and dorsal hand veins. Subsequently, we explored the effect of higher doses on systemic haemodynamics and plasma urotensin II concentrations.

4.3 METHODS

4.3.1 Subjects

These studies were conducted with the approval of the local research ethics committee and the written informed consent of each subject. The investigation conforms to the principles of the Declaration of Helsinki. Fifteen healthy men, mean age 37 ± 4 years (range 22-53), were recruited from a bank of community volunteers held by the Clinical Research Centre at the Western General Hospital in Edinburgh. Four subjects took part in pilot studies and eleven in the definitive vein and forearm studies. Subjects were asked to fast from midnight before each study, and to abstain from caffeine containing drinks, alcohol and smoking over the preceding 24 hours. Subjects mean height was 176 ± 3 cm (range 170-180) and mean weight was 80 ± 8 kg (range 63-92).

4.3.2 Drugs

All drugs were freshly prepared aseptically and dissolved in either saline (0.9% Baxter Healthcare Ltd., Norfolk, UK) or Gelofusine® (Braun Healthcare Ltd., Sheffield, UK). The drugs used were: human urotensin II (Peptide Institute, Osaka, Japan and SmithKline Beecham, Pennsylvania, USA), angiotensin II (ANGII; Clinalfa, Laufelfingen, Switzerland), norepinephrine (NE; Abbott Laboratories, Kent, UK), sodium nitroprusside (SNP; David Bull Laboratories, Warwick, UK) and L-N^G-monomethylarginine (L-NMMA; Clinalfa).

We confirmed the authentic nature of the human urotensin II from both sources by high performance liquid chromatography and microsequencing (in the laboratory of Drs. S.A. Douglas and E.H. Ohlstein, SmithKline Beecham). We also confirmed the biological activity of the urotensin II peptides by showing the anticipated responses, and potency, in the rat proximal aorta.

4.3.3 Study design

Saline was infused at 1 ml/min for a period of 30 min before drug infusion protocols were started to ensure a stable baseline. The total infusion rate was kept constant at 1 ml/min. Throughout the study forearm blood flow was measured simultaneously in both arms by venous occlusion plethysmography [Whitney 1953; Benjamin *et al* 1995; Wilkinson and Webb 2001], as previously described [Love *et al* 1996]. Forearm blood flow was measured over a 3 min period every 6 min, and the last five recordings of forearm blood flow were averaged to determine flow in each arm. Cardiac index was assessed using a validated [Northridge *et al* 1990] transthoracic electrical bioimpedance technique (NCCOM3, BoMed Irvine CA, USA). Both blood pressure and cardiac index were recorded after each forearm blood flow recording was completed. Mean arterial pressure was defined as diastolic pressure plus 1/3 of the pulse pressure. Peripheral vascular resistance was calculated as mean arterial pressure divided by cardiac index and expressed in arbitrary units. Throughout the study continuous electrocardiographic (ECG) monitoring was employed and a full 12 lead ECG recorded at baseline and at the end of the highest urotensin II infusion rate on each study day.

4.3.4 Pilot studies

Human UII (Peptide Institute, Osaka Japan), diluted in 0.9% saline vehicle was given intra-arterially on 3 separate occasions, each in 2 subjects, at rates of 0.001, 0.003 and 0.01pmol/min, 0.03, 0.1 and 0.3pmol/min, and 1, 10 and 30pmol/min. After 30 min saline run-in, each dose of urotensin II was given for 20 min.

4.3.5 Local arterial and systemic haemodynamics (Study 1)

On two occasions, separated by one week, each subject received a 30 min infusion of saline and then either urotensin II (Peptide Institute, Osaka Japan) or saline in a single-blind, randomised manner. Four subjects received 30 and 100pmol/min urotensin II, and 6 subjects 100 and 300pmol/min urotensin II. Each rate was maintained for a total of 20 min and forearm blood flow recorded at 3, 9 and 15 min. After the final forearm blood flow recording during saline baseline infusions and each dose increment, systemic haemodynamic measurements were made (heart rate, blood pressure and cardiac index) and 10mL of venous blood was collected for determination of plasma urotensin II concentration. In addition, forearm blood flow studies were repeated in some of the same subjects. First, we used an alternative batch of urotensin II (SmithKline Beecham, Pennsylvania, USA; dose range 0.1, 1, 10, 30pmol/min: 6 subjects). Second, we used an alternative Gelofusine® vehicle with the original urotensin II (Peptide Institute, Osaka Japan; dose range 1, 3, 30, 300pmol/min: 4 subjects).

4.3.6 Local arterial haemodynamics with inhibition of endothelial mediators (Study 2)

Five of the subjects who took part in study 1 underwent a further study involving a 'nitric oxide clamp' [Verhaar *et al* 1998]. First, saline was infused for 30 min, and followed by L-NMMA infused intra-arterially at 4μmol/min to block endogenous nitric oxide generation [Vallance *et al* 1989]. Forearm blood flow was then restored to within ± 10% of baseline by the co-infusion of SNP, an endothelium-independent nitric oxide donor (mean dose 0.6 nmol/min, range 0.3-1.0). To produce a simultaneous inhibition of prostanoid production, each subject received 600mg aspirin dissolved in 200 ml of water 30 min before the study. At this dose aspirin inhibits bradykinin-stimulated endothelial prostacyclin generation and platelet thromboxane production [Heavey *et al* 1985], but has no direct effect on blood pressure or basal vascular tone. Once forearm blood flow had returned to basal levels, urotensin II was co-infused at 1, 10 and 100 pmol/min, each rate for 20 min. Forearm blood flow and systemic haemodynamics were recorded as for Study 1.

4.3.7 Venous tone (Study 3)

Six subjects made two visits, separated by one week. Each received a 30 min infusion of saline into a selected dorsal hand vein followed by either L-NMMA (100 nmol/min) or saline in a single-blind, randomised manner for 5 min. Human urotensin II was then co-infused at 3, 30 and 300 pmol/min, each rate for 20 min. Saline was then infused for 10 min, followed by ANGII (25 ng/min) for 3 min then saline for a further 10 min and

finally NE (8 ng/min) for 3 min to assess the integrity of the vein. Hand vein diameter was measured every 5 min after a 10 min baseline saline infusion. The total infusion rate was kept at 0.25 mL/min.

4.3.8 Plasma urotensin II concentrations

Venous blood (10 mL) was drawn during the last 2 min of each infusion period from a cannula sited in the non-infused arm. Samples were collected into ethylene diamine tetra-acetic acid, immediately centrifuged at 3,000 *g* for 10 min at 4°C and the plasma stored at -80°C until subsequent analysis. Plasma human urotensin II concentrations were determined by radioimmunoassay using rabbit anti-flounder urotensin II antibody and human urotensin II iodinated by the Iodogen method of Fraker and Speck [Fraker and Speck 1978]. The antibody had equal specificity for human and flounder urotensin II, and there was no cross-reactivity in the assay with endothelin-1, angiotensin II or somatostatin-14 (Sigma Chemical Co, UK). Before assay, plasma samples were subject to reverse-phase chromatographic purification using Sep-Pak C18 cartridges (Millipore UK Ltd) with acetonitrile solvent. The assay protocol was based on that previously described for flounder UII [Winter *et al* 1999]. Briefly, sample extract was incubated with antibody (38,400 dilution) and ^{125}I human urotensin II at 4°C for 24h. Following this, the complexes formed were precipitated by the addition of bovine ν-globulin (Sigma Chemical Co, UK) and polyethylene glycol (Sigma Chemical Co, UK), and the bound fraction was counted for 10 min in a gamma counter (1275 minigamma, Wallac, Finland). A typical standard curve for the human urotensin II radio-immunoassay is

shown in Figure 4.1. Also shown is the parallelism of serial dilutions of human plasma extract with the standard curve established for synthetic human urotensin II, confirming the specificity of the assay and its suitability for measurement of plasma human urotensin II. Recovery of human urotensin II in plasma extracts was 63% and intra- and inter-assay coefficients of variation in our laboratory were 7.6% and 13.3% respectively; the sensitivity of the assay was 1 fmol human urotensin II mL plasma.

4.3.9 Statistical analysis

All results are expressed as mean ± SEM. Data for FBF has been expressed as a percentage change from baseline of the FBF ratio (derived from infused arm value divided by non-infused arm value). Repeated measure ANOVA was used to identify differences in FBF response between urotensin II and saline, urotensin II concentrations during placebo and drug infusion and in the vein studies between presence and absence of urotensin II and L-NMMA co-infusion. For single comparisons, data were analysed using paired Student's *t*-tests. Results were considered significant at $P<0.05$.

4.4 RESULTS

All subjects were symptom free throughout each study. Baseline forearm blood flow, heart rate, cardiac index, blood pressure, plasma urotensin II concentrations and vein diameter were similar on the different study days and there was no significant difference in the basal forearm blood flow between the infused and non-infused arms. Neither continuous single-lead ECG monitoring, nor the full 12-lead ECGs, revealed any changes during the 3 studies.

4.4.1 Pilot studies

There was no significant change in FBF in either arm, or systemic haemodynamics, during infusion of saline or hUII (data not shown).

4.4.2 Local arterial and systemic haemodynamics (Study 1)

Baseline values for the non-infused and infused forearm blood flow were as follows; 3.5 ± 0.9 and 4.5 ± 2 ml/100ml tissue/min respectively for Figure 4.2A and 2.9 ± 0.4 and 2.9 ± 0.7 ml/100ml tissue/min for Figure 4.2B. There was no significant change in forearm blood flow ratio during infusion of saline or urotensin II in either of the dose ranging studies (Figures 4.2A and B). There was no significant change in systemic haemodynamics during infusion of urotensin II at any dose (Table 4.1A and B). However, there was a substantial and significant increase in circulating plasma urotensin II concentrations during urotensin II infusion (Figures 4.3A and B). Studies with urotensin II diluted in Gelofusine® rather than saline, and urotensin II from an

alternative supplier (SmithKline Beecham, Pennsylvania, USA), similarly did not change forearm blood flow or systemic haemodynamics (data not shown).

4.4.3 Local arterial haemodynamics with inhibition of endothelial mediators (Study 2)

Baseline values for non-infused and infused forearm blood flow were 3.1 ± 0.3 and 3.2 ± 0.5 ml/100ml tissue/min respectively. Infusion of L-NMMA resulted in a significant reduction in the forearm blood flow ratio (1 ± 0.1 at baseline compared with 0.6 ± 0.1; P=0.01 Student's t-test) (Figure 4.4). Co-infusion of SNP (mean dose 0.6 nmol/min, range 0.3 to 1.0) returned the forearm blood flow ratio to baseline (1 ± 0.1 at baseline compared with 0.8 ± 0.1, P=0.7 Student's t-test). There was no significant change in the forearm blood flow ratio, or forearm blood flow in either arm, during co-infusion of urotensin II following L-NMMA and SNP (forearm blood flow ratio, P=0.3). Human urotensin II infusion did not significantly alter heart rate, systolic or diastolic blood pressure (P=0.8, P=0.8 and P=0.3 respectively, ANOVA).

4.4.4 Venous tone (Study 3)

Increasing doses of urotensin II had no significant effect on hand vein diameter compared with baseline (P=0.9, ANOVA) (Table 4.2). During co-infusion of urotensin II and L-NMMA, there was also no significant change in hand vein diameter (P=0.8, ANOVA). In contrast, ANGII and NE both induced a substantial venoconstriction (see Table 4.2). The response to ANGII and NE was slightly higher during L-NMMA co-

administration, but these differences from the response with L-NMMA were not significant (P=0.8 and P=0.2 respectively, Student's t-test).

Figure 4.1

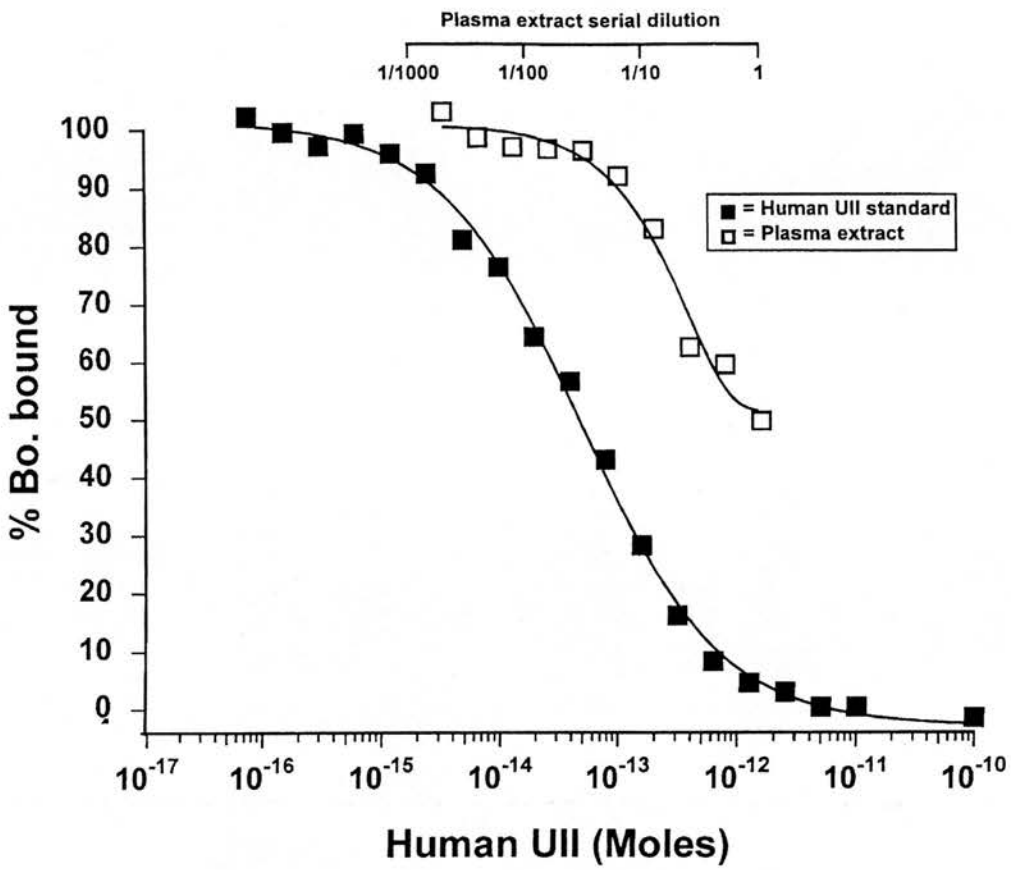


Figure 4.1

Typical radio-immunoassay curve for hUII, with antibody bound 125-I hUII for increasing standard concentrations of synthetic hUII expressed as % the maximum label binding (Bo) measured in the zero standard tubes. Also shown is antibody bound label for assay tubes containing serial dilutions of human plasma extract. The parallelism of the two curves confirms the specificity of the assay for hUII and its suitability for plasma hUII measurements.

Figure 4.2

A Mean percentage change in FBF ratio during 30 and 100 pmol/min UII infusion (●) compared with saline (□). ANOVA P=0.9 (n=4).

B Mean percentage change in FBF ratio during 100 and 300 pmol/min UII infusion (●) compared with saline (□). ANOVA P=0.4 (n=6).

Figure 4.2

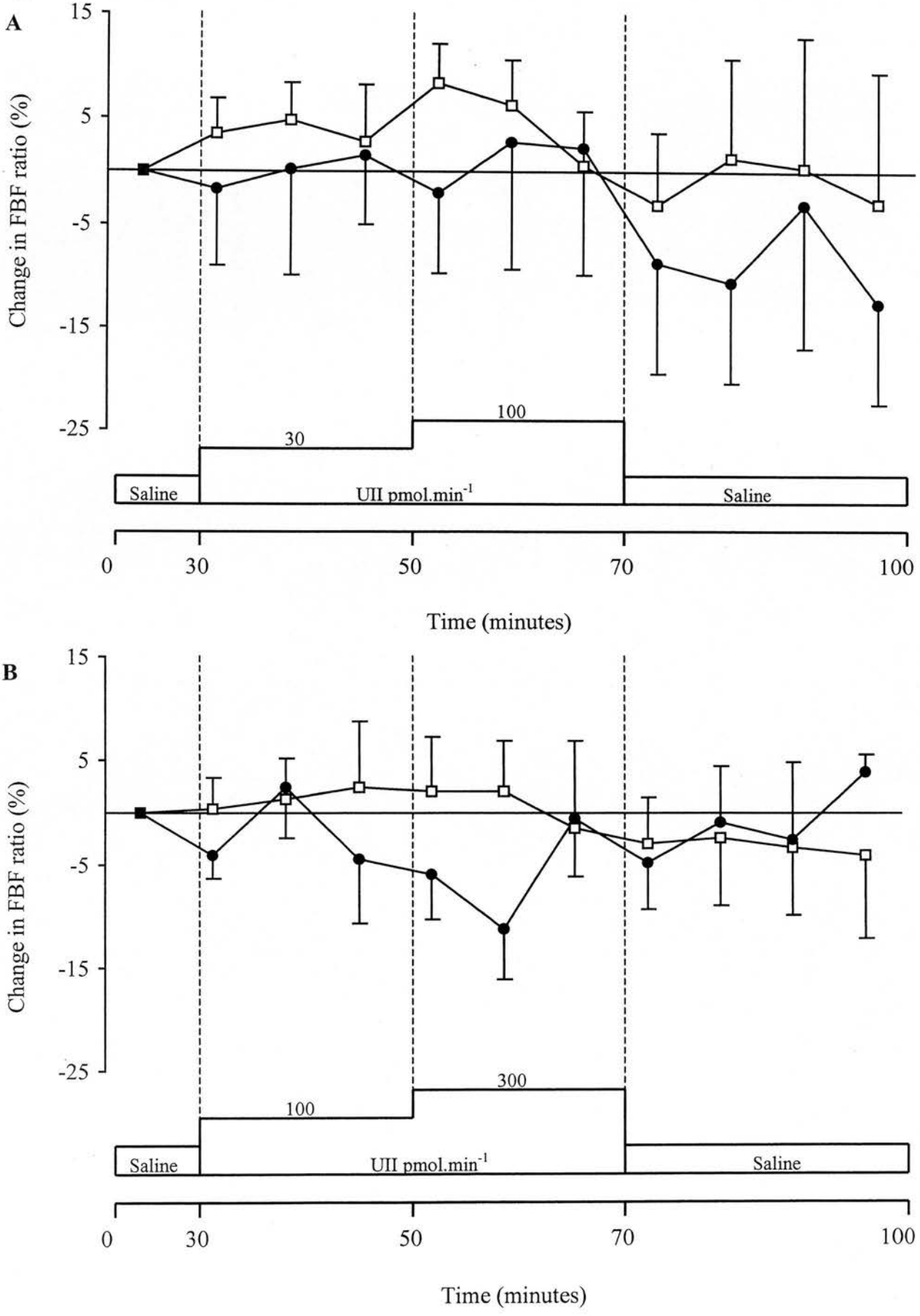

A
15
5
-5
-15
-25
Change in FBF ratio (%)
30
100
Saline
UII pmol.min-1
Saline
0
30
50
70
100
Time (minutes)
B
15
5
-5
-15
-25
Change in FBF ratio (%)
100
300
Saline
UII pmol.min-1
Saline
0
30
50
70
100
Time (minutes)

Table 4.1 A Systemic hemodynamics during UII infusion in study 1.

	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (min^{-1})	*CI* ($L.min^{-1}.m^{-2}$)	*PVR* (arbitrary units)
Saline	136 ± 5	79 ± 3	98 ± 4	63 ± 2	3.4 ± 0.2	29.4 ± 1.9
30 $pmol.min^{-1}$	131 ± 9	81 ± 1	98 ± 3	65 ± 3	3.4 ± 0.1	28.8 ± 1.7
100 $pmol.min^{-1}$	133 ± 7	80 ± 2	98 ± 3	68 ± 4	3.3 ± 0.1	29.7 ± 1.0
Saline	137 ± 8	82 ±4	100 ± 5	66 ± 3	3.2 ± 0.1	31.4 ± 1.3

Results are expressed as mean values ± SEM (n=4). SBP=systolic blood pressure, DBP=diastolic
blood pressure, HR=heart rate, CI=cardiac index, PVR=peripheral vascular resistance.

Table 4.1 B Systemic hemodynamics during UII infusion in study 1.

	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (min^{-1})	*CI* ($L.min^{-1}.m^{-2}$)	*PVR* (arbitrary units)
Saline	124 ± 6	75 ± 6	91 ± 6	63 ± 4	3.7 ± 0.2	24.9 ± 2.5
100 $pmol.min^{-1}$	124 ± 7	77 ± 5	93 ± 5	61 ± 4	3.7 ± 0.2	25.2 ± 2.3
300 $pmol.min^{-1}$	122 ± 6	77 ± 5	92 ± 5	60 ± 3	3.8 ± 0.2	25.6 ± 2.8
Saline	128 ± 5	77 ± 4	94 ± 4	62 ± 3	3.7 ± 0.2	26.2 ± 2.2

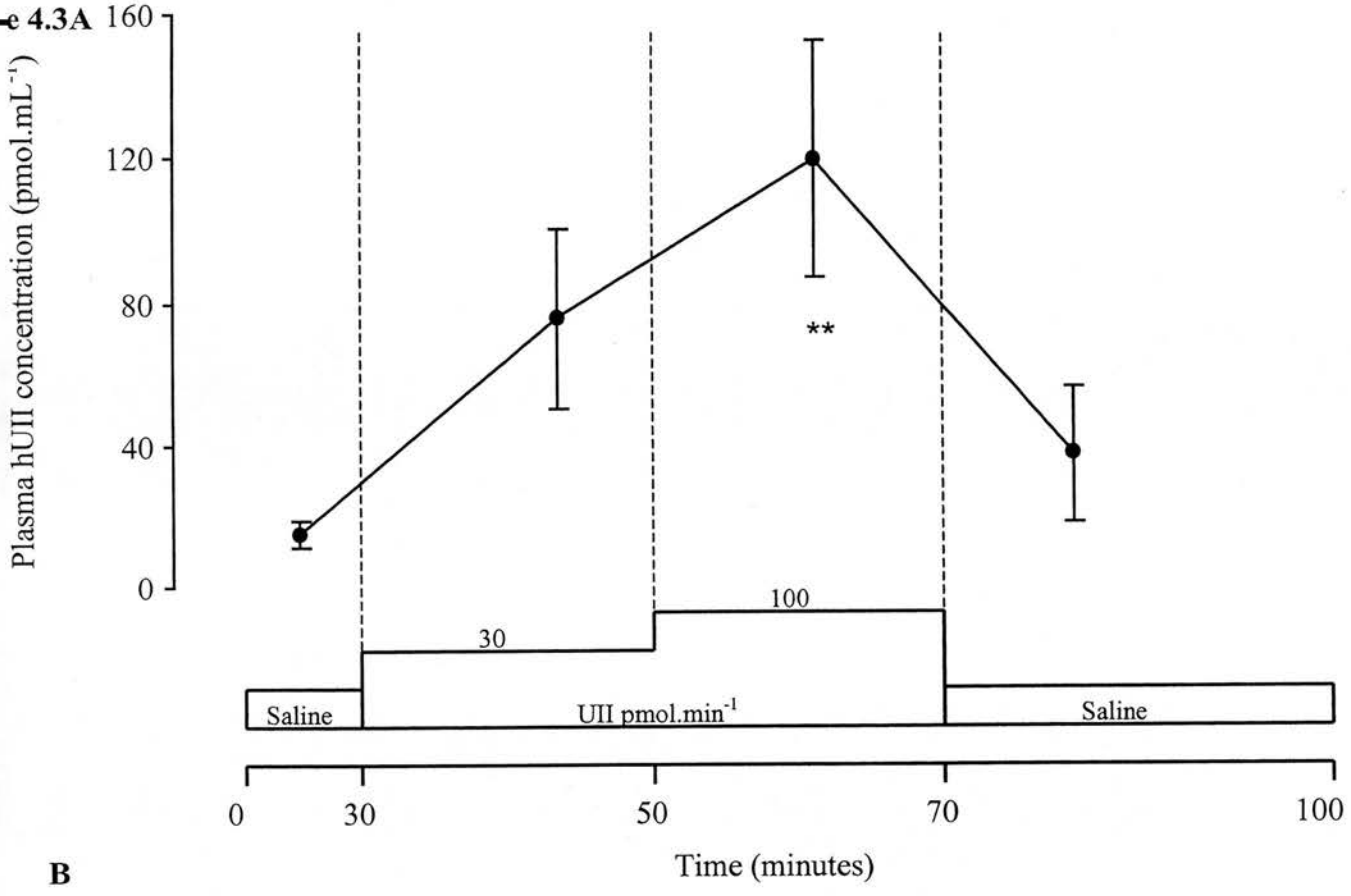
Results are expressed as mean values ± SEM (n=4). SBP=systolic blood pressure, DBP=diastolic blood pressure,
HR=heart rate, CI=cardiac index, PVR=peripheral vascular resistance.

Figure 4.3

A Mean plasma concentration of hUII during 30 and 100 pmol.min^{-1} UII infusion. ** $P<0.01$ compared to baseline saline infusion (n=4).

B Mean plasma concentration of hUII during 100 and 300 pmol.min^{-1} UII infusion. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, Student's *t*-test compared to baseline saline infusion (n=6).

e 4.3A


Plasma hUII concentration (pmol.mL-1)
160
120
80
40
0
**
100
30
Saline
UII pmol.min-1
Saline
0
30
50
70
100
Time (minutes)

B

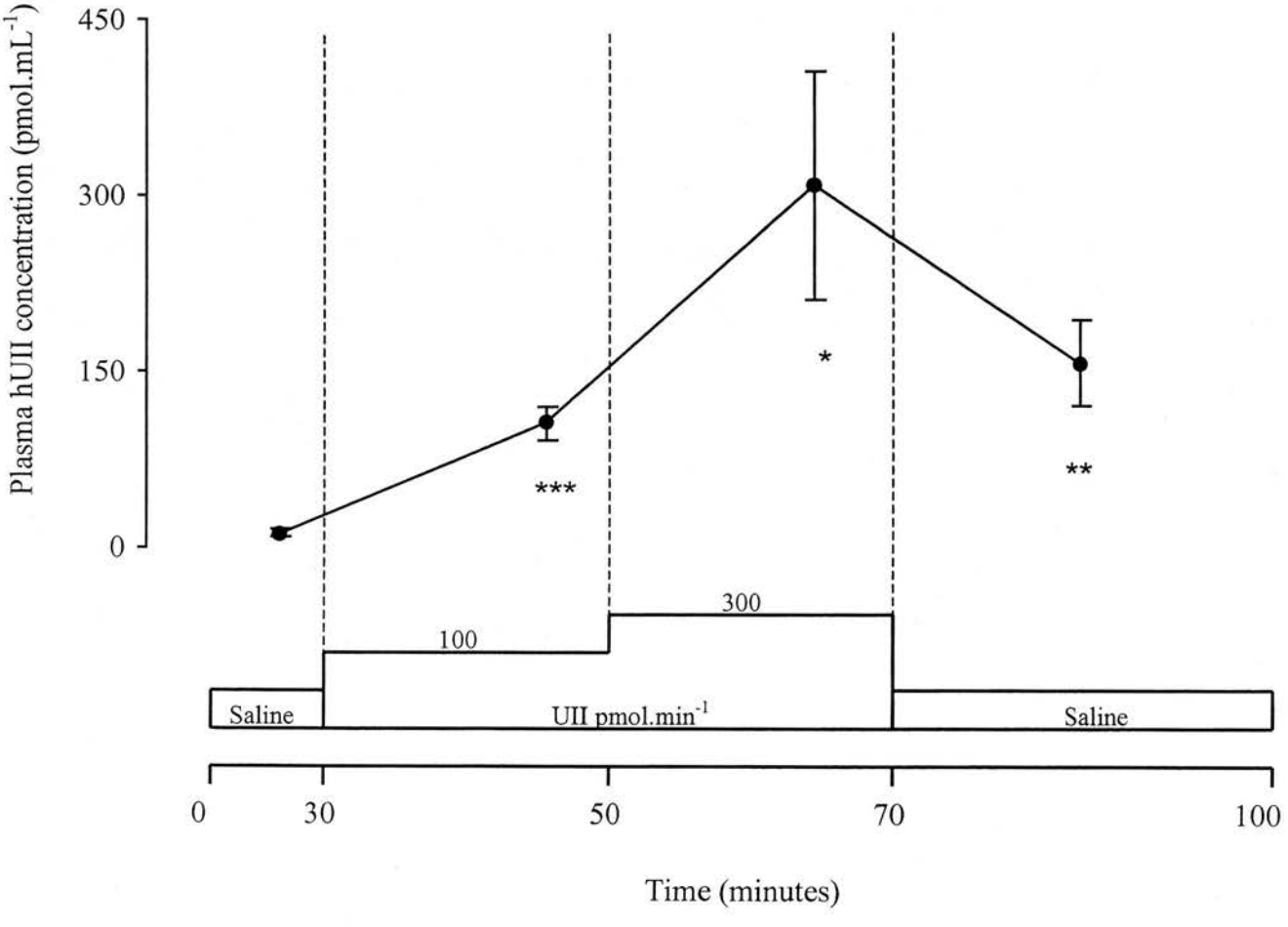

Plasma hUII concentration (pmol.mL-1)
450
300
150
0
***
*
**
300
100
Saline
UII pmol.min-1
Saline
0
30
50
70
100
Time (minutes)

Figure 4.4

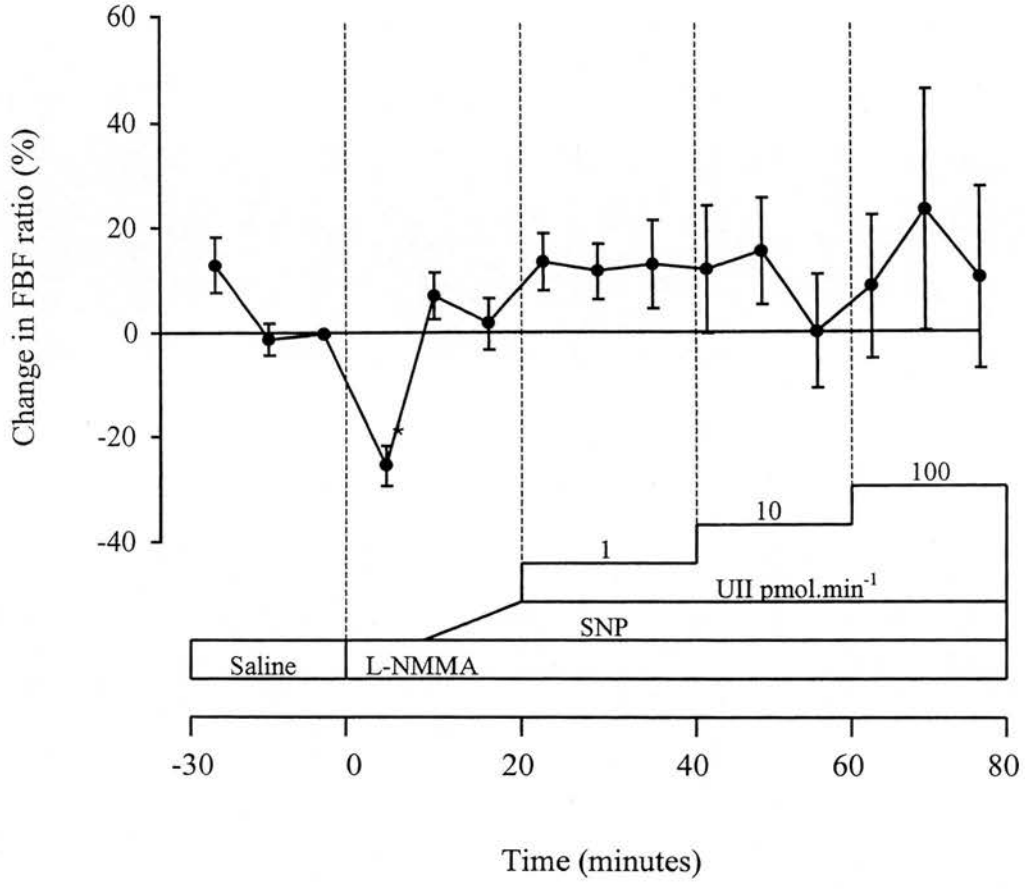


Figure 4.4

Mean percentage change in the FBF ratio after co-infusion of L-NMMA (4μmol.min^{-1}) and co-infusion of UII, * P=0.01 compared to baseline, Student's t-test. ANOVA for UII response was not significant P=0.3 (n=5).

Table 4.2 Percentage change in hand vein diameter during UII infusion with and without co-infusion of L-NMMA in study 3.

	UII 3pmol.min^{-1}	UII 30pmol.min^{-1}	UII 300pmol.min^{-1}	Saline	ANGII 25ng.min^{-1}	Saline	NE 8ng.min^{-1}
Without L-NMMA	0.6 ± 6.9	-0.8 ± 5.5	-0.8 ± 4.4	0.7 ± 2.4	-59.4 ± 8.5***	0.7 ± 4.0	-49.1 ± 10.0**
With L-NMMA	-5.2 ± 3.2	-4.2 ± 3.9	-5.3 ± 5.0	-8.5 ± 4.6	-64.2 ± 15.3**	-25.2 ± 10.8	-70.4 ± 7.1***

Values represent mean percentage change in hand vein diameter ± SEM (n=6), ** $P<0.01$, *** $P<0.001$.

4.5 DISCUSSION

The principal finding in these human studies *in vivo* is that infusion of urotensin II has no effect on arterial or venous tone, or on systemic haemodynamics. In addition, combined inhibition of nitric oxide and prostanoid production did not reveal any vasoactive effects of urotensin II in the forearm arteries or dorsal hand veins. We established the authentic nature of the urotensin II by microsequencing and by showing that it was pharmacologically active *in vitro*. Furthermore, substantial and consistent increases in plasma concentrations of urotensin II confirmed its delivery to the local and systemic circulation.

In isolated human arteries *in vitro*, Maguire *et al.* demonstrated that human urotensin II receptors are present in vascular smooth muscle layers [Maguire *et al* 2000]. In addition, they showed a positive response to urotensin II where the potency of urotensin II in coronary, mammary and radial arteries was 50-fold greater than endothelin-1. However, there were differences in the characteristics of the responses. The maximal responses to endothelin-1 were consistently greater than those to urotensin II, and 30% of the arteries failed to respond to urotensin II, whereas all responded to endothelin-1. Recently, Hillier *et al.* examined a wide range of human arteries and veins of differing calibre *in vitro*, and found no effect of human urotensin II [Hillier *et al* 2001]. The reason for this is not yet clear. Ames *et al.* performed a detailed *in vivo* study of the systemic haemodynamic response to urotensin II in non-human primates [Ames *et al* 1999]. At lower systemic doses of urotensin II, Ames observed positive inotropism,

whereas at higher doses urotensin II induced ischaemic myocardial dysfunction and extreme rises in peripheral resistance. On the basis of early human *in vitro* and the *in vivo* cynomolgus monkey data, we hypothesised that urotensin II would cause constriction of resistance vessels of the human forearm and raise blood pressure. Based on a forearm blood flow of 50mL/min and an infusion rate of 300pmol/min, the estimated local plasma concentration of urotensin II in the infused arm in our study would be 6 nmol/L, similar to those causing vasoconstriction in human *in vitro* studies [Maguire *et al* 2000]. Nevertheless, we found no effect of urotensin II at 300pmol/min for 20 min in either the brachial artery or dorsal hand vein. This contrasts markedly with the local vascular responses in humans to other paracrine and endocrine mediators, such as endothelin-1 and ANGII, both of which cause ~40% reduction in forearm blood flow at only 5pmol/min [Clark *et al* 1989; Haynes and Webb 1994], and suggests that urotensin II does not play an important role in regulating peripheral vascular tone.
Indeed, during intra-arterial infusion of ANGII a 10 fold increases in plasma concentrations of ANGII in the non-infused arm caused mean arterial pressure to rise by ~ 15mmHg [Labinjoh *et al* 2000], whereas 30 fold increases in plasma urotensin II had no effect.

In any negative study it is important to consider the possibility that a real effect on arteriolar tone was missed. This is particularly important given the variability in the responses of isolated human vessels to urotensin II [Maguire *et al* 2000; Stirrat *et al* 2001; Douglas *et al* 2000; Hillier *et al* 2001]. This is unlikely to be the case because there was no suggestion of groups of responders and non-responders from the 15

subjects who received urotensin II over a wide range of doses. In addition, brachial infusion studies are an extremely powerful tool for detecting vasoactive responses, usually requiring no more than 6 subjects to have a high degree of confidence in showing statistically significant effects [Benjamin *et al* 1995].

Previously, Gibson found that fish urotensin II caused endothelium dependent vasodilatation at low dose, prior to vasoconstriction, in rat aortic tissue [Gibson 1987]. This raised the possibility that human urotensin II may induce activation of nitric oxide synthase and subsequent release of nitric oxide. To date, only one *in vitro* study has studied the influence of nitric oxide synthase on responses to urotensin II [MacLean *et al* 2001], using L-N-nitro-arginine methylester (L-NAME) to inhibit nitric oxide synthase in isolated pulmonary vessels [MacLean *et al* 2001]. L-NAME increased maximal responses but not potency of urotensin II in rat main pulmonary artery. L-NAME also enhanced maximal responses to urotensin II in human pulmonary arteries, though only 3 of 10 vessels responded to urotensin II, and then only with very variable contractions. In the current studies, nitric oxide and prostanoid production were inhibited, using standard techniques. Even so, we were unable to unmask vasoconstriction to urotensin II in either human resistance or capacitance vessels *in vivo*.

The lack of response of resistance vessels *in vivo* may be due to low receptor density or poor coupling to signal transduction mechanisms at this site, perhaps as part of inter-species variation. The proximal aorta seems to be most sensitive to urotensin II and it is possible that subtle effects on large arteries are caused by urotensin II but not detected using routine haemodynamic assessment. The *in vivo* effects of urotensin II on human

large arteries merits further investigation. A possible alternative explanation for the lack of effects of urotensin II is high receptor occupancy. Studies with urotensin II antagonists *in vivo* can address this issue, and should allow the physiological role of urotensin II in man to be more clearly defined. In conclusion, we have found no evidence of local or systemic haemodynamic effects of urotensin II *in vivo* despite infusion of urotensin II at doses that increase plasma concentrations 30-fold.

CHAPTER 5

STUDIES OF HAEMODYNAMIC PARAMETERS DURING SYSTEMIC INTRAVENOUS INFUSION OF UROTENSIN II

JT Affolter, IB Wilkinson, MJ Winter, RJ Balment, DE Newby and DJ Webb. No effect on central or peripheral blood pressure of systemic urotensin II infusion in man. *British Journal of Clinical Pharmacology* 2002;**54**:617-621.

5.1 SUMMARY

In rodent and primate studies, urotensin II is an extremely potent vasoconstrictor peptide with effects in the central aortic and arterial vasculature as well as on cardiac function. The aim of the present study was to assess systemic haemodynamic responses to intravenous urotensin II infusion in man. In 10 healthy male volunteers, intravenous urotensin II (3, 30 and 300 pmol/min) and saline placebo were given on separate occasions in a single-blind randomised manner. Systemic haemodynamics and arterial stiffness were assessed by sphygmomanometry, transthoracic bioimpedance, and pulse wave analysis. Plasma urotensin II immuno-reactivity was measured by radio-immunoassay. Intravenous urotensin II infusions were well tolerated with no adverse clinical effects and no electrocardiographic changes. Circulating plasma urotensin II immuno-reactivity increased from baseline of 16 ± 1 to 1460 ± 82 pmol/mL during infusion of urotensin II at 300 pmol/min (P<0.001). However, there were no significant placebo adjusted changes in heart rate (95% confidence intervals: -3.6 to +4.4 /min), mean arterial pressure (-5.8 to +1.7 mmHg) or cardiac index (-0.1 to +0.4 L/min/m^2). There were no changes in augmentation index (-4.1 to +5.2 %) or pulse wave velocity (-1.3 to +0.3 m/s). In conclusion intravenous urotensin II infusion did not affect systemic haemodynamics or arterial stiffness, despite achieving a ~ 100-fold increase in plasma immuno-reactivity. We conclude that urotensin II is unlikely to have a physiological role in the short term regulation of vascular tone or blood pressure in man. Further confirmatory studies with urotensin II receptor antagonists are required.

5.2 INTRODUCTION

Urotensin II is a vasoactive peptide found in the circulation of man and many animal species [Affolter and Webb 2001; Coulouarn *et al* 1998; Ames *et al* 1999; Nothacker *et al* 1999]. In man it has 11 amino acids differentiating it from other species with 12 and 13 amino acids such as the fish and frog [Affolter and Webb 2001]. Urotensin II is the most potent arterial vasoconstrictor yet discovered, having sustained effects in *in vitro* studies in animals [Douglas *et al* 2000]. In addition, it has profound and potentially lethal pressor and vasoconstrictor effects in non-human primates *in vivo* [Ames *et al* 1999].

Human urotensin II was first isolated in man from subgroups of motor neurones in the spinal cord [Coulouarn *et al* 1998]. Outwith the central nervous system, the kidney has the highest expression of human prepro-urotensin II mRNA and this, therefore, appears to be the most likely source of circulating urotensin II in man [Nothacker *et al* 1999]. The distribution of urotensin II receptors has been mapped using immuno-histochemistry, confirming target binding sites in cardiovascular tissues; including coronary arteries, internal mammary arteries and ventricular cardiomyocytes [Ames *et al* 1999; Maguire *et al* 2000]. Thus, it can be considered likely that urotensin II functions as an endocrine hormone with cardiovascular actions [Affolter and Webb].

Both the anatomical location and species appear to dictate the observed vascular response to UII administration [Douglas *et al* 2000]. In the rat, there is a marked

vasoconstrictor response in the proximal aorta with continuous reduction in activity progressively down the arterial tree [Itoh *et al* 1987]. Previous human *in vivo* studies, carried out in our laboratory, show that high concentrations of urotensin II delivered by the intra-brachial route have no effect on local vascular tone in the forearm [Wilkinson *et al* 2002]. This is in contrast to a similar, but not placebo-controlled, study performed recently by Böhm and Pernow [Böhm and Pernow 2002]. However, these studies primarily aimed to assess responses of the resistance arterioles and did not specifically examine the integrated response of the arterial system. This omission may be important because the extreme pressor and myocardial ischaemic responses seen in non-human primates may have resulted from large artery stiffening or vasoconstriction [Ames *et al* 1999].

The arterial pressure waveform alters with progression down the arterial tree. This is due to local variations in vascular stiffness as well as superimposition of the reflected pressure waveform that returns to the central arteries and aorta in diastole [Nichols and O'Rourke 1998]. Augmentation index is dependent on three components: pulse wave velocity, site of wave reflection in the vascular tree and amplitude of the reflected wave. Increased stiffness of small arteries causes an increase in the amplitude of the reflected wave and effectively moves the site of wave reflection proximally. However, increasing large artery stiffness is manifested as a rise in pulse wave velocity. Aortic pulse pressure depends on aortic stiffness and the degree of wave reflection. These stiffness-related effects produce an increase in central aortic pressure and cardiac afterload, and a reduction in coronary perfusion pressure due to the movement of the reflected wave into

systole. By measuring augmentation index a composite measure of central arterial stiffness can be obtained, whereas aortic pulse wave velocity examines the contribution of large arterial stiffness [Nichols and O'Rourke 1998; Wilkinson *et al* 2001; O'Rourke *et al* 2001].

Given the data from studies in non-human primates, we hypothesised that systemic administration of urotensin II would act physiologically as a circulating hormone to increase large arterial stiffness and blood pressure. Our aim was, therefore, to investigate the effects of intravenous urotensin II infusion on a range of systemic haemodynamic parameters including blood pressure and central arterial stiffness, *in vivo* in healthy man.

5.3 METHODS

5.3.1 Subjects

Ten healthy men, mean age 42 ± 4 years (range 22 to 55), were recruited into the study, which was conducted with the approval of the local research ethics committee (Lothian Research Ethics Committee) and the written informed consent of each subject. Subjects abstained from caffeine containing drinks, alcohol and tobacco over the preceding 24 hours and were fasted from midnight prior to the study.

5.3.2 Drugs

Human UII (Peptide Institute, Osaka, Japan) was dissolved in saline (0.9% Baxter Healthcare Ltd., Norfolk, UK) and administered intravenously at 1 mL/min via a constant rate infusion pump (IVAC). Purity and fidelity of human urotensin II from the Peptide Institute was established by high performance liquid chromatography and microsequencing. Biological activity and potency of the human urotensin II peptide was confirmed in the rat proximal aorta (data not shown). Doses used in study protocols were based on our initial studies giving urotensin II via the intra-brachial route [Wilkinson *et al* 2002].

5.3.3 Study design

Augmentation index was determined from the radial artery using the technique of pulse wave analysis (SphygmoCor 2000 version 6.2; PWV Medical PTY Ltd, Sydney, Australia) as previously described [Nichols and O'Rourke 1998]. Pulse wave velocity

was determined using pulse wave analysis (SphygmoCor 2000 version 6.2) combined with electrocardiographic monitoring at the carotid artery (adjacent to the thyroid cartilage) and femoral artery (immediately below the inguinal ligament). The separation of the pulse waveforms was defined as the difference between the distances from the sternal notch to the inguinal ligament and to the thyroid cartilage. All measurements were made in duplicate and mean values used in subsequent data analysis. Recordings with systolic and diastolic variability in excess of 5% were excluded and the measurement repeated. Blood pressure was recorded in the non-infused arm using a validated oscillometric sphygmomanometer (HEM 705CP, Omron, Japan) [O'Brien *et al* 1996]. Cardiac index was assessed using a validated transthoracic electrical bioimpedance technique [Northridge *et al* 1990] (NCCOM3, BoMed Irvine CA, USA). Mean arterial pressure was defined as the diastolic pressure plus a third of the pulse pressure. Systemic vascular resistance index was defined as the mean arterial pressure divided by the cardiac index and then converted from Wood units to dynes.s.m^2/cm^5 on the basis that 1 Wood unit approximates to 80 dynes.s.m^2/cm^5. Throughout the study continuous electrocardiographic monitoring was employed and a 12 lead electrocardiogram (ECG) recorded at baseline, during the last 2 minutes of the 300 pmol/min infusion of urotensin II and at the end of the final saline infusion.

5.3.4 Systemic intravenous infusion protocol

Each subject attended on two occasions at least one week apart and received an initial 30 min saline infusion during which baseline recordings were performed at 15, 22 and 25 min. Baseline bloods and ECG were obtained at 28 min, just prior to urotensin II or

placebo infusion. This was followed by a single blind randomised administration of either urotensin II (3, 30 and 300 pmol/min for 20 min at each dose) or saline for one hour, before a final 30 min saline infusion. Blood pressure, heart rate, cardiac index and augmentation index were recorded at 5 and 12 min, pulse wave velocity at 15 min, and blood samples obtained and a 12 lead electrocardiogram taken at 18 min of each infusion period.

5.3.5 Plasma urotensin II concentrations

Venous blood (10 mL) was drawn during the last 2 min of each infusion period from a cannula sited in the non-infused arm. Samples were collected into ethylene diamine tetra-acetic acid, immediately centrifuged at 3,000 *g* for 10 min at 4°C and the plasma stored at -80°C until subsequent analysis. Plasma levels of urotensin II immuno-reactivity were determined using an acetic acid extraction technique and radio-immunoassay, with rabbit anti-flounder urotensin II, as described previously [Wilkinson *et al* 2002; Winter *et al* 1999] and are expressed in the results as pmol/mL.

5.3.6 Statistical analysis

All results are expressed as mean ± SEM. The AIx and PWV values represent change from baseline. Data were analysed using ANOVA with repeated measures. Statistical significance was taken at the 5% level. Previous studies carried out in our department using noradrenaline infusions had 98% power to detect a change of 7% in 8 volunteers at a significance level of 0.05 [Wilkinson *et al* 2001].

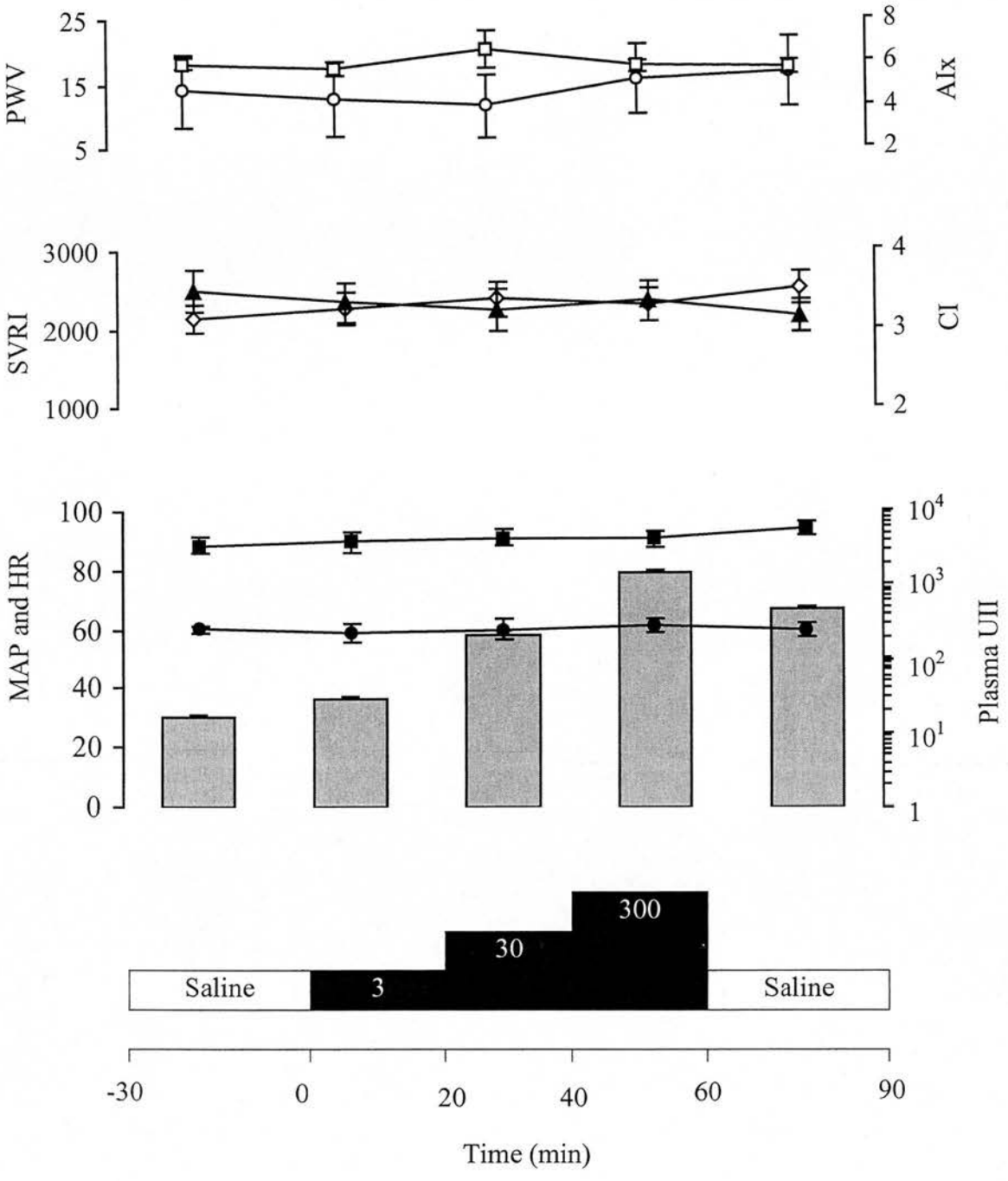
5.4 RESULTS

Baseline heart rate, cardiac index, blood pressure, augmentation index, pulse wave velocity and plasma urotensin II concentrations were similar on the two study days. The baseline augmentation index and pulse wave analysis raw data recordings were 13 ± 4% and 5.9 ± 0.3 m/sec respectively. Both values were consistent with other healthy subjects from our local population with similar demographics. All subjects were symptom-free throughout the studies: specifically, there were no reports of chest pain, headache or abdominal pain. There were also no changes in continuous single lead cardiac monitoring and 12 lead electrocardiograms throughout the studies. There were no significant changes in systemic haemodynamic parameters or central aortic stiffness during either saline placebo or urotensin II infusion (Figure 5.1). At the highest infusion rate of 300 pmol/min, plasma urotensin II immuno-reactivity increased 91-fold (16 ± 1 to 1460 ± 82 pmol/mL) in the systemic venous plasma (n=10; $P<0.001$). Despite this, there were no significant placebo adjusted changes in heart rate (0.4, -3.6 to +4.4 /min: mean difference, 95% confidence interval), mean arterial pressure (-2.0, -5.8 to +1.7 mmHg), cardiac index (0.2, -0.1 to +0.4 L/min/m^2) and systemic vascular resistance index (-160, -396 to +76 dynes.s.m^2/cm^5). Moreover, arterial stiffness was unaffected, with no demonstrable alterations in augmentation index (+0.5, -4.1 to +5.2 %) or pulse wave velocity (-0.5, -1.3 to +0.3 m/s).

Figure 5.1.

Mean pulse wave data, haemodynamic parameters (displayed as lines) and plasma urotensin II concentration (UII; displayed as histogram; pmol/mL) during intravenous urotensin II infusion. Mean augmentation index (AIx; ■; %), pulse wave velocity (PWV; □; m/sec), cardiac index (CI; ○; L/min/m^2), systemic vascular resistance index (SVRI; □; dynes.s.m^2/cm^5), mean arterial pressure (MAP; ●; mmHg), and heart rate (HR; ▲; beats/min).

Figure 5.1


PWV
25
15
5
AIx
8
6
4
2
SVRI
3000
2000
1000
CI
4
3
2
MAP and HR
100
80
60
40
20
0
Plasma UII
10^4
10^3
10^2
10^1
1
300
30
3
Saline
Saline
-30
0
20
40
60
90
Time (min)

5.5 DISCUSSION

This is the first *in vivo* study of which we are aware in which systemic intravenous administration of urotensin II has been used to increase circulating peptide concentrations in man. There were no demonstrable effects of urotensin II on systemic haemodynamics or arterial stiffness, although plasma urotensin II immuno-reactivity increased by nearly 100-fold. This contrasts with the modest 2-fold elevation of plasma urotensin II in renal disease [Totsune *et al* 2001].

Our findings contrast with *in vivo* studies in non-human primates, where urotensin II caused potent pressor and vasoconstrictor effects [Ames *et al* 1999]. Moreover, the *in vivo* human studies done by Böhm and Pernow and ourselves, an intra-arterial urotensin II infusion of 300 pmol/min did not alter systemic blood pressure [Wilkinson *et al* 2002; Nichols and O'Rourke 1998], an intra-arterial infusion rate that we demonstrated to raise systemic plasma urotensin II immuno-reactivity by 30-fold [Wilkinson *et al* 2002]. Such an increase is not always sufficient to cause peripheral haemodynamic effects, as can be seen with vasopressin, which requires a 10 to 100-fold rise in plasma concentrations [Landry and Oliver 2001; Aylward *et al* 1986]. *In vitro* studies have shown that the rat aorta is highly responsive to urotensin II, particularly in its proximal region [Douglas *et al* 2000; Maguire *et al* 2000]. However, against the reproducible *in vitro* pharmacological response to urotensin II in cardiovascular tissues from animals, findings reported in human *in vitro* studies are inconsistent [Affolter and Webb 2001; Hillier *et al* 2001]. Our results may reflect a fundamental difference in species response,

although the *in vitro* human aortic response is currently unknown. As it appears likely that urotensin II is an endocrine hormone with receptors located in human cardiovascular tissues, the question remains as to its function in human vascular physiology.

We have previously demonstrated dose-dependent increases in augmentation index with intravenous infusion of pressor hormones, including angiotensin II and norepinephrine [Wilkinson *et al* 2001]. Moreover, intravenously infused peptides, such as angiotensin II and endothelin-1, cause a significant rise in mean arterial blood pressure for only a 2 and 3-fold rise in plasma concentrations respectively [Motwani and Stuthers 1992; Kaasjager *et al* 1995]. In the present study, we administered 300 pmol/min (total dose of 85 pmol/kg) of urotensin II for 20 minutes and achieved a 91-fold increase in plasma urotensin II immuno-reactivity. This was associated with no symptoms, no electrocardiographic changes, and no alterations in systemic haemodynamic parameters. When given intravenously, urotensin II caused profound ischaemic electrocardiographic changes in non-human primates in association with cardiac dysfunction and even death [Ames *et al* 1999]. Ames *et al* used doses of urotensin II up to 3000 pmol/kg and reported that doses < 30 pmol/kg increased cardiac output, while doses > 30 pmol/kg increased vascular resistance and decreased myocardial function [Ames *et al* 1999]. However, due to safety concerns, we did not use either bolus injections or the higher doses of urotensin II that were used in the non-human primate *in vivo* studies. It may be the case that urotensin II has a role in cardiovascular regulation in man that is not addressed directly in our studies. For instance, it has recently been suggested that

urotensin II may influence atherogenesis by augmenting the mitogenic activity of sub-fractions of oxidised low density lipoprotein [Watanabe *et al* 2001] and even that urotensin II might have a role in the regulation of insulin release [Silvestre *et al* 2001].

In conclusion, we have observed no change in arterial stiffness or systemic haemodynamic parameters, including blood pressure in response to intravenous urotensin II infusion *in vivo* in man despite a nearly 100-fold increase in plasma urotensin II immuno-reactivity. These findings indicate that urotensin II is unlikely to have a major physiological role in the regulation of vascular tone and blood pressure in man. Further confirmatory studies using urotensin II receptor antagonists will be required before firm conclusions can be drawn about the possible role of urotensin II in human vascular physiology and disease.

CHAPTER 6

INTRA-ARTERIAL VASOPRESSIN IN THE HUMAN FOREARM: PHARMACODYNAMICS, REPRODUCIBILITY AND THE ROLE OF NITRIC OXIDE

JT Affolter, SP McKee, AH Salem, R Jones, DE Newby, DJ Webb. Intra-arterial vasopressin in the human forearm: pharmacodynamics, reproducibility and the role of nitric oxide. *Clinical Pharmacology and Therapeutics* 2003;74:9-16.

6.1 SUMMARY

Diverse vascular effects have been ascribed to vasopressin, including the potential to cause vasodilatation, vasoconstriction and nitric oxide release. The study aims were to establish the pharmacodynamics, reproducibility and nitric oxide dependence of its vasomotor actions in the forearm resistance vessels. Blood flow was measured in both forearms of 12 healthy men using venous occlusion plethysmography. Continuous and discontinous doses of intra-brachial vasopressin (1-300 pmol/min) were administered. To assess the contribution of nitric oxide, vasopressin was co-administered with the 'nitric oxide clamp', a balanced co-infusion of L-N^G-monomethylarginine (a nitric oxide synthase inhibitor) and sodium nitroprusside (an exogenous nitric oxide donor) to block endogenous nitric oxide production and restore normal basal blood flow respectively. Vasopressin produced dose-dependent and biphasic change in blood flow with a maximum reduction of 22±5% at 3 pmol/min ($P<0.01$) and increase of 80±30% at 300 pmol/min ($P<0.01$). There were no significant differences in repeated responses obtained either within or between days. Repeated discontinuous dosing did not change the magnitude of the maximum vasoconstriction or vasodilatation, but prolonged continuous infusions produced maximal vasodilatation at 12 min that subsequently underwent substantial tachyphylaxis ($P=0.04$). Although there was no augmentation of vasoconstriction, the 'nitric oxide clamp' abolished vasopressin induced vasodilatation ($P<0.05$). Intra-arterial vasopressin causes a reproducible dose-dependent biphasic change in forearm blood flow. Vasomotor responses are time-dependent with a modest

delay to peak vasodilatation and tachyphylaxis with prolonged sustained infusions. Nitric oxide release provides a major contribution to vasopressin induced vasodilatation but does not directly oppose low dose vasopressin induced vasoconstriction.

6.2 INTRODUCTION

Arginine vasopressin is an endocrine hormone that originates from the posterior pituitary and is primarily secreted in response to osmotic stimuli and hypotension. Outwith the central nervous system, it has physiological effects on arteries and renal collecting ducts to produce vasomotor and anti-diuretic effects respectively [Landry and Oliver 2001]. These actions play a role in the pathophysiology of conditions, such as heart failure and cirrhosis, where high plasma vasopressin concentrations are associated with fluid retention. The development of selective and non-selective vasopressin receptor antagonists may provide a novel therapeutic approach in the treatment of these conditions [Burrell *et al.* 2000]. The physiological study of such antagonists, in local vascular models in man, necessitates insight into vasopressin responses in the chosen study model. To date, studies using the robust methodology of forearm plethysmography and intra-arterial drug infusion, have focused only on dose response relationships and not addressed issues such as onset and offset of effects, the potential for tachyphylaxis or, reproducibility.

The actions of vasopressin are mediated through 3 G-protein coupled receptors: the V1, V2 and V3 receptors. Only the V1 and V2 receptors appear to be present in vascular tissue and these are linked to differing second messenger systems, namely phospholipase C and adenylate cyclase respectively. The V1 receptor is present on vascular smooth muscle and mediates arterial vasoconstriction [Hirsch *et al.* 1989, Imaizumi *et al.* 1992]. Although the physical presence of extra-renal V2 receptors in vascular smooth muscle or

endothelium has yet to be demonstrated by either radio-ligand binding or mRNA expression [Philips *et al* 1990], there is strong pharmacological evidence for their existence. Intra-arterial infusion of desmopressin, a synthetic V2 receptor agonist, and high dose vasopressin both cause vasodilatation in the human forearm [Hirsch *et al* 1989] that can be abolished by selective V2 antagonism [Tagawa *et al.* 1995]. In addition, patients with congenital diabetes insipidus due to a genetic defect in the V2 receptor do not vasodilate with intra-arterial desmopressin and demonstrate only vasoconstriction with vasopressin [Van Lieburg *et al.* 1995]. *In vivo* evidence, using superseded methods, suggested that vasopressin-induced vasodilatation is mediated by the release of nitric oxide [Tagawa *et al.* 1993; Van Lieburg *et al.* 1995]. Furthermore, it has been proposed that V2 receptor mediated vasodilatation to vasopressin has a greater dependence on nitric oxide release than that associated with desmopressin [Van Lieburg *et al.* 1995].

Vasopressin causes profound vasoconstriction of human mesenteric arteries *in vitro* [Ohlstein *et al.* 1986]. However, intravenous vasopressin infusion causes only modest systemic hemodynamic effects in healthy volunteers [Aylward *et al.* 1986]. Examination of the direct vascular responses to vasopressin using intra-arterial infusion and venous occlusion plethysmography has demonstrated variable responses, finding both vasoconstriction and vasodilatation [Susuki *et al.* 1989] or vasodilatation alone [Hirsch *et al.* 1989]. Moreover, intra-brachial vasopressin can simultaneously cause an increase in total limb blood flow but skin pallor by means of a reduction in forearm skin

blood flow [Hayoz *et al.* 1997]. Thus, the vasomotor actions of vasopressin appear to vary with both dose and vascular bed affected.

The aim of the present study was to describe the forearm vascular actions of vasopressin *in vivo* in man to facilitate studies with vasopressin antagonists. We wished to establish the pharmacodynamics and reproducibility of responses to intra-brachial vasopressin, and to assess more reliably the contribution of nitric oxide to its vasomotor actions.

6.3 METHODS

6.3.1 Subjects

These studies were conducted with the approval of the local research ethics committee and the written informed consent of each subject. The investigations conformed to the principles of the Declaration of Helsinki. Twelve healthy men, mean age 42 ± 4 years (range 23-64), were recruited. Subjects were fasted from midnight before each study (water consumption was not restricted) and abstained from caffeine containing drinks, alcohol and smoking for the preceding 24 hours.

6.3.2 Drugs

Arginine vasopressin (Pitressin™, Goldshield, U.K.), sodium nitroprusside (SNP; David Bull Laboratories, Warwick, UK) and L-N^G-monomethylarginine (L-NMMA; Clinalfa, Läufelfingen, Switzerland) were aseptically prepared and dissolved in saline (0.9% Baxter Healthcare Ltd., Norfolk, UK).

6.3.3 Study design

To ensure a stable baseline and maintain cannula patency, saline was infused for 30 min before drug infusion protocols were commenced. The total rate of infusion was kept constant at 1 mL/min throughout all studies. Forearm blood flow was measured simultaneously in both arms by venous occlusion plethysmography [Whitney 1953; Benjamin *et al.* 1995], as previously described [Wilkinson and Webb 2001]. Forearm blood flow was measured over the last 3 min of every 6 min period. In protocols 1 and

3, dose infusion was continued for 4 further min to allow blood pressure and pulse recordings using a validated oscillometric sphygmomanometer (HEM 705CP, Omron Japan) [O'Brien *et al.* 1996]. The last five plethysmographic recordings of forearm blood flow were averaged to determine flow in each arm. Blood pressure and heart rate were recorded over the brachial artery in the non-infused arm. Mean arterial pressure was defined as diastolic pressure plus 1/3 of the pulse pressure.

6.3.4 Protocol 1. Dose-response of vasopressin

On two occasions, at least one week apart, each of 8 subjects received a 30 min saline infusion followed by either vasopressin or saline placebo in a randomised cross-over manner. Vasopressin was infused at 1, 3, 10, 30, 100, 300 pmol/min for 10 min at each dose.

6.3.5 Protocol 2. On and offset of vasopressin action

Based on the results of *protocol 1*, eight subjects attended on two occasions separated by at least a week and received a 24 min infusion of saline followed by either 3 or 300 pmol/min of vasopressin. Subjects received 3 separate infusions of intra-arterial vasopressin for 6, 12 and 18 min, each separated by 24 min of saline infusion. Forearm blood flow recordings were made every 6 min throughout the study. The same subjects re-attended a further occasion and received 48 min of 300 pmol/min intra-arterial vasopressin followed by 48 min of saline.

6.3.6 Protocol 3. Reproducibility

To determine within-day and between-day reproducibility of the vasopressin responses, eight subjects attended on two occasions. Subjects received a 30 min infusion of saline followed by vasopressin at 3, 30 and 300 pmol/min followed by saline for 30 min before receiving repeated infusion of vasopressin at the same doses.

6.3.7 Protocol 4. Vasopressin and the 'nitric oxide clamp'

Six subjects, who originally took part in *protocol 1* participated in a 'nitric oxide clamp' [Verhaar *et al.* 1998]. After 30 min saline infusion L-NMMA was infused intra-arterially at 4 μmol/min to block endogenous nitric oxide generation [Vallance *et al.* 1989] and forearm blood flow was restored to within 10% of baseline by a titrated co-infusion of sodium nitroprusside, an endothelium independent nitric oxide donor (mean dose 0.5 nmol/min, range 0.3-0.8). On restoration of forearm blood flow vasopressin was co-infused at 3, 30 and 300 pmol/min, each for 18 min.

6.3.8 Statistical analysis

All results are expressed as mean ± SEM. Data for forearm blood flow have been expressed as a percentage change from baseline forearm blood flow ratio (the quotient of infused arm and non-infused arm blood flow) [Benjamin 1995]. To assess the offset of vasopressin response in *protocol 2*, the rate of decline in blood flow response per minute was calculated for each response. In *protocol 3*, within-day and between-day reproducibility was assessed using the method of Bland and Altman [Bland and Altman, 1986] and coefficients of repeatability were determined for 95% confidence intervals

using the Student's *t* distribution. The Bland and Altman method considers the reproducibility between repeated measurements of the same subjects, using the same method on different occasions. The coefficient of reproducibility is then calculated as 1.96 times the standard deviation of the differences between the two measurements. Data were analysed using ANOVA and Student's paired *t*-test as appropriate. Statistical significance was taken at $P<0.05$.

6.4 RESULTS

Subjects were caucasian and had a mean age of 42 ± 4 years (range 23-64), mean body mass index of 24 ± 0.5 kg/m^2 (range 21-26) and a mean arterial pressure 85 ± 3 mmHg (range 74-100). All subjects were symptom free and the studies were well tolerated with no significant side effects. Throughout all studies there were no significant changes in non-infused forearm blood flow, mean arterial pressure or heart rate (ANOVA P=0.7, P=0.9, P=0.5 respectively).

6.4.1 Protocol 1. Dose-response of vasopressin

Baseline non-infused and infused forearm blood flows were 3.8 ± 0.7 and 3.7 ± 0.7 mL/100 mL tissue/min respectively. Although unchanged during placebo infusion, forearm blood flow demonstrated a dose-dependent biphasic response to vasopressin (Figure 6.1; ANOVA P<0.001) with a 22.5 ± 4% and 22.5 ± 11% reduction in blood flow during 3 and 10 pmol/min respectively (t-tests, P=0.006 and 0.03 respectively) and an 80 ± 30% increase during 300 pmol/min (t-test, P=0.006). Maximal vasodilatation responses were observed for all 8 subjects during 300 pmol/min, however, maximal vasoconstriction was observed in 4 subjects at 10 pmol/min.

6.4.2 Protocol 2. Onset and offset of vasopressin action

Baseline non-infused and infused forearm blood flows were 2.7 ± 0.3 and 2.7 ± 0.4 mL/100 mL tissue/min respectively. Infusion of repeated doses of 3 pmol/min vasopressin caused a reduction in forearm blood flow of 21 ± 4, 12 ± 4 and 17 ± 5%

(Figure 6.2: P=0.02, 0.01 and 0.003 respectively). Infusion of repeated doses of 300 pmol/min vasopressin increased forearm blood flow by 119 ± 32, 103 ± 21 and 119 ± 31% (Figure 6.2: P=0.01, <0.001 and 0.01 respectively). A consistent maximal increase in forearm blood flow was achieved at 12 min after starting vasopressin infusion regardless of dose duration. Continued infusion of vasopressin from 6 to 12 and 18 min significantly slowed the offset of vasodilatation from 10 ± 1.5 to 7 ± 0.7 and 5 ± 0.3%/min respectively (ANOVA, P=0.001). Although there is a suggestion of a similar trend for the vasoconstrictor effect this did not reach statistical significance (ANOVA, P=0.2).

During prolonged infusion of 300 pmol/min vasopressin, % change in forearm blood flow ratio increased to a maximum of 126 ± 42% after 12 min (Figure 6.3: ANOVA, P<0.001) and fell to 39 ± 15% by 48 min (t-test, P=0.04). After cessation of vasopressin infusion, % change in forearm blood flow ratio remained consistently below baseline flow (t-test, P=0.03).

6.4.3 Protocol 3. Reproducibility

Baseline non-infused and infused forearm blood flows were 3.4 ± 0.6 and 3.6 ± 0.7 mL/100 mL tissue/min respectively. There was good reproducibility of the vasopressin vasomotor responses with no significant differences in within- or between-day responses (Table 6.1).

6.4.4 Protocol 4. Vasopressin and the 'nitric oxide clamp'

Baseline non-infused and infused forearm blood flows were both 2.5 ± 0.2 mL/100 mL tissue/min. Infusion of L-NMMA reduced forearm blood flow ratio by 30 ± 3% (Figure 6.4: ANOVA, P=0.002) and co-infusion of sodium nitroprusside (mean dose 0.5 nmol/min, range 0.3 to 0.8) restored baseline blood flow (2.5 ± 0.2 compared to 2.5 ± 0.2 ml/100ml tissue/min, P=0.9). Subsequent co-infusion of 3 and 30 pmol/min vasopressin reduced forearm blood flow ratio by 19 ± 5 and 22 ± 4% (P=0.03 and P=0.002 respectively), although blood flow returned to baseline during 300 pmol/min vasopressin (P=0.5).

Table 6.1

Within day and between day reproducibility for forearm blood flow responses to 3, 30 and 300 pmol/min vasopressin. Vasoconstrictor responses have been expressed as % change in ratio and the vasodilator responses as a % change in infused arm forearm blood flow [Wilkinson and Webb 2001].

	Within day responses			*Between day responses*		
Vasopressin (pmol/min)	*Mean % change in blood flow*	*Mean of the differences*	*Coefficient of repeatability*	*Mean % change in blood flow*	*Mean of the differences*	*Coefficient of repeatability*
3	-22	6	12	-23	6	12
30	-27	7	17	-28	16	32
300	81	15	39	99	14	27

Figure 6.1

Percentage change in forearm blood flow during vasopressin 1, 3, 10, 30, 100, 300 pmol/min (●) and saline placebo infusion (○). * $P<0.05$, ** $P<0.01$, Student's *t*-test compared to saline placebo infusion.

Figure 6.1

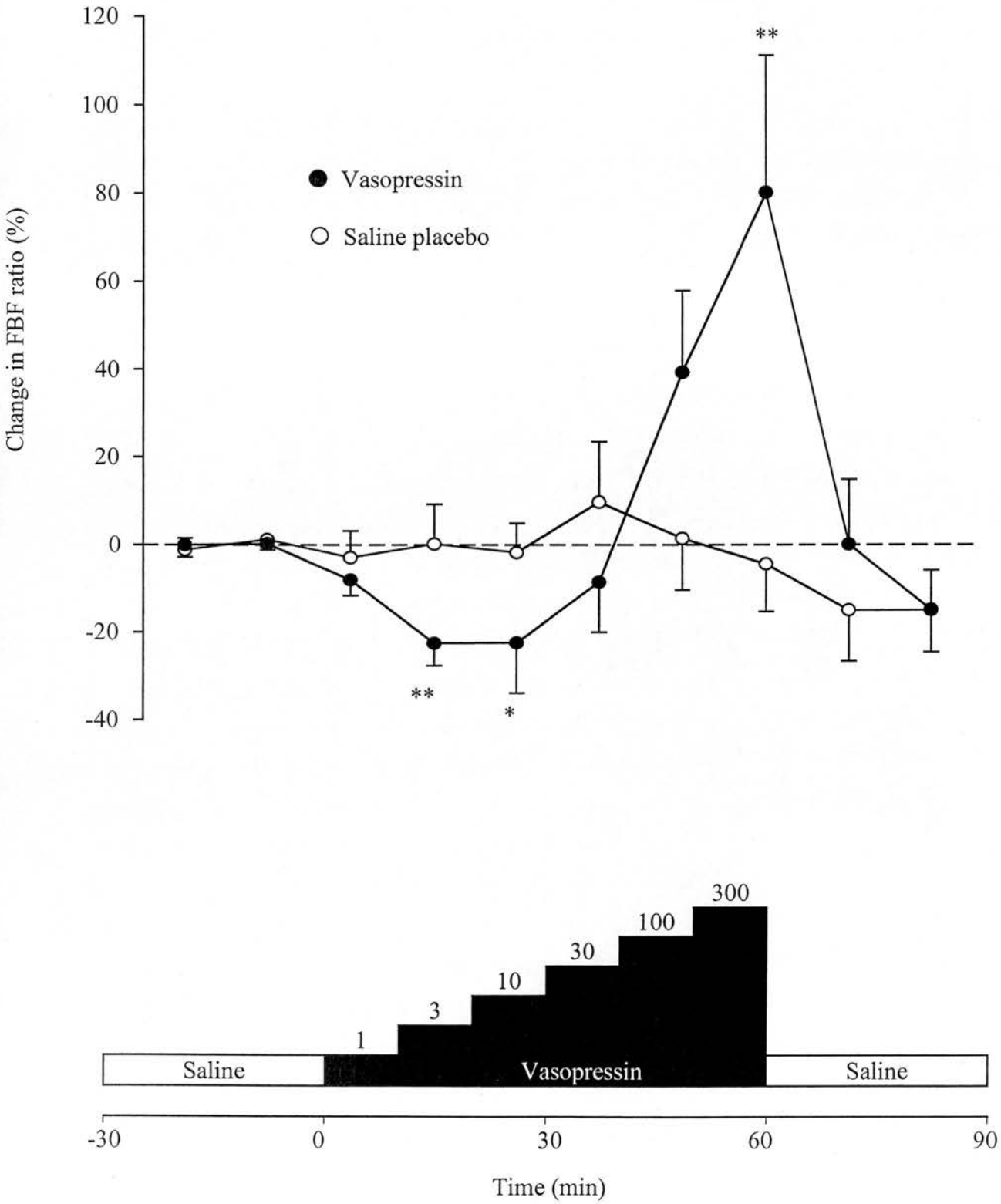

120
100
80
60
40
20
0
-20
-40
Change in FBF ratio (%)
Vasopressin
Saline placebo
**
**
*
300
100
30
10
3
1
Saline
Vasopressin
Saline
-30
0
30
60
90
Time (min)

Figure 6.2

Percentage change in forearm blood flow during infusion of vasopressin for 6, 12 and 18 min of either 3 (●) or 300 (○) pmol/min. * $P<0.05$, ** $P<0.01$, Student's *t*-test compared to saline baseline.

Figure 6.2

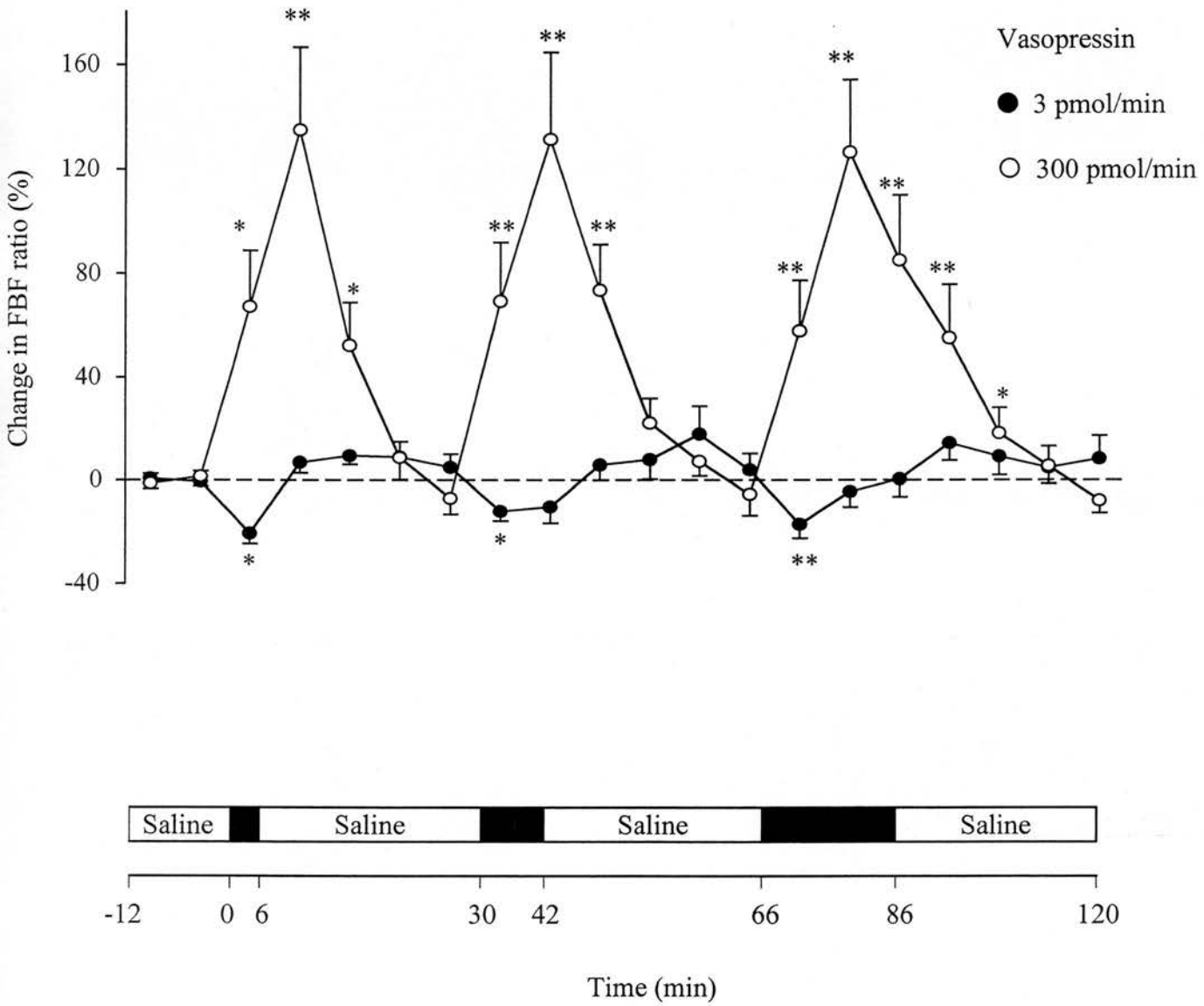

Vasopressin
3 pmol/min
300 pmol/min
Change in FBF ratio (%)
160
120
80
40
0
-40
Saline
Saline
Saline
Saline
-12
0
6
30
42
66
86
120
Time (min)

Figure 6.3

Percentage change in forearm blood flow during infusion of 48 min of 300 pmol/min AVP infusion. * $P<0.05$, ** $P<0.01$, Student's *t*-test compared to saline baseline. Annotated points, peak effect versus last time point during vasopressin infusion, Student's *t*-test $P=0.04$.

Figure 6.3

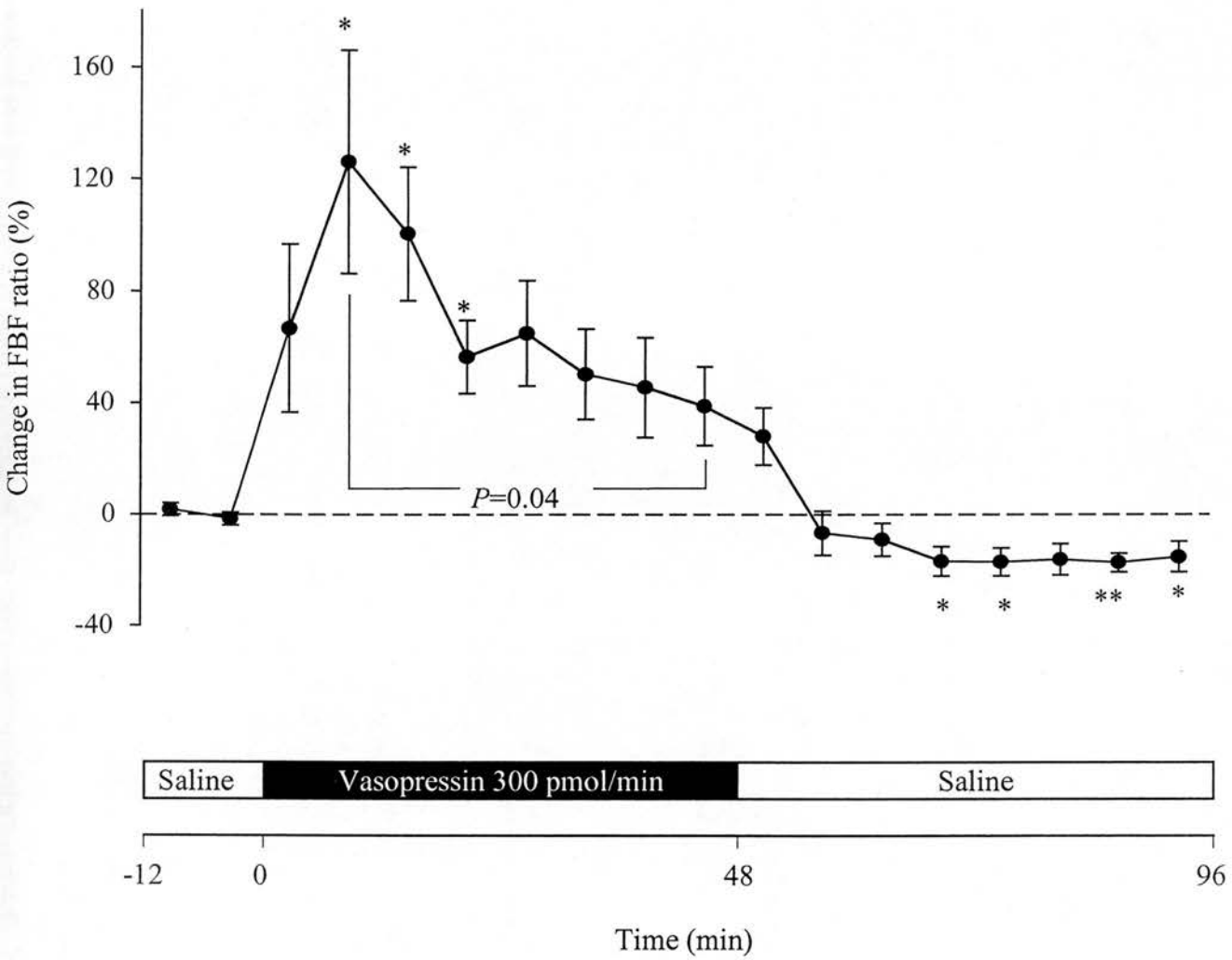
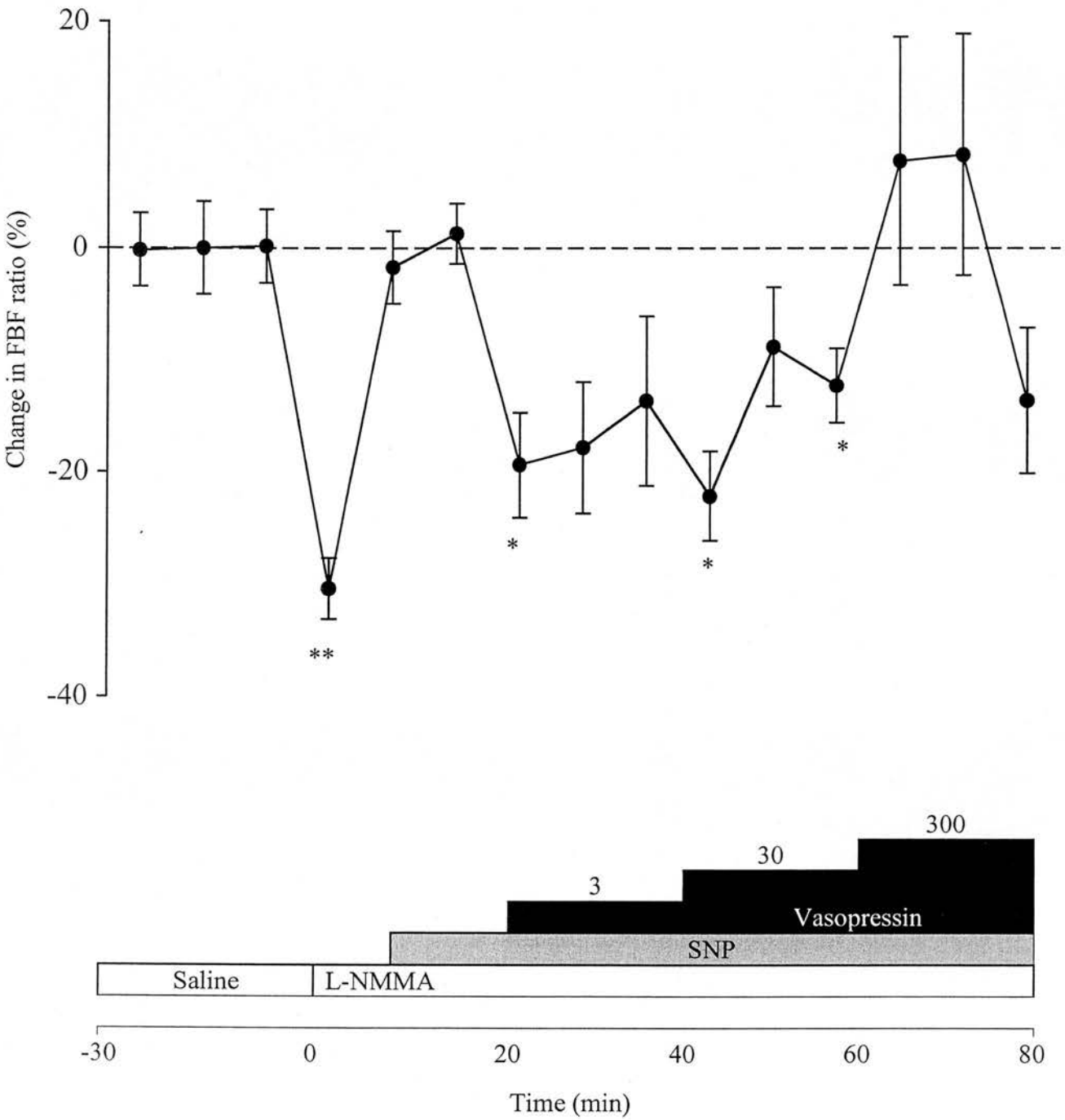

Change in FBF ratio (%)
160
120
80
40
0
-40
*
*
*
P=0.04
*
*
**
*
Saline
Vasopressin 300 pmol/min
Saline
-12
0
48
96
Time (min)

Figure 6.4

Percentage change in forearm blood flow during 3, 30 and 300 pmol/min vasopressin in the presence of the 'nitric oxide clamp'. ** $P<0.01$ compared to saline baseline, Student's *t*-test. * $P<0.05$, Student's *t*-test compared to post SNP baseline (n=6).

Figure 6.4


20
0
-20
-40
Change in FBF ratio (%)
**
*
*
*
300
30
3
Vasopressin
SNP
Saline
L-NMMA
-30
0
20
40
60
80
Time (min)

6.5 DISCUSSION

We have shown that intra-brachial vasopressin infusions are well tolerated, reproducible and cause a dose-dependent biphasic blood flow response with modest vasoconstriction at lower doses (3-30 pmol/min) and substantial vasodilatation at higher doses (>100 pmol/min). The vasomotor response is time-dependent with a modest delay to peak vasodilatation and tachyphylaxis with prolonged sustained infusions. Nitric oxide release provides a major contribution to vasopressin induced vasodilatation but does not directly oppose low dose vasopressin induced vasoconstriction.

Our findings are consistent with previous clinical studies using comparable doses of vasopressin [Suzuki *et al.* 1989, Tagawa *et al* 1993, Tagawa *et al* 1995] that have demonstrated a similar magnitude of vasopressin induced vasoconstriction (28% reduction in flow) and vasodilatation (126% increase in flow). It is likely that vasoconstriction is mediated by a direct action on vascular smooth muscle V1 receptors [Imaizumi *et al* 1992] and that vasodilatation is mediated through vascular V2 receptors. However, receptor affinities for vasopressin do not readily explain the dose-dependent biphasic response since, in comparison to the V1 receptor, vasopressin has near double the affinity for the V2 receptor [Thibonnier *et al* 1998]. In addition, very small changes in plasma vasopressin concentrations have renal V2 receptor mediated anti-diuretic effects and, in contrast to renal responses, vasodilatation was only observed with very high and supra-physiological local vasopressin concentrations. This suggests that there

is a higher threshold for pharmacodynamic responses mediated by the vascular V2 receptor and its second messenger system compared to the renal V2 receptors.

We have demonstrated that maximal vasoconstrictor and vasodilator responses were unchanged with repeated vasopressin infusions. However, peak vasodilatation was achieved at 12 min regardless of the duration of the infusion, even when less than 12 min, and prolonged infusion beyond 12 min was associated with tachyphylaxis to vasopressin. The length of infusion determined the offset of action with a slower fall in response seen with longer infusion times. This profile of the vasomotor responses may relate to the physiology of the vasopressin receptor. When bound by vasopressin, the majority of the V1 and V2 receptor are internalised by the cell and only recycled to the cell surface once the agonist is displaced [Thibonnier *et al.* 2001]. It is likely that the peak response requires the majority of receptors to be present on the cell surface and that waning of the response occurs because of receptor-ligand internalisation. However, during intermittent infusion of vasopressin, the receptors may have time to recycle back to the cell surface ensuring an undiminished maximal response. The delayed peak vasodilatation seen with the brief 6 min vasopressin infusion also suggests some damping or delay in the second messenger pathway that is not seen with other G-protein coupled receptor responses, such as those to bradykinin and substance P. The more protracted offset of the vasodilatation with longer vasopressin infusions may also represent rebinding of receptors that have been recycled back to the cell surface membrane.

Previous *in vitro* [Katusic *et al.* 1984 and 1992] and *in vivo* studies [Tagawa *et al.* 1993, Rector *et al.* 1996] have suggested that nitric oxide contributes to the vasodilatation produced by vasopressin. In the first *in vivo* study intra-arterial L-N^G-monomethylarginine (L-NMMA), a nitric oxide synthase inhibitor, was co-infused with vasopressin and appeared to inhibit the vasodilatation. However, L-NMMA inhibits basal nitric oxide production and thereby causes vasoconstriction and a reduction in basal forearm blood flow. Such basal changes in vessel geometry and blood flow may, therefore, make the interpretation of subsequent responses difficult [Webb 1995; Benjamin *et al* 1995]. Co-infusion of sodium nitroprusside, an exogenous nitric oxide donor, with L-NMMA can be used to restore the baseline blood flow and vessel geometry by replacing endogenous with exogenous nitric oxide; the so-called 'nitric oxide clamp' [Stroes *et al* 1997]. This technique establishes a stable baseline forearm blood flow that can be maintained for up to 120 min and permits the assessment of vascular responses in the absence of endogenous nitric oxide synthesis [Verhaar *et al* 1998]. Using the 'nitric oxide clamp' we have shown abolition of vasopressin-induced vasodilatation, consistent with V2 receptor activation causing endothelial nitric oxide generation. Without further study, it is unclear if the restoration of baseline blood flow with high dose vasopressin is attributable to tachyphylaxis of the vasoconstriction or whether there is some residual nitric oxide independent vasodilatation. Although vasopressin causes vasoconstriction at lower doses, there remains the possibility that even greater vasoconstriction could have been achieved in the absence of the potentially counteracting V2 receptor effects. However, against this, we were unable to

demonstrate potentiation of the vasoconstrictor response during the 'nitric oxide clamp', suggesting that the vascular V2 receptor is unstimulated at these concentrations.

In conclusion, we have found that intra-arterial vasopressin is well tolerated and causes a reproducible dose-dependent and biphasic blood flow response. The assessment of forearm vasomotor responses to vasopressin provides a practical, reliable and sensitive method of assessing the *in vivo* efficacy of selective and non-selective V1 and V2 receptor antagonists in man.

CHAPTER 7

THE STUDY OF VASOPRESSIN AND A SELECTIVE V1 RECEPTOR ANTAGONIST IN THE HUMAN SKIN MICROCIRCULATION

7.1 SUMMARY

Arginine vasopressin is both an anti-diuretic and vasoactive peptide which causes vasoconstriction via the V1 receptor and, in some vascular beds, vasodilatation via V2 like vascular receptors. Using laser Doppler flowmetry, we hypothesised that in the skin microcirculation vasopressin would cause vasoconstriction and that a novel selective peptide V1 receptor antagonist ($d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$) would attenuate this response. We aimed to study the effects of a dose range of vasopressin and the control vasoconstrictor, endothelin-1 (ET-1), on skin blood flow before combination with the V1 antagonist.

Skin blood flow was measured in 15 healthy men using laser Doppler flowmetry coupled with intra-dermal injection of drugs. Comparative dose ranges $3x10^{-14}$ to $3x10^{-11}$ mol/10 μL of both vasopressin and endothelin-1 were administered, followed by an extended dose range of vasopressin 10^{-17} to 10^{-11} mol/10 μL to obtain an EC_{50}. A dose range of $1x10^{-20}$ to $1x10^{-10}$ mol/10 μL $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ was injected prior to combination with $1x10^{-15}$, $1x10^{-14}$, $1x10^{-13}$ mol/10 μL vasopressin with either saline placebo or $1x10^{-12}$ mol/10 μL $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$.

Vasopressin and endothelin-1 caused a dose dependent reduction in skin blood flow (ANOVA $P<0.001$). The EC_{50} for vasopressin was $7 \pm 1.3x10^{-15}$ mol/10 μL. The V1 antagonist, $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ alone, did not change skin blood flow (ANOVA $P=0.3$) nor did it alter vasoconstrictor effects of co-administered vasopressin

(ANOVA P=0.3). Antagonist activity was confirmed in rat biopressor assays and a pA_2 value of 8.07 obtained. We conclude that $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ is not a potent V1 antagonist in vivo in the skin microcirculation in man. The failure of the V1 antagonist to alter the vasoconstrictor responses to vasopressin may reflect a low potency and /or inter-species variation.

7.2 INTRODUCTION

Arginine vasopressin, an endocrine hormone released from the neurohypophysis, is responsible for the regulation of plasma osmolarity, body water and support of blood pressure in response to hypotension and increased plasma osmolarity. Vasopressin has been implicated in disease states such as congestive cardiac failure [Goldsmith *et al* 1983; Szatalowicz *et al* 1981] and end stage cirrhosis [Burmeister *et al* 1983; Akriviadis *et al* 1997] where the vasopressin axis is stimulated and plasma concentrations are high, especially when hyponatraemia is a feature [Szatalowicz *et al* 1981]. Vasopressin antagonists may have a potential therapeutic role in such patient groups.

Extensive studies in healthy human volunteers have been performed to assess the physiology of vasopressin. Intra-arterial infusion in man results in a bi-phasic forearm blood flow responses [Affolter *et al* 2003] where low dose (3 pmol/min) cause vasoconstriction via V1 receptors [Hirsch *et al* 1989; Imaizumi *et al* 1992], and at higher doses (300 pmol/min) a nitric oxide [Affolter *et al* 2003] and V2 receptor mediated vasodilatation [Tagawa *et al* 1995]. However, not all vascular beds show the same pattern of responses and although the composite measure of forearm blood flow increases over the higher dose range of intra-arterial vasopressin, there are vasoconstrictor effects on both the radial artery and skin blood flow for all infusion rates.

From non-peptide antagonist studies, it appears likely there are only V1 receptor mediated effects on skin blood flow [Weber *et al* 1997], although these studies involved systemic dosing with an orally active V1 antagonist. Of the peptide V1 receptor antagonists, there have been numerous problems with poor selectivity and co-antagonism of oxytocin receptors, however $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$, the diaminobutyric acid analogue of $d(CH_2)_5Tyr(Me)AVP$, is highly selective for V1 receptors [Chan *et al* 1996].

The assessment of skin blood flow can be made non-invasively using laser Doppler flowmetry [Holloway and Watkins 1977; Schabauer and Rooke 1994]. This is a reproducible method [Kubli *et al* 2000] dependent on the principle of a Doppler shift in frequency of the reflected He-Ne laser light from flowing red blood cells in the skin microcirculation. Combined with intra-dermal drug injection this method has been successfully used to study vasoactive peptides including endothelin-1 and its antagonists [Wenzel *et al* 1993].

We hypothesised that vasopressin would induce vasoconstriction in skin microcirculation. We also hypothesised that the selective V1 antagonist $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$, would not change basal skin blood flow but would attenuate exogenous vasopressin induced vasoconstriction.

7.3 METHODS

7.3.1 Subjects

Studies were conducted with the approval of the local research ethics committee and the written informed consent of each subject. The investigations conformed to the principles of the Declaration of Helsinki. Fifteen healthy men, mean age 26 ± 2 years (range 21 to 46) were recruited. Subjects were asked to fast from midnight before each study (water consumption was not restricted) and to abstain from caffeine containing drinks, alcohol and smoking over the preceding 24 hours.

7.3.2 Drugs

Arginine vasopressin (Pitressin™, Goldshield, UK), endothelin-1 (Clinalfa, Läufelfingen, Switzerland) and $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ (Clinalfa, Läufelfingen, Switzerland) were aseptically prepared and dissolved in saline (0.9% Baxter Healthcare Ltd.,Norfolk, UK). The antagonist activity of $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ was confirmed using an *in vivo* biopressor assays with pentobarbitone anaesthetised rats.

7.3.3 Study design

Studies were performed with subjects resting supine in a quiet clinical laboratory which was maintained at a constant temperature of 22-24°C. After 20 min rest, 4 injection sites were selected on the volar aspect of each forearm. After baseline recordings sites were injected with 10 μL drug and/or 10 μL saline placebo using a disposable 29.5 SWG 0.5 mL graduated syringe (Becton-Dickinson, Dublin, Ireland). Injections were made

strictly intra-dermally, producing a visible symmetrical wheal, if this was not the case, the injection site was excluded. Recordings were made for 30 seconds at each site immediately after injection, 5 min, 10 min and every 10 min thereafter for 1 hour. The location of both dose and control sites were randomised using a Latin square design in all studies.

7.3.4 Protocol 1. Dose response to vasopressin and endothelin-1

On two occasions, at least 1 week apart each of 8 subjects received either vasopressin or endothelin-1 in the concentration range of $3x10^{-14}$, $1x10^{-13}$, $3x10^{-13}$, $1x10^{-12}$, $3x10^{-12}$, $1x10^{-11}$, $3x10^{-11}$ mol/10 μL.

7.3.5 Protocol 2. Extended dose range for vasopressin

On one occasion, each of 8 subjects received vasopressin in the concentration range of $1x10^{-11}$, $1x10^{-13}$, $1x10^{-14}$, $1x10^{-15}$, $1x10^{-16}$, $1x10^{-17}$ mol/10 μL.

7.3.6 Protocol 3. Dose range of $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$

On one visit 8 subjects received $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ in the concentration range $1x10^{-10}$, $1x10^{-12}$, $1x10^{-14}$, $1x10^{-16}$, $1x10^{-18}$, $1x10^{-20}$ mol/10 μL.

7.3.7 Protocol 4. Co-administration of vasopressin and $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$

On one visit 8 subjects received $1x10^{-15}$, $1x10^{-14}$, $1x10^{-13}$ mol/10 μL vasopressin co-injected with 10 μL saline placebo and the same doses of vasopressin co-injected with

$1x10^{-12}$ mol/10 μL $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$. Controls of saline alone and saline and $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ were used.

7.3.8 Rat biopressor assay

Six adult male Wistar rats (Charles River, Margate, England), mean body weight 327 g, range 245-401 g, were anaesthetised with 60 mg/kg intra-peritoneal pentobarbital and continuous 6 mg/h intra-venous pentobarbitol for steady levels of anaesthesia. Body temperature was maintained at 36 to 37 °C with a heating blanket and a tracheal cannula was inserted with tidal volume and respiratory rate monitored by an electrospirometer (MacLab). The right carotid and femoral arteries were cannulated for blood pressure monitoring and drug administration respectively. Arginine vasopressin (Pitressin™, Goldshield, UK) and $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ (Clinalfa, Läufelfingen, Switzerland) were prepared in and dissolved in saline (0.9% Baxter Healthcare Ltd., Norfolk, UK) and drugs were administered in 0.1 mL volumes then washed in with 0.2 mL saline. Doses of 10, 30, 100, 300, 1000 ng vasopressin and 5, 10, 20, 40 ng $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ were used. After stable anaesthesia was achieved sequential vasopressin doses 10, 30, 100 ng, were administered followed by a 20 minute washout phase and reappraisal of level of anaesthesia prior to antagonist dosing. Five minutes after the antagonist was given a further sequential 30, 100, 300 ng vasopressin was given followed by another 20 minute washout phase. Again, levels of anaesthesia were assessed for stability and a second antagonist dose given before 100, 300, 1000 ng of vasopressin. In the first 3 rats 5 and 10 ng $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ were given before the two sequential vasopressin dose ranges and in the final 3 rats 20 and 40 ng

doses were used. Recording of the mean arterial blood pressure was made in the 15 seconds before drug injection and at the point of maximal change during the 120 seconds immediately following drug injection. The effects of drugs were determined by comparing responses to agonist alone and following administration of antagonist. Data are expressed change in mean arterial blood pressure ± SEM and as EC_{50} values ± SEM. The pA_2 value (the log antagonist dose required to necessitate a doubling of the agonist dose to achieve the same pharmacodynamic response) was obtained from the x axis intercept of a Schild plot of log antagonist dose *vs.* log (dose response-1). The animal studies were performed in accord with European Community guidelines, approved by the institutional ethics committee and licensed under the UK Home office regulations. Studies were done with this investigator present, but not performed by himself due to licensing restrictions on those allowed to perform animal studies.

7.3.8 Statistical analysis

All results are expressed as mean ± SEM. As previously described intra-dermal injection of saline alone causes an increase in skin blood flow [Holloway 1980] that diminished over time. The area under the curve for both saline control and drug injection sites were calculated and used to determine differences between them and expressed in arbitrary perfusion units (PU). The EC_{50} was defined as the concentration of agonist that provoked a response half way between the baseline and maximum response. It was calculated using data from the extended dose range of vasopressin, with Graph Pad Prism™. Statistical analysis was performed using repeated measure ANOVA and for individual comparisons data were analysed using Student's *t*-test.

Statistical significance was taken at $P<0.05$.

7.4 RESULTS

All subjects were symptom free throughout the studies and the intra-dermal injections of all drugs were well tolerated. There was no significant difference in response to saline controls throughout all studies (ANOVA P=0.3).

7.4.1 Protocol 1. Dose response to vasopressin and endothelin-1

Vasopressin caused a reduction in skin blood flow (Figure 7.1; ANOVA P<0.001) for all doses tested. A reduction in skin blood flow was observed for all doses of endothelin-1 (Figure 7.1; ANOVA P<0.001).

7.4.2 Protocol 2. Extended dose range for vasopressin

Vasopressin caused an incremental reduction in skin blood flow (Figure 7.2; ANOVA P<0.001) with significant differences between the lowest dose ($1x10^{-17}$ mol/10 μL) and highest dose ($1x10^{-11}$ mol/10 μL; t-test, P<0.001). The EC_{50} value was calculated to be $7 \pm 1.3x10^{-15}$ mol/10 μL.

7.4.3 Protocols 3 and 4. Dose range of d(CH$_2$)$_5$[Tyr(Me)^2,Dab^5]AVP and co-administration of vasopressin and d(CH$_2$)$_5$[Tyr(Me)^2,Dab^5]AVP

Intra-dermal injection of d(CH$_2$)$_5$[Tyr(Me)^2,Dab^5]AVP caused no significant change in skin blood flow (Figure 7.3; ANOVA P=0.3). The co-administration of antagonist with vasopressin did not alter the response to vasopressin (Figure 7.4; ANOVA P=0.3).

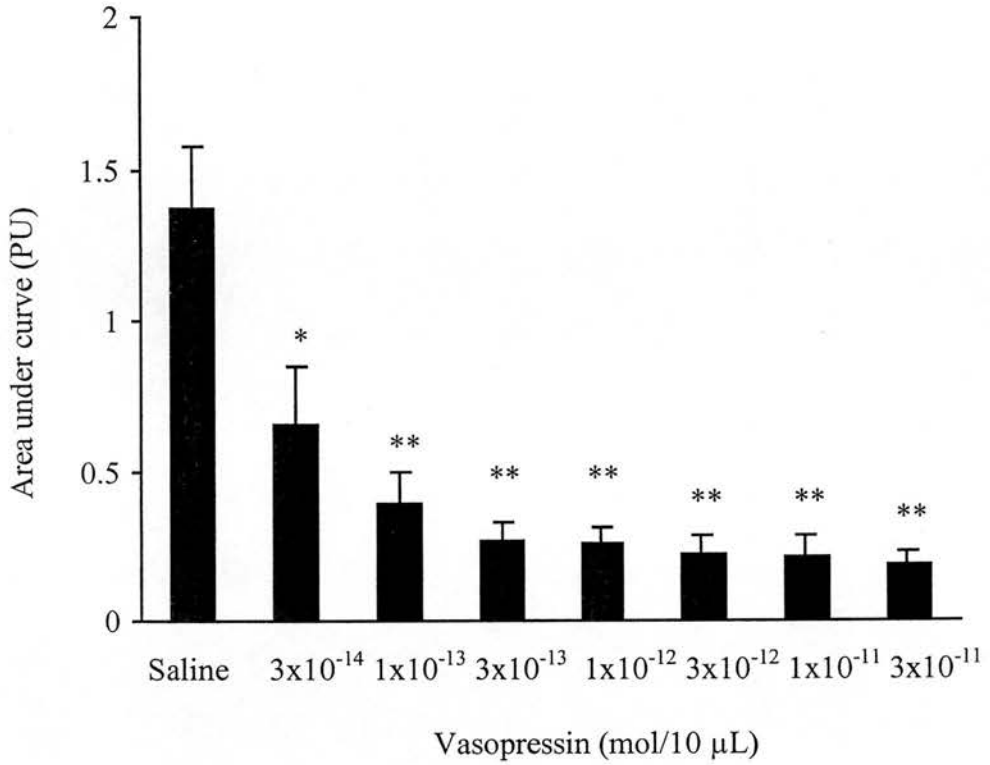
7.4.4 Rat biopressor assay

Vasopressin caused a dose dependent rise in mean arterial blood pressure. Injection of 10 and 100 ng vasopressin increased blood pressure by 3 ± 1 and 16 ± 2 mmHg respectively (P<0.001). Pre-treatment with 40 ng d$(CH_2)_5$[Tyr(Me)^2,Dab^5]AVP reduced the expected rise in mean arterial pressure during 100 ng vasopressin dosing to 6 ± 3 mmHg (P=0.05, n=3). The EC_{50} of vasopressin was 93 ± 20 ng and with 40 ng d$(CH_2)_5$[Tyr(Me)^2,Dab^5]AVP pre-treatment, the EC_{50} rose to 500 ± 157 ng (P=0.05, n=3). The pA_2 was calculated to be 8.07.

Figure 7.1

Skin blood flow responses to a dose range to vasopressin and endothelin-1. * $P<0.05$, ** $P<0.01$, Student's *t*-test compared to saline placebo.

Figure 7.1


2
1.5
1
0.5
0
Area under curve (PU)
*
**
**
**
**
**
**
Saline
3x10-14
1x10-13
3x10-13
1x10-12
3x10-12
1x10-11
3x10-11
Vasopressin (mol/10 µL)

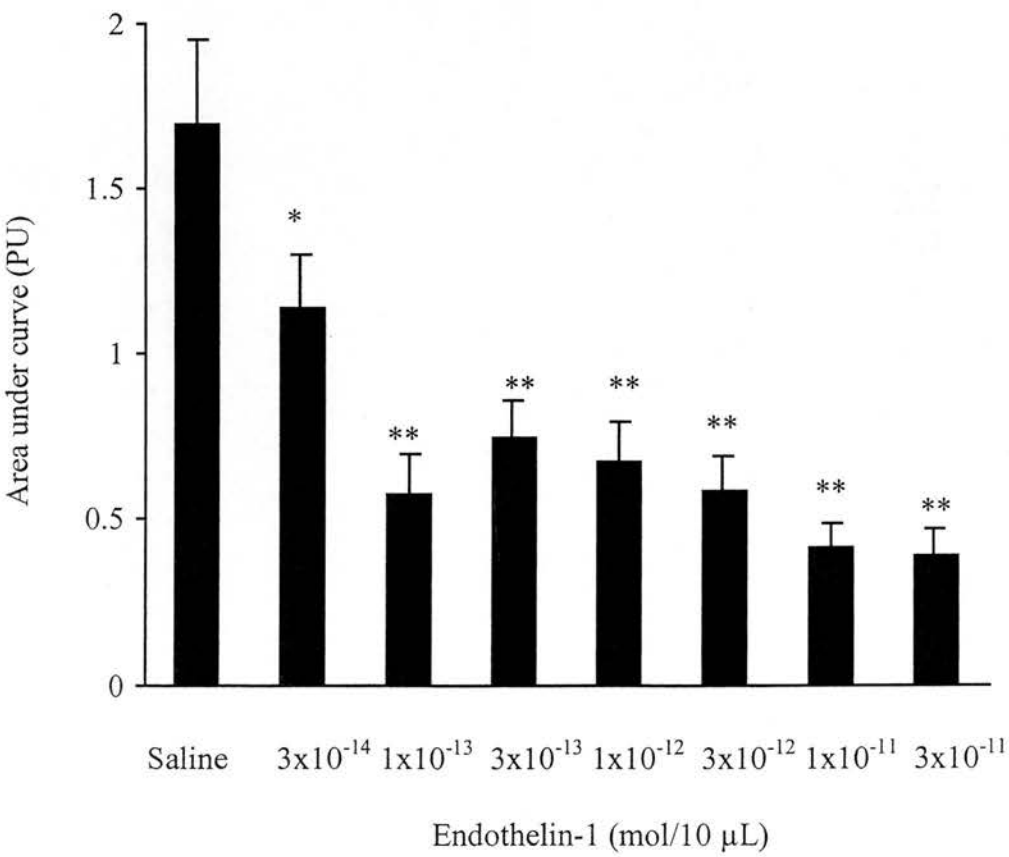

2
1.5
1
0.5
0
Area under curve (PU)
*
**
**
**
**
**
**
Saline
3x10-14
1x10-13
3x10-13
1x10-12
3x10-12
1x10-11
3x10-11
Endothelin-1 (mol/10 µL)

Figure 7.2

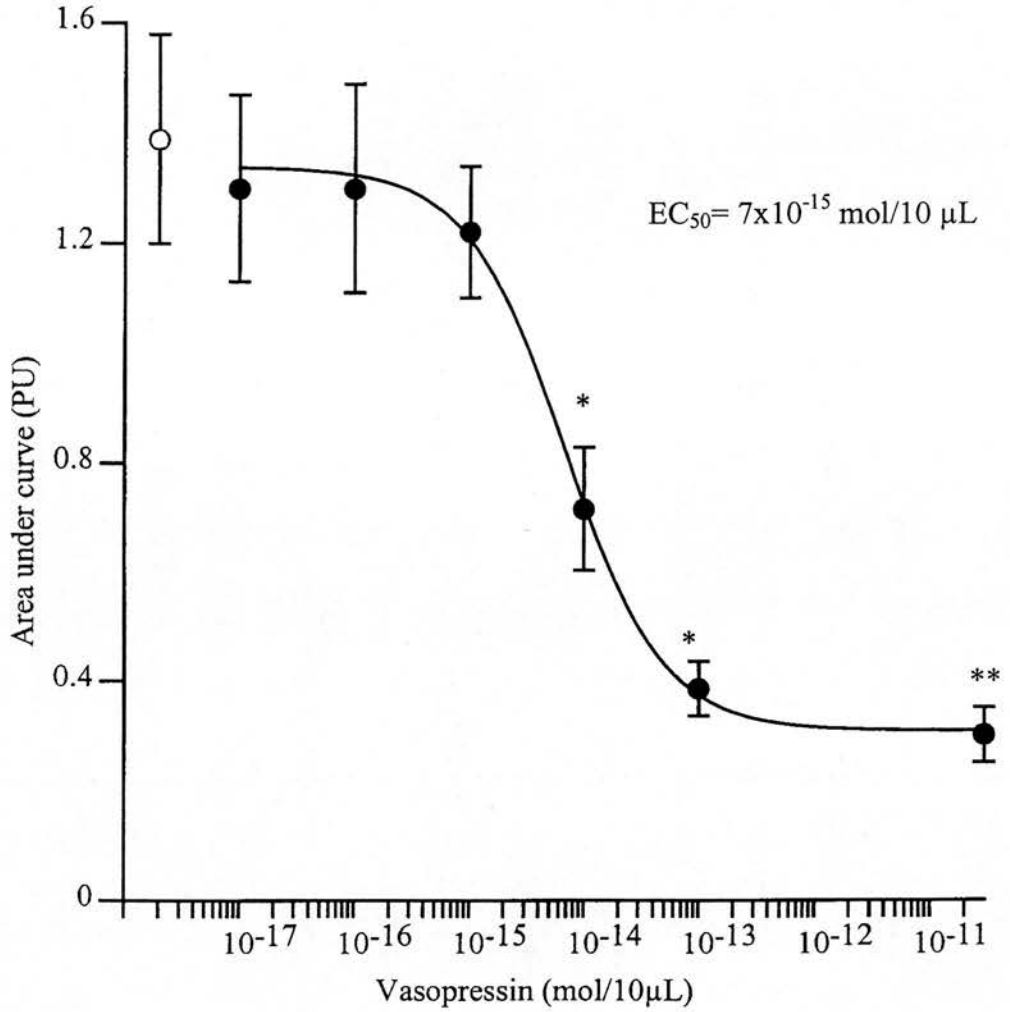


Figure 7.2

Skin blood flow responses to an extended dose range of vasopressin showing EC_{50} of $7x10^{-15}$ mol/10 µL. Saline placebo (○) and vasopressin (●). * $P<0.05$, ** $P<0.01$, Student's *t*-test compared to saline baseline.

Figure 7.3

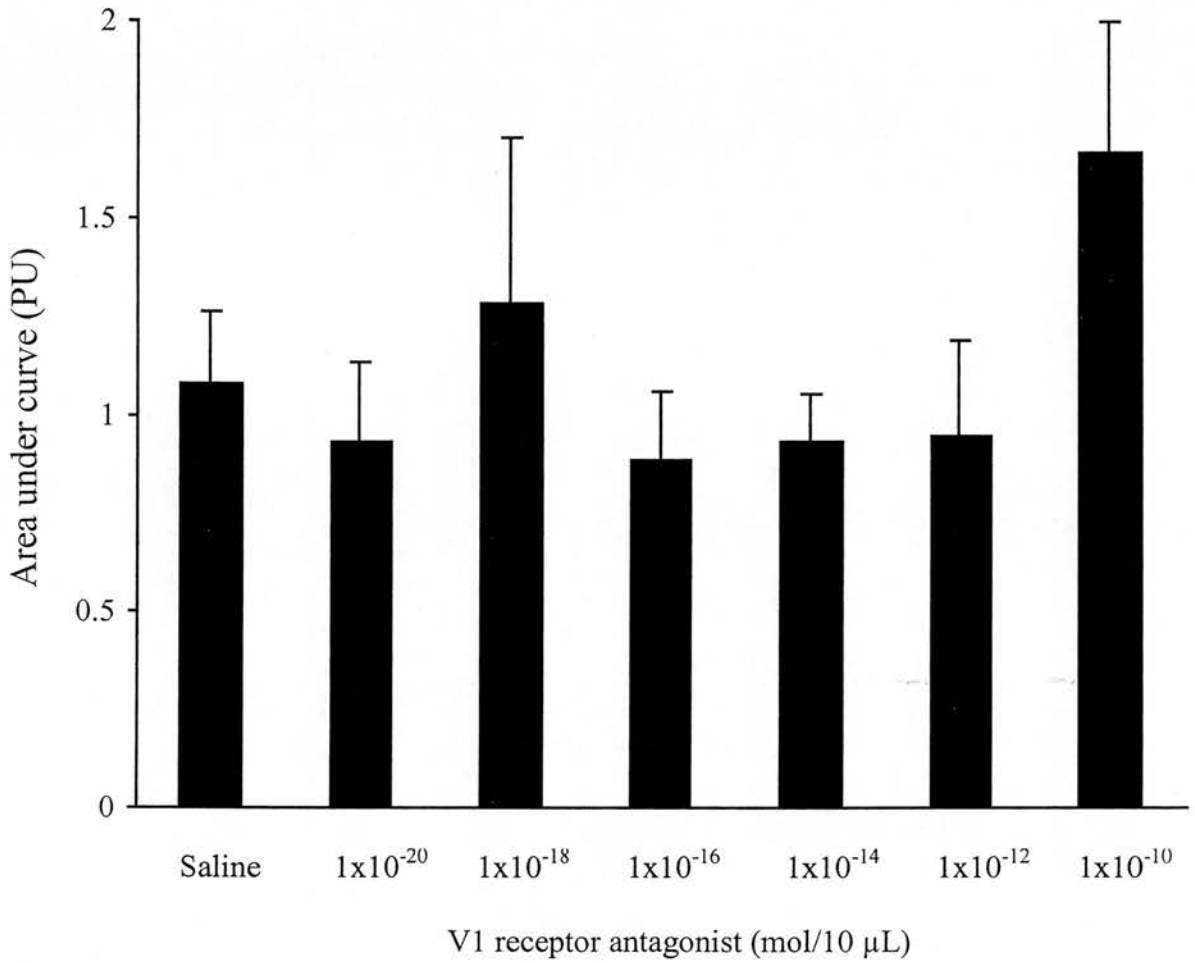


Figure 7.3

Skin blood flow responses to the vasopressin antagonist. ANOVA P=0.3.

Figure 7.4

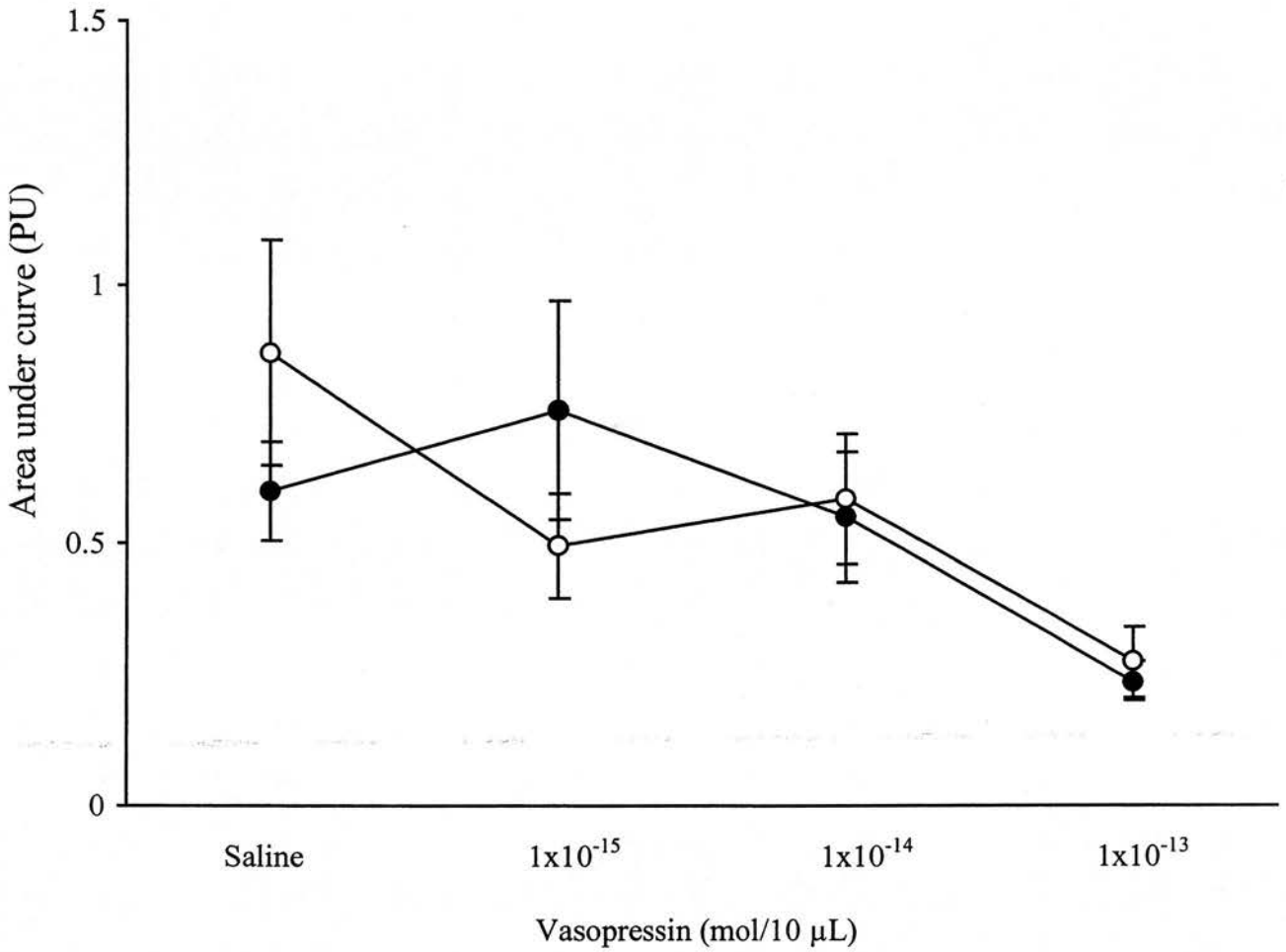


Figure 7.4

Skin blood flow responses to vasopressin with saline placebo (○) and with $1x10^{-12}$ mol/10 µL vasopressin antagonist (●). ANOVA P=0.3.

7.5 DISCUSSION

We have confirmed the potential for both vasopressin and endothelin-1 to cause vasoconstriction in the skin microcirculation of healthy male volunteers and that the EC_{50} for vasopressin is $7x10^{-15}$ mol/10 μL. The V1 receptor antagonist $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ did not alter vasoconstriction associated with vasopressin in man but was found to be a potent inhibitor of the vasopressin pressor effects in rats We conclude that $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ is not a potent V1 antagonist in vivo in the skin microcirculation in man

Both vasopressin and its analogues, such as ornipressin, have been used in similar studies and cause potent vasoconstriction [Fruhstorfer and Heisler 1994; Weber *et al* 1997] in the skin microcirculation. However, we have observed consistent vasoconstriction occurring at doses up to 100-fold lower than previously described [Weber *et al* 1997]. During the study of endothelin-1, we observed a dose dependent reduction in skin blood flow for all doses, including those 30-fold less than used in previous studies [Brain *et al* 1989]. The disparity from previous results may reflect the different equipment used to measure skin blood flow. In our study the Moor Instruments Laser Blood Flow Monitor MBF3D uses near infra-red light as opposed to the red light used by Weber *et al* and Brain *et al*, which in caucasian skin can potentially have half the depth of light penetration [Vongsavan and Matthews 1993]. Drug injection techniques were similar although the injection volumes were 50μL and the drug vehicle differed. We calculated an EC_{50} value based on the amount of vasopressin required to

vasopressin required to generate a 50 % change from baseline when the maximal response had been determined. Other studies do not cite an EC_{50} value for their intra-dermal vasopressin studies so we are unable to directly compare our results with those of others.

Vasopressin release as a response to shock increases circulating vasopressin concentration by up to 28-fold greater than the non-stimulated plasma concentration [Landry and Oliver 2000]. This increase is far greater than that required for anti-diuretic effects at the renal V2 receptors. We hypothesised that the V1 receptor antagonist would not alter basal skin blood flow on the basis that in healthy volunteers, vasopressin would be most unlikely to contribute to basal vascular tone compared to other vasoactive peptides such as endothelin-1 [Haynes and Webb 1994]. As the V1 antagonist did not alter the effect of exogenous vasopressin on skin blood flow we cannot confirm this hypothesis using this antagonist.

Co-administration of vasopressin doses around the EC_{50} concentration with the V1 receptor antagonist $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ unfortunately did not show the predicted antagonism. Inter-species difference may account for the lack of effect and this is the first time $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ has been used *in vivo* in human subjects. Our wish was to use the most selective peptide V1 antagonist. However, during the construction of this antagonist, the amino acid asparagine at position 5 is replaced by diaminobutyric acid, that whilst greatly increases the selectivity for V1 receptors, has a detrimental effect on potency [Chan *et al* 1996]. This could account for

the lack of antagonist effect. To confirm antagonist activity we performed a rat biopressor assay using pentobarbitone anaesthetised Wistar rats and were able to confirm antagonist activity with a pA_2 value of 8.07. The biopressor assay is however limited in terms of the number of animals used. There are at however 20 amino acids that differ within the extra-cellular loops of the rat and human V1 receptors [Thibonnier *et al* 2001]. These residues have been shown to be critical to the inter-species variation in the binding of other V1 receptor antagonists such as the non-peptide V1 receptor antagonist OPC-21268 [Thibonnier *et al* 2000].

Potential limitations in our study include the co-administration of both drug and antagonist in a single preparation. An approach of 2 injections, with the antagonist first could have been performed but the skin trauma of 2 injections at one site may simply increase the error of measurement. Furthermore, this antagonist had never been used in human *in vitro* or *in vivo* studies before and antagonist doses had to be estimated from animal models.

We have shown vasopressin to be a potent vasoconstrictor in the human skin microcirculation. The lack of effect of the antagonist, $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$, may have been due to either insufficient potency or inter-species variation.

CHAPTER 8

THE STUDY OF VASOPRESSIN PEPTIDIC V1 AND V2 RECEPTOR ANTAGONISTS IN THE HUMAN FOREARM CIRCULATION

8.1 SUMMARY

In man arginine vasopressin causes vasoconstriction via the V1 vascular receptor and vasodilatation in some vascular beds via V2 vascular receptors. Vasopressin peptide antagonists have been used with variable success due to selectivity problems. Using the reproducible and sensitive method of bilateral venous occlusion plethysmography coupled with intra-arterial drug infusion, we hypothesised that the V1 receptor antagonist, $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$, would reduce vasopressin induced vasoconstriction and enhance its induction of vasodilatation. Conversely, the V2 antagonist, $d(CH_2)_5[D\text{-}Ile^2\text{-}Ile^4\text{-}Ala^9]AVP$, would attenuate vasopressin induced vasodilatation.

Forearm blood flow was measured in 6 healthy male subjects using bilateral venous occlusion plethysmography during intra-arterial infusion of 1, 3, 10, 30, 100, 300, 1000 and 3000 pmol/min of V1 antagonist, V2 antagonist or saline placebo given in a randomised double blind manner. A further study combined sequential vasopressin dose ranges of 3, 30 and 300 pmol/min with V1 antagonist, V2 antagonist or saline placebo in a randomised double blind manner. Heart rate and blood pressure were recorded through out each study. The study was well tolerated and no significant changes in heart rate, blood pressure or flow in the non-infused arm were observed. Compared to placebo infusion, both V1 and V2 antagonists did not change forearm blood flow (ANOVA, $P=0.6$ and $P=0.9$ respectively). Infusion of vasopressin caused a dose dependent biphasic change in blood flow (ANOVA, $P<0.001$), with vasoconstriction

with 3 pmol/min ($P<0.01$) and vasodilatation with 300 pmol/min. Compared to saline placebo, neither the V1 nor V2 antagonist, co-infused at 20 nmol/min, altered the dose dependent biphasic response pattern to vasopressin (ANOVA, $P=0.1$ for both).

We conclude that neither the V1 nor V2 antagonist affected forearm blood flow, nor did they alter the normal vascular effects of vasopressin. This indicates that $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ and $d(CH_2)_5[D\text{-}Ile^2\text{-}Ile^4\text{-}Ala^9]AVP$ do not appear to be efficacious in blocking V1 or V2 receptors in man *in vivo*. This may reflect inter-species differences in antagonist activity or low potency of the antagonists.

8.2 INTRODUCTION

As an endocrine hormone vasopressin is responsible for osmoregulation and maintenance of blood pressure by means of peripheral arterial vasoconstriction in response to hypotension. An activated vasopressin axis has been implicated in the pathophysiology of heart failure, a condition characterised by increased vascular tone and fluid retention and vasopressin receptor antagonists have subsequently become a focus for therapeutic intervention [Burrell *et al* 2000].

The use of peptidic vasopressin antagonists had a chequered history. Although non-peptide antagonists have been helpful in forwarding pharmacological knowledge, peptide antagonists have suffered from poor selectivity and inter-species variation. From human intra-arterial studies, vasopressin shows a dose dependent biphasic response in forearm blood flow [Suzuki *et al* 1989; Tagawa *et al* 1993, Affolter *et al* 2003], although others have shown only vasoconstriction [Weber *et al* 1997] or vasodilatation [Hirsch *et al* 1989].

From studies using vasopressin antagonists, it is likely that V1 receptors [Hirsch *et al* 1989; Imaizumi *et al* 1992] mediate vasoconstriction and V2 receptors [Tagawa *et al* 1995] mediate nitric oxide dependent vasodilatation [Chapter 6]. However, few studies have directly assessed the effects of V1 antagonists and have instead employed systemic oral or intra-venous antagonist administration prior to a dose range of intra-arterial vasopressin [Hirsch *et al* 1989; Imaizumi *et al* 1992].

Of the peptide antagonists $d(CH_2)_5[Tyr(Me)^2]AVP$ has high antagonist activity against the V1 receptor [Kruszynski *et al* 1980] but it also has equivalent anti-oxyntic effects and mild V2 anti-diuretic agonist effects [Manning and Sawyer 1986] making it less than ideal in terms of selectivity. Although not traditionally associated with cardiovascular regulation, oxytocin receptors are involved with plasma volume regulation and have been located in the rat vena cava and aorta [Jankowski *et al* 2000]. The more recent $d(CH_2)_5[Tyr(Me)^2, Dab^5]AVP$ has no V2 agonist nor oxytocin receptor antagonist effects [Chan *et al* 1996] making it relatively unique amongst peptide antagonists. Slightly less success has been seen in the development of V2 peptide antagonists with persistent problems in selectivity, although the production of $d(CH_2)_5[D\text{-}Ile^2\text{-}Ile^4\text{-}Ala^9]AVP$ [Sawyer *et al* 1987] saw superior selectivity being 83-fold more selective for V2 than V1 receptors. Vasopressin has yet to be studied against the above antagonists in direct vascular studies in man.

We aimed to study the forearm blood flow responses to an intra-arterial dose range of V1 and V2 receptor antagonists before co-infusing vasopressin with each antagonist and a saline placebo. We hypothesised that firstly, intra-arterial infusion of the V1 ($d(CH_2)_5[Tyr(Me)^2, Dab^5]AVP$) and V2 ($d(CH_2)_5[D\text{-}Ile^2\text{-}Ile^4\text{-}Ala^9]AVP$) antagonists alone would not alter forearm vascular tone. Secondly, vasopressin co-infusion with the V1 antagonist would reduce the vasoconstrictor effects of vasopressin and enhance vasodilatation and thirdly, the V2 antagonist would attenuate the vasodilatation response to vasopressin.

8.3 METHODS

8.3.1 Subjects

These studies were conducted with the approval of the local research ethics committee and the written informed consent of each subject. The investigations conformed to the principles of the Declaration of Helsinki. Six healthy men, mean age 35 ± 5 years (range 23-48), were recruited. Subjects were asked to fast from midnight before each study (water consumption was not restricted) and to abstain from caffeine containing drinks, alcohol and smoking over the preceding 24 hours.

8.3.2 Drugs

Arginine vasopressin (Pitressin™, Goldshield, U.K.), V1 antagonist ($d(CH_2)_5[Tyr(Me)^2$, $Dab^5]AVP$; Clinalfa, Läufelfingen, Switzerland) and V2 antagonist ($d(CH_2)_5[D\text{-}Ile^2\text{-}Ile^4\text{-}Ala^9]AVP$; Clinalfa, Läufelfingen, Switzerland) were aseptically prepared and dissolved in saline (0.9% Baxter Healthcare Ltd., Norfolk, UK).

8.3.3 Study design

Studies were performed with subjects resting supine, in a quiet clinical laboratory that was maintained at a constant temperature of 22-24°C. The brachial artery of the non-dominant arm was cannulated with a 27 SWG gauge steel needle (Cooper's Needle Works, U.K.) under local anaesthesia, (1% lignocaine; Astra Pharmaceuticals Ltd., Hertfordshire, UK). This was connected to a constant rate infusion pump (IVAC) via a 16-gauge epidural catheter (Portex Ltd. UK). Saline was infused for 30 min before drug

infusion protocols were commenced to ensure a stable baseline and maintain cannula patency. The total rate of infusion was kept constant at 1 mL/min throughout all studies. Forearm blood flow was measured simultaneously in both arms by venous occlusion plethysmography [Benjamin *et al.* 1995], as previously described [Wilkinson and Webb 2001]. Forearm blood flow was measured over the later 3 min of a 6 min period, the dose infusion was continued for 4 further min to allow blood pressure and pulse recordings using a validated oscillometric sphygmomanometer (HEM 705CP, Omron Japan) [O'Brien *et al.* 1996]. The last five plethysmographic recordings of forearm blood flow were averaged to determine flow in each arm. Blood pressure and heart rate were recorded in the non-infused arm. Mean arterial pressure was defined as diastolic pressure plus 1/3 of the pulse pressure.

8.3.4 Protocol 1. Infusion of vasopressin peptide antagonists

On 3 occasions, at least 1 week apart, each of 6 subjects received in a randomised and double blind manner, saline placebo, V1 or V2 antagonist. Drugs were infused at 1, 3, 10, 30, 100, 300, 1000, 3000 pmol/min for 10 min at each dose.

8.3.5 Protocol 2. Co-infusion of vasopressin and saline placebo, V1 and V2 antagonists

On 3 occasions, at least 1 week apart, the same 6 subjects received vasopressin at 3, 30 and 300 pmol/min for 10 min each, co-infused with saline. This was followed by saline alone for 30 min before receiving a repeated infusion of vasopressin at the same doses with a co-infusion of either saline placebo or 20 nmol/min of V1 or V2 antagonist given

in a randomised and double blind manner. During co-infusions drug and placebo was infused at 0.5 mL/min each.

8.3.6 Statistical analysis

All results are expressed as mean ± SEM. Data for forearm blood flow have been expressed as a percentage change from baseline forearm blood flow ratio in the results and figures (the quotient of infused arm and non-infused arm forearm blood flow) [Chin-Dusting *et al* 1999, Wilkinson and Webb 2001]. Repeated measures ANOVA was used to identify differences in forearm blood flow response. For single comparisons, data were analysed using Student's paired *t*-test. Statistical significance was taken at $P<0.05$.

8.4 RESULTS

All subjects were symptom free and the studies were well tolerated. There were no significant differences in the basal forearm blood flow between infused and non-infused arms and throughout all studies there were no significant changes in non-infused forearm blood flow and mean arterial pressure or heart rate (ANOVA P=0.6, P=0.9 and P=0.5 respectively).

8.4.1 Protocol 1. Infusion of vasopressin peptide antagonists

Baseline forearm blood flow for non-infused and infused arms were 4.7 ± 1.0 and 4.8 ± 1.0 mL/100mL tissue/min respectively. Forearm blood flow was unchanged during infusion of V1 and V2 antagonist compared to placebo (Figure 8.1; ANOVA P=0.6 and P=0.9 respectively).

8.4.2 Protocol 2. Co-infusion of vasopressin and saline placebo, V1 and V2 antagonists

Baseline values for non-infused and infused arms were 3.0 ± 0.6 and 3.4 ± 0.6 mL/100mL tissue/min respectively. During both first and second vasopressin dose ranges co-infused with saline and then saline placebo, vasopressin caused a bi-directional response (Figure 8.2, ANOVA P<0.001 for both) with an 18 ± 2% and 18 ± 7% reduction in blood flow respectively during infusion of 3 pmol/min vasopressin (t-tests, P=0.002 and P=0.06) and blood flow increases of 95 ± 17% and 52 ± 17% respectively during infusion of 300 pmol/min of vasopressin of (t-tests, P=0.02 and

P=0.05). Compared to the co-infusion of saline with the second dose range of vasopressin, co-infusion of 20 nmol/min of either V1 or V2 antagonist did not change the dose dependent biphasic response to vasopressin (Figures 8.3 and 8.4, ANOVA P=0.1 for both).

Figure 8.1

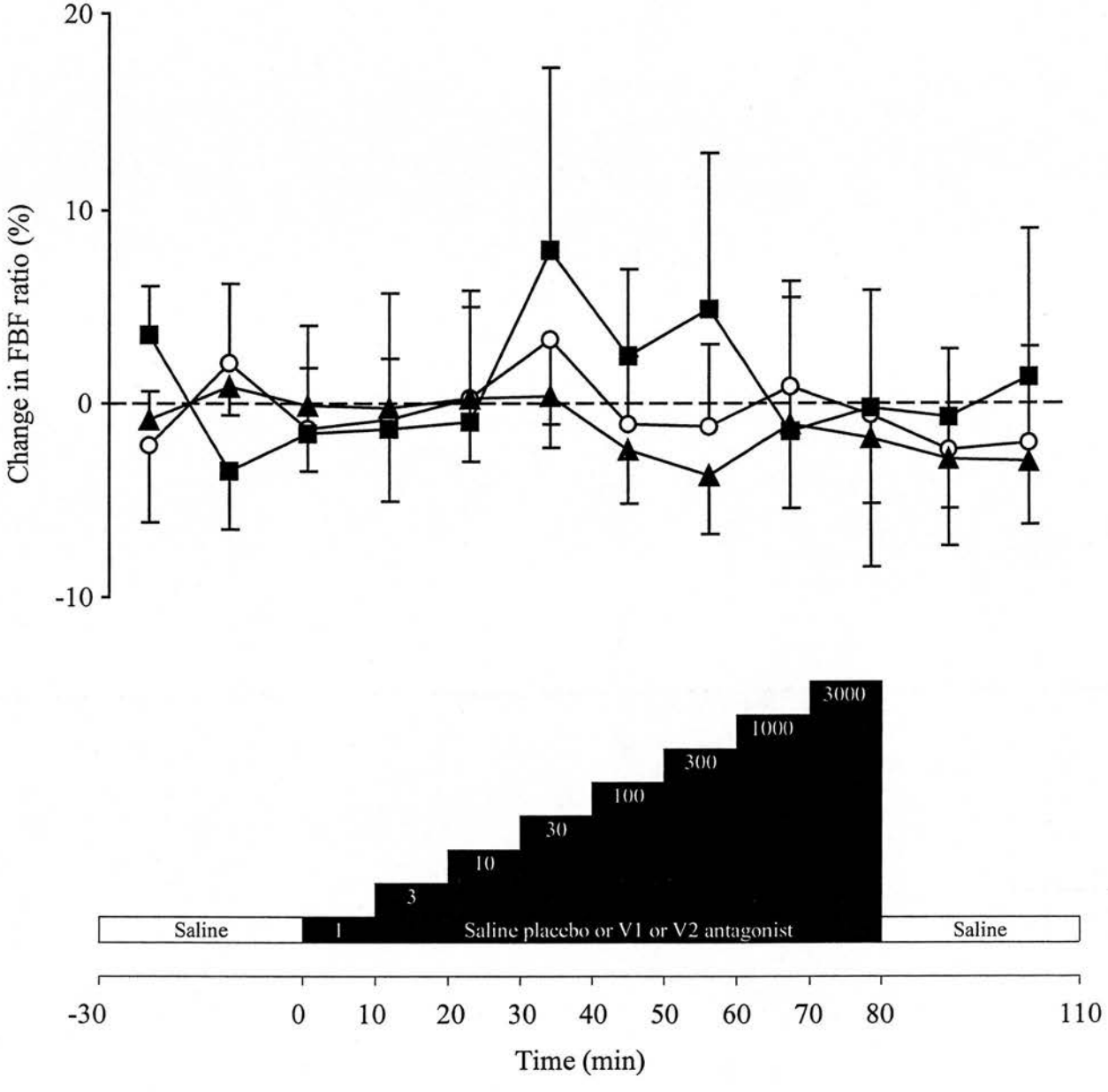


Figure 8.1

Percentage change in forearm blood flow during saline placebo (○), V1 antagonist (▲), V2 antagonist (■) 1, 3, 10, 30, 100, 300, 1000, 3000 pmol/min.

Figure 8.2

Percentage change in forearm blood flow during sequential infusions of vasopressin 3, 30, 300 pmol/min. First dose range –○– with saline and second dose range with placebo —●— * $P<0.05$, ** $P<0.01$, Student's *t*-test compared to saline baseline.

Figure 8.2

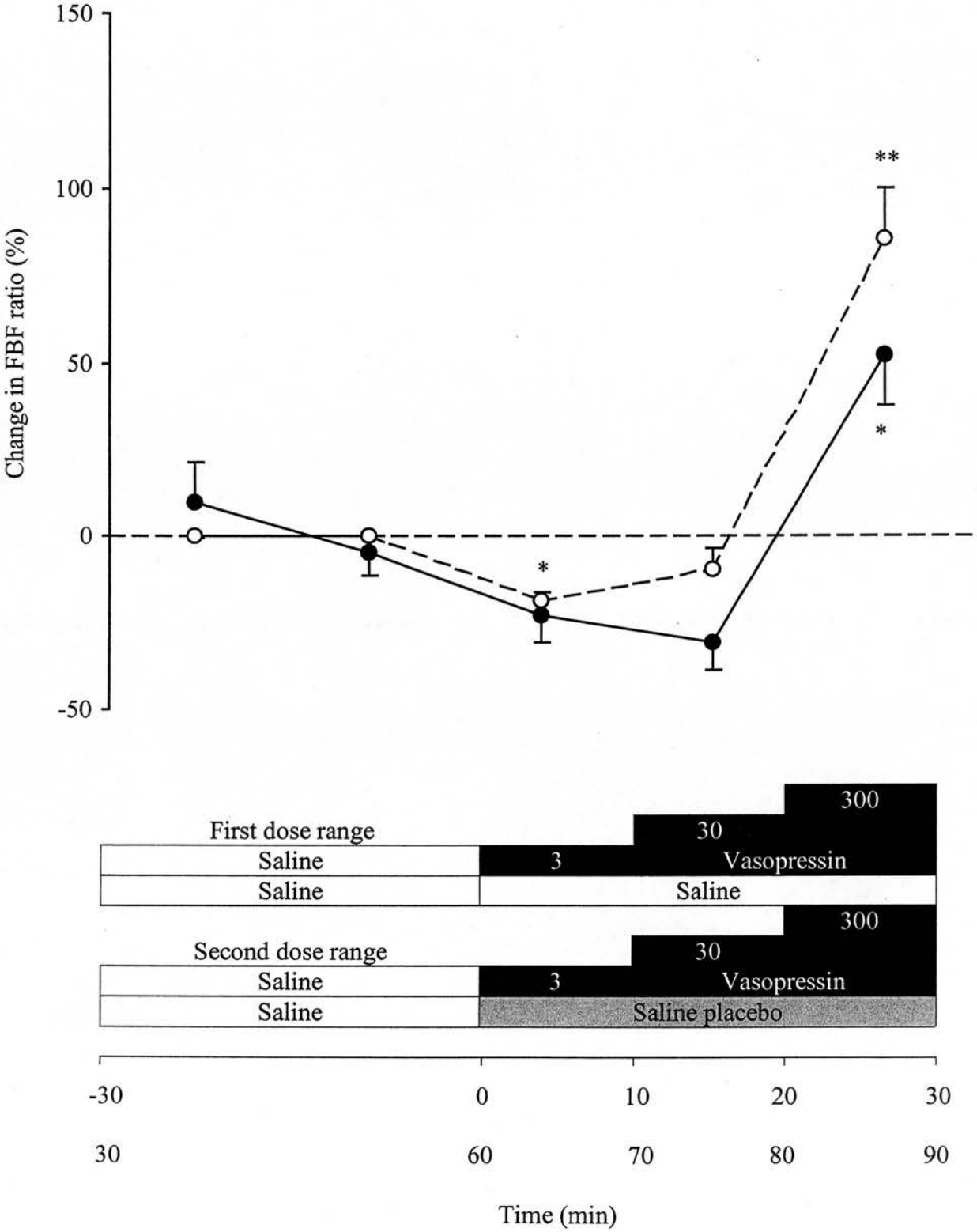

150
100
50
0
-50
Change in FBF ratio (%)
**
*
*
First dose range
Saline
Saline
3
30
300
Vasopressin
Saline
Second dose range
Saline
Saline
3
30
300
Vasopressin
Saline placebo
-30
0
10
20
30
30
60
70
80
90
Time (min)

Figure 8.3

Percentage change in forearm blood flow during sequential infusions of vasopressin 3, 30, 300 pmol/min. First dose range – □ - with saline and second dose range with V1 antagonist —■— and placebo for comparison—●— * $P<0.05$, Student's *t*-test compared to saline baseline.

Figure 8.3

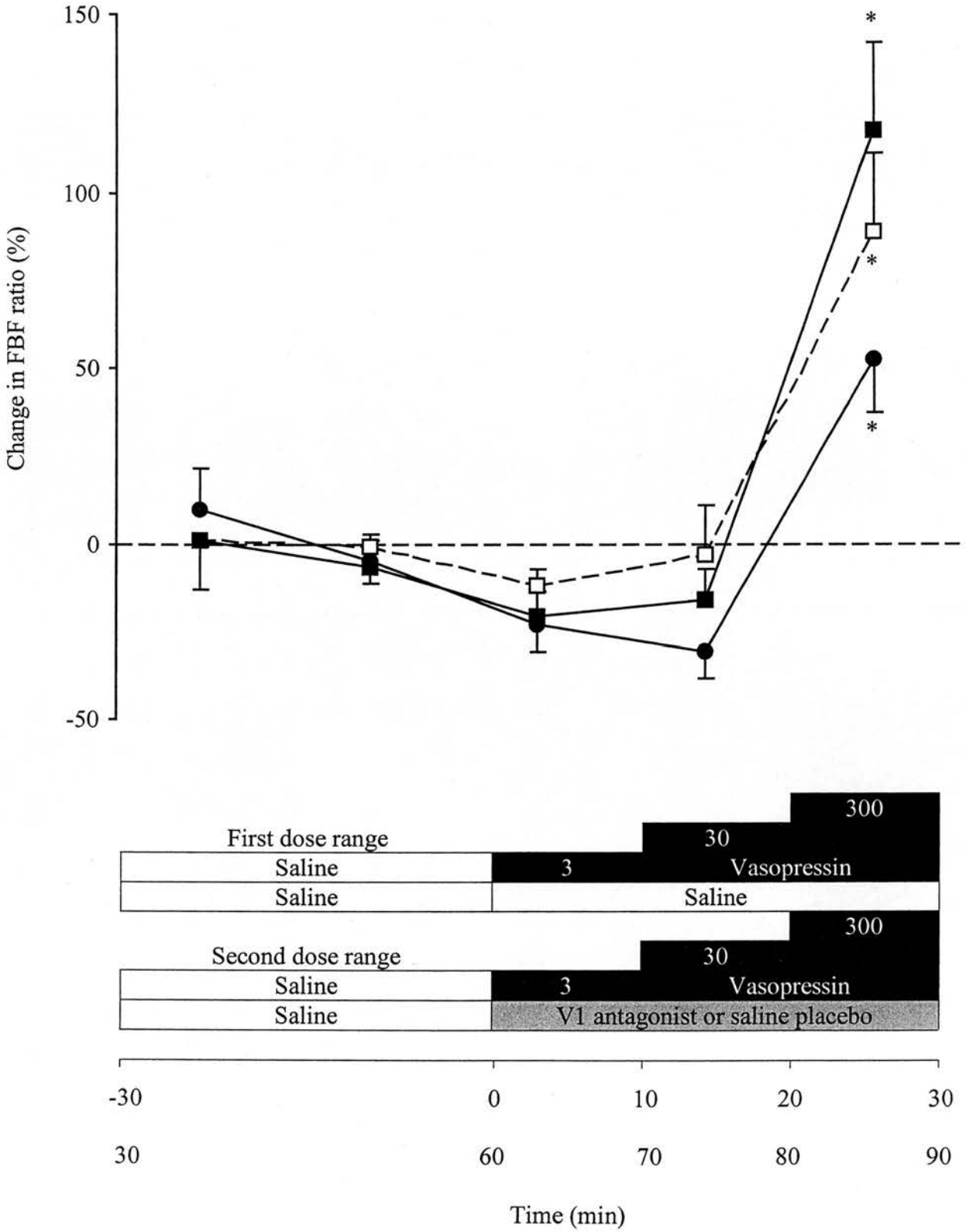
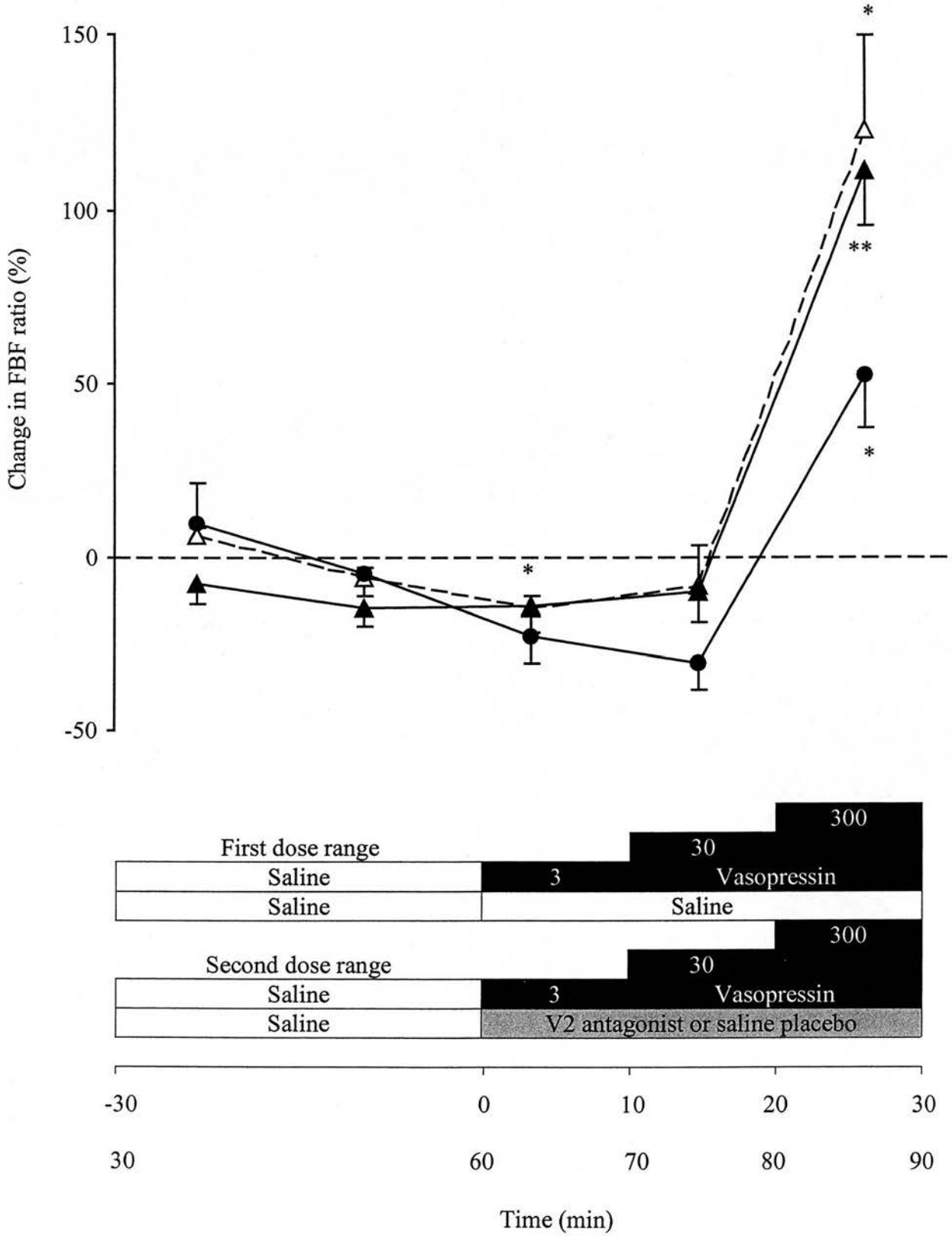

150
100
50
0
-50
Change in FBF ratio (%)
*
*
*
First dose range
Saline
3
30
300
Vasopressin
Saline
Saline
Second dose range
Saline
3
30
300
Vasopressin
Saline
V1 antagonist or saline placebo
-30
0
10
20
30
30
60
70
80
90
Time (min)

Figure 8.4

Percentage change in forearm blood flow during sequential infusions of vasopressin 3, 30, 300 pmol/min. First dose range – △ – with saline and second dose range with V2 antagonist —▲— and placebo for comparison —●— * $P<0.05$, ** $P<0.01$, Student's *t*-test compared to saline baseline.

Figure 8.4


150
100
50
0
-50
Change in FBF ratio (%)
*
**
*
*
First dose range
Saline
3
30
300
Vasopressin
Saline
Saline
Second dose range
Saline
3
30
300
Vasopressin
Saline
V2 antagonist or saline placebo
-30
0
10
20
30
30
60
70
80
90
Time (min)

8.5 DISCUSSION

We have shown that infusions of $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ and $d(CH_2)_5[D\text{-}Ile^2\text{-}Ile^4\text{-}Ala^9]AVP$ peptidic antagonists are well tolerated but did not change forearm blood flow when infused alone or in combination with vasopressin.

We had hypothesised that V1 receptor antagonism would reduce vasoconstriction and enhance vasodilatation induced by co-infused vasopressin whereas V2 receptor antagonism would attenuate the vasopressin induced vasodilatation during co-administration of exogenous vasopressin. Previous human studies using oral systemic non-peptide V1 antagonists such as OPC-21268, decreased the vasoconstrictor response to intra-arterial vasopressin and augmented its vasodilator effects [Imaizumi *et al* 1992]. Unlike the composite forearm blood flow measurement, when specifically observed, radial artery flow is reduced, regardless of the infused intra-arterial dose [Hayoz *et al* 1996; Weber *et al* 1997]. This response has also been antagonised by intra-arterial SR 49059, a later and more specific non-peptide antagonist [Weber *et al* 1997]. Peptide antagonists such as $d(CH_2)_5[Tyr(Me)^2]AVP$ have not been studied directly by intra-arterial infusion against vasopressin, although $d(CH_2)_5[Tyr(Me)^2]AVP$ has been given as a systemic intra-venous dose prior to intra-arterial vasopressin but showed only an enhancement of forearm vasodilatation [Hirsch *et al* 1989]. The peptidic antagonist, des-$Gly(CH_2)_5$D-Tyr(Et)Val-AVP, has been used in human *in vitro* studies and shown to antagonise desmopressin (a human V2 agonist) induced endothelial dependent

vasodilatation in human veins [Aldasoro *et al* 1997]. However, this particular antagonist also has partial V1 antagonist effects.

Despite our studies it remains unclear whether vasopressin contributes to resting vascular tone in healthy subjects given that we were unable to demonstrate effective blockade of the V1 and V2 receptors in man *in vivo* when antagonists were infused alone or co-infused with exogenous vasopressin. It is biologically plausible that vasopressin would not contribute to basal vascular tone as vasopressin induced vasoconstriction is usually part of either a non-sustained physiological response to shock or related to a chronic pathological state, such as heart failure, that is characterised by an abnormally high vascular tone. Moreover, limited systemic studies with the less selective V1 peptide antagonist $d(CH_2)_5[Tyr(Me)^2]AVP$, failed to induce haemodynamic effects in man [Gavras *et al* 1984; Hirsch *et al* 1989].

In making our choice of antagonist, we aimed to use the most selective available vasopressin receptor antagonist, although neither of the antagonists had been used in human clinical studies, and like many before, had only been tested in rats. Using the higher antagonist infusion rate of 20 nmol/min and assuming forearm blood flow to be 20 to 50 mL /min (taking account of vasopressin induced vasodilatation during the dose range), we would have achieved a local tissue concentration of 4 nmol/mL of $d(CH_2)_5[Tyr(Me)^2, Dab^5]AVP$ (at highest forearm blood flow) in the studied vascular bed, thus markedly exceeding the plasma concentration of 0.1 nmol/mL of $d(CH_2)_5[Tyr(Me)^2]AVP$ required to antagonise the effects of vasopressin infused by

systemic intra-venous infusion [Gavras *et al* 1984]. The production of $d(CH_2)_5$[Tyr(Me)^2, Dab^5]AVP involved the substitution of Asn at position 5 with diaminobutyric acid which increases greatly the selectivity but not potency [Chan *et al* 1996], which could be a reason for the lack of an effect. To confirm antagonist activity, we carried out rat biopressor assays using pentobarbitone anaesthetised Wistar rats and were able to confirm antagonist activity and a pA_2 value of 8. However, there are at least 20 amino acids that differ within the extra-cellular loops of the rat and human V1 receptors [Thibonnier *et al* 2001]. These residues have been shown to be critical to the inter-species variation in the binding of the non-peptide V1 receptor antagonist OPC-21268 [Thibonnier *et al* 2000].

The original development of the V2 antagonist $d(CH_2)_5$[D-Ile^2-Ile^4-Ala^9]AVP was directed against renal V2 receptors [Saywer *et al,* 1988] and it is unknown how closely they resemble the vascular V2 like receptors, as no V2 receptors have been directly isolated from human vascular smooth muscle and endothelium to make the comparison.

In conclusion we have detected no change in forearm blood flow during V1 and V2 antagonist infusion alone nor change in the vascular activity of vasopressin. There are a number of possible reasons for the lack of activity including low potency, inter-species variation and that vascular and renal V2 receptors may have different physical characteristics.

CHAPTER 9

CONCLUSIONS AND FUTURE DIRECTIONS

9.1 UROTENSIN II

Overall, it has become clear that urotensin II causes diverse vascular effects in mammals, based not only on species [Douglas *et al* 2000], but also on the vascular region tested [Gibson 1987; Itoh *et al* 1987]. However, during our studies of urotensin II, we were unable to demonstrate changes in either vascular tone or systemic haemodynamics as assessed by local and systemic vascular studies.

Our findings were in contrast to the forearm studies carried out by Böhm and Pernow [Böhm and Pernow *et al* 2002]. When using a similar dose range they demonstrated a 31% reduction in forearm blood flow. There were a number of methodological differences, principally, in their case, not taking into account possible changes in the non-infused arm and not using the forearm blood flow ratio as the overall measurement, the optimal measure of assessing vasoconstriction. In scrutinising our own studies to ascertain why our results were different, we were able to confirm drug delivery to the chosen vascular beds and verify both the pharmacological authenticity, by the drugs amino acid sequence, and pharmacodynamic efficacy of our batch of urotensin II by studying its effects on rat aorta *in vitro*. This was not done by Böhm and Pernow.

It has recently been postulated that such variability, or lack of observed response, could be accounted for by the number of free receptors available for the ligand to exert its action [Douglas 2003]. The basis of Douglas's postulate comes from observations relating to pharmacokinetics of the ligand and receptor. Urotensin II binds with high

affinity {K_d ~ 300 pM) to its receptor with a very slow dissociation time [Ames *et al* 1999; Maguire *et al* 2000]. Thus if the majority of receptors are occupied and perhaps even activated, then no effect may be seen during the addition of exogenous urotensin II. Plasma urotensin II concentrations are in the nanomolar range [Affolter *et al* 2002; Dschietzig *et al* 2002; Heller *et al* 2002] which is well above the *Ki* of urotensin II for its receptor. Furthermore, radioligand studies have shown only sparse binding in human coronary and rodent aortic membranes [Maguire *et al* 2000; Itoh *et al* 1988]. It may well be the case that subtle changes in the numbers, and/or location of receptors expressed, is the primary method of controlling urotensin II vascular effects. Moreover, this mode of control would be less quickly exerted than a rapid rise in circulating hormone in the presence of numerous unoccupied receptors, perhaps making urotensin II a more chronic cardiovascular regulator than our short-term infusion studies have been able to show. It is entirely possible that while the search for vascular effects of urotensin II has not yielded significant results in humans it may have a completely different role in physiology to the one hypothesised on this thesis. Urotensin II is known to have effects in fish on membrane sodium transport, lipid and glucose metabolism [Bern *et al* 1985] and cortisol secretion [Kelsall and Balment 1998]. These observations, taken together with high expression of prepro-urotensin II in the human kidney [Nothacker *et al* 1999] and the recognition of conservation of function under evolutionary pressures, would be consistent with an endocrine role for urotensin II in sodium handling, and perhaps even in the metabolic syndrome.

9.2 UROTENSIN II: CLINICAL RELEVANCE AND FUTURE DIRECTIONS

Shortly after the completion of these healthy volunteer studies, a number of publications reported the plasma concentrations of urotensin II in various disease states. Interestingly several of the conditions seen to have elevated urotensin II concentrations are states associated with fluid overload, namely, cardiac failure [Richards *et al* 2002; Douglas *et al* 2002; Leong *et al* 2002; Dschietzig *et al* 2002], renal failure [Totsune *et al* 2001], cirrhosis and portal hypertension, especially when associated with ascites [Heller *et al* 2002] and hypertension [Cheung *et al* 2004]. Ong et al have recently reviewed other situations associated with high plasma concentrations of urotensin II but not characterised by overt fluid overload such as diabetes mellitus with or without proteinuria , mitogenesis, ischaemic heart disease and pulmonary hypertension [Ong *et al* 2005]. Limited skin blood flow studies have been performed in subjects with hypertension [Sondermeijer *et al* 2005], which is known to be associated with an elevated urotensin II plasma concentration. A comparison was made between hypertensive and normotensive volunteers with respective dose dependent vasoconstrictor and vasodilatation responses observed. This was suggestive of urotensin II having a contributory role in the increased vascular tone seen in those with hypertension.

Although urotensin II has been found in high concentrations, the mode of this increase is unknown. Whether this represents poor clearance or increased production is not clear. The actual site of urotensin II release is likely to be the kidney but little is known about

urotensin II metabolism and excretion. These points are highly relevant for the above conditions, all of which have altered renal function. Some investigators have also looked at the expression of urotensin II mRNA and receptor binding capacity and found these to be increased in the myocardium of the failing human heart [Douglas *et al* 2002], supporting the concept that urotensin II regulation may be at the receptor level.

The use of a highly selective human urotensin II receptor antagonist will no doubt help clarify what role urotensin II plays in health and disease in man. Although not wholly specific, a neuromedian B receptor antagonist, BIM-23127, has proven to be the most potent urotensin II antagonist to date [Herold *et al* 2003]. If indeed receptor numbers control the urotensin II response a well designed study will be required for any human *in vivo* antagonist study. Future studies might include using firstly health volunteers with a selective antagonist infused alone and then co-infusion with urotensin II in the forearm vascular bed. Secondly, antagonist infusion in patient groups with elevated plasma urotensin II concentrations may be revealing and helpful in delineating any pathophysiological role of urotensin II. If such short term studies were to still reveal no effects one may have to wait for an antagonist with a long half life so that temporal differences in vascular responses to antagonist alone and to repeated local and systemic infusion of urotensin II could be studied.

9.3 VASOPRESSIN

In part, our aim in using vasopressin was to increase our knowledge of vasopressin vascular responses before using vasopressin antagonists. There was also the opportunity to assess vasopressin pharmacodynamics, such as, tachyphylaxis, its on and offset, and the role of nitric oxide in vasopressin mediated vasodilatation. As a peptide to which urotensin II could be compared, vasopressin is a vasoactive peptide with control over renal tubular function and hence volume homeostasis, which itself has also been implicated in fluid overloaded states such as heart failure [Goldsmith *et al* 1983; Szatalowicz *et al* 1981] and end stage cirrhosis [Burmeister *et al* 1983; Akriviadis *et al* 1997].

We were able to reproduce the typical biphasic forearm vascular response to vasopressin at equivalent doses to other studies where low intra-brachial infusion rates caused vasoconstriction and higher rates caused a nitric oxide dependent vasodilatation [Affolter *et al* 2003]. During the nitric oxide clamp, enhanced vasoconstriction response to vasopressin was not observed, suggesting that the vascular V2 receptors are not stimulated by low vasopressin concentrations. Tachyphylaxis seen during prolonged infusion of vasopressin had not been demonstrated before. However, the oxytocin receptor, which is very similar to the vasopressin receptors, has been shown to undergo oxytocin induced desensitisation in cultured human myometrial myocytes, although this did take up to 4 hours before 50% of peak effect was lost [Robinson *et al* 2003]. It was interesting to note the possible relationship between the on and offset of forearm blood

flow responses and the known internalisation cycle of the vasopressin receptor/ligand complex [Thibonnier *et al* 2001]. Return to the cell surface after agonist displacement occurs, but at a reduced rate with waning of responses observed.

Although the peak effect is decreased and delayed, there are receptors capable of binding vasopressin, unlike the postulated paucity of free urotensin II receptors. It is also of note that circulating vasopressin concentrations are in the picomolar range [Landry and Oliver 2001], an order of magnitude less than urotensin II. Few data exist as regards the regulation of vasopressin receptor numbers in disease states, where high plasma vasopressin concentrations have been found [Goldsmith *et al* 1983; Szatalowicz *et al* 1981; Burmeister *et al* 1983; Akriviadis *et al* 1997].

The lack of effect of the peptidic antagonists has been a long term problem in vasopressin research. Almost all V1 peptide antagonists have some cross reactivity with V2 receptors and the most selective ones tend to be less potent. The V2 antagonists are even less selective, which creates problems in interpretation. We cannot conclusively say that our dosing protocols or drug preparation was not to blame for the lack of observed effect. Unfortunately the more selective and potent synthetic antagonists are currently not available to us for clinical use.

9.4 VASOPRESSIN: CLINCAL RELEVANCE AND FUTURE DIRECTIONS

Vasopressin antagonism has now become a therapeutic option for conditions such as heart failure, incorporating long acting non-peptidic orally available agents such as Tolvaptan, a selective V2 receptor antagonist which has led to a reduction in body weight and normalisation of serum sodium in patients with hyponatraemia and cardiac failure [Gheorghiade *et al* 2003]. The rationale for the use of a V2 antagonist is that a reduction in the V2 receptor mediated reabsorption of water, i.e. the induction of an aquaresis to accompany the loop diuretic driven natriuesis, would help ameliorate the fluid overload state in cardiac failure. As yet no long term studies have taken place and the most up to date results are tolerability studies. Vasopressin has also extended its role in medicine as an inotrope in critical care where it has been studied with beneficial effect in both haemorrhagic and septic shock [Dries 2003; Dunser *et al* 2002].

By gathering more information on the basic physiology of vasopressin, the methodology of antagonist studies can be refined. Of particular interest will be the effects of vasopressin and its antagonists on the endothelial cytokines such as Il-6, fibrinolytic and haemostatic functions. Vasopressin is known to induce platelet aggregation [Thibonnier *et al* 1999] and stimulation of the vascular V2 receptors causes release of t-PA, vWf, factor VIII:C, IL-6 and TNF [Newby *et al* 2000; Kaufmann *et al* 2000]. With the introduction of vasopressin antagonists to the therapeutic regimens for common cardiovascular conditions it will be important to know how the balance of these factors will change.

In looking to future studies, it seems reasonable to use forearm and skin blood flow models. It would be helpful if the basic vasopressin responses could be assessed in patients with cardiac failure where vasopressin plays a role in the pathophysiology. Ideally safe, non-toxic, efficacious and more receptor-specific vasopressin antagonists could be used to dissect physiology from pathophysiology, initially in healthy volunteers and then in patients with cardiac failure shown to have a supra-normal plasma vasopressin concentration.

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