THE CONTROL OF VASCULAR TONE IN PREGNANCY

Thesis presented for the Degree of PhD
In the Faculty of Neuroscience
Of the University of London

By
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University College London
IN MEMORY OF MY FATHER

1928 – 2003
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ABSTRACT

This thesis examines the regulation of peripheral and renal blood flow during human and rat pregnancy.

In healthy pregnancy, hand blood flow increases in order to release heat generated by metabolic activity. Using venous occlusion plethysmography, inhibition of nitric oxide (NO) synthase with intra-arterial \( \text{NO} \)-monomethyl-L-arginine (L-NMMA) caused a greater reduction of hand blood flow in pregnant compared with non-pregnant subjects. This suggests that increased NO synthase contributes to the gestational increase in peripheral blood flow.

A rise in local temperature also causes an increase in hand blood flow, which was attenuated by inhibition of NO synthase with intra arterial L-NMMA. Furthermore, the sensitivity of hand vasculature to noradrenaline and L-NMMA was temperature-dependent. Noradrenaline was more potent at low compared with high local temperatures and L-NMMA was more potent than noradrenaline at high temperatures.

Pre-eclampsia is a vasoconstricted state, unique to human pregnancy. Using high performance liquid chromatography (HPLC), plasma levels of the endogenous inhibitor of NO synthase, asymmetric dimethyl-L-arginine (ADMA), were higher in pre-eclamptic compared with normotensive pregnant women. ADMA excreted by fetal kidney contributed to rising ADMA levels in amniotic fluid as pregnancy progressed.
The isolated-perfused rat kidney (IPRK) was used to investigate the role of perivascular nerves in the gestational increase in renal blood flow. The vasoconstrictor response to electrical field stimulation (EFS) of perivascular nerves was attenuated in IPRK of late pregnant compared with virgin rats and was associated with a large fall in renal cortical neuropeptide Y levels. EFS of sensory-motor nerves and capsaicin caused renal vasodilatation, but desensitization of the responses to sensory-motor nerve stimulation did not augment the vasoconstrictor response to EFS. The vasodilator response to exogenous calcitonin gene related peptide (CGRP) was greater in kidneys from late pregnant compared with virgin rats. CGRP was identified by immuno-histochemistry in nerve fibres supplying arterioles in both pregnant and virgin rat kidneys.

Increased NOS-III was noted in the coronary arteries of pregnant compared with non-pregnant rats. Increased NOS-I was identified in the macula densa of the kidney and may play a role in altered auto-regulation of renal blood flow during pregnancy.

In conclusion, this thesis provides evidence that diverse vasoactive pathways in the peripheral and renal vasculature adapt to pregnancy and changes in temperature.
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<tr>
<td>AII</td>
<td>Angiotensin II</td>
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<td>ACh</td>
<td>Acetylcholine</td>
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<tr>
<td>ADMA</td>
<td>Asymmetric dimethyl-L-arginine</td>
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<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
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<td>AVP</td>
<td>Arginine-Vasopressin</td>
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<tr>
<td>CGMP</td>
<td>Cyclic guanosine 3,5-monophosphate</td>
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<td>CGRP</td>
<td>Calcitonin Gene Related Peptide</td>
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<tr>
<td>DDAH</td>
<td>Dimethylarginine dimethylaminohydrolase</td>
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<td>EDRF</td>
<td>Endothelial derived relaxing factor</td>
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<td>Electrical Field Stimulation</td>
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<tr>
<td>GFR</td>
<td>Glomerular Filtration rate</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>5'-HT</td>
<td>5-Hydroxytryptamine (serotonin)</td>
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<td>IPRK</td>
<td>Isolated Perfused Rat Kidney</td>
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<tr>
<td>L-NMMA</td>
<td>N⁰-monomethyl-L-arginine</td>
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<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
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<tr>
<td>NA</td>
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<tr>
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<td>Nicotinamide-adenine dinucleotide phosphate</td>
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<td>Substance P</td>
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<tr>
<td>SVR</td>
<td>Systemic vascular resistance</td>
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<td>VIP</td>
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PREFACE

This thesis originated from my interest in the mechanism of cardiovascular change during healthy pregnancy and the vasoconstricted-state of pre-eclampsia. The Introduction (Chapter 1) highlights the haemodynamic changes of healthy human and rat pregnancy and summarises the current understanding of mechanisms that control vascular tone in the non-pregnant and pregnant state. Particular emphasis is placed on the control of peripheral and renal blood flow and the L-arginine-nitric oxide pathway.

Chapter 2 summarises the Methods and Materials used to investigate different vascular control mechanisms. The experimental chapters include studies on humans (Chapters 3, 4 and 5) and on animals (Chapters 6 and 7).

Chapter 3 describes a study that measured the change in hand blood flow following a brachial artery infusion of the nitric oxide (NO) synthase inhibitor L-NMMA and the alpha-adrenoreceptor agonist, noradrenaline in pregnant and non-pregnant women. The greater response to L-NMMA in early- and late-pregnant women suggested that increased NO synthase activity may contribute to increased peripheral blood flow and attenuated vasoconstrictor response to noradrenaline during healthy pregnancy.

Hand blood flow increases in healthy pregnancy in order to liberate excessive heat from increased metabolic activity. I became intrigued by the possibility that changes in local temperature may alter vascular reactivity in a thermo-regulatory organ, such as the hand. In Chapter 4 hand blood flow was measure in healthy non-pregnant volunteers at hot
and cold temperatures to assess whether the response to intra-arterial NO synthase inhibition and noradrenaline differed when local temperature changes.

In Chapter 5, the plasma concentration of the endogenous inhibitor of NO synthase, asymmetrical dimethyl-L-arginine (ADMA) was measured in women with gestational hypertension and those with a normotensive pregnancy. As ADMA appears in high concentrations in urine, it was speculated that fetal urine might contribute ADMA to amniotic fluid. It is speculated that ADMA in amniotic fluid may influence fetal pulmonary vascular resistance and uterine smooth muscle contractions at term.

I set-up a model of the isolated perfused rat kidney (IPRK) to study the role of peri-vascular nerves (sympathetic and sensory-motor nerves) and their neuro-peptides on the increase in renal blood flow during rat pregnancy (Chapter 6). Immuno-histochemistry and biochemistry was used to compare the expression and content of calcitonin gene related peptide (CGRP), substance P and neuropeptide Y in the kidneys of pregnant and virgin rats.

In Chapter 7, the immuno-reactivity of all three isoforms of NO synthase was compared in kidneys and coronary arteries of pregnant and virgin rats. Differences between NOS-I in the macula densa and NOS-III in coronary arteries are highlighted.

In the General Discussion, Chapter 8, the focus is on the implications of the study observations for clinical practice and future research.
CHAPTER 1

INTRODUCTION
1.1 PHYSIOLOGICAL CHANGES DURING PREGNANCY

Since modern Homo sapiens emerged 100,000 years ago, it is estimated that the human population has increased from 50,000 to its current global level of 6,000 million. Such reproductive success has defied gross reproductive inefficiency. Only 20-35% of fertilised ova result in a successful pregnancy. Most fail around the time of implantation with chromosomal abnormalities. A high miscarriage rate prevents the mother from investing major physical resources towards a pregnancy unlikely to result in the propagation of parental genes. Even if a pregnancy is successful, usually only one offspring is produced after 9 months. Indeed, if the fetal brain was not programmed to outgrow the maternal pelvic outlet, anthropological comparisons with the great apes indicate that human pregnancy would last 16 months! (Steer, 1998).

1.2 Preparing for Pregnancy

The female body prepares for pregnancy during every menstrual cycle. It is not only the endometrium that anticipates implantation of a fertilised ovum, but the whole cardiovascular system. During the post-ovulatory or luteal phase of each menstrual cycle there is a decrease in systemic vascular resistance by approximately 20% that leads to a 10% fall in mean arterial pressure (MAP) compared with the follicular phase (Chapman et al, 1997). As a consequence, cardiac output increases by almost 20%. Renal vasodilatation increases blood flow to the kidneys and the glomerular filtration rate (GFR) by approximately 10%. All of these changes resolve with involution of the corpus luteum and onset of menses.
1.3 **Haemodynamic changes in human pregnancy**

If fertilisation is successful, the haemodynamic changes established in the menstrual cycle develop further (Robson et al, 1989; Chapman et al 1998). A progressive fall in systemic vascular resistance (SVR) by up to 40% creates a maximal decrease in MAP by the end of the first trimester (Redman, 1995). Diastolic blood pressure falls between 5-15 mmHg before rising to non-pregnancy levels at term (37 - 42 weeks gestation), while systolic BP remains unchanged throughout pregnancy. A gestational increase of heart rate from approximately 72 to 85 beats per minute and of stroke volume by up to 30%, combines with the reduction in systemic vascular resistance (SVR) and increase in circulating blood volume by approximately 40% (1.2L) to increase cardiac output (Robson, 1989; Brown & Gallery, 1994). By 24 weeks, cardiac output reaches a maximum of 50% above non-pregnant levels, which is sustained until term (Poppas et al, 1997). Left ventricular (LV) wall thickness and LV mass increase progressively throughout pregnancy by up to 30% and 50% respectively (Robson et al, 1989; Mesa et al, 1999). All of these changes are geared towards increasing blood flow to the developing fetus and to support increased metabolic demand of maternal organs.

1.4 **Distribution of increased cardiac output**

Although it is technically difficult to measure blood flow to certain maternal viscera during human pregnancy, it is clear that the timing and extent of changes to blood flow varies between organs (Figure 1.1). There is most agreement concerning renal blood flow that increases maximally by 80% at 20 weeks gestation, before declining in the third trimester to a level still 60% above non-pregnant values (Sturgiss et al, 1994). Conversely,
THE DISTRIBUTION OF INCREASED BLOOD FLOW DURING HEALTHY HUMAN PREGNANCY

Early increase in mammary blood flow (not quantified)

No change in hepatic blood flow

10-fold increase in uterine artery blood flow, mainly after 24 weeks

Small (approximately 10%) and gradual increase in cerebral blood flow

50% increase in cardiac output by 24 weeks

80% increase in renal blood flow by 20 weeks; decline to 60% in the third trimester

Gradual increase in peripheral blood flow. Greater than 200% by term.
uterine artery blood flow changes very little during the first trimester, but after 24 weeks there is a precipitous rise. Overall, uterine artery blood flow increases from 50 to 500ml min\(^{-1}\), proportionately more than any other maternal organ (de Swiet, 1998).

Hepatic blood flow is particularly difficult to measure in vivo, but serial measurements throughout pregnancy suggest it does not change (Robson et al, 1990). Blood flow-velocity within the middle cerebral artery decreases with advancing gestation, suggesting vasodilatation (Serra-Serra, et al 1997). Regional cerebral blood flow in early pregnancy (7-19 weeks) is approximately 10% higher in all areas of the brain, except the occipital cortex, where there is no change (Serra-Serra, et al 1997). However, brain size diminishes during pregnancy, suggesting a fall in cerebral blood flow - that is supposed to return to normal postpartum! (Oatridge et al, 2002). Mammary artery blood flow increases early in pregnancy, but precise measurements are lacking. Breast tenderness and swelling are amongst the first symptoms of pregnancy. Skin blood flow increases gradually throughout pregnancy by at least 200% (Abramson et al, 1943). Warm hands and palmar erythema provide clinical evidence for increased peripheral blood flow. Unravelling the mechanism of these widespread, but heterogeneous vascular changes in human pregnancy has proved challenging.
1.5 Control of vascular tone

As a prelude to discussion of the mechanisms that control vascular tone in pregnancy, a general review of the control of vascular tone is presented.

Vascular tone is a balance between vasodilator and vasoconstrictor influences. These opposing effects on vascular smooth muscle are mediated by neurotransmitters released from peri-vascular nerves and by vaso-active substances acting on the endothelium, which in turn influence vascular smooth muscle tone (Burnstock, 1990; Burnstock, 1993; Ralevic and Burnstock, 1995). Vascular smooth muscle itself has intrinsic myogenic tone, which is characterized by vasoconstriction in response to mechanical changes in pressure (Hill et al, 2001) (Figure 1.2).

**Figure 1.2** The control of vascular tone is a balance between the influences of peri-vascular nerves, the endothelium and myogenic tone within vascular smooth muscle itself. Vasoconstrictor tone is mediated by sympathetic nerves, endothelin, circulating hormones and myogenic tone. Vasodilator influences are mediated by nitric oxide, vasodilatory prostacyclin, endothelial derived hyperpolarizing factor and shear stress (By kind permission from R. McAllister).
1.6 Perivascular nerves

The muscular layer (media) of resistance blood vessels is covered in an outer network of terminal nerve fibres. Terminal axons are rich in varicosities (0.5-2μm in diameter) that contain neurotransmitters, which are released en passant during the conduction of a nerve impulse (Burnstock et al, 1984). Unlike the classic synapse of the skeletal neuromuscular junction, a fixed relationship between varicosities and smooth muscle cells is not a feature of the autonomic neuroeffector junction. The junctional cleft can vary between 50 and 2000nm, depending on the size of the vessel and it has been suggested that the wide cleft predisposes the autonomic neuroeffector junction to both pre- and post-junctional modulatory influences from locally released transmitters from the same or adjacent nerve terminals, or by circulating neurohormones or local agents such as prostanoids, bradykinin, histamine or angiotensin I (Ralevic and Burnstock, 1995).

The characteristics of the peri-vascular network of nerves vary between vascular beds and across species. In addition to the classically recognized noradrenaline (NA) released from sympathetic nerves and acetylcholine (ACh) from parasympathetic nerves (Burnstock, 1987), varicosities contain many more neurotransmitters and neuro-modulators. Neuro-transmitters are packaged into characteristic combinations as NA is usually found with adenosine 5'-triphosphate (ATP) and the neuromodulator neuropeptide Y (NPY) in sympathetic nerves, ACh is found with vasoactive intestinal polypeptide (VIP) in parasympathetic nerves, and substance P (SP) is found with calcitonin gene-related peptide (CGRP) and ATP in sensory-motor nerves (Lincoln and Burnstock 1990). Co-transmission allows enhanced fine control and complexity of neurotransmission via pre
and post-junctional modulatory mechanisms. Neuromodulation can also take place between transmitters released from adjacent terminals of different populations of nerves and this has been termed ‘cross-talk’ (Burnstock, 1993).

1.7 **Sympathetic peri-vascular nerves**

Stimulation of sympathetic nerves classically leads to vasoconstriction via the release of noradrenaline (NA), which activates postsynaptic α-adrenoreceptors (Burnstock, 1986). As the sympathetic nervous system makes up the division of perivascular nerves that mediates vasoconstrictor tone, it has received much attention in understanding hypertension. However, the sympathetic nervous system is strongly influenced by co-release of the neuro-transmitter ATP acting on P2X-purinoreceptors (Meldrum and Burnstock, 1983; Burnstock, 1988). The relative contributions of NA and ATP vary between species, vascular beds (Ralevic and Burnstock, 1998) and according to the frequency of nerve stimulation. Low frequencies favour ATP release and high frequencies stimulate the noradrenergic component (Kennedy et al, 1986). The neuro-modulator NPY is co-stored and co-released with NA and ATP and usually enhances the vasoconstrictor effects of sympathetic nerve stimulation (Hieble, et al, 1989).

1.8 **Parasympathetic peri-vascular nerves**

Parasympathetic nerves typically exert opposite actions to the vasoconstrictor effects of sympathetic nerves. They also release a number of different transmitters, including acetylcholine (ACh), vasoactive intestinal peptide (VIP) and nitric oxide (NO) (Yoshida and Toda, 1997). However, ACh only rarely causes vasodilatation directly on the
vascular smooth muscle, more commonly requiring the endothelium to mediate vasodilatation (Furchgott and Zawadzki, 1980). It is unlikely that ACh released from peri-vascular nerves passes through vascular smooth muscle before entering the endothelium and then mediating relaxation of the vascular smooth muscle (Ralevic and Burnstock, 1995). Rather ACh is synthesized, stored and released from endothelial cells during shear stress to activate receptors on endothelial cells (Parnavelas et al, 1985).

1.9 Sensory-motor peri-vascular nerves

Sensory nerves have been shown to store and release SP, CGRP and ATP (Burnstock, 1993). A network of peri-vascular sensory-motor neurons are so-called because they have an afferent limb that takes sensory impulses to the spinal cord and an efferent limb that elicits immediate motor responses on vascular smooth muscle (Burnstock, 1993; Rubino and Burnstock, 1996). This dual afferent and efferent function is mediated either through an axon-reflex arrangement or by release of neurotransmitters from the same terminal that is excited by the environmental stimulus (Maggi & Meli, 1988).

Sensory-motor neurons are susceptible to the stimulatory and neurotoxic actions of capsaicin (Maggi & Meli, 1988). When capsaicin is applied to a neuro-vascular preparation, it will bind to specific neuronal receptors, causing depolarisation and neurotransmitter release. This is followed by desensitisation and a lack of functional response to a further application of capsaicin or electrical field stimulation (Holzer, 1991). CGRP and SP co-exist within the same granular vesicles in many nerve terminals and are consequently co-released (Gulbenkian et al, 1986). Both neuro-peptides interact with specific receptors
Calcitonin gene related peptide is a 37 amino acid peptide produced from the calcitonin gene in those tissues operating alternative RNA processing from that in thyroidal C cells (Amara et al, 1982). Although CGRP is of similar size to calcitonin (32 amino acids in length) and has a disulfide ring within the terminal amino region, there is little sequence homology between the two peptides. Furthermore, there are discrete receptor sites for both calcitonin and CGRP in the nervous system and peripheral tissues (Goltzman and Mitchell, 1985). However, each peptide can cross-react with the others' receptor (Goltzman and Mitchell, 1985). There are also reciprocal interactions (cross-talk) with both sympathetic and parasympathetic branches of the autonomic nervous system (Rubino and Burnstock, 1996). CGRP is a potent vasodilator. Systemic administration of CGRP to rats reduces MAP and renal vascular resistance, while mediating a rise in GFR (Siren and Feuerstein, 1988; Amuchastegui et al, 1994).

1.10 The endothelium

Until 1980, the endothelium was regarded as an inert barrier between blood and vascular smooth muscle. After the discovery that the endothelium can also modulate vascular tone by producing labile substances that relax (Furchgott and Zawadzki, 1980) and constrict (Yanagisawa et al, 1988) smooth muscle, it was clear that the role of the endothelium was far more complex. The ability of the endothelium to control blood flow is
matched by its ability to maintain a balance between blood fluidity and thrombosis, as well as a regulator of cell growth within the vessel wall (Vallance and Webb, 2000).

Stimulation of endothelial receptors activates endothelial pathways that mediate either relaxation or constriction of vascular smooth muscle. Endothelial responses are triggered by ACh, ATP, serotonin (5-HT), SP, angiotensin II (AII), vasopressin, (AVP), histamine, bradykinin and vasoactive hormones (Vanhoutte and Rimele, 1983; Lincoln and Burnstock, 1990; Hill et al, 2001). Endothelial derived vasoactive factors that then have an influence on vascular smooth muscle tone include, prostaglandins (Moncada et al, 1976; Mombouli and Vanhoutte, 1999), which are both vasodilatory (prostacyclin) and vasoconstrictor (thromboxane); endothelins, which are predominantly vasoconstrictor (Yanagisawa et al, 1988; Bagnall and Webb, 2000); endothelial derived hyper-polarizing factor (EDHF), which is predominantly vasodilator (Chen et al, 1988; Garland et al, 1995), but has still not been fully characterized; and nitric oxide, which is a vasodilator (Palmer et al, 1987; Vallance et al, 1989).

The vessel wall is therefore composed of a highly sophisticated network of perivascular nerves and endothelial factors that influence each other and ultimately underlying vascular tone (Figure 1.3). One of the pathways investigated in this thesis is the L-arginine-nitric oxide pathway and its influence on the control of vascular tone in pregnancy. The physiology of this pathway is elaborated upon further.
Figure 1.3 Regulation of vascular tone by peri-vascular nerves and endothelium.

Neuropeptide Y (NPY), noradrenaline (NA), adenosine 5'-triphosphate (ATP), calcitonin gene-related peptide (CGRP), substance P (SP), and vasoactive polypeptide (VIP) can be released from nerve varicosities in the adventitia (ADV) to act on receptors in the media (MED), causing vasoconstriction or vasodilatation. ATP, acetylcholine (ACh), 5-hydroxytryptamine (5-HT) and SP released from endothelial cells (END) by shear stress or hypoxia act on their receptors on endothelial cells to cause a release of endothelium-dependent relaxing factor/nitric oxide (EDRF/NO) or prostaglandins (PG), which in turn act on the smooth muscle to cause relaxation. Angiotensin II (AII), arginine-vasopressin (VP) and histamine (H) are also contained in and may be released from sub-populations of endothelial cells. In areas denuded of endothelial cells, opposite effects may be produced by receptors on the smooth muscle cells, for example via P2X- and P2Y-purinoceptors and muscarinic receptors (M). (From Burnstock, 1993).
1.11 L-arginine-nitric oxide pathway

Nitric oxide is a labile free radical synthesised from one of the guanidine-nitrogen atoms of L-arginine by three different isoforms of nitric oxide synthase, yielding L-citrulline as a by-product (Palmer et al 1987; Ignarro et al, 1987; Palmer et al, 1988). Nitric oxide (NO) has a short half-life (10-60s) (Knowles and Moncada, 1992) and is rapidly oxidized to nitrite and then nitrate by oxygenated haemoglobin, molecular oxygen and superoxide anions, before being excreted in the urine (Wennmalm et al, 1992; Moncada and Higgs, 1993). Nitric oxide synthases require several co-factors including nicotinamide-adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH₄), calmodulin and oxygen, in order to generate NO from L-arginine (Bredt and Snyder, 1990; Lewis et al, 1993). Nitric oxide derived from endothelium diffuses across into vascular smooth muscle cells where it stimulates guanylate cyclase to generate cyclic guanosine 3,5-monophosphate (cGMP) from guanosine triphosphate (GTP) (Ignarro et al, 1984; Moncada and Higgs, 1993). The second messenger cGMP, mediates most of the biological functions of NO, including control of vascular tone, platelet activation and neurotransmission (Chan and Vallance, 2002; Figure 1.4).

Three NOS isoforms are found within many different mammalian cell types and influence a myriad of physiological processes (Forstermann et al, 1994; Lincoln et al, 1997). They are classified by the place in which they were originally identified and where they are most commonly found. Neuronal NOS (NOS-I) is a low-output enzyme that is constitutively expressed and is regulated by both calcium (Ca²⁺) and calmodulin (Mayer et al, 1990). Inducible NOS (NOS-II) is a high-output enzyme that is upregulated by
cytokines and as it is irreversibly bound to calcium/calmodulin, it is independent of \( \text{Ca}^{2+} \) and is predominantly found in macrophages (Xie et al, 1992). Endothelial NOS (NOS-III) is a low-output enzyme that is constitutively expressed and regulated by \( \text{Ca}^{2+} \) and calmodulin (Forstermann et al, 1994).

![Diagram of the L-arginine - nitric oxide pathway](attachment:image.png)

**Figure 1.4:** The L-arginine - nitric oxide pathway (With permission from Chan and Vallance, 2002).

Endothelial NOS can be activated by several routes. Shear stress is an important physiological stimulus to activation of NOS-III leading to vasodilatation (Davies, 1995).
NOS-III can also be activated by autacoids such as bradykinin (BK) and histamine, platelet derived mediators, such as serotonin and adenine diphosphate and hormones such as oestrogen (Chen and Vallance, 2002). Binding to their receptors on the endothelial surface leads to the influx of calcium, which binds with calmodulin, and triggers activity of the enzyme.

1.12 Endogenous inhibitors of nitric oxide synthase

L-NMMA is a naturally occurring substance in human plasma and urine (Kakimoto and Akazawa, 1970; Park et al, 1988). However, two other methylated analogues of L-arginine, NΩ, NΩ-dimethylarginine (asymmetric dimethyl-L-arginine, ADMA) and its isomer symmetrical dimethylarginine (SDMA), circulate in much higher concentrations than that of L-NMMA (Vallance et al, 1992). These arginine analogues (Figure 1.5) were first identified in human urine in 1970 (Kakimoto and Akazawa, 1970), before the significance of the L-arginine – NO pathway had been recognized.

Purification and infusion of ADMA into animals caused an increase in systemic blood pressure, whereas SDMA was inactive (Vallance, 1992). Local infusion of ADMA into the brachial artery of human volunteers reduced forearm blood flow by the same degree as L-NMMA, an effect that was attenuated by L-arginine (Vallance et al, 1992). Therefore both L-NMMA and ADMA are of similar potency, but in health the circulating concentration of ADMA is ten-fold greater than L-NMMA (Vallance et al, 1992).
Figure 1.5 Structures of endogenous methylarginines compared with the structure of L-arginine (*signifies the terminal guanidine nitrogens of L-arginine).

ADMA is synthesized by human endothelial cells (Fickling et al, 1993; MacAllister et al, 1994) and is metabolized by dimethylarginine dimethylaminohydrolase (DDAH) to L-citrulline and dimethylamine (MacAllister et al, 1996). DDAH is functionally active within blood vessels and is itself inhibited by a structural analogue of ADMA, S-2-amino-4-(3-methylguanodino) butanoic acid, (4124W) (MacAllister et al, 1996) (Figure 1.6).
Inhibition of DDAH leads to an increase in intracellular ADMA concentration and inhibition of NOS (MacAllister et al, 1996). DDAH activity is also itself reversibly inhibited by nitric oxide-induced S-nitrosylation of a reactive cysteine residue (Leiper et al, 2002). Endogenous ADMA and the control of its metabolism, therefore provides further mechanisms by which NOS activity can be regulated (Figure 1.6).

**Figure 1.6**  Model for the regulation of intracellular ADMA and nitric oxide synthase. (adapted from Leiper and Vallance, Cardiovasc. Res., 1999; 43, 542-548).

1.13  **Assessment of Nitric Oxide Synthase Pathway**

It is possible to assess the activity of the L-arginine-nitric oxide pathway by using molecular, biochemical, immuno-histochemical or functional studies. The distribution of NO synthases can be determined using antibodies to the different isoforms of NOS and to
NADPH diaphorase (Forstermann, et al, 1994). Northern blotting can detect messenger RNA for NO synthases and antibodies to NO synthase can be used to measure protein expression by Western blotting.

As NO has a short half-life and is present in small amounts, direct quantification of NOS activity is difficult. Attempts have been made to use a porphyrin-based microsensor in vivo to detect free NO (Vallance et al, 1995), but have not proved reliable to instability of NO in vivo. The presence of NO can be measured by assays that indirectly reflect its production (Archer, 1993). Stable oxidation metabolites e.g. nitrite and nitrate in plasma and urine are limited by their ability to distinguish dietary and other exogenous nitrate from endogenous nitrate (Baylis and Vallance, 1998). This difficulty can be overcome by quantifying $^{15}$N nitrate excretion in urine after intravenous administration of the isotope L-[$^{15}$N]$_2$-guanidino arginine (Forte et al, 1997). Although this method will give no indication to the cellular origin of NOS. Changes in the concentration of the NO second messenger, cGMP have also been widely used, but as cGMP is also the second messenger for atrial natriuretic factor (ANF) it is difficult to interpret results (see later).

Functional in vivo and ex vivo studies have focused on the effects of stimulating and inhibiting NO synthesis. Endothelium dependent agonists such as ACh and BK have been used to assess the role of NO in vasodilatation in animals and humans and these studies in relation to pregnancy will be discussed later. The use of an NOS inhibitor allows direct comparison of NOS activity between groups. Several non-specific NOS inhibitors (e.g. $N^G$ monomethyl-L-arginine (L-NMMA), $N^G$ nitro-L-arginine (L-NOARG) and $N^G$
The gene for each NOS enzyme has now been cloned and mice with one or other of the NOS isoforms genes deleted 'knocked out' have been produced (Shesely et al, 2001). Interestingly, these mice breed well and have uneventful normotensive pregnancies (Shesley et al, 2001). This observation possibly reflects one of the drawbacks of gene 'knock-out' models, in that deletion of one gene often leads to upregulation of compensatory mechanisms. Whether it is possible to knockout all three isoforms of NOS and have a viable litter is unclear.

1.2 CONTROL OF PERIPHERAL VASCULAR TONE IN PREGNANCY

Although the precise mechanism of maternal vasodilatation is likely to be different in different vascular beds, a healthy endothelium is essential for the healthy cardiovascular adaptation to pregnancy. The endothelium-derived vasodilators, nitric oxide, prostacyclin and endothelial derived hyper-polarising factor have all been implicated in the gestational fall of systemic vascular resistance (see later). Conversely, the role of perivascular nerves has been very briefly investigated in pregnancy.
1.21 Perivascular nerves in pregnancy

In 1950, a study was performed on healthy, normotensive pregnant women, (that would be unlikely to receive ethical approval today), in which they were given tetraethylammonium chloride (TEAC) or high spinal anaesthesia in order to cause systemic blockade of the autonomic nervous system (Assali and Prystowsky 1950). The effect was to cause a dramatic fall in blood pressure. Despite the potential non-specificity of this method, this unique study would suggest that sympathetic nerve activity is essential during healthy pregnancy in order to maintain vascular tone. These early observations have been confirmed by direct recordings from peripheral muscle sympathetic nerves, which showed increased sympathetic output during the latter months of healthy pregnancy (Greenwood et al, 2001).

During pregnancy, plasticity of perivascular innervation is evident in the uterine artery of the guinea-pig, where perivascular noradrenergic nerves decrease and sensory-motor innervation increase (Bell and Malcolm, 1978). During rat pregnancy, the sensory-motor neuropeptide CGRP becomes even more potent at reducing MAP and relaxing the uterine artery (Gangula et al, 1999; Nelson et al, 1993). However, in the isolated perfused mesentery sympathetic nerve vasoconstrictor responses are decreased in the late pregnant rat, whereas sensory-motor responses were unchanged (Ralevic and Burnstock, 1996). Furthermore, in ovariectomised rats, administration of either progesterone or oestrogen enhances the fall in MAP to CGRP, suggesting a sex hormone induced increase in vascular sensitivity to the vasodilatory effects of CGRP (Gangula et al, 1999).
1.22 L-arginine-nitric oxide pathway in pregnancy

The study of pregnant women is difficult because of the unknown, but critical vulnerability of a developing fetus to pharmacological manipulation. Many investigators have therefore concentrated upon gestationally related changes in cardiovascular function of a variety of animals - with frequently conflicting results.

There is much evidence from animal studies to suggest that increased activity of the L-arginine - NO pathway contributes to the generalised vasodilatation and attenuated response to exogenous vasoconstrictors characteristic of pregnancy (Chu and Beilin 1993; Conrad et al, 1993; Weiner et al, 1994). Furthermore, the inhibition of nitric oxide synthase in pregnant rats abolishes the refractoriness to vasopressors and produces elevation of the blood pressure and proteinuria; signs of pre-eclampsia (see later) (Yallampalli and Garfield, 1993; Sladek et al, 1997).

Assessment of the L-arginine - nitric oxide pathway in human pregnancy and pre-eclampsia has proved more challenging. Different methodologies and conflicting results present a confusing picture. It is much easier to find evidence that the L-arginine-NO pathway is up-regulated in animal than in human pregnancy. This largely reflects the controlled conditions in which animals live compared with humans. For example, measurements of the stable oxidation products nitrite (NO2) and nitrate (NO3) have revealed a wide range of different results. Efforts to measure 24 hour urinary excretion of NO2 and NO3 (often combined to give NOx), are influenced by nitrogen intake in the diet,
absorption from the gastro-intestinal tract, renal handling of NOx as well as de novo synthesis (National Academy of Sciences, 1981).

1.3 Healthy human pregnancy

1.31 Cyclic guanosine monophosphate (cGMP)

The cyclic nucleotide, cGMP acts as a second messenger for NO (Figure 1.X) and has been used as a surrogate marker for NOS activity. In human pregnancy, there is agreement that urinary concentrations of cGMP increase early in pregnancy and remain elevated until term (Kopp et al, 1977; Chapman et al, 1998). Cyclic-GMP clearance increases as early as the sixth week of gestation, in parallel with a fall in plasma cGMP levels that persists until the end of pregnancy (Chapman et al, 1998). Others have found slight or significant increases in plasma cGMP during normal pregnancy, remaining high until term (Schneider, et al 1996; Boccardo et al, 1996). Boccardo et al, 1996 found cGMP levels and [3H] L-citrulline production within platelets were similar between pregnant and non-pregnant women, suggesting platelets do not contribute to excessive NO production in pregnancy.

Cyclic GMP concentrations in plasma or urine are only an indirect indication of NOS activity as responses to atrial natriuretic peptide (ANP) are also mediated through cGMP. In a meta-analysis of 53 studies it was concluded that the circulating concentration of ANP did not rise until the third trimester (Castro et al, 1994). However, this is long after the increase in urinary cGMP, which coincides with the gestational increase in renal blood flow and glomerular filtration rate (Sturgiss et al, 1994; Roberts et al, 1996). It is possible
that increased NOS activity has a role in vasodilatation during healthy human pregnancy, but it cannot be concluded from the measurement of cGMP alone that increased NOS activity plays a role in the gestational fall in systemic vascular resistance.

1.32 Oxidation products of nitric oxide; nitrite and nitrate

Several investigators have measured the serum concentration of nitrite (NO$_2$) and nitrate (NO$_3$), or their product, NOx, during healthy pregnancy. However, most have ignored the confounding problem that concentrations of these NO metabolites are sensitive to dietary nitrogen intake. Not surprisingly, studies of normotensive pregnant women on uncontrolled nitrogen diets have shown either increased (Seligman et al, 1994) or unchanged (Curtis et al, 1995; Smarason et al, 1997) plasma NO metabolites in comparison with non-pregnant women. In one study, plasma NOx levels were measured after a 12-15 hour fast, and found to be significantly elevated in normotensive pregnancy (from before 12 weeks gestation until term) compared with non-pregnant women (Nobunaga et al, 1996), but this was not supported by another diet controlled study (Conrad et al, 1999). In another carefully controlled study, guanidino [N$^{15}$] L-arginine was infused into five healthy pregnant volunteers after being on a nitrate free diet for the preceding week and a 12 hour fast pre-infusion. Arginine flux and nitrite/nitrate pool turnover were higher in early compared with late pregnancy, suggesting NO production is higher in early pregnancy (Goodrum et al 1996).
1.33 Nitric oxide synthase activity in vivo

*In vivo* functional studies provide the most compelling evidence that NO synthase is upregulated in the maternal circulation during normal pregnancy. L-NMMA induces a non-sustained vеноconstriction in hand veins of healthy women in the early puerperium, but not in the same women 12-16 weeks later (Ford et al, 1996). In the non-gravid state, infusion of L-NMMA, at a dose that maximally inhibits bradykinin, does not produce vеноconstriction of hand veins (Vallance et al, 1989). Isolated endothelial cells from hand veins of pregnant women have been shown to respond to ATP with a large transient increase in intracellular Ca\(^{2+}\) (Mahdy et al 1998). This response was significantly greater in endothelial cells isolated from the hand veins of healthy pregnant women compared to non-pregnant and pre-eclamptic women (Mahdy et al 1998).

The relative effect of an infusion of an NOS inhibitor (L-NMMA) into the brachial artery causes a greater reduction in forearm blood flow of pregnant compared with non-pregnant women (Anumba et al, 1999a). Further studies revealed that forearm blood flow responses to the nitric oxide donors glyceryl trinitrate (GTN) and sodium nitroprusside (SNP) were similar in non-pregnant, pregnant and pre-eclamptic subjects (Anumba, 1999b). Taken together, these observations suggest that increased nitric oxide synthase activity rather than increased sensitivity to nitric oxide is responsible for increased forearm blood flow in pregnancy. However, forearm blood flow responses to serotonin, a nitric oxide dependent vasodilator were diminished in pregnancy compared with non-pregnant subjects (Anumba, 1999b). This may be due to the limited availability of L-arginine at times of high basal nitric oxide synthase activity, or alterations to the signal transduction pathway of
serotonin induced forearm vasodilatation (Anumba et al., 1999b). Anumba et al went on to eliminate the possibility that shear stress might have activated NOS, by reducing the elevated forearm blood flow in pregnant volunteers to the same level of non-pregnant subjects and still found an increased reduction in blood flow to L-NMMA (Anumba et al., 2001).

In this thesis, venous occlusion plethysmography was used to measure changes in hand blood flow during pregnancy. Hand blood flow was studied since it is representative of skin blood flow and, unlike the predominantly muscular forearm vascular bed, flow in the hand has been reported to increase 2-6 fold by late pregnancy (Abramson et al., 1943; Ginsberg and Duncan, 1967). The response to the NOS inhibitor L-NMMA on hand blood flow was compared with the response to an endothelium-independent vasoconstrictor, noradrenaline. Other vasoconstrictors have shown an attenuated pressor response in healthy human pregnancy, in particular angiotensin II (Gant et al., 1973; Benjamin et al., 1991).

1.34 Nitric oxide in the resistance vasculature of the maternal circulation

Limitation in the availability of human tissue is an obvious drawback to investigation in human pregnancies. However, small arteries may be obtained from subcutaneous fat, omentum and myometrium by biopsy at caesarean section. The development of a variety of techniques for reproducible investigation of small artery tension and diameter has greatly facilitated studies in human tissue, as small biopsies may provide adequate material for experiment. The methods most frequently used have been small vessel wire myography and small vessel perfusion myography that allow the
measurement of isometric tension in arteries as small as 150μm internal diameter (Mulvany and Halpern, 1977; Halpern et al, 1984).

Blood flow to the skin is greatly enhanced in human pregnancy (Abramson, 1943) and investigation of this circulation may therefore provide insight into gestationally related mechanisms of dilatation. Small subcutaneous arteries can be obtained by removal of biopsies of subcutaneous fat from consenting women at caesarean section. The first study of these arteries, using the wire myograph, investigated responses to ACh in arteries of mean internal diameter 250-300μm and showed that relaxation to ACh was no different between arteries from pregnant women and those from non-pregnant women obtained during routine abdominal surgery (McCarthy et al, 1994). Interestingly, the NO synthase inhibitor, L-NMMA failed to completely inhibit relaxation to ACh, and indomethacin had little effect. The residual relaxation to ACh in the presence of the NOS inhibitor was greater in the arteries from the pregnant women and could suggest increased synthesis of an endothelium dependent dilator other than NO or PGI₂, potentially an endothelium derived hyper-polarizing factor (EDHF). In a later study, the same group (Knock and Poston, 1996) found that pregnancy was associated with increased relaxation to bradykinin (BK) in small subcutaneous arteries, leading to the suggestion that elements of the signal transduction pathway for BK were preferentially affected by pregnancy.

In contrast, using arteries from the omental circulation Pascoal et al (1996) concluded that neither ACh nor BK-mediated relaxation was different in arteries from term pregnant women and non-pregnant women, although pregnancy was associated with
increase in a novel component of BK-mediated relaxation, possibly a hyper-polarising factor (Pascoal et al, 1996). Another study using the wire myograph has also shown no difference in relaxation to BK in small myometrial arteries from pregnant women and from non-pregnant women obtained during hysterectomy (Ashworth et al, 1997) although structurally these arteries might be expected to be very different from one another. However, Kublickiene et al (1997a, 2000) have found that if mounted on a perfusion myograph, small myometrial arteries from pregnant women respond well to ACh and that this relaxation is greater than relaxation to ACh in omental arteries from term pregnant women, perhaps indicating that enhanced receptor mediated relaxation may contribute to increased myometrial blood flow in pregnancy. Taken together these studies show little consensus regarding the role of agonist stimulated NO synthesis in vasodilatation of pregnancy, perhaps because of the different vascular beds studied.

1.35 Flow mediated vasodilatation

There is more agreement that flow mediated NO synthesis is raised in the resistance vasculature in human pregnancy. Cockell and Poston (1997) have shown that the subcutaneous arteries from pregnant women demonstrate a remarkably increased response to flow compared to those from non-pregnant women, which was totally inhibited by L-NAME. This substantiated earlier work in arteries from pregnant rats (Learmont et al, 1996; Cockell and Poston, 1996) showing enhanced flow mediated dilatation, an observation confirmed by Ahokas et al, (1997). Using the same technique, NO mediated responses to flow have been observed in small myometrial arteries from women at term (Klubickiene et al, 1997b).
Fluid shear stress can also be observed in vivo by applying a distal cuff around the lower arm or wrist. Release of the cuff leads to hyperaemic dilatation and an increase in flow (and shear stress) in the brachial artery. This vasodilatation is predominantly mediated by NO, which may be monitored with high-resolution Doppler ultrasound, or by venous occlusion plethysmography (Anumba et al, 2002). Using this method it has been shown that pregnant women demonstrate an increased response to flow compared with nonpregnant subjects (Veille et al, 1998; Dorup et al, 1999). Taken together these ex vivo and in vivo studies support the hypothesis that an enhanced response to shear stress is an important stimulus to vasodilatation in pregnancy.

1.36 Mechanisms of vasomotor actions of oestrogens during pregnancy

The onset of physiological change during the menstrual cycle suggests that maternal rather than feto-placental factors initiate gestational adaptation. Oestrogen, mainly in the form of 17B-oestradiol is a potent vasodilator (Mendelsohn and Karas, 1999). 17B-oestradiol is produced by the corpus luteum during the luteal phase of each menstrual cycle and for the first 10 weeks of pregnancy. After 10 weeks, the placenta elaborates its own 17B-oestradiol, so that by 40 weeks gestation, maternal oestradiol levels are approximately 250 fold higher than those found during the menstrual cycle (Edouard et al 1998; Chapman et al, 1999).

17B-oestradiol causes vasodilatation by action on both vascular endothelium and smooth muscle cells (Karas et al, 1994; Farhat et al, 1996; Ma et al, 1997). Both vascular
cell types possess two oestrogen receptors, (o)estrogen receptor alpha (ERα) (Walter et al, 1985) and (o)estrogen receptor beta (ERβ) (Kuiper et al, 1996). These receptors are members of the steroid hormone receptor super-family and like all members of this super-family they are transcription factors that alter gene expression when activated. ERα activates the genes that produce prostacyclin synthase, endothelial and inducible nitric oxide synthase, vascular-cell adhesion molecule (VCAM), matrix metalloproteinase 2, collagen and vascular endothelial growth factor (VEGF) (Mendelsohn and Karas, 1999).

On the endothelial NOS gene 5' flanking 'promotor' region there are 11 copies of an incomplete (half palindromic motif) estrogen response element (ERE) (Robinson et al, 1994). In other genes these ‘half motifs’ interact to form a complete ERE and the occupied oestrogen receptor may activate NOS-III by binding to these regions.

ERβ is structurally and functionally different from ERα, but when stimulated by oestrogen it is similarly protective against vascular injury. The level of expression of ERα and ERβ varies between vascular beds, between men and women and between disease states. This provides one explanation for different tissue responses, but cell-specific differences, with the same level of ERα may be explained by differences in receptor-associated co-activator or co-regulatory proteins. These proteins may assemble in unique combinations to influence the response to oestrogen stimulation. In some tissues where both receptors are present the two types of receptor form a heterodimer, which further complicates their action once activated by oestrogen (Mendelsohn and Karas, 1999).
The effect of oestrogen on genes that produce vasodilatory enzymes leads to long-term effects. Oestrogen can also act on the vascular wall to induce rapid non-genomic changes in vascular tone. This rapid response can occur through two predominant systems, either through nitric oxide released from the endothelium (Lantin-Hermoso et al, 1997; Chen et al, 1999) or the opening of calcium-activated potassium channels through a nitric oxide – cyclic GMP dependent pathway in vascular smooth muscle (White et al, 1995).

The mechanism by which oestrogen acutely activates nitric oxide synthase has not been fully elucidated, but it probably occurs through a novel action of ERα on the plasma membrane (Russell et al, 2000) and is blocked by anti-oestrogens (Mendelsohn and Karas, 1999). Indirect, in vivo evidence from non-pregnant women, also supports the observation that oestrogens increase NOS activity. Women with artificially suppressed endogenous oestrogen levels, given oral oestradiol had raised plasma nitrate levels compared with those measured after placebo (Ramsay et al 1995). Furthermore, post-menopausal women given transdermal 17β-oestradiol show enhanced serum levels of nitrite and nitrate (Roselli et al 1995).

Acute endothelium-independent vasodilatory effects of 17β-oestradiol have been observed at supra-physiological levels in isolated coronary arteries from animals (Jiang et al 1991) and humans (Chester et al 1995). The mechanism of action is in part mediated by antagonism of Ca^{2+} influx through voltage-gated channels in vascular smooth muscle (Jiang et al 1992) and in part through potassium channels (White et al 1995). 17β-oestradiol stimulates cGMP-dependent phosphorylation of the Ca^{2+} activated potassium channel,
leading to potassium efflux, membrane repolarisation and relaxation of vascular smooth muscle (White et al 1995).

*In vivo* studies have shown that the acute vasodilator effects of oestrogens are endothelium dependent (Gilligan et al 1994a). 17β-oestradiol infusion potentiated the reduction in coronary artery resistance evoked by ACh in women with coronary artery disease, but did not affect responses to the endothelium independent vasodilators, adenosine and sodium nitroprusside (Gilligan et al 1994a). 17β-oestradiol and oestrogen replacement therapy also potentiates endothelium dependent, nitric oxide (NO) activity in the forearm of post-menopausal women (Gilligan et al 1994b, Majmudar et al, 2000). The immediate effect of 17β-oestradiol in this situation is probably an example of a rapid non-genomic response.

The majority of studies indicate that restoration of oestrogen with Hormone Replacement Therapy (HRT) reduces cardiovascular risk by half (Varas-Lorenzo et al, 2000). About one-third of this oestrogen-induced cardio-protective effect is through their ability to reduce circulating low-density lipoproteins (LDL), to protect these lipids from oxidative stress (Sack et al, 1994) and raise the levels of high-density lipoproteins (HDL). Oestrogen acting through ERα also has an anti-inflammatory effect by antagonising the activity of the pro-inflammatory transcription factor NF-κB (Harnish et al, 2000). 17β-oestradiol may also prolong the half-life of NO by scavenging free radicals, which would otherwise ‘quench’ NO. This antioxidant effect has also been demonstrated in a study in which ethinylestradiol elevated cGMP in cultured bovine aortic endothelial cells, without
increasing NOS activity, NOS protein or mRNA, but simultaneously was shown to decrease synthesis of the superoxide radical (Arnal et al 1996).

Despite these vascular protective properties, the benefit of HRT may only be seen in women with healthy endothelium, expressing normal ERs i.e. for primary prevention and not in women with pre-existing endothelial dysfunction i.e. secondary prevention (Holm et al, 1999; Koh, 2002). Until the results of further clinical studies are available, it has been recommended by the American heart Association, that the use of HRT for the sole purpose of preventing cardiovascular disease is not be recommended (Mosca et al, 2000).

1.37 Prostacyclin in the maternal circulation

Although previously a contentious issue, it is now considered that prostacyclin (PGI₂) is more likely to play a role as a local autacoid than to contribute to the lowering of peripheral resistance in normal pregnancy (Barrow et al, 1983). Since the half-life of PGI₂ is so short, evaluation of synthesis depends on the measurement of stable metabolites. The concentration of the prostacyclin metabolite, (6-oxo-PGF₁α), were too low for PGI₂ to function as a circulating hormone, despite a significant upward trend during pregnancy (Barrow et al, 1983). This conclusion was upheld by studies in pregnant animals and women in which infusion of indomethacin was shown not to affect blood pressure or peripheral resistance (Conrad and Colpoys 1986; Sorensen et al, 1992). Urinary excretion of 2,3-dinor-6-keto-PGF₁α, the major systemic enzymic metabolite of PGI₂, is also raised early during human pregnancy and increases with each trimester (Goodman et al 1982; Fitzgerald et al, 1987). Whilst reflecting overall PGI₂ biosynthesis, measurement of this
metabolite in the urine cannot discriminate between maternal and fetal sources, and may also reflect renal synthesis.

Human pregnancy is also associated with increased synthesis of the constrictor prostanoid, thromboxane (TXA$_2$), as assessed by measurement of its stable systemic metabolite 2,3-dinor-TXB$_2$ (Fitzgerald et al, 1987a). TXA$_2$, which in pregnancy seems to be mainly derived from platelets (Fitzgerald et al, 1987b) increases 3-5 fold during gestation and remains elevated throughout (Fitzgerald et al, 1987a).

1.38 Endothelium derived hyperpolarizing factor (EDHF) in pregnancy

Several studies have suggested that prostaglandin and NO independent, but endothelium-dependent mechanisms of relaxation may be enhanced in human pregnancy (McCarthy et al, 1994; Pascoal et al 1996). This observation has been supported by studies on arteries from pregnant rats (Bobadilla et al, 1997; Gerber et al, 1998; Dalle Luca, 2000). The component of relaxation to ACh, which was insensitive to cyclo-oxygenase blockade or to inhibition of NO synthase, was greater in the pregnant rats than virgin animals and totally inhibited in both groups by slight membrane depolarization (induced by modest elevation of the potassium concentration in the organ bath). This is strongly indicative of a role for enhanced synthesis of the putative endothelium derived hyperpolarizing factor (EDHF) (Garland et al, 1995). The exact nature of EDHF has proven to be elusive, although the various candidates include a cytochrome P450 derived metabolite of arachadonic acid, one of the epoxyeicosatrienoic acids (EETs) and a cannabinoid (Feletou and Vanhoutte, 1999).
1.39 Endothelin in the Maternal Circulation

There are three isoforms of endothelin (ET-1, ET-2 and ET-3) (Bagnall and Webb, 2000). The plasma concentration of ET-1, the most potent vasoconstrictor currently identified, is not affected by normal pregnancy and is very low or undetectable in maternal plasma (Wolff et al, 1997). However, ET-1 causes potent constriction of myometrial arteries from pregnant and non-pregnant women and is more potent than all other vasoconstrictors tested (Fried and Samuelson 1991; Wolff et al, 1993). ET-1 was more potent than ET-3 and potentially plays an important role in the regulation of uteroplacental blood flow (Wolff et al 1993).

1.40 Endothelium derived clotting factors

One of the non-vasoactive properties of the endothelium is to prevent intravascular coagulation. Healthy pregnancy is however a procoagulant state. In anticipation of haemorrhage at childbirth, normal pregnancy is characterised by low grade, chronic intravascular coagulation within both the maternal and utero-placental circulation (Letsky 1995). As a consequence, the risk of thromboembolism increases six fold during pregnancy (Royal College of General Practitioners 1967) and is the most common direct cause of maternal death in the UK (Department of Health 2001). There is evidence for increased levels of clotting factors, especially fibrinogen (Bonnar 1987) and depression of fibrinolysis (Kruithof et al 1987). During the third trimester, plasma levels of endothelium-derived von Willebrand factor are elevated, promoting coagulation and platelet adhesion (Sorensen et al
The procoagulant state of the endothelium does appear to be compensated by upregulation of the fibrinolytic system (Bremme et al 1992; Sorensen et al 1995).

Factors other than a pro-coagulant endothelium contribute to the increased incidence of thromboembolism. In late pregnancy, the gravid uterus partially obstructs the inferior vena cava, causing venous stasis in the lower limbs. Furthermore, occult thrombophilias often present clinically for the first time during pregnancy.

1.5 PRE-ECLAMPSIA

Pre-eclampsia is a multi-system disorder unique to human pregnancy (Williams and de Swiet 1997). It is classically recognised by hypertension, proteinuria and oedema, affects about three percent of primigravidae and almost invariably occurs after 20 weeks gestation. Yet these signs conceal the true identity of a disorder that may be recognised prior to the onset of hypertension and even evolve into eclampsia (convulsions), without hypertension. Pre-eclampsia remains an important cause of maternal death in Western Europe (Department of Health, 2001) and the USA (Kaunitz et al 1990). It is important to recognize that there is another gestational hypertensive syndrome, ‘pregnancy-induced hypertension’ (PIH), which is distinct from pre-eclampsia. Pregnancy induced hypertension affects about 7% of pregnancies in UK and tends to recur in subsequent pregnancies with a trend to larger rather than smaller babies.

Relative to the vasodilated, plasma expanded state of healthy pregnancy, pre-eclampsia is a vasoconstricted, plasma contracted condition with evidence of intravascular
coagulation. The fetus is particularly vulnerable from early onset disease and the mother can succumb following an illness that can include liver haemorrhage and necrosis, acute renal failure, subendocardial necrosis, microangiopathic haemolysis, a consumptive coagulopathy and convulsions. The most common causes for maternal death are adult respiratory distress syndrome (ARDS) and intra-cerebral haemorrhage (Department of Health, 2001). It is of interest that women who have had pre-eclampsia are at increased risk of cardiovascular disease in later life (Sattar and Greer, 2002).

Whereas healthy maternal endothelium is crucial for the physiological adaptation to normal pregnancy, the multiple organ failure characteristic of severe pre-eclampsia is predominantly secondary to widespread endothelial cell dysfunction (Roberts and Redman 1993). It is not yet known whether the endothelium of women destined to develop pre-eclampsia fails to adapt properly, or is damaged by unknown factors during a pre-eclamptic pregnancy. However, there are several sub-clinical events that take place early in a pregnancy destined to develop pre-eclampsia.

1.51 Angiotensin II sensitivity in pre-eclampsia

Gant et al (1973) showed that at 22-26 weeks gestation, prior to the onset of clinically identifiable pre-eclampsia, a group of predominantly black, teenage American women demonstrated greater sensitivity to an infusion of ANG II compared with women who did not develop pre-eclampsia. Compatible with underlying endothelial dysfunction, increased sensitivity to this pressor agent was acclaimed as a useful screening test for pre-eclampsia. However, the positive predictive power of this original study has not been
reproducible in six other populations (Kyle et al 1995). Indeed, the most comprehensive study, involving 495 healthy nulliparous women studied at 28 weeks gestation, revealed a positive predictive value of only 19% (Kyle et al 1995). Differences in the populations studied may explain this disparity, but overall this invasive test, originally heralded as a predictive test for pre-eclampsia is of no clinical uses in predicting pre-eclampsia in an otherwise healthy European population.

Prior to the onset of clinically identifiable disease, women destined to develop pre-eclampsia show evidence of poor placentation (Brosens et al, 1972) and high uteroplacental resistance (Bower et al, 1993). How high placental vascular resistance triggers maternal endothelial dysfunction is still not completely understood.

1.52 Abnormalities of the uterine vasculature

In normal pregnancy, the most striking change to a maternal artery occurs within the small spiral arteries of the uterus. These terminal branches of the uterine arteries are the final pathway by which jets of oxygenated maternal blood are delivered into the intervillous space of the growing placenta. Normally, during the first trimester, the terminal branches of the spiral arterioles are invaded by placental cytotrophoblast cells (Brosens, et al 1967; Pijnenborg et al 1980). The muscular and elastic components of the spiral arteriole wall are replaced by a fibrinoid layer of variable thickness, in which trophoblast cells are embedded. Normally, there is no disruption of the endothelium (Khong et al, 1992). Remodelling of the utero-placental vessels is normally complete by 18 weeks gestation (Matijevic et al 1995), but in women who develop pre-eclampsia, cytotrophoblast invasion of the spiral...
arteries is incomplete and high resistance vessels that retain their muscular wall, persist until term (Khong et al 1986; Zhou et al 1993). This incomplete placentation is considered to play an important role in pre-eclampsia, but as it also occurs in intra-uterine growth retardation, cannot be considered unique to pre-eclampsia. Furthermore, the endothelium of these abnormal utero-placental arteries is disrupted by intraluminal endovascular trophoblast (Khong et al 1992). Prior to any clinical symptoms or signs, Doppler ultrasound of uterine arteries at 20-24 weeks gestation, can aid in the identity of women with high resistance vessels, at risk of pre-eclampsia (Bower et al, 1993).

Animal studies are of little use in the study of pre-eclampsia - a disease unique to humans (certainly primates) in-which many of the normal vascular responses to pregnancy appear to fail.

1.53 The sympathetic nervous system in pre-eclampsia

Although the endothelium is dysfunctional in pre-eclampsia, some investigators have suggested that the pathological increase in vascular tone is secondary to changes in the sympathetic nervous system. A cold pressor test, which is though to raise blood pressure via increased sympathetic nerve activity, has been shown to cause a greater increase in BP in pregnant women at 16-20 weeks who are destined to develop pre-eclampsia compared with those who remain normotensive (Woisetschlager et al, 2000). Women with pre-eclampsia have been found to have increased activity of the sympathetic nervous system in skeletal muscle (Schobel et al, 1996) and higher plasma noradrenaline levels compared with normotensive women (Manyonda et al 1998). In this latter study, tyrosine hydroxylase
activity and mRNA levels were greater in placental tissue from pre-eclamptic compared with normotensive pregnancies. It is proposed that excessive noradrenaline breaks down more triglyceride to free fatty acids, which are then oxidised to lipid peroxides (Manyonda et al 1998). The latter are cytotoxic to endothelial cells (see later). Systemic blockade of the autonomic nervous system with tetraethylammonium chloride (TEAC) or high spinal anaesthesia was much less effective at lowering blood pressure in women with pre-eclampsia compared with normotensive pregnant women (Assali and Prystowsky 1950). This unique study would suggest that the hypertension of pre-eclampsia is mediated by a factor independent of the autonomic nervous system.

1.54 Endothelial function in pre-eclampsia

There is much evidence to support the proposal that the corollary to 'up-regulation' of the endothelium in healthy pregnancy is dysfunctional endothelium in pre-eclampsia. Healthy endothelial cells maintain vascular integrity, prevent platelet adhesion and influence the tone of underlying vascular smooth muscle. Damaged endothelial cells are unable to perform these three functions leading to increased capillary permeability, platelet thrombosis and increased vascular tone (Flavahan and Vanhoutte 1995). These features are found in pre-eclampsia and suggest that the maternal syndrome is, at least in part, an endothelial disorder (Roberts et al 1989; de Groot and Taylor 1993). Evidence of endothelial cell damage prior to clinical manifestation of pre-eclampsia can be demonstrated by the presence of markers of endothelial cell activation. Specifically, levels of fibronectin (Ballegeer et al 1989) and Factor VIII related antigen are elevated (Roberts and Redman 1993). Furthermore, women with endothelial cell damage, secondary to pre-
existing hypertension or other micro-vascular disease, have a higher incidence of pre-eclampsia than normotensive women (Ness and Roberts 1996). Morphological evidence of endothelial damage in pre-eclampsia can be seen in the glomerular capillaries (Figure 1.7), and in the utero-placental arteries - a vasculopathy known as acute atherosis.

1.55 Nitric oxide in pre-eclampsia

The L-arginine-NO pathway is an expected casualty of endothelial cell damage in pre-eclampsia. However, probably because of methodological limitations there is no consensus on whether NOS activity is altered by pre-eclampsia. Most studies have either shown no change (Cameron et al, 1993; Curtis et al 1995; Silver et al, 1996; Conrad et al, 1999b), or an increase (Nobunaga et al 1996; Smarason et al 1997) in circulating or urinary NO metabolites in women with pre-eclampsia, probably reflecting variable dietary nitrate. Only Seligman et al 1994 documented lower plasma NOx concentrations in women with pre-eclampsia compared with normotensive controls. Cameron et al, 1993 and Nobunaga et al 1996 documented a correlation between systolic blood pressure and increasing urinary and plasma concentrations of NOx, respectively. In the latter study, volunteers were starved for 12-15 hours in an attempt to control for dietary nitrogen. However, using an even more strict dietary restriction Conrad et al showed NOx was unchanged in pre-eclamptic women compared with normotensive women.
**Figure 1.7**  

a. Renal biopsy of a healthy glomerulus. The arrows point to patent glomerular capillaries.  
b. Renal biopsy of woman with pre-eclampsia at 26 weeks gestation. Only a few patent capillaries can be seen, the overwhelming majority of glomerular capillaries have been obliterated by swollen endothelial cells. This process is known as endotheliosis and is pathognomic of pre-eclampsia. This lesion provides morphological evidence of endothelial damage in pre-eclampsia. (Renal biopsy taken by myself and histology provided by Dr Meryl Griffiths).
1.56 Nitric oxide in the resistance vasculature in pre-eclampsia

There is wide agreement that agonist mediated NO synthesis is reduced in small arteries from women with pre-eclampsia. Pre-eclampsia is associated with many facets of endothelial cell dysfunction and the abnormal relaxation to agonists is probably yet another indication of a general endothelial cell defect. In small arteries from the subcutaneous circulation, sensitivity to both ACh (McCarthy et al, 1994b) and to BK (Knock and Poston, 1996) is reduced in women with pre-eclampsia. Similarly, in the omental circulation of women with pre-eclampsia, Pascoal et al (1998) found relaxation to ACh was totally absent whilst responses to BK were unaffected when compared to normotensive controls.

In resistance vessels from women with pre-eclampsia, attenuated responses to endothelium dependent vasodilators can be due to reduced NO synthesis (Knock et al, 1996) or NO independent (Pascoal et al; 1988). Flow mediated responses, which are largely NO dependent, are however, severely reduced in small subcutaneous arteries from women with pre-eclampsia (Cockell and Poston, 1997).

A brachial-artery infusion of L-NMMA to women with pre-eclampsia gave the same forearm responses as normotensive pregnant women, even though they were more sensitive to angiotensin II (Anumba et al, 1999a). Furthermore, smooth muscle sensitivity to NO donors was unchanged in pre-eclampsia, suggesting that the pre-eclampsia is unlikely to be associated with a major disruption of the L-arginine-NO pathway (Anumba et al, 1999a).
1.57 Prostanoids in pre-eclampsia

In contrast to normal pregnancy, pre-eclampsia is associated with relative underproduction of PGI\textsubscript{2} and over abundance of TXA\textsubscript{2} (Fitzgerald et al, 1990). The imbalance between the synthesis of these prostanoids formed the rationale for investigations of "low dose aspirin" therapy for prevention of pre-eclampsia. Whereas aspirin in excess of 80 mg/day substantially inhibits both PGI\textsubscript{2} and TXA\textsubscript{2}, low or intermittent doses lead to preferential inhibition of TXA\textsubscript{2} biosynthesis (Ritter et al, 1989), and could redress the imbalance between these prostanoids in pre-eclampsia. Selectivity for TXA\textsubscript{2} may lie in differential access of aspirin to platelet cyclooxygenase in the portal circulation (Pedersen and FitzGerald, 1984) with pre-systemic metabolism preventing access of aspirin to the systemic vasculature and placenta (Dekker and Sibai, 1993) and/or in the differential affinity of aspirin for cyclooxygenase in platelets (Patrignani et al, 1982). Also platelets, being anuclear, lack the capacity to regenerate cyclooxygenase, whereas endothelium can regenerate the enzyme and so maintain PGI\textsubscript{2} production (Ritter et al, 1989). However, the largest randomised trial of low dose aspirin to date, the UK based Collaborative Low Dose Aspirin Study in Pregnancy included 9364 women (CLASP Collaborative Group 1994). Overall there was no evidence of any clinically important benefit in women who took 60mg aspirin rather than placebo.

1.58 Is maternal serum toxic to endothelium?

The effects of sera from pre-eclamptic compared with healthy pregnant mothers on cultured cells has produced conflicting results. These discrepancies are likely to represent differences in experimental method. It is very difficult to draw any other conclusion from
studies that use different concentrations of serum or plasma (ranging from 2% - 30%, made up in different medium), applied to different types of endothelial cells, some from the fetal circulation and some even from other animals.

1.59 Endothelin in pre-eclampsia

Women with pre-eclampsia also have higher plasma endothelin (ET-1) concentrations than women with a normal pregnancy (Wolff et al, 1996a). This perhaps would be anticipated, as a marker of the generalized endothelial dysfunction, but nonetheless could contribute to vasoconstriction. Studies in isolated omental (Vedernikov et al, 1995; Wolff et al, 1996a) and myometrial arteries (Wolff et al, 1996b) have shown similar responses in arteries from normal and pre-eclamptic women.

1.6 Aetiology of maternal endothelial dysfunction in pre-eclampsia

How poor placentation and the resultant poor uterine blood flow leads to the maternal syndrome of pre-eclampsia, characterised by wide spread endothelial cell damage, remains uncertain. Over the years there have been many theories, but currently three or four predominate. Substantial evidence now supports a role for oxidative stress. The imbalance between free radical synthesis and antioxidant capacity may arise from reduced placental perfusion coupled with dyslipidaemia (Branch 1994, Hubel 1996, Barden 1996). Alternative hypotheses include a role for activated neutrophils (Greer 1989), the innate immune system (Redman et al, 1999) pro-inflammatory cytokines (Chen et al 1996) and pro-thrombotic states (Kumpfermine et al, 1999). Others suggest that deported trophoblast fragments may be responsible for maternal endothelial cell damage.
1.61 Oxidative stress and dyslipidaemia in pre-eclampsia

Free radicals may lead directly to endothelial damage through direct cytotoxicity, or indirectly through the synthesis of lipid peroxides. The evidence for oxidative stress in pre-eclampsia is substantial. Raised concentrations of lipid peroxides as judged by elevated antibodies to oxidized low-density lipoprotein (LDL) (Branch, 1994), plasma malondialdehyde levels (Hubel et al, 1996) and of the stable lipid peroxide products, isoprostane, 8-epi-PGF2α (Barden et al, 1996).

The origin of the reactive oxygen species may lie in the placenta or any ischaemic maternal organ, such as the liver. Placental ischaemia accelerates trophoblast cell turnover and so increases the concentration of purines which act as substrate for xanthine dehydrogenase/oxidase (Many et al, 1996). Under hypoxic conditions, xanthine oxidase predominates over xanthine dehydrogenase to produce urate and a reactive oxygen species. This process could explain why hyperuricaemia often precedes clinically recognisable pre-eclampsia and occurs prior to any fall in GFR (Gallery et al 1979).

Towards the end of normal pregnancy, maternal plasma levels of cholesterol and triglyceride increase by 50% and 300%, respectively (Potter and Nestel 1979). Women with pre-eclampsia have even higher circulating levels of triglyceride, free fatty acid and total cholesterol (van den Elzen et al 1996; Hubel et al 1996), with a relative increase in LDL cholesterol. Under conditions of oxidant stress and hypertriglyceridaemia, increased amounts of unsaturated fatty acids will be oxidised to lipid peroxides (Chirico et al 1993). There is also a qualitative change in LDLs in established pre-eclampsia, with a shift
towards small dense particles (Hubel et al 1998), a characteristic, which predisposes the
LDL particle to oxidation (Chait et al, 1993).

1.62 Neutrophil activation

Neutrophils and platelets are activated in normal pregnancy and further activated in
pre-eclampsia (Greer et al 1989; Zemel et al 1990). Activated neutrophils, adhere to
endothelium and mediate vascular damage by the release of proteases and reactive oxygen
radicals. Neutrophil elastase, a specific marker of neutrophil activation, circulates in higher
concentrations in women with pre-eclampsia compared with normotensive pregnant women
(Greer et al 1989). Neutrophil adhesion to the endothelium is mediated through the
expression of cell adhesion molecules on the endothelial cell surface. During endothelial
cell activation expression of certain cell adhesion molecules is increased on both
neutrophils and the endothelium. Neutrophil adhesion is much more marked in women with
pre-eclampsia, as they have increased expression of cell adhesion molecules compared with
healthy normotensive pregnant women (Barden et al 1997), specifically, vascular
endothelial cell adhesion molecule (VCAM-1; Hubel et al, 1998).

The stimulus to neutrophil activation remains unknown, but pro-inflammatory
cytokines can activate neutrophils and simultaneously increase expression of cell adhesion
molecules on endothelial cells (Lyall and Greer 1996). Leucocyte TNF-α gene expression
and circulating levels of TNF-α are enhanced in pre-eclamptic patients compared with
normotensive and non-pregnant women (Chen et al 1996; Kupfermine et al 1994).
Furthermore, in one study the frequency of the TNF1 allele was markedly increased in pre-
eclamptic patients (Chen et al 1996). TNF-α can generate reactive oxygen species, inhibit NOS, favour synthesising thromboxane A2 over prostacyclin, change endothelial cells from an anti-haemostatic to a pro-coagulant state and activate transcription of VCAM-1 (Chen et al 1996). On the basis of its biological properties therefore, TNF-α is a strong candidate for mediating endothelial damage in pre-eclampsia.

A possible unifying hypothesis is that an ischaemic placenta produces excessive proinflammatory cytokines, free radicals, or other toxic agents such as syncytiotrophoblast microvilli (Knight et al, 1998), which activate neutrophils and generate lipid peroxides to mediate maternal endothelial cell damage and hence pre-eclampsia (Conrad and Benyo 1997). There is unlikely to be a single pathway to pre-eclampsia, which has a broad clinical spectrum and a range of pathophysiological triggers, which start a cascade of events that are unstoppable until delivery (Figure 1.8).

Discriminating between true pathogenic factors and innocent para-phenomenon is difficult. Furthermore, attempts to understand the mechanisms of physiological change during healthy pregnancy have been relatively neglected. Interpretation of studies comparing pre-eclamptic with normotensive pregnancies should always be put in context by simultaneous analysis of samples from healthy non-gravid controls. Without this information it is impossible to appreciate how much the physiological baseline has moved during healthy pregnancy. Furthermore, prospective studies that start in early pregnancy, before clinically evident pre-eclampsia should be more helpful at dissecting out the triggers to pre-eclampsia, rather than patho-physiological adaptive phenomenon.
Figure 1.8  Pathophysiology of endothelium during pre-eclampsia. There is endothelial cell damage, mediated by several mechanisms including lipid peroxidation, pro-inflammatory cytokines and possibly other toxins from an ischaemic placenta. Platelets and activated neutrophils become adherent to damaged endothelium leading to further damage, microthrombi and multi-organ ischaemia. This pathology would suggest that endothelial derived relaxing factors would be reduced as a consequence of endothelial damage.
The Endothelium in Pre-eclampsia

Platelet activation → TXA$_2$

- Neutrophil elastase
- IL-6

Leucocyte activation

- Oxidized LDL
- TNFα
- Cellular Dysfunction
- VCAM-1

Shear, ACh, BK

↓PGI$_2$

NO

Endothelin

Fibronectin

vWF

PA-I

↓↓
Prevention of endothelial disruption during pre-eclampsia is a better strategy than treatment of its consequences. Until we understand more about the activity of the L-arginine-NO pathway in pre-eclampsia, trials that supplement the NO synthase substrate L-arginine to women at risk of pre-eclampsia, would be premature. Donors of NO must also be used with caution as they not only relax vascular smooth muscle, but have other, non-vascular actions such as ripening of the cervix. Anti-oxidants acting as free radical scavengers might be successful as prophylaxis against endothelial cell dysfunction. A large multi-centre trial to compare the outcome of pregnancies in women at high risk of pre-eclampsia supplemented with a diet of antioxidants is under way.

In conclusion, the endothelium plays a central role in the maternal adaptation to healthy human pregnancy. The peripheral circulation of the healthy mother is vasodilated and prothrombotic, but prone to endothelial cell dysfunction and pre-eclampsia - whatever it's aetiology. Delivery of the baby is the only definitive way to cure pre-eclampsia.

1.7 Aims of thesis

This thesis aims to study different vascular control mechanisms related to the haemodynamic changes of human and rat pregnancy.

a. To understand the role of the L-arginine-nitric oxide pathway in the peripheral vasodilatation of healthy human pregnancy.

b. To understand the role of the endogenous inhibitor of nitric oxide synthase, asymmetric dimethylarginine (ADMA) during the vasoconstricted state of pre-eclampsia.
c. To understand the role of juxtaglomerular nitric oxide synthase and perivascular nerves towards the increase of renal blood flow during rat pregnancy.

d. To assess the potency of nitric oxide synthase inhibition and noradrenaline at different local temperatures in the human hand.
CHAPTER 2

MATERIALS AND METHODS
2.1 VASCULAR BED PREPARATIONS

2.2 Mechanism of control of local blood flow in vivo

In this thesis, local infusion of drugs into human volunteers was used to investigate mechanisms of blood vessel control. Inhibition of nitric oxide synthase with $N^G$-monomethyl-L-arginine (L-NMMA) was compared with noradrenaline in the vasodilated states of healthy pregnancy and local warming of the hands. Local infusion of drugs allows study of the direct effects of drugs or medicines on blood vessels. Since the doses used are small and do not have any systemic effects it is possible to investigate effects of drugs in the absence of reflex circulatory changes which might complicate the interpretation of the observed responses. Furthermore, the use of small doses minimises the risks associated with using compounds for the first time, especially as this was the first time L-NMMA had been infused into a pregnant mother.

2.3 Venous occlusion plethysmography of the hand

Most studies using venous occlusion plethysmography (VOP) have measured forearm rather than hand blood flow. Indeed, the hand is usually excluded from the circulation when forearm blood flow is measured (Benjamin et al, 1995). The hand was chosen for study as hand blood flow was thought to increase considerably more than forearm blood flow in pregnancy. The principle of measuring hand blood flow with VOP is the same as with forearm blood flow, in that venous occlusion allows arterial blood to enter the hand/forearm, but prevents venous blood from returning to the heart. However, instead of using a strain gauge to measure forearm swelling and hence blood flow, hand swelling is measured as it displaces water out of the sealed box in which it is placed and up a funnel in
which there is an electrode that senses changes in conductivity (Figure 2.1). One venous occlusion cuff is placed on each wrist. Unlike VOP of forearm, there is clearly no need for an arterial cuff when measuring hand blood flow (used to exclude the hand from the circulation when measuring forearm blood flow), which makes the measurement of hand blood flow more comfortable.

Hand blood flow was studied since it is representative of skin blood flow and, unlike the predominantly muscular forearm vascular bed, flow in the hand has been reported to increase 2-6 fold by late pregnancy (Abramson et al., 1943; Ginsberg and Duncan, 1967). Venous occlusion plethysmography of the hand, as opposed to laser Doppler of forearm skin, was used as a co-worker (Dr Fred Imms) had long experience of the use of the plethysmography equipment that was first developed in the Barcroft laboratories more than 50 years ago. It was also felt that the response to intra-arterial drugs would be more comprehensively recorded by measurement of whole hand blood flow, predominantly made up of cutaneous arteriovenous anastamoses, than by the laser Doppler measurement of a patch of skin.

Venous occlusion plethysmography of the hand by water displacement has been used for more than 50 years and has been shown to be accurate in comparison with calorimeters and surface thermo-electric junction (Cooper et al, 1949). As skin blood flow makes up the bulk of hand blood flow (Greenfield et al, 1951) especially at high temperatures, it was decided that this would be the best method for assessing skin blood flow in pregnant women.
Blood flow through skin arterio-venous anastamoses (AVAs) is influenced by the sympathetic nervous system (Hertzman 1959). As a consequence non-thermal, extraneous stimuli will activate sympathetic nerves and influence skin AVA blood flow. However, in subjects with an intact sympathetic nervous system these stimuli will affect both hands equally. There is also a rhythmic fluctuation in the amplitude of hand blood flow that varies according to the local temperature (Burton, 1939). To ensure reliable results when adding a drug to one hand, the thermal conditions, i.e. the water temperature in both plethysmographs, must be identical. It is thought that the rhythmic fluctuations of peripheral blood flow improve the body’s ability to regulate its temperature according to its environment. The amplitude of these fluctuations is greatest when blood flow is in the middle range, less in dilatation and least during constriction.

Subjects lay semi-recumbent and blood flow (ml 100ml hand⁻¹min⁻¹) was measured throughout the study in both hands simultaneously, using water filled plethysmographs placed above the level of the heart (Barcroft and Edholm, 1945). Wrist cuffs were inflated to a venous pressure (40mmHg) for 10s in each 20s cycle. This caused a linear increase in hand volume for 10s followed by a 10s deflation which allowed venous emptying (Figure 2.2). Each dose of drug would be infused for 5min before starting a measurement period of at least five 20s cycles of inflation and deflation, from which a mean increase in hand volume would be derived (see later). The venous occlusion cuff would be deflated until the next measurement period.
All venous occlusion plethysmography studies were performed with the approval of the local committee on ethics at University College Hospital (Chapter 3) and St Thomas’ Hospital (Chapter 4). All subjects gave their informed written consent. Venous occlusion plethysmography studies were performed in collaboration with Dr Fred Imms, senior lecturer, Physiology, GKT.
Figure 2.1 Subject lying with hand plethysmographs at the level of the heart. A 27-gauge needle infuses drugs into the non-dominant brachial artery. Occlusion of the wrist cuff to 40mmHg prevents venous blood leaving the hand, but allows arterial blood to enter. As the hand swells, water is displaced up the chimney at the top of the plethysmograph. The rise in water level is measured with an electrode connected to a polygraph. The slope generated by hand swelling/water displacement reflects arterial blood flow (see Figure 2.2).
Figure 2.2  A tracing from a subject having L-NMMA 2µmol/min infused into the non-dominant brachial artery (lower trace). The arrows signify the point of inflation of the venous occlusion cuffs, approximately for 10s in every 20s. It is clear that the slope is flatter in the lower tracing and this reflects lower blood flow. It is also of note that there are differences in the slope from one inflation to the next. This is typical of hand blood flows, unlike the steady flow found in the forearm. These changes may relate to rhythmic changes in the opening and closing of arteriovenous anastomoses. The important point is that the change in shape of the slope is always relative to the contra-lateral hand to which the infused hand is always compared.
2.4 Venous occlusion plethysmography of the hand in pregnancy

In the study of pregnant women (Chapter 3), three groups of volunteers were studied; women in early pregnancy (9-15 weeks gestation) following completion of arrangements for a therapeutic termination of pregnancy (n=10); women in late pregnancy (36-41 weeks) 24 hours prior to an elective Caesarean section or induction of labour for non-medical reasons (n=10) (Figure 2.3); healthy non pregnant women (n=10). Only healthy, normotensive subjects who were taking no medication were recruited.

Ambient temperature was kept constant during each study (23.2 °C±1.9; mean ± SD). The temperature of the water in the plethysmographs was kept constant within and between experiments (31.8 °C±0.33; mean ± SD). Blood pressure was measured in all subjects and in some subjects from each group during infusion of the maximum doses of noradrenaline and L-NMMA.

Drugs or physiological saline were infused continuously (0.5ml min⁻¹) through a 27 SWG needle inserted into the brachial artery of the non-dominant arm (Vallance et al., 1989). One percent lignocaine solution was used to anaesthetise the skin prior to insertion of the needle. After establishing resting control values of blood flow for 15 min during an infusion of saline, four doses of noradrenaline were infused to produce a dose response curve (60, 120, 240 and 480 pmol min⁻¹, each dose for 5 min). After a further 15 min of saline infusion and when blood flows had returned to baseline four doses of L-NMMA were infused to produce a second dose response curve (1, 2, 4 and 8µmol min⁻¹, each dose for 5 mins). The doses of drugs were selected to produce local changes in the infused arm only.
Figure 2.3; A 38-week pregnant volunteer in venous occlusion plethysmography equipment. This apparatus and the study protocol was well tolerated by almost all volunteers. There were never any systemic symptoms or signs, in particular there were no adverse affects with regards uterine activity or fetal heart rate.
and are below systemically effective doses (Vallance et al., 1989). Whilst the action of noradrenaline is short lived, the duration of action of L-NMMA is up to 1 hour and therefore this drug was always infused last (Vallance et al 1989).

2.5 Analaysis of data (Chapter 3)

Blood flow was expressed as ml 100ml hand$^{-1}$min$^{-1}$ according to the method of Barcroft and Edholm, 1945. Basal blood flows for each group were log transformed to derive normally distributed data and then compared using one-way ANOVA. To determine responses to drugs, blood flow in the infused hand was expressed as a ratio of blood flow in the non-infused hand. Changes in hand blood flow in response to drug infusion were then expressed as a percentage of the ratio during control (saline infusion) periods (Calver et al., 1993). To compare the response to each drug independently between groups a two way ANOVA on log ratio data was used. For comparison of the relative response to both drugs within and between groups, the overall response to each drug in each individual was calculated as the area under each dose-response curve (AUC). The AUC for noradrenaline was subtracted from the AUC for L-NMMA for each individual. The average (mean) difference between drug responses for each group was compared between groups using a series of t-tests.

2.6 Venous Occlusion Plethysmography of the Hand During Changes in Local Temperature (Chapter 4)

Advantage was taken of the ability to change the temperature of water in the hand plethysmographs in order to study whether temperature alters the potency of the L-arginine-
nitric oxide pathway or adrenergic stimulation. The equipment used in Chapter 3 was adapted for this dynamic study where cool water was replaced with warm water half-way through the experiment. The full protocol for all three of the studies performed in this chapter are outlined in Chapter 4. Draining the plethysmographs of cool water and then filling them with warm water, as quickly as possible, was a two-man job. These studies could not have been performed without the assistance of Dr Fred Imms.

2.7 Analysis of data (Chapter 4)

Blood flow was expressed in ml 100ml hand\(^{-1}\) min\(^{-1}\). In experiments 1 and 2, geometric means (GM) of hand blood flow were estimated for each combination of experiment, patient, time period and hand (infused or control), using a linear regression model with hand blood flow as the outcome. Patients were clustered by means of robust variance using the Stata statistical package (StataCorp, 2001). Confidence intervals were calculated for the GMs, and also for various ratios of these GMs, which were selected as summary measures to compare interesting temperature-drug interactions.

For each of the warming experiments, the GM ratio was measured of the infused-hand 30-minute/20-minute flow ratio to the control-hand 30-minute/20-minute flow ratio to compare the post-warming increase in blood flow in the treated hand compared with the increase in blood flow in the control hand. The GM of the 3 post-warming flow measurements for L-NMMA treated hands, L-NMMA control hands, NA treated hands and NA control hands were measured. The grand GM of L-NMMA/NA ratios were calculated
for treated hands and control hands, respectively, in order to compare flow between the 2 drugs.

For the two constant-temperature experiments, the blood flow in each hand of each patient in each experiment at the time of each of the 4 doses of each of the 2 drugs, was divided by the most recent flow under saline infusion for the same hand to derive a treated/saline ratio. For each combination of experiment, patient, hand and drug, the GM treated/saline ratio for the 4 doses was calculated. For each combination of experiment, patient and drug, the ratio of the infused-hand GM treated/saline ratio to the control hand GM treated/saline ratio was calculated to define a GM infused/control ratio (a ratio of ratios). For each patient, 4 ratios of interest between these 4 GM infused/control ratios were then calculated, namely a warm L-NMMA/cold L-NMMA ratio, a warm NA/cold NA ratio, a warm L-NMMA/warm NA ratio, and a cold L-NMMA/cold NA ratio. Finally, for each of these 4 patient-specific ratios of ratios of ratios, the sample geometric mean was calculated, with a confidence interval for the population geometric mean. All of these rather complicated summary measures were chosen in order to detect flow ratios as sensitively as possible, using small samples of patients, against a background of between-patient variability in hand blood flow rates. Dr Roger Newson, Lecturer in statistics at Kings College Hospital, GKT, gave statistical advice.

2.8 Drugs used in both venous occlusion plethysmography studies (Chp 3 & 4)

Noradrenaline (Levophed, Winthrop Laborotories, Guilford, Surrey, UK) and L-NMMA (Wellcome Research Laborotories, Beckenham, Kent, UK) were dissolved in
physiological saline (0.9% NaCl; w:v). L-NMMA was passed through a 'Flowpore' D26 bacterial filter (Sartorius, Gottinggen, Germany) immediately prior to use. Ascorbic acid (one drop of 100mg ml\(^{-1}\)) (Evans Medical Ltd, Horsham, Sussex, UK) was added to the solution of noradrenaline to prevent auto-oxidation. This dose of ascorbic acid has no effect on forearm blood flow in healthy volunteers (Hornig et al., 1998).

2.9 Isolated Perfused Rat Kidney (Chapter 6)

Virgin and first time pregnant, Sprague-Dawley rats, matched for pre-pregnancy weight (271±5g; mean±SEM; University College London), were anaesthetised with 60mg kg\(^{-1}\) i.p. pentobarbitone sodium (Sagatal; Rhone Merieux) and heparin sodium (monaparin; CP Pharmaceuticals Ltd, Wrexham, UK) 500 units kg\(^{-1}\) was given via the left femoral vein (Figure 2.4). Following a mid-ventral and sub-costal incision, the intestines were moved aside and wrapped in a warm, moist swab. A loose ligature was tied around the right renal artery and the right adrenal artery was tied off. The superior mesenteric artery was tied off distally and a bull-dog clip attached proximally. A 21-gauge needle was inserted retrogradely between these points. The bull-dog clip was removed and the needle advanced across the aorta into the right renal artery. The kidney was immediately perfused with a modified Krebs-Henseleit physiological saline (constituents mM; NaCl 133; KCl 4.7; NaHCO3 16.4; MgSO4 0.6; NaH2PO4 1.4; Glucose 7.7; CaCl2 2.5; pH 7.2), gassed with 95% \(O_2\)/5%CO\(_2\) and containing 10mL\(^{-1}\) of an amino acid mixture (Vamin 14; Kabia Pharmacia Ltd, Milton Keynes, Bucks) to give a mixture similar to that recommended by Epstein et al, 1982. The kidney was then rapidly removed into a neighbouring glass vessel warmed with water circulating at 37 °C.
Figure 2.4  a. The anaesthetised rat was anti-coagulated prior to surgery with 200 units of unfractionated heparin (monaparin, CP, UK).

b. Retrograde cannulation of the superior mesenteric artery allowed the perfusion needle to pass into the aorta and then down into the right renal artery. Bulldog clips and sutures around the distal end of the superior mesenteric artery and right adrenal artery prevented blood loss during this procedure. As soon as the needle was in the right renal artery, a suture tied around the artery was tightened so that the needle was secured. The kidney was injected with 1 ml of modified Krebs perfusate and heparin and then removed, before being secured into the perfusion apparatus.
A non-recirculating perfusion system of the isolated rat kidney was used, adapted from the model described by Churchill and Ellis, 1993 (Figure 2.5). The kidney lay on a wire mesh, which was attached by an electrode to a Grass SD9 stimulator. In order to mimic the gestational increase in renal blood flow, we began perfusion of the isolated rat kidney at 10ml min\(^{-1}\). Perfusion flow was then adjusted to give a baseline perfusion pressure of 85mmHg in all three groups. This was maintained for 20-30min before experiments began.

2.10 Calculations and Statistics (Chapter 6)

Results in the text and figures are given as mean ± standard error of mean (SE); n refers to the number of rats included in each experimental group. Pharmacological studies with the IPRK were analysed using two-way ANOVA. Comparison between groups for CGRP immunoreactivity were analysed using ANOVA and Tukey's one way multiple comparison. P <0.05 was taken as being significant.

2.11 Drugs (Chapter 6)

Pentobarbitone sodium was from Sagatal, Rhone Merieux. Heparin sodium was from monaparin, CP Pharmaceuticals Ltd, Wrexham, UK. The ingredients of modified Krebs-Henseleit physiological solution, methoxamine hydrochloride, tetrodotoxin and capsaicin were from Sigma, Poole, England. The amino acid mixture added to Krebs solution was Vamin 14 from Kabia Pharmacia Ltd, Milton Keynes, Bucks, UK. Guanethidine monosulphate (Ismelin) was from Ciba-Geigy, Horsham, West Sussex, UK. Calcitonin gene related peptide was from CRB, UK, Cambridge, England.
The perfusion apparatus was modified from the arrangement described by Churchill and Ellis. As soon as the kidney was removed it was fixed into the perfusion apparatus. Further surgery was usually needed to remove perinephric tissue and this could be performed when the kidney was perfused *ex vivo*. A modified Krebs solution with Vamin 14 (amino acid mixture) improved the viability of the kidney preparation. Further modification of the apparatus allowed electrical field stimulation through the needle perfusing the kidney passing through renal parenchyma to its outer surface – which was in contact with a wire mesh attached to an electrical stimulator.
2.2 BIOCHEMICAL ASSAYS

2.21 Asymmetrical Dimethyl-arginine (ADMA) (Chapter 5)

One millilitre aliquots of plasma were loaded onto pre-conditioned, 2ml Bond Elut benzene sulphonic acid (SCX) columns (Anachem, Beds. United Kingdom). Samples of amniotic fluid or fetal urine were centrifuged and the supernatant was loaded onto SCX columns. The dimethylarginines were eluted with 50% ammonia in methanol and evaporated to dryness at 130°C under nitrogen. The plasma eluate was redissolved in 1ml distilled water and added to a similarly preconditioned Bond Elut carboxylic acid (CBA) column. The dimethylarginines were eluted with 10% ammonia in methanol and again evaporated to dryness. The samples were reconstituted in 100μl distilled water, centrifuged at 12000g for 5 minutes and the supernatants removed for analysis.

Identification of dimethylarginines was by high performance liquid chromatography (HPLC). Separation of ADMA and its symmetric isomer was achieved by injecting 20μl of the extracted sample onto an ODS C18 analytical HPLC column (Spherisorb, Phasesep) using an ion pair based mobile phase. Absorbance of the eluate was determined at 200nm. Concentrations of ADMA in the samples were determined by computerisation integration of peak area and comparison with known standard solutions. These concentrations were corrected for extraction efficiency, estimated by the addition of 10μg L-NMMA added to each sample before the extraction procedure. Inter and intra-assay variation was less than 5%.
2.3 Sensory-motor neuropeptides in pregnant rat kidney

2.3.1 Measurement of CGRP, Substance P and Neuropeptide Y levels within renal cortex

The kidney was bisected and then separated with a sharp blade into four readily identifiable zones, the renal cortex, the outer and inner medulla and renal pelvis. Specimens were immediately stored in liquid nitrogen until peptide extraction. Individual specimens were weighed and the peptides extracted into 0.5 M acetic acid in polypropylene tubes in a boiling water bath for 15 min. The samples were homogenized, centrifuged for 30 min at 3500g and lyophilized.

CGRP was quantified using an inhibition enzyme linked immunosorbent assay as described previously (Belai et al, 1985). Briefly, flat-bottomed polystyrene microtitre plates (Dynatech Laborotories, Inc., Alexandria, Va.) were coated with either 0.075μg ml⁻¹ CGRP (CRB U.K. Ltd. Cambridge, U.K.), NPY conjugated to polyglutamate in 0.1 M carbonate-bicarbonate buffer, pH 9.6, containing 0.02% sodium azide (Sigma Chemical Co., Poole, U.K.) for incubation at 4°C for 18h. After incubation, the contents of the plates were discarded and washed three times with PBS/Tween and incubated for 1 h at room temperature with PBS/Tween (0.02% sodium azide) containing 0.1% gelatin (sample buffer) to prevent non-specific binding. After the plates were emptied by inversion, reconstituted samples and standards (50μl) were added to each well followed by 50μl of antiserum raised in rabbits to either synthetic CGRP, NPY or SP (diluted 1:12 500, 1:12 500 and 1:50 000 respectively) in sample buffer. The plates were covered and incubated over night at 4°C. The plates were then washed three times with PBS/Tween/azide and 100
μl of goat-anti rabbit immunoglobulin conjugated to alkaline phosphatase (Sigma Chemical Co.) was added to each well at a dilution of 1:20000 in sample buffer. The plates were incubated in a humid chamber for 2 h at 37°C. The unbound goat-antirabbit immunoglobulin conjugated to alkaline phosphatase was removed using three washes with PBS/Tween and one wash with glycine buffer, containing 0.001M magnesium chloride and 0.001M zinc chloride (pH 10.4). The chromogenic substrate p-nitrophenyl phosphate, 1 mg/ml in glycine buffer, was added to each well. The colour development was monitored for 4 h at room temperature. The absorbance was read in a Titertek Multiscan automatic spectrophotometer (Flow Laboratories, Rickmansworth, Herts, U.K.) at 405 nm.

2.4 Immunohistochemistry (Chapter 6)

The non-perfused kidney was removed and immersion-fixed for 3-4 h in 4% paraformaldehyde in phosphate buffered saline (PBS). The kidney was then rinsed in PBS three times before being transferred into 7% sucrose in PBS azide for storage at 4°C for 12 hours. The kidney was then frozen using liquid nitrogen cooled iso-pentane and cut at 10μm on a Leika Cryostat 1800.

Slides were incubated with primary antibody, overnight at room temperature. Rabbit polyclonal anti-CGRP and rabbit polyclonal anti-tyrosine hydroxylase were used (Affiniti, Exeter, U.K.). Both antibodies were diluted 1:1000, in an antibody diluting medium; PBS with 0.1% sodium azide, 0.01% bovine serum albumin, 0.1% lysine and 0.1% Triton X-100. Slides were than washed in PBS for 10 min, three times, followed by incubation with 1:250 biotinylated donkey anti-rabbit immunoglobulin (Amersham Life Sciences, U.K.) for 1 hour, at room temperature. Slides were then washed another three times in PBS (10 min
each) followed by incubation with Streptavidin-FITC (Amersham Life Sciences, U.K.) for 1 hour, at room temperature (1:100). Slides were then washed in PBS another three times prior to being coverslipped using Citifluor as a mountant.

2.5 Immunohistochemistry (Chapter 7)

Rats were killed (pregnant rats killed at 12 and 21 days of pregnancy) by an overdose of CO$_2$ gas. The hearts and kidneys were dissected out and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2) for 24h at 4C temperature. The samples were washed in 20% sucrose in PBS + 0.01% sodium azide and stored for 24h at 4C. The samples were frozen in liquid nitrogen and 10µm sections were cut on a cryostat (Reichert-Jung, Cambridge Instruments, G.m.H. Germany) at −25C and mounted on gelatin-coated glass slides.

Tissue sections were incubated for 18h in a humid chamber at room temperature either with rabbit polyclonal antibody to NOS-III (N30030) or antibody (N32030) to inducible isoform of NOS (iNOS/NOS-II) at a concentration of 5µg/ml. Both antibodies were manufactured by Transduction Laborotories, Lexington, USA (distributed by Affiniti, Exeter, UK) and characterized for use and tested in a number of different systems including Western blots; both antibodies were affinity purified. After incubation, the sections were next washed in PBS, incubated for 1h with biotinylated goat anti-rabbit immunoglobulin G serum (Life Science, Amersham, UK) dilution 1:250, washed in PBS and incubated for 1h with streptavidin-flurescein (Life Science) diluted 1:100. The specimens were then washed again in PBS and mounted in Citifluor mountant – glycerol/PBS solution (Chemical
Laboratory, Canterbury, UK). The immuno-labeled tissues were examined with a Zeiss photomicroscope. Immuno-labeling was tested routinely by omission of the primary antibodies and/or replacement of primary antibodies with immunoglobulin G serum; no labeling was observed in these control preparations.

2.6 ELECTRON MICROSCOPY

2.6.1 Animal Treatment

Female, virgin Sprague-Dawley rats and age matched (200-250 g), first time pregnant (12 and 19 day gestation) rats were compared. Rats were anaesthetised with sodium pentobarbitone (Sagatal, 60 mg kg\(^{-1}\) i.p.) and perfused through the heart (left ventricle) with 4% paraformaldehyde and 0.1% glutaraldehyde or 0.045% picric acid (at room temperature) in 0.1M phosphate buffer at pH 7.4 for 15 min. Hearts were removed and immersion-fixed for 3-4 h at 4°C in the same fixative and then transferred to phosphate buffer and stored overnight at 4°C. The following day, 70-100μm vibrotome sections through the route of the aorta, including the origin of both coronary arteries and more distal, intra-myocardial branches were cut. These sections were then processed for pre-embedding peroxidase-antiperoxidase (PAP) immunocytochemistry of NOS-III.

2.6.2 Immunocytochemistry

Coronary arteries were initially exposed for 30 min to 0.3% hydrogen peroxide in 30% methanol for the blocking of endogenous peroxidases, washed in 0.05M TRIS-buffered saline (TBS; Dako, High Wycombe, UK) at pH 7.6 and then exposed to 10% normal goat serum (Nordic Immunology, Tilburg, The Netherlands) diluted 1:30 in TBS
containing 1% sodium azide or 0.5% thiramazole (this buffer was also used for the dilution of primary and secondary antibodies) for 1.5h. After being rinsed in TBS, the specimens were incubated for 48h at 4°C with a mouse monoclonal antibody to NOS-III at a dilution of 1:250-1:400 (1 - 0.625µg antibody per millilitre incubation buffer). After a rinse in TBS, the specimens were then exposed for 1.5 - 16h to goat-antimouse immunoglobulin G serum (reacting with mouse IgG subclass G1, G2a, G2b and G3; Sigma, Poole, UK) diluted 1:40 or bioti-SP-conjugated affinity pure donkey anti-mouse IgG (Jackson, USA) diluted 1:500. After being rinsed in TBS, specimens were incubated for 4h at room temperature with a mouse PAP complex (Sigma), diluted 1:200. Specimens were rinsed in TBS and then treated with 3,3'-di-aminobenzidine (Sigma) and 0.01% hydrogen peroxide. After being rinsed in TBS and phosphate buffer, the specimens were post-fixed in 1% osmium tetroxide for 1h at 4°C, rinsed in phosphate buffer, dehydrated in a graded series of ethanol and then embedded in Araldite or Durcupan (Sigma). The ultrathin circumferential sections were stained with uranyl acetate and lead citrate and subsequently examined with a JEM-1010 electron microscope.

2.63 Control preparations

Mouse monoclonal anti-NOS-III (N30020) and anti-NOS-II (N32020) antibodies were manufactured by Transduction Laboratories, Lexington, USA (distributed by Affiniti, Exeter, UK). The anti-NOS-III antibody (IgG1 isotype) and anti-NOS-II antibody (IgG2a isotype) were raised against bacterially expressed fragments of the relevant proteins. They were characterised for use in a number of different systems, including Western blot analysis, and showed a wide species reactivity, including reactivity with human and rat
NOS (Transduction Laboratories). In the present study, the anti-NOS III and anti-NOS-II antibodies were used at an optimal dilution of $1 \mu g \text{ ml}^{-1}$ incubation medium.

In the Chapter 7, the specificity of the immuno-labeling was tested routinely by omission of the primary antibody and IgG steps, independently, and by the substitution of primary antibodies with non-immune normal mouse serum (Nordic Immunology) at a dilution 1:250-1:400. No labeling was observed in these control preparations.

2.71 Safety and ethical issues regarding experimentation during pregnancy

When testing the hand blood flow response to an intra-arterial injection of L-NMMA in pregnant women, one had to be sure that no harm would be done to either the mother or her fetus. For this reason doses of L-NMMA were used that were known not to have a systemic effect on blood pressure in healthy non-pregnant volunteers (Vallance et al, 1989). It is possible however, that the utero-placental circulation, which is known to be sensitive to NOS inhibition, could be compromised by low doses of L-NMMA. In an attempt to have theoretical reassurance on this issue, it is possible to calculate the maternal plasma concentration of L-NMMA following experimental infusion. An arbitrary margin of safety, up to 100 fold the concentration that would be needed for a therapeutic effect, could then limit the maximum dose used. I did not pursue this course of action, as I felt it would not accurately reflect the in vivo response. However, I did measure maternal BP, fetal heart rate and uterine contractions using cardiotocography (CTG). All of these parameters including perinatal outcome were unaffected by the study.
There are ethical issues surrounding experimentation on pregnant women. In particular, what are the rights of the unborn child? Quite simply, the unborn child does not have any rights in British law. Decisions made by the mother are her prerogative until the child is born. However, if a pregnant mother gives informed consent to be involved in a study and this results in harm to the unborn child that is evident after birth, what claim does this child have against its mother or the investigator? Such a case has not to date been tested in court. However, it is likely that in British law the child has no recourse against its mother. Although no precedent has been made in the research arena, the law has decided that a child cannot pursue a case against its mother for any act that she might have committed during the pregnancy that did harm to the fetus. It is thought injudicious to punish a mother for smoking, drinking alcohol or taking illicit drugs when these acts are known to do harm to the fetus. Up to 30% of women still smoke during pregnancy.

In the context of a research project during pregnancy, a court would have to test specific features of the case, such as did the mother give truly informed consent and did she understand the risks of taking part? It is likely that such a case would lean towards finding the investigator guilty, as he/she will have insurance cover to benefit the child. In this context, a child can sue his/her mother for an injury sustained in a car accident during pregnancy (if she is responsible for the accident), as she will have insurance cover.

Such claims often result in very high financial costs and as a consequence drug companies are very reluctant to be associated with clinical trials of new drugs in pregnancy. This fear of doing harm at such an important stage of human development and the
consequent potential cost if harm is done will hinder therapeutic and medical advances in obstetrics for years to come.
CHAPTER 3

NITRIC OXIDE-MEDIATED

VASODILATATION IN HUMAN PREGNANCY
Abstract

1. The maternal peripheral vasculature dilates during healthy pregnancy. This study investigated the role of nitric oxide synthase towards the gestational increase in hand blood flow.

2. Using venous occlusion plethysmography the change in hand blood flow following a brachial artery infusion of the nitric oxide synthase inhibitor L-NMMA was compared between three groups of women; non-pregnant, early pregnant (9-15 weeks), late pregnant (36-41 weeks).

3. Basal hand blood flow increased significantly during late pregnancy compared with non-pregnant and early pregnant subjects (p=0.007). L-NMMA produced a greater reduction in hand blood flow in both pregnant groups compared with non-pregnant women (p=0.0003). Noradrenaline produced an attenuated response in late pregnancy compared with non-pregnant and early pregnant women (p=0.0029).

4. If other vascular beds respond in the same way as the hand, the gestational increase in L-NMMA response in the hand, implicates a gestational increase of nitric oxide in the fall of peripheral vascular resistance during healthy human pregnancy.
3.1 INTRODUCTION

The mechanism for the primary reduction in total peripheral vascular resistance during pregnancy is unclear, but one possibility is that there is increased production of a vasodilator substance. Peripheral arterial vasculature is maintained in a state of active vasodilatation by continuous synthesis of endothelium-derived nitric oxide (NO) from L-arginine (Vallance et al., 1989). Inhibition of NO synthesis leads to vasoconstriction and a reduction in blood flow, whereas activation leads to further vasodilatation and an increase in blood flow (Vallance et al., 1989). The response to \( \text{L}^6\)-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide synthase (NOS) has been widely used as an index of the contribution made by NO to resting vascular tone (Calver et al., 1992, Knowles and Moncada, 1994,). The constrictor action of this agent is mediated by loss of endothelium-derived NO, not to a direct constrictor action of the drug (Rees et al., 1989).

In this study, venous occlusion plethysmography was used to measure changes in hand blood flow during pregnancy. Hand blood flow was studied since it is representative of skin blood flow and, unlike the predominantly muscular forearm vascular bed, flow in the hand has been reported to increase 2-6 fold by late pregnancy (Abramson et al., 1943; Ginsberg and Duncan, 1967). The response to the NOS inhibitor L-NMMA on hand blood flow was compared with the response to an endothelium-independent vasoconstrictor, noradrenaline in non-pregnant, early and late pregnant women.
3.2 PROTOCOL

The study was approved by the local committee on ethics. Three groups of volunteers were studied after giving informed consent; women in early pregnancy (9-15 weeks gestation) following completion of arrangements for a therapeutic termination of pregnancy (n=10); women in late pregnancy (36-41 weeks) 24 hours prior to an elective Caesarean section or induction of labour for non-medical reasons (n=10); healthy non pregnant women (n=10). Only healthy, normotensive subjects who were taking no medication were recruited.

3.21 Venous Occlusion Plethysmography

Subjects lay semi-recumbent and blood flow (ml 100ml hand⁻¹min⁻¹) was measured throughout the study in both hands simultaneously, using water filled plethysmographs placed above the level of the heart (Figure 2.3). Wrist cuffs were inflated above venous pressure (40mmHg) for 10s in each 20s cycle. Ambient temperature was kept constant during each study (23.2 C ± 1.9; mean ± SD). The temperature of the water in the plethysmographs was kept constant within and between experiments (31.8 C ± 0.33; mean ± SD). Blood pressure was measured in all subjects and in some subjects from each group during infusion of the maximum doses of noradrenaline and L-NMMA.

Drugs or physiological saline were infused continuously (0.5ml min⁻¹) through a 27 SWG needle inserted into the brachial artery of the non-dominant arm (Vallance et al., 1989). One percent lignocaine solution was used to anaesthetise the skin prior to insertion of the needle. After establishing resting control values of blood flow for 15 mins during an
infusion of saline, four doses of noradrenaline were infused to produce a dose response curve (60, 120, 240 and 480 pmol min\(^{-1}\), each dose for 5 mins). After a further 15 mins of saline infusion and when blood flows had returned to baseline four doses of L-NMMA were infused to produce a second dose response curve (1, 2, 4 and 8 µmol min\(^{-1}\), each dose for 5 mins).

3.2.2 Safety of L-NMMA in pregnancy

This was the first time an NOS inhibitor had been infused into a pregnant woman and there was concern about the systemic effect of NOS inhibition, especially with regards to the fetus and uterus. As inhibition of NOS has been found to increase uterine activity in isolated rat myometrium (Yallampali et al., 1993) and decrease blood flow through the isolated human placenta (Myatt et al., 1991), the fetal heart rate and uterine contractions were monitored using a cardiotocograph.

3.3 Statistical Analysis and calculations

See 2.

3.4 Results

The characteristics of each group of women are shown in Table 3.1. There were no changes in uterine activity or fetal heart rate during the studies.
### Table 3.1 Characteristics of study groups

<table>
<thead>
<tr>
<th></th>
<th>Non-pregnant</th>
<th>Early-Pregnancy (9-15 weeks)</th>
<th>Late Pregnancy (36 – 41 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29 ± 6</td>
<td>23 ± 4</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>Age Range (years)</td>
<td>20-38</td>
<td>18 - 31</td>
<td>23 ± 32</td>
</tr>
<tr>
<td>Smokers</td>
<td>1/10</td>
<td>9/10</td>
<td>2/10</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>61.0 ± 7.5</td>
<td>57.0 ± 7.4</td>
<td>61.0 ± 6.8</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>115/80 ± 8/6</td>
<td>105/65 ± 6/5</td>
<td>110/70 ± 9/8</td>
</tr>
<tr>
<td>Forearm volume (ml)</td>
<td>1020 ± 140</td>
<td>935 ± 155</td>
<td>1180 ± 295</td>
</tr>
<tr>
<td>Hand Volume (ml)</td>
<td>320 ± 50</td>
<td>330 ± 55</td>
<td>375 ± 50</td>
</tr>
</tbody>
</table>
3.41 Basal hand blood flow

In all groups, blood flow in the non-infused hand remained stable throughout each experiment. Basal blood flows in non-pregnant women $9.4 \pm 2.3\, \text{ml} \, 100\, \text{ml} \cdot \text{hand}^{-1} \cdot \text{min}^{-1}$ (mean $\pm$ SE) were similar to flows in early pregnancy $7.7 \pm 2.1\, \text{ml} \, 100\, \text{ml} \cdot \text{hand}^{-1} \cdot \text{min}^{-1}$. However, basal blood flows were significantly higher in late pregnancy $20.6 \pm 3.5\, \text{ml} \, 100\, \text{ml} \cdot \text{hand}^{-1} \cdot \text{min}^{-1}$ compared with the other two groups ($p=0.007$; Figure 3.1).

3.42 Response to L-NMMA and Noradrenaline

In all three groups of subjects both L-NMMA and noradrenaline produced a dose dependent reduction in hand blood flow ratio (infused arm:control arm) (Figure. 3.2). Women in both pregnant groups had an increased response to L-NMMA compared with non-pregnant women ($p=0.0003$; Figure 3.2a). In contrast, the response to noradrenaline in late pregnancy was blunted when compared with non-pregnant and early pregnant subjects ($p=0.0029$; Figure 3.2b).

Comparison between drugs within each group demonstrates (i) non-pregnant women appeared to have a greater reduction in hand blood flow with noradrenaline compared with L-NMMA, this did not reach statistical significance ($p = 0.16$; Fig. 3.3a) (ii) women in early pregnancy had almost identical responses to both noradrenaline and L-NMMA ($p = 0.99$; Figure. 3.3b) and (iii) women in late pregnancy had a greater response to L-NMMA than to noradrenaline ($p = 0.0002$; Figure. 3.3c);
Figure 3.1 Basal hand blood flows. Hand blood flow (ml 100ml hand tissue⁻¹ min⁻¹) in non-infused hand throughout each experiment in all groups (mean ± SE). Blood flow was greatest in late pregnant women compared with non-pregnant and early pregnant women (p=0.007).
Hand Blood Flow (ml/100ml hand/min)

- Non-pregnant
- Early Pregnant
- Late Pregnant

* Significant difference
Figure 3.2a Log dose response curve to L-NMMA. Percent reduction in hand blood flow in response to L-NMMA in non-pregnant (diamonds), early pregnant (squares) and late pregnant subjects (triangles). Women in both pregnant groups had an increased response to L-NMMA compared with non-pregnant women (p=0.0003).

Figure 3.2b Log dose response curve to noradrenaline. Percent reduction in hand blood flow in response to noradrenaline in non-pregnant (diamonds), early pregnant (squares) and late pregnant subjects (triangles; n = 10 in each group). Women in late pregnancy had an attenuated response to noradrenaline compared to non-pregnant and early pregnant subjects (p=0.0029). Values expressed as means ± SE.
Figure 3.3   Response to L-NMMA (dashed line) and noradrenaline (solid line) for (a) non-pregnant, (b) early-pregnant and (c) late pregnant subjects (n = 10 in each group): d) Area under dose response curve for noradrenaline was subtracted from area under the dose response curve for L-NMMA, for each individual in each group. The response to L-NMMA increases relative to noradrenaline as pregnancy progresses. The difference between late pregnant and non-pregnant groups was significant (*p=0.0089).
Area under curve
(Arbitrary units)

Non-Pregnant

Early-Pregnant

Late-Pregnant

% reduction in hand blood flow

% reduction in hand blood flow

% reduction in hand blood flow

480 (pmol min⁻¹)

240 (pmol min⁻¹)

120 (pmol min⁻¹)

60 (pmol min⁻¹)

0

L-NMMA

L-NMMA

L-NMMA

Noradrenaline

Noradrenaline

Noradrenaline

Noradrenaline

0 20 40 60

0 20 40 60

0 20 40 60

0 20 40 60
When the AUC for noradrenaline was subtracted from the AUC for L-NMMA for each subject, differences between groups became even more marked. The response to L-NMMA became relatively more pronounced as pregnancy progressed (p=0.0089; Fig 3.3d).

3.5 Discussion

The results of this study indicate that inhibition of nitric oxide synthase with L-NMMA reduces hand blood flow more during pregnancy than in the non-gravid state. This was apparent even though basal blood flow was higher in late pregnancy and therefore the concentration of L-NMMA reaching the resistance vessels would have been lower. Indeed, in contrast to the responses seen in the non-pregnant and early pregnant groups, the response to the highest dose of L-NMMA (8 μmol min⁻¹) in late pregnancy was not at the top of the dose response curve, yet it produced a 62.4% reduction in blood flow (decreasing average basal flow from 20.6 ± 3.5 to 7.8 ml 100ml⁻¹ min⁻¹; (Figure 3.4). In non-pregnant subjects, following the same dose of L-NMMA, average basal blood flow was reduced by 43.8% from 9.4 ± 2.3ml 100ml⁻¹ min⁻¹ to 5.3ml 100ml⁻¹ min⁻¹. The reduced hand blood flow following L-NMMA in late pregnant women (7.8 ml 100ml⁻¹ min⁻¹) therefore lay between the pre- and post-L-NMMA blood flows of non-pregnant women. As late pregnant women did not reach a maximal reduction in hand blood flow following 8 μmol min⁻¹ of L-NMMA, it is possible that an even higher dose would have reduced blood flow even further, to levels found in non-pregnant women post-L-NMMA (i.e. 5.3ml 100ml⁻¹ min⁻¹). Under these circumstances, increased NO mediated vasodilatation would be solely responsible for the gestational increase in hand blood flow. However, I was unwilling to pursue this point by using higher doses of L-NMMA in this group of patients.
The response to the highest dose of L-NMMA (8µmol min⁻¹) in late pregnancy produced a 62.4% reduction in blood flow (decreasing average basal hand blood flow from 20.6 ± 3.5 to 7.8 ml 100ml⁻¹ min⁻¹. In non-pregnant subjects, following the same dose of L-NMMA, average basal blood flow was reduced by 43.8% from 9.4 ± 2.3ml 100ml⁻¹ min⁻¹ to 5.3ml 100ml⁻¹ min⁻¹. As L-NMMA 8 µmol min⁻¹ was not the maximal dose in late pregnancy, it is possible that a higher dose would have reduced hand blood-flow even further to the same levels as non-pregnant women.
When comparing the response to L-NMMA with the response to noradrenaline within each group (a situation in which both drugs would be exposed to the same conditions of basal blood flow and pressure) the L-NMMA response increased relative to that of noradrenaline as pregnancy progressed. This is particularly important since venous occlusion plethysmography is at its most powerful when used to compare relative potencies to different drugs given sequentially in the same experiment (Robinson, 1990). Together these findings indicate an enhanced response to L-NMMA in pregnancy and suggest that basal nitric oxide mediated dilatation is increased. Furthermore, they support the observations of Anumba et al, 1999a who made similar observations with an infusion of L-NMMA into the brachial artery, but measured a greater reduction in forearm blood flow in pregnant as compared with non-pregnant women. This group eliminated the possibility that shear stress might have activated NOS, by reducing the elevated forearm blood flow in pregnant volunteers to the same level of non-pregnant subjects and still found an increased reduction in blood flow to L-NMMA (Anumba et al, 2001). It is of interest that this group also found a diminished response to serotonin, a nitric oxide synthase dependent vasodilator, in pregnant compared with non-pregnant women (Anumba et al, 1999b). Whether this is due to a lack of NOS substrate (L-arginine), when basal NOS activity is high, or gestational changes to the serotonin signal-transduction pathways is unclear.

The response to L-NMMA was enhanced in both early and late pregnancy suggesting that increased basal NO mediated dilatation in the skin occurs early in pregnancy, at a time when cardiovascular changes are starting to occur. The augmented response to L-NMMA in early pregnancy is particularly striking as 9/10 subjects in this
group were smokers, a habit normally associated with endothelial cell damage and a reduced response to L-NMMA (Kiowski et al., 1994).

The response to noradrenaline in late pregnancy was reduced compared with that recorded in early pregnancy and non-pregnant controls. Although a blunted response to direct acting vasoconstrictors has not been a universal finding in studies during human pregnancy (Lumbers, 1970; Ramsay et al., 1992), similar results have been reported in studies on animals in vivo and in vitro (Chu and Beilin, 1993; Nathan et al., 1995; Ralevic and Burnstock, 1996). The pattern of hypo-responsiveness to constrictor agents with exaggerated vasoconstriction to L-NMMA or other NOS inhibitors is similar to that reported in sepsis (Petros et al., 1991) and other vasodilated hypotensive states associated with enhanced generation of NO (Albillos et al., 1995).

This study explored the functional effects of NO in maternal vasculature. Increased NOS activity has been found in platelets from healthy pregnant compared with non-pregnant and pre-eclamptic women (Delacretaz et al., 1995). Indirect biochemical assays of cGMP, the second messenger for NO and a stable oxidation product of NO, NOx have given conflicting results, most probably due to confounding problems (see Introduction) (Conrad et al., 1999; Boccardo et al., 1996).

There are several mechanisms by which NO mediated vasodilatation could be activated in pregnancy. Shear stress increases NO release (Rubanyi et al., 1986) and it is possible that the elevated blood flow in the hand itself stimulates NO activity. Flow
mediated endothelium-dependent vasodilatation is enhanced in pregnancy (Veille et al, 1998; Dorup et al, 1999). However, an increased response to L-NMMA was also found in early pregnancy, before hand blood flow increased this seems an unlikely explanation of our findings. Indeed, the typical response to L-NMMA in early pregnancy might have been underestimated since a large number of women in this group were smokers. Volume expansion or the increase in hand temperature during pregnancy could enhance NO generation (Calver et al, 1992; see chapter 4). Oestrogens up-regulate NOS in animals (Weiner et al., 1994) and therefore the huge rise in circulating oestradiol concentration during early pregnancy (Tulchinsky and Korenman, 1971) could stimulate increased NO synthesis. Consistent with this suggestion, post-menopausal women given transdermal 17 beta-oestradiol show enhanced serum levels of nitrite and nitrate (Rosselli et al., 1995). Furthermore, hand vasculature contains multiple arterio-venous anastomoses (Grant and Bland, 1931). Such vascular networks, which occasionally exist as arterio-venous malformations, enlarge during pregnancy and regress postpartum (Elliott et al., 1985).

This study examined the effect of L-NMMA on mother and fetus. Following local infusion of both L-NMMA or noradrenaline there was (a) no change in uterine activity or fetal heart rate in the late pregnant group and all babies were born healthy; (b) no significant overall change in basal blood flow in the non-infused hand throughout the study in all groups and (c) in those 3-4 subjects from each group in whom blood pressure was measured at the end of the study, no change in blood pressure. These observations confirm previous studies (Calver et al., 1993; Vallance et al., 1989) indicating that local infusion of L-NMMA
in these doses into the brachial artery produces a local effect only and is a safe investigative procedure to explore basal NO activity.

In conclusion, this study demonstrates that noradrenaline (an adreno-receptor agonist) and L-NMMA (a competitive inhibitor of nitric oxide synthase) produce dose dependent falls in hand blood flow in pregnant and non-pregnant women. These results suggest that catecholamines and endogenous nitric oxide synthase activity are able to alter skin blood-flow in healthy pregnant and non-pregnant women. Furthermore, it has been shown that the vascular response to blocking NOS increases in pregnancy, the degree of inhibition appearing to increase further towards term. These results suggest that there is enhanced NO-mediated vasodilatation, at least in the skin of the hand. The observation that L-NMMA returned basal blood flow back to normal in late pregnancy, is consistent with the proposal that NO might mediate the gestational increase in blood flow and contribute to the hypo-responsiveness to vasoconstrictors that occurs in pregnancy. The changes seen in this study also occur in the forearm and may account for the widespread cardiovascular changes of normal pregnancy. Although a failure to increase NO generation in the forearm (Anumba et al, 1999) is not seen in women with pre-eclampsia, it remains to be determined whether this is a ubiquitous finding in the maternal circulation.
CHAPTER 4

NITRIC OXIDE AND ADRENERGIC CONTROL OF HAND BLOOD FLOW DURING CHANGES IN LOCAL TEMPERATURE
Abstract

1. The cutaneous vasculature of the hands is predominantly composed of arteriovenous anastamoses, which play a major role in thermoregulation. The aim of the study was to determine the roles of the vasodilating substance nitric oxide and of the sympathetic vasoconstrictor noradrenaline towards local heat induced vasodilatation of the hands.

2. The possibility that the activity of these two vasoactive pathways is temperature dependent was investigated. Using venous occlusion plethysmography, it was determined whether a brachial artery infusion of the nitric oxide synthase (NOS) inhibitor, \(N^G\)-monomethyl-L-arginine (L-NMMA), could attenuate the increase in hand blood flow following a rise in local temperature from 23.2°C (SE 0.3) to 40.1°C (SE 0.8). In a similar experiment, noradrenaline was infused in an attempt to prevent local heat induced vasodilatation. Two further experiments measured the response to intra-arterial noradrenaline and L-NMMA on hand blood flow, once with cool water (26.2°C, SE 0.2) in the plethysmographs and on a separate occasion with warm water (34.6°C, SE 0.2).

3. A brachial artery infusion of L-NMMA (2 μmol min\(^{-1}\)) attenuated the rise in hand blood flow due to local warming by 5.4 (SE 0.8) ml 100ml hand\(^{-1}\) min\(^{-1}\); (95%CI, 3.4 to 7.3 ml 100ml hand\(^{-1}\) min\(^{-1}\), p<0.0005) compared with the control hand. However, local heat induced vasodilatation was not attenuated by noradrenaline 120 pmol min\(^{-1}\), (a dose equipotent with L-NMMA 2 μmol min\(^{-1}\) at euthermic temperatures).
4. Noradrenaline was \(x1.5\) (SE 0.2, 95%CI 1.1 to 2.0, \(p=0.008\)) more potent at reducing hand blood flow at cool compared with warm temperatures, whereas L-NMMA was equipotent at both temperatures. At high local temperatures L-NMMA was \(x1.14\) (SE 0.06, 95%CI 1.00-1.24, \(p=0.05\)) more potent than noradrenaline, but at low local temperatures, the maximal efficacy of noradrenaline was greater than L-NMMA.

5. These results indicate that increased nitric oxide synthase activity contributes to the vasodilatation of local warming of the hands. The relative potencies of adrenergic and nitrergic pathways change in the circulation of a thermoregulatory organ such as the hand. At high temperatures NO synthase activity predominates over reduced noradrenaline potency, but at low temperatures noradrenaline is more potent.
4. 1 Introduction

It was clear from Chapter 3 that the measurement of hand blood flow in pregnancy, using water displacement venous occlusion plethysmography varied according to local hand temperature. The water temperature in both plethysmographs had to be kept identical to avoid error. The study detailed in Chapter 4 asked whether the potency of vasoactive substances changes at different temperatures in a thermoregulatory organ such as the hand.

Control of the cutaneous vasculature plays a major role in thermoregulation (Clark & Edholm, 1985). In particular, blood flow through acral skin such as that of the ears, hands and feet is sensitive to changes in both core body and local environmental temperature (Joyner & Halliwell, 2000). Autonomic control of hand blood flow is predominantly provided through dense innervation of sympathetic vasoconstrictor neurones to arterio-venous anastomoses (AVAs) (Morris, 1997). A fall in core body temperature reduces blood flow to the fingers through increased sympathetic vasoconstrictor tone (Saumet et al, 1992). A rise in core body temperature leads to increased hand and finger blood flow due to withdrawal of sympathetic tone (passive vasodilatation) (Shepherd, 1963). Following local nerve block, hand blood flow will increase further if it is warmed directly (Roddie & Shepherd, 1956). These findings suggest that a local mechanism, possibly a vasodilator substance independent of the autonomic nervous system, is responsible for the vasodilatation of local hand warming.

The identity of this vasodilator substance has remained elusive since 1927, when Lewis suggested that local heat increased the production of a vasodilator 'H-substance'.
Nitric oxide (NO), an endothelium-derived vasodilator, now appears to be a likely candidate. An increase in local temperature will cause a rise in blood flow to forearm skin, which is reduced by NO synthase inhibition (Warren 1994; Goldsmith et al. 1996; Kellogg et al., 1999, Minson et al. 2001). The cutaneous vaculature of the forearm (non-acral) skin is however both functionally and anatomically different from the palmar surface of the hand. In this respect, the skin of the forearm resembles the non-glabrous skin of the dorsum of the hand rather than the palmar surface. Both forearm skin and dorsal hand skin contain vasodilator nerves that are activated by a rise in core temperature (Saumet et al. 1992, Kellogg et al. 1995, Johnson et al. 1995; Joyner & Halliwell 2000). No such vasodilator nerves innervate the skin of the palm (Johnson et al. 1995).

Further differences between palmar and dorsal hand skin were demonstrated by Noon et al. 1996, who showed that under basal conditions, inhibition of NO synthase reduces blood flow through the pulp of the thumb, but not the dorsal surface of the hand. This suggests that NO has a role in the control of vascular tone in those regions rich in arterio-venous anastamoses that serve a thermoregulatory function. As local temperature rises, 80-90% of hand blood flow is shunted through AVAs, rather than nutritive capillaries (Coffman, 1972).

This chapter investigates whether increased nitric oxide synthase activity plays a role in the increase in hand blood flow following a local rise in temperature. The warming experiment measured the effect of a brachial artery infusion of a nitric oxide synthase inhibitor (L-NMMA) on local heat induced increases in hand blood flow and compared it
with the effect of an infusion of noradrenaline (NA). In a separate experiment the potency of L-NMMA and of noradrenaline on hand blood flow is measured when the hands were cool and on a separate occasion when they were warm.

4.2 Methods

Three sets of experiments were performed.

4.21 Experiment 1; Protocol of warming experiment

Healthy male subjects were studied on two separate occasions at a constant ambient temperature of 24.0°C (SD 0.5). Subjects were lying clothed and lay semi-recumbent and blood flow (ml 100ml hand⁻¹ min⁻¹) was measured throughout the study in both hands simultaneously, using water filled plethysmographs placed at the level of the heart (Figure 2.1; Barcroft & Edholm, 1945). Wrist cuffs were inflated above venous pressure (40mmHg) for 10s in each 20s cycle. The plethysmographs were filled with cool water at 23.2°C (SE 0.3). A thermistor taped to the hand within the glove of the plethysmograph, recorded skin temperature. Core temperature was measured with an insulated thermistor in the external auditory meatus.

When all monitoring equipment was in place, a one percent solution of lignocaine was used to anaesthetise the skin of the antecubital fossa of the non-dominant arm, before insertion of a 27 SWG needle into the brachial artery. An infusion of 0.9% sodium chloride (0.5ml min⁻¹) was commenced. Once skin temperature and blood flow of both hands had stabilised and not less than fifteen minutes after starting the saline infusion, resting control
values of blood flow were measured in both hands over a period of ten minutes. Then, an infusion of either L-NMMA 2μmol min⁻¹ or noradrenaline 120 pmol min⁻¹ was commenced. After 5 min of drug infusion the cool water was rapidly changed to warm 40.1°C (SE 0.8) in both plethysmographs, while continuing the infusion of drug. Blood flow measurements were recommenced and continued for a further 20 min.

4.22 Experiment 2

Nitrergic and adrenergic responses at cool and warm temperatures

Using the same apparatus and techniques as for the warming experiment, blood flow was measured in both hands of 10 volunteers, once with the water in the plethysmographs at 26.2°C (SE 0.2) and on a separate occasion at least one week apart, with the water at 34.6°C (SE 0.2). An ambient temperature of 24.0°C (SD 0.5) was maintained throughout all experiments. Basal hand blood flow values were established for 15 min during an infusion of physiological saline. Then noradrenaline (60, 120, 240 and 480 pmol min⁻¹) followed by L-NMMA (1, 2, 4 and 8μmol min⁻¹) were infused for 5 min each dose into the brachial artery of one arm. Saline was infused for 15 min between the final dose of noradrenaline and the first dose of L-NMMA to allow blood flow to return to baseline. Whilst the action of noradrenaline is short lived, the duration of action of L-NMMA is up to one hour and therefore this drug had to be infused last (Vallance et al, 1989). To determine responses to drugs, blood flow in the infused hand was expressed as a ratio of blood flow in the non-infused hand. Changes in hand blood flow in response to drug infusion were then expressed as a percentage of the ratio during control (saline infusion) periods.
4.23 Experiment 3; Effect of Indirect Heating

As warming of the body or lower legs increases blood flow to the hands ('indirect heating'; Roddie & Shepherd, 1956), a further experiment was performed on two healthy male subjects to exclude the possibility that hand vasodilatation in response to warming was the result of body core warming. Hand blood flow was measured in two healthy male subjects using the same water filled plethysmographs as for previous experiments. In the absence of drugs, blood flow of both hands was measured before and after increasing water temperature in one plethysmograph from 20.1°C (SE 0.3) to 38.8°C (SE 0.9), while keeping the water temperature in the other plethysmograph at 22.0°C (SE 0.2).

4.24 Subjects

For the warming experiments, the L-NMMA study used a sample of 8 subjects, and the NA experiment used a sample of 6 subjects (5 of which were also in the sample for the L-NMMA experiment). The two constant temperature experiments both used the same sample of 10 subjects. The subjects in all samples were healthy male volunteers aged 21-42 years. They all gave informed, written consent to participate in the study that was approved by the local Research Ethics Committee.

4.3. Drugs (see Chapter 2.)

4.4 Statistical Analysis and calculations (see Chapter 2.)
4.5 RESULTS

4.5.1 Warming Experiment; Temperature control

Throughout the study, the temperature of water within the plethysmographs and of hand skin did not differ between the hand infused with drug (L-NMMA or noradrenaline) and the control hand. There was no difference in temperatures between experiments that infused L-NMMA and those that infused noradrenaline (Table 4.1). The rise in skin temperature was well tolerated by all subjects and did not cause pain.

**L-NMMA Infusion**

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<td><strong>Cold</strong></td>
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<tr>
<td>Water (Control Hand)</td>
<td>23.2 ± 0.9°C</td>
<td>40.2 ± 2.4°C</td>
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<tr>
<td>Water (Study Hand)</td>
<td>23.4 ± 1.1°C</td>
<td>39.3 ± 2.5°C</td>
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<tr>
<td>Hand Skin (Control Hand)</td>
<td>24.4 ± 0.1°C</td>
<td>36.2 ± 2.2°C</td>
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<tr>
<td>Hand Skin (Study Hand)</td>
<td>24.2 ± 1.0°C</td>
<td>35.8 ± 2.4°C</td>
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Core Temp was stable (36.8 ± 0.1°C) throughout all studies.

**Noradrenaline Infusion**

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<td><strong>Cold</strong></td>
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<tr>
<td>Water (Control Hand)</td>
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<td>40.7 ± 2.2°C</td>
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<tr>
<td>Water (Study Hand)</td>
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<tr>
<td>Hand Skin (Control Hand)</td>
<td>23.8 ± 0.6°C</td>
<td>36.0 ± 2.3°C</td>
<td></td>
</tr>
<tr>
<td>Hand Skin (Study Hand)</td>
<td>23.9 ± 0.7°C</td>
<td>36.0 ± 1.7°C</td>
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Core Temp was stable (36.9 ± 0.3°C) throughout all studies.

*Table 4.1* Summary of temperature changes to hand skin and plethysmograph water during local heating of the hands
4.52 Effect of L-NMMA infusion

On warming blood flow in the control hand increased from 2.8 (SE 0.8) to 13.5 (SE 0.9) ml 100ml hand\(^{-1}\) min\(^{-1}\). In the hand infused with L-NMMA blood flow increased from 2.7 (SE 0.8) to 8.0 ml (SE 0.9) 100ml hand\(^{-1}\) min\(^{-1}\) (Figure 4.1a). L-NMMA 2\(\mu\)mol min\(^{-1}\) attenuated the rise in hand blood flows due to local heating by 5.4ml 100ml hand\(^{-1}\) min\(^{-1}\) (95% Cl, 3.4 to 7.3 ml 100ml hand\(^{-1}\) min\(^{-1}\); p<0.005).

4.53 Effect of Noradrenaline infusion

In a similar experiment, the response to an infusion of noradrenaline 120 pmol min\(^{-1}\), in place of L-NMMA, was investigated. Replacing cool water with hot, caused blood flow in the control hand to rise from 2.9 (SE 0.6) to 15.0 (SE 1.1) ml 100ml hand\(^{-1}\) min\(^{-1}\). In the hand infused with noradrenaline, blood flow rose from 2.4 (SE 0.5) to 12.5 (SE 1.7) ml 100ml hand\(^{-1}\) min\(^{-1}\) (Figure 4.1b). The rise in blood flow in the noradrenaline treated hand was 2.5 ml 100ml hand\(^{-1}\) min\(^{-1}\) less than the corresponding rise in the control hand (95% CI, -2.2 to 6.2 ml 100ml hand\(^{-1}\) min\(^{-1}\); p = 0.30).

4.54 Comparison between L-NMMA and Noradrenaline Infusions

Blood flow through the control hands at the high temperature was similar when either L-NMMA or noradrenaline was being infused into the study hand; difference in control hand blood flow 1.7 ml 100ml hand\(^{-1}\) min\(^{-1}\) (95% C.I. -2.3 to 5.7; p=0.35). However, at the same high temperatures blood flow through the hand infused with L-NMMA 2\(\mu\)mol min\(^{-1}\) was suppressed by 6.0 ml 100ml hand\(^{-1}\) min\(^{-1}\) compared with blood
Figure 4.1 a Change in hand blood flow due to raising local temperature (water in plethysmographs) from 23.2°C (SE 0.3) to 40.1°C (SE 0.8). Control hand (solid line) and infused hand with nitric oxide synthase inhibitor, L-NMMA 2μmol min⁻¹ (dashed line). After warming, blood flow in the hand infused with L-NMMA rose to only 58% (95% CI 48-71%; p < 0.005) of blood flow in the control hand.

Figure 4.1 b Change in hand blood flow due to raising local temperature (water in plethysmographs) from 23.2°C (SE 0.3) to 40.1°C (SE 0.8). Control hand (solid line) compared with hand infused with noradrenaline 120pmol min⁻¹ (dashed line). After warming, there was no difference in blood flow between the hand infused with noradrenaline and the control hand 94% (95% CI 67-167%; p = 0.79). (Mean ± SE).
flow through the hand infused with noradrenaline 120pmolmin⁻¹ (95% CI, 2.3 to 9.6 ml 100ml hand⁻¹ min⁻¹; p = 0.005).

4.55 Nitrergic and adrenergic responses at cool and warm temperatures

In this study the temperature of water and hand skin was kept the same for both infused and control hands. There was no significant difference in blood flow between the two hands when saline was infused into the non-dominant brachial artery. In cool water, blood flows averaged 6.4 (SE 0.9) ml 100ml⁻¹ min⁻¹ and in warm water were 13.6 (SE 1.0) ml 100ml⁻¹ min⁻¹. Noradrenaline was x 1.5 (SE 0.2) 95% CI 1.1 - 2.0, p = 0.008) more potent in cool compared with warm water (Figure 4.2a). L-NMMA reduced hand blood flow by a similar amount at both warm and cool temperatures (Figure 4.2b).

During the hot study, L-NMMA was x 1.14 (SE 0.06, 95% CI 1.00 to 1.26, p=0.05) more potent than noradrenaline at reducing hand blood flow (Figure 4.3a). At a high local temperature the top dose of L-NMMA (8µmolmin⁻¹) did not appear to give a maximal response. In the cold, both drugs gave a similar reduction in hand blood flow, except that L-NMMA reached maximal efficacy with the third dose (4µmolmin⁻¹) and noradrenaline continued to reduce hand blood flow further (Figure 4.3b).
**Figure 4.2 a** Reduction in hand blood flow (percent change from saline infusion) in response to a brachial artery infusion of noradrenaline at warm (34.6°C SE 0.2; dashed line) and cool (26.3°C SE 0.3; solid line) local temperatures. Noradrenaline was 1.51 (SE 0.2, 95% CI 1.15 to 2.01, p=0.008) more potent at reducing hand blood flow at cool compared with warm temperatures.

**Figure 4.2 b** Reduction in hand blood flow (percent change from saline infusion) in response to a brachial artery infusion of L-NMMA at warm local temperature (solid line) and on a separate occasion at a cool local temperature (dashed line). There was no difference in the potency of L-NMMA at high or low temperatures (p=0.42).
Figure 4.3a Comparison between responses to L-NMMA and noradrenaline of hand blood flow at high local temperature (34.6°C SE 0.2). L-NMMA (solid line) was 1.14 (SE 0.06, 95%CI 1.00 to 1.24, p=0.055) more potent than noradrenaline (dashed line) at a high temperature.

Figure 4.3b Comparison between responses to L-NMMA and noradrenaline of hand blood flow at low local temperature (26.3°C SE 0.3). L-NMMA (dashed line) and noradrenaline (solid line) caused a similar reduction in hand blood flow at low temperatures, but the maximal efficacy of noradrenaline was greater than L-NMMA. (Mean ± SE).
4.56 Actual Hand Blood Flows

The wide range of actual hand blood flows between individuals at the same local and core temperatures confounded direct comparison of this parameter. Furthermore, actual hand blood flows in cold were less than half the flows in the warm. Therefore, the relatively larger response to noradrenaline in the cold was not statistically larger than the response in the hot when actual blood flows were compared (x1.3 (SE 1.1) 95% CI -1.2 – 3.8, p=0.29).

4.57 Effect of Indirect Heating

Local heating of one hand increased blood flow 20 fold in one subject and 12 fold in another subject. At the same time and for the subsequent 20 min, blood flow in the euthermic, contralateral hand remained unchanged in both subjects. No evidence of 'indirect heating' from warming the opposite hand was therefore found.

4.6 DISCUSSION

This study has shown that the increase in hand blood flow in response to local heating is attenuated by a brachial artery infusion of the NO synthase inhibitor, L-NMMA. This suggests that activation of nitric oxide synthase contributes to the increase in hand blood flow due to a rise in local temperature. Whether NO synthase is the only local heat-induced vasodilator enzyme remains unknown. It is likely that a higher dose of L-NMMA would have been even more effective at attenuating hand blood flow as the dose-response curves showed that L-NMMA 2μmol min⁻¹ did not give a maximal reduction in hand blood flow.
An attempt was made to prevent the increase in hand blood flow of local warming with a brachial artery infusion of noradrenaline 120pmolmin\(^{-1}\), a dose that is equipotent with L-NMMA 2μmolmin\(^{-1}\) at 26°C. This dose of noradrenaline did not attenuate the rise in hand blood flow of local warming. This supports the observation of Roddie & Shepherd 1956, who found that local temperature dependent vasodilatation of the hand is mediated through a local vasodilator mechanism independent of withdrawal of adrenergic tone. They showed that blood flow through the nerve blocked hand increased further when heated locally (Roddie & Shepherd, 1956). The noradrenaline dose-response curves show that doses higher than 120pmol min\(^{-1}\) cause a greater reduction in hand blood-flow, therefore it is possible that a higher dose of noradrenaline would have attenuated local hand warming. Indeed, the arterial infusion of noradrenaline could be equated with artificially maintaining adrenergic tone, and therefore a higher dose of noradrenaline would be expected to attenuate the rise in hand blood flow of local warming. This does not however take account of the opposing actions of nitric oxide synthase, which becomes more potent than noradrenaline at reducing hand blood flow as local temperature rises.

Doses of drugs were selected to produce local changes in the infused hand only and are below systemically effective doses. Furthermore, 2μmol min\(^{-1}\) L-NMMA was compared with 120 pmol min\(^{-1}\) noradrenaline, as it has been found that these two doses of drug cause a similar reduction in hand and forearm blood flow at ambient temperatures (Calver et al 1992). A rise in skin temperature from 15°C to 25°C is associated with a very small rise in hand blood flow, but above 25°C hand blood flow increases dramatically (Burton & Edholm, 1955). Temperatures above 42°C cause pain and can stimulate sensory-motor
nerves (Kellogg et al 1999). The experiment was therefore started with cool water in the plethysmographs at 23°C - 24°C and raised to just less than 42°C.

Nitric oxide synthase activity contributes to basal vasodilator tone of the finger pulp (Noon et al, 1996, Coffman, 1994). Finger blood flow is reduced by reflex sympathetic vasoconstriction (body cooling), but is not reduced further by nitric oxide synthase inhibition (Coffman 1994). Furthermore, under basal temperature conditions L-NMMA reduced finger blood flow by approximately 33%, while reflex sympathetic vasoconstriction decreased finger blood flow by 74% (Coffman 1994). It was concluded from these observations that nitric oxide is the most important factor controlling AVA blood flow (Coffman 1994). Noradrenaline caused a greater reduction in hand blood flow at low compared with high local temperatures and that L-NMMA was more potent than noradrenaline at high temperatures. In cool water, L-NMMA and noradrenaline gave similar reductions in hand blood flow, except that L-NMMA reached the top of its dose-response curve when noradrenaline reduced hand blood flow further. Taken together these observations suggest that at cool temperatures, nitric oxide synthase activity in AVAs of the hand is overwhelmed by increased sympathetic tone, but at high local temperatures, when sympathetic tone is withdrawn nitric oxide plays a prime role in opening AVAs further.

Finger blood flow increases on body warming (withdrawal of sympathetic tone) and can then be reduced by local cooling. This reduction in finger blood flow is not changed by nerve block, demonstrating the absence of a neurogenic influence on control of vascular tone in response to changes in local temperature (Arnott & Macfie, 1948). It is possible that
low finger blood flow persists at a low temperature due to reduced NO synthase activity relative to residual noradrenaline activity. Local cooling augments alpha-2-adrenergic vasoconstriction in the finger and isolated hand veins (Bodelsson et al, 1990; Freedman et al 1992) and reduces enzymatic degradation of noradrenaline within the vessel wall (Roberts et al, 2002). Patients with peripheral vaso-occlusive disorders such as scleroderma and Raynauds’ syndrome have increased sensitivity to alpha-2-adrenoceptors (Flavahan, 1991) and can benefit from local nitric oxide delivery systems (Tucker et al, 1999).

The rabbit ear is both functionally and anatomically similar to the palmar aspect of the hand as they share a thermoregulatory function by having a dense population of AVAs close to the surface of the skin (Morris, 1997). The AVA segments of the rabbit ear contain NO synthase and like the hand are densely innervated with sympathetic nerves (Funk et al, 1994; Morris, 1997). Inhibition of NO synthase reduces blood flow through the rabbit ear, but does not prevent the rise in ear blood flow due to whole body warming (Khan et al, 1993). As in the human hand, withdrawal of sympathetic tone plays an important role in the rise of rabbit ear blood flow following whole body warming (Roddie & Shepherd 1956; Bell & Robbins 1997). Blood flow through the rabbit ear is therefore carefully regulated by a balance between adrenergic and nitrergic pathways (Li et al, 1998). I have added to this observation by showing a temperature dependent effect of these two opposing vasoactive pathways on hand blood flow in vivo. At low temperatures, reduced endogenous NO synthase activity may augment the vasoconstrictor response to noradrenaline. Conversely, at high local temperatures increased NO synthase activity may attenuate the action of noradrenaline on hand blood flow.
NO synthase immuno-reactivity is present within epithelioid cells of the tunica media of AVAs of human finger and rabbit ear, but there are very few nitrergic fibres supplying AVAs (Funk, 1994). This is compatible with the physiological observation that vasodilator nerves do not control hand blood flow and our own observations that local warming can induce NO synthase-mediated dilatation. In the rabbit ear, NO synthase inhibition causes a greater degree of vasoconstriction of AVAs compared with arterioles and venules (Li et al, 1998), giving further support to a thermoregulatory role for NO synthase within AVAs situated on the palmar surface of the hand.

The increase in hand blood flow secondary to a rise in core body temperature appears to be entirely mediated by release of sympathetic vasoconstrictor tone and not stimulation of vasodilator nerves (Warren et al, 1942; Arnott & Macfie, 1948; Gaskell, 1956; Roddie et al, 1957). When body temperature is high, hand blood flow is increased further by local heating, but not by local nerve block (Gaskell, 1956, Roddie & Shepherd, 1956). The mechanism for increased hand blood flow in response to a rise in local temperature is therefore independent of either vasodilator nerves or further withdrawal of sympathetic tone. Conversely, vasodilator nerves are present in the forearm, as nerve block prevents vasodilatation induced by body heating and reduces forearm blood flow once elevated by body heating (Edholm et al, 1957). However, nitric oxide does not appear to be the vasodilator substance released from vaso-motor nerves in forearm skin (Dietz et al, 1994). Although there are anatomical and physiological differences between the skin vasculature of the forearm and palm, local heating also increases forearm skin blood flow.
Nitric oxide also mediates vasodilatation secondary to increased blood flow (Joannides et al, 1995). The mechanical stimulus for flow-induced vasodilatation appears to be a change in shear stress exerted on the endothelium, as demonstrated by vasodilatation following increased blood viscosity at constant flow (Melkumyants et al 1989). Blood viscosity increases at low temperatures (Cinar et al, 2001), but in this study the dose-response curve to L-NMMA was similar at both low and high temperatures. It is unlikely that shear stress mediated the increase in NOS activity during the warming experiments, as the L-NMMA infusion was started at low temperatures and at low hand blood flows. L-NMMA attenuated the rise in hand blood flow due to local warming before elevated blood flow had been established, rather than diminish high blood flow once it was elevated. It is therefore most likely that NOS is activated as a consequence of local warming of the hand rather than shear stress-induced activation that follows increased blood flow due to another vasodilator pathway.

Local warming of one hand was not sufficient to increase blood flow in the other euthermic hand. This suggests the absence of 'indirect heating' when such a small area as the contralateral hand is warmed.

A rise in local temperature will induce many physiological enzymes (Dixon & Webb, 1979). This is particularly true for tissues exposed to a large temperature
differential, such as the skin. As the temperature coefficient (change in $V_{\text{max}}$ 10°C$^{-1}$) around physiological temperatures is approximately 2 (Dixon & Webb, 1979), NO synthase activity could have doubled when skin temperature rose from 24.4°C to 35.8°C, as in our first study. Although the percentage reduction in hand blood flow to L-NMMA was equally effective at both high and low temperatures, hand blood flow was more than twice as great in hot compared with cold experiments. It is likely that under hot conditions, when blood flow was high, the concentration of drug arriving at the hand is reduced and therefore the sensitivity of the tissues to L-NMMA is increased, suggesting increased local NO synthase activity. In situations of increased NO synthase activity, the response to L-NMMA is increased and vice versa (Rees et al 1990).

In conclusion NO synthase contributes to the increase in hand blood flow during local warming. The relative potencies of noradrenaline and NO synthase are temperature dependent. Nitric oxide synthase inhibition was more potent than noradrenaline at warm compared with cool temperatures and noradrenaline was more potent at reducing hand blood flow at cool compared with warm temperatures. Blood flow through a thermoregulatory organ like the hand is predominantly controlled by adrenergic tone at cool local temperatures and by nitric oxide synthase at high local temperatures.
CHAPTER 5

ASYMMETRIC DIMETHYL-L-ARGININE IN GESTATIONAL HYPERTENSION AND THE FETAL-AMNIOTIC FLUID CIRCULATION
Abstract

1. This chapter investigates the role of the endogenous nitric oxide synthase (NOS) inhibitor, N\textsuperscript{G}, N\textsuperscript{G}-dimethyl-L-arginine (asymmetric dimethyl-L-arginine; ADMA) in two patho-physiological states: gestational hypertension and the fetal – amniotic fluid circulation.

2. Plasma ADMA levels in non-pregnant women were 0.99 (SE 0.1) µmolL\textsuperscript{-1} and fell to 0.43 (SE 0.06) µmolL\textsuperscript{-1} by the third trimester of pregnancy (p<0.01). Women with pre-eclampsia had higher plasma ADMA levels 1.20 (SE 0.19) µmolL\textsuperscript{-1} compared with normotensive pregnant women (p<0.001) and those who developed pregnancy-induced hypertension 0.56 (SE 0.19) µmolL\textsuperscript{-1} (p<0.05).

3. In human amniotic fluid, the concentration of ADMA was 0.67 (SE 0.06) µmolL\textsuperscript{-1} at 17 ± 1 week gestation and increased to 1.29 (SE 0.09) µmolL\textsuperscript{-1} at term (38 ± 1 week). In one individual, the ADMA concentration in the fetal bladder 1.44µmolL\textsuperscript{-1} was almost 3 fold higher than amniotic fluid 0.55µmolL\textsuperscript{-1}. Further studies in pregnant sheep confirmed that the highest ADMA levels were in fetal urine 18.3 (SE 5.2) µmolL\textsuperscript{-1}, followed by amniotic fluid 16.8 (SE 5.1) µmolL\textsuperscript{-1}, then fetal serum 4.6 (SE 0.81) µmolL\textsuperscript{-1} and maternal serum 1.6 (SE 0.27) µmolL\textsuperscript{-1}.

4. In conclusion, this chapter has found 1) plasma ADMA levels fall in healthy pregnancy, but are elevated in women with hypertension secondary to pre-eclampsia. 2) the fetal kidney excretes ADMA into amniotic fluid in increasing concentrations as pregnancy progresses. ADMA may compete with myometrial NOS to lower the threshold for labour at term and may inhibit NOS in the fetal pulmonary circulation until birth.
5.1 INTRODUCTION

5.12 The role of ADMA in regulation of nitric oxide synthase metabolism

In Chapters 3 and 4, an inhibitor of nitric oxide synthase NG methyl-L-arginine (L-NMMA), was used to investigate the role of NOS in the peripheral vasodilatation of healthy pregnancy and local hand warming. In the first part of Chapter 5, the plasma concentration of an endogenous inhibitor of NOS, asymmetric dimethyl-L-arginine, (ADMA) was measured in four groups of women 1. pregnancy-induced hypertension 2. pre-eclampsia, 3. healthy normotensive pregnancy and 4. non-pregnant volunteers. Women with hypertension that preceded the index pregnancy, chronic hypertensives were excluded from this investigation.

In the second half of this chapter, the concentration of ADMA in amniotic fluid was measured. Amniotic fluid completely engulfs the developing fetus. It is both swallowed and inhaled by the fetus, before being excreted back into the amniotic cavity by the fetal kidneys (Figure 5.1). By 11 weeks gestation, fetal urine makes the greatest contribution to the composition of amniotic fluid (Lind, 1981; Moore, 1982). Consequently, molecules filtered through the fetal kidney, such as creatinine, appear in increasing concentrations as pregnancy progresses (Gulbis et al, 1996).

Adult kidneys also filter ADMA (Vallance et al, 1992). The concentration of ADMA in adult urine is between 50 - 75 times higher than in adult plasma (Kakimoto and Akazawa, 1970); Vallance et al, 1992). In 1982, ADMA was also identified in normal human amniotic fluid (Lou and Chang, 1982). Although no role for ADMA was identified
Figure 5.1  a) Fetal micturition of a 32-week male fetus. Demonstrated by gray-scale ultrasonography (arrow head) and by colour Doppler ultrasound. (With permission, Devesa and Torrents, New Eng J Med, 338; 170)

b) Fetal-amniotic fluid circulation. Arrow shows how fetal urine contributes to amniotic fluid, which is both inhaled and swallowed by the fetus.
at that time, it is now known that NOS has a role in uterine quiescence during pregnancy (Bansal et al, 1997) and also in the pulmonary vasculature (Rairigh et al, 2001). It is possible therefore that ADMA within amniotic fluid has an important influence on the NO-pathway in neighbouring fetal and uterine systems.

This thesis has already provided functional evidence that increased NOS activity contributes to the fall in peripheral vascular resistance of healthy pregnancy. In the first section of this chapter, it is hypothesized that women with gestational hypertension would have higher circulating levels of ADMA than normotensive pregnant women. In the second section of this chapter, it was hypothesized that the growing fetus would excrete increasing concentrations of ADMA into the amniotic fluid.

5.2 METHODS

5.21 Gestational Hypertension

Healthy non-pregnant women and women in the third trimester of pregnancy were recruited from the antenatal clinics and wards of The Obstetric Hospital, University College Hospital and The Department of Obstetrics, St Georges' Hospital. All subjects gave their written, informed consent and the study had the approval of the local Ethics committee at both hospitals. Women who developed a blood pressure consistently greater than 140/90 during pregnancy, in the absence of proteinuria, were defined as having pregnancy induced hypertension (PIH). Those who had a similar rise in blood pressure, associated with proteinuria greater than 500mg/24h were classified as having pre-eclampsia. In all subjects, hypertension and proteinuria had resolved by 6 weeks postpartum. At the time of blood sampling no patients' had received anti-hypertensive medication. Blood pressure was measured in the semi-recumbent position using an appropriate sized cuff and mercury sphygmanometer. The diastolic reading was taken at the fifth Korotkoff sound. Venous blood was taken from a) eight healthy non-pregnant women, b) ten normotensive pregnant
women, c) nine women with pregnancy-induced hypertension and d) eight women with pre-eclampsia. All pregnant women were in the third trimester and matched for gestation. A blood sample (4 ml) for the measurement of ADMA was taken into a tube containing ethylene diaminetetracetic acid (0.054ml of 0.34M EDTA). After centrifugation, the plasma was stored at -20°C until assayed.

5.22 Fetal - Amniotic Fluid Circulation

Amniotic fluid was taken from 9 women in early pregnancy (gestational range 10-18 weeks), at the time of amniocentesis and from ten women in late pregnancy, at the time of elective Caesarean section. All subjects gave their written, informed consent and the study had the approval of the local Ethics committee at University College Hospital. In one patient samples were also taken from the fetal bladder as well as amniotic fluid.

Three chronically catheterized pregnant sheep under the care of Professor Mark Hanson at University College London were also investigated. Aliquots of 4ml of fetal bladder urine, fetal serum, amniotic fluid and maternal serum were removed from indwelling catheters for ADMA analysis.

5.23 ADMA Analysis

Samples were extracted and measured by HPLC, as described in Chapter 2. Statistical significance was calculated using Student’s t-test.
5.3 RESULTS

5.3.1 Gestational Hypertension

In healthy pregnancy plasma ADMA levels fell from 0.99 (SE 0.1) μmolL⁻¹ (non-pregnant) to 0.43 (SE 0.06) μmolL⁻¹ (3rd trimester) (p<0.05). Women with pre-eclampsia had significantly higher levels of ADMA 1.20 (SE 0.19) μmolL⁻¹ compared with gestationally matched normotensive pregnant women (p<0.001), but similar levels to non-pregnant women. The plasma ADMA levels in women with pregnancy-induced hypertension 0.56 (SE 0.19) μmolL⁻¹ were similar to healthy pregnant women, but less than that found in women with pre-eclampsia (p<0.05) (Figure 5.2).

There was no correlation between plasma ADMA concentration and creatinine clearance, but there was a trend for plasma SDMA concentration to rise as creatinine clearance (GFR) fell (Table 5.1).

<table>
<thead>
<tr>
<th>Volunteer (Initials)</th>
<th>Creat Cl. (ml min⁻¹)</th>
<th>ADMA (μmolL⁻¹)</th>
<th>SDMA (μmolL⁻¹)</th>
<th>Ratio (ADMA:SDMA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>22</td>
<td>0.58</td>
<td>1.74</td>
<td>0.33</td>
</tr>
<tr>
<td>JMS</td>
<td>81</td>
<td>0.55</td>
<td>0.51</td>
<td>1.08</td>
</tr>
<tr>
<td>DH</td>
<td>105</td>
<td>0.48</td>
<td>0.31</td>
<td>1.5</td>
</tr>
<tr>
<td>KN</td>
<td>111</td>
<td>0.53</td>
<td>0.62</td>
<td>0.85</td>
</tr>
<tr>
<td>NC</td>
<td>115</td>
<td>0.45</td>
<td>0.31</td>
<td>1.45</td>
</tr>
<tr>
<td>JMO</td>
<td>138</td>
<td>0.53</td>
<td>0.31</td>
<td>1.71</td>
</tr>
<tr>
<td>AS</td>
<td>158</td>
<td>0.47</td>
<td>0.38</td>
<td>1.24</td>
</tr>
</tbody>
</table>

Table 5.1 Plasma ADMA and SDMA levels in relation to renal function (creatinine clearance) during pregnancy.
Figure 5.2  Plasma concentrations of ADMA in non-pregnant controls (NPC), pregnant controls (PC), women with pregnancy-induced hypertension (PIH) and women with pre-eclampsia (PET). Horizontal bars = mean values. Values for women with PET were higher than those for normotensive pregnant women and for those with PIH (p<0.001 in both cases, Student’s t test).
Two normotensive pregnant women who had very different renal function exemplify the rise in SDMA levels with falling renal function. One woman with a renal transplant had a creatinine clearance of 22ml min\(^{-1}\) and plasma SDMA concentration of 1.74\(\mu\text{mol L}^{-1}\), while the other with a creatinine clearance of 138ml min\(^{-1}\) had plasma SDMA concentration of 0.31\(\mu\text{mol L}^{-1}\) (Table 5.1). Both women had similar plasma ADMA concentrations (0.53 and 0.58\(\mu\text{mol L}^{-1}\)).

### Human fetal amniotic fluid circulation

The concentration of ADMA in amniotic fluid at the end of pregnancy (38 ± 1 week gestation) 1.29 (SE 0.09) \(\mu\text{mol L}^{-1}\) (n = 8) was twice the level during the second trimester (17 ± 1 week) 0.67 (SE 0.06) \(\mu\text{mol L}^{-1}\) (n = 11). In one patient a mid-pregnancy sample from the fetal bladder (ADMA 1.44\(\mu\text{mol L}^{-1}\)) was almost three fold higher than from the corresponding amniotic fluid (ADMA 0.55\(\mu\text{mol L}^{-1}\)). This suggests that the fetal kidneys are a source of ADMA that is excreted in to amniotic fluid.

### Sheep Pregnancy

Three chronically catheterized pregnant sheep were sampled on four occasions throughout pregnancy. The highest ADMA levels were in fetal urine 18.3 (SE 5.2) \(\mu\text{mol L}^{-1}\), followe by amniotic fluid 16.8 (SE 5.1) \(\mu\text{mol L}^{-1}\), fetal serum 4.56 (SE 0.81) \(\mu\text{mol L}^{-1}\) and maternal serum 1.59 (SE 0.27) \(\mu\text{mol L}^{-1}\). This shows a similar gradient of ADMA levels to that found in human pregnancy, although the levels in fetal urine and amniotic fluid are much higher in the sheep. It is of note that sheep fetal serum contains x3 fold higher concentration of ADMA compared with maternal serum.
5.4 DISCUSSION

5.41 Gestational Hypertension

This study has demonstrated that circulating plasma ADMA levels fall during healthy, normotensive pregnancy. The reason for these changes was not investigated, but could reflect changes in the rate of ADMA synthesis or metabolism, its release from cells or re-uptake into cells (Fickling et al, 1993; MacAllister et al, 1996a; Leiper et al, 2002).

Circulating ADMA levels might also have fallen secondary to the haemodynamic changes of healthy pregnancy. These include a 40% increase in plasma volume (Brown and Gallery, 1994) and 50% increase in glomerular filtration rate (GFR) (Sturgiss et al, 1994). Changes in haematocrit did not however correlate with circulating ADMA concentration in pregnancy (Holden et al, 1998) and in this study neither did creatinine clearance correlate with ADMA levels. Creatinine clearance did however correlate with circulating levels of the inactive isomer SDMA. Although ADMA accumulates in plasma of patients with renal failure, the accumulation of SDMA is several-fold higher (MacAllister et al, 1996b). SDMA is not metabolized like its active isomer ADMA, but id cleared by renal excretion (MacAllister et al, 1996a). It would therefore seem most likely that during healthy pregnancy, the metabolism of ADMA is increased. The enzyme DDAH is a likely candidate as it co-localises to sites of NOS expression, including vascular endothelial cells and placenta (Kimoto et al, 1995).

In Chapter 3, increased NOS activity was shown to play a role in the peripheral vasodilatation of healthy pregnancy. A gestational fall in plasma ADMA concentration is consistent with that finding. In rat pregnancy, the blunted pressor response to exogenous angiotensin II (AII) can be reversed by NOS inhibition (Ahokas and Sibai, 1992). The pressor response to AII is also attenuated in normotensive human pregnancy, but there is
increased sensitivity to AII in women destined to develop pre-eclampsia (Gant et al, 1974). It is possible that low ADMA levels in normotensive pregnancy facilitate increased NOS activity, which results in an attenuated pressor response to AII and that elevated ADMA levels in women with pre-eclampsia inhibits NOS activity and increases maternal sensitivity to AII.

Pre-eclampsia is a plasma volume contracted state often associated with a reduction in GFR, but for the reasons stated above, it is unlikely that either of these haemodynamic changes play a major role towards the elevation of plasma ADMA levels in pre-eclampsia. Increased circulating ADMA may be responsible for constricting the utero-placental circulation and further compromising placental perfusion. Decreased placental perfusion is a common observation in women with pre-eclampsia (Williams and de Swiet, 1997) and may lead to a reduction in placental DDAH synthesis and consequently higher circulating levels of ADMA. DDAH activity can also be decreased by oxidised low-density lipoprotein (LDL) and the pro-inflammatory cytokine TNF-α, leading to a rise in ADMA levels (Ito et al, 1999). Both oxidised LDL and TNF-α levels are increased in pre-eclampsia (Uotila et al, 1998; Williams et al, 1999). Further studies on placental activity of DDAH would need to clarify this issue.

Inhibition of NOS by ADMA is competitively reversed by L-arginine (Vallance et al, 1992). During healthy pregnancy, plasma L-arginine levels fall, but are five fold lower in women with pre-eclampsia (D’Aniello et al, 2001). The relationship between plasma and intracellular L-arginine is unclear, but it is likely that low circulating L-arginine levels would enhance the inhibitory effect of ADMA on NOS activity.

Another interesting observation was that women with pregnancy-induced hypertension (PIH) had plasma ADMA levels similar to healthy normotensive pregnant
women rather than to pre-eclamptic women. This observation supports the phenotypic
differences between PIH and pre-eclampsia, where the former tends to be a high cardiac
output state with a large placenta and the latter tends to be a low cardiac output state with a
small placenta (Bosio et al, 1999). Women with PIH with a large placenta would be
expected to synthesise more DDAH and consequently have lower circulating ADMA
levels, similar to those found in women with normotensive pregnancies.

5.42 Fetal - Amniotic Fluid Circulation

In this study the concentration of ADMA in amniotic fluid increased as pregnancy
progressed. In one human subject (mother and fetus) ADMA levels were shown to be
higher in fetal bladder than in amniotic fluid, which suggests that the fetal kidney, like the
adult kidney, excretes ADMA. This observation is supported by the results from pregnant
sheep that show a similar gradient of ADMA concentration from fetal urine to amniotic
fluid as in humans. As the fetal kidney makes the greatest contribution to the composition
of amniotic fluid (Lind, 1981), the increasing ADMA concentration may be influential on
neighbouring systems that utilize the L-arginine – NOS pathway.

By 20 weeks gestation uterine quiescence is mediated by increased expression of
inducible nitric oxide synthase (NOS-II) (Bansal et al, 1997). NOS-II activity falls at term
and may therefore allow stimulants of uterine muscle to act unopposed for initiation of
labour (Bansal et al, 1997; Wray, 1993). The results from this study are compatible with
the increasingly mature fetal kidney excreting ever more ADMA into amniotic fluid, until
eventually the concentration is high enough to inhibit myometrial NOS-II. This system may
be an example of fetal maturity dictating the onset of labour. Further studies investigating
changes in the expression of the enzyme that metabolises ADMA (DDAH) in myometrium,
might provide further insight in to the role of the L-arginine – NO pathway towards the
onset of labour. The precise trigger to human labour has yet to be established, but is likely to be a complex, multi-faceted system.

Nitric oxide also plays an important role in reducing pulmonary vascular resistance (Rairigh et al, 2001). The fetal pulmonary circulation is vasoconstricted throughout pregnancy. Oxygenated blood from the placenta is shunted from the right to left side of the fetal heart, bypassing the lungs. During pregnancy the fetal lungs are bathed in amniotic fluid. At the moment of birth, fetal pulmonary vascular resistance falls rapidly and pulmonary blood flow increases 8-10 fold (Rudolph, 1979). It is possible that ADMA within inhaled amniotic fluid keeps the fetal pulmonary arteries vasoconstricted, but when amniotic fluid is expressed from the lungs at birth, pulmonary NOS is no longer inhibited and pulmonary vascular resistance falls.

Further studies are necessary to clarify the role of ADMA as a trigger for labour and as a vasoconstrictor of the fetal pulmonary vasculature in utero. Pathological changes to the metabolism of L-arginine – NO- ADMA pathway may be responsible for pre-term labour and persistent pulmonary hypertension of the newborn.
CHAPTER 6

THE ROLE OF PERIVASCULAR NERVES IN THE CONTROL OF RENAL BLOOD FLOW DURING RAT PREGNANCY
Abstract

1. This chapter investigated the role of peri-vascular nerves in the increase of renal blood flow during pregnancy. The function of sympathetic and sensory-motor nerves was examined in the isolated perfused rat kidney (IPRK) from virgin, 12-day and 21-day pregnant rats.

2. At basal vascular tone, electrical field stimulation (EFS; 80v, 1mS, 15s, 0.5-24Hz) elicited a frequency-dependent increase in renal perfusion pressure (RPP) that was attenuated in IPRK from late pregnant compared with virgin and mid-pregnant rats (p<0.0001). Desensitisation of sensory-motor nerves with acute capsaicin (10⁻⁶M) in late pregnancy did not augment the reduced pressor response to EFS. The alpha-1 receptor agonist, methoxamine caused a rise in RPP that was attenuated in mid-pregnancy when compared with virgin and late pregnant rats (p<0.05).

3. At raised vascular tone and following blockade of sympathetic neurotransmission with guanethidine, the sensory-motor neuropeptide, calcitonin gene related peptide (CGRP) caused a greater fall in perfusion pressure in kidneys from late pregnant compared with virgin and mid-pregnant rats (p<0.02).

4. At raised vascular tone and following blockade of sympathetic neurotransmission with guanethidine, EFS (80v, 0.1mS, 15s, >8Hz) produced vasodilatation that was very similar to that induced by exogenous CGRP and acute capsaicin. EFS-induced vasodilatation was abolished following treatment with capsaicin.

5. The remaining kidney from virgin and pregnant rats was divided into four segments: cortex, inner and outer medulla and renal pelvis. Renal cortical levels of CGRP and substance P (SP) fell during late pregnancy (CGRP, virgin 8.9±0.9, late
pregnant 2.9±0.7 pmol g⁻¹ p<0.01; SP, virgin 13.8±1.7, late pregnant 1.3±1.0 pmol g⁻¹ p<0.001). Renal cortical levels of neuropeptide-Y (NPY) also fell during late pregnancy (virgin 88.99±21.49; late pregnant 6.70±1.66; p<0.003). There were no significant differences in tissue levels of CGRP, SP or NPY in inner medulla or renal pelvis. Plasma levels of CGRP did not change during rat pregnancy.

6. Immunofluorescence revealed regression of perivascular CGRP containing nerves throughout the renal parenchyma during late pregnancy.

7. In conclusion, this study has shown an attenuated vasoconstrictor response to EFS in the late pregnant rat kidney. This reduced vasoconstrictor response is not due to increased activity of sensory-motor nerves. In late pregnancy, the isolated perfused rat kidney becomes more sensitive to exogenous CGRP as the renal cortical concentration of CGRP and SP falls and immunofluorescence reveals regression of perivascular sensory-motor nerves. The reduced response to EFS in late pregnancy coincides with a reduction in renal cortical levels of the sympathetic neurotransmitter NPY. The plasticity of renal perivascular innervation during pregnancy may play a functional role in altered gestational renal blood flow. This study has also shown preliminary evidence that electrical field stimulation of the isolated perfused rat kidney induces vasodilatation by stimulating sensory-motor nerves.
6.1 INTRODUCTION

In the rat, renal blood flow (RBF) and glomerular filtration rate (GFR) reach a maximum of 50% above non-pregnant levels on day 12 and then fall gradually until term (Baylis, 1994; Alexander et al, 1999). A similar pattern of increased RBF and GFR is seen during human pregnancy, although both remain elevated above non-pregnant levels at term (Sturgiss et al, 1994).

Increased NOS activity has a major role in the renal adaptation to rat pregnancy. The gestational rise and fall of RBF in the rat coincides with a rise and fall of nitric oxide (NO) production (Conrad et al, 1993) and can be prevented by NO synthase (NOS) inhibition (Danielson & Conrad 1995). In mid-pregnancy the endothelium mediates a reduction in vascular myogenic reactivity of renal arteries (Gandley et al, 2001). By late pregnancy renal perfusion pressure in the rat returns to non-gravid levels, but vasoconstrictor responses remain depressed despite NO synthase inhibition (Chu and Beilin, 1997). Furthermore, vasodilatory prostaglandins do not contribute to the increased renal blood flow of pregnancy (Conrad and Colpoys, 1986; Baylis, 2002). Endothelial derived vasodilator factors cannot therefore explain all of the changes to renal blood flow in pregnancy.

In the kidney, the endothelium interacts with peri-vascular sympathetic nerves and other paracrine systems to auto-regulate renal blood flow and maintain the glomerular filtration rate (Navar et al, 1996; Di Bona and Kopp, 1997). The kidney is heavily innervated by efferent sympathetic nerves to all its functional components; blood vessels, glomeruli and tubules (DiBona, 2002). Noradrenaline released from sympathetic nerves activates adreno-receptors on effector tissues and mediates vasoconstriction of renal blood vessels (DiBona, 2002). The actions of noradrenaline can be modulated by co-release of neuropeptide Y (NPY) and the co-transmitter adenosine triphosphate (ATP) (Lincoln &
Sensory afferent neurones are also found in the renal parenchyma surrounding blood vessels, but are particularly abundant around the renal pelvis and ureters (Knight et al, 1991). Afferent neurones rich in substance P (SP), ATP and calcitonin gene related peptide (CGRP) (Amara et al, 1982; Brain et al, 1985) are stimulated by mechanoreceptors that sense stretch in the renal pelvis, ureters, arteries and veins (Stella and Zanchetti, 1991). Activated afferent neurones synapse in the sensory dorsal columns and influence central haemodynamics as well as sympathetic nerve activity to the contra-lateral kidney; the reno-renal reflex (Stella and Zanchetti, 1991; DiBona and Kopp, 1997). Stretch receptors in the renal pelvis are stimulated by release of SP, but a physiological role for the potent vasodilator CGRP in sensory-motor nerves is currently unknown (DiBona and Kopp, 1997).

Certain sensory afferent neurones can also exhibit an efferent motor component, by transmitting antidromic impulses to elicit vasodilator responses; sensory-motor nerves (Burnstock 1986; Holzer, 1988; Rubino and Burnstock, 1996). A unique feature of sensory-motor neurones is their acute stimulatory, but chronic neurotoxic response to capsaicin. Application of capsaicin leads to release of CGRP, SP and ATP, followed by desensitisation and a lack of functional response to a further application of capsaicin or electrical field stimulation (EFS) (Holzer, 1991).

Systemic administration of CGRP to rats reduces MAP and renal vascular resistance, while mediating a rise in GFR (Siren and Feuerstein, 1988; Amuchastegui et al, 1994). Capsaicin infused into the renal artery of an isolated perfused rat kidney has
previously been shown to reduce renal perfusion pressure and increase release of CGRP-like immunoreactivity in the venous effluent (Geppetti et al. 1989). In the rat mesentery, capsaicin augments adrenergic vasoconstriction produced by stimulation of periarterial nerves (Kawasaki 1988). This suggests that sensory-motor nerves oppose the vasoconstrictor effects of the sympathetic nervous system in rat mesentery.

The aim of this study was to investigate the functional role of sympathetic and sensory-motor perivascular nerves in the control of renal blood flow in pregnant and non-pregnant rats, using the isolated perfused rat kidney. Immunohistochemical and biochemical techniques were also used to investigate whether there was a gestational change in perivascular innervation within the rat kidney.

6.2 METHODS

6.21 Isolated Perfused Rat Kidney

A non-recirculating perfusion system of the isolated rat kidney was used, adapted from the model described by Churchill and Ellis, 1993 (See Figures 2.4 and 2.5).

6.22 Electrical field stimulation of perivascular nerves before and after capsaicin

Electrical field stimulation (EFS) of the isolated perfused rat kidney (IPRK) was applied with bipolar electrodes using a Grass SD9 stimulator. One electrode was attached to the needle perfusing the renal artery and the other was attached to the wire-mesh on which the kidney lay. Changes in perfusion pressure were measured by a Statham transducer.
(Viggo-Spectramed) attached to a Grass polygraph (Grass Instruments Co, Quincy, Mass, USA). At basal tone, a frequency-response curve to EFS (80v, 1mS, 15s, 0.5-24Hz) was measured. The kidney was then perfused with capsaicin (10^-6M) for 20 min, before the frequency-response curve to EFS was repeated in each of the three groups of rat. The neuronal origin of these vasoconstrictor responses was confirmed, as they were abolished after application of tetrodotoxin (1μM).

6.23 Responses to Exogenous CGRP

Using the same apparatus as for the previous experiment, but with vascular tone of the isolated perfused rat kidney raised with the alpha-adrenoceptor agonist, methoxamine (3x10^-6M), and with the release of neurotransmitters from sympathetic nerves blocked by the addition of guanethidine (5μM), vasodilator responses to doses (100 μl bolus injections) of exogenous CGRP (10^-9mol to 10^-6mol) were measured in virgin, 12-day pregnant and 19-day pregnant rats. The time between doses varied between 3 and 30min depending on the time it took for the tone to return to baseline.

6.24 Electrical field stimulation of sensory-motor nerves

In an attempt to observe the vascular response to EFS of sensory-motor nerves, vascular tone was raised with the alpha adrenoceptor agonist, methoxamine (3x10^-6M) and the release of neurotransmitters from sympathetic nerves was blocked by the addition of guanethidine (5μM) and. At raised tone, the responses to EFS, (80v, 0.1mS, 30s duration, 8-24Hz) were compared between the three groups of rat; virgin, mid-pregnancy (12-day) and late-pregnant (19-day). Under the same conditions, the acute response to a bolus dose of the sensory-motor desensitising agent capsaicin (100μl of 10^-6mol) was also measured.
After at least two injections of capsaicin (100μl of 10^{-6}mol) an attempt to elicit vasodilatation with EFS was repeated.

6.25 Immuno-histochemistry

The non-perfused kidney was removed and immersion-fixed for 3-4 h in 4% paraformaldehyde in phosphate buffered saline (PBS), prior to immuno-staining (see Chapter 2).

6.26 Measurement of CGRP, Substance P and Neuropeptide Y levels within renal cortex

The kidney was bisected and then separated with a sharp blade into four readily identifiable zones, the renal cortex, the outer and inner medulla and renal pelvis. Specimens were immediately stored in liquid nitrogen until peptide extraction. Details of the assays used to measure these peptides are described in Chapter 2.

6.3 RESULTS

6.31 Isolated Perfused Rat Kidney

Characteristics of isolated perfused rat kidneys and parameters for perfusion are given in Table 6.1. When methoxamine (3 x 10^{-6}M) was added to the perfusate, the pressure within kidneys from virgin rats increased by 88 ± 13 mmHg (n = 12), in 12-day pregnant rats by 38 ± 15 mmHg (n = 6) and in 19-day pregnant rats by 81 ± 15 mmHg (n = 11). Mid-pregnant rats had a significantly blunted response to methoxamine compared with virgin and 19-day pregnant rats (p < 0.05).
Table 6.1.
Characteristics of isolated perfused rat kidneys from virgin and pregnant rats (mean ± SE).

<table>
<thead>
<tr>
<th></th>
<th>Virgin (n=12)</th>
<th>12 Day Gestation (n=6)</th>
<th>19 Day Gestation (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat weight (g)</td>
<td>271 ± 5</td>
<td>343 ± 12</td>
<td>390 ± 8</td>
</tr>
<tr>
<td>Kidney wet weight (g)</td>
<td>0.88 ± 0.01</td>
<td>1.02 ± 0.06</td>
<td>0.96 ± 0.03</td>
</tr>
<tr>
<td>Kidney dry weight (g)</td>
<td>0.20±0.01</td>
<td>0.22 ±0.01</td>
<td>0.23 ±0.01</td>
</tr>
<tr>
<td>Perfusion Pressure (mmHg)</td>
<td>85 ± 3</td>
<td>83 ± 4</td>
<td>85 ± 3</td>
</tr>
<tr>
<td>Perfusate Flow Rate (ml min⁻¹)</td>
<td>12.5 ± 0.6</td>
<td>17.9 ± 1.9</td>
<td>15.5 ± 0.6</td>
</tr>
</tbody>
</table>
6.32 Electrical field stimulation of perivascular nerves at basal tone

At basal tone, EFS evoked vasoconstrictor responses that were attenuated in late pregnant rats compared with virgin and 12 day pregnant rats (p<0.0001) (Figure. 6.1a). These responses were abolished following the application of tetrodotoxin (1μM), thus revealing their neural origin. Desensitising sensory-motor nerves by the addition of capsaicin (10^-6M) to the perfusate did not affect the response to EFS in any group of rat (Figure 6.1b). The response to EFS remained attenuated in late pregnant compared with virgin and mid-pregnant rats (p<0.001). This suggests that sensory-motor nerves do not contribute to the reduced vasoconstrictor response to EFS in late-pregnancy.

6.33 Responses to Exogenous CGRP

At raised vascular tone, 100 μl bolus injections of exogenous CGRP (10^-9mol to 10^-6mol) evoked dose dependent vasodilatory responses. Vasodilatory responses were greater in late pregnant compared with virgin rats (p<0.02) (Figure 6.2a). The response to exogenous CGRP was not different between virgin and mid-pregnant rats (Figure 6.2b).

6.34 Electrical field stimulation of sensory-motor nerves

Electrical field stimulation at raised vascular tone and in the presence of guanethidine (5μM) resulted in a prolonged vasodilatation (Figure 6.3a). The prolonged nature of this vasodilatation was compatible with the release of exogenous vaso-active substances. Acute administration of 100μl capsaicin (x10^-6), which desensitises sensory-motor nerves by releasing CGRP, substance P and ATP, produced a short-lived vasoconstriction followed by a prolonged vasodilatation. EFS-induced vasodilatation could not be repeated following acute administration of capsaicin. Capsaicin-induced vasodilatation was almost identical to that produced by EFS and by exogenous administration of CGRP (Figure. 6.3b). The initial transient vasoconstrictor response following capsaicin may have been caused by release of ATP.
**Figure 6.1a** Frequency dose response curve to electrical field stimulation of IPRK in virgin, mid-pregnant (12 days gestation) and late-pregnant (19 days gestation) rats. The increase in perfusion pressure in response to electrical field stimulation was attenuated in IPRK from late pregnant compared with virgin and mid-pregnant rats (p < 0.0001).

**Figure 6.1b** Frequency dose response curve to electrical field stimulation of the IPRK after it was perfused with capsaicin (1x10^-6M) for 20 min in virgin, mid-pregnant (12 days gestation) and late-pregnant (19 days gestation) rats. The vasoconstrictor responses to EFS remained attenuated in comparison to virgin and mid-pregnant rat kidneys (p<0.001).
Figure 6.2. Percent fall in renal perfusion pressure in response to exogenous CGRP in the IPRK. Figure 6.2a; There is a greater response to exogenous CGRP in late pregnant rats compared with virgin rats (p<0.02). Figure 6.2b; There was no difference between virgin and mid-pregnant rats.
**Figure 6.3a,b:** Typical tracing of perfusion pressure from isolated perfused rat kidney (IPRK)

**Figure 6.3a.** Guanethidine $5 \times 10^{-6}$M was added to the perfusate, to inhibit release of transmitter from adrenergic neurons. Vascular tone was raised with the alpha 1-adreno-receptor agonist, methoxamine. A bolus injection of acetylcholine (Ach; $10^{-6}$M), followed by electrical field stimulation (80v, 1mS, 12Hz for 30S) and then a bolus injection of capsaicin $10^{-6}$M all cause vasodilatation. Capsaicin and higher frequency nerve stimulation (not shown in illustration) caused a transient vasoconstriction. It is possible that ATP released from sensory-motor neurones elicited a transient vasoconstriction, before CGRP mediated a longer acting vasodilatation.

**Figure 6.3b.** Dose-response curve to CGRP revealing a similar pattern of vasodilatation as that seen following nerve stimulation or administration of capsaicin. CGRP is released in the venous effluent of the kidney following administration of capsaicin (Geppetti et al, 1989).
(a) Nerve Stimulation
0.1 mV, 16 Hz, 80 V, 30 s
Capsaicin -5 M

100 (mm Hg)
60 s

(b) CGRP 3x10^{-9} M CGRP -8 M CGRP 3x10^{-6} M

100 (mm Hg)
60 s

CGRP 7 M CGRP 3x10^{-7} M CGRP -6 M
6.35 Immuno-histochemistry

CGRP immunofluorescence was noted in peri-vascular nerves throughout the renal parenchyma of pregnant and virgin rats (Figure 6.4a, b). Assessment by a histologist, unaware of the different groups, indicated that the peri-vascular CGRP immunofluorescence was more widespread in the cortex and medulla of kidneys from virgin compared with late pregnant rats. However, the most intense CGRP immuno-reactivity was in the renal pelvis (Figure 6.4c). The intensity of CGRP staining within the renal pelvis did not differ between pregnant and virgin rats. These findings correlate well with the biochemical assay.

Tyrosine hydroxylase immunofluorescence was also found around perivascular nerves and around the collecting ducts, but not in the renal pelvis of all three groups. There was no difference in the intensity of tyrosine hydroxylase staining between virgin and pregnant rats.

6.4 BIOCHEMICAL ASSAY

6.41 Plasma concentrations of CGRP

The plasma concentration of CGRP did not differ between each group of rat; virgin $5.13 \pm 0.62$ pmol l$^{-1}$ (n=8); mid-pregnancy $3.77 \pm 0.64$ pmol l$^{-1}$ (n=6); late pregnancy $5.28 \pm 0.94$ pmol l$^{-1}$ (n=10).

6.42 Renal concentration of CGRP

The concentration of CGRP in kidney homogenate, which contains nerves, endothelial and epithelial cells was greatest in the renal pelvis, in all 3 groups of rat studied.
Figure 6.4. a,b CGRP immunofluorescence in perivascular nerves (arrows) around interlobular arteries within the kidney of late pregnant rats.

c. The strongest immunostaining for CGRP was in the wall of the renal pelvis.
Comparison between the three groups of rat revealed a significant fall in CGRP concentration in the renal cortex as pregnancy progressed (Table 6.2a; Figure 6.5; p<0.01). In the outer medulla, there was a similar trend towards a fall in CGRP concentration, but this did not reach statistical significance. There was no significant difference between groups in the concentration of CGRP in the inner medulla or renal pelvis.

6.43 Renal concentration of Substance P

The concentration of substance P was also highest in the renal pelvis. However, the levels of substance P in the renal pelvis and inner medulla did not change as pregnancy progressed. In the renal cortex there was a significant fall in substance P concentration as pregnancy progressed (Table 6.2b; Figure 6.5, p < 0.001). A significant fall in substance P concentration was also seen in the outer medulla as pregnancy progressed (p < 0.001).

6.44 Renal concentration of Neuropeptide-Y

Comparison between the three groups of rat revealed a significant fall in NPY concentration in the renal cortex as pregnancy progressed (Table 6.2c; Figure 6.5, p<0.003). In the outer medulla, there was a trend towards a fall in NPY concentration. There was no significant difference between groups in the concentration of NPY in the inner medulla or renal pelvis.
### Table 6.2a CGRP levels in rat plasma and kidney throughout pregnancy (n=6; mean ± SE)

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Renal Cortex</th>
<th>Outer Medulla</th>
<th>Inner Medulla</th>
<th>Renal Pelvis</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(pmol l⁻¹)</td>
<td>(pmol g⁻¹)</td>
<td>(pmol g⁻¹)</td>
<td>(pmol g⁻¹)</td>
<td>(pmol g⁻¹)</td>
</tr>
<tr>
<td>Virgin</td>
<td>5.13 ± 0.62</td>
<td>8.87 ± 0.90</td>
<td>14.49 ± 4.39</td>
<td>9.79 ± 0.72</td>
<td>217.3 ± 18.1</td>
</tr>
<tr>
<td>Mid-pregnancy</td>
<td>3.77 ± 0.64</td>
<td>7.92 ± 0.55</td>
<td>12.39 ± 1.82</td>
<td>9.62 ± 1.30</td>
<td>157.4 ± 26.5</td>
</tr>
<tr>
<td>Late-pregnancy</td>
<td>5.28 ± 0.94</td>
<td>2.94 ± 0.65</td>
<td>4.67 ± 1.13</td>
<td>8.67 ± 1.05</td>
<td>131.2 ± 32.2</td>
</tr>
<tr>
<td>Significance</td>
<td>N.S.</td>
<td>p&lt;0.01</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
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</table>

### Table 6.2b Substance P levels in rat kidney throughout pregnancy (n =6; mean ± SE)

<table>
<thead>
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<th>Inner Medulla</th>
<th>Renal Pelvis</th>
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<tr>
<td></td>
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<td>(pmol g⁻¹)</td>
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<td>(pmol g⁻¹)</td>
</tr>
<tr>
<td>Virgin</td>
<td>13.83 ± 1.68</td>
<td>84.72 ± 13.5</td>
<td>2.68 ± 0.93</td>
<td>189.2 ± 52.4</td>
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<tr>
<td>Mid-pregnancy</td>
<td>10.04 ± 0.98</td>
<td>77.88 ± 14.4</td>
<td>2.79 ± 0.72</td>
<td>92.6 ± 11.4</td>
</tr>
<tr>
<td>Late-Pregnancy</td>
<td>1.26 ± 1.02</td>
<td>5.23 ± 1.6</td>
<td>2.70 ± 0.53</td>
<td>77.3 ± 10.5</td>
</tr>
<tr>
<td>Significance</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>N.S.</td>
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</table>

### Table 6.2c Neuropeptide Y levels in rat kidney throughout pregnancy (n=6; mean ± SE)

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<th>Inner Medulla</th>
<th>Renal Pelvis</th>
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<tbody>
<tr>
<td></td>
<td>(pmol g⁻¹)</td>
<td>(pmol g⁻¹)</td>
<td>(pmol g⁻¹)</td>
<td>(pmol g⁻¹)</td>
</tr>
<tr>
<td>Virgin</td>
<td>88.99 ± 0.90</td>
<td>59.89 ± 22.0</td>
<td>3.55 ± 1.47</td>
<td>33.2 ± 6.9</td>
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<tr>
<td>Mid-pregnancy</td>
<td>24.95 ± 2.86</td>
<td>35.12 ± 4.8</td>
<td>5.62 ± 2.22</td>
<td>26.8 ± 5.4</td>
</tr>
<tr>
<td>Late-pregnancy</td>
<td>6.70 ± 1.66</td>
<td>11.23 ± 1.89</td>
<td>1.50 ± 0.36</td>
<td>23.4 ± 11.6</td>
</tr>
<tr>
<td>Significance</td>
<td>p&lt;0.003</td>
<td>N.S.</td>
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</table>
Figure 6.5. Histogram showing the concentration of sensory-motor neuropeptides (CGRP and substance P) and the sympathetic neuromodulator, neuropeptide Y (NPY) in rat renal cortex during pregnancy. There was a gradual fall in CGRP (white bar), substance P (black bar) and NPY (hatched bar) as pregnancy progressed (CGRP, p < 0.01; substance P, p < 0.001; NPY, p < 0.003). For further details see Table 6.2.
6.5 

DISCUSSION

6.5.1 

Perivascular Nerves and the Control of Renal Blood Flow in Pregnancy

The aim of this study was to investigate the role of peri-vascular renal nerves towards the increase of renal blood flow during healthy pregnancy.

**Mid-Pregnancy**

Kidneys isolated from rats at 12 days gestation required a higher flow rate to maintain a perfusion pressure similar to virgin and late pregnant rats. This is in accordance with *in vivo* studies that have shown renal blood flow is maximal on days 12-14 of a 21-day rat pregnancy (Baylis, 1994, Conrad et al, 1993).

In the present study, kidneys from 12-day pregnant rats were resistant to vasoconstriction with the alpha-1-adrenoceptor agonist, methoxamine, but the response to electrical field stimulation (EFS) at this gestation was not attenuated. There are several explanations for this observation. It is possible that during mid-pregnancy there is a transient reduction in adreno-receptor number or function, but this would have to be balanced by increased release of sympathetic neurotransmitter in order to explain the unaltered response to EFS. Another possibility is that the high flow of mid-pregnant rat kidneys induced shear stress. Flow-induced shear stress increases nitric oxide synthase (NOS) activity, a process that is up-regulated in isolated mesenteric arteries from pregnant rats (Cockell and Poston, 1996). The mid-pregnancy rise of renal blood flow coincides with increased nitric oxide production (Conrad et al, 1993) and can be abolished by NOS inhibition (Danielson and Conrad, 1995). Both the pregnancy hormone relaxin and also endothelin acting on endothelial ET$_B$ receptors, can increase renal blood flow in mid-pregnancy by stimulating NOS activity (Conrad et al, 1999; Danielson et al, 2000; Novak et al, 2001).
Another possibility is that attenuated vasoconstrictor responses are secondary to reduced smooth muscle contractility. In isolated small renal arteries from mid-pregnant rats there is an endothelial dependent reduction in myogenic reactivity compared with virgin rats (Gandley et al, 2001). In the current study, which investigated the whole renal vascular bed, changes in myogenic activity alone could not explain the opposing observations of reduced response to methoxamine, but unaltered response to EFS.

**Late Pregnancy**

In late-pregnant rat kidneys the flow rate needed to maintain a basal perfusion pressure of 85mmHg was the same as for virgin kidneys. This correlates with *in vivo* observations that renal blood flow returns to non-gravid levels at term (Conrad et al, 1993). The vasoconstrictor response to methoxamine in late-pregnancy was the same as for virgin rats. In the in-situ perfused kidney, the vasoconstrictor response to noradrenaline was also found to be similar in late-pregnant and virgin rats (Chu and Beilin, 1997). However in the current study, the response to electrical field stimulation in the kidney of late-pregnant rats was attenuated. As the response to methoxamine was unchanged in late-pregnancy, reduced vasoconstriction to EFS is likely to be mediated through pre-junctional changes to perivascular sympathetic nerves. This may involve alterations in sympathetic transmitter content, release, uptake or degradation. The same pre-junctional changes to neurotransmission have also been observed in the mesenteric vascular bed (Ralevic and Burnstock, 1996) and in isolated mesenteric veins (Hohmann et al, 1990) during late rat pregnancy.

Most sympathetic nerves in the rat kidney are immunoreactive for both noradrenaline and the sympathetic co-modulator, neuropeptide-Y (NPY) (Knight et al, 1989). Noradrenergic vasoconstriction is enhanced by NPY (Han et al, 1998), but the release of neurotransmitters from capsaicin sensitive nerves is inhibited by NPY (Giuliani
et al, 1989). It is possible therefore that the fall in renal cortical NPY levels during late pregnancy mediates reduced noradrenaline release. The pharmacology results of the present study make it unlikely that a reduction in NPY levels had a significant effect on release of sensory-motor transmitters, as desensitisation of sensory-motor nerves by acute capsaicin treatment did not augment the response to EFS in late pregnancy. This suggests that sensory-motor nerves do not contribute to the reduced EFS of late pregnancy.

Administration of the sensory-motor neuropeptide CGRP however, caused a greater reduction in renal perfusion pressure in kidneys from late pregnant compared with virgin rats. Gangula et al 2001, used radioactive microspheres to measure renal haemodynamics following systemic administration of CGRP in rat pregnancy and found a greater fall in renal vascular resistance compared with virgin rats. During pregnancy CGRP is also more potent at reducing MAP and uterine artery resistance (Nelson et al 1993; Gangula et al 1999). The sex hormones progesterone and oestrogen enhance the fall in MAP in response to CGRP (Gangula et al, 1999). These observations may partly be explained by up-regulation of CGRP receptors (Yallampalli et al, 2002), but the expression of CGRP receptors has not been investigated in the pregnant rat kidney.

In the kidney, CGRP is dependent on a healthy endothelium and NOS to mediate vasodilatation (Elhawary and Yang, 1995). It is surprising therefore that CGRP is more potent in late pregnancy, at a time when NOS activity is reduced (Conrad et al, 1993). In general however, CGRP evokes endothelium independent vasodilator responses (Rubino and Burnstock, 1996).

In late-pregnancy there were lower levels of CGRP and SP in the renal cortex, which correlated with less intense peri-vascular CGRP immunofluorescence compared with virgin rats. This is compatible with regression of sensory-motor nerves during late-
pregnancy and consequent increased sensitivity to CGRP. Evidence for plasticity of innervation has been documented during adaptation to both physiological and pathological states (Burnstock, 1991). For example, in the pregnant guinea-pig uterine artery, perivascular innervation with noradrenaline decreases while innervation with CGRP and SP increases (Bell & Malcolm, 1978; Mione et al, 1993). This pattern of neuronal plasticity was not observed in the pregnant rat kidney, where sensory-motor nerves regressed and sympathetic vasoconstriction was reduced as pregnancy progressed.

The reason for reduced renal efferent sympathetic nerve vasoconstrictor activity (ESNA) in late pregnancy may relate to the late-gestational fall in MAP. In the rat, MAP does not fall until day 19 of a 21-day pregnancy (Baylis, 1994). Reduced renal ESNA would allow the late-pregnant kidney to auto-regulate appropriately around a new set point for MAP. Auto-regulation of renal blood flow adjusts to new levels of MAP in rat pregnancy (Reckelhoff et al, 1992).

In the current study, plasma levels of CGRP during rat pregnancy did not differ from virgin rats, although others have found higher levels in rat pregnancy (Yallampalli et al, 2002). Blood was collected at the moment the kidney was removed for perfusion and it is possible that blood was diluted with Krebs perfusate, so contaminating the blood samples. In human pregnancy, the circulating concentration of CGRP increases as pregnancy progresses (Stevenson et al, 1986). Furthermore, plasma levels of CGRP are also higher in women taking the combined progesterone and oestrogen contraceptive, suggesting a hormonal influence on its secretion (Valdemarsson et al, 1990). Plasma CGRP levels also increase in response to an elevated plasma volume (Odar-Cederlof et al, 1991), another feature of healthy human and rat pregnancy (Baylis, 1994).
Pre-eclampsia is a syndrome unique to human pregnancy, characterised by endothelial cell damage (Roberts and Redman, 1993). It leads to widespread vasoconstriction, hypertension and organ-ischaemia, including reduced renal blood flow (Williams and de Swiet, 1997). Inhibition of NOS in the pregnant rat leads to a syndrome similar to pre-eclampsia, which can be reversed by co-administration of CGRP (Yallampalli et al, 1996). Sensitivity to chronic CGRP infusion in these animals increased during pregnancy compared with the non-pregnant state. It is possible that a reduced concentration or sensitivity to CGRP in human pregnancy may increase a woman’s vulnerability to pre-eclampsia.

6.52 Preliminary evidence for vasodilator nerves in the kidney

A preliminary, but novel observation from this study was unrelated to pregnancy. At raised vascular tone and in the presence of blockade to sympathetic neurotransmission with guanethidine, electrical field stimulation of the isolated perfused rat kidney (IPRK) caused vasodilatation that could not be repeated following desensitisation of sensory-motor nerves with capsaicin. The slow onset of these vasodilatory responses was very similar to that produced with acute capsaicin and by exogenous CGRP. Capsaicin is neurotoxic to sensory-motor nerves, therefore the absence of vasodilatation to EFS following capsaicin is suggestive that sensory-motor nerves are stimulated by EFS. Capsaicin can also act directly on vascular smooth muscle, but has a vasoconstrictor rather than vasodilator effect (Duckles, 1986). The neuronal origin of vasodilator responses to EFS could be confirmed by the elimination of a response following tetrodotoxin, but this was not performed in this study. However, immunostaining for CGRP in the kidney has only revealed neuronal sites of storage (Geppetti et al, 1989; Knight et al, 1991). It is likely therefore that vasodilatation
following EFS or desensitisation by capsaicin is mediated by release of CGRP from sensory-motor nerves.

Capsaicin infused into the renal artery of an isolated perfused rat kidney has previously been shown to reduce renal perfusion pressure and increase release of CGRP-like immunoreactivity in the venous effluent (Geppetti et al 1989). These observations provide evidence that capsaicin-sensitive, sensory-motor nerves within the resistance vessels of the kidney release CGRP that can decrease renal vascular resistance. The current study has added to these observations by observing a similar pattern of vasodilatation following electrical field stimulation. Studies to collect the venous effluent following nerve stimulation would be needed to identify whether CGRP immunoreactivity or ATP and substance P were in the venous effluent and whether a CGRP receptor antagonist could block the vasodilatation.

Immediately after administration of capsaicin there was a transient vasoconstriction that preceded the long vasodilatation. ATP released from perivascular nerves predominantly acts on P2X receptors that vasoconstrict vascular smooth muscle directly (Burnstock, 1993). It is possible that release of ATP from sensory-motor nerves caused this transient vasoconstriction via P2X receptors. Substance P released from perivascular nerves, as opposed to endothelium, appears to have little vasodilating activity (Burnstock, 1993).

The vasodilatation following EFS at raised vascular tone and in the presence of guanethidine was a reproducible observation, but it varied widely even between kidneys from the same group of animals. This variability did not allow quantitative comparison between pregnant and virgin rat kidneys. The inconsistency of these results may be due to
the solid nature of the kidney, as compared with the vascular mesentery (Kawasaki et al, 1988).

The densest innervation for CGRP was seen within the renal pelvis, which corresponded to the highest biochemical levels of CGRP and supports the immunohistochemical observations of Knight et al, 1991. Sensory nerves have a role in sensing dilatation of a full renal pelvis and ureter and then trigger a reno-renal reflex that causes increased sodium and water excretion in the contra-lateral kidney (DiBona and Kopp, 1997). The renal pelvis dilates during pregnancy (Waltzer, 1981), yet CGRP levels in this structure did not appear to change as pregnancy progressed.

An efferent vasodilator role of sensory-motor nerves in the kidney has not been elucidated, but it is likely to be minor in opposition to the powerful vasoconstrictor effect of sympathetic nerves. Sensory receptors at the cortico-medullary boundary are stimulated by increases in intra renal pressure or kidney volume (Dolezel, 1975). It is tempting to speculate that as the kidney becomes plethoric and distended in pregnancy, there is a reflex sensory-motor vasodilatation of renal vasculature.

There have been many previous attempts to elicit a neuronal vasodilatation of the kidney. These have included electrical stimulation of the canine mid-brain, which increased renal blood flow in the presence of guanethidine that was thought to be due to release of dopamine (Bell and Lang, 1973). However, others have failed to demonstrate functional vasodilator nerves in the kidney by stimulation of peripheral nerves. Many previous investigators studied renal blood flow in anaesthetised dogs (DiSalvo and Fell, 1971; Takeuchi et al, 1971, Gomer and Zimmerman 1972, Holdaas and DiBona, 1984) and did not raise vascular tone in order to facilitate the demonstration of vasodilatation.
In conclusion, this chapter has shown that during late rat pregnancy, sympathetic vasoconstriction is reduced by a pre-junctional mechanism that may relate to reduction in renal cortical levels of the sympathetic neuromodulator NPY. Sensory-motor nerves regress as pregnancy progresses and by late pregnancy vasodilatation to CGRP is increased. The attenuated response to EFS during late pregnancy is not mediated by increased activity of renal sensory-motor nerves. For the first time, this study provides preliminary evidence for the presence of functional vasodilator nerves in the isolated perfused rat kidney.
CHAPTER 7

UPREGULATION OF NEURONAL NITRIC OXIDE SYNTHASE IN RENAL MACULA DENSA AND ENDOTHELIAL NITRIC OXIDE SYNTHASE IN CORONARY ARTERIES DURING RAT PREGNANCY
Abstract

1. This chapter investigates the morphology of the renal juxtaglomerular apparatus and coronary arteries during pregnancy and compares immuno-reactivity of the three isoforms of nitric oxide synthase in the blood vessel wall and peri-vascular structures on day-12 pregnancy and at term.

2. In rat kidneys, NOS-I was present in the macula densa of both virgin and pregnant rats. The intensity of NOS-I staining was greatest in macula densa of 12-day compared with virgin and 21-day pregnant rats. Electron-microscopy identified NOS-I throughout the cytoplasm of the macula densa.

3. Proximal coronary arteries of pregnant rats had a wider diameter and were more intensely labeled with NOS-III compared with virgin rats. NOS-III was present in a sub-population of endothelial cells and perivascular macrophages. Within endothelial cells the NOS-III immuno-precipitate was predominantly found in the Golgi-apparatus. There was no staining for NOS-II in coronary arteries from either pregnant or virgin rats.

4. In conclusion, pregnancy up-regulates activity of NOS-I in macula densa of pregnant rats and NOS-III in coronary arteries. Increased NOS-I in the macula densa during pregnancy could suppress tubulo-glomerular feedback and allow increased renal blood flow. Increased NOS-III activity in the coronary artery of pregnant rats would support the gestational increase in cardiac work.
7.1 INTRODUCTION

The dramatic changes to cardio-vascular and renal function in human and rat pregnancy are outlined in detail in Chapter 1. These changes include an increase in cardiac output, which allows the mother to meet the metabolic demands of pregnancy and an increase in renal function, which allows her to eliminate increased metabolic waste. Immunohistochemistry is used to observe any changes to morphology or NOS immunostaining in the area of the kidney that controls renal blood flow, the juxtaglomerular apparatus (Figure 7.1) and also the coronary arteries of pregnant rats.

![Diagram of the Juxtaglomerular Apparatus](image)

Figure 7.1 The Juxtaglomerular Apparatus (From Thorup and Persson, 1998). The macula densa cells are located in the wall of the thick ascending limb of the loop of Henle, and make contact with the afferent and efferent arterioles close to the parent glomerulus. It is clear how easily vasoactive factors released from the basal surface of the macula densa can act in a paracrine manner to influence vascular smooth muscle tone of the adjacent afferent arteriole.
7.11 The Macula Densa

Renal blood flow is auto-regulated by two main mechanisms 1) baroreceptors that sense stretch in the afferent arteriole and 2) chemoreceptors that sense tubular sodium chloride concentration in the macula densa (Navar et al, 1996). A low perfusion pressure in the afferent arteriole will stimulate the release of renin from juxtaglomerular cells, which in turn activates the angiotensin - aldosterone system (RAAS) to retain salt and water (Navar et al, 1996). A high tubular sodium chloride concentration will activate macula densa cells to release ATP and/or adenosine, which act in a paracrine manner to mediate afferent arteriole vaso-constriction (Inscho et al, 1996; Inscho, 2001; Nishiyama and Navar, 2002); this is known as the tubuloglomerular feedback (TGF) mechanism.

Neuronal NOS (NOS-I) is expressed in macula densa cells and nerves of the kidney (Welch and Wilcox, 2002). Ren et al, selectively inhibited NOS-I and found enhanced vasoconstriction in response to a high sodium chloride load, so demonstrating a role for nitric oxide in TGF. In other words, NO attenuates the vasoconstrictor response of TGF by relaxing the neighbouring afferent arteriole (Ren et al, 2000).

The renal haemodynamic changes are associated with an increase in activity of the renin-angiotensin-aldosterone system (RAAS) (Conrad et al, 1989; Chapman et al, 1998). In mid-pregnancy when renal blood flow is high, it has been suggested that TGF auto-regulation is reset to allow a greater blood flow (Baylis and Blantz, 1985). However it was unclear whether the TGF response, as judged by artificial changes in tubular flow rate, was different from virgin rats (Baylis and Blantz, 1985). Plasma volume expansion has been
reported to reduce the vasoconstrictor capacity of TGF i.e. to suppress TGF (Moore et al, 1980). The precise mechanism that allows the kidney to reset its autoregulatory response around new levels of renal blood flow has not been elucidated. This chapter investigates differences in NOS-I immuno-reactivity in the macula densa of pregnant rat kidneys.

7.12 Coronary Arteries

During pregnancy there is an increase cardiac output (Chapman et al, 1998; Gilson et al, 1997). Although an increase in cardiac pre-load (greater plasma volume) and decrease in after-load (reduction in TPVR) contribute to the gestational increase in cardiac output, the heart itself undergoes ventricular remodeling and increased myocardial contractility (inotropy) during pregnancy (Buttrick et al, 1987; Gilson et al, 1997; Morton et al, 1984). It would be anticipated that increased coronary artery blood flow would be needed to support the gestational increase in cardiac work-load, but this has not previously been investigated.

All three isoforms of NOS have been identified in the heart. Neuronal NOS (NOS-I) and NOS-III have been demonstrated in the endothelium of rat coronary arteries (Bredt et al, 1990; Shochina et al, 1997), whereas myocardial NOS-II expression is mostly associated with disease states (Haywood et al, 1996). During pregnancy, NOS-I and NOS-III mRNA levels are significantly elevated in homogenates of the guinea-pig heart after 50 days gestation (Weiner et al, 1994). In the rat only NOS-III protein expression was increased during pregnancy and not NOS-I or NOS-II (Linke et al, 2002). Increased myocardial NO modulates myocardial oxygen consumption when cardiac output is increased (Linke et al, 2002), such as during pregnancy.
Oestrogen activates NOS-III in cultured coronary artery endothelial cells (Yang et al, 2000) and increases coronary artery blood flow through an NOS dependent mechanism (Guetta et al, 1997). It is possible that high circulating oestrogen levels in pregnancy will similarly increase NOS-III expression and coronary artery blood flow. In the second part of Chapter 7 NOS-III immuno-reactivity is compared between the coronary arteries of pregnant and virgin rats.

7.2 METHODS

Immunohistochemistry for light and electron microscopy

Virgin and first time pregnant Wistar rats were used in this study. Details of the immunohistochemical techniques used for light and electron microscopy are given in Chapter 2.
7.3 RESULTS

7.3.1 Immuno-histochemistry of macula densa during pregnancy

**Light Microscopy**

In the kidney, immuno-reactivity for NOS-I in the macula densa cells of virgin rats did not produce convincing immuno-reactivity (Figure 7.2a), but immuno-staining was intense in macula densa of 12-day pregnant rats (Figure 7.2b and c). The expression of NOS-I immuno-reactivity was contained within macula densa cells of the tubule, although a thin layer of immuno-reactivity was also seen in the glomerular capsule of pregnant kidneys (Figure 7.2b and c). There was no difference in expression of NOS-III in renal vessels in all three groups (data not shown).

7.3.2 Electron microscopy of macula densa during pregnancy

Neuronal NOS was identified in the macula densa of kidneys from virgin rats (Figure 7.3). NOS-III immuno-precipitate was found throughout the cytoplasm of macula densa cells. The most intense NOS-III staining was within the Golgi apparatus, closest to the tubular lumen (Figure 7.3). Unfortunately, the thin (<1μm) sections through kidneys from pregnant rats did not pick up sections of macula densa.
Figure 7.2  a) Renal cortex of virgin rat (control) revealing poor, immuno-fluorescence for NOS-I in macula densa cells. b). Renal cortex of twelve day pregnant rat revealing intense immune-fluorescence for NOS-I in macula densa cells (low power) and c). High power; NOS-I immuno-fluorescence is also evident around glomerular capsule (G) as well as macula densa (MD).
Figure 7.3  Electron micrograph of macula densa cells (MD) in the wall of the thick ascending limb of the loop of Henle. NOS-I immuno-precipitate can be seen in the cytoplasm of the macula densa cells and appears to be strongest close to the tubular lumen (TL). The proximity of macula densa cells to Bowman's Capsule (BC) is also evident.
7.33 Light microscopy of coronary artery during pregnancy

The coronary arteries of both virgin and pregnant rats displayed immuno-reactivity for NOS-III (Figure 7.4 a-d), but not for NOS-II (data not shown). In coronary vessels from virgin rats, the majority of sections revealed moderate immuno-reactivity to NOS-III (Figure 7.4 a). In these vessels, immuno-reactivity was associated with a subpopulation of endothelial cells as well as structures located in the adventitia (Figure 7.4a). Coronary vessels of small diameter were more intensely labeled for NOS-III.

In contrast, the majority of coronary arteries from pregnant animals displayed intense immuno-reactivity for NOS-III in a substantially larger number of endothelial cells (Figure 7.4 b) than was observed in virgin rats. No obvious difference in the intensity of the labeling was seen between 12-day and 21-day pregnant animals (data not shown). Coronary arteries from pregnant rats were so intensely labeled with NOS-III it appeared on light microscopy that the labeling was in endothelium, perivascular nerve fibres, smooth muscle and other cells.

7.34 Electron microscopy of coronary artery during pregnancy

It was also clear on EM that NOS-III immuno-precipitate was more sporadic in coronary artery endothelial cells of virgin rats (Figure 7.5a). There was evidence of NOS-III surrounding the membranes of the cisterns of the Golgi apparatus from both pregnant and virgin animals (Figure 7.5a). The most striking difference between virgin and pregnant rat coronary arteries was that in pregnant animals NOS-III immuno-precipitate was not only found in endothelial cells, but also in peri-vascular macrophages (Figure 7.5 b).
**Figure 7.4** Proximal coronary arteries labelled for NOS-III in virgin (a) and 12 day pregnant (b) rats (lu, lumen; Bar = 30µm). Photomicrograph a) showing NOS-III (small arrows) in a sub-population of endothelial cells and peri-vascular cells (large arrows). The diameter of the pregnant rat coronary artery appears greater b), whereas the vessel wall appears relatively thin walled and shows intense staining for NOS-III.
Figure 7.5  Ultra-structural features of coronary arteries from a) virgin and b) pregnant rats. The coronary artery of a virgin rat a), has endothelial cells that are sporadically stained for NOS-III, especially in the region of the Golgi apparatus. NOS-III staining for advential macrophages was not noted in coronary arteries of virgin rats. b). NOS-III immuno-precipitate (arrows) can be seen throughout the cytoplasm of an endothelial cell and two advential macrophages (M). One macrophage is densely stained for NOS-III and the other has NOS-III related to the Golgi apparatus. Peri-vascular nerves (N) can also be seen, although they do not appear to stain for NOS-III.
7.4 DISCUSSION

7.41 NOS-I immuno-reactivity in the macula densa of pregnant rat kidney

This study provides evidence for increased immuno-reactivity of neuronal NOS (NOS-I) in the macula densa of mid-pregnant rats. Nitric oxide released from NOS-I in the macula densa modulates tubulo-glomerular feedback (TGF) by relaxing the afferent arteriole (Ren et al, 2000). The increased immuno-reactivity of NOS-I in macula densa coincides with maximal renal blood flow of pregnancy. The gestational rise and fall of renal blood flow also correlates with a rise and fall in protein expression for NOS-I in kidney homogenates (Alexander et al, 1999). Furthermore, specific inhibition of NOS-I with 7-nitroindazole decreases renal blood flow and GFR in 12-day pregnant rats, but not in virgin rats (Abram et al, 2001). It is possible that 7-nitroindazole blocked NOS-I in renal nerves around inter-lobar arteries (Abram et al, 2001, Liu and Barajas, 1993), but the observation of increased NOS-I immuno-reactivity in the macula densa in mid-pregnancy suggests a role for NO in modulating TGF and consequently facilitating increased renal blood flow.

Electron microscopy showed that NOS-I immuno-reactivity was present throughout most of the cytoplasm of macula densa cells. The sections cut randomly through the renal cortex at less than 1μm missed the macula densa of pregnant rats. Immuno-reactivity of NOS-I between pregnant and virgin rats with electron microscopy was not compared.

Nitric oxide also plays a role in the control of renin release, which inter-relates with sodium chloride concentration and renal nerve activity (Raij and Baylis, 1995). During rat
pregnancy there is increased activity of the renin-angiotensin-aldosterone system and increased salt and water retention (Nadel et al, 1988; Conrad et al 1989). If the macula densa cells sense a low tubular sodium chloride concentration then NOS-I in the macula densa and renal nerves is activated and renin release rises (Pieruzzi et al, 2002). It is possible that during pregnancy increased NOS-I activity in the macula densa also has a role in stimulating renin release from the juxta-glomerular apparatus.

Increased production of NOS-I in the macula densa has also been observed in a mouse where the gene for renin substrate (angiotensinogen) had been knocked out (Kihara et al, 1997). In this mouse knock-out model, NOS-I production was found in all the cells of the tubular epithelium surrounding the macula densa (Kihara et al, 1997). Such vigorous staining for NOS-I was not observed in the pregnant rat.

Only one study has previously investigated functional aspects of the TGF mechanism in pregnant rats (Baylis and Blantz 1985). Differences between proximal and distal single nephron GFR in the micro-perfused Munich-Wistar rat kidney suggested that the TGF system was suppressed in 12-day pregnant rats (Baylis and Blantz 1985). However, they concluded that the pregnant rat auto-regulates renal blood flow in the same way as a non-pregnant animal (Baylis and Blantz 1985). It is unclear whether these in vitro changes in proximal tubule flow rate are a good surrogate for physiological changes in NaCl concentration: the normal trigger to TGF (Bayliss and Blantz, 1985). Even if TGF is unchanged at its reset level in pregnancy, it is still possible that increased NOS-I in the macula densa provides a more constant vasodilatation of the local afferent arteriole during
pregnancy. This would still allow an appropriate TGF response to changes in tubular sodium chloride concentration.

All three isoforms of nitric oxide synthase have previously been identified in different cell types in the kidney (Wilcox, 2000). As well as neuronal NOS (NOS-I), inducible NOS (NOS-II) is present in macrophages and appears to have constitutive expression (Wilcox, 2000). Endothelial NOS (NOS-III) is constitutively present in the vascular intima and tubular epithelium (Wilcox, 2000). Non-specific inhibition of NOS on day 12 of rat pregnancy prevents the gestational increase in renal blood flow (Danielson & Conrad, 1995). It might be expected that the expression of NOS-III would be maximal on day-12 of rat pregnancy, but it gradually declines as pregnancy progresses (Alexander et al, 1999). The role of renal NOS-II in pregnancy is less clear. However, pregnancy is a pro-inflammatory state and the pregnant rat has an increased NOS-II response to endotoxin which causes glomerular injury and proteinuria (Sakawi et al, 2000; Chou et al, 2002).

7.42 Coronary artery morphology and NOS-III immuno-reactivity in pregnant rats

This study has also demonstrated increased immuno-reactivity for NOS-III in the endothelium and peri-vascular cells of coronary arteries from pregnant rats. Increased NO production may therefore have a role in the gestational vasodilatation of the coronary vasculature. In both mid and late pregnant rats (12 and 21 days gestation), the levels of NOS-III immuno-staining were similar. In virgin rats a few small caliber coronary arteries were labeled for NOS-III with a similar intensity to that found in pregnant rats. The heavily
labeled, vasodilated arteries from pregnant rats were thin walled, suggesting relaxation of vascular smooth muscle rather than hyperplasia.

In this study, NOS-III was present in infiltrating cells that resembled macrophages. NOS-III is found in many cells other than endothelial cells (Forstermann et al, 1999) and has been identified in macrophages of hyper-lipidaemic rabbits (Aliev et al, 2001). Pregnancy is a hyperlipidaemic state and therefore macrophages may respond in a similar way to non-pregnant hyperlipidaemic states. Why they should express NOS-III instead of their usual NOS-II is unclear.

NOS-III has previously been found in perivascular nerves of coronary arteries (Sosunov et al, 1995), suggesting the existence of both NOS-I (Rubino et al, 1999) and NOS-III isoforms in the autonomic nervous system (Loesch and Burnstock, 1998). However, NOS-III immuno-precipitate was not evident in peri-vascular neurons in this study.

The gestational hormone relaxin is produced in increasing concentrations during pregnancy and has a vasodilatory role that is mediated through nitric oxide synthase (Novak et al, 2001). Relaxin administered to the coronary arteries of the Langendorff heart increased coronary artery blood flow and the amount of nitrite, a stable end-product of nitric oxide metabolism, in the perfusate (Bani-Sacchi et al, 1995). It is possible therefore that the increased NOS-III activity observed in this study is triggered by increased circulating concentrations of relaxin.
Non-specific NOS inhibition with L-NMMA during pregnancy has caused hypertension, proteinuria and a small litter size (Yallampalli and Garfield 1993; Molnar et al, 1994). These changes are typical of pre-eclampsia, a condition unique to humans, which can be prevented by providing excess NOS substrate, L-arginine (Yallampalli and Garfield 1993; Molnar et al, 1994). However, when any one isoform of NOS is knocked out, the pregnant mouse does not develop hypertension (Shesely et al, 2001). This would suggest up-regulation of either the remaining NOS isoforms or some other vasodilator pathway. A similar adaptation is noted when NOS is inhibited in the pregnant rat kidney and vasodilatory prostaglandins are recruited (Danielson and Conrad, 1996).

The work described in this chapter provides evidence for up-regulation of the two 'constitutive' isoforms of NOS (I and III) in pregnancy. Increased NOS-I activity in macula densa cells may play a role in modulating TGF and mediating renal afferent arteriole vasodilatation during rat pregnancy. Increased NOS-III immuno-reactivity in coronary arteries suggests a role for NO in augmenting coronary artery blood flow during pregnancy.
CHAPTER 8

GENERAL DISCUSSION
The studies reported in this thesis use different techniques to investigate the control of vascular tone in pregnancy. Performing any pharmacological manipulation in pregnancy is fraught with concerns about harming the unborn fetus and must be conducted with care. This problem makes the study of human pregnancy challenging and therefore the study of pregnant animals has a useful role. Yet animals vary in their utero-placental arrangements and do not appear to suffer pre-eclampsia, although there are reports of gorillas having 'eclamptic' convulsions in late pregnancy, which limits comparison with human pregnancy.

Chapter 3 reports the first study to infuse a nitric oxide synthase (NOS) inhibitor into a pregnant woman. It was therefore conducted with consideration for the welfare of mother and fetus. Furthermore, in the doses given, L-NMMA was shown to have local and not systemic effects. This has led the way for others to perform similar studies to elaborate upon the original findings that suggest increased NOS activity contributes to the fall in peripheral vascular resistance of pregnancy. Venous occlusion plethysmography appears to be a useful tool that with careful consideration of study-doses allows in vivo studies to be performed in pregnancy, without jeopardizing the health of mother or fetus.

Nitric oxide synthase activity was increased in early pregnancy, before a discernable increase in hand blood flow, suggesting it might play a primary vasodilator role rather than be secondary to an increase in flow by other vaso-active pathways. Subsequent studies in pregnancy have reduced forearm blood flow to non-pregnant levels and continued to observe an increased response to L-NMMA supporting the notion that increased NOS activity causes a primary vasodilator effect of pregnancy rather than a secondary response (Anumba et al, 2001).

It would have been most interesting to have studied hand blood flow responses to L-NMMA in a cohort of women with pre-eclampsia, but at the time of the study this was not logistically possible. Such a study has subsequently been done in the forearm and
interestingly did not show any reduction in NOS activity from normotensive pregnant women (Anumba et al, 1999a). However, in this thesis women with pre-eclampsia were found to have increased plasma concentrations of ADMA, which actively competes with L-arginine to inhibit NOS. To reconcile the functional study (no change in NOS activity in pre-eclampsia) with the biochemical study (increased NOS inhibitor in pre-eclampsia), it is possible that NOS activity up-regulates in women with pre-eclampsia to counteract elevated circulating ADMA levels and that other vasoconstrictor mechanisms mediate the primary vasoconstriction. Recent developments in understanding the regulation of ADMA by DDAH and by enzymes that regulate DDAH and the production of ADMA make it difficult to know which is the key regulator of NOS activity in a disease state. The relationship between ADMA and endothelial function in normotensive pregnancy and pre-eclampsia has become an area of my ongoing research. In particular whether judicious supplementation of L-arginine the substrate for NOS (as opposed to large doses of L-arginine which have a non-stereospecific vasodilator effect), might provide a therapeutic option for the vasoconstricted state of pre-eclampsia.

Reflecting on the design of many studies performed in pregnancy and those in this thesis, some important principles of investigation in pregnancy and pre-eclampsia are worth stating. Firstly, in a syndrome such as pre-eclampsia, many vasoactive systems are activated or dysfunctional. It is therefore difficult to know what are primary pathological events and what are secondary events responding to the initial insult. As the duration of pregnancy and pre-eclampsia is relatively short, future studies should follow the progression of a woman from ‘health’ to pre-eclampsia and back to ‘health’ again. In this context ‘healthy’ women may be harbouring sub-clinical disease as follow-up of women who have had pre-eclampsia indicate that they are at greater risk of cardiovascular disease in later life. It is possible therefore that these women were vulnerable to pre-eclampsia before conception and that pregnancy catapulted them into a metabolic and haemodynamic
milieu that they would not normally be exposed to for another 30 years. It is also possible that transient pre-eclampsia causes irreparable cardiovascular damage. Only prospective studies of pregnancy will be able to tease this issue apart. Studies of women with pre-eclampsia also need to consider the major physiological changes of healthy pregnancy by comparing 'study-responses' in a cohort of non-pregnant women to elucidate what is pathological from what is a healthy gestational shift of 'the physiological goal-posts'.

Over the past 20 years, the emphasis of vascular research in pathological states has switched from the perivascular nerves to the endothelium. This probably represents the rush to study the role of new pathways in established disease states. That pre-eclampsia is characterized by endothelial dysfunction is not in doubt, but the role of perivascular nerves appears to have been inappropriately relegated to a lower level of importance by the deluge of studies (some included in this thesis!) to investigate endothelial pathways. Future studies should recognise the integrated control of vascular tone by both endothelium and perivascular nerves (Burnstock, 1993). This thesis provides evidence for changes to perivascular nerves in the rat kidney. The plasticity of renal nerve populations and their ability to change functionally in pregnancy is compatible with even more dramatic changes to perivascular nerves observed in the uterine arteries of guinea-pigs (Mione et al, 1990). It would not be difficult to examine similar changes in the uterine arteries of pregnant women having Caesarean hysterectomies or from maternal sub-cutaneous vessels.

The function of renal perivascular nerves had not previously been investigated in rat pregnancy. The isolated perfused rat kidney (IPRK) is a difficult model to study as the kidney is very sensitive to periods without perfusion, it is prone to thrombosis and dependent on the correct mix of amino acids in the perfusate. Setting-up this model and understanding its sensitivities and requirements in order to achieve reproducible results was
challenging. Furthermore, only one study could be conducted at a time, making studies of comparison between different groups time-consuming. The IPRK model had to be adapted to allow functional studies of peri-vascular renal nerves. Electrical stimulation of a whole renal vascular bed does not allow discrimination between the numerous levels of blood vessels within renal parenchyma that might be activated as a consequence of whole organ stimulation. Conversely, this may be an advantage of this technique as the whole organ is examined and that it is most important to understand the sum of stimulation to all vascular components.

The most striking observation in the adapted IPRK model was reduced sympathetic nerve activity in late pregnancy. There are many possible explanations for this observation, but two possibilities identified in the study included, a pre-junctional reduction in NPY-modulated noradrenaline release or increased sensitivity to CGRP. Sensory-motor nerves did not contribute to the attenuated sympathetic nerve activity. The development of more specific pharmacological agents that can block the actions of CGRP, NPY, SP and ATP would now allow further components of this pathway to be dissected apart.

A tantalising preliminary observation that resulted from the use of the modified IPRK model was the possibility that the rat kidney contains functional vasodilator nerves. The quest for these nerves has been the source of many futile investigations for decades (DiBona, 1997). Capsaicin was shown to abolish this electrically stimulated vasodilatation, suggesting that they originated from sensory-motor nerves, but the neuronal origin of this vasodilatation was not proven conclusively. Further studies focusing on the possibility that renal vasodilator nerves release CGRP rather than dopamine, still occasionally used as a
renal vasodilator in clinical practice, might lead to a useful therapy for improving renal blood flow. This study demonstrated that exogenous CGRP is a potent vasodilator, especially in the kidney during pregnancy. Examination of CGRP receptors in the renal vasculature and the mechanism of their up-regulation in pregnancy, in particular the role of oestrogens, would be another future direction for this work.

The macula densa cells play a key role in the auto-regulation of renal blood flow. It was hypothesized that neuronal NOS in macula densa modified the tubulo-glomerular feedback mechanism in pregnancy, to facilitate increased renal blood flow and plasma volume expansion. Although increased NOS-I was identified in the macula densa of pregnant rat kidneys by immuno-fluorescence, these comparisons are subjective. Future studies could use reverse transcription PCR and Western blotting to more accurately quantitate differences in NOS-I mRNA and protein expression respectively. Similar techniques could also be used to confirm the presence of increased NOS-III immunostaining in coronary arteries of pregnant rats. Interesting functional comparisons of these NOS isoforms could also be made in knock-out mice that have one or other of the NOS isoforms deleted. The problem with this line of research is that animals are able to up-regulate semi-redundant pathways to replace those that are absent or blocked. The extra demands of pregnancy might however expose sub-clinical disease where such adaptations fall short of supra-physiological demands.

This thesis confirms the heterogeneous nature of mechanisms that control vascular tone within different organs and that these different pathways are variously influenced by pregnancy. It is no surprise therefore that each organ adapts its vasculature at a different rate and to a different extent during pregnancy. Clinicians’ often wonder why pre-eclampsia is such a heterogeneous disorder, affecting the liver in one woman, the brain or kidneys in another. It is possible that the reason for this clinical heterogeneity is reflected in
the heterogeneous nature of vascular control mechanism to different maternal organs. A clearer defining of the phenotype and genotype of women with different types of pre-eclampsia is needed to develop this possibility.

The study of hand blood flow in pregnant women generated the hypothesis that the efficacy of vaso-active pathways changes at different temperatures. This field of vascular physiology has gathered a huge following over the last century and spans the development and abandonment of many different techniques used to measure skin and peripheral blood flow. The water plethysmographs used in the study had last been used by Henry Barcroft, 50 years earlier, but still allowed the novel functional studies into hand warming detailed in Chapter 4. The observation that the L-arginine-NO pathway contributes to increased hand blood flow due to warming of the hand, while adrenergic tone is withdrawn contains a simple synergism of effect. Furthermore, NOS inhibition i.e. NOS activity is more potent at high temperatures, while noradrenaline is more potent in the cold. These observations support the use of NO donors or alpha adreno-receptor blockers to overcome peripheral vasospasm. Future studies of hand blood flow could investigate the responses to L-NMMA and noradrenaline at different temperatures in disease states such as Raynauds phenomenon. However, Barcroft's plethysmographs would need to be rebuilt!
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