

From DEPARTMENT OF CLINICAL SCIENCE, DIVISION OF
OBSTETRICS AND GYNECOLOGY
Karolinska Institutet, Stockholm, Sweden

**EXPRESSION AND REGULATION OF
VASOACTIVE SUBSTANCES, SEX
STEROIDS AND THEIR RECEPTORS IN
PLACENTA DURING NORMAL
PREGNANCY AND PREECLAMPSIA**

Josefine Nasiell



Stockholm 2002

All previously published papers were reproduced with permission from the publisher.

Published and printed by Karolinska University Press

Box 200, SE-171 77 Stockholm, Sweden

© , 2002

ISBN 91-7349-154-3

Expression and regulation of vasoactive substances, sex steroids and their receptors in placenta during normal pregnancy and preeclampsia

Josefine Nasiell

Despite intense studies preeclampsia remains enigmatic and a major cause of maternal and fetal morbidity and mortality. It is now widely accepted that the placenta has a central role in preeclampsia; delivery of the placenta is the only known cure. Its manifestations are considered secondary to inadequate trophoblast invasion of the uterine spiral arteries, which leads to placental ischemia and further to the devastating multisystem disorder of the mother and often the fetus. The syndrome is characterised by increased vasoconstriction and endothelial dysfunction.

Several biochemical changes in the placenta are evident. The nitric oxide (NO) system as well as the sex steroid hormones, estrogen and progesterone have been implicated in the aetiology of preeclampsia. However the role and the regulation of these substances is still not clear.

The aim of the present thesis was to study some of the genes expressed in the placenta that could be involved in the pathophysiology of preeclampsia and/or intrauterine growth restriction (IUGR). Consecutive biopsies of fresh placentas were collected from normal and preeclampsia –complicated placentas.

A placental tissue *in vitro* model was set up for experiments. For mRNA studies of *endothelin-1*, *c-fos* and *c-jun*, the progesterone receptor (PR) and the estrogen receptor (ER) we used a solution hybridisation method, which showed elevated expression of *ET-1* and *c-fos* in IUGR placentas. The *c-jun* mRNA was significantly higher in the groups with preeclampsia and/or IUGR compared to controls. Furthermore, *in situ* hybridisation of the PR demonstrated it to be localised in endothelium, in fetal lymphocytes of placental capillaries and occasionally in maternal mononuclear cells. The PR protein content in the different groups showed that the level was decreased in severe preeclampsia but increased in mild preeclampsia compared to healthy controls. Measurement of progesterone content in placental tissue explants showed that addition of the antiprogesterin RU-486 decreased the level of progesterone in the healthy placentas, whereas no such decrease was seen in the placentas from patients with preeclampsia after RU-486 treatment.

Furthermore the effect of the antiestrogen, ICI 182,780 was different in placentas from control and preeclamptic patients. eNOS immunosignal recorded by immunohistochemistry and confocal microscopy was significantly increased in the syncytiotrophoblasts of healthy placentas after ICI treatment but decreased in the preeclampsia placentas.

We suggest that an inadequate supply of progesterone, a deficiency in the mechanism of action of progesterone and/or an altered balance between the sex steroids produced by the placenta could influence not only the immune system but also the NO pathway and hereby contribute to several changes characteristic of preeclampsia.

LIST OF PUBLICATIONS

- I. Faxen, M., Nasiell, J., Lunell, N. O., Blanck, A. (1997) Differences in mRNA expression of endothelin-1, *c-fos* and *c-jun* in placentas from normal pregnancies and pregnancies complicated with preeclampsia and/or intrauterine growth retardation. *Gynecol Obstet Invest*, 44, 93-96.
- II. Faxén, M., Nasiell, J., Lunell, N.-O., Nisell, H., Blanck, A. (1998) Altered placental mRNA expression of the progesterone but not the estrogen receptor in pregnancies complicated by preeclampsia. *Hypertens Pregnancy*, 17, 241-249.
- III. Nasiell, J., Papadogiannakis, N., Carlström, K., Enberg, U., Olovsson, M., Blanck, A., Celsi, G. (2002) Progesterone and progesterone receptors in placentas from women with preeclampsia and from healthy pregnancies. *Submitted*.
- IV. Nasiell, J., Nisell, H., Blanck, A., Lunell, N. O., Faxen, M. (1998) Placental expression of endothelial constitutive nitric oxide synthase mRNA in pregnancy complicated by preeclampsia. *Acta Obstet Gynecol Scand*, 77, 492-496.
- V. Nasiell, J., Blanck, A., Celsi, G., Brismar, H. (2002) Modulation of eNOS expression by ICI 182,780 in placentas from normal and preeclampsia - complicated pregnancies. *Submitted*.

CONTENTS

Introduction	2
The clinical features of preeclampsia.....	2
Risk factors for preeclampsia	3
Fetal consequences of preeclampsia	4
The pathological changes of preeclampsia	5
Biochemical changes in the placenta associated with preeclampsia.....	6
Sex steroid hormones	6
Prostacyclin.....	7
The renin-angiotensin system	7
Nitric oxide	8
Endothelin and endothelin receptors.....	8
Protooncogenes.....	9
Aims of the investigation	10
Patients.....	11
Methodological considerations	12
Placental biopsies	12
mRNA expression	13
Protein expression	14
Placental tissue <i>in vitro</i> model.....	15
Results	18
Discussion	21
Speculations and future perspectives.....	25
Acknowledgements	26
References	27
Paper I-V	32

LIST OF ABBREVIATIONS

DIC	Disseminated intravascular coagulation
IUGR	Intrauterine growth retardation
hCG	Human chorionic gonadotropin
NO	Nitric oxide
ET	Endothelin
PIH	Pregnancy induced hypertension
PR	Progesterone receptor
ER	Estrogen receptor
PGI ₂	Prostacyclin
RAS	Renin-angiotensin system
Ang II	Angiotensin II
eNOS	Endothelial nitric oxide synthase
NOS	Nitric oxide synthase
ET	Endothelin
HELLP	Hemolysis elevated liver enzyme and low platelet syndrome
SGA	Small for gestational age
AGA	Appropriate for gestational age
DME	Dulbeccos modified Eagle medium
TNA	Total nucleic acids
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
RIA	Radio immunoassay
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunoassay
HIF	Hypoxia inducible transcription factor

INTRODUCTION

THE CLINICAL FEATURES OF PREECLAMPSIA

Eclampsia was first described almost 2000 years ago by Celsus, who presented an account of seizures in pregnant women that abated with delivery. This abnormality was given the name *eclampsia*, Greek for "lightning, indicating the rapid and unexpected appearance. For hundreds of years eclampsia was considered a seizure disorder unique to pregnancy (Marsal *et al*, 1996). It was not until the mid 1800s that the similarity to the oedematous eclamptic patient to the patient with acute glomerulonephritis led to the examination for protein in the urine of eclamptic women. It was found that most women with eclamptic seizures had proteinuria and that proteinuria could antedate the seizures. In the late 1800s, the ability to measure blood pressure with the sphygmomanometer made it possible to identify increased blood pressure in women with eclampsia. Over the next several decades, it was recognised that pregnant women who had proteinuria and hypertension, and their infants, were at risk for mortality and morbidity even if the disorder did not progress to convulsions. Thus, after 2000 years, eclampsia was no longer considered a seizure disorder but part of a continuum of a pregnancy-specific, fetal-maternal disorder.

Unfortunately, this reassessment of the disorder led to a new taxonomy, which has also interfered with understanding of the disease. Now no longer a seizure disorder, preeclampsia-eclampsia was termed a hypertensive disorder. It is evident, as one considers the pathological and pathophysiological changes of preeclampsia, that increased blood pressure is a marker, which as a separate entity is not responsible for the multiorgan dysfunction that characterises the disorder (Friedman *et al*, 1991). Additionally, perinatal mortality, which remains five times higher in the infants of preeclamptic women than in those of normal pregnant women, correlates less well with blood pressure than with coagulation changes or changes in uric acid (Roberts and May, 1976). Nonetheless, attention to hypertension, leading to the exclusion of the panoply of other pathophysiological changes of preeclampsia, has largely guided, or even retarded, research in preeclampsia. Definition of preeclampsia based solely on hypertension and proteinuria ignores the wide clinical variability in this syndrome. Women without proteinuria but who have hypertension and other features, such as severe headache or other symptoms like thrombocytopenia, hyperuricaemia, disordered liver function and fetal compromise are likely to have preeclampsia (Higgins and de Swiet, 2001). Eclampsia is a more severe version of preeclampsia, but there is no simple term that covers both presentations. For simplicity the term preeclampsia will be used, which includes all the problems of both syndromes, except for the grand-mal seizures that distinguish eclampsia from preeclampsia. The problem may occur in the second half of pregnancy, during labour or in the first few days after delivery. Maternal hypertension and proteinuria are those signs by which the disease is usually defined. But the syndrome is more extensive, with involvement of the maternal liver, coagulation and the central nervous system, and more polymorph, with considerable variation between cases.

Preeclampsia is a syndrome, dangerous for mother and baby, unpredictable in its onset and progression, and untreatable except by terminating pregnancy. It is a disorder

that only occurs in pregnancy but it also complicates some cases of hydatidiform mole where the uterus contains only disordered placental tissue. Thus a fetus is not necessary, only the placenta (Redman, 1991). The illness always ultimately regresses when the uterus is emptied of placental tissue, which confirms that the placenta is the cause of preeclampsia. It has slowly become clear that the central process in preeclampsia is a relative insufficiency of the uteroplacental circulation. Moreover, evidence is accumulating to support a central role for endothelial dysfunction in preeclampsia (Chambers *et al*, 2001; Roberts *et al*, 1989). However, in spite of many years of research the cause of preeclampsia remains enigmatic.

RISK FACTORS FOR PREECLAMPSIA

Parity

Preeclampsia is more likely to occur in women during her first pregnancy or into the first pregnancy with a new father (Caritis *et al*, 1998; Patrick and Roberts, 1999). Nulliparous women are as much as six to eight times more susceptible than multiparous women (Sibai *et al*, 1995).

Age

Women under 20 or over 35 years of age are reported to be at higher risk (Roberts, 1994). The independent risk of preeclampsia in young women has not been consistently reported. It is possible that the risk for the younger patient may be due to the likelihood that the younger women are primiparous. For the higher age extreme, there is consistent evidence of increased risk for preeclampsia (Roberts, 1994).

Race

Race is associated with increased risk in most studies. Nulliparous black women were shown to be at substantial risk for preeclampsia versus women of other races (Patrick and Roberts, 1999). Severe preeclampsia was more prevalent among women of African American and Hispanic origin as compared to nonpreeclamptic controls (Stone *et al*, 1994)

Familial

Epidemiological evidence indicates a familial tendency to develop preeclampsia. It is more common in sisters and daughters of eclamptic women (Patrick and Roberts, 1999). Previous studies found the incidence of preeclampsia to be 37% in sisters, and 26% in daughters of preeclamptic women. The disease is more common in the daughters of women who have had the disease than in their daughters-in-law. Although a genetic predisposition to preeclampsia is suggested, the exact inheritance pattern has not yet been clarified.

Conditions of Pregnancy

The risk of preeclampsia is increased in the presence of multiple gestations, trophoblastic disease (molar pregnancy), and a hydropic fetus with a large placenta (Patrick and Roberts, 1999).

Associated Medical Conditions

Personal history of diabetes, hypertension, or vascular or renal disease or collagen vascular disease are associated with preeclampsia (Roberts, 1994). Data from several recent epidemiological studies suggest that a woman who experience preeclampsia in her own pregnancy, or who was herself born to a preeclamptic pregnancy, is at reduced risk for breast cancer later in life. Preeclampsia may represent a sensitive indicator of hormonal and related endogenous factors that affects risk for breast cancer (Polednak and Janerich, 1983; Thomson *et al*, 1989).

Obesity

In obese women there is a 3- to 6-fold increase in likelihood to develop preeclampsia (Sibai *et al*, 1995; Stone *et al*, 1994). An increased risk of preeclampsia was noted at a weight approximately 20% above the desirable weight (Sibai *et al*, 1995). In a study of risk factors for severe preeclampsia, (Stone *et al*, 1994) reported that severe obesity (prepregnancy body mass index greater than 32.3kg/m^2 or prepregnancy weight in excess of 84 kg) resulted in an increased risk of 4.9 and 5.1 in primiparous and multiparous women, respectively.

FETAL CONSEQUENCES OF PREECLAMPSIA

About 30% of infants of preeclamptic women are growth restricted by accepted weight criteria from epidemiological studies. Intrauterine growth is a major concern in obstetrics because birth weight is the strongest known indicator of perinatal mortality (Pollack, 1992). The neonatal morbidity associated with intrauterine growth restriction (IUGR) is also significant. Birth asphyxia, meconium aspiration, pulmonary hemorrhage and hypoglycemia are all associated with IUGR. Furthermore, neurodevelopmental problems are increased in IUGR babies. There are many factors associated with the development of IUGR. One can classify the basis for the development of IUGR as being fetal, maternal or placental. The correct classification of IUGR in preeclampsia is not clear. Neither is the reason to why only some of the fetuses in preeclampsia become growth restricted. A characteristic placental abnormality correlated with IUGR and/or preeclampsia has still not been well defined. The ability to use doppler ultrasound has reduced perinatal mortality by about one-third, as a result of subsequent intensive monitoring and elective delivery of those at risk (Divon and Ferber, 2001). Preeclampsia accounts for approximately 40 % of iatrogenic premature deliveries (Chambers *et al*, 2001), which of course is an immense medical and economical dilemma.

THE PATHOLOGICAL CHANGES OF PREECLAMPSIA

Pathological findings in biopsy specimens from preeclamptic women or found at necropsy in women dying from preeclampsia is mainly represented by haemorrhage and necrosis. The findings implicate reduced organ perfusion rather than elevated blood pressure *per se* as the important pathophysiological feature of preeclampsia (Roberts, 1998). These changes are seen in several organs, such as brain, liver, adrenal, heart, kidney and placental bed vessels.

The vessels that supply the intervillous space in the placenta undergo extensive structural remodelling during normal pregnancy (Brosens, 1977). The vessels increase in diameter, and lose many of their structural components. The genesis of this change is secondary to maternal interaction characterising the implantation process, including trophoblast invasion into these vessels. In preeclamptic pregnancies, these changes do not occur normally. This indicates that an important early feature of preeclampsia is reduced placental perfusion. It also emphasises that although preeclampsia is usually first clinically evident in late pregnancy, the root cause is present months earlier.

In addition women with preeclampsia have an increased pressor response, which is well described concerning the response to angiotensin (AbdAlla *et al*, 2001). Moreover the activation of coagulation is indicated by many changes in platelets as well as evidence of consumption of procoagulants (Friedman *et al*, 1991). Frank consumption of procoagulants, i.e. disseminated intravascular coagulation (DIC) is seen only in advanced preeclampsia. Other sensitive indicators of activation of coagulation can also be demonstrated in preeclamptic women. Reduced antithrombin 3 and increased fibrin degradation products are often found in women with severe preeclampsia. Additionally, although sodium retention is a predominant feature of the pathophysiology of preeclampsia, the oedema of preeclampsia is also due to “leaky vasculature” (Roberts *et al*, 1989).

BIOCHEMICAL CHANGES IN THE PLACENTA ASSOCIATED WITH PREECLAMPSIA

That a large number of the biochemical functions of the preeclamptic placenta are altered is undoubted. Several abnormalities have been documented. They include evidence for increased release of placental products into the maternal circulation such as hCG and corticotropin releasing hormone (Redman and Sargent, 1993). Other placental products or activities are reduced in maternal plasma, including placental plasminogen activator activity and placental plasminogen activator inhibitor (Redman and Sargent, 1993). Measurements have also revealed a reduction in the tissue content of pyruvate and lactate with no change in energy charge (Bloxam *et al*, 1987). This observation is consistent with a decrease in glycolysis and suggests that the metabolic adjustment of placental tissue to different conditions of oxygen and nutrient supply can also take place *in vivo* (Schneider, 2000). Plasma free fatty acids constitute the only lipid fraction known to cross the placenta, and could meet most of the fetal requirements. The activity of lipoprotein lipase, which is responsible for the placental transfer, is significantly greater in placentas of women with preeclampsia, perhaps representing a compensatory process. Moreover, there is evidence that the renin-angiotensin system is altered in preeclampsia (Inoue *et al*, 1995). There are also interesting disturbances of prostacyclin and thromboxane, nitric oxide (NO), endothelin (ET), and sex steroids which deserve individual explanation.

SEX STEROID HORMONES

It is well known that the human placenta produces large quantities of progesterone throughout pregnancy (Parker *et al*, 1979; Simpson and MacDonald, 1981). Late in pregnancy, the placenta secretes up to 250 mg or more of progesterone per day. The interaction between progesterone and the placenta during normal and pathological pregnancy remains largely unknown. Chesley found that vascular refractoriness to angiotensin II was significantly lower in women with pregnancy-induced hypertension (PIH) compared to normotensive pregnant women (Chesley, 1966). Everett and associates suggested that 5α -dihydroprogesterone may be important in the maintenance of refractoriness to the pressor effects of angiotensin II during human pregnancy and that a deficit in the production of 5α -dihydroprogesterone may exist in women who develop PIH (Everett *et al*, 1978). The imbalance between thromboxane and prostacyclin, in favour of thromboxane, seen in preeclampsia has been suggested to be due to increased trophoblast progesterone production in preeclampsia which might act by a paracrine mechanism to inhibit prostacyclin synthesis in the placental vasculature (Walsh and Coulter, 1989). As previously mentioned, data from epidemiological studies suggest that a woman who experiences preeclampsia in her own pregnancy, or was herself born to a preeclamptic pregnancy, is at reduced risk for breast cancer later in life. This connection between preeclampsia and breast cancer might be due to hormonal mechanisms, especially the ratio of estrogens to progesterone (Polednak and Janerich, 1983; Thomson *et al*, 1989).

The possible existence of progesterone receptors in the human placenta has long been a matter of debate. Ever since the concept of a critical role for steroids in the regulation of placental function has evolved, the presence of progesterone receptors (PR) as mediators of progesterone action in placental cells has been postulated.

The placenta also produces estrogens in large amounts. Normal pregnancy, near term, is a hyperestrogenic state of remarkable magnitude. In a single pregnancy more estrogen is produced by the placenta than is secreted by the ovaries of 150 ovulating premenopausal women during an entire year (Redman and Sargent, 1993). Estrogen has long been thought to play a role in regulating the vascular system, especially during pregnancy, although the mechanisms are unclear (Myatt, 1992). The effects may be mediated by increases in synthesis or alterations in the action of various vasoactive factors or by direct effects which alter the structure and elastic properties of the vessels themselves.

Early studies showed a significant association between low maternal serum estradiol levels and IUGR in women with severe preeclampsia (Isouard, 1979; Long *et al*, 1979). Whereas no consistent correlation has been found with respect to serum levels of estradiol and preeclampsia Zamudio and coworkers found high levels of progesterone and low levels of estradiol in women with preeclampsia living at high altitude (Zamudio *et al*, 1994). The existence of estrogen receptors (ERs) in the placenta has previously been studied. Most investigations report a low expression or no expression of ERs (Rivera and Cano, 1989; Rossmannith *et al*, 1997).

A recent study demonstrated markedly higher levels of both maternal testosterone and free testosterone in women with preeclampsia than in normotensive controls and this difference was eliminated six weeks after delivery (Serin *et al*, 2001). In the same study no significant effects on e.g. estradiol were observed.

PROSTACYCLIN

In preeclampsia there appears to be a relative deficiency of prostacyclin (PGI₂) by placental tissue when compared with normal pregnancy. Thromboxane A₂ synthesis seems to be increased (Myatt *et al*, 1992). Therefore preeclampsia is characterised by an imbalance in the ratio of thromboxane to prostacyclin relative to that seen in normotensive pregnancy, perhaps indicating an increased thrombotic tendency and accounting for the increased platelet consumption and DIC sometimes seen in this disorder. This imbalance also favours vasoconstriction. It has been speculated that progesterone is able to affect the imbalance between thromboxane and prostacyclin (Walsh and Coulter, 1989), but if this is true and by what mechanisms is not clear.

THE RENIN-ANGIOTENSIN SYSTEM

The components of the renin-angiotensin system (RAS) are expressed in the uteroplacental unit (Poisner, 1998). The expression varies between species, probably due to marked species differences in placental architecture. The conditions for angiotensin II (Ang II) formation exist and Ang II receptors are present in the whole human uteroplacental unit, indicating the presence of a functional local RAS (Nielsen *et al*, 2000). The uteroplacental RAS interacts with other regulatory systems and in this way modulates various aspects of tissue function. It is suggested that the uteroplacental

RAS is important for the regeneration of the endometrium after shedding, and for decidualization, implantation and placentation. The RAS participates in the regulation of the uteroplacental blood flow, prostaglandin synthesis and estradiol secretion. Disturbances of the placental RAS may lead to reduced uteroplacental blood flow in pregnancies complicated by preeclampsia and IUGR.

NITRIC OXIDE

Nitric oxide (NO), a potent vasodilator released by endothelial cells, is a major contributor to the maintenance of low basal vascular tone in the human placenta. The NO radical is generated from the metabolism of L-arginine to L-citrulline by the action of the enzyme endothelial nitric oxide synthase (eNOS). eNOS has been detected by immunostaining in the vasculature and other regions of the placenta (Myatt *et al*, 1993). There is a major controversy regarding the functional status of placental eNOS in preeclampsia, and different studies show enhanced (Grande *et al*, 2000; Kleinert *et al*, 1998; Shaul, 1999), reduced (Al-Hijji and Batra, 1999) or unchanged placental eNOS activity (Arnal *et al*, 1996). What controls NO production in the invading trophoblasts is not known. Perhaps, the NO production may be controlled by sex steroid hormones, i.e. estrogens and/or progesterone (Buhimischi *et al*, 2000; Chan *et al*, 2001).

Several studies have shown the ability of estrogen to regulate NO in different vascular tissues. Most studies indicate that estrogen can enhance eNOS (Grande *et al*, 2000; Shaul, 1999; Van Buren *et al*, 1992), although there are some studies that demonstrate down-regulation of eNOS by estrogen (Al-Hijji and Batra, 1999). It therefore appears that there is a diversity, depending on the tissue, in the mechanism(s) by which estrogen can influence NO and vascular tissue.

The function of estrogen on the placenta and on NO function in the placenta is not clear. In the present thesis one of the aims were to investigate the influence of estrogen on the NO system in the placenta from healthy and preeclampsia pregnancies.

ENDOTHELIN AND ENDOTHELIN RECEPTORS

Besides the potent relaxing factors, prostacyclin and NO, vascular endothelial cells release vasoconstrictor substances. The endothelins (ETs) are a family of three distinct 21 amino acid peptides, ET-1, ET-2 and ET-3, coded for by three separate genes in the human genome. ET-1 is the only ET known to be produced by endothelial cells (Myatt, 1992). Placental tissue express mRNA for both ET-1 and ET-3 (Myatt, 1992). Myatt and co-workers have also shown that ET-1 is a potent vasoconstrictor in the human fetoplacental circulation *in vitro* and there producing the characteristic long-acting vasoconstriction (Myatt *et al*, 1991).

ETs act on at least two distinct subtypes of receptors, ET_A and ET_B receptors. Recent studies indicate that ET-1 acts by way of both ET_A and ET_B receptors in the human placenta (Germain *et al*, 1997; Sand *et al*, 1998). Recent studies have shown that ET-1 levels were higher in placental tissues from women with preeclampsia (Singh *et al*, 2001). Moreover the ET-1 concentrations are increased in preeclampsia, indicating increased ET-1 synthesis (Wolff *et al*, 1996). It has also recently been shown that ET-1 is able to influence the expression of both its own and the NOS isoforms in normal and preeclampsia trophoblastic cultured cells (Napolitano *et al*, 2000). Moreover Sand and

co-workers demonstrated that nitric oxide was able to relax the contraction mediated by ET-1 in placental vessels.(Germain *et al*, 1997; Sand *et al*, 1998). These findings suggest the existence of a functional relationship between ET and NOS isoforms that could constitute the biological mechanism leading to the reduced placental blood flow and increased resistance to flow in the fetomaternal circulation, which are characteristic of the pathophysiology of preeclampsia (Napolitano *et al*, 2000). However, it is still not clear whether an overproduction of ET is important in preeclampsia.

PROTOONCOGENES

Control of gene expression is central to the regulation of cell proliferation and cell function. The transcription of eucaryotic genes is controlled by the interaction of proteins, termed transcription factors, with specific regulatory DNA sequences located in the vicinity of the regulated gene. These regulatory sequences are frequently (but not always) located within several hundred nucleotides upstream of the gene being controlled.

It has long been known that many physiological processes are regulated by transcription factors. For example, the receptors for steroid hormones, thyroid hormones, retinoids, and vitamin D act as transcription factors after binding to the respective ligands. The hormone-receptor complex stimulates or decreases expression of target genes. The *cis*-elements for these receptors are termed hormone response elements. The protooncogenes Fos and Jun are involved in the cascade of sex steroid stimulated events that lead to uterine, and likely also placental, growth and in amplification of the tissue response to estrogen (Stancel *et al*, 1993).

There are studies indicating that the syncytiotrophoblasts are not fully differentiated in placentas from preeclamptic women. Why this occurs is one of the keys to understanding the genesis of preeclampsia. Transcription factors are known to be important in uterine and placental growth and development during pregnancy, and could play a role also in the development of preeclampsia (Knöfler *et al*, 2001).

AIMS OF THE INVESTIGATION

The general purpose of the investigation was to study some of the genes expressed in the placenta, that could be involved in the pathophysiology of preeclampsia and / or IUGR.

The specific aims were to compare placentas from normal pregnancies with placentas from preeclampsia pregnancies, alone or in combination with IUGR, with regard to:

- The expression of *endothelin-1*, *c-fos* and *c-jun*
- Expression and localisation of the progesterone receptor.
- The basal progesterone production and the regulation of progesterone following addition of the antiprogesterin Mifepristone (RU 486).
- The expression and localisation of eNOS. Moreover, to study the regulation of eNOS, by estrogen, as determined by addition of the antiestrogen ICI 182,780 in a placental *in vitro* model.

PATIENTS

Preeclampsia was defined as a blood pressure above 140/90 mm hg, repeated 6h apart and not present before the 20th week in pregnancy, and proteinuria > 0.3g/24 h. Women with mild to moderate PE had a blood pressure between 140/90 and 160/110 mmHg and proteinuria at, or exceeding, 300 mg/24h. Patients with severe preeclampsia had a diastolic blood pressure at or exceeding, 110 mm Hg and proteinuria 3 g / 24h or more. Alternatively, patients with severe preeclampsia, and in one case eclampsia, had manifestations from other organ systems, including the liver or the central nervous system. One patient had a HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome.

In this study new-borns were assigned the diagnosis small for gestational age (SGA) if adjusted birth weight deviation exceeded minus 2 standard deviations (SD) according to Scandinavian growth curves (Marsal *et al*, 1996).

Patients were randomly recruited at the labour department of the Huddinge University Hospital at the time of delivery. Patients were delivered either vaginally or by caesarean section.

Ethical considerations

The local ethics committee at the Huddinge University Hospital approved the studies.

METHODOLOGICAL CONSIDERATIONS

The methods used are discussed in the Material and methods section of each paper. Some general comments on techniques are provided here.

PLACENTAL BIOPSIES

Paper (I, II, IV)

Tissue samples were taken from the central part of the fetal placenta, rinsed from blood under sterile conditions in cold saline and immediately frozen in liquid nitrogen and stored at -70°C until analysed. One investigator performed tissue sampling.

Paper (III)

Placental biopsies were collected immediately after delivery. The biopsies were taken as previously described. Villous vascular trees, including stemvilli and secondary and tertiary branches were dissected.

- a) For mRNA expression studies pieces from each placenta (2x2 cm) were cut under sterile conditions. The tissue was fixed in 4% phosphate-buffered paraformaldehyde and paraffin embedded.
- b) For protein expression studies tissue was collected and frozen in liquid nitrogen as above. Specimens (150 mg) were cut from the frozen biopsies without thawing the tissue and further analysed.
- c) For the progesterone assay fresh placenta was collected as previously described. The specimen was incubated in Dulbecco's modified Eagle medium (DME) supplied with penicillin and streptomycin in 37°C in a humid 5% CO₂ incubator with or without the addition of Mifepristone or RU486 (10⁻⁷M), which is a progesterone receptor antagonist.

Paper (V)

From each fresh placenta 3 samples were dissected and incubated in DME as above with or without the antiestrogen ICI 182,780. For each sample three sections were prepared and used for quantification. From each section 3 villi of similar size were chosen for quantitative analysis of eNOS immunofluorescence.

mRNA EXPRESSION

Solution hybridisation

Solution hybridisation is a method for quantitating different mRNA species, which compared to other methods is very sensitive. Many different samples can be analysed at the same time. Total nucleic acids (TNA) are prepared from the tissue and the concentration is measured spectrophotometrically. A cRNA probe is labelled by S^{35} (approximately 20,000 cpm/incubation) and hybridised to TNA samples in solution at a temperature that for each probe and tissue has been shown to give the most specific hybridisation, keeping an adequate signal intensity. The incubations are performed in duplicate in microcentrifuge tubes in a volume of 40 μ l with 25% formamide. After overnight incubation the samples are treated with 1 ml of a RNase containing solution to digest non-hybridised RNA. Labelled hybrids protected from RNase digestion are precipitated by addition of 100 μ l 6 M trichloroacetic acid, collected on filter and counted in a scintillation counter. In the present study the amount of TNA added to each vial was diluted to a concentration where a linear relationship between amount of TNA and cpm/ μ g TNA was obtained. mRNA expression was given as cpm/ μ g TNA. House keeping genes are mostly measured in parallel to control for e.g. degradation of mRNA in the samples. In this study glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β_2 -microglobulin were used as internal controls.

***In situ* hybridisation**

The principle behind *in situ* hybridisation is the specific annealing of a labelled nucleic acid probe to complementary sequences in fixed tissue, followed by visualisation of the location of the probe. This technique can be used to locate DNA sequences on chromosomes, to detect RNA or viral DNA, to identify endogenous mRNA, to identify sites of peptide synthesis or to study hormonal or drug regulation of gene expression at the single cell level. A critical aspect of these procedures is that the target nucleic acid is retained *in situ*, is not degraded by nucleases, and is accessible for hybridisation to the probe. Unlike the hybridisation of nucleic acids in solution, the target nucleic acid is cross-linked and embedded in a complex matrix that hinders access of the probe and decreases the stability of the hybrids. There are many different protocols depending on the target and probe, whether they are RNA or DNA. In addition, some of the variations are related to the nature of the specimen, whether it is a cell suspension i.e. fluorescent *in situ* hybridisation (FISH), fly embryo or a vertebrate (Wilkinson, 1992).

PROTEIN EXPRESSION

Radioimmunoassay (RIA) / enzyme immuno assay (EIA)

The basic mechanisms of a hormone immunoassay are the reactions between an antigen, the hormone to be measured, and an antibody capable of binding to that antigen. Since the complex that is formed cannot be directly detected, due to its low concentration, an additional reagent needs to be introduced into the reaction, namely the label. The label is coupled to the antigen and produces a signal that can easily be measured. The hormone that is bound in the antigen-antibody complex can thus be detected and quantified. For EIA, an enzyme-linked anti-immunoglobuline (Ig) is added. It will bind to the patient's antigen-antibody complex, thereby starting an enzyme reaction, resulting in a measurable colour change. For RIA the label to be measured is radioactive. The immunoassay procedure is a specific and sensitive method and good concordance with a steroid-binding assay on uterine tissues has been demonstrated (Senekjian et al., 1989) The term EIA and ELISA (enzyme linked immunoassay), which is sometimes used, denotes identical techniques.

Immunohistochemistry

To demonstrate an antigen or protein in a histologic section, one of the reactants (usually the antibody) may be labelled with a substance such as a radioisotope, fluorochrome, or enzyme. The label may then be detected after formation of an immune complex between the antigen and the specific antibody. The labels themselves have various advantages and disadvantages. Although radioisotopes can be detected in very small amounts, their use in histology requires autoradiographic procedures that are time consuming. However, histologic labels such as fluorochromes, with subsequent detection by fluorescence methods, or enzymes, with detection by use of specific substrates in light or electron microscopy, produce excellent localisation of the sites of antigen-antibody reactions. Thus the most common procedures are either fluorescence detection systems (immunofluorescent procedures) or enzymatic detection (immunoenzymic procedures).

Fluorescence procedures

If the fluorescence phenomenon is observed in samples that have not been stained with a fluorescent dye, it is termed *primary fluorescence*, or autofluorescence; *secondary fluorescence*, on the other hand, involves the use of special dyes called fluorochromes.

Primary fluorescence (autofluorescence)

There are many compounds that can fluoresce naturally without the use of fluorochrome dyes. Controls for autofluorescence should be used in any secondary fluorescence reaction. This is accomplished by including an additional tissue section that has been treated similar to the test sample in all respects except actual staining.

Secondary fluorescence

Secondary fluorescence is produced when substances are not naturally fluorescent interact with a fluorochrome dye. A fluorochrome dye is a special kind of dye that can fluoresce when excited with ultraviolet light. A major advantage of secondary

fluorescence techniques is their ability to demonstrate low concentrations of particular components. The method also gives good contrast, and a low-power magnification can be used for screening purposes. A disadvantage of fluorescence methods can be that the localisation of the stained substance appears somewhat imprecise because the fluorescence is given off in all directions.

Attachment procedures in immunofluorescence procedure

There are two major types of attachment procedures used in immunofluorescence methods: *direct staining* and *indirect staining*. Direct methods permit direct visualisation of antigens or antibodies (considered as antigens) in tissue sections by treatment of the sample with a solution containing a fluorochrome-labelled antibody that is specific for the substance to be demonstrated. To perform the direct method, a fluorochrome-labelled antibody specific for each antigen studied is needed.

Indirect procedures also may be used to demonstrate either antigen or antibody localised in a tissue section. With the indirect localisation of antigen by the double-layer technique, the antigen is in the tissue. Samples are first treated with an unlabeled antibody specific for the particular antigen, followed by treatment of the samples with a fluorochrome-labelled antibody specific to the gamma globulin of the first antibody. This last reagent attaches to sites of the specific antibody that has attached to the antigen sites in tissue. The tissue antigen thereby fluoresces and can be detected by fluorescence examination. Sandwich techniques may be used for an indirect localisation of antibody in tissue. The sample is treated first with an unlabeled diluted solution of an antigen that is specific for the antibody present in the tissue and therefore binds to it. The sample then is treated with a fluorochrome-labelled antibody corresponding to the antigen used. The fluorochrome-labelled antibody attaches to the antigen that has, in turn, attached to the antibody present in the tissue. Fluorescence should therefore appear at the sites of the original antibody location.

Confocal microscopy

Confocal microscopy-confocal scanning laser microscopy is a technique for recording fluorescent samples with high spatial resolution. In the confocal microscope only light from the focal plane is detected, light that comes from out of focus regions is attenuated. This results in a well defined depth discrimination in contrast to what is possible with conventional wide-field fluorescence microscopes.

The high depth resolution results in what can be called optical sectioning where approximately 0.5 μm thin slices of the sample are recorded. Correct use of the technique can then permit measurements on a subcellular level and potential staining artifacts e.g. uneven surface binding is avoided.

PLACENTAL TISSUE *IN VITRO* MODEL

Since preeclampsia only occurs spontaneously in primates, the studies on the disease will of necessity be clinical. For ethical reasons the possibilities to perform experimental studies on placental function are therefore limited. To be able to study substances in the placenta I have therefore set-up a placental tissue culture model.

Tissue samples were obtained from fresh placentas. The biopsies were taken from the central part of the fetal placenta and separated from beneath the chorionic plate.

Tissue samples were rinsed from blood under sterile conditions in cold saline. Villous vascular trees including stemvilli, secondary and tertiary branch were dissected and small pieces from each placenta (1x 0,5 cm) were cut under sterile conditions. The tissues were incubated in DME supplemented with penicillin and streptomycin in 37°C in a humid 5% CO₂ incubator for 6 hours with and without the substance to study, i.e. ICI 182,789 (10⁻⁷M) (paper V) or the antiprogesterin RU 486 (10⁻⁷ M) (paper III). The tissue was then fixed in 4% paraformaldehyde, paraffin embedded, sectioned at 5 µm and mounted onto slides for immunohistochemistry or frozen until analyzed by immunoassay.

We first investigated if morphological structures were affected by time in culture. The morphological structure of the placental tissue was well preserved in culture media up to 48 hours. After 72 hours degenerative changes, i.e. fibrosis in villous stroma and reduced stainability of trophoblasts were seen in the tissue. Accordingly, all experiments were performed shortly after starting the incubation.

Under physiological conditions *in vivo* the placenta consumes large amounts of oxygen indicating a very active energy metabolism. This is true for most species and the human placenta certainly is no exception (Schneider, 2000). Together with brain, liver and kidney, it belongs to the organs with the highest requirements of oxygen (Carter, 2000). Oxygen consumption measured *in vitro* using various experimental techniques, such as trophoblast culture, villous explants, slices, or even the dual *in vitro* perfusion of an isolated cotyledon is considerably lower than the estimated *in vivo* values (Schneider, 2000). Yet the morphology of the tissue, including the ultrastructure, is well preserved and energy requiring functions, like the synthesis of proteins and steroids and the active transport of nutrients, are maintained (Schneider, 2000). This is particularly remarkable in view of the exposure of the tissue to warm ischaemia, which may last up to 15-30 min or even longer, before some sort of artificial support of the vital functions can be established. This would suggest that the oxygen dependent metabolism, which *in vivo* runs at a very high rate, is not essential for tissue survival and that there are specific mechanisms of adjustment allowing the support of structure and certain functions under conditions of low oxygen supply (Schneider, 2000). In this respect placental tissue clearly differs from other organs with an equally high consumption of oxygen, which are much more sensitive to a reduction in oxygen supply and in general will quickly show signs of irreparable cell damage (Schneider, 2000).

Isolated cytotrophoblasts have shown a remarkable resistance, with cells remaining viable even when exposed to severe hypoxia with PO₂ levels as low as 12-14 mm Hg for 72 h (Esterman *et al*, 1996). It seems that hyperoxia may have a greater detrimental effect on the tissue than hypoxia. In culture experiments using placental villi, exposure to 95% oxygen was associated with some tissue damage such as an increase in thickness of trophoblasts and villous membranes (Burton *et al*, 1989).

The special tolerance of the placenta to a low oxygen supply, together with an early slowdown in the oxygen dependent metabolism, may be of physiological significance, allowing a redistribution of oxygen provided by the mother in favour of the fetus. Furthermore, it is an important prerequisite for *in vitro* experimentation with placental tissue (Schneider, 2000).

Statistical methods

The Mann-Whitney U test for non-parametric data was used to evaluate differences between the groups. Differences were considered significant at a P value of $< 0,05$. The Spearman R test was used to study correlations. To correct for multiple comparisons the Kruskal-Wallis one-way analysis of variance was used (paper I-IV). Further statistical analysis was performed using a two-way ANOVA with repeated measures (paper V).

RESULTS

Expression of endothelin-1, *c-fos* and *c-jun* (paper I)

The mRNA expression of ET-1 and *c-fos* in women with pregnancies complicated by IUGR was significantly higher than in the control group. There were no significant differences in these mRNA levels between placentas from women with preeclampsia or preeclampsia with SGA compared to those with normal pregnancies.

The *c-jun* mRNA expression level was significantly higher in the groups with preeclampsia and/or SGA as compared to the group with normal pregnancy.

Placental progesterone receptors (Papers II, III)

The PR mRNA expression was found in clusters in the endothelium and in lymphocytes of the fetal capillaries (paper III). Occasionally, positive mRNA signal was detected in the intervillous space, apparently residing in maternal mononuclear cells. There was no difference in localisation of the PR between healthy controls and placentas from women with preeclampsia.

The mRNA expression of PR was significantly higher in the placentas from women with mild/moderate preeclampsia and preeclampsia in combination with SGA, but there was no difference in the mRNA expression in placentas from women with only SGA compared to normal pregnancies (paper II). The same results were also obtained at the protein level, the placentas from women with mild/moderate preeclampsia exhibiting higher levels of PR than those from control pregnancies (paper III). On the other hand, the placentas from women with severe preeclampsia exhibited lower levels of PR than placentas from healthy pregnancies.

There was no correlation between the PR mRNA expression and gestational age in any of the groups studied. The mRNA expression of β_2 -microglobulin was used as a control and did not differ among the groups. When the mRNA levels of PR in individual women were related to the β_2 microglobulin levels, the results were not altered. When comparing the ratio between the PR and ER mRNA expression, the ratios between the control group and the women with preeclampsia were significantly different.

The table below demonstrates the histopathology of placentas from women with severe preeclampsia. Major histopathological findings, gestational age at delivery, apgar score at 1, 5 and 10 minutes and whether the weight of the child was appropriate for gestational age (AGA) or small for gestational age (SGA) are presented.

Patient	Histopathology finding	Gestation age	Apgar score	Fetal weight
1	ablatio, solitaire infarct	29	8, 9, 9	SGA
2	ablatio, infarcts	32	9, 10, 10	AGA
	Signs of hypoxia, small iv thrombosis			
3	small chorangioma, iv thrombosis	28	4, 8, 9	SGA
4	iv hemorrhage, infarcts	32	7, 8, 9	SGA
5	ablatio, signs of hypoxia, iv thrombosis	36	9, 10, 10	AGA
6	signs of hypoxia	28	8, 10, 10	AGA
7	infarcts, iv thrombosis	36	6, 8, 10	SGA
8	small chorangioma	32	6, 9, 10	SGA
9	infarcts	28	9, 10, 10	SGA
10	infarcts, iv thrombosis	28	0, 1, 1	SGA
11	infarcts accelerated villous maturity	29	8, 10, 10	SGA
12	accelerated villous maturity	29	7, 9, 9	SGA
13	infarcts, iv thrombosis	34	8, 9, 10	AGA

Placental progesterone levels (paper III)

- a) The basal progesterone level in placental tissue did not differ between healthy controls and preeclamptic pregnancies.
- b) When adding (RU 486) to the placental explants from normal pregnancy the progesterone level decreased dramatically, whereas addition of antiprogesterin to the placentas from women with preeclampsia did not change the progesterone level.

Expression of the estrogen receptor (paper II)

There was no difference in the mRNA expression level of ER in placentas from women with preeclampsia with or without SGA, or SGA as a single entity compared to normal pregnancies. The ER level was close to the detection level.

Placental endothelial constitutive nitric oxide synthase: expression, localisation and regulation by ICI 182,780, an estrogen receptor antagonist (papers IV, V)

The mRNA expression of placental eNOS was significantly higher in all groups of pathological pregnancies, i.e. preeclampsia, SGA and preeclampsia/SGA, when compared with normal pregnancies. Correction for multiple comparisons did not change the results.

There were no significant differences in the eNOS mRNA expression between the groups of patients with preeclampsia, SGA and preeclampsia together with SGA. The eNOS mRNA expression was not influenced by gestational age. Neither did the mode of delivery i.e. caesarean section or vaginal delivery, affect the mRNA expression. Within the group with normal pregnancies the mRNA levels did not differ significantly due to way of delivery (paper III).

eNOS immunostaining was seen in the syncytiotrophoblasts and in the endothelium of placental fetal capillaries in both placentas from preeclampsia patients and in normal placentas. In basal condition (i.e. without addition of ICI 182,780) the placentas from patients with preeclampsia showed significantly higher intensity in the syncytiotrophoblasts when compared with placentas from healthy subjects. We could detect the same tendency in the endothelium of placental fetal capillaries, although this difference was not statistically significant.

The effect of estrogen, as determined by addition of the antiestrogen ICI 182,780 was different in control and preeclampsia patients. In the healthy placentas the intensity in the syncytiotrophoblasts was significantly increased after antiestrogen ICI 182,780 treatment. ICI 182,780 had an opposite effect on eNOS intensity in the syncytiotrophoblasts in placentas from preeclampsia patients; i.e. a decrease. Therefore, addition of ICI 182,780 resulted in a 8.2-fold lower expression of eNOS in placentas from preeclampsia patients than in similarly treated placentas from control patients. Furthermore, we found the same trend in the endothelium in response to ICI 182,780 but the difference was not statistically significant.

DISCUSSION

A pivotal role of the placenta in preeclampsia is undoubted. However, the exact mechanisms by which a defect placentation leads to such severe maternal and sometimes fetal disease remains unclear.

Given the hypothesis that transcription factors as well as the potent vasomodulator ET-1 might be involved in the pathophysiology of preeclampsia, these factors were studied in placentas from normal and pathological pregnancies. Whereas no significant differences were observed either with respect to *c-fos* or ET-1 the mRNA expression of *c-jun* was significantly higher in the groups with preeclampsia and/or SGA as compared to the group with normal pregnancy. ET-1 stimulates intracellular second messenger events that in turn activate immediate early gene transcription. It has been demonstrated that ET-1 rapidly induces increased RNA levels by increasing transcription of *c-fos* and *c-jun in vitro* (Pribnow *et al*, 1992). Conversely, *in vitro* experiments showed that the ET-1 gene expression was increased by *Fos* and *Jun* (Lee *et al*, 1991). *Jun* might be involved at a very early stage in the pathogenesis of preeclampsia but the altered expression could also represent an unspecific alteration among others in pathological pregnancy.

Minimal information on key regulatory transcription factors, which trigger differentiation or invasion of the trophoblasts, is available. For instance, transcription factors have been shown to play important roles in trophoblast development (Knöfler *et al*, 2001) and could therefore be an essential component in the pathway regulating invasivity of the trophoblasts. It has been shown that another important transcription factor, hypoxia inducible transcription factor (HIF), is upregulated by hypoxia in the placenta (Rajakumar and Conrad, 2000). It is therefore reasonable to assume that placental hypoxia could contribute also to the regulation of *c-jun* during preeclampsia.

The protooncogenes *Fos* and *Jun* are involved in the cascade of sex steroid stimulated events that lead to uterine, and likely also placental, growth and in amplification of the tissue response to estrogen (Stancel *et al*, 1993).

Despite a number of different studies regarding a possible role of aberrant placental production of sex steroid hormones in preeclampsia very little is known about local expression of receptors and dynamic aspects of sex steroids on hormone production and expression of vasoactive factors. Our data regarding placental PR expression support the view of a receptor-mediated action of progesterone on placental function. In the present study the PR was localised in the endothelium of the fetal capillaries, in fetal leukocytes and occasionally, a positive mRNA signal was detected in the intervillous space, apparently residing in maternal mononuclear cells (paper III). Cudeville and co-workers reported presence of the PR in the muscular layer of human term fetoplacental vessels although they did not study vessels smaller than stem villi arteries (Cudeville *et al*, 2000). Conflicting results were reported by (Rossmannith *et al*, 1997). They found the PR localised in the syncytiotrophoblasts, and occasionally in the inner layer of the villi surrounding the vascular tree. Similar to our results Roussev and co-workers described PR positive mononuclear cells in human placental tissue (Roussev *et al*, 1993).

The present study further shows that the PR protein content in the placenta is decreased in placentas from women with severe preeclampsia when compared with healthy controls. As far as we know this is the first study to demonstrate this. The women with severe preeclampsia had of course a shorter gestational length than the healthy controls. As the PR content might vary with gestational age we studied a separate group with prematures that did not have preeclampsia. This group did not differ from the healthy term pregnancy controls regarding PR content. We could hereby exclude that the lower PR protein content in placentas from severe preeclampsia was due to shorter gestational age. On the contrary this study demonstrates that PR expression was increased in mild preeclampsia. The pathophysiological implication of the discrepancy is presently unclear but it might indicate that mild and severe preeclampsia are two distinct disease entities. The present investigation further indicates that the regulation of progesterone is altered in preeclampsia. The marked reduction in progesterone production seen following addition of RU 486 *in vitro* to placentas from normal pregnancies suggests that progesterone exerts a feed-back stimulation on local production of the hormone itself. The effect of blocking the PR seems to be aberrant in placentas from women with preeclampsia. Since progesterone is still produced in preeclampsia placentas the factor(s) that regulate progesterone in preeclampsia remain to be identified. These results therefore indicate that the biological action of progesterone is profoundly altered in preeclampsia. Both autoregulation of progesterone production and expression of progesterone receptors are deranged in mild and severe preeclampsia. Several studies have indeed reported alterations of progesterone -dependent pathways in preeclampsia. For instance, recent studies provide evidence that endogenous progesterone may act as an immunosuppressant by blocking K⁺ channels (Ehring *et al*, 1998). During pregnancy, immunoregulatory mechanisms must operate locally at the placental interface and be readily reversible to preserve the systemic immune competence of the mother. Different mechanisms involving progesterone may contribute to fetal-maternal protection, including altered T cell subsets, or elaboration of immunosuppressive factors (Pepe and Albrecht, 1995). Several authors have proposed a possible link between certain cases of preeclampsia and autoimmune phenomena (Branch, 1991; Brown, 1991). Further studies are needed to evaluate if these alterations in progesterone-dependent responses in preeclamptic placentas are primary to the disease or are part of a complex adaptation to the underlying pathophysiological mechanism.

Progesterone has also been implicated in the regulation of NOS. A preeclampsia-like condition can be induced in pregnant rats by inhibiting nitric NO synthesis (Buhimschi *et al*, 1995). Moreover, there are indications that progesterone partially reverses these effects (Chwalisz and Garfield, 1994). Furthermore, it has been speculated that progesterone may control the vascular adaptation to NO (Wimalawansa and Yallampalli, 1998). We believe that a primary or secondary NO deficiency plays a central role in the pathogenesis of preeclampsia. It has been observed in pregnant rats that the ability of L-arginine (nitric oxide substrate) to relax the uterus diminishes after progesterone withdrawal, both spontaneously at term and after onapristone (antiprogestin) treatment, which strongly suggests that the nitric oxide-dependent-relaxation (nitric oxide synthesis) in the uterus is controlled by progesterone (Yallampalli and Garfield, 1993). Moreover, progesterone treatment partially reversed the L-NAME (NOS inhibitor) - induced hypertension and fetal weight reduction in

pregnant rats (Garfield *et al*, 1994). In addition, postpartum treatment with the progesterone agonist R5020 blocked the postpartum increase in blood pressure seen after L-NAME treatment alone, providing further evidence that progesterone can induce mechanisms which counterbalance the L-NAME- induced hypertension (Chwalisz and Garfield, 1994). Perhaps progesterone stimulates the cGMP effector system in vascular tissues similar to the uterus.

The findings of increased eNOS mRNA expression in the placenta during preeclampsia is in line with several previous studies (Anumba *et al*, 1999; Ranta *et al*, 1999; Shaamash *et al*, 2001). Furthermore, the expression of eNOS in the endothelium and the syncytiotrophoblasts in the placental tissue is in line with studies by Ghabour and co-workers and Myatt and co-workers (Ghabour *et al*, 1995; Myatt *et al*, 1997). The specific cause of the increase in eNOS activity and the true function of it, is not known (Lowe, 2000). Increased shear stress and certain hormones that are increased in pregnancy, such as estradiol and progesterone have been suggested (Wieczorek *et al*, 1995). Most of these studies support the view that eNOS is up-regulated by estrogens (Goetz *et al*, 1999; Shaul, 1999; Stefano *et al*, 2000; Van Buren *et al*, 1992) although a down-regulation of eNOS by estrogen has also been reported (Al-Hijji and Batra, 1999).

As previously mentioned it is possible to stimulate a preeclampsia-like syndrome in rats and mice by blocking production of NO by administering a NOS-inhibiting agent (Yallampalli and Garfield, 1993). This treatment produces similar symptoms as in preeclampsia, suggesting a direct involvement of NO in the mechanism that promotes preeclampsia. This would imply a possible decrease in NO production in the aetiology of preeclampsia, but the measurement of NO and NOS has been contradictory. The most accepted concept concerning NO and preeclampsia is that the increase in NOS must reflect a compensation to the reduced uteroplacental blood flow seen in preeclampsia (Lowe, 2000). If the increased NOS noticed in this condition is of primary significance in the aetiology of the condition is still a matter of debate.

Previous studies have demonstrated that not only progesterone but also estrogen can induce endothelium-dependent relaxation by increasing NO in the mesenteric rat artery (Chan *et al*, 2001). In the present study (paper V) we were able to show that in healthy controls estrogens seem to inhibit eNOS immunostaining intensity, since ICI 182,780 significantly increased eNOS expression. At present, the molecular mechanisms responsible for regulation of eNOS transcription and protein synthesis in the absence and presence of estrogen blockade are unknown. The interplay between estrogens and additional factors may be complex. Most studies indicate that the effect of estrogen on NOS is mediated through the ER, although non-genomic mechanisms have also been implicated (Goetz *et al*, 1999; Shaul, 1999). Furthermore, we found that the placental regulation of eNOS immunostaining seems to be altered in preeclampsia (paper V). Since addition of ICI 182,780 appears to down-regulate eNOS expression, estrogen can be assumed to exert a stimulatory effect in placentas from preeclampsia patients. Further studies are needed to clarify in what way the observed aberrant response of eNOS following antiestrogen treatment is associated with the specific alterations in preeclampsia.

Taken together the results of this thesis might indicate that an inadequate supply of progesterone, a deficiency in the mechanism of action of progesterone and/or an altered

balance between the sex steroids produced by the placenta can influence the NO pathway and maybe other systems, hereby contributing to the complex pathophysiology of preeclampsia.

SPECULATIONS AND FUTURE PERSPECTIVES

The aetiology and the pathophysiological mechanisms behind preeclampsia are still not clear. It is generally accepted that the disease is characterized by alterations in several biochemical pathways, particularly in the placenta. It is likely that many of the alterations observed in preeclampsia are part of a compensatory response that is activated to protect the mother and/or the fetus. However, in spite of many years of research in the field, the pathological change that is primary to the disease is still enigmatic and the therapy has remained basically unchanged during the last decades. The present study has also illustrated some changes occurring in preeclampsia but could not exclude that they represent secondary or compensatory mechanisms.

One possible approach to overcome this problem could be to be inspired by research performed in other biomedical fields, such as for instance in Alzheimer's disease or diabetes research. Here, genetic screening, by using for instance gene polymorphism or microarray technology, has enabled the researchers to identify a small number of gene products that are relevant to the pathology of the disease.

It is therefore likely that similar screening methods may also identify some gene products that are associated with preeclampsia. Transgenic animals and *in vitro* models, such as the method described in this study, may then be successfully used to better characterise the biochemical pathways regulated by these gene products.

ACKNOWLEDGEMENTS

I wish to thank everybody involved in this work, especially:

Agneta Blanck, my supervisor, for personal friendship, for always showing a good mood in spite of a heavy workload and for your talent in writing science.

Gianni Celsi, my supervisor for giving me a room and a laboratory bench at the research laboratory at Astrid Lindgren's Childrens Hospital. For sharing your great genuine knowledge in science, for all the fruitful discussions, and for always having a spare moment even on Christmas day.

Margareta Faxén, my co-supervisor (before you moved to CGR at KI) for teaching me the basics in molecular biology.

Magnus Westgren professor of obstetrics and fetal medicine at Huddinge University Hospital, Karolinska Institute for creating a stimulating academic, exciting atmosphere and for having the ability to arouse enthusiasm.

Britt-Marie Landgren professor at the department of obstetrics and gynecology for being supportive.

George Evaldson for giving me a post at the department of obstetrics and gynecology at Huddinge University Hospital, for believing in my work and for being such a skilful surgeon when I needed it.

Lennart Rosenborg for your hard work in putting together mine and everybody else's schedule and doing your best to fulfil our wishes.

Kerstin Wolff for being such a talented boss, for being my personal obstetrician and most of all for your warm and significant friendship throughout the years.

My co-authors **Nikos Papadogiannakis**, **Hjalmar Brismar**, **Kjell Carlström**, and **Ulla Enberg** for invaluable collaboration and support.

Ann-Christine Eklöf and Professor **Anita Aperia** for providing me with facilities at the laboratory at Astrid Lindgren's Childrens Hospital and for being so supportive.

Henry Nisell for sharing your academic knowledge and for teaching me a lot about preeclampsia.

Ingvar Ek for helping me out with computer problems.

Katarina Englund for encouraging chats according research and other trivial matters.

Ulla Holtbäck, my former studymate in medical school for introducing me to Gianni Celsi who became my tutor and for friendship throughout the years.

Midwives and staff at the delivery ward and outpatient clinics for the fun and pleasant atmosphere during everyday work.

Colleagues at the clinic and at the laboratory for the friendly working atmosphere.

Ingrid Löfgren my goodmother for support and friendship throughout the years.

My father **Gustav** for giving me love throughout childhood, for believing in me and for your never ending ability to amaze.

My mother **Bodil** for your faithfulness and ability too have fun.

Karin my daughter for your drawings when my computer crashed, for your love and all the joy you bring.

Rune my husband, for being a wonderful father, and for being such fun lifepartner.

REFERENCES

- AbdAlla, S., Lother, H., el Massiery, A., Quitterer, U. (2001) Increased AT(1) receptor heterodimers in preeclampsia mediate enhanced angiotensin II responsiveness. *Nat Med*, 7, 1003-9.
- Al-Hijji, J., Batra, S. (1999) Downregulation by estrogen of nitric oxide synthase activity in female rabbit lower urinary tract. *Urol*, 53, 637-641.
- Anumba, D. O., Robson, S. C., Boys, R. J., Ford, G. A. (1999) Nitric oxide activity in the peripheral vasculature during normotensive and preeclamptic pregnancy. *Am J Physiol*, 277, H848-54.
- Arnal, J., Clamens, C., Pechet, C., Negre-Salvayres, C., Allera, J., Girolami, R., Salvayres, R. *et al.* (1996) Ethinylestradiol does not enhance the expression of nitric oxide synthase in bovine endothelial cells but increase the release of bioactive nitric oxide by inhibiting superoxide anion production. *Proc. Natl. Acad. Sci*, 93, 4108-4113.
- Bloxam, D. L., Bullen, B. E., Walters, B. N., Lao, T. T. (1987) Placental glycolysis and energy metabolism in preeclampsia. *Am J Obstet Gynecol*, 157, 97-101.
- Branch, W. (1991) Antiphospholipid syndrome: laboratory concerns, fetal loss, and pregnancy management. *Semin Perinatol*, 15, 230-237.
- Brosens, I. A. (1977) Morphological changes in the utero-placental bed in pregnancy hypertension. *Clin Obstet Gynecol*, 4, 573-93.
- Brown, H. (1991) Antiphospholipid antibodies and recurrent pregnancy loss. *Clin Obstet Gynecol*, 34, 17-26.
- Buhimisch, I., Yallampalli, C., Buhimisch, C., Saade, G., Garfield, R. (2000) Distinct regulation of nitric oxide and cyclic guanosine monophosphate production by steroid hormones in the rat uterus. *Mol Hum Reprod*, 6, 404-414.
- Buhimisch, I., Yallampalli, C., Chwalisz, K., Garfield, R. E. (1995) Pre-eclampsia-like conditions produced by nitric oxide inhibition: effects of L-arginine, D-arginine and steroid hormones. *Hum Reprod*, 10, 2723-30.
- Burton, G. J., Mayhew, T. M., Robertson, L. A. (1989) Stereological re-examination of the effects of varying oxygen tensions on human placental villi maintained in organ culture for up to 12 h. *Placenta*, 10, 263-73.
- Caritis, S., Sibai, B., Hauth, J., Lindheimer, M., VanDorsten, P., Klebanoff, M., Thom, E. *et al.* (1998) Predictors of pre-eclampsia in women at high risk. National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. *Am J Obstet Gynecol*, 179, 946-51.
- Carter, A. M. (2000) Placental oxygen consumption. Part I: in vivo studies--a review. *Placenta*, 21 Suppl A, S31-7.
- Chambers, J. C., Fusi, L., Malik, I. S., Haskard, D. O., De Swiet, M., Kooner, J. S. (2001) Association of maternal endothelial dysfunction with preeclampsia. *Jama*, 285, 1607-12.
- Chan, H., Yao, X., Tsang, S., Chan, F., Lau, C., Huang, Y. (2001) Different role of endothelium nitric oxide in 17beta-estradiol and progesterone- induced relaxation in rat arteries. *Life Sciences*, 69, .
- Chesley, L. C. (1966) Vascular activity in normal and toxemic pregnancy. *Clin Obstet Gynecol*, 9, 871-81.

- Chwalisz, K., Garfield, R. E. (1994) Role of progesterone during pregnancy: models of parturition and preeclampsia. [Review] [80 refs]. *Z Geburtshilfe Perinatol*, 198, 170-80.
- Cudeville, C., Mondon, F., Robert, B., Rebourcet, R., Mignot, T. M., Benassayag, C., Ferre, F. (2000) Evidence for progesterone receptors in the human fetoplacental vascular tree. *Biol Reprod*, 62, 759-65.
- Divon, M. Y., Ferber, A. (2001) Umbilical artery Doppler velocimetry--an update. *Semin Perinatol*, 25, 44-7.
- Ehring, G., Kerschbaum, H., Eder, C., Neben, A., Fanger, C., Khoury, R., Negulescu, P. *et al.* (1998) A Nongenomic Mechanism for Progesterone-mediated Immunosuppression: Inhibition of K⁺ Channels, Ca²⁺ signaling, and Expression in T Lymphocytes. *J Exp Med*, 188, 1593-1602.
- Esterman, A., Finlay, T. H., Dancis, J. (1996) The effect of hypoxia on term trophoblast: hormone synthesis and release. *Placenta*, 17, 217-22.
- Everett, R. B., Worley, R. J., MacDonald, P. C., Gant, N. F. (1978) Modification of vascular responsiveness to angiotensin II in pregnant women by intravenously infused 5 α -dihydroprogesterone. *Am J Obstet Gynecol*, 131, 352-7.
- Friedman, S., Taylor, R., Roberts, J. (1991) Pathophysiology of preeclampsia. *Clin Perinatol*, 18, 661-682.
- Garfield, R., Yallampali, C., Bihimschi, I., Chwalisz, K. (1994) Reversal of preeclampsia symptoms induced in rats by nitric oxide inhibition with L-arginine, steroid hormones and an endothelin antagonist. *Soc Gynecol Invest, 41st. Annual Meeting 1994*, .
- Germain, A. M., MacDonald, P. C., Casey, M. L. (1997) Endothelin receptor mRNAs in human fetal membranes, chorionic vessels, and decidua parietalis. *Mol Cell Endocrinol*, 132, 161-8.
- Ghabour, M. S., Eis, A. L., Brockman, D. E., Pollock, J. S., Myatt, L. (1995) Immunohistochemical characterization of placental nitric oxide synthase expression in preeclampsia. *Am J Obstet Gynecol*, 173, 687-94.
- Goetz, R., Thatte, H., Prabhakar, P., Cho, M., Michel, T., Golan, D. (1999) Estradiol induces the calcium-dependent translocation of endothelial nitric oxide synthase. *PNAS*, 96, .
- Grande, M., Carlström, K., Stege, R., Pousette, Å., Faxén, M. (2000) Estrogens increase the endothelial nitric oxide synthase (ecNOS) mRNA level in LNCaP human prostate carcinoma. *Prostate*, 45, 232-237.
- Higgins, J. R., de Swiet, M. (2001) Blood-pressure measurement and classification in pregnancy. *Lancet*, 357, 131-5.
- Inoue, I., Rohrwasser, A., Helin, C., Jeunemaitre, X., Crain, P., Bohlender, J., Lifton, R. *et al.* (1995) A mutation of angiotensinogen in a patient with preeclampsia leads to altered kinetics of the renin-angiotensin system. *J Biol Chem*, 270, 11430-11436.
- Isouard, G. (1979) Measurement of serum levels of oestriol and human placental lactogen in the management of pre-eclamptic pregnancies. *Med J Aust*, 2, 401-4.
- Kleinert, H., Wallerath, T., Euchenhofer, C., Ihrig-Biedert, I., Li, H., Förstermann, U. (1998) Estrogen increase transcription of the human endothelial NO synthase gene: analysis of the transcription factors involved. *Hypertension*, 31, 582-588.
- Knöfler, M., Vasicek, R., Schreiber, M. (2001) Key regulatory transcription factors involved in placental trophoblast development -a review. *Placenta*, 22, S83-S92.

- Lee, M. E., Dhady, M. S., Temizer, D. H., Clifford, J. A., Yoshizumi, M., Quertermous, T. (1991) Regulation of endothelin-1 gene expression by Fos and Jun. *J Biol Chem*, 266, 19034-9.
- Long, P. A., Abell, D. A., Beischer, N. A. (1979) Fetal growth and placental function assessed by urinary estriol excretion before the onset of pre-eclampsia. *Am J Obstet Gynecol*, 135, 344-7.
- Lowe, D. T. (2000) Nitric oxide dysfunction in the pathophysiology of preeclampsia. *Nitric Oxide*, 4, 441-58.
- Marsal, K., Persson, P. H., Larsen, T., Lilja, H., Selbing, A., Sultan, B. (1996) Intrauterine growth curves based on ultrasonically estimated foetal weights. *Acta Paediatrica*, 85, 843-8.
- Myatt, L. (1992) Vaso-active factors in pregnancy. *Fetal and maternal medicine review*, 4, 15-36.
- Myatt, L., Brewer, A., Langdon, G., Brockman, D. (1991) Attenuation of the vasoconstrictor effects of thromboxane and endothelin by nitric oxide in the human fetal-placental circulation. *Am J Obstet Gynecol*, 165, 1717-1722.
- Myatt, L., Brewer, A. S., Langdon, G., Brockman, D. E. (1992) Attenuation of the vasoconstrictor effects of thromboxane and endothelin by nitric oxide in the human fetal-placental circulation. *Am J Obstet Gynecol*, 166, 224-30.
- Myatt, L., Brockman, D. E., Eis, A. L., Pollock, J. S. (1993) Immunohistochemical localization of nitric oxide synthase in the human placenta. *Placenta*, 14, 487-95.
- Myatt, L., Eis, A. L., Brockman, D. E., Greer, I. A., Lyall, F. (1997) Endothelial nitric oxide synthase in placental villous tissue from normal, pre-eclamptic and intrauterine growth restricted pregnancies. *Hum Reprod*, 12, 167-72.
- Napolitano, M., Miceli, F., Calce, A., Vacca, A., Gulino, A., Apa, R., Lanzone, A. (2000) Expression and relationship between endothelin-1 messenger ribonucleic acid (mRNA) and inducible/endothelial nitric oxide synthase mRNA isoforms from normal and preeclamptic placentas. *J Clin Endocrinol Metab*, 85, 2318-23.
- Nielsen, A. H., Schauser, K. H., Poulsen, K. (2000) Current topic: the uteroplacental renin-angiotensin system. *Placenta*, 21, 468-77.
- Parker, C. R., Everett, R. B., Qirk, J. G., Whalley, P. J., Gant, N. F. (1979) Hormone production during pregnancy in the primigravid patient.
- Plasma levels of progesterone and 5 α -pregnane-3,20-dione throughout pregnancy of normal women and women who developed pregnancy-induced hypertension. *Am J Obstet Gynecol*, 135, 778-782.
- Patrick, T., Roberts, J. M. (1999) Current concepts in preeclampsia. *MCN, Am J Matern Child Nurs*, 24, 193-200; quiz 201.
- Pepe, G. J., Albrecht, E. D. (1995) Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocr Rev*, 16, 608-648.
- Poisner, A. M. (1998) The human placental renin-angiotensin system. *Front Neuroendocrinol*, 19, 232-52.
- Polednak, A., Janerich, D. (1983) Characteristics of first pregnancy in relation to early breast cancer. *J Reprod Med*, 28, 314-318.
- Pollack, D. (1992); Intrauterine growth retardation: Definition, classification, and etiology. In Pitkin (ed), *Clinical obstetrics and gynecology*, Vol 35. Los Angeles: Lippincott, pp 99-107.

- Pribnow, D., Muldoon, L. L., Fajardo, M., Theodor, L., Chen, L. Y., Magun, B. E. (1992) Endothelin induces transcription of fos/jun family genes: a prominent role for calcium ion. *Mol Endocrinol*, 6, 1003-12.
- Rajakumar, A., Conrad, K. (2000) Expression, ontogeny, and regulation of hypoxia-inducible transcription factors in the human placenta. *Biol Reprod*, 63, 559-69.
- Ranta, V., Viinikka, L., Halmesmaki, E., Ylikorkala, O. (1999) Nitric oxide production with preeclampsia. *Obstet Gynecol*, 93, 442-5.
- Redman, C. (1991) Current topic: preeclampsia and the placenta. *Placenta*, 12, 301-308.
- Redman, C. W. G., Sargent, I. L. (1993): *The Human Placenta*: Blackwell scientific.
- Rivera, J., Cano, A. (1989) Oestrogen and progesterone receptors in human term placenta. Measurements by binding assays and immunological methods. *Placenta*, 10, 579-588.
- Roberts, J. (1994): Pregnancy -related hypertension. In Creasy, R., Resnik, R. (eds), *Maternal-fetal medicine: principles and practise*. Philadelphia: W.B. Saunders, pp 804-43.
- Roberts, J. (1998) Endothelial dysfunction in preeclampsia. *Seminar in reproductive endocrinology*, 16, 5-12.
- Roberts, J., May, W. (1976) Consumptive coagulopathy in severe preeclampsia. *Obstet Gynecol*, 48, 163-166.
- Roberts, J., Taylor, R., Musci, T., Rodgers, G., Hubel, C., Mc Laughlin, M. (1989) Preeclampsia: An endothelial cell disorder. *Am J Obstet Gynecol*, 161, 1200-1204.
- Rossmannith, W. G., Wolfahrt, S., Ecker, A., Eberhardt, E. (1997) The demonstration of progesterone, but not of estrogen, receptors in the developing human placenta. *Horm Metab Res*, 29, 604-10.
- Roussev, R., Higgins, N., McIntyre, J. (1993) Phenotypic characterization of normal human placental mononuclear cells. *J Reprod Immunol*, 1, 15-29.
- Sand, A. E., Ostlund, E., Andersson, E., Fried, G. (1998) Endothelin-induced contractions in placental arteries is mediated by both ETA- and ETB-receptors. *Acta Physiol Scand*, 163, 227-34.
- Schneider, H. (2000) Placental oxygen consumption. Part II: in vitro studies--a review. *Placenta*, 21 Suppl A, S38-44.
- Serin, I. S., Kula, M., Basbug, M., Unluhizarci, K., Gucer, S., Tayyar, M. (2001) Androgen levels of preeclamptic patients in the third trimester of pregnancy and six weeks after delivery. *Acta Obstet Gynecol Scand*, 80, 1009-13.
- Shaamash, A. H., Elsonosy, E. D., Zakhari, M. M., Radwan, S. H., El-Dien, H. M. (2001) Placental nitric oxide synthase (NOS) activity and nitric oxide (NO) production in normal pregnancy, pre-eclampsia and eclampsia. *Int J Gynaecol Obstet*, 72, 127-33.
- Shaul, P. W. (1999) Rapid activation of endothelial nitric oxide synthase by estrogen. *Steroids*, 64, 28-34.
- Sibai, B. M., Gordon, T., Thom, E., Caritis, S. N., Klebanoff, M., McNellis, D., Paul, R. H. (1995) Risk factors for preeclampsia in healthy nulliparous women: a prospective multicenter study. The National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. *Am J Obstet Gynecol*, 172, 642-8.
- Simpson, E. R., MacDonald, P. C. (1981) Endocrine physiology of the placenta. *Ann Rev Physiol*, 43, 163-88.

- Singh, H. J., Rahman, A., Larmie, E. T., Nila, A. (2001) Endothelin-1 in feto-placental tissues from normotensive pregnant women and women with pre-eclampsia. *Acta Obstet Gynecol Scand*, 80, 99-103.
- Stancel, G., Baker, V., Hyder, S., Kirkland, J., Loose-Mitchell, D. (1993) Oncogenes and uterine function. *Ocf Rev Reprod Biol*, 15, 1-42.
- Stefano, Prevot, Beauvillain, Cadet, Fimiani, Welters, Fricchione *et al.* (2000) Cell-surface estrogen receptors mediate calcium-dependent nitric oxide release in human endothelia. *Circulation*, 101, .
- Stone, J. L., Lockwood, C. J., Berkowitz, G. S., Alvarez, M., Lapinski, R., Berkowitz, R. L. (1994) Risk factors for severe preeclampsia. *Obstet Gynecol*, 83, 357-61.
- Thomson, W., Jacobson, H., Negrini, B., Janerich, D. (1989) Hypertension, pregnancy, and risk of breast cancer. *J Natl Cancer Inst*, 81, .
- Walsh, S., Coulter, S. (1989) Increased placental progesterone may cause decreased placental prostacyclin production in preeclampsia. *Am J Obstet Gynecol*, 161, 1586-1592.
- Van Buren, G. A., Yang, D.-s., Clark, K. E. (1992) Estrogen -induced uterine vasodilatation is antagonized by L-nitroarginine methyl ester, an inhibitor of nitric oxide synthesis. *Am J Obstet Gynecol*, 167, .
- Wieczorek, K. M., Brewer, A. S., Myatt, L. (1995) Shear stress may stimulate release and action of nitric oxide in the human fetal-placental vasculature. *Am J Obstet Gynecol*, 173, 708-13.
- Wilkinson, D. G. (1992): *In situ Hybridization - A practical approach*.
- Wimalawansa, S., Yallampalli, C. (1998) Pre-eclamptic toxemia: potential new therapy on animal studies. *Ceylon Med J*, 43, 138-46.
- Wolff, K., Nisell, H., Carlstrom, K., Kublickiene, K., Hemsén, A., Lunell, N. O., Lindblom, B. (1996) Endothelin-1 and big endothelin-1 levels in normal term pregnancy and in preeclampsia. *Regul Pept*, 67, 211-6.
- Yallampalli, C., Garfield, R. (1993) Inhibition of nitric oxide synthesis in rats during pregnancy produces signs similar to those of preeclampsia. *Am J Obstet Gynecol*, 169, .
- Zamudio, S., Leslie, K. K., White, M., Hagerman, D. D., Moore, L. G. (1994) Low serum estradiol and high serum progesterone concentrations characterize hypertensive pregnancies at high altitude. *J Soc Gynecol Investig*, 1, 197-205.

PAPER I-V