Phenotypic Characterisation of the Sodium Hydrogen Exchanger and NADPH Oxidase Enzyme in Pre-eclampsia

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Phenotypic characterisation of the sodium hydrogen exchanger and the NADPH oxidase enzyme in pre-eclampsia.

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Although the underlying aetiology of pre-eclampsia remains an enigma, it is only recently that oxidative stress and membrane transport abnormalities associated with sodium have been implicated. These represent two factors that may constitute the pre-eclamptic phenotype proving important in the pathogenesis of the disease.

White cell Na^+/H^+ exchanger activity was increased in pre-eclamptic pregnancy with respect to normotensive pregnant controls and persisted into the post-partum period. This was not due to increased expression of a 97kDa NHE-1 protein but possibly genetically determined due to the persistence of the phenotype in transformed lymphoblasts. Cells isolated from pre-eclamptic women exhibited an intracellular acidosis that again, persisted into the post-partum period. The mechanism(s) responsible were unclear but may have been associated with inhibition of the Na^+K^+ATPase by a digoxin-like factor. Neutrophil NADPH oxidase mediated reactive oxygen species production was measured using a chemiluminescent technique. These results were affected by the intracellular acidosis; consequently measurements were performed in Epstein-Barr virus immortalized lymphoblasts. Upregulated NADPH oxidase activity was identified in pre-eclamptic and post-partum pre-eclamptic cells with tyrosine kinase signal transduction pathways being implicated in their control. Enhanced activity may have been influenced by a genetically determined phenotype resulting in an increased sensitivity of the enzyme.

Collectively, this data adds to our understanding of pre-eclampsia and provides phenotypic characterisation of the Na^+/H^+ exchanger and NADPH oxidase enzyme. These membrane functions may have implications as markers for predisposition to disease or in therapeutic intervention.

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List of Abbreviations

APS	ammonium persulphate
ATP	adenosine triphosphate
ATIII	anti-thrombin III
BCECF-AM	2,7'Bis-(carboxyethyl) 5.6 carboxyfluorescein acetomethyl ester
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CGD	chronic granulomatous disease
CL	chemiluminescnece
CNS	central nervous system
$cPLA_2$	cytosolic phospholipase A_2
CVS	cardiovascular system
DAG	di-acylglycerol
DLF	digoxin-like factor
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DTT	dithiothreitol
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-di-(aminoethyl)-N,N,N',N'-tetraacetic-acid
EPPS	N-[2- Hydroxyethyl] piperazine-N'-[3-Propanesulfonic acid]
ERK	extracellular signal-regulated kinase
FAD	flavine adenine dinucleotide
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GDP	guanine diphosphate
GM-CSF	granulocyte macrophage colony stimulating factor
GTP	guanine triphosphate
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
IL-6	interleukin 6
IP_3	inositol 1,4,5-trisphosphate
IUGR	intrauterine growth retardation
LDL	low density lipoprotein
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate H
NHE	sodium hydrogen exchanger
NO	nitric oxide
NT	normotensive
OxLDL	oxidised low density lipoproteins
PAF	platelet activating factor
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PET	pre-eclamptic
PGI_2/TXA_2	prostacyclin/thromboxane

pH_i	intracellular pH
Phox	phagocytic oxidase
PI3kinase	phosphoinositol-3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLA	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol myristate acetate
PpNT	post-partum normotensive
PpPET	post-partum pre-eclamptic
PTX	pertussis toxin
RLU	relative light units
ROS	reactive oxygen species
rhMIL-8	recombinant human monocyte interleukin-8
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHR	spontaneously hypertensive rat
SOD	superoxide dismutase
STMV	syncytiotrophoblast microvesicles
TC199	tissue culture medium 199
TEMED	N,N,N',N'-Tetramethylethylenediamine
TIRON	4,5-dihydroxy-1,3-benzene-disulfonic acid
TNF	tumour necrosis factor
VCAM-1	vascular cell adhesion molecule-1

Chapter 1

Introduction

1.1 Pre-eclampsia

Pre-eclampsia is a disease unique to pregnancy, and is diagnosed based on a rise in blood pressure and proteinuria that return to pre-pregnancy state post-partum. Eclampsia is the occurrence of generalized convulsions during pregnancy, during labour or within 7 days of delivery, in a woman affected by pre-eclampsia with the exclusion of other causes of fits.

1.1.1. Historical Aspects.

The earliest record of eclampsia is said to have come from the Kahum (Petrie) papyrus dating from around 2200BC [Stevens 1975]. Demanet in 1797 was the first to relate the convulsions of eclampsia with the associated oedema and it was not until 1843 that Lever was credited with the discovery of the proteinuria that was later to become crucial to the diagnosis of the disease. Hypertension was not associated until 1874 when Mahomed interpreted tracings showing arterial hypertension, however, no absolute values could be specified due to the lack of direct measurements of blood pressure [Chesley 1978]. It was not until 1903 that Cook and Briggs documented the association between proteinuria, hypertension and pre-eclampsia with the aid of the improved model of Riva Rocci's sphygmomanometer [Chesley 1978]. Differentiation of pre-eclampsia-eclampsia from renal disease with essential hypertension was established at the end of the 19^{th} century. The development of cellular pathology and histological methods allowed glomerulonephritis to be identified as an entity distinct from eclampsia. Due to an explosion in research, it is now realised that there are progressive pathophysiological changes associated with pre-eclampsia, suggesting that the disease is far more than just hypertension; it is a systemic disease of which hypertension is a feature [Chesley 1978].

1.1.2 Diagnostic Criteria

Pre-eclampsia is characterized by the onset of hypertension and proteinuria in women after 20 weeks gestation. The clinical spectrum is wide and related to the severity of the disease. For the setting of this thesis, pre-eclampsia was diagnosed according to the criteria of Davey and MacGillivray [1988]:

Hypertension in pregnancy
1. Diastolic blood pressure of >=110 mm Hg on any one occasion or 2. A diastolic blood pressure of >= 90 mm Hg on two or more consecutive occasions >= 4 hours apart.
Proteinuria in pregnancy A. One 24-hour urine collection with a total protein excretion of >= 300 mg per 24 hours, or B. Two "clean-catch-midstream" or catheter specimens of urine collected >= 4 hours apart with 1. 1 gm albumin per litre or 2+ or more on reagent strip or sulfosalicylic acid "cold" test, or 2. 0.3 gm albumin per litre or 1+ on reagent strip if specific gravity < 1.030 and pH < 8."

Table 1.1 Diagnostic criteria for pre-eclampsia. B1 does not take into consideration the pH and concentration of the urine.

1.1.3 Epidemiology

Pre-eclampsia occurs at a frequency of approximately 7-10 out of every 100 pregnancies. The incidence of eclampsia in developed countries has declined during the 20th century due to prenatal care becoming universally available. The incidence of pre-eclampsia however has not changed appreciably during the last few decades due to an incomplete understanding of the underlying aetiology and pathophysiology.

There are certain risk factors important to pre-eclampsia. Primiparity is considered to be a strong risk factor for the development of pre-eclampsia. Women are 5-10 times more likely to experience pre-eclampsia during their first pregnancy than in a subsequent pregnancy [MacGillivray 1958]. This implicates the initial placentation with the suggestion that later pregnancies develop a protective mechanism; possibly against paternal antigens, as risk reverts back to that of primiparous women with new paternity [Redman 1991b]. Abortion tends to be protective as the incidence of pre-eclampsia is

significantly lower following the procedure. The results of a study of 9771 women suggest that primiparae with a previous abortion are at a decreased risk for pre-eclampsia, compared to primiparae actually pregnant for the first time [Seidman et al. 1989].

Barrier contraception that prevents exposure to sperm and seminal fluid are associated with an increased risk of developing pre-eclampsia during the subsequent pregnancy. A case-control study was conducted comparing the contraceptive and reproductive histories of 110 primiparous women with pre-eclampsia with 115 pregnant women without pre-eclampsia, aged 15 to 35 years. Unconditional analysis indicated a 2.37-fold increased risk of pre-eclampsia for users of contraceptives that prevent exposure to sperm thus lending support to an immunological mechanism, although the pathogenesis is poorly understood [Klonoff-Cohen et al, 1989].

The risk of pre-eclampsia developing in women who are over 35 years of age is three to four-fold higher than in younger women. It has been suggested that this may reflect collagen replacement of the normal muscle in the walls of the myometrial arterioles and atrophic changes in the vascular microstructure [Zhang et al. 1997].

Raised blood pressure prior to 20-27 weeks gestation is associated with pre-eclampsia in otherwise healthy primiparous women. A study of 700 nulliparous women, performed by Villar and Sibai [1989], suggested that a second trimester mean arterial pressure (MAP) of ≥90mm Hg had a positive predictive value of 23%. A large clinical trial was conducted including a total of 2947 healthy women with a single fetus. Systolic blood pressure at entry was considered a predictor of pre-eclampsia [Sibai et al. 1995].

Pre-existing medical conditions such as hypertension and diabetes pose a substantial risk factor for the later development of pre-eclampsia during pregnancy [Mabie et al. 1986; Garner et al. 1990]. The women with pre-existing hypertension, who were at greatest risk for developing superimposed pre-eclampsia, were those with evidence of more severe underlying blood pressure elevations as demonstrated by left ventricular hypertrophy, a serum creatinine greater than 1.0 mg%, and a diastolic pressure greater than 100 mmHg at less than 20 weeks gestation [Mabie et al. 1986]. A prospective

study of 334 pregnant diabetic women found the incidence of pre-eclampsia to be higher among diabetics (10%) than controls (4%) [Garner et al. 1990].

The incidence of pre-eclampsia is four times greater in a woman if a first-degree relative has suffered [Roberts and Redman 1993]. However, a specific model of inheritance has not yet been established.

Complications associated with increased placental size such as twin pregnancies pose an increased risk of developing pre-eclampsia. This is perhaps due to a reduced placental perfusion associated with an increase in placental size. Zhang et al [1997] demonstrated that women with twin pregnancies were three times more likely to develop complications than women with singletons.

Higher rates of pre-eclampsia are associated with increasing levels of obesity [Sibai 1995]. Obesity has a strong link with insulin resistance. The exact mechanisms by which obesity/insulin resistance are associated with an increased risk for pre-eclampsia are not completely understood. Possible mechanisms include increased shear stress due to the hyperdynamic circulation associated with obesity, dyslipidaemia or increased cytokine mediated oxidative stress and direct haemodynamic effects of hyperinsulinaemia (increased sympathetic activity and increased tubular sodium reabsorption) [Bosio et al. 1999; Dekker and Sibai. 1998].

Studies during pregnancy have found that cigarette smoking is associated with a reduced risk of pre-eclampsia [Sibai et al, 1995]. The mechanisms by which cigarette smoking may protect are unknown. Potentially effective agents in tobacco smoke include thiocyanate, which has a known hypotensive effect and nicotine, which inhibits production of fetal thromboxane A_2, a vasoconstrictor and platelet aggregation stimulator [Darby and Wilson. 1967; Ylikorkala et al. 1989].

Finally, women living at high altitudes seem to be at a greater risk of developing pre-eclampsia [Mahfouz et al. 1994]. Women living at higher altitudes tend to have a lower blood volume and arterial oxygen saturation, suggesting that this observed effect may be mediated by relative hypoxemia [Moore et al. 1982].

1.1.4 Effect of Pre-eclampsia on Organ Systems

Pre-eclampsia is a fascinating clinical syndrome unique to pregnant women. It is characterized by arteriolar vasospasm, increased peripheral resistance, varying aspects of endothelial damage and is reversible following delivery. Virtually every organ system is affected and is therefore termed a "systemic disease".

1.1.4.1 Haematological Changes

Haematological changes develop in some but not all women who have pre-eclampsia. The haematological changes associated with the coagulation system, platelet function and erythrocytes are complex and often conflicting. Evidence for these changes date back to the last century when periportal thrombi were first noted in the liver of women who died from eclampsia [Perry and Martin 1992]. The association of pre-eclampsia with thrombocytopenia has been recognized for the last 70 years and reflects either early platelet consumption or enhanced platelet activation and degranulation [Jones and Goodall 1994]. It has been suggested that women with pre-eclampsia have activated hyper-reactive platelets [Jones and Goodall 1994]. Other changes in platelet structure and function have previously been recognized. Platelet lifespan is reduced [Rakoczi et al. 1979]. Various changes in fibrinolysis and coagulation have been reported. Fibrinolytic activity seems to be reduced whereas fibrin generation increases [McKillop et al. 1977]. Deposits of fibrin-like material are widespread and fibrin degradation products are increased in women with pre-eclampsia [Howie et al. 1971]. The most consistent finding is reduced anti-thrombin III (ATIII) suggestive of increased coagulation [Paternoster et al. 1996]. There is also an increasing interest in plasminogen activator inhibitor-1 (PAI-1) which is said to exhibit higher levels in pre-eclampsia, with the placenta being considered a source, leading to a failure of thrombolysis in both placental and systemic vasculature [De Boer et al. 1988]. Recently the presence of the 4G/5G genotype of the PAI-1 gene has been postulated as one of the risk factors for pre-eclampsia [Yamada et al. 2000]. There is a general agreement that changes such as thrombocytopenia and reduced ATIII are common in severe pre-eclampsia and are thought to be secondary, not causative.

1.1.4.2 Renal Changes

Altered renal function in pre-eclampsia is not fully understood but may relate to altered haemodynamics as well as morphology. Renal perfusion and filtration are decreased in pre-eclampsia but remain above that of non-pregnant values. Uric acid is the end product of purine metabolism and increased levels are associated with pre-eclampsia [Hassan et al. 1991]. Hyperuricaemia can be considered a diagnostic criterion of pre-eclampsia due to its strong correlation with "glomerular endotheliosis", a characteristic of pre-eclampsia [Lancet and Fisher 1956; McFarlane 1963]. Redman et al. [1976] reported that hyperuricaemia is recognised to be an early change in pre-eclampsia and a good marker for severity of disease. They observed at 24 weeks gestation a 20% rise in plasma uric acid above the first measurement made at 17 weeks gestation. The hyperuricaemia of pre-eclampsia can be accounted for by reduced renal clearance [Atallah et al. 1988]. Specifically, the renal clearance of uric acid is decreased mainly because net tubular reabsorption is increased [Hayashi 1956]. Studies by Czaczkes et al. [1958] used Probenecid to inhibit tubular reabsorption of uric acid. The renal clearance and plasma levels were restored in pre-eclamptic women implicating enhanced tubular reabsorption rather that diminished secretion. The aetiology of altered renal tubular re-absorption of uric acid is uncertain but most likely relates to plasma volume depletion [Sica and Schoolwerth 1996]. Presumably, volume contraction leads to enhanced renal absorption of sodium and consequently uric acid reabsorption by the proximal tubule. Increased uric acid production may result from increased xanthine oxidase activity in pre-eclampsia [Many et al. 1996].

Urinary excretion of total protein is exaggerated in pre-eclampsia suggesting a compromise of proximal tubular function [Strober and Waldmann 1974]. Gross albuminuria and excretion of other plasma proteins is secondary to vasoconstriction as well as alterations in molecular size limitations and compromised proximal tubular reabsorption capacity. It remains unresolved that there is reduced reabsorptive capacity for proteins and yet hyperuricaemia is present in pre-eclampsia. This may argue for the role of increased xanthine oxidase activity by factors such as hypoxia-reperfusion, cytokines or increased substrate availability (hypoxanthine and xanthine) resulting in increased production of ROS associated with uric acid production, which would contribute to oxidative stress of pre-eclampsia. [Many et al. 1996].

1.1.4.3 Volume Homeostasis

Pre-eclampsia is associated with early and significant plasma volume contraction largely due to a shift from the intravascular to extravascular space. The fall in plasma volume has been demonstrated several weeks prior to the rise in blood pressure and appearance of other clinical manifestations [Gallery et al. 1984]. The degree of plasma volume contraction is an index of severity and, in severe pre-eclampsia, the plasma volume may decrease by as much as 30-40%. The expanded extracellular fluid (ECF) causes the oedema associated with pre-eclampsia. Sodium is the principal component of the extracellular fluid and changes in total ECF volume are related primarily to sodium handling. Women with pre-eclampsia have abnormal control of sodium balance at several levels including alterations in the renin-angiotensin system, prostaglandins, atrial natriuretic peptide and cellular sodium transport pathways of which the latter will be discussed in detail in section 1.3 [Gordon et al. 1973; Fievet et al. 1988; Goeschen et al. 1993; Miyamoto et al. 1992]. As interstitial volume increases, intravascular space decreases as fluid is inappropriately distributed. As a result pre-eclamptic women become haemoconcentrated and peripheral resistance is increased. In the absence of haemorrhage, the intravascular compartment is not under filled but arteriolar vasospasm and capillary leakage have contracted the intravascular space [Benedetti et al. 1985].

1.1.4.4 Hepatic Changes

Epigastic pain in pre-eclampsia may result from hepatocellular necrosis, oedema and ischaemia that stretch the Glisson capsule. This pain is usually associated with elevated serum liver enzymes and is most likely due to periportal haemorrhagic necrosis in the periphery of the liver lobule [Cunningham 1993]. Increased resistance to hepatic blood flow may play a role in the development of these lesions. Liver involvement is serious and is usually accompanied by other organ involvement such as the kidney and brain along with haemolysis and thrombocytopenia. This is commonly named 'HELLP'(haemolysis, elevated liver enzymes and low platelets) syndrome and occurs in approximately 20% of women with severe pre-eclampsia [Sibai et al. 1993]. Adverse pregnancy outcomes are increased in women with 'HELLP' syndrome [Martin et al. 1993].

1.1.4.5 Cardiovascular Changes

Women with severe pre-eclampsia show markedly increased pulmonary and systemic vascular resistance that, combined with a diminished intravascular volume, can have marked haemodynamic effects. Studies of cardiac output in pre-eclampsia have been conflicting but a study by Lang et al. [1991] concluded a reduced cardiac output in pre-eclampsia as a result of both increased systemic vascular resistance and reduced maternal plasma volume. The latter is due to a shift of fluid from the intravascular to interstitial fluid space, and is accompanied by sodium retention [Gallery. 1982]. Enhanced vascular reactivity most likely contributes to the well-described phenomenon of increased pressure responsiveness to infused vasoconstrictors such as angiotensin II in pre-eclampsia [Gant et al. 1980]. The cross over from high output-low resistance that is observed before the clinical disease to low output-high resistance after the appearance is an emerging concept. It is possible that the initial hyperdynamic and vasodilated state exposes the small arteries and capillaries to haemodynamic forces, damaging the endothelium [Easterling and Benedetti 1989]. However this is unproven. Other mechanisms involving circulating substances from the placenta have been postulated which suggests the possibility of both mechanisms being involved to some degree [Cockell et al. 1997].

1.1.4.6 Central Nervous System Changes

Eclampsia may develop in neglected or fulminant cases of pre-eclampsia and the seizures associated with this tend to be of the grand mal variety. It is not known precisely what effect pre-eclampsia has on cerebral blood flow but it is likely that there is abnormal cerebral perfusion when women with pre-eclampsia present with headache [Belfort et al. 1998]. The evidence is consistent with vasospasm and administration of magnesium sulphate during an eclamptic seizure reverses middle cerebral artery vasoconstriction [Belfort et al. 1998]. It is possible that pre-eclamptic women who convulse have an inherited pre-disposition to do so [Rosenbaum and Maltby 1943].

1.1.5 Two Stage Theory

The widespread organ involvement suggests pre-eclampsia is a true systemic disease. Vasospasm, peripheral resistance and thus reduced organ perfusion are said to account for most of the organ system involvement but placental involvement has long been postulated. Pre-eclampsia only occurs in the presence of a placenta and only after

delivery of the placenta do the signs and the symptoms of pre-eclampsia begin to regress [Redman 1991a]. Pre-eclampsia is believed to be a two-stage phenomenon with defectively implanted trophoblasts leading to placental bed vascular insufficiency. In certain cases, conditions such as hydatidiform moles, multiple pregnancies and vascular disorders pose an increased risk which contribute to the reduced perfusion. During normal pregnancy, the maternal spiral arteries undergo marked remodelling in order to provide a low resistance blood supply to the growing uterus and gestational products [Khong et al. 1986; Gerretsen et al. 1981]. In women with pre-eclampsia the invasion of spiral arteries are confined to their intradecidual pathway without invading the intra-myometrial portion. The non-invaded spiral arteries remain reactive to vasoactive drugs and are significantly narrower, which consequently restricts blood flow. Other pathologic changes also occur. Accumulation of fat-laden macrophages with fibrinoid necrosis (termed acute atherosis), disruption of the basement membranes, platelet deposition, mural thrombi, and proliferation of intimal and smooth muscle cells all decrease the luminal diameter. The narrowed and damaged spiral arteries become thrombosed, resulting in placental infarction and necrosis. Uteroplacental blood flow is then reduced by 50-75% [Chesley 1978]. The primary defect in pre-eclampsia must originate at the maternal-fetal interface (the placenta). Decreased placental perfusion is thought to lead to fetoplacental ischemia. The ischemic placenta may produce a circulating agent, which is currently unidentified, that causes the widespread dysfunction of the maternal vascular endothelium that leads to the systemic manifestations of pre-eclampsia (figure 1.1).

1.1.6 Endothelial Dysfunction

About 12 years ago, Roberts et al. [1989] formally proposed endothelial cell dysfunction as the key event resulting in the diverse clinical manifestations of pre-eclampsia. Initial evidence of alterations in endothelial cells came from the morphological observation characteristic of pre-eclampsia known as glomerular endotheliosis [Altchek. 1964]. These lesions consist of swelling of the cytoplasm of the glomerular capillary endothelial cells with fibrin deposits beneath the basement membrane and within the swollen endothelial cytoplasm. This is the most consistent abnormality in pre-eclamptic women and is not present in any other form of hypertension [Altchek. 1964]. In addition there is evidence of ultrastructural changes in the placental bed and uterine blood vessels [Shanklin and Sibai 1989].

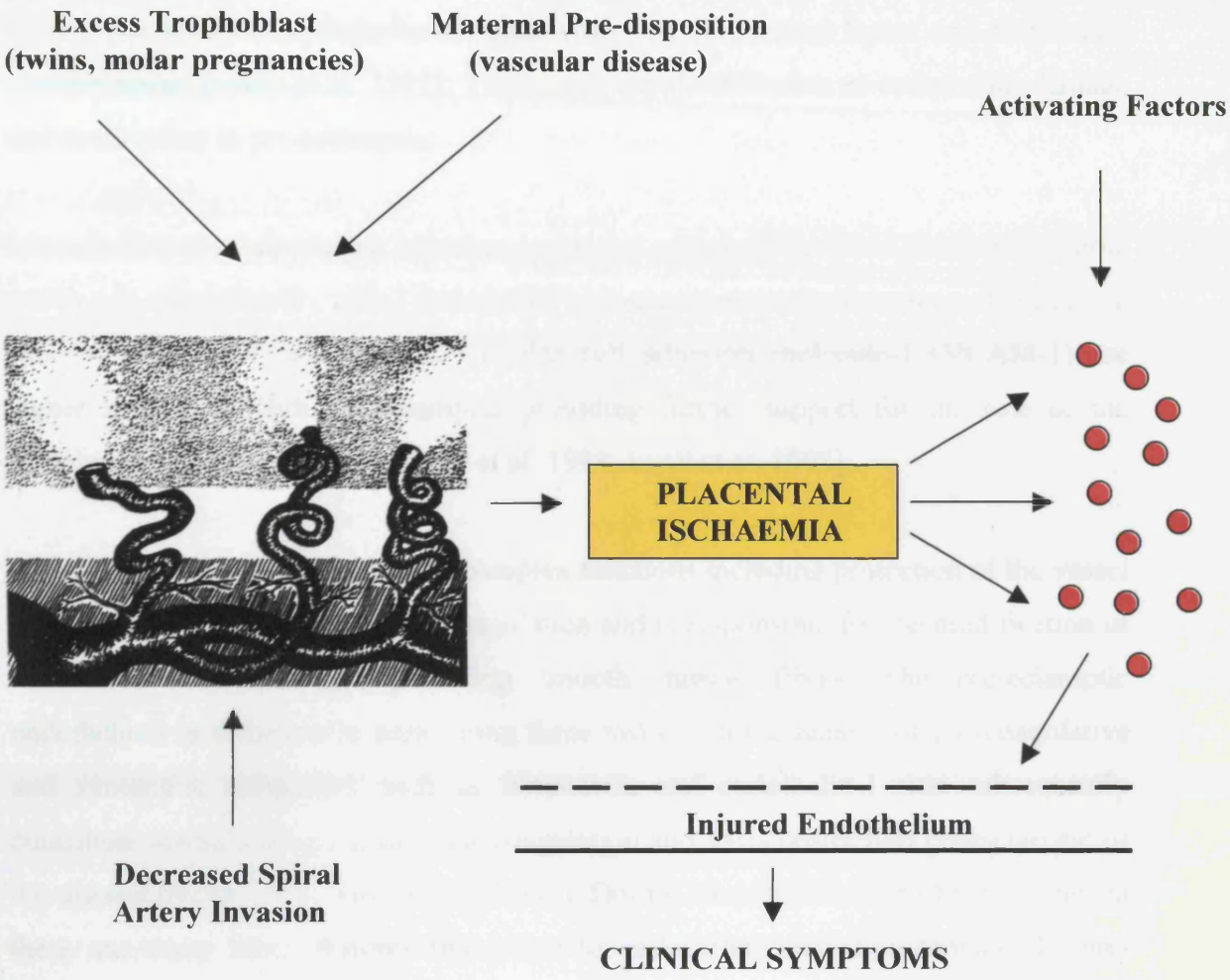


Figure 1.1: Pre-eclampsia setting. First stage: Placental vascularisation defect and ischaemia due to improper placentation. Second stage: Injured endothelium, which may result in clinical symptoms of the disease.

Secondary to these morphological changes are the biochemical changes that point to a central role of endothelial cells in the pathogenesis of pre-eclampsia. Damaged endothelial cells release certain substances into the blood stream such as fibronectin, laminin and von Willebrand factor. The plasma levels of von Willebrand factor and fibronectin were measured in 63 pregnant women with pre-eclampsia and 29 normotensive pregnant women. In pre-eclampsia, the levels of von Willebrand factor and fibronectin were higher than in normal pregnancy [Deng et al. 1994]. The plasma concentration of fibronectin correlates with the amount of proteinuria and uricaemia [Brubaker et al. 1992]. Endothelins are the most potent vasoconstrictors known and a marker of endothelial disease [Yanagisawa et al. 1988]. Levels are increased in

pre-eclampsia, with higher concentrations representing the more severe cases [Nova 1991]. The levels of endothelin correlate with von Willebrand factor and fibronectin concentrations [Greer et al. 1991]. These data are all indicative of endothelial damage and dysfunction in pre-eclampsia.

Increased levels of circulating adhesion molecules represent increased tissue expression, which is associated with endothelial damage and dysfunction. Circulating concentrations of E-selectin and vascular cell adhesion molecule-1 (VCAM-1) are higher in women with pre-eclampsia providing further support for the role of the endothelium in pre-eclampsia [Lyall et al. 1994; Lyall et al. 1995].

Normal endothelium is involved in complex functions including protection of the vessel wall; prevention of intra-vascular coagulation and is responsible for the modification of contractile properties of underlying smooth muscle fibres. The pre-eclamptic endothelium is defective in performing these tasks with the release of pro-coagulative and vasoactive substances such as fibronectin and endothelin-1 that subsequently contribute towards the intra-vascular coagulation and vasoconstriction characteristic of the disease [Nova. 1991; Taylor et al. 1991]. During the manifestation of pre-eclampsia there are many other features that point to endothelial activation/damage. In pre-eclampsia there is believed to be a state of prostacyclin/thromboxane (PGI_2 /TXA_2) imbalance associated with endothelial damage [Zeeman et al. 1992]. Both represent biologically opposite poles of a mechanism that regulates the interaction between platelets and the vessel wall. The principal cyclooxygenase product of arachidonic acid in platelets, TXA_2, is a potent vasoconstrictor and a stimulus to platelet aggregation. The principal cyclooxygenase product of the vascular endothelium, PGI_2 has opposite effects on platelet function and vascular tone. Pre-eclampsia is believed to be a state of PGI_2 deficiency and TXA_2 dominance [Walsh 1985]. Patients with pre-eclampsia have decreased PGI_2 biosynthesis that may result from the endothelial cell damage [Zeeman et al. 1992]. A multicenter prospective study showed that women who developed pre-eclampsia had significantly lower PGI_2 levels throughout pregnancy, even at 13 to 16 weeks' gestation (long before the onset of clinical disease) whereas the TXA_2 levels of pre-eclamptic women were not significantly higher overall [Mills et al. 1999]. Aspirin trials in pre-eclampsia may have failed because an increase in TXA_2 production is not the initial anomaly. Low dose aspirin is believed to inhibit TXA_2, but seeing as the

imbalance is not due to increased levels of TXA_2, the trials have been inconclusive [Mills et al. 1999]. The imbalance between vasodilator prostaglandins and vasoconstrictors, especially in the uteroplacental circulation and the kidney, may explain some of the clinical manifestations of pre-eclampsia. This may contribute to the selective platelet destruction and reduced uteroplacental blood flow. In pre-eclampsia, the intrarenal production of PGI_2 is decreased [Ylikorkala et al. 1986]. This may cause the decrease in effective renal plasma flow, glomerular filtration rate, urate clearance and proteinuria. The deficiency of PGI_2 may result in unopposed intrarenal vascular effects of angiotensin II, causing an impaired ability to secrete sodium. The impaired ability to excrete sodium could cause a shift to the right in renal pressure–natriuresis curve, increasing vascular tone and blood pressure. Although the concept of PGI_2/TXA_2 imbalance explains many clinical features of pre-eclampsia, it has not been proved whether it is a principal pathogenic mechanism.

Nitric oxide (NO) plays an important role in the control of systemic blood pressure as well as being an inhibitor of platelet activation [Knowles and Moncada 1994]. Impairment of its formation through damage to the endothelium would result in vasoconstriction, platelet adhesion, aggregation and release of vasoconstrictor substances, all features relevant to pre-eclampsia. Conflicting evidence exists concerning levels of NO. Oxidised products of nitric oxide were measured and found to be decreased in patients with pre-eclampsia [Seligman et al. 1994]. However, Lyall et al. [1995] found total nitrites to be increased in the fetoplacental circulation in pre-eclampsia which implicates nitric oxide production as a compensatory response to improve blood flow with a putative role in limiting platelet adhesion and aggregation. Its role in pre-eclampsia, therefore, remains controversial. Endothelial cell dysfunction can account for many of the manifestations of pre-eclampsia. However the question still remains as to what precisely causes this endothelial dysfunction and damage.

1.1.7 Oxidative Stress and Pre-eclampsia

Abnormal placentation is implicated in the genesis of pre-eclampsia. Other disorders such as pregnancies with growth-restriction of infants or a subset of pregnancies that result in pre-term birth are also associated with abnormal placentation but do not develop pre-eclampsia. Pre-eclampsia therefore requires more than just a reduced perfusion [Redman 1991a; Knong et al. 1986].

A current hypothesis explaining endothelial alterations in atherosclerosis implicates oxidative stress as being important [Schachter 1997]. The small dense LDL particles are more atherogenic than the lighter fractions [Packard. 1996]. Poor recognition by the LDL receptor-mediated clearance mechanism allows them to stay in the plasma compartment for longer, thereby penetrating the arterial intima more readily. In addition, they are more readily oxidised and may contain less antioxidant, and are more easily taken up by macrophages to create foam cells [Iuliano et al. 2001]. The overall effect is highly atherogenic. These oxidised LDLs are highly reactive and damaging as they alter membrane protein phospholipids and increase the expression of signalling molecules consequently resulting in an increased recruitment of monocytes. The membrane damage alters endothelial function and the macrophages take up the oxidised LDLs to form foam cells that result in the characteristic fatty streak of atherosclerosis.

Oxidative stress is defined as an imbalance between pro-oxidants and antioxidants resulting in a pro-oxidant insult. Pro-oxidants include free radicals and are described as any molecule capable of independent, brief existence that contains one or more unpaired electrons [Grisham and McCord 1986]. Due to this feature they are highly reactive and extremely capable of inducing tissue and cell damage either directly or indirectly. Free radicals are collectively known as reactive oxygen species (ROS), a term used to include the superoxide anion radical, hydroxyl radical, peroxyl radical, hydrogen peroxide and reactive nitrogen species [Halliwell 1993]. Overproduction of ROS in certain disease states is being widely accepted [Datta et al. 2000]. This overproduction can arise from a number of sources (see figure 1.2) [Cadenas and Davies 2000; Wolin 1996; Shatwell and Segal 1996].

Mitochondria have been shown to produce superoxide as a consequence of autooxidation of electron transport chain components [Ksenzenko et al. 1983]. Arachidonate metabolism by cytochrome P450 and lipoxygenase results in production of superoxide [DelMaestro 1980]. Oxidation of hypoxanthine by xanthine oxidase produces superoxide within endothelial cells as well as in the circulation [Parks and Granger. 1986].

Nitric oxide synthase can also generate superoxide as well and hydrogen peroxide, especially if the concentrations of L-arginine are low [Pou et al. 1992]. Factors in the

circulation can produce superoxide that reacts with endothelial cells. Neutrophils are one particular source of superoxide generated in the circulation [Humbert 1975].

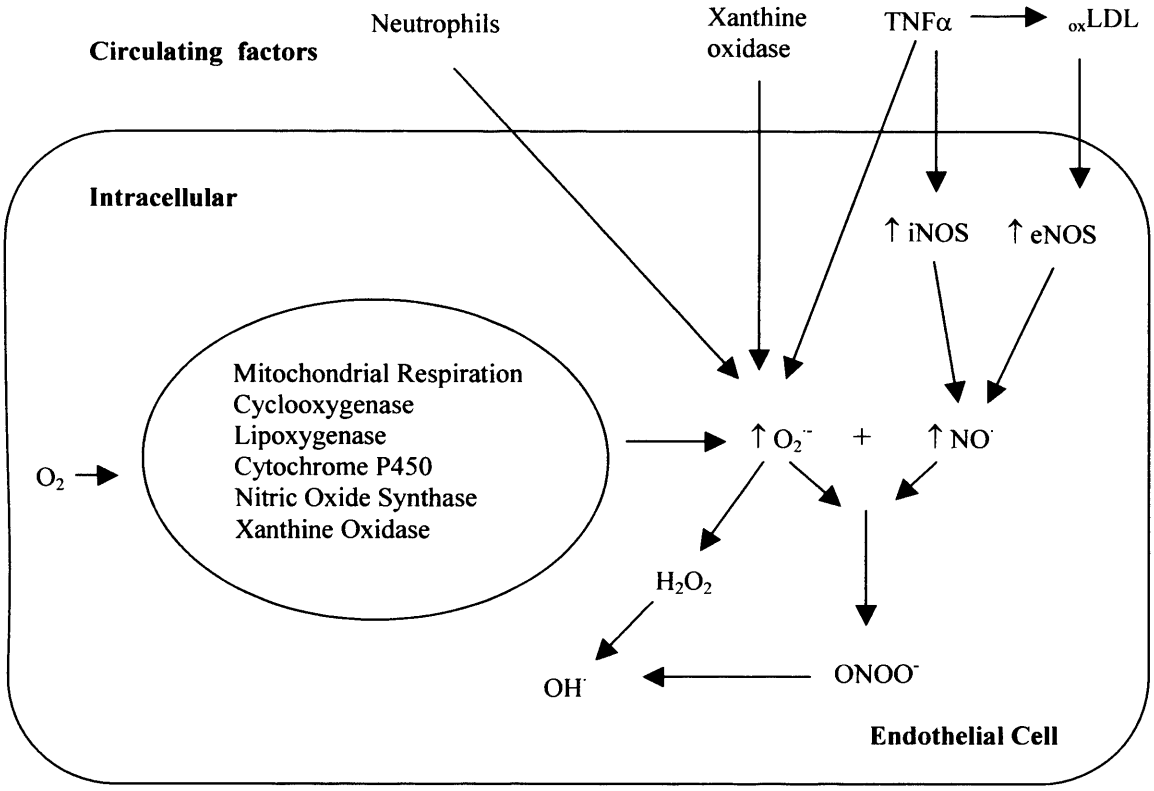


Figure1.2: Oxidant sources that arise from circulating and intracellular sources. Adapted from Davidge 1998.

Cytokines such as TNFα can directly or indirectly initiate oxidative stress. TNFα increases endothelial cell-induced oxidation of LDL and also increases superoxide production through xanthine oxidase [Maziere et al 1994; Page et al, 1997]. These sources of oxidants formed may interact with each other to increase the pro-oxidant insult. Superoxide anions may be reduced to hydrogen peroxide. Hydrogen peroxide may be reduced to form hydroxyl radicals in the presence of transition metals (Fenton reaction). Nitric oxide is produced by the nitric oxide synthase enzymes and may be regulated by circulating factors such as TNFα and LDL. Nitric oxide can react with

superoxide to produce peroxynitrite that can decompose to form the highly reactive hydroxyl radical.

It has been proposed that as with atherosclerosis, oxidative stress is a component of pre-eclampsia that may provide the link between abnormal placentation and the endothelial dysfunction [Roberts and Cooper 2001]. It has been postulated that placental and maternal factors converge to generate this oxidative stress that eventually compromises the function of the vascular endothelium.

Lipid peroxidation has received a great deal of attention in pre-eclampsia [Bayhan et al. 2000; Kharb 2000; Hubel et al. 1989]. This process results in the formation of products that can function in normal physiology but when there is an overproduction of ROS the result can be uncontrolled lipid peroxidation. It involves oxidative conversion of unsaturated fatty acid to primary products known as lipid peroxides and a variety of secondary metabolites. Lipid peroxides can alternatively be formed by cyclooxygenase or lipoxygenase. Indication that lipid peroxidation is implicated in pre-eclampsia comes from measurements of serum malondialdehyde (MDA), a major breakdown product split off from lipid peroxides. Pre-eclamptic patients had higher MDA compared with control; MDA levels were significantly correlated with the systolic and diastolic blood pressure and with serum uric acid levels [Yanik et al. 1999]. Placental tissue pieces were isolated and incubated in serum-free DMEM for 48 h, medium samples were analysed for MDA. At 48 h of incubation, the mean concentrations of MDA were significantly higher for the placentas from pre-eclamptic women than for the placentas from normal pregnant women [Walsh et al. 2000]. Isoprostanes are prostaglandin-like compounds produced specifically by free radical-catalysed peroxidation of arachidonic acid and are found to be a reliable marker of lipid peroxidation and may cause vasoconstriction and platelet activation [Sobal and Sinzinger 2001]. Tissue levels of isoprostane were found to be significantly higher for pre-eclamptic placentas than for normal placentas. Concentrations of MDA were highly correlated with those of isoprostane [Walsh et al. 2000]. The interaction of nitric oxide and superoxide produces peroxynitrite anion, a strong, short-lived oxidant with pronounced deleterious effects that may cause vascular damage. The formation and action of peroxynitrite can be detected by immunohistochemical localization of nitrotyrosine [Roggensack et al. 1999]. Nitrotyrosine residues, an index of oxidative stress arising from peroxynitrite

formation and action, are found in placental vasculature of pregnancies complicated by pre-eclampsia. The intensity of nitrotyrosine immunostaining in pre-eclampsia was significantly greater than that of controls [Myatt et al. 1996]. Immunohistochemical techniques were used to determine increased nitrotyrosine immunostaining in the maternal vasculature of women with pre-eclampsia. Vessels were obtained from a biopsy of subcutaneous fat at the time of Caesarean section from normal pregnant and pre-eclamptic women. There was significantly more staining and with greater intensity for nitrotyrosine in the endothelium of vessels from women with pre-eclampsia compared with that of normal pregnant women and was suggestive of increased peroxynitrite formation [Roggensack et al. 1999].

The increased prevalence of small LDLs, which have several intrinsic properties capable of inducing endothelial dysfunction, are apparent in pre-eclampsia and may contribute to the endothelial dysfunction [Lorentzen and Henriksen 1998]. Enhanced lipid peroxidation may be involved in the foam-cell formation of decidua and in the pathogenesis of pre-eclampsia. Increased autoantibodies to an epitope of oxidized LDL have been described in women with pre-eclampsia. Uotila et al [1998] reported increased titres of serum autoantibodies against copper-oxidised LDL in pre-eclampsia, which may reflect enhanced lipid peroxidation involving circulating lipoproteins. The morphological changes evocative of the atherosclerotic lesions are confined to the decidual vessels but widespread alterations in endothelial dysfunction, similar to that of atherosclerosis, are seen in pre-eclampsia. The mechanisms by which modified LDLs affect vascular function are not well established. Increased tissue production of lipid hydroperoxides and the predominance of TXA_2 in pre-eclampsia consequently result in increased vasospasm with exacerbation of placental ischaemia [Wang et al. 1992]. The detrimental effect of lipid hydroperoxides is due to disruption of lipoprotein and cell membranes and reactive intermediates formed during the peroxidation process or during reaction of lipid hydroperoxides with transition metals [Esterbauer 1993].

Antioxidants protect against oxidative stress. They protect by reacting with radicals faster than radicals react with potential targets. For example α-tocopherol slows lipid peroxidation by scavenging lipid peroxyl radicals, thus breaking the peroxidation chain [Tesoriere et al. 1996]. Ascorbate (vitamin C) regenerates α-tocopherol by reducing tocopherol radicals in the membranes and is thus an ultimate antioxidant nutrient [Frei

et al. 1988]. Decreased plasma ascobate levels are associated with pre-eclampsia. Hubel et al [1997] reported ascorbate concentrations to be 50% lower in pre-eclampsia relative to normal pregnancy plasma. In a study by Kharb [2000], antioxidant vitamins E and C were estimated in 30 pre-eclamptic and 30 normotensive pregnant women. Significantly lowered levels of vitamins E and C were observed in pre-eclamptic women compared to controls. In patients with pre-eclampsia antioxidant nutrients may be utilized to a greater extent to counteract free radical-mediated cell damage, resulting in a reduction in serum antioxidant levels. SOD levels have been analysed in both placenta and red blood cells and found to be lower in pre-eclampsia compared to normotensive pregnancy [Kharb 2000; Wang and Walsh. 2001]. A study by Bowen et al. [2001] implicates uric acid as an antioxidant in pre-eclampsia with the increased levels serving as a protective role against oxidative stress. Pre-eclampsia is associated with an imbalance between lipid peroxides and antioxidant nutrients. The imbalances favour lipid peroxides with the increasing severity of pre-eclampsia [Panburana et al. 2000]. Further evidence to support the role of oxidative stress in pre-eclampsia comes from trials involving supplementation of antioxidants for women with a high risk of developing pre-eclampsia. In one study, 283 women were identified as being at increased risk of pre-eclampsia by abnormal two-stage uterine-artery doppler analysis or a previous history of the disorder and were randomly assigned vitamin C (1000 mg/day) and vitamin E (400 IU/day) or placebo at 16-22 weeks' gestation. The results suggested that supplementation with vitamins C and E may be beneficial in the prevention of pre-eclampsia in women at increased risk of the disease and suggest that oxidative stress is implicated [Chappell et al. 1999].

The plethora of evidence discussed implicates oxidative stress in the pathogenesis of pre-eclampsia. A continuing enigma remains of how the oxidative stress transfers to the systemic circulation and how it is related to the initial placental lesions.

1.1.7.1 Neutrophils

There is increasing evidence to suggest a role for maternal neutrophil activation in the endothelial damage associated with pre-eclampsia. Neutrophil activation has been shown to result in vascular damage in non-pregnant subjects in whom lesions similar to that of acute atherosis of pre-eclampsia are seen [Harlan 1987]. Upon activation neutrophils release a variety of substances capable of inducing vascular injury. They

include ROS that can produce membrane lipid peroxidation, lysis of endothelial cells and increased vascular reactivity, as well as granule contents such as elastase that can destroy the integrity of endothelial cells [Harlan 1987]. In addition neutrophils metabolise arachidonic acid to several metabolites that can degrade various cell components [Klebanoff 1988].

Evidence to support the hypothesis that neutrophil activation is implicated in the genesis of pre-eclampsia is reported next. Plasma neutrophil elastase was measured by radioimmunoassay. There was a significantly higher concentration of plasma neutrophil elastase in both mild/moderate and severe pre-eclampsia than in normotensive pregnancies [Greer et al. 1989]. This was the first technique used to identify neutrophil activation in pre-eclampsia. A significant correlation exists between the levels of plasma neutrophils elastase and von Willebrand factor, a marker of endothelial dysfunction [Greer at al, 1991]. Elastase positive neutrophils are increased in the decidua of the placental bed in women with pre-eclampsia and correlate with plasma urate, a marker of disease activity [Butterworth et al. 1991]. Neutrophil adhesion molecule expression and increased serum soluble L -selectin levels are consistent with activation of peripheral blood neutrophils and document neutrophil involvement. L-Selectin and CD11b surface expression on neutrophils were analysed in 13 women with pre-eclampsia and 17 age and gestation matched control women. Women with pre-eclampsia displayed higher levels of CD11b and soluble L-selectin serum relative to normal values [Sabatier et al. 2000]. Several studies have involved the measurement of ROS produced upon agonist activation of neutrophils in pre-eclampsia. According to one study, fMLP induced superoxide production of neutrophils was significantly higher in pre-eclamptics compared to normal non pregnant and pregnant subjects, suggesting that the neutrophils may have been primed by some factor rendering them more sensitive to agonist stimulation [Tsukimori et al. 1993]. In further support of neutrophil activation, pre-eclamptic serum potentiates the production of ROS in non-pregnant neutrophils stimulated with fMLP whereas a normal and non-pregnant serum does not [Tsukimori et al. 1993]. This may suggest that the serum contains factors that activate or prime neutrophils. However, other studies have been unable to identify evidence of a factor in serum from women with pre-eclampsia [Clark et al. 1997]. Other studies have suggested that pregnancy itself is associated with neutrophil activation with pre-eclampsia exhibiting one extreme of a generalized inflammatory response [Sacks et al.

1998]. Crocker et al [1999] present a conflicting argument that suggests neutrophils in normal pregnancy exhibit a diminished response compared to pre-eclamptic and non-pregnant women. Superoxide anion generation was reduced in the pregnant group compared with non-pregnant controls (fMLP by 51% and zymosan activated serum by 56%) but pre-eclamptic measurements did not show a similar reduction. Pre-eclampsia is by most accounts, associated with neutrophil activation and increased production of ROS.

In addition to bringing about endothelial damage directly through oxidation of lipid membranes and lipid peroxidation, ROS inhibit vascular relaxation *in vivo* and *in vitro* by inactivating endothelium-derived relaxing factor (EDRF) [Hubel et al. 1998; Gryglewski et al. 1986]. It is possible that neutrophil ROS contributes to the prostaglandin imbalance characteristic of pre-eclampsia. Prostacyclin may be reduced because of extensive lipid peroxidation that preferentially inhibits PGI_2 synthase, resulting in reduced vasorelaxation. Studies in rat models of increased ROS show increased PGH synthase dependent vasoconstriction [Davidge et al. 1993]. Peroxynitrite may activate prostaglandin H synthase leading to increased TXA_2. Landino et al [1996] have shown that peroxynitrite can activate PGH synthase and may represent a pathway for activation in women with pre-eclampsia. These pathways are speculative but may contribute towards the prostaglandin imbalance in pre-eclampsia. Evidence suggests that neutrophil activation, characterized by the production of ROS and elastase, is important in promoting the vascular damage in pre-eclampsia [Clark et al. 1998]. However, the mechanism of local neutrophil activation is unclear. These cells could be activated by oxidative stress in the intervillous space and then produce ROS on contact with the endothelium [Roberts and Cooper. 2001]. Alternatively, the hypoxic placenta might produce cytokines that prime or activate the circulating neutrophils [Benyo et al. 1997; Conrad and Benyo 1997]. Activated complement factors C3a and C5a are increased in pre-eclampsia and have chemotactic activating properties on neutrophils [Haeger et al. 1992]. The role of the neutrophil is increasingly being implicated in the pathogenesis of the disease.

1.2 NADPH Oxidase

Neutrophils produce superoxide as part of their bactericidal host defense mechanism [Rosen et al. 1995]. The Nicotinamide adenine dinucleotide phosphate H (NADPH)

oxidases are a group of multi-component, membrane-associated enzymes that are involved in ROS production in neutrophils. The enzyme accepts electrons from NADPH at the cytosolic side of the membrane and donates them to molecular oxygen at the other side of the membrane, either on the outside of the cell or in the phagosome according to the following reaction: -

$$2\ O_2 + NADPH \longrightarrow 2O_2^- + NADP^+ + H^+$$

The superoxide produced is subsequently converted to hydrogen peroxide, hypochlorous acid and other reactive metabolites. A continuous supply of NADPH is generated from glucose via the hexose monophosphate shunt [Rossi 1986]. The enzyme has been recognized in various tissues such as proximal tubules and smooth muscle cells as well as non-phagocytic cells such B-lymphocytes, which may be transformed by the Epstein-Barr virus and propagated indefinitely [Griendling et al. 2000; Maly et al. 1988]. The essential role played by the NADPH oxidase enzyme is in protecting the body from infection. The importance of the NADPH oxidase is illustrated by the inherited condition chronic granulomatous disease (CGD) in which a component of the respiratory burst oxidase is absent or defective. Affected individuals suffer from recurrent, chronic and severe infections due to the inability of their neutrophils to kill microbes [Smith and Curnutte 1991].

1.2.1 Composition

Sbarra and Karnovsky first reported the existence of the NADPH oxidase enzyme over 40 years ago [Babior 1999]. The core oxidase comprises five components: p67-*phox*, p47-*phox*, p40-*phox*, p22-*phox* and gp91-*phox*. In the resting cell, three of these five components-p67-*phox*, p47-*phox* and p40-*phox* exist in the cytosol. The other two are located in the membranes [Morel et al. 1991].

1.2.1.1 Flavocytochrome b_{558}

The main structural element of the enzyme is a membrane bound flavocytochrome known as b_{558} because of the typical absorption peak at 558nm of the haem binding component of the oxidase in the reduced state [Segal and Jones 1979]. This redox core takes electrons from NADPH and passes them, via FAD and haem, to O_2. Flavocytochrome b_{558} comprises two protein subunits. The α subunit is the smaller and

is referred to as p22-*phox*, comprises 194 amino acids, is non-glycosylated and has a derived M_r of 21-22 kDa [Parkos et al. 1987]. The β subunit is larger than p22-*phox* and is known as gp91-*phox*, is 569 amino acids in length, heavily glycosylated and has an approximate M_r of 91 kDa [Parkos et al. 1987].

1.2.1.2 Cytosolic Components

The first indication that factors other that flavocytochrome b are required was when Segal et al [1985] discovered that some patients with autosomal recessive CGD lack a 47 kDa phosphoprotein, p47-*phox*. P47-*phox* is chiefly responsible for the process of translocation during oxidase activation and is absolutely necessary for O_2^- generation by the phagocyte NADPH oxidase [Nunoi et al. 1988]. The cDNA for p47-*phox* has been cloned and the gene has been localized and characterized [Volpp et al. 1990]. The deduced amino acid sequence of p47-*phox* contains 390 residues of which at least six are potential serine phosphorylation sites for protein kinase C. Furthermore, the amino acid sequence contains two SH3 motifs and at least one proline-rich region that probably plays a role in assembly of the oxidase upon activation [Leusen et al. 1996; El Benna et al. 1994]

The function of the p67-*phox* is unclear. However, it is believed to possess a catalytically essential binding site for NADPH [Smith et al. 1996]. The predicted 526 amino acid sequence also contains two SH3 domains and at least one proline-rich region [Leto et al. 1990].

The function of p40-*phox* has been examined and is suggested to be an inhibitory oxidase subunit [De Sathyamoorthy et al. 1997] or a stimulatory subunit [Tsunawaki et al 1996]. P40-*phox* is not essential for activation and is thought to play a role in stabilizing p67-*phox* in intact cells [Leusen et al. 1996]. More recently, Cross [2000] suggested that p40-*phox* can function to promote oxidase activation by increasing the affinity of p47-*phox* for the enzyme approximately 3-fold. The cDNA has been cloned and the predicted 339 amino acid sequence contains one SH3 domain [Wientjes et al. 1993].

Rac2 is a ~22 kDa, GTP-binding protein that has been identified as an NADPH oxidase co-factor essential for optimal superoxide generation [Knaus et al 1991]. It is probable

that Rac2 functions by changing from an inactive, GDP-bound state to an active GTP-bound state, mediating the activation of NADPH oxidase [Knaus et al. 1992; Mizuno et al. 1992].

Finally Rap1A, a low molecular weight ras-related GTP-binding protein has been located in the membranes of resting neutrophils. It was first implicated in the function of NADPH oxidase when it was found to co-purify with cytochrome b_{558} [Quinn et al. 1989]. Rap1A has been shown to bind specifically to cytochrome b_{558} with a one-to-one stoichiometry and become phosphorylated by protein kinase A at the COOH-terminal region [Quilliam et al. 1991]. It is possible that Rap1A regulates the deactivation of NADPH oxidase [Bokoch et al. 1991]

1.2.2 Mechanisms of Activation

The oxidase is normally dormant but can be rapidly activated by a number of stimuli [Rossi 1986]. These include agonists that interact with surface receptors like fMLP, complement fragment (C5a) and opsonized particles as well as protein kinase C agonists such as phorbol myristate acetate (PMA) [for review see Segal and Abo 1993]. Different stimuli activate different proximal pathways. The response to PMA is usually seen after 25 seconds, lasts for many minutes, is calcium independent and blocked by kinase inhibitors. The fMLP response occurs after 5-10 seconds and is of lower intensity, calcium dependent and resistant to inhibitors of protein kinase C. A 'primed' state can be induced and refers to a process whereby the response of neutrophils to an activating stimulus is potentiated, sometimes greatly, by prior exposure to a priming agent [Condliffe et al. 1998]. Priming reduces the lag time and amplifies the response to a subsequent stimulus up to 20-fold in some cases. Although different activation pathways exist, downstream processes converge in their requirements for the cytosolic components described below.

1.2.3 Phosphorylation and Translocation

After activation of NADPH oxidase is initiated, a complex, and as yet only partially understood, series of signal transduction pathways leads to the translocation of the cytosolic factors to the plasma membrane (Figure 1.3). Phosphorylation is an essential element of the activation of NADPH oxidase. Phosphorylation of p47-*phox* has been recognised for many years. However, more recently, the phosphorylation of p67-*phox*

and p40-*phox* have been demonstrated [Babior 1999]. Faust et al [1995] demonstrated that only phosphorylation of ^{379}Ser on p47*phox* is essential for oxidase activity and membrane association. Upon phosphorylation the entire complex docks with the membrane-bound oxidase, leading to an active NADPH oxidase. In vitro binding studies indicate that the entire complex docks via an association between p47-*phox* and the proline rich region at the carboxy-terminus of p22-*phox* [Leto et al. 1994]. It is believed that Rac functions as a "shuttle protein," binding to p67-*phox* and carrying it to the membrane in its GTP-bound state and dissociating in its GDP-bound state and

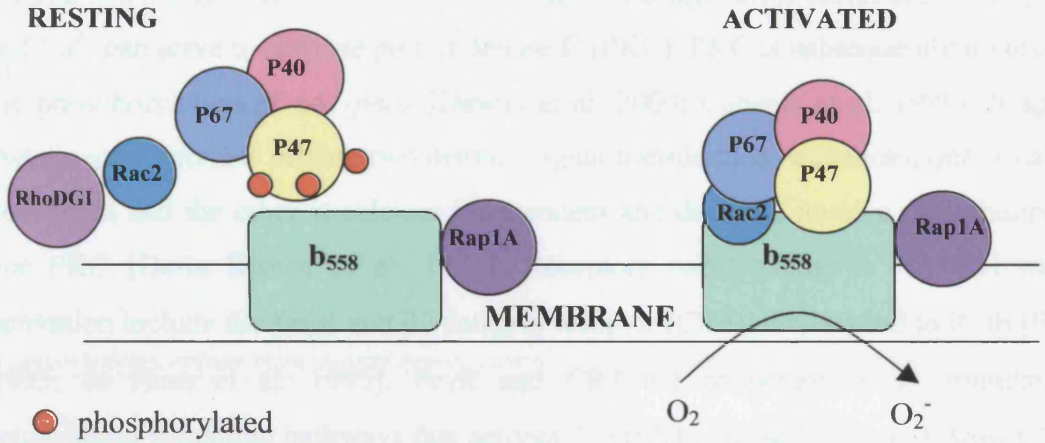


Figure 1.3: Activation of the leucocye NADPH oxidase. In the resting cell, subunits are distributed between the cytosol (p67-phox, p47-phox, p40-phox and Rac2) and the membranes (cytochrome b_{558}, a p22-phox, gp91-phox complex and Rap1A) Rac2 and Rap1A are low-molecular weight guanine nucleotide-binding proteins that function in other processes besides activation. Phox proteins are unique to NADPH oxidase enzyme. When the cell is activated, p47 becomes heavily phosphorylated and the cytosolic complex translocates to the membrane, where they bind to the flavocytochrome b_{558} to assemble the active oxidase.

returning to the cytosol [Leusen et al. 1996]. It has been indicated that binding of Rac2 to p67-*phox* is essential for translocation and thus probably mediates translocation of p47-*phox*, essential for activity of the NADPH oxidase in intact cells. The cytosolic p40-*phox* and Rap1A are not considered essential for oxidase activity when using the cell free system. It is likely that Rap1A in its GTP-bound state activates the oxidase and in its GDP-bound state, deactivates the oxidase [Quilliam et al. 1991].

1.2.4 Signal Transduction

The signal transduction pathways mediating NADPH oxidase activation are the subject of intense investigation and are known to include protein tyrosine kinases, GTP-binding proteins and protein kinase C (PKC). Agonists binding to receptors activate heterotrimeric G protein dissociation. The α subunit, bound to GTP, dissociates and in turn activates a plasma membrane phospholipase C. This enzyme cleaves phosphatidylinositol 4,5-bisphosphate and delivers two second messengers: inositol 1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG). IP_3 mediates rapid Ca^{2+} release, leading to a transient rise in cytosolic free Ca^{2+} concentration [Pozzan et al. 1983]. DAG and Ca^{2+} can serve to activate protein kinase C (PKC). PKC is subsequently involved in the phosphorylation of p47-*phox* [Dewas et al. 2000; Cabanis et al. 1996]. It appears that receptor agonists initiate two distinct signal transduction sequences, one is calcium dependent and the other is calcium independent and does not involve phospholipase C nor PKC [Della Bianca et al. 1993]. Receptors participating in NADPH oxidase activation include the FcγR and β2-integrin receptor (CR3), which bind to iC3b [Brown 1995; de Haas et al. 1995]. FcγR and CR3 act cooperatively to stimulate the intracellular signalling pathways that activate NADPH oxidase [Zhou and Brown 1994]. Cross-linking of FcγR induces the activation of src family tyrosine kinases and tyrosine phosphorylation, triggering the signal transduction pathway [Sanchez-Mejorada and Rosales. 1998]. Cross-linking of FcγR also stimulates tyrosine phosphorylation of phospholipase Cγ, ultimately activating PKC [Rankin et al. 1993]. Stimulation of FcγR and CR3 also activates p38 MAPK in neutrophils [Rose et al. 1997]. Several papers have suggested p38 MAPK involvement in the signalling pathway of NADPH oxidase activation and phosphorylation of p47phox or upstream of it [Hazan et al. 1997; Yamamori et al. 2000]. Research continues to try and unravel the complex signal transduction mechanisms involved in order to fully understand NADPH oxidase activation.

1.3 Membrane Abnormalities in Pre-eclampsia

The complex aetiology along with altered intracellular electrolyte composition and abnormal control of sodium balance mentioned in section 1.1 suggest that cell membrane transport abnormalities may play a role in contributing towards the pathophysiology of pre-eclampsia. Membrane transport abnormalities have previously

been demonstrated in pre-eclampsia and are probably involved in the volume homeostasis and hypertension. Na^+/K^+ATPase activity is altered in erythrocytes from pre-eclamptic patients [Heilmann et al. 1993]. This pump accomplishes the extrusion of cellular sodium and altered activity may contribute towards the high levels of cellular sodium reported in pre-eclampsia [Forrester et al. 1990]. It has been postulated that leucocyte sodium is elevated and potassium depressed as a result of this altered sodium pump activity which may consequently give rise to the hypertension in pre-eclampsia [Forrester and Alleyne 1980]. This defect in sodium extrusion is thought to be partially due to reduced sodium pump expression that has been documented in smooth muscle cells [Maxwell et al. 1998].

Evidence for an endogenous digitalis-like factor in pre-eclampsia has been growing [Graves et al. 1995; Delva et al. 1989; Gregoire et al. 1988; Lopatin et al. 1999]. This substance is characterized by its affinity for the digitalis-binding site and its ability to inhibit Na^+/K^+ATPase [Graves et al. 1995]. This inhibition leads to increased intracellular sodium and thereby calcium as a consequence of alterations in Na^+/Ca^{2+} exchange. Increased intracellular calcium is another characteristic of pre-eclamptic electrolyte composition [Kilby et al. 1990]. The presence of this digitalis–like factor may indirectly raise intracellular calcium and be responsible for the vascular hyper-responsiveness such as increased vascular tone and hence increased peripheral resistance, which characterises pre-eclamptic pregnancies [Gregoire et al. 1988].

The aetiology of the membrane abnormalities in pre-eclampsia is still unresolved. Few studies have examined Na^+/Li^+ counter transport in normal and pre-eclamptic pregnancies but the conclusions are somewhat conflicting. Rutherford et al. [1992] and Miyamoto et al. [1992] could find no differences between normal and hypertensive pregnancies whereas Worley et al. [1982] reported increased activity. However, the physiological role of the Na^+/Li counter transport remains enigmatic.

Surprisingly, little work has focussed on the ubiquitous Na^+/H^+ exchanger. It is feasible that an altered activity of this membrane transport system could contribute towards the high levels of cellular sodium reported in pre-eclampsia [Seon and Forrester. 1989]. Only one study to date has examined the role of Na^+/H^+ exchanger activity in pre-eclampsia [Graham et al. 1997]. No differences were reported between the two study

groups. However, this particular study examined non-proteinuric pre-eclamptic women so may be considered somewhat tenuous. Changes in intracellular pH could also potentially affect free radical generation by the NADPH oxidase since H^+ are released intracellularly by the NADPH oxidase enzyme upon activation (if H^+ are not cleared by an efflux mechanism such as the Na^+/H^+ exchanger, the fall in intracellular pH could limit the activity of the NADPH oxidase enzyme). Finally, although well established as an intermediate phenotype (a surrogate disease marker for genetic predisposition studies, intermediate between the gene and the disease) for essential hypertension, its role in pre-eclampsia has not been extensively explored [Sweeney et al. 1997].

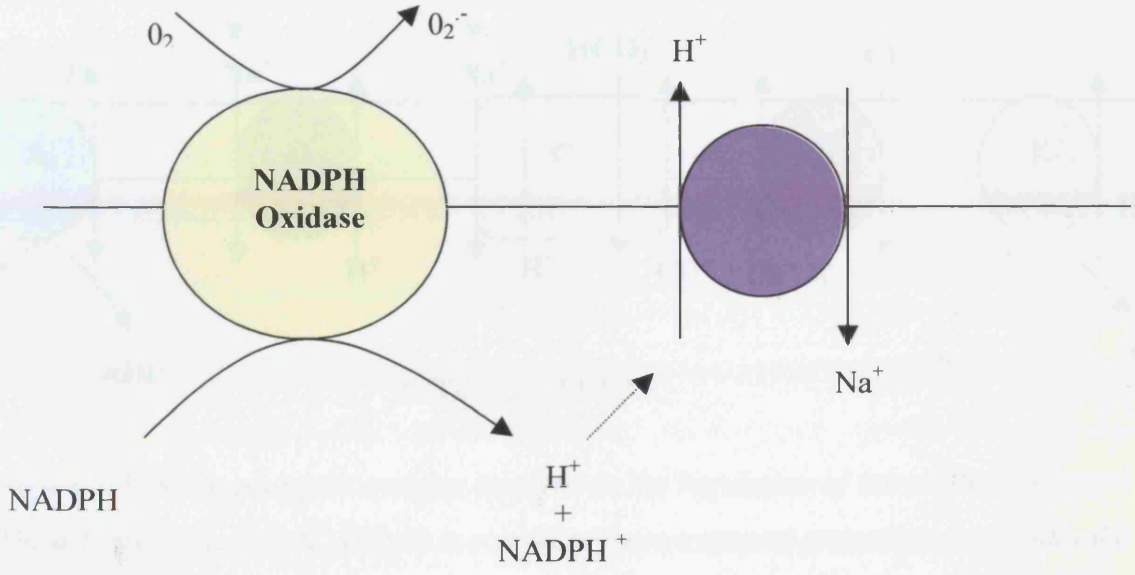


Figure 1.4 Possible associations between NADPH oxidase and NHE-1

1.3.1 Na^+/H^+ Exchanger

The regulation of intracellular pH (pH_i) is dependent on a cellular buffering capacity and the action of several transport proteins that mediate the net flux of acid base equivalents (Figure 1.5). One such transport system is the Na^+/H^+ exchanger, which mediates the electroneutral 1:1 exchange of Na^+ for H^+. The initial presence of the Na^+/H^+ exchanger was experimentally demonstrated in 1976 by Murer et al as a protein that utilises the extracellular to intracellular sodium gradient generated by the Na^+/K^+ATPase, to expel excess protons from the cell [Murer et al. 1976]. During the past three decades, mainly due to the cloning of prokaryotic and eukaryotic Na^+/H^+

exchangers in 1987 and 1989 respectively, understanding and progress in this area has been remarkable [Goldberg et al. 1987; Sardet at al. 1989].

1.3.2 Physiology of the Na^+/H^+ Exchanger

The Na^+/H^+ exchanger is an omnipresent feature of the plasma membrane of mammalian cells. It has been detected in practically every cell type examined: hepatocytes, fibroblasts, smooth, skeletal and cardiac muscle, blood cells including erythrocytes, lymphocytes, neutrophils, platelets and macrophages, and epithelial cells

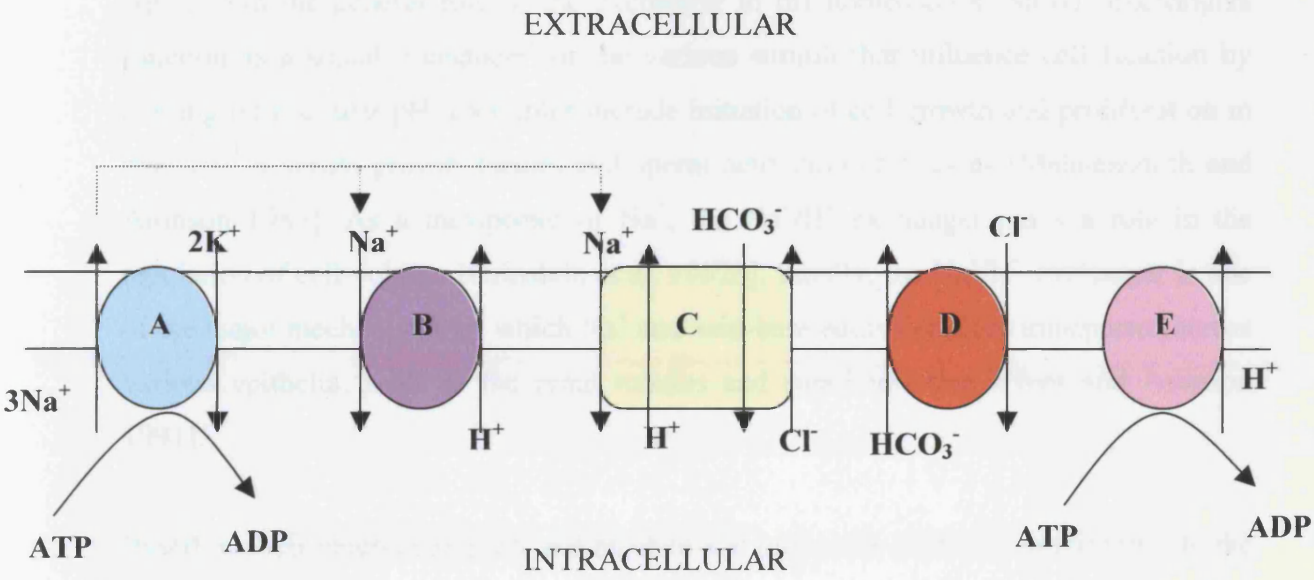


Figure 1.5: Some transport systems involved in the regulation of intracellular pH. A= The activity of the Na^+/K^+ ATPase is crucial to these transport systems as it provides the electrochemical gradient for Na^+ B= Na^+/H^+ exchanger, C= Na^+-dependent bicarbonate transporter, D= Na^+-independent bicarbonate transporter, E= H^+ ATPase.

from the renal tubule and intestine [Aronson 1985]. In the intact cell, the Na^+/H^+ exchanger mediates the extrusion of protons coupled to the flux of Na^+ into the cell. The energy for the extrusion of protons is obtained exclusively from the steep inwardly directed Na^+ gradient that is maintained by the primary active extrusion of Na^+ via the separate plasma membrane Na^+/K^+ATPase system. Thus, Na^+/H^+ exchanger is an example of secondary active transport. The exchanger is normally nearly quiescent when cytoplasmic pH is at the physiological level. At acidic pH_i the Na^+/H^+ exchanger is activated by intracellular protons that are presumed to interact with an internal

allosteric 'modifier site' that is separate from the sites involved in Na^+ and H^+ exchange i.e. protons are not only transported but also control a regulatory site within the protein [Aronson et al. 1982]. Activation without acidification can be modulated by several factors including growth factors, hormones, tumour promoters, chronic extracellular acidification, chemotactic factors and fertilizations of eggs as well as physical factors such as changes in cell volume and cell spreading [Sardet et al. 1991; Orlowski et al. 1997].

Apart from the general role of the exchanger in pH homeostasis, Na^+/H^+ exchangers function as a signal transducer for the various stimuli that influence cell function by altering intracellular pH. Examples include initiation of cell growth and proliferation in response to serum growth factors and sperm activation of oocytes [Mahnensmith and Aronson 1985]. As a transporter of Na^+, the Na^+/H^+ exchanger plays a role in the regulation of cell volume [Grinstein et al. 1992b]. Finally, the Na^+/H^+ exchanger is one of the major mechanisms by which Na^+ and acid-base equivalents are transported across various epithelia, such as the renal tubules and small intestine [Huot and Aronson 1991].

Based on their pharmacological and biochemical properties (different sensitivities to the diuretic compound amiloride and its analogues), studies have presently revealed six isoforms that are expressed to varying degrees in different tissues and have been named according to the chronological order of their molecular identification. All the mammalian Na^+/H^+ exchanger isoforms are similar in primary structure (NHE-1, 815-820 amino acids; NHE-2, 809-813 amino acids; NHE-3, 832-832 amino acids and NHE-4, 717 amino acids) and the predicted secondary structure [Yun et al. 1995]. All are predicted to have two structurally and functionally distinct domains: an N-terminal domain, consisting of 10-12 putative transmembrane α helices that confers allosteric regulation by H^+ and transports Na^+ and H^+ and a long cytoplasmic C-terminal domain that provides regulatory control and contains a number of phosphorylation sites (serine residues) and two calmodulin binding sites (high and low affinity) [Harris and Fliegel 1999]. It has been confirmed that the C-terminals for NHE-1, NHE-2 and NHE-3 are intracellular [Tse at al. 1994]

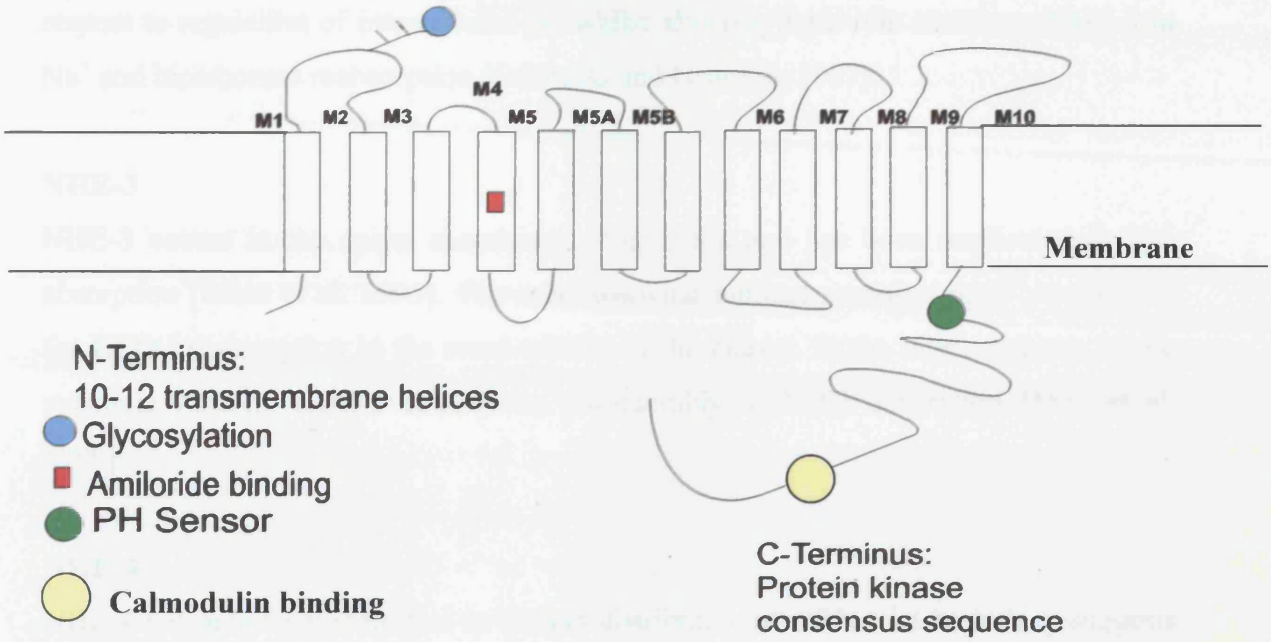


Figure 1.6: Topological model for the Na^+/H^+ exchanger isoforms. Model is derived based on hydropathy profiles and biochemical studies.

NHE-1

NHE-1 comprises of 815 amino acids and has a predicted molecular weight of 91kd. Its actual weight is 110kd because it is N-glycosylated [Sardet et al 1990; Counillon et al 1994]. NHE-1 has been designated the 'housekeeping' isoform due to its widespread distribution throughout the cells of the body [Bookstein et al. 1994a]. It mediates functions such as pH homeostasis, volume regulation and cell growth and proliferation and is expressed on the basolateral surface of several epithelia [Yun et al, 1995]. Growth factors stimulate NHE-1 by a change in the affinity constant for intracellular H^+ [Yun et al, 1995].

NHE-2

Northern blot analysis has identified NHE2 mRNA only in small intestine, prostate, kidney, colon, and skeletal muscle [Malakooti et al.2001]. The precise nature and role of this isoform is not entirely clear, although its potential role in volume regulation in renal inner medullary collecting duct cells has been suggested [Soleimani et al. 1994]. This exchanger has also been reported to behave in a manner similar to NHE-1 with

respect to regulation of intracellular pH whilst also playing a role similar to NHE-3 in Na^+ and bicarbonate reabsorption [Orlowski and Grinstein 1997].

NHE-3

NHE-3 occurs in the apical membrane of epithelia and has been implicated in Na^+ absorption [Brant et al. 1995]. The accompanying luminal secretion of H^+ is essential for HCO_3^- reabsorption in the renal tubules of the kidney. In the later segments of the proximal tubules, NHE-3 contributes considerably to NaCl absorption [Moe et al. 1990].

NHE-4

NHE-4 has only been identified in rat and distribution of mRNA in the kidney suggests that it is localized to the basolateral membranes of the renal inner medullary tubules [Orlowski et al. 1992]. The function of this isoform is largely unknown, but expression correlates with regions of high tissue osmolarity suggesting that it functions as a volume regulatory mechanism [Bookstein et al. 1994].

NHE-5

The presence of NHE-5 has been identified in whole kidney, brain and spleen but relatively little is known about its localizations and function [Moe 1997]

NHE-6

The newly identified NHE-6 has been isolated to the inner membrane of mitochondria [Numata et al. 1998].

1.3.3 Regulation of the Na^+/H^+ exchanger

During the past decade evidence has accumulated suggesting that the Na^+/H^+ exchanger isoform 1 plays a pivotal role in a variety of pathologies including essential hypertension and diabetic nephropathy [Siffert and Dusing 1996]. I shall therefore summarize some aspects concerning the regulation of the Na^+/H^+ exchanger isoform 1. Activity of the Na^+/H^+ exchanger isoform 1 can be modulated by a number of growth factors, hormones and neurotransmitters (via receptor tyrosine kinases and seven-transmembrane receptor coupled G proteins) as well as by mechanical stimuli and hypertonic shrinkage [Fliegel 1996]. Most of these signals that activate Na^+/H^+

exchanger do so by shifting the pH_i activity curve towards the alkaline range, thus stimulating the enzymatic activity of the exchanger at constant pH_i and moving it closer to the maximal rate.

1.3.3.1 Role of Phosphorylation

Even before the molecular characterization of the Na^+/H^+ exchanger, phosphorylation had been proposed as a likely mechanism of activation [L'Allemain et al. 1984]. This is because growth factor activation of Na^+/H^+ exchange requires ATP. Sardet et al. [1990] subsequently showed that epidermal growth factor, thrombin, phorbol esters and serum all induce phosphorylation of the protein. While the exchanger is phosphorylated in unstimulated cells, the extent of phosphorylation on serine residues is increased in parallel with Na^+/H^+ exchanger activation, suggesting phosphorylation as a mechanism for its activation [Sardet et al. 1991]. The phosphorylation sites have been mapped to the C-terminal domain of the Na^+/H^+ exchanger [Bianchini et al. 1997]. The same phosphorylation pattern was determined regardless of which pathway of the cells was stimulated, whether it be via G protein coupled receptors (e.g. thrombin) or receptors with intrinsic tyrosine kinase activity (e.g. EGF) [Sardet et al. 1990; Bianchini et al. 1991; Sardet et al. 1991]. This common phosphorylation pathway has led to the proposition of the existence of a NHE-1 kinase activated pathway integrating extracellular stimuli such as the mitogen-activated protein kinase (MAPK) cascade that finally mediates the phosphorylation of NHE-1 [Siffert and Dusing 1996]. However, the existence of such a kinase remains to be proven. Phosphorylation is not the sole mechanism by which Na^+/H^+ exchange is regulated. This has become clear from mutational and deletion analyses. Half the transport activation remained following deletion of the C-terminus containing all the sites that are phosphorylated in response to activation by a combination of thrombin/insulin [Wakabayashi et al. 1992]. Secondly, deleting the stretch between positions 567 and 635 eliminated transport stimulation but preserved phosphorylation [Wakabayashi et al. 1994]. Important additional signalling elements, which are independent of phosphorylation, participate in activation of the exchanger.

1.3.3.2 Role of Ca^{2+}-Calmodulin

The best characterized and probably the most important phosphorylation independent mechanism involves an interaction between calmodulin and the cytoplasmic domain of

the exchanger protein. A stretch between residues 636 and 656 can bind Ca-calmodulin with a high affinity [Wakabayashi et al. 1994]. In the unstimulated state this region functions as a self-inhibitory domain thereby keeping the exchanger in its resting conformation preventing an alkaline shift of the pH_i activation curve. The inhibitory control is physiologically important, as the continuous overload of Na^+ that would be imposed by an active antiporter would lead to cellular energy expenditure since the Na^+ must be removed by the Na^+/K^+ATPase. When the calcium concentration is elevated and induces calmodulin to bind, the auto-inhibitory effect is abolished allowing the exchanger to counteract the cytosolic acidification. The mechanism of Ca-calmodulin activation is able to account for most, if not all of the Ca-dependent activation of the Na^+/H^+ exchanger. It is possible that increased Ca^{2+} levels could modulate activity of kinases which may be members of the signalling cascades that activate NHE [Frohlich and Karmazyn 1997]. There is evidence for interactions between Na^+/H^+ exchanger and additional proteins besides calmodulin. A Na^+/H^+ exchanger accessory protein of 24kDa has been isolated which is not calmodulin as it could be immunoprecipitated using a NHE-1 deletion mutant lacking the calmodulin binding region [Goss et al. 1996]. One of the larger heat shock proteins, hsp70 has found to be associated with Na^+/H^+ exchange [Silva et al. 1995]. The potential roles of these proteins in modulating the exchanger are not fully elucidated.

1.3.3.3 Role of Cell Volume

A variety of cell types regulate their volume by stimulating Na^+/H^+ exchange. Osmotic challenge activates the Na^+/H^+ exchanger by increasing its sensitivity to intracellular H^+ but in contrast, the induced alkalinization is not associated with a change in phosphorylation status or a change in cytoplasmic Ca^{2+} levels [Grinstein et al. 1992b]. The cytosolic alkalosis normally elicited by hypertonic media was found to be absent in Na+/H+ exchange-deficient fibroblasts. Responsiveness to osmotic challenge was restored by stable transfection of these cells with the cDNA encoding NHE-1. In these transfectants, phosphorylation of the antiporter was also unaffected during osmotic activation [Grinstein et al. 1992b]. While direct phosphorylation does not occur, the ATP-dependence of shrinkage-induced activation suggests involvement of a phosphorylation dependent step involving a kinase [Grinstein et al. 1985]. The signalling pathways that link changes in cell volume with activation of Na^+/H^+ exchange are still unknown. Current evidence suggests that various kinases, including

myosin light chain kinase and one or more stress-activated kinases may be involved. Takeda et al. [1993] presented data showing increased phosphorylation of myosin light chain in glomerular mesangial cells during cell shrinkage. Moreover, ML-7, an inhibitor of MLCK, was found to inhibit shrinkage-induced activation of Na^+/H^+ exchange [Shrode et al. 1995]. Thus, the ATP-dependence of shrinkage-induced activation of the exchanger could be explained by the ATP requirement for MLC phosphorylation. Itoh et al. [1994] suggest that MAPK or MAPK like kinases are activated upon hyperosmotic stress. Further studies are necessary to fully characterize and understand the signalling cascades that lead to stimulation of the exchanger during volume regulation.

1.3.3.4 Role of Metabolic Acidosis

Reusch et al [1993] measured both lymphocyte and platelet Na^+/H^+ exchange activity in patients with decreased renal function and mild metabolic acidosis and in normal control subjects. Metabolic acidosis influenced Na^+/H^+ exchange only in lymphocytes, but not in platelets, it is possible that protein synthesis may be involved in increasing Na^+/H^+ exchange. Experimental metabolic acidosis caused by ingestion of ammonium chloride resulted in a 1.5-fold increase in NHE-1 mRNA in human lymphocytes. These data suggest that the increased Na^+/H^+ exchange activity in metabolic acidosis may be caused by de novo synthesis of antiport protein [Quednau et al. 1994]. It appears that chronic acidosis activates the exchanger through transcriptional control of its expression.

1.3.3.5 Role of NaCl

Extreme changes in NaCl intake have been shown to increase Na^+/H^+ exchanger activity in lymphocytes. Changes in NaCl intake from 20 to 300 mmol/day increased Na^+/H^+ exchanger activity in lymphocytes from normotensive volunteers by approximately 35% however, the mechanisms mediating this increase remain obscure [Gobel et al. 1994]. Resnick et al. [1994] have examined the effect of dietary salt loading and found that salt-sensitive subjects responded with a significant increase in $[Ca^{2+}]_i$ and a decrease in pH_i after a dietary NaCl load. There are two hypotheses concerning mechanisms by which a NaCl load could modify the Na^+/H^+ exchanger. Given the Ca^{2+} dependency of the exchanger, a NaCl induced rise in $[Ca^{2+}]_i$ could directly activate the exchanger. Alternatively, the NaCl induced fall in pH_i could regulate the exchanger in a manner

similar to that observed in metabolic acidosis. Enhanced Na^+/H^+ exchanger activity could be a genetic factor that contributes to salt sensitivity. Transgenic mice that overexpress Na^+/H^+ exchanger protein display a marked increase in blood pressure on NaCl loading [Kuro et al. 1995].

1.3.4 Na^+/H^+ Exchanger and Hypertension

In hypertension, a number of membrane transport abnormalities have been described. An enhancement of Na^+/H^+ exchange in a variety of cells is present in both human essential hypertension and a genetic rat model of hypertension. [Rosskopf et al. 1993; Kelly et al. 1997]. Studies have shown that this enhanced activity persists in immortalized lymphocytes from these patients after prolonged culture, excluding the effect of the environment and implicating genetic control [Rosskopf et al. 1993]. However, evidence strongly argues against a mutation in the encoding gene or over expression of NHE-1 protein. The enhanced exchanger activity in cell lines from hypertensive patients was not accompanied by a corresponding increase in steady-state NHE-1 mRNA transcript levels, which argues against overexpression of antiporter protein in hypertension [Rosskopf et al. 1993]. Siczkowski et al. [1994a] demonstrated that increased Na^+/H^+ exchanger activity in spontaneously hypertensive rat (SHR) cells *in vitro* and *in vivo* is not due to an increased amount of NHE-1 protein in SHR cultured vascular and striated muscle cells using an antibody raised against NHE-1. The complete cDNAs encoding for NHE-1 from immortalized hypertensive and normotensive cell lines with low and enhanced NHE activity have been sequenced by Rosskopf et al. [1993b] and no sequence changes were identified. Linkage analysis has been performed and no mutation in the NHE gene was uncovered in primary hypertension [Lifton et al. 1991]. The enhancement of Na^+/H^+ exchange is associated with increased phosphorylation and an agonist induced rise in cytosolic free Ca^{2+} concentration that are possibly related to up regulation of signalling pathways upstream of MAPK and activation of pertussis toxin (PTX)-sensitive G proteins [Ng et al. 1995; Siffert and Dusing 1996; Sweeney et al. 1997]. Pre-treatment of normotensive and hypertensive cell lines with PTX, which functionally eliminates signalling via G proteins, rendered signal transduction in the two cells identical [Siffert et al. 1995]. Signal transduction via PTX-sensitive G proteins is therefore enhanced in hypertensive phenotype. This genetically fixed overactivity of PTX-sensitive G proteins in hypertension may account for responses such as Ca^{2+} mobilization and MAPK

pathways that precipitate in cell proliferation and hypertrophy [Bogoyevitch et al. 1995; Inglese et al. 1995]. Thus enhanced G protein activation in hypertension would be compatible with enhanced vasoconstriction and hypertrophy frequently observed in patients with essential hypertension.

The kidney plays an essential role in the regulation of blood volume and pressure and within the kidney the proximal tubule reabsorbs the bulk of the filtered Na^+. Increases in NHE-1 activity in the proximal convoluted tubule do not lead to an alteration in Na^+ reabsorption due to the basolateral localisation. However; there may be indirect effects on $NaHCO_3$ reabsorption modulated by altered NHE-1 activity [Good et al. 1995]. The apical location of the NHE-3 in proximal tubules suggests an important role of this isoform in the control of salt reabsorption. NHE-3 exerts a major influence on overall fluid and electrolyte balance and a slight increase in apical membrane NHE-3 activity would increase Na^+ reabsorption considerably, thus increasing fluid reabsorption and blood volume contributing to the initiation of hypertension [Orosz and Hopfer 1996]. The importance of NHE-3 in hypertension has recently been demonstrated in mice using genetic deletion studies. Deletion of NHE-3 in mice reduced the mean arterial blood pressure [Schulthesis et al. 1998]. Studies on proximal convoluted tubule cells from spontaneously hypertensive rats (SHR) have demonstrated increased activity of both NHE-1 and 3 [Kelly et al. 1997]. Whilst enhanced NHE-1 activity is neither due to increased turnover or expression, NHE-3 protein expression is increased [Kelly et al. 1997]. Increased NHE-3 activity would blunt pressure natriuresis, resulting in the set point for maintenance of normal extracellular fluid volume being achieved at a higher renal perfusion which is important in the pathogenesis of hypertension [Guyton et al. 1984].

1.4 Hypotheses

The objective of this study was to examine two membrane functions of white blood cells relevant to pre-eclampsia and the contribution of these to the manifestation of the disease.

This thesis addresses the hypothesis that leucocytes isolated from pre-eclamptic and post-partum pre-eclamptic women may exhibit increased Na^+/H^+ exchanger activity that may contribute to the altered intracellular Na^+ in pre-eclamptic leucocytes that persists into the post-partum period. This altered activity may be implicated in the pathophysiology of pre-eclampsia and therefore serve as an intermediate phenotype in the disease. In contrast, genetic influences contribute to Na^+/H^+ exchanger activity in essential hypertension. We wished to test the hypothesis that differences in Na^+/H^+ exchanger activity in pre-eclampsia are genetically determined by investigating the persistence of elevated activity in pre-eclamptic and post-partum pre-eclamptic immortalized cells compared to their respective controls. This would provide evidence of the genetic influences on this intermediate phenotype in pre-eclampsia.

Another hypothesis that was tested was that neutrophils isolated from pre-eclamptic and post-partum pre-eclamptic women may show enhanced NADPH oxidase mediated ROS production upon agonist stimulation. This could represent an increased sensitivity characterized by the enhanced response of the NADPH oxidase enzyme and may contribute to the oxidative stress and endothelial dysfunction characteristic of the disease. In addition, we wished to examine the hypothesis that there is a genetic component to elevated NADPH oxidase mediated ROS production in pre-eclampsia by investigation of the persistence of raised activity in EBV-immortalized lymphoblasts from both pre-eclamptic and post-partum pre-eclamptic patients since these cells can be removed from the influences of plasma factors *in vivo*.

This thesis also tests the hypothesis that intracellular pH may have a modulating effect on NADPH oxidase activity and the subsequent production of ROS, thus affecting some of the measurements of ROS production in freshly isolated leucocytes from patients.

Finally, the hypothesis that differences or up-regulation in the signal transduction pathways at a point between agonist stimulation and the production of ROS may contribute to the increased sensitivity exhibited was examined.

1.5 Aims of Thesis

The primary aim of this thesis was to examine two membrane functions of white blood cells relevant to pre-eclampsia and to determine genetic and environmental influences on the systems. These membrane functions are Na^+/H^+ exchanger and NADPH oxidase enzyme in particular this study aimed to:

1. Assess the status of the Na^+/H^+ exchanger isoform 1 activity, protein abundance and phosphorylation status in representative nucleated cells from pre-eclamptic and post-partum pre-eclamptic women compared to their respective controls and to evaluate the possible contribution to the pathogenesis of pre-eclampsia.

2. Furthermore, this study aimed to provide further evidence of increased neutrophil ROS production in pre-eclampsia. The study examined neutrophil NADPH oxidase mediated ROS production in pre-eclamptic and post-partum pre-eclamptic women compared to their respective controls matched for age. Baseline and agonist induced NADPH oxidase mediated ROS production was measured using a chemiluminescent technique. The abundance of the different components of the NADPH oxidase enzyme was also examined using Western blotting.

3. The aim was then to conduct measurements in the absence of the influence of plasma (environmental) factors *in vivo* using a model in which the phenotypic changes persist despite transformation and culture *in vitro*. Study using EBV-immortalized lymphoblasts from both 3rd trimester pre-eclamptic and post-partum pre-eclamptic women and their respective control groups would allow identification of genetic influences on the systems studied in this thesis.

4. This study also aimed to assess the presence of inhibitors of the Na^+/K^+ ATPase enzyme in pre-eclampsia and their influence on intracellular pH.

5. Using the present knowledge of signal transduction pathways implicated in activation of NADPH oxidase, we wished to elucidate differences or upregulation in any of the signalling pathways in lymphoblasts with regards to the intermediate phenotype of increased sensitivity to agonists observed in pre-eclampsia.

Chapter 2

Materials and Methods

2.1 Materials

New Zealand White rabbits were supplied by University of Leicester, Biomedical Services Unit.

Sigma-Aldrich Company, Fancy Road, Poole, Dorset, UK.

Bovine serum albumin Fraction V, glutamine, (N-[2-Hydroxyethy]piperazine-N'-[ethanesulfonic acid]) (HEPES), TC199, RPMI, n-(2-Hydroxyethyl)piperazine-N'-(3-propanesulfonic acid) (EPPS), fluorescamine, horse-radish peroxidase conjugate of a goat anti-IgM, polyoxyethylene-9-lauryl ether (C_{12} E_9), phenylmethylsulphonyl fluoride, o-phenanthroline, iodoacetamide, ethylenediaminetetraacetic acid (EDTA), Na fluoride, sodium orthovanadate, sodium pyrophosphate, pepstatin A, leupeptin, sodium molybdate, penicillin, streptomycin, dithiothreitol (DTT), sodium dodecyl sulphate (SDS), bromophenol blue, β-mercaptoethanol, Percoll, Freund's complete adjuvant, monensin, nigericin, N,N,N'N'-Tetramethylethylenediamine (TEMED), ammonium persulphate (APS), bromophenol blue, dextran, superoxide dismutase (SOD), 4,5-dihydroxy-1,3-benzene-disulfonic acid (TIRON), catalase, coloured density marker beads, luminol (5-amino-2,3-dihydro 1,4-phthalazinedione), lucigenin (10,10' dimethyl-9,9'-biacridnium dinitrate, DBA), phorbol-12-myristate-13-acetate (PMA), n-formyl-met-leu-phe (fMLP), Sepharose Q and Sepharose CM columns, ampicillin, histopaque 1077, DH5α bacteria, wortmannin, PD98059, staurosporine, genestein, tyrphostin, adenosine 5'- triphosphatase from porcine cerebral cortex, adenosin 5'-triphosphate, ouabain.

Amersham International

Horseradish peroxidase linked donkey anti rabbit, ECL reagent, Hybond-C supported nitrocellulose membrane

Smith, Klein Beecham

SB203580

Roche Ltd, Welwyn Garden City

RO 318220,

Cambridge Bioscience

bis(carboxyethyl)carboxyfluorescein acetoxymethyl ester (BCECF-AM)

Pharmacia Biotechnologies Ltd.

Protein-A Sepharose CL4B beads,

Life Technologies, Paisley, UK

Trypsin-EDTA, penicillin/streptomycin,

Peninsular Labs Inc San Carlos

C_{18} Sep Pak cartridges

Aventis Pharma

3-methylsulphonyl-4-piperidinobenzoyl (HOE-694)

Melford Laboratories

Isopropyl-β-D-thiogalactopyranoside (IPTG)

Globepharm Ltd, University of Surrey, Guilford, Surrey, UK.

Foetal calf serum

Cayman Chemical Ann Arbor, MI

Methy-arachidonylflurophosphonate (MAFP)

Santa Cruz Biotechnology, Santa Cruz, CA

Rac2 specific antibody

Fisher chemicals /Fisher Scientific, Leicestershire, UK, all other chemicals

Sodium chloride, potassium chloride, potassium dihydrogen orthophosphate, sodium dihydrogen orthophosphate, glucose, calcium chloride, N-methyl D-glucamine (NMG),

magnesium sulphate, ammonium chloride, sodium bicarbonate, sucrose, sodium hydrogen carbonate, copper sulphate, ferrous sulphate, magnesium chloride, zinc sulphate, calcium sulphate, ferrous nitrate, glycine, glycerol, methanol, stannous chloride, trifluoroacetic acid, hydrazine sulphate, sulphuric acid and ammonium molybdate.

2.2 Blood Pressure Assessment

Blood pressure was measured at the time of blood sampling by the phlebotomist. Systolic and diastolic (fifth Korotkoff sound) blood pressures were obtained by averaging two blood pressure measurements taken with a standard cuff 5 minutes apart.

2.3 Cell Separation

2.3.1 Leucocytes

Twenty millilitres of blood was collected from the forearm of patients into syringes containing 1 mM sterile sodium citrate as the anti-coagulant. The patients had consented to this prior to the study. Leucocytes used for this study were isolated by dextran sedimentation according to the method of Baron et al. [1969] and the contaminating erythrocytes were lysed by hypo-osmotic shock. Dextran solution was prepared at 300 mg in 20 ml of TC199 and placed into sterilin tubes. The blood was added, gently mixed and clamped at 45° on a stand. Sedimentation of the erythrocytes took 30 minutes. Plasma and leucocytes were removed and centrifuged for 10 minutes at 482 g. The cell pellet was collected and vortexed with the addition of 9 ml of distilled water for 10 seconds to produce the hypo-osmotic shock necessary to lyse contaminating red cells. Osmolarity was restored by adding 1 ml of 10 x concentrated TC199 solution. Cells were then washed twice in TC199 and used accordingly.

2.3.2 Neutrophils

Forty millilitres of blood was collected from the forearm of patients in syringes containing 1 mM sterile sodium citrate as the anti-coagulant. The blood was subjected to dextran sedimentation as described in the methodology for the isolation of leucocytes. Neutrophils were separated from the other cells using a self-generated continuous density gradient of a colloidal silica solution, Percoll. Five millilitres of iso-osmotic Percoll stock solution was diluted with the addition of 0.8 ml 10 x TC199, 1 M HEPES, 5 M NaCl and 1.84 ml distilled water. The self-generated continuous gradient was obtained by centrifugation of 8 ml of a 30 % Percoll solution at 21982 g for 15

minutes at 4 °C. Centrifugation without the application of brakes was performed to avoid disturbing the gradient during deceleration. The density gradient was checked by the use of coloured density marker beads. Once the red cells had been lysed the cells were re suspended in 1ml of sterile TC199 and applied to the density gradient. The gradient was then centrifuged at 482 g for 15 minutes at 28 °C to separate the neutrophils from the other white cells in the pellet. The neutrophil band formed 2/3rds of the way down the tube and was carefully aspirated into a sterilin. The neutrophils were then washed several times in TC199 to remove all traces of Percoll and viability of the isolated cells was consistently more than 95 %, as demonstrated by the dye exclusion test with trypan blue. Neutrophils were then used according to each experimental protocol.

2.4 Measurement of Intracellular pH

Fluorescent probes such as BCECF-AM are commonly used to assess intracellular pH. This dye is pH sensitive and can be incorporated into the intact cell without causing damage to the membrane [Tsien 1989]. The fluorescence produced is proportional to the dye protonation therefore the greater the pH the higher the fluorescence intensity. The inside of the cell is loaded with the hydrolysable ester form of the BCECF, which permeates the cell membrane and is cleaved by esterases with the subsequent release of the impermeant form [Roos and Boron 1981].

2.4.1 BCECF Loading

Isolated leucocytes were loaded with 5 μM of the pH sensitive fluorescent dye, BCECF-AM in TC199 for 30 minutes at 37 °C. The cells were then washed twice in TC199 and then left for 15 minutes to allow for complete de-esterification of the fluorophore. Determination of pH immediately after dye loading gave lower values due to the continuing de-esterification of BCECF-AM

2.4.2 Intracellular pH

A portion of dyed cells was then used to measure the intracellular resting pH according to the method of Ng et al [1989b]. The cells were incubated at 37 °C in a buffer consisting of NaCl 140 mmol/l, KCl 5 mmol/l, $MgSO_4$ 0.8 mmol/l, $CaCl_2$ 1.8 mmol/l, glucose 5 mmol/l and HEPES 15 mmol/l pH 7.4 for 10 minutes. The intracellular resting pH was obtained by measuring the fluorescence of the trapped intracellular dye.

The measurements were performed in a 37 °C thermostatically controlled sample compartment holder within a dual grating fluorometer (Deltascan: Photon Technology International, South Brunswick, New Jersey, USA) with dual wavelength excitation at 500 and 439 nm (slit width 2.5 nm) and the emission wavelength set at 530 nm (slit width 10 nm). The fluorescence ratio was obtained by dividing the emission signal when excited at 500 nm with that at 439 nm and calibrated by cells suspended in buffers at pH values varying from 6 to 8. Calibration employed a double ionophore technique (nigericin and monensin) [Ng et al. 1989b]. To construct the calibration curve, cells were suspended in isotonic KCl buffer containing nigericin and monensin with the pH manipulated accordingly. The plot against fluorescence ratio gave a curve fitted by a non-linear least square technique. The relationship between the fluorescence ratio F and intracellular pH is sigmoidal and is described by the equation:

$$F = A+B(10^{pH-pK}) / (1+10^{pH-pK})$$

Where F is the fluorescence ratio, A and B are constants extrapolated from the curve and pK is the pK of the intracellular dye (~7.0) [Ng et al. 1990]. The values of A, B and pK were obtained using a curve fitting program. This technique measures intracellular pH independent of the dye concentration, cell density and photobleaching [Grinstein at al. 1986]

2.5 Na^+/H^+ Exchanger

Assessment of Na^+/H^+ exchanger activity employs the use of the fluorescent dye BCECF-AM to measure changes in intracellular pH, which represent the Na^+ dependent H^+ efflux from the cell. Amiloride, a potent inhibitor of the exchanger, can be used to determine the proportion of fluxes attributable to the Na^+/H^+ exchanger [Frelin et al. 1987]. All solutions were bicarbonate free to allow assessment of the exchanger without contributions from other pH regulatory mechanisms [Simchowitz and Roos 1985].

2.5.1 Intracellular pH Clamping

A portion of the dyed cells was used to measure NHE-1 activity. The cells were incubated for 5 minutes in a buffer identical to that used for measuring the resting intracellular pH but with the addition of the ionophores nigericin (2 μmol/l) and monensin (5 μmol/l) adjusted to a pH of 6.0. Nigericin is a K^+/H^+ ionophore that sets

internal pH to equal the same as the external pH with the absence of a K^+ gradient across the cell membrane [Thomas et al. 1979]. Monensin is a Na^+/H^+ ionophore similar to nigericin [Pressman 1976]. In the presence of both ionophores the intracellular pH becomes equal to the extracellular pH with virtually no intracellular Na^+ present. Any influx observed will be due to the exchanger alone. The cells were washed and incubated in a second buffer similar to the previous but with the addition of bovine serum albumin 1 g/L and removal of the ionophores. The albumin binds to the ionophores in the membrane and extracts them leaving the cells clamped at the desired pH of 6.0 and the maximum capacity of the exchanger can be assessed [Frighi et al. 1991].

2.5.2 Measurement of Na^+/H^+ Exchange Activity

The exchanger operates near its maximal transport capacity when its pH is near 6.0 [Ng and Bomford 1989]. The exchanger activity was therefore measured with the intracellular pH clamped at 6.0 as above. One hundred microlitres of the clamped cells were pipetted into a cuvette. The exchanger was then activated by the addition of 2.4 mls of NaCl buffer (133 mmol/l final concentration, pH 7.4). The resulting change in intracellular pH (alkalinisation) was recorded. This process was repeated 3 times to ensure reproducibility of the measurements.

In order to calculate Na^+/H^+ exchanger activity it was necessary to determine Na^+ independent H^+ efflux. To determine this a portion of the clamped cells were subjected to the addition of N-methyl-D-glucamine chloride (NMG) (133 mmol/L final concentration, pH 7.4) NMG is an impermeant ionic substitute for Na^+ allowing the determination of the efflux of H^+ that is not due to the activity of the Na^+/H^+ exchanger [Reuss and Finn 1975]. The rate of change of intracellular pH was recorded as previously described and then subtracted from the rate of change obtained by the addition of Na^+ to calculate the initial rate of change in intracellular pH due to the Na^+/H^+ exchanger. Exchanger activity was finally calculated as the product of the initial rate of change in intracellular pH upon the addition of Na^+ (pH units/min) multiplied by the buffering capacity of the cells (mmoles/l.pH unit). Therefore the units of Na^+/H^+ exchanger activity are mmoles/ l. min [Ng and Dudley 1989]. Coefficients of variation for the same individual on different days were: 11.8% for intracellular pH and 7.3% for Na^+/H^+ exchanger activity.

2.5.3 Buffering Capacity

Intracellular buffering capacity varies with pH and measurement was therefore necessary to calculate Na^+/H^+ exchanger activity [Ng and Dudley 1989]. The buffering capacity was measured when pH was 6.0 by recording the pH change induced by the addition of extracellular NH_4Cl (50 mM final concentration) [Ng and Dudley 1989]. If the pKa of NH_3 inside and outside the cell is assumed to be the same, and only NH_3 crosses the cell membrane rapidly and causes a step change in internal pH, then it is possible to determine pH values by the following calculation derived from the Henderson-Hasselbalch equation:

Buffering Capacity= (change in$[NH^+_4]_{in}$/(change in internal pH)

And the change in $[NH^+_4]_{in}$ is defined as follows:

Total base concentration x $10^{pHo - pHa}/(1 + 10^{pHo - pHa})$

Where pH_o and pH_a are the pH of the buffer and the internal cellular pH, respectively after adding the NH_4Cl. The units are mmoles/l.pH unit.

This measurement was performed in triplicate to again assure reproducibility.

2.6 Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis (PAGE) Analysis of Proteins

Electrophoresis is the migration of charged molecules in solution in response to an electric field. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone - and SDS binds to proteins in a mass ratio of 1.4:1. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with dithiothreitol (DTT). In denaturing SDS-PAGE protein separations, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight. Determination of molecular weight is performed by SDS-PAGE of proteins of known molecular weight along with the proteins to be characterised. A linear relationship exists between the logarithm of the molecular weight of an SDS-denatured polypeptide and its *Rf*. The *Rf* is calculated as the ratio of the distance migrated by the molecule to that migrated by a marker dye-front. Generally the sample is run in a support matrix such as polyacrylamide gel. In a discontinuous system, a stacking gel is layered on top of a resolving gel. The large pore size of the stacking gel allows the samples to become focussed at the interface of the resolving gel. The higher

pH of the running gel allows the samples to move through the gel matrix according to their molecular weight. The proteins are then electroeluted on to supported nitrocellulose paper before detection with the specific antibody of choice.

2.6.1 Preparation of Samples for SDS-PAGE

Leucocytes or neutrophils were washed several times in ice cold phosphate buffered saline (PBS) and then re suspended in extraction buffer consisting of EPPS 20 mM, pH 7.4, NaCl 140 mM, Na_2EDTA 5 mM, polyoxyethylene-8-lauryl ether 1 %, sodium pyrophosphate 10 mM, sodium fluoride 10 mM, sodium molybdate 1 mM, sodium orthovanadate 5 mM with the addition of the 5 protease inhibitors phenylmethylsulfonyl fluoride 1 mM, leupeptin 2 μg/ml, iodoacetamide 1 mM, phenanthroline 1 mM and pepstatin 1 μg/ml less than 10 minutes before use of the buffer. The cells were snap frozen in liquid nitrogen and thawed rapidly to disrupt the membranes and then sonicated for 15 minutes on ice [Deutscher 1990]. After sonication the cells were vortexed and centrifuged and a portion of the supernatant was removed for protein determination.

2.6.2 Protein Determination

Protein concentrations were determined from 5 μl of each extract using a fluorescamine assay. Two millilitres of 50 mmol L^{-1} Na_2HPO_4. pH 8.0 was placed into 10 ml tubes and varying amounts of bovine serum albumin standard protein added to construct a protein curve. The samples were then placed in tubes containing the Na_2HPO_4 and vortexed. 100 μl of 25 mg/ml fluorescamine, dissolved in acetone was added to all of the tubes. The standards and samples were incubated in the dark for 10 minutes to allow interaction of fluorescamine and primary amines to form a fluorescent moiety. The fluorescence was then determined using a fluorometer (Deltascan: Photon Technology International, South Brunswick, New Jersey, USA) with excitation at 398 nm and the emission wavelength set at 420 nm. A standard curve was constructed and the unknown proteins determined.

2.6.3 SDS-PAGE Analysis of Cell Extracts

An equal volume of gel sample buffer (Tris 0.125 mM, pH 6.8, glycerol 20 %, SDS 5 % and bromophenol blue 0.004 %) was added to the supernatant followed by 100 mM

dithiothreitol (DTT) with subsequent boiling for 4 minutes to allow binding of SDS. Total protein extract was then resolved on SDS- polyacrylamide gels (between 7.5 and 15 % depending on the size of the protein to be determined) at 100 V for 1 hour in running buffer. Molecular weight markers were also subjected to SDS-PAGE to allow identification of proteins of interest.

2.6.4 Transfer to Nitrocellulose

After electrophoresis the gel was soaked for at least 10 minutes in chilled transfer buffer (Tris 25 mM, glycine 192 mM and methanol 20 %). Proteins were then electroblotted to supported nitrocellulose. The gels were placed onto nitrocellulose paper between 4 sheets of filter paper. Separated proteins were transferred at 100 V for 1 hour at 22 °C in transfer buffer. Unbound nitrocellulose was then blocked in 5 % low fat milk powder in PBS/0.01 % Tween -20 overnight.

2.6.5 Detection of Proteins

After washing the nitrocellulose, proteins were detected by incubating the blot with 1 μg/ml of the specific antibody in 5 % Marvel in PBS-0.1% Tween-20 for 1 hour. After several washes with PBS-0.1 % Tween-20, the second antibody (1:1500 horseradish peroxidase-linked donkey anti-rabbit IgG for all the experiments in this thesis) was added to the blot in PBS-0.1 % Tween-20 for 1 hour incubation. After several washes the bands of interest were then visualised using enhanced chemiluminescence methodology (ECL) [Ng et al. 1994]. Scanning of the density of the band of interest using a Biorad densitometer enabled determination of protein abundance to be estimated.

2.7 Specificity of G252 Antibody

The G252 antibody specific for NHE-1 was developed in our laboratory [Siczkowski et al. 1995]. The specificity was assessed and is illustrated in figure 2.1. Lymphoblast cell extracts were subjected to SDS-PAGE and then probed with G252 antibody that reacted with a 97 kDa protein. A corresponding blot was probed with G252 antibody that had been pre-incubated with a 2 fold excess of CB28 fusion protein. Staining of the 97 kDa band was diminished.

2.8 Preparation and Characterisation of Phosphoserine Antibody (G174)

The phosphoserine specific antibody G174 raised in rabbits was developed in laboratory according to the method of Heffetz et al [1991]. Phosphoserine was conjugated to dithiothreitol reduced keyhole limpet haemocyanin using bromoacetic acid N-hydrosuccinimide ester as described previously for the production of phosphothreonine specific antibodies by Heffetz et al [1991].

Specificity of the antibody was assessed by subjecting 1 μg, 200 ng and 40 ng of phosphoserine, phosphothreonine and phosphotyrosine conjugated to BSA with bromoacetic acid N-hydroxysuccinimide ester to SDS-PAGE. The blot was then probed with phosphoserine specific antibody (G174) and the cross reactivity assessed. The Western blot was stripped and re-probed using G174 phosphoserine antibody that was pre-incubated with 5 μg/ml of the BSA-phosphoserine conjugate. This would allow the demonstration that the immunoreactivity of the phosphoserine antibody would diminish by co- incubation with the antigen. The specificity of the antibody was checked using phosphoserine, phosphothreonine and phosphotyrosine conjugated to BSA with the bromoacetic acid N-hydroxysuccinimide ester as illustrated in figure 2.2A. Some cross reactivity of phosphoserine G174 to phosphothreonine was observed at 1 μg and 200 ng but no cross reactivity was observed against phosphotryosine. The Western blot from figure 2.2A was stripped and re-probed using G174 phosphoserine antibody that was pre-incubated with the BSA-phosphoserine conjugate. The blot illustrated in figure 2.2B demonstrated that the immunoreactivity of the phosphoserine antibody was diminished by co- incubation with the antigen.

2.9 Determination of NHE-1 Phosphorylation

The degree of phosphorylation of NHE-1 was determined by a technique for the detection of phosphoserine mass on proteins. The cells were thawed rapidly to disrupt the membranes and then sonicated for 15 minutes on ice [Deutscher 1990]. After sonication the sample was centrifuged at 21982 g for 10 minutes. The supernatant was recovered and pre-absorbed with 10 μl of protein A Sepharose CL4B beads for half an hour. NHE-1 G252 antibody produced in our laboratory was then added to the supernatant to a final concentration of 20 μg/ml, and the samples were rotated end over for 16 hours at 4 °C. Immunoprecipitates of NHE-1 were recovered using 10 μl of protein A Sepharose CL4B beads. After rotation for 1 hour the beads; with the attached

NHE-1 immunoprecipitates were washed 5 times in extraction buffer containing 1 μg/ml of BSA. One hundred microlitres of gel sample buffer was then added to the beads along with DTT and then the samples were boiled for 4 minutes and then subjected to SDS-PAGE on a 7.5 % gel.

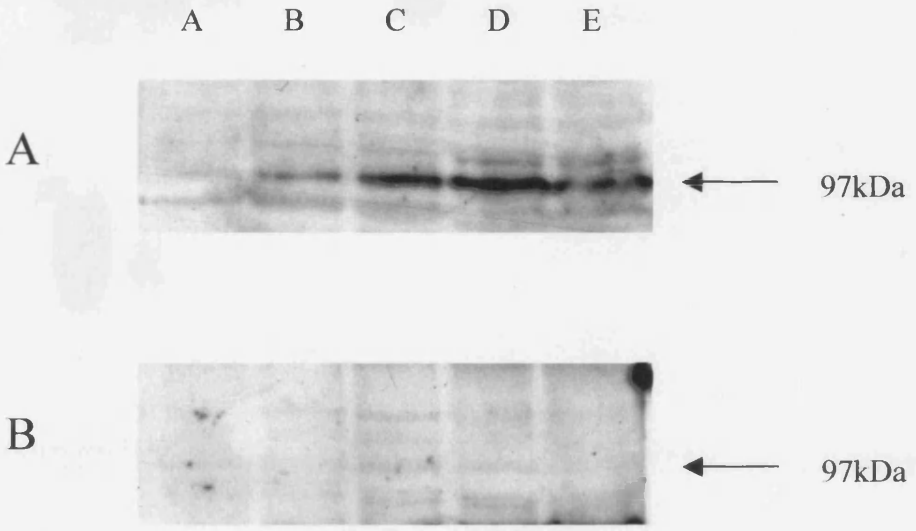


Figure 2.1 Specificity of G252 antibody

A. Western blot shows the specific response of NHE-1 anti-sera G252, to lymphoblast cell extracts (A= 3.125, B= 6.25, C= 12.50, D= 25 and E= 50 μg per track). A protein, corresponding to 97 kDa can be seen clearly.

B. Corresponding blot probed with G252 antibody that had been pre-incubated with a 2 fold excess of CB28 fusion protein. Staining of the 97 kDa band was abolished.

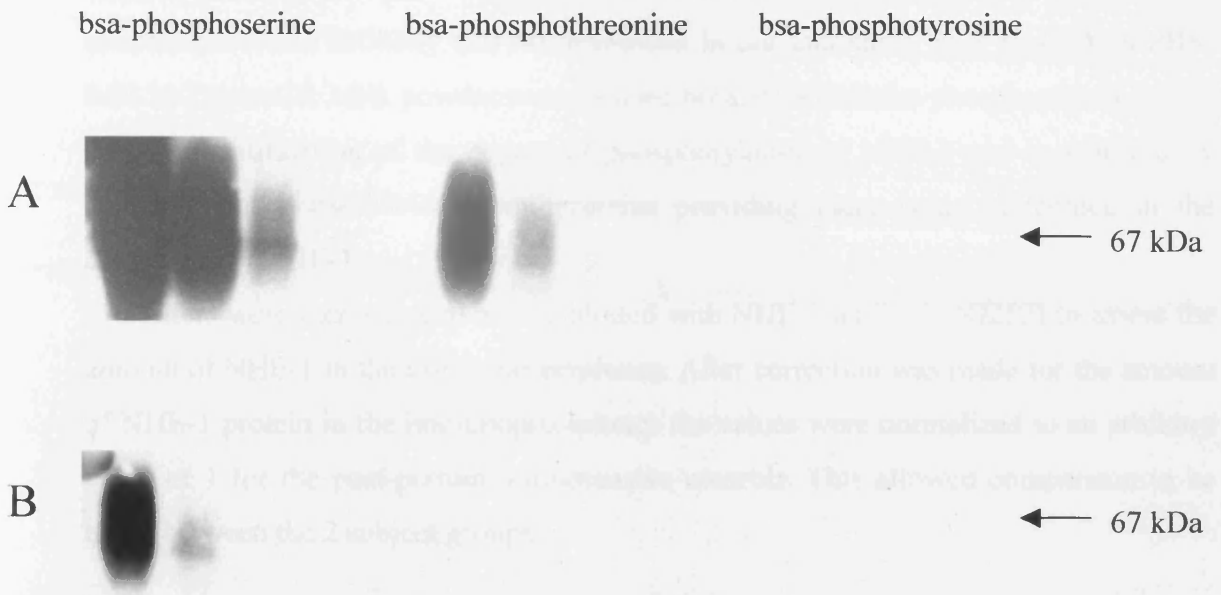


Figure 2.2 Specificity of G174 phosphoserine antibody

A. Western blot showing the specificity of antibody G174 to the phosphoserine-bovine serum albumin antigen at 1 μg, 200 ng and 40 ng of protein. Some cross reactivity of phosphoserine G174 to phosphothreonine was observed at 1 μg and 200 ng but no cross reactivity was observed against phosphotryosine.

B. The Western blot was stripped and re-probed using G174 phosphoserine antibody that was pre-incubated with the BSA-phosphoserine conjugate. The blot demonstrated that the immunoreactivity of the phosphoserine antibody was diminished by co-incubation with the antigen.

The proteins in the gel were then electroblotted onto supported nitrocellulose and blocked overnight in PBS-BSA-0.01 % Tween-20. The filters were probed with antiphosphoserine antibody (G174) developed in our laboratory in 3 % BSA in PBS-0.01 % Tween-20. Milk powder was not used because it contains phosphoproteins. This enables identification of the degree of phosphorylation of NHE-1 and is achieved by detecting phosphoserine mass on proteins providing there is no difference in the abundance of NHE-1.

The filters were then stripped and re-blotted with NHE-1 antibody (G252) to assess the amount of NHE-1 in the immunoprecipitates. After correction was made for the amount of NHE-1 protein in the immunoprecipitates the values were normalized to an arbitrary value of 1 for the post-partum normotensive controls. This allowed comparison to be made between the 2 subject groups.

2.10 Subcellular Fractionation

Cells were washed twice in cold PBS and then resuspended in 2 ml of sucrose buffer which consisted of sucrose 250 mmol/l, EDTA 1 mmol/l and HEPES 5 mmol/l, pH 7.2 with the addition of phenylmethylsulfonyl fluoride 1 mmol/l, leupeptin 2 μg/ml, iodoacetamide 1 mmol/l, phenanthroline 1 mmol/l and pepstatin 1 μg/ml less than 10 minutes before use of the buffer. The cells were then subjected to nitrogen cavitation at 1500 psi for 15 minutes at 4 °C. The lysate was collected and the cavitator washed out with sucrose buffer. The lysate was then spun at 482 g for 10 minutes to remove the nuclei. One hundred microlitres was kept back as the post-nuclear supernatant (PNS) and the remainder subjected to a Percoll gradient to separate the membranes from the cytosol. The lysate was carefully layered on top of the Percoll gradient consisting of 35 % Percoll, sucrose 250 mmol/l, EDTA 1 mmol/l and HEPES 5 mmol/l, pH 7.2. Density marker beads were layered onto an identical gradient to allow identification of the subcellular fractions. The gradients were then spun at 48246 g for 1 hour. The membranes were located at a density of 1.042, at the top of the blue marker bead. The cytosol was located above the Percoll gradient.

2.11 Neutrophil Chemiluminescence.

Reactive oxygen species are generated following activation of the respiratory burst with specific stimuli and the accompanying chemiluminescence (CL) is an energy product of

this activity and represents the photon emission as the excited molecules relax [McNally and Bell 1996]. The application of chemiluminescence is used to measure ROS generation. The quantum yield of these ROS is poor so this CL is amplified by the addition of luminescent substrates such as luminol and lucigenin resulting in a sensitive tool for examining the oxidative reactivity of a variety of different cells. Certain inhibitors such as superoxide dismutase and catalase can be employed and suggest that luminol-derived CL results primarily but not exclusively from the action of H_2O_2. In contrast lucigenin-derived CL is not related to H_2O_2 activity and is inhibited by superoxide dismutase suggesting involvement of $O_2^{-\cdot}$ [Allen 1986]. The luminol-derived chemiluminescence (CL) technique was introduced by Allen and Loose in 1976 and measures both the extracellular release and the intracellular production of oxygen metabolites. The exact chemical species inducing the luminol chemiluminescence have not to date been definitely identified. Lucigenin is a widely used chemiluminescent detector of superoxide anion radical production but care must be taken to use the correct concentration as lucigenin can stimulate the actual production of superoxide at high concentrations. Thus, lucigenin CL still appears to be a valid probe for detecting superoxide by cellular and enzymatic sources when a concentration of less than 50μM was used [Yunbo et al. 1998].

2.11.1 Preparation of the Luminescence Medium

The medium was prepared using NaCl 140 mmol/l, KCl 5 mmol/l, $MgSO_4$ 0.8mmol/l, $CaCl_2$ 1.8 mmol/l, glucose 5 mmol/l and Hepes 15 mmol/l pH 7.4. The medium was then filtered and kept frozen until required.

2.11.2 Preparation of PMA and fMLP

PMA is a cocarcinogen extracted from croton oil that causes specific degranulation and activation of the respiratory burst via protein kinase C in polymorphonuclear leucocytes [Allen 1986]. A 2 mM stock solution was prepared in dimethyl sulphoxide. This stock solution was further diluted with luminescence medium to attain the desired concentration of PMA used for stimulation. fMLP is a synthetic formylpeptide widely used as a model chemoattractant and secretagogue in human neutrophils that binds to the receptor for N-formylated peptides [Brazil et al. 1998]. A 5 mM stock solution was prepared in dimethyl sulphoxide. This stock solution was further diluted with luminescence medium to attain the desired concentration.

2.11.3 Preparation of the Chemiluminogenic Probes

Two different chemiluminogenic probes were employed in the experiments presented in this thesis. Luminol (5-amino-2,3-dihydro 1,4-phthalazinedione) a cyclic hydrazide with a reported quantum yield of 0.01 was prepared as a 50 mM stock in 50:50 DMF and distilled water. The stock was kept frozen in the dark until required. Lucigenin (10,10' dimethyl-9, 9'-biacridnium dinitrate, DBA) is a water soluble acridinium salt with a quantum yield comparable to that of luminol. A 50 mM solution was prepared with water and kept frozen until required.

2.11.4 Determination of Cell Count

Cells were resuspended in 1 ml of TC199, 20 µl of this suspension was placed into 10ml of Isoton and counted using a Beckman ZM Coulter Counter. The current was set at 100 mA and the lower threshold at 5.3 so the cells being counted were determined as being 5.8 µM diameter, which represented the average diameter of a neutrophil. Lymphoblasts were counted using a lower threshold of 14.0 so that cells of 8.0 µM diameter were counted, this represented the average diameter of a cell. The cell counts were performed in triplicate and an average taken.

2.11.5 Lucigenin-Derived Chemiluminescence

400,000 neutrophils or EBV transformed lymphocytes were used to assess superoxide production via NADPH oxidase adapted from the method of Li et al [1998]. Cells were washed twice in luminescence medium containing 50 µM lucigenin final concentration. The cells were then resuspended in 500 µl of the luminescence solution and the baseline measured in a thermostatically controlled Berthold LCB953 tube luminometer set at 37 °C for approximately 10 minutes. The respiratory burst was then stimulated using either 1 µM PMA or fMLP. The response to the agonists was then recorded over a period of 30 minutes using an Autolumat LB953 luminometer, where the standard detector was a photon counter covering a spectral range from 390-620 nm. The background was reduced by cooling the photomultiplier to 8 °C. The cellular luminescence was measured as the light emission of a single sample over a time period. The peak response and area under the curve (AUC) were taken to represent superoxide production when the oxidase was stimulated with fMLP and PMA. Simpson's rule was used to find the area under a curve, also called the integral of the curve.

$h/3\ [f(x[0]) + 4f(x[1]) + 2f(x[2]) + 4f(x[3]) + ... + 2f(x[n-2]) + 4f(x[n-1]) + f(x[n])\ ]$

The values were expressed as relative light units (RLU) and relative light unit seconds (RLU.sec) respectively. Coefficients of variation for the same individual on different days were: 10.8% for fMLP and 12.2% for PMA.

2.11.6 Luminol-Derived Chemiluminescence

400,000 neutrophils were used to assess the H_2O_2 produced from the cells based on the method of Allen and Loose [1976]. Again, cells were washed in solution B containing 5 μM luminol and 6 units per ml HRP. The HRP acts as a catalyst and results in the oxidation of luminol and yields a luminol radical. The cells were then resuspended in 500 μl of the luminescence solution and the baseline measured for approximately 10 minutes. The respiratory burst was than stimulated using either 1 μM PMA or fMLP. The response to the agonists was then recorded over a period of 30 minutes and the peak response and area under the curve (AUC) were taken to represent hydrogen peroxide production when the oxidase was stimulated with fMLP and PMA. The area under the curve was calculated over 0-30 minutes using Simpson's method. The values were expressed as relative light units (RLU) and relative light unit seconds (RLU.sec) respectively. Coefficients of variation for the same individual on different days were: 11.9% for fMLP and 12.8% for PMA

2.12 Production of NADPH Oxidase Antibodies

NADPH oxidase is a multi-component enzyme that catalyses the production of superoxide in neutrophils and EBV transformed B cell lymphocytes amongst other cells [Morel et al. 1992]. The enzyme consists of 5 protein subunits that upon stimulation, translocate to the membrane to form the active oxidase enzyme [Leusen et al. 1996]. For this thesis it has been necessary to produce antibodies against the sub-unit proteins to allow complete assessment of oxidase activity in different subject groups. The antibodies were not available commercially when this work was performed.

Two methodologies were employed to complete production of the antibodies. P47-*phox* and p67-*phox* (cytosolic components of the oxidase) were produced using a baculovirus expression system using SF9 insect cells as the host. P22-*phox* was produced using a bacterial system in which p22 DNA was cleaved with a restriction enzyme and joined in

vitro to the multilinker site of GST plasmid. The resulting recombinant plasmid was then used to transform competent bacteria. Active antibodies were produced by immunising New Zealand White rabbits with 200μg of antigen and allowing an immune response to develop. Gp91-*phox* antibody was kindly donated by Dr D.Roos (University of Amsterdam).

2.12.1 Production of Cytosolic Components of Oxidase using Baculovirus Expression Vector System

Baculovirus expression vector systems are commonly used to express genes from many sources. The expression of the gene can be highly variable dependent on the cell, virus or medium components [Summers and Smith 1987]. The use of log phase SF9 cells (a clonal isolate of *spodoptera frugiperda* IPLB-sf21-AE cells) that are at least 97 % viable is of major importance. The are several groups of baculovirus, the virus used in this method is called nuclear polyhedrosis virus (NPV). These viruses are encased by large protein crystals called occlusion bodies (polyhedra) that protect the virus from proteases. These occlusions are an important part of the natural virus life cycle, providing the means for horizontal transmission of the virus [Summers and Smith 1987]. Once the occlusions are in the host they dissociate to release the infectious virus particles (virion) that penetrate the nucleus and uncoat. The viral DNA is transcribed and replicated and the new virions are formed which bud from the surface to infect other cells.

2.12.2 Growth of *spodoptera frugiperda* Cells (SF9)

SF9 cells are a commercially available insect line derived from the pupal ovarian tissue of a nocturnal moth known as the Spodoptera frugiperda [Defrancesco 1998]. The SF9 cells were obtained from the MRC Toxicology Unit at Leicester University. The cells were seeded into 175 cm^2 tissue culture flasks containing Grace's insect medium supplemented with 10 % foetal calf serum, 200 mM glutamine, 100 units of penicillin and 0.1mg of streptomycin. The cells adhered to the surface of the flask and were incubated at 27 °C for optimal growth. The cells were observed and fed every 2 days.

2.12.3 Insertion of the Virus

Viruses were supplied courtesy of Dr D. Lambeth, Department of Biochemistry, Emory University, Atlanta. Once the SF9 cells were 80 % confluent, 200 µl of the virus containing the p47 or p67 gene encoding sequence was added to the flask. The cells were harvested 96 hours after viral infection using a cell scraper to detach cells into the medium. Five hundred microlitres of the medium was kept back for SDS-PAGE analysis. The medium was then centrifuged at 21982 g at 4 °C for 10 minutes. The medium was poured off carefully to avoid loss of any of the pellet. The pellet was frozen at –80 °C until extraction was performed at a later stage.

2.12.4 Extraction

The cell pellets were defrosted and then resuspended in 10 ml of sterile lysis buffer containing PIPES 5mM, KCl 50mM, NaCl 3mM, $MgCl_2$ 2mM, EDTA 1mM, and protease inhibitors, pH 7.5 and left on ice for 20 minutes. The cell extract was then placed in a nitrogen cavitator and subjected to 1000 psi for 20 minutes to allow the cells to lyse. The nitrogen cavitator was then disassembled and the lysate was collected slowly into a sterilin tube. Five hundred microlitres of lysate was removed and used for SDS-PAGE analysis. The remainder of the lysate was centrifuged at 759 g for 10 minutes to remove cell membranes. The supernatant was collected and then centrifuged again at 48246 g for 1 hour to remove nuclei and mitochondria. The supernatant was then filtered through a 0.2 µm acrodisc to ensure that all particulate matter was removed. The purified sample was then adjusted to pH 7.4 using 6 M NaOH, 2 µl at a time.

2.12.5 Purification of p67 NADPH Oxidase Component

A Sepharose Q fast flow column was obtained which uses ionic charge to bind the protein to the column. This was an anion exchanger. The column was washed and equilibrated with filtered buffer A (low salt buffer) that consisted of Tris-HCl 50 mM, DTT 0.1 mM, pH 7.4. Two hundred microlitres of sample was loaded onto the column at a time (0.2 ml loop, flow rate 1 ml/minute, 10 seconds between injections). Once all 5 ml of the sample was loaded, the column was washed with 20-30 mls of buffer A until the baseline was near zero at 280 nm. The linear gradient program was then started which ran for 50 minutes at a flow rate of 1 ml/min starting with 100 % buffer A and

ending with 100 % buffer B (buffer A + 1M NaCl). Fractions were collected when peaks showed on the chromatogram and immediately placed on ice. The fractions were then resolved on a 12 % SDS-PAGE gel and subsequently stained with Coomassie Blue (glacial acetic acid 10 %, methanol 50 %, Brilliant Blue R 0.5 %) to identify which fraction contained the 67 kDa protein of interest (see figure 2.3).

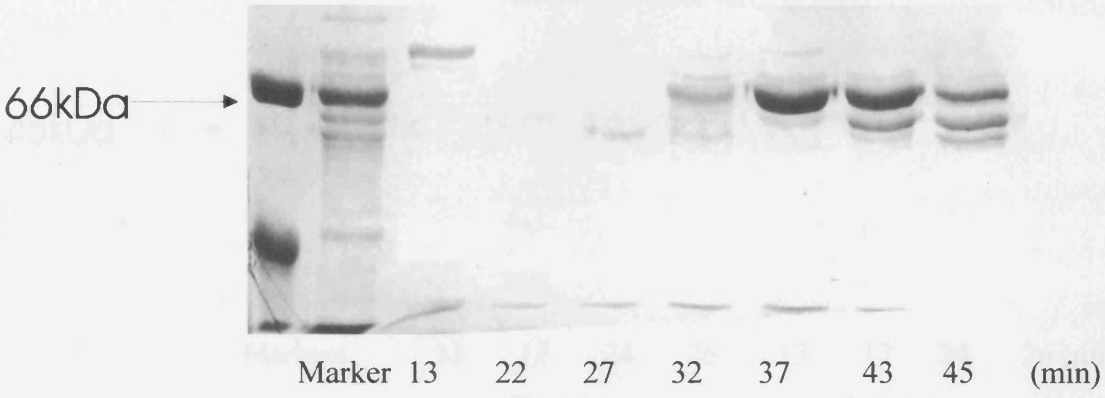


Figure 2.3: 67 kDa fractions retrieved after ion exchange chromatography was performed on the cell lysate. The 67 kDa band represents the p67 phox protein and can be identified in fractions at 37, 43 and 45 minutes of the gradient program. These fractions were pooled and used for immunization of the rabbit.

2.12.6 Purification of p47phox NADPH Oxidase Component

A Sepharose CM fast flow column was used for p47 phox purification. This was a cation exchanger. The column was washed with 20 ml of buffer W which consisted of NaH_2PO_4 20 mM, NaCl 2M, DTT 2mM, pH 7.0 and then equilibrated with 50 ml of buffer A which consisted of NaH_2PO_4 20 mM, DTT 2 mM, pH 7.0. Two hundred microlitres of sample was loaded onto the column at a time (0.2ml loop, flow rate 1 ml/minute, 10 seconds between injections). Once all 5 ml of the sample was loaded, the column was washed with 20-30 ml of buffer A until the baseline was near zero at 280 nm. The linear gradient program was then started which ran for 50 minutes at a flow rate of 1 ml/min starting with 100 % buffer A and ending with 100 % buffer B (NaH_2-PO_4 20 mM, NaCl 500 mM, DTT 2 mM, pH 7.0) as shown in the table below. Fractions were collected when peaks showed on the chromatograph and immediately placed on ice. The fractions were then resolved on a 12 % SDS-PAGE gel and subsequently

stained with Coomassie Blue (glacial acetic acid 10 %, methanol 50 %, Brilliant Blue R 0.5 %) to identify which fraction contained the 47 kDa protein of interest (see figure 2.4).

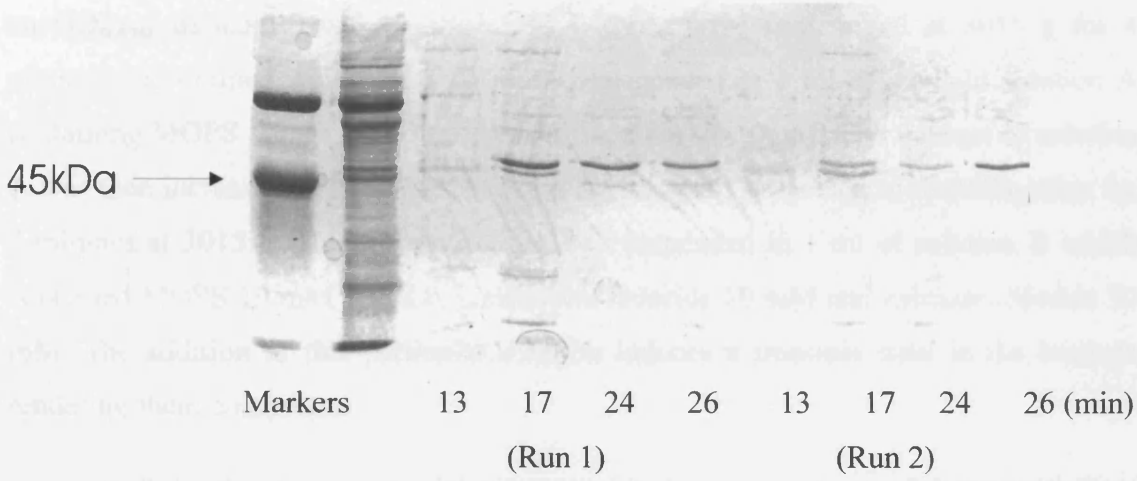


Figure 2.4: 47 kDa fractions retrieved after ion exchange chromatography was performed on the cell lysate. The 47 kDa band represents the p47 phox protein and can be identified in fractions taken at 17, 24 and 26 minutes of the gradient program. These fractions were pooled and used for immunization of the rabbit.

2.12.7 Preparation of p22 Component of NADPH Oxidase Using a Bacterial System

Bacterial plasmids are double stranded circular DNA molecules that range in size from 1 kb to 200 kb. They behave as accessory genetic units that replicate independently of the bacterial chromosome. Plasmid vectors are commonly used for the expression of foreign proteins. In the laboratory, plasmid DNA can be introduced into bacteria by the artificial process of transformation. In this process bacteria are treated with mixtures of divalent cations to make them temporarily permeable to small DNA molecules. Even under the best conditions, plasmids become stably established in only a small minority of the bacterial population. To identify these transformants, selectable markers encoded by the plasmid are used. Common markers include genes that confer resistance to antibiotics such as ampicillin [Davies and Smith 1978].

2.12.8 Production of Competent Bacteria

Competent bacteria were required to transform the bacteria with the plasmid DNA. The treatment explained below induces a transient state of "competence" in the recipient bacteria during which they are able to take up the plasmid DNA. Tryptone broth (TB) was placed in a falcon tube with a stab of DH5α bacteria and shaken overnight at 37 °C. A further 20 ml of TB was added to the pre-culture and allowed to grow at 37 °C until an OD_{600nm} of 0.5-1.0 was reached. The bacteria were centrifuged at 3015 g for 4 minutes and drained well. The cells were resuspended in 1 ml of ice-cold solution A containing MOPS 10mM pH 7.0 and rubidium chloride 10mM. The volume of solution A was then increased to 5 ml with solution A and again, subjected to centrifugation for 5 minutes at 3015 g. The cell pellet was then suspended in 1 ml of solution B which contained MOPS 10 mM at pH 6.5, rubidium chloride 10 mM and calcium chloride 50 mM. The addition of this particular solution induces a transient state in the bacteria rendering them competent.

2.12.9 Transformation of Plasmid DNA into Competent Bacteria

The plasmid (PGEX-2T-GST-11) containing the foreign DNA was kindly donated by Dr D Roos. The plasmid arrived on Whatman 3MM paper and was recovered by adding 50 µl of Tris 10 mM, pH 7.4 to the microfuge tube and vortexing gently. The tube was then centrifuged and the supernatant liquid used to transform the competent bacteria. Two microlitres of plasmid DNA was added to the competent bacteria and incubated at 37 °C for 30 minutes. The bacteria were then subjected to heat shock at 42 °C for 45 seconds and subsequently placed on ice for 2 minutes. Nine hundred microlitres of SOC solution containing tryptone, yeast extract, NaCl 8.5 mM, KCl 25 mM, glucose 20 mM and magnesium chloride 2 M was added to the bacteria and incubated at 37 °C for 1 hour, shaking. The bacterial cells containing the recombinant plasmid were transferred to 2 L of tryptone broth with ampicillin (50 µg/ml) and incubated at 37 °C overnight. Once OD_{600nm} had reached 0.5-1.0, which suggested that the bacteria were growing at log phase, IPTG was added at a concentration of 0.1 mM. The bacteria were then allowed to grow for 3-5 hours at 37 °C, shaking. Five hundred microlitres of pre and post IPTG bacterial cells were harvested to allow SDS-PAGE analysis (see figure 2.5). This would allow identification of the p22-GST protein. The bacteria were then harvested by centrifugation and re-suspended in SPET solution containing 8 % sucrose,

EDTA 1 mM, Triton X-100 1 %, 10X phosphate buffered saline, PMSF 0.5 mM, DTT 2 mM, leupeptin 0.1 mM, pepstatin 1 mM.

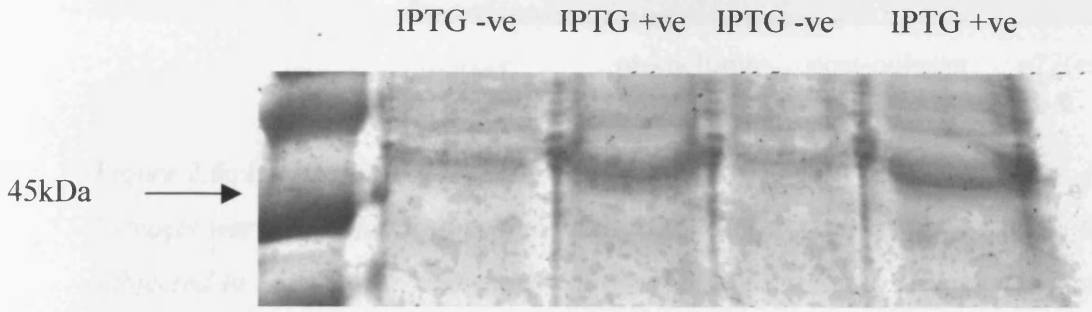


Figure 2.5 Induction of p22-GST protein production. Fractions of bacterial extract were treated with or without IPTG and subsequently subjected to SDS-PAGE on a 12 % gel. Only the area corresponding to the protein of interest was shown. p22-GST protein can be visualized at 50 kDa in the positive tracks. This suggests that the bacteria were induced to produce the protein. There was also a 50 kDa protein present in the negative tracks that represents the p22-GST. Bacteria produce small amounts of protein in the absence of IPTG.

2.12.10 Recombinant p22-GST Protein Purification

The recombinant protein was then extracted from the bacteria. The solution was sonicated on ice for 2-3 minutes to allow bacterial fractionation and then centrifuged. The supernatant was then collected and glutathione Sepharose slurry was added to the tube of lysate and allowed to rotate overnight at 4 °C. The p22-GST protein would then bind to the glutathione Sepharose. The beads were collected by centrifugation and then washed in buffer consisting of PBS, 1 % Triton and DTT 10 mM. The p22-GST was then eluted from the beads using free glutathione 10 mM with Tris 50 mM, pH 8.0. Samples were retained at each step of the purification process to be later subjected to SDS-PAGE analysis (see figure 2.6). This would ensure that the p22-GST protein had been successfully purified.

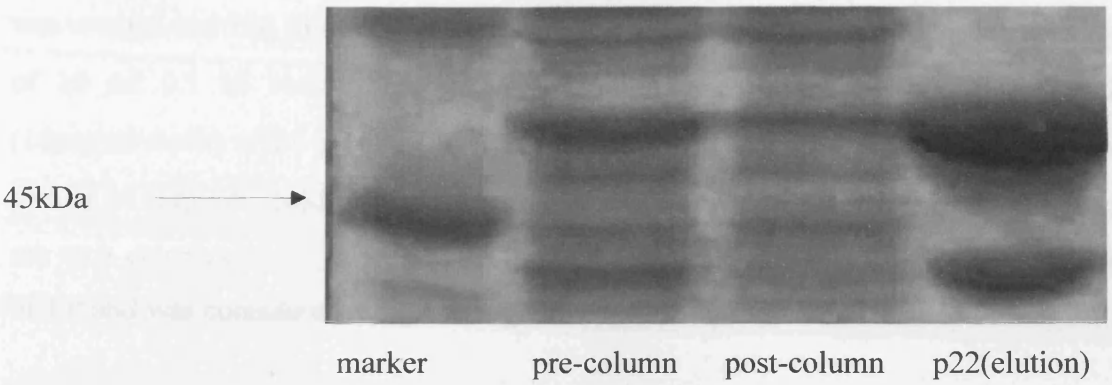


Figure 2.6: Purification of the p22-GST protein.
Extracts were collected and subjected to SDS-PAGE. Extracts have been collected and subjected to SDS-PAGE and then visualized by staining with Coomassie Blue. The gel shows a fraction collected before the extract was put down the column, a fraction of extract collected from the column and a fraction eluted off the column. The fraction collected from the column on elution is purified p22-GST protein that was subsequently used to immunize the rabbit for antibody production. A band can be visualized at approximately 30 kDa and is likely to be free GST that has a molecular weight of approximately 26 kDa.

2.12.11 Preparation of Injections.

The fractions that contained the desired protein were pooled together and purified by filtration through a 0.2 μm filter under sterile conditions into phosphate buffered saline. The first injection volume of 250 μl containing 200 μg of antigen was mixed with 250 μl of Freund's complete adjuvant before injecting into subcutaneous sites of a New Zealand White rabbit. Subcutaneous injections were made at 4 weekly intervals. Test samples of serum were taken from the rabbits at regular intervals and the immune response of the rabbit was tested in-house against the specific protein using the original antigen. ELISA plates were coated with 100 μl of antigen (100ng/100μl) and incubated overnight at 4°C. The plates were washed 5 times with PBS/0.05 % Tween and then blocked in 3 % BSA/PBS overnight at 4 °C. The plates were washed as above and then a dilution made of the rabbit serum (1:1000) in PBS/0.5% Tween. Two-fold dilutions were then made across the plate. The plate was then left overnight at 4 °C. The wash step was repeated and 100 μl of HRP conjugated donkey anti-rabbit IgG (1:5000 in

PBS/0.5% Tween) added to each well and left for 1 hour at room temperature. The plate was washed and 100 µl of colorimetric solution was added to each well which consisted of 10 ml 0.1 M sodium acetate, 75 µl of 3,3',5,5'-tetramethyl-benzidine (TMB) (10mg/ml stock) and 6 µl of 27 % hydrogen peroxide. Once the colour developed 100 µl of 1 M sulphuric acid was added to stop the reaction. The plate was then read at 450 nm on a colorimeter (Dynatech MRX). A 1:1000 dilution of neat serum gave a reading of 1.0 and was considered a high enough titre for the animal to be sacrificed.

2.12.12 Purification of the Antibody using Protein A Column

Once the antibody titres were confirmed sufficiently high, the serum was collected and the antibody of interest was purified using protein A Sepharose. Any IgG in the serum attached to the protein A beads. The column was subsequently washed with Tris 100 mM, pH 8.0 until the OD_{280} was around zero. The antibody of interest was then eluted from the Sepharose column with glycine 100 mM, pH 2.5. The change in pH detaches the IgG. The antibody fractions were immediately neutralized to a pH of 7.5 with 2M Tris (250 µl/ml of eluate). The concentration of antibody was determined at OD_{280} using a spectrophotometer.

2.12.13 Specificity of NADPH Oxidase Antibodies

NADPH oxidase antibodies were tested for specificity using 2 techniques. The specificity of the p47 and p67 antibodies was determined using lymphoblast extracts subjected to SDS-PAGE. Figure 2.7 illustrates the specificity of p47 antibody. Two identical blots were used to examine specificity as stripping and re-using the same blot resulted in reduced intensity due to loss of protein on the blot. A protein corresponding to 47 kDa can be visualised which represents the p47-*phox* protein. A corresponding blot was probed with p47 antibody that was pre-incubated with a 2 -fold excess (2 ug/ml) of p47 antigen. The 47 kDa band was partially abolished but still remained due to the affinity of the primary antibody. P47 antibody was also shown to recognise the p47 antigen directly in Western blots (not shown).

Figure 2.8 illustrates the specificity of the p67 antibody. Lymphoblast extracts were subjected to SDS-PAGE and then probed with p67 antibody. Again, 2 identical blots were examined. A band was visualised at 67 kDa that represented p67-*phox* protein. The corresponding blot was probed with p67 antibody that had been pre-incubated with

a 2-fold excess (2 ug/ml) of p67 antigen. The 67kDa band had been partially abolished due to the affinity of the primary antibody. This band was blocked proportionally more that the other bands shown in figure 4.6 (A=24.52 %, B=70.1 % and C=6 % respectively) following assessment using densitometry.

The specificity of the p22 and gp91 antibodies was determined using a technique that allowed separation of the cytosol from the membranes following fractionation. The p22-*phox* and gp91-*phox* proteins are only present in the membranes and therefore the cytosol fractions should not react with the respective antibodies. Lymphocyte extracts of post nuclear supernatant (PNS), cytosol and membranes were subjected to SDS-PAGE and probed with specific antibodies. Figure 2.9 illustrates the specificity of p22 antibody. Fifty micrograms of protein was loaded into each track following separation of the membranes from the cytosol. The blot was then probed with 1μg/ml p22 specific antibody developed in-house and showed a protein present in the membranes at ~21kDa that was not present in the cytosol. This protein was specific for p22 antibody. In addition, these results were crosschecked with a mouse monoclonal antibody for p22-*phox* (Dr Roos, University of Amsterdam) that reacted with a protein of the same size (not shown). The characterization of this monoclonal antibody has previously been performed [Verhoeven et al. 1989]. Figure 2.10 illustrates the specificity of gp91 antibody. Fifty micrograms of protein was loaded onto each track following separation of the membranes from the cytosol. The blot was then probed with 1 μg/ml gp91 specific antibody and showed a protein present in the membranes at ~91kDa that was only slightly visible in the cytosol. This protein was relatively specific for gp91 antibody and could only give an approximation of gp91 protein abundance. The gp91 antigen was donated and used to immunize the rabbit so it was not possible to determine specificity in the usual way. However, the characterization of an antibody developed using this antigen has previously been performed [Verhoeven et al. 1989].

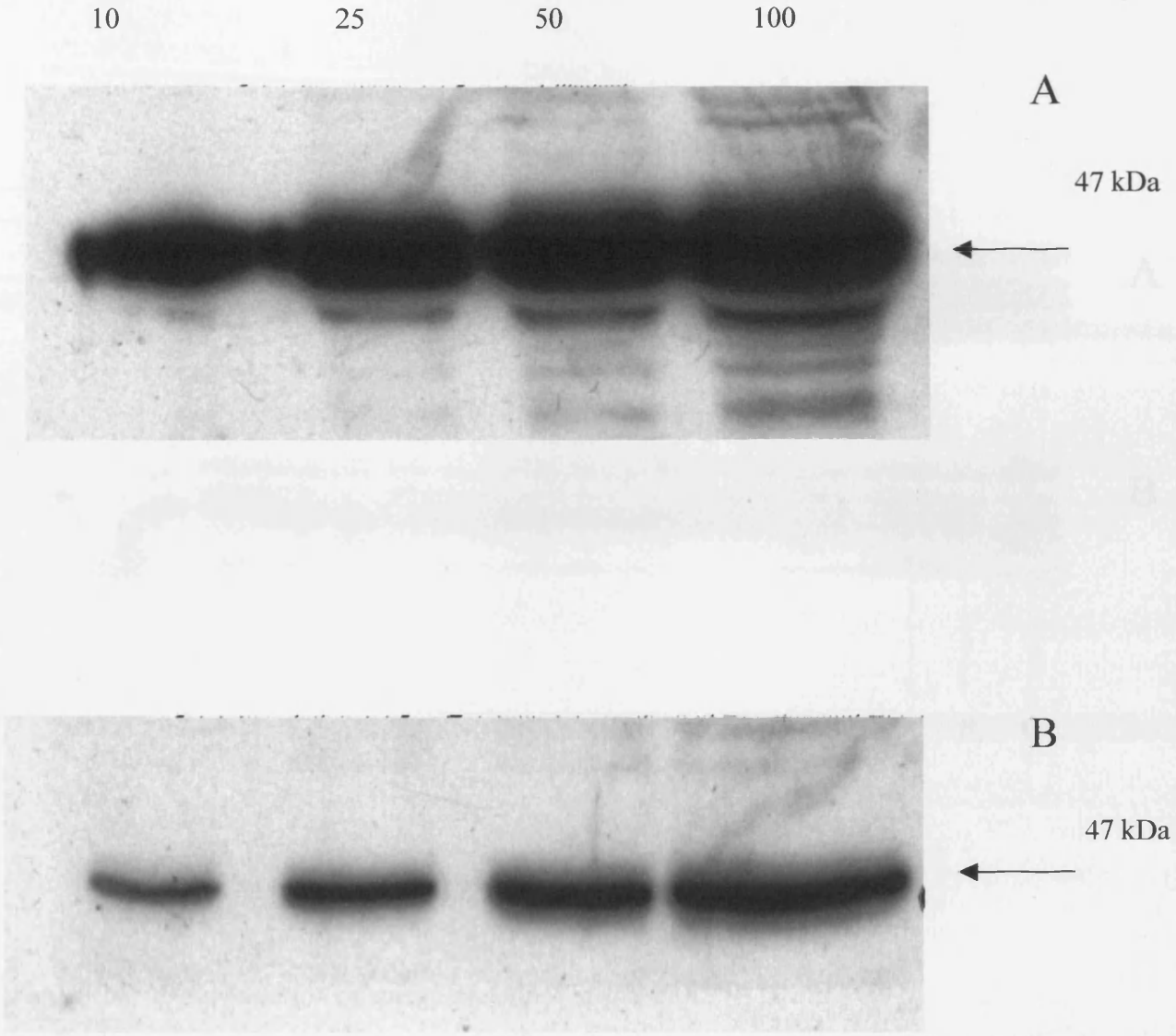


Figure 2.7: Determination of specificity of p47 antibody

A. Demonstrates specific response of p47 antibody to lymphoblast protein extracts (10, 25, 50, 100 μg). A protein corresponding to 47kDa can be visualised clearly.

B. The corresponding blot was probed with p47 antibody that had been pre-incubated with a 2-fold excess (2 ug/ml) of p47 antigen. The 47kDa band was partially abolished but still remained due to the affinity of the primary antibody. The section of the blot representing the area of interest is shown.

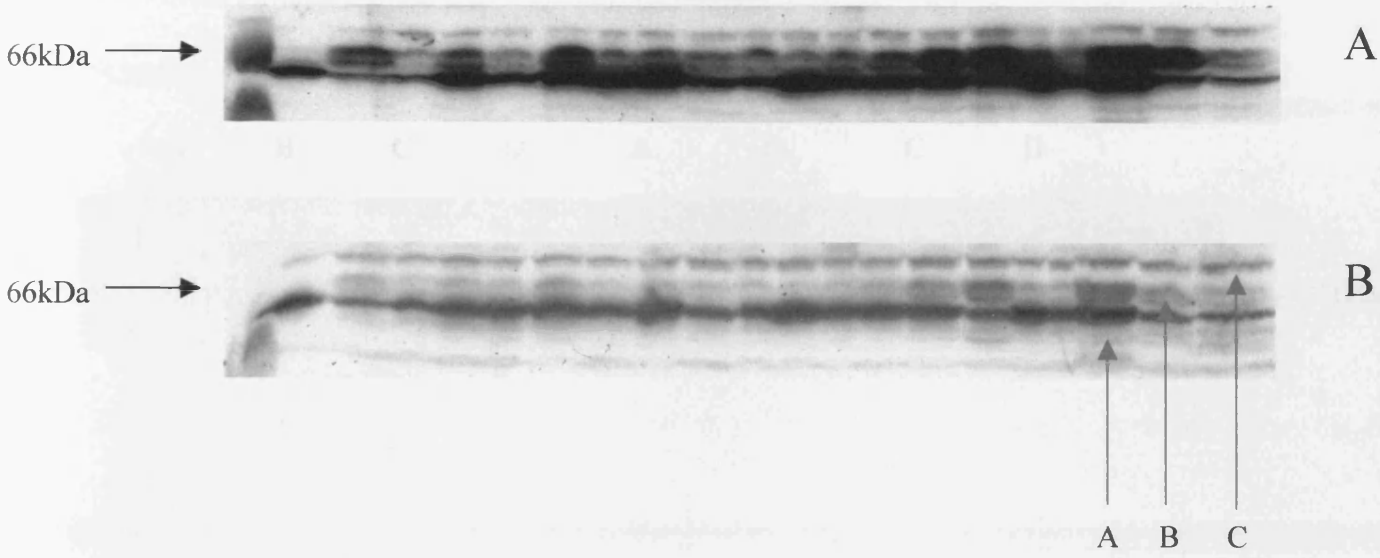


Figure 2.8: Determination of specificity of p67 antibody

A. Demonstrates specific response of p67 antibody to lymphoblast protein extracts (50μg). A protein corresponding to 67kDa can be visualised.

B. The corresponding blot was probed with p67 antibody that was pre-incubated with a 2- fold excess (2 ug/ml) of p67 antigen. The 67kDa band was partially abolished but still remained due to the affinity of the primary antibody. The intensity of the band corresponding to 67 kDa (B) was reduced proportionally more in the block experiment compared to surrounding bands (A and C). The section of the blot representing the area of interest is shown.

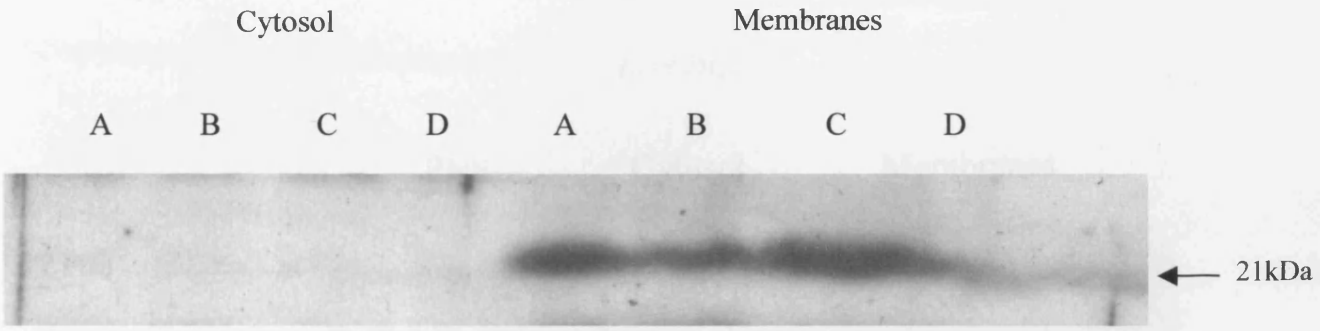


Figure 2.9: Fractionation experiments to validate p22 antibody. Fifty micrograms of protein was loaded into each track following separation of the membranes from the cytosol. The blot was then probed with 1 μg/ml p22 specific antibody developed in-house and showed a protein present in the membranes at 21kDa that was not present in the cytosol. This protein reacted with the p22 antibody. A,B,C and D represent different lymphoblast cell lines. The section of the blot representing the area of interest is shown.

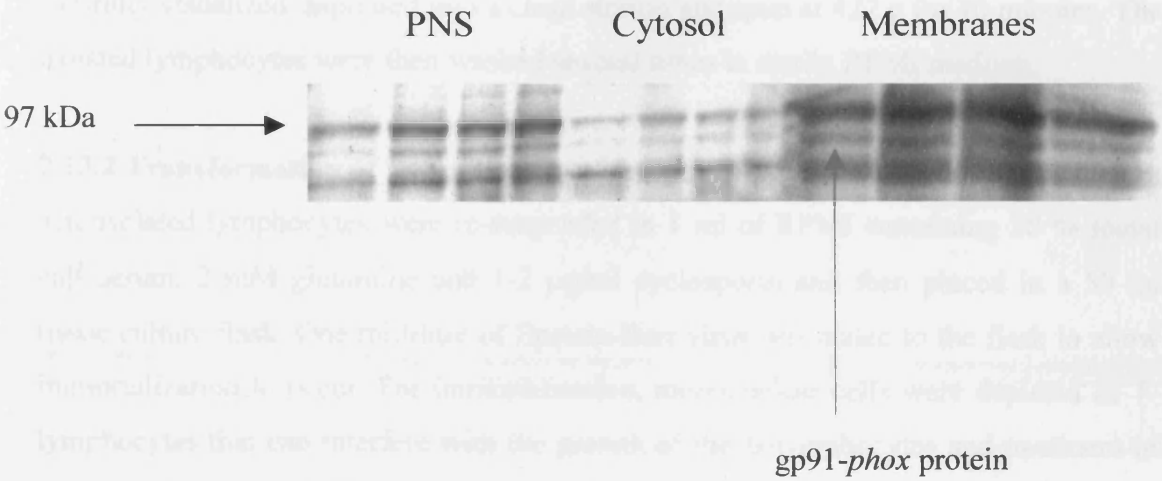


Figure 2.10: Fractionation experiments to validate gp91 antibody. Fifty micrograms of protein was loaded into each track following separation of the membranes from the cytosol. The blot was then probed with 1μg/ml gp91 specific antibody developed in-house and showed a protein present in the membranes at 91kDa that was only minimal in the cytosol. This protein reacts with the gp91 antibody. PNS= post nuclear supernatant. Experiments were performed using 4 different lymphoblast cell lines. The section of the blot representing the area of interest is shown.

2.13 Preparation of EBV Immortalized B Cell Lymphocytes.

2.13.1 Isolation of Lymphocytes

Lymphocytes were isolated from whole blood using histopaque 1077 medium. Five millilitres of blood were collected from the forearm of the patient in a syringe containing 1 mM sterile sodium citrate as the anti-coagulant. The whole blood was then carefully layered onto 5 ml of histopaque 1077 in a sterilin using aseptic techniques. The sterilin was then centrifuged at 919 g for 20 minutes with the brake switched off to allow separation of the lymphocytes from whole blood. The lymphocyte layer was then carefully visualized, aspirated into a clean sterilin and spun at 427 g for 10 minutes. The isolated lymphocytes were then washed several times in sterile RPMI medium.

2.13.2 Transformation of Lymphocytes with Epstein-Barr Virus

The isolated lymphocytes were re-suspended in 1 ml of RPMI containing 20 % foetal calf serum, 2 mM glutamine and 1-2 μg/ml cyclosporin and then placed in a 50 ml tissue culture flask. One millilitre of Epstein-Barr virus was added to the flask to allow immortalization to occur. For immortalization, mononuclear cells were depleted of T-lymphocytes that can interfere with the growth of the B-lymphocytes and treatment of the cultures with cyclosporin A was incorporated for this purpose. The flask was placed in the incubator at 37 °C with 5 % CO_2 and observed daily. After 2-3 weeks colonies of immortalized lymphocytes started to form and the cells were transferred into 100 ml flasks. EBV was grown up from marmoset B95-8 cells in our laboratory at an earlier date. There are several risk factors associated with the use of EBV. Firstly the risk of glandular fever is associated with exposure to EBV. Secondly, the risk of lymphoma if a person is inoculated with their own transformed cells. Therefore is it crucial that cells isolated from colleagues were never transformed with EBV.

2.13.3 Maintenance of Immortalized Cell Lines

Cell lines were fed every 2-3 days with RPMI supplemented with 10 % FCS, 2 mM glutamine, 100 units of penicillin and 0.1 mg streptomycin. Once the growth of cells was continuous a portion of cells was removed and resuspended in cryopreservative (50 % FCS, 10 % DMSO and 40 % RPMI) and stored in liquid nitrogen. This represented a library of patients' immortalized cell lines for later use.

2.14 Assessment of Signal Transduction Pathways Implicated in NADPH Oxidase Activation.

EBV-immortalized lymphoblasts were incubated with various inhibitors of the signal transduction pathways to try and elucidate control mechanisms of enhanced NADPH oxidase activity. The concentrations chosen were the result of performing dose response curves and consulting the literature of previous experiments including the inhibitors [Daniels et al. 1999; Dang et al. 1999; Dent et al. 2000; Mollapour et al. 2001; Chow et al. 2000]. Figure 2.11 presents a representative dose-response curve.

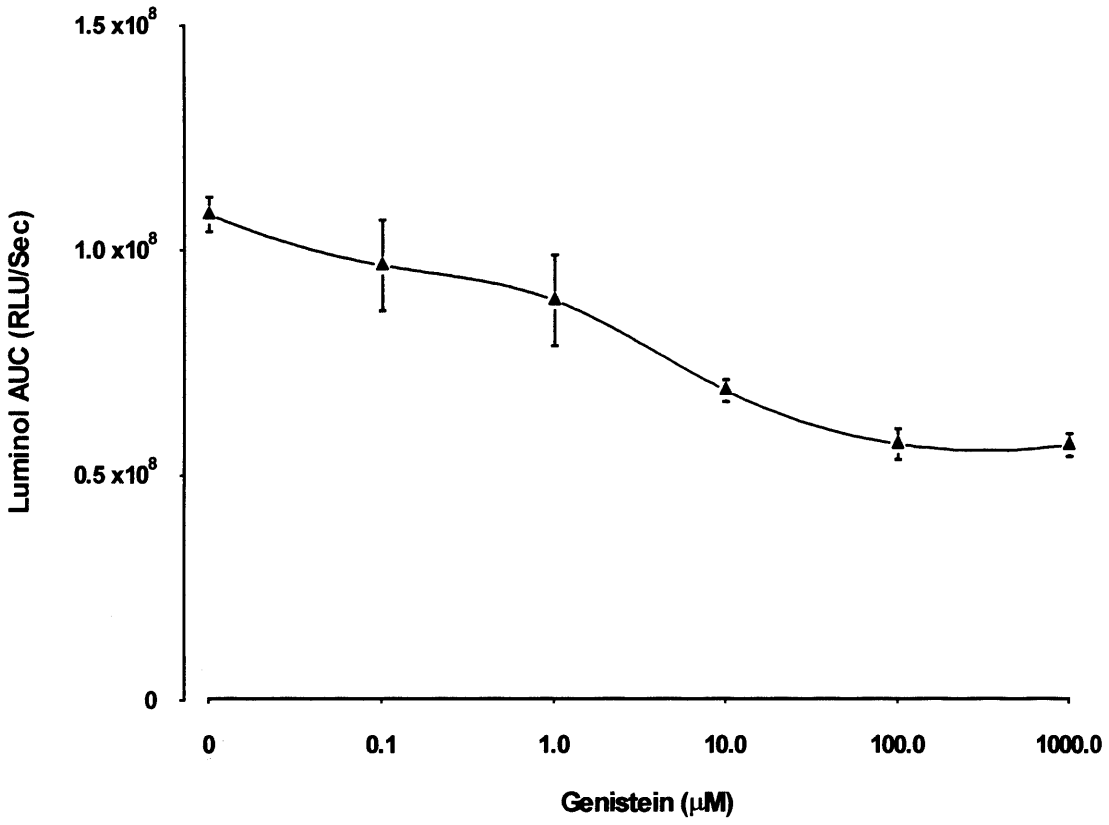


Figure 2.11 Dose response curve for genistein. Varying concentrations were analysed for their inhibitory effect upon NADPH oxidase production of ROS stimulated with the agonist PMA. Mean values and SEM are plotted, n=3.

The table below illustrates the inhibitors, concentration, time and effect. Inhibitors were added and incubated at 37 °C, the inhibitor was then washed off and the cells assayed for production of hydrogen peroxide as described in section 2.8.2

Inhibitor	Effect	Concentration	Time
Wortmannin	Inhibitor of PI-3 kinase	1μM	20 minutes
Genistein	Tyrosine protein kinase	100μM	20 minutes
MAFP	$CPLA_2$	1μM	20 minutes
PD98059	ERK MEK specific	50μM	20 minutes
SB203580	P38 MAPK	1μM	20 minutes
Tyrphostin	Receptor tyrosine kinase	100μM	20 minutes
Staurosporin	PKC	80nM	20 minutes
RO318220	PKC	1μM	20 minutes

Table 2.1 Illustration of inhibitors used to assess NADPH oxidase signal transduction cascades. Concentrations were determined after dose-response curves had been constructed.

2.15 Assessment of a Digoxin-Like Factor in Plasma

2.15.1 Plasma Samples

Peripheral blood samples were drawn from patients into sodium citrate coated tubes. The plasma was collected by centrifugation of the samples at 1709g for 20 minutes. Plasma samples were stored at –20 °C until extraction.

2.15.2 Extraction of Plasma Samples

Disposable C_{18} SepPak cartridges were preconditioned with 4 ml of 25 % acetonitrile made up in distilled water followed by 20 ml of 0.1 % trifluoroacetic acid (TFA). Two millilitres of plasma were passed through the column and then the column was washed with 10 ml of 0.1 % TFA, and eluted with 3 ml of 25 % acetonitrile. Recovered eluate was evaporated to dryness under vacuum and stored at –80 °C until assayed. The rationale for this was to remove any phosphate from the samples.

2.15.3 Measurement of a Digoxin-Like Factor (DLF).

Extracted plasma was reconstituted in 200 µl of ATPase buffer (NaCl 100 mM, KCl 10 mM, $MgCl_2$ 4 mM, EGTA 0.2 mM, Tris/Base 30 mM, Histidine/Base 5 mM pH 7.8 HCl). The ionic composition of this buffer was largely based on the optimal conditions for measurement of leucocyte Na^+K^+ATPase activity reported by Baron and Khan [1985]. One hundred microlitres of reconstituted plasma was incubated with 400 µl of ATPase solution containing 2 milliunits of Na^+/K^+ATPase (pig cerebellum) and left for 20 minutes at 37 °C. One hundred microlitres of water (no plasma) was added to the control tube and represented a sample with no inhibition. One hundred microlitres of reconstituted plasma was added to the test tubes. One hundred microlitres of ATP (buffered to pH 7.8 with Tris/Base) was added to all tubes, making a final volume of 0.6 ml and a final ATP concentration of 4 mmol/L. The tubes were then vortexed and incubated in a waterbath at 37 °C with gentle agitation for 30 minutes. The reaction was stopped by adding 0.4 ml SDS 100 g/L. This stopped the enzyme activity very effectively as the inorganic phosphate did not rise by more than 1 % over the subsequent hour. To measure inorganic phosphate in supernatants a more sensitive version of the Fiske-Subbarow method was adopted [1925]. Stannous chloride was made as follows. Twenty eight millilitres of concentrated sulphuric acid was added to 700 ml of distilled water and left to cool at room temperature. Two grams of hydrazine sulphate was added and the volume made up to 1 litre with distilled water. Two hundred micrograms of stannous chloride was added to the litre and the solution was filtered. The acid molybdate solution was made by adding 35 ml of concentrated sulphuric acid to 700 ml of distilled water. After cooling, 10 g of ammonium molybdate was added and the solution made up to 1 litre with distilled water. One millilitre of stannous chloride was added to the sample and control tubes and the tubes were then mixed. One millilitre of acid molybdate solution was then added. The tubes were left for 15 minutes and then the OD_{640nm} was read on a spectrophotometer. Sample determination was performed by subtracting the OD_{640nm} of the blank control from the OD_{640nm} of the test samples. A standard curve was constructed with dilutions of a 1 mol/L sodium dihydrogen orthophosphate solution. Inorganic phosphate concentration was then read from the standard curve and the results were expressed as nanomoles inorganic phosphate/ml plasma. This represented the degree of inhibition by factors present in the

reconstituted plasma since an inhibited Na^+/K^+ATPase would result in less inorganic phosphate being liberated according to the following reaction:-

$$ATP + H_2O \xrightarrow{ATPase} ADP + P_i$$

2.15 Statistical Analysis

Data was analysed using Minitab (PA). All analyses were performed using un-paired Student's t-tests, paired Student's t-tests or ANOVA (analysis of variance). All data was tested for normality using the Anderson-Darling test. In some cases normalization was performed in order to achieve normal distribution of data. This was performed by taking the mean of the control group and dividing both control and patient data by this value. All patient data was therefore normalized to a mean value of 1.0 in the control group. Statistical analysis was then performed accordingly. This method of normalization was used throughout this thesis. Unless otherwise stated, all data were expressed as mean ± standard error of the mean (SEM). P values >0.05 were considered non significant. Pearson's correlation coefficient was used to measure association between 2 variables.

Chapter 3

Assessment of Leucocyte Intracellular pH and Na^+/H^+ Exchanger Activity in Post-partum Pre-eclamptic Women.

3.1 Introduction

A higher Vmax Na^+/H^+ exchanger is well documented in a wide variety of cell types isolated from patients with essential hypertension and is considered an intermediate phenotype (a characteristic intermediate between the gene(s) causing a disease and the disease itself) [Rosskopf et al. 1993]. These findings suggest that Na^+/H^+ exchanger activity may be a marker for predicting patients who may develop pathological conditions such as hypertension.

It has been established that leucocyte Na^+ content increases during pre-eclamptic pregnancies [Seon and Forrester 1989]. This increase in cellular Na^+ persists post-partum for up to 38 weeks suggesting that the changes in cellular ion homeostasis could be independent of the humoral changes associated with pregnancy and the pre-eclamptic state [Forrester et al. 1990]. Inhibition of the Na^+/K^+ATPase has been reported in pre-eclampsia extending in the post-partum period and may therefore contribute to the increased leucocyte Na^+ content associated with the pre-eclamptic syndrome [Lopatin et al. 1999; Bolton et al. 1994]. The Na^+/H^+ exchanger is a membrane transport system that involves the transport of Na^+ and increased activity of this transport system may contribute to this increased leucocyte Na^+ content.

This chapter tests the hypothesis that leucocytes isolated from post-partum pre-eclamptic women may exhibit increased Na^+/H^+ exchanger activity that may contribute to the altered intracellular Na^+ in pre-eclamptic leucocytes and thus be implicated in the pathological condition.

This study was designed to assess the status of the Na^+/H^+ exchanger isoform 1 in women who had suffered a pre-eclamptic pregnancy and to evaluate the possible contribution of this to pre-eclampsia. The objective was to investigate whether the intermediate phenotype, which is well documented in essential hypertension, may be expressed in nucleated leucocytes from women who have had a pre-eclamptic pregnancy. Leucocytes were analysed as they are easily accessible, representative cells

that contain mRNA and may reflect alterations that occur in other cell types. Investigation during the post-partum state may uncover genetic influences and would avoid the confounding effect of activation of the Na^+/H^+ exchanger due to the physiological acidosis associated with pregnancy [Baricef et al. 1995]. In addition, the mechanisms underlying the enhancement of Na^+/H^+ exchanger activity, such as the abundance of the Na^+/H^+ exchanger protein and phosphorylation of the protein were investigated. The possibility of increased activity due to the presence of other isoforms such as Na^+/H^+ exchanger isoform 3 was also studied.

3.2 Materials and Methods

The methods used for assessing Na^+/H^+ exchanger activity, phosphorylation and abundance are included in chapter two of this thesis.

3.3 Patients

Fourteen post-partum pre-eclamptic women and fourteen post-partum normotensive women were evaluated (Table 3.1). None of the women had a family history of essential hypertension.

The average age of the post-partum pre-eclamptic women was 35 years (range 27-47) and the aged matched post-partum controls was 37 years (range 27-45). Two of the patient group had a family history of pre-eclampsia and three had previously suffered from a pre-eclamptic pregnancy. Blood pressure was significantly higher during pregnancy in the women who suffered from pre-eclampsia (P=0.0001). No differences in the blood pressure at the antenatal booking visit were identified (12-16 weeks gestation). Gestational age at delivery and birthweight were significantly lower in the women who suffered a pre-eclamptic pregnancy (P=0.001). Pre-eclamptic women tend to deliver earlier than their matched controls which will affect the weight of the baby.
Clinical and laboratory details of the women are shown in table 3.1. Note that no laboratory details are illustrated for the controls due to the normal progression of their pregnancy.

TABLE 3.1
CLINICAL DATA

	PET	NT
Women (n)	14	14
Age (years)		
Mean	35 (±1.5)	37 (±1.2)
Range	27-47	27-45
Gestation (weeks)		
Mean	34^{+1} (±1.4)	$40^{+}1$ (±0.31)*
Range	25^{+2}-40^{+1}	38-42^{+2}
Mode of Delivery		
C/S	8	2
Spontaneous	2	10
Induced	4	2
BP booking (mmHg)		
Mean	115/72 (±2.5)	113/66 (±2.0)
Range	100/60-130/85	99/60-120/80
BP highest (mmHg)		
Mean	173/106 (±4.2)	113/65 (±1.8)**
Range	139/72-200/125	100/55-120/70
BP visit (mmHg)		
Mean	127/74 (±2.8)	117/70 (±2.0)
Range	98/65-142/95	99/60-131/79
Smoke		
Yes	1	0
No	13	14
Family History		
PET	2	0
Hypertension	0	0
Previous PET	3	0
Months post-partum at time of sampling		
Mean	6.5 (±0.95)	7 (±0.1.2)
Range	5.5-7.5	5.5-8.5
Parity		
1	8	8
>1	6	6
Sex of Newborn		
Female	7	5
Male	7	9
Weight of Newborn	4lb7oz (±0.60)	7lb7oz (±0.16)**
Mean	1lb7oz-7lb2oz	6lb6oz-8lb9oz
Range		

Protein (urine)		
+	0	
++	4	Nil
+++	9	
++++	1	
Lowest Hb (g/L)		
Mean	10.04 (±0.492)	N/A
Range	7.9-13.2	
Lowest Platelets (10^9/L)		
Mean	109.9 (±20.0)	N/A
Range	49-250	
Highest Urate (mmol/L)		
Mean	394 (±19.9)	N/A
Range	265-539	
Highest Creatinine (μmol/L)		
Mean	88.9 (±4.66)	N/A
Range	58-115	
Highest Urea (mmol/L)		
Mean	4.8 (±0.46)	N/A
Range	2.7-6.5	
Highest ALT (U/L)		
Mean	54 (±10.9)	N/A
Range	8-129	

Table 3.1: Clinical data for the post-partum pre-eclamptic and normotensive control women. Values are means with range and SEM $^{*}P=0.001$, $^{**}P=0.0001$, normotensive versus pre-eclamptic women by Student's t-test.

3.4 Results

Figure 3.1 shows typical results for intracellular pH measurements.

3.4.1 Intracellular pH

The results for intracellular pH at resting conditions are illustrated in Figure 3.2. Analysis using Student's t-test showed that the mean intracellular pH of the groups was significantly different ($P<0.001$). Women who suffered a pre-eclamptic pregnancy had a significantly more acidotic intracellular pH with a mean value of 7.11±0.02 compared to those who had a normal pregnancy 7.31±0.04.

3.4.2 Buffering Capacity

Buffering capacity showed variability and the results are illustrated in Figure 3.3. There was a significant difference between the mean values of the two groups ($P=0.005$). The post-partum pre-eclamptic women had a significantly greater leucocyte buffering capacity (51.1 ±6.5 mmol/l.pH unit) compared to the normotensive (28.7 ±2.4 mmol/l.pH unit) women. Figure 3.4 shows that a negative correlation exists between intracellular pH and buffering capacity for the whole group. Women whose cells were acidotic tended to have a higher buffering capacity.

3.4.3 Na^+/H^+ Exchanger Activity

Figure 3.5 demonstrates the leucocyte Na^+/H^+ antiporter Vmax for both post-partum pre-eclamptic and normotensive women. Analysis using Student's t-test showed that the Na^+/H^+ exchanger activity of the groups was significantly different ($P=0.029$). Women who had a pre-eclamptic pregnancy had a significantly higher activity at pH_i 6.0 (35.8±2.8 mmoles/l.min) compared to the normotensive control women (27.3±2.3 mmoles/l.min). The exchanger activity was calculated as the product of initial change in intracellular pH (pH units/min) multiplied by the buffering capacity (mmoles/l.pH unit). Therefore the units of exchanger activity were mmoles/l.min.

3.4.5 Na^+/H^+ Exchanger Protein Abundance

Figure 3.7 shows a typical Western blot of leucocyte extracts obtained by loading equal amounts of total protein (30 μg per track) for both post-partum pre-eclamptic

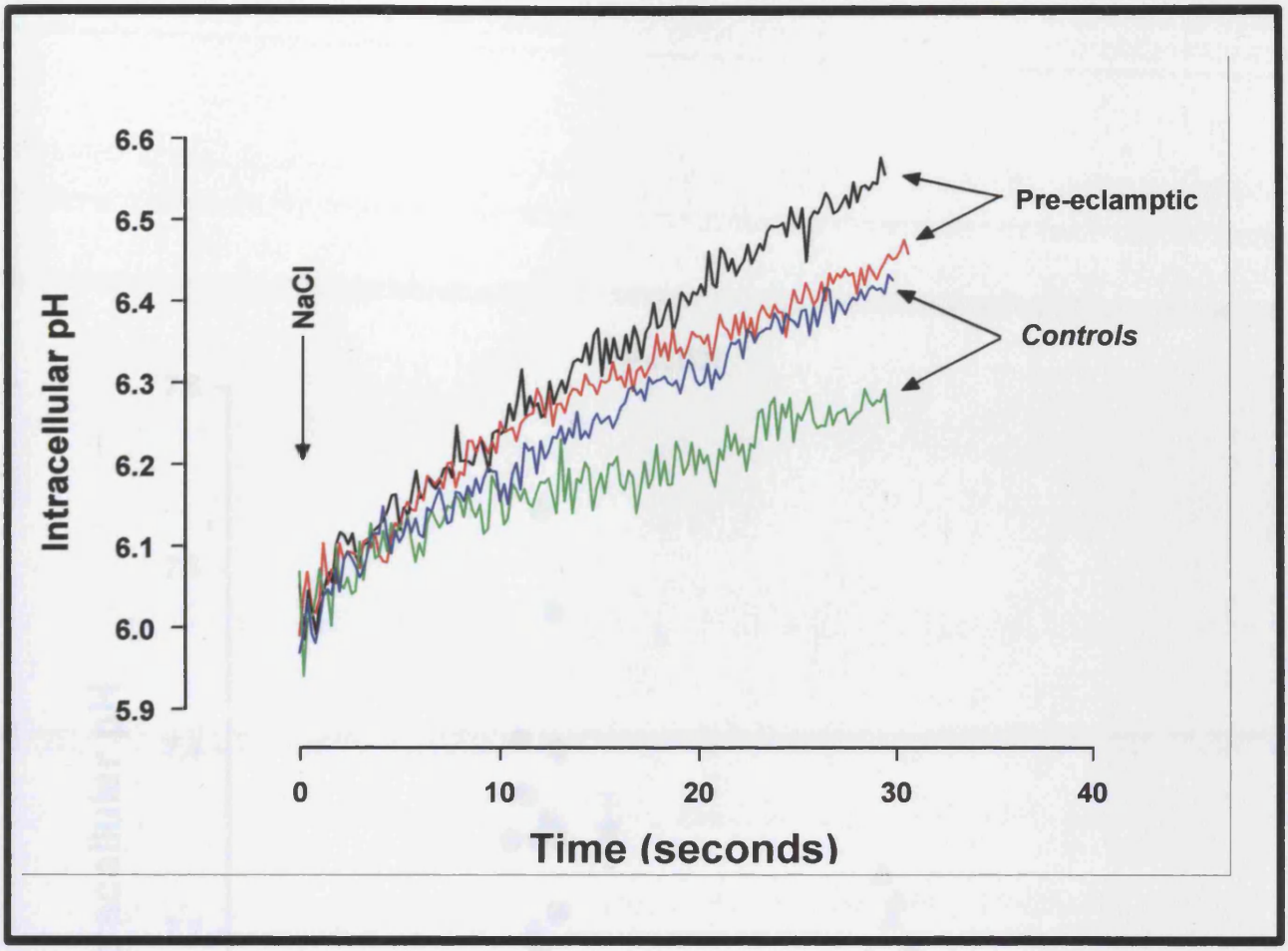


Figure 3.1: Representative leucocyte pH changes due to H^+ efflux from 2 random control and pre-eclamptic subjects. Leucocyte intracellular pH was clamped at 6.0 and then extracellular NaCl was added that initiated alkalinization. The H^+ efflux is mediated by the Na^+/H^+ exchanger. The rate of change in intracellular pH was calculated using the first 20 seconds of the trace.

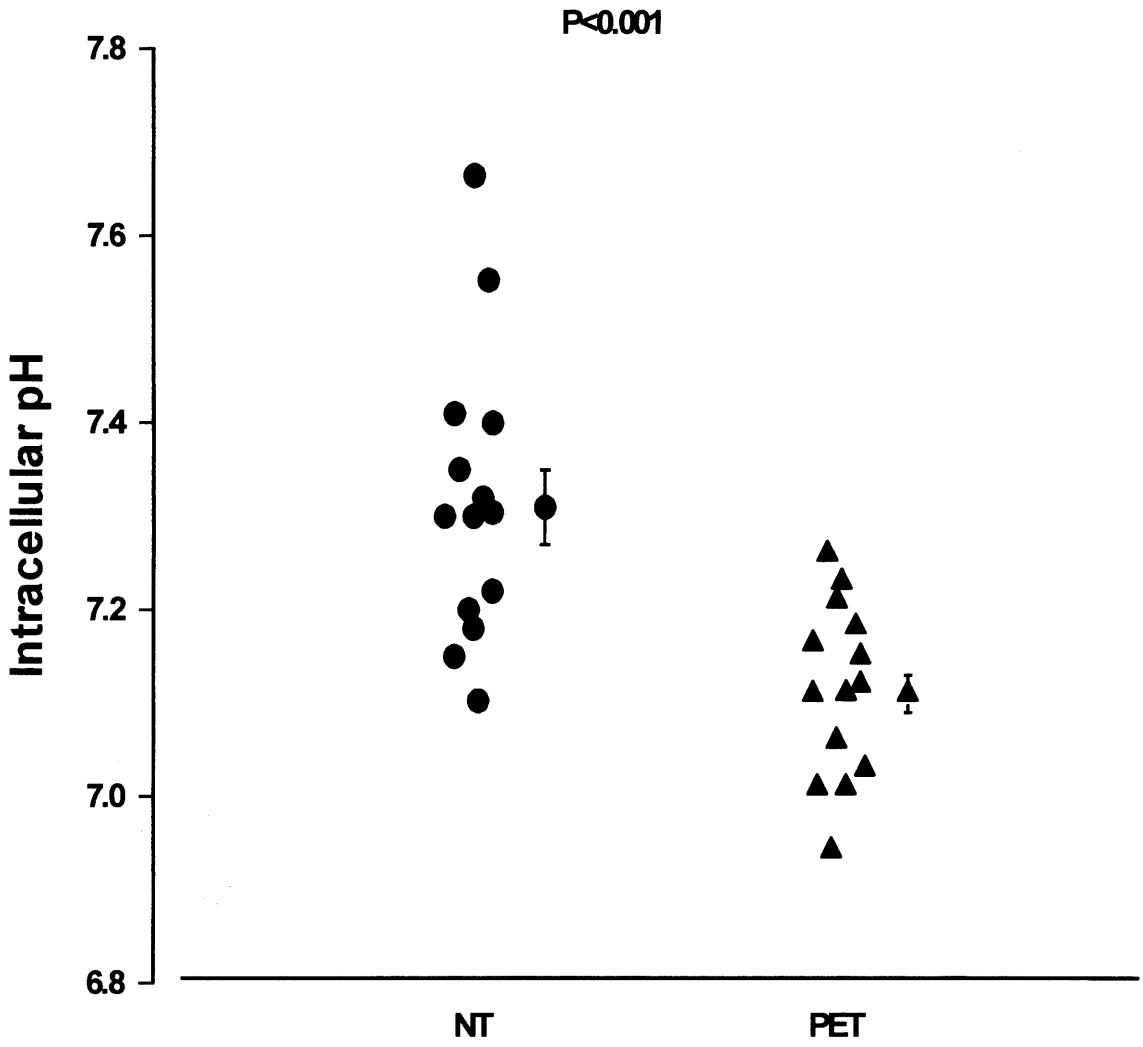


Figure 3.2: Plot of leucocyte intracellular pH in post-partum normotensive (NT) and post-partum pre-eclamptic (PET) women. The mean and SEM are plotted. PET leucocytes exhibited a lower intracellular pH (7.11 ±0.02) compared with NT leucocytes (7.31 ±0.04).

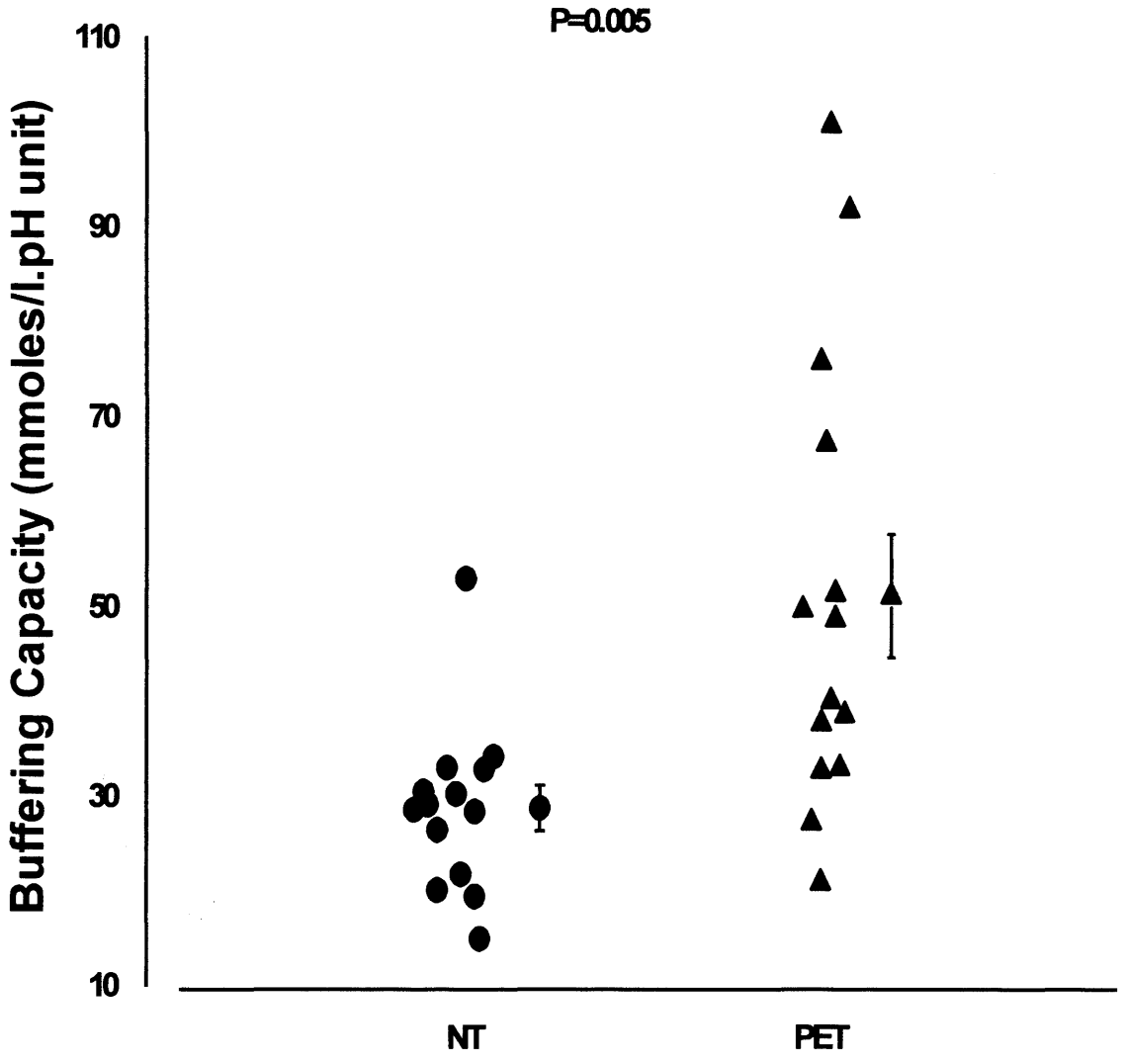


Figure 3.3: Plot of leucocyte buffering capacity measured at pH_i 6.0 in post-partum normotensive (NT) and post-partum pre-eclamptic (PET) women. The mean and SEM are plotted. The buffering capacity of leucocytes from PET women (51.1 ±6.5 mmoles/l.pH unit) was significantly elevated compared with NT women (28.7 ±2.4 mmoles/l.pH unit).

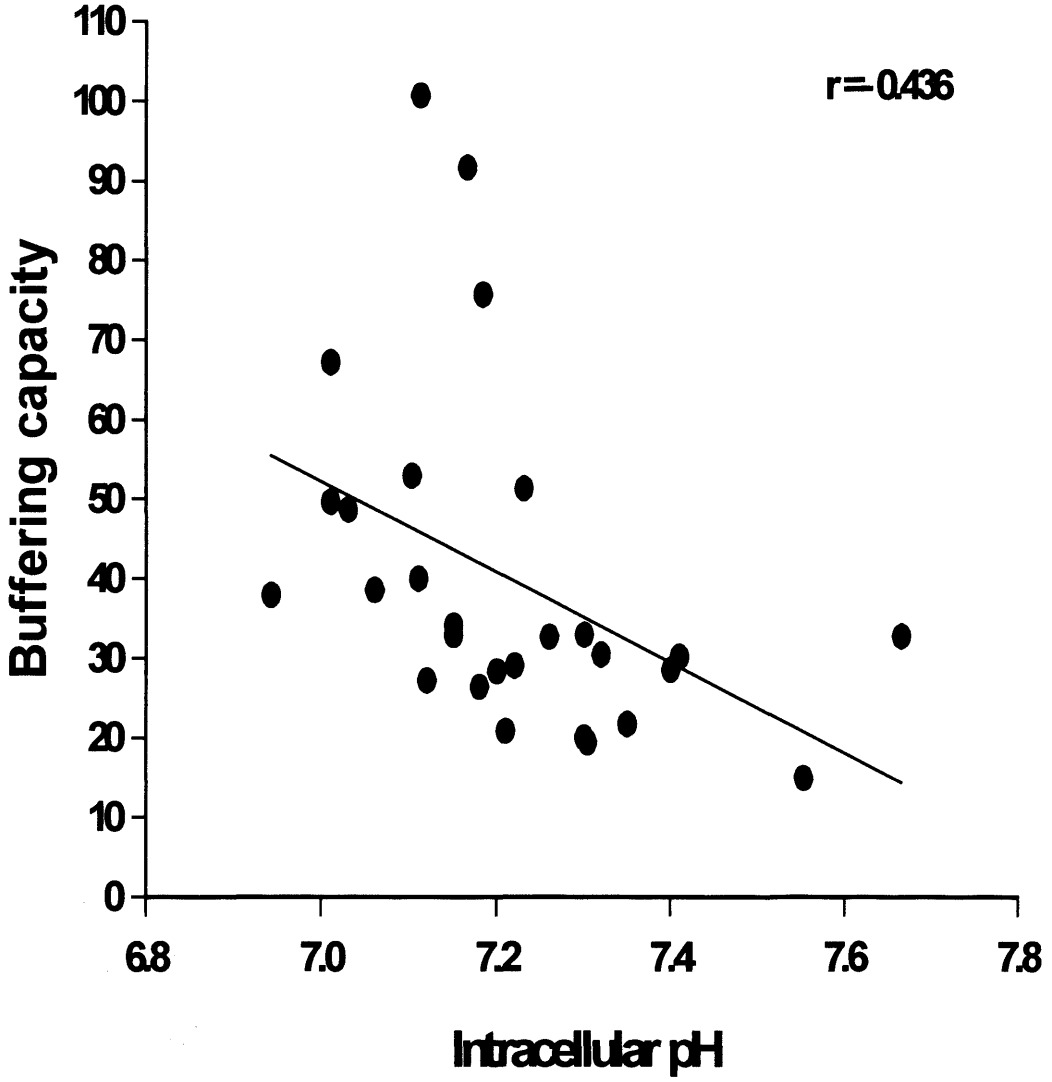


Figure 3.4: Plot of correlation between buffering capacity and intracellular pH for the whole group. A negative correlation exists for the whole group (r=-0436, P=0.02). Women whose cells were acidotic tended to have a higher buffering capacity.

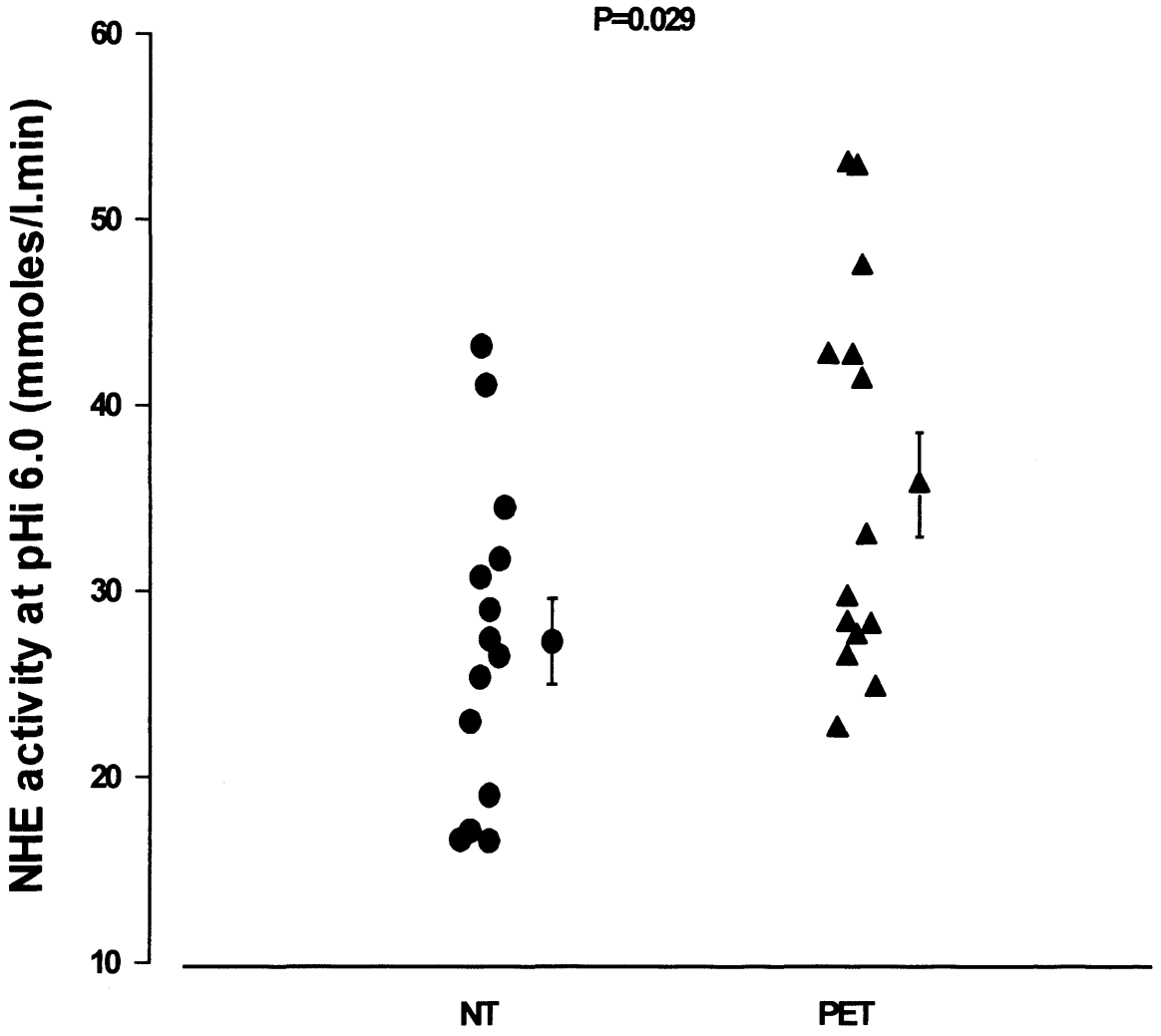


Figure 3.5: Plot of leucocyte NHE activity in post-partum normotensive (NT) and post-partum pre-eclamptic (PET) women. NHE activity was determined after clamping pH_i to 6.0, near the V_{max} of the NHE of leucocytes. The mean and SEM are plotted. The NHE activity of leucocytes from PET women (35.8 ±2.8 mmoles/l.min) was significantly elevated compared with NT women (27.3 ±2.3 mmoles/l.min).

and normotensive controls. The G252 antibody specific for NHE-1 reacted with a 92-114kD protein. The size of this protein correlated with the stated molecular mass of NHE-1 [Sardet et al. 1990]. No significant differences were identified in NHE-1 protein abundance when the 2 subject groups were compared (Table 3.2). No NHE-3 protein was identified in any of the leucocyte extracts from either post-partum pre-eclamptic or post-partum normotensive controls. Fig 3.7B shows the same Western blot as fig 3.7A that has been stripped and re-blotted with NHE-3 specific antibody (G110). G110 antibody should react with an 85kD protein but there is no evidence of NHE-3 in the leucocyte extracts which was to be expected [Kelly et al. 1997]. Both antibodies reacted with their respective target proteins in PCT extracts, as previously described [Kelly et al. 1997].

3.4.5 Na^+/H^+ Exchanger Protein Phosphorylation

Figure 3.9 shows a typical Western blot of leucocyte extracts immunoprecipitated with G252 antibody and subsequently probed with anti-phosphoserine antibody, G174 for both post-partum pre-eclamptic and post-partum normotensive controls. Ten micrograms of CB28 obtained from Prof J Poussegur (University of Nice) was used as a positive control. CB28 was developed in-house and is the C terminal of NHE-1 attached to a maltose binding protein [Sardet et al. 1990]. The CB28 allowed analysis of proteins blotted on different days. The phosphorylated NHE-1 appeared as a 100 kDa band. The density of the bands within each group showed variability but upon analysis involving densitometry, no significant differences were seen in NHE-1 protein phosphorylation (see table 3.2).

3.5 Discussion

Although altered Na^+/H^+ exchanger activity has consistently been reported in essential hypertension, few studies have examined its status in pre-eclampsia. This study examined the activity of the Na^+/H^+ exchanger in a carefully selected group of post-partum pre-eclamptic and post-partum normotensive control women. Study in the post-partum period excluded the possibility of alterations in activity that may have been influenced by the humoral changes associated with pregnancy and the pre-eclamptic state. Alterations in Na^+/H^+ exchanger activity were identified in leucocytes from the post-partum pre-eclamptic women compared to the post-partum normal controls.

	post-partum pre-eclamptics n=14	post-partum normotensives n=14
Intracellular pH	7.11 ±0.02 *	7.31 ±0.04
Buffering capacity (mmoles /l.pH unit)	51.1 ±6.5 [&]	28.7 ±2.4
NHE-1 activity (mmoles /l.min)	35.8 ±2.8[$]	27.3 ±2.3
NHE-1 protein abundance (normalised to 1 in NT)	1.10 ±0.09	1.0 ±0.09
NHE-1 phosphorylation (normalised to 2 in CB28)	2.44±0.28	2.19±0.10

*Table 3.2: Assessment of NHE-1 in post-partum pre-eclamptic and post-partum normotensive women. Data are means ±SEM *P<0.001 compared with post-partum normotensives, [&]P=0.005 compared with post-partum normotensives and [$] P=0.029 compared with post-partum normotensive (unpaired Student's t-test).*

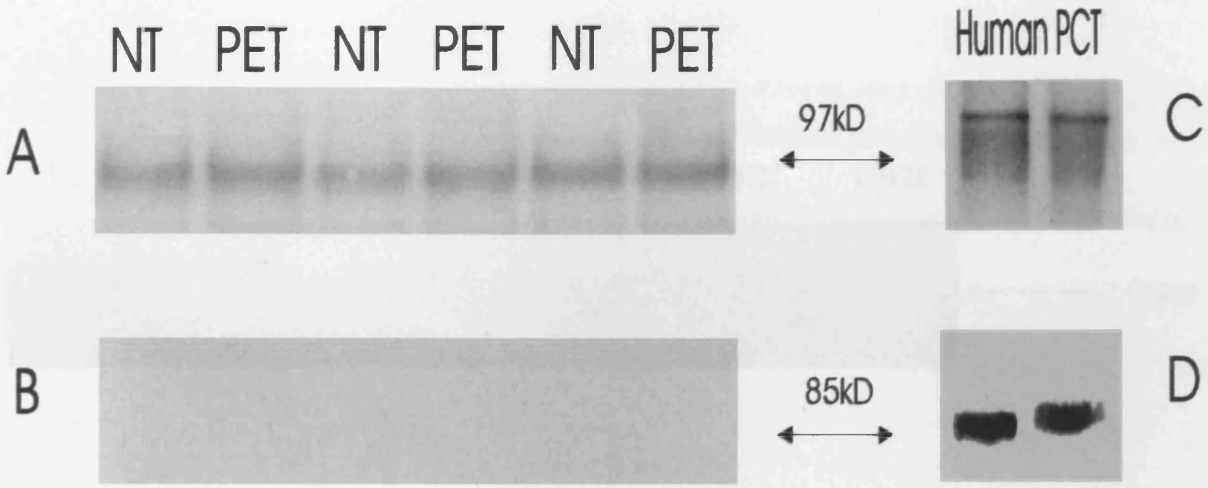


Figure 3.7: Western blot of leucocyte extracts obtained by loading equal amounts (30μg) of protein and probed with specific antibodies.

A .The blot was probed with G252 NHE-1 specific antibody. The NHE-1 protein can be visualised at 97-100 kDa. The molecular weight marker is shown at 97kDa. No difference is evident in NHE-1 protein abundance when comparing post-partum pre-eclamptics with post-partum normotensive controls.

B. The same blot probed with G110 NHE-3 specific antibody. The NHE-3 protein is usually visualised at ~85kDa. No NHE-3 protein was evident in any of the leucocyte extracts.

C. Human proximal convoluted tubule cell extracts subjected to SDS-PAGE and used as positive controls to confirm the reactivity of the NHE-1 specific antibodies. The NHE-1 control protein is visualized at 97-100 kDa.

D. Human proximal convoluted tubule cell extracts subjected to SDS-PAGE and used as a positive control to confirm the reactivity of the NHE-3 specific antibody. The NHE-3 control protein is visualized at 85kDa.

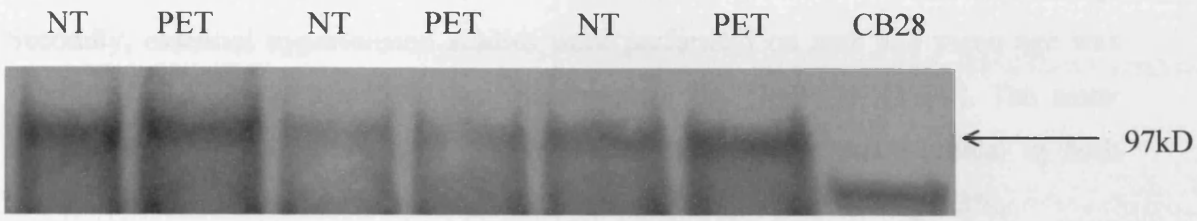


Figure 3.9: Leucocyte extracts immunoprecipitated with G252 antibody. Extracts were resolved on a 7.5% sodium dodecyl sulphate gel, transferred onto nitrocellulose and probed with anti-phosphoserine antibody. Only the band of interest is pictured. Phosphorylated NHE-1 appeared as a 100 kDa band. CB28 was used as a positive control and was the densitometry value to which the data was normalised. The post-partum pre-eclamptic subjects are denoted as PET and the post-partum normotensive controls as NT. The molecular mass marker for 97kDa is shown. There was no significant difference in the amount of phosphorylation of NHE-1 in the extracts.

The values obtained differed from those found in earlier studies that examined the exchanger activity in essential hypertension. The values reported in studies concerning essential hypertension were 48.8 ±2.1 mmoles/l.min for the normotensive subjects and 75.3 ±6.2 mmoles/l.min for the hypertensive subjects [Ng et al. 1990]. The variation in values between studies was possibly for several reasons. Firstly, time between assays was different due to the samples used in this study being collected in the community. Secondly, essential hypertension studies were performed on men and mean age was significantly different being 52 years in the study by Ng et al. [1990]. The assay adopted for the measurement of Na^+/H^+ exchanger activity was identical in both studies and reliable as evidenced from the coefficient of variation being 7.3%.

This was the first time that significant alterations in Na^+/H^+ exchanger activity have been identified in post-partum pre-eclamptics. Matteucci and Giampietro [1997] showed that Na^+/H^+ exchanger activity was elevated in erythrocytes from normal pregnant (1^{st} trimester, 10.0 ±3.5, 2^{nd} trimester, 9.65 ±2.94, 3^{rd} trimester 8.36 ±3.50 mmol h^{-1} l^{-1}) compared to non-pregnant control women (6.28 ±2.55 mmol h^{-1} l^{-1}) with the highest value being observed in the first trimester of pregnancy. These findings may have been due to the intracellular acidosis associated with pregnancy. Another study reported an elevation in platelet Na^+/H^+ exchanger activity in the 3^{rd} trimester of pregnancy (713 ±101 pmol $Na^+/10^6$) compared to non-pregnant women (415 ±20 pmol $Na^+/10^6$). The Vmax of the exchanger in women with non-proteinuric pre-eclampsia in the 3^{rd} trimester (712 ±44 pmol $Na^+/10^6$) did not differ from that of the 3^{rd} trimester pregnant controls [Graham et al. 1997]. Neither study considered the complications that may have been caused by the hormonal changes that occur during pregnancy or considered studying more representative nucleated cells as models; their observations may have been the result of both environmental and genetic influences. The methodology adopted to assess Na^+/H^+ exchanger activity was different for each study and may have contributed to the discrepancies documented.

This study suggested that women who had suffered a pre-eclamptic pregnancy more than 5 months ago had a lower intracellular pH and an enhanced Na^+/H^+ exchanger activity compared to post-partum normotensive controls. Activation of the exchanger in essential hypertension has previously been found to be associated with alkalinization

[Ng et al. 1995]. However, this did not seem to be the case in pre-eclampsia. It could be considered that the increased cellular Na^+ influx was secondary to the intracellular acidosis resulting in an increased exchange of extracellular Na^+ for intracellular H^+ ions as suggested by Resnick et al [1987]. The increased H^+ efflux may have been a primary abnormality as in essential hypertension. The Na^+/H^+ exchanger activity remains upregulated in essential hypertension even when the pH_i is manipulated to pH 6.0 [Ng et al. 1995]. In this study, the intracellular pH was clamped at pH 6.0 to enable analysis of exchanger activity near its Vmax. Therefore, this increased Na^+/H^+ exchanger activity was unlikely to be secondary to the intracellular acidosis as postulated by Resnick et al [1987]. However, the chronic effects of intracellular acidosis, leading to increased Na^+/H^+ exchanger activity from possible changes in protein phosphorylation or alterations in regulatory proteins binding to the exchanger cannot be excluded.
Intracellular pH has been found to be dependent on Na^+ dependent Cl^-/HCO_3^- exchange [Aalkjaer and Cragoe. 1988]. The measurements of intracellular pH were made in bicarbonate free solutions. The absence of bicarbonate in the present experiments enabled study of the Na^+/H^+ exchanger without the influence of Na^+ dependent Cl^-/HCO_3^- exchange. The observed differences in pH_i may not have been as marked *in vivo* due to operation of the Na^+ dependent Cl^-/HCO_3^- exchange. However there was a definite enhanced activity of the Na^+/H^+ exchanger at pH_i 6.0 when confounding factors such as a raised Na_i or lower pH_i were removed.

The lower intracellular pH in pre-eclamptic leucocytes may have been due to an up-regulation of proton producing mechanisms in these cells, e.g. an alteration in cellular metabolic acid production from mitochondria or oxidative stress. Alternatively, an altered set point of Na^+/H^+ exchange may have accounted for the lower pH, although higher Na^+/H^+ exchanger activities are usually associated with an elevated pH set point [Tokudome et al. 1990]. There is increasing evidence to suggest Na^+/K^+ATPase inhibition in pre-eclampsia that is believed to persist into the post-partum period [Lopatin et al. 1999; Bolton et al. 1994]. This inhibition of Na^+/K^+ATPase may have resulted in a lowering of intracellular pH in the post-partum pre-eclamptic cells due to an initial build up of Na^+. These hypotheses remain to be explored in subsequent chapters. There was a marked variability in intracellular pH measurements (figure 3.2) that was probably due to the limitations of the BCECF dye since the correlation between fluorescence and intracellular pH was sigmoidal, thus introducing errors at

the extremes of pH. The relationship between fluorescence ratio and intracellular pH was linear in the range 6.2-7.6 [Ng et al. 1989b]. Studies by Ng et al [1989c] in hypertension suggested that normal leucocytes had an intracellular pH within the range 7.25-7.53 and suggested that hypertensive leucocytes were markedly more alkaline with intracellular pH lying within the range 7.26-7.95.

As well as a raised leucocyte Na^+/H^+ exchanger activity, the post-partum pre-eclamptic cells also possessed a raised intrinsic buffering capacity compared to post-partum normotensive controls. This raised buffering capacity may have been attributable to the lower intracellular pH as it has been demonstrated that if intracellular pH falls below 7.0 there is a significant rise in buffering capacity which may be an important mechanism to protect against severe intracellular acid loads [Ng et al. 1989a]. The raised cellular buffering capacity could have been a response to counteract any up-regulation in intracellular acid production in these cells. The raised leucocyte Na^+/H^+ exchanger activity identified was not attributable to differences in intracellular Na^+ concentration as the cells were clamped to the same intracellular pH of 6.0 and were depleted of intracellular Na^+ (using nigericin and monensin) in order to produce a standard gradient to drive the exchanger. Na^+/H^+ exchanger activity was measured when the intracellular pH was clamped at pH 6.0 and represented the pH at which maximal transport capacity is achieved. However, it has never been established whether pH 6.0 was the Vmax for pre-eclamptic leucocytes and further studies may have proved useful to establish the optimum and provide further information concerning the kinetics of the exchanger in pre-eclamptic cells.

It must be considered that there could have been other potential confounding factors that may have influenced the comparison between post-partum pre-eclamptic and post-partum normotensive controls. Changes in dietary protein content may have influenced the leucocyte Na^+/H^+ exchanger activity. Kinetic studies have revealed evidence for an increased Vmax of the Na^+/H^+ exchanger in rats fed high protein diets compared to a low protein diet [Harris et al. 1984]. This factor could have been taken into consideration when selecting the women and it may have been helpful to control for diet. Secondly, increased Na^+/H^+ exchanger activity appeared to be characteristic in overweight subjects [Harris et al. 1984]. Failure to control for weight in the post-partum pre-eclamptic and post-partum normal control women may have introduced

confounding variables that warrant discussion. Finally, plasma cholesterol has been shown to influence Na^+/H^+ exchanger activity [Graham et al. 1997]. The relationship between Na^+/H^+ exchanger activity and plasma cholesterol concentration was examined and plasma cholesterol was found to correlate positively with the Vmax of the Na^+/H^+ exchanger in normal pregnancy [Graham et al. 1997]. It would have been interesting to measure this parameter in the plasma of the women included in this study and examine the effect of plasma cholesterol and the contribution of this to the reported increased exchanger activity in post-partum pre-eclamptic cells.

Previous studies investigating essential hypertension and diabetes have assessed Na^+/H^+ exchanger protein abundance using a specific Na^+/H^+ exchanger antibody (G252) [Ng et al. 1995]. This technique was applied to the leucocyte extracts from both post-partum pre-eclamptic and post-partum normotensive control women. No significant differences were identified suggesting that this increased Na^+/H^+ exchanger activity was not due to differences in NHE-1 protein abundance. These findings were similar to those described in essential hypertension [Ng et al. 1995]. However, it remains that the antibody may have been insufficiently sensitive to show alterations in abundance of the protein and it must be noted that ECL methodology has detection limitations. It is possible that the presence of other isoforms may have contributed to the enhanced Na^+/H^+ exchanger activity observed in the post-partum pre-eclamptics. The abundance of Na^+/H^+ exchanger isoform 3 was examined as specific antibodies to the isoform were readily available. No Na^+/H^+ exchanger isoform 3 protein was identified in any of the leucocyte extracts confirming that enhanced Na^+/H^+ exchanger activity in the post-partum pre-eclamptics was not due to the presence of Na^+/H^+ exchanger isoform 3. However, it seemed unlikely that this protein would be present since Na^+/H^+ exchanger isoform 3 is confined to the apical location in the proximal tubules of the kidney suggesting its importance in the control of salt reabsorption [Kelly et al. 1997].

Post-translational processes have been shown to alter Na^+/H^+ exchanger activity. It was unlikely that N-linked glycosylation was responsible for the enhanced activity as no significant differences were identified in the molecular weight glycoform bands between post-partum pre-eclamptics and post-partum normotensives. In addition, Counillon et al. [1994] analysed the potential outcome of deglycosylation by site-

directed mutagenesis at the N-glycosylation site (Asn 75). The cDNA containing the mutation was generated and expression of the cDNA in antiporter deficient PS120 fibroblasts showed that all of the transporters had transport rates similar to the wild type molecule thus indicating that N-linked glycosylation of the Na^+/H^+ exchanger may not alter its activity [Counillon et al. 1994]. Secondly, phosphorylation is a post-translational process that can alter activity of the exchanger. It was possible that altered Na^+/H^+ exchanger activity was due to enhanced phosphorylation of the protein as observed in essential hypertension [Kelly et al. 1997]. Increased phosphorylation can be due to enhanced signal transduction upstream of the Na^+/H^+ exchanger protein. Siffert et al [1998] have identified enhanced signal transduction via pertussis toxin sensitive G proteins with the detection of a novel polymorphism in exon 10 of the gene encoding the beta 3 subunit of G proteins that may be associated with enhanced Na^+/H^+ exchanger activity. However, no differences in the phosphorylation of Na^+/H^+ exchanger protein were identified between post-partum pre-eclamptic and post-partum normotensive extracts as the antibody may have been insufficiently sensitive especially in identifying very small changes in phosphorylation of serine residues.

However, phosphorylation is not the only control mechanism of Na^+/H^+ exchange since mutational and deletion analyses show that half the transport activity in response to insulin/thrombin remains following deletion of the phosphorylation sites [Wakabayashi et al. 1992]. A further mechanism that may be considered is the calcium calmodulin binding domain, with a rise in intracellular calcium stimulating Na^+/H^+ exchanger activity [Siczkowski et al. 1997]. However, investigation of this hypothesis would require intracellular calcium to be measured in leucocytes to assess the contribution of this parameter to increased activity of the exchanger in women who have suffered a pre-eclamptic pregnancy. Increased Na^+/H^+ exchanger activity in the post-partum pre-eclamptic cells may have been dependent on other accessory proteins interacting with the C-terminal domain of the exchanger that have been suggested by Silva et al. [1995] and Goss et al [1996]. However, before this hypothesis could be investigated these proteins need to be identified and characterised with regards to the activation of the Na^+/H^+ exchanger.

3.6 Conclusion

In conclusion, post-partum pre-eclamptic women had a lower leucocyte intracellular pH when measured under bicarbonate free conditions. The Na^+/H^+ exchanger activity and buffering capacity were increased in cells isolated from post-partum pre-eclamptic women compared to post-partum normotensive controls. The increased Na^+/H^+ exchanger activity was not due to differences in protein abundance and no difference in the phosphorylation of the exchanger protein was identified. However, phosphorylation is not the sole control mechanism of the exchanger. Non-phosphorylation-dependent mechanisms of control may have been responsible for alterations in activity in the post-partum pre-eclamptic cells. Such mechanisms include altered intracellular calcium with the direct binding to calmodulin and interactions with accessory proteins. The enhanced Na^+/H^+ exchanger activity may contribute to the increased leucocyte Na^+ content found in post-partum pre-eclampsia as hypothesised and may be considered an intermediate phenotype in the pathogenesis of the pre-eclamptic pregnancy itself.

Chapter 4

Assessment of Neutrophil Intracellular pH and Na^+/H^+ Exchanger Activity in Pre-eclampsia.

4.1 Introduction

In the leucocyte, higher cellular Na^+ levels have been demonstrated in pre-eclampsia compared to normal pregnant controls which suggests the possibility of alterations in cellular sodium transport pathways [Seon and Forrester, 1989]. Cellular cation handling, especially in the kidney, plays an important role in both volume homeostasis and control of blood pressure and may therefore be important in the pathogenesis of pre-eclampsia. Changes have been described in Na^+/K^+ATPase, Na^+/K^+, Na^+/Li^+ and Na^+/H^+ exchange activity with regards to normal pregnancy [Matteucci and Giampietro 1997; Worley et al. 1980; Heilmann et al. 1993; Lindheimer and Katz 1971]. Sodium electrochemical gradients across cell membranes are sustained primarily by the Na^+/K^+ATPase. This pump maintains low intracellular sodium and high intracellular potassium concentrations, while in the kidney, the same enzyme system implements tubular sodium reabsorption. In normotensive pregnancy, the function of this enzyme is increased and results in a lowered intracellular sodium concentration and aids in gradual renal sodium retention [Gallery et al. 1988; Tranquilli et al. 1988]. Pre-eclampsia is associated with the presence of a circulating inhibitor of this pump, consequently resulting in a reduction in pump function and an increase in intracellular sodium. [Kaminski and Rechberger 1991; Miyamoto et al. 1992]. Based on the results reported in the previous chapter, it seems that Na^+/H^+ exchanger activity may also contribute to alterations in cellular sodium transport in pre-eclampsia providing the differences are present in the actual disease state.

This chapter tests the hypothesis that neutrophils isolated from pre-eclamptic women may show alterations in Na^+/H^+ exchanger activity that contribute to the increased intracellular sodium recognised in the disease. This altered activity may be implicated in the pathophysiology of pre-eclampsia and thus serve as an intermediate phenotype in the disease.

Only one study exists that assessed the role of the Na^+/H^+ exchanger in pre-eclampsia. No differences were reported when considering pre-eclamptic and normotensive pregnancies [Graham et al. 1997]. This study was complicated by the use of 'non proteinuric' pre-eclamptics as the study group and the use of platelets as models [Graham et al. 1997]. The work in this chapter aimed to investigate a putative role for Na^+/H^+ exchanger activity in the pathogenesis of pre-eclampsia using well-defined 3rd trimester pre-eclamptic women and representative nucleated cells. The previous chapter reported the presence of changes in the post-partum period and suggests that the abnormality perhaps stems from the pre-eclamptic pregnancy or even before which may suggest a genetic basis to this transport abnormality. The presence of a lower intracellular pH in post-partum pre-eclamptic cells (chapter 3) was established and this study aimed to identify the presence of this phenotype in pre-eclampsia. Neutrophils were used instead of a mixed leucocyte pellet as subsequent experiments needed pure neutrophils to assess the activity of the NADPH oxidase enzyme. A leucocyte pellet consists of approximately 70% neutrophils and since comparisons were not intended between 3rd trimester and post-partum women, the use of a different cell type was undertaken. In addition, Frighi et al. [1991] found that the intracellular pH and Na^+/H^+ exchanger activity of neutrophils was very similar to that of a mixed leucocyte pellet. The correlation between Na^+/H^+ exchanger activity in mixed leucocytes and neutrophils was significant (r_s=0.94, P<0.01) [Frighi et al. 1991].

Time course experiments were performed to assess the effect of environmental (plasma) influences on Na^+/H^+ exchanger activity and intracellular pH. Na^+/H^+ exchanger protein abundance and phosphorylation were not analysed in this study. Protein abundance was unlikely to show any alterations considering the results obtained in chapter 3 and differences that may exist in phosphorylation of the Na^+/H^+ exchanger are difficult to show using non-radioactive immunoprecipitation protocols (see chapter 3).

4.2 Materials and Methods

The methods used for assessing Na^+/H^+ exchanger activity and measuring intracellular pH are included in chapter two of this thesis.

4.3 Patients

Seventeen well defined pre-eclamptic women and seventeen normotensive pregnant women matched for age and gestation were evaluated. None of the women had a family history of essential hypertension. (Table 4.1). The average age of the pre-eclamptic women was 28 years (range 22-40), the aged-matched controls was 29 years (range 23-41). The mean age of both the 3rd trimester pre-eclamptic women and the age-matched controls was lower that the mean age of both post-partum pre-eclamptic women and the age-matched controls. This had no effect on the results since there was no correlation found between age and Na^+/H^+ exchanger activity and age and intracellular pH in either of the groups. Two of the pre-eclamptic women had previously suffered from a pre-eclamptic pregnancy. Blood pressure was significantly higher in the women who suffered from pre-eclampsia (P=0.001). No differences in blood pressure measurements at the antenatal booking visit were identified (12-16 weeks gestation). Gestational age at delivery and birthweight were significantly lower in the women who suffered a pre-eclamptic pregnancy (P=0.002 and P=0.035 respectively). Pre-eclamptic women tended to deliver earlier than the normotensive pregnant controls, which would have affected gestational age and the birth weight. Clinical and laboratory details of the subjects are shown in table 4.1. Note that no laboratory details are illustrated for the control women due to the normal progression of their pregnancy.

4.4 Results

4.4.1 Intracellular pH

The results for intracellular pH are illustrated in figure 4.1. Unpaired Student's t-test suggested that the mean intracellular pH of the groups was significantly different ($P<0.001$). The mean value for the normal control cells was 7.36 ± 0.027. Women with pre-eclampsia possessed cells that were significantly more acidotic, with a mean intracellular pH of 7.15 ± 0.050.

4.4.2 Buffering Capacity

The results for buffering capacity are illustrated in figure 4.2. The results were analysed using an unpaired Student's t-test. The buffering capacity of neutrophils from pre-eclamptic women (40±3.90 mmoles/l.pH unit) was significantly elevated compared to normotensive women (26±1.3 mmoles/l.pH unit P=0.005).

TABLE 4.1
CLINICAL DATA

	PET	NT
Patients (n)	17	17
Age (years)		
Mean	28 (±1.02)	29 (±1.2)
Range	22-40	23-41
Gestation (weeks)		
Mean	34^{+4} (±1.0)	39^{+5} (±0.24) *
Range	26-40	38-41
Mode of Delivery		
C/S	9	4
Spontaneous	0	11
Induced	8	2
BP booking (mmHg)		
Mean	115/70 (±3.6)	112/65 (±1.9)
Range	100/60-140/85	100/60-120/80
BP highest (mmHg)		
Mean	160/106 (±2.9)	112/66 (±1.5) **
Range	140/94-186/131	100/160-120/70
Gestational age at sampling (weeks)		
Mean	35^{+4} (±1.0)	36^{+1} (±1.9)
Range	$27\text{-}40^{+2}$	$31^{+2}\text{-}41^{+4}$
Smoke		
Yes	2	5
No	15	12
Family History		
PET	0	0
Hypertension	0	0
Previous PET	2	0
Parity		
1	10	9
>1	7	8
Sex of Newborn		
Female	7	9
Male	10	8
Weight of Newborn		
Mean	6lb4oz (±0.64)	8lb (±0.29) ***
Range	1lb6oz-9lb8oz	6lb3oz-10lb4oz

Urine (protein)		
Mean 24 hour	2.013 (±0.38)	
Range	0.3-4.5	Nil
Lowest Hb (g/dl)		
Mean	10.87 (±0.409)	N/A
Range	8.2-13	
Lowest Platelets (10^9/L)		
Mean	193.8 (±18.5)	N/A
Range	34-262	
Maximum Urate (mmol/L)		
Mean	365 (±20.8)	N/A
Range	236-499	
Maximum Creatinine (μmol/L)		
Mean	77.9 (±5.44)	N/A
Range	59-143	
Maximum Urea (mmol/L)		
Mean	4.4 (±0.54)	N/A
Range	2.4-9.6	
Maximum ALT (U/L)	59 (±11)	N/A
Mean	7-131	
Range		

*Table 4.1: Clinical data for the 3rd trimester pre-eclamptic and 3rd trimester normotensive control. Values are means with range and SEM *P=0.002, **P=0.001, ***P=0.035, normotensive versus pre-eclamptics by Student's t-test. NA=not applicable.*

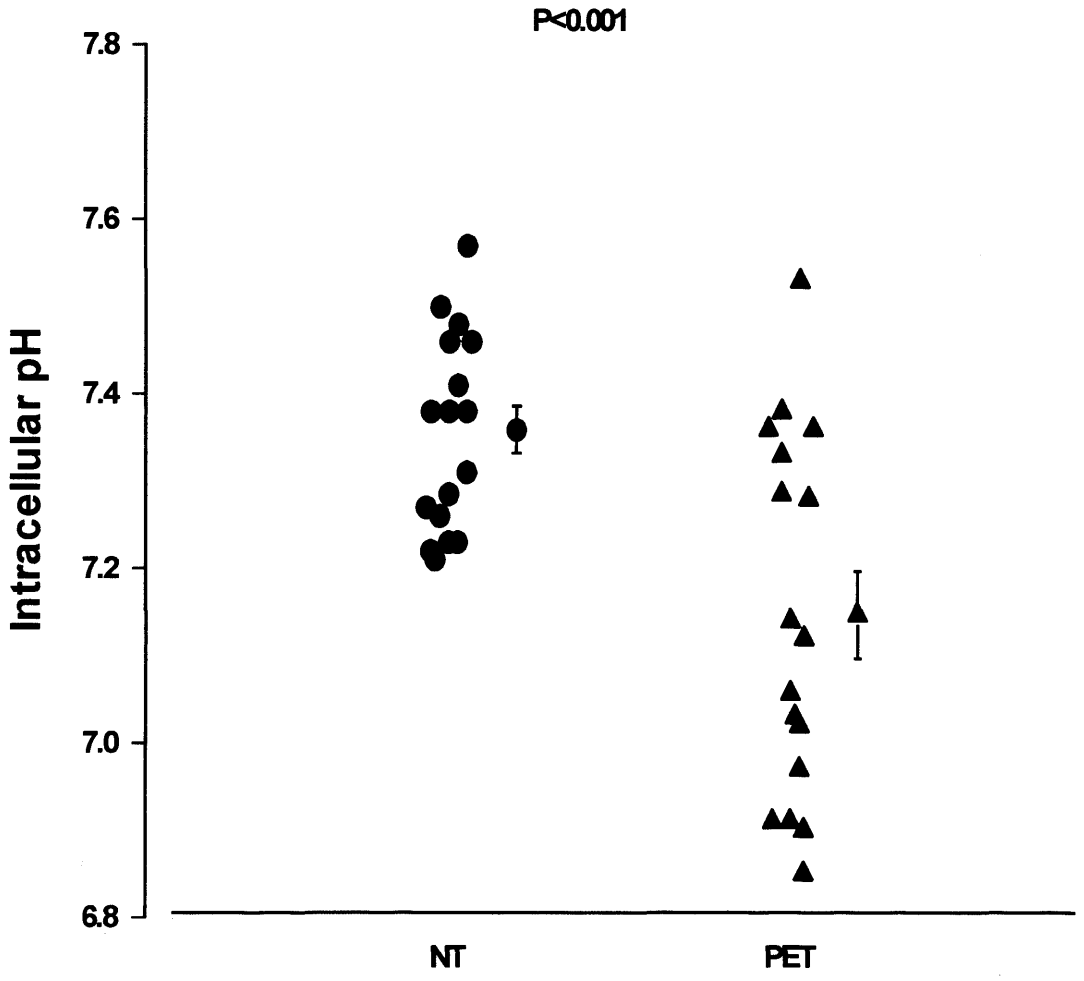


Figure 4.1: Plot of neutrophil intracellular pH from 3rd trimester normotensive (NT) and pre-eclamptic (PET) women. The mean and SEM are plotted. PET women exhibited a lower intracellular pH (7.15 ± 0.050) compared with NT women (7.36 ± 0.027, P<0.001, Student's t-test).

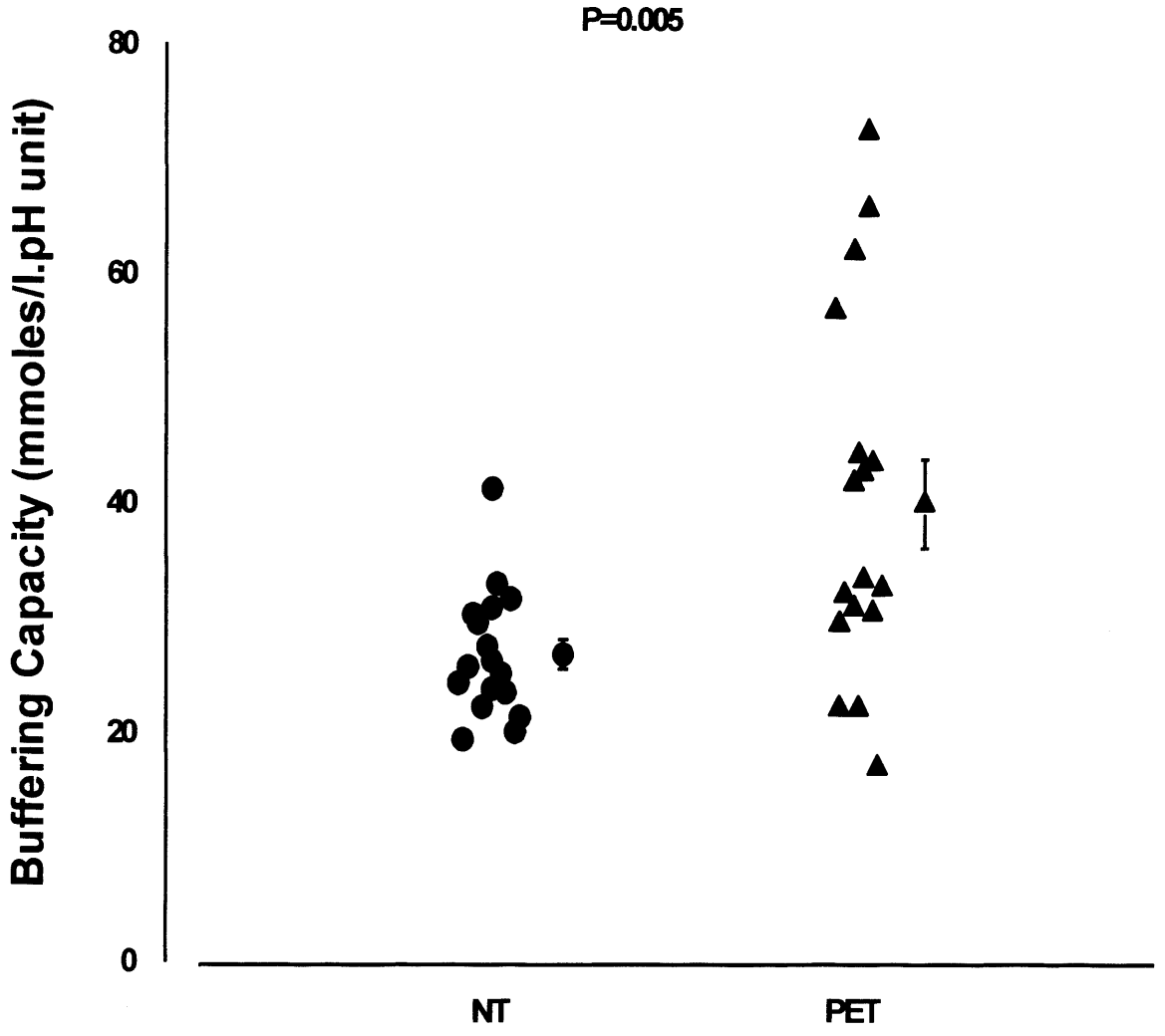


Figure 4.2: Plot of neutrophil buffering capacity measured at pH_i 6.0 from 3rd trimester normotensive (NT) and pre-eclamptic (PET) women. The mean and SEM are plotted. The mean buffering capacity of neutrophils from PET women (40±3.9 mmoles/l.pH unit.) was significantly elevated compared with NT women (26.82±1.3 mmoles/l.pH unit, P=0.005, Student's t-test).

4.4.3 Na^+/H^+ Exchanger Activity

Figure 4.3 demonstrates Na^+/H^+ exchanger activity of neutrophils after clamping intracellular pH to 6.0, near the Vmax. The mean maximal transport capacity was significantly higher in the pre-eclamptic cells, with a Vmax of 32.35 ± 1.9 mmol/l.min compared to a control Vmax of 27.09 ± 1.6 (P=0.038) using an unpaired Student's t-test.

4.4.4 Influence of Plasma Factors on Intracellular pH and Na^+/H^+ Exchanger Activity.

After the initial assessment of the intracellular pH and Na^+/H^+ exchanger activity, the cells were incubated in TC199 medium at 37 °C for 2 hours and then analysed once more. This allowed the cells to be examined further away from the influence of "plasma factors". Differences between initial and 2 hour measurements may have indicated the influence of plasma factors on both intracellular pH and exchanger activity. Figure 4.4 illustrates the effect of plasma factors on intracellular pH. Intracellular pH was measured immediately after neutrophil isolation and 2 hours later in both 3rd trimester pre-eclamptic and 3rd trimester control neutrophils. Mean 3rd trimester control intracellular pH showed no significant differences when analysed using a paired Student's t-test whereas mean pre-eclamptic neutrophil intracellular pH increased from 7.11±0.049 to 7.25±0.043. Therefore cells isolated from pre-eclamptic women showed a significant rise in intracellular pH when measured 2 hours after isolation from whole blood. Buffering capacity was also analysed using the same rationale. Neutrophil mean buffering capacity did not alter significantly in either 3rd trimester pre-eclamptic or 3rd trimester pregnant control neutrophils. Figure 4.5 illustrates this data. Finally, Na^+/H^+ exchanger activity was assessed in the same way and showed no significant differences in either 3rd trimester pre-eclamptic or 3rd trimester pregnant control patient neutrophils. Figure 4.6 illustrates these findings. Plasma was re-added to the neutrophils to assess the effect on the intracellular pH measurements; however, these experiments proved unsuccessful and gave the opposite results to those expected since HCO_3 present in the plasma buffered the intracellular pH to higher values.

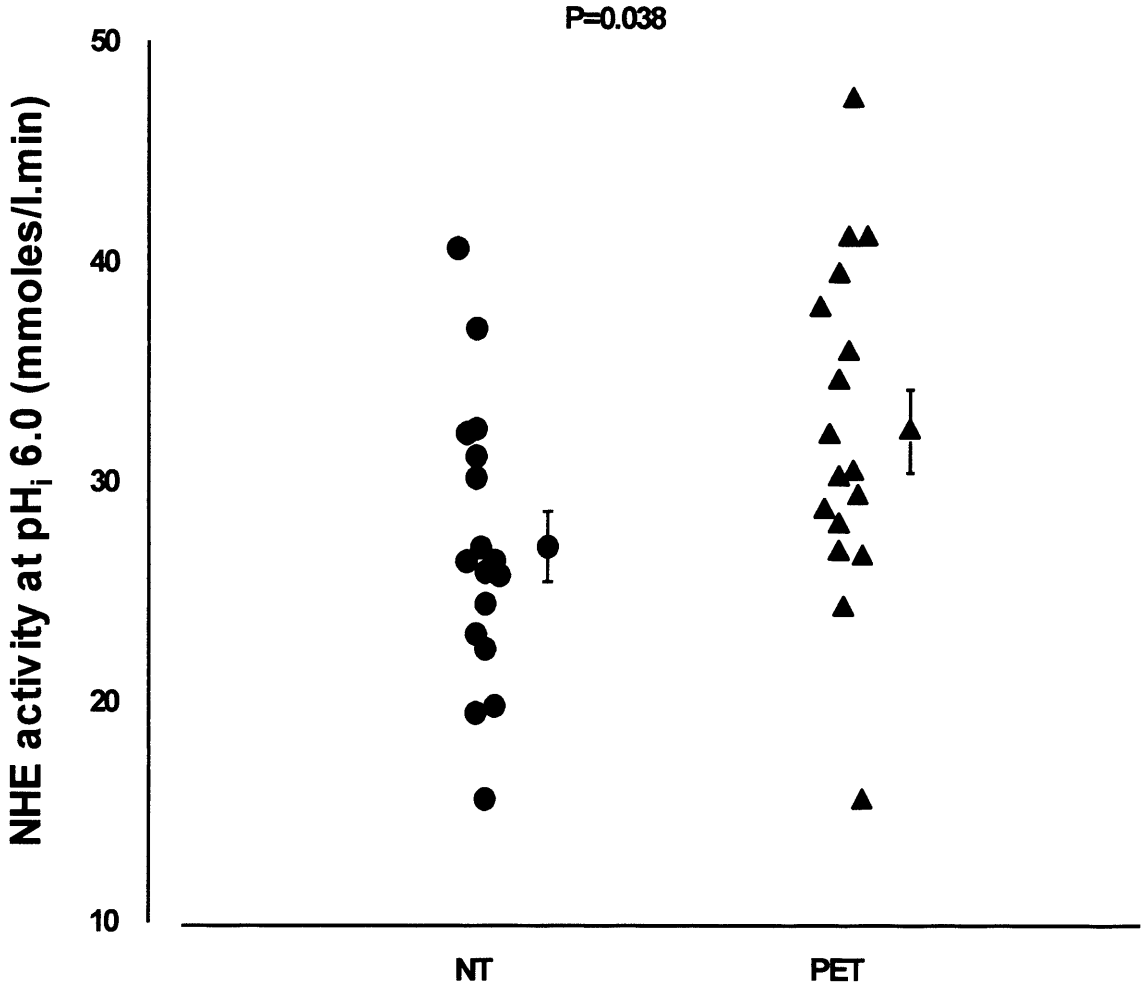


Figure 4.3: Plot of neutrophil Na^+/H^+ exchanger activity in 3rd trimester normotensive (NT) and pre-eclamptic (PET) women. Na^+/H^+ exchanger activity was determined after clamping pH_i to 6.0, near the Vmax of the exchanger. The mean and SEM are plotted. The mean Na^+/H^+ exchanger activity of neutrophils from PET women (32.35 ±1.9 mmol/l.min) was significantly elevated compared with NT women (27.09 ±1.6 mmol/l.min, P=0.038, Student's t-test).

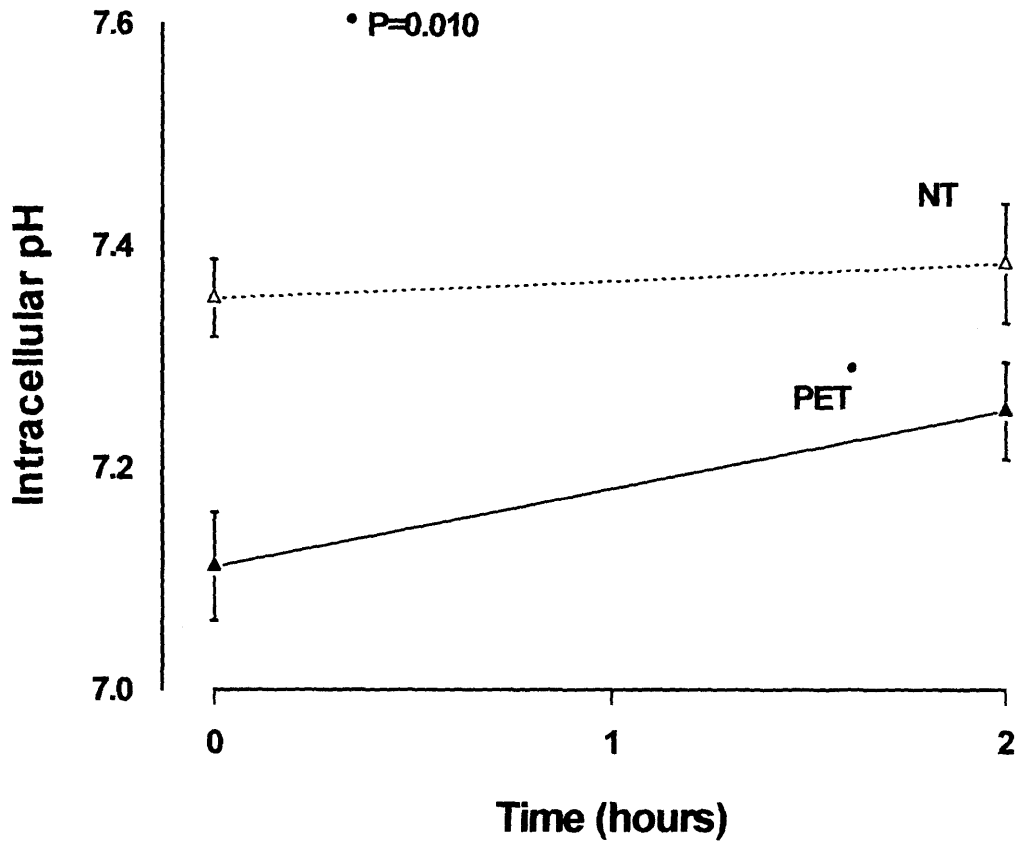


Figure 4.4: Neutrophil mean pH_i of both 3rd trimester normotensive (NT) and pre-eclamptic (PET) women is plotted against time after cell isolation. There was a significant increase in pH_i from 0 – 2 hours in the PET women (7.11 ± 0.0490 vs 7.25 ± 0.0438, P=0.010, paired Student's t-test, n=17).

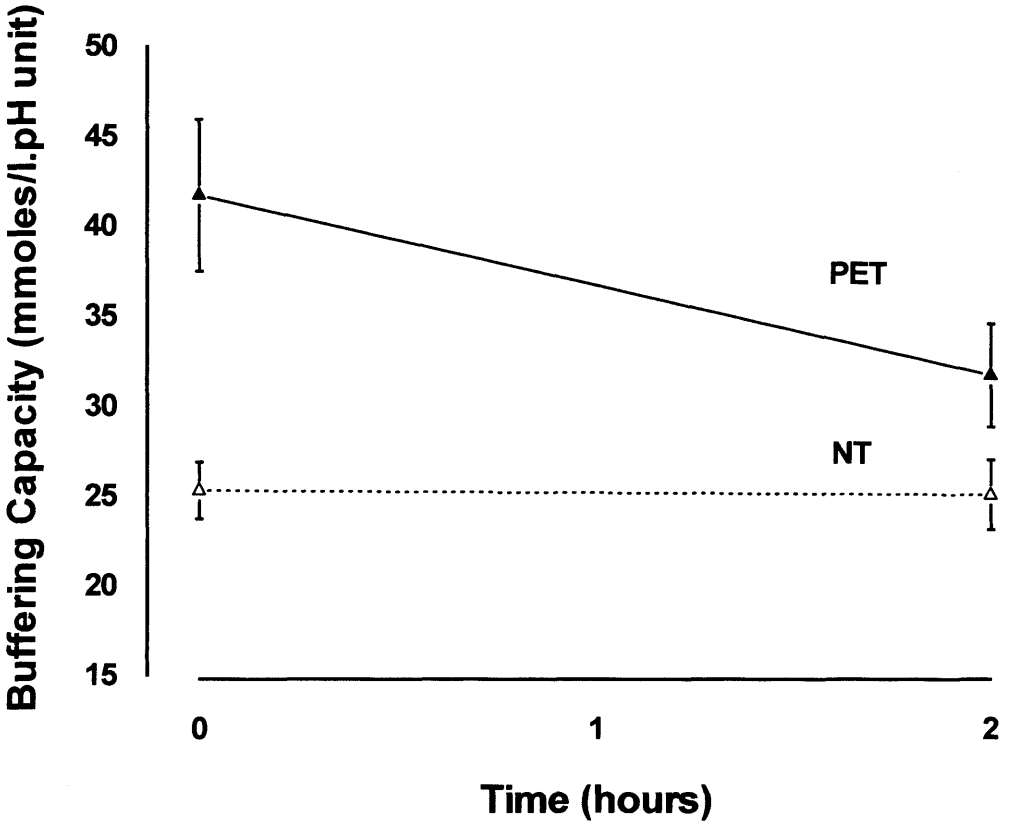


Figure 4.5: Neutrophil mean buffering capacity of both 3rd trimester normotensive (NT) and pre-eclamptic (PET) women is plotted against time after cell isolation. There were no significant differences present in buffering capacity from 0 – 2 hours in the PET women or NT women.

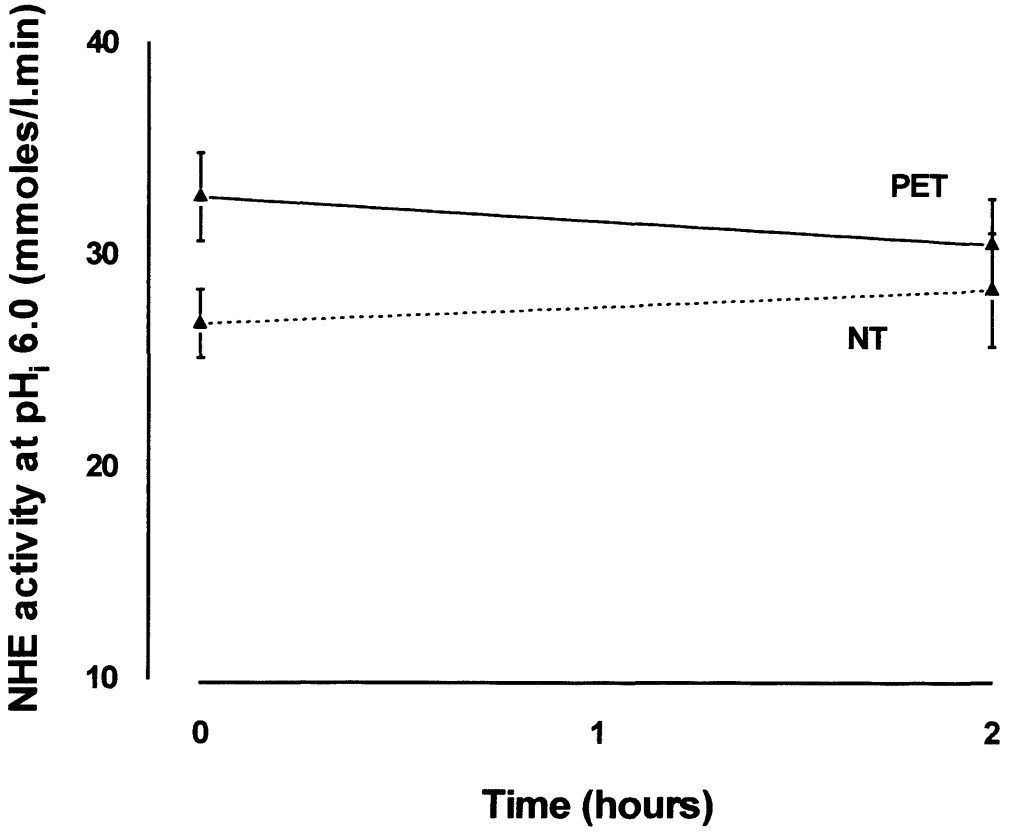


Figure 4.6: Neutrophil mean Na^+/H^+ exchanger activity of both 3rd trimester normotensive (NT) and pre-eclamptic (PET) women plotted against time after cell isolation. There were no significant differences present in Na^+/H^+ exchanger activity from 0 – 2 hours in the PET women or NT women.

4.5 Discussion

The data presented in this chapter provides evidence that Na^+/H^+ exchanger activity is elevated in neutrophils isolated from pre-eclamptic women with respect to cells isolated from normotensive pregnant controls. This is the first time that this phenomenon has been demonstrated in women with pre-eclampsia. Only one study to date exists concerning the activity of the exchanger in pre-eclampsia and as mentioned previously, no differences were identified between the pre-eclamptic and control women [Graham et al. 1997]. These finding were in disagreement with the findings of this study since significant differences were established. The reason for the discrepancies was perhaps due to the inclusion criteria of the patient group. The study by Graham et al. [1997] considered whether Na^+/H^+ exchanger activity was altered in non-proteinuric pre-eclampsia. Pre-eclamptic women examined in this study were selected according to the tightly defined criteria of Davey and MacGillivray [1988]. This study group also included multiparous as well as primiparous women. Barden et al. [1999] suggested that studies investigating the pathophysiology of pre-eclampsia should include multiparous women as investigations performed did not indicate a different pathophysiology.

The cause of the enhanced Na^+/H^+ exchanger activity in cells isolated from pre-eclamptic women is unknown but possible mechanisms concerning the enhanced Na^+/H^+ exchanger activity observed in the post-partum pre-eclamptic group were briefly discussed in chapter 3. The buffering capacity was increased in pre-eclamptic cells and the intracellular pH was found to be more acidotic, findings consistent with the results presented in chapter 3. Once more, the raised cellular buffering capacity could be a response to counteract any up-regulation in intracellular acid production in these cells. The 3rd trimester pre-eclamptic cells were more acidotic than the 3rd trimester controls. Again, it was unclear which mechanisms were responsible for this acidosis.

Experiments were performed to assess the effect of plasma factors on the measurement of Na^+/H^+ exchanger activity, intracellular pH and buffering capacity. Neutrophils were isolated and separated from whole blood. Measurements were performed immediately and after 2 hours. This small study provided some interesting results concerning the nature of the intracellular acidosis reported in pre-eclamptic neutrophils. The results suggested that factor(s) in the pre-eclamptic plasma may have influenced the

intracellular pH as pH started to approach values near to that obtained for normotensive controls. Possible plasma factors that influence intracellular pH and the relevance of these to pre-eclampsia are still unknown at present. The results, however, may have been influenced by the incubation medium. The TC199 may have had constituents that contributed to the increase in intracellular pH in the pre-eclamptic neutrophils. Therefore the constituents may have been replaced that had been lacking in the pre-eclamptic plasma, thus causing the intracellular pH to increase. Add back experiments would have proved useful and may have provided further support for the presence of a plasma factor(s) influencing intracellular pH. These were attempted but were unsuccessful upon performance due to the presence of HCO_3 in the plasma samples.

It seemed that plasma factors had some influence on both buffering capacity and Na^+/H^+ exchanger activity in pre-eclamptic neutrophils although the changes were non-significant. Some of these changes may have represented variation in measurements (especially in Na^+/H^+ exchanger activity) as indicated by the standard error of the mean perhaps resulting from damage to the neutrophils due to incubation time away from the *in vivo* environment.

Pre-eclampsia is associated with the presence of an endogenous digoxin-like factor that circulates in the plasma [Lopatin et al. 1999]. HPLC purified immunoreactive material from pre-eclamptic plasma inhibited Na^+/K^+ATPase activity. The study suggested, that in pre-eclampsia, plasma concentrations of this digoxin-like factor were enough to substantially inhibit the sodium pump in cardiovascular tissues, and were in accordance with the views attributing endogenous digitalis-like factors a pathogenic role in the pre-eclamptic hypertension [Lopatin et al. 1999]. It is possible that the presence of this factor in the pre-eclamptic plasma may have been associated with the lower intracellular pH. Evidence to strengthen this argument comes from the work of Souza et al. [2000] who examined the involvement of intracellular pH and Ca^{2+} in Na^+/K^+ATPase inhibition in cardiac myocytes. They reported that the addition of 100 μM ouabain (an inhibitor of the Na^+/K^+ATPase) to myocytes resulted in a large increase in $[Ca^{2+}]_i$ (200%) and a decrease in pH_i. The intracellular acidification was prevented by the removal of extracellular calcium and inhibition of the Na^+/H^+ exchanger had no effect on the ouabain-induced acidification. This suggested that the intracellular acidification was mediated by an initial Ca^{2+} influx via Na^+/Ca^{2+} exchanger that may have activated

the Ca^{2+}/H^{+} exchange system [Souza et al. 2000]. Intracellular pH and calcium had no effect on Na^{+}/K^{+}ATPase activity and are therefore likely a consequence rather than a cause of pump inhibition [Ehrenfeld et al. 1992]. Although it is a little too ambitious to attribute the presence of this digoxin-like factor accountable for the acidotic pH it is certainly an attractive hypothesis that needs to be further examined.

4.6 Conclusion

In conclusion, pre-eclamptic women had a lower neutrophil intracellular pH when measured under bicarbonate free conditions compared to normotensive pregnant controls. Intracellular pH seemed to be influenced by a plasma factor(s). The Na^{+}/H^{+} exchanger activity and buffering capacity were increased in pre-eclamptic neutrophils compared to neutrophils isolated from normotensive pregnant women as hypothesised. The Na^{+}/H^{+} exchanger activity in pre-eclamptic neutrophils was not significantly influenced by removing the cells from their surrounding environment. This suggested that the intermediate phenotype of increased exchanger activity was mainly influenced by genetic rather than environmental (plasma) factors. These hypotheses were further evaluated by examination of the phenotype in continuous cell lines derived from the women studied in chapter 3 and 4.

Chapter 5

Examination of Genetic Influences on Intracellular pH and Na^+/H^+ Exchanger Activity in Pre-eclampsia Using Epstein–Barr Virus Immortalized Lymphoblasts as Models

5.1 Introduction

It has been postulated that the intermediate phenotype of increased Na^+/H^+ exchanger activity is determined by genetic factors, since in essential hypertension, diabetes and rat models of hypertension the increased exchanger activity persists in cultured skin fibroblasts, lymphoblasts, cultured proximal convoluted tubule cells and vascular smooth muscle cells, where cells are removed from the influences of plasma factors in vivo [Siczkowski et al. 1994b; Sweeney et al. 1995; Ng et al. 1995; La Pointe et al. 1997; Ng et al. 2000]. Thus, such ion transport abnormalities associated with pre-eclampsia and the post-partum may be determined by genetic factors rather than the extracellular milieu present *in vivo*. The possibility remains that the differences in intracellular pH observed in pre-eclamptic cells, persisting into the post-partum period may be partially plasma mediated as suggested by the results obtained in chapter 4. This chapter tests the hypothesis that the acidotic intracellular pH associated with pre-eclampsia which extends into the post-partum period may be influenced by plasma (environmental) factors and therefore would not persist after immortalization of cells isolated from these women.

In contrast, genetic influences contribute to Na^+/H^+ exchanger activity in essential hypertension. This chapter also tests the hypothesis that differences in Na^+/H^+ exchanger activity may persist in immortalized cells from pre-eclamptic and post-partum pre-eclamptic women compared to their respective controls. This would provide further evidence of the genetic influences on this intermediate phenotype in pre-eclampsia.

The objective was to conduct measurements in the absence of the influence of plasma (environmental) factors *in vivo* using a model in which the phenotypic changes may persist despite transformation and culture in vitro (Figure 7.1). Study using EBV

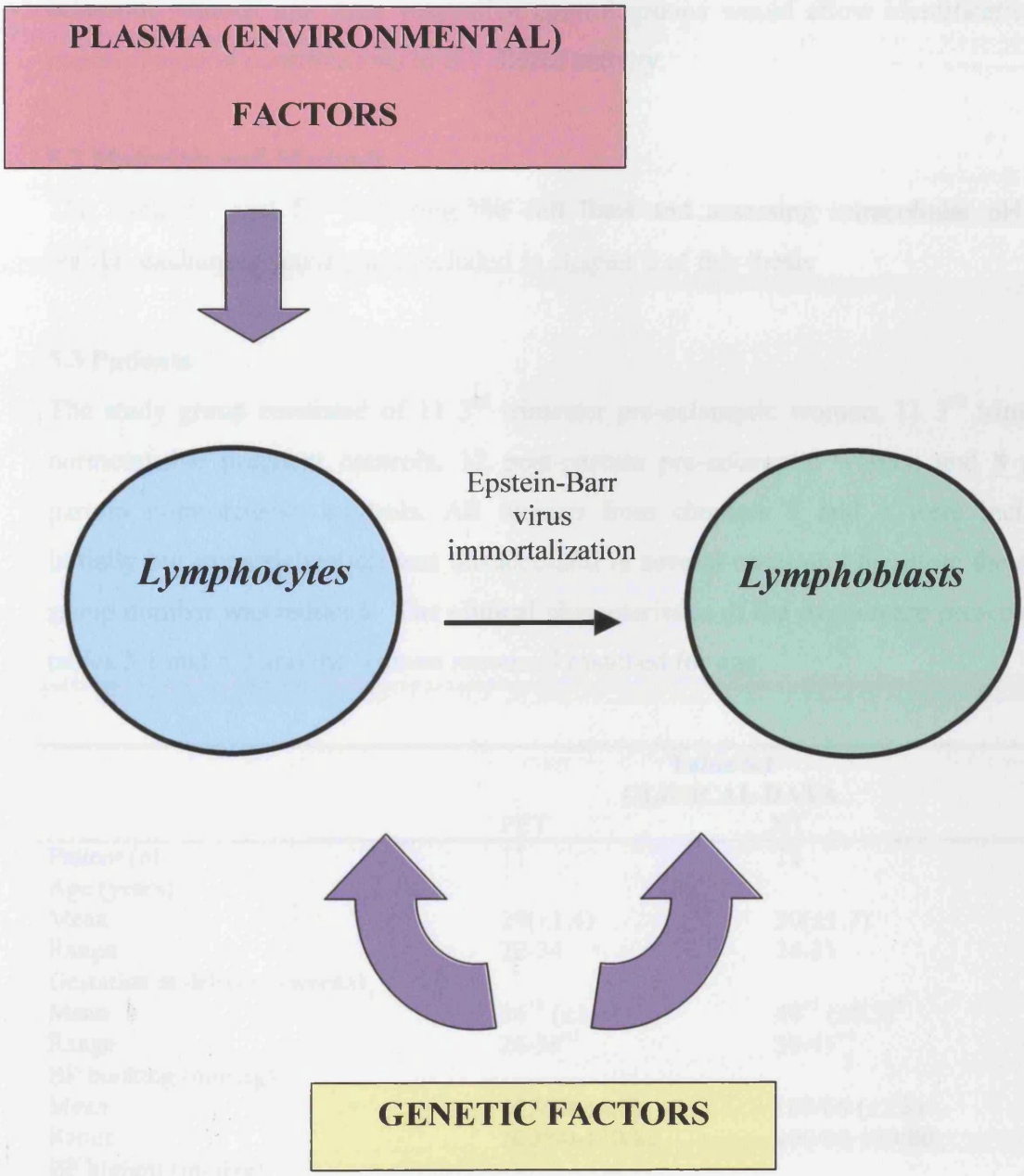


Figure 5.1: Schematic representation of the hypothesis underlying the study presented in this chapter. Genetic and plasma (environmental) factors interact to determine cell function in relation to manifestations of disease. Transforming the cells into a continuous cell line with the use of Epstein-Barr virus removed the environmental effects and allowed assessment of the contributing genetic influences.

immortalised lymphoblasts from both 3rd trimester pre-eclamptic and post-partum pre-eclamptic women and their respective control groups would allow identification of genetic/humoral contributions to the altered activity.

5.2 Materials and Methods

The methods used for preparing the cell lines and assessing intracellular pH and Na^+/H^+ exchanger activity are included in chapter 2 of this thesis

5.3 Patients

The study group consisted of 11 3rd trimester pre-eclamptic women, 11 3rd trimester normotensive pregnant controls, 12 post-partum pre-eclamptic women and 8 post-partum normotensive controls. All women from chapters 3 and 4 were included initially but immortalization was unsuccessful in several cases and therefore the study group number was reduced. The clinical characteristics of the women are presented in tables 5.1 and 5.2 and the women remained matched for age.

Table 5.1
CLINICAL DATA

	PET	NT
Patient (n)	11	11
Age (years)		
Mean	29(±1.4)	30(±1.7)
Range	22-34	24-31
Gestation at delivery (weeks)		
Mean	34^{+3} (±1.6)	40^{+1} (±0.3)*
Range	$26\text{-}38^{+5}$	$39\text{-}41^{+4}$
BP booking (mmHg)		
Mean	115/70(±4.5)	109/65 (±2.8)
Range	100/60-130/85	100/60-120/80
BP highest (mmHg)		
Mean	159/ 105(±4.8)	114/66 (±2.2)**
Range	140/94-180/113	102/60-120/70
Gestational age at sampling (weeks)		
Mean	33^{+1}(±1.20)	33.6(±1.3)
Range	$26\text{-}37^{+2}$	$27\text{-}39^{+1}$
Parity		
1	6	5
>1	5	6

*Table 5.1: Clinical data for the 3rd trimester pre-eclamptic and 3rd trimester normotensive control. Values are means with range and SEM *P=0.011, **P=0.0001 normotensive versus pre-eclamptics by Student's t-test.*

	Table 5.2 CLINICAL DATA	
	ppPET	ppNT
Patient (n)	12	8
Age (years)		
Mean	36 (±2.0)	36(±1.6)
Range	27-47	27-45
Gestation at delivery (months)		
Mean	34^{+1} (±1.5)	40^{+2} (±0.3)*
Range	$28\text{-}40^{+3}$	$39\text{-}41^{+2}$
BP booking (mmHg)		
Mean	116/71(±2.0)	115/64 (±2.1)
Range	100/60-130/80	100/60-120/80
BP highest (mmHg)		
Mean	174/ 114(±5.1)	117/66 (±1.6)**
Range	150/100-200/120	100/60-120/70
BP visit (mmHg)		
Mean	128/79 (±4.6)	119/71 (±3.5)
Range	101/ 68-142/95	110/66-130/79
Months post-partum at time of sampling		
Mean	8.5 (±0.7)	8.0 (±0.8)
Range	7.5-9	7-9
Parity		
1	6	4
>1	5	4

*Table 5.1: Clinical data for the post-partum pre-eclamptic and post-partum normotensive control. Values are means with range and SEM *P=0.002, **P=0.0001 normotensive versus pre-eclamptics by Student's t-test.*

5.4 Results

5.4.1 Intracellular pH and Na^+/H^+ Exchanger Activity in EBV-Immortalized Lymphoblasts from 3rd Trimester Pre-eclamptic and 3rd Trimester Normal Control Women.

EBV immortalized lymphoblast cell lines were used to further assess membrane transport abnormalities in pre-eclampsia. Intracellular pH and Na^+/H^+ exchanger activity were assessed and the results are illustrated in figure 5.2. In this figure, data from women with pre-eclampsia and normal control women are shown. It is apparent that the pre-eclamptic cells had a greater exchanger activity (20.77 ±0.92 mmol/l.min) compared to the normal pregnant controls (15.22 ±0.92 mmol/l.min, $P<0.001$, Student's t-test). Assessment of the intracellular pH was performed and no differences were identified between pre-eclamptic cells (7.54 ±0.02) and normal pregnant controls

(7.65 ±0.04, Student's t-test). Buffering capacity was analysed and no differences were found between pre-eclamptic cells (25.19 ±0.96 mmol/l.pH unit) and normal pregnant control cells (24.20 ±0.92 mmol/l.pH unit, Student's t-test).

5.4.2 Intracellular pH and Na^+/H^+ Exchanger Activity in EBV Immortalized Lymphoblasts from Post-partum Pre-eclamptic and Post-partum Normal Control Women.

EBV-immortalized lymphoblasts from post-partum pre-eclamptic and post-partum normal control women were analysed and the results are presented in figure 5.3. Statistical analysis using an unpaired Student's t-test showed that the mean Na^+/H^+ exchanger activity was significantly higher in the post-partum pre-eclamptic cells (15.59 ±0.59 mmol/l.min) compared to the normal post-partum control cells (13.52 ±0.49 mmol/l.min P=0.043). Assessment of the intracellular pH was performed and no differences were identified between post-partum pre-eclamptic cells (7.33 ±0.02) and normal post-partum controls (7.28 ±0.02, Student's t-test). Buffering capacity was analysed and no differences were found between post-partum pre-eclamptic cells (23.13 ±0.60 mmol/l.pH unit) and normal post-partum controls (22.45 ±0.83 mmol/l.pH unit, Student's t-test).

5.5 Discussion

In chapter 4 it was demonstrated that neutrophil Na^+/H^+ exchanger activity was elevated in pre-eclamptic women compared to normotensive pregnant controls and may represent an intermediate phenotype similar to that identified in essential hypertension [Kelly et al. 1997]. Furthermore, chapter 3 presented data complementing a role for this intermediate phenotype, as the abnormality persisted into the post-partum period suggesting the possibility of genetic influences. The differences in Na^+/H^+ exchanger activity may have been attributed to environmental (plasma) factors. However, evidence against this is presented as measurement in EBV-immortalized lymphoblasts from pre-eclamptic and post-partum pre-eclamptic women showed that the phenotypic changes persist despite transformation and culture *in vivo*. The increased activity of the Na^+/H^+ exchanger was thus likely to be genetically determined

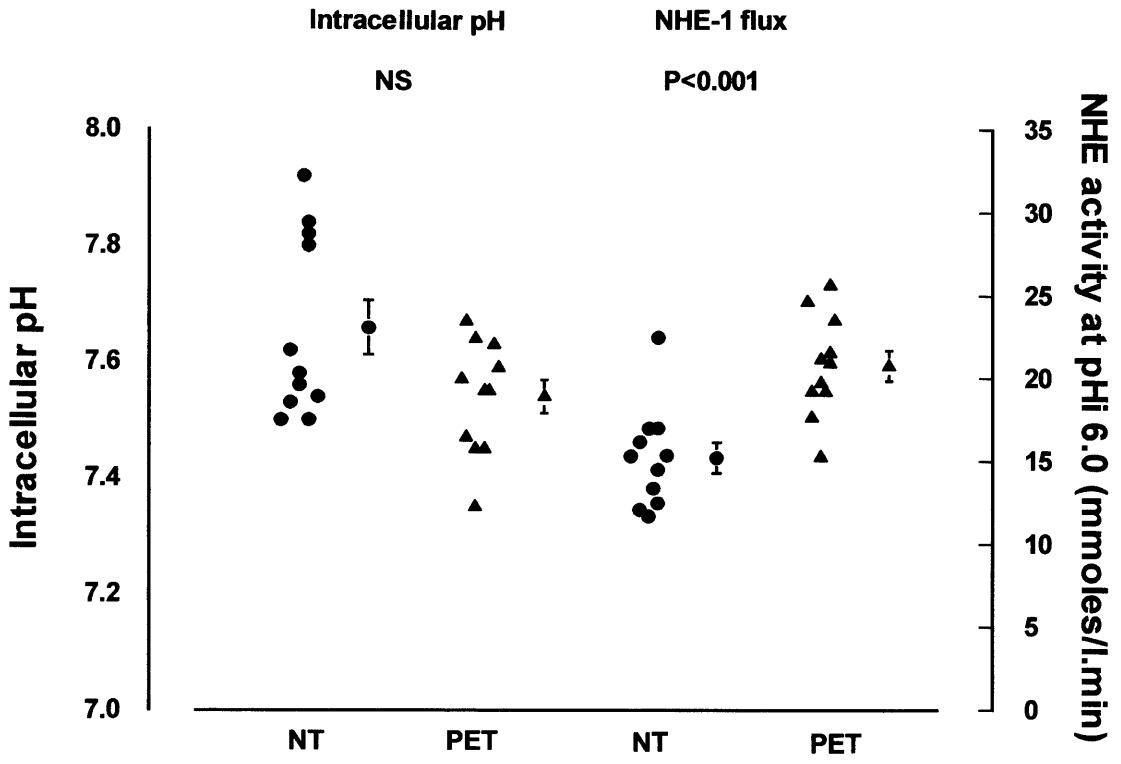


Figure 5.2: Plot of EBV immortalized lymphoblast Na^+/H^+ exchanger activity and intracellular pH in 3rd trimester normotensive (NT) and pre-eclamptic (PET) women. NHE activity was determined after clamping pH_i to 6.0, near the Vmax of the Na^+/H^+ exchanger of cells. The mean and SEM are plotted. The Na^+/H^+ exchanger activity of EBV immortalized lymphoblasts from PET women (20.77±0.92 mmoles/l.min) was significantly elevated compared with NT women (15.22 ±0.92 mmoles/l.min). There was no significant difference in intracellular pH.

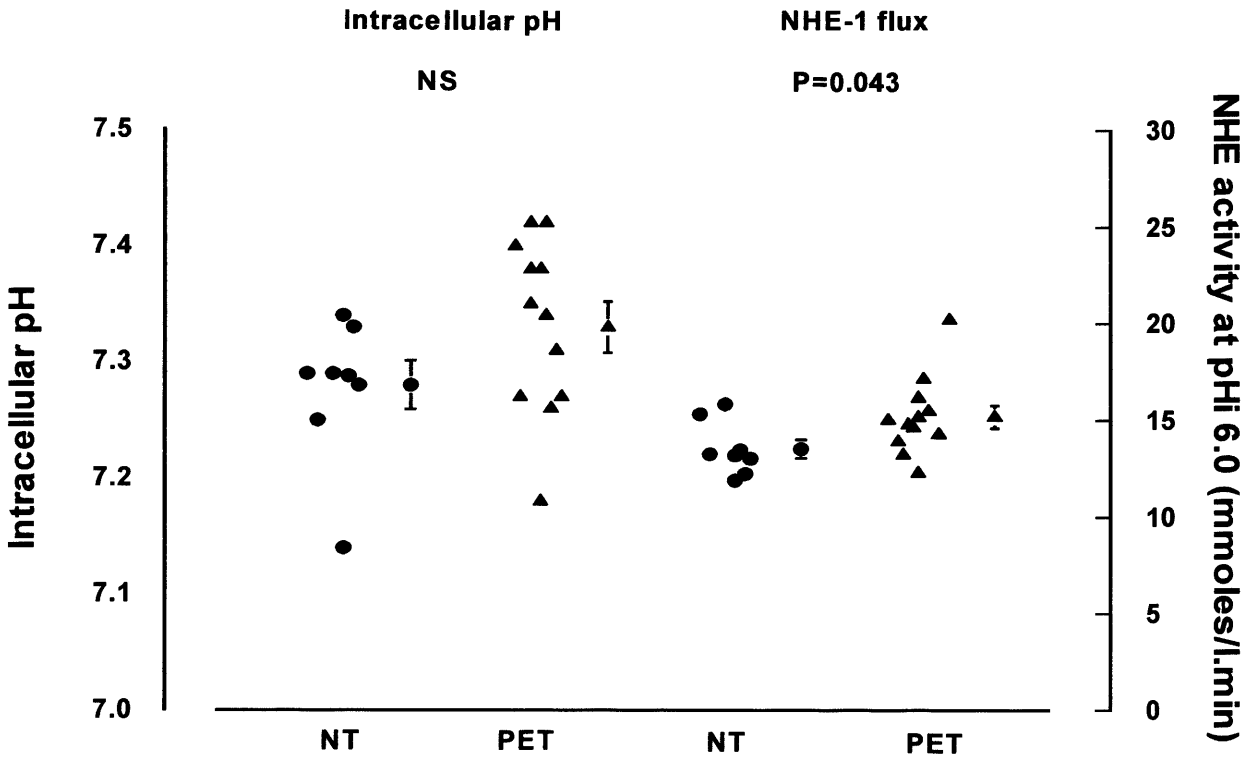


Figure 5.3: Plot of EBV immortalized lymphoblast Na^+/H^+ exchanger activity and intracellular pH in post-partum normotensive (NT) and post-partum pre-eclamptic (PET) women. NHE activity was determined after clamping pH_i to 6.0, near the Vmax of the Na^+/H^+ exchanger of cells. The mean and SEM are plotted. The Na^+/H^+ exchanger activity of EBV immortalized lymphoblasts from PET women (15.19±0.59 mmoles/l.min) was significantly elevated compared with NT women (13.52 ±0.49 mmoles/l.min). There was no significant difference in intracellular pH.

as in essential hypertension and diabetes mellitus [Ng et al. 1994]. It may seem that normal pregnant women had a mean Na^+/H^+ exchanger activity similar to that presented for post-partum pre-eclamptic women. However, the results obtained from the 3rd trimester study cannot be directly compared with the results obtained from the post-partum study as the cells were not transformed into immortalized lines at the same time and were therefore in different passages at the time of analysis; this may have affected the results between studies. It is unlikely that the differences in results between studies was due to the differences in mean age since age had no correlation with either intracellular pH or Na^+/H^+ exchanger activity (not shown).

It remains unclear how the presence of this intermediate phenotype would be implicated in the pathogenesis of pre-eclampsia. If the activity was increased in renal tissue, Na^+ retention could result requiring that the kidney be perfused by a greater than normal arterial pressure in order to maintain salt balance [Mahnensmith and Aronson 1985]. Furthermore, if such changes occurred in vascular smooth muscle, vascular tone may be increased as any factor that elevates intracellular Na^+ will tend to elevate intracellular $Ca^{2+,}$ causing an elevated peripheral resistance [Wray 1988; Blaustein 1984]. It is at present unknown whether these changes exhibited in white blood cells represent similar changes that occur in other cell types in pre-eclamptic women. Due to the evidently genetic basis of the intermediate phenotype in pre-eclampsia, it is possible that a gene encoding control of NHE-1 is involved and since this particular isoform is ubiquitous, it is likely that changes exist in all cell types. However, this relies on the control mechanism being ubiquitous also. A functional single-nucleotide variant of the gene encoding the beta3 subunit of heterotrimeric G proteins (Gbeta3 C825T), associated with enhanced G-protein activation and increased activity of the Na^+/H^+ exchanger, has been implicated in the development of hypertension and may exist in pre-eclampsia [Schorr et al. 2000].

Intracellular pH was found to be lower in both pre-eclamptic and post-partum pre-eclamptic cells compared to their respective normotensive controls as illustrated in chapters 3 and 4. Furthermore, evidence presented in chapter 4 suggested the contribution of plasma factors to this unique phenomenon. In order to further investigate this hypothesis, intracellular pH was examined in EBV-immortalized lymphoblasts and no significant differences were found between post-partum pre-

eclamptic and post-partum normotensive cells or 3rd trimester pre-eclamptic and 3rd trimester normotensive cells. In fact, intracellular pH was slightly higher in the post-partum pre-eclamptic cells in keeping with the alkalosis associated with essential hypertension [Ng et al. 1989c]. Once more, results between studies were not compared since the 3rd trimester and post-partum lymphocytes were immortalized at different times and were therefore analysed in different passages. This would have affected the results between studies and was therefore a limitation of the investigation. Results between 3rd trimester pre-eclamptics and 3rd trimester normotensives remained legitimate since immortalization was performed at the same time. The same applied for the post-partum cells. The findings presented argue against the possibility of genetic influences associated with intracellular acidosis in pre-eclamptic cells. What remains somewhat confusing is the persistence of leucocyte intracellular acidosis into the post-partum period as documented in chapter 3. This suggested that the 'plasma' influences continued to have an effect into the post-partum period when most of the abnormalities whether, haematological or biochemical have supposedly reverted back to normal. However, there is evidence to suggest that abnormalities may remain after the pre-eclamptic episode. Spaanderman et al. [2000] suggested that haemodynamic parameters and volume homeostasis in a symptom-free subgroup of women with a history of pre-eclampsia were different from those in healthy parous controls. Others have reported persistent endothelial dysfunction with ensuing dysregulation of blood pressure, haemostatic perturbation and dyslipoproteinaemia after pre-eclampsia that may have indicated a proneness to future cardiovascular disease [van Pampus et al. 1999; He et al. 1999]. It is therefore biologically plausible that plasma (environmental) induced alterations in intracellular pH may have continued into the post-partum period but were removed upon EBV-immortalization of cells into a continuous line. Once more, results between studies were not compared since the 3rd trimester and post-partum cells were immortalized at different times and were therefore analysed in different passages. This would have affected the result and again was a limitation of the study. The relevance of this intracellular acidosis in the pathogenesis of pre-eclampsia remains unclear along with the factor(s) responsible for its initiation. These questions remain to be investigated in subsequent chapters. It seems fair to conclude that the increased Na^+/H^+ exchanger activity and low intracellular pH associated with pre-eclampsia are not a direct consequence of one another as increased Na^+/H^+ activity persists after immortalization when the intracellular acidosis is no longer present. It

was considered in previous chapters that the increased exchanger activity might have been a mechanism of dealing with the chronic effects of increased intracellular acid load. This hypothesis needs revision in light of the present chapter.

5.6 Conclusion

The intermediate phenotype of increased Na^+/H^+ exchanger in pre-eclampsia is partly determined by genetic factors, since increased exchanger activity persists in EBV-immortalized lymphoblasts obtained from both pre-eclamptic and post-partum pre-eclamptic women. The intracellular acidosis associated with pre-eclampsia and the post-partum appeared to be independent of genetic factors and more likely due to the presence of plasma factors as the phenomenon did not persist after immortalization of cells into continuous lines. The factors responsible for this intracellular acidosis remain to be identified.

Chapter 6

The Contribution of a Digoxin-like Factor to the Regulation of Intracellular pH in Pre-eclampsia.

6.1 Introduction

Lopatin et al [1999] suggested the presence of a digoxin-like factor (DLF) in the plasma of women with pre-eclampsia and suggested that it may have a pathogenic role in the disease through its ability to inhibit the Na^+/K^+ATPase. DLF inhibits Na^+/K^+ATPase activity and could result in a build up of protons triggered by an initial build up of sodium. This build up of Na^+ may activate Na^+/Ca^{2+} exchange in reverse mode resulting in a build up of Ca^{2+}. This Ca^{2+} influx, via the Na^+/Ca^{2+} exchanger, may activate the Ca^{2+}/H^+ exchange system resulting in the subsequent build up of protons [Souza et al. 2000]. Intracellular acidosis has been associated with pre-eclampsia, persisting into the post-partum period. This chapter tests the hypothesis that a lower intracellular pH in pre-eclampsia may be due to the presence and effect of a circulating digoxin-like factor in the pre-eclamptic and post-partum pre-eclamptic plasma since acidosis did not persist once cells were immortalized. The effects of plasma factors would have declined in culture. The presence of a DLF may not only contribute towards this acidosis but may contribute towards the increased sensitivity of the vascular bed to pressor hormones and hypovolemic hypertensive state as well as causing vasoconstriction and increased blood pressure [Gregoire et al. 1988].

This study aimed to assess the presence of a putative DLF by collecting extracts from whole plasma. Plasma was incubated with a known amount of Na^+/K^+ATPase (pig cerebellum) and then free ATP was added. The Na^+/K^+ATPase would catalyse the breakdown of the ATP to form ADP and free phosphate that was subsequently measured by colorimetry. The more DLF present in the extracts (inhibits Na^+/K^+ATPase) the less free phosphate would result and be subsequently measured.

6.2 Materials and Methods

The assay developed to assess the presence of a digoxin-like factor in extracted plasma samples is presented in chapter 2.

6.3 Patients

Patients assessed for a DLF were selected from those examined in previous chapters. Selection was made based on severity of the pre-eclamptic episode to allow the best chance of detecting significant differences in DLF between the patients and controls. The groups remained matched for age as shown in tables 6.1 and 6.2.

Table 6.1
CLINICAL DATA

	PET	NT
Patient (n)	6	6
Age (years)		
Mean	28 (±1.6)	29(±1.4)
Range	22-33	24-32
Gestation at delivery (weeks)		
Mean	33^{+2} (±1.9)	40^{+1} (±0.4) *
Range	28-40	$39\text{-}41^{+6}$
BP booking (mmHg)		
Mean	120/70(±4.1)	110/70 (±2.2)
Range	110/65-130/55	100/60-120/80
BP highest (mmHg)		
Mean	160/ 105(±4.2)	110/65 (±2.0)**
Range	140/94-180/113	100/60-120/70
Gestational age at sampling (weeks)		
Mean	33^{+4}(±1.0)	33^{+6}(±1.21)
Range	$26\text{-}37^{+2}$	$27\text{-}39^{+1}$
Parity		
1	3	3
>1	3	3

*Table 6.1: Clinical data for the 3rd trimester pre-eclamptic and 3rd trimester normotensive control. Values are means with range and SEM *P=0.02, **P=0.0001 normotensive versus pre-eclamptics by unpaired Student's t-test.*

	Table 6.2 CLINICAL DATA	
	ppPET	ppNT
Patient (n)	6	6
Age (years)		
Mean	34 (±1.7)	36(±1.2)
Range	29-40	32-40
Gestation at delivery (weeks)		
Mean	33^{+2} (±2.1)	40 (±0.5) *
Range	28-40+5	$38\text{-}41^{+2}$
BP booking (mmHg)		
Mean	121/73(±2.0)	114/61 (±2.1)
Range	120/70-130/80	100/60-120/65
BP highest (mmHg)		
Mean	171/ 111(±5.8)	113/66 (±3.3) **
Range	150/105-190/120	100/60-120/70
BP visit (mmHg)		
Mean	124/76 (±3.5)	115/72 (±4.2)
Range	110/ 70-133/82	99/65-130/79
Months post-partum at time of sampling		
Mean	8.0 (±0.8)	8.5 (±0.9)
Range	7.5-8.5	7-9
Parity		
1	4	4
>1	2	2

*Table 6.1: Clinical data for the post-partum pre-eclamptic and post-partum normotensive controls. Values are means with range and SEM *P=0.03, **P=0.0001 normotensive versus pre-eclamptics by Student's t-test.*

6.4 Results

6.4.1 Measurement of DLF

Figure 6.1 illustrates the ability of extracted plasma from both 3rd trimester pre-eclamptic and 3rd trimester normal pregnant women to inhibit the Na^+/K^+ATPase mediated hydrolysis of ATP. The presence of a DLF was reflected by the amount of inorganic phosphate liberated from ATP with less phosphate representing inhibition of the enzyme and hence more DLF. Inorganic phosphate was significantly lower when extracted plasma from the pre-eclamptic women was incubated with the enzyme compared to extracted plasma from normotensive pregnant women (54.9 ±2.6 nmol/ml of plasma vs 63.91 ±1.7 nmol/ml of plasma, n=6, P=0.018 unpaired Student's t-test).

Figure 6.2 illustrates the ability of extracted plasma extracted from both post-partum pre-eclamptic and post-partum normal pregnant women to inhibit the Na^+/K^+ATPase mediated hydrolysis of ATP. The inorganic phosphate liberated was measured and no

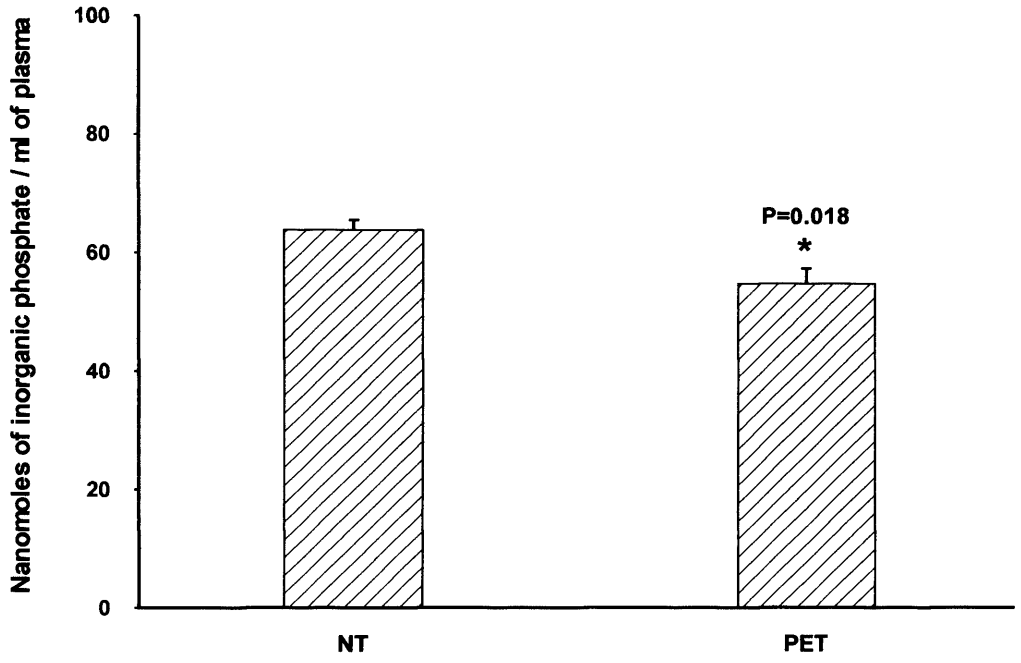


Figure 6.1 DLF activity presented as the amount of inorganic phosphate liberated when plasma was incubated with the Na^+/K^+ATPase isolated from pig cerebellum. Plasma from normotensive and pre-eclamptic pregnant women was analysed and there was a significant decrease in the inorganic phosphate measured when pre-eclamptic plasma was incubated with the enzyme compared to plasma from normotensive pregnant women (54.9 ±2.6 nanomoles/ml of plasma vs 63.91 ±1.7 nanomoles/ml of plasma, n=6, P<0.018 unpaired Student's t-test).

significant differences between post-partum pre-eclamptics (61.7 ±2.3 nmol/ml of plasma) and post-partum normotensives (65.02 ±4.0 nmol/ml of plasma, n=6, unpaired Student's t-test) were found.

6.5 Discussion

This study suggested that a factor, characterised by its ability to inhibit Na^+/K^+ATPase, was present in plasma from normal pregnant women but was significantly increased in pre-eclamptic plasma. These findings complement the work of Gregoire et al [1988] and Lopatin et al [1999] and may suggest a mechanism that contributed towards the lower intracellular pH reported previously in this thesis. Lopatin et al demonstrated the existence of a digoxin-like compound that was significantly increased in pre-eclamptic plasma. The concentrations of this compound were enough to substantially inhibit the sodium pump [Lopatin et al 1999]. However, it seems unlikely that the presence of a DLF was the only mechanism contributing towards this low intracellular pH as no differences were identified in the inorganic phosphate liberated when plasma extracts from post-partum pre-eclamptic women compared to their respective controls were incubated with ATP. This suggested that there was a factor present that inhibited Na^+/K^+ATPase but the levels were similar in the 2 groups. Secondly, the results presented in chapter 4 are indicative of another mechanism that is unlikely to be plasma borne as intracellular pH values in pre-eclamptic neutrophils do not reach those of the control neutrophils, remaining slightly more acidic [figure 4.4]. This unknown mechanism may possibly be present in both pre-eclamptic and post-partum pre-eclamptic women and thus contribute towards the presented acidosis.

There is increasing evidence to suggest a reduction of magnesium in the pathogenesis of pre-eclampsia. Twenty women with pre-eclampsia and 20 control women matched for gestational age were examined and low cellular magnesium levels were suggested in women with pre-eclampsia [Adam et al. 2001]. Kisters et al. [2000] examined plasma, cellular, and membrane (erythrocytes) magnesium content by atomic absorption spectroscopy and reported lowered plasma, intracellular, and membrane magnesium concentrations in pre-eclampsia. Hypomagnesaemia has been associated with pre-eclampsia and is linked to cellular pump failure. Hypomagnesaemia may contribute towards the intracellular acidosis in pre-eclampsia as a deficiency of magnesium causes

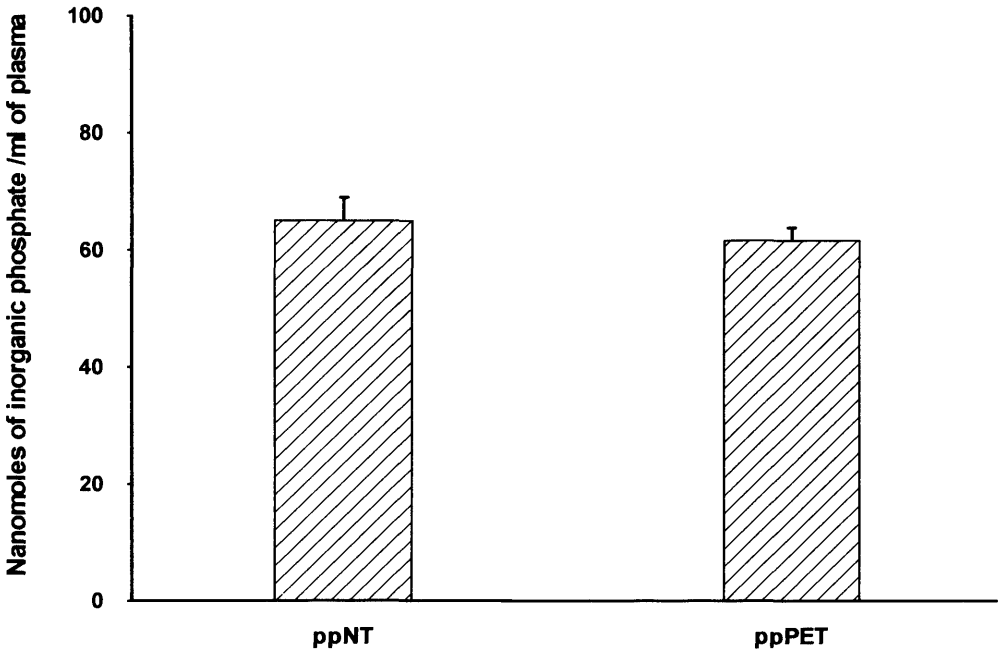


Figure 6.2 DLF activity presented as the amount of inorganic phosphate liberated when plasma was incubated with the Na^+/K^+ATPase isolated from pig cerebellum. Plasma from post-partum normotensive and post-partum pre-eclamptic pregnant women was analysed and no significant differences between post-partum pre-eclamptic (61.7 ±2.3 nanomoles/ml of plasma) and post-partum normotensive (65.02 ±4.0 nanomoles/ml of plasma, n=6, unpaired Student's t-test) were found.

magnesium-requiring enzymes such as Na^+/K^+ATPase to become impaired [Newman and Amarasingham 1993]. Impairment of Na^+/K^+ATPase would consequently result in increased calcium and subsequent intracellular acidosis as seen in cells from pre-eclamptic and the post-partum pre-eclamptic women. It is possible that the presence of an increased DLF combined with hypomagnesaemia could be considered sufficient to cause this intracellular acidosis.

6.6 Conclusion

Most of the conclusions drawn from this chapter are a little presumptuous. However, the presence of an increased DLF in extracted pre-eclamptic plasma has been identified by its ability to inhibit Na^+/K^+ATPase and may, in conjunction with some other factor, contribute towards the intracellular acidosis in pre-eclampsia. It remains unclear how this acidosis persists into the post-partum period as no differences are found between post-partum normal and pre-eclamptic groups. It is possible that magnesium may contribute. These hypotheses warrant further investigation to have any scientific validity.

Chapter 7

NADPH Oxidase Activation and the Production of Reactive Oxygen Species in Pre-eclampsia

7.1 Introduction

Neutrophil activation is believed to contribute towards the pre-eclamptic syndrome and initial evidence to support this comes from studies examining plasma levels of granular components such as elastase [Greer at al. 1989]. Elevated plasma levels of this substance reflect neutrophil activation *in vivo* and may contribute to the endothelial damage [Greer at al. 1989]. Neutrophils have been implicated in the pathogenesis of pre-eclampsia through their ability to produce ROS. The production of ROS has been used to document *in vivo* neutrophil activation [Sacks et al. 1998]. It is believed that increased ROS production plays a role in the vascular endothelial damage and dysfunction associated with pre-eclampsia [Dekker and Kraayenbrink 1991].

Published studies have produced conflicting results concerning neutrophil ROS production in pre-eclampsia and the significance of this production is not completely clear [Tsukimori et al. 1993; Crocker et al. 1999]. This chapter tests the hypothesis that neutrophils isolated from pre-eclamptic women may show enhanced NADPH oxidase mediated ROS production upon agonist stimulation. This would represent an increased sensitivity characterized by the enhanced response of the NADPH oxidase enzyme and may contribute to the oxidative stress and endothelial dysfunction characteristic of the disease.

The aim of this study was to provide conclusive evidence of increased neutrophil ROS production in pre-eclampsia. The study examined neutrophil NADPH oxidase mediated ROS production in 3rd trimester pre-eclamptic and 3rd trimester normotensive pregnant controls matched for age. The work presented in this chapter complements that cited in the literature and may provide evidence for an intermediate phenotype of increased NADPH oxidase activity in the pathogenesis of pre-eclampsia, reflecting a cell that is more sensitive to agonist stimulation. This increased sensitivity may involve both genetic and environmental (plasma) factors. NADPH oxidase protein sub-unit

abundance was also analysed and may contribute towards the increased activity postulated.

7.2 Materials and Methods

The methods for assessing ROS produced upon agonist stimulation of the NADPH oxidase enzyme are included in chapter 2 of this thesis along with the techniques used to assess NADPH oxidase protein abundance.

7.3 Patients

The clinical details of the patient groups are illustrated in table 4.1 of chapter 4 since the patient groups remained identical.

7.4 Results

Figure 7.1 and 7.2 show typical traces for the chemiluminescent response to both luminol and lucigenin, respectively. The time required for a set number of neutrophils, in this case 400,000, to reach a peak response was recorded. Velocity was dependent on the type of stimulus employed. As the ratio of stimulus to cell was increased, the time to reach peak velocity was decreased and is illustrated in figures 7.1 and 7.2. The temporal pattern of lucigenin-derived chemiluminescence following stimulation with PMA was different to that observed when stimulating with fMLP. Except for the use of different stimuli, the CL represented in fig 7.2 was obtained under the same conditions of testing. The temporal pattern of luminol-derived CL following stimulation with different agonists was similar. The response to each stimulus was measured as both area under the curve using Simpson's rule (AUC) and peak reading and expressed as relative light units seconds (RLU.sec) or relative light units (RLU), respectively. Peak and AUC results correlated closely for both lucigenin-derived (fMLP r =0.983, PMA r =0.993) and luminol-derived chemiluminescence (fMLP r =0.930, PMA r =0.989).

7.4.1 Lucigenin-Derived Chemiluminescent Response of Neutrophils

Neutrophil NADPH oxidase was stimulated with the agonists' fMLP and PMA to produce ROS. The ROS produced in response to each stimulus was measured as both area under the curve and peak response and recorded as RLU.sec or RLU respectively

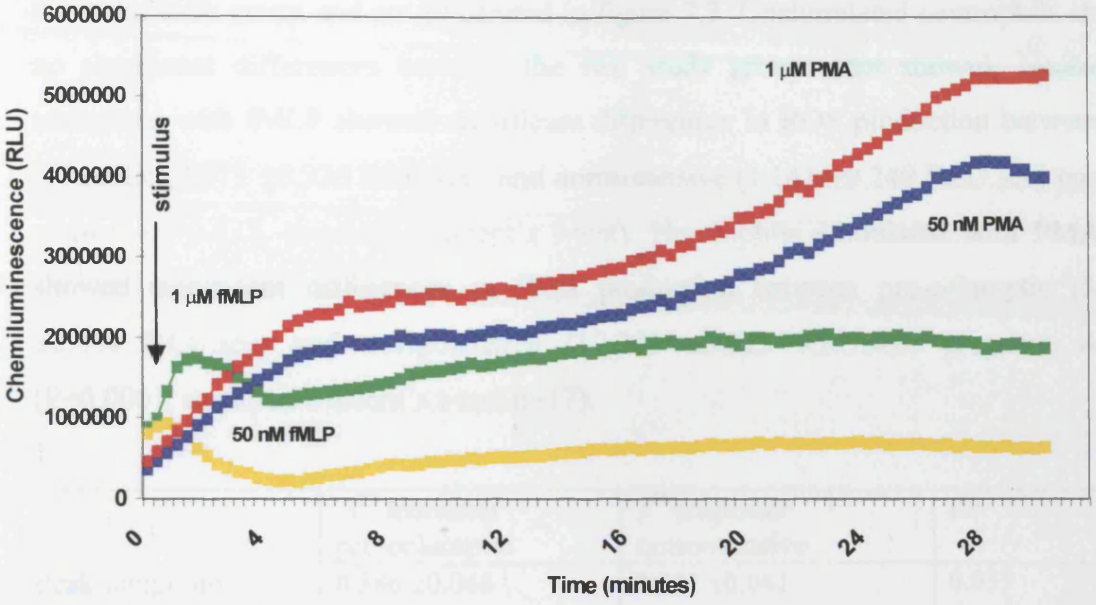


Figure 7.1: T*ypical traces for the neutrophil chemiluminescent response of lucigenin stimulated with both n-formyl-met-leu-phe (fMLP) and phorbol myristate acetate (PMA)*

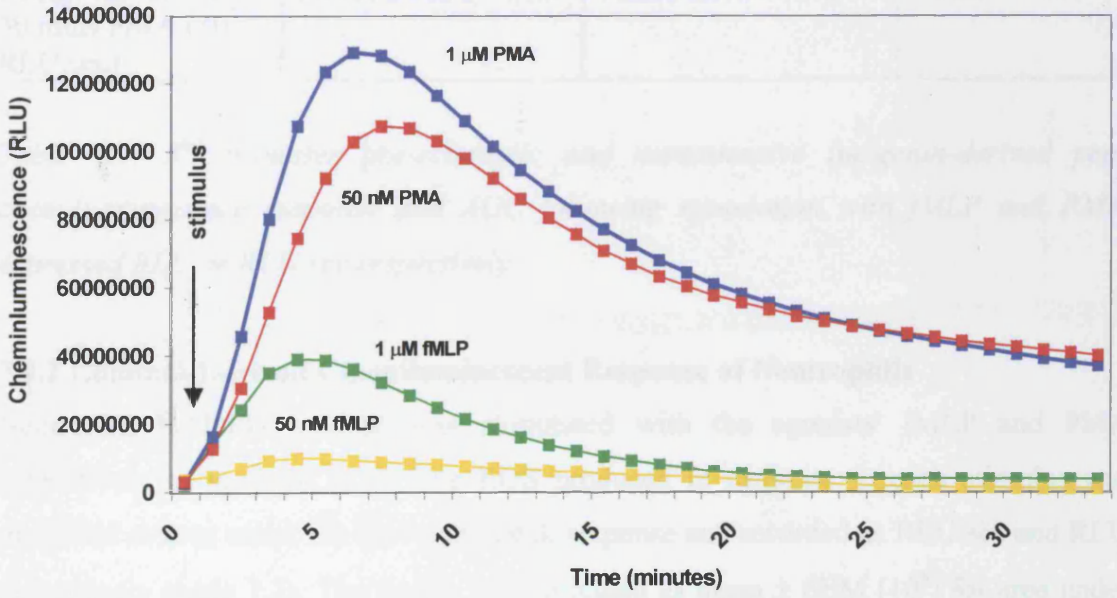


Figure 7.2: Typical traces for the neutrophil chemiluminescent response of luminol stimulated with both n-formyl-met-leu-phe (fMLP) and phorbol myristate acetate (PMA)

(table 7.1). The results are expressed as mean ± SEM (10^7) value for area under the curve for each group and are illustrated in figure 7.3. Unstimulated neutrophils showed no significant differences between the two study groups (not shown). Neutrophils stimulated with fMLP showed significant differences in ROS production between pre-eclamptic (2.071 ±0.336 RLU.sec) and normotensive (1.141 ±0.249 RLU.sec) pregnant women (P=0.035, unpaired Student's t-test). Neutrophils stimulated with PMA also showed significant differences in ROS production between pre-eclamptic (34.954 ±2.634 RLU.sec) and normotensive (17.208 ±3.325 RLU.sec) pregnant women (P=0.0001, unpaired Student's t-test n=17).

	3rd trimester pre-eclamptic	3rd trimester normotensive	P=
Peak lucigenin signal fMLP (10^7 RLU)	0.386 ±0.046	0.241 ±0.041	0.033
Lucigenin AUC 0-30 mins fMLP (10^7 RLU.sec)	2.071 ±0.336	1.141 ±0.249	0.035
Peak Lucigenin signal PMA (10^7 RLU)	2.174 ±0.166	1.388 ±0.212	0.007
Lucigenin AUC 0-30 mins PMA (10^7 RLU.sec)	34.956 ±2.634	17.208 ±3.325	0.0001

Table 7.1: 3rd trimester pre-eclamptic and normotensive lucigenin-derived peak chemiluminescence response and AUC following stimulation with fMLP and PMA, expressed RLU or RLU.sec respectively.

7.4.2 Luminol-Derived Chemiluminescent Response of Neutrophils

Neutrophil NADPH oxidase was stimulated with the agonists' fMLP and PMA respectively to produce ROS. The ROS produced in response to each stimulus was measured as area under the curve and peak response and recorded as RLU.sec and RLU respectively (table 7.2). The results are expressed as mean ± SEM (10^8) for area under the curve for each group and are illustrated in figure 7.4. Unstimulated neutrophils showed no significant differences between the two study groups (not shown). Neutrophils stimulated with fMLP showed significant differences in ROS production between pre-eclamptic (1.955 ±0.316 RLU.sec) and normotensive (1.058 ±0.191

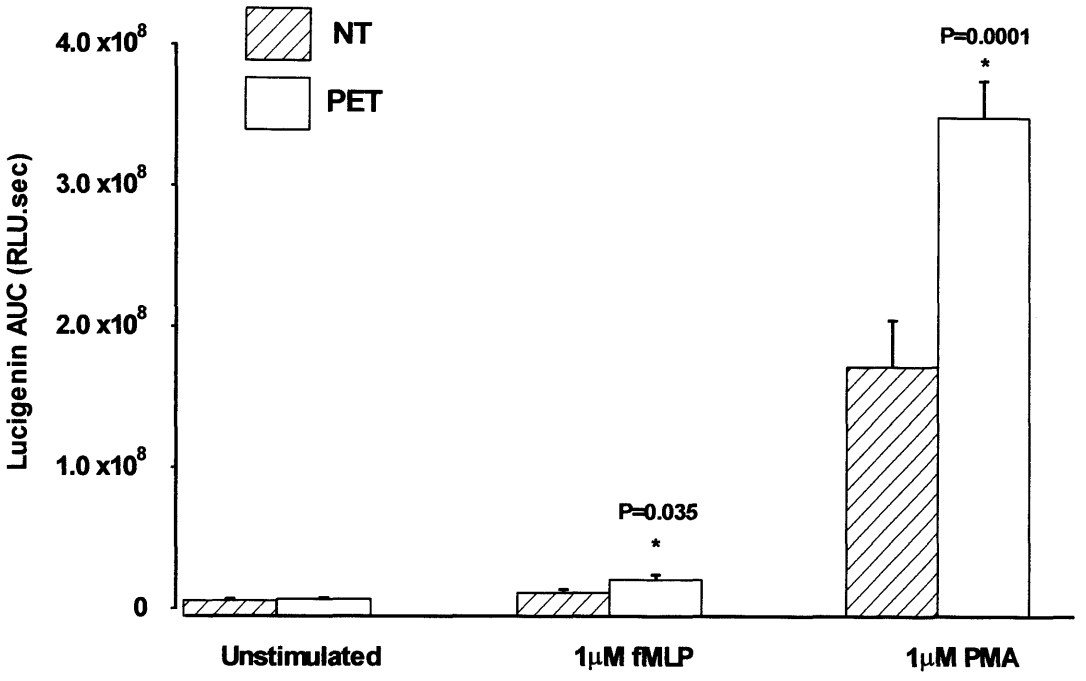


Figure 7.3: Lucigenin-derived chemiluminescence responses of neutrophils from normotensive and pre-eclamptic patients stimulated with n-formyl-met-leu-phe (fMLP) and phorbol myrsitate acetate (PMA). Chemiluminescence output was recorded as RLU.sec. The results represent mean AUC and the SEM are shown. There were significant differences present between groups with both fMLP and PMA stimulation of neutrophils, n=17, unpaired Student's t-test.

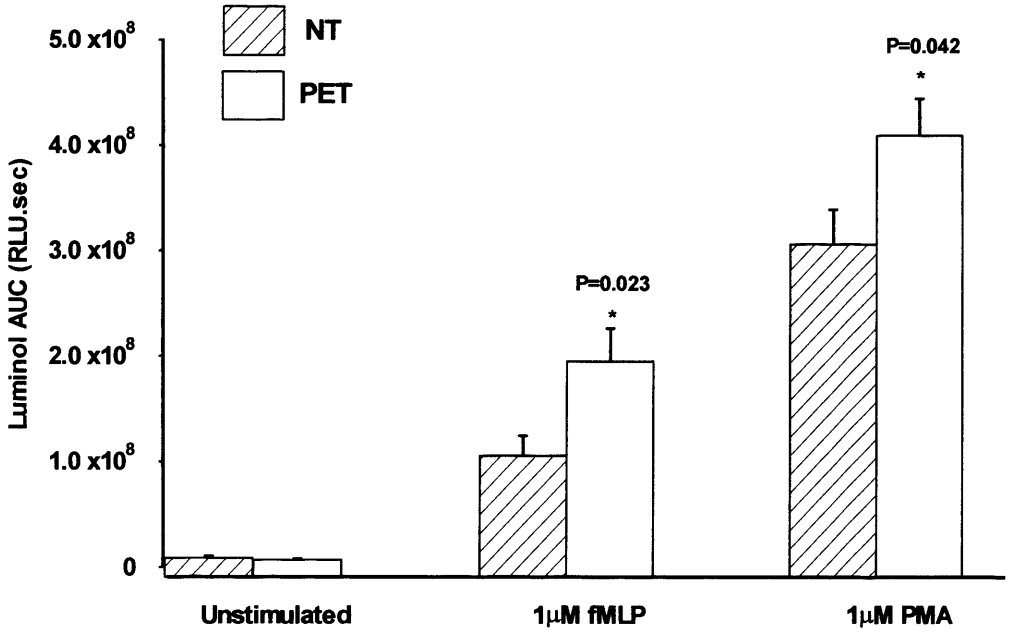


Figure 7.4: Luminol-derived chemiluminescence responses of neutrophils from normotensive and pre-eclamptic patients stimulated with n-formyl-met-leu-phe (FMLP) and phorbol myrsitate acetate (PMA). Chemiluminescence output was recorded as RLU.sec. The results represent mean AUC and the SEM are shown. There were significant differences present between groups with both fMLP and PMA stimulation of neutrophils, n=17, unpaired Student's t-test.

RLU.sec) pregnant women (P=0.023, unpaired Student's t-test, n=17). Neutrophils stimulated with PMA also showed significant differences in ROS production between pre-eclamptic (4.108 ±0.351 RLU.sec) and normotensive (3.073 ±0.332 RLU.sec) pregnant women (P=0.042, unpaired Student's t-test, n=17).

	3rd trimester pre-eclamptic	3rd trimester normotensive	P=
Peak luminol signal fMLP (10^8 RLU)	0.677 ±0.102	0.487 ±0.0839	N/S
Luminol AUC 0-30 mins fMLP (10^8 RLU.sec)	1.955 ±0.316	1.058 ±0.191	0.023
Peak luminol signal PMA (10^8 RLU)	0.934 ±0.079	0.816 ±0.067	N/S
Luminol AUC 0-30 mins PMA (10^8 RLU.sec)	4.108 ±0.351	3.073 ±0.332	0.042

Table 7.2: 3rd trimester pre-eclamptic and normotensive luminol-derived peak chemiluminescence response and AUC following stimulation with fMLP and PMA, expressed RLU or RLU.sec respectively.

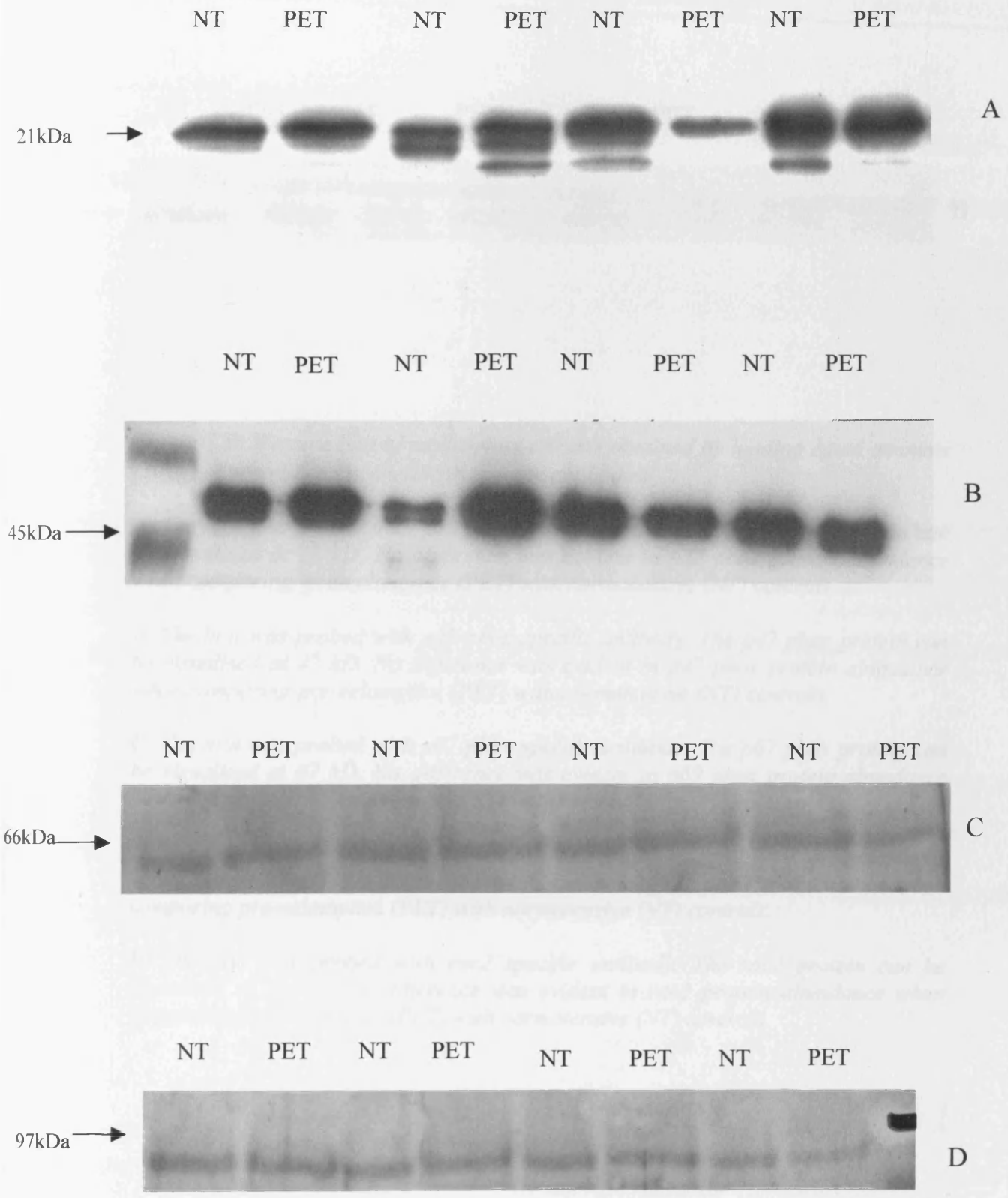
7.4.3 Specificity of Techniques

Certain scavengers were employed to confirm the specificity of the techniques. Superoxide dismutase (SOD) and 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron) have been included to verify that lucigenin is indeed measuring superoxide production and catalase has been included to ensure the measurement of hydrogen peroxide by luminol. Treatment with the pharmacological scavenger of superoxide, Tiron (10mM), completely abolished the lucigenin response produced upon stimulation with both fMLP and PMA thus confirming the specificity of lucigenin for measuring superoxide production [Hein and Kuo 1998] (not shown). SOD scavenges superoxide by catalysing the conversion of superoxide to hydrogen peroxide. Treatment with SOD (100 units/ml) only reduced the lucigenin response to both PMA and fMLP by ~90% (not shown). Tiron has advantages over SOD as SOD acts as a pro-oxidant in the presence of hydrogen peroxide, thus explaining why superoxide was incompletely scavenged. Catalase (500 units/ml) had no effect on the lucigenin response following stimulation with both fMLP and PMA (not shown).

To examine the role of luminol in specifically measuring hydrogen peroxide, cells were treated with the cell impermeable scavenger, catalase (500 units/ml) and then stimulated with PMA and fMLP. The luminol response was ~70% inhibited by catalase but not by SOD (not shown). This suggested that luminol was not completely specific and measured metabolites in addition to hydrogen peroxide. Since catalase and SOD were expected to reduce only extracellular generated oxidative metabolites it seems that some of the metabolites produced were consequently of intracellular origin [Dahlgren 1987]. However, the use of luminol as a chemiluminogenic probe gives us an indication of the production of ROS that are produced upon agonist stimulation.

7.4.4 Analysis of NADPH Oxidase Protein Sub-component Abundance

Neutrophil extracts (30μg per track) for both 3rd trimester pre-eclamptic and 3rd trimester normotensive controls were subjected to SDS-PAGE. Figure 7.5 illustrates typical Western blots obtained by loading equal amounts of cell extract. The blots were probed with antibodies against the different components of the oxidase enzyme. The antibody specific for p22 *phox* reacted with a 22 kDa protein. The size of this protein correlated with the stated molecular mass of p22 *phox* protein subcomponent [Ginsel et al. 1990]. The protein bands were analysed using densitometry and values were normalized as described in chapter 2. No significant differences were seen in p22 *phox* protein abundance when we compared the 2 subject groups (see figure 7.6). The antibody specific for p47 *phox* reacted with a 47 kDa protein, the size of this protein correlated with the stated molecular mass of p47 *phox* protein [Rodaway et al. 1990] Again, no significant differences were seen when the two groups were compared. The antibodies specific for p67 *phox* and gp91 *phox* reacted with a 67 kDa and a 91kDa protein, respectively. The size of these proteins correlated with the stated molecular mass in the literature [Heyworth et al. 1996; Lambeth et al. 2000]. No significant differences were seen in the abundance of these proteins. Finally the abundance of the small GTP binding protein, Rac2 was analysed using a commercial antibody (Santa Cruz Biotechnology). This antibody reacted with a 22 kDa protein the size of which correlated with the quoted molecular mass of Rac2 [Pick et al. 1993]. No differences were identified. All comparisons were made using an unpaired Student's t-test.


NT PET NT PET NT PET NT PET
21kDa
A
NT PET NT PET NT PET NT PET
45kDa
B
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66kDa
C
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97kDa
D

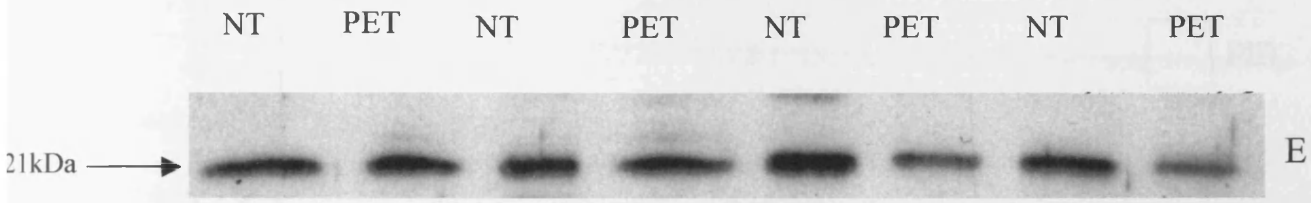

Figure 7.5: Western blot of neutrophils extracts obtained by loading equal amounts (30μg) of protein and probed with specific antibodies

A. The blot was probed with p22 phox specific antibody. The p22 phox protein can be visualised at 22 kD. No difference was evident in p22 phox protein abundance when comparing pre-eclamptics (PET) with normotensive (NT) controls.

B. The blot was probed with p47 phox specific antibody. The p47 phox protein can be visualised at 47 kD. No difference was evident in p47 phox protein abundance when comparing pre-eclamptics (PET) with normotensive (NT) controls.

C. The blot was probed with p67 phox specific antibody. The p67 phox protein can be visualised at 67 kD. No difference was evident in p69 phox protein abundance when comparing pre-eclamptics (PET) with normotensive (NT) controls.

D. The blot was probed with gp91 specific antibody. The gp91 phox protein can be visualised at 91 kD. No difference was evident in gp91 protein abundance when comparing pre-eclamptics (PET) with normotensive (NT) controls.

E. The blot was probed with rac2 specific antibody. The rac2 protein can be visualised at 21 kD. No difference was evident in rac2 protein abundance when comparing pre-eclamptics (PET) with normotensive (NT) controls.

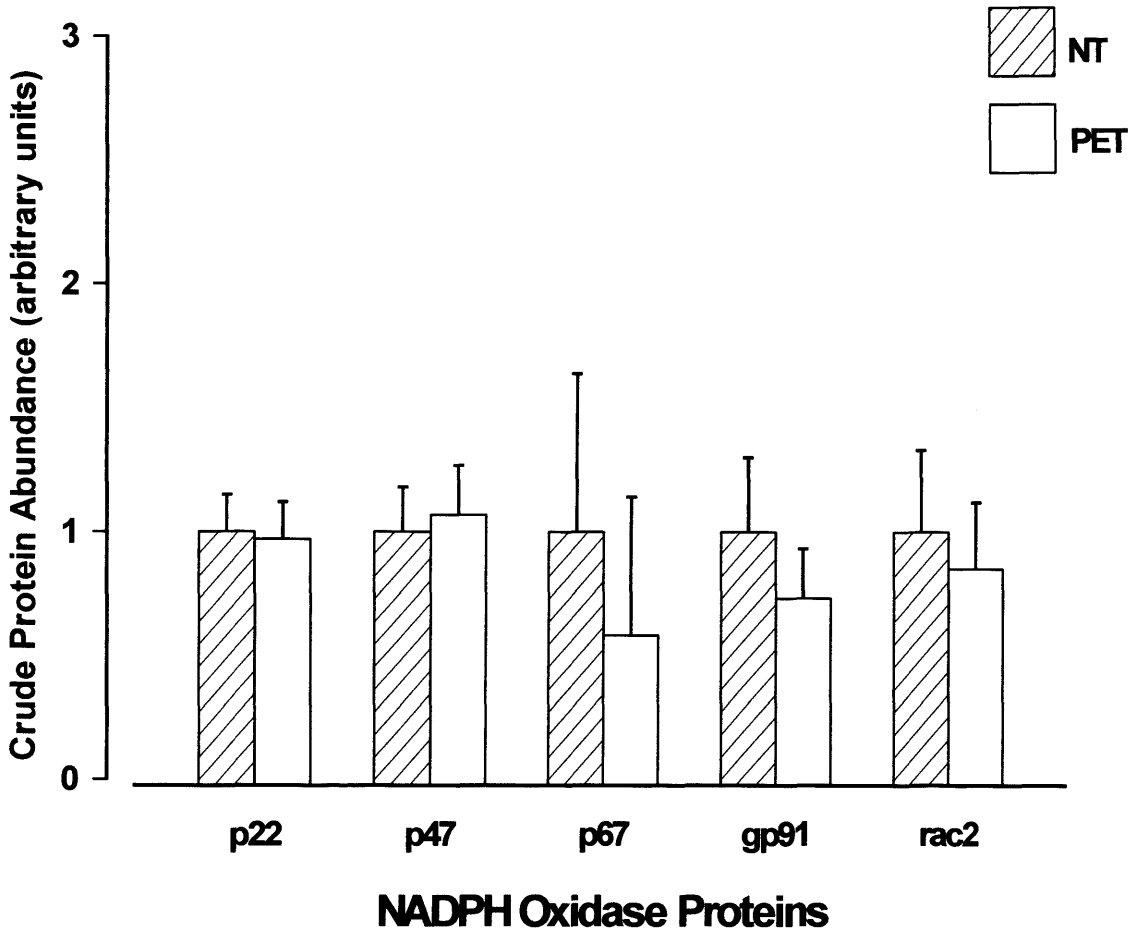

Figure 7.6: Crude protein abundance of NADPH oxidase sub-units from normotensive (NT) and pre-eclamptic (PET) subjects. Values were normalized to a value of 1 in the control group (described in section 2.16). The results represent mean values and SEM are plotted. No significant differences existed, n=17, unpaired Student's t-test.

7.5 Discussion

There is increasing evidence to suggest endothelial cell damage and dysfunction in the pathogenesis of pre-eclampsia as described in chapter 1 [Roberts et al. 1989]. The actual cause of this endothelial damage is unknown, although neutrophils through their ability to produce ROS have been implicated as a likely candidate. Superoxide anions have been shown to inactivate endothelium-derived relaxing factor (EDRF) and reduce the release of prostacyclin, thus favouring vasoconstriction [Whorton et al. 1985 and Drexler et al. 1999]. High concentrations of superoxide have been found to reorientate the arachidonic acid pathway in cells towards the production of thromboxane A_2, which is a potent stimulator of vasoconstriction and platelet aggregation [Cosentino et al. 1994]. The imbalance between prostacyclin and thromboxane A_2 is a well-documented observation in pre-eclampsia [Liu et al. 1998; Mill et al. 1999]. Furthermore, superoxide anions can initiate lipid peroxidation, which is known to result in endothelial cell lysis [Radi et al. 1991]. In pre-eclampsia, blood levels of lipid peroxidation products are elevated and the vascular endothelium shows morphological alterations suggesting the presence of lipid peroxidation as a mechanism of endothelial injury [Walsh 1994; McCarthey 1969]. It seems plausible that the increased neutrophil ROS may be important in mediating the endothelial damage seen in pre-eclampsia.

The data presented in this chapter suggested that although unstimulated responses remained unaltered, agonist stimulated NADPH oxidase activity was enhanced in pre-eclamptic neutrophils compared to normotensive controls when considering lucigenin and luminol-derived chemiluminescent probes. These results suggest that agonist stimulated ROS production was increased in neutrophils isolated from pre-eclamptic women. Therefore the pre-eclamptic neutrophils were considered more sensitive to agonist stimulation as hypothesised. The specificity of the two probes was assessed using scavengers. Luminol measured metabolites in addition to hydrogen peroxide and Tiron (10mM) completely abolished the lucigenin response produced upon stimulation with both fMLP and PMA thus validating the specificity of lucigenin for measuring superoxide production [Hein and Kuo 1998].

The results presented in this chapter complement the work of Tsukimori et al [1993]. Their group suggested that superoxide generation was enhanced in response to the agonist fMLP but not PMA. The study presented in this chapter demonstrated enhanced

ROS production from pre-eclamptic neutrophils in response to both fMLP and PMA. This may have been a consequence of using different techniques to assess neutrophil NADPH oxidase mediated ROS production. Our study incorporated the well-validated lucigenin-derived chemiluminescent technique that may have detected differences in PMA mediated stimulation that were not detectable using other assays. Von Dadelszen et al [1999] also presented results it agreement with the present study. They analysed activation of maternal peripheral blood leucocytes by flow cytometric measurements of intracellular free-ionised calcium and confirmed activation in pre-eclampsia. The observed changes in $[Ca^{2+}]_i$ were thought to be causally related to the increased production of ROS since there may have been a $[Ca^{2+}]_i$ threshold for NADPH oxidase activation. The work presented in this chapter, although consistent with earlier studies of superoxide production in pre-eclampsia and pregnancy, does conflict with studies by Crocker et al. [1999]. They suggested a reduction in the production of superoxide by cells in normal pregnancy and detected no functional differences between these cells and cells from either non-pregnant or pre-eclamptic women using a similar technique to that adopted in this thesis. The reason for this discrepancy was unclear but may have been due to differences in the neutrophil isolation procedure since they used a different method to the one adopted in this thesis. Their particular findings do not fit with the theory associating pre-eclampsia with neutrophil activation but suggested a protective effect of normal pregnancy that was lacking in pre-eclampsia.

In addition to the studies cited above, a study by Barden et al. [1997] concerning neutrophils in pre-eclampsia suggested the presence of increased activation that complements the findings of this study. Neutrophil activation was examined in 22 women with pre-eclampsia and 22 age- and gestation-matched control women using whole-blood flow cytometry to assess basal and platelet-activating factor stimulated CD11b and CD18. Basal neutrophil CD11b expression was significantly increased in women with pre-eclampsia compared with normal pregnancy and was indicative of increased neutrophil activation [Barden et al. 1997]. Neutrophil activation was assessed by Berge et al. [1988] through measuring the release of ROS from neutrophils upon agonist stimulation. In pre-eclampsia, a higher ROS production was seen [Berge et al. 1988]. Von Dadelszen et al. [1999] measured the response of phagocytes to stimulation with fMLP. The peak responses of monocytes to stimulation with 10 nmol/L fMLP was greater in samples isolated from pre-eclamptic women, and suggested evidence of an

increased sensitivity. The study reported in this chapter suggested an increased sensitivity of neutrophil NADPH oxidase activity in response to the agonists' fMLP and PMA. This increased sensitivity may have been achieved by modulating a signal transduction pathway where the pathways may converge or even by two distinct pathways. The enhanced signal transduction may have resulted in an increased phosphorylation of p47phox on specific serine residues and suggest the involvement of a pathway regulated by protein-tyrosine kinase and phosphatidylinositol 3-kinase as well as re-distribution of oxidase components [Dang et al. 1999; DeLeo et al. 1998]. These cells did not show altered abundance of any of the *phox* protein sub-units as no differences were identified when analysing neutrophil extracts following SDS-PAGE analysis. It remains that the conclusions drawn from studies involving the antibodies developed against the components of the NADPH oxidase proteins may be considered tenuous. There was considerable non-specific staining by several of the antibodies on Western blots of neutrophil extracts. However, the antibodies were examined for specificity and the target polypeptides were selected on the basis of expected molecular weight, peptide block, comparison with previously characterized antibodies and fractionation techniques that allow separation of membranes from cytosol. This should be considered sufficient evidence to draw conclusions as to the relative abundance of the NADPH oxidase phox proteins in the 2 groups

The question of what causes this increased sensitivity to agonist stimulation in neutrophils from pre-eclamptic women remains to be elucidated. Both environmental (plasma) and genetic factors may be involved to some extent. Environmental (plasma) factors have been suggested since incubation studies have shown that pre-eclamptic serum enhanced superoxide generation in non-pregnant neutrophils upon agonist stimulation [Kobayashi et al. 1998]. These environmental (plasma) factors are postulated to increase the sensitivity (prime) of neutrophils for subsequent superoxide release upon agonist stimulation and thus may contribute to the oxidative stress and endothelial dysfunction characteristic of pre-eclampsia. Various plasma factors have been cited in the literature. Amongst them are elevated plasma levels of tumour necrosis factor alpha (TNFα), some cytokines, and syncytiotrophoblast microvesicles (STMV) [Vince et al. 1995; von Dadelszen et al. 1999]. Tumour necrosis factor alpha and some cytokines are certainly capable of increasing the sensitivity (priming) of circulating neutrophils *in vitro* and may therefore be important in the increased sensitivity of

neutrophils in pre-eclampsia [Berkow et al. 1987; Daniels et al. 1992]. It was also suggested that the factor that may 'prime' neutrophils is a heat labile protein since the enhancement of superoxide production upon incubation with pre-eclamptic plasma was destroyed by incubation at 100 °C and by trypsin treatment [Tsukimori et al. 1993]. The hypothesis for a circulating plasma factor that 'primes' cells is however, not supported by others. Barden et al. [2001] examined whether circulating factors in the plasma of women with pre-eclampsia caused neutrophil activation and found no evidence since neutrophil CD11b and CD18 expression was not differentially altered after incubation with pre-eclamptic plasma.

In addition, a genetic component may be implicated and this hypothesis was investigated in subsequent chapters. It may have been beneficial to undertake experiments to investigate the presence of a factor in the plasma of pre-eclamptic women capable of 'priming' neutrophils for subsequent release of ROS. However, examination of NADPH oxidase mediated ROS production in post-partum pre-eclamptic neutrophils and EBV-immortalized lymphoblasts may provide evidence for genetic influences on this increased sensitivity.

7.6 Conclusion

In summary, this study provided further evidence implicating the role of neutrophil activation in the pathophysiology of pre-eclampsia and suggested increased NADPH oxidase activity as an intermediate phenotype. Pre-eclampsia was characterised by enhanced NADPH oxidase mediated ROS production upon agonist stimulation compared to normal pregnant women. This represented an increased sensitivity characterized by the enhanced response of the NADPH oxidase enzyme to agonist stimulation and possibly contributes to the oxidative stress and endothelial dysfunction characteristic of the disease. This increased sensitivity was unlikely to be due to increased abundance of the *phox* components as no differences were identified between groups.

Chapter 8

NADPH Oxidase Activation and Reactive Oxygen Species Production in Post-partum Pre-eclamptic Women.

8.1 Introduction

A lot of attention has been directed towards the role of oxidative stress in pre-eclampsia [Davridge 1998; Hubel 1999]. It has been postulated that this oxidative stress contributes towards the endothelial dysfunction characteristic of pre-eclampsia resulting in the clinical manifestations of the disorder with placental factors being implicated [Hubel 1999]. There is evidence to suggest that activated neutrophils are involved in generating this oxidative stress through the production of reactive oxygen species (ROS) that can affect endothelial function directly or contribute indirectly through production of lipid peroxides. In addition to the findings presented in the previous chapter, Tsukimori et al. [1993] reported that fMLP induced superoxide production of neutrophils was significantly higher in pre-eclamptics compared to normal pregnant subjects.

NADPH oxidase is an enzyme responsible for the production of ROS in a variety of cells of mesodermal origin. The most thoroughly studied of these is the leucocyte NADPH oxidase, which is found in phagocytes and B-cell lymphocytes [Babior 1999; Maly et al. 1988]. This enzyme consists of protein subunits that on stimulation with certain agonists, translocate to the membrane to form the active oxidase that promotes the reduction of molecular oxygen to superoxide and hydrogen peroxide which are collectively know as ROS [Jones 1994]. Activation of this enzyme, present in neutrophils, is believed to contribute towards the production of ROS in pre-eclampsia [Wang et al. 2001]. However, most studies concerning pre-eclampsia have been conducted during pregnancy, and it has not been possible to separate maternal (genetic) from the environmental (plasma) mechanisms underlying the development of the disease. Examination in the post-partum period would allow examination of maternal (genetic) factors associated with the increased sensitivity of NADPH oxidase and subsequent ROS release. This chapter tests the hypothesis that the intermediate phenotype of increased sensitivity of the NADPH oxidase enzyme may be present in post-partum pre-eclamptic neutrophils. Neutrophils isolated from post-partum pre-eclamptic women may have increased production of ROS upon agonist stimulation of

the NADPH oxidase enzyme which could represent a predisposition that is genetically controlled and may contribute to the oxidative stress and vascular endothelial dysfunction of the disease.

The work presented in this chapter aimed to assess baseline and agonist induced NADPH oxidase mediated ROS production in neutrophils isolated from post-partum pre-eclamptic and post-partum normotensive control patients using a chemiluminescent technique. The abundance of the different components of the NADPH oxidase enzyme were examined using Western blotting techniques.

8.2 Materials and Methods

The methods for assessing ROS produced upon stimulation of the NADPH oxidase enzyme are included in chapter 2 of this thesis. Methods for the production of polyclonal antibodies against the sub-components of the NADPH oxidase enzyme are described along with the techniques used to assess total protein abundance.

8.3 Patients

Eleven of the 14 patients included in chapter 3 were studied with their corresponding age-matched controls. The reduction in patient number was due to volunteer drop out as this study was performed after the study presented in chapter 3. The clinical details of the patient groups are as shown in table 8.1. Once more the mean age of both the 3rd trimester pre-eclamptic women and the age-matched controls was lower than the mean age of both post-partum pre-eclamptic women and the age-matched controls. This had no effect on the results since there was no correlation found between age and lucigenin-derived chemiluminescence and age and luminol-derived chemiluminescence in either of the groups.

8.4 Results

8.4.1 Lucigenin-Derived Chemiluminescent Response of Neutrophils

Isolated neutrophils were stimulated with the agonists' fMLP and PMA. The ROS produced in response to each stimulus was measured as area under the curve and peak response and recorded RLU.sec and RLU respectively. The results were expressed as mean values (10^7) for area under the curve for each group and are illustrated in figure 8.1. Post-partum pre-eclamptics produced similar ROS (0.869 ±0.106 RLU.sec)

compared to the normotensive controls (0.612 ±0.102 RLU.sec) when stimulated with fMLP. The post-partum pre-eclamptic women also exhibited similar ROS production in response to PMA (7.517 ±1.656 RLU.sec) when compared to the post-partum normotensive controls (6.354 ±1.536 RLU.sec). Statistical analysis using an unpaired Student's t-test showed no significant differences between the two groups using either peak response or AUC (see table 8.2). Figure 8.1 also shows that no significant differences exist between ROS levels when the neutrophils were in an unstimulated (baseline) state.

Table 8.1
CLINICAL DATA

	PET	NT
Patient (n)	11	11
Age (years)		
Mean	36 (±1.9)	38 (±1.5)
Range	27-47	27-45
Gestation at delivery (weeks)		
Mean	33.1 (±1.6)	40 (±0.3)*
Range	$28\text{-}40^{+5}$	$38\text{-}41^{+2}$
BP booking (mmHg)		
Mean	116/71(±2.0)	115/64 (±2.1)
Range	100/60-130/80	100/60-120/80
BP highest (mmHg)		
Mean	173/ 110 (±5.9)	115/66 (±2.2)**
Range	150/105-200/120	100/60-120/70
BP visit (mmHg)		
Mean	129/75 (±2.2)	116/70 (±3.0)
Range	120/ 70-142/95	99/65-130/79
Months post-partum at time of sampling		
Mean		
Range	8 (±0.8)	8.5 (±0.95)
	7-9	7-9.5
Parity		
1	6	6
>1	5	5

*Table 8.1: Clinical data for the post-partum pre-eclamptic and post-partum normotensive control. Values are means with range and SEM *P=0.004 **P=0.0001 normotensive versus pre-eclamptics by Student's t-test.*

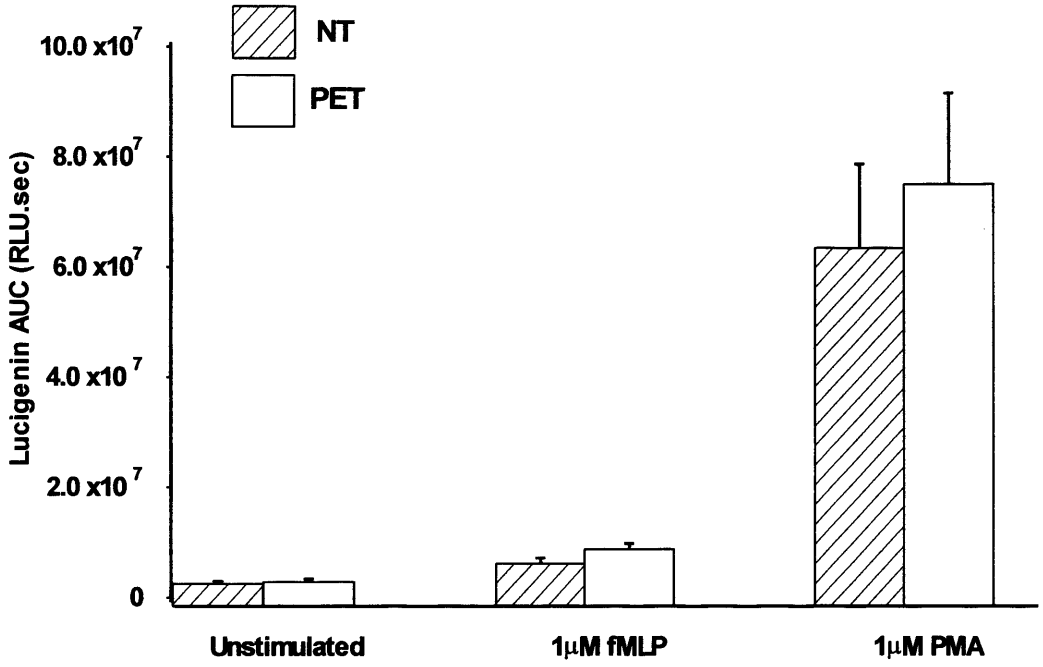

Figure 8.1: Lucigenin-derived chemiluminescence response of neutrophils from post-partum normotensive and post-partum pre-eclamptic women stimulated with n-formyl-met-leu-phe (fMLP) and phorbol myristate acetate (PMA). Chemiluminescence output was recorded as relative light units per second (RLU.sec) and was expressed as area under the curve (AUC) derived using Simpson's rule. The results represent means and the SEM are shown. There were no significant differences present, p=N/S, n=11.

	ppPET	ppNT	P=
Peak lucigenin signal fMLP (10^7 RLU)	0.384 ±0.225	0.098 ±0.020	N/S
Lucigenin AUC 0-30 mins fMLP (10^7 RLU.sec)	0.869 ±0.106	0.612 ±0.102	N/S
Peak lucigenin signal PMA (10^7 RLU)	8.741 ±1.726	6.088 ±1.741	N/S
Lucigenin AUC 0-30 mins PMA (10^7 RLU.sec)	7.517 ±1.656	6.354 ±1.536	N/S

Table 8.2: Post-partum pre-eclamptic (ppPET) and normotensive (ppNT) lucigenin-derived peak chemiluminescence response and AUC following stimulation with fMLP and PMA, expressed as RLU or RLU.sec.

8.4.2 Luminol-Derived Chemiluminescence Response of Neutrophils

Isolated neutrophils were stimulated to produce ROS with the agonists' fMLP and PMA. The ROS produced in response to each stimulus was measured as area under the curve and peak response and recorded as relative light units. The results expressed as mean values (10^8) for area under the curve for each group are illustrated in figure 8.2. Post-partum pre-eclamptics produced slightly more ROS (2.083 ±0.316 RLU.sec) compared to the normotensive controls (1.594 ±0.259 RLU.sec) when stimulated with fMLP. The post-partum pre-eclamptic women also exhibited slightly more ROS production in response to PMA (4.143 ±0.303 RLU.sec) compared to the post-partum normotensive controls (3.840 ±0.299 RLU.sec). Statistical analysis using an unpaired Student's t-test shows no significant differences between the two groups using either peak response or AUC (see table 8.3). Figure 8.2 also shows no significant differences between ROS levels when the neutrophils are in an unstimulated (baseline) state.

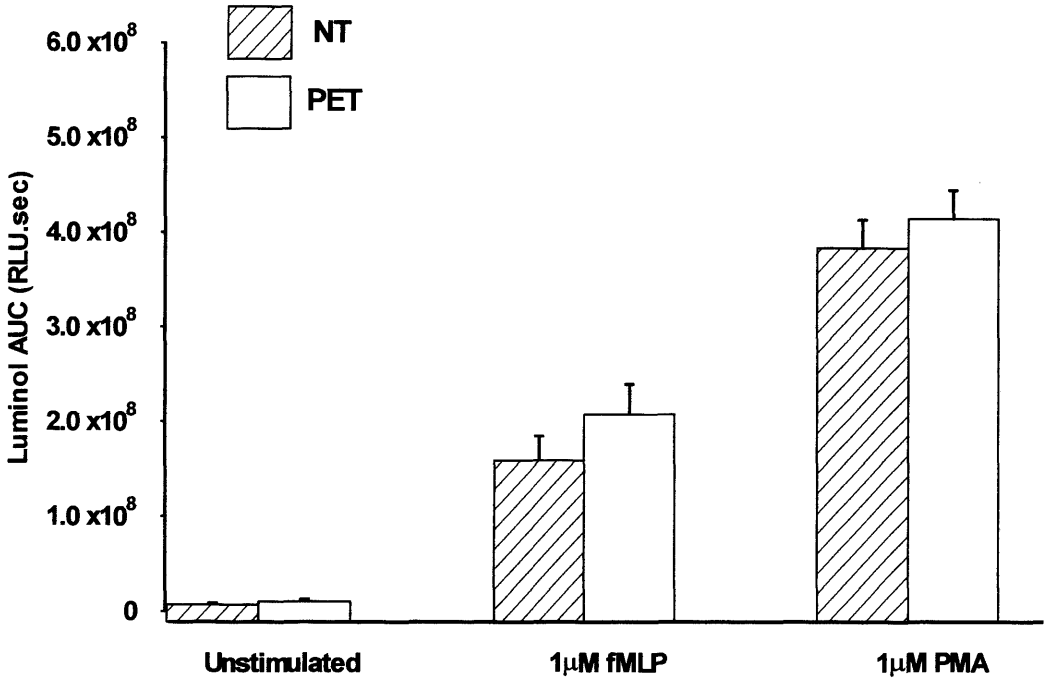

Figure 8.2: Luminol-derived chemiluminescence response of neutrophils from post-partum normotensive and post-partum pre-eclamptic women stimulated with n-formyl-met-leu-phe (fMLP) and phorbol myristate acetate (PMA). Chemiluminescence output was recorded as relative light units per second (RLU.sec) and was expressed as area under the curve (AUC) derived using Simpson's rule. The results represent means and the SEM are shown. There were no significant differences present, P=N/S, n=11.

	ppPET	ppNT	P=
Peak luminol signal fMLP (10^8 RLU)	0.737 ±0.102	0.687 ±0.101	N/S
Luminol AUC 0-30 mins fMLP (10^8 RLU.sec)	2.083 ±0.316	1.594 ±0.259	N/S
Peak luminol signal PMA (10^8 RLU)	0.942 ±0.088	0.866 ±0.092	N/S
Luminol AUC 0-30 mins PMA (10^8 RLU.sec)	4.143 ±0.303	3.840 ±0.299	N/S

Table 8.3: Post-partum pre-eclamptic (ppPET) and normotensive (ppNT) luminol peak chemiluminescence response and AUC following stimulation with fMLP and PMA, expressed as RLU and RLU.sec respectively.

8.4.3 Analysis of NADPH Oxidase Protein Sub-component Abundance

Neutrophil extracts (30μg per track) for both post-partum pre-eclamptic and post-partum normotensive controls were subjected to SDS-PAGE. Figure 8.3 illustrates typical Western blots obtained. The blots were probed with antibodies against the different *phox* components of the oxidase enzyme. The protein bands were analysed using densitometry and values were normalized to a value of 1 in the normal controls. No significant differences were seen in protein abundance of any of the oxidase components when the two subject groups were compared (see figure 8.4). All comparisons were made using an unpaired Student's t-test.

8.5 Discussion

The results presented in this chapter show the production of ROS in response to agonist stimulation of neutrophils isolated from post-partum pre-eclamptic and post-partum normotensive control women. ROS were measured using both lucigenin and luminol as chemiluminogenic probes and no significant differences were identified in either unstimulated or stimulated cells when analysing both peak response and area under the curve. This was not that surprising since the data tends to exhibit large standard error of the mean suggesting a large variation within the groups.

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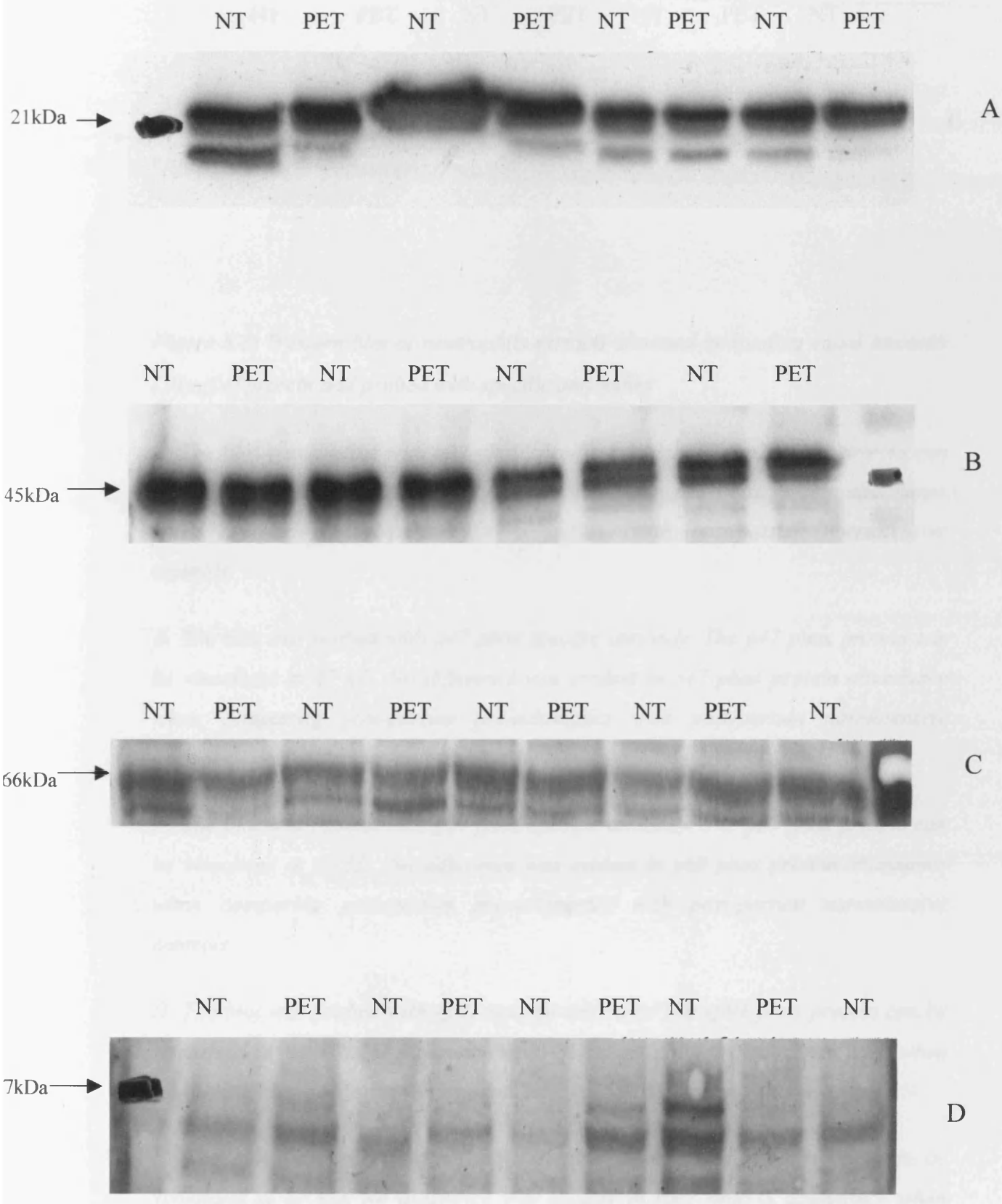
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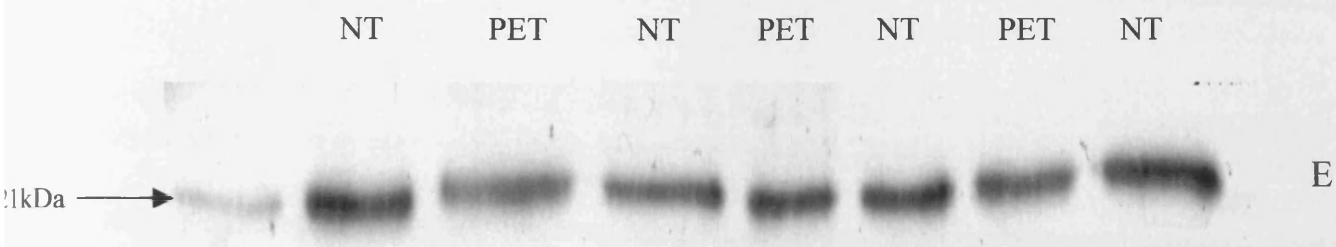


Figure 8.3: Western blot of neutrophils extracts obtained by loading equal amounts (30μg) of protein and probed with specific antibodies

A. The blot was probed with p22 phox specific antibody. The p22 phox protein can be visualised at 22 kD. No difference was evident in p22 phox protein abundance when comparing post-partum pre-eclamptics with post-partum normotensive controls.

B. The blot was probed with p47 phox specific antibody. The p47 phox protein can be visualised at 47 kD. No difference was evident in p47 phox protein abundance when comparing post-partum pre-eclamptics with post-partum normotensive controls.

C. The blot was probed with p67 phox specific antibody. The p67 phox protein can be visualised at 67 kD. No difference was evident in p69 phox protein abundance when comparing post-partum pre-eclamptics with post-partum normotensive controls.

D. The blot was probed with gp91 specific antibody. The gp91 phox protein can be visualised at 91 kD. No difference was evident in gp91 protein abundance when comparing post-partum pre-eclamptics with post-partum normotensive controls

E. The blot was probed with rac2 specific antibody. The rac2 protein can be visualised at 21 kD. No difference was evident in rac2 protein abundance when comparing post-partum pre-eclamptics with post-partum normotensive controls.

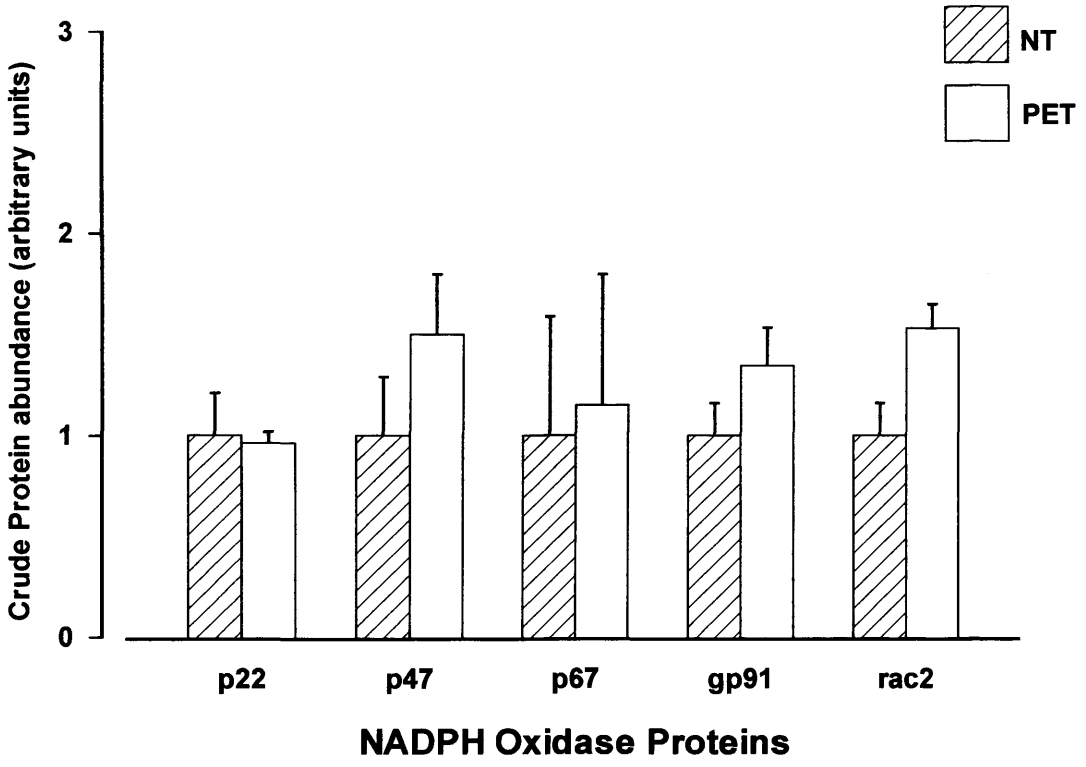


Figure 8.4: Crude protein abundance of NADPH oxidase sub-units from post-partum normotensive (NT) and pre-eclamptic (PET) subjects. Values were normalized to a mean value of 1 in the control group. The results represent mean values and SEM are plotted. No significant differences exist, n=11.

In this study the production of ROS from neutrophils was measured upon stimulation with agonists using both lucigenin, a chemiluminescent probe selective for extracellular superoxide anions and luminol, a probe that measures hydrogen peroxide production as well as other oxygen metabolites [Vladimir and Wilhelm 1989]. The validity of lucigenin has previously been questioned as a chemiluminogenic probe for detecting superoxide anions since lucigenin itself may act as a source of superoxide via autoxidation of the lucigenin cation radical in the NADPH oxidase system of superoxide production [Li et al. 1998]. To detect superoxide, lucigenin must first be reduced by one electron to produce the lucigenin cation radical [Allen. 1986]. The lucigenin cation then reacts with the superoxide to yield an unstable dioxetane intermediate. This decomposes to produce two molecules of N-methylacridone, one of which is in an electronically excited state, which upon relaxation to ground state, emits a photon [Allen. 1986]. However, at a concentration of 50μM and below, lucigenin fails to undergo redox cycling in the superoxide generating system [Li et al. 1998]. Therefore, experimental error due to production and measurement of superoxide from the redox cycling of lucigenin may be ruled out. This phenomenon does not contribute to the ROS produced in post-partum pre-eclamptic and post-partum normotensive neutrophils. Neutrophils are very susceptible to stimulation, even in the absence of agonists. The utmost care was taken to minimize stimulation during the isolation procedure. All solutions used were sterile and endotoxin free but the possibility of experimental error due to inappropriate stimulation during the isolation steps cannot be ruled out. It has previously been shown that the neutrophil isolation procedure affects ROS production and in some cases may cause a misinterpretation of results. Rebecchi et al. [2000] reported on the use of lucigenin- and luminol-derived chemiluminescence to estimate the production of ROS from intact cells. The results were reported to be significantly higher for cells isolated by density gradient both in the absence and presence of added stimuli. The use of a more stable cell line free from the artefacts of the isolation procedure would perhaps provide more conclusive results.

EBV-immortalized lymphoblasts would act as a more suitable model to study agonist stimulated ROS production in post-partum pre-eclampsia. The plasma (environmental) influences would decline further during culture and these cells express the NADPH oxidase enzyme [Kobayashi et al. 1990]. This would allow genetic influences to be examined. The inconclusive nature of the study may have been due to the instability of

neutrophils as models. It is not ambitious to postulate a trend towards significance had the number of women in each group been increased. The results presented in this study contrast to those reported in the 3rd trimester neutrophils and may represent the study being underpowered with respect to the post-partum women. Power analysis was performed and the number of women calculated (n) for a power of 90% to detect a difference at P=0.05 level. An n of 40 would have been required to detect a difference at P=0.05 when considering lucigenin-derived chemiluminescence upon agonist stimulation with fMLP.

The results presented in this chapter may be suggestive that environmental (plasma) influences are indeed important since the differences in sensitivity and subsequent ROS production that were present during the 3rd trimester pre-eclamptic neutrophils were no longer present in the post-partum pre-eclamptic cells. This may suggest that the effects do not persist into the post-partum period and are therefore associated with the pre-eclamptic pregnancy (plasma mediated) and not genetically influenced as postulated.

The presence of antioxidants in pre-eclampsia may have been important for data interpretation. Values obtained for ROS measured by luminol-derived chemiluminescence techniques showed similar values for both 3rd trimester and post-partum pre-eclamptic neutrophils (tables 7.2 and 8.3) which suggests the possibility that this study in post-partum pre-eclamptic neutrophils was underpowered. By contrast, lucigenin-derived measurements of ROS production between studies were not similar (Tables 7.1 and 8.2). This may have been due to the confounding effects of a lack of superoxide dismutase that has been associated with pre-eclamptic pregnancies [Mutlu-Turkoglu et al. 1998]. This would prevent superoxide being converted to hydrogen peroxide therefore one would expect to see higher values for lucigenin-derived chemiluminescent measurement of ROS compared to luminol-derived chemiluminescence since lucigenin measured superoxide and luminol mostly hydrogen peroxide. The correction of the SOD levels in the post-partum period may have resulted in less ROS production measured using lucigenin-derived chemiluminescence since it would have been converted to hydrogen peroxide, catalysed by SOD. Again, examination of these hypotheses using EBV-immortalized lymphoblasts would perhaps provide more conclusive results since environmental (plasma) influences decline during culture. It was unlikely that the differences in values obtained between studies was due

to the differences in mean age since no correlations were found between age and lucigenin-derived chemiluminescence and age and luminol-derived chemiluminescence in either the pre-eclamptic or control women.

There were no differences in the abundance of the various components of the NADPH oxidase enzyme using Western blot analysis. This result was not surprising since no significant differences were uncovered in the agonist-stimulated production of ROS in either group.

8.6 Conclusion

The fact that post-partum pre-eclamptic cells may be more sensitive to agonist stimulation resulting in enhanced ROS production cannot be ruled out. The difference between groups was small and the study underpowered to detect differences in ROS production. It cannot be ruled out that the increased sensitivity of the oxidase demonstrated in neutrophils isolated from 3rd trimester pre-eclamptic women did not persist into the post-partum period and may therefore be environmentally (plasma) influenced rather than genetically influenced. Further examination of these hypotheses is presented later in this thesis using a more stable and robust cell model that overcomes the isolation difficulties and the group size that posed a problem in this study. No differences in abundance of the various *phox* proteins were identified but the specificity of the antibodies used to probe for these proteins remains questionable.

Chapter 9

Examination of Genetic Influences on NADPH Oxidase Activity in Pre-eclampsia Using Epstein–Barr Virus Immortalized Lymphoblasts as Models

9.1 Introduction

The problem of obtaining a sufficient number of cells with which to perform detailed biochemical studies continues to exist. Immortalization of B-cell lymphocytes with Epstein-Barr virus allows cells to be propagated indefinitely and provides a copious supply of cells to examine the NADPH oxidase activity in pre-eclampsia. EBV-immortalized lymphoblasts are therefore a useful model for study of the intermediate phenotype of increased NADPH oxidase activity and subsequent production of ROS in pre-eclampsia. Like neutrophils, EBV immortalized lymphoblasts express all constituents of the NADPH oxidase complex necessary to generate ROS [Furukawa et al. 1992]. The composition of the NADPH oxidase in EBV-immortalized lymphoblasts appears to be identical to that of phagocytic cells and is therefore a good model to study NADPH oxidase activity [Jones and Wood 1996]. Research concerning NADPH oxidase was initially focussed around chronic granulatomous disease, a disease associated with a deficiency of one of the *phox* components of the oxidase enzyme. A defect in the regulation of this enzyme in these subjects persisted in EBV-immortalized lymphoblast cell lines and strongly suggested that ROS produced by lymphoblasts is via the same NADPH oxidase system as is used by neutrophils [Porter et al. 1992]. In addition, two-dimensional phosphopeptide mapping analysis of p47-phox from EBV-immortalized B lymphocytes and human neutrophils showed that the same peptides were phosphorylated in both cells [El Benna et al. 1996] Consequently, EBV-immortalized lymphoblasts provide a robust and stable cell line without the problems of activation associated with the isolation procedure since only one step is required to obtain the cells from whole blood samples. However, it could be argued that the model has its drawbacks since the cells contain no receptors for fMLP and ROS production is only 5% of that measured from neutrophils and might be the result of the small amount of expressed cytochrome b558 [Batot et al. 1998 and Volkman et al. 1984]. However, since NADPH oxidase produces ROS via the same system regardless of the cell type, B lymphoblasts provide an excellent model system for studies of the NADPH oxidase in pre-eclampsia [Volpp and Lin 1993].

This study tests the hypothesis that examination of NADPH oxidase mediated ROS production in EBV-immortalized lymphoblasts from pre-eclamptic and post-partum pre-eclamptic patients may allow identification of a genetically influenced increased sensitivity of NADPH oxidase enzyme in pre-eclampsia since cells are removed from the influences of plasma factors *in vivo*.

The study aimed to examine EBV-immortalized lymphoblasts from both 3rd trimester pre-eclamptic, post-partum pre-eclamptic women and their respective control groups with respect to NADPH oxidase mediated ROS production using luminol as the probe and PMA as the agonist. This would allow the identification of genetic/humoral influences on NADPH oxidase mediated ROS production in pre-eclampsia.

9.2 Materials Methods

The methods used for preparing the cell lines and assessing ROS produced upon stimulation of the NADPH oxidase enzyme are included in chapter 2 of this thesis along with the techniques used to assess *phox* protein abundance.

9.3 Patients

The study group consisted of patients examined in chapter 5. The clinical characteristics of the patients are presented in tables 5.1 and 5.2.

9.4 Results

9.4.1 Reactive Oxygen Species Production in EBV-Immortalized Lymphoblasts From 3rd Trimester Pre-eclamptic and 3rd Trimester Control Women.

EBV-immortalized lymphoblast cell lines were used to assess the production of ROS in response to the agonist PMA. Figure 9.1 illustrates the results obtained from the 3rd trimester cell lines. The pre-eclamptic cells produced significantly more ROS in response to PMA stimulation of NADPH oxidase compared to the normal 3rd trimester control cells. The mean pre-eclamptic measurement (10^7) was 2.890 ±0.522 RLU.sec compared to a mean value of 1.419 ±0.268 RLU.sec recorded for the normal control cells (P=0.025, Student's t-test). Baseline measurements were assessed and no significant differences were present between the 2 groups (not shown).

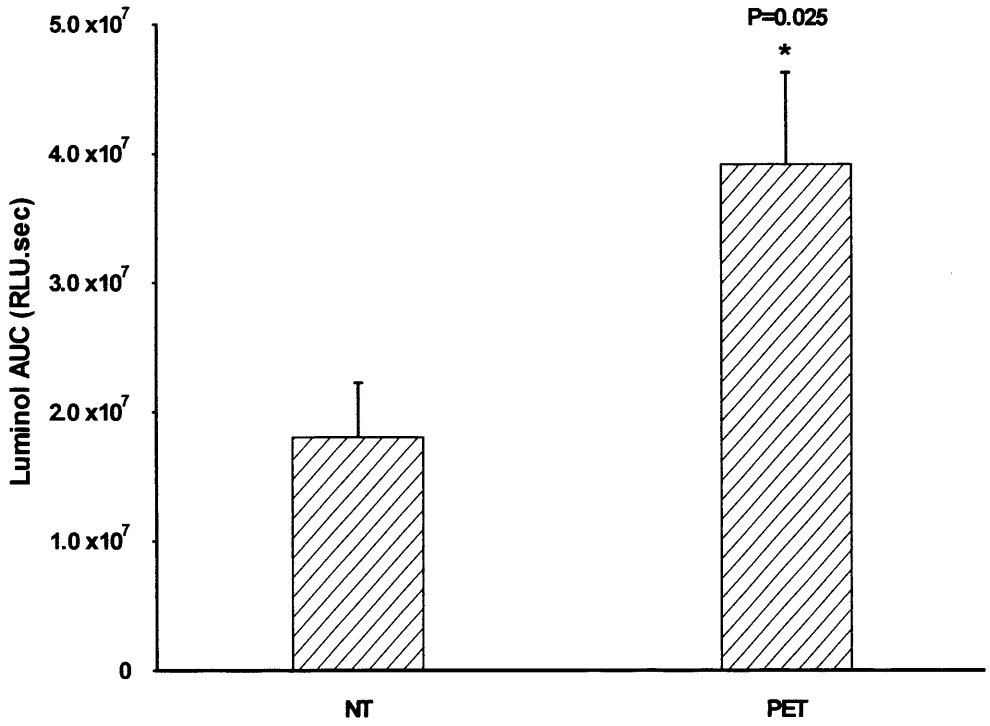


Figure 9.1: Luminol-derived chemiluminescence responses of EBV immortalized lymphoblasts from 3rd trimester normotensive (n=11) and pre-eclamptic (n=11) patients stimulated with phorbol myrisitate acetate (PMA). Chemiluminescence output was recorded as relative light units (RLU.sec). The results represent mean values of 3 separate experiments and SEM are shown (10^7). The EBV lymphoblast chemiluminescence response was significantly enhanced in the PET (2.890 ±0.522 RLU.sec) compared to the NT (1.419 ±0.268 RLU.sec) patient group (P=0.025 Student's t-test).

9.4.2 Reactive Oxygen Species Production in EBV-Immortalized Lymphoblasts From Post-partum Pre-eclamptic and Normotensive Control Women

Figure 9.2 illustrates the results obtained when cells from the post-partum women were subjected to stimulation of the NADPH oxidase enzyme with the agonist PMA. Cells produced significantly different amounts of ROS in response to stimulation. The mean value obtained for the post-partum pre-eclamptic group was 3.923 ±0.710 RLU.sec compared to 1.807 ±0.421 RLU.sec obtained for the post-partum normotensive controls (P=0.021, Student's t-test). There were no differences in baseline measurements obtained before stimulation (not shown).

9.4.3 Analysis of NADPH Oxidase Protein Sub-component Abundance in EBV Immortalized Lymphoblasts.

The post-partum EBV-immortalized lymphoblasts revealed the most significant differences between groups when assessing the production of ROS in response to PMA. Therefore, these cell lines were used to assess whether any differences existed in the abundance of the membrane bound and cytosolic sub-components of the oxidase enzyme. Figure 9.3 illustrates typical Western blots obtained by loading 50μg of cell extract. The blots were probed with antibodies against the different *phox* components of the oxidase enzyme. Figure 9.4 illustrates the results of the densitometry analysis of the lymphoblast extracts and represents the abundance of a particular sub-component. Values were normalized to a value of 1.0 in the control group as described in chapter 2. No significant differences were seen in abundance of any of the *phox* components of the oxidase enzyme.

9.5 Discussion

EBV-immortalized lymphoblasts derived from the four patient groups were used to further examine the role of NADPH oxidase activity as an intermediate phenotype in pre-eclampsia. ROS production was measured upon agonist stimulation of the NADPH oxidase enzyme. Luminol was incorporated as the chemiluminogenic probe with which to measure the response as lucigenin responses were not sensitive enough to measure the ROS produced (not shown). Secondly, EBV-immortalized lymphoblasts did not possess the receptors for fMLP induced stimulation, so PMA was chosen as the agonist

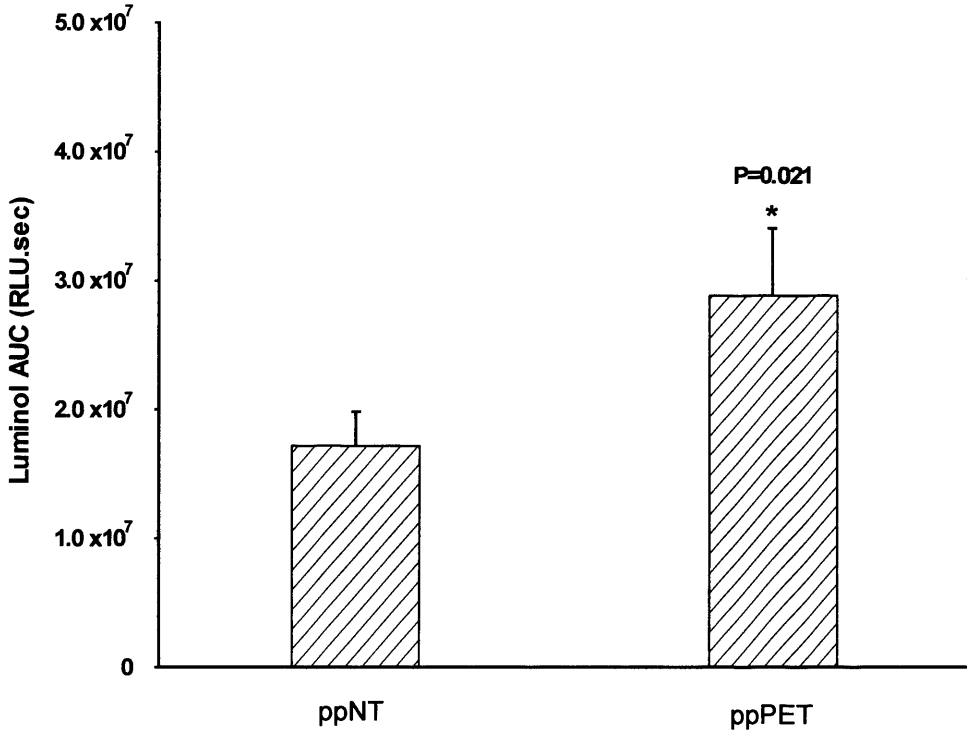
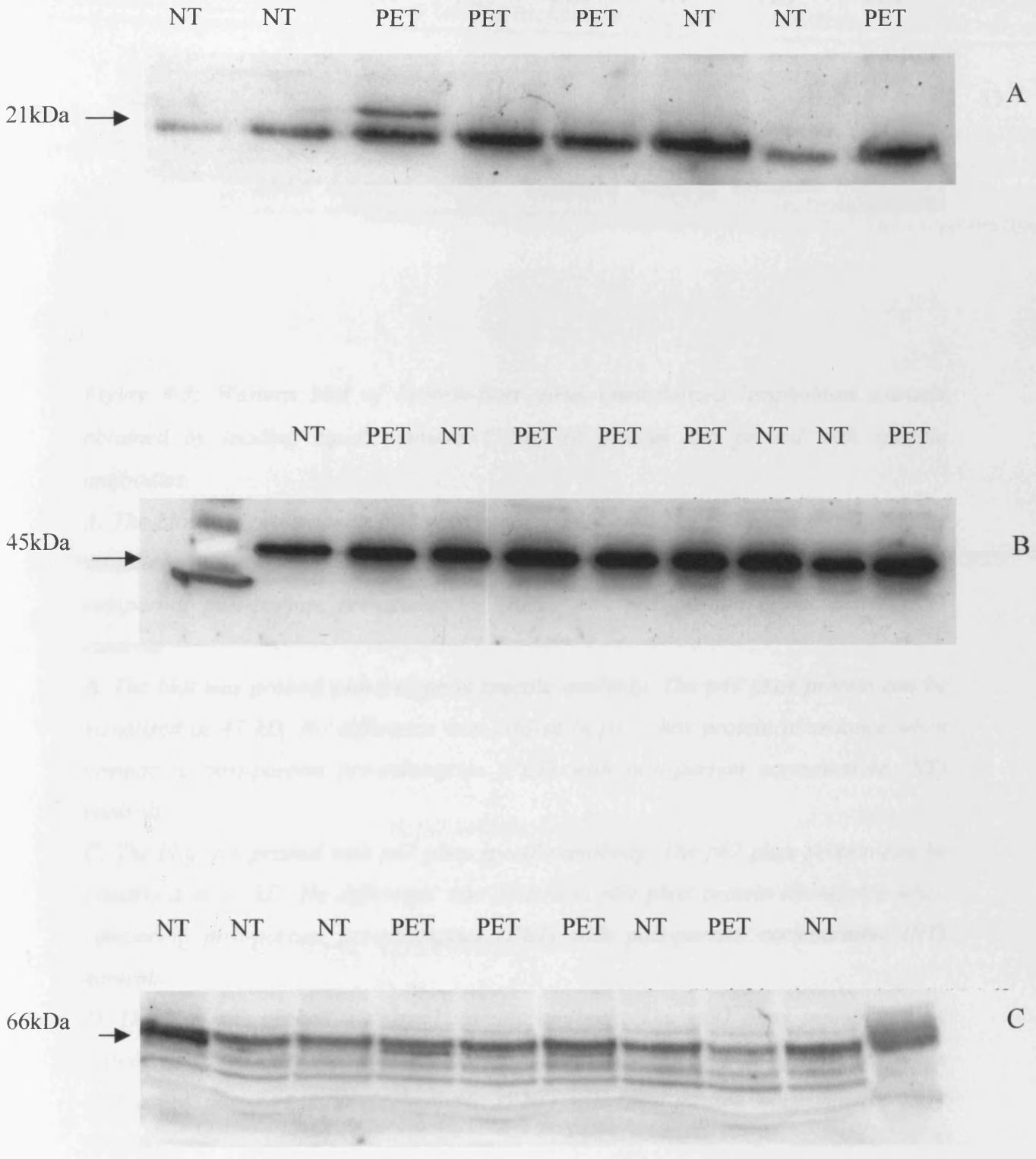


Figure 9.2: Luminol derived chemiluminescence responses of EBV immortalized lymphoblasts from post-partum normotensive (n=8) and pre-eclamptic (n=12) patients stimulated with phorbol myrsitate acetate (PMA). Chemiluminescence output was recorded as relative light units (RLU.sec) The results represent mean values of 5 separate experiments and SEM are shown. The EBV lymphoblast chemiluminescence response was significantly enhanced in the PET 3.923 ±0.710 compared to the NT 1.807 ±0.42 patient group (P=0.021, Student's t-test).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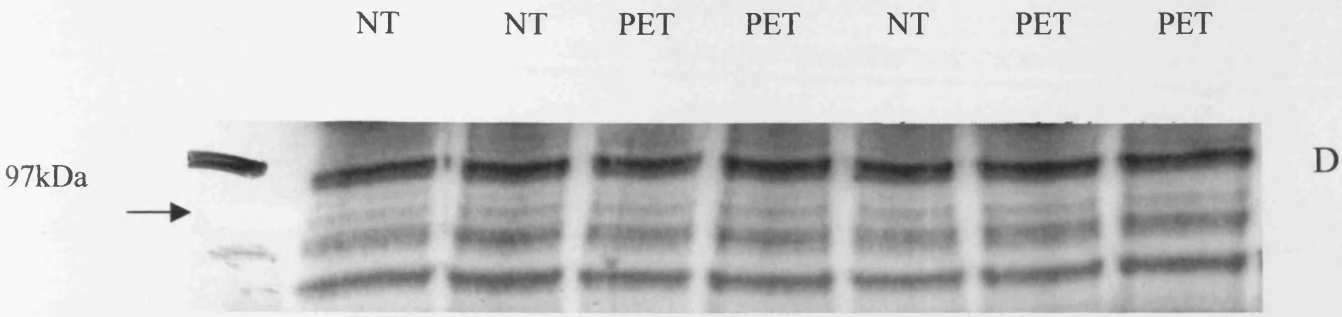


Figure 9.3: Western blot of Epstein-Barr virus immortalized lymphoblast extracts obtained by loading equal amounts (50μg) of protein and probed with specific antibodies.

A. The blot was probed with p22 phox specific antibody. The p22 phox protein can be visualised at 22 kD. No difference was evident in p22 phox protein abundance when comparing post-partum pre-eclamptics (PET) with post-partum normotensive (NT) controls.

B. The blot was probed with p47 phox specific antibody. The p47 phox protein can be visualised at 47 kD. No difference was evident in p47 phox protein abundance when comparing post-partum pre-eclamptics (PET) with post-partum normotensive (NT) controls.

C. The blot was probed with p67 phox specific antibody. The p67 phox protein can be visualised at 67 kD. No difference was evident in p69 phox protein abundance when comparing post-partum pre-eclamptics (PET) with post-partum normotensive (NT) controls.

D. The blot was probed with gp91 specific antibody. The gp91 phox protein can be visualised at 91 kD. No difference was evident in gp91 protein abundance when comparing post-partum pre-eclamptics (PET) with post-partum normotensive (NT) controls.

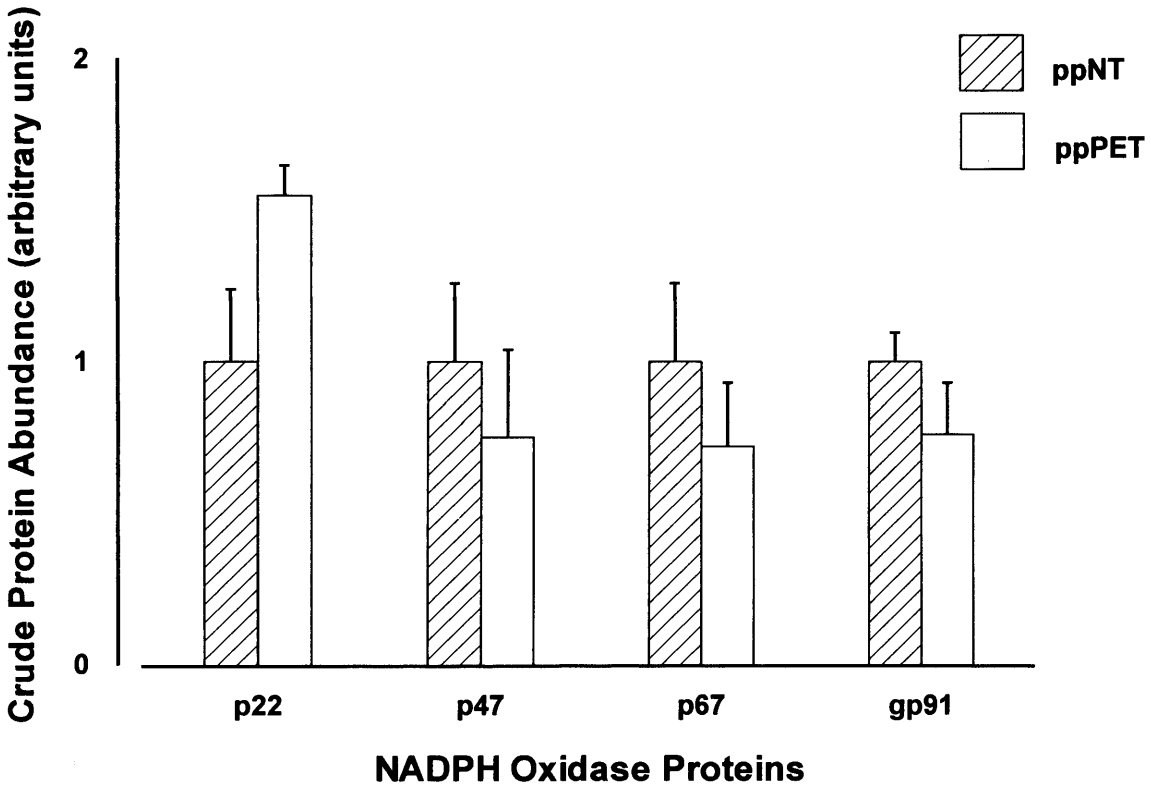


Figure 9.4: Crude protein abundance of NADPH oxidase sub-units from EBV lymphoblasts of post-partum normotensive (ppNT) and pre-eclamptic (ppPET) subjects, measured as detailed in the legend from figure 7.6. Values were normalized to a value of 1 in the controls. The results represent mean values and SEM are plotted (n=6). No significant differences exist, unpaired Student's t-test, P=N/S.

[Volkman et al. 1984]. The agonist stimulated production of ROS was increased in both 3rd trimester pre-eclamptic and post-partum pre-eclamptic cells with respect to their normal controls. This increased sensitivity may have been influenced by genetic factors and would account for the results demonstrated in EBV-immortalized lymphoblasts since transformation eliminates the influence of environmental (plasma) factors and allows the examination of genetic influences on a system. However, this hypothesis does not take into the consideration the data that suggests pre-eclampsia is associated with a factor present in pre-eclamptic plasma that renders cells more sensitive to subsequent agonist stimulation [Tsukimori et al. 1993].

There is evidence in the literature to suggest that pre-eclamptic cells are 'primed' by some factor in the plasma, rendering them more sensitive to agonist stimulation with increased ROS production [von Dadelszen et al. 1999]. TNFα, PAF and syncytiotrophoblast microvesicles (STMV) are among the factors that may contribute [von Dadelszen et al. 1999; Wang et al. 1999; Clark et al. 1998]. However, the results presented in this chapter do not favour this hypothesis since the increased sensitivity persisted in EBV-immortalized lymphoblasts where the environmental (plasma) factors were removed. This suggests that women with pre-eclampsia may be genetically predisposed to possess neutrophils that exhibit an increased sensitivity similar to that of a 'primed' cell. This predisposition may contribute towards the oxidative stress and endothelial dysfunction since upon agonist stimulation these cells produced subsequently more ROS than cells from normal pregnant women. It is therefore postulated that the pre-eclamptic environment may contain the actual stimulus for this activation but a pre-disposition exists. It must be taken into consideration that this increased sensitivity in EBV-immortalized cells isolated from pre-eclamptic and post-partum pre-eclamptic women was only based on luminol-derived chemiluminescent measurement of ROS stimulated with the agonist PMA and was therefore a limitation of using these cell lines as a model.

The abundance of the NADPH oxidase sub-components were examined in 6 post-partum pre-eclamptic and 6 post-partum normotensive cell lines. This experiment was repeated in EBV-immortalized lymphoblasts even though previous results suggested no difference in abundance in the various *phox* proteins in neutrophils. The reasons for this were two-fold. Firstly, different cell types were analysed and secondly, Western blotting

confirmed that lymphoblasts do indeed possess all the necessary components of the oxidase as cited by Batot et al [1998]. No differences in the abundance of any of the sub-components were identified providing evidence that the enhanced sensitivity was not due to altered abundance of the components of the NADPH oxidase enzyme. These findings supports the findings presented in the previous chapters. However, all components of the oxidase were indeed present.

Finally, this chapter showed increased NADPH oxidase mediated ROS production in EBV-immortalized cells from post-partum pre-eclamptic women compared to their normal controls and represented an increased sensitivity of the enzyme to agonist stimulation. This increase was not observed in the experiments reported in chapter 8 involving neutrophils and using both luminol and lucigenin as probes. This may have been due to the isolation procedure that may have resulted in unwanted stimulation. Measurements using EBV immortalized cells ruled out this artefact as cultured cells were not subjected to the isolation procedure. Alternatively, there is evidence to suggest that acidosis inhibits ROS production [Simchowitz 1985]. The effect of alterations in intracellular pH on ROS production may have contributed to the inconsistency in differences identified between studies. This hypothesis was examined in the next chapter.

9.6 Conclusion

In conclusion, pre-eclampsia was associated with an increased sensitivity of the NADPH oxidase enzyme using EBV-immortalized lymphoblasts as models. This increased sensitivity was demonstrated through increased ROS production upon agonist stimulation with PMA, measured using luminol-derived chemiluminescence. This increased sensitivity persisted in the post-partum pre-eclamptic cell lines. This effect may have been due to genetic factors since environmental (plasma) were removed. The increased sensitivity may contribute to the neutrophil activation in pre-eclampsia through its ability to produce subsequently more ROS upon agonist stimulation. The enhancement of ROS generation may be important in mediating the endothelial dysfunction seen in pre-eclampsia.

Chapter 10

Effect of Intracellular pH on Reactive Oxygen Species Production.

10.1 Introduction

Previous workers presented conflicting arguments concerning the effect of intracellular pH on NADPH oxidase activity and the production of superoxide and hydrogen peroxide. Simchowitz [1985] provided evidence that alkalinization of intracellular pH facilitated the generation of ROS in the presence of fMLP. Geffner et al [1993] examined the effect of manipulating the pH on immune complex induced NADPH oxidase activation and found the production of ROS to be significantly inhibited at pH 6.2 compared to 7.4. Trevani et al [1999] suggested that an acidotic pH enhanced oxygen dependent cytotoxic responses such as cytoskeletal responses and the production of hydrogen peroxide but inhibited superoxide production.

This chapter tests the hypothesis that acidotic intracellular pH may have a modulating effect on NADPH oxidase activity and the subsequent production of ROS. This may help to explain the discrepancy in results obtained between neutrophils and EBV-immortalized lymphoblasts isolated from post-partum pre-eclamptics when considering NADPH oxidase mediated ROS production. The acidotic intracellular pH in post-partum pre-eclamptic neutrophils may have inhibited NADPH oxidase mediated ROS production whereas the more alkaline intracellular pH values in EBV-immortalized lymphoblast lines may have permitted full expression of this intermediate phenotype of increased NADPH oxidase mediated ROS production.

In this study luminol and lucigenin-derived chemiluminescence were adopted to examine the effect of manipulating intracellular pH on NADPH oxidase mediated ROS production in various cell types. Intracellular pH was manipulated to the desired value using the double ionophore method used to clamp intracellular pH to 6.0 when assessing Na^+/H^+ exchanger activity. The effect of blocking Na^+/H^+ exchange on the production of ROS in EBV immortalized lymphoblasts using an NHE-1 specific inhibitor called HOE694 was examined.

10.2 Materials and Methods.

Cells were isolated from normal volunteers recruited from within the laboratory and clamped at various intracellular pH's using the double-ionophore method described in chapter 2. Sodium was substituted for NMG in the chemiluminescence medium to prevent activation of the Na^+/H^+ exchanger. The methods for measuring the production of ROS in response to agonists were also presented in chapter 2. The effect of Na^+/H^+ exchanger blockade upon ROS production was examined by incubating EBV immortalized lymphoblasts with 5μM HOE694.

10.3 Results

Figure 10.1 illustrates the effects of manipulating the intracellular pH on luminol-derived chemiluminescence. Normal control EBV-immortalized lymphoblasts were stimulated with PMA and the ROS produced was measured. The results showed that NADPH oxidase activity, measured as ROS production, increased significantly as the intracellular pH became increasingly alkaline (ANOVA, P=0.001, n=6).

Figure 10.2 illustrates the effects of manipulating intracellular pH on luminol-derived ROS production in neutrophils isolated from normal controls. Cells were clamped to various pH's ranging from 6.8 to 7.8 and then NADPH oxidase was stimulated with PMA. At acidotic pH the production of ROS was significantly lower than that produced at a more alkaline pH (ANOVA, P=0.001 n=6). A sharp increase in ROS was measured between pH 7.2 and 7.6 indicative of optimal pH for NADPH oxidase activation.

Figure 10.3 illustrates the effects of manipulating intracellular pH on lucigenin-derived measurement of ROS in neutrophils in response to PMA stimulation of NADPH oxidase. There was an increase in the production of ROS at alkaline pH compared to acidotic pH. Towards the acidotic range, the production of ROS remained low but started to increase linearly with a sharp rise between pH 7.6 and 7.8. At acidotic pH the production of ROS was significantly lower than that produced at a more alkaline pH (ANOVA, P=0.001, n=6).

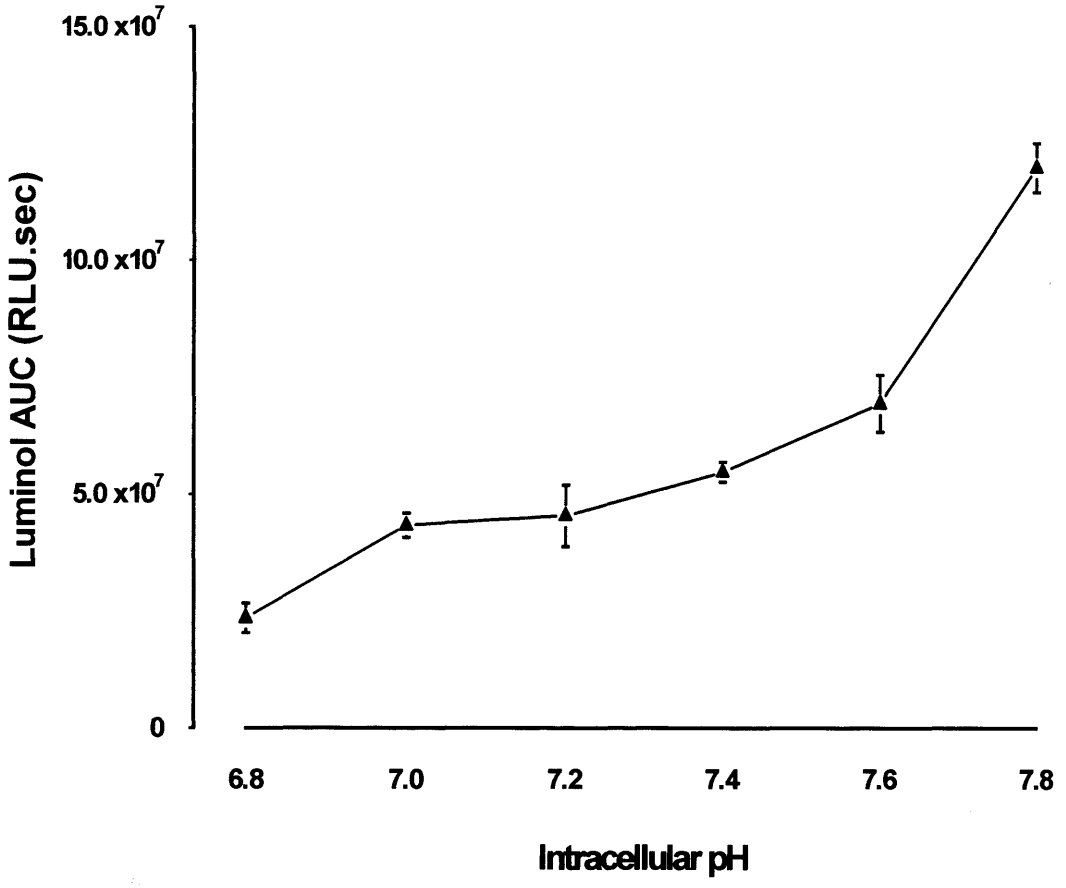


Figure 10.1: The effect of manipulating intracellular pH on luminol-derived reactive oxygen species production in EBV-immortalized lymphoblasts derived from normal controls. The results are expressed as mean ± SEM (10^7 RLU.sec) for area under the curve at each pH (ANOVA, P=0.001, n=6).

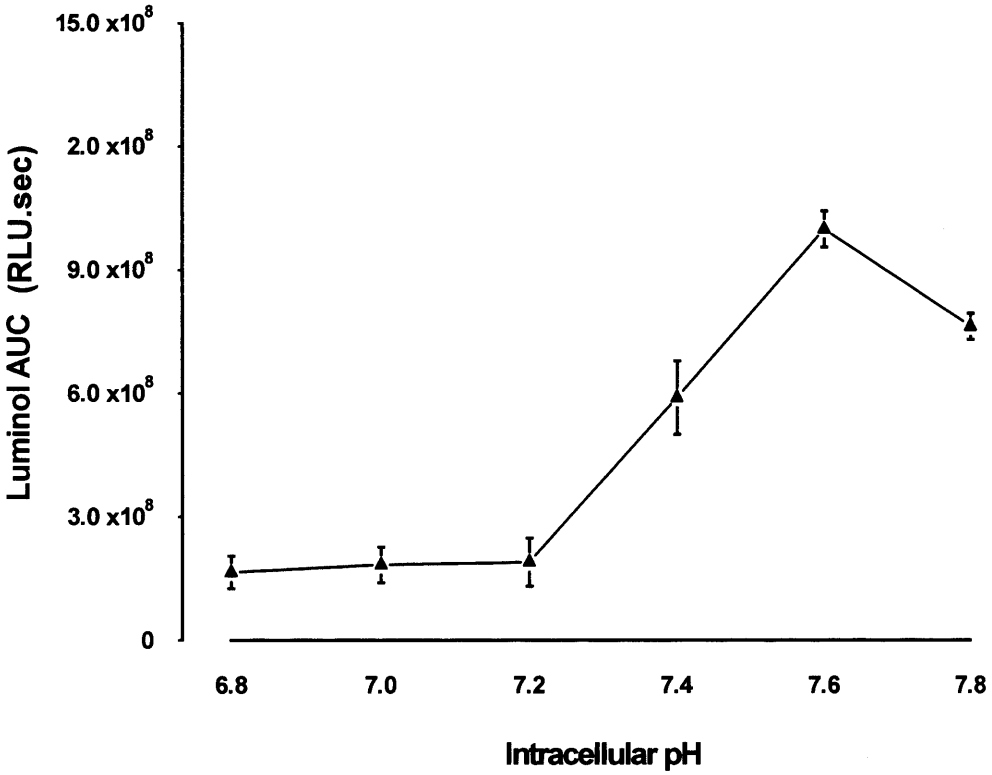


Figure 10.2: The effect of manipulating intracellular pH on luminol-derived reactive oxygen species production in neutrophils isolated from normal controls. The results are expressed as mean ± SEM (10^7 RLU.sec) for area under the curve at each pH (ANOVA, P=0.001, n=6).

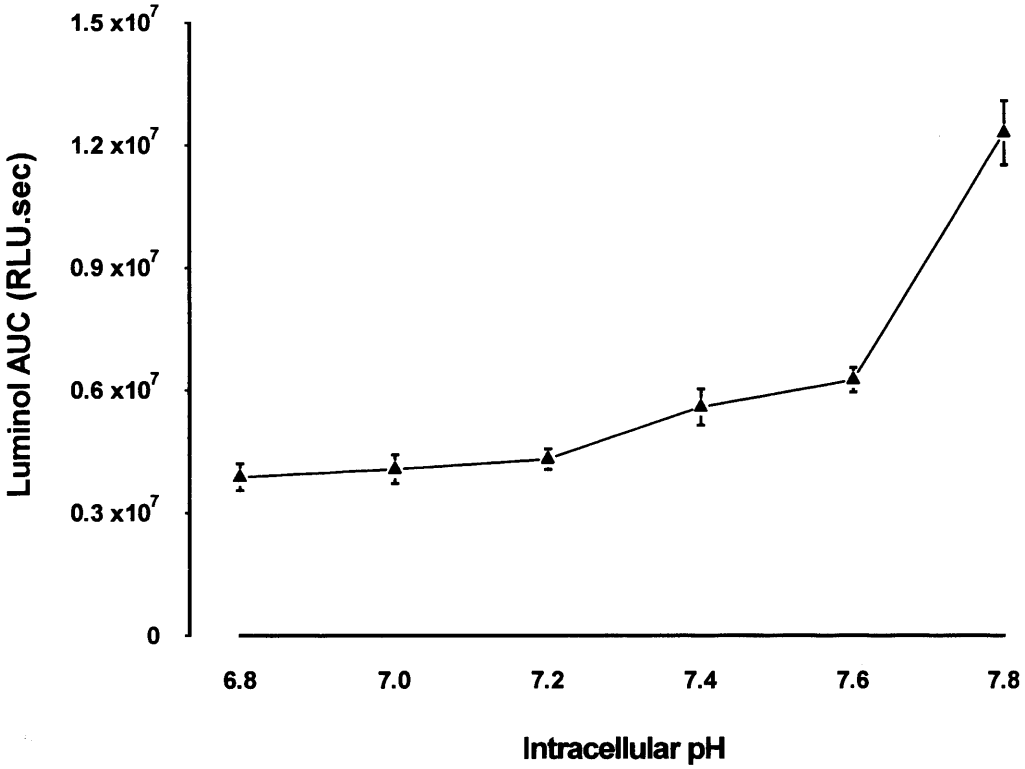


Figure 10.3: The effect of intracellular pH on lucigenin-derived reactive oxygen species production in neutrophils isolated from normal controls. The results are expressed as mean ± SEM (10^7 RLU.sec) for area under the curve at each pH (ANOVA, P=0.001, n=6).

Figure 10.4 represents PMA induced production of ROS in both post-partum pre-eclamptic and normotensive EBV-immortalized lymphoblasts in the presence and absence of HOE694, a drug which blocks Na^+/H^+ exchange. The extent of release was approximately half when the cells were incubated with HOE694 but was found to be statistically significant in the post-partum pre-eclamptic group (12.500 ±7.200 inhibited to 7.400 ±2.855 RLU.sec P=0.05) but not the post-partum normotensive group (3.880 ± 2.440 inhibited to 2.191 ± 1.343 RLU.sec) using a paired Student's t-test, n=6.

10.4 Discussion

The regulation of intracellular pH has previously received considerable attention due to the importance of intracellular pH in a number of cellular functions. pH has been shown to play a role in egg fertilization, properties of muscle, cell growth and division and epithelial transport and secretion [Roos and Boron 1981]. Numerous cellular metabolic events have been shown to be pH sensitive. These include intracellular calcium, protein and DNA synthesis, contractile apparatus activity, membrane permeability to ions and the activity of a large number of hydrolytic enzymes [Busa and Nucciteli 1984]. Some of these appear to play a critical role in activation of NADPH oxidase and suggest that several functions of neutrophils may be regulated by their pH [Segal et al. 1981].

The results of the present study demonstrated a direct relationship between intracellular alkalinization and the amount of ROS generated when NADPH oxidase was stimulated with the agonist PMA. PMA was chosen as the stimulus since both neutrophils and EBV-immortalized lymphoblasts respond and the time course of ROS production in response to the stimulus was greater. The effects of manipulating intracellular pH on the production of ROS in both neutrophils and EBV immortalized lymphoblasts were examined and consistent findings reported. The findings of this study complemented those reported by Simchowitz [1985] who demonstrated most superoxide generation in response to agonist stimulation when the pH_i rose to its most alkaline value of 7.84. Trevani et al [1999] showed a partially conflicting result and suggested that superoxide production was enhanced at alkaline pH and H_2O_2 inhibited, favouring an acidotic environment to provide maximal activation in response to agonist stimulation. The data presented in this chapter indicated a modulating effect of intracellular pH on luminol

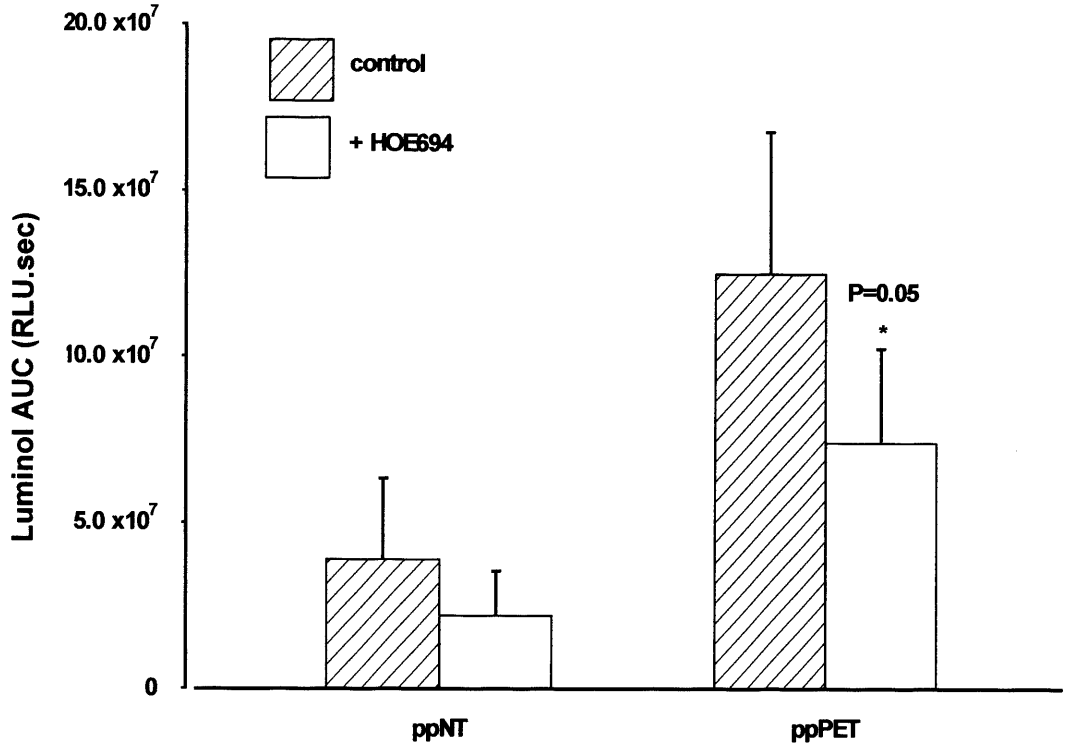


Figure 10.4 Effect of blocking the Na^+/H^+ exchanger with HOE694 on PMA agonist induced production of reactive oxygen species in EBV immortalized lymphoblasts from post-partum pre-eclamptic (ppPET) and normotensive controls (ppNT). Performed in medium of pH 7.4. The results are expressed as mean ± SEM (10^7 RLU.sec) for area under the curve. HOE694 inhibited the production of ROS in both group but was only statistically significant in the post-partum pre-eclamptic group (P=0.05, Student's t-test, n=6).

and lucigenin-derived ROS generated by PMA induced activation of neutrophil and EBV-immortalized lymphoblast NADPH oxidase. These observations may help to explain the apparently conflicting results obtained in chapters 7,8 and 9. Neutrophil intracellular pH was manipulated and ROS production assessed after stimulation with PMA using luminol-derived chemiluminescent detection. Little ROS was detected between pH 6.8 to 7.2 with a sharp increase in production occurring at pH 7.2 to 7.6. These findings may suggest why no significant differences were obtained in the luminol-derived chemiluminescent analysis of NADPH oxidase mediated ROS production in cells isolated from post-partum pre-eclamptic women compared to their respective controls (figure 8.2). Post-partum pre-eclamptic women possess pH_i values (pH 7.11) within the range that exhibit limited ROS production in response to agonist stimulation whereas their respective controls exhibit a pH_i that falls within the range that show moderate ROS production. Yet when the effects of acidotic intracellular pH are removed as seen in EBV immortalized cells isolated from these groups of women, differences in ROS production were unmasked (figure 9.2) implicating the importance of intracellular pH on agonist induced ROS production. This phenomenon would also have had an effect in pre-eclampsia since intracellular pH 7.14 falls within the range of lower ROS production. However, significant differences were seen (figure 7.4). Therefore some other mechanism must be counteracting this inhibitory effect of low pH on ROS production in the 3rd trimester pre-eclamptic cells. A similar effect can be seen with respect to lucigenin-derived chemiluminescent detection of ROS following agonist stimulation of NADPH oxidase with PMA. No significant differences were seen in lucigenin-derived ROS production in post-partum pre-eclamptic compared to their normal controls (figure 8.1) possibly due to the modulating effect of intracellular pH on the measurement of NADPH oxidase mediated ROS production using lucigenin methodology. However, once the effect of different intracellular pH was removed as seen in EBV-immortalized lymphoblasts from the same women, differences in ROS production could be seen (figure 9.1). Once more, differences were seen in 3rd trimester neutrophils (figure 7.3) and may suggest the presence of a mechanism that counteracts or overcomes the inhibitory effect of intracellular acidosis on NADPH oxidase mediated ROS production in pre-eclampsia.

The potentiation of superoxide production demonstrated at alkaline pH_i values may represent the titration of a critical functional group(s) on a protein(s) in a pathway

leading to superoxide generation and subsequent hydrogen peroxide generation. This protein may be expected to reach pH optimum in the region of 8.0 [Simchowitz 1985]. Our range did not cover that spectrum as none of the intracellular pH measurements in either neutrophils, leucocytes or EBV-immortalized lymphoblasts reached that value. The results were suggestive that optimum pH was within this alkaline region due to the sharp increase in production seen between pH 7.6 and 7.8. When assessing the effect of pH_i on neutrophil luminol-derived ROS production, increases were recorded with progressive alkalinization and reached a maximum at pH 7.6 with a decline noted above that value. An increased rate of dismutation at acidic pH_i may have accounted for this finding. Previous studies have demonstrated that spontaneous dismutation of superoxide occurs more rapidly at acidic pH [Rabani and Nielsen 1969]. The production of H_2O_2 is downstream of superoxide and therefore superoxide produced at alkaline pH may not have been dismutated as readily to H_2O_2 due to the optimal pH of SOD being at a more acidic value. This would result in a lower production of H_2O_2 at a value of pH 7.8 (see figure 8.5).

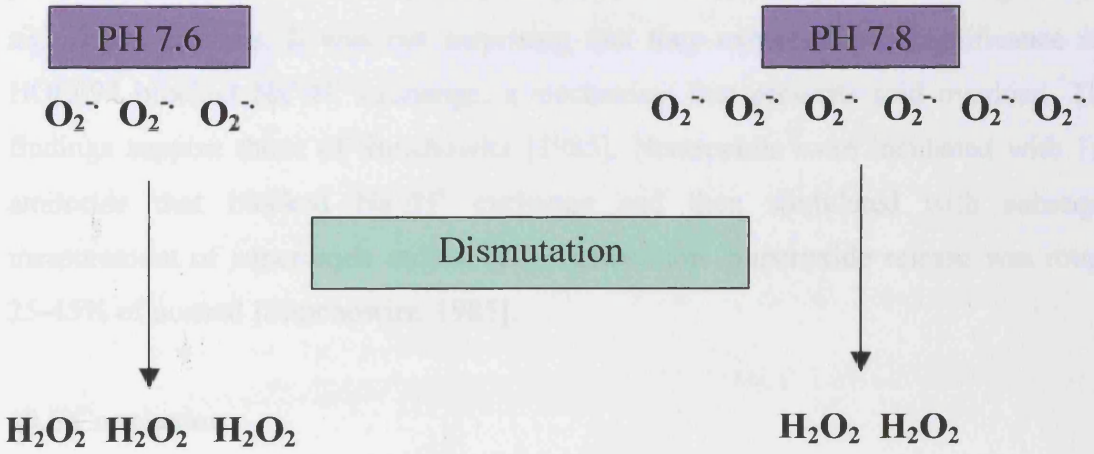


Figure 8.5: The effects of dismutation of hydrogen peroxide production at pH 7.6 and 7.8.

It must also be mentioned that luminol-derived chemiluminescence is sensitive to intracellular pH and the increased production of ROS at alkaline pH range may have been associated with the technique adopted to assess production. Luminol has an optimal pH of 9.5 [Misra and Squatrito 1982]. The effect of intracellular pH on ROS

production may be have been due to the pH sensitivity of the detection system as luminol measured extracellular and well as intracellular production of ROS. However, this seems unlikely since the extracellular pH was set to 7.4 whilst manipulating intracellular pH. Most of the ROS detectable by luminol was extracellular because catalase abolished 70% of the stimulated response (not shown).

The hypothesis that acidotic intracellular pH may have had a modulating effect on NADPH oxidase activity and the subsequent production of ROS was also demonstrated by experiments involving HOE694. HOE694 was added to inhibit Na^+/H^+ exchanger activity and lymphoblasts were then stimulated to produce ROS. This resulted in a build up of protons in the cytosol that would have subsequently acidified the intracellular environment. The amount of ROS produced in the presence of HOE694 was lower compared to that produced in the absence of HOE694. This may have been due to the effect of intracellular acidification on NADPH oxidase mediated ROS production. The inhibition of ROS production achieved by blocking Na^+/H^+ exchange was only significant in the post-partum pre-eclamptic cell lines possibly due to the higher initial production of ROS and consequently the production of more protons leading to a more significant acidosis. It was not surprising that they exhibited this significance since HOE694 blocked Na^+/H^+ exchange, a mechanism that prevents acid overload. These findings support those of Simchowitz [1985]. Neutrophils were incubated with 1mM amiloride that blocked Na^+/H^+ exchange and then stimulated with subsequent measurement of superoxide radical (O2-) generation. Superoxide release was roughly 25-45% of normal [Simchowitz. 1985].

10.5 Conclusion

In conclusion, it appears that intracellular pH plays a modulating or regulatory role at one or more steps leading to superoxide and hydrogen peroxide release in both neutrophils and EBV immortalized lymphocytes stimulated with PMA. It is likely that this phenomenon is not due to the pH sensitivity of the detection system. However, the mechanism by which pH_i exerts its regulatory role is unclear but may be postulated to play a protective role in pre-eclamptic pregnancies, possibly counteracting the enhanced NADPH oxidase activation and, hence, overproduction of ROS.

Chapter 11

Signal Transduction Pathways Implicated in the Regulation of NADPH Oxidase Mediated ROS Production in Pre-eclampsia.

11.1 Introduction

NADPH oxidase is activated by a variety of agents including fMLP and PMA. These stimuli trigger signal transduction pathways leading to the phosphorylation of several proteins of the NADPH oxidase system, such as p47-*phox* [Heyworth and Badway 1990]. The signal transduction pathways controlling NADPH oxidase activation are still poorly documented. There are understood to be PKC dependent and independent pathways of activation that are either calcium dependent or independent [Della Bianca et al. 1993; Dewas et al. 2000; Cabanis et al. 1996]. One of these pathways involves activation of members of the mitogen-activated-protein-kinase family (MAPK). Recent studies have demonstrated that MAPK pathways such as p38MAPK and extracellular-signal regulated kinases (ERK1/2) are activated in neutrophils stimulated with fMLP, PMA, TNFα and GM-CSF [McLeish et al. 1998; El Benna et al. 1996; Thompson et al. 1994]. Phospholipases play an integral role by generating secondary messengers. $cPLA_2$ is believed to be important in the signal transduction pathway to NADPH oxidase mediated superoxide production and the archidonate cleaved may play a role in activation [Lennartz 1999]. A role also exists for the involvement of PI-3 kinase and PLD in activation of NADPH oxidase with various agonists. [Yasui and Komiyama. 2001]. It was suggested that PI3-kinase and PLD play a pivotal role in the signal transduction pathway of the chemo-attractant-receptor involved neutrophil activation. These enzymes produce second messengers that are required for subsequent superoxide production in human neutrophils.

There is now increasing evidence implicating the role of tyrosine phosphorylation as an integral part of the signalling pathway as tyrosine phosphorylation of several polypeptides is increased in neutrophils stimulated with fMLP and PMA [Huang et al. 1990]. A 40-42 kDa polypeptide is one of the primary targets of tyrosine phosphorylation in stimulated neutrophils and happens to be the molecular mass of ERK [Grinstein and Furuya. 1992a].

Preceding chapters demonstrated enhanced NADPH oxidase activation in response to both PMA and fMLP in pre-eclamptic neutrophils. Analysis of NADPH oxidase activation in EBV-immortalized lymphoblasts showed an enhanced response to PMA in both the pre-eclamptic and post-partum pre-eclamptic cell lines compared to their respective controls that represented an increased sensitivity that was influenced by genetic factors.

This chapter tests the hypothesis that differences or upregulation in the signal transduction pathways at a point between agonist stimulation and the production of ROS may contribute to the increased sensitivity exhibited by pre-eclamptic EBV-immortalized lymphoblasts.

Using the present knowledge of signal transduction pathways implicated in activation of NADPH oxidase, this study aimed to elucidate differences or upregulation in any of the signalling pathways with regards to the intermediate phenotype of increased sensitivity to agonists observed in pre-eclampsia. EBV-immortalized lymphoblasts were used as models as large quantities of cells were required with which to assess the signal transduction pathways implicated. Cell lines from the post-partum groups were incubated in the presence of inhibitors of the various signalling pathways and then stimulated to produce ROS. This cohort of patients showed the greater difference in ROS production compared to their respective control (chapter 9). Inhibition of ROS production may provide an indication as to the signalling pathway responsible for the increased sensitivity exhibited by pre-eclamptic and post-partum pre-eclamptic cells.

11.2 Materials and Methods

For detailed methodology on luminol-enhanced chemiluminescent measurement of NADPH oxidase mediated ROS production, please refer to chapter two. Measurements were performed in the presence and absence of various inhibitors of the signal transduction pathways. The inhibitors incorporated are detailed in table 2.3 and dose-response curves had been performed previously to elucidate the required dose.

11.3 Patients

Six representative post-partum pre-eclamptic and six representative post-partum normotensive post-partum controls were taken from the library of continuous cell lines

derived for the purpose of this thesis and analysed accordingly. The patients were selected on the severity of the pre-eclamptic episode and matched for age with the respective control group. Table 6.1 shows the clinical characteristics of the study groups.

11.4 Results

11.4.1 Protein Kinase C

Staurosporine inhibited NADPH oxidase mediated ROS production almost completely following stimulation with PMA in both post-partum pre-eclamptic (8.26 ±1.60 versus 0.82 ±0.45 10^7 RLU.sec) and post-partum normotensive control (3.49 ±1.42 versus 0.09 ±0.04 10^7 RLU.sec) cell lines (figure 11.1). RO318220, a PKC inhibitor, also inhibited NADPH oxidase mediated ROS production completely following stimulation with PMA in both post-partum pre-eclamptic (10.03 ±1.99 versus 0.001 ±0.0007 10^7 RLU.sec) and post-partum normotensive control (3.97 ±1.56 versus 0.0007 ±0.0002 10^7 RLU.sec) cell lines (figure 11.2).

11.4.2 Protein Tyrosine Kinase

The effect of genistein on inhibition of NADPH oxidase activation with PMA is illustrated in figure 11.3. Genistein inhibited NADPH oxidase mediated ROS production in both the post-partum pre-eclamptic (10.30 ±2.82 versus 4.05 ±1.24 10^7 RLU.sec) and post-partum normotensive control group (2.77 ±1.28 versus 0.77 ±0.36 10^7 RLU.sec). The difference between mean uninhibited and inhibited ROS values was only significant in the post-partum pre-eclamptic group (P=0.020). Mean uninhibited (control) values were compared to mean inhibited values in both groups using a paired Student's t-test.

The effect of tyrphostin A_{20} on inhibition of NADPH oxidase activation with PMA is illustrated in figure 11.4. Mean uninhibited (control) values were compared to mean inhibited values in both groups using a paired Student's t-test. Tyrphostin A_{20} inhibited NADPH oxidase activation in both the post-partum pre-eclamptic (8.92 ±2.59 versus 5.875 ±2.257 10^7 RLU.sec) and post-partum normotensive (2.03 ±1.10 versus 0.604 ±0.38 10^7 RLU.sec) control groups. The difference between mean uninhibited and inhibited ROS values was only significant in the post-partum pre-eclamptic group (P=0.007).

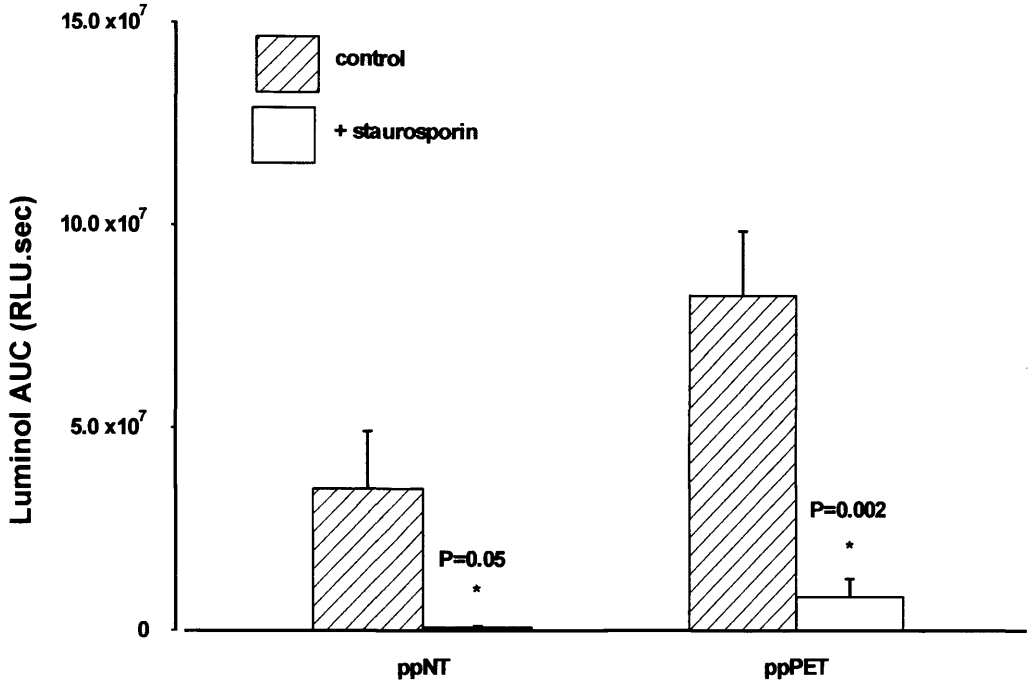


Figure 11.1 Effect of staurosporin ROS production stimulated by PMA. EBV immortalized lymphoblasts were incubated in the absence and presence of staurosporine (80nM) in 1ml of TC199 for 20 mins at 37℃. Cell suspensions were then analysed for ROS production after activation of NADPH oxidase with 1μM PMA. Staurosporin inhibited NADPH oxidase activation in both groups. Responses were abolished in each group (n=6, paired Student's t-test).

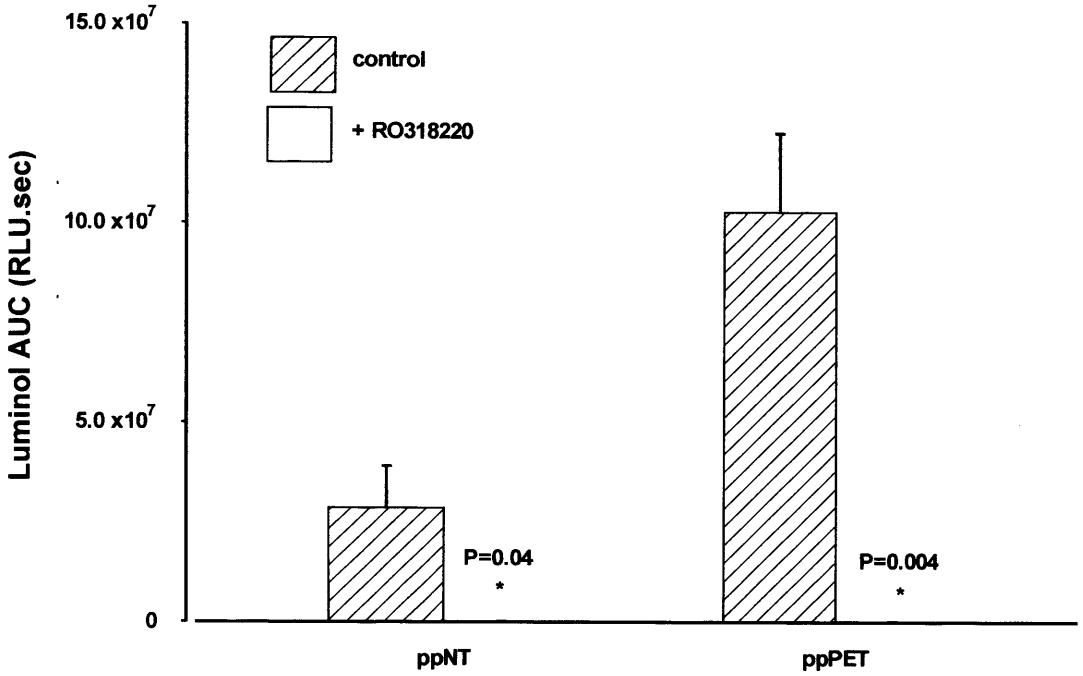


Figure 11.2 Effect of RO318220 on induction of ROS production by PMA. EBV immortalized lymphoblasts were incubated in the absence and presence of RO318220 (1μM) in 1ml of TC199 for 20 mins at 37℃. Cell suspensions were then analysed for ROS production after activation of NADPH oxidase with 1μM PMA. RO318220 inhibited NADPH oxidase activation in both groups. Responses were abolished in each group (n=6, paired, Student's t-test).

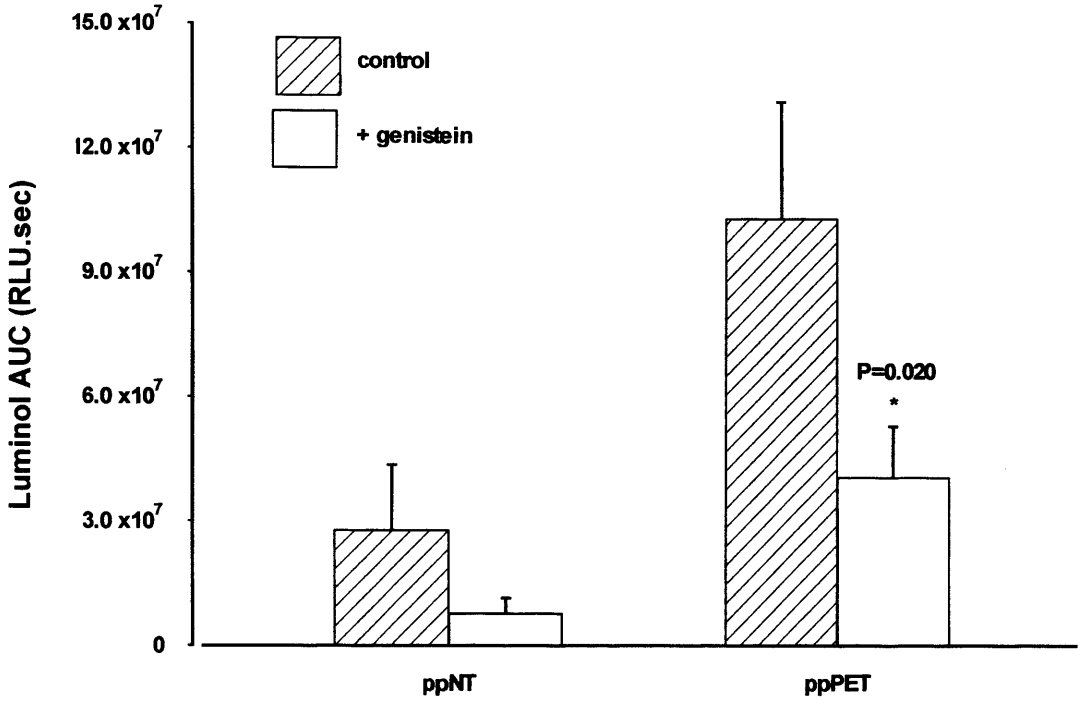


Figure 11.3 Effect of genistein on induction of ROS production by PMA. EBV immortalized lymphoblasts were incubated in the absence and presence of genistein (100μM) in 1ml of TC199 for 20 mins at 37℃. Cell suspensions were then analysed for H_2O_2 production after activation of NADPH oxidase with 1μM PMA. Genistein inhibited NADPH oxidase activation in both groups. The difference between mean un-inhibited and inhibited ROS values was only significant in the post-partum pre-eclamptic group. (n=6, paired, Student's t-test).

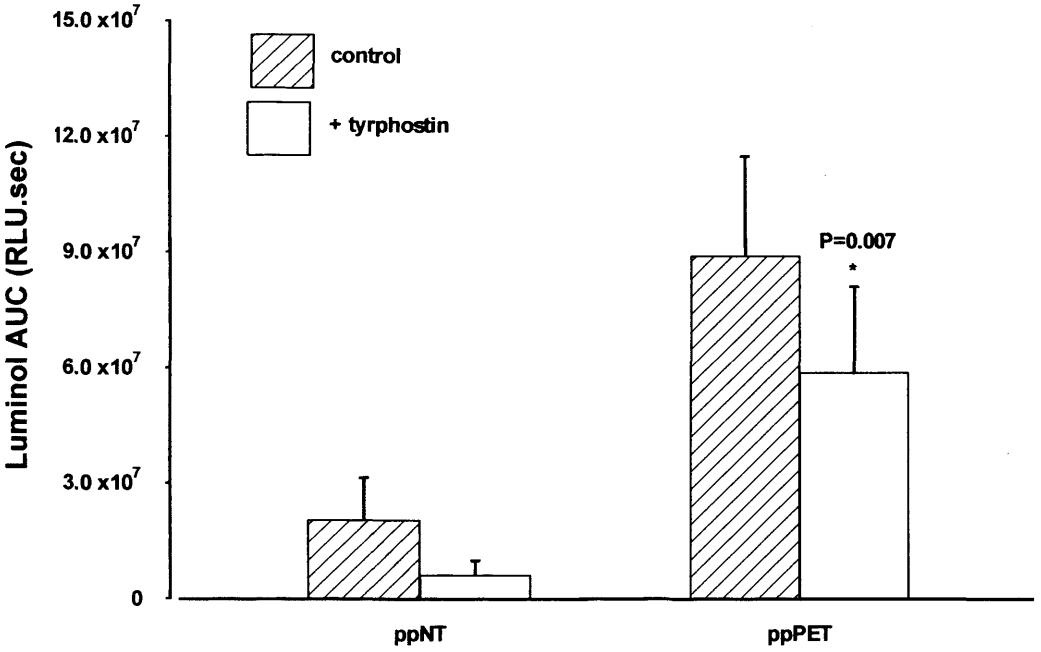


Figure 11.4. Effect of tyrphostin on induction of ROS production by PMA. EBV immortalized lymphoblasts were incubated in the absence and presence of tyrphostin (100μM) in 1ml of TC199 for 20 mins at 37℃. Cell suspensions were then analysed for H_2O_2 production after activation of NADPH oxidase with 1μM PMA. Tyrphostin inhibited NADPH oxidase activation in both groups. The difference between mean un-inhibited and inhibited ROS values was only significant in the post-partum pre-eclamptic group. (n=6, paired Student's t-test).

11.4.3 MAPK

The effect of MEK inhibitors on NADPH oxidase activation was analysed. EBV immortalized lymphoblasts were incubated for 20 minutes with PD98059 (50μM), an inhibitor of MEK or SB203580 (1μM), an inhibitor of p38MAPK, then stimulated with PMA. PD98059 had no inhibitory effect on NADPH oxidase mediated ROS production upon agonist stimulation with PMA in either post-partum pre-eclamptic (9.95 ±2.33 versus 10.60 ±2.69 10^7 RLU.sec) or normotensive (2.52 ±1.07 versus 3.24 ±1.35 10^7 RLU.sec) cell lines. SB203580 also had no inhibitory effect on post-partum pre-eclamptic (10.29 ±1.96 versus 11.80 ±1.85 10^7 RLU.sec) or normotensive (3.34 ±1.37 versus 3.54 ±1.29 10^7 RLU.sec) cell lines.

11.4.4 PI3-kinase and $cPLA_2$

The effect of PI3-kinase and $cPLA_2$ inhibitors on NADPH oxidase activation was analysed. Wortmannin, a PI3-kinase inhibitor, did not inhibit NADPH oxidase mediated ROS production to any measurable degree in either post-partum pre-eclamptic (11.00 ±2.57 versus 10.04 ±2.60 10^7 RLU.sec) or normotensive (2.70 ±1.24 versus 1.90 ±0.83 10^7 RLU.sec) control cells. MAFP, a $cPLA_2$ inhibitor, showed no inhibitory effect in either post-partum pre-eclamptic (10.39 ±2.89 versus 9.29 ±2.56 10^7 RLU.sec) or normotensive (2.49 ±1.27 versus 1.89 ±12.20 10^7 RLU.sec) control cells.

11.5 Discussion

PMA mediated stimulation of the respiratory burst was dependent on PKC activation in EBV-immortalized lymphoblasts since both staurosporine, which is a potent inhibitor of phospholipid/calcium dependent protein kinase with some selectivity for PKC and RO318220, which is another PKC specific inhibitor, inhibited the production of ROS in both post-partum pre-eclamptic and post-partum normotensive cell lines. These findings were in agreement with PMA mediated stimulation of neutrophils [Cox et al. 1985]. Neutrophils were stimulated with PMA via activation of protein kinase C, which in turn activated either a regulatory constituent or the NADPH-oxidase directly in the plasma membrane to generate an active superoxide generating system [Cox et al. 1985]. In addition, the PKC inhibitor, staurosporine, dose-dependently inhibited the production of superoxide induced by PMA [Kessels et al. 1993]. It was later demonstrated that PKC activated constituents of the NADPH oxidase since inhibition of PKC prevented

phosphorylation of several phox proteins and, hence, translocation of the cytosolic complex to the membrane preventing the respiratory burst from occurring [Benna et al. 1997 and Yamaguchi et al. 1996]. P38MAPK did not seem to be implicated in the signal transduction pathways leading to NADPH oxidase activation in EBV-immortalized lymphoblasts since the inhibitor SB203580 did not have any effect on ROS production in either post-partum pre-eclamptic or post-partum normotensive cell lines. These finding contrast with the idea that P38MAPK was found to be implicated in the activation of the NADPH oxidase by PMA in neutrophils as well as macrophages and T-cells. Lal et al [1999] found that activation of the oxidase was partially suppressed by SB203580. In contrast others have suggested that P38 MAPK may only be involved in the signal transduction pathway implicated in receptor-mediated activation of NADPH oxidase in neutrophils. [Yamamori et al. 2000 and Rane et al. 1997]. PKC has been shown to simultaneously activate ERK1/2 and contribute to activation of NADPH oxidase upon stimulation with PMA. However, the role of ERK 1/2 seems to be minor in PMA stimulated activation as the respiratory burst and subsequent production of ROS was only slightly inhibited by inhibitors of ERK1/2 [Dewas et al. 2000]. This was in keeping with results showing that mutation of P47-phox at serines within MAPK recognition sequence decreased the respiratory burst in transfected B-cell lymphoblasts stimulated with PMA [Faust et al. 1995]. The role of ERK 1/2 in PMA induced NADPH oxidase activation of EBV-immortalized lymphoblasts was assessed using the inhibitor PD98059. This is an inhibitor of MEK that was reported to catalyse the phosphorylation of ERK1/2 upon tyrosine^{204} and threonine^{202} [Daniels et al. 1999]. This inhibitor had little effect on NADPH oxidase activation measured by the production of ROS.

The ROS released in response to PMA agonist stimulation of NADPH oxidase in EBV immortalized lymphoblasts was independent of phosphatidylinositol 3-kinase (PI3-kinase) because the inhibitor wortmannin did not affect the response. Karlsson et al [2000] suggested that PMA induced a wortmannin-sensitive NADPH-oxidase activation, however, not resulting in release of superoxide but in the intracellular production of the radical. This indicated that two pools of NADPH-oxidase, one localized in the plasma membrane and the other in the granule membranes, may be separately regulated and the signal transduction pathways leading to activation of these pools differ regarding involvement of PI3-kinase. EBV-immortalized lymphoblasts

were used as models and do not possess granule membranes and therefore may explain the absence of involvement of PI 3-kinase in PMA activation and subsequent ROS release in this cell type. This does not rule out the possibility that PI-3 kinase is involved in regulation of neutrophil NADPH oxidase activation in the pre-eclamptic phenotype

Cytosolic phospholipase A2 (cPLA2) did not appear to be involved directly in the PMA mediated activation of NADPH oxidase in EBV immortalized lymphoblasts as the inhibitor MAFP had no effect on ROS production in these cells from either post-partum pre-eclamptic or normotensive cell lines. This supports the hypothesis that arachidonate production is not exclusively required for the respiratory burst in PMA stimulated cells. Both genistein and tyrphostin, inhibitors of tyrosine kinases, partially blocked the production of NADPH oxidase mediated ROS upon activation with PMA in both sets of cell lines but to a significant degree in the post-partum pre-eclamptic group. These finding suggested the importance of a tyrosine kinase regulatory pathway involved in enhanced NADPH oxidase activity associated with the pre-eclamptic phenotype as pre-eclamptic cells showed significant inhibition with respect to both genistein and tyrphostin. Similar findings have been documented in neutrophils. Utsumi et al [1992] suggested that tyrosine kinase activity was involved in the 'priming' linked generation of superoxide. However, the role of tyrosine kinase in this increased sensitivity and activation remains elusive.

The findings presented in this chapter implicate that NADPH oxidase activation may invoke a mechanism in addition to PKC that may involve an unknown protein tyrosine phosphorylation. Evidence has accumulated suggesting that tyrosine phosphorylation is an integral part of the signal transduction pathway triggered in response to stimuli. Thus tyrosine phosphorylation of several polypeptides is increased in neutrophils stimulated with fMLP and PMA [Berkow and Dodson 1990; Huang et al. 1990]. Studies have suggested that a 40-42 kD polypeptide is one of the primary targets of tyrosine phosphorylation in PMA stimulated neutrophils [Gomez-Cambronero et al. 1989].

Tyrosine kinase and increased phosphotyrosine formation has been implicated in neutrophil 'priming' by cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and TNFα.[Utsumi et al. 1992]. Due to the inhibition with genistein

and tyrphostin, a role for tyrosine kinase has been implicated the intermediate phenotype of increased NADPH oxidase activity and increased sensitivity in pre-eclampsia.

11.6 Conclusion

PKC and tyrosine kinase dependent activation of NADPH oxidase in EBV immortalized lymphoblasts was elucidated. Tyrosine kinase dependent mechanisms may be implicated in the increased sensitivity of the NADPH oxidase associated increased ROS production with pre-eclampsia. At present it is difficult to draw a clear picture of how the signal cascades were integrated. Critical areas for further investigation concern the identification of low abundance specific proteins that could be tyrosine phosphorylated and the involvement of these proteins in the signal transduction pathways in pre-eclampsia. The use of EBV-immortalized lymphoblasts as models has its drawbacks as well as advantages. PMA mediated activation of NADPH oxidase was examined and therefore alterations with regards to receptor mediated control of the intracellular signalling pathways in pre-eclampsia were not examined.

Chapter 12
General Discussion

Pre-eclampsia is a disease that may have a heterogeneous cause with manifestations that affect most of the organs of the body. However, the pathophysiological basis for the disease is not yet fully understood. Most disease states result from either genetic or environmental influences or indeed a combination of the two. By investigating two membrane functions of white blood cells it was hoped that defects may be uncovered allowing a better understanding of the basis of the disease. This thesis aimed to assess environmental and genetic influences on Na^+/H^+ exchanger activity and NADPH oxidase enzyme activation in pre-eclampsia using white blood cells and EBV-immortalized lymphoblasts as models and to determine how these intermediate phenotypes, through various mechanisms may contribute to the pathophysiology of the pre-eclampsia.

12.1 Blood Cells as Models

Nucleated blood cells serve as a convenient and accessible model to study membrane transport abnormalities. All of the studies performed in this thesis make assumptions that *in vitro* modifications reflect disturbances *in vivo* and that changes in one cell type reflect changes in other cell types or tissues. Therefore, blood cells provide an indication as to what may be occurring in other cells within the body i.e. vascular smooth muscle cells (VSMCs) and proximal convoluted tubule cells (PCTs). Further investigations in these more physiologically relevant cells would be desirable. A number of studies have been performed using EBV-immortalized cells and there are numerous advantages and disadvantages of using such a model. For example measurements may be conducted in the absence of the influence of plasma (environmental) factors *in vivo* using a model in which the phenotypic changes persist despite transformation and culture *in vitro*. Therefore genetic influences could be examined on the system being studied. B-cell lymphocytes immortalized with Epstein-Barr virus can be propagated indefinitely and provide a copious supply of cells to examine membrane abnormalities in pre-eclampsia. However, it must be remembered that EBV-immortalized cells do not contains receptors for formyl peptides and the systems studied are taken out of their natural environment [Volkman et al. 1984]. It may

be valid to extrapolate values from the cells under study to the wide syndrome of pre-eclampsia since in essential hypertension, diabetes and rat models of hypertension the increased exchanger activity persists in cultured skin fibroblasts, lymphoblasts, cultured proximal convoluted tubule cells and vascular smooth muscle cells, where cells are removed from the influences of plasma factors *in vivo* [Siczkowski et al. 1994b; Sweeney et al. 1995; Ng et al. 1995; La Pointe et al. 1997; Ng et al. 2000].

12.2 Membrane Transport Abnormalities in Pre-eclampsia

Membrane transport abnormalities associated with the transport of Na^+ have previously been implicated in the pathogenesis of pre-eclampsia [Lopatin et al. 1999]. Most work has focussed on the Na^+K^+ATPase that is a membrane transport system that maintains cellular sodium levels. Altered activity of this sodium pump has been postulated. Na^+/K^+ATPase activity was found to be decreased in erythrocytes from pre-eclamptic patients [Heilmann et al. 1993]. Decreased activity of this pump may contribute towards the high levels of intracellular sodium reported in leucocytes isolated from pre-eclamptic women [Seon and Forrester. 1990]. This decreased activity is associated with the presence of a digoxin-like factor (DLF) that may inhibit Na^+/K^+ATPase. [Graves et al. 1995; Delva et al. 1989; Gregoire et al. 1988; Lopatin et al. 1999]. The presence of a DLF was examined in chapter 9 and an increased presence of this factor was demonstrated in pre-eclamptic plasma and may have been sufficient to cause inhibition of Na^+/K^+ATPase in pre-eclampsia. This increase in the presence of a DLF was not demonstrated in the post-partum pre-eclamptic plasma samples and therefore could not account for the increased leucocyte Na^+ levels that persist for up to 38 weeks post-partum [Forrester et al 1990]. Altered activity of this pump in pre-eclampsia may therefore contribute to the increased cellular Na^+ distinctive of pre-eclampsia and subsequently be implicated in vascular hyper-responsiveness such as increased vascular tone and hence increased peripheral resistance, which characterises pre-eclamptic pregnancies [Seon and Forrester. 1989; Gregoire et al. 1988].

12.3 Na^+/H^+ Exchanger Activity as an Intermediate Phenotype

The Na^+/H^+ exchanger is another membrane transport system that involves the transport of sodium and mediates the electroneutral 1:1 exchange of Na^+ for H^+. The work presented in this thesis provides evidence of increased Na^+/H^+ exchanger activity that may contribute to the increased cellular Na^+ that is characteristic of pre-eclamptic

pregnancies [Seon and Forrester 1989]. The increased activity persists into the post-partum period and could account for the observations made by Forrester et al [1990] who demonstrated increased cellular Na^+ levels for up to 38 weeks post-partum. Increased Na^+/H^+ exchanger activity may, therefore, be considered as a useful intermediate phenotype in pre-eclampsia that appears to be genetically determined due to the persistence of the phenotype in the post-partum cell after immortalization into a continuous line. Studies in EBV-immortalised cells have helped to clarify the role of genetic influences since the cells are removed from the influences of environmental factors.

The results presented in this thesis on Na^+/H^+ exchanger activity in pre-eclampsia do not support the work of Graham et al. [1997]. This is the only other study to date that exists concerning the activity of the exchanger in pre-eclampsia and no differences were observed between the pre-eclamptic and control women [Graham et al. 1997]. Some of the reasons to account for the discrepancies documented between this study and theirs could be that nucleated cells were used to examine exchanger activity in this study rather than platelets. Pre-eclamptic women examined in this thesis were selected according to the tightly defined criteria of Davey and MacGillivray [1988] whereas the study by Graham et al. [1997] used non-proteinuric pre-eclamptic women.

The reasons for this intermediate phenotype of increased Na^+/H^+ exchanger activity were examined. It was unlikely to be due to an increased abundance of the NHE-1 protein since no differences were identified using NHE-1 specific antibody: this correlates with findings identified in essential hypertension [Ng et al. 1995]. However, the detection limits of ECL may not have been sensitive enough to detect the subtle alterations in abundance of the protein. Also, NHE-1 protein abundance was only examined in the post-partum cell extracts, no data was presented on NHE-1 protein abundance in 3rd trimester pre-eclamptic extracts.

There are several possibilities that could explain the increased activity observed. Phosphorylation is one likely mechanism and it has been demonstrated that the phosphorylation of serine residues increases parallel to exchanger activation making it a likely mechanism for increased activity [Sardet et al. 1991]. Phosphorylation of the exchanger is increased in essential hypertension; however, no differences in the

phosphorylation of Na^+/H^+ exchanger protein were identified in this study. The antibody used in these experiments may have been insufficiently sensitive in identifying very small changes in phosphorylation of serine residues.

Other mechanisms exist that may be responsible for the increased exchanger activity. A rise in intracellular calcium acting through the calcium calmodulin binding domain has been shown to stimulate Na^+/H^+ exchanger activity [Siczkowski et al. 1997]. Therefore, increased activity may be due to differences in calcium. Increased Na^+/H^+ exchanger activity may have been dependent on other accessory proteins interacting with the C-terminal domain of the exchanger, these accessory proteins were postulated by Silva et al. [1995] and Goss et al [1996]. Further investigation is, therefore, required to elucidate mechanisms of control. There is increasing evidence to suggest upregulation of signal transduction pathways leading to the phosphorylation of NHE-1 protein in essential hypertension. Upregulation of signalling pathways upstream of mitogen-activated protein kinase (MAPK) and activation of pertussis toxin (PTX)-sensitive G proteins have been implicated and may suggest areas for further study in pre-eclampsia [Ng et al. 1995; Siffert and Dusing 1996; Sweeney et al. 1997]. Although increases in Na^+/H^+ exchanger activity have been observed, the exact mechanism has still not been elucidated.

12.4 Na^+/H^+ Exchanger Activity and Pre-eclampsia

Pre-eclampsia is a heterogeneous disorder that is characterised by hypertension and abnormal sodium balance amongst other complications. Sodium is the principal cation of the extracellular space and is, thus, important in maintaining volume homeostasis. Changes in extracellular fluid volume are related primarily to sodium handling and therefore, maintain a balance between factors promoting renal retention and excretion, although this balance is poorly understood in pre-eclampsia. Following development of the disease, pre-eclamptic women behave like sodium-depleted individuals with avid sodium retention and slow excretion. There is a decrease in plasma volume and an increase in sodium retention to correct the plasma volume. Sodium handling, especially in the kidney, plays an important role in volume homeostasis and control of blood pressure, however, increases in Na^+/H^+ exchanger activity in pre-eclampsia may not lead to alterations in Na^+ reabsorption due to its basolateral location.

Inhibition of the Na^+/K^+ATPase and an increase in Na^+/H^+ exchanger activity would result in an increase in intracellular sodium. This Na^+ overload could result in increased intracellular Ca^{2+} as a consequence of reversal of Na^+/Ca^{2+} exchange. If this occurred in vascular smooth muscle cells it could result in an increase in vascular tone and peripheral resistance possibly contributing to the vasoconstriction and thus hypertension associated with pre-eclampsia.

It is important to consider the placenta with regards to pre-eclampsia. NHE-1,2 and more recently NHE-3 have been located in microvillous plasma membrane (MVM) of the placental syncytiotrophoblast and increased activity may be important with regard to the growth of the fetus [Hughes et al. 2000]. The pharmacological profiles of these exchangers indicate that the placental brush border membrane possesses NHE-1, whereas the basal membrane possesses the epithelial or apical type NHE-2 [Kulanthaivel et al. 1992].

To assess the significance of these findings Na^+/H^+ exchanger activity should be assessed in other tissues such as proximal convoluted tubules and vascular smooth muscle cells and further characterisation of Na^+/H^+ exchanger isoforms in pre-eclampsia should be sought. There may be the potential for Na^+/H^+ exchange activity to be used as a clinical marker in pre-eclampsia and due to the genetic influences on it, it may be useful in predicting women destined to develop the disorder.

12.5 Intracellular pH

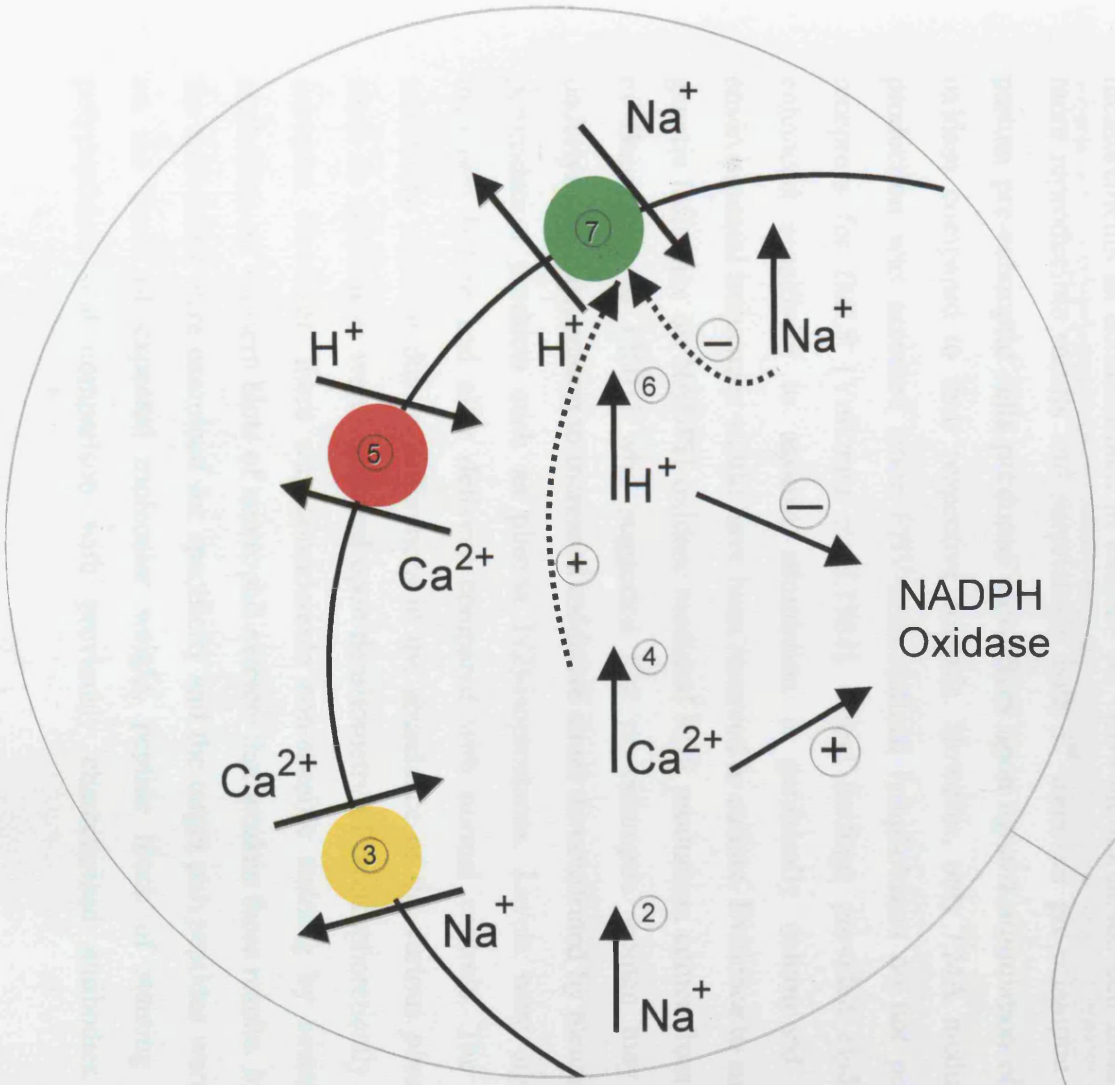
It was demonstrated in chapters 3 and 5 that pre-eclampsia and post-partum pre-eclampsia were associated with an acidotic intracellular pH in which environmental (plasma) factors were the stimulus to the acidosis. Various hypotheses have been addressed in this thesis concerning this intracellular acidosis that although not genetically determined, persisted into the post-partum period. Inhibition of the Na^+/K^+ATPase is probably the most documented membrane abnormality concerning pre-eclampsia [Forrester and Alleyne 1980; Heilmann et al. 1993; Maxwell et al. 1998]. This acidosis could have been triggered by an initial build up of sodium followed by a Ca^{2+} influx via Na^+/Ca^{2+} that may have activated Ca^{2+}/H^+ exchange as demonstrated in figure 12.1 [Souza et al. 2000]. The presence of increased levels of a DLF demonstrated

in pre-eclamptic plasma, complements the work of others. Lopatin et al [1999] suggested the presence of a digoxin-like factor (DLF) in the plasma of women with pre-eclampsia and suggested that it may have a pathogenic role in the disease through its ability to inhibit the Na^+/K^+ATPase. Gregoire et al. [1988] also demonstrated the presence of a DLF that may not only contribute towards this acidosis but may contribute towards the increased sensitivity of the vascular bed to pressor hormones and hypovolemic hypertensive state as well as causing vasoconstriction and raised blood pressure.

This factor was not unlike ouabain in that it inhibited Na^+/K^+ATPase. Therefore the increased amount of a DLF in pre-eclamptic plasma may have contributed towards the intracellular acidosis seen in pre-eclamptic cells. It was interesting that no differences in the presence of a DLF were found in plasma extracts isolated from post-partum pre-eclamptic and post-partum normotensive women, the contribution of a DLF to intracellular acidosis was therefore doubtful when considering this group of patients, suggesting the possibility of other contributory mechanisms.

One interesting possibility is the existence of hypomagnesaemia in pre-eclampsia. There is increasing evidence to suggest a reduction of magnesium (hypomagnesaemia) in the pathogenesis of pre-eclampsia [Seeling 1980]. More recently, Adam et al. [2001] showed low cellular magnesium levels in women with pre-eclampsia. This hypomagnesaemia associated with pre-eclampsia has been linked to cellular pump failure and may contribute towards the intracellular acidosis in pre-eclampsia as a deficiency of magnesium causes enzymes such as Na^+/K^+ATPase to become impaired [Newman and Amarasingham 1993]. This impairment of Na^+/K^+ATPase could consequently result in increased Ca^{2+} and intracellular acidosis as seen in cells isolated from pre-eclampsia and post-partum pre-eclamptic women.

It is important to recognise that measurements of pH_i were performed in bicarbonate free solutions and pH_i *in vivo* is likely to be different considering the participation of anion/bicarbonate exchangers in the intracellular acid-base homeostasis [Thomas 1989].



Summary of events

1. ↑ DLF & ↓ Mg^{2+} → ↓ Na^+K^+ATPase
2. → ↑ Na_i^+
3. → ↑ Ca/Na
4. → ↑ Ca_i^{2+}
5. → ↑ Ca/H
6. → ↑ H^+
7. → ↑ NHE-1

Possible sequence

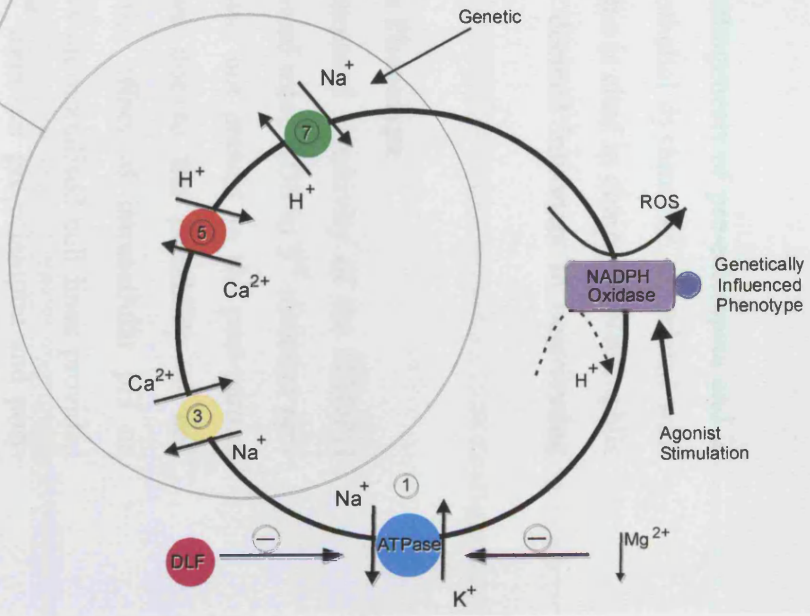


Figure 12.1
Predicted pre-eclamptic phenotype

12.6 Oxidative Stress in Pre-eclampsia

Oxidative stress has long been implicated in the pathogenesis of pre-eclampsia and is believed to contribute towards the widespread endothelial dysfunction associated with the disease [Hubel 1999]. The evidence to support this is cited in chapter 1. Neutrophils through their ability to produce ROS, have been deemed important in contributing towards this oxidative stress [Clark et al. 1998].

12.7 NADPH Oxidase Activity as an Intermediate Phenotype

The results presented in this thesis suggest an increased sensitivity of the NADPH oxidase enzyme that upon agonist stimulation produced more ROS in 3rd trimester pre-eclamptic neutrophils. This increased sensitivity was not present in the post-partum cells. Neutrophil measurements were inconclusive due to the possibility of cell stimulation during the isolation procedure and the effect of intracellular pH on measurements as demonstrated in chapter 10. EBV-immortalised cell lines provided more reproducible results and surprisingly both 3rd trimester pre-eclamptic and post-partum pre-eclamptic cells produced more ROS upon agonist stimulation of NADPH oxidase compared to their respective controls. However, only PMA mediated ROS production was assessed since EBV-immortalized lymphoblasts do not possess the receptors for fMLP [Volkman et al.1984]. These findings provided evidence that enhanced sensitivity to agonist stimulation is genetically determined since all environmental influences would have been removed in culture. Evidence to support this genetic influence on NADPH oxidase mediated ROS production comes from the work of Barden et al [2001] who suggested that pre-eclamptic women may have an underlying predisposition to increased oxidative stress demonstrated by measuring lipid peroxidation products such as plasma F(2)-isoprostanes. Levels were significantly increased before and after delivery compared with normal controls. The increased sensitivity was not due to differences in the abundance of the various *phox* proteins since no differences were identified upon densitometry of electrophoretically separated subunits. However, there was considerable non-specific staining by several of the antibodies on Western blots of neutrophil extracts that weaken these results. In defence, the antibodies were examined for specificity and the target polypeptides were selected on the basis of expected molecular weight, peptide block of staining of target polypeptides, and comparison with previously characterized antibodies. It was,

therefore, concluded that the results were valid and altered activity of NADPH oxidase mediated ROS production in pre-eclampsia was not due to variations in the abundance of the protein subcomponents of the enzyme. In summary, it is concluded that there is an increased sensitivity of the NADPH oxidase enzyme that may be considered a genetically 'programmed' intermediate phenotype in pre-eclampsia.

12.8 Environmental Influences

The inconsistency between ROS measurements in different cell types may be defended and was perhaps the results of both genetic and environmental influences on NADPH oxidase activity (figure 12.1). Significant differences between pre-eclamptic and normotensive controls were seen when NADPH oxidase was stimulated in 3rd trimester neutrophils but no differences were seen when examining post-partum neutrophils. This may have been due to the stimulation of cells during the isolation procedure and the relatively small number of women included in the study. However, there was more ROS produced in 3rd trimester neutrophils compared to those from post-partum suggesting that some other mechanism was probably responsible for the discrepancy. Once cells were immortalized with EBV, differences were seen between pre-eclamptic and normotensive controls in both 3rd trimester and post-partum cells and the values obtained were similar between groups. It was hypothesised that environmental influences may have played a part to some degree. Intracellular pH had a modulating effect with a lower pH resulting in less ROS production upon agonist stimulation. The effect of acid environment was only studied for PMA stimulation. The lower intracellular pH identified in pre-eclamptic and post-partum pre-eclamptic cells was not found to persist once cells from these women were immortalized and yet differences in NADPH oxidase mediated ROS production were more evident. This suggested that intracellular pH may have had a modulating effect in native cells that was protective. Secondly, environmental (plasma) factors are deemed important in the activation of neutrophils. These environmental (plasma) factors have been suggested since incubation studies have shown that pre-eclamptic serum enhanced superoxide generation in non-pregnant neutrophils upon agonist stimulation [Kobayashi et al. 1998]. These environmental (plasma) factors were postulated to 'prime' neutrophils for subsequent superoxide release upon agonist stimulation and, therefore, render cells more sensitive and, thus, contribute to the oxidative stress and endothelial dysfunction characteristic of pre-eclampsia [Kobayashi et al. 1998]. Various environmental (plasma) factors have

been cited in the literature and are certainly capable of priming circulating neutrophils *in vitro* and may, therefore, be important in the priming of neutrophils in pre-eclampsia [Vince et al, 1995, von Dadelszen et al. 1999; Daniels et al. 1992]. Berkow et al [1987] demonstrated that brief exposure to TNFα was able to enhance or prime the neutrophil oxidative burst in response to a second stimulus such as fMLP. Daniels et al [1992] demonstrated that recombinant human monocyte interleukin-8 (rhMIL-8) primes human neutrophil responses to fMLP. Human neutrophils were preincubated for 10 min with 10^{-8} M rhMIL-8 and then stimulated with fMLP. The neutrophils produced an enhanced release of superoxide anions. These plasma factors may have an effect on 3rd trimester cells that in addition to the genetically determined increased sensitivity counteract the modulating effect of the acidosis. The decreased pH in the post-partum pre-eclamptic cells was possibly enough to inhibit the ROS production to levels similar to that of post-partum normotensive cells. However, during the pre-eclamptic pregnancy, the contribution of environmental (plasma) factors may have counteracted the modulating effect of the acidotic intracellular pH on NADPH oxidase activity, thus allowing a difference in ROS generation to be observed between the 3rd trimester pre-eclamptic and 3rd trimester normotensive women. This suggested that perhaps both genetic and environmental influences are important in determining NADPH oxidase sensitivity to agonist stimulation. Therefore, pre-eclampsia is likely the result of both environmental and genetic influences that converge to give rise to the disease and its manifestations. Once the environmental influences are removed by immortalization of the cells, the genetic influences contributing towards increased sensitivity are still present and are sufficient to show differences in ROS production upon agonist stimulation of the oxidase in both pre-eclamptic and post-partum pre-eclamptic cells compared to their respective controls. The plasma effects should be assessed by cross-incubation studies and are likely to provide some interesting information.

12.9 Tyrosine Kinase Activity

The underlying mechanisms responsible for this increased sensitivity have begun to be investigated. The importance of tyrosine kinase regulatory pathways involved in enhanced NADPH oxidase activity associated with the pre-eclamptic phenotype is beginning to be unveiled. This mechanism exists in addition to PKC and may involve an unknown protein tyrosine phosphorylation. Tyrosine kinase and increased

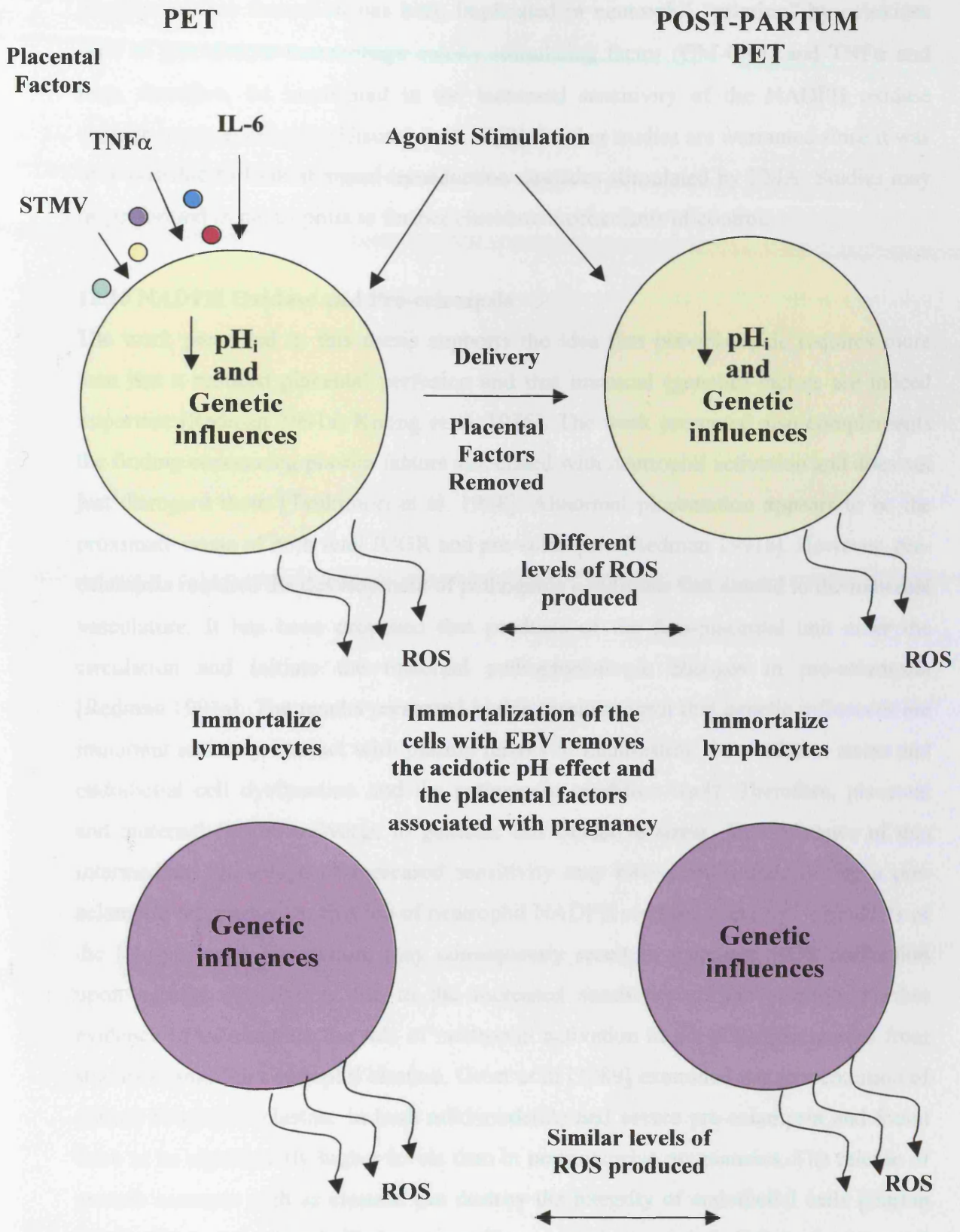


Figure 12.2 Hypothesised genetic and environmental influences on NADPH oxidase mediated ROS production in pre-eclampsia.

phosphotyrosine formation has been implicated in neutrophil "priming" by cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and TNFα and may, therefore, be implicated in the increased sensitivity of the NADPH oxidase enzyme in pre-eclampsia [Utsumi et al. 1992]. Further studies are warranted since it was only possible to look at signal transduction cascades stimulated by PMA. Studies may be performed in neutrophils to further elucidate mechanisms of control.

12.10 NADPH Oxidase and Pre-eclampsia

The work presented in this thesis supports the idea that pre-eclampsia requires more than just a reduced placental perfusion and that maternal (genetic) factors are indeed important [Redman 1991a; Knong et al. 1986]. The work presented also complements the finding concerning plasma factors associated with neutrophil activation and does not just disregard them [Tsukimori et al. 1988]. Abnormal placentation appears to be the proximate cause of both fetal IUGR and pre-eclampsia [Redman 1991a]. However, pre-eclampsia requires the development of pathogenic conditions that extend to the maternal vasculature. It has been proposed that products of the feto-placental unit enter the circulation and initiate the maternal pathophysiologic changes in pre-eclampsia [Redman 1991a]. The results presented in this thesis suggest that genetic influences are important and may interact with plasma factors in manifesting the oxidative stress and endothelial cell dysfunction and the pathogenic condition itself. Therefore, placental and maternal factors converge to generate this oxidative stress. The existence of this intermediate phenotype of increased sensitivity may have implications during a pre-eclamptic pregnancy. Activation of neutrophil NADPH oxidase, possibly by products of the feto-placental circulation, may consequently result in increased ROS production upon agonist stimulation due to the increased sensitivity of the enzyme. Further evidence to substantiate the role of neutrophil activation in pre-eclampsia comes from studies examining neutrophil elastase. Greer et al [1989] examined the concentration of plasma neutrophil elastase in both mild/moderate and severe pre-eclampsia and found there to be significantly higher levels than in normotensive pregnancies. The release of granule contents such as elastase can destroy the integrity of endothelial cells [Harlan 1987]. This mechanism is likely to contribute towards the endothelial dysfunction and damage consistently seen in pre-eclampsia. In addition to elastase, neutrophil activation may be shown by neutrophil adhesion molecule expression. Increased serum soluble L -

selectin levels are consistent with activation of peripheral blood neutrophils and document neutrophil involvement in pre-eclampsia [Sabatier et al. 2000].

The vascular endothelium is prone to damage by ROS and granule contents that are released from activated neutrophils since the vascular endothelium interfaces with blood. Overproduction of ROS in pre-eclampsia not only damages endothelium directly but may result in uncontrolled lipid peroxidation that is damaging through oxidation of lipid membranes [Hubel et al. 1998]. Indirect support exists for the role of oxidative stress in pre-eclampsia in that many of the endothelial abnormalities described can be reproduced by lipid peroxidation in experimental systems [see table 12.1]. These findings suggest that oxidative stress is a central feature of pre-eclampsia rather than just an epiphenomenon. The fact that increased sensitivity of NADPH oxidase is genetically influenced in pre-eclampsia supports this statement.

In addition to bringing about endothelial damage directly through oxidation of lipid membranes and lipid peroxidation, ROS inhibit vascular relaxation *in vivo* and *in vitro* by inactivating endothelium-derived relaxing factor (EDRF) [Hubel et al. 1998; Gryglewski et al. 1986]. It is possible that neutrophil ROS contributes to the prostaglandin imbalance characteristic of pre-eclampsia. Prostacyclin may be reduced because of extensive lipid peroxidation that preferentially inhibits PGI_2 synthase, resulting in reduced vasorelaxation. Landino et al [1996] have shown that peroxynitrite can activate PGH synthase and may represent a pathway for reactivation of PGI_2 synthesis in women with pre-eclampsia.

It must be remembered that there are multiple possible sources of oxidative stress in pre-eclampsia. At one extreme, generation of ROS could be solely due to the increased sensitivity of NADPH oxidase in various cells including neutrophils and placental syncytiotrophoblasts. However, a marked deficiency in antioxidant protection alone could precipitate oxidative stress. The results presented in this thesis suggested that it is more likely a combination of these variables that summate to generate the oxidative injury. Such interactions could contribute to the observed heterogeneity of pre-eclampsia.

Dysfunction in pre-eclampsia	**Peroxidation in experimental model**
1. Evidence of endothelial injury	Exposure to lipid peroxides damages endothelial cells
2. Proteinuria	Intrarenal infusion of hydrogen peroxide induces proteinuria in rats
3. Convulsions	Convulsions in rats fed lipid peroxides day 13 gestation
4. Vasoconstriction	Lipid peroxides and OxLDL increase artery sensitivity to agonists
5. Reduced prostacyclin production by vessels	Increased lipid peroxidation decreases prostaglandin production
6. Haemolysis	Lipid peroxidation promotes osmofragility and haemolysis
7. Pre-eclampsia plasma increases endothelial production of nitric oxide	OxLDL increases nitric oxide production from endothelial cells in culture

Table 12.1 Endothelial dysfunction and damage in pre-eclampsia mimicked by experimental lipid peroxidation. Adapted from Hubel, 1999.

12.11 Intermediate Phenotypes of Increased NADPH Oxidase Activity and Na^+/H^+ Exchanger Activity in Later Life

The implications of these genetically influenced intermediate phenotypes may also have an impact in later life. There is evidence of increased risk of future cardiovascular complications in women who suffer a pre-eclamptic pregnancy [Fisher et al. 1981]. This may be due to the fact that pre-eclamptic neutrophils are more sensitive to agonist stimulation. Neutrophil activation is emerging as an important factor in hypertension, cardiovascular disease and diabetes amongst other diseases where increased sensitivity may be important [Griendling et al. 2000; Kristal et al. 1998]. In addition, Henrik et al [2001] examined the long-term mortality of mothers after pre-eclampsia and suggested an increased risk of cardiovascular disease in later life if a woman had suffered a pre-eclamptic pregnancy. They suggested that genetic factors that increase the risk of cardiovascular disease (perhaps related to the NHE-1 and NADPH oxidase phenotypes defined in this thesis) could also be linked to pre-eclampsia. Therefore, increased NADPH oxidase and NHE-1 not only have implications with regard to the pre-eclamptic pregnancy but may also be a marker of predisposition to early cardiovascular disease in these women.

12.12 Conclusion

Throughout this thesis two membrane functions of white blood cells have been examined with regard to pre-eclampsia. It has been determined that these may represent intermediate phenotypes in pre-eclampsia and may contribute to the manifestations of the disease through both oxidative stress and endothelial dysfunction as well as sodium handling.

Intracellular acidosis may have as yet undefined implications in the pathogenesis of the disease but may also have a modulating effect on ROS production. In conclusion, the possibility of the Na^+/H^+ exchanger and the NADPH oxidase enzyme as phenotypic markers in pre-eclampsia has been established with possible implications in later life. Further work is needed to elucidate the tyrosine kinase pathways that lead to this increased sensitivity of the NADPH oxidase phenotype, and the basis for the increased Na^+/H^+ exchanger isoform 1 activation needs to be unravelled.

Further Work

The data presented in this thesis has made a significant contribution to the understanding of membrane transport abnormalities and NADPH oxidase activity in pre-eclampsia. However, there are several areas that arise in which further work would enhance our understanding of the topics addressed including:

1. It would be interesting to examine of the contribution of hypomagnesaemia to the intracellular acidosis that has been documented in the pre-eclamptic and post-partum pre-eclamptic cells. This may be performed by initially measuring the magnesium levels in both the blood and white blood cells from suitable subjects.

2. It may be beneficial to examine the relevance of Na^+/K^+ATPase activity in pre-eclampsia by measuring its activity in cells from pre-eclamptic subjects.

3. The role of plasma factors needs further evaluation and it would be important to examine the role of these factors by performing add-back experiments to elucidate effects on sensitivity of NADPH oxidase mediated ROS production and intracellular pH in pre-eclampsia.

4. Further analysis of relevant signal transduction pathways using pharmacological dissection with activators and inhibitors to define the signalling pathways leading to increased NADPH oxidase activity would enhance our understanding of this area.

5. Examination of NHE-1 and NHE-3 in other cell types (e.g. proximal convoluted tubule cells) may help to assess the contribution to Na^+ handling in the kidney with regards to pre-eclampsia.

6. Examination of candidate genes implicated in the increased sensitivity of the NADPH oxidase enzyme associated with pre-eclampsia using microarray techniques would be a novel way to progress. RNA from lymphoblasts from both pre-eclamptic and normotensive patients may be pooled and subjected to

microarray analysis. This would allow identification of any up or down regulated genes that may be associated with oxidative stress and pre-eclampsia.

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Publications arising from this thesis

Papers

Lee VM, Halligan AWF, Ng LL. *Leucocyte intracellular pH and Na^+/H^+ exchanger isoform-1 activity in post-partum women with pre-eclampsia.* Br J Obstet Gynaecol, 2001. **108**, 615-622

Lee VM, Halligan AWF, Ng LL. *Neutrophil Intracellular pH and Na^+/H^+ Exchanger Activity in Pre-eclampsi*a. Submitted to Br J Obstet Gynaecol, Sept 2001.

Lee VM, Quinn PA, Jennings SC, Halligan AWF, Ng LL. *Altered Na^+/H^+ exchanger isoform 1 activity in immortalized lymphoblasts from women with pre-eclampsia. Evidence for an intermediate phenotype.* Submitted to Clin Sci, Jan 2002.

Lee VM, Quinn PA, Jennings SJ, Ng LL. *Neutrophil activation and reactive oxygen species production in pre-eclampsia.* Submitted to Br J Obstet Gynaecol Feb 2002.

Abstracts

Lee VM, Halligan AWF, Ng LL. *Leucocyte Na^+/H^+ exchanger isoform 1 (NHE-1) activity and phosphorylation in post-partum pre-eclamptic women.* Abstract accepted for Blair Bell Society Meeting 1999, published in Br J of Obstet Gynaecol.

Lee VM, Halligan AWF, Pettit AI, Ng LL. *Enhanced reactive oxygen species production in Epstein-Barr virus immortalized lymphocytes from women who have had pre-eclamptic pregnancies. The role of priming?"* Abstract accepted for Blair Bell Research Society Meeting 2000, published in Br J Obstet Gynaecol.