# IMMUNOLOGICAL MARKERS, ANTIOXIDANTS AND PROSTAGLANDINS IN PREGNANCY-INDUCED HYPERTENSION

by

GONG CHEN

M.B., Master of Medicine

University Department of Medicine,

Royal Infirmary,

Glasgow

A thesis submitted for the degree of Doctor of Philosophy

to the University of Glasgow

August 1993



ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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**Acknowledgements**

I should like to thank the following people and organizations without whose contributions this work would have been impossible:

Professor James H. McKillop and Dr. Rhoda Wilson, University Dept. of Medicine, Glasgow Royal Infirmary, for their guidance, advice, support and encouragement throughout.

Drs. J.J. Walker and G. Cumming, University Dept. of Obstetrics and Gynaecology, Glasgow Royal Infirmary, for their organisation and supply of samples, and also for Dr. Walker's advice throughout.

Professor W.E. Smith, Dept. of Pure and Applied Chemistry, University of Strathclyde, for his advice on the antioxidant work.

Professor R.H. Burdon and Dr. P. Boyd, Dept. of Bioscience & Biotechnology, University of Strathclyde, for access to molecular biology facilities and their advice on the molecular work.

Dept. of Statistics, University of Glasgow, for the advice on the statistical methods used.

Staff at University Dept. of Medicine, Glasgow Royal Infirmary, and Staff at Molecular Laboratory, Dept. of Bioscience & Biotechnology, University of Strathclyde, for their valuable help.

University of Glasgow and Overseas Research Students Awards (London) for their financial support.

Finally, I would like to thank my parents and my wife without whose help and understanding this thesis could never have been written.

## Declaration

The work described in this thesis was performed in the University Department of Medicine, Glasgow Royal Infirmary (Chapter 2,3,4,5,7) and Department of Bioscience and Biotechnology, University of Strathclyde (Chapter 6), between January 1991 and August 1993. The detailed planning of the work and its execution were my individual responsibility, and except where indicated, the work was entirely personally performed.

Most of the work presented herein has already been published, presented or been submitted for publication.

1. Chen G, Wilson R, Cumming G, Walker JJ and McKillop JH. Prostacyclin, thromboxane and antioxidant levels in pregnancy-induced hypertension. Eur J Obstet Gynecol Reprod Biol (Accepted).

2. Chen G, Wilson R, Cumming G, Smith WE, Walker JJ and McKillop JH. Intracellular and extracellular antioxidant buffering levels in erythrocytes from pregnancy-induced hypertension (PIH). J Human Hypertension (Accepted).

3. Chen G, Wilson R, Cumming G, Walker JJ and McKillop JH. Production of prostacyclin and thromboxan A2 in mononuclear cells from preeclamptic women. Am J Obstet Gynecol (Accepted).

4. Chen G, Wilson R, Cumming G, Smith WE, Walker JJ and McKillop JH. (1992). Effects of atenolol, labetalol and methyldopa on antioxidant agents *in vitro*. Scottish Med. J., 37:156 (abstract).

5. Chen G, Wilson R, Cumming G, Walker JJ and McKillop JH. Immunological changes in pregnancy-induced hypertension. (Submitted).

6. Chen G, Wilson R, McKillop JH and Walker JJ. The role of cytokines in production of prostacyclin and thromboxane A2 in human mononuclear cells. (Presented at 3rd European Congress Prostaglandins in Reproduction, Edinburgh, 1993).

7. Chen G, Wilson R, Cumming G, Walker JJ and McKillop JH. Calcium channel blockers and antioxidant activity. (Submitted).

8. Chen G, Wilson R, Cumming G, Smith WE, Walker JJ and McKillop JH. Antioxidants and immunological markers in essential hypertension and pregnancy-induced hypertension. (Submitted).

9. Chen G, Wilson R, Boyd P, McKillop JH, Walker JJ and Burdon RH. Normal superoxide dismutase (SOD) gene in patients with preeclampsia: is the decreased SOD activity a secondary phenomenon? (Submitted).

10. Chen G, Wilson R, Cumming G, Walker JJ and McKillop JH. Interleukin-2 and Pregnancy-induced hypertension. (Submitted).

## Abbreviations

| | |
|---|---|
| $^3H$ | Tritium |
| $^{\bullet}OH$ | Hydroxyl radical |
| ANOVA | Analysis of variance |
| bp | Base pair |
| BSA | Bovine serum albumin |
| cAMP | 3'5'-cyclic adrenosine monophosphate |
| CAT | Catalase |
| cDNA | Complementary or copy DNA |
| Ci | Curie |
| ConA | Concanavalin A |
| cpm | Counts per minute |
| dATP | Deoxyadenosine triphosphate |
| dCTP | Deoxycytidine triphosphate |
| $ddH_2O$ | Distilled/deionised water |
| dGTP | Deoxyguanosine triphosphate |
| DNA | Deoxyribonucleic acid |
| DTNB | 5,5'-dithio-bis-(2-nitrobenzoic acid) |
| dTTP | Deoxythymidine triphosphate |
| EDRF | Endothelium-derived relaxing factor |
| EDTA | Ethylenediaminetetra-acetate |
| EIA | Enzymeimmunoassay |
| ELISA | Enzyme-linked immunosorbant assay |
| ESSE | 5,5'-dithio-bis-(2-nitrobenzoic acid) |
| FCS | Foetal calf serum |
| GFR | Glomercular filtration rate |
| GM-CSFC | Granulocyte-macrophage colony stimulating factor |
| GSH-ed | Glutathione reductase |
| GSH-px | Glutathione peroxidase |
| GSHl | Lysate glutathione |
| GSHp | Plasma glutathione |
| GSSG | Oxidized glutathione |
| $H_2O_2$ | Hydrogen peroxide |
| HCl | Hydrochloric acid |
| HEPES | N-[2-Hydroxyethyl] piperazine-N-[2-ethanesulphonic] acid |
| HETEs | Hydroxy acids |
| HLA | Human leucocyte antigen |
| HPETEs | Hydroperoxy eicosatetraenoic acids |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IL-2R | Interleukin-2 receptor |
| INF | Interferon |
| Kb | Kilobases |

| | |
|---|---|
| LSH | Lysate thiol |
| MCH | Mean corpuscular haemoglobin |
| MCHC | Mean corpuscular haemoglobin concentration |
| MCV | Mean corpuscular volume |
| MHC | Major histocompatibility complex |
| mRNA | Messenger ribonucleic acid |
| MSH | Membrane thiol |
| NADP | Nicotinamid adenine dinucleotide phosphate |
| NADPH | Nicotinamid adenine dinucleotide phosphate (reduced form) |
| NK cell | Natural killer cell |
| $O_2^{-\bullet}$ | Superoxide anion |
| $O_2^{2-}$ | Peroxide ion |
| OD | Optical density |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffer saline |
| PCV | Haematocrit |
| PGE | Prostaglandin E |
| PGF | Prostaglandin F |
| PGI2 | Prostacyclin |
| PHA | Phytohaemagglutinin |
| PHS | Pooled human serum |
| PIH | Pregnancy-induced hypertension |
| PKC | Protein kinase C |
| PNP | P-Nitrophenyl phosphate,phosphatase substrate |
| PPi | Sodium pyrophosphate |
| PPO | 2,5 Diphenyloxazole |
| PSH | Plasma thiol |
| PVP | Polyvinylpyrrolidone |
| PWM | Pokeweed mitogen |
| RBC | Red blood cells |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| ROS | Reactive oxygen species |
| SDS | Sodium dodecyl sulphate |
| SOD | Superoxide dismutase |
| SSA | Sulphosalicylic acid |
| TGFβ | Transforming growth factor β |
| TMP | Tetramethylbenzidine |
| TNB | 5-thio-2-nitrobenzoic acid |
| TNF | Tumour necrosis factor |
| TXA2 | Thromboxane A2 |
| TXB2 | Thromboxane B2 |
| V | Volt |

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## Summary

Pregnancy-induced hypertension (PIH) is still a major cause of obstetrical and perinatal morbidity and mortality, and no breakthrough has yet been achieved in the understanding of its pathogenesis. The present *in vitro* work studied the pathogenesis of PIH in three aspects: immunology, antioxidants and prostaglandins using various immunological, biochemical and molecular genetic methods.

An increased cellular immunological activity was detected in patients with PIH, especially in those with proteinuria, as the proliferation of peripheral blood mononuclear cells (PBMC) with PHA stimulation and without mitogens was increased and the IL-2 activity was elevated compared to normotensive pregnant women. The hypersecretion of IgG from B lymphocytes was also found in patients with PIH. The increased immunological activity is in accordance with some important changes seen in PIH, such as an increased intracellular calcium, the presence of a blood-borne mitogenic factor and a decreased prostaglandin E series. These findings support the hypothesis that PIH may result from the imbalance between fetal antigenic load and maternal production of immunological blockage.

Intracellular (lysate thiol, lysate glutathione and lysate superoxide dismutase (SOD) in red blood cells) and extracellular (plasma thiol, plasma glutathione and red blood cell membrane thiol) antioxidant buffering levels were investigated in patients with PIH and both of them were decreased in the patients, suggesting an occurrence of increased reactive oxygen species (ROS) activity in this disorder. It was also found that there were significant positive correlations between the levels of prostaglandins and antioxidant activity. It was possible that the changes of prostaglandin production resulted from the imbalance of decreased antioxidant buffering levels and increased ROS formation in PIH. The cause of the decreased antioxidant levels in PIH is not clear. However the reduced SOD (CuZn) activity seems to be an acquired phenomenon since its gene structure and expression in PIH did not differ from that in normal subjects.

Production of prostacyclin (PGI2) and thromboxane A2 (TXA2) in mononuclear cells was determined in patients with PIH and the effects of PIH serum on this production were investigated. The results showed that the imbalance of decreased PGI2 and increased TXA2 production occurred in mononuclear cells from PIH patients and thus the ratio of TXA2 to PGI2 was increased. Serum from PIH patients with proteinuria slightly reduced PGI2 synthesis in normal pregnant women but

markedly increased TXA2 production, producing a ratio of TXA2 to PGI2 similar to that found in PIH. This result suggested that there was a factor(s) in serum from PIH patients with proteinuria to contribute, at least in part, to the imbalance between PGI2 and TXA2 seen in PIH.

PIH and essential hypertension in pregnancy have an important clinical feature in common, high blood pressure, however, the cause of this remains unclear. The immunological markers and the antioxidant agents were evaluated in both diseases. The results showed that immunological changes found in PIH did not occur in essential hypertension in pregnancy. Antioxidant agents were decreased in both diseases, however the types of antioxidants involved were different. There results suggested that different pathogenetic mechanisms may associated with these two disorders.

The effects of antihypertensive drugs (atenolol, labetalol, methyldopa, nifedipine and nimodipine) on antioxidants were also studied and the results indicated that atenolol and labetalol *in vitro* possessed some antioxidant activity as they can significantly raise the levels of plasma thiol and membrane thiol. The other three drugs were not found to have any antioxidant property.

Taken together, the work in this thesis suggests that the cause of PIH is complicated and multifactorial. The disorder occurred in the immune system might be an important factor in triggering the development of PIH. The immunological maladaptation could lead to the imbalance between antioxidant levels and ROS activity. The balance of PGI2 and TXA2 was shifted to the TXA2 dominance and this change could contribute to many if not all of the pathophysiological features found in PIH. The decreased antioxidants and increased ROS activity may damage enthothelial cells and cause an imbalance of PGI2 and TXA2 production, and thus it forms the link between the immune system and endothelial dysfunction.

# CHAPTER ONE GENERAL INTRODUCTION

## 1.1 PIH———A Review

### 1.1.1 The Early References

It is difficult to say who first discovered pregnancy-induced hypertension (PIH). The signs and symptoms of this fascinating disease were mentioned in the ancient Egyptian, Chinese, Indian and Greek medical literatures and but were not differentiated from epilepsy (Chesley 1974). However, Chesley (1974) believes that it was the ancient Greeks (before the time of Hippocrates) who provided the first bona fide documentation of recognizing PIH as a separate disease. There are two relevant aphorisms in the Cona Prognosis: "In pregnancy drowsiness with headache accompanied by heaviness and convulsions is generally bad."; "In pregnancy the onset of drowsy headaches and heaviness is bad; such cases are perhaps liable to some sort of fits at the same time".

In 1694, Mauriceau (1694) attributed gestational convulsions to irritation of the uterine cervix and in cases of fetal death, to noxious vapours arising in the uterus. He did not make a clear distinction between epilepsy and PIH, but his specification of causes suggests that he had recognized a difference. He also set forth several quotations dealing with convulsion in pregnancy. Among them were: No. 229, "Primigravidas are at far greater risk of convulsions than are multiparas."; No. 230, "convulsions during pregnancy are more dangerous than those beginning after delivery.". Mauriceau (1694) observed that the convulsions often cease with delivery and he recommended prompt termination of pregnancy as the best treatment.

It was not until 1739 that De Sauvages differentiated epilepsy from convulsions of acute cause, which he called "eclampsia" (Chesley 1978). However, it was soon followed by the confusion of PIH with nephritis, which lasted for more than a century. Lever (1843) discovered the proteinuria of PIH when he looked for it because the clinical picture of PIH resembled that of glomerulonephritis. He also noted that eclamptic proteinuria abated quickly after delivery and concluded that PIH was a different disease, but others were not so astute. To them, PIH still meant renal disease which was confused with essential hypertension at that time. Corwin & Herrick (1927) made a major contribution in recognizing essential hypertension as a frequent component of the hypertensive disorders in pregnancy and as the usual form

of hypertension found at follow-up. Herrick and Tillman (1936) wrote: "When these are fully delineated it is our opinion that we shall find nephritis concerned in but a small fraction of the toxemias; that the larger number, including the eclampsia, the preeclampsia, and the variously designated milder types of late toxemia will be found to have unit characteristics based upon cardiovascular disease with hypertension.". Since then, obstetricians gradually realized that PIH is often mistaken for essential hypertension and renal disease.

### 1.1.2 Incidence and Epidemiology

The incidence of PIH has been variously given as 2.6% to 15% by different centres (Roberts 1984; Saftlas *et al.* 1990; DHSS 1991). However, it is believed that in the Western world, PIH affects 5% to 10% of all pregnancies.

PIH is predominantly a disease of primigravid patients (about 75% of all cases). When PIH affects multiparous patients it is usually superimposed upon essential hypertension, or less often is associated with one of the features described below.

1. Twin pregnancies: Twin pregnancies are especially prone to be complicated by PIH, the incidence being increased by two or three times, or even more (Scholtes 1975).
2. Diabetes: Diabetic women are at increased risk for developing PIH during pregnancy. A study by Garner et al. (1990) concluded that PIH is twice as common in diabetic pregnancies compared with normal controls. The incidence of PIH in diabetic patients is even higher if the glycemic levels are not well controlled (Siddiqi *et al.* 1991).
3. Hydatidiform mole: The greatest increase in incidence occurs in cases of hydatidiform mole, in which up to 50% of pregnancies may show evidence of PIH (Studd 1977).

Race seems to be a significant factor associated with PIH. Black women have been shown to be at greater risk for developing PIH (Chesley *et al.* 1984). They have a higher incidence of essential hypertension than white women and the increased risk of PIH in the black may be due to mistaking essential hypertension for PIH and thus including those with essential hypertension into the PIH group (Chesly *et al.* 1984). Recently, a case-control study, however, excluded that the increased susceptibility for PIH in black women can be explained by the inclusion of patients with essential hypertension (Eskenazi *et al.* 1991).

Although the incidence of PIH is not related to socioeconomic status (Eskenazi *et al.* 1991), women working during pregnancy have a significantly higher rate of PIH than those not working (Marcoux *et al.* 1989; Klebanoff *et al.* 1990). It is postulated that pregnant women who work may be more stressed and have different levels of physical activities than those who do not work.

Young pregnant women are more susceptible to PIH (Cunningham *et al.* 1989). Saftlas *et al.* (1990) found that teenagers were at significantly higher risk than women 25 to 34 years of age. However, a recent study (Eskenazi *et al.* 1991) cast some doubt on this as it was observed that the increased incidence of PIH in teenagers disappeared when parity was controlled for.

Cigarette-smoking is hazardous to the fetus, however, it is paradoxically associated with a reduced incidence of PIH, as is alcohol consumption (Eskenazi *et al.* 1991). Adelstein and Fedrick (1980) found that smokers had a lower average pre-pregnant weight than the non-smokers. This difference in weight might account for the different observed incidences of PIH in different smoking status because heavy-set women tend to develop PIH (Chesley 1984). Another explanation for this is that smoking and drinking women appear to have lower blood pressure than abstainers (Friedman *et al.* 1982). Although tobaco and alcohol consumption may tend to protect against PIH, the well-known risk of both of these agents to the fetus outweighs the slight benefit.

### 1.1.3 Classification, Definition and Diagnosis

In reviewing the literature on hypertension in pregnancy, one often faces two problems. One is the inconsistency in the classification used to categorize it. Another is that there are different criteria used to define PIH or the degree of it.

1. Classification

There are a multitude of classifications used to categorize high blood pressure during gestation. It is reported that there are more than 60 names in English and 40 in German to describe PIH (Davey & MacGillivray 1986). For example, gestational hypertension, gestational proteinuric hypertension, gestosis, pregnancy-associated hypertension, pregnancy-induced hypertension and toxemia are among the terms used to describe PIH. The National High Blood Pressure Working Group (1990) recommended the approach proposed in 1972 by the American College of

Obstetricians and Gynecologists (Hughes 1972), which is still the most concise and practical of all current classifications. This schema classifies the following four categories of hypertension associated with pregnancy.

I. Pregnancy-induced hypertension
    1. Preeclampsia
        a. mild
        b. severe
    2. Eclampsia
II. Chronic hypertension (of whatever cause)
III. Chronic hypertension with superimposed PIH
    1. Superimposed preeclampsia
    2. Superimposed eclampsia
IV. Late, or transient hypertension

2. Definition

In the above classification, PIH falls into two categories: preeclampsia and eclampsia. Preeclampsia is defined as hypertension induced by pregnancy after 20 weeks' gestation, concurrent with proteinuria or edema or both. It defines hypertension as a diastolic pressure of at least 90 mmHg, a systolic pressure of at least 140 mmHg, or a rise from baseline of 15 mmHg diastolic pressure or 30 mmHg systolic pressure. The elevated blood pressure reading must occur on at least two occasions at least 6 hours apart. Eclampsia, a severe complication of PIH, is defined as the occurrence of convulsions unrelated to coincidental neurologic disease in a woman with PIH.

3. Diagnosis

The diagnostic criteria used by Klonoff-Cohen *et al.* (1989), which is based on the American College of Obstetricians and Gynecologists (1986) (Table 1.1) is more pratical and better one.

### 1.1.4 Pathophysiology

1. Hypertension

Blood pressure readings in women with PIH are characteristically labile and are often associated with a reversal of the circadian blood pressure rhythms that normally occur in pregnant and non-pregnant populations, *i.e.* morning peaks and nocturnal nadirs. In PIH, the rhythm may be abolished or even reversed, with the highest levels occuring at night (Murnaghan *et al.* 1980; Cunningham & Lindheimer

**Table 1.1 Diagnostic criteria for PIH***

**Mild Preeclampsia**

All of the following signs after 20 weeks of gestation:

1. An increase in systolic pressure to 140 mm Hg or an increase in systolic pressure of >30 mm Hg above usual, taken twice 6 hours apart at bed rest.
2. An increase in diastolic pressure to 90 mm Hg or an increase in diastolic pressure of 15 mm Hg more than usual, taken 2 times 6 hours apart at bed rest.
3. Presence of ≥300 mg of protein in clean-catch urine in at least 2 random urine specimens collected 6 hours apert.
4. Edema of the face or hands of >1+ or a gain of ≥5 lb in 1 week.

**Severe Preeclampsia**

One or more of the following signs:

1. Systolic pressure of 160 mm Hg or diastolic pressure of 110 mm Hg recorded 2 times 6 hours apart with patients at bed rest.
2. Proteinuria, 5 g in 24 hours, or a 3 to 4+ protein on dipstick.
3. Oliguria, urinary output of <400 ml in 24 hours.
4. Cerebral or visual disturbances, including eye changes.
5. Pulmonary edema or cyanosis.
6. Epigastric pain.
7. Evidence of hemolysis, abnormal result from liver function test, and falling platelet count (HELLP syndrome).

**Eclampsia**

Generalized convulsions and/or coma.

* From Klonoff-Cohen *et al.* **(1989).**

1992). This labile blood pressure reflects the intense sensitivity of the vasculature to endogenous pressor hormones and autacoids and represents a reversal of the marked refractory state to the pressor effects of angiotensin II characteristic of normal pregnancy (Aalkjaer *et al.* 1985). The increased vascular sensitivity is all the more remarkable because it occurs while circulating catecholamines remain unaltered and angiotensin II levels may even decrease (Aalkjaer *et al.* 1985; Davision & Lindheimer 1988).

The cause of the increased pressor responsiveness in PIH is obscure, but research in two areas show promise. One popular hypothesis is the imbalance between vasoconstrictor prostaglandins (mainly TXA2) and vasodilating prostaglandins (mainly PGI2). A relative or absolute decrease in prostagladin I or E series results in the development of hypertension in pregnancy (Walsh 1985; Friedman 1988). Another hypothesis is vascular endothelial cell dysfunction (Roberts *et al.* 1991). Endothelial cell injury can cause alterations of several circulting substances including mitogenic factors, endothelin and serotonin, which lead to vasoconstriction and hypertension (Musci *et al.* 1988; Weiner 1990; Schiff *et al.* 1992).

2. Renal Dysfunction

Glomercular filtration rate (GFR) decreases in women with PIH, but because GFR is normally higher in pregnancy, values may still be at or above non-pregnant levels. Decrease in GFR is partly due to a characteristic renal lesion that involves swelling of the intracapillary glomerular cell termed glomerular endotheliosis (Fisher *et al.* 1981; Gaber *et al.* 1987). The increased protein excretion almost always accompanies glomerular endotheliosis. Urate clearance also decreases, and often to a greater degree than does GFR; thus, hyperuricemia can be an early indication of PIH. The ability to excrete sodium is also decreased, but the degree to which this occurs varies and severe disease can occur in the absence of edema——"dry PIH" (Lindheimer & Katz 1992). The underlying pathophysiology responsible for these changes may be vasospasm and an increased permeability to large molecules such as albumin.

3. Cardiovascular and Hematologic Changes

a. Blood Volume

One of the most significant pathophysiologic changes in PIH is substantial hemoconcentration (Lindheimer & Katz 1992). By approximately 34 weeks, the normal pregnant woman has expanded her blood volume by about 40% to 50%

above the non-pregnant level (Pritchard 1965). In contrast, women with PIH manifest a decrease in blood volume (Cunningham & Pritchard 1978).

b. Cardiac

A wide variety of hemodynamic changes has been noted in women with PIH. Before treatment, it appears that cardiac output ranges from normal to a hyperdynamic state, that systemic vascular resistance is increased, and that both central venous pressure and pulmonary capillary wedge pressure are normal to low (Cunningham *et al.* 1989).

c. Hematologic

The major hematologic change seen in some women with PIH is thrombocytopenia (Cunningham *et al.* 1989). Other relatively uncommon hematologic abnormalities include overt hemolysis, elevated fibrin-degradation products, decreased plasma fibrinogen, and a protracted thrombin time. Intense vasospasm may induce endothelial damage (Pritchard *et al.* 1976). The endothelial injury exposes the underlying vascular basement membrane, resulting in platelet adherence and fibrin deposition. Platelets are decreased and red blood cells are torn and fragmented in these fibrin plugs. This process has termed "microangiopathic hemolytic anemia" (Cunningham & Pritchard 1978).

d. Placental Perfusion

A major pathophysiological event in PIH is a decrease in uteroplacental perfusion (Kaar *et al.* 1980; Lunell *et al.* 1984). Worley *et al.* (1975) reported that the placental clearance rate of dehydroisoandrosterone sulfate was initially increased in women who were destined to develop hypertension, but perfusion was uniformly decreased by the time overt hypertension developed. Others (Everett *et al.* 1980; Fritz *et al.* 1985) confirmed that the placental clearance rate of this hormone accurately reflected maternal placental perfusion. The causes of poor uteroplacental perfusion in PIH are unclear. However, it is believed that the causes have a key role in the pathogenesis of PIH (Stirrat 1987; Walsh 1990).

e. Calcium Metabolism----Hypocalciuria

Although research on calcium metabolism in PIH has attracted less attention than on the other fields, some recent findings (Belizan *et al.* 1988; Belizan *et al.* 1991; Sanchez-Ramos *et al.* 1991) have suggested that calcium may have a role in the development of gestational hypertension. Calcium metabolism during pregnancy is characterized by minor changes in the serum levels of total and ionized calcium; whereas urinary calcium excretion increases markedly during normal pregnancy (Gertner *et al.* 1986). Serum calcium levels in PIH appear no different from the values in normotensives, but urinary calcium excretion is considerably reduced (Sanchez-Ramos *et al.* 1991). Intracellular calcium concentrations in erythrocytes

and platelets were reported to be higher in women with PIH (Haller *et al.* 1989; Sowers *et al.* 1989). The cell's responsiveness to pressure stimuli is increased by a high concentration of intracellular calcium (Belizan *et al.* 1991). Thus, the increased intracellular calcium could be a factor which contributes to the high blood pressure seen in PIH.

There is evidence that hypocalciuria in PIH may be due to increased distal tubular reabsorption of calcium (Taufield *et al.* 1987). Belizan *et al.* (1991) observed that a decreased incidence of PIH occurred in pregnant women who received calcium supplements. They postulated that a high calcium intake increased levels of serum calcium, lowered parathyroid hormone concentrations, decreased renal calcium reabsorption (i.e. increased urinary calcium excretion), therefore, reduced intracellular calcium levels, and finally diminished cell responsiveness to pressure stimuli and lowered blood pressure.

### 1.1.5 Clinical Aspects

PIH occurs most often in nulliparous women, almost always after 20 weeks' gestation and more commonly towards term. It is associated with hypertension, proteinuria, edema, and at times, coagulation and/or liver function abnormalities (Sibai *et al.* 1982). Eclampsia is characterized by these abnormalities along with generalized convulsions. The signs and symptoms of PIH usually become apparent at a relatively late stage in pregnancy, often in the third trimester, whereas the underlying cause of the pathophysiologic mechanisms that are thought to be responsible for the disease process appears to occur much earlier in pregnancy, between 8 and 18 weeks' gestation (Robertson & Khong 1987). The occurrence of the signs and symptoms listed in Table 1.2 is particularly ominous.

Preeclampsia can progress rapidly to eclampsia, one of the most dramatic and life-threatening complications of pregnancy. Convulsions are usually preceded by premonitory symptoms and signs, including severe frontal or occipital headaches, epigastric pain, hyperreflexia, hemoconcentration and/or visual disturbance, but occasionally convulsions appear suddenly and without warning in an asymptomatic woman known only to have mild hypertension. There is also a variant of the disease whose clinical appearance may be misleading, because patients begin with minimal elevations in blood pressure and liver enzymes, a small decrease in platelet counts, and little or no renal dysfunction. Such patients, however, may progress rapidly to a life-threatening syndrome characterized by hemolysis and marked signs of liver

**Table 1.2 Ominous signs and symptoms in women with PIH***

1. Blood pressure ≥160 mm Hg systolic or ≥110 mmHg diastolic.
2. Proteinuria of new onset at a rate of ≥2 g per 24 hours or ≥100 mg per deciliter in a randomly collected specimen.
3. Increasing serum creatinine levels (especially >177 umol per liter or 12 mg per deciliter, unless the level was known to be elevated previously.
4. Platelet count $<10 \times 10^9$ per liter or evidence of microangiopathic hemolytic anemia (e.g., schistocytes or increase in lactic acid dehydrogenase and direct bilirubin levels).
5. Upper abdominal pain, especially epigastric and right-upper-quadrant pain.
6. Headache, visual disturbances, or other cerebral signs.
7. Cardiac decompensation (e.g., pulmonary edema, usually associated with underlying heart disease or chronic hypertension).
8. Retinal hemorrhage, exudates, or pappiledema (these signs are extremely rare in the absence of other indicators of severity and, when present, almost always indicate underlying hypertension).
9. Fetal growth retardation.

* From Cunningham and Lindheimer (1992).

dysfunction, as well as coagulation abnormalities. This uncommon form of PIH, called HELLP syndrome (Hemolysis, Elevated Liver enzymes, and Low Platelet count), requires prompt termination of the pregnancy (Killam *et al.* 1975; Sibai *et al.* 1986).

PIH typically regresses rapidly after delivery, and its signs and symptoms usually abate within 48 hours. Occasionally, late postpartum eclampsia, characterized by hypertension, proteinuria, and convulsions, occurs within 10 days after delivery (Brown *et al.* 1987; Sibai 1988).

### 1.1.6 Treatment

The aims of treatment should be to protect the mother from an excessive rise in blood pressure, and prevent the disease progressing to eclampsia or other maternal complications. Risks to the fetus should be minimised and it should be delivered by the easiest, safest way when the risks of continuing the pregnancy outweigh the risks of delivery. Undue delay for fetal reasons has been criticised as a cause of maternal mortality (DHSS 1991).

1. Prophylaxis

Although prophylaxis of PIH is difficult since there is currently no suitable predictive test for it, several approaches to preventing PIH have been recommended, including salt restriction, prophylactic diuretic therapy, calcium supplements and low-dose aspirin (Brown 1990).

It is questionable if restriction of salt or diuretic therapy has any beneficial effect on the prevention of PIH and some researchers have suggested that dietary salt restriction or prophylactic use of diuretics is unnecessary (Zuspan 1978; Collins *et al.* 1985).

The idea of supplemental calcium results from the facts that there is an inverse relation between calcium intake and the degree of PIH and calcium supplementation lowers blood pressure in both pregnant and non-pregnant women (Villar *et al.* 1983 and 1987). Furthermore, several studies have demonstrated that pregnant women who receive calcium supplemantation after the 20th week of pregnancy have a reduced risk of PIH (Lopez-Jaramillo *et al.* 1990; Villar & Repke 1990; Belizan *et al.* 1991).

The use of aspirin in prevention or treatment of PIH was first proposed by Goodlin *et al.* (1978). He reported that a patient with recurrent PIH seemed to benefit from aspirin. Since then growing evidence has demonstrated that low-dose aspirin (60 to 81 mg per day) reduces the incidence of PIH (Wallenbury *et al.* 1986; Benigni *et al.* 1989; Walsh 1990). Aspirin irreversibly acetylates cyclooxygenase, reducing cyclic endoperoxide synthesis from arachidonic acid, and decreasing production of both PGI2 and TXA2. Interestingly, if aspirin is used in lower doses, it only selectively inhibits platelet cyclooxygenase, reducing TXA2 synthesis without inhibiting endothelial cell synthesis of PGI2. An alternative explanation for this selective inhibition of cyclooxygenase is related to the pharmokinetics of low-dose apsirin. Absorption of non-ionized aspirin occurs in the stomach. Aspirin is then hydrolysed to salicylic acid in the liver so that platelets passing through the portal circulation are exposed to a higher concentration of aspirin than endothelial cells in the systemic circulation. The lower concentration of aspirin in the systemic circulation may be insufficient to inhibit endothelial cell cyclooxygenase activity. Recently, Walsh *et al.* (1992) proposed another hypothesis for the selective inhibition of aspirin. Platelets, which are the main productive site of TXA2, have a limited life span of only 8 to 10 days and do not have a nucleus. Therefore once platelet cyclooxygenase is inhibited, it is inhibited for the life span of the platelet. On the other hand, endothelial cells, which are the main productive site of PGI2 do have a nucleus and so can resynthesize cyclooxygenase after inhibition by aspirin. After a single dose of aspirin platelet TXA2 production is suppressed for 2 days and only gradually recovers (Burch *et al.* 1978), but endothelial cell production of PGI2 completely recovers within 36 hours (Jaffe & Weksler 1979).

## 2. Management

If the diagnosis of PIH is suspected, hospitalization should be considered (Cunningham *et al.* 1989) and the intervention required depends upon stage of gestation and disease severity. Management is outlined in Table 1.3.

The antihypertensive agents most frequently used in the management of PIH are listed in Table 1.4. At present there is no good evidence that any one of these drugs is preferable to another (Collins & Wallenburg 1989). The drug of choice is the one with which the obstetrician is most familiar and of which the maternal and fetal side effects are best understood.

**Table 1.3 The management of PIH***

| Clinical Condition | Therapy |
|---|---|
| **A.** PIH when the fetus is mature | Definitive:<br>1. Prevent convulsions<br>2. Control blood pressure<br>3. Deliver |
| **B.** PIH when the fetus is premature but there is<br>1. Severe preclampsia or superimpressure posed preclampsia<br>2. Fetal growth retardation<br>3. Fetal jeopardy<br>4. Thrombocytopenia, liver dysfunction, progressive renal dysfunction and premonitory signs of eclampsia | Definitive:<br>1. Prevent convulsions<br>2. Control blood<br>3. Deliver |
| **C.** Eclampsia, whether the fetus is mature or premature | Definitive:<br>1. Treat convulsions<br>2. Control blood pressure<br>3. Stabilize mother<br>4. Deliver |
| **D.** PIH when the fetus is premature | Expectant:<br>1. Ambulatory<br>2. Hospitalization |

* Modified from Gant N.F. & Worley R.J. (1980). Hypertension in Pregnancy: Concepts and Management. New York: Appleton-Century-Crofts.

**Table 1.4 Antihypertensive drugs used in the management of PIH***

| Drugs | Modes of action | Cautions |
|---|---|---|
| Methyldopa (α-Adrenergic receptor agonist) | Methlydopa (after conversion to methyl-noradrenaline in nerve endings) stimulates alpha receptors in the medulla and reduces sympathetic outflow. | Causes postural hypo-tension. Avoid in active hepatic disease and HELLP syndrome. |
| Atenolol, oxprenolol pindolol, propranolol (β-Adrenergic receptor antagonists) | Inhibit beta-adrenergic activity. Initial fall in blood pressure due to decrease in cardiac output. May therefore reduce uteroplacental perfusion. | Atenolol has been associated with intrauterine growth retardation. |
| Labetolol (α- & β-Adrenergic receptor antagonist) | Competitive inhibition of alpha- and beta-adrenergic activity. Decreased peripheral vascular resistance without reducing cardiac output and uteroplacental perfusion. | |
| Nifedipine (Calcium channel blocker) | Inhibits calcium ion flux in vascular smooth muscle. Decrease in peripheral vascular resistance. Little, if any, reflex tachycardia. | Side effects include edema and headache. |
| Hydralazine (Arteriolar vaso-dilator) | Direct action on vascular smooth muscle causing decrease in peripheral vascular resistance. Reflex tachycardia often marked. | Side effects include headache |

* Modified from Davies N.J. (1992). Hypertensive disorders of pregnancy for the trainee. Br. J. Hosp. Med., 47:613-619.

### 1.1.7 Theories of Aetiopathogenesis

Outside the Chicago Lying-in Hospital the pedestal reserved for the person who establishes the cause of PIH remains vacant in spite of many avenues of research investigated by obstetricians and other scientists. Too many hypotheses or factors concerning the aetiology of PIH have been produced over the past century. In fact, the theories became so numerous that PIH was termed "the disease of theories", as Chesley (1971) has pointed out: "Everyone from allergist to zoologist has proposed hypotheses and suggested rational therapies based upon them, such as mastectomy, oophorectory, renal decapsulation, trephination, alignment of the patients with the earth's magnetic field with her head pointing to the earth's pole, and all sorts of medical regimens.".

A rational aetiologic hypothesis should develop from epidemiological, clinical or experimental observations. Here, some important facts associated with aetiologic considerations are listed in Table 1.5.

Taken together, it seems likely that PIH results from multiple aetiologic factors and/or interactions among them rather than a single one. The five most promising areas are immunological factors, antioxidant or reactive oxygen species (ROS) factors, eicosanoid factors, genetic factors and placental factors. Each of these is discussed in detail in the appropriate following section.

## 1.2 **Immunological Factors**

The concept that PIH may be an immunological disorder was first proposed in 1902. The hypothesis suggested that PIH reflected either a partial breakdown of the mechanisms responsible for the normal exemption of the immunological response directed against tissue- or organ-specific antigens associated with the placenta (Beer 1988). Since then increasingly sophisticated investigations that have parallelled new developments in immunology have furthered our understanding of immunobiology in the maternal-placental interface and thereby this condition.

### 1.2.1 Evidence

The evidence supporting the concept that immunological components may play a role in the development of PIH is listed in Table 1.6.

**Table 1.5 Observations associated with aetiologic implications in PIH**

| Observations | Aetiologic Implications |
|---|---|
| Decreased incidence with previous abortion; previous blood transfusions; previous frequent exposure to same seminal fluid. | Immunological factor |
| Increased incidence with primigravid state; paternal change; previous use of a barrier contraception method. | Immunological factor |
| Decline in uteroplacental blood flow precedes PIH by 1 to 3 weeks. | Uteroplacental hypoxia<br>ROS factor? Eicosanoid factor? |
| Increased sensitivity to angiotensin II. | Eicosanoid factor |
| Protection by low-dose aspirin. | Eicosanoid factor |
| Familial predisposition. | Genetic factor |
| Black women more susceptible. | Genetic factor |
| Occurs in abdominal pregnancy; in the absence of fetus (e.g. mole). | Not a uterine or fetal factor.<br>Placental factor? |
| Cured by delivery of placenta. | Placental factor |
| Higher incidence in patients with chronic vascular disease (essential hypertension, diabetes). | Uteroplacental ischemia<br>ROS factor? |
| Increased frequency with large amounts of trophoblast (mole, twins, etc.). | Uteroplacental hypoxia<br>ROS factor |

**Table 1.6. Evidence supporting the implication of immune mechanisms in the pathogenesis of PIH**

| |
|---|
| I. Conditions associated with increased incidence |
| 1, Primigravid state |
| 2, Increased trophoblastic mass |
| 3, Pregnancies with a different partner |
| 4, Previous use of a barrier contraceptive method |
| 5, Pregnancies after oocyte donation |
| II. Conditions associated with decreased incidence |
| 1, Previous pregnancy by same partner |
| 2, Previous abortion |
| 3, Previous frequent exposure to seminal fluid |
| 4, Pregnancies after previous blood transfusions |
| 5, Pregnancies after leucocyte immunization |
| 6, Pregnancies in consanguineous marriages |

The demographic and epidemiologic studies indicate that the degree of unprotected coital exposure experienced by the mother-to-be prior to the first pregnancy may relate to her risk of developing PIH (Klonoff-Cohen *et al.* 1989). The greater the exposure to same semen (an allogeneic antigen for women), the less the likelihood that she will develop PIH. This type of reasoning is also supported by data that parity has a protective effect against the development of PIH (MacGillivray 1958; Campbell *et al.* 1985; Seidman *et al.* 1989), whereas, the risk of PIH increases when a multiparous woman is exposed to new or different paternal antigens (Feeney 1980; Serhal & Cragt 1987). A study of inbred communities also found that PIH was commoner when mother/conceptus immunogenetic disparity was greatest (Need 1975). Feeney *et al.* (1977) studied the incidence of PIH in women who previously received blood transfusions and discovered that previous blood transfusions protected against its development, suggesting that it might be analagous to the beneficial effect of blood transfusions on renal allograft survival. Studies also indicated that the incidence of PIH was significantly lower after an induced abortion (1.70%) but not after a previous spontaneous abortion (2.33%) (Seidman *et al.* 1989). Taken together, there has been sufficient demographic and epidemiologic evidence to propose that immunological mechanisms are involved in the pathogenesis of PIH.

### 1.2.2 Immunological Maladaptation

Reproduction in an outbred population normally involves the mating of genetically different members of the same species. This results in intimate contact between cells which are allogeneic to one another. The maternal genital tract is exposed to male semen which contains spermatozoa and some mononuclear leukocytes bearing antigens of the male (Alexander & Anderson 1987; Wolff & Anderson 1988; Anderson & Hill 1989; Jassim 1990) and the conceptus which implants in the uterus expresses male-derived histocompatibility antigens against which the maternal immune system can react (Smith *et al.* 1978; Zuckermann & Head 1986; Billington & Burrows 1989). These alloantigens are particularly important during the first trimester and the early second trimester when uteroplacental vascular circulation is being established.

Extensive morphologic and physiologic changes take place in the uteroplacental vasculature which is necessary for the development of hemochorial placentation, characteristic of human pregnancy. These vascular changes are thought to be induced by the interaction of the fetal-derived trophoblasts with maternal tissues at the time of implantation. The human placenta receives its blood supply from

numerous uteroplacental arteries which are developed by the action of migratory interstitial and endovascular trophoblasts into the walls of spiral arteries (Gerretsen *et al.* 1983; Hustin *et al.* 1983; Althabe *et al.* 1985). During normal pregnancy the arteries are eroded by the invading trophoblasts to such a great extent that entire portions of the spiral arteries in the endometrium and even into the myometrium are eliminated (Ramsey & Donner 1980). As a result of these trophoblast-induced changes, foreign fetal antigens that are expressed on these various trophoblast cells are directly exposed to maternal blood that contains both cellular and humoral immune factors. Therefore, the maternal ability to mount immune responses and react against non-self antigen, *ie,* the immunological tolerance between maternal and fetal tissues, is important and necessary for normal conception and placentation.

In PIH a state of imbalance between the ratio of maternal blocking antibodies (alloantibodies) and fetal antigenic load may occur. The antigenic dose presented by the trophoblast overwhelms the maternal immune system to such an extent that adequate immunological coexistence between host and graft is not achieved or, alternatively, that the immune-response genes governing the mother's immunological reactivity are not sufficiently stimulated to produce an adequate and effective immunological blockade to the fetal antigens presenting. Factors responsible for placental immunoprotection may be produced in insufficient levels to camouflage the invasive trophoblast and to restrain the reactivities of effector lymphocytes (effector blockade). This process can be termed 'immunological maladaptation'. It is tempting to propose that such immunological maladaptation results in a disturbance of trophoblast invasion in the spiral arteries, which are left with a non-pregnant architecture and fail to dilate (Robertson & Khong 1987).

Since the morphologic and physiologic changes in the maternal-placental interface occur at the time of the first trimester (Roberts *et al.* 1989; Zeeman *et al.* 1992), much earlier than the overt disease, the development of mutual immunological tolerance in the first and early second trimesters of pregnancy is thought to have a decisive effect on whether or not PIH occurs.

### 1.2.3 Major Histocompatibility Complex (MHC) and Cytokines

In fact, pre-implantation embryos and villous syncytiotrophoblasts do not express class I or class II MHC antigenic determinants and syncytiotrophoblasts are covered by a highly charged sialomucin coat (Desoye *et al.* 1988; Lata *et al.* 1990). The covering of the syncytiotrophoblast surface with a sialomucin coat results in many of

the other surface antigens being either hidden from recognition or impervious to immune attack. Only cells of the extravillous/spongiform trophoblast, which eventually come in direct contact with decidua, express a novel HLA (human leucocyte antigen), HLA-G (Ellis 1990). HLA-G is probably involved in some way in interactions with the cells in the decidua. Although the function of HLA-G is not completely understood, it may have another role in allowing recognition by maternal graft rejection effector cells. The lack of classical HLA expression by the trophoblasts precludes the possibility that maternal T cells, cytotoxic or helper, can respond directly to the trophoblasts, however, it may increase the susceptibility of the trophoblasts to nonadaptive immunity, for example, mediated by natural killer cells (Lijunggren & Karre 1990). Thus it is likely that the immune interaction between mother and fetus is more analogous to that of a host-tumor than host-graft relationship.

Placental cells especially decidua and placental villi have been found to be a source of several cytokines (Jaattela *et al.* 1988; Casey *et al.* 1989; Kauma 1989; Romero *et al.* 1989b, 1989c and 1990; Berkowitz *et al.* 1990; Dudley 1990). So far there is little knowledge on the direct implication of cytokines in the development of PIH. However, in view of the fact that some cytokines have the ability to stimulate local prostacyclin production (Romero *et al.* 1989a and 1989b; Mitchell 1991), it is reasonable to believe that cytokines may be involved in the development of PIH.

It is believed that the maternal decidua constitutes a distinct immunological microenvironment (Dudley 1990). Decidual cells have been shown to be a source of several cytokines such as IL-1, IL-3, TNF and granulocyte-macrophage colony stimulating factor (GM-CSFC) (Athanassakis *et al.* 1987; Wegmann 1987; Casey *et al.* 1989; Romero *et al.* 1989b; Dudley *et al.* 1990; Vince 1992). Cytokines could exert immunotrophic effects on the maternal-placental interface by acting to stimulate trophoblast growth (Wegmann 1988). According to this model, a deficient immune response could lead to subnormal levels of growth factors and in turn reduce the ability of trophoblasts to destroy the muscular layer and the autonomic innervation of the spiral arteries. Consequently, the spiral arteries are thicker, their lumens are narrower, their vascular tone is higher than the vessels of normal pregnancy, and blood flow through the uterus and into the intervillous space of the placenta is thus reduced (DeWolf 1975; Ramsey 1980; Gerretsen 1983; Lunell 1984).

### 1.2.4 Autoimmunity

While a major focus of the development of PIH has been on the maternal ability to mount immune responses and react against non-self antigens, autoimmunity needs to be considered. In fact, a number of investigators have suggested that some cases of PIH are associated with clinical autoimmune phenomena (Griffin & Wilson 1979; Foidart *et al.* 1986; Kochenour *et al.* 1987; Rote *et al.* 1987). Since the syncytial trophoblast is an immunologically privileged tissue, the focus of the immune attack by the effector cells or their products appears to be the placental cytotrophoblastic component of the arterioles that are physiologically and morphologically altered during placentation (Labarrere 1988). This autoreactive process may result in immune vasculitis pathognomonic of PIH and thus may play a role in contributing to the well-defined consequences: choriodecidual damage and liberation of tissue thromboplastin, fibrin, fibrinogen and the vasoactive mediators (including prostaglandins). To date abnormal levels of autoantibodies towards trophoblast antigens (Hytten & Brinton 1963; Bieglmayer *et al.* 1986), amniotic glycoprotein, placental and renal antigens (Gaugas & Curzen 1974), lymphocytes (Mekori *et al.* 1981), laminin (Foidart *et al.* 1986), and endothelial cells (Rappaport *et al.* 1990) have been reported in the sera of women with PIH.

### 1.2.5 Complement and Immune Complexes

Circulating immune complexes may have some relevance in the tissue injury that occurs in PIH. Unfortunately, there is no agreement between different investigators on this issue (Masson *et al.* 1977; Stirrat *et al.* 1978; Gleicher and Theofilopoulos 1979). Some workers have not detected immune complexes in either normal pregnant or PIH sera, whereas others have found increased concentrations in association with PIH (Redman & Sargent 1986; Haeger *et al.* 1991). The increase in immune complexes may have a role in stimulating macrophages and platelets to synthesize prostaglandins and also in inducing the release of cytokines and the production of ROS from monocytes (Hansch *et al.* 1987). Although there is some suggestions that complement activation and immune complexes are involved in the preeclamptic process, there is no direct evidence that any of these factors actually cause this condition. Clearly, more detailed investigations are needed.

## 1.3 Reactive Oxygen Species (ROS)

ROS are also known as oxygen free radicals. However, because some biologically important oxidants are not radicals, for example, hydrogen peroxide, hypochlorous acid, ozone, singlet oxgen, it is appropriate to speak of ROS instead of oxygen free radicals.

ROS have played an essential role in the origin of aerobic life forms and radical reactions are an integral part of the homeostasis in cellular processes. The catalytic action of many cellular enzymes and energy producing electron transport processes involve one-electron transfers that produce reactive intermediate oxygen species. Molecular oxygen, present in all aerobic organisms, readily accepts electrons, resulting in the formation of ROS. ROS can become highly destructive to cells and tissues if their production is not tightly controlled.

### 1.3.1 Chemistry of Reactive Oxygen Species

The molecule of ROS has one or more unpaired electrons. Molecular oxygen ($O_2$) has two unpaired electrons, each located in different orbitals. The reactivity of molecular oxygen is increased by the addition of a single electron, forming the superoxide anion ($O_2^-$). Addition of a second electron produces the peroxide ion ($O_2^{2-}$). At physiologic pH, the peroxide ion will immediately be protonated to form hydrogen peroxide ($H_2O_2$). Hydrogen peroxide is also generated in aqueous solution by the spontaneous dismutation of the superoxide anion. While the superoxide anion and hydrogen peroxide are both ROS and are able to cause direct cellular damage, they are weak oxidants in aqueous solution. Their pathophysiologic significance relates to their ability to serve as substrates for the formation of the very reactive hydroxyl radical ($^{\bullet}OH$) (Wispe & Roberts 1987).

Fenton first proposed the existence of the hydroxyl radical in 1894 and suggested that it was formed as a product of oxidation of $Fe^{2+}$ or other metals ($Ti^{3+}$, $Cu^{1+}$, $Co^{2+}$) by hydrogen peroxide (Reaction 1.1):

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \qquad \text{(Reaction 1.1)}$$

The modified Fenton reaction includes the reduction of $Fe^{3+}$ by superoxide anion in the two-step Haber-Weiss reaction (Reactions 1.2 and 1.3):

$Fe^{3+} + O_2^- \longrightarrow Fe^{2+} + O_2$ (Reaction 1.2)

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$ (Reaction 1.3)

The Fenton or Haber-Weiss reactions are important because they provide mechanisms by which the limited reactivity of superoxide anion and hydrogen peroxide can be increased. The hydroxyl radical is highly reactive and capable of reacting with almost every type of cellular molecule.

ROS broadly have the following properties:

1. High reactivity with a consequent extremely short life span (measured in μs).
2. Self-perpetuating (autocatalytic) and diverse chemical reactivity.
3. Low chemical specifity.
4. Generated both *in vivo* and *in vitro*.

### 1.3.2 Possible Sources of ROS

It is now well established that ROS are continuously produced *in vivo*. There are two main sources of ROS: cellular and environmental (Table 1.7). Increased ROS levels have been documented in various diseases including PIH (Ishihara 1978; Maseki *et al.* 1981; Wickens *et al.* 1981; Dekker & Kraagenbrink 1991; Wang *et al.* 1992). There are several fundamental pathological changes in PIH which may contribute to the increased production of ROS.

1. Placental hypoxia

The spiral arteries in PIH fail to undergo a physiological change and can not become refractory to vasomotor agents (Brosens 1977; Ramsey & Donner 1980; Cooper *et al.* 1988). In addition, many vessels are occluded by fibrinoid material and exhibit adjacent foam cell invasion (atherosis) (Robertson & Khong 1987). The placental maladaptations would result in poor perfusion, placental hypoxia and concomitant placental damage. This concept is supported by the observations that a decrease in uteroplacental blood flow occurs in PIH (Kaar *et al.* 1980; Lunell *et al.* 1984) and that many of the ultrastructural changes of PIH placental tissue closely resemble alterations seen in placental tissue when placed in hypoxic organ culture (Tominaga & Page 1966; Fox 1970). Tissue hypoxia appears to be a promoting factor for ROS production (Figure 1.1). Poor perfusion or ischemia can lead to a rapid decrease in tissue ATP (Hems & Brosnan 1970) and a rise in the ATP degradation products

**Table 1.7 Sources of ROS in biological systems***

| Sources | | Examples |
|---|---|---|
| Cellular | Auto-oxidation | Adrenaline<br>Dinucleotide<br>Flavin<br>Flavin adenine<br>Melanin thiols<br>Mononucleotide<br>Reduced riboflavin |
| Metabolism | Cell proliferation and activation | |
| | Eicosanoid metabolism | |
| | Endoplasmic reticulum oxidation | |
| | Enzymic activity | NADPH oxidase<br>Xanthine oxidase |
| | Mitochondrial electron transport | |
| | Phagocytosis | |
| Environmental | Cytotoxics | Bleomycin<br>Doxorubicin |
| | Drugs | Halothane<br>Paracetamol |
| | Pesticides | Paraquat |
| | Photochemical air pollutants | |
| | Radiation | X-ray<br>Light |
| | Tobacco smoke | |

* Modified from Sinclair *et al.* (1990).

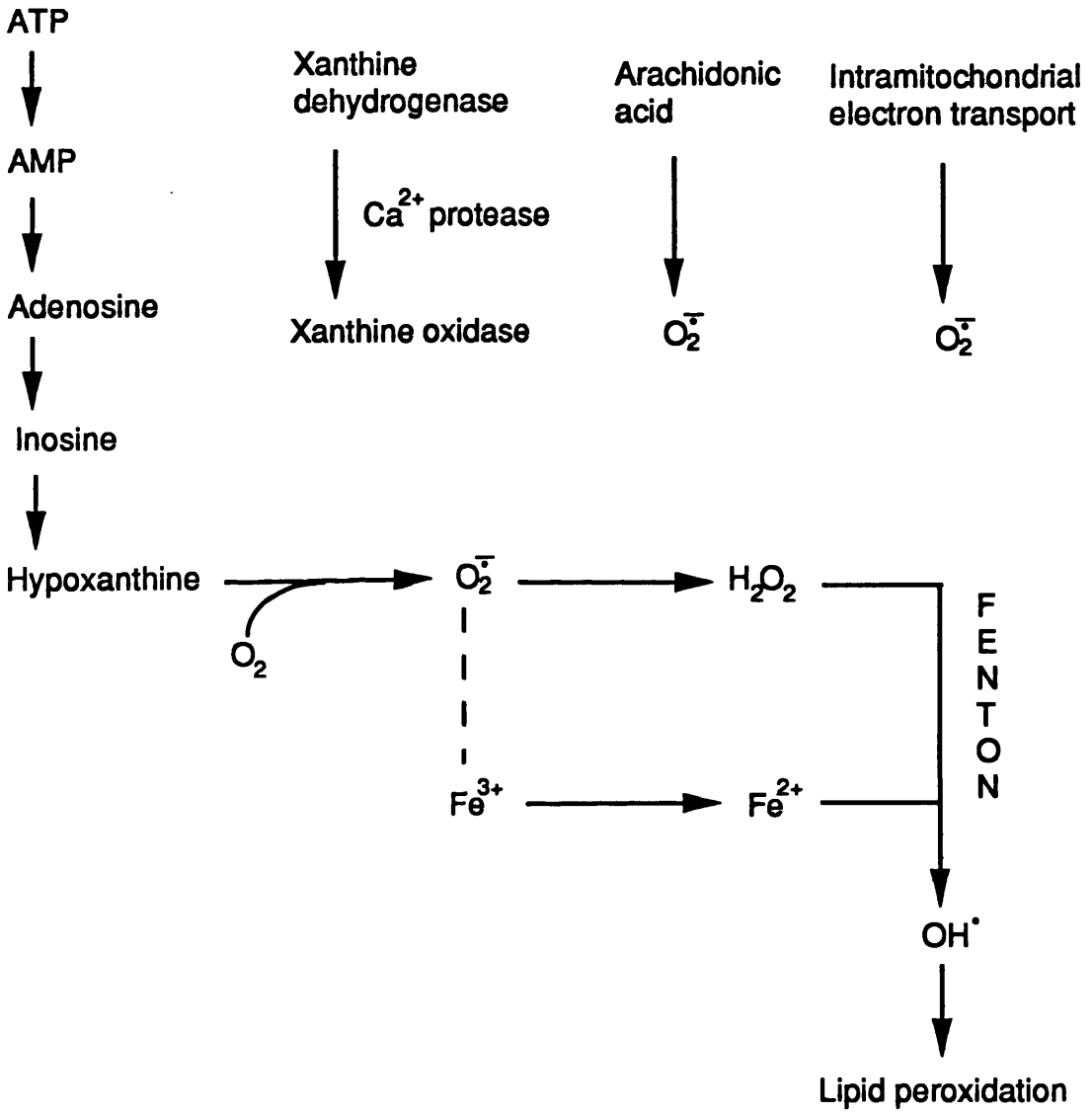


**Figure 1.1 Generation of ROS during tissue hypoxia and ischemia**

adenosine, inosine and hypoxanthine (Osswald *et al.* 1977; Miller *et al.* 1978). The loss of adenosine from cells by degradation during poor perfusion or ischemia is believed to result in the depletion of adenine nucleotides. The enzyme xanthine dehydrogenase normally catalyses the reaction (Reaction 1.4):

$$\text{Hypoxanthine} + H_2O + NAD^+ \longrightarrow \text{Xanthine} + NADH + H^+ \quad \text{(Reaction 1.4)}$$

But in conditions of hypoxia, activation of a calcium-dependent protease induces a transformation of the enzyme leading to predominantly oxidase activity (Parks & Granger 1986). During hypoxia, xanthine oxidase will use any available oxygen as an electron acceptor and convert xanthine or hypoxanthine to uric acid, with the production of the superoxide radical. Superoxide radical and its reduction products, hydrogen peroxide and hydroxyl radical, can produce cellular injury through lipid peroxidation of mitochondrial, lysosomal and plasma membranes (Kellogg & Fridovich 1975). A role for the participation of ROS in injury during hypoxic insult has been found in the brain, heart and intestine of experimental animals (Flamm *et al.* 1978; Guarnieri *et al.* 1980; Granger *et al.* 1981; Kogure *et al.* 1982).

2. Arachidonic acid metabolism

Arachidonic acid metabolism through eicosanoid biosynthesis is accompanied by the generation of ROS (Figure 1.2). The first step in the biosynthesis of prostaglandins is the oxygenation of arachidonic acid to the hydroperoxy endoperoxide $PGG_2$ (Crawford 1983). This reaction is catalyzed by a heme-containing oxygenase, called cyclooxygenase, that requires no external source of electrons (Hemler *et al.* 1976; Miyamoto *et al.* 1976; Van der Ouderaa *et al.* 1977). The hydroperoxy group of $PGG_2$ is reduced to a hydroxy group ($PGH_2$) by a peroxidase that utilizes a wide variety of compounds to provide the requisite pair of electrons (Miyamoto *et al.* 1976; Ogino *et al.* 1978) and this process produces a radical species (Ox) which has many of the properties of the hydroxyl radical, although it is known not to be this species.

An alternative pathway of arachidonate metabolism is an oxidation controlled by lipoxygenase enzymes. The fatty acid is converted into hydroperoxy derivatives (hydroperoxy eicosatetraenoic acids; HPETEs) which can readily be reduced to the corresponding hydroxy acids (HETEs) by glutathione peroxidase. The conversion of hydroperoxy derivatives to the hydroxy fatty acid yields oxygen radicals (Ox) (Figure 1.2), such as peroxy radical (Taylor & Morris 1983).

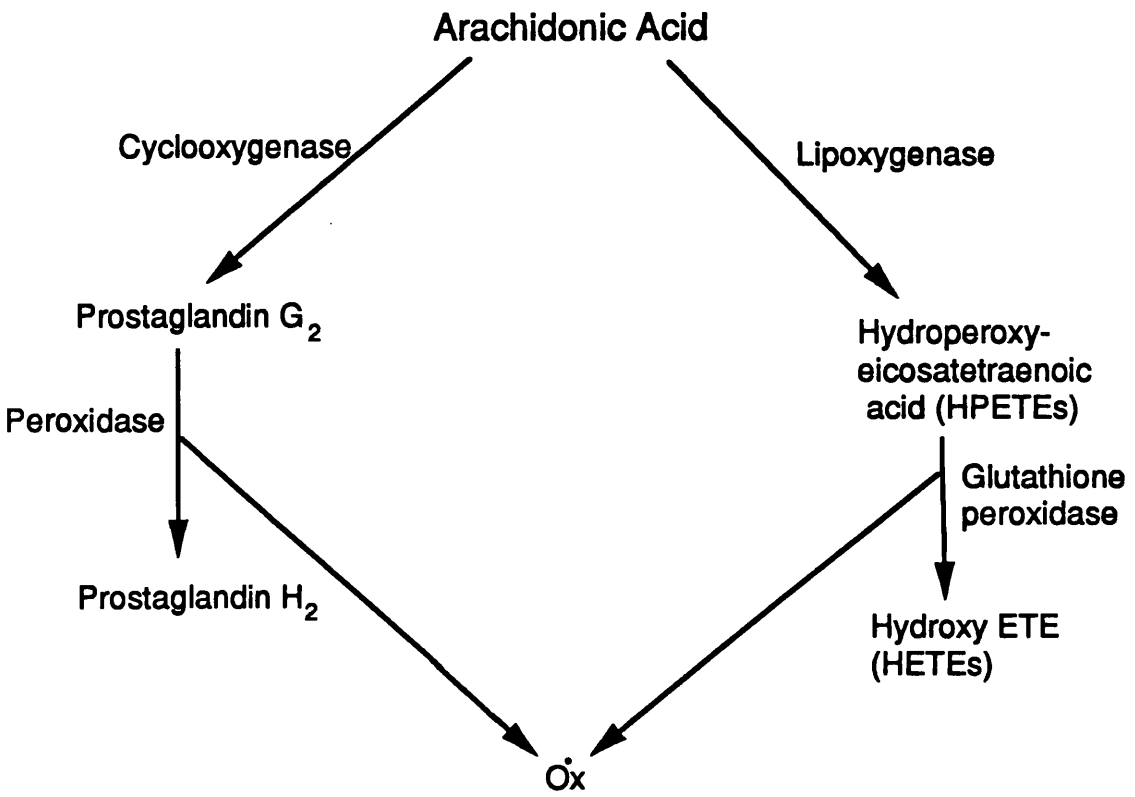


**Figure 1.2 Generation of ROS during eicosanoid metabolism**

There is evidence suggesting that both cyclooxygenase and lipoxygenase activities are changed in patients with PIH (Saeed & Mitchell 1983; Ogburn *et al.* 1984; Satoh *et al.* 1991; Walsh *et al.* 1992). Therefore, it is reasonable to consider eicosanoid metabolism as a possible source of ROS production in PIH.

3. Leukocytes

The superoxide anion is produced by activated neutrophils and macrophages. Oxygen uptake in activated neutrophils and macrophages is due to the action of a NADPH-oxidase complex associated with the plasma membrane, the electrons released on oxidation of NADPH reducing oxygen to superoxide radical as part of the "respiratory burst" (Babior 1978). During the respiratory burst, myeloperoxidase is released into the phagocytic vacuole and reacts with hydrogen peroxide to produce reactive byproducts including hypochlorous acid (Reactions 1.5 & 1.6), which can oxidise many biological molecules, especially reduced thiol groups (Babior 1978; Marletta 1989). For example, hypochlorous acid in low concentrations has been shown *in vitro* to inactivate rapid α1-antiproteinase, the main inhibitor of proteolytic enzymes in extracellular fluids. The extent to which inactivation could occur *in vivo* depends on the environment of the neutrophil, as in plasma, hypochlorous acid reacts preferentially with albumin.

$$2H^+ + 2O_2^- \xrightarrow{SOD} H_2O_2 + O_2 \qquad \text{(Reaction 1.5)}$$

$$H_2O_2 + Cl^- \xrightarrow{\text{myeloperoxidase}} HOCl + OH^- \qquad \text{(Reaction 1.6)}$$

It has now been estabilshed that the development of PIH is associated with neutrophil activation as indicated by the increasing plasma levels of neutrophil elastase (Greer 1989 and 1991). The concentration of neutrophil elastase in human plasma is normally very low and independent of neutrophil count, thus, elevated levels reflect neutrophil activation and degranulation *in vivo* (Weissman *et al.* 1980; Janoff 1985).

### 1.3.3 Injury to Cells and Tissues

Among the major cellular and extracellular targets for ROS are proteins, unsaturated fatty acid components of lipids and lipoproteins, and DNA constituents including carbohydrates, as depicted in Table 1.8.

**Table 1.8 The major cellular targets for ROS**

| TARGET | DAMAGE | CONSEQUENCE |
|---|---|---|
| DNA | scission on deoxyribose ring; base damage; strand breaks; cross-linking. | mutations; translational errors; inhibition of protein synthesis. |
| PROTEIN | aggregation & cross-lonking; fragmentation & breakdown; modification of thiol groups. | modified ion transport; increased calcium influx; modified enzyme activity. |
| POLY-UNSATURATED | loss of unsaturation; formation of reactive metabolites (MDA and 4-HNE). | altered lipid; fluidity and membrane permeability; effects on membrane-bound enzymes. |

1. Lipids

The phospholipid component of the cellular membrane is a highly vulnerable target due to the susceptibility of its polyunsaturated fatty acid sidechains to oxidative damage termed lipid peroxidation. This can lead to changes in the membrane permeability characteristics and its ability to maintain transmembrane ionic gradients (Slater 1984). The initiating ROS extracts a hydrogen atom from the carbon chain generating a lipid carbon-centered radical ($L^{\bullet}$, Reaction 1.7). This lipid radical reacts with molecular oxygen to yield a hydroperoxyl radical ($LOO^{\bullet}$, Reaction 1.8) that can propagate the oxidizing chain reaction by abstracting electrons from other susceptible fatty acids to form another lipid radical and lipid hydroperoxide (LOOH, Reaction 1.9).

$$LH + {}^{\bullet}OH \longrightarrow H_2O + L^{\bullet} \qquad \text{(Reaction 1.7)}$$

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet} \qquad \text{(Reaction 1.8)}$$

$$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet} \qquad \text{(Reaction 1.9)}$$

In the presence of transition metals and metal complexes, particularly iron ($Fe^{2+}$, $Fe^{3+}$), hydroperoxides yield another series of reactive lipid species including alkoxyl radicals ($LO^{\bullet}$, Reaction 1.10) and peroxyl radicals ($LOO^{\bullet}$, Reaction 1.11). (O'Brien 1969; Davies & Slater 1987).

$$LOOH + Fe^{2+}\text{-complex} \longrightarrow LO^{\bullet} + Fe^{3+}\text{-complex} + OH^{-} \qquad \text{(Reaction 1.10)}$$

$$LOOH + Fe^{3+}\text{-complex} \longrightarrow LOO^{\bullet} + Fe^{2+}\text{-complex} + H^{+} \qquad \text{(Reaction 1.11)}$$

Alkoxy and peroxyl radicals can initiate new rounds of lipid peroxidation and propagate further radical chain reactions thus amplifying the initial damage. Cleavage of the carbon bonds during lipid peroxidation reactions results in the formation of (Figure 1.3):

1), Alkanals, such as malondialdehyde, which interact with protein thiols, cross link amino groups of lipids and proteins and give rise to chromolipids and aggregated proteins (Tappel & Dillard 1981).

2), Alkenals, such as 4-hydroxy nonenal, which are very biologically active. They inhibit platelet aggregation, modify adenyl cyclase activity and are a substrate for glutathione transferases (Esterbauer 1985).

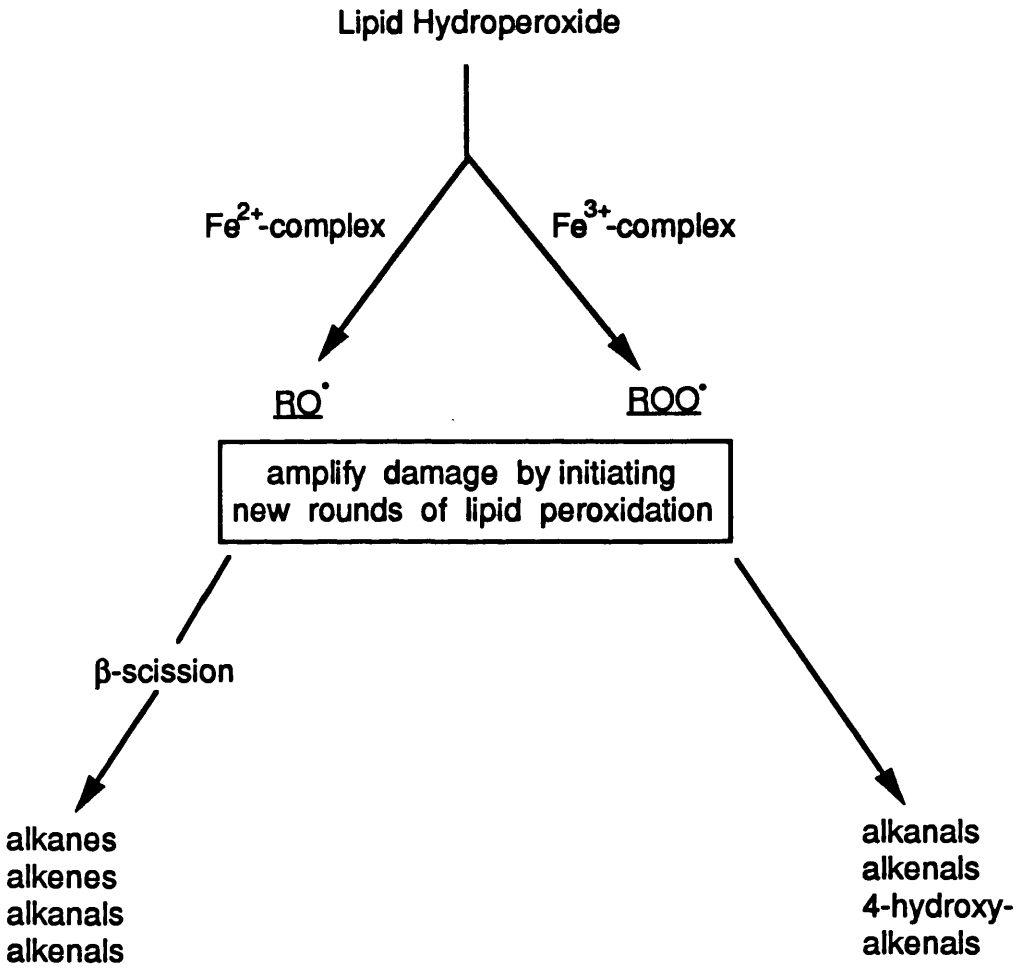


**Figure 1.3 The degradation of lipid hydroperoxides**

3), Alkanes *e.g.* pentane, produced by this mechanism as an end-product of the oxidation of linoleic and arachidonic acid, and ethane from linolenic acid (Tappel & Dillard 1981).

The production of ROS leads to primary reactions (lipid peroxidation) which are close to the site of formation. Secondary products of such peroxidative events such as lipid peroxyl radicals and lipid hydroperoxides may diffuse in the plane of the membrane before reacting further, thereby spreading the biochemical lesion. Such processes therefore not only affect the structural and functional integrity of the membrane, its fluidity and permeability, but also the breakdown products of lipid peroxidation can further damage cellular function. Some lipid peroxidation products can escape from the membrane and cause disturbances at a distance. Therefore a reaction that originally produces a radical which interacts within its microenvironment may produce a sequence of later events that direct disturbances throughout the cell, its membrane and, in some instances into the extracellular domain.

Therefore, these lipid peroxidation reactions, once initiated, can result in chain reactions, with injuries at sites distant from the initial site of ROS generation (Wispe & Roberts 1987).

2. Protein

Proteins are susceptible to ROS injury through oxidation of their sulphydryl groups, which may alter protein structure or inactivate catalytic sites (Wolff *et al.* 1986). Oxidation reactions may also cause degradation or cross-linking of proteins into large aggregates. Oxidative modification of proteins also renders them more susceptible to proteolytic attack and enzymic hydrolysis (Davies 1987; Wolff *et al.* 1986). Reactions of ROS with proteins also generate protein radical byproducts that can propagate the initial damage. Extracellular proteins with a large proportion of disulphide bridges (e.g. IgG or albumin) appear to be particularly vulnerable to hydroxyl and peroxy radical attack.

3. Nucleic acids

Nucleic acids are also susceptible to ROS-mediated alterations. It seems to be generally accepted to date that the hydroxyl radical is the main source of damage to DNA. Hydroxyl radical may penetrate the cell, thereby gaining access to DNA (Cochrane 1991). The hydroxyl radical damages DNA by hydroxylating the purine and pyrimidine bases and by scission, nicking, and cross-linking of DNA strands.

Injury to cellular DNA (ROS-induced chromosomal aberrations) subsequently causes mutations, alterations in growth, and inhibition of protein synthesis (Schraufstatter *et al.* 1988).

### 1.3.4 Defence against ROS —— Antioxidants

Biological systems are continuously challenged by pro-oxidants which are either exogenously generated (*e.g.* directly from xenobiotics) or generated from endogenous sources such as phagocytes. In the normal course of events cells have adequate anti-radical defence mechanisms, both those synthesised *in vivo* and those taken up in the diet (Halliwell 1990). Thus oxidative stress occurs when the balance between pro-oxidants and antioxidants is shifted in favour of pro-oxidants (Sies 1986), or, alternatively, when a measurable shift occurs in one or more redox couples to a more electron deficient (oxidised) steady state or equilibrium (Smith 1991). Any situation which increases the turnover of the antioxidant cycle whether increased oxidative stress or modified anti-radical defences can lead to progressive cellular and membrane damage. There is some evidence to show that ROS activity is increased in PIH (Ishihara 1978; Maseki *et al.* 1981; Wickens *et al.* 1981; Dekker & Kraagenbrink 1991; Wang *et al.* 1992). However, knowledge regarding the antioxidant status in PIH is limited.

The antioxidants synthesised in the body include a range of proteins, enzymes and other molecules (bilirubin and urate). The range of antioxidants in the different locations in the body, which are located either intracellular, extracellular or bound to the membranes, is shown below.

#### 1.3.4.1 Extracellular Antioxidant Defences

1. Transferrin

Oxidant damage to cell organelles and membranes is markedly potentiated by the presence of iron (Halliwell & Gutteridge 1984; Weiss & LoBuglio 1982). Apotransferrin binds iron (III) for transport and delivery to cells. It is its capacity as an iron binding protein which enables it to function as an antioxidant by making iron (III) unavailable for participation in iron-catalysed radical reactions.

2. Caeruloplasmin

This copper-containing protein is regarded as a physiological inhibitor of lipid peroxidation (Gutteridge 1978). In this one of its many roles, it acts as an

antioxidant by virtue of its ferroxidase activity, converting iron (II) to iron (III) by electron transfer.

3. Albumin

Albumin, one of the most important proteins in human plasma, is able to bind copper (II) tightly and iron weakly. Copper (II) bound to albumin is still effective in generating ra`ical species in the presence of hydrogen peroxide. Thus macromolecules functioning by this mechanism are called sacrificial antioxidants since the hydroxyl radical is generated locally on the protein and reacts at the specific site (Halliwell 1988). The binding of copper ions to albumin may represent a protective mechanism overall, since not only can the damaged albumin be quickly replaced but it can also absorb hypochlorous acid (Halliwell 1988). Another aspect of the antioxidant action of albumin may be its ability to scavenge some hydroxyl and peroxy radicals (Wayner *et al.* 1985; Gutteridge 1986), which may partly account for its reported ability to decrease lipoxygenase activity (Duniec & Robak 1984).

4. Haptoglobin/Haemogopexin

These proteins bind free haemoglobin/haem, thus protecting delocalised haemoglobin from influences which might otherwise activate the protein or destablise the haem ring and promote iron release (Gutteridge & Smith 1988).

5. Urate

Urate has the potential for chelating iron and copper rendering them unreactive and thus inhibiting lipid peroxidation. It also reacts with singlet oxygen, peroxy radicals and hydroxyl radicals (Ames *et al.* 1981; Grootveld & Halliwell 1987).

6. Vitamin E (α-Tocopherol)

This is a membrane-bound (lipid soluble) chain-breaking antioxidant which reacts with peroxyl and other reactive radicals (Esterbauer *et al.* 1989; MaCay 1985). Its lipid-soluble nature allows it to concentrate preferentially within the phospholipid bilayer of cell membranes and within blood lipoproteins (Lambert & Mourot 1984). Therefore, vitamin E is one of the major lipid-soluble chain-breaking antioxidants which protect membrane fatty acids from oxidant-induced lipid peroxidation (Ozawa *et al.* 1978).

7. Vitamin C (Ascorbic acid)

Although at relatively low levels in human plasma, many tissues contain ascorbate in millimolar concentrations. This antioxidant nutrient scavenges singlet oxygen and reacts rapidly with the hydroxyl radical, the superoxide anion and the hydrogen peroxide. It has also been proposed that it acts synergistically with vitamin E (MaCay 1985). Reaction of vitamin C with superoxide, peroxide and hydroxyl radicals forms, via a semidehydroascordate intermediate, dehydroascorbic acid (Bates 1981). Vitamin C is reported to be the most effective aqueous-phase antioxidant in human plasma, protecting against oxidants released from polymorphonuclear leukocytes and against lipid soluble peroxyl radicals (Levine 1986; Frei *et al.* 1990).

8. β-Carotene

This carotenoid scavenges singlet oxygen and peroxyl radicals (Burton & Ingold 1984; Vile & Winterbourn 1988).

9. Lycopene

Lycopene is a low concentration, core carotenoid antioxidant in lipoproteins (DiMascio *et al.* 1989; Esterbauer *et al.* 1989).

10. Metallothionein

The metallothioneins are generated in response to high concentrations of certain metal ions. They bind these ions extremely efficiently such that they are rendered harmless and can be removed from the body (Halliwell & Gutteridge 1984).

11. Bilirubin

Bilirubin has been proposed to be an effective antioxidant in terms of its capacity to protect albumin-bound polyunsaturated fatty acid (Stocker *et al.* 1987).

1.3.4.2 Intracellular Antioxidant Defences

1. Superoxide dismutase (SOD)

SOD is the major intracellular antioxidant enzyme in aerobic cells and there are two forms of SOD (Weisiger & Fridovich 1973). One is a cytosolic copper-zinc enzyme (CuZn-SOD) and the other a manganese-centred enzyme (Mn-SOD) found in the mitochondria. These two dismutases are structurally very different proteins, however, they both catalyze the dismutation of superoxide radical to hydrogen peroxide and oxygen very rapidly and specifically, and with equal efficiency

(Reaction 1.12) (Fridovich 1978). Its antioxidant activity is about 350000 times more effective than caeruloplasmin (Markland 1980). SOD activity is high in tissues with high oxygen utilization.

$$2O_2 + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad \text{(Reacton 1.12)}$$

2. Thiols

Thiol-containing compounds, such as cysteine, glutathione (GSH) and proteins with sulphydryl groups, stop ROS reactions by donating electrons from their sulphydryl group (Freeman & Crapo 1982) and they play an important role in scavenging ROS and in suppressing oxidative injury in cells and tissues exposed to hypoxia (Weinberg *et al.* 1987). The tripeptide glutathione (-Glu-Cys-Gly) is widely distributed in most mammalian cells (Meister & Anderson 1983). It is typically present in high (0.1-10 mM) levels and is thus the pivot in various protective systems (Meister 1988). In the GSH redox cycle, GSH is used by glutathione peroxidases (GSH-px) to reduce hydrogen peroxide and organic peroxides and form innocuous end products and oxidized glutathione (GSSG) (Reactions 1.13 & 1.14). To protect cells and tissues from ROS-mediated damage, high concentrations of GSH are essential for maintaining the GSH redox cycle. Cells are thus equipped with NADPH-dependent glutathione reductase (GSH-rd) to reconvert GSSG to the reduced GSH (Reaction 1.15).

$$2GSH + H_2O_2 \xrightarrow{GSH\text{-}px} GSSG + 2H_2O \qquad \text{(Reaction 1.13)}$$

$$2GSH + LOOH \longrightarrow GSSG + LOH + H_2O \qquad \text{(Reaction 1.14)}$$

$$GSSG + NADPH + H^+ \xrightarrow{GSH\text{-}rd} 2GSH + NADP \qquad \text{(Reaction 1.15)}$$

3. Catalase

Catalase reacts very rapidly with hydrogen peroxide, converting it to water and oxygen (Reaction 1.16). It is located in the cytosol, mitochondria and other subcellular organelles, such as peroxisomes. Normally, the low concentrations of hydrogen peroxide production are efficiently reduced in cells by glutathione peroxidase. However, if the concentration of hydrogen peroxide is raised, catalase becomes important (Southorn & Powis 1988).

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2 \qquad \text{(Reaction 1.16)}$$

4. Coenzyme Q (Ubiquinone)

As well as its function as a transporter in the mitochondrial electron transfer chain and in energy conservation, coenzyme Q has been reported to function as an antioxidant in its hydroquinone form (the majority of tissue coenzyme Q molecules exists in the reduced state) (Baum 1991; Beyer 1990). The mechanism of action is unclear, but several studies in subcellular systems, intact animals and human subjects in a clinical context all support one of the functions of coenzyme Q as a membrane, and possibly LDL, protectant against ROS damage.

#### 1.3.4.3 Secondary Protection

In addition, cells contain mechanisms for repairing DNA after attack by ROS, systems for degrading proteins damaged by ROS (Marcillat *et al.* 1988) and erythrocytes contain oxyhaemoglobin which is capable of protecting the cell membranes against peroxidative damage (Rice-Evans *et al.* 1985).

In general, intracellular antioxidants are systems for removing ROS, whereas among the extracellular components are those which additionally serve the essential function of keeping the transition metal catalysts under control.

## 1.4 Eicosanoid System

The increasing interest in the eicosanoid system in PIH reflects a growing recognition of its importance in the process of this disease. The functional imbalance between vasodilator and vasoconstrictor eicosanoid products may play an important role in the pathophysiologic mechanisms involved in the development of the various signs and symptoms of PIH (Dekker 1989, Friedman 1988). Two of the most important eicosanoids are prostaglandins and leukotrienes.

### 1.4.1 Biosynthesis

Neither the prostaglandins nor the leukotrienes are stored in tissues but are biosynthesized from fatty acid precursors upon cell stimulation. Arachidonic acid is the common precursor for products of both the cyclooxygenase (prostaglandins, prostacyclin and thromboxanes) and lipoxygenase (leukotrienes) pathways. The amount of free arachidonic acid within cells is very low but there is a comparatively large amount esterified in cell membrane phospholopids and glycerides. Therefore,

the initial and rate limiting step in the biosynthesis of eicosanoids is the enzymic liberation of free arachidonic acid from the ester pools. The main source of this precursor acid is the phospholopids. Arachidonic acid is located predominantly at the position of phospholipids and its release is controlled either by phospholipase A2 or the combined action of phospholipase C and a diglyceride lipase (Van den Bosch 1980; Irvine 1982; Flower & Blackwell 1983). Arachidonic acid released from the phospholipids reacts with oxygen, and cyclizes in a cyclooxygenase-catalysed reaction to give the prostaglandin endoperoxide PGG2. This endoperoxide is converted into a second endoperoxide, PGH2, in a peroxidase-catalysed reaction. Various synthetases then catalyse and convert PGH2 into other prostaglandins, prostacyclin and thromboxanes. Arachidonic acid may also be converted by lipoxygenase into a hydroperoxy derivative from which leukotrienes are biosynthesized (Figure 1.4).

The critical factor that determines the particular arachidonic acid metabolites produced by a given cell type is which enzymes of the arachidonic acid cascade are present in that cell type (Stenson & Parker 1984). Thus platelets, which contain cyclooxygenase and thromboxane synthetase, produce TXA2 and endothelial cells, which contain cyclooxygenase and prostacyclin synthetase, produce PGI2, whereas neutrophils, which contain lipoxygenase, leukotriene A synthetase, and leukotriene A hydrolase, produce leukotrienes.

### 1.4.2 Prostaglandins

The prostaglandins are a groups of 20-carbon unsaturated fatty acids that are similar in structure, yet varied in function. The term prostaglandin was introduced by early investigators who discovered the compounds in human semen and believed the prostate gland to be a major source. This suggestion is now known to be incorrect but the name has been retained. There are four most relevant prostaglandins, that is prostaglandin E (PGE), prostaglandin F2 (PGF2), prostacyclin (PGI2) and thromboxane A2 (TXA2). Their functions and main sites of production places are listed in Table 1.9.

#### 1.4.2.1 Prostacyclin

PGI2 is a potent vasodilator and inhibitor of platelet aggregation (Goodman *et al.* 1982; Walsh *et al.* 1985; Schiff *et al.* 1989). It is the most abundant arachidonic acid metabolite and is synthesized primarily in the endothelium but other tissues such as

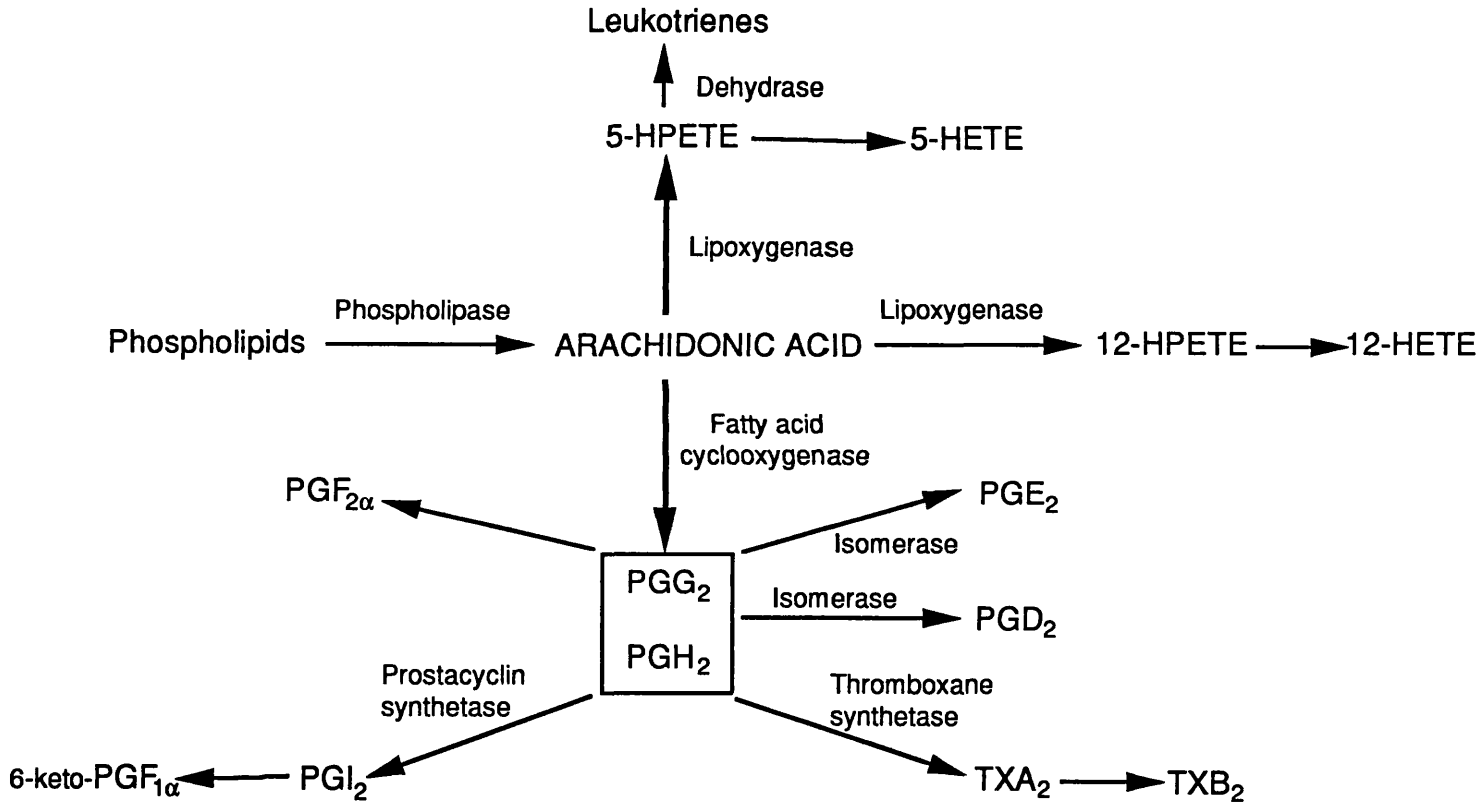


**Figure 1.4 Scheme of the major metabolic transformations of arachidonic acid**

**Table 1.9 The functions and production places of prostaglandins**

| | Vasocon-strictor | vaso-dilator | platelet aggregation stimulator/inhibitor | Main production place | Main in-activation place | cyto-protection |
|---|---|---|---|---|---|---|
| PGE1 | - | + | -/+ | | lung | ++ |
| PGE2 | - | ++ | -/++ | renal medulla | | ++ |
| PGF2 | + | - | +/- | | lung | ++ |
| PGI2 | - | +++ | -/+++ | endothelial cells renal cortex, leukocytes trophoblast, placenta | not lung | ++ |
| TXA2 | +++ | - | +++/- | platelet, lung, placenta, spleen | | |

myometrium and placenta also produce some PGI2 (Omini *et al.* 1979; Jogee *et al.* 1983; Rakoczi *et al.* 1983; Walsh & Parisi 1986; Walsh 1987). Trophoblastic production of PGI2 might function to prevent platelet-clumping in the intervillous space, whereas vascular PGI2 would maintain (placental) vasodilation. PGI2 is chemically unstable, with a half-life of three minutes at physiological pH and temperature (Dusting *et al.* 1978). Because its instability, PGI2 is frequently quantified by measuring its stable degradation product 6-keto-PGF1α.

Studies examining PGI2 levels in normal pregnant and preeclamptic women have yielded conflicting results and there seems to be no general agreement about how prostaglandin production is best assessed. In studying the maternal compartment, including plasma, urine and blood vessels, the evidence weakly suggests that the levels of PGI2 may be increased in normal pregnancy and decreased in PIH (Goodman *et al.* 1982; Walsh *et al.* 1985; Walsh 1985; Yamaguchi & Mori 1985; Friedman 1988; Schiff *et al.* 1989). Walsh *et al.* (1985) reported that the production of PGI2 in placental tissue obtained from women with PIH was significantly lower than that from normotensive women. Ylikorkala *et al.* (1981) also reported decreased levels of prostacyclin in amniotic fluid obtained from women with PIH, as compared with normotensive pregnant women.

Other investigators have preferred to study prostaglandin production in fetal rather than maternal tissues. The justification for this point of view is that fetal tissues are apparently capable of synthesizing greater amounts of prostacyclin than maternal tissues (Kawano & Mori 1983; Remuzzi *et al.* 1979). The literature on the fetoplacental unit largely agree about the decrease in PGI2 production in PIH (Friedman 1988).

#### 1.4.2.2 Thromboxane A2 (TXA2)

TXA2 opposes the action of PGI2. It is a potent vasoconstrictor and a stimulant of platelet aggregation (Ellis 1976; Bhagwat 1985). TXA2 is primarily produced by platelets in nonpregnant patients (Fitzgerald *et al.* 1987; Reilly & Fitzgerald 1987). Like PGI2, TXA2 is too unstable for isolation and measurement. Its biologic half-life in the blood at 37°C is approximately 30 seconds (Moncada & Vane 1979), and it is therefore usually measured as its stable hydration product TXB2 (Granstrom *et al.* 1982). TXA2 synthesis is increased in normal pregnant women; this is likely due to increased production by either the placenta (Walsh *et al.* 1985) or platelets (Fitzgerald *et al.* 1987).

### 1.4.2.3 Imbalance between PGI2 and TXA2

Since both prostacyclin and thromboxane A2 are increased during normal pregnancy, it has been thought that a major mechanism involved in the pathophysiology of PIH is a change in the ratio of prostacyclin to thromboxane production (Ylikorkala *et al.* 1981; Walsh 1985), with a change in "the direction of thromboxane A2 dominance." (Ylikorkala *et al.* 1981). In fact, Walsh (1985) reported that the placentae of women with PIH produced seven times more thromboxane A2 than prostacyclin. The selective inhibition of thromboxane A2 and sparing of prostacyclin by low-dose aspirin administration has led to several clinical trials attempting to prevent or forestall the development of PIH (Wallenburg *et al.* 1986; Wallenburg & Rotmans 1988; Benigni *et al.* 1989; Schiff *et al.* 1989).

The cause of the imbalance of TXA2 and PGI2 is still unclear. Walsh and Coulter (1989) indicated that progesterone, either alone or in combination with estradiol, could suppress PGI2 production by the human placenta, but it did not affect placental TXA2 production. However, this can not explain the decreased PGI2 found in the maternal circulation in PIH because the circulating concentrations of progesterone do not differ from those found in normal pregnancy (Parker *et al.* 1979; Pedersen *et al.* 1983). It has been suggested that ROS may cause the imbalance of TXA2 and PGI2. Lipid peroxides can activate cyclooxygenase and impair endothelial PGI2 synthetase (Higgs & Vane 1983). Therefore the increased lipid peroxide levels in PIH favor production of platelet-derived TXA2 above vascular PGI2 production. There is evidence showing that 12-HPETE can inhibit PGI2 synthesis (Turk *et al.* 1980; EI Tahir & Williams 1981). Ogburn *et al.* (1982 & 1984) also speculated that elevated production of lipoxygenase products by the uteroplacental unit could be responsible for the inhibition of PGI2 production.

## 1.4.3 Leukotrienes

Studies have suggested that PGI2 is not the only physiologic vasodilator in normotensive pregnancy. Spitz *et al.* (1988) reported that treatment of normotensive pregnant women, who exhibit an increased vascular sensitivity to angiotensin-II, with low-dose aspirin did not completely restore vascular refractoriness to angiotensin-II. The incidence of PIH in pregnant women using high doses of prostaglandin systhetase inhibitors is not increased (Dekker 1989; Briggs *et al.* 1990). It has been suggested that PGI2 production merely functions as a rescue

mechanism, especially during periods of ischemia and hypoxia (Spokas *et al.* 1983). Vasodilatory prostaglandin is evoked locally in an attempt to re-establish normal function by regulating tissue perfusion and metabolism. It might be that other autocoids such as leukotrienes are involved in the physiologic vasodilation in pregnancy. Those considered the most important physiologically are leukotriene B4, leukotriene C4 and leukotriene D4. In general, leukotriene B4 causes chemotaxis and increased vascular permeability (in the presence of neutrophils). Leukotriene C4 and leukotriene D4 induce vasoconstriction and increase vascular permeability (Piper 1984; Stenson & Parker 1984). Saeed and Mitchell (1983) have demonstrated that human uterine and intrauterine tissues are capable of producing 5-HPETE and 12-HPETE. 5-HPETE is the labile precursor of the leukotrienes and it undergoes enzymatic conversion into leukotrienes A4, C4, D4 and E4 (Piper 1984; Stenson & Parker 1984). Furthermore, Ogburn *et al.* (1984) have presented some data suggesting that the production of lipoxygenase products, including 12-HPETE is elevated in PIH.

## 1.5 Genetic Factors and HLA system

The discovery of the occurrence of PIH to be familial suggests that susceptibility to PIH may be inherited. There is, therefore, an important genetic component in the disease. However, the exact mode of inheritance has not yet been understood. In principle, susceptibity could be controlled by the maternal genetype alone, or the fetal genotype alone, or by a genotype-by-genotype interaction between the mother and her fetus.

Most of the simple pedigree data point to a major role for the single maternal genotype in the recessive inheritance. Sutherland *et al.* (1981) compared the frequency of PIH in mothers versus mothers-in-law of PIH index cases and found that the incidence of severe PIH was much higher among the former than the latter. Subseqently, Chesley and Cooper (1986) studied the incidence of PIH in 147 sisters, 248 daughters, 74 granddaughters, and 131 daughters-in-law of women who had eclampsia. The authors observed an increased frequency of PIH in the daughters and granddaughters, but not in daughters-in-law, of women who themselves had a history of eclampsia.

Some evidence does suggest a fetal genetic contribution to PIH as shown in the following references.

1. Discordance of PIH in identical twin sisters has been found (Thornton & Sampson 1990).
2. There is a higher incidence of PIH with male fetuses and in pregnancies conceived by partners of dissimiliar race (Alderman *et al.* 1986; James 1987).
3. The fetal chromosomal abnormalities of triploidy and trisomy 13 can be associated with PIH (Toaff & Peyser 1976; Bower *et al.* 1987; Feinberg *et al.* 1991).
4. It has been reported that there are various kinds of association between susceptibility and HLA types and this also implicates the fetal genetic contribution (Redman *et al.* 1978; Kilpatrick *et al.* 1987 & 1989a).

Arngrimsson *et al.*(1990) postulated that a dominant gene with incomplete penetration may contribute to the development of PIH. It now seems likely that susceptibility depends on a combination of maternal and fetal genotypes involving a single recessive gene shared by mother and fetus. This gene may be associated with HLA-DR4 (Kilpatrick *et al.* 1989a), but the association needs to be further confirmed (Wilton *et al.* 1990).

HLA may be a marker for a closely linked gene (or genes) that predispose to or cause PIH and account for its familial incidence. It has been reported that there is an association between the presence of HLA-DR4 and increased incidence of PIH ( Simon *et al.* 1988; Kilpatrick *et al.* 1989a). This finding suggests that the genetic susceptibility to PIH is associated with HLA-DR4. The association probably also indicates an underlying tendency to autoimmune disease with which HLA-DR4 is linked. However, subsequent studies by others have make this association remain in some doubt (Hoff *et al.* 1990; Wilton *et al.* 1990). It has also been suggested that there is a defective genetic coding for normal placental prostacyclin and/or thromboxane production in women with PIH from the time of conception (Walsh 1990).

## 1.6 Placental Factors

The facts that the symptoms and lesions of PIH disappear soon after termination of pregnancy (Chesley 1978; Roberts 1984) and that the disease can occur in abdominal and molar (without a fetus) pregnancy (Page 1939; Rote 1985) suggest that uterine and fetal factors are not required and that the placenta is a very important component and can provide key information relating to the pathogenesis of PIH.

During the establishment of uteroplacental maternal circulation, the denervation of spiral arteries and the production of eicosanoids take place. The uterine vasculature is richly supplied with adrenergic nerves (Owman *et al.* 1967), so erosion of the spiral arteries in normal pregnancy decreases the uterine content of sympathetic neurotransmitters, producing a functional denervation (Thorbert *et al.* 1979; O'Shaughnessy *et al.* 1983; Robertson & Khong 1987). At the same time the inner lining, endovascular trophoblast and endothelium, of these so-called uteroplacental arteries, produce vasodilating prostaglandins (Friedman 1988) and perhaps other vasodilating autocoids, such as endothelium-derived relaxing factor (EDRF) (Klebanoff 1988; Hubel *et al.* 1989). As a result of these morphologic and physiologic changes, the uteroplacental blood vessels have markedly decreased vascular tone and are maximally dilated. Consequently a low-resistance, low-pressure, high flow syspem is formed. Immunological mechanisms are also believed to involved in the development of poor placentation in PIH (see Section 1.2.2).

In PIH, the maladaptation at the maternal-placental interface results in the trophoblast failing to destroy the spiral arteries and results in placentation with poor uteroplacental circulation, which in turn leads to placental ischemia. Histopathologic and epidemiologic data have shown a decrease in uteroplacental blood flow in PIH (Dixon *et al.* 1963, Kaar *et al.* 1980, Lunell *et al.* 1984). In addition, experimental animal models of PIH also indicated that reduction of placental and uterine perfusion was a common feature (Chesley 1978). Many of the ultrastructural changes of PIH placental tissue closely resemble alterations seen in placental tissue when it is placed in hypoxic organ culture (Tominaga & Page 1966; Fox 1970). The poor placentation has been correlated with the increased incidence of placental infarction, fetal distress and fetal growth retardation that often accompany PIH (Chesley 1978; Ramsey & Donna 1980; Gerretsen *et al.* 1983; Sibai 1984; Redman 1987). Therefore, fundamental defects of placentation and uteroplacental vascular development in PIH may relate to poor perfusion and placental hypoxia, which are the earliest and most consistent changes in PIH (Chesley 1978; Roberts *et al.* 1989).

Placental hypoxia is thought to cause an increased production of ROS ( Klebanoff 1988; Hubel *et al.* 1989; Roberts *et al.* 1990). ROS, which are increased in PIH (Ishihara 1978; Maseki *et al.* 1981; Wickens *et al.* 1981; Dekker & Kraagenbrink 1991; Wang *et al.* 1992), have several adverse effects which set in motion a dysfunction cascade of coagulation, vasoconstriction, and intravascular fluid redistribution that results in the clinical syndrome of PIH (see Section 1.3).

The precise cause of poor placentation and the effects of poor placentation on the pathogenesis of disease are still not completely understood, but there is enough evidence to regard poor placentation as a final common step resulting in PIH. PIH is primarily a placental disease.

Several lines of studies have suggested that human placental cells have an immunoregulative function by the production of cytokines and other immunological substances (Wolf 1988; Pockley & Bolton 1990; Kettel *et al.* 1991; Vince *et al.* 1992) (see Section 1.2.3). Considering that ROS are able to damage cell membranes (Hubel *et al.* 1989; Croustein 1991) and they have an important role in the immune response (Gallaghen & Curtis 1984; Fidelus 1988; Sekkat *et al.* 1988), placental hypoxia which leads to production of ROS will undoubtedly affect the immune system, at least the local immune response in maternal-placental interface. Although the research on the immunolocical changes at the local maternal-placental interface in PIH is limited, an exciting discovery by Greer and his coworkers (1990) has shown that neutrophil activation in PIH is localized in part to the placental bed. Activated neutrophils are capable of destroying the integrity of endothelial cells, vascular basement membranes and subendothelial matrix by releasing neutrophil granules such as elastase and other proteases (Harlan 1987; Greer *et al.* 1990). In addition, activation of neutrophils also produce ROS and leukotrienes, both of which may contribute to the necrotizing arteriopathy seen in PIH (Sacks *et al.* 1978; Weiss *et al.* 1981; Pelusi *et al.* 1990).

# CHAPTER TWO IMMUNOLOGICAL FACTORS

## 2.1 Introduction

### 2.1.1 Why are immune mechanisms involved in the pathogenesis of PIH

Immunological components may play a role in the development of pregnancy-induced hypertension (PIH). This conclusion arises from the following facts:

1, The greater the exposure to semen (an allogeneic antigen for women), the less the likelihood that PIH will occur (Klonoff-Cohen *et al.* 1989).

2, PIH is most likely to arise in a woman who changes partners (Feeney 1980).

3, Previous blood transfusion protects against PIH development (Feeney *et al.* 1977).

### 2.1.2 Cell-mediated immunity

Immunological reactions are conveniently divided into two mechanistically different types: humoral (or antibody-mediated), and cellular (or cell-mediated) immunity. The former type is mediated by specific antibodies and the latter by immune lymphocytes. There are two lymphocyte populations: T lymphocytes, responsible for cell-mediated immunity and B lymphocytes, concerned in the synthesis of circulating antibodies. Approximately 80% of normal blood lymphocytes are T cells. They emerge from a period of development and differentiation in the thymus as cells capable of 1), undergoing blast transformation in response to certain mitogenic substances; 2), producing many soluble mediators of immune reactivity; 3), serving as memory cells of previous antigenic exposure; 4), being helper and suppressor cells in many B-cell responses; and 4), having the special capacity to kill foreign cells through direct cytotoxic mechanisms (Roitt *et al.* 1989).

There are a number of publications describing the changes in cell-mediated immunity in PIH. Moore *et al.* (1983) studied the various lymphocyte subsets and showed a significant increase in helper T cells. However, others found a significantly lower proportion of helper T cells (Bardeguez *et al.* 1991) or a proportional decrease in both helper and suppressor T cells (Sridama *et al.* 1983). Although the findings of T cell subset analysis are inconsistent, the results do suggest that abnormalties of T cell function might exist.

### 2.1.3 Immunoglobulins

Antibody activity is associated with the classical γ-globulin fraction of serum. With the recognition of heterogeneity in the types of molecules which can function as antibodies, it has now become customary to use the general term 'immunoglobulin'. In each species, the immunoglobulin (Ig) molecules can be subdivided into different classes on the basis of the structure of their 'backbone' (rather than on their specificity for given antigens). Thus, five major structural types or classes in the human can be distinguished: IgG, IgM, IgA, IgD and IgE. The ability to secrete Igs is the distinguishing functional characteristic of B lymphocytes. Therefore, B lymphocyte function or activity can be monitored by its ability to produce and secrete Igs.

Clinical autoimmune phenomena in patients with PIH are manifested by production of autoantibodies toward lymphocytes, endothelial cells, trophoblast antigens and placental and renal antigens (Rappaport *et al.* 1990; Sibai 1991). As stated above, the main function of B lymphocytes is to produce antibodies (Igs). The appearance of these abnormal antibodies suggests that it is very likely that B cell function or activity is altered. So far, the production of Igs from lymphocytes has not yet been studied in patients with PIH.

### 2.1.4 Cytokines

There are two ways in which T lymphocytes can influence the activities of other cells. One involves cell contact, recognition and the subsequent destruction by T lymphocytes with cytotoxic properties. The other, more versatile way in which T lymphocytes communicate and influence other cells is by the production and secretion of a variety of potent mediator molecules, termed lymphokines or more generally 'cytokines'. T lymphocytes are a particularly rich source of cytokines. At the last count they are able to produce over 10 cloned products, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-9, IL-10, IL-13, TNF, IFN, GM-CSF and TGFβ. It appears that the majority of T cell effects are mediated by the production of cytokines.

As stated in Sections 2.1.2 and 2.1.3, PIH might be associated with T and B lymphocyte abnormalties. If this is true, one possible mechanism involved is the roles of cytokines. Among cytokines, IL-2 acts as an activating factor and a growth factor for T, B and NK (natural killer) cells (Smith 1984). Studies on IL-2 acitvity

have been carried out in several kinds of diseases associated with T and B lymphocyte disorders (Huang *et al.* 1988; Kahaleh & LeRoy 1989). However, this issue has not been investigated in PIH previously.

### 2.1.5 Cytokines and prostaglandins

The generation of immune responses involves a complex series of cellular events, each of which may be regulated by antigen-specific and nonspecific signals. One of the antigen nonspecific immunoregulatory mechanisms results from the action of prostaglandins. The effects of prostaglandins on T lymphocyte function and cytokines have been well studied (Kingston *et al.* 1985; Hwang 1989). Conversely, there is only limited information on the role of cytokines in the production of prostaglandins especially PGI2 and TXA2, which play an important role in the development of PIH (Friedman 1988; Walsh 1990). Increasing our understanding of the interaction between cytokines and prostaglandins (PGI2 and TXA2) could help us to understand how and why PIH develops.

### 2.1.6 Aims of the study

The aims of the study were to determine the following parameters in women with PIH and normotensive pregnant women.

1, T lymphocyte function (mitogenic activity).
2, B lymphocyte function (IgG and IgM production).
3, IL-2 production from PBMC (peripheral blood mononuclear cells).
4, The effects of IL-1, IL-2 and TNF on the production of PGI2 and TXA2 from PBMC.

## 2.2 Materials and Methods

### 2.2.1 Study Population

Three groups of subjects were included in the study of T and B lymphocyte function and IL-2 production. 1, 37 normotensive pregnant women; 2, 19 PIH women without proteinuria; 3, 9 PIH women with proteinuria. None of the pregnant women were taking any anti-hypertensive drugs before sampling. Clinical data is shown in Table 2.1.

**Table 2.1 Demographic Characteristics of Study Population**

| Group | N | Age (year) | Gestational Age (weeks) | Diastolic Blood Pressure (mmHg) |
|---|---|---|---|---|
| Normotensive Pregnancy | 37 | 27.9±4.9 | 35.2±3.7 | 73.1±9.5 (n=24) |
| PIH without Proteinuria | 19 | 26.5±4.3 | 35.2±3.7 | 93.9±4.7* |
| PIH with Proteinuria | 9 | 25.8±4.1 | 35.4±5.2 | 110.0±9.3 |

All data were expressed as mean ± SD.

*$p<0.001$ versus PIH with proteinuria.

The study of the effect of cytokines (IL-1β, IL-2 and TNFα) was carried out in 7 normal non-pregnant women.

PIH was defined by the standard recommended by the American College of Obstetricians and Gynecologists (For details see Chapter 1).

### 2.2.2 Major reagents

Fetal calf serum (FCS) was supplied by Northumbria Biologicals Ltd., Cramlington, UK. PBS and lymphoprep™ were purchased from Oxoid Limited, England and Nyegaard and Co., Oslo, Norway respectively. RPMI 1640 (10x) was obtained from Gibco, BRL., UK. $^3$H-thymidine (TRK418, specific activity 40-60 Ci/mM) was supplied from Amersham International plc., UK.

Three distinct mitogens were used in study of mitogenic lymphocyte activity, phytohaemagglutinin (PHA), Concanavalin A (ConA, Type IV) and pokeweed mitogen (PWM) and all of them were purchased from Sigma Chemical Company Ltd., UK. PWM for Ig production was obtained from Gibco Ltd., UK and PHA for IL-2 production from Wellcome Diagnostics, UK. Human IgG/IgM, anti-human IgG/IgM, anti-human IgG (γ-chain specific)/IgM (μ-chain specific) alkaline phosphatase conjugate and PNP were from Sigma Chemical Company, USA.

Purified recombinant human TNFα and human IL-1β were products of Cistron Biotechnology, Pine Brook, NJ07058, USA. TNFα had specific bioactivity of 5.6 x $10^7$ Units of TNFα per mg of protein. The specific activity of IL-1β is 1 unit per nanogram of IL–1β. Human IL-2, with specific activity of $1x10^6$ U/mg, was obtained from Cell Biology Boehringer Mannheim GmBH, Germany. 1 unit is defined as the amount of IL-2 that is required to support half-maximal $^3$H-thymidine incorporation into CTLL-6 cells.

The enzyme immunoassy (EIA) systems for TXB2 and 6-keto-PGF1α (the stable metabolites of TXA2 and PGI2, respectively) were purchased from Amersham International plc., Amersham, U.K.

### 2.2.3 Culture medium and mitogen preparation

The culture medium (completed RPMI 1640) was prepared according to the manufacturers instructions. Distilled/deionised water ($dH_2O$) was autoclaved for 20

minutes at a temperature of 120 °C and a pressure of 1.2/cm$^2$. The $dH_2O$ was allowed to cool before the RPMI 1640 (10x) was added. Sodium bicarbonate (7.5%, Gibco, BRL) was added to give a final concentration of 2 g/l where it is to act as a $CO_2$ buffer as well as a nutrient or to give a final concentration of 0.85 g/l where N-[2-Hydroxyethl] piperazine-N-[2 ethanesulphonic] acid (HEPES) was the $CO_2$ buffer of choice. HEPES (1M, Gibco, BRL) was added to the media to give the concentration of choice (usually 10 mM). Finally the various supplements as detailed elsewhere in the text, were added prior to use. The bottle of medium was then adjusted to the correct pH (between pH 7.2 and pH 7.4) by addition of either HCl or NaOH. Glutamine (200 mM) was added to the bottle of medium immediately prior to use. The reason for this is that glutamine has a half-life in the order of 19 days at 4°C. Therefore it is important to replenish the glutamine lost. After 7 days at 4 °C only 80% of the original glutamine will remain. It is for this reason that glutamine was added every week to bottles of media in use for longer than a week to make up for this shortfall. Glutamine is an essential nutrient and energy source for the growing cell. Stock glutamine was for this reason stored at -20°C.

PHA (for lymphocyte mitogenic activity) was resuspended in sterile distilled water to give a stock solution of 1000 µg/ml. PHA stock was aliquoted and stored at -20 °C. Prior to use an aliquot of stock PHA was further diluted in completed RPMI 1640 to give a working concentration of 10 µg/ml (a 100-fold dilution).

ConA was resuspended in sterile distilled water to give a stock solution of 5 mg/ml. ConA stock was aliquoted and stored at -20 °C. Before use an aliquot of the stock was further diluted in completed RPMI 1640 to produce a working stock of 100 µg/ml (a 50-fold dilution).

PWM (for lymphocyte mitogenic activity) was prepared at a concentration of 50 µg/ml in completed RPMI 1640, aliquoted and stored at -20 °C. Prior to use an aliquot of the stock was further diluted to give a working concentration of 0.2 µg/ml (a 250-fold dilution) in completed RPMI 1640.

PWM (for Ig production) was made up as the manufacturers instructionson by adding 10 ml of PBS. This solution was then aliquoted and stored at -20 °C. Prior to use an aliquot of the stock was diluted in completed RPMI 1640 containing 20% FCS at 1:250 as the working concentration.

PHA (for IL-2 production) was resuspended in sterile distilled water to give a stock solution of 1000 μg/ml. PHA stock was aliquoted and stored at -20 °C. Prior to use an aliquot of stock PHA was further diluted in completed RPMI 1640 to give a working concentration of 2 μg/ml

### 2.2.4 PBMC preparation

Venous blood was added as aseptically as possible to a universal container with preservative-free heparin such that the final concentration was 30 units/ml. PBMC were separated by density gradient centrifugation over lymphoprep™, washed three times in PBS and resuspended in 1 ml RPMI 1640 media. The cells were counted and adjusted to the required concentration.

### 2.2.5 Lymphocyte mitogenic activity assay

The lymphocyte culture was set up in 96 well, U-shaped microtiter plates (Greiner Labortechnik Ltd., UK). All cultures were set up in triplicate and in a final volume of 200 μl. Each well contains 100 μl of cells and 100 μl of mitogen (in control wells RPMI 1640 instead of mitogen). Cells were used at the concentration of 1 $x10^6$ cells/ml for PHA and ConA stimulation. When PWM was the mitogen, the cells were resuspended to the concentration of 2 x $10^6$ cells/ml. The culture were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$/air for a total of 72 hours. All mitogens were used at optimal concentrations (Wilson *et al.* 1989).

The most commonly used method of detecting lymphocyte activation is the incorporation of $^3$H-thymidine into DNA, which correlates well with the number of lymphocytes in the S-phase of the cell cycle. The working stock contains 50 μCi $^3$H-thymidine, 50 μM 'cold' thymidine (stock 5 mM) (Sigma Chemical Company, UK) and completed RPMI 1640. The equivalent of 1 μCi was added to each well (20 μl) at the time of completing 68-hour incubation. The cultures were incubated in the presence of the label for further four hours.

After 72 hour incubation, the microtiter plates were harvested on a Titertek Cell Harvester (Flow Laboratories). The cell harvester transfers the cells from the wells to clearly defined discs on a glass-fibre filter mat (Skatron Ltd., UK) and unbound $^3$H-thymidine passes through the filter into the reservoir. The discs containing the radioactivity were removed from the filter mats and placed in clearly labelled minivials (Pony Vials, Canberra Packard International S.A., Switzerland) and

allowed to dry. The scintillation fluid was prepared by added 4 grams of 2.5 diphenyloxazole (PPO, Scintillation Grade, United Technologies Packard, UK) to one litre of methylbenzene (Pronalys A.R. (toluene), May & Baker, UK). Three mls of scintillation fluid were added to each vial, the vials were then counted on a LKB 1216 Rackbeta II liquid scintillation counter (Phamacia, UK). The counter was programmed to measure low energy beta emissions. The results were expressed as cpm.

### 2.2.6 Ig production and ELISA for IgG and IgM

Ig production from lymphocyte was set up in 24 well microtiter plates (Greiner Labortechnik Ltd., UK). All cultures were set up duplicate wells and at a final volume of 2 ml. Each well contains 1 ml of cells (1 x $10^6$) and 1 ml of PWM (1:250 dilution). Control wells consisted of cells and culture media which replace PWM. This control was set up for each sample to account for spontaneous release of Igs. The plate was incubated at 37°C in a humidified atmosphere of 5% $CO_2$/air for a total of 7 days. After culture, the medium was pipetted off into 10 ml plastic conical tubes and centrifugated at about 700 x g for 10 minutes. The supertanants were obtained and stored at -20°C until assay.

The amount of IgG and IgM was determined by ELISA (Wilson *et al.* 1988). A 96 well microtitre plate was coated with 200 µl (1:1000 dilution) goat anti-human IgG/IgM and incubated for one hour at 37°C. The plate was then washed four times with washing buffer (0.5% BSA, 0.05% Na Azide and 0.05% Tween 20 in PBS), using a plate washing machine (Ultrawash II, Dynatech Laboratories Ltd., England). 200 µl of diluted buffer (0.5% BSA and 0.05% Na Azide in PBS) was then added and incubated for further 30 minutes at 37°C. The plate was then shaken out and blotted dry. 100 µl samples and standard IgG/IgM (3.125, 6.25, 12.5, 25, 50, 100 and 200 ng/ml) were added to the plate and incubated for two hours at 37°C. The plate was washed four times as before. 100 µl (1:1000 dilution) of the anti-human IgG/IgM conjugate was now added to the plate and incubated for another two hours at 37°C. The plate was washed four times again as before. 100 µl of PNP substrate was added to the plate and incubated for final 30 minutes at 37°C. The substrate was prepared beforehand by dissolving 4 tablets (5 mg/tablet) of P-nitrophenyl phosphate substrate (PNP) in 20 ml substrate buffer (2.2 ml of 0.2 M $Na_2CO_3$, 2.8 ml of 0.2 M $NaHCO_3$, 2.0 ml of 0.01M $MgCO_2$, 14 ml of $dH_2O$). The reaction was stopped by adding 100 µl of 3 M NaOH. The plate was now read at 490 nm on a Dynatch MR 700 microplate reader (Dynatech Laboratories Ltd., England).

### 2.2.7 IL-2 production and assay

The method for IL-2 production and assay has previously been described by Wilson et al. *(1989)* and Smith *et al.* (1990).

The lymphocyte culture for IL-2 production was set up in 24 well microtiter plates (Greiner Labortechnik Ltd., UK). The culture system was carried out at a final volume of 1 ml completed RPMI 1640 with 4% FCS. Each well contains 4 x $10^6$ cells and 2 μg of PHA. The control well for each sample was set up to examine the spontaneous release of IL-2 and therefore it contained cells without PHA. The plate was incubated at 37 °C in a humidified atmosphere of 5% $CO_2$/air for a total of 48 hours. After incubation, the culture medium was centrifuged at 700 x g for 10 minutes. The supernatants were obtained and stored at -20°C until assay.

The IL-2 assay is a bioassay which relies on measuring the increase in proliferation of a dependent cell line, HT-2 cells. This cell line is derived from murine T-helper cells and requires IL-2 for continuous growth. HT-2 cells are maintained in RPMI 1640 containing 10% FCS and 2 x $10^{-5}$M 2-mercaptoethanol (conditional medium). Cell cultures are fed every 48 hours with the conditional medium, this corresponds to a concentration of approximately 20 international units of IL-2 per ml (Cell Biology Boehringer Mannheim GmBH, Germany). After two days when the cell density is approximately 2 x $10^5$ cells, cultures are split to 2 x$10^4$ cells per ml and re-fed with IL-2.

The protocol for bioassay for IL-2 and calculation of unitage is as follows:

1, Harvest the HT-2 cells 2 or 3 days after feeding with IL-2. Wash the cells twice by centrifugation in RPMI 1640.

2, Determine viability of the cells by Trypan Blue dye exclusion (cells should be > 80% viable) and resuspend cells to a final concentration of 1.5 x $10^5$ cells/ml in RPMI 1640 containing 20% FCS

3, Titrate the IL-2 standard in triplicate in a 96 well microtitre plate. Start the titration at 100 Unit/ml IL-2 and then make serial twofold dilutions down to 0.195 unit/ml IL-2. Prepare dilutions of the samples (twofold serial dilutions) in triplicate. The negative control is culture medium without IL-2. Each well should contain a volume of 100 μl.

4, Add 100 μl of the cell suspension to each well and incubate the plates for 20 hours at 37 °C in a humidified $CO_2$ incubator.

5, Add 1 μCi of tritiated thymidine to each well and return the plates to the incubator for further 4 hours.

6, Harvest the contents of each well onto filter mats, using MicroMate$^{TM}$ 196 Cell Harvester (Packard Instrument Company, CT, USA), and determine the radioactivity incorporated into DNA by a computer-programmed Matrix 96$^{TM}$ Direct Beta Counter (Packard Instrument Company, CT, USA).

7, The raw data for proliferation (cpm) is first transformed to a percentage of maximum counts. A rough estimate of relative potency can be obtained for comparing the dilution at which 50% maximum counts are obtained for each sample.

8, Transformed counts are then converted to log values, as are the dilutions for each sample, and plotted graphically.

9, The unitage of each sample, relative to the standard, is calculated from the distance between the straight line portions for each sample. These straight lines should be parallel if the molecule responsible for the activity in samples/standards is the same.

### 2.2.8 Cell culture for the effects of cytokines on prostaglandin production and EIA for PGI2 and TXA2

To assess the effects of cytokines on TXB2 and PGI2 biosynthesis, 2 x $10^5$ PBMC were placed in 96 well, U-shaped microtitre plates (Greiner Labortechnik Ltd., UK) and incubated at 37 °C in a humidified atmosphere of 5% $CO_2$/air for 30 minutes to 24 hours with the specified cytokine dilution. After incubation, the supernatants were obtained and stored at -20°C until assay.

Control samples consisted of PBMC processed in the same manner as those treated with cytokines. Controls were set up at each time point to account for spontaneous release of TXA2 and PGI2. The culture medium was tested for interference with the assay system and found to contain 6-keto-PGF1α and TXB2 concentrations of less than 5% and 4% respectively of the concentrations detected in supernatants of the background PBMC culture.

The levels of 6-keto-PGF1α and TXB2 in supernatants were determined by the EIA method and performed as outlined in the protocol supplied with the system (for details see Chapter 4). Briefly, 50 μl of sample was added to a 96-well plate which was coated with donkey anti-rabbit IgG and then 50 μl of rabbit anti-6-keto-PGF1α or rabbit anti-TXB2 was added. After incubation by shaking, the samples were

reacted with 6-keto-PGF1α- or TXB2-horseradish peroxidase for 1 hour. The wells were washed four times with washing buffer by a plate washing machine (Ultrawash II, Dynatech Laboratories Ltd., England) and then 150 µl enzyme substrate (TMB) dispensed into the wells. The reaction was stopped by 1.0 M sulphuric acid and read at 450 nm on a Dynatch MR 700 microplate reader (Dynatech Laboratories Ltd., England) within 30 minutes. The assay sensitivity, defined as the amount of TXB2 or 6-keto-PGF1a needed to reduce zero dose binding by two standard deviations was 3.6 pg/ml and 3.0 pg/ml respectively. The intra and inter-assay variations for TXB2 assays were 2.5% and 9.9% respectively and for 6-keto-PGF1α 4.5% and 14.8%.

### 2.2.9 Statistics

Data for prostaglandins are expressed as medians and ranges since prostaglandin levels are not normally distributed (Moodley *et al.* 1984). Kruskal-Wallis analysis was performed to determine whether comparisons among groups were valid and differences between groups were analyzed for by Mann-Whitney test. Other data are expressed as mean±SD. Differences between groups were tested for by Student's *t* test. All statistical analyses were done by using Minitab Statistical Software. A p value of less than 0.05 was considered to indicate statistical significance.

## 2.3 Results

### 2.3.1 Lymphocyte mitogenic activity

Figure 2.1 shows mitogenic activity. In the absence of mitogenic stimulation $^{3}H$ thymidine uptake was significantly greater in PBMC from PIH women without proteinuria compared to normotensive pregnant women and PIH women with proteinuria (Figure 2.1D). There was no significant difference between the latter two. In the presence of mitogenic stimulation, PBMC from patients suffering from PIH with or without proteinuria showed enhanced $^{3}H$ thymidine uptake when stimulated by PHA (Figure 2.1A) but not by ConA (Figure 2.1B) and PWM (Figure 2.1C) compared to normotensive pregnant women. $^{3}H$ thymidine uptake in stimulated PBMC of PIH did not differ significantly between women with and without proteinuria (Figure 2.1A to 2.1C).

# Figure 2.1. Lymphocyte mitogenic activity

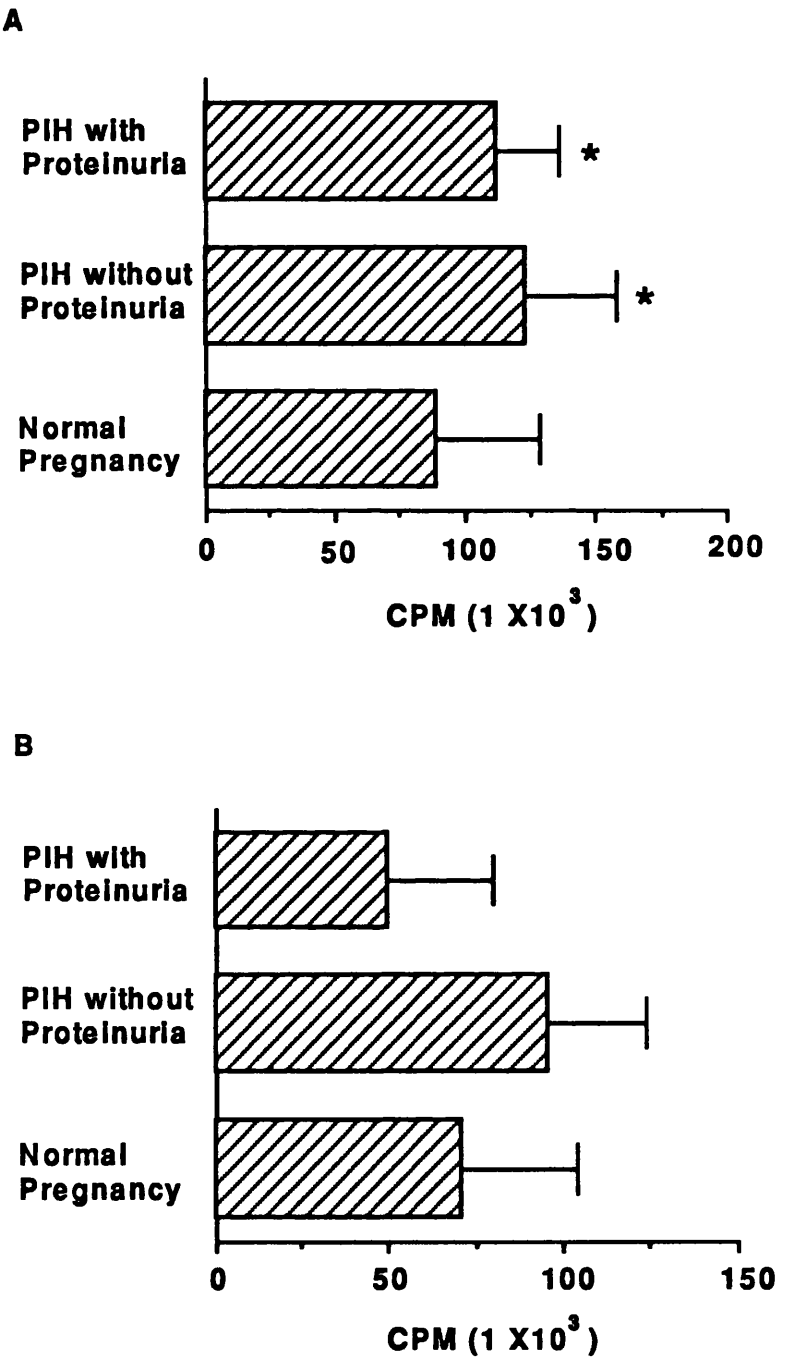


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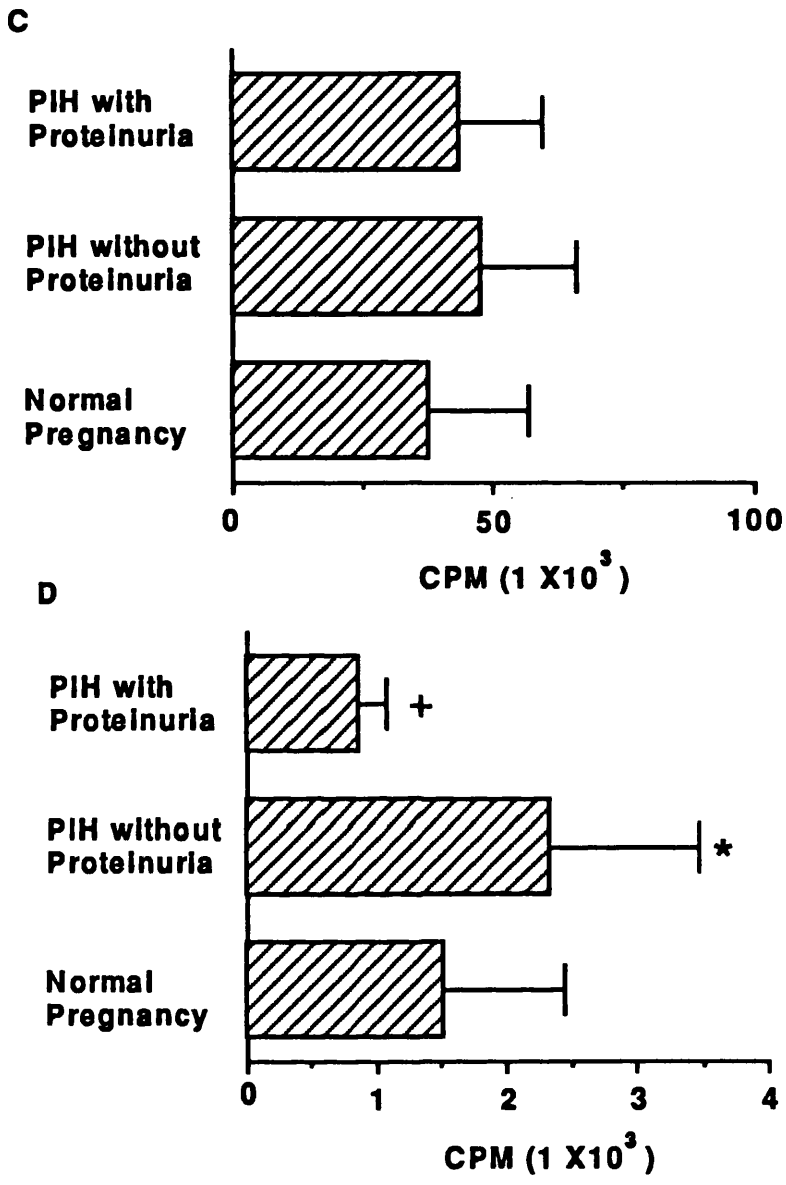
*Figure 2.1. continuing*




PMBC were incubated at 37°C for 72 hours in the presence of mitogens (A: PHA; B: ConA; C: PWM) or in the absence of mitogens (D). At the time of 68 hour incubation, $^3$H-thymidine was added. The incorporation of $^3$H-thymidine into DNA was measured after incubation. The data are represented as mean of samples with standard deviation. Statistical significance is given by asterisks and crosses: *$p<0.05$ compared with normal pregnant women; +$p<0.05$ compared with PIH women without proteinuria.

### 2.3.2 IgG and IgM Production

The amount of immunoglobulins (IgG and IgM) produced by PWM mitogen-stimulated PMBC is shown in Figure 2.2. Compared with normotensive pregnant women, IgG production was significantly raised in PIH women with proteinuria, but not in those without proteinuria. There was no significant difference in IgG production between PIH women with proteinuria and those without proteinuria. IgM production did not differ significantly amongst the three tested groups.

### 2.3.3 IL-2 activity

The results are shown in Figure 2.3. IL-2 activity was significantly higher in PIH patients with proteinuria than normal pregnant women ($p<0.01$) and PIH patients without proteinuria ($p<0.05$). There was no significant increase in IL-2 production in PIH patients without proteinuria ($p=0.087$).

### 2.3.4 Effects of TNFα on 6-keto-PGF1α and TXB2 production

The results are illustrated in Figure 2.4. The effects of various concentrations of TNFα on 6-keto-PGF1α and TXB2 production were studied in four PBMC preparations. The effects of TNFα on 6-keto-PGF1α and TXB2 generation were tested at various doses of TNFα (0.2 to 20 ng/ml) and in all cases a bell-shaped dose response curve were obtained. PBMC synthesized the largest amount of 6-keto-PGF1α (mean: 544, range: 506-618 pg per $10^6$ cells) at 2 ng/ml of TNFα and TXB2 (mean: 694, range: 652-768 pg per $10^6$ cells) at 0.2 ng/ml of TNFα. It was found that PBMC produced a mean of 189 (range: 159-225) pg 6-keto-PGF1α per $10^6$ cells in the conditioned media of the control sample and a mean of 420 (range: 398-440) pg 6-keto-PGF1α per $10^6$ cells in the tested medium treated with 0.2 ng/ml of TNFα, representing a 2.2-fold enhancement whereas PBMC produced a mean of 544 (range: 506-618) pg TXB2 per $10^6$ cells in the conditional media of sample control and a mean of 694 (range: 652-768) pg TXB2 per $10^6$ cells in the tested medium with 0.2 ng/ml of TNFα representing only 1.3-fold enhancement, which was much less than that of 6-keto-PGF1α ($p<0.05$). TNFα at the concentration of 20 ng/ml inhibited both 6-keto-PGF1α and TXB2 generation from PBMC.

**Figure 2.2. B lymphocye function (Ig production)**

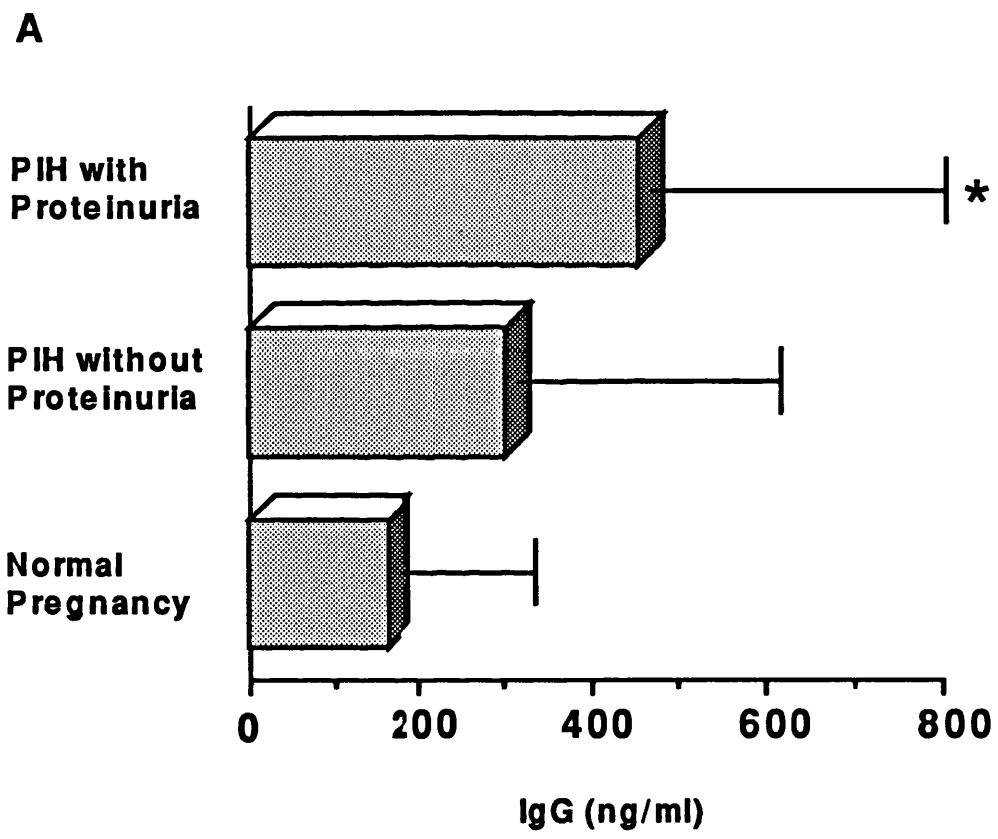



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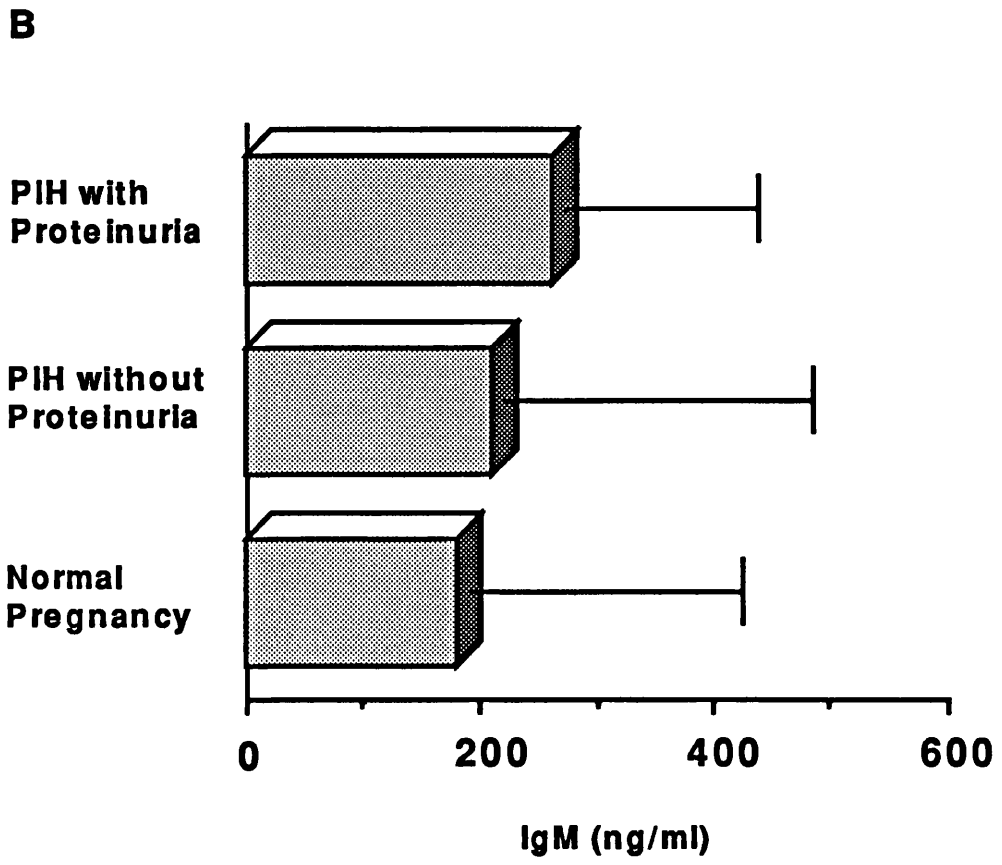
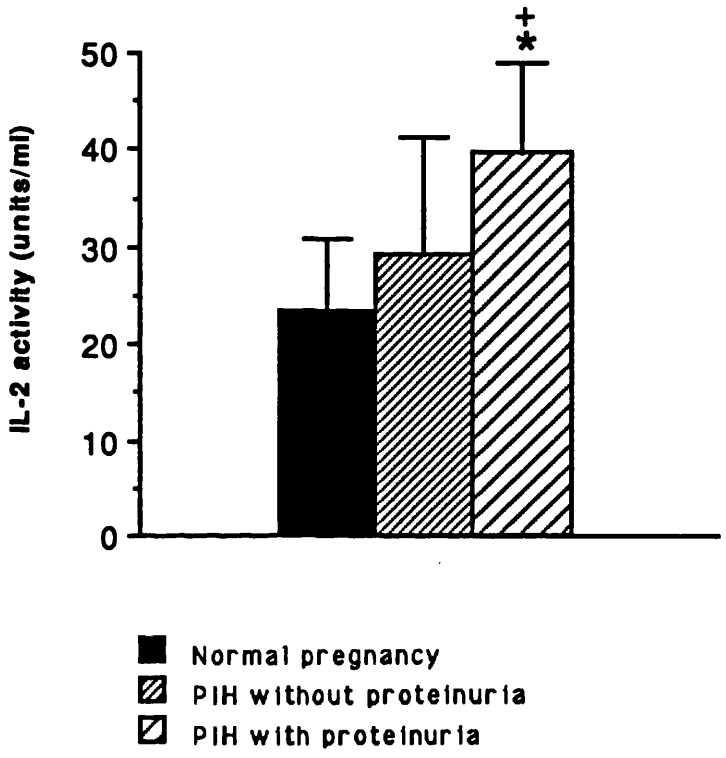
*Figure 2.2. continuing*



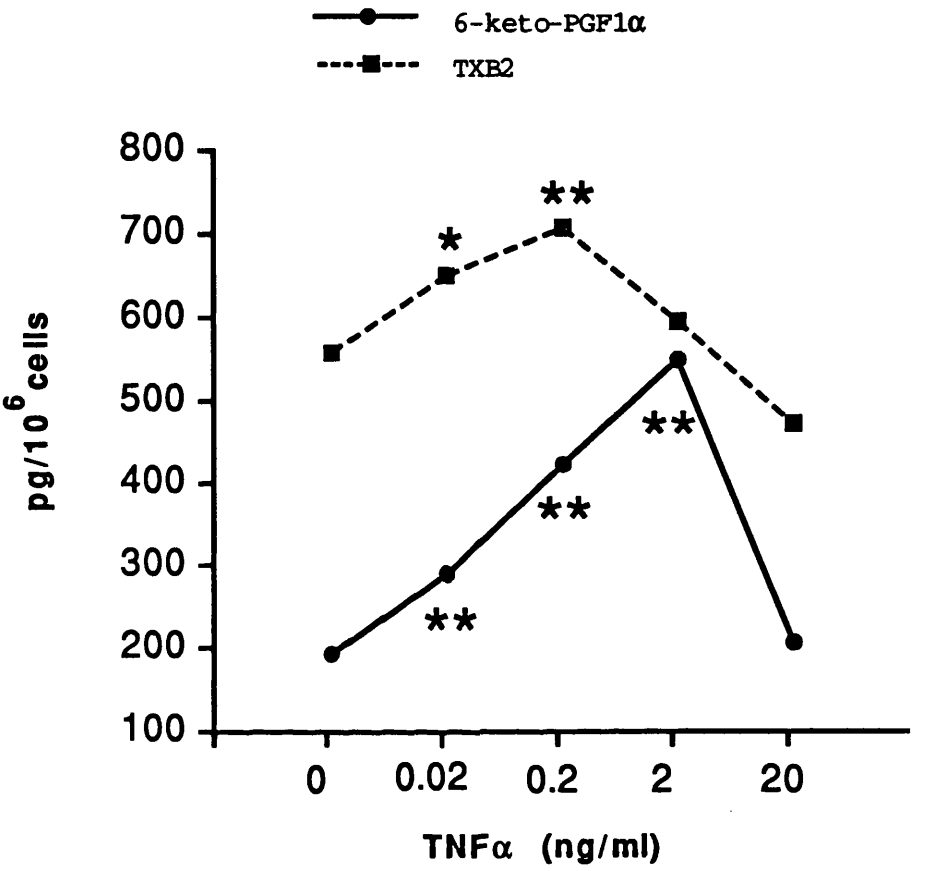

PMBC were incubated at 37°C for 7 days at the presence of PWM and culture supernatants were obtained for the determination of Igs. IgG and IgM were measured by ELISA. The data are represented as mean of samples with standard deviation. Statistical significance is given by asterisks: *$p<0.05$ compared with normal pregnant women.

## Figure 2.3 IL-2 activity




IL-2 containing supernatants were obtained by culturing PBMC in the presence of PHA for 48 hours. IL-2 activity was determined by a bioassay using HT-2 cells as IL-dependent cell lines. The data are represented as mean of samples with standard deviation. Statistical significance is given by asterisk: *$p<0.01$ compared with normal pregnant women, +$p<0.05$ compared with PIH patients without proteinuria.

## Figure 2.4. Effects of TNFa on 6-keto-PGF1a and TXB2 production




PBMC were incubated with varying concentrations of TNFα for 24 hours. The levels of 6-keto-PGF1α and TXB2 in the conditional media were determined by EIA after incubation. Each point represents median of samples (n=4). Statistical significance of a point versus no TNFα is given by asterisks, *p<0.05, **p<0.01. TNFα significantly affects both 6-keto-PGF1α and TXB2 synthesis, both p<0.01, Kruskal-Wallis analysis for the effects of different concentrations of TNFα.

### 2.3.5 Effects of IL-1β and IL-2 on 6-keto-PGF1α and TXB2 production

The production of 6-keto-PGF1α from PBMC was increased by both IL-1β and IL-2. IL-1β significantly enhanced 6-keto-PGF1α production at the concentration ≥ 0.025 ng/ml (Figure 2.5A). IL-2 appeared to be less effective, as 25 ng/ml of IL-2 was required to produce a significant increase in 6-keto-PGF1α production. IL-2 had no effects on TXB2 production although IL-1β significantly increased its production at the concentrations ≥ 0.25 ng/ml (Figure 2.5B).

### 2.3.6 The time course study of 6-keto-PGF1α and TXB2 production induced by TNFα, IL-1β and IL-2

Generally, enhancement of both 6-keto-PGF1α and TXB2 by the three cytokines was time-related. It was noted that the stimulation by TNFα reached a significant increase after only a 30-minute incubation. Both IL-1β and IL-2 also increased 6-keto-PGF1α and TXB2 production but this did not reach a statistical significance until 8 hours after the start of the incubation (Figure 2.6).

### 2.3.7 The ratio of TXB2 to 6-keto-PGF1α

Figures 2.7 shows the changes in the ratio of TXB2 to 6-keto-PGF1α during the formation of prostaglandins stimulated by TNFα, IL-1β and IL-2 respectively. Apart from TNFα at the concentration of 20 ng/ml (Figure 2.7A), the ratio of TXA2 to PGI2 gradually decreased in PBMC treated with cytokines (TNFα: Figure 2.7A; IL-1β and IL-2: Figure 2.7B) compared with untreated controls.

**Figure 2.5 Effects of IL-1β and IL-2 on 6-keto-PGF1a and TXB2 production**

A

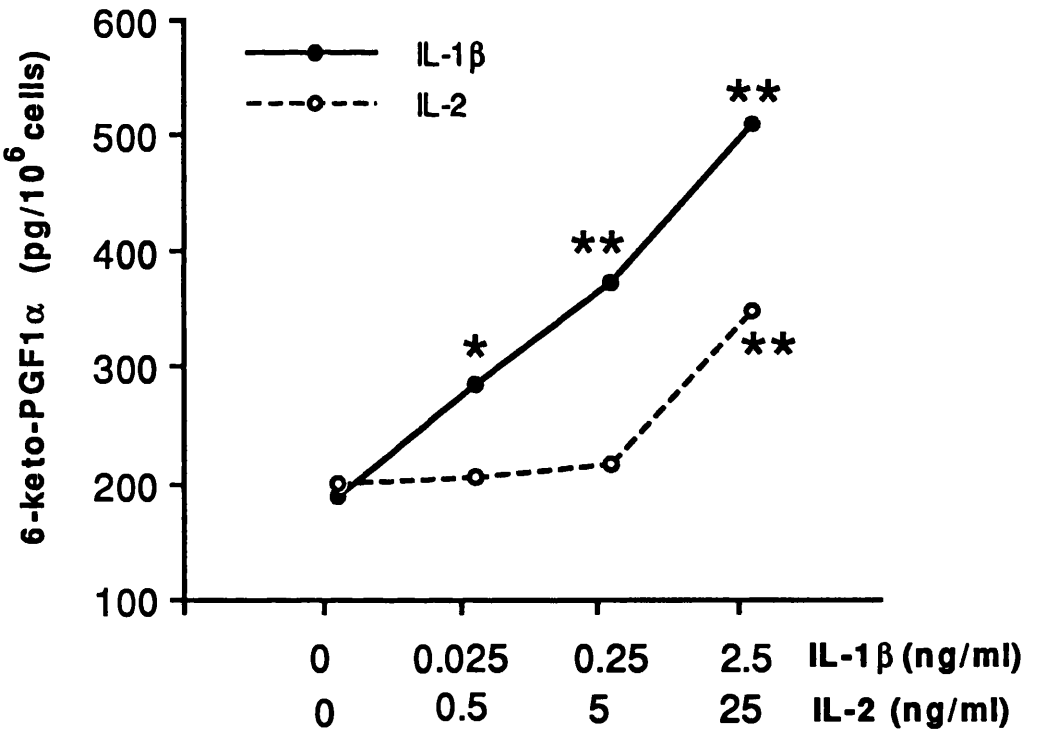



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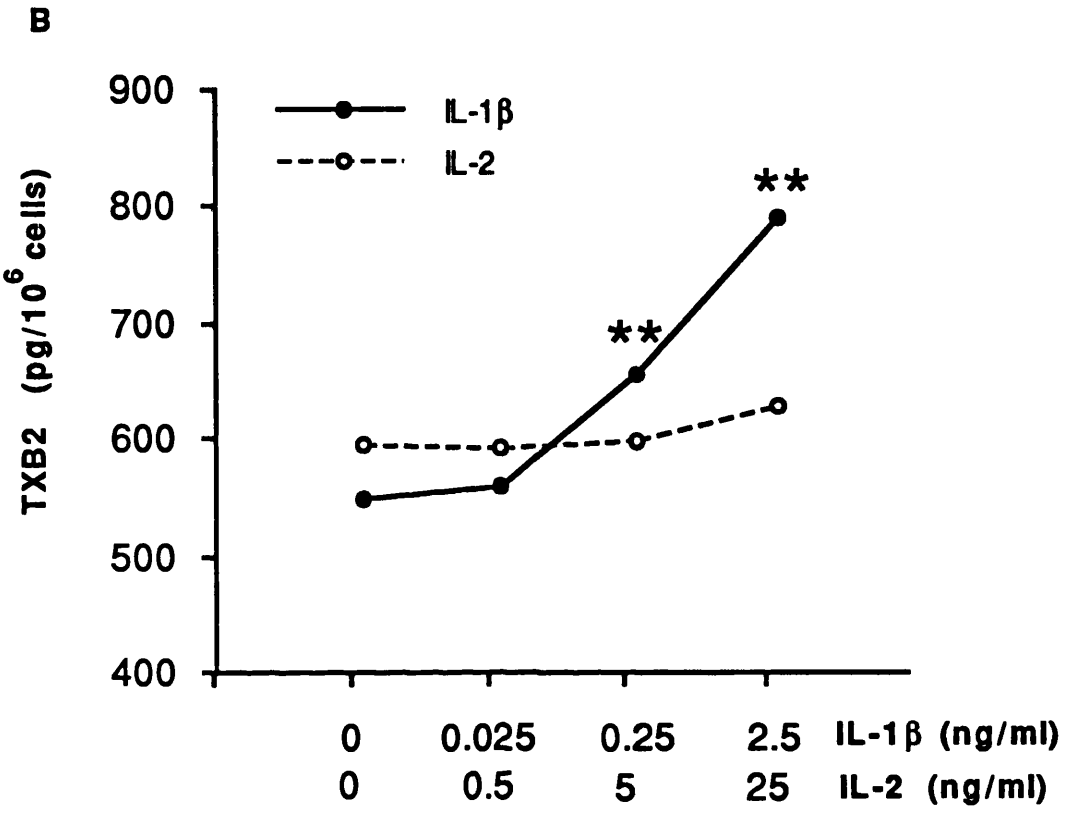
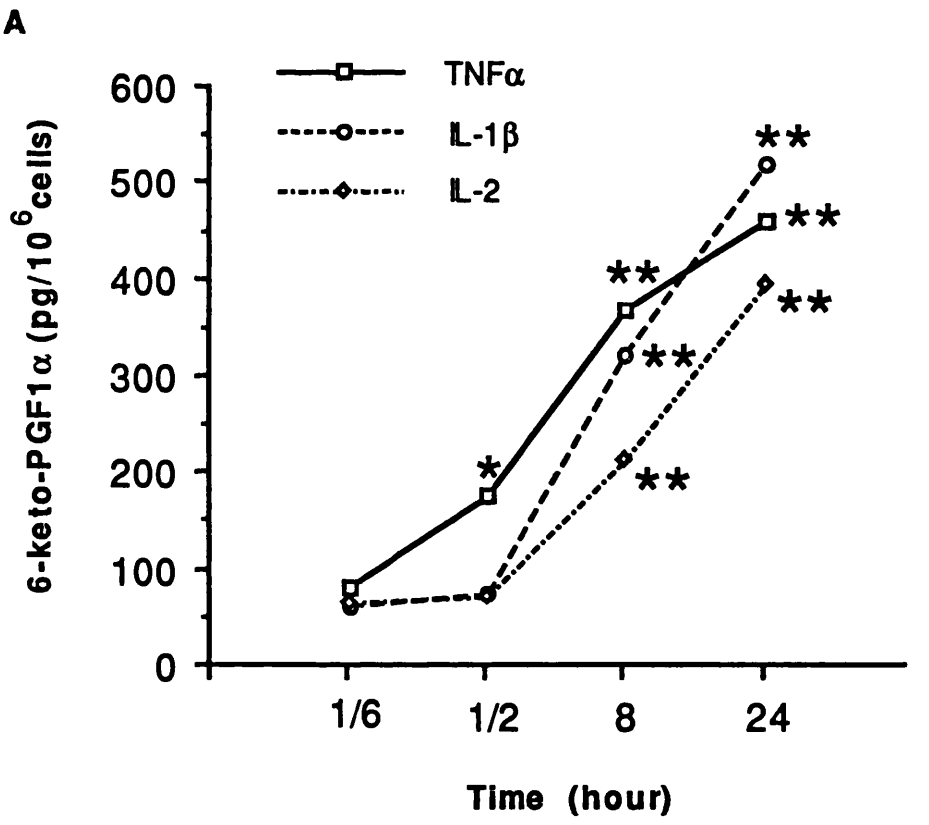
*Figure 2.5. continuing*



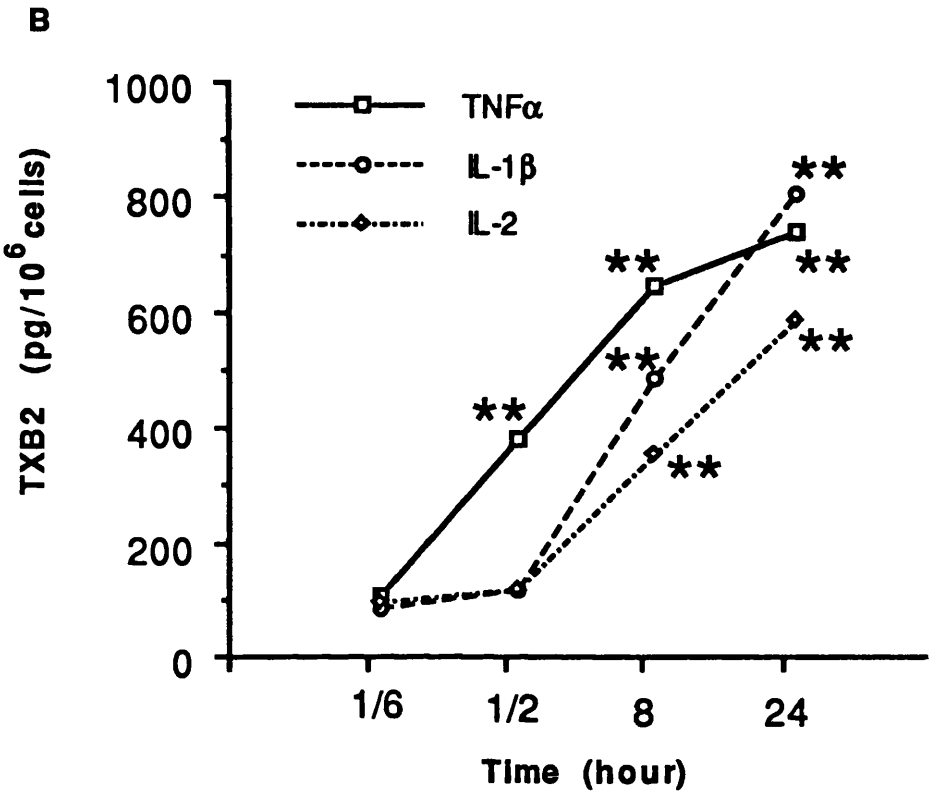

PBMC were incubated with varying concentrations of IL-1β or IL-2 for 24 hours. The levels of 6-keto-PGF1α and TXB2 in the conditional media were determined by EIA after incubation. Each point represents median of samples (n=4). Statistical significance of a point versus no IL-1β or IL-2 is given by asterisks, *$p<0.05$, **$p<0.01$. IL-1β significantly affects both 6-keto-PGF1α and TXB2 synthesis, both $p<0.01$, Kruskal-Wallis analysis for the effects of different concentrations of IL-1β. IL-2 significantly affects 6-keto-PGF1α synthesis, $p<0.05$, Kruskal-Wallis analysis for the effects of different concentrations of IL-2.

**Figure 2.6** **The levels of 6-keto-PGF1α (A) and TXB2 (B) in PBMC conditional media after different incubation times**



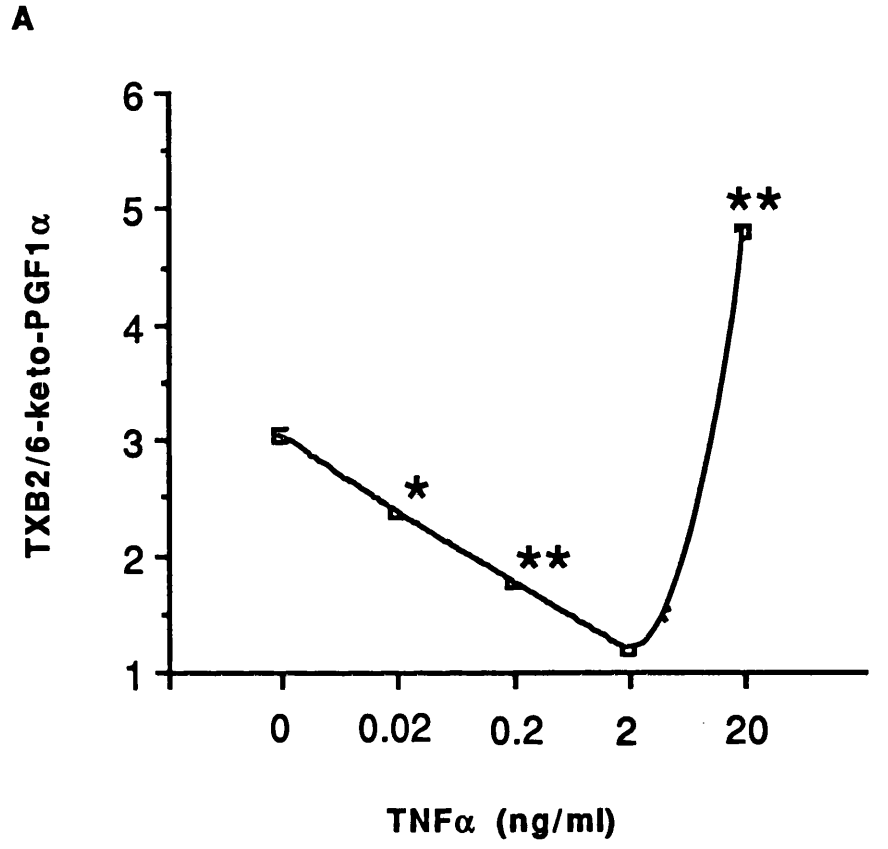

*Continuing*

*Figure 2.6 continuing*



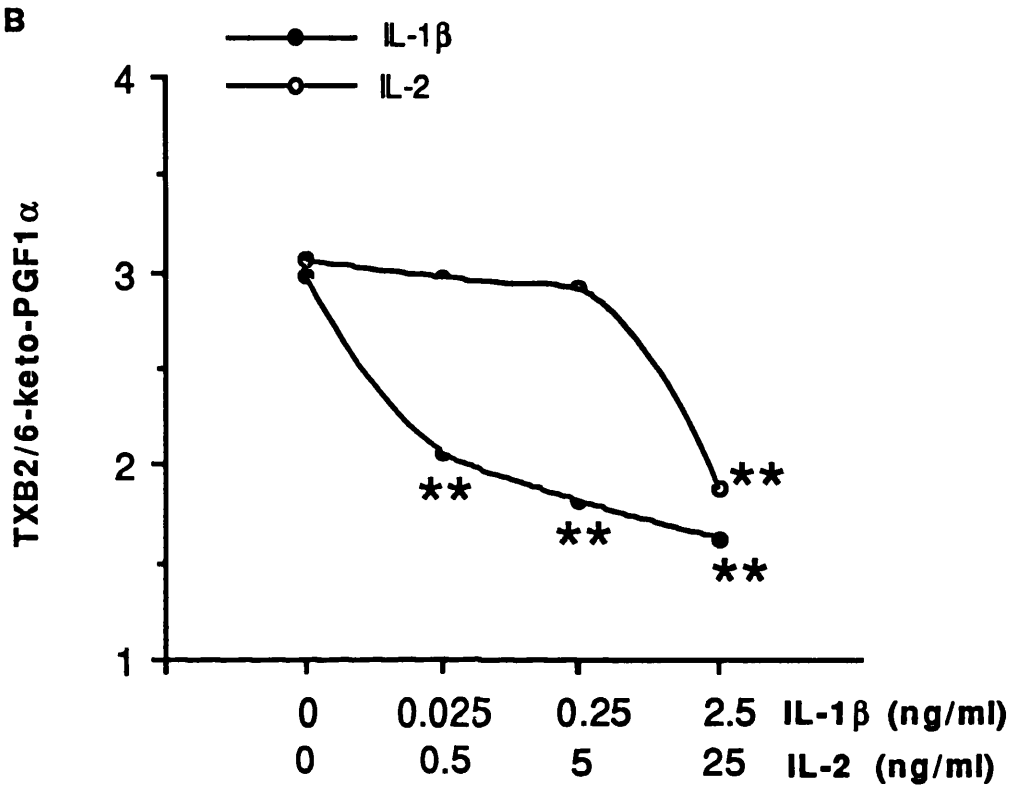

PBMC were incubated with 0.2 ng/ml of TNFα, 2.5 ng/ml of IL-1β and 25 ng/ml of IL-2 respectively. The supematants were obtained after different incubation times and the levels of 6-keto-PGF1α and TXB2 were determined. Each point represents result (median) of three PBMC preparations. Statistical significance of a point versus 1/6-hour incubation is given by asterisks, *$p<0.05$, **$p<0.01$. The effects of TNFα and IL-1β on the production of 6-keto-PGF1α and TXB2 at the different incubation times were significantly different, all $p<0.01$, Kruskal-Wallis analysis for the effects of TNFα and IL-1β at the different time point. The effects of IL-2 on the production of 6-keto-PGF1α at the different incubation times were significantly different, $p<0.05$, Kruskal-Wallis analysis for the effects of IL-2 on the production of 6-keto-PGF1α at the different time point.

**Figure 2.7 The changes of ratio of TXB2 to 6-keto-PGF1α after cytokine treatment**




*Continuing*

*Figure 2.7 continuing*




PBMC were incubated with varying concentrations of TNFα (A), IL-1β or IL-2 (B) for 24 hours. The levels of 6-keto-PGF1α and TXB2 in the conditional media were determined by EIA after incubation and the ratio of TXB2 to 6-keto-PGF1α was calculated. Each point represents result (median) of four PBMC preparations. Statistical significance of a point versus no cytokines is given by asterisks, *$p<0.05$, **$p<0.01$. TNFα, IL-1β and IL-2 significantly affects the ratio, $p<0.001$, $p<0.001$ and $p<0.01$ respectively. Kruskal-Wallis analysis for the effects of different concentrations of cytokines.

## 2.4 Discussion

The results of this study have demonstrated that lymphocyte activity or its response to the mitogen PHA was increased in some groups of patients with PIH. This increased activity did not seem to be affected by the status of patients' proteinuria since lymphocyte response to PHA was not different between PIH patients with and without proteinuria. The reason why increased PBMC proliferation in the absence of mitogenic stimulation was observed only in PIH patients without proteinuria and not in those with proteinuria remains unclear. Increased mitogenic activity in PIH may result from intrinsic lymphocyte abnormalities. A number of facts, however, suggest it may be due to some intracellular and extracellular mechanism associated with the disease. Firstly, increased intracellular calcium has been noted in patients with PIH (Haller *et al.* 1989; Sowers *et al.* 1989). The increase in intracellular calcium is an essential signal for T cell activation (Tsien *et al.* 1982). Secondly, changes of prostaglandin pattern can greatly affect lymphocyte activity or proliferation. It is reported that the PGE series and PGI2 have a suppressive effect on the immune system (Goodwin & Webb 1980, Kingston *et al.*, 1985) whereas TXA2 is suggested to have a positive effect (Kelly *et al.* 1979). The imbalance of increased TXA2 and decreased PGI2 and PGE has been well documented in PIH (Friedman 1988; Lindheimer & Katz 1989; Walsh 1990). Thirdly, a blood-borne mitogenic factor from patients with PIH has recently been discovered (Taylor *et al.* 1990). Although the patient serum was separated from PBMC in this study, the possibility that the mitogenic factor could have been secreted from PBMC cannot be excluded. Fourthly, studies have indicated that ROS are able to activate lymphocytes and subsequently to enhance lymphocyte proliferation (Fidelus 1988; Sekkat *et al.* 1988) and the acitivity of ROS is increased in PIH (Dekker *et al.* 1991; Wickens *et al.* 1981). Lastly, an increase in IL-2 activity has been shown in PIH by this study. It is well known that IL-2 can stimulate lymphocyte proliferation via the activation of PKC (Watson & Mochizuki 1980; Smith 1984).

Previous investigators have found some alterations in the human immune system during PIH. Among the changes are elevated levels of circulating immune complexes (Haeger *et al.* 1991) and the presence of antibodies to trophoblast antigens, amniotic glycoprotein, placental and renal antigens, and endothelial cells (Rappaport *et al.* 1990; Sibai 1991). Here, we demonstrated that the amount of IgG secreted from PBMC in culture was elevated, suggesting an increased B lymphocyte function in PIH. The B lymphocyte hyperactivity could contribute to increased antibodies toward fetal or maternal tissues seen in PIH. Some of the factors which

favour the increase of PBMC in response to mitogens, may also affect B lymphocyte activity. Most autoantibodies belong to the IgG class and this may explain why the production of IgM is not altered in PIH. However, at present it is unclear why increased IgG production was only observed in PIH women with proteinuria but not in those without proteinuria. A very recent study has shown that the IgG fraction prepared from plasma containing antiphospholipid antibody can cause increased placental TXA2 production without altering PGI2 production (Peaceman & Rehnberg 1992). It appears that the TXA2 level is increased and antiphospholipid antibody occurs frequently in PIH (Branch *et al.* 1988; Friedman 1988; Kilpatrick *et al.* 1989b; Triplett 1989; Walsh 1990). It is therefore plausible that the observation of increased IgG production is one of the factors which may result in increased TXA2 in PIH.

IL-2 plays a pivotal role in the study of lymphocyte function *in vitro* (Morgan *et al.* 1976; Smith 1984). It is produced after T lymphocytes are activated by an antigen or mitogen and IL-2 binding to its receptors leads to T-lymphocyte proliferation and expansion in an antigen-independent manner. Although the cause of PIH is still not fully understood, it is likely to be an immunologically mediated process (Redman 1991). Endothelial cell damage has been deemed to have such a key role in the pathogenesis of PIH that it is regarded as final common pathway in the development of PIH (Roberts *et al.* 1989 & 1991). Endothelial cell injury has been closely linked with IL-2-mediated mechanisms. Firstly, IL-2 administration is associated with vascular changes, especially the capillary leak syndrome (Rosenstein *et al.* 1986; Klausner *et al.* 1989a). Endothelial compromise and damage may result from IL-2 stimulating lymphocytes directly or indirectly. IL-2, in a dose-dependent manner, induces lymphocytes to adhere to vascular endothelium but not to other cell types. Activated lymphocytes (cytokine activated killer cells) are highly cytotoxic to endothelial cells (Damle *et al.* 1987). Damage is probably mediated by the release of soluble mediators such as lymphotoxin, TNFα and INFγ (Fletcher & Goldstein 1987; Nedwin *et al.* 1985). Secondly, IL-2 is able to induce ROS production and ROS are known to be toxic to endothelium (See Chapter 3). Lymphocytes, neutrophils and macrophages can respond to IL-2 stimulation and generate ROS (Nathan 1987; Staub 1988). Endothelial cells, although not directly affected by IL-2 (Klausner *et al.* 1989a), can produce and secrete ROS in response to other cytokines such as IL-1 and INF-γ (Matsubara & Ziff 1986), which are known to be released after IL-2 administration (Damle *et al.* 1987). Thirdly, IL-2 is also known to induce TXB2 production. This was demonstrated by the present study (See below) and others (Klausner *et al.* 1989b; Welbourn *et al.* 1990 & 1991). TXB2 can adversely affect

endothelial cells (Welles *et al.* 1985; Klausner *et al.* 1988) and is responsible in a large part for IL-2-induced the early increase in permeability of endothelial cells (Klausner *et al.* 1989b). On the other hand, TXB2 itself is a potent vasoconstrictor and its level in PIH is increased (Friedman 1988). Thus it plays an important role in the pathophysiology of PIH (See Chapter 4).

A explanation has long been sought for survival of the fetus without immunological rejection in normal pregnancy. A major focus of this issue has been on the female's ability to mount an immune response and react against non-self antigens. A change in the balance of maternal immunoregulatory cells towards greater suppression of immune responses would contribute to the immunoregulation of normal pregnancy. As a result, there is a balance between fetal antigenic load and maternal production of blockage (alloantibodies). The immunological suppression in normal pregnancy is evident by the fact that the response of maternal lymphocytes to PHA and mixed lymphocyte reactions are depressed (Petrucco *et al.* 1976; Tomoda *et al.* 1976; Stankova & Rola-Pleszczynski 1984). Conversely, in PIH as in recurrent spontaneous abortions (Christiansen *et al.* 1990; Redman 1991) the immune mechanism may not be able to adjust the balance of immunoregulatory cells in favor of immune suppressive function. Therefore, an excessive fetal antigenic load or inadequate production of immunological blockade to the fetal antigens presenting might lead to development of PIH (Zeeman & Dekker 1992). Fetal antigens are immunological and can induce a typical cell-mediated response. Locally, the decidual cellular immunity is stimulated to limit the trophoblastic invasion and this results in the failure of the endovascular trophoblast to destroy the muscular layer and the autonomic innervation of the spiral arteries (Zeeman *et al.* 1992). Consequently, the spiral arteries are left with a non-pregnant architecture and fail to dilate. Systemically, the immune system could be activated to a certain degree. It has been reported that the activities of neutrophils, macrophages and T-cell lymphocytes are increased in women with PIH (Greer *et al.* 1991; Zeeman & Dekker 1992). The findings of increased PBMC response to PHA, increased IL-2 activity and elevated IgG production from PBMC are the further evidence of this hypothesis.

Previous blood transfusion protects against PIH development (Feeney *et al.* 1977), suggesting PIH may be analagous to the beneficial effect of blood transfusion on renal allograft survival. Blood transfusions could lead to the presence of immune-blocking factors in the host and thus develop mutual immunological tolerance between the host and fetal or renal allograft (Persijn *et al.* 1979; Proud 1980). Interestingly, the findings of increased immune responses in PIH are also noted in

renal allograbt (Kirkpatrick & Rowlands 1992), suggesting that a similar immunological mechanism might exist in both conditions.

Recent developments in the study of pathogenetic aspects of PIH seem to suggest that this enigmatic disease is mulitifactoral (Hubel *et al.* 1989; Walsh 1990; Redman 1991), involving the immune, eiscosanoid and ROS systems. From the systemic point of view, it is important to understand the relationship between them. We have demonstrated that there is an imbalance of increased TXA2 and decreased PGI2 (See Chapter 4) as well as an increased IL-2 activity in PIH. Therefore, it is important to study the effect of immune mediators such as IL-1β, IL-2 and TNFα on the production of PGI2 and TXA2 in normal persons. Although IL-1β and TNFα have not yet been investigated in PIH, the close link between IL-2 and other cytokines including IL-1β and TNFα was the reason that their effects on prostaglandins were also studied.

It has been documented that cytokines are able to stimulate prostaglandin production from several kinds of cells (Kawakami *et al.* 1986; Akahoshi *et al.* 1988; Frasier-Scott *et al.* 1988; Raz *et al.* 1988). However, there is little data available regarding the effects of cytokines on the formation of prostaglandins from PBMC. This experiment has demonstrated that TNFα, IL-1β and IL-2 could stimulate the synthesis of PGI2 and TXA2 in a time- and dose-dependent manner. The induction of prostaglandin production was exquisitely sensitive to the concentrations of IL-1β; as little as 0.025 ng/ml and 0.25 ng/ml produced a clear stimulation of PGI2 and TXA2 syntheses respectively. The effects of TNFα on prostaglandin synthesis was biphasic. Culture of PBMC with 0.2 to 2 ng/ml of TNFα up-regulated the production of prostaglandins while at the concentration of 20 ng/ml, TNFα exerted down-regulation of prostaglandin production. The stimulatory activity of TNFα was rapid. It was observed that both PGI2 and TXA2 production increased after only 30-minute incubation of PBMC with TNFα. This is quite different from the effect of IL-1β and IL-2, in which no significant effects on PGI2 and TXA2 synthesis were recorded after 30-minute incubation but were present after a 8-hour incubation. This observation suggests that the effect of TNFα and interleukins on prostaglandin production is somewhat different. The regulatory effects of TNFα is unlikely to involve protein biosynthesis, at least at the early stage of the prostaglandin synthesis.

Regulation of prostaglandin synthesis can be obtained by either increasing the supply of arachidonic acid or altering the cyclooxygenase activity or the specific prostaglandin synthetases. Our findings raise a number of possibilities. Firstly, the

content of arachidonic acid in membrane lipid of PBMC is very high and more importantly the release of arachidonic acid from PBMC is increased when the cells are activated (Hwang 1989). As cytokines are able to activate PBMC, one putative role of the cytokines on the up-regulation of prostaglandin production in this study might be through their activation of PBMC and increasing arachidonic acid release. Secondly, cytokine-induced augmentation of cell activation is associated with generation of ROS (Meier *et al.* 1989). Although the high concentrations of ROS can be damaging to cyclooxygenase, it is believed that a lower levels of ROS activate the enzymes (Taylor *et al.* 1983). On this basis, it is possible that cytokines may increase the production of prostaglandins by inducing the activity of ROS. Thirdly, the production of cyclooxygenase and specific prostaglandin synthetase have been reported to be increased by the stimulation of cytokines in several cell systems. Frasier-Scott *et al.* (1988) proved that IL-2 induced *de novo* synthesis of the 70-KD subunit of prostaglandin H synthase in endothelial cells and Raz *et al.* (1988) reported that IL-1 could enhance cyclooxygenase synthesis in a time- and dose-dependant fashion in dermal fibroblast. However, there is, so far, no data available to demonstrate whether this is the case in PBMC. Since the significant actions of IL-1β and IL-2 were observed at a quite late stage of incubation (8th hour), it is believed that the increase of cyclooxygenase or other related enzyme synthesis in PBMC induced by interleukins could be considered to be a possible mechanism. Finally, as the time of action between the TNFα and interleukins (IL-1β and IL-2) differs, as stated above, the mechanism of TNFα induction of prostaglandin production may differ, at least in part, from that of the interleukins. Unlike TNFα, it is impossible that the increased release of arachidonic acid from cell membrane or the induction of cyclooxygenase activity by the increase of ROS is a major mechanism of IL-1β and IL-2.

It has been established that TNFα has an immunoregulatory activity on PBMC (Balkwill 1989). Paradoxically, it is also a potent cytotoxin for cells. In addition to generation of ROS, there is now evidence that TNFα can interfere with the intracellular antioxidant buffering capacity such that cells or enzymes become more sensitive to oxidant-mediated injury (Ishii *et al.* 1992). Although the biphasic effect of TNFα can not be directly explained by the present experimental data, overproduction of ROS or damage of antioxidant ability could be one of explanations for this interesting phenomenon. Increased ROS levels or deficient antioxidant ability have been known to occur in PIH (See Chapter 3). However, whether there is an elevated TNFα level in PIH to account for these changes remains an interesting question.

Both PGI2 and TXA2 are derived from arachidonic acid through the action of cyclooxygenase. It is worth noting that the increasing rate of prostaglandins induced by cytokines is quite different between the PGI2 and TXA2 and it depends on the type and concentration of cytokines involved. At lower concentrations of TNFα (0.02 to 2 ng/ml), the enhancement of PGI2 is much greater than that of TXA2, resulting in a decreasing ratio of TXA2 to PGI2 (Figures 2.7A). Conversely, at higher concentrations of of TNFα (more than 2 ng/ml), the increase in TXA2 is more obvious than that in PGI2 and this leads to a increasing ratio of TXA2 to PGI2. IL-1β at the concentrations of 0.025 to 2.5 ng/ml and IL-2 at 25 ng/ml decrease TXA2 to PGI2 ratio (Figure 2.7B). The mechanisms for these complicated effects are unclear. It is contradictory that there is an increased IL-2 activity (about 32 units/ml) and an elevation of TXA2 to PGI2 ratio in PIH and that IL-2 at the concentration of > 25 ng/ml (about 30 units/ml) can reduce this ratio in an *in vitro* experiment. However, this may suggest that IL-2 itself is not a major factor contributing to the prostaglandin changes in PIH. Nevertheless, IL-2 may affect this ratio indirectly via its effects on other cytokine release such as TNFα.

Evidence has shown that PGI2 inhibits immunological function in a manner analogous to PGE2 (Kingston *et al.* 1985), a well known immunosuppressant, whereas TXA2 has been suggested to have an action on lymphocyte activation opposite to that of PGE2 (Kelly *et al.* 1979). From this point of view, the increased IL-2 activity and lymphocyte hyperactivity in PIH are in accordance with the imbalance of increased TXA2 and decreased PGI2. The interactions between the cytokines and prostaglandins need to be further investigated and should be considered to be of potential importance in the development of PIH.

## 2.5 Conclusions

1. PIH is associated with increased B lymphocyte function including the hypersecretion of IgG and increased T lymphocyte function such as hyper-response to mitogenic stimulation and elevation of IL-2 activity.
2. Increased IL-2 activity together with other cytokines such as TNFα may partially contribute to the imbalance of increased TXA2 and decreased PGI2 found in PIH.

# CHAPTER THREE ANTIOXIDANTS

## 3.1 Introduction

### 3.1.1 Reactive oxygen species and antioxidants

The role of reactive oxygen species (ROS) in the pathogenesis of PIH has recently attracted attention (Hubel *et al.* 1989; Zeeman *et al.* 1992). Produced by most human cells, ROS may act as mediators in the development of hypertension in pregnancy by producing various noxious effects due to the changes in cell membrane phospholipids, with consequent changes in cell integrity, fluidity and permeability (Cronstein 1991; Rice-Evans & Bruckdorfer 1992). Other toxic effects include damage to nucleic acids and proteins. Lipid peroxidation production and glutathione peroxidase activity are increased suggesting an active role of ROS activity in PIH (Hubel *et al.* 1989), although the mechanism responsible is unclear.

The body has a multilayered antioxidant system including enzymatic and nonenzymatic components to cope with elevated or adventitious ROS production. This enables the body to use ROS biochemistry without the risk of uncontrolled reactions affecting physiology. However, when balance between ROS generation and the protective mechanisms is shifted in favour of pro-oxidants, then excessive ROS can be damaging.

Antioxidant buffers are essential in the protection against the deleterious effects of ROS, but to date knowledge about antioxidant buffering level in PIH is limited. Since red blood cells (RBC) can synthesize thiols and have only a limited ability to repair (Chilles *et al.* 1990) and since thiols are effective antioxidants, their concentrations can reflect any stress which has occurred. Therefore, RBC provide a useful model for the *in vitro* study of antioxidants or ROS.

### 3.1.2 Reactive oxygen species and prostaglandins

Research has suggested that the activity of ROS is closely associated with the production of prostaglandins. One important phenomenon which has been noted is the selective inhibition of PGI2 synthetase by ROS. In contrast, TXA2 synthetase is not influenced by comparable concentrations of such reactive molecules (Moncada *et al.* 1976; Salomon *et al.* 1978; Ham *et al.* 1979; Warso and Lands 1983; Schimke *et al.* 1992). This view is in line with some recent studies dealing with the influence of

ROS on eicosanoid metabolism in cells. Vercellotti *et al.* (1991) and Whorton *et al.* (1985) demonstrated a $H_2O_2$-induced dose-dependent inhibition of PGI2 formation in cultured endothelial cells. PGI2 and TXA2 are involved in the control of vascular tone and hemostasis via a reciprocal function in blood-endothelium interactions. The production of TXA2 is increased in PIH, while the production of PGI2 is relatively or absolutely decreased (Friedman 1988; Walsh 1990; See Chapter Four). This leads to an increase in the ratio of TXA2 to PGI2. It has been demonstrated that changes in this ratio play an important role in PIH (Friedman 1988; Walsh 1990). If ROS change the ratio of TXA2 to PGI2 formation, it would diminish the protective function of the endothelium in hemostasis and vascular tone.

Since both ROS activity and prostaglandin levels appear to be changed in patients with PIH (Friedman 1988; Hubel *et al.* 1989; Walsh 1990; Zeeman *et al.* 1992), it is hypothesized that the alterations in the levels of prostaglandins may result from increased ROS activity or decreased antioxidant ability in PIH. To our knowledge, there is no evidence so far available to link prostaglandin levels and antioxidants in PIH.

### 3.1.3 Aims of the study

The first aim of the present study was to investigate the possible changes of intracellular and extracellular antioxidant buffering levels in RBC from patients with PIH and to analyse their roles in association with pathological features of PIH. Three extracellular antioxidant markers were measured: plasma thiol (PSH), plasma total glutathione (GSHp) and membrane thiol (MSH) and three intracellular antioxidant markers in RBC: lysate thiol (LSH), lysate total glutathione (GSHl) and lysate superoxide dismutase (SOD).

The second aim was to investigate the antioxidant state and the prostaglandin system simultaneously and to analyse relations between them. For this reason the antioxidant agents, TXA2 and PGI2 were chosen to be measured in the same sample of peripheral blood.

## 3.2 Materials and Methods

### 3.2.1 Subjects

Four groups of subjects were studied:

1, 25 healthy nonpregnant women.
2, 36 normotensive pregnant women.
3, 27 PIH women without proteinuria.
4, 8 PIH women with proteinuria.

All pregnant women were in their third trimester of pregnancy. The detailed clinical information is listed in Table 3.1.

PIH women were sampled after diagnosis but before any anti-hypertensive therapy had been instituted.

PIH is defined as a persistent or recurrent diastolic blood pressure of 90mmHg which developed during pregnancy after 20 weeks of gestation, and resolved by six weeks post partum. Proteinuria is defined as the persistent presence of protein in the urine of $\geq$ + on urine 'dipstick' testing, or >300 mg excreted in 24 hours. A more detailed discussion of these criteria has been given in Section 1.1.3 of Chapter One. Patients who had a history of hypertension before the twentieth week of pregnancy are designated as essential hypertension and were not included in this study.

### 3.2.2 Blood sample collection and separation of plasma and RBC

For the measurement of antioxidant markers, 10 ml of heparinized (30 units/ml blood) peripheral venous blood was obtained. This was then immediately centrifuged at 1200 x g for 10 minutes at 4°C and the plasma and RBC were separated.

For measurement of TXB2 and 6-keto-PGF1$\alpha$, 10 ml of peripheral venous blood was obtained and anticoagulated with 3.2% trisodium citrate (0.8ml/10 ml blood). This was immediately centrifuged at 1200 x g for 10 minutes at 4°C. Plasma was then separated and rapidly stored at -20°C until assay.

**Table 3.1 Clinical and Laboratory Data**

| | Age(y) | Blood pressure(mmHg) Systolic | Diastolic | RBC number count($x10^{12}$/ml) | Hemoglobin (g/L) | PCV (%) | MCV (g/dL) | MCH (g/dL) | MCHC (g/dL) | Platelet ($x10^9$/ml) |
|---|---|---|---|---|---|---|---|---|---|---|
| PIH women with proteinuria | 23.9±4.4 | 158±16 | 109±11 | 3.81±0.53 | 11.2±1.03 | 33.2±3.3 | 87.6±8.7 | 29.3±2.75 | 33.3±0.55 | 207±61 |
| Statistics | NS | ** | ** + | NS | NS | NS | NS | NS | NS | * ++ |
| *n* | (7) | (7) | (7) | (7) | (7) | (7) | (7) | (7) | (7) | (7) |
| PIH women without proteinuria | 26.9±4.4 | 145±10 | 99±5 | 3.95±0.33 | 11.8±1.12 | 35.5±2.8 | 89.0±4.6 | 29.9±2.15 | 33.3±0.83 | 298±87 |
| Statistics | NS | ** | ** | NS | NS | NS | NS | NS | NS | NS |
| *n* | (25) | (25) | (25) | (25) | (25) | (25) | (25) | (25) | (25) | (25) |
| Normal pregnant women | 27.3±5.0 | 116±11 | 72±10 | 3.85±0.28 | 11.5±0.96 | 34.9±2.9 | 90.2±4.9 | 30.4±1.99 | 33.5±0.99 | 280±55 |
| *n* | (33) | (33) | (33) | (34) | (34) | (25) | (25) | (25) | (25) | (34) |

Data are expressed as mean±SD.

Compared with normal pregnant women, *$p<0.05$, **$p<0.01$; compared with PIH women without proteinuria, +$p<0.05$, ++$p<0.01$.

NS: no statistical significance ($p\geq0.05$).

### 3.2.3 Preparation of RBC lysate

All steps were carried out at 4°C. 1 ml of RBC pellet was resuspended in 1 ml of ice-cold distilled water in a 10 ml glass tube, mixed well and put on ice for 2 hours. During this period the sample was stirred occasionally. 800 µl of cooled absolute ethanol and chloroform mixture (3:5, v/v) was then added and mixed thoroughly with a glass pipette until the mixtures became thicker and the colour turned brick red. The mixtures were left on ice for a further 10 minutes and mixed occasionally. Three more drops of cooled absolute ethanol and chloroform mixture (3:5, v/v) and 300 µl of distilled water were then added and mixed thoroughly. The mixtures were centrifuged at 1200 x g for 10 minutes at 4°C. The pale yellow supernatants were removed using a pipette and constituted the RBC lysate. If the separated supernatants still had pink or brick red colour, one more drop of cooled absolute ethanol and chloroform mixture (3:5, v/v) was added and the pellet was mixed thoroughly. The mixtures were centrifuged as above and the supernatants were obtained.

### 3.2.4 PSH and LSH assay

Assays of LSH and PSH concentrations have been described previously by Banford *et al.* (1982a & 1982b). This assay requires the following reagents:

1, Sodium phosphate buffer, 0*1 M, pH 7.6 at room temperature (25° C).
2, DTNB, 0.01 M, in 0.1 M sodium phosphate buffer (kept in ice and used within one hour of preparation).

Cuvettes (3 ml) were set up as follows at room temperature.

| A | B | C |
|---|---|---|
| reagent blank | plasma blank | lysate blank |
| **A1** | **B1 = B2** | **C1 = C2** |
| reagent test | plasma test | lysate test |

To the reagent blank cuvette (A) are added 3 ml of sodium phosphate buffer and to reagent test (A1) 2.5 ml of sodium phosphate buffer and 0.5 ml of DTNB. The reaction, which is carried out at room temperature, is started by the addition of

DTNB and monitored spectrophotometrically by the increase in absorbance at 440 nm against the reagent blank (A) exactly five minutes after adding DTNB. This value is the reagent blank. To plasma and lysate blank cuvettes (B and C) are added 2.8 ml of sodium phosphate buffer and 0.2 ml of sample (plasma or RBC lysate). To plasma and lysate cuvettes (B1, B2 and C1, C2) are added 2.3 ml of sodium phosphate buffer and 0.2 ml of the sample (plasma or RBC lysate). The reaction is carried out as above except comparison is against the respective blanks (B or C), to yield the plasma and RBC lysate test values.

The results are calculated by subtracting the blank value from the average of the test value and multiplying by a correction factor to correct for the dilution in cell volumes. The correction factor for PSH is 1471 and for LSH 2297 (Banford *et al.* 1982a & 1982b). Values are expressed in μmol/L, as follows.

PSH(μmol/L) = [(B1 + B2)/2] x 1471
LSH(μmol/L) = [(C1 + C2)/2] x 2297

The intra and inter-assay variations for LSH assays in our laboratory were 1% and 7% respectively and for PSH 1.2% and 1.7%.

### 3.2.5 SOD assay

SOD activity was measured by the method of Misra and Fridovich (1977) based on the increase in the rate of photo-oxidation of 0-dianisidine. This assay requires the following reagents and materials:

1, Potassium phosphate buffer, 0.01 M, pH 7.5 at room temperature (25°C).
2, Riboflavin solution*, 1.3 x $10^{-5}$ M, in 0.01 M potassium phosphate buffer.
3, 0-dianisidine solution*, 0.01 M, in ethanol. 0-dianisidine is obtained from Sigma Chemical Company LTD.
4, SOD standard solution. SOD (from bovine RBC) is purchased from Sigma and stored at -20°C. It can not be used if the SOD powder turns green.
5, Light box, 8 W Philip fluorescent tubes mounted 6 inches apart, box size: 648 $cm^2$, 38 cm high.
6, 3 ml quartz cuvettes.

*: Riboflavin solution and 0-dianisidine solution are light sensitive and should be covered with foil.

The spectrophotometer was zeroed on air at 460 nm. Cuvettes were covered with foil. For the reagent blank, 2.94 ml of riboflavin solution and 0.06 ml of 0-dianisidine solution were added to the cuvette and mixed well by inversion. For the standard and samples, 2.8 ml of riboflavin solution, 0.06 ml of 0-dianisidine solution and 0.06 ml of the standard or sample were added to the cuvette and mixed well by inversion. The absorbance at 460 nm was measured. The cuvette was then illuminated for exactly 4 minutes in the light box. Absorbance at 460 nm was remeasured. Results are calculated by subtracting the blank value from the standard or sample value and multiplying by a correction factor (78.125) (Misra & Fridovich 1977). The amount of SOD was plotted against the corresponding absorbance forming a standard curve and used to determine the SOD in the unknown samples. The value is expressed in μmol/L. The intra and inter-assay variations in our laboratory were 4.6% and 7.0%.

### 3.2.6 GSH assay

Many worker have used the DTNB-GSSG reductase recycling procedure to measure GSH in biologic samples. This was first reported by Owens and Belcher (1965) and later modified by Tietze (1969). The modification described here is basically that of Anderson (1985). The recycling assay for total GSH (GSH + GSSG, in GSH equivalents) is a sensitive and specific enzymatic procedure. As indicated in equation 3.1, GSH is oxidized by DTNB to give GSSG with stoichiometric formation of TNB. GSSG is reduced to GSH by the action of the highly specific glutathione reductase (GSSG reductase) and NADPH (equation 3.2). The rate of TNB formation is followed by spectrophotometry at 412 nm and is proportional to the sum of GSH and GSSG present.

$$2\,GSH + DTNB \longrightarrow GSSG + TNB \qquad \text{(Equation 3.1)}$$

$$GSSG + NADPH + H^{+} \xrightarrow{\text{GSSG reductase}} 2\,GSH + NADP^{+} \qquad \text{(Equation 3.2)}$$

This assay requires the following reagents:

1, Stock buffer: 0.143 M sodium phosphate plus 6.3 mM EDTA (pH 7.5 at 25°C), stored at 4°C.

2, Working buffer: 0.248 mg NADPH per milliliter of stock buffer (prepared daily and stored at 4°C).

3, DTNB solution, 6 mM in stock buffer (stored at -20°C).

4, GSSG reductase, 50 U/ml in stock buffer (Sigma type VII, stored at 4°C).

5, Glutathione standards, diluted to desired concentrations daily from a frozen stock solution (1 mM GSH, prepared weekly).

6, Sulphosalicylic acid (SSA) (BDH Chemical Ltd., England), 5% (W/V).

Plasma or RBC lysate was immediately deproteinized by adding one half volume of 10% (W/V) SSA. Before assay, all reagents are warmed up to room temperature (25°C). 700 µl of the working buffer, 100 µl of DTNB solution, 175 µl of distilled water and 25 µl of sample or standard (containing the same amount of SSA as the samples) were pipetted into a quartz cuvette (1.5 ml). The sample is added with mixing by inversion and the assay is initiated by addition of the GSSG reductase solution (10 µl, 50 U/ml). The spectrophotometer is set to 0.5 absorbance units full scale and zeroed on air at 412 nm. The background rate is determined from a blank containing the same amount of SSA as the sample. The amount of GSH is determined from a standard curve in which the GSH equivalents present are plotted against the rate of change of absorbance at 412 nm. A standard curve for each test is prepared. Values are reported in µmol/L. The intra and inter-assay variations were 5.7% (n=7) and 9.8% (n=8) respectively.

### 3.2.7 MSH assay

ESSE (5,5'-dithio-bis-(2-nitrobenzoic acid), which was first synthesized by Ellman (1959), is a reagent widely used for the determination of sulphydryl groups. ESSE exchanges with sulphydryl groups and can be used to determine the number of sulphydryl groups on the exofacial surface of the intact erythrocytes (Hoey 1987). ESSE undergoes a thiol-disulphide exchange reaction with free sulphydryl groups to produce a mixed disulphide and an anionic species (equation 3.3 and 3.4). The generation of $ES^-$, an intensely chromophoreic product, can be monitored spectrophotometrically at 412 nm, while unreacted ESSE absorbs at 325 nm. ESSE can induce cell lysis when used at high concentrations and when prolonged incubation times are employed. This can be overcome however by careful control of experimental conditions.

$$ESSE + RES \longrightarrow ES\text{-}SR + ESH \qquad \text{(Equation 3.3)}$$

$$ESH \longrightarrow ES^- + H^+ \qquad \text{(Equation 3.4)}$$

The RBC pellet was washed with PBS three times in 15 ml-centrifuge tube (1000 x g, 10 minutes, 25°C). 1 ml of the RBC pellet was added to a 15 ml-centrifuged tube

containing 9 ml of ESSE solution ($10^{-4}$ M). The tube was inverted a number of times to ensure sufficient mixing had occurred and then incubated at 37°C for 30 minutes. After incubation, the sample was centrifuged at 1000 x g for 10 minutes. The supernatant was pipetted into a cuvette. The spectrum of the supernatant was recorded over the range of 300 to 600 nm, referencing the sample against PBS. Absorbance values were taken at wavelengths of 412 nm and 541 nm respectively. Absorbance values obtained at 541 nm was to ensure that cell lysis could be monitored. The sample should be discarded if cell lysis occurs. MSH was calculated by the following formula and the values were expressed in number of thiols per RBC as shown below. The intra and inter-assay variations were 6.6% and 13.5% respectively.

$$\text{MSH(No of thiols/cell)} = \frac{[ES^-] \times \text{volume} \times \text{Avogadro's No.}}{\text{RBC number} \times 1000 \times 13600}$$

where [ES-] = Absorbance of $ES^-$ at 412 nm.
Volume = 10.
Avogadro's No. = $6.02 \times 10^{23}$.
1000 = Concentration factor
13600 = Molar absorptivity of ($ES^-$) at 412 nm.
RBC number is calculated as follows.

A RBC count was determined by taking an aliquot (10 µl) of freshly washed RBC and adding to PBS (90 µl). This suspension was mixed thoroughly. Another aliquot (10 µl) was removed from this suspension and added to PBS (90 µl) and mixed well. The cells were treated in this manner until a 1:100,000 dilution of the cell suspension was achieved. This diluted suspension (10 µl) was then transferred underneath a microscope slide placed over a haemocytometer. Using this method, it was then possible to calculate the number of cells present within a 4 x 4 matrix. The calculation was done by using the following formula.

Number of cells = 1/dilution factor x $10^4$/ml ($10^4$ is the factor due to haemocytometer).

### 3.2.8 Measurement of 6-keto-PGF1α and TXB2

Both TXA2 and PGI2 are extremely labile in the circulation, undergoing rapid hydration of the oxange ring to yield more stable prostanoids, thromboxane B2 (TXB2) and 6-keto-prostaglandin F1α (6-keto-PGF1α) respectively. Therefore, we monitored TXB2 and 6-keto-PGF1α as a measure of the levels of TXA2 and PGI2 respectively.

6-keto-PGF1α and TXB2 concentrations in plasma were measured using an enzymeimmunoassay (EIA) system (Amersham International plc, Amersham, U.K.). For details see Section 4.2.6 in Chapter 4.

### 3.2.9 Whole blood counts

Complete whole blood counts including RBC number, hemoglobin, haematocrit (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) and platelet count were measured using an automated Coulter Counter.

### 3.2.10 Statistical analysis

Data for clinical parameters and antioxidants are presented as mean±SD. Statistical differences between groups were tested by Student's *t* test. Since 6-keto-PGF1α and TXB2 levels are not normally distributed (Moodley *et al.* 1984), their data are described by median and ranges and to determine whether comparisons among groups were valid, a Kruskal-Wallis analysis was performed. Where permitted, differences between groups were tested for by the two-tailed Mann-Whitney (Siegel & Castellan 1988).

The relation between variables was assessed using the Pearson coefficient of correlation. Variance was compared using the F test. A p value of less than 0.05 was considered to indicate statistical significance. All statistical analyses were done by using Minitab Statistical Software.

## 3.3 Results

### 3.3.1 Clincal and laboratory data

Clinical and laboratory data are summarized in Table 3.1. Blood pressure was significantly greater in the both PIH groups and the diastolic blood pressure in PIH women with proteinuria was higher than in those without proteinuria. RBC number count, hemoglobin concentrations, PCV, MCV, MCH and MCHC in the PIH groups were not significantly different from those in the normal pregnant controls. Platelets did not differ significantly between the PIH women without proteinuria and the normal pregnant women. However the levels were much lower in the PIH women with proteinuria compared both to those without proteinuria and to normal pregnant women as well.

### 3.3.2 Extracellular antioxidant buffering level

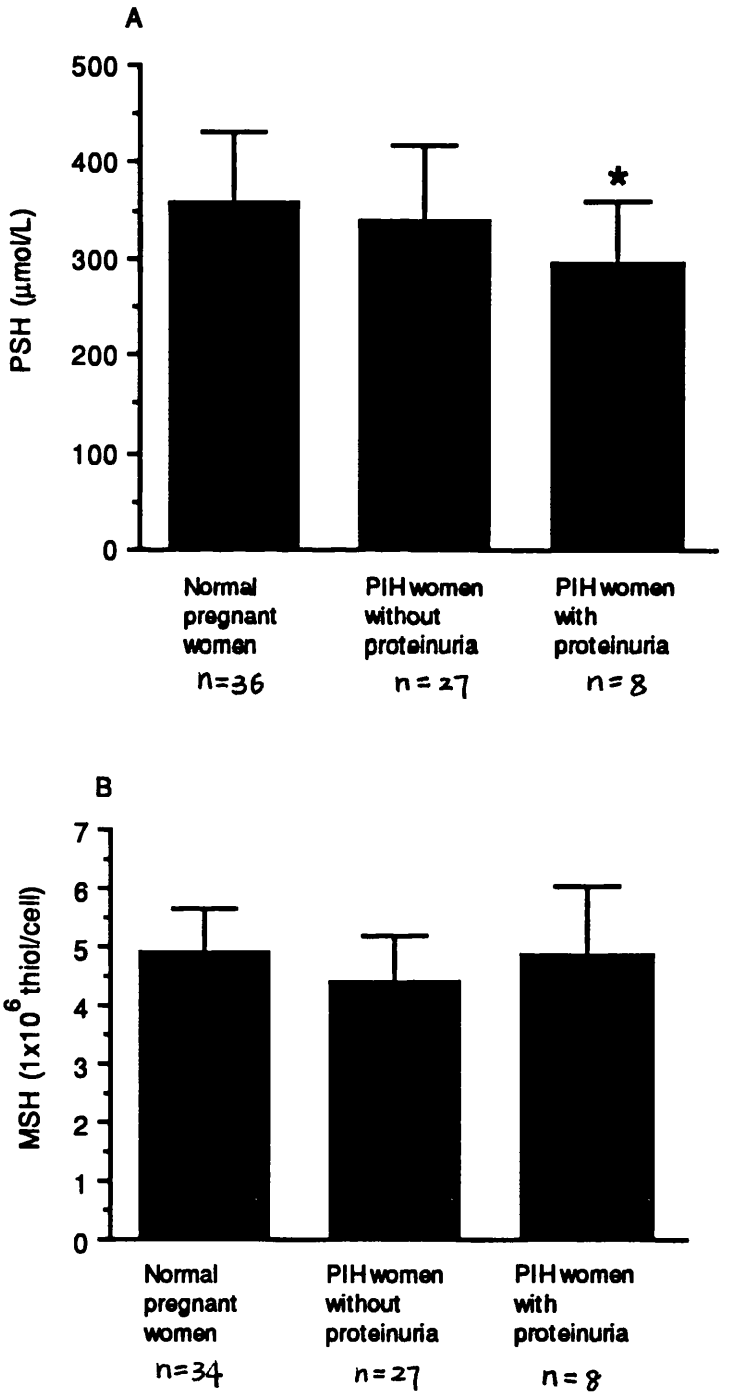
The concentrations of PSH in PIH women with proteinuria were lower than that in normal pregnant women (294±67 vs 358±74 μmol/L, $p<0.05$) (Figure 3.1A). Although the concentrations of PSH in PIH women without proteinuria were also lower than that in normal pregnant women, these did not differ significantly (338±79 vs 358±74 μmol/L, $p>0.05$). The levels of MSH did not differ significantly between the three groups (Figure 3.1B). GSHp in PIH women with proteinuria was significantly decreased compared to those without proteinuria (9.3±2.5 vs 18.4±11.3 μmol/L, $p<0.01$) and to normal pregnant women (9.3±2.5 vs 16.4±8.1 μmol/L, $p<0.001$) (Figure 3.1C). However, the levels of GSHp between normal pregnant women and PIH women without proteinuria did not differ significantly.

### 3.3.3 Intracellular antioxidant buffering level

The PIH women with proteinuria had a significant reduction in the intracellular antioxidant buffering level as compared with the normal pregnant women. This was shown by the decreased GSHl levels (233±42 vs 322±69 μmol/L, $p<0.01$) and SOD activity (31.5±8.7 vs 44.6±20.2 μmol/L, $p<0.05$) (Figure 3.2A and 3.2B). However, the levels of LSH were unchanged compared to those without proteinuria and to normal pregnant women (Figure 3.2C). The concentrations of GSHl in PIH women with proteinuria were also lower than that in those without proteinuria (233±42 vs 298±80 μmol/L, $p<0.05$). The levels of LSH and SOD in PIH women with proteinuria were not significantly different from that in those without proteinuria.

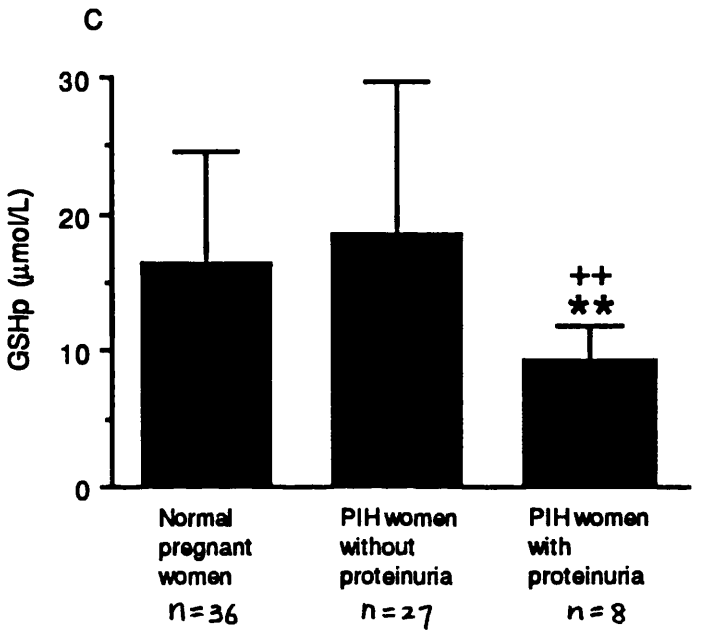
The concentrations of all three intracellular antioxidant markers in PIH women without proteinuria were not different from those in the normal pregnant women, though the levels of GSHl and SOD tended to be lower (Figure 3.2A, 3.2B and 3.2C).

Figure 3.1 Extracellular antioxidant buffering activity in women with PIH and normal pregnant women



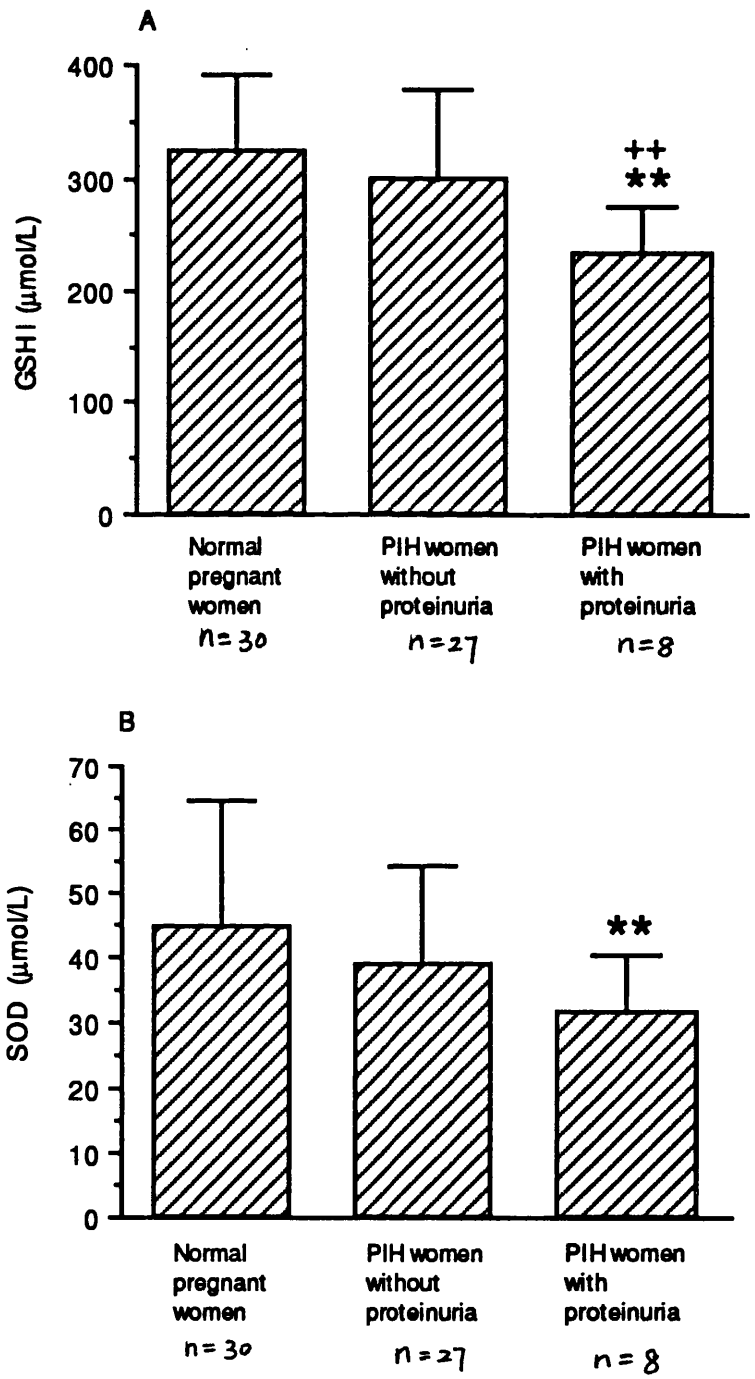

*Continuing*

*Figure 3.1 continuing*



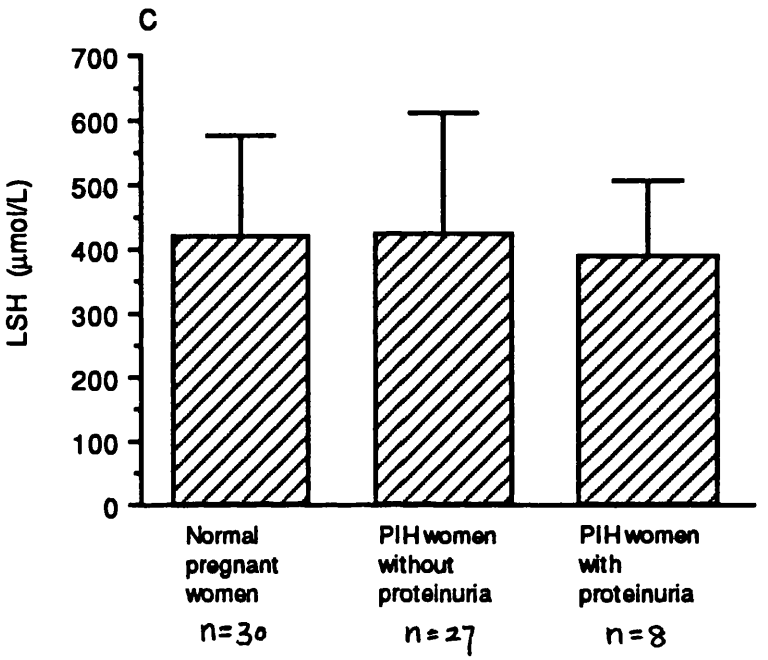

Plasma and RBC (red blood cells) were separated from peripheral venous blood. PSH (A, plasma thiol) and GSHp (C, plasma total glutathione) were measured in plasma and MSH (B, membrane thiol) was measured on the membrane of RBC. Results are represented as mean ± SD. Statistical significance is expressed as: *$p<0.05$, **$p<0.01$, compared with normal pregnant women; ++$p<0.01$, compared with PIH women without proteinuria.

Figure 3.2 Intracellular antioxidant buffering activity in women with PIH and normal pregnant women




*Continuing*

*Figure 3.2 continuing*




Lysate was prepared from RBC of peripheral venous blood and GSHl (A, lysate total glutathione), SOD (B, superoxide dismutase) and LSH (C, lysate thiol) were measured. Results are represented as mean ± SD. Statistical significance is expressed as: **p<0.01, compared with normal pregnant women; ++p<0.01, compared with PIH women without proteinuria.

### 3.3.4 Correlation between the antioxidant buffering levels and the blood pressure.

Results were shown in Table 3.2. Blood pressure readings were taken at time of sample. None of the pairs showed a significant correlation.

### 3.3.5 Plasma levels of TXB2 and 6-keto-PGF1α

The plasma concentrations of TXB2 and 6-keto-PGF1α in PIH patients are shown in Figure 3.3 and 3.4 respectively. The levels of TXB2 were increased in normal pregnant women (402(216-902) pg/ml) and in PIH women both with (1091(772-1370) pg/ml) and without (779(565-1047) pg/ml) proteinuria, as compared with nonpregnant women (301(187-397) pg/ml). The increased plasma concentration of TXB2 in PIH women with proteinuria was 262% of nonpregnant women, in PIH women without proteinuria 159%, and in normal pregnant women 34% (Table 3.3). When compared with PIH women without proteinuria (779(565-1047) pg/ml), we also found that the levels of TXB2 were much higher in the PIH women with proteinuria (1091(772-1370) pg/ml) ($p<0.05$). Nonpregnant women (145(91.6-192) pg/ml) produced less 6-keto-PGF1α than normal pregnant women (217(119-499) pg/ml) ($p<0.01$) (Figure 3.4). There were, interestingly, no significant differences in the concentrations of 6-keto-PGF1α between the nonpregnant women (145(91.6-192) pg/ml) and PIH women with (149(101-185) pg/ml) or without (145(52-388) pg/ml) proteinuria ($p=0.65$ and 0.99 respectively). However, the levels of 6-keto-PGF1α in PIH women both with and without proteinuria were significantly lower than that in normal pregnant women (217(119-499) pg/ml) (both $p<0.01$).

### 3.3.6 The ratio of TXB2 and 6-keto-PGF1α

The ratio of TXB2 to 6-keto-PGF1α is an index of the relative activity of the opposing stimuli that modulate vascular tone and platelet activation. This ratio was markedly elevated in PIH women with (7.75(5.91-8.90)) and without (5.68(2.43-19.9)) proteinuria, as compared with normal pregnant women (1.75(1.18-2.63)) (both $p<0.01$) and nonpregnant women (2.08(2.04-2.38)) (both $p<0.01$) (Figure 3.5). The ratio in normal pregnant women did not differ significantly from that in nonpregnant women. There was no significant difference in the ratio between PIH women with proteinuria and those without proteinuria.

**Table 3.2 Correlation between the antioxidant buffering levels and the blood pressure in patients with PIH.**

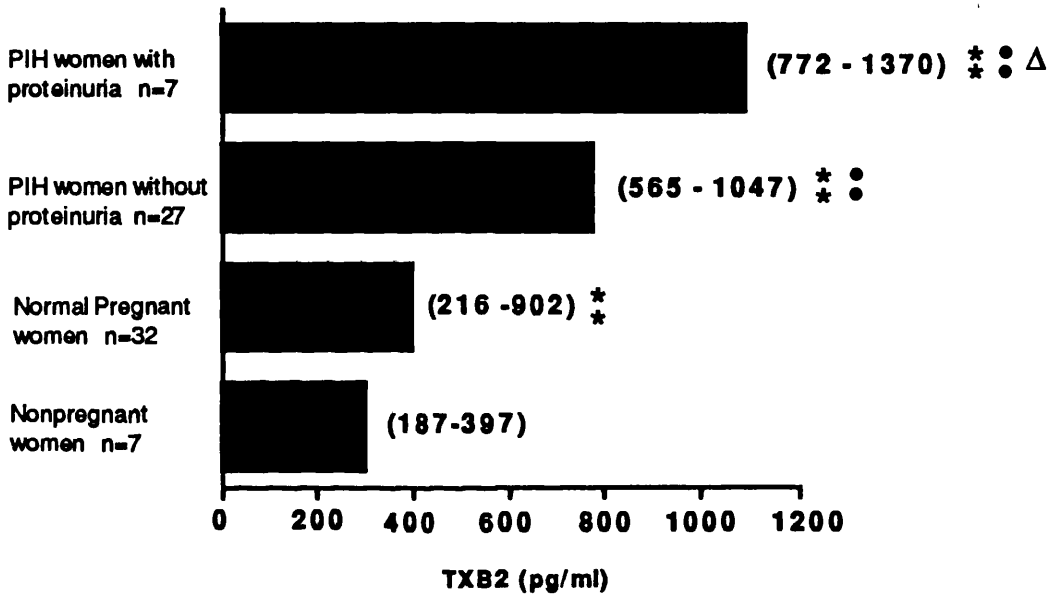
A. PIH patient without proteinuria

| | | PSH | MSH | GSHp | GSHl | SOD | LSH |
|---|---|---|---|---|---|---|---|
| Systolic blood pressure | r | -0.298 | 0.273 | -0.409 | 0.397 | -0.063 | -0.274 |
| | t | 0.300 | 0.780 | 0.540 | 1.310 | 1.140 | 1.590 |
| | p | 0.770 | 0.456 | 0.598 | 0.226 | 0.281 | 0.142 |
| Diastolic blood pressure | r | 0.040 | 0.176 | -0.094 | -0.014 | -0.096 | -0.452 |
| | t | -1.930 | -1.390 | -0.640 | -0.430 | -0.010 | -0.780 |
| | p | 0.083 | 0.194 | 0.535 | 0.676 | 0.995 | 0.452 |

B. PIH patients with proteinuria

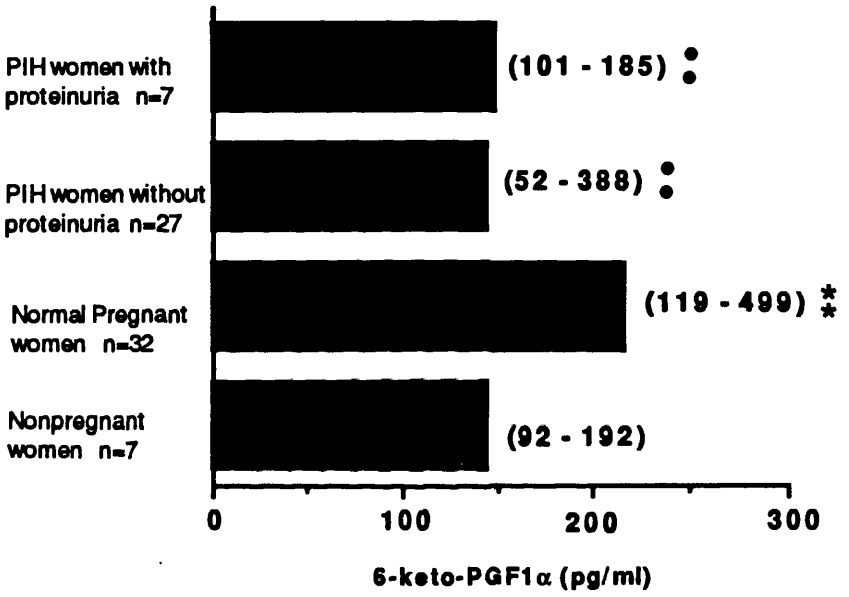
| | | PSH | MSH | GSHp | GSHl | SOD | LSH |
|---|---|---|---|---|---|---|---|
| Systolic blood pressure | r | -0.298 | 0.346 | 0.332 | 0.381 | 0.263 | 0.699 |
| | t | -1.470 | 1.420 | -1.050 | 0.920 | 0.740 | 1.590 |
| | p | 0.380 | 0.391 | 0.485 | 0.424 | 0.593 | 0.142 |
| Diastolic blood pressure | r | 0.115 | 0.537 | 0.048 | 0.058 | 0.021 | 0.472 |
| | t | -9.460 | 11.130 | -9.890 | -0.550 | 10.750 | -0.780 |
| | p | 0.067 | 0.057 | 0.064 | 0.621 | 0.059 | 0.452 |

Figure 3.3 Plasma concentrations of TXB2 in nonpregnant women, normal pregnant women and women with PIH




Plasma was separated from peripheral venous blood and TXB2 was measured by immunoenzymeassay (EIA). Results were represented as the medians with the ranges in parentheses. Statistical significance was expressed as: **$p<0.01$, compared with nonpregnant women; ••$p<0.01$, compared with normal pregnant women; $\Delta p<0.05$, compared with PIH women without proteinuria.

Figure 3.4 Plasma concentrations of 6-keto-PGF1α in nonpregnant women, normal pregnant women and women with PIH




Plasma was separated from peripheral venous blood and 6-keto-PGF1α was measured by immunoenzymeassay (EIA). Results were represented as the medians with the ranges in parentheses. Statistical significance was expressed as: **p<0.01, compared with nonpregnant women; ••p<0.01, compared with normal pregnant women.

**Table 3.3 Increasing rate (%)* of TXB2 and 6-keto-PGF1α in PIH patients and normal pregnant women**

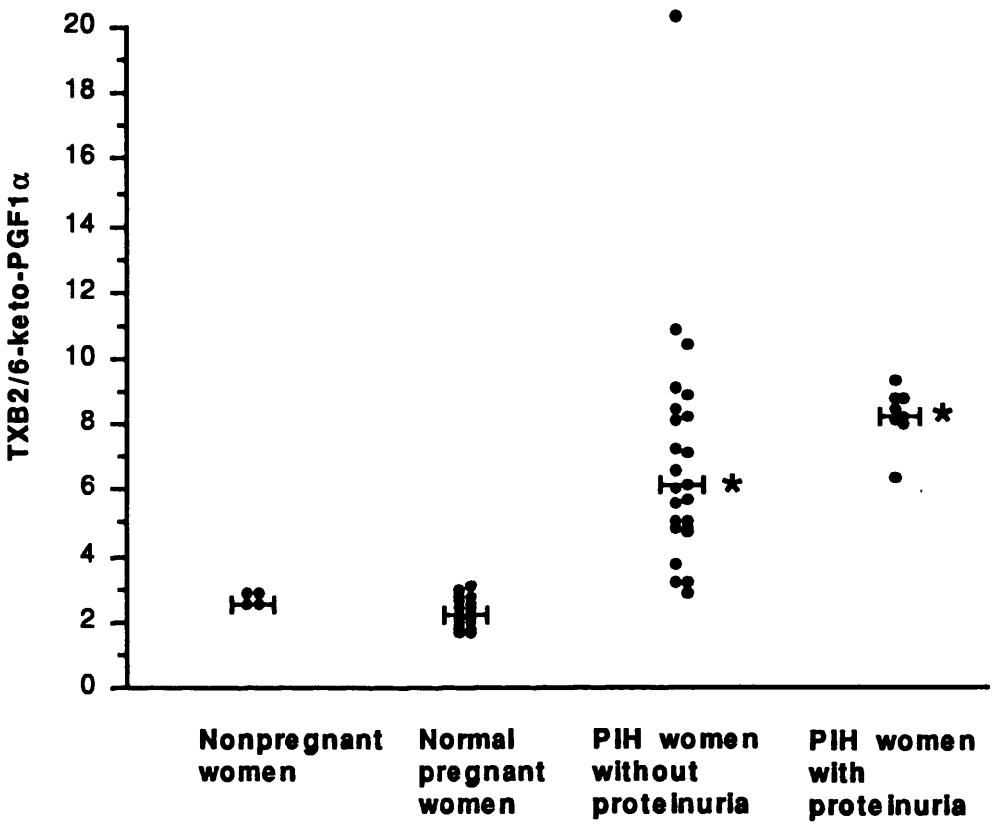
| TXB2 | 6-keto-PGF1α | TXB2/6-keto-PGF1α |
|---|---|---|
| 34 | 50 | -16 |
| 159 | 3 | 173 |
| 262 | 3 | 273 |

* Increasing rate (%) = (A-B)/B x 100

where A: value from normal pregnant women or PIH patient without proteinuria or PIH patient with proteinuria.

B: value from nonpregnant women.

Figure 3.5 The ratio of TXB2 to 6-keto-PGF1α in nonpregnant women, normal pregnant women and women with PIH




TXB2 and 6-keto-PGF1α in plasma were measured by immunoemzymeassay and the ratio of TXB2 to 6-keto-PGF1α was calculated. The individual data and the group medians were represented as: "•" and "⊢⊣" respectively. Statistical significance was expressed as: *$p<0.01$, compared with nonpregnant women and normal pregnant women.

### 3.3.7 Correlation between the antioxidant buffering level and prostaglandins.

Correlation and regression adjustment analyses demonstrated that there were significant positive correlations between the several pairs of markers. The results of the correlations were given in Table 3.4.

**Table 3.4 Correlation and regression between antioxidant markers and TXB2 or 6-keto-PGF1α in patients with PIH.**

| | Pairs | r | t | P |
|---|---|---|---|---|
| Patients without proteinuria | PSH and 6-keto-PGF1α | 0.464 | 5.77 | 0.026 |
| | SOD and 6-keto-PGF1α | 0.700 | 20.21 | 0.001 |
| | SOD and TXB2 | 0.417 | 4.42 | 0.048 |
| Patients with proteinuria | PSH and 6-keto-PGF1α | 0.969 | 76.9 | 0.001 |
| | SOD and 6-keto-PGF1α | 0.765 | 7.04 | 0.045 |

All other correlations were not significant ($p>0.05$).

## 3.4 Discussion

The changes in TXB2 and 6-keto-PGF1α levels in patients with PIH have been well documented (Friedman 1988; Walsh 1990). However none of the studies carried out previously have considered these changes in relation to ROS activity. In this study where TXB2 and 6-keto-PGF1α levels were measured in the same blood sample at one particular point in time, we found the concentrations of TXB2 to be increased and 6-keto-PGF1α to be decreased in PIH patients. These findings are in agreement with previous studies.

A major pathological event in PIH is systemic vasospasm which may lead to multiorgan dysfunction in extreme cases. PGI2 is a potent vasodilator and inhibitor of platelet aggregation. Conversely, TXA2 opposes the actions of PGI2 being a potent vasoconstrictor and platelet aggregator. It is therefore proposed that the most likely cause of vasospasm in PIH is an imbalance between TXA2 and PGI2 (Friedman 1988). This is discussed in detail in Chapter 4 and is not the topic of this Chapter.

In the present study, we have also demonstrated that both extracellular and intracellular antioxidant buffering levels were decreased in the PIH women with proteinuria. While antioxidant buffering levels in PIH women without proteinuria were not significantly different to that seen in the normal pregnant women, levels of four out of the six antioxidant markers tested tended to be lower in the PIH women without proteinuria. This suggests that while the antioxidant buffering level in these patients may be sufficient to scavenge ROS, its reserve capacity may be reduced.

Antioxidants function as blockers of radical processes. The intracellular antioxidant buffering level is considered to be the mechanism for removal of adventitious ROS, whereas the extracellular one serves to control the release of ROS for essential functions (Rice-Evans & Bruckdorfer 1992). Among the intracellular antioxidant systems, GSH, a non-protein free thiol, is thought to be the most important component. It works to reduce hydrogen peroxide to water through the action of GSH peroxidase (Sinclair *et al.* 1990) and traditionally, GSH has been viewed as the major component responsible for maintaining and regenerating protein-thiol groups that are susceptible to disulfide oxidation in RBC. SOD which catalyzes the dismutation of the superoxide anion radical to water and oxygen (Sinclair *et al.* 1990), serves as a major antioxidant enzyme in RBC where superoxide radicals are continuously generated by the autoxidation of hemoglobin (Misra & Fridovich

1972). Therefore, GSH and SOD work together to form a substantial defensive network to protect protein and lipid from ROS attack in RBC.

The levels of PSH in PIH women with proteinuria are very low. The thiol group on albumin accounts for most of the available thiol in plasma and is one of the most likely groups to be attacked by excess ROS. Although most cells are susceptible to oxidative injury, RBC seem to be more at risk because of their high iron content and exposure to high oxygen tension as well as oxidative xenobiotic agents (Cohen *et al.* 1964; Goldberg & Stern 1976; Hebbel *et al.* 1982). While attack by ROS is likely, a more probable route of attack is by membrane damage or even cell lysis. Release of GSH into the plasma would make GSH available to react with caeruloplasmin (CP) to form oxidized glutathione (GSSG) (Figure 3.6). However the assay for GSH which actually measures GSSG + GSH, gave a low result suggesting that some glutathione has been removed. If the plasma is oxidized in PIH women with proteinuria, as suggested by the low PSH, then it may be that GSH is removed by exchange. The levels of SOD were significantly reduced in PIH women with proteinuria. Given that ROS are produced continually in RBC (Misra & Fridovich 1972; Hebbel *et al.* 1982) and there is a lower level of intracellular GSH, the reduced SOD levels may therefore be the result of intracellular attack.

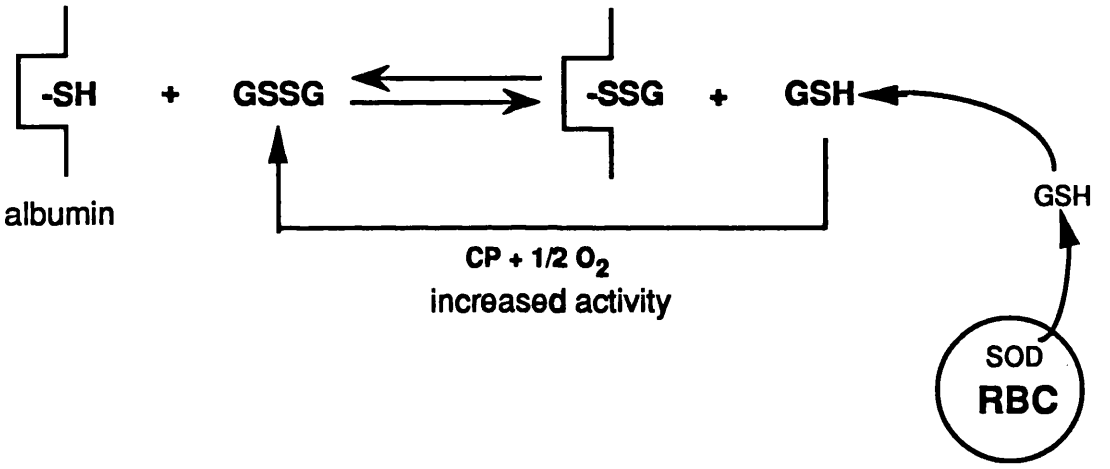
Taken together, a decreased level of antioxidants can mean either an overproduction of ROS which produced a decrease in the antioxidant level or an original antioxidant deficiency which subsequently leads to increased ROS activity. Whatever the mechanism, the balance between antioxidants and ROS is shifted to ROS dominance in PIH. Although we have no direct data to show an increased ROS activity in PIH, this conclusion is in accordance with the findings of others that the formation of ROS and lipid peroxidation were increased in this disease (Maseki *at al.* 1981; Wickens *at al.* 1981; Hubel *at al.* 1989; Sane *at al.* 1989; Roberts *at al.* 1990; Dekker & Kraayenbrink 1991).

Oxidative stress can produce major interrelated derangements of cell metabolism, including DNA strand breakage, rises in intracellular "free" calcium, damage to membrane transports, cell-surface receptors, and other specific proteins especially thiol-containing proteins (for details see Section 1.3 in Chapter 1). These abnormalities may associated with the following pathological changes seen in PIH:

1, Elevation of intracellular calcium in RBC (Sowers *et al.* 1989).

2, Reduced RBC deformability (Cunningham *et al.* 1985; Anceschi *et al.* 1992).

Figure 3.6 Scheme of the glutathione (GSH) and oxidized glutathione (GSSG) pathway




-SH, thiol groups; CP, caeruloplasmin, -SSG, compounds that react with GSH to form GSSG; SOD, superoxide dismutase; RBC, red blood cells.

3, Inhibition of endothelium-derived relaxing factor (EDRF) (Sarrel *et al.* 1990; Pinto *et al.* 1991).

4, Increased immunological activity (See Chapter 2).

5, Imbalance between decreased TXA2 and increased PGI2 (Friedman 1988; Walsh 1990).

6, Damage in endothelial cells (Roberts *et al.* 1989; Zeeman *et al.* 1992).

The mechanisms for each point listed above are given as follows.

Firstly, the $Ca^{2+}$-ATPase and hormone receptors involved in maintaining $Ca^{2+}$ homeostasis contain an essential thiol group (Haenen *et al.* 1989). The impairment of this thiol moiety could lead to increased intracellular levels of calcium (Bast *et al.* 1991). The elevation of intracellular calcium is considered to have a causal role in the enhanced vascular reactivity in PIH (Sowers *et al.* 1989).

Secondly, the composition and organization of biological membranes are important factors that determine the fluidity, permeability and deformability of cells. Biomembrane phospholipids are composed largely of unsaturated fatty acids. ROS attacking the membrane can lead to the oxidative destruction of unsaturated fatty acids in the well-documented process termed lipid peroxidation. It is also known that ROS can damage membrane proteins by causing fragmentation, cross-linking and amino acid modification (Rive-Evans & Bruckdorfer 1992) and furthermore, RBC are more likely to be attacked by ROS (Hebbel *et al.* 1982). Studies have shown that the increased ROS activity results in RBC membrane peroxidation and reduced RBC deformability (Hirayama *et al.* 1986; Davies & Goldberg 1987) and on the other hand that antioxidant agents are important in maintaining RBC deformability (Powell *et al.* 1989). Decreased RBC deformability may result in decreased microcirculatory flow as a result of the RBC, with a normal diameter of 7 μm, no longer being able to deform and negotiate the capillaries, which have an average diameter of 4.5 μm. Thus, in PIH, reduction in RBC deformability may contribute to the decreased placental perfusion and inadequate oxygen supply to the fetus (Cunningham *et al.* 1985; Anceschi *et al.* 1992).

Thirdly, EDRF, released by vascular endothelium, is a potent vasodilator and plays an even more prominent role in the regulation of vascular responsiveness and tone than PGI2 (Vane *et al.* 1990). It is reported that EDRF is protected from breakdown by SOD and is inactivated by $Fe^{2+}$ (Gryglewski *et al.* 1986). $Fe^{2+}$ can damage

EDRF by generation of hydroxyl radicals from $H_2O_2$ in the Haber-Weiss and Fenton reaction (Freeman & Crapo 1982).

Fourthly, increasing data have been documented to support the hypothesis that antioxidants inhibit immunological response whereas ROS activate it via several different ways. Research by Gallagher & Curtis (1984) suggested that ROS could stimulate lymphocytes mitogenically. Others indicated that antioxidants could suppress the expression of receptors for IL-2 and transferrin and production of IL-2 itself (Chaudri *et al.* 1986 & 1988; Dornand & Gerber 1989). Oxidation of lymphocyte surface thiol groups has been shown to result in impairment of suppressor cell activity in patients with rheumatoid arthritis (Brown & Hall 1988). However, ROS have also been reported to inhibit lymphocyte function (Nishida *et al.* 1981; Allan *et al.* 1987). It seems that effects of ROS or antioxidants on the immune system are complicated and the net result depends on their concentrations, types, target cells and other related factors such as calcium and cytokines. Nevertheless, an increased immunological activity has been found in PIH (See Chapter 2). Although there are no direct data yet available to support that decreased antioxidant levels have a causive role in it, there is a good theoretical basis for believing some links may exist.

Fifthly, increased ROS can change the pattern of prostaglandin production in favour of TXA2 synthesis (Moncada *et al.* 1976; Salomon *et al.* 1978; Warso & Lands 1983; Schimke *et al.* 1992). Although the cyclooxygenase (an enzyme essential for PGI2 production) and PGI2 synthase require low levels of hydroperoxides for activation and continued catalysis, high concentrations of hydroperoxide are inhibiting (Ham *et al.* 1979; Markey *et al.* 1987). The activity of cyclooxygenase is also limited by a self deactivation (Brotherton & Hoak 1983). Whorton *et al.* (1985) demonstrated a hydrogen peroxide-induced dose-dependent inhibition of the PGI2 formation in cultured endothelial cells. The role of ROS and antioxidants in changing production of TXA2 and PGI2 in PIH is reinforced by a recent study which showed increased lipid peroxides and decreased vitamin E levels in PIH maternal blood associated with an imbalance between TXA2 and PGI2 (Wang *et al.* 1991). The observations of positive correlation between the ROS scavengers and 6-keto-PGF1$\alpha$ in the present study is another piece of evidence to support this theory.

Sixthly, it has been well established that the increased intracellular GSH protects endothelial cells against oxidative damage whereas decreased intracellular GSH sensitizes endothelial cells to oxidative cytolysis (Harlan *et al.* 1984; Tsan *et al.*

1985). Endothelial cell injury is so important in the development of PIH that PIH has been characterised as an endothelial disorder (Roberts *et al.* 1989). RBC circulate within the vascular system and it is possible that the change of antioxidant levels in RBC reflects an occurrence of such an alteration in the vascular system including the vascular endothelial cells. This suggestion is further supported by our observation that extracellular antioxidant buffering level is also decreased in patients with PIH.

Decreased antioxidant buffers can be associated with several important features of PIH. However, what is the exact role played by antioxidants or ROS in the development of PIH? Unfortunately, at present, there is not enough evidence to give a completely satisfactory answer to this question. However, it seems that increased ROS activity or decreased antioxidants may form the link between the placental immunological maladaptation and the injury of endovascular trophoblast and endothelial cells in PIH. Immunological maladaptation can lead to placental hypoxia which occurs in PIH (Kaar *et al.* 1980; Lunell *et al.* 1984). As a result of hypoxia, ROS formation is increased (See Section 1.3.2 in Chapter 1) and subsequently damage to endovascular trophoblasts and endothelial cells occurs. Since PIH is primarily a placental disease with disappearance of its symptoms and lesions soon after termination of pregnancy (Chesley 1978; Roberts 1984; Redman 1991), the placental hypoxia is likely to be the major cause of increased ROS or decreased antioxidant levels. Arachidonic acid metabolism and leukocytes can also attribute to this changes (See Section 1.3.2 in Chapter 1). Work presented later in this thesis (Chapter 6) will indicate that the a reduced SOD activity is an acquired phenomenon as its gene structure and expression is normal, but whether there is a genetic factor involved in the other defective antioxidant agents still needs further studied (Walsh 1990).

Although the extracellular antioxidant buffering level is less important in removing ROS than the intracellular one, its state can modulate intracellular antioxidant capability. Tsan *et al.* (1989) has reported that exogenous GSH can enhance intracellular concentrations of GSH. From this point of view, the decreased extracellular antioxidant buffering level in patients with PIH could be regarded as the reduction of the reserve capacity of intracellular antioxidant buffering level.

Plasma concentrations of ROS oxidation products are reported to have a correlation with the level of the blood pressure in PIH (Zeeman *et al.* 1992). However, no correlation was found between the antioxidant buffering level and blood pressure in

the present study. This observation could suggest that the decreased antioxidant ability may not be the direct cause of raised blood pressure in PIH.

If the reduced antioxidant buffering level and/or the increased ROS activity did damage RBC by changing RBC deformability and fragility, it is possible that the RBC count could be reduced in patients with PIH. However, the RBC count in PIH women either with proteinuria or without proteinuria was found to be no different from that in the normal pregnant women in this study, although it has been reported by others (Skajaa 1929).

PSH, an extracellular antioxidant agent, is a thiol group located mainly on serum albumin. Therefore, one question arises from this fact. Does an increment in associated protein excretion due to renal dysfunction contribute to the decreased antioxidant level found in PIH women with proteinuria? To date, there is no direct data to prove or exclude this possibility. However, since the reduced antioxidant levels in PIH appear to be both intracellular and extracellular and the decreased antioxidant agents are both proteinous and non-proteinous (such as GSH and SOD). Thus it is unlikely that the renal protein loss can be attributed to the decreased antioxidant state in PIH. However, further studies are needed to clarify this question.

Finally, the emerging appreciation of the role of ROS or antioxidants in the pathogenesis of PIH suggests that a possible strategy for therepeutic intervention. A wide range of antioxidants, both natural and synthetic (Table 3.5), has been proposed for use in the treatment of human disease (Greenwald 1991) and there is clear evidence accumulating that the presence of antioxidants such as vitamins E and C in the blood may have a protective role against cardiovascular diseases (Rice-Evans & Bruckdorfer 1992). Although, until now, antioxidant features have not been a factor to be considered by clinicians to choose drugs for treatment of PIH, in fact, some anti-hypertensive drugs have been recently found to be antioxidant (Mak & Weglicki 1988; Nayler & Britnell 1991; See Chapter 5). Whether using those anti-hypertensive drugs with antioxidant property or other antioxidant agents is more or less beneficial to PIH patients remains an interesting question.

**Table 3.5 Some antioxidants available for therapeutic use**

| Naturally-occurring molecules and related substances | Synthetic molecules |
|---|---|
| SOD (isolated or recombinant) | Thiols (e.g., N-acetylcysteine, mercaptopropionylglycine) |
| α-Tocopherol | |
| Ascorbic acid | Synthetic chelators (e.g.,hydroxypyridones) |
| Adenosine | |
| Transferrin | Xanthine oxidase inhibitors (e.g., allupurinol, oxypurinol, amflutizole) |
| Lactoferrin | |
| Glutathione and its percursors | |
| Carotenoids, other plant pigments | Inhibitors of phagocyte activation |
| Deferoxamine | Lipid-soluble chain-breaking antioxidants (e.g., probucol) |
| | Trolox C |

## 3.5 Conclusions

1, Both extracellular and intracellular antioxidant buffering levels were decreased in patients with PIH, especially in those with proteinuria. This probably reflects an occurrence of increased ROS activity in this disorder.

2, The reduction of the antioxidant buffering levels may account for several important pathological feathers seen in PIH and thus play a role in the development of PIH.

3, Plasma PGI2 level was decreased in PIH whereas TXA2 increased, resulting in an increased ratio of TXA2 to PGI2. These changes may result from the imbalance between decreased antioxidant buffering levels and increased ROS formation in PIH.

Postscript to Chapter 3

The concentrations of 6-keto-PGF1α and TXB2 found here are much higher than others (FitzGeral *et al.* 1983. Circ 67:1174-1176; Ritter *et al.* 1983. Lancet 1:317-319; McLaren *et al.* 1985. Thrombosis Res. 37:177-183; Greer *et al.* 1985. Br J Obstet Gynaecol 92:581-585). In the present study an enzymeimmunoassay was applied to detect these two substances but most of others used a radioimmunoassay method. Although the different methods used may account for the differences, other factors could not be completely excluded, such as cross-reaction with other substances especially 11-dehydro-thromboxane B2, 6-keto-prostaglandin E1 and E2 because the cross-reaction for these prostaglandins has not been checked with the enzymeimmunoassay. Therefore a further investigation is needed.

# CHAPTER FOUR PROSTACYCLIN AND THROMBOXANE

## 4.1 Introduction

### 4.1.1 Importance of PGI2 and TXA2 in PIH

PIH is characterized by increased blood pressure, general vasoconstriction, and platelet hyperactivity (Pritchard *et al.* 1976; Lindheimer & Katz 1992). Theoretically, these changes could be caused by an imbalance between PGI2, a potent vasodilator and an inhibitor of platelet aggregation, and TXA2, a potent vasoconstrictor and a stimulant of platelet aggregation. A deficiency of PGI2 could account for endothelial cell injury through impairment of its cytoprotective function, whereas a predominance of TXA2 could account for the vasospasm and activation of intravascular coagulation (Friedman 1988). It is now well established that a major mechanism involved in the pathophysiologic changes of PIH is an alteration of the ratio of TXA2 to PGI2, with a change in the direction of TXA2 dominance (Friedman 1988; Walsh 1990). The importance of the change of this ratio in PIH has been further proved by the studies showing decreased PGI2 biosynthesis preceding the development of clinical disease (Fitzgerald *et al.* 1987) and the reduced incidence of PIH produced by low-dose aspirin treatment (Wallenberg *et al.* 1986; Wallenberg & Rotman 1988; Benigni *et al.* 1989; Schiff *et al.* 1989), which can selectively suppress the synthesis of platelet TXA2 without inhibiting the production of vascular PGI2 (Walsh 1990; Walsh *et al.* 1992). In addition, it has been demonstrate` that urinary excretion of TXB2 metabolites correlates with the severity of PIH (Fitzgerald *et al.* 1990). Considerable attention has been focused on the pathologic role of PGI2 and TXA2 in PIH and the clinical significance of them.

### 4.1.2 Unresoloved questions

Although it has been well documented that the changes of PGI2 and TXA2 play an important role in the development of pathophysiologic and clinical features of PIH (Friedman 1988; Walsh 1990), it remains questionable as to whether PGI2 deficiency and/or TXA2 overproduction are a primary change and thus perhaps a cause of PIH, or whether they reflect some other, more basic changes, such as an increase in ROS which has been known to occur in PIH women and can specifically inhibit the synthesis of PGI2 (Higgs & Vane 1983; Wang *et al.* 1989; Wang *et al.* 1992; Warso & Lands 1983, also see Chapter 3).

The incidence of PIH in pregnant women is not increased using a high dose of inhibitors of prostaglandin synthesis (Dekker 1989; Briggs *et al.* 1990) and there is at least one study suggesting that PGI2 production merely functions as a rescue mechanism, especially during periods of ischemia and hypoxia (Spokas *et al.* 1983). On the other hand, another study has shown that a decrease in PGI2 production precedes the onset of PIH (Fitzgerald *et al.* 1987), suggesting that the changes in prostaglandin production might play a aetiological role in the development of PIH. Furthermore, it is reported that infusion of exogenous PGI2 to PIH women and pregnant animals, drastically reduces maternal blood pressure, diminishes platelet consumption and increases the uterine blood flow (Clark *et al.* 1982; Clark & Harrington 1982; Fidler *et al.* 1980; Lewis 1983; Walker *et al.* 1982).

The cause of the changes of PGI2 and TXA2 production in PIH remains unclear. Previous studies designed to assess the direct effects of PIH serum on several features of PIH demonstrated that there was a factor(s) 1, cytotoxic to human endothelial cells (Rodgers *et al.* 1988); 2, mitogenic to fibroblasts (Musci *et al.* 1988); 3, stimulating platelet-derived growth factor synthesis (Taylor *et al.* 1991b); 4, inducing a selective activation of endothelial cell procoagulant protein production (Taylor *et al.* 1991a). These data strongly suggest that there is a specific circulating factor(s) existing in PIH patients and functioning to induce the features of PIH.

### 4.1.3 Aims of the study

The aim of this study was to determine the production of PGI2 and TXA2 in human peripheral blood mononuclear cells (PBMC) and to investigate whether PIH serum is relevant to this event. The rational for using PBMC instead of umbilical vein endothelial cells as the study model was several fold. Firstly, the advanced assay systems for prostaglandins have proved that human lymphocytes which are the major component of PBMC can produce detectable amounts of prostaglandins (Aussel *et al.* 1987). Secondly, most of previous studies concerning the prostaglandins in PIH were carried out on the umbilical vein endothelial cells or placental tissue. The production of PGI2 and TXA2 by PBMC has not been examined in PIH. Thirdly, if PIH is associated with a specific factor(s) in the serum, the factor must circulate systemically rather than being present only in local tissues such as the placenta. The prostaglandins are considered to be local mediators, generally exerting their biologic effects within a micrometer of their site of synthesis (McCormack *et al.* 1991). As the PBMC are the major functional cells in the circulation, their production of prostaglandins is potentially much more important than that produced by other cells.

Lastly, unlike placenta or umbilical vein endothelial cells, PBMC are available easily from the patients with PIH throughout the whole period of pregnancy. Therefore, the use of PBMC as the study material is more practical.

## 4.2 Materials and Methods

### 4.2.1 Reagents

Fetal calf serum (FCS) was supplied by Northumbria Biologicals Ltd., Cramlington, UK. PBS and lymphoprep$^{TM}$ were purchased from Oxoid Limited, England and Nyegaard Co., Oslo, Norway respectively.

RPMI 1640 (10x) was obtained from Gibco, BRL and the culture medium was prepared according to the manufacturers instructions. For details see Section 2.2.3 in Chapter 2.

The enzymeimmunoassy (EIA) systems for TXB2 and 6-keto-PGF1$\alpha$ were purchased from Amersham International plc., Amersham, U.K.

### 4.2.2 Subjects

Three groups of subjects were studied: 1, 16 normotensive pregnant women, age: 27.3±4.6 (mean±SD) years, gestational length: 34.8±3.8 weeks. 2, 9 PIH women without proteinuria, age: 26.6±4.1 (mean±SD) years, gestational length: 35.3±4.2 weeks. 3, 6 PIH women with proteinuria, age: 25.9±4.1 (mean±SD) years, gestational length: 35.1±4.9 weeks. All were primigravidas. The PIH women were sampled after diagnosis but before any anti-hypertensive therapy.

PIH is defined as a persistent or recurrent diastolic blood pressure of ≥90mmHg developing during pregnancy after 20 weeks of gestation, and resolving by six weeks post partum. Proteinuria is defined as the persistent presence of protein in the urine of ≥ + on urine 'dipstick' testing, or >300 mg excreted in 24 hours. The latter measurement is preferred where available. Patients who had a history of hypertension before the twentieth week of pregnancy were designated as essential hypertension. (see Section 1.1.3 in Chapter 1).

### 4.2.3 PBMC preparation

Blood was obtained from the three group patients stated above by venipuncture and collected into a sterile universal container with 300 units of preservative-free heparin (final concentration in blood: 30 units/ml, Leo Laboratories). PBMC were separated by density gradient centrifugation using a modification of the method originally described by Boyum (Boyum, 1968). Heparinised blood was layered on to an equal volume of lymphoprep$^{TM}$ in 15 ml plastic conical tubes (diameter 12mm). Following centrifugation at 500 x g for 30 minutes at room temperature (25°C), the mononuclear cell bands at the lymphoprep/plasma interface were removed using a Pasteur pipette. The bands/cells were washed three times in PBS by centrifugation at 400 x g for 10 minutes and the cell pellet resuspended in RPMI 1640 containing 2mM L-glutamine, sodium bicarbonate, penicillin and streptomycin. The cell number was assessed in a 10 µl aliquot of cell suspension stained with 90 µl of white cell staining solution (2% glacial acetic acid, a few grains of crystal violet in PBS) and then counted in a hematocytometer (Improved Neubauer). Their viability, checked with trypan blue staining, was routinely higher than 95%.

### 4.2.4 Serum preparation

Blood was centrifuged at 2000 rpm for 10 minutes and serum was obtained. Serum was frozen at -70°C until use.

### 4.2.5 Cell culture

The modified method described here is originally from that of Jakob et al.(1990). 2 x $10^5$ PBMC were incubated in 96 well-U-shaped microtitre plates, at 37°C, 5% $CO_2$ for 48 hours. 62.5 ug/ml of ConA (Sigma Chemical Company Ltd., UK) was added at the initiation of the culture. To test the effects of serum on the production of PGI2 and TXA2 from normal pregnant PBMC, serum was added to cells to give a concentration of 10% at the beginning of the culture. After incubation the supernatants were obtained and assayed for thromboxane B2 (TXB2) and 6-keto-prostaglandin F1α (6-keto-PGF1α) levels (the stable metabolites of TXA2 and PGI2, respectively). The sample controls consisted of PBMC processed in the same manner as those treated with ConA and serum. This control was set up to take account of spontaneous release of TXB2 and 6-keto-PGF1α. The medium controls were tested for medium intereference with the assay system and found to contain 6-

keto-PGF1α and TXB2 concentrations of less than 5% and 4% respectively of the concentrations of detected in supernatants of the background PBMC culture.

### 4.2.6 Measurement of TXB2 and 6-keto-PGF1α

The levels of 6-keto-PGF1α and TXB2 (the stable metabolites of TXA2 and PGI2, respectively) in supernatants were determined by the EIA method, performed according to the instructions. Briefly, 50 µl of sample was added to a 96-well plate which was coated with donkey anti-rabbit IgG and then 50 µl of rabbit anti-6-keto-PGF1α or rabbit anti-TXB2 was added. After incubation by shaking at room temperature, the samples were reacted with 6-keto-PGF1α- or TXB2-horseradish peroxidase for 1 hour. The wells were washed four times with wash buffer and then 150 µl enzyme substrate (TMB) was dispensed into the wells. The reaction was stopped by adding 100 µl of 1.0M sulphuric acid and read at 450nm within 30 minutes. The assay sensitivity, defined as the amount of TXB2 or 6-keto-PGF1a needed to reduce zero dose binding by two standard deviations was 3.6 pg/ml and 3.0 pg/ml respectively. The intra and inter-assay variations for TXB2 assays were 2.5% and 9.9% respectively and for 6-keto-PGF1α 4.5% and 14.8%. The cross-reactivity for TXB2 and 6-keto-PGF1α was performed with a number of related compounds and the results shown in Table 4.1.

### 4.2.7 Result Adjustment

Serum 6-keto-PGF1α levels are lower and TXB2 levels higher in preeclamptic women (Friedman 1988). Therefore in those experiments where the effects of serum on prostaglandin production are considered the results need to be adjusted according to 6-keto-PGF1α and TXB2 levels in the serum used. In this experiment 10% serum was added to a final volume of 0.2 ml per culture well to give a 0.02 ml of serum per well. The determination of serum levels of 6-keto-PGF1α and TXB2 showed that 0.02 ml of serum from preeclamptic women with proteinuria contained 13.78 (median) pg more TXB2 and 1.36 (median) pg more 6-keto-PGF1α than the same volume of serum from normal pregnant women and serum from those without proteinuria contained 7.54 (median) pg more TXB2 and 1.44 (median) pg more 6-keto-PGF1α. Therefore, 13.78 for TXB2 and 1.36 for 6-keto-PGF1α should be subtracted from the results using proteinuric-preeclamptic serum. The same principle also applies to the data from using non-proteinuric-preeclamptic serum.

**Table 4.1 The cross-reactivity for TXB2 and 6-keto-PGF1α+**

| Compound | % Cross-reactivity TXB2 | 6-keto-PGF1α |
|---|---|---|
| Thromboxane B2 | 100.0 | 0.03 |
| 6-keto-prostaglandin F1α | ND* | 100.0 |
| 11-dehydro-thromboxane B2 | 0.10 | ND |
| 2,3-dinor-6-keto-prostaglandin F1α | <0.40 | 10.5 |
| 2.3-dinor-thromboxane B2 | 60.50 | ND |
| 6,15-diketo-13,14-dihydro-prostaglandin F1α | <0.01 | 0.2 |
| 6-keto-prostaglandin E1 | ND | 9.2 |
| 6-keto-prostaglandin E2 | <0.01 | ND |
| Arachidonic acid | 0.01 | 0.01 |
| Prostaglandin D2 | 0.18 | 0.5 |
| Prostaglandin E2 | <0.01 | 2.8 |
| Prostaglandin F1α | 1.6 | 2.1 |
| Prostaglandin F2α | 0.06 | 1.4 |

* The cross-reactivity was not done.

+From the instruction of enzymeimmunoassay system for TXB2 and 6-keto-PGF1α (Amersham International plc., Amersham, UK).

### 4.2.8 Statistical Analysis

Since the levels of TXB2 and 6-keto-PGF1α are not normally distributed (Moodley *et al.* 1984), their data are expressed as medians and ranges. To determine whether comparisons among groups were valid, we first performed a Kruskal-Wallis analysis. Where permitted, the two-tailed Mann-Whitney was then used to test statistical difference (Siegel & Castellan 1988). All statistical analyses were done by using Minitab Statistical Software. A p value of less than 0.05 was considered to indicate statistical significance.

## 4.3 Results

### 4.3.1 Production of 6-keto-PGF1α

Figure 4.1 shows that the production of 6-keto-PGF1α from PBMC tended to be lower in PIH patients both with and without proteinuria than in normal pregnant women (257, 218 and 324 pg per $10^6$ cells, respectively), although this did not reach the statistical significance ($p=0.153$ and $p=0.054$ respectively). There was no significant difference between the PIH patients with proteinuria and those without proteinuria ($p>0.05$).

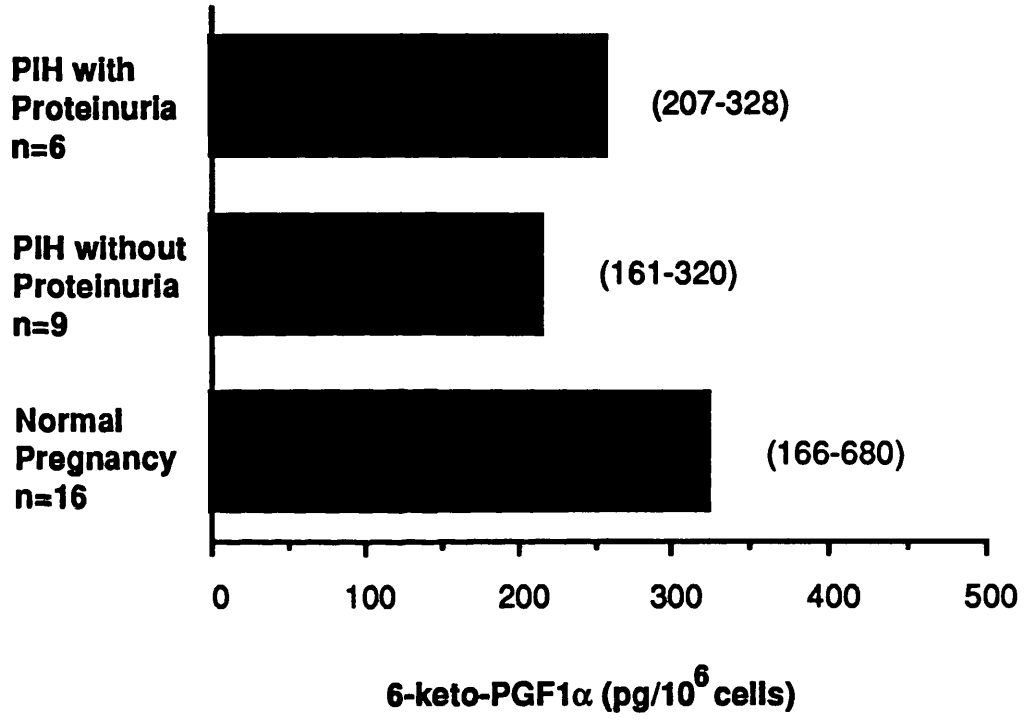
### 4.3.2 Production of TXB2

Figure 4.2 shows that the concentrations of TXB2 from PBMC were significantly higher in PIH patients both with and without proteinuria than in normal pregnant women (1397, 1065 and 897 pg per $10^6$ cells respectively and both $p<0.01$). The levels of TXB2 in PIH patients with proteinuria was significantly increased compared with those without proteinuria ($p<0.05$).

### 4.3.3 Ratio of TXB2 to 6-keto-PGF1α

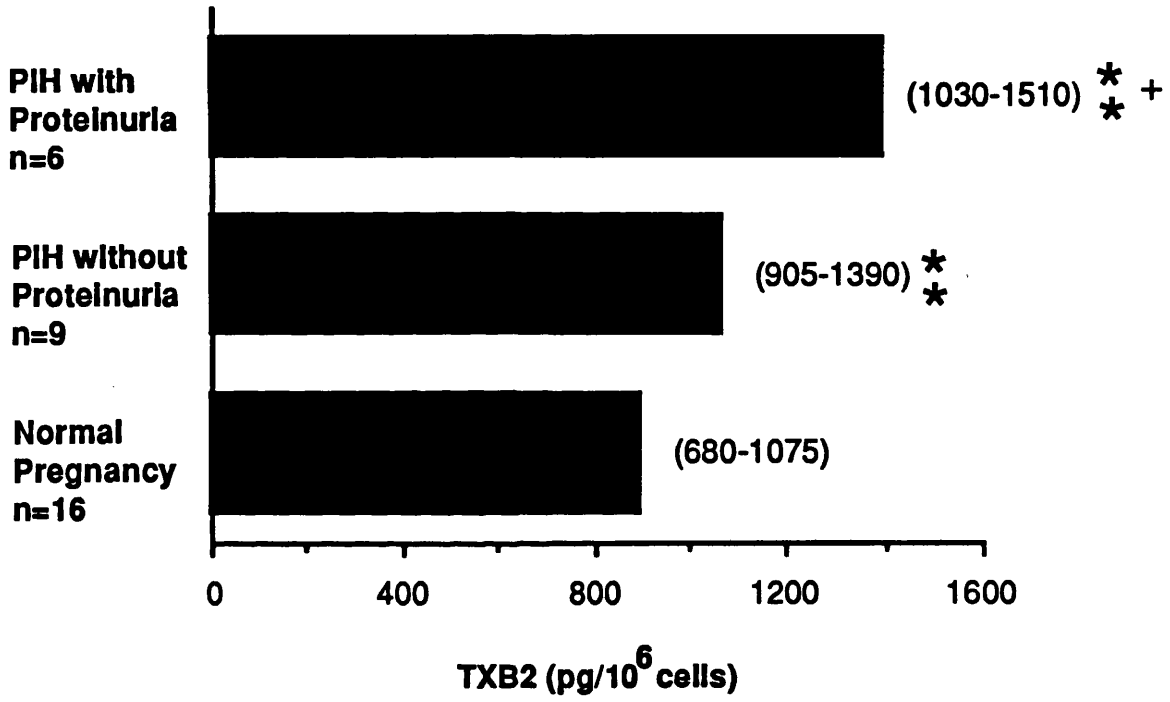
Figure 4.3 shows the change of the ratio of TXB2 to 6-keto-PGF1α. This ratio reflects the relative concentrations of TXB2 and 6-keto-PGF1α. Therefore, its value is an index of the relative activity of the opposing stimuli that regulate vascular tone and other functions. This ratio was significantly elevated in PIH patients both with and without proteinuria compared to that in normal pregnant women (5.30, 5.16 and 2.46 respectively and both $p<0.01$). However, there was no significant difference between the PIH patients with proteinuria and those without proteinuria ($p>0.05$).

**Figure 4.1.** **The production of 6-keto-PGF1α in PBMC from women with PIH and normal pregnant women**



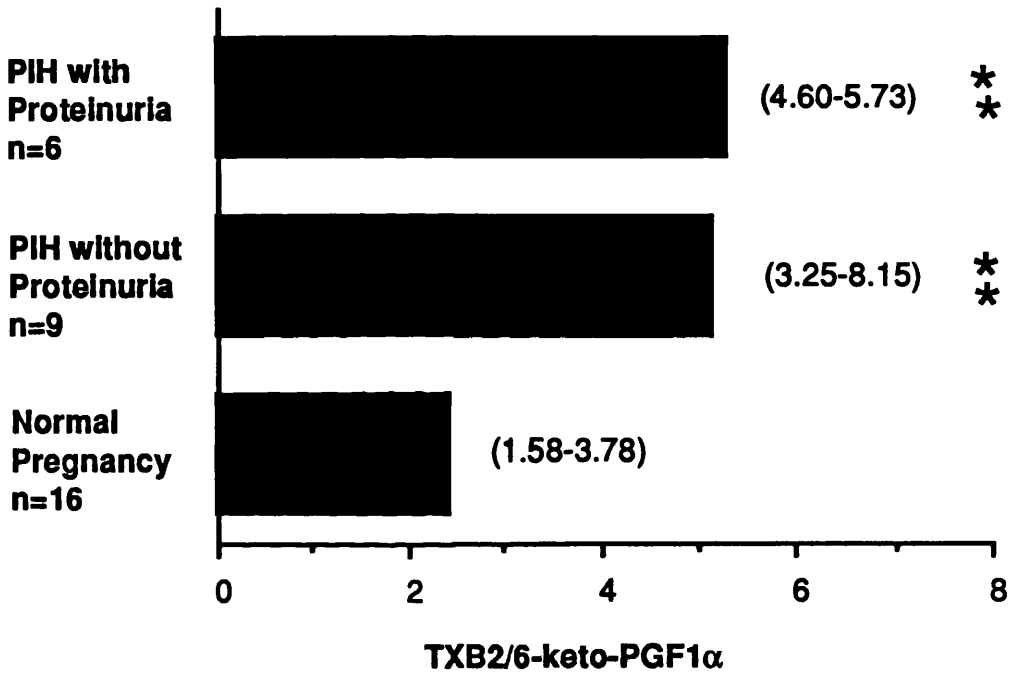

PBMC were stimulated with ConA and incubated for 48 hours. 6-keto-PGF1α in the supernatants was measured by the method of enzymeimmunoassy. The data are represented as the medians with the ranges in parentheses.

**Figure 4.2.** **The production of TXB2 in PBMC from women with PIH and normal pregnant women**




PBMC were stimulated with ConA and incubated for 48 hours. TXB2 in the supernatants was measured by the method of enzymeimmunoassy. The data are represented as the medians with the ranges in parentheses. Statistical significance is expressed as: **p<0.01, compared with nomal pregnant women; +p<0.05 compared with PIH women with proteinuria.

**Figure 4.3.** **The ratio of TXB2 to 6-keto-PGF1α in women with PIH and normal pregnant women**




PBMC were stimulated with ConA and incubated for 48 hours. TXB2 and PGF1α in the supernatants was measured by the method of enzymeimmunoassy and the ratio of TXB2 to 6-keto-PGF1α was calculated. The data are represented as the medians with the ranges in paretheses. Statistical significance is expressed as: **$p<0.01$, compared with nomal pregnant women.

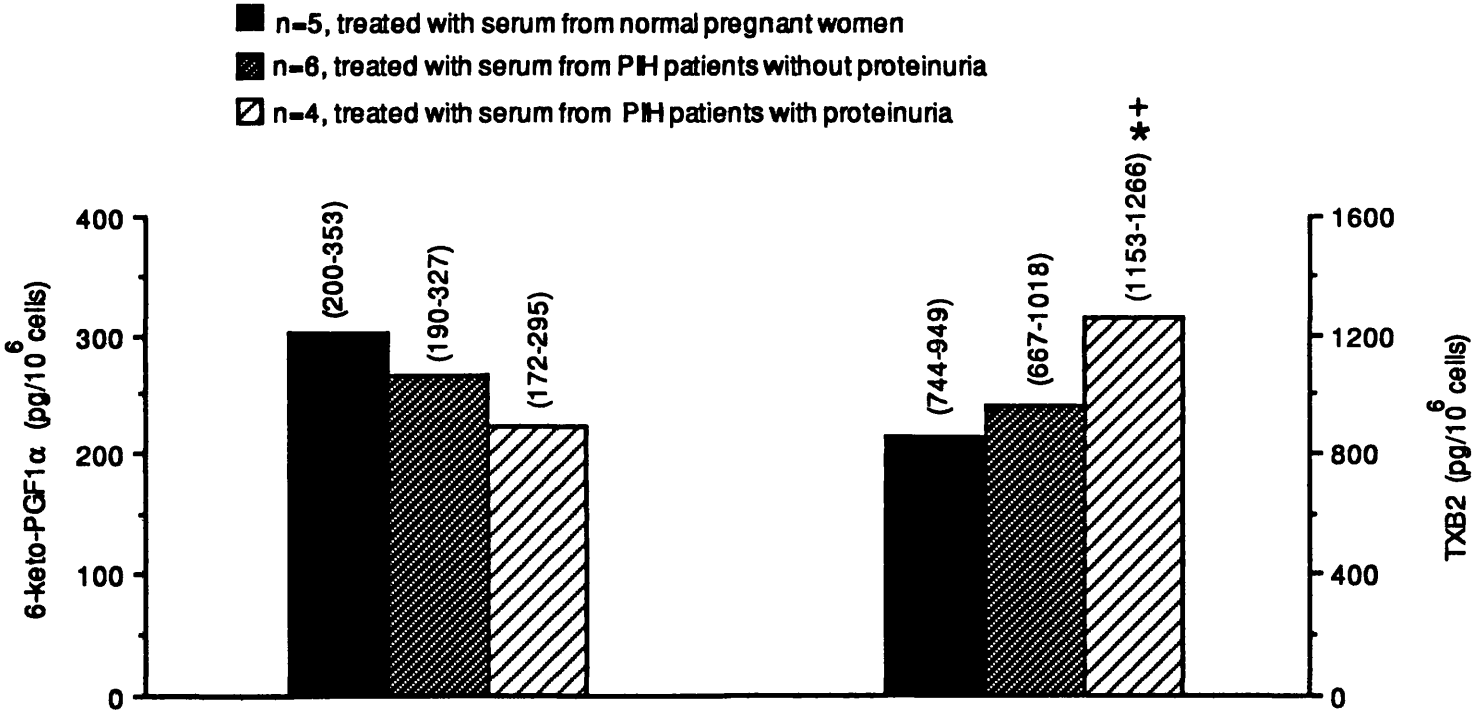
### 4.3.4 Effect of PIH sera on 6-keto-PGF1α and TXB2 production

The effects of sera on the production of 6-keto-PGF1α and TXB2 are illustrated in Figure 4.4. PBMC from normal pregnant women exposed to sera from normal pregnant women release 301 pg 6-keto-PGF1α per $10^6$ cells into their conditioned media, whereas conditioned media from identical cells treated with serum from PIH women with proteinuria contained 223 pg 6-keto-PGF1α per $10^6$ cells. Serum from PIH women with proteinuria significantly increased the production of TXB2 as compared with identical cells treated with normal pregnant sera (1241 vs 844 pg per $10^6$ cells, $p<0.05$) or treated with non-proteinuric-PIH serum (1241 vs 950 pg per $10^6$ cells, $p<0.05$). However, serum from PIH women without proteinuria did not significantly affect either 6-keto-PGF1α or TXB2 formation. Cells treated with serum from PIH women with proteinuria had a significantly increased ratio of TXB2 to 6-keto-PGF1α, compared to cells treated with sera from either normal pregnant women (5.54 vs 2.81, $p<0.01$) or from PIH women without proteinuria (5.54 vs 3.19, $p<0.01$) (Figure 4.5). Although the ratio of TXB2 to 6-keto-PGF1α from cells treated with PIH patients without proteinuria tended to be higher than that of cells treated with normal pregnant sera (3.19 vs 2.81), this difference did not reach statistical significance ($p>0.05$).

### 4.3.5 6-keto-PGF1α and TXB2 production from PBMC with and without sera treatment.

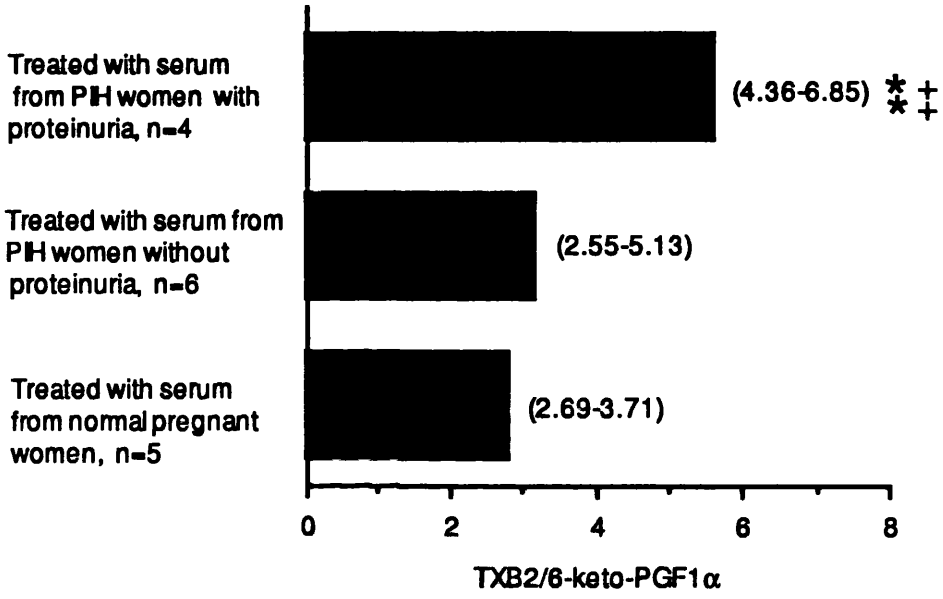
When production of 6-keto-PGF1α and TXB2 from PBMC without serum treatment was compared to that with treatment, it was found that TXB2 levels from normal pregnant PBMC treated with the serum from PIH women with proteinuria was similar to that from proteinuric-PIH PBMC which had not received serum treatment (1241 vs 1398 pg per $10^6$ cells, $p>0.05$). The ratio of TXB2 to 6-keto-PGF1α in normal pregnant PBMC treated with proteinuric-PIH serum was also similar to that of proteinuric-PIH PBMC without serum treatment (5.54 vs 5.30, $p>0.05$) and was significantly higher than that of normal pregnant PBMC without serum treatment (5.54 vs 2.46, $p<0.01$).

**Figure 4.4. The effects of sera on the production of 6-keto-PGF1α and TXB2**




PBMC were stimulated with ConA and incubated for 48 hours. Test sera (10%) were added to cells at the beginning of incubation. 6-keto-PGF1α and TXB2 in the supernatants were measure after 48-hour incubation. The data are represented as the medians with the ranges in parentheses. Statistical significance is expressed as: *$p<0.05$ compared with PBMC treated with normal pregnant serum; +$p<0.05$ compared with PBMC treated with serum from PIH women without proteinuria.

**Figure 4.5.** **The ratio of TXB2 to 6-keto-PGF1α after serum treatment**




PBMC were stimulated with ConA and incubated for 48 hours. Test sera (10%) were added to cells at the beginning of incubation. TBX2 and 6-keto-PGF1α in the supernatants were measured after culture and the ratio of TXB2 to 6-keto-PGF1α was calculated. The data are represented as the medians with the ranges in parentheses. Statistical significance is expressed as: **$p<0.01$, compared with PBMC treated with nomal pregnant serum; ++$p<0.01$ compared with PBMC treated with serum from PIH women without proteinuria.

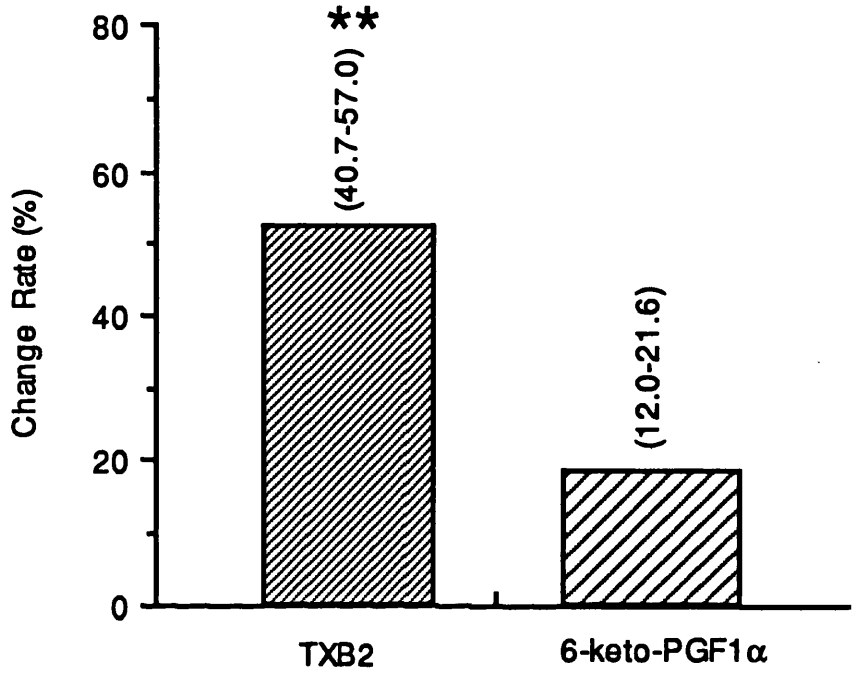
### 4.3.6 A comparison of the effect of serum from proteinuric PIH patients on TXB2 production with that on 6-keto-PGF1α production

It was noted that although sera from PIH patients with proteinuria affected both TXB2 and 6-keto-PGF1α formation, the effects on TXB2 was greater than that on 6-keto-PGF1α. In order to assess this difference, change rate was calculated as follows.

$$\text{Change Rate (\%)} = \frac{\text{Sample treated with PIH serum} - \text{Sample treated with normal pregnant serum}}{\text{Sample treated with normal pregnant serum}} \times 100\%$$

The results of this showed that when PBMC were treated with sera from PIH patients with proteinuria, the change rates of TXB2 is significantly greater than that of 6-keto-PGF1α (Figure 4.6).

**Figure 4.6. Comparison the effect of serum on TXB2 production with that on 6-keto-PGF1α production**




PBMC were stimulated with ConA in the presence of 10% test sera. After 48-hour incubation, 6-keto-PGF1α and TXB2 in the supematants were measured. Change rate was calculated as follows:

$$\text{Change Rate (\%)} = \frac{\text{Sample treated serum from PIH women with proteinuria} - \text{Sample treated with serum from normal pregnant wome}}{\text{Sample treated with serum from normal pregnant women}} \times 100\%$$

The data are represented as the medians with the ranges in parentheses. Statistical significance is expressed as: **$p<0.01$, compared with 6-keto-PGF1α.

## 4.4 Discussion

In recent years, evidence from maternal plasma, maternal urine, fetal plasma, fetal vessels, amniotic fluid and fetoplacental units has supported the concept that PIH is associated with a functional imbalance between PGI2 and TXA2 (Friedman 1988; Walsh 1990). Using PBMC as a study model, we have also confirmed that changes of PGI2 and TXA2 production occur in PIH. The balance between PGI2 and TXA2 shifted to a dominance of TXA2. In normal nonpregnant women production of PGI2 is below the threshold for biological activity (Fitzgerald *et al.* 1981). However, in view of its enhanced production in normal pregnancy (Brash *et al.* 1983), PGI2 may regulate blood pressure. Thus, a reduction in PGI2 biosynthesis may play a pathophysiological role in PIH through its effects on vascular smooth muscle. In addition to PGI2 deficiency, TXA2 overproduction could exaggerate vasoconstriction in PIH, because the balance between PGI2 and TXA2, rather than either agent alone, may be a factor resulting in vascular dysfunction and thereafter hypertension and probably a reduction of uterine and placental blood flow (Lunell *et al.* 1984)

Alternatively, PGI2 and TXA2 may play a role in PIH through their effects on platelets. A decreased PGI2 biosynthesis and increased TXA2 production may result in increased platelet activation, either systemically or in the uterine vascular bed, leading to enhanced vascular tone through release of platelet vasoactive mediator or obstruction of the placental vasculature by platelet aggregates (Moncada & Vane 1979; Lindheimer & Katz 1981; Editorial 1986). Consistent with platelet activation in this disease, a decrease in platetlet count, an increase in platelet aggregation and an excessive release of beta-thromboglobulin and other platelet factors have been observed in patients with PIH (Inglis *et al.* 1982; O'Brien *et al.* 1986).

Of primary importance, this study has demonstrated that the sera from PIH women with proteinuria contained a factor(s) which could suppress the PGI2 production and enhance TXA2 synthesis in PBMC. It is unlikely that this factor(s) is solely responsible for the imbalance of PGI2 and TXA2 in PIH as no significant effects were observed in the cells treated with sera from PIH patients without proteinuria. These patients also showed the changes in prostaglandin metabolism albeit to lesser degree than those with proteinuria.

The cause of the imbalance between PGI2 and TXA2 seems to be complicated and multifactorial. To date, there are several substances which have been reported to be

changed in PIH and are also known to affect the production of prostaglandins in humans.

The first of them is progesterone. Progesterone is capable of inhibiting PGI2 production (Walsh 1988) and is found to be increased in PIH placentas (Walsh 1989). However, as progesterone does not affect TXA2 production and as its circulating concentration is not significantly different between normal and PIH women (Pedersen *et al.* 1983), it is unlikely that the effects described here can be attributed to progesterone.

The second is reactive oxygen species. It has been reported that the activity of reactive oxygen species is increased in PIH (Ishihara 1978; Maseki *et al.* 1981; Wickens *et al.* 1981; Dekker & Kraagenbrink 1991; Wisdom *et al.* 1991; Wang *et al.* 1992; also see Chapter 3). Increased reactive oxygen species can change the pattern of prostaglandin production in favour of TXA2 synthesis (Moncada *et al.* 1976; Salomon *et al.* 1978; also see Chapter 3). At present, it is not shown that the serum factor(s) belongs to reactive oxygen species, but the possibility does remain.

The third is the mitogenic factor(s). The mitogenic factor(s), which was recently discovered in the serum from PIH patients by Taylor and his colleagues, has the ability to stimulate fibroblasts and is classified as a growth factor (Musci *et al.* 1988; Taylor *et al.* 1990). It has been documented that the process of activation of cells induced by growth factors or mitogens can generate reactive oxygen species (Meier *et al.* 1989). If the reactive oxygen species is overproduced, the imbalance of PGI2 and TXA2 as stated above would occur. Therefore, there is a possibility that the serum factor(s) we have found here is the mitogenic factor(s).

The fourth is lipoxygenase products. Lipoxygenase products have been suggested to suppress PGI2 synthesis (Turk *et al.* 1980) and are known to be increased in PIH (Friedman 1988). Since the role of lipoxygenase products on TXA2 production is not known, it is unclear at present whether the serum factor(s) is related to them.

The last possibility is a completely new substance which is at present totally unkown to us.

The effect of PIH sera on TXA2 formation is much more intense than that on PGI2 production as it is proved by the calculation of change rate (Figure 4.6). Both PGI2 and TXA2 are derived from arachidonic acid through the action of cyclooxygenase.

If the the serum factor(s) described here only affects the level of cyclooxygenase then the change rate of PGI2 and TXA2 should be similar. From this point of view, the site of action of this serum factor(s) may locate on the levels of TXA2 and PGI2 synthase rather than on the levels of cyclooxygenase or prostaglandin H synthase. This suggestion is similar to that of Satoh *et al.* (1991) who analysed the TXA2 and PGI2 synthesizing activity in PIH and found that TXA2 synthesis was significantly increased whereas PGI2 was not.

The mechanism leading to the changes in TXA2 and PGI2 in PIH is complicated. The serum factor(s) found here can not fully account for such changes, as serum from PIH patients without proteinuria failed to exert such an effect. It seems that a number of factors may work in combination to contribute to the imbalance of TXA2 and PGI2. In addition to the above four substances, a defective genetic coding for TXA2 and PGI2 production should be also considered (Walsh 1990). The occurrence of altered prostacyclin biosynthesis long preceding the development of PIH clinical signs (Fitzgerald *et al.* 1987) and the induction of similar pathological changes and hypertension in pregnant rats by a diet deficient in polyunsaturated fatty acids (McKay *et al.* 1967) implicate abnormalities in prostanoid formation in the genesis of the human disease. Obviously, further and more comprehensive studies are needed to extrapolate the mechanism of abnormal prostaglandin production in PIH and the present study must be regarded as preliminary.

Several pharmacological approaches to the prevention and treatment of PIH can stem from the possible role of PGI2 deficiency and exaggerated TXA2 generation in the development of PIH. Firstly, dietary factors (composition of precursor fatty acids, antioxidants such as vitamin E) may modify the PGI2 and TXA2 balance (Lands 1981), but their implication in the synthesis of PGI2 and TXA2 during pregnancy has not been thoroughly studied. Secondly, evidence has shown that the infusion of exogenous PGI2 increases and inhibition of endogenous PGI decreases the uterine blood flow in pregnant animals (Clark & Harrington 1982; Clark *et al.* 1982). More important, preliminary data showed that synthetic PGI2, when given intravenously to PIH women, significantly reduced maternal blood pressure and platelet consumption (Fidler *et al.* 1980; Walker *et al.* 1982; Lewis 1983). However, these provocative trials need to be confirmed in controlled studies, the performance of which is hampered by the knowledge that it may cause a bleeding tendency (Fidler *et al.* 1980). Thirdly, besides giving dietary factors and exogenous PGI2, the most promising therapeutic intervention in this regard is small doses of aspirin. Low doses of aspirin would inhibit TXA2 formation while sparing PGI2 synthesis (Walsh

1990; Walsh *et al.* 1992) and have been successful in reducing the incidence of PIH and improving the clinical signs in several trials (Wallenberg *et al.* 1986, Wallenberg & Rotman 1988; Benigni *et al.* 1989, Schiff *et al.* 1989, Walsh 1990). However, large-scale clinical trials are still needed to comfirm the efficacy and safety of this treatment protocol in the wide population of pregnant women and to establish the precise nature of the relation between the aspirin-induced decrease in the ratio of TXA2 to PGI2 and the reduction in the incidence of PIH.

## 4.5 Conclusions

1. PIH is associated with an imbalance of decreased PGI2 and increased TXA2 production in PBMC.
2. A factor(s) from PIH patients with proteinuria was discovered to contribute to, at least in part, the abnormal pattern of prostaglandin production.

# CHAPTER FIVE EFFECTS OF ANTIHYPERTENSIVE DRUGS ON ANTIOXIDANTS

## 5.1 Introduction and aim of the study

The treatment of PIH with antihypertensive drugs is controversial, but methyldopa, atenolol, labetalol and calcium-channel blockers all have been used in treatment of this disorder (Fuerst 1982; Moretti *et al.* 1990; Blake & Macdonald 1991). Toxic oxygen metabolites such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) and the hydroxyl radical ($OH^-$), commonly called reactive oxygen species (ROS), have been implicated in the development of cardiovascular diseases including PIH (Wilson 1990; Krzanowski 1991; Rice-Evans & Bruckdorfer 1992; See Chapter three). Furthermore, there is some evidence that there is protection from cardiovascular diseases from the administration of nonenzymatic antioxidant agents such as vitamin E (Guarnieri *et al.* 1980) and glutathione (Menasche *et al.* 1986) or enzymatic antioxidant agents (Bernier *et al.* 1989). Therefore, from a therapeutic standpoint, it is clearly of great interest to find if antihypertensive drugs have favourable effects on ROS scavengers.

The effects of methyldopa on blood pressure is related to the stimulation of $\alpha_2$-adrenergic receptors. It used to be believed that methylnoradrenaline, the metabolite of methyldopa, had a weaker action than noradrenaline but in fact its pressor properties are hardly less than those of noradrenaline. It is now clear that methylnoradrenaline produces its antihypertensive effect by stimulating $\alpha_2$-adrenergic receptors in the brainstem. Such stimulation results in decreased sympathetic outflow from the central nervous system (Bobik *et al.* 1986; Reid 1988). Atenolol binds with high affinity to β-adrenergic receptor sites and subsequently blocks the catecholamine stimulation of adenylate cyclase (Lefkowitz 1976). Labetalol is a β-blocker with $\alpha_1$-adrenoceptor antagonist properties. $\alpha_1$-receptor blockade leads to relaxation of arterial smooth muscle and β-receptor blockade limits the reflex sympathetic stimulation of the heart and causes peripheral blood vessel vasodilation (Gold *et al.* 1982, Louis *et al.* 1984). Binding to the α or β receptors can result in changes of cellular signals such as 3'5'-cyclic adrenosine monophosphate (cAMP) and calcium. Since recent studies have shown that ROS activity is associated with cellular signals (Lim *et al.* 1983, Vercellotti *et al.* 1991), it is possible that these three agents might have antioxidant biochemical properties.

Published literature has indicated that β-blockers are able to provide significant protection against ROS-mediated sarcolemmal membrane lipid peroxidation (Mak & Weglicki 1988), but the literature investigating the relation between β-blockers and antioxidant agents in peripheral blood is limited. Likewise, relatively few studies have been considered the effects of methyldopa on ROS or antioxidant agents in peripheral blood.

Calcium channel blockers constitute a structually and pharmacologically diverse group of organic compounds which share the common action of reducing the movement of calcium ions into cells. They have become a widely accepted therapy in hypertension and angina pectoris (Cohn & Braunwald 1984; Kiowski *et al.* 1989). Recently, calcium channel blockers have become particularly attractive as possible intervention agents in peroxidative damage (Henry 1991; Nayler & Britnell 1991). However, the mechanism accounting for their antiperoxidative protection is not fully understood.

The aim of the present study was to determine *in vitro* the effects of atenolol, labetalol and methyldopa on antioxidant levels and to see *in vitro* whether calcium channel blockers (nifedipine and nimodipine) exert their antiperoxidative effect by changing the levels of antioxidant activity. Six antioxidant markers were investigated. They were red cell membrane thiol (MSH), plasma thiol (PSH), red cell lysate thiol (LSH), plasma total glutathione (GSHp), lysate total glutathione (GSHl) and red cell lysate superoxide dismutase (SOD).

## 5.2 Materials and Methods

### 5.2.1 Reagents and Supplies

5,5'-Dithio-bis (2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), glutathione, glutathione reductase (type III), O-dianisidine, riboflavin and superoxide dismutase were purchased from Sigma Chemical Company LTD, Dorset, England. Sulphosalicylic acid (SSA) was from BDH Chemical Ltd, Poole, England. Atenolol was supplied by ICI Pharmaceuticals, Macclesfield, England, labetalol by Glaxo and Methyldopa by Merck Sharp and Dohme. Nifedipine and nimodipine were supplied by Bayer UK Limited, Berkshire, UK.

### 5.2.2 Drug solution preparation

Atenolol (0.1-100 μg/ml), labetalol (0.1-100 μg/ml) and methyldopa (0.1-100 μg/ml) were dissolved in PBS. Nifedipine (1-1000 ng/ml) and nimodipine (1-1000 ng/ml) were dissolved in PBS containing 0.04% ethanol (ethanol is required to dissolve both drugs). All these solutions were shown to have no significant effect on cell viability by trypan blue staining. The cell viability was found to be greater than 95%.

### 5.2.3 Preparation of cells

Heparinized peripheral blood (30 units of heparin/ml) was taken from 14 healthy normal subjects (12 females, 2 males, mean±SD age: 33.6±6.8) who had no family history of hypertension or autoimmune disease. Peripheral blood was incubated with atenolol, labetalol, methyldopa, nifedipine and nimodipine respectively. After incubation, RBC and plasma were separated by the centrifugation of peripheral blood at 4°C for 10 minutes.

### 5.2.4 Preparation of RBC lysate

All steps were carried out at 4°C. 1 ml of the RBC pellet was resuspended in 1 ml of ice-cold distilled water, mixed and stood in ice for 2 hours. 800 μl of cooled absolute ethanol and chloroform mixture (3:5, v/v) was then added and mixed thoroughly. Lysate was obtained after centrifugation (For details see Chapter 3)

### 5.2.5 Assays for antioxidant agents (For details see Chapter 3)

The methods for the assays of SOD and PSH have been previously described by Banford *et al* (1982a, 1982b). LSH activity was measured according to the method of Misra and Fridovich (1977), based on the increase in the rate of photo-oxidation of O-dianisidine. The glutathione content of the plasma or lysate was measured enzymatically at 25°C by the method of Anderson (1985). MSH was measured using Ellman's reagent. Ellman's reagent can be used to induce an oxidative stimulus on the exofacial membrane sulfhydryl group of the human red cell, thus making it a chemical probe of the sulphydryl population (Hoey 1987). MSH was calculated by the following formula:

$$\text{MSH(No of thiols/cell)} = \frac{[ES^-] \times \text{volume} \times \text{Avogadro's No.}}{\text{number of cells} \times 1000 \times 13600}$$

### 5.2.6 cAMP assay

After peripheral blood was treated with atenolol, labetalol and methydopa respectively as stated above, plasma was separated and kept in -70°C until assay. cAMP was measured using an in-house radio-imunoassay after acetylation of the samples with triethylamine/acetic anhydride (2:1). The cAMP antiserum has been described previously (O'Reilly *et al.* 1986). The assay has a detection limit of 30 pmol/l and a between-batch coefficient of variation of 10-15% over the working range of the assay. All samples were assayed in duplicate and performed by Dr. W.D. Fraser, Dept. of Biochemistry, Liverpool Royal Infirmary, UK.

### 5.2.7 Statistical analysis

All statistical analyses were made with statistical analysis package for microcomputers (Minitab). Effects of atenolol, labetalol, methyldopa, nifedipine and nimodipine were analyzed for drug effect by analysis of variance (ANOVA). In cases in which a significant ANOVA was demonstrated, a paired Student's *t* test was used to determine which of the data point(s) differed. Results are expressed as mean ± SD. A p value less than 0.05 was considered to be significant.

## 5.3 Results

### 5.3.1 Statistical results of effects of antihypertensive drugs on antioxidant acitivity by ANOVA

0.1-100 μg/ml of atenolol, labeltalol and methyldopa and 1-1000 ng/ml of nifedipine and nimodipine were incubated with peripheral blood samples respectively for 120 minutes. After incubation, the antioxidant markers were measured. The data were subjected to ANOVA to test whether these drugs had effects on antioxidant levels. This results were presented in Table 5.1. Atenolol has significant effects on PSH and MSH and labetalol has significant effects on PSH only. However these two drugs had no effects on other antioxidant markers. Methyldopa, nifedipine and nimodipine did not affect any of the parameters tested.

**Table 5.1 ANOVA results of effects of antihypertensive drugs on antioxidant levels**

**A.**

| | Atenolol | | | Labetalol | | | Methyldopa | | |
|---|---|---|---|---|---|---|---|---|---|
| | DF* | F | P | DF | F | P | DF | F | P |
| PSH | 8/45 | 3.39 | **0.004** | 8/45 | 3.71 | **0.002** | 7/41 | 1.37 | 0.243 |
| LSH | 8/44 | 1.32 | 0.260 | 8/45 | 1.61 | 0.148 | 7/42 | 0.41 | 0.893 |
| SOD | 7/44 | 0.88 | 0.529 | 7/38 | 0.76 | 0.680 | 7/38 | 0.42 | 0.886 |
| GSHp | 6/36 | 0.61 | 0.722 | 6/36 | 1.38 | 0.249 | 6/36 | 1.01 | 0.437 |
| MSH | 8/42 | 2.96 | **0.010** | 8/43 | 2.15 | 0.051 | 7/40 | 0.13 | 0.995 |

*DF is expressed as DF Factor/DF Error.

**B.**

| | Nifedipine | | | Nimodipine | | |
|---|---|---|---|---|---|---|
| | DF* | F | P | DF | F | P |
| LSH | 2/42 | 0.33 | 0.718 | 2/42 | 0.47 | 0.629 |
| SOD | 2/78 | 0.86 | 0.427 | 2/51 | 0.11 | 0.900 |
| GSHI | 2/60 | 3.08 | 0.053 | 2/51 | 2.94 | 0.062 |
| MSH | 2/33 | 0.69 | 0.507 | 2/33 | 1.27 | 0.293 |

*DF is expressed as DF Factor/DF Error.

### 5.3.2 Effects of nifedipine and nimodipine on MSH, LSH, GSHl and SOD respectively

Tables 5.2 and 5.3 were the detailed data of antioxidant markers after nifedipine or nimodipine treatment. Compared to an untreated control group both drugs at the concentrations of 10-1000 ng/ml incubated with peripheral blood for 120 minutes tended to increase the levels of the four tested antioxidant agents. However, these increases did not reach statistical significance (all $p>0.05$, *t* test).

### 5.3.3 The time course study of effects of nifedipine and nimodipine on antioxidant levels

Results are shown in Tables 5.4 and 5.5. 100 ng/ml of both drugs at different incubation times (0-120 minutes) had no effect on antioxidant levels.

### 5.3.4 Effects of atenolol, labetalol and methyldopa on PSH, LSH, SOD, MSH and GSHp respectively

As shown in Figure 5.1A, atenolol at concentrations from 1 to 50 μg/ml and labetalol at concentrations from 0.1 to 25 μg/ml increased PSH levels significantly. The dose response curves were bell shaped with maximum effect being detected at a concentration of 25 μg/ml for both atenolol and labetalol. As shown in Figure 5.1B atenolol and labetalol at various concentrations produced no significant changes in LSH levels. Atenolol at concentrations of 1 to 50 μg/ml and labetalol at concentrations of 0.1 to 25 μg/ml had significant effects on MSH (Figure 5.1C). Atenolol, labetalol and methyldopa showed no effects on SOD activity (Figure 5.1D) and GSHp content (Figure 5.1E). There was no significant effect of methyldopa on any of the tested antioxidant agents (Figures 5.1A-C,E).

### 5.3.5 The time course study of effects of atenolol and labetalol on antioxidant levehs

Time course studies (Figure 5.2) showed that 25 μg/ml of atenolol and labetalol exerted significant effects on PSH and MSH after 60 to 120 minutes of incubation with peripheral blood, but not after 15 and 30 minutes of incubation.

**Table 5.2 Effects of nifedipine on antioxidant activity**

| | n | Nifedipine Concentrations (ng/ml) 0 | 1 | 10 | 100 | 1000 |
|---|---|---|---|---|---|---|
| MSH (No. of thiols x $10^6$/cell) | 5 | 4.80±0.50 | 4.72±0.48 | 4.91±0.52 | 5.10±0.72 | 5.00±0.52 |
| LSH (μmol/L) | 5 | 483±145 | 490±174 | 482±121 | 499±135 | 491±102 |
| GSHl (μmol/L) | 5 | 318±78 | 309±45 | 334±76 | 366±67 | 354±54 |
| SOD (μmol/L) | 5 | 30.6±7.3 | 31.8±8.0 | 34.1±10.1 | 34.0±8.1 | 34.5±6.2 |

**Table 5.3 Effects of nimodipine on antioxidant activity**

| | n | Nimodipine Concentrations (ng/ml) 0 | 1 | 10 | 100 | 1000 |
|---|---|---|---|---|---|---|
| MSH (No. of thiols x $10^6$/ cell) | 5 | 4.78±0.48 | 4.82±0.72 | 4.97±0.52 | 4.80±0.61 | 4.81±0.51 |
| LSH (µmol/L) | 5 | 479±87 | 488±104 | 490±112 | 494±132 | 487±112 |
| GSH1 (µmol/L) | 5 | 310±36 | 300±68 | 342±72 | 344±70 | 331±52 |
| SOD (µmol/L) | 5 | 28.7±8.2 | 31.2±10.3 | 32.7±9.9 | 33.6±7.3 | 31.8±9.4 |

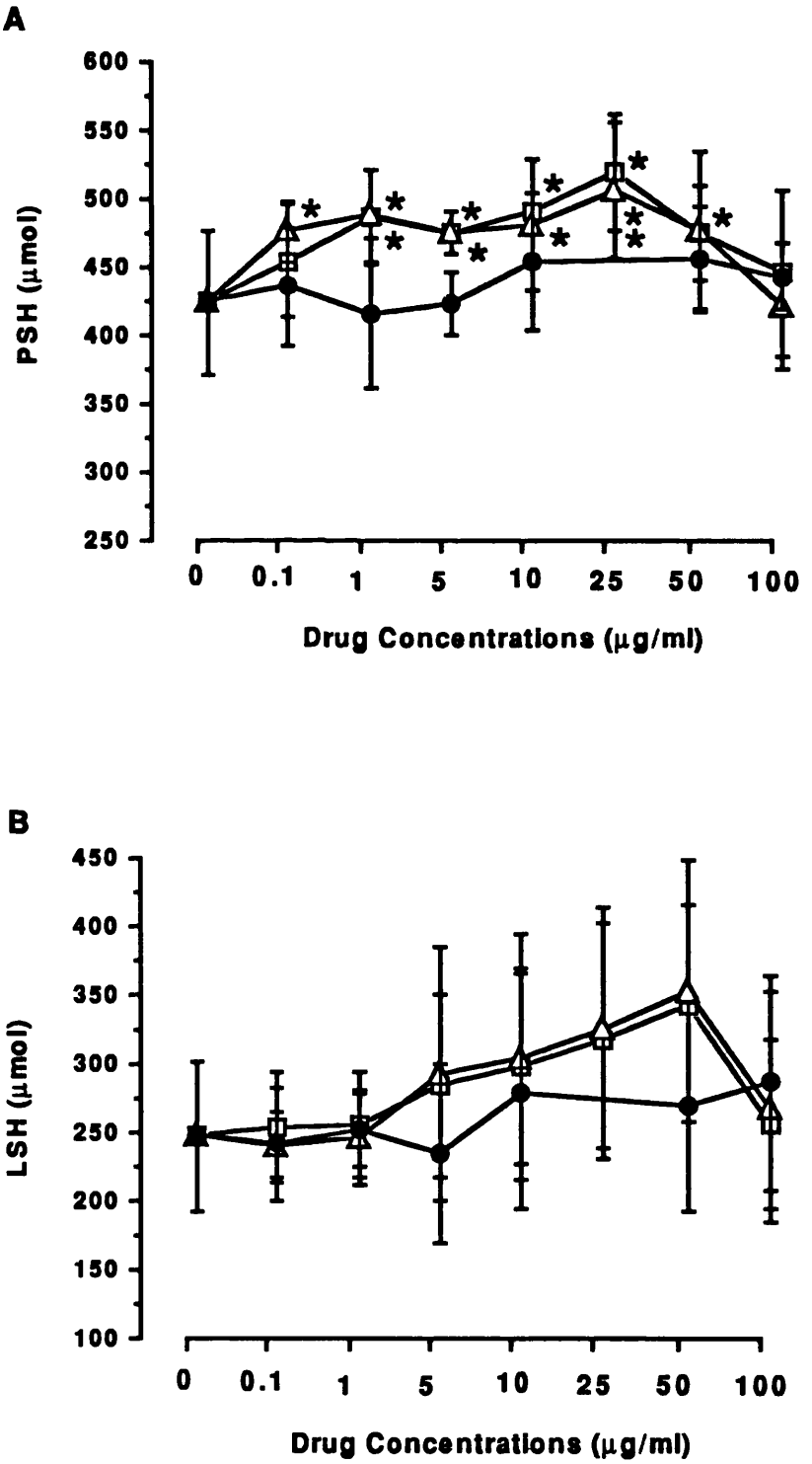
**Table 5.4 Effects of nifedipine on antioxidant activity at different incubation times**

| | n | Time (minutes) 0 | 30 | 60 | 120 |
|---|---|---|---|---|---|
| MSH (No. of thiols x $10^6$/cell) | 4 | 4.65±0.82 | 4.71±0.62 | 4.98±0.73 | 4.82±0.36 |
| LSH (μmol/L) | 4 | 463±145 | 461±136 | 481±108 | 489±167 |
| GSHl (μmol/L) | 4 | 320±65 | 333±62 | 329±48 | 328±67 |
| SOD (μmol/L) | 4 | 30.6±9.2 | 28.7±6.7 | 31.7±10.1 | 33.5±12.0 |

**Table 5.5 Effects of nimodipine on antioxidant activity at different incubation times**

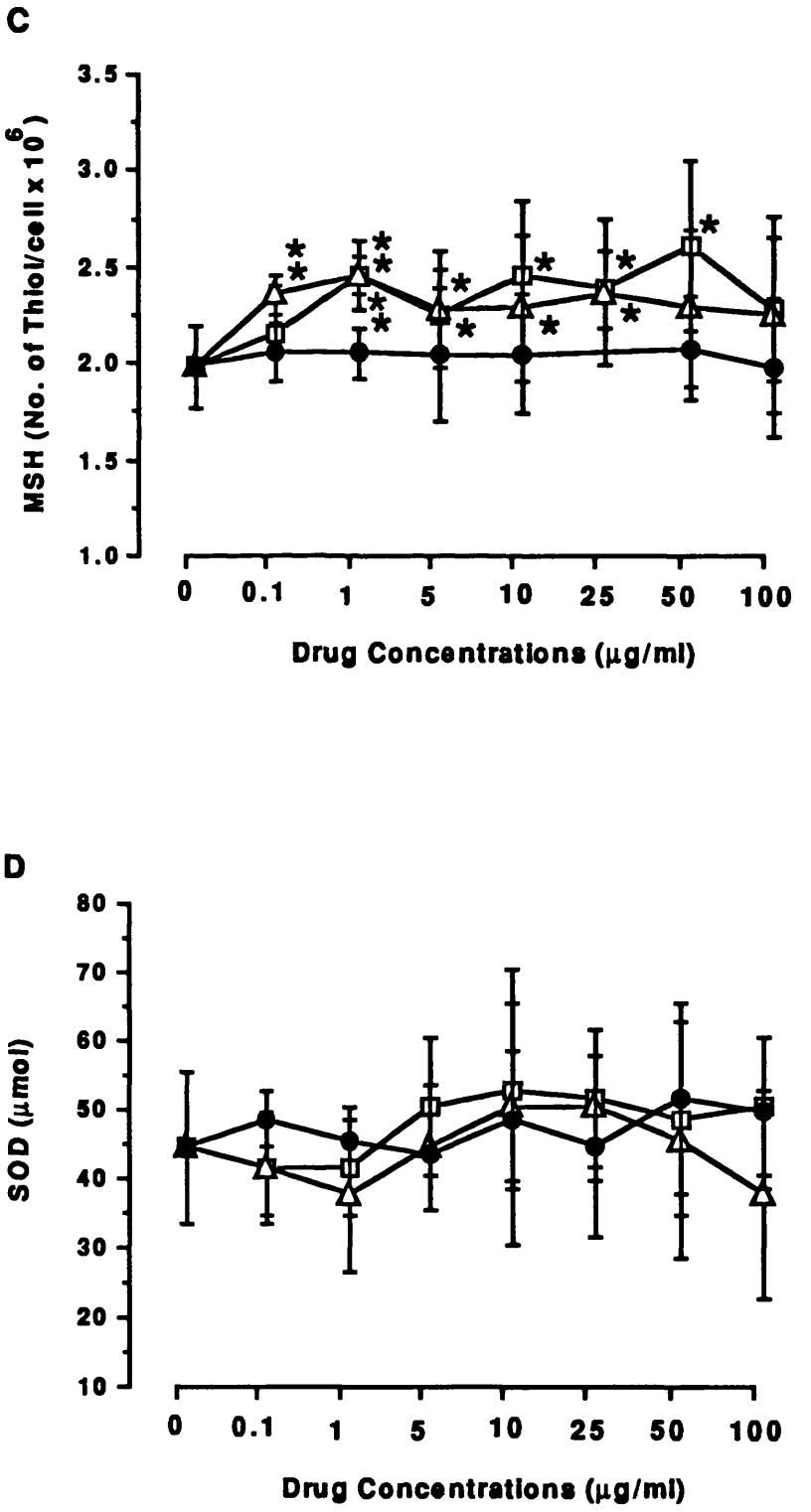
| | n | Time (minutes) 0 | 30 | 60 | 120 |
|---|---|---|---|---|---|
| MSH (No. of thiols x $10^6$/cell) | 4 | 4.76±0.67 | 4.60±0.58 | 4.91±0.82 | 4.93±0.70 |
| LSH (µmol/L) | 4 | 472±136 | 480±180 | 482±157 | 490±139 |
| GSHl (µmol/L) | 4 | 321±80 | 324±55 | 332±65 | 346±81 |
| SOD (µmol/L) | 4 | 32.7±8.8 | 29.8±5.4 | 32.8±9.2 | 33.9±11.2 |

**Figure 5.1.** **Effects of atenolol, labetalol and methyldopa on antioxidant agents**



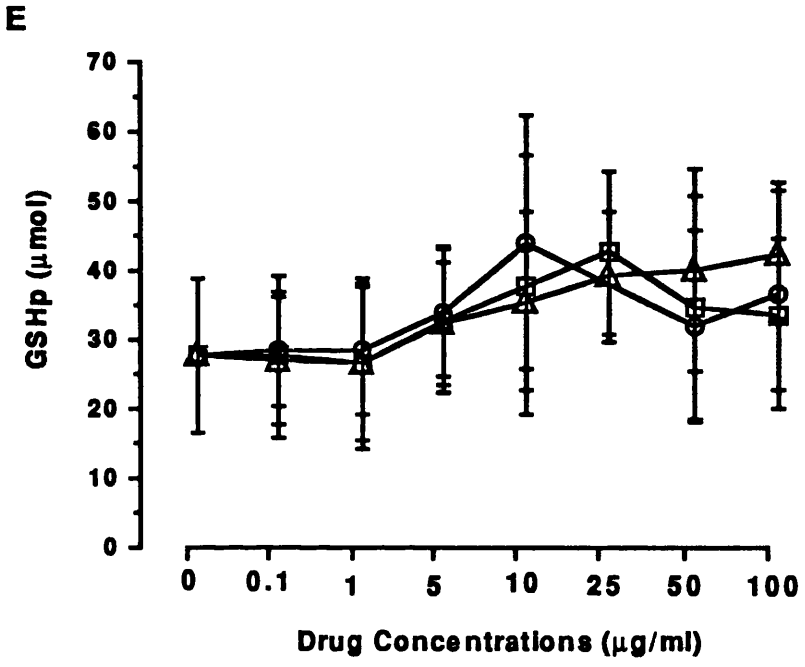

*Continuing*

*Figure 5.1 continuing*



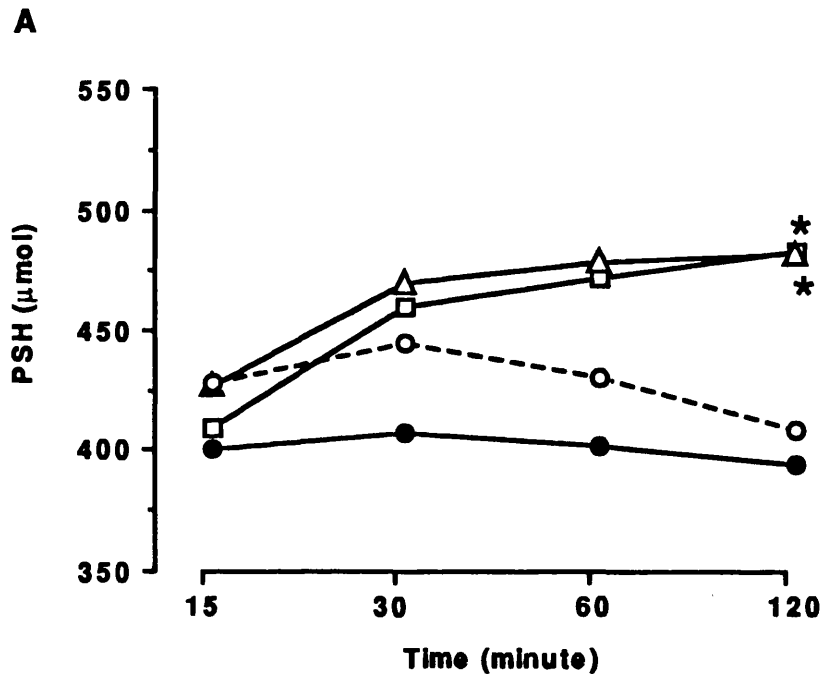

*Continuing*

*Figure 5.1 continuing*



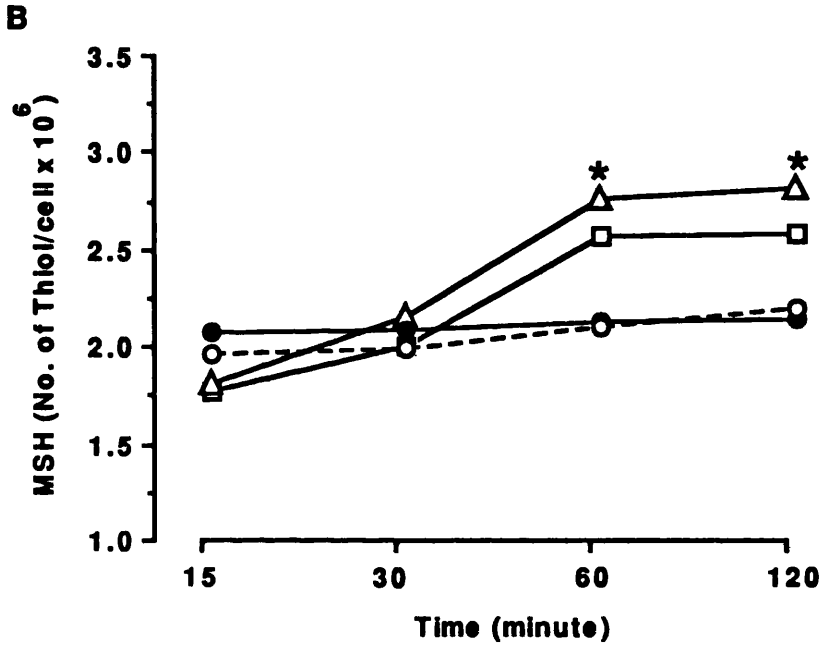

Peripheral blood was incubated with atenolol (—□—), labetalol (—△—) and methyldopa (—●—) respectively. After incubation, plasma and red cell lysate were prepared and antioxidant markers: PSH (**A**, n=8), LSH (**B**, n=8), MSH (C, n=8), SOD (D, n=8) and GSHp (E, n=7) were measured. The data are expressed as mean ± SD. Statistical significance of a point versus no drug is given by asterisks: *$p<0.05$, **$p<0.01$. A. Atenolol and labetalol significantly raise the levels of PSH, $p<0.004$ and $p<0.002$, ANOVA for effects of atenolol and labetalol respectively. C. Atenolol and labetalol significantly raise the levels of MSH, $p<0.01$ and $p<0.05$, ANOVA for effects of atenolol and labetalol respectively.

**Figure 5.2.** **Changes of antioxidant levels after incubation of peripheral blood with atenolol, labetalol and methyldopa respectively at different times**




*Continuing*

*Figure 5.2 continuing*




Peripheral blood was incubated with atenolol ( —□— , n=4), labetalol (—△— , n=4), methyldopa ( —●— , n=4) and PBS (controls: - - -o- - -, n=4) respectively. After incubation, plasma and red cell lysate were prepared and antioxidant markers: PSH (A) and MSH (B) were measured. The data are expressed as mean ± SD. Statistical significance of a point versus no drug is given by asterisks: *$p<0.05$. A. Atenolol and labetalol significantly affect the levels of PSH, $p<0.03$ and $p<0.049$, ANOVA for effects of atenolol and labetalol respectively. B. Atenolol and labetalol significantly affect the levels of MSH, $p<0.05$ and $p<0.012$, ANOVA for effects of atenolol and labetalol respectively.

### 5.3.6 Determination of PSH and MSH in the solution of atenolol and labetalol

Solutions of atenolol and labetalol *per se* at different concentrations were determined for PSH and MSH activity in order to find out whether the chemical forms of these physiological antioxidant agents exist in the drugs. The levels of PSH and MSH in drug solutions were not significantly different from those in phosphate-buffered saline solution (PBS) alone (all $p>0.05$, ANOVA) (Table 5.6).

### 5.3.7 Additive effect of atenolol and labetalol

An additive effect of atenolol and labetalol on antioxidant agents was observed at 5 μg/ml of atenolol plus 5 μg/ml of labetalol, but not at 50 μg/ml of atenolol plus 50 μg/ml of labetalol (Table 5.7).

### 5.3.8 Plasma cAMP levels after antihypertensive drug treatment.

Following the incubation of whole blood with atenolol (0.1-50 μg/ml), labetalol (0.1-50 μg/ml) and methyldopa (0.1-50 μg/ml) for 60 and 120 minutes respectively, no significant difference in plasma cAMP levels were found in any group (Table 5.8).

**Table 5.6 Determination of PSH and MSH in PBS and a solution of atenolol (A) and labetalol (B) respectively**

**A.**

| | Atenolol(μg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| | PBS | 0.1 | 1 | 5 | 10 | 50 | 100 |
| PSH(μmol/l) | 3.4 ±1.7 | 3.3 ±1.4 | 3.5 ±1.3 | 6.0 ±2.0 | 3.4 ±1.5 | 6.1 ±1.4 | 8.0 ±2.1 |
| MSH(No.of Thiols/Cell X $10^6$) | 1.74 ±0.12 | 1.72 ±0.11 | 1.66 ±0.09 | 1.68 ±0.08 | 1.76 ±0.14 | 1.77 ±0.11 | 1.77 ±0.15 |

**B.**

| | Labetalol(μg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| | PBS | 0.1 | 1 | 5 | 10 | 50 | 100 |
| PSH(μmol/l) | 3.4 ±1.2 | 8.2 ±2.9 | 3.6 ±1.5 | 4.0 ±1.8 | 10.3 ±3.6 | 1.1 ±0.8 | 9.9 ±3.1 |
| MSH(No.of Thiols/cell X $10^6$) | 1.74 ±0.09 | 1.63 ±0.13 | 1.80 ±0.19 | 1.69 ±0.16 | 1.72 ±0.18 | 1.77 ±0.21 | 1.74 ±0.19 |

**Table 5.7 Effects of atenolol and labetolol alone or combination of them on PSH and MSH**

***A.***

| | | PSH | MSH |
|---|---|---|---|
| Atenolol | 5 µg/ml | 455±40 | 224±21 |
| Labetalol | 5 µg/ml | 448±38 | 232±39 |
| Atenolol<br>Labetalol | 5 µg/ml+<br>5 µg/ml | 506±21 | 254±39 |
| Atenolol | 10 µg/ml | 482±40 | 247±40 |
| Labetalol | 10 µg/ml | 473±48 | 236±39 |

***B.***

| | | PSH | MSH |
|---|---|---|---|
| Atenolol | 50 µg/ml | 479±31 | 229±17 |
| Labetalol | 50 µg/ml | 458±41 | 255±25 |
| Atenolol<br>Labetalol | 50 µg/ml+<br>50 µg/ml | 472±34 | 252 30 |

**Table 5.8 Plasma cAMP after blood was incubated with atenolol, labetalol and methyldopa respectively.**

| Concentration (µg/ml) | 0.1 | | 25 | | 50 | |
|---|---|---|---|---|---|---|
| Incubation Time (minutes) | 60 | 120 | 60 | 120 | 60 | 120 |
| Atenolol (n=7) | 18.7±6.1 | 16.3±5.9 | 16.3±7.2 | 19.6±5.5 | 15.0±5.3 | 15.2±4.6 |
| Labetalol (n=7) | 19.8±7.2 | 18.3±8.1 | 15.5±7.2 | 17.8±4.2 | 16.7±3.4 | 17.1±5.8 |
| Methyldopa (n=7) | 20.3±8.4 | 17.3±6.5 | 19.3±8.2 | 18.9±7.8 | 17.3±4.5 | 18.4±8.8 |

## 5.4 Discussion

In this study, we have shown that when incubated with whole blood, atenolol and labetalol at certain concentrations are able to raise the levels of the ROS scavengers: PSH and MSH, suggesting that they possess some antioxidant properties. The dose-effect curve of atenolol and labetalol on LSH (Figure 1B) seems to show that both agents can raise the level of LSH. However, statistical analysis demonstrated no significant difference. This maybe due to the wide variation of the data. The increase in the level of the ROS scavengers could arise from increased production of these scavengers or decreased formation of ROS.

Our study has excluded the possibility that atenolol and labetalol *per se* contain chemical forms of PSH and MSH observed in this study because no significant difference in the values of these scavengers was found between the solutions of drugs and PBS.

Both atenolol and labetalol are β-blockers, while methyldopa, nifedipine and nimodipine, which showed no antioxidant effects in these experiments, are not. On this basis, it can be inferred that the antioxidant effects of β-blockers may occur by a mechanism involving the β-receptor. One of the major pharmacological mechanisms of β-adrenoceptor blockers is to decrease intracellular cAMP through the inactivation of adenosine cyclase (Lefkowitz *et al.* 1984). Elevation of cAMP levels or reduction of adenosine levels has been suggested to increase ROS production in other studies (Meltzer *et al.* 1989; Yukawa *et al.* 1989). Therefore, it is possible that by decreasing cAMP levels and subsequently reducing ROS formation, β-blockers may take the place of the physiological ROS scavengers thus causing the levels of PSH and MSH to rise. Since PSH and MSH whose levels are changed in this study are not intracellular antioxidants, the measurement of plasma cAMP was chosen to test whether plasma cAMP is relevant to the changed antioxidants found here. Plasma cAMP levels after administration of β-blockers (which changed PSH and MSH) were not different from that of methyldopa (which did not change PSH and MSH). Therefore, the possibility that the changes of PSH and MSH found in this study might result from the alteration of plasma cAMP is excluded.

To avoid possible cell activation consequent to the purification procedure (Shappel *et al.* 1990), Blood components has not been purified in this study. By incubating the drugs with whole blood, the effects of β-blockers on ROS were studied. It has been documented that blood cells such as lymphocytes can not only produce ROS

but can also modulate ROS production via cytokines including interferon-γ and interleukin-2 (Salisbery *et al.* 1990; Klausner *et al.* 1991). Therefore, it is also possible that the β-blockers reduce ROS activity by altering cytokine production or release. However, the time course of blood cell activation to produce cytokines is measured in hours or days (Gupta *et al.* 1987; Oppenheim *et al.* 1991). In the present experiment, the significant effects of β-blockers were detectable as early as 60 minutes after the beginning of incubation. Thus, any change in cytokines, caused by the β-blockers, is unlikely to be the main mechanism by which β-blockers exert antioxidant effects.

It has been reported that the concentration of atenolol in plasma ranges from 0.3 to 2.2 μg/ml (mean value 1.0 μg/ml) and the concentration of labetalol from 0.093 to 0.271 μg/ml (mean value 0.191 μg/ml) (McNeil *et al.* 1979; Cruickshank 1980). Therefore, the clinical concentrations of both agents appear to be the concentrations at which the antioxidant activity was found in this study. Concentrations well above the range needed clinically to block β-adrenergic receptors also showed antioxidant effects, although their effects did not always increase with drug concentration. At concentration 100 μg/ml no effects were observed. In addition, the data also showed that 5 μg/ml of atenolol plus 5 μg/ml of labetalol produced similar effects to those observed with 10 μg/ml of atenolol or 10 μg/ml of labetalol ($P>0.05$), and was more effective than 5 μg/ml of atenolol or 5 μg/ml of labetalol alone ($p<0.05$) (Table 5.7A). However, 50 μg/ml of atenolol plus 50 μg/ml of labetalol show no such additive effects (Table 5.7B). These results support the concept that the effects could be due to the interaction of the drugs with specific membrane receptors, probably β receptors.

It is known that the antioxidant mechanisms for conventional chainbreaking agents are related to their phenols or aromatic amines which are capable of stabilizing trapped radicals (Burton & Ingold 1981). Although the intrinsic chemical properties of β-blockers are quite diverse, they all contain this kind of aromatic resonance rings (Connolly *et al.* 1976). However, it is impossible to attribute the antioxidant effects of atenolol and labetalol found in this study to their common structural features of aromatic resonance rings, as the calcium channel blockers which also have aromatic resonance rings( Mak & Weglicki 1990) showed no antioxidant effects in the present experiments.

Mak & Weglicki (1988) and Mak *et al.* (1989) have demonstrated that β-blockers can interact with the membrane hydrophobic components and subsequently inhibit

ROS propagation in the membrane. Concomitantly, they protect the membranes against ROS-induced lipid peroxidation. Our study found that atenolol and labetalol can exert antioxidant activity either directly or indirectly by raising the levels of ROS scavengers PSH and MSH. Although the mechanism responsible for the observed effects of β-blockers on ROS scavengers remains to be further elucidated, the potential therapeutic significance of these drugs has been deemed to be of scientific interest.

Jones *et al.* (1981) have demonstrated that methyldopa stimulates the conversion of the primary catalase peroxide complex (the active form of the enzyme) to the catalytically inactive secondary catalase peroxide complex, leading them to conclude that methyldopa may initiate a ROS process. Using ROS scavengers as indirect indexes of ROS activity, our study showed that methyldopa had no significant effects on ROS, producing neither augmentation nor reduction of ROS levels.

Previous studies have suggested that the movement of calcium ions is important in the coupling of extracellular messages to intracellular response and this process results in cell activation (Rasmussen & Goodman 1977; Abbas *et al.* 1991). It is reported that the activation of cells is associated with an increase in the production of ROS (Meier *et al.* 1989) and moreover, the calcium ionophore A23187 has been shown to stimulate the formation of ROS (Lim *et al.* 1983). On the above basis, it is seems reasonable to assume that calcium channel blockers such as nifedipine and nimodipine may possess the ability to increase antioxidant levels by inhibiting the formation of ROS. However, this is not supported by the present study which found that neither nifedipine nor nimodipine had any effect on the antioxidant agents measured in this study. Although this finding is unexpected, it may suggest that calcium is not a major factor contributing to the production of ROS in the present test system.

Several studies have claimed that calcium channel blockers possess antiperoxidative effects (Henry 1991; Nayler & Britnell 1991). Using an *in vitro* model of sarcolemmal membrane lipid peroxidation, Mak *et al.* (1990) demonstrated that calcium channel blockers significantly protected the cell from death by decreasing the levels of superoxide and hydroxyl radicals. This effect was believed to be due to the specific structure of aromatic resonance rings in calcium channel blockers rather than the blockade of calcium influx as the aromatic resonance rings are capable of stabilizing trapped radicals and calcium is omitted in the test system. Therefore, it is concluded that calcium channel blockers can directly scavenge the ROS *in vitro*.

The data from the present study did not provide any evidence to show that calcium channel blockers themselves have a radical scavenging effect, but the possibility that they might serve as indirect scavengers by increasing the levels of antioxidant activity *in vitro* is excluded. Very recently, an *in vivo* study by Ding *et al.* (1992) proved that the calcium channel blocker, isradipine had no effect on ROS. It should be noted that these studies on the relation of calcium channel blockers, ROS and antioxidant levels were carried out on differejt systems. Therefore, whether or not calcium channel blockers possess an antioxidant effect *in vitro* or *in vivo* needs to be further studied.

The pharmacological serum concentrations of nifedipine and nimodipine have been reported to be 47±20 ng/ml and 39±26 ng/ml respectively (Gilman *et al.* 1990; Tartara *et al.* 1991). These concentrations are well within the range examined by this study. Therefore it is concluded that calcium channel blockers at pharmacological concentrations or above are unable to increase the physiological antioxidant level *in vitro*. If these drugs have any antiperoxidative effect *in vitro*, it is impossible that that action is due to the elevation of antioxidant levels.

## 5.5 Conclusions

1, Atenolol and labetalol *in vitro* possess some antioxidant activity as they can significantly raise the levels of plasma thiol and membrane thiol.

2, Atenolol and labetalol at lower concentrations tend to have additive effects on antioxidant agents but at higher concentration do not, suggesting the antioxidant property may associated with specific membrane receptors, probably β receptors.

3, Calcium channel blockers are unable to elevate the levels of physiological antioxidant agents *in vitro*.

# CHAPTER SIX SUPEROXIDE DISMUTASE GENOMIC POLYMORPHISM AND GENE EXPRESSION

## 6.1 Introduction and aim of the study

Superoxide dismutase (SOD) catalyses the dismutation of the superoxide anion into oxygen and hydrogen peroxide and is thought to be an important component of the cellular defense repertoire against oxidative damage mediated by superoxide radicals produced as a by-product of oxygen metabolism (Fridovich 1978; Getzoff *et al.*, 1983).

In eukaryotic cells, there are two distinct types of SOD (Fridovich 1978; Getzoff *et al.*, 1983). The predominant SOD is a soluble cytosolic enzyme containing copper and zinc at the catalytic site (CuZn-SOD). The second type is the manganese-containing SOD (Mn-SOD) found in the matrix of the mitochondria. The Mn-SOD differs substantially from the CuZn-SOD in primary structure (Steinman & Hill, 1973). Although extracellular SOD, which is similar to CuZn-SOD, has been reported, the physiological significance of this type of SOD remains unknown (Marklund 1984a). The gene for human CuZn-SOD is on chromosome 21 and that for the Mn-SOD on chromosome 6; the chromosomal location for the extracellular SOD gene has not yet been reported.

In recent years, increased superoxide formation or lipid peroxidation products have been described in patients with pregnancy-induced hypertension (PIH) (Hubel *et al.*, 1989; Wang *et al.* 1992; Tsukimori *et al.* 1993). Decreased antioxidant levels or activities including SOD have also been found in this disorder (See Chapter three). Therefore, an imbalance between pro-oxidation and anti-oxidation has been suggested to be implicated in the pathogenesis of PIH. Considering that PIH is well known to be familial and a genetic predisposition has been proposed (Cooper *et al.*, 1988; Kilpatrick *et al.*, 1989; Arngrimsson *et al.*, 1990), one might speculate that a mutation in a critical region of the SOD gene or its abnormal expression could result in an alteration of SOD activity. To determine whether reduced SOD activity found in patients with PIH is associated with molecular genetic abnormalities, the CuZn-SOD genomic DNA and mRNA expression were analysed in the present work.

## 6.2 Materials and Methods

Reagents described in this section were supplied by BDH Laboratory Supplies, Merck Ltd., UK, unless indicated. The 0.6 Kb *Pst* I insert from pSOD1, a plasmid containing a CuZn-SOD cDNA was kindly supplied by Dr. Y Groner, Weizmann Institute of Science, Rehovot, Israel and used as the probe (Lieman-Hurwitz *et al.* 1982; Danciger *et al.* 1986).

### 6.2.1 Subjects

Eight PIH patients with mean age 27.5±5.0 years and mean gestational age 36.3±4.2 weeks were included in the study. Four of these patients had proteinuria, and four did not. Seven normal pregnant women with mean age 25.2±6.1 years and mean gestational age 36.1±5.2 weeks and five normal nonpregnant women with mean age 28.8±5.3 served as controls.

The diagnosis of PIH was made using the criteria of the American College of Obstetricians and Gynecologists (For details see Chapter 1). Briefly, PIH is defined as a persistent or recurrent diastolic blood pressure of ≥90mmHg developing during pregnancy after 20 weeks of gestation, and resolving by six weeks post partum. Proteinuria is defined as the persistent presence of protein in the urine of ≥ + on urine 'dipstick' testing, or >300 mg excreted in 24 hours. The latter measurement is preferred where available. Patients who had a history of hypertension before the twentieth week of pregnancy were designated as essential hypertension.

### 6.2.2 White cell preparation

30 ml of peripheral blood was collected, and 10 ml was placed in each of three sterile conical tubes containing EDTA (1 mg/ml blood) (Pharmacia Ltd., Bucks, UK). 10 parts EDTA blood were mixed with 1 part 6%(w/v) Dextran 500 and this was left to settle. When the white cell band in the upper plasma layer was visible, it was transferred to another tube.

### 6.2.3 Genomic DNA isolation (Darbre 1988; Sambrook *et al.* 1989)

1. The white cells (from 10 ml of blood) were pelleted by centrifugation at 1000 x g for 10 minutes and the cell pellet was washed twice with cold PBS. The cells were adjusted to a concentration of 5 x $10^7$ cells/ml in TE buffer and

mixed with an equal volume of 2x lysis buffer for 30 minutes to lyse the cells and nuclei.

2. An equal volume of water-saturated phenol was added to the tube and mixed gently by inversion for 10 minutes. This was then centrifuged for 10 minutes at 2500 x g to separate the layers.
3. The top phase was transferred to another tube and Step 2 was repeated once.
4. Two volumes of 100% ethanol were added and the tube was inverted several times until the solution was thoroughly mixed. The nucleic acid precipitate should be visible at this stage.
5. The nucleic acid precipitate was fished out with a glass rod and allowed to air dry for a few minutes. The precipitate was then dissolved in 2.8 ml of 1x lysis buffer.
6. RNase (BRL, Gibco Ltd., UK) was added to a final concentration of 50 µg/ml and incubated in a water bath (37°C) for 1 hour.
7. SDS and proteinase K (Boehringer Mannheim GmbH., German) were then added to final concentrations of 0.5% and 100 µg/ml respectively and incubated for a further 2 hours in a water bath (55°C).
8. Repeat Step 2.
9. Repeat Step 2 but using phenol:chloroform:isoamyl alcohol instead of water-saturated phenol.
10. Repeat Step 4.
11. The DNA was spooled onto a glass rod, allowed to air dry and DNA was then put in a microtube in 100-500 µl of TE buffer. This was left at 4°C for some days to be dissolved.
12. The absorbance of the DNA at 260 nm and 280 nm was measured. the ratio of $A_{260}$ to $A_{280}$ should be greater than 1.75. A lower ratio is an indication that a significant amount of protein remains in the preparation. In this case, Steps 7-11 were repeated.
    Amount of DNA (µg/ml) = O.D(260nm) x 50 x dilution factor.
13. Store DNA samples at 4°C.

### 6.2.4 RNA isolation (Chomczynski & Sacchi 1987)

1. The white cells (from 20-30 ml of blood) were mixed with an equal volume of red cell lysis buffer for 15 minutes at room temperature to lyse the remaining red cells. The pellets were washed twice with cold PBS and sedimented by centrifuging for 10 minutes at 2500 x g.
2. Add 5 ml of prechilled denaturing solution and mix thoroughly by inversion.

3. Add 0.5 ml of 2M sodium acetate, pH 4.0 and mix thoroughly by inversion.
4. Add 5 ml of phenol:chloroform:isoamyl alcohol mixture, mix by inversion and shake vigorously for 10 seconds. Chill on ice for 15 minutes.
5. Transfer this mixture to a 30 ml tube (DEPC-treated) and centrifuge at 10,000 x g for 20 minutes at 4°C. Either a fixed-angle or swinging bucket rotor may be used.
6. Carefully remove the top aqueous phase which will contain the RNA and transfer it to a fresh DEPC-treated tube. DNA and proteins will remain in the organic phase at the interface. Be careful to avoid taking material from the interface.
7. Add an equal volume of isopropanol and incubate the sample at -20°C overnight to precipitate the RNA.
8. Pellet the RNA by centrifugating at 10,000 x g for 15 minutes at 4°C.
9. Repeat Steps 2-8.
10. Resuspend the RNA pellet ij 5 ml of denaturing solution and vortex until the RNA dissolves.
11. Add an equal volume of isopropanol and precipitate the RNA as described in Step 7.
12. Pellet the RNA by centrifugating at 10,000 x g for 15 minutes at 4°C. Wash the pellet with ice-cold 75% ethanol and centrifuge as above. A minimum of 10 ml of 75% ethanol should be used.
13. Dry the pellet in air.
14. Resuspend the RNA in DEPC water.
15. The absorbance of the RNA at 260 nm and 280 nm was measured. the ratio of $A_{260}$ to $A_{280}$ should be greater than 1.70.
    Amount of RNA (µg/ml) = O.D(260nm) x 40 x dilution factor.
16. Store RNA samples at -20°C.

### 6.2.5 Electrophoresis of DNA through agarose gel (Sambrook *et al.* 1989)

Agarose (Sigma Chemical Co. ltd., England) was dissolved in E buffer by boiling in a microwave oven. The agarose solution was allowed to cool to about 50°C. While the gel was cooling, the open ends of the tray were sealed with masking (autoclaving) tape. The gel solution was then poured onto the gel tray. Insert the comb and leave to set for about 30 minutes. When the agarose had set, the comb and the tape were removed and the gel tray was placed under the E buffer in the gel electrophoresis tank. 1/10 volume of stop mix was added to each sample. The stop mix (1). helps the samples sink into the wells and not float away (Ficoll); (2). stops

any enzymatic reactions from occurring (EDTA) and (3). tracks the position of the sample in the gel (orange G). The gel was run at constant voltage and towards the positive electrode. Once the gel had run *i.e.* the orange dye had reached the end, the power pack was switched off and the gel was stained in a solution of ethidium bromide (1 mg/L) for 10 minutes. The gel was then viewed over a UV light. A photograph of the gel was then taken with Polaroid camera using a fast film (Polaroid Type 667).

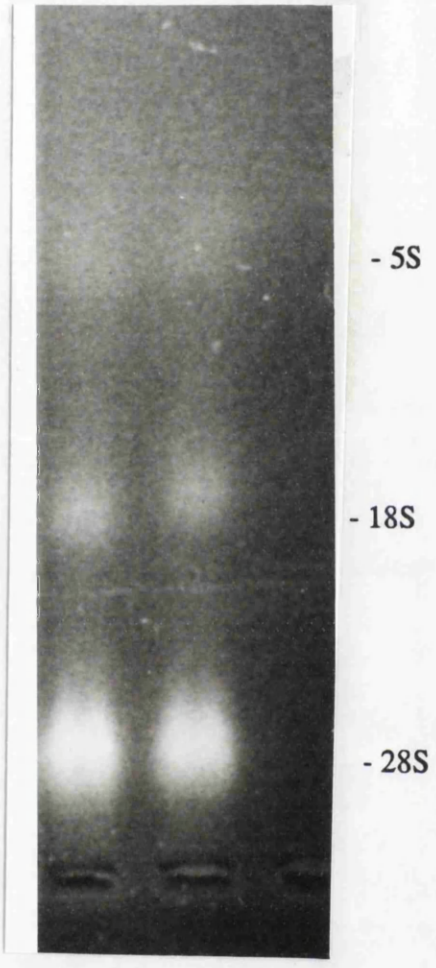
### 6.2.6 Electrophoresis of RNA through gel containing formaldehyde (Sambrook *et al.* 1989)

1. Prepare the gel by melting the appropriate amount of agarose in water, cooling it to 60°C. In chemical hood, add 5x formaldehyde gel-running buffer and formaldehyde to give final concentrations of 1x and 2.2 M respectively. Mix well and immediately cast the gel. Allow the gel to set for at least 30 minutes.
2. Prepare the sample by mixing the following in a sterile microfuge tube:

| | |
|---|---|
| RNA (up to 30 µg) | 4.5 µl |
| 5x formaldehyde gel-running buffer | 2.0 µl |
| formaldehyde | 3.5 µl |
| formamide | 10.0 µl |

3. Incubate the samples for 15 minutes at 65°C, and then chill them on ice. Centrifuge the samples for 5 seconds to deposit all of the fluid in the bottom of the microfuge tubes.
4. Add 2 µl of sterile, DEPC-treated formaldehyde gel-loading buffer.
5. Before loading the samples, prerun the gel for 5 minutes at 5V/cm and then immediately load samples into the lanes of the gel.
6. Run the gel submerged in 1x formaldehyde gel-running buffer at 3-4V/cm. When the bromophenol blue has migrated approximately 8 cm the gel can be used for photography and blotting. A representative RNA gel photograph is shown in Figure 6.1.

## Figure 6.1 RNA Electrophoresis




A representative electrophoresis of RNA through agarose gel containing formaldehyde is shown. Each lane was load with 10 µg of RNA. The gel was stained with ethidium bromide.

### 6.2.7 DNA polymorphisms

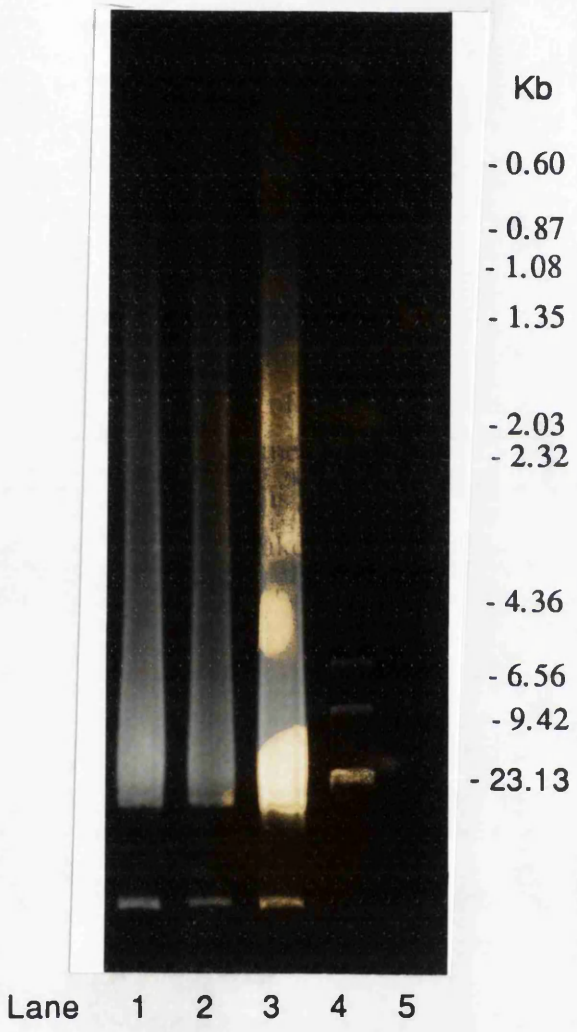
In order to make genes easier to study, DNA needs to be cut into mangeable fragments by enzymes isolated from bacteria called restriction endonucleases. Plasmid DNA is usually digested with 1 unit enzyme/μg DNA. Enzyme activity is defined as the amount of enzyme required to cut lambda DNA. For genomic DNA it is normal to use about 2 units enzyme/μg DNA. Reaction volume is about 1 μg in 10 μl for plasmid DNA and normally 1 μg DNA in 5 μl final volume for genomic DNA. Restriction enzyme *EcoRI* (BRL, Gibco Ltd., UK) was used for digesting genomic DNA in this experiment.

1. Calculate what volume correspond to 1 mg DNA.
2. Set up a reaction mix containing

| | |
|---|---|
| 10x buffer | 1/10 of final volume |
| DNA | volume equivalent to 1 μg |
| *EcoRI* | 2 units |
| Sterile $dH_2O$ | to make the volume to 5 μl |

3. Incubate at 37°C for 1 hour for plasmid DNA or 4 hours (or overnight) for genomic DNA.
4. After incubation, add at least 1/10 volume stop mix and run on a gel as described in section 6.2.5. A representative genomic DNA gel photography is shown in Figure 6.2.

**Figure 6.2 DNA Electrophoresis**




A representative ethidium bromide stained agarose gel containing genomic DNA that has been completely digested with the restriction endonuclease, *EcoRI* (lanes 1-3). Each lane was load with 6 µg of DNA sample. Lanes 4 and 5 are *HindIII*-digested *lambda*-DNA marker and *HaeIII*-digested *phi X174*-RF DNA marker respectively. Sizes are in kb.

### 6.2.8 Transfer of DNA and RNA from agarose gels to solid support

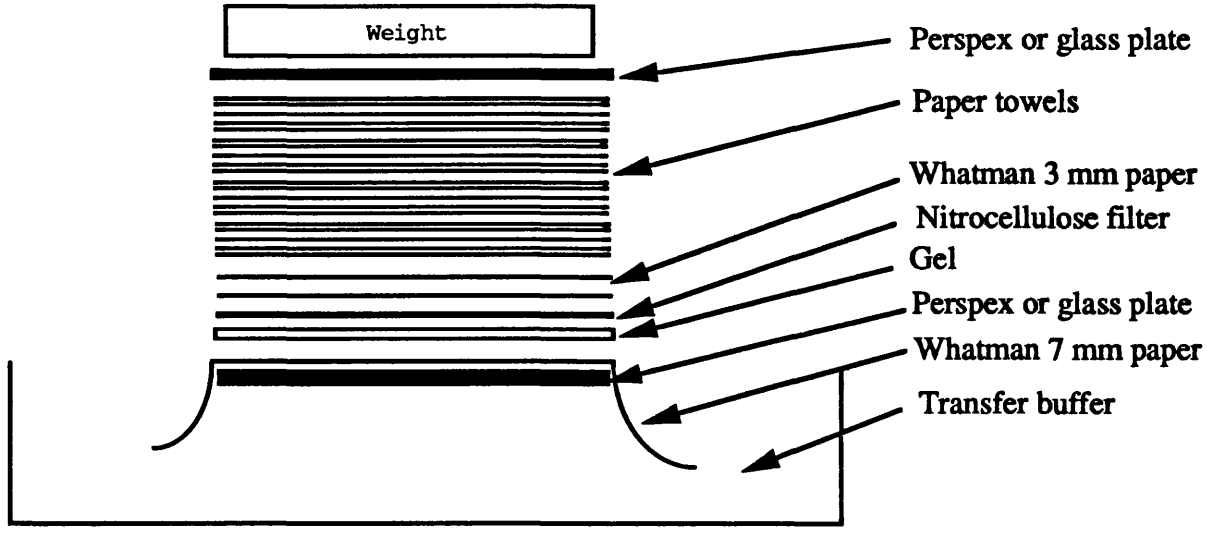
#### 6.2.8.1 Southern blotting for DNA transfer (Southern 1975)

1. Soak the gel in denaturing buffer for 1 hour.
2. Wash the gel with $dH_2O$ briefly and immerse it in neutralizing buffer for another 1 hour.
3. Put the gel on transfer apparatus (Figure 6.3). Now cover all edges of the gel using four pieces of clingfilm, tucking excess under the tray but taking care not to cover any areas of the gel.
4. Cut a piece of nitrocellulose (Amersham International plc. UK) to the same size as the gel, wet it in $dH_2O$ and then transfer to 20x SSC for at least 5 minutes.
5. Place nitrocellulose on top of the gel. Using a pipette, roll out any air bubbles between the nitrocellulose and the gel.
6. Briefly wet a piece of Whatman 3MM filter paper (Whatman International Ltd., England) which had been cut to the size of the gel and place it on the nitrocelluse. Again remove any air bubbles between the 3MM paper and the gel.
7. Place a dry piece of Whatman 3MM filter paper on top and then paper towels (about 8 cm). On the top of the pile place a weight (about a kilogram). Leave overnight.
8. Disassemble blot the next day. Carefully mark positions of wells. Remove nitrocellulose, place it betwen glass plates and bake in a vacuum oven at 80°C for 2 hours. The filter may then be stored until required.
9. Stain the gel with ethidium bromide and look at under UV illumination to ensure that all the DNA has transferred.

#### 6.2.8.2 Northern blotting for RNA transfer (Sambrook *et al.* 1989)

1. Soak the gel for 20 minutes in 0.05 N NaOH and then rinse the gel in DEPC-treated water.
2. Soak the gel for 45 minutes in 20x SSC. Then carry out Steps 3-9 as described in section 6.2.8.1.

## Figure 6.3 Diagram of apparatus used for Southern blotting*




Capillaey transfer of DNA from agarose dels. Buffer is drawn from a reservoir and passes through the gel into a stack of paper towels. The DNA is eluted from the gel by the moving stream of buffer and is deposited on a nitrocellulose filter. A weight applied to the top of the paper towels help to ensure a tight connection between the layers of material used in the transfer sysytem.
* adopted from Sambrook *et al.* (1989).

### 6.2.9 Preparation and labelling of probes

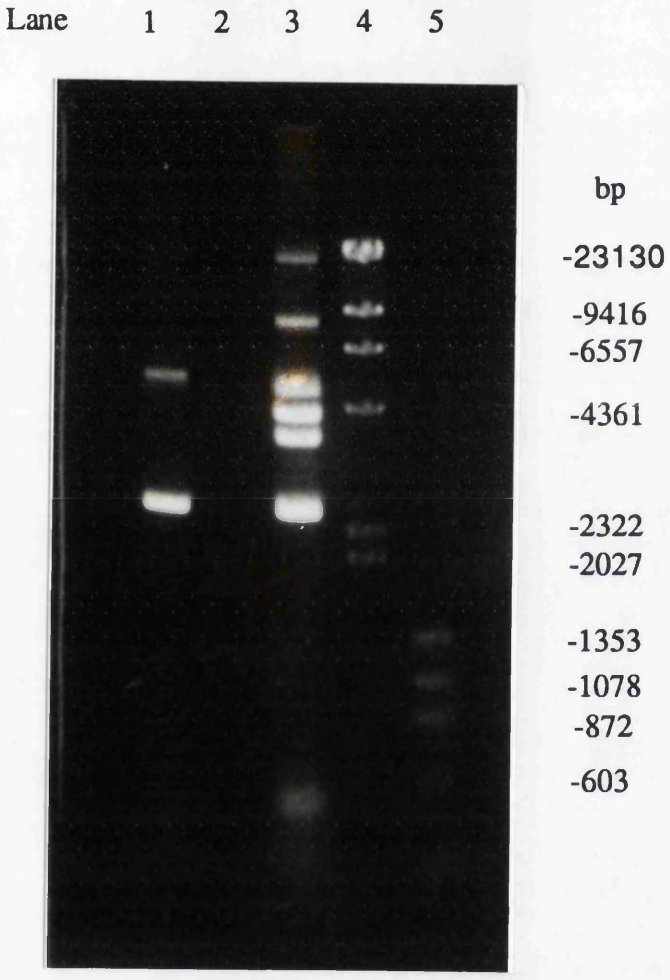
#### 6.2.9.1 Small scale plasmid preparation (Sambrook *et al.* 1989)

1. Single colonies are removed from the antibiotic-containing plates using a sterile cocktail stick and placed in 10 ml of L Broth.
2. Incubate at 37°C overnight on an orbital shaker.
3. Spin 500 x g 4°C, discard the supernatants.
4. Add 200 µl cold Solution I, votex well.
5. Add 400 µl fresh Solution II, mix by inversion and leave on ice for 5 minutes.
6. Add 300 µl cold solution III votex gently and leave on ice for 5 minutes.
7. Transfer to a microtube and spin 13,000 x g for 5 minutes.
8. Remove supertanants to a fresh microtube and add approximately 600 µl chloroform/phenol (1:1). Votex well.
9. Spin 13,000 x g for 5 minutes and transfer top phase to another microtube.
10. Add 600 µl iso-propanol and allow to stand 5 minutes at room temperature or in ice for 30 minutes.
11. Spin 13,000 x g for 5 minutes and discard the supernatants.
12. Wash the pellet with cold 75% ethanol and allow the pellet to dry in the air for 10 minutes.
13. Add 50 µl TE buffer containing DNAase-free pancreatic RNase (20 µg/ml, BRL, Gibco Ltd., UK). Votex briefly and store at -20°C.

#### 6.2.9.2 Isolation of fragment from low melting agarose gels for random priming (Parker & Seed 1980; Struhl 1985)

1. Digest plasmid (pSOD1) DNA with restriction emzyme, *Pst I* (BRL, Gibco Ltd., UK) (see section 6.2.7). *Pst I* is known to cut out the CuZn-SOD fragment (600 bp) (Lieman-Hurwitz *et al.*, 1982; Danciger *et al.*, 1986).
2. Run sample on 0.8% low melting point agarose (Sigma Chemical Co. Ltd., England) gel.
3. Stain the gel with ethidium bromide (see section 6.2.3) and examine gel briefly on a transilluminator. A photograph was taken as this stage and it showed that there was a band at 600 bp (Figure 6.4).
4. Cut out the band (600 bp) using scalpel into a pre-weighed 1.5 ml microtube.
5. Add sterile distilled water at a ratio of 2 ml/g of the gel.
6. Melt the gel by boiling the tube for 10 minutes.
7. Store DNA/agarose mixture at -20°C until use.

**Figure 6.4 Digestion of pSOD1 plasmid DNA and siza analysis**




pSOD1 plasmid DNA was isolated by the method of small scale plasmid preparation. 5 µg of plasmid DNA was applied to 0.8% low melting point agarose gel and run at 32V for 4 hours. Lane 1: undigested plasmid DNA. Lane 3: plasmid DNA was digested by restriction enzyme *Pst I*. There was a band at 600 bp in length which contained the CuZn-SOD insert. Lane 4: lambda DNA/*Hind III* fragments (molecular weight marker). Lane 5: phi X174 RF DNA/*Hae III* fragments (molecular weight marker).

6.2.9.3 Labelling by Random Priming (Feinberg & Vogelstein 1983,1984)

1. Set up reaction as follows:

| | |
|---|---|
| Oligonucleotide reaction mix | 10 µl |
| BSA (Sigma Chemical Co. Ltd., England) | 2 µl |
| $\alpha$-$^{32}$P-dATP(Amersham International plc., UK) | 2.5 µl |
| Klenow fragment | 2 units |
| DNA/agarose mixture* | 20 µl |
| Sterile $dH_2O$ | add to 50 µl |

Incubate at room temperature in a lead pot overnight.

* this mixture needs to be boiled for 10 minutes and kept at 37°C (in water bath) for at least 10 mins before use.

2. Terminate the reaction by addition of 50 µl oligonucleotide stop mix.
3. The incorporated dNTPs was separated from the unincorporated triphosphates by passing through a 7 cm Sephadex G-50 (DNA Grade, Pharmacia LKB, Sweden) column equilibrated with 1 x CB, 0.02% (w/v) SDS.
4. Collect incorporated dNTPs in a tube and leave it on ice.

6.2.10 Preparation of denatured salmon sperm DNA (Sambrook *et al.* 1989)

Salmon sperm DNA (type II sodium salt, Sigma Chemical Co. Ltd., England) was dissolved in 0.5 M NaOH at a concentration of 10 mg/ml and boiled for 30 minutes. When it cooled glacial acetic acid was used to adjust pH to 6.0. This mix solution was then diluted 2 fold and precipitated with 2 volumes cold ethanol at -20°C overnight. The precipitation was obtained by centrifuging at 10,000 x g 4°C for 5 minutes and dissolved in sterile water at a concentration of 10 mg/ml. The $OD_{260}$ of the solution was then determined and the exact concentration of the DNA was calculated as follows.

Denatured salmon sperm DNA (µg/ml)=$OD_{260}$ x dilution factor x 50

6.2.11 Hybridization with labelling probe and autoradiograph (Sambrook *et al.* 1989)

1. The filter was placed in heat-sealable polyethylene bag with 25-30 ml of prehybridization/hybridization mix and the air bubbles in the bag was removed as much as possible. The bag was placed in a shaking water bath at 68°C for at least 1 hour.

2. Boil the labelling probe for 10 minutes immediately before use and plunge it into ice.
3. The bag was carefully opened by cutting a hole near the top and the labelling probe was added to the solution in the bag. The solution was then mixed thoroughly.
4. Hybridization was carried out overnight at 68°C in the shaking water bath.
5. When hybridization was complete the filter was removed from the bag and washed quickly with 300 ml of washing solution (pre-heated to 68°C) for 5-10 minutes. Wash was repeated 3 more times.
6. The filter was blotted dry between 2 pieces of 3MM Whatman paper, then placed on a dry 3MM Whatman paper and covered with clingfilm.
7. The filter was put into an X-ray cassette and coverd with a X-ray film (Kodak XAR 5) in the dark. The film was exposed overnight at -70°C and developed the next day.

### 6.2.12 Solutions used in molecular genetic work

1. 0.05% DEPC water
   Add DEPC to $dH_2O$, leave at room temperature overnight and then autoclave.

2. 20 x Denhardts
   0.4% Ficoll
   0.4% Polyvinylpyrrolidone (PVP)
   0.4% Bovine serum albumin (BSA)
   Filter through 0.45 µm filter.

3. 20 x SSC, pH 7.4
   0.3 M Sodium citrate
   3.0 M Sodium chloride

4. 1x lysis buffer
   0.05 M Tris, pH 7.9
   0.5 mM EDTA
   10 mM Sodium chloride

5. 2x lysis buffer
   0.1 M Tris, pH 7.9
   1.0 mM EDTA
   20 mM Sodium chloride
   4% SDS

6. 5x Formaldehyde gel-running buffer
   0.1 M MOPS (pH 7.0)
   40 mM Sodium acetate
   5 mM EDTA (pH 8.0)

7 10 x column buffer, pH to 5.0 with glacial acetic acid
3 M sodium chloride
0.1 M sodium acetate
0.2% (w/v) SDS

8. CSB buffer
42 mM Sodium citrate
0.83% N-lauryl sarcosine
0.2 mM b-mercaptoethanol

9. Denaturing solution (for Southern blotting)
0.5 M Sodium hydroxide
1.5 M Sodium chloride

10. Denaturing solution (for RNA isolation)
25g Guanidine thiocyanate (4M final) (Fluka AG, Switzerland)
33ml CSB buffer
Mix throrough until components are completely dissolved.

11. E buffer, pH 8.2 with glacial acetic acid
40 mM Tris
20 mM Sodium acetate
1 mM EDTA

12. Formaldehyde gel-loading buffer
50% Glycerol
1 mM EDTA (pH 8.0)
0.25% Bromophenol blue

13. L Broth, pH 7.2, for 1000 ml:
10 g Tryptone
5 g Teast extract
5 g Sodium chloride
1 g Glucose

14. Neutralising solution, pH 7.4 (for Southern blotting)
1 M Tris
2 M sodium chloride

15. Oligonucleotide reaction mix
Solution A
Solution B
Solution C
in ratio 100:250:150.

16. Oligonucleotide stop mix
20 mM sodium chloride
20 mM Tris, pH 7.5
2 mM EDTA
0.25% (w/v) SDS

15. Phenol:chloroform:isoamyl alcohol
Mix at the ratio of 25:24:1
Store at 4°C

16. Prehybridization/hybridization mix (for DNA)
5x SSC
4x Denhardts
10% Dextran sulphate (Sigma Chemical Co. Ltd., England)
0.1% SDS
0.1% Sodium pyrophosphate (PPi)
100 µg/ml Denatured salmon sperm DNA (see section 6.2.8)

17. Prehybridization/hybridization mix (for RNA)
5x SSC
4x Denhardts
0.1% SDS
0.1% Sodium pyrophosphate (PPi)
100 µg/ml Denatured salmon sperm DNA (see section 6.2.8)

18. Proteinase K (Fungal)
Make up a 10 mg/ml solution in 1x lysis buffer + 0.05% SDS and store at -20°C.

19. Red cell lysis buffer, pH 7.4
155 mmol/L Ammonium chloride ($NH_4Cl$)
10 mmol/L Patassium hydrogen carbonate ($KHCO_3$)
100 µmol/L $Na_2$-EDTA

20. Ribonuclease A (RNase A) (BRL, Gibco Ltd., UK)
Make up a 10 mg/ml solution, boil for 5 minutes and store at -20°C.

21. Sephadex SP-50
4 g SP-50
100 ml 1 x column buffer
0.2% (w/v) SDS

22. Solusion I (for plasmid DNA preparation)
50 mM Glucose
25 mM Tris (pH8.0)
10 mM EDTA (pH8.0)

23. Solution II (for plasmid DNA preparation)
0.2 N NaOH
1% SDS

24. Solution III (for plasmid DNA preparation)

| | |
|---|---|
| 5 M Potassiou Acetate | 60 ml |
| Glacial acetic acid | 11.5 ml |
| Water | 28.5 ml |

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

25. Solution A (for random primer labelling of DNA)
1 ml solution O
18 µl β-mercaptoethanol
5 µl 100 mM dCTP (BRL, Gibco Ltd., UK)
5 µl 100 mM dTTP (BRL, Gibco Ltd., UK)
5 µl 100 mM dGTP (BRL, Gibco Ltd., UK)
Store at -20°C.

26. Solution B, pH 6.6 with 4 M NaOH (for random primer labelling of DNA)
2 M Hepes titrated (Sigma Chemical Co. ltd., England)

27. Solution C (for random primer labelling of DNA)
Hexadeoxyribonucleotides dissolved in TE at 90 OD units/ml.

28. Solution O, pH 8.0 (for random primer labelling of DNA)
1.25 M Tris
0.125 M magnesium chloride

29. Stop mix, pH 8.1
15% Ficoll (Sigma Chemical Co. Ltd., England)
0.2 M EDTA
0.01% (w/v) Orange G

30. TE buffer, pH 8.0
10 mM Tris-HCl
1 mM EDTA

31. Washing solution
2x SSC
0.1% SDS
0.1% PPi

### 6.2.13 SOD activity assay

SOD activity in red blood cells was determined by a photochemical augmentation method described by Misra and Fridovich (1977) (For details see Chapter 3). Briefly, 1 ml of red cell pellet was resuspended in 1 ml of ice-cold distilled water, mixed and put in ice for 2 hours. 800 µl of cooled absolute ethanol and chloroform mixture (3:5, v/v) was then added and mixed thoroughly. Lysate was obtained after centrifugation. All steps were carried out at 4°C. The SOD activity was then assayed based on the increase in rate of photo-oxidation of 0-dianisidine.

### 6.2.14 Statistical analysis

Data are expressed as mean ± S.D., with differences between groups being assessed by Student's *t* test.

## 6.3 Results

The SOD activity was significantly decreased in patients with PIH as compared to either normal pregnant women (p=0.036) or normal nonpregnant women (p=0.017) (Table 6.1). There was no difference between the normal pregnant women and normal nonpregnant women (p=0.37).

Genomic DNA from white cells of the eight women with PIH was treated with endonuclease (*EcoRI*) and hybridized to a CuZn-SOD probe. Representative experiments are shown in Figure 6.5. The frequencies of the 5.26 and 0.74 Kb fragments did not differ between patients with PIH and the control groups as all three groups showed all of these bands. There was also no difference between PIH patients with proteinuria and those without. However, there seems to be an extra band in lane 7 whose size is close to 5.26 Kb. This patient was known to be complicated with asthma and nephrotic syndrome.

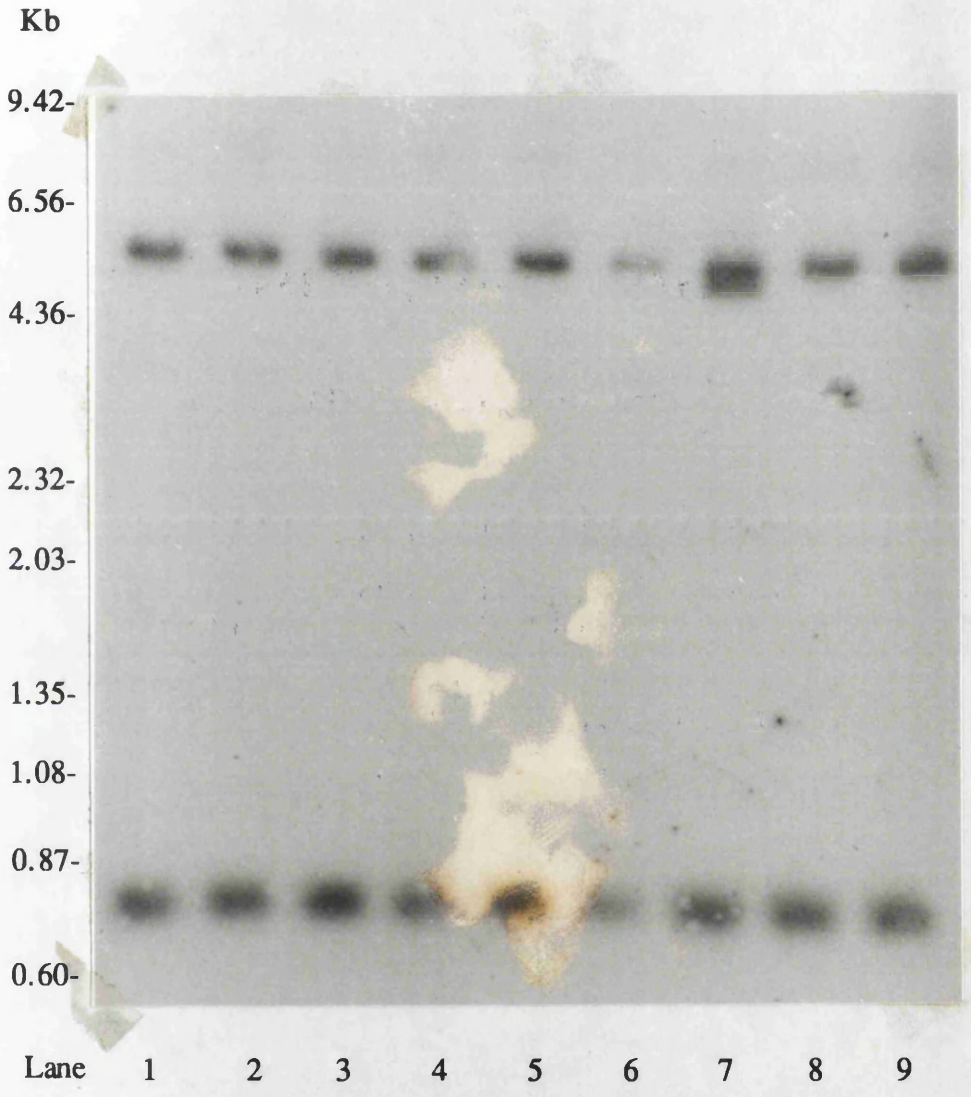
Figure 6.6 shows the Northern blot analysis of RNA isolated from white cells. The RNA was hybridized to a cDNA probe of CuZn-SOD. A band, estimated (by the 0.24-9.5 Kb RNA Ladder) to be 0.67 Kb, was detected. This is similar size of the CuZn-SOD mRNA in human cells reported by others (Lieman-hurwitz *et al.* 1982; Sherman *et al.* 1984; Delabar *et al.* 1987). The results obtained in the Northern blot experiments were quantified by the densitometry on a Microcomputer Imaging Device (Imaging Research INC., Canada). No significant changes in expression of the CuZn-SOD gene was found between the patients with PIH and controls ($p>0.05$). There was also no difference in CuZn-SOD gene expression between PIH patients with proteinuria and those without.

**Table 6.1. SOD activity in patients with PIH and controls**

| | n | SOD(μmol/L) | p* |
|---|---|---|---|
| PIH | 8 | 37.0±7.1 | |
| Normal pregnancy | 7 | 44.1±4.5 | 0.036 |
| Normal nonpregnancy | 5 | 47.0±5.5 | 0.017 |

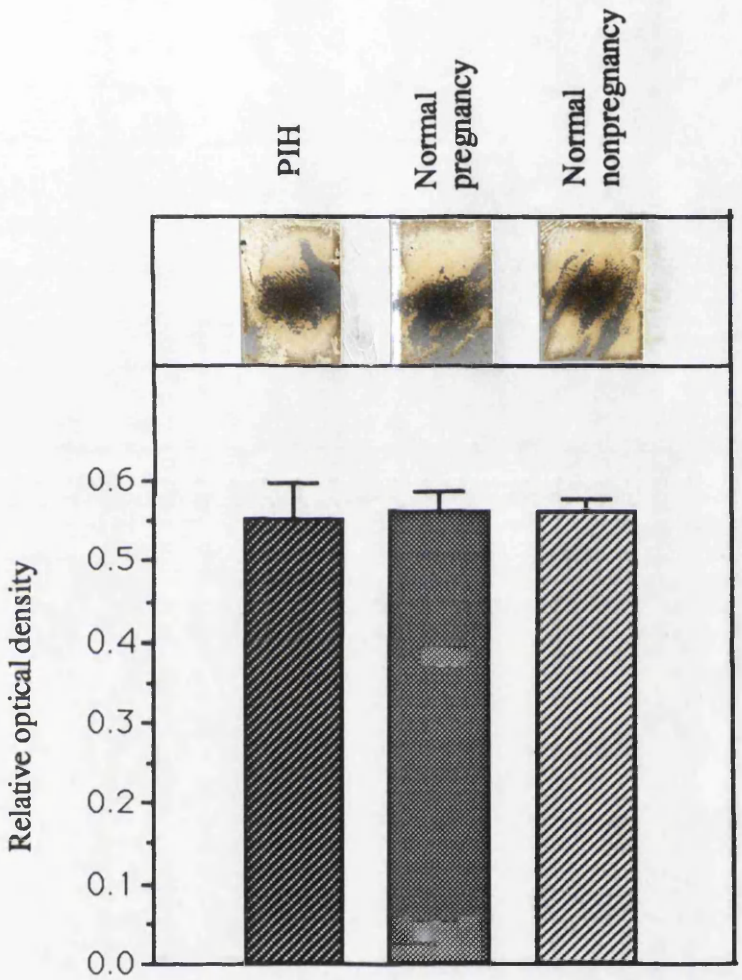
* Compared with PIH.

**Figure 6.5 Southern blot analysis of DNA**




Southern blot analysis of genomic DNA from white cells of PIH patients (lane 3-7), normal pregnant women (lane 8, 9) and normal nonpregnant women (lane 1, 2). DNA was digested with restriction enzyme *EcoRI* and the filter was hybridized using a human CuZn-SOD probe. The frequencies of 5.26 and 0.74 kb fragments did not differ between PIH patients and the control groups.

**Figure 6.6 Northern blot analysis of RNA**




Northern blot analysis of RNA from white cells of PIH patients (n=7), normal pregnant women (n=7) and normal nonpregnant women (n=5). Blots were hybridized with a human CuZn-SOD probe. The intensities of autoradiographic images were quantified by the Microcomputer Imaging Device. The upper panel shows the autoradiography of a representative experiment. The graph represents the mean ± SD of the independent experiments.

## 6.4 Discussion

The assay for SOD in this study is based on the property that SOD can increase the rate of the aerobic photo-oxidation of dianisidine, sensitized by riboflavin (Mira and Fridovich 1977). Although this assay is convenient, reproductive and free of serious interferences, it can not indicate the type of SOD. It could be both CuZn-SOD and Mn-SOD. However, the SOD measured in the present experiment is the SOD in peripheral red blood cells, this type of SOD is considered to be the CuZn-SOD as the mature red blood cells contain no mitochondria (Lewis 1989), which is home of Mn-SOD (Fridovich 1978; Getzoff *et al.* 1983). Therefore the decreased SOD activity found in this study model should correspond to CuZn-SOD.

CuZn-SOD is a major SOD in mammalian tissue. A decreased CuZn-SOD activity in PIH is not surprising because the disease has been associated with increased superoxide production and lipid peroxidation (Hubel *et al.* 1989; Wang *et al.* 1992; Tsukimori *et al.* 1993). Superoxide is known to exert a hypertensive effect by changing the pattern of prostaglandin production (Wang *et al.* 1992; also see Chapter 4), inactivating the endothelium-derived vascular relaxing factor (Gryglewski *et al.* 1986), and directly contracting smooth muscles (Katusic & Vanhoutte 1989). Conversely, injection of SOD has been shown to have a hypotensive effect (Nakazono 1991).

The inheritance of a low CuZn-SOD activity has been documented in some human diseases such as amyotrophic lateral sclerosis (Rosen *et al.* 1993). There is increasing evidence to show that a genetic factor plays a role in the pathogenesis of PIH (Cooper *et al.* 1988; Kilpatrick *et al.* 1989; Arngrimsson *et al.* 1990), but a study of the genetic aspects of antioxidant enzymes in PIH has not been investigated before. Although it is reasonable that there could be a link between the low SOD activity and the its gene mutation or defective expression, this possibility seems unlikely in the present study as both the DNA and mRNA related to CuZn-SOD in PIH did not differ from that in normal pregnancy and normal nonpregnant women. However, one patient (lane 7 in Figure 6.5) with the exception. An extra band whose size is close to 5.26 Kb appeared in this patient. Since this PIH patient was also suffering from asthma and nephrotic syndrome at time of sampling, whether this possible extra band was relevant to asthma or/and nephrotic syndrome remains to be further studied. It is possible that some women with PIH may be like the PIH patient suffered from asthma and nephrotic syndrome and show abnormalities of the SOD gene whereas others may not.

It is known that hydrogen peroxide, a product of SOD itself, can effectively inactivate CuZn-SOD enzyme by a Fenton-type reaction of $H_2O_2$ with $Cu^{++}$ at the centre of the enzyme, forming a reactive intermediate that destroys an essential liganding histidine residue (Blech & Borders 1983; Marklund 1984b). Hydrogen peroxide is mainly metabolized by a selenium-dependent glutathione peroxidase at the expense of glatathione (GSH) (Sinclair *et al.* 1990). A dietary lack of selenium has been suggested to contribute to a high incidence of PIH (Lu *et al.* 1990) and, furthermore, lower GSH levels occurred in patients with PIH (see Chapter 3). In ad`ition, activation of cells, especially white blood cells, is known to induce hydrogen peroxide formation (Curnutte *et al.* 1987; Klockars & Savolainen 1992). Neutrophils and mononuclear cells also appear to be activated in PIH (Greer *et al.* 1991; also see Chapter 2). Therefore, it is likely that increased hydrogen peroxide levels may result in the decreased SOD activity in PIH. A number of cytokines such as TNF and IL-1 have been reported to affect SOD activity *in vitro* (White *et al.* 1989). However, so far, their state in PIH is not known. Finally, the SOD deficiency can also occur at some time during the course of its protein synthesis.

Although the SOD activity in white cells has not been measured in this study, it can be inferred that its activity may be also lower in PIH patients. The increased activity of white cells in PIH patients (Greer *et al.*, 1991; also see Chapter 2) favors formation of hydrogen peroxide, which is a powerful chemical to inactivate SOD (Blech & Borders, 1983; Marklund, 1984b). And there is also a study which indicates that SOD activity in while cells is parallel to that in red cells (Feher *et al.* 1988).

In the absence of size abnormality of both mRNA and major structural rearrangements in genomic DNA, it is likely that the decreased SOD activity may be a secondary phenomenon which develops during the course of the disease, though whether there are subtle changes such as microdeletion/insertion or point mutation needs further investigation. Finally, it needs to be mentioned that absence of a genetic abnormality in CuZn-SOD does not exclude the possibility of a genetic induced deficiency of other antioxidant enzymes or proteins.

## 6.5 Conclusions

This study seems to indicate that there is not a genetic cause for the decreased SOD (CuZn) activity in PIH, suggesting that decreased SOD activity is an acquired phenomenon which occurs in the development of PIH.

# CHAPTER SEVEN PIH AND ESSENTIAL HYPERTENSION

## 7.1 Introduction and Aim of the Study

Hypertension in pregnancy is defined as a blood pressure of 140/90 mmHg or greater on two separate occasions. There are several possible causes of high blood pressure during pregnancy (Table 7.1). Among them, PIH and essential (chronic) hypertension are the most common. Although both PIH and essential hypertension in pregnant women have similar clinical phenomena, it is important to distinguish between them because they have different aetiological and pathophysiological mechanisms and thus require different treatments.

PIH is a placental disease. Its pathological features and clinical signs and symptoms disappear soon after delivery (Roberts 1984) and it can develop in abdominal and molar pregnancy (Page 1939; Rote 1985). All these suggest that the placenta is a vital factor in the pathogenesis of this disorder. It has been thought that immune system, ROS and eicosanoids contribute significantly to the aetiology of PIH (See Chapters 2, 3, 4).

The cause of essential hypertension is unclear. Alterations in systemic hemodynamics, the renal handling of salt and water, the renin-angiotensin-aldosterone system, sympathetic activity, genetic mechanisms and diet have all been implicated to varying degrees in the pathogenesis of essential hypertension. Although considerable attention has been focused on these elements, there is some evidence suggesting that in many forms of experimental as well as human essential hypertension, alterations in ROS and the immune system may have a role in the development of the disease.

As these two diseases have similar clinical features and some similar pathophysiological changes, it was the aim of this study to determine whether they show differences in the levels of antioxidant agents and in the immune system. This kind of study has not previously been carried out in hypertension during pregnancy.

**Table 7.1 Causes of hypertension in pregnancy***

| |
|---|
| 1. Pregnancy-induced hypertension |
| 2. Essential hypertension |
| 3. Renal disease |
| Renal artery stenosis |
| Chronic pyelonephritis |
| Acute and chronic glomerulonephritis |
| Polycystic renal disease |
| 4. Adrenocortical hyperfunction |
| Cushing's syndrome |
| Hyperaldosteronism |
| Congenital adrenogenital syndromes (17-and 11-hydroxylase defects) |
| 5. Pheochromocytoma |
| 6. Hypo- and hyperthyroidism |
| 7. Hypercalcemia |

* Adapted from Swartz *et al.* (1981).

## 7.2 Materials and Methods

### 7.2.1 Subjects

Three groups of sujects were enroled in this study. Group 1, 38 normotensive pregnant women; Group 2, 34 women with PIH; Group 3, 5 pregnant women with essential hypertension. The demographic characteristics of the study population are detailed in Table 7.2.

### 7.2.2 Diagnosis

The diagnosis of essential hypertension in pregnancy is usually based on any of the following findings (Hughes 1972):

1, A history of hypertension before pregnancy.

2, Persistent blood pressure elevations of at least 140 mmHg (systolic) or 90 mmHg (diastolic) before the 20th week of gestation.

3, Evidence of persistent hypertension beyond the 42nd day postpartum.

PIH was defined by the standard recommended by American College of Obstetricians and Gynecologists. Specifically, hypertension was defined as the presence of a blood pressure of 140/90 mmm Hg or more. Preoteinuria was defined as excretion of >300 mg of urinary protein/24 hours or ≥ + on urine 'dipstick' testing (For details see Chapter 1).

### 7.2.3 Peripheral blood mononuclear cells (PBMC) preparation and red blood cell (RBC) lysate preparation

Blood was obtained by venipuncture and collected into a universal containing 300 units of preservative-free heparin (for 10 ml of blood). A total of 60 ml of blood was obtained. PBMC were obtained by density gradient centrifugation using a modification of the method originally described by Boyum (1968) (For details see Chapter 2). RBC were disrupted by distilled water and lysate was extracted by using absolute ethanol and chloroform mixture (For details see Chapter 3).

### 7.2.4 Mitogenic activity assay

Briefly, PBMC were prepared in RPMI 1640 containing 10% heat inactivated human serum and made up to a concentration of $1x10^6$ cells/ml for PHA and ConA and

**Table 7.2 Clinical information on study population**

| Group | Age (year) | Gestational Age (week) | Diastolic Blood Pressure (mmHg) | Systolic Blood Pressure (mmHg) |
|---|---|---|---|---|
| Normal pregnancy | 27.9±4.9 | 35.2±3.7 | 73.1±9.5 | 116.3±12.0 |
| No.of observations | 38 | 38 | 24 | 24 |
| PIH | 26.2±4.2 | 35.3±4.5 | 102.4±7.9* | 150.0±15.0*+ |
| No.of observations | 34 | 34 | 34 | 34 |
| Essential hypertension | 28.8±5.8 | 33.4±7.7 | 90.8±3.8* | 136.6±5.9* |
| No.of observations | 5 | 5 | 5 | 5 |

All data were expressed as mean ± SD.

*$p<0.001$ versus normal pregnancy.

+$p<0.01$ versus essential hypertension.

$2x10^6$ cells/ml for PWM. All mitogens were obtained from Sigma Chemical Company and used at optimal concentrations. PHA was used at 10 μg/ml; ConA at 100 μg/ml and PWM at 200 μg/ml. Cells were grown for 72 hours and 1 μCi of $^3$H-thymidine (TRK418, Amersham) was added to each well for the final four hours of incubation. The cells were harvested using a Dynatech cell harvester and the incorporated tritiated thymidine was measured by a beta counter(LKB) (For details see Chapter 2).

### 7.2.5 IL-2 production

IL-2 containing supernatants were obtained by culturing PBMC at 4 x $10^6$ cells/ml in the presence of PHA (Wellcome) for 48 hours at 37 °C in 5% $CO_2$/95% air.

A murine IL-2-dependent cell line, HT-2 (a gift from Cetus Corporation) was used in a bioassay to assess IL-2 production. Reciprocal dilutions of the IL-2 containing supernatants were carried out in a microtitre plate. 1.5 x $10^4$ HT-2 cells were added to each of the reciprocal dilutions in triplicate. The cells were cultured at 37 °C in 5% $CO_2$/95% air for 20 hours. 1 μCi of $^3$H-thymidine (TRK418, Amersham) was then added to each of the wells and the plates were incubated for a further 4 hours. The cells were harvested by MicroMate™ 196 Cell Harvester (Packard Instrument Company, CT, USA) onto glass fibre paper. The $^3$H-thymidine incorporation was determined by a computer-programmed Matrix 96™ Direct Beta Counter (Packard Instrument Company, CT, USA). For quantitation of activity (units) in unknown samples, a curve was plotted for the percentage of maximum counts versus the dilutions for each of the samples/standards and then test results were compared with standard curve (For details see Chapter 2).

### 7.2.6 Immunoglobulin production and assay

The ability of B cells to secrete IgG and IgM in culture medium was determined. PBMC were made up to $1x10^6$ cells/ml in RPMI-1640 containing 10% FCS and cultured for 7 days in presence or absence of PWM(1:250 dilution). The amount of immunoglobulins in culture medium was measured using an ELISA assay as described in Chapter 2.

### 7.2.7 Antioxidant agent assay (For details see Chapter 3)

The methods for the measurement of superoxide dismutase (SOD) and plasma thiol (PSH) have been previously described by Banford *et al* (1982a, 1982b). Lysate thiol (LSH) level was measured according to the method of Misra and Fridovich (1977), based on the increase in the rate of photo-oxidation of O-dianisidine. The glutathione content of the plasma (GSHp) was measured enzymatically at 25°C by the method of Anderson (1985). Red cell membrane thiol (MSH) was measured by using Ellman's reagent. Ellman's reagent can be used to induce an oxidative stimulus on the exofacial membrane sulphydryl group of the human red cell, thus making it a chemical probe of the sulphydryl population (Hoey 1987). MSH was calculated by the following formula:

$$\text{MSH(No of thiols/cell)} = \frac{[ES^-] \text{ x volume x Avogadro's No.}}{\text{number of cells x 1000 x 13600}}$$

### 7.2.8 Statistical analysis

Results and data are expressed as mean±SD. Statistical comparisons between groups were carried out by Student's *t* test. Correlations between parameters were determined by univariate linear regression and the Pearson coefficient of correlation. A p value of less than 0.05 was considered significant.

## 7.3 Results

### 7.3.1 Mitogenic activity

Figure 7.1 shows mitogenic activity. There was no significant difference in any of the four parameters tested between normal pregnant women and women with essential hypertension. However, compared to both normal pregnant women and women with essential hypertension, PBMC from patients suffering from PIH showed enhanced $^3H$ thymidine uptake when stimulated by PHA (both $p<0.01$). Since the mean systolic blood pressure in patients with essential hypertension was significantly lower than in those with PIH (Table 7.2), the question arises whether the different mitogenic activity was due to the different blood pressure levels found in these two groups. A paired Student's *t* test was perfomed to see if these differences still exist after their blood pressure was matched for. Five PIH patients with 137.6±7.3 (mean±SD) mmHg of systolic blood pressure were compared to five essential

hypertensive patients with 136.6±5.9 mmHg of systolic blood pressure. After match, there was no significant difference in blood pressure between the two groups. The results showed that PHA-induced mitogenic activity was still lower in patients with essential hypertension than in those with PIH (62146±35593 vs 108870±47291 cpm, n=5, $p<0.05$).

### 7.3.2 IL-2 activity

IL-2 activity in patients with PIH (26.9±8.6 units/ml) was higher than in normal pregnant women (23.3±7.4 units/ml) or in pregnant women with essential hypertension (22.1±9.9 units/ml), however, a significant difference was found only between PIH and normal pregnancy ($p<0.01$) not between PIH and essential hypertension ($p=0.093$) (Figure 7.2).

### 7.3.3 Immunoglobulins

The amount of immunoglobulins (IgG and IgM) produced by PWM mitogen-stimulated PMBC is shown in Figure 7.3. IgG production in both PIH women and women with essential hypertension was significantly higher than that in normal pregnant women (both $p<0.05$). The levels of IgG were not significantly different between women with PIH and those with essential hypertension. IgM production was not different between the three groups.

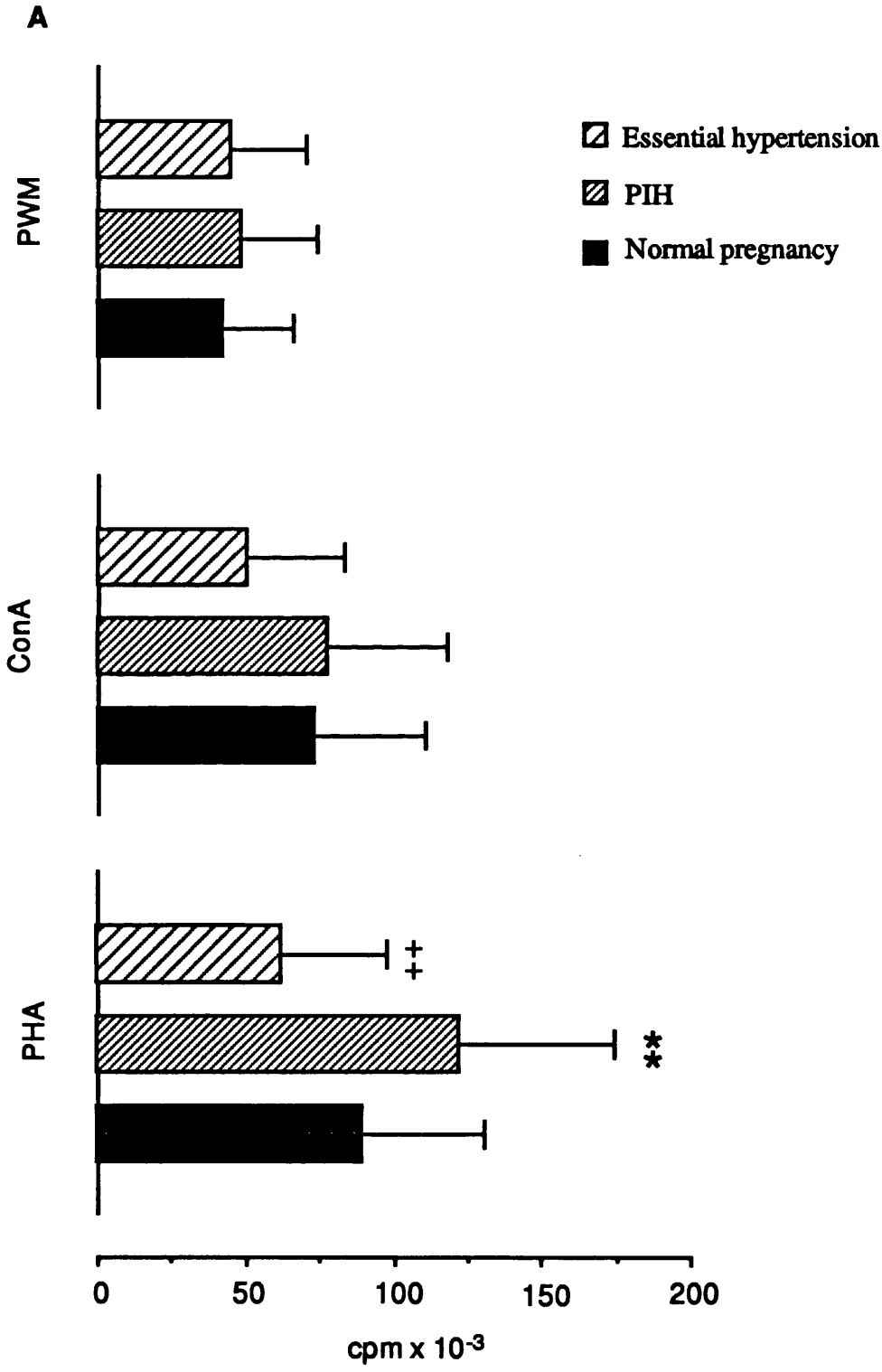
### 7.3.4 Antioxidant levels

The results for antioxidant activity are presented in Table 7.3. In patients with PIH, the levels of PSH were significantly reduced compared with normal pregnant women. The levels of LSH in pregnant women suffering from essential hypertension were much lower than those in normal pregnant women and PIH women. After blood pressure in both patient groups was matched for, the levels of LSH in PIH patients were 395±89 μmol/L which was still higher ($p<0.05$, paired Student's *t* test) than that in patients with essential hypertension (222±87 μmol/L). There was no significant differences in other antioxidant markers between the three groups.

### 7.3.5 Correlation

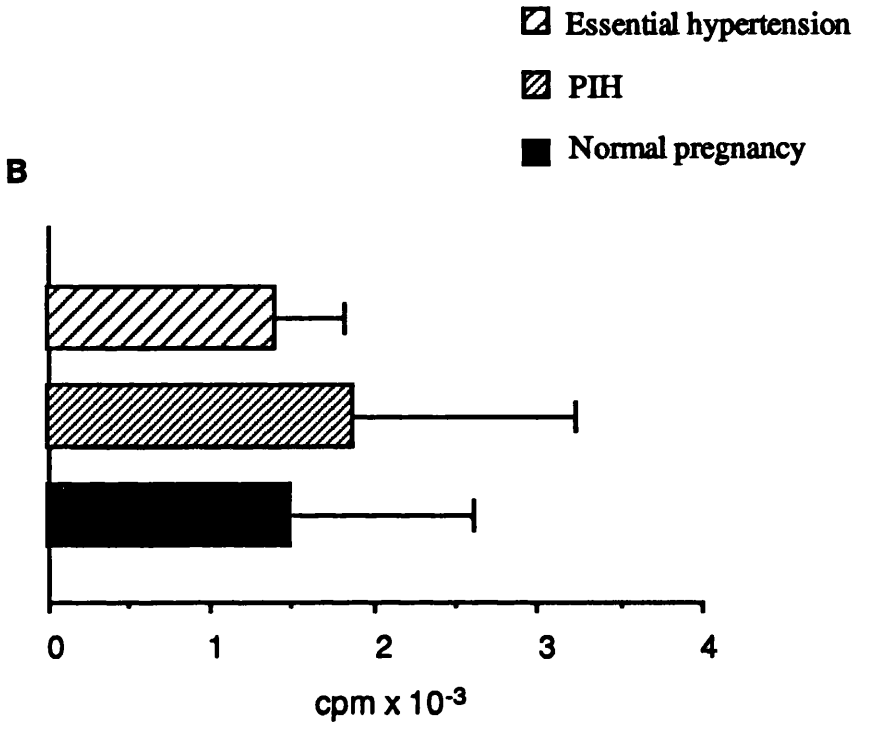
No significant correlation was found between blood pressure (systolic and diastolic) and any of the parameters tested in the investigated groups.

**Figure 7.1 Lymphocyte activity**



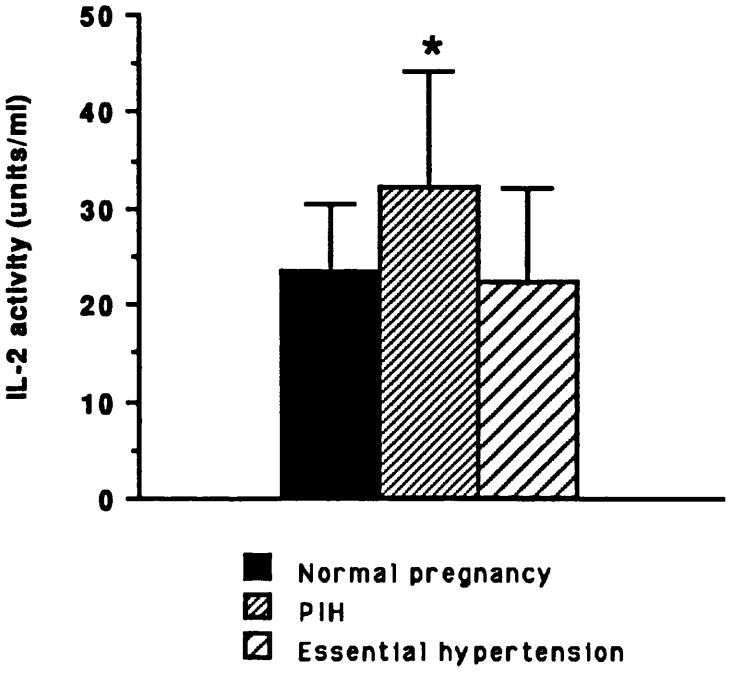

*Continuing*

*Figure 7.1 continuing*



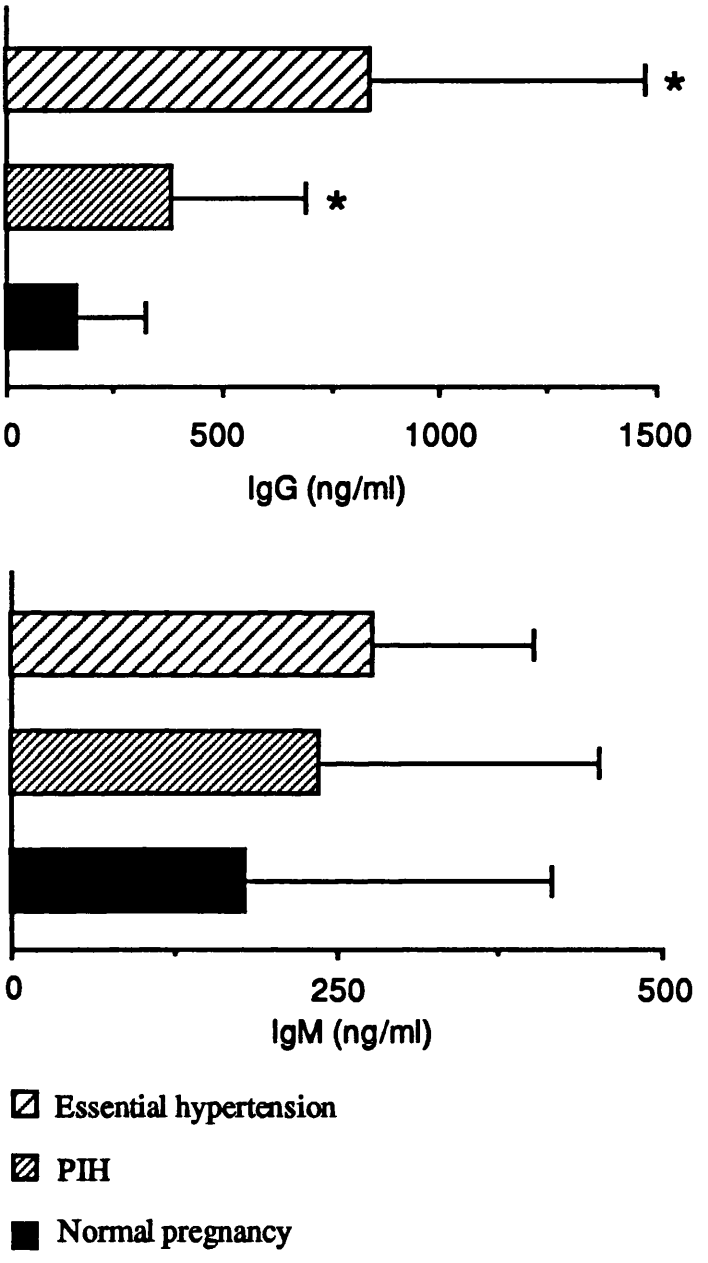

PBMC were incubated for 72 hours in the presence of mitogens (**A**, PHA, ConA and PWM) or in the absence of mitogens (**B**). The data are represented as mean ± SD. Statistical significance is expressed as: **p<0.01, compared with normal pregnant women; ++p<0.01 compared with women with PIH.

## Figure 7.2 IL-2 activity




IL-2 containing supernatants were obtained by culturing PBMC in the presence of PHA for 48 hours. IL-2 activity was determined by a bioassay using HT-2 cells, an IL-dependent cell line. The data are represented as mean of samples with standard deviation. Statistical significance is given by asterisk: *$p<0.01$ compared with normal pregnant women.

## Figure 7.3 Production of immunoglobulins by lymphocytes




Immunologlobulins were secreted from PBMC stimulated by PWM after 7 day incubation. IgG and IgM were measured by ELISA. The data are represented as mean ± SD. Statistical significance is expressed as: *$p<0.05$ compared with normal pregnant women.

**Table 7.3 Antioxidant levels**

| | PSH (μmol/L) | LSH (μmol/L) | SOD (μmol/L) | GSHp (μmol/L) | MSH ($1x10^6$ thiol/cell) |
|---|---|---|---|---|---|
| Normal pregnancy | 358±74 | 425±161 | 44.4±19.9 | 16.4±8.0 | 4.90±0.75 |
| No.of observations | 36 | 36 | 37 | 38 | 31 |
| PIH | 300±70* | 411±174 | 37.0±13.5 | 14.0±11.2 | 4.46±0.90 |
| No.of observations | 34 | 33 | 34 | 34 | 34 |
| Essential hypertension | 346±66 | 222±87*,+ | 53.2±40.5 | 12.4±8.2 | 4.82±0.59 |
| No.of observations | 5 | 5 | 5 | 5 | 5 |

All data were expressed as mean ± SD.

*$p<0.05$ versus normal pregnancy.

+$p<0.05$ versus PIH.

## 7.4 Discussion

Although PIH and essential hypertension have the most important clinical feature in common, that is, high blood pressure, the cause of these conditions remains unclear. Recently, it has been suggested that a disorder of the endothelial cells may play an important role in the regulation of blood pressure and hence contribute to the development of both diseases in some similar and/or different ways (Tesfamariam & Halpern 1988; Koga *et al.* 1989; Roberts *et al.* 1989; Pinto *et al.* 1991).

Endothelial cells, which lie between the circulating blood and vascular smooth muscle cells, are a rich source of prostacyclin and other endothelium-derived relaxing factors (EDRF) (Moncada *et al.* 1977; Luscher 1988). The control of vascular function by the endothelium, however, proved to be more complex than anticipated as the cells not only release different vasodilator substances but also mediate contraction of the underlying vascular smooth muscle. Endothelium-dependent contractions are known to be elicited by a number of substances such as arachidonic acid, thrombin, acetylcholine, nicotine, norepinephrine and physical stimuli (quick stretch and pressure) (DeMey & Vanhoutte 1982; Harder 1987; Katusic *et al.* 1987; Katusic *et al.* 1988; Shirahase *et al.* 1988). Recently, immunological factors and antioxidant factors have also been suggested to mediate endothelial functions in several ways.

Activated polymorphonuclear leukocytes, monocytes and lymphocytes can adhere to endothelial cells and damage them by generating toxic substances such as ROS. In this microenvironment, the production of cytotoxic products derived from the metabolism of molecular oxygen, including superoxide anion, hydrogen peroxide, hydroxyl anion and hypochlorous anion, inevitably leads to an increase in vascular permeability and myocyte damage and a decrease in vasodilator substance (such as PGI2) production by the endothelium. Cytokines are mediators of the complex bidirectional interactions between leukocytes and vascular cells. Cytokines produced by activated lymphoid and mononuclear phagocytes elicit a complex spectrum of responses in endothelium. IL-1 is able to induce PGI2 production from endothelial cells (Rossi *et al.* 1985) and IL-1, IL-2 and TNF can cause endothelial cells to become markedly adhesive for leukocytes and lymphocytes (Cavender *et al.* 1986; Cotran 1987). Furthermore, it has been documented that IL-2 therapy could prevent the development of hypertension and lower blood pressure to normotensive levels in hypertensive rats, though conflicting reports existed (Tuttle & Boppana 1990).

In the present study, lymphocyte mitogenic activity and IL-2 production were found to be increased in patients with PIH but not in patients with essential hypertension, suggesting that in the pathogenesis of these two diseases cellular immunological factors may only be associated with the former but not with the latter.

It is well known that ROS can damage endothelium and conversely, antioxidants can prevent endothelial injury from ROS (For details see Chapter three). A decreased levels of antioxidants may indicate that the balance between pro-oxidation and anti-oxidation is shifted to the former. Both PGI2 and EDRF are readily degraded by ROS (Prabha *et al.* 1990). In the present, series evidence of reduction in antioxidant levels was found in both PIH and essential hypertension though the types of antioxidants involved were somewhat different. The levels of PSH was decreased in PIH while LSH remained unchanged. In contrast, the levels of PSH was unchanged and LSH was decreased in essential hypertension. The levels of GSH and MSH were similar in both diseases. Although the mechanisms underlying these differing changes is unknown, the findings indicate that antioxidant/ROS factors could be relevant to the development of hypertension in both diseases.

A common finding in patients with PIH or essential hypertension is an elevated level of IgG production. However, the significance of this change may be different for these two diseases. As elevated levels of autoantibodies to trophoblast antigens, amniotic glycoprotein, placental and renal antigens, and endothelial cells are evident in PIH (Rappaport *et al.* 1990; Sibai 1991) and increased lymphocyte mitogenic activity and IL-2 production have been found in PIH, the higher IgG production may reflect an increased B cell function in PIH and therefore represent the involvement of the humoral components of the immune system in the pathogenesis of PIH. In essential hypertension, there is evidence to suggest that elevated levels of IgG are secondary to vascular damage since immunologlobulin levels were highest after a vascular event (Kristensen & Solling 1983). However, elevated immunoglobulin levels were also found in patients with essential hypertension who had not experienced a vascular pathology (Suryaprabha *et al.* 1984). Therefore, the possibility that humoral immunological factors are involved in the onset of essential hypertension cannot be excluded.

It has been reported that the levels of serum IgG correlated positively with blood pressure in essential hypertension (Kristensen 1978). However, no significant correlations were found between blood pressure and any immunological or

antioxidant markers in this study. Therefore it is possible that there is no direct causal link between blood pressure elevation and the immunological or antioxidant factors tested in this study.

## 7.5 Conclusions

Although both PIH and essential hypertension in pregnant women have similar clinical features, the mechanisms underlying them are different. Immunological factors might be involved in the pathogenesis of PIH, but it is unlikely that this is the case of essential hypertension. Antioxidant changes are seen in both diseases.

# CHAPTER EIGHT FINAL DISCUSSION

This study like other previous ones suggests that the cause of PIH is multifactorial. The immune system changes, ROS, eicosanoids and genetic factors all may have a role in the development of this disorder.

The occurrence of PIH in abdominal and molar pregnancies indicates that uterine and fetal factors are not required (Page 1939; Rote 1985). Two areas of direct contact between the mother and fetus are thus located: the syncytiotrophoblast, bathed by maternal blood, and the extravillous cytotrophoblast within the decidua. Trophoblastic cells are heterogeneous. They express paternal antigens, and these antigens can elicit a graft rejection response (Toder *et al.* 1982; Foglia *et al.* 1986). Furthermore fetal antigens may circulate in the maternal circulation as early as the 18th week of gestation (Beer 1988). On the other hand, trophoblast supernatant can suppress mononuclear cell transformation (Silver *et al.* 1990). The ability of the first-trimester trophoblast to inhibit maternal T-cell proliferation and the balance between the fetal antigenic load and maternal blocking antibodies may contribute directly to fetal survival and it is also believed that they may play an important role in triggering the abnormal immunological characteristics seen in PIH, such as an increased cellular immunity and an excess production of autoantibodies (For details see Chapter 2).

Because immunological responses are determined genetically, genetic predisposition also appears to be one of the major contributing factors to the development of PIH (Kilpatrick *et al.* 1987 & 1989a; Arngrimsson *et al.* 1990). A delicate balance between the maternal immune responses and the fetal genotype may regulate the process of trophoblastic invasion that is necessary for normal placentation. A disturbance in this balance by either maternal or fetal factors may preclude the normal hypertrophy of placental arteries in synchrony with the growing uterus (poor placentation) (Robertson & Khong 1987; Roberts *et al.* 1989). Under these circumstances, stretching and tension on the placental artery will produce lesions of the arterial wall, initiating biochemical maladaptations in the maternal vasculature. These changes include a decreasing PGI2 production, platelet aggregation and overproduction of TXA2 (Friedman 1988). Other maladaptational changes involve the renin-angiotensin-aldosterone system and the kallikreikinin system (Hanssens *et al.* 1991). These changes would be followed by placental hypoxia, leading through a chain of events to the development of PIH.

Endothelial cell injury and altered endothelial cell function play an important role in the pathogenesis of PIH. The damaged vascular endothelium expresses antigens that make these cells themselves an immunological target. Antibodies to human vascular endothelial cells are present in PIH (Rappaport *et al.* 1990). Binding of these antivascular endothelial cell antibodies and immune complexes to resting endothelial cell monolayers may be involved in altered PGI2 secretion, increased platelet adherence, activation of the complement cascade, and disruption of the monolayers. A cytotoxic circulating factor also contributes to human endothelial cell injury (Rodgers *et al.* 1988). In addition, mitogenic activity is increased in predelivery blood obtained from PIH patients (Musci *et al.* 1988). It has been hypothesized that this increase in mitogenic or growth factor activity was a result of the direct release of mitogens from injured endithelial cells or from platelet activation by perturbed endothelium (Musci *et al.* 1988; Taylor *et al.* 1990).

Reactive oxygen species (ROS) and antioxidants may establish the link between the immunological mechanisms and the injury of endothelial cells in PIH. Immunological maladaptation can result in placental hypoxia and activation of leukocytes both of which are capable of inducing ROS formation and reducing antioxidant ability (Babior 1978; Flamm *et al.* 1978; Granger *et al.* 1981; Kogure *et al.* 1982; Marletta 1989). In addition, the active arachidonic acid metabolism seen in PIH is also a promoting factor in ROS production (Miyamoto *et al.* 1976; Ogino *et al.* 1978). The ROS are well known to be cytotoxic to cells by oxidative conversion of membrane unsaturated fatty acids and damaging protein and nucleic acid (Wolff *et al.* 1986; Wispe & Roberts 1987; Schraufstatter *et al.* 1988). In PIH, both the immunological maladaptation and the hypoxic environment occur subsequently in the placental vascular bed and thus set the stage for the imbalance of increased ROS formation and damaged antioxidant levels which lead to the endothelial cell injury. Increased ROS can also (1), change the pattern of prostaglandin production in favour of TXA2 synthesis (Moncada *et al.* 1976; Salomon *et al.* 1978; Wang *et al.* 1992; also see chapter 3 & 4); (2), inactivate EDRF (Gryglewski *et al.* 1986); (3), cause a decrease in endothelin levels (Dekker *et al.* 1991). Although, it is believed that increased ROS formation results from immunological abnormalities and placental hypoxia, other mechanisms may also have a role to play. The normal CuZn-SOD gene structure and expression indicates that reduced SOD activity may not be primary event (see Chapter 6). However, this does not exclude the possibility that genetic factors may associated with other antioxidant agents. Indeed, the incidence of PIH is higher in a Norwegian family in whom there is a failure of the NADH ubiquinone oxidoreductase step in the mitochondrial electron chain (Torbergsen *et*

*al.* 1989), still giving a suggestion that there could be a genetic factor in imbalance of ROS and antioxidants found in PIH.

**Hypothesis**

Extensive morphological and physiological changes take place in the uteroplacental vasculature which is necessary for the development of hemochorial placentation, characteristic of human pregnancy. A low-resistance, low-pressure, high-flow placental vascular system is therefore developed (Ramsey & Donner 1980). These vascular changes are thought to be induced by the interaction of the fetal-derived trophoblasts with maternal tissues at the time of implantation. In PIH, immunological maladaptation results in poor placentation in which the endovascular system remains a non-pregnant architecture and fail to dilate (Robertson & Khong 1987). The proposed abnormal maternal immunological activity and the placental hypoxia may induce an imbalance of increased ROS formation and decreased antioxidant levels. ROS are probably produced by various leukocytes and other local cells and they can not only impair endothelial cells but also directly cause vasoconstriction (Gryglewski *et al.* 1986; Katusic & Vanhoutte 1989). The antigens on the damaged endothelial cells may be attacked by the immune system. Such alterations can cause the imbalance of increased TXA2 production and decreased PGI2 formation, damage other vasodilators such as EDRF and endothelin, and probably lead to the release of lipoxygenase products. These changes, in turn, result in the clinical and laboratory signs of PIH (Figure 8.1).

As the story unfolds, it is becoming apparent that endothelial dysfunction is the final common pathway in the aetiology and pathogenesis of PIH. However, there are some critical questions remain to be answered. What is the exact role of the trophoblasts in PIH? Does a genetic factor have a role to play in the development of PIH? What is the nature of the special circulating factor(s) in PIH? And where do they come from? Definitive studies of this disorder are urgently needed if we wish to diminish this leading cause of maternal morbidity and mortality, intrauterine growth retardation, and perinatal morbidity and mortality.

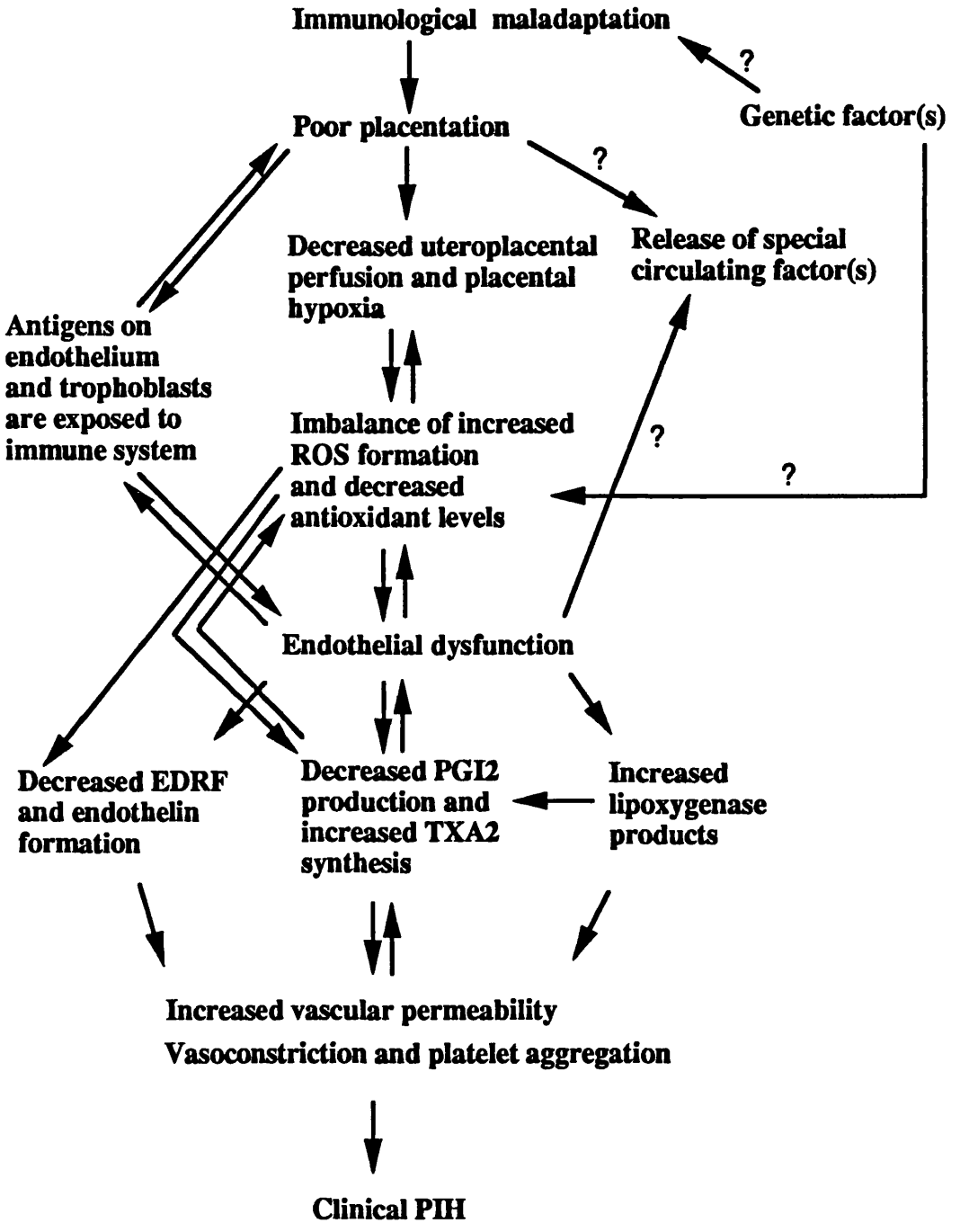



**Figure 8.1** **Proposed model to explain the aetiology and pathogenesis of PIH**

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