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PLACENTAL PROSTACYCLIN PRODUCTION

IN PREGNANCY INDUCED HYPERTENSION

presented by

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Major professor

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PLACENTAL PROSTACYCLIN PRODUCTION

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By

Michael James Behr

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ABSTRACT

PLACENTAL PROSTACYCLIN PRODUCTION IN PREGNANCY INDUCED HYPERTENSION

By

Michael James Behr

Production of prostacyclin, a potent vasodilator and inhibitor of platelet aggregation, was investigated by RIA of its stable metabolite, 6-keto-$PGF_{1\alpha}$ in normal and toxemic placentas. Tissues (300mg) were incubated for 48h at 37°C with 95% O_2, 5% CO_2 in a metabolic shaker. Prostacyclin production was significantly reduced in toxemia (toxemic vs. normal, 2.72 $\pm$ 0.49 vs. 7.22 $\pm$ 0.49 pg/mg/h, mean $\pm$ SE, $p<0.01$). In normal and toxemic placentas indomethacin (5 or 50uM) inhibited prostacyclin production whereas arachidonic acid (5 or 100uM) had no effect ($p>0.10$). In a low oxygen environment (20% O_2) only production by normal placentas was significantly reduced ($p<0.01$). Amnionic and chorionic production was not affected by toxemia ($p>0.10$). Both tissues had similar production rates ($p>0.01$), approximately 1/7th that of the normal placental rate. These findings demonstrate a decrease in placental prostacyclin production in toxemia and underscore the possible involvement in the etiology and associated complications of this disorder.

The preeclamptic patient "has been blistered, bled, purged, packed, lavaged, irrigated, punctured, starved, sedated, anesthetized, paralyzed, tranquilized, rendered hypotensive, drowned, been given diuretics, had mammectomy, been dehydrated, forcibly delivered, and neglected." (Zuspan and Ward, 1964)

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I. INTRODUCTION

Each year more than 45,000 infants die in the United States. Premature births account for 30,000 of these deaths. Despite the success of the United States at reducing infant mortality by approximately 55% over the last 20 years, it is surprising to note that we still have a higher infant death rate than thirteen other countries (Jaquet and Bodde, 1983). According to the National Institute of Child Health and Human Development a major reason for this statistic lies in the relatively large number of "low birth weight" babies that are delivered in this country. An infant whose weight at birth is less than 5.5 pounds is considered too small (James and Adamsons, 1982). One out of every 13 newborns fall into this category (Jaquet and Bodde, 1983; Walsh, 1979). Low birth weight in children is attributed to one of two factors, premature delivery or fetal growth retardation while in utero. Neither condition, however, is mutually exclusive (Jaquet and Bodde, 1983).

What challenges does a premature infant face in his new environment? Not only is he more likely to die before his first birthday than is his counterpart delivered at term, but he is also more likely to experience long-term developmental disabilities (Jaquet and Bodde, 1983). For example, respiratory distress syndrome which is prevalent among premature infants often leads to chronic respiratory complications. Also of major concern is the increased risk of

intracranial hemorrhage which can lead to hydrocephalus and mental retardation.

To improve the odds of infant survival, investigators have attempted to elucidate the various mechanisms involved in inducing fetal growth retardation and premature delivery. This thesis is concerned with the etiology of the clinical syndrome preeclampsia-eclampsia, also known as toxemia of pregnancy or pregnancy induced hypertension. Toxemia occurs in approximately five percent of all pregnancies and is a major cause of morbidity in the mother, newborn and fetus (Dennis et al., 1982).

The three principle pathophysiological changes that characterize toxemia are increased vasoconstriction, increased platelet aggregation, and reduced uteroplacental blood flow (Chesley, 1978). Clinically, these abnormalities manifest themselves by high blood pressure, edema, proteinuria, central nervous system hyperactivity, and disseminated intravascular coagulation (Dennis et al., 1982). The most advanced stage of the disease, known as eclampsia, is distinguished from preeclampsia by the appearance of maternal convulsions. Eclampsia along with hemorrhage and infection constitute the three leading causes of maternal fatalities (Pritchard and Macdonald, 1976; Dennis et al., 1982).

II. LITERATURE REVIEW

A. Background

The dangers associated with toxemia of pregnancy have been recognized for centuries. Although the term "eclampsia" does not appear in the literature until 1619, references to the symptomatology of the disease dates back to Hippocrates (Dexter et al., 1941; Theobold, 1955). An attempt to accurately outline ancient writings on the subject is thwarted by the frequent lack of documentation in early review articles (Chesley, 1978). As Chesley writes, this problem is exacerbated by, "errors that live on in [these] second-, third-, and nth hand reviews."

During the 17th and 18th century in France, physicians began introducing into the literature a more definitive description of what would eventually be known as eclampsia (Chesley, 1978). Francois Mauriceau (1637-1709) made important contributions in this respect (Dexter et al., 1941; Chesley, 1978). In his textbook on obstetrics he noted that there is an increased chance of maternal and fetal death if between convulsions the mother does not remain conscious; that primagravidas are more susceptible to seizure than are multiparas; and that convulsions following delivery are not as life threatening as as those which occur during pregnancy (Chesley, 1978).

A century later T. Denman wrote in his Essays on the Puerperal Fever and on Puerperal Convulsions (Dexter et al.,

1941) that "When convulsions seize a woman at the full time of pregnancy, almost all authors whom I have consulted, have recommended the use of every method in our power to hasten delivery, a speedy delivery being, in their opinion, the only chance to preserve the life of the mother and child."

A major obstacle in defining eclampsia as a disease state was the inability to distinguish the disorder from epilepsy (Chesley, 1978). Differentiation between the two conditions appeared in Pathologia Methodica published by Bossier De Sauvages in 1739. De Sauvages defined convulsions of acute causation as eclampsia and attributed seizures that recurred over extended periods of time to epilepsy. This definition of eclampsia was the standard for more than 200 years (Chesley, 1978). Unfortunately, unless convulsions appeared, clinical diagnosis was extremely difficult. Even today a significant number of preeclamptics are misdiagnosed or go unrecognized.

The following four sections will briefly describe the primary clinical criteria presently used in determining preeclampsia and also discuss coagulation changes that occur with the disease.

B. Clinical Signs

1. Hypertension

The existence of hypertension with eclampsia is a relatively recent finding. In the late 19th century clinicians were able to obtain estimates of blood pressure with the use of crude sphygmographic tracings (Chesley, 1978). Ballantyne (1889) showed an increase in the blood pressure of two women with eclampsia and one with severe preeclampsia (Chesley, 1978). When use of the modern sphygmomanometer, which was perfected by Rocci, became widespread, the changes in blood pressure during normal pregnancy were clearly defined (Andros, 1945). The appearance of hypertension during the third trimester was then recognized as a major indicator of toxemia.

During normal pregnancy the mean blood pressure will generally decrease slightly during the second trimester and then return to its pre-pregnancy level as term approaches. When taking the blood pressure with the mother in the lateral position, one might typically see a systolic pressure of 98 mmHg and a diastolic pressure of 55 mmHg during the first trimester; a systolic pressure of 91 mmHg and a diastolic pressure of 49 mmHg during the second trimester ; and a systolic and diastolic pressure of 95 and 50 mmHg respectively, during the final trimester (Quilligan, 1982).

Preeclampsia is diagnosed when the blood pressure during the third trimester rises to 140/90 mmHg or above.

With severe preeclampsia these values often reach levels of 170/110 mmHg or higher. A distinguishing characteristic of toxemia is the relatively rapid return of the blood pressure to normotensive levels following delivery (Dennis et al., 1982).

2. Proteinuria

In addition to hypertension, another principle feature of preeclampsia is protein in the urine or proteinuria. Proteinuria is indicated if the urinary concentration in a 24 hour collection is more than 300 mg/liter (Dennis et al., 1982). Lever in 1843 is credited with recognizing the association of proteinuria with eclampsia (Chesley, 1978). He reported its occurrence in 9 of 10 woman suffering from seizures. A postmortem on the one subject who failed to show protein in her urine revealed that the original diagnosis of eclampsia was probably incorrect.

In the normal pregnant state approximately 150 mg/day of protein is excreted by the kidney (Quilligan, 1982). With preeclampsia this value can vary from 150 mg/day to 5 or more g/day (Dennis et al., 1982). Renal histological changes associated with proteinuria generally begin healing soon after delivery (Dennis et al., 1982).

3. Edema

In addition to hypertension and proteinuria, the third clinical sign used to determine preeclampsia is edema.

Because a rise in fluid retention is common in normal and toxemic pregnancies, the appearance of edema alone does not always indicate the presence of the disease. A more important index is the rate at which edema occurs, particularly during the third trimester. A weight gain in excess of 2 kg/week is considered abnormal (Dennis et al., 1982).

4. Central Nervous System Hyperactivity

Central nervous system hyperactivity is also typical of a preeclamptic patient. Generally, a mother's reflexes become more sensitive to stimulation as the disease progresses (Dennis et al., 1982). The knee jerk reflex is commonly used to assess the degree of hyperflexia present. Special care is taken to keep the patient calm which helps to minimize the possibility of convulsions.

5. Coagulation Abnormalities

Although not used in the first line of diagnosis, coagulation changes with preeclampsia have been well documented (Hathaway and Bonnar, 1978; Bonnar et al., 1971). While such changes are no longer considered a primary cause of the disease (Pritchard et al., 1976), some level of disseminated intravascular coagulation (DIC) is likely to accompany the clinical condition (Chesley, 1978).

There are two mechanisms responsible for these coagulation changes. The first process involves thromboplastins (Figure 1). These are substances which activate thrombin

Figure 1

Flow diagram showing the pathway from thromplastins to stable fibrin.

DISSEMINATED INTRAVASCULAR COAGULATION FIBRIN PATHWAY

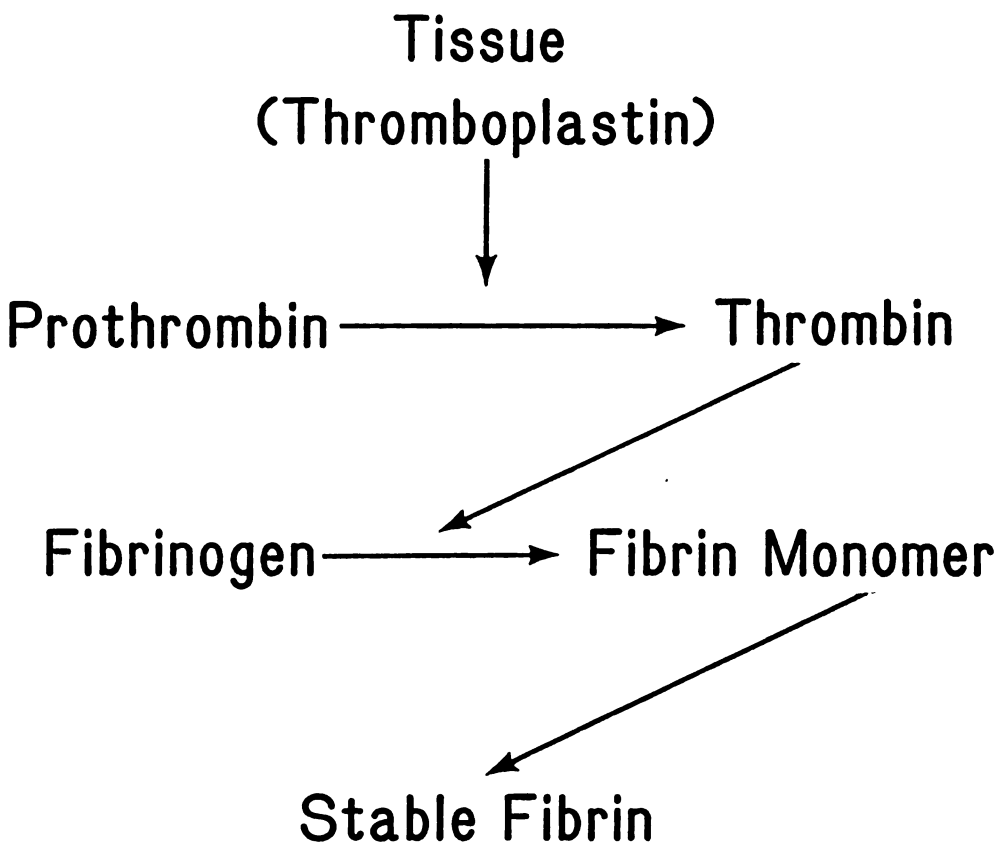

Figure 1

formation from prothrombin and ultimately lead to the deposition of fibrin in the microcirculation. The second mechanism is induced by endothelial cell damage and culminates in platelet clumping (Figure 2). Important to this process is the intravascular relationship between prostacyclin and thromboxane. Both the blood vessel wall and the platelets have their own stores of arachidonic acid. Within the vessels, the arachidonic acid cascade leads primarily to the production of prostacyclin and, within platelets, arachidonic acid leads to the production of thromboxane. Clumping occurs when, following vessel wall damage, thromboxane and other mediators of platelet aggregation overwhelm the antiaggregatory effects of prostacyclin (Terrango, 1980).

The magnitude of the five clinical symptoms described above allow the physician to assess the severity of toxemia. This disorder is a pregnancy-induced condition and generally following the delivery of the placenta the blood pressure of the mother returns to normotensive levels and her condition improves significantly.

C. Placental Involvement in Toxemia

Because the placenta is the largest endocrine organ, weighing approximately 500 grams, and because it is unique to pregnancy, it is not surprising that many investigators have attempted to assess its involvement in toxemia. Maternal blood enters from the spiral arteries of the uterus into

Figures 2

Schematic illustration of aggregation balance between prostacyclin and thromboxane.

PLATELET AGGREGATION

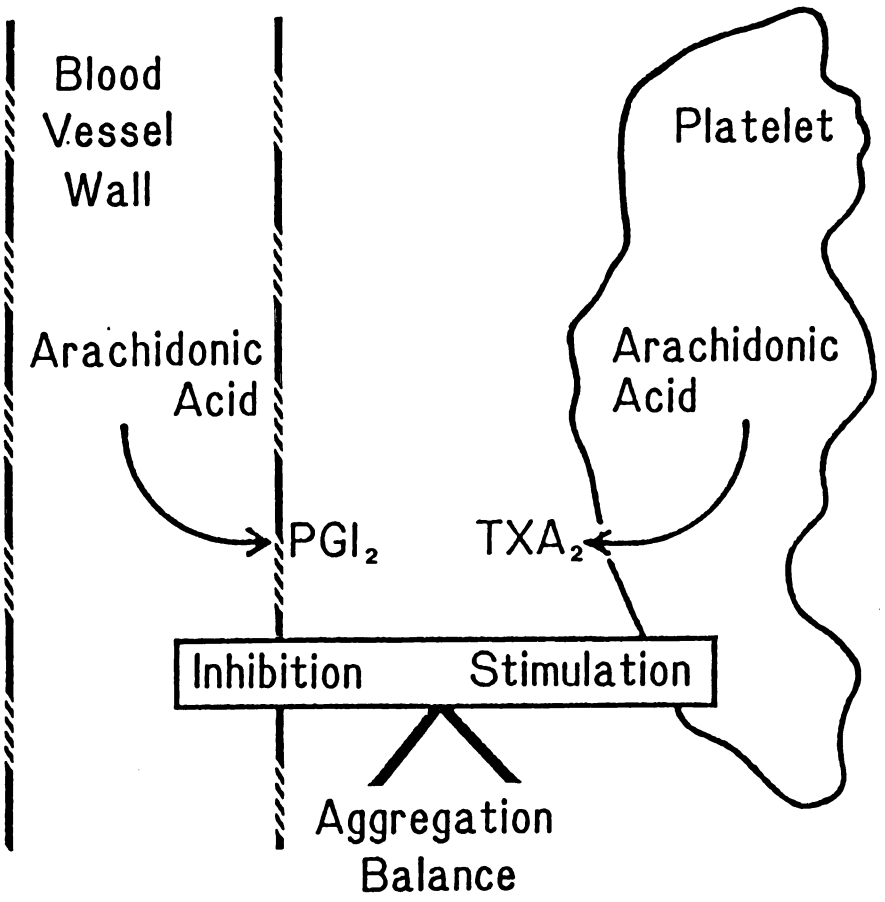

Figure 2

the intervillous space of the placenta where it constantly baths a vast network of tiny villi (Figure 3). It is across these villi that the exchange of nutrients and gases occur. Because of the ongoing interaction between the maternal blood and the villi, it is not unusual for placental cell fragments or even whole villi to break off and enter the maternal circulation (Kaiser, 1982).

In toxemia there is an increased release of these placenta cells into the maternal circulation when compared to the normal pregnant state. Because these cells have been reported to have the highest thromboplastic activity per weight of any tissue, some of the coagulation changes that occur in toxemia have been attributed to their elevated levels in the maternal blood (Dennis et al., 1982).

Investigators have observed a higher incidence of infarcts in toxemic placentas than in normal placentas (Bartholomew, 1932; Hathaway and Bonnar, 1978). This might explain the increased release of placental cells in toxemia since dead tissue is probably more prone to detachment. The occurrence of infarcts in normal placentas of late gestation are not uncommon (Aladjem, 1968) and seem to reflect the natural aging process of the organ. However, some placental lesions are often attributed specifically to preeclampsia (Jones and Fox, 1980; Chesley, 1978). While it is likely that the cause of these lesions is due to hypoxia, other undefined mechanisms may be involved. Clinically, a correlation has been made between the severity of fetal growth

Figure 3

A diagram of the human placenta.

HUMAN PLACENTA

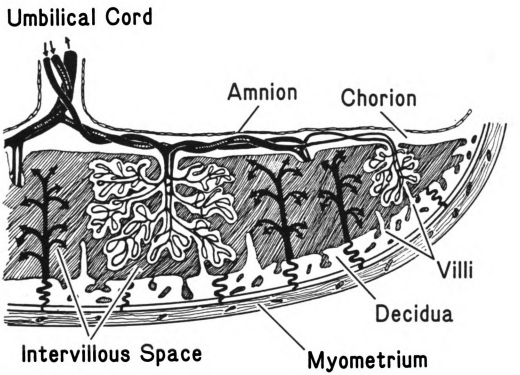

Figure 3

retardation and the extensiveness of placental infarcts (Hathaway and Bonnar, 1978).

Frequently toxemia is associated with a decrease in placental perfusion (Speroff, 1973; Chesley, 1978; Dennis et al., 1982). Whether uteroplacental ischemia predisposes a patient to preeclampsia or whether it is simply an effect of the disease has not been clearly established. Despite this uncertainty the result of a diminished blood flow to the placenta leads to an increased risk of fetal complications. As preeclampsia progresses such complications often necessitate premature delivery which in itself is undesirable but may save the life of both the mother and the child.

Although the placenta was once considered to be simply a physical barrier between the mother and fetus, today it is recognized as an active interface capable of many specific functions. As more information about the organ is uncovered it seems inevitable that we will also be closer to unraveling the complexity of preeclampsia.

D. Etiology of Toxemia

1. Background

Early studies examining toxemia were concerned primarily with the etiology of eclampsia rather than preeclampsia because of the more dramatic manifestations of eclampsia as evidenced by maternal convulsions. Today it is generally recognized that eclampsia is a more pronounced

stage of preeclampsia (Dennis et al., 1982).

One of the first of the modern explanations of eclampsia, proposed by Mauriceau in the seventeenth century, was that toxic agents originating from a dead and decomposing fetus circulated in maternal vessels where they fostered the disease (Dexter et al., 1941). Since that time the variety of theories developed to explain the etiology of toxemia have been numerous, often reflecting the fashion in research at the time that they were proposed. As is often the case clinical treatment of the disorder found itself subject to similar trends. Zuspan and Ward (1964) addressed this issue when they wrote that the preeclamptic patient "has been blistered, bled, purged, packed, lavaged, irrigated, punctured, starved, sedated, anesthetized, paralyzed, tranquilized, rendered hypotensive, drowned, been given diuretics, had mammectomy, been dehydrated, forcibly delivered, and neglected." Reference has even been made to a physician who attempted to cure toxemia by alligning the mother's head with the north pole (Pritchard and Macdonald, 1976).

Although successful treatment of preeclampsia has improved significantly over the past twenty years, prevention of the disease will not be realized until there is a clearer understanding of the underlying mechanisms involved. Unfortunately, despite rapid advancements in medicine and science, investigators have still been unable to provide convincing evidence for any one hypothesis.

The following sections provide a brief summary of more recent theories that have been proposed to explain the etiology of toxemia. The literature review ends with a more in depth accounting of the prostaglandin story.

2. Malnutrition

Some studies have postulated a causative role for diet in the pathogenesis of toxemia. They attribute the clinical appearance of preeclampsia in the third trimester of pregnancy to a depletion of maternal reserves (Brewer, 1966; Davies, 1971).

If this theory were true, then one would expect a greater occurrence of the disease in women of lower socioeconomic classes. However, studies have found a significantly higher incidence of preeclampsia in primigravida of all classes with no statistical difference between groups (Woodhill, 1955).

The World Health Organization Expert Committee concluded that "there seems to be no scientific basis for believing that deficiency or excess of any essential nutrients predisposes to eclampsia or preeclampsia....except to say that the nutritional status of the patient may possibly modify the course of the disease" (Davies, 1971).

It is difficult to argue against the importance of a well balanced diet, but there is no evidence to indicate that it is the cause of toxemia.

3. An Immunological Disorder

Since the turn of the century an immunologic approach to the study of toxemia has been considered (Kitzmiller, 1977). The attractiveness of this school of thought is apparent. The feto-placental unit which invades the maternal reproductive system would seem likely to invoke an immune response. Fortunately, however, during normal pregnancy the fetus is able to thrive without succumbing to rejection. The pathogenesis of toxemia might then be explained as a pregnancy-induced immune response to the fetus and fetal membranes.

While investigations continue, studies to date have not provided convincing evidence for the immunological hypothesis (Scott and Beer, 1976; Balasch et al., 1981). Confusion within the literature is evidenced by several recent review articles in which the authors have found it difficult to draw any general conclusions from available data (Scott and Beer, 1976; Jenkins, 1976; Kitzmiller, 1977).

Many progenitors of theories on this subject presuppose that toxemia results from an abnormal immune reaction (Kitzmiller, 1977). Perhaps this cause and effect relationship is the contrary, however. That is, the immunological abnormality may not be the cause of the disease but rather an effect of it. It is easier to account for the inconsistent immunological findings reported in the literature if they are not considered to be responsible for the disorder.

For example, the increase in placental trophoblasts observed in the maternal circulation of preeclamptics (Scott and Beer, 1976) could both impinge on an individual's immune system and induce disseminated intravascular coagulation, depending on the amount of tissue released by the placenta in each case. While some workers have found disseminated intravascular coagulation to be prevalent among preeclamptics, others have not (Chesley, 1978; Gant and Worley, 1980). The same inconsistency holds true for studies which have looked for immune disorders in preeclamptics (Scott and Beer, 1976; Kitzmiller, 1977). Thus, it would appear that immune reactions are secondary to the disease rather than causative. The normal variation in human response might explain some of the conflicting observations that have been documented.

4. Uteroplacental Ischemia

When immunological studies began appearing in the literature, another attractive hypothesis was introduced by Young in 1914 (Speroff, 1973). Young postulated that a decrease in uteroplacetal blood flow was the primary cause of eclampsia.

Beker, between 1925 and 1948 (Bastiaance, 1954), completed several studies from which he concluded that an increased resistance in uterine blood vessels during pregnancy sensitized the patient to eclampsia. He showed, using barium injections, that in multiparous cows of two months

gestation uterine arteries were significantly more dilated than the same vessels of primagravida cows (Speroff, 1973). Later he confirmed this finding in humans. He attributed the greater incidence of toxemia in primigravidas to their higher uterine resistance associated with first pregnancies and their less extensively developed uterine vascular system. The hypertension accompanying the disease was thought to be an adaptive mechanism to maintain the blood flow to the placenta and fetus. Page, Bastiaance and others (Bastiaance, 1954) who adopted the theory of uteroplacental ischemia felt that in the event of a multiple pregnancy, hydatiform mole or enlarged fetus, the associated increase in resistance of vessels supplying the affected uterus might account for the higher number of toxemics that occur in these groups. wThe ischemia theory was expanded when Page and Ogden (1939) suggested that the release of a pressor agent from the placenta or kidney was secondary to the decrease in uterine blood flow. Although presented in new terms their hypothesis brought the focus of study back to Mauriceau's concept of a circulating toxin. The search for such a toxic agent attracted many researchers with renewed enthusiasm (Hunter and Howard, 1960; Dunlap and Ryan, 1966).

Gross (1964) identified a renin-like substance that was produced by the rabbit placenta and uterus. Subsequently the release of a renin-like protein by the human placenta was also demonstrated (Hodari et al., 1967), as well as renin production by human chorionic and myometrial tissue <u>in vitro</u>

(Skinner, 1968). Interest in renin with respect to preeclampsia was twofold: first, renin is the proteolytic enzyme responsible for the conversion of angiotensinogen into angiotensin I, which is in turn converted to angiotensin II (A-II), the most potent vasoconstrictor known and a stimulator of aldosterone secretion (Figure 4); and second, renin inhibits bradykinin vasodilating activity by hydrolysis.

Studies which implicate a cause and effect relationship between uteroplacental ischemia and the activity of the renin-angiotensin system in preeclampsia have not been conclusive, nor has the cause and effect relationship between utero-placental ischemia and toxemia been clearly defined. Originally what some researchers expected to find in the toxemic patient was an increase in circulating levels of renin which would in turn elevate the levels of A-II enough to produce the high blood pressure observed clinically (Hodari et al., 1967). As described in the next section this is not the case.

5. Renin-Angiotensin System

During normal human pregnancy maternal plasma levels of renin, aldosterone (Pedersen, 1982) and A-II (Speroff, 1973) are increased from the nonpregnant state. Pregnancy is also characterized by a depressed vascular responsiveness to A-II (Talledo, 1967).

In toxemia, on the other hand, it has been shown that

Figure 4

Flow diagram of the renin-angiotensin system.

RENIN–ANGIOTENSIN SYSTEM

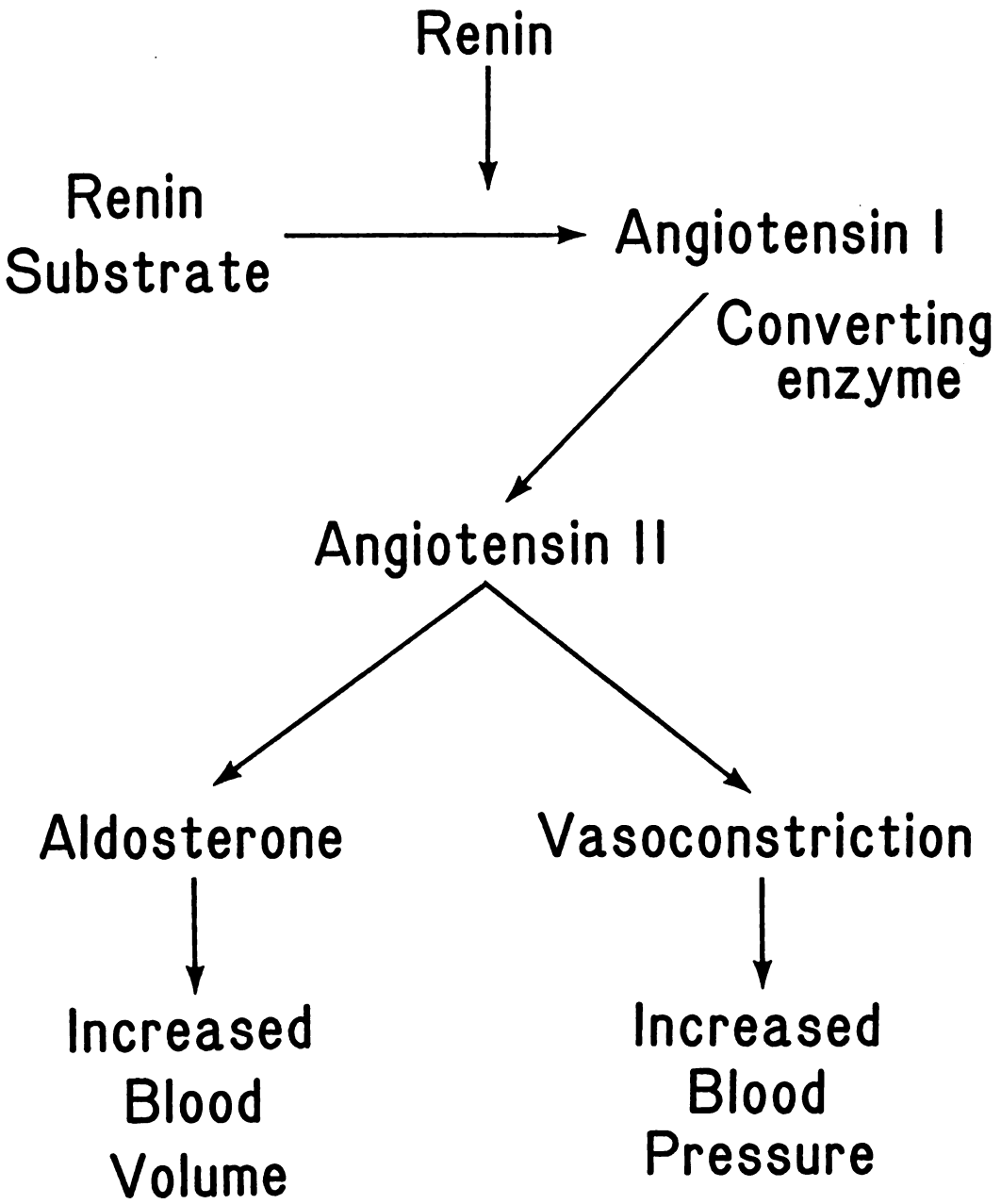

Figure 4

there is a decrease in the plasma concentrations of renin (Pedersen, 1982), A-II and aldosterone (Pedersen et al., 1982). Despite the lower levels of A-II in toxemia there appears to be an increased sensitivity to its pressor effects (Chesley, 1965). A role for A-II in the etiology of preeclampsia, then, might still be indicated.

In an important study by Gant et al. (1974) it was shown that as early as the 22nd week of gestation it is possible to detect patients destined to become preeclamptic by assessing their pressor response to an A-II infusion. Clinical symptoms usually do not appear until the 34th week. The pressor response to angiotensin II was determined by recording the dose required to raise the diastolic blood pressure by 20 mmHg. Gant and colleagues found that as pregnancy progressed the amount of angiotensin II needed to increase the diastolic blood pressure by 20 mmHg was significantly less in toxemic women than in normally pregnant women (Figure 5). They proposed that during normal pregnancy prostaglandins might desensitize the maternal vasculature to the pressor effect of A-II, and that in toxemia, this prostaglandin mechanism might be defective.

Strong evidence for prostaglandin involvement in toxemia was demonstrated in a subsequent study done by Everett et al. (1978). They found that the administration of a prostaglandin inhibitor (indomethacin or aspirin) to normally pregnant women resulted in a marked increase in sensitivity to infused A-II, a sensitivity similar to that

Figure 5

Pressor response to angiotensin II in normal and toxemic patients.

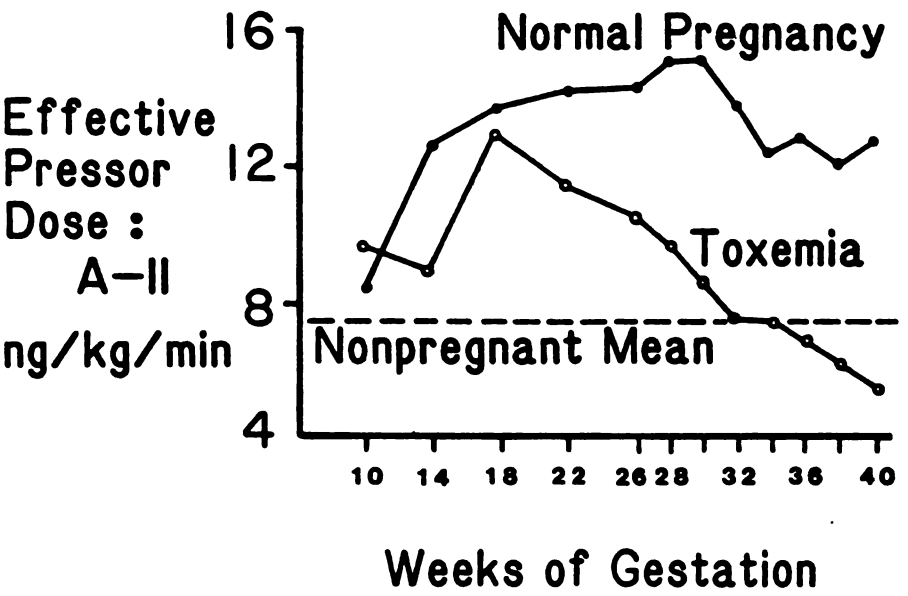
16
12
8
4
Effective
Pressor
Dose :
A–II
ng/kg/min
Normal Pregnancy
Toxemia
Nonpregnant Mean
10
14
18
22
26
28
32
36
40
Weeks of Gestation

Figure 5

seen in preeclamptic patients (Figure 5). In other words, following treatment with a prostaglandin inhibitor normal women required a significantly smaller dose of angiotensin II to raise their diastolic blood pressure by 20 mmHg than they required before treatment.

Since the discovery of prostaglandins many investigators have tried to elucidate the possible role of these substances in the pathogenesis of the disease. A review of these studies follows.

E. Prostaglandins

1. History

In 1930 Kurzrok and Lieb reported that samples of human seminal fluid either enhanced or diminished the motility of human uterine strips *in vitro* depending on the sample. Three years later Von Euler and Goldblatt independently found that sheep seminal fluid injected into test animals caused smooth muscle contraction and a lowering of blood pressure. Subsequently, Von Euler identified the active ingredients of seminal fluid as polyunsaturated fatty acids. Because he considered the prostrate gland to be the source of these fatty acids he named them "prostaglandins". This term although still used today is a misnomer because it is now known that these compounds are synthesized by tissues throughout the body.

Further work with the prostaglandins was interrupted by

World War II and it was not until the late fifties that their chemical isolation was undertaken by Bergstrom and Sjuvall (1957). The first two prostanoids to be purified, prostaglandin E_1 and prostaglandin $F_{1\alpha}$, were obtained from sheep vesicular glands. Eventually, prostaglandins related to PGE_1 and PGF_1 were identified in human seminal fluid. Since that time a number of prostaglandins and prostaglandin-like substances have had their chemical structures elucidated, the most recent ones being prostacyclin (Moncada et al, 1976), thromboxane (Hamberg et al, 1975) and the leukotrienes (Samuelsson et al, 1975).

2. Biosynthesis

The biosynthesis of the prostaglandins can begin with any one of three essential polyunsaturated fatty acids; linoleic acid, linolenic acid, or arachidonic acid. In man, the major precursor of the prostaglandins is arachidonic acid which is obtained either directly from the diet or by the conversion of linoleic acid (Figure 6). Within the body, the majority of arachidonate is found either covalently bound via an ester linkage to the C2 position of phospholipids, or esterified to cholesterol. A small percentage of arachidonic acid is also located in the triglycerides of adipose tissue and plasma. Free arachidonic acid makes up only 1%-2% of the total free fatty acid concentration in plasma.

The release of arachidonic acid from the C2 position of

Figure 6

Flow diagram outlining the production of the major prostaglandins and thromboxane from arachidonic acid.

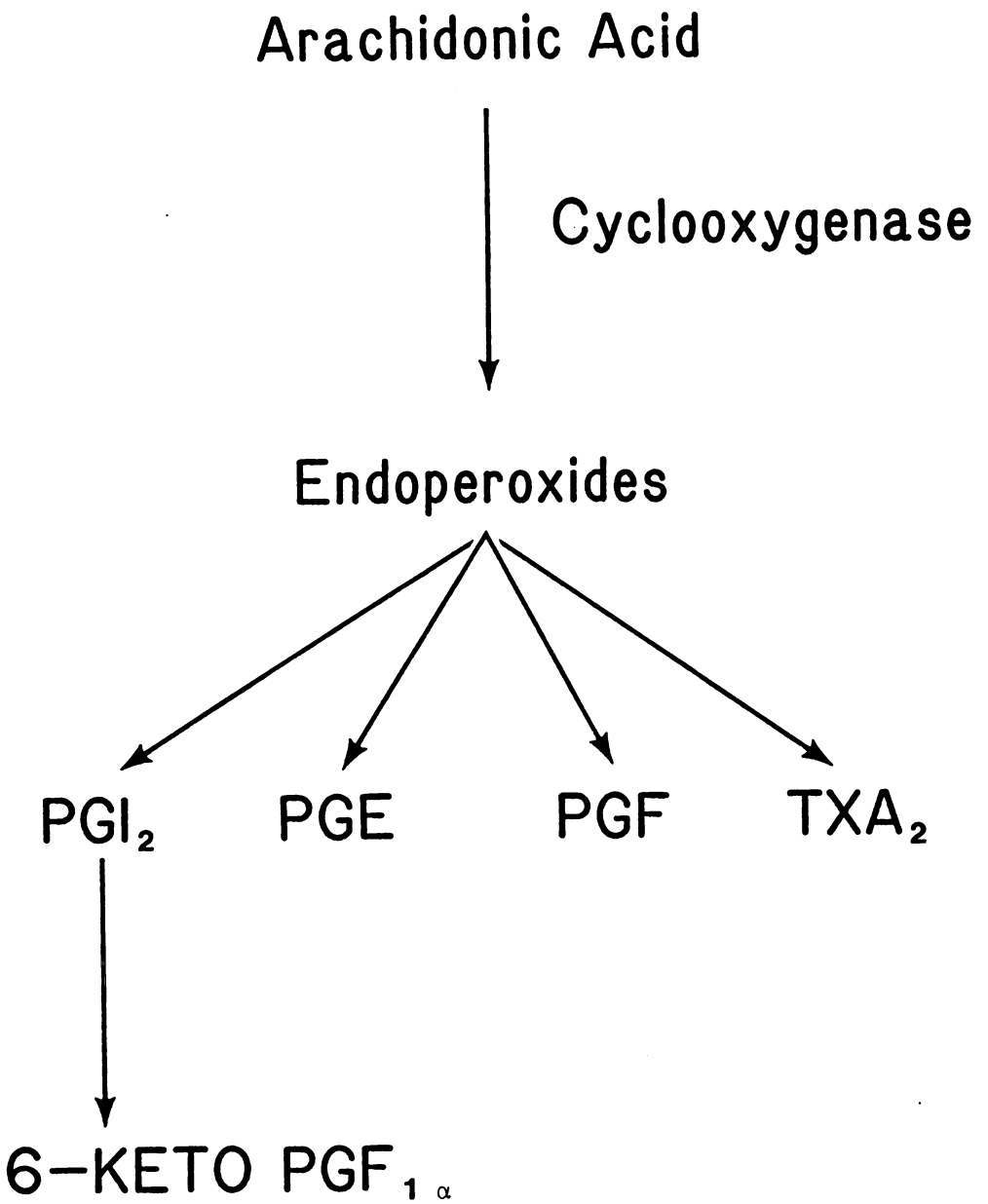
Arachidonic Acid
Cyclooxygenase
Endoperoxides
PGI2
PGE
PGF
TXA2
6-KETO PGF1α

Figure 6

phospholipids is the rate limiting step in the production of prostaglandins. The enzyme responsible for this process is phospholipase A_2. Some of the various stimuli capable of inducing phospholipase A_2 include: burns, infusions of hyper- and hypotonic solutions, thrombi and small particles, endotoxin, snake venom, mechanical stretching, catecholamines, bradykinin, angiotensin II and the sex steroids (Ramwell, 1980). Once released, two different pathways are available to arachidonic acid, the cyclo-oxygenase pathway or the lipoxygenase pathway.

3. Products of the Arachidonic Acid Cascade

The products of the lipoxygenase pathway include 12-hydroxyeicosatetraenoic acid (12-HETE) and the more recently discovered leukotrienes. While the physiological significance of 12-HETE is undetermined, the leukotrienes have been shown to induce bronchospasm in conjunction with anaphylaxis. Undoubtedly, a more thorough understanding of the lipoxygenase products is forthcoming.

In contrast, the physiological importance of the prostaglandins and the prostaglandin-like metabolites of the cyclooxygenase pathway has been more firmly established. This pathway produces the PG's of the A, B, E, and F series, prostacyclin and the thromboxanes. These substances play a fundamental role in maintaining homeostasis within the body. Their ability to modify the production of cyclic adenosine monophosphate appears to be the underlying mechanism respon-

sible for their influence on a variety of biological systems (Karim, 1975). Some of the more important activities affected by the prostanoids include renal blood flow, lipid metabolism, central nervous system activity, vasoconstriction or dilation of the blood vessels, platelet aggregation, gastrointestinal motility and secretion, and cardiovascular function (Karim, 1975).

During pregnancy prostaglandins are produced by the placenta, amnion, chorion and umbilical cord, as well as, by the uterus (Tsang, 1982; Remuzzi, 1979; Mitchell, 1978; Willman, 1978; Mitchell, 1977; Keirse, 1976). Findings not only suggest a role for the prostaglandins in some of the physiological changes which occur in the pregnant mother, but also in the development of the fetus. Specifically, these substances are thought to be important in maintaining uteroplacental blood flow (Tuvemo, 1980), fetal and umbilical vascular tone (Remuzzi, 1979; Cassin, 1980; Tuvemo, 1980) and patency of the fetal ductus arteriosus (Coceani, 1980). In addition, studies have implicated their involvement in the initiation of labor (Ylikorkala, 1981; Turnbull, 1980; Mitchell, 1978; Schwarz, 1976; Schultz, 1975). Because normal pregnancy appears to be dependent on the biological activity of the prostaglandins and their potent vasoactive properties, some investigators hypothesize that these substances are associated with the pathogenesis of toxemia.

4. Prostaglandins in Toxemia

Originally the E and F prostaglandins were the focus of attention. It was believed that in toxemia the production of PGE, a vasodilator, might be reduced or that the production of PGF, a vasoconstrictor, might be increased (Speroff, 1973). Findings reported in the literature, however, have been inconsistent.

In 1973, Alam et al., using thin layer chromatography, demonstrated that the metabolism of tritiated PGE_1 by homogenized human placental tissue was depressed in direct proportion to the severity of the disease. They postulated that PGE_1 has vasoconstrictor effects in the placenta and that its reduced metabolism might explain the fall in blood flow to the placenta. In addition, a decrease in the metabolism of PGE_1 might lead to lower than normal levels of PGA, a metabolite of PGE_1 with vasodilator activity. In contrast to these findings, Demers and Gabbe (1976) reported a significant reduction in the toxemic placental tissue concentration of PGE and a significant elevation in the tissue concentration of PGF. These levels were obtained by radioimmunoassay (RIA) of frozen placental tissues which had been thawed and then extracted by column chromatography.

Conflicting information concerning placental PGE and PGF levels in toxemia continued to appear in the literature. Hillier (1981) found that the concentrations of these prostaglandins were not significantly different in preeclamptic and normal placentas. Column chromatography and RIA were

again employed to determine prostaglandin levels, but different reagents were used in the extraction process. The differences in methodologies or perhaps random variation might explain the inconsistent results.

Measuring prostaglandin concentrations with radiometric techniques, Valenzuela et al. (1980) were unable to demonstrate a decrease in placental metabolism of PGE as previously shown, but did observe a significantly reduced metabolism of PGF. In a follow up study, Valenzuela and coworkers (1983) examined PGE and PGF levels in maternal blood samples taken from the radial artery, the uterine vein, and a peripheral vein of both toxemic and normotensive patients. Venous PGE and PGF levels were always higher in toxemic women in labor when compared to normotensive women in labor, but only the increase in PGE was statistically significant. Arterial PGF levels were also significantly higher in toxemic woman. Valenzuela suggested that a decreased catabolism of PGF by the lungs coupled to an increase in its uterine production may be responsible for the high blood pressure associated with toxemia. Any vasodilation due to elevated venous PGE levels was not enough to offset the greater levels of the vasoconstrictor PGF.

Measurements of urinary prostaglandin levels in toxemia and normal pregnancy, as estimated by RIA, did little to alleviate the confusion already established within the literature. While urinary excretion of PGE was significantly

lower in toxemia than in normal pregnancy, levels of PGF in the urine did not differ (Pedersen, 1983).

Studies to date have not convincingly demonstrated a role for PGE or PGF in the etiology of toxemia. Some authors have reported increases, others decreases, and still others no changes in the levels of these prostaglandins. Such inconsistency has made it difficult to accept any one finding. With the recent discovery of prostacyclin, however, interest in the prostaglandins with respect to toxemia has been renewed.

5. Placental Prostacyclin Production in Toxemia

Prostacyclin is a potent vasodilator and an inhibitor of platelet aggregation. It is released by the lungs and blood vessel walls into the circulation. Because of prostacyclin's short half-life of approximately three minutes, its levels are generally estimated by a highly specific radioimmunoassay of one of its stable metabolites 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$). Unlike the other primary prostaglandins prostacyclin does not appear to be extensively metabolized by the lungs (Gerkins et al., 1978), a property that allows it to act as a circulating hormone. Prostacyclin inhibits the aggregation of platelets and their adhesion to the vascular wall which prevents vascular wall damage, thromboembolism and disseminated intravascular coagulation (Terrango, 1980). Because toxemia is characterized by increased vasoconstriction, increased platelet

aggregation and decreased uterine blood flow, several investigators have suggested that a decrease in prostacyclin's production, or perhaps a loss of response to it, might play a causative role in this disorder.

During normal pregnancy the production of prostacyclin is increased because maternal plasma and urinary metabolite concentrations are elevated when compared to the nonpregnant state (Lewis et al., 1980; Goodman et al., 1981). The source for this increase in prostacyclin during pregnancy is unknown. One possibility is the placenta. Studies have shown that the placenta, amnion and chorion are capable of producing prostacyclin (Mitchell, 1978; Myatt, 1977). The large mass of the placenta alone suggests that it could be a major contributor to the higher levels of prostacyclin.

During toxemia, the production of prostacyclin is apparently decreased in comparison to normal pregnancy. Early investigations examined the biological activity of this prostanoid by assaying platelet aggregation. Remuzzi et al. (1980) determined that prostacyclin activity in placental veins and umbilical arteries was depressed in this disorder. Bussolino et al. (1980) reported similar findings in placental veins, as well as in maternal subcutaneous and uterine vessels. Two groups found that prostacyclin activity was also reduced in the amniotic fluid of preeclamptic women (Bodzenta et al., 1980; Ylikorkala et al., 1981).

Downing et al. (1980) measured the enzyme activity of prostacyclin synthetase from umbilical arteries of

preeclamptic and normal woman. Although enzyme function was normal in toxemic women, less total enzyme was found. Makila et al. (1983) showed a direct correlation between lower concentrations of 6-keto-$PGF_{1\alpha}$ found in umbilical arteries and a decrease in uterine blood flow. Lewis and colleagues (1981) reported a case in which prostacyclin levels decreased in the maternal blood prior to a worsening of hypertension, the appearance of proteinuria and the onset of fetal growth retardation. Urinary metabolite concentrations of prostacyclin have also been shown to be depressed during toxemia (Goodman et al., 1982).

Carreras et al. (1981) were the first to look at the production of prostacyclin in toxemia. They examined its production from placental vein and umbilical artery fragments which were incubated in a tris buffer. Aliquots of incubation media were collected every four minutes over a twenty-four minute period and a radioimmunoassay was then used to determine the concentration of 6-keto-$PGF_{1\alpha}$ in the collected samples. In toxemia the production of prostacyclin by the umbilical artery was significantly reduced from normal pregnancy levels, but no change was measured in its production by toxemic placental veins. The addition of arachidonic acid, the precursor for the prostaglandins, to the incubation media did not change prostacyclin levels which indicates that prostacyclin synthesis is probably not substrate limited. Because concentrations of prostacyclin at each sample time were not reported, one cannot determine

what effect injury to the tissues as they were prepared for incubation may have had on these results. Damage to tissue as it is prepared for incubation might cause a release of phospholipase A_2 from lysosomes, that could in turn stimulate prostacyclin synthesis by increasing the availability of arachidonic acid.

The above investigations strongly implicate prostacyclin in the etiology of toxemia. Because the placenta is unique to pregnancy and is a likely source for the increase in prostacyclin during normal pregnancy, and because previous studies have consistently reported a decrease in prostacyclin levels in toxemia, this thesis was undertaken to compare the production of prostacyclin by normal and toxemic placentas and their associated structures. Several factors differentiate this study from previous investigations.

A drawback to earlier work is that much of it appears in the literature as letters to the editor. These letters provide only minimal information and interpretation of data is difficult. In most of these studies a lack of specific patient information leaves several questions unanswered. For example, three studies show a reduction in prostacyclin levels in toxemia, but do not report a mean gestational age for the normal and toxemic woman who were examined (Remuzzi et al., 1980; Downing et al., 1980; Carreras et al., 1981). Although the investigators for these studies associate

decreased prostacyclin levels with toxemia, their findings may have been influenced by differences in the gestational age of the fetuses. That gestational age may not be a factor is supported by one group (Stuart et al., 1981) who reported that decreased fetal umbilical production of prostacyclin in toxemia is not related to gestational age, an observation based on the amount of radio-labeled arachidonic acid converted to 6-keto-$PGF_{1\alpha}$ by umbilical arteries of various gestational ages. However, this study did not investigate the effects of gestation on maternal levels of prostacyclin. Therefore, a gestational effect can not be ruled out if normotensive women who deliver at term are compared to toxemic women who deliver prematurely. The present studies were designed to minimize the effects of variables such as gestational age, by adopting strict criteria for assessing normal and toxemic pregnancies.

Several authors have reported a decrease in the production of prostacyclin (Remuzzi et al., 1980; Downing et al., 1980; Stuart et al., 1981; Goodman et al., 1982) but only two groups, Carreras et al. (1981) and Makila et al. (1983), have reported their findings in units of time (pg/mg/hr and ng/g/min, respectively). The results from the other studies are given as pg/mg. In addition, previous investigations have only examined <u>vascular</u> production rates (Carreras et al., 1981; Makila et al., 1983). This study is the first to look at <u>placental</u> production of prostacyclin in toxemia. Because the placenta is highly vascular it is likely that

some prostacyclin is generated from its vessels but the syncytiotrophoblast is also a likely source. The fact that the amnion produces prostacyclin (Mitchell et al., 1978) demonstrates that avascular tissue can produce prostacyclin.

F. Objectives

The specific research objectives for this thesis were the following:

1) Measure the prostacyclin production rates by the normal and toxemic placenta, amnion and chorion.

2) Demonstrate inhibition of prostacyclin synthesis to validate that prostacyclin is being produced in vitro rather than simply being released.

3) Evaluate the production of prostacyclin over an extended period of time (48 hours). The longest any previous study had looked at prostacyclin's production rate was 24 minutes (Carreras et al., 1981).

4) Compare production rates of prostacyclin from tissue incubated in a high oxygen environment and low oxygen environment because a high oxygen environment has been suggested as possibly being toxic to the placenta (Tuvemo, 1981) and a low oxygen environment has been suggested as a

possible stimulus for prostacyclin production in the fetus and in the heart.

5) Test the *in vitro* model for substrate limitations to insure enough substrate is present in both normal and toxemic placentas for the 48 hours of incubation and to insure that prostacyclin production by toxemic placentas is not substrate limited.

6) Investigate mild as well as severe preeclamptics. Earlier works have examined primarily severe preeclamptics.

III MATERIALS AND METHODS

A. Patients

Fresh term placentas from twelve healthy and twelve toxemic woman were used. All specimens were obtained from E. W. Sparrow Hospital (Lansing, Michigan) in cooperation with the Department of Obstetrics and Gynecology and the Labor and Delivery Unit. The following criteria were used to assess normal pregnancy: 1). maternal blood pressure less than 110/70 mmHg. 2). no proteinuria. 3). gestational length of 36 to 43 weeks. 4). labor duration less than 18 hours for prima gravida and less than 12 hours for multigravida. 5). no overt maternal medical complications such as diabetes mellitus, infection or metabolic disorders. 6). no neonatal abnormalities. 7). birth weights of 2.5 to 4.5 Kg. 8). no third trimester bleeding. 9). no gross placental abnormalities. 10). no oligo- or polyhydramnios. 11) the mother had to be a non-smoker and a non-alcohol drinker during pregnancy.

The criteria used to assess toxemic pregnancies were the same as those used to evaluate normal pregnancies except that maternal blood pressure had to be $\geq$ 140/90 mmHg and proteinuria had to be present (urinary protein $\geq$ 0.3 g protein/24 hr). In addition, we were willing to accept the presence of some neonatal abnormalities or complications but in the present study all fetuses were born healthy.

B Experimental Protocols

1. General Tissue Preparation

Following delivery, each placenta was placed into a sterile bag, put on ice and immediately transported to the laboratory. Processing of the tissues began within one hour of delivery, and all experiments were performed under sterile conditions. To insure maximum viability of the tissue, fragments of amnion, chorion, and placental tissues were separated and then gased with 95% oxygen and 5% carbon dioxide in 15 mls of Dulbecco's Modified Eagles Media (Gibco, Grand Island, NY). Pieces of tissue were removed from the oxygenated buffer and prepared for incubation as needed.

Amnion, chorion, and placental tissues were processed in quadruplicate. Approximately 300 mg of each tissue was washed repeatedly with buffer, minced and then incubated in 5 mls of Dulbecco's Modified Eagles Media. Incubation was carried out for 48 hours at 37^{o} centigrade, with 95% oxygen and 5% carbon dioxide, in a Dubnoff incu-shaker (Lab-line, Melrose Park, IL). Some tissues were incubated in a low oxygen environment (20% O_2) to assess the relationship between oxygen tension and prostacyclin production. An acid-base analyzer (PHM 72MK2, Radiometer) was used to measure the pH, the pO_2 and the pCO_2 of the incubation media at 0 hours and again at 48 hours. Partial pressures and hydrogen

ion concentrations in the incubation media were consistent at approximately PO_2= 250 mmHg, PCO_2= 20 mmHg and pH = 7.50. The production of prostacyclin was estimated by determining the concentration in the incubation media of one of its stable metabolites 6-keto-prostaglandin $F_{1\alpha}$. A highly specific radioimmunoassay was used. Samples of media (120 ul) were collected at 0 hours, 8 hours, 20 hours, 32 hours, and 48 hours, mixed and then frozen until assayed.

Prostacyclin production by both normal and toxemic placentas were also analyzed in media enriched with either indomethacin or arachidonic acid (Sigma, St. Louis). Two doses of indomethacin, 5 and 50 uM, and two doses of arachidonic acid, 50 and 100 uM, were used.

2. Cell Viability

Trypan blue exclusion staining was used to determine tissue viability at 0 hours and again at 48 hours. A small fetal artery to the placenta was cannulated and flushed with sterile saline. Approximately 1.0 gram of blanched placental tissue was minced with a razor blade and placed in a 15 ml Corning centrifuge tube (Corning, NY). The cells were suspended in 10.0 mls of a 0.25% trypsin-EDTA solution (GIBCO, Grand Island, NY) and then incubated for 30 minutes at 37°C with 95% O_2 and 5% CO_2 in a Dubnoff Incu-Shaker. The cell suspension was then mixed with a 10.0 ml glass pipet

and placed back in the incubator for another 30 minutes. After the second incubation period (total incubation time of one hour) the cells were centrifuged in an IEC clinical centrifuge (model no. 428-16706) at a setting of 2. The supernatent was decanted and discarded. The pellet of cells was resuspended in 0.5 ml of a 0.1 M phosphate buffered saline (PBS). Trypan blue (120 ul of a 0.4% solution, GIBCO, Grand Island, NY) was added to the cell suspension and allowed to incubate at room temperature for five minutes. This solution was centrifuged for five minutes in the clinical centrifuge at the same setting of 2 and the supernatent was decanted. The pellet was again resuspended in 0.5 ml PBS. Cells were examined under a Leitz Ortholux microscope (Chicago, IL) at 400X and a hemocytometer was used to count viable cells.

C. Radioimmunoassay

A highly specific radioimmunoassay (RIA) was used to analyze the stable breakdown product of prostacyclin, 6-keto-prostaglandin $F_{1\alpha}$. A brief description of this RIA will be given here while a detailed description and the validation of the assay are provided in the Appendix. Standards with known amounts of 6-keto-$PGF_{1\alpha}$ (0.025-1.25 ng/50 ul, U51787, Upjohn Co., Kalamazoo, MI) were used in each assay to generate a standard curve from which unknown quantities of this substance in samples could be calculated. Each standard (in triplicate) and sample (0.05 ml) was

diluted with 0.15 ml of a 0.1 M phosphate buffer (PBS) in a 12x75 mm glass tube. Rabbit antiserum to 6-keto-$PGF_{1\alpha}$ (0.05 ml of a 1:35,000 titer, Seragen®, Boston, MA) and of tritium labeled 6-keto-$PGF_{1\alpha}$ (0.05 ml containing approximately 6000 cpm, Amersham, Arlington Heights, IL) were added to each tube bringing the reaction volume to 0.3 ml. After vortex-mixing the tubes they were incubated for one hour at room temperature followed by another 18-24 hours of incubation at 0-4°C.

To remove protein not bound to the antibody, 1.0 ml of dextran-coated charcoal, pre-cooled to 0-4°C (0.5% charcoal: 0.05% dextran), was added to all of the tubes except the total count tubes. Charcoaled tubes were mixed and allowed to incubate for 12 minutes at 0-4°C and then centrifuged at 2000xg (Palo Alto, CA). The supernatent was decanted into scintillation vials containing 15 ml Scintillation cocktail (Safety Solve, Mount Prospect, IL) and the radioactivity was counted on a Packard Tri Carb 300C (Packard Instrument Co., Downers Grove, IL) liquid scintillation counter for ten minutes or until 2.0 percent statistical accuracy was achieved.

D. Statistical Analysis

Differences between normal and toxemic placental hormone production were statistically assessed by linear regression and analysis of variance with Student-Newman-Kuels test (Linton and Gallo, 1975). Data were expressed as

pg/mg wet tissue weight/hour.

IV RESULTS

The two groups of patients investigated, toxemic and normal, each consisted of eight vaginal deliveries and four cesarean sections with two of the women not yet in labor. The time in labor for the other women was 6.7 $\pm$ 0.9 vs. 8.8 $\pm$ 1.2 h (normal vs. toxemia, n=10, $p>0.01$). Prostacyclin production was not correlated with time in labor ($r = 0.002$). Eight of the normal patients and only one of the toxemic patients were multigravid. Three of the toxemic patients had severe toxemia (systolic/diastolic > 170/110 mmHg). Clinical data revealed no significant differences ($p>0.01$) between normal and toxemic pregnancies for placental weights (544 $\pm$ 37 vs. 521 $\pm$ 53 g, respectively, mean $\pm$ SE, n=12), fetal weights (3.55 $\pm$ 0.17 vs. 3.04 $\pm$ 0.14 kg), weeks of gestation at delivery (40 $\pm$ 1 vs 39 $\pm$ 1), ages of the mothers (24 $\pm$ 2 vs. 26 $\pm$ 2 yrs.), weight gain of mothers (31 $\pm$ 5 vs 38 $\pm$ 6 lb.), and apgar scores of the fetuses (9 $\pm$ 1 vs. 9 $\pm$ 1). All babies were born alive and healthy. Tissue viability, determined after 48 hours of incubation with trypan blue exclusion staining, was greater than 95% for both normal and toxemic placentas.

The production of prostacyclin by normal placentas, as estimated by 6-keto-$PGF_{1\alpha}$ is shown in Figure 7. The concentration of 6-keto-$PGF_{1\alpha}$ in the incubation medium increased progressively and in a linear fashion during the 48 hours of incubation. Concentrations were significantly

Figure 7

Production of prostacyclin by normal placentas, as estimated by 6-keto-$PGF_{1\alpha}$. Data represent mean $\pm$ SE (n=12).

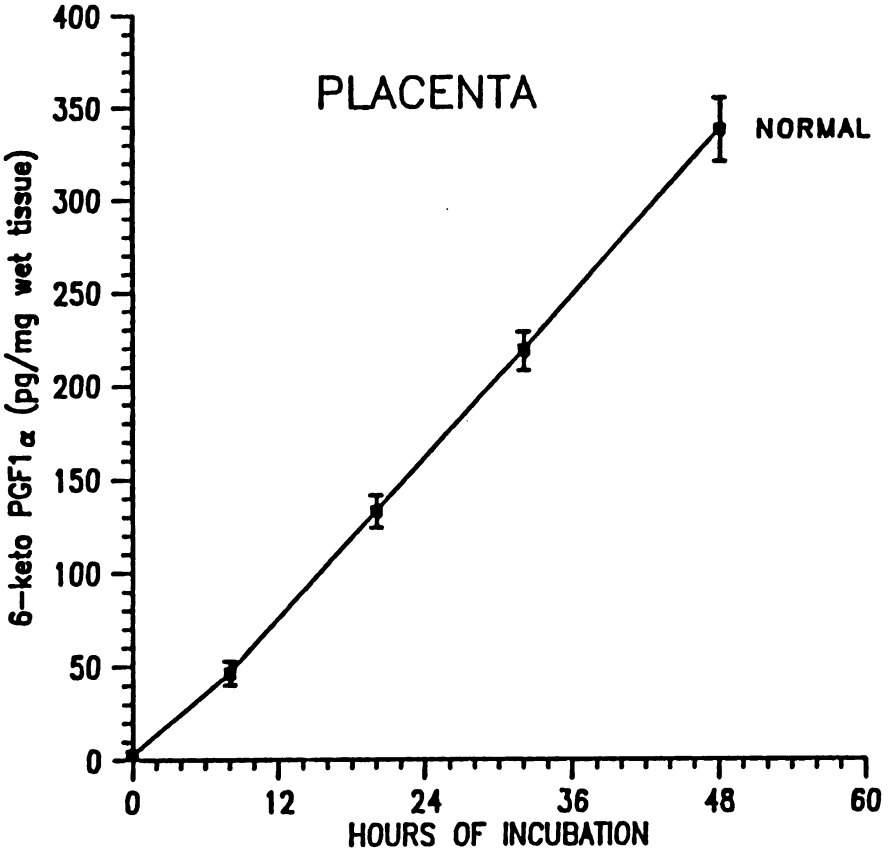
PLACENTA
NORMAL
6-keto PGF1α (pg/mg wet tissue)
400
350
300
250
200
150
100
50
0
0
12
24
36
48
60
HOURS OF INCUBATION

Figure 7

elevated at 8 h, and again at 20 h, 32 h and 48 h. Peak levels after 48 h averaged 346 pg/mg. The production rate was 7.22 $\pm$ 0.44 pg/mg/h (mean $\pm$ SE, n=12) or approximately 94 ug/day.

The effects of adding indomethacin to the incubation medium are seen in Figure 8. Addition of 50 uM indomethacin completely inhibited prostacyclin production in both normal and toxemic placentas (n=4). No differences were observed between normal and toxemic tissues ($p<0.01$). Incubation in a 5 uM indomethacin medium showed a slight production rate of 0.27 $\pm$ 0.14 pg/mg/h (n=4). After 48 hours 6-keto-$PGF_{1\alpha}$ levels averaged only 13 $\pm$ 6 pg/mg. Again, no differences were observed between normal and toxemic tissues ($p<0.01$). Figure 9 shows the effects of enriching the incubation medium of normal placentas with arachidonic acid. The production rate of prostacyclin was not significantly changed ($p>0.01$) by the addition of this substrate (5 or 100 uM). No significant differences were noted between doses. The production rates with and without arachidonic acid were 4.38 $\pm$ 0.19 vs. 6.33 $\pm$ 1.26 pg/mg/h (n=4). Peak hormone levels after 48 hours in samples with and without arachidonic acid averaged 230 $\pm$ 8 pg/mg vs. 309 $\pm$ 62 pg/mg. A significant decrease in prostacyclin production by normal placentas was observed when they were incubated in a low oxygen environment (20% O_2 vs. 95% O_2, 3.09 $\pm$ 0.39 vs. 7.55 $\pm$ 0.54 pg/mg/h, n=5) (Figure 10).

In contrast to healthy tissues, a considerably lower

Figure 8

Production of prostacyclin by normal placentas in media with 5 uM indomethacin. Data represent mean ± SE (n=4).

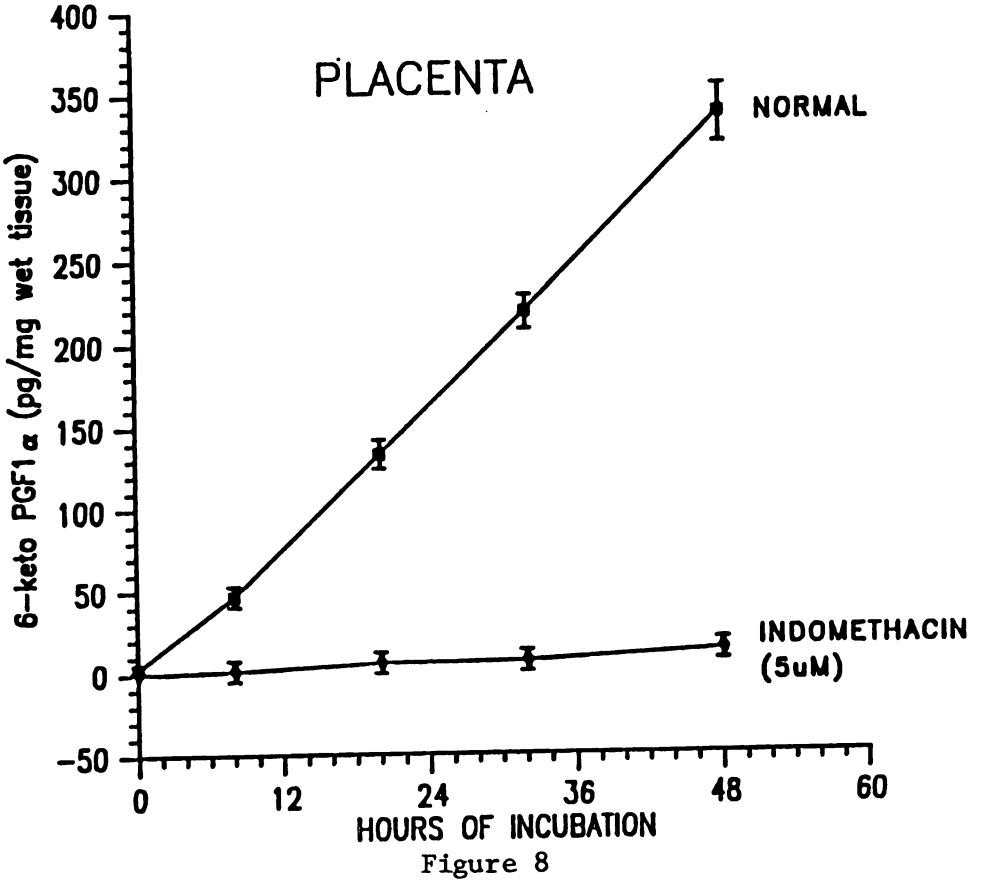
PLACENTA
NORMAL
INDOMETHACIN
(5uM)
6-keto PGF1α (pg/mg wet tissue)
400
350
300
250
200
150
100
50
0
-50
0
12
24
36
48
60
HOURS OF INCUBATION

Figure 8

Figure 9

Production of prostacyclin by normal placentas with 5 or 100 uM arachidonic acid added to the incubation media. Data represent mean ± SE (n=4).

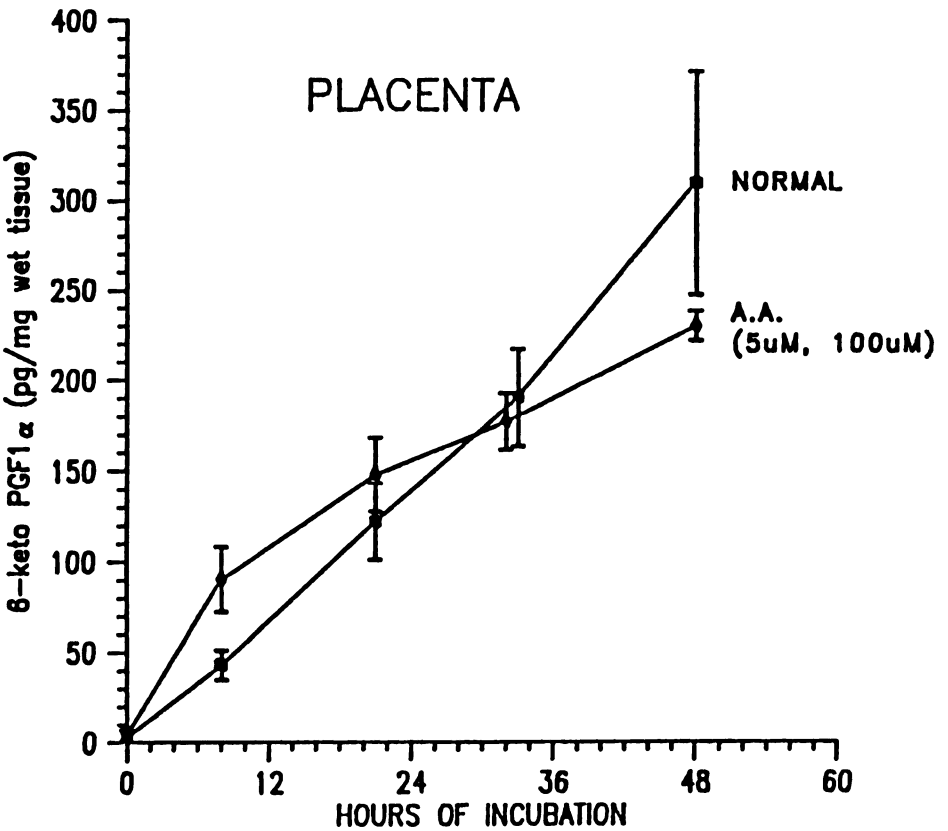
PLACENTA
NORMAL
A.A.
(5uM, 100uM)
8-keto PGF1α (pg/mg wet tissue)
400
350
300
250
200
150
100
50
0
0
12
24
36
48
60
HOURS OF INCUBATION

Figure 9

Figure 10

Production of prostacyclin by normal placentas in a two different oxygen environments (20% vs. 95% O_2). Data represent mean $\pm$ SE (n=5).

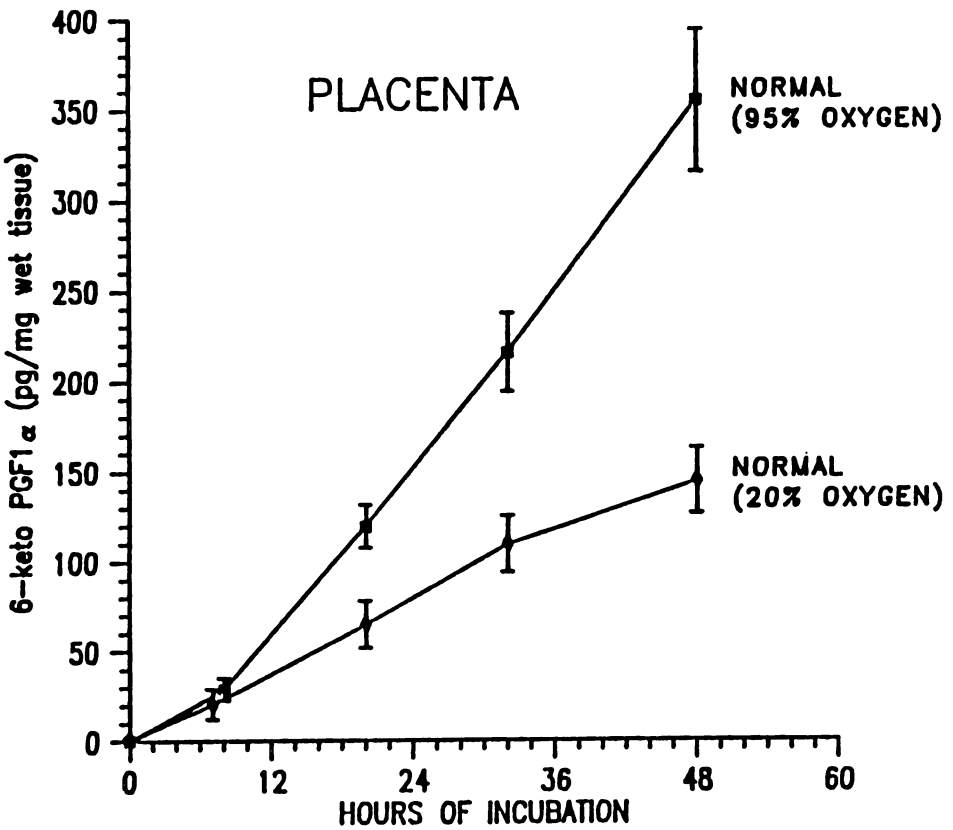
PLACENTA
NORMAL
(95% OXYGEN)
NORMAL
(20% OXYGEN)
6-keto PGF1α (pg/mg wet tissue)
400
350
300
250
200
150
100
50
0
0
12
24
36
48
60
HOURS OF INCUBATION

Figure 10

production rate of prostacyclin was found in toxemic placentas (Figure 11). The concentrations of 6-keto-$PGF_{1\alpha}$ were significantly less than those from normal placentas at 8 h, 20 h, 32 h and 48 h ($p<0.05$). At 48 hours peak levels averaged only 128 $\pm$ 20 pg/mg compared to 346 $\pm$ 21 pg/mg for normal placentas. A production rate for toxemic placentas of 2.72 $\pm$ 0.49 pg/m/h was significantly reduced from a rate of 7.22 $\pm$ 0.44 pg/mg/h for normal tissues ($n=12$, $p<0.05$). Figure 12 shows the production of prostacyclin by toxemic placentas incubated in either 5 or 100 uM arachidonic acid. No significant differences were noted between doses nor was a significant difference found between tissues with or without added arachidonic acid (3.62 $\pm$ 0.63 vs. 3.02 $\pm$ 0.68 pg/mg/h, $n=5$, $p>0.01$). The production rate of prostacyclin by toxemic placentas was not affected by a low oxygen environment (20% O_2 vs. 95% O_2, 1.80 $\pm$ 0.45 vs. 2.08 $\pm$ 0.54 pg/mg/h, $n=5$) (Figure 13).

Prostacyclin production by the amnion is shown in figure 14. The concentrations of 6-keto-$PGF_{1\alpha}$ in the incubation medium increased progressively, reaching a peak concentration of 50 $\pm$ 19 pg/mg at 48 hours. No differences were observed in the production rates between normal and toxemic amniotic tissues (0.98 $\pm$ 0.29 vs. 0.82 $\pm$ 0.17 pg/mg/h, $n=8$, $p>0.01$). The same finding was true for chorionic tissues (Figure 15). The production rates were not significantly different between normal and toxemic tissues (0.95 $\pm$ 0.38 vs. 0.69 $\pm$ 0.21 pg/mg/h, $n=8$, $p>0.01$). The concentrations of

Figure 11

Production of prostacyclin by normal and toxemic placentas. Data represent mean ± SE (n=12).

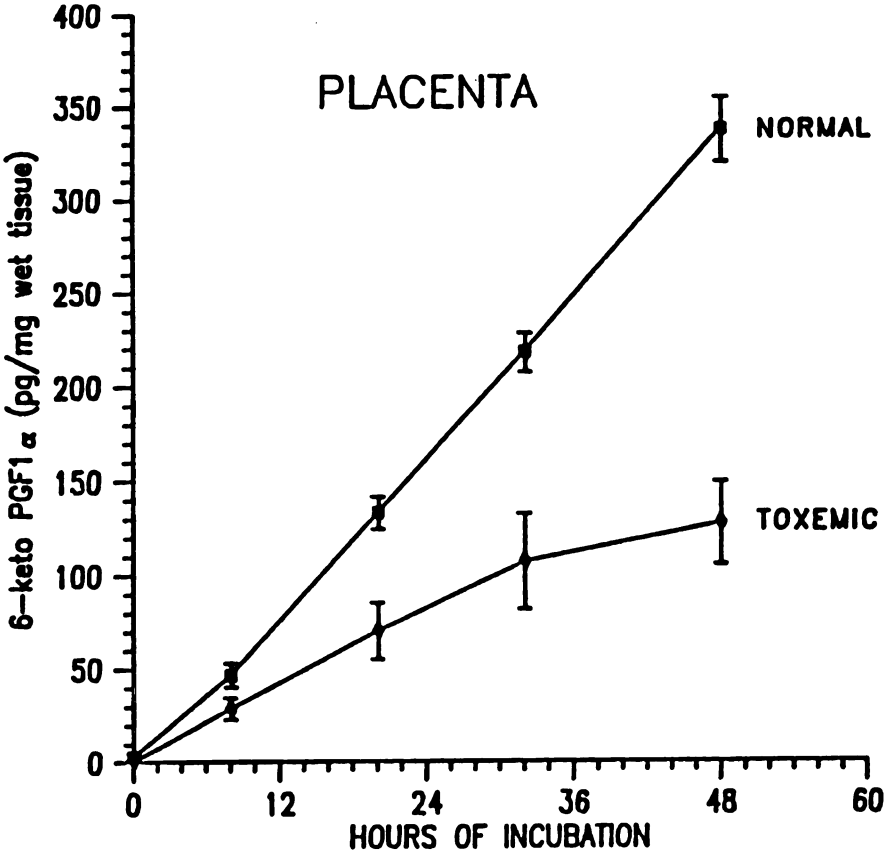
PLACENTA
NORMAL
TOXEMIC
6-keto PGF1α (pg/mg wet tissue)
400
350
300
250
200
150
100
50
0
0
12
24
36
48
60
HOURS OF INCUBATION

Figure 11

Figure 12

The production of prostacyclin by toxemic placentas with 5 or 100 uM arachidonic acid added to the incubation media. Data represent mean ± SE (n=5).

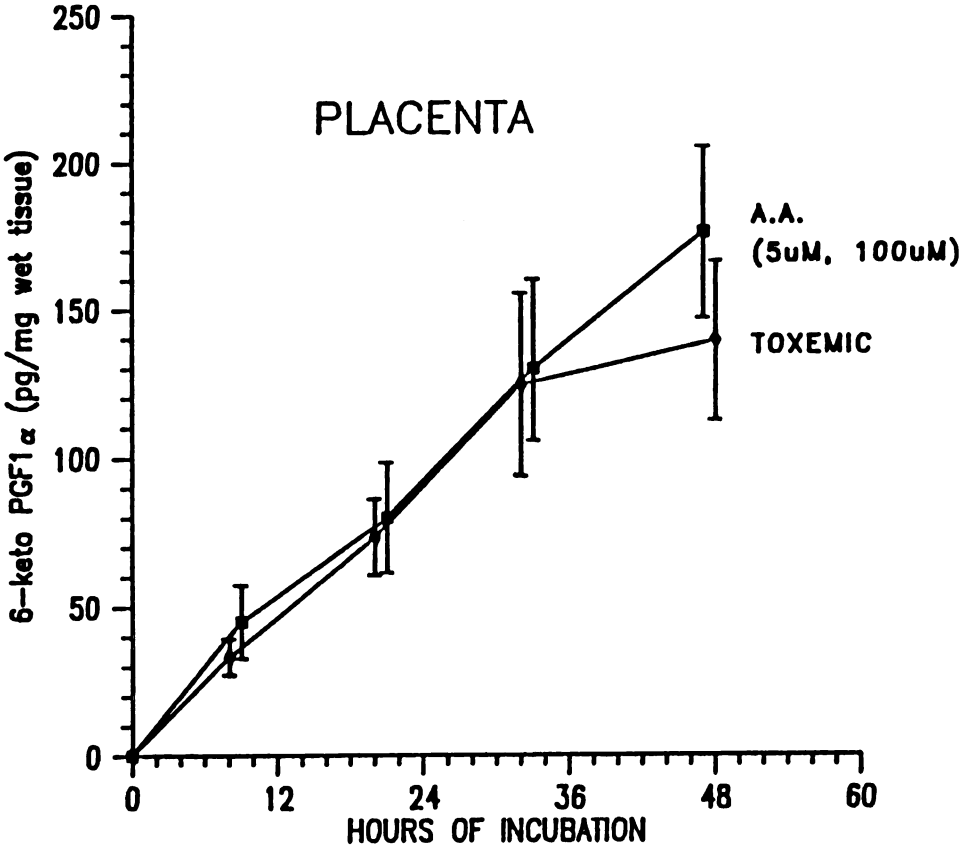
PLACENTA
6-keto PGF1α (pg/mg wet tissue)
250
200
150
100
50
0
0
12
24
36
48
60
HOURS OF INCUBATION
A.A.
(5uM, 100uM)
TOXEMIC

Figure 12

figure 13

The production of prostacyclin by toxemic placentas in two different oxygen environments (20% vs. 95% O_2). Data represent mean $\pm$ SE (n=5).

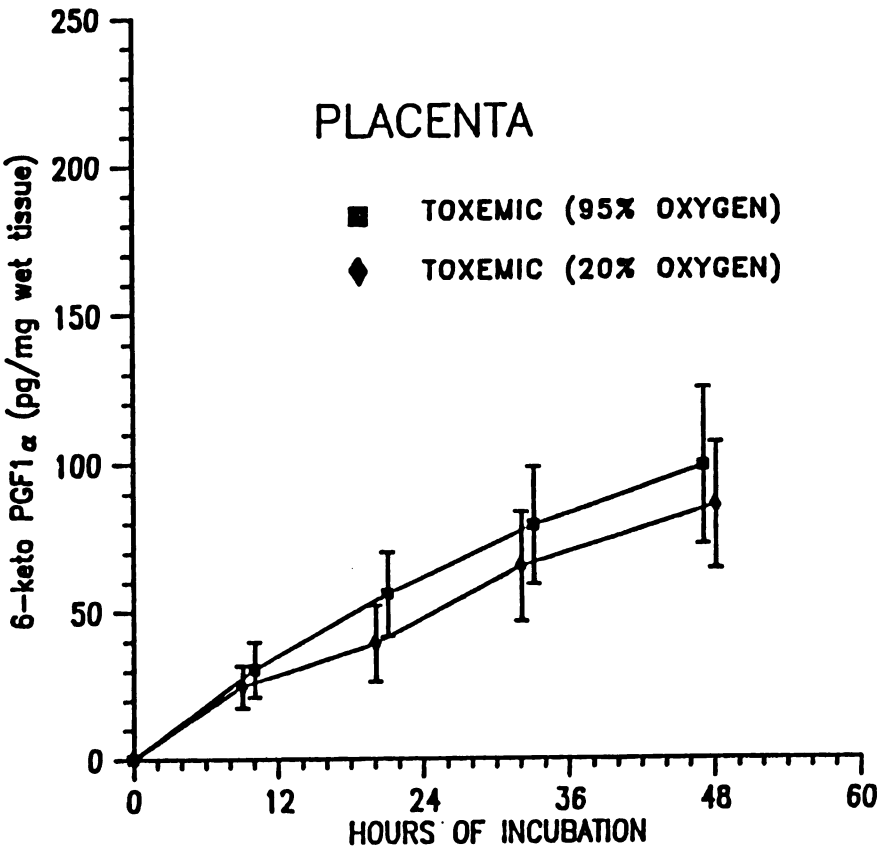
PLACENTA
TOXEMIC (95% OXYGEN)
TOXEMIC (20% OXYGEN)
6-keto PGF1α (pg/mg wet tissue)
250
200
150
100
50
0
0
12
24
36
48
60
HOURS OF INCUBATION

Figure 13

Figure 14

The production of prostacyclin by normal and toxemic amnionic tissues. Data represent mean ± SE (n=8).

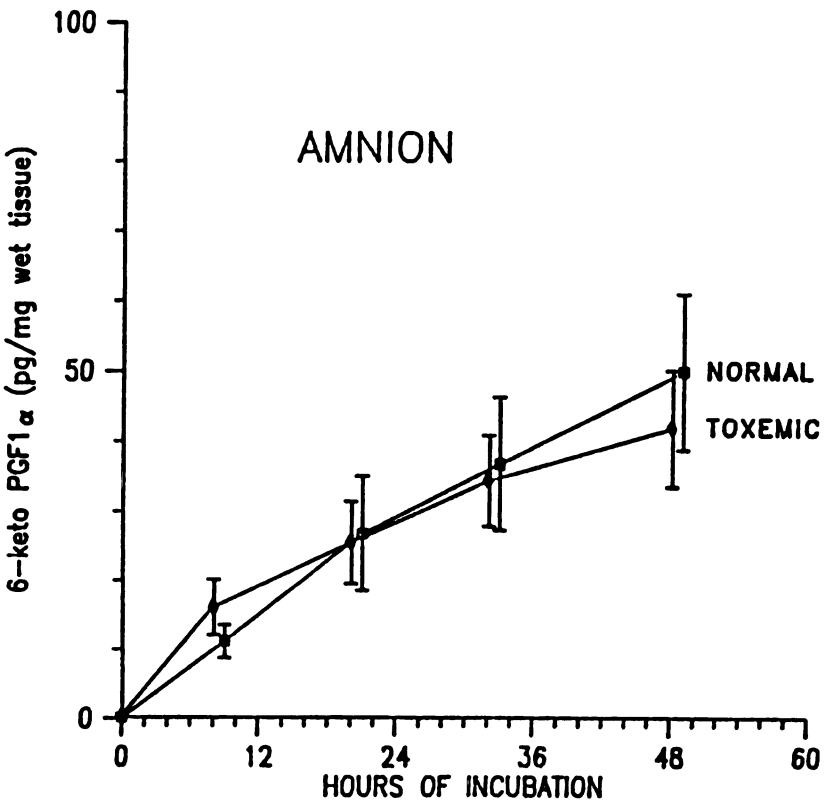
AMNION
6-keto PGF1α (pg/mg wet tissue)
100
50
0
0
12
24
36
48
60
HOURS OF INCUBATION
NORMAL
TOXEMIC

Figure 14

Figure 15

The production of prostacyclin by normal and toxemic chorionic tissues. Data represent mean ± SE (n=8).

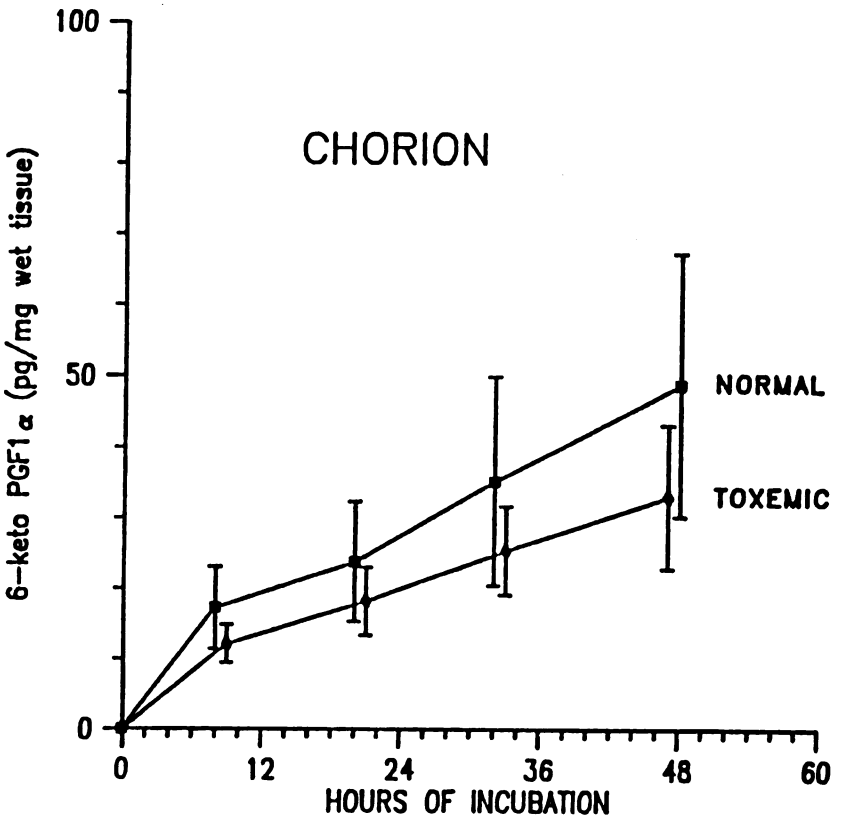
CHORION
6-keto PGF1α (pg/mg wet tissue)
100
50
0
0
12
24
36
48
60
HOURS OF INCUBATION
NORMAL
TOXEMIC

Figure 15

6-keto-$PGF_{1\alpha}$ increased progressively, reaching a peak concentration of 47 $\pm$ 13 pg/mg after 48 hours. Amnion and chorion production rates were not significantly different from each other ($p>0.01$), but they were less than the placental production rates ($p<0.01$). For example, the production rate of normal amnion and chorion was only 1/7th the rate of the placenta.

V DISCUSSION

The present study is the first to show that prostacyclin production by toxemic placentas is significantly less than that by normal placentas (Figure 11). Toxemic placental extracts incubated *in vitro* generated prostacyclin at a rate of 2.72 pg/mg/h compared to a rate of 7.22 pg/mg/h for control tissues. Previous investigators have reported decreased production of prostacyclin by umbilical arteries and placental veins (Carreras et al., 1981), reduced prostacyclin activity in amniotic fluid (Ylikorkala et al., 1981), low urinary metabolite concentrations of prostacyclin (Goodman et al., 1982) and correlation between depressed prostacyclin concentrations in umbilical arteries and decreased uterine blood flow (Makila et al., 1983).

Prostacyclin is a circulating hormone with potent vasodilating and platelet antiaggregatory properties. Because normal pregnancy is characterized by a five-fold increase in maternal prostacyclin levels (Lewis et al., 1980), it has been suggested that a deficiency in its production may be a causative factor preeclampsia (Remuzzi et al., 1980). Although the source of this increase is unknown, it is probable that the placenta is a major contributor to the elevation in maternal prostacyclin levels. We focused our research on the placenta for several reasons: the placenta is unique to pregnancy, it generates prostacyclin, and it performs an active role during pregnancy as

the interface between the maternal and fetal circulations. Reduced placental production of this prostaglandin might account for the increased vasoconstriction, increased platelet aggregation and decreased uteroplacental blood flow noted in toxemia.

To evaluate possible variation in our study due to factors other than toxemia a number of clinical indices were compared in the normal and toxemic groups. No significant differences were found between normal and toxemic pregnancies for weeks of gestation at delivery, time in labor, number of vaginal versus cesarean section deliveries, weight gain of mothers, age of mothers, placental weights, fetal weights, tissue viability and apgar score of the fetuses. All babies were born alive and healthy. Therefore, aside from expressing the clinical symptoms of toxemia the normal and toxemic women were similar. The decrease in placental prostacyclin production, then, could be attributed to the disorder and not to nonspecific variation in the pregnancies of the two groups.

Placental tissues from women with uncomplicated pregnancies were examined first to establish the controls for this investigation. The concentration of 6-keto-$PGF_{1\alpha}$ in the incubation medium, expressed as pg/mg of wet tissue, increased progressively and in a linear fashion during the 48 hours of incubation (Figure 7). Peak levels after 48h averaged 346 pg/mg. Because the synthesis of prostacyclin is dependent on the release of phospholipase A_2 from lysosomes,

we were concerned that damage to the tissue during its preparation would initially result in a large breakdown of lysosomes and a surge of prostacyclin production. The linear increase in prostacyclin production from the onset of incubation, however, suggests that tissues were minimally affected during their preparation.

One way to demonstrate production of a substance is to show inhibition of that substance. To confirm that prostacyclin was produced by our placental extracts in vitro and to eliminate the possibility that we were simply measuring its release from tissue stores, we added indomethacin to the incubation medium. This agent acts on the cyclooxygenase enzyme, preventing the conversion of arachidonic acid to endoperoxides thereby blocking the synthesis of all the prostaglandins (Figure 6). Two concentrations of indomethacin were investigated. A 50 uM dose completely abolished the production of prostacyclin by both normal and toxemic tissues, while a 5 uM dose reduced the production rate to only 0.27 pg/mg/h (Figure 8). These results indicated that we were measuring the production of prostacyclin and not its release.

The production of prostacyclin is dependent on the availability of arachidonic acid, the precursor for prostacyclin (Figure 6). With an abundance of phospholipids in cellular walls throughout the placenta, one would speculate that the supply of arachidonic acid is not a limiting factor. Carreras et al. (1983) found that fragments of umbili-

cal arteries and placental veins in arachidonic acid enriched medium did not effect the production rate of prostacyclin by these vessels. However, no one has examined this substrate's availability in vitro over a 48 hour period. If stores are depleted or become inaccessible, the measurements of prostacyclin concentrations obtained late in the experiment may not be representative of actual values. To address this question two concentrations of arachidonic acid were added to the incubation medium, 5 and 100 uM. Levels of 6-keto-$PGF_{1\alpha}$ following treatment with arachidonic acid were not significantly different from control values during 48 hours of incubation (Figure 9). Therefore, the production of prostacyclin does not appear to be substrate limited in our preparation.

Although exogenous arachidonic acid did not influence the production rate of prostacyclin by normal placentas, a decrease in this precursor might still account for the reduced placental prostacyclin production noted in toxemia. The addition of arachidonic acid to the incubation medium of toxemic tissues, however, did not increase the production rate of prostacyclin (Figure 12). This finding rules out the possibility that this disorder is the result of arachidonic acid limitations.

Prostacyclin production in a low oxygen environment was investigated to see what effect low oxygen might have on placental production. It has been suggested that a high oxygen environment is toxic to the placenta (Tuvemo, 1980),

while a decreased oxygen supply to the fetus or heart stimulates prostacyclin synthesis. Gasing the incubation media with 20% oxygen significantly reduced rather than increased the production of prostacyclin (Figure 10). The production rate was was only 3.09 pg/mg/h compared to 7.55 pg/mg/h for normal tissue gased with 95% oxygen. In the low oxygen environment the decrease in prostacyclin production by toxemic placentas was not statistically significant (Figure 13).

The placenta is not the only fetal tissue to produce prostacyclin. Our data confirms that the amnion and chorion generate prostacyclin, but at 1/7th the rate of the placenta. This finding is in contrast to the observations of Mitchell et al., (1978) who reported that the amnion produced twice as much of this prostaglandin as did the chorion or placenta. The reason for this discrepancy is unknown, but the placenta with its rich vascular network and large mass seems more likely to be the major producer. We found the production rate by amnion and chorion to be 0.98 and 0.82 pg/mg/h, respectively, compared to a rate of 7.55 pg/mg/h by normal placentas. There were no significant differences between normal and toxemic amniotic or chorionic tissues (Figures 14 and 15). These findings indicate that the placenta rather than the amnion or chorion, is principally involved in the pathogenesis of toxemia.

An attractive hypothesis for the pathogenesis of toxemia is that a drop in the concentration of prostacyclin

leaves thromboxane, a potent vasoconstrictor and a stimulator of platelet aggregation, biologically unopposed (Figure 2). In the vascular system, thromboxane synthesis occurs in platelets, usually in response to vascular wall damage, and its release and subsequent action stimulates the platelets to clump and plug the lesion. Under normal conditions prostacyclin inhibits this process to maintain flow through the vessels. In toxemia, lower prostacyclin levels mean a less effective inhibition of platelet aggregation. This imbalance in activity might explain some of the coagulation abnormalities that occur with this disease, such as, disseminated intravascular coagulation. As a vasoconstrictor thromboxane may also be involved in the regulation of local blood flow. A major reason for intrauterine growth retardation in toxemia is a 30-40% decrease in the blood supply to the uterus which is likely maintained during uncomplicated pregnancies by the elevated concentration of prostacyclin. With a fall in circulating prostacyclin levels enhanced thromboxane activity could contribute to uteroplacental ischemia. The decrease in delivery of oxygen to the placenta could also account for the greater number of infarcts observed at term in toxemic placentas compared to normal placentas.

During normal pregnancy thromboxane levels in the maternal blood are increased from the nonpregnant state (Mitchell et al., 1978). The source of this increase is unknown but may involve fetal tissues because it is produced

by the amnion, chorion, decidua and placenta (Mitchell et al., 1978; Tuvemo, 1980). Preliminary studies support the hypothesis that the normal balance between thromboxane and prostacyclin in uncomplicated pregnancy is disturbed in toxemia (Walsh and Fenner, 1984). These studies demonstrated that in addition to a decreased production of prostacyclin by the toxemic placenta, there is also a significant increase in thromboxane production by this tissue when compared to the normal placenta. The ratio of thromboxane production to prostacyclin production by the toxemic placenta was shown to be increased approximately ninefold from a 1:1 ratio observed in normal pregnancy. Wallenburg and Rotmans (1982) found enhanced platelet activation and platelet aggregation in normotensive and toxemic pregnant woman with small-for-gestational age infants. Another group (Ylikorkala et al., 1981) reported reduced amniotic levels of prostacyclin in toxemia but no change in the levels of thromboxane. The production of thromboxane by umbilical arteries was also found to remain normal during toxemia (Makila et al. 1983; Stuart et al., 1983). Investigators speculate that the surprising effectiveness of small doses of aspirin in treating some preeclamptics is due to the selective inhibition of thromboxane production (Crandon and Isherwood, 1979; Masotti et al., 1979). Reducing thromboxane production might reestablish the thromboxane-prostacyclin balance at a lower activity level, thereby improving the condition of the mother. Unfortunately, aspirin is capable

of irreversibly blocking the synthesis of all the prostaglandins, including prostacyclin. Administration of such prostaglandin synthetase inhibitors can also cause premature closure of the ductus arteriosus (Coceani and Olley, 1980). Large variation in the effective dose needed to selectively act on the thromboxane synthetase enzyme makes aspirin too unpredictable for this particular use. The development of specific inhibitors of thromboxane could prove to be beneficial clinically.

Thromboxane is probably not the only vasoactive substance responsible for inducing hypertension in toxemia. Other vasoconstrictors such as angiotensin II and the catecholamines, are likely to be involved as well. Gant (1973) demonstrated that enhanced sensitivity of toxemic women to infused angiotensin II can appear as early as the twenty-second week of gestation. He found that as pregnancy progressed, the dose of angiotensin II necessary to increase diastolic blood pressure by 20 mmHg was significantly less in toxemic patients than in normal pregnant woman (Figure 5). This study was strengthened by Everett et al. (1978) who found that administration of indomethacin or aspirin to normal pregnant women resulted in an increased sensitivity to infused angiotensin II, an enhanced sensitivity similar to that seen in toxemic women. Therefore, the decrease in prostacyclin not only affects the normal prostacyclin-thromboxane balance but may also disturb its relationships with other substances.

What is the underlying mechanism responsible for the decrease in prostacyclin production? Preliminary work suggests that its low levels might be due to a decrease in enzyme availability in tissues unique to pregnancy. An enzyme deficiency is indicated by the lower concentrations of prostacyclin synthetase that are found in the umbilical arteries of toxemic women (Downing et al., 1980). Localization of this defect in tissues of fetal origin would explain why toxemia is a pregnancy-induced condition. Because thromboxane synthesis by the placenta is increased in toxemia (Walsh and Fenner, 1984), the enzyme deficiency may be specific for prostacyclin while other prostaglandins are either not affected or, like thromboxane, show increased are increased.

In conclusion, our study not only confirms that prostacyclin is likely to be involved in the etiology of toxemia, but also for the first time indicates that a decrease in its production by the placenta may be an important event in the pathogenesis of the disorder. Unquestionably, the ultimate goal of this and other investigations is to provide effective treatment for women suffering from toxemia and eventually its total prevention. As mentioned above, the use of aspirin for treatment is too unpredictable. Possibly, an alternative form of treatment in the future will be the use of specific thromboxane synthase inhibitors. It is hoped that their administration to women with pregnancy-induced hypertension will reestablish the

normal balance between prostacyclin and thromboxane.

VI. SUMMARY AND CONCLUSIONS

(1) Compared to the production of prostacyclin in the normal placenta, production in the toxemic placenta was significantly reduced.

(2) Prostacyclin production in the normal and toxemic placenta can be inhibited with indomethacin, which would indicate that prostacyclin is not simply released from the placenta but is synthesized there.

(3) The substrate arachidonic acid did not affect the production of prostacyclin in either the normal or toxemic placenta, suggesting that production is not substrate limited.

(4) Prostacyclin production in the normal placenta was significantly reduced in a low oxygen environment whereas no effect was noted in the toxemic placenta. A high oxygen environment does not appear to be toxic to placental tissues *in vitro*.

(5) The amnionic and chorionic prostacyclin production rates were not affected by toxemia. Both tissues produced prostacyclin to a much lesser degree than the placenta.

(6) Therefore, this study supports the hypothesis that a decrease in the production of prostacyclin by the placenta may be an important link in the etiology and associated hypertension and disseminated intravascular coagulation of toxemia.

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Prostacyclin Radioimmunoassay (RIA)

Placental production of prostacyclin was estimated by assaying the concentration of a stable breakdown product, 6-keto-prostaglandin F_1 alpha (6-keto-$PGF_{1\alpha}$) in the incubation media. 6-keto-$PGF_{1\alpha}$ is generated by nonenzymatic degradation of prostacyclin. The half-life of prostacyclin is approximately 3 minutes (Moncada et. al 1976).

REAGANTS:

All reagents used in the assay were diluted with phosphate buffered saline (PBSG - 0.01 phosphate, pH 7.4, 0.09% sodium chloride and 0.1% gelatin) (Table 1). Sodium azide (0.01 percent) was added as perservative and PBSG was used for up to one week. Standards (2.44 pg/0.05 ml - 1.25 ng/0.05 ml) were made by adding known amounts of 6-keto-$PGF_{1\alpha}$ to PBSG in 15 ml polypropylene centrifuge tubes. Fresh standards were made every six months. Disposable glass tubes (12mm x 75mm) were used for the RIA. Rabbit antibody (Seragen, Boston, MA) highly specific for 6-keto-$PGF_{1\alpha}$ (Table 2) was diluted as obtained from Seragen 1:22.5 in buffer (final titer 1:35,000). The original volume of radioactive 6-keto-$PGF_{1\alpha}$ (Amersham, Arlington Heights, IL; 0.25 ml of acetonitrile:water, 9:1, with 0.025 mCi of labeled 6-keto-$PGF_{1\alpha}$) was diluted with an additional 1.0 ml of acetonitrile:water (9:1) to minimize loss of the radioactive prostaglandin due to evaporation and adsorbtion on the

Table 1

0.1M Phosphate Buffer

2.76 grams $NaH_2PO_4 \cdot H_2O$

11.35 grams Na_2HPO_4

9.0 grams NaCl

0.1 grams Na azide

5.0 grams gelatin

1000.0 ml grade A water

Charcoal Solution

2.5 grams Norit A charcoal

(Fisher Scientific Co., Fair Lawn, NJ)

0.25 grams Dextran T

(Pharmacia Fine Chemicals, Uppsala, Sweden)

500.0 ml of 0.1M PBSG

Table 2

Rabbit-anti-6-keto-$PGF_{1\alpha}$

Cross Reactivity at 50% B/B_o

6-keto-$PGF_{1\alpha}$	(100%)
$PGF_{1\alpha}$	10.7%
$PGF_{2\alpha}$	2.3%
6-keto-PGE_1	1.5%
PGE_1	0.4%
PGE_2	0.06%
PGA_1, PGA_2	<0.01%
PGB_1, PGB_2	<0.01%
Thromboxane	<0.01%
PGD_2	<0.01%
15-keto-$PGF_{1\alpha}$	<0.01%
Dihydro 15-keto-$PGF_{2\alpha}$	<0.01%
15-keto-PGE_2	<0.01%
Dihydro 15-keto-PGE_2	<0.01%

Amersham vial during storage. For each assay, trace was diluted in PBSG to a concentration of approximately 6000 cpm/0.05 ml solution.

PROCEDURE:

A Micrometic automatic pipet was used to add all reagants to the glass tubes. Fifty microliters of sample or standard were pipeted into tubes and diluted with 150.0 ul of PBSG. The final incubation volume (300 ul) was reached by simultaneously adding 50 ul of antibody and 50 ul of labeled hormone to each tube. The total amount of radioactivity placed in each tube was determined by the counts per minute (CPM) produced by 50 ul of trace diluted in 250 ul of buffer (TCT tube; table 3). Total counts were approximately 6000 per minute. A zero dose tube (B_o tube) was made by adding 50 ul of antibody and 50 ul of trace to 200 ul of PBSG (Table 3). Nonspecific binding (NSB tube) in the assay was determined from the CPM generated by charcoal stripped total count tubes (Table 3). All tubes were vortexed and incubated at 4°C for 18-24 hours.

Following incubation 1.0 ml of dextran-coated charcoal (0.5% charcoal: 0.05% dextran), pre-cooled to 0-4°C, was added to all the tubes except total count tubes at a rate of 40 tubes per minute using the Micrometic automatic pipette. The total count tubes received 1.0 ml PBSG so that quenching was equal for all tubes. Charcoal was used to separate labeled 6-keto-$PGF_{1\alpha}$ that was not bound by the antibody during the incubation period. The charcoal was kept on ice

Table 3

Radioimmunoassay Protocol

Tube*	Buffer (ul)	Tracer (ul)	Antibody (ul)	Receive charcoal on day 2 of assay
Total count tube (TCT)	250	50	--	No
Zero dose tube (B_o)	200	50	50	Yes
Non-specific binding (NSB)	250	50	--	Yes
6-keto-$PGF_{1\alpha}$ standards (50 ul)	150	50	50	Yes
Samples (50 ul)	150	50	50	Yes

*Tubes run in triplicate

and mixed continuously. After charcoaling, all tubes were vortexed. Fifteen minutes after the last tube received charcoal they were centrifuged at 3000 rpm (2000 x g, Beckman Model J-6B, Palo Alto, CA) for 12 minutes. The supernatent was decanted into scintillation vials containing 15 ml scintillation cocktail (Safety Solve, Mount Prospect, IL) and the radioactivity was counted on a Packard Tri Carb 300C liquid scintillation counter for ten minutes or until 2.0 percent statistical accuracy was achieved. One ml of each total count tube was pipeted into a scintillation vial and counted.

Standards were plotted as a percentage of the zero dose tubes (B_o) (figure 16). The sample concentration could then be determined from the standard curve using the percent binding of each sample obtained experimentally. Most samples ranged between 30-40 percent binding (B/B_o).

Figure 16

Representative binding of 6-keto-prostaglandin F_1 alpha standards. Plotted are the percentage of the total binding for each of the eight 6-keto-prostaglandin F_1 alpha standards, mean $\pm$ SD, n=10. Also graphed are the serial Dulbeco's Modified Eagles media dilutions: P_{25}=100 ul, P_{50}=50 ul, and P_{50}=25 ul.

STANDARD CURVE

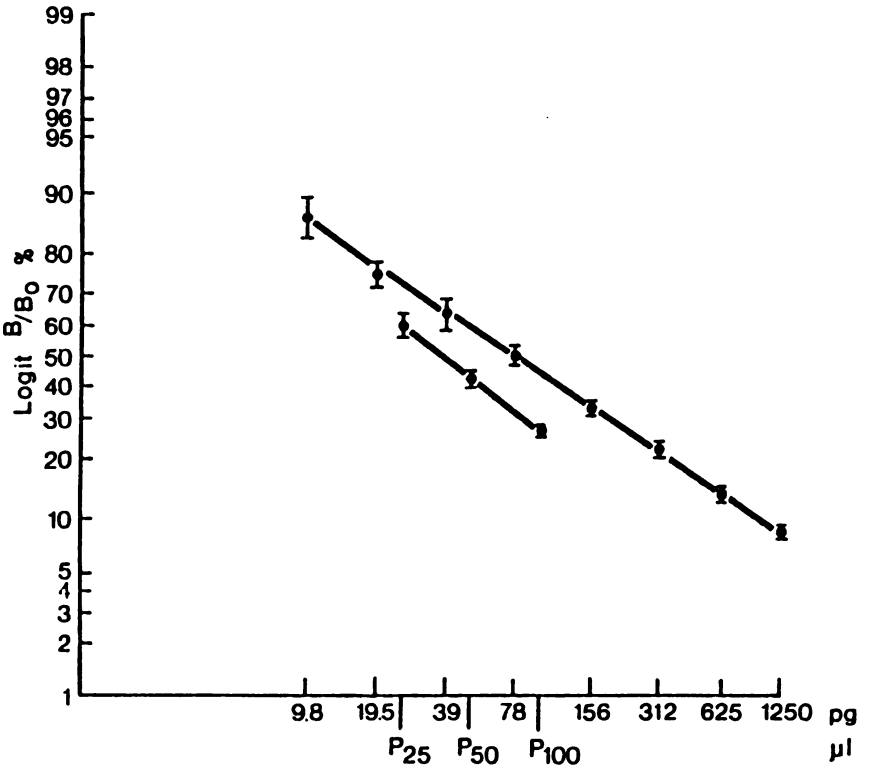

Figure 16

Reliability of the Assay

Specificity: A highly specific antibody for 6-keto-$PGF_{1\alpha}$ was used in this assay (Table 2). Therefore, sample extraction was not necessary and the incubation media was assayed directly.

Test of parallelism: Three serially diluted samples of Dulbecco's Modified Eagles Media (DMEM) were processed in each assay. They were plotted along with each standard curve and were assessed for parallelism to the curve (Figure 16).

Sensitivity: The least detectable concentration (LDC, the concentration two standard deviations away from the zero dose level) was 6.14 $\pm$ 3.0 (mean $\pm$ SD, n=5).

Precision of the assay: Within- and between- assay variations were calculated according to the methods of Rodbard for each of the three serial DMEM dilutions (Table 4). Within-assay variations ranged from 13.6 to 15.0% and between-assay variation ranged from 5.12 to 13.9%.

Accuracy: To determine the accuracy of the assay, varying concentrations of 6-keto-$PGF_{1\alpha}$ (19.5, 39.0, 78.0, 156.0, 312.0, and 625 pg, Figure 17) were added to DMEM. Unspiked DMEM samples were also run in each assay and were equivalent to a zero dose response (mean $\pm$ SE, 104.5 $\pm$ 2.3%, n=5). Recovery values for the spiked DMEM were as follows: 19.5 pg, 71.0%; 39.0 pg, 85.0%; 78.0 pg, 99.0%; 156.0 pg, 93.0%; 312.0 pg, 97.5%; 625.0 pg, 95.0%.

Table 4

Precision of the 6-keto-PGF_1 alpha Radioimmunoassay

Volume of sample assayed (ul)	Mean (pg)	df	Within-assay SD	Within-assay variation = CV	Between-assay SD	Between-assay variation = CV
25	49.5	5	7.0	13.7	16.0	13.9
50	112.0	5	17.0	15.0	13.0	11.7
100	242.0	5	33.0	13.6	12.4	5.12

Figure 17

Test of accuracy of 6-keto-prostaglandin F_1 alpha assay. Known amounts of 6-keto-prostaglandin F_1 alpha added to phosphate buffer are plotted against amount recovered from the assay, mean $\pm$ SD.

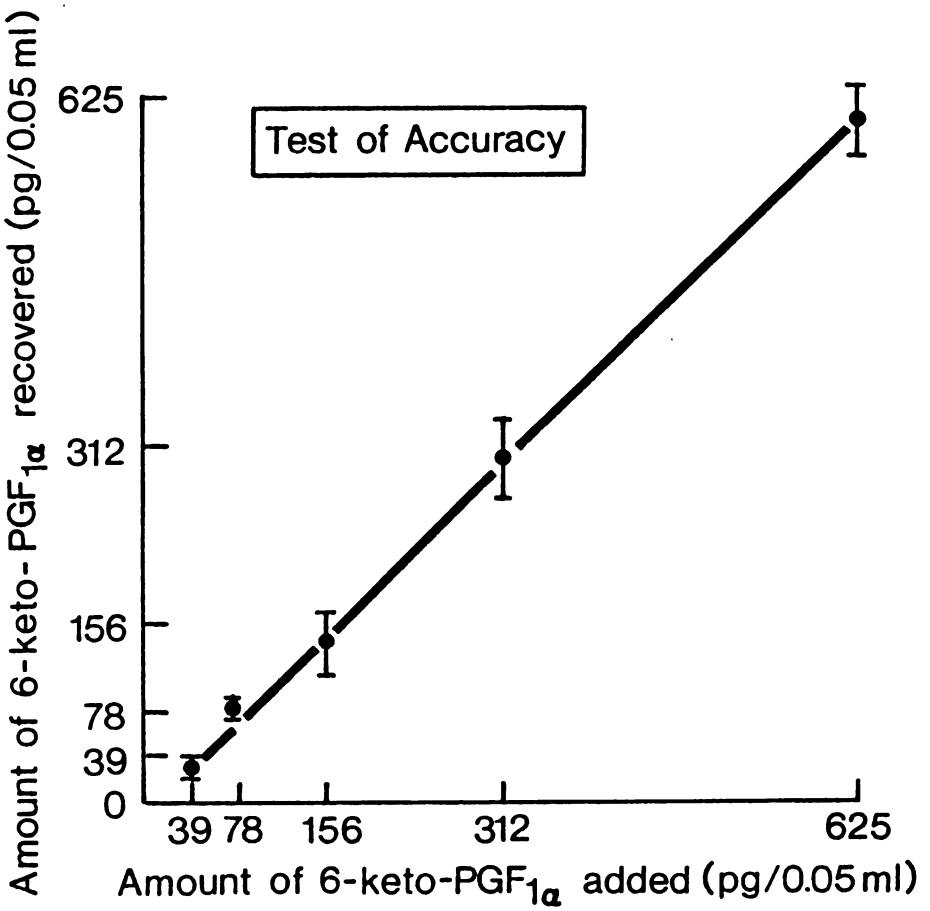
Test of Accuracy
Amount of 6-keto-PGF1α recovered (pg/0.05 ml)
625
312
156
78
39
0
39 78 156 312 625
Amount of 6-keto-PGF1α added (pg/0.05 ml)

Figure 17