DEVELOPMENT OF METHODOLOGY FOR THE MEASUREMENT OF PROSTACYCLIN AND THROMBOXANE PRODUCTION: POSSIBLE RELEVANCE OF PROSTACYCLIN/THROMBOXANE BALANCE IN CLINICAL CONDITIONS

by

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DECLARATION

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Some of the work in this thesis has already been published:-

(1) McLaren M, Belch J J F, Forbes C D, Prentice C R M
Development of a radioimmunoassay for the measurement of prostacyclin metabolites in unextracted plasma
Thrombosis Research 37: 177 - 183 (1985)

(2) McLaren M, Greer I A, Walker J J, Forbes C D
Umbilical artery perfusion : a method to measure prostacyclin prostacyclin production in vitro
Progress in Lipid Research 25: in press (1986)

(3) McLaren M, Belch J J F, Forbes C D
Development of a radioimmunoassay for the measurement of prostacyclin metabolites in unextracted plasma
In: Advances in Prostaglandin, Thromboxane and Leukotriene Research Vol 13 Neri Serneri (Editor): 41-42 (1985)

(4) Belch J J F, McLaren M, Anderson J, Lowe G D O, Sturrock R D, Capell H A, Forbes C D

Increased prostacyclin metabolites and decreased red cell deformability in patients with systemic sclerosis and Raynaud's syndrome

Prostaglandins Leukotrienes and Medicine 17: 1-9 (1985)

(5) Belch J J F, McLaren M, Lowe G D O, McIntyre D E, Forbes C D, Prentice C R M

Effect of Des-Amino-D-Arginine Vasopressin (DDAVP) on plasma levels of platelet and endothelial cell release products - Reply

Thrombosis and Haemostasis (Stuttgart) 49: 65 (1983)

(6) Greer I A, McLaren M, Belch J J F, Lowe G D O, Forbes C D

Endothelial stimulation by DDAVP in von Willebrand's disease and haemophilia

Haemostasis 16: 15-19 (1986)

(7) Greer I A, Walker J J, McLaren M, Bonduelle M, Cameron A D, Calder A A, Forbes C D

Immunoreactive prostacyclin and thromboxane metabolites in normal pregnancy and the puerperium

British Jounral of Obstetrics and Gynaecology 92:

581 - 585 (1985)

(8) Greer I A, Walker J J, Cameron A D, McLaren M, Calder A A, Forbes C D

A prospective longitudinal study on immunoreactive prostacyclin and thromboxane metabolites in normal and hypertensive pregnancy

Clinical and Experimental Hypertension 134: 167-181 (1985)

(9) Greer I A, Walker J J, McLaren M, Calder A A, Forbes C D
Inhibition of thromboxane and prostacyclin production in whole blood by adrenoceptor antagonists
Prostaglandins, Leukotrienes and Medicine 19: 209-217 (1985)

(10) Greer I A, Walker J J, McLaren M, Lang G D, Serle E, Calder A A, Forbes C D
Thromboxane A_2 and prostacyclin levels in molar pregnancy
British Journal of Obstetrics and Gynaecology 92: 652 (1985)

This thesis describes the historical development of our knowledge of prostaglandins and thromboxane and the synthesis, metabolism and actions of prostacyclin and thromboxane. Chapter 3 then presents work on the development and validation of a radioimmunoassay to measure plasma prostacyclin metabolite levels. Comparisons are made of results using a tritiated and an iodinated radioactive label and also of results obtained using different antibodies. Results are also presented of an assay to measure prostacyclin stimulating factor in normal plasma and serum. A method developed to measure endothelial cell prostacyclin production using a human umbilical artery perfusion model is described.

Chapter 4 describes the various methods which may be used to measure production of thromboxane A_2. Firstly a radioimmunoassay is described which measures thromboxane B_2 the stable metabolite of thromboxane A_2 and results using this assay for both plasma and serum samples are produced. Methods for measuring thromboxane generation by platelets in response to aggregation with 4 ug/ml of collagen and also serum thromboxane B_2 levels in bleeding time blood are described.

The second part of the thesis is concerned with an attempt to validate the relevance of the prostacyclin/thromboxane balance in clinical situations.

Possible ways of altering the thromboxane/prostacyclin balance which may have clinical implications were explored.

Plasma levels of prostacyclin metabolites and thromboxane A_2 before and after ingestion of drugs known to alter arachidonic acid metabolism are shown. Studies on the effect of dietary fat intake and smoking on the prostacyclin/thromboxane balance are presented.

Chapters 6 and 7 examine the thromboxane/prostacyclin balance in a range of vascular diseases and in bleeding disorders respectively. A Chapter is then devoted to the possible relevance of the thromboxane/prostacyclin balance in pregnancy and associated disorders of pregnancy. Chapter 9 summarises the results of this work and also discusses the implications for future research.

All the statistics in this thesis unless otherwise stated, use Wilcoxon's rank sum tests on paired or unpaired data as described in: Statistics at Square One Ed T D V Swinscow, British Medical Association, London

CHAPTER 1 - HISTORICAL INTRODUCTION AND OVERVIEW

1.1 Discovery of Prostaglandins

In 1930 in New York a gynaecologist Dr Raphael Kurzrok, whose particular interest was artificial insemination, discovered that lipid fractions isolated from human semen increased the contractile response of the uterine muscle. Kurzrok and his colleague Lieb carried out a series of *in vitro* experiments using human uterine strips obtained after hysterectomy. They discovered that the same sample of semen was able to produce contraction in one uterine strip but not another. Moreover they found that one uterus contracted in response to one semen sample but dilated on exposure to another. They were also able to find correlations between the contractile response and the patient's history of infertility. Other workers soon became interested in these experiments. In 1935 Goldblatt attributed the biological activity of seminal plasma to a lipid or lipid like substance (Goldblatt 1935). Von Euler (1936) was the first to call this active substance prostaglandin supposing, wrongly as it later turned out, that it was produced by the prostrate gland. Von Euler (1936) also reported similar activity in extracts prepared from seminal vesicles of sheep. He proposed that the active component had the properties of a fatty acid. Sheep seminal vesicles still remain the best biological source of prostaglandin intermediates and end products.

At this stage of development the work was unable to progress much further mainly due to the lack of sophisticated analytical techniques and equipment.

It had to wait another thirty years until Bergström and colleagues (Bergström 1964) in association with van Dorp in Holland (Van Dorp 1964) were able to isolate the first pure prostaglandin and elucidate its structure. Initially Bergström designated a fraction as "active" if it induced smooth muscle contraction in rabbit duodenum and caused the rabbit's blood pressure to drop. However the introduction in the 1960's of gas-liquid chromatography and mass spectometry (GCMS) (Ryhage 1960) allowed further characterisation of these "active" prostaglandin fractions. It soon became apparent that prostaglandins were ubiquitous, their precursors being identified by Bergström (1964) and by Van Dorp (1964) as unsaturated fatty acids such as arachidonic acid.

Synthesis of prostanoids can apparently take place in all tissues from arachidonic acid incorporated in the phospholipids of cell membranes. The products are formed and released as a result of various stimuli: mechanical, chemical, immunological and others. It was found that many of the prostanoids were unstable and measurement of stable metabolites using sensitive assay methods could often give a clearer picture. Several of these end products were themselves found to possess biological activity.

After having reached the circulatory system most prostaglandins are rapidly metabolised, the main inactivation occurring in the lungs and the liver by the action of a dehydrogenase. Prostaglandin A_2 however is not attacked by this dehydrogenase and thus exerts a prolonged action.

One of the most significant discoveries in the prostaglandin story was the demonstration by Vane and colleagues that prostaglandin synthesis could be inhibited by aspirin (Vane 1971).

This provided a new tool in the study of these compounds. Valuable information can be provided on the action of a biological substance by its inhibition and this greatly extended knowledge of the biological role of prostaglandins.

Figure 1 shows the pathways of arachidonic acid metabolism in human platelets and endothelial cells.

1.2 Nomenclature of Prostaglandins

There are nine classes of naturally occurring prostaglandins which have been discovered up to this time. Figure 2 shows the structure of each of these nine classes.

The first successful purification of a prostaglandin was achieved by partition of the crude mixture between ether and phosphate buffer. Prostaglandin E was obtained from the ether phase and prostaglandin F from the aqueous phase (fosfat). Treatment of prostaglandin E with acid gave prostaglandin A and treatment with a base gave prostaglandin B. When other prostaglandins were discovered they were given appropriate letters to complete and extend the sequence. The subscripts 1, 2 etc are used to signify the number of double bonds in the side chains of the prostaglandin. The numbering of prostaglandins is conventional, the number one being given to the carbon atom of the principal group. In the F series of prostaglandins the subscript refers to the configuration of the hydroxyl group attached to C-9.

The thromboxanes have an oxacyclohexane ring in place of the cyclopentane ring of the prostaglandins.

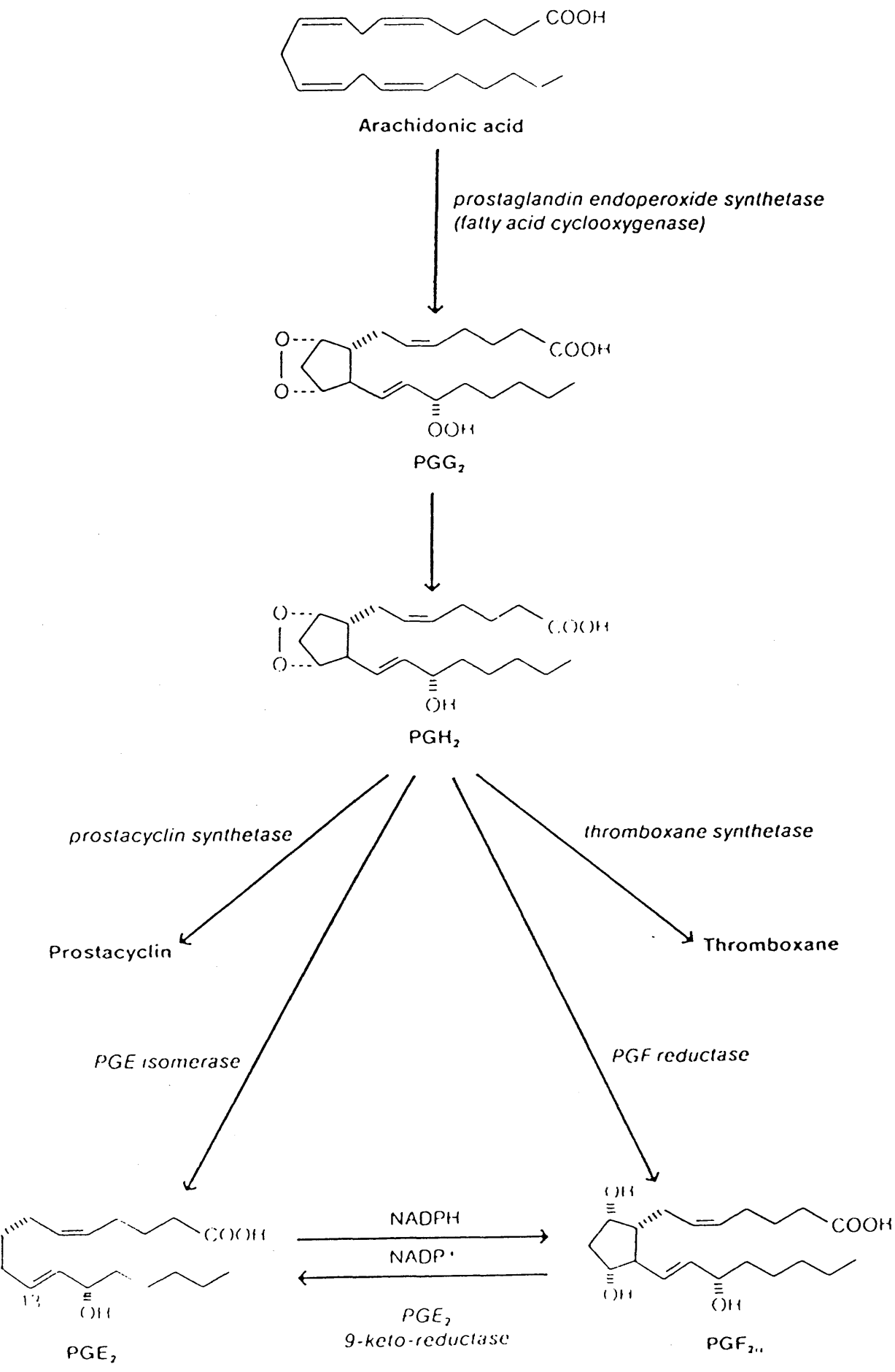
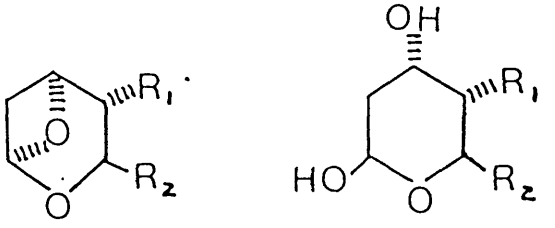


Figure 1.1 : Arachidonic acid metabolism

Two classes of thromboxane have been isolated and their structure is shown in Figure 3.



Thromboxane A Thromboxane B

Figure 1.3 - Structure of thromboxanes

1.3 Discovery of thromboxanes

Piper and Vane (1969) first demonstrated the release from guinea pig isolated perfused lungs of a previously unknown substance which potently contracted rabbit aortic strips and many other isolated vascular smooth muscle preparations. They first called it rabbit aorta contracting substance or RCS. This RCS was found to have a half life of less than two minutes at 37°C and its release was caused by a variety of stimuli including antigen challenge of sensitised lungs. The release could be blocked by aspirin-like drugs which suggested that RCS was from the same family as the prostaglandins. As the unstable prostaglandin endoperoxides also contract rabbit aorta it was first suggested that RCS was an endoperoxide. However its half life was shorter than that of the endoperoxides (about 5 minutes).

Furthermore the amounts of endoperoxide present in lung effluent were insufficient to account for the rabbit aorta contracting ability. It was found that RCS was released by aggregating platelets (Hamberg 1975) and this led to the discovery of its structure. Hamberg and colleagues (1975) isolated the highly unstable intermediate in the conversion of prostaglandin H_2 to thromboxane B_2 (TXB_2) and called it thromboxane A_2 (TXA_2). It has a half life of only about 30 seconds at $37^{o}C$ and is more active than PGH_2 in contracting rabbit aorta. It is the major constituent of the previously discovered RCS. Thromboxanes were so called because of their ability to produce thrombosis via aggregation of blood platelets. The structure of TXB_2 has been fully elucidated but the structure of TXA_2 is based only on chemical and mass spectral data.

1.4 Discovery of prostacyclin

Prostacyclin (PGI_2) was first described by Moncada and Vane (1976). The discovery arose from experiments designed to investigate the theory that damaged blood vessels might produce TXA_2 which could act synergistically with that produced by platelets. It was found however that when blood vessels were incubated with endoperoxides it was not TXA_2 that was produced but a substance with opposite properties. It caused inhibition of platelet aggregation and induced relaxation of vascular smooth muscle. This substance was initially called PGX before its structure was elucidated in collaboration with scientists from the Upjohn Company (Johnson 1976) when it was renamed prostacyclin.

PGI_2 is generated by blood vessel microsomes or fresh vascular tissue from the prostaglandin endoperoxide PGH_2 (Bunting 1976, Moncada 1976). Later it was shown that fresh vascular tissue could also utilise arachidonic acid as a substrate for PGI_2 production though the endoperoxides are much better substrates (Bunting 1976). It has been suggested that the vessel wall is able to synthesise PGI_2 from its own endogenous precursors or from prostaglandin endoperoxides released by the platelets. This would indicate biochemical co-operation between the platelets and the vessel wall (Gryglewski 1976). Tansik (1978) showed that lysed aortic smooth muscle cells could be supplied with prostaglandin endoperoxides by lysed human platelets. Marcus (1978) also showed that undisturbed monolayers of endothelial cells readily transform PGH_2 to PGI_2. Needleman (1978) however showed that perfused rabbit hearts rapidly converted arachidonic acid to PGI_2 but that PGH_2 was not readily transformed and they concluded that vascular damage was necessary for the endoperoxide to be utilised by PGI_2 synthetase.

CHAPTER 2 - SYNTHESIS, ACTION AND METABOLISM OF THROMBOXANE AND PROSTACYCLIN

2.1 Introduction

Prostaglandins are a family of oxygenated fatty acids synthesised by nearly every tissue in the body which have been detected in the venous effluent from numerous mammalian organs. Prostaglandin release can be stimulated by hormones, nerve stimulation, mechanical damage and decreased oxygen tension. Prostaglandins are thought to act as local hormones which are synthesised near to their site of action and then rapidly inactivated (autacoids).

Arachidonic acid, the precursor of all bis-enoic prostaglandins can be obtained direct from the diet or by anabolic desaturation and chain elongation from the dietary essential fatty acid, linoleic acid. In cell membranes arachidonic acid is incorporated as a structural component of phospholipids in all body tissues. The general structure of a phospholipid is shown in figure 2.1.

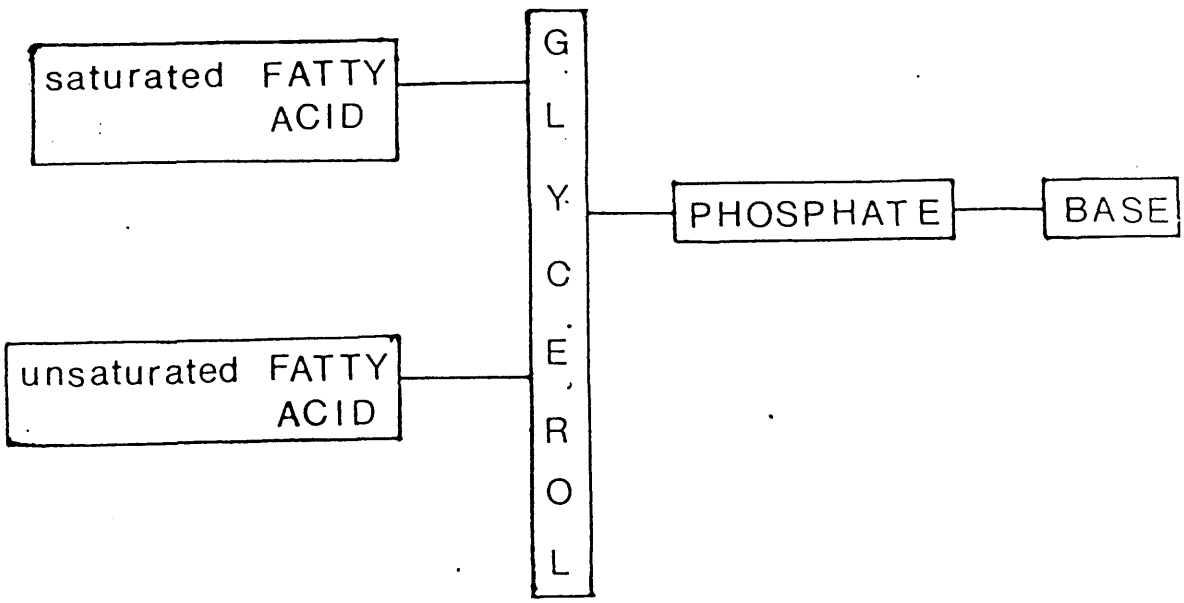


Figure 2.1 - General Structure of a Phospholipid

The three major membrane lipids are phospholipids, glycolipids and cholesterol. Phospholipids are derived either from glycerol or sphignosine, a more complex alcohol. Phospholipids which are derived from glycerol are more specifically called phosphoglycerides. There are a variety of phospholipases which act at different sites on the phospholipid. Phospholipase A_2 (PLA_2) catalyses the hydrolysis of the ester bond of the 2-acyl group, the source of the unsaturated fatty acid arachidonic acid. Cyclic AMP inhibits PLA_2 formation and it is stimulated by thrombin and collagen. Phospholipases can be activated by changes in the chemical environment or by mechanical stimulation. Figure 2 shows diagramatically arachidonate release and subsequent formation of oxygenated products.

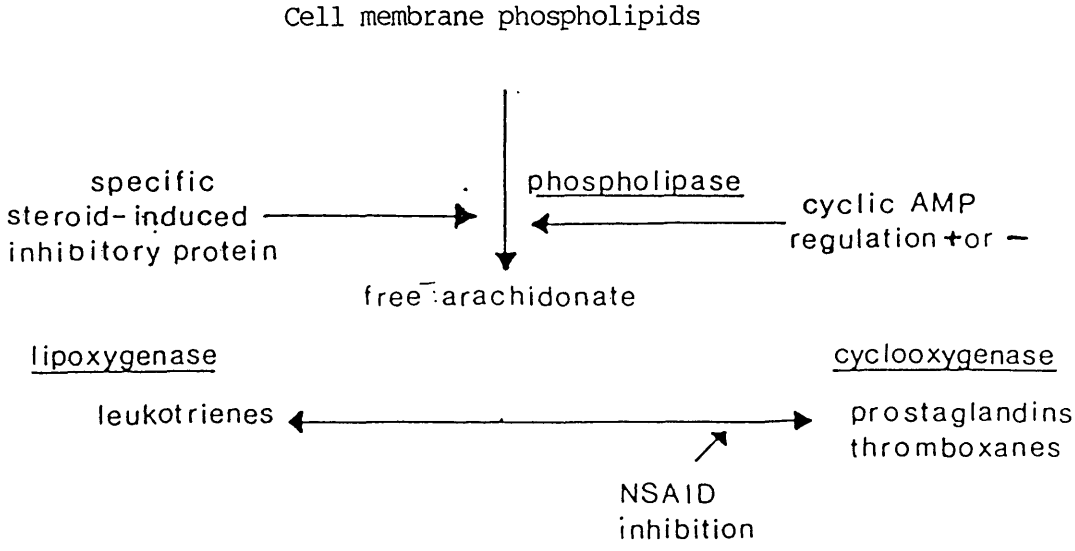


Figure 2.2 - Arachidonate release from membrane phospholipids and subsequent formation of oxygenated products

2.2 Essential Fatty Acids

Polyunsaturated fatty acids from which prostaglandins are derived were known to be essential to the diet before the discovery of prostaglandins and were thus termed essential fatty acids (EFA's). In the late nineteen twenties Burr and Burr (1929) found that rats which were fed on a diet devoid of the fatty acid fraction of fats developed a deficiency syndrome. This led them to conclude that the active principle was linoleic acid. Similar results were obtained with γ-linoleic acid and arachidonic acid. It has since been shown that the metabolism of arachidonic acid leads to prostaglandins of the 2-series in mammalian systems. The corresponding 1-series prostaglandins are derived from dihomo-γ-linoleic acid and the 3-series from 5, 8, 11, 14, 17 - eicosapentaenoic acid. So far as is known the 2 and 3 series prostaglandins are biosynthesised by analogous pathways to the arachidonic acid pathway. Arachidonic acid is stored in cells in various esters. The phospholipids of cell membranes contain large amounts of arachidonic acid as the phosphatidyl esters of choline, inositol, serine and ethanolamine. Release of arachidonic acid is calcium dependent and achieved by the action of specific phospholipases. Type I phospholipases are bound to cellular membranes and their enzymatic activity is related to the membrane stability. Type II enzymes are soluble and found in many mammalian cells. Interestingly these type II enzymes are similar to phospholipase A_2 enzymes found in the venoms of certain poisonous snakes and in bees and wasps. It may be that this leads to the release of vasodilatory prostaglandin which speeds up the release of the poison into the circulation.

PLA_2 has also been found in the lung, intestine, thyroid, seminal fluid and placenta. Certain activating and inhibitory factors are known to influence the activity of PLA_2 enzymes.

2.3 Cyclo-oxygenase Pathway

When arachidonic acid is released it activates both the cyclo-oxygenase and lipoxygenase enzymes. Cyclo-oxygenase, which is the prostaglandin synthetase, is really a group of enzymes which convert arachidonic acid or other 20-carbon unsaturated fatty acids into an endoperoxide configuration. These endoperoxides are the key precursor molecules for all subsequent prostaglandin and thromboxane production (see Figure 1.1).

The first step in endoperoxide formation involves the interaction of one molecule of arachidonic acid with two molecules of oxygen in the prostaglandin cyclo-oxygenase enzyme system to form the bicyclic endoperoxide PGG_2. Peroxidase activity results in the cleavage of the C-15 hydroperoxide to from the hydroxyl group present in PGH_2. The cyclo-oxygenase activity is still not fully understood but appears to catalyse the destruction of the enzyme itself. Nearly all tissues synthesise PGG_2 and PGH_2. There is still controversy as to whether the further conversion of PGH_2 to the so-called "primary prostaglandins": PGD_2, PGE_2 and PGF_2 is catalysed by an enzyme. It is believed that PGF_2 is a non-enzymatic product of the endoperoxides and might therefore be expected to be a constant component of any mixture of cyclo-oxygenase products especially where the enzyme pathways are blocked.

The actual mixture of products formed shows marked cell and tissue specificity. Different cell types in culture have different patterns of cyclo-oxygenase products. In some tissues such as kidney, uterus and lung different areas form different mixtures of cyclo-oxygenase products (Downing 1980, Schulman 1982, Sraer 1982). As well as cell and tissue specificity it has been shown that there are species differences. In rabbits and in humans the major cyclo-oxygenase product in the lungs is prostacyclin (Alabaster 1980), whereas in the guinea pig lung it is thromboxane A_2 (Uotila, 1980). This species specificity means that it is inappropriate to compare results obtained from animal models to the human situation.

2.4 Synthesis of Thromboxane

In most biological systems more than one prostaglandin together with thromboxane A_2 (TXA_2) is the normal mixture of cyclo-oxygenase products. Generally at higher substrate concentrations TXA_2 synthesis will increase disproportionately to prostacuclin (PGI_2) synthesis. The synthesis of TXA_2 from the endoperoxides is catalysed by a thromboxane synthetase enzyme which is found primarily in the microsomal fraction of virtually every cell type except the erythrocyte in mammalian species. TXA_2 differs in structure from the prostaglandins in that the cyclopentane ring is replaced by a six-membered oxane ring (see Figure 1.3). A variety of aggregating agents such as thrombin, collagen and epinephrine stimulate TXA_2 formation by platelets. The aggregating effects of low, but not of high concentrations of these aggregating agents can be blocked by cyclo-oxygenase inhibitors.

Work by Serneri and colleagues (1983) indicates that human arterial and venous vessel walls are able to synthesise TXA_2. They showed progressive generation of TXB_2, when measured by radioimmunoassay for up to forty minutes incubation time. They also showed that TXA_2 production was lower in arteries from women than those from men, which is the opposite from what has been found with PGI_2 production (Masotti 1981). It appears that TXA_2 is produced by the media and PGI_2 by the endothelial cells. More recent work by Orlandi (1985) using platelet free monocytes from human peripheral blood has shown that activated monocytes are capable of producing at least as much thromboxane as activated platelets, but it is not yet clear whether it is TXA_2 or TXB_2 which is produced. TXB_2 has also been shown to be synthesised by parenchymal cells prepared from diseased livers removed after partial hepatectomy but not by cells from normal livers (Spolarics 1984). This supports the theory that prostaglandins and thromboxanes may be involved in liver regeneration.

2.5 Action of Thromboxane

The short half life of TXA_2 and difficulty of preparation means that studies on its actions on whole animals are very limited. TXA_2 aggregates platelets, being more potent than PGG_2, and PGH_2 and it would seem likely that under normal circumstances *in vivo* these endoperoxides cause platelet aggregation through conversion to the more potent TXA_2 (Moncada 1979).

TXA_2 powerfully contracts guinea pig and human airway smooth muscle *in vitro* and bronchoconstriction following intravenous administration to the anaesthetised guinea pig has been shown (Svensson 1977). Guinea pig lung readily produces TXA_2 and it

is possible that TXA_2 mediates part of the bronchoconstriction of anaphylaxis in this species. It appears that human lung does not produce TXA_2 though human airways respond to it. It is therefore difficult to see how TXA_2 could contribute to asthmatic broncho-constriction although increased levels of TXB_2 have been reported during asthmatic attacks. TXA_2 has a weak contractile action relative to PGE_2 and PGF_2 and on gastrointestinal smooth muscle. It has also been reported to contract human and guinea pig, but not rat, uterine smooth muscle in vitro.

TXB_2 has been reported to have weak contractile activity on some isolated smooth muscle preparations but as it is very many times less potent than TXA_2 this is unlikely to be of any physiological significance.

2.6 Metabolism of Thromboxane

TXA_2 is very labile, having a half life of approximately thirty seconds in aqueous media at $37^{o}C$. The hydrolyses of TXA_2 occurs spontaneously but may be enzyme assisted in vivo. In man β-oxidation is the major metabolic pathway by which TXB_2 is degraded and 2,3 - dinor - TXB_2 is the major urinary metabolite (Roberts 1977).

2.7 Sites of Prostacyclin Synthesis

After the initial studies of PGI_2 synthesis with arterial and venous strips (Raz 1977, Johnson 1976) or with arterial microsomes prepared from homogenates of whole arterial walls (Bunting 1976), Moncada and colleagues examined differential formation by layers of rabbit aorta (Moncada 1977).

They found that although the intima comprised only 5% by weight of the rabbit aorta it produced 40% of the PGI_2, with the media coming next and adventitia last. Porcine (McIntyre 1978) and bovine (Weksler 1977) aortic endothelium and human umbillical vein endothelium (Weksler 1977) have all been shown to produce PGI_2. In most species tested arteries produced more than veins by weight of tissue (Kent 1981). Foetal tissue has been shown to be more active than adult tissue in the conversion of arachidonic acid to PGI_2 (Terragno 1978). The placenta is also capable of producing large amounts of PGI_2 (Myatt 1977). It has also been shown that cultured endothelial cells can produce more PGI_2 than smooth muscle cells (Boenziger 1977 and 1980).

2.8 Actions of Prostacyclin

2.8 (a) Action on Platelets

PGI_2 is the most potent known naturally occurring inhibitor of platelet aggregation being up to forty times more potent than PGE_1 and PGD_2 *in vitro*. Not only does PGI_2 inhibit platelet aggregation, but it can also disaggregate already aggregated platelets (Ubatuba, 1979). PGI_2 inhibits platelet activation at several metabolic steps: it elevates cyclic AMP thus activating a cyclic AMP dependent protein kinase that phosphorylates myosin kinase which in turn decreases the phosphorylation of myosin, reduces actin-myosin interaction and inhibits the release reaction. Aggregation of platelets by all known aggregating agents is inhibited by PGI_2 and secretion of platelet vasoactive mediators is also inhibited (Bunting 1981).

Ristocetin-mediated agglutination of washed, fixed platelets is not inhibited by PGI_2 whereas the ristocetin induced aggregation mediated by the release reaction is inhibited (Moake 1981). Inhibition of aggregation is dose dependent on the concentration of PGI_2. Patients receiving infusions of PGI_2 have prolonged bleeding times (Ubatuba, 1979), but although PGI_2 has a profound effect on platelet aggregation it has not been found to have any direct action on blood coagulation (Blasko, 1979). PGI_2 also prevents shape change in platelets (Ehrman 1980) but has less effect on platelet adherence to the subendothelium. Adhesion is only impaired at concentrations >100nM (Weiss 1979, Czervizoke 1978, Curwen 1980, Adelman 1981). However aggregation of platelets on a single adherent layer is readily inhibited by PGI_2.

2.8 (b) Vasodilation

With the exception of pig coronary artery, PGI_2 causes vasodilation and relaxes smooth muscle in all mammalian species tested. The degree of vasodilation and relaxation varies from species to species and with the vascular bed. When PGI_2 is administered to humans, flushing is often apparent within one minute of the start of the infusion and, in some subjects, may remain for some time after the infusion is stopped. PGI_2 has potent actions on the kidney, causing vasodilation, increased urine formation and electrolyte excretion (Baer 1979, Hill 1979). PGI_2 dilates the blood vessels of the stomach and is the major cyclo-oxygenase product of the gastric mucosa of several species (Gerkens 1978, Whittle 1980, Salvati 1981).

2.8 (c) Receptors

In 1905 Langley observed that cells contain a 'receptive substance' as well as the 'chief substance' concerned with secretion or contraction and that the receptive substance affects the metabolism of the chief substance and that not only neuromuscular drugs but also internal secretions probably act upon receptive substances (Langley 1905). A few years later Ehrlich proposed that highly specific side chains are present in the cell protoplasm and these chemically unique structures could serve as receptors with which chemotheraputic drugs combine (Ehrlich 1913). Since that time there has been great expansion of knowledge of receptor structure and function but these basic concepts have remained essentially unchanged. In 1956 it was discovered that 'spare' receptors are available for drugs and hormones (Stephenson 1956). It has been shown that [^{3}H] PGI_2 binds to a particulate fraction of platelets from various species including man (Siegl 1979, Miller 1979, Schillinger 1980).

PGI_2 receptors have also been demonstrated on red blood cells (Willems 1981). On platelets PGI_2 and PGE_1 occupy the same receptor site. 6-keto-prostaglandin F1α, the stable hydrolysis product of PGI_2, does not bind to the PGI_2 receptor site and therefore does not interfere with the binding of PGI_2 to platelets (Miller 1979). PGI_2 prevents the expression of fibrinogen receptors on the platelet surface (Hawiger 1980).

2.9 Metabolism of PGI_2

In biological fluids PGI_2 has a half life of only three minutes (Dusting 1978), being hydrolysed to the stable 6-keto-prostaglandin F1α (6-keto-PGF1α). This is the metabolite

which is most commonly measured as as index of PGI_2 production. In vitro PGI_2 is a substrate for the 15-hydroxyl-PG-dehydrogenase enzyme of lung tissue and blood vessels (McGuire 1978, Sun 1978) but 6-keto-PGF1α is a much poorer substrate (McGuire 1978), perhaps due to the hemiketal structure limiting accessibility for the enzyme. At the tissue level any PGI_2 generated may therefore be metabolised through a bicyclic 15-keto derivative and thus estimations of tissue PGI_2 output may be misleading if based on 6-keto-PGF1α measurements alone. Unlike the primary prostaglandins where greater than 90% is inactivated in one passage through the lungs (Ferreira 1967), PGI_2 suffers no loss in biological activity (Bolger 1978, Dusting 1978). Furthermore the lung has been found by some workers to produce PGI_2 and release it into the arterial circulation (Gryglewski 1978, Hensby 1979). This together with the lack of pulmonary inactivation has been used as evidence to support the theory that PGI_2 is a circulating hormone (Moncada 1978). Other investigators however have failed to obtain evidence of pulmonary production (Smith 1978, Pace-Asciak 1980). More recent work measuring urinary metabolites also suggests that PGI_2 is not a physiologically important circulating hormone in normal man (Fitzgerald 1981). However even if this is true it does not preclude an important role for PGI_2 in the local regulation of platelet-vessel wall interaction. In other organs such as the liver and kidneys metabolism of PGI_2 seems to be similar to that of the primary prostaglandins (Dusting 1978, Ferreira 1978).

Although PGI_2 is not metabolised enzymatically in the lungs, as shown in experiments with rats (Sun 1978), there may be substantial enzymatic metabolism in the blood vessels. Measurement of urinary metabolites in rats has shown that any PGI_2 enzymatically metabolised is rapidly hydrolysed to 6-keto-PGF1α even when infused over an extended period (Sun 1978). There has been less work done on human PGI_2 metabolism than in animals. However what has been done suggests that PGI_2 infused into the systemic circulation is not degraded by the 15-hydroxyl-PG-dehydrogenase initially, due to poor specificity for tissue uptake systems (Hawkins 1978). The suggestion from the rat experiments (Sun 1978) of substantial enzymatic metabolism in the blood vessels does not appear to be true in man. Using a radioimmunoassay to measure 13, 14-dihydro-6, 15-dioxo-PGF1α it would appear that little of this compound is produced during infusions of either PGI_2 or 6-keto-PGF1α (Myatt 1981). However during prolonged low dose infusions or from endogenous production, PGI_2 may be locally metabolised enzymatically before appearing in the circulation. In vivo this may be a major source of 13, 14-dihydro-6, 15-dioxo-PGF1α and therefore measurements of 6-keto-$PGF_1\alpha$ in the systemic circulation will not take this into account. The enzyme 9-hydroxyl PG dehydrogenase which converts PGF compounds into the corresponding PGE compounds has been identified in both rabbit and rat kidney (Hoult 1978, Pace-Asciak 1975). The contribution, if any, of this enzyme to the metabolism of PGI_2 in man is not yet known.

CHAPTER 3 - MEASUREMENT OF PROSTACLYCLIN AND ITS METABOLITES

3.1 Introduction

Since the discovery of PGI_2 workers have been trying to determine its role in various pathological conditions in man. In order to try to elucidate its contribution, ways must be found to determine whether or not PGI_2 is present in the body tissues or fluids in question and to try to measure its production. The general characteristics of the various methods which can be used will be discussed in this chapter and detailed descriptions of the methodology developed for this thesis will be given.

3.2 Bioassay

Any reproducible, dose dependent biological response can be used as the basis for a bioassay. As with any other assay, the relative usefulness of a bioassay depends on its sensitivity, specificity, reliability and simplicity. Although theoretically possible, *in vivo* bioassay techniques are now seldom used for ethical reasons. Originally bioassay was the method used for the detection of PGI_2 due to its potent inhibitory action on platelet aggregation and its ability to disaggregate already aggregated platelets. The method has contributed much to our knowledge of prostaglandins in general and made possible the original discovery of PGI_2. Bioassay has the ability to detect unexpected biologically active substances in an experimental situation which is rarely true of chemical methods. There has been much interest in the use of isolated preparations of arteries and veins because of their ability to differentiate between the various prostaglandins and thromboxanes. If the assay organ is carefully chosen a characteristic response to the test substance can be shown.

Although sensitive, bioassay methods can however never be absolutely specific and are difficult to use quantitatively. The method also has a low sample capacity. Bioassay is of use mainly when looking for the presence of biologically active compounds or when dealing with substances of an unknown nature.

3.3 Gas chromatography and mass spectometry

Techniques for the measurement of prostaglandins based on gas chromatography and mass spectometry (GCMS) were developed in order to provide methods with greater specificity. The basis of the method is the addition of a deuterated compound to a sample. After purification and derivatisation the ratio between deuterated and non-deuterated molecules is measured with the mass spectrometer. From knowing the amount of added deuterated compound the amount of unlabelled compound can easily be calculated. Since the deuterated molecules have physical properties almost identical to the unlabelled molecules they co-chromatograph during most purification systems and therefore the labelled molecules serve as carriers during purification and derivatisation and as internal standards during the final gas chromatographic mass spectometric analysis. The preparation of good carrier molecules is of vital importance.

A highly specific method for the measurement of 6-keto-PGF1α using negative-ion chemical ionization mass spectrometry has been developed (Barrow 1982) and has been used to measure 6-keto-PGF1α concentrations in human plasma (Blair 1982). Using this method they found normal concentrations to be in the range 0.5 - 2.5 pg/ml suggesting that PGI_2 is not a circulating hormone in man under normal conditions, but a locally acting substance released in response to local stimuli (Ritter 1983).

However it has been shown that only a proportion of PGI_2 metabolites are 6-keto-PGF1α (Rozencranz 1981) and therefore GCMS may not be the best method when examining variations in PGI_2 production rather than absolute levels of 6-keto-PGF1α. Although the use of sophisticated techniques such as GCMS often raise the confidence in a particular result, care must be taken in the interpretation of results. The method is also susceptible to methodological problems. For example the electron impact ionisation is dependent on the gas phase collision between the molecule being analysed and a high energy electron. During the ionisation process, excess internal energy is imparted to the molecule. The energy is dissipated by the fragmentation reactions which occur in the source of the mass spectrometer. This can cause loss of sensitivity or specificity in the anlysis of prostaglandins (Blair 1983). Also the gas chromatography method of analysis generally uses prostaglandin analogues as internal standards and this can also lead to problems with specificity. However in many laboratories perhaps the biggest problem with this method of analysis is the availability of equipment. Highly sophisticated and expensive equipment is required which is not always available. The method also has a low sample capacity and requires a fairly large initial sample volume in comparison to other methods.

3.4 Radioimmunoassay - Introduction

Radioimmunoassay (R.I.A) has advantages over other quantitative methods : it is possible to obtain high sensitivity with very low limits of detection; specificity may be considerable;

large numbers of samples can be assayed within a reasonably short period of time; the precision and accuracy compare favourably with other methods and, apart from a radioactivity counter, no sophisticated laboratory equipment is necessary. As our interest was in trying to determine the role of PGI_2 and thromboxane in various pathologic conditions and this involved measurement of levels of these substances in large numbers of samples, we put considerable effort into the development of a specific, sensitive and reproducible RIA for the measurement of PGI_2 metabolites.

3.5 History of Radioimmunoassay

Radioimmunoassay may be said to have developed as a by-product in the development of nuclear weapons. With many radioactive isotopes the energy of their emission is so great that it is possible to detect a few atoms with relatively simple equipment. If such atoms are attached to another molecule then it can also be detected in equally small amounts. In the mid 1950's Yalow and Berson were studying ^{131}I-labelled insulin and showed that insulin requiring diabetics almost always have a circulating insulin-binding protein (Berson et al 1956). At the same time as demonstrating the binding of ^{131}I insulin by this binding protein they also showed that the labelled tracer could be displaced from the binding sites on the antibody by the addition of large amounts of unlabelled insulin. They then showed that the degree of binding of the tracer was quantitatively related to the amount of unlabelled insulin present. This observation led to the first radioimmunoassay for insulin (Yalow & Berson 1971) after the development of a specific antisera to human insulin by the immunisation of guinea-pigs (Yalow & Berson 1960).

Originally iodination involved handling large amounts of radioactive isotopes and could therefore only be carried out by highly specialised laboratories. The development of the chloramine T iodination procedure by Greenwood et al (1963) was one of the most important in the advancement of RIA to the stage where it is at today with numerous commercial kits available for the measurement of many different compounds.

3.6 Principle of Radioimmunoassay

RIA is based on the competition between labelled and unlabelled molecules of a particular compound for binding sites on an antibody directed against that compound as shown in figure 3.1.

The amount of labelled compound is known and constant in all the tubes but the amount of unlabelled compound is known and varied to obtain a standard curve. The sample tubes contain constant volumes with unknown amounts of the compound in question, sample values can later be extrapolated from the standard curve. Each assay must also have tubes with no antibody present to give non-specific binding (NSB) and also tubes with no unlabelled compound to give maximum binding. In the presence of few unlabelled molecules a large proportion of the labelled ones are bound to the antibody. As the amount of unlabelled molecules increase the amount of labelled molecules bound decreases quantitatively. The situation can be described by the law of mass action which states that : at equilibrium, the ratio of the products of the concentrations on the two sides of the equation will be a constant K.

$$\frac{[Ag\ Ab]}{[Ag]\ [Ab]} = K$$

Where [Ag], [Ab] and [Ag Ab] are the concentrations of the free antigen, free antibody and antigen-antibody complex respectively. The k value for any system provides a measure of the energy of the reaction between the antigen and antibody. Given an unvarying quantity of antibody of fixed k value, the ratio of bound to free ligand at equilibrium will be quantitatively related to the total amount of ligand present. This is the basic principle of all radioimmunoassays.

3.7 Development of a Radioimmunoassay for Prostacyclin Metabolites

3.7 (a) Problems

Because of the short half life of PGI_2 most workers have attempted to measure 6-keto-PGF1α the stable hydrolysis product when looking for an index of PGI_2 production. In the literature there are vast differences in the published 'normal' plasma levels. Even when examining results obtained with GCMS assays, which is considered to be highly specific, results range from 200 ng/l (Hensby 1979) to as little as 3ng/l (Blair 1982). When results of assays using RIA are compared there are still large differences in 'normal' plasma levels as is shown in Table 3.1.

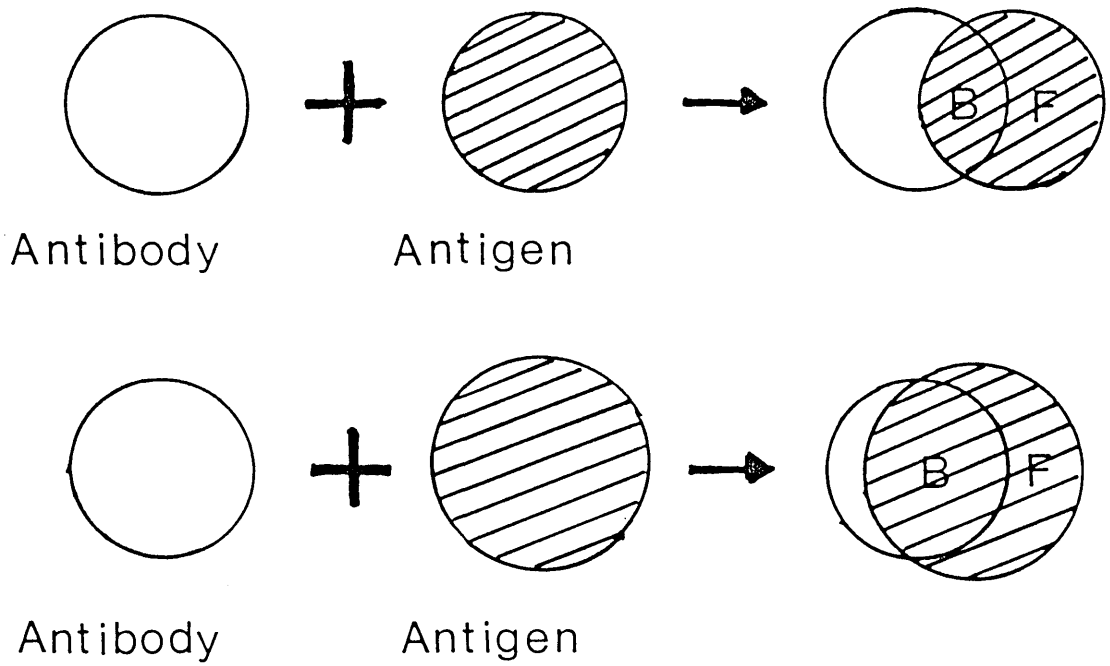


FIGURE 3.1 : The basic principle of immunoassay. If given amounts of antigen and antibody are allowed to react together (above) then at equilibrium they will form an antigen - antibody complex (the overlapping area B) together with a proportion of both the antibody and the antigen (F) which remain free. If the amount of antibody is held constant while the total amount of antigen is increased (below) then at equilibrium the amount of antigen-antibody comples (B) is increased; however the increase in the free fraction (F) is relatively greater and thus yields a lower bound to free ratio.

6-keto-PGF1 levels (pg/ml)	Authors	
115 ± 24	Mitchell	1978
123 ± 22	Lewis	1980
690 ± 386	Kinney	1981
69.6 ± 11.6	Gullner	1982
93 ± 42.3	Ylikorkala	1982
4.7 ± 3.2	Seiss	1982
7.5	Patrono	1982
64 ± 14	Roy	1983
13.7 ± 1.8	Gotoh	1983
270 ± 14	Uehara	1983
36 ± 4	Brandt	1984
27 ± 3	Swartz	1985

Table 3.1 : Range of published 'normal' levels of plasma 6-keto-PGF1α

3.7 (a) (i) Extraction Procedures

In radioimmunoassay, samples may often require to be extracted before the performance of the assay. There are two reasons for extraction : firstly to improve the sensitivity, the endogenous ligand may be concentrated, and secondly, if the assay is not sufficiently specific, the endogenous ligand must be separated from other materials. Extraction procedures can be divided into two common types : those in which the ligand in the sample is absorbed to particulate material and is thus easily separated from unadsorbed components; and those in which the sample is treated with an immiscible organic solvent which extracts the ligand.

Absorption methods are normally used where the ligand must be separated from other components in the sample. The majority of methods described for the assay of plasma 6-keto-PGF1α involve an extraction step prior to RIA. However it has been shown by Greaves et al (1982) that organic extraction and to a lesser extent, silicic acid chromotagraphy may themselves introduce artefact. Measurable levels of 6-keto-PGF1α were found in a sample of distilled water which was subjected to the same extraction procedures as used on plasma samples.

They found that this blank value could be reduced by dilution of the sample but the curve obtained did not match the standard curve for the assay. Thus any attempt to "correct" for the blank by simply subtracting it from the sample is completely invalid.

Unless extensive purification is carried out after extraction these procedure blanks are almost invariably seen (Granström 1978). Greaves (1982) found that three separate purification steps were necessary after extraction before the blank value was removed. Therefore if absolute values of 6-keto-PFG1α are to be measured it is vital that this extensive purification is performed on every sample prior to assay and that a procedure blank is always included. This is impracticable where large numbers of samples are to be assayed, which is one of the big advantages of RIA. However it has been shown that only about 40% of PGI_2 is metabolised to 6-keto-PGF1α (Myatt 1981) and little work has been published on the remaining 60% of metabolites such as 13,14-dihydro-6,15 diketo prostaglandin F1α. It is now generally accepted that to measure only one metabolite may give a false picture as for example with PGE_2. The term "prostacyclin

metabolite assay" may therefore be more accurate when using a polyclonal antibody. The term "metabolite assay" is routinely used in the measurement of other prostaglandins such as PGE_2. We therefore decided to attempt to develop a method for a radioimmunoassay on unextracted plasma. Work published by Forder (1983) confirmed that direct assay methods could be of use provided only the linear portion of the standard displacement curve was used for measurement.

3.7 (a) (ii) Sensitivity

Although fairly high levels of plasma 6-keto-PGF1α are still being published it is now generally accepted that normal levels are in the low picogram range. Thus a very sensitive assay must be developed in order to be able to measure these very low levels. As will be shown in a later section of this chapter some of the available commerical kits are not sufficiently sensitive for measuring plasma levels.

3.7 (b) Method of Sampling

It has been shown by Morris (1981) that variables in sampling procedure and preparation of samples can affect the results obtained from prostaglandin assays. A traumatic venepuncture may cause increased levels due to endothelial cell damage. It is now thought that white cells can produce PGI_2 (Orlandi 1985) and thus it is important that further *in vitro* generation of PGI_2 after sampling is abolished. In our laboratory venous blood is collected using a 19 gauge butterfly needle. Blood is immediately put into cold plastic tubes containing 1:9 vol 3.8% trisodium citrate containing 3×10^{-5}M indomethacin added to prevent *in vitro* generation of PGI_2 and thromboxane.

The tubes are kept in ice and centrifuged within one hour of sampling at $4^{o}C$ and 2500g for fifteen minutes. The plasma is separated and stored in aliquots at $-70^{o}C$ until assay.

3.7 (c) Buffer

A wide range of buffers are used in RIA. The choice of buffer has been shown to make no difference to the assay provided it is within one unit of neutrality and has a molarity in the range 0.01 - 0.1 (Chard 1981).

Care must also be taken to ensure that the buffer does not become contaminated by the ligand or by tracer. It is very easy to cause buffer contamination with pipette tips or other laboratory equipment and because levels being measured in this particular assay are so low it is easy to turn the buffer into a standard solution. Protein is commonly added to help to stabilise the buffer and also to prevent absorption losses on to the surfaces of tubes, pipette tips or other equipment used in the assay. We use a 0.1M phosphate buffer PH 7.0 containing 0.1% bovine albumin.

3.7 (d) Standards

The preparation of the standards for any assay is one of the most important steps in ensuring the reliability of the results. The ideal situation for any assay is to have a single standard for distribution to all laboratories concerned with a particular assay. The National Institute for Biological Standards is the principle agency in the United Kingdom for this purpose. However to the present time no such international standard is available for 6-keto-PGF1α and each laboratory must prepare their own standards.

The usual procedure for setting up a standard is to obtain a sufficiently large quantity of the substance in question and to dispense identical volumes of a known solution in several hundred tubes which can then be stored at low temperatures. Ideally each individual standard should be prepared independently from this stock solution rather than performing doubling dilutions where any error in an initial step will affect all subsequent steps. However in the case of 6-keto-PGF1α where standards must be of such low concentrations practicality demands the use of doubling dilutions. Unless very large quantities of the original standard were available the preparation of individual standards would necessitate the measurement of such small volumes that the chance of error would be greater than with the doubling dilution method. However it is important that great care is taken when preparing each set of standards and appropriate controls are included in every assay as a check. We obtained a standard from Mr Simon Thomas, Medical Sciences Liaison Officer for the Upjohn Company. This standard was originally diluted in buffer to give a solution of lng/ml, aliquots of which were stored at $-70^{o}C$. Working dilutions as set out in the results section are prepared each week and these are stored at $-4^{o}C$. A zero standard of buffer alone is stored under the same conditions as the other standards. As a control, a pool of normal plasma is collected in the same way as sample plasmas and aliquots are stored for up to six months and included with every assay.

3.7 (e) Labelled Ligand

In any binding assay there must be some means of determining the final distribution between the bound and free fractions.

In a radioimmunoassay this 'tracer' is a radioactive isotope. There are two types of tracer : those which have an existing atom in the ligand molecule replaced by a radioactive isotope of that atom for example ^{3}H for ^{1}H; and those with an external label where an atom or atoms of a radioactive isotope such as ^{125}I are substituted for an existing atom on the ligand molecule. This type of external label is thus not identical to the unlabelled ligand, though in practice its behaviour should be undistinguishable from the unlabelled molecule. Tracers with an internal label are usually prepared on a commercial scale. Tritium labelled 6-keto-PGF1α (^{3}H-6-keto-PGF1α) was obtained from Amersham International and diluted with buffer as required.

3.7 (f) Antibody

Probably the most important component of any RIA is the antibody or binder which is used. The specificity of the antibody dictates how reliable the assay will be. The affinity of the antibody for the antigen is also important since this determines the energy of the reaction and the time taken to reach equilibrium. The production of an antibody of the required specificity with a high affinity or K value is a time consuming and problematical process. We were fortunate enough to receive a supply of antibody to 6-keto-PGF1α from Dr Eric Preston, Department of Haematology, Royal Hallamshire Hospital, Sheffield. We also compared results using this antibody with results using other antibodies and this will be discussed later in chapter 3. Table 3.2 shows a list of the cross reactivities of the Sheffield antibody. With prostaglandin assays, where there are so many closely related compounds, it is very important to the

specificity of the assay that any cross reactivity is as low as possible. Table 3.2 shows the cross reactivities of the Sheffield antibody.

Compound	% cross reactivity
6-keto-PGF 1α	100.0
13,14-dihydro-6-keto-PGF 1α	1.1
$PGF_1\alpha$	0.35
PGD_2	0.25
$PGF_2\alpha$	0.20
PGE_1	0.075
13,14-dihydro-6,15-diketo-PGF1α	0.075
6,15-diketo-PGF 1α	0.075
13,14-dihydro-PGF 2α	0.020
PGA_2	0.015
PGA_1	0.004
Thromboxane B_2	0.004
13,14-dihydro-PGE_1	0.004
PGB_1	0.002
13,14-dihydro-PGE_2	0.001
13,14-dihydro-15-keto-$PGF_2\alpha$	0.001
13,14-dihydro-15-keto-PGE_1	0.001
Arachidonic acid	0.001

Table 3.2 - Cross reactivities with available prostaglandins

3.7 (g) Separation Method

When the assay has reached equilibrium it is necessary to determine the distribution of the radioactive ligand between the bound and free fractions. A variety of techniques have been

described for this purpose. The principal criteria for a separation procedure are efficiency and practicality. For example electrophoresis (Hunter 1964) and gel column filtration (Haber 1965) have been described as techniques for separating bound and free fractions. However these techniques are very time consuming, especially where large numbers of samples are being assayed and are therefore impracticable and rarely used nowadays. We tried fractional precipitation using polyethylene glycol. In terms of practicality this method is simple, fast and inexpensive. The polyethylene glycol is added at a concentration in which the bound fraction is insoluble and therefore precipitates while the free fraction remains in solution. The precipitate is packed by centrifugation and the radioactivity of the supernatant (free fraction) can be counted. However we found that this method yielded a very high assay blank of about 15% on average and reproducibility was poor. This may be partly due to physical trapping of the tracer in the interstices of the bulky precipitate.

The double antibody technique of separation is now widely used especially where the second antibody is coupled to an insoluble matrix such as cellulose. The technique of double antibody separation methods was first described by Utiger (1962) and Morgan (1963). The second antibody is specific to the globulin of the species in which the first antibody was raised. This causes precipitation of the bound fraction which can once again be packed by centrifugation. We used "Sac-cel" cellulose coupled anti-rabbit antibody made by Wellcome Reagents, Beckenham, Kent. We were unable to obtain satisfactory separation using this method. The non-specific absorption of biological molecules to

particle surfaces is widely used as a separation method. Figure 3.2 shows diagramatically the principle of the method. The most commonly used absorption material is charcoal which was first described by Herbert (1965). Originally it was recommended that the charcoal be pretreated with dextran to block any larger pores which might absorb the binder. However it has since been shown that this is unnecessary (Binoux 1973). By using free ligand only in the concentration used in our assay we showed that 300 µl of a 0.5% suspension of acid-washed charcoal (BDH) was sufficient to allow complete separation. It is important that the separation is performed using ice-cold charcoal and with the assay tubes in melting ice. This is important if absorption of the bound complex is to be avoided.

For the same reason the time taken to perform the addition, mixing and centrifugation of tubes must be standardised. In our method the tubes are vortex mixed and centrifuged at exactly 9±1 minute from the addition of charcoal to the first tube. The tubes are centrifuged at $4^{o}C$ for 10 minutes at 2500g. The supernatant is decanted into 10 mls scintillant (Packard Insta-Gel) and counted in a Packard scintillation counter (Model 460, Packard Inc, USA).

3.7 (h) Performance of the Radioimmunoassay

Originally we set up an assay using the antibody at the recommended final dilution of 1:10,000. Using labelled ligand to give approximately 10000 cpm total counts and incubating the mixture for 18-20 hours at $4^{o}C$ we were only able to achieve a detection limit of 50 pg/ml. This did not bring our standard plasma within the limit of sensitivity of the assay.

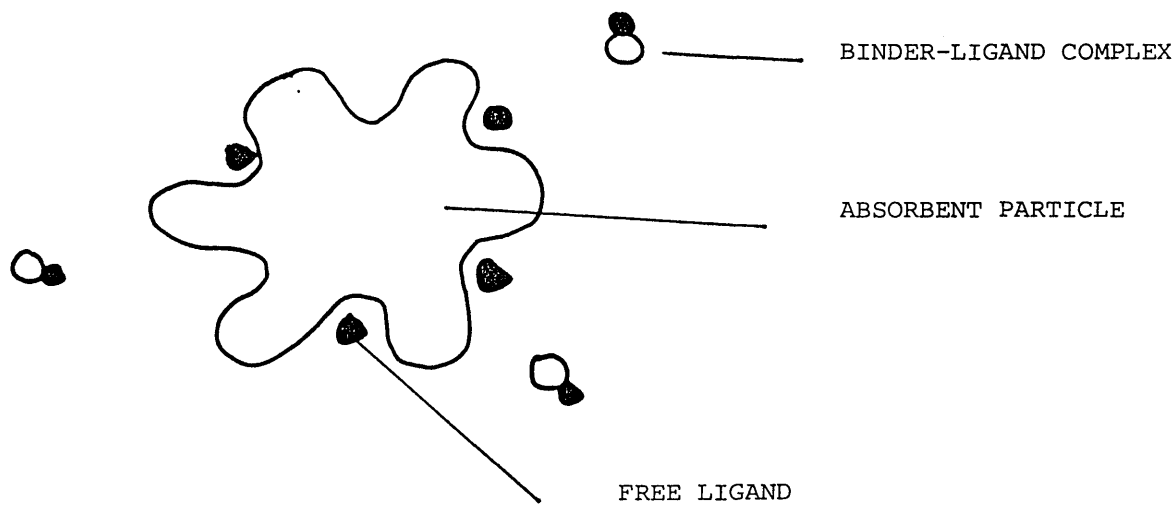


FIGURE 3.2 Separation by the use of an absorbent. Free ligand can enter 'crevices' on the particle and is firmly absorbed. Bound ligand cannot enter the crevices and thus remains in the liquid phase.

The most common method of increasing the sensitivity of an assay is to reduce the amount of tracer (Chard 1981). It is however wrong to assume that sensitivity can be increased indefinitely simply by reducing the amount of tracer. There is always a limiting concentration below which there is no significant increase in sensitivity. We set up a series of standard curves with standards varying from 2.0 pg/ml up to 1000 pg/ml together with a sample of our standard pool plasma. We also added known amounts of 6-keto-PGF1α and varied the amount of tracer used in each. The results are shown in Table 3.3.

Amount of Tracer cpm	Incubation time & temp	STD plasma (pg/ml)	% recovery	Detn. limit (pg/ml)
10,000	Overnight 4°C	<50	68	50
7,000	"	<50	93	50
5,000	"	<50	105	50
4,000	"	<30	88	30
3,000	"	<60	84	60

Table 3.3 - Results obtained when decreasing amounts of tracer were used

As can be seen we were unable to bring our standard plasma within the limits of sensitivity simply by reducing the amount of tracer used.

The use RIA's which are interrupted by separation of bound and free before equilibrium is reached is becoming more common with commercial kits. This can often appear to increase the sensitivity but may only be because the zero binding is reduced. We tried various incubation times and temperatures, but, as can be seen in Table 3.4 we were still unable to bring our standard plasma within the limits of detection of our assay.

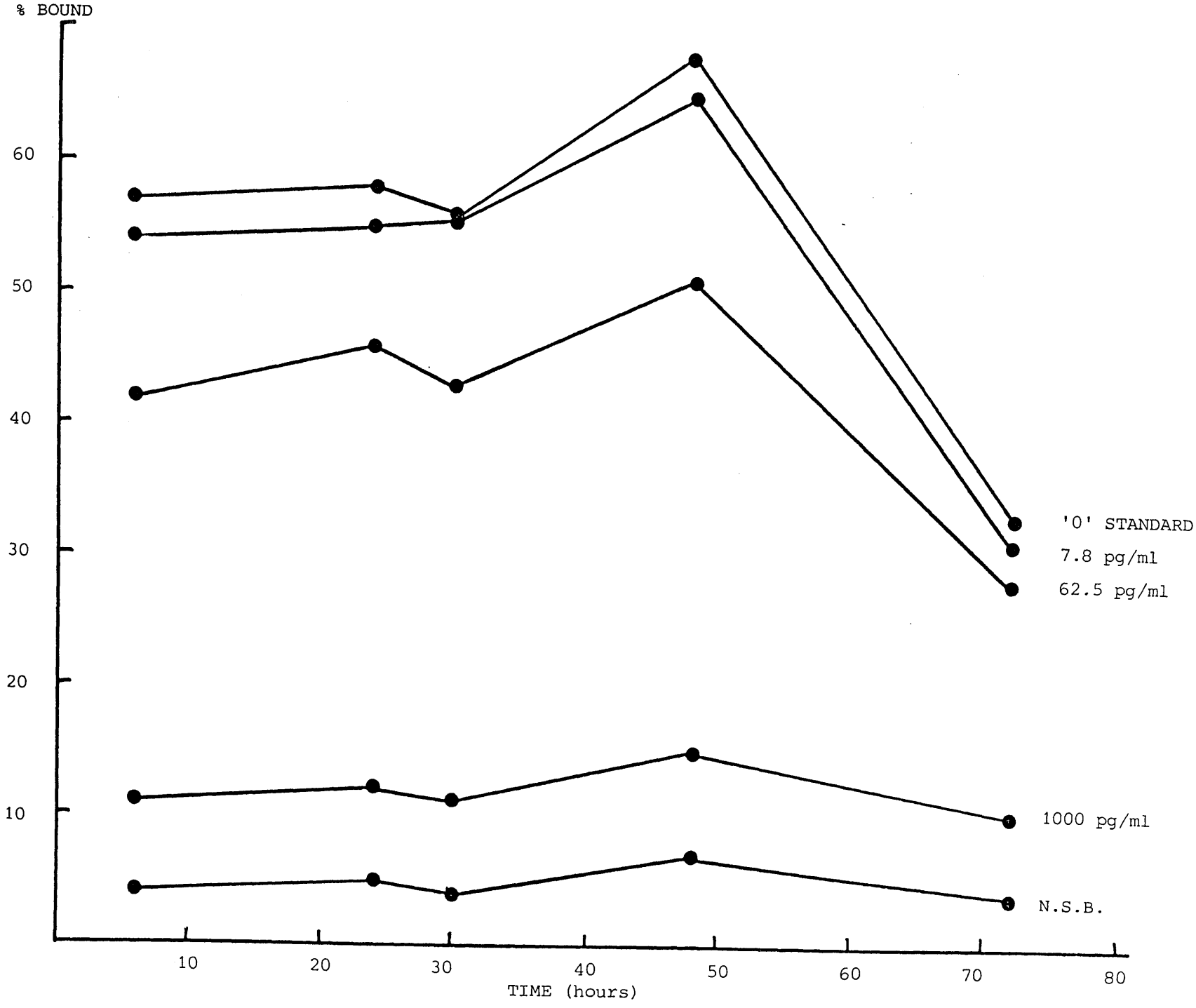
Amount of Tracer cpm	Incubation time & temp	STD plasma (pg/ml)	% recovery	Detn. limit (pg/ml)
10,000	2 hours 4°C	20	70	20
8,000	"	50	75	50
6,000	"	50	88	50
3,000	"	40	80	40
6,000	1 hour 37°C	30	42	30
3,000	"	30	60	30

Table 3.4 - Results obtained with various incubation times and temperatures

Normally reagents are added in the order sample - tracer - binder thus giving both labelled and unlabelled ligand equal access to the binding site. However it has been shown by complicated mathematical analysis (Samols 1963) that if the order is changed to sample - binder - tracer and if the addition of the tracer is delayed, sensitivity may be increased. However we were unable to increase the sensitivity of our assay using this 'late-addition' method.

It has been shown that reducing the amount of binder used can increase the sensitivity of an assay (Chard 1981). There must however be a balance between the apparent increase in sensitivity and the confidence limits of replicates. Where the zero binding falls below 20% any apparent increase in sensitivity is negated by the decrease in precision. We reduced the final concentration of our antibody to 1:35000 using tracer of 2-3000 cpm. By increasing our incubation time we were able to increase our sensitivity to 10 pg/ml thus bringing our standard plasma within the limits of sensitivity of the assay. It can be seen from figure 3.3 that by increasing the incubation time to 48 hours in order to allow our assay to reach equilibrium we were able to obtain zero binding of greater than 60%.

FIGURE 3.3 PGI_2 METABOLITE ASSAY - TIME TAKEN TO REACH EQUILIBRIUM



The method used therefore is to mix 200µl of standard or sample, 100µl tracer to give approx 2-3000cpm and 50µl antibody giving a final dilution of 1:35000. The mixture is incubated for 48 hours at 4°C and then separation of bound and free is achieved using charcoal as described. The exact protocol for the assay is shown in Table 3.5. Standards of 2, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 pg/ml are used.

Tube	Total Count	Non specific binding Buffer	Non specific binding Plasma	Stds 0-1000 pg/ml	Std plasma	Sample
Buffer	250	250	50	-	-	-
Standard Plasma	-	-	200	-	200	-
Standard or sample	-	-	-	200	-	200
3H PGF1	100	100	100	100	100	100
Antibody	-	-	-	50	50	50
	Mix and incubate for 48 hours at 4°C					
Buffer	300ul	-	-	-	-	-
Charcoal Suspension	-	300	300	300	300	300

Spin at exactly 9 ± 1 minute after start of charcoal addition and centrifuge at 4°C and 2500 g for 10 minutes. Decant supernatant into 10 ml scintillant and count.

Table 3.5 - Protocol for RIA of PGI_2 metabolites (all volumes in µl)

3.7 (i) Results

Figure 3.4 shows the standard curves obtained over ten assays using the method described. The horizontal axis shows the concentration of 6-keto-PGF1α used in the standards and the vertical axis the percentage binding with the zero binding taken as 100%.

Taking the limit of sensitivitiy as being two standard deviations from zero binding the lower limit of sensitivity for the assay is 10 pg/ml. It should be noted however that this is the average figure for sensitivity. The minimal detection limit of any radioimmunoassay is likely to vary from one assay to another and also from one operator to another. If the replicates for the zero standard are poor the lower limit of sensitivity may be greatly increased. Conversely the sensitivity will increase with greater precision.

Over ten assays the inter and intra assay variation for the method is 4.9 and 4% respectively.

The mean recovery over ten assays of added known amounts of 6-keto-PGF1α is 95 $\pm$ 9% (SD). The normal range for the assay is 10 to 22 pg/ml (x = 13.1 pg/ml).

3.7 (j) Validation of Assay

In order to validate our assay by showing that we are measuring PGI_2 metabolites, we allowed known quantities of PGI_2 (Wellcome Laboratories) to hydrolyse at 37^{o}C for 30 minutes in phosphate buffer pH 7.0. Figure 3.5 shows the curves obtained. The curves show parallelism over the linear portion of the curve showing that the difference between the two materials is solely in their affinity for the antibody.

Figure 3.4 PROSTACYCLIN METABOLITE ASSAY Standard curve over ten assays using ^{3}H tracer

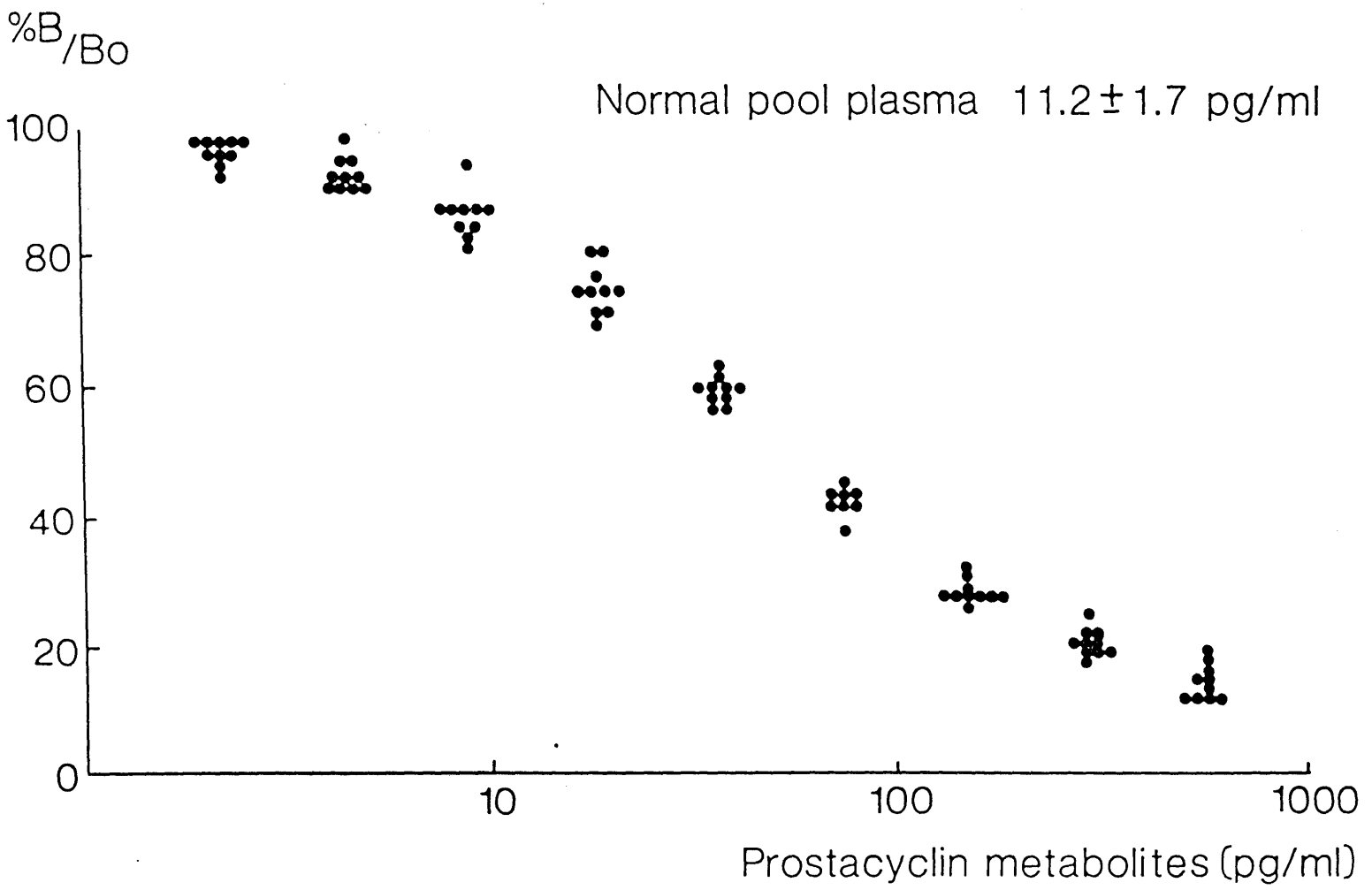
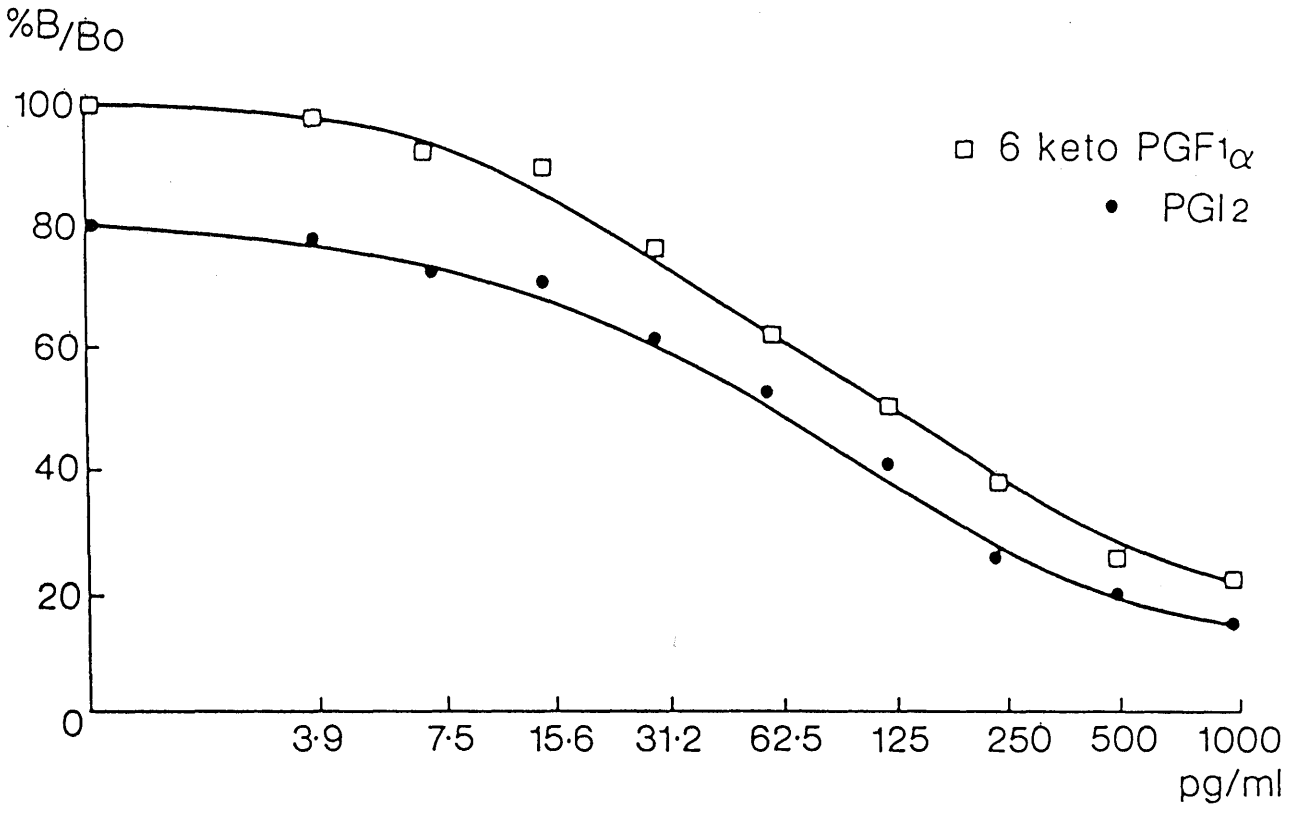


Figure 3.5 6 Keto $PGF1_{\alpha}$ levels compared to levels obtained for same quantities of hydrolised PGI2



Parallelism should also be demonstrated by diluting an unknown sample several times and measuring these dilutions against the standard curve. After multiplication by the dilution factor all dilutions should give a similar value.

However due to the low normal levels of PGI_2 metabolites in plasma this is not usually possible. However this has been done with plasma from a patient receiving an infusion of PGI_2 and the results obtained are shown in Table 3.6.

Dilution	PGI_2 metabolite value (pg/ml)
Undiluted	170
1/2	81
1/4	38

Table 3.6 - PGI_2 metabolite levels on diluted sample

We also have validated the assay by adding known amounts of 6-keto-PGF1α to our standard pool plasma. As already shown in the results section 3.7(i), the mean recovery was 95 $\pm$ 9% (SD).

As another means of validating our assay we gave an infusion of PGI_2 to a normal volunteer. Blood was sampled by separate venepunctures at each of the times shown. The samples were processed as previously described and the plasma assayed. Results obtained are shown in Table 3.7.

time	Dose of PGI_2 (µg)	PGI_2 metabolites (pg/ml)
0	0	5
15	4	50
30	8	70
45	12	139
60	16	220
15 min post-infusion	0	5

Table 3.7 - Metabolite concentrations during infusions of PGI_2

This shows that as the dose of PGI_2 was increased the levels of plasma PGI_2 metabolites increased correspondingly.

3.8 Comparison of ^{3}H-6-Keto-PGF1α and ^{125}I 6-keto-PGF1α

Higher specific activity can be obtained with an iodinated label compared to a tritiated label. We were able to obtain a commercial kit (N.E.N.) which used an iodinated label for the measurement of 6-keto-PGF1α . Initially we used the protocol described for the kit and, as with our initial assays, found that the limit of sensitivity of the assay did not bring our standard plasma within measurable limits. However we modified the method by using half the amount of antibody - 50ul instead of 100ul per tube and half the amount of label. As with our own method we increased the incubation time to 48 hours. As can be seen in figure 3.6 we were able to obtain a more sensitive assay, with slightly better sensitivity than when using tritiated tracer. This however is the result of only one assay using this iodinated tracer, and as can be seen from figure 3.6 the assay was not much more sensitive than our own method. Unfortunately because of the very high cost of the kit we were unable to perform any validation experiments with it. For the same reason the sensitivity of the method was based on only one assay and may have improved on subsequent occasions. However if any assay is to be viable in a clinical situation cost must be a prime consideration. Any increase in sensitivity would not be worth the considerably higher cost per sample.

3.9 Results using different antibodies

Viinikka and Ylikorkala have shown that 6-keto-PGF1α assays performed with two antibodies of apparently similar specificity gave different results (Viinikka 1982). This may have been due to some so far undiscovered closely related compound cross reacting with only one of these antibodies.

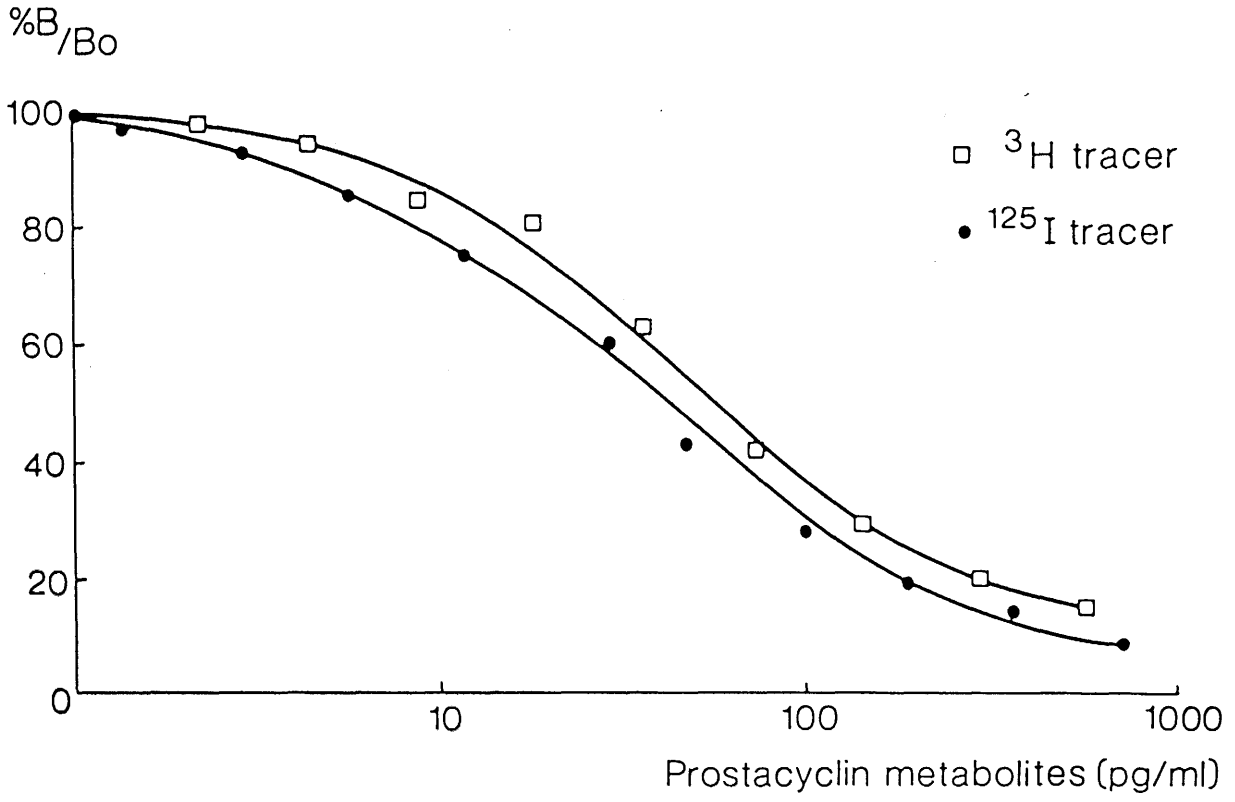


Figure 3.6 : Standard curves obtained using iodinated and trititated labels

We tested antibodies from several different sources. We used our own methodology with these antibodies all of which had very similar cross reactivities. Results are shown in Table 3.8.

Source of Antibody	Tracer	Standard plasma (pg/ml)
Walker Laboratories	^{125}I	12
New England Nuclear	"	11.5
Upjohn Company	3H	10
Bioanalysis	"	9
Walker Laboratories	"	8

Table 3.8 - Results using different antibodies

As can be seen the results varied little from the value of 11.2 $\pm$ 1.7 pg/ml for the standard pool plasma using our original antibody. As all these figures are the results from only one assay they cannot be compared exactly with our own value which was the mean of ten assays. However there is no evidence of any significant difference as has been observed by Viinikka.

3.10 Prostacyclin Stimulating Factor

3.10 (a) Introduction

PGI_2 is the most potent inhibitor of platelet aggregation yet discovered. Its production by the vascular endothelium is thought to be one mechanism by which the vessel wall maintains a patency (Moncada 1976, Gryglewski 1976). *In vitro* cultures of vascular endothelial cells synthesise and release PGI_2 which can then be measured by RIA (Weksler 1977, McIntyre 1978). Several researchers have reported the existence of factors in plasma which might regulate the production of PGI_2 by the vascular endothelium (McIntyre 1978, Remuzzi 1979 a, Defreyn 1980).

A marked decrease in this stimulation factor has been found in the haemolytic ureamic syndrome (Remuzzi 1978 b); immediately after actue myocardial infarction (Yui 1984) and in sickle cell anaemia (Stuart 1981). Much of the work done trying to measure this PGI_2 stimulating factor has used rat aortic rings which are exhausted of spontaneous PGI_2 production and then stimulated to produce PGI_2 once more (Yui 1984, Jorgensen 1981, Remuzzi 1979). However the use of human tissue would seem to be preferable and therefore we have developed a method which uses human umbilical artery rings.

3.10 (b) Method

Human umbilical cords are obtained from normal term deliveries. The cords are immediately placed in ice cold Ringer's buffer. Within two hours of delivery the arteries are dissected free from the surrounding tissue and cut into approximately 1mm rings. The rings are incubated at $37^{o}C$ for 30 minutes in Ringer's buffer and the buffer is then renewed. This process is repeated until no antiaggregatory activity is detectable in the supernatant. This is tested by adding 100 µl of supernatant to 200 µl platelet rich plasma (PRP) in the cuvette of an aggregometer (Malin Electronics, Green Street, Ayr).

Aggregation is induced by adding 2 ug/ml collagen. Approximately 15 mg (wet weight) aliquots of these exhausted rings are then placed in plastic tubes with 1 ml Ringer's buffer and placed in melting ice. Rings from different arteries must be kept seperate and tested individually for 'exhaustion'. Ringer's buffer is removed from one aliquot of rings and replaced by 1 ml of normal pool plasma collected from 10 male and 10 female donors, the

blood being anticoagulated (1:9) with 3.2% trisodium citrate. The rings and plasma are incubated at 37°C and 100 µl aliquots removed at five minute intervals. This is added to 200 µl PRP in the aggregometer and collagen 2 µg/ml added. The experiment is then repeated on an aliquot of rings from the same artery using sample plasma. In each experiment, as a control, 100 µl of either pool or sample plasma which has not been incubated with artery rings is added to the PRP and aggregation induced as before. The inhibition of aggregation shown by the plasma incubated with rings is expressed as a percentage of this control value.

Some researchers have shown that serum causes greater stimulation of PGI_2 release than plasma (Ritter 1983, Seid 1983) whereas other workers failed to find this (Vergara-Dauden 1985). We therefore performed similar experiments incubating the rings with serum in place of platelet poor plasma.

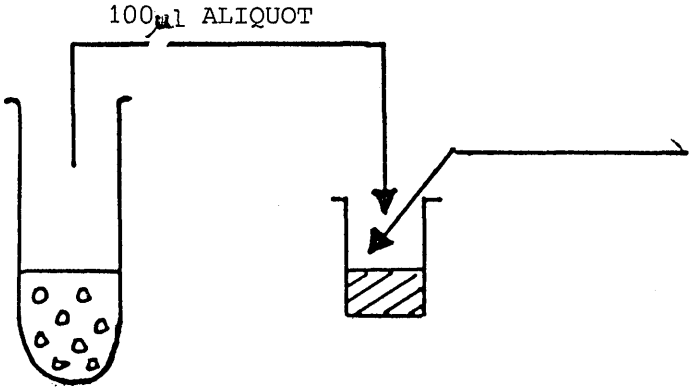
The principle of the assay method is shown diagramatically in figure 3.7.

3.10 (c) Results

The results obtained incubating umbilical artery rings with normal platelet poor plasma for increasing lengths of time are shown in figure 3.8(a).

The results obtained in similar experiments using normal serum instead of platelet poor plasma are shown in figure 3.8(b).

1. ASSAY



2. CONTROL

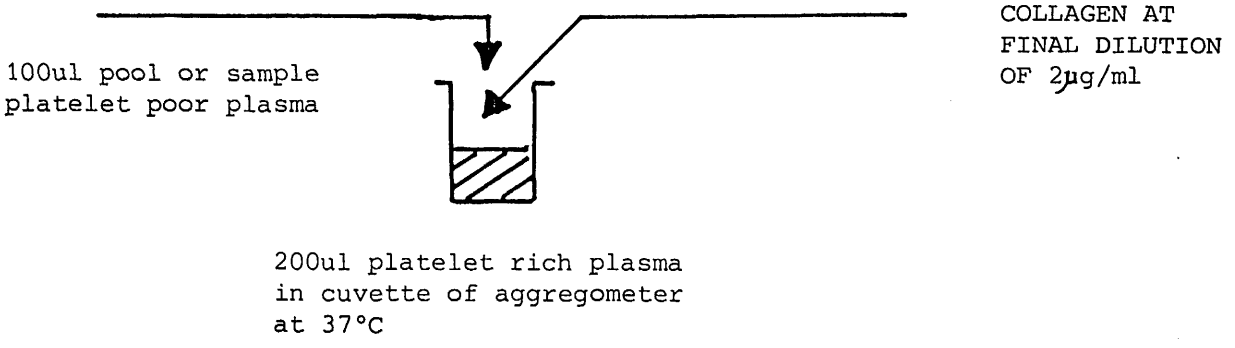
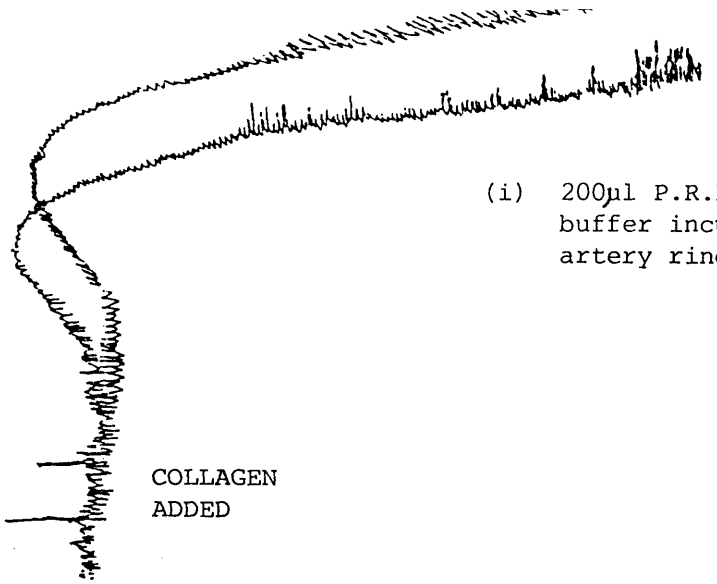
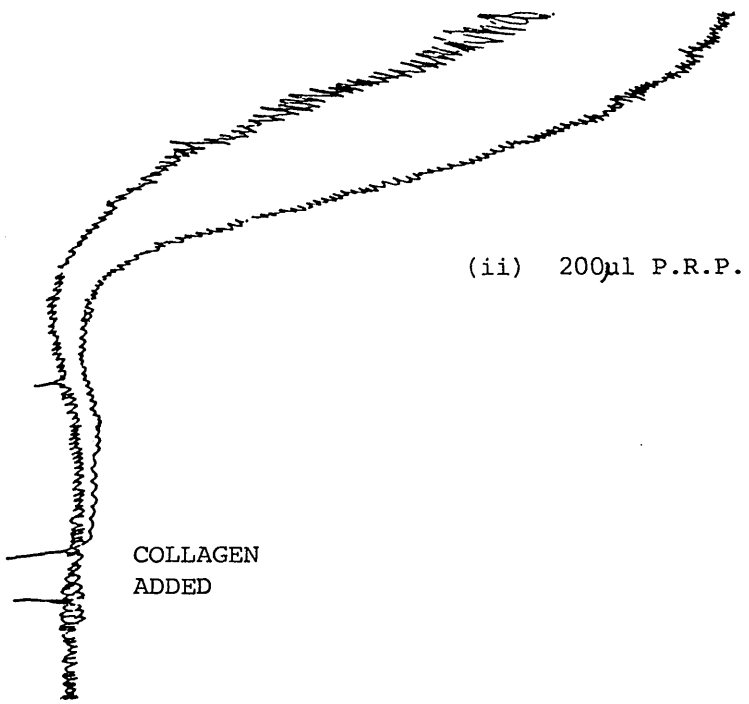


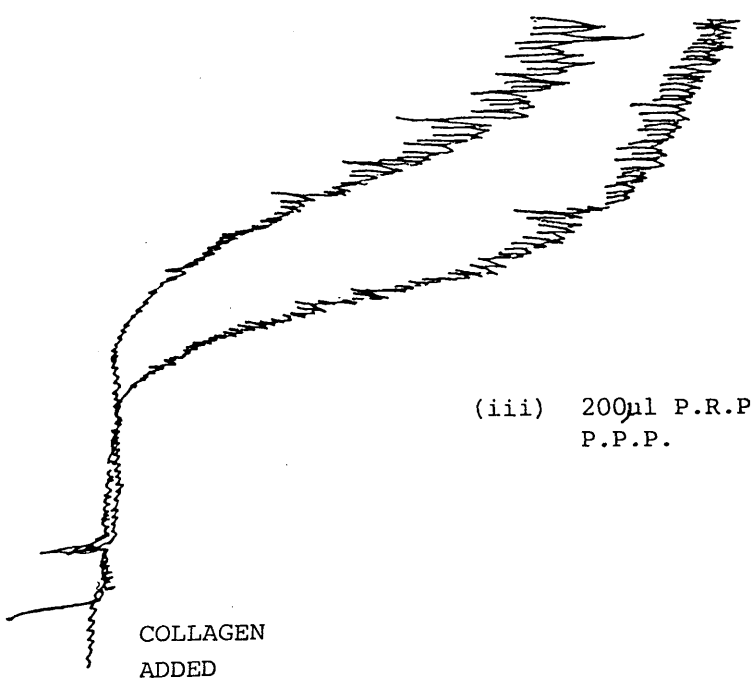
FIGURE 3.7: METHODOLOGY FOR PROSTACYCLIN STIMULATING FACTOR ASSAY



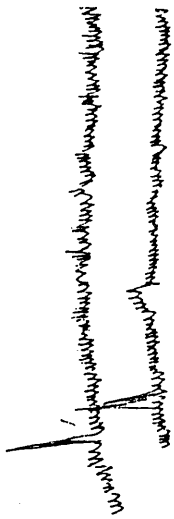
(i) 200µl P.R.P. + 100µl supernatant buffer incubated with umbilical artery rings for 5 minutes



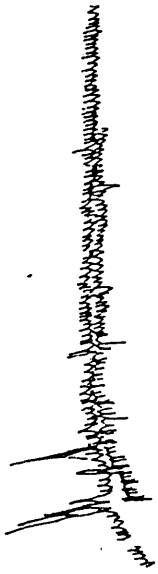
(ii) 200µl P.R.P. + 100µl buffer



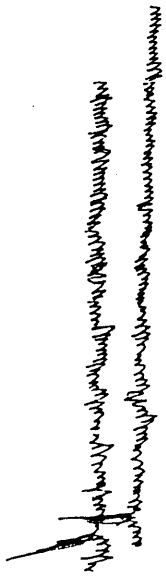
(iii) 200µl P.R.P. + 100µl pool P.P.P.



(iv) 200µl P.R.P. + 100µl supernatant pool P.P.P. incubated with umbilical artery rings for 5 minutes

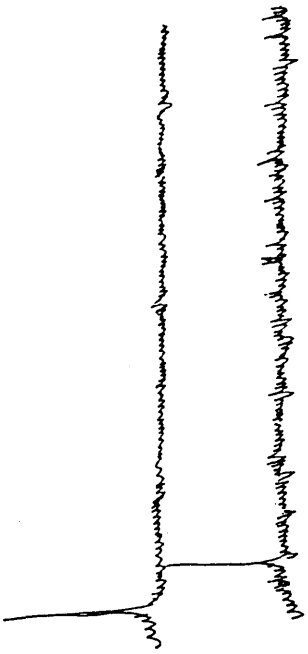


(v) 200µl P.R.P. + 100µl supernatant pool P.P.P. incubated with umbilical artery rings for 10 minutes



(vi) 200µl P.R.P. + 100µl supernatant pool P.P.P. incubated with umbilical artery rings for 15 minutes

FIGURE 3.6 (a)

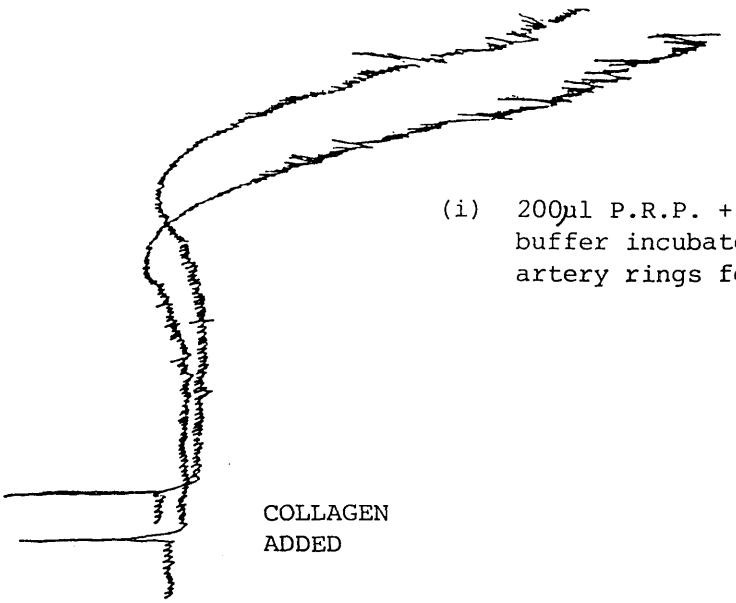


(vii) 200µl P.R.P. + 100µl supernatant pool P.P.P. incubated with umbilical artery rings for 20 minutes

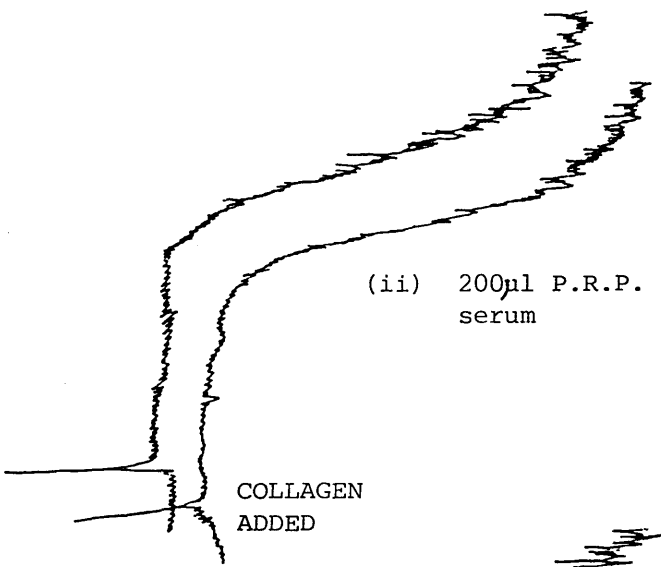
COLLAGEN ADDED

(viii) 200µl P.R.P. + 100µl supernatant pool P.P.P. incubated with umbilical artery rings for 30 minutes

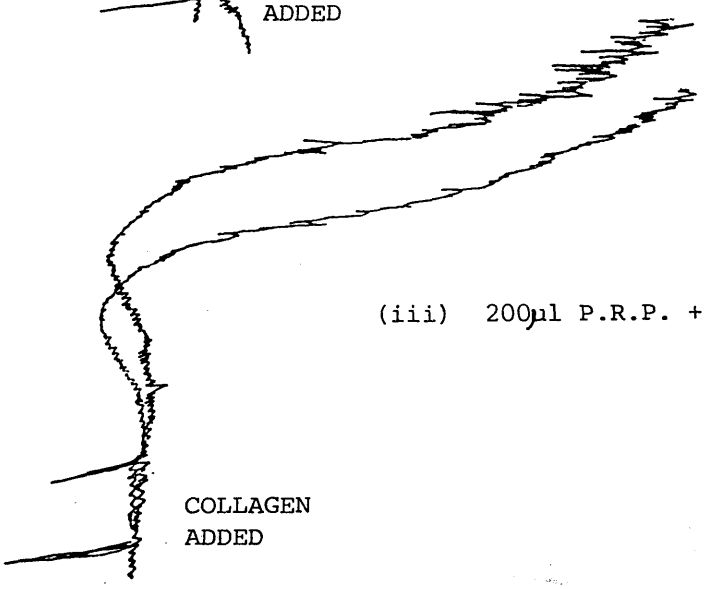
COLLAGEN ADDED



(i) 200µl P.R.P. + 100µl supernatant buffer incubated with umbilical artery rings for 5 minutes



(ii) 200µl P.R.P. + 100µl normal serum



(iii) 200µl P.R.P. + 100µl buffer

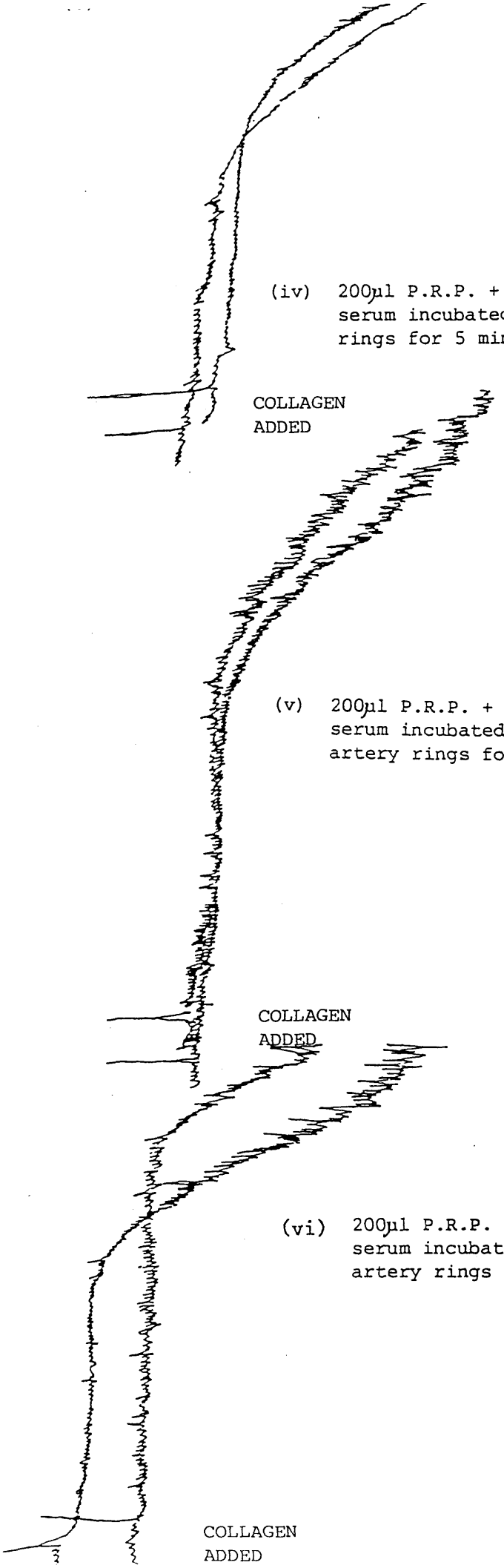
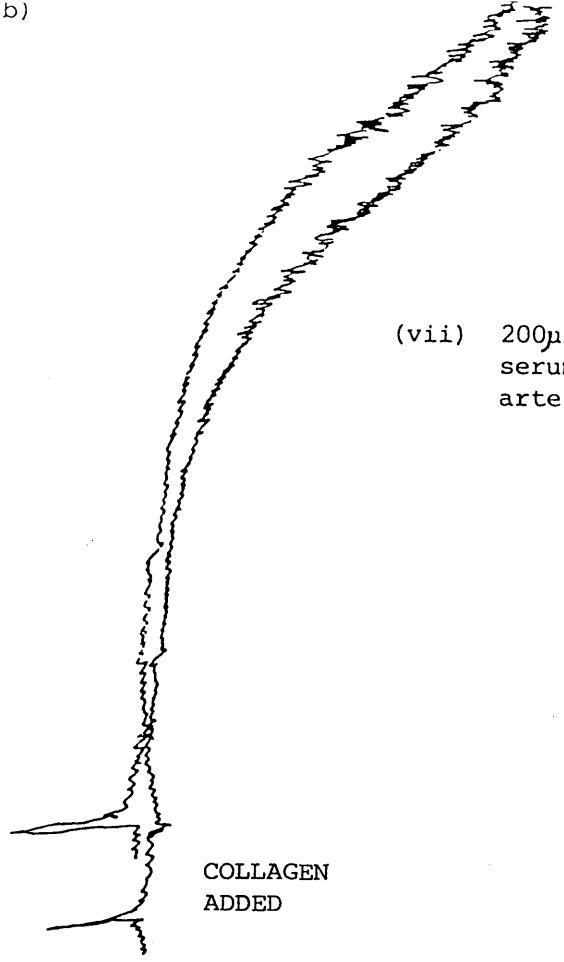
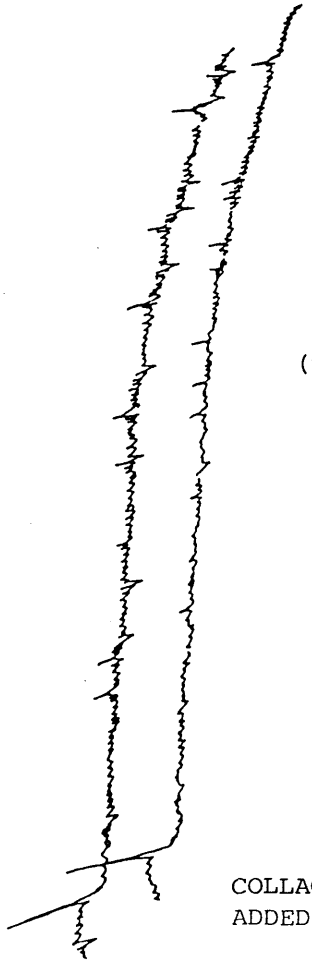

(iv) 200µl P.R.P. + 100µl supernatant serum incubated with umbilical artery rings for 5 minutes
COLLAGEN ADDED
(v) 200µl P.R.P. + 100µl supernatant serum incubated with umbilical artery rings for 10 minutes
COLLAGEN ADDED
(vi) 200µl P.R.P. + 100µl supernatant serum incubated with umbilical artery rings for 15 minutes
COLLAGEN ADDED

FIGURE 3.8 (b)



(vii) 200µl P.R.P. + 100µl supernatant serum incubated with umbilical artery rings for 20 minutes



(viii) 200µl P.R.P. + 100µl supernatant serum incubated with umbilical artery rings for 30 minutes

3.10 (d) Discussion

These results show that when platelet rich plasma is incubated with umbilical artery rings for only five minutes aggregation is completely inhibited and this effect is still present after 30 minutes incubation. When serum from a normal female was used in place of platelet poor plasma aggregation was not completely inhibited until 30 minutes incubation time. We therefore found that both normal platelet poor plasma and serum inhibited aggregation completely, but serum required incubation with the artery rings for a considerably longer period of time than did plasma for this effect to be observed. We obtained similar results using serum from another normal female donor. However since the platelet poor plasma used was from a pool of normal plasma obtained from 10 male and 10 female volunteers the results could possibly be different if a pool serum was used.

3.11 Umbilical Artery Perfusion Model

3.11 (a) Introduction

The use of rat aortic rings to measure PGI_2 production by endothelial cells (Yui 1984, Jorgensen 1981) has been well documented. Our method using umbilical artery rings showed inhibition of platelet aggregation from normal plasma which had been incubated with artery rings. However these methods have certain disadvantages. Firstly, as previously discussed, the use of human tissue would seem to be preferable to rat aortic rings. Even when using human umbilical artery rings there are still disadvantages. Layers of subendothelium are exposed using artery rings and because the entire surface area of endothelium in the

experiment is so small these subendothelial layers could play a significant part. It has been shown (Serneri 1983) that the medial layers of arteries are capable of producing thromboxane. As this has directly opposing actions to PGI_2 it could influence the results of the experiments. It is also almost inevitable that endothelial cells are lost from the artery rings during repeated washings. This may not be a constant loss from one aliquot to the next and therefore this also could influence the results. During preparation of the rings the tissue is also severely traumatised and it has been shown (Mehta 1982 a) that mechanical trauma may result in the release of TXA_2 from intact human umbilical vein though in much lesser quantities than PGI_2. Vessel ring models also lack the pulsatile flow of normal circulation. As the aim of any in vitro model is to resemble the in vivo situation as closely as possible we decided to try to develop an in vitro model which would resemble the in vivo situation more closely. Other researchers have used continuously perfused umbilical vein models (Mehta 1982 a, Mehta 1982 b). However it has been shown that arterial tissue is capable of producing more PGI_2 than venous tissue (Kent 1981). We have therefore developed an umbilical artery perfusion model to measure endothelial cell PGI_2 production.

3.11 (b) Materials and Methods

We use umbilical cords from normal term deliveries which have not been subjected to trauma, the placenta being delivered by maternal effort. Cords from elective sections at term are also used.

The cords are detached from the placenta and immediately placed in Ringer's buffer at $4^{o}C$. In our original experiments we used the complete cord, cannulating one artery within 2-3 hours of delivery. The artery was continuously perfused and fractions collected for measurement of PGI_2 metabolites by radioimmunoassay (R.I.A.). However using the whole cord we found large variations between individual cords of equal length and also between two halves of the same cord as shown in figure 3.10. This may be due to different surface area of endothelium in equal lengths of cord caused by twisting of the cord. Another disadvantage of this method was that about four/fifths of the cords used were unsuitable due to extravasation of the perfusate into the surrounding tissue; this being not always immediately visible. We now use a dissected vessel model. The artery is carefully dissected free from the surrounding Wharton's jelly and is then cannulated with a size 16 Medicut cannula and flushed through with buffer to clear any residual blood.

The vessel is cut into two equal halves, one half acting as control, and is supported in a bath of oxygenated Ringer's buffer at $37^{o}C$. Each half is connected to a peristaltic pump which gives a pulsatile flow similar to normal circulation. The pumps are calibrated to deliver a flow of 1 ml per minute. One half of the cord is perfused with oxygenated Ringer's buffer to act as control while the other half is perfused with drug or plasma as required. 5ml fractions are collected using a fraction collector and stored at -70^{o} for assay for PGI_2 metabolites using R.I.A. as described in 3.7. Figure 3.11 shows a photograph of the apparatus used.

FIGURE 3.10 PERFUSION OF NORMAL UMBILICAL ARTERIES

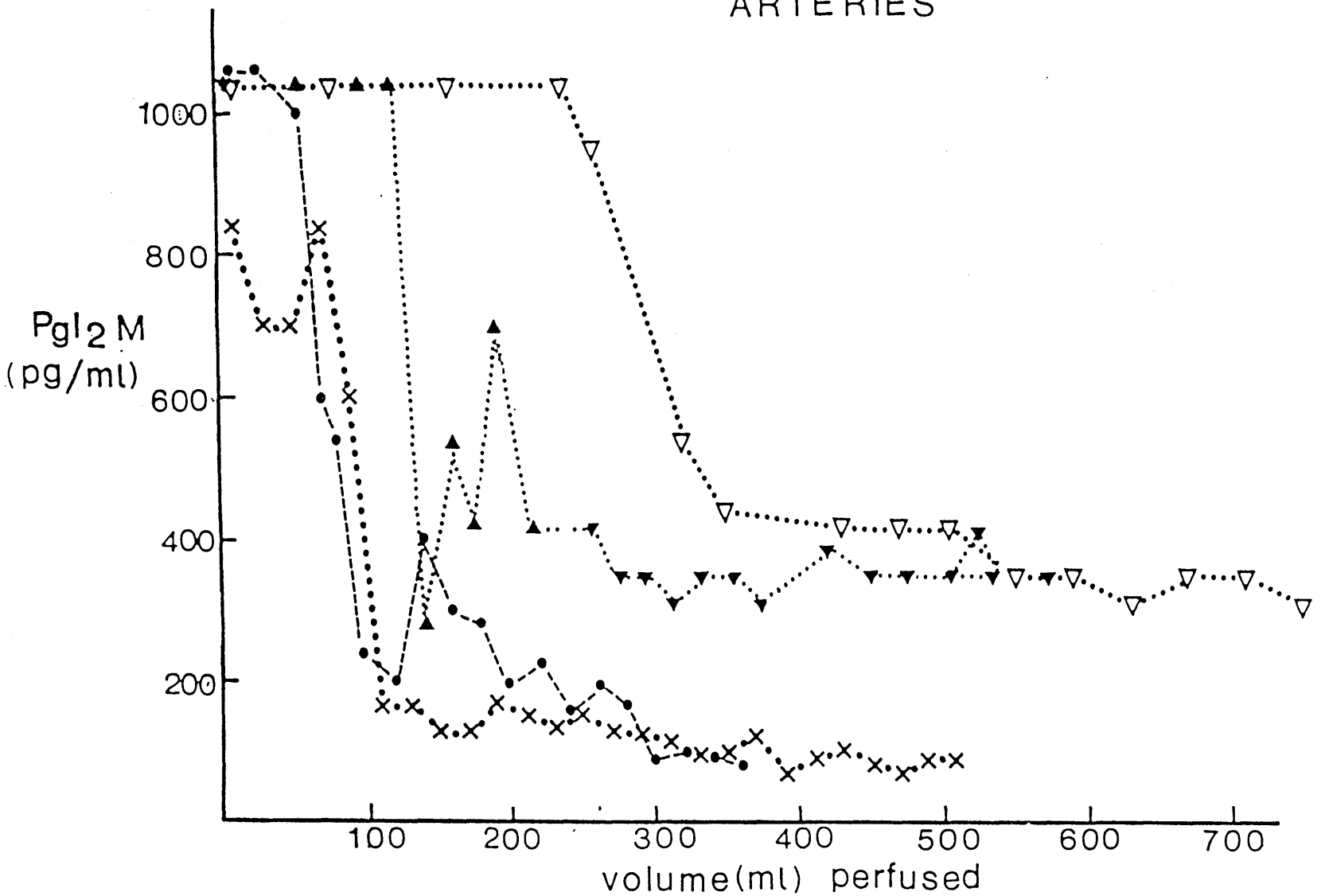


Figure 3.11 - The apparatus used

To show that we had healthy, functioning endothelium we perfused one artery with whole blood followed by 1% glutaraldehyde in buffer as fixative. This was then prepared for scanning electron microscopy by dehydration and critical point drying. The experiment was performed on two halves of the same vessel: the first after perfusion with 50 ml of buffer and the second after overnight perfusion.

3.11 (c) Results

The electron micrograph scans show that in the first case where the vessel had been perfused with only 50 ml of buffer there is healthy endothelium and no visible platelet clumps (Fig 3.12).

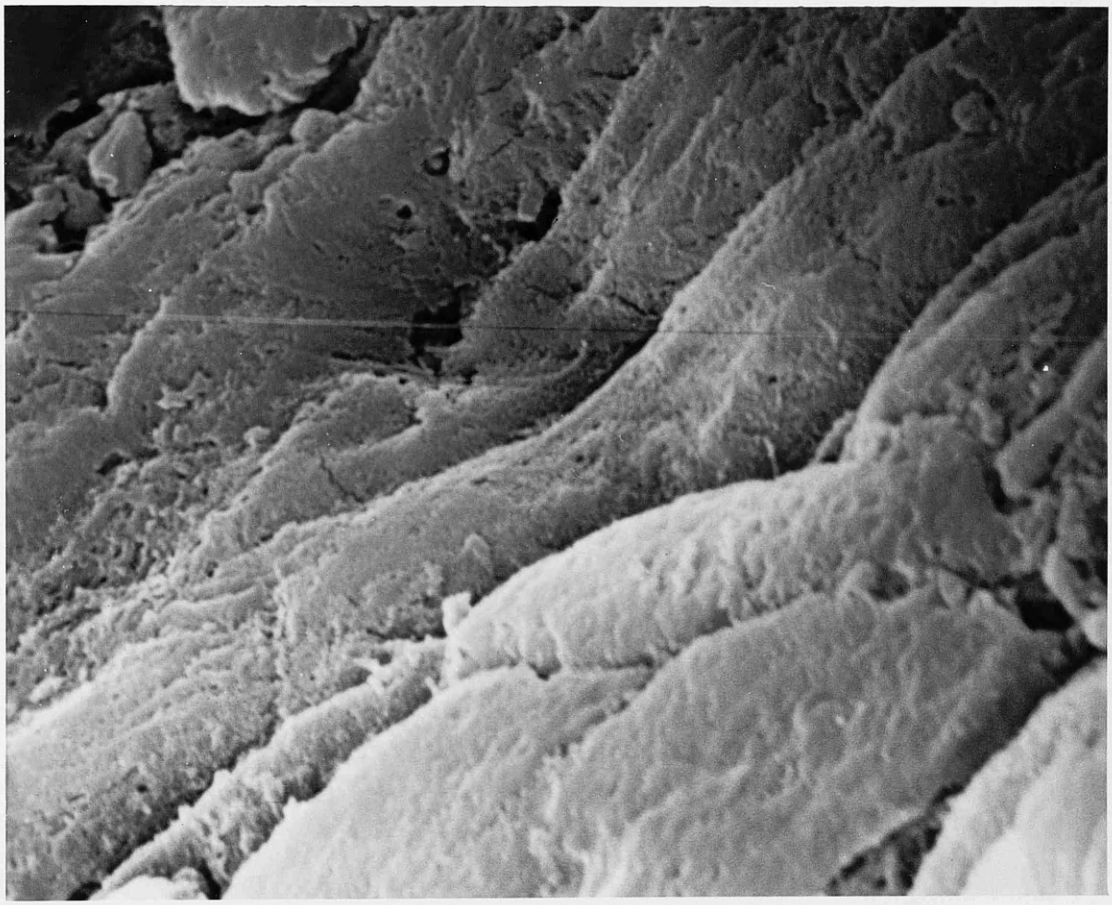


Figure 3.12 - Electron micrograph prior to exhaustion (x 2500)

However after overnight perfusion there are large platelet aggregates visible (Figure 3.13) after exhaustion of PGI_2 production.

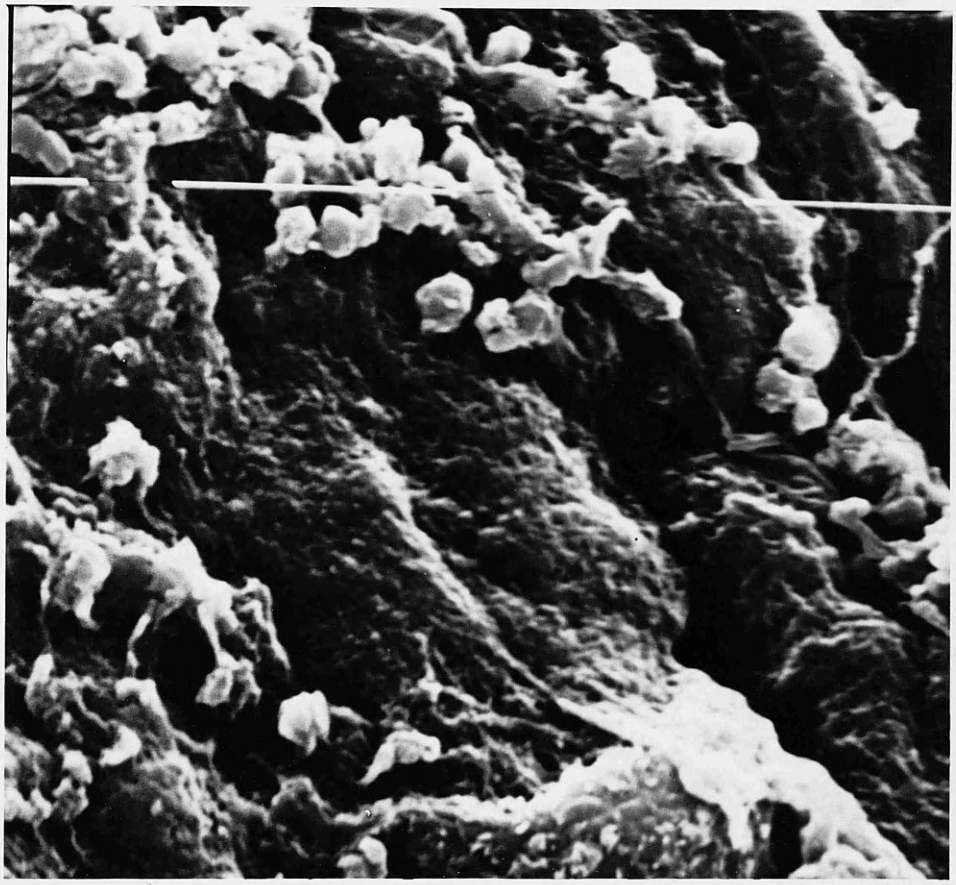


Figure 3.13 - Electron micrograph post exhaustion (x 2500)

Figure 3.14 shows a graph of PGI_2M levels from two halves of the same artery perfused simultaneously with Ringer's buffer. After perfusion of approximately 150 ml (150 minutes) levels of PGI_2 metabolites reached a steady baseline level of 100 pg/ml. There is a correlation of 0.95 between levels of PGI_2 metabolites in the two halves of the vessel.

We also wanted to show that PGI2 production could be inhibited. We were able to show inhibition both with aspirin 250 µg/ml (Figure 3.15) and indomethacin 1 pg/ml (Figure 3.16).

It was also important to show that our endothelium could be stimulated to produce more PGI_2 after reaching a steady baseline level. In order to show this we used platelet poor plasma which has been shown to contain a factor or factors capable of stimulating endothelial cell PGI_2 production (McIntyre 1978, Remuzzi 1979 a, Defreyn 1980). After perfusing both halves of the same artery with Ringer's buffer until a steady baseline level was reached we perfused one half with 5ml of normal pool platelet poor plasma and continued to perfuse the other half with buffer. As can be seen in Figure 3.17 levels of PGI_2 metabolites rose in the half perfused with plasma while the control levels remained steady.

3.11 (d) Discussion

Using this dissected umbilical artery model we have shown, by the use of electron microscopy, that we have healthy functioning endothelium. Using normal platelet poor plasma we have shown that it is capable of stimulation to produce more PGI_2 after having reached a steady baseline level. We have also shown that PGI_2 production can be inhibited both by aspirin and by indomethacin.

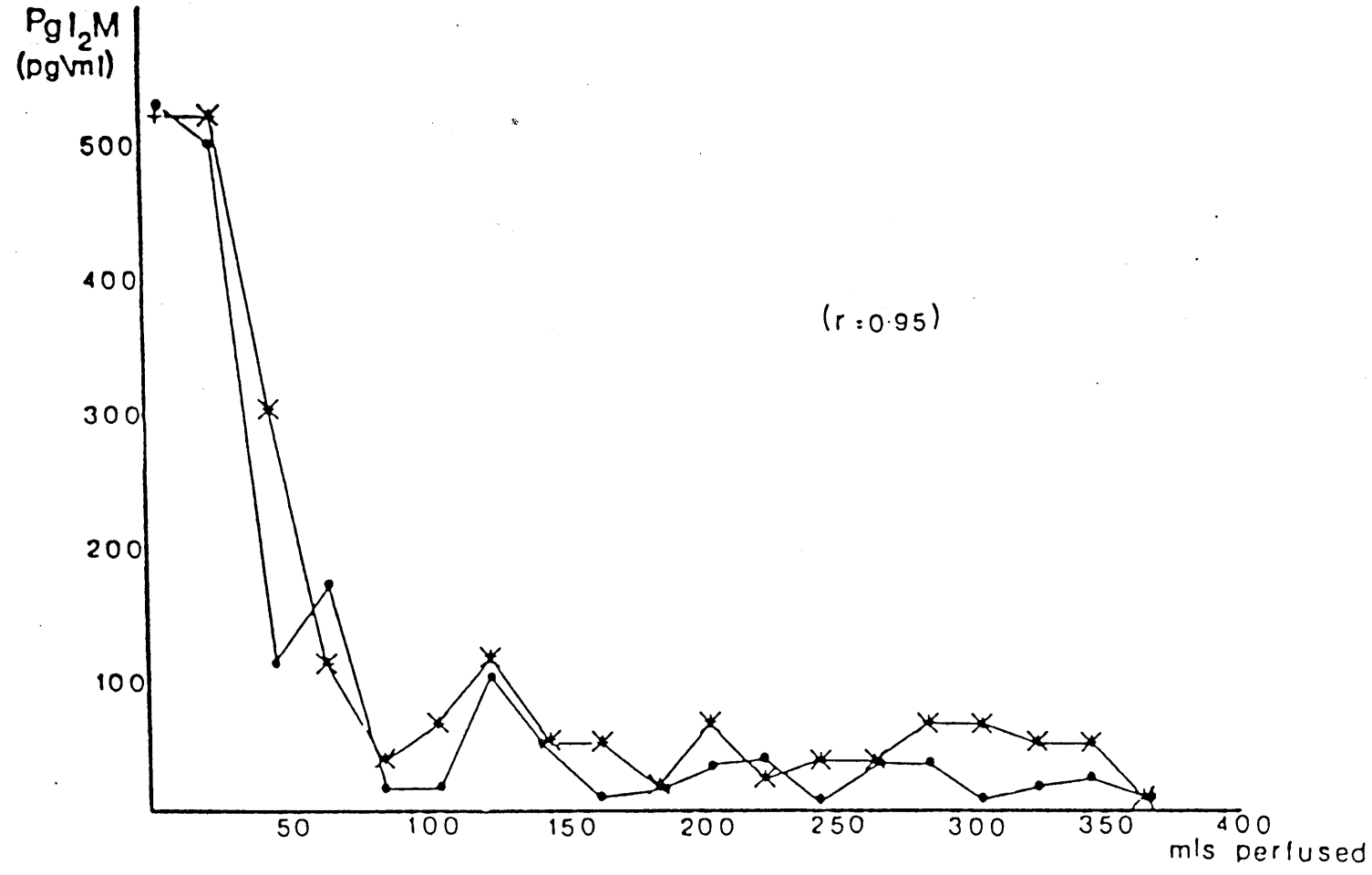


Figure 3.14 Perfusion of Two Halves of One Artery With Buffer Alone

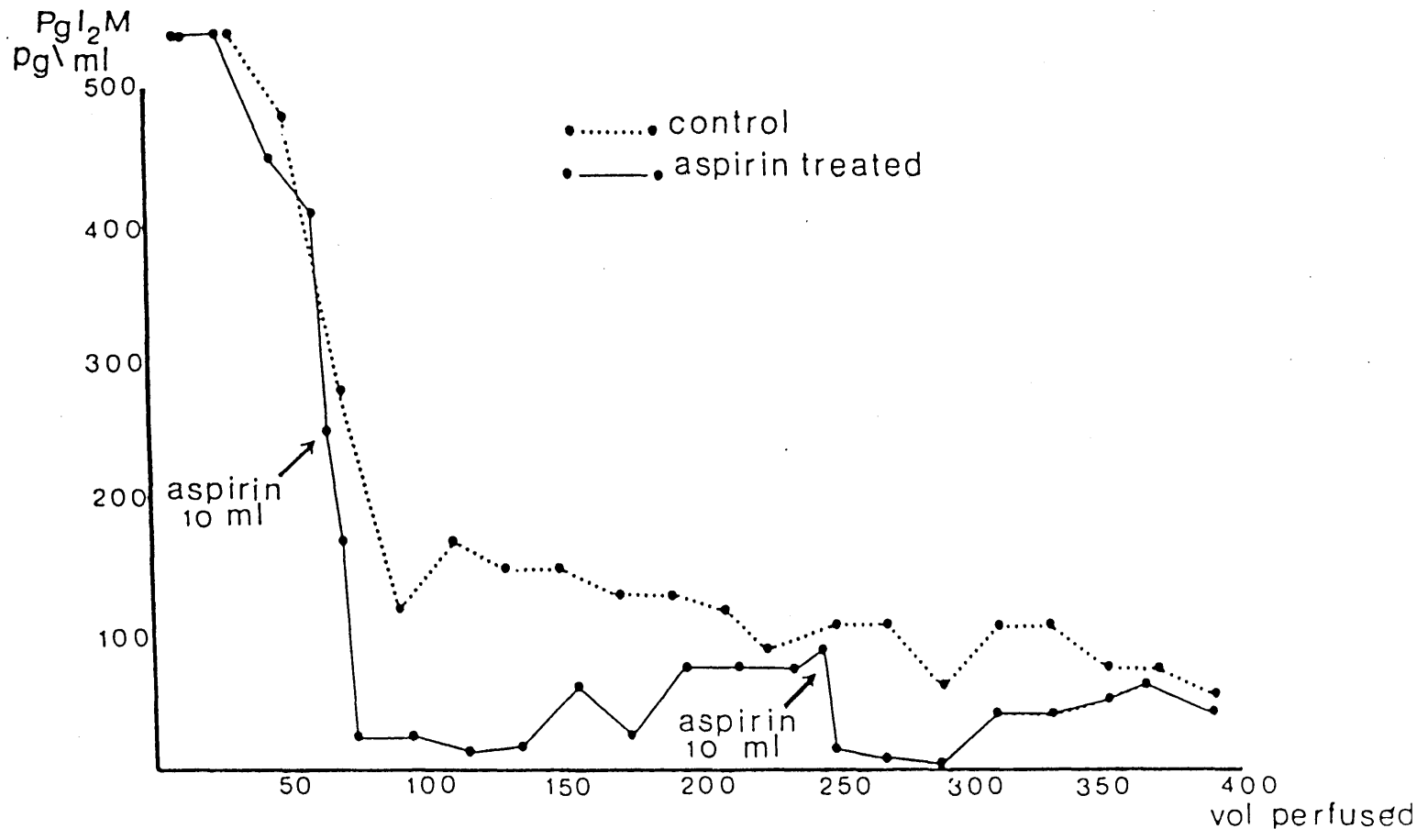


Figure 3.15 Perfusion With Aspirin 250µg\ml

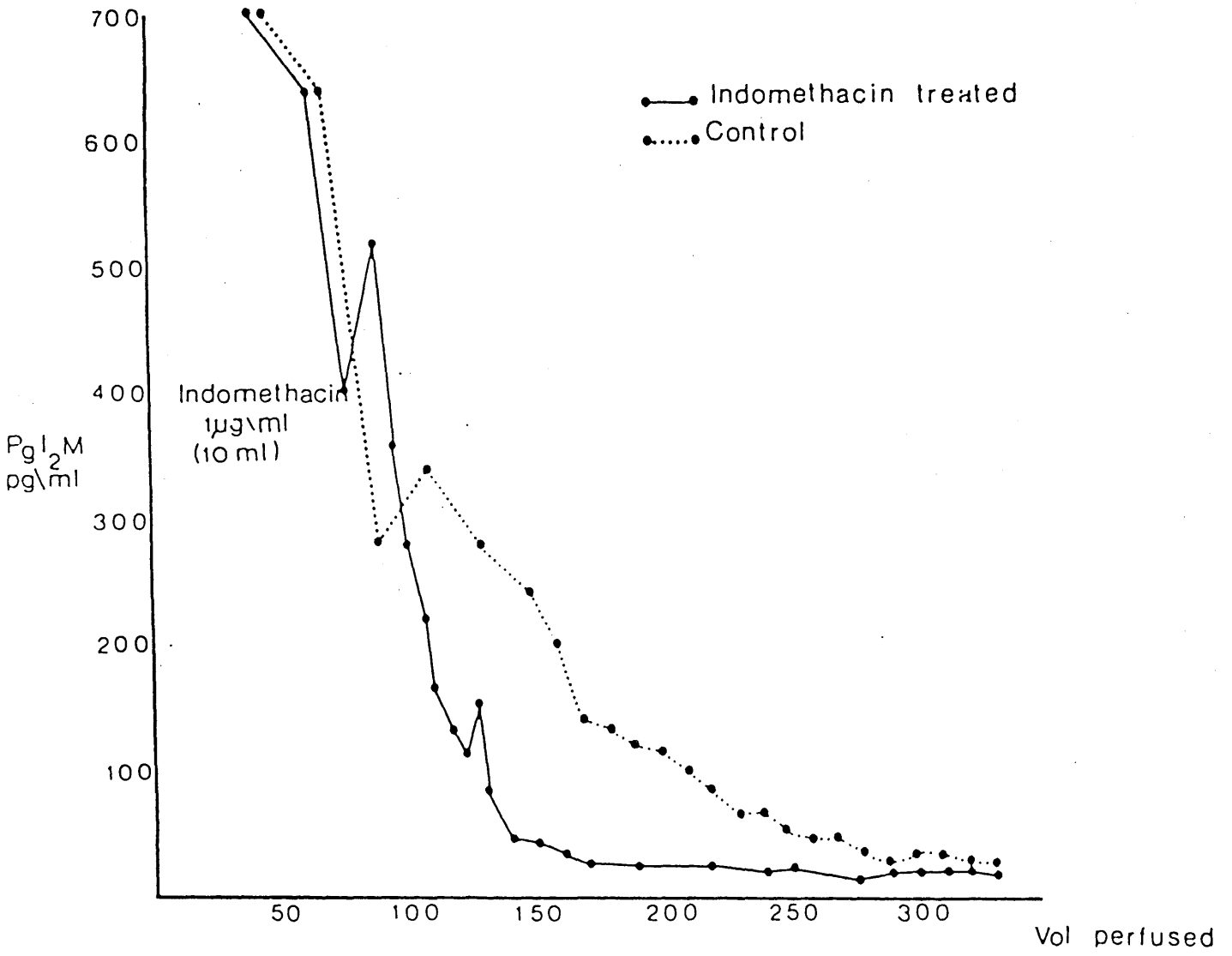


Figure 3.16 Perfusion with Indomethacin 1µg\ml

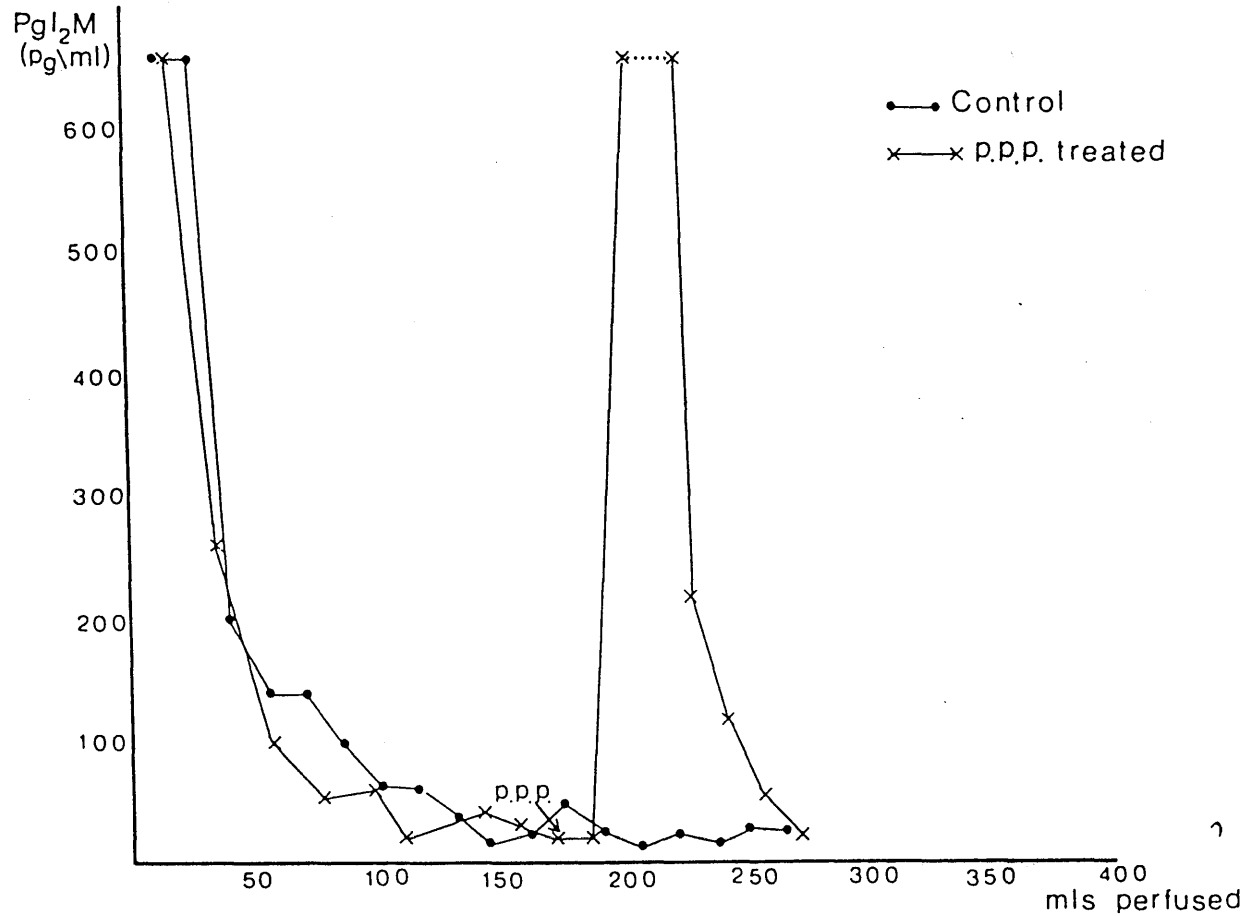


Figure 3.17 Perfusion With Platelet Poor Plasma (5 ml)

Although the flow rate and pressure are less than in the in vivo situation we feel that our model resembles the in vivo situation more closely than models using artery rings. There is also the advantage that umbilical cords are ready available. We feel that it is a useful model to assess the effects of drugs on PGI_2 production. Although it would be impracticable to use this model to assay large numbers of samples for PGI_2 stimulating factor it would be useful to test plasma samples which were found to have low PGI_2 stimulating factor using the ring model. We also hope to be able to use this model to see if there is decreased endothelial cell PGI_2 production in cords obtained from mothers with pregnancy induced hypertension. It has also recently been shown that PGI_2 production was decreased in the umbilical artery from a mother who delivered a baby with Down's syndrome (Mäkilä 1985). Decreased PGI_2 production has also been shown in smoking mothers (Dadak 1982) and may be a factor for reduced foetal placental blood flow or foetal growth retardation. Our model may be a useful tool in the further elucidation of these conditions.

CHAPTER 4 - MEASUREMENT OF THROMBOXANE

4.1 Introduction

As with PGI_2, much research has been carried out to try to determine what the role of thromboxane is in health and disease. Since its discovery (Piper 1969) various methods of quantifying thromboxane have been developed. Because of the very short half life of TXA_2 many of these techniques use TXB_2, the stable breakdown product, as an index of TXA_2 production. In this chapter the various methods will be described and advantages and disadvantages discussed.

4.2 Bioassay

The ability of TXA_2 to contract vascular smooth muscle which first led to its discovery is still used as a bioassay technique. The ability of TXA_2 to cause aggregation of platelets may also be used to detect thromboxane-like activity. However the very short half-life of only 36 seconds in aqueous media causes much difficulty when using either of these methods of detection. As TXB_2, the stable breakdown product, has only very weak biological activity bioassay techniques are not suitable for its measurement. The disadvantage of bioassay of thromboxane therefore, as well as those discussed previously in 3.2, is mainly its very short half life.

4.3 Gas chromotography mass spectometry

TXB_2 was shown to be the stable hydrolysis product of TXA_2 using electron impact mass spectometry (Hamberg 1975).

The urinary metabolites of infused TXB_2 in man were identified by gas chromotography - electron impact mass spectometry (Roberts 1981) which in turn led to the development of quantitative assays for TXB_2 based on analysis of a urinary metabolite (Fitzgerald 1983). However when measuring plasma levels of TXB_2, RIA is the method most commonly used. The disadvantages of GCMS as discussed in 3.3 for PGI_2 apply also to thromboxane.

4.4 Radioimmunoassay

4.4 (a) Introduction

One of the first RIA's for TXB_2 was developed by Granström and colleagues (Granström 1978) and since then RIA's for TXB_2 have been developed by many other groups (Viinikka 1980, Mitchell 1978, Kaar 1983, Orlandi 1983, Lieb 1983). Unlike plasma 6-keto-PGF1α the range of reported normal values has not been so great as is shown in Table 4.1.

Plasma TXB_2 (pg/ml)	Author
70.1 $\pm$ 26.7	Viinikka 1980
276 $\pm$ 80.2 (age 19.9 $\pm$ 4.1 yrs)	Kaar 1983
118	Ouwendijk 1983
101 $\pm$ 34.4 (age 11.7 $\pm$ 3.8 yrs)	Orlandi 1983
64.2 $\pm$ 29.4	Lieb 1983

Table 4.1 - Range of reported normal levels of plasma TXB_2

The arguments for and against extraction of samples prior to R.I.A. as discussed in 3.7(a) in relation to 6-keto-PGF1α apply equally to TXB_2. The measurement of plasma levels of TXB_2 has also received much criticism due to the potential for

artefactually high levels. Since TXA_2 is generated by platelets, any trauma associated with venepuncture will activate the platelets and so give rise to artificially high levels. Unless an inhibitor of TXA_2 generation is added to the anticoagulant there will also be a further generation of thromboxane by the platelets during processing of the sample. However provided a standard venepuncture technique is adopted and samples discarded where there was difficulty experienced in obtaining blood, the in vitro generation of TXA_2 should be minimised. The processing and handling of samples must also be carefully standardised for the same reason.

4.4 (b) Sampling procedure

Venous blood is collected using a 19 gauge butterfly needle. Samples are discarded where there is any trauma associated with the venepuncture. The blood is placed immediately into ice cold plastic tubes containing 1:9 vol/vol 3.8% sodium citrate with indomethacin added to give a final concentration of 3×10^{-5}m to prevent in vitro generation of TXA_2. The samples are kept on ice and centrifuged within one hour at 2500g and $4^{o}C$ for 15 minutes. The plasma is stored in aliquots at $-70^{o}C$ until assay. We have compared results obtained using different sampling techniques:

(i) Citrate/indomethacin anticoagulant in the syringe and blood collected directly into this.

(ii) Adding UK 38,485 (Pfizer Ltd) a thromboxane synthetase inhibitor to the anticoagulant instead of indomethacin.

The results of these experiments are shown in table 4.2.

Mean TXB_2 (pg/ml) (SD) n=6	Method (i)	Method (ii)	Original Method
	95 ± 16.4	102.7 ± 18.6	85.2 ± 9.8

Table 4.2 - Results obtained using different sampling techniques

4.4 (c) Radioimmunoassay Procedure

Antibody to TXB_2 and TXB_2 for use in making up standards were gifted by Mr Simon Thomas, Medical Science Liaison Officer, Upjohn Ltd, Crawley. The same criteria as discussed in 3.7(d) for the preparation of standards for 6-keto-PGF1α were observed. The standard obtained was diluted in assay buffer to give a stock solution of 1ng/ml. Aliquots of this standard are stored at -70°C together with aliquots of a zero standard as for 6-keto-PGF1α. Working dilutions ranging from 3.9 to 1000 pg/ml are prepared freshly each week and stored at 4°C. The pool of normal plasma used as a standard for PGI_2 metabolites is also used for TXB_2. ^3H labelled TXB_2 was obtained from Amersham International (Amersham, Buckinghamshire). The method used for the RIA is exactly as described for the antibody by F A Fitzpatrick of the Drug Metabolism Research Department, Upjohn Company Ltd, Kalamazoo, except that we use the same buffer as for the PGI_2 metabolite assay. To appropriately labelled tubes (Luckham LP_3) are added 100µl standard or sample, 100µl ^3H TXB_2 to give total counts of approximately 3000cpm and 100µl antibody (final dulition 1:10,000). Both plasma and buffer non-specific binding are determined using pool plasma or the highest standard respectively. The tubes are incubated for 20-24 hours at 4°C. After incubation the tubes are placed in a bath of melting ice and 300µl of 0.5% acid washed charcoal (BDH) in buffer is added to all except total count tubes.

All tubes are centrifuged at exactly 9 ± 1 minute from the start of addition of charcoal at 4°C and 2500g for 10 minutes. The tubes are replaced in the ice bath and the supernatant decanted into 10 mls scintillant (Packard Insta Gel) and counted in a Packard scintillation counter.

The exact protocol for the assay is shown in Table 4.3.

Tube	Total Count	Non specific binding Buffer	Non specific binding Plasma	Stds 0-1000 pg/ml	Std plasma	Sample
Buffer	200	100	100	-	-	-
Standard Plasma	-	-	100	-	100	-
Standard or sample	-	100	-	100	-	100
3H TXB_2	100	100	100	100	100	100
Antibody	-	-	-	100	100	100
	Mix well and incubate at 4°C for 20-24 hours					
Buffer	300	-	-	-	-	-
Charcoal Suspension	-	300	300	300	300	300

Centrifuge at exactly 9 ± 1 minute after start of additional of charcoal at 4°C and 2500 rpm for 10 minutes. Decant supernatant into 10mls scintillant and count.

Table 4.3 - Protocol for TXB_2 assay (all volumes in µl)

4.4 (d) Results

Figure 4.1 shows the results for the standard curve obtained over ten assays using the method described. The horizontal axis shows the concentration of TXB_2 in the standards and the vertical axis the percentage binding taking zero binding to be 100%.

Taking the limit of sensitivity as being two standard deviations from zero binding the average lower limit of sensitivity for each assay is 15.6 pg/ml.

Over ten assays the inter and intra assay variations are 10% and 4% respectively.

4.5 Serum Thromboxane Measurement

4.5 (a) Introduction

Because of methodological difficulties in blood sampling when measuring plasma TXB_2 as discussed in 4.4(a), thrombin-induced TXA_2 production in whole blood, as reflected by serum TXB_2 measurements, is becoming widespread for human studies in health and disease. The method described here employs an endogenous stimulus i.e. generated thrombin to platelets in whole blood. Thrombin induced TXA_2 production in whole blood appears to accurately reflect the inhibition of platelet TXA_2 biosynthesis by cyclooxygenase inhibitors *in vivo* (Patrignani et al 1982, Weksler et al 1983) and by thromboxane synthetase inhibitors (Fischer et al 1983, Patrignan et al 1984). As the volume of blood required is much less than for measurements of plasma TXB_2 this allows for long term studies on the same subject with negligible blood loss. The final measurement of serum TXB_2 levels is performed using the R.I.A. previously described in 4.3.

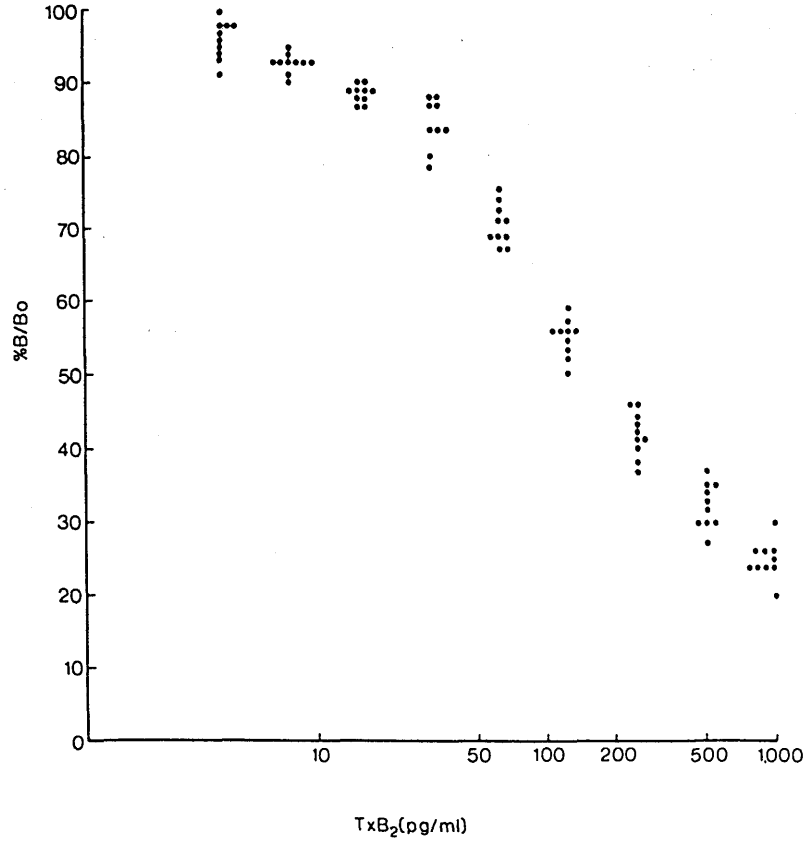


FIGURE 4.1 : Plasma TXB_2 assay - Standard curve over ten assays

However the handling of the blood sample prior to incubation at 37°C may be the cause of variations in reported normal values (Mahoney et al 1983, Codde et al 1984). Because of this we looked initially at the effects of incubation of the blood at different temperatures and for different lengths of time on the serum TXB_2 levels.

4.5 (b) Method

Venous blood was taken from healthy volunteers who had taken no drugs over the previous two weeks. Venepuncture was carried out using a 19 gauge butterfly needle with minimal venous compression. The blood was immediately divided into 1ml aliquots and placed in glass tubes and these were allowed to stand at room temperature (approximately 24°C) for different lengths of time i.e. 10, 30, 60, 90 and 120 minutes. Thereafter the blood was incubated in a constant temperature room at 37°C for a standard one hour period. The tubes were then centrifuged at 2500g for 15 minutes to separate the serum which was stored at -70°C. In a second series of experiments blood from the same volunteers was taken and the aliquots were immediately incubated at 37°C for varying times i.e. 60, 90, 120 and 150 minutes. The serum was prepared as before, and was assayed in duplicate for TXB_2 using R.I.A. as previously described.

4.5 (c) Results

As can be seen from Table 4.4 there was a statistically significant fall in the recorded values of serum TXB^2 with increasing standing time at room temperature (correlation 0.89).

Standing Time at Room Temp (min) Prior to 1 hr at 37°C	TXB_2 (ng/ml) n=10 (x ± S.E.M.)
0	179.6 ± 13.8
30	90.0 ± 7.0
60	56.8 ± 3.5
90	45.6 ± 3.0
120	39.2 ± 1.8

Table 4.4 - Serum levels of TXB_2 with increasing standing time at room temperature prior to incubation for one hour at 37°C

Table 4.5 shows the results when the samples were incubated immediately after venepuncture but left for varying times at 37°C.

Time at 37°C		TXB_2 (ng/ml) x + S.E.M. n = 10
A	60	176.5 ± 16.0
B	90	112 ± 5.7 *
C	120	103 ± 8.2
D	150	119.5 ± 5.7

* Different from A p 0.05

Table 4.5 - Serum TXB_2 levels with increasing time of incubation at 37°C

This shows a statisically significant decrease in recorded TXB_2 levels between 60 and 90 minutes incubation but no statistical difference thereafter. Our normal range of serum TXB_2 levels when samples are placed immediately into a temperature of 37°C and left to incubate for one hour is from 156 - 400 ng/ml with a mean of 311 ± 112 (S.D.) ng/ml.

4.5 (d) Discussion

These results agree with work done by Patrono (1980) but also show that more important than the actual incubation time is the time the sample is left at room temperature prior to incubation at 37°C. Even if blood is left to stand at room temperature for 30 minutes prior to incubation at 37°C levels fall by 50%. Our results agree with work done by Hall and colleagues (Hall 1984). They conclude that since platelet rich plasma can be kept at 37°C for several hours and still have the ability to produce TXB_2 from arachidonate at 37°C the reduction in TXB_2 after standing room at temperature is probably not due to instability of the enzyme. However whether or not PRP still retains this ability after standing at room temperature rather than 37°C has still to be determined.

Much more work would require to be carried out in order to demonstrate the exact mechanism for this phenomena. However from the practical point of view this means that it is imperative that, immediately after withdrawal, blood is incubated at 37°C. Therefore if blood sampling is performed outwith the laboratory for example in wards or at clinics there must be facilities available for immediate transfer of samples to a temperature of 37°C. For this purpose we use a protable water bath. Provided care is taken in the handling of the samples in the way described, serum TXB_2 levels would seem to give a reliable measurement of the ability of platelets to generate thromboxane in response to an endogenous stimulus. This may be of more relevance than circulating levels as measured by plasma TXB_2. Some patients may generate very high amounts of TXA_2 in response to stimuli and this may predispose them to a

thrombotic tendency. Conversely patients with certain bleeding disorders may have an impaired ability to generate thromboxane. This will be discussed in a chapter 7.

4.6 Thromboxane Generation

4.6 (a) Introduction

The measurement of serum throxboxane levels measures the generation of TXA_2 by platelets in response to an endogenous stimulation: thrombin generated during blood clotting. We have also examined thromboxane generation in response to an exogenous stimulus: collagen, added at a final dilution of 4ug/ml.

4.6 (b) Method

Platelet rich plasma (PRP) is prepared by centrifugation of whole blood at 180g for 10 minutes. The platelet count if greater than 400 x 10^9/litre is adjusted to approximately 300x 10^9/litre with the same platelet poor plasma. 200µl of PRP are placed in the cuvette of an aggregometer (Malin Electronics) at 37°C and aggregation induced by the addition of collagen (Hormon-Chemie, Munich) at a final concentration of 4 µg/ml using the method of Born (1962). ADP at a final concentration of 4µm may also be used. Aggregation is allowed to continue with constant stirring for exactly 4 minutes. The reaction is then terminated by the addition of 200µl of absolute alcohol. The samples are stored at -70°C until RIA for TXB_2. Prior to assay the samples are thawed and centrifuged at 2500g for 15 minutes. The supernatant is removed and diluted 1/400 with assay buffer.

4.6 (c) Results

Figure 4.2 shows the dose response curve we obtained using various concentrations of collagen.

Figure 4.3 shows the dose response curve obtained using various concentrations of ADP.

In order to demonstrate inhibition of Tx generation using this method we incubated PRP for five minutes with indomethacin prior to addition of collagen 4 µg/ml. The results obtained are shown in Table 4.6.

Concentration of indomethacin	TXB (ng/ml)
Control (n = 6)	96 ± 9.8
Indomethacin 100 ng/ml	99.3 ± 23.0
Indomethacin 1 µg/ml	5.4 ± 3.2

Table 4.6 - TX generation after prior incubation with indomethacin

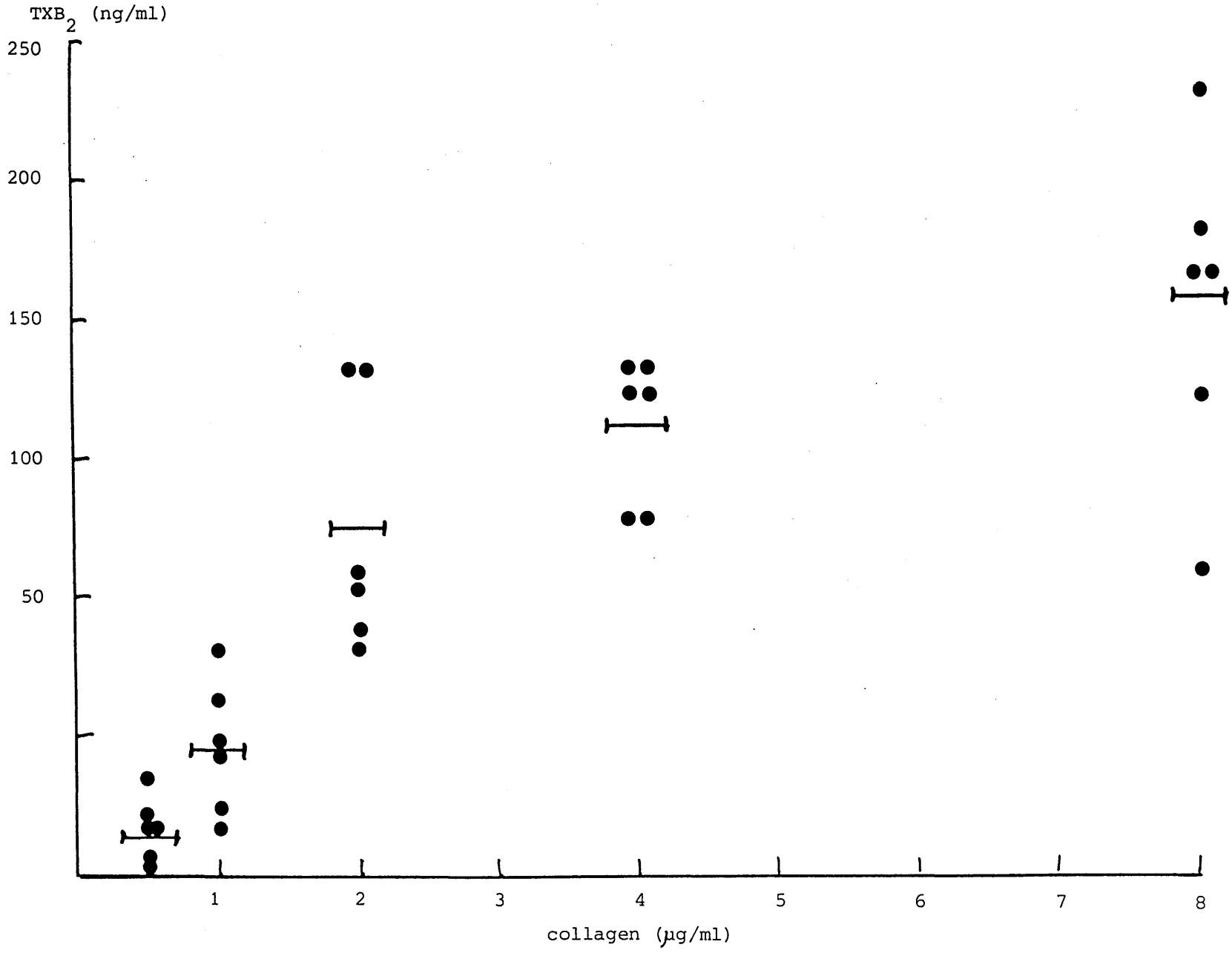
Our normal range for thromboxane generation using 4µg/ml collagen is 80-180 ng/ml with a mean of 118 ± 30 ng/ml (SD).

4.7 Thromboxane generation in Bleeding Time Blood

4.7 (a) Introduction

Some familial bleeding disorders have been associated with deficiencies in the synthesis of thromboxane A_2 by platelets (Defreyn 1981). The presence of TXA_2 in circulating blood as measured by plasma TXB_2 levels is perhaps of less relevance in these disorders than serum TXB_2 levels or thromboxane

FIGURE 4.2 : Thromboxane generation - Collagen dose response curve



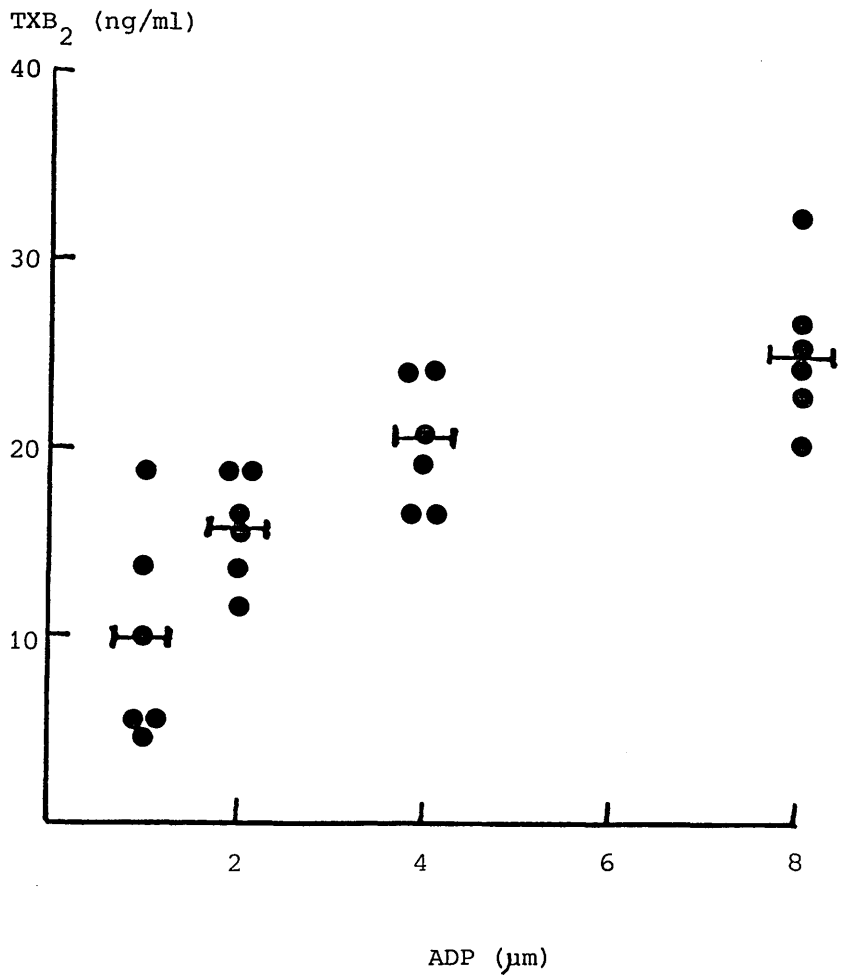


FIGURE 4.3 : THROMBOXANE GENERATION DOSE RESPONSE CURVE FOR ADP

generation in response to an exogenous stimulus as described in 4.6. We thought that it would be of interest to be able to get some indication of amount of TXA_2 generated at the site of injury. There have been studies on the measurement of TXA_2 generation from bleeding time blood by Thorngren (1983). Although work done by Kakkar (1981) suggests that normally TXA_2 plays only a minor role in haemostasis it may be important where there is a bleeding tendency. As a significant part of the work in our department is concerned with bleeding disorders we felt that a method to measure TXA_2 production in bleeding time blood would be of use when used in conjunction with the other measurements of TXA_2 production already described in this chapter. Thorngren (1983) collected the blood at intervals of one minute from the skin incision. The blood was collected into capillary tubes anticoagulated with EDTA and indomethacin. We attempted this method but found that the volume of blood was so small that there was insufficient plasma after centrifugation to enable a plasma assay to be performed reliably. Another problem was in obtaining a constant volume of blood at each sampling which is important because of the standard amount of anticoagulant in each tube.

4.7 (b) Method

The bleeding time is measured using a simplate II bleeding time device (General Diagnostics, Science Park, Milton Road, Cambridge). The blood from one incision is used to measure the bleeding time in the usual way. The blood from the second incision is collected at intervals of one minute into capillary

blood sampling tubes (Sarstedt, Boston Road, Leicester). The tubes are immediately placed in a water bath at 37°C and left to incubate for one hour. The samples are centrifuged at 2500g for 15 minutes and the supernatant serum diluted 1/400 with assay buffer. The diluted serum is mixed and stored at -70°C until RIA for TXB_2. Because of the much higher levels of TXB_2 present in serum the RIA can be performed on a very much smaller sample than with plasma.

4.7 (c) Results

Figure 4.4 shows the levels of TXB_2 obtained from normal individuals. The horizontal axis gives the bleeding time in minutes and the vertical axis the TXB_2 levels in ng/ml.

The mean level of TXB_2 in the first two minutes was 255.5 $\pm$ 99.2 ng/ml (SD) with a range of 106-400ng/ml which is similar to our levels for serum TXB_2.

We calculated the correlation between the mean level of TXB_2 for the first two minutes and the bleeding time and found a significant correlation ($r = 0.79$). This is shown in Figure 4.5.

4.7 (d) Discussion

In theory one would expect that the more TXA_2 generated the shorter the bleeding time, giving a negative correlation. At first sight it therefore seemed surprising to obtain a positive correlation. However on re-examination of the data it was seen that in many cases volunteers with fairly long bleeding times had high levels of TXB_2 at each time interval whereas one volunteer from whom we were able to obtain only one sample at two minutes had undetectable levels of TXB_2 as is shown in table 4.7.

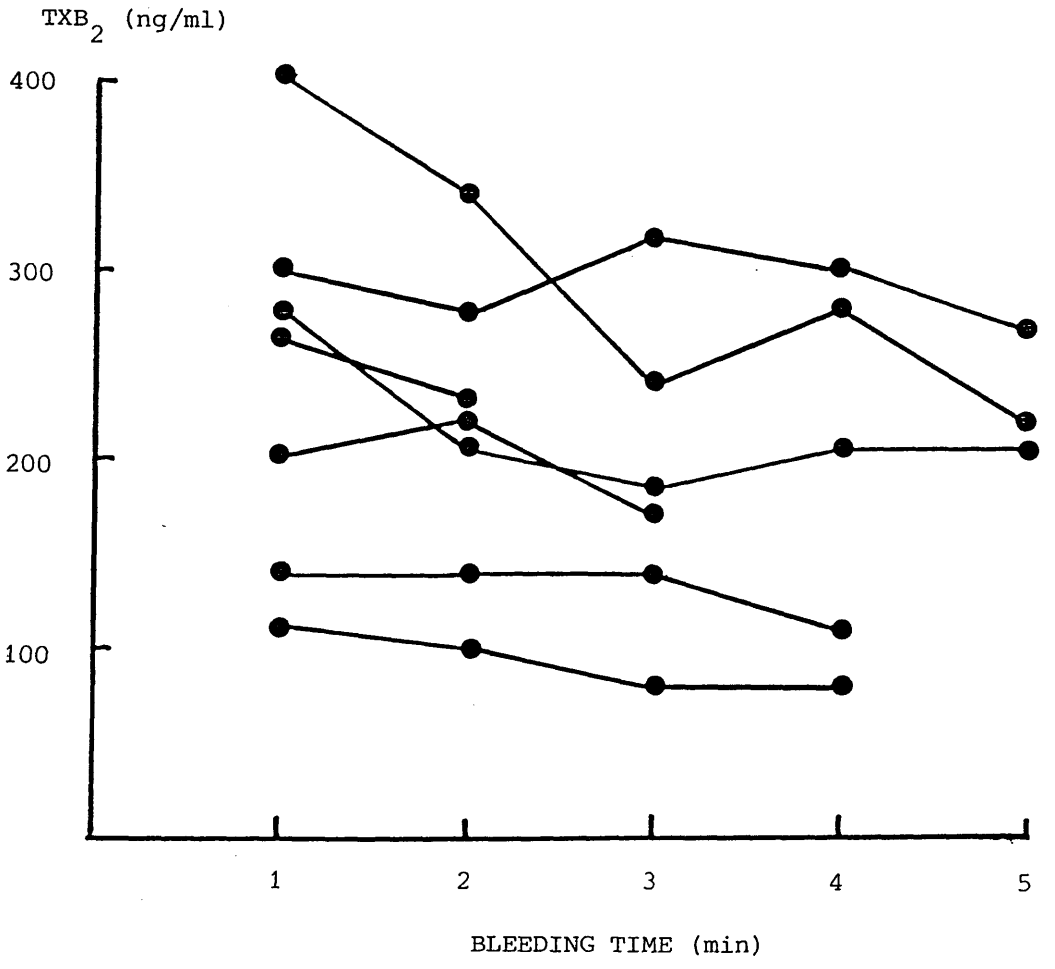


FIGURE 4.4: THROMBOXANE GENERATION IN BLEEDING TIME BLOOD IN SEVEN NORMAL INDIVIDUALS

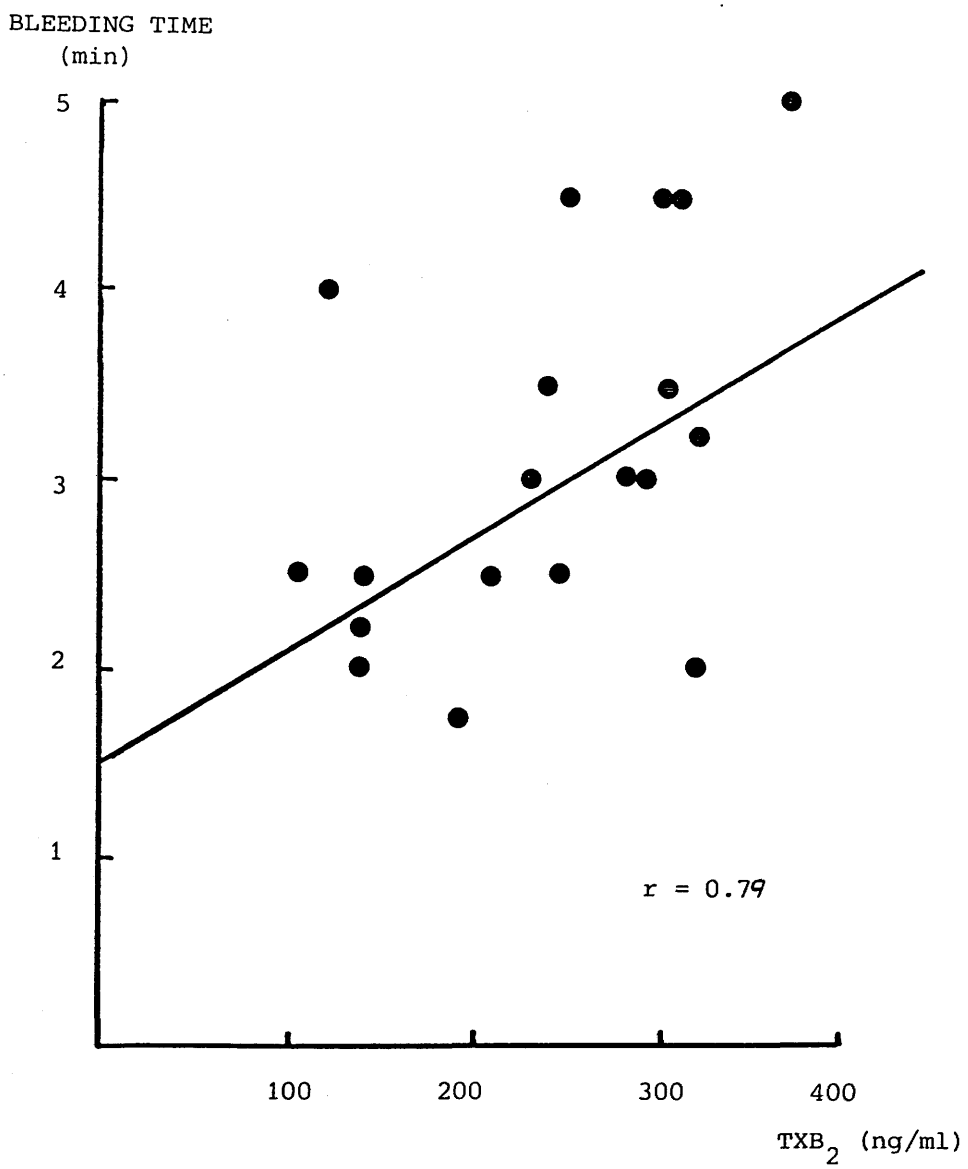


FIGURE 4.5 : CORRELATION BETWEEN BLEEDING TIME AND THROMBOXANE GENERATION IN BLEEDING TIME BLOOD

Volunteer A		Volunteer B	
Time (min)	TXB_2 (ng/ml)	Time (min)	TXB_2 (ng/ml)
1	400	1	no sample
2	400	2	4
3	400		
4	400		
5	400		
6	400		
7	400		
8	400		

Table 4.7 - Bleeding Time serum TXB_2 levels in two normal volunteers

We thought therefore that the levels we were measuring may be simply a reflection of the platelet count in the sample.

If the bleeding time is short, platelets will adhere to the surface of the incised vessel and therefore there will be fewer platelets in the sample to generate TXA_2 during the incubation period. In order to try to prove this we repeated the bleeding time TXA_2 generation test on six new volunteers. This time we sampled TXB_2 serum levels as before but took blood from one of the incisions into a 10µl capillary to perform a whole blood platelet count using an Ultra Flow 100 whole blood aggregometer. The results of this experiment are shown in Figure 4.6.

This experiment did show a significant positive correlation (0.52). However many of the values for serum TXB_2 were greater than the maximum limit of detection of the assay which is 400ng/ml. Since such a small initial volume of serum is obtained using this method it was therefore impossible to repeat these samples at a higher dilution which may have given a closer correlation.

We feel that this new technique is a useful method to measure the amount of thromboxane produced by platelets in response to vascular injury. This will be further discussed in Chapter 9.

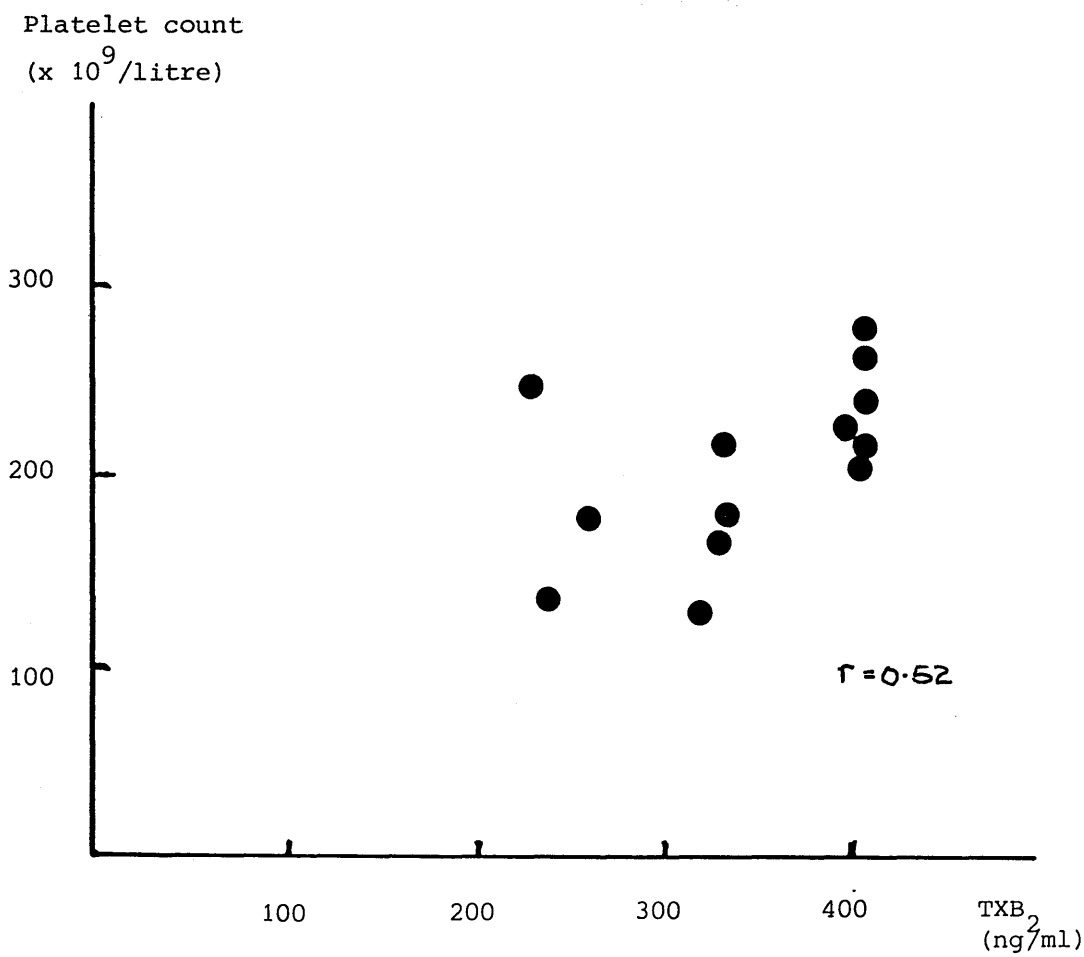


Figure 4.6 : Serum TXB_2 in bleeding time blood
(average of first two minutes)

CHAPTER 5 - CLINICAL SIGNIFICANCE OF PROSTACYCLIN/THROMBOXANE BALANCE. EFFECTS OF SOME VARIABLES ON THIS BALANCE

5.1 Introduction

Since it was discovered that prostacyclin (PGI_2) and thromboxane A_2 (TXA_2), both products of arachidonic acid metabolism, have directly opposing biological actions there has been much interest in the role each plays in the clinical field. TXA_2 is a powerful vasoconstictor and induces platelet aggregation while PGI_2 is a potent vasodilator and inhibitor of platelet aggregation. It would thus seem logical to suppose that manipulation of the balance between the two substances could offer a possible approach to the treatment and prevention of various diseases. To date several diseases have been shown to be associated with an imbalance in the PGI_2/TXA_2 system. As far back as 1977 Legarde showed that platelets from patients with arterial thrombosis, deep vein thrombosis or recurrent venous thrombosis produce more prostaglandin endoperoxides and TXA_2 than normals and that their platelets also have a shorter life span than normals. Shimamoto and colleagues (Shimamoto 1978) have shown that platelets from rabbits in which atherosclerosis has been induced by dietary manipulation produce more TXA_2 and are more sensitive to aggregating agents than platelets from control rabbits. The same has also been claimed in patients who have survived myocardial infarction (Szczeklik 1978). More recently (Yui 1984) it has been shown that there is a significant reduction in serum PGI_2 stimulating factor in patients with acute myocardial infarction compared to patients with angina pectoris or normals. They also found that this activity was much lower immediately after infarction than 80 $\pm$ 17 hours later.

Hirsh (1981) measured TXB_2 levels in coronary sinus blood from patients with unstable angina. They found local TXA_2 release to be associated with recent attacks of angina but were unable to determine whether this was cause or effect. Harrison (1978) showed that diabetic rats had increased release of TXA_2 from their platelets and reduced PGI_2 production from their blood vessels. This was shown to be reversible after long term treatment with insulin. Johnson (1979) has also shown decreased PGI_2 production by blood vessels from diabetic patients. In diabetics complicated by proliferative retinopathy circulating levels of 6-keto-PGF1α when measured by GCMS have been shown to be reduced (Dollery 1979). However Davis et al (1981) failed to show this decrease in circulating levels of 6-keto-PGF1α when measured by RIA, though they were able to confirm the decreased PGI_2 production from blood vessels. Discrepancies in results of circulating levels of 6-keto-PGF1α are not uncommon and are probably due to differences in methodology as discussed in chapter 3. Takahashi (1985) have shown that the activity of thromboxane synthesis as measured by the conversion by platelets of exogenously added ^{14}C-arachidonic acid into ^{14}C-TXB_2 was significantly higher in diabetic patients than in controls. Although there was no significant difference there was a trend towards increased synthesising activity as diabetic retinopathy developed. This activity was shown to decrease significantly on treatment with insulin whereas patients treated by diet or by sulphonylureas maintained their elevated thromboxane synthesising activity. Sensaki (1985) examined the ability of plasma from diabetic patients to stimulate PGI_2 production from "exhausted" rat aortic tissue.

Diabetics showed a significant reduction in this PGI_2 stimulating factor compared to controls. Patients with diabetic retinopathy also showed a significant decrease when compared to patients without this complication. The abnormality appeared to be reversible and related to the degree of diabetic control.

Urinary levels of 6-keto-PGF1α have been shown to be greatly increased in patients with Bartter's syndrome (Gulner 1979). Urinary excretion of 6-keto-PGF1α has been shown to be decreased in patients with essential hypertension (Grose 1980) whereas enhanced PGI_2 production has been reported in blood vessels from spontaneously hypertensive rats (Pace-Asciak, 1978). Urinary levels could however reflect reduced PGI_2 production from the kidney itself rather than from the whole body. A reduction in plasma levels of 6-keto-PGF1α has however been shown in patients with essential hypertension compared to controls (Uehara 1983). They found a significant negative correlation between mean blood pressure and plasma levels of 6-keto-PGF1α . It has been suggested that increased TXA_2 production due to the effects of bilirubin may contribute to the haemostatic disturbancies in neonatal hyperbilirubinemia (Kaapa 1985). Newborns suffering from idiopathic respiratory distress syndrome have been found to have significantly elevated levels of plasma 6-keto-PGF1α (Kaapa 1982, Hutchison 1985). Defreyn (1981) has shown a link between a familiar bleeding tendency and a partial deficiency of thromboxane synthetase. Prostacyclin and thromboxane have also been reported to be associated with various pathological conditions of pregnancy but this will be discussed separately in chapter 8.

From this selection of the hundreds of papers written on PGI_2 and TXA_2 in various disease states it is apparent that there is much interest in the subject. It would seem possible that in diseases where there is a tendency for the development of thrombosis, PGI_2 production is decreased or TXA_2 production increased or both. The opposite may be true in diseases associated with a bleeding tendency.

5.2 Inhibition of Cyclo-oxygenase by Acetylsalicylic Acid

5.2 (a) Introduction

One of the most important discoveries in the development of our knowledge of prostaglandins was when Vane and colleagues (Vane 1971) demonstrated that PGI_2 synthesis could be inhibited by acetylsalicylic acid (aspirin (ASA)). This gave a method by which it was theoretically possible to manipulate the cyclo-oxygenase pathway. However ASA binds covalently to the active site of cyclo-oxygenase and therefore inhibits TXA_2 production as well as PGI_2 (Steiner 1970). Since platelets are unable to synthesise new proteins the enzyme action is inhibited for the entire lifespan of the platelet. Recovery of thromboxane generation only takes place gradually as platelets are renewed and complete recovery takes approximately 10 days. Inhibition of vascular cyclo-oxygenase may however persist for a much shorter period because of the ability of the vasculature to produce new enzyme. There has been much interest shown in the possible theraputic value of prolonged treatment with low dose ASA as an antithrombotic agent. The inhibition of platelet thromboxane generation while still sparing cyclo-oxygenase in the systemic peripheral vasculature therefore represents the pharmacological goal for solving the so-called "aspirin dilemma".

O'Grady and Moncada (1978) showed that in healthy volunteers aged 20-34 years, ingestion of a single dose of aspirin of 0.3gm significantly prolonged the bleeding time, but a dose of 3.9gm had no significant effect on the bleeding time. The same group (Amezuca 1979) showed that following a single high dose of 3.9gm of aspirin given orally the bleeding time was unchanged 2 hours later, although there was marked inhibition of platelet function and TXA_2 release was demonstrable. Twenty four and seventy two hours afterwards the bleeding time was significantly increased returning to normal one week after the administration of aspirin. This suggests that shortly after a high dose of aspirin the production of both TXA_2 and PGI_2 were blocked. As the endothelial cyclo-oxygenase recovery was quicker than the release of new platelets, the ratio of PGI_2 to TXA_2 was changed in favour of PGI_2 at 24 and 72 hours after treatment, thus explaining the increased bleeding time and its slow recovery of up to one week.

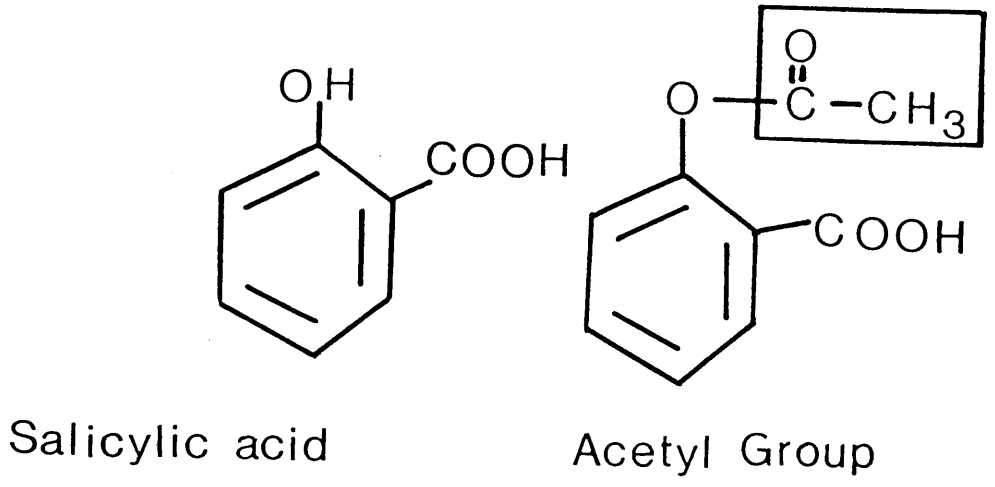
Patrono et al (1980) found that with low doses of aspirin : 0.33 - 0.40 mg/kg body weight there was a remarkable degree of individual susceptibility to the inhibitory effect of the drug on serum TXB_2 levels. This is consistent with the wide variability of the inhibitory effect in vitro (Patrono, 1978). Patrono (1980) also found that a dose dependent reduction of the inhibitory effect of aspirin could only be seen below a threshold dose of 2 mg/kg bodyweight. Thus it would appear that in a normal healthy subject a single oral dose of 2 mg/kg gives a maximal effect on TXA_2 generation by platelets. Burch et al (1978) however found that a maximal effect of 95% was obtained only after a single dose of 650 mg.

It has been suggested that perhaps some of the dilemma is caused by wide inter-individual variability of aspirin pharmacokinetics (Cerlitti 1985). It has also been suggested that an enteric coated preparation of aspirin may deliver little or no aspirin to the peripheral circulation (Ali 1980, Brantmark 1982). The inhibitory effects on platelet cyclo-oxygenase would be the same but vascular PGI_2 synthesis would be spared in the absence of detectable peripheral aspirin levels. However Cerletti et al (1985) found that aspirin levels were undetectable in plasma from three out of the 6 subjects receiving the compressed form of aspirin (320mg) and in 2 out of 6 receiving the enteric coated preparation (800mg) two hours after compressed aspirin and four hours after the enteric coated preparation supporting the suggestion of inter-individual viarability. However because of the potential for erratic absorption of aspirin especially in enteric coating, sampling may require to be more prolonged after ingestion. Rao et al (1985) showed that by 6 hours post ingestion plasma samples from all patients taking 650 mg of enteric coated aspirin showed significant levels of aspirin in their plasma and the only difference between the enteric coated and compressed preparations was in the initial delay in the inhibitory effect due to the slow release from the enteric coated formulations. It may be however that if there is in fact wide inter-individual differences in aspirin pharmacokinetics, if aspirin were to be successfully used as a long term anti-thrombotic drug, each individual would require to be assessed to find the optimum personal dose which would inhibit platelet thromboxane generation while sparing vascular cyclo-oxygenase.

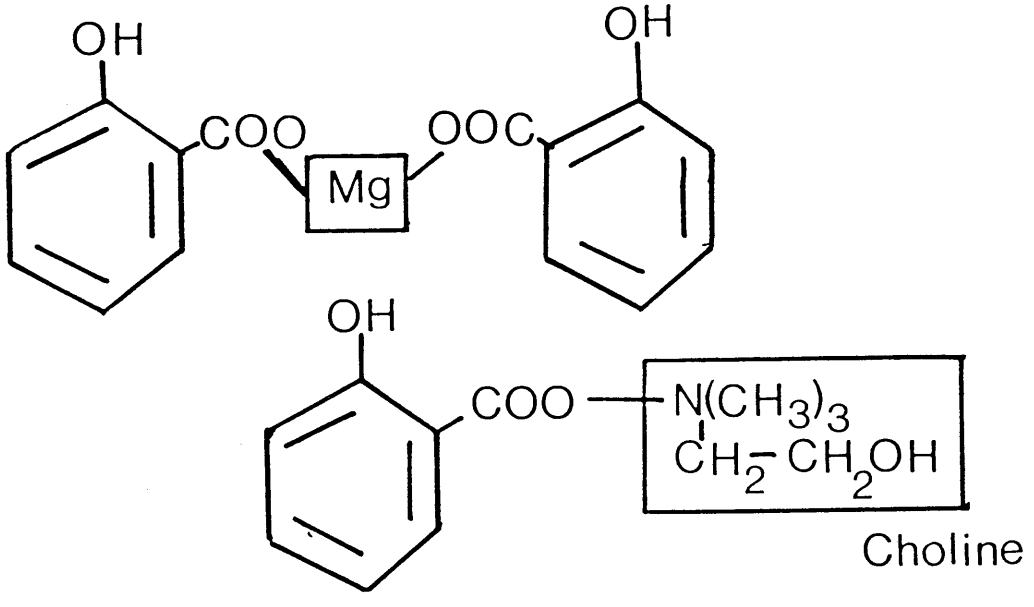
Recently Heavey (1985) showed that when PGI_2 production was stimulated in normal volunteers by an intravenous infusion of bradykinin an oral dose of 600 mg aspirin caused rapid and substantial inhibition of PGI_2 production. Recovery however occurred within six hours showing that even with this dose of aspirin PGI_2 synthesis would be spared most of the time if dosing once daily.

5.2 (b) Importance of the acetyl group in inhibition of cyclo-oxygenase

It is thought that it is the acetyl group in ASA that binds to cyclo-oxygenase thus inhibiting its action. The most well known use of ASA is as an anti-inflammatory drug. In some situations it is important that there is no anti platelet action when anti-inflammatory drugs are required for example in diseases associated with a bleeding tendency. In order to try to demonostrate whether or not the acetyl group is important in inhibition of cyclo-oxygenase we carried out a study to compare the effects of ASA with choline magnesium trisalicylate (CMT) on the cyclo-oxygenase pathway. CMT lacks the acetyl group of aspirin and the chemical structure of each drug is shown in figure 5.1.



ACETYLSALICYLIC ACID (Aspirin)



CHOLINE MAGNESIUM TRISALICYLATE (CMT)

Figure 5.1 : Structure of aspirin and CMT

5.2 (b) (i) Design of study

In our study ten healthy volunteers 6 males and 4 females aged 20 - 24 were given either 625mg aspirin which contains 500mg of salicylate or an equivalent salicylate dose of CMT. Each volunteer received both drugs with an interval of at least two weeks between. Samples were taken immediately prior to and 24 hours post ingestion of the drug. The parameters we examined were plasma and serum TXB_2 levels; TXA_2 generation in response to aggregation with 4ug/ml collagen; serum TXB_2 in bleeding time blood; bleeding times and plasma levels of PGI_2 metabolites. The methodology for all these tests is described in chapters 3 and 4.

5.2 (b) (ii) Results

The following figures shows results for the various parameters measured.

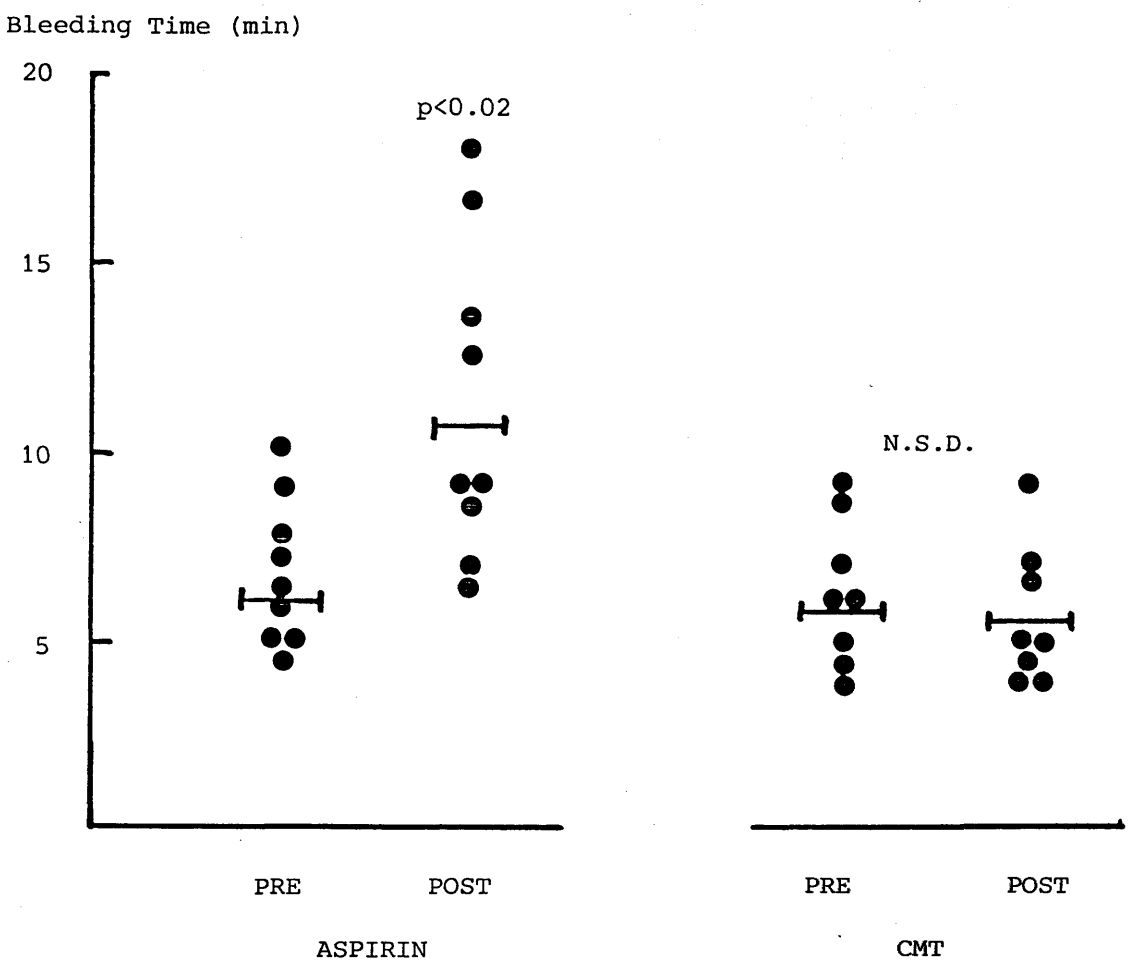


FIGURE 5.2 : BLEEDING TIMES PRE AND POST ASPIRIN AND CHOLINE MAGNESIUM TRISALICYLATE

FIGURE 5.3: PLASMA THROMBOXANE B_2 LEVELS PRE AND POST ASPIRIN AND CHOLINE MAGNESIUM TRISALICYLATE

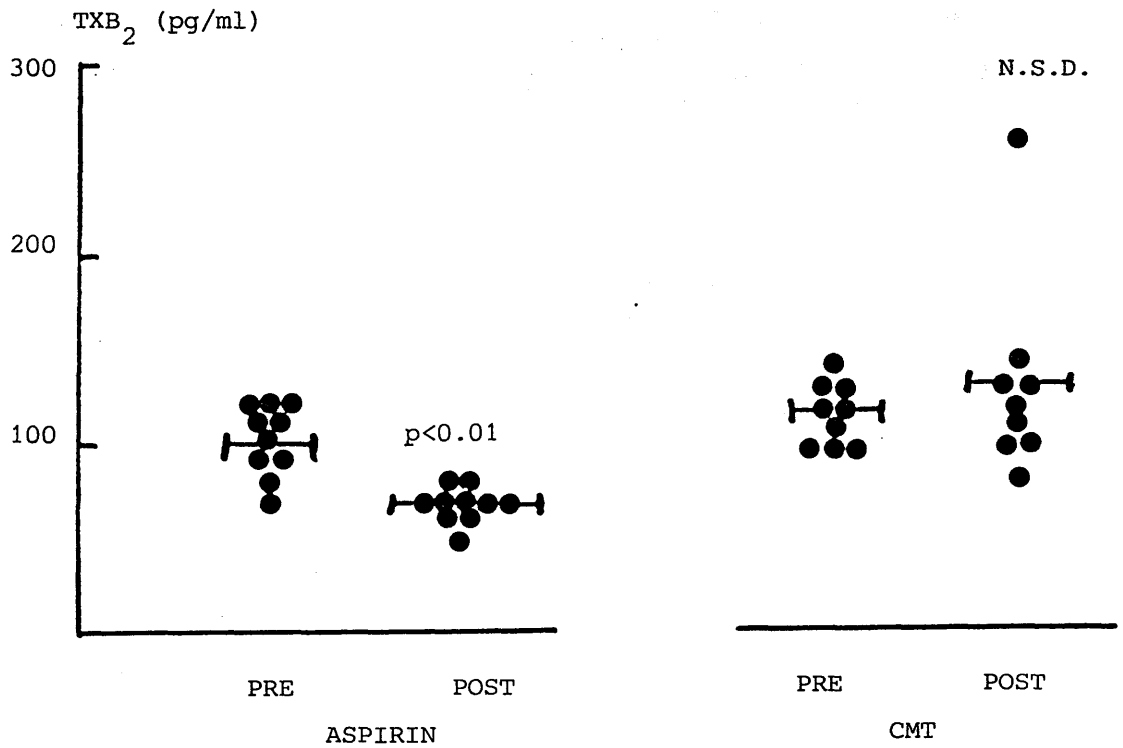
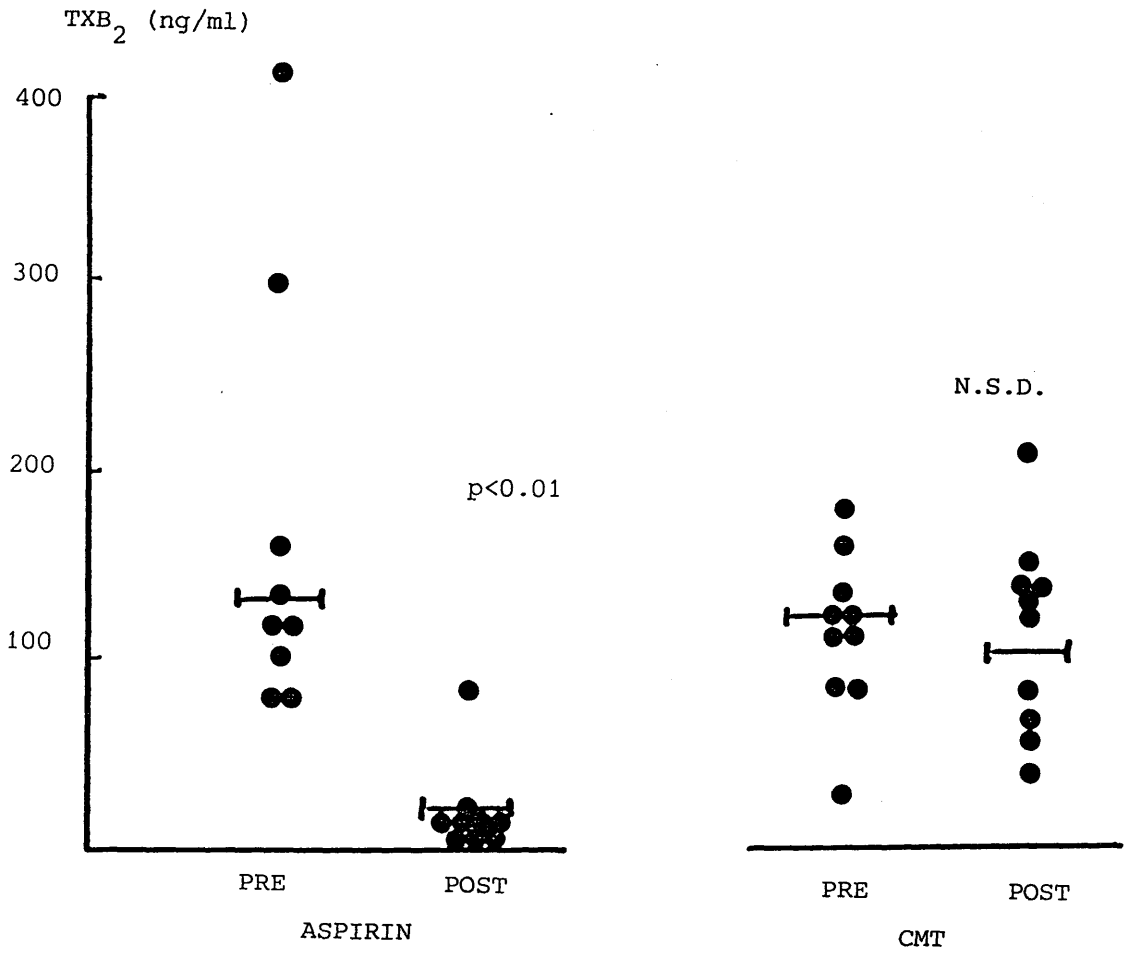


FIGURE 5.4 : THROMBOXANE GENERATION PRE AND POST ASPIRIN AND CHOLINE MAGNESIUM TRISALICYLATE



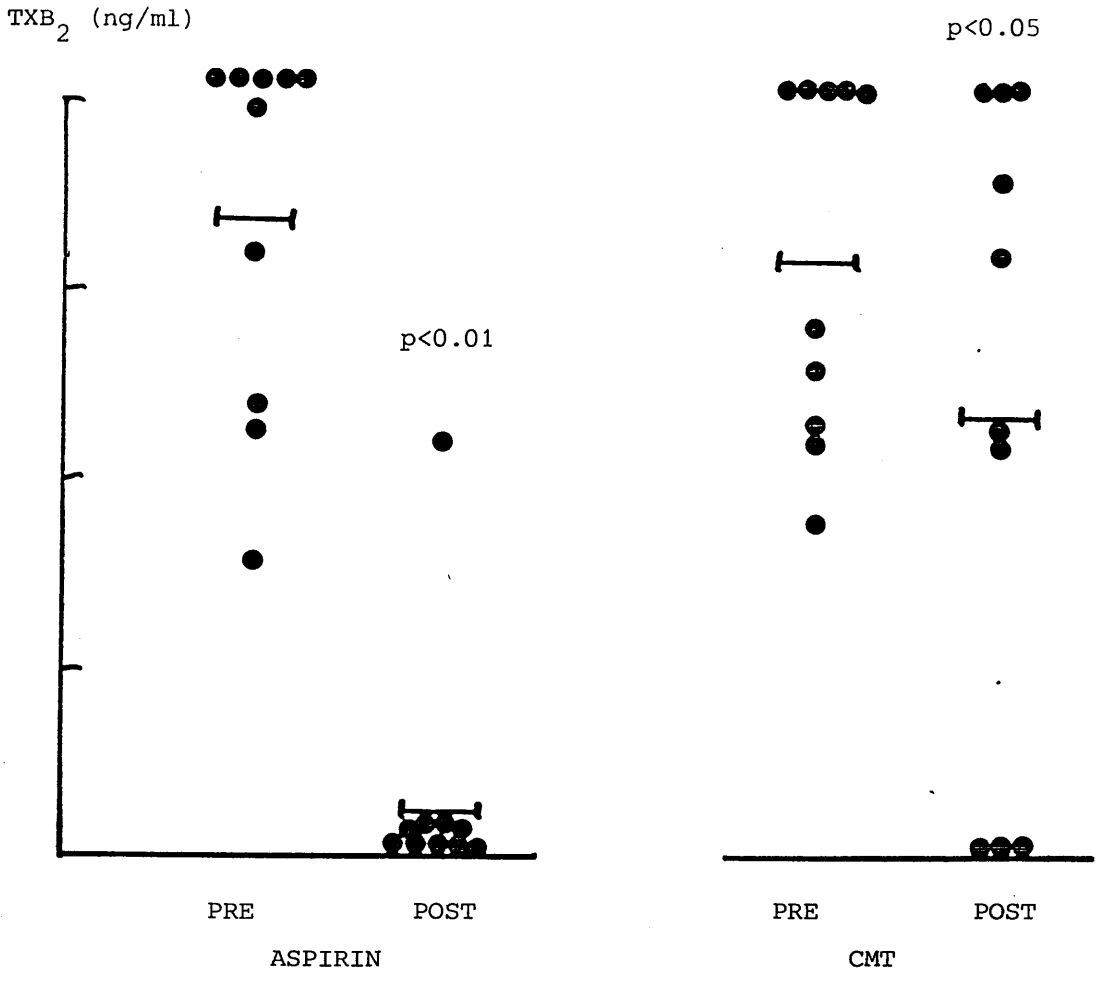


FIGURE 5.5 : SERUM THROMBOXANE B_2 PRE AND POST ASPIRIN AND CHOLINE MAGNESIUM TRISALICYLATE

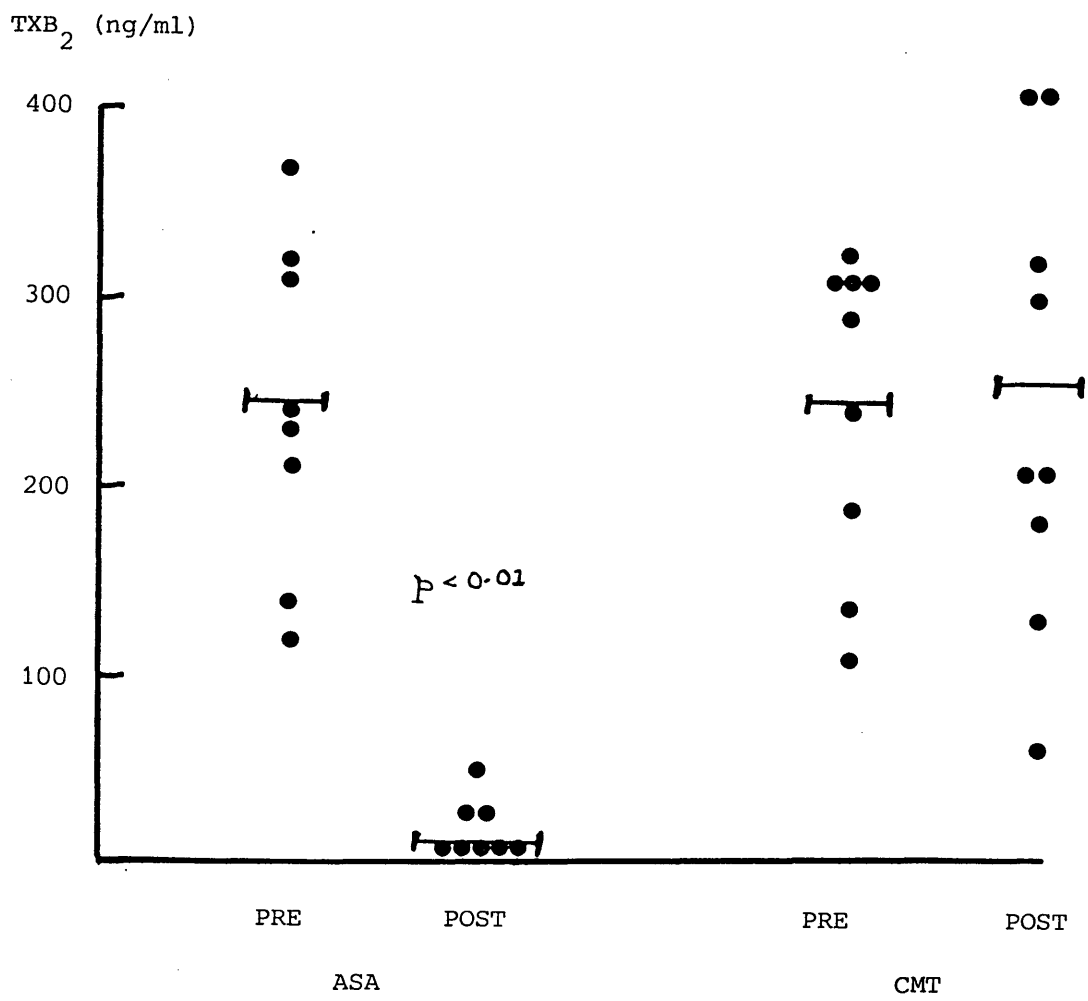


Figure 5.6 : Serum TXB_2 in bleeding time blood pre and post aspirin and CMT

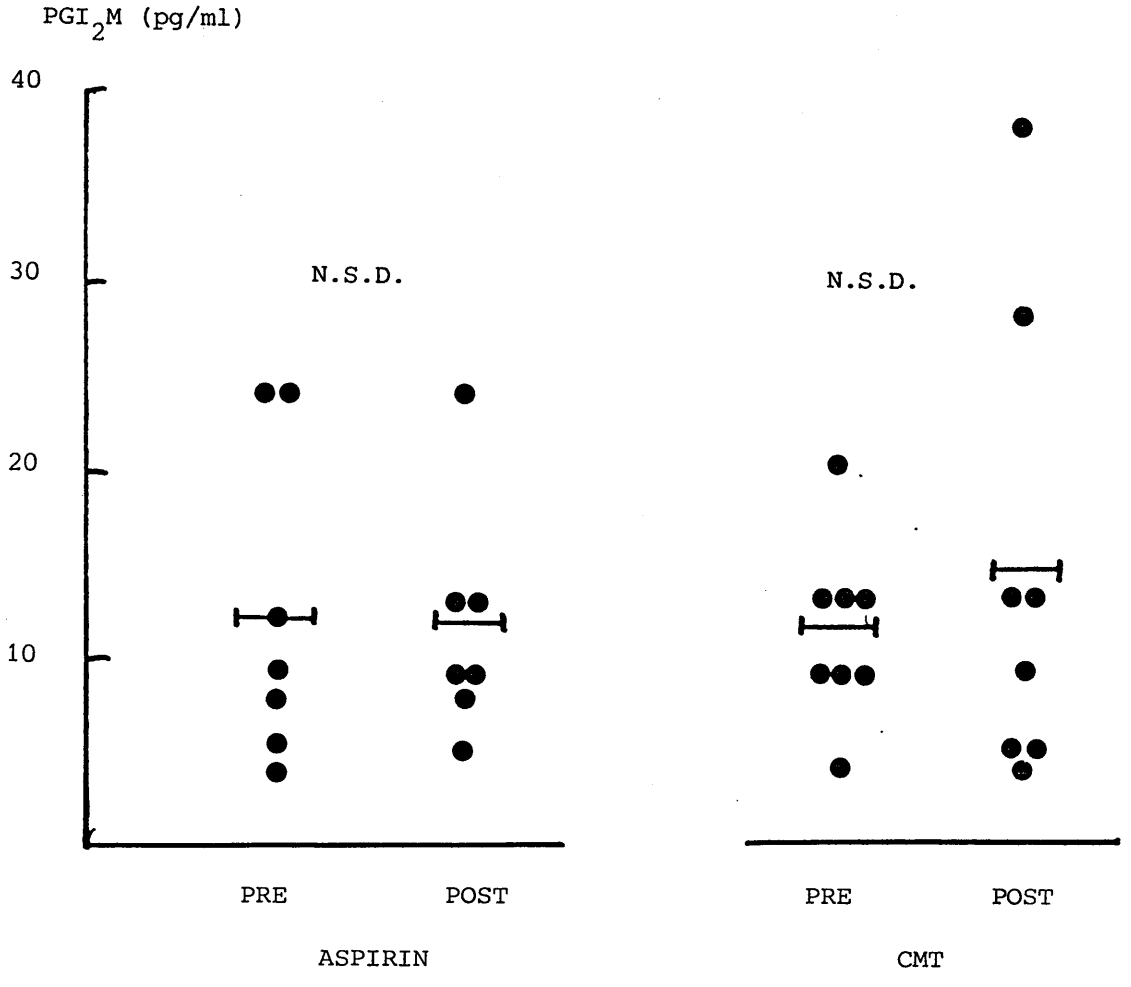


FIGURE 5.7 : PLASMA PGI_2M METABOLITE LEVELS PRE AND POST ASPIRIN AND CHOLINE MAGNESIUM TRISALICYLATE

5.2 (b) (iii) Conclusions

Our results show significant inhibition of thromboxane synthesis twenty-four hours after ingestion of a single oral dose of 625mg aspirin. After an equivalent salicylate dose of CMT however there was no significant differences in any of the parameters measured except serum levels of TXB_2 which were significantly decreased at the 5% level twenty four hours after CMT. Plasma levels of PGI_2 metabolites were not significantly different after either aspirin or CMT. However since normal levels of PGI_2 metabolites are very close to or below the limits of sensitivity of our assay it is not possible to use this measurement to detect decreases in PGI_2 production. Since CMT lacks the acetyl group of aspirin it is therefore unable to block the arachidonic acid pathway by acetylation of the enzyme cyclo-oxygenase. If analgesics are required by patients in whom an anti platelet effect is undesirable, for example those with a bleeding disorder, a salicylate such as CMT which lacks the acetyl group would seem to be preferable.

5.3 Inhibition of cyclo-oxygenase by indomethacin

5.3 (a) Introduction

Indomethacin is an anti-inflammatory drug which is also known to inhibit prostaglandin and thromboxane synthesis. The structure of indomethacin is shown in figure 5.8.

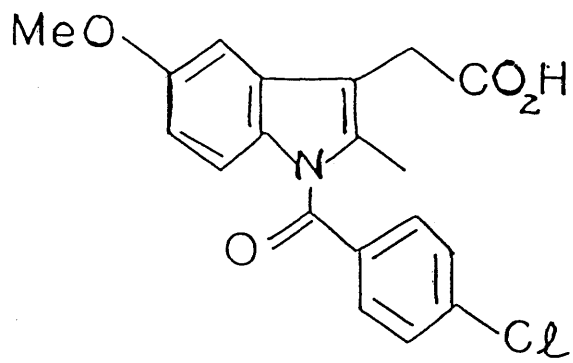


Figure 5.8 - Structure of indomethacin

5.3 (b) Methods

In order to demonstrate inhibition by indomethacin using our methodology we allowed the blood to clot in the presence of indomethacin. A series of glass tubes were prepared containing either indomethacin at varying concentrations or saline. Blood was taken from a normal volunteer and 1ml aliquots were immediately put into the previously prepared glass tubes, mixed and left to incubate for one hour at 37°C. The tubes were then centrifuged at 2500 and 4°C for 15 minutes. The serum was separated and aliquots stored at -70°C for RIA for PGI_2 metabolites and TXB_2 as described in Chapters 3 and 4 respectively. The experiment was repeated eight times at each concentration.

5.3 (c) Results

Figures 5.9 and 5.10 show the levels of serum TXB_2 and plasma PGI_2 metabolites respectively at each concentration of indomethacin (mean $\pm$ SD).

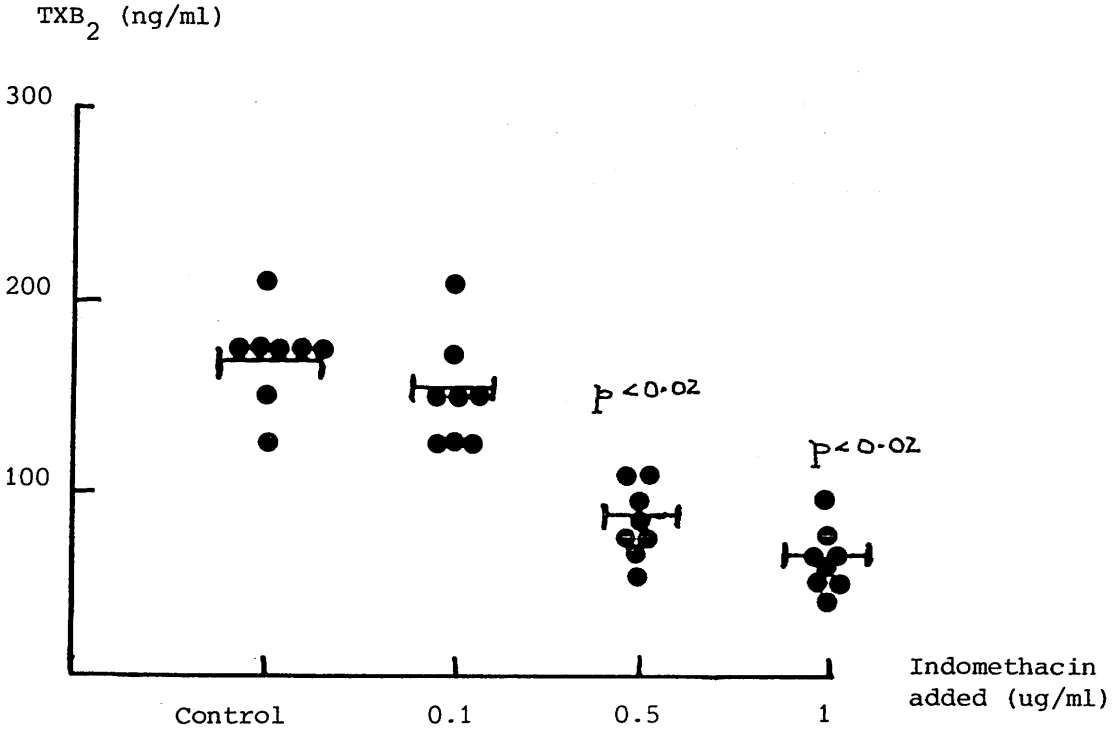


FIGURE 5.9 Whole blood clotted with indomethacin - TXB_2 levels

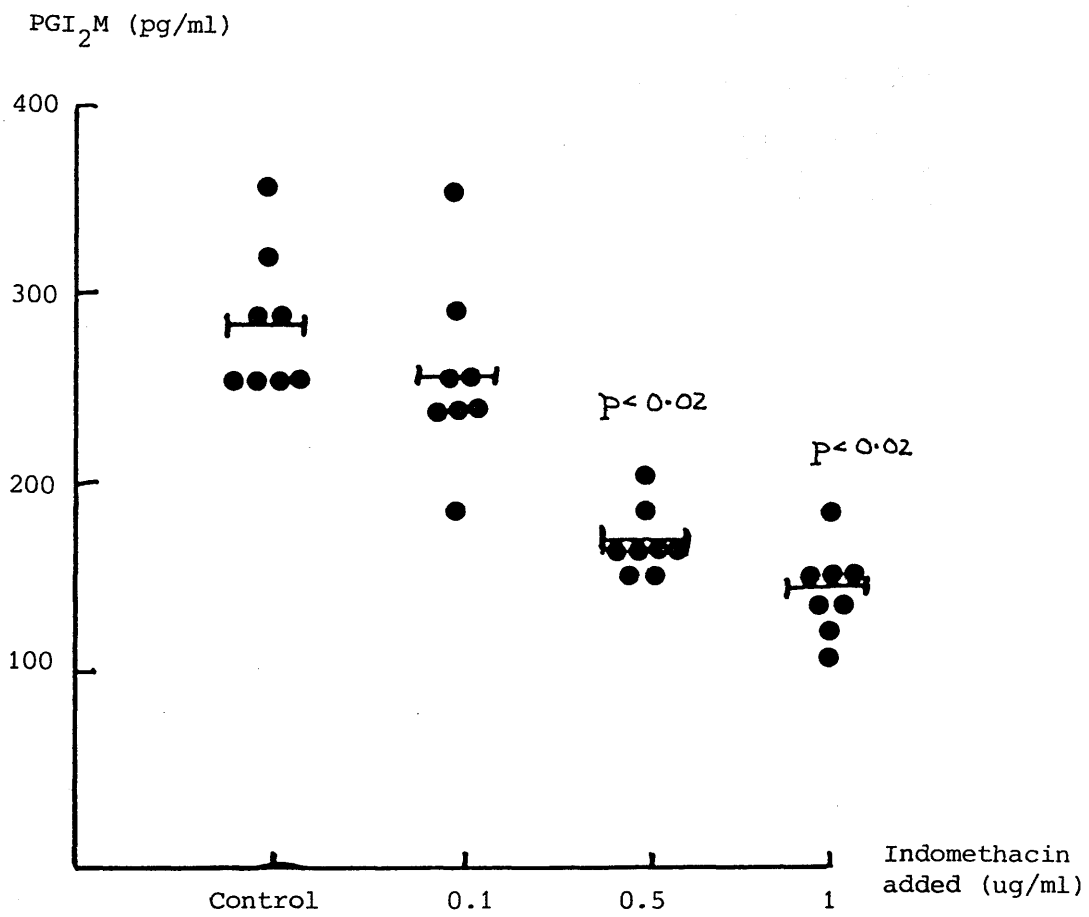


FIGURE 5.10 Whole blood clotted with indomethacin - PGI_2 metabolite levels

5.4 Prostaglandins and Essential Fatty Acids

In the 1920's it was discovered that when rats were fed on a purified, fat-free diet a deficiency syndrome developed with completely different symptoms from those of vitamin E deficiency. By feeding the rats different fats it was shown that the substance in the fats which prevented the deficiency syndrome was in the fatty acid fraction. Linoleic acid, γ-linoleic acid and arachidonic acid gave similar results and the generic term essential fatty acid (EFAS) was given (Burr et al 1930).

At the same time as knowledge of the EFA's grew, knowledge of the prostaglandins increased and gas-liquid chromatography enabled the structure of the primary prostaglandins to be elucidated. The presence of the double bonds in the prostaglandins led Bergström et al 1964 and Van Dorp et al (1964) to suspect that the prostaglandins had their origin in the EFA's and both groups provided evidence that this was in fact true. This work led to the elucidation of the arachidonic acid cascade (Hamberg et al 1974).

Originally it was thought that because the amounts of prostaglandins synthesised were so small and the amounts of the EFA precursors available in cells were comparatively large there could be no relationship between the EFA's and the regulation of PG biosynthesis. However studies on both humans and rats (Epstein 1981, Hornstra et al 1975) have provided evidence that dietary linoleic acid can augment prostaglandin production. When rats are given labelled arachidonic acid the label is found in the cell membrane phospholipids and in the prostaglandins relatively quickly suggesting the source of the PGs to be in the cell membrane.

However this conflicts with evidence from studies done on rabbits (Hassam et al 1979) where rabbits fed on EFA deficient diets did not show any significant change in AA levels in membrane phosphoglycerides over a four month period. The rabbits appeared to conserve the AA and lose the docosahexaenoic acid. However the synthesis of PGE_1, E_2 and F_2 dropped suggesting that there was a relationship between adipose stores of the precursor as opposed to cell membrane components. It has therefore been suggested that there are two sources of PG precursor : endogenous synthesis derived from a metabolic pool and stimulated synthesis derived from the cell membrane pool. The synthesis of PGs relates back ultimately to a dietary source of EFA but it would seem possible that there could be two levels at which the PG-EFA link operates: one in which the metabolism of dietary linoleic acid results in a PG precursor immediately available for PG synthesis the other using membrane AA released by a lipase as a direct percursor for PG synthesis. Whether these two sources lead to production of the same PG, if in fact the concept is correct, has still to be established. The membrane pool of PG precursor would be expected to be more stable and therefore susceptible only to long term changes in diet, whereas the metabolic pathway would be more likely to be directly influenced by dietary changes in EFA's and other fatty acids. If these theories are correct it gives the clinician another way of influencing physiological responses regulated by the prostaglandins and leukotreines using dietary manipulation.

5.5 Dietary Studies

5.5 (a) Introduction

The enzyme systems for the metabolism of fatty acids in general are shared, which means that different fatty acids will compete with each other. It has been shown that saturated fats suppress essential fatty acid activity (Holman, 1977). It has been concluded from whole life studies that during the period in our evolution when man was a hunter-gatherer, food would have had a low fat content, but what fat there was would have been relatively rich in essential fatty acids (Crawford 1968). The fat in the meat of wild animals or fish is mainly structural fat rich in phosphiglycerides. Plant food is also generally rich in essential fatty acids. In our modern domestic animals, which have been bred to produce meat, there is a higher proportion of saturated non essential fats. This is also true of much of our modern diet. The balance of fats in man's diet has therefore changed to the situation where non essential and saturated fatty acids dominate. In Western countries approximately 40% of the dietary energy comes from fat and about 4% of this is derived from essential fatty acids. It would thus seem likely that the essential fatty acids have significant competition with the saturated fats for the enzyme systems necessary for their metabolism. It may be that the high incidence of atherosclerosis and thrombosis in Western society is due to the high levels of saturated fat in our diet which lead to a relative deficiency of essential fatty acids. Thus the requirements for essential fatty acids in a Western diet could be higher than expected because of this competition with the non-essential fats.

It is now generally accepted that the type and amount of dietary lipids may alter the thrombotic tendency of the blood. (Lowe et al 1979, Johnston et al 1982) and over a long term with the development of atherosclerosis (Heyden et al 1975). Epidemiological studies (Renaud 1979, Kannel 1970) indicate that a diet rich in saturated fats has a distinct negative influence on coronary artery disease, whereas a polyunsaturated diet might have a protective effect (Horstra 1973). In several other conditions associated with thrombosis and arterial disease platelet behaviour is influenced by alterations in dietary lipids (Nordoy 1971) and it has been well documented that alimentary hyperlipidaemia alone may cause platelet activation (Lowe et al 1979, Johnston 1982, O'Brien 1976). That this may be linked to clinical disease is suggested by work done both by Regan (1961) and Kuo (1955) who demonstrated decreased oxygen delivery to the myocardium after a fat meal and an increased frequency of angina attacks in fat fed patients with ischaemic heart disease. The mechanism by which a saturated fat diet produced this platelet activation may be related to an alteration in prostanoid production and in particular to an increase in production of thromboxane A_2 (Hornstra 1982). Because of this we decided to look at the effects on prostacyclin and thromboxane production of a saturated fat meal in healthy male volunteers. If the effects of saturated fat on platelets are mediated through an increase in TXA_2 it might be reasonable to suppose that selective inhibition of TXA_2 might normalise platelet behaviour. UK 38, 485 (Pfizer Ltd) is an imidazole derivative which has been shown to inhibit thromboxane synthetase. Inhibition may have the added benefit of augmenting PGI_2 production (Parry 1983) which should further protect against thrombosis.

We therefore repeated the fat feeding experiment on the same subjects one month later after pretreatment with a thromboxane synthetase inhibitor.

5.5 (b) Subjects and Methods

Ethical permission was obtained. 10 healthy male volunteers of average age 28 ± 7.1 years (SD) were enrolled in the study after giving informed consent. The protocol was similar to that used previously (Lowe 1979, Johnston 1982). The subjects fasted for 12 hours overnight after which baseline blood samples were taken. 150ml of double cream were then ingested (fatty acid content see Table 5.1).

Further blood samples were taken by separate venepuncture 2 and 4 hours later and again after an overnight fast, 24 hours later. After a one month washout period the procedure was repeated but this time the subjects had received pretreatment with 100 mg of UK 38 485 twenty four, twelve and one hour before ingestion of the cream. TXB_2 and PGI_2M were measured in plasma samples as described in chapters 3 and 4. Serum lipids were measured for us in the routine biochemistry laboratory.

Table 5.1 shows the fatty acid composition of double cream.

Saturated	28.8g/100g cream
Monosaturated	15.4g/100g cream
Polyunsaturated	1.3g/100g cream

Table 5.1 - Fatty Acid Composition of Double Cream

5.5 (c) Results

The results obtained are shown in Table 5.2. The first line of each pair is the control value and the second the results of pre treatment with a thromboxane synthetase inhibitor.

	PRE	2 hrs	4 hrs	24 hrs	
Triglyeride	1.1 ± 0.1	2.0 ± 0.1 **	2.7 ± 1.6 **	1.0 ± 0.1	- UK
mmol/l	1.1 ± 0.1	2.0 ± 0.1 **	3.1 ± 1.9 **	1.0 ± 0.1	+ UK
Cholesterol	5.9 ± 1.0	5.8 ± 1.0	5.8 ± 1.0	5.7 ± 1.3	- UK
mmol/l	5.9 ± 1.3	6.0 ± 1.3	6.0 ± 1.4	5.8 ± 1.3	+ UK
PGI_2M	11.9 ± 3.2	12.1 + 4.3	12.6 ± 4.6	13.4 ± 4.1	- UK
pg/ml	14.3 ± 3.9	14.4 ± 4.7	12.4 ± 4.2	16.6 ± 13.9	+ UK
TXB_2	137 ± 28	129 ± 24	154 ± 108	164 ± 82	- UK
pg/ml	119 ± 27 *	122 ± 26	119 ± 33	173 ± 111	+ UK

* $p < 0.05$ student t test

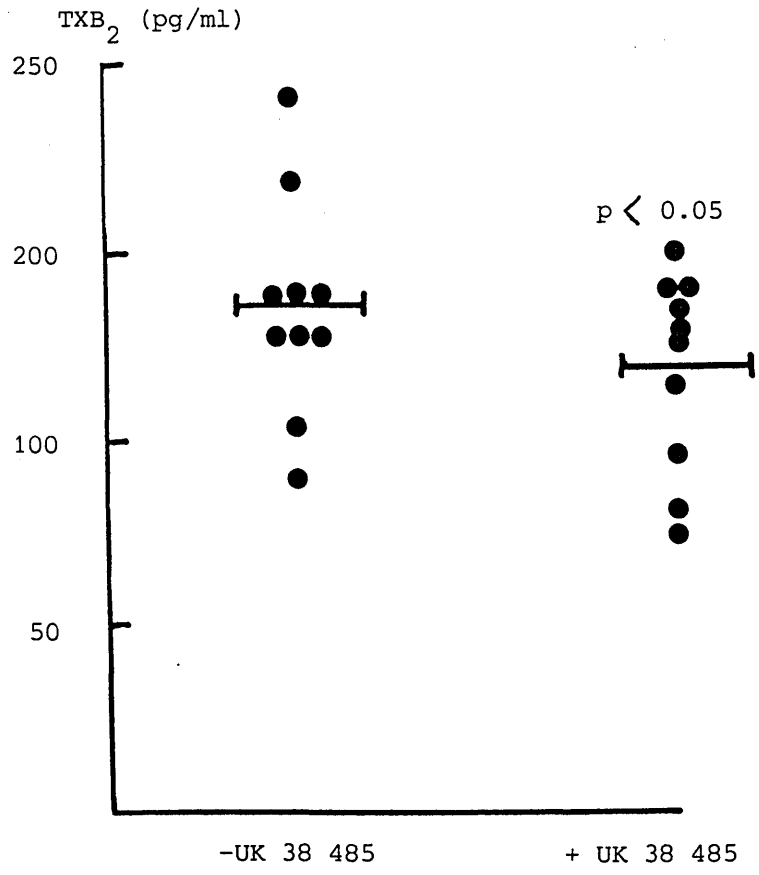
** $p < 0.001$ paired t test

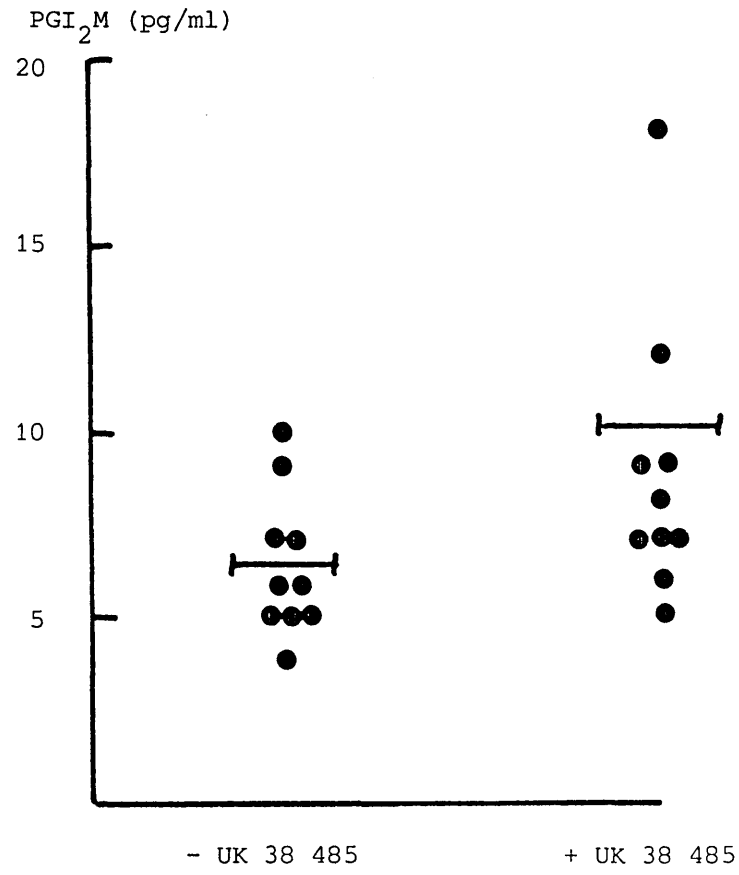
Table 5.2 - Plasma levels of triglyceride, cholesterol, PGI_2 metabolites and TXB_2 with and without pretreatment with a thromboxane synthetase inhibitor

5.5 (d) Discussion

The only result to reach statistical significance was the baseline level of TXB_2 after ingestion of UK 38 485. This is shown graphically in Figure 5.11. Baseline PGI_2M levels were higher after the UK 38 485 was given but this did not reach statistical significance. Our results therefore provide no evidence to suggest that the mechanism by which a saturated fat diet produces platelet activation is related to any alteration in the prostacyclin/thromboxane balance.

FIGURE 5.11 : Plasma levels of PGI_2 Metabolites and TXB_2 before and after thromboxane synthetase inhibitor UK 38 485





5.6 Dietary Studies - Orally administered evening primrose oil to normal volunteers

5.6 (a) Introduction

'Efamol' capsules manufactured by Efamol Ltd were taken by ten normal healthy volunteers at a dose of 12 capsules per day for a ten week period. Ethical committee permission and informed consent had been obtained. Each capsule contained 500mg evening primrose oil which supplies approximately 40mg gamma-linoleic acid and 350mg linoleic acid in a shell composed of gelatin and glycerin. 10mg of natural Vitamin E is added to each capsule. Since prostaglandins of the 1-series can be obtained by anabolic desaturation and chain elongation from dihomo-γ-linoleic acid this might be expected to show a shift in metabolism away from the 2-series prostaglandins. Blood samples were taken prior to and at fortnightly intervals during ingestion of the Efamol capsules. Blood was handled and RIA of plasma PGI_2 metabolites was performed as described in Chapter 3.7. In this study we also measured plasma PGE_2 metabolites using a RIA kit supplied by Amersham Ltd, Amersham, Buckinghamshire.

5.6 (b) Results

The results of this study on PGI_2 metabolites are shown graphically in Figure 5.12 and on PGE_2 metabolites in Figure 5.13.

5.6 (c) Discussion

Seven out of the ten individuals showed an increase in plasma levels of PGI_2 metabolites at some point during the study although at no time did levels reach statistical significance.

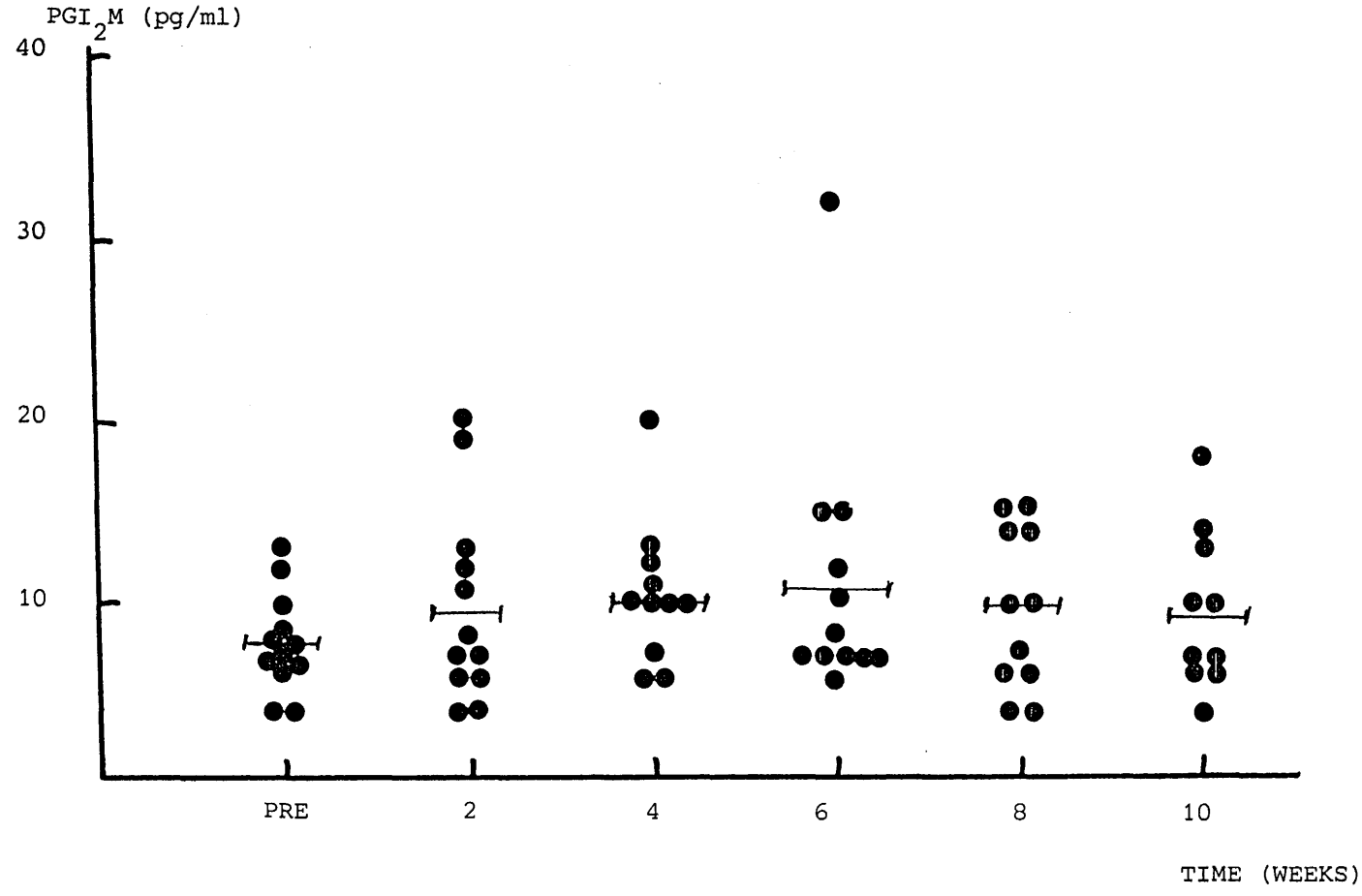


FIGURE 5.12 : PLASMA PGI_2M METABOLITE LEVELS IN NORMAL VOLUNTEERS TAKING 'EFAMOL' CAPSULES OVER A TEN WEEK PERIOD

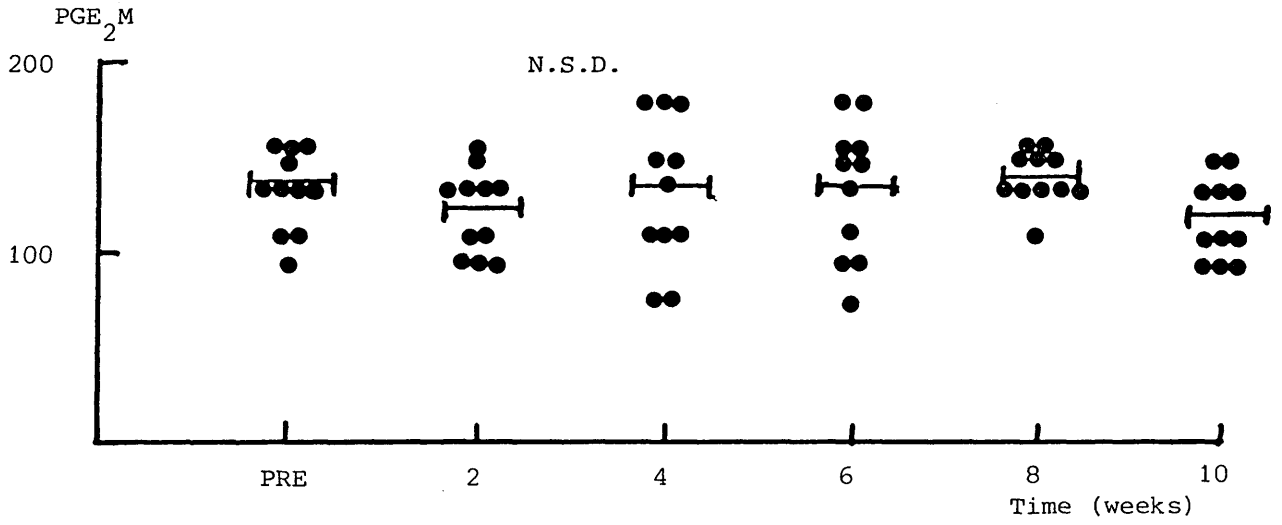


Figure 5.13: Plasma PGE_2 metabolite levels in patients taking 'Efamol' capsules

There was also no obvious sex difference: of the three who showed no increase in levels of PGI_2 metabolites two were male and one was female. PGE_2 metabolite levels also show no significant difference. These results would therefore appear to show no evidence of a shift in prostaglandin synthesis away from the 2-series prostaglandins. However since normal levels of PGI_2 metabolites in plasma are at or near the lower limit of detection of the assay it is very unlikely to give statistically significant results where decreased PGI_2 production would be expected. This will be discussed more fully in chapter 9 of this thesis. However PGE_2 metabolite levels also showed no significant differences. In this study further investigation of the 1-series prostaglandins might have shown evidence of a shift from 2-series to 1-series prostaglandins.

5.7 Cigarette smoking and PGI_2/TXA_2 production

5.7 (a) Introduction

The incidence of ischemic heart disease is significantly increased in cigarette smokers (Fredrickson 1977) the risk being proportional to the number of cigarettes smoked. Although the exact relationship between the two is not properly understood, enhanced platelet aggregability has been reported by several investigators (Glynn 1966, Levine 1973, Bierenbaum 1978). Some evidence has been published that this could be due to the direct effect of nicotine (Werle 1965, Saba 1975) but this is not confirmed by others (Brinson 1974). However more recently the effect of cigarette smoking on platelet aggregation has been questioned (Seiss 1982, Laszlo 1983) and platelet hyperaggregability following acute cigarette smoking has been shown to be an immediate effect disappearing within half an hour (Davis 1979, Hawkins 1972).

Cigarette smoking has also been shown to increase circulating platelet aggregates by some workers (Davis 1979) while others were unable to confirm this effect (Hillborn 1985). Smoking has also been shown to increase platelet count (Seiss 1982). In the earlier studies platelet count was not controlled and increasing platelet count could have been the reason for the supposed increased platelet reactivity. A number of studies have reported an inhibitory effect of smoking on prostacyclin production (Nadler 1983 , Pittilo 1982 , Isotel 1982). It has also been shown that brief periods of smoking in non-smokers leads to increased TXA_2 production but only a slight increase in PGI_2. In contrast, similar periods of smoking in habitual smokers is not accompanied by increases in either TXA_2 or PGI_2 (Mehta 1982).

Nicotine has been shown to inhibit the release of PGI_2 in human and in laboratory animals' blood vessels (Alster 1981, Wennmalm 1983) whereas other workers have been unable to show this (Hartiala 1982). Jeremy et al (1985) have shown that high concentrations of up to 1g/l of nicotine were unable to inhibit in vitro synthesis of PGI_2 by human umbilical artery or by rat or rabbit aorta or rat lung. However they found that aqueous and ethanolic cigarette smoke extracts did inhibit PGI_2 synthesis and release in these tissues. These extracts contained a maximum of 26mg/l of nicotine and therefore this excluded nicotine as the inhibitory component of the cigarette smoke extract. It would appear therefore that although cigarette smoking is well recognised as a major risk factor in the pathogenesis of vascular disease (Murphy 1966, Dawber 1959, Spain 1970), the evidence as to the mechanisms involved is still conflicting. Also evidence of the involvement of PGI_2 and TXA_2 is unclear and inconclusive.

5.7 (b) Design of Study

We measured plasma PGI_2 metabolite levels in six healthy male volunteers after acute smoking. Each subject smoked two cigarettes in succession and blood samples were taken at 0, 30, 60 and 120 minutes thereafter as described in chapter 3.7(b). The volunteers were of average age 31.4 $\pm$ 4.6 years (SD) and all were regular smokers.

5.7 (c) Results

The results for each individual are shown graphically in figure 5.14.

5.7 (d) Discussion

Although the numbers are very small in this study four out of the six individuals studied showed an increase in plasma levels of PGI_2 metabolites from the baseline level. Mehta (1982) showed a slight increase in plasma 6-keto-PGF1α levels after smoking in non-smokers although they were unable to detect this in habitual smokers. A study using larger numbers of volunteers would have been preferable and also a study of the effects of smoking on non-smokers. However we found it difficult to find larger numbers of habitual smokers to take part in the study. It also seemed unethical to persuade non-smokers to participate. However if this is in fact a true result it could be a defense mechanism to counteract the platelet hyperagregability found by some researchers (Glynn 1966, Levine 1973, Bierenbaum 1978).

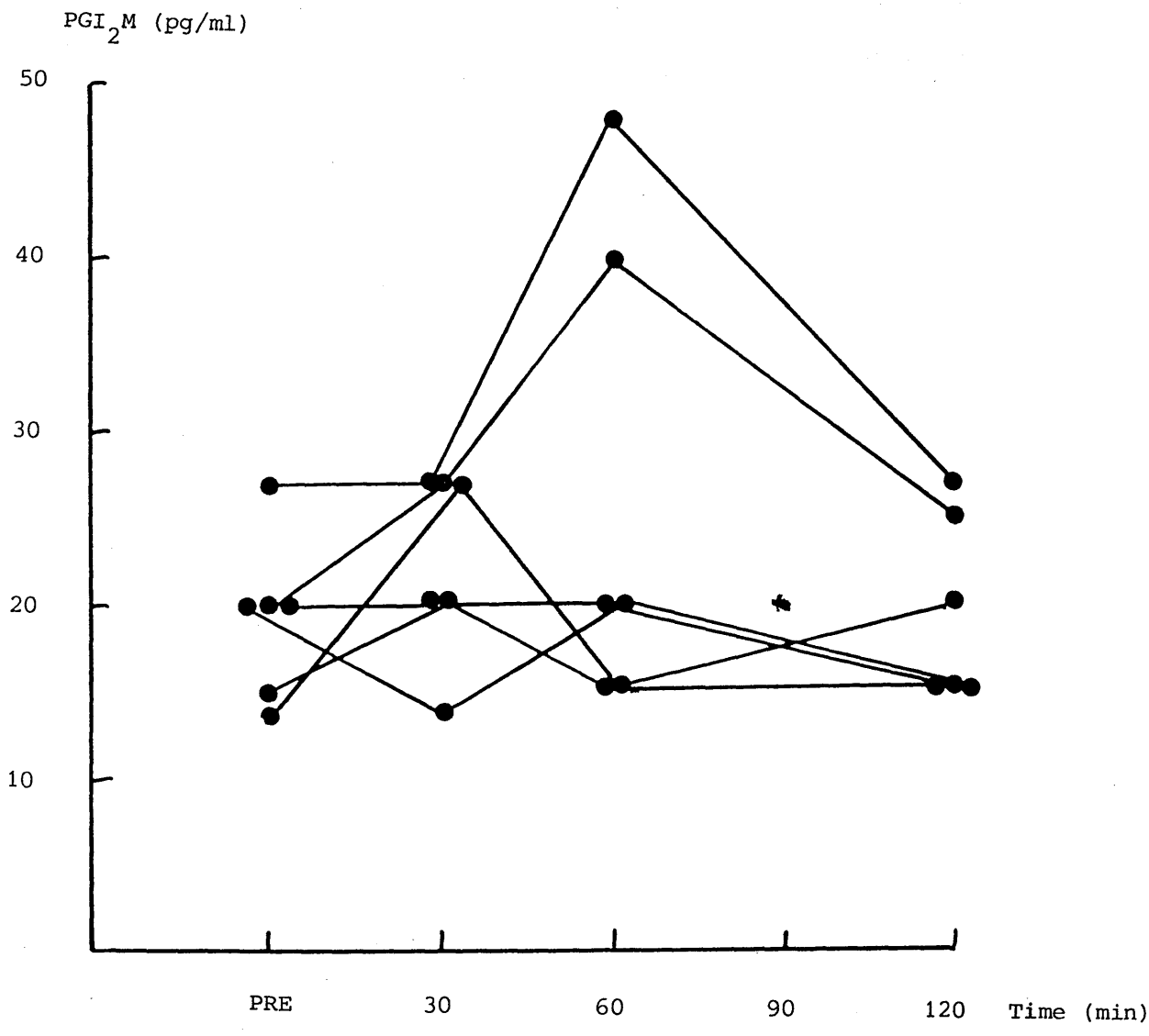


FIGURE 5.14 : Levels of plasma PGI_2 metabolites after smoking

5.8 Conclusion

The studies presented in this chapter have shown that, using the methodology described in chapters 3 and 4, we can show inhibition of thromboxane production by aspirin and that the acetylation of the cyclo-oxygenase enzyme by the acetyl group of aspirin appears to be the mechanism by which this inhibition occurs. We have also shown inhibition of thromboxane and prostacyclin in whole blood incubated with indomethacin. The dietary studies on ingestion of double cream and also of 'Efamol' capsules show no changes in the thromboxane/prostacyclin balance after dietary manipulation.

There is some indication that the balance of thromboxane prostacyclin may be altered in favour of prostacyclin after smoking in some subjects but a larger study would be necessary to show whether this is of clinical significance in somking related diseases and this is discussed in chapter 9.

CHAPTER 6 - PROSTACYCLIN AND THROMBOXANE IN SOME VASCULAR DISEASES

6.1 Raynaud's Syndrome

6.1 (a) Introduction

Raynaud's phenomenon is the name given to a peripheral vascular disturbance consisting of spasmodic contraction of the digital arteries, which is precipitated by cold and emotion. Primary Raynaud's phenomenon is commonest in young women and is an exaggerated physiological response to cold. Secondary Raynaud's occurs in response to various factors one of which is associated with certain occupations in which the arms and hands are exposed to vibrating tools e.g. pneumatic drills, chain saws, polishing tools etc. Secondary Raynaud's may also occur in connective tissue disorders especially scleroderma. In the early uncomplicated stages there are no pathological changes, however thrombosis in the lumen may develop later leading to ischaemic changes in the skin of the fingers and in the nails, followed by superficial necrosis and finally gangrene (De Takatas 1962). Fingers are usually more affected than toes and the clinical presentation would be numbness, tingling, burning and then pain. Sensitivity to cold may be extreme and disabling. In varying degrees of severity Raynaud's phenomenon exists in 5-10% of the population (Blunt 1981). However in the vast majority of cases it requires only avoidance of cold and the wearing of gloves to relieve the symptions. Various methods of treating the severe cases of Raynaud's syndrome have been tried: intra-arterial reserpine (Elbaor 1971) oral vasodilators (Leyroy 1971) sympathectomy (De Takatas 1962), fibrinolytic enhancement (Jarrett 1981) and plasma exchange (Zahavi 1980) have been used with varying effect.

6.1 (b) Prostacyclin and Thromboxane in Raynaud's syndrome

Recently treatment with PGI_2 has been shown to be of benefit to patients suffering from Raynaud's phenomenon (Belch 1981) as has treatment with prostaglandin E_1 (Clifford 1980). Intravenous infusions of these prostaglandins have been shown to increase blood flow for up to six weeks after the infusion. The clinical improvement observed after infusion would suggest either inadequate production of the vasodilatory prostaglandins or resistance to normal or even increased production. Platelets from patients with Raynaud's phenomenon have been found to be less sensitive to the anti-aggregatory effects of PGE_1 and PGI_2 when cooled to $27^{o}C$ (Mikhailidis 1981). It has also been shown that lymphocytes (Inoshita 1981) and endothelial cells (Whicker 1980) from patients with systemic sclerosis are more resistant than normal to the effects of the E-series prostaglandins. In systemic sclerosis collagen deposition in vessels may contribute to the development of Raynaud's phenomenon. Fiocco (1982) and Horrobin (1983) have reported increased prostaglandin levels in patients with systemic sclerosis. These studies provide evidence of cellular resistance to rather than inadequate production of the vasodilatory prostaglandins.

6.1 (c) Plasma levels of PGI_2 metabolites in patients with Raynaud's syndrome

6.1 (c) (i) Patients and Methods

Using the methodology described in chapter 3 we measured plasma levels of PGI_2 metabolites in fifteen patients with Raynaud's syndrome alone and in fifteen patients with systemic sclerosis and Raynaud's syndrome.

None of the patients was taking any drug known to alter prostaglandin production and none were on the contraceptive pill. Fifteen age and sex matched controls were also studied. Patients were left to sit in a warm room for at least half an hour prior to blood sampling to ensure that no blood sample was taken during a Raynaud's attack. The samples were coded so that the assays were performed blind.

6.1 (c) (ii) Results

The levels of plasma PGI_2 metabolites in each group are shown in table 6.1

	Primary Raynaud's phenomenon	Raynaud's + system sclerosis	Controls
PGI_2 metabolites (pg/ml)	11 ± 2	51 ± 5 *	16 ± 2

* $p < 0.01$

Table 6.1 - Plasma PGI_2 metabolite levels in patients with Raynaud's phenomenon and systemic sclerosis, Raynaud's phenomenon and controls (+ SD)

6.1 (d) Raynaud's phenomenon associated with vibratory injury

6.1 (d) (i) Introduction

In some patients with Raynaud's phenomenon the disease is associated with certain occupations in which the arms and hands are exposed to vibrations from machinery used at work such as pneumatic drills or polishing tools. The term secondary Raynaud's phenomenon is used to describe this type of clinical syndrome.

PGI_2 metabolites and plasma TXB_2 levels were measured in fifteen patients with this type of secondary Raynaud's phenomenon. We compared them to age and sex matched controls and also to fifteen patients with primary Raynaud's phenomenon. The methodology used is described in chapters 3 and 4.

6.1 (d) (ii) Results

Plasma levels of PGI_2 metabolites in fifteen patients with Raynaud's syndrome caused by vibratory injury are shown in table 6.2 compared to patients with primary Raynaud's phenomenon and controls.

	Secondary Raynauds	Primary Raynauds	Controls
PGI_2 metabolites (pg/ml)	* 24.9 ± 16.2	11 ± 2	16 ± 2
TXB_2 (pg/ml)	** 131.8 ± 32.9	104.1 ± 38.2	95.9 ± 25.7

* significantly different from controls $p < 0.05$ and from secondary Raynaud's $p < 0.01$

** significantly different from primary Raynaud's and controls $p < 0.005$

Table 6.2 - Plasma levels of PGI_2 metabolites in patients with secondary Raynaud's associated with vibratory injury compared to patients with primary Raynaud's and controls

6.1 (e) Discussion

This study has shown increase levels of plasma PGI_2 metabolites in patients with systemic sclerosis and Raynaud's phenomenon and also in patients with secondary Raynaud's phenomenon associated with vibratory injury. Since it has previously been shown that intravenous infusions of PGI_2 are beneficial in Raynaud's syndrome (Belch 1981, Dowd 1982) this result was unexpected. However this increase PGI_2 production may be a compensatory mechanism. Vascular damage is known to occur in systemic sclerosis. In the early stages this is suggested by an increase in factor VIII released from the endothelium (Kahaleh 1981) and later microscopic changes can be demonstrated (Kahaleh 1979). Since the endothelium produces PGI_2 in response to noxious, chemical or mechanical stimuli (Ritter 1983) repeated vasospastic attacks could lead to a chronic increase in PGI_2 production. Since the blood vessels would then be chronically subjected to these high levels of PGI_2 the muscle cells may have become desensitised to the action of PGI_2. Another possibility is that the cells are resistant to the effects of PGI_2. PGI_2 increases cyclic AMP and it is thought that this acts as a negative feedback to PGI_2 production (Horrobin 1980). If this increase in cyclic AMP fails to occur due to cellular resistance an excess of PGI_2 will be produced. This cellular "resistance" could be an inherited defect or it may be due to blockage of cell membrane receptor sites by immune complexes (Moretta 1978). Pharmacological doses of PGI_2 have been shown to normalise the cyclic AMP levels in systemic sclerosis lymphocytes and this "resistance" may therefore be overcome by infusions of PGI_2.

Plasma TXB_2 levels are significantly increased in patients with secondary Raynaud's phenomenon ($p < 0.005$) when compared to either controls or to patients with primary Raynaud's phenomenon. This indicates increased platelet activation in these patients which could be a contributory factor to the degree of severity of the disease with platelets causing blockage of small vessels. The increased levels of plasma PGI_2 metabolites may be a compensatory mechanism in an attempt to counteract the high levels of TXB_2.

6.2 Vascular disorders in diabetics

6.2 (a) Introduction

Vascular disease is the major long term problem in diabetes. In diabetics over the age of fifty years arterial diseases are the commonest cause of death, the mortality rate being much higher than would be expected in a non-diabetic population of this age (Garcia 1969). Diabetic retinopathy is the most important systemic disease causing blindness in those aged between 30 and 64 years of age. Atherosclerosis occurs commonly and extensively in diabetics being more widespread and occurring earlier than in non diabetics (Garcia 1974). Thus diabetics are prone to intermittent claudication and grangrene and myocardial infarction prematurely. Diabetics are also more likely to develop thrombosis than non-diabetics (Timperley 1970).

6.2 (b) Involvement of prostacyclin and thromboxane in diabetes

Depressed vascular PGI_2 production in vitro was first shown in diabetic rats treated with streptozocin (Harrison 1978).

In diabetic patients depressed PGI_2 production was found from arterial segments removed at operation (Johnson 1979) and from samples obtained at elective vein biopsy (Silberbauer 1979). Treatment with insulin has been shown to restore vascular PGI_2 production to normal (Harrison 1980). Uncontrolled diabetes in man is linked with the development of both large vessel (Garcia 1974) and small vessel (Jarrett 1976) disease and decreased vascular PGI_2 production could be associated with this. There is conflicting evidence as to whether diabetics with microangiopathy have lower PGI_2 levels than normal non-diabetic controls or diabetics without microangiopathy (Dollery 1979, Mitchell 1978, Davis 1981).

Decreased PGI_2 production has been shown from aortic tissue from chronically diabetic pregnant rats which appears to be related to the duration of the disease (El Tahir 1982). Betteridge (1982) showed that platelets from 30% of diabetic patients showed a marked decreased in their sensitivity to the antiaggregation action of PGI_2. Those diabetics with vascular disease had significantly decreased platelet sensitivity to PGI_2. Those without vascular disease did not have a significant decrease in platelet sensitivity.

More recently (Takahashi 1985) showed a trend towards increased thromboxane synthetase activity as diabetic retinopathy develops through these differences did not reach significance. However in all diabetics studied the thromboxane synthetase activity was significantly higher than in age and sex matched controls. Other studies have shown increased thromboxane production in diabetics using a variety of exogenous aggregating agents (Gensini 1979, Halushka 1981).

However Carter (1986) has shown that the synthesis of TXB_2 by clotted whole blood from diabetics was significantly lower than from controls with no difference between those taking oral hypoglycaemic drugs than that in those receiving insulin. They also showed that TXB_2 synthesis is influenced by the degree of diabetic control.

It has been shown that plasma from diabetic patients showed a decreased ability to stimulate PGI_2 release from exhausted rat aortic tissue compared to controls and that patients with diabetic retinopathy showed decreased stimulatory activity. When compared to those without this complication (Sensaki 1985).

6.2 (c) Plasma levels of PGI_2 metabolites and TXB_2 in diabetics

6.2 (c) (i) Patients and Methods

We measured plasma levels of PGI_2 metabolites in 27 diabetics with varying degrees of vascular involvement. There were 17 males and 10 females in the group, the ages ranging from 21 - 75 years with an average age of 51.7 $\pm$ 14.4 years (S.D.) We compared these patients with 27 age and sex matched controls.

None of the patients or controls were taking any drugs known to alter prostaglandin production. The methodology used for sample preparation and assays was as described in Chapters 3 and 4.

6.2 (c) (ii) Results

The plasma levels of PGI_2 metabolite and TXB_2 showed that diabetic patients had higher levels of PGI_2 metabolites and lower levels of TXB_2 than controls. The results are shown in table 6.3.

	Diabetics	Controls
PGI_2M (pg/ml)	18.6 ± 10.0 *	9.9 ± 3.9
TXB_2 (pg/ml)	74 ± 24.6 *	102.7 ± 21.3

Significantly different from controls $p < 0.001$

Table 6.3 - Plasma levels of PGI_2 metabolites and TXB_2 in diabetic patients and controls (mean + SD)

We examined the results further by dividing the diabetic patients into those with juvenile onset diabetes mellitus on insulin treatment (Type I) and those with non-insulin dependent diabetes treated with diet and/or oral hypoglycaemic drugs (Type II). We found that Type II diabetics had significantly higher levels of PGI_2 metabolites than those with Type I diabetes ($p < 0.005$) but there was no significant difference in plasma TXB_2 levels. These results are shown in table 6.4.

	Type I (i.d.)	Type II (n.i.d.)
PGI_2M	15.2 ± 6.1 *	26 ± 12.7
TXB_2 (pg/ml)	68.1 ± 21.5	81.7 ± 27.3
	(Means ± S.D.)	

* significantly different from Type II $p < 0.005$

Table 6.4 - Plasma PGI_2 metabolite and TXB_2 levels in Type I and Type II diabetics

When we examined patients of both types who had vascular complications and compared them to those diabetics with no vascular complications, we found no significant differences in plasma levels of PGI_2 metabolites or TXB_2.

6.2 (d) Discussion

The increased levels of PGI_2 metabolites we have found in diabetic patients compared to controls would seem to be in disagreement with the findings of depressed PGI_2 production from arterial (Johnson 1979) and venous (Silberbauer 1979) human tissue, and from rat aortic tissue (El Tahir 1982). However this, together with our finding of increased plasma levels of PGI_2 metabolites in patients with secondary Raynaud's syndrome and patients with Raynaud's phenomenon and systemic sclerosis (6.2) indicates that higher levels of PGI_2 metabolites may be associated with vascular diseases.

The finding of decreased platelet sensitivity to PGI_2 in some diabetic patients (Betteridge 1982) would perhaps indicate that the platelets had been subjected to chronically high levels and thus become desensitised. The decreased plasma levels of TXB_2 which we have found in diabetic patients when compared to controls is supported by the recent finding of decreased serum TXB_2 levels in diabetics (Carter 1986). Most other researchers have however shown increased thromboxane production and further study of thromboxane generation is required in an attempt to clarify this.

6.3 Prostacyclin and Thromboxane in Cardiovascular Disease

6.3 (a) Introduction

The potent and contrasting effects of PGI_2 and TXA_2 on vascular tone and platelet function have led researchers to suppose that the balance between the two may play an important role in human cardiovascular disease.

Some of the first work published on plasma TXB_2 levels from patients with variant angina due to coronary artery spasm found detectable levels of TXB_2 in these patients whereas levels were undetectable in healthy volunteers (Lewy 1979 a). They were also able to show beneficial clinical effects in these patients with aspirin therapy concluding that thromboxane generation appears to be a pathogenic factor in Prinzmetal's angina (Lewy 1979 b). In 1981 Robertson and colleagues (Robertson 1981) reported that peripheral venous levels of TXB_2 at baseline and during an episode of chest pain were almost undetectable, whereas levels were increased several minutes after the vasopastic event. In 7 out of 8 episodes of coronary vasospasm, coronary sinus levels of TXB_2 increased to detectable levels within 2 minutes of the end of the attack. It would seem reasonable to suppose that since coronary arterial flow is diminished because of vessel constriction during an attack, a mediator such as TXA_2 may not appear in the coronary venous effluent until the end of the vasospastic attack. Other researchers have concluded that since treatment with aspirin or indomethacin failed to influence either the frequency or the severity of attacks of angina, thromboxane does not play a role in the disease.

However, as discussed in chapter 5.2, aspirin and indomethacin block the cyclo-oxygenase pathway unselectively and it may be possible that there is an optimum dose for each patient which blocks the thromboxane pathway while allowing recovery of endothelial cell production of PGI_2. It may be that the dosage given to these patients was blocking production of PGI_2 as well as thromboxane.

It has been tentatively suggested that PGI_2 may be involved in the etiology of variant angina. However this is based only on clinically significant benefit being observed in one out of nine patients treated with an infusion of PGI_2 (Chierchia 1982).

Tada (1981) reported increased TXB_2 production during rapid cardiac pacing in patients with chronic stable angina. However Martin (1983) found no intracoronary release of TXB_2 during rapid cardiac pacing in patients with coronary artery disease, but did show TXB_2 release immediately after pacing in those patients with evidence of myocardial lactate production. Hirsh (1981) studied 52 patients with chronic stable angina subjected to rapid cardiac pacing and observed no increase in coronary sinus or transcardiac levels of TXB_2 despite the production of myocardial ischemia in many of these patients. It has been suggested that in addition to the influence of sampling techniques and assay methodology on results as discussed in chapter 4 another reason for conflicting results may be the diagnostic criteria used by various investigators for patient classification.

6.3 (b) Plasma levels of PGI_2 metabolites and TXB_2 in patients with coronary artery disease

6.3 (b) (i) Patients and Methods

We measured plasma levels of PGI_2 metabolites and TXB_2 in 6 patients with coronary artery disease who had had a previous myocardial infarction and compared them to six patients who had coronary artery disease without myocardial infarction and six normal age and sex matched controls. Coronary heart disease was defined as >50% occlusion of a major coronary vessel shown by coronary angiography.

The methodology used for sample preparation and assays was as described in chapters 3 and 4. None of the patients had taken any drugs known to alter prostaglandin production during the previous two weeks.

6.3 (b) (ii) Results

The results for all coronary artery disease patients compared to age and sex matched controls are shown in table 6.5.

	CAD Patients	Controls
PGI_2M	15.8 ± 5.2	11.9 ± 4.0
TXB_2	127.1 ± 39.2	103.8 ± 36.6

Table 6.5 - Plasma PGI_2 metabolite and TXB_2 levels (pg/ml) in patients with coronary artery disease compared to age and sex matched controls (means + SD)

The results for plasma PGI_2 metabolites and TXB_2 levels in patients with and without previous myocardial infarction are shown in table 6.6.

	Myocardial Infarction	CAD but no myocardial infarction	Controls
PGI_2M	15.2 ± 6.8	14 ± 6.6	10 ± 4.1
TXB_2	103.8 ± 29.2 *	149.2 ± 41.2	109.2 ± 16.6 *

* diff from CAD but No MI $p < 0.05$

Table 6.6 - Plasma levels of PGI_2 metabolites and TXB_2 (pg/ml) in patients with myocardial infarction, coronary artery disease but no myocardial infarction and controls (means + SD)

6.3 (c) Plasma levels of PGI_2 metabolites and TXB_2 after rapid cardiac pacing in patients with chest pain

6.3 (c) (i) Patients and Methods

Using the methodology described in Chapters 3 and 4 we measured plasma levels of PGI_2 metabolites and TXB_2 in patients before and immediately after rapid cardiac pacing.

Twenty four males with ages ranging from 40 - 60 years (mean 51) gave their informed consent. Patients being routinely evaluated for chest pain with exercise electrocardiography were selected provided they did not have valvular heart disease. All patients were subjected to rapid cardiac pacing by exercise on an upright bicycle ergometer pedalling at a constant speed. A graded exercise protocol was used with an initial workload of 300 kilopond meters/minute (300kpm = 50 watts).

A positive exercise test was defined as one producing ST depression of 2mm persisting 0.08 seconds after the J point in three consecutive cardiac cycles and/or the development of typical cardiac chest pain on exertion. All exercise tests were performed between 10 - 11 a.m. and venous blood was taken from separate venepuncture sites prior to exercise with the patients seated at rest and within one minute of peak exercise. Blood was processed and assays performed as described in chapters 3 and 4.

6.3 (c) (ii) Results

The results of plasma PGI_2 metabolite and TXB_2 levels in patients with positive and negative exercise tests are shown in table 6.7 (means $\pm$ SD).

	+ve test (n = 9)		-ve test (n = 15)	
	PRE	POST	PRE	POST
PGI_2M (pg/ml)	14.3 $\pm$ 5.9	20.4 $\pm$ 7.8 *	14.8 $\pm$ 6.1	19.2 $\pm$ 9.4
TXB_2 (pg/ml)	113.3 $\pm$ 44.1	169.7 $\pm$ 84.4	128.5 $\pm$ 31.1	135.8 $\pm$ 51.6

* different from PRE values $P < 0.02$

Table 6.7 - Plasma PGI_2 metabolites and TXB_2 before and after rapid cardiac pacing in patients with positive and negative exercise tests

6.3 (d) Discussion

Although results did not reach statistical significance plasma PGI metabolite levels tended to be increased in patients with coronary artery disease compared to age and sex matched controls. The results still did not reach statistical significance when split up into those patients with and without a previous myocardial infarction but this was probably due to the smaller numbers. A study using much larger numbers is necessary to determine whether levels of PGI_2 metabolites are in fact higher in patients with coronary artery disease. However the tendency of higher levels noted would indicate that higher PGI_2 metabolite levels may be associated with severe vascular disease as discussed in 6.1 in Raynaud's syndrome patients and in diabetics (6.2). TXB_2 levels are significantly higher in patients with CAD who have had no previous myocardial infarction ($p < 0.05$) than in either patients with previous myocardial infarction or controls. This would indicate greater platelet activation in these patients which could contribute to the disease. However why patients who have had a previous myocardial infarction do not show increased levels of plasma TXB_2 is unclear.

Those patients having a positive exercise test showed a statistically significant increase in plasma levels of PGI_2 metabolites ($p < 0.002$). This may be a compensatory mechanism to try to produce increased vasodilation in the diseased vessels whereas these patients with a negative exercise test were able to cope with the increased blood flow produced by exercise without the need for this increased vasodilation. Plasma TXB_2 levels did not show a statistically significant increase in either group which is in agreement with the results of Hirsh (1983).

6.4 Conclusions

From these studies it would appear that PGI_2 metabolite levels are increased in a wide range of severe vascular disease states. Whether this is a compensatory mechanism or due to some other unknown factor remains unclear.

CHAPTER 7 - BLEEDING DISORDERS: RELEVANCE OF THROMBOXANE AND PROSTACYCLIN

7.1 Introduction

Under this heading of bleeding disorders are included a large number of conditions which are all characterised by an abnormal bleeding tendency. In this chapter the possible role of thromboxane and prostacyclin in bleeding disorders will be discussed. The results obtained for plasma levels of PGI_2 metabolites after infusion of 1-deamino-8-d-arginine vasopressin in von Willebrand's patients will be presented and a patient will be described who appears to have a deficiency of thromboxane synthetase.

7.2 The Coagulation System

For centuries it has been known that shed blood turns from a free-flowing liquid into a jelly-like mass. The mechanism by which this transformation occurs has been the source of much work and wonder and has only recently been elucidated. Plato and Aristotle felt that blood clotted because it cooled on exposure to air causing its "fibers" to congeal (quoted in Jewett 1892, Lee 1952). In 1731 Petit presented the idea that blood clotting might stop the flow of blood from wounds. The role of platelets in the arrest of bleeding by adhesion to the walls of injured vessels was first put forward in 1882 (Bizzozero 1882). Since then our knowledge of the coagulation system and its relationship to platelet function has developed extensively and is still developing. Figure 7.1 shows diagrammatically a scheme of blood coagulation.

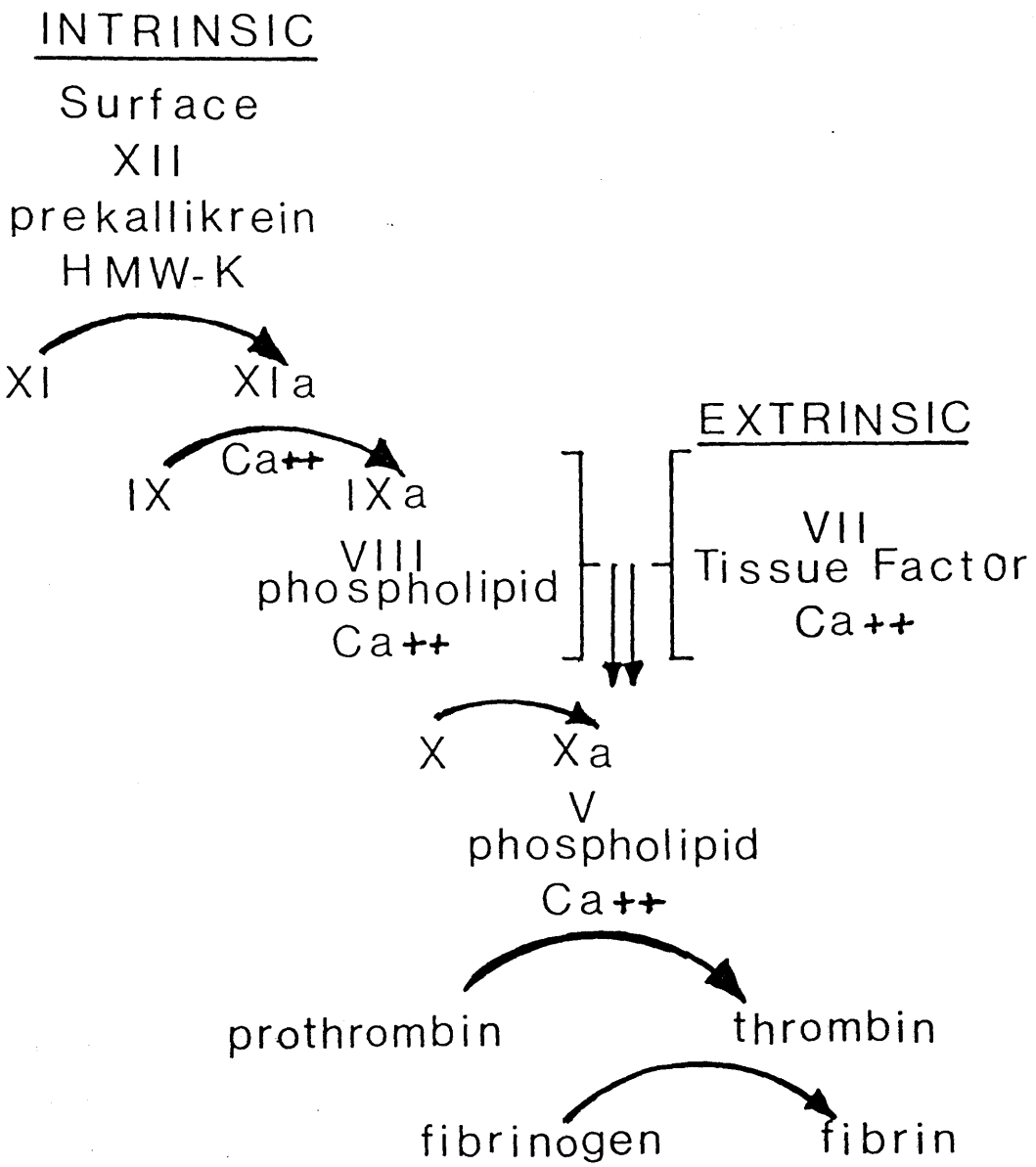


FIGURE 7.1 : A scheme of blood coagulation

The factors involved in the arrest of bleeding are complex and include:

(i) Contraction of damaged small vessels

(ii) Plugging of damaged vessels by adhesion and aggregation of platelets

(iii) Coagulation of blood.

An essential feature of haemostasis is the active contraction of the capillaries in response to injury. This, together with plugging of the bleeding points by the platelet thrombus, allows time for the coagulation of the blood to take place. A normal platelet count is essential for the arrest of haemorrhage. The number of platelets in normal circulating blood is 150 - 250 x 10^9 per litre. Platelets are small round anuclear cells and have a diameter about one-third that of red cells. They are produced by megakaryocytes in the bone marrow. Platelets have a lifespan of about ten days in the blood and do not adhere to normal vascular lining or to each other in normal individuals. It has been suggested that PGI_2 produced by the endothelial cells may prevent this adhesion. Damage to the endothelium thus allows platelets to contact subendothelial structures especially collagen, and initiates platelet adhesion. As well as forming a haemostatic plug at the site of injury, platelets contribute to blood coagulation by providing the procoagulant activity, platelet factor 3 which is not demonstrable in intact platelets and is apparently generated during platelet aggregation.

All schemes of blood coagulation distinguish between an intrinsic system which involves factors in the blood and an extrinsic system which is activated by a tissue factor (or factors) either in the laboratory or possibly in the body as a result of tissue injury particularly in the lungs, placenta or brain.

Each clotting factor circulates in the plasma as an inactive precursor. Once the clotting process is initiated an active enzyme is formed which catalyses the next reaction hence the term "coagulation cascade" or "waterfall sequence". When blood is exposed to tissue extracts factor X is rapidly generated via the extrinsic pathway by the interaction of a plasma protein, factor VII, and a tissue lipoprotein, tissue factor (or tissue thrompoplastin). The intrinsic pathway is initiated by contact of blood with a surface other than the normal vascular lining and circulating blood cells. Thus, the earliest steps in this pathway are called the 'contact phase'. Recently links have been found between the extrinsic and intrinsic pathways but the physiological significance of these links remains unclear. Within the mesh of the fibrin clot formed, red and white cells together with platelets are entrapped.

Fibrinolysis is the term used to describe the dissolution of these fibrin clots. This is essentially a defense mechanism to prevent occlusion of blood vessels.Figure 7.2 shows a simplified view of the human fibronolytic system.

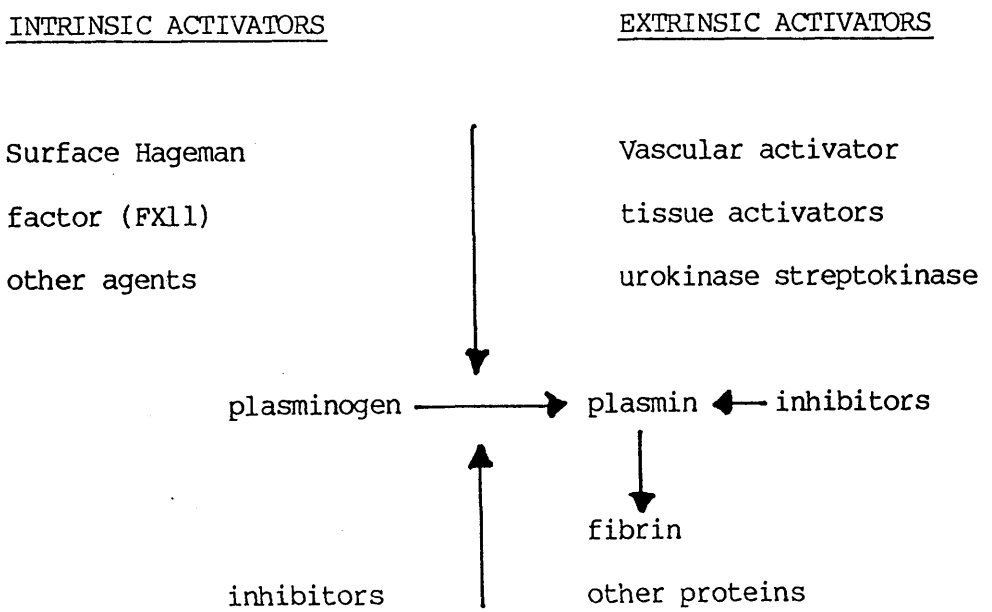


Figure 7.2 - A scheme of the fibrinolytic system

Plasminogen is a plasma protein and is also found to a lesser extent in most body fluids. Plasminogen is converted to plasmin by the action of specific enzymes: plasminogen activators. Plasmin may digest fibrinogen, factor V and factor VIII as well as fibrin. Plasminogen activators can be classified into intrinsic and extrinsic activators. Intrinsic activators are endogenous to blood and may convert plasminogen to plasmin when blood comes in contact with a foreign surface. All intrinsic activators known are much less active than extrinsic activators in their ability to activate plasminogen. Extrinsic activators are widely distributed in almost all body tissues including vascular endothelium and this endothelium derived plasminogen activator is thought to play an important role in plasminogen activation because of its close proximity to blood. The activator urokinase is apparently produced by the kidney and excreted into urine.

7.3 Role of Thromboxane and Prostacyclin in Bleeding Disorders

It is speculated that since TXA_2 produces platelet aggregation and vasoconstriction and PGI_2 inhibits platelet aggregation and produces vasodilation an imbalance in the production of one or other of these substances could be of importance in bleeding disorders: either a decreased production of TXA_2 or increased production of PGI_2 or both. In contrast to the vast amount of literature which has been published on the roles of PGI_2 and TXA_2 in ~~thromboxane~~ thrombotic vascular disorders there has been very little work published on their roles in disorders associated with a bleeding tendency.

Brief reports have been published of two cases where a deficiency in platelet thromboxane synthetase was presumed (Weiss 1977, Mestel 1980). These patients had impaired platelet aggregation to arachidonic acid and to prostaglandin G_2. Defreyn (1981) reported a familial bleeding tendency associated with partial platelet thromboxane synthetase deficiency. Three members of a family each from successive generations presented with a moderate bleeding tendency and a functional platelet defect. The patients had reduced serum TXB_2 levels and also increased plasma levels of 6-keto-PGF1α. The commonest bleeding disorders of genetic origin are the haemophilias (A and B) and von Willebrand's. I report studies on TXB_2/PGI_2 balance in these conditions.

7.4 von Willebrand's disease

7.4 (a) Introduction

In 1926 von Willebrand first described a congenital haemorrhagic disorder which he observed in several members of one family. He found 23 cases among 66 members of the family. The condition was found in both men and women and several children from the family had died prematurely from intestinal bleeding or following minor injury. von Willebrand found that patients suffering from the disease had a prolonged bleeding time, a normal clotting time and normal platelet count. Their platelets were morphologically normal. The disease was first called pseudo-haemophilia because of the similarity to that disorder. The disease has been shown to be inherited as a simple autosomal dominant.

Later studies have shown that platelet retention by a column of glass beads is abnormal in von Willebrand's disease (Salzman 1963) as is ristocetin induced platelet aggregation (Howard 1971) and ex vivo interaction of platelets with the subendothelial surface of segments of blood vessels (Tschopp 1974). von Willebrand's disease can now be subdivided into at least four major subgroups (Types 1 - 4).

Type I has a reduction in all the factor VIII von Willebrand factor complex activities. Type II is characterised by discordant reductions in the factor VIII von Willebrand factor complex activities, with VIII : RCo being more reduced than VIII : C or VIII R : Ag. At least three subgroups have been recognised. In type IIa the patients may have mild to severe clinical symptoms with inheritance being most compatible with autosomal dominant transmission. Ristocetin-induced aggregation of platelets in platelet-rich plasma is reduced as is FVIII:RCo. Platelets from patients of type IIb have the ability to aggregate in response to much lower concentrations of ristocetin than do normal platelets. Type IIb also appears to be inherited as an autosomal dominant. As technology advances various new subtypes are being described.

There is also a group of patients who clinically have a bleeding disorder similar to von Willebrand's disease but since the defect in this group is thought to be primarily in the platelet and not in the von Willebrand factor, it has been termed platelet-type or pseudo Von Willebrand's disease (Miller 1982). With the information available to date it is impossible to identify with certainty the precise abnormality in any of the types of von Willebrand's disease.

7.4 (b) 1-deamino-8-d-arginine vasopressin (DDAVP) and von Willebrand's disease

7.4 (b) (i) Introduction

DDAVP has been used effectively to treat bleeding episodes in patients with von Willebrand's disease (Lowe 1977, Mannucci 1977, Ockelford 1980, Warrier 1983). Uremic patients with haemmorrhagic tendencies have also been shown to correct their bleeding tendency after DDAVP (Mannucci 1983). DDAVP stimulates the vascular endothelium to release FVIIIR : Ag and plasminogen activator (Cash 1974).

It has been shown that, after an infusion of FVIII in haemophilia there is increased platelet aggregation to ADP and also a rise in the number of circulating platelet aggregates (Grigani 1979). However when endogenous FVIII levels are increased after an infusion of DDAVP this increase in circulating platelet aggregates is not observed (Belch 1982). This could be explained if DDAVP also stimulated endothelial cell release of PGI_2, which could also explain the flushing observed during DDAVP infusion.

There has been controversy in the literature as to whether or not DDAVP does in fact stimulate release of PGI_2 from endothelial cells. Our laboratory previously published evidence showing that plasma levels of 6-keto-PGF1α were raised following DDAVP infusion (Belch 1982). However studies with an improved assay showed that the levels of 6-keto-PGF1α were unrealistically high, probably due to extraction methods used (see 3.7). Barrow (1983) has since reported that DDAVP infusion did not increase circulating levels of PGI_2 or TXA_2 in normal volunteers.

They measured absolute values of 6-keto-PGF1α using negative ion chemical ionisation mass spectometry. Using an umbilical vein perfusion model Barnhart (1983) could find no increase in levels of 6-keto-PGF1α when measured by R.I.A., after DDAVP. All these studies are based on measurement of 6-keto-PGF1α . After the development of our assay for PGI_2 metabolites we repeated our earlier work measuring plasma levels of PGI_2 metabolites before and after infusion of DDAVP using this improved methodology. We also studied haemophiliac patients and patients with von Willebrand's disease receiving infusions of DDAVP.

7.4 (b) (ii) Patients and Methods

Seven normal healthy subjects, 11 severely affected haemophiliacs with baseline factor VIII coagulant activity (FVIIIc) or factor IX less than 1% (7 with haemophilia A and 4 with haemophilia B) and 8 patients with von Willebrand's disease (7 with type 11a and 1 with type 1) were studied after informed consent had been obtained. No subject had taken any drugs for a minimum of two weeks previously. Venous blood was collected as described in chapter 3.7 for measurement of plasma levels of PGI_2 metabolites and TXB_2.

DDAVP was then given intravenously at a dose of 0.3ug/kg body weight over 15 minutes using a Vickers Treonic IP3 syringe pump. The volume of infusion was kept constant at 25 ml for all subjects by making up the DDAVP in 0.9% sodium chloride. A further blood sample was taken, as before, immediately after termination of the infusion. Plasma PGI_2 metabolites and TXB_2 levels were measured by R.I.A. as described in chapters 3 and 4 respectively.

7.4 (b) (iii) Results

The results are shown in Table 7.1

The basal levels of PGI_2 metabolites were significantly lower in the von Willebrand patients than in the normals and haemophiliacs. There was no difference in the basal levels of PGI_2 metabolites between the normal and haemophiliac groups. All three groups showed a significant increase in PGI_2 metabolites following infusion of DDAVP. No significant difference in the per cent increase was found in the three groups. The post-DDAVP levels of PGI_2 metabolites were still significantly lower in the von Willebrand group than in the normal group.

All the normal volunteers and the haemophiliacs showed marked facial flusing during the infusion but this was not observed in any of the von Willebrand subjects. There was no statistically significant difference in basal levels of plasma TXB_2 between any of the groups nor were there any statistically significant differences post - DDAVP. Although the results do not form part of this thesis FVIIIR:Ag, FVIIIC and plasminogen activator are included in this table to substantiate the theory of endothelial cell stimulation by DDAVP. Methodology for these assays is as described by Greer et al (1986).

	Normal		Haemophilia		vWd	
	pre-DDAVP	post-DDAVP	pre-DDAVP	post-DDAVP	pre-DDAVP	post-DDAVP
PGI_2M pg/ml	17.5 ±2.4	29.0** ±9.0	16.3 ±5.7	22.6** ±9.8	11.5**** ±1.7	15.2****** ±4.0
TXB_2 pg/ml	145.0 ±38.2	195.0 ±52.6	108.0 ±33.0	118.0 ±32.0	92.0 ±40.7	117.1 ±97.4
FVIIIR : Ag % normal pool	71.1 ±30.4	132.0** ±30.8	118.6 ±30.4	185.6** ±53.4	45.1 ±18.7	95.5** ±37.0
FCIIIc % normal pool	139.6 ±41.9	334.3** ±82.6	-	-	41.5 +15.8	129.9** ± 67.2
FPLA mm^2	60.8 ±11.5	97.1* ±28.0	52.7 ±14.3	77.8*** ±18.4	68.4 ±25.6	96.5** ±29.7
n		7		11		8
Male:female		2:5		11:0		2:6

*$p < 0.05$:**$p < 0.02$:***$p < 0.005$ compared with pre-DDAVP measurement for same group;****$p < 0.005$ compared to pre-DDAVP measurement for normal and haemophilia group:****p 0.002 compared to post-DDAVP measurement for normal group.

Table 7.1 - Effect of DDAVP infusions on plasma TXB_2 and PGI_2 metabolites (mean + SD)

7.4 (b) (iv) Discussion

These results confirm our previous findings that DDAVP stimulates PGI_2 production in normal and haemophiliac patients. It has been shown by Ritter (1983) that physical stimulation of vascular endothelium can release PGI_2.

It has recently been shown that production of PGI_2 by cultured endothelial cells depends on the number of platelets in the flowing blood and also on the wall shear rate (Grabowski 1984).

Deficiency of von Willebrand factor results in shear dependent reduction in platelet adhesion to the vessel wall (Weiss 1978). It is possible therefore that the lower basal and post DDAVP levels of PGI_2 metabolites found in von Willebrand's disease may be due to decreased platelet interaction with the endothelium resulting from von Willebrand factor deficiency.

Although TXB_2 tended to be lower in vWd, the difference was not statistically significant. Since patients with vWd have normal platelet aggregation to physiological stimuli and normal arachidonic acid metabolism, it is perhaps not surprising that TXB_2 is not reduced. The mechanism for the action of DDAVP on PGI_2 production is not entirely clear. Previous in vitro studies have shown DDAVP to have a direct stimulatory effect on PGI_2 production from arterial tissue (Belch 1982) while there is mounting evidence that the effect of DDAVP on FVIIR : Ag release is mediated via a secondary messenger (Cash 1978, Booyse 1981). In the absence of any change in TXB_2 levels following DDAVP, the stimulatory effect on PGI_2 production may be mediated through an endothelial cell receptor rather than a general activation of the arachidonic acid cascade.

The failure of the vWd subjects to flush is also of interest, since this general vasodilatation is seen in normal and haemophilic subjects and has been shown to be abolished by aspirin (Belch 1982). It is likely therefore to be due to the potent vasodilator action of PGI_2 or failure to reach a "flush threshold", as the vWd subjects had lower post-DDAVP levels of PGI_2 than normal subjects.

7.4 (c) Perfusion of umbilical artery with DDAVP

7.4 (c) (i) Introduction

Previous work from our laboratory showed evidence of PGI release from rat aortic rings after incubation with DDAVP (Belch 1982). However using an umbilical vein perfusion model Barnhart (1983) was unable to detect a similar increase. In order to try to confirm our previous work using difference methodology we used our umbilical artery perfusion model described in 3.11.

7.4 (c) (ii) Methods

The umbilical artery perfusion model was set up as described in 3.11 using a normal umbilical artery. DDAVP 10^{-9}M was perfused through the vessel after prior perfusion with 650 mls of Ringer's buffer. Fractions were collected and stored at -70^{o}C for R.I.A. for PGI_2 metabolites as described in 3.7.

7.4 (c) (iii) Results

The results of the perfusion of a normal umbilical artery with DDAVP on levels of PGI_2 metabolites are shown in figure 7.3 Levels of PGI_2 metabolites showed an increase from the level of 100 pg/ml prior to the perfusion of DDAVP to a peak level of 580 pg/ml after DDAVP was perfused.

7.4 (c) (iv) Discussion

This experiment although performed only on one occasion does confirm our earlier work using rat aortic rings in which we showed increased levels of 6-keto-PGF1α from the rings after incubation with DDAVP (Belch 1982). Together with the perfusion studies in normal volunteers it would appear that DDAVP acts as a

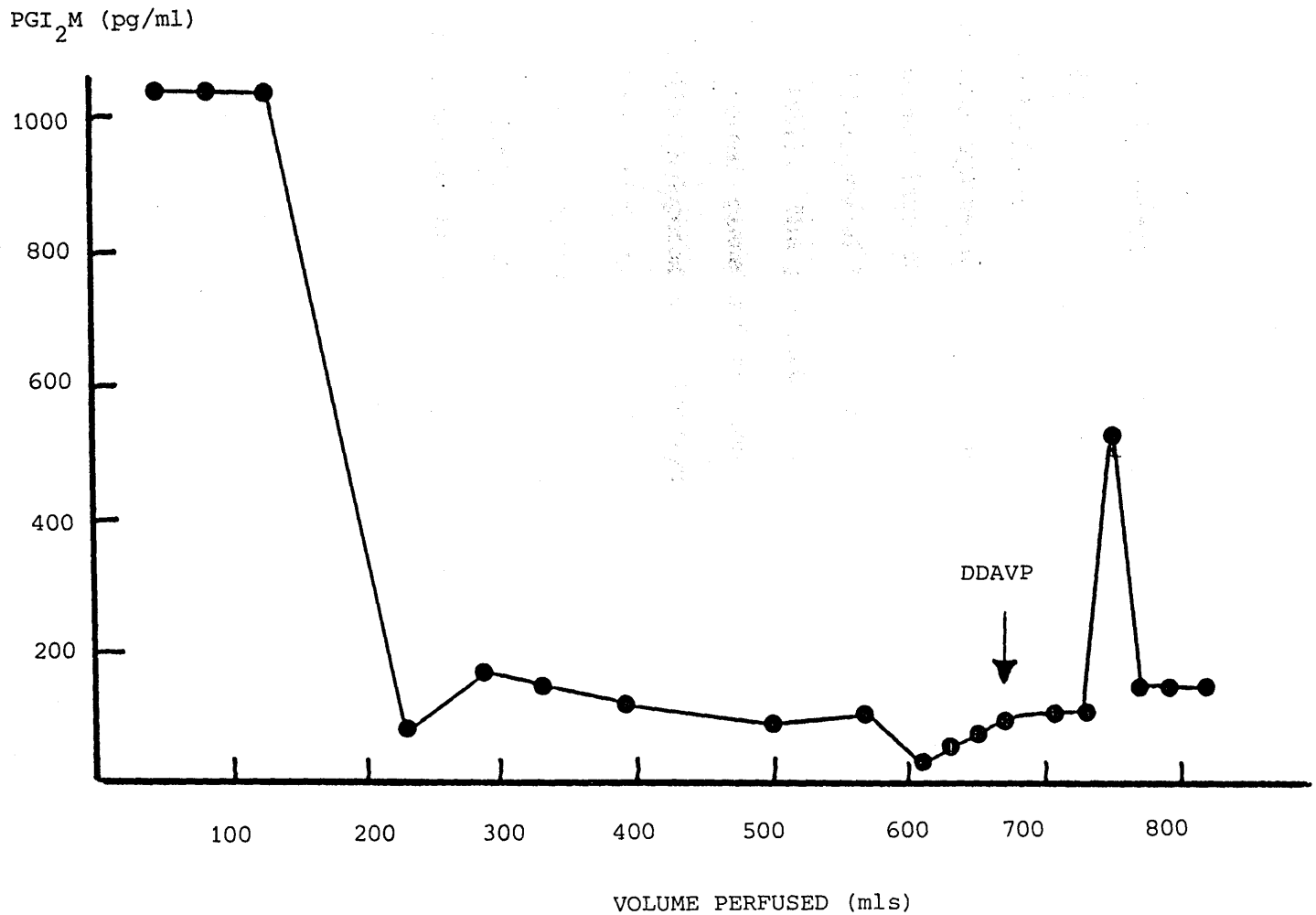


FIGURE 7.3 : UMBILICAL ARTERY PERFUSION WITH DDAVP 10^{-9}M

general endothelial cell stimulant, stimulating release of PGI_2 as well as FVIIIC, FVIIIR : Ag and plasminogen activator. However the underlying mechanisms for this remain unclear.

7.5 Report on Patient with a possible thromboxane synthetase deficiency

7.5 (a) Introduction

Thromboxane synthetase is the enzyme that converts the cyclic endoperoxides into thromboxane A_2. To date only two families with a deficiency of this enzyme have been reported (Mestel 1980, Defreyn 1981). The platelets from these patients do not form TXA_2 as normal platelets do, even when they are supplied with the cyclic endoperoxides. Defreyn noted increased levels of plasma 6-keto-PGF1 however their normal levels of 181 $\pm$ 46 pg/ml would appear to be very high thus leaving their assay methodology open to question. However the increased levels of 6-keto-PGF1 could be due to the cyclic endoperoxides produced by the platelet gaining access to the endothelium where they are converted to PGI_2. We report results on one patient who would appear to have a possible deficiency of thromboxane synthetase.

7.5 (b) Patient and Methods

Our patient suffered heavy bleeding after all dental extractions and also had mennhoragia. She had post-partum haemorrhage after all three deliveries and had a probable secondary haemorrhage after a tonsillectomy at 21 years of age. She had however undergone an apparently uneventful appendectomy. She had a normal thrombin time, prothrombin time and activated partial thromboplastin time. Factor FVIIIC and factor VIIIR : Ag were

within the normal range. Aggregation to ristocetin and ADP were normal but collagen aggregation was reduced. She had a bleeding time of 14 minutes 15 seconds. Using the method described in Chapter 4 we measured serum TXB_2 thromboxane generation in response to aggregation with collagen 4ug/ml and serum TXB_2 in bleeding time blood. We also measured plasma PGI_2 metabolites as described in 3.7.

7.5 (c) Results

The results are shown in table 7.2.

	Patient	Normal
Serum TXB_2 (ng/ml)	12	311 $\pm$ 112 SD
Serum TXB_2 in BT blood (ng/ml) (average of first two minutes)	4	400
TX generation	12	118 $\pm$ 30 SD
Plasma PGI_2 metabolites (pg/ml)	14	13.1 $\pm$ 1.2

Table 7.2 - Results of thromboxane levels in patient with suspected thromboxane synthetase deficiency

(d) Discussion

The results of serum TXB_2 and serum TXB_2 in bleeding time blood are very much lower than our normal levels. Thromboxane generation in response to aggregation with 4 ug/ml collagen was also much reduced. However since the patient had reduced response to collagen aggregation, when this patient returns a blood sample should be taken to measure the thromboxane generation in response to ADP.

Since ADP platelet aggregation was normal we would then be able to measure the amount of thromboxane generated in response to maximal aggregation and this was not true for collagen. There is no evidence of increased levels of PGI_2 metabolites. There is much more study of this patient and her family necessary to show that this is a case of thromboxane synthetase deficiency. However such low levels of serum TXB_2 and serum TXB_2 from bleeding time blood have not been seen in any other patients we have studied except when treated with cyclo-oxygenase inhibitors or thromboxane synthetase inhibitors.

CHAPTER 8 - PROSTACYCLIN AND THROMBOXANE IN PREGNANCY AND RELATED CONDITIONS

8.1 Introduction

The maternal and foetal circulations are characterised by low vascular resistance and vasodilation which may or may not be due to prostacyclin. Tissues such as myometrium (Bamford et al 1980) decidua, chorion and amnion (Mitchell et al 1978) and trophoblast (Rakoczi et al 1983) all pregnancy associated tissues, have been shown to produce PGI_2 *in vitro*. Various studies (Mitchell et al 1978, Harper et al 1983, Nikolaides et al 1983, Makila et al 1984) have reported PGI_2 production by placental tissues. However Dembele-Duchesne et al (1982) claim that this anti-aggregatory factor is neither PGI_2 nor a PGI_2 metabolite. Various studies using human umbilical vessels and different *in vitro* models (Hamberg et al 1979, Remuzzi et al 1979, Ritter et al 1982) and Stoel et al 1982) have reported PGI_2 from arteries and veins with arterial tissue having the ability to produce the greater amounts (Kent 1981). Increased release of PGI_2 has also been demonstrated from the uterine vessels of pregnant women (Bussolino et al 1980). This ability to produce PGI_2 by both foetal and maternal tissues may be of importance in normal pregnancy and may be implicated in some pathological conditions of pregnancy. The results of studies measuring levels of 6-keto-PGF1α, the stable hydrolysis product of PGI_2, in maternal peripheral blood have been conflicting. Lewis et al (1980) showed an increase during late normal pregnancy and the puerperium whereas Bolton et al (1981) showed that levels reached a peak at 18-22 weeks of pregnancy with mean values from 26-42 weeks being significantly lower.

In order to carry out any study of PGI_2M and TXB_2 levels in pathological disorders of pregnancy it is necessary to have normal levels with which to do comparisons. The aim of our first study therefore was to determine the values both of PGI_2 and of TXA_2 metabolites throughout normal pregnancy and the puerperium.

8.2 Plasma PGI_2 metabolites and TXB_2 in normal pregnancy and the puerperium

8.2 (a) Patients and Methods

The study included 155 women; 44 normal non-pregnant, 29 in the first trimester of pregnancy, 31 in the second trimester, 29 in the third trimester and 21 on the third day after delivery. All pregnancies were uncomplicated and no subject had taken aspirin-like drugs for at least two weeks prior to sampling. Blood sampling and RIA's for PGI_2 and TXA_2 metabolites were performed as already described in 3.6 and 4.3 respectively.

8.2 (b) Results

Results for PGI_2M are shown in Figure 8.1, and for TXB_2 in Figure 8.2. We found a significant difference in PGI_2M levels in the first trimester ($p < 0.01$) when compared with those in the non-pregnant group. While there were no significant differences between non-pregnant values and those in the second or third trimesters, we noted a trend towards lower concentrations of PGI_2M on the third postnatal day, but this did not reach significance. Levels of PGI_2M were significantly lower in the second and third trimester than in the first trimester group. There was a progressive reduction in plasma TXB_2 concentrations from the second trimester, through the third trimester and into the puerperium. Table 8.1 gives a fuller statistical analysis.

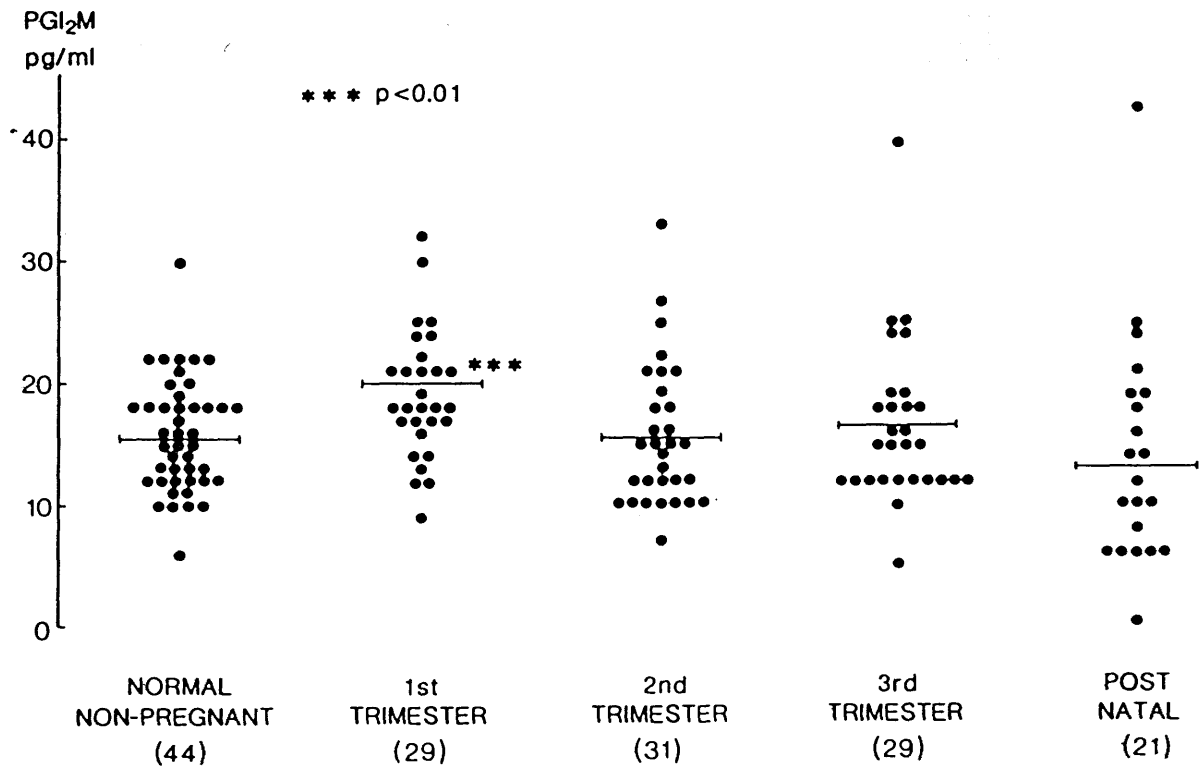


Figure 8.1 : Plasma PGI_2 metabolite levels in normal pregnancy

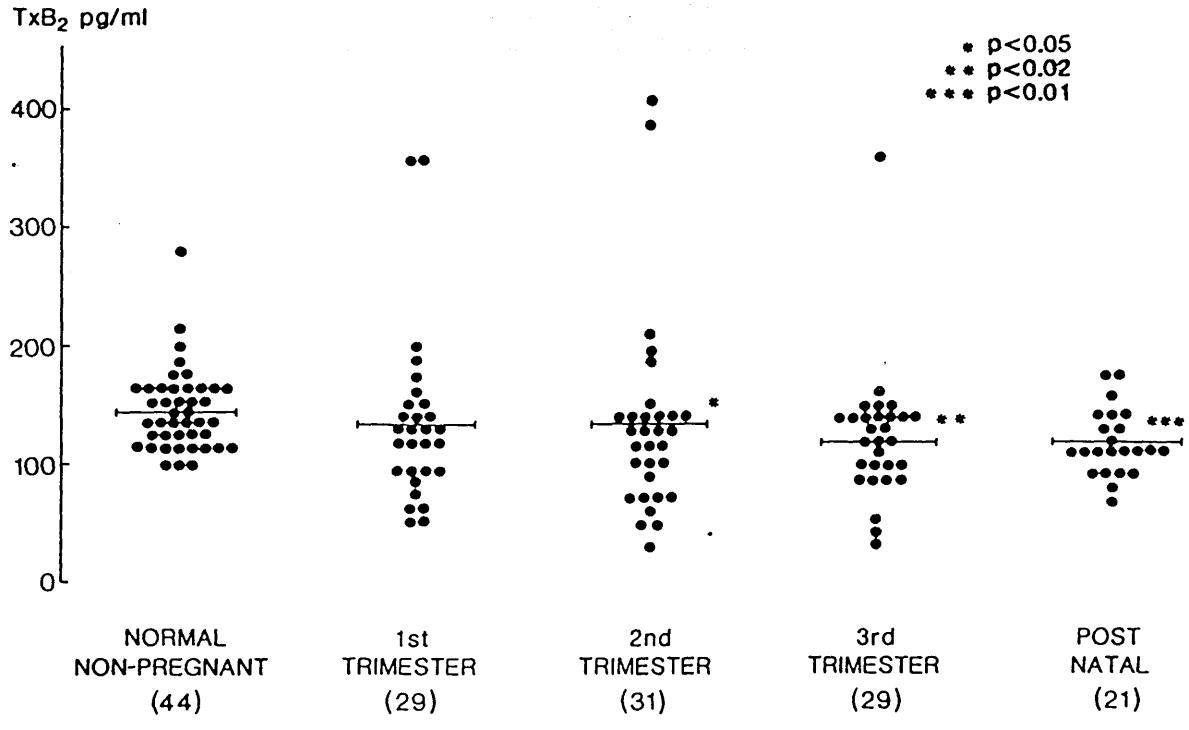


Figure 8.2 : Plasma TXB_2 levels in normal pregnancy

	Non pregnant	1st trimester	2nd trimester	3rd trimester	Postnatal
		***			
PGI_2M	15.9(0.68)	19.9(0.96)	15.5(1.05)	16.4(1.22)	13.6(1.99)
TXB_2	142(4.9)	131(14.2)	133(14.9)*	123(10.7)**	119(6.3)***
n	44	29	31	29	21

*** $p < 0.01$, ** $p < 0.02$, * $p < 0.05$ compared with non pregnant,
*** $p < 0.05$, ** $p < 0.01$, compared with 1st trimester
Results are mean (SEM) values

Table 8.1 - Plasma levels of prostacyclin metabolite (PGI_2M and thromboxane B_2 (TXB_2) in non-pregnant women, the three trimesters of pregnancy and the puerperium

8.2 (c) Discussion

Despite using larger numbers we did not confirm the work of Lewis et al (1980) who showed a higher concentration of 6-keto-$PGF_1\alpha$ in the late pregnancy and the puerperium. Neither could we confirm the work of Bolton et al (1981) as our peak levels of PGI_2M occurred in the first trimester, and not in mid-pregnancy as they reported, although they did not record levels before 14 weeks gestation.

We found a significant difference in the first trimester compared with values in the non-pregnant and second and third trimesters. This early increase may be involved in the physiological vasodilation and insensitivity to angiotensin II (Grant et al 1973) which are characteristic of early pregnancy.

It has recently been shown that trophoblast from early pregnancies has a high capacity to synthesise PGI_2 and it has been suggested that colonisation of maternal blood vessels by trophoblast is facilitated by PGI_2 which may prevent platelet aggregates from halting this process (Lewis 1982). This theory might explain our findings of elevated PGI_2M levels in early pregnancy.

Failure of this response may possibly result in threatened or spontaneous abortion, and may even lead to fetal growth retardation in later pregnancy, a condition where low PGI_2 metabolites have previously been recorded (Jogee et al 1983; Stuart et al 1981).

Theoretically one might expect high levels of PGI_2 in late pregnancy, due to the potential for increased production from the uterus and fetoplacental circulation unit. We have not found any increased levels at this stage.

This may be explained by the findings of Remuzzi et al (1981), who found a reduced level of prostacyclin-stimulating factor in late normal pregnancy, possibly a homeostatic mechanism. Since the capacity to produce PGI_2 is increased at this stage, less prostacyclin-stimulating factor is required, and PGI_2 levels would remain constant as we have described. Conversely when the ability to produce PGI_2 is low, as in pregnancy-induced hypertension, prostacyclin-stimulating factor rises in an attempt to increase PGI_2. This change has also been noted by Remuzzi et al (1981). In the postnatal group we observed a trend towards reduced levels which contradicts the work of Lewis et al (1980). Our sampling was performed only on the third postnatal day, while Lewis et al (1980) sampled between the first and seventh postnatal days. If many of the samples were obtained soon after delivery they may have a high 6-keto-$PGF_1\alpha$ level due to vascular injury. Also, increased in-vitro production of 6-keto-$PGF_1\alpha$ from sheep cervical tissue taken at parturition has been reported (Ellwood et al 1981). Since the fetoplacental unit, which has enormous capacity to synthesise PGI_2 is removed at birth, a reduction in 6-keto-$PGF_1\alpha$ levels postnatally might be expected.

We have observed a progressive fall in plasma TXB_2 concentrations through pregnancy and into the puerperium. This may reflect increased platelet stability, or the diversion of TXB_2 precursors into the formation of other prostaglandins, such as PGD_2 or PGE_1. Other possible explanations of this may be related to a change in activity of thromboxane synthetase or decreased substrate availability, but further studies are required to elucidate this.

We noted several very high levels of TXB_2 (Figure 2) which we cannot explain; they were levels normally associated with traumatic venepuncture or haemolysis, but we discarded all samples in which either of these problems were apparent. We would suggest that these may be due to subclinical trauma at the time of venepuncture with resultant platelet activation.

8.3 Pregnancy Induced Hypertension

8.3 (a) Introduction

Approximately 10 per cent of all pregnancies are complicated by some degree of hypertensive disease. The level of blood pressure is closely related to foetal well-being and if the blood pressure is persistantly elevated during pregnancy there is an associated increase in perinatal mortality. The pathogenesis of pregnancy induced hypertension (PIH) is not yet fully understood but some workers suggest the involvement of prostaglandins: PGI_2 has been shown to be produced by the placenta (Carreras et al 1981) and as it is not metabolised in the lungs (Gerkens et al 1978) placentally produced PGI_2 could have local and systemic effects.

Downing et al (1980) have found PGI_2 synthetase activity to be the same in cords from normal and hypertensive pregnancies however the cords from pregnancies complicated by hypertension contained less of the enzyme. Carreras et al (1981) compared PGI_2 production by umbilical arteries and veins found decreased production in hypertensive patients. The production of PGI_2 from placentae obtained from toxaemic pregnancies were found to produce approx one third of that produced by placentae from uncomplicated pregnancies (Walsh et al 1985). This agreed with earlier work by Lewis et al (1981) who had found decreased production by both placental and umbilical vessels. When measuring plasma levels of 6-keto-$PGF_1\alpha$, the stable hydration product of PGI_2, conflicting reports appear in the literature. Ylikorkala et al (1981) report no difference between pre-eclamptic and normal pregnancies. Whereas Yamaguchi et al (1985) and Lewis et al (1980) both report lower levels in pre-eclamptics compared to normal patients. As was discussed in chapter 3, the methodological problems associated with the assay of plasma levels of 6-keto-$PGF_1\alpha$ make comparisons between different groups difficult. Lewis et al (1981) also relate falling levels of 6-keto-$PGF_1\alpha$ to the worsening of the disease. Beneficial effects of PGI_2 infusions have been reported (Fidler et al 1980). Thromboxane A_2 has also been implicated in the etiology of PIH (Ylikorkala et al 1980, 1981). High levels of TXA_2 would cause increasing blood pressure and falling platelet count which are both features of fulminating pre-eclampsia. It would seem therefore that the interaction in the roles of PGI_2 and TXA_2 are important and worth investigation in this disease.

8.3 (b) A longitudinal study of plasma PGI_2M and TXB_2 levels in normal and hypertensive pregnancies

8.3 (b) (i) Introduction

Bolton (1981) has produced the only prospective longitudinal sutdy of maternal plasma 6-keto-$PGF_1\alpha$ levels. They found that in 12 normal pregnancies levels peaked between 18 and 22 weeks gestation. However they did not take samples during the first trimester nor did they measure TXB_2 levels. Various cross sectional studies have been published giving conflicting results as discussed in 8.1. We performed a prospective longitudinal study of PGI_2 metabolites and TXB_2 in maternal plasma throughout normal pregnancy.

However six of our fourteen randomly selected patients subsequently became hypertensive and we were thus able to study PGI_2 metabolites and TXB_2 in both normal and hypertensives. There was no apparent reason for this high incidence of pregnancy induced hypertension in our randomly selected group. The normal incidence of pregnancy induced hypertension in our population is 5-10% and the high incidence appears to be totally coincidental.

8.3 (b) (ii) Patients and Methods

Fourteen randomly selected primigravidae patients were selected who had no previous history of hypertension and no other medical disorder. None of the subjects took any non-steroidal anti inflammatory drugs for at least two weeks prior to blood sampling. Samples were taken in the first trimester between 9 and 12 weeks gestation; in the second between 17 and 24 weeks gestation and in the third between 32 and 36 weeks gestation.

In the patients who became hypertensive further samples were taken where possible between 36 weeks and term. Mild to moderate pregnancy induced hypertension was defined as a persistent diastolic blood pressure of 90mm mercury. None of the six who became hypertensive developed significant platelet consumption or proteinuria. All the patients had vaginal deliveries and all delivered healthy babies of normal birth weight.

Blood sampling and radioimmunoassay was performed as described in chapters 3 and 4.

8.3 (b) (iii) Results

Figure 8.3 shows the plasma PGI_2 metabolite and TXB_2 levels in normal pregnancies. PGI_2 metabolite and TXB_2 levels were significantly lower in both the second and third trimesters than in the first.

Figure 8.4 shows the plasma PGI_2 metabolite and TXB_2 levels in hypertensive pregnancies. This group showed no significant differences in PGI_2 metabolite levels in the three trimesters also levels were not significantly different from the normal group. However all the hypertensive patients developed unrecordable levels of $< 5pg/ml$ in the third trimester when they were hypertensive. None of the normal group developed unrecordable levels at any stage.

As with the normal group TXB_2 levels decreased significantly in the second and third trimesters when compared to the first. There were also no significant differences at any stage when compared to the normal group.

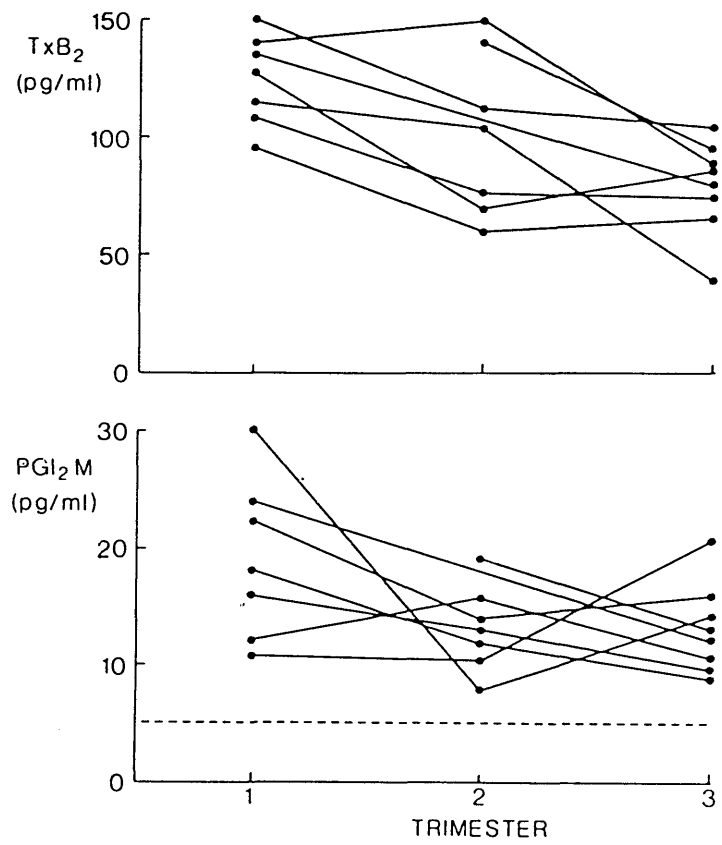


Figure 8.3 : Plasma TXB_2 and PGI_2 metabolit
levels in normal pregnancy

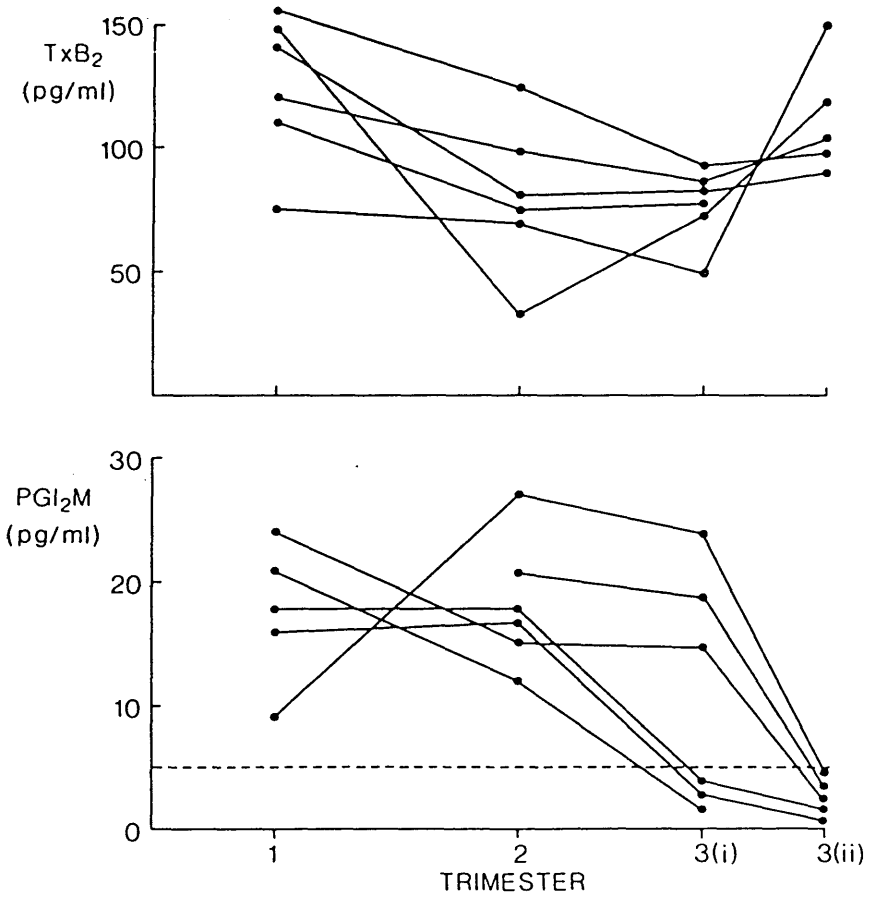


Figure 8.4 : Plasma TXB_2 and PGI_2 metabolite levels in hypertensive pregnancy

8.3 (b) (iv) Discussion

In 5 out of the 6 hypertensive patients the hypertension had developed prior to the sample which gave unrecordable levels. Therefore it was impossible to determine which came first: the hypertension or the unrecordable levels of PGI_2 metabolites. One patient however had a third trimester unrecordable level prior to the onset of hypertension and levels remained unrecordable thereafter. These results are in agreement with work published by Lewis (1981) who reported falling levels of plasma 6-keto-$PGF_{1\alpha}$ in one patient when she developed hypertension. Goodman (1982) measured urinary levels of PGI_2 metabolites and found significantly reduced levels in hypertensive patients compared to controls.

Since one patient showed unrecordable levels of PGI_2 metabolites prior to the development of hypertension it is possible that this may be a means of detecting the onset of hypertension.

However much more work must be carried out in order to prove this. TXB_2 levels were not significantly different in the hypertensive group when compared to control and therefore would not appear to be related to pregnancy induced hypertension.

The results in the normal group, although numbers are small, agree with our cross sectional study (8.2).

8.3 (c) Umbilical artery perfusion model: comparison of vessels from normal and hypertensive pregnancies

8.3 (c) (i) Materials and Methods

We obtained umbilical cords from two patients suffering from pregnancy induced hypertension defined as persistent diastolic blood pressure of > 90mm of mercury. One patient had underlying renal pathology which could lead to hypertension but she had shown no signs of this before becoming pregnant.

The arteries were dissected and the perfusion model set up as described in 3.11. After initially perfusing with 150 - 200 mls of Ringer's buffer, 5 mls of normal platelet poor plasma was perfused through the artery then perfusion with Ringer's buffer was continued. 5ml fractions were collected and stored at -70^{0}C for RIA of PGI_2 metabolites as described in 3.7.

8.3 (c) (ii) Results

The results we obtained from the two hypertensive cords were compared with the same experiment we had previously done using an artery from an uncomplicated pregnancy. We have repeated the same experiment on normal umbilical arteries on several occasions and an increase in the levels of PGI_2 metabolites after perfusion of normal platelet poor plasma is always seen. However as can be seen from the results shown in figure 8.5 neither of the arteries from the hypertensive patients showed any significant increase in levels of PGI_2 metabolites.

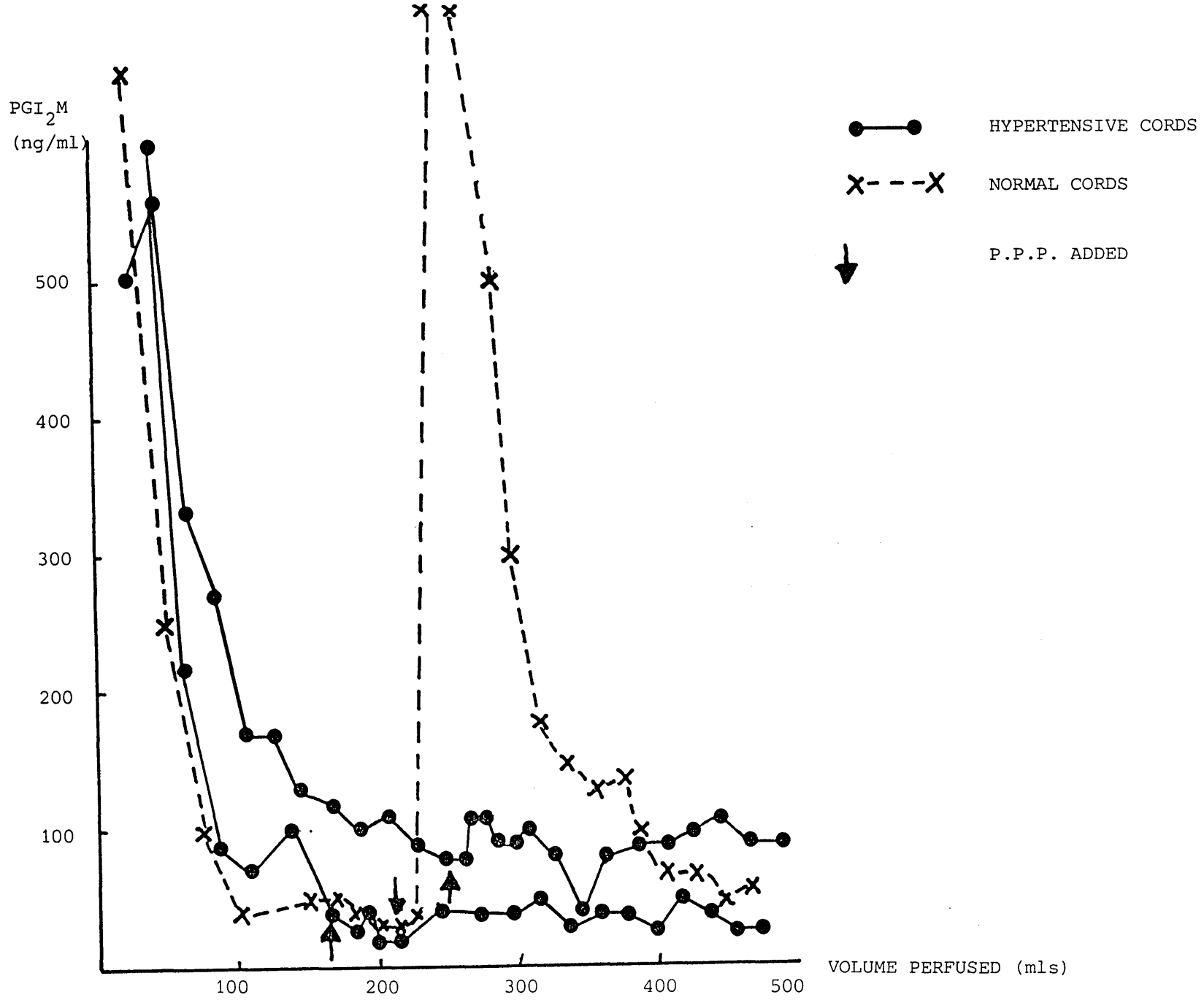


FIGURE 8.5: PERFUSION OF UMBILICAL ARTERIES FROM HYPERTENSIVE PATIENTS

8.3 (c) (iii) Discussion

Since this is the result of only two experiments it is necessary to repeat the perfusion on many more arteries from pregnancies complicated by pregnancy induced hypertension. It has been shown that normal plasma contains a factor capable of stimulating endothelial cell producion of PGI_2 (McIntyre 1978, Remuzzi 1979a, Defreyn 1980) and we have shown (3.11 c) that using our umbilical artery perfusion model we can stimulate PGI_2 production using platelet poor plasma. However in both arteries studied from complicated pregnancy this stimulation of PGI_2 production was not seen. It would seem therefore that our finding of unrecordable levels of PGI_2 metabolites in hypertensive pregnancies (8.3) could be related to defective endothelial cell production.

8.4 Labetalol in Pregnancy Induced Hypertension

8.4 (a) Introduction

Labetalol is a combined α and β adrenergic blocking agent which has been used extensively in the treatment of pregnancy induced hypertension (PIH). Labetalol has been shown to be of value in controlling the blood pressure with resulting improved foetal survival (Michael et al 1979, Lamming et al 1979, Walker et al 1982). Labetalol also reduces proteinuria (Lamming et al 1979, Walker et al 1982) and platelet consumption (Walker et al 1982a) as well as having a vasodilator action. In patients with severe pregnancy induced hypertension with active platelet consumption, thrombocytopenia and microangiopathic haemolytic anaemia, treatment with labetalol reverses these parameters returning the platelet count to normal (Walker et al 1982b).

The mechanism behind the action of Labetalol is unclear. We have performed studies to examine the in vitro affect of Labetalol on PGI_2 and TXA_2 production.

8.4 (b) Affect on thromboxane and prostacyclin production in whole blood of incubation with Labetalol

8.4 (b) (i) Introduction

There have been several studies published which show that β-adrenoceptor antagonists can reduce platelet aggregation and TXA_2 production from platelet rich plasma stimulated by thrombin and arachidonic acid. (Heinroth 1983, Brandt 1984, Mehta 1983). Most of these studies however have concentrated on propanolol which is a β_1, β_2 blocker. There has however been little work on the effect on whole blood. This may be a more physiological method to use since it leaves the platelets in contact with red and white cells which can influence platelet behaviour by release of ADP (Small 1982) and PGI_2 (Orlandi 1985) respectively.

8.4 (b) (ii) Method

Blood was obtained without stasis from healthy volunteers who had taken no drugs for a minimum of two weeks previously. 1 ml aliquots were placed into previously prepared glass tubes containing either Labetalol in varying concentrations or its vehicle. The tubes were immediately put to incubate at $37^{o}C$ for one hour. The serum was separated by centrifugation at 2500g for 15 minutes at $4^{0}C$. Aliquots were stored at $-70^{o}C$ for RIA for PGI_2M and TXB_2 as described in Chapters 3 and 4 respectively. We had previously shown that inhibition of PGI_2M and TXB_2 could be measured using this technique (see 5.3).

8.4 (b) (iii) Results

Concentration of Labetalol (μg/ml)	Serum TXB_2 (ng/ml)	Serum PGI_2M pg/ml
Control n = 8	117.8 ± 12.5	413.8 ±
1.0 n = 8	99.4 ± 27.7 *	340.0 ± *
10.0 n = 8	74.6 ± 30.3 **	303.8 ± **
100.0 n = 8	48.0 ± 21.2 **	251.2 ± **

* $p < 0.05$
** $p < 0.02$ different from control values

Table 8.2 - Levels of serum TXB_2 and PGI_2M (mean + SD) after incubation with Labetalol

8.4 (c) Thromboxane generation - prior incubation with Labetalol

The reduction observed in PGI_2M and TXB_2 in whole blood allowed to clot with the addition of Labetalol was in response to an endogenous stimulus i.e. thrombin. We also studied the amount of TXB_2 generated in response to an exogenous aggregating agents, in this case collagen at a final concentration of 4 μg/ml. The method used was the method described for thromboxane generation in 4.6 but the PRP was incubated with 100μl of either saline or labetalol for 5 minutes prior to the addition of the aggregating agent.

Table 8.3 shows results of TXB_2 generation after prior incubation with Labetalol

Conc of Labetalol (μg/ml)	TXB_2 (ng/ml)
Control n = 10	105.5 ± 7.1
1 n = 10	78.5 ± 10.8 *
12.5 n = 10	69 ± 16.5 **
25 n = 10	52 ± 15.2 *

* $p < 0.001$
** $p < 0.005$ different from control values

Table 8.3 - TXB_2 generation levels after prior incubation with Labetalol (Means + SD)

8.4 (d) PGI_2 Production from umbilical artery perfusion model treated with Labetalol

We used our umbilical artery perfusion model described in chapter 3.11 to measure levels of PGI_2 metabolites after perfusion with Labetalol. Initial experiments using rat aortic rings to detect release of a PGI_2-like substance by bioassay proved inconclusive. Because of the limitations of the artery ring method as discussed in 3.11 we decided to use the more physiological artery perfusion model. If pregnancy induced hypertension is mediated partly through PGI_2 deficiency (Bodzenta 1980) stimulation of PGI_2 release from the endothelium would help to explain the beneficial effects described after treatment with Labetalol.

25 mls of Labetalol (100 µg/ml) in Ringer's buffer were perfused through the vessel after prior perfusion with 175 mls of buffer alone. The artery was in two halves with one half acting as control. 5ml fractions were collected and stored at $-70^{o}C$ until RIA for PGI_2 metabolites as described in Chapter 3.7. The results we obtained are shown in figure 8.6.

8.4 (e) Discussion

Inhibition of both TXB_2 and PGI_2M was observed when whole blood was left to clot with Labetalol. This would suggest that the inhibition may be due to a block in the arachidonic acid pathway at either the phospholipase or the cyclooxygenase steps. The inhibition of TXA_2 may be clinically beneficial in view of the evidence implicating TXA_2 in vascular disease (Cocker 1983, Robertson 1981, Hirsch 1981, Cocker 1981). However the inhibition of PGI_2 is potentially harmful in view of its vasoprotective effect. This is especially true in toxemic

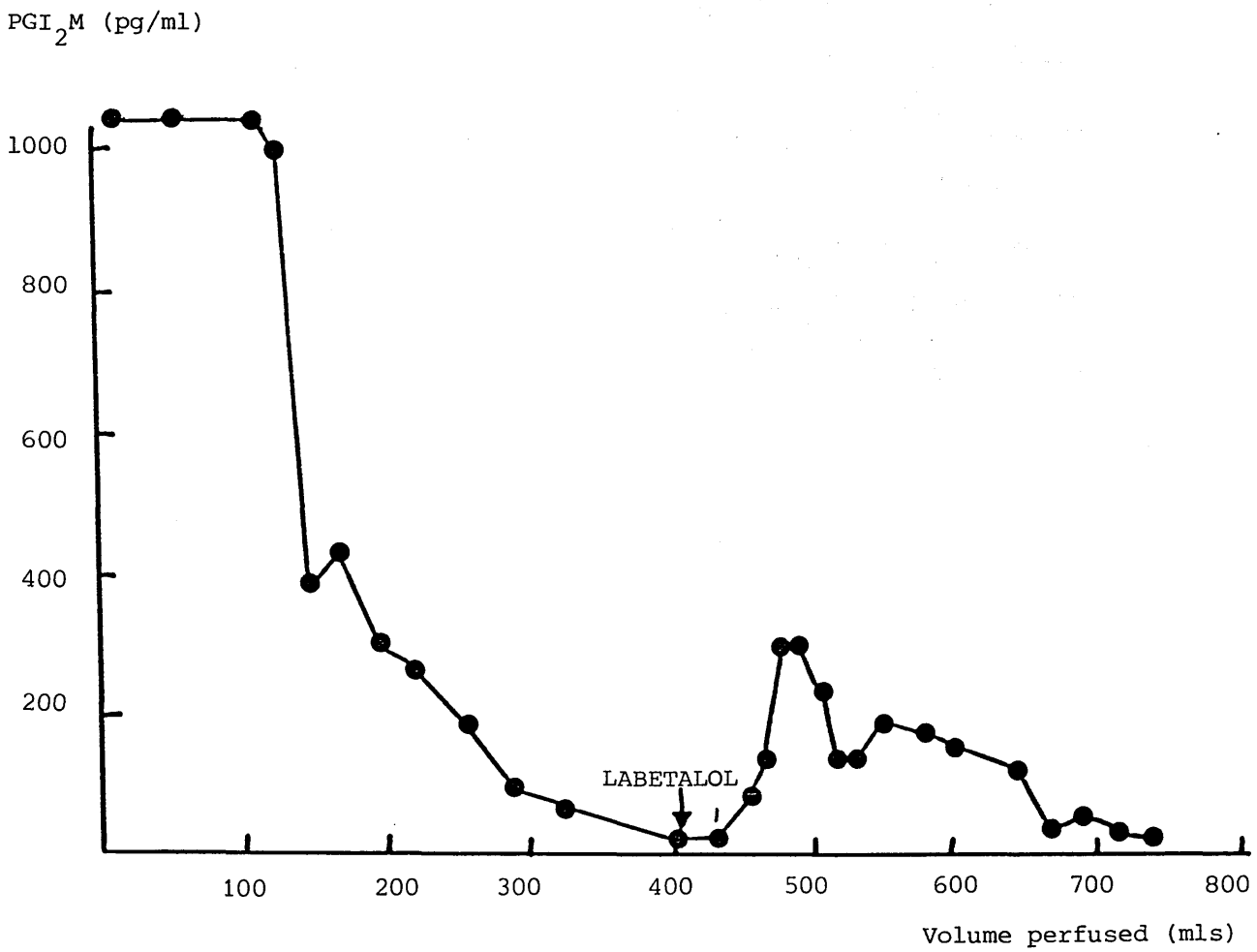


FIGURE 8.6: UMBILICAL ARTERY PERFUSION WITH LABETALOL 100µg/ml

pregnancies in which reduced PGI_2 production has been implicated (Remuzzi 1980, Walsh 1985). Any further reduction therefore could worsen the disease. However the reduction in serum PGI_2 levels was only an average of 39.2% whereas serum TXB_2 levels fell by an average of 59.3% after incubation with Labetalol. Our initial study using rat aortic rings suggested that Labetalol stimulated release of a PGI_2 like substance. When Labetalol 100 µg/ml was perfused through our umbilical artery there was no evidence of inhibition of vascular PGI_2 production there was in fact a slight increase in PGI_2 metabolite levels. However this experiment must be repeated using more umbilical arteries.

It has been shown by Campbell (1981) that propranalol had no effect on PGI_2 production from cultured vascular endothelial cells. We showed however that propranalol inhibited serum PGI_2 after incubation with whole blood (Greer 1985). It may be therefore that vascular PGI_2 production is less sensitive to the effect of Labetalol and other adrenoceptor antagonists such as Labetalol than is PGI_2 production by whole blood which may be affected by white cells. Further work on white cells PGI_2 production must be done before this can established. Whether or not these observations on TXA_2 and PGI_2 production are of any clinical significance must be established by further <u>in vivo</u> studies.

8.5 Amniotic Fluid PGI_2 metabolites

8.5 (a) Introduction

Many pregnancy associated tissues such as the uterus, decidua, placenta and fetal membranes and vessels produce PGI_2 and TXA_2 *in vitro* (Tuvemo 1980). Consequently these tissues could be a source of the increased production of these prostanoids during gestation. Since the amniotic fluid is in contact with many of these tissues its concentrations of PGI_2 metabolites and TXB_2 could reflect intrauterine production of PGI_2 and TXA_2 *in vivo*. Previous studies have found levels of 6-keto-PGF1α in amniotic fluid at term pregnancy to be 444 pg/ml and TXB_2 100 pg/ml (Mitchell 1978, 1979). Ylikorkala et al (1981) found normal levels of 6-keto-PGF1α of 171.8 $\pm$ 9.0 and TXB_2 35.0 $\pm$ 5.7 at approximately 38 weeks of pregnancy. All samples they tested from both normal and complicated pregnancies had detectable amounts of both 6-keto PGF1α and TXB_2. Roy et al found detectable levels of PGI_2 metabolites in amniotic fluid in the second trimester with a mean of 34 $\pm$ 17 (SD) pg/ml (Roy 1984). As the levels of 6-keto-PGF1α, all measured by radioimmunoassay, varied considerably in the published work we decided to use our radioimmunoassay to measure amniotic fluid levels of PGI_2M in amniotic fluid. The involvement of PGI_2 in pre-eclampsia and other pregnancy complications characterised by fetoplacental vasoconstriction is poorly understood and to be able to measure levels in amniotic fluid as well as in plasma would perhaps be valuable. In view of the apparent lack of both synthesis and degradation of prostaglandins in amniotic fluid (Keirse 1979) levels in this body fluid may be considered to reflect the state of prostaglandin metabolism within the uterus and feto-placental unit.

8.5 (b) Patients and Methods

We took samples of amniotic fluid from 27 patients who were undergoing amniocentesis for genetic reasons or for αfetoprotein levels to be measured. Amniotic fluid was immediately placed in ice cold tubes containing 1:9 vol 3.8% trisodium citrate with 3 x 10^{-5}M Indomethacin. The samples were kept on ice and centrifuged within one hour for 15 minutes at 4°C and 2500g. Aliquots were stored at -70°C until RIA for PGI_2 metabolites as described in 3.7.

8.5 (c) Results

We could find no detectable levels of PGI_2 metabolites in any of the samples tested except for one patient who had a level of 62 pg/ml. Her plasma PGI_2 metabolite level was 170 pg/ml. This patient was found to have a foetus with extensive open neural tube defects and subsequently had her pregnancy terminated. She was the only patient in the group studied to have a positive open neural tube defect.

8.5 (d) Discussion

The reason for the measurable level of PGI_2 metabolites in amniotic fluid and also for the very high level of plasma PGI_2 metabolites is unclear. It may be related to exposure of damaged highly vascular, neural tissue to the amniotic fluid and to placental transfer of the PGI_2 metabolites. Further work is needed in order to substantiate this finding. However it is possible that high PGI_2 metabolite levels may be a marker for such defects.

8.6 Conclusions

These studies indicate involvement of the prostacyclin thromboxane balance in pregnancy induced hypertension. It has however yet to be determined whether or not the reduced levels of PGI_2 metabolites observed in the hypertensive patients appeared before the onset of the hypertension and oedema.

The initial observations of failure of platelet rich plasma to cause stimulation of PGI_2 production from the endothelium of umbilical arteries taken from hypertensive patients indicate the possibility of an endothelial cell defect.

CHAPTER 9 - DISCUSSION

9.1 Introduction

When I first started to plan this thesis in 1981 there was much excitement and enthusiasm in the prostaglandin field. The discovery of prostacyclin (PGI_2) was still fairly recent and many people saw it as the answer to many clinical thrombotic problems. It was thought that since PGI_2 stopped platelets aggregating, caused vasodilation and relaxed smooth muscle, reduced production might be the cause of many vascular and thrombotic diseases. Equally, increased production of thromboxane A_2, which has the directly opposite biological actions to PGI_2, might be implicated in many disease states. Because of this much time and effort by many researchers all over the world was invested in attempts to measure production of PGI_2 and TXA_2. As discussed in Chapters 3 and 4 various methods were used to measure plasma levels of these two substances. Many papers have been published relating imbalance of one or other with various disease states. Clinical trials were also being performed in many centres using PGI_2 infusions in various pathological conditions. It almost seemed as if PGI_2 was the 'flavour of the eighties'. However as more and more work was published it soon became apparent that widely differing so-called 'normal' plasma levels were being quoted especially for 6-keto-PGF1α, the metabolite of PGI_2. Over the five years that it has taken me to complete this work thoughts have changed from the euphoric state at the beginning to the present, where many people believe that since plasma levels of 6-keto-PGF1α are now known to be in the low picogram range it is a waste of time trying to measure it at all.

Also, since there is such potential for artefactually high levels of plasma TXA_2 to be generated during blood sampling and processing, the measurement of plasma levels of TXB_2, the stable metabolite, is also worthless. In this discussion I will try to justify my five years of work on these two substances by looking at the value of all the methodology described and the results obtained using that methodology. I will also discuss future work I see resulting from this thesis.

9.2 Value of the methodology developed for the thesis

9.2 (a) Plasma PGI_2 metabolite assay

This is probably the measurement that has received most criticism over the past few years. It is now generally believed that PGI_2 does not function as a circulating hormone. However since it is known that endothelial cells produce PGI_2 in response to noxious, chemical and mechanical stimuli (Ritter 1983) this does not preclude an important local role for PGI_2. For the reasons discussed in Chapter 3 we have developed an assay for the measurements of PGI_2 metabolites in unextracted plasma. Undoubtedly absolute values of 6-keto-PGF1α can best be measured by negative ion gas chromatography mass spectometry assays. it is now generally accepted with PGE_2 that measurements of only one metabolite gave an abnormal picture and the same may be true for PGI_2. It has been shown that only 40% of PGI_2 is metabolised to 6-keto-PGF1α (Myatt 1981) and there has been little published work on the remaining 60% of metabolites such as 13,14-dihydro-6,15 diketo-PGF1α .

We have validated our assay as described in 3.7 and believe that we are measuring metabolites of PGI_2. Since normal levels have been shown to be very near to the lower limit of detection of the assay it is not possible to detect any decrease in PGI_2 production using this method. However in situations where elevated levels of PGI_2 are to be expected it would seem to be a potentially useful measurement. The elevated levels we have found in certain vascular diseases as discussed in Chapter 6 may help our understanding of the pathology of these diseases. Except possibly in the case of pregnancy induced hypertension it is not of use as a diagnostic assay but, in retrospect, the time spent in the development of the assay has I feel not been wasted.

9.2 (b) Prostacyclin stimulating factor assay and umbilical artery perfusion model

As discussed in detail in Chapter 3, the PGI_2 stimulating factor assay using umbilical artery rings has limitations and hence justified the development of our umbilical artery perfusion model. However if one wishes to examine several samples for the presence of this PGI_2 stimulating factor, the umbilical artery ring method is much quicker and allows selection of samples which do not contain stimulating factor. Such selected samples can then be assayed more effectively and accurately using the umbilical cord perfusion model. The umbilical artery perfusion method is a more physiological model than the rings and is more useful in the assessment of the effects of drugs on endothelial cell PGI_2 production. Perhaps its principal limitation is in the number of samples generated for RIA of PGI_2 metabolites which is an expensive assay to perform and hence the reason for

many of our experiments using this model not being repeated as often as one would wish. Our initial findings of the failure of normal platelet poor plasma to stimulate endothelial cell production of PGI_2 in arteries from patients with pregnancy induced hypertension deserves further study using this model.

9.2 (c) Plasma TXB_2 assay

Measurement of plasma levels of TXB_2 is liable to give artefactually high levels due to platelet activation at venepuncture. It is open to question whether what we are measuring is in fact purely thromboxane A_2 produced in response to venepuncture. Like PGI_2 it is now generally thought that TXA_2 does not act as a circulating hormone but is produced in response to local stimuli. It would seem that, if we wish to measure the ability of platelets to produce TXA_2, serum TXB_2 levels would give a more reliable index Since it is less liable to give artefactually high levels. However all samples used for this thesis were taken using a standard venepuncture technique and all samples associated with traumatic venepuncture were discarded. In future studies however serum TXB_2 levels would seem to be a more reliable measurement to use.

9.2 (d) Serum TXB_2, Serum TXB_2 in bleeding time blood and thromboxane generation

As discussed in 4.5, provided blood is immediately incubated at 37 C after venepuncture, serum TXB_2 levels give a reliable measurement of the ability of platelets to generate thromboxane A_2 in response to the endogenous stimulus of thrombin generated during blood clotting. The thromboxane generation assay gives a measurement of the ability of platelet to generate thromboxane

A_2 in response to the exogenous stimulus of collagen 4 µg/ml. It would perhaps appear from the dose response curve we obtained for collagen (figure 4.2) that 8 µg/ml of collagen would be a more suitable dose to use in order to obtain maximum aggregation. However results at this dose are much more variable and 4 µg/ml would seem to be a more satisfactory stimulant.

The measurement of serum TXB_2 from bleeding time blood would appear to give no more information than normal serum levels. However it gives us an indication of serum TXB_2 levels in response to injury rather than when blood is simply left to clot in vitro. It could therefore be argued that it is a more physiological method to use. However the disadvantage of subjecting the patients to a bleeding time test must be weighed against this. When a patient's bleeding time has to be measured for other reasons it would seem to be worthwhile to measure the serum levels of TXB_2 from the site of the incision especially in the case of unexplained bleeding disorders.

9.3 The importance of the prostacyclin/thromboxane balance in the clinical situation

Since a large part of this thesis is concerned with development and evaluation of methodology for the measurement of PGI_2 and TXA_2 production we must therefore decide in retrospect whether or not it was worthwhile. The initial normal volunteer study on diet indicates no involvement of PGI_2 or TXA_2 in the mechanism by which a saturated fat diet produces platelet activation. Although negative findings are usually disappointing to the researcher they still contribute to our knowledge albeit negatively. In the study on smoking we measured only plasma PGI_2 metabolite levels and showed an increase of PGI_2

metabolites after smoking, though the small numbers prevent this being statistically significant. It would have been interesting to measure TXB_2 levels in this study but we were unable to obtain a sufficient volume of blood. This initial study however warrants further investigation of the effect of smoking on the thromboxane/prostacyclin balance.

Our studies on vascular disease showed the perhaps unexpected results of increased levels of PGI_2 metabolites associated with severe vascular disorders. This finding points to the need for further studies to try to determine whether this is a compensatory mechanism; whether there is cellular resistance or whether there is decreased platelet sensitivity to PGI_2 due to exposure to persistently high levels of PGI_2. In these vascular studies we decided initially to measure plasma TXB_2 levels. Since most of these patients were having blood samples taken for other biochemical tests we were unable to obtain sufficient blood for both tests.

In retrospect measurement of serum levels would perhaps have been of more value and we have a study in progress where serum levels of TXB_2 are being measured.

The results from the studies on bleeding disorders show lower levels of plasma PGI_2 metabolites in patients with von Willebrand's disease and also a lower percentage increase after infusion of DDAVP when compared to normals or haemophiliacs perhaps indicating some defect in endothelial cell production. The report on the patients with a possible thromboxane synthetase deficiency is of interest and warrants further study of the patient and her family.

Our studies on pregnancy and associated diseases indicate involvement of the prostacyclin, thromboxane balance in pregnancy induced hypertension and our preliminary findings of the inability of normal platelet poor plasma to stimulate PGI_2 production from the endothelium is of great interest.

9.4 The way ahead

Perhaps the one thing I have noticed most of all when writing the results of this work is the need for more work. As our knowledge of a subject develops so does our quest for yet more knowledge and we may never reach the end. From this viewpoint alone therefore the work presented in this thesis would seem to be worthwhile. The initial development and evaluation of the methodology has enabled us to see which methodology is the most useful for future studies. I feel a larger study on smoking would be worthwhile using large numbers to try to discover whether or not there are any differences in PGI_2 metabolite levels in smokers who develop smoking related diseases and those who do not. It could conceivably be that the suggestion of increased PGI_2 metabolite levels we have noticed after smoking in some smokers is a defence mechanism which fails in some people thus leading to vascular and other disorders. There is much research being performed at present by drug companies in the development of PGI_2 analogues suitable for oral and transdermal administration. Measurement of plasma PGI_2 metabolites in patients using these preparations would give some indication of absorption. A study of thromboxane generation both by endogenous stimulus in serum and generation by the exogenous stimulus of

collagen would be worthwhile in patients with various vascular diseases and this has in fact been started. The use of the various indices of thromboxane generation described in Chapter 4 will also be useful in the future investigation of patients suffering from unexplained bleeding disorders.

More work with larger numbers must be done to discover if our initial findings suggesting a defect in endothelial PGI_2 production associated with pregnancy induced hypertension is in fact true and whether this could also be associated with other types of hypertension.

Adelman B, Stemerman M B, Mennell D
The interaction of platelets with aortic subendothelium Inhibition of adhesion and secretion by PGI_2
Blood 58: 198-205 (1981)

Alabaster V A
Metabolism of arachidonic acid and its endoperoxide (PGH_2) to myotropic products in guinea-pig and rabbit isolated lungs
British Journal of Pharmacology 69(3): 479-489 (1980)

Ali M, McDonald J W D, Thiessen J J, Coates P E
Plasma acetylsalicylate and salicylate and platelet cyclo-oxygenase activity following plain and enteric-coated aspirin
Stroke 11: 9-13 (1980)

Alster P, Wennmalm A
Effects of nicotine on the formation of prostaglandins in the rabbit kidney
Acta Physioligica Scandinavia (Suppl) 479: 49-51 (1981)

Amezuca J L, O'Grady J, Salmon J A, Moncada S
Prolonged paradoxical effect of aspirin on platelet behaviour and bleeding time in man
Thrombosis Research 16: 69-79 (1979)

Asmussen K, Kjeldsen K
Intimal ultrastructure of human umbilical arteries. Observations on arteries from newborn children of smoking and non-smoking mothers
Circulation Research 36: 579-589 (1975)

Baenziger N L, Dillender M J, Majerus P
Cultured human skin fibroblasts and arterial cells produce a labile platelet-inhibitory prostaglandin
Biochemical and Biophysical Research Communications 78: 294-300 (1977)

Baenziger N L, Becherer P R, Majerus P W
Characterisation of prostacyclin synthesis in rats cannot be completely dissociated.
British Journal of Haematology 42: 425-431 (1979)

Baer P G, McGiff J C
Comparison of effects of prostaglandins E_2 and I_2 on rat renal vascular resistance
European Journal of Pharmacology 54(4): 359-363 (1979)

Bamford D S, Logee M, Williams K I
Prostacyclin formation by pregnant human myometrium
British Journal of Obstetrics and Gynaecology 87: 215-218 (1980)

Barnhardt M I, Chen S-te, Lusher J M
DDAVP: Does the drug have a direct effect on the vessel wall?
Thrombosis Research 31: 239-253 (1983)

Barrow S E, Heavey D J, Jacqz E, Blair I A, Dollery C T
Des-Amino-D Arginine Vasopressin does not increase circulating levels of prostacyclin or Thromboxane A_2 during infusion in man
Thrombosis and Haemostatis 50(2): 662 (1983)

Barrow S E, Waddell K A, Ennis M, Dollery C T, Blair I A
Analysis of picomolar concentrations of 6-oxo-prostaglandin F_1 alpha in biological fluids
Journal of Chromatography 239: 71-80 (1982)

Beguin F, Bygdeman M, Green K, Samuelsson B, Toppozoda M, Wiqvist N
Analysis of prostaglandin F_2 and metabolites in blood during constant intravenous infusion of PGF_2 in the human female.
Acta Physiol Scana 86: 430-432 (1972)

Belch J J F, McLaren M, Anderson J, Lowe G D O, Sturrock R D, Capell H A, Forbes, C D
Increased PGI_2 metabolites and decreased red cell deformability in patients with systemic sclerosis and Raynaud's syndrome.
Prostaglandins Leukotrienes and Medicine 17: 1-9 (1985)

Belch J J F, Newman P, Drury J K, Lieberman P, Capell H, Forbes C D, Prentice C R M
Successful treatment of Raynaud's syndrome with prostacyclin
Thrombosis and Haemostatis 45(3): 255-256 (1981)

Belch J J F, Small M, McKenzie F, Hill P A, Lowe G D O, McIntyre D E, Forbes C D, Prentice C R M
DDAVP: Stimulates prostacyclin production
Thrombosis and Haemostasis 47(2): 122-123 (1982)

Berberich F R, Cuene S A, Chard R L J
Thrombotic thrombocytopenic purpura. Three cases with platelet and fibrinogen survival studies
Journal of Paediatrics 84: 503-509 (1974)

Bergstrom S, Samuelsson B
Isolation structure and actions of the prostaglandins.
Prostaglandins, Nobel Symposium 2
New York Interscience Publishers: 21-30 (1967)

Bergstrom S, Danielsson H, Samuelsson B
The enzymatic formation of prostaglandin E_2 from arachidonic acid
Biochim Biophys Acta 90: 207-210 (1964)

Berson S A, Yalo R S
Insulin-I-131 metabolism in human subjects: demonstration of insulin binding globulin in circulation of insulin treated subjects
Journal of Clinical Investigation 35: 170-190 (1956)

Bertele V, Cerletti C, Schieppati A, Di Minno G, de Gaetano G
Inhibition of thromboxane synthetase does not necessarily prevent platelet aggregation
Lancet: 1057-1058 (1981)

Betteridge D J, El Tahir K E H, Reckless J P D, Williams K I
Platelets from diabetic subjects show diminished sensitivity to prostacyclin
European Journal of Clinical Investigation 12: 395-398 (1982)

Bierenbaum M D, Fieischman A I, Stier A, Sornol H, Watson P B
Effect of cigarette smoking upon in vivo platelet function in man
Thrombosis Research 12: 1051-1057 (1978)

Bionoux M A, Odell W D
Use of dextran coated charcoal to separate antibody-bound from free hormone. A critique
Journal of Clinicial Endocrine Metabolism 36: 303-310 (1973)

Bizzozero J
Ueber eine neuen Formbestandtheil des Blutes und desen Rolle bei der Thrombose und der Blutgerinnung Virchows Archives
(Pathol Anat) 90: 261-332 (1882)

Blair I A, Barrow S E, Waddell K A, Lewis P J, Dollery C T
Prostacyclin is not a circulating hormone in man
Prostaglandins 23(4): 579-589 (1982)

Blair I A, Barrow S E, Waddell K A, Lewis P J, Dollery C T
Specific quantitative determination of 6-oxo-prostaglandin F_1 alpha in human plasma
Advances in Prostaglandin Thromboxane and Leukotriene Research 11: 197-205 (1983)

Blackwell G J, Flower R J
Inhibition of phospholipase
British Medical Bulletin 39: 260-264 (1983)

Blackwell G J, Flower R J, Russell-Smith N, Salmon J A, Thorogood P B, Vane J R
1-n-Butylimidazole : a potent and selective inhibitor of "thromboxane synthetase"
British Journal of Pharmacology 64(3): 435P (1978)

Blasko G
Effects of PGI_2 on the inactivation of thrombin, factor xa and plasmin by antithrombin III and heparin
Prostaglandins 18: 3-9 (1979)

Blunt R J, Porter J M
Raynaud's syndrome
Seminars in Arthritis and Rheumatology 10(4): 282-308 (1981)

Bodzenta A
Prostacyclin activity in amniotic fluid in pre-eclampsia
Lancet: 650-652 (Sept 1980)

Bolger P M , Eisher G M, Ramwell P W, Slotkoff L M, Corey E J
Renal actions of prostacyclin
Nature 271: 467-469 (1978)

Bolton P J, Jogee M, Myatt L, Elder M G
Maternal plasma 6-oxo-PGF1α levels throughout pregnancy: a longitudinal study
British Journal of Obstetrics and Gynaecology 88: 1101-1103 (1981)

Booyse F M, Osikowicz G, Feder S
Effects of various agents on ristocetin-Willebrand factor activity in long term cultures of von Willebrand and normal human umbilical ven endothelial cells
Thrombosis and Haemostasis 46(3): 668 (1981)

Booyse F M, Osikowicz G, Quarfoot A J
Effects of chronic oral consumption of nicotine on the rabbit aorta endothelium
American Journal of Pathology 102(2): 229-238 (1981)

Born G V R
Aggregation of blood platelets by adenosine diphosphate and its reversal
Nature, London 194: 927-929 (1962)

Brain M C, Hourihane D O
Microangiopathic haemolytic anaemia : the occurrence of haemolysis in experimentally produced vascular disease
British Journal of Haematology 13: 135-142 (1967)

Brandt R, Seppala E, Nowak J, Vapriatalo H
Effect of propanolol, practolol and atenolol on human platelet thromboxane formation and plasma levels of prostaglandins 6-keto-F1α and E_2
Prostaglandins Leukotrienes and Medicine 16(2): 191-203 (1984)

Brantmark B, Wahlin-Boll E, Melander A
Bioavailability of acetysalicyclic acid from rapid and slow - release formulations and in combination with dipyridamol
European Journal of Clinical Pharmacology 22: 309-314 (1982)

Brinson K
Effect of nicotine on human blood platelet aggregation
Atherosclerosis 20: 137-140 (1974)

Bunting S, Gryglewski R, Moncada S, Vane J R
Arterial walls generate from prostaglandin endoperoxide a substance (PGX) which relaxes strips of mesenteric and coeliac arteries and inhibits platelet aggregation
Prostaglandins 12: 897-913 (1976)

Bunting S, Simmons P M, Moncada S
Inhibition of platelet activation by PGI_2. Possible consequence in coagulation and anticoagulation
Thrombosis Research 21: 89-101 (1981)

Burch J W, Stanford N, Majerus P W
Inhibition of platelet prostaglandin synthetase by oral aspirin
Journal of Clinical Investigation 61: 314-319 (1978)

Burr G L, Burr M M
A new deficiency disease produced by the rigid exclusion of fat from the diet
Journal of Biological Chemistry 86: 587-621 (1930)

Bussolino E, Benedetto C, Massobrio M, Camussi G
Maternal vascular PGI_2 activity in preeclampsia
Lancet 2: 702 (1980)

Campbell W B, Johnson A R, Callahan K S, Graham R M
Anti-platelet activity of beta-adrenergic antagonists: inhibition of thromboxane synthesis and platelet aggregation in patients receiving long-term propranolol treatment
Lancet: 1382-1384 (1981)

Carey F, Menashi S, Crawford N
Localization of cyclo-oxygenase and thromboxane synthetase in human platelet intracellular membranes
Biochemical Journal 204 (3): 847-851 (1982)

Carreras L O, Defreyn G, Van Houtte E, Vermylen J, Van Assche A
Prostacyclin and pre-eclampsia
Lancet: 442 (1981)

Carter A J, Jones E W
The effect of the degree of diabetic control on thromboxane synthesis in clotted whole blood
Thrombosis Research 41: 137-141 (1986)

Cash J D, Gader A M A, Da Costa J
Release of plasminogen activator and factor VIII in response to LVP, AVP, DDAVP, angiotensin II and oxytocin in man
British Jounral of Haematology 27: 363-364 (1974)

Cash J D, Gader A M, Mulder J L, Cort J H
Structure-activity relations of the fibrinolytic response to vasopressins in man
Clinical Science and Molecular Medicine 54(4): 403-409 (1978)

Cerletti C, Latini R, Del Maschio A, Galleti F, Dejana E, de Gaetano G
Aspirin kinetics and inhibition of platelet Thromboxane Generation-Relevance for a solution of the "Aspirin dilemma"
Thrombosis and Haemostatis 53(3): 415-418 (1985)

Chard T
An introduction to radioimmunoassay and related techniques
North Holland, Amsterdam Editors Work TS, Work E (1981)

Chierchia S, Patrono C, Crea F, Giabattoni G, Caterina R, Ginotti G, Distante, Maseri A
Effects of intravenous prostacyclin in variant angina
Circulation 65: 470-477 (1982)

Clifford P C, Martin M F R, Sheddon E J, Kirby J D, Baird R N, Dieppe P A
Treatment of vasospastic disease with prostaglandin E_1
British Medical Journal 281: 1031-1034 (1980)

Codde J P, Barden A, Vandongen R, Beilin W
Validation of the direct radioimmunoassay of prostanoids in serum and kidney
Prostaglandins Leukotrienes and Medicine 14: 55-64 (1984)

Coker S J, Parratt J R
Effects of dazoxiben on arrthythmias and ventricular fibrillation induced by coronary artery occlusion and reperfusion in anaesthetised greyhounds
British Journal of Clinical Pharmacology (Supplement) 15: 87S-95S (1983)

Coker S J, Parratt J R, Ledingham M C A, Zeitlin I J
Thromboxane and prostacyclin release from ischaemic myocardium in relation to arrhythmias
Nature 291: 323-324 (1981)

Crawford M A
Fatty-acid ratios in free-living and domestic animals
Lancet: 1329-1333 (1968)

Curwen K D, Gimbrone M A, Handin R I
In vitro studies of thromboresistance: The role of prostacyclin (PGI_2) in platelet adhesion to cultured normal and virally transformed human vascular endothelial cells
Laboratory Investigation 42: 366-374 (1980)

Czervionke R L, Hoak J C, Fry G L
Effect of aspirin on thrombin-induced adherence of platelets to cultured cells from the blood vessel wall
Journal of Clinical Investigation 62: 847-856 (1978)

Dadak C H, Leithner C H, Sinzinger H, Silverbauer H
Diminished prostacyclin formation in umbilical arteries of babies born to women who smoke
Lancet: 94 (1982)

Davis J W, Davis R F
Acute effect of tobacco cigarette smoking on the platelet aggregate ratio
American Journal of Medical Science 278: 139-143 (1979)

Davis T M E, Brown E, Finch D R, Mitchell M D, Turner R C
In-vitro venous prostacyclin production, plasma 6-keto-prostaglandin $F_{1\alpha}$ concentrations and diabetic retinopathy
British Medical Journal 282: 1259-1262 (1981)

Dawber T R, Kannel W B, Revotskie N, Stokes J, Kagan A, Gordon T
Some factors associated with the development of coronary heart disease. 6 years follow up experience in the Framgingham study
American Journal of Public Health 49: 1359-1360 (1959)

Defreyn G, Dauden V, Machin S J, Vermylen J
A plasma factor in uraemia which stimulates PGI_2 release from cultured endothelial cells
Thrombosis Research 19: 695-699 (1980)

Defreyn G, Machin S J, Carreras L O, Vergara Dauden M, Chamone D A F, Vermylen J
Familial bleeding tendency with partial platelet thromboxane synthetase deficiency: reorientation of cyclic endoperoxide metabolism
British Journal of Haematology 49: 29-41 (1981)

Dembele-Duchesne M J, Thaler-Dao H, Chavis C, Crastes de Paulet A
The human placental anti-aggregating factor is neither PGI_2 nor a PGI_2 metabolite.
Prostaglandins 24: 701 (1982)

De Takatas G, Fowler E F
Raynaud's phenomenon
Journal of The American Medical Association 179: 1-8 (1962)

Dollery C T, Friedman L A, Hensby C N, Kohner E, Lewis P J, Porta M, Webster J
Circulating prostacyclin may be reduced in diabetes
Lancet 2: 1365 (1979)

Dowd P M, Kovacs I B, Bland C J, Kirby J D T
Effect on PGI_2 and E_1 on red cell deformability in patients with Raynauds phenomenon and systemic sclerosis
British Medical Journal 283: 350 (1981)

Dowd P M, Martin M F R, Bowcock S A, Cooke E, Dieppe P A, Kirby J D T
Therapy of Raynauds phenomenon by intravenous infusion of prostacyclin
British Journal of Dermatology 106: 81-89 (1982)

Downing I, Shepherd G L, Lewis P J
Reduced PGI_2 production in pre-eclampsia
Lancet: 1374 (1980)

Downing I, Williams K I
A comparison of arachidonic acid metabolism by microsomes from pregnant rat decidua and myometrium
Archives of International Pharmacodynamics and Theraputics 247 (1): 4-20 (1980)

Dusting G J, Moncada S, Vane J R
Prostacyclin (PGX) is the endogenous metabolite responsible for relaxation of coronary arteries induced by arachidonic acid
Prostaglandins 13: 3-15 (1977)

Dusting G J, Moncada S, Vane J R
Disappearance of prostacyclin (PGI_2) in the circulation of the dog
British Journal of Pharmacology 62: 414P-415P (1978)

Editorial: Haemolytic uraemic syndrome of young women
Lancet: 943-944 (1976)

Ehrlich P
Chemotheraputics: scientific principles, methods and results
Lancet: 445-451 (1913)

Ehrman M L, Jaffe E A
PGI_2 inhibits the development in human platelets of ADP and arachidonic acid-induced shape change and procoagulant activity
Prostaglandins 20: 1103-1116 (1980)

Elbaor J E, Farrington E, Downey J A, Leroy A C
The effect of reserpine on cold-induced vasoconstriction in Raynaud's syndrome and scleroderma
Arthritis and Rheumatology 14: 381-382 (1971)

Ellwood D A, Anderson A B M, Mitchell M D, Murphy G, Turnbull A C
Prostanoids, collagenase and cervical softening in the sheep.
In: The Cervix in Pregnancy and Labour (Ellwood D A and Anderson A V N eds) Churchill Livingstone Edinburgh: pp 57-73 (1981)

Epstein M, Lifschitz M, Rapport K
Augmentation of prostaglandin production by linoleic acid in man
Clinical Science 63: 565-571 (1982)

El Tahir K E H, Williams K I, Betteridge D J
The effect of experimental diabetes on prostacyclin production by tissues from pregnant rats
Prostaglandins, Leukotrienes and Medicine 8: 429-435 (1982)

Farr M J, Roberts S, Morley A R, Dewar P J, Roberts D F, Uldall P R
The Haemolytic Uraemic Syndrome-A family study
Quarterly Journal of Medicine 174: 161-188 (1975)

Ferreira S H, Vane J R
Prostaglandins: their disappearance from and release into the circulation
Nature 216: 868-873 (1967)

Ferreira S H, Nakamura M, de Abreu Castro M S
The hyperalgesic effects of prostacyclin and prostaglandin E_2
Prostaglandins 16(1): 31-37 (1978)

Fidler J, Bennett M J, De Swiet M, Ellis C, Lewis P J
Treatment of pregnancy hypertension with prostacyclin
Lancet: 31-32 (1980)

Fiocco U, Calo L, Piccoli A, Del Torre M, Cantaro S, Baggio B, Glorioso S,
Borsatti A, Todesco S
Urinary prostaglandins in scleroderma p 768 in
Abstracts of International Prostaglandin Meeting: Florence (1982)

Fischer S, Struppler M, Bohlig B, Bernutz C, Wober W, Weber P C
The influence of selective thromboxane synthetase inhibition with a novel imidazole derivative, UK 38, 485 on prostanoid formation in man
Circulation 68: 821-826 (1983)

Fitzgerald G A, Brash A R, Falardeau P, Oates J A
Estimated rate of PGI_2 secretion into the circulation of normal man
Journal of Clinical Investigation 68: 1272-1276 (1981)

Fitzgerald G A, Maas R L, Lawson J A, Oates J A, Roberts L J, Brash A R
In: Advances in Prostaglandin Thromboxane and Leukotriene Research 11:
pp 265-266 (1983)

Forder R A, Carey F
Measurement of human venous plasma orostacyclin and metabolites by radioimmunoassay-a reappraisal
Prostaglandins, Leukotrienes and Medicine 12(3): 323-346 (1983)

Frederickson D S
In: Harrison's Principles of Internal Medicine p 1306 Thorn G(Ed) New York, McGraw-Hill (1977)

Friedman G D, Petitti D B, Bawol R D, Siegelaub A B
Mortality in cigarette smokers and quitters
New England Journal of Medicine 304: 1404-1410 (1981)

Garcia M J, McNamara P M, Gordon T, Kannell W B
Morbidity and mortality in diabetics in the Framingham population
Diabetes 23: 537-546 (1969)

Garcia M J, McNamara P M, Gordon T
Morbidity and mortality in diabetics in the Framingham population. Sixteen year follow-up study.
Diabetes 23: 105-111 (1974)

Gasser C, Hitzig W H
Hamolytisch-uramische syndrome
Schweiz Med Wchn-Schr 85: 905-909 (September 20, 1955)

Gasser V C, Gautier E, Steck A, Siebenmann R E, Oechslin R
Hamolytisch-uramische syndrome: Bilaterale Nierenrindennekrosen bei akuten erworbenen hamolytischen Anamien
Schweiz Med Wochenschr 85: 905-909 (1955)

Gensini G F, Abbate R, Favilla S, Serneri G G N
Changes of platelet function and blood clotting in diabetes mellitus
Thrombosis and Haemostasis 42: 983-993 (1979)

Gerkens J F, Friesinger G C, Branch R A, Shand D G, Gerber J G
A comparison of the pulmonary, renal and hepatic extractions of PGI_2 and PGE_2-PGI_2 a potential circulating hormone
Life Science 22(20): 1837-1842 (1978)

Gianantonio C A, Vitacco M, Mendilaharzu F
The haemolytic-ureamic syndrome. Renal status of 76 patients at long term follow up
Journal of Paediatrics 72: 757-765 (1968)

Giessen I W J, Wolsman E Z, Verheught F W A, Hoor F, Quadt F J A
Effect of nicotine on production of prostacyclin in human umbilical artery
British Heart Journal 48: 493-496 (1982)

Glynn M F, Mustard J F, Buchanan M R, Murphy E A. Cigarette smoking and platelet aggregation.
Canadian Medical Association Journal 95: 549-553 (1966)

Goldblatt M W
Properties of human seminal plasma
Journal of Physiology (London) 84: 208-218, 1935

Goodman R P, Killam A P, Brash A R, Branch R A
Prostacyclin production during pregnancy and pregnancy complicated by hypertension
American Journal of Obstetrics and Gynaecology 142: 817 (1982)

Gotoh S, Ogihara T, Nakamaru M, Masuo K, Hata T, Kumahara Y
Levels of plasma 6-keto-PGF1α in normotensive and essential hypertensive males with and without a family history of hypertension
Prostaglandins Leukotrienes and Medicine 10(1): 27-32 (1983)

Grabowski E F, Weksler B B, Jaffe E A, Tack-Goldman K, Gersham S L
Platelets augment prostacyclin (PGI_2) production by cultured endothelial cells exposed to flowing blood
Circulation 70: 11-57 (1984)

Grant N F, Daley G F, Chard S, Whalley P J, McDonald P C
A study of angiotensin II pressor response throughout primigravid pregnancy
Journal of Clinical Investigation 52: 2682-2689 (1973)

Grantstrom L, Kindahl H
Radioimmunoassay of prostaglandins and thromboxanes
In: Advances in prostaglandin and thromboxane research: p 119-210
Editor
Frolich J C (1978)

Greaves M, Preston F E
Plasma 6-keto-PGF1α fact or fiction
Thrombosis Research 26(3): 145-157 (1982)

Greenwood F C, Hunter W M, Glover J S
The preparation of T-131-labelled human growth hormone of high specific radioactivity
Biochemical Journal 89: 114-123 (1963)

Greer I A, Walker J J, McLaren M, Calder A A, Forbes C D
Inhibition of thromboxane and prostacyclin production in whole blood by adrenoceptor antagonists
Prostaglandins Leukotrienes and Medicine 19: 209-217 (1985)

Gresele P, Van Hautte E, Arnout J, Deckmyn H, Vermylen J
Thromboxane synthetase inhibition combined with thromboxane receptor blockade: a step forward in antithrombotic strategy
Thrombosis and Haemostasis 52(3): 364 only (1984)

Grignani G, Gamba G, Ascari E
'In vivo' platelet aggregates after replacement therapy in patients with haemophilia A
Thrombosis and Haemostatis 42: 813-814 (1979)

Grose J H, Lebel M, Gbeassor F M
Diminished urinary prostacyclin metabolite in essential hypertension
Clinical Science (supplement) 59(6): 121s-123s (1980)

Gryglewski R J, Bunting S, Moncada S, Flower R J, Vane J R
Arterial walls are protected against deposition of platelet thrombi by a substance (Prostaglandin x) which they make from prostaglandin endoperoxides
Prostaglandins 12: 685-713 (1976)

Gryglewski R J, Korbut R, Ocetkiewicz A C
Generation of prostacyclin by lungs in vivo and its release into the arterial circulation
Nature 273: 765-767 (1978a)

Gryglewski R J, Korbut R, Ocetkiewcz A C, Stachwa T
In vivo method for quantification of antiplatelet potency of drugs
Nauyn - Schmiedeberg's Archives of Pharmacology 302: 25-30 (1978 b)

Gullner H-G, Cerletti C, Bartter F C, Smith J B, Gill J R
Prostacyclin overproduction in Bartter's syndrome
Lancet. 767-768 (1979)

Gullner H-G, Gill J R, Mitchell M D
Plasma 6-keto prostaglandin F1α in disorders involving hypokalemia
Prostaglandins Leukotrienes and Medicine 9(5): 473-478 (1982)

Haber E, Page L B, Richards F F
Radioimmunoassay employing gel filtration.
Annals of Biochemistry 12: 163-172 (1965)

Hall E R, Wu K K, O'Brien R
The reduction of potential TXB_2 levels when serum is preincubated at room temperature
Prostaglandins Leukotrienes and Medicine 3: 279-280 (1984)

Halushka P V, Rogers R C, Loadholt C B, Colwell J A
Increased platelet thromboxane synthesis in diabetes mellitus
Journal of Laboratory and Clinical Medicine 97: 87-96 (1981)

Hamberg M, Svensson J, Samuelsson B
Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides
Proceedings of the National Academy of Science USA 72: 2994-2998 (1975)

Hamberg M, Tuverno T, Svensson J, Johnsson C-E
Formation and action of PGI_2 in the isolated human umbilical artery
Acta Physiol Scand 106: 289-292 (1979)

Hamberg M, Svensson J, Wakabayashi T
Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation
Proceedings of the National Academy of Science USA 71: 345-349 (1974)

Hammarstrom S, Falardeau P
Resolution of prostaglandin endoperoxide synthetase and thromboxane synthetase of human platelets
Proceedings of the National Academy of Science USA 72: 3691-3695 (1977)

Harper M J K, Khodr G S, Valenzuela G
Prostaglandin production by human term placentasin vitro
Prostglandins Leukotrienes and Medicine 11: 121-127 (1983)

Harrison H E, Reece A H
Decreased vascular prostacyclin in experimental diabetes
Life Science 23: 351-356 (1978)

Harrison H E, Reece A H, Johnson M
Effect of insulin treatment on prostacyclin in experimental diabetes
Diabetologia 18: 65-68 (1980)

Hartiala J, Simberg W, Votila P
Exposure to carbon monoxide or to nicotine does not inhibit PGI_2 formation by rat arterial rings incubated with human platelet rich plasma
Artery 10: 412-419 (1982)

Hassam A G, Willis A L, Denton J P, Stevens P, Crawford M A
The effect of essential fatty acid-deficient diet on the levels of prostaglandins and their fatty acid precursors in the rabbit brain
Lipids 14: 78-80 (1979)

Hawiger J, Parkinson S, Timmons S
PGl inhibits mobilisation of fibrinogen binding sites on human ADP and thrombin treated platelets
Nature 283: 195-197 (1980)

Hawkins H J, Smith J B, Nicolaou K C, Eling T E
Studies of the mechanisms involved in the fate of prostacyclin (PGl_2) and 6-keto-PGF1α in the pulmonary circulation
Prostaglandins 16: 871-884 (1978)

Hawkins R I
Smoking, platelets and thrombosis
Nature 236: 450-452 (1972)

Heavey D J, Barrow S E, Hickling N E, Ritter J M
Aspirin causes short lived inhibition of bradykinin-stimulated prostacyclin production in man
Nature 318: 186-188 (1985)

Heinroth I, Block H U, Ponicke K, Forster W
Influence of propanolol, pindolol, practolol and talinolol on arachidonic acid (AA) and U46619-induced aggregation and on AA-induced thromboxane A_2 formation in platelets
Prostaglandins Leukotrienes and Medicine 12: 189-206 (1983)

Hensby C N, Barnes P J, Dollery C T, Dargie H
Production of 6-oxo-$PGF_1\alpha$ by human lung in vivo
Lancet: 1162-1163 (1979)

Hensby C N, Fitzgerald G A, Friedman L A, Lewis P J, Dollery C T
Measurement of 6-oxo-PGF_1 alpha in human plasma using gas chromatography-mass spectometry
Prostaglandins 18(5): 731-736 (1979)

Herbert V, Lau K-S, Gottlieb C W, Bleicher S J
Coated charcoal immunoassay of insulin
Journal of Clinical Endocrine Metabolism 25: 1375-1384 (1965)

Heyden S
Epidemiological data on dietary fat intake and atherosclerosis with an appendix on possible side effects
In: The Role of Fats in Human Nutrition pp 43 - 113 Academic Press, London: Vergrosser A J (Editor) (1975)

Hill T W, Moncada S
The renal haemodynamic and excretory actions of prostacyclin and 6-oxo-PGF1 alpha in anaesthetised dogs
Prostaglandins 17(1): 87-98 (1979)

Hillborn M, Muuronen A, Lowbeer C, Anggard E, Beving H, Kangasaho M
Platelet thromboxane formation and bleeding time is influenced by ethanol withdrawal but not by cigarette smoking
Thrombosis and Haemostasis 53(3): 419-422 (1985)

Hirsch P D, Hillis L D, Campbell W B, Firth B G, Willerson J T
Release of prostaglandins and thromboxane into the coronary circulation in patients with ischaemic heart disease
New England Journal of Medicine 304: 685-691 (1981)

Holman R T
In: Function and biosynthesis of lipids: 515 Eds Bazan N G, Bremner R R, Guisto N M
Plenum Press New York and London: (1977)

Hornby E J, Skidmore I F
Evidence that prostaglandin endoperoxides can induce platelet aggregation in the absence of thromboxane A_2 production
Biochemical Pharmacology 31: 1158-1160 (1982)

Hornstra G, Lewis B, Chait A, Turpeinten D, Karvonen M J, Vergrossen A J
Influence of dietary fat on platelet function in men
Lancet: 1155-1157 (1973)

Hornstra G, Lussenberg R N
Relationship between the type of dietary fatty acid and arterial thrombotic tendency in man
Atherosclerosis 22: 449-516 (1975)

Hornstra G, Haddeman E, Don J A
Blood platelets do not provide endoperoxides for vascular prostacyclin production
Nature 279: 66-68 (1977)

Hornstra G
Dietary fats, Prostanoids and Arterial Thrombosis
In: Developments in Haematology and Immunology Hornstra G(ed)
Nijhoff publishers, The Hague 4: 138-181 (1982)

Horrobin D F
The regulation of prostaglandin biosynthesis: negative feedback mechanisms and the selective control of formation of 1 and 2 series prostaglandins: relevance to infammation and immunity
Medical Hypotheses 6: 687-709 (1980)

Horrobin D F, Jenkins K, Manku M S
Raynaud's phenomenon, histamine and prostaglandins
Lancet: 747-748 (1983)

Hoult J R, Moore P K
Enzyme in rabbit kidney converts prostaglandin F_2 directly to prostaglandin E_2 in vitro [proceedings]
British Journal of Pharmacology 63(3): 415P-416P (1978)

Howard M A, Firkin B G
Ristocetin-a new tool in the investigation of platelet aggregation
Thrombosis et Diathesis Haemorrhogica 26: 362-369 (1971)

Hunter W M, Greenwood F C
Studies on the secretion of human-pituitary-growth hormone
British Medical Journal 5386: 804 - 807 (1964)

Hutchison AA, Ogletree M L, Palme C J H, Leheup B P, Barrett J M, Fiescher A C, Stahlman M T, Brigham K L
Plasma 6-keto-$PGF_{1\alpha}$ and TXB_2 in sick preterm neonates
Prostaglandins Leukotrienes and Medcine 18(2): 163-181 (1985)

Inoshita T, Whiteside T L, Rodman G P, Taylor F H
Abnormalities of T lymphocyte subsets in patients with progressive systemic sclerosis (PSS, scleroderma)
Journal of Laboratory and Clinical Medicine 97 (2): 264-277 (1981)

Istoel, Giessen W J vd, Wolsman E Z, Waverheugt F, ten Hoor F, Quadt F J A, Hugenholtz P G
Effect of nicotine on production of prostacyclin in human umbilical artery
British Heart Journal 48: 493-496 (1982)

Jarret R J, Keen H
Hyperglycaemia and diabetes mellitus
Lancet: 1009-1012 (1976)

Jarret P E M, Morland M, Browse N L
Treatment of Raynaud's phenomenon by fibrinolytic enhancement
British Medical Journal ii: 523-525 (1981)

Jeremy J Y, Mikhailidis D P, Dandona P
Cigarette smoke extracts, but not nicotine, inhibit prostacyclin (PGI_2) synthesis in human, rabbit and rat vascular tissue
Prostaglandins Leukotrienes and Medicine 19: 261-270 (1985)

Jewett B (ed)
The dialogues of Plato (edition 3)3: 339-543 Macmillan New York (1892)

Jogee M, Myatt L, Elder M G
Decreased PGI_2 production by placental cells in culture from pregnancies complicated by fetal growth retardation
British Journal of Obstetrics and Gynaecology 90: 247 250 (1983)

Johnson M, Harrison H E, Raftery A T, Elder J B
Vascular prostacyclin may be reduced in diabetes in man
Lancet: 325-326 (1979)

Johnson R A, Morton D R, Kinner J H, Gorman R R, McGuire C, Sun F F, Whittaker N, Bunting S, Salmon J, Moncada S, Vane J R
The chemical structure of prostaglandin x (prostacyclin)
Prostaglandins 12: 915-928 (1976)

Johnston R V, Giner J V, Lowe G D O, Forbes C D, Prentice C R M
Prevention of lipid induced platelet aggregation by aspirin
Thrombosis Research 27: 235-239 (1982)

Jorgensen K A, Pedersen R S
Familial deficiency of PGI_2 production stimulating factor in the haemolytic uremic syndrome of childhood
Thrombosis Research 21: 311-315 (1981)

Kaapa P
Immunoreactive TXB_2 and 6-keto-PGF1α in neonatal hyperbilirubinemia
Prostaglandins Leukotrienes and Medicine 17(1): 97-105 (1985)

Kaapa P, Koivisto M, Viinikka L
Increased plasma immunoreactive 6-keto-PGF1α levels in newborns with idiopathic respiratory distress syndrome
Pediatric Research 16: 827-829 (1982)

Kaapa P, Koivisto M, Viinikka L, Ylikorkala O
Increased plasma immunoreactive 6-keto-prostaglandin F_1 alpha levels in newborns with idiopathic respiratory distress syndrome
Paediatric Research 16(10): 827-829 (1982)

Kaar M L, Kaapa P, Viinikka L, Ylikorkala O
Prostacyclin and thromboxane in diabetic children
Prostaglandins Leukotrienes and Medicine 11(3): 339-345 (1983)

Kahaleh M B, Osborn I, Le Roy E C
Increased factor VIII/Von Willebrand factor antigen and von Willebrand factor activity in scleroderma and in Raynaud's phenomenon
Annals of Internal Medicine 94(1): 482-484 (1981)

Kahaleh M B, Le Roy E C
Progressive systemic sclerosis: kidney involvement
Clinical Rheumatic Diseases 5: 167 (1979)

Kannel W B
Results of the epidemiologic investigation of ischaemic heart disease
In: de Haas, Hemker and Snellen (eds) Ischaemic Heart Disease
University Press Leiden: 272-310 (1970)

Kent R S, Kitchell B B, Shand D G, Whorton A R
The ability of vascular tissue to produce prostacyclin decreases with age.
Prostaglandins 21(3): 483-490 (1981)

Kierse M J N C
Endogenous prostaglandins in human parturition
In: Human Parturition pp 102-142 eds Kierse M J N C, Anderson A B M, Gravenhorst J B
University Press, The Netherlands: (1979)

Kinney E L, Domers L M
Plasma 6-keto PGF1∝ concentrations in Raynauds phenomenon
Prostaglandins and Medicine 7: 389-393 (1981)

Kulkarni P S, Roberts R, Needleman P
Paradoxical endogenous synthesis of a coronary dilating substance from arachidonate
Prostaglandins 12: 337-353 (1976)

Kurzrok R, Lieb C C
Biochemical studies of human semen
II The action of semen on the human uterus
Proceedings of the Society for Experimental and Biological Medicine 28: 268-272 (1930)

Kuo P T, Joyner C R
Angina pectoris induced by fat ingestion in patients with coronary artery disease
Journal of the American Medical Association 158: 1008-1013 (1955)

Lamming G D, Symonds E M
Use of labetalol and methyldopa in pregnancy induced hypertension
British Journal of Clinical Pharmacology 8: 2175-2225 (1979)

Langley J N
On the reaction of cells and nerve-endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and curare
Journal of Physiology 33: 374-415 (1905)

Laszlo E, Kaldi N, Kovacs L
Alterations in plasma proteins and platelet functions with ageing and cigarette smoking in healthy men
Thrombosis and Haemostasis 49: 150 (1983)

Lee H D P (translator) : Aristotle : Meteorologica
Leob Classical Library Cambridge, Mass Harvard University Press (1952)

Legarde M, Dechavanne M
Increase of platelet prostaglandin cyclic endoperoxides in thrombosis
Lancet: 88 (1977)

Leroy E C, Downey J A, Cannon P J
Skin capillary blood flow in scleroderma
Journal of Clinical Investigation 50: 930-939 (1971)

Levine P H
An acute effect of cigarette smoking on platelet function
Circulation 48: 619-623 (1973)

Lewis P J
The role of PGI_2 in preeclampsia
British Journal of Hospital Medicine 28: 393-395 (1982)

Lewis P J, Boyland P, Friedman L A, Hensby C N, Downing I
Prostacyclin in pregnancy
British Medical Journal 280: 1581-1582 (1980)

Lewis P J, Shepherd G L, Ritter J, Cham S M T, Bolton P J, Jogee M, Myatt L, Elder M G
PGI_2 and pre-eclampsia
Lancet: 559 (1981)

Lewy R, Smith J, Silver M, Said J, Walinsky P, Wiener L
Detection of thromboxane B_2 in peripheral blood of patients with Prinzmetal's angina
Prostaglandins and Medicine 2: 243-248 (1979)

Lieb J, Karmali R, Horrobin D
Elevated levels of prostaglandin E_2 and thromboxane B_2 in depression
Prostaglandins Leukotrienes and Medicine 10(4): 361-368 (1983)

Lock S P, Dormandy K M
Red-cell fragmentation syndrome : a condition of multiple aetiology?
Lancet: 1020-1023 (1961)

Lowe G D O, Johnston R V, Drummond M M, Forbes C D, Prentice C R M
Induction of circulating platelet aggregates in healthy subjects by a saturated fat meal
Thrombosis Research 16: 565-568 (1979)

Lowe G, Pettigrew A, Middleton S, Forbes C D, Prentice C R M
DDAVP in haemophilia
Lancet 2: 614-615 (1977)

Lowry O H, Rosenbrough N J, Farr A L, Randall R J
Protein measurement with the folinphenol reagent
Journal of Biological Chemistry 193: 265-275 (1951)

Mahoney D P, Barden A, Beilin L J, Vandongen R
Radioimmunoassay of prostaglandins and thromboxane B_2 in extracted and unextracted urine and serum using an iodinated ligand.
Prostaglandins Leukotrienes and Medicine 12: 11-20 (1983)

Makila U-M, Viinikka L, Ylikorkala O
Decreased prostacyclin production in Down's Syndrome
Prostaglandins Leukotrienes and Medicine 17: 347-348 (1985)

Makila U-M, Viinikka L, Ylikorkala O
Increased TXA_2 production but normal PGI_2 by the placenta in hypertensive pregnancies
Prostaglandins 27: 87-95 (1984)

Mannucci P M, Canciani M T, Roto L, Donovan B S
Response of factor VIII/von Willebrand factor to DDAVP in healthy subjects and patients with haemophilia A and von Willebrand's disease
British Journal of Haematology 47: 283-293 (1981)

Mannucci P M, Ruggeri Z M, Pareti F I,Capitanio A
1-Deamino-8-d-arginine vasopressin: a new pharmacological approach to the management of haemophilia and von Willebrand's disease
Lancet: 869-872 (1977)

Mannucci P M, Remuzzi G, Pusineri F, Lombardi R, Valsecchi C, Mecca G, Zimmerman T S
Deamino-8-d-arginine vasopressin shortens the bleeding time in uremia
New England Journal of Medicine 308: 8-12 (1983)

Marcus A, Weksler B B, Jaffe E A
Enzymatic conversion of prostaglandin endoperoxide PGH_2 and arachidonic acid to PGI_2 by cultured human endothelial cells
Journal of Biological Chemistry 253: 7138-7144 (1978)

Marcus A J, Broekman M H, Weksler B B, Jaffe E A, Safier L B, Ullman H L, Tack-Goldman K
Interaction between stimulated platelets and endothelial cells in vitro
Philos Trans Royal Society of London (Biology) 294 (1072): 343-353 (1981)

Masotti G L, Poggesi L, Galanti G, Trotta F, Neri Serneri G G
In: Clinical Pharmacology of PGI_2 pl eds P J Lewis and J O'Grady
Raven Press New York: (1981)

Masotti G, Poggesi L, Galanti G, Trotta F, Gastone G, Serneri N
Prostacyclin production in man
In Clinical Pharmacology of Prostacyclin eds Lewis P J, O'Grady J
pp 9-23
Raven Press New York (1981)

McGuire J C, Sun F F
Metabolism of PGI_2 oxidation by rhesus monkey lung 15-hydroxyl prostaglandin dehydrogenase
Arch Biochem Biophys 189: 92-96 (1978)

McIntyre D E, Pearson J D, Gordon J L
Localisation and stimulation of prostacyclin production in vascular cells
Nature (London) 271: 549-551 (1978)

Mehta P, Mehta J
Effects of smoking on platelets and on plasma thromboxane-prostacyclin balance in man
Prostaglandins Leukotrienes and Medicine 9: 141-150 (1982)

Mehta P, Mehta J, Crews F, Roy L, Ostrowski N, Horalek C
Comparison of umbilical vein models for measurement of relative prostacyclin and thromboxane production
Prostaglandins 24 (6): 743-749 (1982 b)

Mehta P, Mehta J, Hay D
Thromboxane and prostacyclin generation by intact human vessels in response to baloon catheter trauma
Prostaglandins Leukotrienes and Medicine 9: 539-548 (1982 a)

Mehta J, Mehta P, Ostrowski N
Influence of propanolol and 4-hydroxypropanolol on platelet aggregation and thromboxane A_2
Pharmacological Theraputics 34: 559 (1983)

Mestel F, Oetliker O, Beck E, Felix R, Imbach P, Wagner H P
Severe bleeding associated with defective thromboxane synthetase (Letter)
Lancet: 157 (1980)

Michael C A
Use of labetalol in the treatment of severe hypertension during pregnancy
British Journal of Clinical Pharmacology 8: 2115-2155 (1979)

Mikhailidis D P, Hutton R A, Dondona P
Effect of cooling of platelet aggregation
Clinical Science 61: 28 (1981)

Miller J A, Castella A
Platelet-type von Willebrand's disease : Characterisation of a new bleeding disorder
Blood 60: 790-794 (1982)

Miller O V, Gorman R R
Evidence of distinct PGI_2 and D_2 receptors in human platelets
Journal of Pharmacological and Experimental Theraputics 210: 134-140 (1979)

Mitchell M D
A sensitive radioimmunoassay for 6-keto-prostaglandin $F1\alpha$: preliminary observations on circulating concentrations
Prostaglandins and Medicine 1: 13-21 (1978)

Mitchell M D, Bibby J G, Hicks B R, Redman C W G, Anderson A B M, Turnbull A C
Thromboxane B_2 and human parturition: concentrations in the plasma and production in vitro
Journal of Endocrinology 78: 435-441 (1978)

Mitchell M D, Bibby J B, Hicks B R, Turnbull A C
Possible role for PGI_2 in human parturition
Prostaglandins 16: 931-937 (1978)

Mitchell M D, Keirse M J N C, Anderson A B M, Turnbull A C
Thromboxane B_2 in amniotic fluid before and during labour
British Journal of Obstetrics and Gynaecology 85: 442-445 (1978)

Mitchell M D, Keirse M J N C, Brunt J D, Anderson A B M, Turnbull A C
Concentrations of the prostacyclin metabolite 6-keto prostaglandin $F1\alpha$ in amniotic fluid during late pregnancy and labour
British Journal of Obstetrics and Gynaecology 86: 350-353 (1979)

Miura Y, Fukui N
Prostaglandins as a possible trigger for liver regeneration
Cellular and Molecular Biology 25: 179-184 (1979)

Moake J L, Tang S S, Olson J D et al
Platelets von Willebrand factor and PGI_2
American Journal of Physiology 241" 1154-1159 (1981)

Moncada S, Buntin S, Mullane K
Imidazole : a selective inhibitor of thromboxane synthetase
Prostaglandins 13(4): 611-618 (1977)

Moncada S, Gryglewski R J, Bunting S, Vane J R
An enzyme from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation
Nature (London) 263: 663-665 (1976)

Moncada S, Herman A G, Higgs EA, Vane J R
Differential formation of prostacyclin (PGX or PGI_2) by layers of arterial wall. An explanation for the anti-thrombotic properties of vascular endothelium
Thrombosis Research 11: 323-344 (1977)

Moncada S, Higgs E A, Vane J R
Human arterial and venous tissues generate prostacyclin (PGX), a potent inhibitor of platelet aggregation
Lancet: 18-20 (1977)

Moncada S, Korbut R, Bunting S, Vane J R
Prostacyclin is a circulating hormone
Nature 273: 767-768 (1978)

Moncada S, Needleman P, Bunting S
Prostaglandin endoperoxide and thromboxane generating systems and their selective inhibition
Prostaglandins 12(3): 323-335 (1976)

Moncada S, Vane J R
Pharmacology and endogenous roles of prostaglandin endoperoxides, TXA_2 and PGI_2
Pharmacological Review 30: 293-331 (1979)

Moretta L, Mingari M C, Romanzi C A
Loss of FC receptors for IgG immune complexes
Nature 272: 618-620 (1978)

Morgan C R, Lazarow A
Immunoassay of insulin: two antibody system. Plasma insulin levels of normal sub-diabetic and diabetic rats.
Diabetes 12: 115-126 (1963)

Morris H G, Sherman N A, Shepperdson F T
Variables associated with R.I.A. of prostaglandins in plasma
Prostaglandins 21(5): 771-787 (1981)

Morrison A R, Nishikawa K, Needleman P
Thromboxane A_2 biosynthesis in the ureter obstructed isolated perfused kidney of the rabbit
Journal of Pharmacological Experimental Theraputics 205(1): 1-8 (1978)

Murphy E A, Mustard J F
Tobacco and thrombosis
American Journal of Public Health 56: 1061 (1966)

Myatt L, Jogee M, Lewis P J, Elder M C
Metabolism of Prostacyclin and 6-oxo-PGF1α in Man
Clinical Pharmacology of Prostacyclin pp 25 - 35
Raven Press, New York eds Lewis P J, O'Grady J (1981)

Nadler J L, Velasco J S, Horton R
Cigarette smoking inhibits prostacyclin formation
Lancet: 1248-1250 (1983)

Needleman P, Bryan B, Wyche A, Bronson S D, Eakino K, Ferrendelli J A, Minkes M
TX synthetase inhibitors as pharmacological tools : differential biochemical and biological effects on platelet suspensions
Prostaglandins 14(5): 879-907 (1977)

Needleman P, Bronson S D, Wyche A, Sivakoff M, Nicolaou K C
Cardiac and renal prostaglandin I_2
Journal of Clinical Investigation 61: 839-849 (1978)

Needleman P, Moncada S, Bunting S, Vane J R, Hambert M, Samuellson B
Identification of an enzyme in platelet microsomes which generates thromboxane A_2 from prostaglandin endoperoxides
Naure 261: 558-561 (1976)

Nikolaides K H, Graven D J, Kirby D S, Filshie G M, Symonds E M
Production of PGI_2 by tissue from human fetoplacental unit
In: Lewis P J, Moncada S, Vane J (eds) PGI_2 in pregnancy
Raven Press, New York: 25-30 (1983)

Nilsson I M, Holmberg L, Aberg M, Vilhardt M
The release of plasminogen activator and factor VIII after injection of DDAVP in healthy volunteers and in patients with von Willebrand's disease
Scandanavian Journal of Haematology 24: 351-359 (1980)

Nordoy A, Rodset J M
Platelet function and platelet phospholipids in patients with hyperbetalipoproteinaemia
Acta Med Scan 189: 385-389 (1971)

O'Brien J R, Etherington M D, Jamieson S
Acute platelet changes after large meals of saturated and unsaturated fats
Lancet: 879-880 (1976)

O'Grady J, Moncada S
Aspirin, a paradoxical effect on bleeding time
Lancet: 780 (1978)

O'Grady J, Warrington S, Moti M J et al
Effects of i/v infusions of PGI_2 in man
Prostaglandins 19: 319-327 (1980)

Ockleford P A, Chandrasekharamenon N, Berry E W
Clinical experience with arginine vasopressin (DDAVP) in von Willebrand's disease and mild haemophilia
New Zealand Medical Journal 92: 375-378 (1980)

Orlandi M, Davi G, Triolo G, Tomasi V, Strano A
Normal plasma thromboxane B_2 and 6-keto-PGF1α levels in childhood diabetes
Prostaglandins Leukotrienes and Medicine 12(4): 437-438 (1983)

Orlandi M, Bartolini G, Chiricolo L, Minghetti L, Franceschi C, Tomasi V
Prostaglandin and thromboxane biosynthesis in isolated platelet-free human monocytes I.A. modified procedure for the characterization of the prostaglandin spectrum produced by resting and activated monocytes.
Prostaglandins Leukotrienes and Medicine 18(2): 205-216 (1985)

Ouwendjik R J T, Zizlstra F J, Wilson J H P, Bonta I L, Vincent J E
Raised plasma thromboxane B_2 levels in alcoholic liver disease
Prostaglandins Leukotrienes and Medicine 10(2): 115-122 (1983)

Pace-Asciak C, Nashat M
Catabolism of an isolated, purified intermediate of prostaglandin biosynthesis by regions of the adult rat kidney.
Biochim Biophysical Acta 388(2): 243-252 (1975)

Pace-Asciak C R, Carrara M C, Levine L, Nicolaouk C
PGI_2 specific antibodies administered in vivo suggest against a role for endogenous PGI_2 as a circulating vasodepressor hormone in the normotensive and spontaneously hypertensive rat
Prostaglandins 20: 1053-1060 (1980)

Pace-Asciak C R, Carrera M C, Rangaraj G, Nicolaou K C
Enhanced formation of PGI_2, a potent hypotensive substance by aortic rings and homogenates of the spontaneously hypertensive rat
Prostaglandins 15(6): 1005-1012 (1978)

Pace-Asciak C, Wolfe L S
A novel prostaglandin derivative formed from arachidonic acid by rat stomach homogenates
Biochemistry 10: 3657-3669 (1971)

Parry M J
Effects of thromboxane synthetase inhibition on arachidonate metabolism and platelet behaviour
British Journal of Pharmacology 15: 235-295 (1983)

Patrignani P, Filabozzi P, Calella F, Pugliese F, Patrono C
Differential effects of dazoxiben, a selective thromboxane synthetase inhibitor, on platelet and renal prostaglandin endoperoxide metabolism
Journal of Pharmacological and Experiemental Theraputics 228: 427-477 (1984)

Patrignani P, Filabozzi P, Patrono C
Selective cumulative inhibition of platelet thromboxane production by low dose aspirin in healthy subjects
Journal of Clinical Investigation 69: 1366-1372 (1982)

Patrono C, Ciabattoni G, Pinca E, Pugliese F, Castrucci G, De Salvo A, Satta M A, Peskar B A
Low dose aspirin and inhibition of thromboxane B_2 production in healthy subjects
Thrombosis Research 17: 317-327 (1980)

Patrono C, Pugliese F, Ciabattoni G, Patrignani P, Maseri A, Chierchia S, Peskar B A, Cinotti G A, Simonetti B M, Pierucci A
Evidence of a direct stimulatory effect of prostacyclin on renin release in man
Journal of Clinical Investigation 69: 231-239 (1982)

Patrono C, Ciabattoni G, Venuti A, Pugliese F, Schiavino D, Patriarca G
Aspirin intolerance unaltered susceptibility of platelet cyclo-oxygenase to inhibition by aspirin in vitro
Journal of Allergy and Clinical Immunology 62: 271-275 (1978)

Petit J L
Dissertation sur la manniere d'arrester le sang dans les hemorrhagies
Member of Academy of Royal Sciences

Piper P J, Vane J R
Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs
Nature 223: 29-35 (1969)

Pittilo R M, Mackie W, Rowles P M, Machine S J, Woolf N
Effects of cigarette smoking on the ultrastructure of rat thoracic aorta and its ability to produce prostacyclin
Thrombosis and Haemostasis 48: 173-176 (1982)

Rakoczi I, Tihanyi K, Falkay C, Rozsa I, Demaler J, Gati I
PGI_2 production in trophoblast
In: Lewis P J, Moncada S, O'Grady J (eds) PGI_2 in pregnancy
Raven Press, New York: 15-23 (1983)

Rao G H, White J G
Disaggregation and reaggregation of "irreversibly" aggregated platelets: a method for more complete evaluation of anti-platelet drugs
Agents Actions 16(5): 425-434 (1985)

Regan T J, Binak K, Gordon S, De Fazio V, Hellems H K
Myocardial blood flow and oxygen consumption during postprandial lipemia and heparin induced lipolysis
Circulation 23: 55-63 (1961)

Remuzzi G, Marchesi D, Mecca G, Misiani R, Livio M, De Gaetano G
Haemolytic uremic syndrome deficiency of plasma factor(s) regulating PGI_2 activity?
Lancet: 871-872 (1978)

Remuzzi G, Misiani R, Mecca G, de Gaetano G, Donati M B
Thrombotic thrombocytopenic purpura: A deficiency of plasma factor regulating platelet-vessel-wall interaction?
New England Journal of Medicine 299: 311 (1978 a)

Remuzzi G, Misiani R, Marchesi D, Livio M, Mecca G, de Gaetano G, Donati M B
Treatment of haemolytic uraemic syndrome with plasma
Clinical Nephrology 12(6): 279-284 (1979)

Remuzzi G, Marchesi D, Zoja C
Reduced umbilical and placental vascular prostacyclin in severe preeclampsia
Prostaglandins 20: 105-110 (1980)

Remuzzi G, Zoja C, Marchesi D, Schieppati A, Mecca G, Misiani R, Donati M B, De Gaetano G
Plasmatic regulation of vascular PGI_2 in pregnancy
British Medical Journal 282: 512-514 (1981)

Remuzzi G, Marchesi D, Misiani R, Mecca G, De Gaetano G, Donati B
Familial deficiency of a plasma factor stimulating PGI_2 activity
Thrombosis Research 16: 517-525 (1979)

Remuzzi G, Misiani R, Muratore D
PGI_2 and human feotal circulation
Prostaglandins 18: 341-348 (1979)

Renaud S, Morazian R, McGregor L, Baudier F
Dietary fats and platelet function in relation to atherosclerosis and coronary heart disease
Haemostasis 8: 234-251 (1979)

Ritter J M, Barrow S E, Blair I A, Dollery C T
Release of PGI_2 in vivo and its role in man
Lancet: 317-319 (1983)

Ritter J M, Orchard M A, Lewis P J
Stimulation of vascular PGI_2 production by human serum
Biochemical Pharmacology 31: 3047-3050 (1982)

Ritter J M, Ongari M A, Orchard M A, Lewis P J
PGI_2 synthesis is stimulated by a serum factor formed during coagulation
Thrombosis and Haemostasis 49: 58-60 (1983)

Ritter J M, Ongari M A, Barrow S E, Orchard M A, Blair I A, Lewis P J
Prostanoid synthesis by human umbilical artery
Prostaglandins 24: 881-886 (1982)

Roberts L J, Sweetman B J, Payne N A, Oates J A
Metabolism of thromboxane B_2 in man
Biological Chemistry 252: 7415-7417 (1977)

Roberts L J 2nd, Sweetman B J, Oates J A
Metabolism of thromboxane B_2 in man. Identification of twenty urinary metabolites
Journal of Biological Chemistry 256: 8384-8393 (1981)

Robertson R M, Robertson D, Roberts W
Thromboxane A_2 in vasotonic angina pectoris
New England Journal of Medicine 304: 998-1003 (1981)

Rosenkranz B, Fischer C, Wiemer K, Frolich J
Metabolism of prostacyclin and 6-keto-prostaglandin $F_{1\alpha}$ in man
Journal of Biological Chemistry 255: 10194-10198 (1980)

Roy L, Mehta J, Mehta P
Increased plasma concentrations of prostacyclin metabolite 6-keto-PGF1α in essential hypertension. Influence of therapy with labetalol
American Journal of Cardiology 51: 464-467 (1983)

Roy A C, Karim S M M, Kottegoda S R, Ratnam S S
Thromboxane A_2 and prostacyclin levels in molar pregnancy
British Journal of Obstetrics and Gynaecology 91: 908-912 (1984)

Ryhage R, Stenhagen E
Mass spectometry in lipid research
Journal of Lipid Research 1: 361-390 (1960)

Saba S R, Mason R G
Some effects of nicotine on platelets
Thrombosis Research 7(5): 819-824 (1975)

Salmon J A, Smith D R, Flower R J, Moncada S, Vane J R
Some characteristics of the prostacyclin synthesising enzyme in porcine aorta
Biochem Biophys Acta 523: 250-262 (1978)

Salmon J A, Smith D R, Flower R J, Moncada S, Vane J R
Further studies on the enzymatic conversion of prostaglandin endoperoxide into prostacyclin by porcine aorta microsomes
Biochemical and Biophysical Acta 523(1): 250-262 (1978)

Salvati P, Whittle B J
Investigation of the vascular actions of arachidonate lipoxygenase and cyclo-oxygenase products on the isolated perfused stomach of rat and rabbit
Prostaglandins 22: 141-156 (1981)

Salzman E W
Measurement of platelet adhesiveness. A simple in vitro technique demonostrating an abnormality in von Willebrand's disease
Journal of Laboratory and Clinical Medicine 62: 724-735 (1963)

Samols E, Marks V
Insulin assays in insulinomas
British Medicial Journal 5329: 507-510 (1963)

Sands E, Bilkus D
A comparison of insulin immunoassays
Proceedings of the Society for Experimental and Biological Medicine 115: 79-84 (1964)

Schillinger E, Prior G, Speckenbach A, Wellershoff S
Receptor binding in various tissues of PGE_2, PGF_2 alpha and sulprostone, a novel PGE_2-derivative
Prostaglandins 18(2): 292-302 (1979)

Schulman E S, Adkinson N F Jr, Newball H H
Cyclo-oxygenase metabolites in human lung anaphylaxis : airway vs parenchyma
Journal of Applied Physiology 53(3): 589-595 (1982)

Seid J M, Jones P B B, Russell R G G
The presence in normal plasma serum and platelets of factors that stimulate the production of PGI_2 by cultured endothelial cells
Clinical Science 64: 387-394 (1983)

Seiss W, Dray F
Very low levels of 6-keto-prostaglandin F1α in human plasma
Journal of Laboratory and Clinical Medicine 99(3): 388-397 (1982)

Sensaki S, Okuma M, Uchino H
Decreased plasma prostacyclin regenerating activities in diabetics
Prostaglandins Leukotrienes and Medicine 20: 197-208 (1985)

Serneri G G N, Abbate R, Gensini G F, Panetta A, Casolo G C, Carini
TXA_2 production by human arteries and veins
Prostaglandins 25(6): 753-766 (1983)

Shimamoto T, Kobayashi M, Takahashi T, Takashima Y, Sakamoto M, Morooka S
An observation of thromboxane A_2 in arterial blood after cholesterol feeding in rabbits
Japanese Heart Journal 19(5): 748-753 (1978)

Siegl A M, Smith J B, Silver M J et al
Selective binding site for 3H PGI_2 platelets
Journal of Clinical Investigation 63: 215-220 (1977)

Siess W, Lorenz R, Rotn P, Weber P C
Plasma catecholamines, platelet aggregation and associated thromboxane formation after physical exercise, smoking or norephinephrine infusion
Circulation 66: 44-48 (1982)

Silberbauer K, Schernthaner G, Sinzinger H et al
Decreased vascular prostacyclin in juvenile-onset diabetes
New England Journal of Medicine 300: 366-367 (1979)

Silberbauer K, Schernthaner G, Sinzinger H, Piza-Katzer H, Winter M
Decreased vascular prostacyclin in juvenile-onset diabetes
New England Journal of Medicine 300(7): 366-367 (1979)

Small M, Douglas J T, Aherne G W, Orr M, Lowe G D O, Forbes C D, Prentice C R M
Effects of the non-selective beta-adrenoceptor blocking agent, carteolol on platelet function, blood coagulation and viscosity
Thrombosis Research 25: 351-360 (1982)

Smith J S, Leter M L, Nicolaou K C
Antibodies which antagonise the effects of PGI_2
Nature (London) 274: 64-65 (1978)

Spain D M, Bradess V A
Sudden death from coronary heart disease: survival time, frequency of thrombi and cigarette smoking
Chest 58: 107 (1970)

Spolarics Z, Tanacs B, Garzo T, Mandl J, Mucha I, Antoni F, Machovich R, Horvatn I,
Prostaglandin and thromboxane synthesising activity in isolated murine hepatocytes
Prostaglandins Leukotrienes and Medicine 16(3): 379-388 (1984)

Sraer J, Siess W, Moulonguet-Doleris L, Oudinet J P, Dray F, Ardaillou R
In vitro prostaglandin synthesis by various rat renal preparations
Biochemic Biophysica Acta 701 (1): 45-52 (1982)

Steer M L, MacIntyre D E, Levine L, Salzman E W
Is prostacyclin a physiologically important circulating anti-platelet agent?
Nature 283: 194-195 (1980)

Steiner M
Platelet protein synthesis studied in a cell free system
Experentia 26: 786 (1970)

Stephenson R P
A modification of receptor theory
British Journal of Pharmacology and Chemotherapy 11: 379-393 (1956)

Stoel I, Giessen W, Zwolsman E, Verheugh F W A, Ten Hoor F, Quadt F J A, Hugenholtz P G
Effect of nicotine on production of PGI_2 in human umbilical artery
British Heart Journal 48: 493-496 (1982)

Stuart M J, Sills R H
Deficiency of plasma PGI_2 regenerating ability in sickle cell aemia
British Journal of Haematology 48: 545-550 (1981)

Stuart M J, Sunderji S G, Yambo T, Clark D A, Allen J B, Eldrad H, Slott J H
Decreased PGI_2 production: a characteristic of chronic placental insuffiency syndromes
Lancet: 1126-1128 (1981)

Sun F F, McGiff J C, Wong P Y K
Metabolism of PGI_2 and 6-keto PGF1α in blood vessels
Federal Proceedings 37: 916 (1978)

Svensson J, Strandberg K, Tuverno T, Hamberg M
Thromboxane A_2: effects on airway and vascular smooth muscle
Prostaglandins 14(3): 425-436 (1977)

Swartz S L, Willett W C, Hennekens C H
A randomised trial on the effects of vitamin E on plasma prostacyclin (6-keto-PGF1α) levels in healthy adults
Prostaglandins Leukotrienes and Medicine 18: 105-111 (1985)

Symonds E M
Management and therapy in the hypertensive disorders of pregnancy
Presented at 3rd World Congress of the International Society for the Study of Hypertension in Pregnancy Dallas (1982)

Szczeklik A, Gryglewski R J, Musical J, Grodzinska L, Serwonska M, Marcinkiewicz E
Thromboxane generation and platelet aggregation in survivals of myocardial infarction
Thrombosis and Haemostasis 40(1): 66-74 (1978)

Tada M, Kuzuya T, Inoue M, Kodama K, Mishima M, Yamada M, Inui M, Abe H
Elevation of thromboxane B_2 levels in patients with classic and variant angina Pectoris
Circulation 64(6): 1007-1015 (1981)

Takahashi H
Studies on the pathophysiology and treatment of von Willebrand's disease. Properties of factor VIII after DDAVP infusion in variant von Willebrand's disease
Thrombosis Research 21: 357-365 (1981)

Takahashi R, Shiraki M, Morita I, Ito H, Morota S, Orimo H
Platelet thromboxane synthesising activity in non insulin dependent diabetes: correlation with diabetic retinopathy and diabetic treatment
Prostaglandins Leukotrienes Medicine 17(2): 149-158 (1985)

Tansik R L, Namm D H, White H L
Synthesis of prostaglandin 6-keto-F_1 alpha by cultured aortic smooth muscle cells and stimulation of its formation in a coupled system with platelet lysates
Prostaglandins 15(3): 399-408 (1978)

Terragno N A, Terragno A, McGiff J C, Rodriguez D J
Synthesis of prostaglandins by the ductus arteriosus of the bovine fetus
Prostaglandins 14(4): 721-727 (1977)

Thorngren M, Shafi S, Born G V R
Thromboxane A_2 in skin-bleeding-time blood and in clotted venous blood before and after administration of acetylsalicyclic acid
Lancet: 1075-1978 (1983)

Timperley W R, Ward J D, Preston F E, Duckworth T, O'Malley B C
A reassessment of small vessel disease in diabetic neuropathy
Diabetologia 12: 237 (1976)

Tschopp T P, Weiss H G, Baumgartner H R
Decreased adhesion of platelets to subendothelium in von Willebrand's disease
Journal of Laboratory and Clinical Medicine 83: 296-300 (1974)

Tuvemo T
Role of prostaglandins prostacyclin and thromboxanes in the control of the umbilical-placental circulation
Seminars in Perinatology 4: 91-95 (1980)

Tuvemo T, Strandberg K, Hambert M
Maintenance of the tone of human umbilical artery by prostaglandin and thromboxane formation
Advances in Prostaglandin and Thromboxane Research 1: 425-428 (1976)

Tyler H M
Thromboxane synthetase inhibition
Lancet: 1313-1314 (1981)

Ubatuba F B, Moncada S, Vane J R
The effect of PGI_2 on platelet behaviour, thrombus formation in vivo and bleeding time
Thrombosis and Haemostasis 41: 425-435 (1979)

Uehara Y, Ishii M, Ikeda T, Atarashi K, Takeda T, Murao S
Plasma levels of 6-keto-prostaglandin F1α in normotensive subjects and patients with essential hypertension
Prostaglandin Leukotrienes Medicine 10(4): 455-464 (1983)

Uotila P, Hittunen A, Makila K, Mannisto J, Toivonen H, Hartiala J
The metabolism of arachidonic acid in isolated hamster, rat and guinea-pig lungs
Prostaglandins and Medicine 5(3): 149-162 (1980)

Utiger R D, Parker M L, Daughaday W H
Studies on human growth hormone I A radio-immunoassay for human growth hormone
Journal of Clinical Investigation 41: 254-261 (1962)

Van Dorp D A, Beerthuis R K, Nugteren D H, Vonkeman H
The biosynthesis of prostaglandins
Biochem Biophys Acta 90: 204-207 (1964)

Viinikka L, Ylikorkala O
Different results of plasma 6-keto-prostaglandin $F_{1\alpha}$ measurements utilising two antisera with apparently similar specificity
Prostaglandins Leukotrienes and Medicine 9: 1-7 (1982)

Von Euler U S
On the specific vasodilating and plain muscle stimulating substances from accessory genital glands in man and certain animals (prostaglandin and vesiglandin)
Journal of Physiology 88: 213-234 (1936)

Vane J R
Inhibition of prostaglandin synthesis as a mechanism of action of aspirin-like drugs
Nature New Biology 231: 232-235 (1971)

Vergara-Dauden M, Balconi G, Breviario F, Chiabrando C
Further studies on the mechanism of action of human plasma in stimulating PGI_2 production by rat smooth muscle cells
Thrombosis and Haemostasis 53(3): 372-376 (1985)

Viinikka L, Ylikorkala O
Measurement of thromboxane B_2 in human plasma or serum by radioimmunoassay
Prostaglandins 20(4): 759-766 (1980)

Walker J J, Crooks A, Erwin L, Calder A A
Labetalol in pregnancy induced hypertension: foetal and maternal effects: Investigation of Labetalol in the management of hypertension in pregnancy
In: Proceedings of Symposium (Eds) Riley A, Symonds E M
International Congress Series 591, Excerpta Medica (1982 a)

Walker J J, Belch J J F, Erwin L, McLaren M, Lang G D, Forbes C D, Prentice C R M, Calder A A
Labetalol and platelet function in pre-eclampsia
Lancet: 279 (1982 b)

Walsh S W, Behr M J, Allen N H
Placental PGI_2 production in normal and toxemic pregnancies
American Journal of Obstetrics and Gynaecology 151(1): 110-115 (1985)

Warrier A I, Lusher J M
DDAVP: A useful alternative to blood components in moderate haemophilia A and von Willebrand's disease
Journal of Paediatrics 102: 228-233 (1983)

Weiss H J, Lages B A
Possible congenital defect in platelet thromboxane synthetase (letter)
Lancet: 760-761 (1977)

Weiss H J, Turitto V T
PGI_2 inhibits platelet adhesion and thrombus formation on subendothelium
Blood 53: 244-250 (1979)

Weiss H J, Turitto V T, Baumgartner H R
Effect of shear rate on platelet interaction with subendothelium in citrated and native blood.
I Shear-dependent decrease of adhesion in von Willebrand's disease and the Bernard-Soulier syndrome
Journal of Laboratory and Clinical Medicine 92: 750-764 (1978)

Weksler B B, Gillick M, Pink J
Effects of propanolol on platelet function
Blood 49: 185-186 (1977)

Wise W C, Cook J A, Halushka P V, Knapp D R
Protective effects of thromboxane synthetase inhibitors in rats in endotoxic shock
Circulation Research 46(6): 854-859 (1980)

Weksler B B, Marcus A J, Jaffe E A
Synthesis of prostaglandin I_2 (prostacyclin) by cultured human and bovine endothelial cells
Proceedings of the National Academy of Science USA 74:
3922-3926 (1977)

Weksler B B, Pett S B, Alonso D, Richter R C, Stelzer P, Subramanian V, Tack-Goldman K, Gay W A Jnr
Differential inhibition by aspirin of vascular and platelet prostaglandin synthesis in atherosclerotic patients
New England Journal of Medicine 308: 800-805 (1983)

Wennmalm A
Effects of nicotine on cardiac prostaglandin and platelet thromboxane synthesis
British Journal of Pharmacology 64: 559-563 (1978)

Wennmalm A, Alster P
Nicotine inhibits vascular prostacyclin but not platelet thromboxane formation
General Pharmacology 14: 189-191 (1983)

Werle E, Schievelbein H
Activity of nicotine and inactivity of kallikrein and kallidin in aggregation of blood platelets
Nature 207: 871-872 (1965)

Whicher J T, Martin M F R, Dieppe P A
Absence of prostaglandin stimulated increased in acute phase proteins in systemic sclerosis
Lancet: 329 (1980)

Whittle B J R
In: Gastro-intestinal mucosal blood flow pp 180-191
Churchill Livingstone, Edinburgh Ed Fielding L P (1980)

Willems C, Van Mourik J A, Stel H V et al
Binding and metabolism of PGI_2 by erythrocytes (abstract)
Thrombosis and Haemostasis 46: 206 (1981)

Yalow R S, Berson S A
In: Principles of competitive-binding protein assays
(Editors) W Odell, Daughaday W H, Lippincott, Philadelphia Pa (1971)

Yalow R S, Berson S A
Immunoassay of endogenous plasma insulin in man
Journal of Clinical Investigation 39: 1157-1175 (1960)

Yamaguchi M, Mori N
6-keto-PGF1α , TXB_2 and 13, 14-dihydro-15-keto PGF concentrations of normotensive and pre-eclamptic patients during pregnancy, delivery and the post-partum period
American Jounral of Obstetrics and Gynaecology 151(1): 121-127 (1985)

Yarger W E, Schocken D D, Harris R H
Obstructive nephropathy in the rat : possible roles for the renin-angiotensin system, prostaglandins and thromboxanes in postobstructive renal function
Journal of Clinical Investigation 65(2): 400-412 (1980)

Ylikorkala O, Makula U-M, Viinikka L
Amniotic fluid PGI_2 and thromboxane in normal, pre-eclamptic and some other complicated pregnancies
American Journal of Obstetrics and Gynaecology 141: 487-490 (1981)

Ylikorkala O, Kirkinen P, Viinikka L
Maternal plasma PGI_2 concentrations in pre-eclampsia and other pregnancy complications
British Journal of Obstetrics and Gynaecology 88: 968-972 (1981)

Ylikorkala O, Osterman T, Linden I-B, Viinikka L
The effect of age on circulating 6-keto-prostaglandin F1α in humans
Prostaglandins Leukotrienes Medicine 9: 569-575 (1982)

Ylikorkala O, Viinikka L
TXA_2 in pregnancy and puerperium
British Medical Journal 2: 1601-1602 (1980)

Yoshimoto T, Yamamoto S, Okuma M, Hayaishi O
Solubilization and resolution of thromboxane synthesizing system from microsomes of bovine blood platelets
Journal of Biological Chemistry 252: 5871-5874 (1977)

Yui Y, Takatsu Y, Hattori R, Sakaguchi K, Susawa T, Ikeda N, Kawai C
Decreased serum levels of a factor stimulating PGI_2 synthesis in acute myocardial infarction
Prostaglandins Leukotrienes Medicine 16: 389-394 (1984)

Zahavi J, Hamilton W A P, O'Reilly M J G, Leyton J, Cotton L T, Kakkar V V
Plasma exchange and platelet function in Raynaud's phenomenon
Thrombosis Research 19: 85-93 (1980)