PROSTAGLANDIN CONTROL OF PLATELET BEHAVIOUR IN HEALTHY AND HYPERTENSIVE PREGNANCY

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by

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Declaration

I declare that the research described in this thesis and the preparation and writing of the manuscript were carried out by myself.

E.H. Horn

ABBREVIATIONS

AA	Arachidonic acid
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APH	Antepartum haemorrhage
ATP	Adenosine triphosphate
β-TG	Beta-thromboglobulin
$[Ca^{2+}]_i$	Intracellular free calcium ions
cpm	Counts per minute
CTG	Cardiotocograph
cyclic AMP	Cyclic adenosine 3′,5′-monophosphate
cyclic GMP	Cyclic guanosine 3′,5′-monophosphate
DAG	Diacylglycerol
DIC	Disseminated intravascular coagulation
dpm	Disintegrations per minute
EC_{50}	Concentration of an agonist giving half maximal response
EDRF	Endothelium derived relaxing factor
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FDP	Fibrin(ogen) degradation products
FPA	Fibrinopeptide A
G protein	Guanine nucleotide binding protein
GDP	Guanosine diphosphate
G_i	Inhibitory G protein

$G_{i\alpha}$	Alpha subunit of inhibitory G protein
$G_{i\beta\gamma}$	Beta/gamma subunit of inhibitory G protein
G_s	Stimulatory G protein
GTP	Guanosine triphosphate
Hb	Haemoglobin
HCl	Hydrochloric acid
HELLP	Haemolysis elevated liver enzymes and low platelets
12-HETE	12-hydroxyeicosatetraenoic acid
HP	Healthy pregnant group
12-HPETE	12-hydroperoxyeicosatetraenoic acid
5-HT	5-hydroxytryptamine
IP_3	Inositol trisphosphate
ITP	Immune thrombocytopenia
K_d	Dissociation constant of an antagonist
mmHg	Millimetres of mercury
MPV	Mean platelet volume
NECA	5-N-ethylcarboxyaminoadenosine
NP	Non pregnant women
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PCV	Packed cell volume
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PDW	Platelet distribution width
PF_4	Platelet factor 4
PGD_2	Prostaglandin D_2

PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
$PGF_{1\alpha}$	Prostaglandin $F_{1\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PI	Phosphatidylinositol
PIH	Pregnancy induced hypertension
PIP_2	Phosphatidylinositol bisphosphate
PLC	Phospholipase C
PPP	Platelet poor plasma
PRP	Platelet rich plasma
RP	Pregnant women at risk of pre-eclampsia
SAFP	Serum alphafetoprotein
SEM	Standard error of the mean
SIN-1	3-morpholinosydnonimine
SVD	Spontaneous vaginal delivery
TCA	Trichloroacetic acid
TOP	Termination of pregnancy
TSI	Thromboxane synthetase inhibitor
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
U/S	Ultrasound scan
VASP	Vasodilator stimulated phosphoprotein
WBC	White cell count

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SUMMARY

Platelet activation in vivo is a feature of normal pregnancy and occurs to a greater degree in pre-eclampsia, a hypertensive disorder of pregnancy. Furthermore, there is increasing evidence that platelet activation plays a key role in the pathophysiology of pre-eclampsia. Increased platelet reactivity in vitro has been previously reported in healthy pregnancy, but variable results have been obtained by different investigators conducting in vitro studies of platelet reactivity in pre-eclampsia.

Platelet behaviour is controlled by a complex interaction of regulatory mechanisms, some of which promote and some of which inhibit platelet activation. Prostaglandins are involved in both stimulatory and inhibitory regulatory pathways. This thesis has examined platelet responses to inhibitory and pro-aggregatory prostaglandins in healthy pregnancy and pre-eclampsia, with an emphasis on the effects on platelets of inhibitory prostanoids.

These studies have shown that during healthy pregnancy, platelets were less sensitive, compared with those from non pregnant women, to the inhibitory effects in vitro of a wide range of prostaglandins including prostacyclin, prostaglandin D_2 and prostaglandin E_1. This was associated with a reduction in accumulation in platelets of the inhibitory second messenger cyclic AMP, in response to these agents. The data presented are consistent with a reduction in platelet adenylate cyclase activity during healthy pregnancy. Longitudinal studies suggested changes in platelet cyclic AMP responses occurred early in the first trimester of pregnancy.

Platelets from primigravid women with established pre-eclampsia showed reductions both in platelet sensitivity to the inhibitory effects of prostaglandins

and in platelet cyclic AMP accumulation, of a similar degree to those demonstrated in platelets from healthy pregnant women. On the other hand, women with a previous history of pre-eclampsia, when studied longitudinally during a subsequent pregnancy, showed a more marked loss of platelet sensitivity to such prostaglandins, and this was accompanied by lower stimulated cyclic AMP levels compared with those in platelets from healthy pregnant women studied in parallel.

In contrast with the loss of sensitivity to inhibitory prostaglandins, platelet aggregation was increased during healthy pregnancy, compared with that in non pregnant women, in response to an agonist at the thromboxane receptor (U46619). This was not due to enhanced endogenous thromboxane synthesis, as differences were maintained between pregnant and non pregnant women in the extent of U46619 induced aggregation in blood incubated with aspirin in vitro. Furthermore, platelet thromboxane B_2 production was not increased during pregnancy, when measured either following spontaneous clotting of whole blood in vitro or in response to arachidonic acid in platelet rich plasma.

It was therefore concluded that enhanced sensitivity of platelets to thromboxane is likely to contribute to increased reactivity of platelets in vitro, during pregnancy, particularly in response to weak agonists.

The simultaneous reduction in platelet responses to inhibitory prostaglandins and increase in platelet sensitivity to pro-aggregatory prostaglandins are likely to interact to play a substantial role in platelet activation in vivo in healthy pregnancy. Further platelet activation in vivo in pre-eclampsia is readily explicable by the well established changes in balance of in vivo biosynthesis of pro-aggregatory and inhibitory prostanoids in pregnancy induced hypertension, on the background of physiological, pregnancy associated, alterations in responses to these prostaglandins which have been demonstrated

in this thesis. Intrinsic differences in platelet behaviour in women at risk of pre-eclampsia, may, however, also promote in vivo platelet activation in this condition.

CHAPTER 1

INTRODUCTION

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1. GENERAL INTRODUCTION.

Pregnancy is associated with a physiological hypercoagulable state, of which platelet activation in vivo is one feature.[1,2,3,4,5] The changes in the haemostatic system during pregnancy are appropriate for the limitation of blood loss at parturition, but they may also contribute to the aetiology of a number of complications of pregnancy such as thromboembolism, disseminated intravascular coagulation and pre-eclampsia. There is evidence in particular for a role for platelets in the pathophysiology of pre-eclampsia.

The regulation of platelet behaviour is complex and is governed by a large number of interacting factors. Prostaglandins make an important contribution to the mechanisms controlling platelet behaviour, either as agents which promote or those which inhibit platelet activation. This thesis is concerned with changes in platelet responses to prostaglandins in normal and hypertensive pregnancy. The introductory chapter describes the normal mechanisms and regulation of platelet function and on this background provides a review of the literature on platelet behaviour in normal pregnancy and in pre-eclampsia.

2. NORMAL MECHANISMS OF PLATELET ACTIVATION

2.1. Platelet Production and Morphology

Platelets are anucleate blood cells whose function is in primary haemostasis. Activated platelets form a plug at sites of vascular injury, thus limiting blood loss. In the resting state platelets have the shape of biconvex

discs[6,7] with a diameter of 2-4um[6]. Platelets are produced by fragmentation of the cytoplasm of megakaryocytes, large polyploid cells found in bone marrow. The process of platelet formation from megakaryocytes may either occur in the marrow sinusoids[8] or megakaryocytes may leave the marrow to enter the blood stream and platelet production subsequently occurs in the pulmonary circulation[9]. The mechanisms involved in the control of platelet production from megakaryocytes are only beginning to be understood, and are likely to involve megakaryocyte colony stimulating activity and "thrombopoiesis" stimulating activity of a number of haemopoietic growth factors[8].

Platelets circulate in the blood for 9-11 days[10] and during that time freely exchange between the general circulation and the splenic circulation. At any given time, approximately one third of platelets are found in the spleen[11]. The normal range for the peripheral blood platelet count is 150-400 x 10^9/l. At the end of their lifespan platelets are destroyed by the reticuloendothelial system.

The platelet has been shown to have four distinct ultrastructural regions revealed by electron microscopy : the peripheral zone, the sol-gel zone, the organelle zone, and the membrane systems[6,7]. Important components of the peripheral zone are the surface membrane glycoproteins, many of which act as receptors for various agents involved in platelet activation[12,13], and the phospholipid bilayer of the surface membrane which has a role in accelerating blood coagulation[14] and also contains transmembrane proteins including adenylate cyclase[15,16]. The sol-gel zone comprises the cytoplasmic matrix of the platelet and contains a system of microtubules and microfilaments involved in platelet contractile processes which play a role in platelet activation responses such as shape change and secretion[6,7]. Actin is the most important contractile protein in platelets and platelet activation is associated with polymerisation of monomeric

G-actin to F-actin[17,18]. The organelle zone contains mitochondria, and the storage granules. Three types of storage granules can be identified in platelets : the alpha granules, dense granules, and the lysosomes[19,20]. Release of the contents of storage granules is a key feature of platelet activation responses and will be discussed in detail later. The internal membrane systems in platelets consist of the surface-connected canalicular system and the dense tubular system[7]. The former represents complex invaginations of the surface membrane into the interior of the cell and can be externalised, possibly exposing active sites when platelets are stimulated[6]. The open canalicular system also serves as a conduit for the contents of the secretory granules to the external medium[7], as granule membranes fuse with this surface-connected membrane when a release reaction is initiated. The dense tubular system is analogous to the sarcoplasmic reticulum in smooth muscle[7,21]. It acts as the major storage site for intracellular calcium. Mobilisation of calcium from the dense tubular system to the cytosol is a key mechanism in stimulus response coupling in platelets[22].

2.2 Basic Physiology of Platelet Plug Formation In Vivo.

When the integrity of vascular endothelium is breached, platelets adhere to components of the exposed subendothelium including collagen[23]. This process is facilitated by von Willebrand factor, particularly at high vessel wall shear rates[24]. Von Willebrand factor, is a multimeric protein synthesised by endothelial cells[25] and circulates in association with the coagulation factor, factor VIII. Von Willebrand factor binds to the subendothelial collagen and to glycoprotein Ib on the platelet surface[26] thus promoting adhesion. Recently, major advances in the understanding of the molecular basis of the platelet adhesive process have been made. It has become clear that a wide variety of adhesive proteins are involved in supporting platelet attachment to extracellular matrix and these are associated

with distinct receptors on the platelet membrane. These receptors most commonly belong to the integrin family, consisting of proteins with a heterodimeric structure with alpha and beta subunits[27]. These include the fibrinogen receptor, glycoprotein IIb-IIIa, the collagen receptor, glycoprotein Ia-IIa, and the fibronectin receptor, glycoprotein Ic-IIa. Fibronectin is found in the extracellular matrix of the vessel wall,in plasma, and in platelet alpha granules, and probably facilitates platelet spreading over the subendothelium[28]. Some integrins such as glycoprotein IIb-IIIa can act as receptors for more than one type of adhesive protein. Furthermore, not all platelet receptors for adhesive proteins belong to the integrin family. Exceptions are glycoprotein Ib-IX, the receptor for von Willebrand factor, and glycoprotein IIIb-IV[27], the receptor for thrombospondin, a protein found in the endothelial basement membrane and platelet alpha granules.

Adherent platelets change shape from discs to spheres with pseudopods and then spread out over the exposed subendothelium[23]. The activated platelets then release the contents of their storage granules and aggregate together building up a haemostatic plug at the site of vascular injury[23]. The pseudopods formed during shape change facilitate platelet-platelet contact and the released granule contents such as adenosine diphosphate (ADP) and 5-hydroxytryptamine (5-HT) recruit further activated platelets[19,20]. Fibrinogen plays a key role in platelet aggregation[20,29,30] as it forms a bridge between adjacent platelets by binding to receptors on glycoprotein IIb-IIIa, which are exposed on platelet activation.[27,31,32,33] Platelet activation during formation of a haemostatic plug is also accompanied by the synthesis of the unstable prostanoid thromboxane A_2 by platelets.[34,35] This in itself is a powerful platelet activator and vasoconstrictor, thus providing another positive feedback mechanism for further platelet aggregation.[20,34,35,36]

The coagulation cascade becomes activated at sites of vascular injury as a result of contact activation and release of tissue thromboplastin. This results in the production of thrombin which, in addition to catalysing the conversion of fibrinogen to fibrin, is a powerful platelet activating substance.[20] Further interactions between platelets and the coagulation cascade occur, as platelets provide a phospholipid surface for localisation and acceleration of many of the reactions in the coagulation system such as the initiation of the intrinsic pathway,[37] the conversion of Factor X to X_a by Factor IX_a[38] and the conversion of prothrombin to thrombin by Factor X_a.[14,39] The latter is sometimes referred to as platelet prothrombinase activity.[14] Ultimately the platelet plug is stabilised by cross-linked fibrin, the end product of the coagulation cascade.

In vivo, a large number of interacting factors are simultaneously involved in promoting platelet activation and responses of platelets occur in concert with one another. Many of the details of individual platelet responses and of the effects of individual platelet agonists have been elucidated from in vitro studies where it is possible to focus on particular aspects of platelet behaviour or upon particular stimuli. These advantages are to some extent offset by the disadvantages of studying platelet behaviour in an unphysiological environment. Nevertheless the bulk of current knowledge of the mechanisms of platelet responses has been gleaned from such in vitro studies. Platelet behaviour in vitro has been investigated in platelet rich plasma (PRP), washed platelet suspensions and in whole blood.

2.3 Aspects of Platelet Behaviour In Vitro.

Platelet activation responses in vitro in suspensions of platelets are similar to those in vivo and consist of shape change, aggregation, secretion, and thromboxane synthesis.[20,40] The responses elicited depend upon the nature of the agonist and upon its concentration.[15,20,40,41,42] All platelet agonists act on specific

receptors on the platelet membrane to initiate the platelet activation process.[40] In some cases these receptors have been identified whilst in others their nature remains obscure. Agonists of physiological relevance which can be used to activate platelets in vitro are ADP, collagen, thrombin, adrenaline, 5-HT, platelet activating factor (PAF) and prostaglandins. Thromboxane A_2 is the key prostaglandin involved in platelet activation in vivo,[34,35,36] but this is too unstable for in vitro use. Thromboxane mediated platelet behaviour may be studied in vitro by using the endoperoxide analogue 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619), which is thought to act at the same receptor as thromboxane.[40] Alternatively, exogenous arachidonic acid, the substrate for thromboxane synthesis,[34,35,36] can be added to platelets in vitro to produce thromboxane dependent platelet responses. Substances which activate platelets can be categorised as weak or strong agonists.[20,43,44] ADP, adrenaline and PAF are weak agonists, whereas thrombin and high concentrations of collagen act as strong agonists.[20,43,44]

Platelet shape change is seen with all physiological agonists except adrenaline.[20,45] Shape change occurs independently of extracellular calcium,[20] requires the weakest stimulus and is the response most resistant to inhibition.[44] Shape change is detected in vitro in platelet rich plasma or washed platelet preparations by an increase in optical density of the suspension.[20,40]

Platelet aggregation is dependent on the binding of fibrinogen to platelets.[29,30] Fibrinogen receptors become exposed on activated platelets [40,46] by induction of a conformational change in the glycoprotein IIb/IIIa complex.[33,40] Monoclonal antibodies, for example PAC-1, have been described which bind to glycoprotein IIb-IIIa only when platelets have been activated.[47] These are a useful tool for assessment of fibrinogen receptor exposure. Following the initial

description of the central role of fibrinogen in ADP induced aggregation,[29,30] it was unclear whether released ADP was responsible for mediating platelet aggregation in response to other agonists. Subsequently, most physiological agonists have been shown to be capable of inducing fibrinogen receptor exposure[40,48,49,50] but some controversy remains about whether collagen is capable of doing this directly, or whether collagen induced aggregation is a consequence of thromboxane A_2 synthesis and ADP release.[41,46] Fibrinogen binding to its receptor and therefore aggregation are dependent upon extracellular calcium.[30] Aggregation is also dependent on close contact between platelets which can be achieved in vitro by stirring the platelet suspension.[30] Aggregation in vitro can be monitored by following reductions in optical density of platelet rich plasma or washed platelet suspensions as aggregation proceeds.[51] Platelet aggregation in whole blood can be monitored either by counting single platelets in small aliquots of blood[52,53] or by measuring changes in electrical impedance.[54]

Platelet aggregation in vitro may be reversible (primary) or irreversible depending on the strength and concentration of the agonist.[20,40,42,43,44] Weak agonists may elicit primary aggregation at low concentrations, and then at higher concentrations a second wave of aggregation (secondary aggregation) occurs which is irreversible and associated with a release reaction and with the synthesis of thromboxane A_2.[20,40,43,44] Secondary, but not primary aggregation in response to weak agonists can be inhibited by preventing thromboxane synthesis with drugs such as aspirin. Strong agonists can elicit a full irreversible aggregation response which is not thromboxane dependent.[20,40,43,44]

As a weak agonist, ADP at low concentrations produces primary aggregation in PRP, followed by secondary aggregation if the concentration of ADP is increased.[42,46] The secondary wave of aggregation in response to ADP in

PRP is thought, however, to be an artefact induced by the unphysiologically low calcium concentrations in plasma anticoagulated with citrate.[55] Platelet aggregation in response to collagen in vitro occurs in a single phase after an initial lag period and is irreversible.[43,56] At low concentrations of collagen, the aggregation response is thromboxane dependent, but at higher concentrations can occur even when thromboxane synthesis is inhibited. As adrenaline induces little or no platelet aggregation in PRP anticoagulated with the thrombin inhibitor hirudin,[57] there has been controversy as to whether adrenaline can itself cause platelet aggregation or whether it merely synergises with other agonists, such as trace amounts of thrombin in citrated PRP. Evidence has emerged, however,for a direct effect of adrenaline, mediated by alpha$_2$ receptor stimulation.[20,40,50,58] Aggregation in response to the addition of exogenous arachidonic acid in vitro occurs in a single phase and is irreversible.[59] By contrast, prostaglandin endoperoxides and agonists at the thromboxane receptor available for in vitro use are of intermediate potency and induce reversible aggregation when present at low concentrations, the response becoming irreversible when their concentration is increased.[59] Thrombin is the strongest platelet agonist and induces irreversible platelet aggregation.[20,40,43,44] Thrombin binds to high and low affinity sites on the platelet membrane,[20,40] but it has been difficult to show which of these sites is the functional thrombin receptor. Recently, a functional platelet thrombin receptor has been cloned and it has been shown that the proteolytic effects of thrombin are necessary for activation of its receptor.[60,61] Aggregation studies in vitro using thrombin require the preparation of washed platelets to remove fibrinogen so that fibrin formation does not interfere with the measurement of aggregation.[56]

Platelet secretion or the release of contents of storage granules can be divided into three phases.[19,20] The strength of stimulus required for alpha granule

release is less than that required for dense granule release which in turn is less than the stimulus required for lysosomal release.[20] Alpha granule release results in the secretion of platelet specific proteins such as platelet factor 4, β-thromboglobulin and platelet derived growth factor, together with coagulation factors such as factor V, and fibrinogen and proteins involved in cell adhesion such as von Willebrand factor, fibronectin and thrombospondin.[20] Platelet factor 4 has a high affinity for heparin[62] and heparan sulphate on endothelial cells. Platelet factor 4 may have a role in vivo in preventing the inactivation of thrombin by antithrombin III, which is greatly accelerated by endothelial heparan sulphate.[63] The biological effects of β-thromboglobulin are less well characterised but it has been shown to bind to endothelial cells and inhibit prostacyclin production[64] and to have chemotactic activity for fibroblasts.[65] Platelet derived growth factor is mitogenic for smooth muscle cells[66,67] and may be involved in the smooth muscle proliferation seen in atheromatous lesions.[67] Alpha granule release also results in the appearance of the alpha granule membrane protein, GMP-140, on the surface membrane of the platelet.[68] GMP-140 is an adhesion molecule of the selectin family which acts as a receptor for neutrophils and monocytes, thus facilitating white cell/platelet interaction.[68] Measurement of plasma platelet factor 4 and β-thromboglobulin levels by radioimmunoassay can provide a useful marker of platelet alpha granule release in vivo, provided blood is taken under conditions which minimise platelet activation during venepuncture and subsequent handling in vitro.[56]

Platelet dense granules contain calcium ions, adenosine triphosphate (ATP), ADP, pyrophosphate and 5-HT.[19,20] ADP and 5-HT are themselves platelet agonists contributing to positive feedback loops promoting further platelet activation. Dense granule contents in general do not exchange with nucleotides or calcium in the

cytoplasm or in the plasma,[20] but there is an active transport system which allows uptake of 5-HT from plasma across the plasma membrane and dense granule membranes.[69,70] Dense granule release can be measured easily in vitro either by labelling the dense granules with radioactive 5-HT which is subsequently released upon platelet stimulation[69] or by continuous monitoring of chemiluminescence using the luciferin-luciferase reaction which measures ATP release during platelet activation.[20]

All physiological agonists are capable of stimulating alpha granule and dense granule release but in the case of weak agonists this occurs in an aggregation dependent manner and is also dependent on thromboxane synthesis.[43,44] If aggregation or thromboxane synthesis is blocked, no release reaction takes place. In the case of strong agonists the release reaction can be triggered independently of aggregation and thromboxane synthesis.[43,44,71]

The third category of secretory granules in platelets, the lysosomes, contain acid hydrolase enzymes, the function of which is unclear, but they may have a role in clearing of platelet thrombi, heparin inactivation and possibly anti-inflammatory mechanisms.[20] Lysosomal secretion only occurs when platelets are stimulated with the strongest agonists, and even then release is incomplete.[20,44]

2.4 Arachidonic Acid Liberation: The Synthesis and Role of Platelet Prostaglandins.

When platelets become activated, various pro-aggregatory prostanoids are synthesised by the activated platelets, the most important of which is thromboxane A_2 which was first identified by Hamberg in 1975.[34,35,36] All physiological agonists are capable of initiating thromboxane synthesis in vitro but the extent of this varies with the strength and concentration of the agonist.[15,41,56]

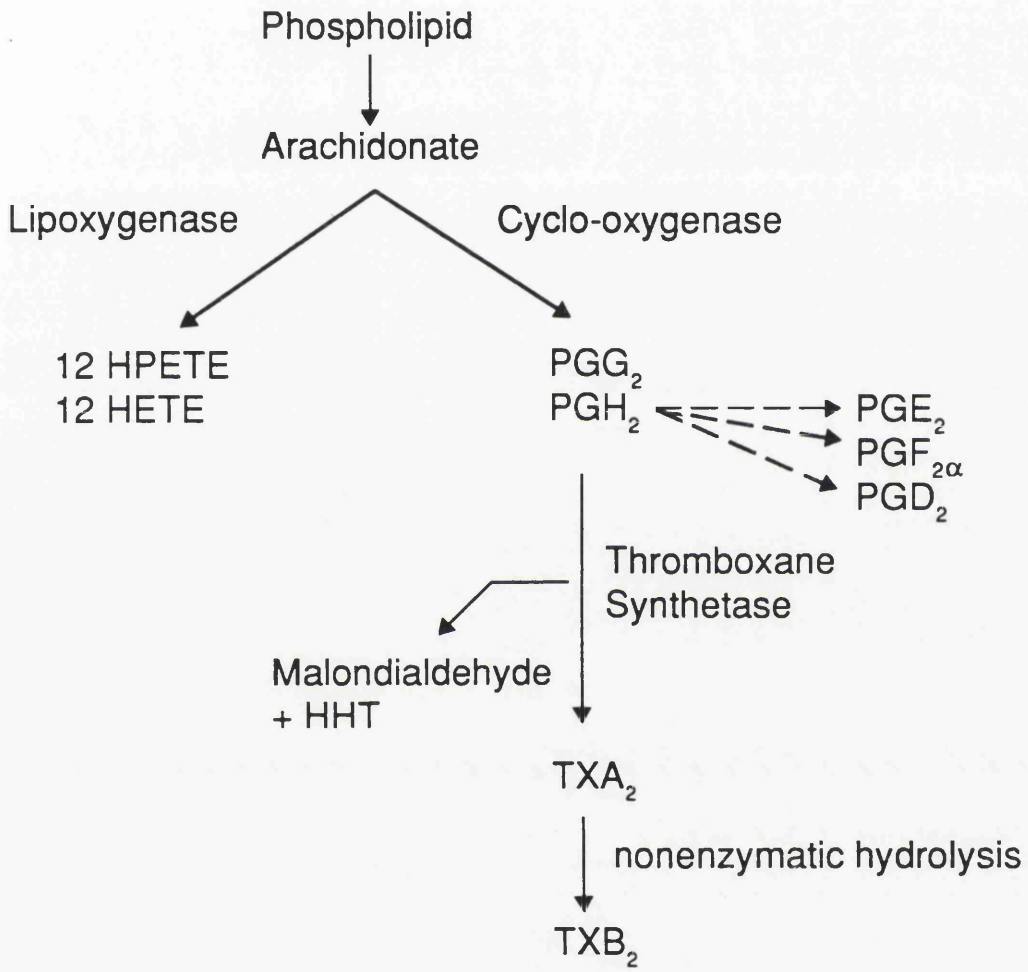
The substrate from which prostaglandins are synthesised is arachidonic acid, a 20-

carbon fatty acid, derived from linoleic acid. Unless added exogenously in vitro, all of the arachidonic acid utilised by platelets for prostaglandin synthesis is released by hydrolysis from platelet membrane phospholipids.[35,36,72] At least two pathways are involved in arachidonic acid liberation. In the first, free arachidonic acid is produced mainly from phosphatidylcholine and phophatidylethanolamine, by the action of phospholipase A_2.[72,73,74] In the second pathway, phospholipase C acts specifically on phosphatidylinositol resulting in diacylglycerol production.[75] Free arachidonic acid is then liberated from diacylglycerol by diglyceride lipase.[76] Free arachidonic acid is metabolised via two main pathways: the cyclooxygenase pathway and the lipoxygenase pathway.[35,36] This is illustrated in Figure 1.1. In activated platelets the cyclooxygenase pathway is of greater importance.

Cyclooxygenase itself catalyses the conversion of arachidonic acid to the cyclic endoperoxides prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2).[77] PGG_2 and PGH_2 are normally rapidly converted by thromboxane synthetase to thromboxane A_2.[34,78] Malondialdehyde and 12-hydroxyheptadecatrienoic acid are formed as byproducts of this reaction.[34] As discussed above, thromboxane synthesis represents an important positive feedback mechanism in platelets and plays a key role in platelet responses to weak agonists.[20] In addition to its pro-aggregatory effects on platelets, thromboxane A_2 is also a potent vasoconstrictor.[35,36,78] In vivo, it appears to exert its effects on platelets and vascular tissue by local rather than systemic action.[79] Thromboxane A_2 is extremely unstable, having a half life of 30 seconds in aqueous solution, and spontaneously hydrolyses to thromboxane B_2.[34,78] In vivo, it is further metabolised giving rise to 2,3 dinor-thromboxane B_2[80] and 11-dehydro-TXB_2[81], which are excreted by the kidney and may be measured in the urine.[82] Measurement of these metabolites in urine by gas chromatography-mass spectrometry (GCMS) is the

Figure 1.1

PLATELET ARACHIDONIC ACID METABOLISM



12-HPETE = 12-hydroperoxyeicosatetraenoic acid
12-HETE = 12-hydroxyeicosatetraenoic acid
HHT = 12-hydroxyheptadecatrienoic acid

most accurate means of assessing thromboxane biosynthesis in vivo, as plasma measurements of thromboxane B_2 are subject to artefact as a result of thromboxane formation from platelet activation during venepuncture and sample handling.[82] Measurement of serum thromboxane B_2 levels, however, represents a useful method of assessing platelet thromboxane production during spontaneous clotting of whole blood in vitro.[56,83]

Although the intermediates PGG_2 and PGH_2 are usually very quickly metabolised to thromboxane A_2, these prostaglandins are themselves pro-aggregatory[84] and probably act at the same platelet receptor as thromboxane.[85] The normal route of metabolism of the cyclic endoperoxides is via thromboxane synthetase, but PGG_2 and PGH_2 can also be converted to other prostaglandins such as PGE_2, PGD_2 and $PGF_{2\alpha}$.[35,36] These prostaglandins are formed only in small amounts under normal circumstances, but their effects on platelet behaviour are worthy of consideration. PGD_2 inhibits platelet activation and may serve as a negative feedback mechanism to limit platelet activation.[86] PGE_2 at low concentrations can synergise with other agonists to promote platelet activation,[87,88,89] whilst at higher concentrations it is inhibitory.[89,90]

The lipoxygenase pathway involves the conversion of arachidonic acid to 12-hydroperoxyeicosotetraenoic (12-HPETE) and 12-hydroxyeicosotetraenoic acid (12-HETE).[35,79] 12-HETE has chemotactic activity for neutrophils,[79] and there is evidence that lipoxygenase products can inhibit platelet aggregation in vitro.[91] It is unlikely, however, on the basis of current evidence, that these agents have a major role in the regulation of haemostasis.

2.5 Stimulus Response Coupling Mechanisms in Platelet Activation.

As in many cell types, cytosolic calcium ions and the products of breakdown of inositol phospholipids act as important stimulatory second

messengers in platelets , linking receptor occupancy with an agonist to platelet responses such as shape change, aggregation, secretion, and thromboxane synthesis.

Intracellular Free Calcium

Evidence for the role of calcium ions as intracellular messengers has been derived from studies showing that calcium ionophores such as A23187 can activate platelets,[92,93] giving rise to responses very similar to those observed with physiological agonists. Permeabilised platelets can also be activated by addition of extracellular calcium in concentrations in the micromolar range,[94,95] and intracellular calcium antagonists, for example some local anaesthetics, have been shown to inhibit platelet activation induced by calcium ionophore or thrombin.[96] Furthermore, in experiments using intracellular calcium probes such as the fluorescent dyes quin-2, and fura-2 or the chemiluminescent photoprotein, aequorin, an increase in intracellular free calcium can be demonstrated upon platelet stimulation by a wide range of physiological agonists.[97,98,99] In many tissues, calcium flux across the plasma membrane plays a role in the increase in cytosolic calcium observed upon excitation of the cell. As platelets can become activated in the absence of extracellular calcium,[100] it was proposed that calcium was mobilised from intracellular storage sites to increase cytosolic free calcium in response to a stimulus. Large amounts of calcium are sequestered by the dense tubular system in resting platelets[101] and the membranes of the dense tubular system contain a calcium-ATPase pump.[102,103] The dense tubular system has therefore been considered to be the major source of stimulus induced increases in cytosolic calcium in platelets. Furthermore, it has been shown that inositol trisphosphate which is released from membrane phospholipids following stimulation of platelets by physiological agonists, is capable of releasing calcium

from isolated calcium transporting internal platelet membranes.[22,104] At least for some agonists, however, an influx of calcium across the plasma membrane via receptor operated calcium channels appears to contribute to the increase in cytosolic calcium observed upon platelet activation.[41,105] This is especially relevant to ADP induced platelet activation.[106,107] Platelets do not appear to possess voltage operated calcium channels.[41,108]

There have been several attempts to correlate concentrations of intracellular free calcium with platelet responses using calcium ionophore treated platelets[97] and permeabilised platelets.[95] Such studies show that shape change requires a lesser rise in intracellular free calcium than aggregation and secretion, and phosphatidylserine exposure leading to prothrombinase activity requires the greatest rise in cytosolic calcium. Studies with quin-2, fura-2 and aequorin in platelets stimulated with physiological agonists have indicated, however, that responses predicted by the rise in intracellular free calcium following stimulation do not completely correlate with the actual platelet responses elicited by the agonist.[41,95,97] Part of the explanation of this lies in the fact that aequorin may be measuring localised calcium transients, and quin-2 may itself quench larger increments in intracellular free calcium,[109,110] but some observations cannot be accounted for in this way. For example stimulation of platelets with collagen, in the presence of cyclooxygenase inhibitors, results in a negligible rise in intracellular free calcium, measured with fura-2,[111] but collagen is a strong agonist capable of eliciting all platelet responses including lysosomal release and prothrombinase activity.[20] By contrast, calcium signals measured by aqueorin in response to collagen could account for all the observed platelet responses to collagen,[41,110] but predicted and observed responses for other agonists such as ADP, on the basis of aqueorin data, fail to correlate.[41] All the data can only be reconciled by the

involvement of other second messenger systems in the signalling of platelet responses to agonists. ADP, thrombin, and thromboxane analogues are good mobilisers of calcium, whereas collagen and adrenaline are poor calcium mobilisers.[41,110]

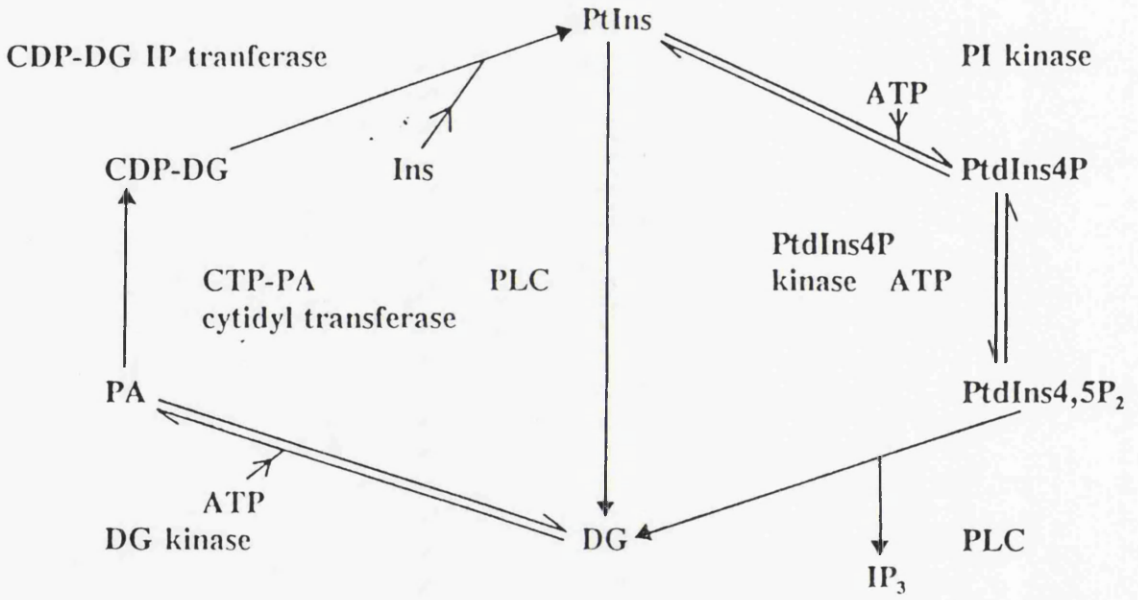
Various targets for cytosolic calcium which are of relevance to the execution of platelet responses have been identified. Platelets have been shown to contain calmodulin,[112] a calcium binding protein which in many tissues mediates calcium regulation of cellular responses. In platelets calmodulin has been shown to regulate the activity of myosin light chain kinase.[113] Moreover, phosphorylation of a 20 Kilodalton (20K) protein[114] subsequently identified as the light chain of myosin occurs during platelet activation with physiological agonists.[115] Contractility of actomyosin is dependent on myosin phosphorylation,[116] therefore calcium dependent activation of myosin light chain kinase ultimately elicits contractile processes in the platelet which may be linked with responses such as shape change and secretion.[114,115] The enzyme phospholipase A_2 represents another target for cytosolic calcium.[74,117] Activation of this enzyme by calcium results in the liberation of arachidonic acid from membrane phospholipids and ultimately prostaglandin synthesis.[74]

Phosphoinositide Metabolism.

As has been shown above, stimulus response coupling in platelets cannot be entirely explained by intracellular calcium transients. There is substantial evidence that platelet activation by many physiological agonists is accompanied by an increased turnover of membrane phosphatidylinositol,[75,98,118,119] together with the inositol polyphosphates, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate (PIP_2).[120,121] Hydrolysis of these phospholipids is catalysed by the enzyme phospholipase C yielding diacylglycerol

and inositol phosphate, inositol bisphosphate or inositol trisphosphate (Figure 1.2).[41,44,75,121] The cycle is completed by the actions of diacylglycerol kinase and two transferases, regenerating phosphatidylinositol which can in turn undergo phosphorylation rendering the inositol polyphosphates.[44,121] Time course studies in thrombin stimulated platelets have shown that phospholipase C induced hydrolysis of PIP_2 is the initial event in this cycle and is closely coupled to receptor occupancy.[120] PIP_2 hydrolysis is linked to receptor occupancy by a putative G protein "G_p".[122] This G protein has not been isolated but evidence for its existence comes from studies showing that addition to permeabilised platelets of GTP analogues, which bind to G proteins, and convert them to their active state, can induce metabolism of polyphosphoinositides,[123] and potentiate platelet responses such as 5-HT secretion.[124] The diacylglycerol generated acts as a second messenger by activating protein kinase C[125] which in turn phosphorylates a 40 kilodalton (40K) regulatory intracellular protein.[40,125,126] Protein kinase C also requires calcium for activation but diacylglycerol greatly reduces the calcium threshold at which the enzyme will become activated.[40,44,125,127] Evidence for the role of diacylglycerol and of protein kinase C in platelet activation comes from the observations that addition to platelet suspensions of exogenous analogues of diacylglycerol or of phorbol esters which activate protein kinase C directly, can elicit platelet responses such as aggregation and secretion.[127] Whilst phosphorylation of the 40K protein, the substrate for protein kinase C, has been consistently observed upon platelet activation by physiological agonists such as thrombin,[114,125] its ultimate function and its relevance to particular platelet responses remain uncertain. Phosphorylation of the 40K protein has been associated with platelet secretion[114] but other studies have found the correlation of the two events to be incomplete.[128]

THE PHOSPHATIDYL INOSITOL CYCLE.



PTIns = phosphatidylinositol; PtdIns4P = phosphatidylinositol-4-phosphate; PtdIns4,5P_2 = phosphatidylinositol-4,5-bisphosphate; IP_3 = inositol trisphosphate; DG = diacylglycerol; PLC = phospholipase C; PA = phosphatidic acid; CDP-DG = cytidine diphosphoryl diacylglycerol.

Figure 1.2

Inositol trisphosphate (IP_3) generated by the metabolism of inositol phospholipids also has a role as a second messenger in platelets. Inositol trisphosphate acts as link between receptor activation and calcium mobilisation. Inositol trisphosphate has been shown to cause dose dependent release of calcium from calcium transporting membrane vesicles derived from platelets.[22,104] The membrane vesicles prepared in these experiments had the biochemical composition of dense tubular system membranes. Furthermore, IP_3 binding sites which have the characteristics of receptors have been demonstrated on highly purified platelet intracellular membranes.[129] There is recent evidence, however, for the existence of an IP_3 insensitive intracellular calcium pool[130]

Different agonists display differing abilities to activate phospholipase C. Thrombin,[75,120] thromboxane,[98] platelet activating factor[119] and vasopressin[118] have all been shown to increase diacylglycerol and IP_3 formation in platelets, whereas the levels of these second messengers are little affected by ADP[41,131] and adrenaline.[40,41] Some very recent studies suggest that thromboxane A_2 may activate phospholipase C via a G protein distinct from "G_p".[132] If thromboxane synthesis is suppressed, platelet stimulation with collagen results in phosphatidate formation[111] but there is little IP_3 formation. Hydrolysis of phosphatidylcholine seems to provide some of the diacylglycerol formed after platelet stimulation with collagen.[111] A phospholipase D, which probably hydrolyses phosphatidylcholine has been identified in platelets[133,134] and it is possible that the collagen receptor is coupled to this.

As has been discussed above, adrenaline is a poor calcium mobiliser and also does not seem to be coupled to activation of phospholipase C.[111] The mechanism of platelet activation by adrenaline therefore remains difficult to

explain. Alpha$_2$ adrenoreceptors are linked to a G protein (G_i) whose activation inhibits adenylate cyclase,[135] the enzyme responsible for the synthesis of cyclic AMP, an inhibitory second messenger in platelets.[136] Whilst a reduction in the basal level of cyclic AMP has not itself been shown to cause platelet activation,[137] reduction in elevated levels of cyclic AMP can antagonise the effects of platelet inhibitors acting via this second messenger.[138] It is conceivable that the coupling of adrenaline to G_i is involved in an as yet undefined manner in the mechanism of adrenaline induced platelet activation, and may be more important in vivo than in vitro. The role of cyclic AMP as an inhibitory second messenger and the control of adenylate cyclase will be discussed in detail in subsequent sections of this chapter.

3. PATHOLOGICAL PLATELET ACTIVATION

In vivo, platelet plug formation is normally limited to an extent appropriate to securing haemostasis by protective mechanisms in which vascular endothelium has a major role to play. When platelet activation occurs inappropriately thrombus formation may occur resulting in pathological sequelae. Circumstances which contribute to thrombus formation are, as first described by Virschow in 1856:[139] 1) damage to the vessel wall, which may lead to failure of normal endothelial function, 2) an abnormal pattern of blood flow, which may itself result from vessel wall damage, and 3) abnormalities of the blood itself, including changes in platelets and coagulation factors. Clearly any shift in the balance between factors which promote platelet activation and those which limit it could lead to a pro-thrombotic state. Platelets have been particularly implicated in arterial thrombosis, as thrombi forming in the fast flow conditions found in arteries are rich in platelets and leucocytes, whereas red cells and fibrin are predominant components of

venous thrombi.[140] Arterial thrombi are also generally associated with disease in the vessel wall.[141] Platelet activation has therefore been implicated in a variety of clinical conditions in which arterial thrombosis plays a part, such as myocardial infarction, ischaemic stroke, transient ischaemic attacks, and occlusion of vascular grafts.[141] Furthermore, platelets have also been implicated in the development of atherosclerosis, and several possible mechanisms have been proposed. One hypothesis[67,142] suggests that endothelial injury or dysfunction results in adherence of platelets and monocytes to the vessel wall. Adherent platelets release platelet derived growth factor which is a mitogen for smooth muscle. Ultimately, intimal thickening and atherosclerosis develop. Pathological platelet activation has also been implicated in the development of other clinical conditions in which vascular occlusion is a feature, for example the microvascular complications of diabetes,[143] migraine,[144] and pre-eclampsia.[145] The role of platelets in pre-eclampsia is discussed in detail in a subsequent section of the introduction. It is relevant here to review the normal physiological mechanisms which limit platelet activation. Some pharmacological means of inhibiting platelet behaviour, aimed at preventing pathological platelet activation and thrombosis will then be described.

4.INHIBITION OF PLATELET ACTIVATION:

THE PHYSIOLOGICAL ROLE OF ENDOTHELIUM.

4.1 General Aspects of Endothelial Function.

In intact blood vessels platelet activation is normally prevented by the endothelium. This is partly because the endothelium forms a physical barrier from the thrombogenic surface of the underlying basement membrane, and the negative charge on both endothelial cells and platelets makes them mutually repulsive.[146] The endothelium, however, plays a much more active role in the regulation of

platelet activity by the synthesis and release of some very powerful platelet inhibitory substances. These consist of inhibitory prostaglandins and endothelium derived relaxing factor. Furthermore, endothelium is capable of promoting catabolism of ADP and also contributes to the inactivation of thrombin. Each of these functions will be considered in turn.

4.2 Synthesis of Prostaglandins which Regulate Platelet Behaviour

The ability of vascular tissue to generate an unstable prostaglandin product which was a potent inhibitor of platelet aggregation was first described by Moncada et al in 1976.[147] This was later identified as prostacyclin.[148] Prostacyclin is synthesised by endothelial cells from arachidonic acid by a pathway identical to that for thromboxane synthesis in platelets except for the final step in which the enzyme prostacyclin synthetase catalyses the conversion of cyclic endoperoxides to prostacyclin (PGI_2).[35,147] Prostacyclin is the most potent known platelet inhibitory agent[35,36] and is also a powerful vasodilator.[149] It is unstable in vitro[147] and, both in vitro and in vivo is rapidly broken down by non enzymatic hydrolysis to 6-ketoprostaglandin$F_{1\alpha}$ ($PGF_{1\alpha}$).[148] In vivo, further metabolism by enzymatic degradation takes place resulting in the excretion of 2,3 dinor-6-keto$PGF_{1\alpha}$ and 6,15-diketo-13,14 dihydro-2,3 dinor $PGF_{1\alpha}$.[150] Prostacyclin is thought to act locally, as circulating levels are too low to have biological activity.[151,152] It is capable of inhibiting all platelet responses, irrespective of the inducing agonist,[20,44] but is less efficient as an inhibitor of adhesion than of other aspects of platelet behaviour.[153] Prostacyclin synthesis by endothelial cells is stimulated by thrombin, bradykinin, changes in shear stress, and products derived from activated platelets such as ADP and ATP.[154] Furthermore PGG_2 and PGH_2 released from activated platelets may be utilised by the endothelium for prostacyclin synthesis.[36,155] The stimulation of prostacyclin synthesis by substances from activated platelets

provides a means of limiting thrombus formation. A rise in intracellular free calcium in the endothelial cell is the main second messenger driving prostacyclin synthesis.[156] Prostacyclin release is subject to rapid homologous desensitisation, even in the continued presence of the stimulating agonist.[157]

It has been proposed that an imbalance between the pro-aggregatory and vasoconstrictor effects of thromboxane and the anti-aggregatory vasodilator effects of prostacyclin may be a key factor predisposing to pathological platelet activation and thrombosis.[35,36] Furthermore, some evidence of such an imbalance has been found in conditions associated with thrombosis such as diabetes,[35,141] hyperlipidaemia[141] and cigarette smoking,[36] and in pregnancy induced hypertension.[158,159,160] The subject of thromboxane/prostacyclin imbalance in hypertensive pregnancy is discussed in detail in a subsequent section of this chapter. On the other hand, increased excretion of urinary metabolites of both thromboxane and prostacyclin have been described in other conditions associated with platelet activation such as severe atherosclerosis and unstable angina.[161,162] The increase in prostacyclin synthesis may represent a compensatory response by vascular tissue to platelet activation.

Whilst prostacyclin is the major prostaglandin synthesised by the endothelium of large and medium sized blood vessels, PGE_2 is the main metabolite of arachidonic acid in microvascular endothelium.[163,164] Unlike prostacyclin, PGE_2 at low concentrations potentiates platelet aggregation induced by other agonists, whilst at high concentrations inhibits platelet behaviour in response to a wide range of agonists.[87,88,89,90]

The prostaglandins produced by endothelial cells and indeed PGD_2 produced by platelets themselves, act as inhibitors of platelet behaviour by increasing the levels of the inhibitory second messenger cyclic AMP in platelets.[165,166] The

inhibitory prostaglandins act on specific surface receptors which are linked to stimulation of adenylate cyclase, the enzyme which catalyses the synthesis of cyclic AMP from ATP.[166] As cyclic AMP is capable of inhibiting most platelet responses at the level of intracellular signal processing, the inhibitory effects of agents which act via cyclic AMP are not agonist specific.[44]

4.3 Endothelium Derived Relaxing Factor.

Endothelium produces a highly labile agent with powerful vasodilator activity which became known as endothelium derived relaxing factor (EDRF)[167] and was subsequently identified as nitric oxide.[168] EDRF inhibits platelet behaviour in response to a wide range of agonists,[169,170,171] and unlike prostacyclin, is moderately active as an inhibitor of platelet adhesion.[172] Like prostacyclin, EDRF, as a highly labile agent, is likely to exert its effects on platelets and blood vessels at a local level.[173,174] EDRF is synthesised from L-arginine by the soluble enzyme nitric oxide synthetase.[175] The activity of nitric oxide as an inhibitor of platelet behaviour and as a vasodilator is prolonged by superoxide dismutase and is inhibited by haemoglobin to which nitric oxide binds.[169,171,174] EDRF synthesis in endothelium is stimulated by acetylcholine,[167] substance P,[173,176,177] shear stress on the endothelium,[173] and factors released during thrombus formation such as ATP, ADP, 5-HT, and thrombin.[173,176,177] This again could act as a negative feedback mechanism limiting thrombus extension. EDRF acts as an inhibitor of platelet behaviour by raising the level in platelets of cyclic GMP,[171,178,179] which like cyclic AMP, acts as an inhibitory second messenger in platelets.[41,44,180,181] Nitric oxide increases cyclic GMP by stimulating the platelet soluble guanylate cyclase, the enzyme responsible for the synthesis of cyclic GMP from GTP.[171,178,179] The inhibitory effects on platelet behaviour of prostacyclin and EDRF are markedly

synergistic,[182] but the exact mechanism of the synergism has not been completely elucidated.

4.4 Other Mechanisms By Which Endothelium Limits Haemostasis.

ADP and ATP released from platelets and damaged vessel walls are catabolized by endothelial ectonucleotidases to yield adenosine.[183] Adenosine is also capable of acting on specific receptors on the platelet membrane to raise platelet cyclic AMP levels and to inhibit platelet behaviour.[184] The activity of adenosine is limited by its rapid uptake into red cells and endothelial cells.[183,185] Endothelial cells also have a surface protein called thrombomodulin which binds thrombin with a high affinity.[186] Binding of thrombin to thrombomodulin inhibits its ability to cleave fibrinogen and to activate platelets and Factor V. Furthermore, thrombin mediated activation of the naturally occurring anticoagulant Protein C is greatly accelerated by thrombin binding to thrombomodulin.[186,187] Activated Protein C then inhibits the activity of Factor V_a and Factor $VIII_a$ thus limiting thrombin and fibrin formation.[187]

The second anticoagulant, as opposed to antiplatelet, property of endothelium resides in the presence of substances on the endothelial surface which have heparin like activity and are thus able to accelerate the activity of plasma antithrombin III.[63] In addition to inhibiting thrombin, antithrombin III has activity against Factor X_a and other serine protease coagulation factors.[188]

4.5 Inhibitory Second Messengers in Platelets

Cyclic AMP and cyclic GMP perform the function of inhibitory second messengers in platelets.[44,136,166] Increased levels of these cyclic nucleotides lead to inhibition of most aspects of platelet behaviour. The importance of the cyclic nucleotides lies in their key role as mediators of inhibition of platelet behaviour by physiologically relevant regulators of haemostasis such as prostacyclin[165] and

other inhibitory prostanoids in the case of cyclic AMP[135,138,166,180] and EDRF in the case of cyclic GMP.[171,178,179]

Cyclic AMP.

The inhibitory effects on platelet behaviour of cyclic AMP and its dibutyryl derivative were first described by Marcus and Zucker in 1965.[136] Basal levels of cyclic AMP in platelets are very low and exert little effect on platelet behaviour.[189] On the other hand only a slight elevation of cyclic AMP is required to obtain a marked inhibitory effect.[41,138,190] Cyclic AMP is synthesised from ATP by the enzyme adenylate cyclase in response to receptor occupation by specific ligands.[166] The metabolism of cyclic AMP to 5'-AMP is brought about by phosphodiesterase enzymes.[166]

Adenylate cyclase is a high molecular weight transmembrane enzyme[15,16,191] which consists of a catalytic subunit closely associated with two guanine nucleotide binding proteins (G proteins): the stimulatory G protein (G_s) and the inhibitory G protein (G_i).[40,41,44,191] Indirect evidence for the involvement of G proteins in the regulation of platelet adenylate cyclase activity came from observations that GTP and its analogues could influence adenylate cyclase activity.[135,166] Binding of a ligand to a receptor linked with G_s leads to stimulation of adenylate cyclase, whilst occupancy of a receptor linked to G_i leads to inhibition of the enzyme.[135] Both G proteins consist of alpha, beta, and gamma subunits.[44,122,191] The alpha subunit contains the site which binds to guanine nucleotides. In the inactive state the G proteins bind GDP, but when stimulated by receptor activation, lose their affinity for GDP and bind GTP tightly. This leads to dissociation of the alpha subunit from the other subunits and subsequent stimulatory or inhibitory action on the catalytic component of adenylate cyclase.[40,122]

Substances which have been shown to interact with surface receptors leading to stimulation of adenylate cyclase include prostacyclin (PGI_2),[165] prostaglandin E_1 (PGE_1),[192,193] PGD_2,[194] PGE_2 in high concentrations,[90] adenosine,[184] and $beta_2$ adrenergic agonists.[193] PGI_2, PGE_1 and PGE_2 share a common receptor on the platelet membrane,[195,196] whilst PGD_2 has a distinct receptor.[195,197,198,199] The catalytic subunit of adenylate cyclase can also be stimulated directly with forskolin, a diterpene isolated from plants.[200,201] PGI_2 is the most potent stimulator of adenylate cyclase and gives rise to a more sustained elevation of cyclic AMP than that produced by PGE_1 and PGE_2.[166] All of the prostaglandins which stimulate adenylate cyclase, however, give rise to a rapid increase in cyclic AMP which then decreases to a variable extent to reach a plateau.[202] Whilst metabolism of cyclic AMP by phosphodiesterase contributes to this, there is evidence that this effect also results from prostaglandin induced activation of G_i via receptors on the platelet surface distinct from the prostaglandin receptors linked with G_s.[202] The relative affinity of prostaglandins for receptors linked with G_s and those linked with G_i varies and for each prostaglandin its effects on adenylate cyclase inhibition are both time and concentration dependent.[202] There is good evidence that PGE_2 at low concentrations is capable of reducing platelet cyclic AMP levels raised by other adenylate cyclase stimulators, whilst at higher concentrations PGE_2 stimulates the enzyme resulting in an increase in cyclic AMP.[88,89,203] A number of other agents can inhibit adenylate cyclase by binding to receptors linked to G_i. These include many aggregating agents such as ADP,[204] adrenaline (acting at the $alpha_2$ adrenoreceptor),[135] and thrombin.[205] Conflicting evidence exists regarding the ability of thromboxane A_2 to inhibit adenylate cyclase.[206,207] Reduction in cyclic AMP from basal levels by these agents, however, appears <u>not</u> to be the mechanism by which they induce platelet aggregation.[137,138,180,193] Furthermore, in

the case of ADP, there is evidence that the receptor which mediates inhibition of adenylate cyclase is distinct from that mediating aggregation.[40,208]

Platelets contain several subtypes of the phosphodiesterase enzymes which break down cyclic nucleotides.[209,210] Type III phosphodiesterase (PDE III) is specific for cyclic AMP and is active at low concentrations of the cyclic nucleotide. PDE II hydrolyses both cyclic AMP and cyclic GMP and requires higher concentrations of cyclic nucleotide for activity, and PDE I preferentially hydrolyses cyclic GMP. Cyclic AMP may be raised by drugs which inhibit cyclic AMP phosphodiesterase.[88,166,192] Inhibition of platelet behaviour occurs as a result of a smaller increment in cyclic AMP with phosphodiesterase inhibitors in comparison with adenylate cyclase stimulators.[44,211,212] Moreover, phosphodiesterase inhibitors and adenylate cyclase stimulators have synergistic effects on platelet cyclic AMP levels and on inhibition of platelet behaviour.[166,192]

An increase in cyclic AMP in platelets inhibits most morphological and biochemical aspects of platelet behaviour, suggesting that cyclic AMP acts at a point in the signal transduction mechanism close to stimulus response coupling. Cyclic AMP appears to increase the threshold stimulus required for activation rendering platelets more resistant to activation,[44] but cyclic AMP is also capable of causing platelet disaggregation.[44] The exact mechanism of action of cyclic AMP has not been fully elucidated but multiple effects have been reported. Cyclic AMP has been shown by some to increase calcium re-uptake by platelet internal membranes, an effect which would reduce cytosolic calcium,[213] but others have disputed this effect.[214] Inhibition of agonist stimulated phospholipase C activation has also been reported.[40,75,215] This may result from enhancement by cyclic AMP of GTP_{ase} activity of the putative G protein "G_p" implicated in receptor-phospholipase C coupling thus converting the G protein back to the inactive (G_p-

GDP) state.[124] Another group of investigators have shown that cyclic AMP is capable of inhibiting intracellular calcium mobilisation by IP_3, an effect distinct from enhancement of calcium re-uptake.[216] It has also been shown, however, that inhibition of platelet responses such as aggregation and release occurs at lower cyclic AMP concentrations than those required to inhibit phophoinositol breakdown, suggesting that cyclic AMP may additionally act at sites distal to phosphoinositol metabolism.[190] Inhibition of protein kinase C by cyclic AMP has been reported by some,[217] but not confirmed by other investigators,[218] and cyclic AMP dependent phosphorylation and consequent inhibition of myosin light chain kinase has been demonstrated.[219]

Many of the effects of cyclic AMP are dependent on the action of a cyclic AMP dependent protein kinase, referred to as protein kinase A.[220] Indeed, phosphorylation of a number of intracellular proteins has been demonstrated following treatment of platelets with agents which increase cyclic AMP or with dibutyryl cyclic AMP. Such proteins include a 22kDa, a 24kDa, a 39kDa and a 50kDa protein.[181,221,222] Some of these proteins have very recently been identified. The 24kDa protein is the beta chain of glycoprotein 1b,[223] and the 22kDa protein has been identified as rap 1b, a low molecular weight GTP binding protein[224,225] which shares homology with the product of the ras oncogene. The exact function of rap 1b is only beginning to be elucidated. Some reports have suggested that its phosphorylation by protein kinase A may not be involved in the inhibition of platelet aggregation and secretion by cyclic AMP.[221] By contrast, Lapetina[226] has suggested that, in thrombin stimulated platelets, rap 1b can bind to another platelet protein, rasGAP, which is constitutively associated with phospholipase C. Binding of rasGAP to rap1b facilitates hydrolysis of membrane inositol phospholipids by phospholipase C. Phosphorylation of rap1b, by cyclic

AMP dependent protein kinase, induces dissociation of rap1b from the membrane and therefore inhibits the action of phospholipase C on inositol phospholipids. The 50kDa protein phosphorylated by protein kinase A has also recently been purified and has become known as vasodilator stimulated phosphoprotein (VASP).[227] The phosphorylation of this protein correlates well with platelet inhibition by cyclic nucleotides.[228] A simplified scheme of the interaction of cyclic AMP and second messenger systems involved in platelet activation is shown in Figure 1.3.

Cyclic GMP.

An increase in the level of cyclic GMP in platelets leads to inhibition of their behaviour.[40,41,44,178-181,191] Cyclic GMP, like cyclic AMP can inhibit platelet responses to a wide range of agonists, and appears to be a better inhibitor of platelet adhesion than cyclic AMP.[172] Cyclic GMP is synthesised from GTP by the action of guanylate cyclase, which is a soluble enzyme found in the platelet cytoplasm.[179,191] Types I and II phosphodiesterase are capable of hydrolysing cyclic GMP.[209,210] As discussed above, EDRF stimulates guanylate cyclase, but the enzyme may also be stimulated by nitrovasodilators such as sodium nitroprusside,[178,229] glyceryl trinitrate and 3-morpholinosydnonimine (SIN-1), a metabolite of molsidomine.[230] It is thought that these compounds stimulate guanylate cyclase by releasing nitric oxide.[173,178,230] Cyclic GMP levels may also be raised by inhibiting the phosphodiesterase enzymes responsible for its metabolism, and some compounds exist which are relatively specific for the type I phosphodiesterase.[210] Platelet aggregation has been reported to increase cyclic GMP levels.[180,189,231] This may be mediated by contact of platelets with each other or a matrix such as collagen [189,231] and may act as a negative feedback mechanism. It has recently been shown that platelets themselves possess an

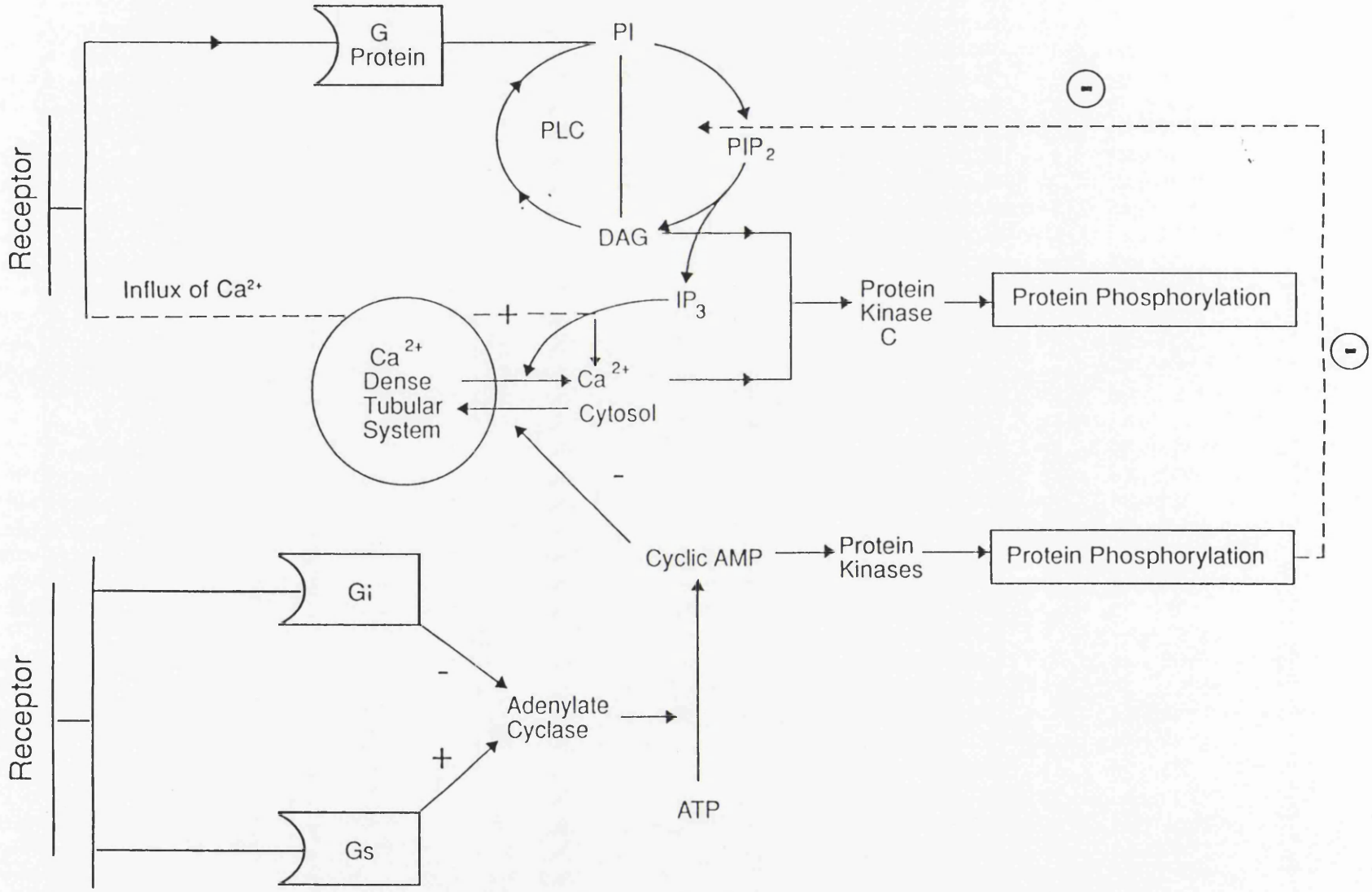

G
Protein
PI
PLC
PIP2
DAG
Receptor
Influx of Ca2+
IP3
Protein
Kinase
C
Protein Phosphorylation
Ca 2+
Dense
Tubular
System
Ca 2+
Cytosol
Cyclic AMP
Protein
Kinases
Protein Phosphorylation
Gi
Adenylate
Cyclase
Receptor
Gs
ATP

Figure 1.3

L-arginine-nitric oxide pathway, and this is likely to be involved in the mechanism of aggregation-induced increments in cyclic GMP levels.[232,233]

Relatively little is known of the mechanisms by which cyclic GMP inhibits platelet behaviour but treatment of platelets with sodium nitroprusside or with 8-bromo-cyclic GMP has been reported to result in phosphorylation of the VASP 50kDa protein.[40,181,222,227,228] Phosphorylation of VASP by both protein kinase A and cyclic GMP dependent protein kinase may be involved in the synergism of platelet inhibitors acting via these cyclic nucleotides,[228] but other mechanisms may also come into play. For example, it has been shown that cyclic AMP phosphodiesterase is inhibited by cyclic GMP.[234]

5.PHARMACOLOGICAL MEANS OF INHIBITING PLATELET BEHAVIOUR.

To date therapeutic agents which have been used clinically as inhibitors of platelet behaviour have mainly been targeted at the platelet arachidonic acid-thromboxane pathway. Aspirin is the most widely used antiplatelet drug and the agent for which there is the most evidence of clinical efficacy in thrombotic disease. Benefit of aspirin has been shown in myocardial infarction,[235,236] unstable angina,[236] ischaemic stroke,[236] transient ischaemic attacks,[236] prevention of occlusion of coronary artery bypass grafts[237,238] and large trials are currently underway in pregnancy induced hypertension. Aspirin inhibits cyclooxygenase, preventing endoperoxide production.[239,240] This inhibition is irreversible as aspirin acetylates cyclooxygenase[241] and, platelets, being anucleate, are unable to regenerate cyclooxygenase activity. Recovery of prostaglandin production is therefore dependent upon production of new platelets from megakaryocytes.[242] It is noteworthy that as an inhibitor of cyclooxygenase aspirin also reduces endothelial prostacyclin synthesis. Advantage can be taken of the capacity of the

endothelium to regenerate cyclooxygenase activity, the relative sensitivities of the platelet and endothelial enzymes, and the extensive pre-systemic hydrolysis of aspirin to salicylate, to limit the effects of aspirin on prostacyclin synthesis.[243,244] Low doses of aspirin are relatively selective in inhibiting platelet thromboxane production whilst sparing prostacyclin production;[243] no dose of aspirin, however, is completely selective.[245]

Drugs which inhibit thromboxane synthetase are capable of suppressing thromboxane production selectively.[246] The main mechanism of action of thromboxane synthetase inhibitors, however, is thought to reside in the diversion of the metabolism of endoperoxides formed in platelets to give rise to PGD_2,PGE_2 and $PGF_{2\alpha}$ in much larger amounts than would normally be formed.[247,248] Inhibition of platelet behaviour is consequently achieved by stimulation of adenylate cyclase by PGD_2.[247,249] Furthermore, there is evidence that when thromboxane synthetase is blocked, PGG_2 and PGH_2 can be utilised by endothelium resulting in enhanced prostacyclin synthesis.[250] Despite such potentially favourable pharmacological effects, thromboxane synthetase inhibitors have given disappointing clinical results.[251] One explanation for this may lie in the fact that incubation of platelets in vitro with thromboxane synthetase inhibitors fails to result in inhibition of platelet aggregation in some individuals.[247-249,252] These individuals have been referred to as non responders and have been shown to have platelets which are more sensitive to the pro-aggregatory effects of endoperoxides and PGE_2.[253,254] Moreover, platelets from non responders produce less cyclic AMP in the presence of thromboxane synthetase inhibitors and in response to PGD_2.[249,253,254] Poor clinical results with thromboxane synthetase inhibitors may also result from their short duration of action.[251]

More recently, thromboxane/endoperoxide receptor blockers have been developed which also offer the potential advantage over aspirin of specifically targeting thromboxane mediated platelet responses, whilst sparing PGI_2 production.[251,255,256,257] These drugs have as yet to be proven superior to aspirin in clinical trials in thrombotic disease. There are good theoretical grounds for believing that the best method of manipulating the platelet arachidonic acid-thromboxane pathway would be the combined use of a thromboxane receptor blocker and a thromboxane synthetase inhibitor.[257] The receptor blocker would overcome the problem of remaining pro-aggregatory activity of endoperoxides with thromboxane synthetase inhibitors and the combination would offer the potential advantage of enhanced inhibitory prostanoid availability which would not be provided by the use of a thromboxane receptor blocker alone.[257,258]

All of the pharmacological methods of manipulating the platelet thromboxane pathway, however, have the drawback that pathways of platelet activation which are not thromboxane dependent are unaffected and are able to remain active to promote thrombus formation. The use of drugs which increase the level of cyclic AMP in platelets can overcome this problem, as cyclic AMP renders platelets more resistant to activation by all agonists. Cyclic AMP can be increased by the use of an adenylate cyclase stimulator or a phosphodiesterase inhibitor.[166,192] Prostacyclin itself has been used in some clinical conditions associated with platelet activation, and although efficacy was demonstrated in Raynaud's phenomenon,[259] peripheral arterial disease,[260] and cardiopulmonary bypass,[261] its use was limited by its instability, and a high incidence of adverse effects such as flushing and hypotension.[262] Furthermore, rebound platelet hyperactivity has been described both during infusions and when the drug was discontinued.[263,264] Stable prostacyclin analogues which can be administered either

orally or by infusion are currently being evaluated. Phosphodiesterase inhibitors inhibit platelet behaviour at lower levels of cyclic AMP than adenylate cyclase stimulators[44,211,212] and have the potential of synergising with endogenous adenylate cyclase stimulators such as prostacyclin.[166,192] The existence of phosphodiesterase subtypes which are to some extent tissue specific also makes this class of drugs an attractive option in antithrombotic therapy.[209,210] No phosphodiesterase inhibitors with specific effects on platelet cyclic AMP are available for clinical use to date. Dipyridamole, which has been used therapeutically as an antiplatelet agent for many years, has been shown to have activity as a phosphodiesterase inhibitor.[265] It is more likely, however, that its main antiplatelet effects reside in its ability to inhibit adenosine uptake into cells such as red cells and vascular tissue.[265] Adenosine therefore becomes more available to act on surface platelet receptors linked to stimulation of adenylate cyclase. Clinical trials, however, have shown that dipyridamole alone has little efficacy as an antithrombotic agent.[236,265]

Several new approaches to manipulating platelet behaviour, aimed at treatment of disease in which platelet activation plays a role, have been developed recently and are undergoing evaluation. Specific monoclonal antibodies directed at the fibrinogen binding site on glycoprotein IIb/IIIa have become available[266] which offer the possibility of blocking "the final common pathway" of platelet aggregation.[267] These agents appear to be promising but require more extensive clinical evaluation. Potential problems include allergic reactions and bleeding. Synthetic peptide analogues containing an 'RGD' (arginine-glycine-asparagine) sequence which is found in many adhesion proteins[268] have been synthesised. These are also capable of competing with fibrinogen for binding to platelets by attaching to the appropriate site on glycoprotein IIb/IIIa.[269] Ticlopidine is an

antiplatelet drug which inhibits platelet aggregation induced by a wide range of agonists, probably by impairing fibrinogen binding to platelets.[270] Although adverse effects such as neutropenia, diarrhoea and skin rashes may limit its use, it has recently been shown to compare favourably with aspirin in a large double blind randomised trial in ischaemic stroke.[271] Finally a thrombin inhibitor, recombinant hirudin, has become available which offers the possibility of combined antiplatelet and anticoagulant action.[272,273] Until these newer agents have undergone extensive clinical trials it is likely that aspirin will remain the cornerstone of antiplatelet therapy in clinical practice, and any novel antithrombotic treatment should be shown to compare favourably with aspirin before being adopted for therapeutic use.

6.PLATELET BEHAVIOUR IN NORMAL PREGNANCY.

6.1 General Aspects of Haemostasis During Pregnancy.

The physiological hypercoagulable state which occurs during normal pregnancy consists of changes in both platelets and coagulation factors. Documented changes in the coagulation system include an increase in factors VII, VIII, IX, X and a particularly marked increase in plasma fibrinogen.[1,4,5,274] Others have noted an increase in high molecular weight fibrin/fibrinogen complexes in plasma,[275] and some, but not all, investigators have reported a rise in levels of fibrinopeptide A,[276] indicating thrombin cleavage of fibrinogen. Deposits of fibrin are found in normal placentae, indicating local activation of the coagulation system during pregnancy.[3]

Complex changes in fibrinolysis also occur, including a reduction in plasma plasminogen activator activity,[277] and an increase in plasminogen activator inhibitor (PAI) activity, with the appearance of a novel PAI, named PAI II[277,278]. The overall

result is a reduction in fibrinolysis.[277] Despite this, FDP and D dimer have been shown to be elevated, presumably reflecting activation of coagulation, fibrin deposition, and subsequent catabolism.[276,277] Studies have given rise to varied findings regarding the naturally occurring inhibitors of coagulation in normal pregnancy, particularly with regard to antithrombin III. Some authors have reported a slight reduction in antithrombin III during normal pregnancy,[274] whilst others have reported no change.[279] Most authors agree, however that levels of Protein S fall during pregnancy.[280]

All of these changes favour a pro-coagulant state, and there is strong evidence that platelets also make an important contribution to the physiological hypercoagulability of pregnancy. There is evidence of platelet activation in vivo during healthy pregnancy and increased platelet reactivity in vitro has also been documented. The changes in platelet behaviour which have been described in normal pregnancy will now be reviewed in detail.

6.2 Evidence of Platelet Activation in Vivo in Pregnancy.

Evidence of platelet activation in vivo during pregnancy comes from studies of platelet indices in peripheral blood, measurement of platelet secretory products in plasma, and studies of prostaglandin metabolites in urine.

Platelet Indices in Peripheral Blood in Pregnancy

Since the widespread introduction of multi-parameter automated electronic counters in haematology laboratories there have been several studies investigating platelet count and indices of platelet size during pregnancy. Some studies have reported a reduction in platelet count in late pregnancy[281,282,283], whilst others have not confirmed this.[284,285,286,287] Criteria for patient selection have varied and it was not made clear by some authors whether all subjects with complications of pregnancy which may lead to a reduction in platelet count had been excluded. The

authors of one longitudinal study point out that there is a great deal of variation between individual patients in the direction of change in their platelet count during healthy pregnancy.[288] From all of the available evidence it seems reasonable to conclude that overall no substantial change in platelet count occurs in normal pregnancy.

Reports of changes in mean platelet volume (MPV) and platelet distribution width (PDW) during pregnancy are more consistent. Most report an increase in both of these indices, indicating the presence of a population of larger platelets in the circulation of pregnant women.[281,286,287] Increases in indices of platelet size occur in conditions in which there is peripheral platelet consumption or destruction,[289] but there is some debate as to whether this is simply because of an increase in the proportion of 'young' platelets circulating[290] or whether platelet size is determined by changes in the pattern of platelet production from megakaryocytes.[289] Irrespective of the mechanism, the increase in MPV and PDW associated with normal pregnancy supports the occurrence of peripheral platelet consumption, which could result from platelet activation in the circulation and the formation of platelet microthrombi. In normal pregnancy, platelet consumption is compensated by increased platelet production by megakaryocytes, resulting in no significant change in the peripheral blood platelet count.

Platelet Lifespan

In the light of the above evidence a reduction in platelet lifespan would be expected during pregnancy. As radiolabelled platelets cannot be used during pregnancy to study platelet lifespan, this has been investigated by following the recovery of platelet malondialdehyde production following the administration of a single dose of aspirin. Malondialdehyde is formed as a byproduct during thromboxane synthesis from cyclic endoperoxides.[35] When platelet

cyclooxygenase is irreversibly inhibited by aspirin, no endoperoxides are available for malondialdehyde synthesis until a new population of platelets enter the circulation. The recovery of platelet malondialdehyde synthesis is therefore a reflection of platelet lifespan. Such studies of platelet lifespan have, however, revealed only small non significant reductions during pregnancy.[291,292] It is possible that the administration of even a single dose of aspirin, by inhibiting platelet activation, prolongs platelet lifespan and therefore masks any pregnancy associated reduction which may be evident if it were possible to make measurements by another method.

Beta-thromboglobulin and Platelet Factor 4

A number of studies have shown higher plasma levels of the platelet alpha granule protein β-thromboglobulin (β-TG) in third trimester pregnant women compared with non pregnant controls.[292,293,294,295] This supports the occurrence of platelet activation in vivo during the later stages of pregnancy. Interestingly, Douglas et al showed, in a study measuring platelet release products and fibrinopeptide A, that the increased levels of β-TG in pregnancy were not associated with increased thrombin generation.[293] This implies that the platelet activation which occurs in vivo in pregnancy is not thrombin dependent. β-TG is cleared from plasma by the kidney, therefore alterations in plasma levels can occur as a result of changes in renal function.[296] Reduced clearance, however, could not explain the findings in pregnancy as the glomerular filtration rate increases in pregnant women.[297] Furthermore, a study measuring both β-thromboglobulin and platelet factor 4, an alpha granule protein whose rapid clearance from plasma is by binding to endothelium rather than by renal excretion,[296] showed that the level of both proteins increased in parallel in plasma in third trimester healthy pregnant subjects.[294]

6.3 Prostaglandin Biosynthesis and Platelet Activation in Vivo During Pregnancy.

Reproductive tissues synthesise a variety of prostaglandins and this increases during pregnancy.[298,299] The placenta is a major site for prostaglandin synthesis during pregnancy. In vitro, placental tissues are capable of generating PGE_2, PGD_2, $PGF_{2\alpha}$, and probably thromboxane A_2,[298,299,300] but prostacyclin is the major placental eicosanoid product.[298] Many studies have shown that prostaglandin biosynthesis in vivo is increased in pregnant compared with non pregnant women. Historically, increased PGE_2 and $PGF_{2\alpha}$ formation were first to be described.[298] The exact physiological role of these prostanoids in pregnancy is not known, but it has been reported that PGE_2, whilst itself capable of constricting human umbilical vessels,[301] blunts the vasoconstrictor effects of angiotensin II on isolated umbilical arteries.[302] It is also thought that PGE_2 and $PGF_{2\alpha}$ play a role in induction of labour.[298,303,304] As described in the previous section, PGE_2 can either activate or inhibit platelets[87,88,89,90] and the relationship of the changes in PGE_2 biosynthesis to platelet activation in vivo during pregnancy has not been studied. Prostacyclin and thromboxane biosynthesis have subsequently also been shown to increase during healthy pregnancy.[298] Plasma levels of derivatives of these prostaglandins have been shown by some[298,305,306,307] but not all[300,308] studies to be elevated. More significantly, studies measuring urinary metabolites of thromboxane and prostacyclin, which give a more reliable indication of in vivo synthesis, have confirmed increased production of both PGI_2 and TXA_2 during pregnancy.[160,309,310] Excretion of the prostacyclin metabolite 2,3-dinor-6-keto-$PGF_{1\alpha}$ increases 6-10 fold in normal pregnancy.[298,310] Increased prostacyclin synthesis is present during the first trimester and persists throughout gestation.[160] There is

some evidence that the placenta is the main source of this increased prostacyclin production.[298]

Urinary excretion of the thromboxane metabolites 2,3-dinor-thromboxane B_2 and 11-dehydro-thromboxane B_2 rise markedly earlier than 20 weeks gestation in healthy pregnancy and remain elevated throughout the gestational period.[309] There is good evidence that platelets are the source of increased thromboxane biosynthesis in pregnancy. Following treatment of pregnant subjects with doses of aspirin sufficient to cause 97% inhibition of platelet thromboxane production ex vivo, whilst preserving cyclooxygenase dependent prostaglandin synthesis by other tissues, Fitzgerald showed that urinary 2,3,dinor-thromboxane B_2 was almost completely suppressed and its recovery parallelled the recovery of platelet thromboxane B_2 production.[309]

The physiological significance of alterations in thromboxane and prostacyclin biosynthesis during pregnancy relate to control of both vascular reactivity, particularly in the placental circulation, and platelet reactivity. Prostacyclin seems likely to have an important role in lowering resistance in the placental vascular bed.[311] Furthermore, increased prostacyclin production may be the mechanism which leads to a reduced systemic pressor response to infused angiotensin II which is observed during healthy pregnancy.[312,313] There is evidence of the presence of thromboxane receptors in the placental vasculature[314] and endoperoxide analogues have been shown to constrict human umbilical arteries[315] and increase resistance in isolated perfused human cotyledon.[316]

In relation to platelets, the increase in urinary thromboxane metabolites in pregnancy is of significance firstly as a further marker of in vivo platelet activation during pregnancy, and secondly because increased biosynthesis of thromboxane may itself promote platelet activation. It cannot be determined from these in vivo

studies whether there are primary alterations in the platelet biosynthetic pathway for thromboxane which give rise to the increase in urinary thromboxane metabolites during pregnancy.

The changes in prostaglandin biosynthesis which have been described raise the question of why increased platelet activation in vivo occurs during normal pregnancy in the presence of increased prostacyclin production. Even if the increase in prostacyclin formation is insufficient to exert a systemic antiplatelet effect, this is unlikely to explain the apparent lack of efficacy on in vivo platelet reactivity during pregnancy as the placenta has been proposed as an important site of platelet activation and intravascular coagulation in pregnant women[3,5] and is also the main source of prostacyclin formation. Intrinsic alterations in platelet reactivity during pregnancy, including altered responses to the prostaglandins which regulate their behaviour could theoretically provide an explanation for the observations.

6.4 Studies of Platelet Behaviour In Vitro.

In vitro studies of platelet behaviour during pregnancy support the hypothesis that platelets from pregnant women are intrinsically more active compared with those from non pregnant females. The literature on platelet behaviour in vitro during pregnancy includes studies of a wide variety of aspects of platelet behaviour and platelet second messengers. Each will be reviewed in turn.

Studies on Platelet Aggregation in Platelet Rich Plasma

There are many published studies on platelet aggregation in platelet rich plasma (PRP) during pregnancy. These have given rise to varied results, probably because of differences in experimental technique, the choice of parameter analyzed, the type and concentration of agonists employed and the patients

selected. One early study reported no change in platelet aggregation patterns in PRP[317] and two studies have reported reduced platelet aggregation responses associated with pregnancy.[318,319] Studies investigating minimum threshold concentrations of agonists required to induce a full aggregation response have, however, generally shown increased platelet reactivity during pregnancy. A number of investigators have shown that the minimum concentration of ADP required to induce irreversible aggregation in PRP is reduced in the third trimester of pregnancy.[295,320,305] One early study also showed that secondary aggregation was observed more frequently in response to low concentrations of adrenaline in PRP from pregnant subjects compared with non pregnant controls.[320]

Two published studies provide evidence of increased platelet reactivity in PRP in response to arachidonic acid (AA) in healthy pregnant women. The first was a cross-sectional study, which compared healthy mothers at delivery with their neonates and with non pregnant female controls[321] and the second was a longitudinal study which showed that platelets were more sensitive to AA from 16 weeks gestation in normal pregnancy.[322] Platelet behaviour returned to a pattern similar to that of non pregnant controls by the sixth postnatal week.

The influence of factors such as changes in haematocrit and plasma albumin must be taken into account when interpreting platelet aggregation responses during pregnancy. One study showed that when the citrate concentration in PRP was adjusted for haematocrit, enhanced responsiveness of platelets to adrenaline, which had been demonstrated in standard citrated PRP from pregnant subjects, was no longer evident.[323] Other studies have shown no correlation between haematocrit and platelet aggregation parameters.[322] As AA binds to albumin, reductions in plasma albumin during pregnancy[324] could result in an increase in free AA being available for platelet activation. Studies showing

changes in AA induced platelet aggregation responses have, however, shown no correlation with plasma albumin levels.[321,322]

Platelet Aggregation In Whole Blood

All of the studies described so far have investigated platelet aggregation in PRP. Some investigators have extended the observations on platelet behaviour during pregnancy to the investigation of platelet responses in whole blood. One of the first published studies of platelet aggregation in whole blood investigated spontaneous aggregation and the effect of different anticoagulants in vitro.[325] The study was longitudinal in design and used a platelet counting technique.Spontaneous platelet aggregation (SPA) in whole blood anticoagulated with heparin was increased from 16 weeks gestation, and increased further as pregnancy progressed. The increase in SPA, although still significant, was less marked in citrated blood. Heparin did not have the effect of increasing SPA in blood from non pregnant controls. In addition to providing information on platelet reactivity during pregnancy, this study also defined the importance of effects of different anticoagulants and concluded that heparin would not be a suitable anticoagulant for studies of platelet behaviour during pregnancy. The implications of these observations for the use of heparin therapeutically in the treatment of thromboembolic disease during pregnancy are not clear, but this would be worthy of further investigation.

Agonist induced platelet aggregation in whole blood has been measured during pregnancy by the electrical impedance method, showing increased platelet aggregation in response to ADP, collagen and AA.[326] A longitudinal study in normal pregnancy of platelet reactivity in whole blood, employing the single platelet counting technique, has been published by Louden et al.[327] Increased platelet aggregation in response to 0.2mM arachidonic acid was noted from 16

weeks gestation onwards, and platelets were more responsive to 1uM adrenaline from 28 weeks gestation. Six weeks following delivery, there was no statistically significant difference in platelet behaviour compared with the preconceptional parameters, but the extent of platelet aggregation in response to AA and adrenaline had not fully returned to baseline values.

Platelet Release Reaction

There are less data available on the platelet release reaction in vitro during pregnancy than on platelet aggregation. Nevertheless, some studies have investigated dense granule release and the results generally reflect the increased platelet reactivity observed in platelet aggregation experiments. In one study, platelets from pregnant women underwent a release reaction in response to lower concentrations of AA than those from non pregnant controls, but the extent of the release reaction did not differ between groups of subjects.[322] Studies conducted in whole blood, however,showed increased ^{14}C-5HT release in response to adrenaline from 28 weeks gestation onwards, reflecting the increased platelet aggregation observed with this agonist.[327]

General Patterns of Platelet Reactivity During Pregnancy as Assessed by Platelet Aggregation and Release Experiments

Evidence has been presented from studies conducted in PRP and in whole blood of increased platelet reactivity during pregnancy. Longitudinal studies suggest that at least some of the changes in platelet reactivity are detectable early in the second trimester and persist throughout pregnancy, reverting to the non pregnant pattern between 6 and 12 weeks postnatally.

In general, pregnancy related alterations in platelet behaviour are most evident when agonists are chosen which have a thromboxane dependent component in their mechanism of action, for example arachidonic acid,[321,322,327] adrenaline,[320,327]

and the secondary phase of aggregation in response to ADP.[295,305,320] Furthermore in studies conducted in whole blood, cyclooxygenase inhibition by the administration of low dose aspirin to healthy pregnant women inhibited platelet behaviour ex vivo in response to arachidonic acid and adrenaline at times during the gestational period when increased platelet reactivity to these agents was demonstrable in a group of healthy pregnant women who were randomised to receive placebo.[328] This provides further evidence that thromboxane A_2 has a role in the increased platelet reactivity observed during pregnancy. It cannot be determined from simple studies of platelet aggregation and release whether this is due to increased synthesis of thromboxane by platelets or increased sensitivity of platelets to thromboxane. The data already discussed relating to thromboxane production in vivo[309] suggest that in this situation platelet thromboxane production is increased, but this does not provide information about the intrinsic reactivity of the platelet thromboxane pathway, and studies of platelet thromboxane production in vitro have yielded conflicting results.

Platelet Thromboxane Production.

Published studies have measured serum levels of thromboxane B_2 (TXB_2), the stable hydrolysis product of thromboxane A_2. This reflects platelet thromboxane production under conditions of spontaneous clotting of whole blood in vitro. A cross sectional study comparing pregnant women at various gestations with non pregnant and puerperal women showed significantly higher serum TXB_2 in pregnant women, regardless of gestation, and in puerperal women than in controls.[329] A longitudinal study from the same centre did not confirm increased serum TXB_2 during pregnancy,[292] but, as subjects were not studied prior to conception, this may have been missed if changes in platelet thromboxane production occur early in the gestational period and take some time to return to

pre-pregnancy levels in the puerperium. A subsequent longitudinal study which included preconceptional women, however, has also failed to confirm changes in serum TXB_2 during healthy pregnancy.[327] The question of the contribution of the absolute amount of thromboxane synthesis by platelets to increased platelet reactivity during pregnancy remains unresolved and the possibility of altered platelet sensitivity to thromboxane should not be discounted.

Platelet Sensitivity To Agents Which Inhibit Their Behaviour

There have been some reports of a reduction in platelet sensitivity to inhibition of aggregation by prostacyclin and its stable analogues in vitro during pregnancy.[330,331,332]

Outwith the context of pregnancy, it has been shown that incubation of PRP in vitro with thromboxane synthetase inhibitors (TSIs) leads to inhibition of platelet behaviour in some individuals ('responders') but not in others ('non-responders').[249,251-254] One study has shown that the majority of healthy pregnant women are non responders to thromboxane synthetase inhibitors, whilst most non pregnant women in the child bearing age group are responders.[322] This is of interest since inhibition of platelet behaviour by thromboxane synthetase inhibitors is mediated by PGD_2 produced from cyclic endoperoxides by a diversion pathway operating when thromboxane synthetase is blocked.[247,248] Although acting on distinct surface receptors on the platelet membrane, prostacyclin and PGD_2 both exert their inhibitory effects on platelets via an increase in cyclic AMP. These data raise the possibility of a defect in cyclic AMP dependent inhibitory pathways in platelets during pregnancy.

6.5 Platelet Second Messenger Systems In Pregnancy.

In recent years investigators have studied platelet second messenger systems during pregnancy in an attempt to elucidate the mechanisms underlying

the observed changes in platelet behaviour. Attention has been mainly directed at intracellular free calcium $[Ca^{2+}]_i$. The first study investigating platelet $[Ca^{2+}]_i$ during pregnancy showed no change in either basal $[Ca^{2+}]_i$ or in transient increases in $[Ca^{2+}]_i$ upon platelet stimulation with ADP or 5-HT, when third trimester healthy pregnant women were compared with non pregnant women.[333] A subsequent publication reported a "slight increase" in basal $[Ca^{2+}]_i$ in platelets from pregnant women, with no difference in stimulated platelet $[Ca^{2+}]_i$ in response to thrombin and angiotensin II when compared with platelets from non pregnant women.[334] Both of these studies, employed the calcium sensitive indicator quin-2, which is known to significantly quench increases in platelet $[Ca^{2+}]_i$ resulting from platelet stimulation.[109,110,335] Furthermore, the method used to prepare washed platelets in the first study may have led to significant loss of the most active platelets.

The calcium sensitive fluorophore fura-2 has the advantage of weaker calcium chelating properties than quin-2.[335] A more recent study using fura-2 to measure intracellular free calcium has shown a significant increase in platelet basal $[Ca^{2+}]_i$ in the third trimester of pregnancy in comparison with data from non pregnant female volunteers.[336] This has subsequently been borne out by a prospective longitudinal study by the same authors which confirmed a significant increase in basal $[Ca^{2+}]_i$ in platelets by the 28th week of pregnancy, which returned to baseline by 6 weeks postpartum.[337] These data are extremely interesting and may throw some light on the mechanism of increased platelet reactivity observed during pregnancy. One could speculate, for example, that platelets which have elevated basal intracellular free calcium require a lesser rise of $[Ca^{2+}]_i$ upon stimulation by an agonist in order to reach a certain threshold required for a response such as aggregation or release. It is also noteworthy in this respect that phospholipase A_2, the enzyme involved in mobilising arachidonic acid from the

platelet membrane for thromboxane A_2 synthesis, is highly calcium dependent.[74,117] Some questions regarding the role of intracellular free calcium in platelet activation during pregnancy, however, remain unresolved. The significance of basal $[Ca^{2+}]_i$ in determining patterns of platelet behaviour is largely ill defined; studies outwith the context of pregnancy have shown poor correlations between basal $[Ca^{2+}]_i$ and platelet reactivity.[338] It is also quite possible that the increased platelet basal $[Ca^{2+}]_i$ in vitro reflects a degree of platelet activation in vivo, and is not a direct mediator of the increased platelet reactivity observed during pregnancy. Nevertheless, the study of platelet intracellular free calcium may make a significant contribution to knowledge in the area of platelet behaviour in pregnancy, and further studies on the role of $[Ca^{2+}]i$, particularly if including parallel measurements of platelet behaviour and examining stimulated $[Ca^{2+}]_i$ would be useful.

There have been few studies of the inhibitory second messengers in platelets during pregnancy. Two studies have investigated platelet cyclic AMP levels in pregnancy. Goser et al in 1980 showed no change in basal platelet cyclic AMP over the gestational period, except in association with the use of beta agonists to suppress labour.[339] Long-term use of such agents resulted in a reduction in basal platelet cyclic AMP levels. Stimulated cyclic AMP levels were not measured in this study, and a radioimmunoassay was employed, which is less sensitive than some other established methods of measuring platelet cyclic AMP. A later investigation of platelet cyclic AMP during pregnancy, which employed a more sensitive assay and examined basal and PGE_1 stimulated platelet cyclic AMP levels, also demonstrated no change in platelets from pregnant women compared with those from non pregnant controls.[323] This study, however, included only 5 pregnant subjects all of whom were near term.

7.THE ROLE OF PLATELETS IN PRE-ECLAMPSIA

7.1 Classification of Hypertension in Pregnancy and Definition of Pre-eclampsia.

Pre-eclampsia is a complication of pregnancy characterised by hypertension, proteinuria, and oedema. The syndrome occurs most frequently in primigravidae, and is associated with significant maternal and fetal morbidity and mortality. Before reviewing the literature on platelet behaviour in pre-eclampsia, it is appropriate to consider the definition of pre-eclampsia and pregnancy induced hypertension in relation to the classification of the hypertensive disorders of pregnancy and also to describe the clinical, epidemiological, and pathophysiological features of pre-eclampsia.

Research and clinical practice in the hypertensive disorders of pregnancy has been complicated by the use of different classifications and diagnostic criteria by different clinicians and investigators. In 1986 Davey and M^{c}Gillivray proposed the following classification,[340] which has been adopted by the International Society for the Study of Hypertension in Pregnancy:

Chronic Hypertension: Hypertension occurring in pregnancy in a woman with pre-existing hypertension. This situation may arise in essential hypertension, in chronic renal disease or in other less common forms of secondary hypertension.

Pregnancy Induced Hypertension: Hypertension developing during pregnancy and resolving following delivery in a woman previously known to be normotensive.

Pre-eclampsia: The combination of pregnancy induced hypertension and significant proteinuria

Eclampsia: Generalized tonic/clonic seizures usually but not necessarily occurring in a woman with previous pre-eclampsia in the absence of any other neurological explanation for the seizures.

As this thesis is concerned with the role of platelets in pregnancy induced hypertension (PIH) and pre-eclampsia, rather than chronic hypertension all subsequent discussion refers to those hypertensive disorders developing during pregnancy.

The definition of the hypertensive disorders specific to pregnancy follows on from the above classification. Again, varying criteria for the definition of raised blood pressure and significant proteinuria have often been used. The definition of pregnancy induced hypertension recommended by the American College of Obstetricians and Gynaecologists will be used for the purposes of this thesis.[341] PIH is defined as being present when a systolic blood pressure of greater than 140 mmHg or a diastolic blood pressure of greater than 90 mmHg, using Korotkov phase IV, is recorded after the 20th week of pregnancy, or if there is a rise of greater than 30 mmHg in systolic or greater than 15mmHg in diastolic pressure, compared with the blood pressure recordings in the first trimester of pregnancy. Blood pressure recordings should be confirmed by two readings at least 6 hours apart. The advantage of this definition is that it allows a rise in pressure to be taken into account rather than solely relying on an arbitrary blood pressure level to be reached.

A widely accepted definition of pre-eclampsia is the fulfilment of the above blood pressure criteria together with the excretion of > 0.3g of protein in the urine in a 24 hour period.[340] As otherwise healthy pregnant women may occasionally demonstrate proteinuria in excess of 0.3g per 24 hours, others have considered the use of 0.5g proteinuria in 24 hours as a more suitable threshold for

the definition of significant proteinuria and therefore for the definition of pre-eclampsia.[342,343] The latter has been used for the purposes of this thesis. Pre-eclampsia may follow on from PIH, but patients may also present de novo with proteinuric hypertension.

Although these definitions are somewhat arbitrary it has been shown that there is an increased risk to the fetus when the maternal diastolic pressure rises above 90mmHg[344] and to both mother and fetus when significant proteinuria develops.[345,346]

7.2 Clinical Features of PIH and Pre-eclampsia.

Pregnancy induced hypertension uncomplicated by proteinuria is usually asymptomatic, and the raised blood pressure is often the only clinical abnormality. The main significance of pregnancy induced hypertension is as a precursor of pre-eclampsia.[347] Furthermore, there is an increased incidence of placental abruption in PIH.[346,348,349]

Pre-eclampsia may also be asymptomatic, but is often accompanied by clinical features other than hypertension and proteinuria. Oedema is classically listed as one of the clinical signs of pre-eclampsia, but simple dependent oedema is so common in healthy pregnancy that this sign is of no discriminatory value.[350] Facial oedema, which usually does not occur in normal pregnancy, however, may occur in pre-eclampsia.[350] A raised serum urate is a common feature of pre-eclampsia, and occurs as a result of reduced renal clearance.[351] A raised urate is useful both diagnostically and prognostically, being associated with a greater perinatal mortality for any given level of blood pressure.[352] A modest fall in platelet count is an early feature of pre-eclampsia,[353] but the development of clinically significant thrombocytopenia indicates a severe form of the condition. In such cases activation of the coagulation cascade and disseminated intravascular

coagulation may occur.[354] In severe pre-eclampsia, hepatic congestion giving rise to epigastric pain may occur. This is usually associated with raised hepatic transaminases, and is a sign of impending eclampsia.[350] A variant of pre-eclampsia, in which microangiopathic haemolysis, elevated liver enzymes, and low platelets are the predominant features has been described and referred to as the HELLP syndrome.[355] This syndrome carries a particularly bad prognosis for mother and fetus. Other less common clinical features which may occur in severe pre-eclampsia are uraemia,[348] pulmonary oedema,[356] cerebral haemorrhage[348,357,358] and even transient blindness.[359] The ultimate maternal complication of pre-eclampsia is the occurrence of eclamptic seizures, thought to arise as a result of cerebral ischaemia and cerebral oedema.[358] Vasospasm may make a contribution to the cerebral ischaemia of eclampsia.[358,360]

7.3 Management and Prognosis of Pre-eclampsia.

By definition, the clinical features of pre-eclampsia and eclampsia resolve after delivery of the fetus, and delivery therefore remains the most effective treatment of pre-eclampsia.[348] The timing of delivery must be optimised to minimise risks to both mother and fetus. Clearly in some cases delivery must be undertaken in the maternal interest when the fetus is still immature and therefore at risk of all the complications of prematurity in the neonatal period. Unfortunately as the primary cause of pre-eclampsia remains unknown little effective medical treatment is available and the mainstay of medical management is supportive. Such supportive measures include the administration of drugs to lower blood pressure, for example hydralazine,[348,361] the prevention of seizures using either phenytoin[343,348] or magnesium sulphate,[362] optimal intravenous hydration,[363,364] and the correction of coagulation abnormalities using fresh frozen plasma and

occasionally cryoprecipitate and platelet transfusions in the presence of bleeding due to disseminated intravascular coagulation or thrombocytopenia.[365]

In uncomplicated PIH and in mild pre-eclampsia, a double blind placebo controlled trial conducted by Rubin et al showed that the administration of the beta blocker atenolol lowered maternal blood pressure, reduced hospital admissions, reduced the development of proteinuria in those with uncomplicated PIH, and was not associated with any adverse effects on the fetus.[342] There was also a reduction in the incidence of neonatal respiratory distress syndrome, but larger trials would be required to demonstrate any difference in perinatal mortality in atenolol versus placebo treated pregnancies. Agents such as atenolol are particularly useful in cases of PIH and mild pre-eclampsia when the fetus is relatively immature and risks to the neonate may be improved by prolonging the pregnancy.

Small clinical trials have suggested a possible beneficial effect of aspirin in the prophylaxis of pre-eclampsia in those at risk,[366,367,368,369] and larger multicentre studies are currently in progress to attempt to determine definitively whether aspirin is useful in the prevention or treatment of pre-eclampsia.

PIH has been reported to be associated with increased fetal death when the maternal diastolic blood pressure is above 90mmHg,[344] but other large studies have disagreed, suggesting that perinatal mortality is unaffected by non proteinuric PIH.[370] Maternal prognosis is not altered by uncomplicated PIH. Predictably, pre-eclampsia carries a higher perinatal mortality than PIH[345,346,370] and a worse prognosis for the mother. Pre-eclampsia and eclampsia are still amongst the most common causes of maternal death. In the U.K., pre-eclampsia and eclampsia accounted for 12.1 deaths/million pregnancies in the period from 1985-1987.[371] The perinatal death rate was reported as 33.7/1000 in pregnancies complicated

by pre-eclampsia and eclampsia in the British Births Survey of 1970.[370] Intrauterine fetal growth retardation and the need for premature delivery in the maternal interest contribute both to fetal mortality and morbidity.[345,372]

7.4 Epidemiology of Pre-eclampsia.

Early studies by MacGillivray in Aberdeen suggested that pre-eclampsia was more common in primigravidae.[347,373] In this series, 24% of nulliparae developed PIH and 5.5% developed proteinuric pre-eclampsia, compared with figures of 10-12% and 1% respectively for multiparae. This was confirmed in the DHSS Report on Inpatient Inquiry for England and Wales (1973-1976).[374] Furthermore, the incidence of renal biopsy lesions characteristic of pre-eclampsia, has been shown to be greater in primigravidae with hypertension and proteinuria than in multiparae.[375] For this reason it has been considered that studies relating to pre-eclampsia which include primigravid women only are less likely to have inadvertently selected women with other pathology. Parous women with new partners, however, may have an increased risk of pre-eclampsia.[376,377] There is also an increased incidence of pre-eclampsia in subsequent pregnancies in women who have developed pre-eclampsia or eclampsia in their first pregnancy.[373]

The incidence of pre-eclampsia increases with maternal age and there is a particular increase in maternal mortality from pre-eclampsia in women over the age of 35.[376] Interestingly, women less than 20 also show an increased incidence of pre-eclampsia. There may be a relationship in this age group with lack of antenatal care and concealed pregnancies.[376]

There is now considerable evidence that pre-eclampsia is under genetic control, and it has been postulated that it may be an autosomal recessive condition with variable penetrance.[378,379]

There has been some controversy as to whether race influences the incidence of pre-eclampsia. Carefully conducted studies in the USA have shown that the incidence of pre-eclampsia is similar in caucasian and in black Americans.[376] There is some evidence, however, that pre-eclampsia is more common in Indian women than in women from other racial groups.[380]

Other maternal factors which have been associated with pre-eclampsia include underlying essential hypertension and diabetes.[343,376] In both conditions it is difficult to separate out cases of hypertension complicated by renal disease from true pre-eclampsia.

Some fetal factors have been associated with an increased risk of pre-eclampsia. These include conditions associated with hyperplacentosis such as twin pregnancy, fetal hydrops, and fetal triploidy.[343,376,381] The presence of a fetus is not a pre-requisite for the occurrence of pre-eclampsia, as the condition has an association with hydatidiform mole.[381] Molar pregnancy should be suspected in any patient presenting with clinical signs of pre-eclampsia before 24 weeks gestation.[350]

7.5 Pathophysiological Mechanisms in Pre-eclampsia.

Despite much research having been directed at the subject, the primary cause of pre-eclampsia remains unknown. A substantial amount of knowledge of the secondary pathophysiological mechanisms involved in the condition, however, has been accumulated over the years, and changes in the cardiovascular system, kidneys, and haemostatic and endocrine systems have been well documented. The "gold standard" for the pathological diagnosis of pre-eclampsia is the presence of a characteristic lesion on renal biopsy referred to as glomerular endotheliosis.[382,383] This lesion consists of swelling of the glomerular capillary endothelial cells, an increase in size and number of intercapillary cells and the

deposition of fibrinoid material within the cells and beneath the basement membrane. Immunofluorescent techniques have demonstrated that this fibrinoid material contains fibrinogen or its derivatives.[384] Proteinuria and hyperuricaemia are related to these histological changes.[343,352,385] Renal plasma flow and glomerular filtration rate are both reduced in pre-eclampsia.[343,383] Although the renal lesion described is characteristic of pre-eclampsia, it is almost certainly a secondary phenomenon and there is no evidence that pre-eclampsia is primarily a renal disease.

Vasoconstriction and a reduction in intravascular volume contribute to the reduced perfusion of the kidneys and other vascular beds supplying major organs in pre-eclampsia, but it is uncertain whether the contracted plasma volume stimulates compensatory vasoconstriction or whether widespread vasoconstriction shuts off plasma volume expansion.[386] It has been shown, however, that the contracted plasma volume does not, as expected, lead to stimulation of the renin-angiotensin system,[313,343] as measured by plasma renin activity and plasma angiotensin II concentrations. Both of these hormones have been shown to be present in reduced concentrations in plasma in proteinuric PIH compared with healthy pregnancy.[313,343] The changes in the renin-angiotensin system in pre-eclampsia appear complex as there is some evidence of a role for renin synthesised locally in the uteroplacental circulation in pre-eclampsia.[313] Furthermore, it is established that women destined to develop pre-eclampsia show an enhanced pressor response to infused angiotensin II as early as 22 weeks gestation.[387,388] Altered vascular responsiveness to angiotensin II could therefore contribute to widespread vasoconstriction and to raised blood pressure.

The combination of reduced intravascular volume and arteriolar vasoconstriction in pre-eclampsia is reflected in haemodynamic parameters

measured using a Swan-Ganz catheter which have shown a reduced pre-load, reduced cardiac output and elevated left ventricular afterload.[363] Raised blood pressure is consequent upon arteriolar vasoconstriction.[363]

Reduced uteroplacental blood flow plays a central role in the fetal complications of pre-eclampsia. Placental ischaemia and infarction have been well documented but are non specific findings.[349] Furthermore in the early stages of placentation there is failure of invasion by the trophoblast of the myometrial segments of the spiral arteries.[389,390] The latter therefore retain their thick muscular wall and fail to convert to thin walled high flow/low resistance uteroplacental vessels. This failure of the normal physiological processes involved in placentation is thought to play a key role in reducing maternal blood flow to the placenta.[349]

Reduced perfusion of renal, uteroplacental and other vascular beds in pre-eclampsia is closely tied in with changes in the haemostatic system and with the mechanisms that control primary haemostasis. It has been documented from as far back as the late 19th century that eclampsia is associated with widespread thrombosis in the microcirculation.[391] In 1953 M^{c}Kay described the presence of fibrin deposits in the vasculature of the placenta and the kidney in pre-eclampsia.[3] "Acute atherosis" of the arterial vessels supplying the placenta has been subsequently described in cases of pre-eclampsia and intrauterine growth retardation.[392] This lesion consists of fibrinoid necrosis and accumulation of fat laden macrophages in the vessel wall often associated with intraluminal deposition of fibrin and platelets. In severe cases of pre-eclampsia consumption of coagulation factors and platelets may occur to such a degree that clinically overt disseminated intravascular coagulation may occur.[354,393] Much research has therefore been directed towards delineating changes in the coagulation factors and in platelets in pre-eclampsia. The principle changes which occur in coagulation

factors and their inhibitors consist of a reduction in the ratio of Factor VIII:C to von Willebrand Factor antigen,[394] an increase in fibrin degradation products[395] and in fibrinopeptide A levels,[293] and a reduction in antithrombin III levels.[2,396] There also appears to be a net reduction in fibrinolysis.[2] Most of these changes are consistent with increased fibrin generation with consequent consumption of coagulation factors. The reduction in antithrombin III is also likely to reflect consumption. The primary stimulus resulting in the activation of the coagulation system has not been revealed by such studies.

In recent years the study of the haemostatic system in pre-eclampsia has focused on platelets and the mechanisms regulating primary haemostasis. There is good evidence of platelet consumption early in the course of the disease, often before clinical manifestations occur.[353] This has led to the suggestion that platelets may play a key role in the pathophysiology of the disease. The publication of some small studies showing that the administration of aspirin as an antiplatelet agent may be useful in the prophylaxis of pre-eclampsia in those at risk has lent further support to this hypothesis.[366-369] Finally, a further attraction of a hypothesis involving a central role for platelets is that changes in haemostasis could be tied in with some of the changes in vascular reactivity which have been described. Activated platelets produce substances which are powerful vasoconstrictors such as thromboxane A_2 and 5-hydroxytryptamine. 5HT has been shown to synergise with angiotensin II in terms of vasoconstrictor effects.[2] Studies of prostaglandin urinary metabolites in pre-eclampsia have revealed increased thromboxane production in vivo[158,159] together with reduced prostacyclin synthesis.[160,298,310] Isolated placental and umbilical blood vessels from pregnancies complicated by pre-eclampsia have also been shown to produce reduced amounts of prostacyclin.[397,398] Studies have also shown that placental tissues from patients

with pre-eclampsia produce reduced vasodilatory PGE_2 and increased vasoconstrictory $PGF_{2\alpha}$.[399] Taken together, these changes in prostaglandin synthesis would favour both platelet activation and vasoconstriction. The alterations in prostaglandins in themselves are unlikely to account for the widespread systemic vasoconstriction which results in raised blood pressure in pre-eclampsia, as circulating levels are too low to exert a generalised systemic effect,[151,152,298] but they probably exert an important influence on vascular control at a local level particularly in the placental circulation,[298,301,311,314,316] thus contributing to reduced placental blood flow. Furthermore, relative deficiency of prostacyclin may be responsible for the observed enhancement of vascular sensitivity to angiotensin II in pre-eclampsia, as it has been shown that the pressor effects of angiotensin II can be modulated by vasodilator prostaglandins.[312,313] Studies relating to platelet behaviour in pre-eclampsia will now be reviewed in detail.

7.6 Evidence of Platelet Activation in Vivo in PIH and Pre-eclampsia.

Platelet Count and Indices of Platelet Size.

Most authors agree that the platelet count falls in peripheral blood in pre-eclampsia.[286,353,400-404] Some have also noted a reduction in mean platelet count in uncomplicated PIH[400,401] in comparison with normal pregnancy, but others disagree.[286] Redman et al[353] showed that in a group of women at risk of pre-eclampsia because of chronic hypertension, platelet count fell early in the evolution of the disease in women who developed "pre-eclampsia" as determined by a sustained rise in the serum urate level. Only 37% of the "pre-eclamptic" patients had proteinuria, but raised serum urate has been shown to correlate with the appearance of lesions typical of pre-eclampsia on renal biopsy.[343,352,385]

Despite the well documented reductions in platelet count in both pre-eclampsia and PIH, only 15% of those with pre-eclampsia and 6% of those with PIH develop thrombocytopenia as defined by a peripheral blood platelet count of less than 150 x 10^9/l.[400]

Indices of platelet size in PIH and pre-eclampsia show an increase in MPV beyond that occurring in normal pregnancy.[286,400,401,403] Studies vary in their conclusions regarding PDW, some reporting an increase in PIH beyond that occurring in normal pregnancy,[403] and others suggesting no difference in PDW in PIH compared with healthy pregnancy.[286] On the whole, these indices suggest a megakaryocyte response to peripheral platelet consumption.

Platelet Lifespan

Platelet lifespan, determined by the non radioisotopic technique as described for the studies in normal pregnancy, has been shown to be significantly reduced in pre-eclampsia,[291] although one longitudinal study disagreed[292] and another investigating women with PIH, only showed a reduction in platelet lifespan in those who also demonstrated poor fetal growth.[405]

Plasma Levels of Platelet Alpha Granule Proteins

Plasma β-thromboglobulin levels increase significantly in PIH and to a greater degree in pre-eclampsia, compared with healthy pregnant controls.[293,294,406-408] This implies that platelet alpha granule release, already occurring in vivo in normal pregnancy is further enhanced in PIH and pre-eclampsia. One study showed that, in contrast with normal pregnancy, this was accompanied by an increase in fibrinopeptide A in both PIH and pre-eclampsia, but there was no correlation between plasma levels of β-TG and FPA.[293] This suggests that although significant thrombin generation occurs in vivo in hypertensive pregnancy, mechanisms other than thrombin mediated platelet stimulation are at least in part

responsible for platelet activation in vivo in this condition. Changes in plasma platelet factor 4 appear to be less marked than for β-TG, one study showing an increase which just failed to reach significance in comparison with normal pregnancy,[294] and another showing no difference between healthy and hypertensive groups.[408] As β-TG but not PF4 is cleared by the kidneys,[296] a contribution of impaired renal function to the changes in β-TG in pre-eclampsia cannot be excluded.

Very recently, a novel approach has been used to detect platelet activation in vivo in pre-eclampsia. Studies employing whole blood flow cytometric analysis in conjunction with fluorescein conjugated specific monoclonal antibodies, detected increased fibrinogen binding together with increased surface expression of a lysosomal membrane marker in resting platelets from women with pre-eclampsia compared with those from healthy pregnant women.[409] There is therefore considerable evidence of platelet activation in vivo in PIH and pre-eclampsia, which occurs to a greater degree than in normal pregnancy. Furthermore, increased platelet production can no longer fully compensate for the increased peripheral platelet activation and reduced platelet lifespan, and a reduction in platelet count results.

7.7 Prostaglandins and Platelet Activation in Vivo in Pre-eclampsia.

Further evidence of platelet activation in vivo beyond levels occurring in normal pregnancy comes from studies of urinary metabolites of thromboxane. Fitzgerald et al have shown that there is a marked increase in urinary excretion of 2,3-dinor-thromboxane B_2 and 11-dehydro-thromboxane B_2 in pre-eclamptic subjects compared with healthy third trimester pregnant subjects.[158] In the pre-eclamptic subjects, urinary thromboxane metabolites fell rapidly following delivery.

This group also carried out a prospective longitudinal study of urinary thromboxane metabolites in each trimester, but could not demonstrate any significant difference between healthy pregnant women and those who subsequently developed pregnancy induced hypertension.[298] Another group of investigators, however, have claimed that urinary thromboxane metabolites are increased before the development of clinical signs of pre-eclampsia in pregnant women at risk of the disease on the basis of an enhanced pressor response to angiotensin II.[159]

Fitzgerald et al have also shown a reduction in the urinary excretion of the prostacyclin metabolite 2,3,-dinor-$PGF_{1\alpha}$ in pre-eclamptic compared with healthy pregnant subjects.[160,298,310] This ties in with evidence that isolated placental and umbilical vessels from pre-eclamptic subjects show reduced prostacyclin synthesis.[397,398] Furthermore, in the case of prostacyclin, there is good evidence that reductions in biosynthesis precede the development of clinical features of pre-eclampsia, being detectable as early as the first trimester of pregnancy.[160]

The significance of alterations in thromboxane and prostacyclin biosynthesis in relation to the vascular changes in pre-eclampsia has already been discussed. Clearly, these changes would also shift the balance in the vasculature to conditions favouring platelet activation, but it cannot be determined at present whether thromboxane/prostacyclin imbalance is the primary factor triggering platelet activation in pre-eclampsia. Whilst the evidence suggests that prostacyclin deficiency, perhaps resulting from endothelial damage, occurs early, there may be intrinsic changes in platelet reactivity in women who develop pre-eclampsia over and above those occurring in normal pregnancy. A great many studies have been carried out to investigate platelet reactivity in vitro in pre-eclampsia and PIH, the majority of which have examined platelet aggregation.

7.8 Studies of Platelet Behaviour in Vitro in Pre-eclampsia.

Platelet Aggregation Studies in PRP in Pre-eclampsia and PIH.

In 1971, Howie et al[404] showed a reduced platelet aggregation response to ADP in vitro in PRP in severe pre-eclampsia. No differences were noted in this respect between patients with non proteinuric PIH, and healthy pregnant controls. Reduction in ADP induced platelet aggregation in severe pre-eclampsia was later confirmed by Maki.[410] It has also been reported that a significant reduction in maximum rate of platelet aggregation occurs in PRP in severe pre-eclamptics in response to arachidonic acid compared with healthy pregnant controls and in response to collagen and vasopressin compared with non pregnant women.[317]

By contrast, a study investigating the threshold concentration of ADP giving rise to secondary aggregation in PRP showed no change in pre-eclampsia compared with healthy pregnancy.[295] Socol et al found that platelet reactivity in response to collagen was unchanged in pre-eclampsia.[408] O'Brien et al showed no significant differences in maximal platelet aggregation in PRP in response to a range of agonists in pre-eclampsia compared with normal pregnancy, but this study was rather unusual in being amongst the few reporting reduced platelet aggregation in healthy pregnancy.[319]

Morrison et al[322] studied platelet sensitivity to arachidonic acid in PRP in PIH as measured by the lowest concentration of arachidonic acid giving rise to visible aggregates and a release reaction. Platelets from women with PIH showed an increased sensitivity to AA compared with those from healthy pregnant women, and subgroup analysis showed that this difference was confined to subjects with a raised serum urate. Interestingly, the increased sensitivity to AA was still evident in PRP from this group of women 6 weeks after delivery, a time at which changes in platelet reactivity had resolved in healthy pregnant women.

Platelet Aggregation in Whole Blood in Hypertensive Pregnancy

Spontaneous aggregation in whole blood from women with PIH does not differ from that observed in blood from healthy pregnant women.[325]

A more recent study of agonist induced platelet aggregation in whole blood, measured by a platelet counting technique, showed reduced platelet responses to ADP in pre-eclampsia compared with normal pregnancy.[411] The method employed by these investigators, however, had some disadvantages as centrifugation was undertaken prior to platelet counting. A study by Louden et al[412] using a more standard platelet counting technique of measuring platelet aggregation in whole blood showed no significant differences in agonist induced platelet aggregation from women with either PIH or pre-eclampsia compared with healthy pregnant controls, except when adrenaline was used to stimulate platelets. In the case of adrenaline induced responses, there was a significant reduction in platelet aggregation in pre-eclampsia, and a similar trend was noted in PIH which did not reach statistical significance. A trend towards reduced aggregation to arachidonic acid was also noted in pre-eclamptic subjects, but this also failed to reach statistical significance.

Platelet Release Reaction In Hypertensive Pregnancy

Morrison et al[322] showed no overall change in the extent of ^{14}C-5HT release in PRP in response to arachidonic acid (AA) in patients with PIH, some of whom met the criteria for pre-eclampsia, compared with both healthy pregnant and non pregnant women.In whole blood it has been shown that platelets from women with pre-eclampsia release less ^{14}C-5HT in response to adrenaline, than those from healthy pregnant women, mirroring the reduced platelet aggregation in response to this agonist in pre-eclampsia.[412]

General Patterns of Platelet Reactivity In Pre-Eclampsia

Although studies of platelet behaviour in PIH and pre-eclampsia have differed in their findings, many have shown reduced platelet reactivity in vitro when compared with healthy pregnancy.[317,404,410-412] Full clinical details have not been provided by all investigators, but close examination of those available suggest that the studies showing reduced platelet responsiveness in vitro in hypertensive pregnancy have included patients with moderate to severe pre-eclampsia and therefore more severe disease than those which have demonstrated either no change or increased platelet reactivity. This may arise because, in severe pre-eclampsia, sufficient platelet activation in vivo has taken place to result in the circulation of exhausted platelets[413] which are subsequently hypoaggregable when tested in vitro. This is supported by the findings of one study which showed a reduction in intraplatelet 5-HT content in patients with severe pre-eclampsia who demonstrated platelet hyporesponsiveness in vitro.[317] The possibility of platelet activation during preparation of PRP also cannot be discounted as a factor contributing to these findings. Studies in whole blood, however, have tended to support a reduction in platelet responsiveness in vitro in pre-eclampsia.[411,412] If reduced platelet reactivity in vitro occurs as a result of platelet activation in vivo, then in vitro studies of platelet behaviour in established pre-eclampsia may not yield data which will elucidate whether alterations in intrinsic platelet reactivity, over and above those occurring in normal pregnancy, contribute to the pathophysiology of pre-eclampsia. The study by Morrison et al[322] is interesting in this respect since it does suggest that in women with PIH whose disease is less severe, platelets may be more responsive to AA than in normal pregnancy, and this difference was still detectable postnatally, when the clinical signs of PIH had resolved.

Platelet Thromboxane Production in Hypertensive Pregnancy.

The data relating to thromboxane production in vivo which have already been discussed above,[158,298] suggest that thromboxane A_2 is produced in greater amounts in pregnancies complicated by pre-eclampsia, than in healthy pregnancy. Studies of platelet thromboxane production in vitro have, as in normal pregnancy, been conflicting. As a measure of reactivity of the platelet thromboxane pathway, Wallenburg et al[405] measured malondialdehyde formation in PRP following stimulation with thrombin, and found this to be increased in PIH, but only when the pregnancy was also complicated by intrauterine fetal growth retardation. By contrast, Pekonen et al[292] found no difference in serum thromboxane B_2 levels between women with pre-eclampsia, and healthy third trimester women. Some of the pre-eclamptic subjects in this study were receiving treatment with antihypertensive drugs, but a more recent study has also shown unaltered levels of thromboxane B_2 in serum from untreated women with PIH and pre-eclampsia.[412]

Platelet Sensitivity to Inhibitory Agents in Hypertensive Pregnancy.

As in normal pregnancy, platelets from women with PIH and pre-eclampsia have been shown to be less susceptible to inhibition of their behaviour by prostacyclin and by thromboxane synthetase inhibitors.[322,331,332] Two reports suggest that reduction in prostacyclin sensitivity in platelets from pre-eclamptic women may be more marked than in those from healthy pregnant women.[331,332]

7.9 Platelet Second Messengers in PIH and Pre-eclampsia.

As in the case of healthy pregnancy, studies of platelet second messengers in PIH and pre-eclampsia have concentrated on intracellular free calcium.

The first report on platelet intracellular free calcium, using quin-2 showed no difference in basal or ADP stimulated platelet intracellular free calcium levels in PIH or in pre-eclampsia compared with healthy pregnancy.[333] There was,

however, a significant reduction in 5-HT stimulated $[Ca^{2+}]_i$ in platelets from pre-eclamptic women.[333] The reasons for this latter finding are unclear, but 5-HT induced responses in platelets are known to be particularly easily suppressed as a result of prior platelet activation.[414]

Two subsequent studies have shown an increase in basal intracellular free calcium levels in platelets from women with pre-eclampsia, over and above that observed in healthy pregnancy.[334,336] This was not the case in non proteinuric PIH.[336] The differing conclusions of the studies of platelet $[Ca^{2+}]_i$ in hypertensive pregnancy probably result from differences in methodology as described in the discussion of platelet $[Ca^{2+}]_i$ in healthy pregnancy. Again, it is difficult to ascertain from the data available whether the increase in basal platelet $[Ca^{2+}]_i$ is a reflection of the presence of a population of partially activated platelets or is in itself a cause of altered platelet reactivity in pre-eclampsia. There is some evidence, however, from a recently published study, that alterations in stimulated platelet $[Ca^{2+}]_i$ precede the development of clinical signs of pre-eclampsia. The study was prospective and investigated a group of primiparous women from a population with a high incidence of pre-eclampsia. Women who subsequently developed pre-eclampsia showed higher platelet $[Ca^{2+}]_i$ levels following stimulation with arginine vasopressin as early as the first trimester of pregnancy, but basal platelet $[Ca^{2+}]_i$ was unaltered.[415]

7.10 ANGIOTENSIN II and Platelets in Pre-eclampsia.

Angiotensin II receptors on platelets represent a new area of research in the field of platelet studies in pre-eclampsia. The aim of these studies is to further elucidate the role of the renin angiotensin system in pre-eclampsia by using platelets as a model for vascular smooth muscle. The relevance of the data emerging from such studies in relation to haemostasis and platelet reactivity is at

present unclear. Nevertheless, preliminary findings are of interest and will therefore be described briefly.

Platelets possess small numbers of angiotensin II binding sites. Baker et al[416] have recently shown that platelets from normal pregnant women exhibit reduced angiotensin II binding, and preliminary data suggests that this reverts towards non pregnant binding patterns in pre-eclampsia.[417] These results are of interest as they mirror the pressor effects of intravenously infused angiotensin II in the clinical situation, and therefore the sensitivity of vascular smooth muscle to angiotensin II. In normal pregnancy, reduced pressor effects of angiotensin II are observed.[387] An increase in the pressor response to infused angiotensin II occurs prior to the onset of clinical signs of pre-eclampsia,[387,388] and has been used as a means of predicting pre-eclampsia, although this would be impractical for widespread clinical use. Clearly, if platelet angiotensin II binding proved to be of predictive value, this could be very useful in the clinical management of patients thought to be at risk of pre-eclampsia.

The role of angiotensin II binding sites in relation to platelet behaviour is unknown, but there are reports suggesting that angiotensin II may enhance platelet aggregation responses to ADP and adrenaline.[418,419] Unpublished work in the Department of Medicine, University of Nottingham, however, has not reproduced these findings (Fox and Heptinstall, personal communication). On the other hand, a role for angiotensin II in platelet activation is supported by a study showing that platelet intracellular free calcium levels could be raised by angiotensin II in vitro, and this rise was greater in platelets from women with pre-eclampsia.[334] This ties in with the findings of an enhanced platelet $[Ca^{2+}]_i$ response to the pressor agent arginine vasopressin in pre-eclampsia.[415]

7.11. The Role of Antiplatelet Drugs in Pre-eclampsia.

As there is considerable evidence of thromboxane/prostacyclin imbalance in vivo in pre-eclampsia, there is a rationale for the use of low dose aspirin in the prophylaxis and treatment of the condition. A number of small clinical studies have suggested that low dose aspirin may indeed be of benefit in preventing pre-eclampsia in patients deemed at risk either on clinical grounds or on the basis of angiotensin II induced pressor responses. Although a total of four such studies have now been published[366-369] the numbers of patients involved are small, and the results of ongoing multicentre studies in the U.K. and in the U.S.A. should be awaited before the use of aspirin in pre-eclampsia is adopted into routine clinical practice. Furthermore, the possibility of harmful effects of aspirin, such as suppression of maternal prostacyclin production and effects on haemostasis in the neonate should be borne in mind. Available data, however, are encouraging in this respect, as one study showed that administration of 60mg daily of aspirin did not reduce urinary excretion of the prostacyclin metabolite 6-keto-prostaglandin $F_{1\alpha}$,[369] and several investigators have shown that lose dose aspirin has little effect on neonatal platelet reactivity and thromboxane production.[328,369,420] The clinical studies on aspirin in pre-eclampsia also lend support to a role for platelets in the pathophysiology of the disease.

Other antiplatelet agents have been used clinically in pre-eclampsia, but their evaluation has been even more limited than in the case of aspirin. Beta blockers are sometimes administered to women with pre-eclampsia to control blood pressure. There is some evidence that the combined alpha/beta blocker labetolol can inhibit ADP induced platelet aggregation ex vivo in subjects with PIH.[421] This may be due to its membrane stabilising properties and beta blockers which are less lipid soluble would not be expected to affect platelet behaviour.

Thromboxane synthetase inhibitors[422] and infusions of prostacyclin[423,424,425] have also been used as therapeutic agents in pre-eclampsia. Reports of the use of both of these agents are anecdotal, and often involve patients with very advanced disease. It is therefore difficult to draw any conclusions about efficacy. It would be surprising, however, in view of the in vitro data with thromboxane synthetase inhibitors,[322] if these agents were beneficial if used alone, but a case could be made for investigating their effects in combination with a thromboxane receptor blocker. In the case of prostacyclin there are good theoretical grounds for its therapeutic use in pre-eclampsia. On practical terms, however, its role is limited by vasodilator side effects,[423,424,425] and outwith the context of pre-eclampsia, rebound platelet hyperactivity has been described.[263,264] Newer, more stable prostacyclin analogues have not been used to date in pre-eclampsia.

8.RATIONALE OF THESIS

In the foregoing sections of this introductory chapter, evidence has been presented of profound changes in platelet behaviour both in normal pregnancy and in pre-eclampsia. Prostaglandins have an important role in regulating platelet behaviour in vivo. Studies to date have shown that biosynthesis of both pro-aggregatory and anti-aggregatory prostanoids increases markedly in healthy pregnancy, and in pre-eclampsia there is some evidence for a disturbance in the balance of these prostanoids, favouring both vasoconstriction and platelet activation. Studies of platelet behaviour in vitro in healthy pregnancy have demonstrated increased reactivity of platelets towards agonists which have a thromboxane dependent component in their mechanism of action, but conclusive data regarding the intrinsic reactivity of the thromboxane pathway in platelets during pregnancy is lacking. Furthermore, results of published studies to date have

also failed to fully explain the occurrence of increased platelet activation in vivo in healthy pregnancy in the context of a very marked increase in the synthesis of inhibitory prostaglandins, particularly prostacyclin. Whilst a reduction in sensitivity of platelets from pregnant women to prostacyclin has been demonstrated in vitro, the mechanisms underlying this observation have not been elucidated, nor is it known whether sensitivity to other inhibitory prostaglandins is altered. The studies described in the following chapters of this thesis were designed to test the hypothesis that alterations in platelet responses to inhibitory and pro-aggregatory prostaglandins may be involved in determining platelet reactivity during pregnancy.

A further aim was to elucidate the mechanism of any such changes in platelet responses to the inhibitory prostaglandins by studying the accumulation of platelet cyclic AMP, the second messenger involved in signal transduction linking interaction of these agents with receptors on the platelet membrane and response of the cell.

Finally, on the background of physiological data relating to platelet responses to prostaglandins in healthy pregnancy, studies have been conducted to determine whether any further alterations in such parameters occur in platelets from women suffering from or at risk of pre-eclampsia. Ultimately, a fuller understanding of the mechanisms underlying the pathological platelet activation which occurs in pre-eclampsia may lead to a more rational basis for treatment of this important obstetric complication.

CHAPTER 2.

MATERIALS AND METHODS

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1. MATERIALS

Anticoagulants.

For platelet studies, blood was anticoagulated with trisodium citrate dihydrate, supplied by BDH. A 3.13% solution was prepared in distilled water and stored tightly capped at 4°C. The solution was replaced every 4 weeks. For estimation of full blood count, pre-prepared tubes containing tripotassium ethylene diamine tetra-acetic acid (EDTA) were purchased from Becton Dickinson Ltd.

Agents For In Vitro Investigation.

Platelet Agonists: Arachidonic acid was supplied by the Sigma Chemical Company Ltd in 1 gram quantities as the 99% pure acid on dry ice. This was weighed out accurately into 10ml of absolute alcohol to give an approximately 300mM stock solution. The concentration of each batch of stock solution was calculated, and the solution stored in 0.2ml aliquots in tightly capped tubes at -70°C. Immediately before use, arachidonic acid was converted to the sodium salt by adding 0.5g/100ml sodium carbonate (BDH Ltd) in 0.15M saline (Polyfusor saline for intravenous infusion). The amount of sodium carbonate solution added was adjusted according to the concentration of the stock solution of arachidonic acid.

9,11 dideoxy-11 alpha, 9 alpha epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) was manufactured by the Upjohn Company and supplied by the Sigma Chemical Company, as a powder. 10mg was dissolved in 1ml ethanol, and 1.854ml of phosphate buffered saline (Dulbecco's Formula, supplied by Flow Laboratories) was added to make a 10mM stock solution. This was stored in aliquots at -70°C. Further dilutions were made in phosphate buffered saline immediately prior to use.

Platelet Inhibitors: Iloprost (0.1mg/ml) was supplied as a gift from Schering Health Care Ltd. This was stored at 4°C. Dilutions were made in 0.15M saline (Polyfusor) and a stock solution (1ug/ml) was stored in aliquots at -70°C. Further dilutions in normal saline were made on the day of each experiment.

Prostaglandins D_2, E_1, and E_2 were supplied by the Sigma Chemical Company and stored as solutions in ethanol (1mg/ml) at -70°C. In each case, further dilutions were made in 0.15M saline (Polyfusor) immediately prior to use.

Forskolin was purchased from Calbiochem and stored as a 5mg/ml solution in ethanol at -70°C. Further dilutions were made in normal saline on the day of each experiment.

The phosphodiesterase inhibitor AH-P719 was supplied as a gift from Boehringer Ingelheim in the form of a salt. A 10mM stock solution was prepared in normal saline and stored protected from light in aliquots at -70°C. On the day of each experiment further dilutions were made in saline.

Adenosine was purchased from the Sigma Chemical Company. Adenosine was dissolved in normal saline to a concentration of 2.5mM and stored in aliquots at -70°C. Adenosine deaminase, used to remove adenosine in some experiments, was of type VI from calf intestinal mucosa and was purchased from Sigma. The 2,000U/ml solution was stored at 4°C. Immediately prior to use, a working solution was prepared by dilution in normal saline.

The thromboxane synthetase inhibitor dazmegrel was donated by Pfizer Central Research as a white powder. Fresh solutions were prepared for each experiment by dissolving 25mg of dazmegrel in 1ml of 0.1M sodium hydroxide (BDH). Further dilutions were made in 0.15M saline (Polyfusor).

The thromboxane receptor antagonist ICI 192605 was a gift from ICI Pharmaceuticals. A 0.1M stock solution was prepared in ethanol and stored at

4°C. On the day of each experiment further dilutions were prepared in normal saline (Polyfusor).

Other Solutions for Platelet Aggregation and Release Experiments.

Aspirin was purchased from the Sigma Chemical Company. Solutions were prepared in distilled water and stored at room temperature for no longer than 7 days.

The fixing solution for measurement of platelet aggregation in whole blood was prepared from formalin (Timstar Laboratory Supplies Ltd), 0.15M saline (Polyfusor), disodium EDTA (Sigma), potassium dihydrogen phosphate (Eagle Scientific), disodium hydrogen phosphate (Fisons), and sodium chloride (M&B). Phosphate buffered saline (pH 7.4) was prepared by dissolving 9g sodium chloride, 0.5427g potassium dihydrogen phosphate, and 1.5846g disodium hydrogen phosphate in 100ml distilled water. A 0.077M solution of disodium EDTA was prepared in distilled water and formalin was diluted to 4% in distilled water. The solutions were then mixed in the following proportions: 3ml 0.077M disodium EDTA, 5ml 4% formalin, 2ml phosphate buffered saline, 10ml distilled water, and 30ml normal saline.

Cyclic AMP Assay.

"Cold" adenine was used as a diluent for ^{3}H-adenine, and was supplied by Sigma. A stock solution was prepared in distilled water. The concentration of the stock solution varied depending on the adenine content of each batch of ^{3}H-adenine, such that a 1 in 10 dilution of radioactive adenine in cold adenine gave a final adenine concentration of 1.2uM when 10ul of this solution was added to 440ul of platelet rich plasma.

Solutions For Column Chromatography.

Concentrated hydrochloric acid was supplied by Fahrenheit Lab Supplies Ltd, Nottingham, and diluted to a concentration of 0.5M with distilled water.

Sodium hydroxide was supplied by BDH and dissolved in distilled water to a concentration of 0.5M.

Trichloroacetic acid (BDH) was dissolved to 10% and 15% weight/volume solutions in distilled water.

Ammonium formate was purchased from Sigma and 0.2M solutions were prepared in distilled water.

Potassium phosphate buffer was prepared by making 1mM solutions of dipotassium hydrogen phosphate (M&B) and sodium dihydrogen phosphate (BDH), and adding a sufficient quantity of the latter to the 1mM dipotassium hydrogen phosphate to result in a final solution of pH 7.3. The working solution was prepared freshly for each experiment.

Imidazole was purchased from Sigma and 0.1M solutions were prepared in distilled water. On the day of each experiment the pH of the solution was adjusted to 7.5 by adding a few drops of 0.5M hydrochloric acid.

Materials For Column Chromatography.

Dowex (50 x 8-400, 8% cross linked, 200-400 dry mesh , H^+ form) was supplied by Sigma. Before use, it was prepared according to the manufacturer's instructions by washing 50g of the resin sequentially with 300ml distilled water, 300ml 0.5M sodium hydroxide, 300ml 0.5M hydrochloric acid, and a further 300ml distilled water. During storage the prepared resin was kept under distilled water in a covered container at 4°C.

Dowex columns were made from 14cm glass Pasteur pipettes (Bilbate Ltd). The end of each pipette was plugged with glass wool and Dowex resin prepared

as above was added until the packed volume was 1.5ml. Distilled water was placed over the resin and the columns capped off when not in use.

Neutral alumina (type WN-3, activity grade 1) was purchased from Sigma. Alumina columns were prepared using glass pipettes (10ml 'E-Mil' B, 0.8 x 30cm). The end of each pipette was plugged with glass wool and 1.5g of dry alumina was added and allowed to settle at the bottom of the pipette Alumina columns were stored dry at room temperature.

Reagents for the Thromboxane B_2 Assay.

"Cold" thromboxane B_2 was used as a thromboxane standard and was purchased from Sigma. A 1mg/ml stock solution was prepared in ethanol and stored at -70°C.

Tris-saline buffer was prepared by dissolving 8.19g sodium chloride (M&B Ltd) and 0.9g trizma hydrochloride (Sigma) in 1 litre distilled water. The pH was adjusted to 7.4 with hydrochloric acid. The composition of Tris-saline/gelatine was as above except 3g of gelatine (Fisons) was added to each litre of buffer.

Charcoal (BDH) was mixed with dextran T70 (Pharmacia) and with tris-saline and tris-saline gelatin buffers on the day of each experiment in the following proportions: 200ml of tris-saline gelatin, 100ml of tris saline, 1.5g charcoal, 0.15g dextran.

Radiochemicals.

^{14}C-5hydroxytryptamine creatinine sulphate complex was purchased from Amersham International. The specific activity was 56mCi/mM. The solution was stored as 50ul aliquots at -20°C in a freezer reserved for radiochemicals. A fresh aliquot was used for each experiment.

[adenine-U-^{14}C]-adenosine 3',5'-cyclic phosphate, ammonium salt was purchased from Amersham International (specific activity 306mCi/mmol). A 1 in

100 dilution in distilled water was prepared and the resultant stock solution stored in 250μl aliquots at -20°C in the radiochemical freezer. A fresh aliquot was used for each experiment.

[2,8-^{3}H]-adenine, specific activity 17-31 Ci/mmol, was purchased from New England Nuclear and was stored at 4°C in a refrigerator reserved for radiochemicals. A fresh solution was prepared on the day of each experiment by dilution of the ^{3}H-adenine by a factor of 10 in cold adenine.

5,6,8,9,11,12,14,15-(n)-^{3}H-thromboxane B_2 (specific activity 210 Ci/mmol) was purchased from Amersham International. This was stored at -20°C for no longer than 2 months. On the day of each experiment, a working solution was prepared by adding 10ul ^{3}H-thromboxane B_2 to 33ml tris-saline gelatin buffer.

Liquid Scintillant and Vials.

Packard-199 scintillant was supplied by Packard Instrument company Inc. Optiphase-Hisafe II was supplied by LKB/Pharmacia. Plastic and glass scintillation vials were also purchased from LKB/Pharmacia Ltd.

Reagents for Running the Coulter Counter (model Z_m)

Isoton II was used as a diluent for platelet rich plasma prior to platelet counting. This diluent, together with the calibrating and control reagents for the counter (S-Cal calibrator, and CTC-3 thrombocyte control) were purchased from Coulter Electronics Ltd.

Test Tubes and Miscellaneous Disposables.

PAP-4 microsiliconised glass test tubes for measurement of platelet aggregation in platelet rich plasma, paper for the PAP-4 aggregometer, and micro stir bars were purchased from LEP Scientific Ltd. General purpose polystyrene test tubes (75x12mm) and polystyrene test tubes (64x11mm) for the platelet release and whole blood platelet aggregation assays were obtained from LIP Equipment

and Supplies Ltd. Polypropylene test tubes (75x10mm) for the cyclic AMP assay were purchased from Sarstedt Ltd. "Eppendorf" polypropylene tubes were purchased from Elkay products Inc.

Polystyrene 30ml universal containers for blood collection were supplied by Media Disposables Ltd, 19 gauge butterfly needles by Abbott Ireland Ltd, and 20ml "Plastipak" polypropylene syringes by Becton Dickinson. Plastic 3 way taps were from Nipro Medical Industries Ltd.

2. CLINICAL METHODS

2.1 Subjects

All studies were approved by the ethical committee at the University Hospital, Nottingham. All subjects gave informed consent. The broad criteria used for subject selection will be described here, but further detail on individual study design and subjects included will be provided at the beginning of each subsequent chapter.

Healthy non pregnant women were selected as controls from staff and students at the University Hospital Nottingham. All were between the ages of 16 and 35 and had not been on an oral contraceptive for at least 12 weeks prior to the study date. Phase of menstrual cycle was not used as a criterion for selection, but the first day of the last period was noted.

Healthy pregnant women aged 16-35 years were selected randomly at the antenatal clinic. All of these women were primigravidae. For cross sectional studies, primigravid women in the third trimester of pregnancy (after the 24th week of gestation) were chosen and studied on one occasion only. Any woman thought to be in early labour was excluded.

A small group of healthy pregnant women was studied longitudinally through pregnancy. Most of these subjects were selected pre-conceptionally by asking for volunteers from female hospital staff and female blood donors who were planning their first pregnancy, and by referral of such women from general practitioners. A minority of healthy pregnant subjects in the longitudinal study were chosen from those referred to hospital before the 12th week of pregnancy for antenatal care. Subjects in longitudinal studies were asked to donate blood at the time of selection, as soon as pregnancy was confirmed, at 12, 24, and 36 weeks gestation, and after the 6th postnatal week.

All subjects acting as non pregnant controls and those classified as healthy pregnant women were questioned regarding their past medical history. Any women with chronic illnesses or on chronic drug therapy (with the exception of iron and vitamin supplements) were excluded. For example, women suffering from diabetes, hypertension, epilepsy, chronic asthma or renal disease did not meet the criteria for selection. Women with a past history of thromboembolic disease were also excluded. On the day of study a drug history was taken, and any subject who had ingested any drug likely to alter platelet function within a period of 14 days was excluded. Subjects were questioned specifically about ingestion of aspirin and other non steroidal anti-inflammatory drugs. Blood pressure was measured prior to venepuncture and no subject was included whose blood pressure was greater than 140/90 or, in the case of pregnant subjects, if any of the criteria for a diagnosis of pregnancy induced hypertension were fulfilled (see below).

Women with pregnancy induced hypertension (PIH) or pre-eclampsia were selected from those admitted to hospital with these diagnoses. The definition of PIH was a rise in diastolic blood pressure of greater than 15mmHg compared with the level recorded in the first trimester of pregnancy, and an absolute blood pressure of greater than 140/90.[341] In the case of women with non proteinuric PIH, only those women in whom raised blood pressure was confirmed by two measurements at least 6 hours apart were included.

To be classified as pre-eclamptic, greater than 0.5g protein had to be excreted in the urine over a 24 hour period in addition to meeting the above blood pressure criteria.[342,343] As a few women in this group required urgent delivery, confirmation of blood pressure recordings after 6 hours and quantification of proteinuria were not possible on every occasion. In such cases, the clinical

evidence of pre-eclampsia was indisputable, and all had heavy proteinuria on "dipstick" testing of random urine specimens. Most subjects with PIH or pre-eclampsia were primigravid; a minority were not, but either had a previous history of pre-eclampsia, or had a new partner for the pregnancy studied.[377] The majority of subjects with PIH or pre-eclampsia had received no drug treatment at the time of study. A small number of critically ill subjects had received medication, the details of which are provided in the relevant chapter. Subjects with PIH or pre-eclampsia who had ingested aspirin or other non steroidal anti-inflammatory drugs were excluded, and no woman with evidence of essential hypertension was included.

A small group of multiparous women considered to be at risk of pre-eclampsia because of a previous history of the condition, was studied longitudinally during pregnancy. These women were selected from the combined medical/obstetric clinic run at the University Hospital, Nottingham, to supervise the antenatal care of high risk pregnancies. Most were recruited in the second trimester of pregnancy. Drug treatment was recorded and subjects taking aspirin were excluded.

2.2 Measurement of Blood Pressure.

Blood pressure was measured over the brachial artery after 5 minutes rest in the supine position, using a standard sphygmomanometer. Phase IV of the Korotkov sounds was used to determine the diastolic blood pressure.

2.3 Blood Collection.

Blood was withdrawn from a large vein in the antecubital fossa using a 19 gauge butterfly needle for venepuncture. A tourniquet was applied just prior to venepuncture, but excessive stasis was avoided. Depending on the studies conducted, between 30ml and 90ml blood was withdrawn using 20ml polystyrene

syringes and a 3 way tap. For platelet studies, blood was immediately dispensed into polystyrene universal containers, containing 3.13% trisodium citrate dihydrate, 9 parts blood to 1 part anticoagulant. The tubes were mixed gently by inverting three times. For each subject, blood was also placed in a tube containing EDTA for measurement of a full blood count and, where appropriate blood was dispensed into a glass tube and allowed to clot to prepare serum samples suitable for estimation of serum thromboxane B_2 or for measurement of urea and electrolytes and uric acid.

2.4 Collection of Urine Specimens.

Subjects with PIH or pre-eclampsia were asked to supply a complete 24 hour collection of urine, unless the clinical condition demanded urgent delivery of the baby. Subjects were asked to empty their bladder of residual urine and then to place all urine passed over the next 24hours in a large plastic bottle containing mercurothiolate preservative.

Healthy pregnant women and those women with pre-eclampsia in whom a 24 hour urine collection was impractical were asked to provide a mid stream specimen of urine which was tested for protein using "Multistix" (Bayer Diagnostics).

3. LABORATORY METHODS

All platelet studies were completed within 3 hours of venepuncture. Platelet studies were carried out either in platelet rich plasma or in whole blood.

3.1 Preparation of Platelet Rich Plasma.

Platelet rich plasma was prepared by centrifugation of whole citrated blood at 150g for 10 minutes at room temperature. The upper layer of platelet rich plasma (PRP) was carefully aspirated off using a plastic Pasteur pipette and placed

in a tightly capped polystyrene tube or universal container. The remaining blood was immediately further centrifuged at 2000g for 10 minutes at room temperature to prepare platelet poor plasma (PPP). The platelet count in PRP was measured as detailed below, and the count adjusted to 250 x 10^9/l using the autologous PPP. The adjusted PRP was covered with a layer of 5% CO_2 in air and tightly capped to prevent pH changes.

Measurement of Platelet Count in PRP.

A Coulter counter (model Z_m) was used to measure the platelet count in PRP. Prior to counting, PRP was diluted by a factor of 1 in 3,000, by measuring 6.67ul of PRP using a positive displacement pipette into 20ml "Isoton II" and mixing thoroughly by inversion. The background particle count in the Isoton was previously checked and the diluent was discarded and replaced with fresh solution if the background count was greater than 1 particle/ul. The platelet count in the diluted PRP was measured 3 times, the mean calculated, and the background particle count subtracted. The Coulter counter was set to measure platelets as particles whose volume was between 3 and 36 fl, and the machine was calibrated regularly by the half count method[426] against a red cell preparation with a known MCV (Coulter S_{cal}). The calibration was checked against Coulter platelet standards (CTC 3).

3.2 Platelet Aggregation in PRP.

Platelet aggregation was estimated in PRP by a turbidometric method as first described by Born,[51] using a Biodata PAP 4 platelet aggregometer with an in-built chart recorder. This method is dependent on detecting an increase in light transmission by PRP as platelets form aggregates and the optical density of the plasma suspension changes to resemble that of platelet poor plasma. The 100% baseline (representing the optical density of the solution if all platelets formed

aggregates) was set using autologous platelet poor plasma and the 0% baseline was set using untreated PRP before inducing platelet aggregation with an agonist. The temperature in the aggregometer wells was maintained at 37°C throughout experiments. Experiments were carried out in microtubes made of siliconised glass, and samples were stirred at 1,000 r.p.m. using metal stir bars placed in the base of the tubes before adding PRP. 200ul of PRP was used in each experimental tube and 20ul of the appropriate platelet agonist was added to induce aggregation after a 2 minute pre-incubation period in the aggregometer. Hamilton syringes were used to obtain accurate measurement of agonist solutions and to ensure that the agonist was dispensed directly into the PRP at the base of the tube. Where drugs were added to inhibit platelet aggregation, 20ul of the appropriate solution was placed in the experimental tubes before the PRP was added. In such cases, the volume of PRP in each tube was adjusted to 180ul. Aggregation reactions were always allowed to proceed for 6 minutes after addition of the agonist in each case. Maximum % aggregation and slope (the slope of the tangent taken to the steepest part of the aggregation curve) could be read off the tracing produced by the in-built chart recorder on the PAP 4 aggregometer. The coefficient of variation for maximum aggregation was between 2% and 10% depending on the particular agonist and its concentration, and for the slope was between 6% and 9%.

3.3 Platelet Release Reaction.

Platelet dense granule release in PRP was measured by using a ^{14}C-5-Hydroxytryptamine (5-HT) labelling technique, based on the method described by Heptinstall et al.[427] This method was used to compare the effect, in vitro, in different subject groups, of various pharmacological agents which would inhibit platelet behaviour. In all cases the effect of these agents on arachidonic acid induced platelet dense granule release was studied.

Platelet dense granules were labelled with ^{14}C-5-HT by placing 6ul of radiolabelled 5-HT (specific activity 57.5 mCi/mmol) in the anticoagulant prior to collecting the blood. ^{14}C-5-HT is rapidly and extensively taken up into platelet dense granules and is available for subsequent release upon stimulation by an agonist.[69] 460ul of adjusted PRP was added to polystyrene tubes containing 20ul of the inhibitory agents under investigation and a metal stir bar. In control tubes the inhibitory pharmacological agents were replaced with saline. On the addition of the PRP, each tube was immediately placed in a waterbath at 37°C over a magnetic stirring base set to ensure a constant stirring speed of 1,000 rpm. After 2 minutes equilibration platelet aggregation and release were induced by the addition of 20ul of arachidonic acid (final concentration 1mM) or 20ul saline/sodium carbonate was added to control tubes. Incubation and stirring were continued for a further 6 minutes, at which time 50ul of a supersaturated solution (2.5mg/ml) of aspirin was added and the tubes cooled rapidly by placing on ice, thus ensuring that no further release took place. The presence or absence of visible aggregates was noted and the samples were then centrifuged at 2°C at 4,000g for 10 minutes to deposit the platelets and aggregates, leaving released ^{14}C-5-HT in the supernatant. Duplicate aliquots of 50ul of the supernatant were then pipetted into plastic vials, 10ml scintillation fluid was added (Packard 199), and radioactivity in the vials counted using an LKB 1215 Rackbeta liquid scintillation counter, on the ^{14}C single isotope program. The counting efficiency for ^{14}C at the quench of the samples was 90%.

The extent of the release reaction was then calculated , by expressing ^{14}C-5HT in the supernatant as a percentage of the total ^{14}C-5HT taken up into the platelet dense granules. The latter was calculated from the counts present in untreated and uncentrifuged PRP, and those present in platelet poor plasma

prepared by centrifuging the control tube to which saline/sodium carbonate only had been added. The formula used to calculate ^{14}C-5-HT release, together with a worked example are shown in Appendix 1. In practice, a computer program based on this formula, prepared by Dr A Kelman, University of Glasgow, was used to calculate the percentage release in all experiments. The intra-assay coefficient of variation for the extent of ^{14}C-5-HT release induced by 1mM arachidonic acid in the absence of an inhibitor was 6.8%.

3.4 Measurement of Platelet Cyclic AMP.

Principles of the Assay.

Cyclic AMP is formed in platelets from ATP by the action of the enzyme adenylate cyclase. Cyclic AMP can be assessed in platelets by measurement of absolute cyclic AMP concentrations by a protein binding assay[428] or by radioimmunoassay.[429] Alternatively, radioactive cyclic AMP can be measured in platelets prelabelled with ^{3}H-adenine, which is incorporated into the metabolic adenine nucleotide pool and subsequently converted to ^{3}H-cyclic AMP.[430] Cyclic AMP can be extracted by lysing the platelets with trichloroacetic acid.[430,431] The radiolabelled cyclic AMP in the extract is then separated from other nucleotides by column chromatography. This type of assay does not measure total amounts of cyclic AMP, but gives information about changes in the rates of synthesis and steady state levels. Its main advantage is that it excludes extracellular cyclic AMP, the levels of which are significant in plasma, and this type of assay is therefore more sensitive to small physiologically relevant changes in cyclic AMP, the baseline levels of which are very low.

Adenine Nucleotide Labelling.

^{3}H-adenine added to PRP is actively taken up by platelets.[432] Adenine metabolism does not occur in plasma, so all of the adenine added to PRP is

available for uptake.[433] It has been shown that the rate of uptake of ^{3}H-adenine by platelets is dependent on the platelet count,[254] the incubation temperature,[254] the total adenine concentration,[433] and the extent of uptake on the incubation time.[254] The ideal platelet count for uptake of adenine appears to be 300 x 10^{9}/l but uptake is little affected unless the platelet count falls below 200 x 10^{9}/l.[254] An initial total adenine concentration of 1.2uM and an incubation temperature of 37°C also give rise to maximum rates of uptake.[254,433] It was shown by Gray that the rate of uptake of ^{3}H-adenine by PRP is initially rapid, and thereafter slows, reaching a plateau by 90 minutes.[254]

^{3}H-adenine taken up by platelets is converted to ^{3}H-AMP by adenine-phosphoribosyl transferase.[433] The ^{3}H is then distributed amongst the metabolic pool of adenine nucleotides by the action of adenylate kinase giving rise to ^{3}H-ADP and ^{3}H-ATP.[433,434] It has been shown that ^{3}H is distributed amongst AMP, ADP and ATP in proportion to the endogenous amounts of these metabolically active adenine nucleotides.[434] Adenine nucleotides in the storage pool are not labelled when ^{3}H-adenine is incubated with PRP.[434] The labelled adenine nucleotides are catabolized in platelets to inosine monophosphate and hypoxanthine.[434] Variable amounts of ^{3}H-ATP are converted to ^{3}H-cyclic AMP by action of adenylate cyclase. At basal adenylate cyclase activity the rate of conversion of ATP to cyclic AMP is very low[189] but increases considerably in the presence of adenylate cyclase stimulators. The amount of ^{3}H-cyclic AMP formed at a given time point will also be increased above basal levels if the rate of metabolism of cyclic AMP to 5'-AMP is reduced in the presence of a phosphodiesterase inhibitor.[192]

Isolation of ^{3}H-cyclic AMP.

The sensitivity and accuracy of a ^{3}H-adenine labelling assay for cyclic AMP is dependent on the efficiency of the isolation procedure. In 1968, Krishna et al

showed that cyclic AMP could be separated from ATP by column chromatography on Dowex cation exchange resin, followed by precipitation with zinc sulphate and barium hydroxide.[435] Subsequently, White and Zenser,[436] and Ramachandran[437] showed that cyclic AMP separation from ATP could be achieved using columns packed with alumina ion exchange resin. Alumina is an amphoteric ion exchange resin, absorbing either anions or cations, depending upon the pH.[437] The separation of cyclic AMP on such columns is dependent on the fact that cyclic AMP is monovalent at neutral pH and can therefore be eluted off the column, whereas ATP, ADP, and AMP are polyvalent and are retained on the alumina.[437]

In 1974, using an adenylate cyclase assay based upon labelling of hepatocytes with ^{32}P-ATP, Salomon showed that improved separation of cyclic AMP from ATP could be achieved using both Dowex and alumina columns.[438] In 1981, Haslam and M^{c}Clenaghan showed that a double column separation technique using alumina and Dowex was applicable to the separation of ^{3}H-cyclic AMP from other ^{3}H labelled products in an assay of cyclic AMP in washed platelets incubated with ^{3}H-adenine.[430] This assay, with only minor modifications, was then used for the measurement of platelet cyclic AMP in PRP by Gray, who extensively evaluated the technique.[254] This isolation method involved the application of a trichloroacetic acid extract of PRP to a column of neutral alumina primed with 10% trichloroacetic acid. The alumina column was then washed with 10% trichloroacetic acid, which gives improved purification of the plasma extract[431] and delays the elution of the cyclic nucleotides.[436] The column was subsequently washed with distilled water to elute the bases[436] (adenine and hypoxanthine) which are non ionic at neutral pH. Finally, cyclic AMP was displaced from the column by exchange with the formate anion ($HCOO^{-}$) in ammonium formate (pH 6.0).[431,436,437] To ensure complete separation of cyclic

AMP, particularly from ATP,[435,438] the eluate from the alumina column was then applied to a column of Dowex 50W ion exchange resin which had been previously primed with hydrochloric acid and distilled water. The cyclic AMP was eluted from this column with potassium phosphate buffer (pH 7.3). The final eluate was then freeze dried to reduce sample volume prior to liquid scintillation counting.
Gray showed by thin layer chromatography that the final column eluate appeared to contain no other substances except cyclic AMP.[254] As significant loss of cyclic AMP occurs during the isolation procedure in this assay, a recovery label must be used in each sample. The use of ^{14}C-cyclic AMP, added to the trichloroacetic acid used to carry out the extraction of cyclic AMP from platelets, is a convenient method of monitoring recovery, provided facilities are available for dual label liquid scintillation counting.[430]

In view of the improved sensitivity of this assay compared with protein binding and radioimmunoassays, I chose to use this method for measurement of platelet cyclic AMP. Furthermore the choice of an assay based on pre-labelling with ^{3}H-adenine allowed the sensitive measurement of platelet cyclic AMP in PRP without the necessity of preparing washed platelets.

The experiments carried out to validate the technique, which was set up by myself for the first time in the Department of Therapeutics, Nottingham University, and was based upon the work of Gray[254] and Haslam and M^{c}Clenaghan[430] will now be described in detail.

Validation of the Assay of Platelet Cyclic AMP.

Uptake of ^{3}H-adenine by Platelets.

PRP in which the platelet count had been adjusted to 250 x 10^{9}/l was incubated with [2,8-^{3}H]-adenine (specific activity 23Ci/mmol) which had been previously diluted by a factor of 10 in a solution of cold adenine immediately prior

to use. The concentration of the cold adenine solution was such that the total final concentration of adenine (radiolabelled and unlabelled) in PRP was 1.2uM when the solution was added to the PRP at a 1 in 45 dilution (10ul of adenine solution added for every 440ul of PRP). Incubation of the PRP with adenine was carried out at 37°C for 90 minutes. These incubation conditions corresponded to those used by Gray[254] except for the platelet count which was adjusted to 250 x 10^9/l instead of 300 x 10^9/l. 250 x 10^9/l was chosen as it was anticipated there may be difficulty in achieving a final adjusted PRP count of 300 x 10^9/l in some pre-eclamptic subjects. Aliquots of 450ul of PRP were removed after 90 minutes and added to polypropylene tubes containing 50ul of saline (corresponding with the volume of additions to PRP in experiments where incubation of ^{3}H-adenine labelled PRP with pharmacological agents was subsequently carried out). These tubes were immediately centrifuged at 4,000g at 2°C for 10 minutes to deposit a platelet pellet. 50ul of supernatant was then carefully removed from each tube and dispensed into liquid scintillation vials labelled "^{3}H uptake" to which 15ml of Optiphase Hisafe II scintillation cocktail was added. 10ul of the ^{3}H-adenine/cold adenine solution was pipetted in triplicate into scintillation vials labelled "^{3}H totals" and scintillation cocktail added as before. 3 vials containing scintillation cocktail only were also prepared to determine the background radioactivity. All vials were then counted on a Beckman scintillation counter (model LS 8000) with automatic quench correction on a dual isotope program, set so that all ^{3}H counts appeared in channel 1. Percentage of ^{3}H-adenine remaining in the supernatant was

calculated from the formula :

% ^{3}H-adenine not taken up by platelets =

$$\frac{\text{Mean cpm in totals - mean background}}{\text{Mean cpm in uptakes - mean background cpm}} \times 10^{*} \times 100$$

* to correct for counting of 50ul out of a total of 500ul in "uptake vials".

The result of the above calculation was then subtracted from 100% to give the % uptake of ^{3}H-adenine by platelets.

During pilot studies uptake of ^{3}H adenine was determined using this protocol in 12 healthy individuals of both sexes, aged 18-40 years,on no medication. The mean and standard deviation of uptake of ^{3}H-adenine by platelets in PRP was 74.2% +/- 9.6%, with a range of 56.8% to 92.5%. As these figures corresponded closely to those quoted by Gray[254] it was decided to accept this incubation protocol for use in subsequent studies. It is noteworthy that there is a large amount of variation between individuals in the % uptake of ^{3}H-adenine by platelets. It is therefore necessary to express the conversion of ^{3}H-adenine to ^{3}H-cyclic AMP by platelets as a percentage of ^{3}H-adenine taken up by the platelets. As the subsequent drug incubation protocols of pharmacological agents with labelled PRP took a maximum of 34 minutes to carry out, it was necessary to ensure that uptake of ^{3}H-adenine did not alter significantly over this time period. During pilot experiments, uptake of ^{3}H-adenine was therefore determined after the 90 minute incubation period of ^{3}H-adenine with PRP, immediately before drug incubations were carried out and again at the end of a 34 minute drug incubation protocol. It was found in a study of 12 subjects that uptake of ^{3}H-adenine only varied by 1.37% $\pm$ 1.79% over this short period.

Determination of Elution Profile of Cyclic AMP from Alumina and Dowex Columns.

The elution profile of cyclic AMP from alumina and Dowex columns was determined using ^{14}C-cyclic AMP (specific activity 276mCi/mmol). Alumina and Dowex columns were prepared exactly as described by Gray.[254] The alumina columns were made by adding 1.5g of neutral alumina (type WN3) to 10ml graduated glass pipettes (see materials section) plugged with glass wool. Dowex ion exchange resin was prepared, as recommended by the supplier, by washing 50g of the Dowex (50x8-400; 8% cross linked; 200-400 dry mesh, H^+ form) in a Buchner flask under vacuum with 330ml distilled water, then 330ml 0.5M sodium hydroxide, followed by 330ml 0.5M hydrochloric acid, and finally a further 330ml distilled water. Glass Pasteur pipettes (length 14cm) were plugged with glass wool and filled with the prepared Dowex so that each column contained 1.5ml packed volume of ion exchange resin. The alumina and Dowex columns were primed as described by Gray[254] and by Haslam and M^{c}Clenaghan[430] with 10ml 10% trichloroacetic acid (TCA) for the alumina columns and with 10ml 0.5M hydrochloric acid (HCl) followed by 15ml distilled water for the Dowex columns. The elution profile of ^{14}C-cyclic AMP on the alumina columns was then determined using the protocol for separating cyclic AMP described by Haslam and M^{c}Clenaghan[430] as follows. A solution of ^{14}C-cyclic AMP in 15% trichloroacetic acid was prepared containing approximately 10,000 cpm/ 2ml TCA. 2ml of this solution (corresponding to the acid platelet extract in the assay of platelet cyclic AMP) was then applied to each of four alumina columns which were washed with solutions as illustrated in the flow chart (Figure 2.1). 1ml fractions of the eluate from the columns were collected in glass scintillation vials and 15ml Optiphase Hisafe II scintillation cocktail was added. Vials labelled "totals" were prepared containing scintillation cocktail and 200ul of the solution of ^{14}C-cyclic AMP in

FIGURE 2.1

ELUTION OF CYCLIC AMP FROM ALUMINA COLUMNS

APPLY ^{14}C-cAMPin 2ml TCA

↓

WASH WITH 9ml 10% TCA

↓

WASH WITH 9ml DISTILLED H_2O

↓

WASH WITH 7ml 0.2M AMMONIUM FORMATE (pH 6.0)

↓

COLLECT 1ml FRACTIONS of ELUATE

THROUGHOUT PROCEDURE

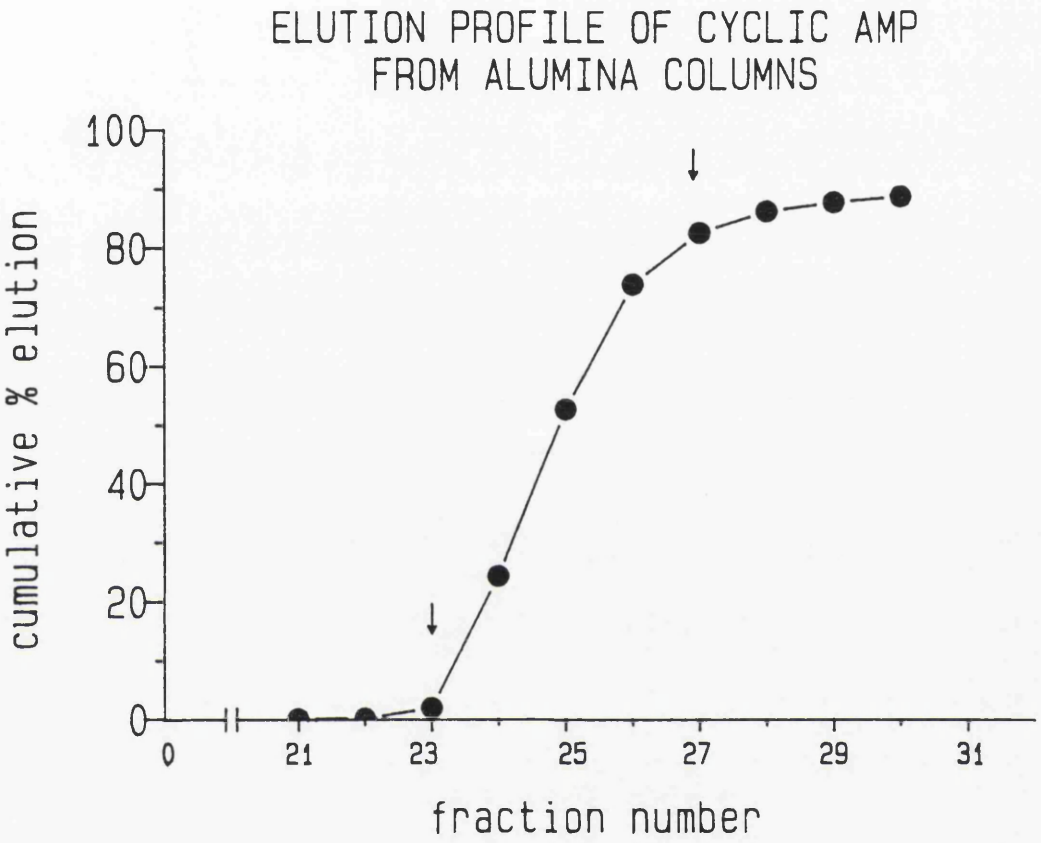


FIGURE 2.2

The figure shows the mean cumulative elution of ^{14}C-cAMP as a % of the total applied to 4 alumina columns, plotted against fraction number in mls of the column eluate. The arrows indicate the fraction numbers between which most of the cyclic AMP was eluted and which were subsequently chosen as those to be collected during routine use of the assay.

TCA. The radioactivity in all of the vials together with blanks was counted on the Beckman LS8000 scintillation counter on a program in which the parameters had been optimised for ^{14}C. Total ^{14}C-cyclic AMP applied to each column was calculated by multiplying the counts in "totals" vials by 10 after subtracting the background radioactivity. The mean counts in each 1ml fraction were then plotted against fraction number, as illustrated in Figure 2.2. It was found that 82.5% of the cyclic AMP added to the columns was eluted in the 3rd to the 7th ml inclusive of ammonium formate added to the columns (corresponding to fraction numbers 23-27). This corresponded exactly to the findings of Gray,[254] but differed slightly from those of Haslam and M^{c}Clenaghan,[430] who noted that the cyclic AMP was eluted in the 3rd to 5th ml inclusive of ammonium formate added to the columns.

The elution profile of ^{14}C-cyclic AMP from the Dowex columns was then determined by a very similar procedure, but in this case ^{14}C-cyclic AMP was diluted in 0.2M ammonium formate to give approximately 10,000 cpm per 5ml ammonium formate. 5ml of this solution (corresponding to the fraction of the eluate from the alumina columns shown to contain cyclic AMP) was applied to each of four Dowex columns which were then washed as shown in Figure 2.3. The potassium phosphate buffer used to elute the cyclic AMP was prepared by titrating a solution of 1mM K_2HPO_4 with 1mM NaH_2PO_4 until the pH reached 7.3. 1ml fractions of the column eluates were collected as before and the radioactivity of these samples counted together with totals and blanks. In this case the totals were prepared using 0.5ml of the ammonium formate/^{14}C-cyclic AMP solution. The mean counts in each 1ml fraction were then plotted against fraction number as illustrated in Figure 2.4. It was shown that 89.4% of the added ^{14}C-cyclic AMP was eluted in the 4th to 11th ml inclusive of potassium phosphate buffer added to the columns. Again, these findings corresponded exactly with those of Gray,[254] but differed from those of Haslam and

FIGURE 2.3

ELUTION OF CYCLIC AMP FROM DOWEX COLUMNS

APPLY ^{14}C-cAMP in 5ml AMMONIUM FORMATE

↓

WASH WITH 11ml POTASSIUM PHOSPHATE BUFFER (pH 7.3)

↓

COLLECT 1ml FRACTIONS of ELUATE

THROUGHOUT PROCEDURE

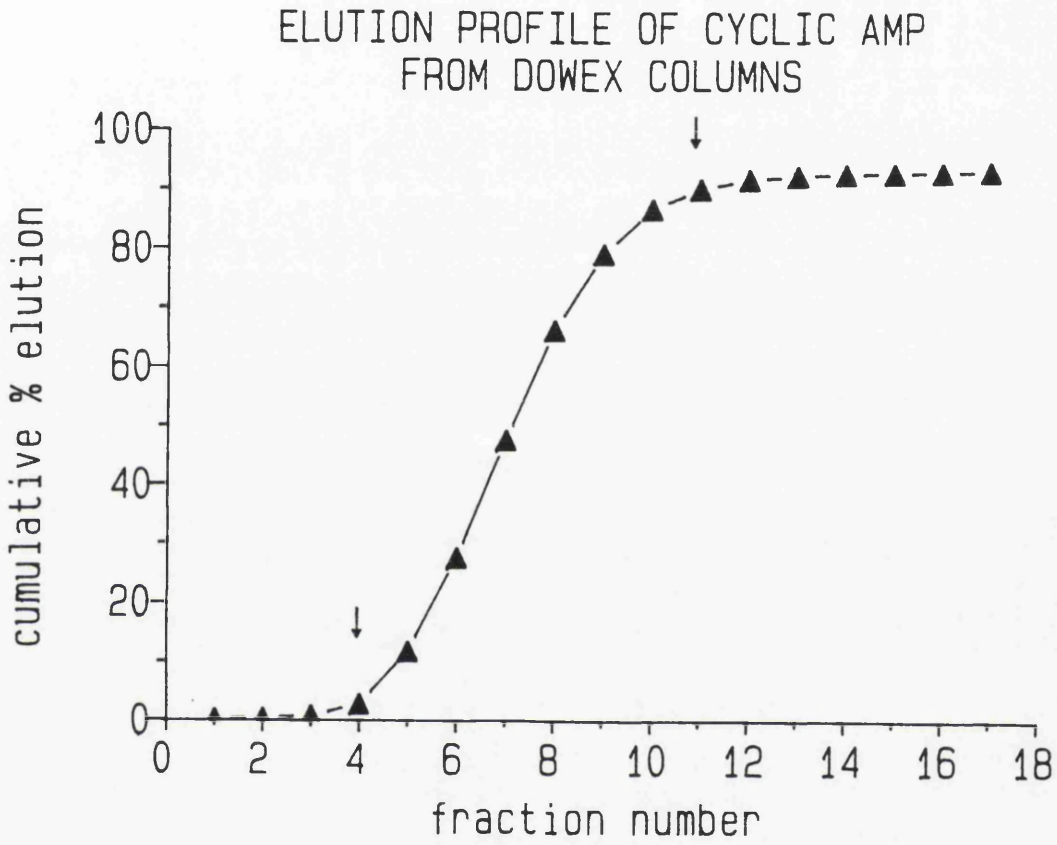


FIGURE 2.4.

The figure shows the mean cumulative elution of ^{14}C-cAMP as a % of the total applied to 4 Dowex columns, plotted against fraction number in mls of the column eluate. The arrows indicate the fraction numbers between which most of the cyclic AMP was eluted and which were subsequently chosen as those to be collected during routine use of the assay.

M^{c}Clenaghan,[430] who found that cyclic AMP was eluted in the 7th to 15th ml of potassium phosphate added.

In view of the above findings it was decided in subsequent experiments for assay of platelet cyclic AMP to adhere to the sequence of elution buffers for each set of columns as described, and to collect the fraction eluting from the alumina columns on the addition of the 3rd to 7th ml of ammonium formate, which would then be applied to the Dowex columns. The fraction eluting from the latter on the addition of the 4th to 11th ml of potassium phosphate buffer would subsequently be collected.

Further Modification of Procedure for Isolating Cyclic AMP.

Although the final column eluate using the isolation procedure described had been shown in the past by thin layer chromatography to be free of contaminating substances, I found during the establishment of the platelet cyclic AMP assay that on some occasions, basal levels of cyclic AMP were higher than those documented by previous investigators using this technique. This was noted to occur more frequently with a particular batch of ^{3}H-adenine, and was particularly marked when the uptake of ^{3}H-adenine by the platelets was lower than average. In view of this, contamination of the cyclic AMP containing fraction of the column eluate with a tritiated compound present in the ^{3}H-adenine, or with ^{3}H-adenine itself, was suspected. This was confirmed by the following experiment. Counts in collected fractions of eluate from columns to which 2ml 15% TCA containing no radioactivity had been applied in place of a sample were equivalent to counts in blank vials. By contrast, the application of 400,000 cpm ^{3}H-adenine diluted in 2ml TCA (approximately equivalent to the amount of ^{3}H-adenine remaining in plasma if uptake by platelets was 50%) applied to columns resulted in the presence of 219 cpm in the final eluate collected. Although this represents a small amount of contamination in percentage terms, this is significant as basal levels of cyclic AMP are very low

(equivalent to approximately 200 ^{3}H counts) and the error introduced would therefore be considerable. Details of the chemical purity of the ^{3}H-adenine supplied by the manufacturer showed that the ^{3}H-adenine was 97% pure. It was therefore very likely that a low level contaminant in the ^{3}H-adenine was eluting along with the cyclic AMP fraction on column chromatography. It is improbable that its exact nature could have been easily identified as it was present in extremely small amounts in chemical terms. It was therefore decided to modify the isolation procedure in an attempt to further purify the final column eluate, rather than to attempt to positively identify the contaminant. In the dual column cyclic AMP assay described by Salomon,[438] cyclic AMP was isolated by applying the sample first to a Dowex column, followed by an alumina column, which was developed by washing with imidazole. A possible means of increasing the purity of the isolation procedure in the present assay was to add on a third stage to the column chromatography procedure, by applying the eluate from the Dowex to a further alumina column and using the method of Salomon[438] to elute cyclic AMP from this column. Columns containing 1.5g of alumina were prepared as previously described and washed with 10ml 0.1M imidazole (pH 7.5). 8ml aliquots of potassium phosphate buffer (equivalent to the eluate from the Dowex columns) containing either ^{3}H-adenine or ^{14}C-cyclic AMP were then applied to these columns, which were subsequently washed with 6ml 0.1M imidazole. 1ml fractions of the column eluate following the application of these samples were collected and the elution profile of ^{3}H counts and ^{14}C counts determined. The results are illustrated in Figure 2.5. It can be seen that the majority of ^{3}H counts were eluted as the sample ran through, whereas ^{14}C-cyclic AMP was retained on the column and eluted with the imidazole wash. The addition of this step to the dual column chromatography described by Haslam and M^{c}Clenaghan was likely to improve the isolation procedure considerably. This was borne out in subsequent formal comparison of assay results

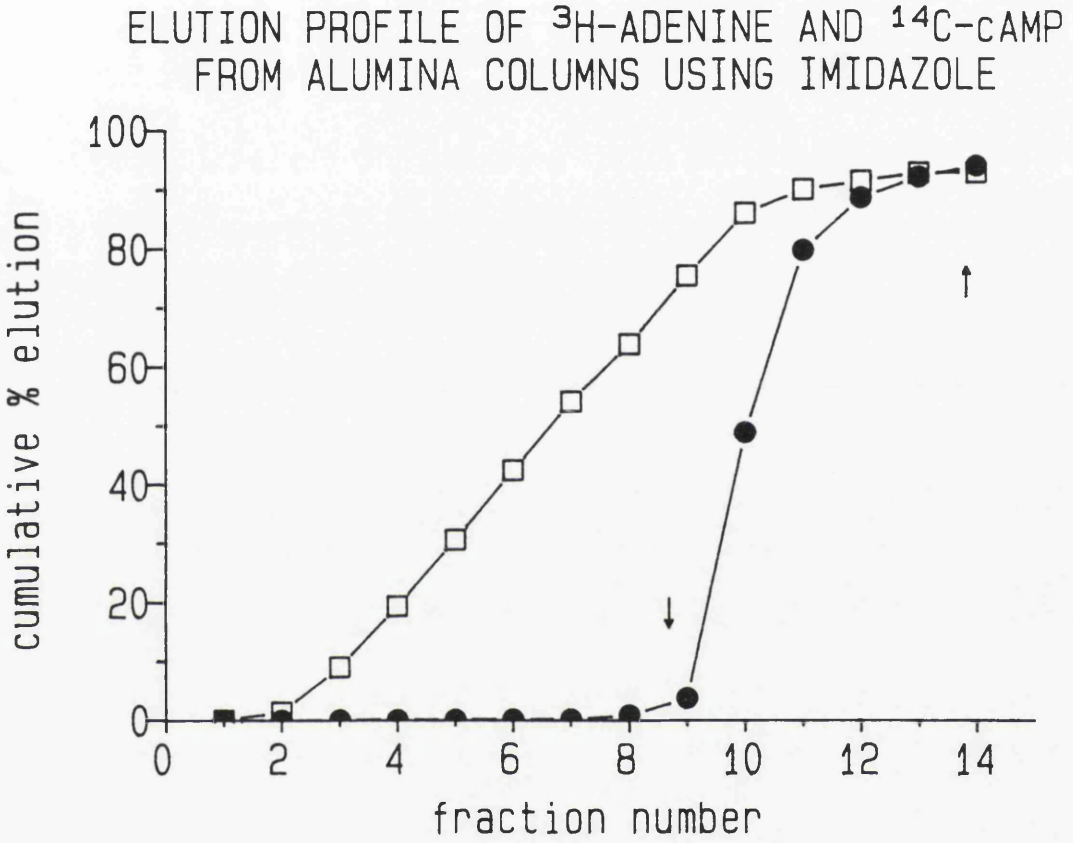


FIGURE 2.5

The figure shows the mean cumulative elution of ^{3}H-adenine (□) and ^{14}C-cAMP (●) from 4 alumina columns primed with and then washed with 0.1M imidazole, as a % of the total ^{3}H and ^{14}C counts applied to the columns. The radiolabelled adenine and cyclic AMP were applied to the columns in 8ml potassium phosphate buffer. The arrows indicate the fraction numbers between which most of the cyclic AMP was eluted from the column, and which were subsequently chosen as those to be collected during routine use of the assay. It is evident that most of the ^{3}H counts were eluted earlier than the cyclic AMP.

TABLE 2.1 COMPARISON OF TWO AND THREE COLUMN METHODS OF SEPARATING PLATELET CYCLIC AMP

^{3}H "totals"	868066.56
^{3}H uptake	78.56%
^{14}C spillover	29.28%

Drug Additions	Method	Channel 1 counts	Channel 2 counts	Recovery of cAMP	% Conversion ^{3}H-adenine to ^{3}H-cAMP
Saline	2 column	236.4	240.8	33.64%	0.0516
Saline	2 column	329.8	355.0	50.43%	0.0479
Saline	3 column	209.7	255.2	37.84%	0.0282
Saline	3 column	254.0	291.5	43.57%	0.0339
0.5ng/ml iloprost	2 column	255.7	256.1	35.89%	0.0537
0.5ng/ml iloprost	2 column	256.9	227.0	31.61%	0.0671
0.5ng/ml iloprost	3 column	254.1	219.4	32.19%	0.0605
0.5ng/ml iloprost	3 column	298.4	276.1	41.14%	0.0542

The table shows the raw and calculated data for assay of platelet cyclic AMP using two and three column separation methods simultaneously. It can be seen that, although calculated results are similar for stimulated cyclic AMP, basal levels are higher using the two column method. In fact, the calculated results using the latter method show little difference between basal levels and cyclic AMP levels in the presence of a low concentration of iloprost. By contrast results based on the three column method show an approximately twofold rise in cyclic AMP under these conditions. Such small increments in cyclic AMP may be physiologically relevant; it is therefore important to use an assay of sufficient sensitivity to detect them.

using dual and triple column chromatography. An example of counts obtained and calculated results of basal platelet cyclic AMP levels in PRP from one individual assayed by both methods simultaneously is shown in Table 2.1. As well as providing a purer cyclic AMP isolate the triple column method was practically more convenient. The final eluate volume was lower, allowing its direct uptake by Optiphase Hisafe II scintillant obviating the need for freeze drying to reduce sample volume. This also gave rise to improved cyclic AMP recovery as freeze drying resulted in considerable cyclic AMP loss. Using the triple column method, recovery was generally between 40% and 80% and varied considerably from column to column. As described by Haslam and M^{c}Clenaghan,[430] 1,000 dpm ^{14}C-cyclic AMP was added to each experimental sample in the TCA used to lyse the platelets and "stop" the reaction after incubation of PRP with pharmacological agents. This was achieved by preparing a solution containing 18ul of a 1 in 10 dilution in distilled water of stock ^{14}C-cyclic AMP per 2ml 15% TCA. Percentage conversion of ^{3}H-adenine to ^{3}H-cyclic AMP in each sample could then be corrected for cyclic AMP recovery, by comparison of ^{14}C counts in samples of column eluate with total ^{14}C-cyclic AMP originally added to each sample.

Column Washing and Regeneration.

After elution of the cyclic AMP, the first set of alumina columns were washed with 5ml of 0.2M ammonium formate, followed by 5ml 10% TCA, the Dowex columns were washed with 5ml 1mM potassium phosphate buffer, and the final alumina columns with 10ml 0.1M imidazole. The purpose of this was to ensure complete elution of any remaining cyclic AMP to prevent carry over between experiments. When ^{14}C-cyclic AMP was added to columns and eluted as described, 2.8% of ^{14}C counts were recovered on washing the Dowex with 5ml potassium phosphate buffer, and further washing did not elute any more cyclic AMP. For the

first set of alumina columns, 6% of ^{14}C counts were recovered in the first 3ml the ammonium formate wash, and further washing resulted in no further recovery of ^{14}C-cyclic AMP. For the second set of alumina columns, ^{14}C counts in the 10ml imidazole wash were equal to the background count.

Before use on each occasion columns were primed as follows: the first set of alumina columns with 10ml 10% TCA as described by Gray; Dowex columns with 10ml 0.5M HCl and 15ml distilled water as recommended by the manufacturer; and the final alumina columns with 10ml 0.1M imidazole as described by Salomon.[438]

All columns were replaced every 3 months, and with this protocol no deterioration in cyclic AMP recovery or any increase in basal cyclic AMP levels was noted in successive experiments.

Counting of Samples.

All samples were counted in glass vials on a Beckman LS 8000 counter after adding 15ml Optiphase Hisafe II liquid scintillant. The windows were set so that virtually all of ^{3}H emissions were counted in channel 1 with negligible spillover to channel 2 (Channel 1 = 0-450, ^{3}H spillover to channel 2 = 0.18%). The majority of ^{14}C emissions were counted in Channel 2 (450-655), but, on average, 33% spillover of ^{14}C counts to Channel 1 occurred, making it necessary to correct for spillover of ^{14}C counts to channel 1 when results were calculated. Two vials containing only ^{14}C-cyclic AMP were used as crossover controls. The Beckman LS 8000 was calibrated using a series of quenched standards for ^{3}H and ^{14}C supplied by the manufacturer. The counting efficiencies for ^{3}H and for ^{14}C respectively using the dual isotope program, at the quench of the experimental samples, were 51% and 94%. The program used incorporated an automatic quench control which adjusted the cpm in each channel for sample quench. Uniformity of quench in samples and totals in the cyclic AMP assay was in any case ensured by adding 6ml imidazole to samples used

to assess ^{3}H and ^{14}C totals, those used to assess ^{14}C crossover, and those used to assess uptake of ^{3}H-adenine, as cyclic AMP in experimental samples was eluted from the columns in 6ml imidazole.

In each assay, the following totals and controls were counted along with experimental samples. Vials containing 10ul of the ^{3}H-adenine solution originally added to the PRP were counted in triplicate to assess the total ^{3}H-adenine added to each sample. In order that ^{3}H-adenine uptake could be calculated, 2 vials containing 50ul of platelet poor plasma were counted. The platelet poor plasma was prepared at the end of the 90 minute incubation period of PRP with ^{3}H-adenine, as described in the section above on ^{3}H-adenine uptake. Total ^{14}C counts added to each sample were assessed by counting 18ul of the solution of ^{14}C-cyclic AMP prepared by diluting the stock radiochemical by a factor of 10 in distilled water. Crossover controls were prepared by adding 18ul of the undiluted stock ^{14}C-cyclic AMP to scintillation vials. Finally, blank vials containing only imidazole and 15ml of scintillation cocktail were counted to enable correction of all counts for background radiation.

Calculation of Results.

The procedure used for calculating % conversion of ^{3}H-adenine to ^{3}H-cyclic AMP is summarised in the Appendix 2 together with a worked example of the calculation of results from raw data. In practice, a computer program written by Dr A. Kelman, University of Glasgow was used to calculate results in each experiment in the studies undertaken. The program was validated by comparing results calculated by myself from raw data with those obtained using the program, and showing that results obtained by both methods were identical.

Final Protocol For Measurement of Platelet Cyclic AMP.

A flow chart summarising the final protocol used for the assay of platelet cyclic AMP is illustrated in Figure 2.6. Centrifugation of the ^{3}H labelled PRP for measurement of ^{3}H-adenine uptake was carried out at 2°C, at 4,000g for 10minutes. Details of drug incubation protocols for each study are given in the relevant chapters. In all cases samples in each subject studied were assayed in duplicate. Samples were thoroughly vortex mixed for 1 minute following the addition of the trichloroacetic acid to lyse the platelets and extract cyclic AMP. Subsequent centrifugation to deposit platelet debris was carried out at 2°C, at 4,000g for 30 minutes for the first spin, and 5 minutes for the second spin. The details of column chromatography are not shown in Figure 2.6, but were exactly as described in the foregoing text.

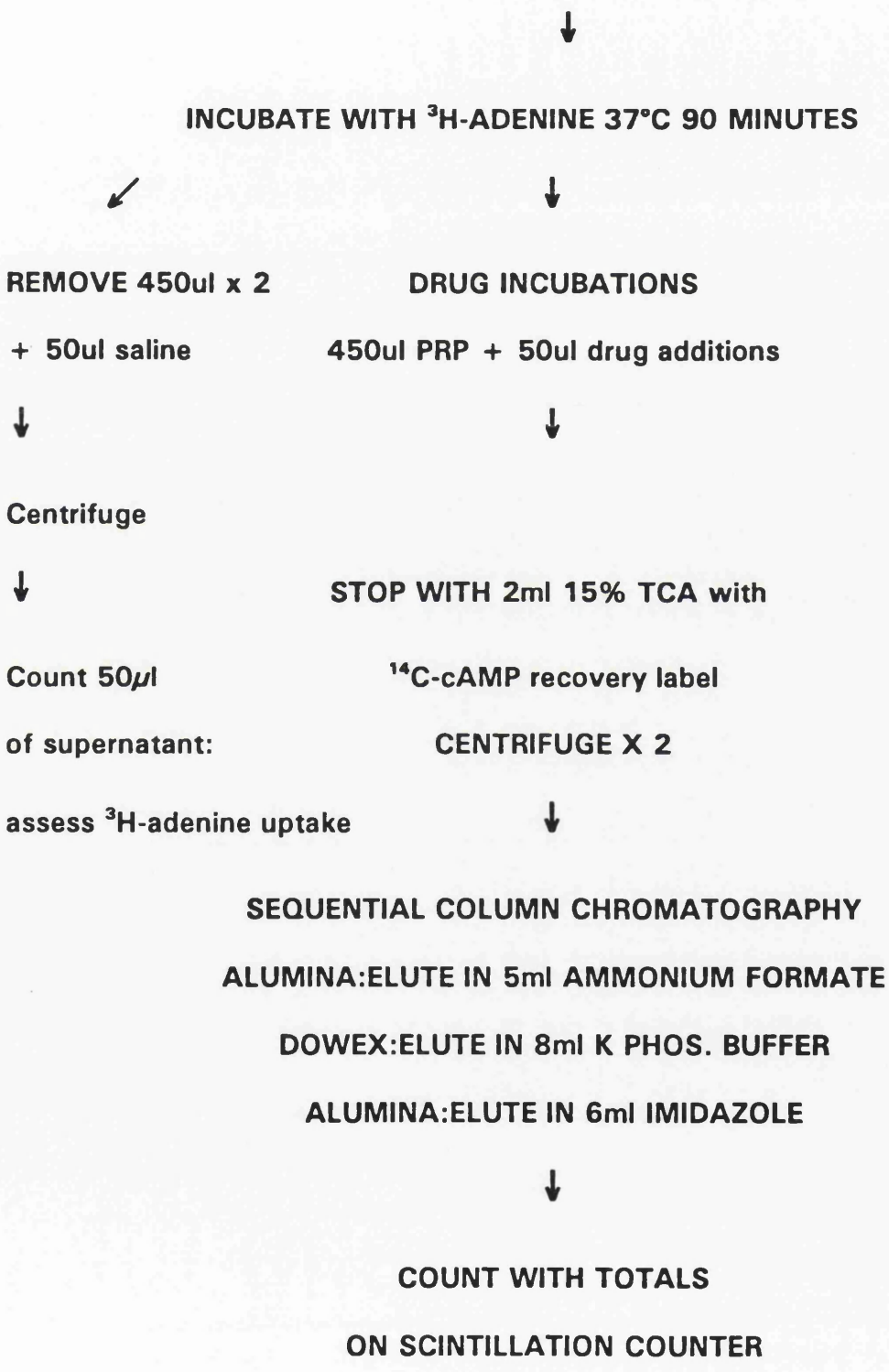
Intra-Assay Variation.

Using the final protocol described, the intra-assay variation was 6.5% for measurement of basal cyclic AMP levels, 5.2% for measurement of platelet cyclic AMP in the presence of maximal concentrations of the phosphodiesterase inhibitor AH-P719, and 5.8%-9.6% for measurement of cyclic AMP when platelets were incubated with an adenylate cyclase stimulator, depending on the nature of the pharmacological agent and its concentration.

Determination of Platelet Cyclic AMP Responses to Adenylate Cyclase Stimulators and a Phosphodiesterase Inhibitor.

Some pilot experiments were carried out to ensure that dose dependent increases in cyclic AMP could be detected using the ^{3}H-adenine pre-labelling assay when platelets were incubated with standard adenylate cyclase stimulators and with a phosphodiesterase inhibitor. The results of these experiments were also taken into account when choosing concentrations of pharmacological agents for subsequent study protocols. Examples of dose response curves obtained when platelets from

FIGURE 2.6 FINAL PROTOCOL FOR MEASUREMENT OF PLATELET CYCLIC AMP



healthy subjects were incubated with the PGI_2 analogue iloprost, with PGD_2, and with the phosphodiesterase inhibitor AH-P719 are illustrated in Figures 2.7-2.9.

3.5 Assay of Thromboxane B_2 in Serum and in PRP.

Sample Preparation.

For estimation of serum thromboxane B_2 blood was taken into a glass tube containing no anticoagulant and was allowed to clot at 37°C for 30 minutes. The sample was then centrifuged at 2,000g at room temperature for 10 minutes and the upper layer of serum carefully aspirated off using a plastic Pasteur pipette. The serum was immediately frozen at -70°C and, within 3 months, was subjected to radioimmunoassay for thromboxane B_2, the stable hydrolysis product of thromboxane A_2.

Platelet Thromboxane Production.

For measurement of platelet thromboxane B_2 production, adjusted PRP was incubated and stirred at 1,000rpm in the Biodata PAP 4 aggregometer for 2 minutes before adding arachidonic acid, final concentration 1mM. Platelet aggregation was then allowed to proceed for 4 minutes, at which time 200ul PRP was pipetted out and immediately added to a plastic Eppendorf tube containing 200ul of ice cold absolute alcohol. The tube was thoroughly vortex mixed. This procedure served to "stop" the reaction and to lyse the platelets, releasing the thromboxane B_2. The sample was then immediately frozen at -70°C and radioimmunoassay for thromboxane B_2 carried out within 3 months.

Thromboxane B_2 Radioimmunoassay.

This assay depended upon competition between 'cold' thromboxane in the unknown sample or standard with ^3H-thromboxane B_2 for binding to a specific anti-thromboxane B_2 antibody.

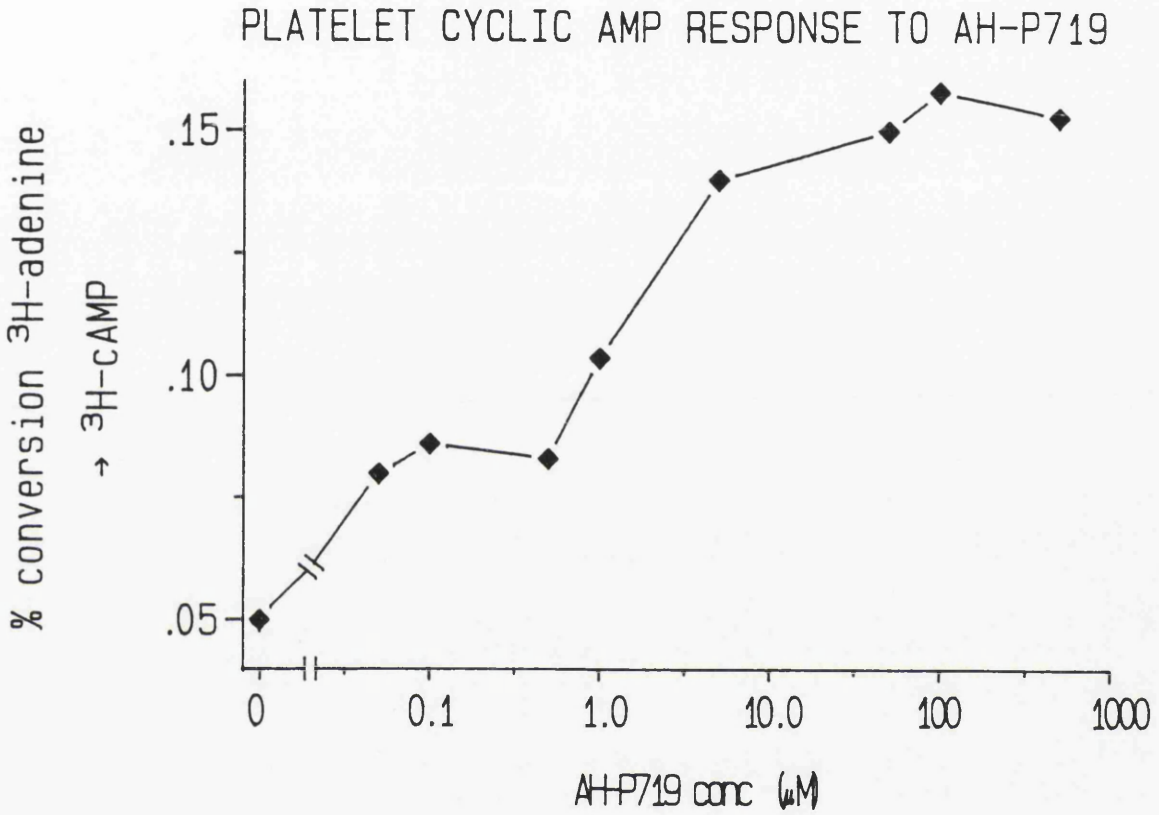


FIGURE 2.7.

A concentration-response curve of platelet cyclic AMP levels following treatment of PRP with AH-P719 is shown. The data were obtained from a single experiment. The x axis has a log scale. PRP was incubated with the phosphodiesterase inhibitor for 8 minutes. AH-P719 appears to exert its maximum effect at a concentration of 100μM.

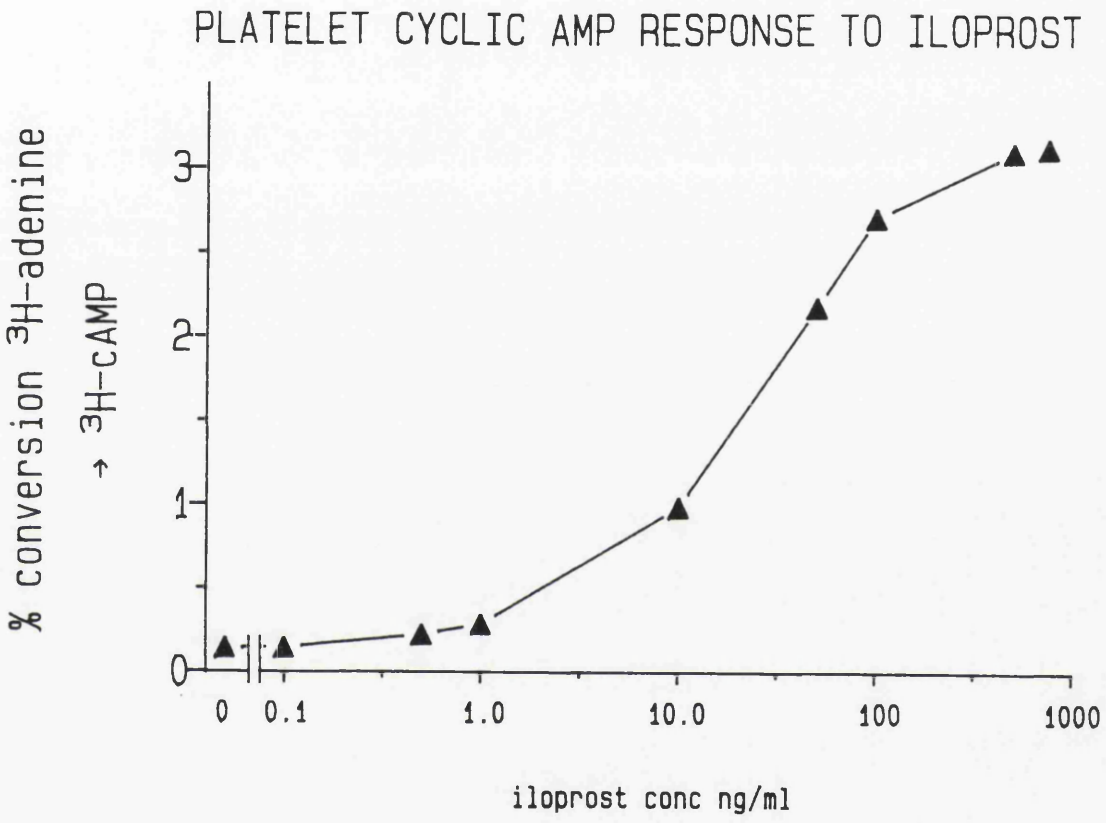


FIGURE 2.8.

A concentration-response curve of platelet cyclic AMP levels following treatment of PRP with iloprost in the presence of AH-P719 (100μM) is shown. The data illustrated were obtained from a single experiment. The x axis has a log scale. PRP was incubated with the phosphodiesterase inhibitor for 2 minutes before adding iloprost; incubation was then continued for a further 6 minutes.

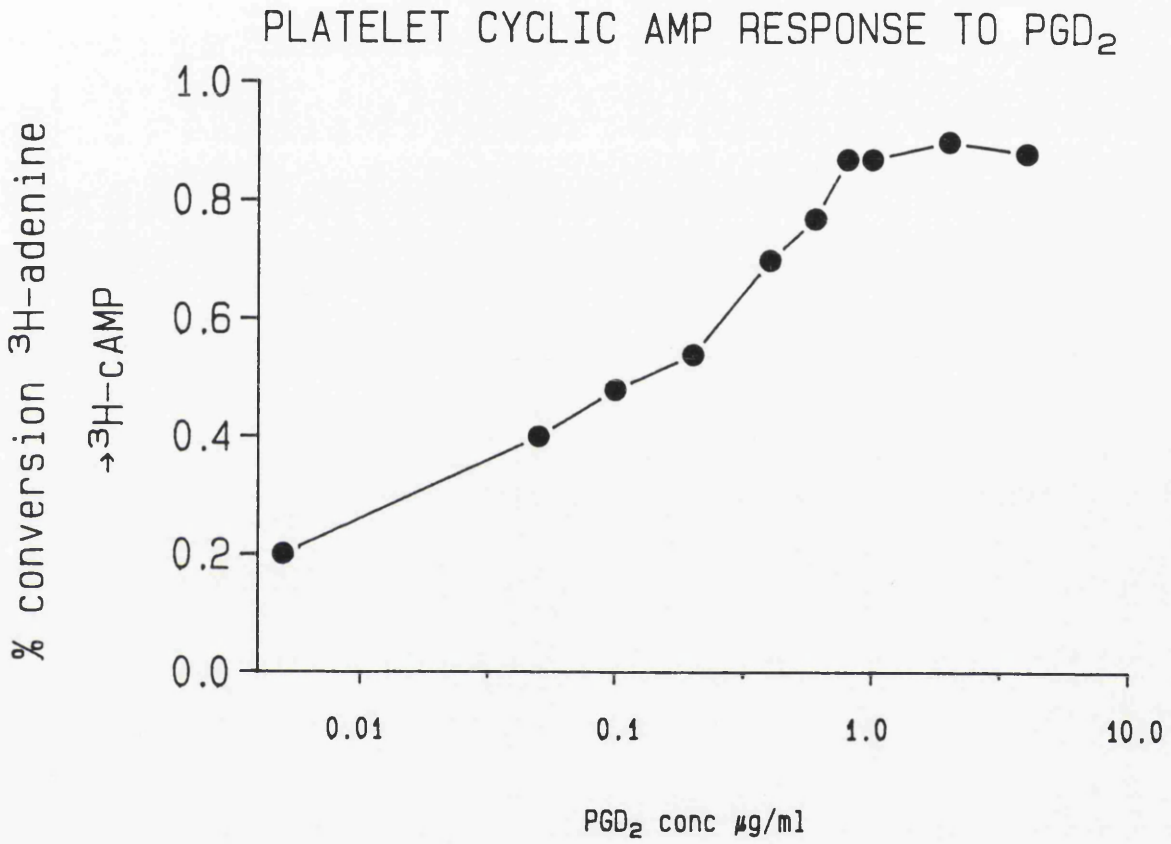


FIGURE 2.9.

A concentration-response curve of platelet cyclic AMP levels following treatment of PRP with PGD_2 in the presence of AH-P719 (100μM) is shown. The data were obtained from a single experiment. The x axis has a log scale. PRP was incubated with the phosphodiesterase inhibitor for 2 minutes before adding PGD_2, and incubation was then continued for a further 6 minutes.

On the day of performing the assay serum and platelet samples were allowed to reach room temperature. The platelet samples were centrifuged at full speed in a microfuge (Eppendorf Centrifuge 5414 S) for 3 minutes to deposit platelet debris, and the supernatant was carefully pipetted off. All samples were then diluted in Tris-saline assay buffer. Serum samples were diluted 1 in 500 and 1 in 1,000. Platelet samples were diluted 1 in 1,000, 1 in 2,000 and 1 in 5,000. Cold thromboxane B_2 standard was diluted in tris-saline assay buffer to concentrations ranging from 1pg/100ul to 100pg/100ul. An internal quality control standard, prepared from a single batch of cold thromboxane B_2, was also run with each assay. The ^{3}H-thromboxane B_2 (specific activity 210Ci/mmol, chemical concentration 0.476M) was diluted by adding 10ul of the radiochemical to 33ml Tris-saline with gelatin. The anti-thromboxane B_2 antibody was diluted from stock to a final dilution of 1 in 80,000 in Tris-saline with added gelatin. This was shown in an antibody dilution curve to result in 50% binding of ^{3}H-thromboxane B_2 when conditions were as standard in the assay, except that Tris-saline buffer replaced cold thromboxane B_2. The antibody dilution curve is illustrated in Figure 2.10. One hundred microlitres of each of the following was then added to assay tubes in duplicate: 1) diluted unknown sample or diluted standard, 2) diluted ^{3}H-thromboxane B_2, 3) diluted antiserum. The following control tubes were also set up in duplicate: 1) B_0 tubes containing ^{3}H-thromboxane B_2, and antiserum, but with Tris-saline in place of unknown sample, 2) 'totals' (CT) and 'non specific binding' (NSB) tubes containing ^{3}H-thromboxane B_2 with assay buffer in place of antiserum and unknown sample. All the tubes were then vortex mixed, capped and left at 4°C overnight. The following day, 1ml of a solution of charcoal was added to all tubes except the 'totals', to adsorb the remaining unbound ^{3}H-thromboxane B_2 in the sample. 1ml of a mixture of 1 part Tris-saline buffer and 2 parts Tris-saline gelatin was added to the 'totals' tubes. All tubes were allowed to stand for 12 minutes at

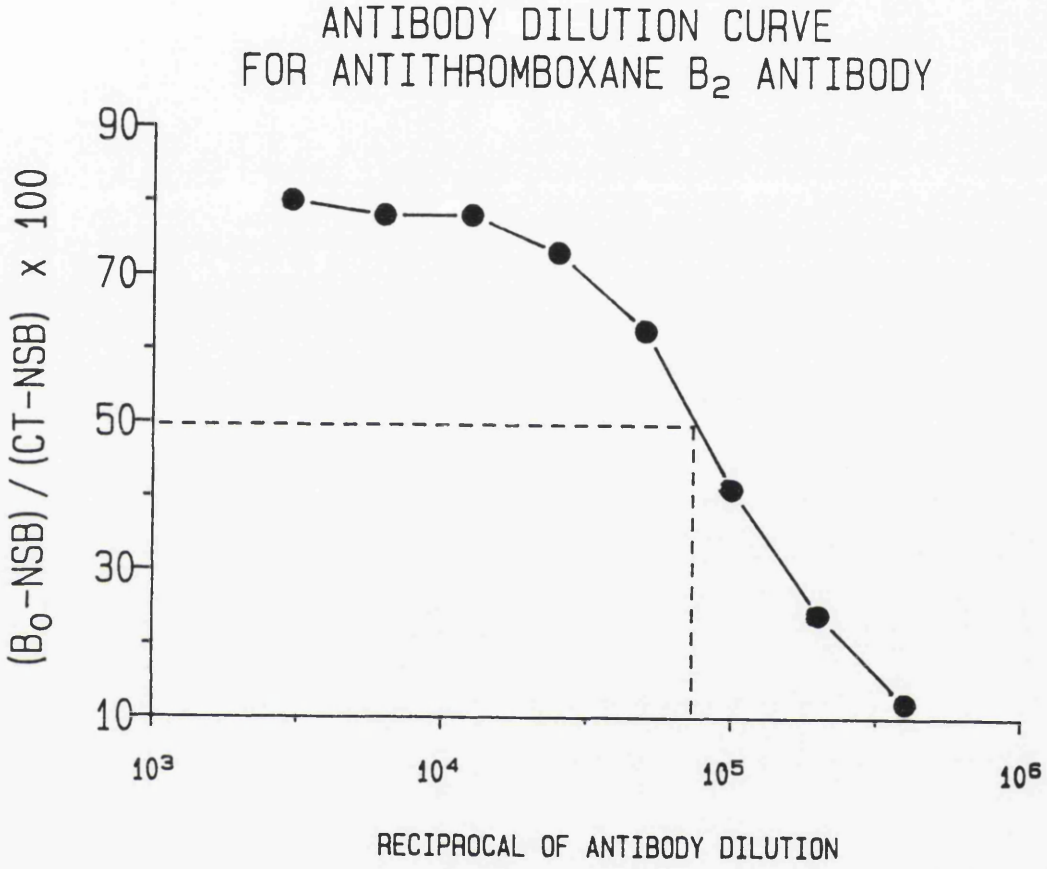


FIGURE 2.10

The % specific binding of ^{3}H-thromboxane B_2 to the anti-thromboxane B_2 antibody is plotted against the reciprocal of the antibody dilution. The scale on the x axis is a log scale. No "cold" thromboxane B_2 was present in this experiment. The antibody bound 50% of the ^{3}H-thromboxane when it was at a dilution of 1:80,000. B_0 = dpm in tubes containing ^{3}H-TXB_2 and antibody, following adsorption of unbound ^{3}H-TXB_2; CT = total ^{3}H-TXB_2 added to tubes; NSB (non specific binding) = dpm in tubes containing ^{3}H-TXB_2, but no antibody, in which unbound ^{3}H-TXB_2 had been adsorbed.

4°C and then all tubes except 'totals' were centrifuged at 4,000g for 10 minutes at 4°C. The supernatant was then carefully decanted into scintillation vials and 10ml of Packard 199 scintillation cocktail added. The entire contents of the 'totals' vials were also placed in scintillation vials to which scintillation fluid was subsequently added. All vials were counted for 5 minutes on an LKB 1215 Rackbeta scintillation counter on a single label program whose parameters were optimised for counting tritium. The counting efficiency for tritium at the quench of the samples was 32.6%. The mean of duplicate dpms for each sample was calculated, and a standard curve constructed by plotting % ^{3}H-thromboxane B_2 binding to antibody in standard samples against concentration of standard. Percentage binding was calculated as follows:

$$\frac{\text{dpm of standard - dpm in NSB}}{\text{dpm in } B_0 \text{ - dpm in NSB}}$$

% binding in unknown samples was then calculated and thromboxane B_2 concentration read from the standard curve. The quality of the assay was monitored 1) by calculating the percentage binding of the ^{3}H-thromboxane B_2 to antibody in the absence of competition from cold thromboxane (dpm B_0/dpm CT x 100); 2) by calculating the % non specific binding (dpm NSB/dpm CT); 3) by ensuring that the internal quality control standards lay on the standard curve when values of % binding in these samples were plotted against their concentration. Values of % B_0/CT were considered acceptable if they lay between 35% and 50%. % NSB was acceptable if it was below 4%. Any assay run not fulfilling these criteria was repeated. The coefficient of variation of the assay, calculated by assessing the mean and standard deviation of values obtained for a serum sample assayed four times within the same assay run, was 9.9%.

3.6 Platelet Aggregation In Whole Blood.

Measurement of platelet aggregation in whole blood offers the advantages that subpopulations of active platelets are not lost, as they may be during preparation of PRP, and platelet behaviour may be examined in a more physiological milieu. Two techniques are available for assessment of platelet aggregation in whole blood. The first depends upon changes in electrical impedance when platelets form aggregates,[54] the second involves the measurement of the reduction in number of single platelets in whole blood as aggregation proceeds. I have chosen to use the platelet counting method, as first described by Fox et al.[52] For such studies, blood collected into 3.13% trisodium citrate (1 part citrate : 9 parts blood) was allowed to equilibrate at 37°C in a waterbath for 30 minutes. 0.48ml aliquots of whole blood were then pipetted out and placed in polystyrene tubes containing a metal stir bar. These tubes were immediately placed in a circulating waterbath at 37°C over a magnetic stirring base set to ensure a constant stirring speed of 1,000 rpm. After 2 minutes 20ul of a solution of an appropriate platelet agonist was added to induce aggregation, and incubation and stirring continued for a further 8 minutes. 15ul subsamples of blood were removed before placing the experimental tubes in the waterbath, at 0.25, 0.5, 0.75, 1, 2, 4, and 8 minutes following the addition of the agonist. These 15ul subsamples were immediately placed in polystyrene tubes containing 30ul of a platelet fixing solution (see materials section). When experiments designed to assess the effects of inhibitors upon platelet aggregation in whole blood were carried out, 20ul of a solution of the inhibitor was placed in the experimental tubes before adding the blood, and the volume of blood added was adjusted to 0.46ml, ensuring that the final volume in each experimental tube was always 0.5ml.

The platelet count in the subsamples was measured within 3 hours using the Ultraflo-100 which measures the platelet count in whole blood by a technique

dependant on counting the ratio of platelets to red cells. The machine was primed using intravenous infusion saline (0.15M), and an arbitrary red cell count was dialled in. 30ul of fixed blood was added to plastic Ultraflo vials filled with 9.1ml of infusion saline, the vials mixed by inversion, and counted in duplicate. The background count in a vial containing saline only was previously checked and the solution discarded if the count was greater than 2. The percentage reduction in single platelets in each original experimental sample was calculated from the platelet counts in each timed subsample and those in the subsample taken before stirring and incubation was begun. A worked example of the calculation is shown in the Appendix 3. In practice a computer program prepared by Dr A. Kelman, University of Glasgow, based upon this calculation, was used to calculate experimental results. When an arbitrary red cell count is dialled into the Ultraflo, the true platelet count in each subsample can be determined by multiplying the count obtained by the ratio of true red cell count/arbitrary red cell count. Although the true red cell count was determined for each experimental subject, the platelet counts obtained from the Ultraflo were not corrected in this way as the final results were expressed as the percentage decrease in platelet count, and this was unaffected by correction for true red cell count. The intra-assay coefficient of variation for measurement of platelet aggregation in whole blood following stimulation with 0.3uM U46619 was between 5% and 11% for all time points except at 15seconds after the addition of the agonist, when the coefficient of variation was 18%.

3.7 Haematology and Biochemistry.

Full blood counts were performed on blood collected into EDTA from each subject in all studies. This was carried out in the Department of Haematology, University Hospital, Nottingham, using a Coulter counter model S plus IV for the majority of samples. For the studies described in Chapters 6 and 7, full blood counts were

performed using a Sysmex NE8000 multiparameter counter. Both machines were subject to regular internal and external quality control.

For those studies in which patients with PIH or pre-eclampsia formed one of the study groups, serum samples were analyzed in all subjects for urea and electrolytes and uric acid, and urinary protein content was quantified in subjects with PIH or pre-eclampsia. These analyses were carried out in the Department of Clinical Chemistry, University Hospital, Nottingham. Serum urea, creatinine, and urate were analyzed on a Hitachi 712 analyzer (Boehringer Mannheim Ltd) using diacetyl monoxime, alkaline picrate and uricase methods respectively. Urine total protein was measured by nephelometry following precipitation with a mixture of sulphosalicylic acid (30g/l) and sodium sulphate (140g/l).

4. STATISTICAL ANALYSIS

As much of the data generated from platelet studies were not normally distributed, non parametric statistical tests were generally used unless otherwise stated. For cross sectional studies involving 2 subject groups, the Mann-Whitney U test was employed, and for those where the objective was to compare 3 subject groups, the Kruskal-Wallis one way analysis of variance was used. In general, p values less than 0.05 were considered statistically significant, but for studies of platelet aggregation in whole blood, only p values less than 0.01 were considered significant as the design of the experiments demanded that repeated two sample tests were performed on data from the same subjects, and after taking statistical advice, no suitable non parametric analysis of variance could be found. For clarity of illustration results are shown as means $\pm$ standard error of the mean (SEM) unless otherwise stated. All statistical analysis was carried out using a computer program provided by Dr A. Kelman, University of Glasgow (Statis 3, Clydesoft Statistical and Scientific Software).

CHAPTER 3.

A CROSS SECTIONAL STUDY OF THE EFFECT OF INHIBITORY PROSTAGLANDINS AND OTHER CYCLIC AMP MANIPULATORS ON PLATELET BEHAVIOUR IN HEALTHY AND HYPERTENSIVE PREGNANCY

A CROSS SECTIONAL STUDY OF THE EFFECT OF INHIBITORY PROSTAGLANDINS AND OTHER CYCLIC AMP MANIPULATORS ON PLATELET BEHAVIOUR IN HEALTHY AND HYPERTENSIVE PREGNANCY

1. INTRODUCTION

There is some evidence that platelets during pregnancy may be less sensitive to the inhibitory effects of prostacyclin in vitro,[330,331,332] and that this effect may be more marked in pre-eclampsia.[331,332] Furthermore, one study showed that the thromboxane synthetase inhibitor dazoxiben was less effective as an inhibitor of platelet behaviour in vitro in PRP from healthy and hypertensive pregnant women.[322] As discussed in the introductory chapter, prostacyclin produces its inhibitory effects on platelets by acting on a specific receptor[195,196] to stimulate adenylate cyclase,[165,166] resulting in an increase in the level of intracellular cyclic AMP. The inhibitory effects of thromboxane synthetase inhibitors are also at least in part mediated by cyclic AMP, as PGD_2 is synthesised from cyclic endoperoxides by a diversion pathway when thromboxane synthetase is blocked.[247,248,249]

The study reported in this chapter was designed to investigate whether the loss of platelet sensitivity to prostacyclin during pregnancy could be demonstrated in vitro with other pharmacological agents which inhibit platelet behaviour by increasing cyclic AMP, and also to determine whether there were further differences in platelet responses to these agents in PIH and pre-eclampsia. A wide range of cyclic AMP manipulators which act at different points in the pathway has been investigated with the purpose of gaining some insight into the mechanism of any changes demonstrated in platelet responses to inhibitory prostaglandins.

2. METHODS

2.1 Subjects.

The study was cross sectional in design with a total of 84 subjects who were divided into three groups: 1) 26 healthy non pregnant women, age 26$\pm$0.9 years (mean $\pm$ SEM); 2) 27 healthy third trimester primigravid women, age 23.2$\pm$0.96 years; 3) 31 women with PIH or pre-eclampsia , age 24$\pm$0.75 years. The criteria for subject selection were as outlined in Chapter 2. Twenty-four women in the PIH/pre-eclampsia group met the criteria for pre-eclampsia, while seven had non proteinuric PIH. Twenty-seven of the PIH/pre-eclampsia group were primigravid, four patients were multiparous, three of whom had a previous history of pre-eclampsia, and one had a new partner for the pregnancy studied. Twenty-six patients in the hypertensive group had received no treatment, three patients had received labetolol, one had received phenytoin for seizure prophylaxis, and one had received intravenous hydralazine some hours before the study. In all of these cases clinical signs of severe pre-eclampsia were present at the time of study. Subjects were placed in the supine position and after 5 minutes rest blood pressure was measured and blood withdrawn for platelet studies, full blood count, serum thromboxane B_2 and urea and electrolytes and uric acid.

2.2 Laboratory Methods.

Platelet studies were conducted in PRP in which the platelet count had been adjusted to 250 x 10^9/l. In eight subjects, all of whom had pre-eclampsia, it was not possible to conduct experiments at a final adjusted PRP count of 250 x 10^9/l. All of these subjects had whole blood platelet counts less than 200 x 10^9/l, and six had thrombocytopenia as defined by a whole blood platelet count less than 150 x 10^9/l. The final adjusted PRP counts in these cases ranged from 85 x 10^9/l

to 245 x 10^9/l. There was, however, no overall difference between subject groups in the platelet count in adjusted PRP ($p=0.2908$).

The effect of cyclic AMP manipulators on platelet ^{14}C-5HT release induced by 1mM arachidonic acid was examined. The pharmacological agents used were as follows: the stable prostacyclin analogue iloprost at final concentrations of 0.1-10ng/ml, PGD_2 0.01-0.5ug/ml, forskolin 0.2-4.0ug/ml, the cyclic AMP specific phosphodiesterase inhibitor AH-P719 0.005uM-1uM, and the thromboxane synthetase inhibitor dazmegrel at final concentrations of 100uM and 500uM. PRP was incubated for 2 minutes at 37°C with these inhibitory agents in all cases before inducing aggregation and release with 1mM arachidonic acid (AA). Details of the laboratory methods used for measuring ^{14}C-5HT release from platelet dense granules, and for estimation of serum thromboxane B_2 have already been documented in Chapter 2.

For the purpose of statistical analysis, subjects with PIH and pre-eclampsia were treated as one group, since no difference in laboratory results could be demonstrated between subjects in these categories when treated separately. All comparisons between non pregnant, healthy pregnant, and hypertensive pregnant groups were carried out using Kruskal-Wallis one way analysis of variance by ranks. The Mann-Whitney U test was used to check similarity of gestation in healthy pregnant and hypertensive pregnant groups. The Fisher exact test was used to analyze the responder status to the thromboxane synthetase inhibitor in the three subject groups.

TABLE 3.1

PERIPHERAL BLOOD HAEMATOLOGY

The table shows some haematological data from the non pregnant subjects (NP), the healthy pregnant subjects (HP) and the subjects with PIH or pre-eclampsia (PIH). Values are means ± SEM for all parameters

	NP	HP	PIH	p value	Comparison
PCV	0.380 ± 0.005	0.355 ± 0.0009	0.362 ± 0.008	<0.01 <0.05	NP v HP NP v PIH
Platelet Count ($x10^9/l$)	265 ± 12	272 ± 15	202 ± 14	<0.01 <0.01	NP v PIH HP v PIH
MPV (fl)	8.81 ± 0.19	8.36 ± 0.32	10.52 ± 0.37	<0.01 <0.01	NP v PIH HP v PIH
PDW	16.27 ± 0.08	17.15 ± 0.14	17.3 ± 0.14	<0.01 <0.01	NP v HP NP v PIH

TABLE 3.2

BIOCHEMICAL PARAMETERS OF RENAL FUNCTION

The table shows some biochemical parameters for the non pregnant subjects (NP), the healthy pregnant subjects (HP) and the subjects with PIH or pre-eclampsia (PIH). Values are means ± SEM for all parameters NA = not applicable

	NP	HP	PIH	p value	Comparison
Proteinuria*	-	-	2.64 ± 0.70†	NA	-
Serum urate (μmol/l)	206 ± 10.12	230 ± 14	378 ± 24.6	<0.01 <0.01	NP v PIH HP v PIH
Serum urea (mmol/l)	4.06 ± 0.16	3.15 ± 0.15	4.29 ± 0.29	<0.01 <0.01	NP v HP HP v PIH
Serum creatinine (μmol/l)	83.2 ± 2.28	73.4 ± 2.49	87.8 ± 4.5	<0.05 <0.05	NP v HP HP v PIH

*Proteinuria was not quantified in non pregnant and healthy pregnant subjects as none showed proteinuria on urine dip-stick testing. In six out of 31 hypertensive pregnant subjects the requirement for urgent delivery of the baby precluded quantification of proteinuria. All six showed unequivocal proteinuria on dip-stick testing. Data are shown for the remaining 25 subjects

† 7 out of 25 patients had non proteinuric PIH

3.RESULTS

3.1 Clinical Data.

The normal pregnant patients were at 35.4±0.73 weeks gestation at the time of study, and those with hypertension at 34.1±0.74 weeks ($p = 0.1988$). None of the subjects was in labour at the time of study. Diastolic blood pressure was significantly higher ($p<0.01$)in the hypertensive pregnant group (96.4±1.92 mmHg) than in the healthy pregnant (70.5±2.0 mmHg) and non pregnant groups (71.5±1.88 mmHg). The majority of the hypertensive group met the criteria for pre-eclampsia.

3.2 Haematology and Biochemistry.

Haematological and biochemical data are illustrated in Table 3.1 and 3.2. Packed cell volume (PCV) was significantly lower in normal and hypertensive pregnant subjects compared with non pregnant subjects. There was no difference in platelet count in whole blood between non pregnant and pregnant subjects, but the platelet count was significantly lower in the hypertensive pregnant group in comparison with both other groups, and seven subjects with pre-eclampsia were thrombocytopenic, as defined by a whole blood platelet count less than 150 x 10^9/l. Mean platelet volume (MPV) was likewise significantly higher in the hypertensive group, and platelet distribution width (PDW) was significantly and similarly increased in both healthy and hypertensive pregnant subjects compared with that in non pregnant women. Uric acid was raised in the hypertensive pregnant subjects in comparison with healthy pregnant and non pregnant women.

3.3 Arachidonic Acid Induced Platelet Dense Granule Release.

In the absence of inhibitors, the extent of ^{14}C-5HT release was greater in response to stimulation with 1mmol/l arachidonic acid (AA) in PRP from pregnant individuals than in PRP from non pregnant women (Fig 3.1). There was no

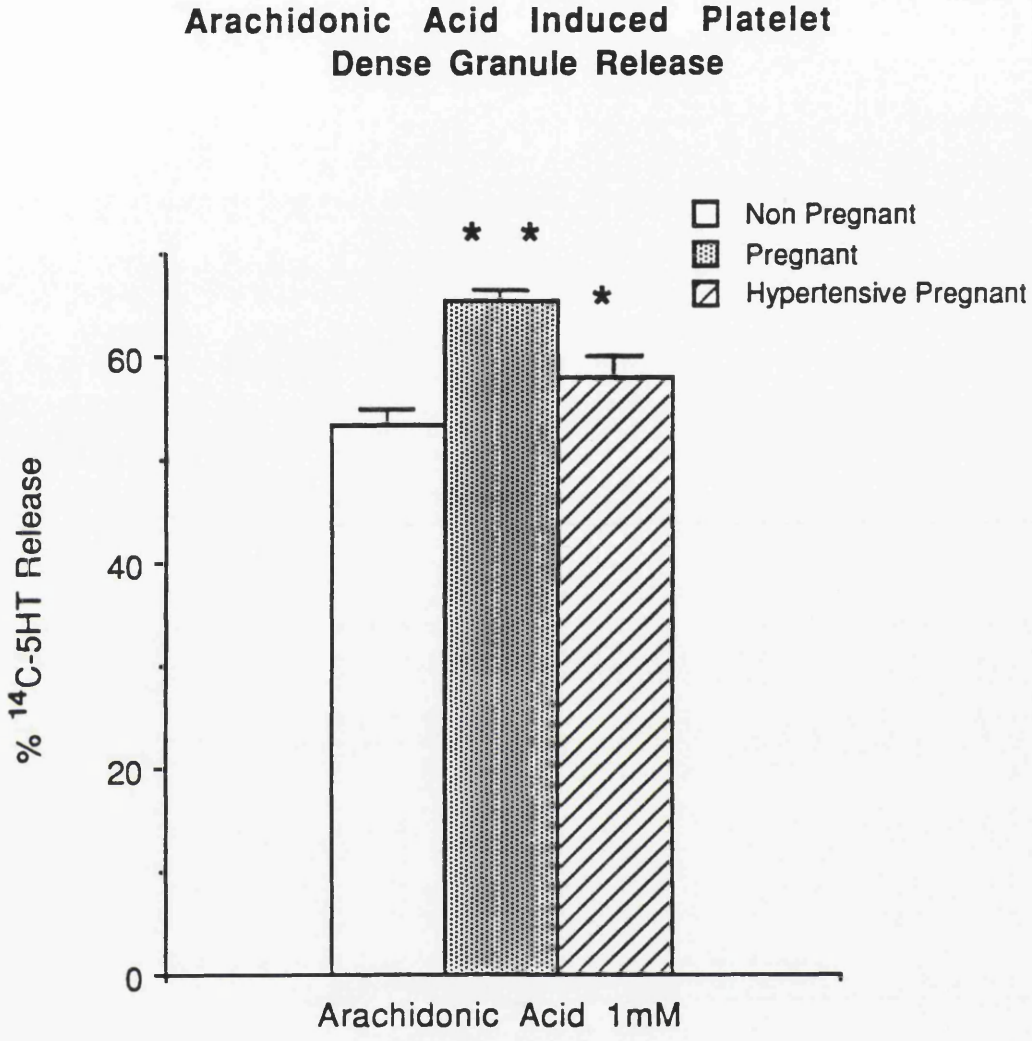


Figure 3.1

The figure shows the extent of the platelet release reaction on stimulation with 1mmol/l arachidonic acid in the absence of inhibitors of platelet behaviour in the three subject groups. Hypertensive pregnant = pregnancy induced hypertension and pre-eclampsia. Means and standard errors are illustrated. ** = $p < 0.01$, comparing non pregnant with pregnant; * = $p < 0.05$ comparing healthy pregnant with hypertensive pregnant subjects.

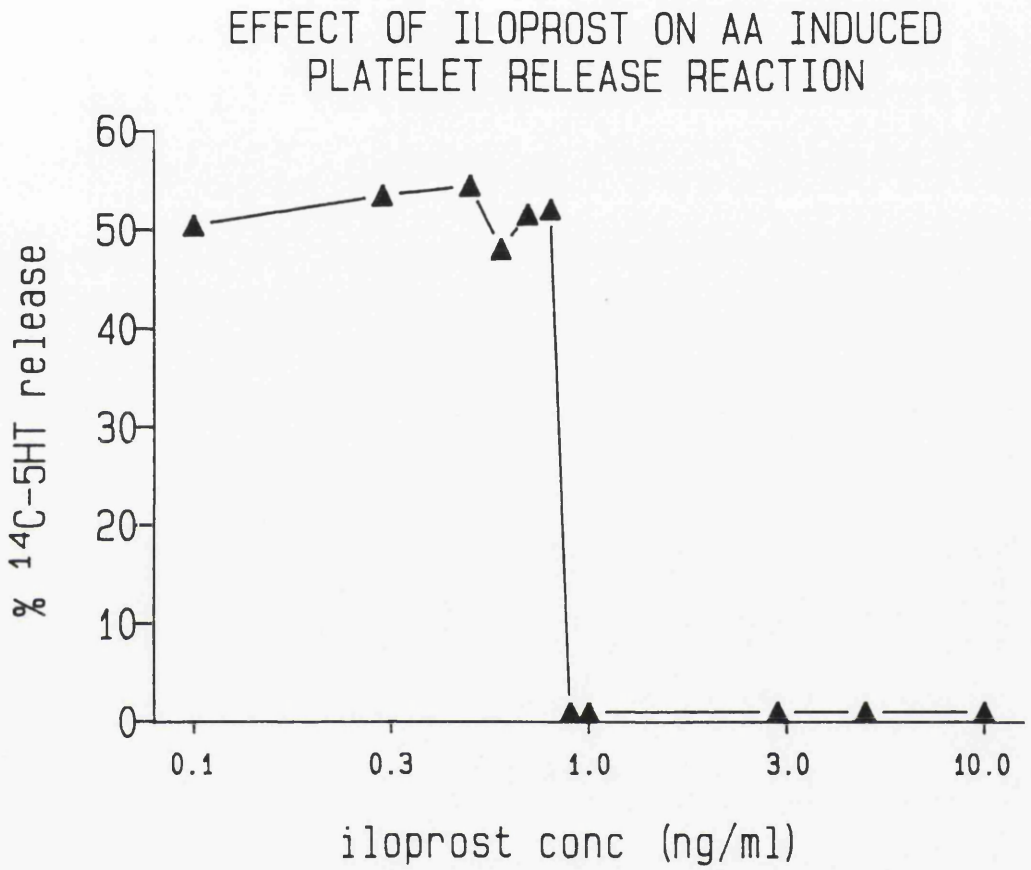


Figure 3.2

The figure shows the effect on the arachidonic acid induced platelet release reaction of increasing concentrations of iloprost in PRP from one subject. The x axis has a log scale. In this case the release reaction is abolished at or above 1ng/ml iloprost. Concentrations less than 1ng/ml have little effect on the extent of the release reaction. The "threshold" concentration of iloprost is therefore 1ng/ml.

difference between non pregnant and hypertensive pregnant women, but the latter demonstrated significantly less dense granule release than the healthy pregnant group. Some of the patients with severe pre-eclampsia showed particularly reduced secretory responses, three subjects with fulminating disease releasing <40% ^{14}C-5HT following stimulation with 1mmol/l arachidonic acid. Although coagulation studies were not formally included in this study, coagulation screens were carried out as part of the clinical investigation in the majority of subjects in the PIH/pre-eclampsia group. These data were retrieved retrospectively in view of the possibility that the reduction in dense granule release was due to platelet 'exhaustion' secondary to disseminated intravascular coagulation (D.I.C.). Two out of the three pre-eclamptic patients demonstrating < 40% ^{14}C-5HT release had evidence of D.I.C. with thrombocytopenia, prolonged thrombin clotting times, raised fibrin degradation products and microangiopathic haemolysis.

3.4 Inhibition of Arachidonic Acid Induced Platelet Behaviour by Cyclic AMP Manipulators.

When PRP from all subjects was incubated in the presence of iloprost, PGD_2, forskolin, and AH-P719, as the concentration of the inhibitor was increased, a clear point was reached at which total abolition of platelet aggregation and release occurred. This was termed the threshold concentration of the inhibitor and was noted for each of these agents in each subject. An example of how the threshold was determined for iloprost is shown in Figure 3.2.

Inhibition of Platelet Behaviour by Adenylate Cyclase Stimulators Acting via Surface Receptors.

The threshold concentrations of iloprost and PGD_2 required to abolish platelet aggregation and release are shown in Figures 3.3 and 3.4. Significantly higher concentrations of both prostaglandins were required to inhibit platelet

"Threshold" Concentration of cAMP Manipulators Required to Abolish Platelet Aggregation and Release

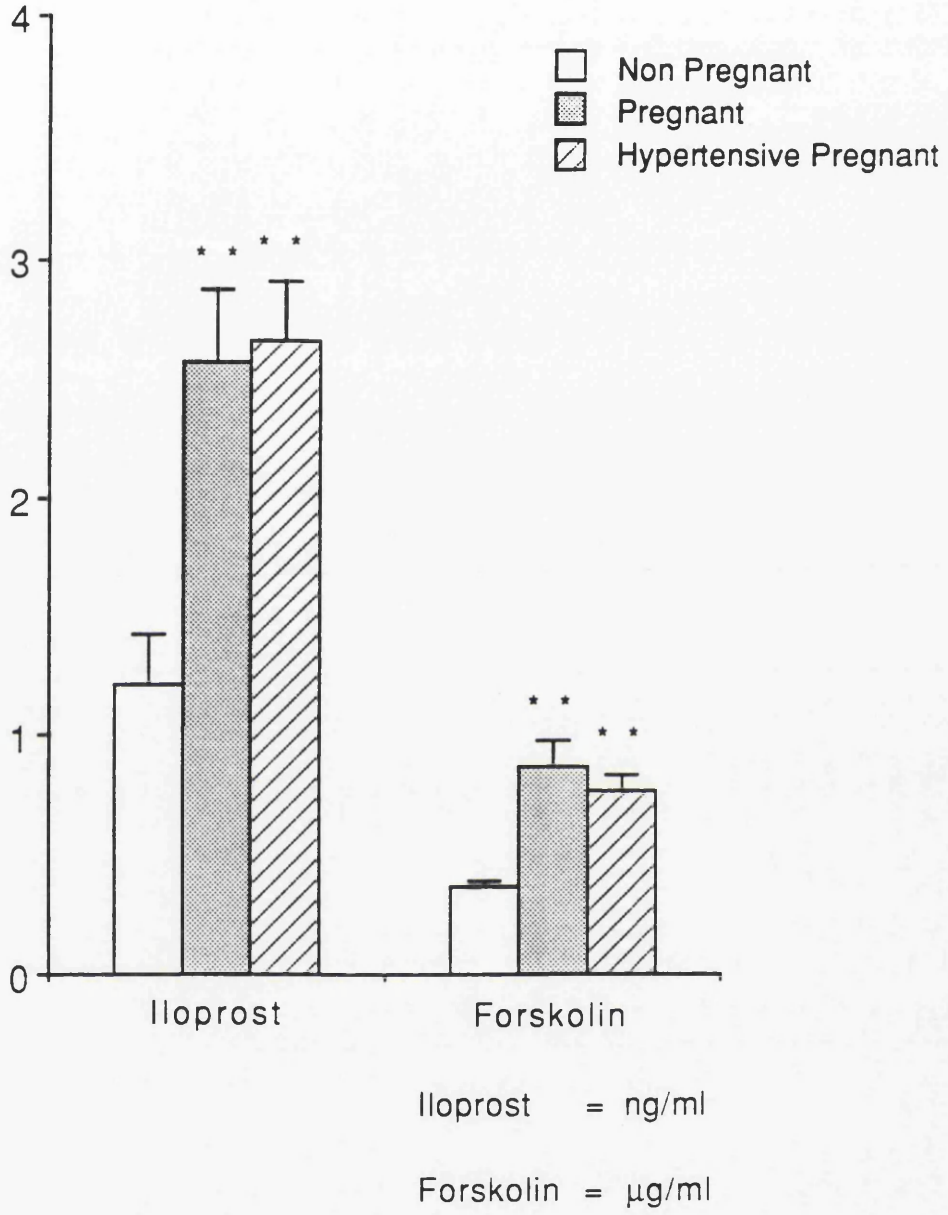


Figure 3.3

The figure shows the lowest concentration of iloprost and of forskolin at which AA induced platelet aggregation and release were totally abolished. Values are means with bars indicating S.E.M. In each case, comparison of non pregnant with healthy pregnant, and non pregnant with hypertensive pregnant subjects yielded a p value of <0.01.

"Threshold" Concentration of cAMP Manipulators Required to Abolish Platelet Aggregation and Release

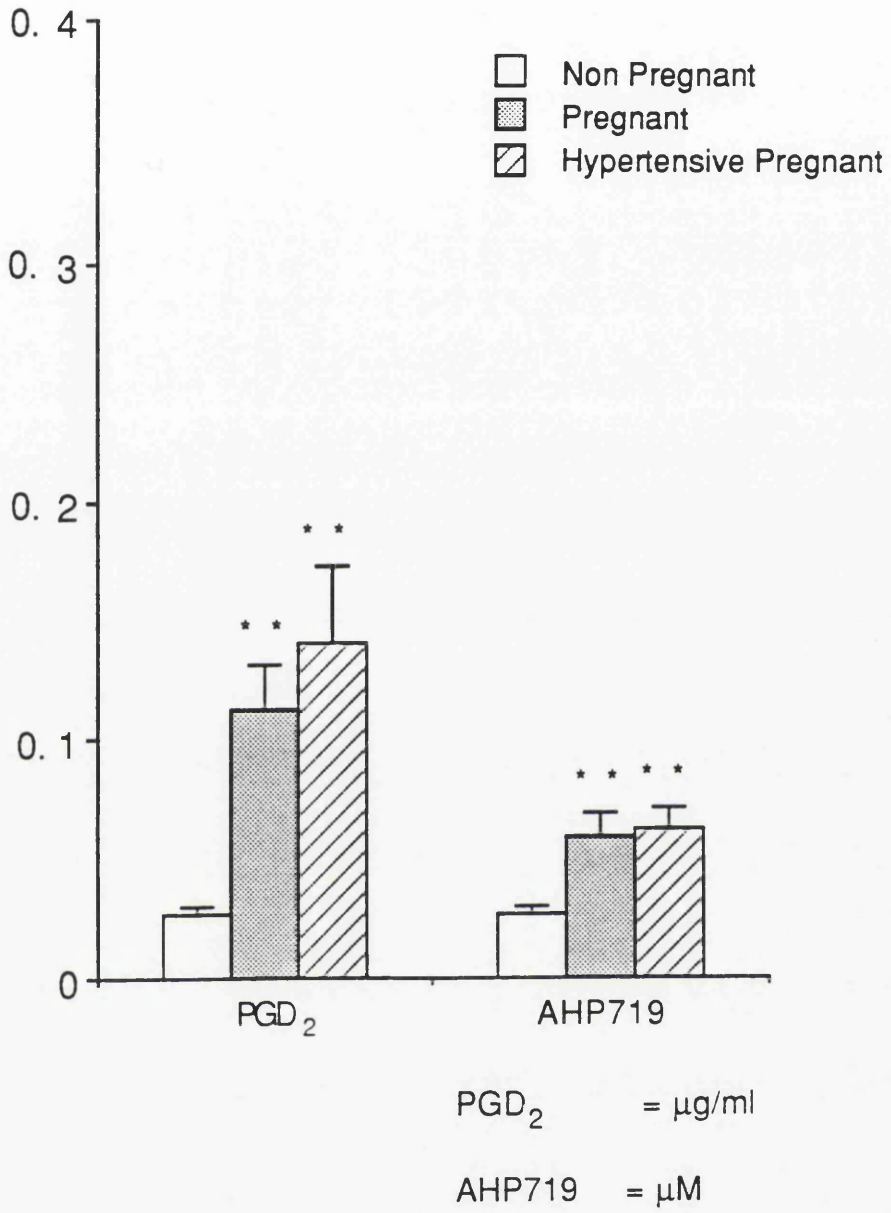


Figure 3.4

The figure shows the lowest concentration of PGD_2 and of AH-P719 at which AA induced platelet aggregation and release were totally abolished . Values are means with bars indicating S.E.M. In each case, comparison of non pregnant with healthy pregnant, and non pregnant with hypertensive pregnant subjects yielded a p value of <0.01.

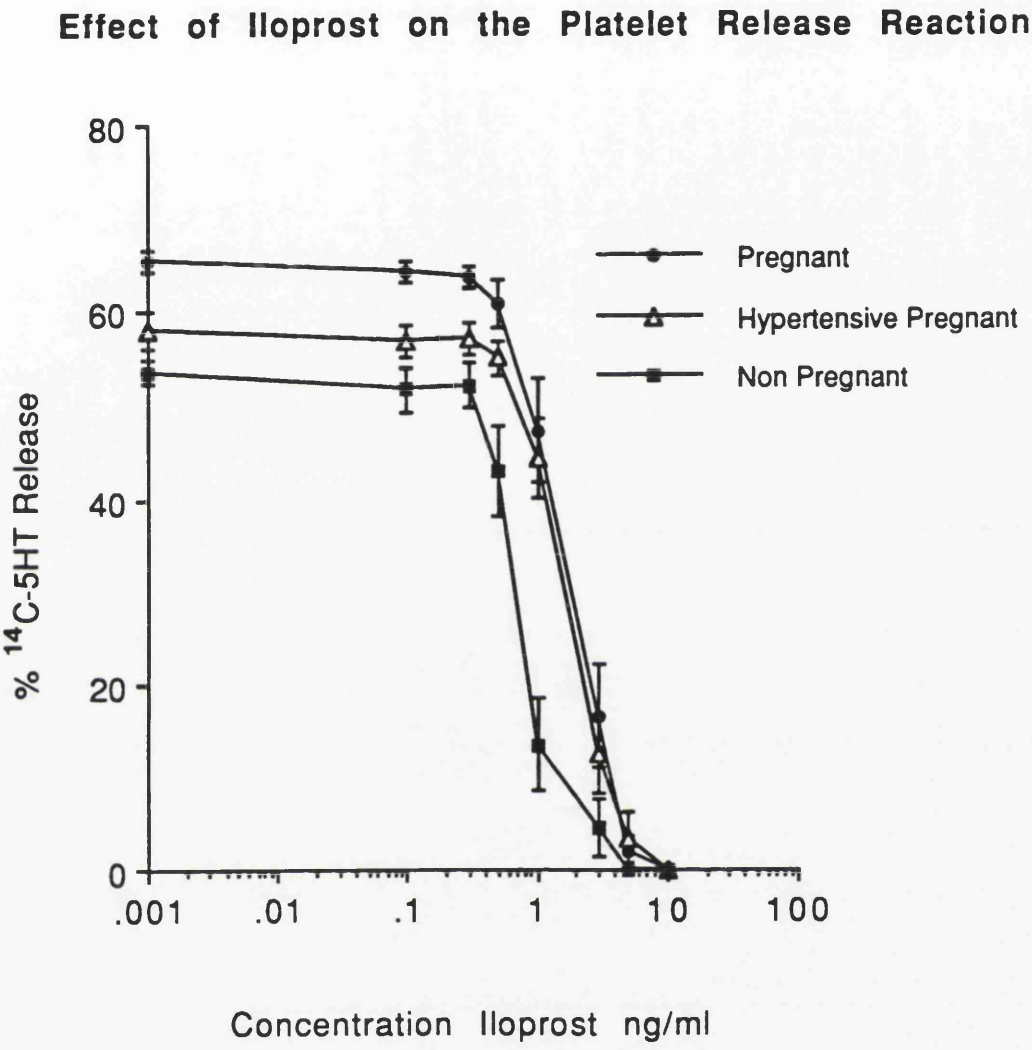


Figure 3.5

Percentage release of ^{14}C-5HT is plotted against concentration of iloprost for each subject group. The x axis has a log scale. All values are means $\pm$ S.E.M. The concentration-response curve is shifted to the right in both healthy and hypertensive pregnant subjects compared with non pregnant subjects.

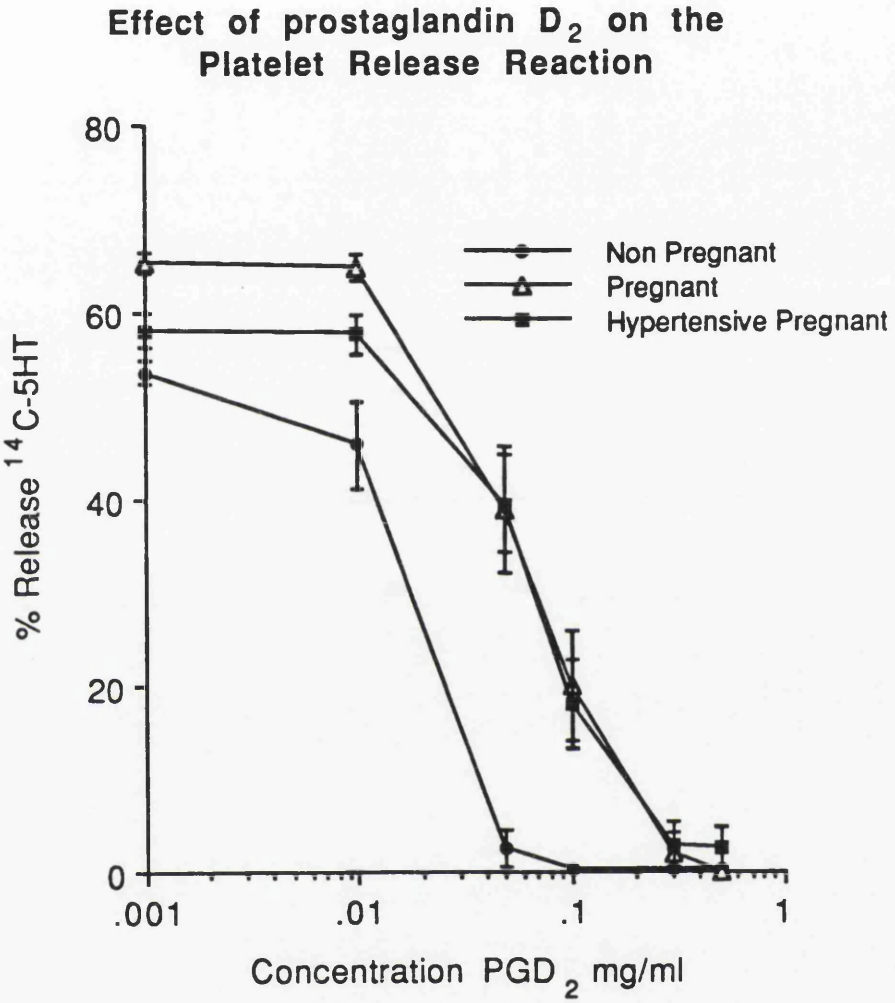


Figure 3.6

Percentage release of ^{14}C-5HT is plotted against concentration of PGD_2 for each subject group. The x axis has a log scale. All values are means $\pm$ S.E.M. The concentration-response curve is shifted to the right in healthy and hypertensive pregnant subjects, compared with non pregnant subjects.

behaviour in both healthy pregnant and hypertensive pregnant groups compared with the non pregnant group. There was no difference between normal primigravidae and women with PIH or pre-eclampsia. The loss of platelet sensitivity to inhibition of their behaviour by these prostaglandins during pregnancy could also be demonstrated by a shift in the concentration response curve to the right as illustrated in Figure 3.5 and 3.6.

Inhibition of Platelet Behaviour by Forskolin.

A similar pattern was seen when the effects of forskolin on arachidonic acid induced platelet dense granule release were investigated. Figure 3.3 shows that a higher concentration of forskolin was required to abolish platelet aggregation and release in healthy and hypertensive pregnant women compared with non pregnant controls. Again, there was no difference between normal pregnant subjects and those in the PIH/pre-eclampsia group. The loss of sensitivity to forskolin is also evident from the concentration response curve illustrated in Figure 3.7.

Inhibition of Platelet Behaviour by the Phosphodiesterase Inhibitor AH-P719.

Threshold concentrations of the PDE inhibitor AH-P719 required to abolish platelet aggregation and release in each subject group are illustrated in Figure 3.4 and the concentration response curve for this agent is shown on Figure 3.8. It can be seen that platelet sensitivity to AH-P719 was again significantly and similarly reduced in both pregnant groups of subjects compared with non pregnant controls, although this effect was less marked than in the case of the adenylate cyclase stimulators.

Inhibition of Platelet Behaviour By Dazmegrel.

It was not appropriate to analyze the data relating to the thromboxane synthetase inhibitor dazmegrel in terms of an inhibitory threshold concentration as

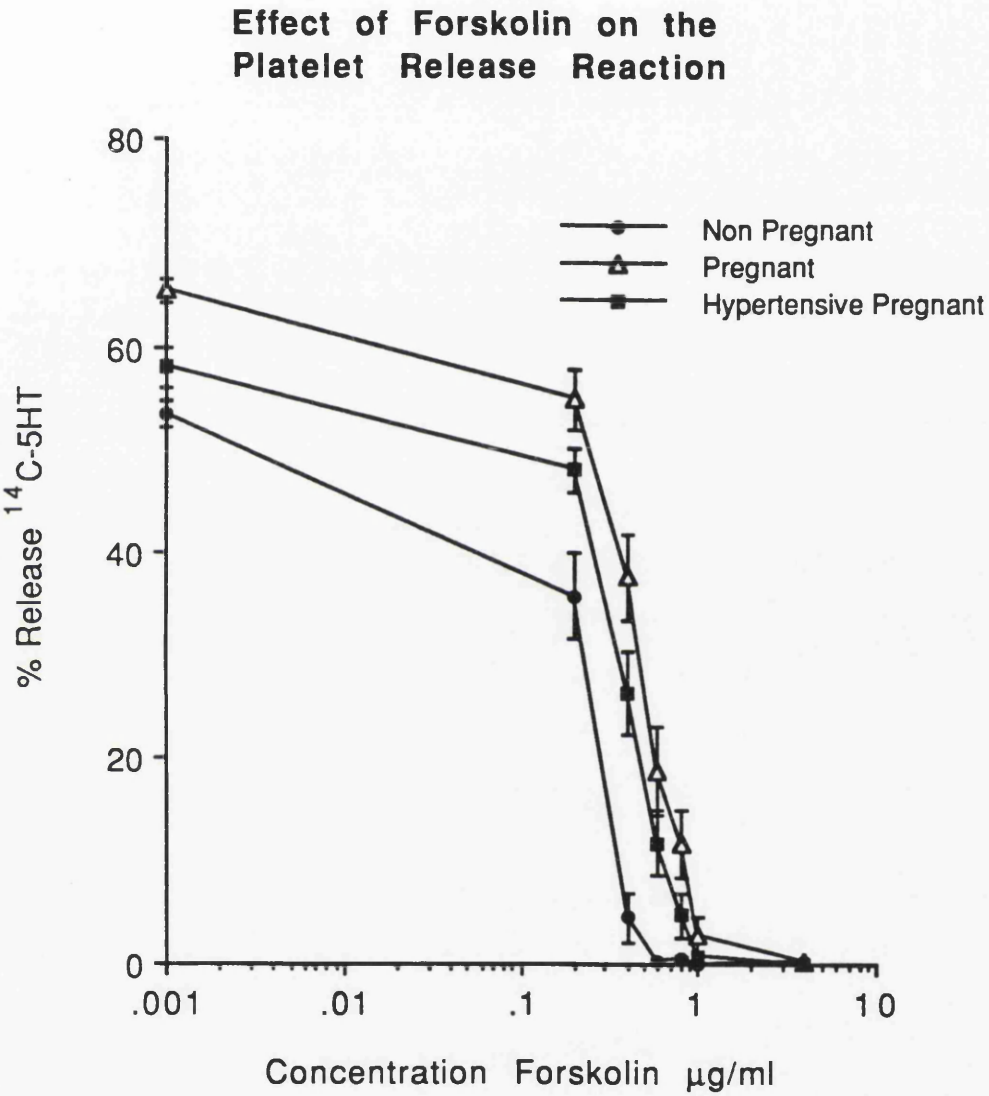


Figure 3.7

Percentage release of ^{14}C-5HT is plotted against concentration of forskolin for each subject group. The x axis has a log scale. All values are means $\pm$ S.E.M. The concentration-response curve is shifted to the right in healthy and hypertensive pregnant subjects, compared with non pregnant subjects.

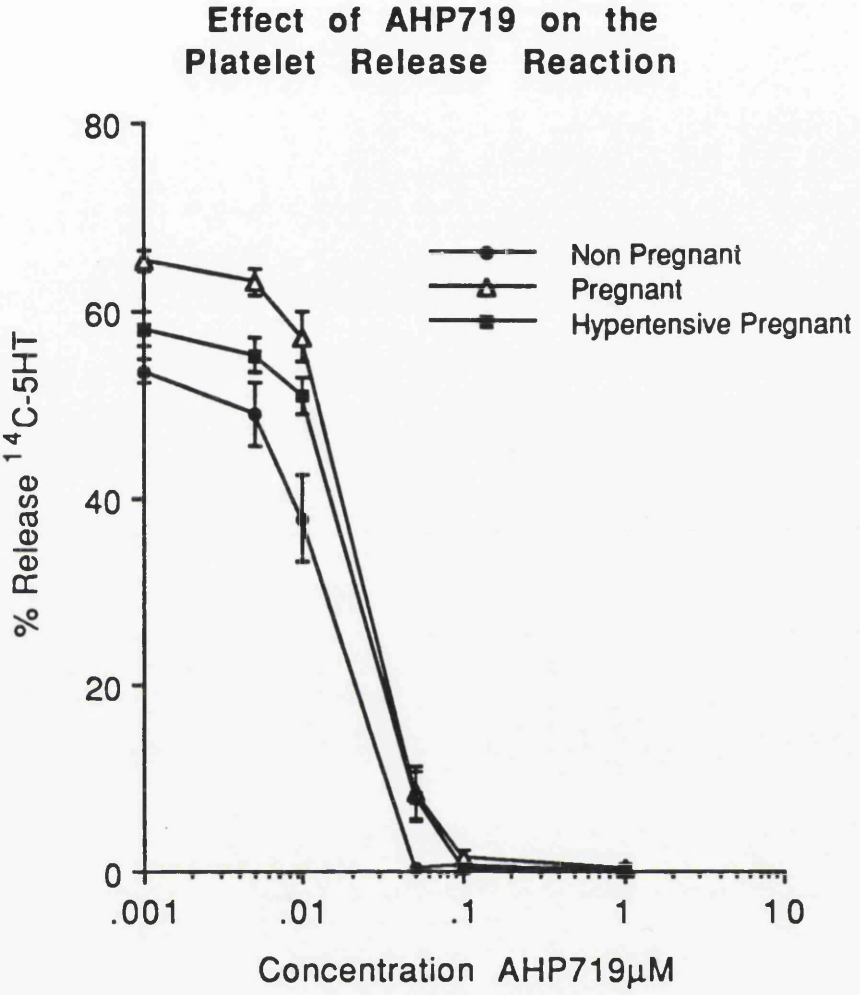


Figure 3.8

Percentage release of ^{14}C-5HT is plotted against concentration of AH-P719 for each subject group. The x axis has a log scale. All values are means $\pm$ S.E.M. The concentration-response curve is slightly shifted to the right in healthy and hypertensive pregnant subjects, compared with non pregnant subjects.

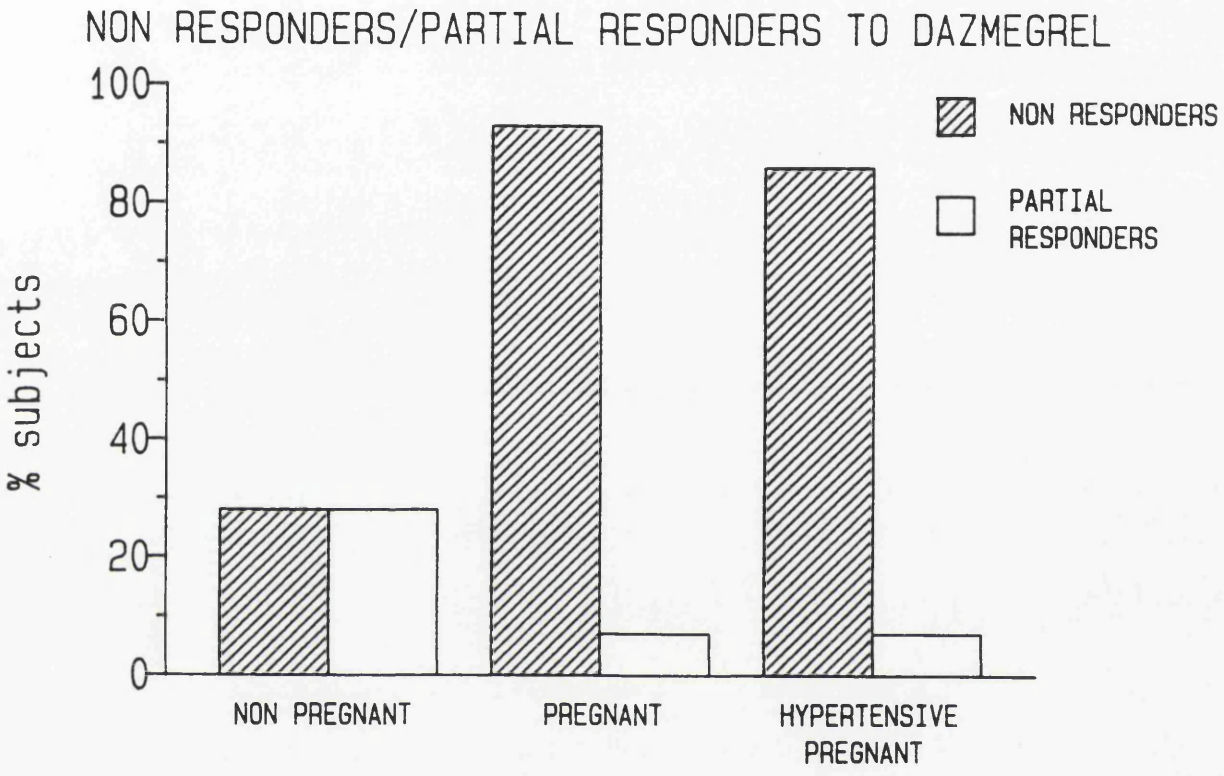


Figure 3.9

The bar graph shows the proportion of subjects in each group who were "non responders" to the thromboxane synthetase inhibitor dazmegrel. The shaded bars represent those subjects whose platelet behaviour was not completely inhibited by either 100μM or 500μM dazmegrel, and the unshaded bars represent subjects whose platelet behaviour was completely inhibited by 500μM but not by 100μM dazmegrel. The number of non responders to dazmegrel was significantly increased in both healthy and hypertensive pregnancy ($p < 0.0001$ for 100uM dazmegrel, and $p < 0.00001$ for 500μM dazmegrel, Fisher exact test).

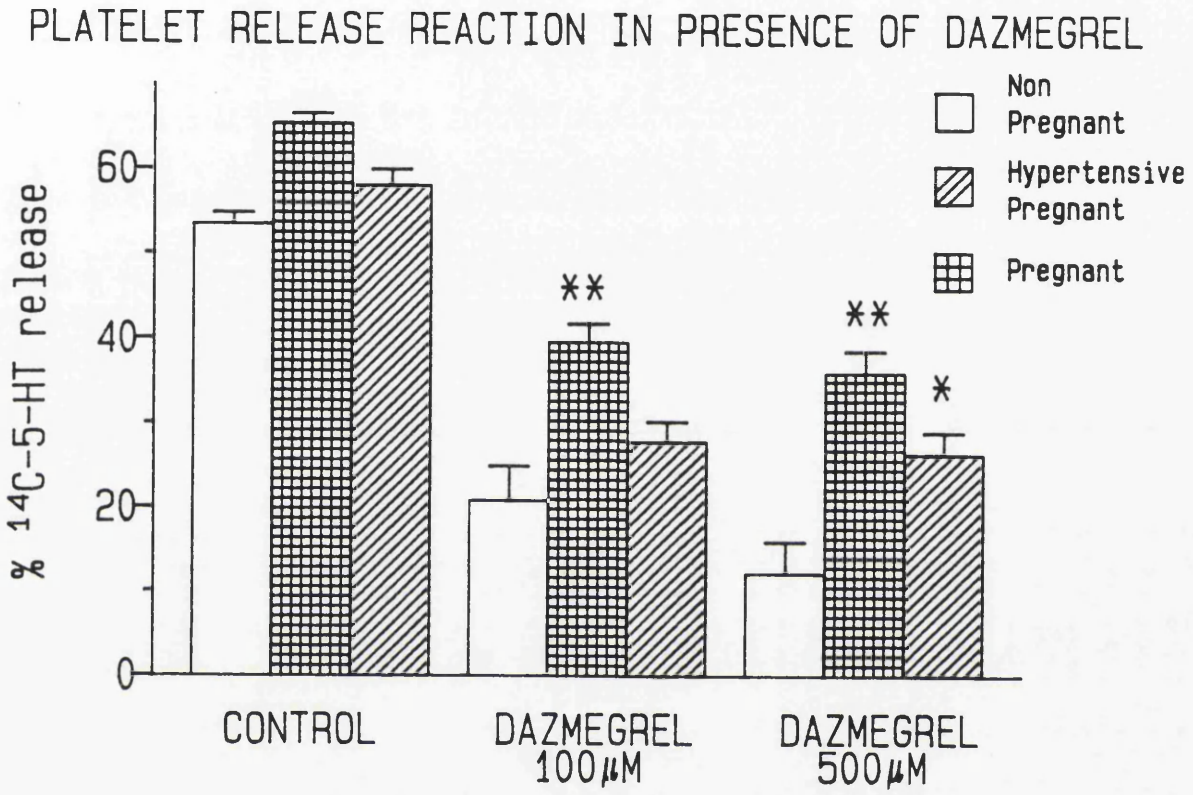


FIGURE 3.10

The bar graph shows the effect on the extent of the arachidonic acid induced platelet release reaction of the thromboxane synthetase inhibitor dazmegrel. It is evident that the release reaction was inhibited to some extent in all groups of subjects by both concentrations of dazmegrel. In the presence of 100μM dazmegrel, the p value for Kruskal-Wallis ANOVA was 0.0002, and extent of the release reaction was significantly greater in pregnant compared with non pregnant and hypertensive pregnant subjects ($p < 0.01$). In the presence of 500μM dazmegrel, the Kruskal-Wallis ANOVA p value was < 0.00001, and the extent of the release reaction was significantly greater in pregnant ($p < 0.01$) and hypertensive pregnant ($p < 0.05$) subjects compared with non pregnant women. In this case comparison of the pregnant and hypertensive pregnant groups revealed no significant difference.

TABLE 3.3

SERUM THROMBOXANE B_2

The table shows means and SEM of levels of thromboxane B_2 in serum in the three subject groups. There was no significant difference in results between groups ($p = 0.968$).

GROUP	SERUM THROMBOXANE B_2 pmoles/10^7 platelets
Non Pregnant	33.86 ± 3.20
Healthy Pregnant	33.72 ± 3.46
Hypertensive Pregnant	42.48 ± 7.75

the effects of only two concentrations were investigated. It is well established outwith the context of pregnancy that individuals can be divided into 'responders' or 'non responders' to thromboxane synthetase inhibitors depending upon whether complete inhibition of their platelet behaviour is observed in vitro in the presence of the drug.[247-249,252,253] In the present study, when subjects were divided into responders and non responders for each concentration of dazmegrel there was a significantly increased proportion of subjects in both healthy and hypertensive pregnant groups who fell into the category of non responders ($p < 0.0001$ for 100uM dazmegrel, $p < 0.00001$ for 500uM dazmegrel). There was no difference in the distribution of responder status between healthy and hypertensive pregnant subjects. Response to dazmegrel in each subject group is illustrated in Figure 3.9. As some degree of inhibition of dense granule release occurred even in non responders to the thromboxane synthetase inhibitor, data were also analyzed with respect to the extent of the release reaction. The effect of dazmegrel on ^{14}C-5HT release induced by 1mM arachidonic acid is shown in Figure 3.10.

3.5 Serum Thromboxane B_2.

There was no difference between subject groups in serum thromboxane B_2 levels, although the levels were more variable in hypertensive pregnant women. The data are shown in Table 3.3.

4. DISCUSSION

It has been previously reported that platelets from pregnant women are less sensitive to inhibition of their behaviour by prostacyclin.[330,331,332] This study has extended these observations to other cyclic AMP modulators, and has compared non pregnant women, healthy pregnant women and those with PIH or pre-eclampsia.

The study has shown that during pregnancy, platelets release more 5-HT in response to stimulation with arachidonic acid than those from non pregnant women. Although it has been previously reported that, during pregnancy, platelets are more sensitive to arachidonic acid,[321,322,327] no change in the extent of the release reaction in PRP was found in these studies.[321,322] In the present study, in the hypertensive pregnant group, the trend reverted and there was a significant reduction in the release reaction compared with results in healthy pregnant women. These findings differ from other studies of platelet behaviour in PIH,[322] but are in keeping with those of an early study by Whigham et al, in which, as in the present study, the majority of hypertensive pregnant subjects had pre-eclampsia.[317] Some of the present pre-eclamptic subjects, two of whom had disseminated intravascular coagulation (DIC), demonstrated a markedly reduced release reaction, which may be a result of the presence of exhausted platelets, as described in other clinical conditions in which in vivo platelet activation and DIC occur.[413,439]

The results presented in this chapter also indicate that platelets in healthy and hypertensive pregnancy are generally less sensitive to the inhibitory effects of a wide range of agents which act by increasing cyclic AMP. There was no difference in this respect between healthy pregnant women and those with PIH or pre-eclampsia. This is unlikely to be due to patient selection as the subjects in the PIH/pre-eclampsia group demonstrated a number of features associated with significant disease such as proteinuria and raised serum uric acid, both of which have been associated with other changes in platelet behaviour in previous studies.[322,353] Furthermore, the hypertensive subjects had a reduced platelet count and indices of platelet size in keeping with a degree of platelet activation in vivo.[289,290]

Reduction of platelet sensitivity during pregnancy to inhibitory prostaglandins which stimulate adenylate cyclase via a receptor and G protein dependent mechanism[40,166,195-198,202] has been demonstrated, but similar results were obtained with forskolin which can act directly on the catalytic subunit of adenylate cyclase.[200] Although slightly less marked, a statistically significant reduction in platelet sensitivity to the cyclic AMP specific phosphodiesterase inhibitor also occurred in both healthy and hypertensive pregnant groups. Furthermore, findings reported in a previous study[322] have been confirmed by the demonstration of an increase in proportion of non responders to the thromboxane synthetase inhibitor dazmegrel in both pregnant groups. This is in keeping with the reduction in platelet sensitivity to PGD_2 during pregnancy as there is evidence that this prostaglandin, synthesised in platelets when thromboxane synthetase is blocked, plays a role in inhibition of platelet behaviour by thromboxane synthetase inhibitors.[247,248,249] Indeed, outwith the context of pregnancy, platelets from individuals who were non responders to the thromboxane synthetase inhibitor dazoxiben, have been shown to synthesise less cyclic AMP in response to both PGD_2 and the combination of dazoxiben and AA.[249,254]

When considering the effects of any inhibitory agent on arachidonic acid induced platelet behaviour, the influence of the activity of the thromboxane biosynthetic pathway must be taken into account. Any increase in thromboxane A_2 generation in healthy or hypertensive pregnant subjects could theoretically contribute to a reduction in efficacy of cyclic AMP manipulators in these subject groups. In agreement with some previous studies,[292,327] however, the present study has demonstrated no change in levels of thromboxane B_2, the stable hydrolysis product of thromboxane A_2, in serum in either healthy or hypertensive pregnancy. Serum thromboxane B_2 reflects platelet thromboxane production under

conditions of spontaneous clotting of whole blood in vitro. Other investigators, by contrast, have shown evidence of increased platelet thromboxane production both in vivo[309] and in vitro[329] in pregnancy. The findings of the present study would not, however, support a major role for alterations in thromboxane A_2 synthesis in the observed reduction in sensitivity to cyclic AMP manipulators during pregnancy.

On the basis of the data presented, the hypotheses could be formed that platelets from pregnant women are either less sensitive to the inhibitory effects of cyclic AMP itself or that less cyclic AMP is synthesised in response to a wide range of pharmacological agents capable of increasing the level of this inhibitory second messenger. The measurement of platelet cyclic AMP levels was carried out to address this question, and the resulting data are presented in the following chapter.

CHAPTER 4.

A CROSS-SECTIONAL STUDY OF PLATELET CYCLIC AMP IN HEALTHY AND HYPERTENSIVE PREGNANCY.

CHAPTER 4.

A CROSS-SECTIONAL STUDY OF PLATELET CYCLIC AMP IN HEALTHY AND HYPERTENSIVE PREGNANCY.

1.INTRODUCTION.

The data presented in Chapter 3. demonstrated that platelets from healthy and hypertensive pregnant women were less sensitive to the inhibitory effects of not only prostacyclin, but of a wide range of agents which act by increasing cyclic AMP. In order to determine whether this results from reduced synthesis of cyclic AMP by platelets during pregnancy, platelet cyclic AMP was measured in a cross-sectional study carried out in parallel with that investigating the effects on platelet behaviour of cyclic AMP manipulators.

2. METHODS.

Subjects corresponded exactly with those who took part in the study presented in the previous chapter, and were divided as before into non pregnant, healthy pregnant, and hypertensive pregnant groups. Blood was taken at the same time, using the same venepuncture as for the studies reported in Chapter 3. Trisodium citrate dihydrate 3.13% w/v was again used as the anticoagulant (9 parts blood : 1 part citrate).

Platelet rich plasma in which the platelet count was adjusted to 250 x 10^9/l was assayed for basal and stimulated cyclic AMP by the ^{3}H-adenine labelling method described in Chapter 2. Following the 90 minute incubation period with ^{3}H-adenine, PRP was incubated in duplicate aliquots of 0.45ml at 37°C in an unstirred system with various pharmacological agents that raise cyclic AMP levels

or with saline for measurement of basal platelet cyclic AMP. Samples were pre-incubated for 2 minutes in the water bath at 37°C in the presence of either 25ul saline or 25ul of the phosphodiesterase inhibitor AH-P719, before adding 25ul of an adenylate cyclase stimulator. Incubation was continued for a further 6 minutes before stopping the reaction and lysing the platelets with 2ml ice cold 15% trichloroacetic acid containing ^{14}C-cyclic AMP as a recovery label. Following centrifugation, the assay was completed by column chromatography on the platelet supernatants as described in Chapter 2.

The same pharmacological agents were used as in the release reaction experiments but final concentrations were as follows: iloprost was used at 0.5, 5, and 50ng/ml; PGD_2 at 0.05, 0.5, and 5ug/ml, forskolin at 4ug/ml, and dazmegrel at 500uM. Concentrations of these agents were chosen from pilot experiments in which concentration response curves of their effect on platelet cyclic AMP levels were constructed (see Chapter 2). The concentration of dazmegrel was chosen to ensure maximal inhibition of thromboxane synthetase, based on published data[440]. Measurements of cyclic AMP in response to adenylate cyclase stimulators were generally made both in the presence and absence of inhibition of phosphodiesterase with 100uM AH-P719. This concentration of the phosphodiesterase inhibitor was at the top of the concentration response curve when platelet cyclic AMP was assayed in the presence of a range of concentrations of AH-P719 in pilot experiments (see Chapter 2). To investigate the effect of the thromboxane synthetase inhibitor, dazmegrel (25ul) was placed in the tubes before the PRP and, after 2 minutes, 1mM AA (25ul) was added. No phosphodiesterase inhibitor was included in this case. Cyclic AMP was also assayed in PRP to which only saline and 1mM AA was added. The effect of AA on iloprost stimulated cyclic AMP levels was examined by measuring the cyclic

TABLE 4.1

PLATELET CYCLIC AMP LEVELS UNDER CONDITIONS OF BASAL ADENYLATE CYCLASE ACTIVITY AND ON STIMULATION WITH LOW CONCENTRATIONS OF PROSTAGLANDINS

The Table shows means and standard errors of platelet cyclic AMP levels in the three groups of subjects. All cyclic AMP levels are expressed as % conversion of ^3H adenine to ^3H cyclic AMP. NP = non pregnant; P = healthy pregnant; PIH = pre-eclampsia and pregnancy induced hypertension; NS = not significant.

Reagent	NP	P	PIH	p value	Comparison
Saline*	0.0345±0.0031	0.0400±0.0037	0.0349±0.0034	NS	All comparisons
AH-P719$	0.1389±0.0419	0.1317±0.0419	0.1353±0.0436	NS	All comparisons
0.5ng/ml Iloprost	0.0457±0.0037	0.0571±0.0044	0.0486±0.0040	NS	All comparisons
0.5ng/ml Iloprost + AH-P719$	0.2994±0.0113	0.3067±0.0116	0.2874±0.0118	NS	All comparisons
0.05μg/ml PGD_2	0.1213±0.0109	0.1165±0.0089	0.0952±0.0065	NS	All comparisons
0.05μg/ml PGD_2 AH-P719$	0.5738±0.0292	0.5109±0.0212	0.4548±0.0201	$p<0.01$	PIH v NP

* Basal cyclic AMP levels

$ AH-P719 was always used at a concentration of 100 μM

AMP response to 5ng/ml iloprost in the presence of 1mM AA. The drug incubation protocol is shown in detail in Appendix 4.

Results were expressed as % conversion of ^{3}H-adenine to ^{3}H-cyclic AMP and were corrected both for uptake of ^{3}H-adenine for each subject and for cyclic AMP recovery in each sample. There was no difference in uptake of ^{3}H-adenine between subject groups ($p=0.5928$).

For the purposes of statistical analysis, subjects with PIH and pre-eclampsia were again treated as one group as no differences existed between them when treated separately. All comparisons between the three subject groups were, as before, by Kruskal-Wallis one way analysis of variance by ranks. The Wilcoxon matched pairs signed rank test was used to test the effect within subject groups of the presence of arachidonic acid on iloprost induced production of cyclic AMP by platelets.

3.RESULTS

Clinical, haematological and biochemical data for the three groups of subjects have already been documented in Chapter 3.

3.1 Platelet Cyclic AMP Levels Under Conditions of Basal Adenylate Cyclase Activity.

In the absence of any agents which stimulated adenylate cyclase, there was no difference in cyclic AMP generation in PRP from the three groups of subjects. This was the case whether or not the phosphodiesterase inhibitor AH-P719 was present. The results are shown in Table 4.1.

3.2 Platelet Cyclic AMP Levels Stimulated by Receptor Dependent Mechanisms

In response to 0.5ng/ml of iloprost, the rise in cyclic AMP was very small

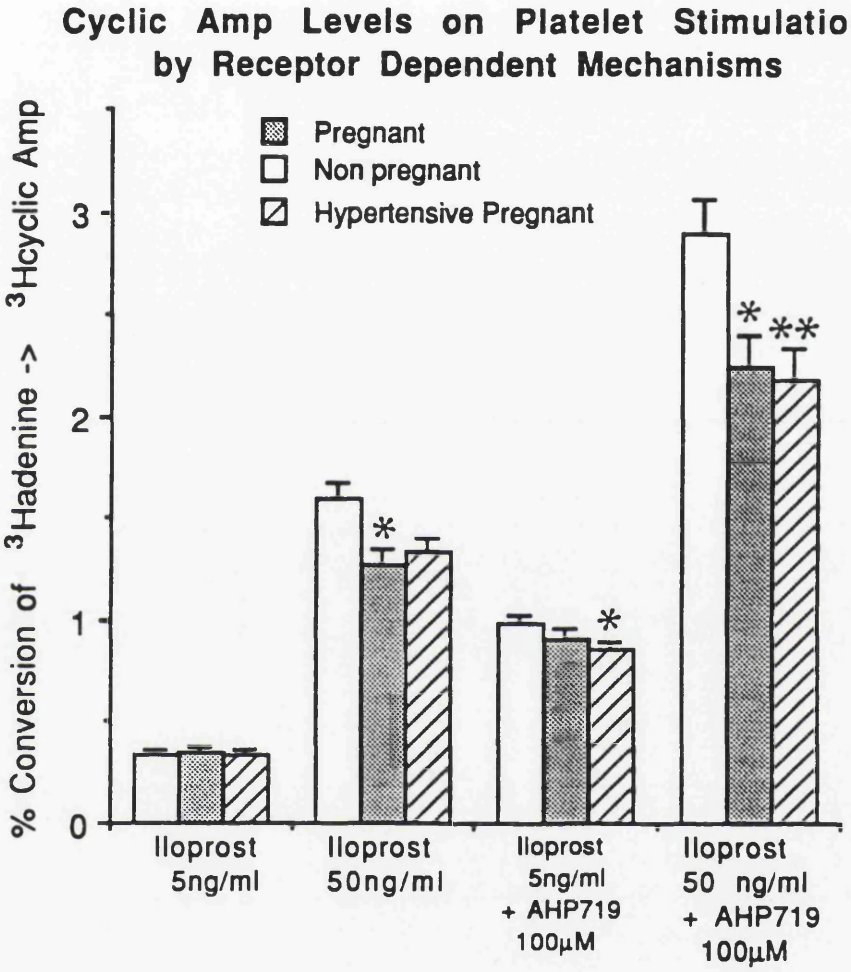


Figure 4.1

The bar graph shows mean and S.E.M. cyclic AMP levels in platelets incubated with iloprost. * signifies a p value <0.05, ** signifies $p<0.01$. In each case one way ANOVA was used and significant differences were obtained by comparison with the non pregnant group.

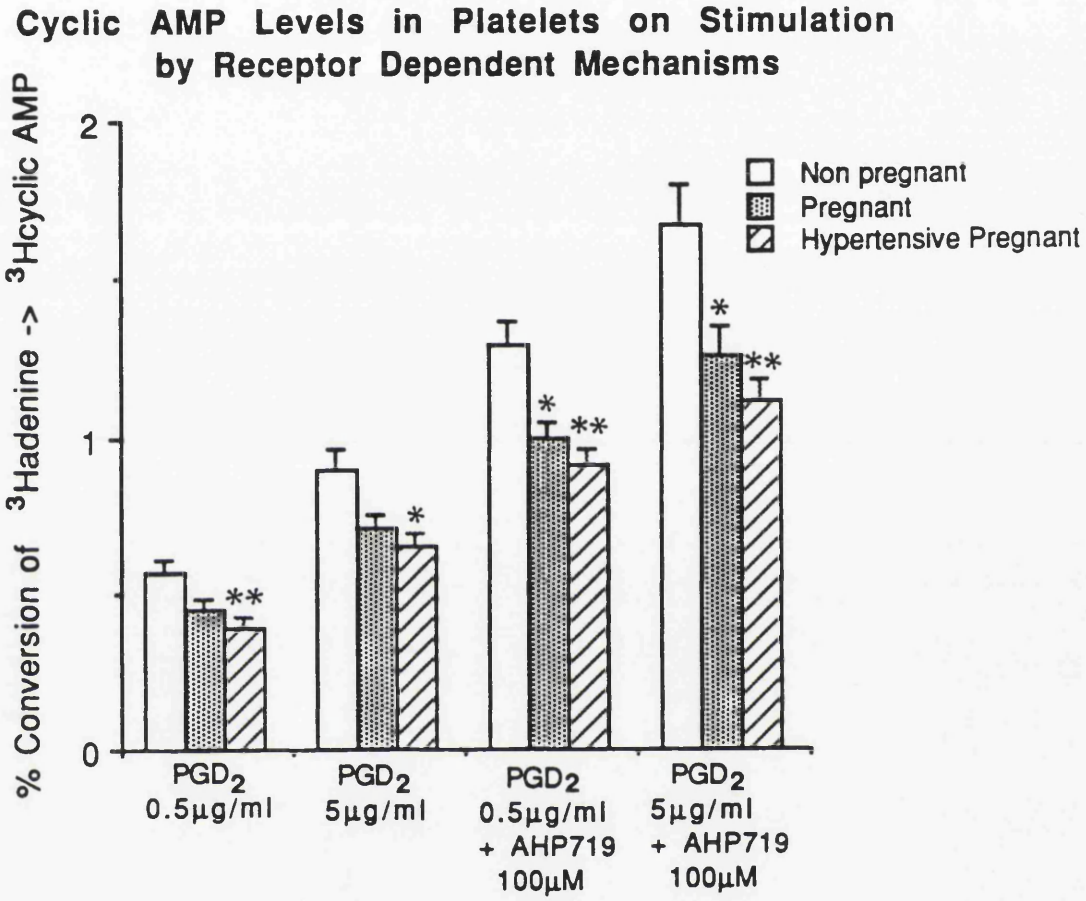


Figure 4.2

The figure shows platelet cyclic AMP responses to PGD_2. * indicates statistical significance at $p<0.05$, ** indicates $p<0.01$. Comparisons were made by one way ANOVA and all significant values refer to the comparison of both healthy and hypertensive pregnant groups with the non pregnant group.

in all subjects, and there was no difference between groups. There was marked synergism between iloprost and the phosphodiesterase inhibitor AH-P719 in all subjects, but cyclic AMP levels remained similar in all three groups of women when platelets were stimulated with 0.5ng/ml iloprost in the presence of AH-P719 (Table 4.1). When platelets were incubated with 0.05ug/ml PGD_2, cyclic AMP accumulation was reduced in the hypertensive pregnant group, compared with the healthy pregnant and non pregnant groups. This difference reached statistical significance when the phosphodiesterase inhibitor was also present ($p<0.01$ in comparison with the non pregnant group, Table 4.1)

Significantly less cyclic AMP was generated by platelets from hypertensive pregnant women in response to 5ng/ml iloprost when metabolism of the cyclic nucleotide was inhibited by AH-P719 (Figure 4.1). When the concentration of iloprost was further increased tenfold, there was also a significant reduction in platelet cyclic AMP levels in healthy pregnancy, whether or not phosphodiesterase was inhibited (Figure 4.1).

Figure 4.2 shows platelet cyclic AMP responses to PGD_2 when present at concentrations higher than those generally required to inhibit platelet behaviour. A repeating pattern of reduction in stimulated platelet cyclic AMP levels in both healthy and hypertensive pregnant subjects was demonstrated.

Although slightly more marked in pregnant women with hypertension, the reduced platelet cyclic AMP response to both prostanoids was primarily a pregnancy effect. Subgroup analysis of the hypertensive group revealed no significant difference in basal or stimulated platelet cyclic AMP levels between women with pre-eclampsia and those with non proteinuric PIH. It is also noteworthy that the observed differences between subject groups were more marked when phosphodiesterase was inhibited with AH-P719.

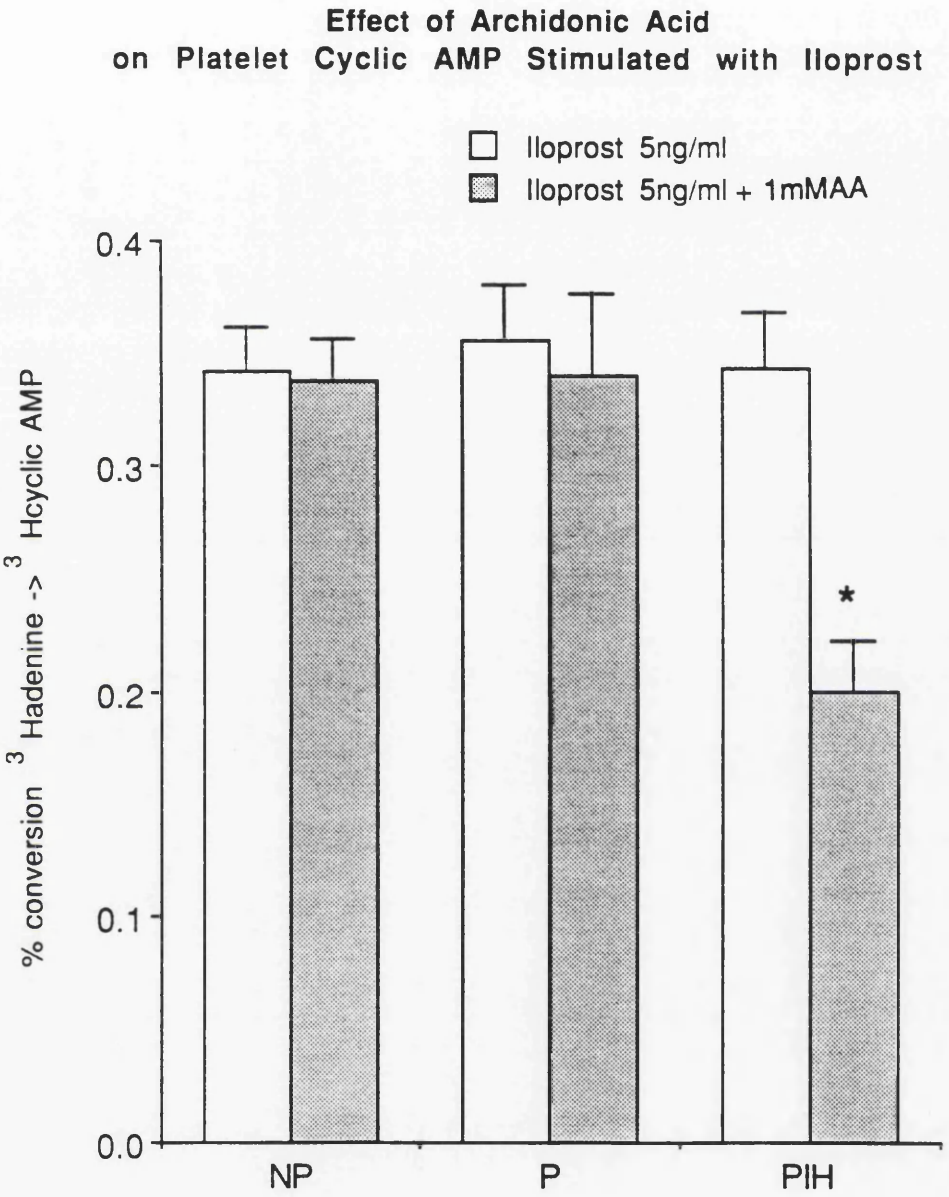


Figure 4.3

Platelet cyclic AMP responses to 5ng/ml iloprost are shown in the presence and absence of 1mmol/l AA. Means and standard errors are illustrated. In the hypertensive pregnant group (PIH) only, AA reduced cyclic AMP accumulation in response to iloprost ($p<0.0001$ using the Wilcoxon matched pairs signed rank test). ANOVA comparing cyclic AMP levels in the presence of ilprost and AA in the three subject groups showed that less cyclic AMP was generated in the hypertensive pregnant group compared with healthy pregnant (P) and non pregnant (NP) subjects ($p<0.05$).

TABLE 4.2

PLATELET CYCLIC AMP LEVELS IN PRESENCE OF DAZMEGREL AND ARACHIDONIC ACID

The table shows means and SEM of platelet cyclic AMP in the three groups of subjects. All cyclic AMP levels are expressed as % conversion of ^{3}H adenine to ^{3}H cyclic AMP. Abbreviations are identical to those in Table 4.1.

REAGENT	GROUP			p value	Comparison
	NP	HP	PIH	(ANOVA)	
AA (1mM)	0.0605 ± 0.0036	0.0762 ± 0.0061	0.0681 ± 0.0039	0.1742	all NS
Dazmegrel (500μM) + AA(1mM)	0.1168 ± 0.0084	0.1499 ± 0.0145	0.1267 ± 0.0087	0.2567	all NS

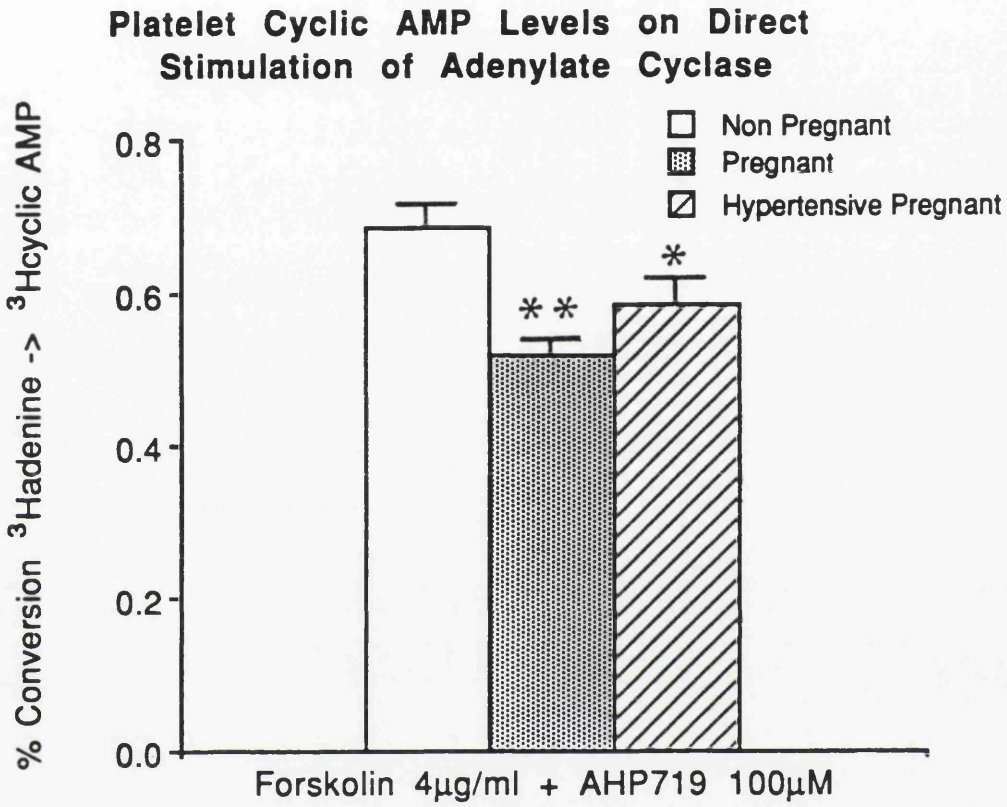


Figure 4.4

The bar graph shows mean cyclic AMP generation in platelets incubated with forskolin and AH-P719. ANOVA was used to compare subject groups. ** indicates $p<0.01$ for the comparison of the pregnant with the non pregnant group; * indicates $p<0.05$ for the comparison of hypertensive pregnant with non pregnant subjects.

3.3 Influence of Arachidonic Acid on Platelet Cyclic AMP Stimulated with Iloprost.

Platelet cyclic AMP responses to 5ng/ml iloprost were also measured in the presence of 1mmol/l arachidonic acid. The phosphodiesterase inhibitor was not included in this case and the samples were unstirred so that no aggregation or release reaction took place. AA had no effect on iloprost induced platelet cyclic-AMP responses in non pregnant and healthy pregnant subjects. In pregnant subjects with hypertension there was a significant reduction in cyclic AMP levels on stimulation with iloprost in the presence of AA when compared both with measurements made under the same conditions in healthy pregnant and non pregnant women and with platelet cyclic AMP responses to iloprost alone in the hypertensive pregnant group (Figure 4.3).

3.4 Platelet Cyclic AMP Levels in the Presence of Dazmegrel and Arachidonic Acid.

AA alone produced a slight rise in platelet cyclic AMP above basal levels but there was no difference between subject groups. Dazmegrel and AA together increased cyclic AMP to a greater extent than AA alone, but, again, there was no difference in platelet cyclic AMP levels under these conditions between the three groups of subjects.(Table 4.2)

3.5 Platelet Cyclic AMP Levels on Direct Stimulation of Adenylate Cyclase.

Platelet cyclic AMP responses to forskolin were examined only in the presence of AH-P719. Results are shown on Figure 4.4. There was again a significant reduction in cyclic AMP generation in both healthy and hypertensive pregnant subjects.

4.DISCUSSION

This cross-sectional study has compared basal and stimulated cyclic AMP levels in healthy third trimester pregnant women, those with PIH or pre-eclampsia and healthy non pregnant women. There was no difference between groups under conditions of basal adenylate cyclase activity, whether or not phosphodiesterase was inhibited. In contrast, platelets from healthy and hypertensive pregnant women produced less cyclic AMP in response to all the adenylate cyclase stimulators studied. These results are broadly in keeping with the generalised reduction in platelet sensitivity to inhibition of their behaviour by cyclic AMP manipulators during pregnancy as demonstrated in the previous chapter.

The reduction in stimulated levels of platelet cyclic AMP, although more marked in the hypertensive group, was primarily a pregnancy effect. This is also in keeping with the data relating to inhibition of platelet ^{14}C-5HT release discussed in Chapter 3. The data show no differences between platelet cyclic AMP levels in women with non proteinuric PIH compared with women with pre-eclampsia. The few existing and more limited studies of platelet cyclic AMP levels in pregnancy report no change[323,339], except in association with the administration of beta$_2$ agonists to suppress labour, which when used long term caused a reduction in platelet cyclic AMP[339]. No patient included in this study had received treatment with any tocolytic drug. Previous studies have concentrated on basal platelet cyclic AMP or have not used a range of types and concentrations of cyclic AMP modulators. Furthermore, the assay employed by the larger of the two published studies[339] was a radioimmunoassay, which is less sensitive than the type of method used in the present study.

It is likely that a complex relationship exists between changes in platelet cyclic AMP levels during pregnancy and the reduction in sensitivity to the

inhibitory effects on platelet behaviour of cyclic AMP manipulators. Pregnancy associated reductions in platelet cyclic AMP levels occurred only when the concentrations of adenylate cyclase stimulators were higher than those generally required to inhibit platelet activation or in the concomitant presence of the phosphodiesterase inhibitor. The relative lack of efficacy of the cyclic AMP manipulators in pregnant subjects in the release reaction experiments described in Chapter 3 would have been determined not only by the level of platelet cyclic AMP reached but also by production of thromboxane A_2 and possibly other pro-aggregatory AA metabolites. Although the results of the studies reported in Chapter 3 would not support an increase in the absolute amount of thromboxane produced in vitro in pregnancy, the efficacy of cyclic AMP manipulators as inhibitors of platelet behaviour could be influenced by platelet sensitivity to the pro-aggregatory effects of thromboxane and possibly by inhibition of adenylate cyclase by prostaglandins derived from AA. The evidence regarding the effect on adenylate cyclase activity of thromboxane is, however, conflicting, with some studies suggesting an inhibitory effect,[206] whilst others show no such action.[207] Nevertheless, the results of the study described in this chapter show that in the presence of arachidonic acid, the platelet cyclic AMP response to 5ng/ml iloprost was reduced in the hypertensive pregnant group. This occurred even although aggregation and release was deliberately prevented as the PRP was not stirred. The explanation for this observation is unclear, but may lie in a difference in the effects on adenylate cyclase of AA metabolites such as thromboxane or prostaglandin E_2.

There was no difference between subject groups in platelet cyclic AMP production in the presence of dazmegrel and AA, despite the clear loss of response during pregnancy to the inhibitory effects of dazmegrel on AA induced

platelet behaviour demonstrated in Chapter 3. One interpretation is that cyclic AMP production is not a major determinant of responder status to thromboxane synthetase inhibitors during pregnancy, and factors such as sensitivity to the pro-aggregatory effects of endoperoxides may be more important. A study outwith the context of pregnancy has, however, shown that platelets from non responders to dazoxiben demonstrated a lesser rise in cyclic AMP in response to this thromboxane synthetase inhibitor and AA than those from responders.[249] Measurements in this case, in contrast with the present study, were made in the presence of a phosphodiesterase inhibitor. It is therefore conceivable that small to moderate differences between subject groups were missed in the present study, as the rise in cyclic AMP above basal levels in response to dazmegrel and AA was small in the absence of the potentiating effect of a PDE inhibitor.

The reduction in platelet cyclic AMP response to iloprost and to PGD_2 in healthy and hypertensive pregnant women was most marked in the presence of the phosphodiesterase inhibitor AH-P719. The most likely interpretation is that the changes noted are due to differences in stimulated adenylate cyclase activity in platelets during pregnancy, and that these differences are emphasized by inhibiting the breakdown of cyclic AMP. The possibility of differences in metabolism of cyclic AMP in platelets from pregnant women cannot, however, be completely excluded, as the effects of AH-P719 on the type II phosphodiesterase enzyme are unknown. At the concentration used in these experiments, AH-P719 is thought to be a highly selective phosphodiesterase inhibitor for the type III enzyme, and has no effect on cyclic GMP metabolism (personal communication in writing from Dr Hans Weisenberger, Karl Thomae GmbH, subsidiary of Boehringer Ingelheim). Metabolism via type III phosphodiesterase is the more usual route for breakdown of cyclic AMP, the type II enzyme having a lower affinity for cyclic AMP. When

cyclic AMP levels are raised by adenylate cyclase stimulators, however, metabolism via the type II enzyme can occur.[209,210] Ongoing metabolism of cyclic AMP in the presence of AH-P719 therefore cannot be excluded. This phosphodiesterase inhibitor is, however, one of the most potent inhibitors of cyclic AMP metabolism in platelets when compared with other PDE inhibitors,[254] and, in this study, was used in concentrations shown to result in maximal inhibition of phosphodiesterase.[249]

Given that a change in stimulated adenylate cyclase activity in pregnancy is the most likely cause of the observed reduction in cyclic AMP levels, this could result from changes in receptors, changes in G proteins, or alterations in the enzyme itself. As the reduction in platelet cyclic AMP in pregnancy occurred not only with those adenylate cyclase stimulators which act via surface receptors and the stimulatory G protein (G_s), but also with forskolin which is capable of acting directly on the catalytic subunit of the enzyme,[200,201] down regulation of platelet prostaglandin receptors could not explain these findings. A change in the catalytic component of adenylate cyclase could entirely account for the results. A G protein mediated effect could not be completely ruled out, however, as the effects of forskolin may be modulated by G proteins.[201] Adenylate cyclase is controlled by a stimulatory (G_s) and an inhibitory G protein (G_i).[40,41,44,135,191] Many prostaglandins interact with a population of receptors linked to G_i in addition to those linked to G_s.[202] Reduced activity of platelet G_s or increased activity of G_i during pregnancy could be postulated. The data regarding forskolin, however, would suggest that any changes in the G proteins would have to be independent of prostanoid control. In the case of G_i, there is evidence from studies in other tissues that the α subunit mediates inhibition of ligand stimulated adenylate cyclase activity, whilst the β/γ subunit mediates inhibition of basal activity.[441] Both subunits may mediate

inhibition of forskolin stimulated adenylate cyclase activity.[441] As platelet cyclic AMP accumulation was unaltered during pregnancy, under conditions of basal adenylate cyclase activity, a change in the G_i β/γ subunit is unlikely. A reduction in the activity of the catalyst itself or alterations in the activity of the α subunits of either G protein therefore appear to be the most likely mechanisms accounting for the reduction in platelet cyclic AMP responses during pregnancy. This will be explored in more detail in Chapter 6.

The data on indices of platelet size presented in Chapter 3 would support a degree of platelet activation in vivo in the healthy pregnant subjects[289] which was more marked in those with PIH and pre-eclampsia. Whether the biochemical changes demonstrated in this study of platelet cyclic AMP precede or result from platelet activation in the circulation of pregnant women cannot be determined from the available data. If, however, reductions in platelet cyclic AMP responses, similar to those demonstrated in vitro, do occur in vivo, this could render platelets less responsive to important physiological regulatory mechanisms and contribute to platelet activation in vivo in healthy and hypertensive pregnant women. The relative prostacyclin deficiency in pre-eclampsia[160,298,310] could add to the physiological pregnancy associated reduction in platelet sensitivity to inhibitory prostaglandins, to promote more extensive platelet activation in vivo than in normal pregnancy where prostacyclin production is increased.[160,298,310]

In summary, the studies presented in this chapter have demonstrated that reduced platelet cyclic AMP accumulation is associated with the reductions in response of platelets from pregnant women to inhibitory agents including prostaglandins which play an important regulatory role in vivo.

CHAPTER 5.

LONGITUDINAL STUDIES OF PLATELET CYCLIC AMP DURING PREGNANCY.

CHAPTER 5.

LONGITUDINAL STUDIES OF PLATELET CYCLIC AMP DURING PREGNANCY.

1. INTRODUCTION.

In the previous two chapters, I have shown in cross sectional studies that platelets from healthy third trimester pregnant women produce less cyclic AMP in response to stimulation of adenylate cyclase than those from non pregnant women. This is accompanied by a reduction in efficacy of prostaglandins and other cyclic AMP manipulators as inhibitors of platelet behaviour. This chapter deals with studies which have been carried out to validate the findings in a prospective longitudinal manner and to determine the relationship of these changes in platelets to gestation as pregnancy progresses. The first study was carried out in healthy primigravid women, and the second in women considered at risk of pre-eclampsia because of a history of the condition in a previous pregnancy. Women with a previous history of pre-eclampsia have a 25% incidence of recurrence of the condition in subsequent pregnancies.[373]

2. METHODS.

2.1 Subjects.

For the first study, subjects were recruited initially from women planning their first pregnancy, including volunteers from hospital staff, those referred to me from their general practitioners, and volunteers recruited from regular blood donors. At least 8 weeks had elapsed from the time of last ingestion of an oral contraceptive for all subjects studied pre-conceptionally. Only 5 women recruited

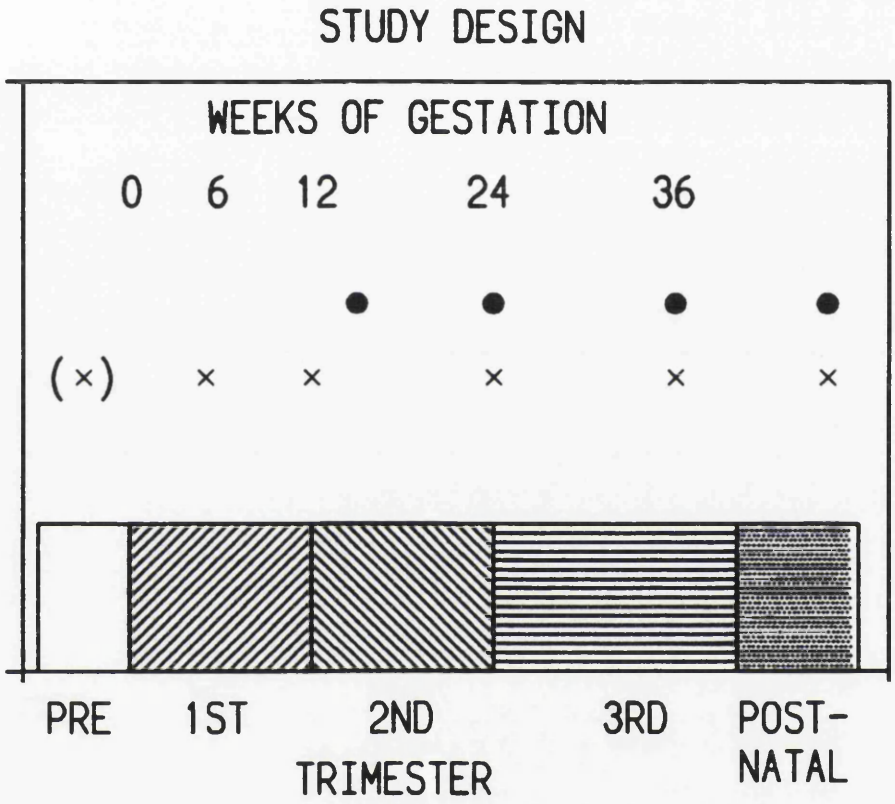


FIGURE 5.1.

The figure summarises the study design. × represent times for platelet studies in healthy pregnant subjects (HP), whilst ● represent those for subjects at risk of pre-eclampsia (RP).

TABLE 5.1

HEALTHY PREGNANT SUBJECTS

Table 5.1 shows some demographic details relating to the healthy pregnant women studied longitudinally. TOP = Termination pregnancy. S.A. = Spontaneous Abortion.

Subject	Age	Parity	Time of Recruitment	Postnatal Study time
1	32	0 + 0	Preconceptional	Not studied (emigrated)
2	31	0 + 0	Preconceptional	6 weeks
3	25	0 + 2 (S.A. 10/40, TOP 12/40)	5 weeks gestation	7 weeks
4	30	0 + 0	Preconceptional	13 weeks
5	31	0 + 0	Preconceptional	13 weeks
6	25	0 + 0	8 weeks gestation	9 weeks
7	30	0 + 1 (TOP 10/40)	11 weeks gestation	12 weeks
8*	28	0 + 0	Preconceptional	6 weeks

* This subject was excluded from the data analysis as she developed the HELLP syndrome at 38 weeks gestation.

in this manner subsequently became pregnant within the time the study was running, and so a further three women were recruited from healthy primigravid women who either volunteered for the study in the very early weeks of pregnancy or who presented to the hospital antenatal clinic at 12 weeks gestation or less. Subjects were studied pre-conceptionally where possible, then as soon as pregnancy was confirmed using an ELISA for human chorionic gonadotrophin on a random urine sample (Hybritech Icon Kit). The mean gestation for the first study time point during pregnancy for those subjects recruited pre-conceptionally was 5 weeks gestation. All subjects were subsequently studied at 12, 24, and 36 weeks gestation, and finally after the 6th postnatal week. The study design is summarised in Figure 5.1. One subject was excluded from the data analysis in the healthy pregnant (HP) group as she developed the H.E.L.L.P. syndrome[355] at 38 weeks gestation, but data for this subject will be presented separately. Some demographic details relating to these subjects are presented in Table 5.1. No subject ingested any drug except iron and vitamins throughout the period of the study.

For the second study, 5 women with a previous history of pre-eclampsia were recruited from referrals to the medical obstetric clinic for high risk pregnancies at the University Hospital, Nottingham. No such women were seen until early in the second trimester. Subjects were studied immediately on recruitment and then subsequently at 24, and 36 weeks gestation and after the 6th postnatal week. Demographic details relating to these subjects are shown on Table 5.2. No subject in the "at risk" group (RP) ingested any drug throughout the period of the study, with the exception of iron and folate supplements.

On each study day subjects were placed in the supine position, and after 5 minutes rest, blood pressure was recorded and venepuncture performed as

TABLE 5.2

SUBJECTS AT RISK OF PRE-ECLAMPSIA

Table 5.2 shows some demographic data relating to the subjects studied longitudinally who were considered to be at risk of pre-eclampsia

Subject	Age	Parity	Time of Recruitment	Previous Obstetric History	Postnatal Study Time
1	29	1 + 0	13 weeks	Severe pre-eclampsia at 35 weeks G_1	6 weeks
2	18	1 + 1	18 weeks	Fulminating pre-eclampsia 35 weeks G_2; spontaneous abortion at 12 weeks G_1	26 weeks
3	34	1 + 0	18 weeks	Pre-eclampsia at 35 weeks G_1	10 weeks
4	33	1 + 0	13 weeks	Severe pre-eclampsia at 30 weeks in G_1	7 weeks
5*	26	1 + 1	24 weeks	Pre-eclampsia immediately postpartum in G_2; spontaneous abortion at 12 weeks G_1	6 weeks

* No "early pregnancy" data were available for this subject

described in Chapter 2. Blood was taken on each occasion for platelet studies, full blood count, serum thromboxane B_2 measurement, and estimation of urea, electrolytes and uric acid.

2.2 Laboratory Methods.

Platelet studies were conducted in citrated PRP in which the platelet count had been adjusted to 250 x 10^9/l. The effect of cyclic AMP manipulators on platelet ^{14}C-5HT release induced by 1mM arachidonic acid was examined using the method described in Chapter 2, and exactly the same reagents and laboratory protocol detailed in Chapter 3.

Platelet cyclic AMP was measured by the ^{3}H-adenine labelling method described previously, using exactly the same reagents and laboratory protocol as detailed for the cross-sectional study (see Appendix 4). All results were expressed as % conversion of ^{3}H-adenine to ^{3}H-cyclic AMP and were corrected for ^{3}H-adenine uptake by the platelets. Platelet uptake of ^{3}H-adenine did not change throughout the period of the study in either subject group.

Serum thromboxane B_2 was measured by radioimmunoassay as outlined in Chapter 2.

2.3 Statistical Methods

Comparison of data at different time points within subject groups was carried out by Friedman two way analysis of variance. As pre-conceptional data was not available for some patients in the healthy pregnant group, for the purposes of statistical analysis postnatal data in such cases were grouped together with pre-conceptional data in the remaining subjects to form a "non pregnant" category. In those cases where both pre-conceptional and postnatal data were available, comparable results were obtained at these times for all parameters. Furthermore, previous studies have suggested that parameters of

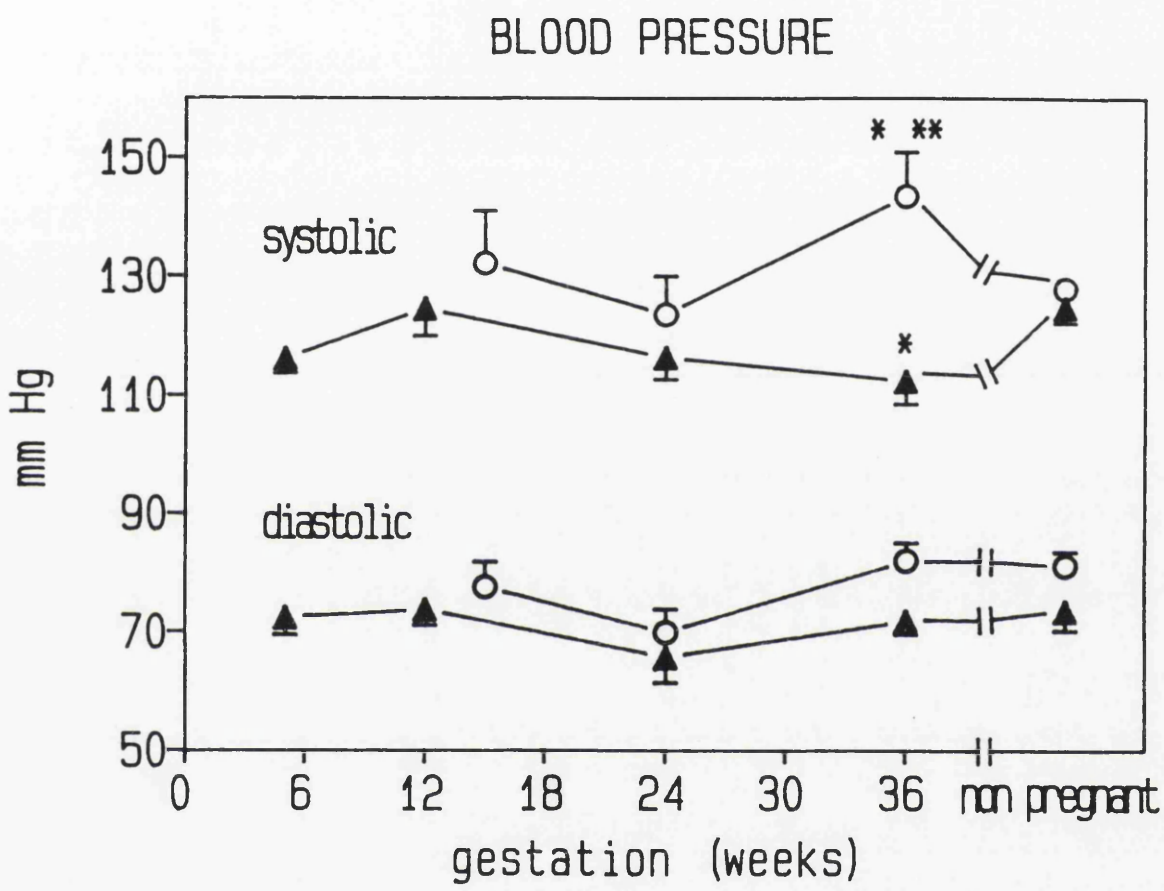


FIGURE 5.2.

The figure shows systolic and diastolic blood pressure data for both groups of subjects throughout the period of the study. Data for HP is shown as ▲ and data for RP as ○. Friedman ANOVA revealed an overall p value of 0.0235 for HP for systolic blood pressure, with a significant reduction in systolic pressure at 36 weeks compared with 12 week data ($p < 0.05$, *). In RP, systolic pressure rose significantly in late pregnancy (Friedman ANOVA $p = 0.0395$, with $p < 0.05$ for the comparison of 36 week with 24 week data, *). Comparison of HP and RP showed a significantly higher systolic pressure in RP at 36 weeks (** $p = 0.0044$). There was no significant change in diastolic pressure throughout the study in either group of subjects, and no significant difference between subject groups.

TABLE 5.3

HEALTHY PREGNANT PATIENTS: PREGNANCY OUTCOME

Subject	Antenatal	Labour	Delivery	Baby (sex and weight)	Postnatal
1	Uncomplicated	Spontaneous 40 + weeks	SVD	♂ 3.7 kg	uncomplicated
2	Uncomplicated	Induced 42 weeks	Forceps under GA (persistent OP presentation)	♀ 3.53 kg	uncomplicated
3	Raised SAFP normal detailed u/s scan	Spontaneous 38 + weeks	Forceps	♂ 3.34Kg(cleft lip)	uncomplicated
4	Uncomplicated	Spontaneous 38 weeks	SVD	♂ 2.88 kg	uncomplicated
5	Anaemic at 33 weeks small APH at 38 weeks	Induced 38 weeks	SVD	♂ 3.85 kg	uncomplicated
6	Uncomplicated	Spontaneous 36 weeks	SVD	♂ 3.53 kg	uncomplicated
7	Uncomplicated	Spontaneous 39 weeks	Forceps	♂ 2.94 kg	uncomplicated

SAFP = serum alphafetoprotein; U/S = ultrasound; APH = antepartum haemorrhage; SVD = spontaneous vaginal delivery.

platelet behaviour return to non pregnant values by the 6th postnatal week following healthy pregnancy.[322]

Comparison of the healthy pregnant group with the "at risk" group was made by two way analysis of variance with repeated measures, supplemented by the Mann-Whitney U test when overall significant p values were obtained using the analysis of variance. As the times of study in the early part of pregnancy did not exactly correspond for the two groups of patients, analysis was facilitated by dividing the data into four groups as follows: "early pregnancy" (12 week data in the healthy pregnancy group and data at first presentation in the at risk group), "mid pregnancy" (24 week data for both groups), late pregnancy (36 week data for both groups) and "non pregnant" (pre-conceptional or postnatal data for the healthy pregnant group and postnatal data for the at risk group).

3. RESULTS.

3.1 Clinical Data.

All of the seven subjects eligible for inclusion in the healthy pregnant group had pregnancies free from major complications. One subject laboured at 36 weeks gestation by dates but the baby appeared fully mature at birth. Another subject had a small antepartum haemorrhage at 38 weeks. All women had babies of normal birth weight for gestational age. Clinical details are shown in Table 5.3, and blood pressure data in Figure 5.2. There was no significant change in diastolic blood pressure over the period of the study, but there was a significant late pregnancy fall in systolic pressure.

In the at risk group three of the subjects had uncomplicated pregnancies, but two developed mild labile non proteinuric hypertension in late pregnancy which settled with rest in both cases and did not require treatment with drugs. All "at

TABLE 5.4

SUBJECTS AT RISK OF PRE-ECLAMPSIA: PREGNANCY OUTCOME

Subject	Antenatal	Labour	Delivery	Baby(Sex and Weight)	Postnatal
1	Uncomplicated	Spontaneous 41 weeks	Kielland's forceps	♀ 3.1 kg	Anaemic (Responded to $FeSO_4$)
2	Mild labile hypertension (third trimester) Breech presentation	-	Elective Caesarean section at 39 weeks	♀ 3.48 kg	uncomplicated
3	Labile hypertension (third trimester)	Spontaneous 40 weeks	SVD	♂ 3.06 kg	uncomplicated
4	Uncomplicated	Induced 40 weeks	Emergency Caesarean section (Fetal distress)	♀ 3.66 kg	uncomplicated
5	Uncomplicated	Spontaneous 40 weeks	SVD	♀ 3.26 kg	uncomplicated

risk" women had babies with normal birth weight for gestational age and birth weights did not differ between the two groups of subjects. Clinical data are shown in Table 5.4, and blood pressure data in Figure 5.2. In the "at risk" group, there was a trend towards a rise in diastolic pressure in late pregnancy which just failed to reach significance, but systolic pressure rose significantly compared with values in mid pregnancy. Two way analysis of variance showed that systolic blood pressure was significantly higher at 36 weeks gestation in women at risk of pre-eclampsia compared with healthy primigravidae. When subjects were not pregnant, all were normotensive and there was no difference between groups in either systolic or diastolic blood pressure.

3.2 Haematology and Biochemistry.

Haematological data are shown in Table 5.5 and biochemical data in Table 5.6. In the healthy pregnant group, there was a significant mid pregnancy reduction in packed cell volume (PCV), but in the group at risk of pre-eclampsia PCV did not change over the period of study. Direct comparison of the two groups, however, showed no significant difference between women at risk and healthy women. There was no significant change in platelet count nor in mean platelet volume (MPV) during pregnancy in either subject group, although the at risk group showed a trend towards a mid pregnancy fall in platelet count.

Serum urea was significantly lower in the healthy pregnant group at 24 and 36 weeks gestation compared with non pregnant values. Serum creatinine reflected these results at 24 weeks gestation. In the group at risk of pre-eclampsia, urea was slightly but significantly lower at 36 weeks gestation compared with postnatal values but creatinine did not change throughout the study period. Serum urea was significantly higher in women at risk of pre-eclampsia compared with healthy women when subjects were not pregnant, but

TABLE 5.5

HAEMATOLOGY PARAMETERS

The table shows means ± SEM for some haematological parameters for both groups of subjects throughout the study. p values are for comparisons within groups. There was no significant difference between groups for any of the parameters shown. HP = healthy pregnant subjects. RP = subjects at risk of pre-eclampsia.

Parameter	Group	Non pregnant	5 weeks	12/15* weeks	24 weeks	36 weeks	Friedman p value	Individual p values
PCV	HP	0.387 ± 0.006	0.364 ± 0.015	0.352 ± 0.007	0.322 ± 0.009	0.345 ± 0.008	0.008	<0.01 NP vs 24 weeks
	RP	0.386 ± 0.012	-	0.366 ± 0.016	0.346 ± 0.015	0.359 ± 0.012	0.165	NS
Platelet Count ($\times 10^9$/l)	HP	247 ± 15	274 ± 23	260 ± 22	261 ± 15	247 ± 20	0.779	NS
	RP	292 ± 24	-	248 ± 25	225 ± 18	248 ± 28	0.0752	NS
MPV (fl)	HP	9.1 ± 0.55	8.1 ± 0.34	8.7 ± 0.46	8.9 ± 0.56	9.4 ± 0.58	0.2977	NS
	RP	9.9 ± 0.47	-	9.2 ± 0.33	9.5 ± 0.43	9.4 ± 0.52	0.2988	NS

* 12 weeks for HP and 15 weeks for RP

TABLE 5.6

RENAL FUNCTION

Parameter	Group	Non Pregnant	12/15 weeks	24 weeks	36 weeks	Friedman p value	Individual p values
Serum urate	HP †	200 ± 10.64	133 ± 7.76	148 ± 6.66	200 ± 12.53	0.0070	p < 0.05 12 vs 36 weeks
(μmol/l)	RP	273 ± 26.08	172 ± 13.82	183 ± 12.84	242 ± 30.36	0.0129	p < 0.05 15vs 36 weeks p < 0.05 15 vs NP
Serum urea	HP •	4.02 ± 0.12	3.48 ± 0.16	2.74 ± 0.16	2.46 ± 0.27	0.0029	p < 0.05 24 weeks vs NP p < 0.01 36 weeks vs NP
(mmol/l)	RP	5.1 ± 0.25	3.03 ± 0.18	3.08 ± 0.29	2.53 ± 0.21	0.0440	p < 0.05 36 weeks vs NP
Serum Creatinine	HP	77.4 ± 4.13	61.2 ± 5.35	55.6 ± 2.54	58.2 ± 5.08	0.0242	p < 0.05 24 weeks vs NP
(μmol/l)	RP	81.5 ± 3.57	60.8 ± 9.24	64.8 ± 9.53	56.0 ± 3.49	0.2123	NS

The table shows means ± SEM for some biochemical parameters of renal function for the two groups of subjects throughout the study. p values are for comparisons within groups.

† Urate was significantly lower at non pregnant ($p = 0.05$) and early pregnancy time points ($p = 0.03$) in the healthy pregnant compared with the 'at risk' group.

• Urea was significantly lower in the healthy group when subjects were not pregnant, compared with the 'at risk group' ($p = 0.014$).

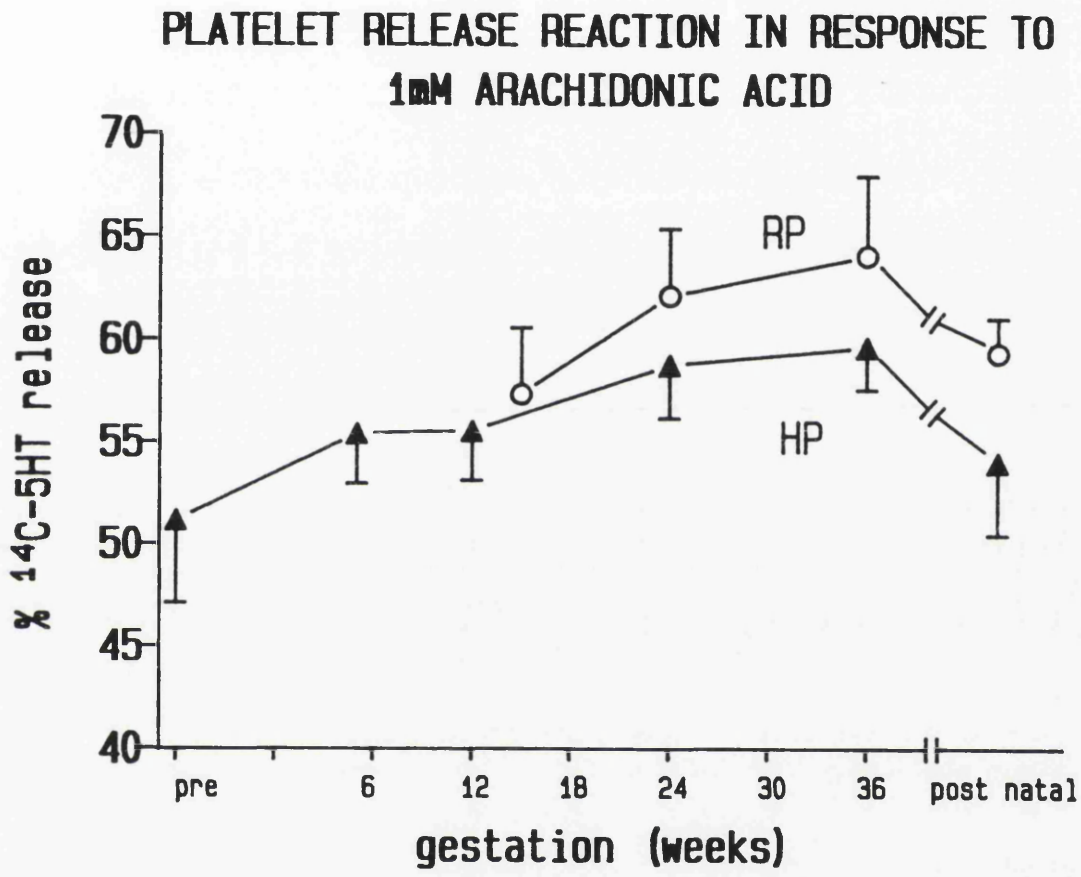


FIGURE 5.3.

The graph shows % ^{14}C-5HT release plotted against gestation following stimulation of PRP with 1mM arachidonic acid in the absence of inhibitors. ▲ illustrates results for HP and ○ illustrates those for RP. Although there was a tendency for the extent of the release reaction to increase as pregnancy progressed, there was no statistically significant difference between results at different time points nor between the two groups of subjects.

did not differ during pregnancy. There was no difference between groups in serum creatinine at any time. No subject "at risk" had a serum urea above the upper limit of the normal range. In the healthy pregnant group, serum urate tended to be lower in early and mid pregnancy compared with results in late pregnancy and when subjects were not pregnant. The difference between urate levels at 12 weeks and 36 weeks gestation was statistically significant. In the "at risk" group, serum urate was significantly lower early in the second trimester when compared with postnatal and 36 week gestational data. Comparison of the two subject groups revealed that urate was higher in women "at risk" when subjects were not pregnant and in early pregnancy, but results were similar in mid and late pregnancy. Urate levels lay within the normal range for all subjects at all time points.

3.3 Arachidonic Acid Induced Platelet Dense Granule Release.

In the absence of inhibitors ^{14}C-5HT release in response to 1mM arachidonic acid tended to increase slightly as pregnancy progressed in both healthy and "at risk" patients but this did not reach statistical significance. Comparison of the healthy pregnant group with the "at risk" group showed no difference between the two groups in the extent of the release reaction at any time during the study. The data are shown in Figure 5.3.

3.4 Inhibition of Platelet Behaviour by Cyclic AMP Manipulators.

During healthy pregnancy there was a gradual reduction in sensitivity of platelets to inhibition of their behaviour by all cyclic AMP manipulators tested, when compared with non pregnant data. This effect was statistically significant in the case of the inhibitory prostaglandins iloprost and PGD_2, and the phosphodiesterase inhibitor AH-P719, and just failed to reach statistical significance with forskolin. The effect reached a maximum at 36 weeks gestation,

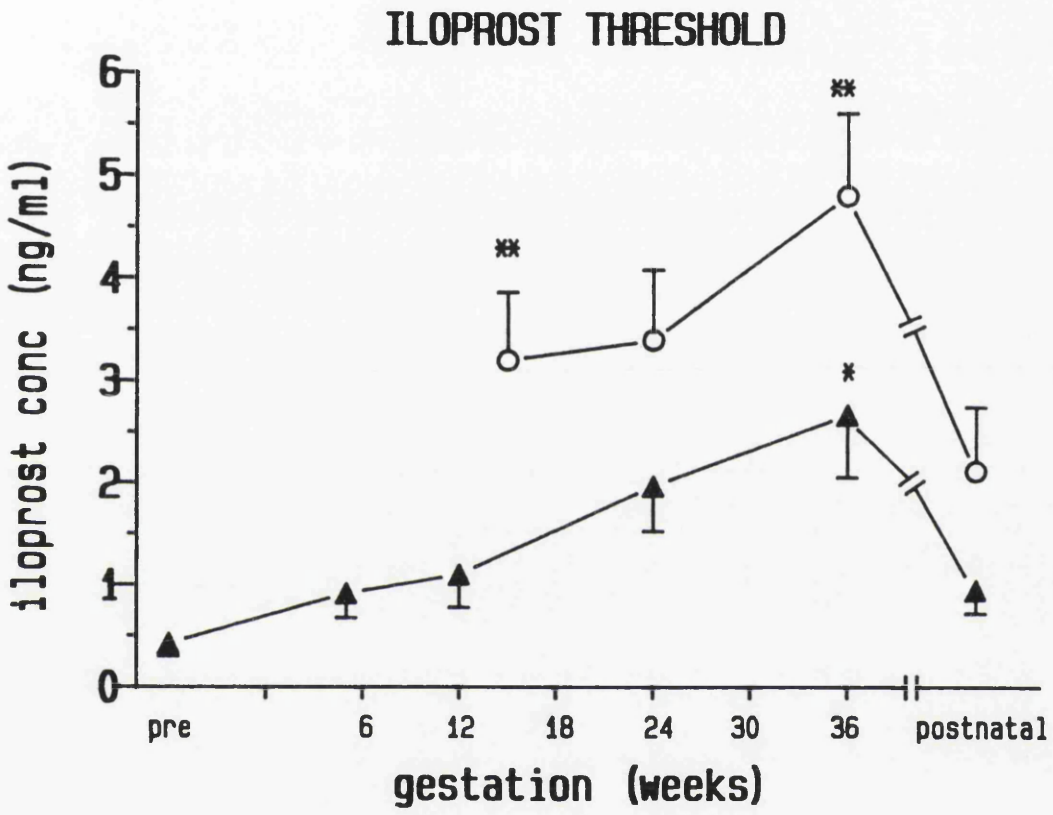


FIGURE 5.4

This shows the threshold concentrations of iloprost required to completely inhibit AA induced platelet dense granule release plotted against gestation. Results in HP are indicated by ▲ and in RP by ○ . In both groups of subjects there was a gradual loss of sensitivity to the effects of iloprost as pregnancy progressed. * indicates statistical significance for comparison of results at different time points by Friedman ANOVA within subject groups and ** indicates statistical significance for comparison of results between groups of subjects.

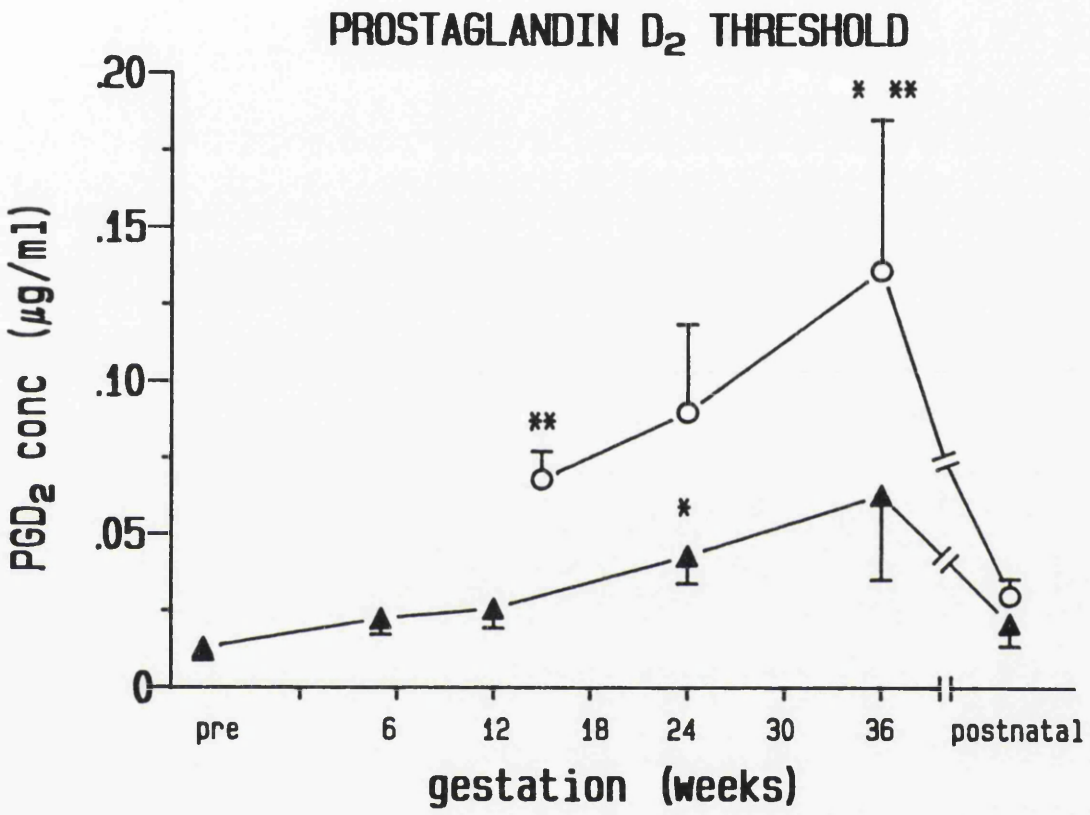


FIGURE 5.5

The graph shows the threshold concentrations of PGD_2 required to abolish AA induced ^{14}C-5HT release throughout the period of the study. An increase in threshold was again noted in both HP (▲) and RP (○) as pregnancy progressed. * indicates statistical significance for comparison of time points within subject groups and ** indicates statistical significance for comparison of data at the same time point between subject groups.

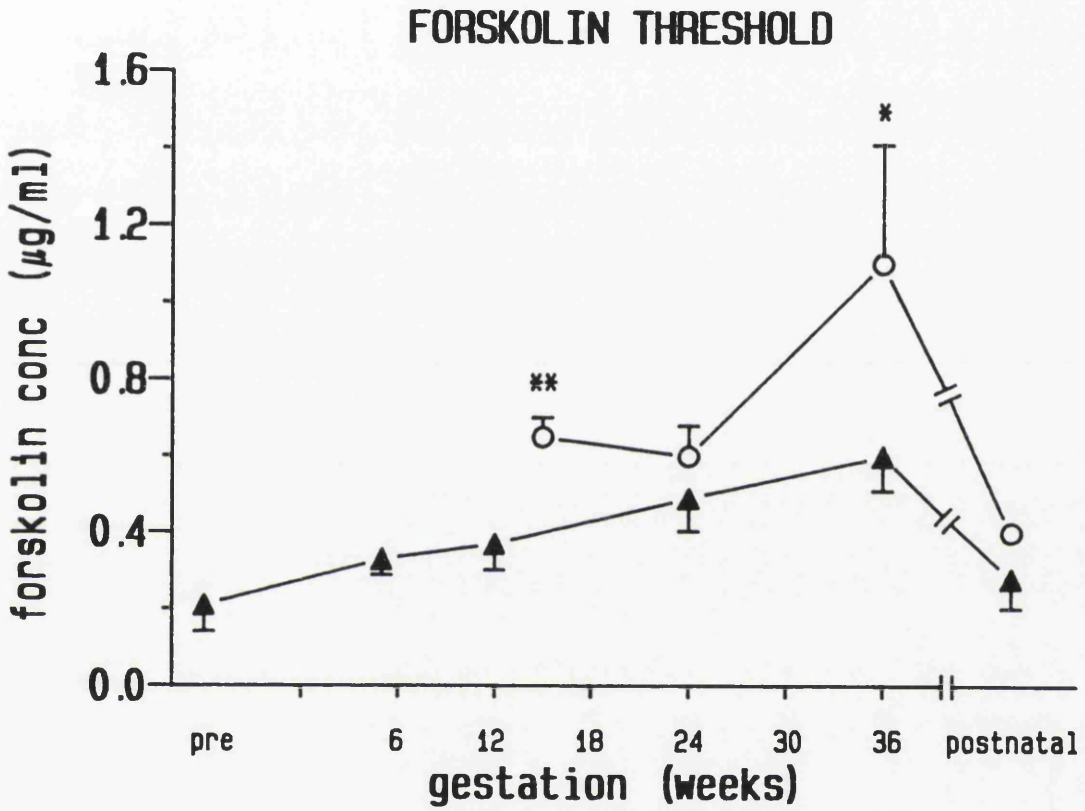


FIGURE 5.6.

The figure shows threshold concentrations of forskolin required to completely inhibit AA induced platelet dense granule release. As before, loss of sensitivity to forskolin occurred as pregnancy progressed ; in this case the effect was more marked in RP (○) than in HP (▲). * indicates statistical significance for comparison within groups of subjects and ** indicates significance for comparison between subject groups.

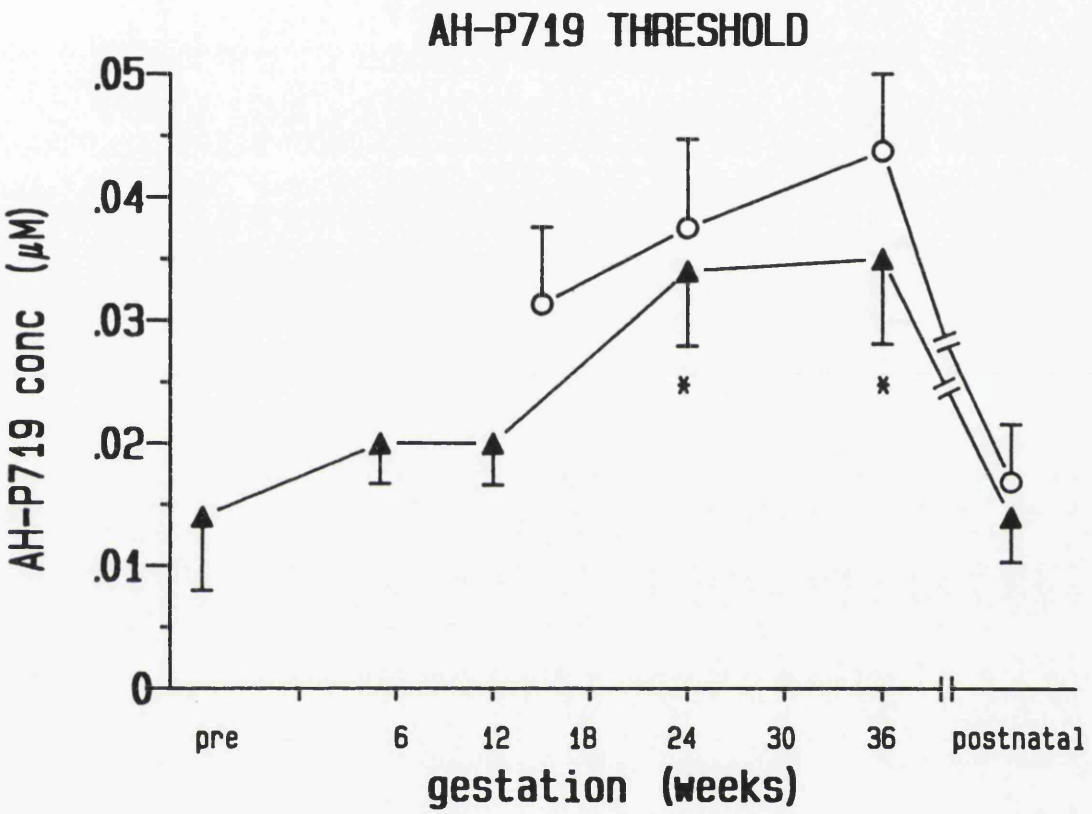


FIGURE 5.7.

The graph shows threshold concentrations of the PDE inhibitor AH-P719 required to abolish AA induced ^{14}C-5HT release. In HP (▲) there was a significant increase in AH-P719 threshold at 24 and 36 weeks gestation (*) compared with postnatal data. Data for RP is indicated by ○ .

TABLE 5.7

Summary of Statistics for Platelet Sensitivity to Inhibitors: Within Group Comparisons

Reagent Threshold	Group	Overall p value (Friedman)	Multiple Comparisons
Iloprost	HP	0.001	5 wks v 36 wks $p<0.05$ 36 wks v PN $p<0.01$
	RP	0.08	all NS
PGD_2	HP	0.005	24 wks vs PN $p<0.05$
	RP	0.01	36 wks vs PN $p<0.01$
Forskolin	HP	0.06	all NS
	RP	0.03	36 wks vs PN $p<0.05$
AH-P719	HP	0.03	24 wks vs PN $p<0.05$ 36 wks vs PN $p<0.05$
	RP	0.19	all NS

The table shows the results of Friedman two way analysis of variance comparing threshold concentrations of the various cyclic AMP manipulators required to abolish AA induced platelet ^{14}C-5HT release at different study time points. The data are illustrated in Figures 5.4 - 5.7. As before HP indicates the healthy pregnant group and RP the group at risk of pre-eclampsia.

TABLE 5.8

SUMMARY OF STATISTICS FOR PLATELET SENSITIVITY TO INHIBITORS:
BETWEEN GROUP COMPARISONS

Reagent Threshold	Overall p value (Two Way ANOVA) HP vs RP	Individual Comparisons of HP vs RP (Mann-Whitney-U)			
		Early	Mid	Late	Non pregnant
Iloprost	0.0001	0.01	0.10	0.03	0.08
PGD_2	0.0003	0.007	0.10	0.04	0.25
Forskolin	0.001	0.05	0.44	0.07	0.25
AH-719	0.0001	0.21	0.70	0.56	0.92

The table shows the results of statistical analysis comparing results in the healthy pregnant (HP) and at risk (RP) groups. Overall p values taking all time points into account were obtained using two way analysis of variance with repeated measures and p values for individual time points were obtained using the Mann-Whitney U test.

and was well established by 24 weeks. Statistically significant changes were not present before 24 weeks gestation in this small study, but the pattern of results suggests that the changes may begin around 12 weeks gestation. Results at pre-conceptional, 5 week gestational, and postnatal time points were generally closely comparable. The data are illustrated in Figures 5.4-5.7 and the statistical results are documented in Table 5.7.

In the "at risk" subjects, a similar pattern of loss of platelet sensitivity to the inhibitors was evident when data during pregnancy were compared with postnatal results. This was statistically significant for forskolin and PGD_2 and just failed to reach significance for iloprost. The maximal change again occurred at 36 weeks gestation. Comparison of data at individual time points did not show any statistically significant changes prior to 36 weeks, but the overall pattern of results as shown in Figures 5.4-5.7 would suggest that changes in platelet sensitivity to cyclic AMP manipulators had started to develop before the second trimester when these patients were entered into the study.

The data suggest that loss of sensitivity to cyclic AMP manipulators may be more marked during pregnancy in women at risk of pre-eclampsia than in healthy primigravidae. This was confirmed for all of the pharmacological agents by comparing data from the two subject groups by two way analysis of variance with repeated measures. In general the differences between groups were most marked in late pregnancy, but were detectable in "early" pregnancy. No difference was apparent at mid pregnancy. There was also a trend towards reduced sensitivity to iloprost postnatally in the at risk group when compared with non pregnant data from healthy subjects, but this just failed to reach statistical significance. The statistical results of comparison between subject groups are illustrated in Table 5.8.

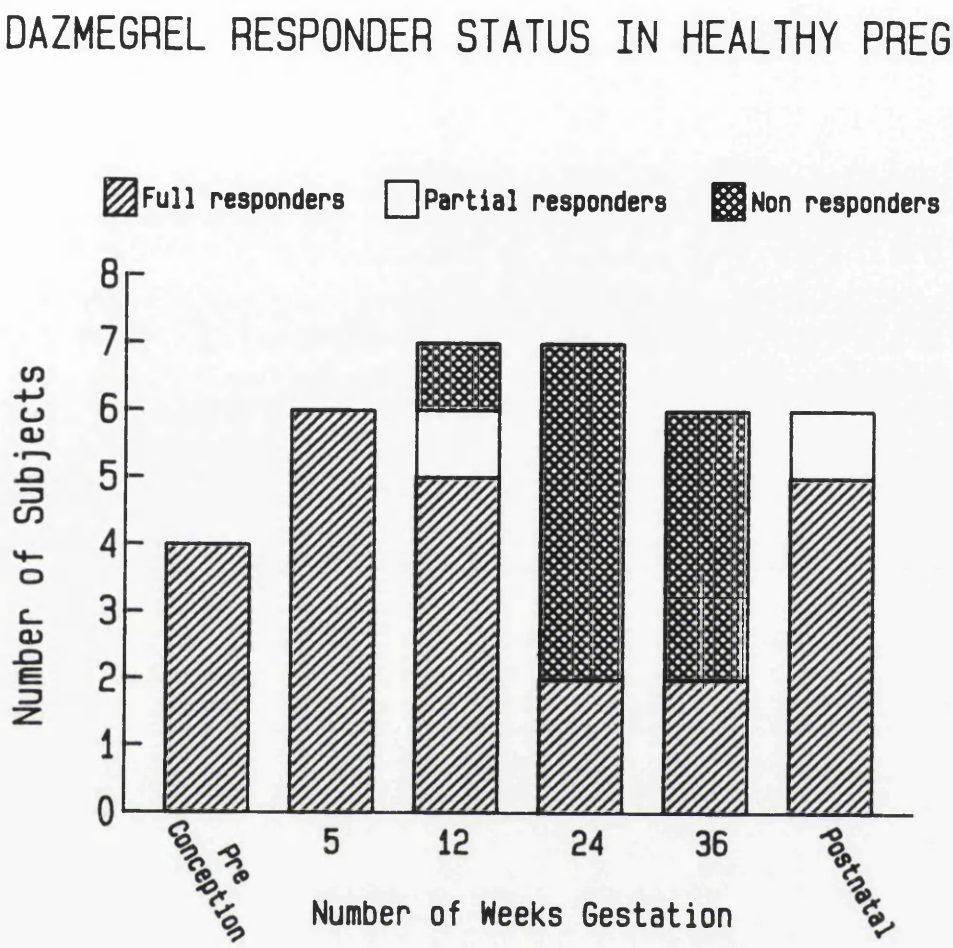


FIGURE 5.8.

The graph shows the number of responders, non responders, and partial responders to the thromboxane synthetase inhibitor dazmegrel in healthy pregnant subjects at each phase of the study. Full responders were those in whom AA induced platelet ^{14}C-5HT release was completely abolished by both 100μM and 500μM dazmegrel, partial responders were those in whom only 500μM dazmegrel resulted in complete inhibition of platelet behaviour and those in whom the release reaction was not abolished by either concentration of the thromboxane synthetase inhibitor were non responders.

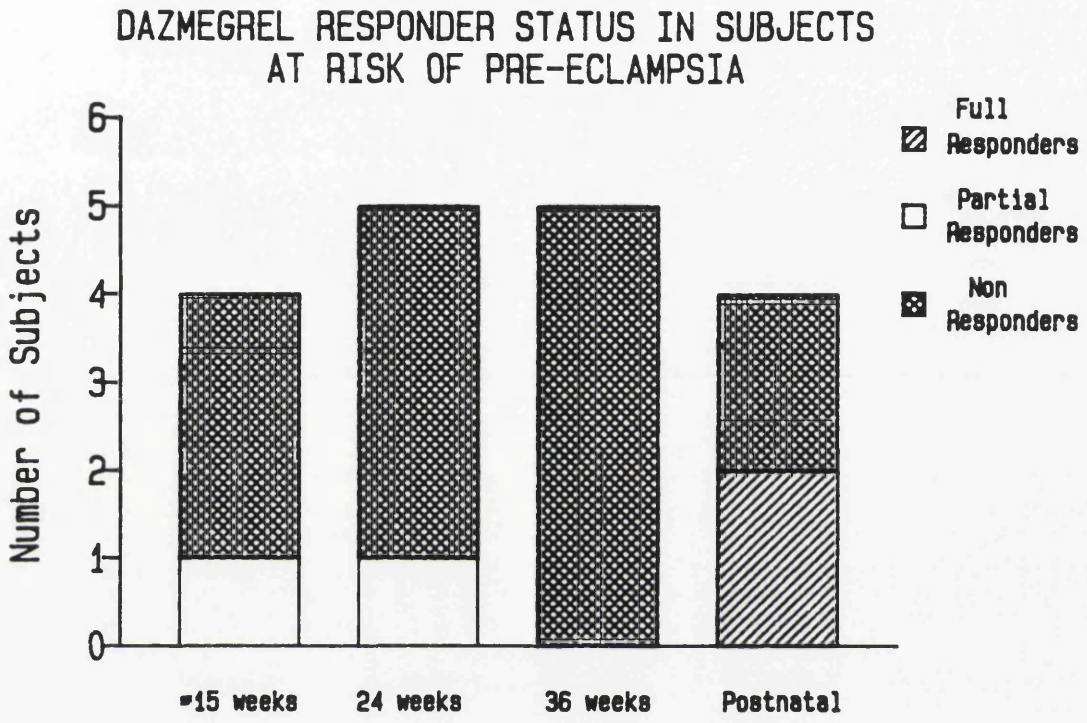


FIGURE 5.9.

The figure shows the numbers of responders, non-responders and partial responders to dazmegrel in patients at risk of pre-eclampsia at each phase of the study. The definitions of responder status were as stated in the legend to FIGURE 5.8 and in the text.

TABLE 5.9

Serum Thromboxane B_2

Gestation(weeks)

Group	NP	5	12/15*	24	36
HP	40.94 ± 6.91	32.71 ± 3.88	30.94 ± 5.29	37.36 ± 2.78	43.71 ± 0.93
RP	29.68 ± 1.90	-	35.32 ± 2.09	33.77 ± 6.85	33.57 ± 2.03

* 12 weeks for HP and 15 weeks for RP.
All values = pmoles/10^7 platelets. Means ± SEM are shown.
NP = non pregnant
HP = healthy pregnant subjects
RP = subjects at risk of pre-eclampsia

3.5 Responder Status to the Thromboxane Synthetase Inhibitor Dazmegrel.

During healthy pregnancy there was a gradual loss of response to the thromboxane synthetase inhibitor dazmegrel. Pre-conceptionally, arachidonic acid induced platelet behaviour was completely abolished by both 100uM and 500uM dazmegrel in all subjects, but by 36 weeks only one third were full responders, the remaining two thirds being complete non-responders. The data are shown in Figure 5.8. It is apparent that changes in responder status were beginning to become apparent at 12 weeks gestation and were established by 24 weeks. Postnatally, 83% of women were full responders to dazmegrel, and in the remainder AA induced platelet behaviour was abolished by 500uM but not by 100uM dazmegrel ("partial responders").

In the "at risk" group there were no complete responders at entry to the study. This remained the case throughout pregnancy, but postnatally, half of subjects were responders (Figure 5.9).

3.6 Serum Thromboxane B_2

The serum levels of thromboxane B_2, the stable hydrolysis product of thromboxane A_2, were similar at all study time points for both the healthy and "at risk" groups. Furthermore, there was no difference in serum thromboxane B_2 between the subject groups for any phase of the study. The data are shown in Table 5.9.

3.7 Platelet Cyclic AMP Levels.

Basal adenylate cyclase activity.

In the absence of any adenylate cyclase stimulators, platelet cyclic AMP levels were similar at all study time points in both healthy and "at risk" subjects. This was the case whether or not the phosphodiesterase inhibitor AH-P719 was present. Furthermore, platelet cyclic AMP levels under these conditions were

TABLE 5.10

Platelet Cyclic AMP Levels Under Conditions of Basal Adenylate Cyclase Activity

	Gestation (weeks)				
Group	NP	5	12/15*	24	36
HP (sal)	0.0354 ± 0.0027	0.0398 ± 0.0054	0.0377 ± 0.0044	0.0370 ± 0.0037	0.0302 ± 0.0033
RP (sal)	0.0292 ± 0.0058	- -	0.0283 ± 0.0049	0.0359 ± 0.0063	0.0375 ± 0.0054
HP (AH-P719)	0.1661 ± 0.0081	0.1404 ± 0.0067	0.1531 ± 0.0062	0.1573 ± 0.0067	0.1406 ± 0.0069
RP (AH-P719)	0.1436 ± 0.0054	-	0.1420 ± 0.0061	0.1375 ± 0.0053	0.1443 ± 0.0060

* 12 weeks for HP and 15 weeks for RP
All values = % conversion ^{3}H-adenine to ^{3}H-cAMP. Means ± SEM are shown.

NP = non pregnant
HP = healthy pregnant subjects
RP = subjects at risk of pre-eclampsia

TABLE 5.11

PLATELET CYCLIC AMP RESPONSE TO ILOPROST

Conc Iloprost ± AHP719	Group	Non Pregnant	At diagnosis of pregnancy	Early	Mid	Late	Friedman p value	Individual p values
0.5ng/ml	HP	0.1092 ± 0.0285	0.0519 ± 0.0088	0.0688 ± 0.0157	0.0517 ± 0.0054	0.0514 ± 0.0129	0.0780	NS
+ Saline	RP	0.0716 ± 0.0346	-	0.0403 ± 0.0068	0.0548 ± 0.0107	0.0545 ± 0.0044	0.2925	NS
5ng/ml	HP	0.6409 ± 0.1313	0.3370 ± 0.0559	0.4116 ± 0.0557	0.3846 ± 0.0556	0.3769 ± 0.0744	0.0116	<0.05 np vs 5 wks
+ saline	RP	0.3890 ± 0.1173	-	0.2519 ± 0.0486	0.3320 ± 0.0375	0.3033 ± 0.0415	0.2725	NS
0.5ng/ml	HP	0.3758 ± 0.0251	0.3032 ± 0.0055	0.3471 ± 0.0136	0.3324 ± 0.0288	0.2864 ± 0.0169	0.0164	<0.05 np vs 36wks
+AHP-719	RP	0.2860 ± 0.0233	-	0.2636 ± 0.0144	0.2965 ± 0.0260	0.3018 ± 0.0126	0.2123	NS
5ng/ml	HP	1.4065 ± 0.1140	0.9745 ± 0.0868	1.1166 ± 0.1471	1.1118 ± 0.1312	1.0499 ± 0.1569	0.1019	NS
+AH-P719	RP	0.9765 ± 0.1432	-	0.7583 ± 0.0469	0.8764 ± 0.0757	0.8469 ± 0.0817	0.3080	NS

TABLE 5.12

PLATELET CYCLIC AMP RESPONSE TO PGD_2

Conc PGD_2 ± AHP719	Group	Non Pregnant	At diagnosis of pregnancy	Early	Mid	Late	Friedman p value	Individual p values
0.05μg/ml	HP	0.1568 ± 0.0251	0.1275 ± 0.0186	0.1422 ± 0.0208	0.14020.0172	0.1052 ± 0.0217	0.1894	NS
+ Saline	RP	0.1174 ± 0.0191	-	0.0831 ± 0.0158	0.1285 ± 0.0114	0.1316 ± 0.0176	0.0576	p< 0.05 for 15wks vs NP 24, 36 wks
0.5μg/ml	HP	0.8517 ± 0.0671	0.5113 ± 0.0986	0.6026 ± 0.1106	0.6028 ± 0.0887	0.4956 ± 0.1074	0.0868	NS
+ saline	RP*	0.6022 ± 0.0688	-	0.4070 ± 0.0715	0.4964 ± 0.0948	0.4787 ± 0.0678	0.0384	p<0.05 15wks vs NP
0.05μg/ml	HP	0.7765 ± 0.0695	0.6168 ± 0.0616	0.6422 ± 0.0527	0.6572 ± 0.0820	0.5735 ± 0.0607	0.3233	NS
+AHP-719	RP	0.5715 ± 0.0448	-	0.4387 ± 0.0414	0.5430 ± 0.0636	0.5325 ± 0.0380	0.1448	NS
0.5μg/ml	HP	1.8943 ± 0.2060	1.3551 ± 0.1651	1.3627 ± 0.1488	1.4743 ± 0.2241	1.2755 ± 0.1782	0.0729	NS
+AH-P719	RP	1.3316 ± 0.1405	-	0.9277 ± 0.1020	1.1146 ± 0.1597	1.0615 ± 0.1363	0.1272	NS

* Two way analysis of variance revealed an overall significant difference between HP and RP for cyclic AMP responses to 0.5μg/ml PGD_2 ($p = 0.0001$). Direct comparison of HP and RP at each time point revealed a significant reduction in cyclic AMP levels in platelets from RP under these conditions when subjects were not pregnant ($p = 0.0328$, Mann Whitney - U test).

comparable in both subject groups. The data are shown in Table 5.10.

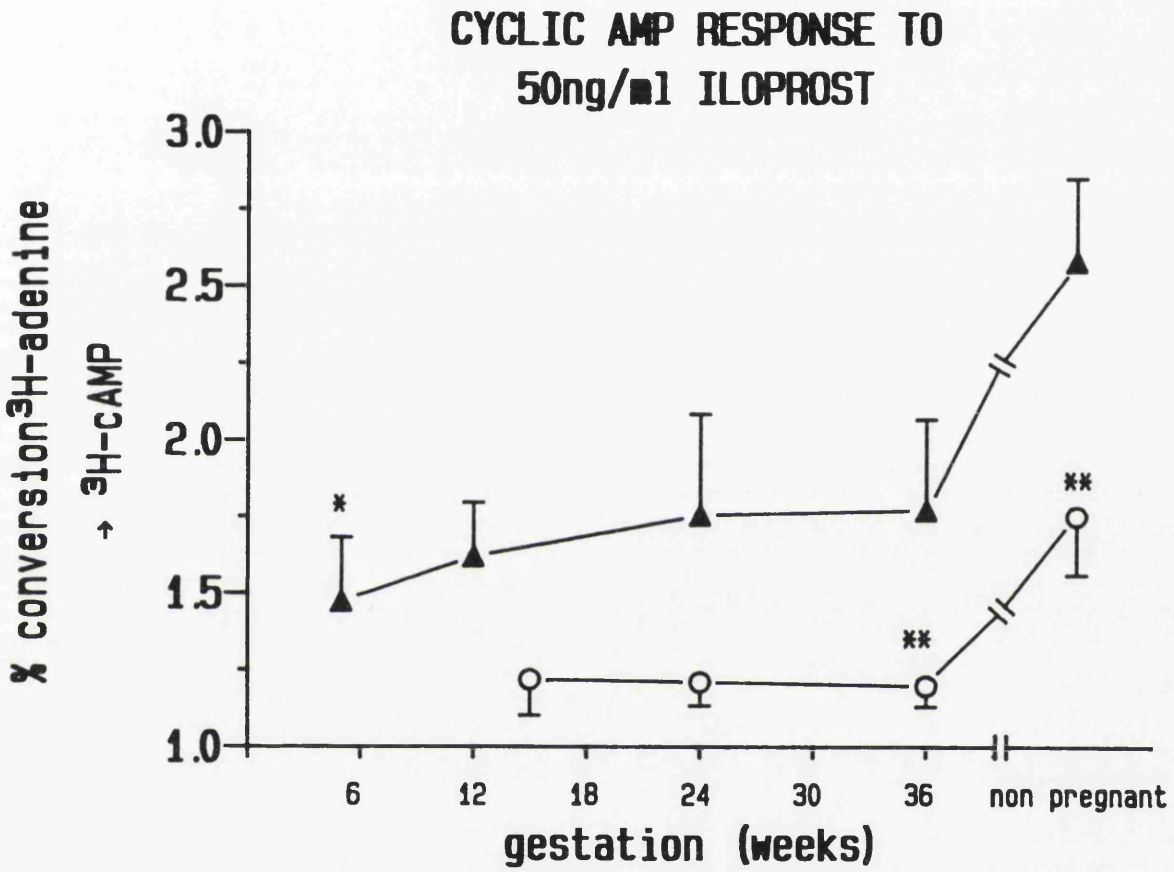
Platelet Cyclic AMP Levels Stimulated by Receptor Dependent Mechanisms.

In the healthy pregnant group there was a reduction in the platelet cyclic AMP response to iloprost and PGD_2 throughout pregnancy compared with non pregnant data. This effect was most pronounced when PRP was incubated with relatively high concentrations of the prostaglandins. The results are shown in Tables 5.11 and 5.12 and Figures 5.10-5.13. Reduced cyclic AMP responses to these agents were evident very early in pregnancy; in some cases as early as 5 weeks gestation. Platelet cyclic AMP responses to both iloprost and PGD_2 appeared to increase slightly in mid pregnancy but there was no statistically significant difference between gestations.

In the group at risk of pre-eclampsia the pattern of results was similar to that in healthy pregnancy, but the reduction in platelet cyclic AMP levels during pregnancy compared with non pregnant levels in this group appeared less marked, just failing to reach statistical significance for iloprost, but reaching significance for PGD_2. Results during pregnancy were similar for all gestations. Furthermore, platelet cyclic AMP responses to iloprost and to PGD_2 were generally significantly smaller in subjects "at risk" at early and late gestational periods compared with healthy pregnant women. There was no difference between the two groups in mid pregnancy. There was also a significant reduction in non pregnant platelet cyclic AMP responses to 50ng/ml iloprost and to 0.5ug/ml and 5.0ug/ml PGD_2 in the women with a history of pre-eclampsia compared with the healthy women.

Platelet Cyclic AMP Levels on Direct Stimulation of Adenylate Cyclase.

In the healthy pregnant group there was a significant reduction in the platelet cyclic AMP response to forskolin during pregnancy compared with non pregnant data. This effect reached statistical significance at 5 weeks and 36



FIGURES 5.10

Platelet cyclic AMP accumulation is plotted against gestation following stimulation of adenylate cyclase with 50ng/ml iloprost. In HP(▲) platelet cyclic AMP levels tended to be lower at all gestational time points compared with data when subjects were not pregnant. Friedman analysis of variance showed an overall p value of 0.0146, with $p < 0.05$ for the comparison of 5 week data with non pregnant data. In RP (○) a similar pattern of results was obtained but the difference between gestational and postnatal data just failed to reach statistical significance (Friedman ANOVA p value = 0.0658). Comparison of HP and RP showed significantly lower cAMP values in RP in late pregnancy and when subjects were not pregnant ($p = 0.05$). Statistical significance for comparisons within subject groups is denoted by * and between subject groups by **.

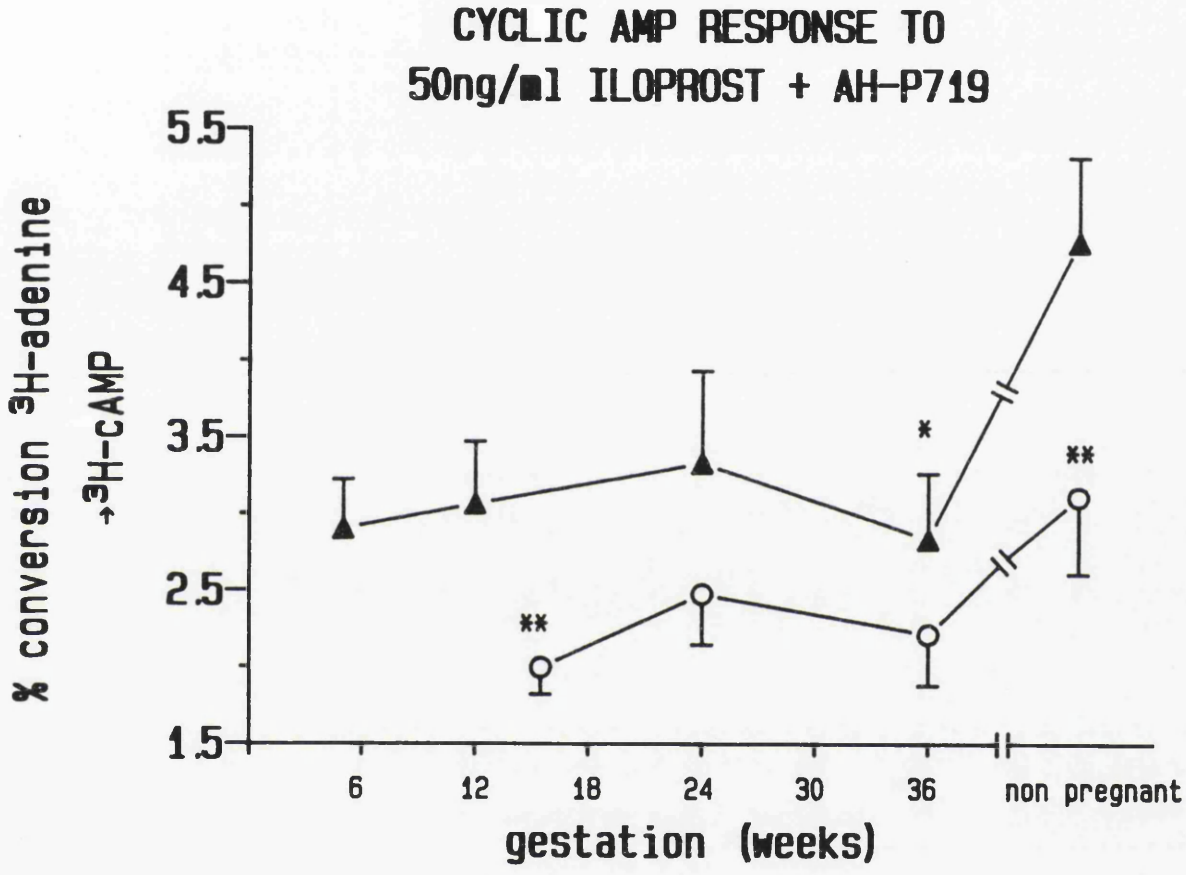


FIGURE 5.11.

The figure shows platelet cyclic AMP accumulation plotted against gestation following stimulation of adenylate cyclase with 50ng/ml iloprost in the presence of the phosphodiesterase inhibitor AH-P719. Once again, in HP (▲), platelet cyclic AMP levels tended to be lower at all gestational time points, compared with non pregnant data. Friedman ANOVA showed $p = 0.0306$, with $p < 0.05$ for 36 week data compared with non pregnant data (*). In RP (○), the pattern of results was similar but the Friedman ANOVA p value was 0.0858. Comparison of HP and RP showed significantly lower cyclic AMP levels in RP in early pregnancy and when subjects were not pregnant (** $p = 0.05$)

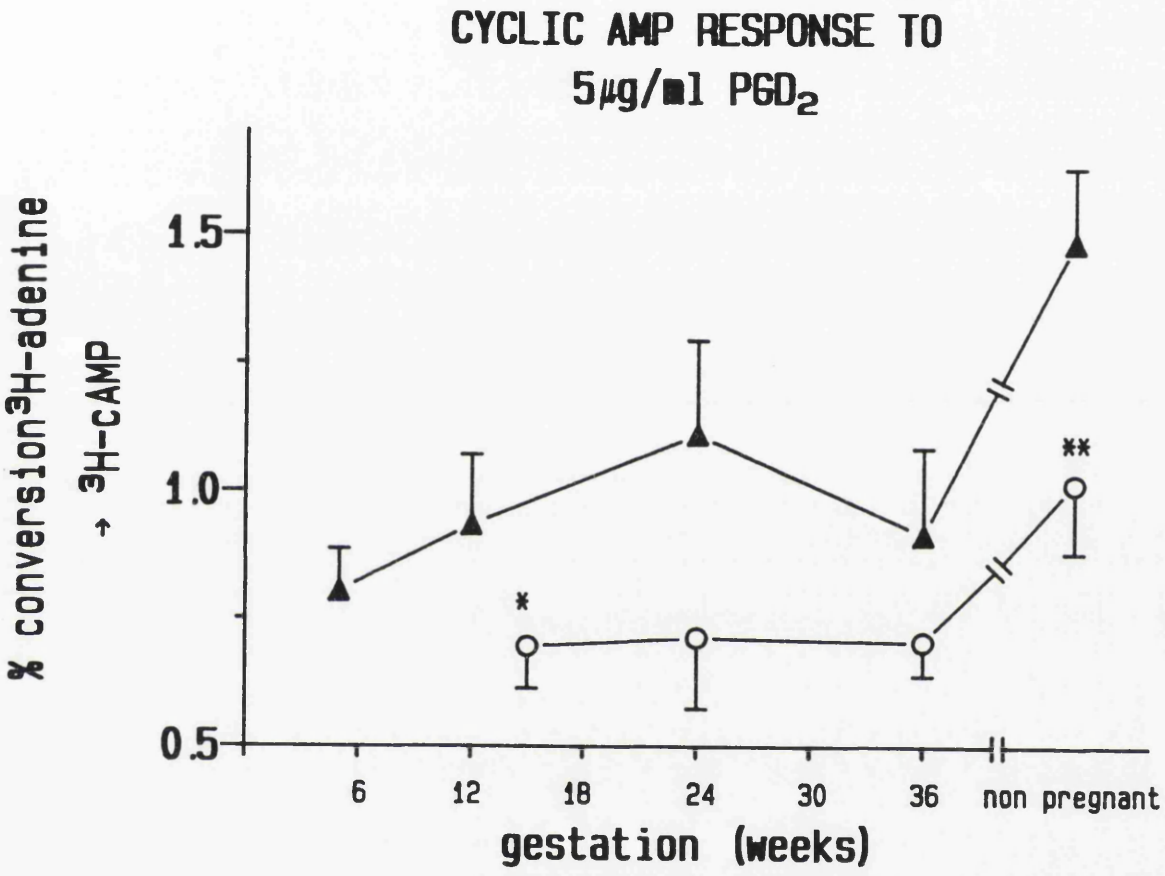


FIGURE 5.12

The figure shows platelet cyclic AMP accumulation following stimulation of adenylate cyclase with PGD_2. The pattern of results was very similar to those generated following exposure of platelets to iloprost. In HP (▲) the overall p value for Friedman ANOVA was 0.0979, and in RP (○) $p = 0.0320$, with $p < 0.05$ for comparison of early pregnancy and postnatal data (*). Comparison of HP and RP showed a significant reduction in cAMP in RP (ANOVA $p = 0.0001$), with $p = 0.0328$ when subjects were not pregnant (**).

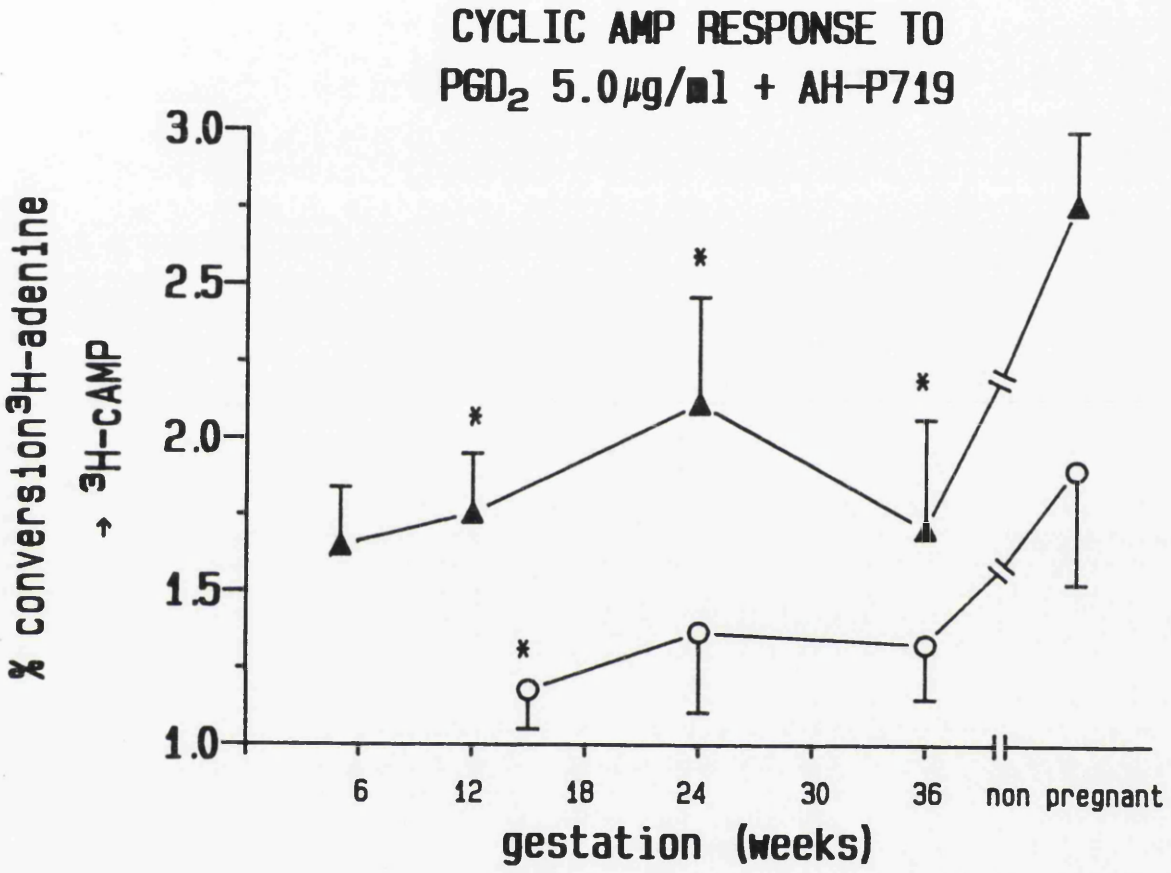


FIGURE 5.13.

The graph shows platelet cyclic AMP accumulation following stimulation of adenylate cyclase with PGD_2 in the presence of AH-P719. In HP (▲) cyclic AMP was lower during pregnancy than when subjects were not pregnant (Friedman ANOVA $p = 0.0440$) with $p < 0.05$ for comparison of data at 12, 24, and 36 weeks with non pregnant data (*). In RP (○) the pattern of results was similar (Friedman ANOVA $p = 0.0455$), with $p < 0.05$ for comparison of data in early pregnancy with postnatal data(*). In this case, comparison of HP and RP revealed no statistically significant difference in results.

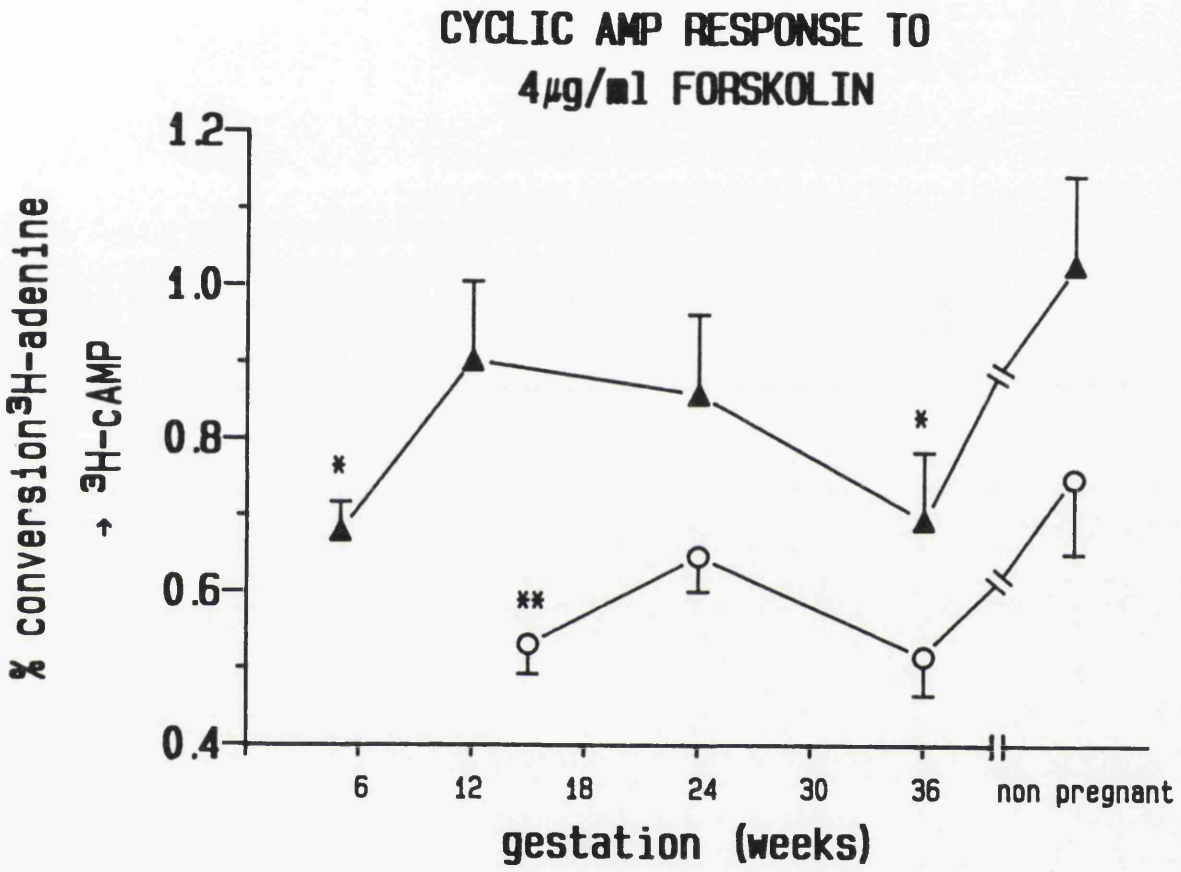


FIGURE 5.14

The figure illustrates platelet cyclic AMP responses to forskolin in the presence of the phosphodiesterase inhibitor AH-P719. There was a significant reduction in platelet cyclic AMP levels in HP (▲) during pregnancy (Friedman ANOVA $p = 0.0092$), with $p < 0.05$ (*) at 5 weeks and 36 weeks gestation compared with non pregnant data. In RP (○) platelet cyclic AMP responses to forskolin showed a similar pattern but there was no significant difference between gestational and postnatal data. In early pregnancy, women at risk of pre-eclampsia showed a significantly reduced platelet cyclic AMP response to forskolin compared with healthy primigravidae ($p = 0.0188$, **) and the difference between groups approached significance for non pregnant data ($p = 0.0813$).

weeks gestation (Figure 5.14). A trend towards a mid pregnancy rise was again noted but, as with the response to the prostaglandins, there was no statistically significant difference between the data at different gestations.

In the "at risk" group, the pattern of response was similar but the difference between the postnatal platelet cyclic AMP response to forskolin and results during pregnancy was not statistically significant.

When the two groups of subjects were compared, there was a significant reduction in the platelet cyclic AMP response to forskolin in early pregnancy in subjects at risk of pre-eclampsia. Although forskolin-stimulated platelet cyclic AMP levels appeared lower postnatally in this group compared with healthy women, this difference just failed to reach statistical significance.

Arachidonic Acid and Platelet Cyclic AMP.

In the healthy patients, arachidonic acid alone led to a slight rise in platelet cyclic AMP above basal levels. This was markedly enhanced in the presence of the thromboxane synthetase inhibitor dazmegrel. Following incubation of PRP with AA alone platelet cyclic AMP levels tended to be lower in early and mid pregnancy compared with late pregnancy and non pregnant data (Figure 5.15). This trend, however, did not reach statistical significance. In the presence of the thromboxane synthetase inhibitor, the pattern of results was similar, but in this case the difference between early pregnancy and 36 week and non pregnant data reached significance (Figure 5.16). It is noteworthy that these data differ from the platelet cyclic AMP responses to individual prostaglandins and to forskolin in the similarity of the late pregnancy response to non pregnant data.

In women at risk of pre-eclampsia there was a trend during pregnancy towards a reduction in platelet cyclic AMP levels in the presence of AA alone and AA and dazmegrel together compared with postnatal data, but statistical analysis

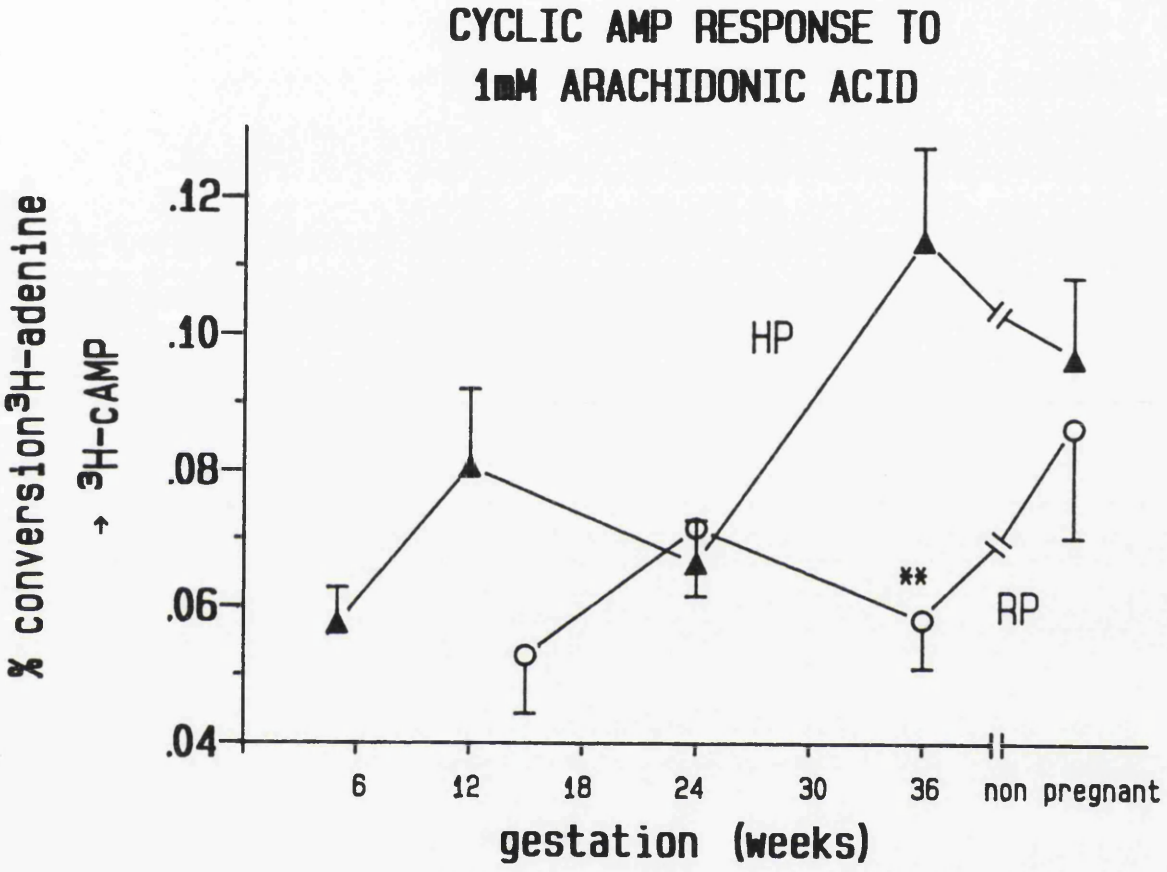


FIGURE 5.15.

The graph shows platelet cyclic AMP levels in the presence of 1mM arachidonic acid throughout the study period. There was no statistically significant difference between results at different phases of the study in either HP (▲) or RP (○). The Friedman ANOVA p value for HP was 0.0868, and for RP was 0.2988. Note , however that a tendency towards a rise in cyclic AMP levels occurred in late pregnancy in HP, at which time platelet cyclic AMP levels were significantly lower in RP (**, p = 0.0328).

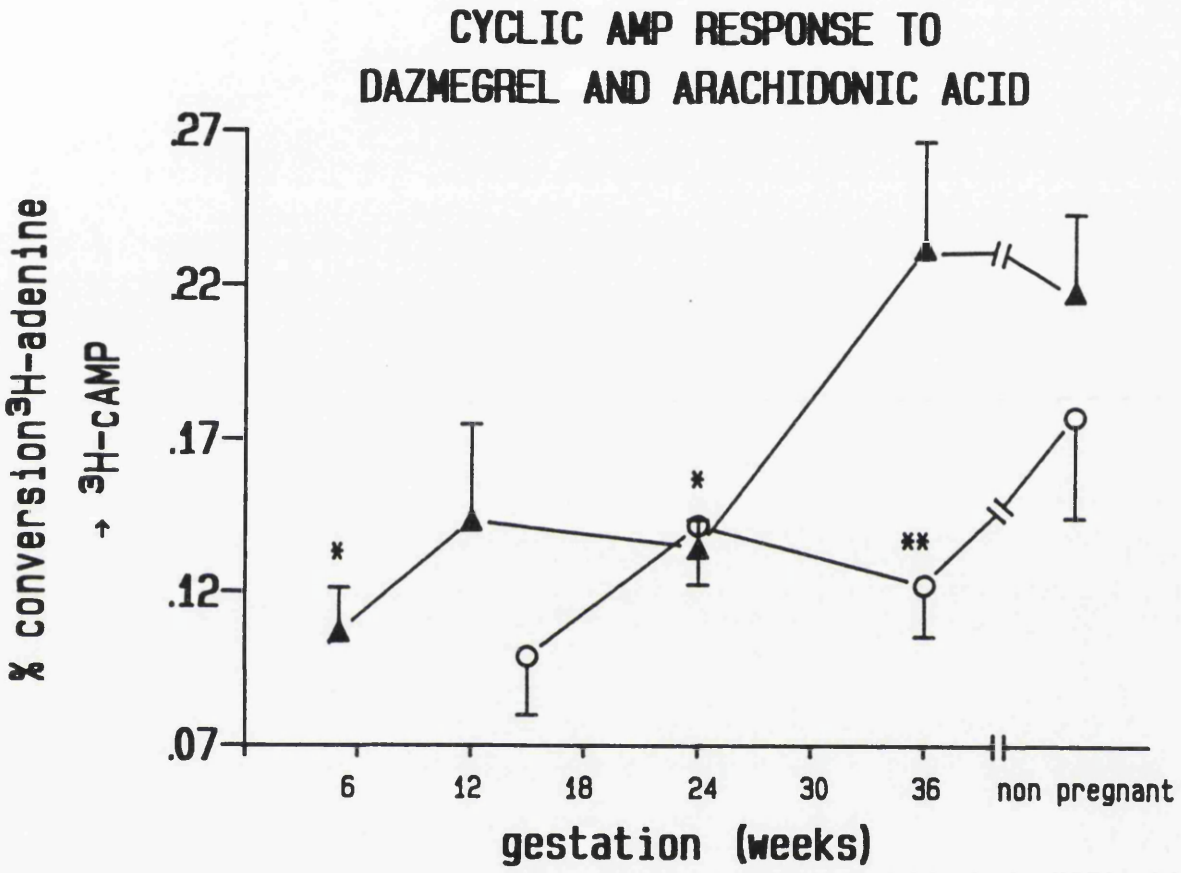


FIGURE 5.16.

The figure shows platelet cyclic AMP levels in the presence of arachidonic acid together with the thromboxane synthetase inhibitor dazmegrel. In HP(▲) platelet cyclic AMP levels were significantly lower in very early and mid pregnancy compared with non pregnant data. There was a significant late pregnancy rise in the cyclic AMP response. The overall p value for Friedman ANOVA was 0.0452, with $p < 0.05$ for comparison of non pregnant with 5 and 24 week data and comparison of 5 week and 36 week data (*). In RP (○) the late pregnancy rise in cyclic AMP responses under these conditions did not occur, and the difference between HP and RP was statistically significant at this time point ($p = 0.0328$, **).

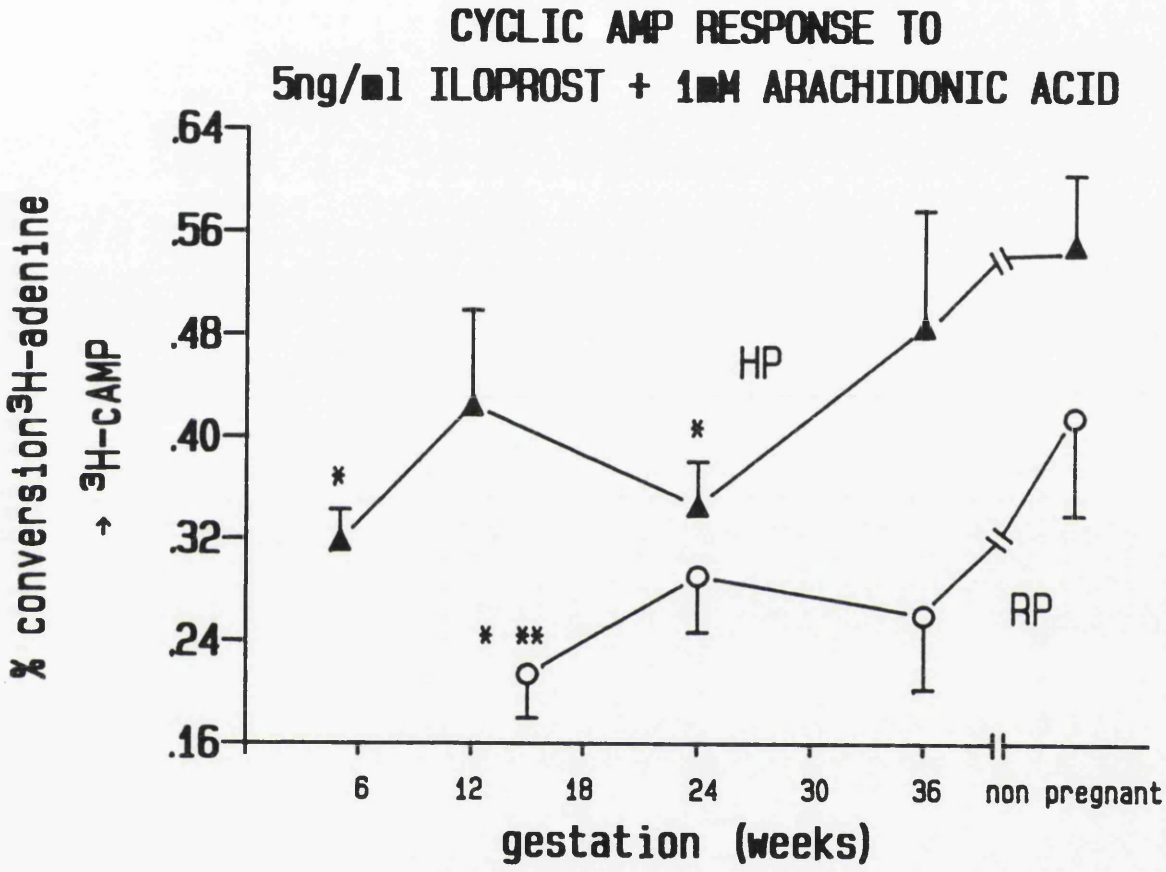


FIGURE 5.17.

The figure shows platelet cyclic AMP accumulation in the presence of 5ng/ml iloprost together with 1mM AA. The pattern of results resembles those in the presence of AA alone and AA and dazmegrel as shown in Figures 5.15 and 5.16. Friedman ANOVA revealed $p = 0.0382$ for HP (▲), with $p < 0.05$ for 5 week and 24 week data compared with non pregnant data. In RP (○), Friedman ANOVA yielded an overall p value of 0.0503, with $p < 0.05$ for comparison of data in early pregnancy and postnatally. Comparison of platelets from HP and RP under these conditions showed significantly less cyclic AMP accumulation in those from RP in early pregnancy (**, $p = 0.0188$).

in both cases revealed no significant difference between study time points. It is noteworthy, however, that the late pregnancy increase in cyclic AMP in response to arachidonic acid and dazmegrel demonstrated by healthy subjects did not occur in the "at risk" group. Indeed comparison of the two groups of subjects by two way analysis of variance showed that platelet cyclic AMP levels in the presence of both AA alone and AA and dazmegrel together were significantly lower in late pregnancy in subjects at risk of pre-eclampsia compared with healthy subjects. There was no difference between groups in results in early and mid pregnancy and when subjects were not pregnant.

Influence of Arachidonic Acid on Platelet Cyclic AMP Response to Iloprost.

In the healthy primigravidae, platelet cyclic AMP responses to iloprost and AA together were significantly reduced at 5 and 24 weeks gestation compared with non pregnant data. There was, however a significant rise in the platelet cyclic AMP levels in the presence of AA and iloprost at 36 weeks compared with those at 5 weeks gestation, mirroring the pattern of results with AA alone and AA and dazmegrel (Figure 5.17).

In women at risk of pre-eclampsia there was a reduction in the cyclic AMP response to iloprost and AA during pregnancy compared with results in the same patients postnatally, reaching statistical significance in the early second trimester. In this group of subjects there was no increase in cyclic AMP in the presence of iloprost and AA in late pregnancy. Comparison of the two subject groups revealed that platelets from subjects at risk of pre-eclampsia produced significantly less cyclic AMP in response to the combination of AA and iloprost in early pregnancy and there was a similar trend in late pregnancy and non pregnant data which just failed to reach statistical significance.

3.8 Platelet Behaviour in Relation to Cyclic AMP Studied Longitudinally in a Primigravida who Developed the HELLP Syndrome.

Case History.

The patient was a 28 year old primigravida who had no past medical history of note and was a regular blood donor. She volunteered for the study when she was planning pregnancy. At the pre-conceptional visit her blood pressure was 125/79. Seven weeks following the pre-conceptional study day, she reported that her menstrual period was seven days late. A sensitive pregnancy test confirmed pregnancy, which was estimated at 5 weeks gestation by dates, and she donated blood for platelet studies. Her blood pressure was 136/79. She booked at the antenatal clinic of another local hospital at 15 weeks gestation by dates, and gestation was confirmed by ultrasound scan. She donated blood for the study at 12, 24, and 36 weeks gestation. At all of these visits and all antenatal clinic visits she was well, with a normal blood pressure, negative urinalysis, and adequate fetal growth by both clinical and/or ultrasound assessment.

At 38 weeks gestation she was admitted under the care of her consultant obstetrician to her local hospital following an antenatal clinic visit because of the "incidental finding" of thrombocytopenia on a routine full blood count (Hb 11.7g/dl, WBC 5.24 x 10^9/l, platelets 49 x 10^9/l). All previous platelet counts were within the normal range. Examination of the peripheral blood film showed that red cell morphology was normochromic normocytic, and the thrombocytopenia was confirmed. Prothrombin time, activated partial thromboplastin time, and thrombin clotting times were normal. The blood pressure at the time of admission was 150/86 and urinalysis was negative for protein. A bone marrow aspirate was carried out, which showed plentiful megakaryocytes, indicating that the thrombocytopenia was due to peripheral platelet consumption,

and a provisional diagnosis of autoimmune thrombocytopenia was made. The patient was treated for three days with high dose intravenous immunoglobulin. Over this period, diastolic blood pressures of 90 mmHg (a rise of 24 mmHg compared with the diastolic pressure at 12 weeks) were recorded on several occasions. Serum urea and electrolytes were normal, but the urate was raised at 393umol/l and liver function tests showed a raised ALT at 339IU/l, with a slight elevation of bilirubin at 40umol/l.

On the third day following admission, proteinuria was recorded on dipstick testing, but was not quantified. The platelet count fell to 27 x 10^9/l, and haemoglobin (Hb) dropped to 10.5g/dl. A reticulocyte count was not done and there was no record of the blood film having been examined for schistocytes at this stage. Clotting times remained normal, but plasma fibrinogen levels and serum FDP were not measured. Later the same day the patient complained of epigastric pain, and diastolic blood pressure was recorded as 100. Brisk reflexes and clonus were documented in the case record. The cardiotocograph was noted to be unreactive with marked prolonged decelerations. A decision was made to perform an emergency Caesarean section for "fetal distress and ITP". During the procedure the estimated blood loss was 1 litre and the patient was transfused with 4 units of blood and 6 units of platelets. A live male baby weighing 3.8kg was delivered with Apgar scores of 9 at 1 minute and 10 at 5 minutes. Following delivery, the blood pressure fell within 24 hours, and there was a rapid resolution of the thrombocytopenia, the platelet count having reached 231 x 10^9/l by the second postnatal day. The postoperative Hb was 8.5g/dl. Liver function tests gradually improved, the ALT reaching a normal value one week following delivery. Mother and baby were both well at the 6 week postnatal visit.

The diagnosis of the HELLP syndrome was made postnatally by the

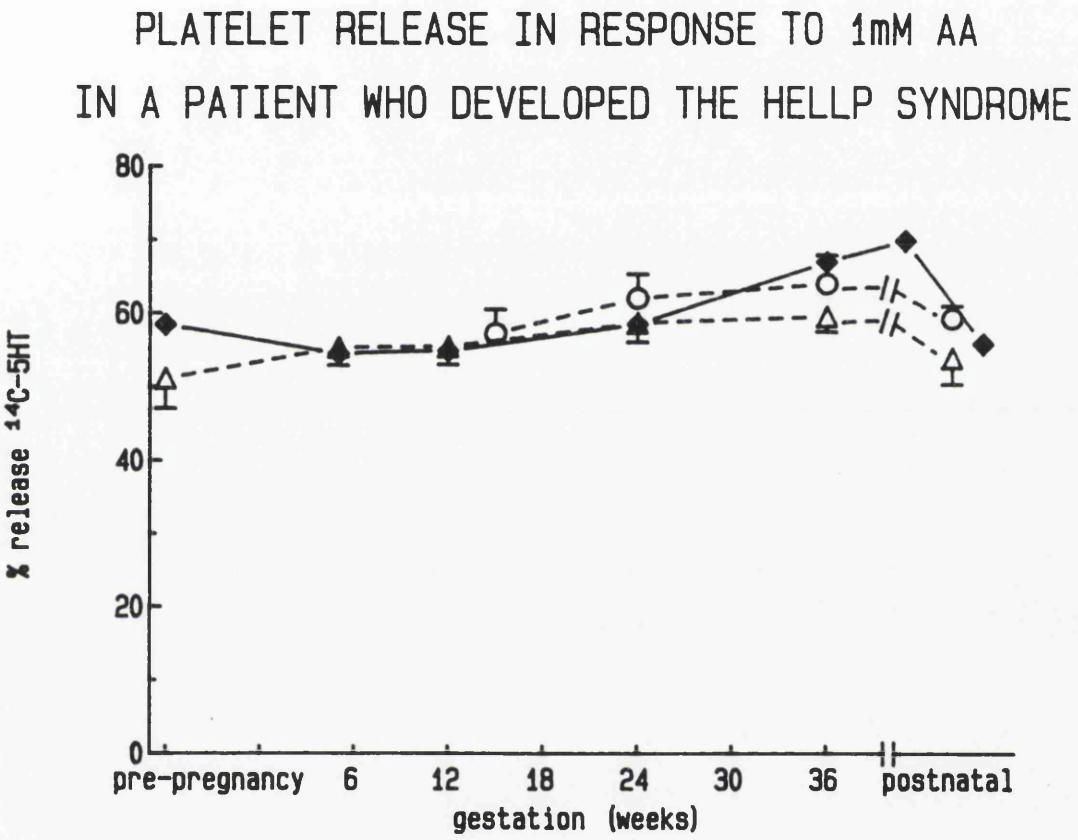


FIGURE 5.18.

The graph shows % release ^{14}C-5HT plotted against gestation in the patient with the HELLP syndrome (—◆—). For comparison, results in the healthy pregnant group studied longitudinally are shown (···△···), together with those in the group at risk of pre-eclampsia (···○···).

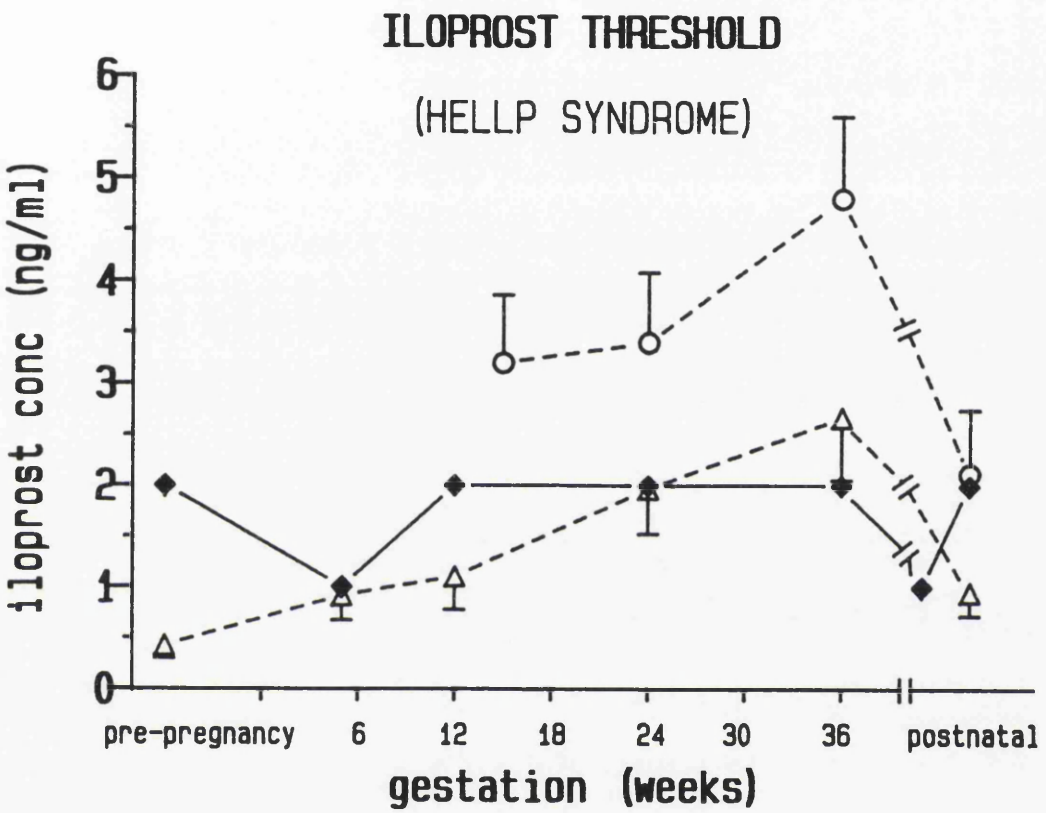


FIGURE 5.19.

The figure shows platelet sensitivity to iloprost for the patient(—◆—), as measured by threshold concentration of the inhibitor resulting in complete inhibition of arachidonic acid induced 5HT release. Results in HP (···△···) and RP (···○···) are shown for comparison. Although the results for the patient did not differ from those in healthy pregnancy at gestational time points, it is noteworthy that platelet sensitivity to iloprost remained fairly constant over the whole study period.

TABLE 5.13

PARAMETERS OF PLATELET BEHAVIOUR IN A PATIENT WHO DEVELOPED THE HELLP SYNDROME

Parameter	Pre-pregnancy	GESTATION 5 weeks	12 weeks	24 weeks	36 weeks	POSTNATAL 4 days	6 weeks
PGD_2 Threshold (μg/ml)	0.02	0.03	0.02	0.03	0.03	0.04	0.02
Forskolin Threshold (μg/ml)	0.6	0.8	0.4	0.8	0.6	0.2	0.6
AH-P719 Threshold (μM)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Dazmegrel Responder Status	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR/R
Serum TXB_2 (pmoles/10^7 platelets)	24.93	26.01	33.04	30.04	31.11	-	30.22

consultant obstetrician in charge of the case. There is no doubt that the patient had low platelets, elevated liver enzymes, and features of pre-eclampsia. The blood pressure was never markedly elevated, but the increment in diastolic pressure met the criteria for PIH and serum urate was elevated. Proteinuria developed as a late feature. From the clinical features documented in the case record, it is apparent that the patient had impending eclampsia just prior to delivery. The evidence for microangiopathic haemolysis is less convincing as red cell morphology was reputedly normal when thrombocytopenia was first identified, but evidence of haemolysis was never specifically sought, and the perioperative drop in haemoglobin was greater than expected taking account of estimated blood loss and transfusion.

Platelet Studies.

Blood was taken for platelet studies pre-conceptionally, at 5, 12, 24, and 36 weeks gestation, and at 4 days and 6 weeks postnatally. Unfortunately there was no opportunity to study the patient at the time when she developed the features of the HELLP syndrome as she was admitted to another hospital and the diagnosis was made in retrospect.

Arachidonic Acid Induced Platelet Behaviour.

The % ^{14}C-5HT release in PRP from the patient is shown in Figure 5.18. There was a slight increase in dense granule release in late pregnancy which had returned to pre-conceptional levels by the 6th postnatal week.

Sensitivity to Cyclic AMP Manipulators.

Iloprost thresholds for the patient, compared with those in healthy pregnant women and those at risk of pre-eclampsia, are illustrated in Figure 5.19. Threshold concentrations of each of the other inhibitors leading to complete abolition of the release reaction in the patient's PRP are shown on Table 5.13. Although the

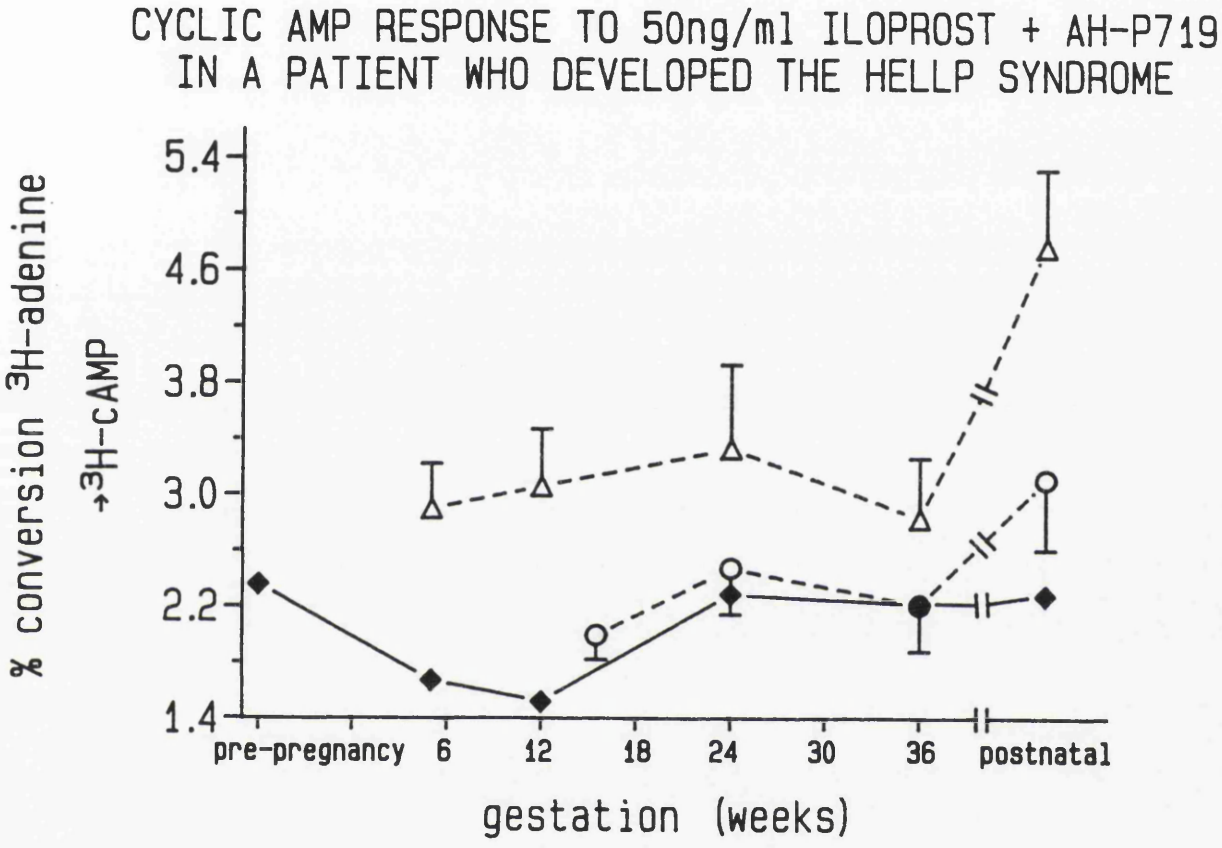


FIGURE 5.20

The figure illustrates platelet cyclic AMP levels in the presence of iloprost and the phosphodiesterase inhibitor for the patient who developed the HELLP syndrome (—◆—). Results for HP (···△···) and RP (···○···) are again shown for comparison. It is noteable that results for the patient closely correspond to those for the group of patients at risk of pre-eclampsia.

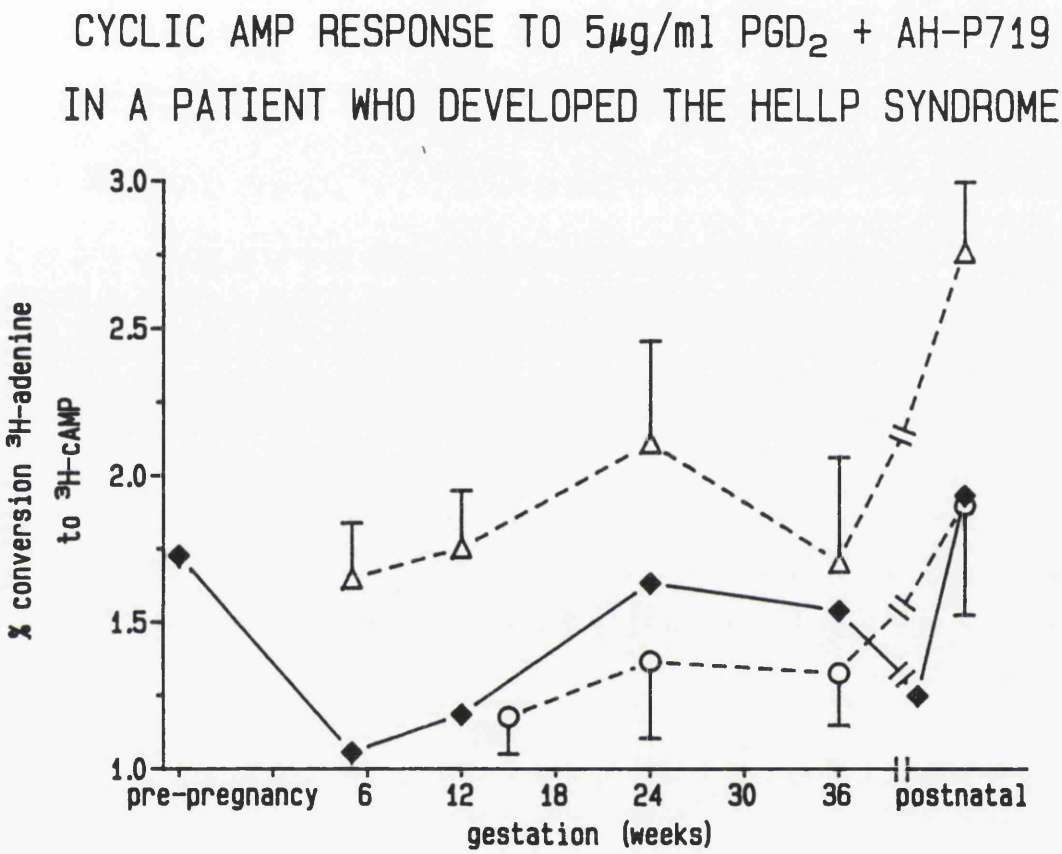


FIGURE 5.21

The figure illustrates platelet cyclic AMP levels in the presence of PGD_2 and the phosphodiesterase inhibitor for the patient who developed the HELLP syndrome (—◆—). Results for HP (···△···) and RP (···○···) are again shown for comparison.

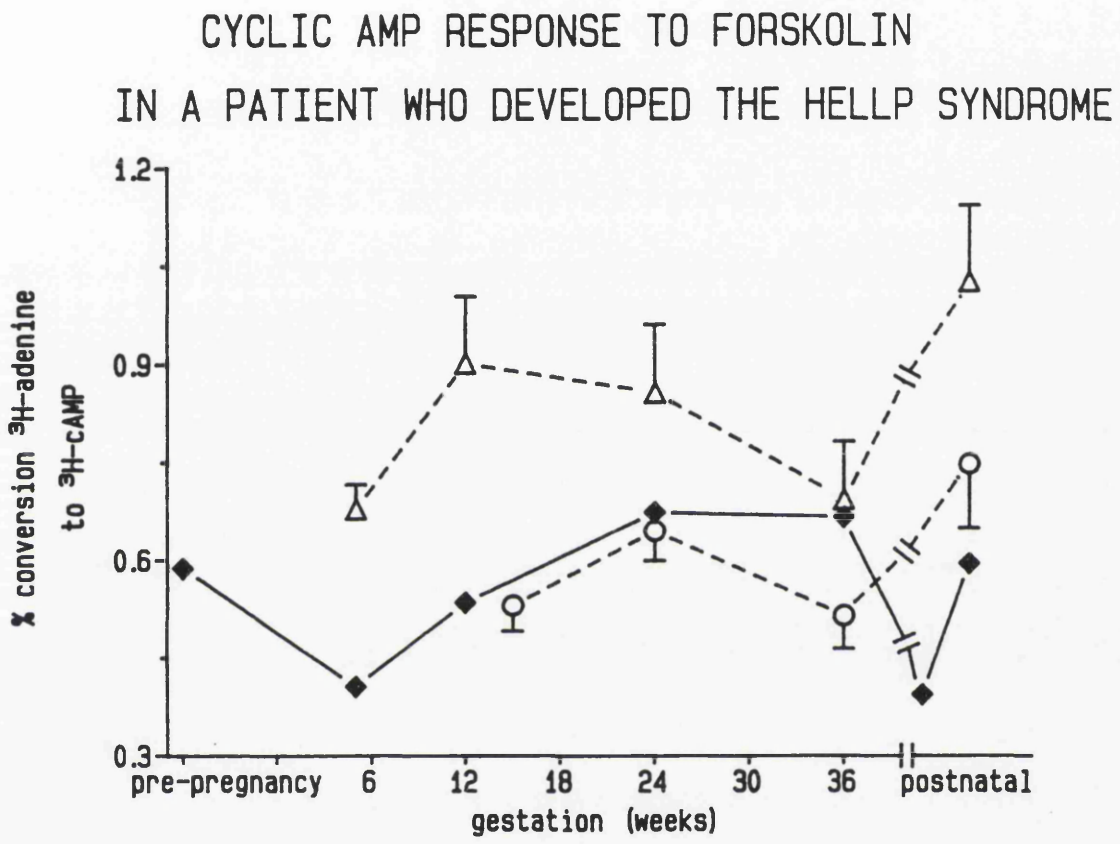


FIGURE 5.22

The figure illustrates platelet cyclic AMP levels in the presence of forskolin and the phosphodiesterase inhibitor for the patient who developed the HELLP syndrome (—◆—). Results for HP (···△···) and RP (···○···) are again shown for comparison.

degree of sensitivity of the patient's platelets to the inhibitors did not clearly differ from values obtained in healthy pregnancy, it is noteworthy in each case that there was little change in sensitivity as pregnancy progressed and results in the pre-conceptional and postnatal phases tended to lie closer to those in the "at risk" group in the case of iloprost and forskolin. Furthermore the patient was a complete non responder to dazmegrel pre-conceptionally, at all gestational time points and at 4 days postnatally. At 6 weeks postnatally she was a partial responder.

Serum Thromboxane B_2

Thromboxane B_2 levels in the patient's serum changed little over the period of the study and were similar to those recorded in healthy pregnancy. The results are shown in Table 5.13.

Platelet Cyclic AMP Levels.

Basal platelet cyclic AMP levels and those in the presence of AH-P719 alone did not change throughout the study period. Examples of platelet cyclic AMP responses to iloprost, PGD_2 and forskolin are shown in Figures 5.20-5.22. The data shown demonstrate the typical pattern obtained in the patient's PRP with all concentrations of these agents with or without the PDE inhibitor. It is noteworthy that the results in the subject who developed the HELLP syndrome, more closely resemble those of the "at risk" group than the healthy pregnant group, but in late pregnancy, just prior to the onset of clinical signs and symptoms, this trend partially reversed, especially in the case of PGD_2 and forskolin. Platelet cyclic AMP accumulation in PRP from the patient in the presence of arachidonic acid in combination with a)dazmegrel, and b)iloprost is shown in Figure 5.23 and 5.24. Again, the pattern for the patient resembled that shown by the women at risk of pre-eclampsia, and in particular, the patient showed no late pregnancy rise in the cyclic AMP response under these conditions, as

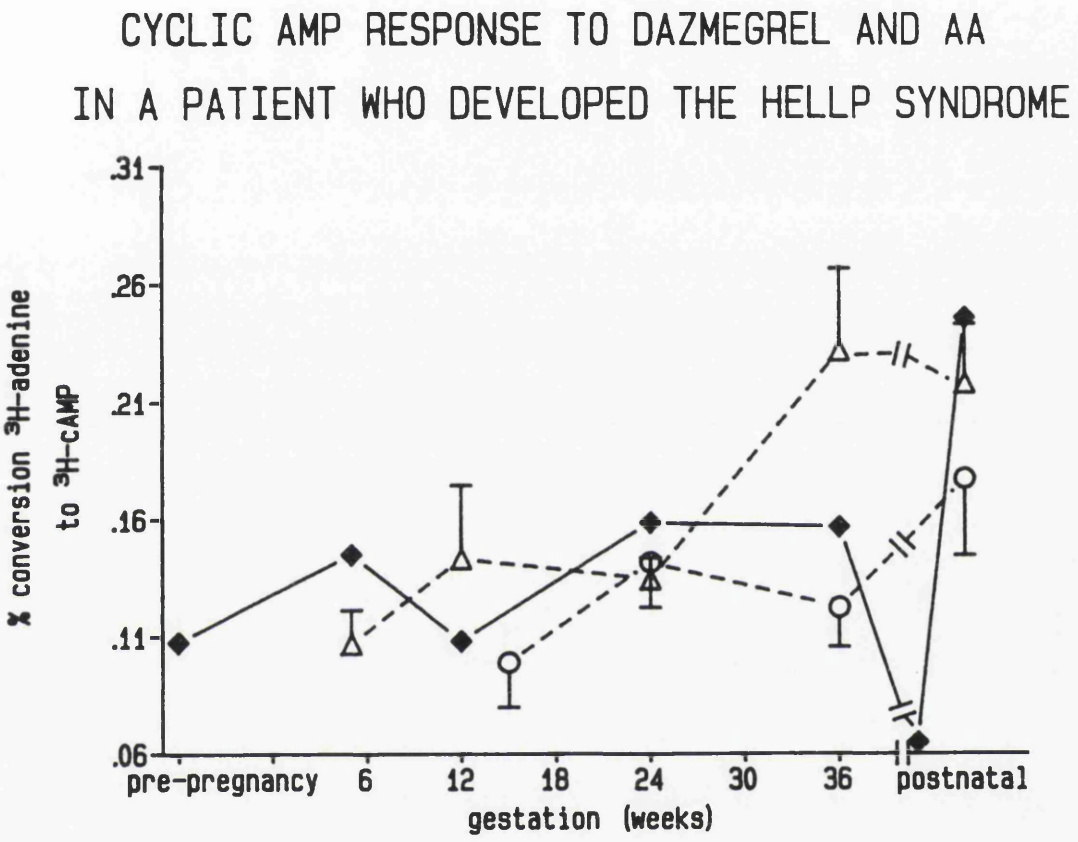


FIGURE 5.23.

The graph shows platelet cyclic AMP accumulation in the presence of dazmegrel 500μM and 1mM AA in the patient with the HELLP syndrome (—◆—), together with results in HP (···△···) and RP (···○···). Note that platelets from the patient, as for RP showed no late pregnancy rise in cyclic AMP under these conditions.

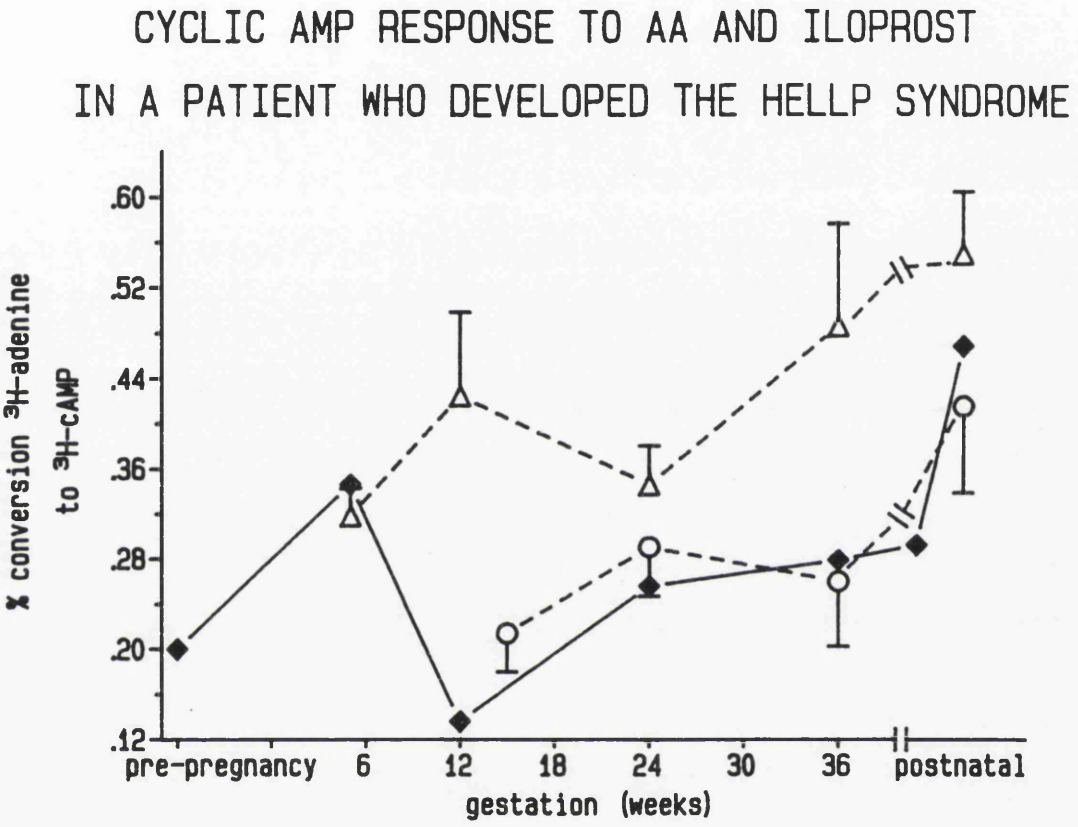


FIGURE 5.24.

The graph shows platelet cyclic AMP accumulation in the presence of AA 1mM and 5ng/ml iloprost in the patient with the HELLP syndrome (—◆—), together with results in HP (···△···) and RP (···○···). Note that platelets from the patient, as for RP showed no late pregnancy rise in cyclic AMP under these conditions.

demonstrated by healthy primigravidae. It is notable that, when studied 4 days postnatally, the patient's cyclic AMP responses to all adenylate cyclase stimulators were much lower than at any other time during the study. The significance of this is uncertain as no other subjects were studied at this time. Furthermore, the patient had received a platelet transfusion at delivery.

4.DISCUSSION.

This study has confirmed the findings of the cross sectional studies reported in Chapters 3 and 4 by showing that, during healthy pregnancy, platelets are less sensitive to the inhibitory effects of a wide range of agents which act via cyclic AMP. This was again associated with a reduction in cyclic AMP production during pregnancy in PRP in response to all adenylate cyclase stimulators tested, compared with results in PRP from the same women when they were not pregnant. As before there was no change in cyclic AMP levels under conditions of basal adenylate cyclase activity, whether or not phosphodiesterase was inhibited. Changes in cyclic AMP responses were observed with a wide range of adenylate cyclase stimulators including forskolin which can act directly upon the adenylate cyclase enzyme.[200,201] These data, taken together with the results of the cross sectional study, suggest that a reduction in adenylate cyclase activity occurs during pregnancy.

The longitudinal data showed that reduction in efficacy of cyclic AMP manipulators as inhibitors of platelet behaviour became gradually more marked as pregnancy progressed, reaching a maximum at 36 weeks gestation. The pattern of cyclic AMP responses with gestation was somewhat different, with reduction in stimulated cyclic AMP levels becoming apparent in the very earliest weeks of pregnancy, and subsequently remaining reduced throughout the gestational period.

In healthy pregnant women changes in platelet sensitivity to cyclic AMP manipulators and cyclic AMP responses reversed postnatally. It would, however, be difficult to ascertain the timing of this as, although all women were studied after the sixth postnatal week, the exact timing of postnatal samples varied widely for practical reasons.

The differing time courses of changes in platelet cyclic AMP responses compared with changes in the efficacy of the adenylate cyclase stimulators as inhibitors of platelet behaviour, suggest that factors other than absolute amounts of cyclic AMP play a role in determining inhibitor efficacy. The extent of thromboxane production would not seem to be implicated, as serum thromboxane B_2 levels did not vary throughout the study. As proposed in the discussion of the cross sectional data, the hypothesis could be put forward that an increase in sensitivity to the pro-aggregatory effects of thromboxane, or to other arachidonic acid metabolites could contribute to the reduction in efficacy of the inhibitors as pregnancy progressed. The platelet cyclic AMP results in the presence of arachidonic acid are also noteworthy in this respect. The pattern of results in the healthy pregnant group in the presence of AA alone, or in combination with dazmegrel or iloprost was different compared with those when adenylate cyclase stimulators were tested in the absence of AA. Healthy pregnant patients showed a significant late pregnancy rise in platelet cyclic AMP levels when AA alone, AA and dazmegrel and AA and iloprost were investigated. The interpretation of the biochemical mechanisms responsible for these observations can only remain speculative, but it is pertinent that the late pregnancy increase in cyclic AMP in the presence of AA in healthy pregnancy occurred at a time when the majority of patients showed a "non responder" status with dazmegrel and when platelets were least sensitive to inhibition of their AA induced behaviour by adenylate

cyclase stimulators in general. It is possible that in late pregnancy cyclic AMP itself is less effective as an inhibitor of platelet behaviour, and these observations again raise the possibility that factors such as sensitivity to pro-aggregatory prostanoids contribute to the determination of efficacy of cyclic AMP manipulators as inhibitors of platelet behaviour including dazmegrel responder status.

In contrast with the data from the cross sectional study, the longitudinal study has revealed interesting differences between healthy pregnant women and those with a previous history of pre-eclampsia. In women with high risk pregnancies, stimulated cyclic AMP levels during pregnancy were consistently lower than those in healthy pregnant women and platelets were also less sensitive to inhibition of the release reaction by all adenylate cyclase stimulators. The late pregnancy rise in platelet cyclic AMP accumulation in the presence of arachidonic acid was not noted in high risk pregnancies, suggesting that a mechanism potentially capable of limiting the pro-aggregatory platelet response to arachidonic acid metabolites, which was present at 36 weeks in healthy pregnancy, appeared to be absent in high risk pregnancies. Furthermore, the primigravid patient who developed the HELLP syndrome,[355] who was recruited independently from the high risk women, showed platelet cyclic AMP responses which were similar under most experimental conditions to those women recruited to the "at risk" study. Although the numbers of subjects in each study group were small, the consistency of results in the gestational period together with the results for the patient with the HELLP syndrome suggest that the differences between healthy women and those at risk of pre-eclampsia are genuine, at least during the gestational period. The difference between groups of women in parameters such as blood pressure, serum urate and pattern of platelet count during pregnancy also give credence to the observed alterations in platelet behaviour in relation to cyclic AMP. The

interpretation of the data in the non pregnant phase must be approached more cautiously, as all the "at risk" subjects were studied postnatally, and no pre-conceptional data were available for this group. Nevertheless, the timing of postnatal sampling did not differ significantly between the two subject groups. The possibility that a longer time is required for pregnancy associated changes in platelet cyclic AMP to reverse postnatally in high risk women cannot, however, be excluded. The hypothesis could therefore be put forward that, at least during pregnancy, there is a reduction in the responsiveness of the platelet adenylate cyclase system in some women, over and above that which occurs in healthy pregnancy, which is associated with a risk of pre-eclampsia. It is possible that this difference also exists when such subjects are not pregnant.

The differences in platelet responses between the two groups during pregnancy cannot be explained by the effects of pre-eclampsia upon platelet behaviour as none of the women recruited into the high risk group developed clinically overt pre-eclampsia during the pregnancy studied. As the cross-sectional study showed that platelet parameters in pre-eclamptic women did not differ significantly from those in healthy primigravidae, it is even possible that the onset of pathological changes in blood vessels which occur when pre-eclampsia is clinically detectable could alter platelet behaviour and biochemistry in a way which would make differences between healthy women and those at risk of the disease more difficult to detect when platelets were studied in vitro. Although caution must be applied to the interpretation of data from one subject, this hypothesis is supported by the results presented for the patient with the HELLP syndrome whose platelet sensitivity to inhibitors acting via cyclic AMP changed little over the gestational period. Furthermore, with the exception of responses in the presence of arachidonic acid, platelet cyclic AMP accumulation in this patient,

although more closely resembling the pattern of responses in the at risk group in early and mid pregnancy, was less easily distinguishable from that in healthy primigravidae at 36 weeks gestation. One possible explanation for these observations is that platelet consumption in vivo in pre-eclampsia[291,293,353,400] leaves only a subpopulation of platelets available for in vitro study. Reduction in placental production of vasodilatory prostaglandins in pre-eclampsia[160,298,310,397-399] may also alter the profile of platelet sensitivity to inhibitory prostaglandins and platelet cyclic AMP accumulation in response to these agents, potentially masking any intrinsic differences in such parameters in women at risk of the disease. The lack of change in efficacy of prostaglandins as inhibitors of platelet behaviour as pregnancy progressed in the patient with the HELLP syndrome would lend support to the lack of a factor in pre-eclampsia which is responsible for physiological alterations in platelet behaviour in normal pregnancy. This raises the question of whether the changes in platelet responses to inhibitory prostaglandins observed during healthy pregnancy could result from heterologous desensitisation of platelet adenylate cyclase as a result of exposure to prostaglandins synthesised by the placenta. If this were the case, reduced placental synthesis of prostaglandins may result in desensitisation being less marked when pre-eclampsia develops.

CHAPTER 6.

EXAMINATION OF THE MECHANISM OF CHANGES IN STIMULATED PLATELET CYCLIC AMP DURING HEALTHY PREGNANCY.

CHAPTER 6.

EXAMINATION OF THE MECHANISM OF CHANGES IN STIMULATED PLATELET CYCLIC AMP DURING HEALTHY PREGNANCY.

1.INTRODUCTION.

The studies reported in Chapters 4 and 5 showed that platelet cyclic AMP accumulation following stimulation of adenylate cyclase was lower in healthy pregnant women compared with non pregnant women. This was the case not only when adenylate cyclase was stimulated by agents acting through specific membrane receptors linked to the stimulatory G protein G_s, but also when the enzyme was stimulated in a non receptor-dependent manner[200,201] with the diterpene forskolin. No change was demonstrated in platelet cyclic AMP levels in pregnant women under conditions of basal adenylate cyclase activity. Furthermore, the reduction in stimulated cyclic AMP was more marked when phosphodiesterase was inhibited with AH-P719. Theoretically, reduction in cyclic AMP accumulation could result either from reduced activity of adenylate cyclase or from increased activity of phosphodiesterase. If increased metabolism of cyclic AMP via phosphodiesterase occurred in platelets during pregnancy, then the presence of a phosphodiesterase inhibitor would be expected to reduce rather than enhance differences in cyclic AMP accumulation between pregnant and non pregnant subjects. These observations therefore suggest that a change in phosphodiesterase activity is unlikely to explain the pregnancy associated reduction in stimulated platelet cyclic AMP levels. The situation is, however, complicated by the presence of three isoforms of phosphodiesterase in platelets,[209,210] some of which are cyclic AMP specific, whilst others are cyclic

GMP specific, and others can hydrolyse both cyclic AMP and cyclic GMP. At the concentrations used in the studies reported in Chapters 4 and 5, AH-P719 is thought to be a specific inhibitor of the type III phosphodiesterase which preferentially hydrolyses cyclic AMP (personal communication in writing from Dr Hans Weisenberger, Karl Thomae GmbH, subsidiary of Boehringer Ingelheim and reference 249). Although AH-P719 is purported not to affect cyclic GMP levels, its effects on all of the PDE isoforms have not been fully documented, and it is therefore possible that metabolism of cyclic AMP was occurring even in the presence of AH-P719.

If changes in adenylate cyclase activity do, however, account for reductions in stimulated platelet cyclic AMP levels during pregnancy, then it is worthwhile considering the possible underlying mechanisms. As cyclic AMP levels were lower in platelets from pregnant subjects in response to agents acting via different receptors and in response to forskolin, it is unlikely that down regulation of prostaglandin receptors could account for the observations. Forskolin is capable of stimulating the catalytic subunit of adenylate cyclase directly but its effects on cyclic AMP accumulation may be modulated by the G proteins.[201,442] Recent studies have suggested that control of adenylate cyclase activity by G_i may involve a dual mechanism.[441] All G proteins have a heterotrimeric structure with alpha, beta, and gamma subunits.[443,122] There is evidence that ligand stimulated adenylate cyclase activity can be reduced by activity of the dissociated alpha subunit of G_i ($G_{i\alpha}$) when it binds GTP, whereas the beta-gamma subunit ($G_{i\beta\gamma}$) inhibits basal adenylate cyclase activity.[441] Forskolin stimulated adenylate cyclase activity can be inhibited both by $G_{i\alpha}$ and by $G_{i\beta\gamma}$.[441] A putative mechanism which could explain the observations on platelet cyclic AMP during pregnancy is therefore an increase in $G_{i\alpha}$ activity. Whilst the study of adenylate cyclase activity

in platelet membranes together with studies of G protein activity in membranes treated with cholera or pertussis toxin[443] would offer a direct approach to the investigation of the mechanism of changes in cyclic AMP accumulation in platelets from pregnant women, such techniques also have inherent disadvantages. The necessity to prepare washed platelets and the subsequent manipulation involved in isolating the membranes may result in the loss of subpopulations of platelets during preparation, which may be all the more important when it could be anticipated that platelets from pregnant women may be more subject to activation during handling. Furthermore, most published techniques for measuring adenylate cyclase activity employ ^{32}P labelled ATP[438], and the use of this isotope has numerous laboratory safety implications. It was therefore decided to use an indirect approach in the first instance to further investigate the possible mechanisms of reduction in platelet cyclic AMP levels during pregnancy.

Three studies have been carried out to address the questions of phosphodiesterase activity and G_i mediated control of adenylate cyclase activity in platelets from healthy pregnant women. The first study involved the comparison of the time course of changes in platelet cyclic AMP following stimulation of adenylate cyclase with PGE_1, which acts at the prostacyclin receptor.[195,196] It has been shown that following exposure to most prostaglandins, platelet cyclic AMP levels rise to a peak and then fall off to reach a plateau.[202] It is felt that this reduction in cyclic AMP results partly from metabolism of the cyclic nucleotide, and partly as a result of activation of G_i, following binding of the prostaglandin to receptors distinct from those linked to stimulation of adenylate cyclase.[202] The affinity and time course of binding to G_i linked receptors differs for each prostaglandin. Binding of PGE_1 to the latter type of receptor occurs later than its binding to receptors linked to G_s.[202] Comparison of the time course of the platelet

cyclic AMP response to prostaglandins in pregnant and non pregnant women may give insight as to whether a difference exists in the ability of platelets to initially generate cyclic AMP in response to stimulation or whether differences occur in the subsequent rate of fall off of cyclic AMP as a result of differences in metabolism or differences in G_i activation.

The second set of experiments were carried out with the aim of establishing whether significant cyclic AMP metabolism occurred in the presence of the PDE inhibitor AH-P719, and whether this differed in pregnant and non pregnant women. Studies in cells other than platelets have shown that cyclic AMP levels, when raised by receptor mediated stimulation of adenylate cyclase, fall rapidly following removal of the agonist, even in the presence of a phosphodiesterase inhibitor.[444]

The third study was conducted to further investigate the effects on platelet cyclic AMP of activation of G_i. PGE_2 has been shown to have dual effects on both platelet behaviour[87-90] and platelet cyclic AMP levels. High concentrations of PGE_2 can raise cyclic AMP by stimulating adenylate cyclase, possibly via the PGI_2 receptor.[88,89,203] By contrast, exposure of platelets to low concentrations of PGE_2 leads to a reduction in cyclic AMP levels, only if these have previously been raised by stimulation of adenylate cyclase via another receptor dependent mechanism.[88,89,203] The latter effect is thought to occur by activation of G_i.[89] PGE_2 could therefore provide a suitable tool for the investigation of G_i mediated events. Furthermore, the examination of the effect on platelet cyclic AMP levels of PGE_2 is of physiological relevance as PGE_2 is the major prostaglandin synthesised by the microvascular endothelium,[163,164] and PGE_2 synthesis also takes place in the placenta.[298,300]

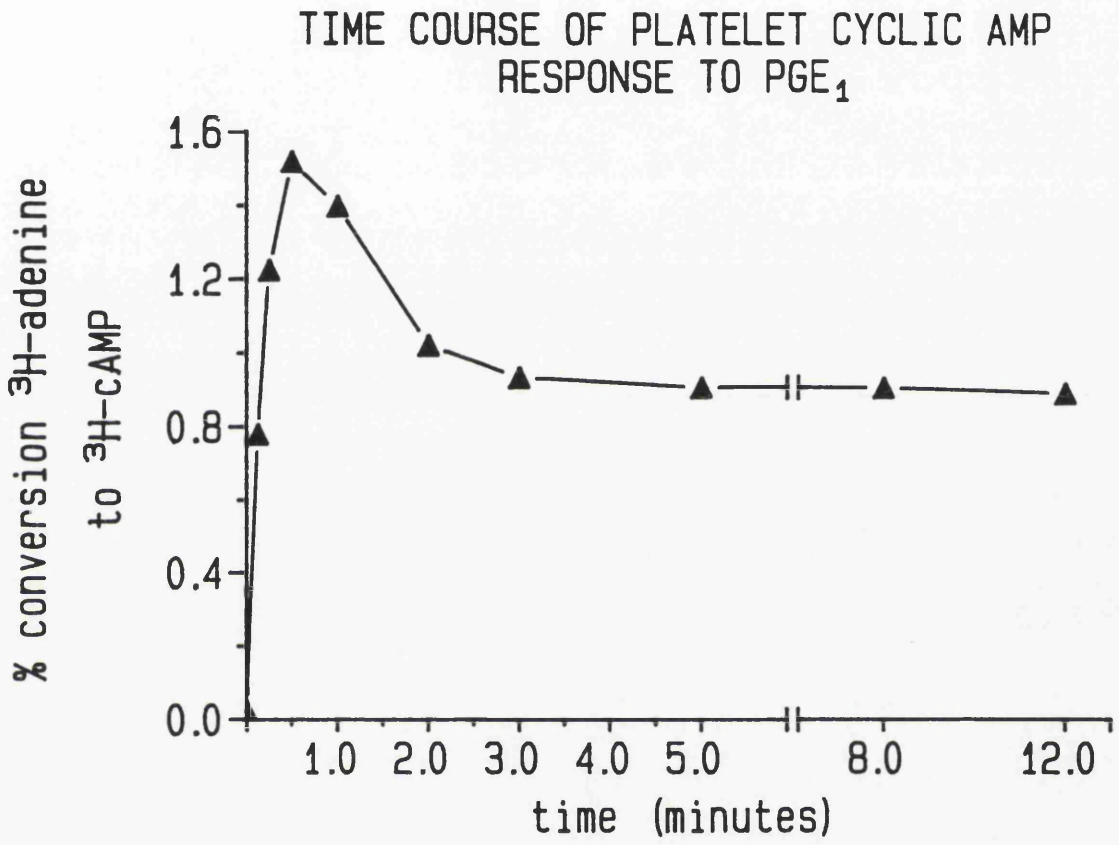


FIGURE 6.1.

The graph shows % conversion of ^{3}H-adenine plotted against time for PRP from a single donor following the addition of 30μM PGE_1. A similar "peak and plateau" time course pattern was obtained in all subjects.

2.METHODS.

2.1 Time Course of Platelet Cyclic AMP Response to PGE_1

Eleven healthy non pregnant women and nine healthy third trimester primigravidae donated blood for the study. Previously described criteria for subject selection were strictly followed. All subjects were normotensive and gave no history of relevant drug ingestion. PRP was prepared from citrated blood, the platelet count adjusted to 250 x 10^9/l and labelled with ^{3}H-adenine. Aliquots of PRP (450ul) were then incubated with 25ul of saline at 37°C and stirred at 1,000rpm for 2 minutes. 25ul PGE_1 (30uM) or saline (for measurement of basal cyclic AMP) was then added and the reaction stopped with 2ml 15% TCA at times varying between 7 seconds and 12 minutes. Assay of cyclic AMP was completed as documented in Chapter 2. A graph of % conversion ^{3}H-adenine to ^{3}H-cyclic AMP against time was drawn for each subject. These graphs showed a typical peak and plateau pattern (Figure 6.1). When log time was plotted against percentage of peak cyclic AMP for time points following the peak response, a sigmoid curve was obtained. An example is shown in Figure 6.2. Points subsequent to the peak for each subject were therefore fitted to a sigmoid curve using computerised non linear regression analysis ("Graphpad Inplot", Graphpad Software Inc, San Diego) and values for the downward slope of each graph were generated. Comparison of the slopes for each subject group was carried out using the Mann-Whitney U test. Overall comparison of cyclic AMP levels in response to PGE_1 in pregnant and non pregnant women was carried out using two way analysis with repeated measures, supplemented with unpaired t tests, as data at each time point were shown to be normally distributed using the Kolmogorov-Smirnov cumulative distribution test.

Sigmoid curve (log scale)
A = bottom, B = top, C = log(EC50), D = 'Hill' Slope

Final Results. Sum of Squares = 9.185E-06 (df = 2)
Goodness-of-fit assessed using actual distances; R squared = 1.000.

Parameter	Value	Approx. SE	%Error(CV)
A	72.63993	1.524E-03	<0.1%
B	100	(Constant)	
C	.04959364	4.209E-04	0.8%
D	-11.32642	.09570017	0.8%

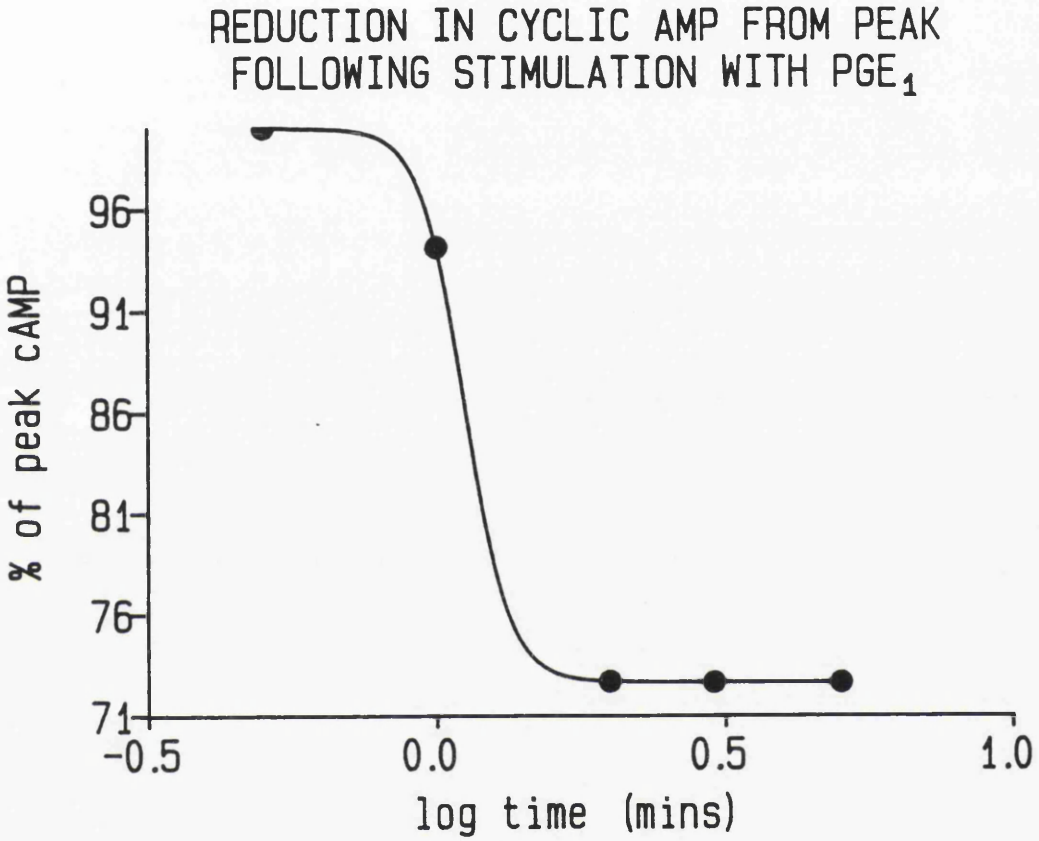


FIGURE 6.2

The figure shows platelet cAMP levels, at time points following the peak achieved in response to 30μM PGE_1, expressed as a % of this peak level, and plotted against the $\log_{10}$ of the time in minutes following addition of PGE_1. The curve fitting data using the Hill equation are shown below the graph. The top of the curve was always fixed at 100% 'D' represents the slope and this value was used in each subject as a measure of the rate of reduction in cyclic AMP following peak levels. In the example the value of 1 for R squared represents a perfect fit of the data to the Hill equation. For all subjects R squared was > 0.9.

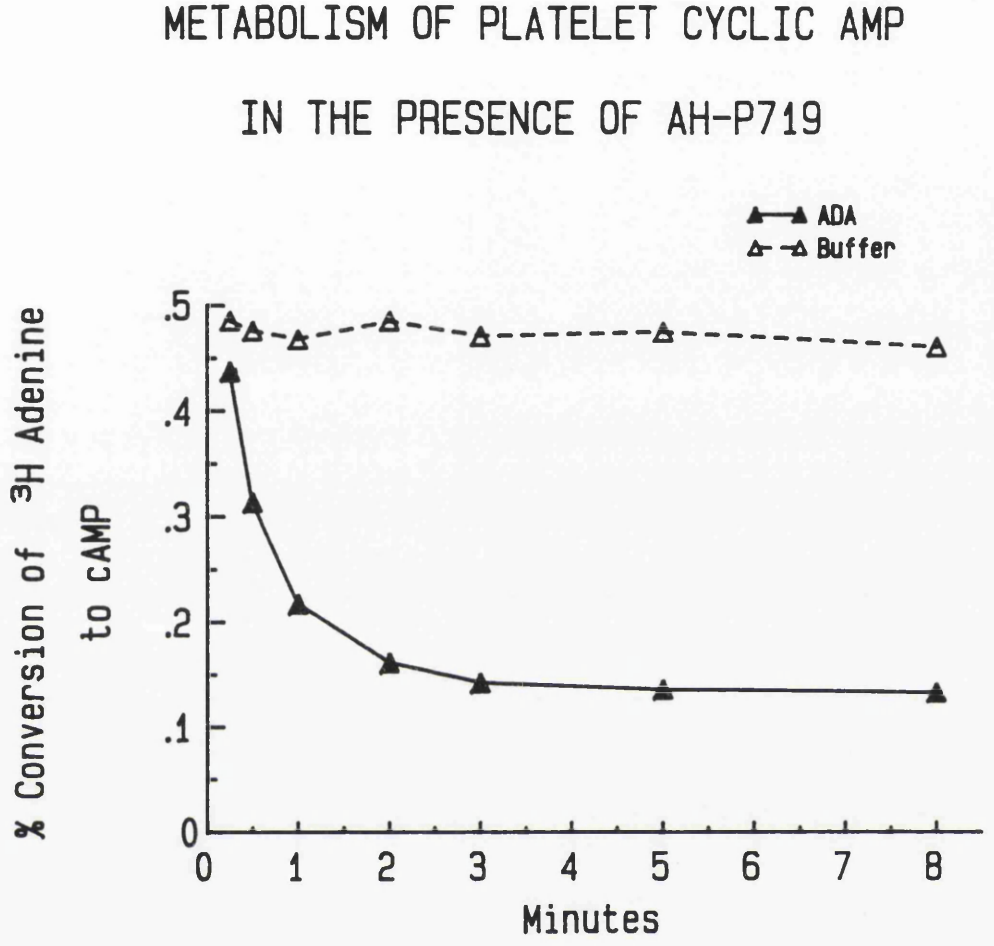


FIGURE 6.3

The figure shows data from a single experiment. The phosphodiesterase inhibitor AH-P719 had been added to PRP 4 minutes before time 0 on the graph, followed by 0.1mM adenosine 2 minutes before time 0. Adenosine deaminase 1.2U/ml (or saline in control tubes) was added at time 0, and platelet cyclic AMP levels were measured at the intervals shown.

2.2 Investigation of Phosphodiesterase Activity in Platelets in the Presence of AH-P719.

As reduction in cyclic AMP levels following interaction of a ligand with a receptor linked to adenylate cyclase is often not purely dependent on phosphodiesterase activity, but also on activation of G_i,[202] it was necessary to design an experiment in which platelet adenylate cyclase could be activated, in the presence of AH-P719, by a ligand which could subsequently be easily removed. Any reduction in cyclic AMP levels with time could only result from metabolism of cyclic AMP. Adenosine is known to raise cyclic AMP levels in platelets by stimulating adenylate cyclase,[184] and can also be removed by the addition of the enzyme, adenosine deaminase.[444,445] An experiment was therefore set up as follows: 440ul aliquots of ^{3}H-adenine labelled PRP were incubated at 37°C with 20ul of AH-P719 (100uM) for 2 minutes. 20ul of a solution of adenosine (0.1mM) was then added to each tube and incubation continued for a further 2 minutes, at which time either 20ul adenosine deaminase (1.2u/ml) or 20ul of saline was added. The reaction was then "stopped" by the addition of 2ml 15% trichloroacetic acid, at time points varying between 15 seconds and 8 minutes following the addition of the adenosine deaminase (ADA). The assay of cyclic AMP was then completed by column chromatography as described previously. A graph was then drawn of % conversion ^{3}H-adenine to ^{3}H-cyclic AMP against time for ADA treated and control platelets. Results of a pilot experiment is shown in Figure 6.3. It is clear that ongoing metabolism of cyclic AMP was taking place in the presence of AH-P719, and it was therefore necessary to repeat the above experiment in a series of healthy third trimester pregnant women with non pregnant women as controls. 10 non pregnant women and 11 healthy primigravidae donated blood for such studies. Criteria for subject selection

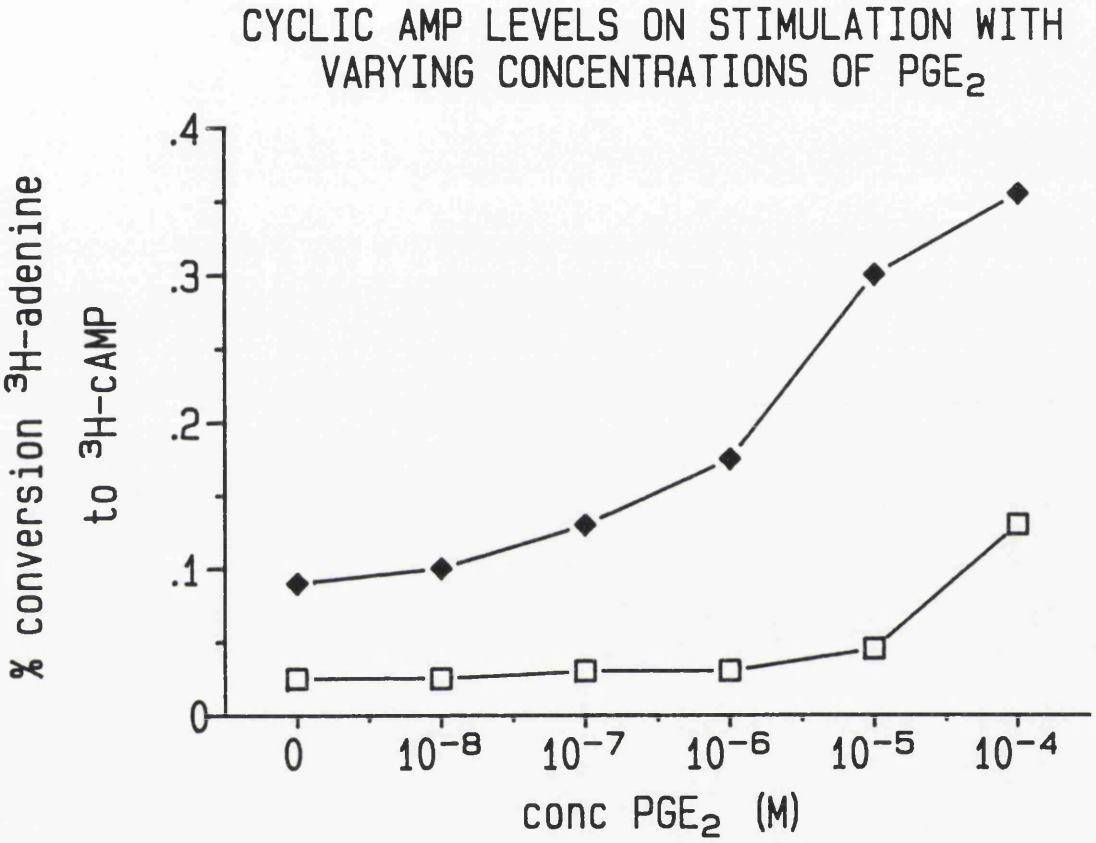


FIGURE 6.4.

The figure shows data from a single experiment. The concentration-response relationship for the effect on platelet cyclic AMP levels of PGE_2 (□) is shown. The experiment was also carried out in the presence of AH-P719 (◆).

previously set out in detail were strictly adhered to.

Graphs were constructed for each subject of % conversion ^{3}H-adenine to ^{3}H-cyclic AMP against time. As can be seen in Fig 6.3 cyclic AMP levels were fairly constant in control tubes, but in tubes where adenosine was removed, there was an initial rapid reduction in cyclic AMP levels, which then fell more slowly after 1 minute, reaching a plateau by 3 minutes. The slope of the initial rapid reduction in cyclic AMP was calculated for each subject by linear regression analysis using a computer program devised by Dr A Kelman, University of Glasgow. The slopes were then compared for pregnant and non pregnant subjects, using a Mann Whitney-U test. Cyclic AMP accumulation in control tubes, to which saline was added in place of ADA, was compared for each subject group using the Mann Whitney-U test. This gave an indication of the "steady state" response to adenosine in the presence of the PDE inhibitor.

2.3 Investigation of the Effect Upon Stimulated Platelet Cyclic AMP Levels of PGE_2.

It was necessary to define a concentration of PGE_2 which could reproducibly activate G_i, whilst itself having a minimal effect on G_s. An experiment was conducted in which platelet cyclic AMP levels in response to a range of concentrations of PGE_2 were measured, by the method previously described, using blood from a healthy non pregnant female volunteer. PRP was pre-incubated for 2 minutes at 37°C, before adding PGE_2 and incubation was continued for 6 minutes thereafter. A dose response curve was constructed, as shown in Figure 6.4. Cyclic AMP levels remained similar to basal levels, until the concentration of PGE_2 was raised above 10^{-5}M when a significant rise was noted. At concentrations above 10^{-7}M, however, PGE_2 synergised with the PDE inhibitor AH-P719. The concentration response curve in the presence of AH-P719 100μM is

TABLE 6.1

EFFECT OF DIFFERENT CONCENTRATIONS OF PGE_2 ON ILOPROST-INDUCED INCREASES IN cAMP

Experiment No	Reagent added after 2 minutes	Saline	5ng/ml iloprost
1	Saline	0.0298 ± 0.0012	0.2980 ± 0.0376
	10^{-8}M PGE_2	0.0363 ± 0.0013	0.2700 ± 0.0133
2	Saline	0.0289 ± 0.0049	0.3190 ± 0.0098
	10^{-7}M PGE_2	0.0348 ± 0.0011	0.1360 ± 0.0069
3	Saline	0.0239 ± 0.0005	0.4550 ± 0.0098
	10^{-6}M PGE_2	0.0395 ± 0.0027	0.1290 ± 0.0064

The table shows the results of three sets of experiments to define the effect of different concentrations of PGE_2 on iloprost induced platelet cyclic AMP increments. Each entry in the table represents mean and SEM % conversion ^{3}H-adenine to ^{3}H-cAMP calculated from duplicate observations in each of two subjects.

also shown in Figure 6.4. Experiments were then carried out in PRP from healthy non pregnant female donors to examine the effect on iloprost stimulated cyclic AMP of 10^{-8}M, 10^{-7}M, and 10^{-6}M PGE_2, as at these concentrations, PGE_2 had no appreciable effect itself upon platelet cyclic AMP. The concentration of iloprost was fixed at 5ng/ml, and PRP was exposed to iloprost for 2 minutes at 37°C before adding PGE_2. Saline only was added to PRP for measurement of basal cyclic AMP levels, and controls for each assay consisted of tubes to which PGE_2 alone was added to PRP, and those to which iloprost alone was added. The final volume in each tube was constant at 500ul. The total incubation time was again 8 minutes, when the reaction was stopped with 15% TCA. Table 6.1 shows the results of these pilot experiments. It was apparent that 10^{-8}M PGE_2 caused very little reduction in iloprost stimulated cyclic AMP, whilst with 10^{-7}M PGE_2 caused a substantial decrease. The latter concentration was therefore chosen as appropriate for use for further investigation of PGE_2 mediated activation of G_i.

Healthy non pregnant women who were not on an oral contraceptive and healthy third trimester pregnant women were recruited as described previously. Blood from 12 pregnant and 12 non pregnant subjects was used to study the effects on cyclic AMP of PGE_2 in the absence of a phosphodiesterase inhibitor and a further 18 primigravidae and 18 non pregnant women donated blood for the investigation of the effect of PGE_2 in the presence of AH-P719. On the day of study, blood pressure was measured for each subject, and blood was withdrawn for platelet studies and for measurement of full blood count.

Citrated PRP was prepared and the platelet count adjusted to 250 x 10^9/l. After incubation with ^{3}H-adenine, PRP was incubated in aliquots with PGE_2 alone, iloprost alone or with both prostaglandins, in the presence or absence of AH-P719. Measurements of basal platelet cyclic AMP were also made in each experiment.

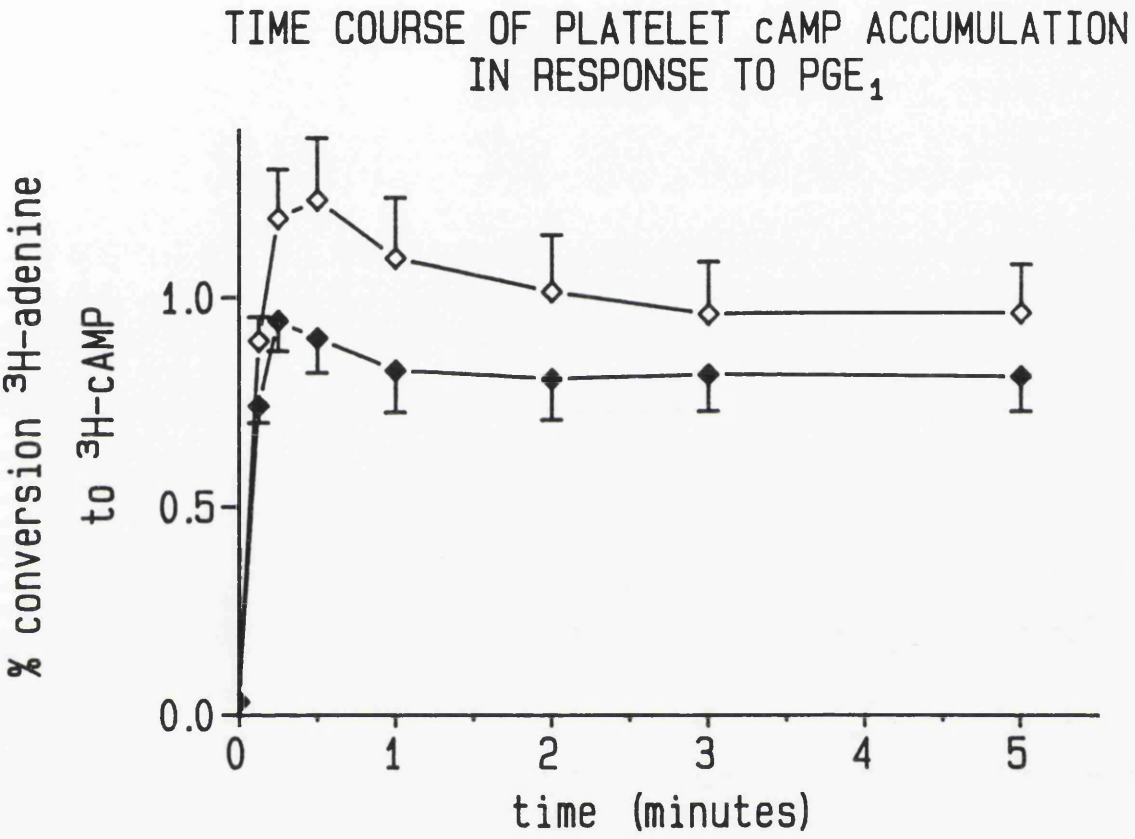


FIGURE 6.5

The graph shows the time course of the platelet cyclic AMP response to 30μM PGE_1 in a group of 11 non pregnant (◇) and 9 third trimester pregnant (◆) subjects. Mean and S.E.M. for the group is shown for each time point. ANOVA revealed a highly significant difference between the two subject groups ($p = 0.0013$). Unpaired t tests revealed a significant reduction in cAMP in pregnant subjects at 7 seconds ($p = 0.037$) and at 30 seconds ($p = 0.046$).

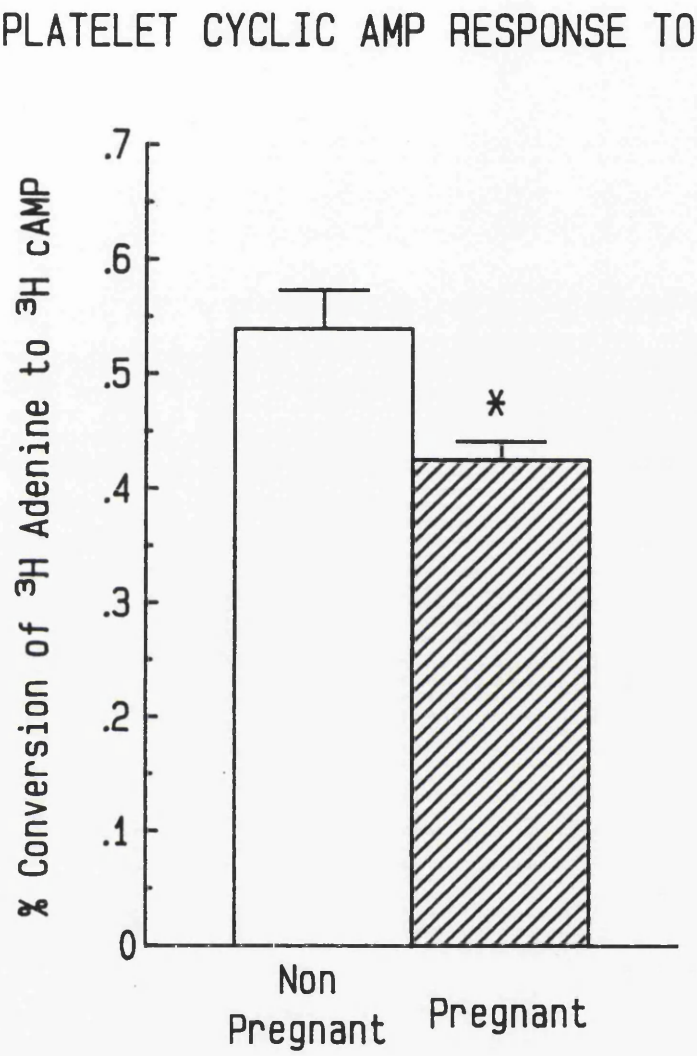


FIGURE 6.6

Mean and S.E.M. platelet cyclic AMP levels in response to 0.1mM adenosine and 100μM AH-P719 are shown. Data are from a group of 11 non pregnant women and a group of 10 third trimester primigravidae. The cyclic AMP measurements illustrated were made following incubation of PRP for 2 minutes with the PDE inhibitor, followed by incubation for a further 2 minutes 15 seconds with adenosine. No adenosine deaminase was added to the PRP in which these measurements were made. Significantly less cyclic AMP was generated in PRP from pregnant women under these conditions ($p = 0.0074$, Mann-Whitney U test).

An example of the protocol is shown in Appendix 5. The cyclic AMP assay was completed by column chromatography on platelet extracts as previously described. The Mann-Whitney U test was used for the comparison of cyclic AMP levels in the two subject groups in response to each agent.

3.RESULTS.

3.1 Time Course of Platelet Cyclic AMP Response to PGE_1.

Figure 6.5 illustrates the time course of platelet cyclic AMP responses to PGE_1 in pregnant and non pregnant women. It was apparent that cyclic AMP levels were lower in pregnant subjects, particularly at early time points. Analysis of variance revealed a highly significant difference between the two groups of subjects ($p=0.0013$) and between time points ($p=0.0001$). Individual comparisons showed that platelets from pregnant women generated significantly less cyclic AMP at 7 seconds ($p=0.037$) and 30 seconds ($p=0.046$) and the difference just failed to reach significance at 15 seconds ($p=0.069$). Analysis of the gradients of the downward slopes following the peak cyclic AMP response revealed no difference between groups of subjects. The means (and standard errors) of the gradients of the downward slopes following the peak cyclic AMP responses were -14.02 ($\pm$ 3.86) for non pregnant subjects and -13.75 ($\pm$2.80) for pregnant subjects ($p = 0.9045$).

3.2 Platelet Cyclic AMP Response to Adenosine.

The baseline cyclic AMP response to adenosine was significantly lower in pregnant compared with non pregnant women. The data are illustrated in Figure 6.6.

3.3 Rate of Metabolism of Cyclic AMP in the Presence of AH-P719.

The rate of metabolism of cyclic AMP in platelets, following removal of

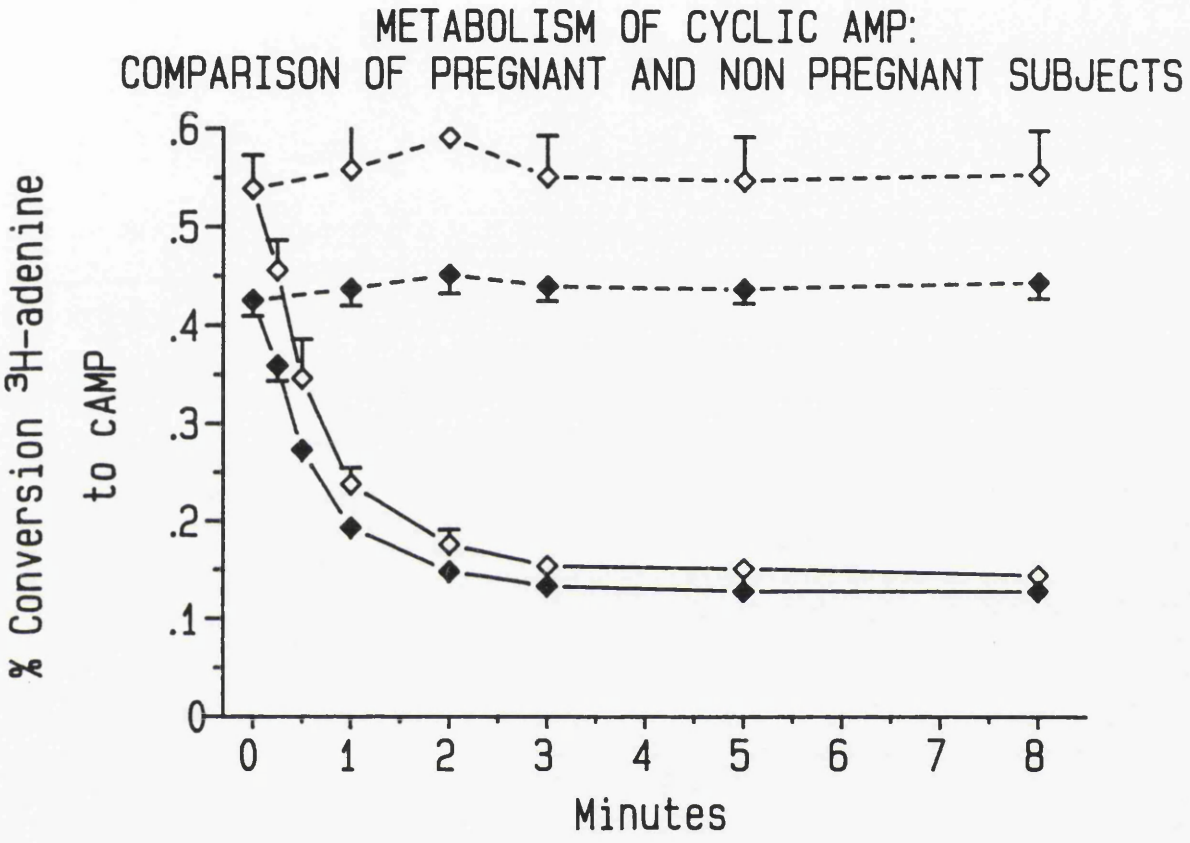


FIGURE 6.7.

The graph shows mean and S.E.M. of cyclic AMP levels plotted against time in PRP from 11 non pregnant (◇) and 10 healthy pregnant women (◆). PRP had been incubated with AH-P719 for 4 minutes and with adenosine (0.1mM) before time 0 on the graph. At time 0 adenosine deaminase (___) or saline as a control (...) was added, and cyclic AMP measured at the intervals illustrated thereafter. Analysis of the downward slopes of the curves yielded values of - 3.86 $\pm$ 0.185 in the pregnant group and -5.023$\pm$0.321 in the non pregnant group. The Mann-Whitney U test revealed that the decrease in the rate of metabolism of cyclic AMP in pregnant subjects was significant ($p = 0.0057$).

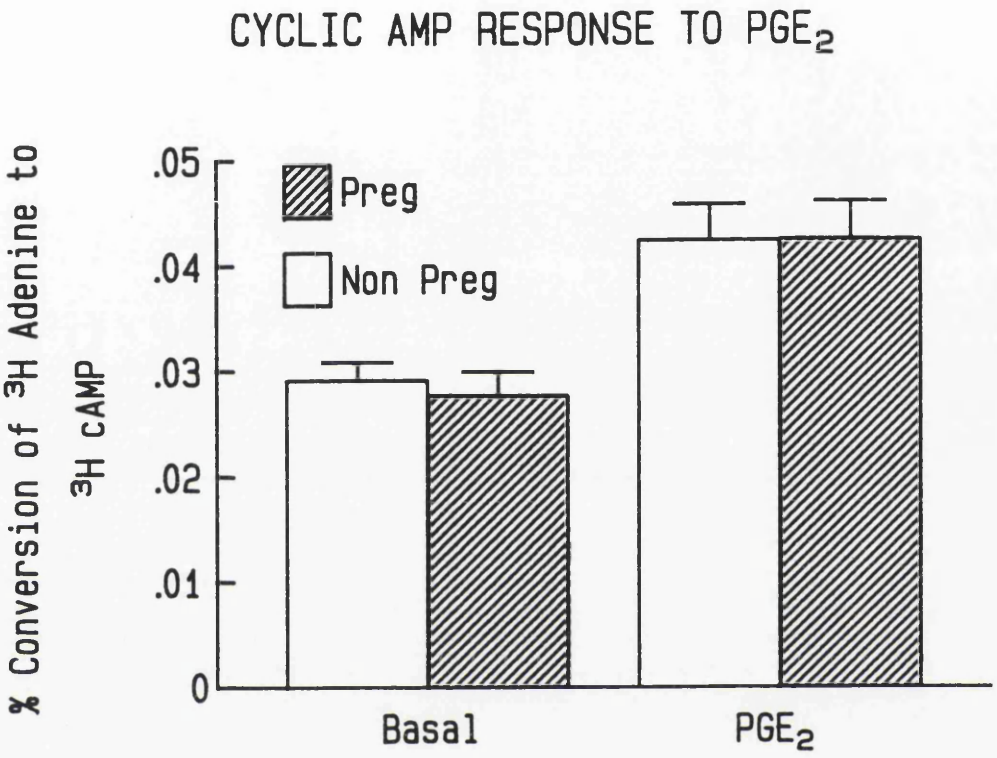


FIGURE 6.8.

The figure shows basal levels of cyclic AMP and those in the presence of 10^{7}M PGE_2 in 12 pregnant and 12 non pregnant women. Means and S.E.M.s are illustrated. There was no difference in results between subject groups.

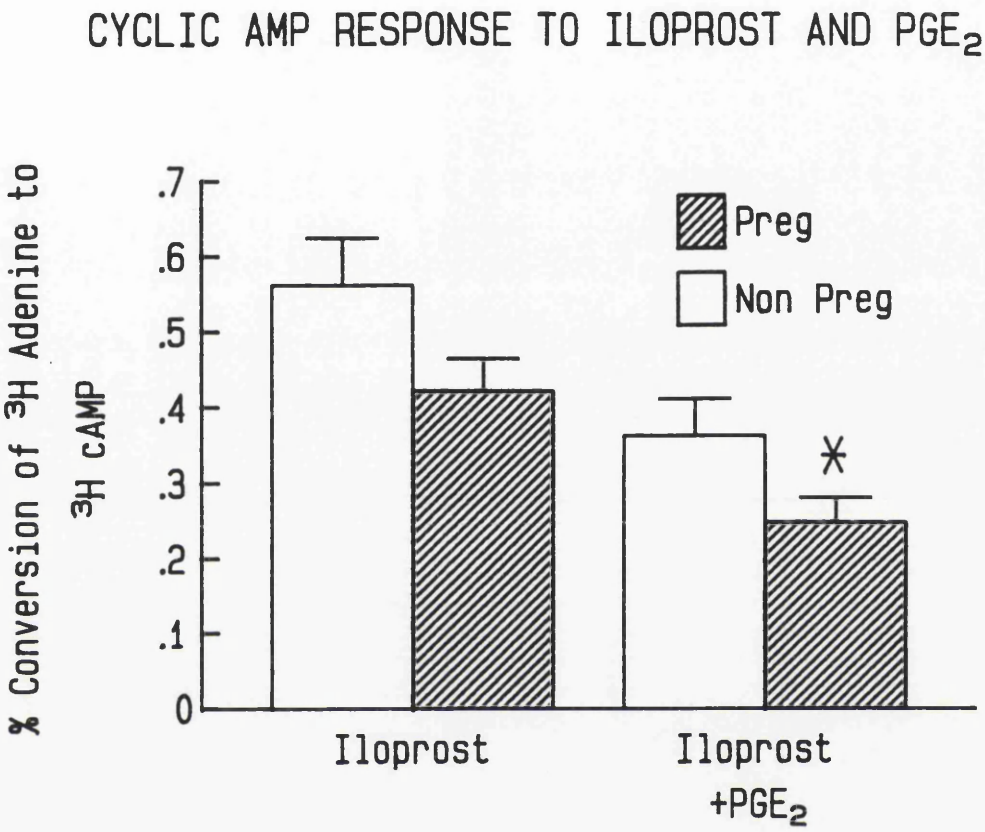


FIGURE 6.9

The figure shows mean ± S.E.M. cyclic AMP levels in 12 third trimester pregnant and 12 non pregnant subjects in the presence of iloprost 5ng/ml alone and in the presence of both iloprost 5ng/ml and PGE_2 10^{-7}M. There was a strong trend towards reduced cyclic AMP levels in pregnant subjects in the presence of iloprost alone, which just failed to reach statistical significance ($p = 0.0727$). In both groups of subjects, PGE_2 decreased the cyclic AMP response to iloprost, and under these conditions, cyclic AMP levels were significantly lower in pregnant compared with non pregnant women (* $p = 0.0206$).

adenosine with ADA, in the presence of AH-P719, is shown for both groups of subjects in Figure 6.7. The gradient of the downward slope of the graph obtained when platelet cyclic AMP was plotted against time was significantly lower in pregnant compared with non pregnant subjects.

3.4 Effect on Platelet Cyclic AMP of PGE_2.

Basal levels of cyclic AMP were similar in platelets from non pregnant and pregnant subjects. 10^{-7}M PGE_2 alone produced a very small increase in platelet cyclic AMP levels but, again, there was no difference between subject groups. The data are shown in Figure 6.8. Iloprost (5ng/ml) substantially raised platelet cyclic AMP levels and there was a strong trend towards a reduced cyclic AMP response in platelets from pregnant subjects, which just failed to reach statistical significance (Figure 6.9). The addition of PGE_2 (10^{-7}M) significantly reduced the platelet cyclic AMP response to 5ng/ml iloprost and under these conditions, cyclic AMP levels were significantly lower in platelets from pregnant compared with non pregnant women (Figure 6.9). The extent of reduction, however, was similar in both subject groups.

There was no difference in platelet cyclic AMP levels between pregnant and non pregnant women when the phosphodiesterase inhibitor AH-P719 was present alone. When PGE_2 (10^{-7}M) was present together with AH-P719, platelet cyclic AMP levels were higher than when AH-P719 alone was added, but results were again similar in both groups of subjects (Figure 6.10). Platelet cyclic AMP levels were significantly reduced in pregnant compared with non pregnant women in response to the combination of 50ng/ml iloprost and AH-P719, but there was no difference between subject groups when platelets were exposed to 5ng/ml iloprost together with the phosphodiesterase inhibitor (Figure 6.11). The addition of 10^{-7}M PGE_2 reduced the cyclic AMP response to both 5 and 50ng/ml iloprost in both

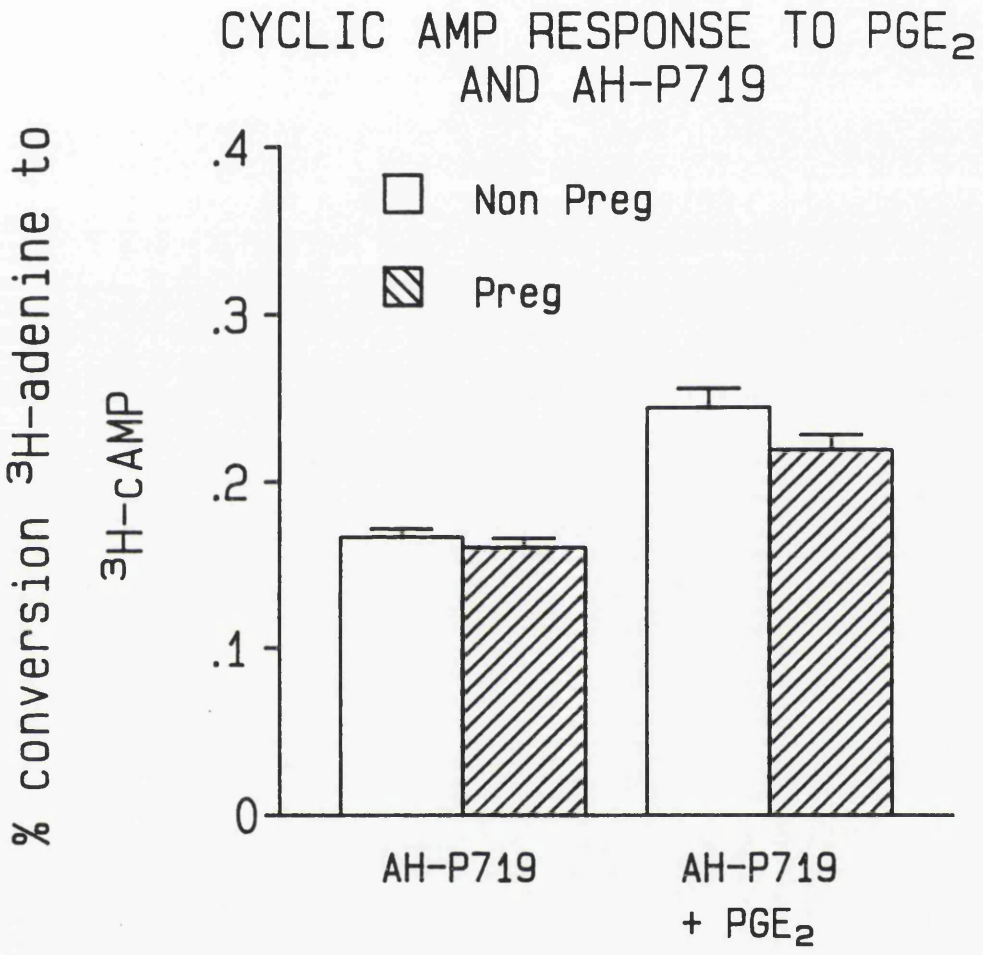


FIGURE 6.10

The bar graph shows cyclic AMP levels in the presence of 100μM AH-P719 alone and together with 10^{-7}M PGE_2. Means $\pm$ S.E.M.s of 18 observations in each group are shown in each case. There was no difference between pregnant and non pregnant women in cyclic AMP levels under these conditions ($p = 0.2150$ for AH-P719 data, and 0.1130 for AH-P719 + PGE_2)

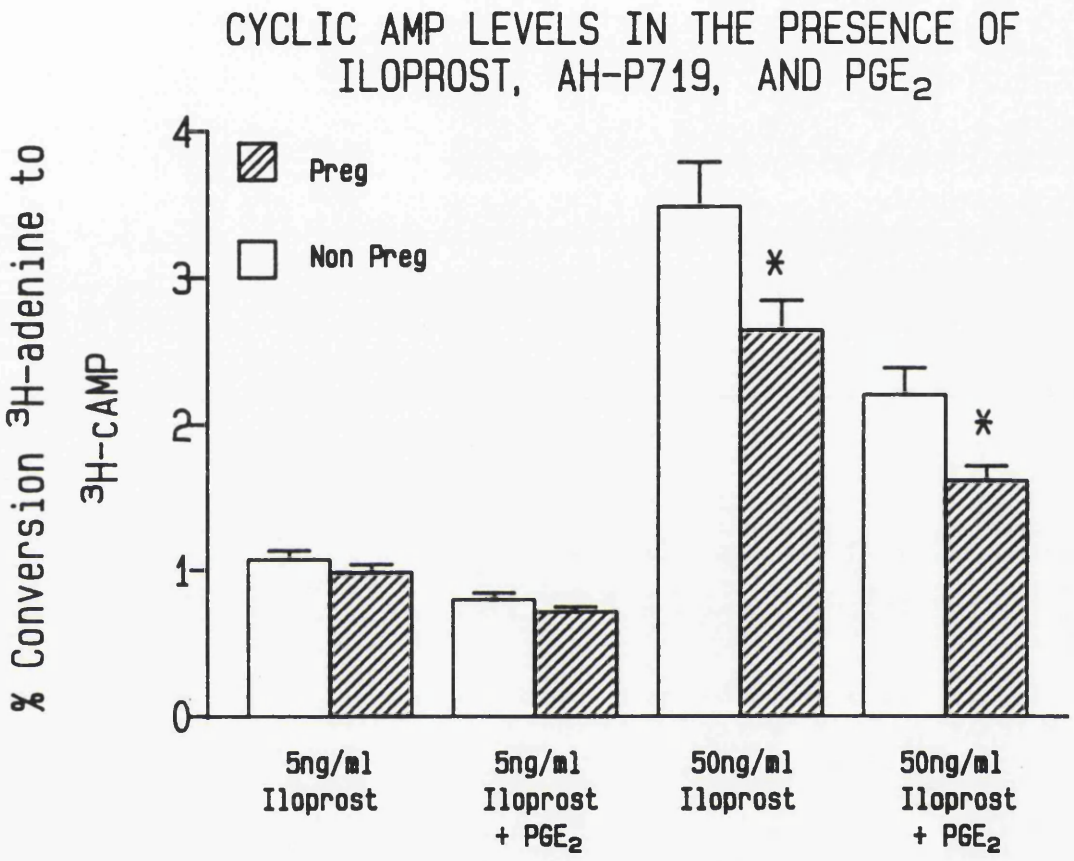


FIGURE 6.11

The bar graph shows platelet cyclic AMP levels in the presence of 100μM AH-P719, together with iloprost and combinations of iloprost and PGE_2 10^{-7}M. Means and S.E.M.s of 18 observations are shown in each case. Platelets from pregnant women accumulated significantly less cyclic AMP in the presence of 50ng/ml iloprost (* $p = 0.0312$). PGE_2 again reduced the cyclic AMP response to iloprost in both groups of subjects, and platelet cyclic AMP levels remained significantly lower in the pregnant group in response to the combination of iloprost 50ng/ml and PGE_2 (* $p = 0.0085$).

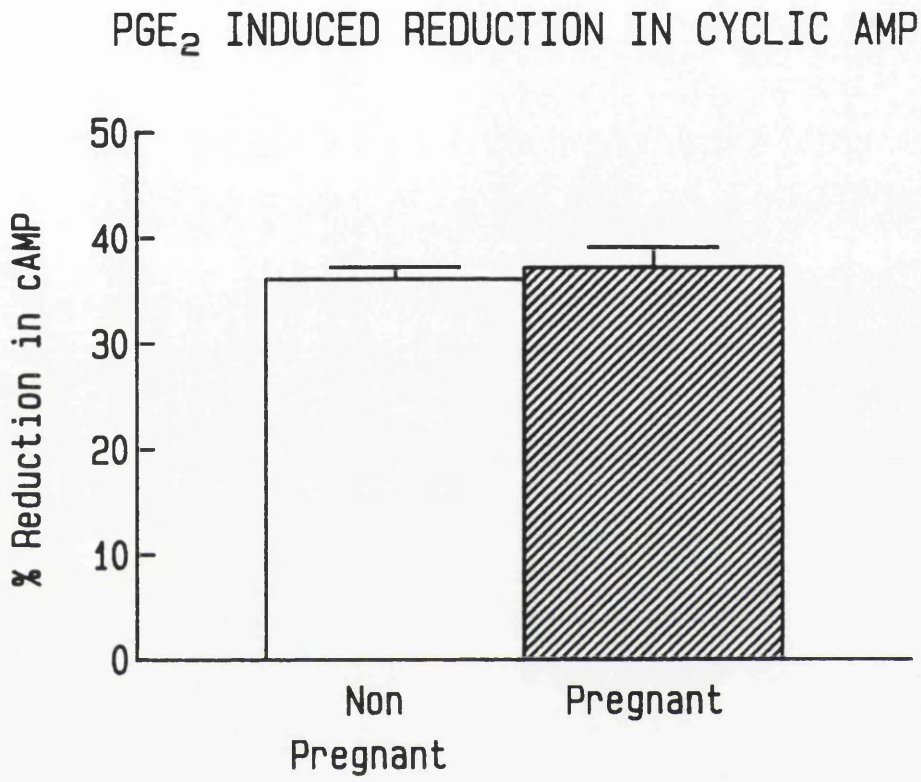


FIGURE 6.12

The graph shows the % reduction in cyclic AMP levels in response to iloprost 50ng/ml, in the presence of the phosphodiesterase inhibitor, resulting from the addition of PGE_2 10^{-7}M to the incubation mixture. These data were derived from the data illustrated in Figure 6.11. Means and S.E.M.s of 18 observations are again shown for each subject group. It is clear that there was no difference between pregnant and non pregnant subjects in the extent of the PGE_2 effect ($p = 0.4839$).

groups, and platelet cyclic AMP levels were significantly lower in pregnant compared with non pregnant women in the presence of the combination of 50ng/ml iloprost, 100uM AH-P719, and 10^{-7}M PGE_2. There was, however no difference between the two groups of subjects in the percentage reduction in cyclic AMP induced by PGE_2 (Figure 6.12).

4.DISCUSSION

Studies of the time course of platelet cyclic AMP responses to PGE_1 showed that a peak and plateau type response, as described by previous investigators,[202] was observed in both pregnant and non pregnant women. Platelets from pregnant women produced less cyclic AMP in response to this prostaglandin at very early time points following exposure. After the response had reached a peak, however, the difference between pregnant and non pregnant subjects was less marked and there was no difference in the rate of fall off of cyclic AMP levels from peak. These data support a reduction in adenylate cyclase activity rather than an increase in phosphodiesterase activity during pregnancy and suggest that changes in PGE_1 induced stimulation of G_i do not occur in pregnant women.

Time course studies following removal of adenosine, in the presence of the phosphodiesterase inhibitor AH-P719, showed that AH-P719 permits ongoing metabolism of cyclic AMP. In platelets from pregnant women cyclic AMP was metabolised at a slower rate under these conditions than in non pregnant women. Increased metabolism of cyclic AMP could not, therefore, account for the reduction in cyclic AMP accumulation in platelets from pregnant subjects under the conditions of the experiments described in preceding chapters. These data would again support the hypothesis that a change in the ability of the platelet adenylate

cyclase enzyme to respond to stimulation occurs during healthy pregnancy. Furthermore, this study has shown that this holds true when adenylate cyclase is stimulated with adenosine, which acts through a receptor distinct from any of the prostaglandin receptors, probably an A_2 adenosine receptor.[184,446]

When the effects on platelet cyclic AMP of low levels of PGE_2 were investigated, this prostaglandin had only a small effect upon platelet cyclic AMP, but produced a substantial reduction in cyclic AMP levels when already raised by another adenylate cyclase stimulator. This effect occurred in both non pregnant and pregnant subjects and provided a tool for the investigation of control of platelet adenylate cyclase by hormone stimulated G_i.[89] The results confirmed the findings of previous studies reported in Chapters 4 and 5 by showing a reduced platelet cyclic AMP response to iloprost during pregnancy, both in the presence and absence of PGE_2. There was no difference, however, in the extent of the reduction in the iloprost response induced by PGE_2 in pregnant compared with non pregnant women. Consequently, it is unlikely that any change in $G_{i\alpha}$ activity occurs in platelets during pregnancy. The similarity of basal platelet cyclic AMP levels in pregnant and non pregnant subjects tends to rule out a pregnancy related change in $G_{i\beta\gamma}$ subunit activity.[441]

In conclusion, the results of studies presented in this Chapter lend support to the hypothesis that, during pregnancy there is a reduction in activity of the catalytic portion of platelet adenylate cyclase enzyme but changes in the regulation of adenylate cyclase by G_s cannot be ruled out.

CHAPTER 7.

PLATELET SENSITIVITY TO THE ENDOPEROXIDE ANALOGUE U46619 DURING HEALTHY PREGNANCY.

CHAPTER 7.

PLATELET SENSITIVITY TO THE ENDOPEROXIDE ANALOGUE U46619 DURING HEALTHY PREGNANCY.

1. INTRODUCTION

During healthy pregnancy there is considerable evidence of platelet activation in vivo and there are also well documented changes in platelet reactivity in vitro. This has been discussed at length in the general introductory chapter but it is appropriate here to review some aspects of platelet behaviour in vitro during pregnancy. Increased platelet aggregation in PRP or in whole blood has been demonstrated by other investigators using arachidonic acid,[321,322,327] ADP,[295,305,320,] and adrenaline[320,327] as agonists. Furthermore, the cross sectional study reported in Chapter 3 showed that platelet dense granule release in response to 1mM AA, was slightly but significantly increased in healthy third trimester pregnant women compared with non pregnant controls. Platelet responses to all of these agonists are at least partially dependent on thromboxane A_2 production. This raises the question of whether increased platelet thromboxane production occurs during pregnancy or whether platelet sensitivity to thromboxane is enhanced. Whilst there is sound evidence that platelet derived thromboxane metabolites are present in urine in increased amounts during pregnancy,[309] data on platelet thromboxane production in vitro are conflicting,[292,327,329] and measurement of in vivo thromboxane production cannot determine whether alterations in the activity of the platelet thromboxane pathway are the primary cause of the observed changes in platelet behaviour.

Both cross-sectional and longitudinal data on serum thromboxane B_2

presented in preceding chapters suggest that platelet thromboxane production in vitro, at least under conditions of spontaneous clotting of whole blood, does not change during healthy pregnancy. These results were generated in pregnant subjects who simultaneously demonstrated increased arachidonic acid induced platelet behaviour, and amongst whom the majority were non responders to the thromboxane synthetase inhibitor dazmegrel. In studies outwith the context of pregnancy it has been shown that platelets from subjects who are non responders to thromboxane synthetase inhibitors show increased sensitivity to the pro-aggregatory effects of PGE_2 and of the endoperoxide analogue U46619,[253,254] which acts at the same receptor as thromboxane.[447]

When all of these observations are taken together, the hypothesis could be put forward that platelets from pregnant women may be more sensitive to thromboxane. As thromboxane A_2 is extremely unstable,[35] in practice this hypothesis must be tested using the endoperoxide analogue U46619 as an agonist to induce platelet activation.

2.METHODS.

2.1 Study Design.

The studies conducted were of cross sectional design comparing healthy third trimester primigravidae with healthy non pregnant women who were not on an oral contraceptive. The criteria for subject selection were as outlined in Chapter 2. No subject had ingested any drug within two weeks of donating blood except iron and vitamin supplements. Four separate studies were conducted as follows and subjects were distinct for each : 1) platelet aggregation in response to U46619 in PRP was studied and the effect of the thromboxane receptor antagonist ICI 192605[448-450] in vitro was tested 2) U46619 induced platelet

aggregation in whole blood was investigated in the absence and presence of ICI 192605; 3) U46619 induced platelet aggregation in whole blood treated with aspirin was investigated; 4) platelet thromboxane production in PRP stimulated with arachidonic acid was measured.

On the day of study subjects were placed in the supine position, and after 5 minutes rest blood pressure was measured and venepuncture performed as described in Chapter 2. Blood was taken into 3.13% trisodium citrate dihydrate (9 parts blood to 1 part anticoagulant) for platelet studies, and into EDTA for measurement of the full blood count.

2.2 Laboratory Methods.

Platelet aggregation studies in PRP were conducted by preparing PRP with an adjusted platelet count of 250 x 10^9/l as described previously. Platelet aggregation was then induced in a PAP-4 Biodata four channel aggregometer using the method of Born as detailed in Chapter 2. Experimental tubes containing 180ul of PRP and 20μl of saline or the thromboxane antagonist ICI 192605 (final concentration 10^{-8}M or 10^{-7}M) were placed in the aggregometer wells and stirred and incubated for 2 minutes before inducing platelet aggregation with 20μl U46619 (0.1uM-200uM). Aggregation was allowed to proceed for 6 minutes, at which time percentage aggregation was noted. Graphs of concentration of U46619 against % aggregation were then constructed and the EC_{50} of the agonist calculated.

Platelet aggregation in whole blood was measured by the counting method described in Chapter 2, following incubation of citrated blood at 37°C for 30 minutes. For whole blood experiments, aliquots of 460μl of whole blood were added to polystyrene tubes containing a metal stir bar and 20μl of saline or ICI 192605 (final concentration 10^{-7}M or 10^{-6}M) and incubated at 37°C whilst stirring

for 2 minutes. 20μl U46619 (final concentration 0.3μM or 3μM) or saline was then added and aggregation allowed to proceed for 8 minutes. 15μl subsamples of blood for subsequent platelet counting were taken from each tube and placed in 30μl of fixative at 0, 0.25, 0.5, 0.75, 1, 2, 4, and 8 minutes after addition of the agonist.

In those studies in whole blood in which the effect of aspirin was investigated, duplicate samples of citrated whole blood containing 0.56mM aspirin or saline as a control, were incubated at 37°C for 30 minutes. Platelet aggregation in response to 1mM arachidonic acid was shown to be completely abolished by treating with aspirin in this way. Triplicate 480ul aliquots of aspirin treated and of control blood were then pre-incubated and stirred in the water bath for 2 minutes, when 20ul U46619 (0.3μM or 3μM) or saline was added to each tube and aggregation allowed to proceed for 8 minutes. 15ul subsamples of blood were removed and placed in fixative at the times detailed above and used for later platelet counting.

For all experiments in whole blood, percentage aggregation was calculated as detailed in Chapter 2, and graphs of percentage aggregation against time were constructed.

Platelet thromboxane production in response to 1mM AA was measured by pre-incubating 500ul adjusted PRP in siliconised glass tubes in the aggregometer wells for 2 minutes whilst stirring at 1,000rpm. Aggregation was then induced with 50ul 1mM AA and the reaction allowed to proceed for 4 minutes at which time 200ul of PRP was removed and immediately placed in a plastic Eppendorf tube containing 200ul ice cold absolute alcohol. The tubes were then thoroughly vortex mixed and immediately placed at -70°C. Radioimmunoassay for thromboxane B_2 was then performed within 3 months as described in Chapter 2.

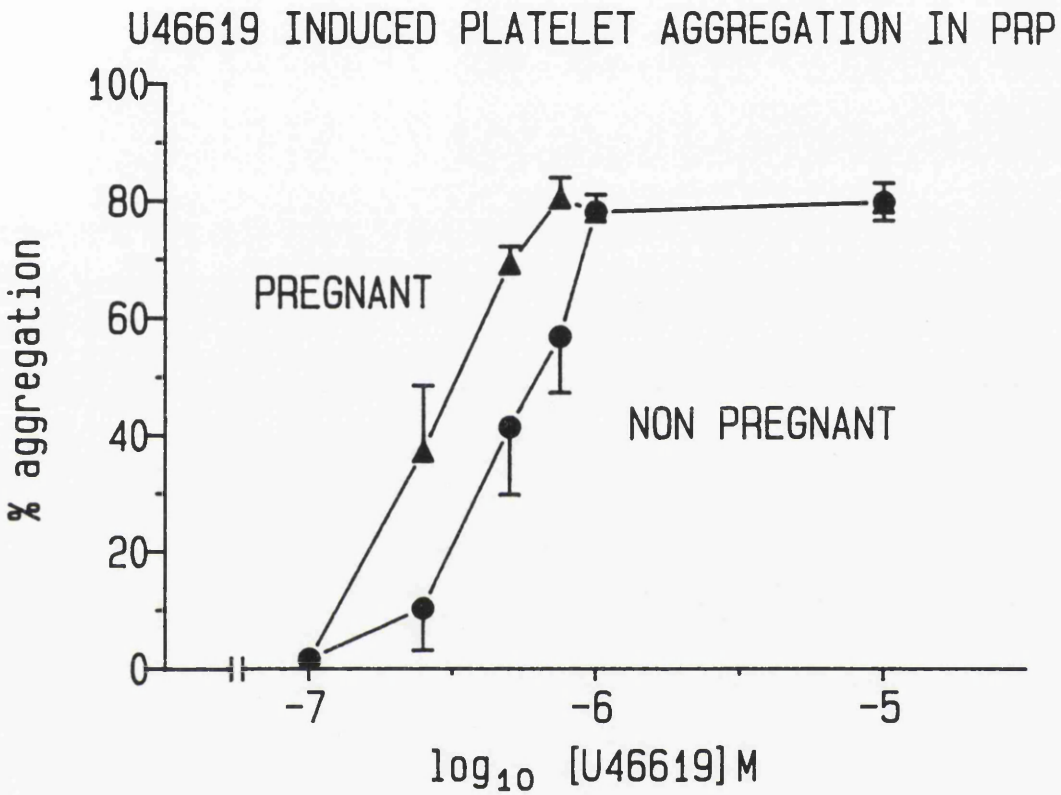


FIGURE 7.1.

The figure shows data from 10 non pregnant and 10 healthy third trimester pregnant women. The concentration response curves for U46619 induced aggregation in PRP are illustrated. Means and S.E.Ms for each group of subjects are illustrated. The curve is shifted to the left in pregnant compared with non pregnant subjects.

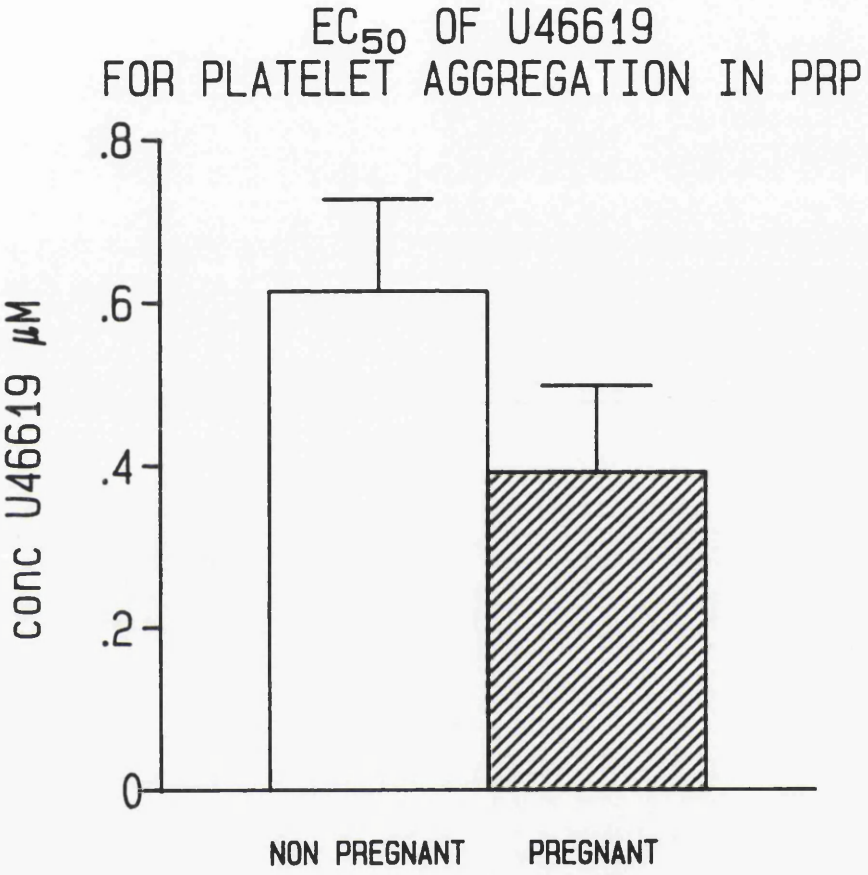


FIGURE 7.2

The bar graph illustrates means + S.E.M.s of EC_{50} values for U46619 induced platelet aggregation in PRP. The data were derived from those illustrated in Figure 7.1. There was a significant reduction in EC_{50} for U46619 in pregnant subjects (* $p = 0.05$).

2.3 Data Analysis.

For the experiments in PRP, EC_{50} values for U46619 induced aggregation were compared in pregnant and non pregnant subjects using a Mann Whitney-U test. The dissociation constant (K_d) values for the thromboxane antagonist ICI 192605 were estimated for each subject using the equation:

$$K_d = \frac{\text{Concentration of antagonist}}{[EC_{50}U46619(2)/EC_{50}U46619(1)]-1}$$

where $EC_{50}(1)$ was in the absence of the antagonist and $EC_{50}(2)$ was in the presence of the antagonist.

K_d values for pregnant and non pregnant subjects were then compared using the Mann Whitney-U test. Statistical significance was defined as $p<0.05$. For all experiments in whole blood, percentage aggregation for each time point was compared using the Mann Whitney-U test and statistical significance was defined as $p < 0.01$ as repeated tests were performed on data from the same subjects. Thromboxane B_2 radioimmunoassay data were analyzed using the Mann-Whitney U test.

3.RESULTS.

3.1 Platelet Aggregation in Response to U46619 in PRP.

In the absence of the thromboxane antagonist, EC_{50} values for U46619 for platelet aggregation in PRP were significantly lower in pregnant than in non pregnant women. Figure 7.1 illustrates the concentration response curves for U46619 induced aggregation in PRP, and Figure 7.2 illustrates the EC_{50} values for U46619. In the presence of 10^{-8}M ICI 192605, there was a small and highly variable shift in the U46619 dose response curve, and there was no statistically significant difference in the EC_{50} values for U46619 for pregnant ($0.97\pm0.34\mu$M) and non

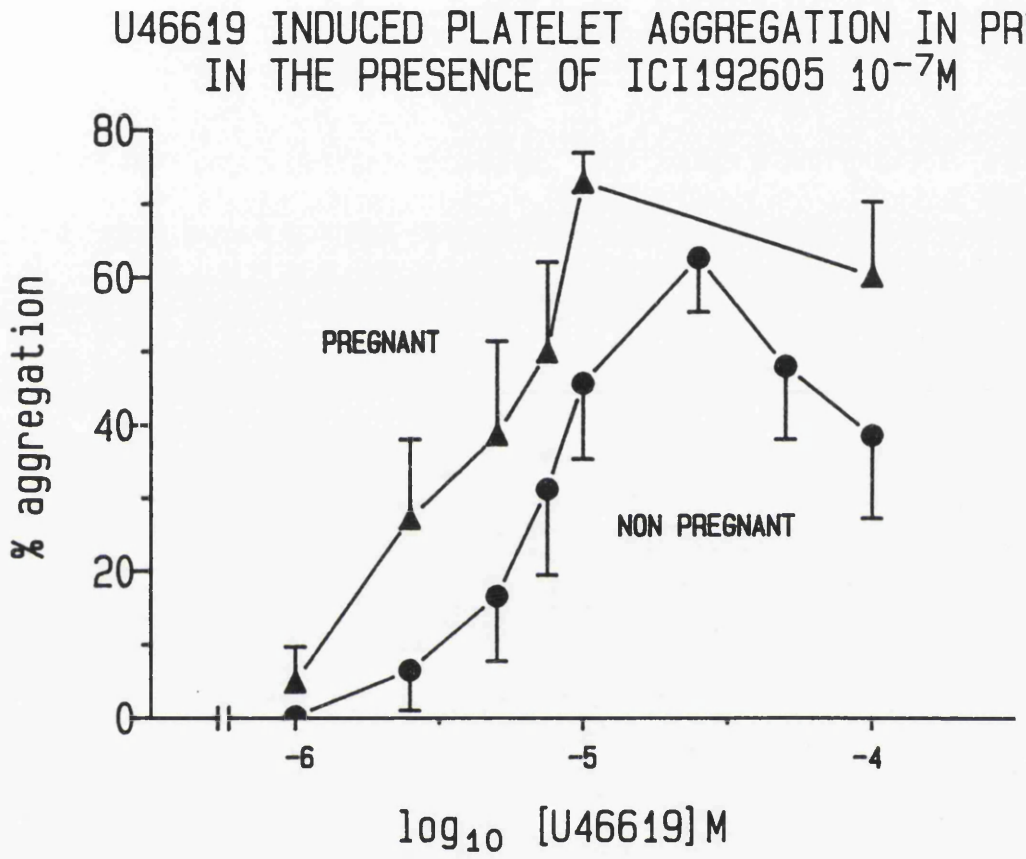


FIGURE 7.3.

The figure illustrates the concentration response curve for U46619 induced platelet aggregation in PRP in the presence of the thromboxane antagonist ICI 192605 (10^{-7}M). Means and S.E.M.s of 10 observations are shown for each data point. The concentration-response curve was again shifted to the left for the pregnant subjects.

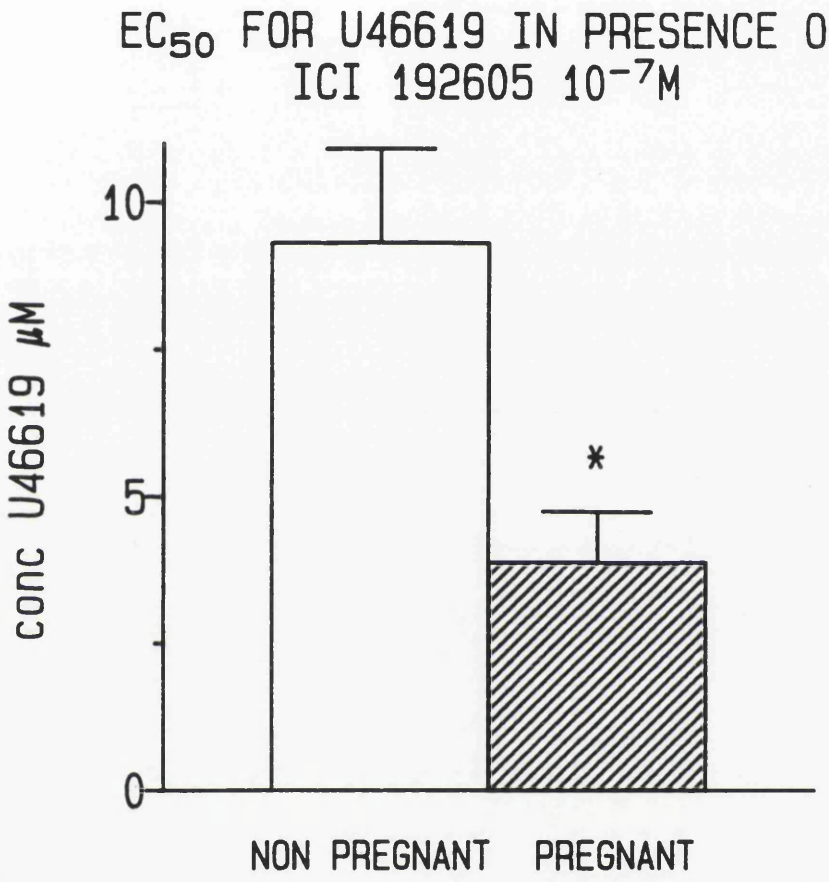


FIGURE 7.4.

The bar graph shows means and S.E.M.s of EC_{50} values for U46619 induced platelet aggregation in PRP. The data are derived from those illustrated in Figure 7.3. There was a significant reduction in the EC_{50} for U46619 in pregnant subjects (* $p = 0.01$).

pregnant subjects (1.68$\pm$0.42μM). When the thromboxane antagonist was present at a concentration of 10^{-7}M, there was an approximate 10 fold shift to the right in the U46619 dose response curve, and there was again a significant reduction in EC_{50} values in pregnant compared with non pregnant subjects. The data are illustrated in Figures 7.3 and 7.4. In the presence of ICI 192605, the extent of platelet aggregation in response to high concentrations of U46619 appeared to decrease. The effect was less marked in pregnant subjects. Estimation of the K_d values for ICI 192605 for each subject showed a mean value of 10.19$\pm$2.78nM for non pregnant subjects and 13.85$\pm$2.54nM for pregnant subjects ($p = 0.15$).

3.2 Platelet Aggregation in Whole Blood in Response to U46619

In the presence of saline alone, a degree of "spontaneous" platelet aggregation was noted in both pregnant and non pregnant subjects. This was maximal at 8 minutes in both subject groups, and whilst there was a tendency for spontaneous aggregation to be more marked in pregnant subjects, this did not reach statistical significance. The data are shown in Figure 7.5.

When platelet aggregation was induced with 0.3uM U46619, the pattern of response in pregnant subjects differed from that in the non pregnant group. In the majority of non pregnant women, rapid but submaximal platelet aggregation took place which was partially reversible by 1 minute, and this was followed by a slow increase in aggregation from 2 to 8 minutes. By contrast, in pregnant women, the general pattern of response was rapid maximal aggregation which was sustained thereafter. The data are shown on Figure 7.6. The increase in platelet aggregation in response to 0.3uM U46619 in the pregnant group was statistically significant at all time points except 8 minutes.

When 3uM U46619 was used as the agonist, a maximal platelet

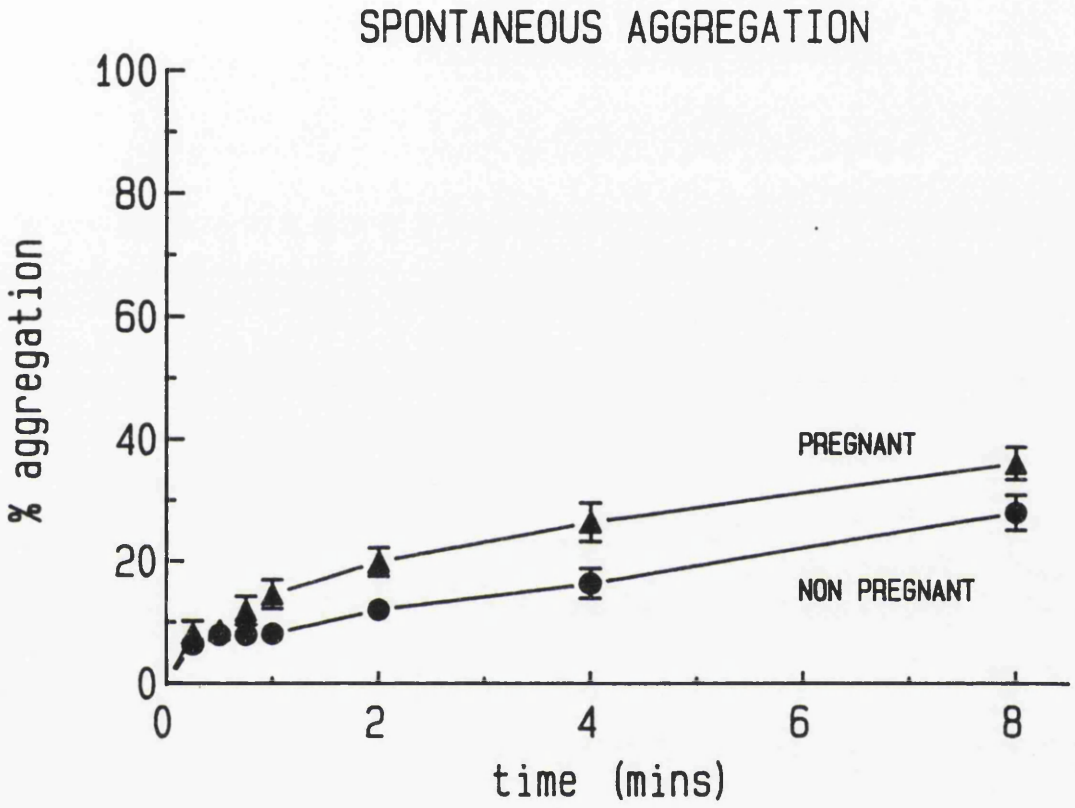


FIGURE 7.5.

The figure shows the extent of "spontaneous" platelet aggregation in whole blood plotted against time. Each data point represents mean $\pm$ S.E.M. of 14 observations for the non pregnant group (●), and 15 observations for the pregnant group (▲). There was a slight trend towards increased spontaneous aggregation in pregnant subjects but this was not statistically significant.

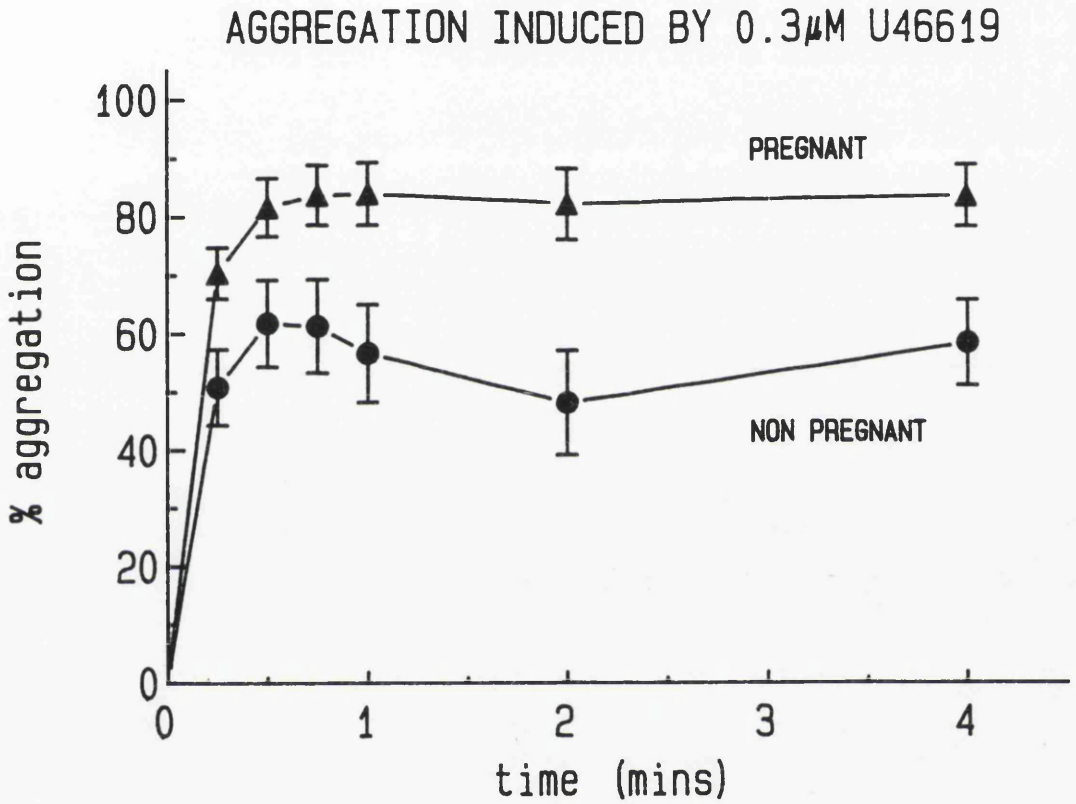


FIGURE 7.6.

The figure shows % platelet aggregation in whole blood plotted against time for pregnant (▲) and non pregnant (●) subjects using 0.3μM U46619 as the agonist. Means and standard errors of data from 15 pregnant and 14 non pregnant subjects are shown. There was a significant increase in platelet aggregation in response to 0.3uM U46619 in the pregnant group at all time points except at 8 minutes when the effects of " spontaneous " platelet aggregation enhanced the response to U46619 in the non pregnant group. (8 minute time point not shown).

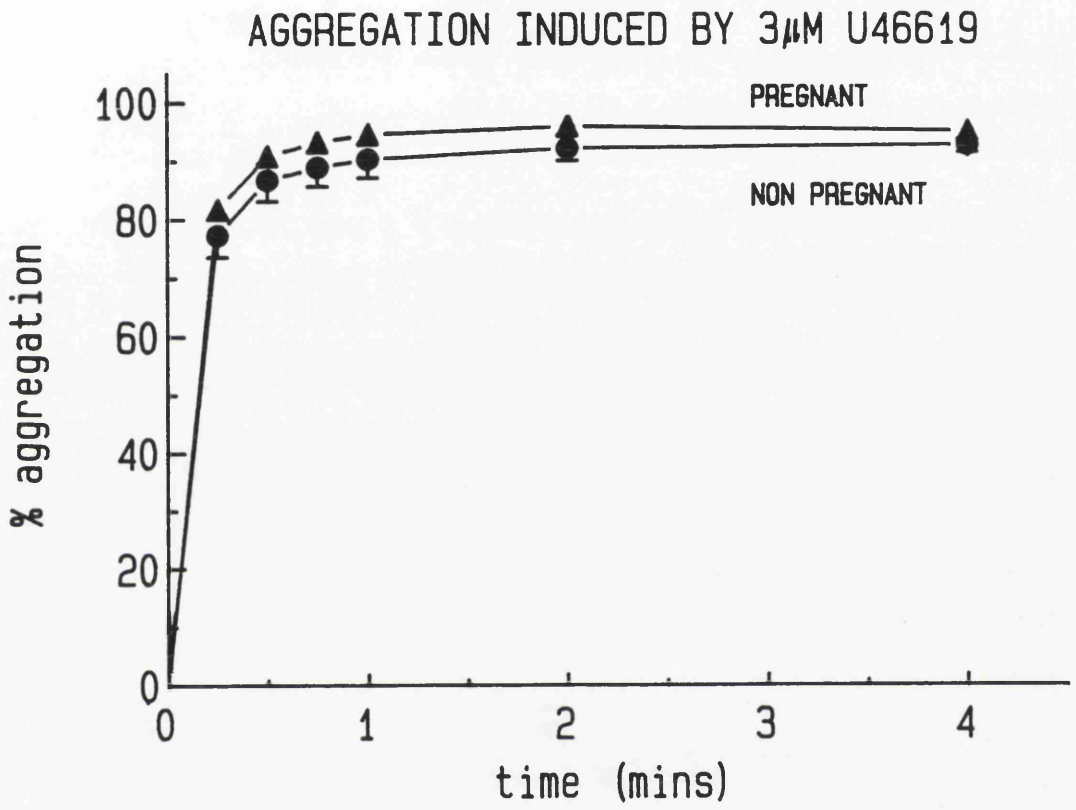


FIGURE 7.7.

The figure shows % platelet aggregation in whole blood plotted against time in response to 3μM U46619. Means $\pm$ S.E.M.s of data from 14 non pregnant (●) and 15 pregnant (▲) subjects are shown. Statistical analysis revealed no difference between subject groups.

TABLE 7.1

U46619 Induced Platelet Aggregation in Whole Blood

Conc U46619		15s	30s	45s	1 minutes	2 minutes	4 minutes	8 minutes
	NP	50.83 ± 6.49	61.74 ± 7.38	61.29 ± 7.95	56.66 ± 8.33	48.19 ± 8.9	58.42 ± 7.27	74.88 ± 5.10
0.3μM	P	70.38 ± 4.36	81.57 ± 4.96	83.72 ± 5.16	83.96 ± 5.33	82.21 ± 6.12	83.71 ± 5.29	84.15 ± 3.72
	p value	0.0087	0.0067	0.0039	0.0029	0.0039	0.0048	0.0836
	NP	77.34 ± 3.72	86.79 ± 3.58	88.95 ± 3.25	90.29 ± 3.17	92.19 ± 2.17	92.67 ± 1.33	90.61 ± 0.91
3μM	P	81.83 ± 1.15	90.90 ± 0.36	93.28 ± 0.33	94.53 ± 0.19	95.95 ± 0.27	95.03 ± 0.40	92.66 ± 0.75
	p value	0.582	0.908	0.509	0.772	0.060	0.105	0.131

The table shows means and S.E.Ms of % platelet aggregation in whole blood in response to 0.3μM and 3μM U46619 in pregnant and non pregnant subjects. p values for statistical comparison of pregnant and non pregnant groups using the Mann Whitney-U test are shown.

aggregation response was obtained in all subjects and there was no difference between pregnant and non pregnant groups (Figure 7.7). The statistical analysis of data relating to U46619 induced platelet aggregation in whole blood is shown in Table 7.1.

3.3 Effect of the Thromboxane Antagonist on U46619 Induced Platelet Aggregation in Whole Blood.

In the presence of 10^{-7}M ICI 192605, platelet aggregation in response to 0.3uM U46619 was inhibited in both subject groups, but the extent of aggregation was still significantly greater in the pregnant group at all time points except 0.25 minutes and 8 minutes. The results are shown in Figure 7.8. 10^{-7}M ICI 192605 had only very minor inhibitory effects upon platelet aggregation induced by 3uM U46619 in both non pregnant and pregnant subjects and there was little difference between groups in this respect as shown in Table 7.2.

In the presence of 10^{-6}M U46619, platelet aggregation in response to 0.3uM U46619 was reduced to levels similar to "spontaneous" aggregation, and there was no difference between subject groups in the extent of aggregation under these conditions. The data are shown in Table 7.2. 10^{-6}M ICI 192605 also significantly inhibited platelet aggregation induced by 3uM U46619. In non pregnant subjects, a pattern similar to "spontaneous" aggregation was obtained under these conditions, but in pregnant subjects, aggregation was greater at early time points than in the presence of saline alone. In the pregnant group the extent of aggregation was significantly greater than in non pregnant women at all time points except 0.25 and 8 minutes (Figure 7.9). The statistical analysis relating to U46619 induced platelet aggregation in whole blood in the presence of the thromboxane antagonist is summarised in Table 7.2.

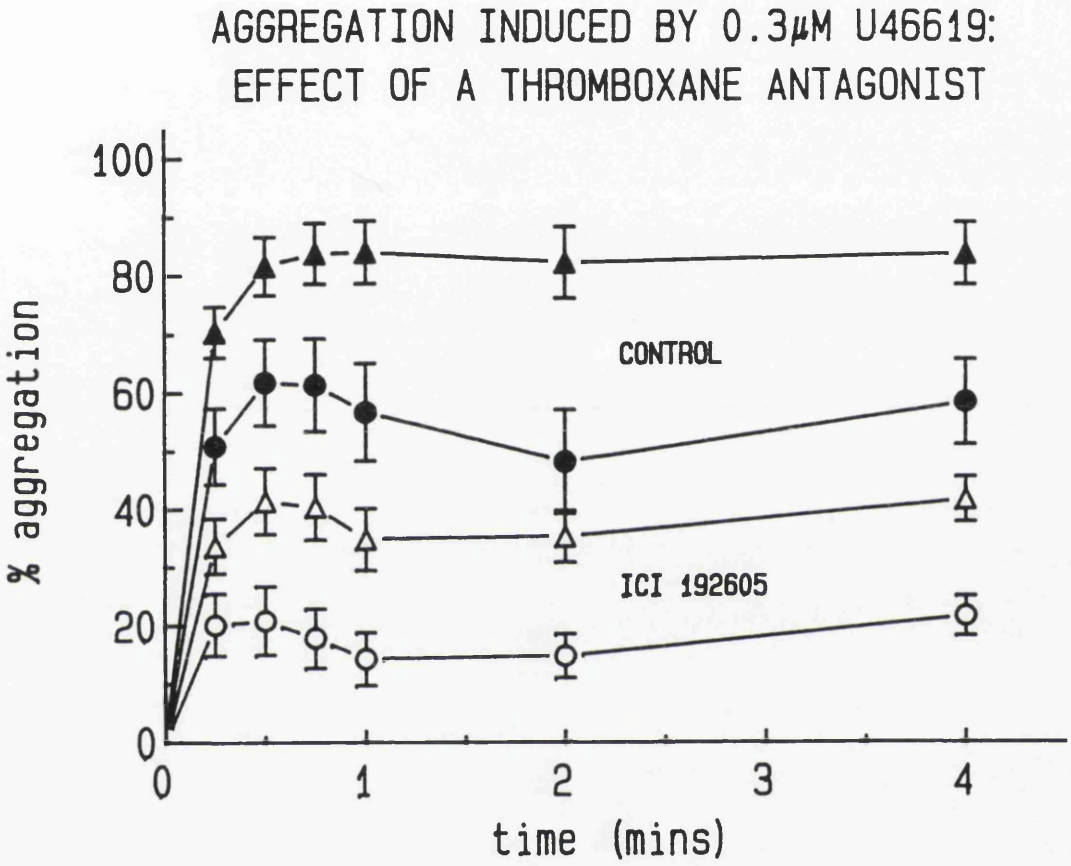


FIGURE 7.8.

The graph shows the effect of the thromboxane antagonist ICI 192605 (10^{-7}M) on platelet aggregation in whole blood induced by 0.3uM U46619. Means and standard errors of data from 15 pregnant (△) and 14 non pregnant subjects (○) are shown. Control data in the absence of the thromboxane antagonist are illustrated with filled symbols (▲ = pregnant, ● = non pregnant).

The thromboxane antagonist inhibited U46619 induced platelet aggregation in both subject groups, but platelet aggregation occurred to a significantly greater extent at all time points except 0.25 and 8 minutes in pregnant subjects in the presence of ICI 192605.

TABLE 7.2

U46619 INDUCED PLATELET AGGREGATION IN WHOLE BLOOD IN THE PRESENCE OF ICI 192605

Conc U46619	Conc ICI 192605		TIME 15s	30s	45s	1 minutes	2 minutes	4 minutes	8 minutes
0.3μm	10^{-7}M	NP	20.08±5.28	20.71±5.84	17.8±5.07	14.22±4.54	14.73±3.77	21.59±3.40	37.49±4.25
		P	33.63±4.72	41.35±5.69	40.37±5.64	34.79±5.33	35.30±4.46	41.73±3.91	50.53±3.39
		p value	0.0542	0.0077	0.0032	0.0039	0.0014	0.0017	0.0320
0.3μM	10^{-6}M	NP	4.71±1.06	4.81±1.11	5.29±0.96	6.93±1.07	7.92±1.08	12.34±2.28	20.8±2.49
		P	5.39±0.93	6.13±1.09	7.68±1.49	9.46±1.33	12.91±1.39	19.43±2.07	31.11±2.56
		p value	0.6136	0.3334	0.3030	0.1429	0.0193	0.0229	0.0166
3μM	10^{-7}M	NP	63.81±3.45	73.49±4.0	76.58±4.17	76.28±4.39	85.1±3.66	88.99±2.79	85.41±1.57
		P	66.84±3.9	80.17±3.11	85.11±2.56	87.87±2.26	91.66±1.32	93.2±0.38	88.53±1.04
		p value	0.3789	0.1153	0.0518	0.0087	0.0153	0.1153	0.1052
3μM	10^{-6}M	NP	6.72±1.47	10.20±2.17	12.67±3.0	11.95±2.97	15.76±3.67	21.45±4.2	36.89±4.65
		P	15.19±2.52	24.01±3.45	29.27±3.58	31.93±3.71	35.69±4.11	39.29±4.19	49.31±4.21
		p value	0.0203	0.0022	0.0026	0.0009	0.0014	0.0029	0.0357

The table shows % platelet aggregation in whole blood in response to U46619 in the presence of ICI 192605. Means ± SEMs of data from 15 pregnant and 14 non pregnant subjects are shown. p values were obtained by comparison of data in pregnant and non pregnant women using the Mann Whitney-U test.

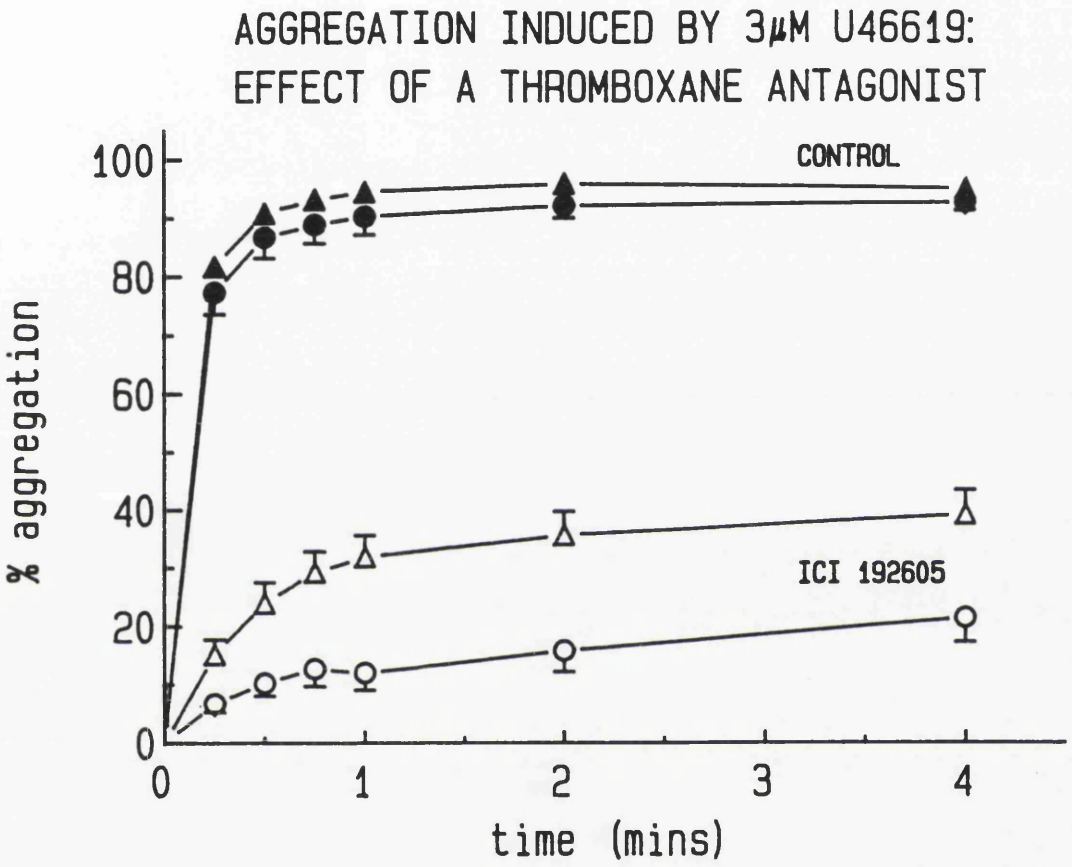


FIGURE 7.9.

The effects of 10^{-6}M ICI 192605 on platelet aggregation in whole blood induced by 3uM U46619 are shown. Means and standard errors of data from 15 pregnant (△) and 14 non pregnant (○) subjects are illustrated. Filled symbols (▲ = pregnant, ● = non pregnant) are used for control data in the absence of the thromboxane antagonist.

The thromboxane antagonist inhibited platelet aggregation in both subject groups. Although there was no difference between groups in the extent of platelet aggregation in response to 3uM U46619 in control tubes, aggregation occurred to a significantly greater extent in blood from pregnant subjects at all time points except 0.25 and 8 minutes in the presence of the thromboxane antagonist.

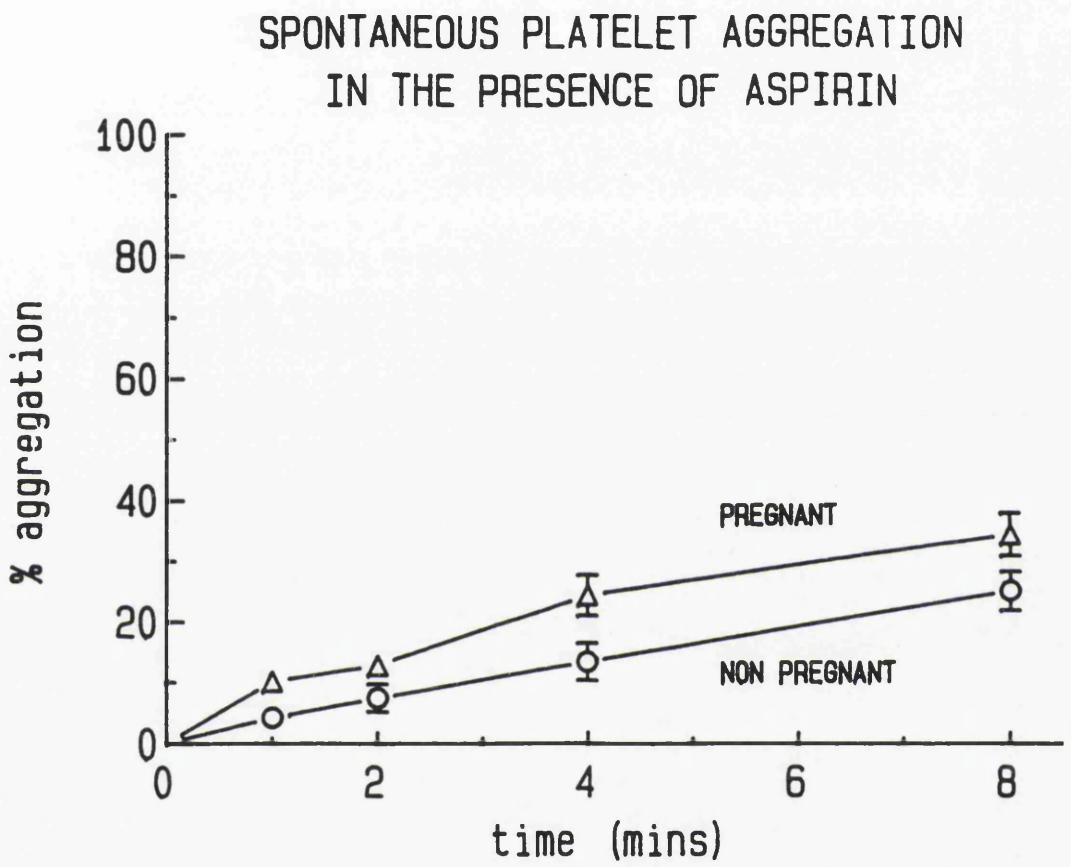


FIGURE 7.10.

The figure shows the extent of "spontaneous" platelet aggregation in whole blood pre-incubated with aspirin (0.56mM). Means $\pm$ S.E.M.s of data from 11 pregnant and 10 non pregnant subjects are illustrated. Although there was a slight trend in aspirin treated platelets to increased spontaneous platelet aggregation in pregnant subjects, this only reached statistical significance at one time point (1 minute). For clarity spontaneous aggregation in control blood is not illustrated here.

3.4 Platelet Aggregation in Whole Blood in the Presence of Aspirin.

Aspirin slightly inhibited "spontaneous" platelet aggregation in both groups of subjects. "Spontaneous" aggregation was slightly but significantly greater in pregnant compared with non pregnant subjects, at the 45 second, 1 minute, and 2 minute time points in control blood, but the increase only reached significance at 1 minute in aspirin treated blood (Figure 7.10).

Aspirin did not inhibit, and in fact, rather surprisingly, in non pregnant subjects, significantly enhanced platelet aggregation induced by 0.3uM U46619. A significant increase in platelet aggregation in response to 0.3uM U46619 was present in control and aspirin treated blood from pregnant compared with non pregnant subjects at all time points except 8 minutes. The results are shown in Figure 7.11 and the statistics are documented in Table 7.3.

3.5 Platelet Thromboxane Production in Platelet Rich Plasma Stimulated with Arachidonic Acid.

Platelet thromboxane B_2 production in PRP stimulated with 1mM AA is shown in Figure 7.12. There was no statistically significant difference between pregnant and non pregnant subjects.

DISCUSSION.

These studies have shown that platelet aggregation in response to the endoperoxide analogue U46619, which acts at the thromboxane receptor,[40,447] is enhanced in healthy pregnant women. The effect appeared more marked in whole blood than in PRP but was statistically significant under both conditions. It is possible that the loss of some active platelets during the preparation of PRP made the effect more obvious in whole blood but differing experimental design in the two studies may also have contributed. The choice of a concentration of U46619

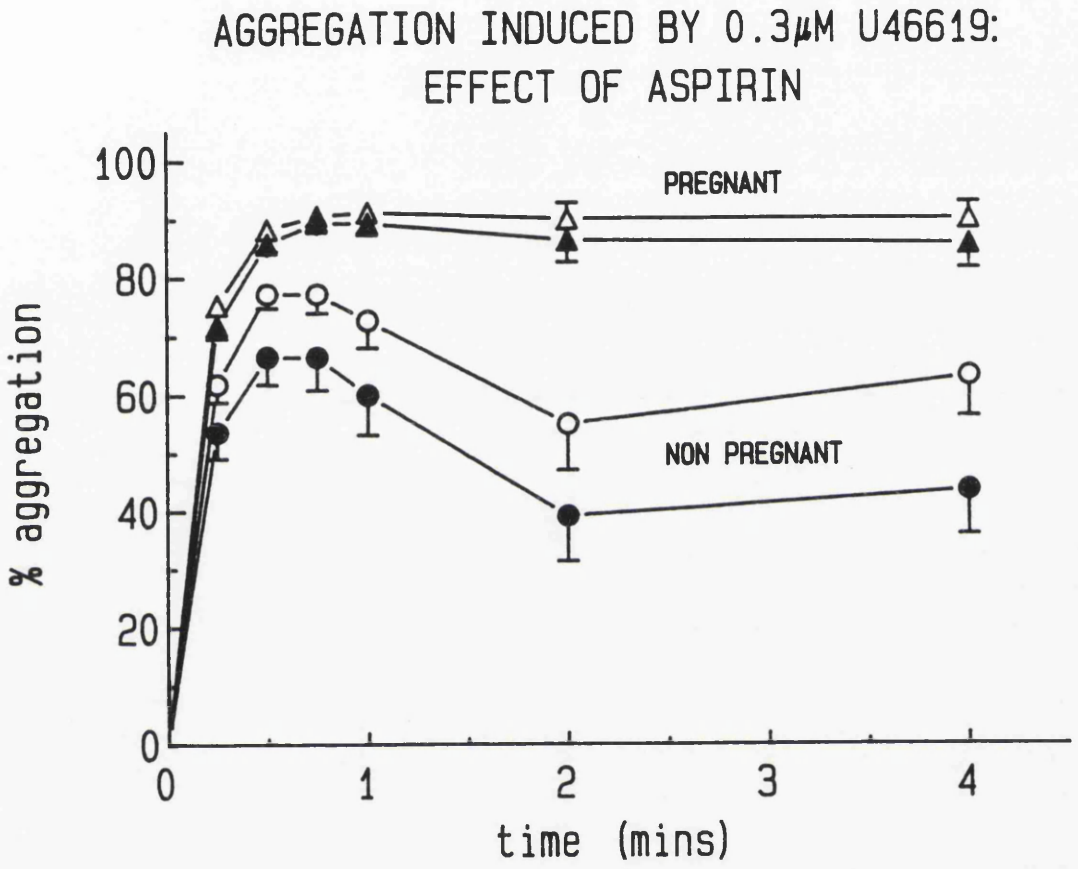


FIGURE 7.11.

The effect of aspirin on platelet aggregation in whole blood in response to 0.3uM U46619 is shown. Means and standard errors of data from 11 pregnant and 10 non pregnant subjects are illustrated. Control data are indicated by filled symbols (▲, ●) and data in the presence of aspirin by open symbols (△, ○).

Aspirin did not inhibit, and in fact, in non pregnant subjects, significantly enhanced U46619 induced platelet aggregation ($p < 0.01$ at all time points, Wilcoxon matched pairs test). A significant increase in platelet aggregation in response to 0.3uM U46619 was present in control and aspirin treated blood from pregnant subjects at all time points except 8 minutes. (8 minute data not shown.

TABLE 7.3

U46619 INDUCED PLATELET AGGREGATION IN WHOLE BLOOD IN THE PRESENCE OF ASPIRIN

Conc U46619	Conditions		TIME 15s	30s	45s	1 minutes	2 minutes	4 minutes	8 minutes
0.3μM	Aspirin	NP	61.82 ± 3.02	77.2 ± 2.38	77.2 ± 3.25	72.61 ± 4.61	54.97 ± 7.83	63.25 ± 6.91	78.73 ± 5.5
		P	75.39 ± 1.64	88.3 ± 1.11	90.58 ± 1.26	91.18 ± 1.57	89.99 ± 2.74	90.05 ± 2.83	87.02 ± 2.94
		p value	0.0021	0.0031	0.0039	0.0048	0.0039	0.0100	0.2585
0.3μM	Control	NP	53.58 ± 4.5	66.49 ± 4.71	66.44 ± 5.61	59.95 ± 6.85	39.16 ± 7.77	43.75 ± 7.71	61.55 ± 7.20
		P	71.93 ± 2.27	85.83 ± 1.36	89.45 ± 1.4	89.27 ± 1.97	86.36 ± 3.75	85.74 ± 4.13	83.39 ± 4.32
		p value	0.0039	0.0039	0.0021	0.0034	0.0019	0.0017	0.0285
3μM	Aspirin	NP	75.7 ± 2.11	87.45 ± 0.86	89.93 ± 0.73	92.09 ± 0.66	93.32 ± 0.52	92.66 ± 0.81	86.06 ± 2.58
		P	78.78 ± 1.57	88.58 ± 0.92	91.71 ± 0.64	93.13 ± 0.70	95.21 ± 0.28	93.62 ± 0.52	89.42 ± 1.81
		p value	0.3054	0.3054	0.0776	0.1691	0.0009	0.4151	0.3054
3μM	Control	NP	81.97 ± 1.39	91.01 ± 0.55	93.6 ± 0.41	94.43 ± 0.36	94.64 ± 0.34	92.56 ± 0.43	86.29 ± 1.92
		P	84.52 ± 0.68	91.56 ± 0.45	94.04 ± 0.24	95.05 ± 0.22	95.42 ± 0.18	93.8 ± 0.3	87.87 ± 1.23
		p value	0.3762	0.5687	0.6171	0.2891	0.0776	0.0568	0.4354

The table shows mean ± SEM % platelet aggregation in control and aspirin treated whole blood in pregnant and non pregnant subjects. Data were obtained in 11 healthy third trimester pregnant women and 10 non pregnant women. p values were derived by comparison of the two groups of subjects using the Mann-Whitney U test. Comparison of data for control and aspirin treated blood within subject groups using the Wilcoxon matched pairs test revealed that platelet aggregation in response to 0.3μM U46619 was significantly greater at all time points in aspirin treated blood in non pregnant subjects ($p < 0.01$). This was not the case in pregnant subjects.

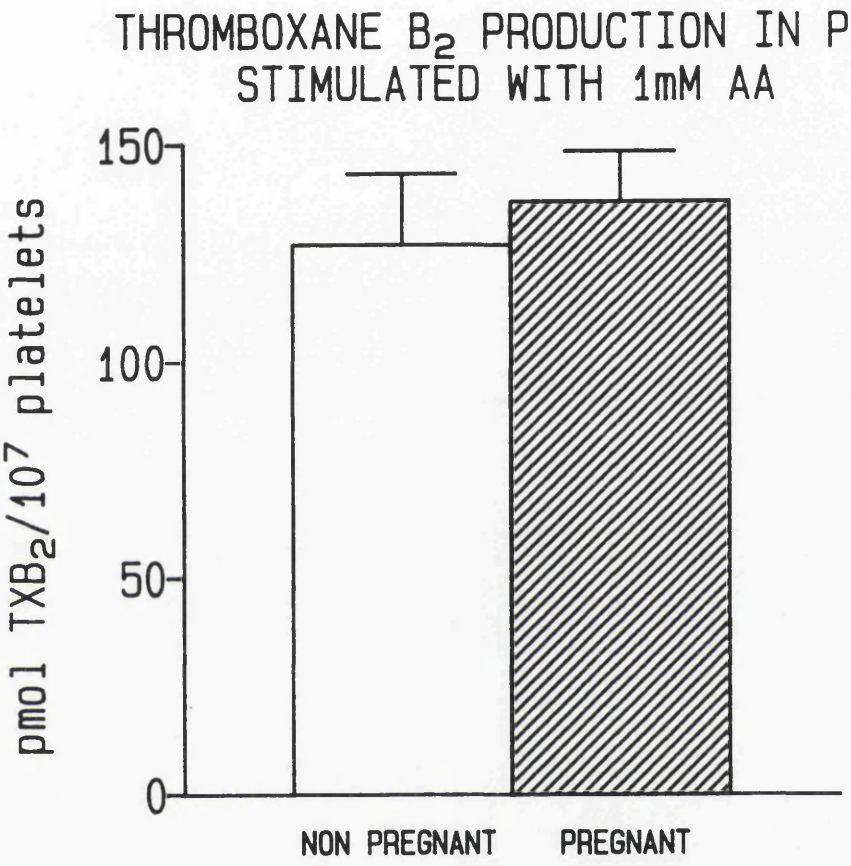


FIGURE 7.12.

The bar graph shows mean and S.E.M. of thromboxane B_2 production in PRP stimulated with 1 mM AA in 13 healthy third trimester pregnant and 13 non pregnant subjects. Statistical analysis revealed no difference between subject groups.

near to the EC_{50} in whole blood experiments would tend to bring out the differences between the two subject groups.

The enhanced aggregation response to U46619 in pregnant women is unlikely to be due to non specific effects such as changes in haematocrit and plasma fibrinogen, as there were only minimal differences in spontaneous platelet aggregation in whole blood in pregnant compared with non pregnant subjects, and these differences were reduced when thromboxane synthesis was inhibited by aspirin.

In both PRP and in whole blood, an enhanced platelet response to U46619 was still detectable in the presence of ICI 192605, an antagonist at the thromboxane receptor.[448-450] Differences in EC_{50} values for U46619 in pregnant and non pregnant subjects in the presence of ICI 192605 in PRP cannot be explained by differing receptor affinity of the antagonist as the estimated K_d values for ICI 192605 were similar for both subject groups. Furthermore, the specificity of the effects of U46619 in pregnancy are supported by the abolition of differences between subject groups in the presence of an excess of the thromboxane antagonist relative to U46619 in whole blood. The reason is unclear for the unusual shape of the concentration response curve for U46619 in PRP in the presence of the thromboxane antagonist. A non competitive component to the antagonism by ICI 192605 should be considered but the shape of the curve was not typical of such an effect, and previous studies have suggested that this agent is a competitive antagonist at the thromboxane/endoperoxide receptor.[448,449] It is possible that thromboxane receptor blockade unmasked an inhibitory effect of very high concentrations of U46619, possibly mediated by binding of the endoperoxide analogue to another prostaglandin receptor. It is interesting in this respect that the decrease in aggregation in response to high concentrations of U46619 when

the antagonist was present, was less marked in pregnant subjects.

In whole blood treated with aspirin, at concentrations shown to abolish aggregation dependent upon platelet thromboxane synthesis, platelet sensitivity to U46619 remained enhanced in pregnant women. Endogenous thromboxane A_2 production does not therefore contribute to the enhanced platelet response to U46619 during pregnancy. In addition, no enhancement of platelet thromboxane synthesis in PRP in response to arachidonic acid was detectable in pregnant subjects, thus casting doubt upon the importance of the amount of thromboxane synthesised as a determinant of increased platelet sensitivity to weak agonists and to arachidonic acid in vitro during pregnancy.

It is of interest that platelets in aspirin treated blood from non pregnant subjects were more sensitive to U46619 than control platelets. It is possible that production of inhibitory prostanoids from white cells in whole blood[440] was inhibited by aspirin, resulting in an increased platelet response to U46619. This must, however, remain speculative, as experiments were not carried out in aspirin treated PRP, nor was prostaglandin production measured. The corollary is that enhanced platelet responses to 0.3uM U46619 in whole blood during pregnancy cannot result from reduced responses to inhibitory prostanoids synthesised by white cells and platelets.

These data taken together suggest that platelets from pregnant women either possess greater numbers of thromboxane/endoperoxide receptors than those from non pregnant women, or that occupancy of fewer thromboxane receptors is required to elicit aggregation as a result of tighter stimulus response coupling. Consequently, enhanced platelet sensitivity to thromboxane A_2 may make a major contribution towards increased platelet reactivity during pregnancy to agonists whose mechanism of action has a thromboxane dependent component. As platelet

thromboxane B_2 production in arachidonic acid stimulated PRP and indeed serum thromboxane B_2 levels (reported in Chapters 3 and 5) were similar in pregnant and non pregnant women, sensitivity to thromboxane during pregnancy is probably a more important determinant of platelet reactivity to thromboxane dependent agonists in vitro than the extent of thromboxane synthesis. It is also possible that enhanced reactivity towards thromboxane contributes to platelet activation in vivo during pregnancy. It has been suggested that the pro-aggregatory effects of PGE_2 at low concentrations may be mediated by the endoperoxide receptor.[89] If this were confirmed, the results of the studies presented here would imply that platelet activation in vivo may also be promoted during pregnancy by PGE_2 synthesised by the placenta.[298]

There is recent evidence that two distinct thromboxane/endoperoxide binding sites exist on human platelets, one of which mediates calcium influx across the cell membrane leading to platelet shape change, and the other of which is linked to platelet aggregation and secretion via the activation of phospholipase C.[85,451] It has also been proposed that activation of phospholipase C by thromboxane and endoperoxides is mediated by a G protein distinct from G_p,[132] the putative GTP binding protein involved in linking polyphosphoinositol hydrolysis with platelet activation responses to thrombin, PAF and vasopressin.[122] Platelet shape change and cytosolic calcium were not measured in these studies, so changes in this aspect of response to thromboxane during pregnancy cannot be ruled out. It is evident, however, that alterations at or beyond the thromboxane receptor mediating aggregation and phospholipase C activation do occur in platelets from healthy pregnant women, but further studies would be required to determine whether this results from increased receptor numbers, changes at G protein level or alterations in phospholipase C itself.

It is interesting to note that both protein kinase C activation[85,452,453] and G protein mediated effects[85,454] have been implicated in the regulation of thromboxane induced platelet activation, including the desensitisation of this response.[85,454] Previous reports have suggested that prior exposure of platelets to thromboxane agonists results in a rapid desensitisation phenomenon mediated by reduction in receptor numbers[455] and uncoupling between the receptor and the G protein.[85,454] As it is established that thromboxane production is increased in vivo during pregnancy,[298,309] other mechanisms must come into play which prevent desensitisation from taking place and paradoxically permit enhancement of platelet responses to thromboxane. Protein kinase C activation could possibly play such a role.[85,452,453] Oxidation or reduction of sulphydryl/disulphide groups on the thromboxane receptor has been shown to cause, respectively, up- and down-regulation of the thromboxane/endoperoxide receptor in human platelet membranes.[456] It has been proposed that exposure of platelets to locally generated oxidants may increase platelet thromboxane receptor numbers in patients with acute myocardial infarction,[85,457] but it would be difficult to envisage that such mechanisms could contribute to platelet sensitivity to thromboxane during healthy pregnancy.

CHAPTER 8.

GENERAL DISCUSSION.

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This thesis has examined the effect on platelet behaviour in healthy pregnancy and in pre-eclampsia of prostaglandins which inhibit and those which promote platelet activation and has explored some aspects of intracellular signalling which mediate the platelet response to these agents.

The results relating to healthy pregnancy are in themselves of considerable interest as an understanding of the pathophysiology of disease can only be obtained on the basis of sound physiological data. The studies presented have demonstrated significant alterations during normal pregnancy in platelet responses in vitro to a variety of prostaglandins which play a role in regulating platelet behaviour in vivo. Platelets from healthy pregnant women have been shown to be less sensitive to inhibition of their behaviour by iloprost, the stable prostacyclin analogue, and by PGD_2, compared with those from non pregnant young women. This was associated with a reduction in platelet cyclic AMP accumulation in response to these agents and other adenylate cyclase stimulators. On the other hand, during pregnancy, platelets were more sensitive in vitro to the pro-aggregatory effects of the endoperoxide analogue U46619, which acts at the thromboxane A_2/PGH_2 receptor.[40,447]

Reduced cyclic AMP accumulation in response to inhibitory prostanoids occurred very early in the first trimester and was sustained throughout gestation. The time course of alterations in sensitivity to pro-aggregatory prostanoids with respect to gestation was not investigated in these studies, but other authors have described enhanced platelet reactivity to agonists dependent on thromboxane for their action from 16 to 24 weeks gestation onwards.[322,327]

The possible mechanisms underlying the observations on platelet responses to inhibitory prostaglandins merit further discussion. The results of the studies presented in this thesis leave little doubt that reduction in platelet cyclic AMP accumulation during pregnancy at least contributes to loss of platelet sensitivity to prostanoids such as prostacyclin. Indirect evidence has been presented in favour of a change occurring during pregnancy in the ability of the platelet adenylate cyclase enzyme to respond to stimulation, perhaps residing in alteration of the catalyst or its regulatory G proteins. This evidence comes firstly from the demonstrated reduction during healthy pregnancy in platelet cyclic AMP responses to a wide range of adenylate cyclase stimulators including forskolin (Chapters 4 and 5), excluding changes in receptors as a likely underlying mechanism. Secondly,increased platelet cyclic AMP metabolism in pregnancy has been excluded under the experimental conditions used (Chapter 6). Furthermore, reduced platelet cyclic AMP responses to PGE_1 during pregnancy were observed at very early time points following exposure of platelets to this agent, suggesting an alteration in the generation of cyclic AMP.

Some caution must be exercised in interpreting the data regarding forskolin, as this agent has been shown to have a number of pharmacological effects other than stimulation of adenylate cyclase, for example it modulates potassium channels in some tissues and it inhibits the glucose transporter in many cell types including platelets.[458] There is no evidence, however, that any of these other actions are likely to affect cyclic AMP levels. It therefore seems reasonable, in the context of the rest of the data presented here, to conclude that the difference in cyclic AMP accumulation in platelets from pregnant women in the presence of forskolin is likely to result from a reduction in adenylate cyclase activity. Although the action of forskolin on adenylate cyclase does not require the stimulatory G

protein, the diterpene has been shown to interact in a complex manner with adenylate cyclase, and its actions may be modulated by G_s and G_i.[201,442] Involvement of G proteins in the pregnancy associated reductions in platelet cyclic AMP accumulation cannot therefore be ruled out, although the data presented suggest that prostaglandin stimulated G_i activity is not altered. Similarity was demonstrated in the extent of reduction in stimulated cyclic AMP levels on activation of the inhibitory G protein by PGE_2 in platelets from pregnant and non pregnant women. Assay of adenylate cyclase activity together with studies of G proteins in platelet membranes may clarify the exact nature of the changes in platelet adenylate cyclase during pregnancy.

It is interesting to speculate on how changes in adenylate cyclase in platelets may be brought about in pregnancy. The synthesis of steroid hormones, principally oestrogen and progesterone, rise markedly during pregnancy,[459] and these may have effects on platelets. Steroid hormones have classically been thought to influence cellular function by entering the nucleus and exerting effects on protein synthesis.[460] As platelets are anucleate, an effect at megakaryocyte level would be required to explain any steroid mediated effect on platelet behaviour. It is possible to speculate that steroid hormones could for example program changes in the synthesis of adenylate cyclase or the stimulatory G protein by megakaryocytes. It is interesting to note that gonadal steroids have been shown to influence the adenylate cyclase system in a variety of tissues from other species. In particular, oestradiol has been shown to reduce cyclic AMP accumulation in CNS preparations from experimental animals.[461]

Another possible mechanism by which pregnancy may bring about changes in the platelet cyclic AMP accumulation is by exposure of platelets to vasodilatory prostaglandins synthesised by the placenta,[160,298,308,310] resulting in heterologous

desensitisation of adenylate cyclase. Desensitisation of platelet responses to inhibitory prostaglandins has been previously reported following both in vitro and in vivo exposure. Initial reports of desensitisation of platelet cyclic AMP accumulation in response to PGI_2 and PGD_2 in vitro suggested that this was agonist specific.[198] As a result of evaluation of infusion of PGI_2 as a therapeutic agent in conditions such as peripheral vascular disease, it became apparent that rebound platelet hyperactivity and desensitisation of platelet responses to PGI_2 in terms of inhibition of platelet behaviour and cyclic AMP accumulation occurred following prolonged exposure to PGI_2 in vivo.[263,264,462,463] Whilst most investigators examined only desensitisation to PGI_2 itself, some reported heterologous desensitisation with respect to PGD_2 following infusion of PGI_2 or PGE_1, but these reports did not include measurement of cyclic AMP.[462,464] One study designed to investigate the effects of intravenous PGI_2 infusion in rabbits showed that platelets were also refractory to the antiaggregatory effects of adenosine as well as prostaglandins, and it was proposed that impairment of the platelet adenylate cyclase regulatory subunit may be the underlying mechanism.[465] Since then, a large amount of data regarding both homologous and heterologous desensitisation of adenylate cyclase in many cell types has accumulated and has been reviewed.[466] Most studies involve cells other than platelets, although some have examined the mechanism of homologous and heterologous desensitisation of platelet adenylate cyclase following prolonged in vitro exposure of platelets to iloprost.[467-469] Homologous desensitisation was associated with reduced ^{3}H-iloprost binding to platelets,[467,468] but heterologous desensitisation, leading to reduced platelet membrane adenylate cyclase activity following stimulation with the adenosine analogue 5-N-ethylcarboxyaminoadenosine (NECA) also occurred.[469] Some evidence was presented that this may be due to loss or modification of the

α subunit of G_s.[469] This hypothesis is supported by work in other tissues which has implicated modification of both G_s and G_i in heterologous desensitisation of adenylate cyclase, but modification of the catalyst itself has not been entirely excluded in these systems.[466] Interestingly, a recent publication showed that in the S49 lymphoma cell line, a simultaneous increase in $G_{i\alpha}$ and decrease in $G_{s\alpha}$ expression resulted from prolonged stimulation of adenylate cyclase by either forskolin or by agonists at the β-adrenergic receptor.[470] There is evidence from this and other studies that cyclic AMP itself or cyclic AMP dependent protein kinase may be implicated in heterologous desensitisation.[466,470]

Although not classifiable as a desensitisation phenomenon, protein kinase C has also been implicated in the modulation of adenylate cyclase activity.[471,472] Some reports suggest that activation of protein kinase C prevents agonist induced inhibition of adenylate cyclase in platelet membranes by phosphorylation of G_i.[471] A more recent report, however suggests that the predominant effect of activation of protein kinase C is inhibition of adenylate cyclase.[472]

The results of the studies presented in this thesis would be in keeping with the development during pregnancy of heterologous desensitisation of platelet adenylate cyclase, mediated either by reduction or modification of G_s or the catalytic subunit of adenylate cyclase itself. Evidence of altered activity of G_i has not been found. A protein kinase C mediated reduction in adenylate cyclase activity could not be excluded.

Irrespective of the underlying biochemical mechanisms, the changes demonstrated in vitro in platelet sensitivity to the inhibitory prostaglandins may play a role in contributing to platelet activation in vivo during pregnancy. There is a great deal of evidence supporting a degree of in vivo platelet activation in healthy pregnancy and it has been open to question why this occurs in the context

of increased production of prostaglandins which inhibit platelet behaviour.[473] This can clearly be at least partly explained by the blunting of the platelet cyclic AMP response to such prostanoids.

The significance of the very early change in platelet cyclic AMP responses to prostaglandins during the gestational period also merits discussion. In other species, platelet activation has been shown to play a role in implantation, probably via mechanisms involving the secretion of growth factors such as PDGF and release of adhesion molecules such as fibronectin.[474] Furthermore, 5-HT released from platelet dense granules is thought to contribute to the maintenance of the corpus luteum in mammalian species such as cattle.[474] If similar mechanisms were involved in the early establishment of human pregnancy, down-regulation of platelet inhibitory mechanisms at this time could help to permit any platelet activity involved in the early establishment of pregnancy.

Another important determinant during pregnancy of platelet reactivity both in vivo and in vitro, however, may be the enhanced platelet aggregatory response to thromboxane and endoperoxides as demonstrated in Chapter 7. Whilst it is well established that platelet reactivity in vitro is enhanced particularly in response to agents with a thromboxane dependent component in their mechanism of action,[295,305,320-322,327] results of studies investigating platelet thromboxane production in vitro have been inconsistent.[292,327,329] The data presented in Chapter 7 suggest that platelet sensitivity to thromboxane during pregnancy may be a more important determinant of platelet responses to thromboxane dependent agonists in vitro than platelet thromboxane synthesis. It is possible that enhanced sensitivity to pro-aggregatory prostanoids also contributes to platelet activation in vivo, and increases in excretion of thromboxane metabolites may be a secondary phenomenon. Increased platelet reactivity towards endoperoxides may

also be an important contributing factor to the predominance of non responder status to thromboxane synthetase inhibitors in vitro in pregnant women. Furthermore, an interplay between effects of pro-aggregatory and inhibitory prostanoids is likely to be important in determining platelet responses both in vitro and in vivo. Such an interplay could offer an explanation for the later development during the gestational period of loss of efficacy of iloprost and PGD_2 as inhibitors of arachidonic acid induced dense granule release on the background of the cyclic AMP response to these agents being reduced from very early pregnancy.

The mechanisms underlying the enhanced platelet responses to pro-aggregatory prostanoids have not been investigated in the studies presented in this thesis and therefore must remain entirely speculative. Changes at the level of receptor, G protein, or phosphoinositol metabolism are each possible. The recent recognition of the likely linkage of the thromboxane/endoperoxide receptor to a unique G protein[132] and reports that protein kinase C activation modulates sensitivity to thromboxane may be of relevance.[85] It is of particular note that increased platelet reactivity to thromboxane/endoperoxides occurs during pregnancy when increased thromboxane production has been demonstrated in vivo.[309] Under such circumstances, current data on regulation of thromboxane mediated events, suggest that desensitisation of platelets to thromboxane would be expected.[85,454,455] Further investigation of the mechanisms regulating platelet responses to pro-aggregatory prostanoids in the context of pregnancy would therefore be of interest.

This thesis set out not only to examine the physiological changes in platelet responses to prostaglandins during pregnancy, but to explore the pathological changes relating to pre-eclampsia. Even without invoking a further intrinsic change in platelet behaviour in pre-eclampsia, increased platelet activation in vivo is readily

explicable by the well established changes in the balance of in vivo biosynthesis of pro-aggregatory and inhibitory prostanoids[158-160,298,310] occurring on the background of platelets "primed" by the pregnancy associated changes in platelet responses to these prostaglandins which have been demonstrated. The studies presented here have shown that, in established pre-eclampsia, there was no significant alteration, compared with normal pregnancy, either in platelet sensitivity to inhibitory prostanoids or in cyclic AMP accumulation in response to these agents. By contrast, pregnant women at risk of pre-eclampsia, who did not develop the condition in the index pregnancy, appeared to demonstrate reduced platelet sensitivity to inhibitory prostaglandins and reduced stimulated platelet cyclic AMP accumulation compared with healthy pregnant women. Furthermore, platelet cyclic AMP increments in response to iloprost and PGD_2 were reduced in women at risk when studied after the 6^{th} postnatal week in comparison with results from healthy women at non pregnant time points. Some caution must be exercised in interpreting these data, firstly because the number of subjects in the longitudinal study was small, and secondly because of the lack of pre-conceptional data in the women at risk of pre-eclampsia. Nevertheless, the consistency of the pattern of results during pregnancy suggest that genuine differences in platelet responses to inhibitory prostaglandins exist between women at risk and healthy women during the gestational period. It is quite possible however that differences demonstrated postnatally could result from platelet responses taking longer to revert to non pregnant patterns in women at risk of pre-eclampsia compared with healthy women. A larger study comparing women who have had pre-eclampsia with those who have had healthy pregnancies at a time more remote from pregnancy, for example 6 months postnatally, may help to resolve the issue of whether an intrinsically less responsive platelet adenylate cyclase system is

associated with pre-eclampsia. Until then, it could not be recommended that platelet sensitivity to inhibitory prostaglandins or platelet cyclic AMP responses could be used to predict pre-eclampsia.

It is of interest to speculate on the reasons why the findings of the cross-sectional and longitudinal studies were in contrast with one another regarding women with, or at risk of, pre-eclampsia. Statistical chance is a possible explanation as subject numbers were small in the longitudinal study. Secondary effects of the disease itself could, however, have influenced results in the cross-sectional study. For example, platelet activation in vivo as a result of pre-eclampsia could have resulted in only a subpopulation of platelets being available for study in vitro. Secondly, if prior exposure to inhibitory prostaglandins in vivo is important in regulating platelet sensitivity to these agents, reductions in prostaglandin synthesis by the vasculature of the placenta and/or damaged systemic vascular endothelium secondary to the establishment of pre-eclampsia[160,298,310,397-399] could offset any physiological desensitisation of platelet adenylate cyclase, thus masking any intrinsic reductions in the activity of the system in women at risk of pre-eclampsia. Perhaps platelet insensitivity to inhibitory prostanoids constitutes a risk factor for the disease which subsequently becomes manifest when placentation is abnormal.[390] This is, however, a highly speculative hypothesis.

As there is increasing interest in the potential therapeutic role of antiplatelet agents in pre-eclampsia, it is worth considering the implications of the data resulting from studies presented here for the prophylaxis or treatment of the condition with such drugs. Aspirin is the only agent being currently evaluated.[366,367,368,369] The demonstration of enhanced platelet sensitivity to pro-aggregatory prostanoids during pregnancy with reduced sensitivity to inhibitory

prostaglandins implies that the choice of a dose of aspirin with optimum selectivity for suppressing thromboxane production whilst preserving prostacyclin synthesis would have more importance in the context of pregnancy than in non obstetric practice. As complete selectivity is difficult to achieve with aspirin,[245] a good argument could be put forward for the evaluation of the combination of a thromboxane synthetase inhibitor with a thromboxane receptor blocker. Such combinations offer the potential of maintaining prostacyclin synthesis, whilst selectively antagonising the effects of thromboxane and possibly enhancing the availability of inhibitory prostanoids.[257,258] Although a number of thromboxane synthetase inhibitors and receptor blockers have undergone investigation in clinical trials in non obstetric practice,[251] there is no proof as yet of clinical superiority to aspirin and caution would be required in the investigation of any relatively new agents in the context of pregnancy.

Another possible approach to antiplatelet treatment in pre-eclampsia would be the use of agents which inhibit platelet behaviour by increasing cyclic AMP. The advantage of the use of such agents is their ability to inhibit platelet activation irrespective of the pathway by which this has been stimulated. Anecdotal reports of the use of prostacyclin in pre-eclampsia,[423,424,425] however have yielded disappointing results, probably partly because of its use in late stages of the disease but also because it was poorly tolerated. The demonstrated reduction in sensitivity of platelets to adenylate cyclase stimulators during pregnancy may increase the prostacyclin dose requirement for an antiplatelet effect, thus reducing efficacy and patient tolerance. Further desensitisation and rebound platelet hyperactivity would also represent likely problems.[263,264,462,463] Although some loss of sensitivity to phosphodiesterase inhibitors was also demonstrated in late pregnancy in the present studies, there was no difference in the platelet cyclic

AMP response to these agents in pregnant women, and so such drugs may represent a better means of manipulating cyclic AMP dependent inhibition of platelet behaviour in the context of pregnancy. There is also a future potential for selectively inhibiting the platelet cyclic AMP phosphodiesterase enzyme whilst having no effect on those in cardiac and smooth muscle,[209,210] thus offering the possibility of inhibition of platelet behaviour without the cardiovascular effects of adenylate cyclase stimulators such as prostacyclin. Such drugs, however, are in early phases of evaluation. It is unlikely that they will be available for clinical use in the near future and caution in their investigation in pregnancy would in any case be imperative. It is interesting to note that stimulators of guanylate cyclase such as nitric oxide and adenylate cyclase stimulators synergise in terms of their inhibitory effects on platelets,[182] but preliminary reports suggest that synergism of their relaxant effects on smooth muscle does not occur.[475] This may offer the possibility of using combinations of these agents to limit the adverse cardiovascular effects of drugs such as prostacyclin or its analogues whilst enhancing antiplatelet effects. The effects on platelets of guanylate cyclase stimulators alone and in combination with cyclic AMP manipulators would therefore be worthy of study in healthy pregnancy and in PIH.

Finally, in conclusion, this work has shown that study of the stimulus response coupling mechanisms which regulate platelet responses to agents such as prostaglandins can be fruitful in elucidating the mechanisms underlying alterations in platelet behaviour during pregnancy. It is likely that further studies, not only of the adenylate cyclase system but also of other platelet signal transduction systems would be of value in contributing to a fuller understanding both of physiological changes in primary haemostasis during pregnancy and of the pathophysiology of pre-eclampsia.

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APPENDICES

APPENDIX 1.

CALCULATION OF EXTENT OF PLATELET RELEASE REACTION

The extent of the platelet release reaction was measured using the assay of ^{14}C-5-HT release described in Chapter 2. % ^{14}C-5-HT was expressed as a percentage of ^{14}C-5-HT taken up by platelet dense granules. The following formula was used for each experimental sample:

$$\% \text{ Release} = \frac{\text{sample count - saline count}}{\text{T' count - saline count}} \times 100$$

Sample count = dpm in 50ul of the supernatant platelet poor plasma of an experimental sample in which the release reaction was induced by an agonist e.g. arachidonic acid in the presence or absence of an inhibitor.

Saline count = the dpm in 50ul of the supernatant platelet poor plasma of a sample to which saline/sodium carbonate had been added in place of arachidonic acid.

$$\text{T' count} = \frac{\text{"Totals" dpm x volume of PRP in each sample}}{\text{total volume of each sample}}$$

"Totals" dpm = dpm in 50ul of untreated ^{14}C-5-HT labelled PRP.

All dpm were corrected for background count, measured in vials containing only liquid scintillant.

WORKED EXAMPLE

Consider an experiment where 460ul of ^{14}C-5-HT labelled PRP was incubated with the following reagents, and reactions were stopped by the addition of 50ul of aspirin.

Sample No	Pre-addition	Agonist
1	20ul saline	20ul $NaCl/NaCO_3$
2	20ul saline	20ul arachidonate
3	20ul iloprost	20ul arachidonate

The following ^{14}C counts were obtained:

			Mean	Background Corrected
Background counts:	21.3	20.4	20.85	-
Sample 1	359.0	359.6	359.3	338.45
Sample 2	1629.0	1801.8	1715.4	1694.55
Sample 3	359.4	360.2	359.8	338.95
"Totals"	2977.7	3065.7	3021.7	3000.85

$$T' = \frac{3000.85 \times 460ul}{550ul} = 2509.8$$

T' count -saline count = 2509.8 - 338.45 = 2171.35

$$\% \text{ release in sample 2} = \frac{1694.55 - 338.45}{2171.35} \times 100 = 62.45\%$$

$$\% \text{ release in sample 3} = \frac{338.95 - 338.45}{2171.35} \times 100 = 0.02\%$$

APPENDIX 2
CALCULATION OF % CONVERSION ^{3}H-ADENINE TO ^{3}H-CYCLIC AMP.

1) Subtract mean background cpm channel 1 (^{3}H) and channel 2 (^{14}C) from all samples and "totals" and mean all duplicates.

2) Calculate uptake of ^{3}H-adenine by platelets:

$$^3\text{H-adenine uptake} = 100 - \left\{ \frac{\text{cpm in "uptake tubes"} \times 10^* \times 100}{\text{cpm in } ^3\text{H "totals" tubes}} \right\}$$

* multiply by 10 to correct for counting 50ul of plasma in uptake samples.

3) Correct ^{3}H "totals" for uptake:

$$\text{Uptake corrected } ^3\text{H totals} = \text{cpm in } ^3\text{H "totals"} \times \% \text{ uptake}$$

4) Correct sample counts for crossover:

(i) crossover of ^{14}C to channel 1 =

$$\frac{\text{channel 1 cpm crossover control}}{\text{channel 1 cpm} + \text{channel 2 cpm crossover control}} \times 100$$

(ii) adjust channel 1 and channel 2 counts in samples for crossover:

$$\text{True } ^{14}\text{C count} = 100 \times \frac{\text{actual channel 2 cpm}}{100 - \% \text{ crossover}}$$

$$^{14}\text{C counts in channel 1} = \text{true } ^{14}\text{C count} - \text{actual channel 2 cpm}$$

$$\text{True } ^3\text{H count} = \text{actual channel 1 count} - ^{14}\text{C counts in channel 1.}$$

5) Calculate cyclic AMP recovery in each sample:

$$\% \text{ recovery} = \frac{\text{true } ^{14}\text{C count in sample}}{\text{cpm in } ^{14}\text{C "totals"}} \times 100$$

6) Correct ^{3}H counts for recovery of cyclic AMP:

$$\text{recovery corrected } ^3\text{H cpm} = \frac{\text{true } ^3\text{H cpm} \times 100}{\% \text{ recovery}}$$

7) Calculate % conversion ^{3}H-adenine to ^{3}H-cyclic AMP:

$$\frac{\text{recovery corrected } ^3\text{H cpm in sample} \times 100}{\text{uptake corrected } ^3\text{H "totals"}}$$

WORKED EXAMPLE OF CALCULATION OF PLATELET CYCLIC AMP RESULTS

Consider an assay with the following data output from the scintillation counter. The procedure outlined above has been applied to calculate % conversion ^{3}H-adenine to ^{3}H-cyclic AMP.

Test Data:

	Channel 1	Channel 2
Backgrounds	26.1	13.2
	23.8	10.4
^{14}C totals	264.07	535.18
	273.16	555.09
^{14}C crossover controls	3569.75	7740.56
	3201.91	6996.16
^{3}H totals	858288.51	
	883479.75	
^{3}H uptake	12485.5	
	11894.6	
Sample 1	383.6	387.3
Sample 2	2998.2	282.6

1) Background corrected counts:

	Channel 1	Channel 2
^{14}C totals	239.12	523.38
	248.21	543.29
^{14}C crossover controls	3544.8	7728.76
	3176.96	6984.36
^{3}H totals	858263.56	
	883454.8	
^{3}H uptake	12460.55	
	11869.65	
Sample 1	358.65	375.5
Sample 2	2973.25	270.8

2) Uptake of ^{3}H-adenine by platelets =

$$100 - \left\{\frac{12165.1}{870859.18} \times 10 \times 100\right\}$$

$$= 100 - 13.97$$
$$= 86.03\%$$

3) Uptake corrected ^{3}H totals $= \frac{870859.18 \times 86.03}{100}$

$$= 749200.15$$

4) (i) crossover of ^{14}C to channel 1 =

$$\frac{3360.88}{7356.56 + 3360.88} \times 100$$

$$= 31.36\%$$

(ii) true ^{14}C count sample 1 $= \frac{100 \times 375.5}{68.64} = 547.06$

sample 2 $= \frac{100 \times 270.8}{68.64} = 394.52$

^{14}C counts in channel 1, sample 1 = 547.06 - 375.5 = 171.56

sample 2 = 394.52 - 270.8 = 123.72

true ^{3}H counts, sample 1 = 358.65 - 171.56 = 187.09

sample 2 = 2973.25 - 123.72 = 2849.53

5) Cyclic AMP recovery =

sample 1, $\frac{547.06 \times 100}{533.34 + 243.67} = 70.4\%$

sample 2, $\frac{394.52 \times 100}{533.34 + 243.67} = 50.77\%$

6) recovery corrected ^{3}H counts =

sample 1, $\frac{187.09 \times 100}{70.4} = 265.75$

sample 2, $\frac{2849.53 \times 100}{50.77} = 5612.63$

7) % conversion ^{3}H-adenine to ^{3}H-cyclic AMP =

sample 1, $\frac{265.75 \times 100}{749200.15} = 0.0355\ \%$

sample 2, $\frac{5612.63 \times 100}{749200.15} = 0.7491\%$

APPENDIX 3
CALCULATION OF PLATELET AGGREGATION IN WHOLE BLOOD

1) Mean all duplicate platelet counts

2) Subtract mean count at time x after addition of agonist from mean count at time 0 (equivalent to platelet count before the addition of the agonist)

3) Divide the difference in time x and time 0 counts by the time 0 count and express as a percentage.

A worked example based on the following raw data is shown overleaf.

Times	Saline	0.3uM U46619
0 minutes	170 176	178 182
15 seconds	164 169	78 75
30 seconds	160 166	46 43
45 seconds	168 161	38 36
1 minute	172 167	42 41
2 minutes	158 164	35 34
4 minutes	152 158	18 20
6 minutes	135 140	10 13

Time	Saline			0.3uM U46619		
	Mean count	Count t_0-t_x	% agg	Mean count	Count t_0-t_x	% agg
0 min	173			180		
15 sec	166	7	4.0	76	104	57.5
30 sec	163	10	5.8	44	136	75.3
45 sec	164	9	5.2	37	143	79.4
1 min	169	4	2.3	41	139	76.9
2 min	161	12	6.9	34	146	80.8
4 min	155	18	10.4	19	161	89.4
6 min	137	36	20.8	11	169	93.6

APPENDIX 4: PROTOCOL FOR DRUG INCUBATIONS FOR MEASUREMENT OF PLATELET CYCLIC AMP

Time	Tube	Add	Time	Tube	Add	Time	Tube	Add
0.00	1	PRP	6.00	-	-	12.00	17	PRP
0.25	2	PRP	6.25	-	-	12.25	18	PRP
0.50	3	PRP	6.50	-	-	12.50	19	PRP
0.75	4	PRP	6.75	-	-	12.75	20	PRP
1.00	5	PRP	7.00	13	D_2 0.5	13.00	13	TCA
1.25	6	PRP	7.25	14	D_2 0.5	13.25	14	TCA
1.50	7	PRP	7.50	15	D_2 5.0	13.50	15	TCA
1.75	8	PRP	7.75	16	D_2 5.0	13.75	16	TCA
2.00	9	PRP	8.00	1	TCA	14.00	17	I 0.5
2.25	10	PRP	8.25	2	TCA	14.25	18	I 0.5
2.50	11	PRP	8.50	3	TCA	14.50	19	I 5.0
2.75	12	PRP	8.75	4	TCA	14.75	20	I 5.0
3.00	5	I 0.5	9.00	5	TCA	15.00	21	PRP
3.25	6	I 0.5	9.25	6	TCA	15.25	22	PRP
3.50	7	I 5.0	9.50	7	TCA	15.50	23	PRP
3.75	8	I 5.0	9.75	8	TCA	15.75	24	PRP
4.00	9	I 50.0	10.00	9	TCA	16.00	25	PRP
4.25	10	I 50.0	10.25	10	TCA	16.25	26	PRP
4.50	11	$D_2$0.05	10.50	11	TCA	16.50	27	PRP
4.75	12	$D_2$0.05	10.75	12	TCA	16.75	28	PRP
5.00	13	PRP	11.00	-	-	17.00	21	I 50
5.25	14	PRP	11.25	-	-	17.25	22	I 50
5.50	15	PRP	11.50	-	-	17.50	23	$D_2$0.05
5.75	16	PRP	11.75	-	-	17.75	24	$D_2$0.05

The drug incubation protocol shown here and overleaf was used for the experiments described in Chapters 4&5. I = iloprost, D_2 = prostaglandin D_2, F = forskolin, AA = arachidonic acid. All times are in minutes. Final concentrations of reagents are indicated in the additions columns. For iloprost all final concentrations are in ng/ml, for PGD_2 and forskolin in ug/ml, and AA was at a final concentration of 1mM. The proportions of additions to each tube were as follows: 450ul of ^{3}H-adenine labelled PRP, 50ul of drug solutions, and 2ml TCA. Each drug solution was added in a volume of 25ul. Those pre-added to the tubes are detailed overleaf.

Time	Tube	Add	Time	Tube	Add	Time	Tube	Add
18.00	25	D_2 0.5	24.00	25	TCA	30.00	-	-
18.25	26	D_2 0.5	24.25	26	TCA	30.25	-	-
18.50	27	D_2 5.0	24.50	27	TCA	30.50	-	-
18.75	28	D_2 5.0	24.75	28	TCA	30.75	-	-
19.00	-	-	25.00	31	PRP	31.00	-	-
19.25	-	-	25.25	32	PRP	31.25	-	-
19.50	-	-	25.50	33	PRP	31.50	-	-
19.75	-	-	25.75	34	PRP	31.75	-	-
20.00	17	TCA	26.00	35	PRP	32.00	-	-
20.25	18	TCA	26.25	36	PRP	32.25	-	-
20.50	19	TCA	26.50	37	PRP	32.50	-	-
20.75	20	TCA	26.75	38	PRP	32.75	-	-
21.00	-	-	27.00	31	F 4.0	33.00	31	TCA
21.25	-	-	27.25	32	F 4.0	33.25	32	TCA
21.50	29	PRP	27.50	33	AA 1.0	33.50	33	TCA
21.75	30	PRP	27.75	34	AA 1.0	33.75	34	TCA
22.00	-	-	28.00	35	AA 1.0	34.00	35	TCA
22.25	-	-	28.25	36	AA 1.0	34.25	36	TCA
22.50	-	-	28.50	37	AA 1/l 5	34.50	37	TCA
22.75	-	-	28.75	38	AA 1/l 5	34.75	38	TCA
23.00	21	TCA	29.00	-	-			
23.25	22	TCA	29.25	-	-			
23.50	23	TCA	29.50	29	TCA			
23.75	24	TCA	29.75	30	TCA			

The following were pre-added to tubes prior to starting the incubation protocol. In each case final concentrations are stated.
Tubes 1 & 2: 50ul saline; Tubes 3 & 4: 25ul AH-P719,100uM and 25μl saline; Tubes 5-16 and 33 & 34: 25ul saline
Tubes 17-28, and 31 & 32: 25ul AH-P719, 100uM
Tube 29: dazmegrel diluent; Tube 30: AA diluent; Tubes 35 & 36: dazmegrel, 500uM
Tubes 37 & 38: nil.

APPENDIX 5.
PROTOCOL FOR DRUG INCUBATIONS FOR MEASUREMENT OF THE EFFECT ON PLATELET CYCLIC AMP OF PGE_2

Time (min)	Tube	Add	Time (min)	Tube	Add	Time (min)	Tube	Add
0.00	1	PRP	8.00	1	TCA	13.00	11	PGE_2 10^{-7}M
0.25	2	PRP	8.25	2	TCA	13.25	12	PGE_2 10^{-7}M
0.50	3	PRP	8.50	3	TCA	13.50	12	PGE_2 10^{-7}M
0.75	4	PRP	8.75	4	TCA	13.75	14	PGE_2 10^{-7}M
1.00	5	PRP	9.00	5	TCA			
1.25	6	PRP	9.25	6	TCA			
1.50	7	PRP	9.50	7	TCA			
1.75	8	PRP	9.75	8	TCA			
2.00	9	PRP	10.00	9	TCA			
2.25	10	PRP	10.25	10	TCA			
2.50	-	-	10.50	-	-			
2.75	-	-	10.75	-	-			
3.00	5	Saline	11.00	11	PRP	19.00	11	TCA
3.25	6	Saline	11.25	12	PRP	19.25	12	TCA
3.50	7	Saline	11.50	13	PRP	19.50	13	TCA
3.75	8	Saline	11.75	14	PRP	19.75	14	TCA
4.00	9	PGE_2 10^{-7}M	12.00					
4.25	10	PGE_2 10^{-7}M	12.25					
4.50	-	-	12.50					
4.75	-	-	12.75					

The protocol is detailed for drug incubations with PRP for the experiments described in Chapter 6, designed to determine the effect on platelet cyclic AMP of PGE_2. The protocol shown is that used in the presence of the phosphodiesterase inhibitor. The proportions of additions were as follows: 450μl ^{3}H-adenine labelled PRP, 50μl drug solutions, 2ml TCA. During the timed protocol, all drug solutions were added in a volume of 20μl. Those pre-added to the tubes are detailed overleaf.

Pre-additions to Tubes.

Tubes 1 & 2	50ul saline
Tubes 3 & 4	40ul saline and 10ul AH-P719 (100μM)
Tubes 5 & 6	20ul iloprost (5ng/ml) and 10ul AH-P719 (100 uM)
Tubes 7 & 8	20ul iloprost (50ng/ml) and 10ul AH-P719 (100 uM)
Tubes 9 & 10	20ul saline and 10ul AH-P719 (100 uM)
Tubes 11 & 12	20ul iloprost (5ng/ml) and 10ul AH-P719 (100 uM)
Tubes 13 & 14	20ul iloprost (50ng/ml) and 10ul AH-P719 (100 uM)

All concentrations stated are final concentrations.

For the measurement of the effect on platelet cyclic AMP of PGE_2 in the absence of AH-P719, a very similar but more simple protocol was used. In this case, the assay consisted of only 8 tubes. The timing of additions was as for the first 8 tubes in the protocol on the foregoing page. Pre-additions were as follows:

Tubes 1 & 2	50ul saline
Tubes 3 & 4	25ul iloprost (5ng/ml)
Tubes 5 & 6	25ul saline
Tubes 7 & 8	25ul iloprost (5ng/ml)

25ul of drug solutions were then added during the timed protocol. Saline was added to Tubes 3 and 4 at 2.5 and 2.75 minutes respectively, and PGE_2 to tubes 5-8 at the times shown on the protocol on the previous page.

PUBLICATIONS

The following is a current list of publications which have resulted from the work presented in this thesis:

Full Papers.

Platelets in Normal and Hypertensive Pregnancy : A Review E.H. Horn. Platelets, 2 : 183-195, 1991.

A cross sectional study of platelet cyclic AMP in normal and hypertensive pregnancy. E.H. Horn, J. Cooper, E.Hardy, S.Heptinstall, P.C. Rubin. Clinical Science, 80: 549-558, 1991.

Abstracts.

Preliminary studies on the effects of cyclic AMP manipulators in vitro in pregnancy. Horn EH, Heptinstall S, and Rubin PC. Platelets, 1: 51, 1990.

Platelet adenylate cyclase activity in normal and hypertensive pregnancy. Horn EH, Cooper J, Hardy E, Heptinstall S, Rubin PC. British Journal of Haematology, 76, Suppl 1: 32, 1990.

Platelet Sensitivity to the Endoperoxide Analogue U46619 during Pregnancy. E.H. Horn, J. Cooper, E. Hardy, S. Heptinstall, P.C. Rubin. British Journal of Haematology, 77, Suppl 1: 79, 1991.

U46619 Induced Platelet Aggregation in Whole Blood During Pregnancy. E.H. Horn, J Cooper, E Hardy, S Heptinstall, P.C. Rubin. Thrombosis and Haemostasis, 65 (6): 1229, 1991.

A Longitudinal Study of Platelet Cyclic AMP in Healthy Primigravidae and in Pregnant Women at Risk of Pre-eclampsia. E.H. Horn, J Cooper, E Hardy, S Heptinstall, P.C. Rubin. British Journal of Haematology 80 (Supplement 1): 10, 1992.