VASCULAR AND PLATELET EICOSANOIDs IN THE AETIOLOGY AND TREATMENT OF CARDIOVASCULAR DISEASE

A thesis presented to the University of London in part fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

by

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ABSTRACT

Methods for the study of receptor-linked prostacyclin (PGI$_2$) synthesis in blood vessels (from the rat, rabbit and man) and thromboxane A$_2$ (TXA$_2$) synthesis in human platelets were developed. The role of calcium, G proteins and protein kinase C in mediating eicosanoid synthesis in these tissues was also studied. These methods were applied in the investigation of: 1) PGI$_2$ synthesis following hypothermic storage of blood vessels in preservation solutions used in organ transplantation, 2) the site of action of drugs known to modulate eicosanoid synthesis; for example, phosphodiesterase inhibitors (PDEIs), non-steroidal anti-inflammatory drugs (NSAIDs) and copper chelators, 3) the mechanisms underlying altered synthesis of vascular PGI$_2$ in diabetes mellitus (DM) and hepatic portal hypertension (HPH) in the rat. There were marked variations in the characteristics of receptor-linked PGI$_2$ release between species (human, rat and rabbit) whereas receptor-linked PGI$_2$ release in different arteries and veins of the same species was remarkably similar. Hypothermic storage of blood vessels (rat, rabbit and human) in preservation solutions exerted minimal effects on PGI$_2$ synthesis, a finding of relevance to transplant surgery. Apart from their accepted sites of action, NSAIDs (cyclooxygenase inhibitors) and PDEIs (cAMP elevators) were also found to inhibit eicosanoid synthesis at the signal transduction level. Copper chelators inhibited eicosanoid synthesis at the level of cyclooxygenase and lipoxygenase. In rats with DM, there were marked differential alterations of receptor-linked PGI$_2$ synthesis in different vessels and in other non-vascular tissues (gastrointestinal tract, urinary bladder, trachea). In rats with HPH,
there were marked changes in receptor-linked PGI$_2$ release by the aorta and mesenteric vasculature, indicating an adaptive series of events involving PGI$_2$. The systems developed in this thesis are a convenient means of investigating not only eicosanoid synthesis but also drug action and the aetiology of vascular disease.
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<tr>
<td>AA</td>
<td>Arachidonic acid</td>
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<tr>
<td>ACE(I)</td>
<td>Angiotensin converting enzyme (inhibitor)</td>
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<tr>
<td>Ach</td>
<td>Acetyl choline</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>Ang</td>
<td>Angiotensin</td>
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<tr>
<td>[Ca^{2+}]_e</td>
<td>Extracellular calcium</td>
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<td>[Ca^{2+}]_i</td>
<td>Intracellular calcium</td>
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<td>cAMP</td>
<td>Adenosine-3'5' cyclic monophosphate</td>
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<td>cGMP</td>
<td>Guanosine-3'5' cyclic monophosphate</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>DAG (DG)</td>
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<td>Diethyldithiocarbamic acid</td>
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<td>DHLA</td>
<td>Dihomo-γ-linolenic acid</td>
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<td>DM</td>
<td>Diabetes mellitus</td>
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<td>DMDTCA</td>
<td>Dimethyldithiocarbamic acid</td>
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<td>EDRF</td>
<td>Endothelium-derived relaxing factor</td>
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<td>EDTA</td>
<td>Ethylene glycol tetraacetic acid</td>
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<td>EFA</td>
<td>Essential fatty acid</td>
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<td>EGTA</td>
<td>Ethylenetetraacetic acid</td>
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<td>GC MS</td>
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<td>G protein</td>
<td>GTP binding protein</td>
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<td>Guanosine triphosphate</td>
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<td>High density lipoprotein</td>
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<td>HETE</td>
<td>Hydroxyeicosatetraenoic acid</td>
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<td>HOTMECs</td>
<td>Human omental tissue microvascular endothelial cells.</td>
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<td>HPETE</td>
<td>Hydroperoxyeicosatetraenoic acid</td>
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<td>HPH</td>
<td>Hepatic portal hypertension</td>
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<td>5-HT</td>
<td>5-Hydroxytryptamine</td>
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<td>H7</td>
<td>Isoquinolinylsulfonylmethylpiperazine.</td>
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<td>HUVECs</td>
<td>Human umbilical vein endothelial cells.</td>
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<td>Isobutylmethylxanthine</td>
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<td>IHD</td>
<td>Ischaemic heart disease</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>myo-inositol 1,4,5-trisphosphate</td>
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<td>KPS</td>
<td>kidney preservation solution</td>
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<td>KRB</td>
<td>Kreb's Ringer bicarbonate buffer</td>
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<td>LDL</td>
<td>low density lipoprotein</td>
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<td>LT</td>
<td>leukotriene</td>
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<td>MEM</td>
<td>Minimum Essential Medium</td>
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<tr>
<td>NaF</td>
<td>sodium fluoride</td>
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<td>NDGA</td>
<td>nordihydroguaretic acid</td>
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<td>NSAID</td>
<td>non-steroidal antiinflammatory drug</td>
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<tr>
<td>PDBU</td>
<td>phorbol 12,13-dibutyrate</td>
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<tr>
<td>PEI</td>
<td>polyethylimine cellulose</td>
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<td>PDE(I)</td>
<td>phosphodiesterase (inhibitor)</td>
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<td>PI</td>
<td>phoshatidyl inositol</td>
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<td>PG</td>
<td>prostaglandin</td>
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<tr>
<td>PK</td>
<td>protein kinase</td>
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<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>PLC</td>
<td>phospholipase C</td>
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<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
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<td>PRP</td>
<td>platelet rich plasma</td>
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<td>PVD</td>
<td>peripheral vascular disease</td>
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<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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<tr>
<td>TETD</td>
<td>triethylthiuram disulphide</td>
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<td>TLC</td>
<td>thin layer chromatography</td>
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<td>thromboxane</td>
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<td>UW</td>
<td>University of Wisconsin Solution</td>
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<td>VSMC</td>
<td>vascular smooth muscle cells</td>
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CHAPTER 1. INTRODUCTION

1.1. Historical overview of eicosanoid research

In the 1930s, seminal fluid was found to be a potent in vitro stimulator of mammalian smooth muscle contractility and to markedly reduce blood pressure, in vivo (Kurzok and Lieb, 1930; Goldblatt, 1933, 1935; von Euler, 1935). Since von Euler (1937) also found that extracts of prostate glands had similar pharmacological effects as seminal fluid and were lipid in nature, these bioactive substance(s) were christened prostaglandins (PGs). It is ironic that von Euler also studied extracts of seminal vesicles which he found to be bioactive and as such termed vesiglandins (von Euler, 1937). Thus, if it had been decided to opt for this latter term, we would now be using such terms as vesanoids and vesicyclin! In 1940, Kellaway and Trethewie, reported the existence of a substance that was released in response to anaphylaxis and contracted airways tissue. This substance, termed "slow reacting substance of anaphylaxis", we now know to be leukotrienes (LTs; Samuelsson, 1982). In the same decade Burr and Burr (1930) established the existence of essential fatty acids (EFA), which over 30 years later were to be recognised as precursors for all eicosanoids.

After their discovery, little work was carried out on PGs until the late 1950s when Bergstrom (1957) isolated, in a pure crystalline form, two primary PGs: PGE\(_1\) and PGF\(_{1\alpha}\). In the 1960s, chemical characterisation of PGs was made possible by two major methodological advances: gas-liquid chromatography (GLC) and mass-spectrometry (MS). Thus, it was established that all PGs contain 20 carbon atoms, a 5-membered ring and have the same basic carbon skeleton (Bergstrom et al., 1968; fig. 1.1.). In 1964, Van Dorp et al. and Bergstrom et al. described the biosynthesis of PGs from dihomo-\(\gamma\)-linolenic acid
(DGLA) and arachidonic acid (AA) when incubated with homogenates of sheep vesicular glands. The transformations involved peroxidation, the incorporation of molecular oxygen and cyclisation of the fatty acids to produce PGE$_1$ and PGE$_2$. These unique transformations led to the term cyclooxygenase to describe the enzyme that catalysed these reactions. It was also determined that PGs can be of the 1, 2 or 3 series depending on the fatty acid from which they are derived: monoenoic (1 series), dienoic (2 series) and trienoic (3 series) PGs are derived from DGLA, AA, and eicosapentaenoic acid (EPA), respectively (Bergstrom et al., 1968; fig. 1.1.). The PGs of the 1, 2 and 3 series possess markedly different properties which has led to therapeutic attempts to alter PG synthesis by the manipulation of dietary intake of oils (e.g. fish oil, evening primrose oil).

Throughout the 1960's a large number of different PGs were characterised (Bergstrom et al., 1968). It was also found that PGs are potent modulators of smooth muscle activity, alter metabolic functions (including lipid and carbohydrate metabolism) and exert effects on neurotransmission (Bergstrom et al., 1968). Since then, PGs have been shown to be involved in the activity of virtually every mammalian cell type.

In the 1970's, there were considerable breakthroughs in eicosanoid research. Although theoretically postulated to exist by Samuelsson in 1965, the endoperoxides PGG and PGH were actually isolated in 1973 (Hamberg and Samuelsson 1973, Nugteren and Hazelhof, 1973; Fig. 1.2.). These endoperoxides are the immediate precursors of both the thromboxanes (TXs) and PGs (Hamberg et al., 1975; fig.1.2.). Cyclooxygenase was also isolated and purified by several different groups (Miyamoto et al., 1976; Hemler et al., 1976). It was also
Fig. 1.1. Di-homo-γ-linolenic acid and arachidonic acid are converted into PGs of the 1 series (one double bond) and 2 series (two double bonds), respectively. These fatty acids and their precursor, linoleic acid, are members of the family of n-6 fatty acids, characterised by an end segment of 6 carbons (at the opposite end from the -COOH). Eicosapentaenoic acid, coming from α-linolenic acid (n-3 family) is converted to 3-series PGs (three double bonds). The characteristic end segments of n-6 and n-3 families are represented in the figure by thick lines.
found that haem (and the iron it contains) is required for cyclooxygenase activity (Hemler and Lands, 1976). Thus, via iron-mediated redox reactions, cyclooxygenase elicits the abstraction of hydrogen atoms from the fatty acid forming radical structures followed by the incorporation of two oxygen molecules (fig. 1.2. Deby, 1988). A cyclic molecule is produced, PGG, which under the peroxidasic activity of cyclooxygenase is reduced to an endoperoxide, PGH, the immediate precursor of PGs and thromboxanes, depending on the synthase/isomerase present in any given tissue (fig. 1.2.).

Lipoxygenase was then discovered in platelets and leucocytes (Nugteren, 1975; Borgeat and Samuelsson, 1979). Lipoxygenase is also a haem-containing enzyme, which abstracts a hydrogen atom and incorporates an oxygen molecule into fatty acids at the C12, C5 or C15 position, producing hydroperoxyeicosatetraenoic acid (HPETE; fig. 1.2. Deby, 1988). HPETE can either be converted to leukotrienes (LT) by LT synthases (principally in leucocytes) or spontaneously degrade to hydroxyeicosatetraenoic acid (HETE), liberating at the same time a superoxide ion (Deby, 1988). Apart from iron, copper has been proposed as being a co-factor of cyclooxygenase and lipoxygenase (Fernandez-Madrid, 1989). One of the objectives of this thesis was to explore the role of copper in mediating the activity of these enzymes in platelets (see introduction of chapter 4 for a more comprehensive rationale for these studies).

In the mid-1970s, prostacyclin (PGI$_2$) was discovered. In a now classic series of experiments, it was found that blood vessels, in vitro, released a substance that was a vasodilator, a potent inhibitor of platelet aggregation, had a very short half-life and whose synthesis was inhibited by aspirin (a cyclooxygenase inhibitor;
Moncada et al., 1977; Bunting et al., 1976; Gryglewski et al., 1977). This hitherto "undiscovered" PG (termed PGX in its neonatal period) was subsequently characterised and called prostacyclin (PGI₂ [now epoprostenol]) due to its unique molecular structure (see fig. 1.2.). In further studies it was suggested that vascular endothelial cells are the main source of PGI₂ in blood vessels (Moncada et al., 1977; Boeynaems et al., 1985). Around the same time it was established that platelets synthesise thromboxane A₂ (TXA₂; Hamberg et al., 1975; Hammarstrom and Falardeau, 1977). TXA₂ was found to have a short half-life, to be a potent stimulator of platelet aggregation and to be vasoconstrictor (Moncada and Vane, 1979). Since blood vessels and platelets synthesise large quantities of eicosanoids with diametrically opposite actions, an intense era of research was initiated, aimed primarily at evaluating the interaction of PGI₂ and TXA₂ in the aetiology of cardiovascular disease (see section 1.5.).

Although it has long been recognised that phospholipase A₂ (PLA₂) is a key enzyme that limits PG formation (Vogt et al., 1969), it was not until the late 1970s that the importance of PLA₂ was fully recognised (Flower and Blackwell, 1976). AA is stored, incorporated as an ester at the sn-2 position (fig. 1.3.) of phospholipids (PLs): phosphatidyl serine, phosphatidyl choline, phosphatidyl ethanolamine or phosphatidyl inositol in biological membranes (Irvine, 1982; Waite, 1987). Under the influence of specific stimulation and intracellular regulation (see sections 1.2 and 1.3) AA is liberated, principally by the hydrolytic action of PLA₂ (Irvine, 1982; Hong and Deykin, 1982). It has also been suggested that AA is derived from diacyl glycerol (DG) by the action of DG lipase (McKean et al., 1981) as well as a PLA₁-lysophospholipase pathway (Martin and Wysolmerski,
"Liberated" AA then undergoes bio-transformations to generate eicosanoids as described above. Several groups established that corticosteroids inhibit eicosanoid formation (Greaves and MacDonald-Gibson, 1972; Kantrowitz et al., 1975; Gryglewski et al., 1975). It was established that this effect of corticosteroids requires receptor occupancy and de novo protein synthesis (Feldman et al., 1972; Thompson and Lippman, 1974; Flower and Blackwell, 1979; Russo-Marie and Duval, 1979). This protein (macrocortin or lipocortin) was isolated and characterised soon after (Blackwell et al., 1980; Hirata et al., 1980; Rothhut et al., 1983; Di Rosa et al., 1984; Flower, 1988).

1.2. Receptor-linked eicosanoid synthesis and the role of calcium

During the last decade attention has focussed on the intracellular mechanisms that govern eicosanoid synthesis. It has long been recognised that agonists which influence the function of any given tissue (e.g. vascular contractility; platelet aggregation) also modulate the local synthesis of eicosanoids. A plethora of vasoactive agents (e.g. histamine, bradykinin, serotonin [5-hydroxytryptamine; 5-HT], LTs, angiotensin II, noradrenaline, purinomimetics and TXA₂ analogues) stimulate the release of PG₂ from intact blood vessels as well as cultured endothelial and vascular smooth muscle cells (for review see Jeremy et al., 1988a). Since the relative potency of these agonists on PG release in blood vessels was similar to the relative potencies on contractility / relaxation [Godfraind et al., 1982]), it was postulated that there was a signal transduction pathway common to both vascular contraction and endogenous PG₂ synthesis (Jeremy et al., 1985a).
The above relationship appears to hold true in other smooth muscle types. For example, in the rat urinary bladder and penis, parasympathomimetics stimulate the synthesis of PGI$_2$, PGE$_2$ and PGF$_{2\alpha}$ (Jeremy et al., 1986a,b), an action blocked by muscarine-receptor antagonists (atropine, gallamine and ipratropium bromide; Jeremy et al., 1986a,b). Again, the relative potency of agonists and antagonists on both contractility / relaxation and eicosanoid synthesis was similar, consolidating the concept of a direct relationship (or common pathway) between the two events in smooth muscle, per se. Furthermore, the fact that all three PGs studied were stimulated to an equal extent pointed to the activation of a phospho-lipase, rather than of cyclooxygenase or of individual synthases or isomerase (Jeremy et al., 1986a,b).

Despite the above apparent general relationship between contractors of smooth muscle and PGI$_2$ synthesis in the rat aorta, there appears to be considerable inter-species variation with regard to the receptor type linked to PGI$_2$ synthesis in blood vessels. For example, acetylcholine (Ach) has no effect on PGI$_2$ synthesis in the rat aorta (Jeremy et al., 1985a) but has been reported to be a potent stimulator in the rabbit aorta (Boeynaems et al., 1985). Similarly, histamine is a potent stimulator of PGI$_2$ synthesis in cultured endothelial (bovine and human) cells (Baezinger et al., 1981) whereas this agonist has no effect on PGI$_2$ release by the rat aorta (Jeremy et al., 1988a). In this context, it is often assumed that agonist-stimulated PGI$_2$ release from whole vessels reflects production by the endothelium. This latter assumption was based on the study of Moncada et al. (1977) and later by Boeynaems et al. (1985), who showed a marked reduction of PGI$_2$ output by aortic rings denuded of
endothelium. The aorta, the most widely used vessel for the study of agonist - PGI₂ relationships, is also a large conduit vessel and as such may vary considerably in its reactive properties from smaller vessels, which in turn may possess different receptor populations. One of the objectives of this thesis, therefore, was to investigate responses to agonists on PGI₂ synthesis in different vessels from the same species and between species (including man). The role of the endothelium in mediating vascular PGI₂ release was also investigated in this thesis. A more complete rationale for these studies is provided in the introduction of chapter 3.

Since Ca²⁺ mobilisation is involved in both activation of PLA₂ (Irvine, 1982; Hong and Deykin, 1982; Martin and Wysolmerski, 1987) and excitation-contraction coupling (Somlyo and Somlyo, 1968), it was deemed possible that Ca²⁺ was the common activator in the two processes (i.e. vasoconstriction and PGI₂ synthesis; Jeremy et al., 1985a,b). Thus, it has been shown that in the rat aorta, rat tail artery, urinary bladder and penis that receptor-linked prostanoid synthesis is inhibited by the removal of extracellular Ca²⁺ with Ca²⁺ chelators, by Ca²⁺ channel blockers such as verapamil, nifedipine and diethylstilbestrol and by lanthanum, a Ca²⁺-binding antagonist (Stewart et al., 1983; Golub et al., 1985; Jeremy et al., 1985a,b). However, in these latter studies, the amounts of Ca²⁺ blockers required to inhibit PG synthesis were greater than those required to inhibit receptor-linked contraction in these tissues. For example, Valloton et al. (1990) found that Ca²⁺ channel blockers at concentrations that inhibit vascular contraction did not inhibit PGI₂ release from cultured vascular smooth muscle cells (VSMCs) in response to angiotensin II (ANG II). More recently, several groups
have measured, concomitantly, PGI$_2$ release and intracellular Ca$^{2+}$ in cultured endothelial cells, using Ca$^{2+}$-sensitive dyes such as Quin-2 and Fura-2 (Hallam et al., 1989; Carter et al., 1989). These groups concluded that the intracellular release of Ca$^{2+}$ is the principal, if not the only, source of Ca$^{2+}$ for PLA$_2$ activation and therefore PGI$_2$ release. Furthermore, in cultured bovine endothelial cells, it has been demonstrated that the release of AA is mediated principally by phospholipase A$_1$-lysophospholipase, the activity of which is not only independent of Ca$^{2+}$, but is actually enhanced by the presence of EDTA (Martin and Wysolmerski, 1987).

In the platelet, aggregation is associated with the concomitant release of TXA$_2$ (Arita et al., 1989). However, the direct relationship between receptors and TXA$_2$ release is complicated by the fact that aggregation, per se, causes the synthesis and release of TXA$_2$ (Arita et al., 1989), making it difficult to dissect out the mechanisms involved in receptor-TXA$_2$ synthesis coupling. This is exemplified by the fact that Ca$^{2+}$ channel blockers (again at high concentrations) inhibit platelet aggregation and concomitant TXA$_2$ release (Jeremy et al., 1985b). However, several studies have demonstrated that TXA$_2$ is synthesised by platelets via Ca$^{2+}$-mediated PLA$_2$ activation (Irvine, 1982; Siess et al., 1983). In an elegant study, Brune and Ullrich (1991) demonstrated that the Ca$^{2+}$ linked to TXA$_2$ synthesis is derived principally from intracellular stores. The involvement of Ca$^{2+}$ and eicosanoid synthesis is represented diagramatically in figs. 1.3. and 1.4. One objective of this thesis therefore was to investigate the release of TXA$_2$ by washed unstirred platelets (see chapter 3 for a more detailed rationale).
**Fig. 1.3.** Schematic representation of the mechanisms controlling vascular PGI$_2$ synthesis. Via G proteins (Gp), vasoconstrictor agonists (e.g. noradrenaline, TXA$_2$) activate phospholipase C (PLC) which generates diacyl glycerol (DAG) and inositol trisphosphate (IP$_3$) from phosphatidylinositol 4,5-bisphosphate (PI). DAG activates protein kinase C (PKC) which may activate Ca$^{2+}$ channels and directly stimulate constriction. IP$_3$ elicits the release of Ca$^{2+}$ ([Ca$^{2+}$]$_i$) from intracellular stores. A net increase in [Ca$^{2+}$]$_i$ enhances constriction (as well as PKC activity) and activates PLA$_2$ (releases arachidonate from phospholipid stores thereby generating PGI$_2$). PGI$_2$ stimulates cAMP synthesis which activates protein kinase A (PKA), an event associated with vasodilation. PKC also down-regulates excitatory receptors. Under similar control, PGI$_2$ release from endothelial cells inhibits platelet and leucocyte activity. How this mechanism relates to cell proliferation and lipid sequestration is given in figs. 1.6., 1.7. and 1.8.
**Fig. 1.4.** Schematic representation of the mechanisms controlling platelet TXA$_2$ synthesis. Via G proteins (Gp), agonists (e.g. collagen, TXA$_2$) activate membrane-associated PLC (mPLC) which generates diacyl glycerol (DG) and inositol trisphosphate (IP$_3$) from phosphatidylinositol 4,5-bisphosphate (PIP$_2$). DG activates protein kinase C (PKC) which in turn enhances secretion, aggregation and PLA$_2$ activity. IP$_3$ elicits the release of intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$) from the dense tubular system (DTS) which in turn activates PLA$_2$ to generate TXA$_2$ and activates calmodulin kinase (CaMK). TXA$_2$ released by the platelet can then further enhance aggregation of neighbouring platelets via the same mechanisms. Elevated [Ca$^{2+}$]$_i$ can also further enhance DG and IP$_3$ generation from phosphatidylinositol (PI) via activation of cytosolic PLC (cPLC) thereby further enhancing the activation of platelets.
1.3. The phosphoinositide cycle and eicosanoid synthesis.

In virtually every cell type hitherto investigated receptor-activation results in the hydrolysis of phosphatidylinositol (PI), an event widely termed 'PI turnover'. It has been long recognised that the initiation of PI turnover is dependent on phospholipase C (PLC) activity (Hokin and Hokin, 1960). PLC hydrolyses PI to generate two intracellular second messengers; 1,2-diacyl glycerol (DG) and inositol 1,4,5-trisphosphate (IP$_3$). PLC activity requires very low (0.1 uM) or no Ca$^{2+}$ (Jackowski et al., 1986). It is therefore generally accepted that Ca$^{2+}$ response following initiation of the PI cycle is secondary rather than being causative. DG, which remains in the plasmalemma, activates protein kinase C (PKC; Nishizuka, 1984, 1988). Essential co-factors for this activation are Ca$^{2+}$ and PS which combine with DAG and PKC to form a quaternary complex within the plasmalemma (Nishizuka, 1988). PKC then acts by phosphorylating (thereby activating and/or deactivating) other intracellular proteins. PKC also down regulates receptors via phosphorylation (Iwamoto et al., 1992).

Experimentally, the DG-PKC system has largely been investigated using the DG-mimetics, phorbol esters (Nishizuka, 1984, 1988; Berridge and Irvine, 1988). In vascular tissue, phorbol esters elicit contraction in vascular smooth muscle (Danthaluri and Deth, 1984; Rasmussen et al., 1984), an effect that is Ca$^{2+}$-dependent and potentiated by A23187. In turn, it was found that phorbol ester stimulates in vitro PGI$_2$ synthesis by the rat aorta, an effect potentiated by noradrenaline and Ca$^{2+}$ ionophore A23187 (Jeremy and Dandona, 1987). This action of phorbol esters was inhibited by Ca$^{2+}$ channel blockers and by the PKC inhibitor, isoquinolinylsulfonyl
methylpiperizine (H7; Jeremy and Dandona, 1987) which also inhibited adrenoceptor-linked PGI$_2$ synthesis (Jeremy and Dandona, 1987). These experiments suggested that adrenoceptor-PGI$_2$ synthesis coupling, at least in the rat aorta, is mediated by PKC and also that PKC elicits Ca$^{2+}$ mobilisation associated with eicosanoid synthesis possibly via activation of Ca$^{2+}$ channels (Jeremy and Dandona, 1987). However, other workers have provided evidence that PKC initiates vascular PGI$_2$ synthesis through direct activation of PLA$_2$ (DeMolle and Boeynaems, 1988). Phorbol esters exhibit dual effects on platelet activity (Nakano et al., 1989): an initial enhancement of platelet responses (aggregation and secretion) followed by inhibition of activity. Phorbol esters cause platelet amine storage granule secretion and hence aggregation without increasing cytosolic Ca$^{2+}$ (Rink et al., 1983). This effect appears to be linked to phosphorylation of P47, the protein responsible for initiating the secretory response (Daniel, 1990). At the outset of this thesis little work had been done on PKC and TXA$_2$ release by platelets.

IP$_3$ binds to specific sites on the endoplasmic reticulum and elicits the release and elevation of intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$) levels (Berridge and Irvine, 1988), which in turn activates Ca$^{2+}$-dependent processes, including contraction (Somlyo et al., 1985) and platelet aggregation (Lapetina et al., 1984; Brass et al., 1985). With regard to blood vessels, receptor-linked PI turnover has been demonstrated in response to vasoactive agents including noradrenaline (Legan et al., 1985; Rapaport, 1987; Villalobos-Molina et al., 1982; Berta et al., 1986) angiotensin II (Griedling et al., 1986), serotonin (Roth et al., 1984), histamine (Resink et al., 1987) and platelet activating factor (PAF; Test and Bang, 1981). Recent
experiments have indicated that IP$_3$-stimulated elevation of [Ca$^{2+}$]$_i$ is the principal mechanism by which PLA$_2$ is activated in vascular tissue (Carter et al., 1988; Hallam et al., 1989).

Considerable attention has been paid to the PI cycle, Ca$^{2+}$ mobilisation and TXA$_2$ synthesis in platelets. Platelet activators which have been shown to induce PI turnover include thrombin, collagen, platelet activating factor (PAF), adenosine diphosphate (ADP), vasopressin and serotonin (Daniel, 1990). It has also been clearly demonstrated that IP$_3$ elicits an increase in intracellular Ca$^{2+}$ and full response in platelets, including TXA$_2$ synthesis (Lapetina et al., 1984; Brass and Joseph, 1985; Brune and Ullrich, 1991). The involvement of DG and IP$_3$ and eicosanoid synthesis is represented diagramatically in figs. 1.3. and 1.4.

### 1.4. GTP binding (G) proteins

G proteins are regulatory guanosine triphosphate (GTP)-binding proteins linking receptor activation to mediators of cell signalling (viz. PLC and adenylate cyclase; Nagata and Nozawa, 1990; Johnson and Dhanasekaran, 1989). G proteins contain three subunits ($\alpha, \beta$ and $\gamma$), bind guanine nucleotides with high affinity and possess GTPase activity and are modulated by toxins (cholera and pertussis; Johnson and Dhanasekaran, 1989). However, between-tissue responses to these toxins can vary markedly and as such caution should be exercised when interpreting toxin-effects. Although well characterised in other tissues, the role of G proteins in mediating vascular and platelet eicosanoid synthesis is not clear. However, in other tissues, it has been firmly established that G proteins are involved in ion channel regulation, exocytotic secretion and PLA$_2$ activation (Nagata and
Nozawa, 1990; Johnson and Dhanasekaran, 1989). Although not proven definitively, there is substantial evidence that a G protein links receptor activation to PLC (Cockcroft, 1987). Haslam and Davidson (1984) showed that GTP and its non-hydrolysable analogue, GTPγS in electrically permeabilised human platelets enhances thrombin-induced DG formation, indicating that G proteins couple receptors to PLC. Other studies have also indicated that there is a GTP-dependent activation of cytoplasmic PLC in platelets (Baldassare, 1986; Deckmyn et al., 1986). In turn, PKC has been postulated to act at the level of G proteins and has been presumed to couple the thrombin receptor with PLC (Halenda et al., 1986). Several studies have indicated that PLA₂ activity is regulated by G proteins (Nakashima et al., 1987). Thrombin-induced AA release was completely inhibited by pertussis toxin treatment whereas PLC activity was decreased by only 20-40% in toxin-treated platelets (Nakashima et al., 1987). These results indicate that AA release by PLA₂ is independent of PLC activation and a pertussis toxin-sensitive GTP-binding protein links receptor activation to PLA₂. cAMP in platelets appears to be regulated by stimulatory (Gs) and inhibitory G proteins (Gi) (Jakobs et al., 1987; Katada et al., 1984).

In vascular tissue, rather than inhibiting PGI₂ release, cholera toxin potentiated agonist-stimulated PGI₂ release from cultured bovine aortic smooth muscle cells, whereas pertussis toxin was inactive (Demolle and Boeynaems, 1989). Sodium fluoride (NaF) has also been used as an activator of G proteins (Gilman, 1987). NaF mimics GTP by forming a fluoro-aluminate complex which then binds to and activates G proteins (Bigay et al., 1985). NaF has been shown to stimulate PGI₂ synthesis by the rat aorta and cultured endothelial
cells (Jeremy and Dandona, 1988; Stansby et al., 1991), an effect blocked by PKC inhibition, Ca^{2+} channel blockade and cholera toxin (Jeremy and Dandona, 1988; Magnusson et al., 1989; Garcia et al., 1991). These data indicate that G proteins linked to vascular PGI_{2} synthesis involves the activation of PLC and Ca^{2+} mobilisation. Little is known of G proteins in the control of PGI_{2}-linked adenylate cyclase in vascular tissue. The possible involvement of G proteins in eicosanoid synthesis is represented diagrammatically in figs. 1.3. and 1.4. Some aspects of G proteins and eicosanoid synthesis in vascular tissue and platelets is further explored in this thesis.

1.5. Vascular PGI_{2} and platelet TXA_{2} in the aetiology of CVD.

Given the diametrically opposing effects of PGI_{2} and TXA_{2} and that these eicosanoids are synthesised by the vascular endothelium and platelet respectively, it was quickly perceived that the relative balance between these two eicosanoids may constitute a fundamental haemostatic mechanism (Moncada and Vane, 1979). Prior to discussing this balance it is pertinent to describe the basic physiology and biochemistry of platelets and the vascular endothelium as they relate to haemostasis.

The physiological task of circulating platelets is to arrest the loss of blood when a blood vessel is damaged. This process involves: a) rapid adhesion to exposed subendothelium, b) platelet to platelet adherence (aggregation) and c) formation of platelet plug (see fig. 1.5.). In vitro, platelets are activated by a wide range of substances (thrombin, collagen, ADP, adrenaline, serotonin, ristocetin, Ca^{2+} ionophore, AA and TXA_{2} analogues; Crawford and Scrutton, 1987). The platelet response to these substances is
characterised by: a) shape change b) aggregation and c) the release of vaso- and thrombo-active substances from the beta and dense granules and d) generation of \( \text{TXA}_2 \) (Crawford and Scrutton, 1987). The dense granules, less numerous and of greater electron density than beta granules, contain nucleotides (viz. ADP), serotonin, \( \text{Ca}^{2+} \) and pyrophosphates. Alpha-granules release platelet derived growth factor (PDGF; mediates tissue repair and wound healing), platelet factor 4 (PF4), fibronectin (forms covalent linkages with collagen and fibrin and subendothelium), beta-thromboglobulin, thromospondin, factor V and von Willebrand's factor (factor VIII). The platelet also synthesises platelet activating factor (PAF; for review see Mikhailidis and Peplow, 1990). A diagrammatic representation of platelet activation sequence is given in fig. 1.5.

The vascular endothelium generates both anti- and pro-thrombotic factors (Sixma 1987; Pearson and Petty, 1989). Endothelium lines the luminal surface of large vessels with a monolayer of polygonal oblong cells stretching their long diameter along the direction of blood flow. In small vessels this "cobblestone" pattern disappears as individual cells fold their cytoplasm to encircle the entire capillary lumen. Prevention of platelet adhesion and aggregation to the healthy vascular endothelium is effected by a physico-chemical interaction of platelets with endothelial surface glycoproteins and the secretion by the endothelium of substances which inhibit platelet activity. These anti-platelet substances include \( \text{PGI}_2 \) (Moncada and Vane, 1979), ADPase (Lieberman et al., 1982) and endothelium-derived relaxing factor (EDRF; Furchgott, 1983), now thought to be nitric oxide (NO; Palmer et al., 1987). Apart from synthesising predominantly, \( \text{PGI}_2 \), the endothelium possesses the capacity to
Fig. 1.5. Principal events in platelet activation, in vivo. Circulating platelets, sensing damage to the endothelium or via stimulation by platelet activating substances, undergo shape change. Platelets then adhere to sites of vascular damage (i.e. where endothelium removal exposes the subendothelium) and release growth factors (e.g. PDGF) which brings about endothelial repair. If vascular damage is more severe, platelets aggregate aggressively in large numbers and form a plug, in and around which fibrin and erythrocytes accumulate, thereby forming a thrombus at the site of vascular injury. Platelets undergo degranulation and release a number of prothrombotic and proaggregatory factors which enhance thrombus formation. These release substances are likely to 'prime' other circulating platelets.
generate other PGs (e.g. PGE$_2$, PGF$_{2\alpha}$ and TXA$_2$ [Stansby et al., 1991]).

In contrast to these anti-platelet factors, platelet adhesion to subendothelium requires the presence of von Willebrand's factor, a glycoprotein synthesised by endothelial cells and stored in organelles known as Weibel-Palade bodies (Wagner et al., 1982). The endothelium also synthesises angiotensin-converting enzyme (ACE; Caldwell et al, 1976), which cleaves angiotensin I to liberate angiotensin II (ANG II; a vasconstrictor, promoter of platelet activity and a mitogen; Taubman et al., 1989). More recently, the endothelium has been shown to synthesise endothelin-1, the most potent vasoconstrictor known (Yanagisawa et al., 1988). Endothelium also produces thrombomodulin (activation of protein C anticoagulant) thromboplastin, tissue plasminogen activating factor (t-PA) and t-PA inhibitor-1 (Sixma, 1987; Petty and Pearson, 1989). Vascular endothelium also possesses the enzyme, lipoprotein lipase, which cleaves fatty acids from lipoproteins, the fatty acids then being taken up for sequestration by vascular tissues (Eckel, 1989; see section 1.8. and fig. 1.6).

1.6. Measurement and assessment of PGI$_2$ and TXA$_2$ synthesis, in vivo and in vitro, in relation to CVD and thromboembolic disease

Both PGI$_2$ and TXA$_2$ break down spontaneously to the stable hydrolysates, 6-oxo-PGF$_{1\alpha}$ and TXB$_2$, respectively (Moncada and Vane, 1979). These latter hydrolysates are further converted by the liver to various metabolites predominantly, 2,3-dinor derivatives (Rosencrantz et al., 1980; FitzGerald et al., 1981; Barrow and Taylor, 1987; Barrow and Ritter, 1988, FitzGerald et al., 1985).
Radioimmunoassay (RIA) of TXB$_2$ and 6-oxo-PGF$_{1\alpha}$ in plasma has been employed, but this method has serious flaws; principally interference by other plasma components (Barrow and Ritter, 1988). Apart from specificity and sensitivity there are other problems with assessing circulating eicosanoid metabolites. At venepuncture activation of just a small number of platelets (there are several 100s of millions in 1ml blood) may artefactually alter the amount of TXB$_2$ in a blood sample. Similarly, venepuncture has been shown to elicit a local large 'trauma-stimulated' release of PGI$_2$ from blood vessels (Jeremy et al., 1984a), potentially altering the amount of PGI$_2$ in a blood sample. The use of gas chromatography-mass spectrometry (GC-MS) has obviated some of these problems (but not of eicosanoid release at time of sampling in the case of measurement in blood) and is now considered the definitive method for assessment of measurement of circulating metabolites of PGI$_2$ and TXA$_2$ (Barrow et al., 1982, FitzGerald et al., 1985; Barrow and Taylor, 1987; Barrow and Ritter, 1988).

It has been established that the actual circulating levels of TXB$_2$, 6-oxo-PGF$_{1\alpha}$ and their hepatic metabolites are in the ng to sub-ng/l range (Barrow and Ritter, 1988) which produces profound problems of sensitivity. These sensitivity problems have been largely obviated by measuring di-nor metabolites in the urine, again using GC/MS (Catella and FitzGerald, 1987; Barrow et al., 1987; Barrow and Ritter, 1988). This approach is advantageous since: a) there are greater concentrations of these metabolites in the urine than in blood, b) urine collection is non-invasive and c) urine is devoid of biological material that can generate these eicosanoids (except, of course, in kidney disease, where blood cells appear in the urine).
The assumption, however, that urinary metabolites reflect vascular and/or platelet-generated eicosanoids must be approached with caution. We know, for instance, that the bladder also possesses the capacity to synthesise PGs (including PGI$_2$ and TXA$_2$) in large quantities (Jeremy et al., 1984b; Mikhailidis et al., 1987b). It was also found that variables such as distension and osmolality of luminal fluid as well as disease states (e.g. diabetes mellitus [DM]) can profoundly alter bladder PG synthesis (Jeremy et al., 1986d). Thus, it was suggested that where a disease state may alter bladder PG synthesis (which, in turn, may alter urinary PG metabolite profiles), then this contribution may distort interpretation of results, if it were assumed that urinary PGs are solely of vascular or platelet origin (Mikhailidis et al., 1987b). In a recent paper, this hypothesis was consolidated when it was demonstrated that the urinary bladder contributes as much as 50% of the total PGs present in urine (Reyes and Klahr, 1990).

TXA$_2$ synthesis by platelets has also been widely studied by measuring the release of this eicosanoid, ex vivo. Problems of sensitivity and specificity are avoided since, platelets possess the capacity to synthesise large quantities of TXA$_2$ (1ml PRP maximally generates microgram amounts of TXA$_2$; Mikhailidis et al., 1983, 1985a, 1985b). Thus, TXB$_2$ has been measured (by RIA) in blood samples which have been allowed to clot (Viinikka and Ylikorkala, 1980) and in samples of PRP following aggregation in an aggregometer (Mikhailidis et al., 1983, 1985a, 1985b). One objective of this thesis was to study the release of TXA$_2$ in washed, unstirred platelets (to obviate aggregation; see introduction of chapter 3 for a more detailed rationale).

Clearly, the study of vascular PGI$_2$ by vessels obtained from
volunteers or patients is not feasible. Thus, most of our knowledge on vascular PGI\(_2\) has been derived from experiments using vessels obtained from laboratory animals. In this approach, vessels are excised, cut into rings, placed and incubated in physiological buffers and PGI\(_2\) release assessed by RIA of 6-oxo-PGF\(_{1\alpha}\). In early experiments, only the spontaneous release of PGI\(_2\) was measured, sometimes by bioassay (inhibition of platelet aggregation, in vitro). However, as was discussed above, PGI\(_2\) synthesis is controlled by complex intracellular mechanisms. Thus, PGI\(_2\) stimulated by different substances can reveal sites at which defects in disease states may occur and also at which sites drugs may exert their action (see section 1.9.). Without doubt for most studies on PGI\(_2\) release vascular tissue has been obtained from cultured vascular cells (endothelial and smooth muscle).

1.7. Eicosanoids and mechanisms of atherogenesis

The presence of advanced atherosclerotic plaques is invariably the common denominator in individuals who develop and die of ischaemic heart disease (IHD) or stroke (Davies and Thomas, 1985; Meade, 1987; Woolf, 1989). In turn, risk factors that predispose to the development of atherosclerosis include diabetes mellitus (DM; Kannel and McGee, 1979; Krowlewski et al., 1985; Pyorala et al., 1987; Betteridge, 1990), hypertension (Hollenberg, 1991; Rau, 1991), cigarette smoking (Meade, 1987; McGill, 1990; Jeremy and Mikhailidis, 1990), gender (i.e. males > females; Kannel and McGee, 1979) and hyperlipidaemia (cholesterol, low density lipoprotein [LDL] and triglycerides; Smith, 1987; Betteridge, 1989a,b; Krauss, 1991).

Key events in the initiation and growth of the atherosclerotic
plaque, are the proliferation of arterial smooth muscle cells, deposition of lipid and accumulation of collagen, elastic fibres and proteoglycans (Haust et al., 1960). It has been proposed that atherogenesis is initiated by endothelial cell injury which, in turn leads to adherence of platelets and subsequent local release of platelet substances (TXA$_2$, serotonin, PAF, histamine and PDGF) all of which are pro-mitogenic, promote further adhesion and aggregation of platelets and attract monocytes and neutrophils (Ross and Glomset, 1976; Spagnuolo et al., 1988). More recently, invasion into vascular tissue of monocytes and their transformation into macrophages has been suggested as the progenitor of the atherosclerotic plaque (Ross et al., 1986; Mitchinson and Ball, 1987). Macrophages, in turn, transform into 'foam cells' which appear at the edges of most advanced atherosclerotic lesions and accumulate lipids, in particular, cholesterol (Gerrity 1981). Macrophages also release a plethora of mitogenic and inflammatory substances (histamine, serotonin, leukotrienes, interleukins (ILs), tumour necrosing factor (TNF), PGs, TXA$_2$, PDGF-like substance, toxic free radicals, and neutral proteases; Mitchinson and Ball, 1987; Woolf, 1989).

As part of their killing (of bacteria) mechanism, neutrophils generate large amounts of superoxide. Apart from the destruction of bacteria, superoxide elicits lipid peroxidation of fatty acids both in the plasma membrane and in LDL, enhancing the atherogenicity of this lipoprotein (Witzum and Steinberg, 1991). Lipid peroxides also inhibit PGI$_2$ synthesis at the endoperoxide synthase level (Warso and Lands, 1983). In turn, PGI$_2$ has been shown to inhibit the infiltration of monocytes into blood vessels, the adhesion of
neutrophils to injured vascular endothelium and the release of pro-atherogenic substances from these cells (Ham et al., 1983; Jones and Hurley, 1984; Weksler and Goldstein, 1980; Belch et al., 1986; Gryglewski et al., 1987).

Notwithstanding the mechanisms that initiate atherogenesis, platelets certainly play a role in the end-stage pathological events that lead to thrombus formation. At end stage, the atherosclerotic lesion becomes calcified and denuded of endothelium and the plaque often ruptures. The vascular lumen is also markedly narrowed (stenosis), which leads to abnormalities in blood flow (Karino and Goldsmith, 1987). Clearly, such a pathologically damaged site constitutes a prime target for platelet adhesion, aggregation and clot formation. Shear stress caused by stenosis has also been widely implicated as a component in platelet activation in atherosclerosis (Karino and Goldsmith, 1987).

Apart from inhibiting platelet and leukocyte adhesion and aggregation, PGI$_2$ and other PGs has been shown to inhibit in vitro VSMC and fibroblast proliferation (Huttner et al., 1977; Nilsson and Olsson, 1984; Owen, 1985; Pomerantz and Hajjar, 1989). In an elegant study, Libby et al. (1988) demonstrated that the cytokine, interleukin-1B (IL-1B), stimulated VSMC and fibroblast proliferation, an effect blocked by PGE$_1$ and PGE$_2$. In turn, both platelet and leucocyte release substances have been shown to stimulate the release of PGI$_2$ and other PGs from endothelial cells, VSMCs and whole vessels (Coughlin et al., 1980, 1981; Baezinger et al., 1981; Van Coevorden and Boeynaems, 1984; Allbrightson et al., 1985; Jeremy et al., 1985c, 1988a; Rossi et al., 1985; Ristimaki and Viinika, 1992) indicating that there is a complex communication between these cells, in which
release substances of opposing actions are the means by which cell proliferation may be controlled in blood vessels (see fig. 1.6.).

PGs have other properties which pertain to the aetiology of atherosclerosis. For example, PGI₂ has been shown to stimulate (via cAMP; Dembinska-Kiec et al., 1980) the activity of cholesteryl ester hydrolase (Hajjar et al., 1982; Pomerantz et al., 1989). In the vascular cell, this latter enzyme converts cholesterol esters (impermeable to cell membranes) to free cholesterol (permeable to cell membranes), which is then liberated into the circulation (see fig. 1.7.). Impairment of cholesteryl ester hydrolase may (possibly involving impairment of PGI₂ synthesis) lead to accumulation of cholesteryl esters, an accepted contributing factor in atherogenesis (Krauss, 1991). Lipoproteins (both high and low density) also stimulate PGI₂ synthesis in vascular tissue, an effect ascribed to the release of AA following the hydrolytic action of lipoprotein lipase (Pomerantz et al., 1984b, 1989). A hypothetical model for the role of PGI₂ in lipid sequestration in vascular tissue is given in fig. 1.7.

1.8. PGI₂ and TXA₂ in IHD and disease predisposing to IHD

a) Atherosclerosis

In arteries, heart, lung and kidney of rabbits with experimental atherosclerosis, there is a decrease in the ex vivo release of PGI₂ (Dembinska-Kiec et al., 1977, Gryglewski et al., 1978). Reduced PGI₂ synthesis by atherosclerotic human blood vessels, obtained post mortem, has been reported (Sinzinger et al., 1979). Larrue et al.,
Fig. 1.6. Possible involvement of PGs in the control of vascular smooth muscle cell proliferation (the pathognomonic lesion of atherosclerosis). Mitogens elicit several early events, in particular, phosphatidyl inositol 4,5-bis-phosphate (PIP$_2$) hydrolysis which in turn results in protein phosphorylation by kinase C and calcium/calmodulin-dependent kinases (caM-PK). These events induce expression of two genes: c-fos and c-myc, thus initiating DNA synthesis and cell division. Elevation of cAMP is associated with inhibition of mitosis. cAMP activates PKA which phosphorylates proteins that counter the effects of mitogens. Thus, the antimitogenic effect of certain endogenous PGs, is mediated by activation of adenylate cyclase.
Fig. 1.7. Possible involvement of PGI₂ in lipid sequestration by vascular cells. Low density lipoprotein (LDL) is taken up by endothelial and/or vascular cells where arachidonic acid (AA) is liberated by lipoprotein lipase from triglycerides contained in the LDL. AA is then converted to PGI₂ which stimulates cAMP synthesis which activates cholesteryl ester hydrolase (CEH). In turn, CEH hydrolyses esterified cholesterol (impermeable to cell membranes) to free cholesterol which can then be removed from the cell (possibly following incorporation into high density lipoprotein HDL). Atherosclerotic lesions are associated with an accumulation of esterified cholesterol (in droplets). Thus, any reduction of the activity of the enzymes involved with PGI₂ synthesis (e.g. by excess cellular cholesterol) may impair the normal egress of cholesterol from cells and as such contribute to atherogenesis.
(1980) also reported diminished PGI₂ release by cultured VSMCs derived from atherosclerotic rabbit aortae. Platelets also adhere to atherosclerotic vessels more readily than normal vessels (Dembinska-Kiec et al., 1977). TXA₂ production by platelets from patients with atherosclerosis and peripheral vascular disease (PVD) is increased (FitzGerald et al., 1986; Mikhailidis et al., 1985a). Patients with severe atheromatous disease excrete greater amounts of 2,3-dinor metabolites of 6-oxo-PGF₁α and TXB₂ than healthy controls (Fitzgerald et al., 1984). As was mentioned earlier, damage to vessels causes enhanced release of PGI₂, which may explain these increased levels of PGI₂ metabolites. Platelets, which are probably activated in vivo by damaged atherosclerotic vessels would also explain the increased levels of TXA₂ metabolites in patients with severe atheromatous disease. However, Ritter et al. (1986), found no elevation of PGI₂ metabolites in plasma from patients with severe atheromatous disease. This, may be explicable by an 'exhaustion' of PGI₂ substrate due to prolonged activation of output in diseased vessels.

b) Diabetes Mellitus (DM)

Patients with diabetes mellitus (DM) are at greater risk than the healthy population of developing cardiovascular disease (CVD; Banga and Sixma, 1986; Mikhailidis et al., 1988; Betteridge, 1989a, 1990). Of the many aetiological factors associating CVD with DM, a marked attenuation of prostacyclin (PGI₂) synthesis by blood vessels from diabetic laboratory animals (Harrison et al., 1978; Rogers et al., 1981; Jeremy et al., 1987a) and from vascular biopsies obtained from diabetic patients (Johnson et al., 1979; Silberbauer et al., 1979) has been reported. The precise mechanisms that determine
reduced vascular PGI₂ synthesis in diabetes mellitus (DM) are not clearly defined, although a reduction in substrate (arachidonate) levels in blood vessels has been suggested (Holman et al., 1983; Jeremy et al., 1987a, Dang et al., 1988). As was discussed earlier, vascular PGI₂ synthesis is stimulated by vasoactive agonists, which in turn is mediated by G proteins, PKC and IP₃ (Griendling et al., 1986; Hallam et al., 1988; Jeremy et al., 1988a). The net result of activation of these systems is transmembrane Ca²⁺ influx and an increase in intracellular Ca²⁺ ([Ca²⁺]ᵢ) which then activates PLA₂. A major objective of this thesis was to further elucidate the mechanisms that determine diminished vascular PGI₂ synthesis in DM, using a range of stimulators that act at different sites in the receptor activation-PGI₂ synthesis pathway in the streptozotocin-induced diabetic rat (for a more complete rationale see the introduction of chapter 5).

In general, disturbances of platelet function are seen only in diabetic patients where micro- or macroangiopathy is present (Hendra and Betteridge, 1989). Possibly the area of greatest disagreement is that of TXA₂ release by platelets from diabetic patients. Increased, decreased an unchanged release of TXA₂ by platelets from diabetic patients has been reported (Hendra and Betteridge, 1989; Mikhailidis et al., 1988). These disparities may be methodological. For example, when one assesses TXA₂ release following aggregation, it must be remembered that the more pronounced the aggregation the greater the release of TXA₂ (Arita et al., 1988). If one looks at release in non-aggregated platelets (elicited by freeze fracturing and/or sonication), then one actually sees a reduction in TXA₂ release from platelets taken from diabetics when compared to controls (Jeremy et
Fig. 1.8. Possible interrelationships between blood vessels, platelets and leucocytes in atherogensis. Damage to the endothelium results in platelet and neutrophil adhesion and monocyte infiltration where they transform into macrophages (progenitors of foam cells). All these cells release substances which are mitogenic, increase vascular permeability and further enhance leucocyte and platelet accumulation. However, these release substance also stimulate vascular PG synthesis which counteract these effect (i.e. diminish platelet and leucocyte adhesion, release reactions and inhibit cell proliferation). PG release thus constitutes a defence mechanism by which atherogenic events are endogenously counteracted by blood vessels. In turn, any impairment of normal PG release may render a blood vessel susceptible to these atherogenic events.
(1988b). This latter point may also indicate that platelet dysfunction in DM is not due to an increased activity of the TXA$_2$ synthesising pathway but is a consequence of increased platelet activity.

In contrast to DM, there is virtually universal agreement that in patients with peripheral vascular disease (PVD), there is a marked increase in platelet activity. Aggregation (in platelet rich plasma and in whole blood) and concomitant TXA$_2$ release is markedly enhanced in platelets from patients with PVD (Mikhaildis et al., 1985a). The general consensus is that increased platelet activity follows rather than precedes PVD. Certainly diseased and damaged vessels are likely to cause an excitation of circulating platelets, since their innate function is to recognise and be activated by vascular damage.

c) Hypertension

Intravenous infusion of PGI$_2$ decreases blood pressure in normotensive and hypertensive patients (Papanikolaou, 1988), indicating that PGI$_2$ plays a role in controlling vascular tone. Although it may be expected that vascular PGI$_2$ would be reduced in hypertension, the majority of studies indicate an increase in PGI$_2$ output by vessels from hypertensive animals (Pace-Asciak, 1978; Botha et al., 1979; Rioux et al., 1985). In experimental portal hypertension, PGI$_2$ synthesis by the hepatic portal vessel is markedly enhanced (Hamilton et al., 1981,1982; Guarner and Soraino, 1993). Furthermore, circulating PGI$_2$ metabolites are increased in patients with portal hypertension (Sitzmann and Li, 1991). Thus, it has been suggested that increased PGI$_2$ release may constitute an adaptive response aimed at diminishing hypertension (Hamilton et al., 1982).
One objective of this thesis was to investigate changes of receptor-linked \( \text{PGI}_2 \) synthesis in both the aorta and mesenteric vasculature of the rat with experimental portal hypertension and possibly to pinpoint sites at which alterations of mechanisms common to both \( \text{PGI}_2 \) synthesis and vasoactivity may occur. A more complete rationale is given in the introduction of chapter 5.

Given that \( \text{TXA}_2 \) is a vasoconstrictor, it has been suggested that platelet hyperactivity may play a role in hypertension (Nyrop and Zweifler, 1988). As in DM, changes in platelet function from hypertensive patients are equivocal. It appears that the median arterial pressure must be \( > 120 \text{ mm Hg} \) before increases in platelet activity are manifest (Nyrop and Zweifler, 1988). Interestingly, it has been demonstrated that there is increased adhesion and migration of monocytes (which also produce \( \text{TXA}_2 \); Davies, 1986) into blood vessels of hypertensive rats (Haudenschild, 1979).

d) Cigarette smoking

Cigarette smokers are at far greater risk of developing atherosclerosis than non-smokers (Kannel, 1981; Wilhelmsen, 1988; McGill, 1990). The effect of cigarette smoking on platelet activity, \( \text{TXA}_2 \) synthesis and vascular \( \text{PGI}_2 \) is an area of considerable difference of opinion. That platelet aggregation and / or release is increased, decreased or unchanged in smokers has been reported by different groups (Kutti, 1990). An increase in 2,3-dinor metabolites of \( \text{TXA}_2 \) in the urine of smokers has been reported (Murray et al., 1990; Barrow et al., 1989). In a recent study the author reported that cigarette smoke condenstates are potent inhibitors of \( \text{TXA}_2 \) release, in vitro (Jeremy and Mikhailidis, 1990). There are reports
that nicotine inhibits vascular PGI$_2$ synthesis, in vitro (Wennmalm and Alster, 1983), whereas other studies have disputed this (Jeremy et al., 1985e). More recently, cigarette smoke extracts have been shown to produce a biphasic effect on vascular PGI$_2$ synthesis, in vitro: stimulation at low concentrations and inhibition at higher concentrations of extract (Jeremy and Mikhailidis, 1990). These findings indicate that another component(s) of tobacco smoke (other than nicotine) is responsible for altered vascular PGI$_2$ synthesis, at least in vitro. It is, however, unequivocal that plasma fibrinogen concentrations are elevated in smokers and that this coagulation factor is a firm predictor of pending IHD (Meade, 1987b). Smoking also causes profound morphological damage to the endothelium (Pittilo et al., 1982, 1984, 1990; Bull et al., 1988) as well as enhancing platelet adhesion to the endothelium (Pittilo et al., 1984). Thus, smoking does not appear to increase the risk of developing IHD through a chronic activation of platelets but possibly via damage to the endothelium. This latter situation may eventually result in hyperactive platelets in response to vascular damage, as in PVD (a condition that has a well established association with smoking).

e) Gender

IHD is more prevalent in age-matched men than pre-menopausal women, but in post-menopausal women this decreased incidence of IHD returns to that of age-matched men (Castelli, 1988). It has been suggested that estrogens confer this protective effect to women (Jeremy and Mikhailidis, 1991). In contrast, women who use the contraceptive pill (containing synthetic estrogens / progestagens)
are more likely to develop thrombosis than non-users (Upton, 1990). Data are scant on the effects of estrogens and androgens on vascular PGI$_2$ synthesis. However, androgens have been shown to inhibit vascular PGI$_2$ synthesis (Chang et al., 1982), whereas estrogens (and progesterone) appear to have no effect (Jeremy and Dandona, 1985). With regard to platelet TXA$_2$ synthesis, few studies have been carried out to elucidate the role of the sex hormones on platelet activity. This is surprising since CVD is overtly gender-linked.

1.9. Eicosanoids and drug action

Apart from the roles played by PGs and TXs in normal cellular function and in the pathophysiology of CVD, these eicosanoids are important mediators of drug action. This mediation can either be by a direct action on eicosanoid synthesising enzymes or by an indirect effect on systems that also control eicosanoid synthesis. The classic example of direct acting drugs are non-steroidal antiinflammatory drugs (NSAIDs) such as aspirin and indomethacin, which inhibit cyclooxygenase activity (Vane, 1971; Flower, 1974). Inhibitors of 5- and 12-lipoxygenases and drugs which act at the synthase level have also been developed (most notably ridogrel, a combined TXA$_2$ synthase inhibitor and TXA$_2$ receptor antagonist; Rainsford, 1988; Sanner, 1988). PLA$_2$ inhibitors include non-steroidal drugs such as mepacrine and quinacrine as well as corticosteroids (via stimulation of lipocortin), such as dexamethasone, betamethasone prednisolone (Flower, 1978; Rainsford, 1988). Eicosanoid receptor antagonists, particularly against LTs and TXA$_2$, have also been developed and have met with some therapeutic success (Sanner, 1988).

Apart from directly acting drugs, other classes of drugs have
been shown to modulate eicosanoid synthesis at sites other than enzymes (PLA₂, synthases, cyclooxygenase, lipoxygenase and/or receptors; Boeynaems, 1988). Put another way, any given drug may act at different sites in the eicosanoid synthesising pathway. For example, although \( \alpha \) - adrenoceptor antagonists (e.g. prazosin, yohimbine, rauwolscine) inhibit noradrenaline or adrenaline-stimulated PGI₂ release from blood vessels, they have no effect on A23187- or AA-stimulated PGI₂ synthesis (Jeremy et al., 1985a). This latter effect is readily explicable by a specific action on adrenoceptors which would, ipso facto, block adrenaline-stimulated PGI₂ synthesis but not the activity of other systems involved in adrenoceptor - PGI₂ synthesis coupling. In contrast, Ca\(^{2+}\) channel blockers such as nifedipine and nimodipine, inhibit rat aortic PGI₂ synthesis when stimulated by \( \alpha \) - adrenoceptor agonists (Stewart et al., 1984; Golub et al., 1985, Jeremy et al., 1985a, 1986c), A23187 (Jeremy et al., 1986c), phorbol ester (Jeremy and Dandona, 1987) and NaF (Jeremy and Dandona, 1988) but not arachidonate or trauma-stimulated PGI₂ release (Jeremy et al., 1986c). This latter effect is explicable since PLA₂ is Ca\(^{2+}\)-activated (Irvine, 1982; Hong and Deykin, 1982) and receptor activation, PKC and G proteins precedes the PLA₂ step whereas AA-stimulated and trauma stimulated synthesis by-pass the Ca\(^{2+}\) mobilising step. In this context, the angiotensin converting enzyme inhibitor (ACEI), captopril, was shown to inhibit rat aortic PGI₂ synthesis when stimulated by adrenaline, U46619 and A23187 but not by AA or trauma (Jeremy et al., 1988e). It was concluded that ACEIs may therefore exert their hypertensive effect not only via inhibition of ANG II generation, but also via inhibition of Ca\(^{2+}\) mobilisation (essential for vasoconstriction; Somlyo and
Somlyo, 1968). Thus, the accepted mode of action of any one class of drug may not be its only site of action. Furthermore, since PGI$_2$ and TXA$_2$ synthesis involves a number intermediary steps, it is therefore possible to detect alternative sites of action of any given drug using different stimulators which act at separate sites in the receptor-eicosanoid synthesis coupling pathway. A major objective of this thesis, therefore, was to apply this principal in the exploration of alternative sites of action of some common drugs used in the treatment of CVD. A more complete rationale is given in the introduction of chapter 4.
AIMS OF THE THESIS

1) To further investigate receptor-linked PGI$_2$ synthesis in blood vessels of the rat, rabbit and man, with particular reference to receptor agonists and the mediatory role of calcium, G proteins and protein kinase C.

2) To investigate the variation of receptor-linked PGI$_2$ release between species and between different blood vessels (arteries and veins) in the same species. Studying the role of the endothelium in mediating PGI$_2$ release by blood vessels was also an objective.

3) To study the effect of hypothermic storage of blood vessels in organ preservation solutions in relation to the complications encountered in organ transplantation.

4) To develop a method for the study of platelet TXA$_2$ synthesis in a static (non-aggregating) system and to study the role of calcium, G proteins and PKC in mediating TXA$_2$ synthesis.

5) To investigate the site of action of drugs (milrinone, non-steroidal antiinflammatory drugs, copper chelators) using the techniques developed in above, in blood vessels and platelets.

6) To apply the systems developed in (1) above to clarify the mechanisms that lead to the altered synthesis of vascular PGI$_2$ in experimental diabetes mellitus and hepatic portal hypertension.
CHAPTER 2. MATERIALS AND METHODS

2.1. MATERIALS

The following chemicals and drugs were obtained from:

a) Sigma Chemical Co. (Poole, Dorset, UK):

- acetylcholine chloride,
- adenosine cyclic 3'5'-mono phosphate,
- adenosine diphosphate,
- adrenaline bitartrate,
- angiotensin II,
- apyrase,
- arachidonic acid (99% pure),
- bovine serum albumin,
- calcium ionophore A23187,
- carbachol chloride,
- collagen type III,
- diethylstilbestrol,
- Dulbecco's minimum essential medium (MEM),
- fetal calf serum,
- histamine
- N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES),
- ibuprofen
- indomethacin,
- isobutylmethylxanthine
- isoquinolinylsulfonylmethylpiperazine (H7),
- medium 199,
- noradrenaline bitartrate,
- nordihydroguaretic acid,
- DL-penicillamine,
- penicillin G,
- phenylephrine chloride,
- phorbol ester dibutyrate,
- phorbol ester myristate acetate,
- PGs (PGE₁, PGE₂, PGF₂α, 6-oxo-PGF₁α)
- serotonin (5-hydroxytryptamine),
- sodium fluoride,
sodium nitroprusside,
staurosporine,
streptomycin
streptozotocin,
substance P,
thapsigargin,
thromboxane A₂,
Tris (hydroxymethyl) - methylammonium chloride (Tris hydrochloride),
U46619 (thromboxane A₂ analogue)
verapamil.

b) Aldrich Chemical Co. (Gillingham, Dorset, UK):
dimethyldithiocarbamic acid,
tetraethylthiouram disulfide
trientine
triethyldithiocarbamic acid
phenanthroline

c) BDH (Poole, Dorset, UK):
absolute ethanol (Aristar grade)
chloroform
diethyl ether
ethyl acetate
glacial acetic acid
methanol
petroleum ether

Unless otherwise stated, all buffer components and metal salts were purchased from BDH (see 2.3.). BDH also supplied plastic plates for thin layer chromatography (TLC), precoated with silica gel (Kieselgel 60; with fluorescent indicator) or precoated with polyethyylimine (PEI) cellulose.
The following radiochemicals were obtained from New England Nuclear (Dreieich, Germany):

\[^{3}H\]-6-oxo-PGF\(_{1\alpha}\) (120 Ci / mmol),
\[^{3}H\]-TXB\(_{2}\) (120 Ci / mmol),
\[^{3}H\]-PGE\(_{2}\) (120 Ci / mmol),
\[^{3}H\]-PGF\(_{2\alpha}\) (120 Ci / mmol),
\[^{14}C\]-arachidonic acid (20 Ci / mmol),
\[^{3}H\]-adenosine cyclic monophosphate (120 Ci / mmol).

Protein binding assay kits for the measurement of cAMP were obtained from Amersham Radiochemicals (Amersham, UK).

Polyclonal antisera against 6-oxo-PGF\(_{1\alpha}\), TXB\(_{2}\), PGE\(_{2}\) and PGF\(_{2\alpha}\) of high serological specificity were purchased from Capell Laboratories (West Chester, PA, USA) and Cayman Chemical Co. (Ann Arbor, MI, USA).

Polypropylene tubes (2.5 ml) for incubation of tissues and platelets as well as for radioimmunoassays were purchased from Luckham Ltd. (Middlesex, UK), micropipettes and automatic liquid dispensers from Gilson (Anachem Ltd., Luton, Beds, UK), liquid scintillation vials from G & G Chemicals (Berkshire, UK), liquid scintillation fluid from National Diagnostics (Sussex, UK), Multistix from Ames Division (Miles Laboratories Ltd., Stoke Poges, Slough, England) and Percoll from Pharmacia (Uppsala, Sweden).

Pharmaceutical companies supplied the following drugs: milrinone (Sterling Winthrop, Guildford, UK), benzydamine (3M Riker, Loughborough, UK), iloprost (Schering, Burgess Hill, UK), nifedipine (Bayer UK Ltd., Newbury, UK), tiaprofenic acid (Rousell, Uxbridge, UK), nabumetone and 6-MNA (Smith Kline and Beecham, Welwyn Garden City, UK), BW 755C (Wellcome Pharmaceuticals, Beckenham, Kent, UK).
2.2. INSTRUMENTS

a) bench centrifuge (Centra 7R) and sonicator (Soniprep 150): MSE Instruments (Sussex, UK).
b) shaking water bath: Grant Instruments (Cambridge, UK)
c) vortex mixer, vacuum chamber evaporator, magnetic stirrer:
   Gallenkamp Ltd. (Middlesex, UK)
d) pH meter: Corning Science Products, (Middlesex, UK).
e) digital balance: Oertling (Maidstone, Kent, UK).
f) beta particle counter (Rakbeta 1210): LKB Wallac (Turku, Finland).
g) platelet counter; model ZM (Coulter Electronics Ltd., Luton, Beds, UK).

2.3. BUFFER COMPOSITIONS (in mmol / l, unless otherwise stated)

a) HEPES buffer A: 140 NaCl; 2.7 KCl, 1 EDTA, 0.001 PGE₃ (or 100 units / ml apyrase), 3.8 HEPES, 0.1% glucose, 0.1% bovine serum albumin; adjusted to pH 7.6 with Tris base).

b) Ca²⁺ - free HEPES buffer B: 140 NaCl, 2.7 KCl, 8 Na₂HPO₄, 1.5 KH₂PO₄, 0.1 MgCl₂, 1 glucose; adjusted to pH 7.6 with 1M Tris base.

c) Tris-gelatin (RIA): 50 Tris HCl, 1g / l porcine gelatin, adjusted to pH 7.4 with HCl.

d) Dextran coated charcoal (RIA): in Tris gelatin buffer, 6g.1⁻¹ Norit activated charcoal and 100 mg / l dextran (M.W. 5,000).

d) Kreb's Ringer bicarbonate buffer (KRB): 118 NaCl; 4.8 KCl; 1.2 MgSO₄.7H₂O, 1.2 KH₂PO₄, 2.5 CaCl₂.2H₂O; 11 glucose; 24 NaHCO₃.
e) University of Wisconsin preservation solution: 125 KCl; 25 NaCl; 5 MgCl₂, 100 lactobionate, 25 NaH₂PO₄, 5 NaSO₄, 1 allopurinol, 3 reduced glutathione, 30 raffinose, 5 adenosine, pH 7.4.

f) Kidney Preservation solution: 80 KCl, 80 NaCl, 40 MgCl₂, 40 NaSO₄, pH 7.4.

g) Minimum essential medium (MEM): pre weighed dissolved in 1 litre double distilled water, NaHCO₃ added to 24 mmol / l; gassed with 95% O₂ : 5% CO₂.

h) Medium 199: pre weighed preparations dissolved in 1 litre double distilled water, containing 24 mmol / l NaHCO₃, 10mg / l streptomycin sulphate, 10 mg / l actinomycin D, 10% fetal calf serum; gassed with 95% O₂ : 5% CO₂.

i) Phosphate buffer (for cAMP assay): 8 Na₂HPO₄, 5 KH₂PO₄, adjusted to pH 7.6 with HCl and/or NaOH.

2.4. Preparation and incubation of vascular (and other) tissues for assessment of PG synthesis.

Blood vessels were obtained from laboratory rats, rabbits or from humans who were donors for liver transplantation. In the majority of animal experiments, aortae were used. In some animal experiments, mesenteric vasculature, hepatic portal vein, vena cava, femoral and carotid arteries were also used (see chapter 3). The principal human vessels investigated were the iliac vein and iliac artery. These vessels were routinely harvested from donors for liver transplantation, since in some cases they may have been required for anastomosis in the implantation procedure. In the majority of cases,
however, they were not required and were therefore used in the present study. On excision from liver donors, vessels were placed in the University of Wisconsin solution (UW; a preservation solution; see chapter 3) and stored on ice. Since initial studies revealed an unpredictable variation in the release of PGs by human vessels, basic studies on the effect of UW and hypothermic storage on prostanoid release were carried out in vessels from animals, as well as human blood vessels.

In animal experiments, following excision, adventitia and fatty tissue were removed and the vessels cut into 2 mm rings with a scalpel blade on a teflon block. Human vessels were opened longitudinally with scissors, cut laterally into 2mm strips and further into 2mm squares with a scalpel blade on a teflon block, care being taken to avoid touching the endothelium. Human blood vessel segments were then processed in the same way as for animal vessels. Where the effect of endothelium removal was investigated, endothelium was removed either by rubbing an opened vessel with a tissue or in intact vessels with a pipe-cleaner.

In all experiments involving stimulation of PG synthesis with agonists, vascular tissue was pooled and placed in Dulbecco's minimum essential medium (MEM) for up to 9 h to allow trauma - stimulated release of prostanoids to subside (Jeremy et al., 1984a, 1985a, 1985b, 1985c). In some comparative experiments, urinary bladders and tracheal rings (chapters 4 and 5) were also investigated. The bladders were cut longitudinally into four strips and each strip into four further segments and whole trachea into 4 mm rings. Tissues were then pooled and randomised in MEM and incubated for up to 9h in MEM, at 37°C, with changes of medium every 30 min, again to allow
prostanoid release elicited by preparative handling to subside (Jeremy et al., 1984a, 1985a; see fig. 2.1. for explanatory notes).

2.5. Effect of agonists, antagonists and other modulators of intracellular second messengers on vascular PG synthesis

Following pre-incubation of vessels as described above, vessel segments or rings were placed in 2.5ml polypropylene tubes containing MEM. Direct stimulatory effects of any given agent were tested by adding the agents over a concentration range and the tissues further incubated for 30 min to 1h, at 37°C. Following incubation, aliquots of supernatant were taken and stored in a freezer at -70°C prior to radioimmunoassay (RIA; see section 2.9.) of PGs. An example of PG release over 6h pre-incubation and following stimulation with noradrenaline and antagonism with yohimbine (an alpha adrenoceptor antagonist) is shown in fig. 2.1.

2.6. Effect of drugs on PGI₂ synthesis

For studies on the effect of drugs on PG synthesis, tissues (following pre-incubation) were placed in 1 ml MEM containing varying concentrations of drugs (in triplicate for each concentration). The tissues were then incubated for up to 1 h, at 37°C, to allow equilibration of tissues with drugs. The media was then aspirated and replaced with fresh media containing the same concentrations of drugs. Prostanoid synthesis was then elicited by the addition of known stimulators (see result section of chapter 3 for list of stimulators used). The tissues were then further incubated for 30 min or 1 h, at 37°C, in a shaking water bath and aliquots of supernatant taken for estimation of PG concentrations by radioimmunoassay (RIA;
Fig. 2.1. Pattern of PGI$_2$ (as 6-oxo-PGF$_{1\alpha}$ measured by RIA) release from rat aortic rings incubated in MEM at 37°C, illustrating the principal of the method employed to study vascular PGI$_2$ release. The figure shows the gradual subsidence of PGI$_2$ release initially caused by the 'trauma' of preparation. Thus, when PGI$_2$ has reached a basal output a stimulator is added (in this case noradrenaline [NA]) which stimulates PGI$_2$ release linearly over a period of 1h. In turn, this effect is antagonised in a dose-dependent fashion by the addition of increasing concentrations of yohimbine (an alpha-blocker). This response is evidence for a receptor-linked process.
see section 2.9.). Trauma-stimulated PG synthesis was elicited by both freeze fracturing and sonication of tissues. Tissues were sequestered into tubes and pre-equilibrated with drugs as above. 

**Freeze thawing:** the media was then aspirated and tubes placed in a freezer at \(-70^\circ C\) for 3 h, after which time medium containing the same concentrations of drugs were added and the tissues incubated for 1 h, at 37°C. 6-oxo-PGF$_{1\alpha}$ (and/or other PG) concentrations were measured in the supernatant, as above. **Sonication:** following preincubation with drugs and replacement of buffer containing drugs, a sonicator probe was placed in the MEM containing the vascular tissue. The tissues were then subjected to 3 x 10 sec bursts of sonication (20 microns) and the tissues incubated as above and samples of MEM taken for RIA of PGs.

### 2.7. Preparation of washed platelets

Blood was drawn from volunteers or patients who had not ingested any drugs for at least 7 days prior to phlebotomy. One volume of trisodium citrate (3.8%) was added to 9 volumes of blood and platelet-rich plasma (PRP) prepared by centrifugation at x 160g for 15 min. PRP was collected and used in some experiments. Where washed platelets were used, platelet pellets were first prepared by centrifugation at 550 g for 10 min and washed with HEPES buffer A. The washed platelets were resuspended in Ca$^{2+}$ - free HEPES buffer B and adjusted with HEPES buffer to a final count of $2 \times 10^{11}$ platelets / l.
2.8. Platelet TXA$_2$ synthesis by unstimred washed platelets.

a) Effect of platelet activators

Aliquots (100 μl) of PRP or washed platelets were placed in polypropylene tubes and 100 μl of various concentrations of stimulators of platelet activity (collagen, calcium ionophore A23187, NaF, phorbol ester myristate acetate, thapsigargin, arachidonic acid, ADP, adrenaline, U46619) added. Following gentle agitation, the tubes were incubated at 37°C for 30 min and the reaction stopped with 400 μl absolute ultra-pure ethanol. Preliminary experiments detected no change in platelet count after the incubation with stimulators. It was therefore deduced that significant aggregation (a stirring-dependent event) had not taken place. Following vortex-mixing and centrifugation, aliquots of ethanolic supernatant were placed into polypropylene RIA tubes and evaporated in a vacuum chamber. Residues were reconstituted in Tris assay buffer and TXB$_2$ concentrations measured by radiommuoassay. In studies on the spontaneous release of TXB$_2$, platelets were incubated for 2h at 37°C without addition of stimulatory agents. Reactions were stopped with ethanol and platelets processed for the measurement of TXB$_2$ as described in section 2.9. For studies on effect of stimulators on PRP, the only difference in procedure was that PRP was used instead of washed platelets.

b) Effect of drugs on stimulated TXA$_2$ synthesis

Where the effect of drugs and / or modulators of TXA$_2$ synthesis were investigated washed platelets (or PRP) were pre-incubated with drugs for 10 min, at 37°C. Stimulators, at pre-determined doses were added in 10 μl aliquots. The tubes were processed for assessment of TXA$_2$ release as described above in 2.8a.
2.9. Measurement of TXB₂, 6-keto-PGF₁α, PGE₂ and PGF₂α by RIA

In all assay procedures described in this section, Tris HCl-gelatin buffer was used. Platelet extracts were reconstituted with 100 µl TRIS-RIA buffer. Aliquots of supernatant from vascular incubates (usually 5-20 µl) was adjusted to 100 µl with Tris-gelatin assay buffer. Standard curves comprised of 100 µl of known concentrations of eicosanoids over a range of 0-1 ng. To these 100 µl volumes, containing known and unknown quantities of eicosanoid, 200 ul diluted antisera containing 25 nCi [³H]-eicosanoid was added. The tubes were incubated at 4°C overnight. Bound and unbound eicosanoid was separated by the addition of 0.5 ml dextran coated charcoal in Tris buffer to each tube, followed by 10 min incubation at 4°C. Tubes were then centrifuged and supernatants decanted into liquid scintillation vials. Liquid scintillation fluid (10 ml) was added to each vial and counted for radioactivity. Standard curves were compiled and unknown eicosanoid concentrations calculated by extrapolation.

2.10. Validation of radioimmunoassays.

In the RIA of eicosanoids in the present studies, no extraction or purification steps were carried out. This is possible in the present in vitro systems as eicosanoids are generated in large quantities by platelets and vessels (unlike the minute quantities of eicosanoid metabolites found in urine or plasma). Furthermore, there are minimal interfering substances (unlike the situation in urine or plasma) present in the incubation buffers. Nevertheless, a series of validation procedures were carried out. Firstly, antisera were checked for cross reactivity. It was found that there was
considerable variation (as one might expect for polyclonal antibodies) between antisera purchased from different companies and between-batch variation of antisera from the same company. In order to obviate inter-batch variation, large amounts of antisera of the same batch were purchased. Cross reactivities of various PGs with the antisera employed are given in table 2.1. Inter- and intra-assay coefficients of variation are given in table 2.2. In order to establish further the accuracy and possible interference of other endogenous substances within the incubation systems employed, known amounts of 6-oxo-PGF$_1\alpha$ and TXB$_2$ were added to supernatants from vessel and platelet incubations, which were then measured by RIA (see fig. 2.2. for further details). These experiments consolidated the high specificity and reliability of the incubation and RIA methods employed.

2.11. Measurement of platelet cAMP phosphodiesterase activity

Platelet phosphodiesterase activity was assayed as previously described (Jeremy et al, 1988). Aliquots (100µl) of washed platelets were pre-incubated with various concentrations of drug for 10 min, at 37°C. Unlabelled cAMP (10 µmoles [10 µl cAMP solution in HEPES in 90 µl washed platelets]) was also added so as to give approximately 50% conversion of cAMP to AMP under the present incubation conditions. 100 nCi [$^3$H]-cAMP was then added, the platelets rapidly agitated on a vortex mixer and incubated for 5 min at 37°C. The reaction was stopped by the addition of 400 µl ice cold absolute ethanol, containing excess concentrations of cAMP and AMP (both 100 µM). Following vortex-mixing, sonication for 2 x 10 sec and centrifugation, the supernatant was evaporated, in vacuo. Residues
Fig. 2.2. Validation of RIA methods: A = platelet TXB\(_2\) and B = rat aortic 6-oxo-PGF\(_{1\alpha}\)3 aortic rings and 1 ml washed platelets were incubated for 30 min with 10 μM A23187. [O = indomethacin - treated; △ = untreated]. Following incubation and centrifugation, supernatants were taken off and divided into 100 μl aliquots. To each aliquot was added known quantities of TXB\(_2\) or 6-oxo-PGF\(_{1\alpha}\) and further aliquots taken for RIA. In the samples not treated with drugs, zero values were subtracted from the values obtained with added eicosanoid. As can be seen the values measured equate precisely with the amounts added. Thus, it is concluded that: 1) there are no interfering substances released by platelet or vessels that may distort measurements by RIA and 2) there is linearity in immunoreactive TXB\(_2\) and 6-oxo-PGF\(_{1\alpha}\) over a wide concentration range.
were resuspended in ethanol/water (50 / 50; v/v) and cAMP and AMP separated by thin layer chromatography (TLC) on polyethylimine (PEI) cellulose plates, developed in 50 mM KCl (Jeremy et al., 1988c). Unchanged cAMP and AMP were detected under UV light and each band removed from the plate and placed in a liquid scintillation vial. KCl (1 ml; 100 mmol / l) was added and left overnight in a shaking water bath at 40°C to extract the nucleotides from the PEI cellulose. Liquid scintillation fluid was added to each vial and radioactivity counted from which the % inhibition of conversion of cAMP to AMP was calculated.


Aliquots of washed platelets were pre-incubated with various concentrations of drug and/or modulator for 10 min, at 37°C. PGE₁ or iloprost was then added over a pre-established stimulatory concentration range (see fig. 2.3. for dose-response curves) and incubated for 10 min, at 37°C. The reaction was stopped by the addition of 400 ul ice cold absolute ethanol. Following vortex-mixing, sonication for 2 x 10 sec and centrifugation, aliquots of supernatant were evaporated in vacuo. Residues were resuspended in cAMP assay buffer and cAMP concentrations measured by cAMP binding protein radioassay (Amersham International plc), as previously described (Jeremy et al., 1988c).
Fig. 2.3. Effect of PGE₁ (〇) and iloprost (□) on cAMP synthesis by washed human platelets. Each point = mean ± SD, n = 7.
Table 2.1. Cross reactivities of some major PGs with antisera employed in this thesis.

cross reactivity = mass of PG required to displace 50% $[^3H\text{-PG}]$

PG = PG against which antisemum has been raised (e.g. 6-oxo-PGF$_{1\alpha}$) and PG$_X$ = other PG being tested for cross reactivity (e.g. PGE$_1$)

% Cross reactivity

<table>
<thead>
<tr>
<th>Eicosanoid</th>
<th>6-oxo-PGF$_{1\alpha}$</th>
<th>TXB$_2$</th>
<th>PGE$_2$</th>
<th>PGF$_2\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-oxo-PGF$_{1\alpha}$</td>
<td>100</td>
<td>0.001</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>TXB$_2$</td>
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<td>100</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>6-oxo-PGE$_{1\alpha}$</td>
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<td>0.005</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>PGF$_2\alpha$</td>
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<td>0.002</td>
<td>0.02</td>
<td>100</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>0.003</td>
<td>0.005</td>
<td>100</td>
<td>0.1</td>
</tr>
<tr>
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<td>0.003</td>
<td>0.1</td>
<td>0.003</td>
</tr>
<tr>
<td>PGE$_1$</td>
<td>0.001</td>
<td>0.01</td>
<td>8.0</td>
<td>0.05</td>
</tr>
<tr>
<td>PGB$_2$</td>
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<td>0.003</td>
<td>0.05</td>
<td>0.003</td>
</tr>
<tr>
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<td>0.005</td>
<td>0.03</td>
<td>0.002</td>
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<td>Arachidonic acid</td>
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<td>&lt; 0.001</td>
<td>0.01</td>
<td>0.001</td>
</tr>
<tr>
<td>Linolenic acid</td>
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<td>&lt; 0.0001</td>
<td>0.001</td>
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</table>
Table 2.2. Inter-assay and intra-assay coefficients of variation (CV) of eicosanoids measured by RIA. CVs were obtained from 10 measurements of the same sample in the same assay (intra-assay CV [%]) and in 10 separate assays (inter-assay CV [%]).

<table>
<thead>
<tr>
<th>Eicosanoid</th>
<th>intra-assay CV</th>
<th>inter-assay CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-oxo-PGF$_{10\alpha}$</td>
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<td>3</td>
</tr>
<tr>
<td>TXB$_2$</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>PGE$_2$</td>
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<td>4</td>
</tr>
<tr>
<td>PGF$_2$</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

2.13. Platelet lipoxygenase activity

Platelets contain the enzyme lipoxygenase that converts AA to 12-HETE. Thus, the lipoxygenase assay is based on the incubation of platelets with radiolabelled AA ([$^{14}$C-AA]) followed by separation and isolation of 12-HETE by TLC. Aliquots (100 $\mu$l) of PRP were centrifuged at 2,500 rpm for 10 min. The plasma supernatants were discarded and the pellets washed with HEPES buffer B, to remove any residual plasma. HEPES B (100 $\mu$l) containing varying concentrations of drug/modulator was added to the pellets (in triplicate for each concentration of drug) and agitated vigorously to resuspend the platelets. The tubes were incubated for 15 min at 37°C to allow equilibration of the drug with the platelets. To each tube, 100 nCi of [$^{14}$C]-arachidonic acid was added to the tubes which were then vortexed and incubated for a further 30 min at 37°C. The incubates were then transferred to glass tubes and 500 $\mu$l diethyl ether: hexane
(50:50, v/v) added. The tubes were vortexed vigorously, neutral lipids (viz. AA and 12-HETE) being extracted into the organic phase. Tubes were centrifuged and supernatants removed and placed in glass tubes. The remaining aqueous phase was then extracted with 500 ul Folch reagent (chloroform / methanol; 3 / 2, v/v) and vigorously vortexed and centrifuged. The subnatant (Folch is denser than aqueous HEPES) was removed and added to the ether / hexane extract. The pooled extracts were then evaporated in a vacuum chamber. Residues were then redissolved in 50 µl Folch solution and spotted onto the origin of precoated (with silica gel) plastic TLC plates. The plates were then placed in chromatography tanks containing 100 ml diethyl ether: hexane: acetic acid (50 : 50 : 1; v/v). The solvent moved up the plate and when at the top was removed and dried in air. The plate was then be divided laterally into 1 cm bands with a pencil and these bands cut sequentially from the origin upward, placed in vials and liquid scintillation fluid added. The vials were then agitated vigorously and radioactivity counted in a beta-particle counter. The separation profile were then compiled (see fig 2.4.). Both the lipoxygenase inhibitors, NDGA and BW755c (but not indomethacin) inhibited conversion of AA to HETE (fig. 2.4.), consolidating that the present method reflects lipoxygenase and not cyclooxygenase activity.

2.14. PGI₂ synthesis by cultured human umbilical endothelial cells (HUVECS) human omental tissue microvascular endothelial cells (HOTMECs)

HUVECs were prepared by Ms Neelima Shukla of the Department of Surgery (Royal Free Hospital), using the method of Jaffe et al.
Fig. 2.4. A. Running positions of phospholipids, 12-HETE and arachidonic acid (AA) on TLC plates coated with silica gel G and developed in solvent mix: hexane / diethyl ether / acetic acid (50 / 50 / 1; v / v / v).

B. Effect of NDGA (○; lipoxygenase inhibitor), BW755c (●; lipoxygenase inhibitor) and indomethacin (▲; cyclooxygenase inhibitor) on conversion of $[^{14}\text{C}-\text{AA}]$ to $[^{14}\text{C}-12\text{-HETE}]$ by washed platelets (each point = mean ± S.D.), $n = 6$. 
Umbilical cords were obtained from women who delivered normal healthy babies spontaneously and who experienced no complications during pregnancy. Following flushing of the umbilical vein with MEM, the cord was clamped at one end and MEM containing 1% collagenase inserted into the venous lumen and the cord clamped at the other end. The cord was then immersed in KRB and incubated at 37°C, for 30 min. The clamp was removed and the fluid containing endothelial cells decanted into a flask. Cells were washed with MEM and centrifuged at 1,000 rpm and the supernatant discarded. This procedure was carried out three times. The washed cells were then placed in a 100ml culture flask containing 5 ml medium 199. Following initial culture in medium 199 (containing 20% fetal calf serum [FCS]) for 5 days, cells were treated with MEM containing trypsin (10 Units / ml) which dislodged the cells which adhere to the flask bottom. The dislodged cells were seeded into 96-well tissue culture plates and further grown in medium 199 containing 20% FCS. A modification of the method of Kern et al., (1983) was used to prepare HOTMECs for culture. Omental fat (20-75g) was obtained from patients undergoing routine surgery. The fat was incubated in 1% collagenase for 30 min at 37°C. the digest was filtered, allowed to settle and adipocytes removed. The remaining solution rich in vessel fragments were centrifuged in 45% Percoll for 20 min, the vessel fragments forming a layer near the top of the density gradient. This endothelial rich layer was then removed and placed in M199 containing 20% FCS and cultured as above for HUVECs. When confluent (2-5 days), the cells were washed with pre-warmed MEM and MEM containing stimulators (± drugs) added. The plates were then incubated at 37°C in an incubator and the supernatants from each
well removed and stored at -70°C for radioimmunoassay of 6-oxo-PGF₁α
and other PGs as described above.

2.15. Ex vivo experiments on rats with diabetes (pair fed and fed ad
libitum) or hepatic portal hypertension.

2.15a. Diabetes

All experiments were carried out using male Sprague Dawley rats
(initial body weight of 250 g). Non-ketonuric, hyperglycaemic
diabetes was induced by injecting streptozotocin intravenously (tail
vein; 65 mg / kg body wt.). These rats developed glycosuria but not
ketonuria or haematuria. In some experiments, diabetic rats were fed
either ad libitum or pair fed with Diet 41B (Grain Harvester, Kent,
UK) and allowed free access to water. In the pair fed experiments,
all animals were given 25g rat chow per day. This is the average food
intake of normal age-matched rats (Thompson et al., 1990). Insulin
(10 i.u., twice daily) was administered to one group of diabetic rats
fed ad libitum. Seven of each group of rats were sacrificed after 8
weeks of diabetes and the results compared with seven age matched
controls. Rat urine was monitored for glucose, ketone bodies and
proteins with Multistix (Ames Diagnostics, Stoke Poges). At the time
of sacrifice, blood was collected by cardiac puncture for measurement
of blood glucose.

After 8 weeks rats were anaesthetised with pentobarbitone (90 mg
/ kg intraperitoneally; Sagatal: May and Baker Ltd., Dagenham, UK). A
midline incision was made into the abdominal cavity and the hepatic
portal vein, aortae, carotid arteries, gastrointestinal (GI) tract,
urinary bladders and trachea were rapidly excised and placed in MEM.
Given the small size of the vasculature in the mesenteric bed and the
presence of large amounts of adipose tissue, venous and arterial mesenteric vessels were not dissected from each other. The intestinal segments were opened by longitudinal incision and rinsed free of residual food. Mucosa was separated from the muscular portion of duodenal, jejunal and ileal tissue with a glass slide (Debnam and Levin, 1975) and placed in MEM. Stomach mucosa and muscularis were separated as previously described (Jeremy et al., 1987). The muscular portion was cut into 2mm wide strip with a scalpel blade and then into 2mm squares; 4 squares in duplicate for each animal were placed in polypropylene tubes containing MEM and incubated in a shaking water bath for 30 min. Mucosal scrapes were washed three times in MEM followed by centrifugation. The mucosal scrape from each intestinal portion and the gastric mucosal discs (in duplicate for each animal) were placed in 1ml MEM and incubated and processed as for muscularis tissue. Following incubation the tubes were centrifuged at 200 rpm, for 10 min and aliquots of supernatant stored at -70°C prior to measurement of 6-oxo-PGF$_{1\alpha}$, PGE$_2$, PGF$_{2\alpha}$ and TXB$_2$ by RIA.

Mesenteric vessels were dissected away from adherent adipose tissue and cut into 1mm lengths of vessel for each animal. Hepatic portal vein was cut into approximately 1mm squares. Aortae and carotid arteries were cut into 1mm rings. Approximately 10 mg vascular tissue (in duplicate for each animal) were incubated in 1ml MEM for 1h at 37°C. Aliquots of supernatant were stored at -70°C for measurement of 6-oxo-PGF$_{1\alpha}$, PGE$_2$, PGF$_{2\alpha}$ and TXB$_2$ by RIA.

2.15b. Hepatic portal hypertension.

Male Sprague Dawley rats of body weight 250g were used. Portal hypertension was induced surgically by partial ligation of the portal
vein (Cummings et al., 1986). These procedures were carried out by Dr Aiden McCormick of the Department of Medicine, Royal Free Hospital. This form of experimental portal hypertension differs from other means of eliciting this state (e.g. by administration of carbon tetrachloride) in that hypertension develops without significant liver dysfunction. Control rats comprised of sham-operated animals. After 7 days, animals were anaesthetised with pentobarbitone (90 mg / kg, i.p.) and portal blood pressure measured following cannulation of the superior mesenteric vein. Vascular tissue was not collected from sites of cannulation or ligation. Thoracic aortae and mesenteric vasculature were excised and processed for assessment of PG\textsubscript{I\textsubscript{2}} release as described above.

2.16. Statistics

Throughout this study, statistical significances between mean values was determined by paired or unpaired Student's two-tailed t test. Values are expressed as mean \(\pm\) SEM or SD.
CHAPTER 3
STUDIES ON PGI$_2$ AND OTHER PROSTANOIDS RELEASED BY BLOOD VESSELS FROM THE RAT, RABBIT AND MAN AND ON TXA$_2$ RELEASE BY HUMAN PLATELETS.

3.1. INTRODUCTION

As outlined in the general introduction, the release of PGI$_2$ from vascular tissue is elicited by a range of vasoactive agonists. This process, in turn, is mediated by intracellular 2nd messengers, including G proteins, PKC, and Ca$^{2+}$. However, few systematic studies between species and between different vessels of the same species have been carried out. This is of importance since data obtained from any one given vessel may not be representative of other vessels in the same species. This point is exemplified by the often-raised objection against using the aorta from laboratory animals as a representative tissue for studies on CVD or drug action (e.g. Tomlinson et al., 1992). It has been suggested that as a conduit vessel, the aorta lacks the sensitivity of other smaller vessels (viz. arterioles and microvascular beds; Tomlinson et al., 1992).

Secondly, receptors and linked mechanisms controlling PGI$_2$ synthesis in a vessel from any given species may vary considerably from other species. This is of importance since the mechanisms underlying a given biofunction in a single species often differ when compared to the general picture obtained from other species. Furthermore, since the results of experiments in animal models/tissues (at least in clinical research) are often extrapolated to man, it is clearly important that the properties of a 'model' vessel should in fact, resemble that of man. In order to examine these areas further, PGI$_2$ release, in response to a range of receptor agonists and
intracellular mediators by different blood vessels from the rat, rabbit and man were investigated. The role of the endothelium in mediating vascular PGI$_2$ release in these vessels was also investigated since there is some confusion as to the relative contribution of the endothelium to 'total output' of PGI$_2$.

The studies on human vessels were complicated by several factors. Firstly, since blood vessels were obtained from donors for liver transplantation, the donors were invariably the victims of traffic accidents (head injuries) or cerebrovascular events (aneurysm). Thus, these unfortunate victims had been sustained on life support systems for variable times and had received a variable range of drugs. Secondly, the vessels, following excision, were placed in preservation solutions (University of Wisconsin; see below) and stored on ice for up to 18h prior to experimentation. In order to determine the effects of hypothermic storage in preservation solutions on vascular PGI$_2$ release, systematic studies on the effect of cold preservation of blood vessels (rat and rabbit) were also carried out. These studies are also of fundamental interest to transplant surgery for several reasons. Primary graft dysfunction, following orthotopic liver transplantation, occurs in about 10% of cases (Maddrey et al., 1988; Starzl et al., 1989; Busuttil et al., 1987; Krom et al., 1989; Quiroga et al., 1991) and is a major cause of morbidity and mortality. The cause of primary graft dysfunction is multifactorial but "storage injury" may be an important contributory factor in a subset of patients (Kazikoe et al., 1990). Studies using rat livers have demonstrated that significant morphological damage to endothelial and Kupffer cells occurs following reperfusion after hypothermic storage of the organs.
in Euro-Collins solution and to a lesser degree in University of Wisonsin (UW) solution (Caldwell-Kenkel et al., 1989; 1990). The functional significance of these morphological changes is unknown. However, the vascular endothelium influences haemostasis and vasoactivity, in part, through the secretion of PGI$_2$, as well as endothelium derived relaxing factor (EDRF; is obligatory for relaxation and also inhibits platelet activity; Furchgott, 1983; Ignarro, 1989; Rubanyi, 1990). Damage to the endothelium and disruption of EDRF and PGI$_2$ release may therefore result in vasoconstriction or thrombosis, which in turn would endanger graft function. Indeed there is a considerable body of evidence that ischaemic injury is a major cause of early graft failures (Maddrey et al., 1988; Starzl et al., 1989; Busuttil et al., 1987; Krom et al., 1989; Quiroga et al., 1991).

TXA$_2$ synthesis by platelets obtained from patients, has largely been investigated either by: a) allowing blood to clot and measuring TXA$_2$ released into the serum by radioimmunoassay (Viinikka and Ylikorkala, 1981) or b) following in vitro aggregation (carried out in plasma) elicited by known aggregating agents (e.g. adrenaline, ADP, collagen; Diczfalusy et al., 1977; Mikhailidis et al., 1983, 1985a, 1985b). In terms of determining the aetiological role of TXA$_2$ in any given disorder, both of these methods have drawbacks. Firstly, components of blood, (e.g. fatty acids, glucose, lipoproteins, etc.), have been shown to influence aggregation and therefore TXA$_2$ release (Hendra and Betteridge, 1988). Although the effect of blood components on aggregation and or TXA$_2$ synthesis and release is in itself of importance one is still confronted by the question as to whether TXA$_2$ release is primary or secondary to
platelet activation. With regard to the study of TXA$_2$ released in response to aggregating agents, we have recently established that in platelets from patients with PVD, which are known to be hyperaggregable and which liberate more TXA$_2$ than controls (Mikhailidis et al., 1985a), that TXA$_2$ synthesising capacity is actually diminished (Jeremy et al., 1988b). This latter finding implies that TXA$_2$ may not play an aetiological role in hyperaggregability of platelets but rather is secondary to the enhanced aggregation.

In order to dissect out TXA$_2$ synthesis from aggregation and to obviate the variable of effects of plasma/serum factors, a method for the study of TXA$_2$ synthesis in unstimred, washed platelets was developed and the following studies carried out to investigate: 1) the effect of pro-aggregatory agonists, ADP, adrenaline, collagen, arachidonic acid and A23187 on TXA$_2$ synthesis, 2) the role of Ca$^{2+}$ in mediating stimulation of TXA$_2$ synthesis using Ca$^{2+}$ chelators, Ca$^{2+}$ channel blockers, high potassium, and thapsigargin (mobilises intracellular Ca$^{2+}$; Thastrup et al., 1990), and 3) the role of PKC using phorbol ester and staurosporine. The methods described in chapter 2 were applied.

3.2. RESULTS

3.2a. Effect of endothelium removal on receptor-linked PGI$_2$ release by the rat aorta.

At 3h post-endothelium removal, concentration-PGI$_2$ response curves of the stimulatory agents investigated (adrenaline, noradrenaline, PDBU, NaF) were shifted markedly to the left (Fig. 3.1.). At 6h post-endothelium removal, however, there were no
differences between these dose-response curves from intact aortae or from aortae with endothelium removed (Fig. 3.1.). Substance P, acetylcholine and nitroprusside were without significant effect on de novo rat aortic PGI₂ synthesis, irrespective of incubation time or the presence of the endothelium (data not shown). At 3h post-endothelium removal, the inhibitory action of yohimbine, prazosin and nifedipine on adrenaline- on noradrenaline- stimulated PGI₂ synthesis were markedly diminished (fig. 3.2.) whilst those of control aortae were similar to previously described data (Jeremy et al., 1985a). At 6h post-endothelium removal the inhibitory action of yohimbine, prazosin and nifedipine were partially restored, although still significantly diminished, whilst at 9h post-endothelium removal, it was completely restored in aortae without endothelium (fig. 3.2.). Relaxors of rat aortic tissue; substance P, Ach, carbachol and nitroprusside (all up to 3 x 10⁻⁴ M) were without significant effect on noradrenaline-stimulated PGI₂ synthesis in aortae with and without endothelium (data not shown).

3.2b. Comparative studies on PGI₂ release by arteries and veins from the rat and rabbit.

In all blood vessels (other than the aorta) investigated in the rat (carotid and hepatic portal vein), PGI₂ synthesis was stimulated by adrenaline, PDBU, NaF, A23187 and thapsigargin (fig. 3.3.), whereas histamine, Ach, carbachol, 5-HT, nitroprusside and KCl were all without significant effect (data not shown). In the rabbit, phenylephrine (an α₁- adrenoceptor agonist), Ach, A23187, AA, NaF and PDBU all stimulated PGI₂ release by the aorta (fig. 3.4.). In the carotid artery, femoral artery and hepatic portal vein of the
rabbit, PGI$_2$ synthesis was stimulated by PDBU, phenylephrine, adrenaline, Ach, A23187, NaF, carbachol and thapsigargin (fig. 3.5.). Histamine and 5-HT were without effect in all rabbit vessels. Removal of the endothelium from the rabbit aorta markedly reduced the release of PGI$_2$ in response to phenylephrine, Ach, A23187, AA, NaF, PDBU (fig. 3.4.).

3.2c. Studies on PGI$_2$ release by human vessels, HUVECs and HOTMECs

Responses in the human vessels (iliaic artery and vein), as one might expect, were variable. In some samples, clear cut dose-response curves were obtained for the various stimulators investigated, whereas in vessels from other individuals, responses were virtually non-existent or displayed excessive background release of PGI$_2$ (as one encounters in damaged vessels [e.g. by freeze-thawing]). This inter-individual variation is exemplified by the responses obtained with the same stimulators in iliac arteries from six consecutive donors (the first six studied; fig. 3.6.). In concomitant experiments on contraction-relaxation in an organ bath using segments of the same human vessels examined for PG release, there were also marked inter-individual variations ranging from reasonable dose-response curves to excessive spontaneous activity, spasmogenic responses to agonists and absolutely no activity at all (Karatapanis and Jeremy, unpublished observations). Therefore, for the compilation of dose-response curves, data from individuals where there was a zero response or where responses resembled vessels where membrane function had been disrupted, were excluded. Thus, in both iliac artery and vein, PGI$_2$ synthesis was stimulated by: phenylephrine, adrenaline, Ach, carbachol, histamine, 5-HT
(fig. 3.7), PDBU, NaF, thapsigargin, A23187 and AA (fig. 3.8) in
dose-dependent manners. Using single maximal doses of selected
stimulators, removal of endothelium in human iliac artery and vein
had minimal effect on PGI$_2$ synthesis (table 3.1.). In cultured
HUVECs, PGI$_2$ synthesis was stimulated with A23187, AA, NaF, PDBU,
histamine and thrombin but not 5-HT, carbachol, or noradrenaline
(fig. 3.9a) and in HOTMECs with A23187, AA, NaF, PDBU but not by
histamine, thrombin, 5-HT, carbachol or noradrenaline (fig. 3.9b).

3.2d. Effect on PGI$_2$ release following hypothermic storage of aortae
(rats and rabbits) and human iliac artery in organ preservation
solutions.

Incubation of rat aorta in either UW or KPS completely inhibited
the release of PGI$_2$ elicited by noradrenaline, PDBU, A23187 and NaF
(fig. 3.10). In contrast, AA-stimulated PGI$_2$ release was not
significantly different from controls (fig. 3.10). Following cold
storage of aortic tissue in MEM, UW, or KPS for 12h, transferral of
all tissues to MEM and immediate incubation in the presence of
noradrenaline, at 37°C for 1h, there were marked enhancements of
both basal and noradrenaline-stimulated PGI$_2$ release (fig. 3.11.)
These enhanced responses reversed to zero time (i.e. without cold
storage) responses (fig. 3.11.). Identical patterns of enhancement
and reversal were also found following storage for 24h and 48h (data
not shown). Following cold storage in UW, kidney preservation
solution or MEM for 24h and 48h after 1h incubation in MEM,
responses to noradrenaline were similar to those in fresh tissue
(fig. 3.12.). Following cold storage for 72h (and after 1h
incubation in MEM) the response to noradrenaline was significantly
diminished when compared to the zero time dose-response curve for all 3 solutions (fig. 3.12.). Following cold storage of intact rabbit aortic rings in UW, MEM or KPS for 24 h and 48h, PG\textsubscript{I}\textsubscript{2} release in response to Ach, phenylephrine, PDBU, NaF, A23187 and AA was unaffected by storage in UW, MEM or KPS (table 3.2.). Following cold storage of intact human iliac artery and vein segments in UW, MEM or KPS for 24 h and 48h, PG\textsubscript{I}\textsubscript{2} release in response to acetylcholine, PDBU, NaF, A23187 and AA was unaffected by storage in UW, MEM or KPS (table 3.3.).

3.2e. Basic studies on platelet TXA\textsubscript{2} synthesis.

TXA\textsubscript{2} synthesis by washed, unstirred platelets was stimulated in dose-dependent manners by collagen, NaF, A23187, phorbol ester myristate acetate (PMA), thapsigargin and AA but not by adrenaline, ADP, PAF, U46619 or 5-HT (fig. 3.13). Response to stimulators was rapid and reached maximal at 10 min after addition of stimulators (fig. 3.14b). TXA\textsubscript{2} synthesis was directly proportional to the number of platelets present, irrespective of the stimulator used (fig. 3.14a). The Ca\textsuperscript{2+} channel blockers nifedipine (3.15a) and diethylstilbestrol (3.15b) inhibited platelet TXA\textsubscript{2} synthesis when stimulated by NaF, PMA and A23187 but not when stimulated by AA or freeze fracturing. The PKC inhibitor, staurosporine inhibited platelet TXA\textsubscript{2} synthesis when stimulated by NaF and PMA but to a lesser degree when stimulated by A23187 but not with AA or freeze fracturing (fig. 3.15c).
Fig. 3.1. Effect of endothelium removal on: adrenaline-, noradrenaline-, phorbol ester dibutyrate- and sodium fluoride- stimulated rat aortic PGI₂ (as 6-oxo-PGF₁α) release at 3h and 6h post-removal of endothelium. Intact aorta (▲); aorta with endothelium removed (●). Each point represents mean ± SD (n=6).
Fig. 3.2. Effect of endothelium removal on antagonism of adrenaline- 
($3 \times 10^{-6}$ M) stimulated PGI$_2$ synthesis at 3h, 6h and 9h following 
removal of endothelium. Intact aorta (▲); endothelium removed (●). 
Each point represents mean ± SD, n = 6. (rat)
Fig. 3.3. Effect of various agonists on PGI$_2$ synthesis by different blood vessels of the rat. A = femoral artery, B = carotid artery, C = hepatic portal vein. Phorbol ester dibutyrate (●), adrenaline (▲), A23187 (▼), NaF (■), thapsigargin (◆), acetylcholine (○), carbachol (●), 5-HT (○) and histamine (○). Each point = mean ± S.D., n = 6.
Fig. 3.4. Effect of a range of vasoactive substances on PGI$_2$ (as 6-oxo-PGF$_{1 \alpha}$) release by rabbit aortic rings. Intact (O); endothelium removed (△). Each point = mean ± SD, n = 8.
Fig. 3.5. Effect of various stimulators on PGI\(_2\) synthesis by different blood vessels of the rabbit.

Phorbol ester (○), phenylephrine (◇), adrenaline (▲), acetylcholine (◆), A23187 (□), NaF (●), carbachol (□) and thapsigargin (△). Each point = mean ± S.D., n = 6.
Fig. 3.6 Effect of various agonists on PGI$_2$ synthesis by human iliac artery from six individuals studied consecutively. Acetylcholine (▽), phenylephrine (◇), phorbol ester (○), A23187 (●), 5-HT (□), histamine (△).
Each point = mean of triplicate measurements.
Fig. 3.7. Effect of various agonists on PGI\(_2\) synthesis by the iliac artery and vein of man. Phenylephrine (△), adrenaline (■), acetylcholine (▽), carbachol (◆), 5-HT (○) and histamine (◇). Each point = mean ± S.E.M., n = 15.
Fig. 3.8. Effect of a range of intracellular second messenger activators on PGI₂ synthesis by A) human iliac artery and B) human iliac vein.

phorbol ester (O), A23187 (V), thapsigargin (□), NaF (△), AA (△) and basal (●).

Each point = mean ± SEM, n=15.
Fig. 3.9. PGI$_2$ (as 6-oxo-PGF$_{1\alpha}$) synthesis by A) HUVECs and B) HOTMECs in response to various stimulators (concentrations are given in parentheses). Each histogram represents mean ± S.D., n = 6.
Table 3.1. Effect of endothelium removal on PGI₂ synthesis (pg 6-oxo-PGF₁α / mg tissue / 30 min; mean ± SD, n = 8) by human iliac artery elicited by a range of stimulators.

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>Intact vessel</th>
<th>with endothelium removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylephrine (10 μM)</td>
<td>138 ± 37</td>
<td>124 ± 22</td>
</tr>
<tr>
<td>Acetylcholine (10 μM)</td>
<td>128 ± 38</td>
<td>118 ± 35</td>
</tr>
<tr>
<td>Histamine (10 μM)</td>
<td>75 ± 19</td>
<td>65 ± 16</td>
</tr>
<tr>
<td>5-HT (10 μM)</td>
<td>45 ± 9</td>
<td>40 ± 14</td>
</tr>
<tr>
<td>NaF (10 mM)</td>
<td>150 ± 60</td>
<td>135 ± 28</td>
</tr>
<tr>
<td>PDBU (1 μM)</td>
<td>179 ± 26</td>
<td>166 ± 28</td>
</tr>
<tr>
<td>A23187 (10 μM)</td>
<td>168 ± 36</td>
<td>150 ± 22</td>
</tr>
<tr>
<td>AA (10 μM)</td>
<td>175 ± 19</td>
<td>170 ± 24</td>
</tr>
<tr>
<td>thapsigargin (3 μM)</td>
<td>73 ± 18</td>
<td>68 ± 16</td>
</tr>
</tbody>
</table>
Fig. 3.10. Effect of incubation of rat aortic rings in minimum essential medium (MEM), University of Wisconsin solution (UW), kidney preservation solution (KPS) on PGI$_2$ synthesis when stimulated with nor-adrenaline (NA; 10 $\mu$M), NaF (10 mM), phorbol ester dibutyrate (PE; 1 $\mu$M), Ca$^{2+}$ ionophore A23187 (A23187; 3 $\mu$M), arachidonic acid (AA; 3 $\mu$M). Each histogram represents mean $\pm$ S.D., n = 6.
Fig. 3.11. Effect of cold storage in MEM (●), KPS (▼), or UW (▲) for 12h followed by incubation of rat aortic rings in MEM (for i) zero time, ii) 30 min and iii) 1 h) prior to stimulation of PGI₂ with noradrenaline (NA). Each point represents mean of 4 determinations. (○) represents the NA-PGI₂ dose-response curve obtained in fresh (not cold-stored) aorta.
Fig. 3.12. Effect of cold storage in MEM (●), KPS (◇) or UW (△) for: i) 24 h, ii) 48 h and iii) 72 h followed by incubation of rat aortic rings in MEM for 1 h prior to stimulation of PGI₂ with NA. Each point represents mean of 4 determinations. (○) represents the NA-PGI₂ dose-response curve obtained in fresh (not cold-stored) aorta.
Table 3.2. Effect of hypothermic storage of rabbit aortic rings in preservation solutions (University of Wisconsin's [UW] and kidney preservation solution [KPS]) and culture medium (MEM) for 24 h and 48 h on PGI$_2$ synthesis (pg 6-oxo-PGF$_{1\alpha}$ / mg tissue / min; mean ± S.D., n = 8) elicited by a range of stimulators (phenylephrine [PE], acetylcholine [Ach], NaF, PDBU, A23187 and AA).

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>MEM</th>
<th>UW</th>
<th>KPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 time</td>
<td>24h</td>
<td>48h</td>
</tr>
<tr>
<td>PE</td>
<td>44±7</td>
<td>42±8</td>
<td>36±11</td>
</tr>
<tr>
<td>Ach</td>
<td>38±8</td>
<td>33±6</td>
<td>31±8</td>
</tr>
<tr>
<td>NaF</td>
<td>120±21</td>
<td>115±14</td>
<td>108±14</td>
</tr>
<tr>
<td>PDBU</td>
<td>84±9</td>
<td>76±8</td>
<td>78±12</td>
</tr>
<tr>
<td>A23187</td>
<td>118±26</td>
<td>120±22</td>
<td>126±28</td>
</tr>
<tr>
<td>AA</td>
<td>135±16</td>
<td>145±25</td>
<td>148±22</td>
</tr>
</tbody>
</table>
Table 3.3. Effect of hypothermic storage of human iliac artery in preservation solutions (University of Wisconsin's [UW] and kidney preservation solution [KPS]) and culture medium (MEM) for 24 h and 48 h on PGI₂ synthesis (pg 6-oxo-PGF₁α / mg tissue / 30 min; mean ± SD, n= 12) elicited by a range of stimulators (phenylephrine [PE], acetylcholine [Ach], NaF, PDBU, A23187 and AA.

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>MEM</th>
<th>UW</th>
<th>KPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 time</td>
<td>24h</td>
<td>48h</td>
</tr>
<tr>
<td>PE</td>
<td>128±27</td>
<td>122±32</td>
<td>110±11</td>
</tr>
<tr>
<td>Ach</td>
<td>118±28</td>
<td>123±26</td>
<td>111±18</td>
</tr>
<tr>
<td>NaF</td>
<td>130±41</td>
<td>125±18</td>
<td>104±24</td>
</tr>
<tr>
<td>PDBU</td>
<td>184±29</td>
<td>176±38</td>
<td>178±26</td>
</tr>
<tr>
<td>A23187</td>
<td>198±46</td>
<td>180±32</td>
<td>176±28</td>
</tr>
<tr>
<td>AA</td>
<td>185±26</td>
<td>175±25</td>
<td>168±32</td>
</tr>
</tbody>
</table>
Fig. 3.13. Effect of different stimulators of platelet aggregation on TXA$_2$ (measured as TXB$_2$) generation by washed human platelets: A23187 (Δ), phorbol ester myristate acetate (●), AA (▲), collagen (▲), NaF (◇), thapsigargin (○), adrenaline (□), ADP (□), U46619 (◇), 5-HT (□) and PAF (□). Each point = mean ± S.E.M., n = 7 healthy volunteers.
Fig. 3.14. TXA₂ (as TXB₂) release in response to various stimulators by: A) different numbers of platelets and B) by same number of platelets over 10 min. Each point = mean ± S.D., n = 6. 1 μM Phorbol ester (□), 3 μM A23187 (◇), 3 μM AA (●), 10 μM thapsigargin (○), 10 mM NaF (△).
Fig. 3.15. Effect of a) nifedipine, b) diethylstilbestrol and c) staurosporine on TXA$_2$ synthesis stimulated by phorbol ester myristate acetate (●), NaF (◇), A23187 (□), arachidonate (○) and freeze-fracturing. Each point = mean ± S.E.M., n = 6. (△)
3.3. DISCUSSION

3.3a. Basic studies on blood vessels

The first point to emerge from this study is the remarkable similarity of responses of different vessels within the same species. Thus, although absolute output of PGI$_2$ varied, almost identical patterns of response to the same substances were obtained for the aorta, carotid and hepatic portal vein of the rat and rabbit. Also in the human vessels, responses were basically similar in both the iliac artery and iliac vein. Thus, although it has often been suggested that the aorta is an inappropriate vessel to use as a model of the vasculature (Tomlinson et al., 1992), the present data indicate that the aorta constitutes an adequate representative model of at least other larger vessels within the same species. It is conceded, however, that such may not be the case in smaller vessels, including arterioles, venules and capillaries (i.e. the microvasculature). From an investigative point of view, the study of microvasculature is fraught with methodological pitfalls. Perfusion of organs with dense microvasculature (e.g. lung) and measurement of PGs in the perfusate following addition of vasoactive agents has been used to assess PGI$_2$ output by microvascular beds (e.g. Stevens et al., 1992). However, it is impossible to exclude the contribution of surrounding tissue to the PGI$_2$ measured in a perfusate. For example, the lung consists of large amounts of bronchial smooth muscle. In turn, airways tissue releases large amounts of PGI$_2$ in response to agonists which do not elicit PGI$_2$ release in blood vessels. For example, in the trachea of the rat, parasympathomimetics (but not sympathomimetics) stimulate PGI$_2$ release,
(Jeremy et al., 1989) whereas the opposite is the case for vascular tissue (this thesis). This point is further considered in the analysis of PGI\(_2\) release from vasculature, trachea and lungs of diabetic rats in chapter 5. Perhaps in order to fully clarify the intra-species variation of PGI\(_2\) release by different vessels, studies on larger animals, where sufficient amounts of small vessels are available, would further clarify this question.

The second point to emerge from the present study was the inter-species differences in responses. The most notable variations were seen with different receptor agonists. Although all species, including man, responded to adrenergic stimulation, the rat and rabbit failed to release PGI\(_2\) in response to histamine or 5-HT. In contrast, histamine and 5-HT were potent stimulators of PGI\(_2\) in vessels from the human vessels. The most striking finding was that Ach, a relaxor of blood vessels (Furchgott, 1983) had no effect on PGI\(_2\) in rat vessels and yet was a potent stimulator of PGI\(_2\) release by rabbit and human vessels. It had previously been suggested that only activators of constriction (noradrenaline, histamine, 5-HT, angiotensin II) cause the release of PGI\(_2\) by blood vessels (Jeremy et al., 1985a; 1998a). The rabbit and human data however, confound this proposal since Ach is a classic relaxor of vascular tissue. In contrast, other relaxors (nitroprusside, substance P) were without effect on PGI\(_2\) release in these vessels which would seem to exclude a consistent relationship between vasodilators and PGI\(_2\) release. Further studies are currently underway by the author to establish which mechanisms are involved in Ach-stimulated PGI\(_2\) release from vessels but due to the constraints of time cannot be included in this thesis.
In contrast to the vasoactive agonists there was universal stimulation of PGI$_2$ release with phorbol ester (activates PKC; Nishizuka, 1984), NaF (a non-specific G protein activator; Gilman, 1987), A23187 (creates artificial Ca$^{2+}$ channels; Reed and Lardy, 1972), thapsigargin (mobilises intracellular Ca$^{2+}$; Tharstrup, 1990) and AA (PGI$_2$ substrate; Bergstrom et al., 1968) by all vessels (as well as in cell cultures) investigated. Thus, the mechanism derived from the rat aorta: that PGI$_2$ synthesis is mediated sequentially by G proteins, PKC, Ca$^{2+}$ mobilisation, PLA$_2$ activation, AA release and conversion of AA to PGI$_2$ (see fig. 1.3. in chapter 1) appears to be universally valid for all vascular tissue. This is of importance since in any one study (e.g. in dissecting out alterations of mechanisms in a disease model, ex vivo or in vitro or in the study of sites of drug action) it is reasonable to extrapolate data obtained using these modulators in a vessel from a laboratory animal model to man.

The third point to emerge from the present study was the role of the endothelium in mediating PGI$_2$ release by whole vessels. In the rat aorta, the endothelium contributes negligible amounts of PGI$_2$ to the total output of this prostanoid, at least in response to the stimulatory agents investigated. The main source of PGI$_2$ in this vessel appears, therefore, to be of medial and/or intimal origin. PGI$_2$ synthesis was observed in response to adrenaline, noradrenaline, U46619, phorbol ester and fluoride in aortae without endothelium compared to vessels with intact endothelium. Similarly, at 3h post-endothelium removal there was a marked reduction in the inhibition of noradrenaline-stimulated PGI$_2$ synthesis by yohimbine and prazosin and by Ca$^{2+}$ channel blockade. Previous studies have
shown that removal of the endothelium results in diminished responsiveness to vasodilators (Furchgott, 1983) and increased response to vasoconstrictors (Egleme et al., 1984) which has led to the concept that vascular reactivity is controlled by EDRF (Furchgott, 1983). The data at 3h post-endothelium removal would therefore seem to indicate that the stimulation of rat aortic PGI₂ synthesis and inhibition of PGI₂ by specific alpha-adrenergic antagonism and Ca²⁺ channel blockade are dependent on the secretion of EDRF. However, protracted incubation (6h and 9h) of the aorta without endothelium normalised these changes of response to stimulators and inhibitors of PGI₂ synthesis, despite continued absence of the endothelium (as assessed by scanning EM). These findings therefore suggest that removal of the endothelium per se rather than the secretion of EDRF, determines these initial (at 3h) changes. Since endothelium removal constitutes a severe trauma to the vessel, it is possible that this mechanical injury sensitises the vessel, thereby enhancing and diminishing responsiveness to stimulators and inhibitors, respectively. The author is aware of no systematic study on mechanical injury and vascular responsiveness in vitro, but it is known that trauma (sustained by wounded soldiers in the 1st World War) causes prolonged spasm in human arteries (Kinmonth et al., 1962), although this would seem to warrant further investigation.

In complete contrast to the rat aorta, removal of the endothelium in the rabbit aorta abolished PGI₂ release in response to both receptor agonists (phenylephrine, Ach), intracellular modulators (NaF, PDBU, A23187) and substrate (AA). These data indicate that in the rabbit the endothelium is the major source of
PGI₂. That this release is mediated by EDRF (as is relaxation of this vessel; Furchgott, 1983; Ignarro et al., 1990) is unlikely since in intact vessels nitroprusside (degrades to form NO which in turn is considered to be EDRF; Palmer et al., 1989) was without effect on PGI₂ release. In contrast, responses in the human iliac artery were relatively unimpaired by removal of endothelium. Thus, of the species investigated the rabbit appears to be anomalous vis a vis the contribution of the endothelium to the total output of PGI₂ by conduit arteries. It should be stressed that the author is not proposing that endothelial PGI₂ is not biologically relevant. Rather, that when assessing output of PGI₂ by blood vessels, particularly in response to agonists and/ or other stimulators, the PGI₂ measured should not be solely ascribed to the endothelium but rather to the smooth muscle component of the vessels (with the exception of the rabbit). This point is exemplified by the data obtained from human endothelial cell cultures (HUVECs and HOTMECs). In HUVECs, PGI₂ release was stimulated by thrombin, histamine, phorbol ester, NaF, A23187 and AA and in HOTMECs by phorbol ester, NaF, A23187 and AA but not by histamine, thrombin, carbachol, adrenaline or 5-HT. In contrast, adrenaline, carbachol and 5-HT all elicited PGI₂ release by whole human vessels. This differential may reflect the separate functions of the two vascular zones: haemostasis at the endothelial-blood interface and contractility in the smooth muscle component. Put another way, it is important to realise that when assessing PGI₂ release by whole vessels in any given experimental scenario (e.g. ex vivo PGI₂ release in rats with artificially-induced pathologies), that the PGI₂ measured mainly reflects that derived from the smooth muscle component rather than
the endothelium. However, because in the rabbit aorta the endothelium is the major source of PGI₂, then this species could be used to investigate changes of PGI₂ at the endothelium. This facet of the rabbit aorta has been exploited in the studies on hypothermic storage of vessels in preservation solutions (below).

3.3b Studies on hypothermic storage in organ preservation solutions

Apart from the mechanistic and logistic aspects discussed above, the rationale derived from these experiments was applied to the study of the effect of hypothermic storage in preservation solutions on vascular viability. This, in turn, is of relevance to transplantation as outlined in the introduction.

The first finding was that incubation of rat aorta in both organ preservation solutions investigated (UW and KPS) resulted in a potent inhibition of rat aortic PGI₂ release after stimulation with noradrenaline, NaF, A23187 or phorbol ester, whereas AA-stimulated PGI₂ was unaffected. Since noradrenaline, NaF and phorbol ester-stimulated PGI₂ synthesis are inhibited by Ca²⁺ channel blockade (Jeremy et al., 1988a), these data indicate that these solutions are acting on the Ca²⁺ pools associated with PGI₂ synthesis (via activation of PLA₂). AA, however, acts directly as substrate for the cyclooxygenase holoenzyme, indicating that the inhibitory effect of preservation solutions is not cyclooxygenase-mediated. Since KPS contains citrate, a Ca²⁺ chelator (Lages et al., 1977), this may explain the inhibitory effect of this solution. UW contains lactobionic acid, allopurinol, raffinose pentahydrate and reduced glutathione. Which of these latter components exerts a direct inhibitory effect on Ca²⁺-dependent PGI₂ release will require
studies on effects of these individual components.

In contrast, it was demonstrated that washing the vessels with MEM normalised the inhibition of PGI₂ following hypothermic storage. Thus, in vessels from the rat and rabbit, storage in UW or KPS elicited no appreciable reduction in PGI₂ in response to any of the stimulators. These data suggest that preservation solutions exert minimal effects on smooth muscle PGI₂ synthesis (rat aorta) or endothelial PGI₂ synthesis (rabbit). The integrity of endogenous PGI₂ synthesis is of relevance to graft function since this eicosanoid is a vasodilator (Moncada and Vane, 1979), exerts a cytoprotective effect (albeit poorly understood; Lefer et al., 1978), reduces leucocyte sequestration within damaged tissue (Jones and Hurley, 1984) and inhibits platelet adhesion and aggregation (Moncada and Vane, 1979). Given these diverse, yet relevant properties, iloprost (a stable PGI₂ analogue) has also been incorporated as an additive to preservation flush solutions (Klepetko et al., 1989; Tamaki et al., 1988). The stimulators of PGI₂ employed in the present study are also modulators of other vascular functions (NaF, phorbol ester and A23187). Thus, hypothermic storage in UW or KPS appear to exert minimal effects on intracellular signal transduction systems which mediate not only PGI₂ synthesis but also vascular contractility (Danthaluri and Deth, 1984). This concept was consolidated by in vitro experiments on rat and rabbit aorta, in which hypothermic storage of blood vessels resulted in minimal effects on contraction/relaxation patterns even at up to 72 h storage (Karatapanis et al., 1990). Since contraction/relaxation is exquisitively sensitive to endothelial damage, these latter data consolidate that hypothermic storage of blood vessels in
organ preservation solutions elicits minimal deleterious effects to the endothelium, per se.

The present results are of relevance to liver transplantation in several respects. A recent study reported that the first post-operative month constitutes the period of greatest risk for graft failure after transplantation (Quiroga et al., 1991). Major contributory factors to early graft failure were vascular complications, including thrombosis and ischaemia (Quiroga et al., 1991). The mechanisms underlying these complications are unknown but endothelial injury induced by preservation solutions and cold storage have been suggested as playing a role (Kazikoe et al., 1990; Caldwell-Kenkel et al., 1989; 1990; Blankensteijn and Terpstra, 1991). However, the results of the present study would seem to indicate that cold storage in organ preservation solutions results in minimal effects on endothelial integrity. However, Caldwell-Kenkel et al. (1989, 1990), found that hypothermic storage of rat livers flushed with Euro Collins solution (similar in composition to KPS) followed by reperfusion of the organ with KRB resulted in loss of viability and denudation of sinusoidal endothelial cells as well as activation of Kupffer cells. UW solution, however, caused less damage than Euro Collins solution (Caldwell-Kenkel, 1989; 1990). Thus, it is possible that the vascular endothelium at different sites (e.g. intra-hepatic as opposed to large arteries) may be differentially susceptible to these solutions. Alternatively, since the present experiments did not include a re-perfusion component, it is also possible that reperfusion (e.g. via shear forces and/or anchorage of endothelial cells to the subendothelium), in combination with cold storage, elicits endothelial injury and
denudation. Notwithstanding these possibilities, it is unquestionable that hypothermic storage and reperfusion of transplanted organs causes damage to the vascular endothelium. Certainly, disturbances of other endothelial functions and interactions (e.g. with coagulation factors, cytokines, neutrophil adhesion and release of reactive oxygen species) following hypothermic storage and reperfusion have been demonstrated (Blankensteijn and Terpstra, 1991). What the present data therefore indicate is that EDRF and PGI\textsubscript{2} release are relatively resistant to disruption by hypothermic storage whereas other key endothelial functions may be more vulnerable.

It is also apparent that UW and KPS exerted no clear advantage over the more conventional physiological solution, MEM, in terms of effects on contractility and PGI\textsubscript{2} release. The question therefore arises as to why specialised fluids such as UW are preferred as perfusates in cold-stored organs for transplantation. Many studies have demonstrated that UW enhances viability / integrity of other, perhaps more susceptible biosystems, than those determining contractility and relaxation (Jamieson et al., 1988; Marsh et al., 1991; Guyomard, 1990). UW was designed originally to prevent cell swelling and free radical injury in organs including the liver, kidney and pancreas (Wahlberg, 1987). Rigorous studies have shown that UW, compared to culture media and solutions like KRB produces less deleterious effects on cells including hepatocytes (Ontell et al., 1988; Jamieson et al., 1988; Marsh et al., 1991; Guyomard, 1990). Thus, the similarity of effects of UW with MEM reported here does not bring into question the usefulness of preservation solutions such as UW.
In conclusion, cold storage of blood vessels in preservation flush solutions used in liver transplantation results in minimal effects on PGI₂ release by the rat and rabbit aorta. Experiments on the human vessels consolidate that hypothermic storage and/or preservation solutions cannot explain the variable responses of blood vessels taken from human donors. It is notable that the concomitant assessment of contractility/relaxation of these human vessels also displayed marked variability: ranging from spasmogenic and zero responses to reasonable responses obtained in approximately the same number of samples as for PGI₂ release (Karatapanis et al., unpublished observations). Thus, the variation can be ascribed to: a) inter-individual (e.g. age, sex) differences, b) the drugs administered to the donors, c) nature of the injuries and/or cause of death of donors, d) infection (known to markedly alter eicosanoid synthesis; Feuerstein and Ramwell, 1981) and e) length of time maintained on life-support systems. A retrospective analysis of these variables in relation to the responses obtained was logistically impossible and at the very best would be anecdotal. Nevertheless, given the importance of vascular patency in the success of transplant surgery (as outlined above), the present data, in conjunction with the organ bath experiments, indicate that further consideration of variables encountered in donors warrants investigation. Such a study could comprise of the assessment of circulating factors (e.g. platelet and white cell activity and their release substances) as well as the other variables outlined above and to relate these parameters to the outcome of transplantation success as well as to basic vascular functions.
3.3c. Basic platelet studies

With regard to platelets, the present study firstly demonstrates that of the pro-aggregatory substances investigated, PMA, NaF, collagen, AA, thapsigargin and A23187, but not ADP, adrenaline, 5-HT, PAF or U46619, stimulated TXA₂ synthesis by unstirred platelets. Thus, it would appear that TXA₂ is secondary to aggregation (i.e. is a consequence of) rather than being a primary event following challenge with adrenaline, ADP, 5-HT, U46619 and PAF. It is notable that even when TXA₂ release is assessed following stimulation of aggregation these agonists generate small amounts of TXA₂ (Barradas, Jeremy and Mikhailidis, unpublished observations) whereas agents such as A23187 generate large quantities of TXA₂. Activators of platelets can be considered as either 'strong' or 'weak' (Crawford and Scrutton, 1988). The differential effects observed here may therefore be explained by this variation in 'strength' of agonist.

With regard to the role of Ca²⁺ in controlling the synthesis of platelet TXA₂, the channel blockers, nifedipine and diethylstilbestrol inhibited TXA₂ synthesis when stimulated with collagen, indicating, by convention, that extracellular Ca²⁺ plays a role in the coupling of these agonists to TXA₂ synthesis. This conclusion is supported by the inhibition of TXA₂ synthesis by the Ca²⁺ chelator, EGTA, which is purported not to enter cells (Baker, 1972). Furthermore, A23817, which creates artificial Ca²⁺ channels was a potent stimulator of TXA₂ synthesis. It has also been reported that intracellular Ca²⁺, released by IP₃, control TXA₂ synthesis in platelets (Knezevic et al., 1992). These latter authors demonstrated that following insertion of IP₃ (by saponification)
into platelets, an increase in intracellular Ca\(^{2+}\) and TXA\(_2\) synthesis preceded secretion of ADP and aggregation. The stimulation of TXA\(_2\) synthesis by thapsigargin, a mobiliser of intracellular Ca\(^{2+}\) (Tharstrup, 1990), supports this latter view. However, using Ca\(^{2+}\)-sensitive dyes, several studies have shown that the agonists that did not stimulate TXA\(_2\) synthesis in this study (ADP, 5-HT, PAF, 5-HT) actually elevate intracellular Ca\(^{2+}\) (Rink and Sage, 1990) and would seem to mitigate against a role for the elevation of intracellular Ca\(^{2+}\) in mediating TXA\(_2\) synthesis. The nature of Ca\(^{2+}\) channels in platelets is obscure (Rink and Sage, 1990). Certainly high concentrations of blockers are required to inhibit platelet aggregation (Jeremy et al. 1985b) as well as TXA\(_2\) synthesis. Furthermore, high concentrations of K\(^+\) (classically activate voltage-dependent channels in smooth muscle, cardiac and neural tissue) fail to elicit both aggregation and TXA\(_2\) synthesis in platelets (Barradas, Jeremy, Mikhailidis, unpublished observations). Thus, it has been suggested that platelets do not possess Ca\(^{2+}\) channels similar to those found in other tissues (Rink and Sage, 1990). It is certainly possible that Ca\(^{2+}\) blockers are acting at other Ca\(^{2+}\)-requiring steps (e.g. PKC and PLA\(_2\)). Others have suggested that Ca\(^{2+}\) channel blockers inhibit platelet activity via non-Ca\(^{2+}\) channel mediated mechanisms. It is also possible that there are sufficient Ca\(^{2+}\) stores located in the plasmalemma and glycocalyx which can supply the required Ca\(^{2+}\) for PLA\(_2\) activation. Such a possibility warrants further investigation.

With regard to signal transduction, both NaF (a non-specific G protein activator; Gilman, 1987) and PMA (a PKC activator; Nishizuka, 1984) stimulated TXA\(_2\) synthesis. In turn, both these
stimulators were inhibited by Ca$^{2+}$ channel blockers and by staurosporine (a PKC inhibitor). Thus, the sequence of events postulated to occur for receptor-linked PGI$_2$ in vascular tissue appears to hold true for TXA$_2$ synthesis in the platelet.

Using the data obtained from the above experiments, a rationale for studying drug action and changes in vascular tissue in selected pathologies using a range of stimulators was developed (see fig. 3.16.). The background and data obtained from this approach are presented in the ensuing chapters.
Fig. 3.16. Scheme of sites of action of different stimulators of PGI₂ synthesis by isolated vascular tissue. The same principle applies to TXA₂ release by platelets. Receptor (Rc) agonists (e.g. adrenaline in the rat, Ach in the rabbit, thrombin in the platelet) act via activation of Rcs. NaF activates G proteins (Gp) which in turn activates phospholipase C, thereby generating diacyl glycerol and inositol trisphosphate (IP₃). Phorbol esters activate protein kinase C (PKC). A23187 creates artificial Ca²⁺ channels, thereby eliciting influx of extracellular Ca²⁺ whereas thapsigargin mobilises intracellular Ca²⁺. The net increase in Ca²⁺ activates PLA₂ which hydrolyses and liberates AA from endogenous phospholipid stores. AA is converted to eicosanoid by cyclooxygenase. This scheme is applied to the study of sites of action of drugs (Chapter 4) and assessment of eicosanoid synthesis in selected pathologies (Chapter 5).
CHAPTER 4.

DRUG ACTION: STUDIES ON VASCULAR PROSTACYCLIN AND THROMBOXANE A2 SYNTHESIS BY HUMAN PLATELETS

4.1. Introduction.

The general background to drugs and eicosanoid synthesis has been discussed earlier (1.8.). The objective of the ensuing studies was to ascertain the site(s) of action (if any) of some important drugs used not only in the treatment of CVD but also in other eicosanoid mediated disorders (viz. inflammation). This objective was achieved by employing a range of stimulators which act at different sites in the receptor-eicosanoid synthesis pathway. The rationale of this approach was derived from data obtained and discussed in the previous chapter (also fig. 3.16). Although the author has investigated several different drugs during the course of this thesis (e.g., benzydamine, ridogrel and nabumetone), the results obtained from three disparate drug types are presented in this thesis: NSAIDs, PDE inhibitors (PDEIs) and copper chelators, as these illustrate the applicability of the methods developed for the study of drug action. Apart from eicosanoid synthesis, other aspects of drug effects were also assessed (e.g. phosphodiesterase [PDE], adenylate cyclase and lipoxygenase activity; as described in Chapter 2). For the sake of clarity, the background for each drug type is described separately.

4.1a. NSAIDs

Non-steroidal antiinflammatory drugs (NSAIDs) are the principal drugs used in the treatment of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, gout, pain and thrombotic disease (Rainsford,
1988). However, NSAIDs also elicit deleterious side effects, including gastric erosion (Atwater et al., 1965; Roth and Bennett, 1987), nephrotoxicity (Lipsett and Goldman, 1954; Swainson, 1984) and hypertensive effects (Durao et al., 1977; Brown et al., 1986). Since NSAIDs reduce prostanoid synthesis through inhibition of cyclooxygenase activity (Vane, 1971; Flower, 1974), their actions and side-effects are largely interpreted as being prostanoid-mediated. However, there are many reports that NSAIDs exert effects on other biosystems, including PDE (Weinryb et al., 1972), Ca\(^{2+}\) binding at the plasmalemma (Northover, 1971) and phospholipase C (Bomalaski, 1986). NSAIDs have also been shown to inhibit tumour growth (Hial et al., 1976; 1977) and cell proliferation through a non-prostanoid mediated mechanism (De Mello et al., 1980). In turn, the G\(_1\) phase of cell division (signal transduction phase that precedes DNA synthesis [S phase]) involves Ca\(^{2+}\) mobilisation, PKC, G proteins and phospholipases (Rosengurt, 1984; Wickremasinghe, 1988). Since, as has been expounded in this thesis, PG synthesis is mediated by these aforementioned mechanisms, possible effects of NSAIDs (indomethacin, tiaprofenic acid and ibuprofen) on these systems in the rat aorta was investigated using a range of stimulators (see fig. 3.16). In order to test "tissue specificity", effects of NSAIDs on PGI\(_2\) release by the rat urinary bladder was also investigated.

4.1b. Milrinone and IBMX (PDE inhibitors)

Milrinone [2-methyl-5-cyano-(3,4'-bipyridin)-6(1H)-one] is a non-glycoside bipyridine derivative that has positive inotropic and vasodilating activity (Alousi et al., 1983; Jaski et al., 1985; Borrow et al., 1985). Milrinone has proved effective in the treatment of
congestive cardiac failure (DiBianco et al., 1989). Milrinone probably exerts its beneficial action principally via inhibition of cyclic adenosine-3'5' monophosphate (cAMP) phosphodiesterase (PDE), thereby elevating intracellular cAMP concentrations and reducing intracellular Ca^{2+} concentrations (Earl et al., 1986; Olson et al., 1987; Mylotte et al., 1985). cAMP-PDE inhibitors (PDEIs) have also been shown to inhibit platelet aggregation (Lindgren et al., 1990; Simpson et al., 1988; Tang et al., 1980). In this context, amrinone, a similar drug to milrinone, inhibits platelet aggregation of rabbit and human platelets (Lippton et al., 1985; Pattison et al., 1986). It is clear that the inhibition of platelet activity in patients with congestive cardiac failure by milrinone would constitute a desirable additional property since: a) platelets are key components in thrombus formation (Chesterman and Berndt, 1984; Meade, 1985) and patients with congestive heart failure have an increased risk of developing thrombotic episodes if ischaemic heart disease (IHD) is involved (Trip et al., 1990; Mikhailidis et al., 1990) and b) platelet release substances (viz. TXA\(_2\)) are potent vasoconstrictors (as well as being proaggregatory) and as such have been widely implicated in the aetiology of CVD (Meade, 1985; Mikhailidis et al., 1990).

In light of the above, the effects and properties of milrinone on various aspects of human platelet function, in vitro were investigated:

1) cAMP PDE activity, as assessed by the conversion of \(^{3}H\)-cAMP to \(^{3}H\)-AMP and iloprost (a PGI\(_2\) analogue)-stimulated cAMP synthesis.

2) TXA\(_2\) synthesis in unstirred platelets (spontaneous and when stimulated by collagen, Ca^{2+} ionophore A23187, NaF, PMA, AA and
freeze-fracturing) and 3) given that vascular PGI$_2$ is also mediated by fundamentally the same mechanisms, the effect of this drug on rat aortic, endothelial cell culture was also investigated.

**4.1c. Copper chelators**

Copper imbalance is associated with both cardiovascular (Klevay, 1975; Cunnane et al., 1979; Swift et al., 1978) and inflammatory disease (Denko, 1989). However, there is some ambivalence as to the precise role of copper in these diseases. For example, it has been demonstrated that copper and the copper binding protein, caeruloplasmin, is elevated in both the serum and synovial fluid of patients with rheumatoid arthritis (Youssef et al., 1983; Scudder et al., 1978a,b). Similarly, copper chelators such as DL-penicillamine and cupralene have been used to treat inflammatory disease (Fernandez-Madrid, 1989). In contrast, complexing of drugs with copper has also been shown to markedly improve the efficacy of anti-inflammatory drugs, including aspirin (Sorenson, 1989). In CVD, copper deficiency rather than copper excess is associated with cardiovascular disorders (Klevay, 1975). However, copper catalyses lipid peroxidation, which in turn is regarded as a major contributory factor in the aetiology of both atherogenesis (Steinberg et al., 1989) and inflammation (Cuthbert et al., 1989).

It has been widely suggested that platelets and their release substances (viz. TXA$_2$) play a role in the pathophysiology of inflammatory (Lewis, 1984) and vascular disease (as has been discussed previously). In turn, copper has been shown to modulate eicosanoid synthesis in macrophages (Elliot et al., 1987), blood vessels (Mitchell et al., 1985), cardiac tissue (Cunnane et al.,
1988) and seminal vesicle homogenates (Maddox, 1973). However, little is known of the role of copper in mediating platelet cyclooxygenase and lipoxygenase activity. In order to investigate this area further, the effect of copper chelators on platelet TXA<sub>2</sub> synthesis and lipoxygenase activity was investigated using systems employed previously to investigate the role of iron with iron chelators (Jeremy et al., 1988; Barradas et al., 1989). The chelators investigated were: dimethyldithiocarbamic acid (DMDC), diethyldithiocarbamic acid (DEDCA), tetraethylthiuram disulphide (TETD), DL-penicillamine, phenanthroline and trientine. Different stimulators of TXA<sub>2</sub> synthesis (NaF, PDBU, AA, Ca<sup>2+</sup> ionophore A23187 and freeze-fracturing) were employed in order to establish sites of action of the chelators. Interactions of chelators with metal salts (Cu<sup>2+</sup>, Cu<sup>+</sup>, Zn<sup>+</sup>, Mn<sup>2+</sup>, Fe<sup>2+</sup>, Fe<sup>+</sup>, Al<sup>3+</sup>, Sn<sup>2+</sup>), superoxide dismutase (SOD) and catalase were also investigated.
4.2. RESULTS

4.2a NSAIDS

Indomethacin inhibited equipotently the synthesis of aortic PGI\(_2\) synthesis when stimulated with noradrenaline, PDBU and A23187 (fig. 4.1.; for IC\(_{50}\)s see figure legend). In contrast, there was a marked right shift (30 fold) in the inhibitory potency of indomethacin when PGI\(_2\) synthesis was stimulated with AA, sonication or freeze-fracturing (fig. 4.1.) The same pattern was found for ibuprofen (fig. 4.1.) and tiaprofenic acid (fig. 4.1.). In the aorta, inhibition of PGE\(_2\), PGF\(_2\) and TXA\(_2\) synthesis by median doses of indomethacin (derived from NSAID IC\(_{50}\)s in PGI\(_2\) synthesis above) were identical to the degree of inhibition of PGI\(_2\), when stimulated with noradrenaline, A23187, AA or sonication (table 4.1.). Similar results were obtained for ibuprofen and tiaprofenic acid. Since there were no differences in the degree of inhibition of each PG when stimulated with AA and since AA-induced PG release is an index of cyclooxygenase and / or PG synthase/isomerase activity, the data indicate that the NSAIDs studied are not acting differentially on individual PG/TX synthases / isomerases. In the urinary bladder, carbachol- and A23187-stimulated PGI\(_2\) synthesis was inhibited by indomethacin, ibuprofen and tiaprofenic acid (fig.4. 2.) in a similar manner to the aorta. Again, there was a marked right shift in the inhibitory dose-response curves when PGI\(_2\) synthesis was elicited by sonication or AA (fig. 4.2.).
**Fig. 4.1.** Effect of indomethacin, ibuprofen and tiaprofenic acid on PGI₂ synthesis by the isolated rat aorta when stimulated with: noradrenaline (■), phorbol ester dibutyrate (▲) A23187 (●), arachidonate (○), freeze fracturing (△) and sonication (□). Each point represents mean ± SEM, n = 6.
Fig. 4.2. Effect of indomethacin, ibuprofen and tiaprofenic acid on PGI\textsubscript{2} synthesis by the isolated rat urinary bladder when stimulated with: carbachol (●), A23187 (▲), arachidonate (○) and freeze fracturing (△). Each point represents mean ± SEM, n = 6.
### Table 4.1. Effect of median inhibitory concentrations of indomethacin (INDO) on rat aortic $\text{PGI}_2$ (as 6-oxo-$\text{PGF}_{1\alpha}$), $\text{PGE}_2$, $\text{PGF}_{2\alpha}$ and $\text{TXB}_2$ (pg / mg tissue / min [% inhibition]) when stimulated with noradrenline (NA), A23187, arachidonate (AA) and sonication (trauma).

<table>
<thead>
<tr>
<th></th>
<th>NA</th>
<th>A23187</th>
<th>AA</th>
<th>TRAUMA</th>
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<tbody>
<tr>
<td>6-oxo-$\text{PGF}_{1\alpha}$ zero</td>
<td>160 (0%)</td>
<td>70 (0%)</td>
<td>190 (0%)</td>
<td>140 (0%)</td>
</tr>
<tr>
<td>INDO</td>
<td>84 (48%)</td>
<td>33 (33%)</td>
<td>80 (58%)</td>
<td>65 (54%)</td>
</tr>
<tr>
<td>$\text{PGE}_2$ zero</td>
<td>88 (0%)</td>
<td>44 (0%)</td>
<td>95 (0%)</td>
<td>70 (0%)</td>
</tr>
<tr>
<td>INDO</td>
<td>43 (51%)</td>
<td>24 (45%)</td>
<td>40 (58%)</td>
<td>33 (47%)</td>
</tr>
<tr>
<td>$\text{PGF}_{2\alpha}$ zero</td>
<td>32 (0%)</td>
<td>18 (0%)</td>
<td>34 (0%)</td>
<td>26 (0%)</td>
</tr>
<tr>
<td>INDO</td>
<td>13 (51%)</td>
<td>9 (45%)</td>
<td>16 (58%)</td>
<td>12 (47%)</td>
</tr>
<tr>
<td>$\text{TXB}_2$ zero</td>
<td>16 (0%)</td>
<td>7 (0%)</td>
<td>18 (0%)</td>
<td>14 (0%)</td>
</tr>
<tr>
<td>INDO</td>
<td>8 (50%)</td>
<td>4 (53%)</td>
<td>9 (50%)</td>
<td>7 (50%)</td>
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</table>
4.2b. Effect of PDE inhibitors on TXA$_2$ synthesis.

Milrinone was a potent inhibitor of spontaneous TXA$_2$ synthesis by intact platelets ($IC_{50} = 3 \times 10^{-7}$ M; fig. 4.3). Milrinone was also a potent inhibitor of platelet TXA$_2$ synthesis when stimulated by median stimulatory doses of collagen ($IC_{50}: 1 \times 10^{-7}$ M; fig. 4.3), NaF ($IC_{50}: 3 \times 10^{-7}$ M; fig. 4.3) and PMA ($IC_{50}: 2.2 \times 10^{-7}$ M; fig. 4.3). In contrast, at stimulatory doses of A23187 and AA there was a marked decrease in the potency of milrinone in inhibiting TXA$_2$ synthesis (fig. 4.3). Milrinone had a weaker effect on TXA$_2$ synthesis when elicited by freeze-fracturing (fig. 4.3). Increasing concentrations of milrinone elicited a parallel right shift in the PMA concentration - TXA$_2$ response curve (fig. 4.4), indicating a competitive inhibition of protein kinase C by milrinone. In all experiments IBMX was a weaker inhibitor of TXA$_2$ synthesis (fig. 4.5).

4.2c. Milrinone effects on phosphodiesterase activity and iloprost-stimulated cAMP synthesis

Both milrinone and IBMX inhibited platelet cAMP-PDE activity in concentration-dependent manners when assessed by the conversion of [$_3^H$]-cAMP to [$_3^H$]-AMP (fig.4.6a). IC$_{50}$s were: milrinone; $2 \times 10^{-6}$ M and IBMX; $4.6 \times 10^{-6}$ M. In turn, milrinone and IBMX increased cAMP levels in platelets when elicited by a median stimulatory concentration of iloprost (fig. 4.6b). EC$_{50}$s (concentration of drug at which iloprost-stimulated cAMP synthesis was stimulated to 50% of maximal) were: milrinone, $5.6 \times 10^{-5}$ M; IBMX, $3 \times 10^{-5}$ M. Neither milrinone or IBMX (up to $1 \times 10^{-4}$ M) elicited a measurable increase in platelet cAMP concentrations (data not shown).
Fig. 4.3. Effect of milrinone on platelet TXA₂ (measured as TXB₂) synthesis when stimulated by (final concentrations): 10 μg/ml collagen (○), 1 μM PMA (◇), 5 mM NaF (◆), 1 μM A23187 (●), 1 μM AA (△), freeze-fracturing (▲) and spontaneous release (□).
Each point = mean ± S.E.M., n = 6.
Fig. 4.4. Effect of different concentrations of milrinone on phorbol ester concentration - TXA₂ (as TXB₂) response curve: (●) + zero milrinone, (▲) + 3 x 10⁻⁷ M milrinone; (♦) + 1 x 10⁻⁶ M milrinone; (■) + 3 x 10⁻⁶ M milrinone. Each point = mean; n = 4.
Fig. 4.5. Effect of IBMX on platelet TXA$_2$ (measured as TXB$_2$) synthesis when stimulated by (final concentrations):
10 µg/ml collagen (O), 1 µM phorbol ester (◇), 5 mM NaF (□), 1 µM A23187 (△), 1 µM AA (●), freeze-fracturing (▲).
Each point = mean ± S.E.M., n = 6.
Fig. 4.6. a. Effect of milrinone (●) and IBMX (▲) on the conversion of \(^{3}\text{H}\)-cAMP to \(^{3}\text{H}\)-AMP by washed human platelets. Each point = mean ± S.E.M., n=6.

b. Effect of milrinone (○) and IBMX (◇) on iloprost (1 µM) - stimulated cAMP synthesis by washed human platelets. Each point = mean ± S.E.M., n=6.
4.2d. Effect of copper chelators on AA-stimulated TXA₂ synthesis and lipoxygenase activity

The chelators studied inhibited conversion of AA to TXA₂ (fig. 4.7a) in this rank order of potency: DMDCA > TETD > DEDCA > trientine > phenanthroline >> DL-penicillamine. These chelators also inhibited lipoxygenase activity, the rank order of potency being similar to that of TXA₂ synthesis (fig. 4.7b).

The degree of inhibition by DMDCA of TXA₂ synthesis when stimulated with A23187, NaF, phorbol ester and AA were similar for all stimulators (fig. 4.8; IC₅₀s for various stimulators: AA, 33uM; A23187, 35uM; NaF, 34uM; phorbol ester, 30uM; freeze fracturing, 28uM). The similarity in the IC₅₀s for each stimulator is indicative of an effect of chelator on cyclooxygenase, rather than on other enzymes or systems (PLA₂, protein kinase C, Ca²⁺ mobilisation). This was confirmed using maximal inhibitory doses of the other chelators (table 4.2.). The inhibitory effect of all copper chelators on both TXA₂ synthesis and lipoxygenase activity was reversed by the addition of approximately equimolar concentrations of Cu²⁺ and to a lesser extent by Cu⁺ and Zn²⁺ but not by Fe³⁺, Fe²⁺, Al³⁺, Mn²⁺ or Sn²⁺ (figs. 4.9a and 4.9b). The addition of neither SOD nor catalase to incubates affected chelator-inhibited platelet cyclooxygenase or lipoxygenase activity.
Fig. 4.7. Effect of copper chelators on A) platelet TXA$_2$ synthesis (stimulated with 10 μM arachidonic acid) and B) lipoxygenase activity (conversion of $^{14}$C-AA to $^{14}$C-HETE). Each point = mean ± S.E.M., n = 7. dimethyldithiocarbamic acid (O), tetraethylthiuram disulphide (●), diethylidithiocarbamic acid (△), phenanthroline (□), trientine (◇), DL-penicillamine (▽).
Fig. 4.8. Effect of dimethyldithiocarbamic acid on TXA$_2$ synthesis by washed human platelets when stimulated with: 5 mM NaF (○), 1 μM PMA (▽), 1 μM arachidonate (◇), 1 μM Ca$^{2+}$ ionophore A23187 (△), freeze fracturing (□).

Each point = mean ± SEM, n = 8
Fig. 4.9. Effect of various metal (chloride) salts on (a) DMDTCA (100 μM)-inhibited TXA₂ synthesis (stimulated with 10 μM AA) and (b) DMDTCA (100 μM) -inhibited lipoxygenase activity (conversion of $^{14}$C-AA to $^{14}$C-HETE by washed human platelets. Each point = mean ± S.D., n = 6.

CuCl₂ (●), CuCl (▲), ZnCl (■), FeCl₂ (△), FeCl₃ (◇), MnCl₂ (○), AlCl₃ (♦). CuCl₂ alone (▼), NaCl alone (▽).
**Table 4.2.** Effect of different copper chelators on TXA$_2$ synthesis (% inhibition; mean ± SD; n = 6) elicited by a range of different stimulators.

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<thead>
<tr>
<th>Chelator</th>
<th>NaF (10 mM)</th>
<th>PMA (1 μM)</th>
<th>AA (3 μM)</th>
<th>spontaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMDCA (100 μM)</td>
<td>93 ± 8</td>
<td>94 ± 8</td>
<td>92 ± 7</td>
<td>96 ± 5</td>
</tr>
<tr>
<td>TETD (100 μM)</td>
<td>90 ± 6</td>
<td>89 ± 8</td>
<td>88 ± 10</td>
<td>88 ± 13</td>
</tr>
<tr>
<td>DEDCA (100 μM)</td>
<td>60 ± 7</td>
<td>59 ± 8</td>
<td>63 ± 8</td>
<td>63 ± 7</td>
</tr>
<tr>
<td>phenanthroline (2 mM)</td>
<td>80 ± 7</td>
<td>83 ± 6</td>
<td>82 ± 8</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>trientine (5 mM)</td>
<td>75 ± 6</td>
<td>73 ± 7</td>
<td>74 ± 8</td>
<td>75 ± 8</td>
</tr>
<tr>
<td>DL-penicillamine (10 mM)</td>
<td>3 ± 0.3</td>
<td>5 ± 0.4</td>
<td>6 ± 0.5</td>
<td>4 ± 0.2</td>
</tr>
</tbody>
</table>
4.3. Discussion.

4.3.a NSAIDS

This study demonstrates that there is a marked disparity in the inhibitory potencies of NSAIDs on vascular PGI$_2$ synthesis when elicited by stimulators which act at different sites in the receptor activation - PGI$_2$ synthesis sequence. The similarity of effect between the aorta and bladder indicates that this inhibition is not confined to vascular smooth muscle and/or adrenoceptors. The IC$_{50}$s of the NSAIDs on trauma- and AA-stimulated PGI$_2$ synthesis were identical but there were marked left shifts in the inhibitory potencies when PG synthesis was stimulated with NA, NaF, PDBU and A23187. The IC$_{50}$s obtained from these stimulators also being identical for each NSAID. As discussed earlier, it has been established that receptor agonist, NaF and A23187 stimulation of PGI$_2$ synthesis in the rat aortae is inhibited by conventional Ca$^{2+}$ channel blockers, such as verapamil and nifedipine (Jeremy et al., 1985a, 1988a). In contrast, AA-or trauma-stimulated PGI$_2$ synthesis are unaffected by Ca$^{2+}$ channel blockers (Jeremy et al, 1986c). Trauma elicits PGI$_2$ synthesis in smooth muscle through exogenous Ca$^{2+}$ activation of PLA$_2$ (i.e. Ca$^{2+}$ contained in incubation media), probably due to destruction of cell membrane integrity thereby by-passing Ca$^{2+}$ channels (Jeremy et al., 1986c). Since AA is the substrate for PG synthesis, stimulation of PGI$_2$ synthesis with exogenous AA, as with trauma, also bypassed the Ca$^{2+}$ mobilisation-PLA$_2$ step that liberates endogenous AA. The present data therefore points to NSAIDs exerting an effect on Ca$^{2+}$ mobilisation linked to exogenous AA release as well as an effect on cyclooxygenase.

Such a proposal is not without precedent. It has been suggested
that a major source of activator Ca^{2+} linked to both contraction and PG synthesis is derived, in part, from Ca^{2+} stores located in the plasma membrane and glycocalyx (Villamil et al., 1973; Loutzenheiser and van Breemen, 1983; Jeremy et al., 1988a). In this context, Northover (1971) demonstrated that NSAIDs inhibit the uptake and binding of Ca^{2+} by plasma membranes of vascular endothelial cells and that ibuprofen and indomethacin inhibits [^{45}Ca^{2+}] uptake by human platelets (Gill et al., 1990). NSAIDs also inhibit other membrane-associated processes, including that of superoxide and anion generation by the cell-free NADPH oxidase system of neutrophils (Biemond et al., 1986), mononuclear cell PLC activity (Bomalaski et al., 1986) and 12-HETE peroxidase of the lipoxygenase pathway in platelets (Siegel et al., 1986). In this context, Abramson and Weissman (1989) demonstrated that the NSAIDs, aspirin, indomethacin and piroxicam are also potent inhibitors of the early steps of receptor-activated neutrophil function, including the uptake and mobilisation of Ca^{2+} at the plasma membrane of these cells.

Disparities of the inhibitory potencies of NSAIDs on PG synthesis have also been reported in other systems. For example, NSAIDs (including indomethacin and ibuprofen) are potent inhibitors of phorbol ester-stimulated PG synthesis by macrophages at concentrations far less (up to 100 fold) than are required to inhibit PG synthesis by isolated seminal vesicle microsomes (Brune et al., 1981). The IC_{50}s of indomethacin and ibuprofen on PG synthesis by seminal vesicles are almost identical to the IC_{50}s reported here for trauma- and AA- stimulated PGI_{2} synthesis whereas the IC_{50}s of the NSAIDs on PG synthesis by macrophages are similar to IC_{50}s derived here for agonist-, NaF-, PDBU- and A23187- stimulated PGI_{2} data.
Thus, notwithstanding the mechanistic implications of the present study, it is clear that the inhibitory potencies of NSAIDs on PG synthesis depend on the methods used and awareness of this may be of importance in evaluating and comparing NSAID efficacy.

The present findings may be of relevance not only to the efficacy of NSAIDs as inhibitors of inflammation but also to their side effects. Firstly, the reduction of inflammation by NSAIDs may be due to inhibition of Ca$^{2+}$ mobilisation linked not only to PG synthesis but also to other Ca$^{2+}$-requiring pro-inflammatory systems (Westwick and Poll, 1986). It is also of interest that NSAIDs exert significant effects on the metabolism of bone and cartilage that are probably not dependent on cyclooxygenase (Famaey et al, 1975; Arumugham and Bose, 1982). The possibility that the side effects of NSAIDs (gastric erosion and ulceration, nephrotoxicity and hypertension), are mediated not only by the inhibition of PG synthesis but also through Ca$^{2+}$-associated signal transduction warrants further investigation.

### 4.3.0 MILRINONE

The concentrations of milrinone that inhibited PDE activity in the present study were approximately 30 times greater than those which inhibit in vitro platelet aggregation (Barradas et al, 1992). In contrast, the concentrations of milrinone that inhibited TXA$_2$ synthesis in the present study were similar to those that inhibited in vitro platelet aggregation (Barradas et al, 1992). Furthermore, the amounts of milrinone required to inhibit PDE activity in the present study are somewhat greater than the accepted therapeutic concentrations (Benotti and Hood, 1984). It is unlikely, therefore,
that the inhibition of PDE activity mediates the anti-aggregatory effect of milrinone. Ex vivo, resting intraplatelet concentrations of cAMP are virtually undetectable and in vivo are probably not tonically stimulated, since it is now accepted that PGI$_2$ is not continually secreted by blood vessels (Blair et al., 1982; Barrow and Ritter, 1988). Thus, effects of milrinone on PDE activity, and therefore intraplatelet cAMP levels, are unlikely to come into play in the circulating, non-activated platelet. However, at the site of a mural thrombus or platelet aggregate, the situation may be quite different. On adhesion and aggregation, platelets release a number of bioactive substances (TXA$_2$, serotonin, platelet derived growth factor) all of which have been shown to be potent stimulators of PGI$_2$ synthesis by vascular tissues (Jeremy et al., 1988a). It was suggested that this platelet-stimulated release of PGI$_2$ may constitute a haemostatic mechanism by which the size of a thrombus is limited at the site of vascular injury (Ritter et al., 1983; Jeremy et al., 1985a; 1985c). In this latter scenario, locally high concentrations of PGI$_2$ will elicit elevation of intraplatelet cAMP concentrations. Thus, milrinone may limit thrombus size/elongation via enhancement of PGI$_2$-stimulated cAMP in the platelets involved in thrombus formation.

In contrast to PDE inhibition and cAMP modulation, milrinone was a potent inhibitor of TXA$_2$ release when stimulated with collagen, NaF and PDBU and to a lesser extent when stimulated with A23187, exogenous arachidonate or freeze-fracturing. This differential potency of inhibition of TXA$_2$ synthesis may be indicative of several possible sites of action of milrinone since the stimulators used to elicit TXA$_2$ synthesis act at different sites in the sequence leading from surface receptor activation to TXA$_2$ synthesis. Thus, given the
disparities in the inhibitory potencies to different stimulators, the present data indicates that milrinone is exerting its most potent effect on the signal transduction mechanism linking receptor activation with TXA_2 synthesis in the platelet. Since milrinone inhibited $[^{45}\text{Ca}^2+]$ uptake at concentrations similar to those that inhibited PMA-, NaF- and collagen-stimulated as well as spontaneous TXA_2 synthesis (Jeremy et al., 1993), the inhibitory effect of milrinone on TXA_2 synthesis may occur at any of the Ca$^{2+}$-dependent steps: viz. activation of PKC (Nishizuka, 1988) and PLA_2 (Irvine, 1982). A similar conclusion was reached by Block et al., (1990), who suggested that amrinone inhibits platelet PLA_2 activity. The greater potency of milrinone on phorbol ester-stimulated TXA_2 and the parallel right shift of the phorbol ester dose-response curve, elicited by milrinone, is indicative of a competitive inhibition of PKC activity. Since PKC activity requires Ca$^{2+}$ (Nishizuka 1988), the present data may reflect a disruption of the binding/association of Ca$^{2+}$ to PKC. In turn, we have previously suggested that the uptake of $[^{45}\text{Ca}^2+]$ in response to agonists in the platelet reflects Ca$^{2+}$ sequestration at the membrane and not changes in intracellular Ca$^{2+}$ (Gill et al., 1991, 1992a). Since PKC, when activated, moves into the plasma membrane (Nishizuka, 1988; Iwamoto, 1992), the present effects of milrinone may reflect localised events at the cell surface. Such a proposal is not without precedent. A recent study demonstrated that milrinone exerts a potent 'membrane effect' on platelets as demonstrated by its inhibitory action on the binding of monoclonal antibodies to platelet membrane glycoprotein Ib (Jackson et al., 1989). Milrinone has been shown to also inhibit Ca$^{2+}$ - ATPase (a Ca$^{2+}$ - stimulated enzyme) in isolated heart plasma membranes
(Mylotte et al, 1987). Such effects on platelet membranes may also explain the inhibitory effect of milrinone on platelet aggregation, since membrane associated Ca\(^{2+}\) is essential for cell-cell stickiness (i.e. aggregation; Gerrard et al, 1982; Rink and Sage, 1990).

It is notable that IBMX, although less potent, elicits a similar pattern of response as milrinone. Structurally, IBMX is not dissimilar to milrinone. It is also of interest that the classic inhibitor of Ca\(^{2+}\) influx, nifedipine, possesses structural similarities to milrinone (milrinone is a dipyridine; nifedipine is a monopyridine). Whether milrinone (and structurally similar PDE inhibitors) also act via disruption of Ca\(^{2+}\) binding to effector proteins and what structural component determines these properties warrants further investigation.

Apart from the inhibition of platelet aggregation, the present findings may be of relevance to the effect of milrinone on other target tissues, viz. blood vessels and the heart. It has been established that milrinone is a potent vasodilator and increases atrial tension and beat rate (Alousi et al., 1983; Grant et al., 1985). Both vascular reactivity and heart function are dependent on the mobilisation and resequestration of Ca\(^{2+}\) and recent studies have also demonstrated the obligatory role of the G proteins, PLC and PKC in these functions (Edes and Kranias, 1990; Danthaluri and Deth, 1984; Rasmussen et al, 1984). It is also of interest that milrinone is synergistic with captopril on cardiac performance and complements effects of captopril on the peripheral circulation (LeJemtel et al, 1985). In turn, it has been demonstrated that captopril also inhibits adrenaline-stimulated \(^{45}\)Ca\(^{2+}\) uptake by human platelets in vitro and ex vivo (Gill et al., 1989; Gill et al., 1992b). The synergistic /
complementary relationship between captopril and milrinone points to a common site of action, possibly Ca\(^{2+}\) mobilisation / transport at the plasma membrane. The possibility that milrinone exerts its vasodilator and inotropic actions via the mechanisms postulated to occur in the platelet in this study warrants further investigation.

### 4.3c Copper chelators

The present study demonstrates that copper chelators, in particular DMDCA, DEDCA and TETD inhibits platelet TXA\(_2\) synthesis and lipoxygenase activity. Both systems were notably inhibited in a similar rank order of potency, indicating a similar mechanism of action. The inhibition of TXA\(_2\) synthesis appears to be at the cyclooxygenase and/or TXA\(_2\) synthase level rather than at other sites since the inhibitory potencies of the chelators was similar for all stimulators of TXA\(_2\) synthesis (NaF, A23187, AA and phorbol ester. The inhibitory action of the chelators was reversed by the presence of Cu\(^{2+}\) and to a lesser extent by Zn\(^{2+}\) and Cu\(^{+}\) but not Fe\(^{2+}\), Fe\(^{3+}\), Mn\(^{2+}\) or Al\(^{3+}\). Since, Zn\(^{2+}\) has only one valence state and such is unlikely to exert effects on cyclooxygenase or lipoxygenase activity it would appear that the present effects are mediated principally via interactions with Cu\(^{2+}\). The reversal of the effect may also be due to negation of drug activity since active sites would be effectively abolished by chelation with metals.

Mechanistically, cyclooxygenase catalyses the oxygenation and peroxidation of AA to yield the endoperoxide, PGG\(_2\), which is then converted to PGH\(_2\) by peroxidase action (Deby, 1988). Since Cu\(^{2+}\) catalyses peroxidation (Cuthbert et al., 1989) and the present data demonstrates a clear inhibition of AA peroxidation, it is possible
that the copper chelators act by inhibiting the peroxidase activity of cyclooxygenase. Alternatively, it has been proposed that TXA₂ synthase catalyses a dismutase-type reaction (Anderson et al., 1978). Given that copper is an essential co-factor for SOD activity (Marklund, 1982), it is possible that the effects of copper chelators on TXA₂ synthesis are mediated by effects on copper-mediated dismutation of PGH₂ to TXA₂. Furthermore, copper-chelator complexes possess dismutative activity in their own right (Lengfelder and Elstner, 1978), which may contribute to the effect of chelators seen here. However, the lack of effect of SOD on chelator-inhibited TXA₂ synthesis does not support this possibility.

Although it is well established that cyclooxygenase holoenzyme contains iron (Kulmacs and Lands, 1984), there is no evidence that cyclooxygenase is a copper-containing enzyme. However, several studies have demonstrated that copper mediates eicosanoid synthesis in other tissues. Maddox (1973) demonstrated that the presence of divalent copper in homogenates enhances the conversion of AA to PGF₂ but inhibits concomitant PGE₂ generation and concluded that Cu²⁺ exerts differential effects on different synthases. It is also notable that although Cu²⁺ chelators, benzhydroxamic acid and 8-hydroxyquinoline reversed these effects of Cu²⁺, whereas DL-penicillamine had an opposite effect (Maddox, 1973). In the present study, it was also found that DL-penicillamine has little effect on platelet TXA₂ synthesis or lipoxygenase activity synthesis. It is difficult to rationalise why penicillamine differs from other chelators, since it possesses high affinity binding capacity for copper. However, it has been demonstrated that penicillamine augments platelet aggregation (Mikhailidis et al., 1988b) whereas other copper
chelators inhibit platelet aggregation (Vargaftig et al., 1974). It would appear therefore that penicillamine is anomalous in the present context. Other extensive studies carried out by Vargaftig et al. (1974) consolidate the present findings since a range of copper chelators were found to inhibit platelet aggregation, an effect also reversed by the presence of copper and zinc. It is notable that Vargaftig et al. (1974) predicted that these chelators were acting via inhibition of eicosanoid synthesis.

Apart from platelets, copper has been shown to markedly influence eicosanoid synthesis in other tissues/cells. Copper deficiency in rats has been shown to reduce by about 50% PGI$_2$ synthesis by aortic rings compared to those for copper adequate animals, implicating an obligatory role for copper in mediating the synthesis of this eicosanoid (Mitchell et al., 1988). It is notable that aortic SOD activity was also reduced by approximately 50% in copper deficient compared to copper adequate animals (Mitchell et al., 1988). However, in the present study, neither SOD nor catalase influenced chelator-inhibited TXA$_2$ synthesis. In another study, hearts from rats fed a low copper or a copper supplemented diet were perfused and secreted less 6-keto-PGF$_{1\alpha}$ than hearts from the copper-supplemented group (Cunnane et al., 1988). It was suggested that copper deficiency allows increased lipid hydroperoxide production which is sufficient to inhibit PGI$_2$ synthase activity and hence reduce PGI$_2$ formation. Elliot et al. (1987) demonstrated that copper stimulated the release of both TXA$_2$ and $^{14}$C-AA release from macrophages and concluded that copper acts on PLA$_2$ rather than on cyclooxygenase in these cells. The present data, however, do not concur with effects of copper chelators on PLA$_2$. 
Clinically, little is known of the relationship between copper imbalance, eicosanoid synthesis and the aetiology of disease. Wilson's disease (a condition characterised by copper overload) is associated with premature osteoarthritis with biopsies of symptomatic joints showing chronic inflammatory cell infiltrates (Denko, 1989). Copper and the copper-binding protein, caeruloplasmin has also been shown to be elevated in serum and in the synovial fluid of patients with rheumatoid arthritis (Youssef et al., 1983; Scudder et al., 1978a,b). In turn, eicosanoids are key mediators of inflammation, including rheumatoid arthritis (Rainsford, 1988; Brune, 1988). Furthermore, copper chelators have been used to treat inflammatory disease (Fernandez-Madrid, 1989). However, this drug type elicits a number of severe side effects and as such have been largely rejected as a major therapy for inflammatory disease (Fernandez-Madrid, 1989). Nevertheless, copper chelators may prove invaluable tools in elucidating the interrelationship between copper, eicosanoid synthesis and lipid peroxidation in the pathophysiology of disease. Development of less toxic chelators may also prove effective as therapeutic agents.
CHAPTER 5.
STUDIES ON PGI₂ RELEASE BY VASCULAR AND OTHER TISSUES FROM RATS WITH EXPERIMENTAL DIABETES MELLITUS OR HEPATIC PORTAL HYPERTENSION.

5.1. Introduction
5.1a Diabetes Mellitus

As was discussed in the general introduction, DM is characterised by a reduction in vascular PGI₂ synthesis (Harrison et al., 1978; Rogers et al., 1981; Jeremy et al., 1987a). The precise mechanisms that determine reduced vascular PGI₂ synthesis in DM are not clearly defined, although a reduction in substrate (arachidonate) levels in blood vessels has been suggested (Holman et al., 1983; Jeremy et al., 1987a, Dang et al., 1988). As was discussed earlier, vascular PGI₂ synthesis is stimulated by vasoactive agonists, which in turn is mediated by G proteins, PKC and IP₃ (Griedling et al., 1986; Hallam et al., 1988; Jeremy et al., 1988a). The net result of activation of these systems is transmembrane Ca²⁺ influx and an increase in intracellular Ca²⁺ ([Ca²⁺]ᵢ) which then activates PLA₂, liberating AA from phospholipid stores (Irvine, 1982). In order to further elucidate the mechanisms that determine diminished vascular PGI₂ synthesis, the effect of a range of stimulators that act at different sites in the receptor activation-PGI₂ synthesis pathway in the streptozotocin-induced diabetic rat were investigated. The stimulators employed were: adrenaline (mediated by heterogenous α-adrenoceptors; Jeremy et al., 1985a) angiotensin II (Ang II; Hassid and Williams, 1983; Stevens et al., 1992), PDBU (a PKC activator), thapsigargin (elevates intracellular Ca²⁺; Thastrup et al., 1990; Levine and
Watanabe, 1991) Ca\(^{2+}\) ionophore A23187 (creates artificial Ca\(^{2+}\) channels; Reed and Lardy, 1972) and arachidonic acid (AA; substrate for PGI\(_2\) synthesis; Hamberg, 1968).

Reports that vascular PGI\(_2\) synthesis is diminished in DM are not universal. Fujii et al., (1986) reported that in perfused rat mesenteric bed taken from diabetic rats there was a marked increase in the output of PGI\(_2\) compared with controls. Indeed, increased blood flow in gastrointestinal (GI) vessels is also associated with the early stages of DM (Tooke et al., 1986). Aetiologically, these disorders have been largely ascribed to diabetic autonomic neuropathy (Lincoln et al., 1984a,b). However, it is also well established that the pathophysiology of various GI disorders in non-diabetics involves endogenous PGs (Branski et al., 1986). With regard to the GI tract in DM, the author is aware of no systematic studies on PG synthesis in man or in experimental animal models. However, it has been demonstrated that caloric deprivation results in differential changes in PGI\(_2\) and TXA\(_2\) synthesis by the small intestine and mesenteric vasculature of the rat (Jeremy et al., 1987c). Since these PGs influence mesenteric blood flow and gut motility (Mailman, 1982), it was suggested that these changes in PG synthesis may play a role in the adaptation of the small gut in response to fasting through modulation of gut motility and mesenteric blood flow (Jeremy et al., 1987c). It is also well established that in experimental DM, the GI tract and associated vasculature of the rat undergoes marked adaptive changes of structure (mucosal and muscular hyperplasia; Jervis and Levin, 1966; Schedl et al., 1982) and function (increased nutrient uptake; Lorenz-Meyer et al., 1977).
In order to investigate a possible role for PGs in the adaptation and pathology of DM in the splanchnic vasculature, the release of PGI$_2$, TXA$_2$, PGE$_2$, PGF$_2\alpha$ by the mesenteric vessels and hepatic portal vein was investigated. PG synthesis by the aorta and carotid artery of the same animals were also measured to assess vascular PG synthesis in 'post-hepatic' vessels. Since diabetic rats are markedly hyperphagic we also examined the effect of pair feeding (amount of food given to diabetic rats per day adjusted to the average daily intake of age-matched controls) on PG synthesis by the above tissues. The effect of insulin administration to diabetic rats was also investigated. Since gut motility affects blood flow (Winne, 1980) and fasting has been shown to markedly alter PG synthesis by the GI tract (Jeremy et al., 1987c), PG synthesis by the mucosal and muscular portions of the GI tract were also assessed.

In order to examine whether other non-vascular smooth muscle tissues are affected in a similar or disparate manner as the aorta and splanchnic vasculature, PGI$_2$ synthesis by the urinary bladder and trachea was investigated. It is also notable that the urinary bladder of the diabetic rat releases more PGI$_2$ per unit weight than controls (Jeremy et al., 1986a). Bladder PGI$_2$ synthesis was stimulated with Ach (PGI$_2$ synthesis in the urinary bladder is mediated by muscarinic receptors; Jeremy et al., 1986b) as well as PDBU, thapsigargin, A23187 and AA.

5.2. Hepatic portal hypertension

Hepatic portal hypertension (HPH) is a complication of liver cirrhosis which sometimes results in severe consequences, such as
bleeding from esophageal varices and ascites formation (Bosch et al., 1992). HPH is always initiated by an increased vascular resistance to portal blood flow (Bosch et al., 1992; Murray et al., 1958; Kowalski et al., 1953; Siegel et al., 1974; Lee, 1989). However, when HPH is fully established there is also reduced splanchnic arteriolar resistance and a marked increase in portal blood inflow, which contributes to the maintenance of portal hypertension. This hyperdynamic splanchnic circulation is accompanied by increased cardiac output together with reduced arterial pressure and peripheral vascular resistance (Bosch et al., 1992; Murray et al., 1958; Kowalski et al., 1953; Siegel et al., 1974; Lee, 1989).

Similar circulatory changes are seen in experimental models including the hepatic portal vein-ligated rat (Bonzam and Blundis, 1987). Although the mechanisms underlying these circulatory phenomena are still undefined, changes in vascular reactivity in response to adrenergic agonists (Ramond et al., 1986; Gerbes et al., 1986; Pizcueta et al., 1990), 5-HT (Kaumann et al., 1986; Cummings et al., 1986), angiotensin II (Murray and Paller, 1985, 1986) as well as prostacyclin (PGI$_2$) synthesis (Hamilton et al., 1983; Ritter et al., 1986; Sitzmann and Li, 1991; Guarner and Soriano, 1993) have been suggested as being involved. It has also been proposed that an increase in the synthesis of vascular NO, as a vasodilator, may play a key role in the vascular adoptions to HPH (Vallance and Moncada, 1991; Whittle and Moncada, 1991; Pizcueta et al., 1992; Lee et al., 1992), although this has recently been contested (Sogni et al., 1992). Some time ago, Murray and Paller (1985, 1986) proposed that there was a post-receptor defect underlying the vascular reactivity
changes in HPH.

With regard to PGI₂, a marked increase in output by the hepatic portal vein in both the laboratory rat and man has been reported (Hamilton et al., 1983; Sitzmann and Li, 1991; Guarner and Soriano, 1993). However, the mechanisms controlling vascular PGI₂ synthesis in HPH are again not clearly defined and little is known of PGI₂ synthesis and release in systemic vasculature in HPH. In order to elucidate the mechanisms that may determine altered vascular PGI₂ synthesis in HPH, the effect of a range of stimulators that act at different sites in the receptor activation-PGI₂ synthesis pathway in the aorta (and mesenteric vasculature) was investigated in rats with HPH using an approach described above for the diabetic rat model.

5.2. RESULTS

5.2.a. Diabetes Mellitus

Body weights (median [range]) of the animal groups at the time of sacrifice were: controls, 475 g (449-516; n = 35); diabetic, 239 g (220-280; n = 35). At sacrifice, blood glucose concentrations (mmol/l; median [range]) were: controls, 8.4 (6.8-10.3); diabetic, 30.3 (28.2-32.7).

Aortic PGI₂ release (expressed as pg 6-oxo-PGF₁α liberated per mg wet tissue per min) was significantly reduced in diabetic rats compared to controls in response to adrenaline (fig. 5.1.), Ang II (fig. 5.2.), PDBU (fig. 5.3.) and A23187 (fig. 5.4.). There were no differences in PGI₂ release in response to thapsigargin (fig. 5.5.) or AA (fig. 5.6.). Since there is good evidence that this reduction of PGI₂ is due to diminished AA stores in vascular tissue of...
diabetic rats, data was transformed to % maximal response to obviate this variable. Transformation of data to % maximal response revealed a marked right shift in the adrenaline-\(\text{PGI}_2\) dose-response curve in aortae from DM rats (EC\(_{50}\) = \(4.1 \times 10^{-6}\)M) compared to controls (EC\(_{50}\) = \(5.4 \times 10^{-7}\)M; fig 5.1). Similarly, there was a marked right shift in the ANG II dose response curves (% maximal response) in aortae from diabetic rats (EC\(_{50}\) = \(2.9 \times 10^{-6}\) M) compared to controls (EC\(_{50}\) = \(4.9 \times 10^{-7}\) M; fig. 5.2.). Following transformation of data to % maximal response, there was also a marked right shift the PDBU-\(\text{PGI}_2\) dose-reponse curve (EC\(_{50}\) = \(1.4 \times 10^{-6}\)M) compared to controls (EC\(_{50}\) = \(1.9 \times 10^{-7}\)M; fig. 5.3.), whereas there were no differences in the EC\(_{50}\)s (% maximal response) of thapsigargin (fig. 5.4), A23187 (fig. 5.5), or AA (fig. 5.6).

In contrast to the aorta there was a marked increase in \(\text{PGI}_2\) release in response to ACh from the urinary bladder of diabetic rats compared to controls (fig. 5.7). Following transformation of data to % maximal response there was a marked left shift in the Ach-\(\text{PGI}_2\) dose-response curve in urinary bladders from diabetic rats (EC\(_{50}\) = \(5.8 \times 10^{-7}\)M) compared to controls (EC\(_{50}\) = \(2.2 \times 10^{-6}\) M; fig. 5.7). Although there was a generalised increase in \(\text{PGI}_2\) output in the urinary bladders from diabetic rats there were no differences in the EC\(_{50}\)s of PDBU (fig. 5.8), thapsigargin (fig. 5.9), A23187 (fig. 5.10.) or AA (data not shown) between diabetic or control rats following tranformation of data to % maximal response. In the trachea of the diabetic rat (compared with controls) \(\text{PGI}_2\) release in response to ACh was significantly diminished (fig. 5.11.).

The synthesis of \(\text{PGI}_2\) (as 6-oxo-\(\text{PGF}_{1\alpha}\)) was significantly elevated in the mesenteric vessels and hepatic portal veins of the
diabetic rats fed ad libitum or pair fed compared with controls (fig. 5.12.). In the diabetic rats, fed ad libitum and pair fed, PGI₂ synthesis by aortae and carotid artery was significantly diminished (fig. 5.12.). TXB₂, PGE₂ and PGF₂α release was similarly altered by blood vessels in all experiments (table 5.1.). Insulin administration prevented all changes of vascular PG synthesis found in diabetic animals (fig.5.12., table 5.1.). 6-oxo-PGF₁α, TXB₂, PGE₂ and PGF₂α synthesis were all unaltered in the mucosal and muscular regions of the stomach, duodenum, jejunum and ileum, from diabetic rats, whether fed ad libitum or pair fed (table 5.2.). PG synthesis by the GI tract following insulin administration was also not significantly different from controls (table 5.2.). It is notable that the relative proportions of the various PGs studied varies from tissue to tissue. Thus, in vascular tissue the average ratio of 6-oxo-PGF₁α : PGE₂ : PGF₂α : TXB₂ was 40: 15: 3: 1 whereas in GI tissue it was 9: 4: 2: 1. This probably reflects the different roles of PGs in these disparate tissues. Analysis of data also revealed that these ratios were not altered by DM.

5.2b. Hepatic portal hypertension

12 portal vein-constricted rats and 12 sham operated (control) rats were studied. Portal venous pressure was significantly elevated in the portal vein constricted rats compared to controls: 13.3 ± 0.8 mm Hg vs 6.3 ± 0.8 mm Hg (p < 0.01). Spleen weights were also increased in portal vein constricted rats compared to controls: 1.04 ± 0.07g vs 0.79 ± 0.08 g (not significant). Spontaneous release of PGI₂ following freeze-thawing and sonication (an index of substrate and synthesising enzymes [PLA₂, cyclooxygenase and synthase] and
substrate availability) was not significantly different between aortae from portal hypertensive rats (8.8 ± 0.86 ng 6-oxo-PGF$_{1\alpha}$/mg tissue/h) and sham-operated animals (9.2 ± 0.89 6-oxo-PGF$_{1\alpha}$/mg tissue/h) but was significantly (p < 0.001) elevated in mesenteric vasculature from portal hypertensive rats (14.2 ± 0.96 ng 6-oxo-PGF$_{1\alpha}$/mg tissue/h) and sham-operated animals (8.2 ± 1.29 6-oxo-PGF$_{1\alpha}$/mg tissue/h).

PGI$_2$ release by aortae from rats with portal hypertension was enhanced in response to adrenaline compared to sham-operated animals (fig. 5.13). In contrast, PGI$_2$ release by the aortae from portal hypertensive rats was reduced in response to PDBU (fig. 5.14), A23187 (fig. 5.15) and thapsigargin (fig. 5.16) compared to controls. There were no differences in aortic PGI$_2$ output in response to AA between rats with portal hypertension and control rats (fig 5.17). PGI$_2$ release by the mesenteric vasculature from HPH rats compared to controls was enhanced in response to adrenaline (fig. 5.13), PDBU (fig. 5.14), A23187 (5.15), thapsigargin (fig. 5.16) and AA (fig. 5.17)

5.3. DISCUSSION

5.3a Diabetes

Although it is well established that PGI$_2$ synthesis by the rat aorta is diminished in experimental DM (Harrison et al., 1978; Rogers et al., 1981; Jeremy et al., 1987a), the mechanisms that determine this reduction are not clearly defined. However, it has been shown that AA stores in vascular tissue, as well circulating AA, are reduced in experimental DM (Holman et al, 1983; Thomson 1980; Dang et al., 1988). This probably accounts for the generalised
Fig. 5.1. Adrenaline-stimulated PGI₂ release by aortae from diabetic (△) and control rats (○). Data are expressed in terms of: a) absolute amounts of PGI₂ released (as 6-oxo-PGF₁α) and b) as % of maximal response. Each point = mean ± S.D., n = 7. *p < 0.001.
Fig. 5.2.Angiotensin II-stimulated PGI2 release by aortae from diabetic (Δ) and control rats (O). Data are expressed in terms of: a) absolute amounts of PGI2 released (as 6-oxo-PGF1α) and b) as % of maximal response. Each point = mean ± S.D., n = 7. * p < 0.001.
Fig. 5.3. Phorbol ester-stimulated PGI$_2$ release by aortae from diabetic (Δ) and control rats (O). Data are expressed in terms of: a) absolute amounts of PGI$_2$ released (as 6-oxo-PGF$_{1α}$) and b) as % of maximal response. Each point = mean ± S.D., n = 7. *p < 0.001.
Fig. 5.4. Ca\(^{2+}\) ionophore A23187 - stimulated PGI\(_2\) release by aortae from diabetic (△) and control rats (○). Data are expressed in terms of: a) absolute amounts of PGI\(_2\) released (as 6-oxo-PGF\(_{1\alpha}\)) and b) as % of maximal response. Each point = mean ± S.D., n = 7. *p < 0.001.
Fig. 5.5. Thapsigargin - stimulated PGI₂ release by aortae from diabetic (△) and control rats (○). Data are expressed in terms of: a) absolute amounts of PGI₂ released (as 6-oxo-PGF₁α) and b) as % of maximal response. Each point = mean ± S.D., n = 7. * p < 0.001.
Fig. 5.6. Arachidonate-stimulated PGI$_2$ release by aortae from diabetic (△) and control rats (O). Data are expressed in terms of: a) absolute amounts of PGI$_2$ released (as 6-oxo-PGF$_{1\alpha}$) and b) as % of maximal response. Each point = mean ± S.D., n = 7. *p < 0.001.
Fig. 5.7. Acetylcholine - stimulated PGI₂ release by urinary bladders from diabetic (Δ) and control rats (O). Data are expressed in terms of: a) absolute amounts of PGI₂ released (as 6-oxo-PGF₁α) and b) as % of maximal response. Each point = mean ± S.D., n = 7. *p < 0.001.
Fig. 5.8. Phorbol ester dibutyrate - stimulated PGI₂ release by urinary bladders from diabetic (Δ) and control rats (O). Data are expressed in terms of: a) absolute amounts PGI₂ released (as 6-oxo-PGF₁α) and b) as % of maximal response. Each point = mean ± S.D., n = 7. *p < 0.001.
Fig. 5.9. Ca$^{2+}$ ionophore A23187 - stimulated PGI$_2$ release by urinary bladders from diabetic (△) and control rats (O). Data are expressed in terms of: a) absolute amounts of PGI$_2$ released (as 6-oxo-PGF$_{1\alpha}$ ) and b) as % of maximal response. Each point = mean ± S.D., n = 7. * p < 0.001.
Fig. 5.10. Thapsigargin - stimulated PGI<sub>2</sub> release by urinary bladders from diabetic (△) and control rats (O). Data are expressed in terms of: a) absolute amounts of PGI<sub>2</sub> released (as 6-oxo-PGF<sub>1α</sub>) and b) as % of maximal response. Each point = mean ± S.D., n = 7. * p < 0.001.
Fig. 5.11. Acetylcholine - stimulated PGI$_2$ release by the trachea from diabetic (Δ) and control rats (○). Data are expressed in terms of: a) absolute amounts of PGI$_2$ released (as 6-oxo-PGF$_{1\alpha}$) and b) as % of maximal response. Each point = mean ± S.D., n = 7. * p < 0.001.
Fig. 5.12. PGI₂ (as 6-oxo-PGF₁α) synthesis by different vascular tissues of the rat, eight weeks after induction of diabetes with streptozotocin. CON = control, DM = diabetic fed ad libitum, DM-PF = pair fed diabetic, INS-DM = insulin-treated diabetic fed ad libitum. Each histogram represents the mean ± SEM, n = 7.

*= p < 0.001 compared with controls.
Table 5.1 PGE$_2$, PGF$_{2\alpha}$ and TXB$_2$ synthesis (pg / mg wet wt tissue / min; mean ± SEM, n = 7) by vascular tissue from diabetic (fed ad libitum, pair fed, insulin-treated) and control rats eight weeks after induction of diabetes with streptozotocin. * = p < 0.001 compared with controls

<table>
<thead>
<tr>
<th></th>
<th>Controls (fed ad libitum)</th>
<th>diabetic (fed ad libitum)</th>
<th>diabetic (pair fed)</th>
<th>diabetic (insulin-treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. mesenteric vessels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>65 ± 6</td>
<td>168 ± 20*</td>
<td>135 ± 15*</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>22 ± 3</td>
<td>78 ± 15*</td>
<td>82 ± 10*</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>TXB$_2$</td>
<td>5 ± 0.5</td>
<td>15 ± 1*</td>
<td>14 ± 2*</td>
<td>8 ± 2</td>
</tr>
<tr>
<td><strong>2. hepatic portal vein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>55 ± 5</td>
<td>140 ± 16*</td>
<td>105 ± 12*</td>
<td>39 ± 5</td>
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<tr>
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<td>16 ± 3</td>
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<td>57 ± 10*</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>TXB$_2$</td>
<td>6 ± 0.5</td>
<td>22 ± 3*</td>
<td>18 ± 3*</td>
<td>8 ± 1</td>
</tr>
<tr>
<td><strong>3. aorta</strong></td>
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</tr>
<tr>
<td>PGE$_2$</td>
<td>88 ± 16</td>
<td>58 ± 12*</td>
<td>54 ± 11*</td>
<td>76 ± 14</td>
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<tr>
<td>PGF$_{2\alpha}$</td>
<td>22 ± 3</td>
<td>13 ± 2*</td>
<td>12 ± 2*</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>TXB$_2$</td>
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<td>2 ± 0.5*</td>
<td>2 ± 0.3*</td>
<td>6 ± 1</td>
</tr>
<tr>
<td><strong>4. carotid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>78 ± 15</td>
<td>55 ± 10*</td>
<td>50 ± 12*</td>
<td>66 ± 20</td>
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<tr>
<td>PGF$_{2\alpha}$</td>
<td>18 ± 4</td>
<td>10 ± 1*</td>
<td>11 ± 2*</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>TXB$_2$</td>
<td>6 ± 0.3</td>
<td>3 ± 0.3*</td>
<td>3 ± 0.3*</td>
<td>7 ± 0.5</td>
</tr>
</tbody>
</table>
Table 5.2. PGI\textsubscript{2} (as 6-oxo-PGF\textsubscript{1\alpha}), PGE\textsubscript{2}, PGF\textsubscript{2\alpha} and TXA\textsubscript{2} (as TXB\textsubscript{2}) synthesis [pg. mg\textsuperscript{-1} wet wt tissue. min\textsuperscript{-1}; mean ± SEM, n = 7] by GI tissue from diabetic (fed ad libitum, pair fed, insulin-treated) and control rats eight weeks after induction of diabetes with streptozotocin.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>diabetic (ad libitum)</th>
<th>diabetic (pair fed)</th>
<th>diabetic (insulin-treated)</th>
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<td><strong>1. Stomach muscularis</strong></td>
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<tr>
<td>6-oxo-PGF\textsubscript{1\alpha}</td>
<td>136 ± 18</td>
<td>146 ± 28</td>
<td>145 ± 24</td>
<td>130 ± 19</td>
</tr>
<tr>
<td>TXB\textsubscript{2}</td>
<td>13 ± 1</td>
<td>12 ± 4</td>
<td>16 ± 3</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>PGE\textsubscript{2}</td>
<td>50 ± 15</td>
<td>57 ± 18</td>
<td>58 ± 12</td>
<td>66 ± 20</td>
</tr>
<tr>
<td>PGF\textsubscript{2\alpha}</td>
<td>24 ± 3</td>
<td>26 ± 8</td>
<td>28 ± 13</td>
<td>23 ± 3</td>
</tr>
<tr>
<td><strong>2. Stomach mucosa</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-oxo-PGF\textsubscript{1\alpha}</td>
<td>22 ± 2</td>
<td>23 ± 3</td>
<td>24 ± 4</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>TXB\textsubscript{2}</td>
<td>4 ± 0.5</td>
<td>4 ± 1</td>
<td>5 ± 0.5</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>PGE\textsubscript{2}</td>
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<td>6 ± 1</td>
<td>7 ± 1</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>PGF\textsubscript{2\alpha}</td>
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<td>4 ± 0.4</td>
<td>3 ± 1</td>
<td>3 ± 0.3</td>
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<td><strong>3. Duodenum (muscularis + mucosa)</strong></td>
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<tr>
<td>6-oxo-PGF\textsubscript{1\alpha}</td>
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<td>120 ± 22</td>
<td>125 ± 18</td>
<td>115 ± 28</td>
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<td>12 ± 2</td>
</tr>
<tr>
<td>PGE\textsubscript{2}</td>
<td>65 ± 8</td>
<td>62 ± 14</td>
<td>51 ± 16</td>
<td>56 ± 11</td>
</tr>
<tr>
<td>PGF\textsubscript{2\alpha}</td>
<td>54 ± 10</td>
<td>64 ± 8</td>
<td>58 ± 13</td>
<td>44 ± 12</td>
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<td><strong>4. Jejunal muscularis</strong></td>
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<tr>
<td>6-oxo-PGF\textsubscript{1\alpha}</td>
<td>148 ± 22</td>
<td>153 ± 24</td>
<td>160 ± 33</td>
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<tr>
<td>TXB\textsubscript{2}</td>
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<tr>
<td>PGE\textsubscript{2}</td>
<td>75 ± 9</td>
<td>84 ± 14</td>
<td>60 ± 15</td>
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<td>PGF\textsubscript{2\alpha}</td>
<td>24 ± 3</td>
<td>30 ± 5</td>
<td>28 ± 7</td>
<td>27 ± 4</td>
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<td><strong>5. Jejunal mucosa</strong></td>
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<td>6 ± 1</td>
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<tr>
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<td>10 ± 5</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>PGF\textsubscript{2\alpha}</td>
<td>6 ± 1</td>
<td>7 ± 2</td>
<td>5 ± 2</td>
<td>3 ± 3</td>
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<tr>
<td><strong>6. Ileal muscularis</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>6-oxo-PGF\textsubscript{1\alpha}</td>
<td>165 ± 38</td>
<td>170 ± 48</td>
<td>170 ± 18</td>
<td>154 ± 33</td>
</tr>
<tr>
<td>TXB\textsubscript{2}</td>
<td>23 ± 3</td>
<td>21 ± 2</td>
<td>22 ± 2</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>PGE\textsubscript{2}</td>
<td>85 ± 19</td>
<td>82 ± 14</td>
<td>92 ± 25</td>
<td>78 ± 22</td>
</tr>
<tr>
<td>PGF\textsubscript{2\alpha}</td>
<td>23 ± 3</td>
<td>29 ± 5</td>
<td>27 ± 6</td>
<td>20 ± 3</td>
</tr>
<tr>
<td><strong>7. Ileal mucosa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-oxo-PGF\textsubscript{1\alpha}</td>
<td>18 ± 4</td>
<td>16 ± 3</td>
<td>20 ± 4</td>
<td>19 ± 4</td>
</tr>
<tr>
<td>TXB\textsubscript{2}</td>
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<td>6 ± 1</td>
<td>7 ± 1</td>
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<tr>
<td>PGE\textsubscript{2}</td>
<td>10 ± 3</td>
<td>12 ± 1</td>
<td>9 ± 2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>PGF\textsubscript{2\alpha}</td>
<td>7 ± 1</td>
<td>6 ± 2</td>
<td>5 ± 2</td>
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</tbody>
</table>
Fig. 5.13. Adrenaline-stimulated PGI₂ release by a) aortae and b) mesenteric vasculature from portal vein-constricted rats (●) and sham-operated rats (◇).

Each point = mean ± S.D., n = 12. * p < 0.01.
Fig. 5. Phorbol ester dibutyrate-stimulated PGI$_2$ release by a) aortae and b) mesenteric vasculature from portal vein-constricted rats (●) and sham-operated rats (◆).
Each point = mean ± S.D., n = 12. * p < 0.01.
Fig. 5. 15. Ca^{2+} ionophore A23187-stimulated PGI_{2} release by a) aortae and b) mesenteric vasculature from portal vein-constricted rats (●) and sham-operated rats (◇). Each point = mean ± S.D., n = 12. * p < 0.01.
Fig. 5. 16. Thapsigargin - stimulated PGI$_2$ release by a) aortae and b) mesenteric vasculature from portal vein-constricted rats (●) and sham-operated rats (◆). Each point = mean ± S.D., n = 12. * p < 0.01.
Fig. 5. 17. Arachidonate-stimulated PGI$_2$ release by a) aortae and b) mesenteric vasculature from portal vein-constricted (○) and sham-operated rats (◆).

Each point = mean ± S.D., n = 12. * p < 0.01.
reduction of PGI₂ release by aortae from diabetic rats, irrespective of the stimulator used. The exception to this was AA-stimulated PGI₂ synthesis, in which no difference in the output of PGI₂ was seen between control and diabetic aortae. This consolidates previous conclusions that there is no alteration of either cyclooxygenase or PGI₂ synthase activity in blood vessels from diabetic rats (Jeremy et al, 1987a). It is also notable that there were no significant differences in PGI₂ output between diabetic and control aortae in response to thapsigargin and overall absolute output was markedly less than that derived from A23187 or adrenaline. This may indicate that intracellular Ca²⁺ elevation liberates AA from distinctly different phospholipid stores than other stimulators. Such a possibility warrants further investigation.

Following transformation of data from absolute output of PGI₂ per unit weight of tissue to % maximal response, differential changes in the dose-response curves emerged for the different stimulators used. Firstly, there were no differences in the dose-response curves for thapsigargin and A23187 between diabetic and control aortae. Since thapsigargin mobilises intracellular Ca²⁺ (Tharstrup, 1990; Levine and Watanabe, 1991) and A23187 elicits entry of extracellular Ca²⁺ via the creation of artificial Ca²⁺ channels (Reed and Lardy, 1972), these data indicate that Ca²⁺ pools and/or PLA₂ activity are unaltered by DM in the rat. In contrast, there were marked right shifts in the dose-PIG response curves for both adrenaline, ANG II and phorbol ester. Since receptor-linked PGI₂ synthesis in the rat aorta is mediated by PKC (Jeremy et al, 1988a), these data indicate that the defect in receptor-linked PGI₂ synthesis in the rat aorta may be due to alterations of PKC activity.
rather than to changes of affinity and/or number of receptors linked to PGI$_2$ synthesis (viz. $\alpha$-adrenoceptor and ANG II receptors). It is also notable that in the rat aorta, the endothelium contributes negligible amounts of the total PGI$_2$ generated by this vessel in response to agonists, phorbol ester and ionophores (Chapter 3). The present data therefore reflects PGI$_2$ released by the vascular smooth muscle component of the aorta rather than that generated by the endothelium.

Recent data consolidates the proposal that vascular PKC activity is altered in DM. Lee et al. (1988) demonstrated a marked reduction in cytosolic (but an increase in membrane-associated) PKC in aortae of diabetic rats, an effect mimicked in vitro by high concentrations of glucose. Under normal conditions, receptor activation by agonists elicits the activation of cytosolic PKC (Iwamoto et al., 1992). Activated PKC phosphorylates effector proteins and also moves into the membrane where it down-regulates receptor activity (Iwamoto et al., 1992). The data of Lee et al. (1988) therefore points to a generalised movement of PKC into the plasma membrane of vascular cells in diabetic rats which in turn would serve to diminish receptor-linked responses, including PGI$_2$ synthesis. A reduction in cytosolic PKC would also account for the diminished response to phorbol ester reported in this study, since phorbol esters elicit responses by activating cytosolic PKC (Nishizuka, 1988; Iwamoto et al., 1992). In terms of other receptor-linked functions, both attenuation and potentiation of receptor-linked contraction of aortae from diabetic rats has been reported (for review see Tomlinson et al., 1992). How PKC relates to these variable alterations in contractility warrants consideration.
In direct contrast to the aorta, there were marked increases of PG synthesis by mesenteric vessels and hepatic portal veins of diabetic rats whereas, in the same animals, PG synthesis by the aorta and carotid arteries was again significantly diminished. Since the increase in PG synthesis by the mesenteric and hepatic portal vessels was not reversed in the pair fed animals these data indicate that DM per se and not the amount of food ingested determines these changes. This conclusion is consolidated by the prevention by insulin administration of changes in PG synthesis, body weights and food intake in diabetic rats. The present findings are also in agreement with the studies of Fujii et al (1986) who demonstrated an increase in PG release by isolated perfused mesenteric vasculature from diabetic rats.

It has been established that experimental DM results in increased GI and splanchnic blood flow (Korthuis et al., 1987; Rosenblum et al., 1985) and a marked reduction in contractile responsiveness of the mesenteric vascular bed (Longhurst et al., 1985). Given that splanchnic blood flow is a key mediator of nutrient uptake (Mailman, 1982) and that PGs are potent modulators of mesenteric and hepatic portal contractility (Traverso et al., 1984; Kondo et al., 1980) the marked increase in PG synthesis may constitute an adaptative response that serves to enhance nutrient uptake. Quantitatively, the dominant PG released by the mesenteric and hepatic portal vessels is PGI₂. In turn, PGI₂ is a vasodilator of the rat splanchnic vascular region (Hadhazy et al., 1988) and of the rat hepatic portal vein (Traverso et al., 1984). Furthermore, although PGE₂, PGF₂α and TXA₂ are vasoconstrictors of mesenteric vessels in the rat (Hadhazy, 1988), they have also been shown to
inhibit norepinephrine release from presynaptic terminals in this vascular region (Kuriyama and Makita, 1982). Since norepinephrine is a vasoconstrictor in the mesenteric vascular bed (Kondo et al., 1980), increased local synthesis and release of these vascular PGs may therefore enhance the tendency towards vasodilation and increased blood flow through suppression of noradrenaline release.

The increased synthesis of PGs in mesenteric and hepatic portal veins may be determined by a dietary component(s) or is consequent to other adaptive changes of these vessels. The most likely candidate for a dietary factor is arachidonic acid (AA), the precursor substrate for the synthesis of PGs. Fatty acid absorption across the small gut is known to be enhanced in experimental DM (Thomson, 1980). Thus, greater quantities of AA in "prehepatic" blood may enhance PG synthesis in the mesenteric and hepatic portal vein. The decrease in prostanoid synthesis by "post hepatic" vessels, in turn, may be attributable to the documented changes in circulating AA (Holman et al., 1983). Thus, although greater quantities of AA may be presented to the liver, reduced "free" AA may actually enter the systemic blood stream of the diabetic rat. Furthermore, other dietary components, in particular glucose, have been shown to enhance vascular PGI₂ synthesis (Fujii et al., 1986) and as such may also play a role in these local changes of vascular PG synthesis.

Also in contrast to the aorta, there was a generalised increase in the release of PGI₂ by the urinary bladder from diabetic rats, irrespective of the stimulator used. However, following transformation of data to % maximal response there was a marked left shift in ACh-PGI₂ dose-response curves obtained from diabetic
bladders when compared with controls. In contrast, no shifts were seen in the dose-response curves for phorbol ester, thapsigargin, A23187 or AA. Thus, in the case of the bladder, there appears to be a specific increase in muscarine receptor number/activity but no changes in PKC, Ca\(^{2+}\) stores, PLA\(_2\), cyclooxygenase or synthase enzymes. These conclusions are in agreement with a recent study in which marked left shifts of the dose-response curves for Ach-stimulated contraction were found in urinary bladders from diabetic rats (Latifpour et al., 1992). The diametrically opposite pattern of receptor-linked PGI\(_2\) synthesis by the urinary bladder and the aorta can be ascribed to the particular stresses imposed on the bladder in experimental DM. In diabetic rats, the urinary bladder is markedly enlarged, a result of chronic distension caused by polydypsia and polyuria (Lincoln et al, 1984). Thus, the up-regulation of muscarine receptors (and the increase in PGI\(_2\) output) in the urinary bladder of diabetic rats is probably a specific adaptive event relating to increased micturition rate. Furthermore, DM does not appear to elicit a per se up-regulation of muscarine receptors linked to PGI\(_2\) synthesis, since Ach-stimulated PGI\(_2\) synthesis was significantly diminished in the trachea from diabetic rats compared with controls. In fact, the trachea more resembles the aorta in terms of diabetes-induced alterations in receptor-linked PGI\(_2\) synthesis. It is perhaps notable that DM is not particularly associated with disorders of the airways in man (e.g. an increased incidence of obstructive airways disease). It may, however, be of interest to study airways function in the diabetic rat, which in turn may provide insights into the role of PGs in basic airways physiology.

Thus, it appears that changes of PGI\(_2\) synthesis in DM are
organ-specific. For example, there are no changes in PGI₂ synthesis in any region of the GI tract (also markedly enlarged due to hyperphagia), whereas there is a three-fold increase in PGI₂ synthesis by the splanchnic vasculature and hepatic portal vein of the diabetic rat. There is also a marked output of PGI₂ from perfused mesenteric vessels from diabetic rats (Fujii et al., 1986). Decreases in PGI₂ have been reported in all regions of the brain and the penis (Jeremy et al., 1985a, 1987b). In contrast, Stevens et al. (1992) found no significant differences in PGI₂ release stimulated by ANG II from perfused lungs of diabetic rats. Taken together, these experimental findings indicate that large arteries appear to be more susceptible to DM than the microvasculature. This phenomenon may in turn be of importance from a clinical point of view since vascular complications (viz. atherosclerosis in large arteries) are a common cause of death in DM. Thus, apart from mediating PGI₂ release, PKC also controls other vascular functions including contractility (Danthaluri and Deth; 1984; Rasmussen et al., 1984) and cell proliferation, the pathognomonic lesion of atherosclerosis (Berridge, 1984). PKC also modulates the generation of vascular nitric oxide (NO; Lewis and Henderson, 1987; Smith and Lang, 1990), an important vasodilator and inhibitor of platelet aggregation (Palmer et al., 1987; Radomski et al., 1987). Thus, the mechanisms and consequences of changes in PKC in vascular tissue from diabetic animals warrants further investigation.

5.3b Hepatic portal hypertension.

The present study firstly demonstrates that there is no
fundamental increase in the activity of PGI₂ synthesising enzymes (PLA₂, cyclooxygenase, PGI₂ synthase) in the aortae of rats with HPH, whereas in mesenteric vessels there is a marked increase in PGI₂ synthesising capacity. These latter findings concur with the previous reports of others: that PGI₂ synthesising enzymes are increased in the splanchnic vasculature of rats with HPH (Hamilton et al., 1982; Sitzmann and Li, 1991; Guarner and Soriano, 1993). It is concluded that in mesenteric vessels, PGI₂ may contribute to reduced vascular reactivity in experimental HPH, whereas it is unlikely to play a similar role in the aorta. This fundamental difference in the aorta and mesenteric vasculature probably reflects the different stresses to which the vessels are subjected in this particular experimental scenario. Following ligation of the hepatic portal vein, there is a marked increase in blood pressure in the splanchnic region and therefore distension of blood vessels. In turn, distension has been shown to increase PGI₂ synthesising capacity, not only in blood vessels, (including the rat hepatic portal vein [Hamilton et al., 1982] and human aorta [Tsang et al., 1988]) but also in other tissues (e.g. urinary bladder of the diabetic rat [Jeremy et al., 1986d]). As was outlined above, this phenomenon may be related directly to hyperplasia, an event known to occur in blood vessels in response to hypertension in both man and the laboratory animal (Swales et al., 1991). We are unaware of any study that demonstrates hyperplasia in the splanchnic or systemic vasculature in experimental HPH, but this proposition would seem to warrant investigation.

In the aorta of HPH rats, enhanced PGI₂ release, in response to adrenaline, but reduced output of PGI₂ in response to phorbol ester,
A23187 and thapsigargin cannot be ascribed to changes in substrate or synthesising enzymes since the total synthesising capacity of PGI$_2$ (index of endogenous substrate and enzymes) was not different between aortae from rats with HPH or sham-operated animals. Therefore, there appears to be a specific enhancement of adrenoceptor-linked PGI$_2$ synthesis in the aortae from rats with HPH which does not seem to reflect changes of PKC activity or Ca$^{2+}$ mobilisation. In a parallel study on aortic contractility in the same animals, adrenoceptor-stimulated contraction was significantly reduced in HPH (Karatapanis et al., 1992), indicating that the effect on adrenaline-stimulated PGI$_2$ cannot be ascribed to an increase in alpha adrenoceptors, per se. On this particular point, it has been previously suggested that there is a direct relationship between contraction of blood vessels and PGI$_2$ (i.e. that agonists which stimulate contraction also stimulate PGI$_2$ via a shared pathway; Jeremy et al., 1985a, 1988a). However, the present data mitigates against this. One possible explanation for this disparity is that adrenoceptors may be linked directly to PLA$_2$ via G proteins (DeMolle and Boeynaems, 1989); effectively by-passing PKC and Ca$^{2+}$ mobilisation. Due to the overall marked increase in PGI$_2$ synthesising capacity by mesenteric vessels from rats with HPH, it was not possible to discern any differential effects of the various stimulators used. However, the biochemical measurement of PKC and Ca$^{2+}$ in the mesenteric vessels would answer whether this vascular tissue resembles the aorta (vis a vis PKC and Ca$^{2+}$).

The apparent reduction of PKC activity and of activator Ca$^{2+}$, as detected by diminished PGI$_2$ release in response to phorbol ester, A23187 and thapsigargin, may explain (at least in part) the well-
established diminished contractility of aortae in rats with HPH.
That $\text{Ca}^{2+}$ is essential for excitation-contraction coupling in vascular tissue is well established (Somlyo and Somlyo, 1968). PKC activation (by phorbol esters) also elicits in vitro vasoconstriction of blood vessels (an effect enhanced by $\text{Ca}^{2+}$ [Danthaluri and Deth, 1984; Rasmussen et al., 1984]). In turn, virtually every pressor agent hitherto investigated elicits generation of diacyl glycerol (DAG) and IP$_3$ in vascular tissue. DAG activates PKC (which is a $\text{Ca}^{2+}$-dependent enzyme) and IP$_3$ liberates intracellular stores of $\text{Ca}^{2+}$, thereby elevating cytosolic $\text{Ca}^{2+}$ levels. Since both events are associated with vasoconstriction, it follows that any attenuation of these events would result in a tendency toward vasodilation. In this context, KCl-stimulated contraction of aortae from rats with HPH (KCl elicits vasoconstriction via mobilisation of extracellular $\text{Ca}^{2+}$) was found to be diminished in aortae of rats with HPH (Karatapanis et al., 1992). It is also of interest that phorbol ester inhibits NO synthesis in vascular tissue (Lewis and Henderson, 1987). Thus, a reduction in PKC activity in vessels would be expected to increase NO synthesis. It would be of interest to study the converse relationship (i.e., the effect of NO on PKC activity) in vascular tissue from rats with HPH. However, it is known that NO inhibits $\text{Ca}^{2+}$ mobilisation in vascular tissue (Ignarro, 1990, Rubanyi, 1989), which in turn may be expected to inhibit PKC activity, since this enzyme is $\text{Ca}^{2+}$-dependent (Nishizuka, 1984). The present data are therefore consistent with a key mediatory role for NO in HPH.

In summary, HPH elicits differential changes of PGI$_2$ synthesis in the aorta and mesenteric vasculature of the rat with experimental
HPH. It appears that, by virtue of the quantities generated, PGI$_2$ may play a vasodilatory role in the splanchnic (but not systemic) vasculature. However, a generalised reduction in PKC activity and Ca$^{2+}$ 'availability' may account, at least in part, for the diminished vasoactivity of the aorta in HPH. Although the mechanisms that govern the adaptive changes of the vasculature in response HPH remain unknown, this phenomenon is likely to be multifactorial (NO, PGI$_2$, PKC, Ca$^{2+}$, G proteins), with each factor interacting with the other factors to engender vasodilation. Furthermore, by using the approach employed in this study, these mechanisms (intrinsic vascular and/or circulating trophic factors) could be dissected out. An understanding of the mechanisms that determine the vascular changes discussed here may be of value in understanding not only the aetiology of HPH but also of other forms of hyper- and hypotension. In turn, this may be of use in the design of drugs that control blood pressure disorders.
CHAPTER 6.
GENERAL DISCUSSION AND CONCLUDING REMARKS

The objective of this thesis was to further explore receptor-linked eicosanoid synthesis in vascular tissue and platelets and to apply these systems to the investigation of drug action and the mechanisms underlying defects of blood vessels in animal models of vascular disorders (viz. diabetes and portal hypertension). Apart from alterations in eicosanoid synthesis (which in themselves are of relevance to drug action and the pathophysiology of disease) the methods developed also provided insights into alternative sites of drug action and possible alterations of vascular function in experimental pathologies. Since detailed discussions on each topic have been presented at the end of each chapter, this discussion centres on some logistic aspects and possible future directions for the work presented in this thesis.

Firstly, using the approach developed in this thesis (eicosanoid release in response to a battery of stimulators) several possible alternative sites of action (viz. Ca$^{2+}$ dependent processes; PKC, and phospholipase activity) were revealed for milrinone and NSAIDs. It is not suggested that this methodological approach supplant other established methods (e.g. platelet aggregation, shape change or vascular contractility in organ baths) but rather that it may prove useful as an adjunct to these methods. Thus, by studying the effect of any given drug on eicosanoid release elicited by a battery of different stimulators, one can obtain an indication of the mode of action of the drug under investigation. This information would then
point the way to approaches using functional tests (e.g. aggregation, contraction/relaxation). This point is exemplified by a breakdown of the day to day logistics of the assessment of TXA₂ release by platelets. In any one day, 10 ml of washed platelets (from 30 ml of blood) derived from approximately an individual represents at least 100 separate data points. By reducing the volume of platelet suspensions for each test (drug concentration) this number of data points can easily be boosted to 200 or more. It takes two days to obtain final results. Thus, for each individual, a large number of data points are quickly accessible. In contrast, 10 ml of platelet rich plasma from a single individual allows for 25 aggregation tests, at most. Whole blood aggregation, however, requires no preparative stage and uses only 12 ul per test. Although platelet function tests (aggregation, shape change) are ultimately "the bottom line" with regard to drug action, preliminary studies with TXA₂ release in washed unstirred platelets could help in planning approaches to functional tests. The same principal applies to vascular tissue from a laboratory animal. From 10 rats, for example, one can obtain as many as 300 aortic rings (as well as large amounts of tissues from other organs). Both the large number of samples that can be processed and their homogeneity reduces inter- and intra-individual variation thereby reducing the necessity for large numbers of separate tests.

It should be emphasised that although the monitoring of eicosanoid release in response to different stimulators may be suggestive of the site of action of a drug, this method is not definitive. Thus, in order to rigorously elucidate sites of drug action, the present approach should be backed up with other
biochemical endpoints: viz. PI turnover, the measurement of PKC, phospholipase assays and Ca\(^{2+}\) dynamics. In this latter context, a fellow PhD student at this laboratory, Ms Jasvinder Gill, has developed methods to investigate Ca\(^{2+}\) uptake (with \(^{45}\)Ca\(^{2+}\)) by human platelets and has confirmed that NSAIDs and milrinone inhibit \(^{45}\)Ca\(^{2+}\) uptake (Gill et al., 1990; Jeremy et al., 1993). It should be stressed that \(^{45}\)Ca\(^{2+}\) uptake does not appear to reflect alterations in dynamics of intracellular Ca\(^{2+}\) (routinely assessed using fluorescent dyes), but rather Ca\(^{2+}\)-dependent events at the plasma membrane (PKC and phospholipase activation, Ca\(^{2+}\) pumps; Gill et al., 1990; 1991). Further work with \(^{45}\)Ca\(^{2+}\) therefore holds much promise as a methodological adjunct to eicosanoid release and the measurement of other signal transduction parameters.

The variations in response (PGI\(_2\) release) to agonists by blood vessels from different species is also exploitable when studying drugs, in particular receptor antagonists. For example, where one wishes to study an adrenoceptor antagonist, then any of the species investigated would be applicable. For muscarine (acetylcholine) receptor antagonists vessels from the rabbit or man (but not rat) would be amenable. Histamine and 5-HT antagonists would have to be studied in human vessels. However, recent experiments indicate that 5-HT and histamine are potent stimulators of PGI\(_2\) release in the guinea pig (Jeremy et al., unpublished observations) rendering this species suitable for study of antagonists of these bioamines. On this particular point, the approach developed in this thesis is particularly useful in assessing drugs which are not out and out receptor antagonists. Many drugs used in the treatment of
cardiovascular disease (particularly antihypertensive drugs) have obvious structural similarities (e.g. the presence of pyridine or pirenzepine groups). These drugs include phosphodiesterase inhibitors, \( \text{Ca}^{2+} \) channel blockers, some ACE inhibitors and ACE antagonists. It would be of potential value to formalise a study in which drug action (using the systems described in this thesis) relates to drug structure. In turn, such a study may contribute to drug design and the prediction of toxic side effects.

The ex vivo studies on vascular \( \text{PGI}_2 \) synthesis (elicited with stimulators which act at different sites) in rats with either diabetes or portal hypertension also illustrates the potential applications of the approach to study drug action. Thus, animals could receive any given drug and possible effects at various key sites (\( \text{Ca}^{2+} \) channels, PKC, phospholipase, cyclooxygenase) detected. The methods described in this thesis are suitable for ex vivo studies of drug action of in man using platelet \( \text{TXA}_2 \) release (elicited by a battery of stimulators) as an endpoint. In this latter experimental approach, it would perhaps be preferable to study release not only in washed platelets but also in platelet rich plasma and whole blood. By using these different platelet preparations one could obtain information on a) the amount of drug taken up by the platelet, b) the relative importance of drug bound to plasma proteins and c) drug effects on interactions of platelets with other blood cells (leucocytes and erythrocytes).

In the studies on experimental models of diabetes and portal hypertension, marked alterations in receptors and signal transduction mechanisms linked to \( \text{PGI}_2 \) release became apparent. Using a battery of
stimulators it was possible to detect not only changes in the enzymes involved in the synthesis of PGI2 but also linked systems (receptors, Ca^{2+}, PKC). In the case of portal hypertension, the conclusions derived from this study (that there are alterations of Ca^{2+} mobilisation and PKC activity in peripheral vasculature) were borne out by studies carried out on contractility of aortic rings in organ baths (Karatapanis et al., 1993). It is also apparent from these experiments that a reduction (or increase) in PGI2 can be due to alterations of receptors and signal transduction mechanisms although there are no changes in synthesising enzymes. The method is applicable in other experimental models. For example, in models of atherosclerosis (e.g. hyperlipidaemic Watanabe rabbits), where cell proliferation is a key event in atherogenesis, it would be interesting to monitor alterations of PKC activity (via phorbol ester stimulated PGI2 release) since PKC controls cell proliferation. PKC and Ca^{2+} are also involved in the release of mitogens and vasoconstrictors from macrophages and platelets. Using a battery of stimulators one could also look at the release substances originating from these cells taken from atherosclerotic animals. A similar approach could be applied to animal models of hypertension, homocysteinaemia, cigarette smoking, ageing and gender. Furthermore, should defects be detected in these models (as in rats with diabetes and portal hypertension) it would be feasible to use these systems to investigate the efficacy of novel therapeutic agents. Put another way, the potency and efficacy of drugs could be defined using normalisation of receptor (and signal transduction)-linked eicosanoid release by vessels and blood cells altered by experimental
pathologies. Finally, the present methods are also applicable in the development of improved preservation solutions used in organ transplantation. On this general theme, these methods could also be used to assess viability of blood vessels which have been cryogenically frozen and then thawed for implantation. Thus, improvements in cryogenic methods could be made using receptor-linked PGI₂ release as an endpoint for monitoring tissue integrity. This area is of clinical importance since vessels from donors can be cryogenically stored and then retrieved for implantation in recipients (e.g. in patients undergoing coronary artery replacement) with a similar tissue type. It is hoped by the author of this thesis to address these possibilities in the near future.
PUBLICATIONS DERIVED FROM WORK PRESENTED IN THIS THESIS
(copies of first pages of publications have been placed in the inside cover of this thesis)

A) PEER REVIEWED PAPERS


B) BOOK CHAPTERS, REVIEWS AND EDITORIALS


C) ABSTRACTS


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Effect of endothelium removal on stimulatory and inhibitory modulation of rat aortic prostacyclin synthesis

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1 Removal of the endothelium (DE) enhanced the in vitro release of prostacyclin (PGI$_2$) by rat aortae in response to adrenaline (Ad), noradrenaline (NA), thromboxane A$_2$ analogue U46619, phorbol dibutyrate (PDBU) and sodium fluoride (NaF) when assessed at 3 h post DE. At 6 h post DE, there were no differences between the dose-response curves obtained from aortic rings with or without endothelium.

2 At 3 h post DE the antagonism of Ad- and NA-stimulated PGI$_2$ synthesis by yohimbine and prazosin, and NA-stimulated PGI$_2$ synthesis by nifedipine was markedly reduced in aortae without endothelium when compared with controls. These effects were reversed by protracted incubation of aortic tissue post DE (6 h and 9 h).

3 Acetylcholine, carbachol, substance P and nitroprusside were without effect on de novo or NA-stimulated PGI$_2$ synthesis, whether or not the endothelium was present and irrespective of incubation time, post-DE.

4 These results indicate that: (a) PGI$_2$ synthesis linked to excitatory receptors (α-adrenoceptors, thromboxane A$_2$) and associated systems (G proteins, protein kinase C) in the smooth muscle component of the rat aorta is not influenced by endothelium-derived relaxing factor (EDRF); (b) the changes of response to stimulators and inhibitors of PGI$_2$ synthesis may be due to an increased reactivity of the vessels caused by the trauma of DE; and (c) vasodilators (parasympathomimetics, substance P and nitroprusside) that do not act directly on excitatory receptors do not influence PGI$_2$ synthesis.

Introduction

Removal of the endothelium (DE) of isolated blood vessels results in the abolition of relaxation elicited by vasodilators (Furchgott, 1983) and in enhanced responses to contractile agonists (e.g. Egleme et al., 1984). These observations have led to the concept that vascular endothelium produces a substance (endothelium-derived relaxing factor; EDRF), the release of which is obligatory in mediating the action of vasodilators (Furchgott, 1983).

It has been demonstrated that vasoconstrictors (α-adrenoceptor agonists, thromboxane A$_2$ analogue U46619, fluoride, phorbol ester; Godfraind et al., 1982; Coleman et al., 1980; Martin et al., 1985; Danthaluri & Deth, 1984) also stimulate the synthesis of the endogenous prostanoid, prostacyclin (PGI$_2$), by isolated vascular tissue (Stewart et al., 1984; Golub et al., 1985; Jeremy et al., 1985a,b; 1986a; Jeremy & Dandona, 1987; 1988). Since PGI$_2$ is both a vasodilator (at low concentrations; Moncada & Vane, 1979) and a vasoconstrictor (at high concentrations; Van Dam et al., 1986), and there is a direct relationship between vascular contractility and PGI$_2$ synthesis, the pharmacological properties of blood vessels, in vitro, following removal of the endothelium may be due, in part, to changes in endogenous PGI$_2$ synthesis. To explore this possibility, the effect of DE on a range of known stimulators of rat aortic PGI$_2$ synthesis (adrenaline, noradrenaline, thromboxane A$_2$ analogue U46619, phorbol dibutyrate [PDBU] and sodium fluoride [NaF]; Jeremy et al., 1985a,b; Jeremy & Dandona, 1987; 1988) and known vasodilators (yohimbine, prazosin and the calcium channel blocker, nifedipine; Jeremy et al., 1985a; 1986a) on rat aortic PGI$_2$ synthesis was investigated. Since acetylcholine (ACh) and substance P are established stimulators

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Muscarninic stimulation of prostanoid synthesis by the isolated rat trachea: calcium dependency and effect of cortisol and cigarette smoke

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Tracheal prostanoid synthesis was stimulated by parasympathomimetics: arecoline > carbachol = methacholine > acetylcholine > arecaidine. McN-A343, dimethyl phenyl piperazinium (DMPP), nicotine, potassium and isoprenaline were without effect. Prostanoid synthesis was also stimulated by Ca\(^{2+}\) ionophore A23187 and arachidonic acid (AA). Carbachol-stimulated prostanoid synthesis was inhibited by cholinergic antagonists (atropine > ipratropium bromide > gallamine > pirenzepine); adrenaline and isoprenaline were without effect. Carbachol-stimulated prostanoid synthesis was also inhibited by the Ca\(^{2+}\)-channel blockers, nifedipine, diethylstilboestrol and TMB-8. Hydrocortisone and betamethasone inhibited carbachol-and A23187-stimulated, but not AA-stimulated, prostanoid synthesis following an 18 h tissue culture. Cigarette smoke extracts had a biphasic effect on carbachol-, A23187- and AA-stimulated prostanoid synthesis (potentiation at low concentrations, inhibition at high concentrations of extracts). These data demonstrate (1) that rat tracheal prostanoid synthesis is stimulable by activation of muscarine receptor-linked Ca\(^{2+}\) mobilisation, and (2) that tracheal prostanoid synthesis may be involved in secretion of mucus, the disruption of which by cigarette smoking may be related to the pathophysiology of airway disease.

Trachea; Cigarette smoke; Prostaglandin I\(_2\); Prostaglandin F\(_2\); Prostaglandin E\(_2\); Thromboxane A\(_2\); Cortisol; Muscarinic stimulation; Ca\(^{2+}\)

1. Introduction

In vascular, urinary bladder and penile tissue it has been demonstrated that those agonists which elicit contraction (or erection) also stimulate the synthesis of endogenous prostanoids through mobilisation of a common activator calcium (Stewart et al., 1984; Jeremy et al., 1985a,b; 1986a,b,c; Golub et al., 1985). Furthermore, the actions of these agonists were blocked by specific receptor antagonists and were dependent on calcium mobilisation (Stewart et al., 1984; Jeremy et al., 1985a,b; 1986a,b,c; Golub et al., 1985). It was therefore proposed that the concomitant synthesis of prostanoids on the initiation of receptor-linked contraction may play a role in the subsequent contraction/relaxation phases of smooth muscle and erection/detumescence of the penis (Jeremy et al., 1985a,b; 1986a,b,c). The question also arose as to whether contractile agonism stimulates prostanoid synthesis in other smooth muscle tissues (Jeremy et al., 1985a,b; 1986a,c).

It was therefore of interest to investigate the trachea of the rat since this tissue synthesis prostanoids (Ally et al., 1982) and its contraction is elicited by parasympathomimetics (Frossard and Landry, 1985). An in vitro system for the study of rat tracheal prostanoid synthesis was therefore
Differential changes of adrenoceptor- and muscarinic receptor-linked prostacyclin synthesis by the aorta and urinary bladder of the diabetic rat

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1 The effect of experimental diabetes mellitus (DM; hyperglycaemic, non-ketototic; 2 months duration) in the rat on receptor-linked prostacyclin (PGI₂) synthesis (measured as 6-oxo-PGF₁α by radioimmunoassay) was studied in the aorta and urinary bladder using adrenaline, angiotensin II (AII) and acetylcholine (ACH). Signal transduction systems were studied via stimulation of PGI₂ synthesis with phorbol ester dibutyrate (PDBU; a protein kinase C activator [PKC]), Ca²⁺ ionophore A23187 (A23187) and thapsigargin (both elevate intracellular Ca²⁺, activating phospholipase A₂ [PLA₂] and arachidonate [AA; substrate for PGI₂ synthesis].

2 In response to adrenaline, AII and phorbol ester, aortic PGI₂ release was markedly reduced (all >75%) in diabetic rats compared to controls. EC₅₀ of the dose–response curves for adrenaline, AII and PDBU were also markedly increased in aortas from DM rats compared to controls. Although there was decreased output of PGI₂ in response to A23187 by aortas from diabetic rats compared to controls, there was no difference in the EC₅₀ (mean ± s.e.mean: diabetic, 2.7 ± 0.2 × 10⁻⁴ M; controls 2 ± 0.18 × 10⁻⁴ M). There were no differences in PGI₂ release (or in the EC₅₀) in response to thapsigargin or AA between aortae from diabetic and control rats.

3 In the urinary bladder, there was a marked increase in PGI₂ output in response to ACh and a marked decrease in EC₅₀ for the ACh-PGI₂ dose–response curves in diabetic rats (EC₅₀ = 5.8 ± 0.32 × 10⁻⁷ M) compared to controls (EC₅₀ = 2.2 ± 0.15 × 10⁻⁴ M). Although there was an increase in PGI₂ output in the urinary bladders from diabetic rats in response to A23187, there were no differences in the EC₅₀ (control, 1.8 ± 0.2 × 10⁻⁴ M; diabetic, 1.1 ± 0.15 × 10⁻⁴ M). In the urinary bladders, there were no differences in PGI₂ output (or the EC₅₀) in response to PDBU, thapsigargin or AA between diabetic or control rats.

4 These data indicate that: (i) reduced PGI₂ synthesis coupled to adrenoceptors and AII receptors in the aortae of diabetic rats may be due to diminished PKC activity and not to changes in receptor density and/or affinity, Ca²⁺ stores, PLA₂, cyclo-oxygenase or PGI₂ synthase; (ii) the diametrically opposite effect of DM on ACh-stimulated PGI₂ synthesis is not due to an increase in PKC activity, but possibly to an increase in muscarinic receptor number and/or affinity; (iii) changes in receptor-linked PGI₂ synthesis are not ubiquitous in experimental DM and may be organ-specific.

Keywords: Prostacyclin; diabetes mellitus; rat aorta; rat urinary bladder; adrenoceptors; muscarinic receptors

Introduction

Patients with diabetes mellitus (DM) are at greater risk than the healthy population of developing cardiovascular disease (CVD; Banga & Sixma, 1986; Mikhailidis et al., 1988). Of the many aetiologic factors associated with CVD in DM, a marked attenuation of prostacyclin (PGI₂) synthesis by blood vessels from diabetic laboratory animals have been reported (Harrison et al., 1978; Rogers et al., 1981; Jeremy et al., 1987a). Apart from being a vasodilator and inhibitor of platelet aggregation (Moncada & Vane, 1979), PGI₂ modulates other vascular functions including cell proliferation (Huttner et al., 1977; Marui et al., 1990; Pomerantz & Hajjar, 1989) and metabolism of cholesterol (Huttner et al., 1977). In turn, disruption of these processes have all been implicated in the aetiology of CVD, in particular, atherogenesis and thrombosis (Jeremy & Mikhailidis, 1990). The precise mechanisms that determine reduced vascular PGI₂ synthesis are not clearly defined, although a reduction in substrate (arachidonate) levels in blood vessels has been suggested (Holman et al., 1983; Jeremy et al., 1987a; Dang et al., 1988). It has also been established that vascular PGI₂ synthesis is stimulated by vasoactive agonists (e.g. noradrenaline, histamine, acetylcholine, angiotensin II; Jeremy et al., 1988a) an effect which in turn is mediated by G proteins, protein kinase C (PKC) and inositol triphosphate (IP₃; Griending et al., 1986; Hallam et al., 1988; Jeremy et al., 1988a). The net result of activation of these systems is transmembrane Ca²⁺ influx and an increase in intracellular Ca²⁺ ([Ca²⁺]i). Ca²⁺ then activates phospholipase A₂ (PLA₂) which in turn liberates PGI₂ substrate, arachidonic acid (AA), from phospholipid stores (Irvin, 1982). In this context, experimental DM in the rat is associated with altered receptor-linked functions in vascular tissue (Tomlinson et al., 1992).

Thus, in order to elucidate further the mechanisms that determine diminished vascular PGI₂ synthesis, the effects of a range of stimulators that act at different sites in the receptor activation-PGI₂ synthesis pathway were studied on PGI₂ synthesis by the streptozotocin-induced diabetic rat: adrenaline (mediated by heterogeneous α-adrenoceptors; Jeremy et al., 1985a) and angiotensin II (AII; Hassid & Williams, 1983; Stevens et al., 1992), phorbol ester dibutyrate (PDBU; a PKC activator: Nishizuka, 1984; Jeremy & Dandona, 1986), thapsigargin (elevates intracellular Ca²⁺; Thastrup et al., 1990; Levine & Watanabe, 1991), Ca²⁺ ionophore A23187 (creates artificial Ca²⁺ channels; Reed & Lardy, 1972) and arachidonic acid (AA; substrate for PGI₂ synthesis). In order to examine whether other non-vascular smooth muscle tissues
Effect of hypothermic storage in liver allograft preservation solutions on vasoactivity and prostacyclin synthesis by the rabbit aorta, in vitro


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Primary graft dysfunction following orthotopic liver transplantation has been ascribed to thrombotic and ischemic complications in a high proportion of cases. It has been suggested that hypothermic storage of livers in preservation solutions elicits damage to the vascular endothelium. Since the endothelium controls vasoactivity and hemostasis via release of endothelium derived relaxing factor (EDRF) and prostacyclin (PGI₂), storage injury to the endothelium may predispose the allograft to thrombosis, ischemia and impaired perfusion. In order to test this, the effect of long-term hypothermic storage in modified University of Wisconsin solution (UW), kidney perfusion solution (KPS) and minimum essential medium (MEM) on phenylephrine (PE)-stimulated contraction and acetylcholine (ACh)-stimulated relaxation, as well as PGI₂ release by rabbit aortic rings was investigated. Following cold storage for 24, 48 and 72 h, PE and ACh dose response curves were unaffected by storage in MEM, UW or KPS. Following hypothermic storage for 24 h and 48 h, PGI₂ release (stimulated with PE, ACh, arachidonic acid, fluoride, calcium ionophore and phorbol ester) was not significantly altered from zero time responses. These results demonstrate that hypothermic storage of rabbit aortic rings in both UW and KPS do not influence two key endothelial functions (the release of EDRF or PGI₂) which in turn indicates that endothelial damage associated with reperfusion following hypothermic storage is not causally related to alterations in EDRF and PGI₂ release.

Key words: PLEASE SUPPLY 3–6 KEY WORDS FOR INDEXING

Primary graft dysfunction, following orthotopic liver transplantation, occurs in about 10% of cases (1–5) and is a major cause of morbidity and mortality. The cause of primary graft dysfunction is multifactorial but 'storage injury' may be an important contributory factor in a subset of patients (6). Studies using rat livers have demonstrated significant morphological damage to endothelial cells and Kupffer cells occurs following reperfusion after hypothermic storage of the organs in Euro-Collins solution and to a lesser degree in University of Wisconsin (UW) solution (7,8). The functional significance of these morphological changes is unknown. However, the vascular endothelium controls hemostasis and vasoactivity, in part, through the secretion of prostacyclin (PGI₂; inhibits platelet aggregation and dilates vessels (9–11)) as well as endothelium-derived relaxing factor (EDRF; is obligatory for relaxation and also inhibits platelet activity (9–11)). Damage to the endothelium and disruption of EDRF and PGI₂ may therefore result in vasoconstriction or thrombosis, which in turn may prejudice graft function. Indeed there is a considerable body of evidence that ischemic injury is a major cause of early graft failures (1–5).

We have previously reported that cold preservation of rat aortic rings had significant effects on vascular function and that the effects differed with the preservation solution used (12–14). Rat aortic rings stored for up to

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INHIBITION OF CYCLOOXYGENASE AND LIPOXYGENASE ACTIVITY BY IRON CHELATORS: POSSIBLE USE IN THE TREATMENT OF EICOSANOID-RELATED DISORDERS

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Introduction

Apart from the treatment of iron overload in thalassemia, oral iron chelators have other potential clinical uses where iron-containing enzymes regulate metabolites which contribute to the pathogenesis of disease. Two major iron-containing enzymes are cyclooxygenase (COX) and lipoxygenase (LOX), which generate potent bioactive substances (viz. prostaglandins [PGs], thromboxanes [TXs] and leukotrienes [LTs]). The activity of these enzymes has been widely implicated in the etiology of clinical disorders ranging from inflammatory to cardiovascular disease. Recent studies have also established that iron chelators inhibit the activity of COX and LOX (1, 2). The present paper examines the role of iron in mediating the activity of COX and LOX and summarizes the role of these enzymes in the pathophysiology of disease. In particular, our experimental findings on the effect of iron chelators on COX and LOX are reviewed. Iron status in relation to disorders associated with eicosanoids and the possible use of iron chelators in treating these disorders is also covered.

Eicosanoid Synthesis

Eicosanoid is a generic term for any metabolite derived from the essential fatty acids (principally arachidonic acid; AA). AA is stored as an ester in the phospholipids of biomembranes and is released in response to specific extracellular signals e.g. in vascular smooth muscle, noradrenaline; in platelets, collagen; in white cells, cytokines (3, 4). AA is liberated by the hydrolytic action of phospholipase A2 (PLA2), an event mediated by activation of G proteins, protein kinase C and calcium mobilization (Fig. 1). Liberated AA can then be converted to PGs, TXs, LTs, hydroxyeicosatetraenoic acid (HETE) or hydroperoxy-eicosatetraenoic acid (HPETE), depending on the cell type and the presence of the enzyme which generates these metabolites (Fig. 1). For example, the principal AA metabolite in vascular smooth muscle is prostacyclin (PGI2); in platelets, TXA2 and in leukocytes, LTs, HETE and HPETE (Fig. 1).

COX is a haem containing enzyme which firstly elicits the abstraction of a hydrogen atom from AA, forming a radical structure (5, 6) followed by the incorporation of two oxygen molecules (Fig. 2). Acyclic molecule is produced, PGG2, which under the peroxidative activity of COX is reduced to form an endoperoxide PGH2, the immediate precursor for prostanoid and thromboxane synthesis, depending on the synthase/isomerase present in any given tissue (Fig. 2).

Lipoxygenase is also an iron (non-haem) containing enzyme (7-9). LOX again abstracts a hydrogen atom from AA, forming a radical structure (5, 6) followed by the incorporation of two oxygen molecules (Fig. 2). A cyclic molecule is produced, PGG2, which under the peroxidative activity of COX is reduced to form an endoperoxide PGH2, the immediate precursor for prostanoid and thromboxane synthesis, depending on the synthase/isomerase present in any given tissue (Fig. 2).
Effect of milrinone on thromboxane A2 synthesis, cAMP phosphodiesterase and 45Ca2+ uptake by human platelets

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The phosphodiesterase inhibitors milrinone and isobutylnmetharbcanthine (IBMX) inhibited the conversion of [3H]cAMP to [3H]AMP by washed human platelets in concentration-dependent manners (IC50: milrinone, 2.6 × 10^-6 M; IBMX, 4.6 × 10^-6 M). Milrinone and IBMX increased cAMP levels when stimulated by a single concentration (0.3 μM) of iloprost. EC50: milrinone, 5.6 × 10^-7 M; IBMX, 3.0 × 10^-7 M. Milrinone was a potent inhibitor of platelet thromboxane A2 (TXA2) synthesis when stimulated by median stimulatory doses of collagen (IC50: 3 × 10^-7 M), sodium fluoride (NaF) (a non-specific G protein activator; IC50: 3.0 × 10^-7 M) and phorbol ester myristate acetate (PMA) (a protein kinase C activator; IC50: 2.2 × 10^-7 M). In contrast, at median stimulatory doses of A23187 and arachidonate there was a marked decrease in the potency of milrinone in inhibiting TXA2 synthesis. Milrinone had a weak inhibitory effect on TXA2 synthesis when elicited by freeze fracturing. In all experiments IBMX was a weaker inhibitor of TXA2 synthesis, although the general pattern of effects was similar to milrinone. Milrinone inhibited both collagen- and adrenaline-stimulated 45Ca2+ uptake by human platelets in dose-dependent manners. Since platelet TXA2 synthesis is dependent on Ca2+, and milrinone inhibited 45Ca2+ uptake, it is concluded that milrinone exerts its inhibitory effect on platelet activity, principally through an action on Ca2+ mobilisation/binding to effector proteins (protein kinase and/or phospholipase A2).

Platelets; Milrinone; Phosphodiesterase; Thromboxane A2

1. Introduction

Milrinone (2-methyl-5-cyano-(3,4'-bipyridin)-6(1H)-one) is a non-glycoside bipyridine derivative that has positive inotropic, lusitropic and vasodilating activity (Alousi et al., 1983; Borrow et al., 1985; Jaski et al., 1985). Milrinone has proved effective in the treatment of acute heart failure (DiBianco et al., 1989; Packer et al., 1991). Milrinone probably exerts its beneficial action principally via inhibition of cyclic adenosine-3'5':monophosphate (cAMP) phosphodiesterase (PDE), thereby elevating intracellular cAMP concentrations and reducing intracellular Ca2+ concentrations (Mylotte et al., 1985; Earl et al., 1986; Olson et al., 1987). Amrinone, a similar drug to milrinone, has been shown to inhibit aggregation by rabbit and human platelets (Tang and Fromijnovic, 1980; Lippton et al., 1985; Pattison et al., 1986; Simpson et al., 1988; Lindgren et al., 1990). Clearly, the inhibition of platelet activity in patients with cardiac failure by milrinone would constitute a desirable additional property since: (a) platelets are key components in thrombus formation (Chesterman and Berndt, 1984) and patients with heart failure have an increased risk of developing thrombotic episodes if ischaemic heart disease is involved (Mikhailidis et al., 1990; Trip et al., 1990); and (b) platelet release substances (viz. thromboxane A2) are potent vasoconstrictors (as well as being proaggregatory) and as such have been widely implicated in the aetiology of cardiovascular disease (Mikhailidis et al., 1987, 1990).

In light of the above we further explored the effects and properties of milrinone on various aspects of human platelet function, in vitro. Firstly, cAMP phosphodiesterase activity was assessed by studying the conversion of [3H]cAMP to [3H]AMP and iloprost (a prostacyclin analogue) -stimulated cAMP synthesis. It has also been suggested that the effects of milrinone-type drugs on platelet activity are not mediated by inhibition of PDE (Lippton et al., 1985; Dorigo et al., 1991). The effect of milrinone on thromboxane A2 (TXA2) synthesis was therefore investigated in platelets when synthesis of this eicosanoid was stimulated with colla-
Effect of Liver and Kidney Preservation Solutions on Vascular Viability: Studies on Contraction and Relaxation of Rat Aorta In Vitro


IMMUNOLOGICAL and technological developments have made liver transplantation an accepted therapy for various end-stage and genetic defective liver diseases. The standard technique for hepatic allograft preservation is hypothermic storage of the liver after vascular flush with cold preservation solutions and package of the organ on ice prior to reimplantation of the allograft. Before 1988, using either Collins or Euro-Collins (EC) preservation solutions, the “safe” storage time resulting in acceptable graft function, was 8 hours or less. However, introduction of the University of Wisconsin (UW) preservation solution has extended the safe storage time of livers for transplantation to 15 hours and longer. The UW solution differs from Collins or EC solutions in that it contains the impermanent agents raffinose and lactobionate, and the free radical scavenger allopurinol. The UW also differs considerably from more conventional “physiological” buffers in that it contains supraphysiological concentrations of potassium (K+) and it is calcium-free.

An important aspect of organ transplantation is the maintenance of patent vascular function in the grafted organ, so that its vessels do not tend toward vasoconstriction or thrombosis. When it occurs, hepatic artery thrombosis is a potentially catastrophic early complication in liver transplantation, requiring retransplantation in most cases. The vascular endothelium plays a key role in preventing thrombosis in that it secretes antithrombotic factors, including prostacyclin (PGI₂), the most potent inhibitor of platelet aggregation known, and endothelium-derived relaxing factor (EDRF), which is obligatory in mediating the action of vasodilators and also inhibits platelet adhesion and aggregation. Damage or removal of the endothelium also results in potentional vasoconstriction. Thus, structural and functional integrity of the vasculature of transplanted organs may be important contributory factors to the success of organ transplantation. In vitro studies with rat livers have demonstrated that after prolonged periods of cold storage the most vulnerable tissues to reperfusion injury were sinusoidal endothelial cells, and that UW elicits less morphologic damage than EC.

To further examine whether hypothermic storage in flush solutions influences vascular function, the effect of long-term hypothermic storage (up to 48 hours) in UW solution as well as kidney perfusion solution (KPS), Kreb’s Ringer bicarbonate solution (KRB), and minimum essential medium (MEM) on contraction and relaxation patterns of rat aortic rings was investigated. Contraction was elicited with phenylephrine, and relaxation of phenylephrine-stimulated contraction with acetylcholine (EDRF-release-dependent). The effect of cold storage in UW and KPS on potassium chloride (KCl)-stimulated contraction was also investigated, since KCl elicits vasoconstriction via a depolarisation mechanism, independent of receptor activation and EDRF release.

MATERIALS AND METHODS

Phenylephrine bitartrate, acetylcholine chloride, and Dulbecco’s MEM were purchased from Sigma Chemical Co (Poole, Dorset, United Kingdom) and was comprised of (per mmol/L): K+, 125; sodium (Na+), 25; magnesium (Mg2+), 5; lactobionate, 100; H2PO4⁻, 25; SO4²⁻, 5; raffinose, 30; adenosine 5; and pH 7.4. The KPS contained (per mmol/L): K+, 80; Na+, 80; Mg2+, 40; SO4²⁻, 55; citrate, 55; mannitol, 185; and pH 7.2. The KRB was comprised of (per mmol/L): sodium chloride (NaCl), 118; KCl, 4.8; MgSO4, 1.2; KH2PO4, 1.2; glucose, 11; NaH2PO4, 2.5; ethylenediamine tetraacetic acid, 0.034; NaHCO3, 24; CaCl2; and 2H2O, 2.5.

Male Sprague Dawley rats were killed by a blow to the head and the thoracic aorta excised. Adventitia and fatty tissue was carefully removed and the aorta cut into 2-mm rings. Aortic rings were placed in either UW, KPS, MEM, or KRB solution and stored at 4°C for 0 hours, 24 hours, or 48 hours. Following storage, rings were washed in KRB and mounted in an organ bath. The bath medium was maintained at 37°C, pH 7.4 and gassed with 95% O2 and 5% carbon dioxide. A resting tension of 1.5 g was applied and the aortic rings were equilibrated for 1.5 hours. Responses were recorded isometrically through force displacement transducers. Concentration-response curves for phenylephrine and KCl were obtained by adding the drug directly to the bathing media in cumulative concentrations. Cumulative acetylcholine concentration-relaxation responses were obtained following stimulation of contraction with 10⁻⁷ mol/L phenylephrine.

Calculation and Statistical Analysis

The degree of contraction is expressed in absolute tension (g), and relaxation as % relaxation of phenylephrine-stimulated contraction.
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Differential Changes of Prostanoid Synthesis by the Gastrointestinal Tract, Mesenteric Vasculature and Hepatic Portal Vein of Diabetic Rats: Comparison between Pair and ad Libitum Feeding

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Abstract. The synthesis of the prostaglandins (PG) I₂ (measured as 6-oxo-PGF₁α), E₂, E₃, and thromboxane (TX) A₂ (measured as TXB₂) by the mucosal and muscular portions of the stomach, duodenum, jejunum, ileum, mesenteric vessels, hepatic portal vein and two arteries (carotid and aorta) was investigated in long term streptozotocin-induced diabetes mellitus (DM; fed ad libitum and pair fed). In all regions of the gastrointestinal tract there were no changes in PG synthesis (per unit weight of tissue) in diabetic rats (pair fed or fed ad libitum) compared to controls. However, there were marked increases in PG synthesis (up to 3 fold) by the mesenteric vasculature and hepatic portal vein in diabetic animals fed ad libitum and in pair fed diabetic rats and decreases in the aorta and carotid artery. These data suggest that increases in PG synthesis by the splanchnic vasculature may constitute a specific adaptive response to DM. The similarity of the responses of pair fed rats to those of rats fed ad libitum indicates that DM and not hyperphagia is the likely determinant of these adaptive changes. Given that increased splanchnic blood flow enhances nutrient uptake (both known to occur in DM), the increase in splanchnic vascular PG synthesis, in particular of vasodilatory PG I₂, may contribute to enhanced nutrient uptake.

Diabetes mellitus (DM) in man elicits gastrointestinal (GI) dysfunction including diarrhoea, constipation, delayed gastric emptying and atony, disordered small bowel movement and aony and dilatation of the colon (4). Increased blood flow in GI vessels is also associated with the early stages of DM (28). Aetiological, these disorders have been largely ascribed to diabetic autonomic neuropathy (4). However, it is also well established that the pathophysiology of GI disorders in non-diabetics involves endogenous prostaglandins (PGs; [2]). Furthermore, DM is associated with a marked decrease in PG synthesis in several tissues, including vascular smooth muscle, the penis, brain and platelets and as such has been implicated in the pathophysiology of disorders associated with DM in these tissues (22). With regard to the GI tract in DM, we are aware of no systematic studies on PG synthesis in man or in experimental animal modes. However, it has been demonstrated that caloric deprivation results in differential changes in prostacyclin (PGI₂) and thromboxane A₂ (TXA₂) synthesis by the small intestine and mesenteric vasculature of the rat (12). Since these PGs influence mesenteric blood flow and gut motility (21), it was suggested that these changes in PG synthesis may play a role in the adaptation of the small gut in response to fasting through modulation of gut motility and mesenteric blood flow (12). It is also well established that, in experimental DM, the GI tract of the rat undergoes marked adaptive changes of structure (mucosal and muscular hyperplasia; 13, 15) and function (increased nutrient uptake; 20, 23).

Therefore, in order to investigate a possible role for PGs in the adaptation and pathology of the GI tract in DM, we assessed PG synthesis (PGI₂, TXA₂, PGE₂, PGF₂α) by the mucosal and muscular regions of the stomach, duodenum, jejunum, ileum as well as the mesenteric vasculature, and hepatic portal vein. PG synthesis by the aorta and carotid artery were also measured to assess vascular PG synthesis in "post-hepatic" vessels. Since diabetic rats are markedly hyperphagic, we also examined the effect of pair feeding (amount of food given to diabetic rats per day adjusted to the average daily intake of age-matched controls) on PG synthesis by the above tissues. The effect of insulin administration to diabetic rats was also investigated.

Materials and Methods

All experiments were carried out using male Sprague Dawley rats of an initial body weight of 250 g. Non-ketonuric, hyperglycemic diabetes was induced by an intravenous injection of streptozotocin (65 mg.kg⁻¹ body wt.) which had been dissolved in citrate buffer (pH 4.5). Controls were injected with the same volume of citrate buffer alone. These rats developed glycosuria but not ketonuria or haematuria (for glucose levels
THE EFFECT OF COLD STORAGE OF RAT THORACIC AORTIC RINGS IN ORGAN PRESERVATION SOLUTIONS—A STUDY OF RECEPTOR-LINKED VASCULAR PROSTACYCLIN SYNTHESIS

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An important aspect of organ preservation is the maintenance of intrinsic dilator and antithrombotic mechanisms of blood vessels. Blood vessels synthesize prostacyclin (PGI₂), a potent vasodilator and inhibitor of platelet adhesion and aggregation. PGI₂ synthesis is controlled by complex mechanisms including adrenoreceptor-linked calcium influx and protein kinase C. Since organ preservation solutions may influence these mechanisms, we investigated the effect on in vitro PGI₂ synthesis of cold storage of rat aortic rings in lactobionate-raffinose solution (LRS) and hypertonic citrate kidney preservation solution (KPS) on in vitro PGI₂ synthesis.  

Acute incubation of aortic tissue in both preservation solutions at 37°C (compared with minimal essential medium) completely inhibited PGI₂ synthesis when stimulated with noradrenaline (NA), phorbol ester (a protein kinase C activator), NaF (a G protein activator), or A23187. Following storage of aortic rings at 4°C (for up to 72 hr) in LRS and KPS, subsequent washing and incubation in MEM, PGI₂ synthesis was initially markedly enhanced in response to NA when compared with tissues stored in MEM. These enhanced responses disappeared, and PGI₂ synthesis returned to normal following 1 hr incubation of tissues in MEM at 37°C. These data demonstrate that cold storage in preservation fluids exerts minimal deleterious effects, not only on PGI₂ synthesis, but possibly on other key processes (calcium homeostasis, protein kinase C activity) in blood vessels.

In clinical organ transplantation, hypothermic storage of organs, such as the liver and kidney, is routinely carried out by a vascular flush with cold solutions followed by storage in ice. Until recently, the safe cold-storage time for livers used in clinical organ transplantation was considered to be about 10 hr, using a vascular flush with cold solutions followed by storage in ice. However, since the introduction of new flush solutions has increased the maximum "safe" storage time to 15 hr, hypothermic storage of other organs, such as the liver and kidney, is routinely carried out by the pharmacy at the Royal Free Hospital and consists of the following (in mmol/L): K⁺, 125; Na⁺, 25; Mg²⁺, 5; lactobionate, 100; H₂PO₄⁻, 25; SO₄²⁻, 5; allopurinol, 1; reduced glutathione, 3; adenosine 5; pH 7.4. KPS contained (in mmol/L): K⁺, 80; Na⁺, 80; Mg²⁺, 40; SO₄²⁻, 55; citrate, 55; mannitol, 185; pH 7.2.

Tissue preparation. Male Sprague-Dawley rats (300 g) were decapitated, and their thoracic aortae excised. Having removed the adventitia and fatty tissue, the aortae were cut into 2-mm rings with a scalpel blade on a Teflon block. The rings pooled and randomised in Dulbecco's minimum essential medium (DME, preoxygenated at 95% O₂/5% CO₂) were used to determine the effect of cold storage on PGI₂ synthesis. The rings were then incubated for 6 hr in MEM in stopped flasks at 37°C, with changes of medium every 30 min, to allow prostacyclin release elicited by preconstricting the rings with phenylephrine (10⁻⁸ M) to subside (5-8) after 2 hr. The following experiments were then carried out.

MATERIALS AND METHODS

Reagents. Noradrenaline bitartrate, sodium fluoride, phorbol ester dibutyrate, calcium ionophore A23187, arachidonic acid (calcium salt; 99.9% pure), and Dulbecco's minimum essential medium was purchased from Sigma Chemical Co. (Poole, Dorset, UK). LRS was made up by the pharmacy at the Royal Free Hospital and consists of the following (in mmol/L): K⁺, 125; Na⁺, 25; Mg²⁺, 5; lactobionate, 100; H₂PO₄⁻, 25; SO₄²⁻, 5; allopurinol, 1; reduced glutathione, 3; adenosine 5; pH 7.4. KPS contained (in mmol/L): K⁺, 80; Na⁺, 80; Mg²⁺, 40; SO₄²⁻, 55; citrate, 55; mannitol, 185; pH 7.2.

Abbreviations: AA, arachidonic acid; EDRF, endothelium-derived relaxing factor; KPS, citrate-based kidney preservation solution; LRS, lactobionate-raffinose solution; NA, noradrenaline; NaF, G protein activator; PKC, protein kinase C.
GUEST EDITORIAL

NSAID Efficacy and side-effects: Are they wholly prostaglandin-mediated?

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Non-steroidal anti-inflammatory drugs (NSAIDs) are the principal agents used for the alleviation of inflammation and pain associated with rheumatoid arthritis and osteoarthritis, ankylosing spondylitis and gout. However, NSAIDs also elicit serious side-effects including ‘NSAID gastropathy,’ nephrotoxicity, hypertension and disturbances of the central nervous system [1–4].

Since NSAIDs inhibit the activity of cyclooxygenase [5] (an enzyme involved in prostaglandin synthesis) it is widely held that their therapeutic action is prostaglandin (PG)-mediated. The ability of NSAIDs to inhibit PG synthesis does not often correlate with their anti-inflammatory efficacy or the incidence and severity of side-effects [6]. Thus, it is of interest that there is an increasing body of evidence that NSAIDs exert effects on non-PG-related systems. For example, several recent studies have shown that NSAIDs disrupt calcium mobilisation in biological membranes [7]. It is well established that calcium is a universal second messenger which links receptor activation to endpoint function in virtually every mammalian cell type. The possibility that NSAID effects are mediated, at least in part, through disruption of calcium dynamics is potentially of extreme importance in rationalising NSAID action and future drug design.

NSAIDs readily partition into biological membranes [8] and have been shown to inhibit a variety of membrane-associated processes [7]. NSAIDs were found to inhibit calcium binding to plasma membranes nearly 20 years ago [9]. Although the implications of this observation were overlooked until recently, Abramson et al. [10] demonstrated that aspirin, indomethacin and piroxicam are potent inhibitors of the early steps of receptor-activated neutrophil function, including the uptake and mobilisation of calcium at the plasma membrane of these cells. It was proposed by these authors that NSAIDs may act by disrupting a "trigger" pool of membrane-bound calcium linked to signal transduction mechanisms. These signal transduction mechanisms include the sequential activation of G proteins, phospholipase C and protein kinase C, all of which are modulated by calcium and ultimately determine the response of the neutrophil. In this context, NSAIDs have also been shown to inhibit cell proliferation at the G1 phase through a non-PG mediated mechanism [11]. In turn, the G1 phase of cell division also involves Ca^{2+} mobilisation as well as phospholipases, protein kinase C and G proteins [12]. Further evidence for effects on activator calcium have also been reported in the control of receptor-linked PG synthesis (also mediated by calcium, G proteins, and protein kinase C) [13] in smooth muscle tissues. In these latter studies it was found that indomethacin, ibuprofen and tiaprofenic acid inhibited receptor-PG synthesis coupling at concentrations lower than those required to inhibit cyclooxygenase activity [14,15]. The disruption of membrane-associated calcium was thought to be responsible for this phenomenon. In this context, it is of interest that indomethacin and ibuprofen are also potent inhibitors of calcium uptake by isolated human platelets at lower concentrations than...
Comparison of Prostanoid Synthesis in Cultured Human Vascular Endothelial Cells Derived from Omentum and Umbilical Vein

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Endothelial seeding of vascular grafts may reduce thrombogenicity and significantly improve graft patency. For clinical studies endothelial cells derived from human omentum may be the ideal source of cells as they are obtainable in large numbers. This study was designed to assess their ability to produce the antithrombogenic substance prostacyclin (PGI₂).

Human omentum microvascular endothelial cells (HOTMECs) were grown and their ability to produce prostacyclin (PGI₂), PGE₂, PGF₂α and thromboxane A₂ (TXA₂) in response to a range of agonists was measured by radioimmunoassay. Control experiments were performed using human umbilical vein endothelial cells (HUVECs).

Both HUVECs and HOTMECs synthesised similar quantities of PGI₂ basally and both increased production after adding A23187 (calcium ionophore), arachidonic acid, sodium fluoride and phorbol ester. In contrast, both thrombin and histamine were potent stimulators of PGI₂ release in HUVECs but were without effect on HOTMECs. In both cell types serotonin, carbachol and noradrenaline were without effect. The pattern of release of PGE₂, PGF₂α and TXA₂ were identical to those of PGI₂ in both cell types but the quantities released were lower.

These results show that HOTMECs can produce the antithrombogenic agent PGI₂ in significant quantities and that they do so by mechanisms similar to those of large vessel endothelium. This supports the proposal that they would be a suitable cell source for vascular graft seeding.

Key Words: Endothelium; Microvascular; Prostacyclin; Human.

Introduction

Oclusive arterial disease affecting the lower limb is an increasing problem in our aged population and bypass of occluded arteries in the leg saves many patients from amputation or severe rest pain. Autologous vein is the graft material of choice but unfortunately this is unavailable in a significant number of patients¹ and for bypass below the knee joint long-term patency results are disappointing when prosthetic material is used.² This problem has been ascribed, at least in part, to the absence of the vascular endothelium on the luminal surface of prosthetic grafts and this concept has prompted work with the seeding of endothelial cells onto the luminal surface of artificial graft materials in an attempt to produce an antithrombogenic surface similar to that possessed by normal vessels. Such endothelial seeding has experimentally been shown to reduce thrombogenicity and significantly improve graft patency even in low flow conditions.³

Cells must be derived from the recipient of the graft or immunosuppression is needed and it has been suggested that autologous microvascular endothelial cells derived from human adipose tissue could provide the ideal source of cells for clinical seeding trials in humans.⁴-⁶ Currently, however, little is known about endothelial cells derived from this source, and whether they retain their ability to produce antithrombotic agents such as prostacyclin (PGI₂).

PGI₂ is the predominant prostaglandin released
Differential inhibitory potencies of non-steroidal antiinflammatory drugs on smooth muscle prostanoid synthesis

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In isolated rat aorta and urinary bladder, indomethacin inhibited the synthesis of the prostaglandins (PG) PGI₂, PGE₂, PGF₂α, and TXA₂ equipotently when PG synthesis was stimulated with excitatory receptor agonists (noradrenaline and carbachol), fluoride (a G protein activator), phorbol ester (a protein kinase C (PKC) activator) and calcium ionophore A23187 (a creator of artificial calcium channels). However, there was a marked right shift (30 fold) in the indomethacin concentration-inhibition curves when PG synthesis was stimulated by arachidonate (PG substrate) and trauma (freeze fracturing and sonication). Although less potent than indomethacin, the NSAIDs tiaprofenic acid and ibuprofen showed a similar disparity between the IC₅₀ with the same PG stimulators. Since PG synthesis stimulated by receptor agonists, fluoride, phorbol ester and A23187 is dependent on calcium channel activation whereas trauma and arachidonate-stimulated PG synthesis bypass calcium channel activation, these data indicate that NSAIDs inhibit not only cyclooxygenase but also (and more potently) the mobilisation of Ca²⁺ linked to PG synthesis in these tissues.

Non-steroidal antiinflammatory drugs; Ca²⁺; Prostaglandins; Smooth muscle

1. Introduction

Non-steroidal antiinflammatory drugs (NSAIDs) are the principal drugs used in the treatment of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, gout and pain. However, NSAIDs also elicit deleterious side effects, including gastric erosion (Atwater et al., 1965; Roth and Bennett, 1987), nephrotoxicity (Lipsett and Goldman, 1954; Swainson, 1984) and hypertensive effects (Durao et al., 1977; Brown et al., 1986). Since NSAIDs reduce prostanoid synthesis through inhibition of cyclooxygenase (Vane, 1971), their actions and side effects are largely interpreted as being prostanoid-mediated. However, there are many reports that NSAIDs exert effects on other biosystems, including phosphodiesterases (Weinryb et al., 1972), Ca²⁺ binding at the plasmalemma (Northover, 1971) and phospholipase C (Bomalaski, 1986). NSAIDs have also been shown to inhibit tumour growth (Hial et al., 1976; 1977) and cell proliferation (at the G1 phase) through a non-prostanoid mediated mechanism (De Mello et al., 1980). In turn the G1 phase of cell division involves Ca²⁺ mobilisation, protein kinase C (PKC), G proteins, and phospholipid diesterases (Rosen-gurt, 1984; Wickremasinghe, 1988). In this context, it has recently been established that excitatory receptor prostanoid synthesis coupling in smooth muscle involves the sequential activation of G protein, PKC, Ca²⁺ mobilisation, phos-
SMOKING AND VASCULAR PROSTANOIDS: relevance to the pathogenesis of atheroma and thrombosis

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Abstract
This review covers the current state of knowledge on the role of the vascular prostanoids in the pathogenesis of smoking-related atheroma and thrombosis. The relationship between prostanoids, smoking, and other risk factors for atherogenesis, is discussed. The conclusion is that cigarette smoke contains one or more components which are capable of inhibiting vascular PGI₂ synthesis which, in turn, may influence other systems adversely leading to an increased rate of atherogenesis and incidence of thrombosis among smokers.

Key words: Smoking, coronary heart disease, prostacyclin, thrombosis, atherosclerosis, nicotine, prostaglandins

Introduction
The present review considers the role of vascular prostanoids in the pathogenesis of smoking-related atheroma and thrombosis. As many readers may not be familiar with this field there follows a brief introduction to three topics:

(a) Smoking and the various types of vascular disease;
(b) Mechanisms responsible for atherogenesis and thrombosis;
(c) Synthesis and actions of vascular prostanoids.

(a) Smoking and the various types of vascular disease
Epidemiological evidence has convincingly linked smoking to CHD, but the mechanisms responsible for this association remain undefined [1–7]. This topic is reviewed in the current issue of the Journal (see Doll, page 3). It would appear that several consequences of smoking accelerate atherogenesis and increase the risk of thrombosis. These factors are briefly considered later in this text.

The epidemiological evidence does present some interesting insights into the role of smoking in the pathogenesis of vascular disease. For example, smoking is more strongly associated with PVD than with CHD [8,9]. In contrast, smoking does not appear to be a major risk factor for stroke [10]. Even more surprising is the evidence that smoking is not a risk factor for venous thrombosis [7,11–13]. Smokers may even carry a lower risk of postoperative thromboembolism [11] and myocardial infarction [12,13]. These

The following abbreviations have been used throughout:
AA, arachidonic acid; ADP, adenosine diphosphate; CHD, coronary heart disease; PDGF, platelet derived growth factor; PG, prostaglandin; PGI₂, prostacyclin; PKC, protein kinase C; PLA₂, phospholipase A₂; PVD, peripheral vascular disease; NEFA, non-esterified fatty acids; SMC, smooth muscle cell; TXA₂, thromboxane A₂.

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INTRODUCTION

Vascular tissue synthesizes prostanoids (PGs) that modulate contractility, myocyte and fibroblast proliferation, platelet aggregation and leucocyte function, all of which are key components in the pathophysiology of atherogenesis. Furthermore, platelet and leucocyte release substances have been shown to stimulate the synthesis and release of PGs from vascular cells. It is possible, therefore, that vascular PGs are part of a protective response against atherogenic events. Consequently, any disruption of PG synthesis (viz. as a result of smoking) may accelerate the atherogenic state. Although smoking is a major risk factor in the development of atherosclerosis, the combination of smoking with other risk factor (e.g., diabetes, hypertension, hypercholesterolaemia) markedly increases the likelihood of death from atherosclerotic disease. The present paper therefore discusses the following:

1) How PGs relate to pathophysiology of atherosclerosis
2) Experimental findings on the effects of cigarette smoking on vascular and platelet prostanoid synthesis.
3) How other risk factors may interact with smoking to influence vascular permeability
4) Future directions and emphases on research into eicosanoids and smoking.

Before discussing the effects of cigarette smoking on vascular PG synthesis, it is relevant to briefly review current concepts on the pathogenesis of atherosclerosis and how they may relate to PGs.

The key event in the initiation and perpetuation of the fibrous plaque, the pathognomonic lesion of atherosclerosis, is the proliferation of arterial smooth muscle cells, followed by deposition of lipid and the accumulation of collagen, elastic fibres and proteoglycans (1). Although the trigger for these events is unknown, it has been proposed that atherogenesis is initiated by endothelial cell injury leading to adherence of platelets resulting in local release of platelet constituents including TXA2, platelet derived growth factor (PDGF), platelet activating factor (PAF), serotonin (5HT) and histamine (2).
Copper imbalance is associated with cardiovascular disease (CVD). Divalent copper has also been shown to modulate eicosanoid synthesis (Elliot et al, 1985; Mitchell et al, 1988). Platelet thromboxane synthesis and lipooxygenase activity has been implicated in the pathophysiology of CVD. In order to explore the role of copper in modulating eicosanoid synthesis by platelets, the effect of the Cu²⁺ chelaters dimethylthiourea hydrochloride (DMIC), diethyldithiocarbamic acid (DEtC) and tetraethylthiuram disulphide (TETD) on TXA₂ synthesis and hydroxy-eicosatetraenoic acid (HETE) synthesis by isolated human platelets was investigated. Platelets were obtained from healthy volunteers. Platelets were washed with HEPES containing apyrase and resuspended in HEPES buffer. Aliquots of platelets were incubated with DMIC, DETC and TETD for 15 min at 37°C prior to the stimulation of TXA₂ synthesis with the following stimulators: arachidonic acid (AA, 10 μM), calcium ionophore A23187 (0.2 μM), NaF (20 μM), phorbol ester dibutyrate (1 μM), and spontaneous release. Platelets were further incubated for 20 min at 37°C. The reaction was stopped by addition of ethanol, and aliquots of supernatant taken for measurement of TXB₂ by radioimmunoassay. Conversion of AA to HETE was assessed by the addition of [¹⁴C]-AA to platelets suspended in HEPES containing various concentrations of Cu²⁺ chelators. Following incubation for 15 min at 37°C, the reaction was stopped and HETE and unchanged AA extracted with chloroform/methanol (2:1). HETE was separated from AA by thin layer chromatography, counted for radioactivity and percentage inhibition of AA conversion to HETE calculated.

DMIC inhibited TXA₂ synthesis in a concentration-dependent manner. IC₅₀ (concentration at which DMIC inhibited TXA₂ synthesis by 50%; derived from 10 experiments) were similar for all stimulators (AA, 33 μM; A23187, 35 μM; NaF, 34 μM; phorbol ester, 30 μM; spontaneous, 28 μM). The similarity in the IC₅₀ is indicative of an effect of chelator on cyclooxygenase, rather than on other enzymes or systems (phospholipases, protein kinase C, calcium mobilisation). DMIC also inhibited the transformation of [¹⁴C]-AA to [¹⁴C]-HETE (IC₅₀, 38.5 μM). DEtC and TETD inhibited TXA₂ synthesis and lipooxygenase activity in a similar fashion to DMIC and at similar concentrations. The inhibitory effect of all Cu²⁺ chelators was reversed by the addition of approximately equimolar concentrations of Cu²⁺ but not by Fe²⁺, Fe³⁺, Zn²⁺, Al³⁺ or Sn²⁺. These data indicate that Cu²⁺ (as is the case with Fe²⁺) may play an obligatory role in cyclooxygenase and lipooxygenase activity. The present data also support the concept that copper imbalance may be involved in the pathophysiology of CVD and that eicosanoids may play a role in mediating this relationship.


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We have explored the role of tyrosine kinases in the signal transduction of PAF-activated rabbit platelets. Rabbit platelets were prepared, signal molecules quantitated and dense granule release determined as described previously (Murphy et al, 1991). Tyrosine phosphorylation of rabbit platelet protein was determined by Western blotting of platelet lysates with PY20, a monoclonal anti-phosphotyrosine antibody (Glenneny et al, 1988). Resting platelets exhibited at least four tyrosine phosphorylated (TP) proteins with molecular weights of 52-62 kDa. Preincubation of platelets with genistein (10-300 μM) for 20 min produced a dose-related inhibition of phosphorylation of the 52-62 kDa proteins. Stimulation with 300 nM PAF induced a rapid increase in TP-proteins visible within 5 sec of both low (35-45 kDa) and high (66-90 kDa) molecular weight. Pretreatment of platelets with genistein (10-300 μM) for 20 min produced a dose related inhibition of tyrosine phosphorylation of a number of the 66-150 kDa and 35-45 kDa proteins, although was much less effective against the proteins of 52-62 kDa. Genistein (Akiyama et al, 1987) was an effective inhibitor of PAF-induced calcium elevation ([Ca²⁺]i, ionol 1,4,5-trisphosphate (IP₃) formation, thromboxane (TX) biosynthesis and the release of dense granules ([¹⁴C]-SHI) see table).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[Ca²⁺]i (nM)</th>
<th>IP₃ (pmol/10⁸pl)</th>
<th>TXB₂ (pmol/10⁸pl)</th>
<th>¹⁴C-SHT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>107 ± 12</td>
<td>7.5 ± 1.5</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Vehicle + PAF</td>
<td>490 ± 22</td>
<td>34 ± 5.5</td>
<td>44 ± 3.2</td>
<td>58 ± 1.5</td>
</tr>
<tr>
<td>10 μM Genistein + PAF</td>
<td>434 ± 17.5</td>
<td>34 ± 5.5</td>
<td>25.5 ± 0.31</td>
<td>46 ± 0.7</td>
</tr>
<tr>
<td>30 μM Genistein + PAF</td>
<td>432 ± 15</td>
<td>22 ± 2</td>
<td>25 ± 0.3</td>
<td>39 ± 2.8</td>
</tr>
<tr>
<td>100 μM Genistein + PAF</td>
<td>341 ± 24</td>
<td>15 ± 0.8</td>
<td>&lt;0.1</td>
<td>13.8 ± 2.3</td>
</tr>
<tr>
<td>300 μM Genistein + PAF</td>
<td>135 ± 13</td>
<td>10.2 ± 2.7</td>
<td>&lt;0.1</td>
<td>3 ± 0.5</td>
</tr>
</tbody>
</table>

We have demonstrated that PAF induces a rapid tyrosine phosphorylation of approximately 17 proteins in three molecular weight ranges. This probably involves a number of tyrosine kinases as genistein is an effective inhibitor of only a limited number of proteins. However, these TP proteins appear to be crucial in the very early signal transduction events of PAF activated platelets as genistein is an effective inhibitor of these events.

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OPPOSITE CHANGES OF ADRENOCEPTOR- AND MUSCARINE-RECEPTOR-LINKED PROSTACYCLIN SYNTHESIS
BY THE AORTA AND BLADDER OF THE DIABETIC RAT

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Although diabetes mellitus (DM) is associated with diminished vascular prostacyclin (PGI₂) synthesis, enhanced synthesis of PGI₂ by other tissues (e.g. urinary bladder [UB]) has been reported (Jeremy et al., 1986a). In turn, PGI₂ synthesis by the aorta and UB of the rat is stimulated by activation of α-adrenoceptors and muscarine receptors, respectively (Jeremy et al., 1985, 1986b).

In order to investigate this area further, the authors performed experiments on the rat. They studied the intracellular pathways of PGI₂ synthesis following the stimulation of PGI₂ synthesis with phorbol ester (PE; a protein kinase C [PKC] activator; Ca²⁺ ionophore A23187 (A23187) and thapsigargin (both elevate intracellular Ca²⁺, activating phospholipase A₂ [PLA₂]) and arachidonate (AA; substrate for PGI₂ synthesis).

DM was induced in male Sprague Dawley rats with streptozotocin (65 mg/kg, i.v.). After 8 weeks, rats were killed and aortae and UBs excised. Aortae were cut into 1 mm rings and UBs into approximately 2x2 mm segments and processed for assessment of agonist-stimulated PGI₂ synthesis as previously described (Jeremy et al., 1985, 1986b).

PGI₂ synthesis was stimulated in the aortae with adrenaline and in the UBs by acetylcholine (Ach). PGI₂ release was measured by radioimmunoassay of 6-exo-FPGF₂₀, and dose-response curves compiled.

Transformation of data to % maximal response revealed a marked right shift in the adrenaline-PGI₂ dose-response curve in aortae from DM rats (EC₅₀ = 4.1 x 10⁻⁶ M) compared to controls (EC₅₀ = 5.4 x 10⁻⁷ M). In contrast, there was a marked left shift in the Ach-PGI₂ dose-response curve in UBs from DM rats (EC₅₀ = 5.8 x 10⁻⁷ M) compared to controls (EC₅₀ = 2.2 x 10⁻⁶ M). In the aortae from DM rats there was also a marked right shift in the PE-PGI₂ dose-response curve (EC₅₀ = 1.4 x 10⁻⁶ M) compared to controls (EC₅₀ = 1.9 x 10⁻⁷ M), whereas there were no differences in the EC₅₀s of thapsigargin, A23187 or AA. In the UBs there were no differences in the EC₅₀s of PE, thapsigargin, A23187 or AA between diabetic and control rats.

These data indicate that: (i) reduced PGI₂ synthesis coupled to adrenoceptors in the aorta of the diabetic rat may be due to diminished PKC activity and not to Ca²⁺ mobilising systems, PLA₂, cyclooxygenase or PGI₂ synthase; and (ii) the diametrically opposite effect of DM on muscarine receptor-linked PGI₂ synthesis consolidates that changes in PGI₂ are organ-specific. What determines these marked differential changes in the activity of different receptor types in DM warrants further investigation.

90P A COMPARISON OF THE INHIBITORY EFFECTS OF PROSTANOID EP₂ RECEPTOR AGONISTS AND β₂-ADRENOCEPTOR AGONISTS ON HUMAN MYOMETRIUM FROM PREGNANT DONORS


β₂-adrenoceptor agonists are used clinically to inhibit premature labour, but they are not very effective (Calixto & Cimas, 1984), and have high side-effect liability (Besinger et al., 1991). E-series prostanooids can both contract and relax human isolated myometrium, contraction being mediated by EP₁ and EP₂ receptors, and relaxation by EP₂ receptors (Senior et al., 1991). In the present study, the tocolytic activities of two selective prostanooid EP₂-receptor agonists, butaprost (Gardiner, 1986) and AH13205 (Nials et al., 1990) have been compared with PGE₂ and the β₂-adrenoceptor agonists, isoprenaline (ISO), salbutamol (SAL), terbutaline (TERB) and ritodrine (RIT), on human isolated myometrium.

Human myometrium from consenting pregnant donors (elective Caesarean sections at term, non-labour), was superfused with Krebs solution (2ml min⁻¹) containing 2.8μM indomethacin, and oxygenated with 95% O₂/5% CO₂ at 37°C (Massele and Senior, 1981). Each agonist was infused at a rate of 0.02μl.min⁻¹ over a range of concentrations (0.1μM-10μM) for 15 min. Inhibition was seen as a decrease in frequency (inhibiting myogenic activity), and recovery time (period of inhibition following cessation of agonist infusion until return of control myogenic activity).

All agonists except RIT, inhibited spontaneous contractions in a concentration-dependent manner and none of the agonists caused spasmodic effects. PGE₂, butaprost, ISO and TERB significantly inhibited myogenic contractions at 0.1μM, whereas AH13205 and SAL had no effect at concentrations <1μM. The mean recovery times (min ± SEM) following a 10μM infusion of agonist were: butaprost (> 120, n=7), AH13205 (89.04±11.8, n=11), SAL (73.6±15.2, n=9), TERB (73.3±16.6, n=9), ISO (71.5±15.9, n=8) and PGE₂ (34.1±16.4, n=5). RIT was inactive (n=9).

Both EP₂-receptor and β₂-adrenoceptor agonists, with the exception of RIT, inhibited spontaneous uterine contractions in human myometrium from pregnant donors. The selective EP₂-receptor agonist, butaprost is the most effective of the agonists tested. The fact that EP-receptor agonists cause inhibition of myometrial activity without excitatory effects could have clinical implications for the prevention and/or treatment of premature labour.

Gardiner, P.J. (1986b), Br. J. Pharmacol. 87: 45-56.