SEROTONIN AND PLATELET STUDIES IN VASCULAR DISEASE

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DOCTOR OF PHILOSOPHY

by

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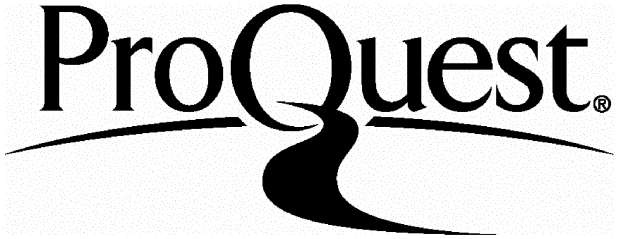

ABSTRACT

The aim of this thesis was to assess platelet function indices in peripheral vascular disease (PVD), diabetes mellitus, hypercholesterolaemia and renal disease in order to improve our understanding of the vascular complications associated with these conditions.

Platelet shape change (PSC) in platelet-rich plasma (PRP) obtained from PVD patients was studied using a new sensitive and reproducible method. 5-hydroxytryptamine (5-HT)-induced PSC was diminished in PVD. This finding may be explained by desensitization, an effect which was reproduced *in vitro* using PRP from healthy subjects. The PSC methodology is likely to be more sensitive than platelet aggregation at detecting the inhibitory effects of drugs (e.g. naftidrofuryl; milrinone).

An optimized method to assess whole blood platelet aggregation in PVD patients revealed enhanced aggregation to several platelet agonists. Aggregation induced by 5-HT and spontaneously was not affected by treatment with low dose aspirin (ASA) or by its addition *in vitro*.

Intraplatelet 5-HT concentration in the above disease states was found to be diminished whereas concomitant plasma concentrations were raised. In renal and hypercholesterolaemic patients, a significant inverse correlation was found between intraplatelet 5-HT and plasma lipids. In hypercholesterolaemic patients, the intraplatelet 5-HT concentration was normalised following treatment with simvastatin, a cholesterol lowering drug. The plasma concentration of 5-HT was markedly elevated in PVD patients who developed graft stenosis; this elevation

was partially corrected by low dose ASA.

Release studies demonstrated that minor platelet activation (e.g. by stirring) causes significant 5-HT release, despite the absence of aggregation. 5-HT uptake was not affected by platelet activation.

These findings may be relevant since 5-HT exerts effects on the vasculature (e.g. vasoconstriction, vascular smooth muscle proliferation). Furthermore, other anti-platelet drugs may be needed in addition to low dose ASA to further inhibit platelet aggregation, PSC and the release of intraplatelet contents.

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ABBREVIATIONS

AA	Arachidonic acid
ABP	Actin binding protein
AC	Adenylate cyclase
ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
ADPase	Adenosine diphosphatase
AMP	Adenosine monophosphate
ASA	Acetyl salicylic acid (aspirin)
ATP	Adenosine triphosphate
ß-TG	Beta-thromboglobulin
Ca^{2+}	Calcium ions
CaI	Calcium ionophore (A12387)
cAMP	Adenosine 3′:5′-cyclic monophosphate
CAPD	Continuous ambulatory peritoneal dialysis
cGMP	Guanosine 3′:5′-cyclic monophosphate
CHF	Congestive heart failure
CPM	Counts per minute
CV	Coefficient of variation
DG	Diacylglycerol
DM	Diabetes mellitus
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
EM	Electron microscopy
ESRF	End stage renal failure
Fc	Final concentration
Fl	Femtolitre
GP	Glycoprotein
G-Protein	Guanine nucleotide-binding protein
GTP	Guanine triphosphate
HbA_1	Glycosylated haemoglobin
HBP	High blood pressure
HD	Haemodialysis
HDL-C	High density lipoprotein cholesterol
HPLC	High performance liquid chromatography
5-HT	5-hydroxytryptamine (serotonin)

IC_{50}	Inhibitor concentration for 50% inhibition
IDDM	Insulin dependent diabetes mellitus
IHD	Ischaemic heart disease
INDO	Indomethacin
IP_2	1,4-inositol bisphosphate
IP_3	1,4,5-inositol trisphosphate
KDa	Kilo-Dalton
KET	Ketanserin tartrate
K_M	Michaelis constant
LDL-C	Low-density lipoprotein cholesterol
MePV	Median platelet volume
MI	Myocardial infarction
MIL	Milrinone
MLCK	Myosin light chain kinase
NAF	Naftidrofuryl
Na^+,K^+-ATPase	Sodium, potassium-adenosine triphosphatase
NaNP	Sodium nitroprusside
NBP	Normal blood pressure
NEFA	Non-esterified fatty acid
NIDDM	Non-insulin dependent diabetes mellitus
NO	Nitric oxide
NS	Nephrotic syndrome
NSAID	Non-steroidal anti-inflammatory drug
5′-NT	5′-nucleotidase
P47	Protein of 47 KDa molecular weight
PA	Phosphatidic acid
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PGE_1	Prostaglandin E_1
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PI	Phosphatidylinositol
PK-A	Protein kinase A
PK-C	Protein kinase C

PPP	Platelet poor plasma
PRP	Platelet rich plasma
PSC	Platelet shape change
PTFE	Polytetrafluoroethylene
PVD	Peripheral vascular disease
RD	Renal disease
RFH	Royal Free Hospital
RGDS	Arginine-Glycine-Aspartate-Serine
RIA	Radioimmunoassay
SEM	Standard error of the mean
SPA	Spontaneous platelet aggregation
TC	Total cholesterol
TG	Total triglyceride
tPA	tissue plasminogen activator
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
VP	Vasopressin (anti-diuretic hormone)
vWF	von Willebrand factor
WB-IA	Whole blood-impedance aggregometry
WB-FPC	Whole blood-free platelet count

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER SUB-INDEX

1.1 Atherosclerosis

Cardiovascular atherosclerotic disease is the chronic condition that accounts for most deaths in the industrialized countries and it is anticipated that by the year 2000 this will be the leading cause of death in many developing countries (Najeeb et al., 1993).

1.2 Atherosclerosis and peripheral vascular disease

At a morphological level, atherosclerosis is characterized by lesions which appear mainly in the coronary, cerebral and lower extremity arteries (Woolf, 1990; Vogt et al., 1992). These lesions which initially may only impair blood flow can progress and develop into occlusive lesions and lead to myocardial infarction (MI), stroke or peripheral vascular disease (PVD; also known as peripheral arterial obstructive disease).

In this thesis, investigations have focused mainly on patients with PVD. In addition, patients with diabetes mellitus (DM), renal disease (RD) and hypercholesterolaemia were studied. DM, RD and hypercholesterolaemia are conditions associated with a greater frequency and severity of atherosclerosis (Lindner et al., 1974; Jay and Betteridge, 1991). These disease states, particularly DM, are also found in association with PVD (Jay and Betteridge, 1991).

In PVD, atherosclerosis occurs in the femoro-popliteal-tibial arteries and less often in the aorto-iliac, carotid and vertebral, splanchnic and renal arteries (Clagett et al., 1992). These patients present with intermittent claudication

which is defined as calf pain present on exercise but not at rest. The disease can progress to pain at rest, as longer arterial segments and more vessels become occluded, and eventually ischaemia can develop. As the disease progresses, superficial skin ulcers may occur, which may give rise to gangrene and the need for amputation (Jay and Betteridge, 1991; Vogt et al., 1992). Epidemiologic studies have documented that 2-3% of men and 1-2% of women, 60 years of age and older, have intermittent claudication (Reunanen et al., 1982, Jelnes et al., 1986). These patients have a 2-4 fold increase in the rate of mortality from cardiovascular and coronary artery heart disease (Criqui et al., 1992).

1.3 Atherosclerotic lesions

The basic lesion of vascular disease is atherosclerosis with raised focal plaques containing lipids and other macromolecules (Ross & Glomset, 1976; Woolf, 1990). The precursors of such lesions are fatty streaks that are thought to progress to mature fibrolipid atherosclerotic plaques, clearly present in middle age and elderly subjects. A plaque consists of a lipid-rich pool with necrotic connective tissue at the base and a fibro-muscular cap on the luminal surface. Beneath the fibrous cap the lesions contain smooth muscle, macrophages and other leukocytes, including monocytes (Ross, 1986). Although the focal distribution of such lesions provides a clue that haemodynamic factors are important, the precise nature of the initial injury and the mode of formation of the plaque remains undefined. Plaque formation can lead to

the development of a soft deformable atheromatous pool which, if excessively large or strained, can result in the rupture of the fibro-muscular cap and precipitate the formation of a thrombus (Harker et al., 1981; Ross, 1986). Plaques may also develop into occlusive lesions or stenosis (narrowing of the artery) that in turn leads to clinical symptoms (Badimon et al., 1992). The process involved in the development of a stenosis is believed to be caused by sub-intimal proliferation of smooth muscle cells derived from the middle layer of the vessel (media) (Woolf, 1990). Despite many studies investigating the mechanisms involved in smooth muscle cell proliferation, the aetiology remains unclear (Woolf, 1990).

1.4 Risk factors for atherosclerosis

Epidemiological studies have identified several risk factors (Table 1.1) for IHD and PVD (Hopkins and Williams, 1981; Kannel et al., 1985; Cortellaro et al., 1992; Fowkes et al., 1992). Tobacco smoking, in particular, has been identified as a major risk factor for atheroma development and appears to be specially important in the peripheral circulation (Fowkes et al., 1992). Smoking is also associated with elevated plasma fibrinogen concentrations, an important risk factor in PVD (Cortellaro et al., 1992). Platelet aggregability has been shown to predict death from IHD in apparently healthy men in a prospective study (Thaulow et al., 1991) and platelet function indices, including platelet aggregability and volume, are risk factors for IHD (Elwood et al., 1990; Martin et al., 1991; Trip et al., 1990).

Unfortunately, large multi-centre epidemiological studies assessing platelet function and platelet release substances have not been carried out in PVD patients. A relatively small study with 147 PVD patients, however, demonstrated increased platelet hyperactivity in this group of patients when compared to healthy younger controls (The PACK Trial Group, 1989). This has been confirmed in a smaller study with age-matched controls (Walters et al., 1993).

TABLE 1.1

Established risk factors associated with ischaemic heart disease (IHD) and peripheral vascular disease (PVD).

RISK FACTORS	
MODIFIABLE	**NOT MODIFIABLE**
Cigarette Smoking	Age
Hyperlipidaemia	Sex
Diabetes	Family History
Hypertension	
Fibrinogen	
Renal Disease	
Platelet Function	

Other risk factors for IHD and PVD include the following which can be measured in blood samples: leukocyte counts, Factor VII and VIII, von Willebrand factor antigen, protein C, anti-thrombin III, fibrinolysis parameters (tPA and PAI-1), protein S deficiency and viscosity.

1.5 The role of platelets in atherogenesis

It has been suggested that platelets play a role in the early stages of development of atherosclerotic lesions and in the thrombotic events associated with plaque rupture. Modest injury, to the endothelial (mono)layer, may leave the endothelium morphologically intact, yet result in platelet adhesion and concomitant release of intraplatelet substances with mitogenic properties that may contribute to atherogenesis (Ross et al., 1974; Ross 1993). In keeping with this are the findings that platelet antigens are present in blood vessels (Woolf and Carstairs, 1967). There is evidence that inhibitors of platelet activity (e.g. sulphinpyrazone and aspirin), can retard the onset of atherosclerosis (Grodzinska and Dembinska-Kiec, 1980; Landymore et al., 1988) which suggests a role for platelets in atherogenesis. On the other hand, it has been difficult to prove a clear association between endothelial denudation (which favours platelet adhesion and aggregation) and the development of atherosclerosis *in vivo* (Nilsson, 1986). The role of platelets in the early and slow stages of atheromatous growth, therefore, remains to be clarified.

It is widely accepted that platelets come into play in the late pathological events that lead to thrombosis. Plaque rupture or endothelial denudation promote platelet adhesion and aggregation, thus, initiating clot formation. Such thrombi have been repeatedly shown to be platelet rich (Woolf and Carstairs, 1967; Harker et al., 1979).

To understand how platelets contribute to atherosclerosis it is important to consider how platelets become activated

(i.e. ready to carry out various functional processes). Basic platelet structure, function and methods used to study platelet shape change, aggregation and "release" are therefore considered below.

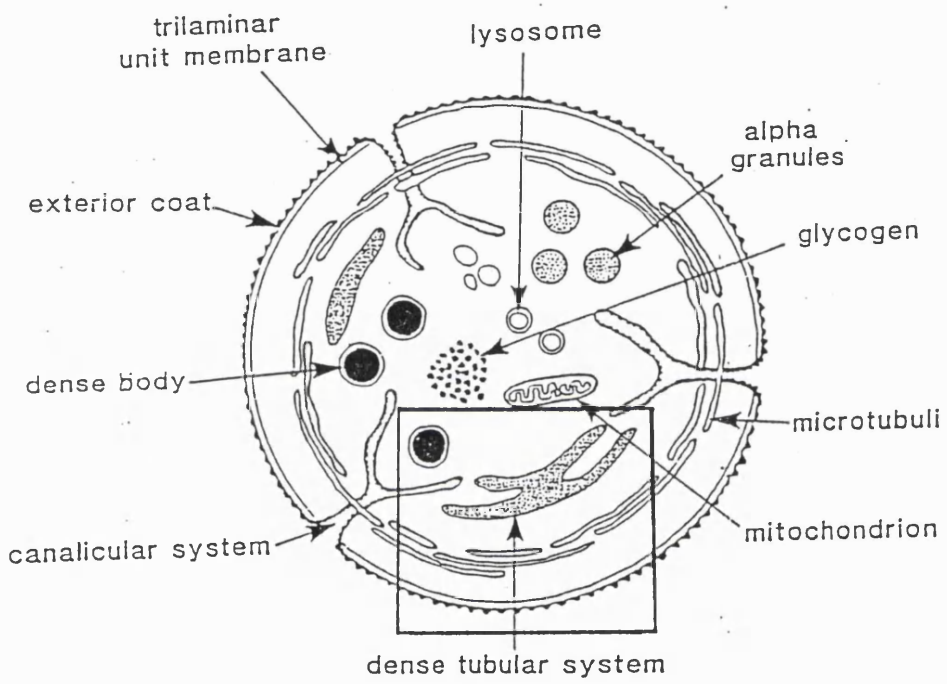
1.6 Platelet structure and function

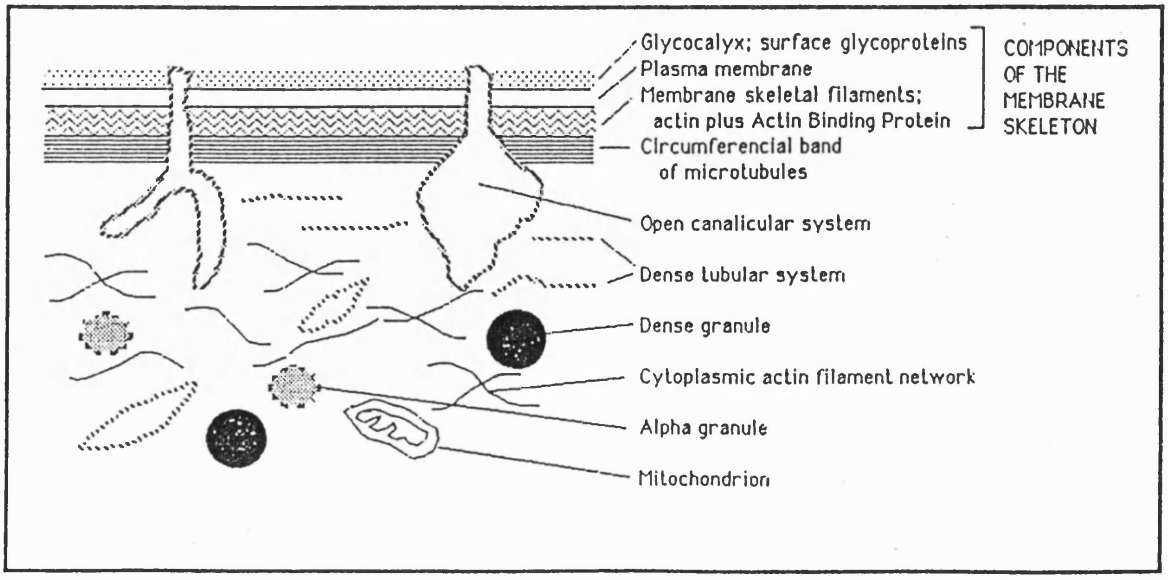
A distinguishing feature of platelets is that they lack a nucleus (Figure 1.1). Therefore, irreversible enzyme inhibition lasts for the duration of the life-span of platelets (7-10 days). Classically, this is illustrated by the inhibition of platelet cyclooxygenase activity (one of the enzymes involved in the synthesis of thromboxane A_2; TXA_2) by aspirin (acetyl salicylic acid; ASA; Webster and Douglas, 1987). Platelets are granular in nature. They contain three types of granules: "dense" granules (calcium rich and therefore opaque to electrons in electron micrographs), α-granules and lysosomes (Table 1.2). The granules contain substances with platelet pro-aggregatory, vasoconstrictory, mitogenic and vascular permeability enhancing effects.

On the surface, platelets possess receptors with a high affinity for adhesive glycoproteins found in the sub-endothelium and in pathological lesions (Coller, 1990). In addition, following activation, the platelet surface facilitates the interaction between coagulation factors (e.g. factors V and XI) to enhance clot formation (Almeida et al., 1990). High affinity specific receptors to a number of substances, e.g. serotonin (5-HT; predominantly 5-HT_2), TXA_2, α-adrenoceptor (predominantly α_2), platelet activating factor

Fig 1.1

Diagrammatic representation of platelet ultrastructure





(PAF) and adenosine diphosphate (ADP) have been characterised on the platelet plasma membrane (Crawford and Scrutton, 1987). The role of these receptors is not defined but the current consensus view is that they mediate platelet function (e.g. platelet shape change and aggregation). In this thesis, studies have been carried out to assess the potential effect of some of the platelet-derived substances shown in Table 1.2. In particular, an attempt has been made to focus on platelet abnormalities associated with 5-HT. This bioamine is stored in large amounts in platelet dense granules and possesses powerful vasoactive effects which could play a role in the pathophysiology of vascular disease.

TABLE 1.2

Intraplatelet Granules

α-Granules	Dense Granules	Lysosomes
ß-TG	Ca^{2+},Mg^{2+}	acid
PF-4	ATP, ADP	hydrolases
PDGF	serotonin (5-HT)	
fibrinogen	histamine	
fibronectin	noradrenaline/adrenaline	
von Willebrand factor		

Note: platelets also possess TXA_2 synthesizing capacity.

1.7 Platelet ultrastructure and early morphological changes

The physiological task of platelets is to arrest the loss of blood from a damaged or severed blood vessel. To achieve this, platelets become activated, change in shape (PSC), adhere to exposed subendothelial layers and clump, forming a platelet plug (Crawford and Scrutton, 1987). "Activation" denotes any or all of the possible physiologic responses by platelets, which include PSC, release of granule contents, expression of surface antigens, changes in the functional status of receptors, expression of coagulant activity and aggregation. During PSC the resting cell, an ellipsoid-like disk, transforms into a sphere, with long and short pseudopods directed outwards. This process is accompanied by extensive reorganization of the internal cell cytoskeleton (Crawford and Scrutton, 1987). Such a process requires contractile elements in the membrane that can change the shape and size of the platelet (Figure 1.1).

1.8 Platelet cytoskeleton and PSC

The major protein components of the platelet membrane cytoskeleton are now recognized as consisting of actin, spectrin, actin binding protein (ABP) and the glycoprotein (GP)-Ib-IX complex which binds von Willebrand factor (vWF; Tuffin, 1991). Studies have also shown that ABP and α-actinin when added together cause platelet actin to gel and cross-link, forming filamentous structures akin to platelet pseudopod formation (White, 1987). Beyond the sub-membrane filaments there is a system of fibres arranged in a bundle,

the microtubules. Because of their location, it was suggested that microtubules might be part of a cytoskeletal support system maintaining the discoid shape of resting cells (White and Rao, 1983). A relationship between the circumferential band of microtubules and the contractile activity of platelets was suggested but dismissed following experiments which showed that microtubular disassembly and reassembly were not required for the centralization of coils and organelles in the aggregating platelet (White and Rao, 1983). Several studies have shown the involvement of actin in early events of platelet activation and PSC (White, 1987; Hartwig, 1992; Heptinstall et al., 1992). The assembly of actin filaments, initially as a random network and then as parallel associations, resembles stress fibres and is considered the earliest physical response of surface-activated platelets (White, 1987). Platelet filaments resemble actin-like filaments found in other cells (e.g. muscle) and extracts of such contractile platelet protein consist of actin and myosin (White 1987). The biochemical mechanisms responsible for the process of actin polymerization remain to be fully elucidated.

1.9 Platelet adhesion and platelet aggregation: role of glycoproteins

Platelet adhesion is crucial in plug formation when a vessel wall is injured and is important in the development of a thrombus. In the latter situation, rupture of an atherosclerotic plaque may expose platelets to subendothelial structures (e.g. collagen) causing

them to adhere and release vasoactive substances. The delayed onset of atherosclerosis in a pig model with vWF deficiency led to the suggestion that platelet adhesion plays a cardinal role in the pathogenesis of atherosclerosis (Fuster et al., 1978). In addition to vWF, other adhesive proteins such as fibronectin, laminin, thrombospondin and vitronectin also support platelet adhesion. It is noteworthy that some of the adhesive proteins such as vWF, fibronectin and fibrinogen are stored in platelet α-granules and may be released during platelet activation (Ejim et al.,1988; Harrison and Cramer, 1993).

Platelet glycoproteins (GPs) play an important role in platelet adhesion and platelet aggregation (Coller, 1990; Peerschke, 1992). These GPs include: GP Ia/IIa, GP Ic/IIa and GP IIb/IIIa which bind collagen, fibronectin and fibrinogen, respectively. Platelet aggregation is thought to be mediated mainly by the platelet integrin GPIIb/IIIa receptor, which unlike other integrins is found only on platelets and megakaryocytes (the precursor cell of platelets). When activated, this receptor can bind several different GPs, including fibrinogen, vWF and fibronectin. The major protein bound to this GP is fibrinogen which, due to its dimeric structure, allows it to interact with two platelets simultaneously giving rise to platelet aggregate formation (Weisel et al., 1992). PSC, which precedes aggregation does not require fibrinogen binding (Bennett et al., 1983).

Platelet aggregation responses induced by agonists such as ADP and adrenaline can often be categorised into two

phases: primary and secondary. The primary response involves the formation of a relatively unstable aggregate. Without further stimulation platelets will dissociate from this loose aggregate (reversible aggregation). Secondary or irreversible aggregation occurs if further stimulation takes place or if the platelets become exposed to another endogenous (e.g. 5-HT and TXA_2) or exogenous stimulus (e.g. collagen). During aggregation there is the release of the contents of storage granules. Such release is thought to contribute to aggregate consolidation by providing autacoids, (e.g. ADP, 5-HT, TXA_2), which recruit further platelets and amplify the initial stimulation (De Chaffoy de Courcelles et al., 1987; Emms and Lewis, 1986).

1.10 Platelet release reaction

The "platelet release reaction", as described above, is a recognized term among "plateletologists" for the extrusion of platelet constituents from storage granules. It is thought that the mechanism for release involves a rise in intracellular Ca^{2+} concentration which triggers the contractile process. During release, the storage granules move and congregate at the centre of the platelet and vacuoles appear in the central mass of the granules. Specific staining has shown that some of these vacuoles are transversely sectioned channels with openings to the outside extracellular space (White, 1987; Kieffer et al., 1992). As expected, the platelet release reaction resembles the steps observed with other secretory cells and a comparison has been made between

platelets and neurons (Barradas and Mikhailidis, 1993a).

There is evidence that the release from the dense and α-granules is governed by different mechanisms (Holmsen and Day, 1970). For example, aspirin has been shown to inhibit the release of dense granule substances (Best et al., 1981; McCabe White et al., 1992; Rinder et al., 1993) whereas it does not always inhibit the release of α-granule contents (Kaplan, 1986; Rinder et al., 1993). It is also not clear whether the release of intraplatelet products such as 5-HT and PDGF require only minor activation or appreciable aggregation. Given the action of intraplatelet products, vis-à-vis, amplification of platelet aggregation, it is important to resolve this issue. If, indeed, platelets release vasoactive and atherogenic substances such as 5-HT and PDGF irrespective of platelet aggregation, then it may become necessary to develop drugs that target this process, in addition to inhibiting platelet aggregation and PSC.

1.11 The biochemistry of PSC and platelet aggregation

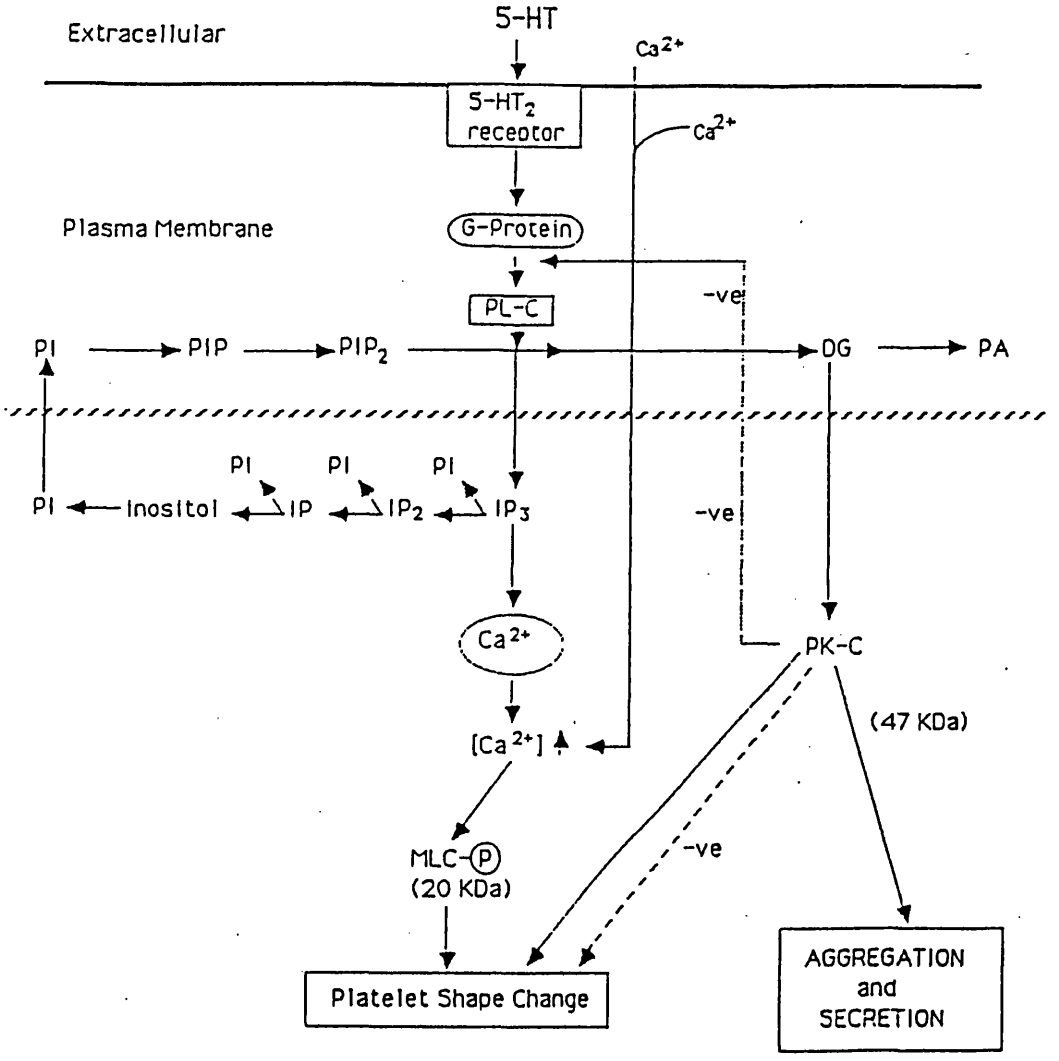
Activation of human platelets involves a complex network of interdependent biochemical processes. A number of signal transduction mechanisms have been suggested to be involved in these responses (Berridge, 1987; Kroll and Schafer, 1989) and others will, no doubt, be described in the future.

The major activating pathway in platelets uses a combination of second messengers derived from enzyme-linked hydrolysis of inositolphospholipids: inositol 1,4,5-trisphosphate (IP_3) and sn-1,2-diacylglycerol (DG). Signal

transduction is initiated by activation of specific receptors present on the cell surface (Figure 1.2). The second messengers involved are generated internally by membrane-associated enzymes (e.g. phospholipase C) through receptor-linked changes in a family of signal-transducing GTP-binding regulatory proteins (known as "G proteins"). In turn, the activated signal-generating enzymes convert highly phosphorylated precursor molecules into intracellular second messengers. Thus, following phospholipase C (PL-C) action, the membrane phospholipid inositol 4,5-bisphosphate (PIP_2) is cleaved into IP_3 and DG which, in turn, exert their intracellular actions by inducing conformational changes on target proteins via protein kinases (Kroll and Schafer, 1989). IP_3 causes release of calcium (Ca^{2+}) which binds to calmodulin causing activation of Ca^{2+}/calmodulin-dependent protein kinases while DG causes activation of protein kinase C (PK-C; Kroll and Schafer, 1989). Both Ca^{2+} mobilization and PK-C activation lead to the diversity of platelet responses (Scrutton and Athayde, 1991). It should be noted that signal transduction pathways involving substances other than IP_3 are also operative. For example there is evidence that ADP can cause a rapid rise in intracellular Ca^{2+} without inositol phosphate turnover (Fisher et al., 1985). Intraplatelet calcium is important for platelet activation and contributes to platelet responses via several mechanisms.These include: (a) Ca^{2+}/calmodulin-dependent protein kinases, (b) Ca^{2+}-dependent proteases, (c) PLA_2 activation, and (d) increased PK-C activity (Kroll and Schafer, 1989; Scrutton and Athayde,

Fig 1.2

Scheme showing biochemical events following platelet activation with 5-HT



The above scheme is based on the current literature (see text).

Abbreviations: **Ca^{2+}**=free calcium; **G-Protein**=guanine nucleotide-binding regulatory protein; **PL-C**=phospholipase C; **PI**=phosphatidylinositol; **PIP**=phosphatidylinositol 4-phosphate; **PIP_2**=phosphatidylinositol 4,5-bisphosphate; **IP_2**= inositol 1,4-bisphosphate; **IP_3**=1,4,5-inositol trisphosphate; **DG**=diacylglycerol; **PA**=phosphatidic acid; **PK-C**=serine/threonine protein kinase C; **47 KDa**=substrate for protein kinase C; **MLC-P**=phosphorylated myosin light-chain.

1991).

These mechanisms and associated platelet processes (PSC, aggregation and release) will be considered, briefly, below.

Biochemically, PSC occurs as a consequence of the interaction between actin and the phosphorylated myosin light chain (20 KDa) (Lapetina, 1984; Daniel et al., 1984). In resting platelets, the level of myosin phosphorylation is low but this protein becomes fully phosphorylated after agonist stimulation. Phosphorylation of the myosin regulatory light chain is carried out by Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK). There are several Ca^{2+}-dependent proteases which are important in platelet activation and possibly PSC. For example, Ca^{2+}-dependent protease cleavage of ABP and of a 235 Kda protein may allow platelet cytoskeletal reorganization during platelet activation (Fox et al., 1985). PLA_2 activation, in contrast to PL-C (Matsuoka et al., 1989), is Ca^{2+}-dependent and subsequent platelet responses are due to the release and metabolism of arachidonic acid (Blackwell et al, 1977; Figure 1.3). Recently it has become clear that the PK-C proteins are important regulators of platelet responses (Berridge, 1987). These proteins require DG, Ca^{2+} and phospholipid for their activity (Kikkawa et al. 1989). Activation of PL-C and generation of DG, for example, by 5-HT, triggers the translocation of inactive PK-C from the cytosol to the platelet membrane (Wang and Friedman, 1990). In the membrane, PK-C is activated in the presence of Ca^{2+} and phosphatidylserine. It is believed that DG increases the affinity of inactive PK-C for Ca^{2+} such that only a slight or

no Ca^{2+} increase is required to induce PK-C activation (Kroll and Schafer, 1989). Under stimulated conditions, intracellular elevated Ca^{2+} and DG are thought to act synergistically to activate PK-C (Wang and Friedman, 1990). The activation of PK-C is associated with the phosphorylation of a platelet protein of 47 KDa (P47) molecular weight (Siess and Lapetina, 1989). As with other aspects of platelet biochemistry it is not completely understood how PK-C causes platelet aggregation and secretion. Nevertheless, recent data shows that phosphorylated P47 but not de-phosphorylated P47, allows elongation of actin elements which contribute to cytoskeletal reorganization during PSC, aggregation and secretion (Hashimoto et al., 1987; Gerrard et al., 1989). In addition, PK-C activation can lead to GPIIb/IIIa binding of fibrinogen which, as described above, is crucial for platelet aggregation (Shattil and Brass, 1987).

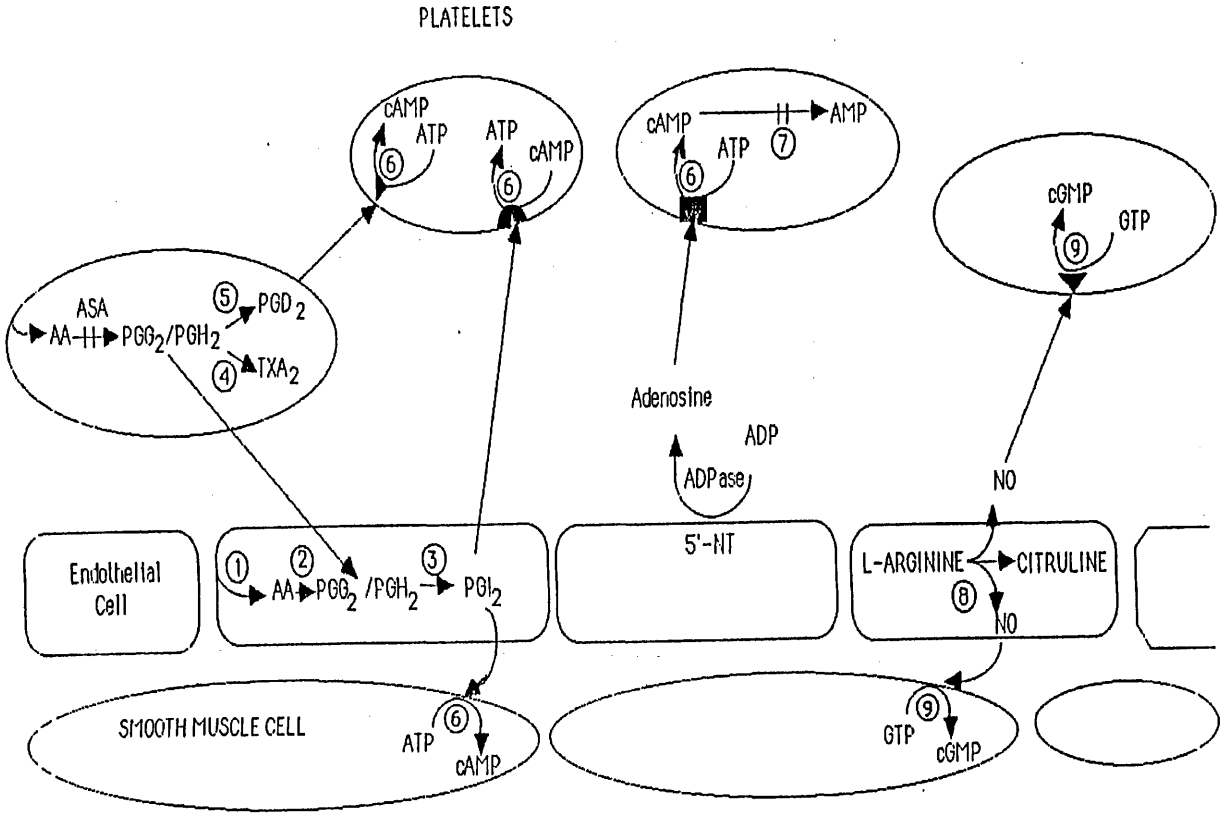
1.12 Inhibition of platelet activation

The endothelium (a unique monolayer of cells lining the vascular wall), the underlying smooth muscle cell layer, circulating cells (e.g. leukocytes), and plasma factors (e.g. coagulation and fibrinolytic enzyme-system) all contribute to the haemostatic balance between "thrombosis" and "bleeding". To achieve such balance, platelet activation must be prevented when it is not necessary. The main factors that are believed to be important in thromboregulation include prostacyclin (PGI_2), endothelium derived relaxing factor (EDRF); and adenosine diphosphatase activity (ADPase) (Ware and Heistad, 1993). PGI_2 is generated by endothelial cells following the

conversion of prostaglandin endoperoxides via prostacyclin synthase (Johnson et al., 1983; Figure 1.3). This compound has a short half-life and acts locally (Mikhailidis et al., 1983c; Ware and Heistad, 1993). EDRF (one form of which is probably nitric oxide, NO) activates soluble guanylate cyclase leading to the accumulation of cGMP in smooth muscle cells and platelets which in turn causes relaxation and inhibition of platelet aggregation (Moncada et al., 1991). At least two products of platelet aggregation, 5-HT and adenine nucleotides, can evoke the release of EDRF(s) by activating endothelial 5-HT receptors and purinergic receptors (Shimokawa and Vanhoutte, 1989). A number of platelet derived-substances and platelet agonists, such as TXA_2, and 5-HT have been shown to cause the release of PGI_2 from blood vessels and vascular cells (Coughlin et al., 1981; Jeremy et al., 1985b). ADPase activity is widely distributed throughout the vascular system, plasma and blood cells. This enzyme degrades ADP to AMP which in turn is further de-phosphorylated by 5'-nucleotidase to adenosine (Arch and Newsholme, 1978; Barradas et al., 1982). The importance of ADPase stems from the fact that ADP is a well established platelet activator and aggregator released by platelets and red cells (Valles et al., 1991) and adenosine is an inhibitor of platelet aggregation and a vasodilator (Arch and Newsholme, 1978; Patelunas et al., 1991). PGI_2 and adenosine stimulate cAMP synthesis, the second messenger involved in the principal inhibitory pathway in platelets (Arch and Newsholme, 1978; Moncada and Higgs, 1987). cAMP activates protein kinase A (PK-A) which leads to the

Fig 1.3

PLATELET-BLOOD VESSEL INTERACTIONS



Arachidonic acid (AA) cleaved from cellular membranes by the action of **PLA_2** (*1*) is converted via **cyclooxygenase** (*2*) into prostacyclin (PGI_2) by **PGI_2 synthase** in endothelial cells (*3*). In platelets prostaglandin endoperoxides (PGG_2/PGH_2) are converted into TXA_2 via **TXA_2 synthase** (*4*) or to PGs, such as PGD_2 via **synthases** (*5*). PGI_2 and PGD_2 stimulate **adenylate cyclase** (AC; *6*) giving rise to platelet inhibition or vasodilation. AC can also be stimulated by adenosine which arises as a result of the conversion of ADP to AMP via **ADPase** and then to adenosine via **5'-nucleotidase** (5'-NT). cAMP is converted to AMP via **phosphodiesterase** (7). This enzyme can be blocked by inhibitors such as milrinone. Platelet activation can also be curtailed following the generation of nitric oxide (NO) by **nitric oxide synthase** (8) which stimulates **guanylate cyclase** (*9*) in platelets (leading to platelet inhibition) or in smooth muscle cells (leading to vasodilation).

phosphorylation of proteins and cellular responses (Hanson and Schulman, 1992). Intracellular cAMP acts as an antagonist of all Ca^{2+}-dependent activation events involved in the agonist-induced signal transduction pathways described above. Thus, cAMP inhibits PLA_2-mediated release of AA (Pannochia and Hardistry, 1987) and the phosphorylation of myosin light chain kinase (important for PSC) (Cox et al., 1984). There is evidence that cAMP influences both the release and uptake of calcium from the platelet dense tubular system via activation of Ca^{2+} pumps (Enouf et al., 1987). In addition, it inhibits PL-C mediated DG and IP_3 formation and appears to directly affect PK-C activity (Knight and Scrutton, 1984).

cGMP is another inhibitory second messenger but much less is known and understood about the way it mediates inhibition of platelet activity. Increasing platelet cGMP indirectly with sodium nitroprusside, NO or 8-bromo-cGMP (a non-hydrolyzable analogue that can penetrate the platelet membrane) inhibits platelet aggregation possibly by inhibiting PL-C activity and/or by influencing the availability of extracellular Ca^{2+} (Matsuoka et al., 1989). There is also evidence that platelet agonists may cause a smaller increase in platelet cGMP due to low level generation of NO (Chirkov et al., 1991). This could serve to dampen the platelet response to agonists.

1.13 Protein kinase C and platelet desensitization

As described above, PK-C becomes activated as a consequence of agonist-induced PIP_2 hydrolysis which in turn can lead to GPIIb/GPIIIa exposure, fibrinogen binding and

platelet aggregation. By using phorbol esters (diterpenes with tumour promoting properties) and synthetic DGs, PK-C activation has been shown to occur independently of PL-C activation (Kikkawa et al., 1989). Furthermore, phorbol esters have been shown to cause aggregation, secretion, release and metabolism of AA (Siess and Lapetina, 1987). In contrast, there is evidence that pre-incubation of platelets with phorbol esters can also result in partial inhibition of subsequent platelet responses to agonists, e.g. thrombin (Zavoico et al., 1985; Yoshida et al., 1986). Thus, PIP_2 turnover, IP_3 generation, Ca^{2+} mobilization and secretion are attenuated by direct PK-C activation prior to thrombin-stimulation (Crouch and Lapetina, 1988). These observation suggest that PK-C may abrogate PL-C-mediated PIP_2 hydrolysis, the principal event which initiates activation. It appears, therefore, that PK-C is not only implicated in positive signals but also in the generation of negative signals contributing to a state of desensitisation in the platelet (Kikkawa et al., 1989).

Desensitization (also referred to as refractoriness, tachyphylaxis and tolerance) is a phenomenon whereby cellular responsiveness is decreased as a result of prolonged exposure to an agonist. In common with other cells, platelets exhibit desensitization on exposure to a wide range of agonists. There are two main types of desensitization: homologous and heterologous (Hallam and Scrutton, 1986). In <u>homologous desensitization</u>, exposure to a given agonist reduces responsiveness to that agonist and either has no effect on, or

enhances responsiveness to other agonists. This type of desensitization limits selectively the extent and/or duration of the response to that agonist. A number of mechanisms have been proposed to account for such desensitization (Table 1.3). In contrast, prolonged exposure to an agonist that causes heterologous desensitization reduces responsiveness both to that agonist and to other agonists that stimulate the cell. Desensitization was first described for human platelet aggregation by O'Brien (1962). These studies showed that incubation with ADP reduced the magnitude of the aggregatory response caused by the addition of a second similar dose of this agonist. Following this report other excitatory agonists i.e. 5-HT, U46619 (a TXA_2 analogue), thrombin, vasopressin, PAF and adrenaline were also shown to possess homologous desensitization characteristics (Hallam et al., 1982). In 1968, Baumgartner and Born reported heterologous desensitization of human platelets induced by 5-HT. It was shown that if the incubation of human platelets with 5-HT was prolonged, then the initial enhancement of the aggregatory response by a second agonist was replaced by inhibition. These findings were later extended and confirmed for other excitatory agonists (Hallam et al., 1982). With the exception of Yoshida et al., 1986, who studied PSC with the "Born-type" optical aggregometer and documented an inhibitory effect by phorbol ester on subsequent ADP and thrombin induced stimulation all other studies (vide supra) assessed platelet aggregation.

TABLE 1.3

PROPOSED DESENSITIZATION MECHANISMS

Mechanism	Type of Desensitization	Example
Decrease in receptor density but no change in receptor affinity	Homologous/Heterologous	Catecholamines[1]
Increase in receptor density and decrease in receptor affinity	Homologous	Platelet Activating Factor[2]
Decrease in receptor density and increase in receptor affinity	Homologous	Catecholamines[3]
Receptor-coupling signal transduction impairment	Homologous/Heterologous	Many agonists[4]
Enhanced rate of removal of agonist by uptake or degradation	Homologous	Serotonin (5-HT)[5]

The following references apply to platelets and are given as examples for the above proposed desensitization mechanisms:

1. Sakaguchi et al., 1986
2. Chesney et al., 1985
3. Gasser et al., 1990
4. Crouch and Lapetina, 1989
5. Hallam et al., 1982

1.14 Methods for studying PSC

Using a photometric technique described for platelet aggregation (see below), MacMillan and Oliver (1965) showed that the rapid morphological change following stimulation of citrated PRP with ADP caused a rapid initial decrease in light transmittance. These changes occurred with low concentrations

of ADP apparently before aggregation had taken place. Later using EDTA, to prevent aggregation, it was shown that 5-HT, collagen and thrombin were able to cause PSC (O'Brien and Heywood, 1966). It is noteworthy, however, that on the basis of theoretical calculations the formation of pseudopods should actually cause a decrease in light scattering and therefore an increase in transmittance (Latimer et al., 1977). Recent studies using citrated PRP also demonstrated that the initial decrease in light transmission was associated with the formation of microaggregates (Thompson et al., 1986).

In the late 1960s, electronic devices measuring electrical resistivity (e.g. Coulter counter) were used for the measurement of particle size (Salzman et al., 1969). Using this equipment it was demonstrated that aggregating agents can cause an approximately 20% increase in apparent volume (Gear, 1981). The issue of whether PSC is associated with actual platelet volume changes, however, is not clear. Theoretical calculations show an <u>apparent volume</u> increase if the axial ratio (i.e. the ratio of thickness to the long diameter) of an ellipsoid is increased without any change in <u>actual volume</u> (Gear, 1981). It is well established that the electrical resistivity measurements carried out with electronic particle counters reflect the orientation and the shape of the particle as it passes through an electrical field in the aperture of the orifice tube and a combination of these parameters is probably what is assessed by a Coulter counter (coupled to a channelyzer) rather than volume changes per se (Born, 1970; Gear, 1981; Lombarts et al., 1986). Scanning electron

microscopy techniques have also been widely used to study PSC. These studies, however, tend to be semi-quantitative and too cumbersome to assess the effect of dose responses following the addition of an agonist or antagonist.

1.15 Methods for studying platelet aggregation

Platelet aggregation has been studied since the early 1960s by the classical turbidometric or light scattering method introduced by O'Brien (1962) and Born (1962). This method requires PRP stirred (usually at 1000 rpm) inside an optical aggregometer. Aggregation is quantified by recording changes in light scatter following the addition of an agonist. The instrument (Born-type aggregometer) is linked to a chart recorder and aggregation is expressed relative to a baseline (pre-stimulation). The light scatter obtained with PRP before agonist addition is assigned as 0% aggregation and platelet poor plasma (PPP) as 100% aggregation. Strictly speaking, this method cannot assess simple particle aggregation which may involve the aggregation of only two platelets. O'Brien (1962) showed that 50% of single platelets had aggregated following ADP stimulation before changes in optical aggregation were detected. More reports have appeared in which poor correspondence between the loss of single particles and optical changes were demonstrated (e.g. Thompson et al., 1986). There are other obvious disadvantages; for example, the absence of red cells and an obligatory centrifugation step for PRP preparation. For these reasons, in the early 1980s new methodological approaches for studying platelet aggregation

were developed. Single platelet counting methods, which were already established in clinical haematology laboratories, became popular among researchers allowing them to study aggregation directly (Fox et al., 1982). Counting could be assessed within very short periods of time (e.g. 10 sec) and in the presence of all the elements of blood. The importance of studying platelet aggregation in whole blood also led Cardinal and Flower (1980) to develop a new method, termed "whole blood impedance aggregometry". This approach depends on changes in impedance as platelets aggregate onto two platinum wires. The method involves the immersion of an electrode assembly into diluted whole blood inside an aggregometer. Initially a monolayer of platelets and possibly other cells adhere to the platinum wires and a baseline is registered on a chart recorder. Following the addition of agonists, an increase in impedance (a fall in conductance) is obtained as more platelets are deposited onto the platinum wires. At the end of the aggregation the electrode must be washed to remove adherent cells (Mackie et al., 1984; Riess et al., 1985).

1.16 Discovery and metabolism of 5-HT

5-HT was isolated in 1948 by investigators who called it "sero-tonin" since this vasoconstrictor substance was found in serum (Rapport et al., 1948). Independently, back in the 1930's, extraction and characterization had been attempted by Erspamer and colleagues (Erspamer, 1954) from enterochromaffin cells of the gastrointestinal mucosa. They discovered a substance that they called "entermine", a gut-stimulating

factor. In the late 1940's it was shown that entermine was present in many tissues. Later, Erspamer & Asero (1952) identified entermine as 5-HT. By this time evidence had accumulated indicating that 5-HT had a wide distribution and possessed many pharmacological actions (Erspamer, 1954). In 1953, Twarog & Page localized 5-HT in the brain and pharmacological studies followed which established 5-HT as a neurotransmitter. Today, 5-HT is also considered an important vasoactive amine (Vanhoutte and Cohen, 1983; Douglas, 1985).

5-HT transport and metabolism by peripheral tissues is complex. Enterochromaffin cells are the major storage site for 5-HT. Other major depots are the brain, peripheral nerves and platelets. 5-HT is also present in the heart, kidney, liver, lung, spleen and thyroid. The exact source and physiological role of 5-HT in these tissues is not known (Tyce, 1990).

Enterochromaffin cells synthesise 5-HT from the essential amino acid, tryptophan. Platelets, however, apparently do not synthesize 5-HT since they lack tryptophan hydroxylase (Morrissey et al., 1977). Platelet 5-HT is thought to be obtained from the circulation following release from enterochromaffin cells (Tyce, 1990). In comparison to histamine, adrenaline and noradrenaline, 5-HT concentrations in the platelet are much higher (e.g. 10 times greater than histamine, 1000 times greater than noradrenaline and >1000 times greater than adrenaline) (Gill et al., 1988; Smith et al., 1992). In the blood, 5-HT is essentially (95%) stored in platelets with only small amounts found in the plasma (Ortiz et al., 1988).

1.17 Pharmacology of 5-HT

The pharmacology of 5-HT receptors is still being established. Recent ligand binding and classical pharmacological studies suggest that there are at least 4 subtypes of 5-HT receptors: 5-HT_1, 5-HT_2 (equivalent to the D receptor), 5-HT_3 and 5-HT_4 receptors. Each of these receptor subtypes can be further subdivided (Roth and Chuang, 1987, Saxena and Villalon, 1991). The molecular biology and the pharmacological, biochemical and physiological properties of the 5-HT_3 and 5-HT_4 receptors are still under investigation. In general terms, the 5-HT_1 receptor "family" is linked to the activation of adenylate cyclase, whereas the 5-HT_2 receptor is linked to phosphoinositol turnover and calcium mobilization (Roth and Chuang, 1987).

1.18 5-HT storage and uptake into platelets

The localization of 5-HT in platelets began in 1951 when Rand and Reid reported that Rapport's serotonin found in serum was contained in platelets. Humphrey and Toh (1954) observed that dog platelets accumulated 5-HT *in vitro*. It was also shown that imipramine (a tricyclic anti-depressant) decreased concentrations of platelet 5-HT due to the specific inhibition of its uptake (Marshall et al., 1960). Later, using electron microscopy and biochemical methods, Tranzer et al. (1966) showed that platelets store 5-HT in subcellular osmiophilic organelles ("dense" granules).

5-HT storage occurs as a result of transport across the platelet plasma membrane and uptake into dense granules.

Plasma membrane uptake is driven by transmembrane gradients of Na^+, Cl^- and K^+ via the action of the electrogenic Na^+,K^+-ATPase (Wolfel et al., 1989). Once in the cytoplasm, there is a separate transport system that moves 5-HT into the dense granule (Rudnick et al., 1980). In the dense granule, a transmembrane H^+ potential (interior of the granule is positive and acidic) provides the driving force for 5-HT transport (Rudnick et al., 1980).

Any substance or disease process that affects platelet 5-HT uptake may promote platelet to platelet interactions and/or influence vascular tone. Drugs such as imipramine, ouabain and N-ethylmaleimide may affect platelet 5-HT uptake by interfering with the 5-HT transporter or by influencing platelet Na^+,K^+-ATPase activity (Andersson and Vinge, 1988; Wolfel et al., 1989).

The effects of platelet stimulators, such as ADP, thrombin and calcium ionophore (A23187; CaI) on radiolabelled 5-HT uptake have been evaluated on human and rat platelets. ADP at low doses (0.3 to 0.8 μmol/L) inhibited 5-HT uptake in rat platelets (Drummond and Gordon, 1976). Thrombin and CaI at high concentrations, resulted in diminished platelet ^{14}C-5-HT with substantial platelet release taking place (Costa and Murphy, 1977). On the other hand, hydrogen peroxide, a substance which activates platelets (Pratico et al., 1992) and is thought to be generated at sites of inflammation and tissue injury, stimulates 5-HT uptake into human platelets (Bosin, 1989).

The disease states linked with platelet 5-HT uptake

disturbances include depression, aging and hypertension (Prina et al., 1981; Faludi et al., 1988; Marazziti et al., 1989). In PVD, 5-HT uptake was found to be unchanged when compared to controls (van Oost et al., 1982).

1.19 Effect of 5-HT on platelet function

In normal healthy subjects, 5-HT is a weak agonist which causes PSC followed by small, and reversible platelet aggregation (De Clerck et al., 1985b). This response is not associated with arachidonate metabolism or the release reaction (De Clerck et al., 1985b). The addition of 5-HT potentiates or amplifies platelet aggregation induced by, ADP, collagen and epinephrine (De Chaffoy de Courcelles et al., 1987; Vanags et al., 1992). Secretion of α-granule constituents and the synthesis of TXA_2 is also augmented by 5-HT (De Clerck et al., 1982). *In vitro*, the importance of endogenous release of 5-HT during aggregation initiated by agonists such as ADP has been demonstrated. These experiments established that the second phase of ADP-induced aggregation can be inhibited by $5\text{-}HT_2$ blockade with ketanserin (Bevan and Heptinstall, 1983; Vanags et al., 1992). Findings such as these have consolidated the view that, *in vivo*, the role of 5-HT is to potentiate platelet responses induced by other agonists (e.g. adrenaline, thrombin, ADP or collagen). This role could be important in the environment of the evolving thrombus.

Binding and functional studies have confirmed the importance of $5\text{-}HT_2$ receptors in mediating 5-HT responses e.g.

platelet aggregation (De Clerck et al., 1984; McBride et al., 1987). Using platelets loaded with calcium indicator-dyes (Quin-2 and Fura-2), 5-HT has been shown to induce the elevation of ionized intracellular calcium (Ca^{2+}) via PIP_2 hydrolysis to IP_3 and DG generation (Figure 1.2). Not surprisingly, 5-HT-induced intracellular Ca^{2+} increase and phosphoinositol turnover can be blocked by ketanserin, suggesting that these events are mediated by $5\text{-}HT_2$ receptors (Kusumi et al., 1991; Rigatti et al., 1992).

1.20 Platelet 5-HT release

Most studies assessing 5-HT release have been carried out using platelets pre-loaded with beta-emitting radiolabelled 5-HT (^3H- or ^{14}C-5HT) and aggregation in the Born-type optical aggregometer. Using this methodology, studies suggest that measurable 5-HT release occurs only after agonist-induced PSC and substantial aggregation (e.g. Packham et al., 1977). On the other hand, studies where aggregation (monitored by counting single platelets) was blocked with EDTA, showed that 5-HT release occurred following stimulation with CaI or thrombin (George et al., 1980; Levy-Toledano et al., 1982). Using quenched-flow aggregometry and radiolabelled 5-HT (^{14}C), it is possible to study the release of this bioamine 100 msec after stimulation with agonists such as ADP, adrenaline and thrombin (Gear and Burke, 1982). Furthermore, it was shown that at high concentrations of thrombin (0.5 U/ml-10 U/ml; citrated PRP), platelet 5-HT release occurred but there was also platelet aggregation. The 5-HT secretion began within 1

sec and was nearly complete by 4 sec. This is in agreement with the kinetics of PSC and associated cytoplasmic calcium changes (Daniel et al., 1984). It was also reported that at low concentrations of thrombin (0.25 U/ml) there was a 40% decrease in single platelets 4 sec after addition with no ^{14}C-5-HT detected outside the platelet. Similar findings were reported for ADP (10 μmol/L) and adrenaline (15 μmol/L; Gear and Burke, 1982). These findings suggest that substantial platelet aggregation is required before radiolabelled 5-HT release can be detected. Another explanation may be that ^{14}C-5-HT methodology is not sensitive enough to show 5-HT release in the early stages of platelet activation. In addition, many workers use ^{14}C-5-HT methodology and washed platelets which involves extensive manipulation during preparation which, as pointed out by Smith et al. (1992), may lead to cellular damage and release of bioamines. In order to avoid these artefacts "cold" methods that use HPLC, radio-immunoassay or enzyme-immunoassay techniques are preferred.

1.21 Effect of 5-HT on vascular tissue

Platelet involvement in the control haemostasis requires platelet aggregation and the release reaction to occur at sites of vascular trauma. Under pathological conditions that promote platelet activation, platelet aggregation and the release reaction may occur within the circulation with the appearance of vasoactive substances such as 5-HT.

In healthy human coronary vessels, 5-HT causes relaxation of vascular smooth muscle and thus vasodilation (Golino et

al., 1991; McFadden et al., 1991). 5-HT is thought to achieve this by interacting with endothelial cells causing the release of EDRF (NO) and PGI_2 (Ware and Heistad, 1993). In isolated vascular smooth muscle preparations 5-HT has been shown to cause contraction. This effect is largely due to the direct activation of $5\text{-}HT_2$ receptors and can be antagonised by $5\text{-}HT_2$ receptor antagonists (e.g. ketanserin and naftidrofuryl) (McGoon and Vanhoutte, 1983; Vashist et al., 1992). There is also evidence that a $5\text{-}HT_1$ receptor(s) may be important in 5-HT-mediated vasoconstrictory effects in human coronaries (Chester et al., 1993). In addition, 5-HT has been shown to have other effects on vascular cells. For example it stimulates the uptake of $^{45}Ca^{2+}$ by human endothelial cells (Gill et al., 1992), causes shape change and contraction of rat aortic myocytes (Bodin & Travo, 1990), possesses mitogenic (Nemecek et al., 1986) and possibly vascular permeability enhancing properties (De Clerck et al., 1985a).

1.22 5-HT, platelet function and vascular disease

As outlined above, the response by vascular tissue to bioavailable 5-HT depends on the vascular bed (in general, veins constrict and arteries dilate). An important additional factor is the condition of the endothelial lining. Endothelial cells from healthy tissues uptake, metabolize and store 5-HT and also trigger the release of vasodilators from the endothelium. However, in blood vessels where endothelium is damaged (e.g. by hypercholesterolaemia or hypertension) 5-HT may become more accessible to vascular smooth muscle thereby

favouring vasoconstriction (Shimokawa and Vanhoutte, 1989).

With regard to platelet function, a number of abnormalities have been reported in PVD. For example, both an increase in platelet aggregation and a shortening of platelet survival have been observed in this condition (Zahavi and Zahavi, 1985). However, whereas in some studies enhanced platelet aggregability in PRP of PVD patients has been documented (Mikhailidis et al., 1985; Zahavi and Zahavi, 1985; The PACK trial group, 1989) in others, no such enhancement could be demonstrated (Cella et al., 1979; Galt et al., 1991). There are at least two factors which may explain this variability. Studies using PRP involve an obligatory centrifugation step that could result in the loss of active platelets during centrifugation (Barradas et al., 1989) or result in centrifugation-induced activation that can lead to desensitization. Studies using whole blood have not been widely carried out in PVD. A study by Catalano et al. (1991) used the whole blood impedance aggregometer. These workers found significantly diminished aggregability to high dose ADP and similar aggregation to high dose collagen when compared to healthy controls. These results are at odds with what would be expected and with the more recent study by Walter et al. (1993) who have recently documented increased platelet reactivity in patients with PVD.

1.23 5-HT bioavailability in vascular disease

In the circulation of normal healthy subjects plasma 5-HT is low. In conditions associated with excess 5-HT production,

e.g. carcinoid syndrome, plasma 5-HT concentrations become appreciable (Kema et al., 1992). There are a number of experimental studies showing that platelets release 5-HT when circulating or activated *in vivo* (Clagett et al. 1980; Osim and Wyllie, 1983; Benedict et al., 1986; Yao et al., 1991; Wester et al., 1992). In addition, a small number of studies have shown raised plasma 5-HT concentrations in patients with cardiovascular disease, renal disease and hypertension (Biondi et al., 1986; Rubanyl et al., 1987; Sebekova et al., 1989; Van den Berg et al., 1989). Plasma 5-HT was also found to be elevated in patients with insulin- and non-insulin dependent diabetes with and without vascular complications (Yoshida et al., 1982; Barradas et al., 1988; Winocour et al., 1990; Pietraszek et al., 1992). Despite the above studies it has not been established whether disease progression/regression or drug therapy can modulate plasma 5-HT concentrations.

1.24 Histamine

Like 5-HT, histamine is a bioamine with potent vasoconstrictor and vasodilator effects (Ginsburg et al., 1984; Douglas, 1985). Histamine has been shown to enhance the leakage of circulating proteins (Harman, 1962; Owens and Hollis, 1979; Hollis and Furniss, 1980; Hollis and Strickberger, 1985; Gill et al., 1990) and increase the inter-endothelial gap space by causing cells to contract (Wu and Baldwin, 1992). These effects are likely to increase the transport of plasma macromolecules (e.g. lipids) into the arterial wall (Ross, 1986). In the blood, histamine is found

mainly in basophils (Graham et al., 1955). Plasma levels of this bioamine are relatively low and platelets store only 5% of the total blood histamine. In conditions associated with platelet hyperaggregability and white cell activation, both cell types have been found to have higher intracellular concentrations of histamine when compared with healthy subjects (Gill et al., 1988). Recent reports also indicate that platelets can synthesize histamine from histidine via the action of histidine decarboxylase (HDC) (McNicol et al., 1989). These workers also showed that intraplatelet histamine makes the integrity of platelet granules more labile to the effect of phorbol ester and have suggested that this effect may contribute to the platelet hyperactivity observed in PVD (McNicol et al., 1989).

1.25 ß-TG

The appearance in the plasma of a platelet-specific ß-globulin was suggested as evidence of *in vivo* platelet activation (Ludlam, 1979). This protein was characterized and named beta-thromboglobulin (ß-TG; Moore et al., 1975). Like PDGF, ß-TG is located in the α-granules and is the most abundant platelet-specific protein (Harrison and Cramer, 1993). The function of ß-TG is not clear whereas PDGF is a well established mitogen and is thought to play a role in the pathogenesis of atherosclerosis (Ross, 1986). In thrombus formation, there may be a burst of release of these proteins at sites where platelet aggregation occurs. On the other hand, in a steady state, platelet release may occur continuously,

reflecting platelet activation and/or destruction.

1.26 Aspirin (acetylsalicylic acid; ASA)

The importance of platelet adhesion and hyperaggregability in the pathophysiology of vascular disorders has led to the widespread use of anti-platelet drugs in cerebral, myocardial or peripheral ischaemia. The most well established and commonly used anti-platelet agent is ASA (Fuster et al., 1993). This drug suppresses platelet aggregation by irreversibly acetylating cylooxygenase hence inhibiting AA metabolism and preventing TXA_2 synthesis (Blackwell et al., 1977; Dahl and Uotila, 1984; Gresele et al., 1991).

A number of large trials examining the effect of anti-platelet drugs on the prevention of further cardiovascular events (secondary prevention trials) have been carried out in the above patient groups (Anti-platelet Trialists' Collaboration, 1988; Fuster et al., 1993). These studies have demonstrated that in survivors of myocardial infarction or stroke and in patients with unstable angina or transient ischaemic attacks, ASA is an effective preventive agent of re-infarction.

The benefit from ASA intake in the inhibition of atherogenesis has not been so favourable and remains controversial (Peto et al., 1988). Clearly, by inhibiting cyclooxygenase activity, ASA blocks the formation not only of platelet activating eicosanoids such as PGG_2, PGH_2 and TXA_2 but probably also that of the platelet inhibitor, PGI_2. This may

be important since PGI_2 has potential anti-atherogenic properties (Moncada and Higgs, 1987). Probably the most important reasons for the limited success of ASA therapy is that only one of the multiple pathways leading to platelet activation is blocked by ASA. Thus, although effective at inhibiting aggregation induced by agonists which mobilize AA for TXA_2 synthesis (e.g. collagen) (Best et al., 1980), ASA is less effective at inhibiting aggregation induced by other agonists (e.g. platelet activating factor, 5-HT, and ADP; Kuster and Frohlich, 1986; De Clerck et al., 1985b; Louden et al., 1992). This has led pharmaceutical companies to develop drugs or examine existing drugs for anti-platelet properties. The possibility that 5-HT may play a role in disease states associated with abnormal platelet function has also raised the interest in anti-5-HT drugs as potential anti-platelet agents (Vanhoutte, 1990; Barradas and Mikhailidis, 1992a).

1.27 Ketanserin and naftidrofuryl

Ketanserin (KET) is a relatively selective 5-HT_2 receptor antagonist with a moderate α_1-adrenoceptor antagonist effect (Fonseca et al., 1984). In its initial clinical evaluation KET appeared to possess anti-hypertensive properties and perhaps, improve symptoms in patients with intermittent claudication and Raynaud's phenomenon (Vanhoutte et al., 1988). Studies assessing the effect of KET on platelets in humans have shown that KET inhibits both *in vitro* and *ex vivo* platelet aggregation induced by 5-HT (Bevan and Heptinstall, 1983; The PACK trial group, 1989; Vanags et al., 1992).

Naftidrofuryl (NAF; Praxilene) is a drug prescribed to PVD patients to improve walking distance (De Felice et al., 1990). This drug has been shown to possess anti-5-HT properties using isolated vascular tissue (Zander et al., 1986; Vashisht et al., 1992). High concentrations of NAF (50-100 μmol/L) were required to inhibit 5-HT induced aggregation in PRP (Davies and Steiner, 1988) and one *ex vivo* study where NAF was infused into healthy volunteers (0.7 mg/Kg) failed to show any effect on 5-HT induced aggregation (Davies and Steiner, 1988). At present, there are no studies examining the effects of NAF on *ex vivo* human platelet aggregation in PVD patients.

1.28 Milrinone

As described above (section 1.12), cAMP modulates platelet aggregation and release by controlling the mobilisation of calcium via effects on agonist-induced signal transduction mechanisms, PL-C and PLA_2. Much interest has been shown in drugs that elevate or maintain intracellular cAMP concentrations. Such drugs include the eicosanoids (e.g. PGE_1, PGI_2), the PGI_2-analogue, iloprost (Ashby, 1990) and cAMP-phosphodiesterase (PDE) inhibitors (Patelunas et al., 1991). PDE inhibitors prevent the hydrolysis of cAMP to AMP which makes them potential inhibitors of platelet hyperaggregability and thrombosis (Hall, 1993). Milrinone, a new PDE inhibitor, is a cardiotonic drug, which until recently was administered to patients in heart failure. This drug is a cAMP-specific phosphodiesterase type-III inhibitor and has been shown to

inhibit various aspects of platelet function including platelet aggregation (Lindgren et al., 1990; Pattison et al., 1990; Patelunas et al. 1991; Ozin et al., 1992; Jeremy et al., 1993). The effect of milrinone on 5-HT-induced platelet aggregation and PSC has not been studied.

1.29 Aims of this thesis

The aim of this thesis was to investigate PSC, aggregation and platelet-derived substances, namely 5-HT, in patients with PVD so as to identify platelet abnormalities that could contribute to the increased incidence of cardiovascular events observed in this condition.

To investigate PSC, a new method was developed that allows the measurement of this parameter in a standardised and reproducible manner. This method was applied to the study of platelet agonists and antagonists in normal subjects and patients with PVD.

A whole blood single platelet counting method was selected to study platelet aggregation in PVD patients. PRP preparation was demonstrated to be associated with a loss of platelets which may explain the conflicting results reported with PRP optical aggregation methods.

Previous studies have shown that 5-HT is released from platelets and it was suggested that this bioamine is an important mediator of platelet aggregation and thrombosis; an attempt was made to substantiate this hypothesis. Plasma and platelet 5-HT concentrations were assessed in patients with PVD, diabetes mellitus, renal disease and

hypercholesterolaemia and these concentrations were related to risk factors and other intraplatelet substances.

Finally, the effects of various antiplatelet drugs and platelet agonists on the uptake and release of 5-HT was studied. This was carried out to establish the role played by such processes on plasma and platelet 5-HT concentrations during treatment with platelet active drugs and in conditions associated with platelet activation.

CHAPTER 2

PLATELET SHAPE CHANGE (PSC) STUDIES

CHAPTER SUB-INDEX

2.1 INTRODUCTION

Various workers have pointed out that platelet shape change (PSC) is important since it favours platelet-to-platelet contacts and platelet-vascular cell interactions (Gear, 1981; Hensler et al., 1992); key features of haemostatic and thrombotic processes (Marcus and Safier, 1993). This aspect of platelet function, however, has not been as intensely studied as other aspects of platelet function, e.g. aggregation and release (Gear, 1981; Thompson and Jakubowski, 1988).

The techniques used to assess PSC include platelet turbidometric aggregometry (using platelet rich plasma; PRP), electron microscopy (EM) and cell counters (using whole blood or platelet rich plasma). The first of these techniques is only semi-quantitative and has often been used in the presence of EDTA in order to prevent platelet aggregation (Born, 1970). EDTA, however, causes platelet swelling and changes in the platelet membrane which could affect PSC (Zucker and Borrelli, 1954). EM visualises PSC which allows the appearance of pseudopodia following activation. Monitoring pseudopod formation is particularly important to assess the effect of agonists that only cause the latter phenomenon but not the extensive internal alterations known as "spheration" (Keraly et al., 1988; Ebbeling et al., 1992). EM, however, is not amenable to rapid and objective quantification of both components of PSC, "spheration" and "pseudopod formation". On the other hand, particle counters coupled to channelyzers (pulse-height analyzers) are technically less demanding than

EM and allow multiple sampling at relatively low cost.

One of the objectives of this thesis was to develop a rapid, sensitive, method to measure PSC. A particle counter (Coulter ZM) coupled to a channelyzer (Coulter C-256) was selected. This apparatus provides at least 4 times the resolution of conventional blood counters used for platelet volume measurements. It was, however, imperative that the methodology allowed the assessment of PSC in the absence of platelet aggregation. Meticulous attention to the platelet count was applied and low agonist concentrations used. PSC was also studied in media which did not cause extensive damage to platelet membranes or inhibit PSC. Following preliminary studies, citrated PRP and low concentrations of agonists were selected together with early sampling (to coincide with maximum PSC) and the absence of stirring during incubations with antagonists . The setting up of a method to examine PSC constitutes Part 1 of this Chapter. Part 2 consists of an application of the PSC methodology in PVD. In Part 3, PSC desensitization phenomena induced *in vitro* are reported. The final part of this Chapter, Part 4, consists of a series of experiments designed to investigate the effects of various anti-platelet and anti-5-HT drugs on PSC.

Part 1

Establishing a new method for measuring PSC

2.2 METHODS (Barradas et al., 1990d, 1992c)

A) Blood collection and subject selection

Blood, from all subjects, was collected by venepuncture

from an antecubital vein. Large vessels and care were used to ensure good blood flow and minimum stasis. Blood was obtained with a G-21 butterfly and 10 ml plastic syringes. In all cases the first 2 ml of blood were discarded. In experiments designed to assess PSC in different anticoagulant media, blood was collected into citrate anticoagulant, citrate + aspirin (ASA) or EDTA solution; 9 volumes blood: 1 volume anticoagulant. In all other experiments assessing PSC, blood sampled was anticoagulated with citrate only. For the composition of these anticoagulant mixtures see Appendix (**Buffers**).

Healthy volunteers that donated blood were staff and students of the Royal Free Hospital School of Medicine. All denied taking any medication for at least 14 days prior to sampling. Where appropriate, the age and sex of volunteers is outlined below.

B) Preparation of platelet-rich plasma (PRP)

Platelet-rich plasma (PRP) was prepared by the centrifugation of anticoagulated blood (150 x g; 15 min at room temperature). The PRP was separated by aspirating gently the top layer avoiding the buffy coat or the red cells. In order to stabilize the PRP and prevent activation due to cooling, PRP was stored capped at 37°C for 30-40 min before use (Mikhailidis et al., 1983a).

C) Agonist addition and sample fixation

Aliquots of PRP (450-470 μl), in glass cuvettes, were

placed in a dual channel optical aggregometer and stirred (using a teflon-coated metal stir bar) at 1000 rpm, at 37°C. For experiments involving antagonists, vehicle or drugs were added (as 5-10 μl volumes) and pre-incubated for 5 min before the addition of agonists. In order to prevent platelet activation due to stirring during the pre-incubation period, the magnetic stirring mechanism in the aggregometers was switched off after the first 30 sec and only switched back on after a 4 min interval. Agonists were added as 10-50 μl volumes to the stirring PRP in order to achieve the final concentrations shown in the Tables and Figures. At the specified times, after agonist treatment, 100 μl aliquots of PRP were removed and mixed with 400 μl of fixative (4% v/v glutaraldehyde in saline).

In preliminary experiments, the possibility of omitting the fixative was investigated. The objective of these experiments was to attempt to simplify the procedure (see below).

D) Platelet counting and particle size analysis

Platelet counts were measured in fixed PRP suspensions diluted 400 fold with Isoton II, at room temperature, using a Coulter ZM coupled to a Coulter C-256 channelyzer. Platelets of size between 2.67 and 19.12 x 10^{-15} L (femtolitre; fl) were counted and channelyzed. The counter was calibrated regularly with platelet volume calibration latex particles of 9 fl obtained from Coulter Electronics Ltd. Particle analysis of the PRP population was carried out by accumulating data to a

maximum of 500 platelets in one of the 256 channels. The results were transferred to an X-Y recorder or displayed on the C-256 screen. The total number of particles accumulated in the 256 channels was 40,000-50,000. Counting of particles "channelyzed" was performed automatically by the equipment. The median value of the size distribution plots, i.e. the median platelet volume (MePV), was the volume of the channel on each side of which 50% of the platelet population was distributed.

Throughout these studies the platelet counts in the PRP in each sample cuvette was monitored and did not change by more than 5-10% of the basal count.

Reproducibility of the methodology was evaluated by calculating the intra- and inter-assay coefficients of variation (CV; for calculation see below).

E) Statistical analysis and presentation of results

Results in Tables are expressed as median and (range). Results are also presented as individual values in scatter diagrams. For diagrammatic purposes data in Figures are occasionally expressed as mean and standard error of the mean (± SEM). Where appropriate, IC_{50s} (concentration of antagonist required to inhibit 50% of platelet shape change induced by an agonist) was estimated graphically. Statistical analysis in this Chapter and subsequent Chapters was always by non-parametric tests since these tests have been recommended for samples of unknown distribution or for samples that do not obey a normal (Gaussian) distribution (Altman et al., 1983).

Results were statistically analyzed using a computer program (C-STAT). Paired values were compared using the Wilcoxon rank sum test (two-tailed). Unpaired data was analyzed by using the Mann-Whitney U-test for non-parametric data (two-tailed).

The coefficient of variation (CV) is defined as :

$$CV = \frac{SD \times 100}{mean}$$

where SD=standard deviation and mean is the average of a number of readings. Standard Deviation is defined as:

$$SD = \frac{d^2}{n-1}$$

where d is the difference between the readings and the mean, and n is the number of readings.

2.3 RESULTS

A) Effect of omitting the fixative

The absence of fixative resulted in larger MePV readings. This difference was considerable both for basal readings (e.g. with fixative: 6.01 fl; without fixative: 6.52 fl) and following the addition of agonists (e.g. MePV 30 sec after adding U46619, 0.027 μmol/l: with fixative: 6.59 fl; without fixative: 7.17 fl). However, these differences are difficult to quantify since the MePV is variable in the absence of fixative. This phenomenon is dependent on the time the platelets remain in contact with Isoton II. For example, the basal MePV in the presence of fixative, changed from 5.40 to 5.37, 5.37 and 5.49 fl after 0, 1, 5 and 15 min incubation (at room temperature in Isoton II), respectively. The corresponding MePVs for the same subject, but in the absence of fixative were: 5.43, 6.03, 6.10 and 6.27 fl, respectively.

This pattern was confirmed in PRP obtained from 2 other subjects. In order to avoid fixative effects the contact time between the PRP and fixative were minimised (less than 10 sec) by rapidly diluting (400 fold) the sample in Isoton II. All experiments were therefore carried out in the presence of fixative in order to obtain stable and reproducible MePV measurements. Unless otherwise indicated, the following experiments were carried out in citrated PRP obtained from healthy volunteers (age range: 20-55 years). The platelet count in PRP ranged from 276-520 x 10^9 platelets/L PRP.

B) Reproducibility of MePV measurements

The intra-assay and inter-assay CV are shown below (Table 2.1 and 2.2). In addition, reproducibility was assessed by adding saline or 0.1 μmol/L 5-HT to freshly prepared PRP samples and to PRP samples that had been standing, at 37°C, in a capped closed plastic tube for 90 min (+/- 30 min). Eleven separate PRP samples were evaluated and the MePV compared (Table 2.3). All subsequent PSC were completed within 2 h of collection of the blood sample.

TABLE 2.1

Intra-assay coefficient of variation

Date	MePV fl	Platelet count x 10^9/L
11/5	5.82	195
11/5	5.73	170
11/5	5.73	146
11/5	5.82	178
11/5	5.75	160
11/5	5.84	181
11/5	5.80	198
11/5	5.82	188
11/5	5.73	180

n=9

MePV: mean=5.78 fl SD=0.05 SEM=0.015
median=5.80 fl Range=5.73-5.84 fl

CV= 0.8%

Platelet count: mean=177x10^9/L SD=17 SEM=6
median=180x10^9/L Range=146-198x10^9/L
CV= 9.4%

TABLE 2.2

Inter-assay coefficient of variation

Date	MePV fl	Platelet count x 10^9/L
15/3	5.70	280
29/3	5.53	284
6/4	6.18	380
20/4	5.88	316
28/4	5.75	213
9/5	5.94	376
16/5	5.75	420
25/5	5.82	368
6/6	5.82	326
15/6	5.82	376

n=10

MePV: mean=5.82 fl SD=0.17 SEM=0.05
median=5.82 fl Range=5.53-6.18 fl
CV= 2.89%

Platelet count: mean=334x10^9/L SD=62 SEM=20
median=347x10^9/L Range=213-420x10^9/L
CV= 18.6%

TABLE 2.3

Effect of keeping PRP at 37°C on basal and 5-HT-induced PSC

Fresh PRP MePV		**90 min (+/- 30min)** MePV	
Sal	5-HT 0.1 μmol/L	Sal	5-HT 0.1 μmol/L
5.45 (4.98-5.71)	5.76* (5.56-6.39)	5.40 (4.98-5.75)	5.82* (5.60-6.34)

Results are expressed in fl.

*P< 0.01: Sal Vs 5-HT for both fresh PRP and PRP kept at 90 min (+/- 30 min).

number of subjects studied=11

Time courses and dose responses

A) Agonist-induced changes in MePV - time courses

Increases in MePV were induced by the addition of: 5-HT (0.1, 1.0 and 100 μmol/L), ADP (0.2 and 0.4 μmol/L), collagen (0.2 and 0.4 mg/L), U46619 (0.027-0.05 μmol/L), AA (0.15-0.25 mmol/L), calcium ionophore (CaI; 2.7 μmol/L) and PAF (0.01-0.02 μmol/L). Agonists had different patterns of MePV expansion which are shown in Figures 2.1 and 2.2 and Table 2.4. These results determined at which time MePV was measured.

Fig 2.1

Time course for PSC induced by various concentrations of 5-HT

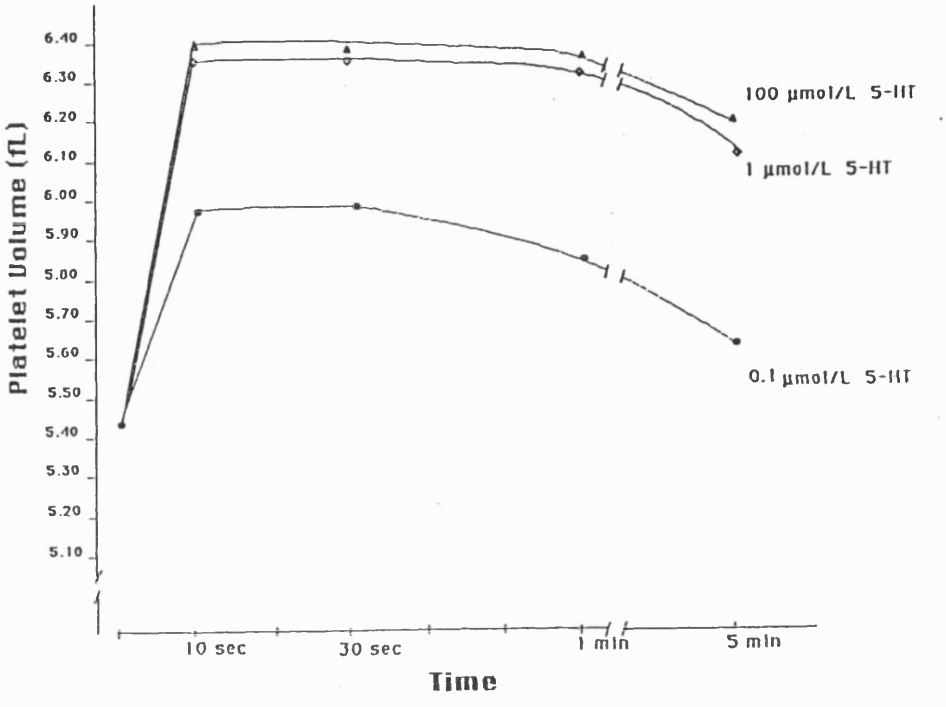


Fig 2.2

Time course for PSC induced by various concentrations of collagen

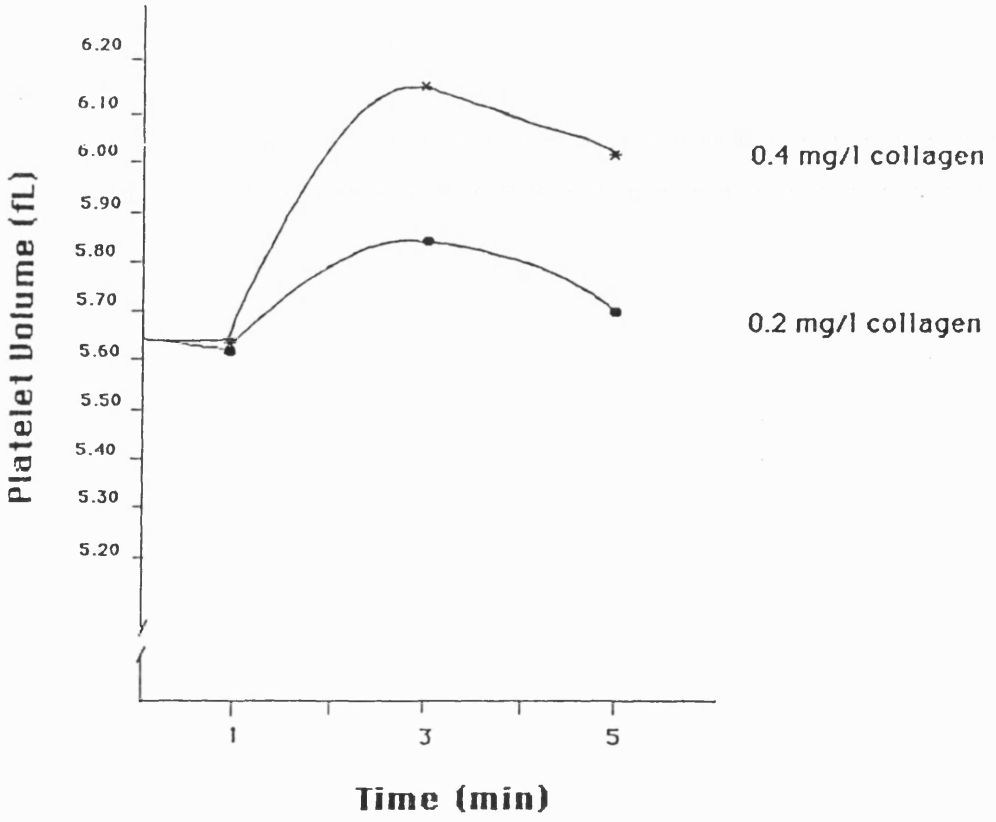


TABLE 2.4

Agonist-induced changes in MePV-Time courses

AGONIST	SAMPLING TIME			
	BASAL	**30 sec**	**1 min**	**5 min**
U 46619 (0.027-0.05 μmol/L)	5.39 (4.92-6.14)	6.01* (5.58-6.78)	6.05* (5.62-6.75)	6.01* 6.72)
AA (0.15-0.25 mmol/L)	5.39 (4.94-6.14)	5.71* (5.58-6.68)	5.75* (5.58-6.71)	5.85* (5.50-6.65)
CaI (A23187) (2.7 μmol/L)	5.50 (4.85-6.14)		5.75* (4.98-6.20)	6.65*$ (5.90-7.04)
PAF (0.01 μmol/L)	5.45 (4.94-6.14)	5.73* (5.26-6.82)	5.75* (5.26-6.84)	5.97* (4.94-6.97)

Results are expressed in fl.

* P< 0.01: MePV compared against BASAL (saline only) value.
$ P< 0.01: Vs. 1 min MePV. All other comparisons were not significant.

number of subjects studied=7.

B) Agonist-induced changes in MePV-Dose responses:

Increases in MePV were induced by the addition of: 5-HT (0.01, 0.1, 1.0 and 100 μmol/L; measured 30 sec after the addition of 5-HT), ADP (0.1, 0.2 and 0.4 μmol/L; 30 sec after the addition of ADP), collagen (0.2 and 0.4 mg/L; 3 min after the addition of collagen), U46619 (0.017, 0.034 and 0.0675 μmol/L; 30 sec after the addition of U46619), AA (0.1, 0.3 and 0.6 mmol/L; 30 sec after the addition of AA), CaI (2.7 μmol/L; 5 min after the addition of the ionophore) and PAF (0.01 μmol/L; measured 30 sec after the addition of PAF). Agonists

had different potencies for MePV expansion which are shown in Table 2.5. The catecholamines, adrenaline and noradrenaline, were also evaluated (Table 2.6).

Agonist-induced changes in MePV - agonist interactions

Two agonists (5-HT and ADP) that induce MePV expansion were combined in order to determine whether synergism or additive effects occur when agonists are added simultaneously. Synergism is defined as a response which is greater than the sum of the individual responses. The results shown in Tables 2.7 and 2.8 suggest that both synergistic and additive effects are possible depending on the initial PSC obtained with each individual agonist. Thus, when agonists added individually yield small increments in MePV, synergism is obtained (Table 2.7) and additive effects are obtained if individual agonists yield large increments in MePV when added alone (Table 2.8).

TABLE 2.5

Median and (range) MePV (fl) following the addition of various agonists

AGONIST	AGONIST CONCENTRATIONS				
5-HT (μmol/L)	**BASAL**	**0.01**	**0.1**	**1.0**	**100**
	5.37 (4.82-5.98)	5.40 (4.66-6.01)	5.76* (5.24-6.91)	5.88* (5.32-6.97)	6.00* (5.52-6.91)
n=11					
ADP (μmol/L)	**BASAL**	**0.1**	**0.2**	**0.4**	
	5.50 (4.82-5.98)	5.57 (4.90-5.88)	5.95* (5.28-7.04)	6.25* (5.75-7.00)	
n=14					
U46619 (μmol/L)	**BASAL**	**0.017**	**0.034**	**0.0675**	
	5.43 (4.72-6.33)	5.64* (4.83-6.44)	5.91* (5.17-6.65)	6.35* (5.49-7.20)	
n=9					
AA (mmol/L)	**BASAL**	**0.1**	**0.3**		
	5.57 (4.92-5.98)	5.85* (4.66-6.33)	6.23* (5.24-6.88)		
n=9					
PAF (μmol/L)	**BASAL**	**0.005**	**0.01**		
	5.67 (4.82-5.98)	5.85* (4.66-6.33)	6.23* (5.24-6.88)		
n=9					

*P< 0.01: MePV compared against BASAL (saline only) value.
n=number of subjects studied.

TABLE 2.6

Effect of adrenaline and noradrenaline on PSC

BASAL	Adrenaline 1.0 μmol/L	Noradrenaline 1.0 μmol/L
5.51 (4.72-5.88)	5.53 (4.72-5.88)	5.72* (4.72-5.88)

Results are expressed in fl.

*P< 0.01: MePV compared against BASAL (saline only) value.

number of subjects studied=12.

TABLE 2.7

Synergistic effect of agonists on PSC

BASAL (Saline)	5-HT 0.01 μmol/L	ADP 0.1 μmol/L	5-HT+ADP
5.37 (4.72-5.94)	5.49 (4.62-6.00)	5.49 (4.72-6.02)	5.75* (4.66-6.14)

Results are expressed in fl.

*P< 0.01: MePV compared against BASAL (saline only) value.

number of subjects studied=7.

TABLE 2.8

Additive effect of agonists

BASAL (Saline)	5-HT 0.1 μmol/L	ADP 0.2 μmol/L	5-HT+ADP
5.37 (4.72-6.33)	5.56* (4.72-6.46)	5.57* (4.73-6.56)	5.77* (4.72-6.85)

Results are expressed in fl.

*P< 0.01: MePV compared against BASAL (saline only) value.

number of subjects studied=11.

Effect of adrenaline and 5-HT on PSC

Adrenaline induces aggregation in citrated PRP obtained from most healthy subjects (but not in neonates and in some adult subjects; Barradas et al., 1986). This bioamine can also potentiate platelet aggregation induced by 5-HT or ADP (Barradas et al., 1990b; Vanags et al., 1992). Using the present methodology it was of interest to determine whether adrenaline could enhance PSC induced by 5-HT since adrenaline on its own did not induce PSC (Table 2.9).

TABLE 2.9

Effect of adrenaline and 5-HT on PSC

BASAL (Saline)	5-HT 0.01 μmol/L	Adrenaline 0.2 μmol/L	5-HT+ Adrenaline
5.45 (4.88-5.70)	5.45 (4.98-5.88)	5.40 (4.87-5.70)	5.64* (5.24-6.01)

Results are expressed in fl. *P< 0.01: MePV compared against BASAL (saline only) value. number of subjects studied=7.

PSC studies in blood collected in different anticoagulant mixture

To assess the effect of different anticoagulants on PSC, blood was collected from healthy subjects (5 male, 5 female; median age and range: 30; 20-55 years).

PRP prepared from blood anticoagulated with EDTA (solution C, Appendix) was associated with the highest platelet counts (Table 2.10) and the largest platelets (Figure 2.3). This anticoagulant strongly inhibited PSC stimulated by 5-HT (Figure 2.3) or ADP. ADP-induced responses (0.4 μmol/L; n=4) were: median and (range) MePV (fl); sal-citrate: 5.45 (5.33-5.75), ADP-citrate: 5.79 (5.33-6.07); sal-citrate+ASA: 5.56 (5.33-5.69), ADP-citrate+ASA: 5.72 (5.49-6.14); sal-EDTA: 6.46 (6.14-6.91), ADP-EDTA: 6.28 (6.14-7.03). Median and (range) platelet counts x 10^9/L for ADP-induced responses were: sal-citrate: 368 (356-452), ADP-citrate: 378 (328-408); sal-citrate+ASA: 389 (352-424), ADP-citrate+ASA: 374 (310-500); sal-EDTA: 436 (408-508), ADP-EDTA: 480 (372-496).

TABLE 2.10

Platelet counts in PRP stimulated with 5-HT

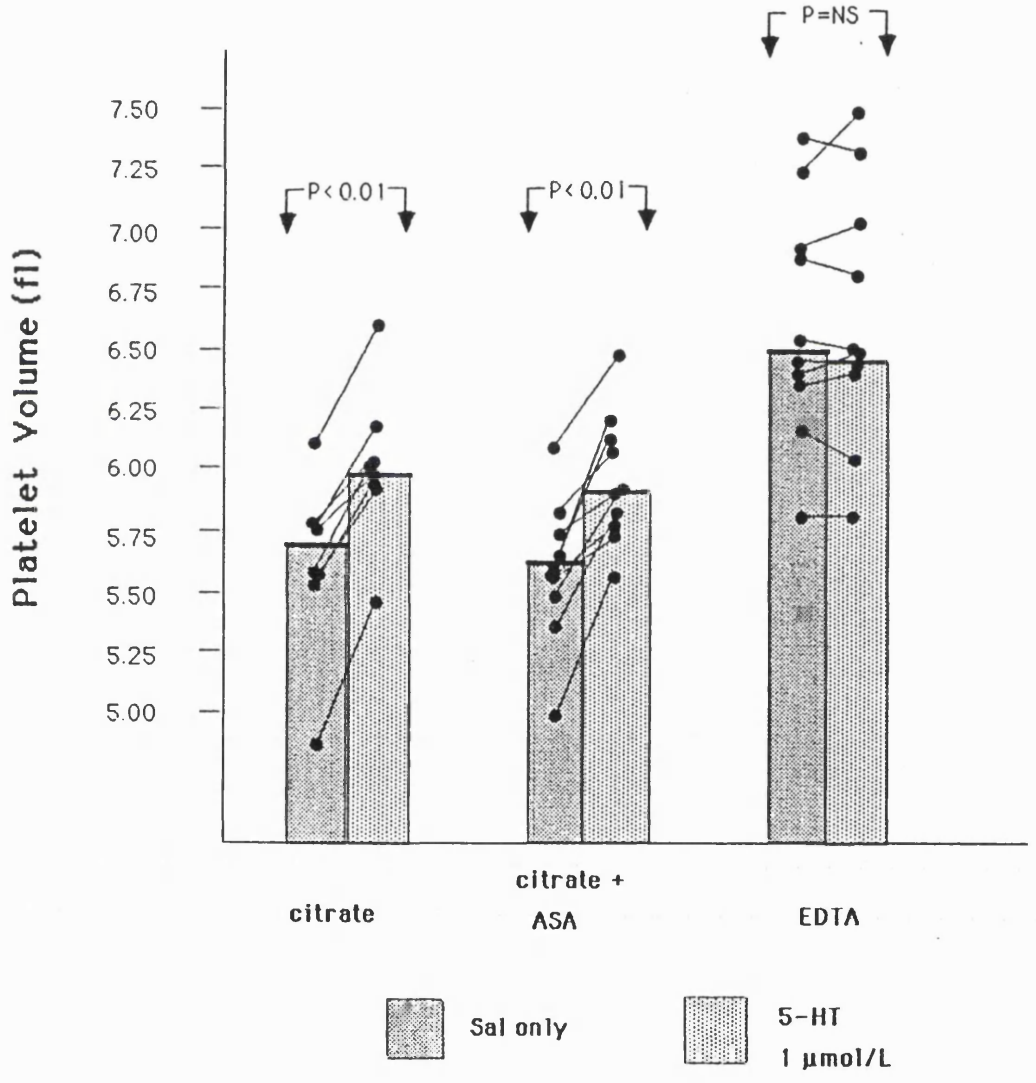
Citrate Sal	Citrate 5-HT 1μM	Citrate + ASA Sal	Citrate + ASA 5-HT 1μM	EDTA Sal	EDTA 5-HT 1μM
374 356-472	398 310-452	390 298-468	410 324-440	486* 408-624	478* 426-548

Platelet counts x10^9/L.

* P=0.005: Vs. corresponding Citrate and/or Citrate + ASA. All other comparisons were not significant. Number of subjects studied=10.

Fig 2.3

5-HT-induced PSC in PRP obtained from blood collected in different anticoagulant mixtures



Solid bars= medians

Final concentrations of mixtures:
citrate=0.38% w/v tri-sodium citrate;
citrate+ASA=0.38% w/v citrate + 1 mmol/L ASA
EDTA=5 mmol/L *di*-sodium EDTA

2.4 DISCUSSION

A simple and highly reproducible method of studying PSC has been described. This method allows the effect of agonists to be examined by measuring the increase in MePV and is amenable to serial measurements for the generation of dose responses. From a methodological point of view, it is important to sample for MePV at specific times after the addition of agonists. For example, the increase in MePV is latent in the case of collagen and CaI and the reversal of PSC occurs over a period of time that varies with each agonist.

Adrenaline's lack of effect on MePV requires clarification. There are other peculiarities when considering this agonist. Platelets from neonates and from some adults do not aggregate in response to this agonist (Barradas et al., 1986) and adrenaline-induced aggregation cannot be measured in whole blood by the impedance method despite the fact that this agonist reduces the free platelet count (Mackie et al., 1984; Barradas et al., 1992b). Furthermore, noradrenaline is a weaker agonist than adrenaline, vis-à-vis, platelet aggregation (O'Brien, 1964) whereas the reverse is true for PSC. The lack of increase in MePV following the addition of adrenaline has previously been reported although this issue is controversial (Gear, 1981; Milton and Frojmovic, 1984; Keraly et al., 1988; Erne et al., 1988). This inconsistency may be attributed to turbidometric and impedance volume measurements primarily reflecting increases in "spheration" rather than "pseudopod formation". Adrenaline, however, does activate platelets and enhances PSC when added in combination with 5-HT

(Table 2.9).

Evidence that synergism/additive effects occur between agonists was also presented. Which of these latter effects is observed, probably depends on whether a maximal (Table 2.8) or submaximal (Table 2.7) response is induced by each individual agonist. These findings suggest that, *in vivo*, low concentrations of agonists, which are present in blood, may combine to increase the MePV. It is, therefore, of interest that significantly higher MePVs have been reported in patients with MI (Cameron et al., 1983; Martin et al., 1983; Sewell et al., 1984; Trowbridge and Martin, 1987). Although in MI "big" platelets may be produced as a result of alterations in thrombopoiesis (Martin and Trowbridge, 1990), acute increases in MePV following direct activatory effects on platelets should not be ruled out. We have documented increases in MePV in various settings, e.g. following the intravenous injection of therapeutic doses of heparin (Mikhailidis et al., 1990); exposure to hypothermia (Escalda et al., 1993) or after the addition (*in vitro*) of bacterial lipopolysaccharide (Whitworth et al., 1989) or to N-formyl-methionine phenylalanine (a bacterial chemotactic peptide; Nystrom et al., 1993).

Part 2

2.5 PSC studies in PVD patients

There are notable studies examining platelet volume in patients with MI (Cameron et al., 1983; Martin et al., 1983; Sewell et al., 1984; Martin et al., 1991). In contrast, there are very few studies examining PSC phenomena. One study

investigated PSC in patients with coronary artery disease and patients with congestive heart failure (Erne et al., 1988). In this study, citrated PRP was diluted (with NaCl but not fixed) before measurements were carried out with a Coulter ZBI coupled to a channelyzer. These workers reported enhanced sensitivity to 5-HT and adrenaline in patients with acute MI and chronic stable angina and a reduced sensitivity to both agonists in patients with congestive heart failure (CHF). Sensitivity to adrenaline but not 5-HT, was reduced in MI patients that were tested 4-7 days after admission when plasma catecholamine concentrations had normalized. These results suggest a possible catecholamine-induced receptor-down regulation in the MI phase which is in agreement with a previously documented reduction in α_2-receptors following MI (Sakaguchi et al., 1986). Diminished responses to 5-HT and adrenaline in CHF also imply a receptor-down regulation possibly due to chronically elevated adrenaline, noradrenaline and 5-HT (the latter bioamine was not measured). A study by Fetkovska et al., (1990) reported unchanged 5-HT-induced PSC in essential hypertensives. In DM, an investigation of ADP and vasopressin-induced PSC reported normal responses for ADP but no PSC following vasopressin (VP; Vittet et al., 1991). In this condition a decrease in the number of VP receptors was also documented. Interestingly, DM patients showed the same extent of platelet aggregation as healthy subjects when higher concentrations of VP were used.

In the present thesis, PSC induced by 5-HT and ADP was examined in patients with PVD and healthy subjects.

2.6 METHODS

A) Selection of PVD patients and healthy subjects

The patients selected were PVD patients attending the vascular clinic at The Royal Free Hospital (RFH). Informed consent was obtained from all patients and healthy subjects before blood sampling. Twenty PVD patients, of which 14 were males and 6 were females volunteered for this study. The median age and (range) of the patients was 61 (45-84) years. Patients selected were drug-free and in a metabolically/ clinically stable condition with no history of recent cardiac events, strokes, transient ischaemic attacks or changes in their claudication distance. None of the patients had undergone any recent surgery, angiography or angioplasty. Twenty healthy subjects were selected; 14 were males and 6 were females. Median age and (range) was 32 (20-58) years. Blood was collected into citrate (solution A; composition in Appendix) and PRP prepared as previously described (Part 1).

B) Platelet counting and particle size analysis

Platelet counting and platelet size analysis were carried out after agonist addition and sample fixation as described above (Part 1). The PRP platelet counts that changed by more than 5-10% of the basal count were deemed to have aggregated and were not analyzed.

2.7 RESULTS

Median platelet counts in PRP of healthy subjects (expressed per 10^9/L) was 312 (range: 224-520) and in PVD

patients: 262 (range: 164-320); this difference was significant (P<0.03). Basal MePV was not different in PVD patients when compared to healthy subjects (Table 2.11 and 2.12). PSC following stimulation with 0.1 μmol/L 5-HT was significantly increased (Vs. basal) in healthy subjects but not in PVD patients. At 1.0 μmol/L 5-HT, PSC achieved by PVD patients was significantly (P<0.04) diminished as compared to healthy subjects. At high concentrations of 5-HT (10 μmol/L), PVD platelets were hyperaggregable. Thus, 4 PVD patients showed appreciable aggregation and were excluded. None of the healthy subjects were excluded (Table 2.11). ADP-induced PSC responses were similar in patients and healthy subjects. Higher concentrations of ADP (0.4 μmol/L) induced appreciable aggregation in all PVD patients and, therefore, sizing was not carried out (Table 2.12).

TABLE 2.11

Effect of 5-HT on PSC in healthy subjects and PVD patients

	Basal MePV	5-HT 0.1 μmol/L	5-HT 1.0 μmol/L	5-HT 10 μmol/L
Healthy Subjects	5.53 (4.85-6.18) n=20	5.75$ (5.05-7.00) n=20	5.94+,$ (5.43-7.15) n=20	6.21$ (5.78-6.8) n=20
PVD Patients	5.49 (4.79-6.23) n=20	5.58 (4.85-6.33) n=20	5.76* (5.17-6.46) n=20	5.93* (5.69-6.8) n=16

Results are expressed in fl. n=number of subjects studied.
Within group comparisons (Wilcoxon rank-sum test):
Healthy subjects (Vs. Basal): $ P< 0.01;
PVD Patients (Vs. Basal): * P< 0.01.
Across group comparisons (Mann-Whitney U-test):
Healthy Subjects Vs. PVD Patients: +P< 0.04. All other comparisons were not significant.

TABLE 2.12

Effect of ADP on PSC in healthy subjects and PVD patients

	Basal MePV	ADP 0.2 μmol/L	ADP 0.4 μmol/L
Healthy Subjects	5.53 (4.85-6.18) n=20	5.88* (5.28-7.04) n=20	6.01** (5.75-7.00) n=10
PVD Patients	5.49 (4.79-6.23) n=20	5.98+ (5.15-7.15) n=20	platelets aggregated n=10

Results are expressed in fl. n=number of subjects studied.
Within group comparisons (Wilcoxon rank-sum test):
Healthy subjects : * P< 0.02; Basal (saline) Vs. 0.2 μmol/L ADP, ** P< 0.01; Basal Vs. 0.4 μmol/L ADP.
PVD Patients: + P< 0.02; Basal Vs. 0.2 μmol/L ADP.

Across group comparisons (Mann-Whitney U-test):
Healthy Subjects Vs. PVD Patients: All comparisons were not significant.

2.8 DISCUSSION

Diminished PSC induced by 5-HT in PVD patients was documented despite the fact that these patients show enhanced platelet aggregability to various agonists including 5-HT (Wu and Hoak, 1976; The PACK trial group, 1989; Ambrus et al., 1990; Walters et al., 1993). On the other hand, it is well established that agonists, at least *in vitro*, may induce a desensitized state, vis-à-vis, platelet aggregation (O'Brien, 1962; Baumgartner and Born, 1968; Hallam et al., 1982). It should be noted, however, that although some agonists are very effective at inducing the refractory state (e.g. 5-HT) others (e.g. collagen) are not (Hallam et al., 1982). In the present study, the patient's platelets appear to have been

desensitized to the effects of 5-HT but not to those of ADP. In the context of 5-HT, it is of interest that plasma 5-HT concentrations are raised in PVD (Barradas et al., 1988; Chapter 4) which may cause down-regulation in receptor numbers or affect intracellular events (see Table 1.3 in Chapter 1 for examples of various desensitization mechanisms). There may also be alterations in signal-transduction mechanisms in PVD platelets. For example, the activation of protein kinase C, by specific agonists or synthetic activators (i.e. phorbol esters; see Chapter 1, Figure 1.2), has an inhibitory effect on platelet responses, e.g. Ca^{2+} mobilization, phosphatidylinositol metabolism and PSC (Zavoico et al., 1985; Yoshida et al., 1986; Crouch and Lapetina, 1988; Kagaya et al., 1990). An additional contributor to the PSC response may be endogenous stores of platelet 5-HT which are decreased in PVD platelets (Barradas et al., 1988 and Chapter 4, Part 1). It is also noteworthy that in conditions associated with activatory stimuli, such as following mental stress (Larsson et al., 1990) or in acute (Siess et al., 1982) and chronic smoking (Meade et al., 1985), diminished platelet aggregation has been documented. However, it is important to note that, in the latter conditions and in PVD, depressed aggregation and PSC responses were observed in PRP, the preparation of which may have removed the more responsive subpopulation of platelets. This issue can be clarified by carrying out PSC studies in whole blood (i.e. eliminating centrifugation).

Part 3

2.9 Desensitization phenomena and PSC

Given that 5-HT-induced responses were diminished and ADP responses were essentially unaltered in PVD, desensitization mechanisms were investigated, *in vitro*, using PRP from healthy subjects and a range of conventional platelet agonists to induce the desensitization state.

2.10 METHODS

A) Selection of healthy subjects and PSC analysis

Seven subjects were selected for these experiments; 5 males, 2 females; median age and (range) was 32 (20-58) years. Blood collection into citrate and PRP preparation were carried out as described in Part 2. Agonist addition, sample fixation, counting and sizing analysis was carried out as described in Part 1.

B) Design of experiments

To elicit the desensitization state, an agonist (1^{st}) was added to a PRP sample in an aggregometer (stirring at 1000 rpm, 37°C). After a 30 sec incubation the stirring mechanism was switched off for 4 min and 30 sec. The sample was then treated with a 2^{nd} agonist and the stirring mechanism switched on for a further 30 sec. MePV was assessed at the end of this latter period (Expt 1 in Figure 2.4). Parallel to this sample, the MePV of PRP that was treated with the 1^{st} agonist only and processed in the same manner as above was also assessed (Expt 2 in Figure 2.4). For comparison, the MePV of PRP that was

treated with the 2nd agonist only (Expt 3) or with saline (sal) was assessed (Expt 4 in Figure 2.4). The PSC responses following desensitizing were calculated as a % and are shown in Table 2.13. Thus,

$$\% \text{ response} = \frac{\text{MePV of } 1^{st}+2^{nd} - \text{MePV of } 1^{st}}{\text{MePV of } 2^{nd} - \text{MePV of sal}} \times 100$$

or

$$\% \text{ response} = \frac{\text{MePV Expt 1} - \text{MePV Expt 2}}{\text{MePV Expt 3} - \text{MePV Expt 4}} \times 100$$

2.11 RESULTS

Significant ($P<0.01$) heterologous desensitization (see Chapter 1, section 1.13) was induced by all agonist studied except for ADP, which did not significantly diminish the 5-HT response. In some subjects (3/7), ADP induced desensitization but in others (4/7) it did not. This variability was not observed with the other agonists studied (see ranges in Table 2.13). Homologous desensitization was induced by 5-HT and ADP, but 5-HT was more powerful than ADP at inducing a desensitization effect (Table 2.13).

2.12 DISCUSSION

These experiments were designed to establish whether PSC desensitization can occur *in vitro* and to offer a potential explanation for the PSC responses observed in PVD (Part 2). The results show that strong desensitization was obtained with several agonists (PAF, AA and U 46619 as 1st agonist) when 5-HT or ADP were used as 2nd agonists. However, when 5-HT was used as 2nd agonist, the PSC response following pre-incubation

Fig 2.4

Design of experiments to assess the effect of pre-incubating an agonist (desensitizing agonist) on subsequent PSC response by the same or different stimulating agonist

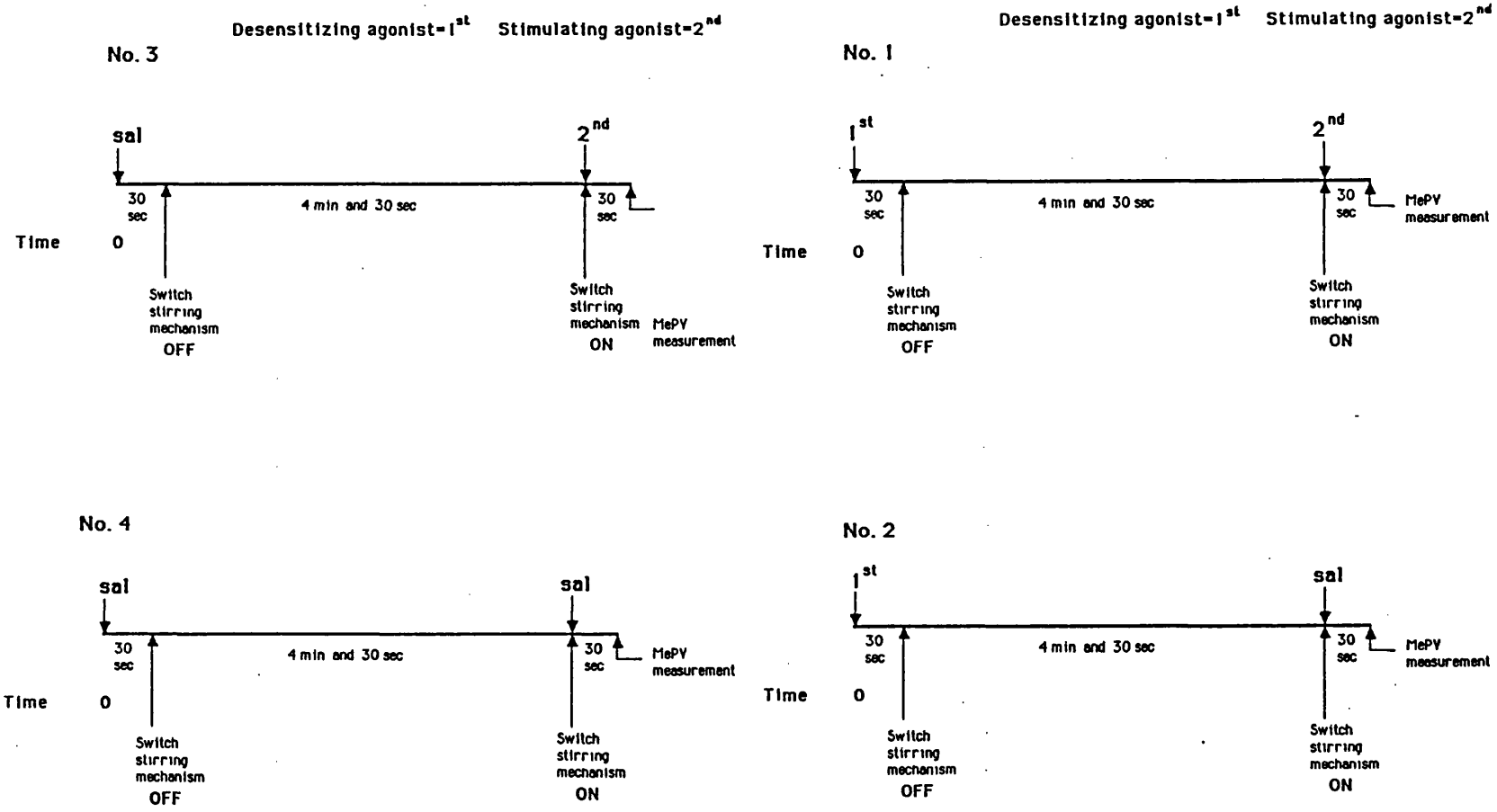


TABLE 2.13

Effect of pre-incubating an agonist (desensitizing) on subsequent PSC response by a stimulating agonist (5-HT or ADP) in healthy subjects

1st agonist	2nd agonist	% Response compared to stimulating agent alone
5-HT (1 μmol/L)	5-HT (1 μmol/L)	26% (1-63)
PAF (0.015 μmol/L)	5-HT (1 μmol/L)	2% (-40-91)
AA (0.25 mmol/L)	5-HT (1 μmol/L)	51% (-22-51)
ADP (0.3 μmol/L)	5-HT (1 μmol/L)	111% (30-159)
U 46619 (0.05 μmol/L)	5-HT (1 μmol/L)	29% (11-55)
5-HT (1 μmol/L)	ADP (0.3 μmol/L)	80% (61-87)
PAF (0.015 μmol/L)	ADP (0.3 μmol/L)	31% (-10-80)
AA (0.25 mmol/L)	ADP (0.3 μmol/L)	57% (-57-91)
ADP (0.3 μmol/L)	ADP (0.3 μmol/L)	33% (20-47)
U 46619 (0.05 μmol/L)	ADP (0.3 μmol/L)	28% (18-56)

Note: **< 100%** response suggests that desensitization occurred. **100 %** response suggests that no desensitization took place; responses **>100%** suggest that stimulation of PSC has occurred. Negative readings are obtained when the MePV obtained with the desensitizing agent added alone is greater than that obtained following the addition of both agonists and suggests that very strong inhibition has taken place.

with ADP was not significantly desensitized. These results suggest that ADP is probably a weak inducer of desensitization phenomena, vis-à-vis, PSC. It is also noteworthy that, in the PVD studies described in Part 2, ADP responses were not significantly impaired when compared to healthy controls. As a result of platelet hyperactivity it is conceivable that in PVD, ADP may, like 5-HT, be released into the plasma pool. However, due to the action of ADPases (Barradas et al., 1990c), ADP may be cleared rapidly from plasma and therefore unable to induce desensitization effects. Notwithstanding that, ADP was very effective at inducing homologous desensitization. The reason for a possible discrepancy between ADP and other agonists may also be related to the way ADP causes platelet activation or to an unknown agent able to recouple responses following ADP exposure. With regard to ADP-induced activation, unlike other agonists, this agonist has been shown to induce activation (e.g. rapid rise in Ca^{2+}) independently of phosphatidylinositol metabolism (PIP_2 conversion to IP_3; Fisher et al., 1985) or strong DG elevation (Scrutton and Athayde, 1991). Given that DG elevation may play a key role in controlling PK-C activity (Kikkawa et al., 1989) and that PK-C activation may exert negative feedback effects on PSC (Yoshida et al., 1986), ADP may not be able to induce desensitization in the same manner as other agonists that cause DG elevation. On the other hand, it is of interest that adrenaline has been shown to restore the responsiveness to thrombin but not to U46619 following thrombin or U46619-induced desensitization (Crouch and Lapetina, 1989). Thus, one

possible interpretation for the lack of effect of ADP as a desensitization agent may be due to the ability of a plasma factor to reverse the desensitization or "recouple" the responsiveness of agonists following ADP exposure. Further work is required to explore these possibilities.

Part 4

2.13 The effect of various drugs on PSC

As outlined in Chapter 1 (section 1.12), the second messengers, cAMP and cGMP, play an important role as intracellular inhibitors of platelet activation (Kroll and Schafer, 1989). In this section the effect of substances which lead to the intracellular elevation of these nucleotides (e.g. PGE_1 and NaNP), were assessed for their effects on PSC. Both of these substances have been shown to inhibit subsequent phases of platelet function (Kahn et al., 1991; Chirkov et al., 1991) at the concentrations used below. Other drugs with anti-platelet activity were also assessed for their effect on PSC. These drugs (indomethacin, ketanserin, naftidrofuryl, imipramine) are either well established drugs (indomethacin blocks TXA_2 synthesis) or are of interest due to their anti-5-HT properties (ketanserin, naftidrofuryl, imipramine). In addition, milrinone (MIL; a phosphodiesterase enzyme inhibitor) with documented anti-platelet effects was also evaluated for the first time (Barradas et al., 1993).

2.14 METHODS

All drugs were used *in vitro* at concentrations previously shown to inhibit platelet aggregation or interfere with 5-HT uptake. The drugs were incubated with PRP (collected from healthy volunteers) for 5 min prior to induction of PSC with agonists. Platelet counting and sizing analysis were carried out as described in Part 1.

Effect of cAMP elevation and cGMP elevation on PSC

To study the effect of elevating cAMP or cGMP on PSC, PGE_1 and NaNP were used. PGE_1 is a stable prostaglandin which inhibits platelet aggregation via cAMP elevation (Moncada and Vane, 1979). NaNP is an NO donor that results in an increase in platelet guanylate cyclase activity and cGMP (Chirkov et al., 1991).

Effect of cyclooxygenase and phosphodiesterase inhibition on PSC (Barradas et al., 1993)

It is well established that indomethacin (INDO) inhibits platelet aggregation by preventing the conversion of AA into TXA_2 (Blackwell et al., 1977; Barradas et al., 1989). This non-steroidal anti-inflammatory agent also inhibits cyclooxygenase more rapidly and at lower concentrations than ASA (Dahl and Uotila, 1984). To assess the effect of cyclooxygenase inhibition on PSC, INDO was therefore selected. 5-HT and AA were used as agonists of PSC. To study the effect of phosphodiesterase (PDE) inhibition, MIL a novel PDE inhibitor (selective inhibitory activity on PDE type-III, an

isoenzyme found in cardiac muscle which hydrolyses cAMP), was selected. In addition, the combined effect of MIL and INDO was investigated to determine whether an additional benefit could be obtained by cyclooxygenase inhibition. It is worth noting that MIL was developed for the treatment of heart failure and such patients may be taking ASA.

Effect of 5-HT_2 antagonists and a 5-HT uptake inhibitor on PSC (Barradas et al., 1990d, 1992c,d)

In this study NAF, a 5-HT_2 antagonist (Zander et al., 1986; Davies and Steiner, 1988) was selected in order to assess whether the PSC methodology could detect inhibitory effects at concentrations below those required to inhibit platelet aggregation. Previously, very high concentrations (50-100 μmol/L; well above peak therapeutic levels: approx. 6 μmol/L) of NAF had been required to inhibit 5-HT-induced platelet aggregation (Davies and Steiner, 1988). Ketanserin was chosen as a blocker of 5-HT_2 receptors (McBride et al., 1987; Vanags et al., 1992) and to determine the relative potency of NAF.

2.15 RESULTS

Effect of cAMP elevation and cGMP elevation on PSC

PGE_1 significantly ($P<0.01$) inhibited PSC induced by 5-HT. The PGE_1 concentrations (30 nmol/L and above; Kahn et al., 1991) required are comparable to those required to raise intraplatelet cAMP concentration and inhibit aggregation induced by ADP. NaNP at concentrations (1 μmol/L and above)

that raise cGMP and inhibit platelet aggregation (Chirkov et al., 1991) significantly ($P<0.01$) inhibited 5-HT-induced PSC (Table 2.14 and 2.15).

Effect of cyclooxygenase and phosphodiesterase inhibition

5-HT-induced PSC was not affected by INDO concentrations (16 μmol/L) previously shown to inhibit platelet aggregation *in vitro* (Mikhailidis et al., 1985). In contrast, AA-induced PSC was markedly inhibited (Figure 2.5).

MIL inhibited significantly ($P< 0.01$) agonist-induced PSC in a dose dependent manner (Figure 2.6). A range of agonists were selected to demonstrate the effects of PDE inhibition on a variety of pathways which lead to PSC. With some agonists (CaI, AA, collagen) MIL was potent at concentrations (IC_{50} : 0.05-0.1 μmol/L) considerably lower than the therapeutic concentrations (approx. 1.5 μmol/L; arrow on Figure 2.6; Multi-Authors, 1991). Higher concentrations of MIL were required to inhibit PSC induced by other agonists: U46619, ADP and 5-HT (IC_{50}s: 0.5, 1.0 and 2.5 μmol/L, respectively). In experiments designed to study the effect of the combined addition of MIL and INDO, concentrations of INDO which block cyclooxygenase (16 μmol/L, Mikhailidis et al., 1985) effectively inhibited AA-induced PSC (Figure 2.5). MIL (0.15 μmol/L) potentiated the inhibitory effect of INDO and abolished AA-induced PSC (Figure 2.5). 5-HT-induced PSC was not affected by INDO and there was no enhancement of the inhibitory effect exerted by MIL alone (2.5 μmol/L; Figure 2.5).

Fig 2.5

Effect of INDO and MIL on 5-HT- and AA-induced PSC

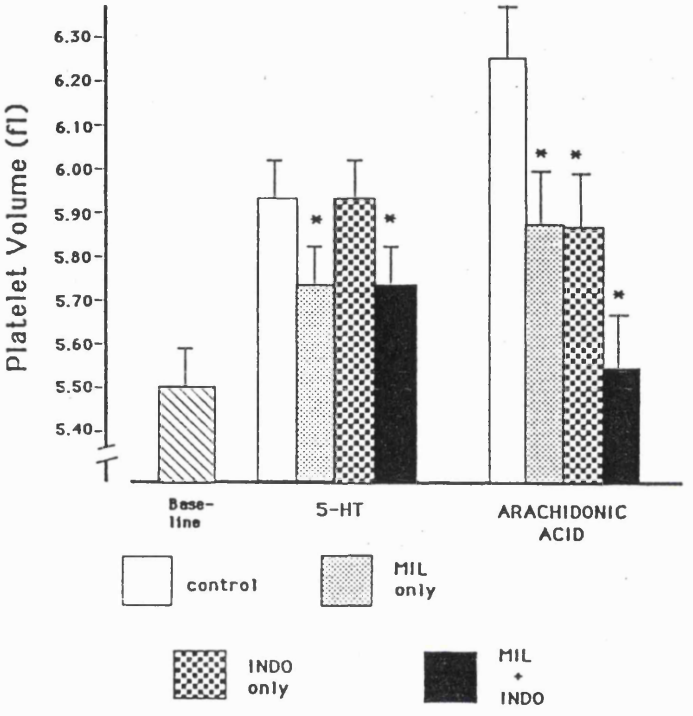


Fig 2.6

Effect of MIL on PSC induced by various agonists

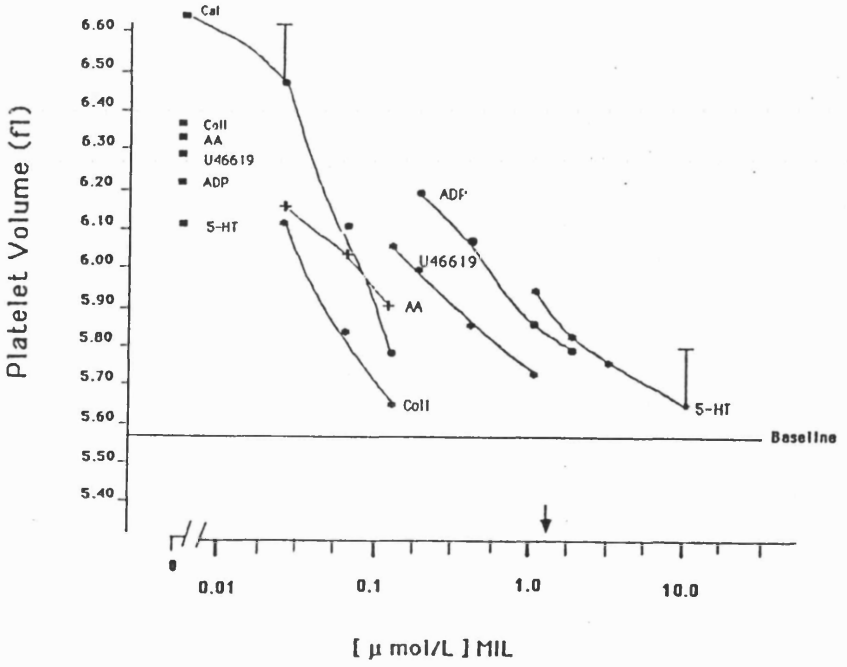


TABLE 2.14

Effect of PGE_1 on 5-HT-induced PSC

Saline	5-HT only	PGE_1 (1.41 nmol/L) + 5-HT	PGE_1 (2.82 nmol/L) + 5-HT	PGE_1 (14.1 nmol/L) + 5-HT	PGE_1 (28.2 nmol/L) + 5-HT
5.56 (5.30-5.75)	5.98^{+} (5.60-6.20)	5.93^{+} (5.60-6.07)	5.85$^{*,+}$ (5.49-5.94)	5.65* (5.40-5.88)	5.58* (5.30-5.82)

* P<0.01: PGE_1+5-HT Vs. 5-HT only

+P<0.01: Vs. saline (vehicle)

Results are expressed in fl.

number of subjects studied=7.

TABLE 2.15

Effect of NaNP on 5-HT-induced PSC

Saline	5-HT only	NaNP (0.1 μmol/L) + 5-HT	NaNP (1.0 μmol/L) + 5-HT	NaNP (10 μmol/L) + 5-HT	NaNP (100 μmol/L) + 5-HT
5.37 (5.30-5.85)	5.88^{+} (5.60-6.13)	5.85^{+} (5.60-6.12)	5.69$^{*,+}$ (5.55-5.98)	5.40* (5.37-5.92)	5.37* (5.30-5.85)

* P<0.01: NaNP+5-HT Vs. 5-HT only

+P<0.01: Vs. saline (vehicle)

Results are expressed in fl.

number of subjects studied=7.

Effect of 5-HT_2 antagonists and 5-HT uptake inhibitor on PSC

The effect of NAF and KET on 5-HT- and ADP-induced expansion in PSC was assessed. KET was a more potent inhibitor of 5-HT-induced PSC (IC_{50}=0.05 μmol/L) than NAF (IC_{50}=3.0 μmol/L) (Figure 2.7). Therapeutic concentrations of KET and NAF are: KET= 0.1-0.2 μmol/L (De Cree et al., 1993) and NAF 1-5 μmol/L (Davies and Steiner, 1988). By using a combination of 5-HT and ADP to induce PSC, NAF at concentrations as low as 1.56 μmol/L significantly (P<0.01) inhibited PSC (Table 2.16).

TABLE 2.16

Effect of NAF (1.56 μmol/L) on PSC induced by 5-HT (0.01 μmol/L) plus ADP (0.1 μmol/L)

Baseline	5-HT only	ADP only	5-HT + ADP	NAF + 5-HT+ADP
5.37 (4.72-5.94)	5.49 (4.72-6.00)	5.49 (4.72-6.00)	5.75+ (4.85-6.40)	5.69* (4.66-6.14)

* P<0.01: NAF+5-HT+ADP Vs. 5-HT+ADP

+P<0.01: 5-HT+ADP Vs. Baseline

Results are expressed in fl.

number of subjects studied=7.

The two 5-HT_2 antagonists (KET and NAF) had similar potencies when PSC was induced by ADP (Table 2.17). The concentration of NAF and KET needed to inhibit ADP-induced PSC was several fold greater than that required to inhibit 5-HT, confirming the specificity of these anti-5-HT agents.

Fig 2.7

Effect of NAF and KET on 5-HT-induced PSC

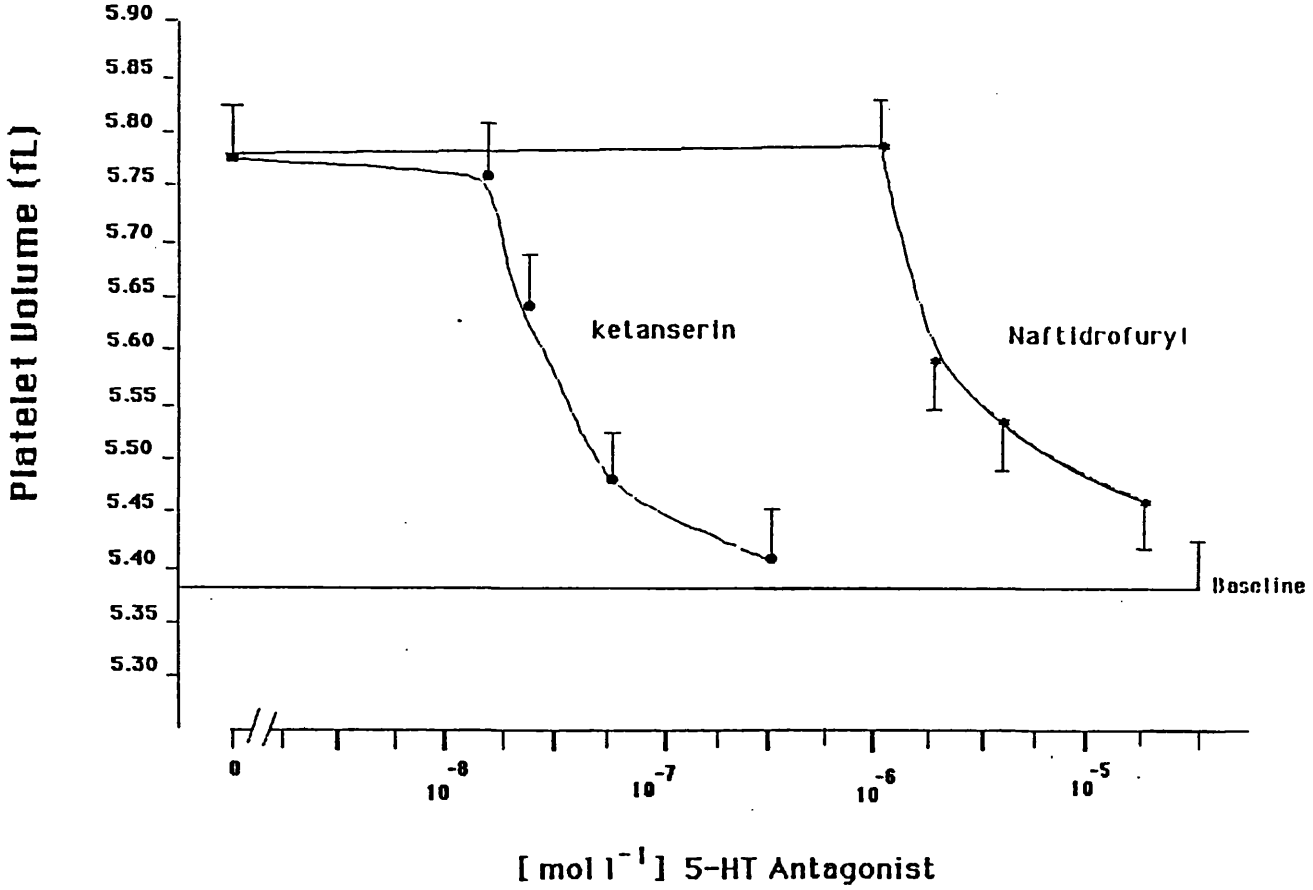


TABLE 2.17

Effect of NAF and KET on ADP-induced PSC

The concentration of ADP was 0.4 μmol/L. Concentrations of antagonists are 100-200 μmol/L.

		ADP+NAF		ADP+KET	
Saline only	ADP only	100	200	100	200
5.56 (4.72-5.94)	6.46 (5.94-6.84)	6.01 (5.43-6.464)	6.04* (4.98-6.46)	6.14* (5.08-6.72)	6.01* (5.11-6.46)

Results are expressed in fl.

*P< 0.01: ADP (NAF) or ADP (KET) Vs. ADP alone.

number of subjects studied=7.

The effect of imipramine (a potent 5-HT uptake inhibitor; Wielosz et al., 1976) on 5-HT induced PSC was investigated to determine whether 5-HT uptake inhibition had any influence on PSC. No effect was detected (Table 2.18).

TABLE 2.18

Effect of Imipramine (IMI) on 5-HT-induced PSC

	Basal MePV (fl)	IMI 1 μmol/L	5-HT 0.1 μmol/L	IMI + 5-HT
Healthy Subjects n= 4	5.43 (4.60-5.75)	5.43 (4.60-5.78)	5.62 (5.11-5.94)	5.61 (5.11-5.88)

For Basal MePV, samples were treated with saline only (vehicle for imipramine).

2.16 DISCUSSION

Effect of cAMP elevation and cGMP elevation on PSC

The measurement of PSC has not been widely used to assess the effect of drugs on platelets. This aspect of platelet function, may nevertheless be of physiological importance. Furthermore, PSC allows the assessment of platelet changes preceding platelet aggregation. Using the method reported in Part 1, both cAMP elevation (via PGE_1) and cGMP elevation (via NaNP) inhibited PSC induced by 5-HT. The concentrations of PGE_1 and NaNP were similar to those required to inhibit platelet aggregation. In the context of platelet suppressive effects, it is perhaps not surprising that cAMP elevation leads to the inhibition of PSC since cAMP influences agonist-induced signal transduction mechanisms (e.g. G-proteins) and exerts inhibitory effects on phospholipase C and phospholipase A_2 activity (Kroll and Schafer, 1989). Although less is known about the way cGMP mediates inhibitory effects on platelets (e.g. an effect on PL-C has been suggested; Matsuoka et al., 1989), it is well established that raising intracellular cGMP concentrations leads to diminished aggregability (Chirkov et al., 1991).

Effect of cyclooxygenase and phosphodiesterase inhibition

Inhibition of PSC following cyclooxygenase inhibition depends on the agonist used and its capacity to synthesize TXA_2. Thus, PSC was inhibited by INDO when induced by AA but not when induced by 5-HT. Interestingly, significantly more inhibitory effect on PSC induced by AA was observed when INDO

and MIL were co-incubated than when either of these drugs was incubated alone. There was no enhancement when both MIL and INDO were added together following 5-HT-induced PSC. These results can be rationalised on the basis that despite adding MIL, the synthesis of TXA_2, itself a powerful excitatory agonist, may oppose the increase in intracellular cAMP (Crawford and Scrutton, 1987) resulting from MIL action. INDO (or cyclooxygenase inhibition) may therefore enhance the inhibitory effects of MIL by preventing TXA_2 availability. The extent of the inhibitory effect exerted by MIL was found to depend on the agonist used. Thus, MIL inhibited AA-induced PSC at concentrations below those achieved during treatment with this drug (1.5 μmol/L) but higher concentrations of MIL were required for a similar degree of inhibition when PSC was induced by 5-HT. Since each agonist transduces its activatory effects via different receptors and biochemical pathways, this suggests that MIL has stronger inhibitory effects on certain pathways leading to platelet activation than on others. This is also in agreement with the conclusion that PDE inhibitors, in addition to reducing cAMP phosphodiesterase activity, also exert other effects on intracellular and extracellular mechanisms which influence platelet activation (Lippton et al., 1985; Jackson et al., 1989; Block et al., 1990; Ozin et al., 1992; Jeremy et al., 1993). Cyclooxygenase inhibitors, such as ASA, although prescribed regularly for IHD have a limited efficacy. On the basis of the above findings, these drugs may be of greater benefit when prescribed in conjunction with MIL or other PDE inhibitors. It would also appear that

MIL, and probably other PDE inhibitors, may be effective at making hyperactive platelets more quiescent. It remains to be shown whether MIL can normalize platelet hyperaggregability and reduce the incidence of thrombosis in disease states associated with platelet hyperaggregability.

Effect of 5-HT_2 antagonists and a 5-HT uptake inhibitor on PSC

The superior sensitivity of the above technique for measuring anti-platelet effects is illustrated by the inhibitory capacity of NAF on PSC as compared to previously reported results for platelet aggregation. Thus, concentrations of NAF of 1.56 μmol/L significantly inhibited PSC whereas, previously, NAF concentrations of 50-100 μmol/L were required to inhibit platelet aggregation (Davies and Steiner, 1988). Peak therapeutic levels of NAF (3.125 μmol/L) also inhibited 5-HT-induced increases in MePV, whereas NAF concentrations as high as 100 μmol/L were required to inhibit ADP-induced increases in MePV. This 33:1 ratio contrasts with a 2:1 ratio when the capacity of NAF to inhibit PRP platelet aggregation induced by these two agonists was compared (Davies and Steiner, 1988). These findings indicate that measuring PSC may be a more sensitive method than PRP aggregation for defining the ability of drugs to block receptors. This advantage may be due to differences in the processes involved in PSC. For example, intracellular calcium mobilization required for PSC may not be as great as that required for platelet aggregation (Scrutton and Athayde, 1991). The low concentrations of agonist required to increase MePV would also

favour reversal by lower antagonist concentrations. The difference in agonist concentrations required to induce PSC and platelet aggregation is especially evident with 5-HT. Thus, 5-HT concentrations of 0.1-1.0 μmol/L induce PSC whereas concentrations of the order of 10 μmol/L are needed to induce aggregation in PRP (Vinge et al., 1988). The conclusion that PSC measurement can be a sensitive indicator of antagonist specificity is also supported by the observation that KET, a relatively specific 5-HT_2-antagonist, was a more potent inhibitor of 5-HT-induced PSC than NAF. However, both NAF and KET were equipotent at inhibiting ADP-induced PSC. KET concentrations that effectively inhibit PSC and PRP aggregation (Vinge et al., 1988) were similar (0.04- 0.08 μmol/L). This suggests that for more specific inhibitors, the increased sensitivity of PSC, compared with PRP aggregation, may not be as evident.

CHAPTER 3

PLATELET AGGREGATION STUDIES

CHAPTER SUB-INDEX

3.1 INTRODUCTION

As described in Chapter 1, platelet aggregation was originally quantified in platelet rich plasma (PRP) using the Born-type aggregometer (Born, 1962; O'Brien, 1962). This technique has been in use for more than three decades but it is limited because it cannot take into account the interaction between platelets and the other cells present in blood. This restriction is a considerable disadvantage since there is evidence that both erythrocytes and leucocytes influence various aspects of platelet function (Valles et al., 1991; Marcus and Safier, 1993). In addition, there is evidence that certain drugs (e.g. dipyridamole and calcium antagonists) require the presence of other blood cells before their effects on platelets are fully manifested (Gresele et al., 1983; Jeremy et al., 1985a). An important consideration in favour of WB methodology was the possible loss of the most active platelets during the centrifugation procedure required for PRP preparation (Barradas et al., 1989). This loss of platelets may account for the conflicting results obtained in PVD studies where platelet aggregation was assessed with PRP aggregometry (see Chapter 1, section 1.22). In this study, it was decided to establish whether the centrifugation procedure, during PRP preparation, leads to a disproportionate loss of platelets in PVD patients when compared to younger healthy subjects. If this is so, then clearly PRP-based methods should be re-considered in favour of WB methodology. There are two main techniques currently used to assess platelet aggregation in WB. These methods, as outlined in Chapter 1, are either

based on the measurement of the platelet free count (WB-FPC) or on impedance changes quantified by the whole blood impedance aggregometer (WB-IA). In the present study, platelet aggregation (in whole blood) was assessed by the WB-FPC method after considering the WB-IA method. The above experiments constitute Part 1 of this Chapter. Having established a WB-FPC aggregometry method, this project proceeded with the assessment of WB aggregation in PVD patients (Part 2).

The use of anti-platelet drugs, in particular ASA, is widespread in IHD and PVD. However, the benefit of taking these drugs in PVD has not been defined (Fuster et al., 1993). Nevertheless, it is worth remembering that ASA has been shown to reduce the incidence of re-infarction and curtail the need for amputations in PVD (Goldhaber et al., 1992). In the context of 5-HT, increased platelet activity may lead to an increased availability of 5-HT to the vasculature and to blood cellular elements which in turn may increase the risk of tissue ischemia. Given this scenario, drugs with platelet anti-aggregatory properties, including 5-HT blocking properties, would be expected to exert a beneficial effect. In this thesis, WB aggregation was assessed in patients taking low dose ASA (150 mg on alternate days) and a number of *in vitro* studies were set up to assess the effect of ASA and/or NAF (a 5-HT_2 antagonist), vis-à-vis, platelet aggregability. Milrinone, a novel PDE inhibitor which inhibits PSC (Chapter 2) and with potential anti-thrombotic action, was evaluated in WB collected from healthy subjects. The studies with ASA, INDO, NAF and MIL constitute Part 3 of this Chapter.

Part 1

3.2 WB-IA and WB-FPC aggregometry

Previous studies carried with WB-IA illustrated the advantages of using whole blood methodology. However, using this method the following was also established: 1) high concentrations of ADP (5 μmol/L and above) and collagen (1 mg/L and above) were required to induce appreciable aggregation (Jeremy et al., 1985a; Barradas et al., 1987b; Greenbaum et al., 1987; Whitworth et al., 1989; Barradas et al., 1990a); b) adrenaline-induced aggregation was rarely observed, except in hyperaggregable states (Greenbaum et al., 1987); c) PAF and spontaneous platelet aggregation were not detected in WB-IA. PAF-induced WB-IA is apparently observed only in "high responders" (Groscurth et al., 1988).

In the context of WB-IA and WB-FPC, only data obtained with respect to 5-HT as platelet agonist is presented and discussed below.

3.3 METHODS

A) Whole-Blood Impedance Aggregometry (WB-IA)

Blood anticoagulated with tri-sodium citrate was collected from 6 healthy volunteers (3 males; 3 females), median age and (range): 26 years (20-52). The objective of these experiments was to assess aggregation to 5-HT. WB-IA was carried out in a whole blood Chronolog aggregometer (model 540) linked to a chart recorder. In order to stabilize the baseline readings and increase the total impedance change, physiological saline (150 mmol/L NaCl) was added to citrated

whole blood (2 volumes saline : 8 volumes citrated blood). This reduced the haematocrit, for citrated blood, from median and (range) 0.375 (0.45-0.37) to 0.290 (0.28-0.32) for the saline-diluted blood and helped considerably to give steeper and less erratic tracings (Mackie et al., 1984; Riess et al., 1985). Using this apparatus, aggregation is quantified in impedance units (Ohms). Impedance is defined as the resistance to the flow of an electric current. An increase in impedance is generated as the platelets (and possibly other cells) are deposited on the platinum electrodes. Impedance is quantified at the end of the aggregation procedure by estimating the size of the curve-deflection obtained and comparing it to a standard 5 Ohm deflection (generated automatically by pressing the "set baseline" button before the addition of agonists).

B) Whole-Blood Platelet Free Count (WB-FPC) Aggregometry (Barradas et al., 1992b)

WB-FPC was assessed using a Coulter model T-890 whole blood counter.

Prior to the addition of 5-HT, blood was pre-incubated for 1 min with a teflon-coated magnet spinning at 1,000 rpm, at 37°C, in a plastic cuvette placed in the same aggregometer that was used for WB-IA studies. By using this aggregometer, identical spinning and temperature conditions were achieved. The number of healthy subjects tested was 10 (5 male, 5 female; median age : 28 years and range: 20-55). The concentrations of 5-HT used are shown in Table 3.1. Samples were withdrawn from the cuvettes using a pipette with a

plastic tip. The time between removing the blood from the aggregometer and aspiration into the blood counter was minimised (<10 sec) so as to prevent disaggregation occurring upon standing. The timing of sampling from the aggregometer is shown in Table 3.1. Using this method, platelet aggregation was calculated on the basis of the number of free platelets remaining after agonist addition and expressed as a percentage of the basal platelet count; thus,

$$\% \text{ WB-FPC} = \frac{\text{Platelet count after adding agonist}}{\text{Basal platelet count}} \times 100$$

The basal count was defined as the count obtained 15 sec after commencing spinning of WB and before the addition of any agonist.

WB-FPC was recorded at 1, 3, 6 and 15 min after adding the appropriate aggregating agent (up to 10 μl volumes). These times were selected because, in our experience, 3 min is usually the time required for maximal aggregation to occur in PRP; 15 min was an arbitrarily selected time beyond which it was felt the methodology would become less practical. In previous work using WB-IA, (Jeremy et al., 1985a; Barradas et al., 1987b; Greenbaum et al., 1987; Whitworth et al., 1989; Barradas et al., 1990a) 3 and 6 min were selected as time points for estimating platelet (impedance) aggregation (Gresele et al., 1983; Mackie et al., 1984; Riess et al., 1986; Groscurth et al., 1988; Sweeney et al., 1989; Elwood et al., 1990; Pattison et al., 1990). All WB aggregation studies were completed within 2 hr of sample collection. Blood was kept at 37°C throughout the study since platelet function may

be influenced by cooling (Mikhailidis et al., 1983a).

B) Effect of 5-HT on WB-FPC:

WB-FPC following the addition of 5-HT was assessed. Concentrations of agonist used and the results obtained are shown in Table 3.1. The objective of these experiments was to compare the results obtained with those reported for WB-IA methodology (see above A).

C) Statistical analysis and presentation of results

Results in Tables 3.1-3.3 are expressed as median and (range). In Figures 3.1-3.7 results are presented as individual values in scatter diagrams with medians shown as solid bars. For diagrammatic purposes certain results are presented as means and ± SEM. Thus, in Figures 3.8 and 3.9 only the largest SEM bars (±) are shown. For statistical analysis, a computer program (C-STAT) was used. Paired values were compared using the Wilcoxon rank sum test (two-tailed). Unpaired values were analyzed using the Mann-Whitney U-test for non-parametric data (two-tailed).

3.4 RESULTS

A) Whole blood platelet impedance aggregometry (WB-IA)

WB-IA to 5-HT was undetectable in blood obtained from healthy subjects (n=5). Thus, median and (range) impedance (in Ohms) 6 min following the addition of 10 μmol/L 5-HT: 0 (0-1); 50 μmol/L 5-HT: 0 (0-1). It is noteworthy that using this technique, previous experiments with the same apparatus,

revealed adrenaline WB-IA only in patients with IHD (Greenbaum et al., 1987) and no spontaneous platelet aggregation (SPA) was detected.

B) Whole blood platelet free count aggregometry (WB-FPC)

It was important to establish if agonists under the same experimental conditions as those used for WB-IA gave similar results when assessed by WB-FPC aggregometry. This study, described in Barradas et al. (1992b), showed that there was no significant difference between the aggregation responses obtained with samples assessed undiluted or diluted saline and with fixative and without fixative.

C) Effect of 5-HT on WB-FPC:

Blood was collected from 6 healthy volunteers (median age and (range): 24 years (20-55); 3 males; 3 females). Basal platelet counts were median: 236; range: 137-292 $x10^9$/L. Results are shown below, in Table 3.1. WB-FPC aggregation to 5-HT (5 μmol/L) was found to reach a maximum 30 sec after agonist addition with a return towards baseline at 1 min. These results are shown below (Figure 3.4).

TABLE 3.1

WB-FPC aggregation in WB

AGONIST	SAMPLING TIME (min)			
	1	3	6	15
5-HT 0.1 μmol/L n = 5	65 (31-93)	80 (65-101)	84 (63-98)	44 (42-69)
5-HT 1.0 μmol/L n = 6	48 (35-76)	76 (54-90)	66 (53-84)	62 (45-72)
5-HT 10 μmol/L n = 6	74 (21-98)	76 (21-96)	67 (32-86)	56 (28-70)

WB-FPC is expressed as a percentage of the basal platelet count (measured 15 sec after commencing spinning and before adding any agonist). Basal WB-FPC=100% (for actual value-see text). Number of subjects tested = n.

D) Reproducibility of the WB-FPC method

The intra-assay variation for platelet counts at 300 x 10^9/L (basal counts) (n=10) was 4% and 18% for platelet counts at 30 x 10^9/L following 10 μmol/L (n=10) ADP. Reproducibility of SPA was also shown to be good with no significant differences between the initial WB-FPC and the same measurement 2 hr later (Barradas et al., 1992b).

3.5 DISCUSSION

WB-IA depends on platelet aggregates attaching to platinum electrodes before an impedance change is registered. "Weak" agonists, however tend to form many small reversible aggregates which never reach the critical size to ensure an

impedance change (Sweeney et al., 1989). Particle counters, on the other hand, measure aggregate formation and because time of sampling can be selected, platelet aggregation can be assessed before platelet clumps disaggregate. Thus, platelet counting techniques are ideally suited to detect the effect of "weak" agonists or phenomena such as SPA.

Previous experience with WB-IA demonstrated that high concentrations of agonists are required before detectable WB-IA is achieved. In addition, adrenaline-induced WB-IA was only observed in IHD patients following the injection of unfractionated heparin, an established stimulator of platelet aggregation (Greenbaum et al., 1987).

The results presented in this thesis show that WB-IA cannot detect aggregation induced by 5-HT whereas WB-FPC aggregometry is able to do this reproducibly. The inability of WB-IA to detect aggregation to agonists such as PAF and SPA, as well as the requirement for high concentrations of other agonists (e.g. collagen) are serious disadvantages (Barradas et al., 1992b).

As outlined in Chapter 1, 5-HT release from activated platelets has been proposed as an important mediator of enhanced platelet and vascular reactivity in conditions associated with atherosclerosis. Clearly, in order to assess the role of platelets and associated mediators in disease states associated with atherosclerosis, platelet aggregation responses to such mediators should be assessed and investigated with a method with optimum sensitivity. Despite the reservations regarding WB-IA outlined above, this

technique was used by Elwood and colleagues (1990) to demonstrate a significant association between ADP-induced WB-IA and IHD. In some circumstances, and with particular agonists, WB-IA is clearly a useful method and may complement other techniques to assess platelet function.

Part 2

3.6 Platelet aggregation in patients with PVD

A reason for abandoning PRP-based optical aggregometry was the widely held view that the centrifugation procedure required for PRP preparation might be associated with the loss of platelets. Furthermore, in hyperaggregable conditions, this centrifugation may affect platelet function in a variable manner resulting in enhanced, diminished or unchanged platelet aggregation. The effect of spinning on platelet yield and function was, therefore, assessed in PVD patients and healthy subjects.

3.7 METHODS

A) Selection of PVD patients

The patients selected were attending the out-patients vascular clinic at the Royal Free Hospital (RFH). These patients had a diagnosis of PVD based on a history of intermittent claudication and an ankle:arm systolic blood pressure ratio below 0.85. In all studies, patients were in a metabolically and clinically stable condition with no history of recent cardiac events, strokes, transient ischaemic attacks or changes in their claudication distance. These patients had

not undergone any recent surgery, angiography or angioplasty. The age, sex, drug history and other known pathology for each patient group is outlined below under each study section. Informed consent was obtained from all patients and healthy subjects before blood sampling.

B) Selection of healthy subjects

Healthy subjects were laboratory and clinical staff. Younger healthy subjects than our patients were selected in this study and in other recent studies (Splawinska et al., 1992; Catalano et al., 1991). It is well documented that there are a number of platelet function changes, including increased platelet aggregation with increasing age in "healthy" subjects (Johnson et al., 1975; Zahavi et al., 1980; Gleerup et al., 1988). These changes may be due to sub-clinical atherosclerosis which is likely to be present with increasing age. However, enhanced aggregability is not always observed with increasing age and may depend on dietary and other factors (Renaud et al., 1991). Platelet counts do not appear to vary with age (Hamilton et al., 1974). In this study, we selected a younger healthy control group since these subjects are more likely to reflect "normal" platelet function. It is appreciated that by using this approach the "age factor" cannot be taken into account.

Sex and age details are provided in each study section. Subjects denied taking medication for 2 weeks prior to sampling or having a history of a major illness.

C) Effect of centrifugation on PRP platelet yield and PRP platelet aggregation

For this study, 10 healthy subjects were selected (5 males; 5 females). Median age and (range) was 32 (20-58) years. 10 PVD patients, of which 8 were males and 2 were females, volunteered for this study. The median age and (range) of the patients was 68 (52-83) years. Only patients that were drug-free were selected for this study.

D) Blood sampling and processing

Blood was withdrawn from patients and healthy subjects between 14.00 and 16.30 h. The blood was collected from an ante-cubital vein with minimum stasis using a G-21 butterfly needle. The first 2 ml of blood were discarded. Blood (9 ml) was added to plastic tubes containing different anticoagulant (1 ml) solutions. The anticoagulant solutions and their final concentrations were: tri-sodium citrate (0.38%; solution A); tri-sodium citrate plus ASA (0.38% + 1 mmol/L; solution B); di-sodium EDTA (5 mmol/L; solution C).

Before centrifugation, a platelet count was performed on each blood/anticoagulant solution using the T-890 counter. PRP was prepared from anticoagulated blood as described in Chapter 2 (section 2.2). The platelet count in each PRP was obtained using the T-890 blood counter.

E) PRP aggregation

PRP was kept at 37°C for 30-40 min before commencing experiments. Aliquots (495 μL) of PRP were placed in a

Chronolog aggregometer (model 540) and mixed by means of a teflon-coated magnetic stir bar (spinning at 1000 rpm). After 15 sec stirring, adrenaline (final concentration: 5 μmol/l; 5 μL aliquots) was added for 3 min. Aggregated platelets in PRP were counted as described above. Adrenaline was chosen as agonist since this agent stimulates thromboxane production and is therefore ideal to test the effectiveness of ASA in preventing platelet aggregation. Furthermore, previous work had shown enhancement of adrenaline-induced PRP aggregation following myocardial infarction, as assessed by the Born-type optical aggregometer (Mikhailidis et al., 1987). 1 mmol/L ASA was selected since this concentration is achieved in serum following therapeutic doses of ASA (Papas et al., 1991) and is not toxic to cells (Valles et al., 1991). This and higher (10 mmol/L) concentrations of ASA have been previously used by others to study platelet aggregation phenomena (Valles et al., 1991; Balduini et al., 1991). It was important to ensure effective inhibition of cyclooxygenase since lower doses of ASA (100 μmol/L), *in vitro*, have been found to be ineffective at inhibiting spontaneous platelet aggregation (SPA) or aggregation induced by agonists (e.g. ADP, PAF) in hyperaggregable states (Wu and Hoak, 1976; Norris et al., 1992).

F) Whole blood platelet aggregation in PVD patients and healthy subjects

24 healthy subjects were selected; 18 were males and 6 were females. Median age and (range) was 28 (20-58) years. 14

PVD patients, of which 11 were males and 3 were females volunteered for this study. The median age and (range) of the patients was 64 (44-83) years. Patients selected were drug-free.

G) Whole blood aggregation

Blood was collected into anticoagulant mixture A and kept at 37°C for 30-40 min. Agonists were added and at specified times (see Table 3.2 and Figures 3.4 and 3.5) aliquots were withdrawn and platelet counts obtained using the T-890 counter. SPA was assessed by spinning blood for 6 and 15 min followed by counting. WB-FPC aggregation in citrated blood is expressed as a % of the basal platelet count.

3.8 RESULTS

A) Effect of centrifugation on PRP platelet yield and PRP aggregation

As shown on Figure 3.1 median WB platelet counts were raised in PVD patients but this did not achieve statistical significance. PRP preparation by centrifugation resulted in platelet yields which were significantly ($P<0.012$) lower in PVD patients than healthy subjects (Figure 3.2). The magnitude of this difference, however, depended on which anticoagulant solution was used. Thus, EDTA (solution C) and citrate+ASA (solution B) were effective at diminishing the loss of platelets during PRP preparation (Figure 3.2). EDTA was also effective at preventing adrenaline-induced platelet aggregation in PRP samples from patients and healthy subjects

(Figure 3.3). ASA was only partially effective at diminishing platelet aggregation induced by adrenaline (Figure 3.3).

B) Whole blood platelet aggregation in patients and healthy subjects

PVD patients exhibited significantly enhanced SPA and platelet aggregation to 5-HT in WB when compared to younger healthy subjects (Figure 3.4 and 3.5). Enhanced aggregation to adrenaline and ADP were observed in PVD patients (Table 3.2).

TABLE 3.2

Adrenaline and ADP-induced WB-FPC aggregation in PVD patients and in healthy subjects

	Platelet Aggregating Agent			
Patients/subjects	Adrenaline 1 μmol/L		Adenosine Diphosphate 2 μmol/L	
	Sampling times		Sampling times	
	1 min	3 min	1 min	3 min
PVD patients n= 14	31%* (9-50)	10%* (5-26)	21% (12-59)	27%* (7-69)
Healthy subjects n= 24	58% (16-87)	49% (10-76)	23% (6-69)	56% (18-90)

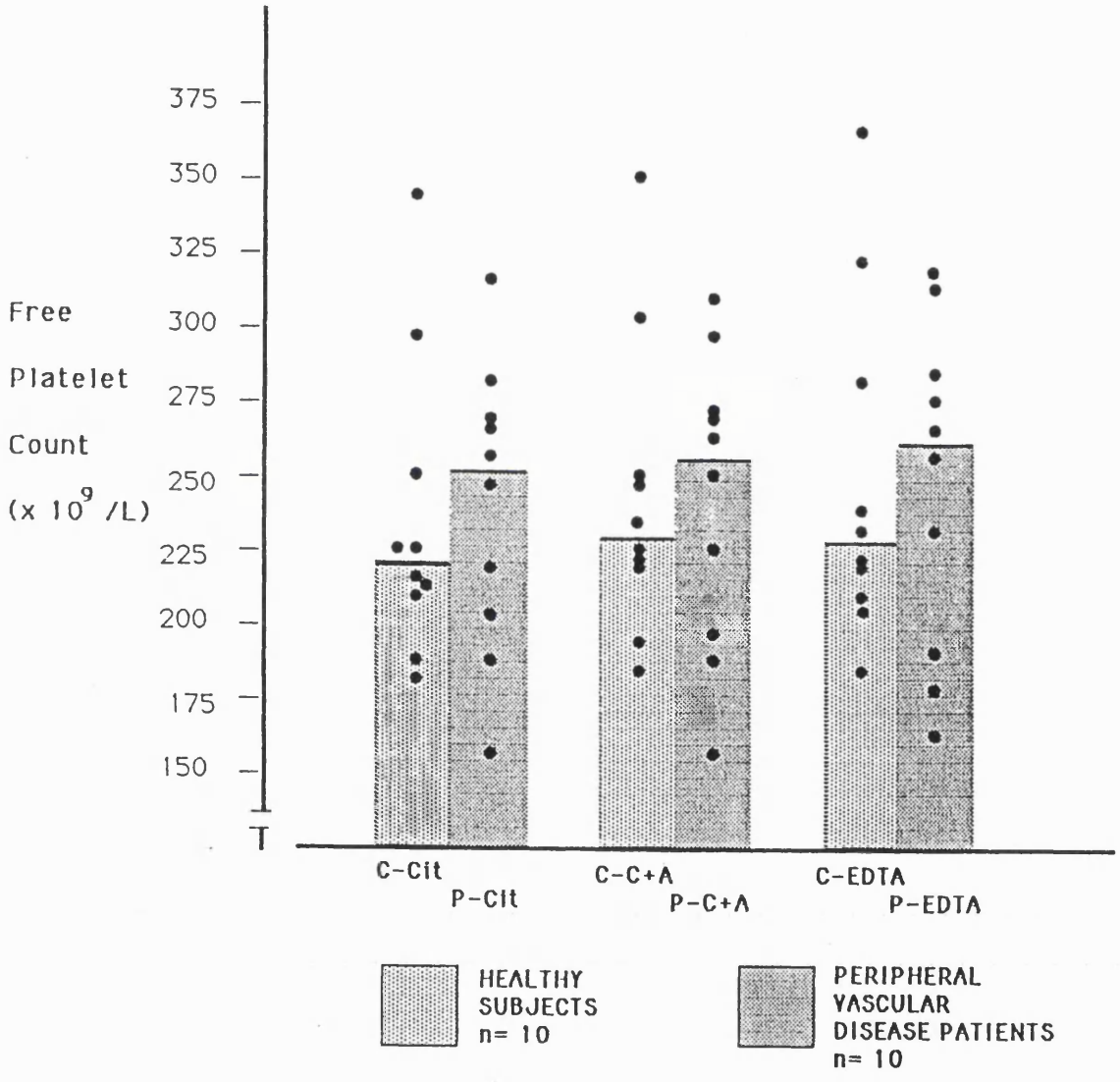
* $P < 0.015$ Vs. healthy subjects

n=number of patients/subjects studied.

Platelet aggregation is expressed as a % of the basal platelet count in whole blood. Results are presented as median and (range).

Fig 3.1

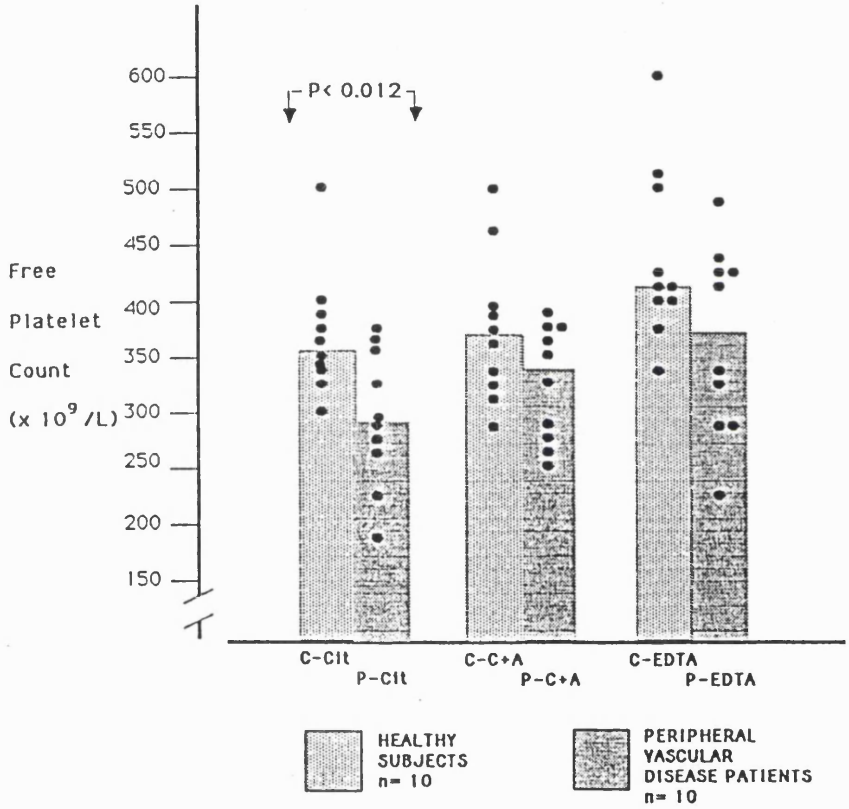
Whole blood platelet counts in healthy subjects and in PVD patients



Blood obtained from healthy subjects (C-) or from patients (P-was anticoagulated with citrate (C-Cit, P-Cit), citrate+ ASA (C-C+A, P-C+A) or EDTA (C-EDTA, P-EDTA). n=number of subjects studied. Statistical comparisons: all comparisons were not significant. Size of columns denotes value of medians. ASA= acetylsalicylic acid; PVD=peripheral vascular disease.

Fig 3.2

Platelet counts in PRP of healthy subjects and in PVD patients



PRP from healthy subjects (C-) or from patients (P-) was prepared from citrate (C-Cit, P-Cit), citrate + ASA (C-C+A, P-C+A) or EDTA (C-EDTA, P-EDTA) anticoagulated blood. n= number of subjects studied. Statistical comparisons: For healthy subjects vs patients for each anticoagulant mixture see Figure. Size of columns denotes value of medians.

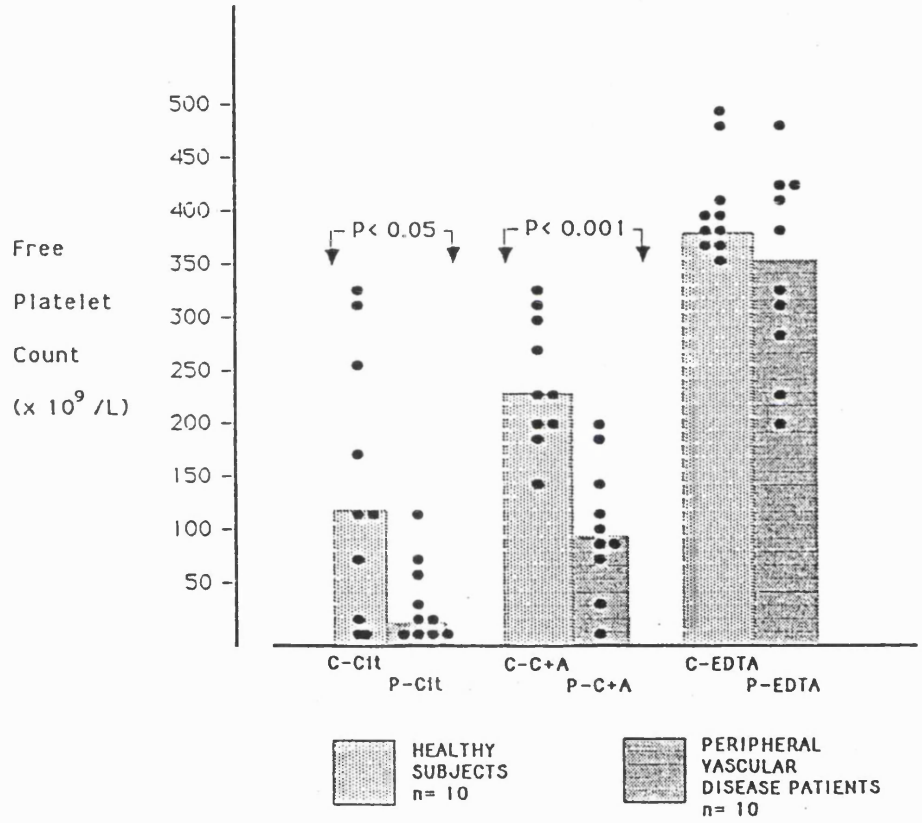
Within group analysis:

Healthy subjects: C-Cit vs C-C+A; P=0.96
C-Cit vs C-EDTA; P< 0.006,
C-C+A vs C-EDTA; P< 0.006.

PVD Patients: P-Cit vs P-C+A; P<0.04,
P-Cit vs P-EDTA; P< 0.01,
P-C+A vs P-EDTA; P< 0.04.

Fig 3.3

Platelet counts in PRP of healthy subjects and of PVD patients following treatment with adrenaline (5 μmol/L)



PRP from healthy subjects (C-) or from patients (P-) was prepared from citrate (C-Cit, P-Cit), citrate + ASA (C-C+A, P-C+A) or EDTA (C-EDTA, P-EDTA) anticoagulated blood and then treated with adrenaline. n= number of subjects studied. Statistical comparisons: For healthy subjects vs patients for each anticoagulant mixture see Figure. Size of columns denotes value of medians.

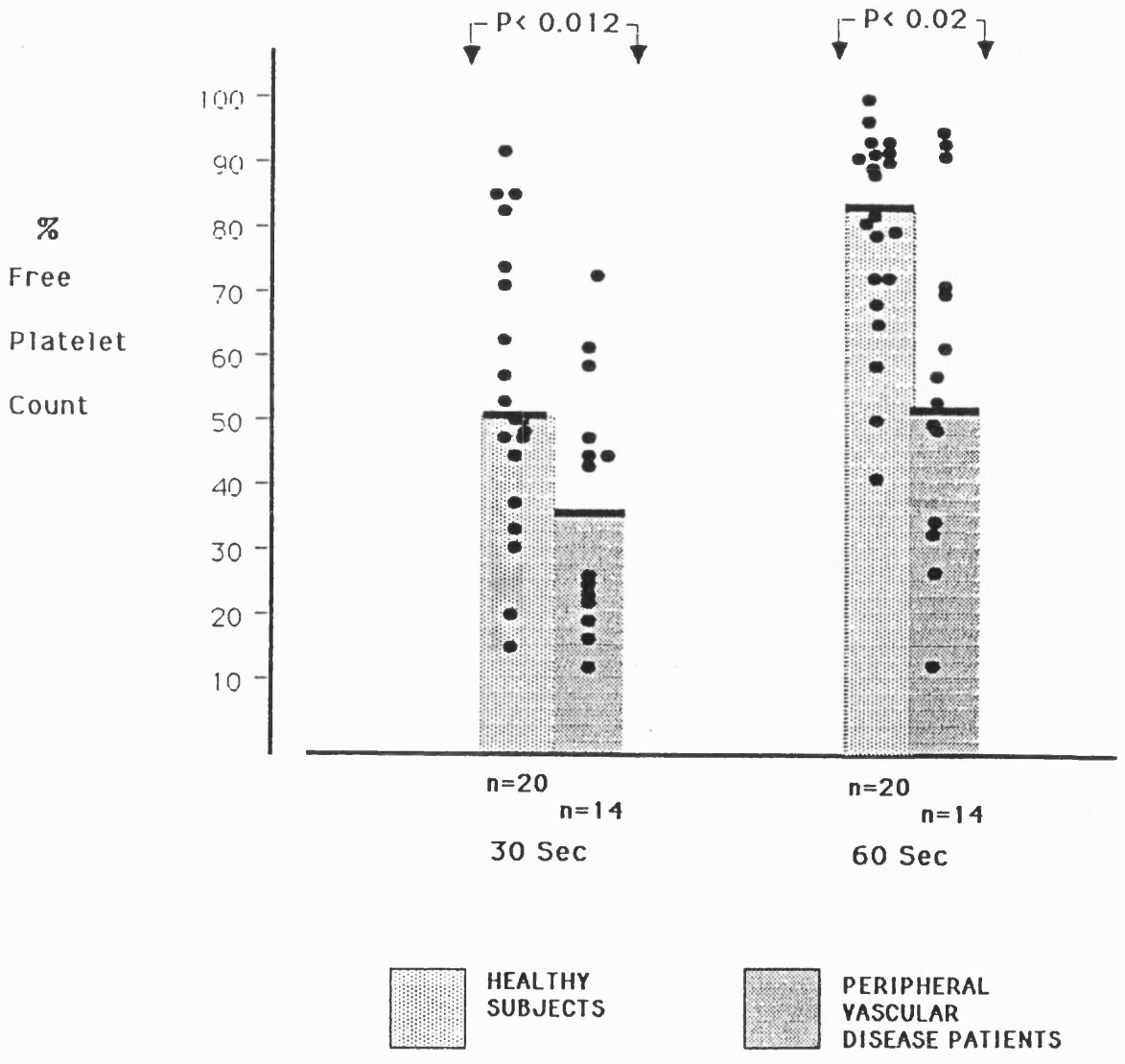
Within group:

Healthy subjects: C-Cit vs C-C+A; P< 0.02
C-Cit vs C-EDTA; P< 0.008,
C-C+A vs C-EDTA; P< 0.008.

PVD Patients: P-Cit vs P-C+A; P< 0.01,
P-Cit vs P-EDTA; P< 0.006,
P-C+A vs P-EDTA; P< 0.008.

Fig 3.4

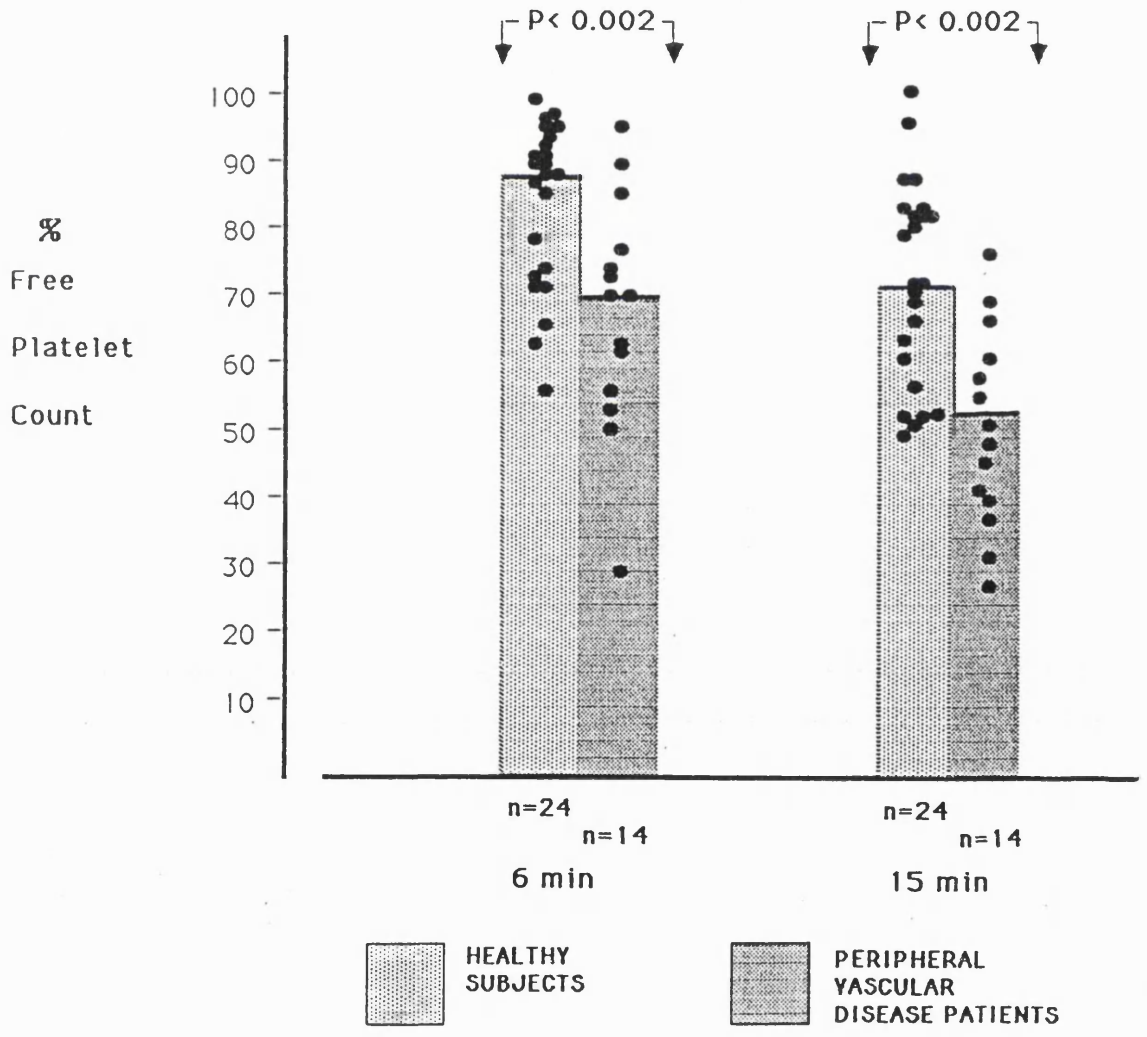
5-HT (5 μmol/L) induced platelet aggregation in WB of healthy subjects and of PVD patients



Aggregation is expressed as a % of the basal free platelet count. WB-FPC aggregation was assessed 30 and 60 sec after addition of 5-HT. n=number of subjects studied.

Fig 3.5

Spontaneous platelet aggregation in WB of healthy subjects and of PVD patients



Aggregation is expressed as a % of the basal free platelet count. WB-FPC Aggregation was assessed 6 and 15 min after starting spinning. n=number of subjects studied.

3.9 DISCUSSION

Centrifugation of WB results in a marked loss of platelets in both healthy subjects and PVD patients. The percentage loss, citrate-PRP Vs. EDTA-PRP, however, was much greater in the case of PVD platelets than younger healthy subjects (26% Vs. 14%, respectively; $P<0.012$). The platelets lost during centrifugation were probably the largest (Denfors et al. 1991) and most active (Barradas et al., 1989). The fact that the addition of ASA (a cyclooxygenase inhibitor) and EDTA (a potent calcium chelator/platelet aggregation inhibitor (Fox et al., 1982) to WB diminished the platelet loss during centrifugation suggests that PRP preparation is associated with platelet activation via cyclooxygenase-dependent (ASA-sensitive) and independent (ASA-insensitive) pathways. EDTA was more effective at preventing platelet aggregation induced by adrenaline implying that ASA intake alone by PVD patients may not provide complete protection against the platelet hyperaggregability observed in this condition. Previous work in our laboratory also reported a platelet-sparing effect when iloprost (a prostacylin analogue and a cAMP elevator/ inhibitor of platelet aggregation) was used during PVD-PRP preparation (Barradas et al., 1989). These findings suggest that the platelets obtained following preparative centrifugation for PRP-turbidimetric studies may not be representative of the *in vivo* platelet population in healthy subjects and PVD patients. Furthermore, centrifugation may result in platelet activation. This effect could contribute to enhanced platelet aggregation or result in a refractory state,

depending on the original degree of activation. These findings may explain why, in some PVD studies, enhanced PRP-platelet aggregation has been observed (De Cree et al., 1985; Mikhailidis et al., 1985; Zahavi and Zahavi, 1985) whilst in others it has not (Cella et al., 1979; Galt et al., 1991). Notwithstanding these contrasting findings which could also be related to patient selection or intake of drugs, in the present study, enhanced PRP aggregation to adrenaline was observed. This agonist has been previously found by us and others to be a sensitive agonist for the detection of hyperaggregable platelets in PRP-based studies (Mikhailidis et al., 1986; Stead et al., 1987; Grace et al., 1987; Gasser et al., 1990).

WB-FPC aggregometry revealed enhanced SPA (a predictor of myocardial infarction; Trip et al., 1990) and aggregation to adrenaline, ADP and 5-HT in PVD platelets. However, our comparisons have been made against drug-free healthy young subjects which are less likely to have sub-clinical (asymptomatic) atherosclerosis than older controls. A study involving older healthy subjects would control for the effect of age. Such a study, however, would require large numbers of controls and invasive techniques in order to assess precisely the degree of asymptomatic atherosclerosis and control for this latter parameter. Platelet aggregation to a range of agonists, has been shown to increase with age (Johnson et al., 1975; Gleerup et al., 1988, De Lorgeril et al., 1991) though this increase is quite variable from study to study. Overall, the increase in aggregability observed within healthy subjects

is not as marked as the changes reported in this Chapter between healthy subjects and PVD patients. In addition, it is worth noting that enhanced platelet aggregability does not always go hand-in-hand with increasing age and may depend on dietary and other factors (Renaud et al., 1991).

SPA and agonist-induced platelet aggregation in WB is likely to be a better predictor of ischaemic events than PRP-based methods (Trip et al., 1990) since the WB method may reflect red cell (Valles et al., 1991) as well as white cell abnormalities (Ciuffetti et al., 1989). Furthermore, there is evidence that SPA in whole blood is largely mediated by ADP released mainly from red blood cells (Fox et al., 1982; Saniabadi et al., 1991). In keeping with this interpretation is the recent finding of an association between prevalent ischemic heart disease and ADP-induced aggregation in WB (Elwood et al., 1990). In contrast, a previous study by Catalano et al. (1991) assessing platelet aggregation in WB of PVD patients reported significantly diminished aggregability to high dose ADP and similar aggregation to high dose collagen when compared to healthy subjects. These workers used WB-IA whereas we have used a WB-FPC method.

Part 3

3.10 The effect of various drugs on platelet aggregation

Evidence indicating enhanced aggregation to 5-HT in blood obtained from PVD patients prompted studies to establish whether therapeutic concentrations of NAF (a 5-HT_2 antagonist)

could inhibit aggregation induced by 5-HT and other agonists. Furthermore, the effect of NAF and ASA, added *in vitro*, was investigated in blood collected from patients who were drug free and patients that were taking low dose ASA. This is an important drug combination to investigate since many PVD patients take ASA. The effect of milrinone and indomethacin on WB-aggregation was also assessed in blood collected from healthy subjects.

3.11 METHODS

A) Effect of NAF on WB-FPC aggregation in samples obtained from drug-free PVD patients

Nine PVD patients, of which 6 were males and 3 were females volunteered for this study. The median age and (range) of the patients was 71 (52-82) years. Patients selected were drug-free. Blood sampled as above was collected into anticoagulant solution A (citrate only) and kept at 37°C for 30-40 min. NAF or vehicle (phosphate buffer pH 7.4 see Appendix ; section on **Buffers and Solutions**) were added to tubes in one of two aggregometer channels. The blood was stirred for 30 sec to ensure adequate mixing and the stirring mechanisms switched off for a further 4 min; 30 sec before commencing WB-FPC aggregation, stirring was restarted and WB-FPC carried out as described above.

B) Effect of NAF on WB-FPC aggregation following the addition of ASA in samples obtained from PVD patients who were drug-free or on ASA

Fourteen PVD patients, 11 males and 3 females, volunteered for this study. The median age and (range) of the patients was 64 (44-83) years. Patients selected were drug-free. Blood was collected into anticoagulant solution A and kept at 37°C for 30-40 min. In experiments where the effect of ASA was being evaluated, blood was collected into the anticoagulant solution B (citrate+ASA). This experimental approach has been used by others (Norris et al., 1992) to study the effect of ASA, *in vitro*, and ensures effective cyclooxygenase inhibition as assessed by agonists that depend on TXA_2 synthesis for aggregation. WB-FPC aggregation was carried out as described above.

Seven PVD patients who were on low dose aspirin (150 mg, once daily, every alternate day) were also studied. Two patients from this group were on frusemide+amiloride, and 1 patient was on isosorbide dinitrate, diclofenac and dipyridamole. One patient was a diabetic on insulin treatment. These patients were selected in order to establish whether NAF was effective in patients taking ASA and other drugs prescribed to PVD patients. Four patients were males and 3 were females. The median age and (range) of the patients was 66 (48-80) years. Blood collected into anticoagulant solution A and anticoagulant solution B was kept at 37°C for 30-40 min. WB-FPC aggregation was carried out as described above (section 3.3 B).

C) Effect of Milrinone (MIL) and Indomethacin (INDO) in blood obtained from healthy subjects (Barradas et al., 1993)

The objective of these experiments was to assess whether MIL, alone or in combination with INDO, could inhibit WB-FPC aggregation at or near therapeutic concentrations. A similar experimental approach was used to assess the effect of these drugs on PSC (see Chapter 2). MIL alone or in combination with INDO was investigated on WB-FPC. The latter drug was selected to represent non-steroid anti-inflammatory (NSAID)-like drugs with cyclooxygenase blocking properties (Blackwell et al., 1977; Dahl and Uotila et al., 1984; Mikhailidis et al., 1985).

Subjects and WB-FPC aggregation

Nine healthy subjects volunteered for this study; 6 males and 3 females. Median age and (range) was 25 (20-58) years. Blood was sampled as described above and collected into anticoagulant solution A and kept at 37°C for 30-40 min.

Milrinone (MIL) was dissolved in a saline/ethanol mixture (final concentration of ethanol less than 0.1% w/v). Experiments with the co-incubation of INDO were carried out with this drug dissolved in phosphate buffer (see Appendix , section on **Buffers and Solutions**). MIL and/or INDO (10 μl volumes) and respective vehicles were incubated with WB for 5 min, at 37°C, before the addition of agonists. WB-FPC aggregation was induced by 5-HT, ADP, U46619, adrenaline, collagen and calcium ionophore (A12387; CaI) and evaluated using the T-890 blood counter. Aggregation was estimated 1 min

after adding agonists, except for collagen (3 min) and for 5-HT (30 sec) as described above.

3.12 RESULTS

A) Effect of NAF on whole blood platelet aggregation in samples obtained from drug-free PVD patients

5-HT-induced WB-FPC aggregation was inhibited significantly ($P<0.01$) by NAF concentrations similar to those which are achieved therapeutically (i.e. 3-6 μmol/L; Davies and Steiner, 1988) (Figure 3.6). At the same concentrations of NAF, SPA and adrenaline-induced WB-FPC, however, were not affected. Thus, median and (range) % WB-FPC-SPA (6 min) control: 68 (35-85), NAF (5 μmol/L): 63 (30-92); SPA (15 min) control: 52 (28-74), NAF: 52 (26-78); adrenaline (1 μmol/L; 1 min) control: 35 (14-55), NAF: 37 (12-52); adrenaline (3 min): control: 12 (7-28), NAF: 15 (5-28).

B) Effect of NAF and/or ASA on WB-FPC in samples obtained from PVD patients

Two groups of patients were studied; patients that were drug-free and patients that were on low dose ASA and other drugs (e.g. isosorbide dinitrate, dipyridamole). In blood treated with or without ASA (obtained from patients that were drug-free) NAF at therapeutic concentrations inhibited significantly ($P< 0.04$) 5-HT-induced WB-FPC aggregation (Figure 3.7). ASA-treated WB-FPC was not less responsive to 5-HT (Figure 3.7) and did not exhibit less SPA when compared to non-ASA-treated blood. Thus, median and (range) % WB-FPC-SPA

(6 min) NO ASA : 64 (32-83), + ASA (1 mmol/L): 63 (34-92); SPA (15 min) No ASA: 55 (24-76), + ASA: 50 (23-77). These findings were also true for 5-HT-induced WB-FPC aggregation in patients that were taking low dose ASA and other drugs (see Table 3.3).

TABLE 3.3

Effect of NAF and ASA on WB-FPC in samples obtained from PVD patients on low dose ASA.

Agonist/Antagonist	Oral ASA only		Oral ASA + ASA added *in vitro*	
	Sampling times		Sampling times	
	30 s	1 min	30 s	1 min
5-HT (5 µmol/L)	48% (23-78)	65% (29-90)	54% (24-79)	68% (32-92)
5-HT (5 µmol/L) + NAF (5 µmol/L)	59%* (28-96)	71% (33-97)	62%* (27-94)	72% (36-97)

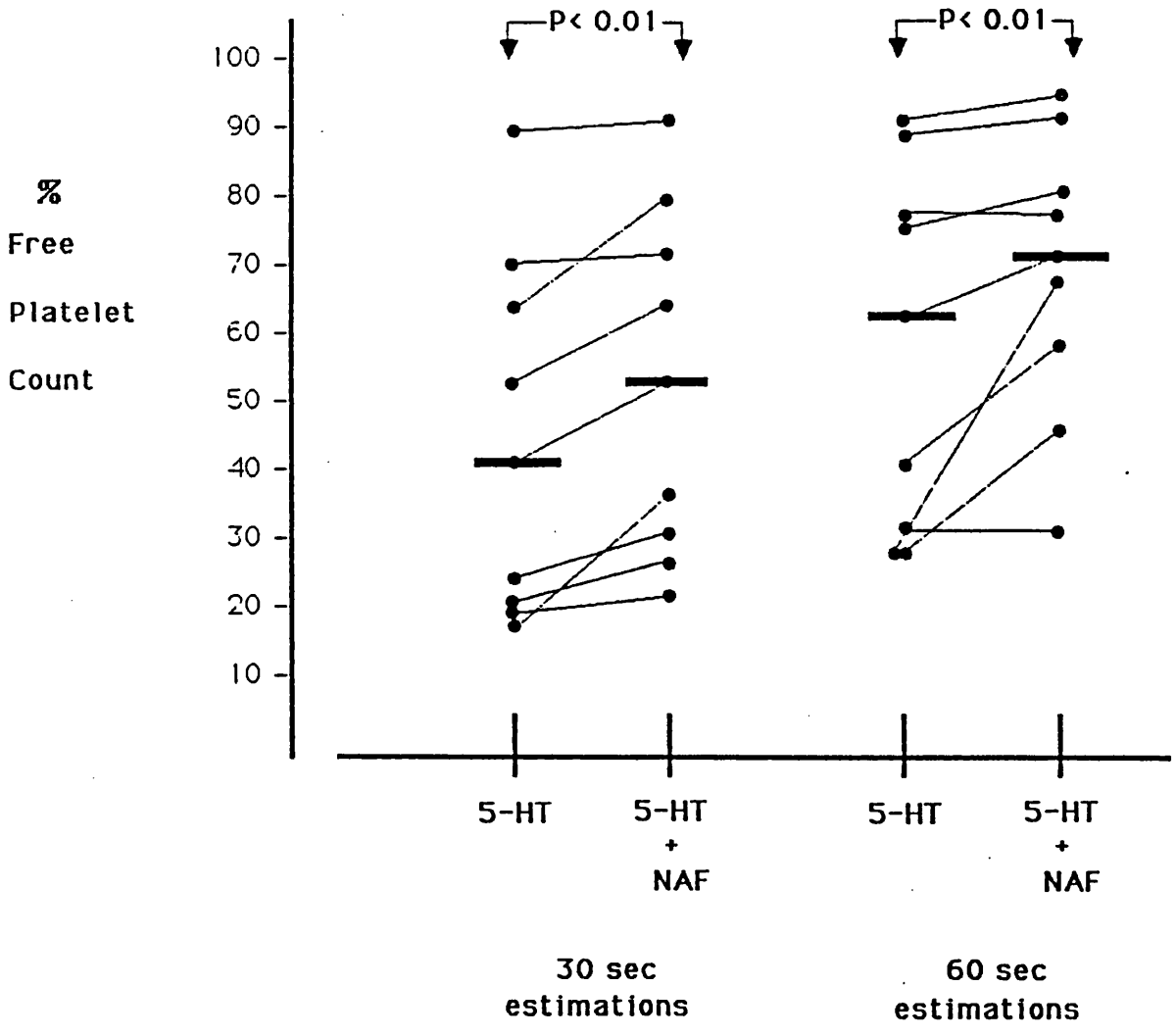
* P < 0.04 5-HT+NAF vs 5-HT only. Comparisons of No ASA treatment Vs. ASA treated blood were not significant.

ASA was added *in vitro* for 35-40 min, at 37°C, before commencing experiments. Oral ASA intake was 150 mg on alternate days. For the *in vitro* addition of ASA, this cyclooxygenase inhibitor was added to achieve a final concentration of 1 mmol/L.

Number of patients studied = 7. Platelet aggregation is expressed as a % of the basal platelet count in whole blood. Results are presented as median (range).

Fig 3.6

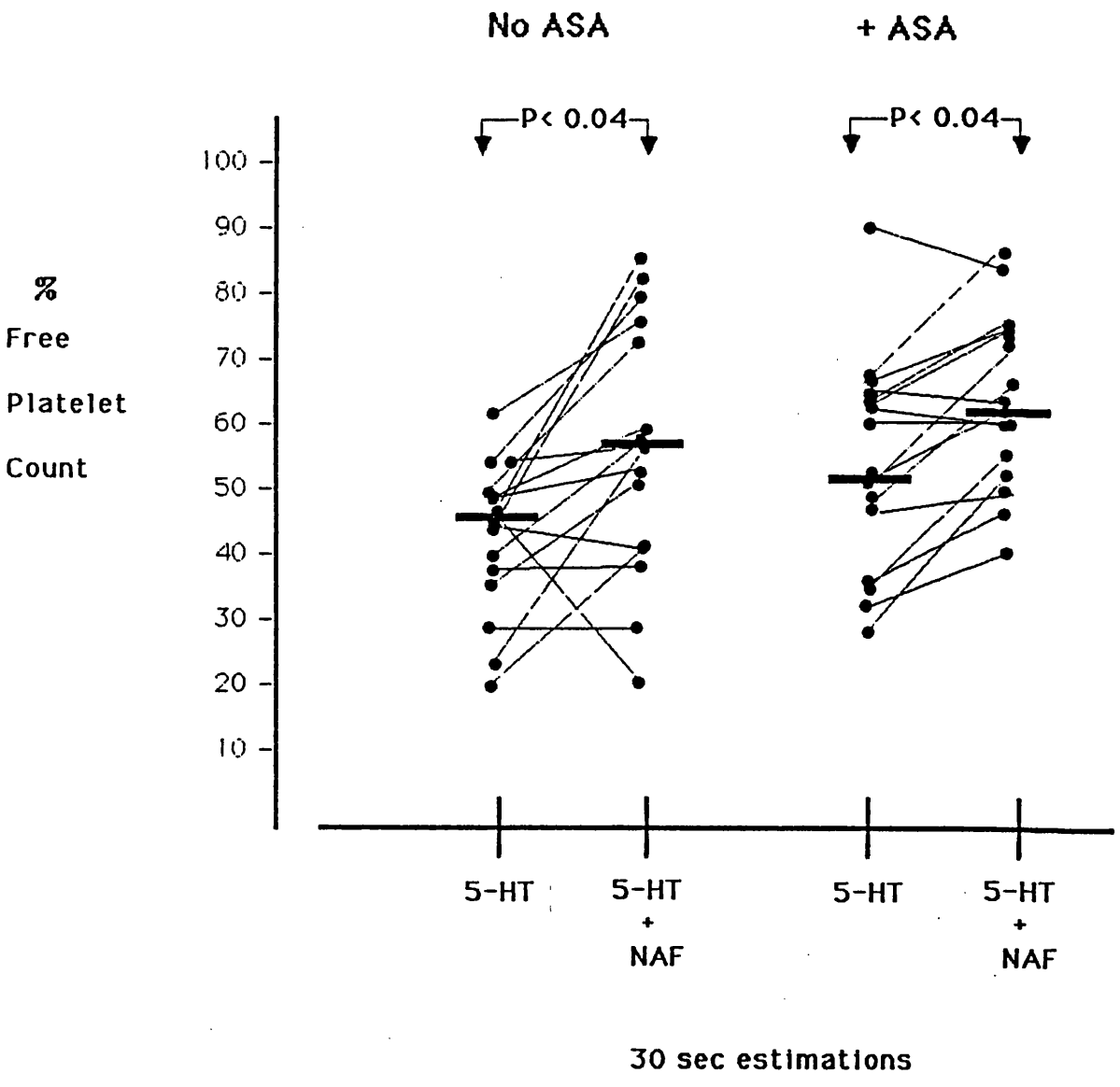
Effect of NAF on WB platelet aggregation in samples obtained from drug-free PVD patients



NAF was pre-incubated for 5 min before addition of 5-HT. Aggregation is expressed as a % of the basal free platelet count. WB-FPC aggregation was assessed 30 and 60 sec after addition of 5-HT (5 μmol/L). Solid bars=medians. 9 subjects were studied.

Fig 3.7

Effect of NAF and ASA on WB platelet aggregation in samples obtained from drug-free PVD patients



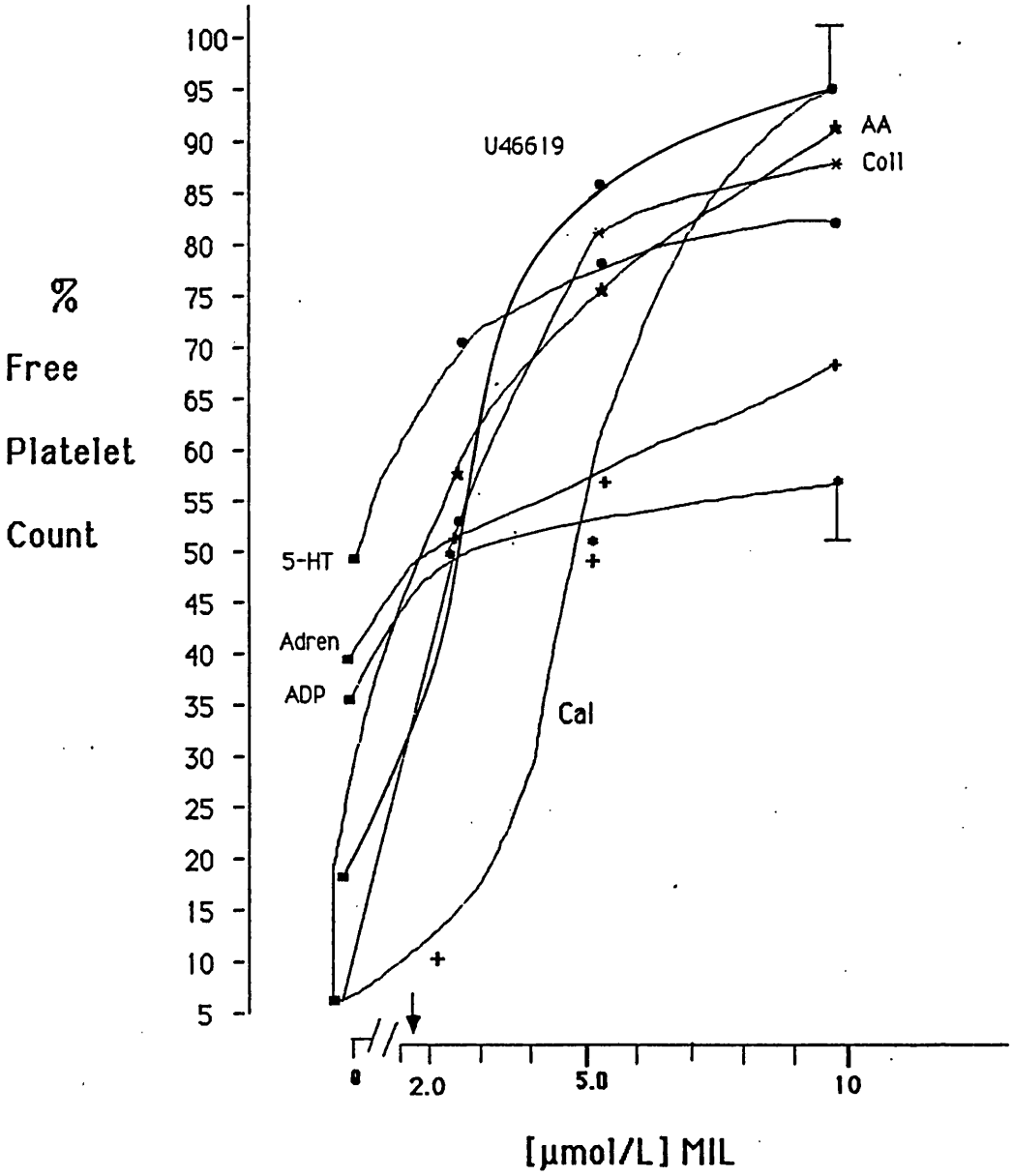
"No ASA" experiments were carried out in blood collected in citrate only. "+ ASA" experiments were carried out in blood collected in citrate+ASA. NAF was pre-incubated for 5 min before addition of 5-HT. Aggregation is expressed as a % of the basal free platelet count. WB-FPC aggregation was assessed 30 sec after addition of 5-HT (5 μmol/L). Solid bars=medians. 14 subjects were studied.

C) Effect of Milrinone (MIL) and Indomethacin (INDO) on WB-FPC in blood obtained from healthy subjects

WB-FPC aggregation induced by 5-HT, AA, U46619 and collagen was significantly inhibited ($P<0.01$) by MIL at 1.25 μmol/L and above. WB-FPC aggregation induced by ADP, CaI and adrenaline was inhibited significantly at MIL concentrations of 2.5 μmol/L and above (Figure 3.8). Collagen-induced WB-FPC was significantly ($P< 0.02$) inhibited by MIL (2.5 μmol/L) and by INDO (16 μmol/L; Figure 3.9). In Figures 3.8 and 3.9 the arrow denotes typical therapeutic concentration of MIL achieved following intravenous infusion in heart failure patients (Multi-Authors, 1991). The combination of MIL and INDO was more effective at inhibiting collagen-induced WB-FPC than either of these agents alone ($P< 0.02$ MIL/INDO vs MIL+INDO). ADP-induced WB-FPC aggregation was not inhibited by INDO and there was no significant additional effect when both drugs were incubated together. WB-FPC aggregation induced by ADP was inhibited significantly ($P< 0.02$) by MIL (2.5 μmol/L) and by MIL+INDO (Figure 3.9).

Fig 3.8

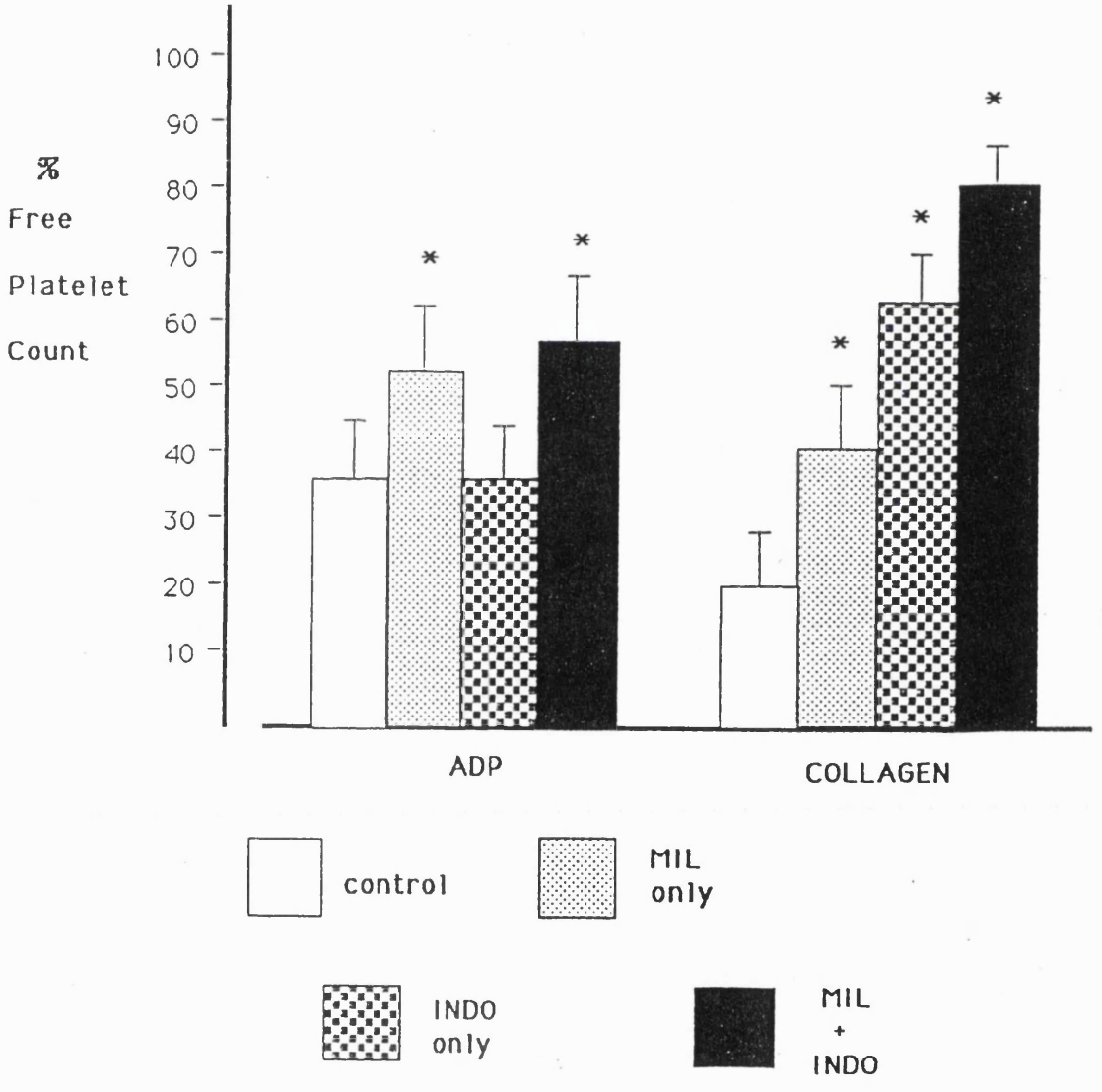
Effect of MIL on agonist-induced WB-FPC aggregation



MIL was pre-incubated for 5 min before addition of agonists. Aggregation was estimated 1 min after adding agonists, except for collagen (3 min) and 5-HT (30 sec). Aggregation is expressed as a % of basal free platelet count. The arrow denotes typical therapeutic concentration of MIL achieved following intravenous infusion in heart failure patients. 9 subjects were studied. Bars= maximal SEM.

Fig 3.9

Effect of MIL and/or INDO on ADP- and collagen-induced WB platelet aggregation



MIL and INDO were co-incubated for 5 min. Aggregation to ADP and collagen was estimated after 1 and 3 min, respectively. 7 healthy subjects were studied. For paired comparisons: * $P<0.02$ vs control. For collagen : MIL or INDO vs MIL + INDO; $P<0.02$.

3.13 DISCUSSION

This study shows that near therapeutic concentrations of NAF inhibit 5-HT-induced WB-FPC in samples obtained from PVD patients. This effect is important since PVD platelets appear to be hyperaggregable in response to 5-HT in both WB (present study) and in PRP (De Cree et al., 1985). NAF probably antagonizes 5-HT-induced effects by virtue of its 5-HT_2 blocking properties (Zander et al., 1986; Davies and Steiner, 1988) although NAF has also been shown to possess other properties (e.g. diminishes red cell aggregation; Nordt et al., 1990). Therapeutic concentrations of NAF, however, did not affect SPA or adrenaline-induced WB-FPC aggregation. A previous study (Davies and Steiner, 1988) with NAF,*in vitro*, showed that 50-100 μmol/L NAF was required to inhibit adrenaline- and 5-HT-induced platelet aggregation in PRP from healthy subjects. In our study, however, concentrations of NAF as low as 5 μmol/L significantly inhibited WB-FPC aggregation induced by 5-HT, in PVD patients. The greater sensitivity of the present system is probably due to the greater reactivity of WB (Valles et al., 1991) and the use of WB-FPC aggregation instead of turbidimetric aggregomety. This study has also demonstrated that the inhibitory effect of NAF is present even when the blood has been treated with ASA and/or the patients have received other drug therapy which inhibits platelets (e.g. isosorbide; Drummer et al., 1991 and dipyridamole; Saniabadi et al., 1991). The results obtained in the presence of ASA suggest that NAF inhibits 5-HT-induced WB-FPC aggregation independently of TXA_2 synthesis. SPA and 5-HT

induced WB-FPC aggregation, were both insensitive to ASA. The fact that SPA was insensitive to ASA is in accord with previous studies which suggest that SPA is due to low-grade release of ADP, probably from red cells (Fox et al., 1982). Low concentration of ADP-induced aggregation is also relatively insensitive to the effects of ASA when added, *in vitro*, to WB (Norris et al., 1992).

The work carried out with MIL in blood collected from healthy subjects shows that inhibition of WB-FPC aggregation can take place at concentrations which are near those achieved therapeutically (1.25 μmol/L). Thus, in the above studies inhibition to stimulation by various agonists was achieved at concentrations of 2.5 μmol/L and above. Previous work in our laboratory has also shown that MIL, in addition to inhibiting platelet aggregation in PRP and concomitant TXA_2 synthesis, also inhibits $^{45}Ca^{2+}$ uptake and sodium transport (Ozin et al., 1992; Jeremy et al., 1993). In this and in other studies (Jeremy et al., 1993), the extent of the inhibitory effect exerted by MIL on platelet aggregation was found to depend on the agonist used. Since each agonist transduces its activatory effects via different receptors and biochemical pathways, this suggests that MIL has differential effects on certain pathways which lead to platelet activation. This proposition is in agreement with the conclusion (Jeremy et al., 1993) that PDE inhibitors, in addition to reducing cAMP phosphodiesterase activity, also exert other effects on intracellular and extracellular mechanisms which influence platelet activation. For example, PDE inhibitors have also been shown to reduce the

expression of GPIb (Jackson et al., 1989) and influence AA release and metabolism (Lippton et al., 1985; Block et al., 1990). Another important aspect to consider is the well established inhibitory effect of PDE-type inhibitors on the cellular uptake of adenosine. Adenosine is a product of purine metabolism, which as described in Chapter 1, may be generated as a result of the action of ADPase and 5'-nucleotidase on ADP and AMP, respectively. Adenosine raises intracellular cAMP concentrations and therefore inhibits platelet activation (Arch and Newsholme, 1978). Drugs such as dipyridamole and other cAMP phosphodiesterase inhibitors prevent the cellular uptake of adenosine, a substance which raises intracellular cAMP concentrations and thus inhibits platelet activation (Arch and Newsholme, 1978). Moreover, Patelunas et al. (1991) have demonstrated that the anti-aggregatory effect of MIL, at concentrations of 5 μmol/L and above, was significantly reduced by the enzymatic removal of plasma adenosine. It is also possible that, in our study, the inhibitory effects due to MIL are enhanced, at least in part, by the presence and accumulation of plasma adenosine (Barradas et al., 1986).

In addition to exerting effects on its own, MIL was found to enhance the inhibitory effect of a cyclooxygenase inhibitor, INDO, on WB-FPC aggregation. This phenomenon, however depends on the agonist used and its capacity to synthesize TXA_2. Thus, a significantly greater inhibitory effect was observed during collagen-induced WB-FPC aggregation when INDO and MIL were co-incubated than when either of these agents was incubated alone. ADP-induced WB-FPC, however, was

not affected by INDO added, *in vitro*, alone and there was no enhancement when both MIL and INDO were added together as compared with MIL alone. These results can be rationalised in the following manner. When aggregation is stimulated with an agonist that is insensitive to cyclooxygenase inhibition (e.g. ADP) then MIL exerts its inhibitory effect but no additional effect is obtained by a cyclooxygenase inhibitor (e.g. INDO). If, on the other hand, aggregation is initiated by an agonist that generates large amounts of TXA_2 (e.g. collagen) MIL exerts its inhibitory effect, but some residual TXA_2 can still be generated which may decrease or reverse the increase in intracellular cAMP (Crawford and Scrutton, 1987) resulting from MIL action. In such a situation, cyclooxygenase inhibition enhances the inhibitory effects of MIL by preventing TXA_2 availability. The present findings are in agreement with the suggestion that PDE inhibitors may be useful potential anti-thrombotic agents (Gresele et al., 1991; Hall, 1993). Evidence is accumulating that suggests that cyclooxygenase inhibitors, such as ASA, although prescribed regularly for IHD have a limited efficacy at inhibiting platelet aggregation in IHD and other platelet hyperaggregable states (De Lorgeril et al., 1991; Louden et al., 1992; Terres et al., 1992; De Lorgeril et al., 1993; Barradas and Mikhailidis, 1993b). On the basis of the above findings, drugs such as ASA may be of greater benefit when prescribed in conjunction with MIL (or other PDE inhibitors) or with NAF (or other $5\text{-}HT_2$ antagonists). *Ex vivo* studies are required to clearly establish these possibilities.

CHAPTER 4

PLATELET AND PLASMA 5-HT STUDIES

CHAPTER SUB-INDEX

4.1 INTRODUCTION

Evidence for increased bioavailability of 5-HT in experimental models and disease states associated with hyperaggregable platelets and atherosclerosis has been presented in Chapter 1. Most studies consist of plasma measurements and platelet involvement has, therefore, been inferred. In the present study, the 5-HT status (platelets and in some cases plasma concentrations) in DM, PVD, renal disease and hypercholesterolaemia was assessed. The influence of factors, such as lipids (cholesterol and triglycerides) or intervention with drugs (e.g. aspirin; ASA) or lipid lowering drugs (simvastatin) was also investigated. For convenience, the studies presented are divided into different parts. Part 1: investigates the effect of ageing, DM and PVD, Part 2 investigates the effects of low dose ASA in PVD patients that have undergone infrainguinal grafting, Part 3 investigates renal disease patients and Part 4 involves hypercholesterolaemic patients studied before and after treatment with simvastatin.

Part 1

4.2 INTRAPLATELET AND PLASMA 5-HT IN HEALTHY SUBJECTS AND IN PATIENTS WITH DM OR PVD (Barradas et al., 1988)

Platelet hyperaggregability (a predictor of IHD; Elwood et al. 1990; Thaullow et al., 1991 and of re-infarction; Trip et al., 1990), and an increased incidence of atherosclerosis have been documented in DM and PVD (Jay and Betteridge, 1991). Many PVD patients are also diabetic and can go on to suffer

MI. The present study was set up to determine the intraplatelet 5-HT concentration in healthy subjects, type I (insulin-dependent) diabetes mellitus patients (IDDM), type II (non-insulin-dependent) diabetes mellitus patients (NIDDM) and PVD. The relationship between intraplatelet 5-HT content and age was investigated in all patient groups. In the diabetic group the relationship between the duration of the disease, glucose concentrations, glycosylated haemoglobin (HbA_1) and intraplatelet 5-HT content was also investigated. In a further study, plasma measurements of 5-HT in samples obtained from DM and PVD patients were also carried out.

4.3 METHODS

A) Healthy subjects and patient selection

Study 1: Two groups of healthy subjects were studied as control populations. One group of healthy subjects, "elderly controls", were attending a local Day Centre and consisted of 16 elderly healthy men and women (8 men, 8 women), median age 72 years (range 56-80). "Young controls" consisted of 18 younger, healthy men and women (11 men, 7 women) members of the medical and laboratory staff, median age 26 years (range: 21-49). For the appropriate age comparisons, the two populations were combined and collectively consisted of 34 "young" and "elderly" subjects (19 men, 15 women; median age 49 years, range 21-80). None of the controls had a history of diabetes, vascular disease or hypertension.

The patients were attending the diabetic and vascular out-patient clinics at The Royal Free Hospital (RFH). The IDDM

group consisted of 23 patients (11 men, 12 women); median age 50 years (range 23-72); median duration of diabetes 6 years (range 2-25); median blood glucose 11.5 mmol/L (range 4-28.3); median HbA_1 9.5% (range 5.8-11.3). The NIDDM group consisted of 23 patients (15 men, 8 women), median age 66 years (range 40-86); median duration of diabetes 10 years (range 1-15); median blood glucose 11.4 mmol/L (range 5.2-13.7); median HbA_1 10.1% (range 7.5-15.1). Some of the diabetic patients selected were hypertensive. Apart from hypertension, DM patients did not have a clinical history of vascular complications (e.g. MI, stroke or PVD). Patients were defined as hypertensive if they had a diastolic blood pressure > 95 mm Hg on at least two separate occasions. The PVD group consisted of 29 patients (18 men, 11 women); median age 73 years (range 47-79). All the patients in the PVD group had intermittent claudication for more than 6 months with ankle:arm systolic blood pressure ratio <0.85. Pain-free walking time was estimated in 14 PVD patients on the day of blood sampling (Fonseca et al., 1988). This test was performed on a motorized treadmill (Powerjog, Birmingham, UK).

Study 2: In this study, both intraplatelet and plasma 5-HT concentrations were measured. The control group consisted of 10 healthy subjects (7 men, 3 women); median age 50 years (range 25-68). The IDDM group consisted of 11 patients (6 men 5 women); median age 53 years (range 23-77); median duration of diabetes 11 years (range 3-40); median blood glucose 9.5 mmol/L (range 4-14); median HbA_1 9.1% (range 6.8-11.0). The NIDDM group consisted of 14 patients (7 men, 7 women); median

age 62 years (range 51-83); median duration of diabetes 11 years (range 2 months-30 years); median blood glucose 10.1 mmol/L (range 6.2-15.5); median HbA_1 9.8% (range 7.3.-12.1). The PVD group consisted of 13 PVD patients (10 men, 3 women); median age 68 years (range 56-83). PVD patients were all current non-smokers.

B) Drugs

Healthy subjects denied taking drugs for at least 2 weeks prior to sampling. Diabetic patients were on standard treatment regimens with insulin/oral hypoglycaemic agents (metformin and glibenclamide) and diet. Hypertensive patients (DM and PVD) were on a combination of nifedipine and bendrofluazide.

C) Blood sample collection and processing

Blood was taken between 9:00 and 11:00 am (fasting subjects) from an antecubital vein with minimal stasis. Nine parts of blood were added to one part of 3.8% w/v trisodium citrate. To prevent release of intraplatelet constituents during processing, ASA at a final concentration of 0.55 mmol/L was added to citrated blood (Gerrard and White, 1976). Plasma for 5-HT measurements was prepared by centrifuging citrated blood for 20 min at 1500 x g at 22°C. The supernatant was frozen immediately and stored at -40°C until assay (within 3 months). PRP and platelet counts were carried out as described in Chapter 3. Following counting, PRP aliquots (1 ml) were centrifuged at 1000 x g for 10 min to prepare platelet

pellets. The pellet was washed with Isoton II and stored at -40°C. Platelet pellets were resuspended in physiological saline (0.9% w/v), and a platelet lysate prepared by freeze thawing 3 times followed by ultrasonication (3 x 10 sec at an amplitude of 18 microns) using an MSE-Soniprep sonicator. In order to ensure full disruption of the platelets, the platelet populations were counted and sized before and after sonication using a Coulter counter coupled to a channelyzer (see Chapter 2). The platelet count following this procedure was reduced to < 5% of the original and the platelet volume became unmeasurable.

D) Intraplatelet 5-HT determination

5-HT was assayed using the method of Drummond and Gordon (1974) and modified according to Dangelmeier and Holmsen (1983). This spectrophotofluorimetric method utilizes the principle that 5-HT forms a fluorophore with O-phthaldialdehyde (OPT). The assay involves the addition of trichloroacetic acid (TCA) to the platelet lysates at a final concentration of 1 mol/L to precipitate proteins. The lysate is then rapidly centrifuged to obtain a clear lysate. The TCA extract (1 part) is added to OPT (0.5% w/v in ethanol) diluted in 8N-hydrochloric acid (4 parts) and placed in a boiling water bath for 10 min and cooled on ice. Finally, the samples are washed twice with chloroform to remove traces of TCA, the aqueous phase removed and the fluorescence read in a Perkin-Elmer MPF-3 fluorescence spectrophotometer (excitation and emission wavelengths of 360 nm and 475 nm, respectively).

Standards and blanks are processed in the same way as platelet lysates. The intra-assay CV was 4 % (n=20) and the inter-assay CV was 12% (n=8).

E) Plasma 5-HT assay

Plasma 5-HT concentrations were estimated using a radioimmunoassay kit purchased from Immunodiagnostics Ltd. This assay is described by Manz et al. (1985). The inter- and intra-assay CV for the measurement of 50 nmol/L 5-HT (n=10) were reported by the manufacturer as 5.1% and 3.1%.

F) Intraplatelet histamine determinations (Gill et al., 1988)

Intraplatelet histamine was determined in a subgroup of healthy subjects, DM and PVD patients. The control group consisted of 19 subjects (12 men, 7 women); median age 46 years (range 25-68). The diabetic group (IDDM+NIDDM) consisted of 34 patients (18 men, 16 women); median age 58 years (range 23-77). The PVD group consisted of 18 PVD patients (12 men, 6 women); median age 67 years (range 57-83). Histamine was assayed in platelet pellets, prepared as described above, with a double-isotope radioenzymatic method (Gill et al., 1988).

G) Blood glucose and glycosylated HbA_1 determination

Blood glucose measurements were carried out using a YSI glucose analyzer (glucose oxidase method). Glycosylated HbA_1 measurements (electrophoretic technique) were performed on whole blood using a glycosylated haemoglobin kit.

H) Statistical analysis and expression of results

Platelet 5-HT is expressed as nmol per 10^9 platelets. Results are presented as individual data points in scatter diagrams with medians shown as solid bars. The Mann-Whitney U-test (two-tailed) was used for comparing data. The Chi-square test was used for the analysis of frequency. Linear regression analysis was carried out using a computer program in use in the Department of Chemical Pathology and Human Metabolism, RFH.

4.4 RESULTS

Study 1

A) Comparison between "young" and "elderly" healthy subjects

In healthy subjects, platelet 5-HT content decreased with increasing age ($n=34$, $r=-0.45$, $P<0.008$; Figure 4.1). Intraplatelet 5-HT was significantly ($P<0.05$) lower in elderly healthy subjects when compared with platelets of younger healthy subjects (Figure 4.2).

B) Comparison between healthy subjects and diabetic patients

Patients with IDDM had significantly ($P<0.001$) lower intraplatelet 5-HT when compared with "young + elderly" healthy subjects (Figure 4.2). Patients with NIDDM had significantly ($P<0.002$) lower intraplatelet 5-HT when compared with "elderly" healthy subjects (Figure 4.2). Different control populations were selected for this study in order to achieve comparability in age.

Intraplatelet 5-HT concentrations in IDDM and NIDDM

patients did not differ significantly. There was no significant correlation between platelet 5-HT and age in either IDDM or NIDDM patients, or when these patients were pooled together (IDDM: n=20, r=-0.3, P<0.9; NIDDM: n=22, r=-0.2, P<0.063; IDDM+NIDDM: n=42, r=-0.004, P<0.79). There was also no significant correlation between platelet 5-HT content and duration of diabetes (n=40, r=-0.04, P<0.87), blood glucose (n=46, r=-0.2, P<0.21) and blood HbA_1 concentration (n=22, r=-0.02, P=0.93). There was no significant difference between IDDM and NIDDM with regard to blood glucose concentrations or HbA_1 levels.

C) Comparison between healthy subjects and patients with PVD

Patients with PVD had significantly (P<0.002) lower intraplatelet 5-HT content when compared with normal subjects of similar ages (Figure 4.2). The presence of hypertension in PVD patients was not associated with a further decrease in intraplatelet 5-HT (see below). There was no correlation between intraplatelet 5-HT content and age in PVD patients (n=26, r=-0.04, P<0.85). On the day of blood sampling pain-free walking time on a treadmill was carried out on 14 PVD patients. Median and range (in secs) pain-free walking time was 96 secs (32-248); median and range intraplatelet 5-HT concentration was 3.0 nmol/ 10^9 platelets (1.15-4.98). There was no correlation between pain-free walking time and intraplatelet 5-HT concentration (n=14, r=0.43, P=0.11). Likewise, the ankle:arm pressure ratio was recorded in 22 PVD patients on the same day of blood sampling. Median and range

ankle:arm pressure ratio was 0.61 (0.31-0.81); median and range intraplatelet 5-HT concentration was 3.04 nmol/ 10^9 platelets (0.94-4.98). There was no correlation between ankle:arm pressure ratio and intraplatelet 5-HT concentration (n=22, r=0.23, P=0.29). The platelet 5-HT content of PVD patients did not differ significantly from that in IDDM or NIDDM patients. Patients (n=8) with both PVD and DM were also studied. These patients had a platelet 5-HT content which was similar to that found in PVD and NIDDM patients, 2.96 nmol/ 10^9 platelets (1.04-5.17).

D) Effect of hypertension on 5-HT content of platelets in patients with DM and PVD

DM (IDDM+NIDDM pooled together) with hypertension had a significantly (P<0.05) lower median intraplatelet 5-HT content than the normotensive DM patients (Table 4.1). Chi-square analysis revealed that the frequency of the hypertension was similar in the NIDDM and IDDM groups. DM patients (IDDM+NIDDM) with and without hypertension had significantly (P<0.001) lower intraplatelet 5-HT than young+elderly control subjects. DM patients with hypertension had significantly (P<0.001) lower intraplatelet 5-HT than elderly control subjects (Table 4.1). Normotensive DM patients had significantly (P<0.003) lower intraplatelet 5-HT than elderly control subjects (Table 4.1). The DM hypertension/no hypertension patients were compared with both "young+elderly" and "elderly" control subjects to achieve better age matching between these groups. There was no significant difference between the platelet 5-HT

content of hypertensive and normotensive PVD patients (Table 4.1). Hypertensive PVD patients had a median intraplatelet 5-HT content that was significantly ($P<0.004$) lower than that in elderly control subjects (Table 4.1). Normotensive PVD patients also had significantly ($P<0.04$) lower intraplatelet 5-HT concentration than that in elderly control subjects (Table 4.1).

E) Histamine concentrations in controls, diabetics and PVD patients

Median and (range) intraplatelet histamine concentrations (per 10^9 platelets) were for healthy control subjects (young+elderly): 18.3 ng (17.3-21); DM patients: 18.4 ng (16.4-21) and PVD patients: 26.15 ng (20.4-28.1). Histamine concentrations were significantly ($P<0.001$) raised in PVD subjects but unchanged in DM patients. In this group of subjects, the intraplatelet 5-HT concentrations prepared from the same platelet lysates as those used for histamine assays (per 10^9 platelets) were for healthy control subjects (young+elderly): 3.92 nmol (2.8-5.9); DM patients: 2.69 nmol (1.26-6.34) and PVD patients: 3.05 nmol (0.9-4.98). Intraplatelet 5-HT concentrations were significantly ($P<0.01$) different between healthy controls and DM or PVD patients. There was no correlation between intraplatelet 5-HT content and intraplatelet histamine concentration in healthy subjects ($n=19$, $r=-0.18$, $P=0.45$), DM patients ($n=35$, $r=-0.006$, $P=0.98$) or PVD patients ($n=18$, $r=-0.10$, $P=0.69$).

Study 2

F) Plasma and intraplatelet 5-HT in healthy subjects and in IDDM, NIDDM, and PVD patients

Plasma 5-HT concentration was significantly raised in NIDDM and in PVD patients when compared with healthy subjects (Table 4.2). Plasma 5-HT concentration in IDDM patients was higher (66% increase) than in healthy subjects but (probably because of scatter) statistical significance was not reached.

Plasma 5-HT concentrations in hypertensive (n=10) and normotensive (n=15) DM (IDDM+NIDDM) patients (170; 56-938 and 199; 63-497 nmol/L, respectively) did not differ significantly. Similarly, plasma 5-HT concentration in the hypertensive (n=6) and normotensive (n=7) PVD patients (230; 165-369 and 205; 170-767 nmol/L, respectively) did not differ significantly. Intraplatelet 5-HT concentrations were similar to those described in study 1.

Fig 4.1

Correlation between age and intraplatelet 5-HT content in healthy subjects

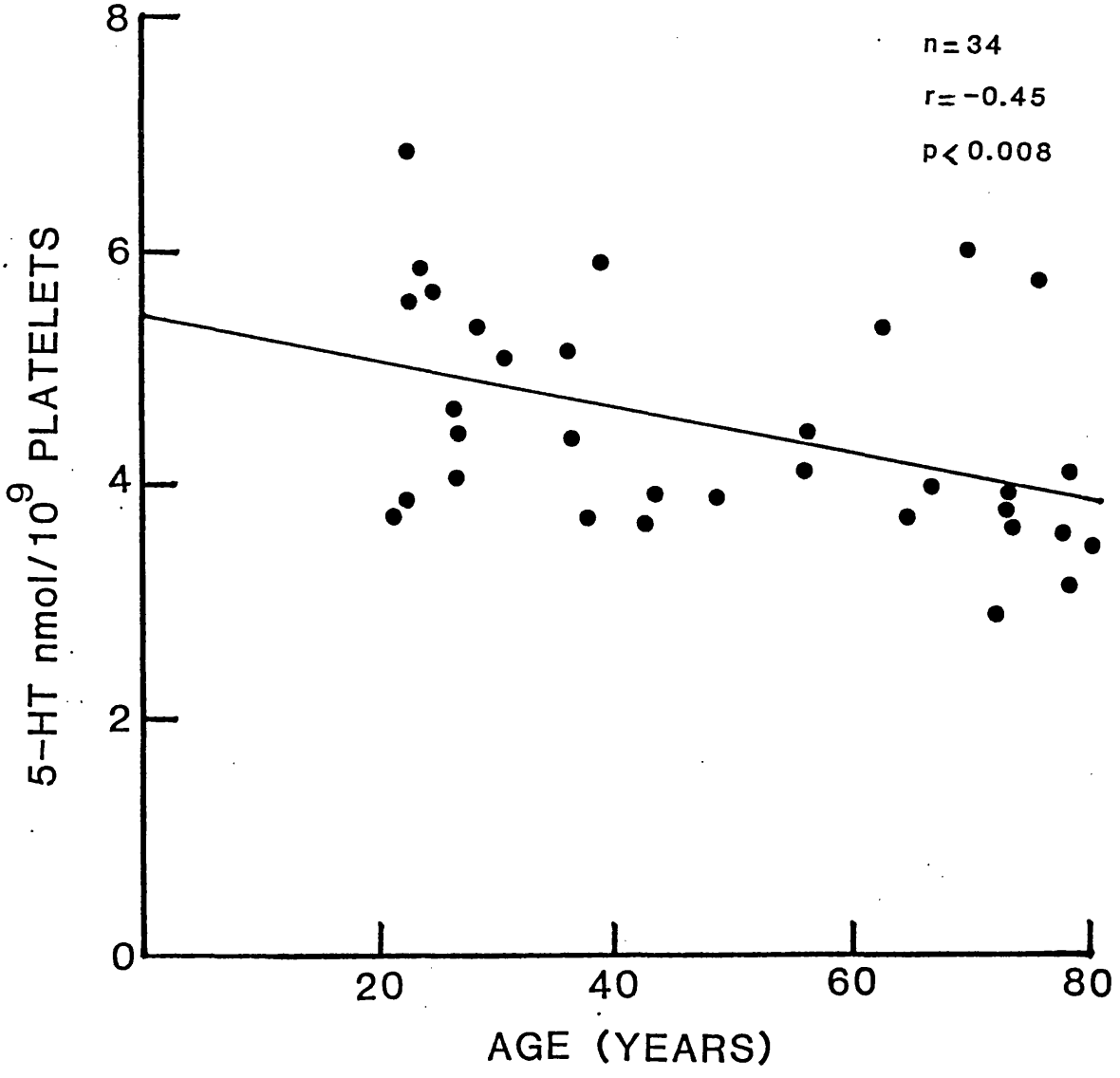


Fig 4.2

Intraplatelet 5-HT content in young, elderly, young + elderly healthy subjects, IDDM, NIDDM, and PVD patients

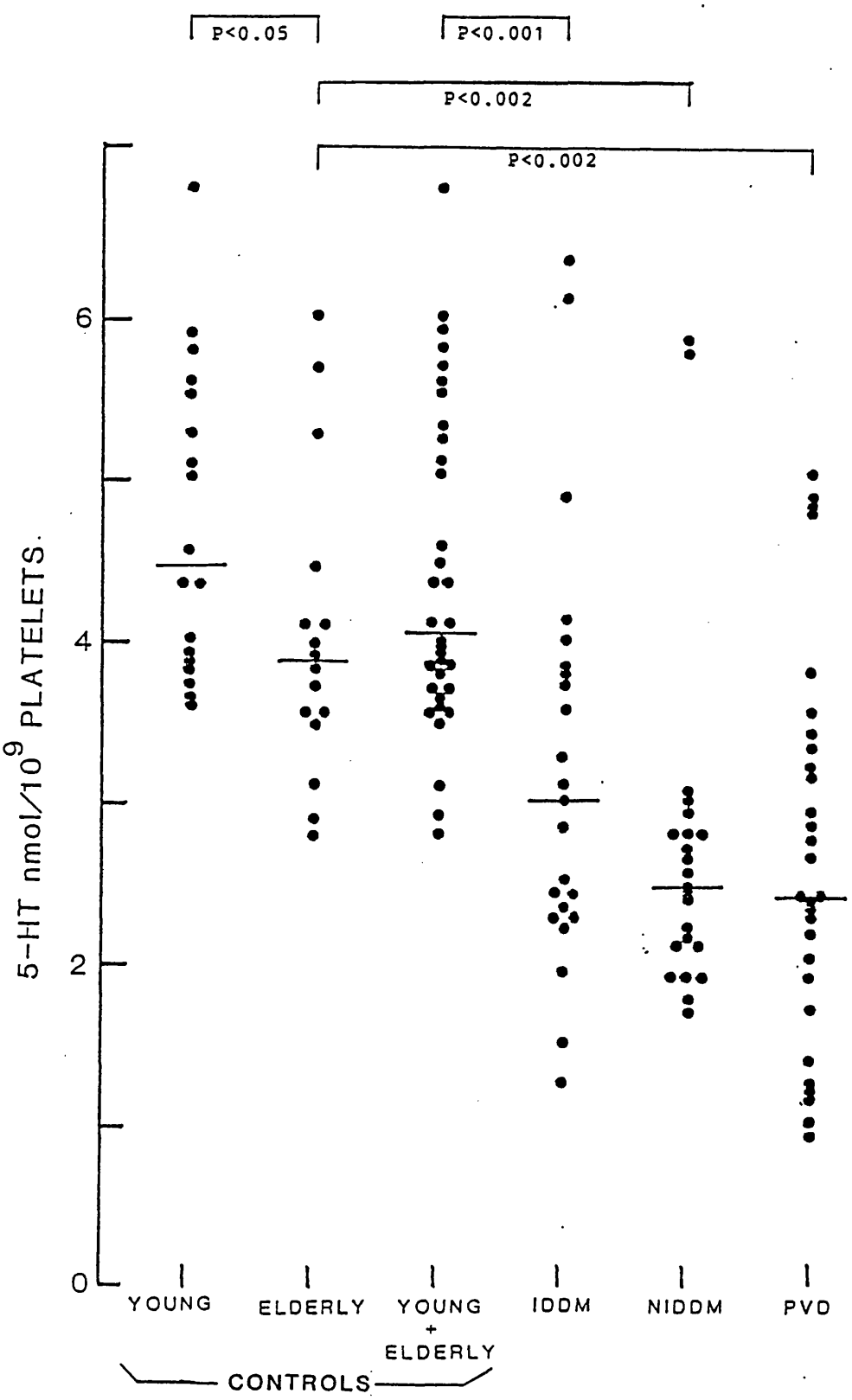


TABLE 4.1

Intraplatelet 5-HT content in healthy subjects, hypertensive and normotensive DM and PVD patients

Control/patient population	No. Patients	Age (years)	Intraplatelet 5-HT (nmol/10^9 platelets)
Healthy (Young+elderly) Subjects	34	49 (21-80)	4.05 (2.8-6.79)
Healthy (elderly) Subjects	16	72 (56-80)	3.87 (2.8-6.0)
DM-NBP	26	56 (31-76)	2.97*,$ (1.92-6.34)
DM-HBP	18	65 (56-73)	2.48+ (1.26-5.81)
PVD-NBP	12	71 (56-78)	2.77# (1.7-4.9)
PVD-HBP	12	74 (56-78)	2.29## (0.6-4.82)

NBP=Normotensive, HBP=Hypertensive

Statistical analysis: * Vs. elderly subjects: P<0.05; $ Vs. P<0.003; + Vs. young+elderly subjects: P<0.001; ** Vs. elderly subjects: P<0.001, # Vs. elderly subjects: P<0.04; ## Vs. elderly subjects: P<0.004.

TABLE 4.2

Intraplatelet 5-HT content and plasma concentrations in control subject, insulin-dependent diabetics (IDDM), non-insulin dependent diabetics (NIDDM) and peripheral vascular disease (PVD) patients

Control/patient population	No. Patients	Age (years)	Intraplatelet 5-HT (nmol/10^9 platelets)	Plasma 5-HT (nmol/L)
Control (Healthy) Subjects	10	50 (25-68)	3.85 (1.8-4.05)	100 (43-284)
IDDM Patients	11	53 (23-77)	2.24* (1.26-3.75)	166 (56-264)
NIDDM Patients	14	62 (51-83)	2.19+ (0.94-3.10)	291# (108-938)
PVD Patients	13	68 (56-83)	2.29* (1.67-4.50)	223# (165-767)

Statistical analysis: * Vs. control subjects: $P<0.05$; + Vs. control subjects: $P<0.01$; # Vs. control subjects: $P<0.002$

4.5 DISCUSSION

5-HT content of platelets in IDDM, NIDDM and PVD patients is significantly lower than that in aged matched healthy subjects. The plasma 5-HT concentration in NIDDM and PVD patients is significantly greater than that in controls.

There is evidence that platelet activation may be enhanced with increasing age in healthy subjects (see Chapter 3). In the present study, platelet 5-HT was found to decrease with increasing age, an observation also made by Shuttleworth and O'Brien (1981). The intraplatelet 5-HT results reported above for IDDM are in agreement with a preliminary communication by Winocour et al. (1987) but not with their more recent work (Winocour et al., 1990). The intraplatelet 5-HT results presented above are consistent with the whole blood 5-HT (Note: 95-99% of whole blood 5-HT is stored in platelets; Ortiz et al., 1988) results obtained for NIDDM and PVD by Pietraszek et al. (1992, 1993). These investigators found significantly diminished whole blood 5-HT concentrations in NIDDM patients (1992) and diminished whole blood 5-HT concentrations in thromboangiitis obliterans (TO) patients (1993). In agreement with the present studies, Pietraszek et al. (1992, 1993) and Winocour et al. (1990) found significantly elevated plasma 5-HT concentrations in IDDM patients with and without complications. It is also noteworthy that Pietraszek et al. has shown that for both NIDDM (1992) and TO patients (1993) there is reduced uptake and increased release of platelet 5-HT. This contrasts with an earlier study by van OoST et al., (1982) who showed 5-HT uptake to be

unchanged in PVD patients and significantly increased in DM patients (IDDM and NIDDM). With regard to hypertension, there is consistent evidence that this condition is associated with decreased intraplatelet 5-HT content (Kamal et al., 1984; Baudouin-Legros et al., 1985). From the present study it would appear that the presence of hypertension in DM further exacerbates the platelet 5-HT imbalance.

Given that platelets do not synthesize 5-HT (Morrissey et al., 1977), the intraplatelet content of this amine is likely to be dependent upon the net balance between uptake and release. It is therefore of interest that there is evidence that platelets obtained from NIDDM patients with vascular complications release more 5-HT than platelets from healthy subjects or diabetics without vascular complications (Peacock et al., 1986; Pietraszek et al., 1992). It is also pertinent to this issue that both DM and PVD are associated with platelet hyperaggregability and an increase in the release of other intraplatelet products (e.g ß-TG; Cella et al., 1979; PF-4; Betteridge et al., 1981 and TXA_2; Mikhailidis et al., 1985, Zahavi and Zahavi, 1985). Even in young IDDM subjects with no obvious vascular complications, platelet hyperaggregability has been demonstrated using whole blood techniques (Jones et al., 1985). Interestingly, platelet noradrenaline concentrations have been found to be significantly lower in IDDM when compared to normal subjects (Smith et al., 1989). Noradrenaline release following thrombin stimulation was also found to be increased in these patients (Smith et al., 1989). It appears, likewise, that the low 5-HT

content of platelets in both DM and PVD may be the result of a combination of decreased uptake and increased release. This conclusion implies an increased plasma pool of 5-HT. This was confirmed in the present study.

The duration of DM and the quality of diabetic control did not influence intraplatelet 5-HT concentrations. Peacock et al. (1986) also found no correlation between the release of platelet 5-HT (measured as ^{14}C-5-HT following stimulation with ADP or adrenaline) and blood glucose, glycosylated haemoglobin and lipid concentrations (cholesterol, HDL-cholesterol and triglycerides). Unfortunately, lipid data was not collected from DM and PVD patients in the present study. Intraplatelet 5-HT status did not correlate with the pain-free walking time or the ankle:arm ratio in PVD patients.

The methodology used to prepare PRP for platelet 5-HT and histamine concentrations in the studies presented above have the disadvantage that higher density platelets (with increased 5-HT granule content) may be lost during the centrifugation procedure required to prepare PRP (Barradas et al., 1989). Although some platelet loss occurs (to the erythrocyte layer) during the centrifugation procedure, the centrifugal (150xg) force used here for PRP preparation has been shown to yield a representative platelet population of whole blood (Denfors et al., 1991). In addition, it is unlikely that the centrifugation influenced the results obtained above since in the same platelet lysates, significantly elevated intraplatelet histamine content was demonstrated in PVD patients (see above and Gill et al., 1988). The elevation in

histamine concentrations in PVD patients is probably due to increased uptake from a plasma rich pool (Gill et al., 1989) since, as shown in our laboratory, platelet histamine uptake can be enhanced when platelets become activated with low doses of agonists (Gill et al., 1987). Given that histamine, like 5-HT, is stored in dense granules (Fukami et al., 1984) an artefact due to centrifugation would be expected to influence the intracellular content of both bioamines in a similar way. Furthermore, using platelet preparative techniques similar to the one outlined in this theses and electron microscopy techniques, van Rensburg and du P. Heyns (1990) demonstrated that elderly controls have the same number of electron dense granules per platelet as young controls, despite releasing more ß-TG and being more responsive to aggregation induced by ADP. In the same study, these workers showed that the dense granules per platelets were markedly reduced (6 versus 8) in PVD patients when compared to controls which may explain why these patients have reduced intraplatelet 5-HT concentrations. There are alternative methodologies for the preparation of platelet populations (e.g. density gradients; Mezzano et al., 1986), these, however, are comparatively more time-consuming and unsuitable for screening large numbers of patients.

The raised plasma 5-HT concentrations observed in DM and PVD is of interest given that the increased bioavailability of 5-HT could result in a tendency to vasospasm and to platelet hyperaggregability which is relevant to structural vascular disease (e.g. proliferation and atherosclerosis) observed in the elderly, diabetics and PVD patients. Increased plasma

concentrations of 5-HT may also be self-perpetuating, since it could result in the activation of platelets and further release of 5-HT from platelets.

Part 2

4.6 PLASMA 5-HT CONCENTRATIONS IN GRAFT PATIENTS RECEIVING LOW DOSE ASA (Cheshire et al., 1992)

Studies investigating the effect of antiplatelet therapy have demonstrated that drugs (e.g. ASA plus dipyridamole, and ticlopidine) are effective at preventing early occlusion but not restenosis (White et al., 1987; Schwartz et al., 1988). On the other hand, it is of interest that in the US Physicians' Health Study the chronic administration of low dose ASA to healthy men reduced the need for peripheral surgery. Following arterial reconstruction for severe leg ischaemia, patients can develop graft stenosis within the first or two years following surgery (Taylor et al., 1990; Grigg et al., 1988). The stenoses, whose aetiology remains to be fully elucidated, are caused by sub-intimal proliferation of smooth muscle cells. In this context, it is also important to note that a number of studies have demonstrated an association between reduced patency of bypass grafts and abnormalities such as platelet activation, hyperfibrinogenaemia and hyperlipidaemia (Wiseman et al., 1989; Wiseman et al., 1990; Campneau et al., 1984; Gavaghan et al., 1990; Yukizane et al., 1991).

Given the opportunity, it was of interest to assess the plasma 5-HT and ß-TG concentrations in patients that had or

were at risk of developing a stenosis and who were also taking low dose ASA with patients that were not receiving antiplatelet therapy.

4.7 METHODS

A) Patient Selection

The patients studied were attending the regional Vascular Unit at St. Mary's Hospital, London. Patients had undergone infrainguinal bypass grafting for severe leg ischaemia with autogenous vein graft or polytetrafluoroethylene (PTFE) grafts in the past 1-48 months before sampling. The grafts were at the femoropopliteal segment or to a single calf artery. Stenosis detection and surveillance in these patients was carried with duplex scanning for at least the first operative year (Grigg et al., 1988). Duplex abnormalities were confirmed by angiography and severe stenoses were corrected by percutaneous dilatation or surgical intervention. Seventeen patients that developed a stenosis and 24 that had not, were studied. Smoking status was ascertained in 44 patients. Fifteen were smokers and 29 reported that they had not smoked in the last 6 months. Non-smoking/smoking status was not confirmed by the measurement of carbon monoxide or nicotine metabolites. As a control population of healthy subjects, seventeen members (12 men, 5 women) of the medical and laboratory staff of the Department of Chemical Pathology and Human Metabolism, RFH, median age 45 years (range 21-65) volunteered for this study. None of the controls had a history of renal disease, diabetes, vascular disease or hypertension.

Healthy subjects denied taking drugs for at leat 2 weeks before sampling.

B) Blood sample collection and processing

Blood was collected as described above (see Part 1, study 1) between 2:00 and 4:00 PM. The first 5 ml were discarded. Blood was collected into plastic tubes containing Na_2EDTA and prostaglandin E_1 at a final concentration of 5 mmol/L and 10 μg/L, respectively (Kellum and Jaffe, 1976). In four healthy subjects (3 male, 1 female), median age and (range) 27 years (20-36), PRP was prepared and aggregation assessed by optical aggregometry in order to confirm that the Na_2EDTA and prostaglandin E_1 anticoagulant mixture effectively prevented platelet aggregation to high doses of agonists: ADP (10 μmol/L), adrenaline (10 μmol/L) and collagen (1 mg/L).

C) Plasma 5-HT assay

Plasma was prepared as described above (see Part 1, study 2). Plasma 5-HT concentrations were estimated using a radioimmunoassay (RIA) kit purchased from Biogenesis Ltd. This 5-HT RIA is identical in principle to the one supplied by Immunodiagnostics Ltd and was used because Immunodiagnostics discontinued the supply of their 5-HT kit. The Biogenesis RIA was also developed by Manz et al., (1985) but is considerably more specific and has a lower detection limit (25 fmol/tube versus 11.4 fmol/tube) than the previous kit.

D) Plasma ß-TG determination

ß-TG determinations on plasma (prepared as described above) were carried out according to the method of Ludlam et al. (1975). Sheep anti-ß-TG and donkey anti-goat serum antibodies were purchased from the Scottish Antibody Production Unit (Carluke, Scotland). ^{125}I-ß-TG and unlabelled ligand were prepared at the Department of Medicine, Western General Hospital, University of Edinburgh, and Scottish National Blood Transfusion Service, Edinburgh, respectively. The inter- and intra-assay coefficients of variation (n=10) for this assay were 14% and 9%.

E) Drugs

Twenty three patients were sampled that were on low-dose ASA (150 mg/alternate days) and 27 that were ASA-free. Patients taking other drugs with anti-platelet effects (nifedipine, dipyridamole, nitrates) were not included. A number of patients (n=7) were on warfarin, 6 were on frusemide+amiloride, 5 were on sleeping tablets (nitrazepam/temazepam), 4 were on cimetidine/ranitidine, 4 were on digoxin, 3 were on allopurinol, 2 were on atenolol, 2 were on thyroxine.

F) Statistical analysis and expression of results

The data was compared using the non-parametric Mann-Whitney U-test (two-tailed). All results were statistically analyzed using a computer program (C-STAT).

4.8 RESULTS

PRP prepared from blood obtained from healthy subjects (n=4) and anticoagulated with EDTA (+PGE_1) was completely unresponsive to aggregation induced by ADP, adrenaline and collagen. Thus, median and range % aggregation assessed using the Born-type aggregometer was 0% (0-0) for ADP (10 μmol/L), adrenaline (10 μmol/L) and collagen (1 mg/L). Aggregation was assessed 3 min after adding the agonist (Barradas et al., 1990a). The complete absence of responsiveness suggests that this anticoagulant prevents platelet activation during PRP preparation.

Plasma 5-HT and ß-TG

Patients had a significantly elevated plasma 5-HT and ß-TG concentrations ($P<0.007$ and $P<0.035$, respectively) when compared to healthy controls (Table 4.3). Patients on ASA had a lower median plasma 5-HT concentration than patients that were drug free (Table 4.3). Due to the scatter of the data this difference did not reach statistical significance. Thus, plasma 5-HT concentrations are 40% lower and only marginally non significant ($P<0.06$) when compared to those patients not taking ASA. Plasma ß-TG was significantly elevated in patients when compared to healthy subjects. ASA, however, did not appear to have a significant effect on plasma ß-TG (Table 4.3). Patients that had been operated for graft insertion and who subsequently developed a stenoses showed a significantly ($P<0.02$) elevated plasma concentration of 5-HT (Table 4.4) when compared to patients that had been operated but who had

not developed a stenosis. Plasma ß-TG, although not significantly (P=0.123) raised in stenotic patients, may have reached statistical significance if a greater number of patients had been studied. The number of stenotic and non-stenotic patients on ASA was similar in both groups (8/17 stenotics and 13/24 non-stenotics were on ASA). Smokers had the highest median plasma 5-HT concentrations when compared to non-smokers, 8.8 (range <1-61) nmol/L and 7.1 (<1-55.1) nmol/L, respectively. This difference did not reach statistical significance (P=0.576). Likewise, plasma ß-TG concentrations were also higher in smokers, 61 (35-107) µg/L and 50 (28-113) µg/L, respectively. This difference did not reach statistical significance.

TABLE 4.3

Effect of ASA-intake on plasma concentrations of platelet release substances

	Healthy Subjects	All PVD Patients	PVD Patients- **No ASA**	PVD Patients- + **ASA**
5-HT nmol/L	2.7 (<1-9.8) n=17	7.1*** (<1-79.6) n=55	7.1** (<1-61) n=27	4.3* (<1-45) n=23
ß-TG µg/L	42 (28-71) n=13	51+ (28-113) n=48	50 (28-109) n=24	66 (35-113) n=22

* P=0.06; Healthy subjects Vs. PVD + **ASA**
** P<0.004; Healthy subjects Vs. PVD **No ASA**
*** P<0.007; Healthy subjects vs. All PVD patients

\+ P<0.035; healthy subjects Vs. All PVD patients
All other comparisons=NS

n=number of subjects studied

TABLE 4.4

Effect of stenotic status on plasma concentrations of platelet release substances

Release Substances	Healthy Subjects	All PVD Patients	**Stenotic** Patients	**Not Stenotic** Patients
5-HT nmol/L	2.7 (<1-9.8) n=17	7.1* (<1-79.6) n=55	13.4* (<1-61) n=17	5.5*,$ (<1-17) n=24
ß-TG µg/L	42 (28-71) n=13	51+ (28-113) n=48	61 (35-113) n=17	47 (28-107) n=21

* P<0.01; Healthy subjects Vs. All PVD patients, **Stenotic** and **Not Stenotic** patients
$ P<0.02; **Stenotic** Vs. **Not Stenotic** patients

+ P<0.035; healthy subjects Vs. All PVD patients

n=number of subjects studied

4.9 DISCUSSION

The data presented above suggest that ASA may have a partial but not complete normalising effect on plasma 5-HT concentrations. The group of patients taking ASA had 40% lower 5-HT in their plasma as compared to patients that were not taking ASA. Plasma ß-TG, a marker of α-granule release was not affected by ASA intake. In view of the fact that ASA and other cyclooxygenase inhibitors (e.g. indomethacin) are unable to significantly inhibit platelet aggregation and PSC (see Chapter 2 for PSC), especially when this is induced by agonists such as 5-HT (see Chapter 3 for platelet aggregation) or sub-maximal doses of ADP (Gerrard and White, 1976), it is not unexpected that ASA had only a limited effect on the

release of both dense granule (5-HT) and α-granule (ß-TG) contents. The findings above also show that patients that develop graft stenoses have significantly elevated plasma 5-HT concentrations. It is worth putting these findings into context. Approximately one quarter of infrainguinal bypass grafts develop a haemodynamically significant stenosis, the majority of which occur within the first twelve months after operation (Taylor et al., 1990). If not actively sought and corrected, these stenoses may be associated with up to 80% of post operative graft occlusions (Taylor et al., 1990). The most common cause of post operative infrainguinal graft stenosis is localized proliferation of sub-intimal smooth muscle cells. These cells are derived from the media of the adjacent artery when anastomotic strictures develop in prosthetic grafts (Clowes et al., 1989; Sottiurai, 1990). The architecture of the lesion consists of proliferated smooth muscle cells in a surrounding matrix of proteoglycan and collagen. Given the above scenario, it is important to highlight that 5-HT has been shown to stimulate the mitogenesis of bovine aortic smooth muscle cells in culture; this effect occurs at concentrations as low as 10 nmol/L (Nemecek al., 1986). Furthermore, although less potent than PDGF, 5-HT significantly enhances the magnitude of the mitogenic response to PDGF (Nemecek et al., 1986). These effects of 5-HT are attenuated by blockade of 5-HT_2 receptors on vascular smooth muscle cells (e.g. with ketanserin; Nemecek et al., 1986).

It is interesting that, in this study, the highest median

plasma 5-HT concentrations were recorded in patients that admitted to being current smokers. Despite this, the difference between smokers and non-smokers was not statistically significant. One reason for this may be that some patients were untruthful and, unfortunately, smoking markers were not assessed. Other factors such as: fibrinogen, lipoproteins (e.g. Lp a), growth factors (e.g. PDGF) clotting factors (e.g. VII, Xa), and others may play a role in the progression of intimal hyperplasia and should be considered in further studies. Nevertheless, the hypothesis that platelet activation and 5-HT play a role in modulating the late development of intimal hyperplasia following vessel injury is strengthened by the recent work of Willerson et al. (1991). These workers found a direct relationship between the degree of platelet activation and the subsequent development of intimal hyperplasia in a chronic canine model of coronary injury. Moreover, they showed that a combined treatment with a dual thromboxane A_2 synthase inhibitor and a 5-HT_2 receptor antagonist prevented platelet activation and markedly attenuated the development of neointimal proliferation (Willerson et al., 1991).

Part 3

4.10 Intraplatelet substances in renal disease (Barradas et al., 1991)

Accelerated atherosclerosis and the risk of thrombosis are widely believed to be associated with renal disease (Lindner et al., (1974). In various forms of renal disease

(e.g. membrano-proliferative glomerulonephritis and glomerulosclerosis) a shortening of platelet survival and enhanced platelet aggregation have been documented (Bang et al., 1973; George et al., 1974). Furthermore, it has been suggested that platelets and/or their release products may contribute to glomerular disease directly (Parbatani et al., 1980; Barnes and Venkatachalam, 1985). The mechanisms underlying glomerular disease and accelerated atherosclerosis in renal disease, however, remain unknown.

Release products from platelets include serotonin (5-HT), ß-thromboglobulin (ß-TG), thromboxane A_2, platelet derived growth factor (PDGF) and histamine which, as highlighted in Chapter 1, have been shown to enhance vascular permeability (e.g. histamine), to possess vasoconstrictor and vasodilator effects (e.g. 5-HT and histamine), and to have platelet proaggregator, chemotactic and mitogenic properties (e.g. 5-HT, ß-TG and PDGF).

The reliability of measuring plasma concentrations as opposed to intraplatelet concentrations of platelet release substances has been questioned for renal disease (Deppermann et al., 1980; O'Brien and Etherington, 1984). Plasma measurements are affected by the presence of impaired renal clearance. Therefore, given the potential role for platelet release substances in renal disease and the controversy over plasma concentrations, the measurement of intraplatelet products (e.g. 5-HT, ß-TG, histamine and total platelet TXA_2 synthesizing capacity) was carried out.

The following subjects were studied: a] healthy

volunteers, b] nephrotic syndrome (NS), c] end-stage renal failure (ESRF), d] patients receiving continuous ambulatory peritoneal dialysis (CAPD) and e] patients receiving haemodialysis (HD). Since no information is available on the effect of plasma lipid concentrations on platelet activity and platelet biogenic amines in renal disease, correlations between these indices and plasma concentrations of albumin, total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) and triglyceride (TG) were also investigated.

4.11 METHODS

A) Patient selection

A total of 53 patients with renal disease in 4 clinical groups were studied.

Nephrotic syndrome (NS) group

This group consisted of 18 patients (11 men, 7 women) with median age 27 years (range 18-60); median duration of nephrotic syndrome was 6 years (range 1-32 years). Nephrotic syndrome was defined as proteinuria > 5 g/24 h and oedema. The diagnosis in these patients was minimal change glomerulonephritis (n=6), membranous glomerulonephritis (n=2), focal sclerosing glomerulonephritis (FSGN) (n=6), systemic lupus erythematosus (n=1) and amyloidosis (n=1). Two patients were hypertensive.

End stage renal failure (ESRF) group

This group consisted of 13 patients (7 men, 6 women); median age 54 years (range 23-62). All patients had a plasma

creatinine > 500 μmol/l. None of these patients had been treated with dialysis. The diagnosis in these patients was chronic pyelonephritis (n=2), FSGN (n=2) and polycystic kidney disease (n=2). The remaining patients presented with hypertension and severe chronic renal failure.

Continuous ambulatory peritoneal dialysis (CAPD) group

This consisted of 9 patients (5 men, 4 women) with median age 57 years (range 41-62). The median duration of dialysis was 1 year (range 6 months to 3 years). The diagnosis in these patients included diabetic nephropathy (n=2), obstructive uropathy (n=2), rapidly progressive glomerulonephritis (n=1), bilateral renal stenosis (n=1), hypertensive glomerulosclerosis (n=4) and end stage renal failure of unknown aetiology (n=2). Three patients had evidence of PVD.

Haemodialysis (HD) group

This group consisted of 13 patients (8 men, 5 women) with median age 65 years (range 50-67) and the median duration of dialysis was 2 years (range 6 months-30 years). The diagnosis in these patients was diabetic nephropathy (n=2), pyelonephritis (n=2), polycystic kidney disease (n=5), polyarteritis nodosa (n=1), obstructive uropathy (n=1), cortical necrosis (n=1) and chronic renal failure of unknown etiology (n=1). Three patients had PVD while pruritus was a problem in 1 patient.

Further biochemical characteristics including plasma creatinine, creatinine clearance rate, plasma albumin, urinary albumin, plasma TC, LDL-C, HDL-C and TG for all patient groups are given in Table 4.5 and 4.6. Full blood counts and PRP

counts are shown in Table 4.7. Informed consent was obtained from all patients who participated in this study.

B) Drugs

The patients were taking the following drugs; (a) ß-blockers (metoprolol, atenolol): 3 patients; ACE inhibitors (captopril) 3 patients; and calcium-channel blockers (nifedipine): 7 patients; digoxin: 4 patients. There were also patients taking allopurinol, insulin, warfarin and thyroxine. Two patients with NS and all other patients (ESRF, CAPD and HD) were on cimetidine or ranitidine to prevent gastrointestinal bleeding. CAPD and HD patients were on alfacalcidol and vitamin supplements. Healthy subjects denied taking drugs 2 weeks prior to sampling.

C) Healthy subjects

Nineteen drug-free healthy men and women (11 men, 8 women) members of the medical and laboratory staff, median age 48 (range 2165) volunteered for this study. None of the controls had a history of renal disease, DM, vascular disease or hypertension.

D) Blood sample collection and processing

Blood was taken as described for Part 1, study 1. The first 5 ml was discarded. In patients on haemodialysis, blood was obtained from the fistula/cannula prior to administration of heparin and the commencement of dialysis since heparin activates platelets (Barradas et al., 1987b). Blood was

collected into plastic tubes containing Na_2EDTA and prostaglandin E_1 as described above (Part 2, section 4.7). PRP was prepared and platelet counts carried out as described above (see Chapter 3). Platelet pellets and lysates were analysed as described below. For each analyte, samples from all groups were assayed in a single batch.

E) Intraplatelet 5-HT determination

Platelet pellets were lysed as described above (see Part 1). The samples were deproteinised by adding perchloric acid/cysteine solution to lysed platelets (100 μl acid to 900 μl of lysate) in a microcentrifuge tube. The tube was vortexed, left at 4°C for 15 min, and then centrifuged at 10 000 x g for 15 min at 4°C. The supernatant was removed and assayed immediately. Intraplatelet 5-HT was assayed using a double-antibody radioimmunoassay (RIA). This assay for platelet 5-HT was preferred to the fluorometric method described above since it was less time consuming, less dangerous (no solvents are used for extraction) and more specific. The reagents supplied were a generous gift from Prof. CRW Edward's Department of Medicine , Western General Hospital, University of Edinburgh. This assay was set up and validated at the above department by Dr Iain Gow (Gow et al., 1987). For the present study, the inter- and intra-assay CV for the measurement of 250 nmol/L 5-HT (n=16) assay were 14% and 12%, respectively.

F) Intraplatelet ß-TG determination

ß-TG determinations on platelet lysates (prepared as described above) were carried out using the RIA technique outlined above (see Part 2, section 4.7).

G) Total platelet TXA_2 synthesizing capacity

This is an established method in use in our laboratory and carried out by Dr JY Jeremy (Jeremy et al., 1988). The method involves the use of sonicated platelets which are incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) at 37°C. Under these conditions, the biologically available endogenous TXA_2 precursor, arachidonic acid is converted into TXA_2 which is measured as TXB_2 (the stable spontaneously hydrolysis product of TXA_2) by a specific radioimmunoassay.

H) Intraplatelet histamine determinations

Intraplatelet histamine was determined using the double-isotope radioenzymatic method described by Gill et al. (1988).

I) Biochemical and haematological analysis

Plasma creatinine, creatinine clearance rate, plasma albumin, urinary albumin, TC, HDL-C and TG were analyzed using standard methods in routine use in the Department of Chemical Pathology and Human Metabolism and the Renal Unit, RFH. Whole blood platelet counts (collected in EDTA; 5 mmol/L) were estimated using a Coulter counter model S in routine use in the Department of Haematology, RFH.

J) Statistical analysis and expression of results

Intraplatelet contents (5-HT, ß-TG, TXA_2 and histamine) are expressed per 10^9 platelets. 5-HT is expressed as nmol, ß-TG as μg and TXA_2 and histamine are expressed as ng per 10^9 platelets. Results are presented as individual data points with medians shown as solid bars in Figures 4.3-4.5. The data was compared using the non-parametric Mann-Whitney U-test (two-tailed). Details of comparisons are included in the legend for each Figure and Table. Spearman rank correlations (r_s) were carried out using a validated computer program in use in the Department of Chemical Pathology and Human Metabolism, RFH.

4.12 RESULTS

A) Intraplatelet content of 5-HT, ß-TG, histamine and TXA_2 synthesizing capacity of platelets

The median intraplatelet 5-HT content in all patient groups (NS, ESRF, CAPD and HD) was significantly ($P < 0.001$) decreased when compared to healthy subjects (Figure 4.3). There was no significant difference between the various patients groups as far as intraplatelet 5-HT content is concerned. Intraplatelet ß-TG was significantly decreased ($P < 0.04$) in NS patients, ESRF ($P < 0.02$) and HD patients ($P < 0.004$) (Figure 4.4). Total platelet TXA_2 synthesizing capacity was increased in ESRF ($P < 0.02$) and HD ($P < 0.001$) patients (Figure 4.5). Intraplatelet histamine concentrations in all patient groups were similar to those of healthy subjects (healthy subjects: median and (range) 8.7 (7.3-9.6) ng/10^9; NS

patients: 8.6 (6.3-12.2) ng/10^9; ESRF patients: 9.0 (6.4-10.1) ng/10^9; CAPD patients: 8.9 (7.6-11.1) ng/10^9; HD patients: 9.6 (6.3-11.9) ng/10^9.

B) Interrelationships between intraplatelet substances in healthy subjects and patients

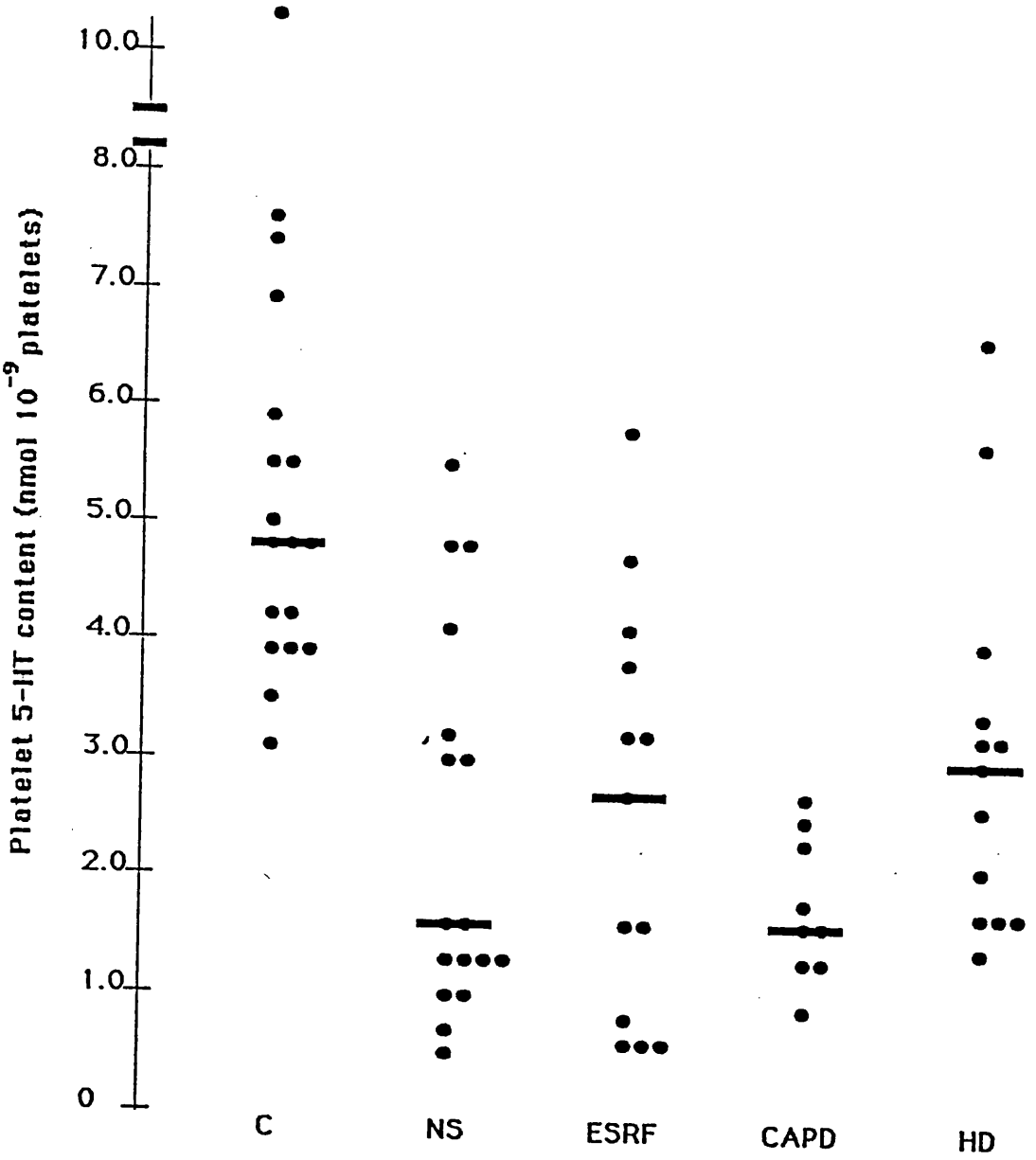
Platelet 5-HT correlated significantly with platelet ß-TG (n=19, r= 0.49, P<0.037). Intraplatelet 5-HT and ß-TG did not correlate with TXA_2 synthesizing capacity (n=19, r=-0.037 and r=-0.016, respectively). In patients undergoing CAPD, platelet 5-HT was strongly correlated with platelet ß-TG (n=9, r=0.83, P < 0.005). No other significant correlation was observed.

C) Comparison of platelet counts in whole-blood and PRP of healthy subjects and patient groups

Median and (range) platelet counts in whole-blood (WB) and in PRP of healthy subjects, NS, ESRF, CAPD and HD patients are shown in Table 4.7. Patients with ESRF and patients undergoing HD had significantly lower WB platelet counts than healthy subjects. NS patients had WB platelet counts which were similar to those in healthy subjects. CAPD patients had platelet counts which tended to be lower than those of healthy subjects, but the difference was not statistically significant (see Table 4.7). Platelet counts in WB correlated significantly with those in PRP preparations (n=37, r=0.88, P < 0.001).

Fig 4.3

Median platelet 5-HT content in healthy control subjects (C), NS, ESRF, CAPD and HD patients

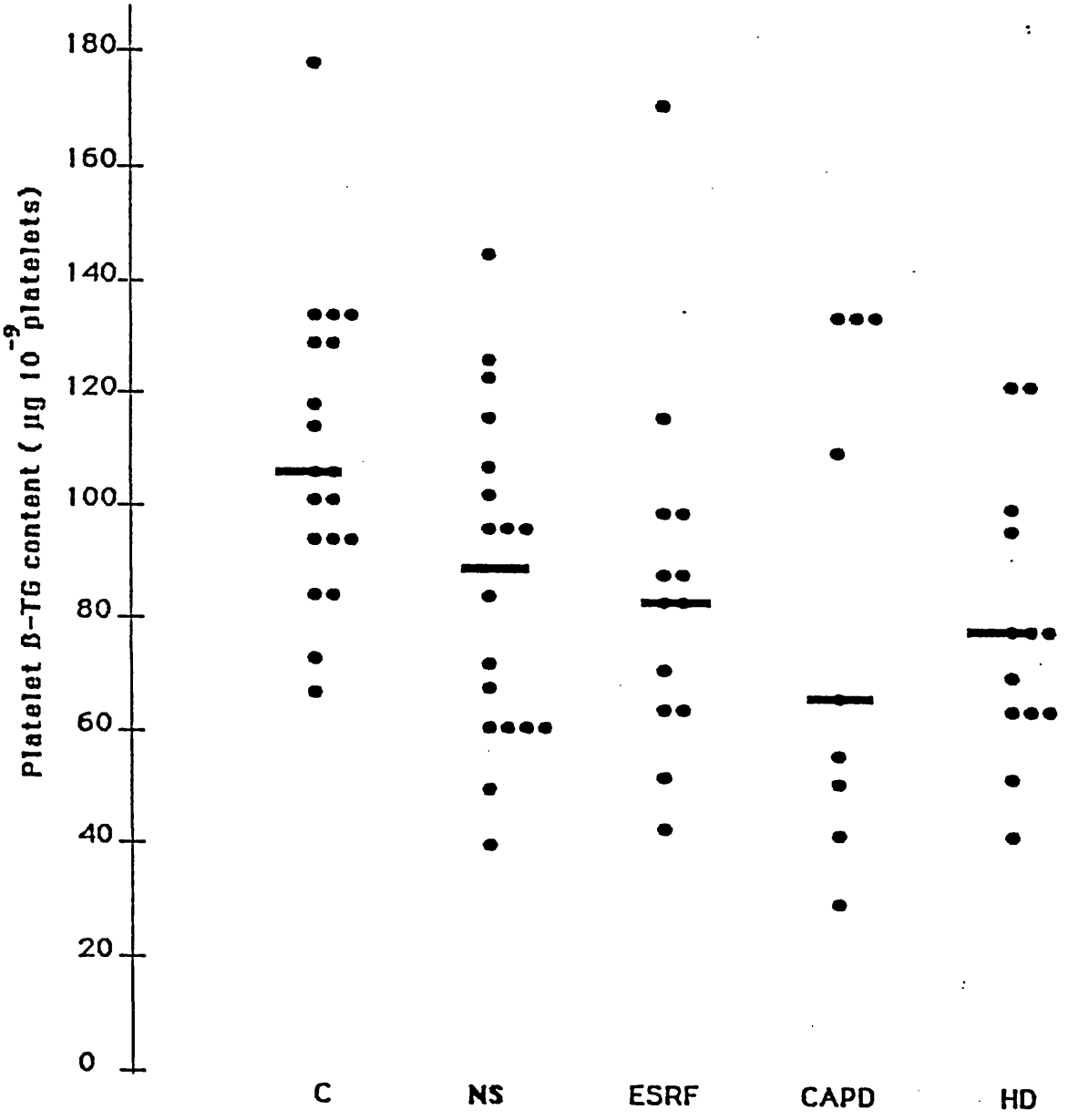


Statistical analysis: C Vs. NS, C Vs. ESRF, C Vs. CAPD, C Vs. HD, P< 0.001. All other permutations = non-significant.

Abbreviations: **C**=healthy controls; **NS**=nephrotic syndrome; **ESRF**=end-stage renal failure; **CAPD**=continuous ambulatory peritoneal dialysis; **HD**=haemodialysis patients.

Fig 4.4

Median platelet ß-TG content in healthy control subjects (C), NS, ESRF, CAPD and HD patients.

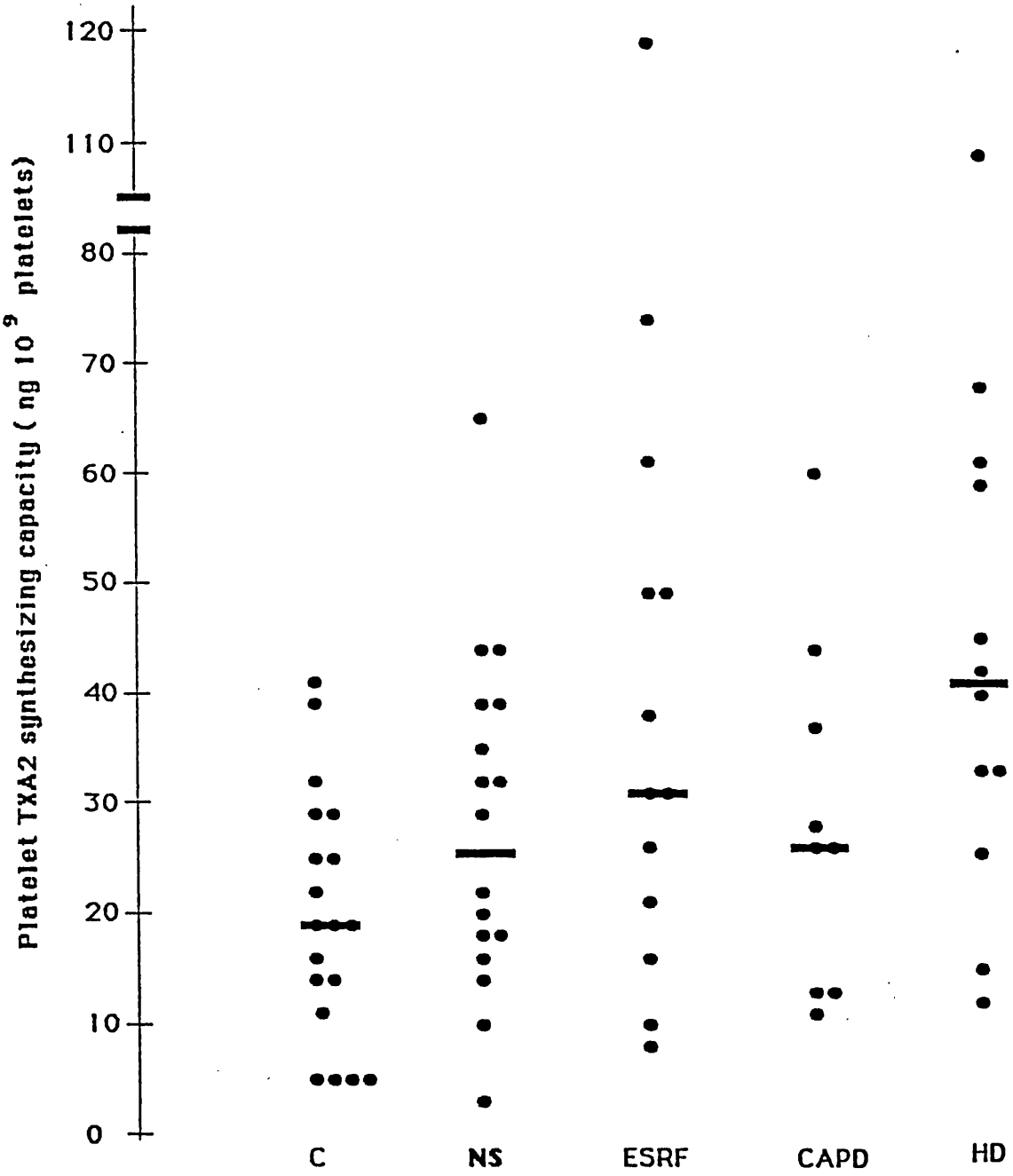


Statistical analysis: C Vs. NS, P<0.004, C Vs. ESRF, P<0.02, C Vs. CAPD, NS, C Vs. HD, P< 0.004. All other permutations= non-significant.

Abbreviations: **C**=healthy controls; **NS**=nephrotic syndrome; **ESRF**=end-stage renal failure; **CAPD**=continuous ambulatory peritoneal dialysis; **HD**=haemodialysis patients.

Fig 4.5

Median platelet TXA_2 synthesizing capacity in healthy control subjects (C), NS, ESRF, CAPD and HD patients



Statistical analysis: C Vs. NS, P<0.004, C Vs. ESRF, P<0.02, C Vs. CAPD, NS, C Vs. HD, P< 0.004. All other permutations= non-significant.

Abbreviations: **C**=healthy controls; **NS**=nephrotic syndrome; **ESRF**=end-stage renal failure; **CAPD**=continuous ambulatory peritoneal dialysis; **HD**=haemodialysis patients.

D) Relationship between plasma TG, TC, LDL-C, HDL-C concentrations and intraplatelet substances in renal patients

Intraplatelet 5-HT correlated significantly and inversely with TG concentrations (see Table 4.6 for plasma lipid concentrations) when patients were considered as a single group ($n=30$, $r=-0.43$, $P < 0.02$; Figure 4.6). Interestingly, other intraplatelet substances were not significantly correlated with TG concentrations. Correlations within each individual renal group were not carried out since lipid details on some patients were not available.

Intraplatelet 5-HT was inversely related to both TC ($n=30$, $r=-0.37$, $P < 0.043$; Figure 4.6) and LDL-C ($n=30$, $r=-0.36$, $P < 0.05$) when all patient groups were considered together. Intraplatelet ß-TG, histamine and total TXA_2 synthesizing capacity did not correlate with plasma TC or LDL-C. Intraplatelet 5-HT did not correlate with plasma HDL-C concentrations but a significant positive correlation between intraplatelet ß-TG and HDL-C concentrations was observed ($n=30$, $r=0.43$, $P < 0.017$).

E) Relationship between plasma albumin, plasma creatinine, creatinine clearance, urinary albumin and intraplatelet contents

Plasma albumin, plasma creatinine, creatinine clearance rate and urinary albumin excretion did not relate to any of the intraplatelet substances considered in this study (see Table 4.5 for actual values).

TABLE 4.5

Biochemical indices of patients with renal disease.

All data are expressed as median and (range).

Group	**Pl Creat** μmol/l	**CC** ml/min	**Pl Alb** g/l	**Ur Alb** g/24
NS n=18	101 (68-208)	115 (27-139)	38 (32-47)	10.3 (5.7-14.2)
ESRF n=13	690 (500-1213)	11.1 (5.4-16)	42 (36-49)	0.6 (0-2.4)
CAPD n=9	784 (375-1229	---	44 (38-50)	---
HD n=13	804 (349-906)	---	44 (38-50)	---
Ref. range	60-120	90-150	40-50	< 1

Abbreviations: **Pl Creat**=plasma creatinine; **CC**=creatinine clearance; **Pl Alb**=plasma albumin; **Ur Alb**=urinary albumin. **NS**= nephrotic syndrome; **ESRF**=End stage renal failure; **CAPD**=patients undergoing continuous ambulatory peritoneal dialysis; **HD**=patients undergoing haemodialysis; n=number of patients studied.

TABLE 4.6

Lipid concentrations in patients with renal disease.

All data are expressed as median and (range).

Group	Pl TC mmol/L	Pl LDL-C mmol/L	Pl HDL-C mmol/L	Pl TG mmol/L
NS n=7	6.1 (3.8-15.7)	5.62 (2.46-12.9)	1.2 (0.62-1.68)	2.33 (0.8-4.7)
ESRF n=10	6.1 (3.8-7.09)	3.23 (1.08-5.18)	0.87 (0.58-1.35)	2.6 (1.5-6.23)
CAPD n=6	6.96 (5.3-8.5)	5.07 (3.08-6.31)	0.79 (0.69-1.35)	2.6 (1.29-3.29)
HD n=7	4.88 (3.3-8.9)	2.9 (1.68-7.72)	0.68 (0.52-0.98)	2.33 (1.08-6.8)
Ref. range	3.0-6.5	< 4.5	1-2.2	0.2-1.5

Abbreviations: **Pl TC**=plasma total cholesterol; **Pl LDL-C**=plasma low density lipoprotein-cholesterol; **Pl HDL-C**=plasma high density lipoprotein-cholesterol; **Pl TG**=plasma triglyceride. **NS**=nephrotic syndrome; **ESRF**=end stage renal failure; **CAPD**=patients undergoing continuous ambulatory peritoneal dialysis; **HD**=patients undergoing haemodialysis; n=number of patients studied. Note: Lipid data was available only in some patients.

TABLE 4.7

Median and (range) platelet counts (10^9/L) in WB and PRP in controls and renal patients

	PATIENT GROUPS				
PLATELET COUNTS	**CONTROLS** n=19	**NS** n=17	**ESRF** n=14	**CAPD** n=9	**HD** n=13
WB platelet counts	275 (200-400)	245 (137-428)	230* (110-377)	256 (192-390)	200* (47-557)
Platelet counts in PRP	528 (328-920)	510 (284-740)	334** (200-544)	380 (216-680)	244* (96-732)

Statistical analysis: Controls Vs. ESRF and controls vs HD: * $P < 0.002$, ** $P < 0.0002$.

Correlation between whole-blood platelet counts and platelet counts in PRP: $r_s=0.88$, $P < 0.001$.

Abbreviations: **NS**=Nephrotic syndrome; **ESRF**=End stage renal failure; **CAPD**=patients undergoing continuous ambulatory peritoneal dialysis; **HD**=patients undergoing haemodialysis; n=number of patients.

Fig 4.6

Correlation (r_s) between plasma triglyceride (TG) concentration and platelet 5-HT content in renal patients

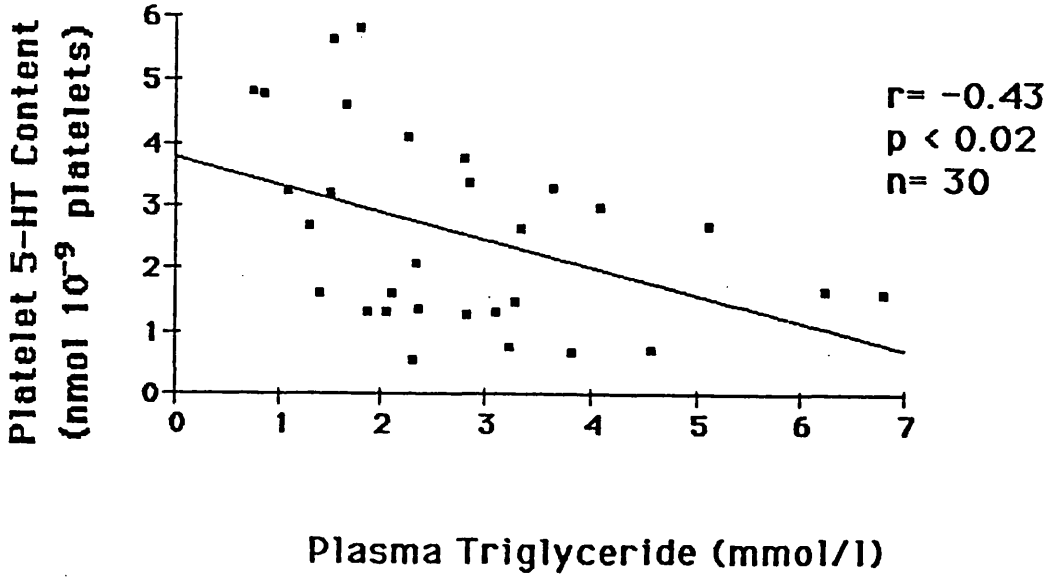
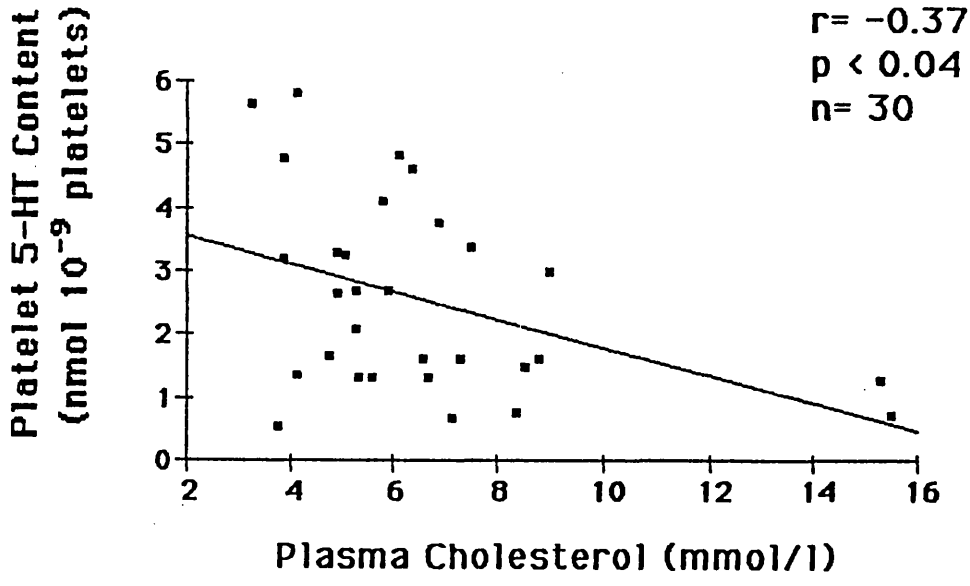


Fig 4.7

Correlation (r_s) between plasma cholesterol (TC) concentration and platelet 5-HT content in renal patients



4.13 DISCUSSION

The data obtained in this study is consistent with the previous observations that platelets in patients with renal disease are hyperactive (Bang et al., 1973; Gordge et al., 1988) and that intraplatelet 5-HT is diminished (Parbatani et al., 1980; Sebekova et al., 1989).

Diminished intraplatelet 5-HT in renal disease and in other conditions associated with platelet hyperaggregability (e.g. DM and PVD) suggests that circulating hyperaggregable platelets release 5-HT, thereby leading to a depletion of this bioamine. This concept is consolidated by the fact that plasma 5-HT concentrations are elevated in diabetic and PVD patients (see above, Part 1) as well as in patients with renal failure (Eknoyan et al., 1981; Minami et al., 1987). Diminished intraplatelet 5-HT concentrations, have been observed in patients with glomerulonephritis, chronic renal failure and patients on haemodialysis (Parbatani et al., 1980; Eknoyan et al., 1981). Previous work provided no information on CAPD.

As described in Chapter 1, ß-TG and 5-HT are stored in separate platelet granules (α- and dense granules, respectively). Thus, a significantly reduced intraplatelet ß-TG concentration in all renal patients (except those in the CAPD group) demonstrates that the contents of both granules are released during platelet activation in this condition. This conclusion is in agreement with previous findings of increased platelet aggregability (Gordge et al., 1988) and elevated ß-TG concentrations found in the plasma of patients with renal disease (Deppermann et al., 1980; Parbatani et al.,

1980; Adler et al., 1980). Platelet ß-TG has not previously been investigated in renal disease. Intraplatelet ß-TG concentrations rather than plasma ß-TG concentrations were measured in the current study since this and other substances may be elevated in plasma due to alterations in plasma clearance (Deppermann et al., 1980; O'Brien and Etherington, 1984).

Since artefacts may be introduced during centrifugation of blood containing hyperactive platelets it was decided to prepare PRP from blood anticoagulated with EDTA and PGE_1. This mixture was found to completely abolish aggregation to high dose ADP, adrenaline and collagen and is therefore likely to inhibit platelet release and preserve intraplatelet contents during PRP preparation. In healthy subjects, intraplatelet 5-HT concentrations were very similar in this study and the study investigating DM and PVD (Part 1).

In agreement with the ß-TG and 5-HT changes found in renal patients, the capacity of platelets to synthesise TXA_2 was increased in the various patient groups. CAPD patients, however, showed a TXA_2 synthesizing capacity which was similar to that of healthy subjects; this was consistent with the absence of a change in ß-TG in these patients and with the smallest decrease in platelet 5-HT observed in the renal groups. Although no data on platelet TXA_2 synthesising capacity in ESRF, CAPD and HD patients is available, a previous study demonstrated that the conversion of [^{14}C]-arachidonate to [^{14}C]-TXA_2 in washed platelets taken from patients with NS was unchanged, while it was elevated in

patients with ESRF (Nakano et al., 1988). Methodologically, the use of exogenous AA to assess TXA_2 synthesis reflects cyclooxygenase and TXA_2 synthase activity which would appear to be enhanced. The present findings, on the other hand, suggest that renal disease is also associated with increased availability of AA as a substrate for platelet cyclooxygenase possibly as a consequence of increased platelet AA content.

Unlike the bioamine 5-HT, intraplatelet histamine concentrations were unchanged in renal patients. Since both 5-HT and histamine are stored in dense granules, these findings suggest that the synthesis, release and/or uptake of these bioamines is controlled by different mechanisms.

In this study, it has also been demonstrated that there is a significant inverse correlation between intraplatelet 5--HT content and plasma TG concentrations. This is of interest since a) an intraplatelet 5-HT-TG interaction has not hitherto been investigated in renal patients and b) although platelet-TG interactions are less well documented, hypertriglyceridaemia may be a risk factor for coronary heart disease (Seymour and Byrne, 1993). In this context, it is of interest that NS patients have been shown to have increased platelet aggregability and TXA_2 release and both of these indices were related to TG concentrations (Jackson et al., 1982). Plasma TC and LDL-C, were inversely related to intraplatelet 5-HT in the present study. These findings are consistent with the known platelet activating properties of cholesterol (Carvalho et al., 1974) and LDL-C (Bruckdorfer, 1989) and are in agreement with the work of Guicheney et al.

(1988) who have shown that plasma TC is inversely related to both intraplatelet 5-HT concentration and the maximal velocity (V_{max}) for 5-HT uptake in essential hypertensive patients. It is possible that 5-HT uptake is also decreased in renal patients and this may contribute to the diminished intraplatelet 5-HT concentrations observed. From these findings it appears that the various mechanisms governing intraplatelet 5-HT concentrations (uptake, release and storage) may be both TG and TC sensitive. The observation that plasma HDL-C concentrations are positively correlated with intraplatelet ß-TG is consistent with the rest of the data and with the previous findings that this lipoprotein subfraction may inhibit agonist-induced platelet aggregation (Desai et al., 1989). It is noteworthy that intraplatelet ß-TG and histamine concentrations, as well as TXA_2 synthesizing capacity, did not correlate with TC and TG concentrations; this may indicate that these substances are influenced by other aspects of renal pathology whereas 5-HT mechanisms are more sensitive to the effects of TC and TG. The significant inverse correlation between intraplatelet 5-HT and plasma TG, TC and LDL-C concentrations suggests that lipid abnormalities in such patients may contribute to platelet activation and to the depletion of 5-HT which may in turn contribute to the pathogenesis of glomerular abnormalities and to accelerated atherosclerosis in renal disease (Lindner et al., 1974; Jacobson, 1991; Klahr, 1991).

Part 4

4.14 The effect of treatment with simvastatin on intraplatelet 5-HT and other platelet function indices in hypercholesterolaemia (Coumar et al., 1991)

It is widely accepted that hypercholesterolaemia is an important risk factor for atherosclerosis and is associated with an increased incidence of IHD (Chobanian, 1991). The mechanisms responsible for this relationship remain to be fully established but it is possible that hypercholesterolaemia mediates some of its effects by adversely influencing the haemostatic system (Miller et al., 1986). With respect to platelets there is evidence that hypercholesterolaemia is associated with platelet hyperactivity (Carvalho et al., 1974; Bruckdorfer, 1989) which is correctable upon lowering cholesterol (Schror, 1990). Furthermore, increasing the amount of cholesterol in platelet membranes, *in vitro*, is associated with enhanced platelet aggregability (Hassall et al., 1983), thromboxane production (Schick and Schick, 1984), Ca^{2+} mobilization (Strano et al., 1982) and phosphoinositide metabolism (Knorr et al., 1988) as well as reduced PGI_2 sensitivity (Strano et al., 1982; Hassall et al., 1983).

The findings presented in the renal study (see above, Part 3) suggest that plasma lipid concentrations may, to some extent, influence intraplatelet 5-HT concentrations. It was, therefore, appropriate to study patients with hypercholesterolaemia that were participating in an open study to investigate the effect of treatment with simvastatin (a 3-

hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitor; MSD Ltd., Hoddesdon, UK) on the lipid profile, platelet aggregation (in PRP and whole blood), TXA_2 release and intraplatelet 5-HT concentration.

4.15 Methods

A) Patient selection

Twelve hypercholesterolaemic patients (serum TC above 6.5 mmol/L) were selected for this study. Nine patients were males and 3 were females. Median age was 56 years (range 32-70). Three patients had symptoms of IHD (angina pectoris) and one had hyperuricaemia with episodes of gout in the past. Previous lipid-lowering medication which was discontinued before the commencement of simvastatin administration consisted of: bezafibrate 200 mg, three times daily (two patients) and cholestyramine, 4-24g, daily (four patients). Other medication which continued for the duration of the trial consisted of: nifedipine, 10 mg three times daily (one patient), nifedipine 5 mg, twice daily (one patient), atenolol, 100 mg daily (one patient), allopurinol, 300 mg daily (one patient). None of the patients were diabetic. Apart from the lipid variables, all patients had normal biochemical and haematological profiles on entry to the trial.

B) Design of trial

Patients were screened and their consent to participating in this study obtained. The screening period for each patient lasted for 6-8 weeks. During this period, patients

discontinued other lipid lowering drugs and were given appropriate dietary advice. Following this preliminary period, a fasting blood sample (Visit 1) was obtained between 9:00 and 11:00 am. Patients were put on a placebo for four weeks and sampled again at the end of this "run in" period. From then on, patients were treated with simvastatin and reviewed every four weeks. The initial dose of simvastatin (10 mg/daily) was administered immediately following Visit 2. After four weeks treatment, the patients were sampled again (Visit 3) and their dose of simvastatin was increased to 20 mg/daily, if the serum TC concentration was above 5.3 mmol/L. After another four weeks treatment, the patients were sampled again (Visit 4) and their dose of simvastatin was increased to 40 mg daily if, as above, the serum TC concentration was above 5.3 mmol/L. The final blood sample was obtained four weeks later (Visit 5). The duration of the total active treatment period was 12 weeks. During the trial period no drugs were added or discontinued and patients did not alter their dietary or smoking habits. The trial had the approval of the ethics committee of the RFH.

C) Tests conducted on blood samples

Lipids: In fasting serum, TC, HDL-C, LDL-C and TG were measured using methods in routine use (Department of Chemical Pathology and Human Metabolism, RFH). Table 4.8 shows the concentrations of lipids at various points during the trial.

Intraplatelet 5-HT determination: Blood processing and platelet lysate preparation was carried out as described above (see Part 2, section 4.7). 5-HT was assayed using a radioimmunoassay kit (Biogenesis Ltd; described in Part 3, section 4.11).

Platelet aggregation and platelet thromboxane A_2 (TXA_2) release: Aggregation was assessed in whole blood using the Chronolog whole blood impedance aggregometer (WB-IA) (as described in Chapter 3) and in PRP (as described in Chapter 2) using the Born-type optical aggregometer (Barradas et al., 1990a). The release of TXA_2 was quantified after inducing aggregation in PRP. A detailed presentation of these results was published separately (Coumar et al., 1991).

D) Statistical analysis: The Wilcoxon rank sum paired test and the Spearman's correlation coefficient (r_s) was used to assess correlations between values. All tests were two-tailed. The results are presented as median and (range).

4.16 RESULTS

A) Tests conducted on blood samples

Lipids: Simvastatin significantly decreased serum TC and LDL-C concentration. These changes occurred within 4 weeks of treatment (Table 4.8). Since there is evidence linking hyperfibrinogenaemia with hyperlipidaemia (Simpson et al., 1983) and plasma fibrinogen concentration is a powerful predictor of IHD (Mead et al., 1986), plasma fibrinogen was

also assessed before and after treatment with simvastatin. This parameter was found to be unchanged by simvastatin intake.

Platelet aggregation and platelet thromboxane A_2 (TXA_2) release: 12 weeks treatment resulted in significant inhibition of PRP aggregation and TXA_2 release (Coumar et al., 1991). When values at Visit 2 (basal value-pretreatment) and those at Visit 5 (12 weeks treatment) were considered together, there was a significant correlation between aggregation induced by ADP (10 μmol/L) and the corresponding TC and LDL-C concentration. Thus, serum TC and ADP aggregation: r_s=0.58, P=0.005; serum LDL-C and ADP aggregation: r_s=0.55, P=0.007. There was a significant fall in the release of TXA_2 induced by platelet agonists (collagen and adrenaline). WB-IA responses were unchanged (Coumar et al., 1991).

Intraplatelet 5-HT determination: There was a significant (P=0.03) increase in the intraplatelet concentration of 5-HT after 12 weeks treatment with simvastatin. Thus, the median intraplatelet 5-HT concentration (nmol/10^9 platelets) was 1.73 (0.38-4.82) at Visit 2 and 3.23 (0.90-5.39) at Visit 5. Intraplatelet 5-HT correlated significantly with serum TC and LDL-C concentration. Thus, for TC and intraplatelet 5-HT: r_s=-0.50, P=0.01; for LDL-C and intraplatelet 5-HT: r_s=-0.65, P=0.001.

TABLE 4.8

Median and (range) lipid concentrations (mmol/L)

	VISIT				
	1	2	3	4	5
TC	8.9 (6.7-12.3)	8.0 (6.5-12.9)	6.5*,$ (4.8-9.1)	6.4*,$ (4.2-8.5)	6.2*,$ (4.6-7.1)
TG	1.5 (0.9-3.8)	1.4 (0.6-4.0)	1.5 (0.9-4.2)	1.1+ (0.8-3.2)	1.2+ (0.5-2.2)
LDL-C	6.7 (4.8-10.1)	6.4 (4.5-10.1)	4.2*,$ (2.9-6.7)	4.4*,$ (2.5-6.7)	4.0 (2.8-6.0)
HDL-C	1.1 (0.6-2.0)	1.1 (0.6-1.5)	1.3 (0.7-2.0)	1.3 (0.9-1.8)	1.1 (0.7-1.8)

On some visits, only 11 of the total 12 samples were available for analysis.

Statistical Analysis:

Cholesterol:
Visit 1 Vs. Visit 2: P=NS
* P<0.005 Vs. Visit 1;
$ P<0.005 Vs. Visit 2. All other comparisons: P=NS.

Triglycerides:
Visit 1 Vs. Visit 2 or 3: P=NS;
+ P<0.02 Vs. Visit 1;
Visit 2 Vs. Visit 3, 4 or 5: P=NS;
Visit 3 Vs. Visit 4: P<0.01. All other comparisons: P=NS.

LDL-C:
Visit 1 Vs. Visit 2: P=NS
* P<0.005 Vs. Visit 1;
$ P<0.005 Vs. Visit 2. All other comparisons: P=NS.

HDL-C:
Visit 2 Vs. Visit 3: P=0.04; Visit 2 Vs. Visit 4: P=0.01
All other comparisons: P=NS.
NS=not significant

4.17 DISCUSSION

The intraplatelet 5-HT concentration results presented above indicate that the correction of hypercholesterolaemia, in addition to normalising platelet hyperactivity, may also normalise the platelet 5-HT imbalance. These results support the notion that abnormalities of platelet function in these patients are secondary to an interaction between platelets and lipids rather than to primary platelet hyperactivity. In support of these suggestions is the observation of a direct interaction between platelets and LDL-C through binding (Virgolini et al., 1993), causing platelet aggregation (Andrews et al., 1987) and PSC (Pletscher et al., 1989). There is ample evidence for platelet activation in patients with hypercholesterolaemia and damaged vascular endothelium (Carvalho et al., 1974; Bruckdorfer, 1989; van Rensburg and du P. Heyns, 1990). The significant correlation between intraplatelet 5-HT and TC/LDL-C observed in this study is also in agreement with the findings of the renal study (Part 3, above). Overall, these observations suggest that TC and possibly membrane cholesterol have a profound effect in modulating platelet 5-HT (uptake/release) and/or platelet activity (aggregation and release). It is important to note that, although platelet aggregation was reduced by various agonists (AA, ADP and collagen) following simvastatin treatment, only ADP-induced aggregation correlated significantly with both TC and LDL-C concentration. This association suggests that cholesterol content does not influence platelet function solely by altering TXA_2 release

since ADP-induced aggregation is largely independent of the synthesis of this eicosanoid (Gerrard and White, 1976; Best et al., 1981; Mikhailidis et al., 1983b). The absence of any significant effect on WB-IA probably reflects a lack of sensitivity of this technique and the high concentrations required to obtain a response (briefly discussed in Chapter 3; Barradas et al., 1992b).

CHAPTER 5

PLATELET 5-HT UPTAKE AND RELEASE STUDIES

CHAPTER SUB-INDEX

5.1 INTRODUCTION

In Chapters 3 and 4, disease states associated with enhanced platelet aggregation/atherosclerosis and increased bioavailability of 5-HT were reported (e.g. PVD). It is not clear, however, how 5-HT becomes available in the above disease states.

In study 1 (below), stirring and the effect of various doses of platelet agonists (ADP, adrenaline, collagen) were used, *in vitro*, to assess their effect on platelet 5-HT uptake. Low doses of agonists have previously been shown by us to enhance histamine uptake (Gill et al., 1987). In addition, these concentrations of agonists may be encountered in pathological situations (e.g. low concentrations of ADP derived from aggregating platelets or ruptured red cells during thrombotic episodes) and subendothelial collagen fibres may become exposed following mechanical injury (e.g. angioplasty), or as a result of endothelium denudation (e.g. following smoking; Pittilo, 1982; 1990). Mild platelet activation may affect 5-HT uptake or release mechanisms altering plasma and intraplatelet 5-HT concentrations. In this Chapter, both these variables were investigated.

The effect of various anti-platelet drugs was assessed for their effect on 5-HT uptake (study 2). Since platelets can release, as well as uptake 5-HT, experiments were set up to assess whether stirring or low-dose (pre-aggregation) concentrations of agonists can induce the release of 5-HT (study 3).

5.2 METHODS

Subjects

Blood collected from 7 healthy volunteers, median age 36 (range 22-43), 5 males, 2 females; was anticoagulated with citrate (solution A, Appendix). PRP was prepared by centrifugation of citrated blood as outlined in Chapter 3. Platelet counts in PRP were estimated using a Coulter ZM.

Study 1: 5-HT uptake experiments

In experiments designed to evaluate the effect of stirring, the stirring mechanism on a Chronolog aggregometer (model 540) was switched off for those experiments not requiring stirring. For all other experiments, PRP was stirred (1000 rpm at 37°C). Agonists (ADP, adrenaline, collagen) were added as 10-30 μL volumes to PRP to achieve the final concentrations (f.c.) shown in the Figures. Radiolabelled 5-HT (^{14}C-5-HT, 57 mCi/mmol; f.c. 200 nmol/L) was added simultaneously with the agonists or 5 min following the addition of agonists. Addition of 5-HT was done at these time points to detect the immediate effects of agonists or those after a period when platelets have undergone PSC and/or aggregation. The uptake was studied over a period of 1 (rapid phase of uptake) and 10 min (storage phase). The concentration of 5-HT (200 nmol/L) was selected since it lies below the K_M for the uptake reaction (K_M=0.6-0.8 μmol/L; Wielosz et al., 1976) and is, therefore, ideal to assess the of 5-HT uptake stimulators and inhibitors. Uptake of ^{14}C-5-HT was terminated by adding a 1/10 volume of ice cold mixture of formaldehyde

(f.c. 1.5% v/v) and indomethacin (f.c. 16 μmol/L). The PRP was placed in microcentrifuge tubes and spun at 10,000 x g for 90 sec. The plasma was discarded and the platelet pellet was washed and sonicated as described in Chapter 4. ^{14}C-5-HT in the pellet was estimated by liquid scintillation in a ß-counter. The concentration (pmol) of 5-HT taken up by the platelet was calculated from the counts per min (cpm), having established the total cpm (12 000) after the addition of 200 nmol/L of ^{14}C-5-HT. Platelet ^{14}C-5-HT uptake was estimated per 10^9 platelets.

Study 2: Effect of anti-platelet drugs on 5-HT uptake

These experiments were set out to study the effect of anti-platelet drugs on 5-HT uptake. Experimental design (i.e. start of ^{14}C-5-HT uptake and termination) was carried out in the same manner as for the experiments described above. The volunteers for study 1 were also used for this study. Anti-platelet drugs were pre-incubated with PRP for 5 min before the addition of 200 nmol/L ^{14}C-5-HT.

Study 3: Effect of platelet activation on 5-HT release

PRP, collected (5 males, 2 females; median age: 34 years; range: 24-43) and prepared as described above. Two sets of experiments were carried out. The first set, utilized PRP that had been pre-incubated with ^{14}C-5-HT for 60 min before commencement of experiments. In the second set, no pre-incubation with ^{14}C-5-HT took place and the PRP was used following a short period (10-15 min) at 37°C to stabilize the

PRP. In both sets of experiments at the end of the pre-incubation phase the PRP was treated with 2 μmol/L imipramine (5 min) to block 5-HT re-uptake (Wielosz et al., 1976). At the end of the time points shown in Tables 5.2 and 5.3, an aliquot (100 μL) of the PRP was taken and treated with glutaraldehyde for counting and sizing analysis (see Chapter 2, for count and size analysis procedure) and the remainder (400 μL) was immediately added to an ice cold 1/10 volume solution of EDTA (f.c. 10 mmol/L) and indomethacin (f.c. 16 μmol/L). The PRP was centrifuged and the plasma collected as described above. The plasma from the first set of experiments, contained ^{14}C-5-HT which was counted using liquid scintillation techniques as described for the 5-HT uptake studies (see above). In the second set of experiments, the plasma containing only endogenous 5-HT was stored at -40°C until assay for 5-HT (within 1 month). 5-HT measurements were carried out using an enzyme immunoassay (EIA) method using kits purchased from Immunotech, France.

5-HT enzyme immunoassay (EIA)

The assay procedure is described in detail by Chauveau et al. (1991). This EIA has the lowest detection limit of any previous RIA and, unlike other techniques, allows the measurement of a large number of samples in approximately 3 hours (Chauveau et al., 1991).

Statistical analysis and presentation of results

Results in Tables are expressed as median and (range).

For diagrammatic purposes results are occasionally presented as means ± SEM. All results were statistically analyzed (C-STAT) using the Wilcoxon rank sum test (two-tailed).

5.3 RESULTS

Study 1: 5-HT uptake experiments

The addition of low doses of agonists to PRP (collected from healthy subjects) did not significantly affect ^{14}C-5-HT uptake whether ^{14}C-5-HT was added simultaneously or after a 5 min pre-incubation period with platelet agonists. Figure 5.1 shows the simultaneous addition of ^{14}C-5-HT and ADP or adrenaline and Figure 5.2 the simultaneous addition of ^{14}C-5-HT for collagen. Identical results to those shown on Figure 5.1 and 5.2 were obtained when ^{14}C-5-HT was added 5 min after the addition of agonists. ^{14}C-5-HT uptake was not significantly different between samples that were stirred (at 1000 rpm) or not stirred. Samples that were stirred, absorbed 125 (range 78-182) pmol/10^9/min and 280 (range 186-431) pmol/10^9/10min of ^{14}C-5-HT. Unstirred samples absorbed 131 (range 80-190) pmol/10^9/min and 275 (range 183-431) pmol/10^9/10min of ^{14}C-5-HT. At higher concentrations of agonists (which induce platelet aggregation and membrane disruption), 5-HT uptake was significantly inhibited (Figure 5.1 and 5.2). Samples that were pre-treated with agonists for 5 min were similarly affected. At the higher concentrations of agonists, microaggregate formation was observed with the naked eye. Counts were not done on these samples so as not to contaminate the platelet counting equipment with

radioactivity. Thus, at 1 μmol/L and 2.5 μmol/L adrenaline, 4 (out of 7 subjects) showed appreciable aggregation; at 5 μmol/L adrenaline, 6 subjects showed appreciable aggregation for both 1 min and 10 min ^{14}C-5-HT uptake. With ADP, appreciable aggregation was observed at 2.5 and 5 μmol/L concentrations for the 1 min uptakes and for 10 min uptakes appreciable aggregation was observed at 1, 2.5 and 5 μmol/L. Six (out of 7) subjects showed appreciable aggregation to collagen at 0.5 and 1.0 mg/L for the 1 min and 10 min uptakes ^{14}C-5-HT.

Study 2: Effect of anti-platelet drugs on 5-HT uptake

Drugs with established anti-platelet activity: prostaglandin E_1 (PGE_1; a cAMP elevator), sodium nitroprusside (NaNP; a cGMP elevator) and milrinone (MIL; a phosphodiesterase inhibitor) did not affect 5-HT uptake at concentrations which inhibit platelet aggregation and platelet shape change (Table 5.1). Naftidrofuryl (NAF) at high concentrations (> 50 μmol/L) significantly inhibited 5-HT uptake (Figure 5.3). In order to establish that the method used could effectively detect enhancement or inhibition of 5-HT uptake, hydrogen peroxide (H_2O_2; for enhancement) and imipramine (for inhibition) were tested under the same experimental conditions as used for the platelet agonists and anti-platelet drugs described above (Figure 5.3).

Study 3: Effect of platelet activation on 5-HT release

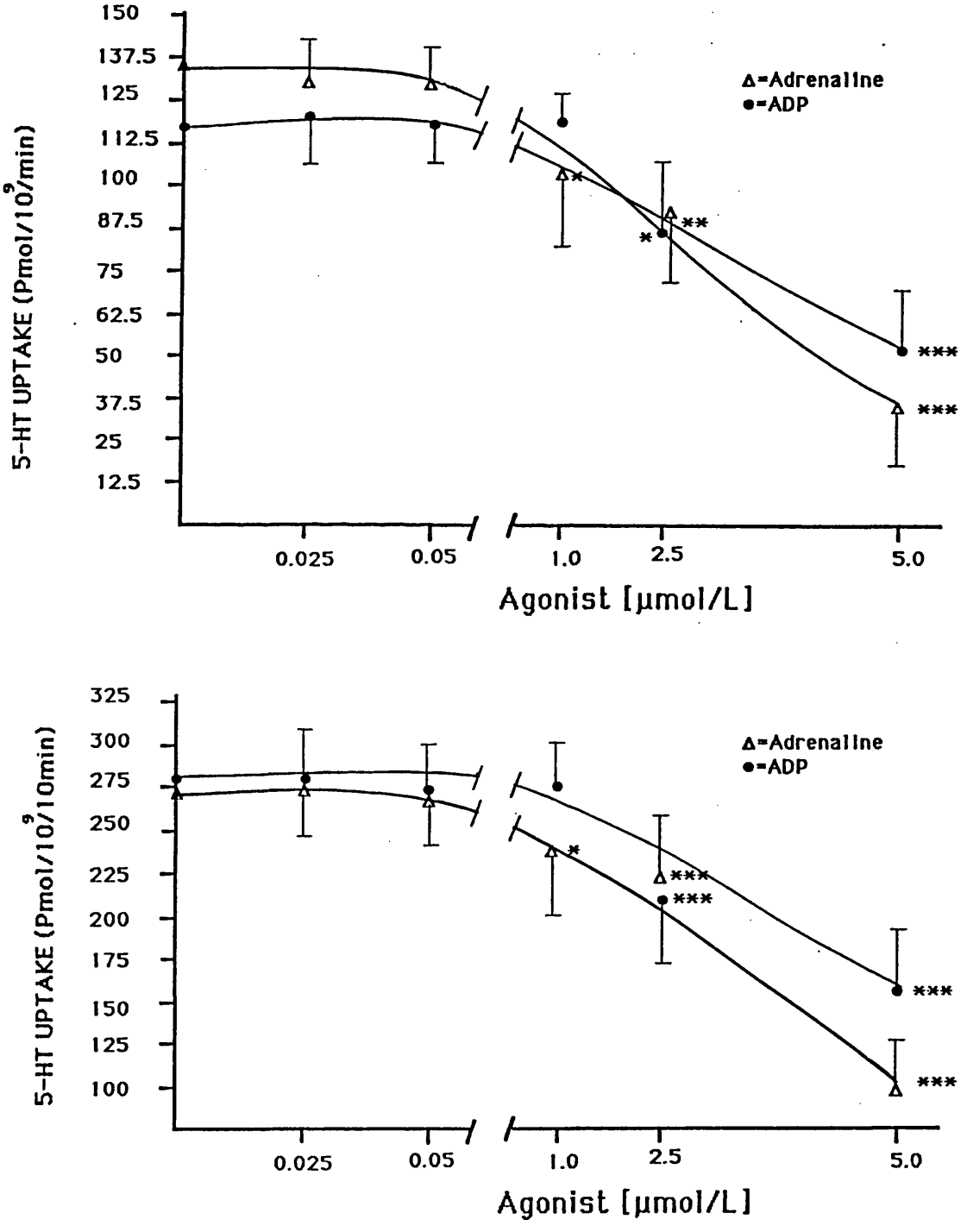
Low concentrations of agonists which cause platelet shape

change (but no aggregation) or stirring only (1000 rpm) induced significant release of endogenous 5-HT (Table 5.2 and 5.3). In contrast, experiments where 5-HT release was assessed by measuring ^{14}C-5-HT efflux showed no significant release following mild stimulation with stirring or agonists. Thus, median and (range) ^{14}C-5-HT release, in pmol/10^9 platelets following saline incubation was: 468 (393-486) ; ADP (0.2-0.25 μmol/L) was: 444 (388-488); U 46619 (0.035-0.05 μmol/L) was: 478 (389-491); collagen (0.2-0.3 mg/L) was 460 (378-488). A total of 4 subjects were studied.

Endogenous 5-HT release varied according to the agonist used (Table 5.2 and 5.3). Stirring alone induced significant release of 5-HT in a time-dependent manner without inducing platelet shape change or aggregation (Table 5.2).

Fig 5.1

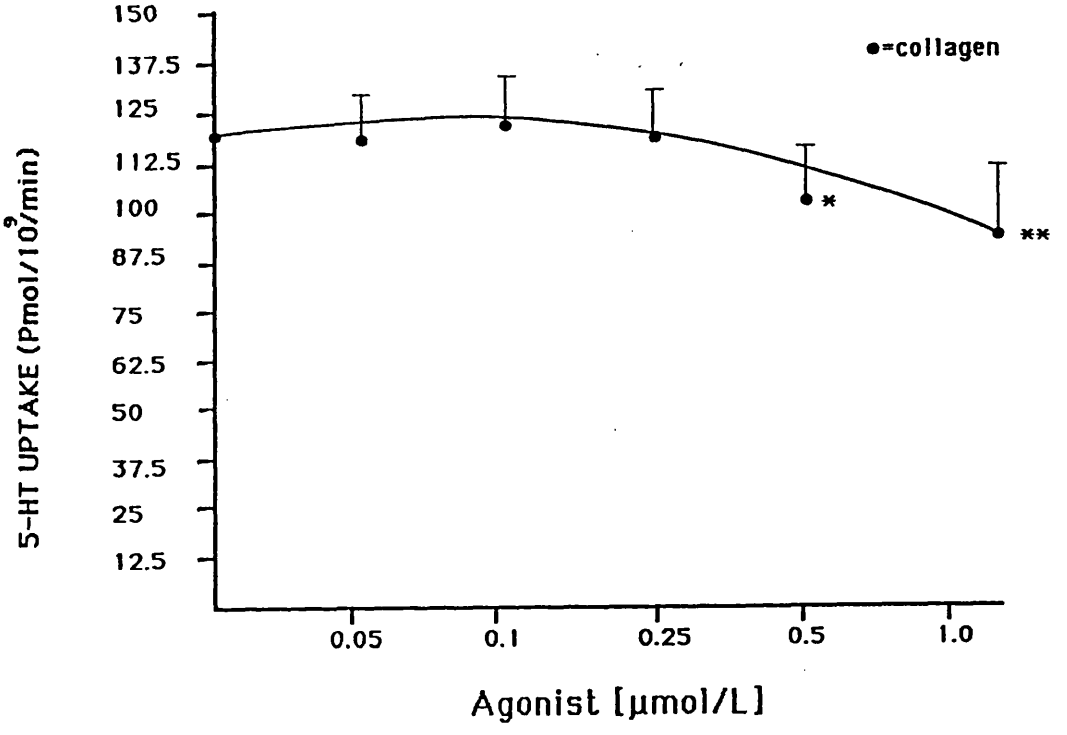
Effect of low and high concentrations of adrenaline and ADP on ^{14}C-5-HT uptake

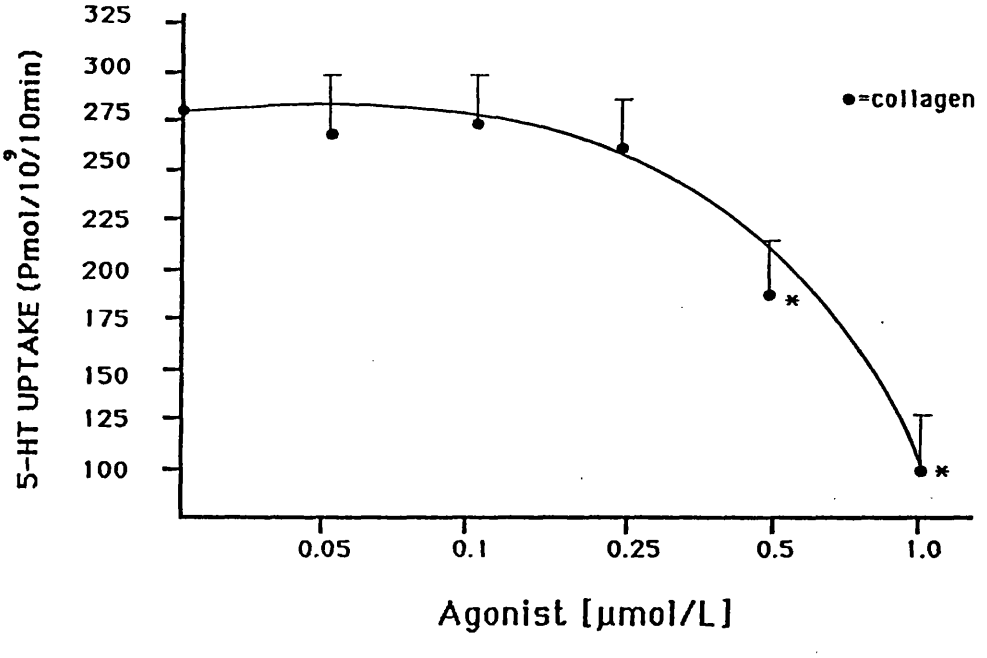


Statistical analysis: * P< 0.03, ** P< 0.02, *** P< 0.01 Vs. saline (control).

Fig 5.2

Effect of low and high concentrations of collagen on ^{14}C-5-HT uptake

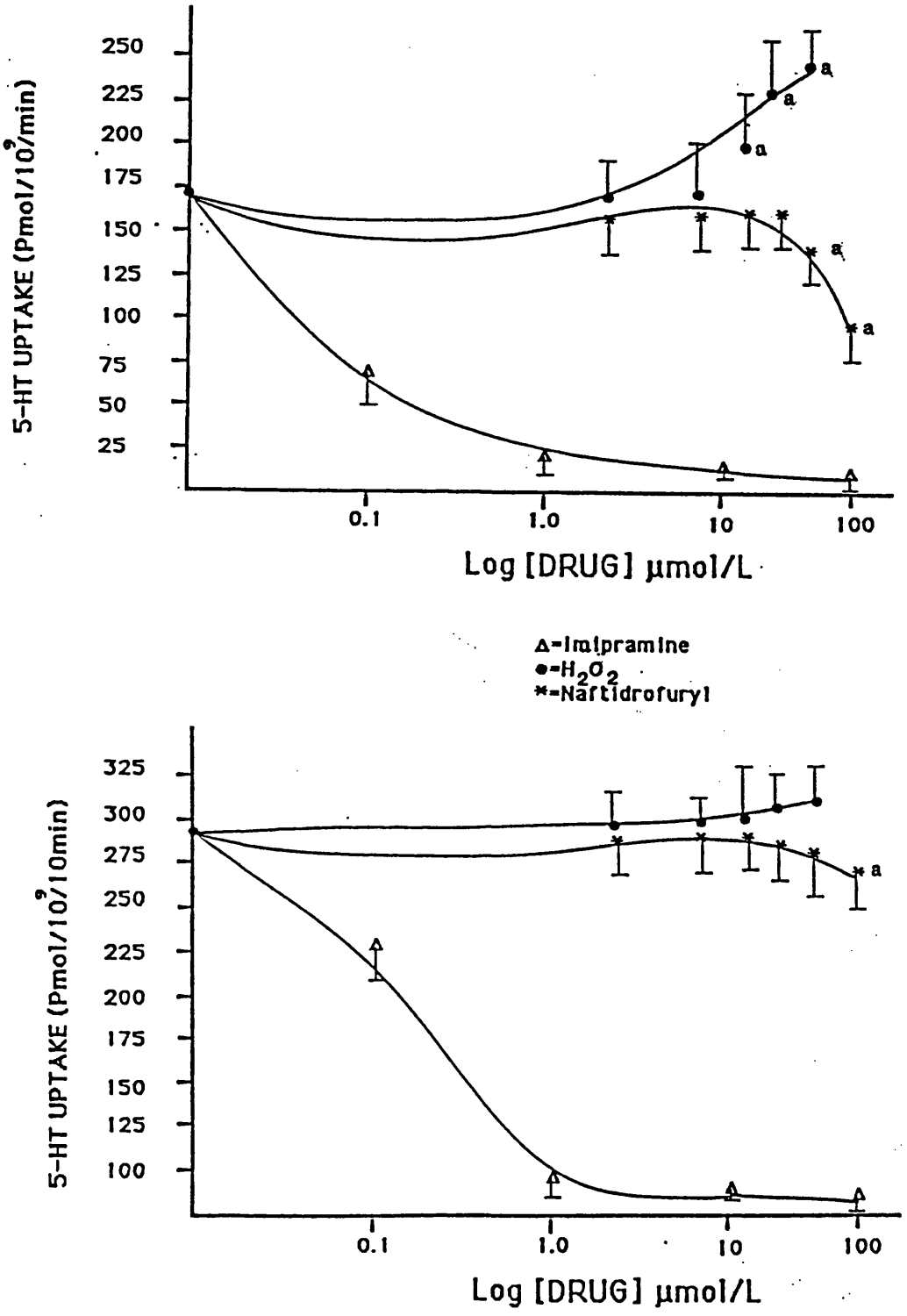




Statistical analysis: * P< 0.02,
** P< 0.01 Vs. saline (control).

Fig 5.3

Effect of various concentrations of imipramine, naftidrofuryl and H_2O_2 on ^{14}C-5-HT uptake



Statistical analysis: a $P< 0.02$ Vs. saline (control). For imipramine, all concentrations $P<0.02$ Vs. saline (control).

TABLE 5.1

Effect of PGE_1, MIL and NaNP on ^{14}C-5-HT uptake

	[μmol/L]	5-HT uptake 1 min pmol/10^9	5-HT uptake 10 min pmol/10^9
PGE_1 n=4	0	166 (132-187)	285 (186-377)
	0.025	167 (135-190)	290 (186-368)
	0.25	170 (132-179)	291 (188-388)
	0.50	166 (133-185)	290 (184-376)
MIL n=7	0	172 (105-185)	288 (180-370)
	1.25	173 (109-187)	295 (178-368)
	2.5	177 (105-188)	298 (181-366)
	5	178 (105-187)	299 (187-366)
	10	177 (103-183)	297 (188-391)
NaNP n=4	0	152 (84-172)	273 (168-345)
	10	158 (88-180)	273 (170-346)
	50	167 (83-171)	271 (171-350)
	100	164 (88-165)	270 (166-344)

PGE_1=prostaglandin E_1; MIL=milrinone; NaNP=Sodium nitroprusside. n=number of subjects studied.

Plasma ^{14}C-5-HT added=200 nmol/L.

Statistical analysis: All comparisons against vehicle-control= not significant.

TABLE 5.2

Effect of stirring on platelet count, platelet volume and 5-HT release

	Platelet Count (X 10^9/L)	Platelet Volume (10^{-15} L)	Plasma 5-HT (nmol/L)
pre-stirring	272 (236-360)	5.56 (5.09-5.88)	14.7 (10.1-16.0)
30 sec stirring	264 (244-328)	5.58 (5.15-5.88)	16.9* (12.0-19.4)
3 min stirring	280 (244-360)	5.52 (5.13-5.94)	21.3* (11.7-24.9)
6 min stirring	264 (240-352)	5.58 (5.00-5.58)	25.8* (15.6-29.3)
15 min stirring	264 (236-325)	5.45 (5.15-5.82)	32.1* (20.9-53.8)

* P< 0.02 Vs. pre-stirring. Number of subjects studied=7

TABLE 5.3

Effect of ADP, U46619 and collagen on platelet count, platelet volume and 5-HT release

	Platelet Count (X 10^9/L)	Platelet Volume (10^{-15} L)	Plasma 5-HT (nmol/L)
Sal (control)	264 (220-356)	5.58 (5.15-5.88)	17.8 (14.3-19.4)
ADP 0.2-0.25 μmol/L	260 (244-352)	6.29* (5.58-6.81)	28.8* (18.8-39.5)
U46619 0.035-0.05 μmol/L	276 (232-380)	6.17* (5.52-6.59)	37.2* (26.6-45.4)
collagen 0.2-0.3 mg/L	272 (220-356)	6.36* (5.86-6.97)	67.5* (52.4-97.7)

* P<0.02 Vs. saline (control; 30 sec stirring). Number of subjects studied=7.

5.4 DISCUSSION

5-HT uptake experiments (studies 1 and 2)

It is important to establish if minor platelet activation has an affect on 5-HT uptake and explain the plasma and platelet imbalance (i.e. more 5-HT in the plasma and less inside platelets) demonstrated in conditions (e.g. DM, PVD, renal disease, hypercholesterolaemia) associated with platelet hyperaggregability (Chapter 4). Previous work in our laboratory has shown that histamine, a vasoactive amine co-stored with 5-HT in platelet dense granules and taken up by platelets (Fukami et al., 1984), could be taken up in significantly larger amounts following the addition of low doses of agonists to PRP (Gill et al., 1987). In contrast, the present results demonstrate that the net 5-HT taken up into platelets is not enhanced by mild activation (stirring, ADP, adrenaline or collagen). It is conceivable that, in the above experimental conditions, mild activation could have enhanced both uptake and release to a similar extent. It was, therefore, important to assess 5-HT release in a situation where uptake could be effectively blocked (e.g. in the presence of imipramine; Wielosz et al., 1976).

The lack of effect of agonists and stirring on 5-HT uptake are consistent with the observations of Frojmovic and Rotman (1980), and those of Costa and Murphy (1977). The former group studied the effects of ADP (pre-incubated for 2 min) on ^{3}H-5-HT uptake in PRP at room temperature, and the latter studied the effect of both thrombin and calcium ionophore added simultaneously with ^{3}H-5-HT in washed platelet

preparations. These studies and the present results, contrast with the findings of Drummond and Gordon (1978), who found ADP (0.3-6.6 μmol/L) to be a potent inhibitor of 5-HT uptake in rat platelets. These workers also found that the effect of ADP disappeared as the length of the pre-incubation period with ADP increased from 1 to 20 min. It is not clear how 5-HT uptake by rat platelets is susceptible to the effects of ADP.

It is of interest that H_2O_2, produced during phagocytic cell activation, ischemia-reperfusion injury, or as a result of redox cycling of drugs and environmental toxins, stimulates 5-HT uptake (Bosin, 1989) and also enhances platelet aggregation induced by collagen (Pratrico et al., 1992). H_2O_2 is normally metabolised by catalase and glutathione peroxidase. If H_2O_2 escapes, the above enzymatic defence mechanisms it may come into contact with platelets promoting platelet activation and 5-HT uptake. How H_2O_2 enhances 5-HT uptake is not clear; it may influence the Na^+K^+-ATPase system which is linked to 5-HT transport or affect a protein (M_r 45,000) present in plasma which appears to act as a modulator of the 5-HT transport complex (Abraham et al., 1987). In the present experiments, H_2O_2 was deliberately chosen in order to include a control substance that enhances 5-HT uptake.

With regard to high concentrations of agonists, the present findings are in agreement with all previous studies showing that agonists diminish net 5-HT uptake. This effect probably occurs due to a) net 5-HT release and b) major membrane disruption as a consequence of aggregate formation (Costa and Murphy, 1977; Drumond and Gordon, 1976; Frojmovic

and Rotman, 1980).

The fact that 5-HT uptake was not affected, *in vitro*, by anti-platelet agents that inhibit platelet aggregation and shape change, demonstrates a separation between these aspects of platelet function. On the other hand, high concentrations of NAF inhibited 5-HT uptake, a phenomenon previously observed with drugs possessing similar membrane stabilizing properties (e.g. popranolol; Grobecker et al., 1973).

Study 3: Effect of platelet activation on 5-HT release

It is important to establish whether platelets can release 5-HT following mild activation. Such a phenomenon may explain, in part, why, in various conditions associated with platelet hyperactivity (e.g. PVD), raised plasma 5-HT and diminished intraplatelet 5-HT concentrations are observed. Secondly, from a physiological point of view, it is important to establish when release/degranulation of 5-HT occurs (i.e. before, during or after platelet shape change and aggregation). This information will have a bearing on future anti-platelet drug development and may help clarify the role of platelets in atherogenesis.

The results presented above demonstrate that platelets release 5-HT in the absence of aggregation and platelet shape change. Activating platelets by simply stirring them at 1000 rpm induces, in a time-dependent manner, a significant release of endogenous 5-HT. These results, obtained with a highly sensitive EIA, contradict the suggestion that platelet 5-HT release is a phenomenon occurring following appreciable aggregation (Packham et al., 1977) and demonstrate that

release/degranulation are events occurring early on, prior to platelet aggregation and shape change, or in tandem with these processes. These results also confirm the concern expressed by previous workers that radiolabelled 5-HT methods lack the sensitivity required to detect 5-HT release following mild activation (Haslam and Rosson, 1972).

In agreement with the proposition that release of intraplatelet substances may occur independently of platelet aggregation is the work of Balduini et al. (1988) and Chronos et al. (1993). The former group showed that, in the absence of stirring and platelet aggregation, thrombin induced ATP release and thromboxane A_2 formation to the same extent as that observed in stirred samples. These workers also showed that, in the presence of stirring and the GPIIb/IIIa antagonist RGDS, aggregation induced by thrombin was markedly inhibited (19% of control response) but ATP and thromboxane A_2 release were only slightly affected (75% and 79% of control, respectively). Chronos and colleagues (1993) used flow cytometry, a sensitive technique which uses fluorescently-conjugated antibodies to study the expression of glycoproteins on the surface of resting and activated platelets. These workers demonstrated that α-granule protein expression and α-granule release (ß-TG) occurs, *in vitro*, following incubation with low osmolar radiographic contrast media used in interventional cardiology. This contrasts with the inhibitory effects that such media have on platelet aggregation (Greenbaum et al., 1987). Overall, these studies suggest that, at least *in vitro*, the release of platelet contents can occur

in situations where aggregation is blocked (e.g. RGDS, contrast media) or prevented (e.g. absence of stirring).

In vivo evidence that platelets release 5-HT whilst circulating has come from the work of Osim and Wyllie (1983). In their study, they used doubly radiolabelled rat platelets (^{14}C-5-HT and ^{111}In oxine) to determine the fate of 5-HT. Platelets and various tissues were analyzed for the tracer compounds in order to determine the recovery and location of platelets and ^{14}C-5-HT. The sampling of tissues revealed that the $^{14}C/^{111}In$ ratio deviated (>1) from that found in platelets. Tissues which accumulated ^{14}C-5-HT, included the adrenals, thyroid, gut and bladder suggesting that under normal conditions platelets deposit 5-HT in these tissues. Osim and Wyllie (1983) concluded that the transfer of 5-HT to specific tissues took place by an active process which did not involve irreversible platelet aggregation. It is also of interest that Larsson et al. (1992) have recently shown that pathophysiological concentrations of adrenaline infused into healthy volunteers was associated with increased plasma non-esterified fatty acids (NEFA), platelet aggregability and plasma ß-TG concentrations. The concentrations of adrenaline achieved in these subjects were comparable to those achieved following myocardial infarction or shock. Since NEFAs undermine the vascular ADPase system (Barradas et al., 1987a) and PGI_2 stability (Mikhailidis et al., 1983c), the release of catecholamines and the subsequent activation of platelets and release of other platelet derived products (e.g. 5-HT) could lead to further thrombotic complications.

CHAPTER SIX

GENERAL DISCUSSION

In this thesis, various aspects of platelet function (e.g. PSC, platelet aggregation and the measurement of platelet-derived substances) were investigated mainly in PVD patients but also in patients with DM, renal disease and hypercholesterolaemia. All of these patients have in common an increased mortality rate from cardiovascular events. The objective of this thesis was to further explore abnormalities in platelet function and in platelet-derived substances, particularly 5-HT, in order to better understand the role of platelets in the pathogenesis of the vascular complications of these conditions. In addition, new methods were established to study alterations in PSC, platelet aggregation and platelet release substances and to improve the evaluation of anti-platelet drugs. Given that the results presented in each Chapter have already been discussed in some detail, this discussion will highlight the more important implications of the findings and offer suggestions for future investigation.

Using a new method, 5-HT-induced PSC was investigated in PRP obtained from PVD patients and shown to be diminished, when compared with young healthy subjects. Bearing in mind that platelet hyperaggregability to 5-HT has been demonstrated in PRP (The PACK study, 1989) and in whole blood (Chapter 3) of PVD patients, diminished PSC responses to this agonist suggests that PSC may be occurring through receptor-linked

mechanisms which are different from those initiating platelet aggregation. Suggestions that platelet aggregation and PSC phenomena occur through different receptors are sparse (see Vittet et al., 1991; Hanasaki and Arita, 1991), but may become more firmly established when PSC phenomena are more widely investigated with sensitive methods such as those presented in Chapter 2 or when specific ligands that trigger PSC and platelet aggregation independently become available. It is possible that hyperaggregable states, such as PVD, cause a desensitization or refractoriness to PSC, particularly that induced by 5-HT (since responses to ADP were unaltered in PVD patients). This phenomenon may represent a defence mechanism which limits further activation in a condition associated with raised plasma concentrations of 5-HT (Chapter 4). This desensitization may result from alterations in intracellular signalling mechanisms or following alterations in receptor counts and/or affinity. In this context, the examination of other hyperaggregable conditions, preferably unrelated to atherosclerosis, may throw light into the relevance of altered bioamine concentrations as modulators of the PSC response. For example, it has been documented that anorexia nervosa patients exhibit marked platelet hyperaggregability to adrenaline and diminished plasma noradrenaline concentrations (Luck et al., 1983). The platelet hyperaggregability was corrected upon weight gain (Mikhailidis et al., 1986). Furthermore, patients with low weight also exhibited significantly increased platelet total α-adrenoceptor counts which was corrected upon weight gain (Luck et al., 1983; Mikhailidis et al., 1986). In

PVD, the mirror image of this phenomena may be taking place with regard to 5-HT. Thus, it would be of interest to determine whether PVD is associated with diminished 5-HT receptor counts as a result of excessive plasma 5-HT concentrations and whether this normalizes following anti-platelet therapy. In relation to intracellular mechanisms, there is now evidence that PK-C mediated feedback mechanisms contribute to diminished calcium mobilization and phosphoinositol metabolism upon re-stimulation with 5-HT (Kagaya et al., 1990). This *in vitro* evidence is in agreement with the desensitization phenomena observed in patients and in the *in vitro* experiments carried out with PRP obtained from healthy subjects (Chapter 2). This line of research, although likely to be important in improving our understanding of the control of platelet activation is presently fraught with problems due to the lack of specificity of existing PK-C inhibitors and the multitude of PK-C isoforms (Wojcikiewicz et al., 1993). A further complication is that the effect of PK-C activation is probably to regulate G-proteins which are themselves a "family" for which specific inhibitors for all elements are lacking. Notwithstanding these current deficiencies, the PSC method presented in Chapter 2 allows the measurement of the early phase of platelet activation with ease and reproducibility which should encourage further studies. In particular, synthetic PK-C stimulators, such as phorbol esters and synthetic diacylglcerol (e.g. oleoylacetylglycerol), should be used to try to pinpoint the intracellular mechanisms leading to the quiescence of

platelets. The benefit of unravelling these mechanisms is that novel drugs could be developed as anti-platelet agents. It should be emphasized, however, that the present PSC method detects only certain aspects of PSC, i.e. "spheration". For example, this method is unable to detect adrenaline-induced PSC which involves mainly pseudopod formation. This drawback may be partially circumvented by using electron microscopy techniques. With regard to anti-platelet effects on PSC, the work carried out with naftidrofuryl and milrinone demonstrates that this is a more sensitive method than platelet aggregation at detecting anti-platelet effects of drugs *in vitro*. PSC may therefore also be an ideal way to detect anti-platelet effects of drugs *ex vivo*. Clinical trials are under way to establish whether this is true.

The platelet aggregation studies presented in this thesis were carried out using a WB-FPC method after extensive experience with the WB-IA method and optimization of the present WB-FPC method. Using this methodology, aggregability to a range of platelet agonists, including 5-HT, was found to be enhanced in PVD patients when compared to younger healthy subjects. These results have been confirmed recently by Walters and colleagues (1993) with age-matched controls. The platelet aggregation results carried out with PVD patients have demonstrated the ineffectiveness of low dose ASA intake (150 mg/alternate days) in preventing aggregability induced by 5-HT. ASA addition, *in vitro*, was also incapable of reducing 5-HT-induced WB-FPC. The fact that other drugs such as naftidrofuryl and milrinone were able to reduce WB-FPC

aggregation strongly suggests that patients with platelet hyperactivity should be prescribed additional or more effective drugs than low dose ASA. Repeated reports in the recent literature suggest an increased interest in evaluating higher concentrations of ASA or other antiplatelet drugs (e.g. ticlopidine; see Walters et al., 1993; De Lorgeril et al., 1993; Barradas and Mikhailidis, 1993b).

In this project, various experiments and studies were carried out in order to determine the 5-HT status in the plasma and platelets of patients (PVD, DM, renal and hypercholesterolaemic) and healthy subjects. The finding of reduced intraplatelet 5-HT concentrations and raised plasma 5-HT concentrations in these patients may explain the vicious cycle of platelet hyperaggregability and ischaemia observed in these conditions. This situation may be due to 5-HT since this bioamine is a potentiator of platelet aggregation with vasoconstrictory, mitogenic and vascular permeability enhancing effects. Given this scenario it is desirable to reduce 5-HT bioavailability either by influencing platelet activity directly or indirectly. It is, therefore, of interest that, having found an association between intraplatelet 5-HT and plasma cholesterol/plasma triglyceride, a second smaller study with hypercholesterolaemic patients documented a diminution in cholesterol accompanied by a diminution in platelet aggregability and an increase in intraplatelet 5-HT following treatment with simvastatin. These findings suggest a close association between 5-HT bioavailability and lipids. Reduced plasma 5-HT concentrations were also found in PVD

patients taking low dose ASA. Overall, these results suggest that control of platelet hyperactivity either by altering the lipid environment or by affecting platelet cyclooxygenase may inhibit 5-HT release from platelets. The uptake and release experiments carried out in this thesis demonstrate that platelet inhibitors (i.e. PGE_1 and NaNP which are cAMP and cGMP elevators, respectively) and platelet stimulators (e.g. ADP, adrenaline and collagen) are unable to stimulate 5-HT uptake. Furthermore, *in vitro* release experiments carried out by measuring endogenous 5-HT with a sensitive EIA demonstrate that 5-HT release may occur not only when aggregation takes place but also when platelets change in shape or when they are activated mildly as with simple stirring (i.e. in the absence of aggregation). This finding has implications for future anti-platelet drug development. Clearly, to prevent the progression of atherosclerosis or the complications associated with restenosis following grafting, one requires a drug that reduces the release of intraplatelet substances such as 5-HT and PDGF. The ineffectiveness of ASA in normalising platelet degranulation and release of such substances may explain why ASA has been disappointing in primary prevention studies assessing IHD or prevention of graft stenosis. It is hoped that, in the near future, the author will be able to investigate the biochemical mechanisms involved in release phenomena perhaps by combining sensitive techniques such as flow cytometry with measurements of release substances (e.g. 5-HT) in PVD patients.

As with other risk factors, such as fibrinogen and

hyperlipidaemia, it would be of value to explore the possibility that plasma/platelet 5-HT is another marker of IHD. This could be achieved by incorporating 5-HT measurements in a large prospective study. Similarly, a study with similar end-points but involving a large number of patients with hypercholesterolaemia undergoing lipid lowering therapy could help establish the relevance of 5-HT in the pathophysiology of vascular disease and its complications.

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APPENDIX : MATERIALS, INSTRUMENTS AND APPARATUS

I Chemicals

Amersham International Plc, Aylesbury, UK

5-Hydroxy [side chain-2-^{14}C] tryptamine creatinine sulphate (57 mCi/mmol)

Bachem Feinchemikalien, Bubesdorf, Switzerland

1-0-Alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (PAF)

BDH Ltd., Dagenham, UK

acetone
acetic anhydride
aspirin (acetylsalicylic acid; ASA)
hydrochloric acid (HCl)
perchloric acid
potassium phosphate, mono-basic
sodium chloride
sodium bicarbonate
di-sodium hydrogen orthophosphate, dihydrate
sodium hydroxide
tri-sodium citrate *di*-hydrate
trichloroacetic acid (TCA)

Biogenesis Ltd., Bournemouth, UK

5-HT radioimmunoassay kit

Coulter Electronics Ltd, Luton, UK

standard latex particles for calibration (Lot 7, 9 fl volume)
Isoton II (a balanced electrolyte solution for blood cell counting and sizing containing: sodium sulphate 9.7 g/L; sodium chloride 4 g/L; dimethylolurea 1 g/L; procaine hydrochloride 0.1 g/L)

German-Hawksley Ltd., Northampton, UK

glycosylated haemoglobin kit

Hormon Chemie, München, Germany

collagen (fibrils from equine tendons)

Immunodiagnostics Ltd., Tyne and Wear, UK

5-HT radioimmunoassay kit

Immunotech SA, Marseille, France

5-HT Enzymeimmunoassay kit

Lipha Pharmaceuticals Ltd., West Drayton, UK
naftidrofuryl oxalate
Scottish Antibody Production Unit, Carluke, UK
sheep anti-ß-thromboglobulin sera
donkey anti-goat sera
non-immune rabbit serum
non-immune sheep serum
Scottish National Blood transfusion Service, Edinburgh, UK
ß-thromboglobulin
Sigma Chemicals Company Ltd., Poole, UK
N-acetylserotonin
adenosine diphosphate (ADP)
adrenaline bitartrate
arachidonic acid (sodium salt; AA)
calcium ionophore (A23187; CaI)
citric acid
cysteine hydrochloride
dibutyryl cAMP (sodium salt)
disodium EDTA
formaldehyde (37% aqueous; w/v)
gelatin
glutaraldehyde (25% aqueous; w/v)
hydrogen peroxide (30% aqueous; w/v)
N-hydroxysuccinimide
human thrombin
indomethacin
ketanserin tartrate
noradrenaline bitartrate
ophthaldialdehyde
serotonin creatinine sulphate (5-HT)
sodium nitroprusside
U46619 (endoperoxide/thromboxane A_2 mimetic)
Sterling-Winthrop, Guildford, UK
Milrinone (2-methyl-5-cyano-(3,4'-bipyridin)-6(1H)-one)
Western General Hospital, Dept. of Medicine, Edinburgh, UK
N-succinamylserotonin-glycyl-[^{125}]-tyrosine
ß-thromboglobulin [^{125}I]

rabbit anti-sera to N-acetyl serotonin

II Buffers and Solutions

A. Citrate anticoagulant solution

Tri-sodium citrate	3.8% w/v

B. Citrate-ASA anticoagulant solution

Tri-sodium-citrate	3.8% w/v
ASA	10 mmol/L

C. EDTA anticoagulant solution

di-sodium EDTA	50 mmol/L

D. Glutaraldehyde fixative solution

glutaraldehyde	4% v/v
saline	150 mmol/L

E. Phosphate Buffer to dissolve drugs

di-sodium hydrogen orthophosphate, dihydrate	116 mmol/L
potassium phosphate, monobasic	20 mml/L
pH 7.4	

F. Saline (physiological)

sodium chloride	150 mmol/L

G. Platelet deproteinisation solution (Gow et al., 1988)

perchloric solution	1.5 mol/L
cysteine	2 mmol/L

H. 5-HT assay buffer (Gow et al., 1988)

citric acid	0.1 mol/L
sodium hydroxide	0.3 mol/L
EDTA	1 mmol/L
gelatin	1 g/L
pH 6.2	

I. ß-TG assay buffer (Ludlam et al., 1975)

di-sodium hydrogen orthophosphate, dihydrate	16 mmol/L
potassium phosphate,	

monobasic	100 mml/L
EDTA	1 mmol/L
gelatin	1 g/L

All buffers and solutions were made up with double distilled water.

III INSTRUMENTS AND APPARATUS

Balance: Sartorius research balance (R160P), Sartorius-Instruments Ltd, GB-Belmont, UK
C-256 channelyzer: Coulter Electronics Ltd, Luton, UK
Centrifuges: Eppendorf micro-centrifuge (5414), Anderman Ltd, UK; ICE CENTRA-7R, International Equipment Company, USA
Chronolog Dual Channel Optical Aggregometer 440: Coulter Electronics Ltd, Luton, UK
Chronolog Whole Blood Aggregometer 540: Coulter Electronics Ltd, Luton, UK
Computer program (statistics): C-STAT, Cherwell Scientific Publishing, Oxford UK
Coulter particle counter ZM (with a 70 µm diameter sampling tube orifice): Coulter Electronics Ltd, Luton, UK
Coulter blood counter T-890: Coulter Electronics Ltd, Luton, UK
Fluorescence spectrophotometer: Perkin-Elmer MPF-3, Hitachi Ltd., Tokyo, Japan
Gamma-Counter: LKB Wallac Compugamma (1282), Pharmacia, UK
Glucose analyzer: YSI Model 23 AM, Yellow Springs Instruments, Yellow Springs, USA
Omniscribe Chart Recorder: Coulter Electronics Ltd, Luton, UK
Liquid scintillation ß-counter: LKB Wallac Rackbeta (1219), Pharmacia, UK
Microplate reader: model MCC/340, ICN Flow, High Wycombe UK
Microplatelet shaker: Titertek, ICN Flow, High Wycombe UK
Microplate washer: Titertek, ICN Flow, High Wycombe UK
Needles: G-21 (butterfly), Abbott Ireland, Rep. of Ireland
pH meter: Corning 140, Corning Science Products, Halstead, UK

Pipettes: Gilson pipetman and Eppendorf Repetitive pipette, Anachem, Luton, UK

Sonicator: M.S.E. Soniprep, Crawley, UK

Syringes: 10 ml, Sherwood Medical, Ballymoney, UK

Water Bath: Shaking water bath, Grant Instruments Ltd, Cambridge, UK

X-Y chart recorder: Coulter Electronics Ltd, Luton, UK

List of Publications

The list below includes papers, reviews and editorials, abstracts, letters published as a result of the work presented in this thesis.

Refereed Papers

Barradas MA, Fonseca VA, Gill DS, Jeremy JY, Varghese Z, Balliod R, Moorhead J, Dandona P. Intraplatelet serotonin, ß-thromboglobulin, and histamine concentrations and thromboxane A_2 synthesis in renal disease. *Am J Clin Pathol* 1991; 96: 504-511.

Barradas MA, Gill DS, Fonseca VA, Mikhailidis DP, Dandona P. Intraplatelet serotonin in patients with diabetes mellitus and peripheral vascular disease. *Eur J Clin Invest* 1988; 18: 399-404.

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Barradas MA, O'Donoghue S, Jagroop A, Mikhailidis DP. Advantages of whole blood platelet aggregation measured by a cell counter (Coulter T-890) in drug evaluation. *J Drug Dev* 1992; 5: 155-166.

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Reviews and Editorials

Barradas MA, Mikhailidis DP. Serotonin, histamine and platelets in vascular disease with special reference to peripheral vascular disease. *Brazilian J Med Biol Res* 1992; 25: 1063-1076.

Mikhailidis DP, Barradas MA. Haemostatic effects of lipid-lowering drugs. *J Drug Dev* 1989; 2: 69-71.

Abstracts

Barradas MA, Cheshire N, O'Donoghue S, Wolfe JHN, Mikhailidis DP. Platelet aggregation and platelet shape change in peripheral vascular disease patients: effect of naftidrofuryl and acetyl salicylic acid. *Platelets* 1992; 3: 114

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Barradas MA, O'Donoghue S, Mikhailidis DP. Shape changes: a novel method for assessing the effect of agonists and antagonists on human platelets? *Br J Pharmacol* 1990; 101: 521P.

Barradas MA, Stansby G, Hamilton G, Mikhailidis DP. Platelet shape change in peripheral vascular disease patients. *16th World Congress of the International Union of Angiology*, Paris, Abstract Book, 1992, pp 330.

Barradas MA, Stansby G, O'Donoghue S, Hamilton G, Mikhailidis DP. Whole blood platelet aggregation in peripheral vascular disease is inhibited by naftidrofuryl. *Clin Hemorheol* 1991; 11: 108.

Cheshire NS, Barradas MA, Wolfe JHN, Mikhailidis DP. Effect of low dose aspirin on platelet aggregation and platelet release substances in peripheral vascular disease. *16th World Congress of the International Union of Angiology*, Paris, Abstract Book, 1992, pp 333.

Letters

Barradas MA, Mikhailidis DP. The effect of age, arteriosclerosis and hypercholesterolaemia on platelet function tests. *Thromb Haemorrh Disorders* 1990; 2: 87-88.

Barradas MA, Mikhailidis DP. Accelerated coronary artery disease after heart transplantation-the role of enhanced platelet aggregation and thrombosis. *J Intern Med* 1993; 234: 433-434.

Barradas MA, Mikhailidis DP. Serotonin as a factor involved in the pathophysiology of thrombangiitis obliterans. *Int Angiol* 1993; in press.

Barradas MA, Mikhailidis DP, Winder AF. Low serum cholesterol and suicide. *Lancet* 1992; 339: 1168.

J. Drug Dev. 1992: 5(3); 155–166

LABORATORY TECHNIQUES IN DRUG DEVELOPMENT (2): ADVANTAGES OF WHOLE BLOOD PLATELET AGGREGATION MEASURED BY A CELL COUNTER (COULTER T-890) IN DRUG EVALUATION

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Abstract

The present study investigates platelet aggregation in whole blood by measuring the platelet free count (WB-PFC). A Coulter T-890, a routine haematology analyser, was used to count platelets, erythrocytes and leucocytes. The results indicate that there is no need for a fixative or for diluting samples. The latter process (which may introduce artifacts) is necessary if aggregation is assessed in WB by the platelet impedance method (WB-PIA) which also requires special equipment. The present study is not a direct comparison of WB-PFC and WB-PIA, but it is possible to contrast some of the differences between these techniques. Thus, spontaneous aggregation, reversibility and adrenalin-induced responses are seldom reported with WB-PIA but are easily elicited with WB-PFC. Furthermore, lower agonist concentrations are needed to elicit responses with WB-PFC than WB-PIA. Of the two WB aggregation techniques, we conclude, from the present findings and the literature on the subject, that WB-PFC is more sensitive, rapid, versatile and simple to use. The simultaneous recording of erythrocyte and leucocyte counts during WB-PFC may also provide added information concerning aggregation in WB. Thus, WB-PFC appears to be the more appropriate of the two techniques for drug development work. The use of routine haematology analysers will also allow the assessment of drug action on platelets even in centres which do not normally conduct such research.

Key words: Whole blood, platelet aggregation, free cell count

Introduction

Assessing the effect of drugs on platelet function is an important component of drug development. Even if the action of a drug is not primarily aimed at modifying platelet function, such actions may be considered as additional advantages or as a relevant side effect.

Platelet aggregation has been assessed in an extensive number of studies. This process was originally quantified in platelet rich plasma (PRP), a technique which did not take into account the presence of other cells in blood [1–26]. This limitation is a considerable disadvantage since there is evidence that both erythrocytes and leucocytes can influence various aspects of platelet function [1–26]. Furthermore, PRP preparation requires centrifugation which delays the assessment of platelet function and may also cause the loss of some platelets [1,3,7,11,27]. It is for

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these and other reasons (reviewed elsewhere [1,3,8] that techniques which assess platelet aggregation in whole blood (WB) have been developed.

There are two techniques currently commonly used to assess platelet aggregation in WB. These methods are either based on the free (i.e. non-aggregated) platelet count (WB-PFC) or on platelet impedance aggregation (WB-PIA) which assesses the number of platelets adhering to platinum electrodes immersed in WB [1,3,8].

It is important to assess and discuss the advantages and disadvantages of these methods when investigating drug action. The purpose of the present study was to consider further methodological modifications and assess some aspects of WB-PFC using a Coulter T-890 and to contrast these findings with those we previously obtained with WB-PIA [12–20]. The present study, however, is not a direct comparison between WB-PFC and WB-PIA using the same samples.

Methods

The present study was divided into two parts with the following objectives:

Study 1: To prepare blood in exactly the same manner as for WB-PIA but to assess platelet aggregation by WB-PFC.

Study 2: To assess various modifications of the WB-PFC method.

Study 1

Blood was collected by venepuncture with minimum stasis from 10 healthy volunteers (seven males, three females). Samples were anticoagulated with 3.8% trisodium citrate (1 volume) added to blood (9 volumes) [12–20]. Physiological saline (0.9%) was then added to citrated whole blood (2 volumes saline: 8 volumes citrated blood) [12–20]. This latter step is required to stabilise the baseline reading of WB-PIA and enhance the responses obtained by this method [1–5,7, 12–20,22,24].

WB-PFC was assessed using a Coulter model T-890 whole blood counter (Coulter Electronics, Luton, Beds., UK). This equipment measures: haemoglobin concentration, red cell count, mean corpuscular volume, mean cell haemoglobin concentration, packed cell volume, platelet count and white cell count. In order to limit the amount of data handled and increase turnover, we only recorded the cell counts that are displayed on the indicator panel of the instrument. These latter measurements are the platelet, white cell and RBC count.

WB-PFC evaluation was initiated within 10 min and completed with 2 h of sample collection. Blood was kept throughout at 37°C since platelet function may be influenced by cooling [28].

Prior to the addition of agonists to induce aggregation, blood was pre-incubated for 1 min with a teflon-coated magnet spinning at 1,000 rpm, at 37°C, in a plastic cuvette placed in a Chronolog WB-PIA aggregometer (Chronolog model 540, Labmedics, Stockport, UK). This is the same aggregometer that we previously used for all our WB-PIA studies [12–20]. These conditions were maintained for the whole duration (15 min) of the assessment of WB-PFC.

The number of subjects tested and the concentration and type of agonists used are shown in Table 1. Samples (150µl) were withdrawn from the cuvettes using a pipette with a plastic tip. The timing of the samples is shown in Table 1. The starting volume of the citrated blood-saline sample in each cuvette was 1ml. The minimum residual volume in the cuvettes was 0.7ml after withdrawal of samples for measurement using the T-890.

Spontaneous platelet aggregation (SPA) was also assessed. This was carried out as described above but no agonist was added.

Throughout the study (unless otherwise stated), platelet aggregation was calculated on the basis of the number of free platelets and expressed as a percentage of the basal platelet count. The basal count was defined as the count obtained 15 sec after commencing spinning of WB and before the addition of any agonist. WB-PFC was recorded 1, 3, 6 and 15 min after adding the appropriate

Table 1. Median (and range) WB-PFC* expressed as a percentage of the basal platelet count (measured 15 sec after commencing spinning and before adding any agonist).

	Sampling time (min)			
Agonist	**1**	**3**	**6**	**15**
Collagen				
0.3125mg/L n=5	72 (17–90)	42 (7–75)	64 (14–83)	51 (28–79)
0.6250mg/L n=7	49 (6–66)	10 (4–48)	28 (7–56)	40 (20–55)
1.2500mg/L n=7	12 (6–88)	8 (2–8)	8 (4–30)	26 (7–46)
ADP				
2μmol/L n=7	27 (9–71)	43 (15–78)	63 (28–76)	61 (40–73)
10μmol/L n=7	11 (4–12)	6 (3–24)	11 (8–50)	18 (13–60)
Adrenaline				
1μmol/L n=7	48 (19–75)	39 (6–68)	26 (5–48)	22 (4–43)
10μmol/L n=7	38 (9–59)	29 (4–57)	30 (4–39)	28 (5–42)
SPA				
n=10	90 (90–92)	84 (70–100)	68 (37–102)	64 (34–85)

* Basal WB-PFC should be considered as 100% when interpreting the data in this table. Citrated blood (8 volumes) was diluted with saline (2 volumes). No fixative was added. Number of subjects tested = n.

aggregating agent (up to 10μl volumes). These times were selected because, in our experience, 3 min is usually the time required for maximal aggregation to occur in PRP; 15 min was an arbitrarily selected time beyond which we felt the methodology would become less practical. We have also reported the 3 and 6 min values when using WB-PIA [12–20]. Others [1–4,6,7,21,22,24,26] have also used similar time intervals for WB-PIA.

The experimental conditions described in this study are exactly the same as those we previously [12–20] used for WB-PIA except that the final step involved the assessment of platelet aggregation by the measurement of the free platelet count instead of by changes in impedance.

Collagen was obtained from Hormon Chemie, Munich, Germany and ADP and adrenaline from Sigma Chemicals, Poole,UK

Study 2

a) Effect of adding a fixative solution

In order to preserve any platelet aggregates formed it could be argued that disaggregation should be prevented by the addition of a fixative. We have, therefore, evaluated this option and compared its performance with that of citrated blood diluted with saline (see Study 1, above) and undiluted citrated blood (see below).

The fixative solution [25] consisted of 150mM NaCl containing 0.16% (w/v) formaldehyde, 4.6mM disodium EDTA,

4.5mM disodium hydrogen phosphate and 1.6mM potassium dihydrogen phosphate, pH 7.4. Fixative (0.3ml) was added to 0.15ml of citrated, saline-diluted, blood (see Study 1) after withdrawal of the sample from the cuvette in which aggregation was proceeding. The experiments were carried out using the same sampling times, experimental conditions and volunteers, as in Study 1. Results are listed in Table 2.

b) Effect of assessing WB-PFC in citrated blood with no dilution and no fixative

In order to simplify the methodology we examined the possibility of recording WB-PFC directly in citrated blood with no saline dilution and no added fixative. WB-PFC was assessed as described in Study 1 and Study 2 (a) but no saline or fixative was added. Results are shown in Table 3. Experiments were carried out using the same volunteers as those described in Studies 1 and 2 (a) above.

c) Effect of WB platelet aggregation on the white cell count (WCC)

During WB-PFC measurements we noted that an increase in WCC appeared to occur concomitantly with the decrease in free platelet count. We were interested in recording this phenomenon because the Coulter T-890 manual warned that platelet aggregates may be read as leucocytes. We therefore attempted to assess the value of the WCC as an index of aggregate size/number. This phenomenon was investigated

Table 2. Median (and range) WB-PFC* expressed as a percentage of the basal platelet count (measured 15 sec after commencing spinning and before adding any agonist).

	Sampling time (min)			
Agonist	**1**	**3**	**6**	**15**
Collagen				
0.3125mg/L n=4	64 (24–77)	47 (9–78)	70 (16–110)	55 (31–80)
0.6250mg/L n=7	65 (9–86)	26 (2–43)	35 (4–55)	32 (14–57)
1.2500mg/L n=7	24 (4–76)	6 (4–10)	12 (2–33)	18 (9–80)
ADP				
2µmol/L n=7	28 (8–74)	57 (10–93)	59 (31–98)	59 (46–83)
10µmol/L n=6	6 (4–24)	6 (2–36)	15 (4–50)	25 (14–55)
Adrenaline				
1µmol/L n=7	47 (31–90)	48 (5–86)	36 (8–48)	32 (2–44)
10µmol/L n=7	54 (9–71)	36 (2–67)	27 (2–48)	26 (2–46)
SPA n=9	90 (90–91)	87 (72–98)	59 (46–95)	58 (50–87)

* Basal WB-PFC should be considered as 100% when interpreting the data in this table. Citrated blood (8 volumes) was diluted with saline (2 volumes) and a fixative solution (see text) was added to aggregated WB at each sampling time.

Table 3. Median (and range) WB-PFC* expressed as a percentage of the basal platelet count (measured 15 sec after commencing spinning and before adding any agonist).

	Sampling time (min)			
Agonist	**1**	**3**	**6**	**15**
Collagen				
0.3125mg/L n=7	63 (11–110)	65 (5–97)	78 (9–89)	67 (28–87)
0.6250mg/L n=7	25 (4–71)	7 (2–99)	25 (2–68)	44 (13–88)
1.2500mg/L n=7	12 (5–14)	6 (4–79)	7 (4–61)	17 (3–83)
ADP				
2μmol/L n=7	47 (21–73)	61 (48–96)	69 (46–116)	66 (61–97)
10μmol/L n=7	8 (4–8)	8 (2–19)	12 (4–60)	38 (16–102)
Adrenaline				
1μmol/L n=7	47 (10–95)	60 (3–77)	57 (4–68)	63 (13–77)
10μmol/L n=7	31 (19–54)	21 (2–35)	13 (2–39)	19 (6–43)
SPA n=10	90 (72–102)	85 (74–98)	73 (54–89)	64 (51–88)

* Basal WB-PFC should be considered as 100% when interpreting the data in this table. Citrated blood was used without any saline diluent or added fixative.

by: a) calculating the correlation (Spearman's) between the free platelet count (expressed as number of platelets per litre of blood) and the WCC (expressed as number of leucocytes per litre of blood), b) determining whether there was a significant increase in the WCC when aggregation in WB occurs, c) assessing the change in WCC in PRP following aggregation induced by various agonists.

The platelet and leucocyte counts were read from the indicator panel of the T-890. For WB experiments, the basal WCC was recorded at the same time as the basal platelet count (for definition, see Study 1 above). In these experiments, blood was prepared with no fixative or diluent. For PRP experiments, the PRP was prepared by centrifugation of citrated blood as previously described [12–14]. Platelet aggregation, in PRP, was induced by various agonists (see Table 6 for details) and the WCC was recorded (using the Coulter T-890) 1, 3 and 6 min after adding the appropriate agonist. These values were compared with those obtained prior to the addition of an agonist but after PRP had been mixed (teflon-coated bar spinning at 1,000 rpm; 37°C) in a Chronolog aggregometer for 1 min.

d) Effect of platelet activating factor (PAF), serotonin (5-HT) and arachidonic acid (AA) on WB-PFC

WB-PFC following the addition of PAF, 5-HT and AA was assessed in small numbers of healthy control subjects. The concentrations used and the results are shown in Table 7. PAF, 5-HT and AA were obtained from Sigma Chemicals, Poole, UK. The blood used in these experiments did not have any diluent or fixative added.

e) Reproducibility of SPA in relation to time, using WB-PFC

SPA was repeated in 10 volunteers 2 h after it had been estimated initially. Only WB collected by the method described in 2 (b) above, was evaluated.

f) Effect of porcine intestinal mucosal (PIM) and bovine lung heparins (BLH) on WB-PFC

PIM, BLH or saline (as control) was added to whole blood from eight healthy volunteers. The blood used in these experiments did not have any diluent or fixative added. The final concentration of heparins was 1U/ml. WB-PFC was measured 1, 3, 6 and 15 min after adding PIM, BLH or saline. Results are expressed as the actual platelet count (x 10^9/L). Heparins were obtained from Sigma Chemicals, Poole, UK.

Statistical analysis

This was performed using non-parametric statistics. The Mann-Whitney test was used for unpaired comparisons, the Wilcoxon test for paired comparisons and Spearman's correlation coefficient for assessing relationships between variables. All tests were two-tailed.

Results

Study 1 (Table 1)

In keeping with previous work [2], the results show that appreciable WB-PFC responses occur at agonist concentrations which are considerably lower than those needed to initiate WB-PIA. For example, using collagen as an agonist, significant WB-PFC responses occurred at concentrations of 0.3125mg/L whereas WB-PIA responses [12–20] require concentrations of approximately 1mg/L. Similarly, using ADP as an agonist, significant responses were noted at concentrations of 2μmol/L whereas WB-PIA responses [12–20] require 5–10μmol/L. On a separate occasion, seven of the volunteers were assessed by WB-PIA; neither SPA nor adrenalin-induced aggregation could be demonstrated. However, such comparisons should be interpreted with caution since the present study does not include the simultaneous evaluation of the same samples by WB-PIA.

In addition, we detected reversibility of aggregation induced by ADP and collagen, adrenalin-induced WB-PFC and SPA, and three processes which are not normally observed with WB-PIA [1–26].

A dose-response pattern was observed with each agonist; the higher the agonist concentration, the lower the % WB-PFC, i.e. the lower the number of free platelets. This conclusion was true when each sampling time was considered separately. The dose-response was not as obvious with adrenalin where aggregation tended to plateau at 6 and 15 min.

Some degree of reversibility was observed with collagen-and ADP-induced WB-PFC. There was no clear pattern of reversibility with adrenalin-induced WB-PFC and SPA. Maximal response occurred at 3 min with collagen- and ADP-induced WB-PIA. With adrenalin-induced WB-PFC, maximal response tended to occur at 6 and 15 min.

SPA resulted in a progressive fall in the percentage WB-PFC. The major reduction in free platelets occurred at 6 and 15 min. There were no significant changes in the RBC count throughout the experiments described above.

Study 2

a) Effect of adding a fixative solution

When compared (Mann-Whitney test) with the results of Study 1 (see Table 1: citrated blood diluted with saline; no fixative added), the addition of fixative solution caused no significant change in WB-PFC when the free platelet count was expressed as a percentage of the basal count. The basal platelet count, however, was significantly (p=0.002) lower (median: 49; range: 42–64 x 10^9/L) than in Study 1 (median: 158; range: 146–191 x 10^9 /L) because the addition of fixative solution caused further dilution of the sample.

b) Effect of assessing WB-PFC in anticoagulated blood with no dilution and no fixative

This method showed dose-responses which are very similar to those observed when

samples were diluted with saline (Study 1) or the fixative solution was added [Study 2 (a)]. The basal platelet counts were, however, higher than with the other two methods. Thus, basal platelet counts were: median: 220; range: 141–283 x 10^9/L. This count was significantly greater when compared with those in Study 1 ($p<0.01$).

c) Effect of WB platelet aggregation on the white cell count (WCC)

i) There was a significant correlation between the platelet and leucocyte counts (Table 4) when WB aggregation was induced by the two highest concentrations of collagen (0.625 and 1.250mg/L). No such correlation was observed when WB was induced by ADP or adrenalin.

ii) There was a small but significant increase in WCC in the 1 and 3 min samples following WB aggregation induced by several agonists (Table 5). There was no significant increase in WCC when WB aggregation was induced by 'weak agonists' like adrenalin 1µmol/L or following SPA.

iii) Aggregation in PRP was associated with small but significant increases in WCC (Table 6). With ADP-and adrenalin-induced aggregation the increase occurred at 1 min. With collagen the increase occurred at 3 and 6 min. SPA was not associated with any change in WCC. There was a general trend for the WCC to rise as the free platelet count fell. Thus, the following significant correlations were obtained between the WCC and the platelet count in PRP:

collagen 0.6250mg/L: n=29; $r_s = -0.42$; $p<0.03$

1.2500mg/l: n=19; $r_s = -0.42$; $p=0.05$

ADP 2µmol/l n=27; $r_s = -0.62$; $p<0.001$

There was no significant correlation when ADP, 10µmol/L, adrenalin, 10µmol/L and SPA were assessed.

d) Effect of platelet activating factor (PAF), serotonin (5-HT) and arachidonic acid (AA) on WB-PFC

The WB platelet aggregation patterns obtained after adding various doses of the above agonists are shown in Table 7.

e) Reproducibility of SPA in relation to time, using WB-PFC

Reproducibility of SPA was very good with no significant differences between the initial WB-PFC and the same measurement 2h later (Table 8).

f) Effect of porcine intestinal mucosal (PIM) and bovine lung heparins (BLH) on WB-PFC

The significant enhancement of WB-PFC is evident with both PIM and BLH (Table 9). This

Table 4. Correlations (Spearman's; r_s) between the WB-PFC and the WCC.

	Agonist						
	Collagen (mg/L)			Adrenaline (µmol/L)		ADP (µmol/L)	
	0.3125	0.6250	1.2500	1.0	10.0	2.0	10.0
R_s	−0.37	−0.40	−0.63	0.02	−0.06	−0.16	−0.25
p	NS	<0.04	0.0001	NS	NS	NS	NS
n	25	28	30	28	27	20	28

NS = not significant; n = number of readings compared;
Both values were analysed as the absolute cell counts (per litre) at each sampling time.

Table 5. Changes in WCC† during platelet aggregation in whole blood.

	Sampling time (min)			
Agonist	**1**	**3**	**6**	**15**
Collagen				
0.3125mg/L	7.0**	7.2*	7.2*	7.1
n=7	(4.4–11.3)	(4.4–10.3)	(4.5–10.2)	(4.8–9.8)
0.6250mg/L	8.4**	7.3*	7.2	7.3
n=7	(4.6–11.0)	(4.6–10.6)	(4.5–10.4)	(4.4–10.4
1.2500mg/L	7.8**	6.9	6.8	6.9
n=7	(5.2–10.5)	(4.4–9.9)	(4.4–10.0)	(4.5–9.7)
ADP				
2µmol/L	7.9**	7.5*	7.2	6.4
n=7	(4.7–11.5)	(4.8–10.1)	(4.8–9.9)	(4.6–10.4)
10µmol/L	7.4**	7.1	7.0	7.0
n=7	(4.6–10.8)	(4.1–10.5)	(4.5–10.5)	(4.5–9.9)
Adrenaline				
1µmol/L	6.5	6.7	7.4	7.2
n=7	(4.3–11.4)	(4.5–9.9)	(4.1–9.7)	(4.8–9.6)
10µmol/L	7.1*	7.2*	7.1	6.6
n=7	(5.2–11.8)	(4.3–10.5)	(3.9–10.2)	(3.7–8.4)
SPA	6.4	6.4	6.7	7.1
n=10	(4.4–9.9)	(4.4–10.3)	(4.5–10.2)	(4.8–9.8)

Comparisons against basal counts: * $p<0.05$; ** $p<0.01$;
† The WCC is expressed as the number of leucocytes ($x10^9$) per litre of citrated blood. No saline or fixative solution was added to the blood sample.
Median basal WCC was 6.4 (4.3–9.8).

enhancement was more evident with BLH. These results contrast with our previous inability to demonstrate enhancement of WB-PIA with PIM [13].

Discussion

Although the present study is not a direct, simultaneous, comparison between WB-PFC and WB-PIA, the findings suggest that the former process occurs at lower agonist (including drugs, such as the two heparins tested) concentrations than those we reported for WB-PIA [12–20]. This impression is in agreement with a previous direct comparison between these methods [2]. However, the latter study had three disadvantages, outlined by the authors [2]: a) WB-PFC was measured at room temperature and WB-PIA at 37°C; b) only the WB-PIA samples were stirred (as we describe); c) only the WB-PIA samples were diluted. We overcame these differences by achieving exactly the same experimental conditions for WB-PFC as we previously used for WB-PIA, except for the final measurement of aggregation.

Dilution is required for WB-PIA to stabilise baseline readings and increase sensitivity [1–5,7,12–20,22,24]. The absence of this step with WB-PFC simplifies methodology and reduces artefactual interference. Our results also indicate that a fixative solution does not necessarily improve results. WB-PFC methodology can be further simplified since standardised spinning may not be needed [2,8–11,25]. Therefore, the WB-PFC technique can be simplified to the extent that the only requirement is operating a WB

Table 6. Median (range) WCC (expressed as number of cells x 10^9 per litre of PRP; top row, smaller numbers) and PFC (expressed as number of cells per litre of PRP; bottom row, larger numbers). Median basal platelet count was 314 (282–484) and median WCC in PRP was 0 (0–0.1) cells/L of PRP.

	Sampling time (min)		
Agonist	**1**	**3**	**6**
Collagen			
0.625mg/L	0.1 (0.0–1.5)	0.3** (0.1–1.1)	0.2** (0.1–0.9)
n=8	230* (126–448)	25** (7–360)	80** (17–354)
1.250mg/L	0.1 (0.0–1.3)	0.3** (0.1–1.1)	0.2** (0.1–0.8)
n=9	283* (43–475)	20** (7–85)	16** (9–154)
ADP			
2µmol/L	1.1** (0.2–1.4)	0.3 (0.0–1.2)	0.2 (0.0–1.4)
n=7	59** (21–238)	123** (10–212)	224** (10–274)
10µmol/L	0.3** (0.0–0.9)	0.1 (0.0–0.9)	0.1 (0.0–0.7)
n=9	14** (8–26)	13** (5–56)	10** (5–53)
Adrenaline			
10µmol/L	0.4** (0.1–1.3)	0.1 (0.0–0.9)	0.1 (0.0–0.8)
n=9	102** (11–327)	20** (7–335)	18** (5–342)
SPA			
n=7	0.1 (0.0–0.1)	0.0 (0.0–0.1)	0.0 (0.0–0.1)
	302 (232–484)	290* (230–463)	275* (119–467)

Comparisons against basal platelet count and WCC in PRP (Wilcoxon test); * $p<0.05$; ** $p<0.01$; Correlations between platelet count and WCC in PRP: see text.

haematology analyser. These analysers also have other applications and preclude the need to measure PCV in order to obtain absolute platelet counts (as when a dedicated platelet counter is used).

Adrenalin-induced aggregation is seldom observed with WB-PIA, unlike with WB-PFC [1,4,12–20,22]. Similarly, PAF-induced WB-PIA is only detected (Reference 26 and our own unpublished observations) in 'high responders' whereas similar concentrations resulted in falls in WB-PFC. However, PIM, AA- and 5-HT-induced aggregation follows the same pattern as the other agonists with lower concentrations needed to elicit WB-PFC responses than to initiate WB-PIA [22,24].

Reversibility of the fall in WB-PFC was observed with several agonists. Reversibility,

Table 7. WB-PFC after addition of arachidonic acid (AA), serotonin (5-HT) and platelet activating factor (PAF).

	Sampling time (min)			
Agonist	**1**	**3**	**6**	**15**
AA				
0.25mmol/L n=4	6.0 (4–9)	4.0 (4–17)	6.0 (3–17)	12.0 (4–13)
0.50mmol/L n=5	6.0 (4–9)	4.0 (4–6)	6.0 (3–8)	10.0 (4–16)
1.00mmol/L n=5	5.0 (3–8)	3.0 (3–4)	3.0 (2–4)	6.0 (4–6)
5-HT				
0.1μmol/L n=5	65 (31–93)	80 (65–101)	84 (63–98)	44 (42–69)
1.0μmol/L n=6	48 (35–76)	76 (54–90)	66 (53–84)	62 (45–72)
10.0μmol/L n=6	74 (21–98)	76 (21–96)	67 (32–86)	56 (28–70)
PAF				
0.01μmol/L n=4	73 (22–80)	72 (64–91)	82 (57–94)	55 (40–98
0.1μmol/L n=4	12 (8–3)	22 (12–80)	55 (15–61)	51 (41–60)
1.0μmol/L n=4	8 (3–18)	10 (5–16)	32 (19–37)	43 (27–63)

however, is not a feature of WB-PIA [1–26]. Consequently, drugs promoting disaggregation may be better evaluated by WB-PFC. It may also be possible to further improve sensitivity to reversibility by prolonging sampling beyond the 15 min we allowed.

SPA is another phenomenon which is not prominent in WB-PIA [1–3,5,17,22,26] but is well reproduced 2 h after initial evaluation using WB-PFC. SPA may predict ischaemic heart disease (IHD) since the incidence of re-infarction was higher in those with excessive SPA, assessed in PRP [29]. The need to establish whether WB-PFC can make similar predictions is reinforced by evidence of increased prevalence of IHD in men with

Table 8. Reproducibility of SPA 2 h after the initial estimation.

Sampling time (min)	Initial SPA	SPA 2.5 h later
1	90(72–102)	85(63–107)
3	85(74–98)	80(48–98)
6	73(54–89)	76(54–85)
15	64(51–88)	68(56–84)

There were no significant differences when initial SPA and SPA 2 h later were compared (Wilcoxon test) at each sampling time.

Table 9. Effect of porcine intestinal mucosal (PIM) and bovine lung (BLH) heparins on WB-PFC.

Sampling time after adding saline/PIM/BLH (min)	Saline	PIM (1U/ml)	BLH (1U/ml)
1	229 (161–295)	227* (146–263)	216**† (92–231)
3	208 (133–258)	201** (89–236)	171**†† (71–202)
6	193 (105–249)	170** (99–225)	147**†† (50–169)
15	151 (76–210)	125** (54–148)	98**†† (29–114)

PIM or BLH versus saline: * $p<0.02$; ** $p<0.01$; *PIM versus BLH:* † $p<0.03$; †† $p<0.01$;
Results are expressed as changes in absolute platelet counts ($x10^9/L$). Blood was obtained from eight healthy volunteers.

more sensitive platelets, as assessed by WB-PIA induced by minimal concentrations of ADP [21]. This latter measurement may be similar to SPA, a process which is probably largely mediated by ADP released from blood cells [8,30]. The possibility that fibrinogen contributes to the initiation of SPA is discussed elsewhere [31,32]. It is also likely that WB-PFC is a more sensitive predictor/indicator of IHD than WB-PIA since low agonist concentrations are more effective and SPA is both detectable and essentially irreversible.

The relationship between platelet aggregation and artefactual alterations in the WCC has not, to our knowledge, previously been systematically investigated. Our evidence linking platelet aggregation and changes in WCC includes a direct correlation between these variables (in WB and PRP) and significant changes in WCC when platelet aggregation occurred (in PRP and WB). The most likely explanation is that platelet clumps were recognised as leucocytes since this potential artefact is mentioned in the Coulter T-890 manual.

The WB-PFC-WCC phenomenon deserves further investigation (for example, by electron microscopy). Such studies may indicate that the artefactual rise in WCC (or its reversal) relates to aggregate size, number or another aspect of the platelet thrombus.

Although we broadly agree with others [2] that WB-PIA and WB-PFC are not mutually exclusive techniques, we feel that one procedure is more advantageous. Thus, WB-PIA, unlike WB-PFC, requires dilution and a dedicated aggregometer. Furthermore, it may be difficult to obtain stable baseline readings and the platinum electrodes are vulnerable, expensive and may need replacement at regular intervals. Adrenalin-, 5-HT- and PAF-induced aggregation, SPA and the reversibility of aggregation are not as easily elicited by WB-PIA as they are when using WB-PFC. The WCC phenomenon we describe may also enhance the value of WB-PFC measurements.

A comprehensive comparison of both methods is beyond our remit but deserves further study since choices will probably have to be made between these techniques.

White [33,34] and red cell [35] aggregation have been documented in the literature. These processes may well interact with platelet aggregation [36] (e.g. by the release of prostanoids, leukotrienes, PAF, bioamines). WB technology using versatile cell counters may be the ideal methodology for assessing these interactions. However, these studies will have to consider the artefactual rise in WCC, described in the present study.

In conclusion, WB-PFC is a practical and sensitive technique for assessing platelet aggregation in WB and the effect of drugs thereon. WB-PFC also has some advantages over WB-PIA. One of these is the use of routine haematology analysers which will facilitate the assessment of the effect of drugs on platelet function, even in centres that do not normally conduct such research.

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SEROTONIN, HISTAMINE AND PLATELETS IN VASCULAR DISEASE WITH SPECIAL REFERENCE TO PERIPHERAL VASCULAR DISEASE

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Cardiovascular disease is a major cause of death. There is evidence that this disease is predicted and its progression influenced by various factors (e.g. hyperlipidaemia). In this review, we consider aspects of platelet structure and function which may explain how this cell type contributes to the pathogenesis of vascular disease. The platelet also contains bioamines (serotonin, 5-HT; histamine) which are potent vasoactive substances. Studies involving patients with peripheral vascular disease (PVD) where abnormalities in platelet function (platelet aggregation and platelet shape change) and in bioamine status (vascular, platelet and plasma bioamine concentrations) are reviewed. We also discuss how platelet activation (*in vitro*) and plasma lipids influence intraplatelet bioamine status. Finally, we report *in vitro* evidence of the effect of two drugs prescribed to PVD patients: aspirin and naftidrofuryl. Aspirin is an ineffective inhibitor of 5-HT-induced whole blood platelet aggregation whereas naftidrofuryl is effective in the presence or absence of aspirin. By identifying and altering the factors which contribute to the pathogenesis of atherosclerosis we will be better equipped to prevent, reverse or retard this process.

Key words: peripheral vascular disease, atherosclerosis, platelets, serotonin, 5-hydroxytryptamine, histamine, platelet shape change, cholesterol.

Introduction

Cardiovascular disease is the major cause of death in the Western world. The disease involves the coronary, cerebral and lower extremity arteries (peripheral vascular diseas, PVD). As more sophisticated non-invasive diagnostic techniques are developed our assessment of the prevalence of vascular diseases has also improved. This is particularly true for PVD for which an increased prevalence has recently been documented (Vogt et al., 1992).

In PVD, the basic lesion is atherosclerosis with raised focal plaques containing lipids and other macromolecules. The precise nature of the initial injury and the mode of formation of the plaque is still undefined. It is clear, however, that plaque formation may eventually develop into an occlusive lesion which in turn gives rise to clinical symptoms. Furthermore, rupture of the atherosclerotic plaque may initiate thrombosis (Harker et al., 1981).

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Patients with PVD complain of local pain on walking and possibly at rest. These patients also have a high mortality from myocardial infarcts (MI) and strokes (Vogt et al., 1992). Lesions tend to occur in the distal portion of the femoral and popliteal arteries. Lesions are also found at bifurcations of vessels (e.g. the aortic bifurcation). At these points, blood flow is more turbulent. Accordingly, this type of disease is rarely seen in the arms and other parts of the microcirculation where blood flow is more uniform.

Current knowledge indicates that atherosclerosis is a multifactorial disease (Hopkins and Williams, 1981). For coronary artery disease, a number of risk factors are of major importance and are being intensely investigated. Table 1 shows some of the more well-established risk factors. This is not an exhaustive list since some 246 risk factors have been recognized for coronary heart disease (Hopkins and Williams, 1981)! A relatively recent addition to this list is platelet function indices. In particular, platelet aggregability (Elwood et al., 1990; Trip et al., 1990; Thaulow et al., 1991) and platelet volume (Martin et al., 1991).

In the present review, we consider some aspects of platelet structure and function which may explain how this cell type contributes to the pathogenesis of vascular disease, with particular emphasis on PVD.

Aspects of platelet structure and function in health and disease

Some of the distinguishing features of platelets are that: 1) They do not have a nucleus. Therefore, irreversible enzyme inhibition with drugs lasts for the duration of the life-span of platelet (7-10 days), as is the case for the inhibition of platelet cyclooxygenase activity by aspirin (acetyl salicylic acid, ASA). Cyclooxygenase is one of the enzymes involved in the synthesis of thromboxane A_2, a powerful vasoconstrictor and platelet aggregant (Webster and Douglas, 1987). 2) The platelet contains two types of granules. Dense granules are opaque to electrons and contain calcium, ATP, ADP and the bioamines, serotonin (5-HT) and histamine (White, 1987). Alpha granules contain platelet factor 4 (PF-4), platelet-derived growth factor (PDGF), beta-thromboglobulin, and other proteins such as fibrinogen and fibronectin (White, 1987). 3) The physiological task of platelets is to arrest the loss of blood from damaged vessels, for example, when a cut is made. To achieve this objective, platelets adhere to exposed subendothelial layers and adhere or aggregate to each other, thus establishing a platelet plug. 4) There are a number of studies involving patients with cardiovascular disease where platelet abnormalities have been documented (De Cree et al., 1985; Mikhailidis et al., 1987; Elwood et al., 1990; Trip et al., 1990; Martin

Table 1 - *Established reversible and irreversible risk factors associated with coronary artery disease.*

Risk factors	
Reversible	Irreversible
Hyperlipidaemia	Age
Hypertension	Sex
Cigarette smoking	Genetic influences
Diabetes	
Fibrinogen	
Platelet function	

et al., 1991). Recent evidence suggests that platelet hyperaggregability (Elwood et al., 1990; Trip et al., 1990; Thaulow et al., 1991) and platelet volume (Martin et al., 1991) can be used to predict cardiovascular disease and associated events (e.g., MI).

Platelets in "early" atheroma

Conceptually, a modest injury to the endothelial monolayer could permit platelets to adhere and subsequently release intraplatelet substances which contribute to atherogenesis (Ross et al., 1974). There is evidence that supports this concept. Platelets contain mitogens (e.g., PDGF, 5-HT) and vascular permeability enhancing substances (e.g., bioamines) which can be released during platelet aggregation (Holmsen, 1985). Platelet antigens have been detected in blood vessels (Woolf and Carstairs, 1967) which suggests that platelet incorporation takes place. There is also evidence that inhibitors of platelet activity can inhibit the onset of atherosclerosis (Grodzinska and Dembinska-Kiec, 1980; Landymore et al., 1988).

Animal models associated with von Willebrand factor deficiency (required for platelet adhesion) have also been found to be associated with diminished atherosclerosis (Fuster et al., 1978).

Platelets in "late" atheroma

Less contentious is the role of platelets in the end-stage pathological events that lead to thrombosis, for example MI (ISIS-2 Collaborative Group, 1988). At the end-stage, the atherosclerotic plaque may rupture or become denuded of endothelium, thus attracting platelets to adhere and aggregate at this site initiating clot formation. It is not surprising therefore, that thrombi have been shown to contain platelets (Woolf and Carstairs, 1967; Harker et al., 1979).

ASA, which has been shown to reduce the incidence of MI in secondary prevention trials is also a well-established platelet inhibitor (Webster and Douglas, 1987; ISIS-2 Collaborative Group, 1988). Furthermore, many conditions considered to be different manifestations of atherosclerosis (e.g. PVD, hypertension, ischaemic heart disease) have been shown to be associated with platelet hyperactivity (De Cree et al., 1985; Zahavi and Zahavi, 1985; De Clerck, 1986; Barradas et al., 1991b).

Bioamines and atherosclerosis

Platelets can uptake, store and release bioamines (5-HT, histamine, epinephrine and norepinephrine) (Da Prada et al., 1981). There is an increasing interest in the role of these bioamines, especially 5-HT, in atherosclerosis and thrombosis (Hillis and Lange, 1991).

Animal models and human studies involving atherosclerosis associated with hyperlipidaemia (Galle et al., 1991), hypertension (Woodman, 1990) and aging (Doyle,

1991), or activation of the haemostatic system (such as cold temperatures; Harker et al., 1991) have shown enhanced vasoconstrictor responses to 5-HT. This bioamine is a well-established activator of platelet aggregation, an effect which appears to be largely independent of cyclooxygenase inhibition (De Clerk et al., 1985b). Moreover, there is evidence that 5-HT may reduce red cell deformability (Nordt et al., 1990). Thus, although the action of 5-HT in the normal circulation (e.g. coronary vasculature) is to increase blood flow, diseased blood vessels as well as effects on platelets and on red cells may lead to an overall diminution in blood flow (Woodman, 1990; Golino et al., 1991; McFadden et al., 1991) in response to this bioamine.

5-HT is the most abundant bioamine found in platelets and more than 95% of blood 5-HT is stored in platelets (Da Prada et al., 1981). This bioamine has also been shown to enhance vascular permeability in animal models (De Clerck et al., 1985a). This effect, which appears to be mediated via 5-HT_2 receptors (De Clerck et al., 1985a), is Ca^{+2} mediated (De Clerck et al., 1981; Gill et al., 1992) and may be relevant in the early stages of atherogenesis (Ross and Glomset, 1976). In this context, another important property of 5-HT is that it exhibits mitogenic properties and enhances the effects of other mitogens, such as PDGF (Nemecek et al., 1986).

5-HT uptake into tissues has also been shown to be diminished in conditions of endothelial injury (Bult et al., 1988) or ischaemia (Oei et al., 1983). This effect, together with an increased release of 5-HT from activated platelets, may be responsible for the observed elevation in plasma 5-HT concentration in various forms of vascular disease (Rubanyl et al., 1987; Barradas et al., 1988; Van den Berg et al., 1989).

Like 5-HT, histamine also has vasoconstrictor effects (Ginsburg et al., 1984) in certain vascular beds and under some circumstances (e.g. inflammation) it exerts vasodilator actions (Wilhelm, 1962).

In man and in numerous animal models, histamine has been shown to enhance the leakage of circulating proteins (Harman, 1962; Owens and Hollis, 1979; Hollis and Furniss, 1980; Hollis and Strickberger, 1985; Gill et al., 1990). There is evidence that histamine increases inter-endothelial gap space by causing endothelial cells to contract (Wu and Baldwin, 1992). These effects are likely to increase the transport of plasma macromolecules (e.g. lipids) into the arterial wall (Ross, 1986). Increased vascular permeability of endothelial cells is regarded as an important event in the initiation of atherosclerosis (Ross and Glomset, 1976).

Most of the histamine in blood is in white cells (e.g. basophils) (Graham et al., 1955). Plasma levels of this bioamine are relatively low and platelets store only some 5% of the total blood histamine. In conditions associated with platelet hyperaggregability and white cell activation, both cell types have been found to have higher intracellular concentrations of histamine when compared with healthy subjects (Gill et al., 1988). These findings may be important since as with platelets, white cell activation results in the adherence of large numbers of leukocytes to endothelial cells (O'Flaherty et al., 1978). Increased adherence of leukocytes followed by migration into the vessel wall is considered to be a key event in initiating endothelial damage (Schwartz et al., 1986).

Recent reports indicate that platelets can synthesize histamine from histidine via the action of histidine decarboxylase (HDC) (McNicol et al., 1989). More importantly, intracellular histamine may make the integrity of platelet granules more labile to the effect of phorbol ester. This effect is thought to contribute to the platelet hyperactivity observed in PVD (McNicol et al., 1989).

Platelet aggregation in peripheral vascular disease

There is considerable evidence that platelet activity is enhanced in PVD. Thus, both an increase in platelet aggregation and a shortening of platelet half-life have been observed (Zahavi and Zahavi, 1985). The enhanced release of intraplatelet products such as beta-thromboglobulin (Cella et al., 1979) and TXA_2 (Mikhailidis et al., 1983; Zahavi and Zahavi, 1985) has also been observed in PVD.

The most common technique used for investigating platelet aggregation *in vitro* has been based on Born's turbidimetric method utilizing platelet-rich plasma (PRP) (Born, 1962). This method, however, is associated with a number of technical limitations and conflicting results have been obtained. Thus, in some studies enhanced platelet hyperaggregability in PRP obtained from PVD patients has been documented (Mikhailidis et al., 1983, 1985; Zahavi and Zahavi, 1985; Barradas et al., 1989) whereas in others no such enhancement could be demonstrated (Cella et al., 1979 ; Galt et al., 1991) .

In our studies we assessed platelet aggregation in whole blood by a free platelet count method (Barradas et al., 1992a) to determine whether PVD is associated with enhanced platelet aggregation. This methodology, unlike PRP preparation, does not require a centrifugation step, which might activate and/or result in the loss of platelets (Denfors et al., 1991). Moreover, it is advantageous to study platelet function in the presence of all blood cells. We showed that whole blood platelet aggregation is enhanced when this process occurs spontaneously or is induced by adrenaline or 5-HT. Spontaneous platelet aggregation (SPA) is important since SPA has recently been shown to be a predictor of MI in PRP (Trip et al., 1990). We believe that SPA and agonist-induced platelet aggregation in whole blood is likely to be an even better predictor of ischaemic cardiac events since this methodology also reflects red cell (Ehaly and Landgaaf, 1981) as well as white cell (Nash and Shearman, 1992) abnormalities observed in PVD and these patients are known to have a high incidence of MI. In keeping with this interpretation is the recent finding of an association between prevalent ischaemic heart disease and ADP-induced aggregation in whole-blood (Elwood et al., 1990).

We also studied the effect of naftidrofuryl (NAF), *in vitro*, on whole blood platelet aggregation. NAF is a drug with 5-HT-blocking properties (Zander et al., 1986; Davies and Steiner, 1988) which has been shown to significantly improve the walking distance in PVD patients (De Felice et al., 1990). This drug was effective in inhibiting aggregation induced by 5-HT at concentrations similar to those achieved during treatment (Barradas et al., 1991b). In contrast, it had been previously shown that supra-therapeutic concentrations of NAF were required to achieve inhibition of platelet

aggregation (Davies and Steiner, 1988). We have also shown that NAF inhibits platelet aggregation in both PVD patients treated with low-dose ASA and in those who had not received ASA (Cheshire et al., 1992). Furthermore, NAF was an effective inhibitor when ASA was added *in vitro* to blood obtained from patients who were not treated with ASA (Barradas et al., 1991b). Although ASA is a widely prescribed drug for patients with ischaemic heart disease, we have shown that it is ineffective in inhibiting whole blood platelet aggregation induced by 5-HT, whether ASA is administered to patients or added *in vitro* (Barradas et al., 1992b; Cheshire et al., 1992).

Platelet and plasma bioamine studies

It remains to be shown whether drugs like NAF can inhibit 5-HT-induced aggregation and platelet release of this bioamine (as well as other vasoactive substances) *in vivo*. This effect would be highly desirable since we have documented low intraplatelet 5-HT and increased plasma 5-HT in PVD and diabetes mellitus (DM) (Barradas et al., 1988). Interestingly, the concentration of this bioamine in platelets may be influenced by plasma lipids. Thus, we, as well as others, have documented an inverse correlation between intraplatelet 5-HT and plasma cholesterol and triglycerides in conditions associated with atherosclerosis such as renal disease (Barradas et al., 1991a) and hypertension (Guicheney et al., 1988).

Plasma cholesterol and triglycerides may exert direct effects on platelets. For example, it has been shown that LDL cholesterol enhances platelet aggregation (Watanabe et al., 1988) and induces platelet shape change *in vitro* (Pletscher et al., 1989). Furthermore, reducing plasma cholesterol in hypercholesterolaemic patients with a lipid lowering drug (simvastatin, an HMGCoA reductase inhibitor) resulted in a reduction in platelet aggregation and a significant increase in intraplatelet 5-HT concentrations (Coumar et al., 1991). This association is further strengthened by a significant correlation between LDL and platelet function indices (Coumar et al., 1991).

In contrast to our findings with platelet 5-HT, platelet histamine was found to be raised in patients with PVD (Gill et al., 1988). Plasma histamine concentrations were also raised in PVD and DM patients (Gill et al., 1989). Our patient studies are in agreement with experimental work in the diabetic rat (Hollis and Strickberger, 1985; Gill et al., 1990) and other models (Owens and Hollis, 1979; Hollis and Furniss, 1980; Skarlatos and Hollis, 1987). Markle and Hollis (1977) showed that HDC (the enzyme responsible for histamine biosynthesis) activity is raised in cells obtained from rabbits fed an atherogenic diet before histologically identifiable lesions are observed. Further feeding results in atherosclerotic lesions. This observation suggests that one of the pre-atherosclerotic metabolic changes is an increased capacity of endothelial cells to form histamine and that at this state the leaky endothelium initiates atherosclerosis.

Very little work has been conducted to investigate alterations in histamine metabolism in human atherosclerotic vascular disease. However, there is one report showing that coronary arteries obtained from patients with a history of ischaemic heart

disease contained twice as much histamine as vessels obtained from controls (Kalsner and Richards, 1984). In contrast, 5-HT concentrations in these blood vessels (Kalsner and Richards, 1984) and in tissues obtained from diabetic rats (Barradas et al., 1990a) were found to be decreased.

Bioamine uptake by human platelets

A well-established function of platelets is to actively take up bioamine into specific storage granules (Stoltz, 1985). It is thought that platelets act as major reservoirs for 5-HT thus preventing the access of this bioamine to the vasculature (Vanhoutte and Cohen, 1983).

We have shown that intraplatelet histamine concentrations are elevated in PVD (Gill et al., 1988). One of the mechanisms which could contribute to this phenomenon is the enhanced uptake of histamine by activated platelets. It is therefore of interest that, using radiolabelled tracer techniques, we demonstrated that histamine uptake by human platelets can be enhanced by various activating stimuli such as stirring or the addition of very low concentrations of platelet aggregating agents (adrenaline, collagen, ADP) (Gill et al., 1987). In contrast, under similar conditions, 5-HT uptake could not be enhanced (M.A. Barradas and D.P. Mikhailidis, unpublished observations).

Other mechanisms leading to an elevated intraplatelet histamine concentration could include an increased uptake as a consequence of the raised plasma histamine levels and the excessive synthesis of histamine in platelets. The enhanced uptake of histamine could be a process which occurs *in vivo* during platelet activation. Moreover, this is likely since stimulatory effects on histamine uptake were observed at concentrations of agonists which do not induce aggregation but which are sufficient for platelets to change in shape (Barradas et al., 1990b).

Platelet shape change

When platelets are activated they change from a discoid to a spheroid particle. This phenomenon is known as the platelet shape change (PSC) (Gear, 1981). PSC precedes platelet aggregation, is reversible and can be induced *in vitro* by low concentrations of agonists (Gear, 1981; Barradas et al., 1990b). It is therefore more likely to be achieved *in vivo*. Although not yet established, PSC may also be associated with the release of bioamines and other vasoactive substances. Using a particle counter (Coulter ZM) coupled to a channelyzer (Coulter C-256; high resolution pulse height-analyzer), we have established time courses and dose responses induced by various agonists (Barradas et al., 1990b). We assessed PSC in PRP prepared from patients with PVD and healthy subjects. Using this technique, we established that PSC induced by low concentrations of 5-HT can be inhibited by low concentrations of 5-HT_2 antagonists (e.g. ketanserin and NAF) (Barradas et al., 1990b). We have shown that PSC responses of platelets obtained from PVD patients were diminished as compared to healthy subjects

when induced by 5-HT but not when induced by ADP (Barradas et al., 1992b). This was observed despite the fact that platelets from the same patients are hyperaggregable when higher concentrations of agonists are used. We have interpreted this desensitization, or refractoriness, as being the manifestation of a defence mechanism to prevent excessive platelet activation *in vivo*. It is well known that platelets (Okada et al., 1978) and other cells (Pauwels et al., 1988) become desensitized when repeatedly stimulated with an agonist. Since PVD platelets may be continuously exposed to relatively high concentrations of endogenous plasma 5-HT this could contribute to a diminished response following the addition of 5-HT *in vitro*.

We are carrying out further studies to determine whether a diminished PSC response in patient platelets is due to a down-regulation in receptors or an uncoupling of signal transduction mechanisms.

Concluding remarks

In the present review, we examined some of the interactions between platelets and the bioamines, 5-HT and histamine, which may be relevant to atherosclerosis and thrombosis.

Figure 1 and Figure 2 are schematic representations of how 5-HT and histamine could contribute to atherosclerosis. Conditions (e.g. hyperlipidaemia, hyper-

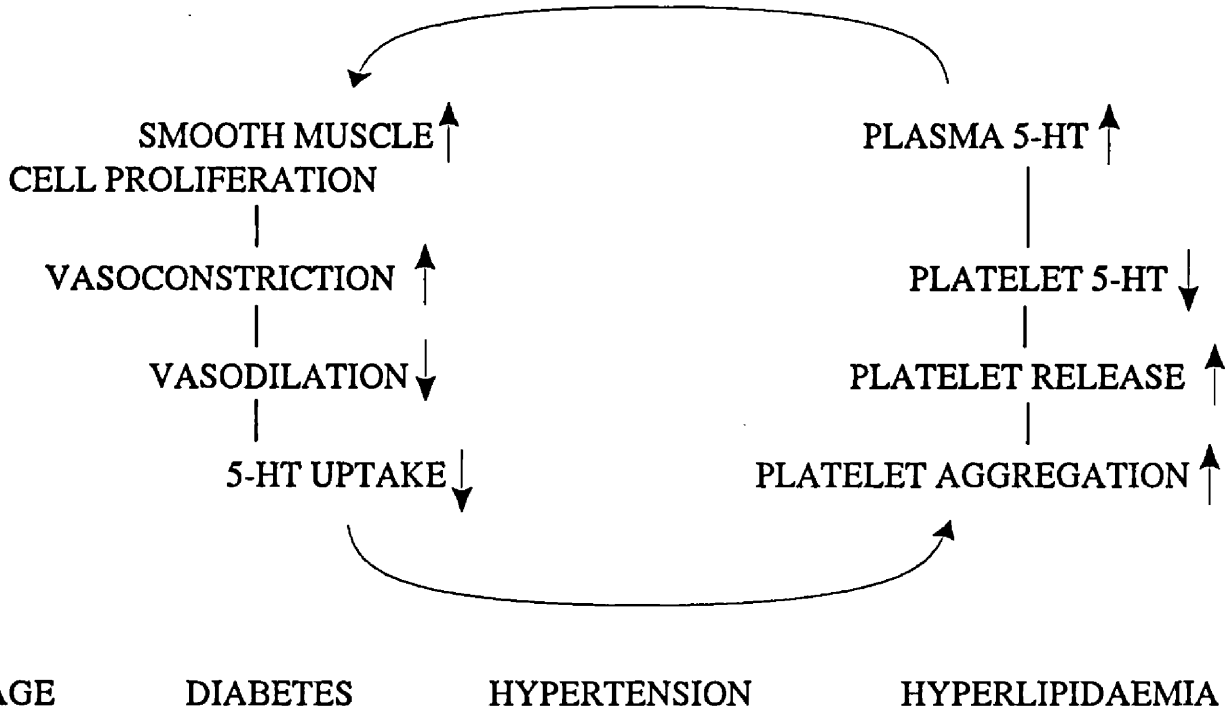

Figure 1 - Disease states such as aging, diabetes mellitus, hypertension, and hyperlipidaemia are associated with increased platelet hyperaggregability, decreased platelet 5-HT and increased 5-HT bioavailability. The elevated plasma 5-HT levels, in turn, may affect cell proliferation and vessel tone as well as reduce tissue 5-HT uptake.

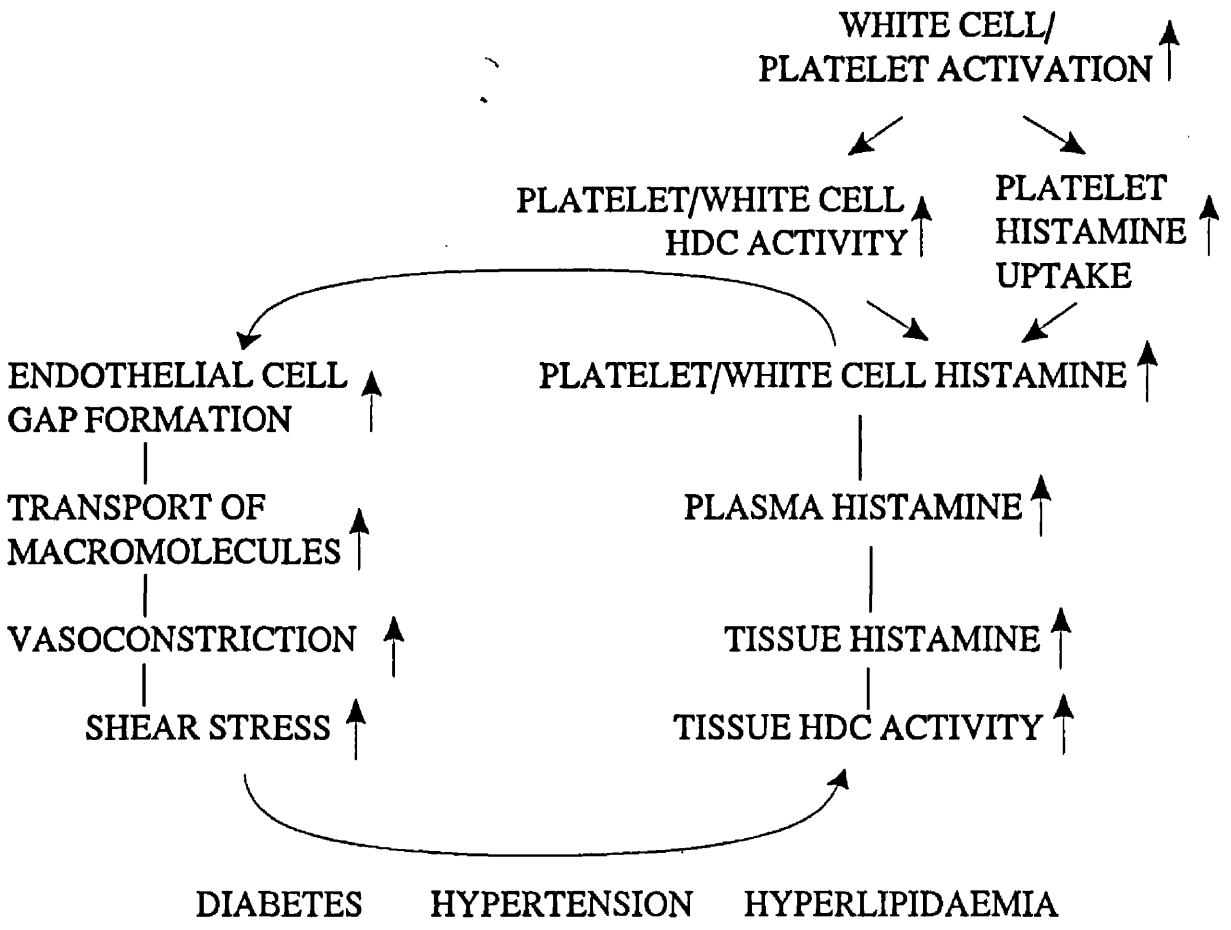

Figure 2 - Disease states such as diabetes mellitus, hypertension and hyperlipidaemia are associated with platelet/white cell activation and increased vascular tissue HDC activity. This, in turn, leads to an increase in plasma and cellular histamine concentrations which can increase vascular permeability and affect vessel tone. Furthermore, increased vasoconstriction and diminished blood flow increases shear stress which raises HDC activity. Increased histamine bioavailability could contribute to the development of atherosclerosis.

tension) which have been shown to be established risk factors for atherosclerosis are also associated with platelet hyperaggregability and enhanced platelet release of substances such as 5-HT. Excess 5-HT in conditions where the vasculature is compromised may enhance vasoconstrictor and diminish vasodilator effects. A balance in favour of vasoconstriction, together with diminished 5-HT uptake into tissues and platelets would leave the vasculature more prone to the mitogenic effects of this bioamine, thus helping the establishment of a vicious cycle and atherosclerosis.

Platelet activation results in increased uptake of histamine from the plasma. In turn, the elevated plasma histamine concentration is probably derived from enhanced tissue synthesis due to increased shear stress. Histamine availability can result in vasoconstriction, increased endothelial gap formation and transport of atherogenic macromolecules.

In conclusion, identifying the various mechanisms which go to create the conditions of an atherosclerotic state will lead to the development of treatment to prevent, reverse or retard this pathological process.

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THE EFFECT OF TREATMENT WITH SIMVASTATIN ON PLATELET FUNCTION INDICES IN HYPERCHOLESTEROLAEMIA

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Abstract

Platelet function indices were evaluated in 12 hypercholesterolaemic patients treated for 12 weeks with the HMGCoA reductase inhibitor, simvastatin. Treatment with simvastatin resulted in the normalisation of the lipid profile and a significant improvement in platelet function (aggregation in platelet rich plasma, release of thromboxane A_2, intraplatelet serotonin concentration). In particular, intraplatelet serotonin concentration increased towards normal values after treatment. This latter change is likely to reflect improved platelet function, *in vivo*. There was no change in plasma fibrinogen concentration throughout the study period. There were no significant changes in haematological and biochemical variables (including creatine kinase and liver function tests). Treatment was well tolerated and all patients completed the trial. In conclusion, simvastatin exerts a dual action on the risk factors that predict ischaemic heart disease. Thus, both the lipid profile and platelet function were significantly improved.

Key words: Hypercholesterolaemia, simvastatin, platelets, serotonin, thromboxane A_2

Introduction

Hypercholesterolaemia is an important risk factor for atherosclerosis and is associated with an increased incidence of ischaemic heart disease (IHD) [1]. The mechanisms responsible for this relationship remain open to debate but it is possible that hypercholesterolaemia mediates some of its effects by adversely influencing the haemostatic system [2–8].

There is evidence that hypercholesterolaemia in patients and in animal models is associated with platelet hyperactivity [3,4,7,9–11]. Furthermore, increasing the amount of cholesterol in platelet membranes, *in vitro*, is associated with enhanced platelet activity [12]. Some of these effects on platelets were reversible on correcting the cholesterol abnormalities. The significance of these findings is enhanced when we consider the role of platelets in thrombogenesis and atherogenesis [1].

In the context of the coagulation system, there is evidence linking hyperfibrinogenaemia with hyperlipidaemia [5,13–15]. This latter relationship is important since plasma fibrinogen, a coagulation factor, is a powerful predictor of IHD [16].

The present study investigates the effect of treatment with a 3-hydroxy-3-methyl-

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glutaryl coenzyme A reductase (HMGCoA reductase) inhibitor, simvastatin, on the lipid profile, platelet function indices and plasma fibrinogen concentration in patients with hypercholesterolaemia.

Patients, materials and methods

Selection of patients

Twelve hypercholesterolaemic patients were selected. Their median age was 56 years (range: 32–70). Nine patients were males and three were females. All had serum cholesterol concentrations above 6.5mmol/l. Three patients had symptoms of IHD (angina pectoris), one had hyperuricaemia with episodes of gout in the past. Previous lipid-lowering medication which was discontinued before the commencement of simvastatin administration consisted of: bezafibrate 200mg, three times daily (two patients) and cholestyramine, 4–24g, daily (four patients). Other medication which continued for the duration of the trial consisted of: nifedipine 5mg, twice daily (one patient), atenolol 100mg daily (one), nifedipine 10mg three times daily (one) and allopurinol, 300mg daily (one). None of the patients were diabetic. All patients had a normal haematological and biochemical profile (apart from the lipid variables) on entry to the trial.

Design of trial

Out-patients were screened and their consent obtained. During this screening period, which lasted 6–8 weeks, the patients were given appropriate dietary advice. Following this preliminary period, a fasting blood sample was obtained (Visit 1) and tested for the variables described below. Patients were then put on placebo for four weeks and sampled again at the end of this period (Visit 2). From then on, patients were treated with simvastatin and reviewed every four weeks. The initial dose of simvastatin (10mg/daily) was administered immediately following Visit 2. After four weeks treatment, the patients were sampled again (Visit 3) and their treatment reviewed. On this visit, the dose of simvastatin was increased to 20mg/daily, if the serum cholesterol concentration was above 5.3mmol/l. After another four weeks treatment, the patients were sampled again and their dose of simvastatin reviewed (Visit 4). If their serum cholesterol did not meet the same criteria as for Visit 3 (see above), the dose of simvastatin was increased to 40mg daily. The final blood sample was obtained four weeks later (Visit 5). Consequently, the duration of the total active treatment period was 12 weeks. Simvastatin was always taken with the evening meal.

No drugs were added or discontinued during the trial period and patients did not alter their dietary or smoking habits.

The trial was approved by the ethics committee of the Royal Free Hospital and Medical School.

Simvastatin was supplied by Merck Sharp & Dohme Ltd., UK.

Tests conducted on the blood samples collected

(a) *Lipids:* The following variables were measured in fasting serum: cholesterol, triglycerides, high density lipoprotein (HDL) and low density lipoprotein (LDL). These variables were measured using methodology in routine use in the Department of Chemical Pathology at the Royal Free Hospital.

(b) *Platelets:* The following variables were assessed:

Platelet count: In whole blood and in platelet rich plasma (PRP). Whole blood counts were performed using a Coulter T-660 (Coulter Electronics, Luton, UK) and those in PRP were performed using a Coulter ZM counter.

Platelet Aggregation: This variable was assessed in whole blood and in PRP. Blood was collected from an antecubital vein with minimum stasis. The blood was anti-coagulated with 3.8% trisodium citrate (9 parts blood; 1 part citrate).

For whole blood aggregation the citrated blood sample was directly evaluated in a Chronolog whole blood

impedence aggregometer (Model 540) [17,18]. Whole blood platelet impedance aggregation (WB-PIA) was induced by various agonists as previously described. The type and concentration of agonists is shown in the appropriate table in the results section.

For PRP aggregation, the PRP was prepared by centrifugation as previously described [17,19]. After separating the PRP, the sample was centrifuged again so as to obtain platelet poor plasma (PPP). The type and concentration of agonists is shown in the appropriate table in the results section.

Platelet thromboxane A_2 (TXA_2) release: The release of this eicosanoid, a vasoconstrictor and potentiator of platelet aggregation was assessed, in PRP, as previously described [17]. Briefly, aggregation was induced by various agonists and after three minutes the PRP was denatured by the addition of absolute ethanol. The sample was then stored, at –40°C, until assay in a single batch. The assay measured TxB_2, the stable and spontaneous breakdown product of TxA_2. The assay has previously been verified for specificity and reproducibility.

Platelet volume: Median platelet volume (MPV) was measured in PRP using a Coulter ZM counter coupled to a C256 channelyzer [18].

Platelet serotonin content: This variable was measured in the 12 patients on Visit 2 and Visit 5. Briefly, platelet pellets were prepared from PRP, lysed with buffer and sonicated as previously described [20]. The lysate was acetylated and assayed for acetylated serotonin using a specific radioimmunoassay [20].

(c) *Plasma fibrinogen concentration* was measured in citrated PPP using an automated ACL 300 Research coagulation system (Instrumentation Laboratory, Warrington, UK).

(d) *Haematological and biochemical profile:* A full blood count was performed and several biochemical variables (plasma potassium, sodium, urea, creatinine, bicarbonate, calcium, phosphate, albumin, total protein, aspartate transaminase, bilirubin, alkaline phosphatase, creatinine kinase) were also monitored using a Technicon SMAC autoanalyzer. Some of the actual values of these variables are listed in Table 5. Blood glucose concentration was estimated by routine methods.

(e) *Side effects:* Patients were asked to report side effects at each visit.

Statistical analysis

The results are presented as median (range) and their statistical significance analysed using non-parametric tests. The Wilcoxon test was used to compare paired values and the Spearman's correlation coefficient (rs) was used to assess correlation between values. All tests were two-tailed.

Results

(a) Lipids (Table 1)

As expected, treatment with simvastatin resulted in a considerable and significant decrease in serum cholesterol and LDL concentration. These changes occurred within four weeks of treatment (at 10mg/day).

(b) Platelets

Platelet count in whole blood and in PRP: There were no significant changes in these variables throughout the study period (results not shown).

(c) Platelet aggregation in PRP (Table 2)

Treatment with simvastatin, for 12 weeks, resulted in significant inhibition of aggregation, in PRP. This inhibition was evident whether values after 12 weeks' treatment were compared with those at the beginning or at the end of the placebo period.

When values at Visit 2 (basal value – pretreatment) and those at Visit 5 (end of 12 weeks' treatment) were considered together

Table 1. Median (range) of lipid variables (mmol/l). On some visits, only 11 of the total of 12 values were available for statistical analysis.

	Visit				
	1	2	3	4	5
Cholesterol	8.9 (6.7–12.3)	8.0 (6.5–12.9)	6.5 (4.8–9.1)	6.4 (4.2–8.5)	6.2 (4.6–7.1)
Triglycerides	1.5 (0.9–3.8)	1.4 (0.6–4.0)	1.5 (0.9–4.2)	1.1 (0.8–3.2)	1.2 (0.5–2.2)
LDL	6.7 (4.8–10.1)	6.4 (4.5–10.1)	4.2 (2.9–6.7)	4.4 (2.5–6.7)	4.0 (2.8–6.0)
HDL	1.1 (0.6–2.0)	1.1 (0.6–1.5)	1.3 (0.7–2.0)	1.3 (0.9–1.8)	1.1 (0.7–1.8)

Statistical analysis of table 1:
Cholesterol:
Visit 1 *vs.* Visit 2: NS; Visit 1 *vs.* Visits 3, 4 or 5: p = 0.005; Visit 2 *vs.* Visits 3, 4 or 5; p = 0.005; All other comparisons: p = NS.

Triglycerides:
Visit 1 *vs.* Visits 2 or 3: p = NS; Visit 1 *vs.* Visits 4 or 5: p<0.02; Visit 2 *vs.* Visits 3, 4 or 5: p = NS; Visit 3 *vs.* Visit 4: p<0.01. All other comparisons: p = NS.

LDL:
Visit 1 *vs.* Visit 2: p = NS; Visit 1 *vs.* Visits 3, 4 or 5: p = 0.005; Visit 2 *vs.* Visits 3, 4 or 5: p = 0.005. All other comparisons: p = NS.

HDL:
Visit 2 *vs.* Visit 3: p = 0.04; Visit 2 *vs.* Visit 4: p = 0.01. All other comparisons: p = NS.

NS = not significant.

Table 2. Median and (range) platelet aggregation (%) in PRP.

	Visit				
Agonist	1	2	3	4	5
AA (n=a) (0.5mmol/l)	91 (78–98)	93 (70–100)	84 (60–100)	82 (0–90)	76*†† (0–90)
ADP (2µmol/l)	45 (15–85)	40 (20–87)	31 (0–100)	18 (0–92)	25 (3–90)
ADP (10µmol/l)	80 (55–100)	83 (62–100)	78 (65–94)	70 (46–100)	75** (50–83)
Collagen (n=8) (0.5mg/l)	70 (8–82)	78 (30–100)	55 (0–95)	55 (0–73)	16††† (0–58)
Collagen (n=8) (1mg/l)	70 (40–95)	83 (15–100)	65 (12–95)	Insufficient numbers tested	72† (0–90)
Adrenaline (1µmol/l)	20 (5–91)	16 (0–76)	15 (0–100)	20 (0-80)	23 (3–85)
Adrenaline (5µmol/l)	54 (8–100)	51 (10–100)	51 (0–88)	46 (0-85)	47 (5–100)

Number of patients/group (n) = 11 unless otherwise stated.

Statistical analysis:
* p=0.02 *vs.* Visit 1; ** p=0.01 *vs.* Visit 1; † p=0.05 *vs.* Visit 2; ††p=0.02 *vs.* Visit 2; ††† p=0.01 *vs.* Visit 2.

(11 patients), there was a significant correlation between aggregation induced by ADP (10μmol/l) and the corresponding serum cholesterol and LDL concentration: serum cholesterol and ADP aggregation: $r_s = 0.58$, $p = 0.005$, serum LDL and ADP aggregation: $r_s = 0.55$, $p = 0.007$.

(d) Platelet aggregation in whole blood (WB-PIA) (Table 3)

There were no significant changes in WB-PIA when the values at Visit 2 were compared with those at Visit 5.

(e) Platelet TxA_2 release (Table 4)

There was a significant fall in the release of TxA_2 (induced by collagen or adrenaline) after 12 weeks' treatment with simvastatin.

Table 3. Median and (range) whole blood aggregation (ohms) measured by the impedance method (WB-PIA).

	Visit	
Agonist	**2**	**5**
Collagen (2mg/l)		
at 3 min	2.0 (0–8.3)	2.8 (0–8.4)
at 6 min	5.8 (0–11.5)	6.3 (0–11.1)
ADP (20μmol/l)		
at 3 min	2.6 (0–7.8)	2.3 (0–7.8)
at 6 min	4.8 (0–10.1)	4.1 (0–8.9)

Statistical analysis:
All comparisons: p = NS.

Table 4. Median and (range) platelet TXA_2 (ng/10_9 platelets) release, in PRP.

	Visit	
Agonist	**2**	**5**
Collagen (1mg/l)	126 (85–204)	75 (32–210)
ADP (10μmol/l)	87 (38–146)	60 (24–118)
Adrenaline (5μmol/l)	86 (36–193)	60 (9–21)

Statistical analysis (Visit 2 vs. Visit 5):
Collagen: p<0.01; ADP: p = NS; Adrenaline: p<0.01.

There was a fall in ADP-induced TxA_2 release but this did not achieve statistical significance.

(f) Median platelet volume (MPV)

There were no significant changes in MPV throughout the study.

(g) Platelet serotonin content

There was a significant (p=0.03) increase in the intraplatelet concentration of serotonin after 12 weeks' treatment with simvastatin. Thus, the median intraplatelet serotonin concentration (nmol/10^9 platelets) was 1.73 (0.38–4.82) at Visit 2 and 3.23 (0.90–5.39) at Visit 5. Intraplatelet serotonin concentration correlated significantly with serum cholesterol and LDL concentration:

cholesterol and intraplatelet serotonin: $r_s = -0.50$, $p = 0.01$
LDL and intraplatelet serotonin: $r_s = -0.65$, $p = 0.001$

(h) Plasma fibrinogen concentration

There was no change in the plasma fibrinogen concentration throughout the study. The corresponding median and (range) fibrinogen concentrations (g/l) were: Visit 1: 2.77 (2.19–4.56); Visit 2: 2.67 (2.05–3.82); Visit 3: 3.01 (2.38–3.83); Visit 4: 3.03 (2.11–4.23); Visit 5: 2.72 (2.28–3.66)

(i) Haematological and biochemical profile (Table 5)

There were no significant changes in the haematological or biochemical variables (for changes in the lipid profile, see section a, above) throughout the study.

(j) Side effects

Treatment was well tolerated. All 12 patients completed the trial. The four patients previously on cholestyramine (see Patients, materials and methods section) did not wish to return to their original medication.

Discussion

The present findings confirm and extend those of previous studies which evaluated the effect of simvastatin administration on platelet function in hyperlipidaemic patients

[2,3]. Thus, we also noted a significant inhibition of various indices of platelet function following treatment with this HMGCoA reductase inhibitor.

Inhibition of platelet aggregation and a reduction in platelet TxA_2 release has been previously documented following four weeks to eight months treatment with simvastatin in type IIa hypercholesterolaemics [2,3]. The suggested mechanisms of action have been changes in the number of platelet prostacyclin (a naturally occurring, potent inhibitor of platelet aggregation) receptors and platelet membrane lipid composition [2,3]. The diminished TxA_2 release following simvastatin administration (observed in earlier studies [2,3] and in the present trial) is in keeping with the evidence that an increase in membrane cholesterol content enhances phospholipase A_2 activity and endoperoxide response in human platelets [7].

It is of interest that we observed a correlation between platelet aggregation induced by the maximal dose of ADP (10μmol/l) and both cholesterol and LDL concentration. The reason why the other agonists did not show a similar correlation may reflect the fact that this dose of ADP tends to result in very reproducible aggregation (unpublished observations). However, this association also suggests that cholesterol content does not influence platelet function solely by altering TxA_2 release since ADP-induced aggregation is not very dependent on this eicosanoid [21]. The absence of a significant effect on whole blood aggregation may reflect a lack of sensitivity of this technique and the high agonist concentrations required to obtain a response.

The present study also indicates that intraplatelet serotonin concentration is normalised following the correction of hypercholesterolaemia. We have previously shown that intraplatelet serotonin concentrations are low in patients with peripheral vascular disease [20]. This abnormality was attributed to platelet hyperactivity (which is known to occur in these patients [20,22] and a resulting imbalance between uptake and release of this bioamine. This conclusion was supported by finding an elevated plasma serotonin concentration in these same patients [20]. We therefore suggest that the serotonin changes observed here are a reflection of a normalisation of platelet activity, *in vivo*. The significant correlation of intraplatelet serotonin with serum cholesterol and LDL concentration has been observed by us in other clinical settings [23]. This association may reflect a role for cholesterol in modulating platelet serotonin uptake and platelet activity.

The absence of an effect on plasma fibrinogen concentration is in agreement with the findings of previous studies [6,24]. However, it would be of interest to investigate the effect of treatment with simvastatin in patients who, unlike our patients, have a combined elevation in both plasma

Table 5. Median and (range) values of biochemical indices.

	Visit				
Test	**1**	**2**	**3**	**4**	**5**
CK (U/l)	106 (20-185)	101 (26–184)	112 (20–159)	91 (22–208)	92 (26–132)
AST (U/l)	33 (16-44)	28 (15–44)	31 (23–66)	28 (17–41)	32 (17–50)
ALP (U/l) (n=11)	87 (50–140)	85 (52–152)	80 (45–143)	80 (51–155)	76 (53–129)
Bil (μmol/l) (n=8)	10 (7–16)	9 (6–17)	12 (6–17)	10 (6–16)	10 (4–16)

Statistical analysis:
All comparisons; p = Not significant.

cholesterol and triglycerides. In this setting, two factors may become relevant in the context of fibrinogen-lowering potential. One factor is that the administration of simvastatin is likely to lower plasma triglyceride concentrations in addition to its well established effect on plasma cholesterol [25]. The second factor is that hypertriglyceridaemia is associated with elevated plasma fibrinogen concentrations [14,15]. The issue of fibrinogen-lowering capacity is of some importance since this coagulation factor enhances platelet aggregation [26] and is an established predictor of IHD [16]. These issues have been discussed elsewhere [27,28].

The findings of the present study are encouraging because they point towards simvastatin exerting a dual action on the factors which increase the risk of myocardial infarction (MI). These factors are hyperlipidaemia and platelet activation. There is considerable evidence linking the first of these factors with atherogenesis and an increased risk of developing IHD [1]. There is also evidence that treating hyperlipidaemias reduces the incidence of cardiovascular events and increases the frequency of regression of coronary lesions [29]. There is also evidence supporting a role for platelets in the pathogenesis of atheroma (e.g. by the release of growth factors [1]. Furthermore, recent evidence indicates that platelet function indices are predictors of MI [30,31]. It is, therefore, of interest that hyperactive platelets have been reported in hyperlipidaemic states, in animal models of hypercholesterolaemia and following the addition of cholesterol to platelets *in vitro* (see Introduction).

Although this is not a double-blind study its findings are in agreement with those of two other studies [2,3] assessing the effect of treatment with simvastatin on platelet function indices.

In conclusion, we have demonstrated that conventional doses of simvastatin, administered over a period of 12 weeks, to patients primarily suffering from hypercholesterolaemia, results in the normalisation of the lipid profile and platelet function. These beneficial effects should be considered when selecting lipid-lowering drugs.

Acknowledgements

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EFFECT OF MILRINONE ON HUMAN PLATELET SHAPE CHANGE, AGGREGATION AND THROMBOXANE A_2 SYNTHESIS: AN IN VITRO STUDY

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Abstract Milrinone (MIL; a cAMP-specific phosphodiesterase type-III inhibitor), added in vitro to achieve concentrations below the therapeutic levels, inhibited agonist-induced platelet shape change (PSC). Arachidonic acid (AA)-induced PSC was significantly more inhibited by a combination of MIL and indomethacin (INDO; a cyclooxygenase inhibitor) than by either alone. PSC induced by 5-hydroxytryptamine was inhibited by MIL but not by INDO; and this effect of MIL was not augmented by INDO. Whole blood-platelet aggregation (WB-PA) and platelet-rich plasma aggregation induced by potent stimulators of thromboxane A_2 (TXA_2) synthesis such as AA and calcium ionophore and by less potent agonists (e.g. ADP and U46619) were inhibited by MIL at or near therapeutic concentrations. WB-PA induced by collagen was significantly more inhibited by the MIL and INDO combination than by either of these agents alone whereas with ADP-induced WB-PA no additional effect could be shown when both MIL and INDO were co-incubated. MIL and similar types of drugs may be of benefit in conditions associated with platelet hyperactivity and some of these effects may be enhanced by cyclooxygenase inhibitors.

Epidemiological evidence suggests that platelet function indices, such as aggregation and platelet volume predict ischaemic heart disease (IHD)(1,2). Platelet hyperactivity (e.g. enhanced aggregability and release of intraplatelet substances) has also been documented in patients with vascular disease (3). Anti-platelet therapy is, therefore, recommended for disease states associated with hyperaggregable platelets (4). Drugs, including aspirin (ASA) and dipyridamole have been widely used clinically and extensively studied (5-8). These drugs are, however of limited anti-platelet efficacy. ASA, for example, although effective at inhibiting cyclooxygenase and aggregation induced by agonists which mobilize arachidonate for

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thromboxane A_2 (TXA_2) synthesis (e.g. collagen) (9,10), is less effective at inhibiting aggregation induced by other agonists (e.g. platelet activating factor (10), 5-hydroxytryptamine (11) and ADP (12)). In the context of platelet-suppressive effects, it is well established that a major inhibitory pathway in platelets depends on the second messenger cAMP (13) which modulates platelet aggregation and release by controlling the mobilisation of calcium (13). It influences agonist-induced signal transduction mechanisms and exerts inhibitory effects on phospholipase C and phospholipase A_2, key enzymes involved in calcium mobilisation (13). Drugs which elevate intracellular cAMP concentrations are therefore of clinical interest. Such drugs include eicosanoids (e.g. PGE_1, PGI_2) and the PGI_2-analogue, iloprost (14). Intraplatelet cAMP levels are also controlled by cAMP-phosphodiesterase (PDE), which hydrolyses cAMP to AMP (15). Thus, PDE inhibitors may be of use in preventing platelet hyperaggregability and thrombosis (15-22). A number of new more specific PDE inhibitors have recently been investigated (15,19,20,22). MIL, a cAMP-specific phosphodiesterase type-III inhibitor, was developed for treatment of heart failure (23). MIL has been shown to inhibit platelet aggregation (19,20) and to have other effects on platelets, for example on $^{45}Ca^{2+}$ uptake (24) and sodium transport (25).

In this study, we investigate the effects of MIL alone and in combination with indomethacin (INDO; a cyclooxygenase inhibitor (26)) on platelet shape change (PSC), platelet aggregation and TXA_2 release following incubations in platelet rich plasma (PRP) and whole blood (WB).

MATERIALS AND METHODS

Blood collected from healthy volunteers was anticoagulated with 3.8% trisodium citrate. PRP and PPP (platelet-poor-plasma) was prepared as previously described (27). MIL was dissolved in a saline/ethanol mixture (final concentration of ethanol less than 0.1% w/v). Indomethacin was dissolved in phosphate buffer (20 mM KH_2PO_4, 116 mM Na_2HPO_4; pH 7.8). MIL and/or INDO (10 μl volumes) and respective vehicles were incubated with blood or PRP for 5 min, at 37 ^{0}C, before the addition of agonists. For PSC studies, PRP (470 μl), in siliconised glass cuvettes, was placed in a Chronolog dual channel optical aggregometer (Coulter Electronics, Luton, UK) and stirred at 1000 rpm, at 37 ^{0}C. Agonists were added as 10-30 μl volumes to the stirring PRP in order to achieve the final concentrations shown in the figures. At the specified times following agonist addition (see figure legends) an aliquot of PRP was removed and fixed with glutaraldehyde (28). Platelet counts were measured in fixed PRP suspensions diluted with Isoton II (Coulter Electronics, Luton, UK) by a Coulter counter ZM (electrical impedance method using a 70 μm diameter sampling tube orifice). For size analysis samples were processed by a C-256 channelyzer (Coulter Electronics, Luton, UK). The median value of the size distribution plots, i.e. the median platelet volume (MPV) was used to assess shifts in platelet size. Throughout this study the platelet count in PRP in each sample cuvette was monitored and did not change by more than 5% of the basal count. The reproducibility of this method has been previously reported (29). Platelet aggregation in PRP (PRP-PA) was quantified using a Chronolog optical aggregometer as previously described (27). Platelet TXA_2 synthesis in PRP, was quantified at the end of the PRP-PA procedure as described earlier (30).

Whole-blood platelet aggregation (WB-PA) was evaluated by a free platelet count method using a blood cell counter (Coulter T-890)(31) with some modifications (32). Calcium ionophore A23187 (CaI), adenosine diphosphate (ADP), epinephrine (epin), 5-Hydoxytryptamine creatinine sulphate (5-HT), human thrombin, arachidonic acid (AA), indomethacin and the TXA_2/endoperoxide mimetic (U46619) were purchased from Sigma Chemical Co., Poole, UK. Collagen (coll) was purchased from Hormon Chemie, Munich, Germany. Milrinone was a gift from Sanofi-Winthrop, Guildford, UK.

Statistical analysis and presentation of results. Results are presented as means and $\pm$ SEM. For improved diagrammatic purposes in figures (Fig) 1,3, 5 and

6 only maximal $^{+}$ SEM bars are shown (i.e. these SEMs were the largest in that specific Fig). Statistical analysis was carried out using the Wilcoxon-test for paired data (two-tailed). For experiments where more than two means are compared (see Fig 2 and 4), Friedmans's 2-way analysis of variance (ANOVA) for overall group comparisons was used to establish a significant interaction before using the Wilcoxon-test (33). IC_{50}s were calculated graphically

RESULTS

MIL inhibited significantly ($P< 0.01$) agonist-induced PSC in a dose dependent manner (Fig. 1). With some agonists (CaI, AA, coll) MIL was potent at concentrations (IC_{50} : 0.05-0.1 μM) considerably lower than the therapeutic concentrations ((23); approx. 1.5 μM; arrow on Fig. 1). Higher concentrations of MIL were required to inhibit PSC induced by other agonists: U46619, ADP and 5-HT (IC_{50}s: 0.5, 1.0 and 2.5 μM).

In experiments designed to study the effect of the combined addition of MIL and INDO, concentrations of INDO which block cyclooxygenase (16 μM, ref. 26) effectively inhibited AA-induced PSC (Fig. 2). MIL (0.15 μM) potentiated the inhibitory effect of INDO and completely abolished AA-induced PSC (Fig. 2). 5-HT-induced PSC was not affected by INDO and there was no enhancement of the inhibitory effect exerted by MIL (2.5 μM; Fig. 2).

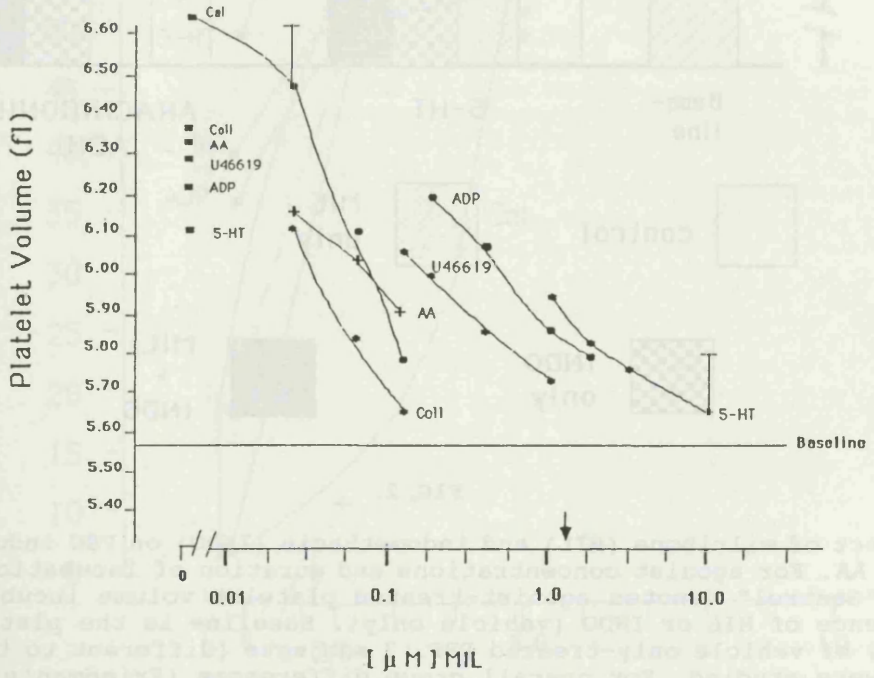

FIG. 1.

Effect of milrinone (MIL) on agonist-induced platelet shape change in PRP. The solid squares on the left-side of the figure denote the median platelet volume following incubation with the agonist and vehicle only. Baseline is the platelet volume in femtolitres (10^{-15} L; fl) of vehicle only-treated PRP (no agonist present). The arrow denotes typical therapeutic concentration of MIL achieved following intravenous infusion in heart failure patients (23). PSC was measured 30 sec following CaI (2.7μM); 3 min after coll (0.2-0.4 mg/L); 30 sec after AA (0.2 mM); 30 sec after U46619 (0.05 μM); 30 sec after ADP (0.2-0.4 μM); 30 sec after 5-HT (1μM). 7 subjects were studied.

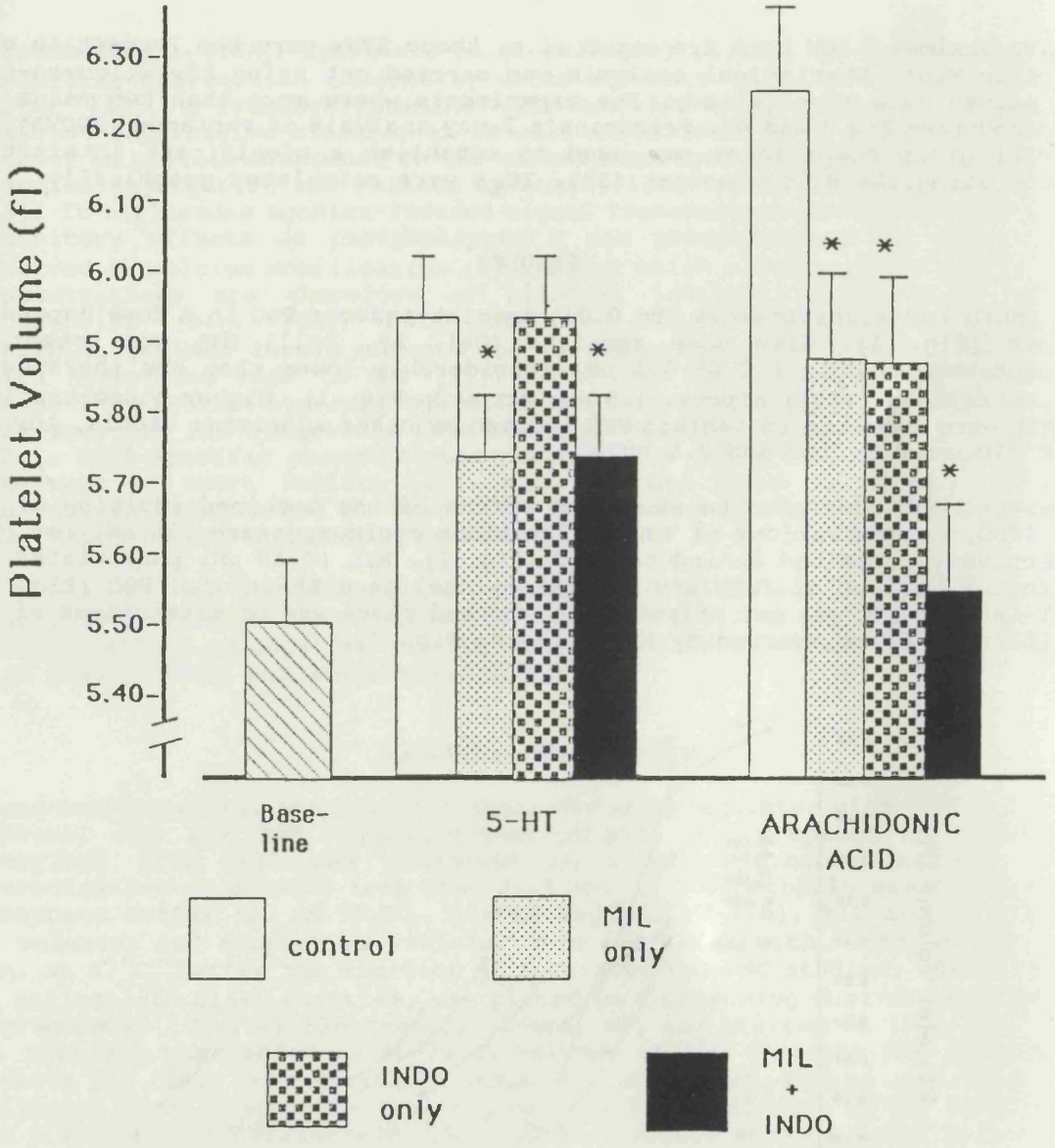

FIG. 2.

Effect of milrinone (MIL) and indomethacin (INDO) on PSC induced by 5-HT and AA. For agonist concentrations and duration of incubations see Fig. 1. "Control" denotes agonist-treated platelet volume incubated in the absence of MIL or INDO (vehicle only). Baseline is the platelet volume (fl) of vehicle only-treated PRP. 7 subjects (different to those in Fig 1) were studied. For overall group differences (Friedman's ANOVA) 5-HT: $P<0.01$; AA: $P<0.001$. For paired comparisons (Wilcoxon-test): * $P<0.02$ vs control. For AA induced PSC: MIL or INDO vs MIL+ INDO; $P< 0.02$.

WB-PA induced by 5-HT, AA, U46619 and collagen was inhibited significantly ($P<0.01$) by MIL at 1.25 μM and above, whereas for WB-PA induced by ADP, CaI and epin, MIL concentrations of 2.5 μM and above were required (Fig. 3). Collagen-induced WB-PA was significantly ($P< 0.02$) inhibited by MIL (2.5 μM) and by INDO (16μM; Fig. 4). Furthermore, the combination of MIL and INDO was more effective at inhibiting collagen-induced WB-PA than either of these agents alone ($P< 0.02$ MIL/INDO vs MIL+INDO). ADP-induced WB-PA was not inhibited by INDO and there was no significant additional effect when both drugs were incubated together. WB-PA induced by ADP was inhibited significantly ($P< 0.02$) by MIL (2.5 μM) and by MIL+INDO (Fig. 4).

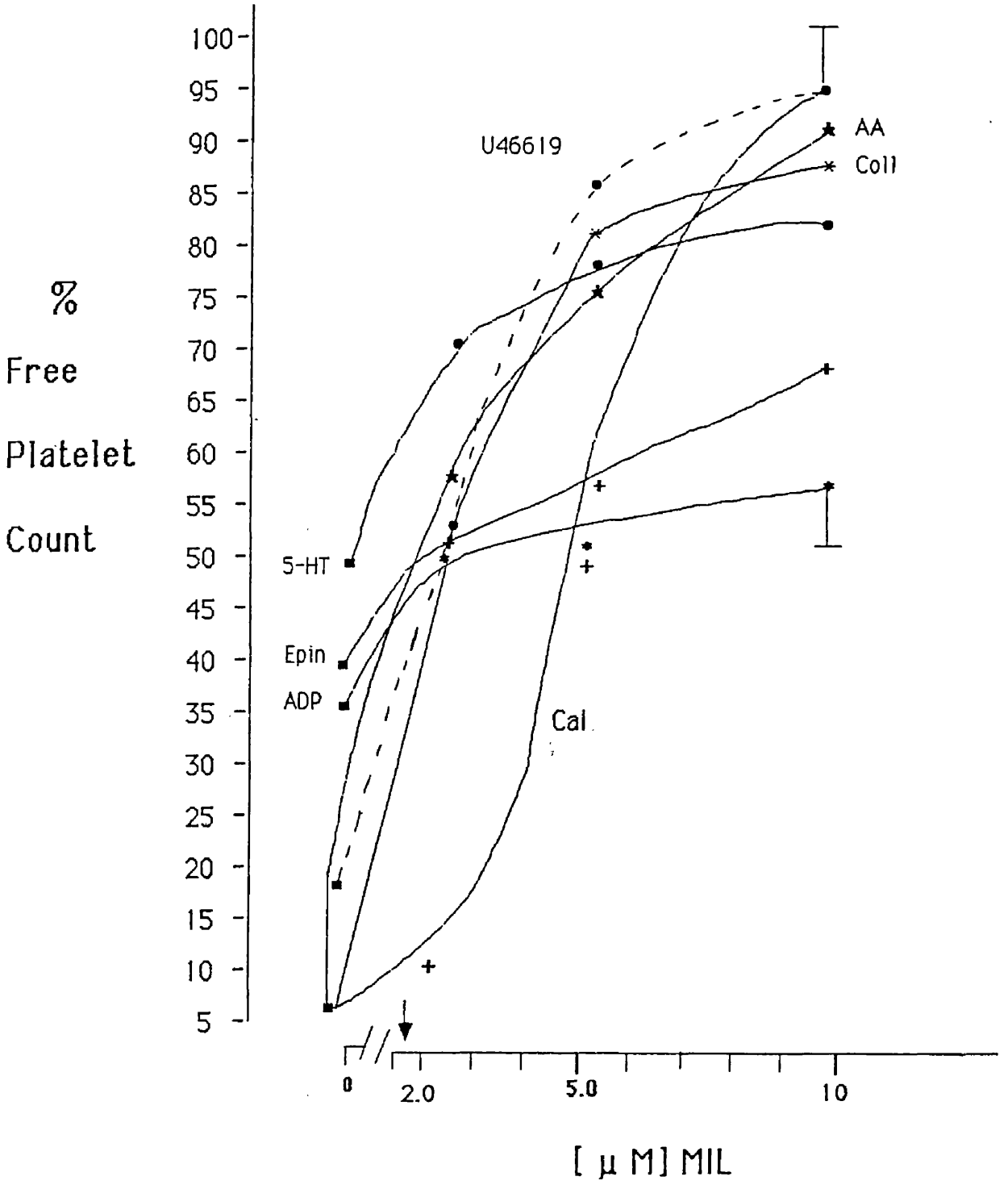

FIG. 3.

Effect of milrinone on agonist-induced whole blood platelet aggregation. Aggregation was estimated 1 min after adding agonists, except for Coll (3 min) and 5-HT (30 sec). Aggregation is expressed as a % of basal (unstimulated) platelet count. The basal count is arbitrarily defined as 100% free platelets. The arrow denotes typical therapeutic concentration of MIL achieved following intravenous infusion in heart failure patients (23). 9 subjects were studied. Bars= maximal SEM.

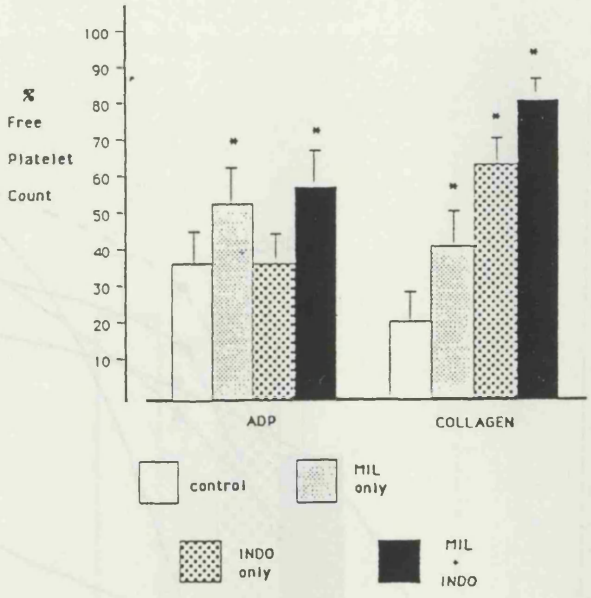

FIG. 4.

Effect of milrinone (MIL) and indomethacin (INDO) on ADP and coll-induced whole blood platelet aggregation. Agonist incubations and aggregation was carried out as outlined in Fig. 3. 7 subjects were studied. For overall group differences (Friedman's ANOVA) ADP: P<0.01; coll: P<0.001. For paired comparisons (Wilcoxon-test):* P<0.02 vs control. For coll: MIL or INDO vs MIL + INDO; P<0.02.

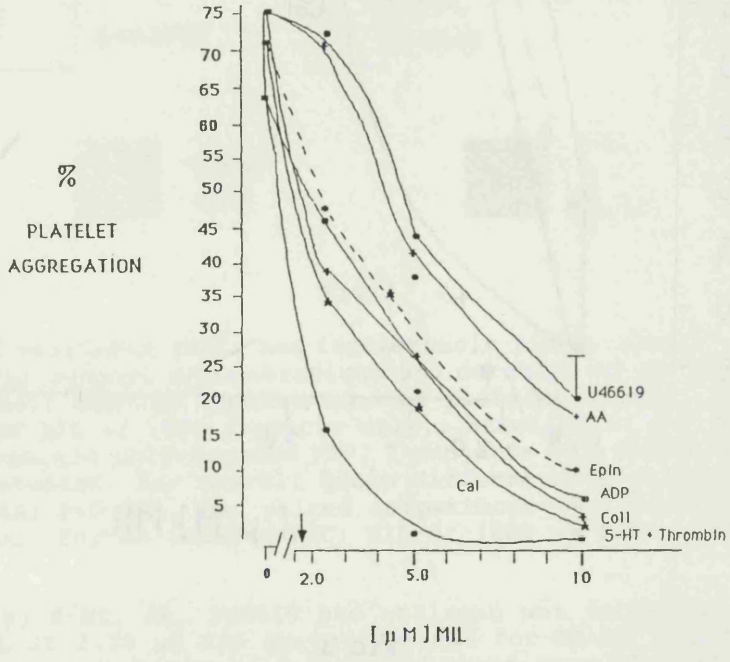

FIG. 5.

Effect of milrinone (MIL) on agonist-induced PRP aggregation. Aggregation was estimated (optically) 3 min after adding agonists. The arrow denotes typical therapeutic concentration of MIL achieved following intravenous infusion in heart failure patients (23). 9 subjects were studied. Bars= maximal SEM.

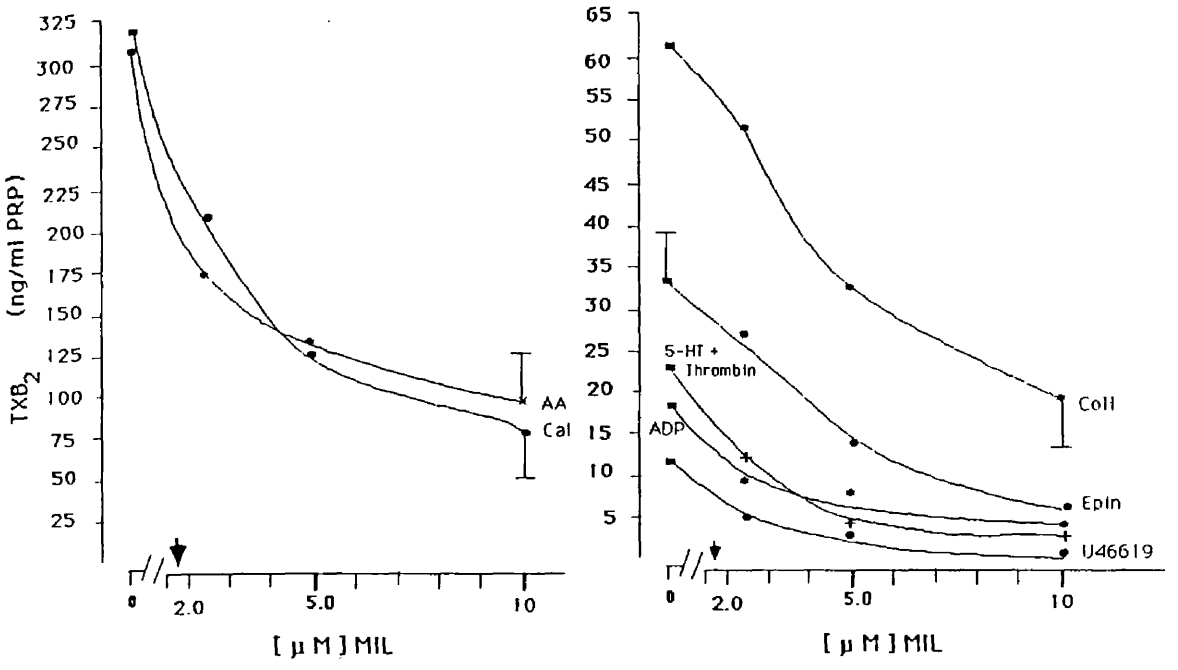

FIG. 6.

Effect of milrinone (MIL) on agonist-induced TXA_2 synthesis in PRP. TXA_2 was measured as TXB_2. The arrow denotes typical therapeutic concentration of MIL achieved following intravenous infusion in heart failure patients (23). 9 subjects were studied. Bars= maximal SEM.

Significant ($P<0.01$) inhibition of PRP-PA was obtained at 2.5 μM of MIL and above for all agonists except coll where significance was achieved at concentrations of MIL of 1.25 μM and above (Fig. 5).

TXA_2 generated by all agonists was significantly inhibited by MIL in a similar fashion to PRP-PA. This inhibitory action was independent of the TXA_2 synthesizing capacity of the agonist used. For some agonists (AA, CaI), lower concentrations of MIL were required to inhibit TXA_2 synthesis than PRP-PA (Fig. 6).

DISCUSSION

The measurement of PSC has not been widely used to assess the effect of drugs on platelets. This aspect of platelet function, may nevertheless be of physiological importance and allows the assessment of platelet changes preceding platelet aggregation (29,34). In this study we have shown that the inhibition of PSC occurs at MIL concentrations below those achieved therapeutically (1.5 μM). MIL may therefore reduce platelet activation which can lead to platelet aggregation and the release of platelet vasoactive substances (e.g. 5-HT; platelet derived growth factor). We have also shown the inhibition of WB-PA and PRP-PA by MIL which is in agreement with previous work (19). Furthermore, we found that MIL potently inhibits TXA_2 synthesis induced by powerful (AA and CaI) or weak (ADP and U46619) inducers of TXA_2 synthesis. The extent of the inhibitory effect on PSC, PA and TXA_2 synthesis, exerted by MIL was found to depend on the agonist used. Since each agonist transduces its activatory effects via different receptors and biochemical pathways, this suggests that MIL has stronger inhibitory effects on certain pathways leading to platelet activation than on others. This is also in agreement with the

conclusion that PDE inhibitors in addition to reducing cAMP phosphodiesterase activity also exert other effects on intracellular and extracellular mechanisms which influence platelet activation (18,24,25,35,36). It is well established, for example, that drugs such as dipyridamole and other cAMP phosphodiesterase inhibitors prevent the cellular uptake of adenosine, a substance which raises intracellular cAMP concentrations and thus inhibits platelet activation (37). Moreover, Patelunas et al (38) have demonstrated that the anti-aggregatory effect of MIL, at concentrations of 5 μM and above, was significantly reduced by the enzymatic removal of plasma adenosine. It is also possible that in our study, the inhibitory effects due to MIL are enhanced, at least in part, by the presence of plasma adenosine. In addition to exerting effects on its own, MIL was also found to enhance the inhibitory effect of a cyclooxygenase inhibitor, INDO, on both PSC and WB-PA. This phenomenon, however depends on the agonist used and its capacity to synthesize TXA_2. Thus, we observed significantly more inhibitory effect on PSC induced by AA when INDO and MIL were co-incubated than when either of these agents was incubated alone. 5-HT-induced PSC, however, was not affected by INDO added in vitro alone and there was no enhancement when both MIL and INDO were added together. We observed a similar phenomenon with WB-PA; collagen-induced WB-PA was significantly more inhibited when INDO and MIL were co-incubated than when either was incubated alone whereas ADP-induced WB-PA was not influenced by INDO alone and no enhancement was observed when both agents were co-incubated. These results can be rationalised on the basis that despite adding MIL the synthesis of large amounts of TXA_2, itself a powerful excitatory agonist, may decrease or reverse the increase in intracellular cAMP (39) resulting from MIL action. INDO (or cyclooxygenase inhibition) may therefore enhance the inhibitory effects of MIL by preventing TXA_2 availability. Cyclooxygenase inhibitors, such as ASA, although prescribed regularly for IHD have a limited efficacy when prescribed alone. On the basis of the above findings these drugs may be of greater benefit when prescribed in conjunction with MIL or other PDE inhibitors. In conclusion, it would appear that MIL and probably other PDE inhibitors may be effective at making hyperactive platelets more quiescent. It remains to be shown, however, whether MIL can normalize platelet hyperaggregability and reduce the incidence of thrombosis in disease states associated with platelet hyperaggregability.

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Measurement of Platelet Volume Using a Channelyzer: Assessment of the Effect of Agonists and Antagonists

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Abstract. *Platelets undergo morphological changes prior to aggregating. This phenomenon is known as the platelet shape change (PSC) and is usually accompanied by at increase in median platelet volume (MePV). We evaluated MePV changes in human platelet rich plasma (PRP) using a high resolution pulse-height analyser («channelyzer»). Increases in MePV were induced by the addition of low concentrations of known aggregating agents. These agonists showed different patterns in terms of potency, duration and reversibility.*

Platelet aggregation is thought to proceed through three stages: platelet shape change (PSC), primary aggregation and secondary aggregation (accompanied by the release reaction)[1,2]. Unlike aggregation, PSC has not been the subject of as many studies as the other stages[1,2]. However, PSC may prove to be as relevant as aggregation and it is therefore important to develop practical methods to assess this process.

PSC can be measured by several techniques which detect the increase in median platelet volume (MePV) which usually occurs concomitantly with morphological changes. These techniques include turbidometric aggregometry (using platelet rich plasma; PRP)[1,2-5], electron microscopy (EM)[5] and resistive-particle counters (using whole blood or platelet rich plasma)[1,2,6,7]. The first of these techniques is at best only semiquantitative (estimation of an increase in optical density representing an expansion in MePV). This technique has often been used with the addition of EDTA to PRP in order to optimise the methology[1,3,4]. EDTA, however, can cause irreversible changes in the platelet membrane glycoproteins and consequently the inhibition of both aggregation[8] and some aspects of PSC[1]. EM visualises PSC (*e.g.* the appearance of pseudopodia with or without a concomitant increase in MePV)[5] following activation. This technique is, however, not amenable to serial sampling or to the objective quantification of the volume changes. Particle counters, which measure changes in "resistive" volume and which reflect both shape and geometrical volume changes, on the other hand, are suited to multiple sampling. Furthermore, if a channelyzer is used, small changes in volume can be reproducibly detected.

The objectives of the present study were to develop a rapid, sensitive method to measure MePV and thereby assess the patterns of PSC which occur following the addition of agonists. We also established the concentration of naftidrofuryl (NAF), added *in vitro,* which is required to inhibit increases in MePV induced by agonists. This drug was selected because the concentration needed to inhibit platelet aggregation in PRP has been shown to be very high (50-100 μmol/1) relative to peak therapeutic levels (up to 6 μmol/1, after intravenous infusion) and there is evidence that NAF has serotonin (5-hydroxytryptamine; 5-HT) antagonist properties[9]. This drug is prescribed to improve walking distance in patients with peripheral vascular disease. Thus we investigated whether therapeutic levels of NAF, added *in vitro,* affect MePV and whether 5-HT selectivity can be demonstrated more definitively by measuring MePV than by assessing turbidometric aggregation.

Materials and Methods

Materials. Adenosine diphosphate, serotonin creatinine sulphate (5-HT), adrenaline bitartrate, noradrenaline bitartrate, ketanserin tartrate, calcium ionophore (A23187), arachidonic acid (sodium salt; AA), U46619 (endoperoxide/thromboxane A_2 mimetic) and glutaraldehyde (25% aqueous (w/v) were obtained from Sigma Chemical Co. Ltd (Poole, Dorset, U.K.). Collagen was obtained from Hormon Chemie (München, Germany). Isoton II was obtained from Coulter Electronics Ltd (Luton, Beds. UK). Naftidrofuryl oxalate (Praxilene) was a gift from Lipha Pharmaceuticals Ltd., West Drayton, Middlesex, UK. Platelet activating factor (PAF; C-18) was obtained from Bachem, Switzerland. All other reagents were of analytical grade and obtained from BDH Ltd (Dagenham, Essex, UK).

Blood collection and preparation of platelet-rich plasma. Blood was collected by venepuncture with minimum stasis from healthy volunteers

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who denied taking any medication for at least 14 days prior to sampling. Platelet-rich plasma (PRP) was prepared by the centrifugation of human blood anticoagulated with 3.8% w/v trisodium citrated (9 volumes blood: 1 volume citrate) as previously described[10]. The platelet count in the PRP was in the range 276-520 × 10^9 platelets/1 PRP. PRP was kept at 37°C, since cooling may affect platelet function[11]. The platelet studies were completed within 2 h of collection of the blood sample.

Agonist addition and sample fixation. Aliquots of PRP (450 µl), in siliconised glass cuvettes, were placed in a Chronolog dual channel optical aggregometer (Coulter Electronics, Luton, Beds., UK) and stirred (using a teflon-coated metal stir bar) at 1000 rpm, at 37°C. For experiments involving antagonists, naftidrofuryl oxalate (NAF), ketanserin tartrate (KET), or phosphate buffer (20 mM KH_2PO_4, 116 mM Na_2HPO_4; used as a vehible) were added (as 5-10 µl volumes) to PRP and pre-incubated for 5 min before the addition of agonists. KET is a relatively specific 5-HT_2 antagonist[12]. In order to prevent platelet activation due to stirring, during this pre-incubation period the magnetic stirring mechanism in the aggregometers was switched off after the first 30 sec and only switched back on after a 4 min interval. Agonists were added as 50 µl volumes to the stirring PRP in order to achieve the final concentrations shown in the Tables and Figures. At the specific times after agonist treatment, 100 µl aliquots of PRP were removed and mixed with 400 µl of fixative (4% v/v aqueous glutaraldehyde).

In preliminary experiments, we investigated the possibility of omitting the fixative. The objective of these experiments was to attempt to simplify the procedure. The results of these experiments are briefly described in the results section.

Platelet counting and particle size analysis. Platelet counts were measured in fixed PRP suspensions diluted 400 fold with Isoton II, at room temperature. These samples were then processed by a Coulter counter ZM (electrical impedance method using a 70 µm diameter sampling tube orifice) coupled to a C-256 channelyzer (pulse-height analyser; Coulter Electronics, Luton, Beds., UK)[7]. The analyzer sample "windows" were set between 2.67 and 19.12 fl. The counter was previously calibrated using platelet volume calibration latex particles of 9 fl volume (Lot. 7, Coulter Electronics, Luton, Beds., UK). For particle analysis, data were accumulated to a maximum of 500 platelets in one of the 256 channels and recorded on an X-Y recorder or displayed on the C-256 screen. The median value of the size distribution plots, *i.e.* the median platelet volume (MePV), was the volume of the channel on each side of which 50% of the platelet population was distributed. MePV was measured rather than the modal or mean platelet volume since this parameter was technically easier to quantify accurately within a short period of time.

Throughout this study, the platelet count in PRP in each sample cuvette was monitored to exclude the possibility of appreciable platelet aggregation which may affect MePV measurements.

Reproducibility was evaluated by calculating the intra- and interassay coefficient of variation (CV). In addition, reproducibility was assessed by adding 0.1 µmol/1 5-HT to PRP samples that had been prepared 90 min (+/− 30 min) earlier. During this time the PRP was kept, at 37°C, in a closed plastic tube. Eleven separate PRP samples were evaluated and the 5-HT-induced increase in MePV after 90 min (+/− 30 min) compared with that initially obtained in the same sample.

Presentation of results and statistical analysis. Results are expressed as median and (range) in Tables I-IV. For diagrammatic purposes the data is represented as means ± SD in Figures 1-3. Paired values were compared using the Wilcoxon rank sum test (two-tailed). The IC_{50} (concentration inhibiting 50% of the platelet shape change induced by 0.1 µmol/1 5-HT) was estimated graphically.

Results

Effect of omitting the fixative. In general, the absence of fixative resulted in larger variable MePV readings than when fixative was present, whether basal or agonist-stimulated measurements were made (results not shown). This phenomenon was dependent on the time the platelets remain in contact with Isoton II. All further experiments were therefore carried out in the presence of fixative in order to obtain stable and reproducible MePV measurements.

Reproducibility of MePV measurement. The intrassay coefficient of variation (CV) was 0.8%. This CV was determined following 9 sequential MePV measurements on a single PRP sample. The MePV (+/− S.D.) recorded was 5.78 (0.05) fl.

The interassay CV was 2.9%. This CV was determined following 10 MePV measurements in PRP samples prepared from the same subject on 10 separate days (spread over a period of 3 months). The MePV recorded was 5.82 (0.17) fl.

The MePV increased from 5.40 (4.92-6.01) to 5.77 (5.43-6.91) fl after the addition of 5-HT (0.1 µmol/1) to PRP (n=4) that had been prepared 90 min (+/− 30 min) earlier. These changes compared very favourably with the MePV, 5.76 (5.56-6.39) fl, achieved after the same dose of 5-HT was added to the same, freshly prepared, PRP. Unstimulated MePVs were also very similar in both samples (results not shown).

Agonist-induced changes in MePV-time courses. Increases in MePV were induced by the addition of: 5-HT (0.1, 1.0 and 100 µmol/1), ADP (0.4 µmol/1), collagen (0.2 and 0.4 mg/1), U46619 (a thromboxane A_2 and endoperoxide analogue; 0.027 µmol/1), AA (0.2 mmol/1), calcium ionophore (2.7 µmol/1) and PAF (0.1 µmol/1). Agonists had different patterns of MePV expansion which are shown in Figures 1 and 2 and Table I. These results determined at which time a single, representative, MePV was measured following the addition of each agonist. these sampling times are shown in the next section. Additional experiments (n=2-4; results not shown) were carried out to ensure that MePV changes followed similar time courses as those at the other concentrations of the agonists mentioned above.

Agonist-induced changes in MePV-dose responses. Increases in MePV were induced by the addition of: 5-HT (0.01, 0.1, 1.0 and 100 µmol/1; measured 30 sec after the addition of 5-HT), ADP (0.1, 0.2 and 0.4 µmol/1; measured 3 min after the addition of collagen), noradrenaline (1 µmol/1; measured 30 sec after the addition of the catecholamine), U46619 (0.017, 0.034 and 0.0675 µmol/1; measured 30 sec after the addition of U46619), AA (0.1, 0.3 and 0.6 mmol/1; measured 30 sec after the addition of AA) and PAF (0.1 µmol/1; measured 30 sec after the addition of PAF). Agonists varied in their potency for causing MePV expansion (shown in Table II).

Adrenaline was also evaluated but an increase in MePV could not be demonstrated (see Table II).

Antagonism of agonist-induced MePV expansion by naftidrofuryl (NAF) and ketanserin (KET). The effect of NAF and

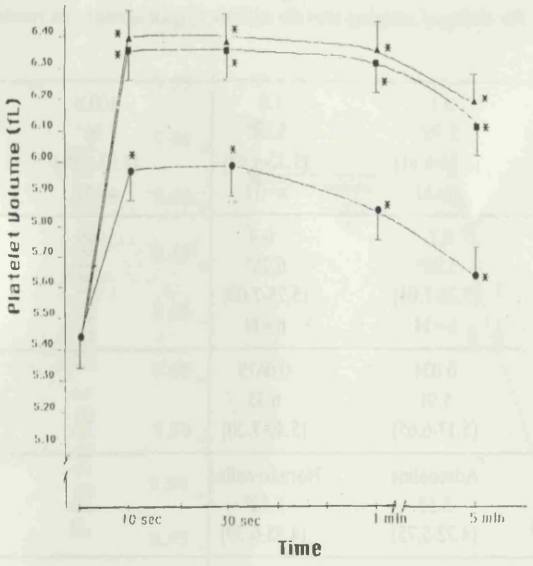

Figure 1. *Median platelet volumes at various time points following the addition of saline (basal) and 0.1 (•), 1.0 (◆) and 100 (▲) μmol/l 5-HT. * P < 0.01: MePV compared against paired control (0) value.*

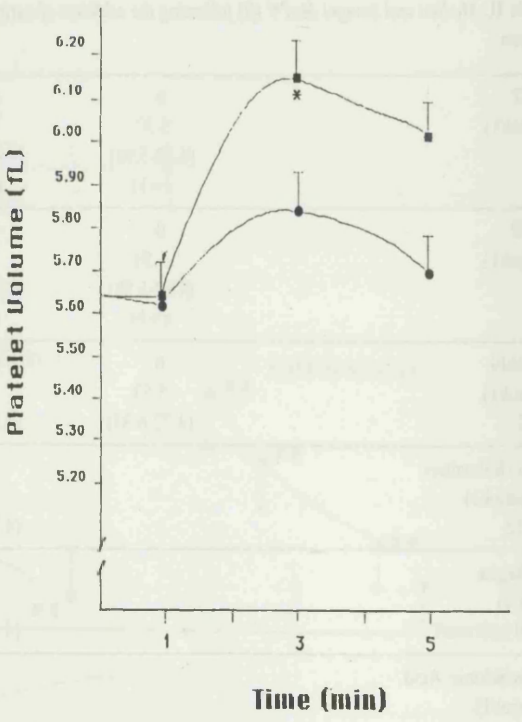

Figure 2. *Median platelet volumes at various time points following the addition of saline (basal) and 0.2 (•) and 0.4 (◆) mg/l collagen. * P < 0.01: MePV compared against paired control (0) value.*

KET on 5-HT- and ADP-induced expansion in MePV was assessed (Figure 3; Table III). KET was a more potent inhibitor of 5-HT-induced MePV expansion (IC_{50} = 0.05 μM) than NAF (IC_{50} = 3.0 μM). The two antagonists had similar potencies when MePV expansion was induced by ADP (Table III). However, the concentration of both NAF and KET needed to inhibit ADP-induced MePV expansion significantly was considerably greater than that required when 5-HT was used as an agonist.

Agonist interactions and the effect of NAF. Two agonists (5-HT and ADP) that induce MePV expansion were combined in order to determine whether synergism occurred. The results in Table IV show a synergistic response between these two agonists. Furthermore, the enhanced sensitivity of this experimental approach resulted in NAF concentrations as low as 1.56 μmol/l having a significant inhibitory effect on MePV expansion (Table IV).

Table I. *Time courses of changes in MePV induced by several agonists. Results are shown as median and (range) MePV (fl). The MePV changes in response to 5-HT and collagen are shown in figures 1 and 2.*

Agonist	Sampling time			
	BASAL	30 sec	1 min	5 min
ADP (0.4 μmol/1) n=5	5.40 (4.85-5.75)	6.14 (5.58-6.72)	6.33 (5.45-6.59)	5.88 (5.20-6.14)
U16619 (0.027 μmol/1) n=3	4.92 (4.85-5.94)	5.69 (5.49-6.59)	5.75 (5.56-6.46)	5.62 (5.56-6.52)
AA (0.2 mmol/1) n=4	5.20 (4.92-5.82)	5.69 (5.28-6.43)	5.59 (5.11-6.35)	5.50 (5.17-6.39)
Calcium ionophore (2.7 μmol/1) n=4	5.46 (4.85-6.39)		5.72 (4.98-6.27)	6.27 (5.04-7.04)
PAF (0.1 μmol/1) n=4	6.01 (5.09-6.39)	6.28 (5.49-6.97)	6.25 (5.69-7.05)	6.11 (5.43-7.04)

Discussion

There is considerable controversy as to how platelet volume is controlled, both in health and disease[13]. It has been proposed that platelet volume is determined in the bone marrow by megakaryocytes, that platelets decrease in size in the circulation as they age, that larger platelets are consumed preferentially following haemorrhage or thrombosis and that activated platelets increase in volume, at least transiently[1,2,6,7,13-18]. These mechanisms have been intensely debated[19].

The present study clearly shows that several agonists (5-HT, ADP, collagen, noradrenaline, AA, PAF, calcium ionophore, U46619) increase the MePV in the absence of any appreciable platelet aggregation. We also present evidence that synergism occurs between agonists. These findings suggest that, *in vivo,* low concentrations of agonists, which are present in blood, combine to increase the MePV. It is therefore of interest that significantly higher MePVs have

Table II. *Median and (range) MePV (fl) following the addition of various agonists. For timing of sampling after the addition of each agonist - see results section.*

5-HT	0	0.01	0.1	1.0	100.0
(μmol/1)	5.37	5.40	5.76*	5.88*	6.00*
	(4.82-5.98)	(4.66-6.01)	(5.24-6.91)	(5.32-6.97)	(5.52-6.91)
	n=11	n=11	n=11	n=11	n=11
ADP	0	0.1	0.2	0.4	
(μmol/1)	5.50	5.57	5.95*	6.25*	
	(4.82-5.98)	(4.90-5.88)	(5.28-7.04)	(5.75-7.00)	
	n=14	n=14	n=14	n=14	
U46619	0	0.017	0.034	0.0675	
(μmol/1)	5.53	5.64	5.91	6.35	
n=2	(4.72-6.33)	(4.83-6.44)	(5.17-6.65)	(5.49-7.20)	
Catecholamines		0	Adrenaline	Noradrenaline	
(1 μmol/1)		5.51	5.53	5.72*	
n=12		(4.72-5.88)	(4.72-5.75)	(4.85-6.39)	
Collagen		0	0.2	0.4	
(mg/1)		5.75	5.74	6.27*	
n=9		(4.72-6.18)	(4.88-6.39)	(5.18-6.80)	
Arachidonic Acid		0	0.1	0.3	
(mmol/1)		5.43	5.75	6.14	
n=6		(4.92-5.94)	(5.28-6.33)	(5.53-6.78)	
PAF		0	0.1		
(μmol/1)		5.34	5.59		
n=2		(4.85-5.82)	(5.30-5.88)		

*P < 0.01: MePV compared against control (0) value.
n = number of subjects sampled.

been reported in situations which are associated with activated platelets[7,10,18,20], for example, in patients with acute myocardial infarction[13-16], following the intravenous injection of therapeutic doses of heparin[7] and after the addition *(in vitro)* of bacterial lipopolysaccharide to normal human platelets[17].

From a methodological point of view, it is important to sample MePV at specific times after the addition of agonists. This is essential because the increase in MePV, and its reversal, occurs over a period of time that varies with each agonist.

The reason for adrenaline not altering the MePV is not clear and remains a controversial issue[1,5,6,23]. There are other peculiarities when considering this agonist. Thus platelets from neonates and from several adults do not aggregate in response to this agonist[21]. Adrenaline-induced aggregation cannot be measured in whole blood by the impedance method, despite the fact that this agonist reduces the free platelet count[22]. Even more perplexing is that noradrenaline is a weaker agonist than adrenaline when platelet aggregation is assessed in PRP[24], whereas the reverse is true for MePV expansion. These phenomena deserve further investigation.

The lack of increase in MePV following the addition of adrenaline has previously been reported although this issue is controversial[1,5,6,23]. This inconsistency may be attributed to both turbidometric and pulse-height analysis primarily reflecting increases in platelet volume rather than morphological changes (*e.g.* pseudopod formation). Thus adrenaline may not induce an increase in MePV although pseudopod formation may occur, albeit at high concentrations (10 μM and above)[5,23,25]. This issue should be resolved by combining electron microscopy and MePV measurements.

The MePV methodology described here appears to be reproducible and amenable to serial measurements. The latter facility allows dose responses to be assessed and

Table III. *Effect of naftidrofuryl and ketanserin on ADP-induced increase in MePV (fl). The concentration of ADP was 0.4 μmol/1. Results are expressed as median and (range). Samples from seven subjects were evaluated. Naftidrofuryl and ketanserin concentrations are expressed in μmol/1.*

		Naftidrofuryl		Ketanserin	
Baseline	ADP	100	200	100	200
5.56	6.46	6.01*	6.04*	6.14*	6.01*
(4.72-5.94)	(5.94-6.84)	(5.43-6.46)	(4.98-6.46)	(5.08-6.72)	(5.11-6.46)

*p < 0.01: Naftidrofuryl + ADP or ketanserin + ADP versus ADP alone.

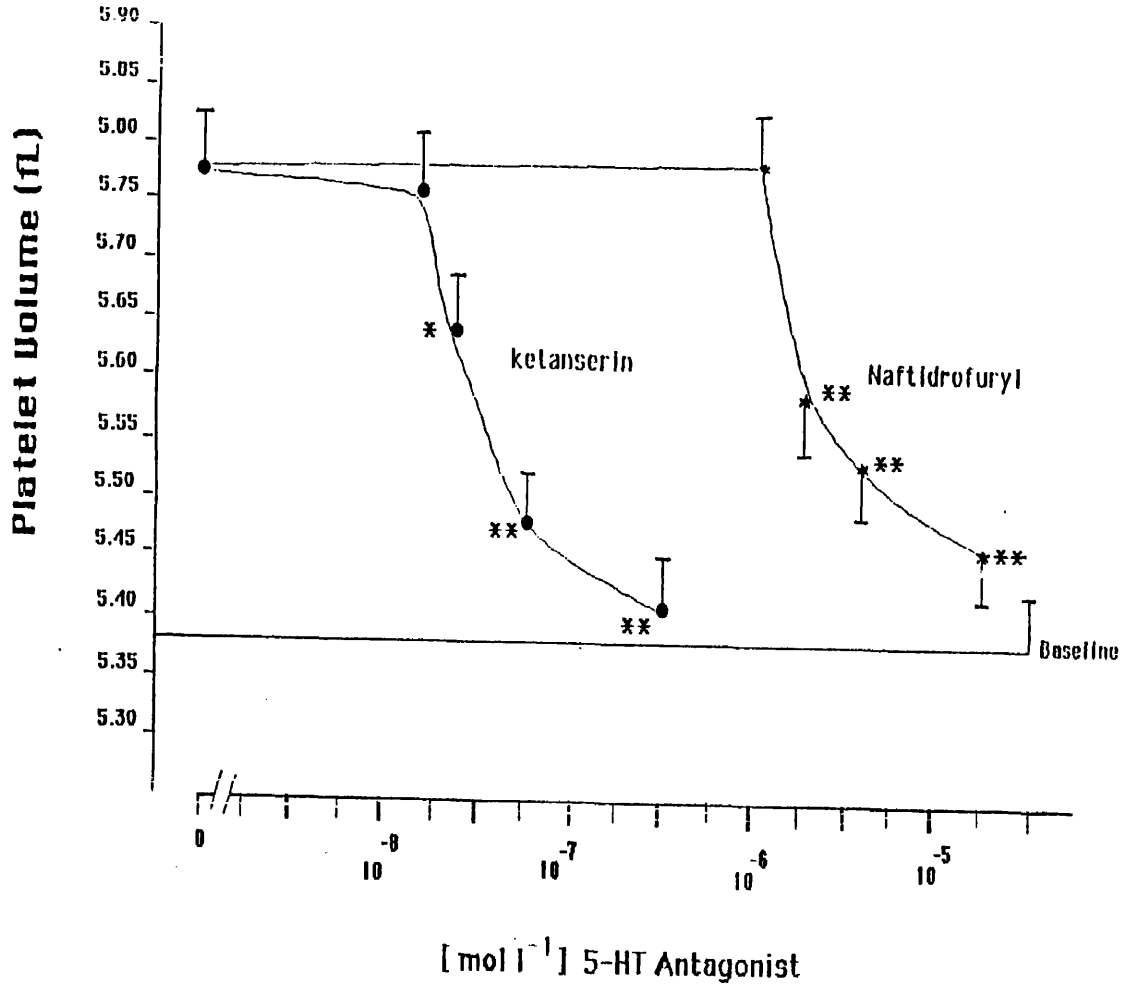

Figure 3. *Mean ± SD platelet volumes 30 s after the addition of saline or 5-HT (0.1 μM) in the presence and absence of NAF and KET (pre-incubated for 5 min). * P = 0.04: ketanserin + 5-HT vs 5-HT only. ** P < 0.01: Naftidrofuryl + 5-HT or Ketanserin + 5-HT vs 5-HT alone.*

antagonists to be evaluated. The MePV technique can also be a more sensitive indicator of the inhibitory capacity of drugs than PRP aggregation. Thus concentrations of NAF as low as 1.56 μmol/l significantly inhibited 5-HT+ADP-induced PSC. This finding suggests that therapeutic concentrations of NAF (up to 6 μmol/l)[9] influence this aspect of platelet function. It was not possible to make this suggestion previously because NAF concentrations as high as 50-100 μmol/l were required to inhibit platelet aggregation, *in vitro,* in PRP[9].

Similarly, therapeutic levels[9] of NAF (3.125 μmol/l) inhibited 5-HT-induced increases in MePV, whereas NAF concentrations as high as 100 μmol/l were required to inhibit ADP-induced increases in MePV. This 33:1 ratio contrasts with a 2:1 ratio when the capacity of NAF to inhibit PRP platelet aggregation induced by these two agonists was compared[9]. These combined findings indicate that measuring MePV may be a more sensitive method than PRP aggregation for defining the ability of drugs to block receptors. These advantages may be due to differences in the processes involved in PSC and in aggregation[26-28] or to the low agonist concentrations which are needed to increase MePV, when compared with those required to induce aggregation. This difference in agonist concentrations is especially evident with 5-HT. Thus 5-HT concentrations of 0.1-1.0 μmol/l increase MePV, whereas concentrations of the order of 10 μmol/l are needed to induce aggregation in PRP[29]. The conclusion that MePV assessment is a sensitive indicator of antagonist specificity is also supported by the observation that KET, a relatively specific 5-HT_2-antagonist[30], was a more potent inhibitor of 5-HT-induced MePV increase than NAF. However, both NAF and KET were equipotent at inhibiting ADP-induced increases in MePV. KET concentrations that effectively inhibit MePV increases and PRP aggregation[30] were similar (0.04-0.08 μmol/l). This

Table IV. *Effect of naftidrofuryl (NAF; 1.56 μmol/l) on increase in MPV (fl) induced by a combination of 5-HT (0.01 μmol/l) and ADP (0.1 μmol/l). The purpose of this experiment is to sensitise the assay to the effect of NAF on 5-HT-induced increases in MePV. Samples from seven subjects were evaluated. Results are expressed as median and (range).*

Baseline	5-HT only	ADP only	5-HT+ADP	NAF+ 5-HT+ADP
5.37 (4.72-5.94)	5.49 (4.72-6.00)	5.49 (4.72-6.00)	5.75^{+} (4.85-6.40)	5.69* (4.66:6.14)

*P < 0.01: NAF + 5-HT + ADP versus 5-HT+ADP
$^{+}$P < 0.01: 5-HT + ADP versus Baseline

observation suggests that, for specific inhibitors, the increased sensitivity of MePV, compared with PRP aggregation, is not as evident.

In conclusion, the methodology described here is amenable to serial and sensitive measurements of MePV. Its widespread use may enhance our understanding of the relevance of PSC in health and disease.

The preliminary findings[31] of this study were presented at the British Pharmacological society meeting, Belfast.

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Original Article

Intraplatelet Serotonin, β-Thromboglobulin, and Histamine Concentrations and Thromboxane A_2 Synthesis in Renal Disease

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Intraplatelet serotonin (5-HT), β-thromboglobulin (β-TG), and histamine content as well as platelet total thromboxane A_2 (TXA_2) synthesizing capacity were measured in 53 patients with chronic renal disease: nephrotic syndrome (n = 18); end-stage renal failure (ESRF; n = 13); continuous ambulatory peritoneal dialysis (CAPD; n = 9); hemodialysis (HD; n = 13). These indices of platelet function were correlated with plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) concentrations. When compared with controls, intraplatelet 5-HT was significantly reduced in all patient groups studied and β-TG was diminished in all patient groups except CAPD. Total platelet TXA_2 synthesizing capacity was increased in ESRF and HD groups. Intraplatelet histamine content was not altered in any of the patient groups studied. There was a significant inverse correlation between intraplatelet 5-HT content on the one hand and plasma TC, LDL-C, and TG on the other. The depletion of intraplatelet 5-HT and β-TG and the increase in total TXA_2 synthesizing capacity are consistent with platelet activation in chronic renal disease. The correlation between these indices of platelet activation and TC, LDL-C, HDL-C, and TG suggests that changes in the concentrations of these lipids may contribute to the activation of platelets in these conditions. (Key words: Platelets; Serotonin; β-Thromboglobulin; Thromboxane A_2; Renal disease; Lipids) Am J Clin Pathol 1991; 96:504-511

There is considerable evidence that platelets and platelet release substances may play a role in glomerular disease and atherosclerosis. Thus, enhanced platelet aggregation has been shown in glomerular disease[1] and a shortening of platelet survival has been observed in various forms of renal disease, including membrane proliferative glomerulonephritis and glomerulosclerosis.[2] Furthermore, accelerated atherosclerosis and thrombosis associated with renal disease also has been reported.[3] Platelet substances released during platelet activation include serotonin (5-HT), histamine, β-thromboglobulin (β-TG), thromboxane A_2, and platelet-derived growth factor (PDGF).[4-6] These substances have been shown to enhance vascular permeability, to possess vasoconstrictor and vasodilator effects, and to have platelet proaggregator, chemotactic, and mitogenic properties.[6-9] Because raised concentrations of intraplatelet substances may contribute to the pathogenesis of glomerular disease and atherosclerosis and the measurement of the concentrations of these indices of platelet activity in plasma is unreliable,[10] we measured intraplatelet 5-HT, β-TG, histamine, and total TXA_2 synthesizing capacity in healthy volunteers and persons (1) with nephrotic syndrome (NS), (2) with end-stage renal failure (ESRF), (3) receiving continuous ambulatory peritoneal dialysis (CAPD), and (4) receiving hemodialysis (HD).

We also investigated possible correlations between these indices and plasma concentrations of albumin, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) because no information is available about the effect of plasma lipid concentrations on platelet activity and platelet biogenic amines in renal disease.

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MATERIALS AND METHODS

Patient Selection

We studied 53 patients with renal disease in 4 clinical groups.

Nephrotic syndrome group. This group consisted of 18 patients (11 men, 7 women) whose median age was 27 years (range, 18–60 years) and the median duration of nephrotic syndrome was 6 years (range, 1–32 years). Nephrotic syndrome was defined as proteinuria level more than 5 g per 24-hour period and edema. The diagnosis in these patients was minimal change glomerulonephritis (n = 5), membranous glomerulonephritis (n = 2), focal sclerosing glomerulonephritis (n = 6), systemic lupus erythematosus (n = 1), and amyloidosis (n = 1). Two patients were hypertensive.

End-stage renal failure group. This consisted of 13 patients (7 men, 6 women) whose median age was 54 years (range, 23–62 years). All patients had a plasma creatinine concentration more than 500 μmol/L. None of these patients had been treated with dialysis. The diagnosis in these patients was chronic pyelonephritis (n = 2), focal sclerosing glomerulonephritis (n = 2), and polycystic kidney disease (n = 2). The remaining patients presented with hypertension and severe chronic renal failure. Kidney biopsy was not performed in these patients because of the severity of renal failure or small kidney size on ultrasound. Hypertensive glomerulosclerosis, glomerulonephritis, or pyelonephritis was probably the cause of renal failure in this group of patients.

Continuous ambulatory peritoneal dialysis group. This consisted of 9 patients (5 men, 4 women) whose median age was 57 years (range, 41–62 years); the median duration of dialysis was 1 year (range, 6 months to 3 years). The diagnosis in these patients was diabetic nephropathy (n = 2), obstructive uropathy (n = 2), rapidly progressive glomerulonephritis (n = 1), bilateral renal stenosis (n = 1), hypertensive glomerulosclerosis (n = 4), and ESRF of unknown etiology (n = 2). Three patients had evidence of peripheral vascular disease, 2 patients were hypertensive, and 2 others were insulin-dependent diabetics.

Hemodialysis group. This group consisted of 13 patients (8 men, 5 women) whose median age was 65 years (range, 50–67 years); the median duration of dialysis was 2 years (range, 6 months to 30 years). The diagnosis in these patients was diabetic nephropathy (n = 2), pyelonephritis (n = 2), polycystic kidney disease (n = 5), polyarteritis nodosa (n = 1), obstructive uropathy (n = 1), cortical necrosis (n = 1), and chronic renal failure of unknown etiology (n = 1). Three patients had peripheral vascular disease and pruritus was a problem in one patient.

Further biochemical characteristics, including plasma creatinine, creatinine clearance rate, plasma albumin, urinary albumin, plasma TC, LDL-C, HDL-C, and TG, for all patient groups are given in Table 1.

Informed consent was obtained from all patients who participated in this study.

Healthy Subjects

Nineteen healthy men and women (11 men, 8 women), members of the medical and laboratory staff (median age, 48 years; range, 21–65 years), volunteered for this study. None of the controls had a history of renal disease, diabetes mellitus, vascular disease, or hypertension.

Drugs

The patients included in this study were taking the following drugs: β-blockers (metoprolol, atenolol), (n = 3); ACE inhibitors (captopril) (n = 3); calcium-channel blockers (nifedipine) (n = 7); digoxin (n = 4). There were also patients taking allopurinol, insulin, warfarin, and

TABLE 1. CLINICAL AND BIOCHEMICAL INDICES OF PATIENTS WITH RENAL DISEASE

Group	Pl Creat μmol/L	CCR mL/min	Pl Alb g/L	Ur Alb g/24	Pl TC μmmol/L	Pl LDL-C mmol/L	Pl HDL-C mmol/L	Pl TG mmol/L
NS (n = 18)	101 (68–208)	115 (27–139)	38 (32–47)	10.3 (5.7–14.2)	6.1 (3.8–15.7)	5.62 (2.46–12.9)	1.2 (0.62–1.68)	2.33 (0.8–4.7)
ESRF (n = 13)	690 (500–1213)	11.1 (5.4–16)	42 (36–49)	0.6 (0–2.4)	6.1 (3.8–7.09)	3.23 (1.08–5.18)	0.87 (0.58–1.35)	2.6 (1.5–6.23)
CAPD (n = 9)	784 (375–1229)	—	44 (38–50)	—	6.96 (5.3–8.5)	5.07 (3.08–6.31)	0.79 (0.69–1.35)	2.6 (1.29–3.29)
HD (n = 13)	804 (349–906)	—	44 (38–50)	—	4.88 (3.3–8.9)	2.9 (1.68–7.72)	0.68 (0.52–0.98)	3.34 (1.08–6.8)
Reference range	60–120	90–150	40–50	0	3.0–6.5	0–5.63	1–2.2	0.2–1.5

Pl Creat = plasma creatinine; CCR = creatinine clearance rate; Pl Alb = plasma albumin; Ur Alb = urinary albumin; Pl TC = total plasma cholesterol; Pl LDL-C = plasma low-density lipoprotein cholesterol; Pl HDL-C = plasma high-density lipoprotein cholesterol; Pl TG = plasma triglyceride; NS = nephrotic syndrome; ESRF = end-stage renal failure; CAPD = patients undergoing continuous ambulatory peritoneal dialysis; HD = patients undergoing hemodialysis; n = number of patients studied.

Lipid data was available only in some patients (NS = 7, ESRF = 10, CAPD = 6, HD = 7, total = 30).

All data are expressed as median and range (in parentheses).

thyroxine. Two patients with NS and all other patients (ESRF, CAPD, and HD) were taking cimetidine or ranitidine to prevent gastrointestinal bleeding. Patients requiring CAPD and HD were taking alfacalcidol and vitamin supplements. Healthy subjects denied taking drugs for at least 2 weeks before sampling.

Blood Sample Collection and Processing

Blood was drawn between 9:00 and 11:00 A.M. (fasting subjects) from the antecubital vein of patients and volunteers with minimal stasis using a G-21 butterfly needle. The first 5 mL was discarded. In patients undergoing hemodialysis, blood was obtained from the fistula/cannula before administration of heparin and the commencement of dialysis. This is important because heparin is known to activate platelets.[11,12] Blood was collected in plastic tubes containing Na_2 ethylene diaminetetraacetic acid and prostaglandin E_1 at a final concentration of 5 mmol/L and 10 μg/L, respectively.[13] Platelet-rich plasma (PRP) was prepared using centrifugation techniques and platelet counts measured using a calibrated Coulter counter ZM (Coulter, Hialeah, FL).[14] Platelet pellets harvested from platelet-rich plasma was stored at −50°C for subsequent analysis, as described below. For each analyte, samples from all groups were assayed in a single batch.

Intraplatelet 5-HT Determination

Intraplatelet 5-HT was assayed using a validated double-antibody radioimmunoassay.[15] The inter- and intraassay coefficients of variation (n = 16) for this assay were 14% and 12%.

Intraplatelet β-TG Determination

Intraplatelet B-TG determinations were performed according to the method of Ludlam and associates.[16] Sheep anti-β-TG and donkey anti-goat serum antibodies were purchased from the Scottish Antibody Production Unit (Carluke, UK). ^{125}I-βTG and unlabeled ligand were a gift from Chris Waugh, Department of Medicine, Western General Hospital, University of Edinburgh, and Dr. Duncan Pepper, Scottish National Blood Transfusion Service, Edinburgh, respectively. The inter- and intra-assay coefficients of variation (n = 10) for this assay were 14% and 9%, respectively, in our experiments.

Total Platelet TXA_2 Synthesizing Capacity

This method established in our laboratory has been previously reported in detail.[17] Briefly, sonicated platelets are incubated in Krebs-Ringer bicarbonate buffer at 37°C. Under these conditions, biologically available endogenous TXA_2 precursor, arachidonic acid, is converted into TXA_2, which is measured as TXB_2 (the stable spontaneously hydrolysis product of TXA_2) by specific radioimmunoassay.

Intraplatelet Histamine Determinations

Intraplatelet histamine was determined using the double-isotope radioenzymatic method using S-[^{3}H]-adenosyl-methionine, rat kidney histamine N-methyltransferase, and [^{14}C]-histamine, as previously described.[18] The inter- and intraassay coefficients of variation for this assay have been described previously.[18]

Biochemical Analysis

Plasma creatinine, creatinine clearance rate, plasma albumin, urinary albumin, total plasma cholesterol, plasma LDL-C, plasma HDL-C, and plasma TG were analyzed using standard methods in routine use in the Department of Chemical Pathology and Human Metabolism and the Renal Unit of our institution.

Statistical Analysis and Expression of Results

Intraplatelet contents (5-HT, β-TG, TXA_2, and histamine) are expressed per 10^9 platelets. Intraplatelet 5-HT is expressed in nanomoles, β-TG in micrograms, and TXA_2 and histamine as nanograms per 10^9 platelets. Results are presented as mean (±2 SD) in Results and as mean ± SEM in Figures 1–3. Because the distribution of the data was nonparametric, the Mann-Whitney U-test (two-tailed) was used for comparing data. Details of comparisons are included in the legend for each figure. Spearman rank correlations were performed using a validated computer program in use in the Department of Chemical Pathology and Human Metabolism of our institution.

RESULTS

Intraplatelet Content of 5-HT, β-TG, Histamine, and TXA_2 Synthesizing Capacity of Platelets

The mean (±2 SD) intraplatelet 5-HT content in healthy subjects was 5.28 (3.61) nmol/10^9 platelets; in NS patients, 2.37 (3.22) nmol/10^9 platelets; in ESRF patients, 2.57 (3.47) nmol/10^9 platelets; in CAPD patients, 1.66 (1.39) nmol/10^9 platelets; in HD patients, 3.10 (3.13) nmol/10^9 platelets. The mean intraplatelet 5-HT content in all patient groups was significantly ($P < 0.001$) decreased when compared to healthy subjects (Fig. 1). There was no significant difference in intraplatelet 5-HT content among the patient groups. The mean (±2 SD) intraplatelet β-TG content in healthy subjects was 109 (27.2) μg/10^9 platelets; in NS patients, 87 (58.8) μg/10^9 platelets; in ESRF patients, 88 (64) μg/10^9 platelets; in CAPD patients, 83.7 (86.6) μg/10^9 platelets; in HD patients, 79.8 (49.8)

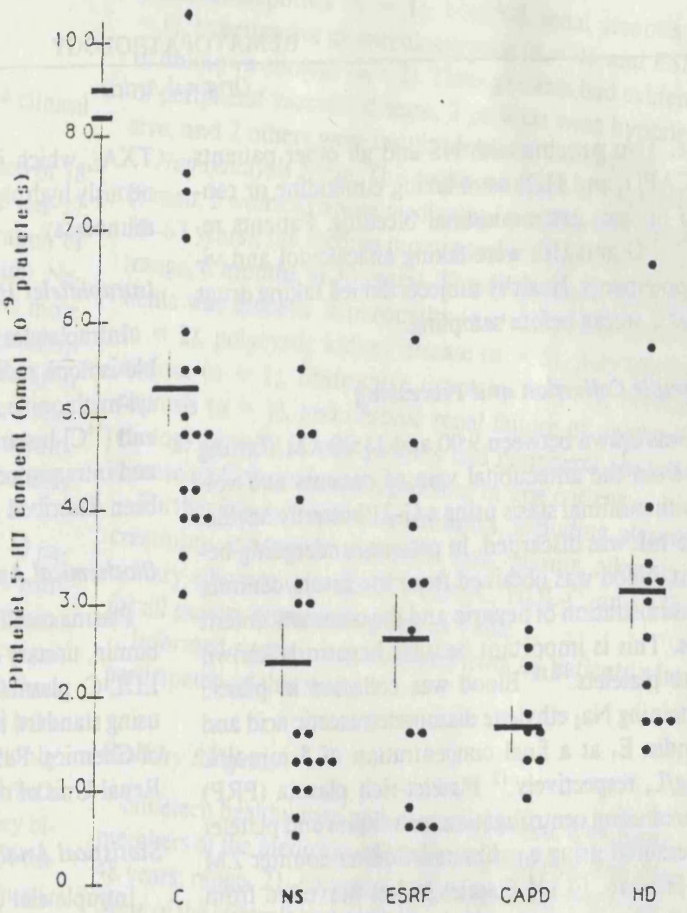

FIG. 1 (*upper*). Mean + SEM platelet 5-HT content in control subjects (C); nephrotic syndrome (NS); end-stage renal failure (ESRF); continuous ambulatory peritoneal dialysis (CAPD); and hemodialysis (HD) patients. C *vs.* NS, C *vs.* ESRF, C *vs.* CAPD, C *vs.* HD, $P < 0.001$. All other permutations are nonsignificant. For statistical analysis, see Materials and Methods.

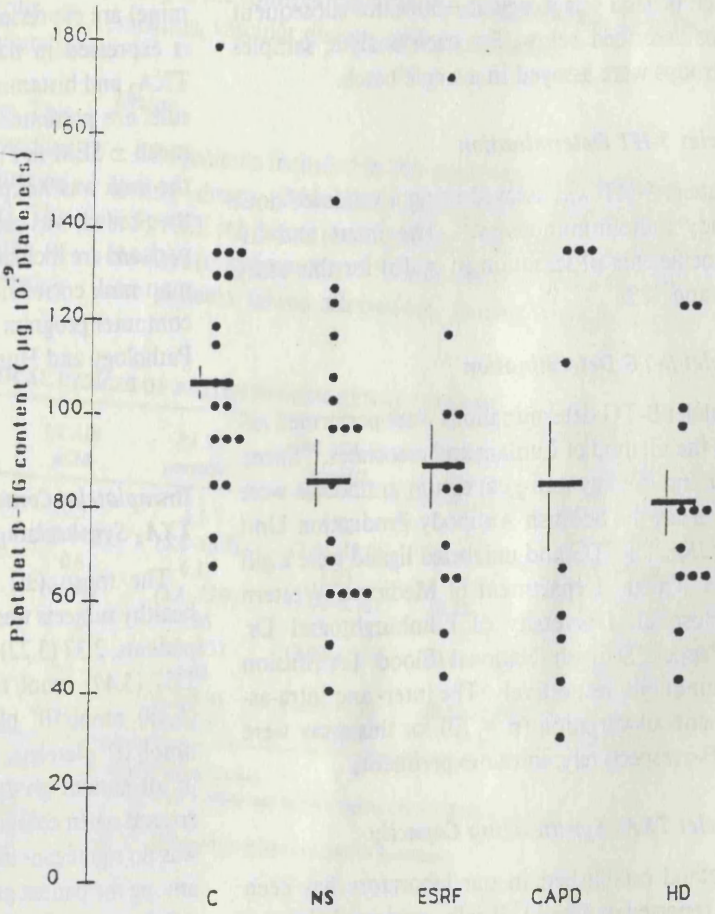

FIG. 2 (*lower*). Mean + SEM platelet β-TG content in control subjects (C); nephrotic syndrome (NS); end-stage renal failure (ESRF); continuous ambulatory peritoneal dialysis (CAPD); and hemodialysis (HD) patients. C *vs.* NS, $P < 0.004$; C *vs.* ESRF, $P < 0.02$; C *vs.* CAPD, not significant; C *vs.* HD, $P < 0.004$. All other permutations are nonsignificant. For statistical analysis, see Materials and Methods.

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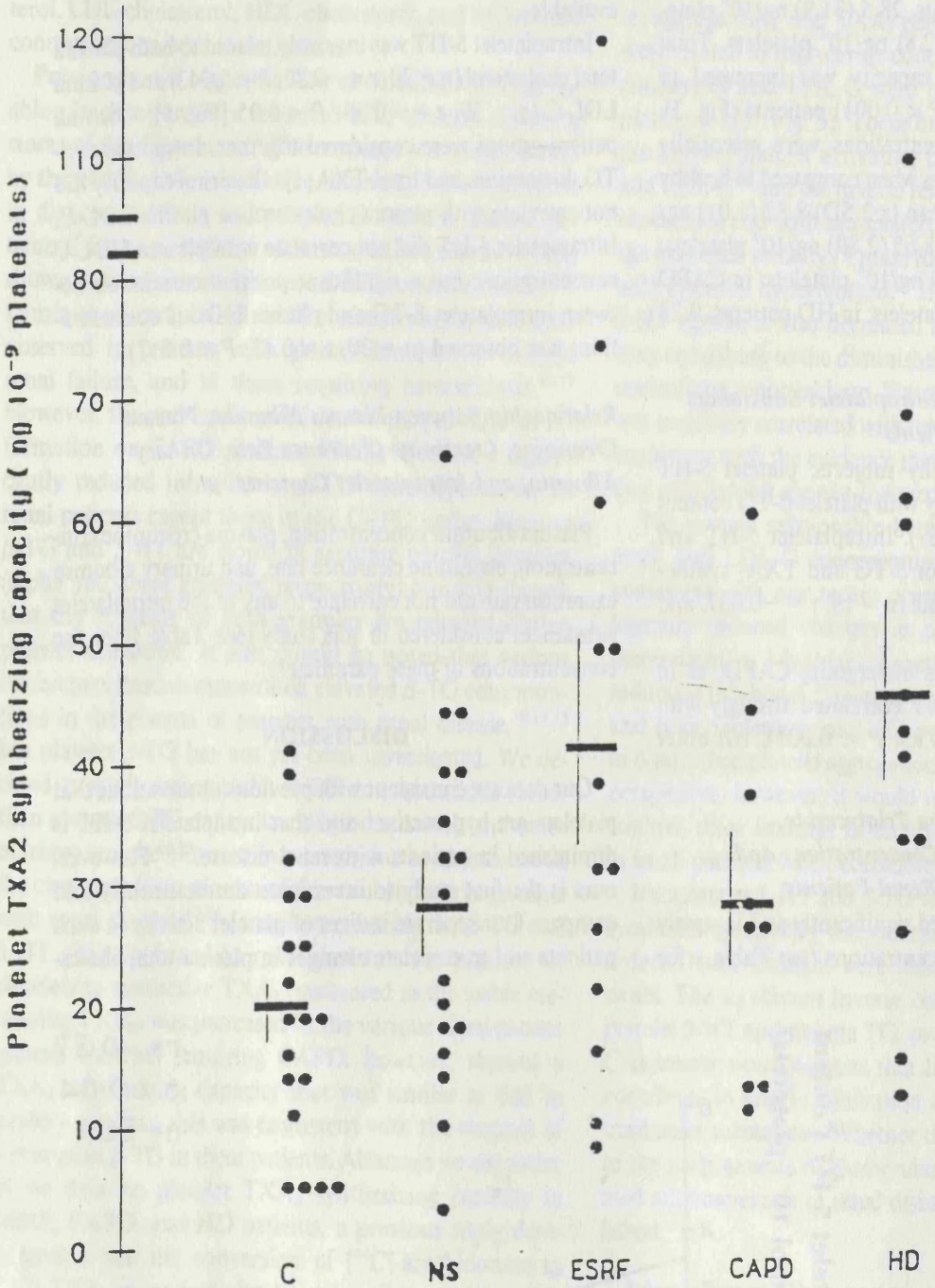

FIG. 3 (*upper*). Mean ± SEM platelet TXA_2 synthesizing capacity in control subjects (C); nephrotic syndrome (NS); end-stage renal failure (ESRF); continuous ambulatory peritoneal dialysis (CAPD), and hemodialysis (HD) patients. C *vs.* NS, not significant; C *vs.* ESRF, $P < 0.02$; C *vs.* CAPD, not significant; C *vs.* HD, $P < 0.001$. All other permutations are nonsignificant. For statistical analysis, see Materials and Methods.

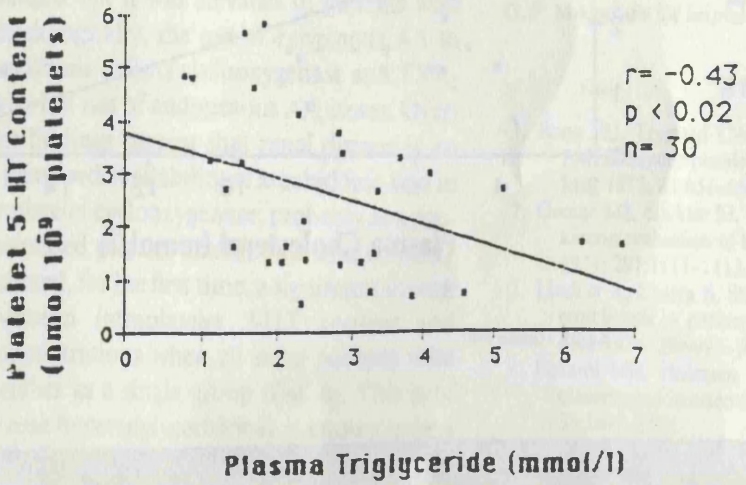

FIG. 4 (*lower*). Correlation between plasma triglyceride concentration and platelet 5-HT content in renal patients from all patient groups.

$\mu g/10^9$ platelets. Intraplatelet β-TG was significantly decreased in NS ($P < 0.04$), ESRF ($P < 0.02$), and HD patients ($P < 0.004$) (Fig. 2). The mean (±2 SD) intraplatelet total TXA_2 synthesizing capacity in healthy subjects was 19.7 (22.1) ng/10^9 platelets; in NS patients, 28.2 (30.9) ng/10^9 platelets; in ESRF patients, 41.1 (61.2) ng/10^9 platelets; in CAPD patients, 28.5 (31.9) ng/10^9 platelets; in HD patients, 45.2 (52.8) ng/10^9 platelets. Total platelet TXA_2 synthesizing capacity was increased in ESRF ($P < 0.02$) and HD ($P < 0.001$) patients (Fig. 3). Intraplatelet histamine concentrations were essentially unchanged in all patient groups when compared to healthy subjects (healthy subjects: mean (±2 SD) 8.82 (1.01) ng/10^9 platelets; in NS patients, 8.65 (2.89) ng/10^9 platelets; in ESRF patients, 8.75 (2.48) ng/10^9 platelets; in CAPD patients, 9.08 (2.08) ng/10^9 platelets; in HD patients, 9.28 (3.76) ng/10^9 platelets.

Interrelationships Between Intraplatelet Substances in Healthy Subjects and Patients

Healthy subjects. In healthy subjects, platelet 5-HT content correlated significantly with platelet β-TG content (n = 19, r = 0.49, $P < 0.037$). Intraplatelet 5-HT and TXA_2 synthesizing capacity or β-TG and TXA_2 synthesizing capacity did not correlate (n = 19, r = −0.037, and r = −0.016, respectively).

Renal patients. In patients undergoing CAPD, as in normal subjects, platelet 5-HT correlated strongly with platelet β-TG (n = 9, r = 0.83, $P < 0.005$). No other correlation was observed.

Relationship Between Plasma Triglyceride, Cholesterol, LDL-C, HDL-C Concentrations and Intraplatelet Substances in Renal Patients

Intraplatelet 5-HT correlated significantly and inversely with plasma triglyceride concentrations (see Table 1 for plasma lipid concentrations) when patients were considered as a single group (n = 30, r = −0.43, $P < 0.02$ [Fig. 4]). Other intraplatelet substances were not significantly correlated with plasma triglyceride concentrations. Correlations within each individual renal group were not carried out because lipid details on some patients were not available.

Intraplatelet 5-HT was inversely related to both plasma total cholesterol (n = 30, r = −0.37, $P < 0.043$) and plasma LDL-C (n = 30, r = −0.36, $P < 0.05$ [Fig. 5]) when all patient groups were considered together. Intraplatelet β-TG, histamine, and total TXA_2 synthesizing capacity did not correlate with plasma cholesterol or plasma LDL-C. Intraplatelet 5-HT did not correlate with plasma HDL-C concentrations but a significant positive correlation between intraplatelet β-TG and plasma HDL-C concentrations was observed (n = 30, r = 0.43, $P < 0.017$).

Relationship Between Plasma Albumin, Plasma Creatinine, Creatinine Clearance Rate, Urinary Albumin, and Intraplatelet Contents

Plasma albumin concentration, plasma creatinine concentration, creatinine clearance rate, and urinary albumin excretion rate did not correlate to any of the intraplatelet substances considered in this study (see Table 1 for the concentrations of these parameters).

DISCUSSION

Our data are consistent with previous observations that platelets are hyperactive[1] and that intraplatelet 5-HT is diminished in patients with renal disease.[19,20] However, ours is the first study to investigate simultaneously and compare four separate indices of platelet activity in such patients and to correlate changes in plasma total choles-

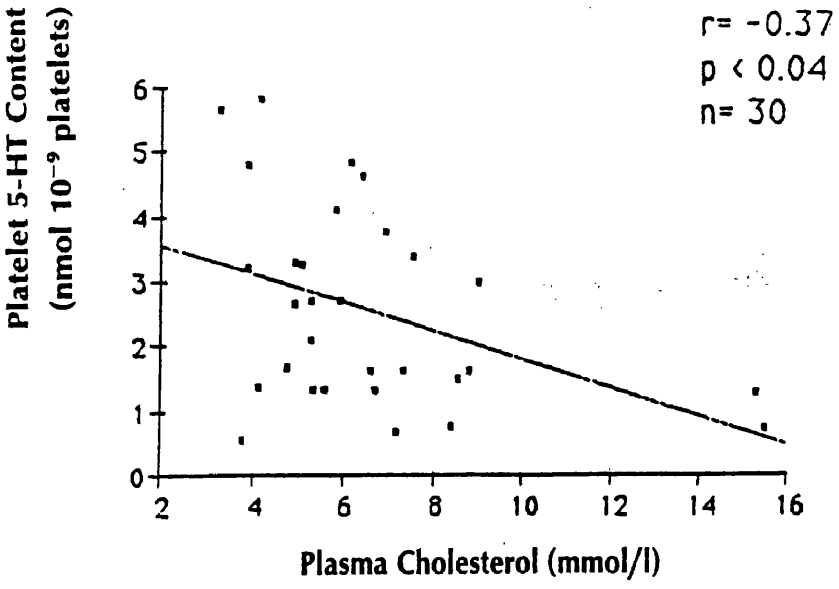

FIG. 5. Correlation between plasma total cholesterol and platelet 5-HT content in renal patients from all patient groups.

terol, LDL-cholesterol, HDL-cholesterol, and triglyceride concentrations with these indices.

Previously, we suggested that circulating hyperaggregable platelets tonically release 5-HT, thereby depleting stores of this bioamine.[14] This concept was consolidated by the fact that plasma 5-HT concentrations are elevated in diabetes mellitus and peripheral vascular disease patients.[14] Recently patients with renal failure also have been shown to have elevated plasma 5-HT concentrations.[21,20] Diminished intraplatelet 5-HT concentrations, have been observed in patients with glomerulonephritis, chronic renal failure, and in those requiring hemodialysis.[22,19] However, the previously published work provides no information on CAPD patients. We have found a significantly reduced intraplatelet β-TG concentration in all renal patients except those in the CAPD group. Because β-TG and 5-HT are stored in separate platelet granules (alpha and dense granules, respectively) our data suggest that the contents of both granules are released during platelet activation. It also should be noted that various researchers have demonstrated elevated β-TG concentrations in the plasma of patients with renal disease,[10,19,23] but platelet β-TG has not yet been investigated. We decided to study intraplatelet β-TG concentrations rather than plasma β-TG concentrations because this and other markers may be elevated in plasma due to alterations in the clearance from plasma of these substances in patients with renal disease.[10,24] In agreement with the β-TG and 5-HT changes found in renal patients, the capacity of platelets to synthesize TXA_2 (measured as the stable metabolite TXB_2) was increased in the various renal patient groups. Patients requiring CAPD, however, showed a TXA_2 synthesizing capacity that was similar to that in healthy subjects; this was consistent with the absence of a change in β-TG in these patients. Although we are aware of no data on platelet TXA_2 synthesizing capacity in ESRF, CAPD, and HD patients, a previous study demonstrated that the conversion of [^{14}C]-arachidonate to [^{14}C]-TXA_2 in washed platelets taken from patients with NS was unchanged but it was elevated in patients with ESRF.[25] Methodologically, the use of exogenous AA to assess TXA_2 synthesis reflects cyclooxygenase and TXA_2 synthase activity but not of endogenous AA stores. Overall, the present findings suggest that renal disease is associated with increased availability of arachidonic acid as a substrate for platelet cyclooxygenase, probably as a consequence of increased platelet arachidonic acid content.

We demonstrated, for the first time, a significant inverse correlation between intraplatelet 5-HT content and plasma TG concentrations when all renal patients were considered together as a single group (Fig. 4). This is of important because hypertriglyceridemia is known to be a risk factor for ischemic heart disease.[26] In this context it is interesting to note that NS patients had increased platelet aggregability and TXA_2 release and that both indices were related to triglyceride concentrations.[27] Plasma total cholesterol and LDL-C were inversely related to intraplatelet 5-HT (Fig. 5). These findings are consistent with the known platelet activating properties of cholesterol[28] and LDL-C[30] and the inverse relationship between plasma cholesterol and both intraplatelet 5-HT concentration and the maximal velocity (Vmax) for 5-HT uptake in patients with essential hypertension.[29] However, it is possible that 5-HT uptake is also decreased in renal patients and this may contribute to the diminished intraplatelet 5-HT concentrations reported here. Plasma HDL-C concentration was positively correlated with intraplatelet β-TG, a finding consistent with the evidence that this lipoprotein subfraction may inhibit agonist-induced platelet aggregation.[30,31]

The inverse relationship between plasma TG, cholesterol, and LDL-C concentrations and platelet 5-HT is consistent with our recent observations that pharmacologically induced changes in plasma cholesterol (with simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor),[32] plasma triglyceride (with fish oil),[33] and both cholesterol and TG (with bezafibrate)[34] results in diminished platelet aggregation. From an epidemiologic perspective, however, it would require many patients to confirm these findings in each patient subgroup as well as in all patients when considered as a single group.

Intraplatelet 5-HT and β-TG were decreased and TXA_2 synthesizing capacity was enhanced in various renal disorders. These changes were least marked in CAPD patients. The significant inverse correlation between intraplatelet 5-HT and plasma TG, total cholesterol and LDL-C concentrations suggests that lipid abnormalities may contribute to platelet activation and the depletion of intraplatelet substances. Whether these changes contribute to the pathogenesis of glomerular lesions and to accelerated atherosclerosis in renal disease remains to be established.

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Intraplatelet serotonin in patients with diabetes mellitus and peripheral vascular disease

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Abstract. Intraplatelet serotonin (5-HT) content was determined in 23 patients with type I (insulin-dependent) diabetes mellitus (IDDM), 23 patients with type II (non-insulin-dependent) diabetes mellitus (NIDDM), 29 patients with peripheral vascular disease (PVD) and 34 age-matched normal subjects. Intraplatelet 5-HT content in normal subjects showed an age-related decline ($r = -0{\cdot}45$; $P < 0{\cdot}008$), as has been previously demonstrated. The median 5-HT content in platelets of the young normal subjects was 4·36 (range: 3·62–6·79) nmol 10^{-9} platelets, while that in the elderly normal subjects was 3·87 (range: 2·8–6·0) nmol 10^{-9} platelets and that in young + elderly subjects was 4·05 (range: 2·8–6·8) nmol 10^{-9} platelets. The median intraplatelet 5-HT content was significantly lower ($P < 0{\cdot}002$) in IDDM patients: 3·0 (range 1·3–6·3), NIDDM patients: 2·5 (range 1·7–5·8), PVD patients: 2·42 (range 0·94–4·98) nmol 10^{-9} platelets than that in all young + elderly healthy subjects. The presence of hypertension in DM patients caused a small but significant ($P < 0{\cdot}05$) decrease in intraplatelet 5-HT content, whilst its presence had no effect in PVD patients. In a smaller study, it was established that NIDDM and PVD patients have significantly ($P < 0{\cdot}002$) greater plasma 5-HT concentrations than controls. Insulin-dependent diabetes mellitus patients had greater plasma 5-HT concentrations but this did not achieve statistical significance despite a 66% increment in its value. We conclude that the diminished 5-HT content in platelets and the increased plasma levels may reflect enhanced release of 5-HT by hyperactive platelets. This increase in plasma 5-HT may contribute to the pathogenesis of atherosclerosis and vasospasm.

Keywords. Platelets, serotonin, diabetes mellitus, vascular disease, hypertension.

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Introduction

Serotonin (5-HT) is a vasoactive amine stored in the electron dense granules of platelets [1]. During activation, platelets may secret 5-HT as well as other vasoactive substances, which may then interact with blood vessels, resulting in vasospasm and increased vascular permeability [2]. Platelets are also thought to contribute to the pathogenesis of atherosclerosis [2]. Since platelet hyperaggregability and an increased incidence of atherosclerosis have been documented in diabetic patients and patients with peripheral vascular disease [3], a study of the intraplatelet content in these disease states may provide an insight into the mechanisms underlying the pathogenesis of platelet hyperaggregability and accelerated atherogenesis in these conditions.

We therefore carried out a study to determine the relationship between intraplatelet 5-HT and age in healthy subjects and in the following disease states: type I (insulin-dependent) diabetes mellitus (IDDM), type II (non-insulin-dependent) diabetes mellitus (NIDDM) and peripheral vascular disease (PVD). We also compared the intraplatelet 5-HT content in these disease states with that in healthy subjects of similar ages.

Patients and methods

Study 1

Two groups of healthy subjects were studied as control populations. One group of healthy subjects, 'elderly controls', attending a local Day Centre consisted of 16 elderly healthy men and women (8 men, 8 women), median age 72 years (range 56–80). 'Young controls' consisted of 18 younger, healthy men and women (11 men, 7 women) members of the medical and laboratory staff, median age 26 years (range 21–49). For the appropriate age comparisons we combined the two populations, which collectively consisted of 34 'young' and 'elderly' subjects (19 men, 15 women; median age 49 years, range 21–80). None of the

controls had a history of diabetes, vascular disease or hypertension.

The patients studied were attending the diabetic and vascular out-patient clinics at The Royal Free Hospital. The IDDM group consisted of 23 patients (11 men, 12 women); median age 50 years (range 23–72); median duration of diabetes 6 years (range 2–25); median blood glucose 11·5 mmol l^{-1} (range 4·0–28·3); median glycosylated haemoglobin 9·5% (range 5·8–11·3). The NIDDM group consisted of 23 patients (15 men, 8 women), median age 66 years (range 40–86); median duration of diabetes 10 years (range 1–15); median blood glucose 11·4 mmol l^{-1} (range 5·2–13·7); median glycosylated haemoglobin 10·1% (range 7·5–15·1). The PVD group consisted of 29 patients (18 men, 11 women); median age 73 years (range 47–79). All the patients in the PVD group had: (i) intermittent claudication for more than 6 months with (ii) ankle:arm systolic blood pressure (SBP) ratio $<0{\cdot}85$. Patients were defined as hypertensive if they had a diastolic blood pressure >95 mmHg on two separate occasions.

Study 2

For this study, a similar but smaller population of patients and healthy subjects were sampled. The control group consisted of 10 healthy subjects (7 men, 3 women); median age 50 years (range 25–68). The IDDM group consisted of 11 patients (6 men, 5 women); median age 53 years (range 23–77); median duration of diabetes 11 years (range 3–40). The NIDDM group consisted of 14 patients (7 men, 7 women); median age 62 years (range 51–83); median duration of diabetes 11 years (range 2 months–30 years). The PVD group consisted of 13 PVD patients (10 men, 3 women); median age 68 years (range 56–83). Patients were diagnosed as PVD according to the criteria described in study 1.

Drugs

Healthy subjects denied taking drugs for at least 2 weeks prior to sampling. Diabetic patients were on standard treatment regimens with insulin/oral hypoglycaemic agents and diet. Hypertensive patients (DM and PVD) were on a combination of nifedipine and bendrofluazide.

Blood sample collection and processing

Blood was taken from the antecubital vein of patients and volunteers with minimal stasis, and nine parts of blood were added to one part of 3·8% w/v trisodium citrate (BDH, Poole, Dorset, U.K.). To each 1 ml of blood 100 μg of acetylsalicylic acid (BDH) were added to prevent any release of intraplatelet constituents [4]. Plasma for serotonin measurements was prepared by centrifuging blood for 20 min at $1500 \times g$ at 22°C. The supernatant was frozen immediately and kept at -40°C until assay. Platelet rich plasma (PRP) was prepared as previously described [5]. Platelet counts were then performed using a Coulter ZM counter (Coulter Electronics Limited, Luton, Bedfordshire, U.K.). Following counting, the PRP was centrifuged at $1000 \times g$ for 10 min to prepare platelet pellets. The pellets were washed with Isoton II (Coulter Electronics Limited) and stored at -20°C until analysis.

Platelet serotonin assay

Platelet pellets were resuspended in physiological saline (0·9° w/v) and a platelet lysate prepared by ultrasonicating the platelet pellet for 3×10 sec at an amplitude of 18 microns using an MSE-Soniprep sonicator (MSE, Sussex, U.K.). In order to ensure that our sonication procedure fully disrupts platelets, we counted, sized and plotted platelet populations before and after sonication. For this purpose, platelets were obtained from healthy subjects and the above parameters were assessed using a Coulter ZM counter with a Channelyzer C-1000 and X-Y Recorder (Coulter Electronics Limited). Following sonication, the platelet count was reduced to $<5\%$ of the original, the mean platelet volume became unmeasurable and the graphical plot obtained with the sonicated platelets became indistinguishable from the plot obtained with platelet diluent (Isoton II). The serotonin content in the platelet lysates was assayed by a modification of the spectrofluorimetric method of Drummond & Gordon [6,7]. Briefly, 6 M trichloroacetic acid (BDH) was added to the platelet lysates to precipitate the proteins. The samples were then centrifuged at $10\,000 \times g$ for 4 min. A portion of the supernatants was removed and transferred to glass test-tubes, and to each of these o-phthaldialdehyde (Sigma Chemical Co., Poole, Dorset, U.K.) in HCl was added, and the mixture heated in boiling water for 10 min. The tubes were then cooled in ice and washed twice with chloroform 'Analar' (BDH) to remove any traces of trichloroacetic acid [8]. The aqueous phase was removed and the fluorescence was read in a Perkin-Elmer MPF-3 (Hitachi Ltd, Tokyo, Japan) fluorescence spectrophotometer, with excitation and emission wavelengths of 360 and 475 nm, respectively. Standards and blanks were processed in the same way as the platelet lysates. The intra-assay coefficient of variation (CV) for the whole procedure was 4·0% ($n=20$) and the interassay CV for the whole procedure was 12% ($n=8$). As an added precaution, samples from each of the groups studied were included in each assay.

Plasma serotonin assay

Plasma serotonin concentrations were estimated using a radioimmunoassay. Antisera, standards and reagents were purchased from Immunodiagnostics Ltd (Washington, Tyne and Wear, U.K.). The intra-assay CV for this method was 3·1% ($n=10$) and the interassay CV was 5·1% ($n=10$).

Blood glucose determination

Blood glucose measurements were carried out in the clinic at the time of blood sampling, using the YSI Model 23 AM glucose analyser (Yellow Springs Instruments, Yellow Springs, U.S.A.).

Glycosylated HbA_1 determination

Glycosylated HbA_1 measurements (electrophoretic technique) were performed on whole blood using the German-Hawksley glycosylated haemoglobin kit (German-Hawksley, Northampton, U.K.).

Statistical analysis and expression of results

Platelet 5-HT content is expressed as nmol per 10^9 platelets. Since the distribution of the data was non-parametric, the results are expressed as medians with the range in parentheses.

The Mann-Whitney *U*-test (two-tailed) was used for comparing unpaired data. Details of comparisons are included in the appropriate tables. The Chi-square test was used for the analysis of frequency. Linear regression analysis was carried out using a validated computer program in use in the Department of Chemical Pathology and Human Metabolism at The Royal Free Hospital.

Results

Study 1

Comparison between 'young' and 'elderly' healthy subjects. In healthy subjects platelet 5-HT content decreased with increasing age ($n=34$, $r=-0{\cdot}45$, $P<0{\cdot}008$). There was a significantly ($P<0{\cdot}05$) lower 5-HT content in the platelets of elderly healthy subjects when compared with platelets of younger healthy subjects (see Table 1 for age range).

Comparison between healthy subjects and diabetic patients. Patients with IDDM had significantly ($P<0{\cdot}001$) lower intraplatelet 5-HT when compared with 'young'+'elderly' healthy subjects (Table 1). Patients with NIDDM had significantly ($P<0{\cdot}002$) lower intraplatelet 5-HT when compared with 'elderly' healthy subjects (Table 1). We selected different control populations in order to achieve comparability in the age range with that of patients.

Intraplatelet 5-HT concentrations in IDDM and NIDDM patients did not differ significantly. There was no correlation between platelet 5-HT and age in either IDDM or NIDDM patients, or when these patients were pooled together (IDDM: $n=20$, $r=-0{\cdot}3$, $P<0{\cdot}9$; NIDDM: $n=22$, $r=-0{\cdot}2$, $P<0{\cdot}63$; IDDM+NIDDM: $n=42$, $r=-0{\cdot}04$, $P<0{\cdot}79$). There was also no significant correlation between platelet 5-HT content and duration of diabetes ($n=40$, $r=-0{\cdot}04$, $P<0{\cdot}87$), blood glucose ($n=46$, $r=-0{\cdot}2$, $P=0{\cdot}21$) and blood HbA_1 concentration ($n=22$, $r=-0{\cdot}02$, $P=0{\cdot}93$). There was no significant difference between IDDM and NIDDM with regard to blood glucose concentrations or HbA_1 levels.

Comparison between healthy subjects and patients with PVD. Patients with PVD had significantly ($P<0{\cdot}002$) lower intraplatelet 5-HT content when compared with normal subjects of similar ages (Table 1). The presence of hypertension in PVD patients was not associated with a further decrease in intraplatelet 5-HT (see below).

The median age and (range) of the PVD patients studied ($n=26$) was 73 (47–79) years and the median and (range) intraplatelet 5-HT (nmol 10^{-9} platelets)

Table 1. Intraplatelet serotonin (5-HT) content (nmol 10^{-9} platelets) in young control subjects, elderly control subjects, young+elderly control subjects, insulin-dependent diabetic (IDDM), non-insulin-dependent diabetic (NIDDM), diabetics (DM: IDDM+NIDDM) with high blood pressure (HBP) and with normal blood pressure (NBP), peripheral vascular disease (PVD) with HBP and with NBP patients

	Control subjects			DM patients				PVD patients		
	Young	Elderly	Young+ elderly	IDDM	NIDDM	DM with NBP	DM with HBP	PVD	PVD with NBP	PVD with HBP
No. subjects	18	16	34	23	23	26	18	29	12	12
Age (years)	26 21–49	72 56–80	49 21–80	50 23–72	66 40–86	56 31–76	65 56–73	73 47–79	71 56–78	74 56–78
5-HT content	4·36* 3·62–6·79	3·87 2·8–6·0	4·05 2·8–6·79	3·01† 1·26–6·34	2·46‡ 1·74–5·83	2·97**†† 1·92–6·34	2·48¶**‡‡ 1·26–5·81	2·42§ 0·94–4·98	2·77§§ 1·7–4·9	3·01¶¶ 0·6–4·82

Values are shown as median and range.

* vs. elderly subjects, $P<0{\cdot}05$; † vs. young+elderly subjects, $P<0{\cdot}001$; ‡ vs. elderly subjects, $P<0{\cdot}002$; § vs. elderly subjects, $P<0{\cdot}002$; ¶ vs. DM with NBP, $P<0{\cdot}05$, ** vs. young+elderly subjects, $P<0{\cdot}001$; †† vs. elderly subjects, $P<0{\cdot}003$; ‡‡ vs. elderly subjects, $P<0{\cdot}001$; §§ vs. elderly subjects, $P<0{\cdot}04$; ¶¶ vs. elderly subjects, $P<0{\cdot}004$.

There were no significant differences in intraplatelet 5-HT content when IDDM, NIDDM and PVD patients were compared with each other.

Table 2. Intraplatelet 5-HT content and plasma concentrations in control subjects, insulin-dependent diabetics (IDDM), non-insulin dependent diabetics (NIDDM) and peripheral vascular disease (PVD) patients

Control/patient population	No. patients	Age (years)	Intraplatelet 5-HT (nmol 10^{-9} platelets)	Plasma 5-HT (nmol l^{-1})
Control subjects	10	50 (25–68)	3·85 (1·8–4·05)	100 (43–284)
PVD patients	13	68 (56–83)	2·29* (1·67–4·50)	223‡ (165–767)
IDDM patients	11	53 (23–77)	2·24* (1·26–3·75)	166 (56–264)
NIDDM patients	14	62 (51–83)	2·19† (0·94–3·10)	291‡ (108–938)

A separate, smaller population of control subjects and patients were sampled for this study. See study 2 in the Patients and methods section.
* vs. control subjects, $P<0·05$; † vs. control subjects, $P<0·02$–$P<0·002$; ‡ vs. control subjects: $P<0·002$.

was 2·42 (0·94–4·98). There was no correlation between intraplatelet 5-HT content and age in PVD patients ($n=26$, $r=-0·04$, $P<0·85$).

A few patients with both PVD and DM were also studied. These had a platelet 5-HT content that was similar to that found in PVD and NIDDM patients. The platelet 5-HT content of PVD patients did not differ significantly from that in IDDM or NIDDM patients.

Effect of hypertension on 5-HT content of platelets in patients with DM and PVD. Diabetes mellitus patients (IDDM+NIDDM pooled together) with hypertension had a significantly ($P<0·05$) lower median intraplatelet 5-HT content than that in normotensive DM patients. Chi-square analysis of the DM-hypertension/no hypertension patients showed no significant difference in the frequency of NIDDM/IDDM in these groups. Diabetes mellitus patients (IDDM+NIDDM) with and without hypertension had significantly ($P<0·001$) lower intraplatelet 5-HT than young+elderly control subjects. Diabetes mellitus patients with hypertension had significantly lower ($P<0·001$) intraplatelet 5-HT than elderly control subjects (Table 1). Normotensive DM patients had significantly ($P<0·003$) lower intraplatelet 5-HT than elderly control subjects (Table 1). The DM hypertension/no hypertension patients were compared with both young+elderly and elderly control subjects to allow for better age matching between these groups. There was no significant difference between the platelet 5-HT content of hypertensive and normotensive PVD patients. Hypertensive PVD patients had a median intraplatelet 5-HT content that was significantly ($P<0·004$) lower than that in elderly control subjects. Normotensive PVD patients also had significantly ($P<0·04$) lower intraplatelet 5-HT concentration than that in elderly control subjects.

Study 2

Plasma and intraplatelet 5-HT in healthy subjects and IDDM, NIDDM and PVD patients. Plasma 5-HT concentration was significantly raised in NIDDM and PVD patients when compared with healthy subjects (Table 2). Plasma 5-HT concentration in IDDM patients was higher (66% increase) than in healthy subjects but did not achieve statistical significance.

Plasma 5-HT concentrations in hypertensive ($n=10$) and normotensive ($n=15$) DM (IDDM+NIDDM) patients (170; 56–938 and 199; 63–497 nmol l^{-1}, respectively) did not differ significantly. Similarly, plasma 5-HT concentration in the hypertensive ($n=6$) and normotensive ($n=7$) PVD patients (230; 165–369 and 205; 170–767 nmol l^{-1}, respectively) did not differ significantly.

Intraplatelet 5-HT concentrations broadly paralleled those described in study 1.

Discussion

Our results show clearly for the first time that the 5-HT content of platelets in NIDDM and PVD patients is significantly lower than that in controls, and that plasma 5-HT concentration in NIDDM and PVD patients is also greater than that in controls. Our observations also demonstrate that the 5-HT content of platelets in IDDM is significantly lower than that in controls; this is consistent with data recently reported in a preliminary communication [9].

Previous studies that considered the uptake [10] or content [9] of 5-HT by DM and PVD platelets are limited by the absence of age-matched controls. This factor was considered in the present study in view of the evidence of an age-related decline in platelet 5-HT content [11]. We have also assessed the effect of hypertension in our patients, since there is evidence

that this factor is associated with decreased platelet 5-HT content [12–14].

Since platelets do not synthesize 5-HT [15], the intraplatelet content of this amine is likely to be dependent upon the net balance of uptake and release. One previous study, although limited by the absence of adequate controls, shows that platelet 5-HT uptake in PVD patients and diabetics with retinopathy is diminished [10]. There is also evidence that hyperaggregable platelets obtained from DM patients with vascular complications release more 5-HT than platelets prepared from healthy subjects or diabetics without vascular complications [16]. It is relevant to mention that both DM and PVD are associated with hyperaggregability and an increase in the release of other intraplatelet products (e.g. β-TG [3,17], PF-4 [3,18] and TXA_2 [19,20]). Even in young diabetics with no obvious vascular complications, platelet hyperaggregability has been demonstrated using more sensitive techniques such as whole blood impedance aggregation [21] or the measurement of platelet lifespan [3,22]. Thus, it is possible that the low 5-HT content of platelets in these two conditions is the result of a combination of decreased uptake and increased release. This conclusion implies an increased plasma pool of 5-HT, which is also known to enhance platelet aggregation [23]. This latter change was reported in the present study. It does not appear that the duration of DM, or the quality of diabetic control, influences intraplatelet 5-HT concentrations.

Our methodology using PRP for platelet 5-HT determination may also have disadvantages: for example, higher density platelets with increased 5-HT granule content may be lost during the centrifugation procedure required to prepare PRP; however, this potential artefact probably does not occur since we found (using identical methodology) a significantly increased and an unaltered intraplatelet histamine content in PVD and DM patients, respectively [24]. This is of relevance since histamine, like 5-HT, is stored in the dense granules [25]. An artefact would therefore be expected to influence the intracellular content of both these bioamines in a similar way. Furthermore, alternative methodologies for homogeneous platelet preparation (e.g. density gradients [26]) are comparatively more time-consuming and therefore unsuitable for screening large numbers of patients.

The increase in plasma 5-HT concentrations observed in the various patient groups could result in a tendency to vasospasm, which is relevant to both structural vascular disease (e.g. atherosclerosis) and to secondary vasospastic disease (e.g. Raynaud's phenomenon in systemic sclerosis). It is also relevant that raised plasma levels of 5-HT have been reported in hypertension [27], another condition associated with low intraplatelet 5-HT [13] and increased platelet hyperaggregability [28]. Increased plasma concentrations of 5-HT may, to some extent, be self-perpetuating, since it could result in the activation of platelets and the consequent further release of 5-HT from platelets [23]. Whether this is true in DM and PVD remains to be determined. Whether the increase in plasma 5-HT concentrations is due to an increase in the synthesis of this amine, or whether it merely reflects a decrease in intraplatelet storage, is not clear. This aspect requires further elucidation.

In conclusion, DM and PVD are associated with diminished platelet 5-HT content over and above the diminution caused by age. This decrease may be of significance in the pathogenesis of atherosclerosis, vasospasm and platelet hyperaggregability in these conditions.

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EFFECT OF NAFTIDROFURYL AND ASPIRIN ON PLATELET AGGREGATION IN PERIPHERAL VASCULAR DISEASE

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Running title: Platelet aggregation in peripheral vascular disease

Abstract. *Platelet aggregation in whole blood (WB) was assessed in healthy subjects and in patients with peripheral vascular disease (PVD) using a WB-free platelet counting (WB-FPC) method. Aggregation induced by stirring and platelet agonists was significantly enhanced in PVD patients. WB-FPC aggregation induced by 5-HT was diminished significantly by incubation with naftidrofuryl (NAF) in WB of PVD patients. In contrast, aspirin (acetylsalicylic acid; ASA), added in vitro, did not significantly affect 5-HT or stirring-induced WB-PFC aggregation in PVD patients. Furthermore, 5-HT-induced WB-FPC was inhibited by NAF in blood collected from PVD patients that were taking low dose aspirin. These findings suggest that NAF may be of benefit to patients with hyperaggregable platelets and elevated plasma 5-HT concentrations, factors thought to predispose to thrombotic complications.*

Introduction

Peripheral vascular disease is associated with a number of rheological disturbances (eg raised plasma and whole blood viscosity and increased plasma concentrations of fibrinogen)[1]. A shortening of platelet survival, increased platelet aggregation and other platelet abnormalities (eg enhanced release of intraplatelet products such as ß-thromboglobulin and thromboxane A_2, TXA_2) have also been observed in this condition[2-6].
In rheological terms, platelet aggregate formation may influence the flow of blood and/or activate white blood cells which may, in turn, occlude nutritive capillaries. Release of intraplatelet

mediators such as serotonin (5-hydroxytryptamine; 5-HT) may influence red cell aggregation which could impair microcirculatory perfusion[7]. In this context, we have previously found raised plasma 5-HT concentrations and diminished intraplatelet 5-HT concentration in PVD patients[8]. Pietraszek *et al* have reported similar findings in patients with thromboangiitis obliterans[9]. Serotonin may be implicated in the pathogenesis of tissue ischaemia by amplifying the response of other platelet agonists (e.g. adenosine diphosphate from red cells) and through direct constrictory effects on atherosclerotic vasculature[10].

The technique most commonly used for investigating platelet aggregation in vitro is based on Born's turbidimetric method utilising platelet rich plasma (PRP)[11]. This method, however, cannot assess the effect of red cell- and white cell-platelet interactions. Furthermore, PRP preparation involves a centrifugation procedure which may lead to the loss of platelets and/or result in alterations in platelet function[12,13]. In this study we have assessed platelet aggregation in whole blood (WB) by a free platelet count method to determine whether PVD is associated with enhanced platelet aggregation when this phenomenon is studied in the presence of all blood cells. We have also determined the effect of naftidrofuryl oxalate (NAF; a drug with 5-HT blocking properties), added in vitro, on WB platelet aggregation in samples obtained from PVD patients on low dose ASA as well as from drug-free PVD patients whose blood was pre-treated, in vitro, with ASA.

MATERIALS AND METHODS

Selection of PVD patients

The patients selected were attending our vascular clinic with a diagnosis of PVD based on a history of intermittent claudication and an ankle:arm systolic blood pressure ratio below 0.85. In all studies, these patients were in a metabolically and clinically stable condition with no history of recent cardiac events, strokes, transient ischaemic attacks or changes in their claudication distance. These patients had not undergone any recent surgery, angiography or angioplasty. The age, sex, drug history and other known pathology for each patient group is outlined below under each study section.

Informed consent was obtained from all patients and healthy subjects before blood sampling.

Selection of Healthy subjects

Healthy subjects were laboratory and clinical staff. Younger healthy subjects than our patients were selected in this study. It is well documented that there are a number of platelet function abnormalities with increasing age in "healthy" subjects[8,14-17]. However, these changes are not consistent[16]. This is probably due to the presence of sub-clinical atherosclerosis. We therefore selected a younger healthy control group since these subjects are more likely to reflect "normal" platelet function[18]. Subjects sex and age are provided in each study section. All subjects denied taking any medication for at least 2 weeks prior to sampling or of having a history of a major illness or vascular

disease.

Blood sampling and processing

Blood was withdrawn from patients and healthy subjects between 14.00 and 16.30 h. The blood was collected from an antecubital vein with minimum stasis using a G-21 butterfly (Abbott Ireland Ltd, Sligo, Rep. of Ireland). The first 2 ml of blood were discarded. Blood (9 ml) was added to plastic tubes containing different anticoagulant mixtures (1 ml). The final concentrations (f.c.) of the anticoagulants were:
anticoagulant mixture A - 0.38 % w/v tri-sodium citrate;
anticoagulant mixture B - 0.38% w/v tri-sodium citrate plus acetylsalicylic acid (ASA; 1 mmol/L). ASA at a concentration of 1 mmol/L was chosen since this concentration is achieved in serum following therapeutic doses of ASA[19] and is not toxic to cells[20]. Lower doses of ASA (100 μmol/L), added in vitro, have previously been found to be ineffective or only partially effective at inhibiting aggregation induced by agonists (such as ADP, PAF[21]) and spontaneously (SPA)[22] in hyperaggregable states. All experiments were completed within 2 h of blood sampling.

Study 1: Whole blood platelet aggregation in PVD patients and healthy subjects

Subjects

24 healthy subjects were selected; 18 were males and 6 were females. Median age and (range) was 28 (20-58) years. 14 PVD patients, of which 11 were males and 3 were females volunteered for this study. The median age and (range) of the patients was

64 (44-83) years. Patients selected were drug-free.

Whole blood Aggregation

Blood was collected into anticoagulant mixture A and kept at 37^{0}C for 30-40 min. WB-platelet aggregation was carried out using a Coulter T-890 routine haematology blood counter as described previously[23]. Briefly, 1 mL aliquots of citrated WB were placed in a Chronolog aggregometer (model 540), stirred (at 1000 rpm) for 15 s and a basal platelet count obtained. Agonists were added and at specified times (see Table I and II and Fig 1-4) aliquots were withdrawn and platelet counts obtained of the remaining free (single) platelets. SPA was assessed by spinning blood for 6 and 15 min and counting as above. WB-platelet aggregation is expressed as a % of the basal free platelet count. The reproducibility and validity of this method of assessing WB platelet aggregation have already been described[23,24].

Study 2a: Effect of NAF on whole blood platelet aggregation in samples obtained from drug-free PVD patients

Subjects

Nine PVD patients, of which 6 were males and 3 were females volunteered for this study. The median age and (range) of the patients was 71 (52-82) years. Patients selected were drug-free. Blood sampled as above was collected into anticoagulant mixture A and kept at 37^{0}C for 30-40 min. NAF at a concentration (5 μmol/L) which is similar to that achieved in the plasma when it is used therapeutically[25] or vehicle (phosphate buffer pH 7.4; 20

mmol/L KH_2PO_4, 116 mmol/L Na_2HPO_4) were added to tubes in one of two aggregometer channels. The blood was stirred (1000 rpm) for 30 sec to ensure adequate mixing; stirring was then switched off for 4 min; 30 sec before commencing WB-platelet aggregation stirring was restarted and WB-platelet aggregation was carried out as described above. Stirring mechanisms were switched off to avoid platelet clumping taking place during the incubation phase.

Study 2b: Effect of NAF and ASA addition on whole blood platelet aggregation in samples obtained from drug-free patients and patients taking low dose ASA

Subjects

14 PVD patients, of which 11 were males and 3 were females volunteered for this study. The median age and (range) of the patients was 64 (44-83) years. Patients selected were drug-free. Blood sampled as above was collected into anticoagulant mixture A and kept at 37^0C for 30-40 min. In experiments where the effect of ASA was being evaluated, blood was collected into the anticoagulant mixture B. This experimental approach has recently been used by others[21] to study the effect of ASA, in vitro, on WB platelet aggregation in normal pregnancy. By allowing a longer contact time (approximately 45 min) between ASA and whole blood the inhibition of platelet cyclooxygenase is obtained[26]. WB-platelet aggregation was carried out as described above.

In a second study, 7 PVD patients who were on low dose aspirin (150 mg, once daily, every alternate day) were sampled. Two patients from this group were on frusemide+amiloride, and 1 patient was on isosorbide dinitrate, diclofenac and dipyridamole.

One patient was a diabetic on insulin treatment. These patients were selected in order to establish whether NAF was effective in patients taking ASA and other drugs prescribed to PVD patients. Four patients were males and 3 were females. The median age and (range) of the patients was 66 (48-80) years. Blood sampled as above was collected into anticoagulant mixture A and anticoagulant B and kept at 37^0C for 30-40 min. WB- platelet aggregation was carried out as described above. All experiments were completed within 2 h of withdrawing the blood.

Drugs and Reagents

Tri-sodium citrate, ASA, adrenaline bitartrate, adenosine diphosphate (ADP), 5-HT creatinine sulphate were obtained from Sigma Chemical Co. Ltd (Poole, Dorset, UK). Naftidrofuryl oxalate (Praxilene) was a gift from Lipha Pharmaceuticals Ltd., West Drayton, UK. Reagents for the Coulter T-890 (unit T-packs) were purchased from Coulter Electronics Ltd (Luton, UK).

Statistical analysis and presentation of results: All results were statistically analyzed using a computer program (C-STAT, Cherwell Scientific Publishing, Oxford, UK). In study 1 and 2 the unpaired data (PVD vs Healthy subjects) was analyzed by using the Mann-Whitney U-test for non-parametric data (two-tailed). Paired data values were compared using the Wilcoxon paired rank sum test (two tailed).

RESULTS

Study 1: Whole blood platelet aggregation in patients and healthy

subjects

PVD patients exhibited significantly enhanced SPA and platelet aggregation to 5-HT in WB when compared to younger healthy subjects (See Fig 1 and 2). Significantly enhanced aggregation to adrenaline and ADP was also observed (Table I).

Study 2a: Effect of NAF on whole blood platelet aggregation in samples obtained from drug-free PVD patients

5-HT-induced WB-platelet aggregation was inhibited significantly ($P<0.01$) by NAF (5 μmol/L)[25] (Fig 3). SPA and adrenaline-induced WB-platelet aggregation, however, were not affected by NAF. Thus, for SPA, median and (range) % WB-FPC at 6 min, control: 68 (35-85), NAF: 63 (30-92); SPA at 15 min, control: 52 (28-74), NAF: 52 (26-78). For adrenaline (1 μmol/L) at 1 min, control: 35 (14-55), NAF: 37 (12-52) and adrenaline at 3 min, control: 12 (7-28), NAF: 15 (5-28).

Study 2b: Effect of NAF and/or ASA on whole blood platelet aggregation in samples obtained from PVD patients

We studied two groups of patients. Patients that were drug-free and patients that were on low dose aspirin plus other drugs prescribed to PVD patients (eg isosorbide dinitrate, dipyridamole) and known to inhibit platelet function[27]. In blood treated or not treated in vitro with ASA obtained from patients that were drug-free, NAF at therapeutic concentrations (5 μmol/L) inhibited significantly ($P< 0.04$), 5-HT-induced WB-platelet

aggregation 30 sec following the addition of 5-HT (Fig 4). ASA-treated WB in vitro was not significantly inhibited when aggregation was induced with 5-HT (Fig 4) or by SPA when compared to non-ASA-treated blood. Thus, for SPA median and (range) % WB-FPC at 6 min, **NO ASA** : 64 (32-83), **+ ASA (1 mmol/L)** : 63 (34-92); SPA at 15 min, **No ASA**: 55 (24-76), **+ ASA** : 50 (23-77). With respect to 5-HT, these findings were also true for patients that were taking low dose ASA and other drugs (see Table II; SPA was not assessed).

DISCUSSION

Using a WB free platelet count method our study shows significant enhanced SPA (a predictor of myocardial infarction[28]) and aggregation to adrenaline, ADP and 5-HT in platelets from patients with PVD. Such enhanced platelet aggregability may contribute to the abnormal rheological properties of the blood of PVD patients either by influencing blood flow directly or via platelet-derived mediators which can affect red cell and white cell function[29]. Our comparisons have been made against a drug-free healthy group of younger subjects who are less likely to possess sub-clinical (asymptomatic) atherosclerosis than older controls. A study involving older healthy subjects would be problematic as it would be necessary to assess and exclude such subclinical atherosclerosis in the control group[18].

Two previous studies assessing platelet aggregation in WB of PVD patients have reported significantly diminished aggregability to ADP[30,31]. In addition, Catalano et al[30] also observed similar aggregation to high dose collagen in healthy

subjects and PVD patients. These workers used the WB-impedance aggregometer (WB-IA) whereas we have used a free platelet count method. The advantages and disadvantages of both methods have been debated previously[23,32]. Briefly, WB-IA is likely to be less sensitive than the free platelet count method requiring higher concentrations and longer durations of stimulation by agonists to achieve detectable readings. In addition Catalano et al[30] left the citrated blood at room temperature for periods of 30-180 min which may have activated platelets and resulted in a refractory state due to cooling-induced activation[33]. Galt et al[31] used flow cytometric analysis to assess platelet function in PVD patients. Some of the patients selected were taking drugs (ß-blockers, nitrates, calcium channel blockers) which they accept may have influenced results. In addition, they collected blood into heparin, which could have activated the platelets of PVD patients[5] and led to a platelet desensitization state[34].

We believe that SPA and agonist-induced platelet aggregation in WB as assessed in the present study is likely to be a better predictor of ischaemic events than PRP-based methods since the WB method may reflect red cell[35] as well as white cell[1] abnormalities present in PVD.

In this study we have demonstrated that concentrations of NAF similar to those found in therapeutic dosages inhibit 5-HT-induced platelet aggregation in PVD patients. This effect may be important since PVD platelets are hyperaggregable in response to 5-HT in both WB (present study) and in PRP[4]. As observed previously, NAF probably antagonizes 5-HT-induced effects by virtue of its 5-HT_2 blocking properties[25], although NAF has also

been shown to possess other properties (see reference 36 for relevant literature). In a previous[25] report, 50-100 μmol/L NAF was required to inhibit 5-HT-induced platelet aggregation in PRP from healthy subjects whereas in the present study concentrations as low as 5 μmol/L significantly inhibited WB-platelet aggregation in PVD patients. The greater sensitivity of our system is probably due to the fact that we have used: (1) WB from PVD patients which is hypersensitive to 5-HT and; (2) by using WB we have a more sensitive system than PRP which is markedly less reactive than WB[20]. It is also of interest that the inhibitory effect of NAF on 5-HT induced WB-platelet aggregation is present even when the blood has been treated with ASA and/or the patients have received other drug therapy which inhibits platelet aggregation (e.g. isosorbide[37] and dipyridamole[38]). These findings suggest that: (a) 5-HT induced aggregation in WB occurs independently of thromboxane A_2 synthesis and; (b) NAF may be of potential benefit, irrespective of ASA intake, in conditions associated with platelet hyperaggregability and raised concentrations of plasma 5-HT (e.g. PVD).

The preliminary findings of this study were presented at the 7th European Conference on Haemorheology[39].

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TABLE I

Whole blood platelet aggregation in peripheral vascular disease patients and healthy subjects. Platelet aggregation is expressed as a % of the basal platelet count in whole blood. Results are presented as median and (range).

Platelet Aggregating Agent

Patients/Subjects	**Adrenaline** 1 μmol/L		**ADP** 2 μmol/L	
	Sampling times		Sampling times	
	1 min	3 min	1 min	3 min
PVD patients n= 14	31%* (9-50)	10%* (5-26)	21% (12-59)	27%* (7-69)
Healthy subjects n= 24	58% (16-87)	49% (10-76)	23% (6-69)	56% (18-90)

* $P < 0.015$ vs healthy subjects

n=number of patients/subjects studied.

TABLE II

Effect of NAF and ASA on whole blood platelet aggregation in samples obtained from PVD patients on low dose ASA. Platelet aggregation is expressed as a % of the basal platelet count in whole blood. Results are presented as median (range).

Agonist/Antagonist	**Oral ASA only**		**Oral ASA + ASA added in vitro**	
	Sampling times		Sampling times	
	30 s	1 min	30 s	1 min
5-HT (5 μmol/L)	48% (23-78)	65% (29-90)	54% (24-79)	68% (32-92)
5-HT (5 μmol/L) + NAF (5 μmol/L)	59%* (28-96)	71% (33-97)	62%* (27-94)	72% (36-97)

* $P < 0.04$ 5-HT+NAF vs 5-HT only. Comparisons of No ASA treatment Vs. ASA treated blood were not significant.

ASA (1 mmol/L) was added in vitro for 35-40 min, at 37°C, before commencing experiments.Number of patients studied = 7.

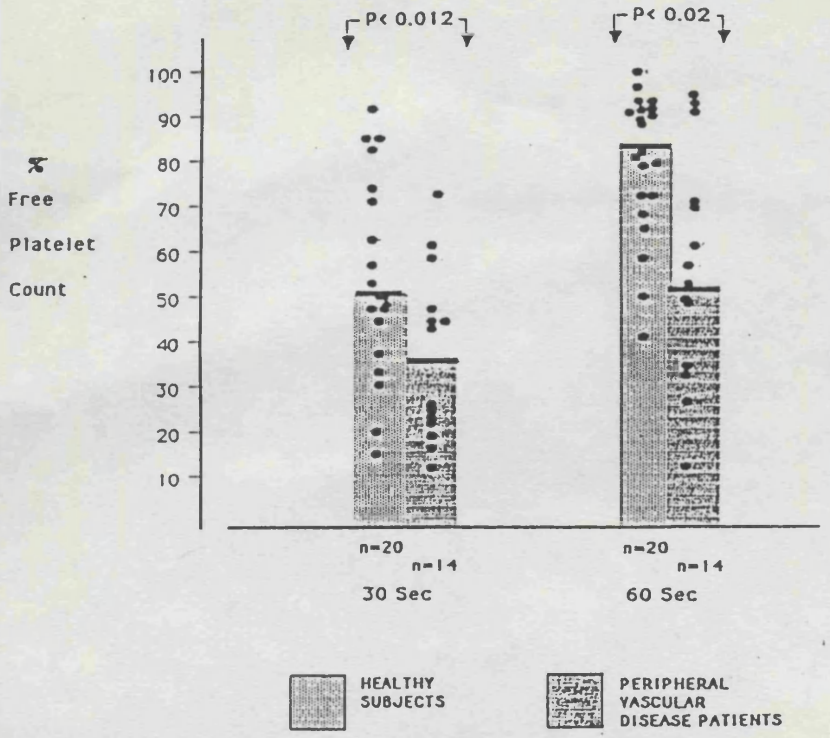
Figure Legends

Figure 1: 5-HT-induced platelet aggregation in whole blood obtained from healthy subjects and peripheral vascular disease patients. Aggregation is expressed as a % of the basal free platelet count. Medians are shown as solid bars.

Figure 2: Spontaneous platelet aggregation in whole blood obtained from healthy subjects and peripheral vascular disease patients. Aggregation is expressed as a % of the basal free platelet count. Medians are shown as solid bars.

Figure 3: 5-HT-induced platelet aggregation in whole blood preincubated with vehicle or NAF for 5 min. Aggregation is expressed as a % of the basal free platelet count. Medians are shown as solid bars.

Figure 4: 5-HT-induced platelet aggregation in whole blood preincubated with vehicle or NAF for 5 min. "No ASA" experiments were carried out in blood collected in anticoagulant A (no acetylsalicylic acid) and "With ASA" experiments blood was collected in anticoagulant B (with acetylsalicylic acid). Aggregation is expressed as a % of the basal free platelet count. Medians are shown as solid bars.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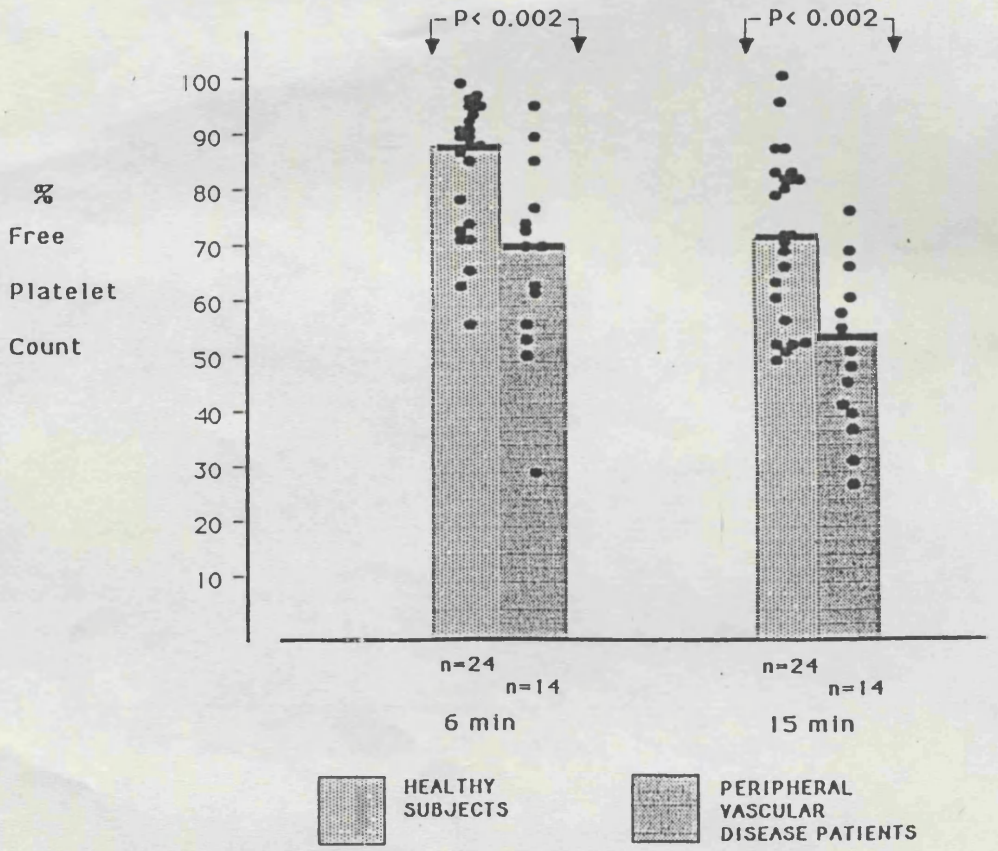
HEALTHY SUBJECTS
PERIPHERAL YASCULAR DISEASE PATIENTS

FIG 1


P< 0.002
P< 0.002
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90
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HEALTHY SUBJECTS
PERIPHERAL YASCULAR DISEASE PATIENTS

FIG 2

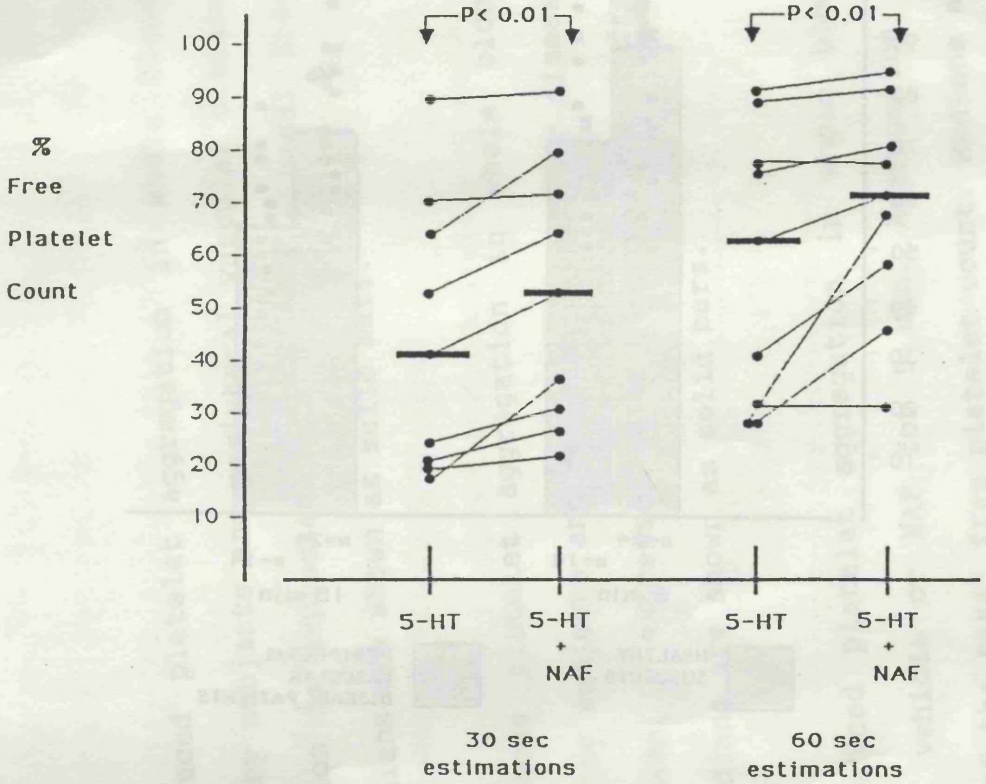
% Free Platelet Count
100
90
80
70
60
50
40
30
20
10
P< 0.01
P< 0.01
5-HT
5-HT + NAF
5-HT
5-HT + NAF
30 sec estimations
60 sec estimations

FIG 3

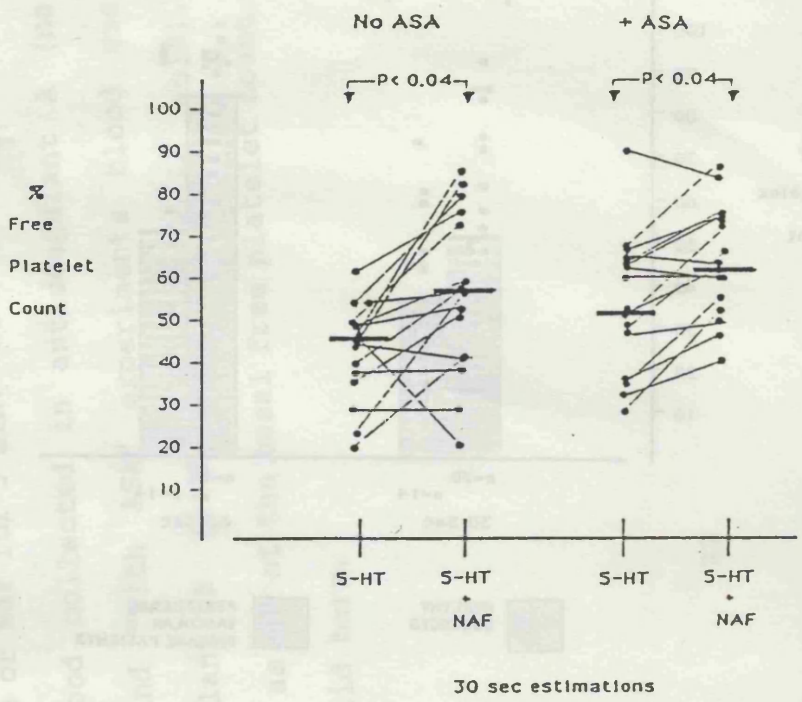
No ASA
+ ASA
% Free Platelet Count
100
90
80
70
60
50
40
30
20
10
P< 0.04
P< 0.04
5-HT
5-HT + NAF
5-HT
5-HT + NAF
30 sec estimations

FIG 4