https://theses.gla.ac.uk/30845/

Theses digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

PROSTACYCLIN ACTIVITY IN PORTAL HYPERTENSION

A thesis submitted for the degree of
Doctor of Medicine
of the University of Glasgow

By

George Hamilton FRCS
University Department of Surgery
Royal Free Hospital
Royal Free & University College School of Medicine
University College London

September 2002

ProQuest Number: 10390622

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10390622

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

GLASGOW
UNIVERSITY
LIBRARY
13006
copy 2

This thesis is dedicated to my wife Maggie,
and children
Gina, Patrick and Sophie

For their continued love and support.

Contents

PROSTACYCLIN ACTIVITY IN PORTAL HYPERTENSION

ABSTRACT

This work was performed between 1979 and 1985 when there was great interest in the role of the recently identified prostacyclin in vascular function and disorders. The discovery of this substance with its powerful vasodilatory and platelet anti-aggregatory powers raised the hypothesis that prostacyclin might be involved in the pathogenesis of portal hypertension, its associated hyperdynamic circulation and typically catastrophic haemorrhage from bleeding oesophageal varices.

Partial portal vein ligation in a rat model of portal hypertension was used because of its simplicity and absence of hepato-cellular dysfunction. This model was found to result in short lived hypertension with return to normal pressure by two weeks. Anatomical studies (using venography and corrosion casting) of the changes to the portal venous circulation after partial portal vein ligation, revealed the development of a dominant portosystemic collateral draining into the left renal vein via the left anterior lumbar vein. Ligation of this collateral at the same time as partial portal vein ligation gave a reliable model of permanent portal hypertension.

Accurate measurement of prostacyclin proved to be difficult. Initially a bio-assay of prostacyclin-like activity was used with success. The rat was found to produce high levels of prostacyclin well within the range of accurate measurement of this assay. Prostacyclin production was shown to increase directly with pressure increase in the portal vein. This direct relationship was confirmed in the acute model where prostacyclin production fell as the portal pressure returned to normal; in the model of chronic portal hypertension, prostacyclin production remained permanently elevated.

A radioimmunoassay for 6 ketoPGF1α, the stable breakdown product of prostacyclin, was developed to allow measurement of prostacyclin in human tissue and serum samples (at this time there were no commercially available RIA kits). Initially the Wellcome antiserum was used with accurate measurement in incubated human tissue samples. These studies confirmed greater intrinsic prostacyclin activity in normal mesenteric and portal vein compared to peripheral venous tissues but failed to show any difference in tissue or plasma levels of portal hypertensive compared to normal patients.

A second antiserum, the Cardeza antiserum, was then used in the radioimmunoassay. Unlike the Wellcome assay, this antiserum did not require a prostanoid extraction process. Comparison of the two assays in identical samples revealed major differences with large quantities of 6 ketoPGF1α being measured using the Wellcome antiserum with its extraction step compared to virtually none detected using the Cardeza antiserum. The extraction step was resulting in production of cross-reacting prostanoid

substances giving falsely high readings. Both radioimmunoassays were abandoned at this stage because of the inaccuracy of the first, and the inability of the second to detect the low levels of 6 ketoPGF1α in human plasma.

The human studies were continued using a highly specific and sensitive assay of 6 ketoPGF1α, namely gas chromatography/negative ion chemical ionisation mass spectroscopy (GC/NICIMS). At the time of this work, this methodology was complex, cumbersome, with limited access and the numbers studied were small.

Very low levels of 6 ketoPGF1α were found in peripheral blood of normal and portal hypertensive patients who were not bleeding from oesophageal varices. In patients without portal hypertension, portal blood levels of prostacyclin were higher compared to peripheral levels, confirming the finding in the rat and pig that prostacyclin activity is higher in the normal portal circulation compared to peripheral vein. Significantly elevated prostacyclin production was found in both the peripheral and portal blood of portal hypertensive patients who were actively bleeding from oesophageal varices.

Portal prostacyclin production was found to be significantly higher in patients with portal hypertension who were actively bleeding compared to normal patients undergoing laparotomy for other conditions. These findings in bleeding patients support the hypothesis that prostacyclin activity is increased in portal hypertension and may play a role in the severity of haemorrhage.

In both the animal and human studies a clear effect of surgical intervention on increased prostacyclin production was found.

These studies demonstrated for the first time increased prostacyclin production in both developing, and established portal hypertension in both the experimental animal situation and in man. High levels of prostacyclin production were found in the portal circulation of portal hypertensive patients undergoing surgery for uncontrolled variceal bleeding.

INDEX

Tables

Figures

Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
cAMP	Cylic adenosine monophosphate
cGMP	Cylic guanosine monophosphate
COX	Cycloxygenase
COX-1	Cycloxygenase 1
COX-2	Cycloxygenase 2
DCC	Dextran coated charcoal
eNOS	Constitutive nitric oxide synthase
ET-1	Endothelin 1
GC/MS	Gas chromatography/mass spectrometry
GTB	Gelatin tris buffer
GTP	Guanosine triphosphate
5HT	5-hydroxytryptamine
iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
PG	Prostaglandin
PGDH	15-hydroxyl prostaglandin dehydrogenase
PGX	Original name for PGI_2
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PPP	Platelet poor plasma
PRP	Platelet rich plasma
RIA	Radioimmunoassay
TBS	Tris buffered saline
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2

Acknowledgements

My first acknowledgement is to Professor Ken Hobbs, my supervisor for this work, and constant source of encouragement. He inspired and stimulated my first surgical and research interest, hepatobiliary disease, during which time the work for this thesis was completed. This support and encouragement continued even after I had deserted hepatobiliary and liver transplantation surgery, to become a vascular surgeon. Without his belief in this work and his mostly gentle goading over the years this thesis would not have been written.

Dr Ron Hutton and Dr Rose Chow of the Katharine Dormandy Haemophilia Centre at the Royal Free Hospital gave superb scientific guidance, support and instruction in the various assays of prostacyclin. I am particularly indebted to Rose Chow without whose expertise in the arcane and difficult field of radioimmunoassay development, this work would not have been possible. I am also indebted to Professor Colin Dollery who gave me access to the resources of his laboratory at the Hammersmith, and to the expertise of Dr Sue Barrow and her colleagues to allow the final exact studies on clinical samples to take place.

The support of several members of the University Department of Surgery must also be acknowledged – Dr Russell Woods for his help with animal work in particular the microsphere experiment, David Haswell, Chief Technician at that time, and Dr Alex Seifalian for his general constructive review and criticism of the data.

My special thanks to the Examination Board of the University of Glasgow who allowed me to submit this thesis a little later than is customary. Finally I thank my long suffering wife and family for their support during the evenings and weekends when this thesis was written.

Statement of Originality of Work

All of the concepts involved in the study design and their execution in this work were my own. Also all surgical procedures were personally performed, as was collection and processing of all tissue and blood samples, both animal and human. The further development of rat models of portal hypertension was my own, helped by Dr Russell Woods who kindly taught me how to prepare microspheres, and Mr Hugh Rogers, who helped me with his expertise in corrosion cast analysis.

Histological examinations and reporting on the rat liver specimens was entirely the work of Dr Margaret Chappell, then Lecturer in Pathology. Splenic venography was performed by myself with the help of the Superintendent Radiographer (once all the patients had gone home) and with the initial advice of Dr Bob Dick, Consultant Radiologist.

Measurement of prostacyclin was performed in collaboration with Dr Ron Hutton and Dr Rose Chow whose guidance and expertise was invaluable. I was involved in the assay procedures continuously throughout this work in the laboratories of the Katharine Dormandy Haemophilia Centre at the Royal Free Hospital under their supervision. My involvement in the development of the radioimmunoassays was as a junior partner of the team but I contributed to the process throughout the work between 1980 and 1983. Prostacyclin assays by the GC/NICIMS method were performed entirely at Professor Colin Dollery's laboratories at the Hammersmith Hospital with no personal involvement beyond the harvesting, preparation and delivery of the samples.

Analysis of all data, interpretation and further study design were all personally performed. All of this thesis was personally written and all figures, tables, illustrations etc were personally prepared.

CHAPTER 1: PORTAL HYPERTENSION

1.1 Introduction

Portal hypertension is an almost invariable complication of chronic liver disease characterised by increased pressure gradients between portal and systemic venous circulations. The normal gradient of 5 mm Hg increases in portal hypertension and becomes clinically significant when it exceeds 12 mm Hg at which level variceal haemorrhage and or development of ascites supervenes.

There are two principal factors implicated in the pathophysiology of portal hypertension. The first is obstruction to portal blood flow most commonly due to diseases of the liver or associated structures. These result in distortion, compression or obliteration of the hepatic vasculature giving increased resistance to portal blood flow leading in turn to portal hypertension. This obstruction is accompanied by portal venous vasodilation and increased mesenteric blood flow, this is known as the hyperaemia of portal hypertension. These developments lead to an abnormal systemic cardiovascular status namely a hyperdynamic circulation characterised by an increased cardiac index and reduced vascular resistance.

Obstruction may occur at different levels. It may be secondary to thrombosis in the hepatic veins commonly known as the Budd-Chiari syndrome, within the liver parenchyma itself, or in the portal vein. The most common cause of

portal hypertension in Europe and North America is cirrhosis which increases intra-hepatic vascular resistance by fibrosis, thrombosis and nodular regeneration. Portal vein obstruction by thrombosis or extrinsic compression is the second most common cause. Hepatic vein occlusion is the third most common but much less frequent cause.

A classification of portal hypertension commonly used is by site of obstruction namely at the pre-sinusoidal, sinusoidal and post-sinusoidal levels. In pre-sinusoidal obstruction, intrinsic liver disease is usually absent and the natural history and prognosis of portal hypertension in this group is therefore relatively good. In patients with a sinusoidal block, which typically occurs in cirrhosis, the degree of hepatocellular damage as a result of the precipitating pathology is the most important factor in the natural history of the disease. Death may result not only from variceal haemorrhage but also from liver failure, malnutrition or infection. Post-sinusoidal obstruction can result in secondary hepatocellular damage particularly in Budd-Chiari syndrome where there is massive congestion of the liver. In all causes of portal hypertension, the underlying degree of hepatocellular damage is the major factor in deciding prognosis.

1.2 Anatomical and Physiological Considerations

The portal venous system is entirely devoid of valves and numerous small tributaries connect the portal and systemic venous system. In portal hypertension these can evolve into major collateral channels. (figs 1.2 (a) & (b))

Fig 1.2(a)

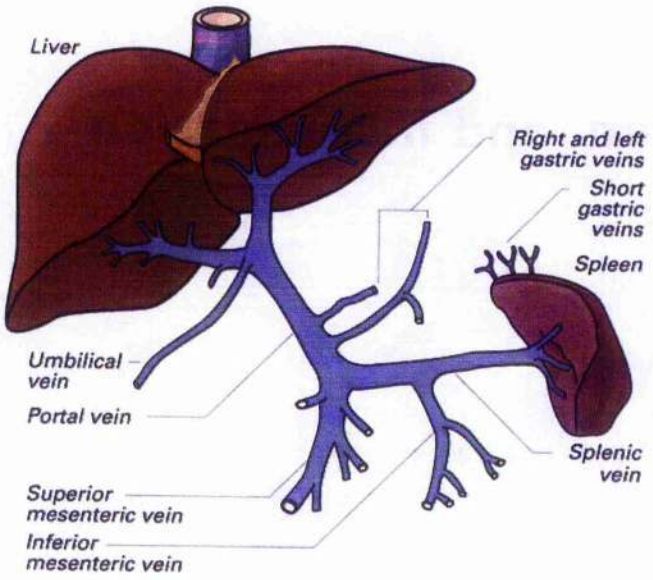


Fig 1.2(b)

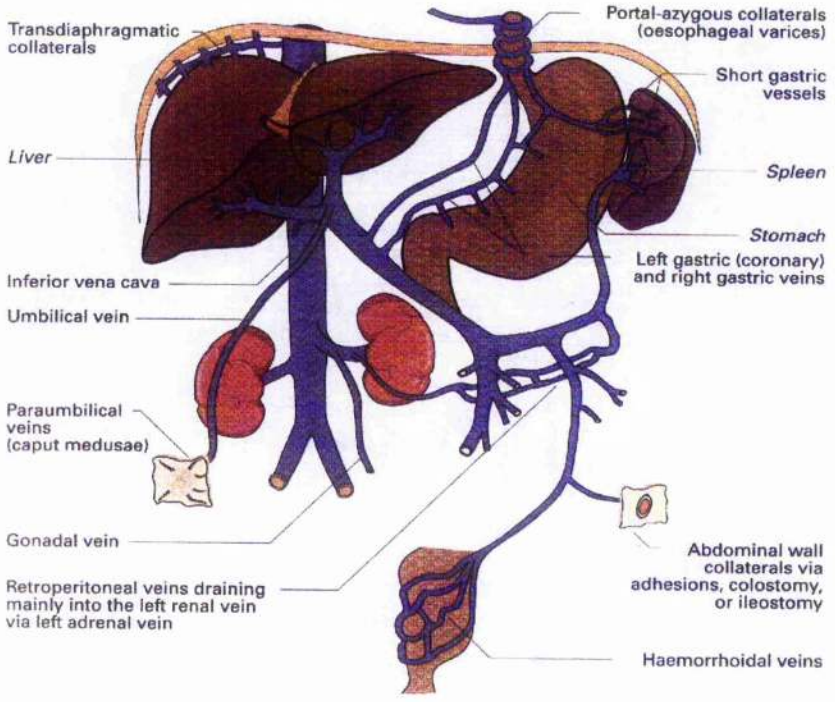


Fig 1.2(a) The Portal Circulation & Fig 1.2(b) Portasystemic Shunts

(from Hamilton.G, Aetiology of Portal Hypertension in Oxford Textbook of Surgery, 2nd Edition, Morris & Wood Eds. Oxford University Press 2000, pp 1713-25).

Clinically the most important channel is the left gastric or coronary vein which connects the oesophagocardiac venous plexus with the splenic or portal vein. Numerous other portal systemic collaterals open up at various sites in the abdomen, across the diaphragm, and into the haemorrhoidal circulation. In addition to these channels, intra-hepatic shunts develop through which a significant portion of the portal venous flow can pass. The opening up of these channels allows up to 80% of the liver's blood supply to bypass the sinusoidal circulation.

The portal vein normally carries 75% of the blood supply to the liver with an average flow of 1,200 ml/min. The hepatic artery supplies the remainder at an average flow of 400 ml/min and also supplies 30-40% of the liver's normal oxygen requirement since portal blood is already partly de-oxygenated. In the presence of compromised flow to the sinusoids a compensatory increase in hepatic arterial inflow takes place to maintain the liver's oxygen requirements. In the presence of intra-hepatic shunting, however, 30% of the hepatic arterial flow may bypass the sinusoidal bed and despite the compensatory increase in arterial flow, an overall significant reduction in hepatic tissue perfusion can occur.

1.3 Haemodynamic Considerations

An appreciation of the basic haemodynamic principles governing flow within blood vessels allows a better understanding of the pathophysiology of portal hypertension. As in all blood vessels, pressure within the portal system is determined by the interaction of flow and vascular resistance. As these parameters change, so does portal pressure. This relationship is expressed by Ohm's law.

$\Delta_p = Q \times R$

where Δ_p is the change in Pressure, Q is flow and R is resistance in a vessel.

Resistance is determined by several factors expressed in Poiseuille's law.

$R = 8nL/\pi r^4$

where n is the coefficient of viscosity, L the vessel length, and r the vessel radius. Within the liver, viscosity and length of vessel are relatively constant and thus changes in the vascular resistance are mainly due to changes in radius. Because the relationship is to the 4^{th} power of the radius, small changes in portal vessel diameter will have profound effects on vascular resistance. By substitution of the equation for resistance into Ohm's equation, it can also be readily seen that pressure changes will also depend on changes in flow.

The normal liver has a very low intra-hepatic resistance which can decrease with increased blood flow due to vascular dilatation. This inherent property of an extremely compliant hepatic circulation can maintain normal portal pressure within a wide range of portal flow. This capacity is lost in liver

disease with dramatic increases in intra-hepatic resistance. Structural disturbances associated with cirrhosis, inflammatory changes in the hepatic venous tree and deposition of fibrous tissue around the terminal hepatic venules and adjacent sinusoids have been described. In cirrhotic livers, intra-hepatic resistance may also be affected by proliferation of myofibroblasts around the sinusoids and terminal hepatic venules, resulting in increased contractility which may raise resistance and contribute to portal hypertension (Rockey DC, 1997). These myofibroblasts are now known as hepatic stellate cells. These cells are star shaped with many branching arms and acquire contractile properties behaving in a similar manner to vascular pericytes. Thus in response to cytokine stimulated release of vasoactive substances, in particular endothelin, these cells by their action on the sinusoids at the microcirculatory level can increase the resistance to portal blood flow. Also sinusoids may be compressed by hepatocyte enlargement in regenerating parts of the liver. This can occur as a result of several toxic, infectious or metabolic insults to the parenchyma and may explain the portal hypertension seen in non-cirrhotic conditions such as alcoholic hepatitis.

1.4 The 'theories' of portal hypertension.

Despite the decompressive effects of the collaterals described above and intra-hepatic shunts, with up to 80% of portal blood flow bypassing the liver, portal pressure remains elevated. Two major hypotheses have been advanced to explain this phenomenon.

The "backward" theory postulates that portal hypertension is due to increased hepatic vascular resistance which develops as a specific response, so that in the presence of normal flow, pressure must increase. Hypertrophy of myofibroblasts or stellate cells within the sinusoidal bed is one possible mechanism by which this could be achieved. Ohm's law suggests that portal hypertension would only develop if normal liver blood flow was maintained, but logically, in the presence of extensive low resistance collaterals, blood flow should be diverted away and thus portal pressure should decrease. In reality, however, the portal and splanchnic circulation becomes not only markedly increased but hyperdynamic, thus negating the likelihood that this theory can provide a complete explanation for portal hypertension.

The "forward" theory was initially postulated by Banti in 1883. He suggested that splenomegaly, portal hypertension, and cirrhosis were the result of increased splanchnic arterial blood flow. The subsequent confirmation of increased splanchnic blood flood as a major feature of portal hypertension has led to further development of this theory (Vorobioff J et al 1983). This now suggests that increased and hyperdynamic flow, together with decreased splanchnic pre-capillary resistance, maintain portal hypertension even in the face of extensive portosystemic shunting. Since Banti first populated this theory, opinions on the relative contribution of increased splanchnic blood flow to the development of portal hypertension have varied. In the early 80s when the studies for this thesis were performed, there continued to be debates regarding the importance of each of these two principal mechanisms. More recently Groszmann, (Gupta TK et al, 1997), and other workers have

increasingly implicated both theories as being involved in the pathophysiology of this condition, perhaps coexisting to different degrees in different conditions.

It is currently believed that the principal and initial abnormalities previously described increase vascular resistance to portal flow and that portal hypertension is then maintained by increased blood flow into the portal circulation, a phenomenon which has been confirmed conclusively both experimentally and clinically. Blood flow to the stomach, spleen, and intestines is increased by 50% in portal hypertension. This is achieved largely by splanchnic vasodilatation and raised cardiac output.

Cirrhosis and other causes of portal hypertension

↓

Increased portal system resistance

↓

Portal hypertension

↓

Increased production of vasoactive substances

↓

Portal collateral formation

↓

Peripheral vasodilatation and increased plasma volume

↓

Hyperdynamic circulation

1.5 Hyperdynamic Circulation of Portal Hypertension

A hyperdynamic systemic circulation is a common feature in patients with portal hypertension. Previously it was thought that the intensity of this phenomenon was related to the degree of hepatocellular dysfunction. However, this status is also seen in patients with extra-hepatic causes of portal hypertension in whom hepatic function is normal. Portosystemic shunting and peripheral vasodilatation are the major determinants in the pathogenesis of this clinical feature of portal hypertension and are not directly related to the status of hepatocellular function. The hyperdynamic circulation is characterised by decreased systemic vascular resistance, decreased mean arterial pressure, increased splanchnic blood flow, increased cardiac index and plasma volume expansion (Murray JF et al, 1958). Three main mechanisms have been identified, namely increased levels of circulating vasodilators, increased endothelial production of local vasodilators, and decreased vascular responses to endogenous vasoconstrictors.

Due to extensive study the pathogenesis of these changes is now becoming better understood (Bendis L and Wong F 2001). The hyperdynamic changes originate in the portal vein and splanchnic venous bed at an early stage secondary to increased resistance. In addition to dilation of this vascular bed and the development of splenomegaly, portal to systemic shunts develop resulting in shunting of portal blood into the systemic circulation. Due to ambulatory sub-clinical sodium retention, expansion of all body fluid compartments develops. Thus initially the hyperdynamic circulation can be detected only after two hours in the supine position probably as a result of

vasorelaxation by suppression of the renin-angiotensin-aldosterone system, and redistribution of excess extracellular fluid into the central blood volume. Similar changes are found in pregnancy where increased blood volume associated with vasodilation and shunting are the fundamental changes which will completely reverse post-partum. After liver transplantation however, where both portal hypertension and liver function are normalised, the hyperdynamic circulation will persist for 6 to 12 months (Piscaglia F et al 1999). This is probably due to a combination of portal venous remodelling, hypertension induced changes to the wall structure of the splanchnic vessels and the continuing presence of portasystemic shunts. With deteriorating liver function leading to severe decompensation, and major activation of the renin-angiotensin-aldosterone system, the systemic hyperdynamic circulation becomes evident at all times and postures. A further important feature of severe decompensation is increasing hyporesponsiveness to increasingly elevated circulating vasoconstrictor substances. The aetiology of this phenomenon is complex but mainly due to the high levels of vasodilators such as nitric oxide and prostacyclin in the systemic circulation acting together with changes to the systemic vascular bed. The net result is resistant systemic hypotension.

Historically several substances have been implicated as hormonal factors which act on both the splanchnic and systemic circulations to produce vasodilation and hyperaemia. The most important of these agents are bile acids, serotonin, glucagons, adenosine, prostaglandins, in particular prostacyclin, and nitric oxide. Elevated levels of bile acids have been

demonstrated in patients with liver disease and implicated as promoters of splanchnic hyperaemia. Their exact role, however, remains uncertain. Glucagon is an important splanchnic vasodilator and is elevated in patients with portal hypertension and probably accounts for 30% of the increased splanchnic blood flow. Indeed the effect of somatostatin in reducing portal pressure and blood flow in the clinical situation may in part result from its inhibitory effect on the release of glucagon, in addition to its direct vasoactive properties. Serotonin has a powerful vasoconstrictor effect on all vascular smooth muscle and is normally released into the portal circulation by the enterochromaffin cells of the gastrointestinal tract. Experimental evidence reveals that selective blockade of serotonin receptors reduces portal pressure thus providing evidence for the role of serotonergic mechanisms in the pathogenesis of portal hypertension.

At the time that the work for this thesis was undertaken, the role of prostacyclin in the aetiology of this phenomenon had not been elucidated. Similarly nitric oxide was not identified and studied until later in the 1980s and its role in the development of the haemodynamic circulation of portal hypertension continues to be investigated. Furthermore, at the time of these studies the central importance of endothelium as a modulator of basic vascular tone was not realised or implicated as perhaps the most important aetiological factor in portal hypertension.

1.5.1. Endothelial Function in Portal Hypertension

Over the last twenty years or so the importance of endothelium above and beyond its traditional role as a barrier between the circulation and the interstitial space has been recognised. Endothelium has a plethora of important physiological functions both metabolic and regulatory. Through balanced control of haemostasis and regulation of vascular tone and blood flow, endothelial function is of central importance to the normal function of all organs. This balance may be disturbed in many disease states, a condition currently known as endothelial dysfunction.

Endothelial dysfunction plays a pivotal role in the pathophysiology of portal hypertension. As soon as portal hypertension is triggered complex changes in the splanchnic and portal endothelium take place. Mechanical forces, namely those of increased pressure and shear stress are sensed by the endothelial cell by the process of mechano-transduction. Powerful vasoactive substances which affect coagulation and vessel tone by vasodilation and vasoconstriction, are released by the endothelial cell to act locally in a paracrine manner within the circulation and also by diffusion to act on vascular smooth muscle cells.

As the disease develops humoral factors also will affect the endothelium including those described previously. In addition agents produced in large quantities by the dysfunctional endothelium will act on other vascular beds in an endocrine manner. Endothelial dysfunction is manifest in the acute situation in disordered function, and in the chronic situation in long term changes to vascular structure.

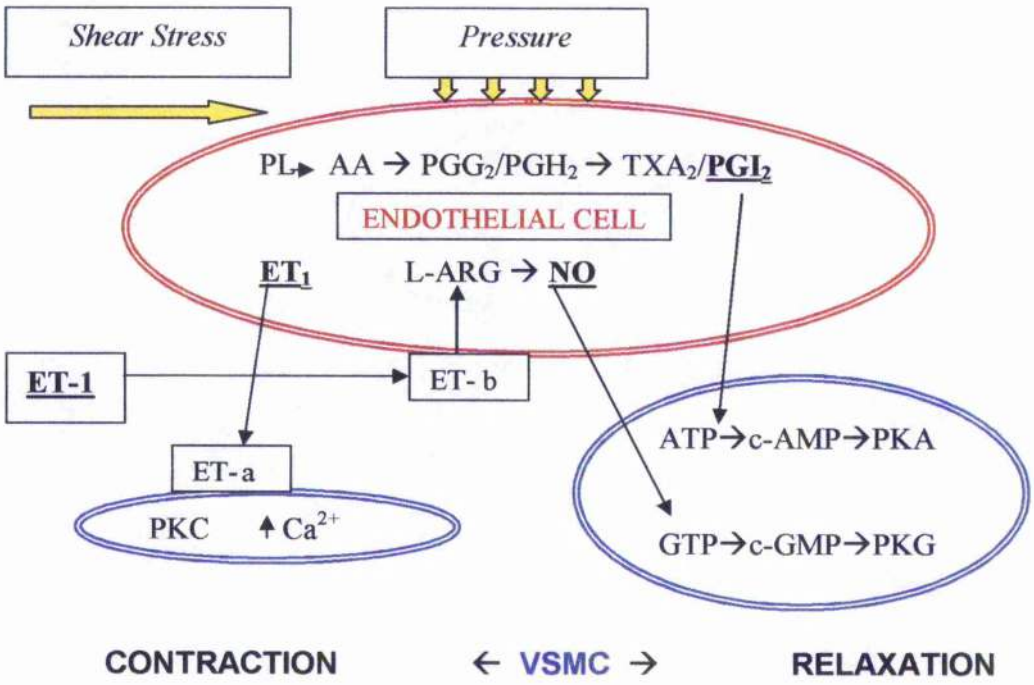


Fig 1.5.1 A simplified schematic of the important mechanisms of modulation of vascular tone by the endothelium:

Mechanical stimuli (shear stress & pressure) are sensed by mechano-transduction which triggers production of vasoactive substances PGI_2 and NO (vasodilators) and ET-1 (vasoconstrictor); these substances act locally in a paracrine manner on the underlying vascular smooth muscle cells (VSMCs) to induce relaxation of the vessel wall and vasodilation, or contraction and vasoconstriction. PGI_2 acts by increasing levels of c-AMP and protein kinase A (PKA): NO acts by raising levels of c-GMP and protein kinase G (PKG); endothelin acts by increasing intracellular Ca2+ and protein kinase C (PKC). 'Cross-talk' between ET-1, NO and PGI_2 takes place via the ET-b receptor and at several levels in both the endothelial and vascular smooth muscle cells.

1.5.1.1 Endothelium derived vasodilators

There are three major vasodilators produced by endothelium, these are prostacyclin, nitric oxide and endothelial-derived hyperpolarizing factor.

Prostacyclin (PGI_2)

PGI_2 is a major product of the cycloxygenase system (COX) in endothelial cells but is also produced in smaller quantities by the rest of the vessel wall. COX is now known to have two isoforms (COX-1 and COX-2) encoded by two separate genes. COX1 is expressed in many tissues including endothelial cells, while COX2 is not constitutively expressed but is fleetingly but rapidly induced in endothelial cells and vascular smooth muscle cells under the stimulus of both physical and pre-inflammatory actions. (Topper J N & Gimbrone M A Jr, 1999), (Marnett L J, et al, 1999). These interactions are described in more detail in chapter 2 (Prostacyclin) but essentially nitric oxide is also released by these same stimuli. The current consensus is that PGI_2 makes a smaller contribution to endothelial derived relaxation than nitric oxide but that these two substances act synergistically to inhibit platelet aggregation (Stoltz J F et al, 1999).

Nitric Oxide (NO)

NO is a labile gas produced in endothelial cells from L-Arginine by NO Synthase (NOS). Endothelial cells have two isoforms of this enzyme, inducible NOS (iNOS) which is calcium dependent, and constitutive NOS (eNOS) which is calcium independent and is abundantly released on induction by cytokines and endotoxin (Moncada S, 1999).

NO produced by endothelial cells diffuses into vascular smooth muscle cells stimulating soluble guanylyl cyclase in the cytosol. This leads to increased levels of cyclic GMP (cGMP), which cause relaxation and vasodilation.

Experimental evidence mainly in the rat model of portal hypertension using partial portal vein ligation, has indicated that nitric oxide is important in the development of portal hypertension. In man, increased levels of constitutive and inducible nitric oxide synthase, secondary to the raised level of endotoxins and cytokines found in cirrhosis, are proposed as a mechanism resulting in production of nitric oxide and thus vasodilatation. Many groups have implicated increased basal production of NO as the major factor in the pathogenesis of the portal hypertension. However, this mechanism remains unproved in patients with cirrhosis, with contradictory results on the circulation found when inhibitors of nitric oxide synthase were administered.

Whole-body production of NO is increased in portal hypertension and increased basal levels of eNOS are found in portal hypertensive animals (Cahill PA et al, 2001). The role of iNOS in portal hypertension is less clear

with most studies unable to localise this enzyme to the splanchnic circulation of portal hypertensive animals. Current work has focused on the primacy of the role of NO in the aetiology and maintenance of the hyperdynamic circulation and of portal hypertension. However, inhibition of NOS was shown not to completely inhibit production of a hyperdynamic circulation (Wu Y et al, 1993). There clearly is a much more complex interplay of vasoactive mediators at work in the pathogenesis of portal hypertension and the hyperdynamic circulation.

Recently endotoxins acting within the portal circulation have been implicated in the development of increased portal pressure. Endotoxin may play a role in stimulating iNOS induction in cirrhosis where portal endotoxaemia is common (Chu C J et al, 1997). A new hypothesis implicates endotoxin release from bacteria which may act by provoking oxidant stress and formation of reactive oxidant species which may be important in the pathophysiology of portal hypertension (Goulis J et al, 1999). Recent work performed within this department and under co-supervision by the author has provided evidence that the hyperdynamic circulation seen in portal hypertension can be prevented in the experimental situation by blocking oxidant stress using N-acetylcysteine (Fernando B et al, 1998).

Endothelial Derived Hyper-Polarising Factor (EDHF)

Endothelium dependent hyper-polarisation and relaxation of vascular smooth muscle is a vasodilator response to shear stress and humoral factors which is

different to that from NO and PGI2. (Campbell W B & Harder D R, 1999). The exact nature of EDHF is unknown but suspected to be potassium (K^+). This ion effluxes through K^+ channels on endothelial cells resulting in elevated myoendothelial K^+ concentrations which hyper-polarise the K^+ channels and stimulates Na^+/K^+ ATPase activity. Several studies indicate that mechanical and/or humoral stimulated EDHF release plays a significant role in modulation of the hyperaemia of portal hypertension. (Cahill et al, 2001). Its precise role in the aetiology of portal hypertension in relation to NO and PGI_2 function remains to be elucidated further, however.

1.5.1.2. Endothelium derived vasoconstrictors

The endothelium can cause vasoconstriction either by reduced production of vasodilators, or by release of diffusible vasoconstrictor substances such as superoxide anions which scavenge NO, thromboxane A_2 (TXA_2) and endothelin (Rubanyi GM. 1991). Several stimuli for endothelial mediated vasoconstriction have been identified including hypoxia, the humoral actions of acetylcholine, 5-hydroxy tryptamine and ADP, and most importantly the production of endothelin. In addition physical stimuli to the vessel wall such as pressure and stretch can cause vasoconstriction. Endothelin-1 (ET-1) is produced by endothelial cells and acts on local vascular smooth muscle cells via surface receptor ET-a causing increased intracellular CA^{2+} and vasoconstriction. Circulating ET-1 also acts on ET-b receptors situated on the endothelial cell surface to stimulate release of vasoactive substances such as NO and PGI_2 under normal physiological conditions. This vasoactive effect however may be reversed in particular vascular beds in pathological

conditions such as portal hypertension. Elevated circulating ET-1 levels have been found both in experimental portal hypertension and in human cirrhotics, and increased responsiveness to ET-1 has been found in experimental portal hypertension and cirrhosis. ET-1 may therefore have an important role in stellate cell contraction and in causing contraction of the portal vein and its intrahepatic branches thus causing increased portal resistance (Cahill PA et al, 2001).

A further possible effect is hyporeactivity to the action of vasopressors on the splanchnic circulation. There is experimental and clinical evidence for decreased responsiveness in portal hypertension and cirrhosis to vasoconstrictor substances such as endothelin, noradrenalin, angiotensin II, and vasopressin (Bendis L & Wong F. 2001). Thus the balance between endogenous vasodilators and vasoconstrictors could be adversely affected in different vascular beds and contribute to vasodilation and the production of a hyperdynamic circulation.

1.5.2. Summary

Considerable evidence now exists to support an important and central role for endothelial dysfunction in the pathophysiology of portal hypertension and the hyperdynamic circulation. The initial increase in pressure within the portal system causes a marked increase in mechanical load to the endothelial cell which can sense and respond to changes in pressure and shear stress by mechano-transduction. The production of vasoactive substances by the endothelium is up-regulated leading to over-production. Later changes

mediated by splanchnic vasodilation and imbalance between dilator and constrictor vasoactive substances result in permanently increased portal venous blood flow. The vasoactive substances produced in the splanchnic circulation are then delivered directly into the systemic circulation via the portosystemic collaterals which carry 80% of the portal blood flow in portal hypertension. These substances remain vasoactive in the systemic circulation, thus inducing a global status of endothelial dysfunction. In addition the underlying vessel wall changes its pressor response leading to a state of vascular hyporesponsiveness. Thus the peripheral systemic vasodilatation seen in these patients probably arises in most part from this 'spill-over' effect.

The plasma volume expansion which takes place in these patients probably follows directly from the vasodilation induced as described above. The proposed mechanism is one of response to the hypovolaemia induced by the dilatation of the systemic and splanchnic circulations. This results in a decreased central blood volume triggering the renin-angiotensin-aldosterone system. The resultant sodium and water retention causes blood volume expansion which, together with peripheral dilatation, results in the hyperdynamic circulation of portal hypertension.

1.6 The Natural History of Portal Hypertension

The natural history and prognosis of portal hypertension depends primarily on the underlying hepatic functional reserve. Conditions causing presinusoidal block but in which hepatocellular function remains good, carry the best

prognosis. Where hepatocellular function is very poor, the prognosis is dismal. A further factor is the extent of the hyperdynamic circulation in different vascular beds which may either increase or decrease as liver disease progresses. Portasystemic shunting generally increases with progression of liver disease as portal resistance also increases. In well compensated cirrhosis portal venous inflow is increased, however in end-stage decompensated cirrhosis portal blood flow may gradually decrease. This results from the high resistance of a fibrous, shrunken cirrhotic liver which together with increased shunting may eventually result in hepatofugal blood flow in 15% of such patients (Gaiani S et al 1991). Further decompensation of liver function results and it is typically in these patients that the severe complications of portal hypertension present.

The major lethal complication for all patients with portal hypertension is haemorrhage from oesophageal varices. In patients with cirrhosis the overall mortality rate from oesophageal variceal bleeding is about 40%. In patients who recover from the acute bleed, the risk of recurrent haemorrhage during the same hospital stay is 60% and this increases to more than 80% at two years. The long-term survival of these patients, therefore, is poor ranging from 6 to 35% at five years. It is important to emphasise, however, that only about 30% of patients with cirrhosis will ever experience such bleeding. The remainder will die of liver failure and infection.

As previously mentioned the outlook in cirrhosis is generally determined by the severity of associated hepatocellular disease. A commonly used

classification of the severity of liver disease was proposed by Child in 1964. This is based on clinical findings and liver function tests and classified into grades A (good liver function), B (moderate impairment of liver function), and C (poor liver function). This classification allows accurate prediction of prognosis with the A and B groups having 70-80% survival rates at one year after oesophageal variceal bleeding, while only 30% of patients in group C will survive for a year. During a major oesophageal bleed, as a result of hypovolaemia and reduced hepatocellular perfusion, there can be further hepatocellular deterioration and commonly a patient in a higher Child's classification can move down into grade C. Thus, variceal bleeding is not only an acute life-threatening event, but may cause acute hepatic decompensation such that a patient in a good prognostic group will enter a lower Child's grade.

1.6.1 Oesophageal Varices

Rupture of oesophageal varices, and less commonly of gastric varices, is the most dramatic complication of portal hypertension. Elegant anatomical studies have revealed in detail the complex and peculiar portasystemic connection which occurs in the distal 2-5 cm of the oesophagus precisely in the zone where varices develop (Kitano S et al, 1986).

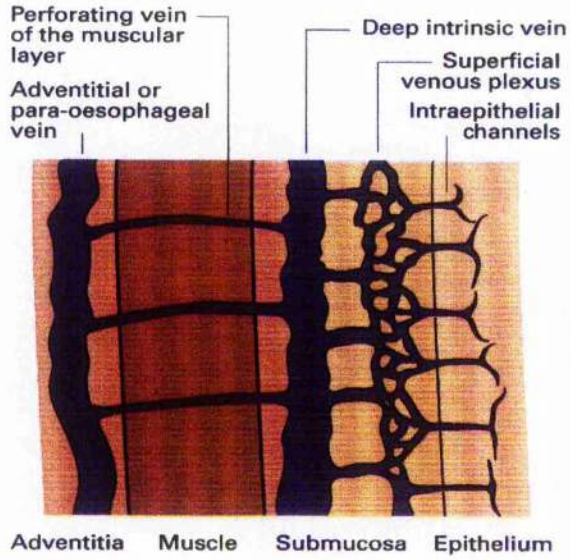


Fig 1.6 Venous drainage of the distal oesophagus: (from Hamilton.G, Aetiology of Portal Hypertension in Oxford Textbook of Surgery, 2nd Edition, Morris & Wood Eds. Oxford University Press, 2000).

Four distinct layers of veins have been found in this zone, extending from the luminal surface to the adventitial layer (fig 1.6). Intraepithelial veins or vascular epithelial channels drain into a superficial venous plexus, which lies just below the oesophageal epithelium. This plexus is in turn connected to a layer of deep intrinsic veins just outside the muscularis mucosae. These deep intrinsic veins connect with the outermost external venous plexus coursing within the oesophageal adventitia via the perforating veins which traverse the muscular layers of the oesophagus. Thus in this distal portion of the oesophagus, venous channels are concentrated largely in the mucosa with venous channels being present actually within the intraepithelial layer itself. In patients with portal hypertension this venous complex situated at the watershed between the portal and systemic circulations, dilates dramatically and forms varices classically running in 3-5 distinct trunks. The intraepithelial

channels dilate to form the cherry red spots which can be seen endoscopically and the presence of these is recognised as a predictor of impending rupture. Less severe variceal bleeds probably result from rupture of the intraepithelial channels while torrential haemorrhage results from rupture of the deeper sub-mucosal high flow intrinsic venous channels. Gastric varices bleed much less commonly probably because of their deeper sub-mucosal situation.

1.6.2 Rupture of Oesophageal Varices

Significant rupture occurs in 30% of patients and the causes remain poorly understood. Two main theories for variceal rupture are commonly advanced. The erosive theory postulates that mucosal damage due to reflux oesophagitis causes erosion into the varix and thus haemorrhage. Reflux oesophagitis is not commonly seen at endoscopy and control trials have found that H_2 blockade has no advantage over placebo in preventing recurrent variceal bleeding. Erosion of the oesophageal mucosa as a cause of rupture therefore seems unlikely.

The second theory – the eruptive theory – proposes that variceal rupture is related to pressure where the varix is in close proximity to the oesophageal lumen. Although increased portal pressure is required for variceal development, (>12mm/Hg), a direct correlation between portal pressure and risk of variceal bleeding has not been found. There is more circumspect evidence to support a relationship between the severity of portal hypertension and risk of variceal haemorrhage. Portal pressure in alcoholic patients following a variceal bleed was higher in those that did not survive. In addition

angiography and blood volume expansion both of which may increase portal pressure, occasionally precipitate haemorrhage. Variceal haemorrhage has also been reported to occur after deep inspiration, coughing and following the Valsalva manoeuvre, all of which cause an increased pressure differential between the oesophageal varix and lumen. Local factors such as variceal wall thickness and that of the overlying mucosa are obviously also of importance.

Variceal size has been reported to be a risk factor for haemorrhage, the larger varices being more likely to bleed. There are several studies relating frequency of haemorrhage to variceal size. Lebrec et al found increased frequency with larger varices, but there was no correlation of variceal size or frequency of haemorrhage with the degree of portal hypertension (Lebrec D et al, 1980). A study by Witzel et al found that varix size increased significantly with the severity of liver disease, and that frequency of haemorrhage also increased significantly with increasing size of varices, (Witzel L et al, 1985). These and other previous studies suggested that large varices are more likely to bleed than small ones, although clinical experience shows that there will be exceptions to this rule, (Baker LA et al, 1959 and Dagradi AE, 1972). The relationship between risk of bleeding from varices and portal pressure remains controversial. Benhamou has proposed that varices develop when portal pressure is more than 10-12mm/Hg over the IVC pressure but once developed, variceal size is not related to the degree of hypertension, (Benhamou JP, 1982). In 1982, Reynolds reviewed the evidence and found that although a few studies directly related higher portal pressure to bleeding,

many more failed to show any significant relationship. In these latter studies portal pressure was measured in patients with severe liver disease in a variety of ways ranging from wedged hepatic venous pressure to trans-hepatic portal pressure, and in all, portal vein pressure could not be found to predict the risk of bleeding, (Reynolds TB, 1982). The most relevant pressure measurement is that of intra-variceal pressure since there may not necessarily be a direct relationship with portal pressure. There are two studies where intra-variceal pressure was measured by direct puncture of the varix above the oesophogastric junction. The first study in 1956 showed no correlation between intra-varix pressure and bleeding risk, (Palmer AD et al, 1956). A study in the 80s by Westaby et al showed a close correlation between intra-abdominal portal pressure as measured by wedged hepatic venous pressure and the oesophageal varix pressure, and furthermore that rises in intra-abdominal pressure caused by direct compression, led to an increased gradient across the varix wall. This manoeuvre did not lead to any immediate haemorrhage, but this study did not address itself to risk of subsequent haemorrhage from variceal puncture because the patients underwent prophylactic injection sclerotherapy, (Westaby D et al, 1985). Of great interest is the fact that in both studies no significant bleeding resulted from needling of the varices. This supports the observation that pressure is indeed not important once the varix has developed, and perhaps coagulation in particular local factors may be of more importance in bleeding from varices.

The lack of relationship between pressure and variceal size reinforces the importance of local factors in varix development however. Polio and

Groszmann have proposed that variceal wall tension may be the unifying predictor of rupture (Polio J & Groszmann RJ, 1986). Tension in the variceal wall can be derived from a modification of Laplace's Law:

$$T=TP \times r/w$$

were T is tension, TP is transluminal pressure, r is vessel radius and w is wall thickness.

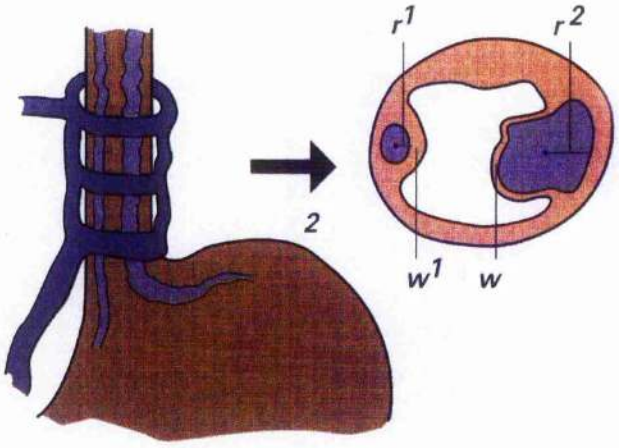


Fig 1.6.1 Variceal wall tension: variceal wall tension is greater where radius (r) is larger and the wall (w) is thinner despite equal transmural pressure (from Hamilton.G, Aetiology of Portal Hypertension in Oxford Textbook of Surgery, 2nd Edition, Morris & Wood Eds, Oxford University Press, 2000)

In oesophageal varices, transluminal tension is the difference in pressure between the oesophageal lumen and that of the varix. This tension is directly

related not only to this pressure difference, but also to the varix radius and is inversely related to variceal wall thickness. These three variables correspond in the clinical situation to variceal size, thickness of epithelium overlying the varix and degree of portal hypertension, all of which have been described as independent predictors of bleeding. When portal pressure is increased the more superficial protruding varix will have less supporting connective tissue and a larger radius than a deeper varix embedded in supporting tissue which is under identical pressure. Wall tension will therefore be higher in these superficial varices and rupture will result when this tension is no longer in equilibrium with the outwardly directed expansile force of portal pressure. When the elastic limit of the vessel has been reached small changes in volume or radius will result in large changes in wall tension and imminent rupture. Reduction of variceal pressure by shunting, disconnection or by pharmacological means will decrease transluminal pressure, vessel radius and wall tension. Sclerotherapy by causing dense perivariceal scarring, will increase the surrounding supporting tissue strengthening the variceal wall and thus decreasing tension.

Once rupture has occurred the severity of haemorrhage will be affected by haemodynamic factors and complicated by the disordered haemostasis that frequently accompanies liver disease. Portal hypertension is frequently complicated by disorders of blood coagulation and haemostasis. The liver plays a central role in these functions, consequently where there is severe compromise of hepato-cellular function, multiple and complex haemostatic defects are invariably present.

Hypersplenism is a common accompaniment to portal hypertension, and the resulting platelet abnormalities compound the coagulatory disorder. The liver is the major site of synthesis of at least 11 blood coagulation proteins, two components of the fibrinolytic system and anti-thrombin. In chronic liver disease significant derangement of haemostasis is most common in the more severe grades of hepato-cellular dysfunction, but with the exception of pro-thrombin, measurement of various components of haemostasis has failed to be useful in prediction of risk of haemorrhage,. Deficiency of the vitamin K dependent factors II, VII, IX and X results in abnormalities of both the extrinsic and intrinsic coagulation pathways and prolongation of the pro-thrombin, and partial thromboplastin times, (PT & PTT). Abnormal PT and PTT can be an early consequence of liver disease, even in the presence of minimally deranged liver function tests, and these tests remain the most useful screening and monitoring measurements for coagulation defects and liver disease. Administration of vitamin K and fresh frozen plasma will correct the PT and PTT in the majority of cases with improved clotting. A minority of patients however continue to bleed despite corrected or improved parameters and this area of deficient haemostasis in severe liver disease remains poorly understood. Thrombocytopaenia is commonly present in liver disease particularly in portal hypertension where hyper-splenism is common, (Toghill PJ et al, 1977). This is usually secondary to platelet pooling and sequestration within the spleen, but platelet survival is normal. In addition platelet function may be deficient in several chronic liver diseases, particularly so in alcoholic disease, (Thomas DP et al, 1967, Ballard HS et al, 1976 and Cowan DH, 1973). In this disease impaired platelet function with prolonged

bleeding times has been shown to be as a result of selective inhibition of TXA_2 synthesis and platelet aggregation, (Cowan DH, 1980, Mikhailidis DP et al, 1986 and Mikhailidis DP et al, 1983).

Another factor in the abnormal coagulation of liver disease is increased fibrinolytic activity. The underlying mechanism of this increased activity remains controversial, but may result from impaired clearance of circulating activators or be secondary to an underlying disseminated intra-vascular coagulation, (DIC), (Fletcher AP et al, 1964). DIC may result because of decreased plasminogen synthesis which has been reported to be low or normal in liver disease, or because of impaired hepatic clearance of activated coagulation productions, (Bloom AL, 1975 and Thomas DP et al, 1970). In portal hypertension, endotoxins absorbed from the gut in to the portal blood, may bypass the liver via porta-systemic collaterals thus entering the systemic circulation unaltered and possibly resulting in platelet aggregation and thrombin formation, (Wilkinson SP, 1977). The importance of endotoxaemia is being realised in recent studies but at the time of the commencement of the work for this thesis in 1980, the importance of endotoxin was not as well defined.

Patients with liver disease may show multiple disorders of haemostasis including deficient production by the liver of the coagulation proteins, thrombocytopaenia secondary to hypersplenism, impaired platelet function, and increased fibrinolytic activity. The relative importance of all these factors in the clinical situation remains unclear. Haemorrhage generally responds to

transfusion of whole, preferably fresh, blood and clotting factors such as fresh frozen plasma, cryoprecipitate and platelets. Empirically, haemodynamic factors may be most important in the development of rupture, while coagulatory factors may be of greater importance once profuse bleeding is established.

In 1979 when the experimental studies described in this thesis were first begun, the major vasodilatory, and more importantly platelet aggregation inhibitory effects of prostacyclin, raised the possibility that excess production of this prostaglandin might have an effect at the site of oesophageal variceal rupture promoting torrential haemorrhage.

CHAPTER 2 : PROSTACYCLIN

Prostacyclin (PGI_2) is a prostaglandin produced mainly by endothelial cells acting in a paracrine manner, and is a very powerful vasodilator and inhibitor of platelet aggregation. Most of this chapter describes what was known about prostacyclin during the years of my experimental and early clinical studies, that is from 1979-1985. Where relevant, up to date knowledge of prostacyclin is reviewed.

2.1 Historical Background

Prostaglandin was a derivative name given to substances initially found in seminal fluid in the 1930s which were found to cause contraction of human uterine muscle, (von Euler US, 1934). The exact nature of the substance began to be defined with advances into the field of lipid chemistry in the 1960s. Several decades before this, linoleic and arachidonic acids had been identified as essential fatty acids. In the 1960s it was established that arachidonate was an important link between the essential fatty acids and prostaglandins when biosynthesis of PGI_2 from aracidonic acid was achieved (Bergstrom S et al, 1964). Prostaglandins were then shown to be 20 carbon unsaturated carboxylic acids with a cyclopentane ring. By this time prostaglandins (PGs) were shown to be present in most body tissues indicating a fundamental role at the cellular level. This led to the proposal that the observed effects of essential fatty acid deficiency might be due to inadequate synthesis of prostaglandins.

The effect of prostaglandins on the circulation was first discovered in 1966 when a prostaglandin subsequently named PGE_1 inhibited ADP-induced platelet aggregation in vivo in the rat, pig and man. Furthermore, infusion of PGE_1 was shown to inhibit

the transient thrombocytopaenia which usually develops after intravenous infusion of ADP (Kloeze J, 1970). In 1973 Smith et al demonstrated that platelets produced stable prostaglandins and also that aspirin was able to inhibit selectively prostaglandin synthesis in human platelets, (Smith JB et al, 1973 and Smith JB et al, 1971). Since it was already known that aspirin inhibited platelet aggregation, the logical conclusion was made that prostaglandins produced by platelets were involved in the process of haemostasis, (Weiss HJ et al, 1968).

In 1973 platelets were shown to be generating several prostaglandins when aggregation was stimulated by addition of arachidonate, and in addition arachidonate was shown to stimulate release of a rabbit aorta contracting substance (RCS), (Vargaftig BB et al, 1973). Production of prostaglandins and RCS were shown to be suppressed by anti-inflammatory drugs such as aspirin. RCS had been previously described by Piper and Vane, (Piper PJ & Vane JR, 1969), as an unstable substance produced in anaphylaxis of isolated guinea pig lungs; it was thought to be an intermediate in prostaglandin synthesis and possibly the cyclic intermediate endoperoxide reported by Samuelsson et al in 1965, (Samuelsson B, 1965). Subsequently, prostaglandins G2 and H2 were produced on incubation of microsomes prepared from sheep seminal vesicles incubated with labelled arachidonic acid. Both of these substances were shown to stimulate platelet aggregation in vitro and to contract rabbit aortic strips, (Hamberg M et al, 1974). A new metabolite was identified in studies with labelled arachidonate incubated with platelets which led to the identification of thromboxane A_2 (TXA_2) (Hamberg M et al, 1974 and Hamberg M & Samuelsson B, 1974). TXA_2 was shown to be a very potent platelet aggregatory agent and vasoconstrictor and also the active component in

rabbit aorta contracting substance, (Hamberg M et al, 1975).

Moncada and Vane studied tissues other than platelets which might produce this substance and while looking at blood vessel homogenates found an unknown prostaglandin which had potent vasodilatory and platelet inhibitory properties which was then named PGX, (Moncada S et al, 1976[a] and Bunting S et al, 1976). At about the same time, other workers independently found this vasodilatory substance PGX on incubation with arachidonate with intact blood vessel preparation (Kulkarni PS et al, 1976 and Raz A et al, 1977). PGX was found to have a striking ability to inhibit platelet aggregation being 20-30 times more potent than PGE_1, and 5-10 times more potent than PGD_2 making it the most powerful naturally occurring anti-aggregatory substance known at that time, (Moncada S et al, 1976[b]). It was found to be highly unstable with its anti-aggregatory activity disappearing after standing for 20 minutes at 22°C in tris-buffer or within seconds after boiling. Its activity could be prolonged by storage at 0°C or at pH10 and preserved by several days if dissolved in dry acetone and stored at -20°C. Its chemical structure was identified as 9-deoxy-6, 9-alpha-epoxy-delta 5 PGF_1 alpha and was finally named prostacyclin or PGI_2, (Whittaker N et al, 1976). This identified PGI_2 as the main metabolite of arachidonic acid in vascular tissues. PGI_2 was probably the same substance identified in 1973 by Saba et al from human umbilical endothelial cells (Saba SR et al, 1973). Pace-Asciak had also previously described a compound called 6-keto PGF_1 alpha which was found to be a product of prostaglandin metabolism in rat stomach, (Pace-Asciak C et al, 1971). Subsequently this substance was confirmed to be the main hydrolytic product of the unstable compound PGI_2.

2.2 Biosynthesis of PGI_2

2.2.1. Release of arachidonate from membrane phospholipids

Arachidonic acid is a polyunsaturated fatty acid which is present in cell membrane phospholipids and other cellular lipids, and is the precursor substrate for all prostaglandins including PGI_2 and TXA_2. Its release is initiated by either mechanical or biochemical stimulation of the cells by the action of phospholipase C and phospholipase A2 and is calcium dependent, being initiated by a rise in intracellular Ca^{2+} (Lands WE, 1979, Bell RL & Majerus PW, 1980 and Broekman MJ et al, 1980).

2.2.2 Synthesis of PGI_2 from arachidonate

There are three pathways for the metabolism of released arachidonic acid, the lipoxygenase, cycloxygenase, or cytochrome P450-dependent epoxygenase mediated pathways. Cycloxygenase converts arachidonate or other 20 carbon chain unsaturated fatty acids into cyclic endoperoxides (prostaglandins and thromboxanes), lipoxygenase converts to the leukotrienes, and cytochrome P450 epoxygenase forms epoxyeicosanoids (Quiroga J, & Prieto J. 1993). These essential fatty acids contain 3, 4 or 5 double bonds which will be metabolised and designated accordingly into groups of prostaglandins namely PG1 (one double bond), PG2 (two double bonds) or PG3 (three double bonds).

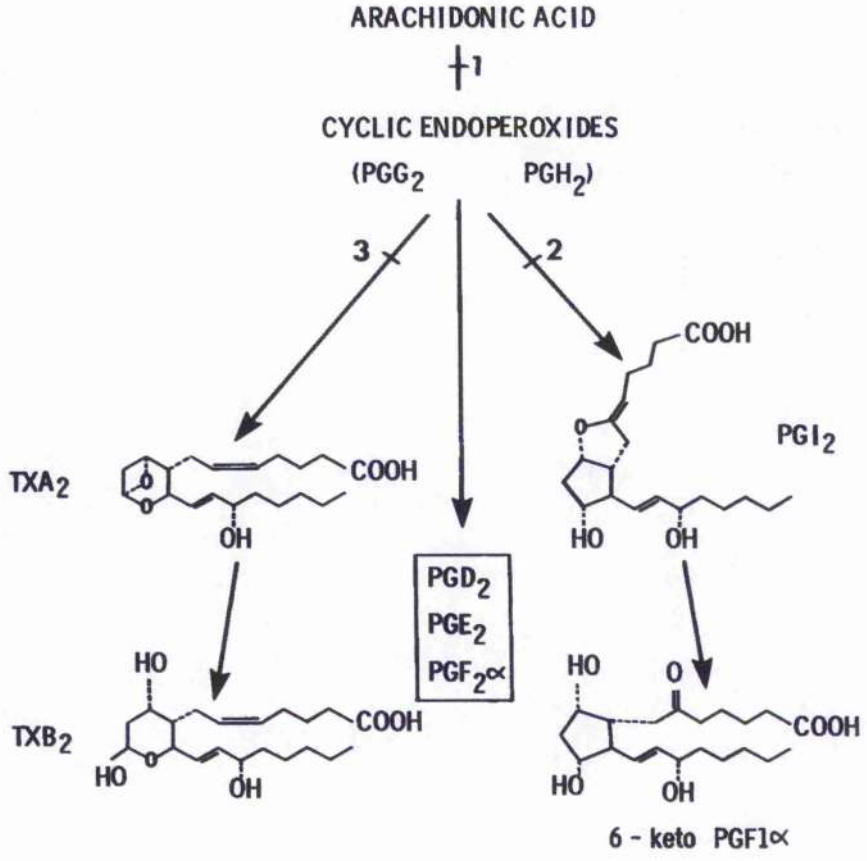


Figure 2.2 Synthesis of prostanoids from arachidonic acid: Arachidonic acid is mobilised from phospholopid pools by the action of phospholipase2; COX-1 and COX-2 act to convert arachidonic acid to cyclic endoperoxides (1): Thromboxane synthase acts at (3) to form TXA_2 and prostacyclin synthase acts at (2) to form PGI_2; the stable end products TXB_2 and 6-keto $PGF_1\alpha$ are formed by hydrolysis.

Cycloxygenase stimulated transformation of arachidonate into PGH_2 is irreversible and is therefore a potentially rate limiting step in prostaglandin production (Thuresson ED et al, 2000). The endoperoxides PGG_2 and PGH_2 thus formed are mainly converted by prostaglandin synthetase in the endothelial cells into PGI_2 or in the platelet by thromboxane synthetase in to TXA_2.

In vitro it was demonstrated that exogenous endoperoxides could be taken up by

endothelial cells and metabolised into prostaglandins such as PGI_2 which could then be released to the exterior (Marcus AJ et al, 1978). This raised the possibility that activated platelets might release endoperoxides which could then be metabolised by endothelial cells to form PGI_2 and thus limit propagation of thrombus formation. Several studies in 1979 however, led to the conclusion that under normal physiological circumstances platelets do not constitute a significant source of endoperoxides for PGI_2 synthesis. The main source of endoperoxides is therefore endogenous from the arachidonic pool of the endothelial cell, (Baenziger NL et al, 1979, Needleman P et al, 1979 and Hornstra G et al, 1979).

PGI2 is a major product of the cycloxygenase system (COX) in endothelial cells but is also produced in smaller quantities by the rest of the vessel wall. COX is now known to have two isoforms (COX-1 and COX-2) encoded by two separate genes. COX-1 is expressed in many tissues including endothelial cells, while COX-2 is not constitutively expressed but is fleetingly but rapidly induced in endothelial cells and vascular smooth muscle cells under the stimulus of both physical and pre-inflammatory actions. (Topper J N, Gimbrone M A Jr, 1999) (Marnett L J, et al 1999). Their products are the same catalyzing a two step reaction producing first PGG_2 then PGH_2. COX-1 is expressed on the endoplasmic reticulum of all cells and is responsible for the constitutive production of prostaglandins in most tissues including platelets. COX-1 dependent prostaglandin production is currently thought to serve physiological housekeeping functions in particular cytoprotection of gastric mucosa, modulation of renal function and production of TXA_2 in the platelet. Its concentration remains relatively stable. COX-2 is virtually undetectable in normal conditions but is induced by cytokines, endotoxins, growth factors or tumour

promoters (Smith WL, Langenbach R. 2001). Thus this inducible conversion of arachidonate to PGH_2 by COX-2 in various pathophysiological settings provides an additional rate limiting step in prostanoid biosynthesis.

In addition to inflammatory modulators mechanical forces, in particular shear stress, are now known to induce COX-2 in vascular endothelial cells (Topper and Gimbrone 1999). Thus increased expression of COX-2 (which is a rapidly occurring phenomenon) is being increasingly implicated in the pathophysiology of several disease processes and indeed may play an important role in the pathogenesis of portal hypertension.

The final step in synthesis of PGI_2 and TXA_2 from PGH_2 involves two similar isomerases, PGI_2 synthase and TXA_2 synthase. These act by a haeme-thyolate mechanism to rearrange the PGH_2 molecule to form the two important vasoactive prostanoids with opposing biological properties, PGI_2 and TXA_2 (Tanabe T, Ullrich V. 1995).

2.2.3. Sites of synthesis of PGI_2

Moncada et al studied the differential production of PGI_2 by different layers of rabbit aorta. It was shown that intima had the greatest PGI_2 activity, the media was intermediate and the adventitia had the least activity, (Moncada S et al, 1977[a]). On quantification of this activity, the intima, although only 5% by weight, was found to produce 40% of the total PGI_2. Later cultured human umbilical vein endothelium and bovine aortic endothelium were shown to produce PGI_2, as were porcine endothelium and smooth muscle cells, (Weksler BB et al, 1977 and Macintyre DE at

al, 1978). Also cultured endothelial cells produced more PGI_2 than cultured smooth muscle cells, (Baenziger NL et al, 1979 and Baenziger NL et al, 1977). In a seminal experiment, Eldor et al demonstrated cessation of PGI_2 production from the luminal surface of rabbit aorta after stripping of the endothelium with a balloon catheter. As a new intima of medial smooth muscle cells developed however, so the capacity for PGI_2 production returned and once fully developed the smooth muscle cells produced as much PGI_2 as normal endothelium, (Eldor A et al, 1981).

Other vessels were subsequently studied, with placenta and umbilical vessels in particular demonstrated to have a very high capacity to synthesise PGI_2. Arteries were shown to produce more PGI_2 than veins on a weight per weight basis, (Johnson AR, 1980). Other cells of non-vascular origin such as macrophages, fibroblasts, mesothelial cells, renal glomerular and gastric fundal mucosal cells were shown also to produce PGI_2 but with no conclusive studies of portal or mesenteric endothelial PGI_2 activity being reported at the time of commencement of the studies to be described in this thesis.

2.2.4 Metabolism of prostacyclin

At physiological pH and temperature, PGI_2 is a very unstable molecule, its hydrolysis being inversely proportional to the pH of its environment. At a pH of 7.55, PGI_2 has a half-life of 3 minutes in buffer solution, but as a result of albumen binding its half-life is greater than 20 minutes in plasma or whole blood at normal pH, (Pifer DD et al, 1981 and Wynalda MA et al, 1980). PGI_2 is at its most stable at an alkaline pH (>9) and at lower temperatures. In physiological media, PGI_2 is rapidly hydrolysed to an inactive derivative 6-keto $PGF_1\alpha$, (Pace-Asciak C et al, 1971). In vivo studies in

animals have shown that PGI_2 metabolism follows metabolic pathways similar to other prostanoids. The in vivo metabolism in humans has been studied by measuring the effect of bolus infusions of PGI_2 or 6-keto $PGF_1\alpha$ into volunteers, (Myatt L et al, 1981). These studies showed that PGI_2 was mainly hydrolysed to 6-keto $PGF_1\alpha$ with a minor degree of dehydrogenation by 15-hydroxyl-PG-dehydrogenase (PGDH) in the lungs. This enzyme inactivates 90% of primary prostaglandins after a single passage through the lungs but PGI_2 survives pulmonary transit without any loss of biological activity, (Ferreira SH & Vane JR, 1967, Dusting GJ et al, 1978 and Bolger PM et al, 1978). In the late 70s, it was reported that the lung released PGI_2 into the arterial circulation leading to the suggestion that PGI_2 might be a circulating hormone (Gryglewski RJ et al, 1978[a], Hensby CN et al, 1979[a] and Moncada S et al, 1978[b]). This proposal, highly contentious and debated at the time, has since been refuted based on later accurate measurements of 6-keto $PGF_1\alpha$ in human blood.

The major clearance of prostaglandins including PGI_2, is by the liver but also by the kidneys and skeletal muscle, (Dusting GJ et al, 1978). There are many urinary metabolites including 2, 3 dinor 6-keto PGF_1 alpha and 6, 15 diketo, 13-14 dihydro, 2-3 dinor $PGF_1\alpha$, (Bizzi A et al, 1977 and Blackwell GJ et al, 1977)

2.2.5. Regulation of PGI_2 synthesis

PGI_2 synthesis is primarily regulated in a rate limiting manner by the availability of arachidonic acid, the preferred fatty acid substrate, by the induction of COX-2 and also by a variety of materials and drugs. COX-2 in turn, is inhibited by glucocorticoids and by the recently available COX-2 inhibitor drugs. In this review

some of the more important factors are described in the regulation of PGI2 regulation which at present remains puzzling but undoubtedly forms part of a complex, highly efficient system of regulatory pathways in cellular function.

Thrombin induces PGI2 production in human endothelial cells suggesting that this might be a physiological mechanism by which the growth of thrombus at the site of a vascular injury might be limited, (Czervionke RL et al, 1979 and Weksler BB et al, 1978). A range of vasoactive substances including bradykinin, histamine, angiotensin II and serotonin induce PGI_2 synthesis from endothelial cells. Histamine has a potent effect on endothelial cell synthesis of PGI_2 occurring at concentrations found during acute allergic reactions. This suggests that this mechanism may play a role in the histamine mediated allergic reactions such as hypotension, (Baenziger NL et al, 1980 and Baenziger NL et al, 1981). Platelet activating factor (PAF) acting via increasing calcium influx has been shown to prime the action of histamine and thrombin in increasing PGI_2 production from human umbilical vein endothelial cells in culture (Weber C, et al 1993). Angiotensin II similarly has a potent stimulatory effect but adrenaline and non-adrenaline do not, (Gimbrone MA, Jr & Alexander RW, 1975, Nolan RD et al, 1981). Serotonin induces synthesis of PGI_2 in smooth muscle cells and its action is potentiated by PDGF, (Coughlin SR et al, 1980 and Coughlin SR et al, 1981). Other plasma substances such as lipoproteins will affect PGI_2 synthesis with high-density lipoprotein (HDL) stimulating, and low density lipoproteins (LDL) suppressing, PGI_2 synthesis in vascular tissue culture, (Beitz J & Forster W, 1980, and Evensen SA, 1979).

Tumour necrosis factor $\propto$ (TNFα) has recently been shown to potentiate angiotensin

II induced PGI_2 synthesis in vascular smooth muscle cells (Askari B, Ferrerib NR. 2001). Both TNFα and interleukin-1 induce slow release of PGI_2 from cultured endothelial cells and this mechanism has been implicated as factor in the hypotension of septic shock (Vane JR, Botting RM. 1995). Human lymphocytes have also been shown to act on cultured human endothelial cells by stimulating PGI_2 production (Merhi-Soussi F, et al 2000).

Superoxide anions ($O_2^{\cdot-}$) are produced by endothelial cells as part of oxygen derived free radical formation and are generally inactivated by scavenging enzymes such as superoxide dismutase. Superoxide anion interacts with nitric oxide very rapidly to form peroxynitrite ($ONOO^-$) which may not only reduce levels of nitric oxide but also powerfully inhibits synthesis of prostacyclin by inhibition of PGI_2 synthase (Katusic ZS. 1996). Increased production of superoxide anion has been implicated in several arterial disease states such as hypertension, resulting in vasoconstriction as a result of the inhibition of both nitric oxide and prostacyclin. There is no data to confirm whether this reaction plays a role in portal hypertension but it is implicated as a factor in endothelial dysfunction (Ullrich V et al 2001). Nitric oxide in the presence of increased superoxide may also therefore be involved in the regulation of prostacyclin synthesis.

Prostacyclin release is significantly stimulated by mechanical forces such as transmural pressure, wall tension and shear stress. In recent years prostacyclin production has been shown to increase biphasically after shear stress loading of HUVECs. This probably results from an initial increased arachidonic acid release and a combination of increased expression of both COXs and PGI_2 synthase (Okahara K, et al 1998).

Current knowledge of the regulation of PGI_2 and TXA_2 synthesis is revealing a most complex interaction involving growth factors, cytokines, other eicosanoids and lipid mediators. At the heart of this complexity is the regulatory coupling of PGI_2 – TXA_2 which is implicated in several disease states. However, in 1980 when the studies to be described were carried out, the regulation of PGI_2 synthesis was poorly understood but recognised to be multi-factorial and complex.

2.3 Pharmacology of PGI_2

The most striking properties of PGI_2 are its potent inhibition of platelet aggregation and its vasodilatory activity (Moncada S et al, 1976a). Its ability to inhibit platelet adhesion and to disaggregate platelets and disperse platelet clumps both in vitro and in the human circulation, has long been recognised (Moncada S et al, 1978[a]) (Szczeklik A, et al 1978). It remains the most potent inhibitor of platelet aggregation yet discovered, much more potent and longer lasting than PGE_1 or PGD_2.

In 1979 receptors had been demonstrated on platelets and red blood cells with the receptor sites on platelets being shared with PGE_1, (Siegl AM et al, 1979 and Miller OV et al, 1979). The nature of these receptors has been further studied particularly with the introduction of TXA_2 synthase blockers which surprisingly showed no inhibition of platelet aggregation. Prostanoid receptors belong to a group of seven-loop transmembrane spanning G-protein coupled receptors. TXA_2 has two receptors but so far only one receptor for PGI_2 has been found and is named the IP-receptor (Ullrich V et al, 2001).

2.3.1. PGI_2 and cyclic AMP

Acting on these receptors, agents which inhibit platelet aggregation increase cAMP,

and agents which stimulate platelet aggregation decrease cAMP levels, (Haslam RJ et al, 1972, Weiss A et al, 1978, Mills DC et al, 1971 and Salzman EW et al, 1971). These observations led to the hypothesis that the biological effects of PGI_2 resulted from stimulation of intracellular adenylate cyclase with subsequent elevation of cAMP levels. This hypothesis was supported when it was shown that PGI_2 was the most potent inducer of platelet cAMP production and at least 10 times more potent than PGE_1, (Gorman RR et al, 1977 and Tateson JE et al, 1977). Inhibitors of adenylate cyclase activation were also shown to prevent the biological actions of PGI_2, (Haslam RJ et al 1978). PGI_2 was shown directly to stimulate adenylate cyclase activity in platelet microsomes causing elevation of cAMP levels, activation of protein kinases and the phosphorylation of other proteins with the direct consequence of inhibition of aggregation (Best LC et al, 1977). It has also been shown to stimulate adenylate cyclase in endothelial cells and similarly PGI_2 has a much more potent effect than PGE_1 or other prostaglandins, (Gryglewski RJ et al, 1978[c]). PGI_2 has also been shown to stimulate cAMP levels in many other types of cells including fibroblasts and arterial rings (Claesson HE, 1980 and Baenziger NL et al, 1980).

The mechanism by which PGI_2 stimulated elevation of cAMP affects platelet aggregability is based on intra-cellular calcium signalling. When TXA_2 is formed in platelets during activation, calcium irons are released from bound intra-cellular sites and are then bound to calmodulin to form the calcium/calmodulin complex. This complex then stimulates phospholipase A2 activity and activates myosin kinase. The activated myosin kinase phosphorylates myosin, which interacts with actin resulting in platelet contraction and the release reaction, (Hathaway DR et al, 1979).

Elevation of cAMP by PGI_2 causes calcium sequestration, decreasing phospholipase A2 activation and release of arachidonate, and activating a cAMP dependent protein kinase. This kinase phosphorylates myosin kinase, thus decreasing phosphorylation of myosin, reducing actin-myosin interaction, and inhibiting the release reaction, (Hathaway DR et al, 1979).

2.3.2 PGI_2 and de-aggregation of platelets

PGI_2 has the ability to de-aggregate platelets initially demonstrated by Moncada et al, who showed reversal of human platelet aggregation by PGI_2 incubated with aortic microsomes, (Moncada S et al, 1977[b]). This activity has also been demonstrated in vivo in man where PGI_2 infusion decreased the percentage of circulating platelet aggregates formed, (Gryglewski RJ et al, 1978[b]).

2.3.3 PGI_2 and platelet adhesion

PGI_2 has been shown to inhibit platelet adhesion but only occurring at much higher concentrations (>100 nM) than those required for inhibition of platelet aggregation (Higgs EA et al, 1978, Weiss HJ et al, 1979 and Adelman B et al, 1981). PGI_2 has also been shown to inhibit changes in platelet shape induced by ADP or arachidonate, and indeed it has been suggested that this might be the mechanism by which platelet adherence is decreased, (Ehrman ML et al, 1980 and Kinlough-Rathbone RL et al, 1970). However, the concentration of PGI_2 required to inhibit adhesion is much higher than that for aggregation and is much greater than that found under normal physiological conditions. It is therefore likely that inhibition of platelet adhesion by PGI_2 is not physiologically important in the circulation but it may be physiologically important at the level of the platelet endothelial cell interface

where the concentrations of PGI_2 are potentially much greater.

2.3.4 PGI_2 and vascular smooth muscle

PGI_2 is a potent vasodilator relaxing vascular smooth muscle in all mammalian species tested. The degree of vasodilation varies with different vascular beds and species but both large and small vessels are affected. The exact mechanism by which PGI_2 achieves this affect was unclear at the time of the study, but was felt to be related to changes in cAMP. It had previously been thought that increases in cAMP levels caused smooth muscle cell relaxation while increased cGMP or decreased cAMP levels caused contraction, (Andersson R et al, 1972, Broekman MJ et al, 1980 and Poch G et al, 1972). However, other studies have shown that smooth muscle relaxation is not dependent on raised cAMP levels, (Collins GA et al, 1975, Diamond J et al, 1975 and Uruno T et al, 1973). PGI_2 like other prostaglandins may have a biphasic action on cAMP production dependent on its concentrations. PGI_2 has been shown to have a duel effect on isolated human ovarian veins with low concentrations causing depression of muscle tone but higher concentrations causing increased tone and inducing phasic contractile cycles, (Borda ES at el, 1979). So a direct relationship between PGI_2 induced changes and cAMP and smooth muscle contractivity could not be demonstrated and at the time of this work, it was thought that PGI_2 acted by altering the levels of cyclic nucleotides thus in turn controlling intra-cellular free calcium iron fluxes, (Andersson R et al, 1972). It is now recognised that Ca^{2+} mediated phosphorylation of myosin light chain is central to relaxation and contraction of vascular smooth muscle (Walsh MP. 1994). Increased intracellular Ca^2 above basal levels causes formation of the Ca^2/calmodulin complex which binds with myosin light chain kinase, enabling

phosphorylation of myosin light chain subunits. Actin-myosin cross-bridging results causing shortening of the muscle fibres and vasoconstriction. Removal of intracellular Ca^2 causes the to Ca^2/calmodulin complex to dissociate, reversing the above process by dephosphorylation of myosin light chain subunits, resulting in muscle relaxation and vessel dilation.

In the clinical situation, infusion of PGI_2 causes rapid cutaneous flushing, lowers mean arterial pressure by lowering diastolic pressure, and causes vasodilatation of coronary, coeliac and mesenteric arterial beds, (O'Grady J et al, 1980). These effects however occur at very high circulating levels of PGI_2, which are not present in the physiological situation. Thus, on the evidence available at the time of this study, the contribution of PGI_2 to normal vascular tone was not clear.

2.4. Prostaglandin Production in Disease

There is substantial evidence that PGI_2 production by vascular tissue may be altered in various disease states, and that increased or decreased production is associated with a range of diseases including chronic renal disease and atherosclerosis.

Venous stasis and mild ischaemia have been shown to increase PGI_2 production and release. This had been demonstrated primarily after temporary peripheral venous occlusion with a sphygmomanometer and also in veins after tourniquet ischaemia for total knee replacement, (Serneri GG et al, 1980 and Zahavi J et al, 1980). In both studies there was a rapid fall in production after release of occlusion. In 1979 a plasma factor was found in patients with uraemia and hepatic failure which stimulated PGI2 release from rat aortic slices and in a separate study a factor was

found in uraemic plasma which stimulated normal endothelial cells to produce PGI_2, (Van Hoof A et al, 1979 and Defreyn G et al, 1980). In acute and chronic uraemia, vascular tissue was found to have increased PGI_2 activity leading to the suggestion that this might be a factor in the severe haemostatic deficiency found in renal failure, (Remuzzi G et al, 1978[a]). This effect of uraemia on PGI_2 production by endothelial cells has been confirmed in vessels from rats with experimentally induced uraemia, (Silberbauer K et al, 1978).

A congenital deficiency of thromboxane synthetase has been demonstrated in three generations of a family with consequent redirection of arachidonic metabolism to formation of PGI_2 and PGD_2. All affected members were found to have prolonged bleeding times with defective platelet function, (Defreyn G et al, 1981). Familial cycloxygenase deficiency has also been reported characterised by a bleeding tendency but paradoxically in this case, both PGI_2 and TXA_2 production were deficient, (Pareti FI et al, 1980).

Bartter's syndrome, (hypertension, hypokalaemia, elevated plasma renin and aldosterone secretion, diminished response to pressor agents), was a focus of a great deal of attention when these patients were shown to have increased urinary 6-keto PGF_1 alpha excretion (Gill JR et al, 1976). In these patients it was shown that there was a plasma factor which had PGI_2 like inhibition of platelets, (Gullner HG et al, 1979 and Jackson EK & Goodman RP, 1981). Treatment with indomethacin, an inhibitor of prostaglandin synthetase, successfully corrected both the blood pressure and platelet aggregation abnormality, (Stoff JS et al, 1980).

Pace-Asciak et al reported increased PGI_2 production by aortic rings from spontaneously hypertensive rats in whom an increased incidence of stroke was shown when PGI_2 synthesis was blocked, (Pace-Asciak C et al, 1976 and Okuma M et al, 1979). In man decreased 6-keto $PGF_1\alpha$ and PGE_2 have been reported in essential hypertension but whether this represents production of these metabolites by the kidney or urinary excretion of metabolites from systemic vascular production is not clear, (Grose JH et al, 1980, Tan SY et al, 1978).

Decreased PGI_2 production appears to be present in arteriosclerosis. In experimentally induced arteriosclerosis in the rabbit, PGI_2 synthesis is decreased in aortic rings, (Dembinska-Kiec A et al, 1977, Sinzinger H et al, 1979 and Gryglewski RJ et al, 1978[c]). In experimental hypercholesteraemia the time taken by aortic neo-intima formed after endothelial trauma to resume PGI_2 synthesis was prolonged when compared to normal (Eldor A et al, 1982). In man both LDL and lipid peroxides similar to those found in atherosclerotic plaques have been shown to inhibit PGI_2 synthesis in normal endothelium, (Nordoy A et al, 1978). In diabetes mellitus reduced PGI_2 synthesis has been demonstrated in man and also in experimentally induced diabetes in rats (Silberbauer K et al, 1979, Davis TM et al, 1981, Harrison HE et al, 1978 and Carreras LO et al, 1980). In diabetic patients, TXA_2 synthesis by platelets has also been shown by several workers to be increased with plasma 6-keto $PGF_1\alpha$ levels appearing to be normal or slightly decreased, (Davis TM et al, 1981, Johnson M et al, 1979, Davis TM et al, 1979 and Halushka PV et al, 1981).

In pre-eclampsia and chronic placental insufficiency syndromes in general, PGI_2 production by the placenta, umbilical artery and the foetus is reduced, (Downing I et

al, 1980, Remuzzi G et al, 1980[a], Remuzzi G et al, 1980[b] and Stuart MJ et al, 1981). Deficiency of a plasma factor stimulating PGI_2 production has been reported in the haemolytic uraemic syndrome and in thrombotic thrombocytopaenic purpura, (Remuzzi G et al, 1978[b], Webster J et al, 1980, Hensby CN et al, 1979[b], Remuzzi G et al, 1979, Jorgensen KA et al, 1981, Wiles PG et al, 1981 and Chen YC et al, 1981). Reduced PGI_2 activity had also been reported in systemic lupus erythematosus, (McVerry BA et al, 1980).

Prostacyclin has been used as a therapeutic agent in several disease states with some success. Prostacyclin infusion (or inhalation) successfully lowers pressure in patients with primary pulmonary hypertension, can significantly improve rest pain in patients with end-stage peripheral vascular disease and relieve the symptoms of Raynaud's disease (Vane RJ and Botting RM, 1995). This therapeutic success in disorders where endothelial dysfunction is a central feature suggests that prostacyclin production is impaired in these disease states.

Thus at the time of this study, there was a considerable volume of data implicating abnormalities of PGI_2 production in vascular and other diseases. There had been very little work on the role of PGI_2 in liver diseases and in particular in hepatic failure and portal hypertension. These observations and other disease states led to the hypothesis to be tested in this thesis.

HYPOTHESIS TO BE TESTED

Alterations in PGI_2 production in portal hypertension may contribute not only to the pathophysiology of portal hypertension but also to deficient haemostasis resulting in the catastrophic haemorrhage which so commonly complicates oesophageal varices.

CHAPTER 3: MEASUREMENT OF PROSTACYCLIN

3.1 Introduction

Because of the evanescence and production in small quantities of most prostanoids, their measurement in the 1970s and 1980s, posed particular difficulties. PGI_2's very short half- life made assay of this prostanoid even more problematical.

PGI_2 was initially assayed by measurement of its biological effects, namely relaxation of vascular smooth muscle and inhibition of platelet aggregation. There are two commonly used applications of these properties in PGI_2 assay. In the cascade perfusion assay PGI_2 is detected by its effect on smooth muscle contractility when continuously perfused with blood or perfusate. Platelet nephelometry on the other hand, measures PGI_2 by the effect of tissue extracts or perfusates on platelet aggregation. More recently, PGI_2 has been assayed by physico-chemical rather than biological methods, namely radio-immunoassay (RIA) and gas chromatography/ mass spectrometry (GC/MS). Both techniques measure 6-keto $PGF_{1\alpha}$ the stable breakdown product of PGI_2.

Over the duration of the study, different assays were used as they became available. Initially a bio-assay was used, then RIAs were developed and applied and in the final part of the study plasma measurements were made using GC/MS. In this chapter the development and application of these assays to PGI_2 measurement are described. With the exception of the GC/MS measurements, this work took place in the Katharine Dormandy Department of Haemophilia at the Royal Free Hospital.

I was fortunate to have access to measurement of PGI_2 working with Dr Rose Chow (who at that time was a Research Scientist in the department) and under the direct supervision and direction of Dr Ron Hutton, Principal Biochemist and expert in

haemostasis.

3.2 Bioassays

3.2.1 Cascade Perfusion

Superperfusion of tissues is a widely used technique for the bioassay of biologically active substances and it was indeed with this technique that Vane first detected PGX or PGI_2, (Bult H et al, 1977 and Vane JR, 1969). The basis for this assay is the smooth muscle relaxing or contracting effect of prostanoids on various animal tissues. PGI_2 characteristically contracts a range of tissues such as rat stomach strip, but relaxes vascular tissue such as rabbit aorta, (Moncada S et al, 1976[a]). This assay's great advantage is the ability to make continuous and instantaneous measurement of a perfused tissue's production of PGI_2. There are several major disadvantages however. Identification of PGI_2 is dependent on an appropriate selection of a range of tissues to be perfused, which will respond in a manner particular to PGI_2, and in order to exclude possible interference from other vaso-active substances appropriate antagonists need to be used. Furthermore accurate measurement of absolute concentrations was not possible. For all these reasons, plus the lack of expertise at the Royal Free Hospital or the Royal Free Hospital School of Medicine in this technique, cascade superfusion was not used to assay PGI_2.

3.2.2 Platelet Nephelometry

This assay exploits the principle that the optical density of platelet rich plasma (PRP) will decrease as platelets aggregate. Born first applied this concept in the development of the platelet aggregometer, (Born GVR, 1962). A modern version of this machine, the Payton dual channel aggregometer, was used in these

experiments. The study was based on methods described and validated by Moncada et al, (Moncada S, 1977[b]). Essentially, the fall in optical density of a test solution containing a fixed amount of platelets after addition of an aggregatory agent is compared to the maximum fall in density occurring in a buffer containing control (fig 3.2.2). The amount of PGI_2 in the sample is calculated by comparing the percentage inhibition measured with a standard curve as described by Hutton et al, 1980.

3.2.2.1 Method

a. Preparation of platelet rich plasma

50ml of blood was taken from the ante-cubital vein of normal subjects who had not taken any prostaglandin synthetase inhibitors (i.e. aspirin or non-steroidal anti-inflammatory drugs) for two weeks. Platelet rich plasma (PRP) was prepared as outlined in Appendix A.

b. Aggregating agents

Adenosine phosphate (sodium salt ADP, Sigma Pharmaceuticals) was used exclusively throughout the study; the final concentration varied from 2 to 10μM made up in Owren's buffered saline, pH 7.35 (Appendix A).

c. Anti-aggregation assay

0.4ml of PRP was incubated at 37^0C for 60 seconds in a siliconised glass cuvette in the Payton aggregometer with 0.05ml of test solution or 0.05ml of control (Tris buffer pH 8.0). 0.05ml of ADP (10μM final concentration) was added and the following optical density registered on a Rikadenky pen recorder (see figure 3.2.2). After 3 minutes the fall in optical density of the PRP was measured from the tracing and expressed as a percentage of that

seen in the buffer control.

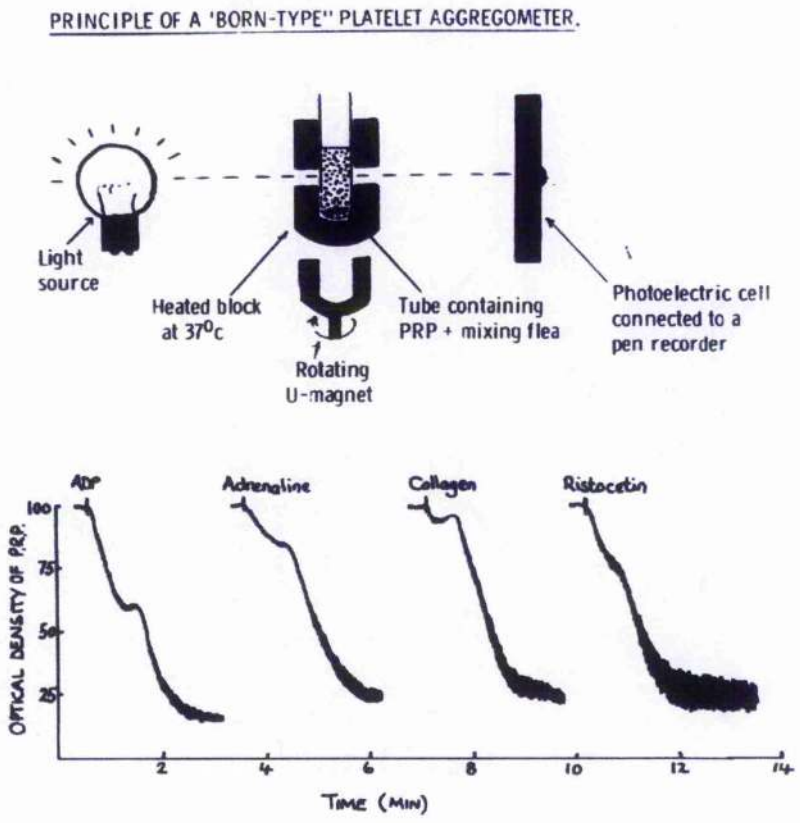


Fig 3.2.2 The Payton Aggregometer exploits the principle that the optical density of stirred plasma decreases as platelets aggregate under the effect of various aggregating agents.

In order to confirm that the inhibitor present was PGI_2 and not another physiologically active substance, all test samples were incubated at 37°C for 20 minutes. This effectively destroys PGI_2 while not affecting other more stable prostaglandin inhibitors or ADP degrading enzymes (ADP-ases). These samples were assayed as above to confirm loss of inhibitory activity.

d. Standard dose response curve for PGI_2

This assay was applied to a range of known concentrations of PGI_2 and inhibition for each concentration was plotted to make a standard dose response curve. Fresh prostacyclin standards were prepared by dissolving crystalline PGI_2 sodium salt in Tris buffer (pH 8.0) at a concentration of 120 ng/ml and serial dilutions with Tris buffer were made. A range of dilutions were therefore made as follows: 0.12, 0.3, 0.6, 1.2, 3.0, 6, 9, 12, 60 and 120 ng/ml. These standard solutions were kept at 4°C to preserve prostacyclin activity.

e. ADP degrading activity

The action of ADP-ases could possibly exist in biological fluids and therefore affect the results. Because ADP was used as the aggregatory agent ADP-ase activity was measured in each sample as follows.

2.0ml of neat or diluted tissue extract was incubated at 37°C with an equal volume of ADP (100μM/l). At 30 to 60 second intervals, 0.1ml of the incubation mixture was added to 0.04ml of PRP, and platelet aggregation by residual ADP was measured as before. A further volume of this incubation mixture was assayed for residual ADP at each interval, using the modified firefly luminescence methods, (Holmsen H et al, 1966 and Hardisty RM et al, 1970).

f. Calculation of PGI_2 Activity

From the aggregation tracing (in the absence of any ADP-ase activity) the percentage inhibition was calculated from the formula

$$\frac{\text{\% aggregation in test sample at 3 min}}{\text{\% aggregation in control at 3 min}} \times 100$$

With this value the amount of PGI_2 activity in the test sample was calculated from the standard curve.

3.2.2.2 Results

The standard dose response curve is shown in figure 3.2.2.2. The lower limit of sensitivity was found to be 1ng/ml. No ADP-ase activity was found.

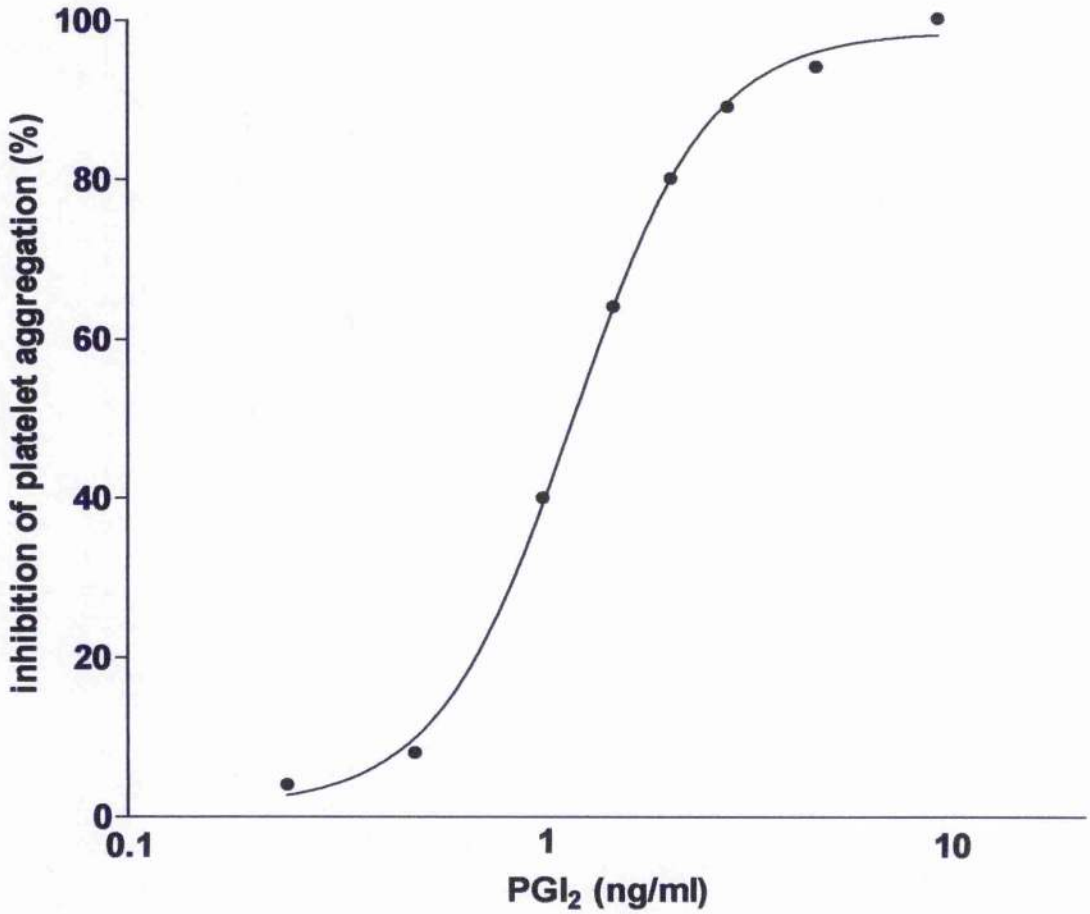


Fig 3.2.2.2 Typical dose response curve for PGI_2

3.2.2.3 Discussion

This method has the major advantage of providing a dynamic physiological assay of biological activity which is relatively straightforward and gives rapid results. There are several problems with the assay however which limits its usefulness.

Firstly the assay specificity is limited, 1ng/ml being the minimum concentration of PGI_2 detectable. Furthermore inhibition of platelet aggregation is not specific to PGI_2 and many other physiological inhibitors such as PGA_2, PGE_1, PGD_2, adenosine and ADP degrading enzymes could be present in biological fluids. By testing for ADP-ase activity and by incubating samples to destroy PGI_2, this possibility can be largely although not totally excluded. A theoretical alternative approach to the problem of specificity would be to use PGI_2 production inhibitors (such as Indomethacin, 15-hydroxy-peroxy-arachidonate [15HPAA] and tranylcypromine) during control incubation of samples to ensure that the substance produced was indeed PGI_2.

Because of the above concern about specificity , the results for PGI_2 measurements were expressed as units of PGI_2-like activity.

There were technical problems related to the need for immediate assay because of the lability of PGI_2. Consequently, the number of samples that could be assayed at any one time was significantly limited. Because of these difficulties and of the need to measure PGI_2 in plasma, a radio-immunoassay (RIA) was also developed.

PHYSICO-CHEMICAL ASSAYS OF 6-keto $PGF_1 \propto$

3.3.1 The Radio-immunoassay of 6-keto $PGF_1 \propto$

A RIA for 6-keto $PGF_1 \alpha$, the stable breakdown of PGI_2, was developed initially using an anti-serum kindly donated by Dr J A Salmon of the Welcome Laboratories. This work was done in collaboration with and under the direct supervision of Dr Rose Chow and Dr Ron Hutton, Department of Haemophilia at the Royal Free Hospital.

3.3.1.1 Sources of antisera and liquids

Antiserum was donated by Dr J A Salmon of the Wellcome Research Laboratories, Beckenham, Kent. Later in the study a second antiserum was supplied by Dr P B Smith of the Cardeza Foundation, Philadelphia, PA, USA. Both Dr Salmon's antiserum and that from Cardeza were raised against 6-keto $PGF_1\alpha$ conjugated to bovine serum albumin and supplied in freeze dried form, (Salmon JA, 1978). Tritiated 6-keto $PGF_1\alpha$ was purchased from the Radiochemical Centre, Amersham, UK.

3.3.1.2 Dilution of the antiserum

The working concentration of an antiserum is normally chosen to give 40-60% binding of a radioactive antibody in the absence of the unlabelled antigen, (Granstrom E et al, 1978). The use of too much antibody results in an assay with diminished sensitivity because there are too many available binding sites. On the other hand, too dilute an antiserum results in a narrowing down of the working range of the standards. It is generally accepted that the greatest sensitivity is obtained if the expected level of the unknown cold ligand or antigen is taken into consideration.

The final working of the serum dilution chosen would be that resulting in maximal displacement between antiserum curves with and without the expected antigen load. The best antiserum dilution for this RIA was determined in the following experiment.

3.3.1.3 Method

A modification of Salmon's method was used, (Salmon JA, 1978), and in order to establish maximum assay sensitivity a loading dose of 100pg 6-keto $PGF_1\alpha$ was included in the assay. A stock solution of 0.5mg/ml of the antiserum was made up in 0.1% gelatin tris buffer pH 7.5 (GTB, Appendix A) and a series of dilutions from 1/10 to 1/1000 were made. Duplicate 0.1ml aliquots were mixed in plastic tubes, (LP3 Sarstadt), with 0.1ml of (H^3) 6-keto $PGF_1\alpha$ (ca 10,000 dpm, Appendix A) and either 0.1ml GTB alone or 0.1ml GTB containing 100pg unlabelled 6-keto $PGF_1\alpha$; total and blank samples were included. After mixing, the mixture was stored at 4^0C for 24 hours. 1ml dextran coated charcoal (DCC) was added, mixed and incubated for 10 minutes. After centrifugation at 3,000g and 4^0C for 15 minutes, 1.0ml of the supernatant was transferred to a vial containing 7.5ml of scintillation liquid (Appendix A) and counted for 10 minutes in a LKB β scintillation counter. This procedure was repeated using different antiserum dilutions and the concentration resulting in the greatest displacement of bound label was determined.

3.3.1.3 Results

The results are shown in figure 3.3.1.3 where percentage bound labelled 6-keto $PGF_1\alpha$ was plotted against concentration of antiserum with and without the loading dose of 100pg unlabelled 6-keto $PGF_1\alpha$. A dilution of 0.0.4mg/ml was found to

produce the greatest displacement between curves at percentage binding of between 32-50%. This was therefore chosen as the antiserum dilution yielding greatest sensitivity.

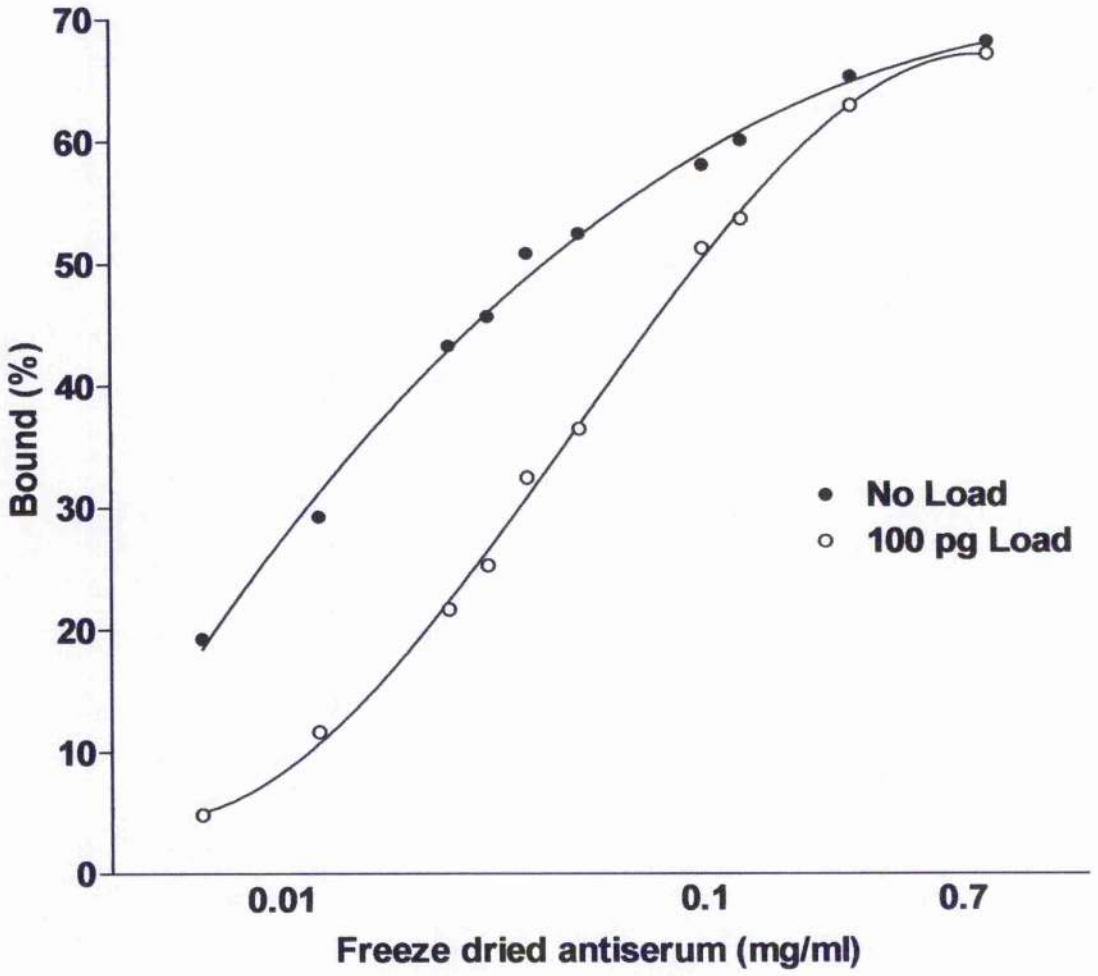


Fig 3.3.1.3 Dilution curve for the Wellcome antiserum with and without 100pg unlabelled 6 keto PGF1α load

3.3.1.4 Standards

Essential characteristics of a standard should be stability and purity. The levels

should be chosen to cover the range of values within which the substance to be measured is expected to fall. Antiserum unlabelled antigen concentration should be chosen accordingly and should ideally concentrate on the most sensitive part of the standard curve, but still extend over the entire range. In addition, upper and lower detection limits should be included in order to identify the range of sensitivity. Sensitivity is measured according to the following principle:

$$Sensitivity = \frac{Sigma}{K}$$

where Sigma = errors and K = affinity constant, (Ekins RP, 1974).

From this, the lowest amount of antigen that can be accurately measured may be derived. Assuming that the relative experimental error Sigma remains constant (i.e. coefficient of variation = 1), then the least detectable amount of antigen = $\frac{1}{K}$

where K is the affinity constant of the antiserum.

3.3.1.4.1 Estimation of affinity constant of the antiserum

The affinity constant (Ka) of an antiserum is derived from the assumption that the rates of association (binding) and disassociation of antigens (Ag) to and from antibodies (Ab) reaches a steady state of equilibrium (i.e. the law of mass action).

$$Ag + Ab \underset{K2}{\overset{K1}{\rightleftharpoons}} Ag/Ab\ complex$$

$$Ka = \frac{K_1}{K_2}$$

where K_1 = the rate of association of Ag/Ab complex

K_2 = the rate of disassociation of Ag/Ab complex

In this case where the antiserum is a heterogeneous mixture of antibodies, each with its own Ka, and the specific activity of the antiserum is not known, the affinity constant was estimated from a saturation curve of bound against free antigen by a method which exploits the Michaelis-Menton equation (Thorell J et al, 1978). From this the Ka in litres/mole (l/M) can be derived from the following equation:

$$Ka = \frac{1x10^{12}}{(FA/MW)xD}$$

where *FA* = pg free antigen at 50% of total load bound

MW = molecular weight and *D* = dilution

3.3.1.4.2 Method

Tritiated 6-keto $PGF_{1\alpha}$ was diluted with GTB pH 7.5 to make a series of concentrations from 4.8 to 81.7pg/ml 6-keto $PGF_1\alpha$. 0.1ml of each dilution was incubated in duplicate with 0.2ml GTB and 0.1ml of antiserum (Appendix A). Duplicate total and blank samples were included for each dilution of labelled antigen. The samples were incubated at 4^0C for 24 hours and then 1ml of DCC was added, incubated for a further 10 minutes and centrifuged at 3,000g for 15 minutes to separate the bound and free fractions. 1ml aliquots of supernatant were counted. The experiment was repeated several times at different dilutions of antiserum to obtain a good spread of points on the saturation curve.

3.3.1.4.3 Results

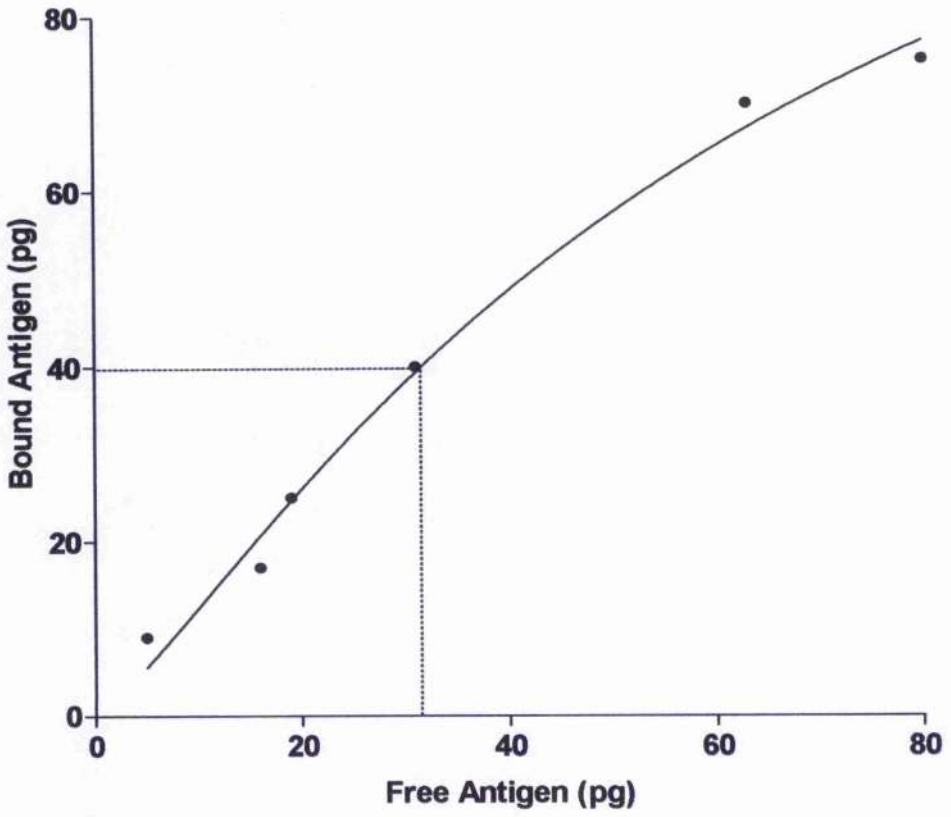


Fig 3.3.1.4.3 Affinity constant of the Wellcome antiserum as determined from the saturation curve

The saturation curve obtained is shown in figure 3.3.1.4.3. A true plateau was not reached: this was therefore assumed to have occurred at the 80pg level. Therefore, 30.5pg free antigen was present at 50% binding of the total load. Thus

$$Ka = \frac{1x10^{12}}{(30.5/380)x2500l} \text{ l/M}$$

$$= \frac{1x10^{12}}{200.7} \text{ l/M}$$

Therefore, the least amount detectable, 1/Ka

$= 200.7 \times 10^{-12}$ M/L

$= 762.7 \times 10^{-14}$ g/0.1ml

$= 7.6 \times 10^{-12}$ g/0.1ml

Thus the lower amount of antigen detectable with this assay was just under 10pg and consequently this was the lowest concentration used in making up the standards.

3.3.1.5 Specificity of the Antiserum

Specificity of an assay is defined as the extent of freedom from interference by substances other than that intended to be measured, (Midgley AR, Jr et al, 1969). RIA supposedly has the major advantage of great specificity but several factors may act adversely to affect this. Non-specific factors such as pH, osmolality, the presence of binding proteins, enzymes and certain cations may affect the antigen-antibody reaction or concentration of available binding sites. Thus the ability of the antibody to detect a particular antigenic determinant may be affected and possibly result in increased cross reactivity with structurally similar substances. It is therefore important to assess the specificity of the antiserum used.

Several prostanoids could theoretically cross react with 6-keto $PGF_{1\alpha}$. Salmon found that the majority of prostaglandins and their metabolites did not cross react with 6-keto $PGF_{1\alpha}$ antiserum, but did report significant cross reaction with PGE_1 (11%), PGE_2 (4.8%), $PGF_{1\alpha}$ (7.5%) and $PGF_{2\alpha}$ (5.4%), (Salmon JA, 1978). Using a different antiserum, another worker found much less cross reactivity (PGE_1 0.09%, PGE_2 0.16%, $PGF_{2\alpha}$ 0.8%), (Mitchell MD, 1978).

3.3.1.5.1 Method

PGA_2, PGE_1, PGE_2, $PGF_{2\alpha}$ (obtained from Sigma Pharmaceuticals) and 6-keto

$PGF_1\alpha$ were prepared in concentrations of 100,000pg, 10,000pg, 1,000pg, 100pg and 50pg in GTB. Each was incubated with 0.1ml antiserum, 0.1ml H^3 – 6–keto PGF_1 alpha (ca 10,000dpm) and 0.1ml GTB. Duplicate samples were made and processed as above. On a separate occasion the method was repeated for PGI_2.

3.3.1.5.2 Results

Standard curves for each agent were plotted using the percentage of total bound ligand against the concentration of ligand used and are shown in figure 3.3.1.5.2 a.

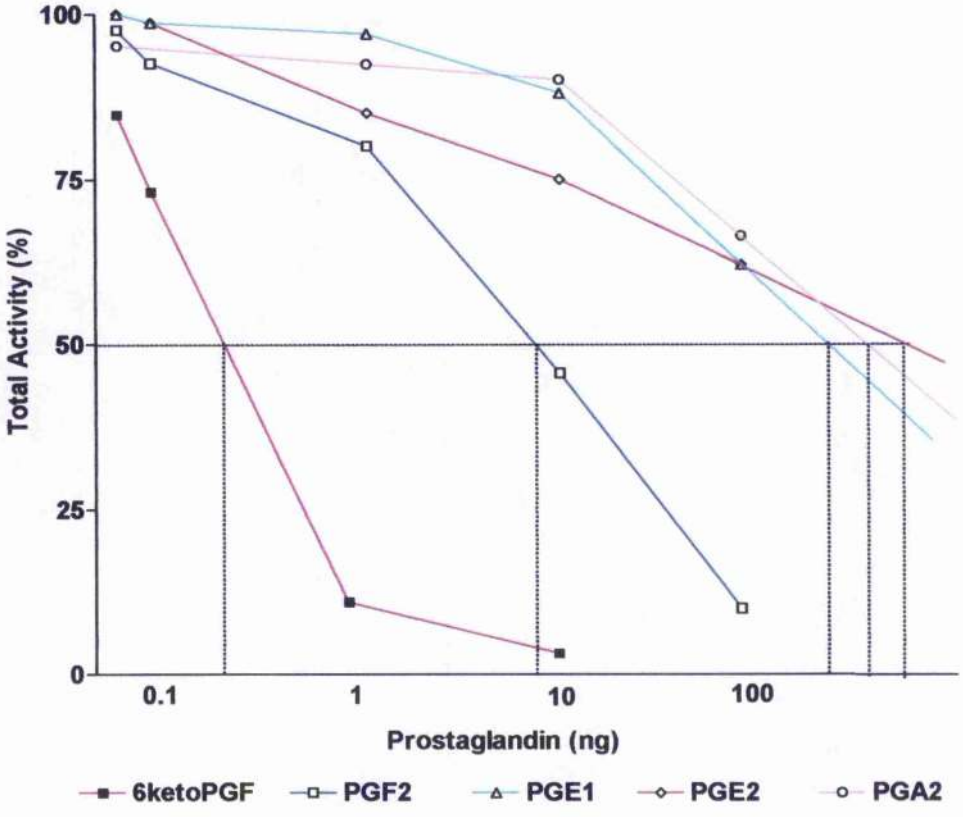


Fig 3.3.1.5.2 a Inhibition of binding of tritiated 6 keto PGF1α to the Wellcome antiserum by unlabelled prostaglandins: antiserum / prostaglandin cross-reactivity is calculated from the intercept with the horizontal axis at 50% total activity

From the above figure the relative cross-reactivity between antiserum and ligands can be calculated from the following formula

$$\% \text{ cross-reaction} = \frac{6ketoPGF1\alpha}{ligand} \text{ concentrations at 50\% total binding.}$$

The data was also plotted using the Logit Plot method which straightens the curves allowing easier comparison (Rodbard D et al, 1970). This method is based on the following equation:

$$\text{Logit (Y)} = \log e \left[\frac{Y}{1-Y}\right]$$

where Y = b/bo

b=fraction of tracer bound, and bo=maximal binding in absence of unlabelled antigen

These results are shown in figure 3.3.1.5.2 b.

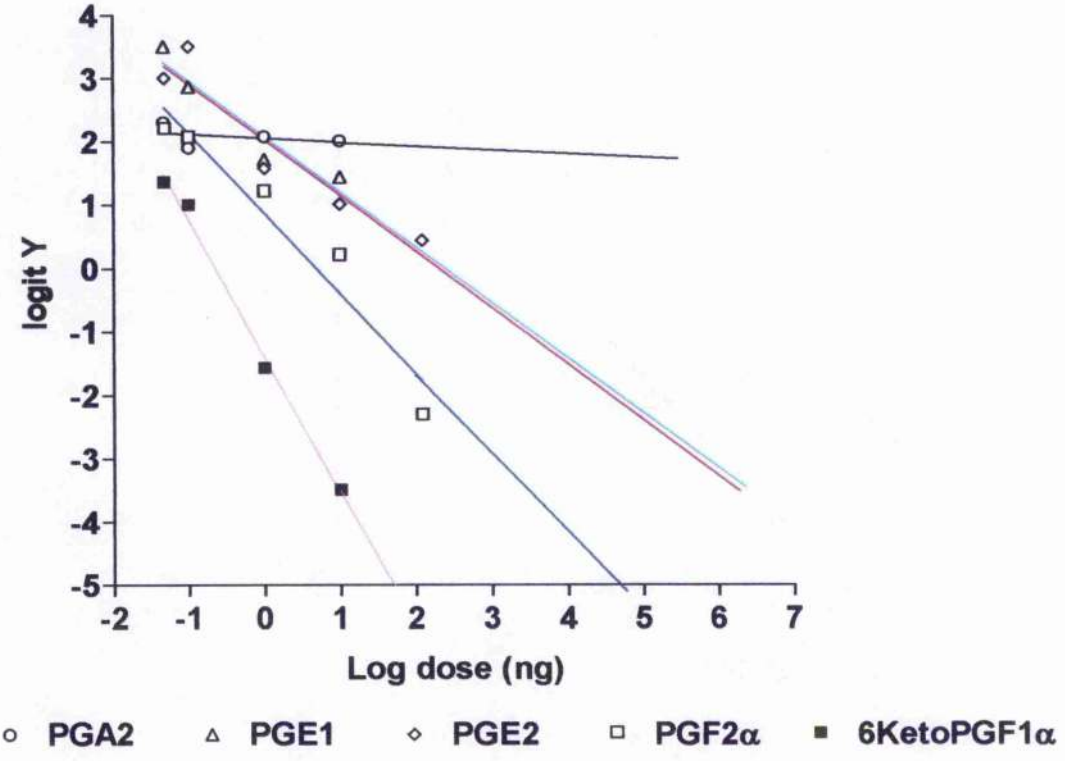


Fig 3.3.1.5.2 b Plots of Logit B/Bo for 6 keto PGF1α and other prostaglandins using the Wellcome antiserum

Calculations of relative cross reactivity are shown in Appendix C and tabulated in table 3.3.1.5.2.

Ligand	Relative Cross-Reaction	
	From curves	From Logit Plots
6ketoPGF$_{1\alpha}$	1	1.0
PGF$_{2\alpha}$	0.03	0.0032
PGE$_1$	0.0009	<0.000004
PGA$_2$	0.0007	<0.000004
PGE$_2$	0.0003	<0.000004
PGI$_2$		0.546

Table 3.3.1.5.2 Specificity of the 6ketoPGF$_{1\alpha}$ Antiserum (See Appendix C for the calculations of the relative cross-reaction to the 6ketoPGF$_{1\alpha}$ antiserum).

Logit plots comparing 6-keto $PGF_1\alpha$ and PGI_2 are shown in figure 3.3.1.5.2 c (6.5).

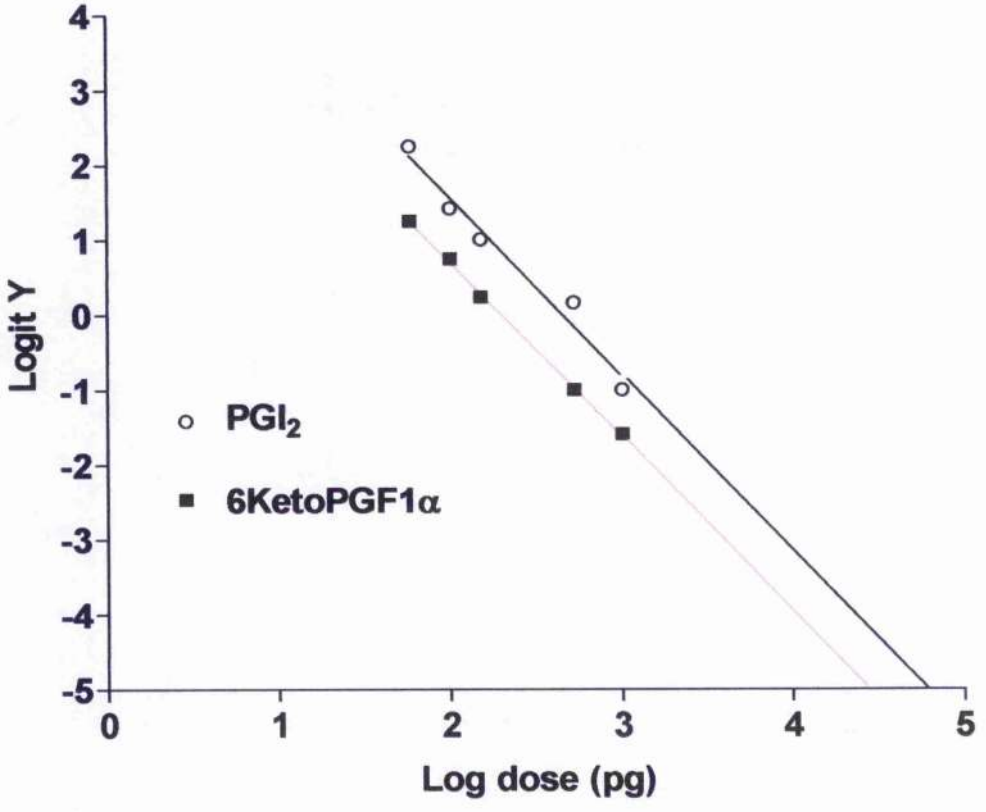


Fig 3.3.1.5.2 c Plot of Logit B/B_0 for 6ketoPGF$_{1\alpha}$ and PGI_2 using the Wellcome 6ketoPGF$_{1a}$ Antiserum

3.3.1.5.3 Conclusion

The relative cross-reactions were found to be higher when calculated by the standard curves rather than by the logit plot method. This difference could be due to the more subjective fitting of the standard curves giving the potential for less accurate measurement of zero binding of the label.

3.3.1.6 Correlation Between 6-keto $PGF_{1}\alpha$ RIA AND PGI_2 Bioassay

Once established the 6-keto $PGF_{1\alpha}$ RIA was correlated with the bio-assay for PGI_2-like activity. This correlation was made using human tissue, namely human umbilical vein which was a readily available and fresh source of vascular tissue.

3.3.1.6.1 Method

Five fresh umbilical cords from normal deliveries were obtained (Ethical Committee Approval and consent obtained) and 10 arteries and 5 veins dissected from them. Vessel rings were prepared as previously described an incubated in TBS pH 7.5 at a concentration of 200pg/ml for 3 minutes at room temperature. The incubation fluid was immediately assayed for PGI_2-like activity by bio-assay. At the same time, aliquots of this fluid were stored at 4^0C and 6-keto PGF_1 alpha assayed by RIA at a convenient time.

3.3.1.6.2 Results

The linear regression plot between these two assays is shown in figure 3.3.1.6.2.

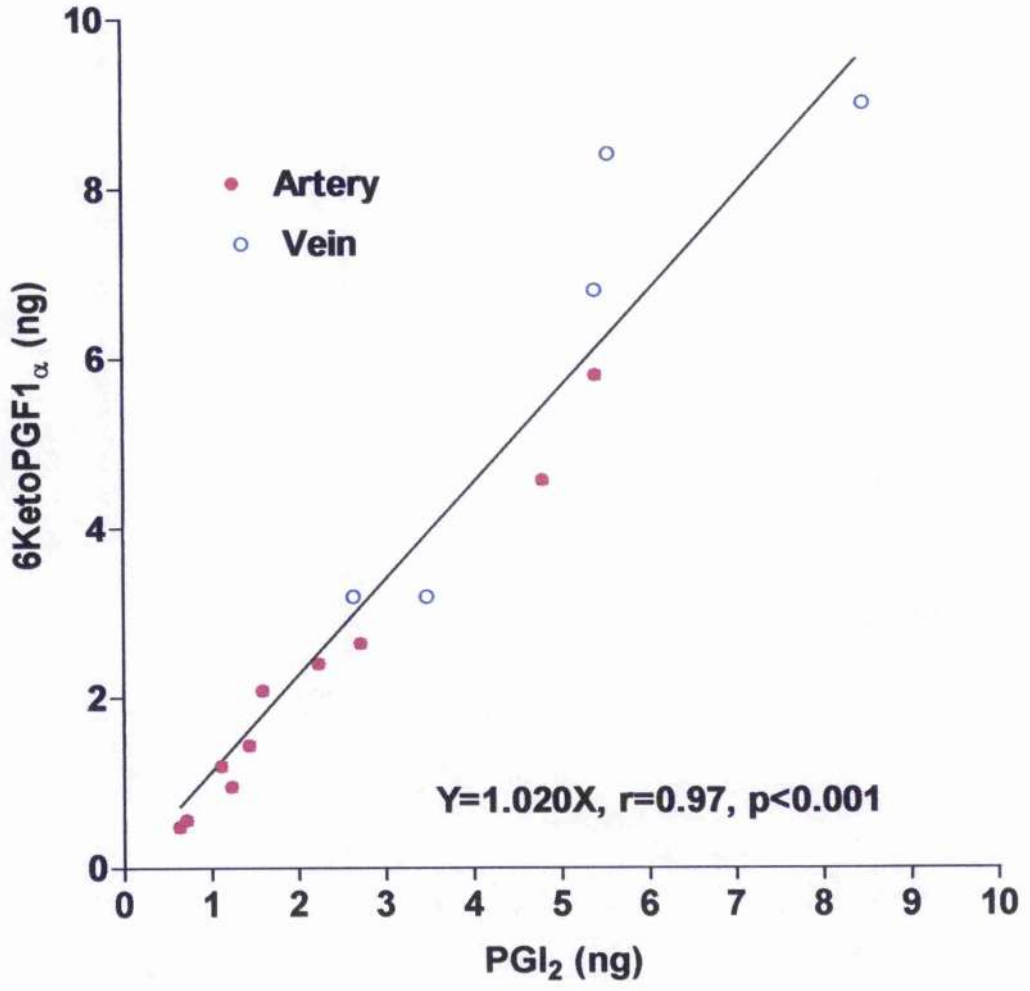


Fig 3.3.1.6.2 Linear regression plot between PGI_2 and 6 keto PGF1α in artery and vein

3.3.1.6.3 Conclusion

There was excellent correlation found between these two assays (R = 0.97), indicating that biological activity measured immediately correlated well with the presence of PGI_2's breakdown products measured at a convenient time later. Thus, RIA for $PGF_{1\alpha}$ using the technique described above is a convenient and reliable method for measuring PGI_2 production in tissue incubates.

3.3.1.7 Discussion

A RIA for 6-keto $PGF_{1\alpha}$ was developed because of several expected advantages over other methods. The major benefits included greater sensitivity and specificity than platelet nephelometry, a lower detection limit and greater precision and accuracy. A further expected advantage was the possibility of carrying out analyses on multiple samples at a convenient time without the need for immediate assay.

In fact, from the above experiments the following criteria for this RIA were established.

a. Maximal sensitivity was achieved at an antiserum concentration of 0.4 mg/ml.

b. The affinity constant Ka of the antiserum was found to be 5×10^9 l/M, with a lower detection limit of 10pg/ml.

c. $PGF_{2\alpha}$ was the only prostanoid of those tested that had any substantial cross reactivity (only prostanoids described at that time as having significant cross-reactivity were studied). The relative cross reaction was low at 0.003 using the Logit Plot method, thus the antiserum was proved to be highly specific for 6-keto PGF_1 alpha.

d. There was very good correlation between 6-keto $PGF_{1\alpha}$ and biological activity as measured by platelet nephelometry.

The obvious disadvantage of the RIA is that it does not directly measure PGI_2 but one of the 2 possible breakdown products. Although 6-keto $PGF_{1\alpha}$ is the main metabolite, it remains unknown whether the balance of metabolites is maintained

under non-physiological conditions. A further problem is that the assay is immunologically based and does not reflect the dynamic physiological function of the substance being measured. Practical disadvantages of the assay were limited to the methodology which proved laborious, and could not be completed within one working day.

The demonstration of the high specificity and excellent correlation between 6-keto $PGF_{1\alpha}$ and biological activity suggests that these possible disadvantages were not of critical importance in the application of the RIA.

SUMMARY

From this work a highly specific RIA for 6-keto $PGF_{1\alpha}$ was developed. In the use of this assay during the study the following method and incubation system was adopted for the RIA of 6-keto PGF_1 alpha using the Wellcome antiserum.

0.1ml gelatin tris buffer pH 7.5

0.1ml standard or sample

0.1ml (H^3) 6-keto $PGF_{1\alpha}$ (ca 10,000dpm)

0.1ml 6-keto $PGF_{1\alpha}$ antiserum (0.4mg/ml)

Standards ranged from 10pg to 10,000pg cold 6-keto $PGF_{1\alpha}$. Total gelatin content was 0.1% with addition of sodium azide 0.1% to inhibit bacterial growth in the gelatin solutions. The samples were stored at 4^0C for 18 to 24 hours before the addition of 1.0ml DCC and a further incubation of 10 minutes at 4^0C. The samples were then centrifuged at 3,000g at 4^0C for 15 minutes and 1.0ml of the supernatant removed by

aspiration for counting.

This RIA provided an accurate and specific method for measuring PGI_2 activity in vascular tissues which formed the basis of the study at this time.

3.3.2 MEASUREMENT OF 6-keto $PGF_{1\alpha}$ BY RIA IN PLASMA

Introduction

Ethically it is difficult to collect human clinical tissue samples either on a single occasion or particularly repeatedly over a period of time for prostaglandin measurements. The ease of collection of peripheral venous samples with the added logistical advantage of repeated sampling made plasma measurement of crucial importance.

An alternative way of measuring prostaglandin production was by assay of urinary metabolites. However, this method only allows measurement of total excretion over a specified period of time (usually 24 hours) and is therefore unable to detect fluctuations in production. At the time, this was felt to be a disadvantage and thus inappropriate to the proposed clinical studies. Furthermore, of more importance to this study, was the inability of this method to determine the source of production of PGI_2. For these reasons, urinary PGI_2 metabolites were not measured.

In this section, the modification and eventual application of the RIA to plasma measurement that took place in this study is described. Ethical Committee approval from the Royal Free Hospital Ethics Committee was obtained for studies using blood samples from volunteers and patients.

Blood Collection

Blood was collected by antecubital venepuncture using 21G needles into plastic tubes containing sodium EDTA and Indomethacin in tris buffer pH 7.5 at final concentrations of 2mg/ml and 10μM respectively. Sodium EDTA, a calcium chelating agent was included to anticoagulate and prevent further activation of the arachidonata pathway while Indomethacin, an inhibitor of cyclo-oxygenase, was used to prevent further formation of PGI_2 or other prostanoids. Blood was collected only from volunteers or patients who had not taken aspirin or non-steroidal anti-inflammatory drugs for 2 weeks prior to sampling. Anti-coagulated samples were stored in tubes placed in iced water for transport. After centrifugation at 3,000G at 4^0C for 15 minutes, plasma was collected in 1.0ml aliquots into plastic tubes and stored at -40^0C until assayed.

3.3.2.1 Measurement of 6-keto $PGF_{1\alpha}$ in Plasma without an extraction step

Because of the small quantities of prostanoids typically present in plasma extraction steps are commonly used to concentrate the substance being studied sufficiently to give levels measurable by the RIA. At the beginning of the study it was not clear whether prostacyclin was present in sufficiently high concentrations to allow measurement in plasma without an extraction step. In order to establish whether the step would be required the following experiments were carried out:

3.3.2.1.1 Experiment i: Direct plasma measurement

Blood was collected from 10 healthy volunteers and processed as above. 0.01ml of

plasma was assayed for 6-keto $PGF_{1\alpha}$ using the standard incubation system. This procedure was repeated using 0.2ml plasma (i.e. double volume) with omission of GTB to maintain an equivalent total volume of 0.04ml; the final gelatin concentration was made up to 0.1%.

Result

6-keto $PGF_{1\alpha}$ levels were all below 10pg/ml even when using the double volume sample.

3.3.2.1.2 Experiment ii: pre-loading plasma before measurement

In an attempt to increase the detection limits of the assay, the RIA was performed in the standard manner but with prior addition of 60pg of cold 6-keto $PGF_{1\alpha}$ in a volume of 0.01ml to each sample; the same volume of GTB was added to the standard.

Result

6-keto $PGF_{1\alpha}$ levels were once again below the lower detection limit of 10pg/ml.

3.3.2.1 Conclusion

From these results, it was clear that this assay could not directly measure 6-keto $PGF_{1\alpha}$ in plasma. It was therefore necessary to include an extraction step in the assay.

3.3.2.2 Extraction of Prostanoids from Plasma

Extraction and further purification steps are very commonly used in RIA of prostanoids. As always this process has disadvantages as well as advantages. The ideal advantage of the extraction process would be in achieving concentration of the

prostanoid in the sample to bring it within the range of the radio-immunoassay. Furthermore, extraction might result in disassociation of most of the prostanoid from the prostanoid/albumin complex which binds most of these substances in plasma, and there was a further perceived potential advantage of removal of unknown factors which might interfere with the RIA.

There are many disadvantages with the extraction process however, including the fact that the recovery of prostanoid can be frequently low and inconsistent, the extraction process itself may modify related substances in the plasma to cross react with the antibody resulting in falsely high results, and impurities in the reagents may be introduced by this step which could interfere with the RIA. Finally, the whole assay procedure becomes more laborious, time consuming and expensive thus severely limiting the number of samples which can be simultaneously processed, (Granstrom E et al, 1978). Many extraction methods for prostaglandins are described, all based on the principle that acidification will render fatty acids insoluble. Prostaglandins are cyclic fatty acids, and as such, the carboxyl group can be altered by changing the pH of the medium. Carboxylic acids have a pKa (negative logarithm of the dissociation constant) of between 4 and 5.

$$\text{Prostanoid} - \text{COOH} \underset{\text{<- Low PH}}{\overset{\text{High pH ->}}{\Leftrightarrow}} \text{prostanoid} - \text{COO}^- + \text{H}^+$$

At low pH the carboxyl moiety is intact and thus insoluble, while at high pH, dissociation into its ionic form takes place and thus becomes soluble in aqueous media. Thus acidification will render the fatty acids insoluble, allowing extraction by organic solvents.

3.3.2.2.1 Standard Method to Extract Plasma

The development of the extraction process presented numerous problems such as emulsification of the samples, poor and variable recoveries and difficulties with extract re-suspension after evaporation. Extraction without further purification by thin layer chromatography (TLC) was used because the recovery was significantly lower after TLC.

Sample number	Recovery after TLC (%) [A]	Recovery without TLC (%) [B]	6ketoPGF1α pg/ml using RIA on [B] samples
1	37.9	73.1	101
2	30.1	62.9	102
3	43.4	60.2	169
4	47.6	85.7	91
5	41	71	167
6	39.9	74.6	91
7	38.1.	69.3	175
8	32.7	72.3	118
9	36.1	64.2	98
10	33.6	68.9	98

Table 3.3.2.2.1 Comparison of recoveries from 10 normal human plasma extractions with and without Thin Layer Chromatography (TLC)

As a result, the method used throughout this part of the work, was as follows:

1ml of plasma was added to a polypropylene tube containing 2ml acetone at -20^{0}C; 0.1ml of H^3 6-keto $PGF_{1\alpha}$ (ca 2,000dpm) was added and the sample vortexed for 30 seconds, centrifuged at 2,000G for 3 minutes, and the supernatant collected into another tube containing 2ml hexane. The protein precipitate was re-extracted with another 1ml of acetone at -20^{0}C. The second collection of supernatant was added to the first, the sample vortexed for 60 seconds and centrifuged. The top hexane layer was then discarded. 0.06ml citric acid (2.87M) and 2ml chloroform were added and the sample vortexed for 60 seconds and centrifuged. The upper layer was aspirated into another tube containing 2ml chloroform and the extraction repeated. The combined bottom layers of chloroform were evaporated with nitrogen at room temperature in a fume cupboard. The residues were re-dissolved in 0.3ml GTB and assayed for 6-keto $PGF_{1\alpha}$.

3.3.2.2.2 Discussion

The levels measured in the 10 samples of normal human plasma (see table 3.3.2.2.1) were 121 +/- 33.1 pg/ml (mean +/- SD) which were within the range of values reported by other workers at that time (Mitchell MD, 1978) (Machin SJ, et al 1981). This assay was therefore applied to measurement of 6-keto PGF1α in plasma studies. However as these studies progressed certain problems with this technique were encountered.

3.3.2.2.3. Problems with the RIA and Extraction Process

As experience with this plasma assay grew, certain inconsistencies and results were found which called into question the reproducibility and variability of the method. Initially, sample extractions were not done in duplicate, but when this was eventually done, the results for duplicate extracted samples were sometimes found to differ markedly. As a result the co-efficient of variation and the reproducibility of the process were determined.

Determination of co-efficient of variation

A large volume of plasma was collected from a normal volunteer. 20 1ml aliquots of plasma was simultaneously extracted and submitted to assay. A corrected measurement of 6-keto $PGF_{1\alpha}$ was derived by converting the value to 100% recovery (i.e. RIA measurement divided by recovery for that sample x 100). Out of a total of 0.3ml of extract for each sample, only 0.1ml was assayed, thus the result was multiplied by 3 to obtain the full corrected quantity per 1ml sample. The co-efficient of variation was then calculated as follows:

Co-efficient of variation (%CV) = 100 (standard deviation / mean)

3.3.2.2.3.1 Results

Sample recovery, RIA readings and corrected readings are shown in table 3.3.2.2.3.1.

Sample recovery %		6ketoPGF$_{1\alpha}$ (pg/ml)	
		Reading from RIA	*Corrected*
1	7.7	19.0	81
2	68.2	12.0	53
3	70.7	13.0	55
4	69.2	15.5	67
5	62.1	14.5	70
6	74.3	12.5	50
7	68.2	17.5	77
8	70.7	12.5	53
9	71.7	22.0	92
10	64.2	13.5	63
11	65.1	16.5	76
12	65.1	25.5	118
13	71.7	11.5	48
14	64.2	17.5	82
15	69.2	20.0	87
16	62.1	16.5	80
17	65.6	16.0	73
18	80.9	16.5	61
19	76.3	20.5	81
20	76.8	11.5	45

Table 3.3.2.2.3.1 6ketoPGF$_{1\alpha}$ in Twenty Samples of the Same Plasma. Each sample contained 1ml Plasma.

There was poor correlation between recovery and the original reading with a correlation co-efficient of –0.39. Recoveries varied from 62.1% to 80.8% (mean

69.4%, SD 5.1). The co-efficient of variation was therefore 7.3%. The corrected levels of 6-keto $PGF_1\alpha$ varied from 45 to 118pg/ml (mean 70.6, SD 17.9). The co-efficient of variation for 6-keto $PGF_{1\infty}$ measurement was therefore 25.4%.

3.3.2.2.3.3 Conclusion

From these results it was clear that there was much variation in the measured levels of replicate extracted samples. The high CV for 6-keto $PGF_{1\infty}$ measurement (CV should not exceed 10%) suggested that the assay system in extracted plasma was likely to be inaccurate.

3.3.2.2.4 Assessment of reliability of the RIA on extracted plasma

This was done by addition of known amounts of unlabelled 6-keto $PGF_{1\infty}$ to plasma before extraction and assay.

Plasma from 6 normal volunteers was pooled. 1ml samples of the pooled plasma were extracted in the standard manner but with prior addition to the samples of known different amounts of cold 6-keto $PGF_{1\infty}$ in tris buffer (amounts added: 400pg, 600pg, and 1,000pg). Triplicate samples of each concentration of 6-keto $PGF_{1\infty}$ or control plasma were then extracted and assayed for 6-keto $PGF_{1\infty}$.

3.3.2.2.4.1 Results

The results are shown in table 3.3.2.2.4.1.

6ketoPGF$_{1\alpha}$ load (pg)	Recovery %	6ketoPGF$_{1\alpha}$ pg/ml plasma	
		Corrected	*mean*
0	87.0	293	
	83.3	286	300
	77.6	321	
400	75.0	482	
	84.0	361	402
	84.0	364	
600	84.3	365	
	83.0	380	405
	81.7	471	
1000	85.7	410	
	73.6	554	454
	77.6	398	

Table 3.3.2.2.4.1 6ketoPGF$_{1\alpha}$ Levels in the Same Sample of Pooled Plasma Where Some Had Known Amounts of Cold 6ketoPGF$_{1\alpha}$ Added

The mean 6-keto PGF$_{1\alpha}$ level in control pooled plasma was higher than that found in the initial study on 10 normal volunteers (see section X: 300pg/ml -v- 121pg/ml respectively); this elevated level may have been the result of the longer time to collect and pool the blood from the 6 volunteers to provide the pooled sample. Mean 6-keto PGF$_{1\alpha}$ levels in the loaded samples were 402, 405, and 454pg/ml for the loads of 400, 600, and 1,000pgs respectively. Assuming the mean control level to be correct, then only 102, 105 and 154pg of 6-keto PGF$_{1\alpha}$ were recovered from

samples containing 400, 600, 1,000pg respectively.

3.3.2.2.4.2 Conclusion

These results confirmed the high variability and unacceptable reliability of this RIA applied to extracted plasma.

3.3.3 A second RIA using a different antiserum to 6-keto $PGF_{1\alpha}$

At this point in the study the application of another RIA technique not requiring an extraction step was explored because of the results obtained previously. An antiserum for 6-keto $PGF_{1\alpha}$ of such high affinity and specificity that an extraction step was not necessary, had been developed at the Cardeza Institute, Philadelphia, USA by Smith and colleagues, (Smith JB et al, 1978). A small supply of this antiserum, hereby denoted Cardeza antiserum, was obtained for comparison with the Wellcome antiserum.

3.3.3.1.1 Method

Previous work with this antiserum had involved reagent measurement in pM rather than pg. For simplicity, this convention was observed with the Cardeza antiserum (conversion factor from pM to pg is 370.47; i.e. 1 pM = 370.47pg).

6-keto $PGF_{1\alpha}$ standards ranging from 0.025pM to 5pM in 0.1ml were prepared in tris buffer containing 1% plasma gamma globulin as a protein carrier (Appendix A). 0.1ml of plasma sample or standard was incubated with 0.1ml of tritium labelled 6-keto $PGF_{1\alpha}$ (ca 10,000dpm). After mixing, 0.05ml of Cardeza antiserum (8µg/ml) was added, mixed and incubated at 37^0C for 1 hour. 0.25ml of saturated ammonium

sulphate solution was added to each tube and then centrifuged at 3,000g for 15 minutes. The entire supernatant containing H^3 6-keto $PGF_{1\alpha}$ of each tube was decanted into a scintillation vial, 7.5ml of scintillation fluid was added and then counted. A standard curve was derived from counts per minute (cpm) of each standard against 6-keto $PGF_{1\alpha}$ concentration.

This new RIA method was applied to a range of different samples and results compared to those obtained using the Wellcome antiserum for RIA following extraction.

The following samples were assayed: 2 plasma samples from normal human volunteers, 2 plasma samples from a dog taken before and immediately after pancreatic surgery, and 2 supernatant fractions from fresh rat portal veins (normotensive rats; vein segments incubated at 20^0C for 3 minutes at a tissue concentration of 50mg/ml tris buffer).

3.3.3.1.2 Results

A typical sigmoidal standard curve was produced by this RIA, from which results for the above test samples could be read (figure 3.3.3.1.2).

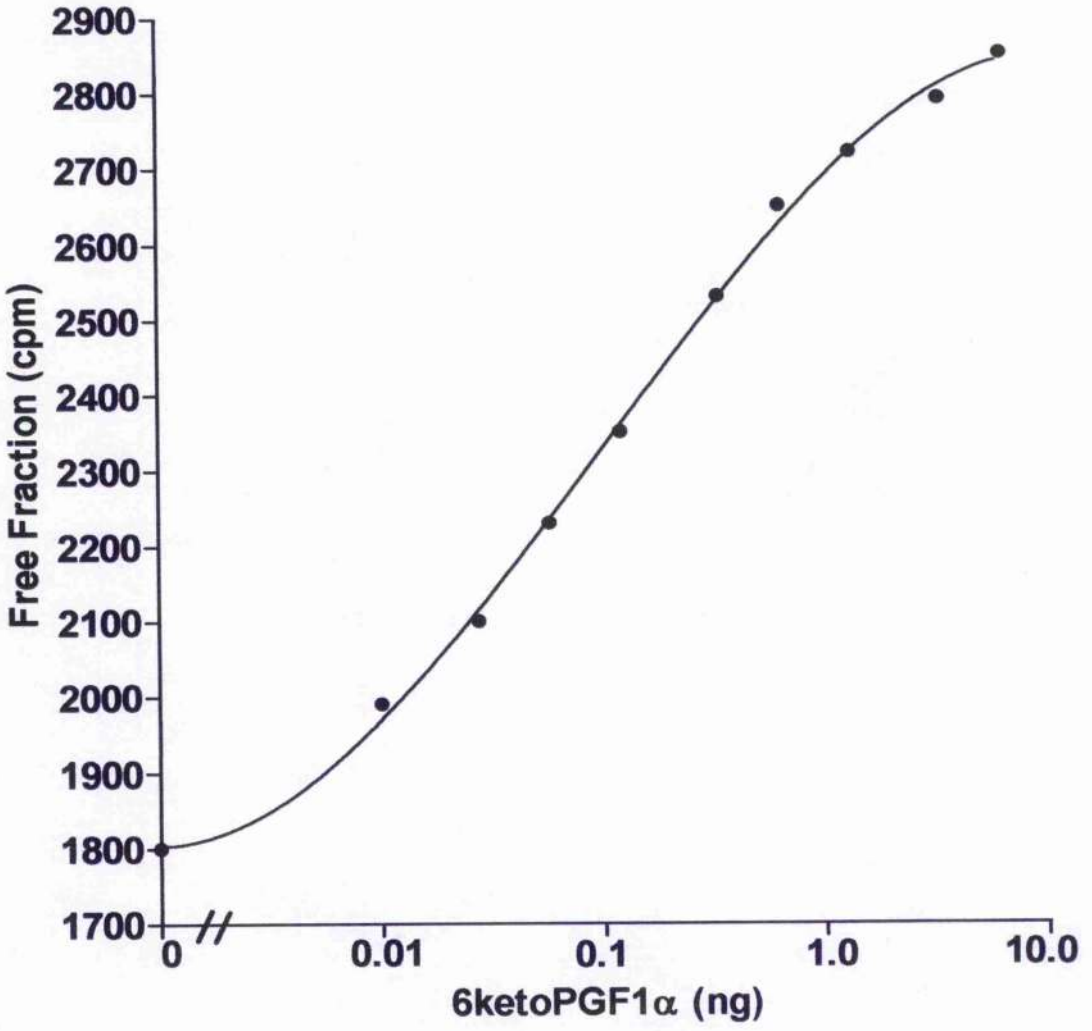


Fig 3.3.3.1.2 Typical standard curve for 6 keto PGF1α RIA using the Cardeza antiserum

The results of 6-keto $PGF_{1\alpha}$ levels in the various plasmas using both Cardeza and Wellcome antisera are listed in table 3.3.3.1.2.

Sample	6ketoPGF $_{1\alpha}$ pg/ml Wellcome antiserum	(mean of duplicate samples) Cardeza antiserum
Normal human plasma 1*	171	0
Normal human plasma 2*	134	0
Dog plasma before surgeryΦ	238	180
Dog plasma after surgery Φ	386	227
SNF from rat vein 1 Φ	128	126
SNF from rat vein 2 Φ	160	167

Table 3.3.3.1.2 Comparison of the 6ketoPGF$_{1\alpha}$ Measured in Various Samples Using the Wellcome and Cardeza Antiserum in the RIAs. SNF = Supernatant Fraction from 3 Minutes Incubation.

*** Extracted samples were used when assayed by the wellcome antiserum.**

Φ Unextracted samples.

3.3.3.1.3 Conclusion

Using the Cardeza antiserum, no 6-keto PGF$_{1\propto}$ could be detected in normal human samples, while using the Wellcome antiserum and extracted plasma these same samples seemingly measured large amounts of 6-keto PGF$_{1\propto}$ (171 and 134pg/ml 6-keto PGF$_1$ alpha). Interestingly both assays were able to measure 6-keto PGF$_{1\propto}$ without an extraction process in both dog samples, but the values obtained were lower using the Cadeza antiserum. In both, more 6-keto PGF$_{1\propto}$ was found after surgery than before. In the rat vein supernatants however, there was good agreement between values obtained with both antisera without an extraction step with 6-keto PGF$_{1\propto}$ being present in ng quantities in these specimens. These results imply that both assays were capable of measuring 6-keto PGF$_{1\propto}$ without an extraction step where the concentrations of 6-keto PGF$_{1\propto}$ are high, but that the

Cardeza antiserum was much more specific.

3.3.3.2 Estimation of lower detection limits of Cardeza antiserum RIA

Since no 6-keto $PGF_{1\alpha}$ could be measured in human plasma in the initial study, two experiments were performed to establish the detection limit of the assay in plasma.

3.3.3.2.1 Experiment 1

This experiment was designed to confirm that the Cardeza antiserum was capable of detecting small amounts of 6-keto $PGF_{1\alpha}$ in plasma.

The assay system was set up as follows:

0.1ml standards in tris buffer or plasma

0.05ml tris buffer

0.05ml (H^3) 6-keto $PGF_{1\alpha}$ (ca 10,000dpm)

0.05ml Cardeza antiserum

The mixtures were well mixed after addition of the tracer. On addition of antiserum, the samples were vortexed, incubated at 37^0 for 2 hours, then precipitated with 0.25ml of saturated ammonium sulphate solution. Tris buffer control standards and normal pooled plasma standards of 6-keto $PGF_{1\alpha}$ were made up and assayed as before.

3.3.3.2.2 Experiment 2

In order to test whether plasma proteins interfered with RIA, tris controlled standards and plasma standards were set up as above. However, after addition of saturated ammonium sulphate solution, 0.1ml of plasma was added to the tris standards and

0.1ml of tris buffer was added to the plasma standards. All samples were centrifuged and the free tracer in the supernatant fraction was counted.

3.3.3.2.3 Results

6-keto $PGF_{1\propto}$ standard curves made in tris buffer or plasma are show in figure 3.3.3.2.3.1.

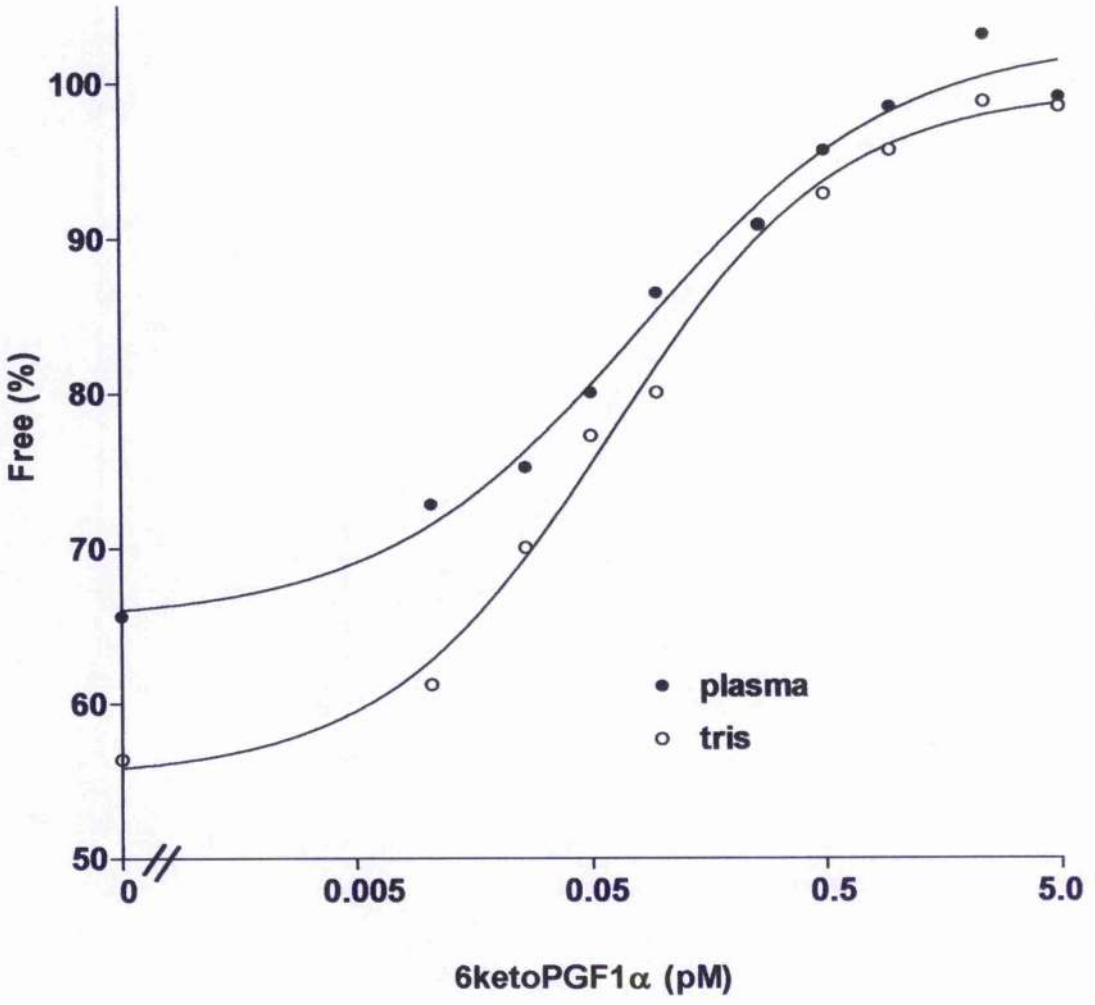


Fig 3.3.3.2.3.1 6 keto PGF1α standard curves made in plasma and tris buffer

Standard curves from the second half of the experiment where the standards were corrected with plasma or tris buffer are shown in figure 3.3.3.2.3.2.

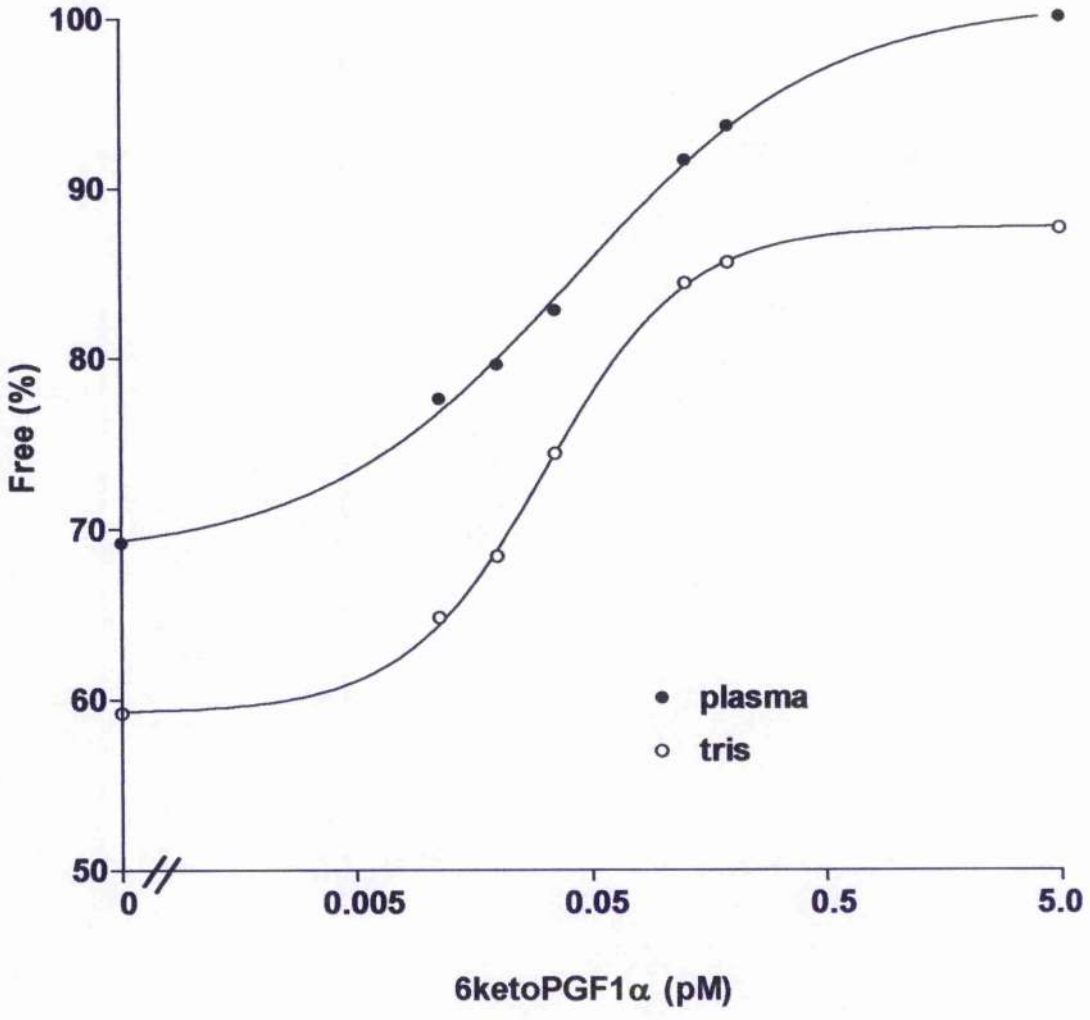


Fig 3.3.3.2.3.2 6 keto PGF1α standards in plasma or tris buffer corrected with tris or plasma respectively, immediately after addition of ammonium sulphate

In both experiments, displacement of plasma standards to the left was found particularly at lower concentrations of 6-keto $PGF_{1\propto}$. The recoveries were all 100%, and of note there was more recovered at 0% binding (i.e. when no 6-keto $PGF_{1\propto}$ was added).

3.3.3.2.4 Conclusions

These results suggest that plasma interferes with binding of antiserum. This could result from the presence of non-specific binding factors in the plasma, or from endogenous 6-keto $PGF_{1\propto}$ being already present in the plasma. More importantly the assay was shown to be capable of measuring small quantities of 6-keto $PGF_{1\propto}$ but it is likely that the plasma proteins do interfere with this assay to a certain extent.

3.3.3.3 Measurement of 6-keto $PGF_{1\propto}$ from plasma after PGI_2 Infusion

A clinical test of the performance of the RIA presented itself shortly after its development. Plasma from patients having PGI_2 infusions for trial management of renal transplant rejection by this treatment was collected for assay. This study was performed with prior approval from the Ethical Committee of the Royal Free Hospital. Peripheral venous samples were collected from one of these patients at intervals before, during and after PGI_2 infusion at the rate of 4ng/Kg/min. Plasma was processed in the standard way and stored at -40^0C until RIA was performed using the Cardeza antiserum.

3.3.3.3.1 Results

These are shown in table 3.3.3.3.1.

Sample (from time of infusion of PGI_2)	6ketoPGF$_{1\alpha}$ pM/0.2ml	Equivalent pg. 6ketoPGF$_{1\alpha}$/ml. Plasma
Pre 1 hour	0.0	0.0
Pre 30 min	0.0	0.0
Pre 10 min	0.0	0.0
Pre 0min	0.0	0.0
Post 1 hour	0.011	20.4
Post 2 hour	0.011	20.4
Post 3 hour	0.011	20.4
Post 4 hour	0.0105	19.4
Control on following day	0.0	0.0

Table 3.3.3.3.1 6 ketoPGF$_{1\alpha}$ in Plasma Samples Collected from a Patient Receiving PGI_2 Infusion.

No 6-keto PGF$_{1\propto}$ was detected in any of the samples before PGI_2 infusion. During the infusion approximately 20pg/ml plasma was detected throughout, but 24 hours post cessation of the infusion, 6-keto PGF$_{1\propto}$ was again undetectable.

3.3.3.3.2 Conclusion

These results clearly show that this RIA is capable of measuring small quantities of 6-keto PGF$_{1\propto}$ directly from plasma during a PGI_2 infusion. The complete absence of detectable 6-keto PGF$_{1\propto}$ before and after the infusion confirms the previous findings in normal human plasma.

3.3.3.4 Comparison of the 2 RIAs in extracted plasma

This experiment was performed to compare performance of both assays in extracted plasma samples.

3.3.3.4.1 Method

Duplicate samples of 8 different plasma samples were extracted and resuspended in the standard way. 0.1ml of each extract were subjected to RIA with both antisera.

3.3.3.4.2 Results

Percentage recovery, absolute and mean values of 6-keto $PGF_{1}\propto$ are shown in table 3.3.3.4.2.

Recovery %	Corrected 6ketoPGF$_{1\alpha}$ (pg/ml)			
	Wellcome Antiserum		Cardeza Antiserum	
	Absolute	Mean	Absolute	Mean
71.4	126	-	193	-
64.3	119	123	534	364
72.6	107	-	157	-
67.9	203	155	142	149
70.2	120	-	145	-
73.8	185	153	323	234
76.2	177	-	48	-
83.3	109	143	187	118
71.4	170	-	279	-
71.4	151	161	341	310
76.2	228	-	248	-
75.0	116	172	138	193
71.4	103	-	182	-
70.2	218	161	410	296
83.3	155	-	313	-
86.9	165	160	383	348

Table 3.3.3.4.2 6 ketoPGF$_{1\alpha}$ from 8 Extracted Plasma Samples as Measured by the Two RIAs.

3.3.3.4.3 Conclusion

6-keto $PGF_{1}\propto$ was detected by both methods but the Cardeza antiserum measured

more 6-keto $PGF_{1\alpha}$ in most samples than the Wellcome antiserum. There was greater variation in measurements with the Cardeza antiserum. These results further confirmed the unreliability introduced to two different assays by the extraction process.

3.3.3.5 Discussion

Direct measurement of 6-keto $PGF_{1\alpha}$ and plasma using the Wellcome antiserum proved unsuccessful. Modifications such as increasing the plasma volume and loading of the samples with known amounts of cold 6-keto $PGF_{1\alpha}$ failed to improve the sensitivity of this assay.

An extraction process to concentrate the amount of 6-keto $PGF_{1\alpha}$ before submission to assay was developed but a variety of technical problems were experienced from the start. Initial problems of emulsification and difficulty in resuspension of the extract after evaporation were overcome by the use of cold acetone and inclusion of a second extraction of the protein precipitate. The use of hexane gave better extraction and elimination of neutral lipids from the organic layer, and addition of gelatin to the tris buffer made resuspension of the lipid extract easier. The best recoveries of 6-keto $PGF_{1\alpha}$ were obtained with chloroform extraction despite the theoretical objection that because of its polarity, 6-keto $PGF_{1\alpha}$ should not easily extract into this non-polar solvent.

Incorporation of these modifications appeared to give recoveries within 60-80%, which compared well with those reported by others, (Machin SJ et al, 1981 and

Salmon JA, 1978). Furthermore 6-keto $PGF_{1\alpha}$ levels measured in normal human plasma fell within a similar range to those previously reported using RIA, (Mitchell MD, 1978 and Machin SJ et al, 1981), and also using measurement by TLC and gas chromatography/mass spectrometry, (Hensby CN et al, 1979 and Hensby CN, 1981). On the basis of these correlations, the Wellcome antiserum was applied to clinical studies reported later in this thesis. After prolonged use of the assay however, certain discrepancies in measurements of duplicate samples lead to questions about the reproducibility of the procedure. Several experiments confirmed this suspicion. The extraction process was found to affect accuracy of 6-keto $PGF_{1\alpha}$ measurement, with an unacceptably high coefficient of variation. Furthermore there was little correlation between recoveries and levels of 6-keto $PGF_{1\alpha}$. In fact it was often found that when recoveries were high the measurements were low, and vice versa. These findings suggested that uneven resuspension of the extracted material was taking place.

It therefore became clear that the extraction method caused major interference with the assay, resulting in poor accuracy and reproducibility in plasma 6-keto $PGF_{1\alpha}$ measurement. At about the same time other workers began to report similar problems with the assay, (Greaves M et al, 1982). In retrospect, perhaps the reproducibility and variability of the assay should have been checked before embarking on these clinical studies but at the time it was the only technique available to undertake these measurements.

Further information to implicate the extraction process subsequently became available. Smith et al using the high affinity Cardeza antiserum could not detect any

6-keto $PGF_{1\alpha}$ in un-extracted serum, but did measure sizable quantities in conventionally extracted plasma. If the currently reported levels of 100pg/ml 6-keto $PGF_{1\alpha}$ were correct, then this high affinity antiserum should have been able to detect significant amounts in 0.1ml plasma without prior extraction. Other workers reported that unextracted plasma treated by TLC always formed a single peak of radioactivity, but that extracted plasma formed 1 to 5 peaks, (Mitchell MD et al, personal communication). This suggested that during the extraction process several related substances to 6-keto $PGF_{1\alpha}$ were formed. The likeliest mechanism in formation of these substances is the acidification step. This is known to convert some prostanoids to those of another series, e.g. PGE to PGA compounds, (Granstrom E et al, 1978).

In our laboratory, the high affinity of the Cardeza antiserum was confirmed, with direct measurement of known added amounts of 6-keto $PGF_{1\alpha}$ to plasma, and in samples taken during PGI_2 infusion. Again, no 6-keto $PGF_{1\alpha}$ could be directly detected in normal human plasma, confirming the findings of Smith et al. Of some concern was the greater than 100% recoveries commonly obtained which suggested that plasma proteins did interfere with this RIA to a certain extent.

All these findings confirmed that all measurements on extracted plasma had to be reassessed. Without question, the levels of 6-keto $PGF_{1\alpha}$ so measured are inaccurate. This data was not totally discarded however since the levels were a reflection of 6-keto $PGF_{1\alpha}$ either in a modified or closely related form. Subsequent measurements were made with the Cardeza antiserum, accepting the possible interference, albeit minor, by plasma proteins. It became clear that 6-keto $PGF_{1\alpha}$ is

not detectable in pg quantities in normal human peripheral plasma.

In conclusion, 2 RIAs were applied to 6-keto $PGF_{1\alpha}$ measurements in this work. Initially the Wellcome antiserum RIA was used until its inaccuracy in extracted plasma was confirmed. Further measurements were made with the reliable and accurate Cardeza antiserum RIA directly on plasma (peripheral and portal venous). Because of the very small levels of 6-keto $PGF_{1\alpha}$ detected, and to a lesser extent, because of potential interference by plasma proteins at the limit of sensitivity of this assay, this too was abandoned in the final part of this study.

3.4 Measurement of 6-keto $PGF_{1\alpha}$ by Gas Chromatography /Mass Spectrometry

Gas chromatography/mass spectrometry (GC/MS) is generally accepted at the present time as the most sensitive and specific method available for measurement of prostanoids present in small quantities. In view of the considerable problems experienced with the RIAs, plasma 6-keto $PGF_{1\alpha}$ measurements in the final stage of this study were made using a more specific and sensitive variation of this method. Essentially this technique became available for use in this study in 1983 in collaboration with Dr Sue Barrow, Clinical Pharmacologist at the Royal Post Graduate Medical School, Hammersmith Hospital, London and by kind permission of Sir Colin Dollery.

3.4.1 Gas Chromatography / Negative Ion Chemical Ionisation Mass Spectrometry

The sensitivity of standard GC/MS in 6-keto $PGF_{1\alpha}$ measurement can be adversely affected by technical problems. These are difficulty in separation of 6-keto PGF1α from other endogenous prostanoids, and of the occurrence of extensive mass spectral fragmentation, (Barrow SE et al, 1982). Furthermore GC/MS assays for 6-keto $PGF_{1\alpha}$ operate close to the limit of sensitivity, (Hensby CN et al, 1979[c]). The range of plasma concentrations measured by such assays (20 to 400pg/ml) was found to be at variance with estimated normal PGI_2 production. Based on urinary excretion rates of 2 of the metabolites of PGI_2, plasma levels were estimated to be in the low pg/ml range, (Oates JA et al, 1981). Professor Dollery's group at the Royal

Post Graduate Medical School, London, concluded that a more specific and sensitive method than standard GC/MS was needed. Thus such an assay based on capillary column gas chromatography coupled with negative ion chemical ionisation mass spectrometry (GC/NICIMS) was developed by this group, and is well described by Blair et al, (Blair IA et al, 1982).

Method of blood collection

Blood was collected by venepuncture of using a 21G needle with prior approval from the Ethics Committee of the Royal Free Hospital. 20ml of blood was collected into ice cold heparin tubes and centrifuged within 15 minutes of collection at 4^0C at 1,000g. After separation of plasma by aspiration, an internal standard of 2ng of tetra deuterated 6-keto $PGF_{1\propto}$ (3, 3', 4, 4'-2H4-6-keto PGF_1 alpha) was added to an extracted volume of plasma (usually 5 or 10ml). Samples were then stored at -20^0C until extraction, derivatisation and assayed by capillary column GC/NICIMS.

3.4.2 Method of GN/NICIMS assay

The method is described in detail by Professor Dollery's group (Blair IA et al 1982) and summarised as follows. After extraction utilising SEP-PAK columns, the residue dissolved in methanol was treated with methoximation of the 6-keto function, then subjected to TLC purification. The purified methoxyamine derivative was submitted to esterification and trimethylsilylation of the 3 hydroxyl groups. Standard curves for 6-keto $PGF_{1\propto}$ in the range 0 to 10pg/ml were prepared by addition of appropriate 6-keto $PGF_{1\propto}$ concentrations to 20ml normal pooled plasma and by processing through the assay procedure. Concentration of 6-keto $PGF_{1\propto}$ was plotted against peak height ratios of protium and deuterium ions to construct calibration curves. A

linear regression equation was calculated to allow determination of the unknown 6-keto $PGF_{1}\propto$ concentrations from the slope of the curve.

3.4.3 Results

A calibration curve for 6-keto $PGF_{1}\propto$ extracted from pooled plasma is shown in figure 3.4.3.1.

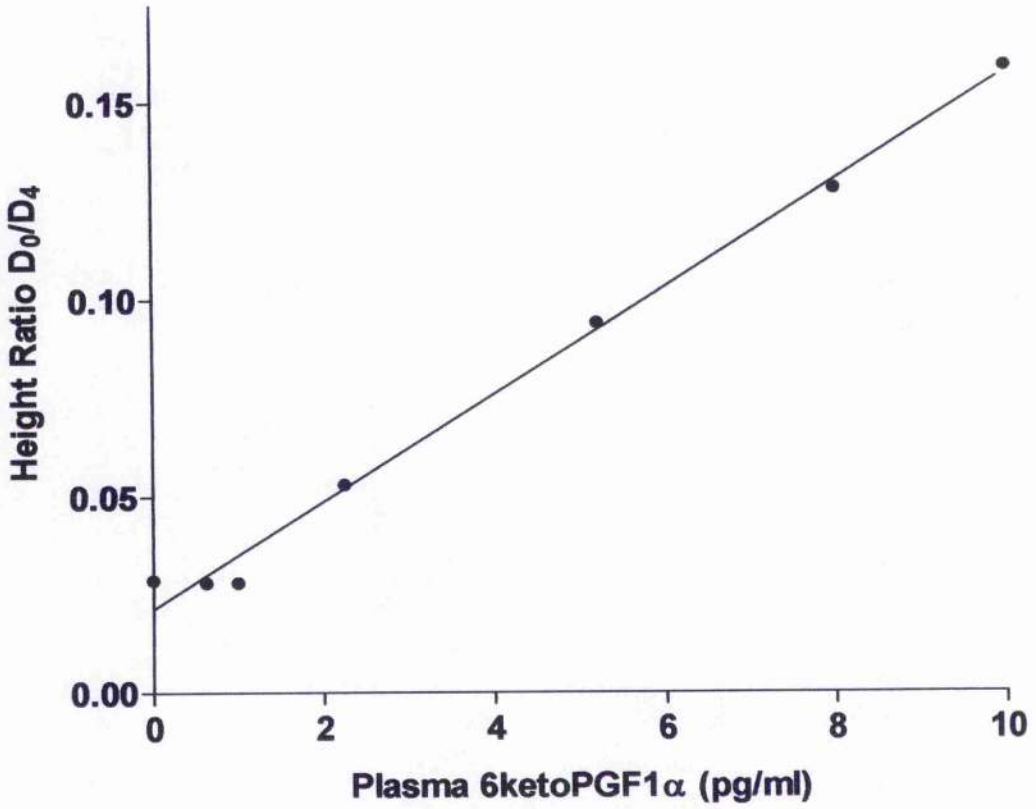


Fig 3.4.3.1 Calibration curve for 6 keto PGF1α extracted from pooled plasma.
$y = 0.014x + 0.017$; $r^2 = 0.995$ (from Blair IA et al 1982)

Typical mass chromatograms for 6-keto $PGF_{1}\alpha$ extracted from plasma are shown in figures 3.4.3.2 and 3.4.3.3.

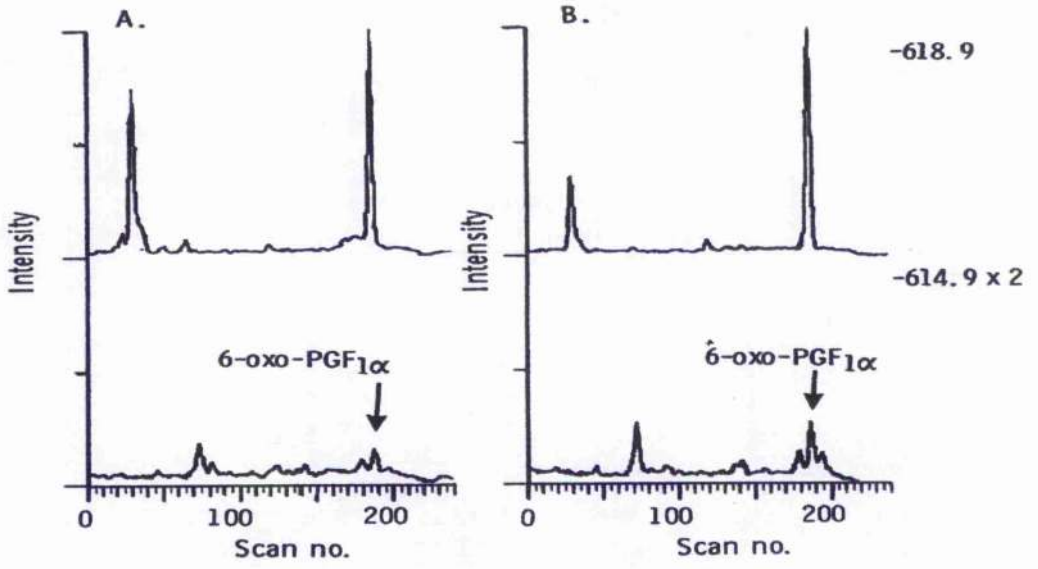


Fig 3.4.3.2. Limited mass chromatograms for 6-keto $PGF_1\alpha$ extracted from plasma. (from Blair IA et al 1982)

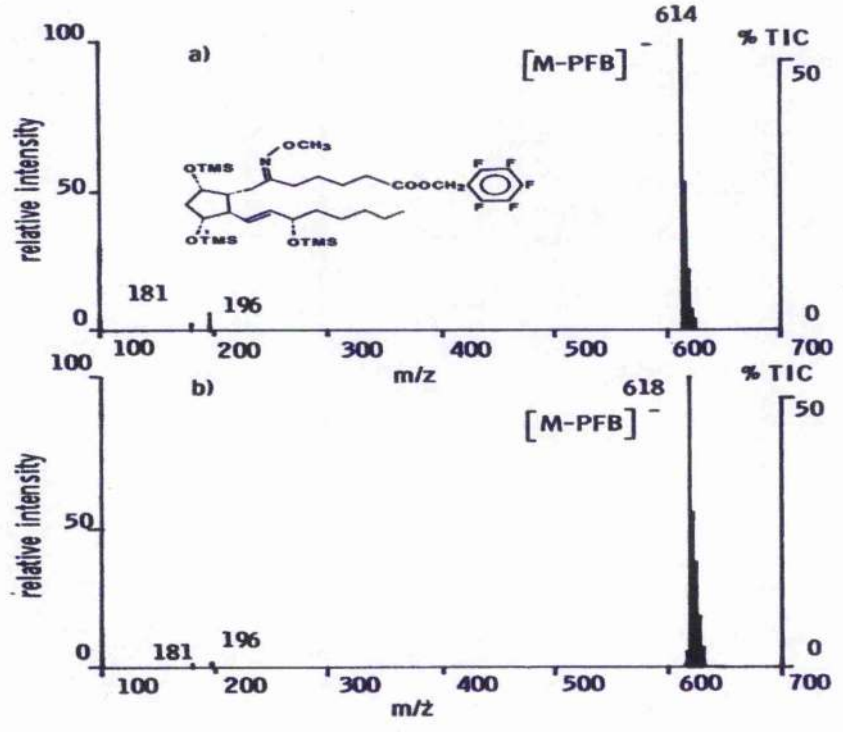


Fig 3.4.3.3. NICI spectra of a) 6-keto $PGF_1\alpha$ and b) radio-labelled 6-keto $PGF_1\alpha$

(from Blair IA et al 1982)

Recovery from this assay was 50%. The assay was shown to be highly accurate with measurements of 0.82pg/ml and 4.92pg/ml being made from plasma samples containing known concentrations of 1pg/ml and 5pg/ml respectively (n = 5). The lower limit of sensitivity of the assay was 0.5pg/ml. Plasma levels in 20 normal volunteers were found to be within a range of < 0.5 to 2.49 pg/ml, (Blair IA et al, 1982).

3.4.4 Conclusion

This assay based on capillary column gas chromatography coupled with negative ion chemical ionisation mass spectrometry is highly specific and sensitive with a much lower detection limit that the Cardeza antiserum (<0.5pg/ml -v- 10pg/ml). The two levels of 6-keto $PGF_{1\alpha}$ in peripheral blood are shown to be much lower than reported with RIA or standard GC/MS measurements.

SUMMARY OF MEASUREMENT OF PGI_2

A constant feature in the history of studies on prostaglandins in general and PGI_2 in particular, was the great problem encountered in accurate measurement of these evanescent substances. Assay of tissue extracts or plasma from species such as the rat which produce PGI_2 in relatively large quantities, has been reasonably straight forward. Unfortunately man produces PGI_2 in very small quantities and the literature on human PGI_2 assay is characterised by a constant search for more accurate methodology. As more accurate assays have been developed, accepted normal human plasma levels have steadily fallen from nanogram to picogram to less than picogram quantities.

Measurements in this study began with platelet nephelometry on rat tissue extracts which, because of the high production of PGI_2 by the rat proved to be acceptably reliable and accurate. The assay however could only measure PGI_2-like activity.

The development of the RIA for 6-keto $PGF_{1\alpha}$ allowed accurate measurements in tissue extracts with good correlation between RIA measurements and PGI_2-like activity. It was only after a considerable period of time that awareness of the inaccuracy of RIA on extracted plasma dawned, both in our laboratory and in the wider scientific community. The development of a more specific antiserum which obviated the need for plasma extraction allowed accurate measurement of 6-keto $PGF_{1\alpha}$. However, the levels found in plasma were found to be around or below the lower level of detection of this method.

The final part of this study made use of GC/NICIMS for plasma measurements. This is the most accurate and sensitive method currently available but is extremely laborious and time consuming with a typical assay taking a week to complete. This assay was carried out in the laboratories of Professor Colin Dollery at the RPMS with heavy demands on their technical expertise and time from many other sources. For these reasons, application of the assay was limited necessarily to relatively small numbers of samples in the final part of the study.

CHAPTER 4: EXPERIMENTAL STUDIES IN PROSTACYCLIN ACTIVITY AND PORTAL HYPERTENSION

Introduction

Haemorrhage is the major complication and cause of death in portal hypertension. As has previously been discussed (chapter 1), once portal hypertension is established a major and important factor in variceal haemorrhage is deficient coagulation. Prompt and massive replacement of clotting factors and blood will improve and even correct the bleeding diathesis as measured by prothrombin time (PT) and activated partial thromboplastin (aPTT). There are situations however where massive bleeding continues despite these measures often even in the presence of normal or near normal clotting studies. In such circumstances, other mechanisms in addition to those of deficient synthesis of coagulation factors, increased fibrinolytic activity and thrombocytopaenia may be of primary importance.

The discovery of prostacyclin's important role in the platelet vessel wall interaction and its function in counteracting the pro-aggregatory property of TXA_2 led many workers to investigate its possible role in many diseases. Alterations in the PGI_2 stimulating or inhibiting activity of plasma from patients with haemorrhagic or thrombotic complications were described in 1978 (Remuzzi G et al, 1978[b]). At about this time van Hoof, Chamone and Vermylen observed that some patients with liver or renal disease had a raised level of PGI_2 stimulating factors in their plasma, (Van Hoof A et al, 1979).

These findings led us to the following hypothesis: in portal hypertension excessive

production of PGI_2 might occur thus contributing to the haemostatic defect which is a major factor in the severity of haemorrhage from oesophageal varices.

To investigate this hypothesis further, an experimental model of acute portal hypertension was needed. This can be achieved in a variety of ways including occlusion of the portal vein using cellophane, by injection of silica or microspheres into the portal vein, portal vein injection of schistosomiases, by various models of induced cirrhosis, and by partial ligation of the portal vein, (Sherlock S, 1981). I chose to use partial ligation of the portal vein in the rat as the simplest technique for inducing portal hypertension without the complicating presence of liver disease in the majority of studies, with microsphere injection used in one set of experiments to mimic pre-sinusoidal obstruction.

4.1 Methods

4.1.1 Experimental Animals

In all experiments, male Sprague-Dawley rats weighing between 150-200g were used. These animals were bred by, and obtained from, the Comparative Biology Unit at the Royal Free Hospital. They were kept in standard cages with a maintenance diet and water ad libitum. Previous studies in our laboratories had shown that the normal portal vein of these animals produced significant levels of PGI_2 as measured by bioassay.

4.1.2 Operative Procedures

All surgical procedures were carried out under light ether anaesthesia (unless otherwise stated) between 0800-1400hrs. The peritoneal cavity was opened by an upper midline incision and abdominal closure was by mass suture of the peritoneum

and muscle layers using 0 chromic catgut, and skin closure with continuous 2/0 silk.

4.1.3 Measurement of Portal Vein Pressure

Pressure was measured by simple manometry and expressed as cm of water, taking the level next to and outside the portal vein at the porta hepatis and at the site of insertion of the needle as 0cm (fig 4.1.3). The portal vein was cannulated with a 25G butterfly needle via a 3-way tap to the manometer and to a reservoir of normal saline at room temperature. Portal pressure was then measured once the hydrostatic column had stabilised. This simple technique proved reliable and without major complication. Haemorrhage after withdrawal of the needle, even from hypertensive veins, was easily controlled by gentle pressure with a gauze swab, and with these precautions was always minimal.

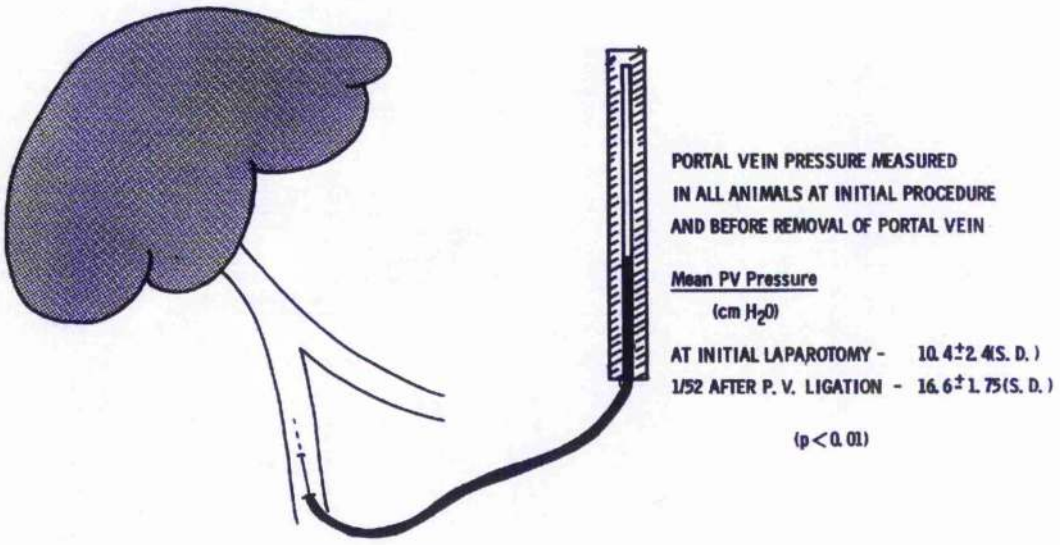


Fig 4.1.3 Portal vein pressure measurement

4.1.4 Induction of Portal Hypertension

The portal vein was mobilised in a standard manner by incision of the peritoneum over the portal structures and careful dissection of the portal vein from the surrounding structures, namely the common bile duct, the hepatic artery and the peri-portal tissues. Portal hypertension was induced by a technique of partial ligation previously described by Halvorsen and Myking, (Myking AO et al, 1973). A metal tube of standard diameter (1.2mm) was placed alongside the portal vein (typically between 2 and 3 mm in diameter) and a 2/0 polyamide ligature (non-absorbable) was tied firmly around both as close to the liver as possible but taking care to keep below the bifurcation of the portal vein. The tube was then gently removed from the ligature leaving the vein constricted to a standard diameter of 1.2mm (figures 4.1.4 a, b, c and d). This is equivalent to inducing a stenosis of >50% of the original diameter. No antibiotics or other drugs were given. Other operative techniques are fully described in the relevant sections.

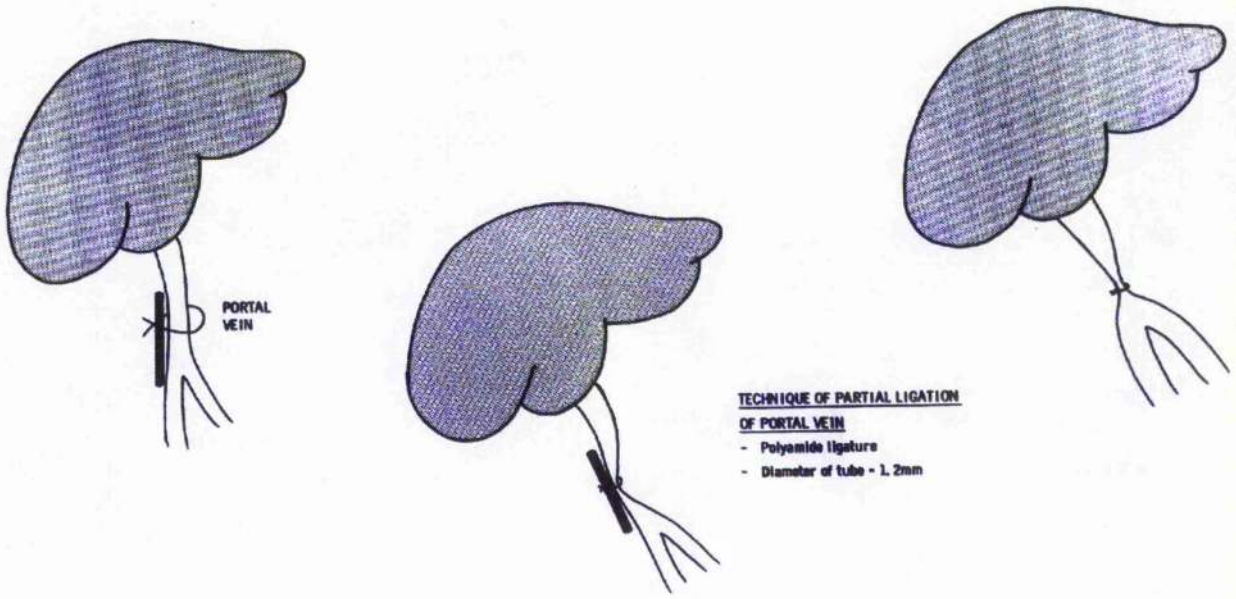


Fig 4.1.4 a. Partial portal vein ligation using 1.2 mm tube

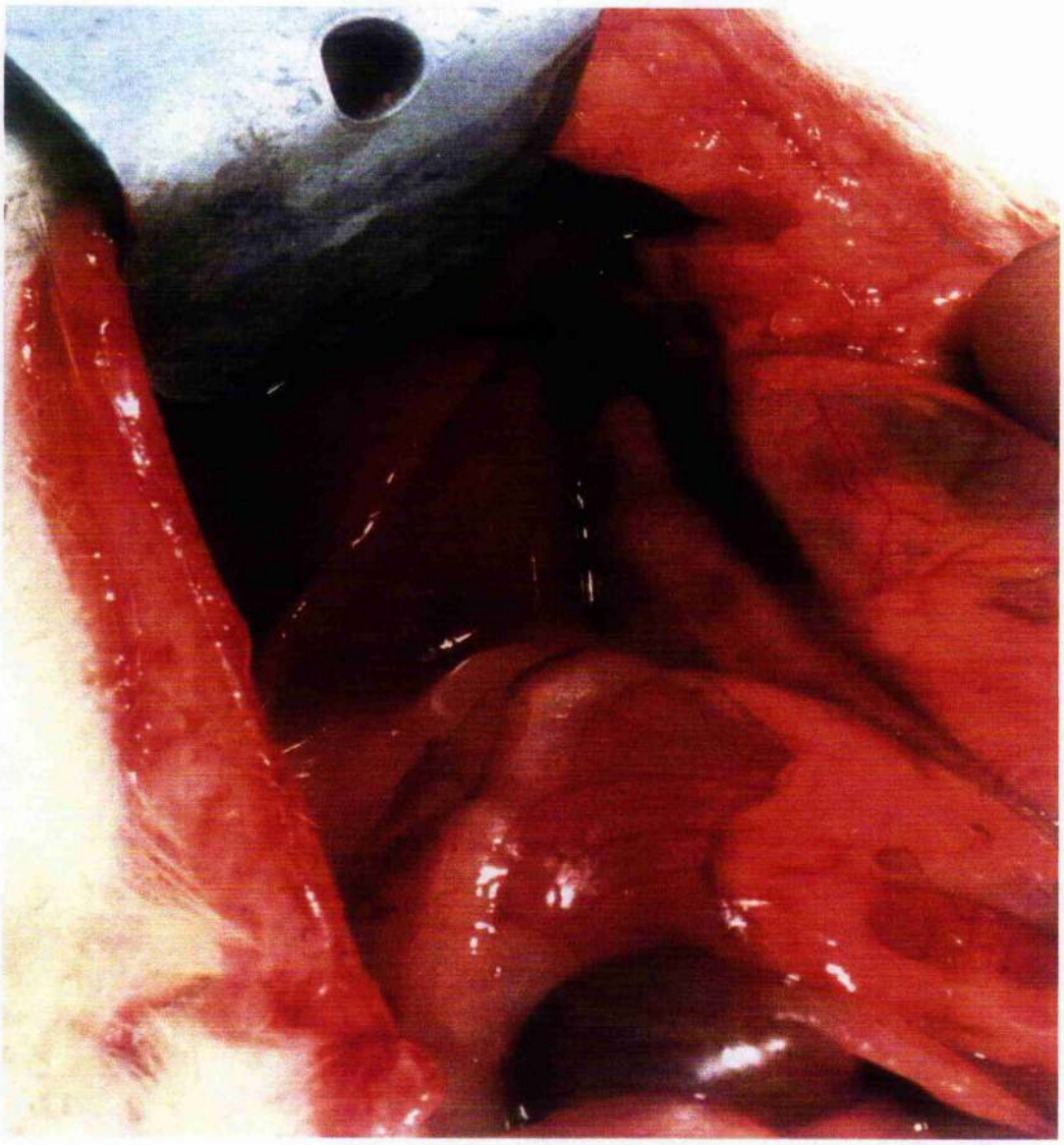


Fig 4.1.4 b. Operative display of the rat portal vein:

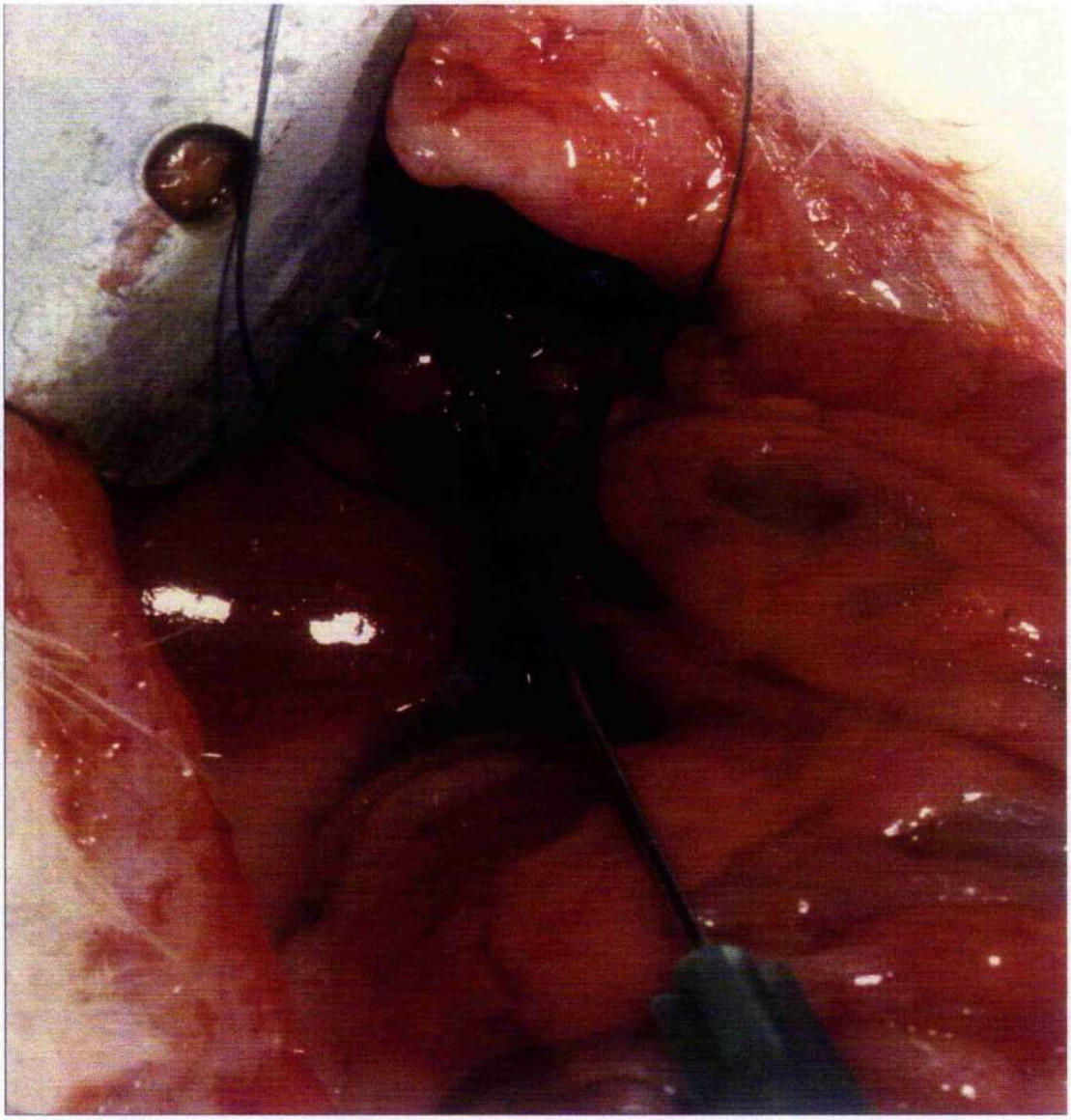


Fig 4.1.4 c. Mobilised portal vein and 1.2 mm diameter needle encircled just below the liver with the non-absorbable ligature

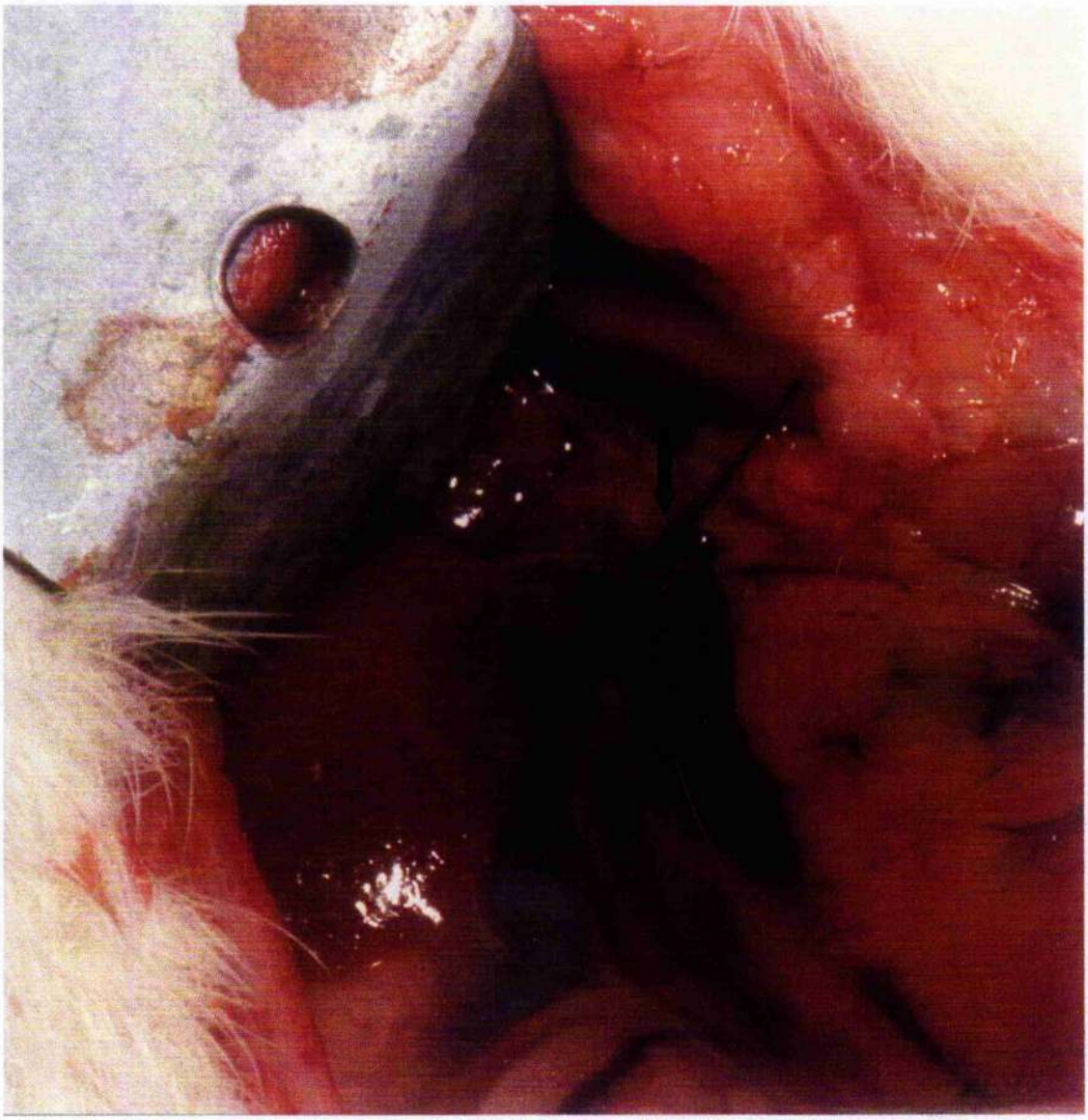


Fig 4.1.4 d. Portal vein partially ligated to diameter of 1.2 mm after removal of the needle

4.1.5 Harvesting and Preparation of Tissues

Segments of portal vein obtained from animals or patients were used to generate PGI_2-like activity. This was measured by bioassay or when it had become available, by measurement of 6-keto $PGF_1\alpha$ using a RIA. All tissues and the tris-buffer in which they were suspended were kept at 4°C prior to testing in order to minimise loss of activity due to the instability of PGI_2.

a). Rat Veins

In rats the portal and superior mesenteric veins and the abdominal inferior vena cava (IVC) were harvested after measurement of pressure. Harvesting was by dissection from surrounding structures and tissues and excision of a 2-3cm section of vein. The specimen was immediately placed in a petri dish at 4°C. After removal of adventitial tissue and fat, the vein was washed with 4°C tris buffer pH 8 (appendix A for preparation of buffer). The vein was then chopped into 1mm rings. These were quickly blotted on filter paper, weighed and re-suspended in the appropriate volume of tris buffer (50mg of tissue / 1ml buffer), and then incubated at 20°C for three minutes. After removal of the rings, the resultant tris buffer solution was stored at 4°C and tested as soon as possible by the platelet anti-aggregation bio-assay, or kept at -40°C until analysed by the 6-keto PGF_1 alpha RIA. All assays were performed within a month of collection of the sample.

b). Human Veins

With prior Ethical Committee approval and patient consent, samples of mesenteric and omental veins, 2-3cm in length, were collected at abdominal surgery as

atraumatically as possible. These samples were collected into ice and immediately transferred to the laboratory where they were processed in the same manner as rat veins. The rings were re-suspended in tris buffer pH 7.5 at a concentration of 25-200mg/ml depending on the amount of tissue available. The suspension was then incubated at 20°C for three minutes using a vortex mixer to maximise mixing during the first 15 seconds of this incubation period. The fluid was then removed by aspiration and either tested immediately or stored at -40°C for later assay.

4.2 EXPERIMENT 1

4.2.1 Introduction

In this set of experiments, portal vein PGI_2 activity was compared in groups of rats that had portal hypertension induced, with various sham operations and controls.

In group 1, 20 rats were anaesthetised with ether, the peritoneal cavity opened in the standard manner and portal vein pressure measured by simple manometry. The portal vein was mobilised, the standard metal tube, 1.2mm in diameter laid alongside the vein and a non-absorbable suture tied firmly around both. The tube was then removed leaving a constriction of 1.2mm. Having ensured haemostasis, the abdomen was closed and the animals allowed to recover.

A further group of 10 rats (group 2) were anaesthetised as before but did not undergo any surgical manipulation or incision. Anaesthesia was maintained for 20 minutes, which is approximately the same time taken for the surgical group's intervention, and the animals then allowed to recover. This group provided baseline control.

Three further control groups of 10 rats each (groups 3, 4 and 5) were set up to investigate the effect of surgical manipulation on portal vein pressure and PGI_2 production. In all these three control groups, the duration of anaesthesia was standardised at 20 minutes and portal pressure was measured in the routine manner.

Group 3 - Ten rats underwent anaesthesia and laparotomy without any manipulation or dissection of the portal vein other than that required for portal vein pressure measurement.

Group 4 - Ten rats underwent anaesthesia, laparotomy and dissection of the portal vein from the portal structures without ligation.

Group 5 - Ten rats underwent anaesthesia, dissection of the portal vein and ligature constriction exactly as described for group 1 but the ligature was removed after 60 seconds, (i.e. a sham ligation).

In two additional groups of six animals each (groups A and B), group A underwent permanent partial ligation of the portal vein, and group B underwent laparotomy and portal vein dissection alone. All groups were allowed to recover and were kept under identical conditions for one week.

In each group, seven days after the original procedure, the following was carried out. Under ether anaesthesia a laparotomy was performed noting in particular the state of the liver and intestines. Portal pressure measurement was carried out in the standard manner, and 2-3cm of the portal venous system was excised taking care not to take the hepatic artery or the bile duct, and excluding the ligatured area. The section excised was the hypertensive part where this had been induced or the anatomically equivalent section in the non-ligated animals. The rat was then killed by opening the thoracic cavity and transecting the IVC. The specimens were immediately collected and prepared for generation of PGI_2 like activity. To minimise observer bias, the samples were coded after harvesting and identified during the assays only by the code number.

Samples from groups A & B were similarly prepared but in addition to bioassay, 6-keto $PGF_1\alpha$ was measured by RIA as described previously (chapter 3.3.1). ADP-ase activity was determined in each sample as described (chapter 3.2).

The results were analysed by Wilcoxon's two-sample test and by linear regression. The results from groups A & B however were analysed using Student's paired t test.

4.2.2 Results

At laparotomy no abnormalities were noted in the liver or intestines, (e.g. necrosis, congestion etc.), in any of the rats. The only consistent finding was a minor degree of adhesion formation around the porta hepatis in all groups.

4.2.2.1 Portal Pressure

Time	Group 1	Group 2	Group 3	Group 4	Group 5
Day 1	10.4 (2.4)	----	9.8 (1)	10.1 (1)	10.2 (1.3)
Day 7	16.6 (1.8)	10.4 (0.9)	10 (2.2)	10.1 (2.3)	10.5 (2.4)

Table 4.2.2.1.Portal pressure (cm H_2O) in surgical groups (mean +/- (SD)) Group 1 – permanent partial portal vein ligation; Group 2 – GA alone; Group 3 – laparotomy without portal vein manipulation; Group 4 – portal vein dissection alone; Group 5 – temporary portal vein ligation (60 seconds).

In the control groups which had undergone laparotomy without permanent ligation of the portal vein, i.e. groups 3, 4 and 5, there was no difference in portal pressure at seven days when compared to the original pressure reading. Neither was there any

difference in the group (group 2) which had undergone anaesthesia alone. In the rats with permanent partial portal vein ligation (group 1) however, there was a highly significant increase in pressure from 10.4 (+/- 2.4) to 16.6 (+/-1.8) cm H20 (mean +/- SD) after 7 days ($p<0.01$). (see table 4.2.2.1)

4.2.2.2 PGI_2–like Activity

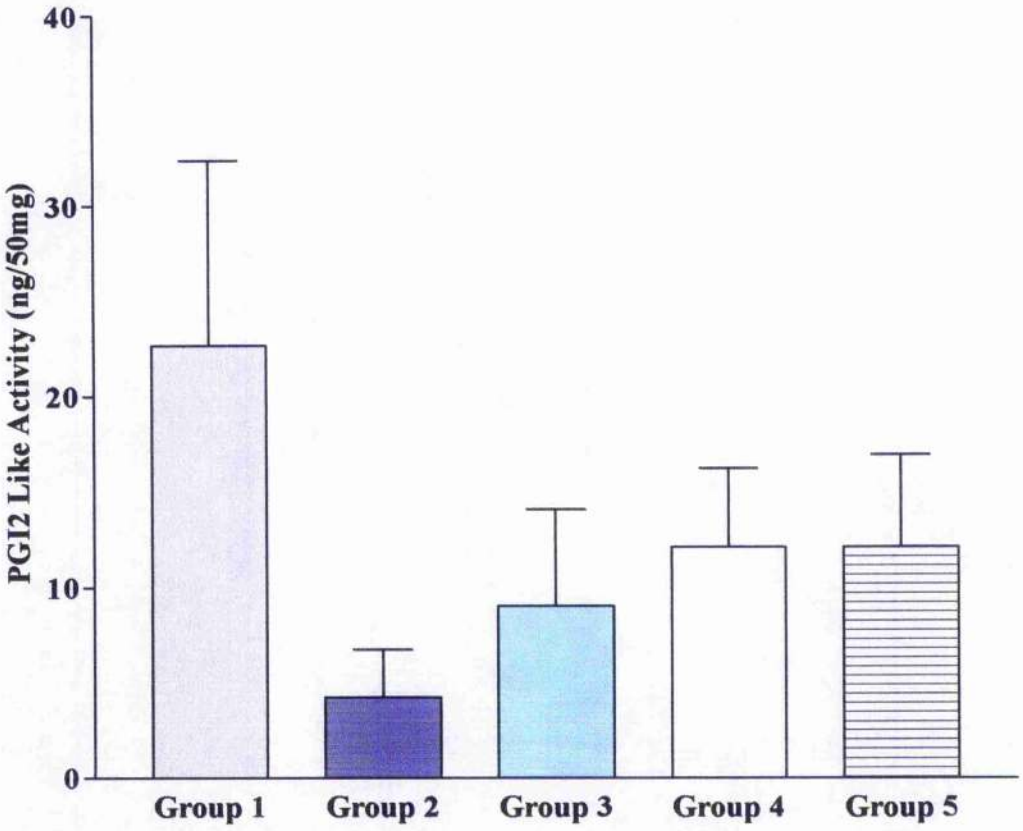


Fig 4.2.2.1 a. PGI2 like activity in rat portal veins after 7 days (mean & SD): Group 1 partial portal vein ligation (22.7 +/- 9.8); Group 2 GA alone (4.2 +/- 2.6); Group 3 laparotomy alone (9.2 +/- 5); Group 4 portal vein dissection without ligation (12.3 +/- 4.2); Group 5 partial portal vein ligation and removal of ligature after 60 seconds (12.3 +/- 4.8).

PGI_2-like activity in the portal veins of the rats which had undergone general

anaesthesia alone, (group 2), had the lowest levels. Compared to this group, all animals which had undergone laparotomy with or without portal vein procedures, (groups 3, 4 & 5), had significantly higher levels of PGI_2-like activity ($P<0.01$ in each group) (see fig 4.2.2.1 a). Between these groups there were no significant differences but group 5 had the highest level of activity. In the animals with permanent portal vein ligation and portal hypertension, (group 1), the greatest PGI_2-like activity of all groups was found, the levels being significantly higher than in any of the four control groups ($p<0.01$).

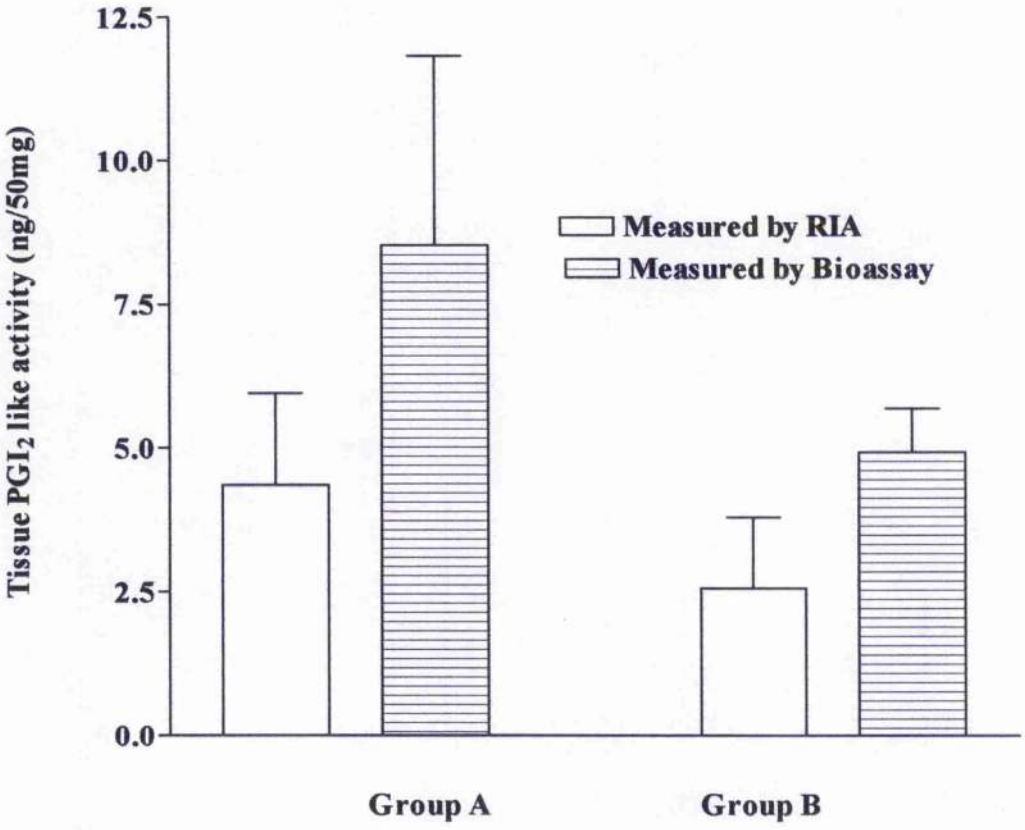


Fig 4.2.2.1 b. Rat portal vein PGI_2-like activity measured by Bio-assay and RIA (mean & SD).

Gp A partial portal vein ligation (RIA 4.4 +/- 1.6; Bioassay 8.5 +/- 3.3)

Gp B sham portal vein ligation (RIA 2.5 +/- 1.3; Bioassay 4.9 +/- 0.8)

Increased production of 6-keto $PGF_1\alpha$ as well as PGI_2-like activity was found in the animals with portal hypertension (see fig 4.2.2.1 b). The results from groups A and B revealed a strong correlation between PGI_2-like activity measured by bioassay and measured by RIA as 6-keto $PGF_1\alpha$ (see fig 4.2.2.1 c).

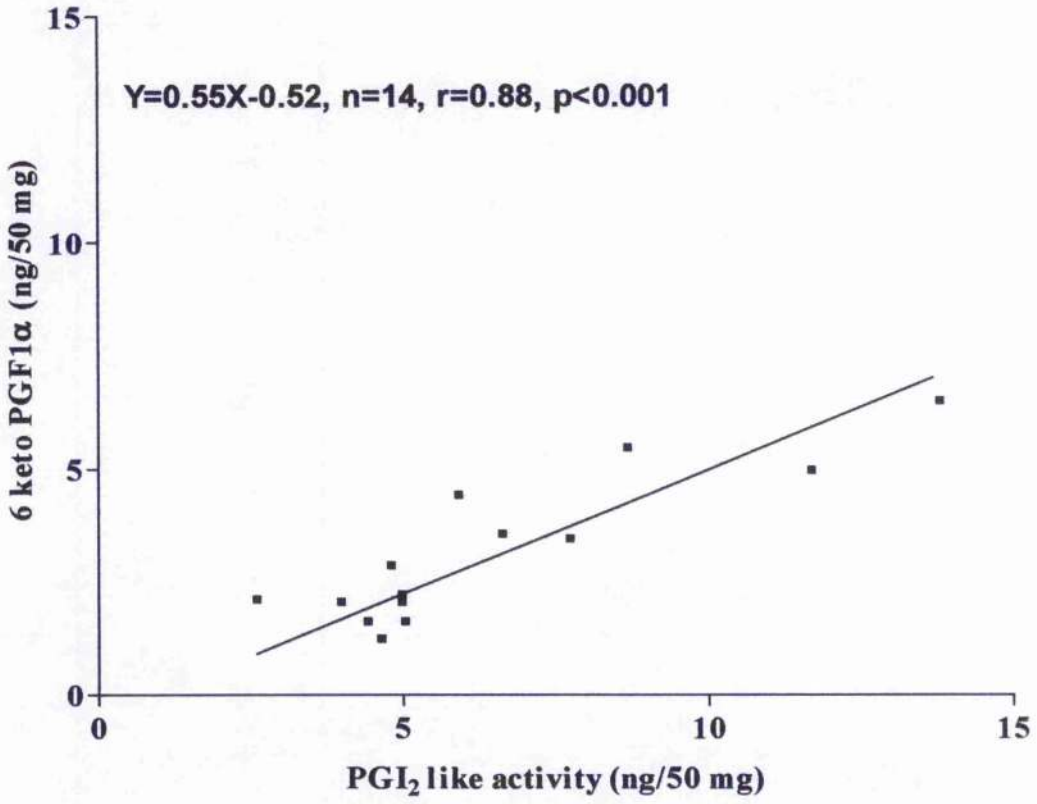


Fig 4.2.2.1.c. Relationship between PGI_2–like activity measured by bio-assay and 6-keto $PGF_1\alpha$ levels in rat portal vein.

Inhibition of platelet aggregation disappeared after incubation at 37°C for 20 minutes, thus ADP-ase activity could not be detected in any of the extracts.

4.2.3 Discussion

Partial ligation of the portal vein proved to be an effective and reliable technique for production of portal hypertension at seven days with minimal morbidity and mortality. Manipulation of the portal vein, including transient ligation, did not result in any increase of pressure above the normal range.

Established portal hypertension of seven days duration was accompanied by a highly significant increase in the production of PGI_2-like activity by the hypertensive segment of the portal vein. Because this activity was destroyed by incubation of the sample at 37°C for 20 minutes, and because there was no ADP degrading activity detected in any of the samples, the conclusion was made that this was true PGI_2-like activity. This observation was confirmed by the strong correlation found between PGI_2-like activity and 6-keto PGF_1 alpha ($R=0.88$, $p<0.001$). The low production of other prostaglandins with similar activity to PGI_2 by vascular endothelium supports the conclusion that the activity detected was due to production of the PGI_2-like substances, (Moncada S et al, 1977 and Moncada S & Vane JR, 1978).

Controls set up to differentiate the effect of the trauma of laparotomy and more importantly of manipulation of the portal vein, from the effect of hypertension, also showed significantly increase PGI_2-like activity compared to animals which had not undergone any surgery at all. This increase however was significantly lower than that produced by portal hypertension ($p<0.01$). The extent of surgical manipulation did not seem to be important since there was no real difference in PGI_2-like activity between the three non-hypertensive surgical groups.

These results indicate that surgery alone results in increased portal vein PGI_2 production. The explanation for this response is not clear but one can speculate that this may be a non-specific inflammatory response to the trauma of laparotomy, handling of the intestines and dissection of the portal vein. Cannulation of veins is known to stimulate release of PGI_2 and another possible explanation is the insertion of the manometry needle into the portal vein.

Over and above this non-specific response to surgery however, there was a significant increase in PGI_2 production by the portal vein associated with portal hypertension. Similar increases in PGI_2 production have been demonstrated in aortic extracts from spontaneously hypertensive rats when compared to normotensive animals (Pace-Asciak CR et al, 1978).

It may be that increased production of PGI_2 by hypertensive vascular tissue is an adaptive response to hypertension, and this would explain the increased activity seen in this model of portal hypertension. Increased local release of PGI_2 would exert a local vasodilatory response, thus tending to reduce hypertension. However, this raises the possibility that the increased production of PGI_2 might also affect platelet function by increased inhibition of platelet aggregation.

In summary, the results of this first experiment supported the hypothesis that increased portal vein PGI_2 production occurs in portal hypertension and raised the possibility that increased inhibition of platelet aggregation resulting from this might contribute to a haemostatic defect.

4.3 EXPERIMENT 2

4.3.1 Introduction

In experiment 1 portal hypertension was successfully achieved by the technique of partial ligation of the portal vein. This unavoidably entails direct mechanical trauma to the portal vein which as a result of the ligature could be chronic and result in inflammatory changes in the portal vein wall. Prostaglandins are known mediators of the inflammatory response and although the role of PGI_2 in this process is not defined, it is possible that the increased PGI_2 activity seen in the experiment could be as a result of inflammatory responses within the portal vein alone.

It is also possible that hepato-cellular function might be compromised in this experiment as a result of diminished portal inflow to the liver. This possibility must be investigated because major clearance of PGI_2 is probably by the liver as well as by the kidneys and skeletal muscle. Altered hepato-cellular function could result in reduced clearance of several vasoactive prostanoids with increased levels being the outcome. In addition, hepato-cellular dysfunction might affect levels of various substances produced by the hepatocyte such as bile acids, and other vasoactive substances known to promote splanchnic hyperaemia which are metabolised by the liver (glucagon, serotonin etc). These potential effects on vasoactive substances which modulate splanchnic blood flow may be involved in the regulation of PGI_2 synthesis. It is therefore important to exclude any adverse effect on hepato-cellular function in this model of portal hypertension before attributing changes in PGI_2 activity to hypertension alone.

Using portal injection of microspheres, a second mode of portal hypertension was developed which involved minimal trauma to the portal vein. Inflammatory change was assessed by histological and electron microscope scanning analysis, and hepato-cellular function was measured in both models of portal hypertension with the specific aim of assessing the contribution of direct trauma, inflammation and hepatic function to changes in PGI_2 production that was measured.

4.3.2 Method: Portal hypertension induced by injection of microspheres

With the collaboration of Dr Russell Woods, Research Scientist in the Academic Department of Surgery at the Royal Free Hospital School of Medicine, an alternative model of portal hypertension was developed. This was achieved by injection of a suspension of glass microspheres – 40 microns of mean diameter – which would result in partial obstruction to portal flow at the pre-sinusoidal level.

4.3.2.1 Preparation of the microsphere suspension

a) buffer

2% gelatin suspension in 0.1M sodium chloride solution was prepared to pH 7.35 using 0.05M potassium diphosphate buffer. The solution was then filtered through a 0.22μm pore filter to remove any gelatin aggregates.

b) microsphere suspension

2.352G of Ballotini glass beads 40 microns mean diameter, (British Glass UK), were suspended in the 2% gelatin buffer. This preparation resulted in concentration of approximately 600,000 spheres/ml as counted on a haemocytometer.

4.3.2.2 Procedures

13 male Sprague-Dawley rats (weight 150-200g) were anaesthetised with ether and the peritoneal cavity opened. Using a 3-way stopcock, portal venous manometry was carried out and then the 2% gelatin/microsphere suspension was injected into the portal vein until portal vein pressure >15cm of water was achieved and confirmed by repeat manometry after five minutes. The abdomen was then closed and the animals allowed to recover. A second group of six rats underwent laparotomy alone and a third group of six rats had partial portal vein ligation, and were allowed to recover.

Seven days later, under ether anaesthesia, the animals underwent laparotomy and any congestion or necrosis of the liver or intestines was noted. Portal venous manometry was then carried out prior to excision and processing for bioassay of the hypertensive segment of the portal vein exactly as described in experiment 1. A segment of liver was also taken for histological analysis by H & E staining. The animal was then killed.

Portal vein pressure and PGI_2-like activity in these rats were compared with levels measured in laparotomy only and partial portal vein ligated animals. Statistical analysis was by Wilcoxon's paired sample test.

4.3.2.3 Measurement of serum hepatic enzyme levels

Blood samples were taken from the tail vein under light ether anaesthesia from three groups of rats. The first group of six rats had undergone laparotomy alone, the second group of six had undergone partial portal vein ligation, and the third group of six had undergone portal injection of microspheres. The samples were collected into lithium heparin tubes before the procedure and at one, three, five and seven days

afterwards. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using an 'optimised standard method' U-V system kit (Boehringer Mannheim Gmbh Diagnostica).

4.3.2.4 Assessment of inflammatory response to partial ligation of portal vein

Six male Sprague-Dawley rats underwent partial ligation of the portal vein under ether anaesthesia and were allowed to recover. At seven days after the procedure, the portal vein was excised and for the purpose of histological and scanning electron microscope analysis was divided into three sections as follows:

a) proximal to the ligature, i.e. the non-hypertensive segment closest to the liver
b) around the ligature, i.e. the segment immediately around and including the ligature
c) distal to the ligature, i.e. the hypertensive segment

These specimens were collected into formalin and coded. Histological analysis was carried out in a 'blinded' manner by Dr Margaret Chappell, at that time Lecturer in Pathology at the Royal Free Hospital, now Consultant Pathologist at the Southend General Hospital.

4.3.3 Results

Using this technique of microsphere injection, a significant elevation of portal pressure was obtained at one week ($p<0.01$) (see fig 4.3.3 a). The degree of hypertension was less than that obtained with partial ligation although this difference was not significant.

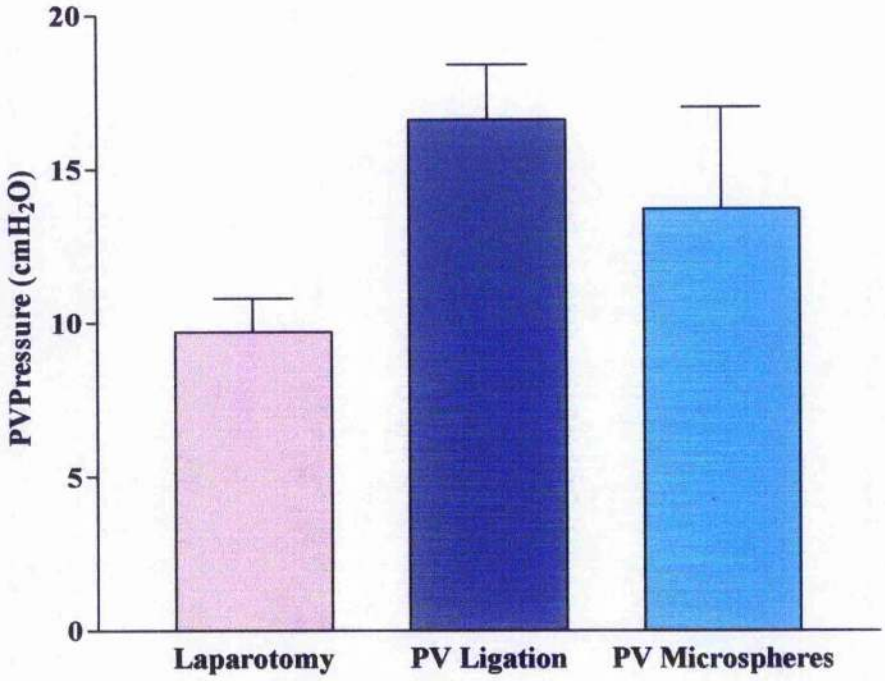


Fig 4.3.3.a Rat portal vein pressure comparing laparotomy alone (n=6; 9.7 +/- 1.1), partial portal vein ligation (n=6; 16.6 +/- 1.8) and portal vein microsphere injection (n=10; 13.7 +/- 3.3) (mean & SD).

There were several problems with this technique however. The main problem was of high mortality – three rats died of venous infarction of the gut in the immediate post-op period, presumably as a result of complete occlusion to the portal inflow by injection of excess numbers of microspheres. There were also technical difficulties in maintaining the microspheres in suspension for longer than 15 minutes after preparation thus severely limiting the time available for induction of anaesthesia, dissection and manometry. More importantly because of this problem the number of animals operated on at any one 'sitting' had to be limited.

PGI_2-like activity was significantly elevated in this group when compared to the group which had undergone laparotomy alone ($p<0.01$) (see fig 4.3.3 b). This increase was comparable to that in the group which had hypertension induced by partial ligation,

and statistically there was no difference in PGI_2-like activity between the two groups.

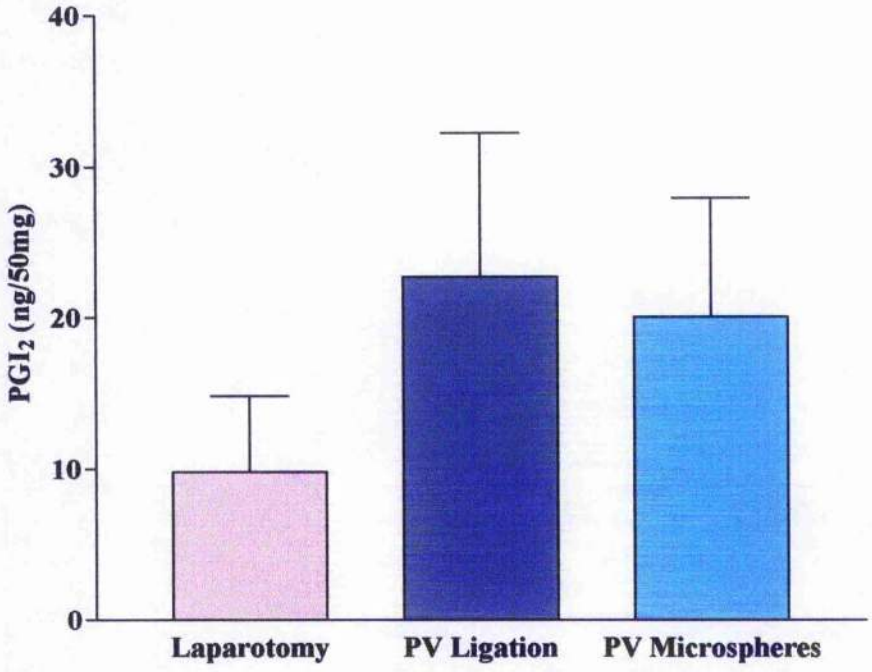
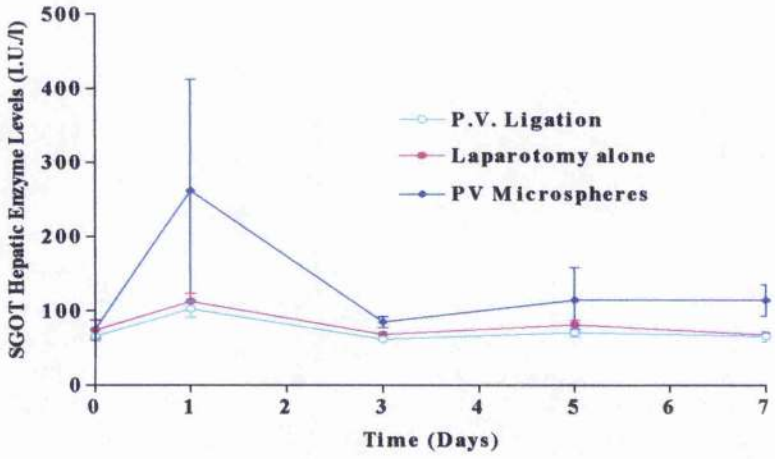
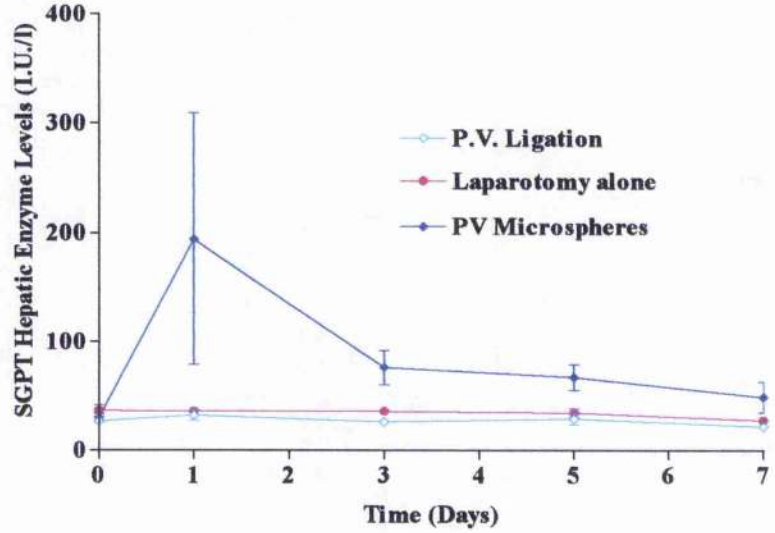


Fig 4.3.3.b Rat portal vein PGI_2 – like production after laparotomy (9.8 +/- 5), partial portal vein ligation (22.7 +/- 9.5) and portal vein microsphere injection (20 +/- 7.9) (mean & SD).

Serum AST levels were elevated on day one in both the laparotomy and partial ligation groups, but thereafter returned to normal (see fig 4.3.3 c). Serum ALT levels showed no elevation at all at any stage after either of these two procedures. In the group which had undergone microsphere injection however, there was striking elevation of both enzymes at day one, but the levels gradually fell approaching normality at day seven. In the animals that did not survive death occurred within hours of microsphere injection and before any post procedure samples could be obtained.



Time (days)	Laparotomy alone	P V ligation	PV microspheres
0	71 (14)	61 (5)	71 (12)
1	107 (11)	100 (10)	250 (143)
3	62 (4)	61 (5)	82 (7)
5	79 (5)	71 (6)	110 (39)
7	64 (2)	64 (3)	111 (21)



Time (days)	Laparotomy alone	P V ligation	PV microspheres
0	37.5 (5)	27.5 (5)	35 (5)
1	35 (2)	32.5 (5)	195 (115)
3	35	25	75 (15)
5	35 (5)	27.5 (5)	67.5 (12.5)
7	27.5	22.5	50 (15)

Fig 4.3.3.c SGOT & SGPT levels during first 7 days after laparotomy, partial portal vein ligation or portal vein microsphere injection (mean & SEM)

4.3.3.1 Histological analysis

Histological analysis of the portal vein seven days after ligation showed no significant inflammatory change either in the intima or other areas of the wall of the portal vein, either proximal, distal to or around the ligature (see fig 4.3.3.1 a & b). There was resolving inflammation of the surrounding connective tissue however, which was compatible with recent dissection.

Histological analysis of liver sections from rats injected with microspheres showed mild inflammatory reaction around the glass microspheres but otherwise normal architecture in the majority of animals (see fig 4.3.3.1 c). In two rats however, very severe chronic inflammatory changes with a prominent lymphocytic reaction and dilated sinusoids were seen. There was no recognisable normal liver tissue seen and haemosiderin laden macrophages were noted. (see fig 4.3.3.1.d)

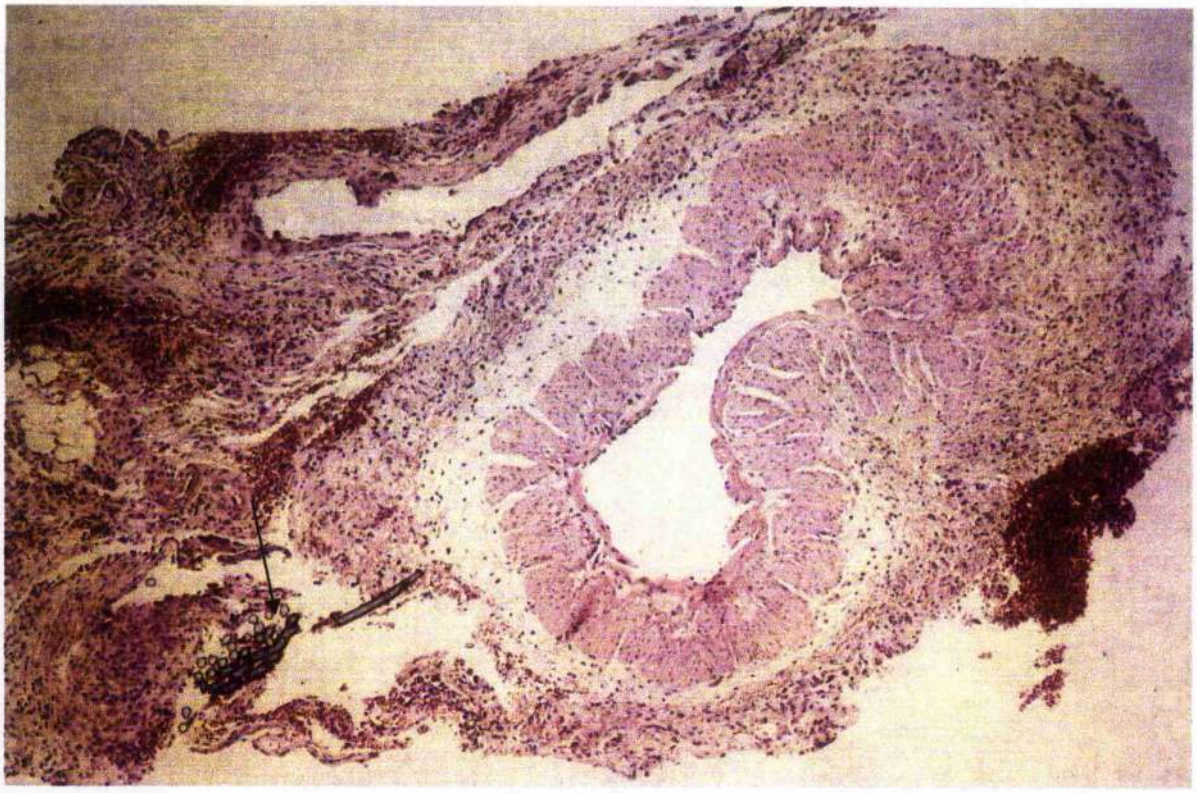


Fig 4.3.3.1.a H&E preparation of partial ligated rat portal vein at site of ligature: arrow marks the fibres of the polyamide ligature; there is perivascular inflammatory and fibrotic activity but the portal vein shows intact morphology with little inflammation.

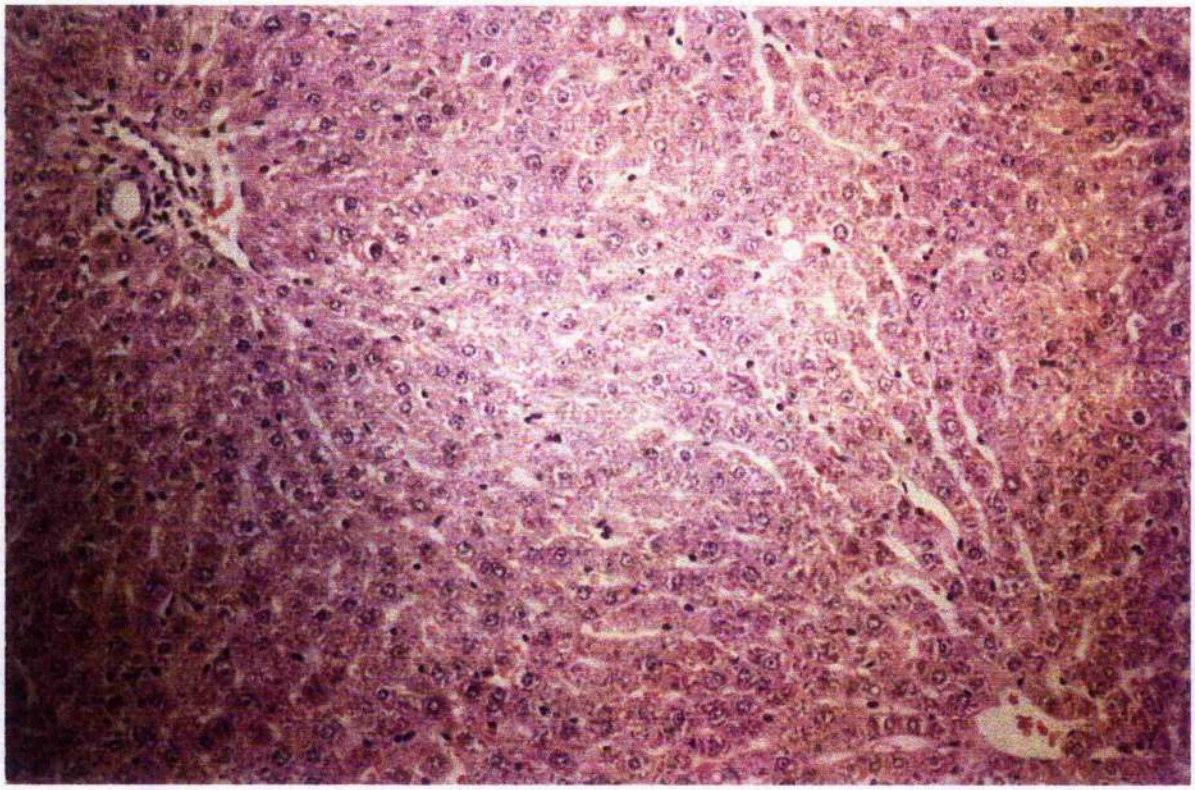


Fig 4.3.3.1.b H&E preparation of liver in partial portal vein ligated rat at 7 days: essentially normal liver structure.

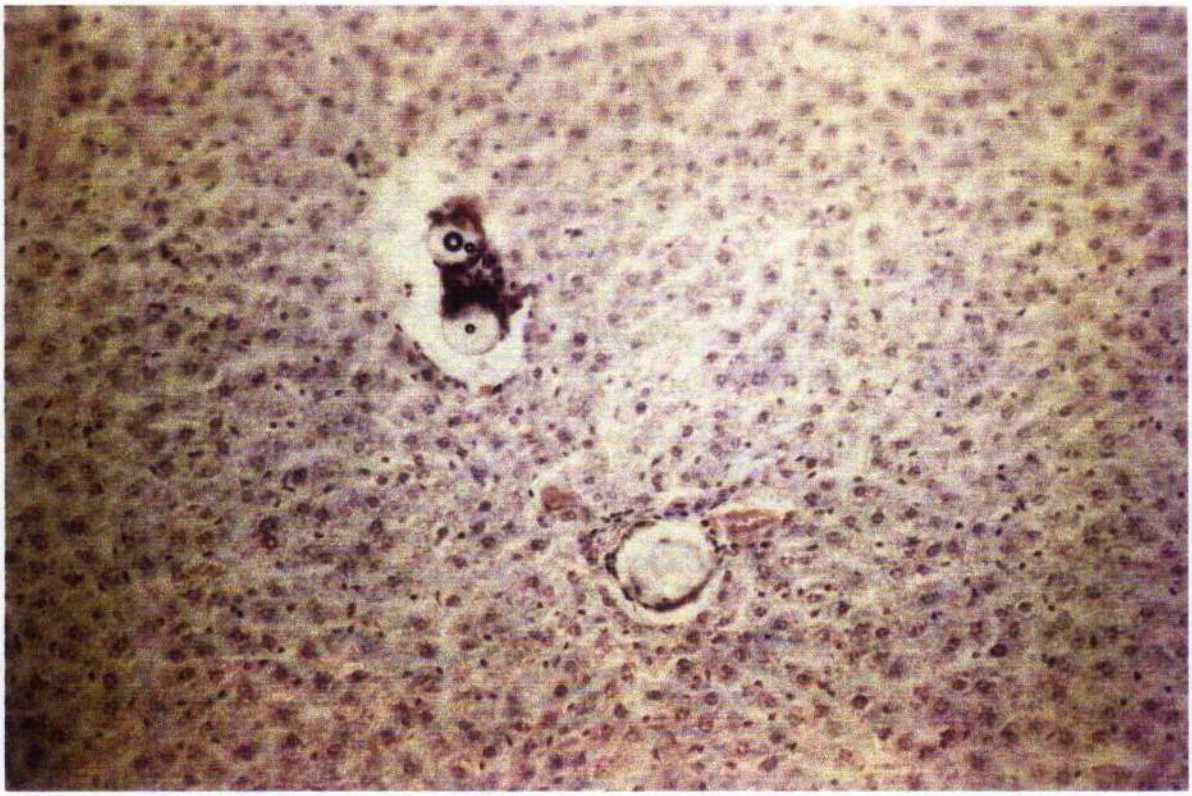


Fig 4.3.3.1.c H&E preparation of rat liver 7 days after portal vein injection of glass microspheres: mild inflammatory reaction around the microspheres but otherwise normal liver architecture was found in most rats.

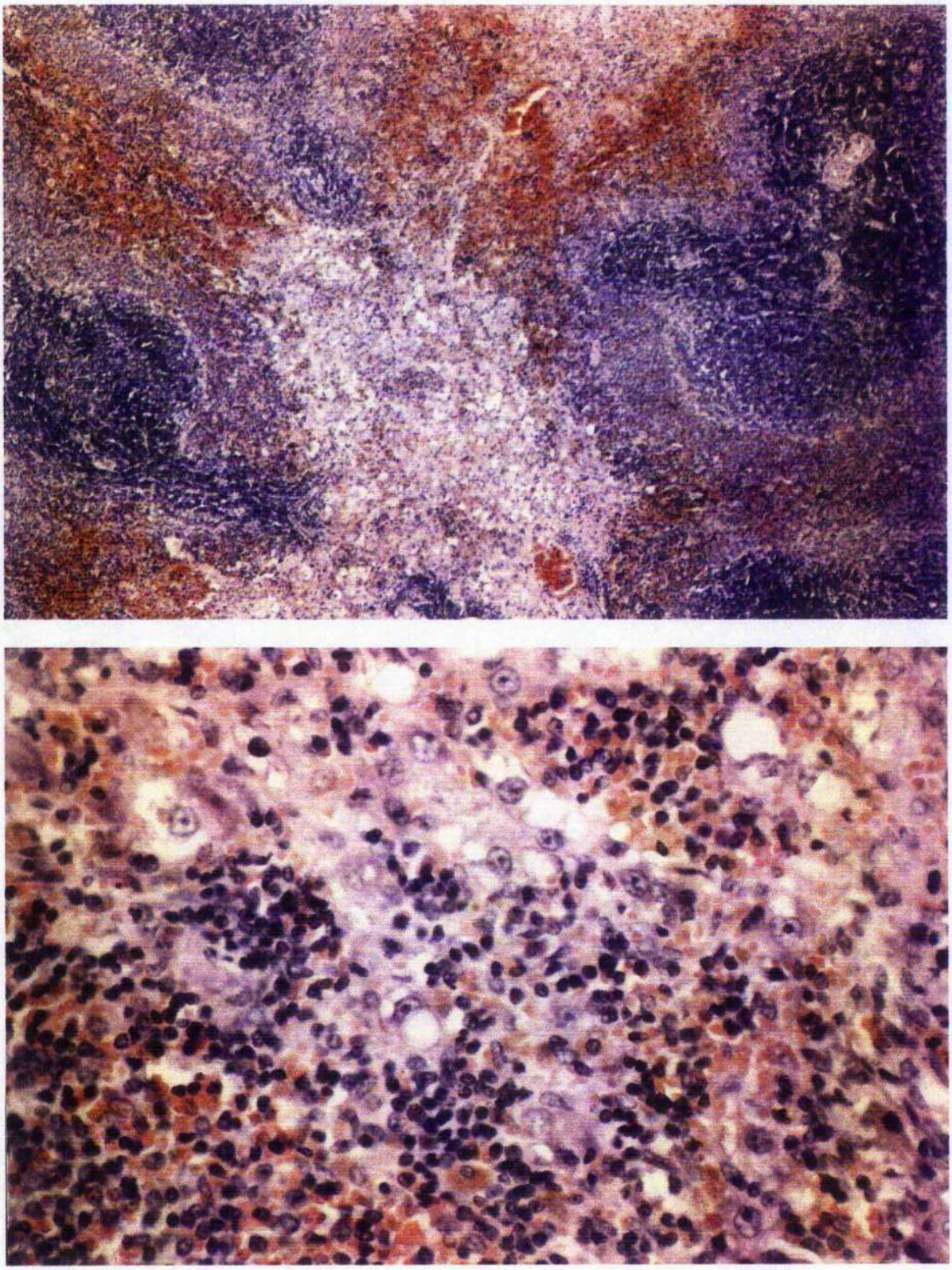


Fig 4.3.3.1.d Very severe liver damage seen in 2 rats 7 days after portal vein microsphere injection

4.3.3.2 Scanning electron microscopy of partial ligated rat portal vein

Scanning electron microscopy confirmed patency at the site of partial portal vein ligation. The hypertensive part of the vein was dilated but with normal structure. The endothelium was intact with no denudation seen in any of the specimens (see figs 4.3.3.2 a & b).

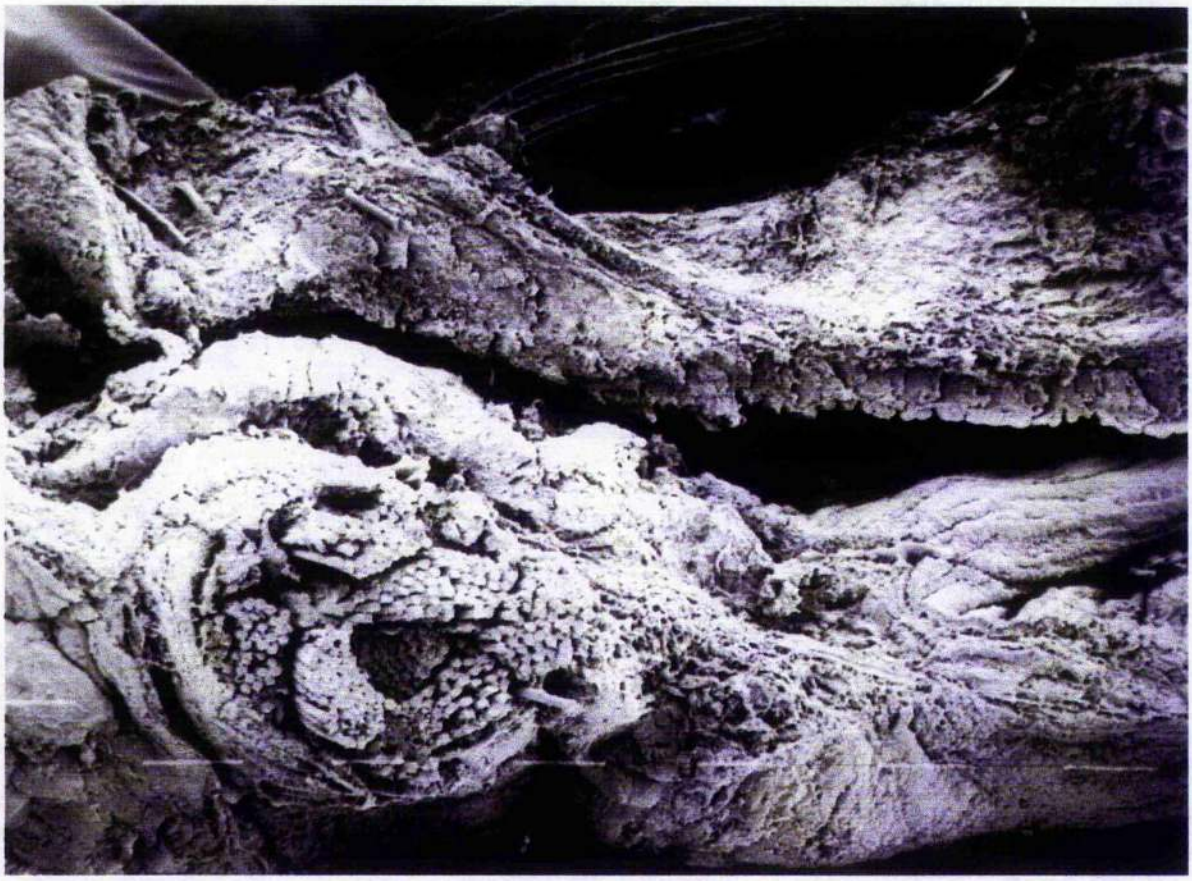


Fig 4.3.3.2.a SEM at site of partial portal vein ligation in the rat after 7 days: the ligature is seen with a constricted but patent vein and downstream dilation; wall structure is normal.

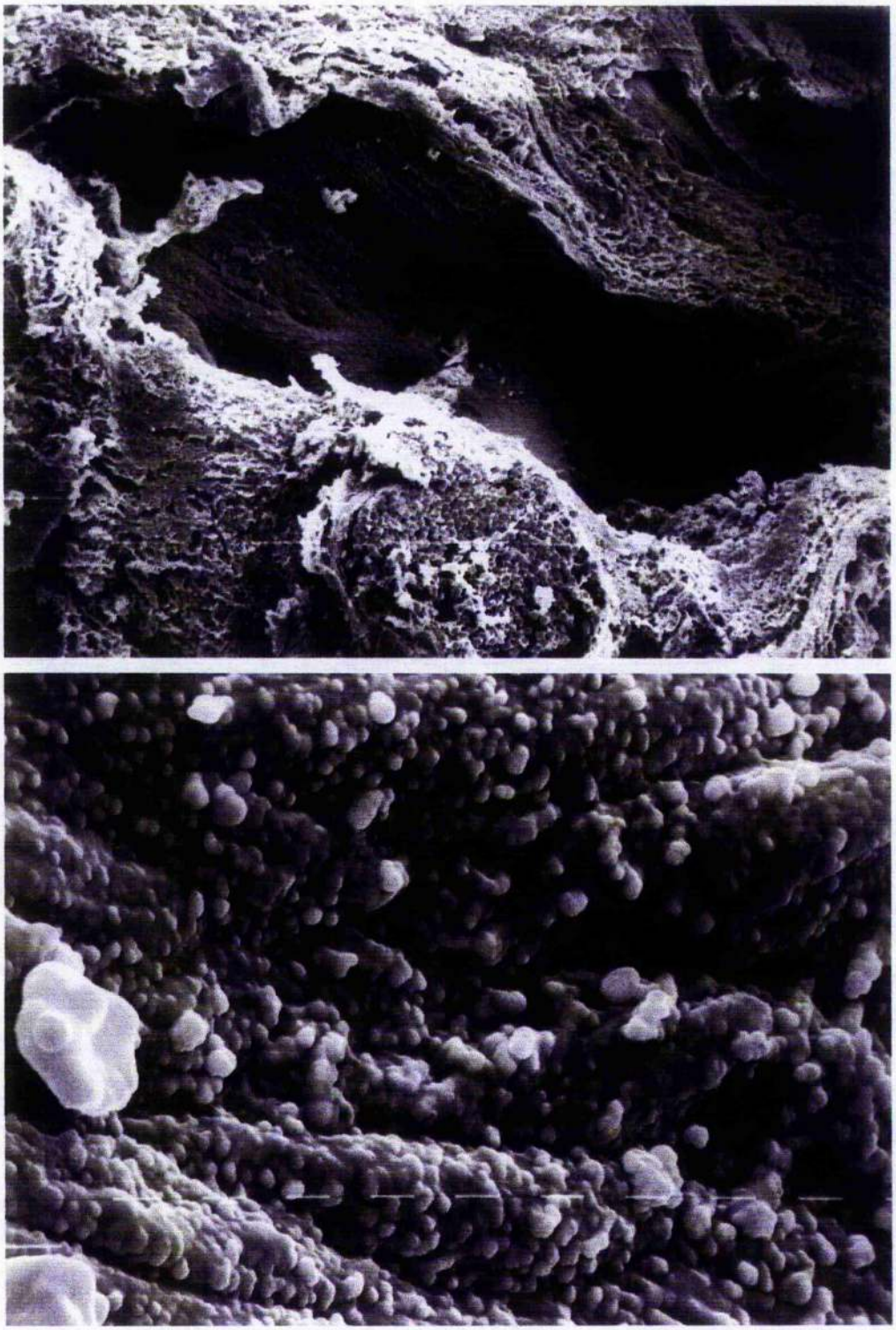


Fig 4.3.3.2.b SEMs showing intact layer of endothelium in the partial ligated portal vein at the ligature (upper scan) and in the hypertensive part (lower scan)

4.3.4 Discussion

Injection of microspheres proved to be an effective means of inducing portal hypertension, but was technically cumbersome and time-consuming. There was an associated high mortality and morbidity as reflected in the elevated serum hepatic enzymes and in the severe liver changes seen in two of the animals.

A similar increase in PGI_2-like activity was found in this model of portal hypertension to that seen after partial portal vein ligation. This finding gives further support to the hypothesis that the increased activity is primarily a response to hypertension. Also the lack of significant inflammatory response within the portal vein wall or endothelium, effectively excludes inflammation as a factor in this activity.

Hepato-cellular function as measured by serum hepatic enzymes, remained normal after the first day in the partial ligation group. This finding effectively excludes hepato-cellular dysfunction as a contributory factor to the increased PGI_2 activity seen in this model of experimental portal hypertension. The degree of hepato-cellular dysfunction seen in the group with microsphere injection is not surprising considering the severity of liver damage seen on histology in a minority of the animals, but even in this group serum enzymes had returned to normal by the time of the PGI_2-like activity assay at seven days. It is unlikely even in this group that hepato-cellular dysfunction played any role in the elevated PGI_2-like activity.

These studies supported the belief that the increased PGI_2-like activity seen in portal hypertension was not related to ligature trauma, inflammation of the portal vein, or

hepato-cellular dysfunction. Further support to the hypothesis that increased PGI_2 is directly related to the increase in pressure was provided by these studies.

4.4 Experiment 3

4.4.1 Introduction

Preliminary studies on various vascular tissues in the rat had suggested that a tissue specific gradient of PGI_2-like activity might exist with least activity found in low pressure vessels such as the IVC, and most in high pressure vessels such as the aorta. These observations together with the conclusions from the preceding experiments, pointed to a theory of PGI_2 production by vascular endothelium being directly related to the pressure within that vessel. Thus, in normotensive vessels there would be a degree of PGI_2 production specific to the pressure within that system, but if hypertension developed within that particular system, the vascular endothelium would respond by increasing its PGI_2 production above normal levels and to a degree relative to the pressure increase.

In the first instance, to investigate this possibility that a tissue specific gradient of PGI_2 production might exist, a range of vascular tissues from the rat and pig were assayed for PGI_2 production by bioassay. An attempt was also made to induce hypertension in the rat IVC and the effect of this on PGI_2 production within this vessel was studied.

4.4.2 Methods

4.4.2.1 Assessment of PGI_2-like activity in various vascular tissues

2-3cm segments of the infra-renal IVC, the aorta, and of the portal vein of Sprague-Dawley rats weighing 150-200g were excised under ether anaesthesia and

processed as previously described for PGI_2-like activity. This was measured by platelet nephelometry and the activities compared.

Segments of the following vessels were removed ante-mortem, from eight pigs which had been used as donors for an experimental liver transplantation programme – portal vein, superior mesenteric vein, pulmonary artery and vein and abdominal IVC. These pigs were 25kg halothane resistant White pigs and had undergone full inhalational anaesthesia with halothane/0_2 induction, intubation and maintenance on N_20/air and halothane mixture with atracurium paralysis. The tissues were harvested from sections of vein which had not been clamped or traumatised during dissection, and collected and processed for bioassay in the standard manner.

4.4.2.2 Induction of hypertension in the IVC in the rat

Under ether anaesthesia, 13 male Sprague-Dawley rats weighing 150-200g underwent partial ligation of the infra-renal IVC (normal diameter 3 to 4.5 mm) to a standard constriction of 1.2mm using the technique of partial ligation described for the portal vein. A control group of seven rats underwent laparotomy and dissection of the IVC without ligation and a third group of six rats underwent general anaesthesia alone. All operated animals had IVC manometry carried out after opening the abdomen. The rats were allowed to recover and one week later under ether anaesthesia, pressure in the infra-renal IVC was measured prior to excision of the infra-renal IVC down to the iliac veins. Tissues were collected and processed as previously described and PGI_2-like activity measured by bioassay. The results were analysed by Wilcoxon's two sample test.

4.4.3 Results

4.4.3.1 PGI_2-like activity in various normotensive vessels

In the rat, PGI_2-like activity was found to be much greater in the aorta than in the portal vein or IVC. Portal vein PGI_2-like activity was greater than in the IVC but this difference was not statistically significant (see fig 4.4.3.1 a).

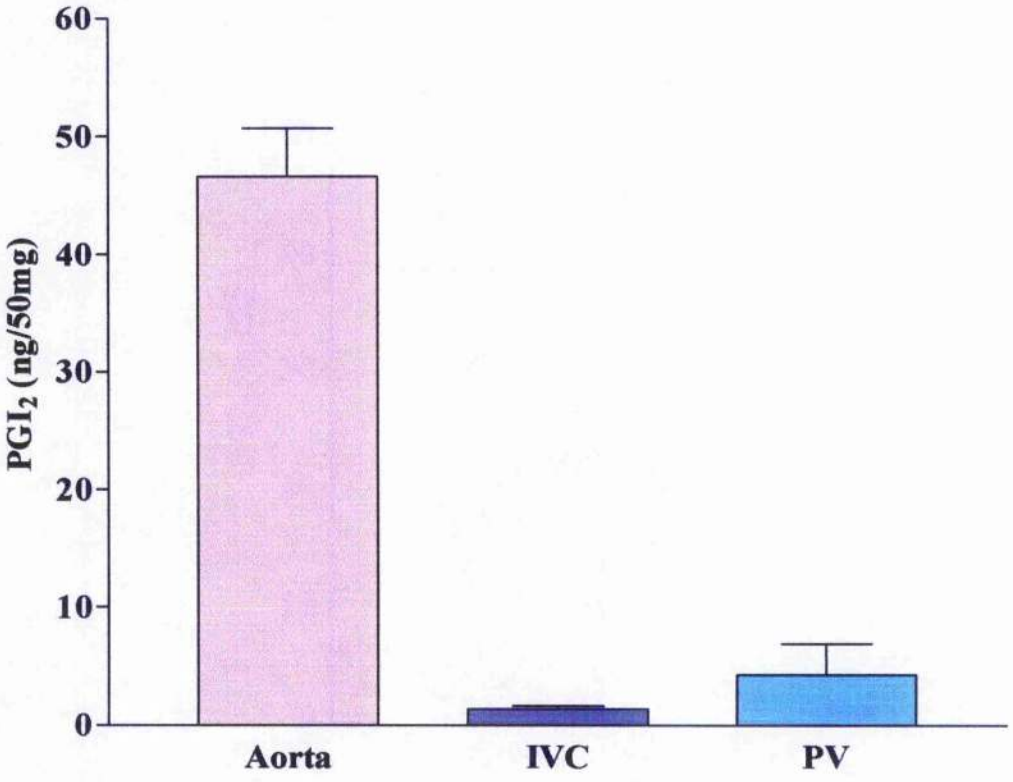


Fig 4.4.3.1.a PGI_2 – like activity in various normal rat vessels (mean & SEM) (Aorta: 47.5 +/- 4); (IVC: 1.4 +/- 0.3); (Portal Vein (PV): 4.4 +/- 2.5).

In the pig, PGI_2-like activity was lowest in the IVC with significantly greater activity in the portal vein, superior mesenteric vein and hepatic veins ($p<0.01$). Although lower than in either the portal or superior mesenteric veins, PGI_2-like activity in the hepatic vein was not statistically different. Activity in the pulmonary circulation was also

significantly greater than the IVC, ($p<0.01$), the artery having significantly more activity than the vein ($p<0.01$) (see fig 4.3.3.1 b).

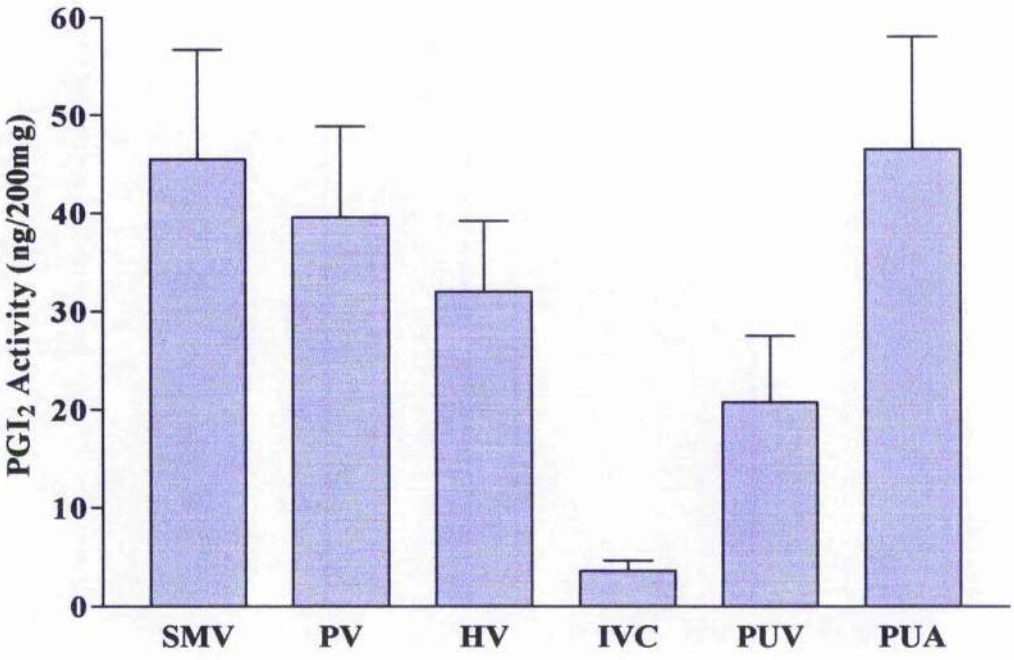


Fig 4.3.3.1.b PGI_2 –like activity in various normal pig vessels (mean & SD): SMV is the superior mesenteric vein (46 +/- 11); PV is the portal vein (39.6 +/- 9.3); HV is the hepatic vein (32.8 +/- 7.4); IVC is the inferior vena cava (3 +/- 0.7); PUV is the pulmonary vein20.7 +/- 10.3); PUA is the pulmonary artery (47 +/- 11.7).

4.4.3.2 Experimental hypertension in the IVC

The basal mean pressure in the IVC (2.3 +/- 2.1) was less than in the portal vein (9.7 +/- 1.1) [cm H_20: mean +/- SD]. One week after partial ligation, the IVC pressure was significantly increased (7.9 +/- 0.8cm H_20) when compared to rats which had undergone anaesthesia alone (2.2 +/- 1.9cm H_20), or laparotomy alone (2.0 +/- 1.6cm H_20). ($p<0.01$) (see fig 4.4.3.2). Portal vein pressure at one week after IVC ligation remained at basal levels in all groups (mean and SD).

PGI_2-like activity was significantly raised in the hypertensive IVC when compared to

non-operated rats ($p<0.01$). PGI_2-like activity was also significantly increased in the normotensive portal veins of rats which had undergone laparotomy alone ($p<0.01$). The trend was to greater activity in the hypertensive compared to normotensive veins but this difference was not statistically significant (see fig 4.4.3.2).

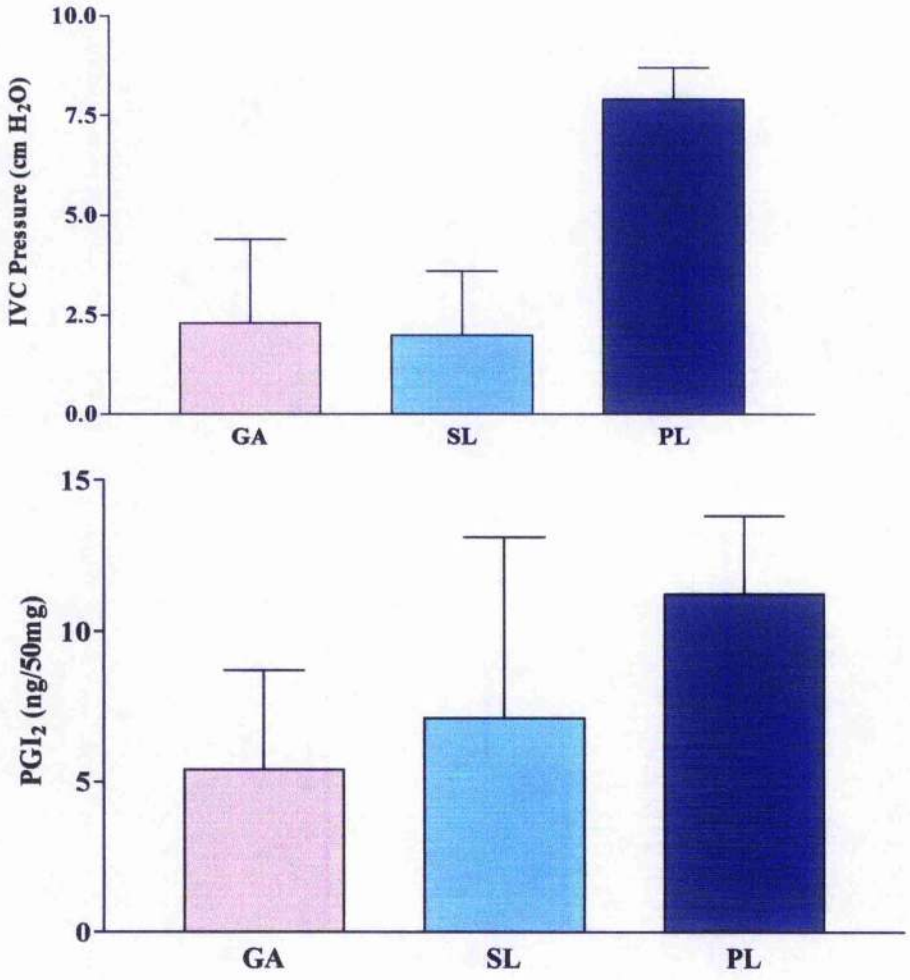


Fig 4.4.3.2 Pressure and PGI_2 - like activity in rat IVC (mean & SD): GA is anaesthesia alone (pressure 2.3 +/- 2.1; PGI_2 5.4 +/- 3.2) ; SL is sham IVC ligation (pressure 2 +/- 1.6: PGI_2 7.1 +/- 6); PL is partial IVC ligation (pressure 7.9 +/- 0.8; PGI_2 11.3 +/- 2.6).

4.4.4 Discussion

Arteries have been shown to produce more PGI_2 than veins on a weight per weight basis, (Johnson AR, 1980). This finding is corroborated by the results of these studies showing maximal activity in arterial tissues in two species. Furthermore, in the normal rat there is indeed a marked and significant difference between high and low pressure systems such as the aorta and IVC. The portal system, also a low pressure vessel, had much less activity than the aorta but showed a trend towards greater activity than in the IVC although this did not reach statistical significance in this small number of animals studied.

Of great interest in the pig was the demonstration not only of significant differences in PGI_2 activity between different veins, but also that the activity reflected the pressure, being greatest in the high pressure veins, (portal, superior mesenteric and pulmonary). The exception to this observation was the relatively high level of PGI_2 production by the hepatic vein which would be at a pressure only 1.2mm/Hg greater than the IVC. Because of the size of the rat, it was not possible to measure PGI_2 activity in veins smaller than the portal, and it remains unknown whether the hepatic vein in this species is similarly active. If the hepatic vein in man also has the property of high production of PGI_2, perhaps deficiencies in this activity might theoretically be a factor in hepatic vein thrombosis.

These findings in normal animals point to the existence of a gradient of PGI_2 activity in venous systems directly related to the ambient pressure. The significance of this finding requires further elucidation but one could postulate that PGI_2's potent vasodilator property might play a role in the regulation of vascular tone.

Hypertension in the IVC was produced successfully by the technique of partial ligation although the pressure obtained was relatively low at 7.9 +/- 0.8cm H_20 (mean +/- SD), which is lower than the normal portal venous pressure. This was a result of the opening up of a rich collateral circulation fed by the many major branches of the infra-hepatic IVC.

Significant and similar increases in PGI_2 production were observed in comparison with non-operated animals in both the hypertensive and normotensive IVCs of operated animals but disappointingly, there was no significant difference between the hypertensive and non-hypertensive groups. This effect was presumably mainly as a result of the surgery since this has been shown previously to increase PGI_2 production in the portal vein. A possible explanation for this lack of response is that the increase in pressure in the IVC was not great enough to elicit the response of increased PGI_2 production. The IVC is a capacitance vessel capable of great dilation and drains many potential collateral vessels. Possibly, insufficient stretch and alteration to the tone of the IVC resulted from the modest increase in pressure.

In summary, the studies in this section have demonstrated a gradient of PGI_2 activity in normal vascular tissues which is directly related to the ambient pressure. Surgery was shown to increase significantly PGI_2 production in the IVC, an effect which has been demonstrated in the portal vein. A significant though modest increase in IVC pressure did not result in a correspondingly significant increase in PGI_2 activity.

4.5 Experiment 4

4.5.1 Introduction

In experiments 1 & 2, increased PGI_2 activity was found seven days after induction of pre-hepatic portal hypertension by two different methods. The study was now directed towards establishing the rapidity of onset and duration of this increased activity. Of the two models for portal hypertension, partial ligation was chosen for these further studies because of the simplicity, reliability and lack of significant morbidity and mortality associated with this technique.

Anti-aggregatory activity was measured in portal hypertensive and control rats at different time intervals over a six week period. An unexpected fall in portal pressure was found after two weeks, and to identify the causes of this fall, splenic venography and subsequently resin cast analysis of the porta-systemic collateral circulation was carried out.

4.5.2 Methods

4.5.2.1 Investigation of onset & duration of PGI_2 response

Portal hypertension was induced in six groups of six rats each. A further six groups of six rats each underwent laparotomy without portal vein ligation, and formed the control groups. The standard technique of ether anaesthesia, abdominal incision and closure was used in all animals. Laparotomy, portal vein manometry and excision of 2-3cm of portal vein was carried out in the ligated and non-ligated groups after 24 hours (group A), four days (group B), seven days (group C), two weeks

(group D), four weeks (group E), and six weeks (group F). The portal vein samples were collected as described previously, coded and processed immediately for measurement of PGI_2-like activity by platelet nephelometry.

The results were expressed as mean and SD for each group, ligated and control, and the portal vein pressures and PGI_2-like activity charted. Statistical analysis was by Wilcoxon's unpaired two sample test. PGI_2-like activity was correlated with portal vein pressure with measurements taken at 4-7 days after the procedure when PGI_2-like activity had previously been shown to be maximal. This data was analysed by calculation of the regression equation for these variables.

4.5.2.2 Splenic venography

Splenic venography was carried out on six rats. The first was on a normal rat, and the remaining five at intervals of 1, 2, 3, 4 and 6 weeks after partial ligation of the portal vein.

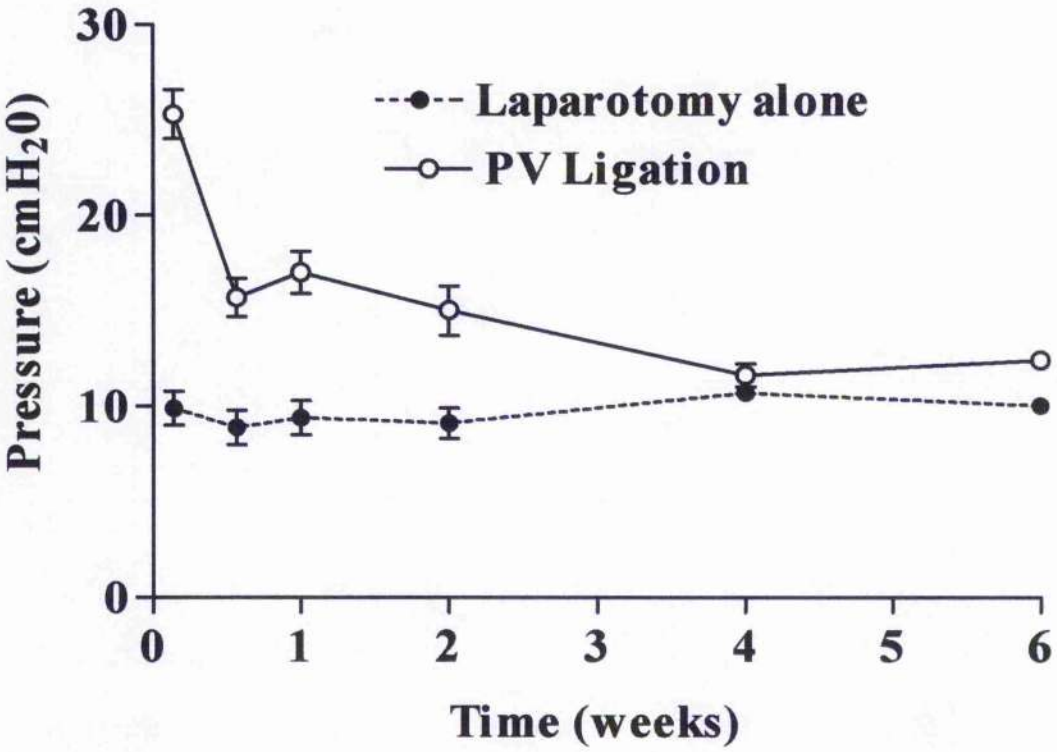
The rats were anaesthetised with intra-peritoneal urethrane, (0.6ml / 100g body weight of 25% solution of urethrane), a long-acting agent allowing transfer of the animals from the laboratory to the Radiology Department without the need for ether anaesthetic and equipment. The abdomen was opened via a left sub-costal incision and the spleen delivered into the wound. 5ml of 45% Hypaque contrast medium was injected into the splenic pulp via a 23g Butterfly needle and serial radiographs taken immediately. These rats were not recovered and were not used for PGI_2 determinations.

4.5.2.3 Resin corrosion cast studies

Corrosion casts were made with the help of Mr Hugh Rogers FRCS, Surgical Research Fellow at that time, by a modification of the method described by Northover, (Northover JM et al, 1980). Under ether anaesthesia, 10ml of resin was injected into the superior mesenteric vein in rats at a monitored pressure of 100 mm Hg, at intervals from five days to five weeks after partial ligation of the portal vein. Four normal un-operated rats were also studied. Injection was carried out while the circulation was still intact. The animal was killed and after the resin had set, an en-bloc resection of the thoraco-abdominal trunk was carried out. The specimen was placed in a bath of 20% NaOH at 37°C and kept in a constant temperature hot room at 37°C. The NaOH solution was renewed at regular intervals until digestion of the tissues was maximal, usually after 2 to 3 weeks. . Using a fine jet of warm water, the cast was then carefully dissected free of residual tissue and the vascular anatomy analysed and compared with the normal.

4.5.3 Results

4.5.3.1 Portal vein pressure

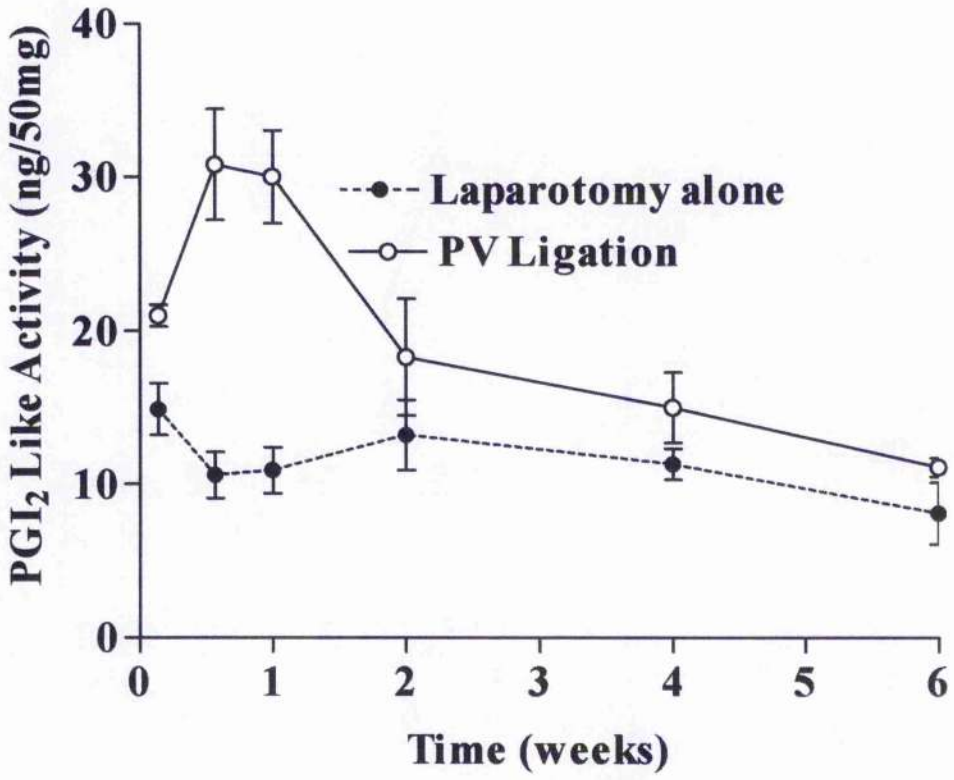


Time	Laparotomy alone	PV Ligation
24 hours	9.9 (0.9)	25.3 (1.3)
4 days	8.9 (0.9)	15.7 (1)
7 days	9.4 (0.9)	17 (1.1)
2 weeks	9.1 (0.8)	15 (1.3)
4 weeks	10.7 (0.4)	11.6 (0.6)
6 weeks	10 (0.4)	12.4 (0.3)

Figure 4.5.3.1 Portal vein pressures in partial portal vein ligated rats and controls over a 6 week period (mean & SD)

Mean portal vein pressure measurements are plotted in fig 4.5.3.1. In the control groups, no significant change of portal venous pressure from the normal was found. There was a significant increase in portal pressure from 24 hours to two weeks which was maximal between 24 hours and seven days ($p<0.01$). After two weeks, the pressure began to fall to the level seen in the control group and had stabilised at normal pressure by six weeks.

4.5.3.2 PGI_2-like activity



Time	Laparotomy alone	PV Ligation
24 hours	14.9 (1.7)	21 (0.7)
4 days	10.6 (1.5)	30.8 (3.6)
7 days	10.9 (1.5)	30 (3)
2 weeks	13.2 (2.3)	18.3 (3.8)
4 weeks	11.3 (1)	15 (2.3)
6 weeks	8.1 (2)	11.1 (0.6

Figure 4.5.3.2 PGI_2-like activity in partial ligated and control rat portal veins over a 6 week period (mean & SD)

Mean PGI_2-like activity measurements in the portal veins of all the groups are plotted in figure 4.5.3.2. These results show significantly increased activity in animals with partial portal vein ligation when compared to control groups between the 24 hour to two weeks period ($p<0.01$). Thereafter, PGI_2-like activity diminishes approaching that of the control animals. This increase was maximal between the fourth and seventh days with a return to control levels by the fourth week.

4.5.3.3 Correlation between portal vein pressure and PGI_2-like activity

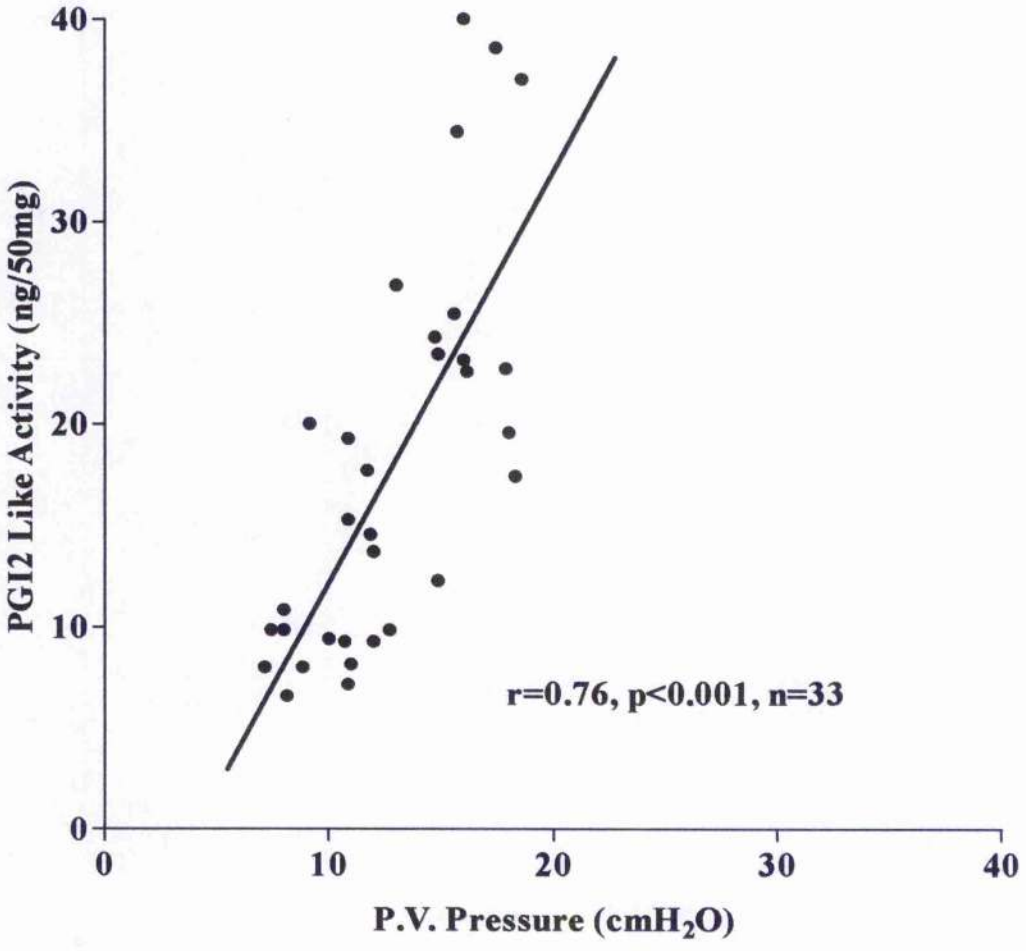


Fig 4.5.3.3 Correlation between portal vein pressure and PGI_2-like activity

From the results above it is obvious that as portal pressure falls, so does the PGI_2-like activity. This relationship is demonstrated in figure 4.5.3.3. Linear regression analysis gives a correlation co-efficient of + 0.76 and demonstrates a strongly positive correlation between pressure and PGI_2-like activity in the portal vein.

4.5.3.4 Splenic venography

Splenic venograms are shown in figures 4.5.3.4 a-d. Venography demonstrated minimal collateral circulation at one week, but progressive development of porta-systemic collaterals after that time. By the second week, spleno-renal, retroperitoneal and peri-oesophageal collaterals are seen. At four weeks, there is a large and well developed collateral circulation with a prominent large spleno-renal vessel draining in to the left renal vein. This collateral was also prominent at six weeks and was found on retrospective analysis of the earlier radiographs.

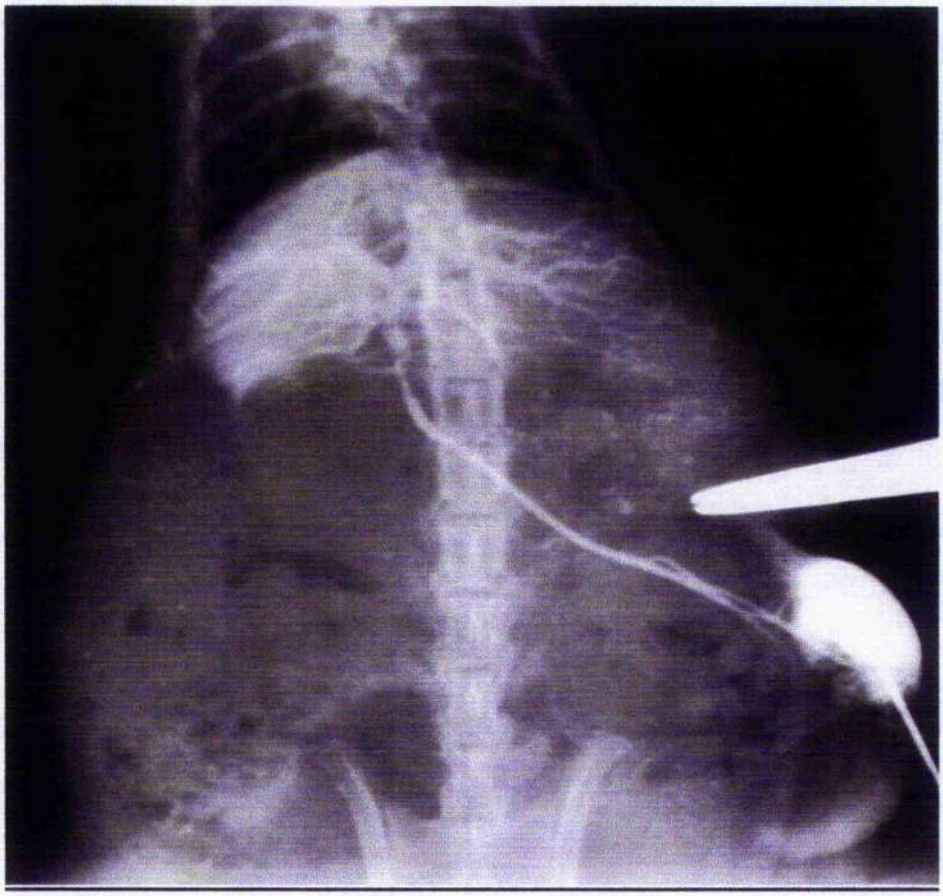


Fig 4.5.3.4.a Splenic venography showing the normal portal system: in all portagrams contrast was injected into the spleen

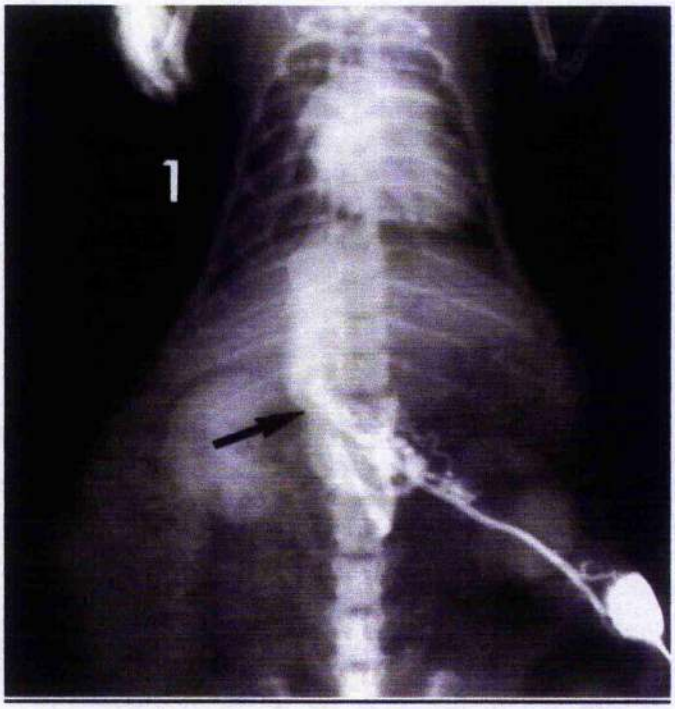


Fig 4.5.3.4.b Splenic portagram in rat one week after partial portal vein ligation: the arrow shows the site of ligature

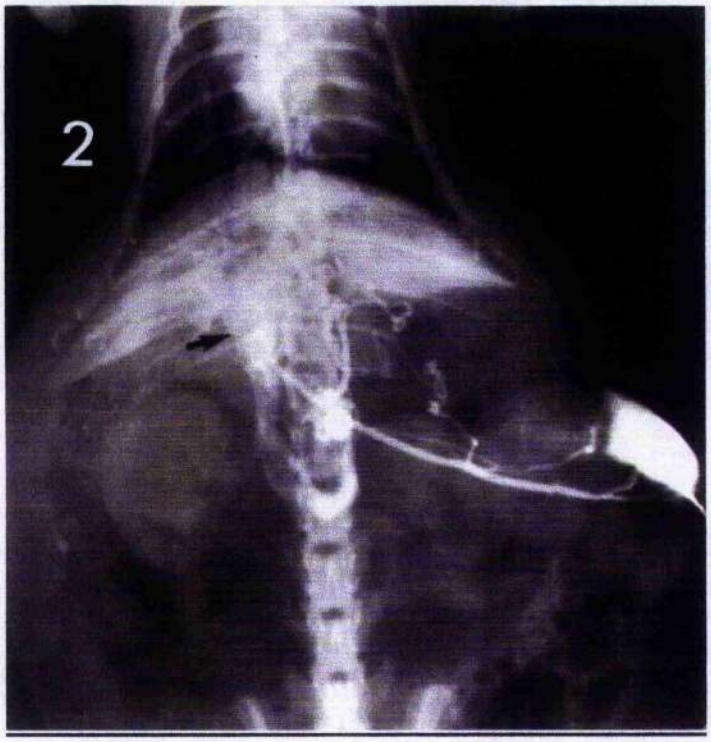


Fig 4.5.3.4.c Splenic portagram in rat 2 weeks after partial portal vein ligation

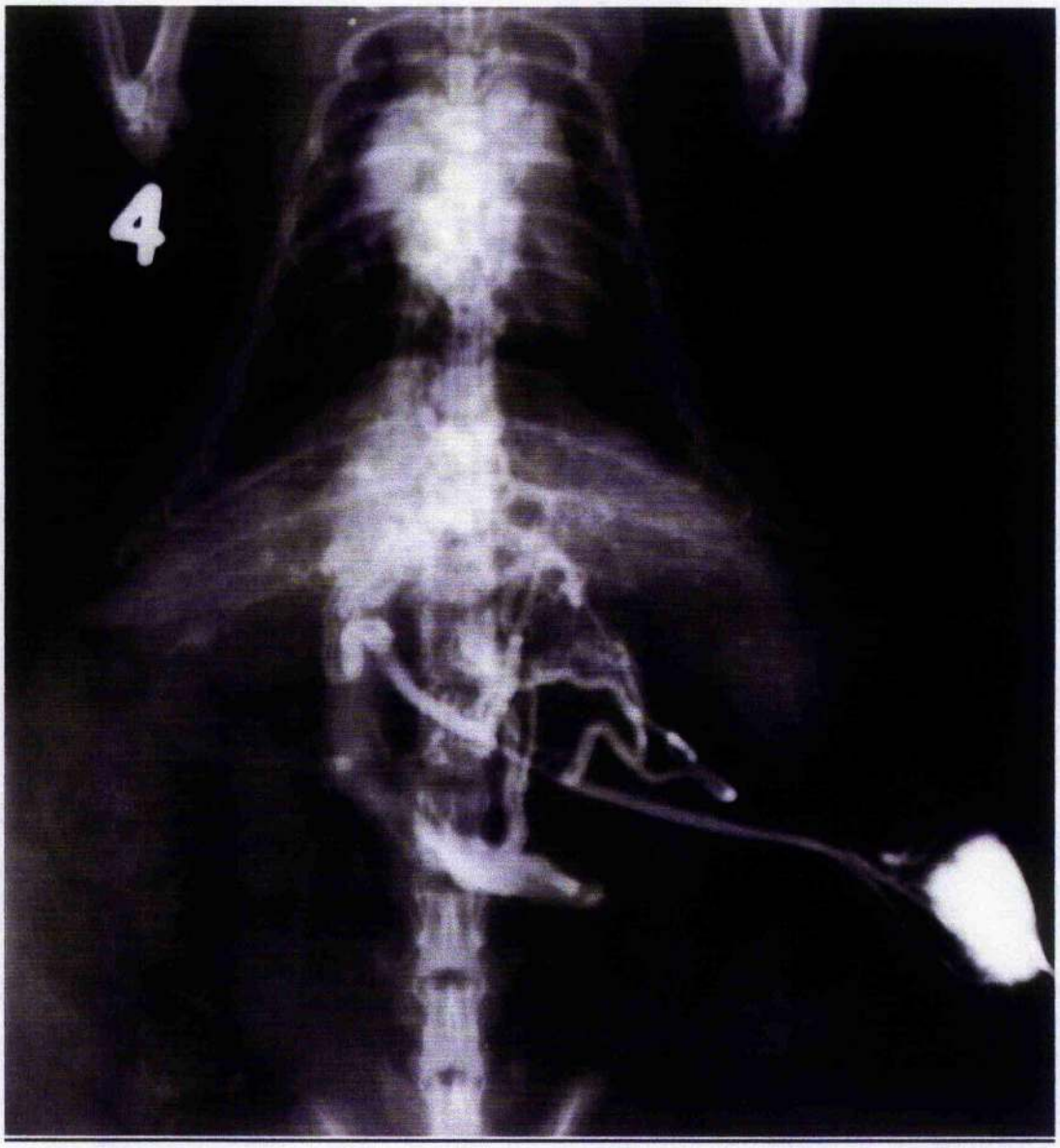


Fig 4.5.3.4.d Splenic portagram in rat 4 weeks after partial portal vein ligation: the arrow marks the site of the ligature; the very large collateral (a hypertrophied left adrenal and anterior lumbar vein) draining the portal bed into the left renal vein and on into the IVC, is easily seen.

4.5.3.5 Corrosion cast studies

In all animals a major porta-systemic collateral pathway was found draining the

gastric, splenic and duodenal veins into the left anterior lumbar vein (LALV).

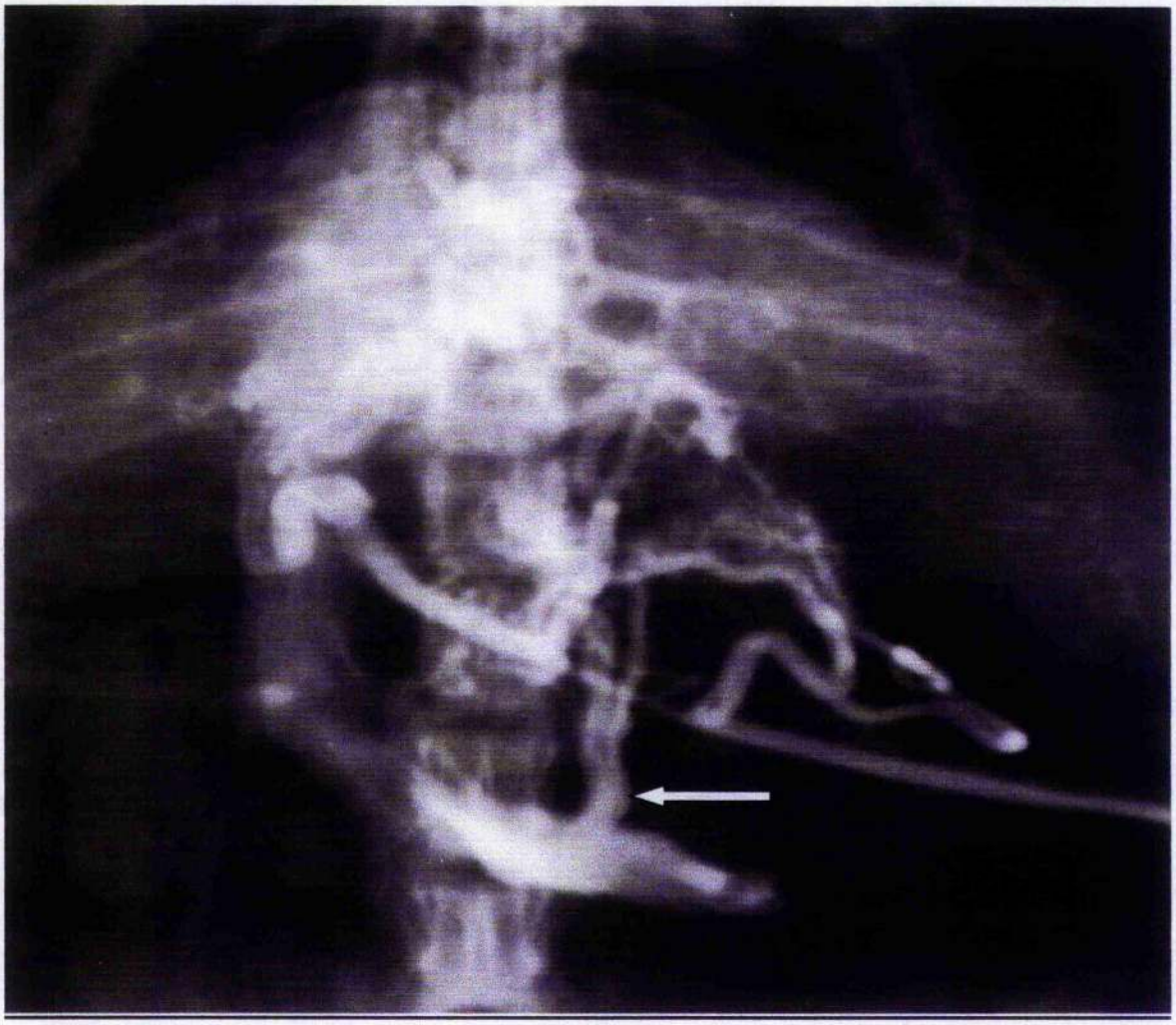


Figure 4.5.3.5 Anatomy of retroperitoneal collaterals found by corrosion cast studies demonstrated on splenic portagram of established portal hypertension in the rat. The white arrow shows the left anterior lumbar vein which was universally found to be the major collateral draining the portal to the systemic circulations.

The left adrenal vein drains into this vessel just before the LALV drains in to the left renal vein. This was found to be the major collateral pathway and all other collateral complexes were much smaller. The LALV was present in the normal rats although of much smaller calibre then in the hypertensive animals. This was the collateral pathway which developed in the portal hypertensive rats and is seen developing in

the splenic portograms (see figures 4.5.3.4 b, c and d).

4.5.4 Discussion

Using the technique of partial portal vein ligation, significant portal hypertension was established within 24 hours. Thereafter, there was a steady decline in portal pressure with a return to control levels by four weeks. Splenic venography confirmed that over this period a porta-systemic collateral circulation became established and over the same time period portal pressure fell to normal levels, presumably as a result of the opening up of this alternative circulation. These findings are in accordance with those of Orda and Ellis who, using a similar model with a constriction of 1.1mm, found that portal hypertension lasted only 10-14 days over which period of time a collateral circulation was shown to have developed, (Orda R et al, 1978).

PGI_2-like activity was significantly increased from day one to week two with the maximal response being between days four and seven. Of great interest is the gradual fall in PGI_2-like activity after day seven which strikingly parallels the fall in portal hypertension. This simultaneous fall, and the demonstration of a strongly positive correlation between the two parameters during the time of maximal pressure and PGI_2 activity, provides further strong evidence to support the hypothesis that increased PGI_2 production results when hypertension is induced. The observation of similar responses related to spontaneous and experimentally induced arterial hypertension in rats leads to the conclusion that increased PGI_2 production by vascular endothelium may be a physiologically important adaptive response. This would result in vasodilation and a tendency to reduction in pressure. In portal

hypertension, PGI_2 may well promote the opening up of collateral channels of embryonic origin, thus also leading to a reduction in pressure.

Splenic venography and the corrosion cast studies demonstrated the development of collateral vessels and in particular the dominant system draining via the LALV into the left renal vein. This pattern of collateral circulation has been described previously in the rat, the dog and man in portal hypertension, (Moritz E et al, 1973, Rousselot LM et al, 1976 and Halvorsen JF & Myking AO, 1974). The LALV in the rat and the supra-renal vein in man are vestigial remnants of the posterior cardinal veins in the embryo, which on the left side largely regress, and on the right side form the IVC, (Grays Anatomy, 1980). In man however, collaterals connecting the gastric, gastro-epiploic and splenic veins to the left renal vein have been demonstrated angiographically in the absence of portal hypertension, (Wexler MJ et al, 1975). This evidence suggests that this collateral pathway results from dilation of pre-existent though vestigial vessels, and not by formation of new channels. While these vessels will dilate as a result of hydrostatic 'back' pressure alone, increased PGI_2 production by its potent vasodilatory effect may have an important role to play in the development and maintenance of the collateral circulation.

4.6 Experiment 5

4.6.1 Introduction

A positive correlation between PGI_2 activity and portal vein pressure has been demonstrated. Portal hypertension induced by the technique of constriction to a diameter of 1.2mm is short-lived lasting just over two weeks. In order to study the effect of prolonged hypertension on PGI_2 production, a model of chronic portal hypertension is necessary.

As a result of the identification of the LALV as the main collateral circulation in the rat, a model of chronic portal hypertension was developed. Chronic portal hypertension was also induced by using a greater degree of constriction of the portal vein. Having established these models PGI_2-like activity at four weeks was assessed by bioassay.

4.6.2 Methods

4.6.2.1 Development of model of chronic portal hypertension

Under ether anaesthesia and via an upper midline incision, portal vein manometry then partial ligation of the portal vein as previously described was carried out in six rats. The LALV was then dissected and completely ligated with a non-absorbable ligature just above its junction with the left renal vein 9see fig 4.6.2.1). Under ether anaesthesia, portal vein pressure was then measured at 9, 17, 25 and 33 days.

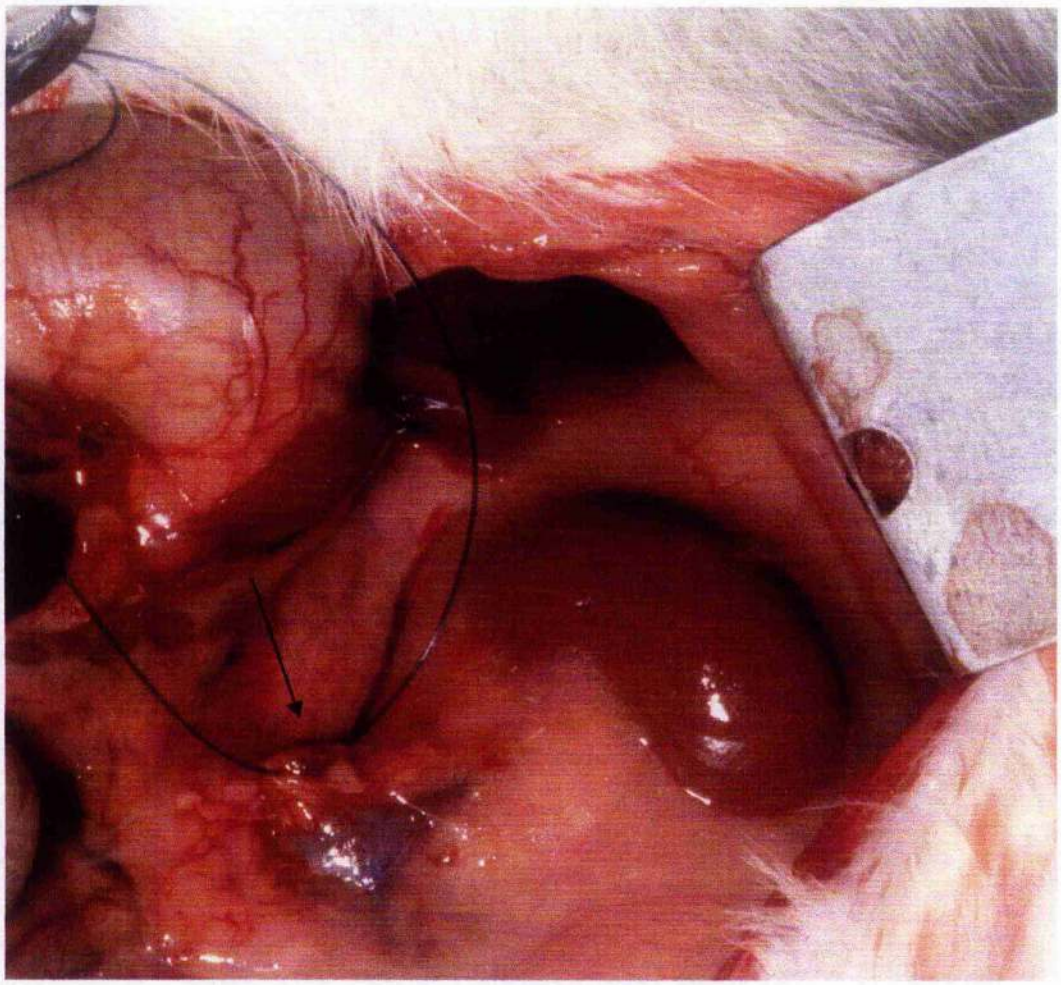


Fig 4.6.2.1 Ligation of the left anterior lumbar vein to cause chronic portal hypertension: the suture shown by the arrow completely ligates the left anterior lumbar vein which normally drains into the left renal vein which can be seen below the ligature

In a further group of six rats, partial portal vein ligation alone was carried out but using a 0.8mm rather than the previously used 1.2mm tube, therefore resulting in a greater degree of portal vein constriction. These rats also underwent portal vein manometry at the same time intervals above. A control group of six rats underwent laparotomy and dissection of the portal and LALV veins without ligation, and

underwent portal vein manometry at the same time intervals.

Because of the repeated laparotomies and portal vein punctures to measure portal pressure, veins from these animals were not harvested for PGI_2 assay.

4.6.2.2 Chronic portal hypertension and PGI_2 activity

Ten rats underwent laparotomy, portal vein manometry and then partial ligation of the portal vein. The LALV was dissected at its junction with the left renal vein and completely ligated. A further group of 10 rats underwent laparotomy, portal vein manometry and then partial ligation of the portal vein to a diameter of 0.8mm. The LALV was not ligated in this group. A third group of 10 rats underwent laparotomy alone.

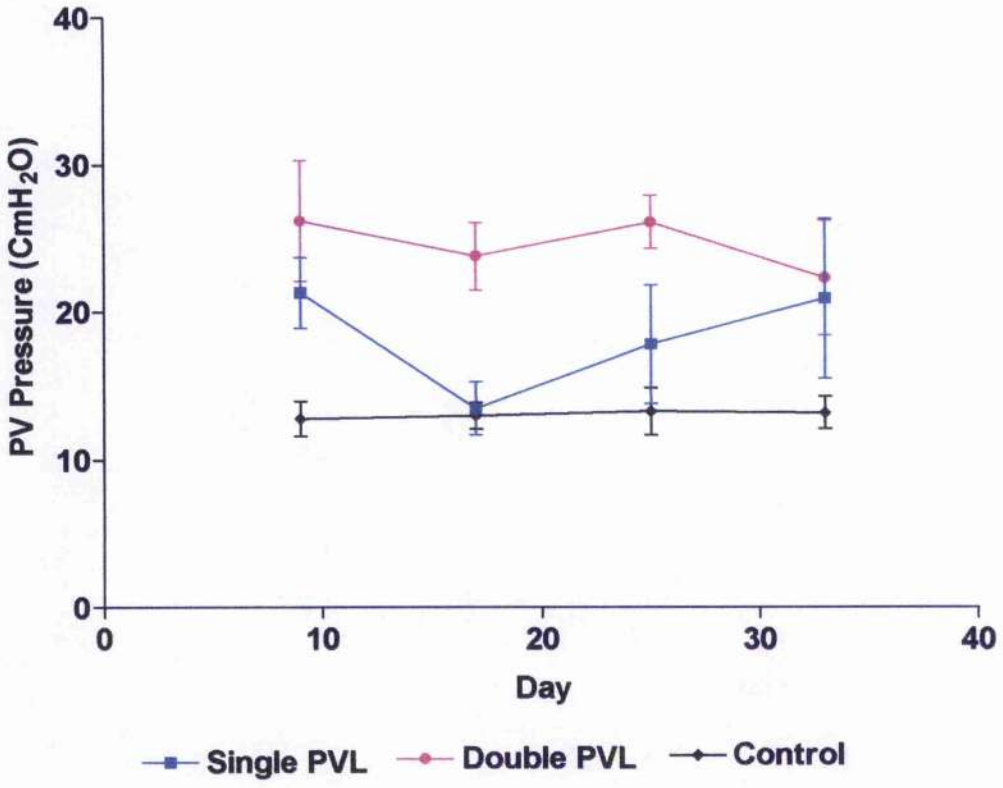
All animals were randomly allocated to these three groups prior to the procedure, allowed to recover afterwards and kept under identical conditions for 28 days after surgery.

On the 28^{th} day, all animals underwent laparotomy and any areas of necrosis or congestion of the liver or intestines was noted. After portal vein manometry, 2-3cm of hypertensive portal vein (or the anatomically equivalent segment in the control group) was excised, collected onto ice and processed in the standard way for immediate for bioassay.

The results were tabulated, and analysed by Wilcoxon's unpaired two sample test.

4.6.3 Results

4.6.3.1 Models of chronic portal hypertension



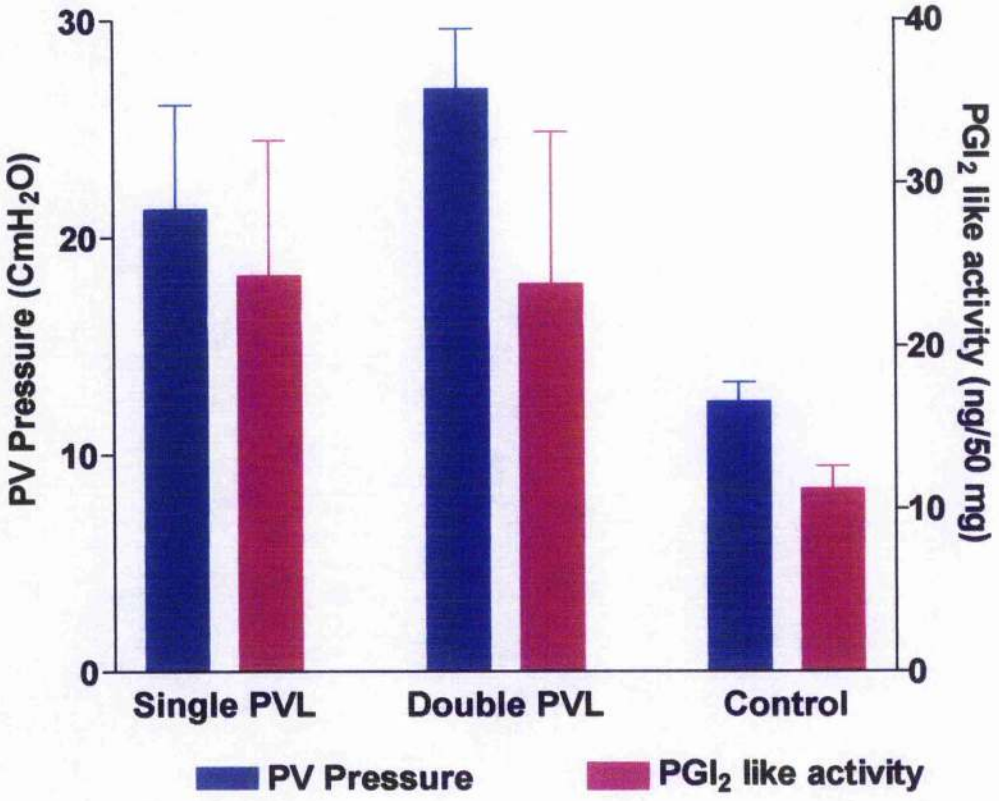
Time	Control	Single PVL	Double PVL
9 days	13 (2.2)	21.8 (2.5)	26.8 (4.2)
17 days	13.2 (1)	13.5 (2)	24.3 (2.2)
25 days	14 (1.5)	18.2 (4)	26.5 (2)
33 days	13.5 (1)	21.5 (5.5)	22.5 (4)

Fig 4.6.3.1 Portal vein pressure over 33 days: single PVL are rats undergoing portal vein ligation to 8mm diameter; double PVL are rats undergoing portal vein ligation to 1.2mm and ligation of the LALV; control animals did not have any vein ligation.

Partial ligation of the portal vein with ligation of the LALV resulted in significant portal hypertension from days 9-33 (see fig 4.6.3.1). Partial ligation of the portal vein alone to a diameter of 0.8mm resulted in a similar increase in portal pressure at day 9. The pressure then fell significantly by day 17 when compared to the double ligation group ($p<0.01$). After this however, the pressure in this group steadily rose until by day 33 there was no significant difference between the two groups which were both significantly hypertensive ($p<0.01$). As expected, the pressure in the laparotomy only group remained stable at normal levels throughout.

4.6.3.2 PGI_2-like activity in chronic portal hypertension

These results are shown in figure and table 4.6.3.2.



Group	Portal vein pressure (cm H2O)	Portal vein PGI_2-like activity (ng/50mg tissue)
Control	12.3 (0.9)	8.4 (1)
Single PVL	21.3 (4.7)	18.2 (6.2
Double PVL	26.7 (2.8)	17.8 (6.8)

Fig 4.6.3.2 Portal vein pressure and PGI_2-like activity in chronic portal hypertension of 28 days duration and controls: single PVL are rats undergoing portal vein ligation to 8mm diameter; double PVL are rats undergoing portal vein ligation to 1.2mm and ligation of the LALV; control animals did not have any vein ligation.

There was no significant difference in portal pressure between the two ligated groups but both developed significantly elevated portal pressures when compared to the control non-ligated animals ($p<0.01$ in both). Also, there was a similar degree of PGI_2-like activity in both hypertensive groups with no statistical difference between the two on analysis. Each of these hypertensive groups however, had significantly increased PGI_2-like activity when compared to the normotensive control group ($p<0.01$).

There were no deaths in any of the groups. There was no significant congestion or necrosis of intestines or liver noted.

4.6.4 Discussion

Two satisfactory models of chronic portal hypertension lasting beyond 28 days were developed. Partial portal vein ligation with complete ligation of the LALV resulted in immediate hypertension which persisted at the same levels throughout. Using the greater degree of constriction (0.8mm), partial ligation alone resulted in a transient hypertension present at nine days followed, as expected, by a fall towards control levels by 17 days. Most surprisingly however, the pressure steadily rose thereafter reaching significant hypertension by 33 days.

This interesting finding is probably best explained by the increasing size and weight of the rat over this period. The initial fall in pressure at 17 days is similar to that seen when using a 1.2mm constriction and is secondary to the development of the porta-systemic collateral circulation. As the rat grows beyond this time with the more

critical portal vein constriction of 0.8mm, the collateral circulation becomes unable to compensate further. Therefore, with increasing splanchnic flow against the critical resistance of the portal constriction, the collateral circulation no longer compensates and the portal pressure rises. Myking and Halvorsen found that a portal vein constriction of 0.9mm in rats weighing 355g (+/- 91g; SD) resulted in a greater than 90% mortality, but that a constriction of 1.2mm was associated with a 75% survival, (Myking AO & Halvorsen JF, 1973). Furthermore, these authors were able to ligate completely the portal vein after a delay to allow a porta-systemic collateral circulation to develop. The rats I used in these experiments weighed only between 150-200g and this explains the lack of mortality with such a severe constriction. By the time the rats had doubled in weight, death did not occur because of the already established collateral circulation, but hypertension did supervene.

Most importantly, PGI_2-like activity was shown in both groups of hypertensive rats to remain significantly elevated in parallel with the pressure at up to 28 days. This evidence confirms the previous finding of a strong correlation between portal vein pressure and PGI_2 release. This effect has been shown to be unrelated to surgical trauma or inflammatory changes within the portal vein itself and persists well after the collateral circulation has become established.

Summary

The results from these experimental studies strongly support the hypothesis that increased PGI_2 production is an adaptive response of vascular tissue to the stimulus of increased intra-luminal pressure. If this response exists in man, PGI_2 production would be elevated in portal hypertension and possibly play a role in the increased splanchnic blood flow commonly seen in this condition. Increased PGI_2 production could also possibly adversely affect the haemostatic mechanisms at a local endothelial interface level and therefore play a role in the severity of haemorrhage seen in bleeding oesophageal varices.

CHAPTER 5: CLINICAL STUDIES

Introduction

Having obtained experimental data to support the hypothesis that prostacyclin activity in the portal vein is related to pressure, evidence of any clinical relevance was now sought. Clinical studies were focused on patients with portal hypertension in comparison to control groups with normal portal venous pressure. Initially venous tissue samples were studied and once the radioimmunoassay was developed, attempts at measurement of serum levels of prostacyclin were made. Latterly, blood levels were measured using gas chromatography / mass spectrometry.

All clinical studies were performed with the prior consent from the patient and approval of the Ethics Committee of the Royal Free Hospital.

5.1 Human vein PGI_2 production measured by bioassay

5.1.1 Introduction

In the rat and pig measurable quantities of PGI_2-like activity were found in a variety of venous tissues (chapter 4). The bioassay for this activity was applied to venous tissues obtained from patients undergoing general surgical procedures and from those undergoing surgical procedures in treatment of portal hypertension. Patients who had taken aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) within two weeks were excluded from the study.

5.1.2 Methods

5.1.2.1 Processing of tissue samples

Tissue samples harvested during surgery were immediately placed in ice for transfer to the laboratory. Processing of samples was exactly as for the animal tissues (chapter 4 introduction). Essentially the vascular rings obtained were re-suspended in tris buffer at pH 7.5 or 8.0 at a range of concentrations depending on the amount of tissue available (range 25-200mg/ml). This was then incubated for 3 minutes at 20°C after initial mixing with a vortex mixer. After centrifugation at 1500g for 30 seconds, PGI_2-like activity in the supernatant was measured using the anti-platelet aggregation assay. Aggregation was induced using ADP in concentrations of 2, 5 and 10μm. PGI_2-like activity was calculated as before (chapter 3).

5.1.2.2 Tissue samples collected

Venous segments from 4 groups of patients (groups 1 to 4) undergoing surgical procedures under full general anaesthesia were obtained from the following sites:

1. Superficial veins from the hip region in patients undergoing total hip replacement (n=19)

2. Proximal long saphenous vein branches in non portal hypertensive patients with varicose veins undergoing ligation of the sapheno-femoral junction (n=7)

3. Mesenteric or omental veins in non portal hypertensive patients undergoing cholecystectomy (n=6)

4. Segments of portal vein, mesenteric vein and IVC from patients with portal hypertension undergoing porto-caval shunting or oesophageal transection for bleeding oesophageal varices (n=6); the vein segments were of equivalent diameter (2-3mm) and length (2cm)

5.1.2.3 Additional tests performed on the tissues

Anticipating low production of PGI_2-like activity from human tissues, the following tests were performed on some vein segments:

1. **Incubation with arachidonic acid**

   Arachidonic acid was added to maximise PGI_2 production in the presence of any increased tissue PGI_2-like activity. Vein segments were re-incubated for 30-120 minutes at 37°C in tris buffer pH 8.0 containing 1mg/ml arachidonic acid. PGI_2-like activity was measured in supernatant specimens.

2. **Incubation in plasma or platelet rich plasma (PRP)**

Plasma factors, which stimulate PGI2 production, have been reported (Defreyn G et al, 1981, MacIntyre DE et al, 1978). Also platelets may provide a source of endoperoxides for PGI_2 synthesis, (Baenziger NL et al, 1977 and Moncada S et al, 1976). Therefore, after prior incubation in tris buffer pH 8.0 at 37°C, vein segments were further incubated with normal plasma and PRP and PGI_2-like activity measured in the supernatant specimens.

3. **Rapid freeze thawing of blood vessels**

Mechanical stress and trauma were thought to stimulate prostanoid production in blood vessels. Vein ring samples incubated in tris buffer at 100mg/ml were rapidly frozen using dry ice and then thawed at 37°C, each cycle being repeated three times. The incubation medium was then tested for PGI_2-like activity.

5.1.3 Results

After simple incubation in tris buffer, no anti-platelet aggregatory activity could be demonstrated in any veins from groups 1, 2 or 4. Two of the mesenteric veins from group 3 (i.e. non portal hypertensive patients undergoing cholecystectomy), produced PGI_2-like activity equivalent to 3ng PGI_2/100mg of tissue. No activity was found in incubates performed at higher tissue concentrations up to 200mg/ml.

1. **Incubation with arachidonic acid**

No PGI_2-like activity could be found in two veins incubated with 1mg/ml arachidonic acid for 30, 60, 90 and 120 minutes. Fifteen further veins were incubated with the same concentration of

arachidonic acid for 60 minutes at 37°C. Four of these produced slight PGI_2-like activity equivalent to 30% inhibition of platelet aggregation or <0.7ng/100mg tissue/60 minutes.

2. **Incubation in plasma or PRP**

Five samples were incubated with normal plasma and PRP after prior incubation in tris buffer. One sample incubated in plasma contained 40% anti-platelet aggregatory activity equivalent to 0.8ng/100mg tissue/10 minutes. The remaining four plasma and five PRP incubated samples did not demonstrate any activity.

3. **Rapid freeze thawing of blood vessels**

Only one sample out of five tested, produced any inhibition of platelet aggregation. This was equivalent to <1ng PGI_2/100mg tissue/15 minutes.

5.1.4 Discussion

With the exception of two normal mesenteric vein samples, no PGI_2-like activity could be detected in tris buffer incubated veins even at higher tissue concentrations. Further attempts to stimulate PGI_2 release by the additional steps outlined failed to shown any appreciable production as measured by the bioassay method. It is well established that arachidonic acid is the primary substrate for PGI_2 in cultured endothelial cells and arterial microsomes, (MacIntyre DE et al, 1978, Weksler BB et al, 1977, Bunting S et al, 1976, Moncada S et al, 1976 and Raz A et al, 1977). Human arteries and veins have subsequently been shown to produce substantial amounts of PGI_2 when incubated with endoperoxides but not with arachidonic acid, (Moncada S et al, 1977). This finding is corroborated in part by this study and

perhaps could have been predicted. At the time of this experiment however, cyclic endoperoxides were not obtainable for these incubation studies which could only be performed with arachidonic acid.

The plasma and PRP incubation studies were prompted by the suggestions that there are factors present in plasma which could stimulate PGI_2 production, (MacIntyre DE et al, 1978). No such effect was demonstrated in this study. Platelet derived endoperoxides could form a significant source of substrate for PGI_2 production, (Moncada S et al, 1978[a] and Bunting S et al, 1976), but this also was not found to any significant extent. An explanation for this could be that only when thromboxane synthetase is inhibited can sufficient platelet derived endoperoxides accumulate to become available as substrate for endothelial PGI_2 production, (Baenziger NL et al, 1979, Needleman P et al, 1979). In this study, neither the patients nor the tissue specimens had been treated with TXA2 synthetase inhibitors thus possibly explaining the lack of PGI_2 production. Similarly disappointing results were found with tissues treated by rapid freeze thawing, a treatment which acts usually by stimulating enzymatic activity although sometimes inhibition may occur instead.

From all of these studies it became obvious that PGI_2-like activity is not as prolific in human tissue as that in the rat or pig. Also it became clear that the bioassay itself was not sufficiently sensitive in detecting this lower level of human PGI_2-like activity.

5.2 Human Vein PGI_2 Production Measured by Radio-Immunoassay

5.2.1 Introduction

Having defined the major limitations of the PGI_2 bioassay in human tissues, its use was abandoned. The timely establishment of a radio-immunoassay for 6-keto $PGF_1\alpha$ at the Royal Free Hospital made possible further study of PGI_2 activity in human vascular tissue. Vein samples were collected from the portal systems of patients with and without portal hypertension. In addition, venous tissue samples from the systemic circulation were collected for measurement of PGI_2 production.

Contemporaneously plasma samples, both peripheral and portal, were collected for 6-keto $PGF_1\alpha$ measurement but these studies on blood samples will be described later in this chapter.

5.2.2 Methods

5.2.2.1 Collection of veins

In all cases vein samples 1-3mm in diameter and 2cm in length were harvested with minimal surgical trauma prior to harvesting. In all the laparotomies, the segments were harvested before extensive handling of the tissues. Patients who had taken aspirin or NSAIDs within the preceding two weeks were excluded. The samples were immediately kept ice cold for transfer to the laboratory for processing in the standard manner.

5.2.2.2 6-keto PGF1α measurement

6-keto $PGF_1\alpha$ was measured in the supernatants obtained after tissue incubation at 20°C for three minutes. The method is described in chapter 3 and the Wellcome antiserum was used without an extraction step, (Saba SR et al, 1973). Results were expressed as mean and standard deviation (SD) and in units of picograms 6-keto $PGF_1\alpha$ /100mg tissue (pg/100mg tissue).

5.2.2.3 Vein samples collected

Sections of veins were collected from four clinical groups of patients as follows:

group I Veins from the hip region in 25 patients undergoing total hip replacement for osteoarthritis; none had taken NSAIDs within two weeks of surgery and there was no concurrent portal hypertension

group II Mesenteric veins from 17 patients undergoing cholecystectomy with no concurrent medical problems or portal hypertension.

group III Mesenteric veins from nine patients with intra-abdominal malignancy with no portal hypertension (carcinoma of stomach, pancreas or colon)

group IV Mesenteric veins from six patients undergoing surgery for bleeding oesophageal varices secondary to portal hypertension. These patients were undergoing the procedure of oesophageal transection to arrest the haemorrhage.

5.2.3 Results

These results are shown in fig 5.2.3.

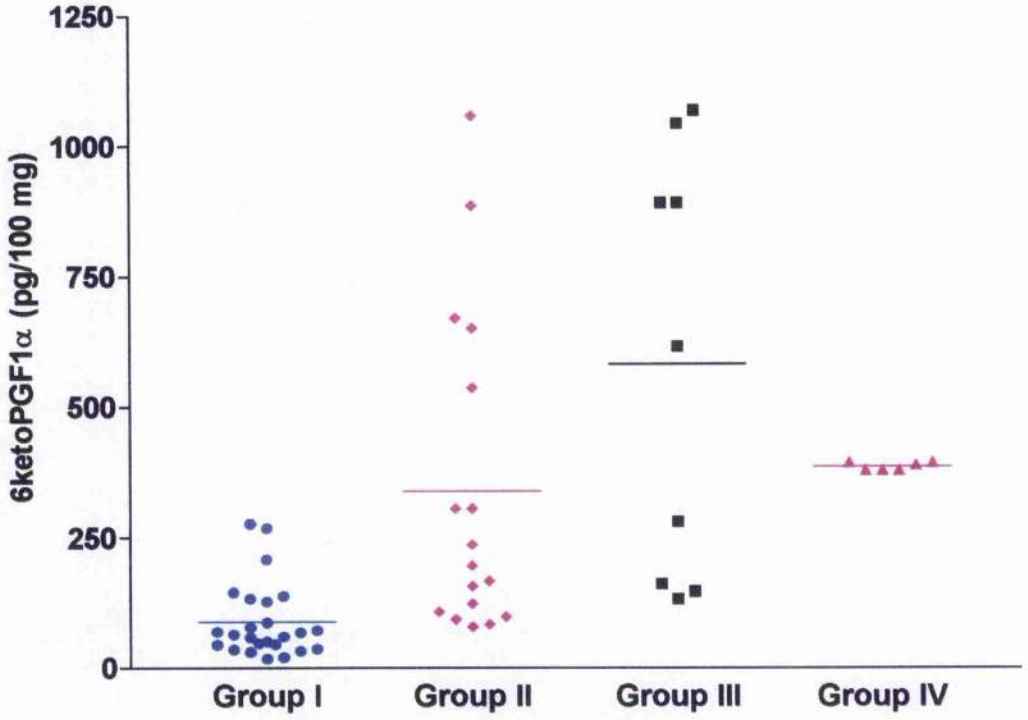


Fig 5.2.3 Scattergram with means of 6-keto $PGF_1\alpha$ measurements for the various groups: group I = 25 hip veins (mean 88); group II = 17 normal mesenteric veins from cholecystectomy patients (mean 350); group III = 9 mesenteric veins from patients with abdominal malignancy (mean 600); group IV = 6 portal hypertensive mesenteric veins (mean 393).

Hip veins (group I) had a mean 66-keto $PGF_1\alpha$ level of 88 +/-74 pg/100mg tissue which was significantly lower than in any of the three other clinical groups, (gp I -v- gp II, $p<0.001$; gp I -v- gp III, $p<0.01$; gp I -v- gp IV, $p<0.00001$). The mesenteric veins in groups II, III and IV showed no statistically significant differences, and specifically no difference between normotensive and hypertensive veins. The standard deviations for values in the normotensive groups were high with a very

small standard deviation found for the mesenteric veins from patients with portal hypertension.

5.2.4 Discussion

The radio-immunoassay using the Wellcome antiserum was clearly sensitive enough to measure 6-keto $PGF_1\alpha$ in incubates of both peripheral (hip) and portal (mesenteric) vein segments. As expected from the studies using the bioassay, the level of PGI_2 activity in man is considerably lower than in the rat and pig, and considerably below the lower threshold of sensitivity for the bioassay.

The striking finding in this study is the highly significantly increased PGI_2 production by mesenteric veins when compared to those from the hip. This finding suggests that there may also exist in man a gradient of PGI_2 activity across the various venous beds. Disappointingly, no difference was found between the hypertensive and normotensive mesenteric veins although there was a marked difference in the range of these values between the two. In fact, the range of 6-keto $PGF_1\alpha$ levels found in the six hypertensive mesenteric veins was remarkably consistent about the mean of 380pg/100mg. These samples were taken from patients actively haemorrhaging from the portal system but who had all been resuscitated to normal systemic pressures to allow the surgery to take place.

The anaesthetic agents used were standard for all of these procedures with all patients being paralysed and ventilated. The portal hypertensive group, (gp IV), was different from the other groups, having had haemorrhagic shock, resuscitation and transfusion with blood, fresh frozen plasma, and in addition treatment with

vasopressin at variable times before the vein harvesting. None of the patients in group IV were on Propranolol for treatment of portal hypertension. There are clearly many clinical differences between group IV and groups II and III and perhaps only a much bigger study with more patients in each group would reveal any underlying difference. The logistics of obtaining a sufficient number of patients with portal hypertension requiring open abdominal surgery are major, thus making a significantly large study impossible.

The trauma sustained by the tissues during harvesting may be another significant factor in explaining the differences. Mesenteric vein harvesting involved equivalent levels of trauma to the tissues in all groups. Technically there is a clear difference in harvesting hip vein however which could help explain the significant difference in 6-keto $PGF_1\alpha$ levels found in these veins. However, the degree of trauma sustained in hip vein harvesting is likely to be greater than that of mesenteric vein and subsequently higher rather than lower levels of 66-keto $PGF_1\alpha$ would have been expected. Assessment of the degree of trauma remains highly subjective and impossible to quantify accurately in this sort of study. Measurement of PGI_2 production in the plasma within these vessels would avoid these confounding factors of trauma from tissue dissection.

5.3 Plasma 6-keto PGF1α Levels Measured by Radio-Immunoassay

5.3.1 Introduction

It is logistically and ethically preferable to measure PGI_2 production from blood rather than tissue. This is particularly true in peripheral blood measurements where repeated sampling can be performed over a time period with minimal inconvenience and trauma to the patient. With the development of the RIA in our laboratory, this methodology was applied to human studies on samples from peripheral and portal blood samples. Initial work was concentrated on peripheral blood measurements in the peri-operative period, then on the first 7-10 days after surgery. Having made these baseline studies, levels in normal portal and hypertensive portal blood were measured.

Most of this work was carried out using the Wellcome antiserum with an extraction step. In the latter part of this study it became clear that the extraction process was resulting in inconsistent results as was previously described. Consequently, a different antiserum was employed which did not require the extraction process.

5.3.2 Methods

5.3.2.1 Studies using the Wellcome antiserum RIA

Samples were collected using 21G needles after the first 2ml of blood were discarded. Only peripheral veins which had not been previously cannulated were used and in portal venous sampling, blood was taken directly either from a

mesenteric, omental or portal vein at the beginning of the operative procedure with a view to minimising possible trauma to the intestines and portal system. The blood was collected into plastic tubes containing sodium EDTA and Indomethacin. Plasma was prepared from these samples, collected into 1.0ml aliquots in plastic tubes, and stored at -40°C until assayed.

Extraction was carried out as outline in chapter 3 (Baenziger NL et al, 1979). The residues were re-dissolved in 0.3ml GTB and assayed for 6-keto PGF_1 alpha using the Wellcome antiserum.

5.3.2.2 6-keto $PGF_{1\alpha}$ levels in peripheral blood of patients undergoing laparotomy or hip replacement

In this initial study blood samples from the antecubital veins were collected from 15 patients without portal hypertension undergoing laparotomy for benign disease, (cholecystectomy, vagotomy). Samples were collected before induction of anaesthesia, 24 hours after surgery and on days 2, 3, 4 and 7 post operation. In collaboration with the orthopaedic surgeons, a similar study was performed on 11 patients undergoing total hip replacement; peripheral venous samples were collected pre-operatively and on days 1, 2, 3, 4, 7 and 10.

5.3.2.3 6-keto $PGF_{1\alpha}$ levels in peripheral and portal blood of non portal hypertensive patients undergoing laparotomy

Blood samples were collected before induction of anaesthesia from 15 patients undergoing laparotomy, 11 for cholecystectomy and four for carcinoma. During the first part of the laparotomy and with minimal disturbance to the intestines, a sample

of portal venous blood was taken from a suitable vein as previously described. Finally peripheral samples were taken 24 hours after the operation.

5.3.2.4 Comparison of 6-keto $PGF_1\alpha$ levels in peripheral and portal vein blood using the Wellcome and Cardeza antisera

Because of marked differences of 66-keto $PGF_1\alpha$ activity found with the two antisera, comparison was made using both methods in the same samples. Blood was collected before induction of anaesthesia in five patients who did not have portal hypertension undergoing laparotomy for benign conditions. Portal samples were collected as previously described and peripheral samples obtained 24 hours after the operation.

Two 1.0ml aliquots from each sample were prepared, one assayed using the Wellcome antiserum after an extraction process, and the other directly assayed using the Cardeza antiserum without the extraction step.

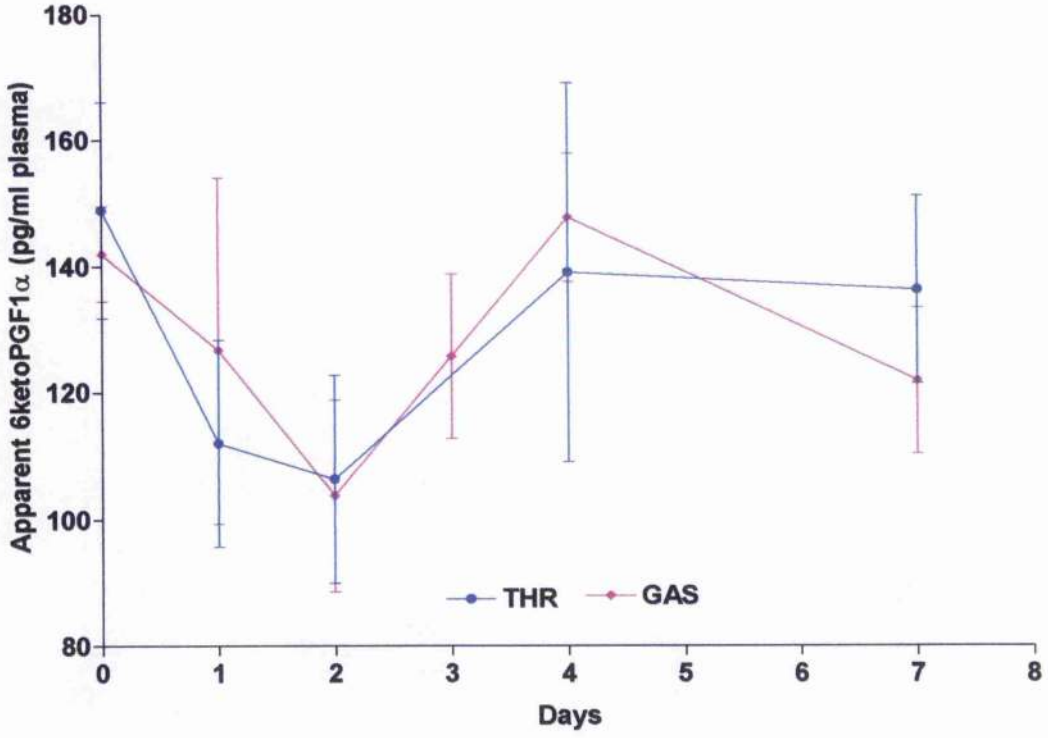
5.3.2.5 6-keto $PGF_{1\alpha}$ levels in portal blood using the Cardeza antiserum

Portal venous samples were obtained at laparotomy from 12 patients undergoing cholecystectomy and three undergoing procedures for bowel carcinoma, all of who did not have portal hypertension. Portal venous samples were also obtained from five patients with portal hypertension undergoing laparotomy for treatment of oesophageal variceal haemorrhage.

5.3.3 Results

5.3.3.1 6-keto PGF1α levels in peripheral blood of patients undergoing laparotomy or hip replacement

Using the Wellcome antiserum after extraction, detectable levels of 6-keto $PGF_1\alpha$ in peripheral blood samples of patients undergoing laparotomy (general abdominal surgery) were found (see fig 5.3.3.1). Overall these ranged from 9 – 196pg/ml of plasma with a pre-operative baseline range of 105 – 181pg/ml. A fall in 6-keto $PGF_1\alpha$ was found on the second post-operative day in the majority and on the third day in one patient, which was significant ($p<0.05$, Wilcoxon paired rank sum test). By one week after surgery, the levels had returned to pre-operative values.



Time	Day 0	Day 1	Day 2	Day 3	Day 4	Day 7
Total Hip Replacement	149 (18)	112 (17)	107 (17)	--------	140 (31)	138 (15)
General Abdominal Surgery	142 (17)	128 (28)	104 (15)	127 (13)	149 (11)	122 (12)

Fig 5.3.3.1 Apparent 6-keto $PGF_1\alpha$ levels in peripheral blood of patients having Total Hip Replacement (THR) or General Abdominal Surgery (GAS) Mean & (SD)

Similar baseline levels of 6-keto $PGF_1\alpha$ were found in the cohort of patients undergoing total hip replacement. There also occurred a similar significant fall in 6-keto $PGF_1\alpha$ on the second post-operative day ($p<0.05$, Wilcoxon paired rank sum

test). Also by day seven, these had returned to the pre-operative levels.

5.3.3.2 6-keto PGF1α levels in peripheral and portal blood of non portal hypertensive patients undergoing laparotomy

Using the Wellcome antiserum major differences were found in 6-keto PGF_1 alpha levels between peripheral and portal venous samples (table 5.3.3.2). This difference was ten to twenty fold with mean portal levels of 3901 (+/- 2304) pg/ml in comparison to mean pre-operative and 24 hour post-operative levels of 182 (+/- 76) and 194 (+/- 130)pg/ml respectively. There was no difference in peripheral plasma 6-keto PGF_1 alpha levels before or one day after surgery, confirming the findings of the preceding study.

Surgical group	Pre-operation peripheral	Post operation peripheral	Intra-operation portal
cholecystectomy	113	302	3830
	171	264	5122
	161	160	5122
	217	334	5036
	349	107	552
	180	199	979
	233	77	5325
	55	291	1054
	142	140	6043
	159	43	3858
	142	89	4071
Carcinoma	97	61	1356
	268	236	8271
	165	514	1652
	273	94	5522
Mean	**182**	**194**	**3901**
SD	**76**	**130**	**2304**

Table 5.3.3.2 Apparent 6-keto $PGF_1\alpha$ measured using the Wellcome antiserum in extracted peripheral and portal plasma from patients without portal hypertension undergoing abdominal surgery

5.3.3.3 Comparison of 6-keto $PGF_{1\alpha}$ levels in peripheral and portal vein blood using the Wellcome and Cardeza antisera

A striking difference in levels of 6-keto $PGF_1\alpha$ detected with the two assay methods was found. As in previous studies considerable quantities were detected with the Wellcome antiserum in extracted samples but no 6-keto $PGF_1\alpha$ at all could be detected with the Cardeza antiserum in non-extracted peripheral specimens (table 5.3.3.3). Low levels of 6-keto $PGF_1\alpha$ were found in four out of five of the portal samples using the Cardeza antiserum with none at all found in the fifth. This difference in levels found between the two assays was of several orders of magnitude with a mean 6-keto $PGF_1\alpha$ of 5955pg/ml in the Wellcome assay versus a mean of 9.34pg/ml in the Cardeza assay.

Patient	Plasma sample	Wellcome antiserum with extraction	Cardeza antiserum without extraction
1	Pre-op	217	0
	Post-op	334	0
	Portal	5036	13
2	Pre-op	113	0
	Post-op	302	0
	Portal	3830	10.4
3	Pre-op	268	0
	Post-op	236	0
	Portal	8271	13.7
4	Pre-op	349	0
	Post-op	107	0
	Portal	552	0
5	Pre-op	142	0
	Post-op	140	0
	portal	6043	9.6

Table 5.3.3.3 6-keto $PGF_1\alpha$ (pg/ml) measured in peripheral and portal blood using both Wellcome and Cardeza antisera

5.3.3.4 6-keto $PGF_{1\alpha}$ levels in portal blood using the Cardeza antiserum

Measurable but much reduced levels of 6-keto $PGF_1\alpha$ were found in all portal samples using this assay (table 5.3.3.4). Levels in the portal hypertensive group were similar to those found in patients with normal portal pressure, i.e. mean 19.9 (+/- 6.7) pg/ml versus 15.5 (+/- 10.4) pg/ml for the cholecystectomy patients and 20.5 (+/- 16.1) pg/ml for the three patients with carcinoma.

Cholecystectomy N=12	**Portal hypertensive patients** N=5	**Patients with carcinoma** N=3
2.9	11.5	8.9
3.7	14.1	13.7
9.6	21.8	38.9
9.6	25.9	
10.3	25.9	
11.5		
12.2		
13		
21.8		
26.8		
29.6		
35.2		
15.5 (10.4)	**19.9 (6.7)**	**20.5 (16.1)**

Table 5.3.3.4 6-keto $PGF_1\alpha$ levels (pg/ml) in portal plasma obtained at surgery measured using the Cardeza antiserum: mean and standard deviation shown in bottom row.

5.3.4 Discussion

Peripheral venous plasma samples contained measurable quantities of 6-keto $PGF_1\alpha$ using the extraction process and the Wellcome antiserum. Studies on orthopaedic and general surgical patients showed a similar pattern of significantly falling 6-keto $PGF_1\alpha$ on the first and second days after surgery with rapid return to normal by seven days. These are intriguing data which could suggest a phenomenon in the early post-operative period of diminished PGI_2 production which could affect platelet aggregability and be implicated in the multi-factorial prothrombotic state seen in the early post-operative period.

These findings were not confirmed using the radio-immunoassay without an extraction step where no 6-keto $PGF_1\alpha$ at all was detected in peripheral plasma samples. This raises the question as to what was been measured by the radio-immunoassay using the extraction procedure? Clearly conclusions regarding PGI_2 production from the extraction RIA cannot be made but the similarity in fall of apparent 6-keto $PGF_1\alpha$ at day two in all types of operation studied is an interesting one with regards to the aetiology of the post-surgical prothrombotic state. As a result of these major discrepancies in measured 6-keto $PGF_1\alpha$ in the same samples using the two radio-immunoassays, the use of the extraction process and the Wellcome antiserum was discontinued after only 15 samples were studied.

Portal vein plasma 6-keto $PGF_1\alpha$ measured by both assays was significantly higher than in peripheral plasma. There could be several explanations for this phenomenon. Firstly, the degree of manipulation or trauma to the intestine or portal

structures may be greater than that involved in taking a peripheral blood sample. However, great care was taken to minimize direct trauma and handling of the tissues prior to blood sampling and in addition, the first 2ml of blood were always discarded. Possibly the trauma of induction of anaesthesia and the laparotomy incision might contribute to this effect. Unfortunately, the design of this study did not involve the taking of peripheral venous samples during anaesthesia or any of the procedures in any of the groups studied. However, in the previously described studies, PGI_2 production from venous tissue samples revealed significantly greater levels produced from portal compared to peripheral veins. It is therefore likely that these elevated portal plasma levels are real and that there is elevated PGI_2 production in the portal system in man. No difference between 6-keto $PGF_1\alpha$ levels in portal normotensive and portal hypertensive patients could be found however in this small group of patients.

In conclusion, the logistics of obtaining portal samples have proved to be major and had limited the sample size of these clinical studies. Furthermore, the levels of PGI_2 produced in man are much lower than pig and rat. Thus reliable measurement using the available radioimmunoassays was also problematical with measured levels without the extraction process being at the very lower range of accuracy of the Cardeza radioimmunoassay. RIA with extraction leads to falsely elevated levels of 6-keto $PGF_1\alpha$ like compounds and RIA without extraction is not sensitive enough to measure levels in peripheral blood and only just so in portal plasma. In order to study PGI_2 production in the portal circulation in man, a more sensitive assay method was required.

5.4 Plasma 6-keto $PGF_{1\alpha}$ Levels Measured by Gas Chromatography / Mass Spectrometry

5.4.1 Introduction

The use of RIA in measuring plasma 6-keto $PGF_1\alpha$ in human samples was abandoned in 1982 because of the poor sensitivity of this assay for prostanoids in the very low levels produced by man. In 1983 a collaboration was set up with the Department of Clinical Pharmacology at the Royal Postgraduate Medical School, London (RPMS) to investigate PGI_2 production measuring 66-keto $PGF_1\alpha$ using gas chromatography / gas spectrometry. Dollery's group at the RPMS had shown that this method could accurately measure 6-keto $PGF_1\alpha$ down to levels of less than 2pg/ml (Blair IA et al, 1982). Samples of peripheral and portal blood from patients at the Royal Free Hospital were collected and transported to the RPMS where 6-keto $PGF_1\alpha$ measurements were made under the supervision of Dr's Sue E Barrow and J M Ritter (now Professor J M Ritter at Guys Hospital).

5.4.2 Methods

5.4.2.1 Subjects

Blood samples were taken from the following groups of patients:

group I Peripheral blood samples were taken from six patients with established cirrhosis and portal hypertension. These patients were stable and were not actively bleeding from oesophageal varices. They were in-patients undergoing investigation of their liver disease.

group II Six patients with portal hypertension and active bleeding from oesophageal varices requiring emergency surgery to stop the bleeding were studied. In three out of the six, pre-operative peripheral blood samples were taken. In all of the six patients, intra-operative peripheral blood samples and portal venous blood samples were collected in the standard manner.

group III Eight patients without liver disease or portal hypertension, but who were undergoing abdominal surgery for benign conditions (peptic ulceration, cholecystectomy), underwent portal venous sampling during surgery to provide a control group to establish 6-keto $PGF_1\alpha$ levels in the portal plasma. Peripheral samples were not taken from these patients during surgery.

5.4.2.2 Blood sampling

Peripheral samples were taken from an antecubital vein. Intra-operative peripheral and portal samples were taken simultaneously immediately on opening the abdomen. The portal samples were taken before any handling of the intestine or other intra-abdominal organs. A 21G needle was used to draw 20ml samples which were placed into ice cold lithium heparin tubes and mixed by gentle inversion. The heparinised tubes were stored in ice and transported to the laboratory for processing within 15 minutes of collection. The blood was immediately centrifuged at 4°C at 1000g. Plasma was aspirated using a plastic pipette and an exact volume (10ml or to the nearest 1 decimal place) was placed in a steralin container containing 2µg of

tetradeuterated 6-keto $PGF_1\alpha$ in distilled water as an internal standard. After mixing well and recording the exact plasma volume and patient's details on the container, the sample was stored at -20°C until transfer for assay. Samples were dispatched from the Royal Free Hospital to the RPMS on dry ice within one week of collection.

5.4.2.3 Assay by GC/MS

The assay method used at the RPMS was gas chromatography / negative ion chemical ionisation mass spectrometry (GC/NICIMS) previously validated as highly specific and sensitive (Blair IA et al, 1982).

Results were analysed using Wilcoxon's non paired rank sum test and for paired samples, Wilcoxon's paired sum rank test.

5.4.3 Results

These results are shown in table 5.4.3.

Patient group	Number	No surgery peripheral	Pre-op peripheral	Intra-op peripheral	Intra-op portal
I Stable	1	<2			
I Stable	2	<2			
I Stable	3	<2			
I Stable	4	<2			
I Stable	5	<2			
I Stable	6	<2			
II Bleeding	7		---	740	796
II Bleeding	8		---	748	1820
II Bleeding	9		---	99	411
II Bleeding	10		25	1842	11485
II Bleeding	11		26	440	177
II Bleeding	12		31	137	217
III Normal	13				51
III Normal	14				1874
III Normal	15				97
III Normal	16				96
III Normal	17				152
III Normal	18				81
III Normal	19				<5
III Normal	20				524

Table 5.4.3 6-keto $PGF_1\alpha$ levels (pg/ml) in peripheral and portal venous blood in stable and bleeding portal hypertensives, and in normal portal venous blood

group I All six stable patients with portal hypertension had levels of 6-keto $PGF_1\alpha$ of <2pg/ml in their peripheral blood.

group II In the pre-surgery samples taken from three patients actively bleeding from oesophageal varices, the peripheral 6-keto $PGF_1\alpha$ levels were significantly higher than the stable portal hypertensive patients (median 26 pg/ml versus <2 pg/ml; $p<0.024$). Very high concentrations were found in both the peripheral and portal venous samples during surgery for the six patients who were actively bleeding. Five out of six of these patients had higher portal levels than peripheral but this difference was not statistically significant.

group III Portal venous samples taken from the eight non portal hypertensive patients undergoing abdominal surgery contained high levels of 6-keto $PGF_1\alpha$ (range 0-1874 pg/ml, median 96.5 pg/ml). These were lower than those found in portal samples from the portal hypertensive patients (median 604 pg/ml – range 177-11485 pg/ml); this difference was significant, ($p<0.04$, Mann-Whitney test).

5.4.4. Discussion

Assay of 66-keto $PGF_1\alpha$ using the GC/NICIMS detected much lower levels in peripheral blood samples with the vast majority of patients having virtually no 6-keto $PGF_1\alpha$ detectable. These results were comparable to those observed in the previous clinical studies using the Cardeza antiserum without the extraction process. This

finding confirms the unreliability of RIA with extraction for prostanoids and confirms the findings of others (Morris HG et al, 1981).

Peripheral 6-keto $PGF_1\alpha$ levels in patients with portal hypertension who were not bleeding and in a stable condition were similarly very low at <2pg/ml. These levels fell within the normal range detected using this assay and were not different from patients with accelerated hypertension or coronary arterial disease in addition (Blair Ia et al, 1982 & Ritter JM et al, 1986). Pre-operative peripheral plasma samples from the three actively bleeding patients however contained higher levels of 6-keto $PGF_1\alpha$ (median 26 pg/ml, range 25 – 31 pg/ml). Although these are very small numbers, comparison with the peripheral plasma levels of the non bleeding portal hypertensive patients reveals that this difference is significant at $p<0.02$. Few conclusions can be made from such a small albeit homogenous sample. This elevation could either result from increased PGI_2 production in the splanchnic circulation in these bleeding patients spilling over into the general circulation, or result from the traumas of resuscitation. These could include central venous cannulation, the administration of blood transfusions and various vaso-active substances, multiple peripheral venous cannulations for blood sampling etc.

The very high levels found during surgery, both in peripheral and portal samples in these acutely bleeding portal hypertensive patients, were not significantly different but the elevation overall was striking. In five out of the six patients, the portal levels were higher than in the peripheral plasma. The 6-keto $PGF_1\alpha$ levels in portal blood in these actively bleeding patients were significantly increased in comparison to portal plasma obtained from patients without portal hypertension undergoing surgery.

This significant difference supports the experimental findings in this work relating increased portal pressure to increasing PGI_2 production

Major surgery seems to increase dramatically peripheral plasma levels of 6-keto $PGF_1\alpha$. This effect was seen not only in these liver patients, but also in patients having coronary artery surgery (Ritter JM et al, 1986). This raises the possibility that the administration of general anaesthetic agents and or the traumas of the various cannulations, incision and handling of tissues, causes a massive release of PGI_2. This could be a direct effect of trauma on endothelial cells and blood vessels since endothelial cells in culture have been shown to produce more PGI_2 when exposed to cyclical stress (Okahara K et al 1998). A further possibility is that PGI_2 is released as a reactive physiological mechanism to 'quench' the coagulation cascade thus preventing thrombus extension from haemostasis occurring in areas of sharp trauma to blood vessels. Alternatively, this may be a counteractive response to the massive platelet activation and release of TXA_2 occurring from the multiple sites of trauma from a major operation. Increased levels of PGI_2 would counteract the resultant vaso-constriction and increase platelet aggregation. This outpouring of PGI_2 during surgery may exhaust temporarily the endothelial cells' capacity for production of PGI_2 and explain the fall in PGI_2 activity found during the first 48 hours after hip and general abdominal surgery. This depletion of PGI_2 production in response to major surgery may be a possible contributor to the post-operative hyper coagulable state.

These studies using a reliable assay of 6-keto $PGF_1\alpha$ and hence PGI_2 production on small numbers of patients, provide clinical evidence to support the hypothesis that PGI_2 production is increased in the portal circulation in portal hypertension in man.

The trauma of surgery contributes to these elevated levels and the true magnitude of the increased PGI_2 production from portal hypertension could only be studied using less invasive methods of portal venous blood sampling.

CHAPTER 6: GENERAL DISCUSSION

Variceal haemorrhage commonly complicates liver diseases which lead to portal hypertension. The haemorrhage, typically spectacular and life threatening, is now dealt with satisfactorily by volume and blood replacement, correction of clotting abnormalities, administration of agents to reduce portal blood flow, and injection sclerotherapy. Long-term control is now achieved by pharmacotherapy with agents such as beta-blockers, and the use of Transjugular Intra hepatic Portasystemic Shunt (TIPS). In the modern age therefore, surgical involvement and treatment of this condition is now limited essentially to liver transplantation. At the time of the production of this work, namely the early 80's, it was quite a different situation. Injection sclerotherapy was in its early stages of clinical introduction and assessment, (Burroughs AK et al, 1989), and the main treatment for acute and chronic variceal bleeding was overwhelmingly surgical.

In the acute situation, this was by emergency portasystemic disconnection procedures such as oesophageal transection, and less commonly emergency porta-caval shunting. The major focus of surgical research into portal hypertension was therefore on the problem of control of haemorrhage in those patients where conservative treatment had failed. In the early 80's, the eruptive theory of explanation of oesophageal rupture was widely accepted with recognition that larger varices were more likely to bleed. It was not clear however whether there was a relationship between higher portal pressure within the varix and bleeding. Reynolds in 1982 reported a review of studies relating portal pressure to oesophageal rupture.

These measurements were mostly made by wedged hepatic venous pressure measurement and most reports showed no significant relationship between high portal pressure and bleeding, (Reynolds TB, 1982). Two further reports actually measured intra -variceal pressure by direct needle puncturing. In one the puncture was in the oesophageal variceal cord above the oesophago-cardiac junction and in the second, the puncture was directly into the varix at its point of potential haemorrhage although in this study the measurement was followed by injection sclerotherapy, (Palmer AD et al, 1956 and Westaby D et al, 1985). The fact that neither of these studies was complicated by significant oesophageal bleeding suggested that the relationship with oesophageal variceal pressure was not important. At the time of the work for this thesis, Groszmann had not yet elaborated the concept of variceal wall tension as the major factor in variceal rupture.

The other major factor implicated in the severity of bleeding from ruptured oesophageal varices was disordered coagulation. In liver disease coagulopathy is frequent and multi-factorial. The patients presenting to the surgeon at the time of this work were those precisely who had undergone aggressive replacement therapy and correction of clotting deficiencies but in whom haemorrhage had not stopped, or where the benefit had only been temporary. Haemorrhage of such severity from a small hole in an oesophageal varix was therefore a major source of perplexity to the surgeon who had to deal with this problem. In the early 80's it was felt that the inadequacies of conservative treatment of this condition reflected the current poor understanding of coagulation defects at that time rather than haemodynamic effects on the variceal wall. The recent discovery at that time of PGI_2 and TXA_2 and the feeling that these prostanoids were important in regulation of platelet aggregability,

possibly coagulation and of vascular tone, led to my investigation of their possible role in the bleeding diathesis of portal hypertension leading to variceal haemorrhage. Indeed, there was widespread interest at that time in the possibility that disturbances in the balance between PGI_2 and TXA_2 might be centrally involved in the pathogenesis of several disorders. Mainly these were disorders of abnormal thrombosis or haemostasis such as arteriosclerosis, diabetes mellitus, the haemolytic uraemic syndrome and others (chapter 1).

Accurate measurement of PGI_2 proved to be the most difficult part of these studies. Because of its evanescent nature, direct measurement of PGI_2 in tissues and fluids was not possible. The initial bioassay for PGI_2-like activity using platelet nephelometry proved to reliable in measuring inhibition of platelet aggregation in tissues. This assay had the advantage of measuring a physiological effect but lacked specificity with regard to measurement of PGI_2 itself. Testing for ADP-ase activity effectively excluded this physiological inhibitor but others such as PGA_2, PGE_1 and PGD_2 could not be excluded. Therefore the major deficiency in our use of this bioassay was in not using specific inhibitors of PGI_2 production in controls in order to establish whether the effect measured was indeed due to the presence of PGI_2. Once the radio-immunoassay for 6-keto $PGF_1\alpha$ was developed, an excellent correlation with PGI_2-like activity was found however, ($R=0.95$), indicating that the activity was indeed most likely due to PGI_2 production by the tissues.

Our development of the radioimmunoassay for 6-keto $PGF_1\alpha$ was driven mainly by the desire to have a more accurate and perhaps less cumbersome method of assay. The development however proved to be technically difficult with considerable

modification of the methodologies required as the studies progressed. This endeavour alone involved a great deal of time and development since these studies predated the advent of commercially available radioimmunoassay kits for 6-keto $PGF_1\alpha$.

The major problem arose in the radioimmunoassay measurement of 6-keto $PGF_1\alpha$ in serum. Initially because of the low levels detected, an extraction step was used which gave measured concentrations similar to many other published reports of that time. This was a time of excitement at the ability to measure significant levels of 6-keto $PGF_1\alpha$ in the serum, leading to the hypothesis that PGI_2 was a circulating hormone modulating vascular tone and coagulation (Moncada S etal, 1978[b]). The finding of elevated levels of 6-keto $PGF_1\alpha$ in portal venous samples also generated excitement in our laboratory which unfortunately proved to be almost as evanescent as the prostanoids. Once it was realised that artefactually high levels were being measured by the RIA, the extraction process was bypassed following the introduction of the high affinity Cardeza anti-serum. Using this anti-serum, 6-keto $PGF_1\alpha$ in normal serum was found to be either undetectable or below 10pg/ml. This finding was corroborated by others, leading to the conclusion that there was very little PGI_2 in the circulating blood and that it did not have a role as a circulating hormone, (Greaves M et al, 1982). In the last part of these clinical studies, plasma measurements were made using GC/NICIMS in Professor Dollery's Laboratories at the Hammersmith Hospital. This proved to be a method with high specificity and sensitivity providing accurate measurements of 6-keto $PGF_1\alpha$ in human serum, (Barrow SE et al, 1982). At that time however, this was a laborious and time-consuming assay involving equipment that occupied two laboratory rooms. Access

to this facility was therefore limited resulting in the last clinical study being constituted of small numbers. Today this equipment is much more convenient and user friendly, occupying one laboratory bench surface. Thus in the early 80's, in the absence of RIA kits and sophisticated spectrometers, the logistical difficulties of performing studies on PGI_2 and 6-keto $PGF_1\alpha$ were considerable.

For this work the model of partial portal vein ligation, (PPVL), to induce portal hypertension was chosen because of its convenience and the availability of the surgical expertise. Furthermore, this model had the advantage of causing portal hypertension without any liver damage, thus removing a possible confounding factor in these early studies. This model had previously been described by Orda and Ellis, (Orda R et al, 1978), and Myking and Halvorsen in the1970s, (Myking AO et al, 1973) who reported that it established portal hypertension by one week after PPVL. There was little else in the literature about this model at the beginning of the work for this thesis. This model was further assessed and developed in this thesis, confirming the rapid establishment of portal hypertension in the size of rat that I chose (150-250g). This effect was established by 24 hours and lasted up to two weeks at the significant level. The gradual return to normal pressure thereafter was confirmed to be due to the opening up of collaterals by studies using splenic portography and resin corrosion cast studies. In particular, the left anterior lumbar vein, (LALV), was found to constitute a major collateral between the portal and systemic circulations. PPVL and simultaneous ligature of the LALV gave a reliable model for chronic portal hypertension. A subsequent study using a tighter stenosis of the portal vein, (8mm versus 1.2mm), also provided a chronic portal hypertension model. Induction of portal hypertension by injection with microspheres was also

developed as a model specifically in order to avoid the potential trauma and inflammation of portal vein ligature. This proved to be effective but was associated with higher mortality and morbidity. Thus, I developed three simple and reliable models of portal hypertension using PPVL and microspheres, one giving acute portal hypertension which was limited to two weeks with return to normal pressure, the second giving chronic portal hypertension lasting over six weeks, and the third used in only one study because of its high morbidity and mortality. This allowed estimation of changes in PGI_2 production in both acute and chronic situations.

The PPVL model has subsequently been used extensively, in particular to study development of the hyper-dynamic circulation of portal hypertension. Grozsmann's group have shown that immediately after PPVL, there was resistance induced portal hypertension characterised by increased portal resistance, increased portal pressure and reduction of portal venous inflow. A series of changes occurred over the following seven days after ligation, namely extensive portasystemic shunting, a return of portal resistance to normal values, and increased portal venous inflow contributing to maintenance of elevated portal pressure, (Sikuler E et al, 1985). Subsequent work by this group confirmed that vasodilatation is the initiating event which together with subsequent plasma volume expansion results in the hyper-dynamic state, (Colombato LA et al, 1992). The experimental studies on rats established a direct relationship between PGI_2 production and pressure in the portal vein. Extensive comparison with controls excluded the effects of anaesthesia, surgical manipulation, and ligature related trauma and inflammation as possible causes. Furthermore, PGI_2 production was shown to be significantly increased by as early as 24 hours. It remained so as long as portal hypertension was present, with

return to normal levels even when previously established portal hypertension had resolved due to collateral development. This was the first report of increased PGI_2 production in portal venous hypertension which other workers later confirmed to develop in rats and rabbits, (Sitzmann JV et al, 1991 and Oberti F et al, 1993).

Further confirmation of the role of prostacyclin in portal hypertension would be provided by studies using cyclooxygenase inhibitors; these would be NSAIDs such as indomethacin or the more recently available COX inhibitors. I did not undertake these experiments, but Sitzmann et al., confirmed that 6-keto $PGF_1\alpha$ levels fell by 50% after indomethacin blockade of PPVL rabbits. Furthermore, infusion of PGI_2 resulted in a return to the previously raised levels of superior mesenteric artery resistance and blood flow seen in indomethacin blocked PPVL rabbits. These studies confirmed the importance of PGI_2 release in experimental portal hypertension and its role in maintaining the haemodynamic changes in the splanchnic circulation. Subsequently increased PGI_2 production was implicated by several workers in the development of porta-systemic collaterals. Once developed the rich collateral circulation may allow up to 90% of portal blood to be shunted away from the liver parenchyma, carrying with it PGI_2 which will not have been inactivated by the liver. Unlike other prostaglandins, PGI_2 is not metabolised by the lung, thus allowing a circulatory vasodilator action to take place in the systemic circulation. Thus, it is probable that a systemic hyperdynamic circulation could only develop once significant shunting of portal blood containing active PGI_2 has taken place.

The final part of my work focused on finding evidence for similar mechanisms in man. A significant difference in PGI_2 production by venous tissue between man and

animals was rapidly established. Bioassay of human venous tissues could not detect any PGI_2-like activity at all, and RIA measurements of 6-keto $PGF_1\alpha$ in venous tissues confirmed lower production of PGI_2. This was lower by an order of magnitude than those found in animal tissues, (pg rather than ng). In man mesenteric vein was found to have significantly greater production of PGI_2 than hip vein, a finding paralleling those in the rat and pig. Thus, in these species and in man, it is likely that a gradient of PGI_2 production in venous tissue exists with the greatest activity being present in veins (such as the portal circulation) exhibiting greater pressure.

No difference in 6-keto $PGF_1\alpha$ production was found between 19 normotensive mesenteric veins from normal patients and six hypertensive mesenteric veins taken from patients with bleeding oesophageal varices. However, there was a considerably increased range of measurements in these normal veins in comparison to the six hypertensive veins. The standard deviation in the normotensive veins was fifty times greater than in the hypertensive veins. This suggests that a difference may be present but this could only be determined by a much larger study than was possible at this time.

Serum levels of 6-keto $PGF_1\alpha$ using the RIA were significantly greater in portal vein compared to peripheral venous samples. This difference was present for both the anti-sera used but with no 6-keto $PGF_1\alpha$ detected in any peripheral samples when using the Cardeza anti-serum. No difference between serum 6-keto $PGF_1\alpha$ levels was found between portal hypertensive and normotensive patients, confirming the finding in the incubated mesenteric human venous tissue samples.

Therefore, these clinical studies confirmed increased PGI_2 production in the portal system but that this activity was at much lower levels than in the rat, rabbit or pig with the average levels being <10pg/ml. After extraction, much greater levels of "apparent" PGI_2 were measured with the intriguing finding of significant falls occurring during the second and third days after hip and abdominal surgery. It was not clear what was being measured in these "apparent" 6-keto $PGF_1\alpha$ samples, but presumably several members of the prostanoid family were being extracted. From all of the experimental and clinical studies, it is apparent that the trauma of surgery and general anaesthesia increases production of PGI_2 and most probably, other prostaglandins. This fall detected in the early post-operative period may signify a degree of exhaustion of prostaglandin metabolism and it would be attractive to investigate this further as a possible contribution to the pro-thrombotic tendency which has long been recognized to occur in the post-operative period. These studies were on tissue and serum, therefore no contribution or effect arising from platelet prostaglandin substrates and in particular TXA_2 can be implicated in this phenomenon. The finding of a major effect of surgery in the studies using gas chromatography/mass spectrometry (GC/MS), confirmed that there is a major outpouring of prostacyclin in major surgical interventions. This further supported the possibility that exhaustion of production of vasoactive prostaglandins does occur in the early post-operative period, an hypothesis which merits further study.

Gas chromatography/negative iron chemical ionisation mass spectrometry (GC/NICIMS) was used in the final part of the human studies to assay PGI_2 production but this was possible only in a limited number of peripheral and portal

venous samples. Once again, levels in samples obtained at laparotomy were found to be much higher than pre-operative peripheral venous samples. Similarly, increased levels of peripheral venous 6-keto $PGF_1\alpha$ were found in patients undergoing cardio-thoracic surgery. The trauma of general anaesthesia for major surgery with its multiple catheterisations, and the surgical handling of tissues is therefore implicated in this massive release of PGI_2. In view of the limited access to this assay during these studies, limited comparison was possible between the normotensive and hypertensive portal venous samples. However, a significant increase in PGI_2 production in portal hypertension was found in portal plasma from patients actively bleeding from oesophageal varices compared to normal. This is an important finding in a small sample of 6 patients which provides clinical evidence to support my hypothesis that increased PGI_2 production may be a factor in the severity of bleeding from ruptured varices.

This highly specific and sensitive method of assaying PGI_2, (Blair IA et al, 1982), confirmed the findings using the Cardeza RIA that in man levels of circulating PGI_2 are extremely low and indeed barely detectable, (<2pg/ml). The finding of increased levels in portal venous and peripheral samples in patients with portal hypertension undergoing surgery, must be interpreted with caution however because of the undoubted major effect of surgical trauma on PGI_2 production. Further studies using this assay, but on samples collected less invasively, perhaps by portal venous cannulation during TIPS procedure would be an area of future study. It would be important in any such studies to exclude the effect of general anaesthesia. General anaesthesia has long been shown to affect splanchnic blood flow causing reductions of up to 30% in this vascular bed with 20-50% reduction in hepatic blood flow,

(Calverley RK et al, 1978, Eger EI et al, 1970 and Gelman SI, 1976). Such a significant reduction in splanchnic blood and hepatic blood flow would reduce portal pressure, thus potentially causing diminished PGI_2 production by the endothelial cells of the portal venous system. Future studies on portal venous tissues in addition to serum would also be of value (accepting the effect of general anaesthesia), but in the modern era the only readily available source of portal hypertensive samples would be in patients in end stage liver failure undergoing liver transplantation. Plasma measurements in such further studies must be using GC/MS methodologies rather than RIA which is undoubtedly unreliable in plasma measurements. As has been emphasized throughout this thesis, these facts were not recognized at the time of the studies and the logistics were such that GC/NICIMS was not readily available for larger studies than the one performed.

Thus the evidence gathered from this work in man indicates high levels of PGI_2 production in the normal portal circulation but it remains unclear whether there is further elevation in portal hypertension. The generally undetectable levels of peripheral PGI_2 in the plasma, even in patients with portal hypertension, would suggest that "spill-over" of PGI_2 from the splanchnic circulation via collaterals is unlikely to contribute in man to the hyperdynamic circulation of portal hypertension. Infusion of PGI_2 for treatment of disorders such as severe lower limb ischaemia is usually given at 8ng/min. This causes marked haemodynamic and platelet effects in man but results in peripheral plasma concentrations of 6-keto $PGF_1\alpha$ of 291-510 pg/ml, (Dollery CT et al, 1983). The levels of <2pg/ml in stable portal hypertensives, and 25-31 pg/ml in patients with bleeding oesophageal varices who are not undergoing surgery, would not be expected to have any major haemodynamic effect.

Possibly in man, PGI_2 may play a role in the initial development of collaterals and the hyperdynamic circulation but not in its maintenance.

In man therefore, PGI_2 most probably acts at a local endothelial level rather than as a systemic hormone. All of my clinical studies have revealed elevated levels of PGI_2 to be present in the portal circulation, both in the presence and in the absence of portal hypertension. These levels are within the range where significant effects on platelet aggregation can occur. Furthermore, there may be surges in PGI_2 production as portal pressure changes. It is well recognised that portal pressure may increase significantly after meals and during infections. A recently proposed hypothesis implicates bacterial infection with portal endotoxaemia in the pathogenesis of variceal bleeding, (Goulis J et al, 1999). Endotoxin may mediate increased release of endothelin causing increased portal hypertension by stellate cell contraction. This may cause increased PGI_2 production which also may be separately simulated by the action of the endotoxin. Furthermore, endothelin may also mediate increased PGI_2 production by the endothelium. Similar effects on increased nitric oxide production result and the combination of increased production of PGI_2 and nitric oxide could inhibit platelet aggregation resulting in disturbance of haemostasis at the site of variceal rupture.

There is scarce human data available to allow conclusions regarding the importance of PGI_2 in development of the hyperdynamic circulation to be made. Further studies are required to investigate possible correlations between haemodynamic markers such as cardiac output, splanchnic blood flow, and accurate circulating PGI_2 measurements.

Since this work was done, several other factors which cause vasodilatation have been proposed and investigated using the model of PPVL. These include glucagon, (Benoit JN et al, 1986, Kravetz D et al, 1988), adenosine (Murakami S et al, 1996), bile acids (Genecin P et al, 1990), endotoxin (Mehta R et al, 1990), nitric oxide (Sieber CC et al, 1992), and tumour necrosis factor (Lopez-Talavera JC et al, 1995). Much current work is focusing on the concept that increased nitric oxide production plays an important role in the development of the vasodilatory changes in experimental portal hypertension. We have recently investigated the hypothesis that formation of reactive oxidant species causes oxidative stress in the portal circulation with activation of NF kappa-β resulting in synthesis of nitric oxide. Nitric oxide produced in the portal circulation and 'spilling over' into the systemic circulation via collaterals, has been implicated as a causative factor in the development of the hyperdynamic circulation (Fernando BS, 1999).

Thus it is likely that the development of the hyperdynamic circulation results from an interplay of several factors which act to maintain it. The majority of evidence is based on experimental models with relatively little human data available at the present time. This accumulated experimental data suggests further future avenues of possible clinical intervention to modulate the development of portal hypertension. Clinical trials of cyclooxygenase inhibitors are ethically difficult because of the likely effect of these agents on renal prostaglandin metabolism leading to hepato-renal syndrome and also because of the coronary complications reported with COX-2 inhibitors. Specific PGI_2 inhibitors which are safe are also not currently available. The proposed prospective double-blinded randomised trial of antibiotic treatment

versus placebo for prevention of variceal bleeding would be of great interest. This would test the hypothesis, (Goulis J et al, 1999), that endotoxaemia is the prime mediator for increases in portal hypertension and release of nitric oxide and prostacyclin which may result in deficient coagulation at the site of variceal rupture. Such a clinical study would involve large numbers of patients and in-vitro platelet studies to establish whether impaired platelet function is present in variceal bleeding would then be possible. If this effect was confirmed, it would also be possible to determine whether this was secondary to the effects of nitric oxide or PGI_2 release. The logistics and ethics of obtaining portal venous samples would be most difficult however. If this study confirms the importance of the two mediators, the development of future inhibitors of nitric oxide synthase or cyclooxygenase may prove to be beneficial in the prevention of variceal bleeding.

Conclusion

Accurate measurement of PGI_2 proved to be difficult from the beginning. The need for evolution of these assays from bioassay, through radioimmunoassay with and without an extraction process, and eventually to the gold standard of GC/NICIMS, in our laboratory paralleled the experience of others. The experimental studies in rat and pig revealed a high level of production of prostacyclin in all tissue studied with a consistent finding of the highest levels of PGI_2 being produced in portal venous tissues.

The model of partial portal vein ligation proved to be reliable and convenient but the initial model in rats of low weight proved to provide portal hypertension of only two

weeks duration. Identification of the important and major collateral channel via the left anterior lumbar vein allowed modification to provide a reliable model of chronic portal hypertension. It was also found that using a more severe degree of partial portal vein ligation also resulted in long lasting portal hypertension.

In the rat PPVL models, production of PGI_2 by portal vein endothelium was found to be directly related to portal hypertension. This relationship was confirmed to be highly significant with significantly increased PGI_2 production being present in all cases when portal hypertension had developed. In the first model, the return of portal vein pressure to normal with the opening up of collateral circulation was accompanied by return of PGI_2 production to normal levels. Correlation between measurements made using the bioassay in these experiments, and those using radioimmunoassay for 6-keto $PGF_1\alpha$ confirmed that the bioassay was measuring PGI_2 production.

A significant but lesser effect on PGI_2 production was confirmed to result from surgical intervention. This increased PGI_2 production was confirmed to also happen in man.

Clinical studies on human venous tissues and plasma confirmed elevated PGI_2 production by portal venous tissue and in the portal venous blood. Very low and in many cases undetectable levels of PGI_2 in peripheral plasma was found in man using the more specific RIA with the Cardeza anti-serum, and the highly specific GC/NICIMS. No difference could be found in portal venous tissue between patients with or without portal hypertension, but higher levels were found in portal

hypertensive venous plasma levels, and the highest levels of all were found in portal plasma samples from actively bleeding patients. The numbers studied however were small.

Several other vasoactive substances are now implicated in the pathophysiology of portal hypertension. The commonest among these currently is nitric oxide which also has an effect similar to prostacyclin, and seems to act synergistically with it. The roles of nitric oxide and prostacyclin in the pathophysiology of portal hypertension and the possible effects on bleeding from oesophageal variceal rupture merit further study. The studies described here were the first to provide insight into the pathophysiology of portal hypertension relating to prostacyclin and endothelial function. Clinical evidence to support the hypothesis that increased prostacyclin release in portal hypertension may play a significant role in the severity of bleeding from oesophageal varices was found albeit in 6 patients. The hypothesis has therefore not been disproved and further clinical study certainly in larger numbers of patients, and possibly with selective COX-2 inhibitor drugs are required.

APPENDIX A

Reagents

Tris Buffer pH 8.0

Tris is Tris (hydroxymethly) methylamine.

Molecular weight = 121.14 (BDH, Analar).

6.057g in 1000ml. Of 0.9% sodium chloride gives 50mM solution.

Its pH, around 11 is then adjusted to ph 8.0 using concentrated hydrochloric acid. The same solution is adjusted to pH 7.5 when indicated.

Gleatin tris buffer pH 7.5 (GTB)

6.057g tris in 1000ml of 0.9% saline

1g sodium azide (Sigma)

1g gelatin (Sigma)

The pH of the solution is adjusted to 7.5 using concentrated hydrochloric acid.

Tris buffer containing 1% plasma γ globulin

0.9ml GTB

0.1ml plasma (blood being collected in 0.38% trisodium citrate).

Tris buffer is used for the 6ketoPGF$_{1\alpha}$ RIA using the Cardeza antiserum.

Owren's buffered saline pH 7.35

20% Owren's barbiturate buffer pH 7.35:

11.75 sodium diethylbarbiturate (Barbitone sodium)

14.67g sodium chloride

1570ml distilled water

430ml 0.1 N hydrochloric acid

80% isotonic saline (0.9% w/v sodium chloride).

Tritiated 6ketoPGF$_{1\alpha}$ (^{3}H) - 6ketoPGF$_{1\alpha}$)

This was bought from the Radiochemical Centre, Amersham, about once every four months and stored at less than -20°C.

Molecular weight with tritium = 380

Specific activity = 150Ci/mMol = 395mCi/mg.

1 Ci (Curie) 2.22 x 10^{12} disintegrations per minute (dpm)

therefore 1mg = 395 x 2.22 x 10^9 dpm

1ng = 395 x 2.22 x 10^9 dpm

1pg = 395 x 2.22 dpm = 876.9 dpm

Activity in the stock = 0.1mCi/ml

0.005ml = 0.0005mCi

Dissolved in 10m Tris = 0.000005mCi

= 0.000005 x 2.22 x 10^9 dpm

= 11.1 x 10^3 dpm

= 11000 dpm

Therefore, to prepare the labelled 6ketoPGF$_{1\alpha}$ solution, 5µl stock (^{3}H) - 6ketoPGF$_{1\alpha}$ was dissolved in 10ml GTB and 0.1ml was used for each sample.

Dextran coated charcoal (DCC)

Charcoal (Norit A, Sigma) was washed several times over a period of days with distilled water, discarding any that did not precipitate and then dried in an oven. This procedure was to ascertain that the washed charcoal would all precipitate when centrifuged during the RIA. Dextran coated charcoal:

0.25g washed charcoal

0.025 Dextran T40 (Pharmacia Fine Chemicals)

100ml GTB

Scintillation Fluid for 6ketoPGF$_{1\alpha}$

20g butyl-PBD (Chemical and Services)

1400ml Toluene (BDH, Analar Grade)

600ml Triton X-100 (BDH, Analar Grade)

Antiserum to 6ketoPGF$_{1\alpha}$ (Wellcome)

The antiserum was in a freeze-dried from and 5mg was weighed out and made up to 10mg in tris buffer pH 7.5. Aliquots of 0.75ml were made and stored at -40°C until used when the aliquot was made up to 9ml with GTB.

APPENDIX B

Procedures

'Total' and 'Charcoal Blank' Samples

A 'total' sample measures the total amount of radioactivity present in each sample.

A 'charcoal blank' sample measures the amount of non-specific binding in the radioimmunoassay system.

Both types of samples have:

0.3ml GTB

0.1ml (^{3}H) - 6ketoPGF$_{1\alpha}$ (ca. 10000 dpm)

However, 1ml of dextran coated charcoal is added to the 'charcoal blank' sample while 1ml of GTB is added to the 'total' sample at the end of the incubation period when bound (^{3}H) - 6ketoPGF$_{1\alpha}$ is separated from free.

Calculation of bound fraction (%)

DCC bound to free ligand in the assay mixture can be precipitated by centrifugation, so that the supernatant fraction consists of the bound ligand which is counted. The LKB scintillation counter converts the count per minute (CPM) to disintegrations per minute (dpm). Expressing the dpm obtained from the sample over the 'total' present having first excluded the amount found in the charcoal blank for both samples, gives the percentage bound.

$$\% \text{ bound} = \frac{\text{dpm in supernatant of sample} - \text{charcoal blank}}{\text{dpm in 'total'} - \text{charcoal blank}}$$

APPENDIX C

Calculations

Calculation on the relative cross-reactions of the Wellcome antiserum to prostaglandins

A) From standard curves (Fig 6.3):

At 50% total activity:

6ketoPGF$_{1\alpha}$	251 pg
PGF$_{2\alpha}$	7244 pg

PGA_2	380189 pg
PGE_2	758578 pg
PGA_1	275423 pg

Therefore relative cross-reaction to

$PGF_{2\alpha}$	=	$\frac{251}{7244}$	=	0.03
PGE_1	=	$\frac{251}{275423}$	=	.0009
PGA_2	=	$\frac{251}{380189}$	=	.0007
PGE_2	=	$\frac{251}{758578}$	=	.0003

B) From Logit Plots (Fig 3.3.1.5.2.b):

At $\log_e$	= -5	
6ketoPGF$_{1\alpha}$	= log 1.6	i.e. 39.8 ng
$PGF_{2\alpha}$	= log 4.1	i.e. 12589.2 ng

PGA_2, E_2 and E_1 are less than 10,000,000

Therefore relative cross-reaction to

$PGF_{2\alpha}$	=		$\frac{39.8}{12589.2}$	=	0.0032
PGA_2, E_2 and E_1	=	<	$\frac{39.8}{10,000,000}$	<	0.000004

C) From Logit Plots (Fig 3.3.1.5.2.c):

At $\log_e$	= -5
6ketoPGF$_{1\alpha}$	= 14700
PGI_2	= 27000

Therefore relative cross-reaction to = $\frac{14700}{27000}$ = 0.54

References

Adelman,B, M B Stemerman, D Mennell, R I Handin, 1981, The interaction of platelets with aortic subendothelium: inhibition of adhesion and secretion by prostaglandin I2: Blood, v. 58, p. 198-205.

Andersson,R, L Lundholm, E Mohme-Lundholm, K Nilsson, 1972, Role of cyclic AMP and Ca++ in metabolic and mechanical events in smooth muscle: Adv.Cyclic.Nucleotide Res, v. 1, p. 213-229.

Askari,B, N R Ferrerib, 2001, Regulation of prostacyclin synthesis by angiotensin II and TNF-alpha in vascular smooth muscle: Prostaglandins, v. 63, p. 175-187.

Awbrey,BJ, J C Hoak, W G Owen, 1979, Binding of human thrombin to cultured human endothelial cells: J.Biol.Chem., v. 254, p. 4092-4095.

Baenziger,NL, M J Dillender, P W Majerus, 1977, Cultured human skin fibroblasts and arterial cells produce a labile platelet-inhibitory prostaglandin: Biochem.Biophys.Res Commun., v. 78, p. 294-301.

Baenziger,NL, P R Becherer, P W Majerus, 1979, Characterization of prostacyclin synthesis in cultured human arterial smooth muscle cells, venous endothelial cells and skin fibroblasts: Cell, v. 16, p. 967-974.

Baenziger,NL, L E Force, P R Becherer, 1980, Histamine stimulate prostacyclin synthesis in cultured human umbilical vein endothelial cells: Biochem.Biophys.Res Commun., v. 92, p. 1435-1440.

Baenziger,NL, F J Fogerty, L F Mertz, L F Chemuta, 1981, Regulation of histamine-mediated prostacyclin synthesis in cultured human vascular endothelial cells: Cell, v. 24, p. 915-923.

Baker,LA, C Smith, Lieberman G., 1959, A natural history of esophageal varices.: Am.J.Med, p. 228-236.

Ballard,HS, A J Marcus, 1976, Platelet aggregation in portal cirrhosis: Arch.Intern.Med., v. 136, p. 316-319.

Barrow,SE, K A Waddell, M Ennis, C T Dollery, I A Blair, 1982, Analysis of picomolar concentrations of 6-oxo-prostaglandin F1 alpha in biological fluids: J.Chromatogr., v. 239, p. 71-80.

Beitz,J, W Forster, 1980, Influence of human low density and high density lipoprotein cholesterol on the in vitro prostaglandin I2 synthetase activity: Biochim.Biophys.Acta, v. 620, p. 352-355.

Bell,RL, P W Majerus, 1980, Thrombin-induced hydrolysis of phosphatidylinositol in human platelets: J.Biol.Chem., v. 255, p. 1790-1792.

Benhamou JP., 1982, Risk factors of gastrointestinal bleeding in alcoholic cirrhosis, in D Westaby, BRD MacDougall, and R Williams (eds), Variceal Bleeding: Pitman Books Ltd, p. 13-19.

Bendis.L, F Wong. The hyperdynamic circulation in cirrhosis: an overview. Pharmacology and Therapeutics 89, 221-231. 2001.
Ref Type: Generic

Benoit,JN, B Zimmerman, A J Premen, V L Go, D N Granger, 1986, Role of glucagon in splanchnic hyperemia of chronic portal hypertension: Am.J.Physiol, v. 251, p. G674-G677.

Bergstrom,S, Danielsson H, Samuelsson B., 1964, The enzymatic formation of prostaglandin E2 from arachidonic acid prostaglandins and related factors 32.: Biochim.Biophys.Acta, p. 207-210.

Best,LC, T J Martin, R G Russell, F E Preston, 1977, Prostacyclin increases cyclic AMP levels and adenylate cyclase activity in platelets: Nature, v. 267, p. 850-852.

Bizzi,A, A M Tacconi, E Veneron, Garattini S., 1977, Pharmocology of steroid oral contraceptive drugs., in S Garattini and HW Berendes (eds), New York, Raven Press, p. 237-249.

Blackwell,GJ, W G Duncombe, R J Flower, M F Parsons, J R Vane, 1977, The distribution and metabolism of arachidonic acid in rabbit platelets during aggregation and its modification by drugs: Br.J.Pharmacol., v. 59, p. 353-366.

Blair,IA, S E Barrow, K A Waddell, P J Lewis, C T Dollery, 1982, Prostacyclin is not a circulating hormone in man: Prostaglandins, v. 23, p. 579-589.

Bloom,AL, 1975, Intravascular coagulation and the liver: Br.J.Haematol., v. 30, p. 1-7.

Bolger,PM, G M Eisner, P W Ramwell, L M Slotkoff, 1978, Renal actions of prostacyclin: Nature, v. 271, p. 467-469.

Borda,ES, M C Agostini, N Sterin-Speziale, M F Gimeno, A L Gimeno, 1979, Spontaneous contractile activity of isolated ovarian human vein. A dual influence of prostacyclin (PGI2): Prostaglandins, v. 18, p. 829-835.

Born GVR, 1962, The fate of 5-hydroxytryptamin in a smooth muscle and in connective tissue.: J.Physiol, p. 67p.

Broekman,MJ, J W Ward, A J Marcus, 1980, Phospholipid metabolism in stimulated human platelets. Changes in phosphatidylinositol, phosphatidic acid, and lysophospholipids: J.Clin.Invest, v. 66, p. 275-283.

Bult,H, M J Parnham, I L Bonta, 1977, Bioassay by cascade superfusion using a highly sensitive laminar flow technique: J.Pharm.Pharmacol., v. 29, p. 369-370.

Bunting,S, R Gryglewski, S Moncada, J R Vane, 1976, Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin X) which relaxes strips of mesenteric and coeliac ateries and inhibits platelet aggregation: Prostaglandins, v. 12, p. 897-913.

Burroughs,AK, G Hamilton, A Phillips, G Mezzanotte, N McIntyre, K E Hobbs, 1989, A comparison of sclerotherapy with staple transection of the esophagus for the emergency control of bleeding from esophageal varices: N.Engl.J.Med, v. 321, p. 857-862.

Butcher,RW, C E Baird, 1968, Effects of prostaglandins on adenosine 3',5'-monophosphate levels in fat and other tissues: J.Biol.Chem., v. 243, p. 1713-1717.

Cahill P.A., Redmond EM, Sitzmann J.V., 2001, Endothelial dysfunction in cirrhosis and portal hypertension.: Pharmacology and Therapeutics, v. 89, p. 273-293.

Calverley,RK, N T Smith, C Prys-Roberts, E I Eger, C W Jones, 1978, Cardiovascular effects of enflurane anesthesia during controlled ventilation in man: Anesth.Analg., v. 57, p. 619-628.

Campbell,WB, D R Harder, 1999, Endothelium-derived hyperpolarizing factors and vascular cytochrome P450 metabolites of arachidonic acid in the regulation of tone: Circ.Res., v. 84, p. 484-488.

Carreras,LO, D A Chamone, P Klerckx, J Vermylen, 1980, Decreased vascular prostacyclin (PGI2) in diabetic rats. Stimulation of PGI2 release in normal and diabetic rats by the antithrombotic compound Bay g 6575: Thromb.Res, v. 19, p. 663-670.

Chen,YC, B McLeod, E R Hall, K K Wu, 1981, Accelerated prostacyclin degradation in the thrombotic thrombocytopenic purpura: Lancet, v. 2, p. 267-269.

Chu,CJ, F Y Lee, S S Wang, R H Lu, Y T Tsai, H C Lin, M C Hou, C C Chan, S D Lee, 1997, Hyperdynamic circulation of cirrhotic rats with ascites: role of endotoxin, tumour necrosis factor-alpha and nitric oxide: Clin.Sci.(Lond), v. 93, p. 219-225.

Claesson,HE, 1980, Prostaglandin I2 synthesis and elevation of cyclic AMP levels in 3T3 fibroblasts: Biochim.Biophys.Acta, v. 618, p. 399-406.

Collins,GA, M C Sutter, 1975, Quantitative aspects of cyclic AMP and relaxation in the rabbit anterior mesenteric-portal vein: Can.J.Physiol Pharmacol., v. 53, p. 989-997.

Colombato,LA, A Albillos, R J Groszmann, 1992, Temporal relationship of peripheral vasodilatation, plasma volume expansion and the hyperdynamic circulatory state in portal-hypertensive rats: Hepatology, v. 15, p. 323-328.

Coughlin,SR, M A Moskowitz, B R Zetter, H N Antoniades, L Levine, 1980, Platelet-dependent stimulation of prostacyclin synthesis by platelet- derived growth factor: Nature, v. 288, p. 600-602.

Coughlin,SR, M A Moskowitz, H N Antoniades, L Levine, 1981, Serotonin receptor-mediated stimulation of bovine smooth muscle cell prostacyclin synthesis and its modulation by platelet-derived growth factor : Proc.Natl.Acad.Sci.U.S.A, v. 78, p. 7134-7138.

Cowan,DH, 1973, Thrombokinetic studies in alcohol-related thrombocytopenia: J.Lab Clin.Med., v. 81, p. 64-76.

Cowan,DH, 1980, Effect of alcoholism on hemostasis: Semin.Hematol., v. 17, p. 137-147.

Czervionke,RL, J B Smith, J C Hoak, G L Fry, D L Haycraft, 1979, Use of a radioimmunoassay to study thrombin-induced release of PGI2 from cultured endothelium: Thromb.Res, v. 14, p. 781-786.

Dagradi,AE, 1972, The natural history of esophageal varices in patients with alcoholic liver cirrhosis. An endoscopic and clinical study: Am.J.Gastroenterol., v. 57, p. 520-540.

Davis,TM, M D Mitchell, R C Turner, 1979, Prostacyclin and thromboxane metabolites in diabetes: Lancet, v. 2, p. 789-790.

Davis,TM, E Bown, D R Finch, M D Mitchell, R C Turner, 1981, In-vitro venous prostacyclin production, plasma 6-keto-prostaglandin F1 alpha concentrations, and diabetic retinopathy: Br.Med J.(Clin.Res Ed), v. 282, p. 1259-1262.

Defreyn,G, M V Dauden, S J Machin, J Vermylen, 1980, A plasma factor in uraemia which stimulates prostacyclin release from cultured endothelial cells: Thromb.Res, v. 19 , p. 695-699.

Defreyn,G, S J Machin, L O Carreras, M V Dauden, D A Chamone, J Vermylen, 1981, Familial bleeding tendency with partial platelet thromboxane synthetase deficiency: reorientation of cyclic endoperoxide metabolism: Br.J.Haematol., v. 49, p. 29-41.

Dembinska-Kiec,A, T Gryglewska, A Zmuda, R J Gryglewski, 1977, The generation of prostacyclin by arteries and by the coronary vascular bed is reduced in experimental atherosclerosis in rabbits: Prostaglandins, v. 14, p. 1025-1034.

Diamond,J, T G Holmes, 1975, Effects of potassium chloride and smooth muscle relaxants on tension and cyclic nucleotide levels in rat myometrium: Can.J.Physiol Pharmacol., v. 53, p. 1099-1107.

Dollery,CT, S E Barrow, I A Blair, P J Lewis, J MacDermot, M A Orchard, J M Ritter, C Robinson, G L Shepherd, K A Waddell, D J Allison, 1983, Role of Prostacyclin, in NE Miller (ed), Atherosclerosis: Mechanisms and Approaches to Therapy: New York, Raven Press, p. 105-123.

Downing,I, G L Shepherd, P J Lewis, 1980, Reduced prostacyclin production in pre-eclampsia: Lancet, v. 2, p. 1374.

Dusting,GJ, S Moncada, J R Vane, 1978, Disappearance of prostacyclin (PGI2) in the circulation of the dog [proceedings]: Br.J.Pharmacol., v. 62, p. 414P-415P.

Eger,Ei, N T Smith, R K Stoelting, D J Cullen, L B Kadis, C E Whitcher, 1970, Cardiovascular effects of halothane in man: Anesthesiology, v. 32, p. 396-409.

Ehrman,ML, E A Jaffe, 1980, Prostacyclin (PGI2) inhibits the development in human platelets of ADP and arachidonic acid-induced shape change and procoagulant activity: Prostaglandins, v. 20, p. 1103-1116.

Ekins,RP, 1974, Radioimmunoassay and saturation analysis. Basic principles and theory: Br.Med Bull., v. 30, p. 3-11.

Eldor,A, D J Falcone, D P Hajjar, C R Minick, B B Weksler, 1981, Recovery of prostacyclin production by de-endothelialized rabbit aorta. Critical role of neointimal smooth muscle cells: J.Clin.Invest, v. 67, p. 735-741.

Eldor,A, D J Falcone, D P Hajjar, C R Minick, B B Weksler, 1982, Diet-induced hypercholesterolemia inhibits the recovery of prostacyclin production by injured rabbit aorta: Am.J.Pathol., v. 107, p. 186-190.

Evensen,SA, 1979, Injury to cultured endothelial cells: the role of lipoproteins and thrombo-active agents: Haemostasis, v. 8, p. 203-210.

Fernando,B, R Marley, S Holt, R Anand, D Harry, P Sanderson, R Smith, G Hamilton, K Moore, 1998, N-acetylcysteine prevents development of the hyperdynamic circulation in the portal hypertensive rat: Hepatology, v. 28, p. 689-694.

Ferreira,SH, J R Vane, 1967, Prostaglandins: their disappearance from and release into the circulation: Nature, v. 216, p. 868-873.

Fletcher,AP, D Biederman, D Moore, N Alkjaersig, S Sherry, 1964, Abnormal plasminogen - plasmin system activity (fibronolysis) in patients with hepatic cirrhosis: J.Clin.Invest, p. 681-695.

Gaiani,S, L Bolondi, B S Li, G Zironi, S Siringo, L Barbara, 1991, Prevalence of spontaneous hepatofugal portal flow in liver cirrhosis. Clinical and endoscopic correlation in 228 patients: Gastroenterology, v. 100, p. 160-167.

Gelman,SI, 1976, Disturbances in hepatic blood flow during anesthesia and surgery: Arch.Surg., v. 111, p. 881-883.

Genecin,P, J Polio, L A Colombato, G Ferraioli, A Reuben, R J Groszmann, 1990, Bile acids do not mediate the hyperdynamic circulation in portal hypertensive rats: Am.J.Physiol, v. 259, p. G21-G25.

Gill,JR, Jr., J C Frolich, R E Bowden, A A Taylor, H R Keiser, H W Seyberth, J A Oates, F C Bartter, 1976, Bartter's syndrome: a disorder characterized by high urinary prostaglandins and a dependence of hyperreninemia on prostaglandin synthesis: Am.J.Med, v. 61, p. 43-51.

Gimbrone,MA, Jr., R W Alexander, 1975, Angiotensin II stimulation of prostaglandin production in cultured human vascular endothelium: Science, v. 189, p. 219-220.

Goldblatt MW., 1935, Properties of human seminal plasma.: J.Physiol, p. 208-218.

Gorman,RR, S Bunting, O V Miller, 1977, Modulation of human platelet adenylate cyclase by prostacyclin (PGX): Prostaglandins, v. 13, p. 377-388.

Goulis,J, D Patch, A K Burroughs, 1999, Bacterial infection in the pathogenesis of variceal bleeding: Lancet, v. 353, p. 139-142.

Granstrom,E, H Kindahl, 1978, Radioimmunoassay of prostaglandins and thromboxanes: Adv.Prostaglandin Thromboxane Res, v. 5, p. 119-210.

Grays Anatomy, 1980, Embryology. Further development of veins., in PL Williams and R Warwick (eds), Gray's Anatomy: p. 193-195.

Greaves,M, F E Preston, 1982, Plasma 6-keto-prostaglandin F1alpha: fact or fiction: Thromb.Res, v. 26, p. 145-157.

Grose,JH, M Lebel, F M Gbeassor, 1980, Variations in urinary metabolites of prostacyclin TXA2 in essential hypertension.: Clin.Res., p. 685A.

Gryglewski,RJ, A Dembinska-Kiec, A Zmuda, T Gryglewska, 1978, Prostacyclin and thromboxane A2 biosynthesis capacities of heart, arteries and platelets at various stages of experimental atherosclerosis in rabbits - *(c)*: Atherosclerosis, v. 31, p. 385-394.

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

Gryglewski,RJ, A Szczeklik, R Nizankowski, 1978, Anti-platelet action of intravenous infusion of prostacyclin in man - ***(b)*** : Thromb.Res, v. 13, p. 153-163.

Gullner,HG, C Cerletti, F C Bartter, J B Smith, J R Gill, Jr., 1979, Prostacyclin overproduction in Bartter's syndrome: Lancet, v. 2, p. 767-769.

Gupta,TK, L Chen, R J Groszmann, 1997, Pathophysiology of portal hypertension: Baillieres Clin.Gastroenterol., v. 11, p. 203-219.

Halushka,PV, R C Rogers, C B Loadholt, J A Colwell, 1981, Increased platelet thromboxane synthesis in diabetes mellitus: J.Lab Clin.Med, v. 97, p. 87-96.

Halvorsen,JF, A O Myking, 1974, The porto-systemic collateral pattern in the rat. An angiographic and anatomical study after partial occlusion of the portal vein: Eur.Surg.Res, v. 6, p. 183-195.

Hamberg,M, J Svensson, T Wakabayashi, B Samuelsson, 1974, Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation - ***(a)***: Proc.Natl.Acad.Sci.U.S.A, v. 71, p. 345-349.

Hamberg,M, B Samuelsson, 1974, Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets - ***(b)***: Proc.Natl.Acad.Sci.U.S.A, v. 71, p. 3400-3404.

Hamberg,M, J Svensson, B Samuelsson, 1975, Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides: Proc.Natl.Acad.Sci.U.S.A, v. 72, p. 2994-2998.

Hardisty,RM, R A Hutton, D Montgomery, S Rickard, H Trebilcock, 1970, Secondary platelet aggregation: a quantitative study: Br.J.Haematol., v. 19, p. 307-319.

Harrison,HE, A H Reece, M Johnson, 1978, Decreased vascular prostacyclin in experimental diabetes: Life Sci., v. 23, p. 351-355.

Haslam,RJ, J A Lynham, 1972, Activation and inhibition of blood platelet adenylate cyclase by adenosine or by 2-chloroadenosine: Life Sci.II, v. 11, p. 1143-1154.

Haslam,RJ, M M Davidson, J E Fox, J A Lynham, 1978, Cyclic nucleotides in platelet function: Thromb.Haemost., v. 40, p. 232-240.

in isolated rat kidney glomeruli: Proc.Natl.Acad.Sci.U.S.A, v. 76, p. 1155-1159.

Hathaway,DR, R S Adelstein, 1979, Human platelet myosin light chain kinase requires the calcium-binding protein calmodulin for activity: Proc.Natl.Acad.Sci.U.S.A, v. 76, p. 1653-1657.

Hensby,CN, G A Fitzgerald, L A Friedman, P J Lewis, C T Dollery, 1979, Measurement of 6-oxo-PGF1 alpha in human plasma using gas chromatography-mass spectrometry - ***(c)***: Prostaglandins, v. 18, p. 731-736.

Hensby,CN, P J Barnes, C T Dollery, H Dargie, 1979, Production of 6-oxo-PGF1 alpha by human lung in vivo - ***(a)***: Lancet, v. 2, p. 1162-1163.

Hensby,CN, P J Lewis, P Hilgard, G J Mufti, J Hows, J Webster, 1979, Prostacyclin deficiency in thrombotic thrombocytopenic purpura - ***(b)***: Lancet, v. 2, p. 748.

Hensby,CN, 1981, Clinical pharmacology of prostacyclin, in PJ Lewis and J O'Grady (eds), New York, Raven Press, p. 37-43.

Herman,AG, M Claeys, S Moncada, J R Vane, 1979, Biosynthesis of prostaglandin (PGI2) and 12L-hydroxy-5,8,10,14- eicosatetraenoic acid (HETE) by pericardium, pleura, peritoneum and aorta of the rabbit: Prostaglandins, v. 18, p. 439-452.

Higgs,EA, S Moncada, J R Vane, J P Caen, H Michel, G Tobelem, 1978, Effect of prostacyclin (PGI2) on platelet adhesion to rabbit arterial subendothelium: Prostaglandins, v. 16, p. 17-22.

Holmsen,H, I Holmsen, A Bernhardsen, 1966, Microdetermination of adenosine diphosphate and adenosine triphosphate in plasma with firefly luciferase system : Anal.Biochem., v. 17, p. 456-473.

Hornstra,G, E Haddeman, J A Don, 1979, Blood platelets do not provide endoperoxides for vascular prostacyclin production: Nature, v. 279, p. 66-68.

Hutton RA, Dandona P, Chow FPR, Craft IL, 1980, Inhibition of platelet aggregation by placental extracts.: Thromb.Res, v. 17, p. 465-471.

Jackson,EK, R P Goodman, 1981, 6-keto-prostaglandin EL and Bartter's syndrome: N.Engl.J.Med, v. 305, p. 287.

Johnson,AR, 1980, Human pulmonary endothelial cells in culture. Activities of cells from arteries and cells from veins : J.Clin.Invest, v. 65, p. 841-850.

Johnson,M, H E Harrison, A T Raftery, J B Elder, 1979, Vascular prostacyclin may be reduced in diabetes in man: Lancet, v. 1, p. 325-326.

Jorgensen,KA, R S Pedersen, 1981, Familial deficiency of prostacyclin production stimulating factor in the hemolytic uremic syndrome of childhood: Thromb.Res, v. 21, p. 311-315.

Katusic,ZS, 1996, Superoxide anion and endothelial regulation of arterial tone: Free Radic.Biol.Med, v. 20, p. 443-448.

Kinlough-Rathbone,RL, M A Packham, J F Mustard, 1970, The effect of prostaglandin E1 on platelet function in vitro and in vivo: Br.J.Haematol., v. 19, p. 559-571.

Kitano S, Terblanche J, Kahn D, Bornman PC, 1986, Venous anatomy of the lower oesophagus in portal hypertension: practical implications.: Br.J.Surg., v. 73, p. 525-531.

Kloeze,J, 1970, Prostaglandins and platelet aggregation in vivo. I. Influence of PGE1 and omega-homo-PGE1 on transient thrombocytopenia and of PGE1 on the LD50 of ADP: Thromb.Diath.Haemorrh., v. 23, p. 286-292.

Kravetz,D, J Bosch, M T Arderiu, M P Pizcueta, R Casamitjana, F Rivera, J Rodes, 1988, Effects of somatostatin on splanchnic hemodynamics and plasma glucagon in portal hypertensive rats: Am.J.Physiol, v. 254, p. G322-G328.

Kulkarni,PS, R Roberts, P Needleman, 1976, Paradoxical endogenous synthesis of a coronary dilating substance from arachidonate: Prostaglandins, v. 12, p. 337-353.

Lands,WE, 1979, The biosynthesis and metabolism of prostaglandins: Annu.Rev.Physiol, v. 41, p. 633-652.

Lebrec,D, P De Fleury, B Rueff, H Nahum, J P Benhamou, 1980, Portal hypertension, size of esophageal varices, and risk of gastrointestinal bleeding in alcoholic cirrhosis: Gastroenterology, v. 79, p. 1139-1144.

Lopez-Talavera,JC, W W Merrill, R J Groszmann, 1995, Tumor necrosis factor alpha: a major contributor to the hyperdynamic circulation in prehepatic portal-hypertensive rats: Gastroenterology, v. 108, p. 761-767.

Machin,SJ, D A Chamone, G Defreyn, J Vermylen, 1981, The effect of clinical prostacyclin infusions in advanced arterial disease on platelet function and plasma 6-keto PGF1 alpha levels: Br.J.Haematol., v. 47, p. 413-422.

MacIntyre,DE, J D Pearson, J L Gordon, 1978, Localisation and stimulation of prostacyclin production in vascular cells: Nature, v. 271, p. 549-551.

Marcus,AJ, B B Weksler, E A Jaffe, 1978, Enzymatic conversion of prostaglandin endoperoxide H2 and arachidonic acid to prostacyclin by cultured human endothelial cells: J.Biol.Chem., v. 253, p. 7138-7141.

Marnett,LJ, S W Rowlinson, D C Goodwin, A S Kalgutkar, C A Lanzo, 1999, Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition: J.Biol.Chem., v. 274, p. 22903-22906.

McVerry,BA, S J Machin, H Parry, A H Goldstone, 1980, Reduced prostacyclin activity in systemic lupus erythematosus: Ann.Rheum.Dis., v. 39, p. 524-525.

Mehta,R, J Gottstein, W P Zeller, R Lichtenberg, A T Blei, 1990, Endotoxin and the hyperdynamic circulation of portal vein-ligated rats: Hepatology, v. 12, p. 1152-1156.

Merhi-Soussi,F, Z Dominguez, O Macovschi, M Dubois, A Savany, M Lagarde, A F Prigent, 2000, Human lymphocytes stimulate prostacyclin synthesis in human umbilical vein endothelial cells. Involvement of endothelial cPLA2: J.Leukoc.Biol., v. 68, p. 881-889.

Midgley,AR, Jr., G D Niswender, R W Rebar, 1969, Principles for the assessment of the reliability of radioimmunoassay methods (precision, accuracy, sensitivity, specificity): Acta Endocrinol.Suppl (Copenh), v. 142, p. 163-184.

Mikhailidis,DP, J Y Jeremy, M A Barradas, N Green, P Dandona, 1983, Effect of ethanol on vascular prostacyclin (prostaglandin I2) synthesis, platelet aggregation, and platelet thromboxane release: Br.Med J.(Clin.Res Ed), v. 287, p. 1495-1498.

Mikhailidis,DP, W J Jenkins, M A Barradas, J Y Jeremy, P Dandona, 1986, Platelet function defects in chronic alcoholism: Br.Med J.(Clin.Res Ed), v. 293, p. 715-718.

Miller,OV, R R Gorman, 1979, Evidence for distinct prostaglandin I2 and D2 receptors in human platelets: J.Pharmacol.Exp.Ther., v. 210, p. 134-140.

Mills,DC, J B Smith, 1971, The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3':5'-cyclic monophosphate in platelets: Biochem.J., v. 121, p. 185-196.

Mitchell,MD, 1978, A sensitive radioimmunoassay for 6-keto-prostaglandin F1alpha: preliminary observations on circulating concentrations: Prostaglandins Med, v. 1, p. 13-21.

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

Moncada,S, R J Gryglewski, S Bunting, J R Vane, 1976, A lipid peroxide inhibits the enzyme in blood vessel microsomes that generates from prostaglandin endoperoxides the substance (prostaglandin X) which prevents platelet aggregation - ***(b)***: Prostaglandins, v. 12, p. 715-737.

Moncada,S, E A Higgs, J R Vane, 1977, Human arterial and venous tissues generate prostacyclin (prostaglandin x), a potent inhibitor of platelet aggregation - ***(b)***: Lancet, v. 1, p. 18-20.

Moncada,S, A G Herman, E A Higgs, J R Vane, 1977, Differential formation of prostacyclin (PGX or PGI2) by layers of the arterial wall. An explanation for the anti-thrombotic properties of vascular endothelium - ***(a)***: Thromb.Res, v. 11, p. 323-344.

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

Moncada,S, J R Vane, 1978, Unstable metabolites of arachidonic acid and their role in haemostasis and thrombosis - ***(a)***: Br.Med Bull., v. 34, p. 129-135.

Moncada,S, 1999, Nitric oxide: discovery and impact on clinical medicine: J.R.Soc.Med, v. 92, p. 164-169.

Moritz,E, W Kreuzer, W G Schenk, Jr., 1973, Studies in experimental canine cirrhosis: hemodynamic alterations with emphasis on degree of spontaneous porto-systemic shunting: Ann.Surg., v. 177, p. 503-506.

Morris HG, Sherman NA, Shepperdson FT, 1981, Variables associated with radioimmunoassay of prostaglandins in plasma.: Prostaglandins , v. 28, p. 771-788.

Murakami,S, J F Bernardo, R A Branch, R Sabra, 1996, Adenosine does not mediate renal sodium retention and peripheral vasodilation elicited by partial portal vein ligation in rats: Hepatology, v. 23, p. 346-352.

Murray,JF, Dawson.A.M., S Sherlock. Circulatory changes in chronic liver disease. Am.J.Med 24, 358-367. 1958.
Ref Type: Generic

Myatt,L, M Jogee, P J Lewis, N G Elder, 1981, NO TITLE, in PJ Lewis and J O'Grady (eds), Clinical Pharmacology of Prostacyclin: New York, Raven Press, p. 25-35.

Myking,AO, J F Halvorsen, 1973, A method for graded portal vein stenosis in rats: survival related to degree of stenosis: Eur.Surg.Res, v. 5, p. 454-457.

Needleman,P, A Wyche, A Raz, 1979, Platelet and blood vessel arachidonate metabolism and interactions: J.Clin.Invest, v. 63, p. 345-349.

Nolan,RD, G J Dusting, T J Martin, 1981, Phospholipase inhibition and the mechanism of angiotensin-induced prostacyclin release from rat mesenteric vasculature: Biochem.Pharmacol., v. 30, p. 2121-2125.

Nordoy,A, B Svensson, D Wiebe, J C Hoak, 1978, Lipoproteins and the inhibitory effect of human endothelial cells on platelet function: Circ.Res, v. 43, p. 527-534.

Northover,JM, E D Williams, J Terblanche, 1980, The investigation of small vessel anatomy by scanning electron microscopy of resin casts. A description of the technique and examples of its use in the study of the microvasculature of the peritoneum and bile duct wall: J.Anat., v. 130, p. 43-54.

O'Grady,J, S Warrington, M J Moti, S Bunting, R Flower, A S Fowle, E A Higgs, S Moncada, 1980, Effects of Intravenous infusion of prostacyclin (PGI2) in man: Prostaglandins, v. 19, p. 319-332.

Oates,JA, P Falardeau, G A Fitzgerald, R A Branch, A R Brash, 1981, Quantification of urinary prostacyclin metabolites in man: Estimates of the rate of secretion of prostacyclin into the general circulation., in PJ Lewis and J O'Grady (eds), Clinical Pharmacology of Prostacyclin: New York, Raven Press, p. 21.

Oberti,F, P Sogni, S Callmail, R Moreau, B Pipy, D Lebrec, 1993, Role of prostacyclin in hemodynamic alterations in conscious rats with extrahepatic or intrahepatic portal hypertension: Hepatology, v. 18, p. 621-627.

Okahara,K, B Sun, J Kambayashi, 1998, Upregulation of prostacyclin synthesis-related gene expression by shear stress in vascular endothelial cells: Arterioscler.Thromb.Vasc.Biol., v. 18, p. 1922-1926.

Okuma,M, Y Yamori, K Ohta, H Uchino, 1979, Production of prostacyclin-like substance in stroke-prone and stroke- resistant spontaneously hypertensive rats: Prostaglandins, v. 17, p. 1-7.

Orda,R, H Ellis, 1978, Self-established porto-caval and porto-pulmonary shunts in mechanically induced portal hypertension. An experimental study: Eur.Surg.Res, v. 10, p. 172-183.

Pace-Asciak,C, L S Wolfe, 1971, A novel prostaglandin derivative formed from arachidonic acid by rat stomach homogenates: Biochemistry, v. 10, p. 3657-3664.

Pace-Asciak,C, 1976, Letter: Isolation, structure, and biosynthesis of 6-ketoprostaglandin F1 alpha in the rat stomach : J.Am.Chem.Soc., v. 98, p. 2348-2349.

Pace-Asciak,CR, M C Carrara, G Rangaraj, K C Nicolaou, 1978, Enhanced formation of PGI2, a potent hypotensive substance, by aortic rings and homogenates of the spontaneously hypertensive rat: Prostaglandins, v. 15, p. 1005-1012.

Palmer,AD, I B Brick, 1956, Correlation between the severity of esophageal varices and portal cirrhosis and their propensity towards haemorrhage.: Gastroenterology, p. 85-90.

Pareti,FI, P M Mannucci, A D'Angelo, J B Smith, L Sautebin, G Galli, 1980, Congenital deficiency of thromboxane and prostacyclin: Lancet, v. 1, p. 898-901.

Pifer,DD, L M Cagen, C M Chesney, 1981, Stability of prostaglandin I2 in human blood: Prostaglandins, v. 21, p. 165-175.

Piper,PJ, J R Vane, 1969, Release of additional factors in anaphylaxis and its antagonism by anti- inflammatory drugs: Nature, v. 223, p. 29-35.

Piscaglia,F, G Zironi, S Gaiani, A Mazziotti, A Cavallari, L Gramantieri, M Valgimigli, L Bolondi, 1999, Systemic and splanchnic hemodynamic changes after liver transplantation for cirrhosis: a long-term prospective study: Hepatology, v. 30 , p. 58-64.

Poch,G, W R Kukovetz, 1972, Studies on the possible role of cyclic AMP in drug-induced coronary vasodilatation: Adv.Cyclic.Nucleotide Res, v. 1, p. 195-211.

Quiroga,J, J Prieto, 1993, Liver cytoprotection by prostaglandins: Pharmacol.Ther., v. 58, p. 67-91.

Raz,A, P C Isakson, M S Minkes, P Needleman, 1977, Characterization of a novel metabolic pathway of arachidonate in coronary arteries which generates a potent endogenous coronary vasodilator: J.Biol.Chem., v. 252, p. 1123-1126.

Remuzzi,G, D Marchesi, M Livio, A E Cavenaghi, G Mecca, M B Donati, G de Gaetano, 1978, Altered platelet and vascular prostaglandin-generation in patients with renal failure and prolonged bleeding
times - ***(a)***: Thromb.Res, v. 13, p. 1007-1015.

Remuzzi,G, R Misiani, D Marchesi, M Livio, G Mecca, G de Gaetano, M B Donati, 1978, Haemolytic-uraemic syndrome: deficiency of plasma factor(s) regulating prostacyclin activity? - ***(b)***: Lancet, v. 2, p. 871-872.

Remuzzi,G, D Marchesi, R Misiani, G Mecca, G de Gaetano, M B Donati, 1979, Familial deficiency of a plasma factor stimulating vascular prostacyclin activity: Thromb.Res, v. 16, p. 517-525.

Remuzzi,G, D Marchesi, G Mecca, R Misiani, E Rossi, M B Donati, G de Gaetano, 1980, Reduction of fetal vascular prostacyclin activity in pre-eclampsia - ***(a)***: Lancet, v. 2, p. 310.

Remuzzi,G, D Marchesi, C Zoja, D Muratore, G Mecca, R Misiani, E Rossi, M Barbato, P Capetta, M B Donati, G de Gaetano, 1980, Reduced umbilical and placental vascular prostacyclin in severe pre- eclampsia - ***(b)***: Prostaglandins, v. 20, p. 105-110.

Reynolds,TB, 1982, Why do varices bleed?, in D Westaby, BRD MacDougall, and R Williams (eds), In: Variceal Bleeding: London, Pitman Books, p. 3-12.

Ritter JM, Hamilton G, Barrow SE, Heavey DJ, Hickling NE, Taylor KM, Hobbs KEF, Dollery CT, 1986, Prostacyclin in the circulation of patients with vascular disorders undergoing surgery.: Clin.Sci., v. 71, p. 743-747.

Rockey,DC. The cellular pathogenesis of portal hypertension: stellate cell contractility and nitric oxide. Hepatology 25, 2-5. 2002.
Ref Type: Generic

Rodbard,D, J E Lewald, 1970, Computer analysis of radioligand assay and radioimmunoassay data : Acta Endocrinol.Suppl (Copenh), v. 147, p. 79-103.

Rousselot,LM, A H Moreno, W F Panke, 1976, Studies in portal hypertension. The clinical and physiopathologic significance of self-established (non-surgical) portal systemic venous shunts.: Ann.Surg., p. 384.

Rubanyi,GM, 1991, Endothelium-derived vasoconstrictor factors: an overview, in U Ryan and GM Rubanyi (eds), Endothelial Regulation of Vascular Tone: New York, Marcel Dekker Inc., p. 375-376.

Saba,SR, W H Zucker, R G Mason, 1973, Some properties of endothelial cells isolated from human umbilical cord vein: Ser.Haematol., v. 6, p. 456-468.

Salmon,JA, 1978, A radioimmunoassay for 6-keto-prostaglandin F1alpha: Prostaglandins, v. 15, p. 383-397.

Salzman,EW, L Levine, 1971, Cyclic 3',5'-adenosine monophosphate in human blood platelets. II. Effect of N6-2'-o-dibutyryl cyclic 3',5'-adenosine monophosphate on platelet function: J.Clin.Invest, v. 50, p. 131-141.

Samuelsson B., 1965, On the corporation of oxygen in the conversion of 8,11,14-eicosatrienoic acid to prostaglandin E1.: J.Am.Chem.Soc., p. 3011-3013.

Semeri,GG, G Masotti, L Poggesi, G Galanti, 1980, Release of prostacyclin into the bloodstream and its exhaustion in humans after local blood flow changes (ischemia and venous stasis): Thromb.Res, v. 17, p. 197-208.

Sherlock,S, 1981, Diseases of the liver and biliary system: Blackwell Scientific Publications, v. 6th Edition, p. 13.

Sieber,CC, R J Groszmann, 1992, Nitric oxide mediates hyporeactivity to vasopressors in mesenteric vessels of portal hypertensive rats: Gastroenterology, v. 103, p. 235-239.

Siegl,AM, J B Smith, M J Silver, K C Nicolaou, D Ahern, 1979, Selective binding site for [3H]prostacyclin on platelets: J.Clin.Invest, v. 63, p. 215-220.

Sikuler,E, D Kravetz, R J Groszmann, 1985, Evolution of portal hypertension and mechanisms involved in its maintenance in a rat model: Am.J.Physiol, v. 248, p. G618-G625.

Silberbauer,K, H Sinzinger, M Winter, 1978, Prostacyclin (PGI2) availability of vascular tissue in experimentally induced acute uraemic rats.: Artery, p. 554-563.

Silberbauer,K, G Schernthaner, H Sinzinger, H Piza-Katzer, M Winter, 1979, Decreased vascular prostacyclin in juvenile-onset diabetes: N.Engl.J.Med, v. 300, p. 366-367.

Sinzinger,H, W Feigl, K Silberbauer, 1979, Prostacyclin generation in atherosclerotic arteries: Lancet, v. 2, p. 469.

Sitzmann,JV, S S Li, N F Adkinson, 1991, Evidence for role of prostacyclin as a systemic hormone in portal hypertension: Surgery, v. 109, p. 149-153.

Smith,JB, A L Willis, 1971, Aspirin selectively inhibits prostaglandin production in human platelets: Nat.New Biol., v. 231, p. 235-237.

Smith,JB, C Ingerman, J J Kocsis, M J Silver, 1973, Formation of prostaglandins during the aggregation of human blood platelets: J.Clin.Invest, v. 52, p. 965-969.

Smith,JB, M L Ogletree, A M Lefer, J C Nicolaou, 1978, Antibodies which antagonise the effects of prostacyclin: Nature, v. 274, p. 64-65.

Smith,WL, R Langenbach, 2001, Why there are two cyclooxygenase isozymes: J.Clin.Invest, v. 107, p. 1491-1495.

Stoff,JS, M Stemerman, M Steer, E Salzman, R S Brown , 1980, A defect in platelet aggregation in Bartter's syndrome: Am.J.Med, v. 68, p. 171-180.

Stoltz,JF, S Muller, X Wang, D Dumas, M Boisseau, S Legrand, V Labrador, 1999, Hemorheology and vascular endothelial cells: Clin.Hemorheol.Microcirc., v. 20, p. 127-139.

Stuart,MJ, D A Clark, S G Sunderji, J B Allen, T Yambo, H Elrad, J H Slott, 1981, Decrease prostacyclin production: a characteristic of chronic placental insufficiency syndromes: Lancet, v. 1, p. 1126-1128.

Sun,FF, B M Taylor, 1978, Metabolism of prostacyclin in rat: Biochemistry, v. 17, p. 4096-4101.

Szczeklik,A, R J Gryglewski, R Nizankowski, J Muslal, R Pieton, J Mruk, 1978, Circulatory and anti-platelet effects of intravenous prostacyclin in healthy men: Pharmacol.Res.Commun., v. 10, p. 545-556.

Tan,SY, P Sweet, P J Mulrow, 1978, Impaired renal production of prostaglandin E2: a newly identified lesion in human essential hypertension: Prostaglandins, v. 15, p. 139-150.

Tanabe,T, V Ullrich, 1995, Prostacyclin and thromboxane synthases: J.Lipid Mediat.Cell Signal., v. 12, p. 243-255.

Tateson,JE, S Moncada, J R Vane, 1977, Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets: Prostaglandins , v. 13, p. 389-397.

Thomas,DP, V J Ream, R K Stuart, 1967, Platelet aggregation in patients with Laennec's cirrhosis of the liver: N.Engl.J.Med, v. 276, p. 1344-1348.

Thomas,DP, S Niewiarowski, A R Myers, K J Bloch, R W Colman, 1970, A comparative study of four methods for detecting fibrinogen degradation products in patients with various diseases: N.Engl.J.Med, v. 283, p. 663-668.

Thorell,J, S M Larson, 1978, Radioimmune assay in related techniques, methodology in clinical applications., St Louis, USA, Mosby, p. 11-31.

Thuresson,ED, K M Lakkides, W L Smith, 2000, Different catalytically competent arrangements of arachidonic acid within the cyclooxygenase active site of prostaglandin endoperoxide H synthase-1 lead to the formation of different oxygenated products: J.Biol.Chem., v. 275, p. 8501-8507.

Toghill,PJ, S Green, F Ferguson, 1977, Platelet dynamics in chronic liver disease with special reference to the role of the spleen: J.Clin.Pathol., v. 30, p. 367-371.

Topper,JN, M A Gimbrone, Jr., 1999, Blood flow and vascular gene expression: fluid shear stress as a modulator of endothelial phenotype: Mol.Med Today, v. 5, p. 40-46.

Uruno,T, I Takayanagi, N Inatomi, K Takagi, 1973, Anti-oxytocin actions of papaverine, aspaminol and isoprenaline on the rat uterus and intracellular cyclin adenosine 3,5-monophosphate level: Jpn.J.Pharmacol., v. 23, p. 897-898.

Van Hoof,A, D A Chamone, J Vermylen, 1979, Plasma from patients with hepatic or renal failure stimulates prostacyclin release from 'exhausted' rat aorta slices.: Thromb.Haemost., p. **43.**

Vane,JR, 1969, The release and fate of vaso-active hormones in the circulation: Br.J.Pharmacol., v. 35, p. 209-242.

Vane,JR, R M Botting, 1995, Pharmacodynamic profile of prostacyclin: Am.J.Cardiol., v. 75, p. 3A-10A.

Vargaftig,BB, P Zirinis, 1973, Platelet aggregation induced by arachidonic acid is accompanied by release of potential inflammatory mediators distinct from PGE2 and PGF2: Nat.New Biol., v. 244, p. 114-116.

Von-Euler,US. Zur kenntnis der pharmakologischen Wirkungen von Nativsekreten und Extrakten mannlicher accessorischer Geschlectsdrussen. Arch.Exp.Pathol.Pharmakol 1034, 78-84. 1934. Ref Type: Generic

Vorobioff,J, J E Bredfeldt, R J Groszmann, 1981, The increased splanchnic blood flow in portal hypertensive rats with cirrhosis and chronic portal hypertension.: Gastroenterology, p. **1353.**

Vorobioff,J, J E Bredfeldt, R J Groszmann, 1983, Hyperdynamic circulation in portal-hypertensive rat model: a primary factor for maintenance of chronic portal hypertension: Am.J.Physiol, v. 244, p. G52-G57.

Walsh,MP, 1994, Regulation of vascular smooth muscle tone: Can.J.Physiol Pharmacol., v. 72, p. 919-936.

Weber,C, H J Kruse, A Sellmayer, W Erl, P C Weber, 1993, Platelet activating factor enhances receptor-operated Ca(++)-influx and subsequent prostacyclin synthesis in human endothelial cells: Biochem.Biophys.Res.Commun., v. 195 , p. 874-880.

Webster,J, A J Rees, P J Lewis, C N Hensby, 1980, Prostacyclin deficiency in haemolytic-uraemic syndrome: Br.Med J., v. 281, p. 271.

Weiss,A, N L Baenziger, J P Atkinson, 1978, Platelet release reaction and intracellular cGMP: Blood, v. 52, p. 524-531.

Weiss,HJ, L M Aledort, S Kochwa, 1968, The effect of salicylates on the hemostatic properties of platelets in man: J.Clin.Invest, v. 47, p. 2169-2180.

Weiss,HJ, V T Turitto, 1979, Prostacyclin (prostaglandin I2, PGI2) inhibits platelet adhesion and thrombus formation on subendothelium: Blood, v. 53, p. 244-250.

Weksler,BB, C E Coupal, 1973, Platelet-dependent generation of chemotactic activity in serum: J.Exp.Med, v. 137, p. 1419-1430.

Weksler,BB, A J Marcus, E A Jaffe, 1977, Synthesis of prostaglandin I2 (prostacyclin) by cultured human and bovine endothelial cells: Proc.Natl.Acad.Sci.U.S.A, v. 74, p. 3922-3926.

Weksler,BB, C W Ley, E A Jaffe, 1978, Stimulation of endothelial cell prostacyclin production by thrombin, trypsin, and the ionophore A 23187: J.Clin.Invest, v. 62, p. 923-930.

Westaby,D, A E Gimson, R Williams, 1985, Esophageal varix pressure measurement: The relationship to portal pressure and the response to changes in intra-abdominal pressure.: GUT.

Wexler,MJ, L D MacLean, 1975, Massive spontaneous portal-systemic shunting without varices: Arch.Surg., v. 110, p. 995-1003.

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

Wiles,PG, L R Solomon, W Lawler, N P Mallick, M Johnson, 1981, Inherited plasma factor deficiency in haemolytic-uraemic syndrome: Lancet, v. 1, p. 1105-1106.

Wilkinson,SP, 1977, Endotoxins and liver disease: Scand.J.Gastroenterol., v. 12, p. 385-386.

Witzel,L, E Wolbergs, H Merki, 1985, Prophylactic endoscopic sclerotherapy of oesophageal varices. A prospective controlled study: Lancet, v. 1, p. 773-775.

Wu,Y, R C Burns, J V Sitzmann, 1993, Effects of nitric oxide and cyclooxygenase inhibition on splanchnic hemodynamics in portal hypertension: Hepatology, v. 18, p. 1416-1421.

Wynalda,MA, F A Fitzpatrick, 1980, Albumins stabilize prostaglandin I2: Prostaglandins, v. 20, p. 853-861.

Zahavi,J, A J Price, J Westwick, M F Scully, S F Al Hasani, A C Honey, M Dubiel, V V Kakkar, 1980, Enhanced in-vivo platelet release reaction, increased thromboxane synthesis, and decreased prostacyclin release after tourniquet ischaemia: Lancet, v. 2, p. 663-667.