OXIDANT STRESS AND THE HYPERDYNAMIC CIRCULATION IN PORTAL HYPERTENSION

Studies performed in the Partial Portal Vein Ligated Rat

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This thesis is dedicated to my parents for their continued love and support.

Abstract

The partial portal vein ligation rat model of portal hypertension is associated with the development of a hyperdynamic circulation characterised by an increased cardiac index and a reduced systemic vascular resistance. Recent studies have shown that tumour necrosis factor-α is involved in the development of the hyperdynamic response. Studies on tumour necrosis factor-α *in vitro* have shown that the signal transduction pathways involve activation of the transcription factor NF-κB by reactive oxygen species and that this can be inhibited by antioxidants. It is not known however, whether involvement of these pathways can be demonstrated *in vivo* in this model, and whether treatment with antioxidants can inhibit these signalling pathways and thus the development of the hyperdynamic circulation.

This thesis concentrated on the signal transduction pathways and the possible role of oxidant stress in the generation of the hyperdynamic circulation in the partial portal vein ligated rat model compared with sham controls. Firstly, a group of compounds known as the F_2-isoprostanes were studied as markers of lipid peroxidation. Secondly, activation of the transcription factor NF-κB was measured in whole liver from different study groups. Levels of plasma TNF-α levels were then measured, as were plasma levels of nitrite and nitrate. Finally, haemodynamic studies were performed in different groups to evaluate the pharmacodynamic effects of different reagents including N-acetylcysteine, pyrrolidine dithiocarbamate and BB-1101.

Partial portal vein ligation is associated with increased lipid peroxidation, activation of hepatic NF-κB and increased nitric oxide synthesis. Furthermore, the hyperdynamic circulation can be inhibited by the chronic administration of N-acetylcysteine, pyrrolidine dithiocarbamate and BB-1101 to PPVL rats. The precise mechanism of action of these reagents remains unclear and further work needs to be performed to answer these questions.

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Abbreviations

BSA,	bovine serum albumin
BSTFA,	bis(trimethylsilyl)trifluoracetamide
DIPEA,	N,N-diisopropylethylamine
DMF,	N,N-dimethylformamide
DTT,	dithiothreitol
EDTA,	ethylene diamine tetraacetic acid
ELISA,	enzyme-linked immunosorbent assay
EMSA,	electromobility shift assay
HEPES,	4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid
LPS,	lipopolysaccharide
NAC,	N-acetylcysteine
NF-κB,	nuclear factor-kappa B
NO,	nitric oxide
NOS,	nitric oxide synthase
PFBR,	pentafluorobenzylbromide
PMSF,	phenylmethylsulfonylfluoride
PPVL,	partial portal vein ligation
ROS,	reactive oxygen species
TLC,	thin layer chromatography
TNF-α,	tumour necrosis factor-α
TPP,	triphenylphosphine

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CHAPTER 1: INTRODUCTION

1.1 The Hyperdynamic Circulation in Portal Hypertension

1.1.1 Background

Portal hypertension is a common complication of chronic liver disease characterised by an increased portal pressure gradient between the portal vein and the hepatic vein. The normal pressure gradient is approximately 5 mmHg but is increased in portal hypertension and becomes clinically significant when it exceeds 12 mmHg since complications such as variceal haemorrhage or the development of ascites occur above this level (Bosch et al. 1989). Portal hypertension is almost invariably accompanied by the development of a hyperdynamic circulation in which there is an increased cardiac index and reduced vascular resistance. It is thought that the hyperdynamic circulation contributes both to the severity and also to the complications of portal hypertension by increasing portal pressure and causing activation of the neuroendocrine systems. These complications include the development of ascites, hepatic encephalopathy, hepato-renal syndrome, enhanced susceptibility to bacterial infections, deranged liver metabolism and most significantly increased portosystemic shunting leading to the development of oesophageal varices. Bleeding from oesophageal varices is associated with a high mortality (about 50%) (Smith and Graham, 1982). For this reason portal hypertension and the pathogenesis of associated haemodynamic changes are of considerable interest.

1.1.2 Methods of Studying Portal Hypertension

Studies of portal hypertension in humans give rise to both technical difficulties as well as ethical limitations. The portal pressure can be measured indirectly using the hepatic venous wedge pressure gradient. This requires the placement of a balloon catheter into a hepatic venous radicle by a transjugular or femoral approach under radiological guidance until it can go no further with the catheter balloon inflated. The "wedged" pressure measurement represents the sinusoidal venous pressure. The catheter is properly positioned if the pressure tracing shows regular oscillations related to transmissions of hepatic arterial pressure and, finally, a small amount of contrast medium is injected and seen to pass against the

predominant flow into the sinusoidal bed. Measurements are then taken with the balloon deflated and reflect the free hepatic venous pressure. The difference between the "wedged" and the "free" pressure is the portal (sinusoidal) pressure. The wedged hepatic venous pressure does not reflect portal pressure in pre-sinusoidal portal hypertension and may even underestimate the true portal venous pressure.

Hepatic venous wedge pressure measurement is relatively easy, safe and can be performed in patients with a bleeding tendency or ascites but it remains an invasive procedure. Hence, much of the current knowledge relating to the pathogenesis of portal hypertension is based on studies in animal models of portal hypertension. The rat is most frequently studied and different procedures can be performed in this animal to induce portal hypertension including partial portal vein ligation, chronic bile duct ligation which gives rise to biliary cirrhosis, or carbon tetrachloride administration which causes a cirrhosis resembling that produced secondary to alcoholic liver disease. Each model may mimic a clinical situation but it must be emphasised that experimental models are not without limitations especially when extrapolating the data to man.

Prehepatic portal hypertension can be induced by partial portal vein ligation (PPVL). Complete occlusion of the portal vein is associated with a high mortality when performed acutely and is therefore not used. Partial ligation however induces portal hypertension with minimal liver damage and a high degree of portosystemic shunting. The clinical counterpart for this model is portal vein thrombosis or cavernomatosis of the portal vein. This model is popular as it is straightforward to perform and is inexpensive. As this model causes portal hypertension without liver damage the responses of different animals tend to be homogenous.

The PPVL model however would be inappropriate to demonstrate changes in intrahepatic resistance or architecture as there is minimal liver involvement. Instead a model of bile duct ligation, or carbon tetrachloride administration in the rat could be used for this purpose as there are marked architectural changes in these models. Chronic bile duct ligation induces portal hypertension and, although often used as a model of cirrhosis, this strictly speaking is not the case. It is associated with fibrotic changes and ductular proliferation in the

liver without any regeneration (Muriel et al. 1994). This is associated with a moderate degree of portosystemic shunting and the responses of different animals tend to be heterogeneous. Carbon tetrachloride treated rats induces cirrhosis over a time period when administered by either inhalation or ingestion but is associated with a high mortality. It is usually necessary to enhance liver injury and thus the chance of developing cirrhosis by inducing the liver enzymes by treating the animals with phenobarbitone (Proctor and Chatamra, 1982). This produces characteristic cirrhotic changes but administering the reagents is time consuming and laborious. This model is associated with a low degree of portosystemic shunting.

1.1.3 Pathogenesis of Portal Hypertension

The portal pressure gradient is the product of the portal venous inflow and resistance to flow. This can be summarised by Ohm's Law:

$$\Delta P = Q \times R$$

where ΔP is the portal pressure gradient, Q is the portal venous blood flow and R is the vascular resistance to portal venous flow.

The vascular resistance, (R) is determined by other factors defined by Pouiselle's Law:

$$R = 8\mu L / \pi r^4$$

where μ is the viscosity of the blood, L is the length of the vessel and r is the radius of the vessel. Thus, the radius of the vessel is the main determinant affecting portal pressure.

Two traditional views have emerged to explain the development of portal hypertension. For many years it was believed that portal hypertension was caused by a "block" to the portal venous system causing congestion with a reduced portal venous inflow. This is known as the backward flow theory of portal hypertension. Support for the backward theory of portal hypertension (Bosch et al. 1989), was the observation that portal blood flow, measured during surgery with electromagnetic flowmeters, is frequently reduced in cirrhotic patients.

However, studies in PPVL rats with portal hypertension have demonstrated that

forward flow may be of importance (Vorobioff et al. 1983). Using radiolabelled microspheres the total portal venous inflow can be measured. This represents the sum of the portal blood flow entering the liver and the portosystemic collateral blood flow. The fraction of the portosystemic shunting is the percentage of portal venous inflow entering the collateral circulation and is determined by injecting a second radiolabelled microsphere into the spleen or mesenteric vein. These studies demonstrated that the portal venous inflow was markedly increased in portal hypertension although most of the portal venous inflow passes through the collateral circulation (as much as 90% in severe portal hypertension) (Vorobioff et al. 1983, Chojkier and Groszmann, 1981).

These observations contradicted the backward flow theory and lead to the development of a forward flow theory (Benoit et al. 1985) which proposes that the increased portal venous inflow plays a central role in the pathogenesis of portal hypertension. The increased portal venous inflow has also been confirmed in other models of portal hypertension, in which there is less portosystemic shunting than PPVL rats, such as carbon tetrachloride-induced cirrhosis and chronic bile duct ligated rats (Pizcueta et al. 1989, Vorobioff et al. 1984).

It is probable that both factors are important and that their significance in the pathogenesis of portal hypertension may vary from disease to disease depending on the underlying aetiology or the severity of liver damage. If portal venous flow remains normal then an increased portal resistance will increase portal pressure. However, in the absence of increased portal vascular resistance an increase in portal venous vascular inflow alone is not sufficient to increase the portal pressure significantly. Thus, both portal vascular resistance and portal venous inflow are important and each theory (backward or forward flow) may coexist to different degrees in different conditions.

1.1.4 Current Understanding of the Partial Portal Vein Ligated Model

Complete ligation of the portal vein however is not a reliable model as it is associated with a high mortality. Instead partial ligation of the portal vein is used.

Following PPVL, there is a reproducible series of typical haemodynamic changes during the first week that have been demonstrated. Since this is the model to be used in the thesis, I have used information from these studies in formulating the hypothesis and this will be discussed further.

It is now established that PPVL is associated with the following changes:

- Increased cardiac index

- Reduced mean arterial pressure

- Reduced systemic vascular resistance

- Increased portal pressure

- Portosystemic shunting

The presence of a hyperdynamic circulation has been demonstrated as being a primary factor for the maintenance of portal hypertension by Vorobioff et al.(Vorobioff et al. 1983). This group studied the systemic and splanchnic haemodynamic changes in partial portal vein ligated and control animals at two weeks. They observed an increase in portal venous inflow with extensive portosystemic shunting following PPVL. This was accompanied by a reduction in the splanchnic resistance and altered systemic haemodynamics including an elevated cardiac index and a reduced mean arterial pressure reflecting the reduced total peripheral resistance. They concluded that the increased blood flow into the portal system may be a major mechanism for the maintenance of chronic portal hypertension in the presence of high-grade portosystemic shunting.

The next question that needed to be answered was how does portal hypertension evolve in this model and how is it maintained? These questions were addressed by Sikuler and colleagues from the same group (Sikuler et al. 1985). Specifically, they investigated the chronological chain of haemodynamic events that occur after stenosis of the portal vein. In particular, the relationship between resistance and flow in the portal system as well as the

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interaction between the development of portosystemic shunting and the appearance of the hyperdynamic circulation. As one could predict on mechanical grounds they found that, immediately after portal vein stenosis, there was a resistance-induced portal hypertension characterised by increased portal resistance, increased portal pressure and a reduction of portal venous inflow. However, over the following seven days a series of circulatory changes occurred which by day eight were maximal. These changes were extensive portosystemic shunting, a return of portal resistance (within the portal vein and collateral circulation) to control values and an increase in portal venous inflow which contributed to the maintenance of an elevated portal pressure. Thus, while portal hypertension initially occurs in this model due to an increase of portal venous resistance, subsequently portal venous inflow increases and becomes dominant in the maintenance of portal hypertension.

Plasma volume expansion was demonstrated by Genecin et al. to be an absolute requirement for the generation of the hyperdynamic state (Genecin et al. 1990b). However, what was not known at this point was the temporal and functional relationship between the two. Specifically, does plasma volume expansion enhance haemodynamic changes or does it occur as a consequence? This was addressed by Colombato et al. who studied the portal vein ligated model over the first four days using Doppler flow studies of the iliac artery and the superior mesenteric artery as well as measurements of haematocrit (Colombato et al. 1992). In this study, they demonstrated that vasodilatation precedes plasma volume expansion. Plasma volume expansion occurs parallel to increases in both regional flow and cardiac index. This confirms other work suggesting that the hyperdynamic circulatory state is dependent on plasma volume expansion. Vasodilatation is the initiating event but requires plasma volume expansion to sustain and develop the hyperdynamic state. These studies showed that the vasodilatation is initially detected in the systemic circulation rather than in the splanchnic circulation. The reason for this is that within the splanchnic circulation there is initial vasoconstriction of the superior mesenteric artery secondary to the outflow block caused by the ligated portal vein. This outflow block is then ameliorated when the portal system forms a

collateral circulation. It is after this that the superior mesenteric artery bed becomes more sensitive to vasodilators.

Different factors that mediate the vasodilatation in this model have been proposed and investigated. These include nitric oxide (Pilette et al. 1996a, Pilette et al. 1996b, Garcia-Pagan et al. 1994, Sogni et al. 1996, Sieber and Groszmann, 1992a, Sieber and Groszmann, 1992b, Lee et al. 1993), prostacyclin (Hamilton et al. 1982, Hamilton et al. 1981, Oberti et al. 1993), glucagon (Benoit et al. 1986, Kravetz et al. 1988), adenosine (Murakami et al. 1996) and bile acids (Genecin et al. 1990a). Studies performed by Lopez-Talvera demonstrated that the proinflammatory cytokine tumour necrosis factor is a major contributor to the development of the hyperdynamic circulation (Lopez-Talavera et al. 1996, Lopez-Talavera et al. 1995). In these studies, inhibition of the actions of TNF-α by an anti-TNF-α antibody prevented the development of the hyperdynamic circulation in PPVL rats.

1.2 TUMOUR NECROSIS FACTOR AND NUCLEAR FACTOR-KAPPA B

1.2.1 Tumour Necrosis Factor-α

Tumour necrosis factor-α (TNF-α) is a 17-kd proinflammatory cytokine that is produced in response to inflammatory stimuli such as injury, infection and neoplasia. It is produced by a variety of cells including monocytes, macrophages, lymphocytes, epithelial and endothelial cells, fibroblasts and parenchymal cells of the gastrointestinal viscera. TNF-α initially exists as a 26-kilodalton surface-associated molecule anchored by an N-terminal hydrophobic domain. It is subsequently processed by proteolytic cleavage to the 17-kilodalton peptide, and three 17-kilodalton molecules become non-covalently associated to form a homotrimeric complex that is biologically active (Smith and Baglioni, 1987). Under normal circumstances, these molecules are not operational or they appear to function at low levels which do not disturb homeostasis. However, soon after an inflammatory stimulus, these mediators are produced both at the site of injury and diversely by cells throughout the body, and they generate a wide range of local and systemic effects incorporating all of the

physiological response systems (ie. cardiovascular, central nervous, pulmonary, gastrointestinal, renal, haemopoietic and endocrine) (table 1).

Lipopolysaccharide (LPS) is an extremely potent stimulus to the synthesis of TNF-α and has been extensively studied. Within 20 minutes of injection of intravenous LPS, there is an increase in concentration of plasma TNF-α that peaks at 90 to 120 minutes. The half-life of TNF-α is 14 to 18 minutes, and levels become undetectable within 4 to 6 hours (Beutler et al. 1985, Hesse et al. 1988). Repeat administration of LPS is followed by a markedly attenuated or absent secondary TNF-α peak. TNF-α interacts with at least two distinct membrane-associated receptors termed TNF-α-R1 (55 kd) and TNF-α-R2 (75 kd) which exhibit similar receptor binding affinities for TNF-α. Both receptors are available on virtually every cell type, except erythrocytes. The two receptors utilise separate pathways for intracellular signal transduction which have been investigated using agonist and antagonist antibodies against each receptor. The pathways activated by p75 are unknown, but the second messenger systems that are activated by p55 include protein kinase C, phospholipase A_2, sphingomyelinase, and phosphatidylcholine-specific phospholipase C (Andrews et al. 1990, Mathias et al. 1993). The TNF-α receptor-ligand complex is internalised and metabolised but this does not appear to be necessary for bioactivity to be expressed since this can occur in the absence of internalisation.

TNF-α receptors also exist in soluble forms that are present in the serum and urine of patients with sepsis, cancer and febrile illness (Van Zee et al. 1992, Digel et al. 1992, Aderka et al. 1991, Kalinkovich et al. 1992). They are produced by the proteolytic cleavage of the extracellular domain of TNF-α receptors. These soluble receptors can bind TNF-α and compete with cellular-associated TNF-α receptors for the cytokine. At high concentrations, soluble TNF-α receptors reduce the bioactivity of TNF-α by binding to it and preventing it from binding to the TNF-α-receptors. At low concentrations however, the soluble TNF-α receptors appear to stabilise the trimeric structure of TNF-α and therefore augments its long term function by slowing down the decay of TNF-α.

The full biological effects of TNF-α can be elicited when only 5% to 10% of the TNF-α receptors are required (Coffman et al. 1988). These effects are related to cellular

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proliferation and differentiation, regulation of cytokine interaction, interaction with other mediators of the stress response, including the prostaglandins and corticosteroids and mediation of the acute metabolic response to injury. With respect to the vascular system TNF-α increases vascular permeability (Stephens et al. 1988), enhances neutrophil adhesion to vascular endothelium (Gamble et al. 1985) promotes neutrophil migration to sites of inflammation and injury (Varani et al. 1988, Gamble et al. 1985) and causes metabolic and structural changes in vascular endothelial cells (Frater-Schroder et al. 1987, Johnson et al. 1991). Furthermore TNF-α causes marked hypotension in mammals (Old, 1985) and several investigators have shown the existence of a cytokine-inducible L-arginine/NO pathway and that the hypotension elicited by TNF-α is reversed after selective inhibition of NO synthesis (Hibbs, Jr. et al. 1992, Kilbourn et al. 1990) suggesting that L-arginine-derived NO is a principal mediator of TNF-α-induced hypotension.

As cytokines are such important mediators in inflammatory processes with significant haemodynamic effects TNF-α levels were investigated in the PPVL model. Lopez-Talavera et al. demonstrated using a bioassay a 5-fold increase in plasma TNF-α levels at day 5 following PPVL and a 10-fold increase at day 14 (Lopez-Talavera et al. 1995). They went on to show that administration of an anti-TNF-α antibody to rats undergoing PPVL attenuated the development of the hyperdynamic circulation. This suggested that TNF-α is a major contributor to the hyperdynamic circulation, and is consistent with other work implicating NO as a mediator causing vasodilatation, since TNF-α is believed to mediate its haemodynamic effects by induction of NO synthesis. A subsequent study involving the administration of thalidomide, which inhibits TNF-α mRNA production, also produced similar results (Lopez-Talavera et al. 1996). What remains unclear is the pathophysiological mechanisms resulting in TNF-α production in this model. Possible mechanisms include the involvement of endotoxin and/or oxidant stress.

Endotoxin and bacterial translocation following PPVL has been studied acutely and chronically following PPVL by Garcia-Tsao et al.(Garcia-Tsao et al. 1993). Following PPVL there is an increase in bacteria in mesenteric lymph node cultures compared to sham rats by 48

hours. However, by seven days there was no significant difference. No detectable circulating endotoxin were detected in the plasma at any time in these studies. Despite this, it is possible that macrophages may be primed in the mesenteric lymph nodes to produce the proinflammatory cytokines but this may only be a short term response as there is no detectable bacterial translocation after 5 days.

Mehta et al. (Mehta et al. 1990) treated rats with antibiotics to see if this could alter the hyperdynamic circulation on the basis that there was bacterial translocation but found that this had no effect on haemodynamic changes.

1.2.2 Nuclear Factor-kappa B

The transcription factor nuclear factor-kappa B (NF-κB) is a key regulator of important inflammatory and immune reactions. It exists as a protein molecule comprising two subunits of different molecular weights (heterodimer). The p-50 and p-65 subunits make up the protein heterodimer which when inactive exists in the cytoplasm of cells of the body bound to an inhibitory protein subunit IκB. When the cells are activated the IκB subunit becomes phopshorylated and dissociates from the heterodimer, NF-κB, (p-50-p65 heterodimer) which then migrates to the nucleus of the cell where it binds to the promoter and enhancer regions of target genes encoding for mRNA transcription (figure 1). The target genes are multiple but include the proinflammatory cytokines TNF-α, IL-6, IL-8 and the inducible form of nitric oxide synthase (iNOS). As there are multiple target genes that can be influenced by activated NF-κB, then, it explains why it is such an important molecule in the acute phase response as well as in immune reactions. NF-κB can be artificially induced in tissue culture by phorbol esters, calcium ionophores, ultra-violet light and various mitogens (Baeuerle and Henkel, 1994, Grilli et al. 1993). *In vivo* activation of NF-κB can be caused by TNF-α (Schutze et al. 1995), IL-1 (Grilli et al. 1993), lipopolysaccharide, viral products such as double stranded RNA (Visvanathan and Goodbourn, 1989) and ROS (Schreck et al. 1991).

System	Effect of TNF-α
Cardiovascular	Shock Myocardial suppression Capillary leakage syndrome Nitric oxide synthesis
Central Nervous	Fever Release of corticotropin-releasing factor, CRF Release of adrenocorticotropic hormone, ACTH Anorexia
Pulmonary	Adult Respiratory Distress syndrome Oedema Hypoxia
Gastrointestinal	Necrosis Biosynthesis of platelet activating factor Hepatic production of acute phase proteins Diarrhoea
Renal	Diuresis followed by oliguric renal failure Acute renal tubular necrosis
Haematopoietic	Suppressed erythropoiesis Increased haemolysis Disseminated intravascular coagulation Neutrophilia or neutropenia
Endocrine	Euthyroid sick syndrome Lactic acidosis Hyperaminoacidaemia

Table 1. Systemic Effects of Tumour Necrosis Factor-α.

Different groups have demonstrated that activation of NF-κB is subject to redox control. *In vitro* studies have shown that prooxidants such as hydrogen peroxide or oxidised glutathione can activate NF-κB (Schreck et al. 1991, Schreck et al. 1992, Galter et al. 1994) and antioxidants such as N-acetylcysteine and pyrrolidine can inhibit NF-κB activation. (Mihm et al. 1991, Staal et al. 1990, Staal et al. 1995). However, in other studies it remains unclear as to whether reactive oxygen species directly activates NF-κB or whether it facilitates activation of NF-κB by other physiological inducers. Hydrogen peroxide is reported to activate NF-κB in Jurkat T cells but it was also observed that it was unable to activate NF-κB without the addition of PMA or TNF-α (Israel et al. 1992, Ziegler-Heitbrock et al. 1993).

TNF-α and other cytokines induce an oxidant stress *in vitro*. This is probably via the generation of reactive nitrogen species. What is interesting is the fact that TNF-α can not only induce an oxidant stress, but can be produced indirectly as a result of oxidant stress through the activation of NF-κB. Therefore, it is possible that a self-perpetuating loop involving oxidant stress and cytokine production may develop.
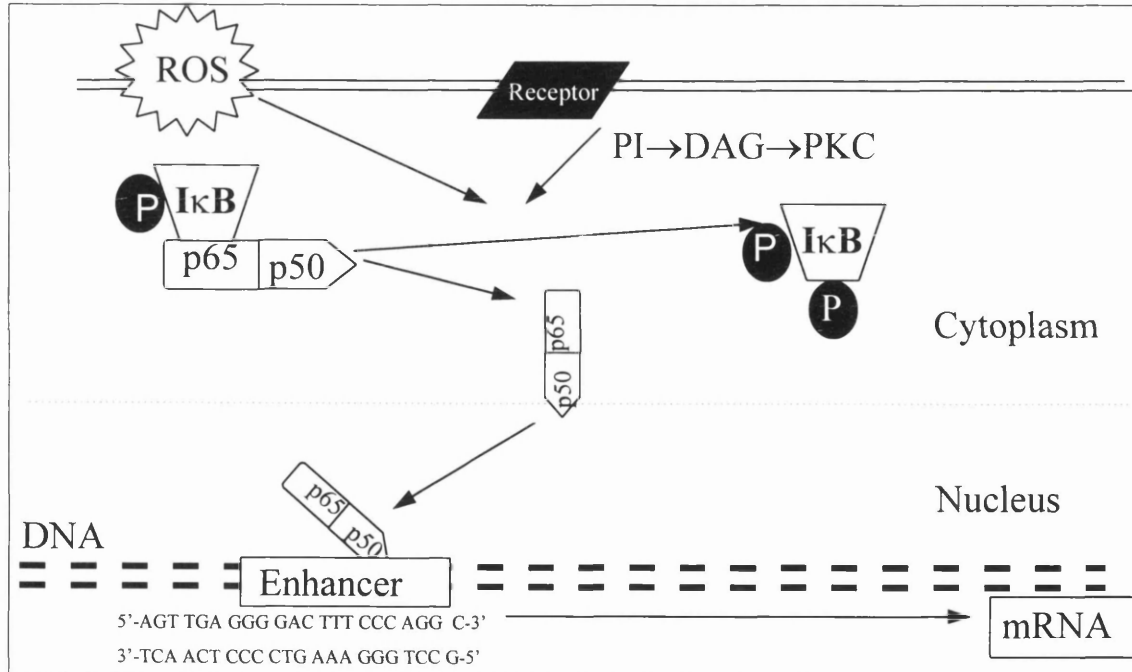
Figure 1. Molecular Actions of NF-κB. In the inactive form it exists in the cytoplasm as a protein heterodimer bound to an inhibitory protein, IκB. When it is activated the IκB subunit dissociates from the protein heterodimer which migrates to the nucleus and binds to enhancer regions of target genes encoding for transcription of specific proteins.

1.3 OXIDANT STRESS

1.3.1 Definition

Oxidant stress is defined as an imbalance between free radical-producing and free radical-scavenging mechanisms resulting in free radical damage. Free radicals are species which contain one or more unpaired electrons and are capable of independent existence. As a result of having an unpaired electron the free radical is chemically more reactive than other molecules as it tries to become stable by either gaining or losing an electron. Biologically important free radicals include reactive oxygen species and reactive nitrogen species. Reactive oxygen species may be defined as any compound derived from molecular oxygen (O_2) which has occurred by chemical reduction of less than four electrons. Reactive nitrogen species include nitric oxide ($NO^\bullet$), nitrogen dioxide ($NO_2^\bullet$), dinitrogen trioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4) and peroxynitrite ($ONOO^-$).

Singlet oxygen

$O\cdot$

$\uparrow$

$O_2 \longrightarrow O_2\cdot^- \longrightarrow H_2O_2 \longrightarrow OH\cdot \longrightarrow H_2O$

| Oxygen | Superoxide anion | Hydrogen peroxide | Hydroxyl radical | Water |
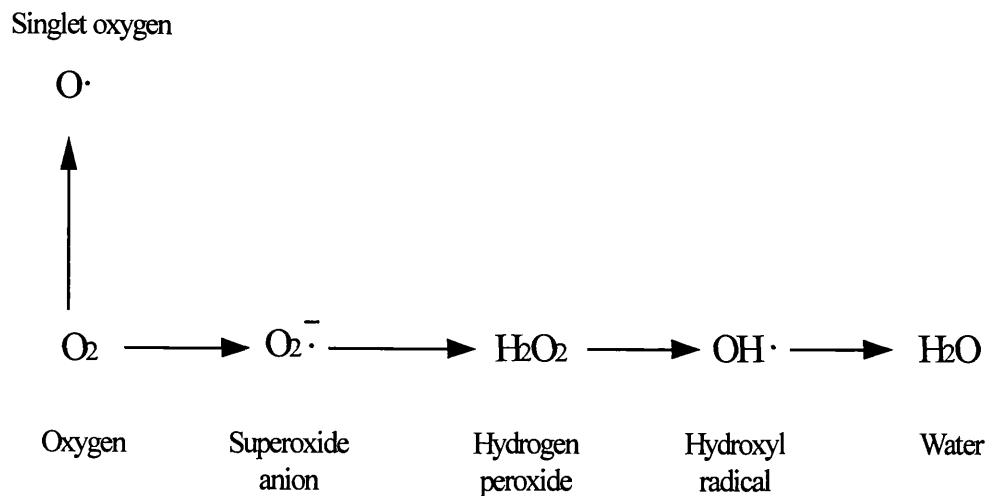
Figure 2. Formation of reactive oxygen species by the four electron reduction of molecular oxygen to water.

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1.3.2 Reactive Oxygen Species

Reactive oxygen species are constantly being formed in the body and are removed by antioxidant defenses. Examples of oxygen-derived free radicals include the superoxide anion ($O_2^{-\bullet}$), singlet oxygen ($O^{\bullet}$), hydroxide ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) species. Examples of ROS which contain an even number of electrons and are thus not free radicals include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and N-chlorinated amines (RNHCl). These different species will be described further.

Superoxide anion is produced by the univalent reduction of molecular oxygen. This is a relatively stable free radical which exists in equilibrium with its conjugate acid, the hydroperoxyl radical ($HOO^{\bullet}$):

$$O_2^- + H^+ \leftrightarrow HOO^{\bullet} \quad (pK = 4.8)$$

At physiological pH virtually all the superoxide will exist as the unprotonated form. However, at the low pH that occurs in the phagolysosome of inflammatory cells such as neutrophils or macrophages or in the phospholipid bilayers of cell membranes the concentration of $HOO^{\bullet}$ may increase dramatically. However, in an aqueous enviroment the superoxide anion will rapidly dismutate to yield H_2O_2 and O_2.

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$
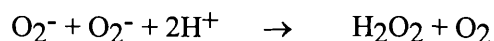
Superoxide can act as an oxidant as well as a reductant. It can oxidise a variety of biomolecules such as ascorbate, sulphydryl-containing compounds and certain catecholamines. Alternatively, superoxide anion may reduce certain compounds including ferric ion (Fe^{3+}), chelates and haemoproteins as well as certain quinones. Additionally, superoxide anion is capable of inactivating a variety of enzymes such as RNase, glyceraldehyde-3-phosphate dehydrogenase and aconitase. However, it is not thought to be highly toxic to cells because of poor permeability but instead its derivatives, including, hydroxyl radical and hydroperoxyl radical, play a more important role in mediating oxidant stress.

Hydrogen peroxide contains no unpaired electrons and is therefore not a free radical. It is however a relatively stable oxidant that sluggishly reacts with most bio-organic molecules. Hydrogen peroxide is lipophilic and will readily cross the lipid bilayer of cell membranes. Once inside the cell it can react with certain transition metals such as iron or copper or with certain haemoproteins to yield reactive free radicals such as hydroxyl radical or ferryl derivatives of haemoproteins. Hydrogen peroxide can also inactivate some enzymes by oxidising essential sulphydryl groups. It has also been suggested that it can affect transforming DNA, inducing chromosomal aberrations and breaking the DNA backbone although these changes are probably mediated by H_2O_2-dependent, metal-catalysed reactions. The hydroxyl radical is produced by the reaction of superoxide anion and hydrogen peroxide with certain transition metals such as iron (Fe^{2+}) or copper (Cu^{2+}) and/or their low molecular chelates to yield the highly reactive $OH^\bullet$.

In this type of reaction iron acts as a catalyst being reduced by superoxide and is then oxidised by hydrogen peroxide. This reaction is known as the Haber-Weiss reaction or the superoxide-driven Fenton reaction.

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$
$$H_2O_2 + Fe^{2+} \rightarrow OH^\bullet + OH^- + Fe^{3+}$$
$$\text{Net: } O_2^- + H_2O_2 \rightarrow OH^\bullet + OH^- + O_2$$
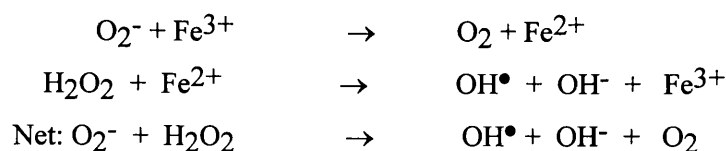
Another mechanism by which the hydroxyl radical is formed *in vivo* has been proposed by Beckman et al. (Beckman and Koppenol, 1996). Their group suggested that nitric oxide combines with superoxide anion to generate the peroxynitrite anion ($ONOO^-$):

$$O_2^- + NO^\bullet \rightarrow ONOO^-$$
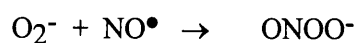
Although peroxynitrite is relatively stable it has a pK_a of 6.6 and therefore at physiological pH it becomes protonated and yields peroxynitrous acid (ONOOH). This compound is very

unstable and it was initially proposed that this rapidly decomposes to yield hydroxyl radical ($OH^\bullet$) and nitrogen dioxide ($NO_2^\bullet$):

$$ONOO^- + H^+ \leftrightarrow ONOOH \rightarrow OH^\bullet + NO_2^\bullet$$

However, current evidence suggests that this sequence does not occur readily, but that peroxynitrite anion itself reacts as a potent radical and is nearly as reactive as the hydroxyl radical. The simultaneous formation of superoxide anion and nitric oxide has been proposed as the mechanism of the superoxide-dependent microvascular injury produced by ischaemia and reperfusion of various organ systems. The hydroxyl radical is an extremely reactive species which can react with virtually all known biomolecules. It reacts in a site-specific manner wherever it is formed because of its high reactivity.

Hydroxyl radicals interact with certain carbohydrates, proteins, nucleotide bases and lipids to produce peroxyl radicals ($ROO^\bullet$) as intermediates. These species are slightly less reactive than hydroxyl radical and therefore would be expected to react at sites distant from those of hydroxyl radical production thereby promoting the toxicity of $OH^\bullet$. Peroxyl radicals can induce peroxidation of polyunsaturated fatty acids (PUFA) initiated by hydroxyl radical. They can also oxidise proteins, carbohydrates and sulphydryl components and haemolyse erythrocytes.

Haemoproteins including myoglobin and haemoglobin may impose an oxidant stress in cells and tissue if released from damaged erythrocytes or if there is enhanced oxidant metabolism in proximity to these haemoproteins. This may occur at three related levels:

• Haemoglobin or myoglobin may interact with small amounts of H_2O_2 or lipid peroxides to yield a haemoprotein-associated oxidant (ferryl-Hb or ferryl-Mb) which is able to peroxidise lipids, degrade carbohydrates, and crosslink proteins.

• These haemoproteins may interact with large amounts of peroxides to produce both the ferryl haemoprotein as well as release the haem iron into the surrounding medium where it is able to perpetuate the oxidant reactions resulting in the generation of free radicals.

- The ferrous form of oxyhaemoglobin and oxymyoglobin can auto-oxidise to yield superoxide anion, hydrogen peroxide and possibly the hydroxyl radical.

Neutrophils contain myeloperoxidase, a haemoprotein in the azurophilic granules. Following activation of neutrophils this enzyme is released into the extracellular medium where it interacts with hydrogen peroxide to form an enzyme substrate complex with a potent oxidising potential. This haemoprotein-associated oxidant is able to oxidise chloride (Cl^-) with the loss of two electrons to form hypochlorous acid:

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$$
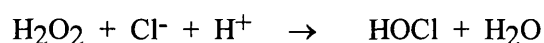
At physiological pH, hypochlorous acid exists in equilibrium with its conjugate base anion hypochlorite (OCl^-). Both hypochlorous acid and hypochlorite anion are both potent oxidising and chlorinating agents that are more reactive than superoxide anion and hydrogen peroxide. They can react with a variety of biological substrates including sulphydryl groups, thioethers, aromatic and aliphatic amino acids, haemoproteins, unsaturated lipids and ascorbate.
Hypochlorous acid can also react especially rapidly with certain primary amines to form derivatives containing the nitrogen-chlorine bond known as N-chlorinated amines.

$$RNH_3^+ \rightarrow RNH_2 + H^+$$
$$RNH_2 + HOCl \rightarrow RNHCl + H_2O$$
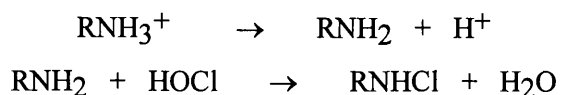
The lipophilic forms of N-chlorinated amines are especially toxic to cells since they can cross the biological membranes and rapidly oxidise the intracellular components such as haemoproteins, thiols and glutathione. Other derivatives have been shown to injure intestinal cells *in vitro* and *in vivo*.

1.3.3 Sources of Reactive Oxygen Metabolites

Non-enzymatic Sources of Reactive Oxygen Species:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^-$$

$$Hb\text{-}Fe^{2+} + O_2 \rightarrow Hb\text{-}Fe^{3+} + O_2^-$$

$$Mb\text{-}Fe^{2+} + O_2 \rightarrow Mb\text{-}Fe^{3+} + O_2^-$$

$$\text{Catecholamines} + O_2 \rightarrow \text{Melanin} + O_2^-$$

$$\text{Reduced flavin} + O_2 \rightarrow \text{Flavin semiquinone} + O_2$$

$$\text{Coenzyme Q (hydroquinone)} + O_2 \rightarrow \text{Coenzyme Q (ubiquinone)} + O_2$$

$$\text{Tetrahydrobiopterin} + O_2 \rightarrow \text{Dihydrobiopterin} + O_2$$

Enzymatic Sources of Reactive Oxygen Species:

Xanthine oxidase

$$\text{Hypoxanthine} + O_2 \rightarrow \text{xanthine} + O_2^- + H_2O_2$$

NADPH oxidase

$$NADPH + 2\,O_2 \rightarrow NADP^+ + 2O_2^- + H^+$$

Amine oxidase

$$R\text{-}CH_2\text{-}NH_2 + H_2O + O_2 \rightarrow R\text{-}CH + NH_3 + H_2O_2$$

Aldehyde oxidase

$$R\text{-}CHO + O_2 \rightarrow RCOOH + O_2^-$$

Dihydro-orotate dehydrogenase

$$\text{Dihydro-orotate} + NAD^+ + O_2 \rightarrow \text{Orotic acid} + NADH + O_2^-$$

Peroxidase

$$H_2O_2 + Cl^- + O_2 \rightarrow HOCl^- + H_2O$$

NADH oxidase reaction:

$$E\text{-}Fe^{3+} + ROOH \rightarrow \text{Compound I} + ROH$$

$$\text{Compound I} + NADH \rightarrow NAD^\bullet + \text{Compound II}$$

$$\text{Compound II} + NADH \rightarrow NAD^\bullet + E\text{-}Fe^{3+}$$

$$2NAD^\bullet + 2O_2 \rightarrow 2NAD^+ + 2O_2^-$$

Table 2. Sources of Reactive Oxygen Species

Reactive oxygen species can be formed from exogenous sources as well as endogenous sources (table 2). Ionising radiation, from the enviroment or administered as medical therapy, is a significant source of reactive oxygen species in biological systems as different products are formed as the water molecule in aqueous solution absorbs energy becoming excited and unstable. The excited water molecule thereby produces the hydroxyl radical and other reactive species including $H^\bullet$, H_2, H_2O_2, and H_3O^+. These species can subsequently react with biological substrates such as protein and carbohydrates. Alternatively, the reactive metabolites can react with each other to generate stable molecular species.

Under physiological conditions the mitochondrial electron transport system is the most important site of free radical production. Normally, more than 95% of all molecular oxygen consumed by cells is reduced by four electrons to form two molecules of water via this mechanism. The enzyme responsible for this reaction is cytochrome oxidase (cytochrome aa_3), the terminal oxidase in the respiratory chain. However, it has been estimated that approximately 1-2% of the electron flow leaks off to form superoxide anion under normoxic conditions. The fact that only hydrogen peroxide and not superoxide can be detected using intact mitochondria suggests that it is dismutated either enzymatically or non-enzymatically to hydrogen peroxide:

$$O_2^- + O_2^- + H^+ \quad \rightarrow \quad H_2O_2 + O_2$$

Within the electron transport chain there are two additional sources of superoxide anion. The major source of superoxide is the partially reduced ubisemiquinone free radical generated by the reduction of ubiquinone during electron transport. Ubisemiquinone interacts with molecular oxygen to form superoxide anion by the following reaction:

$$ubiquinone + e^- \quad \rightarrow \quad ubisemiquinone + O_2 \quad \rightarrow \quad ubiquinone + O_2^-$$
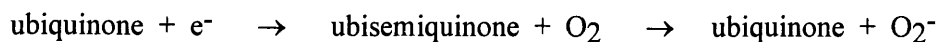
A second source of mitochondrial superoxide formation is from the flavoprotein, NADH dehydrogenase. The flavin moiety of this enzyme is reduced by catalysis to form the flavin semiquinone free radical which interacts with molecular oxygen to form the superoxide anion in a similar reaction to above.

Since mitochondria are rich in superoxide dismutase (SOD), an enzyme which will be discussed in the next section, the majority of the derived superoxide anions are enzymatically dismutated to H_2O_2 and O_2. In certain disease states, however, there is a shift in the redox balance in favour of oxidation. Other enzymatic and non-enzymatic mechanisms for the production of reactive oxygen species are shown in table 2.

1.3.4 Reactive Nitrogen Species

Symbol	Name	Comments
NO	nitric oxide	free radical
NO_2	nitrogen dioxide	free radical, nitrosating agent
N_2O_3	dinitrogen trioxide	nitrosating agent
N_2O_4	dinitrogen tetroxide	dimeric nitrogen dioxide, nitrosating agent
NO_2-	nitrite	produces nitric oxide at acidic pH

Table 3. Reactive Nitrogen Species

Reactive species containing nitrogen (table 3) are also important since they can impose an oxidant stress and influence the redox balance *in vivo* and *in vitro*. Nitric oxide is a colourless gas containing an unpaired electron that is delocalised over the nitrogen and oxygen atom giving the resonance stabilisation structures represented below:

$$:\dot{N}=\ddot{O}: \quad \leftrightarrow \quad :\ddot{N}=\dot{O}:$$

$NO^{\bullet}$ is relatively unstable in the presence of molecular oxygen with an apparent half-life of 3-5 seconds. It rapidly decomposes in the presence of O_2 and H_2O to yield a variety of nitrogen oxides in a complex series of interactions as indicated on the next page:

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$$2NO^\bullet + O_2 \rightarrow 2NO_2^\cdot \text{ (in presence of H}_2\text{O)}$$

$$2NO_2^\bullet \rightarrow N_2O_4 \rightarrow NO_2^- + NO_3^- \text{ (in presence of H}_2\text{O)}$$

$$NO_2^\bullet + N_2O_4 \rightarrow NO^\bullet + 2NO_3^- \text{ (in presence of H}_2\text{O)}$$

$$NO^\bullet + NO_2^\bullet \rightarrow N_2O_3 \rightarrow 2NO_2^-$$
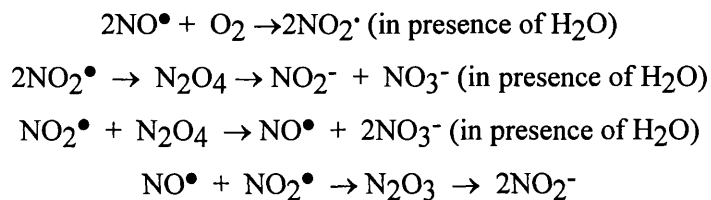
where $NO_2^\bullet$, N_2O_3 and N_2O_4 represent nitrogen dioxide, dinitrogen trioxide and dinitrogen tetroxide respectively. Each of these species are potent N-nitrosating agent (NOX) with the ability to promote the nitrosation of primary and secondary amines to yield potentially carcinogenic nitrosamines:

$$R_2\text{-NH} + NOX \rightarrow R_2\text{N-N=O}$$

where R_2N-N=O represents a nitrosamine. In addition, it has already been mentioned that nitric oxide may interact with superoxide anion to yield peroxynitrite anion (ONOO-). Although peroxynitite is relatively stable it has a pK_a of 6.6 which implies that substantial amounts of this compound will be protonated at physiological pH to yield peroxynitrous acid. This compound is very unstable and is thought to rapidly decompose to yield the hydroxyl radical ($OH^\bullet$) and nitrogen dioxide radical ($NO_2^\bullet$):

$$O_2^- + NO \cdot\rightarrow ONOO^- \rightarrow NO_2^\bullet + OH^\bullet$$

The hydroxyl radical is, as mentioned earlier, an extremely reactive species and tends therefore to react with virtually all biomolecules at the site where it is formed. The nitrogen dioxide radical is also a very reactive radical with the ability to react with alkanes and alkenes via free radical-mediated mechanisms and other studies have shown that nitrogen dioxide can initiate lipid peroxidation *in vitro* (Kikugawa et al. 1994). In addition it has also been shown to react with haemoproteins such as haemoglobin and oxidise thiols and thioethers such as methionine.

1.3.5 Oxidant stress in the PPVL rat model

In the PPVL rat model oxidant stress may occur for a number of reasons. Firstly, there is increased circulating TNF-α which itself may mediate oxidant stress through the induction of nitric oxide and formation of reactive nitrogen species. Secondly, there is increased bacterial translocation acutely following PPVL which presumably causes macrophage activation and a respiratory burst of oxidant stress. Finally, there is a characteristic sequence of haemodynamic changes following PPVL which may give rise to an ischaemia-reperfusion type of injury. In the first few days following PPVL there is a reduced portal venous inflow but by day 4 there is a significant increase in the portal venous inflow. This suggests that within relatively ischaemic areas of the liver, mesentery and portosystemic venous channels the re-exposure to oxygen could subject these areas to an ischaemia-reperfusion type injury and an oxidant stress.

1.3.6 Antioxidants

Antioxidants can be defined as "any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate" (Halliwell and Gutteridge, 1997). These substances form the endogenous defence against free radical mechanisms although they can also be administered as therapeutic agents to protect against oxidant stress. Antioxidants cannot distinguish between radicals that play a role in physiological reactions and those that cause damage. There are different mechanisms by which an antioxidant offers protection. For example, they can prevent lipid peroxidation by:

1) Decreasing localised O_2 concentrations (eg sealing of foodstuffs under nitrogen)

2) Preventing the initiation of the first chain reaction for example by scavenging initiating radicals such as hydroxyl radicals.

3) Binding metal ions in forms that will be unable to generate species that initiate lipid peroxidation such as ferryl, or $Fe^{2+}/Fe^{3+}/O_2$ and/or will not decompose lipid peroxides to

peroxy or alkoxy radicals that can also initiate as well as stimulate the lipid peroxidation process.

4) Decomposing peroxides by converting them to non-radical products such as alcohol instead of the peroxy radicals.

5) By scavenging intermediate radicals such as peroxy and alkoxy radicals to prevent the continued hydrogen abstraction. These are termed chain breaking antioxidants and are often phenols or aromatic amines.

Examples of antioxidants include Vitamin E, Vitamin C, Superoxide Dismutase (SOD), glutathione (GSH), glutathione peroxidase and N-acetylcysteine. These will be discussed further:

Vitamin E is a lipid soluble vitamin and is a mixture of four lipid-soluble tocopherols of which α-tocopherol is the most active. It functions as a chain-breaking antioxidant in that it is able to scavenge the lipid peroxide radical, an intermediate product of peroxidation of PUFAs and prevent further formation of peroxidation chains (Bast et al. 1991):

$$Vit\ E\ +\ LOO^{\bullet}\ \rightarrow\ LOOH\ +\ Vit\ E^{\bullet}$$
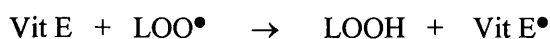
The vitamin E radical is fairly stable due to the delocalisation of its unpaired electron within the aromatic ring. Therefore vitamin E is a good antioxidant. This vitamin can be regenerated by the hydrophilic vitamin C which can also function as a radical scavenger.

Antioxidant enzymes also offer protection against oxidant stress. The primary antioxidant system consists of three enzymes: superoxide dismutases (SOD), catalases (CAT), and peroxidases of which glutathione peroxidase is the most common in mammalian cells. These enzymes are highly compartmentalised. For example, a copper- and zinc-containing-superoxide dismutase (CuZnSOD) is found predominantly in the cytoplasm, whereas a manganese-containing superoxide dismutase (MnSOD) is found primarily in mitochondria. The reaction catalysed by SOD is as follows:

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$$2O_2^- + 2H^+ \quad \rightarrow \quad O_2 + H_2O_2$$
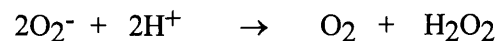
Glutathione peroxidase has at least three different isoenzymes: one found in the cytoplasm, one found in the mitochondria and one found in plasma. The enzyme contains selenium and catalyses the oxidation reaction of reduced glutathione while simultaneously reducing hydrogen peroxide:

$$2GSH + H_2O_2 \quad \rightarrow \quad GSSG + 2H_2O$$
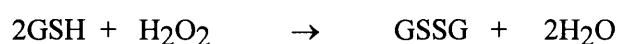
The maintenance of reduced glutathione levels is important and thiol groups (SH) in glutathione are essential in the protection against oxidant injury. The reduced form is regenerated by the reduction of oxidised glutathione using the enzyme glutathione reductase at the expense of NADPH:

$$GSSG + NADPH + H^+ \quad \rightarrow \quad 2GSH + NADP^+$$

Catalase is another important enzyme that contains haem and it transforms hydrogen peroxide into water and molecular oxygen:

$$2H_2O_2 \quad \rightarrow \quad 2H_2O + O_2$$

N-acetylcysteine is an antioxidant that is derived from the amino acid cysteine (figure 3).

Its precise mechanism of action is unclear but it could be by one or more of the following methods:

1) N-acetylcysteine serves as a precursor for glutathione synthesis thereby indirectly acting as a free radical scavenger.

2) It may act directly as a free radical scavenger.

3) It acts as a thiol donor and can therefore react with peroxynitrite resulting in the release of

NO which under normal circumstances behaves as an anti-oxidant.

$$4GSH \;+\; ONOO^- \;\rightarrow\; 2GSSG \;+\; NO \;+\; 2H_2O$$
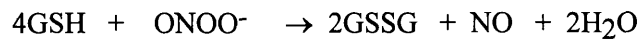
3) The thiol donor action maintains the thiol groups in the reduced state within cells.

4) At high concentrations it may act as an iron chelator.

5) It may prevent accumulation of neutrophils thereby preventing the respiratory burst and the

extracellular formation of reactive oxygen intermediates.
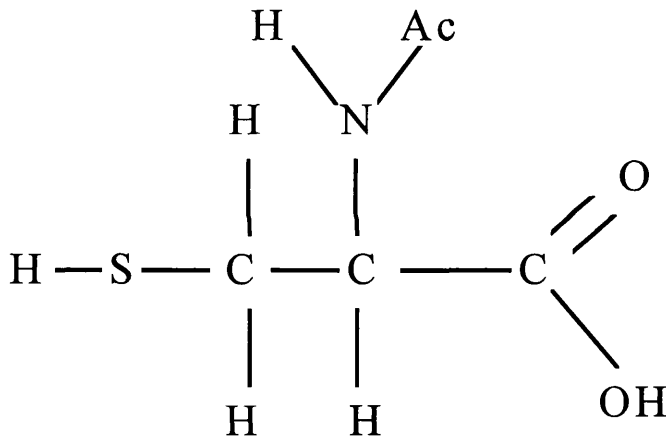
Figure 3. Structure of N-acetylcysteine

Pyrrolidine Dithiocarbamate is an antioxidant which has been shown to inhibit

activation of NF-κB (figure 4). Its precise mechanism of action as an antioxidant is unclear but

it has previously been demonstrated to act as a free radical scavenger and also to be a chelator

of heavy metal ions (Schreck and Baeuerle, 1994).

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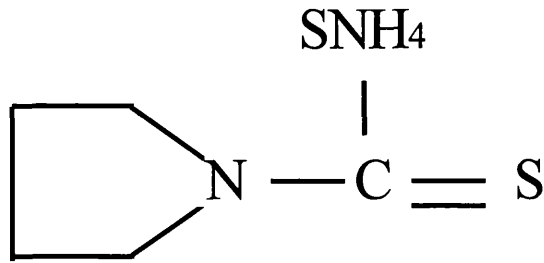
Figure 4. Structure of Pyrrolidine Dithiocarbamate

1.3.7 Demonstration of oxidant stress

Oxidant stress and free radical reactions can be determined directly and indirectly. Technical difficulties arise in demonstrating free radicals since they are highly unstable. The only analytical technique that directly measures free radicals is by electron spin resonance (ESR) spectrometry but it is of limited use *in vivo*. Whole body ESR has been investigated but is not yet developed. Instead ESR has been used to detect free radicals in tissue samples obtained *ex vivo* (Benedetto et al. 1981).

Another method of detecting oxidant stress is to detect the concentrations of antioxidants in tissues. If these are decreased it is likely that there is an increased oxidant stress in tissues. The TRAP assay (Total [peroxyl] Radical-trapping antioxidant Parameter) is a commonly used method for determining an empirical measurement of antioxidant activity in plasma (Wayner et al. 1987). Assessment of the relative contribution of individual antioxidants (ascorbate, urate, α-tocopherol, protein sulphydryls) to the total antioxidant capacity requires different assays. However, measurement of either TRAP or the individual antioxidants are not likely to be useful indices of free radical production as the latter would have to be extensive to disturb the normally steady state levels of antioxidants.

Markers of free radical injury can also be used to demonstrate oxidant stress. These include measurement of lipid peroxidation products, protein damage and DNA damage. Lipid peroxidation is a complex process whereby polyunsaturated fatty acids (PUFAs) in the phospholipids of cellular membranes undergo reaction with oxygen to yield lipid hydroperoxides (LOOH). The reaction occurs through a free radical chain mechanism initiated by the abstraction of a hydrogen atom from a PUFA by a reactive free radical, followed by a complex sequence of propagative reactions. The LOOH and conjugated dienes that are formed can decompose to form numerous other products including alkenals, alkanals, 4-hydroxyalkenals, malondialdehyde and the F_2-isoprostanes. The F_2-isoprostanes are a group of prostaglandin-like compounds which are produced independently of cyclooxygenase and as they are stable by-products they can be used as markers of lipid peroxidation (figure 5 of F_2-isoprostane formation). These compounds will be discussed further in the next section. Lipid peroxidation is an important process for a number of reasons whenever free radicals are formed in biological tissues. The reaction is highly likely to occur due to the abundance of PUFAs and so it is a sensitive marker of free radical reactions. Secondly, the reaction is damaging to the cell causing dysfunction or cell death. Finally, the lipid peroxidation products formed may have biological effects.

Different lipid peroxidation products could therefore be measured to provide an index of ongoing lipid peroxidation. The thiobarbituric acid reacting substances (TBARS) assay is still the most popular and easiest method of demonstrating lipid peroxidation and free radical reactions in biological samples (Esterbauer and Cheeseman, 1990). The assay is based on the reaction of thiobarbituric acid with malondialdehyde. The sample is heated with thiobarbituric acid under acidic conditions and the amount of pink-coloured MDA-TBA adduct product is measured at 532 nm. The test is very simple and quick but the detected concentration of malondialdehyde can be influenced by the incubation time since heating of the specimen may encourage more malondialdehyde to be formed by the decomposition of lipid hydroperoxides. It should be noted that malondialdehyde is a minor product of lipid peroxidation and is rapidly metabolised. This is therefore not an ideal method for measuring lipid peroxidation *in vivo* and

the assay is considered to be non-specific and subject to artefact. Lipid hydroperoxides can be measured using gas chromatography mass spectrometry. This is a sensitive and specific assay and involves reduction of LOOH to the hydroxy acids with triphenylphosphine (Hughes et al. 1986). However, accurate measurement of lipid hydroperoxides are difficult due to their rapid deterioration *in vitro*. It is therefore important to minimise this by the addition of antioxidants and by quick processing of the samples at 4°C which is inconvenient. Other peroxidation products can also be measured including conjugated dienes and other aldehydes but these have not been used extensively *in vivo*. Conjugated dienes may be absorbed in the diet or produced by the metabolism of gut bacteria whereas the methods used for assay of other aldehydes are generally time consuming and expensive.

Measurement of protein damage by free radicals has been less intensely studied than lipid peroxidation due to inherent problems of the presence of a large number of different protein targets and the relatively high number of different amino acid residues. Likewise, the measurement of DNA damage induced by free radicals is limited by the obscurity of the tissue of origin of the products due to interference with other urinary components.

1.3.8 F_2-isoprostanes

In this thesis a group of compounds known as the F_2-isoprostanes were used as an index of lipid peroxidation. The precursor of the isoprostanes is arachidonic acid. The majority of arachidonic acid present *in vivo* exists esterified to phospholipids. It is likely that the oxidation of the lipid-esterified arachidonic acid rather than free arachidonic acid is the most likely source of F_2-isoprostanes *in vivo* and that this is subsequently released by phospholipases (Morrow et al. 1992). A mechanism to explain the non-enzymatic formation of these compounds is outlined in figure 5.
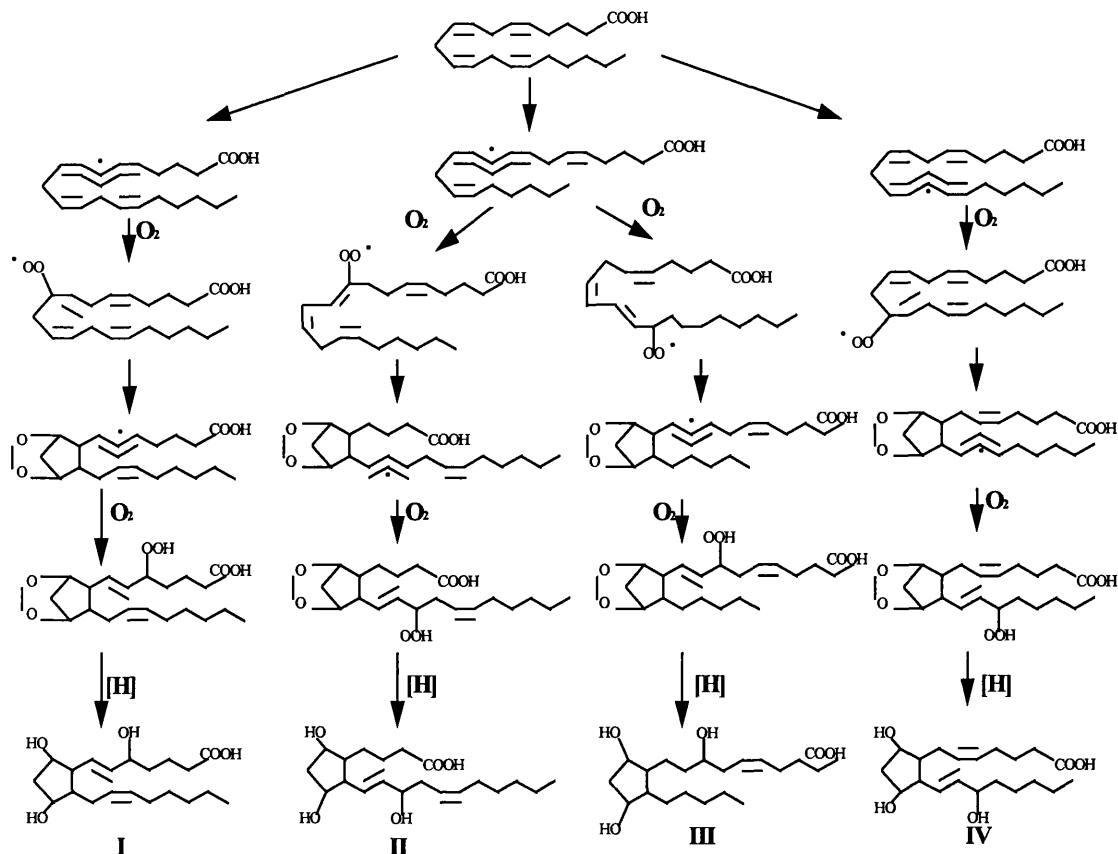
Figure 5. Mechanism of F₂-isoprostane formation.

Arachidonic acid initially undergoes abstraction of a hydrogen atom leading to the generation of one of four isomeric arachidonyl radicals which can each give rise to one of four peroxyl radicals, formed by the addition of molecular oxygen. Subsequently, endocyclisation and addition of another molecule of oxygen yield bicycloendoperoxide prostaglandin-like intermediates that can then be reduced to PGF_2-like regioisomers (I-IV). Theoretically, eight racemic diastereomers for each regioisomer may exist. Thus, sixty-four different compounds can potentially be produced by this mechanism, although the formation of a smaller number of certain isomers is likely to be favoured *in vivo*. It is because these compounds are structurally isomeric with $PGF_{2\alpha}$ that these compounds are collectively referred to as F_2-isoprostanes. Measurement of F_2-isoprostanes has emerged as one of the most reliable and sensitive methods of demonstrating lipid peroxidation and levels can be determined using stable isotope dilution gas chromatography mass spectrometry.

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1.4. THE HYPOTHESIS

1.4.1 The Hypothesis

The hypothesis to be tested in this thesis is that **in partial portal vein ligated rats, oxidant stress induces a hyperdynamic circulation via a cytokine-dependent pathway** (figure 6).

PARTIAL PORTAL VEIN LIGATION

↓

OXIDANT STRESS

↓

NF-kappa B ACTIVATION

↓

TNF-alpha PRODUCTION

↓

NITRIC OXIDE PRODUCTION

↓

HYPERDYNAMIC CIRCULATION
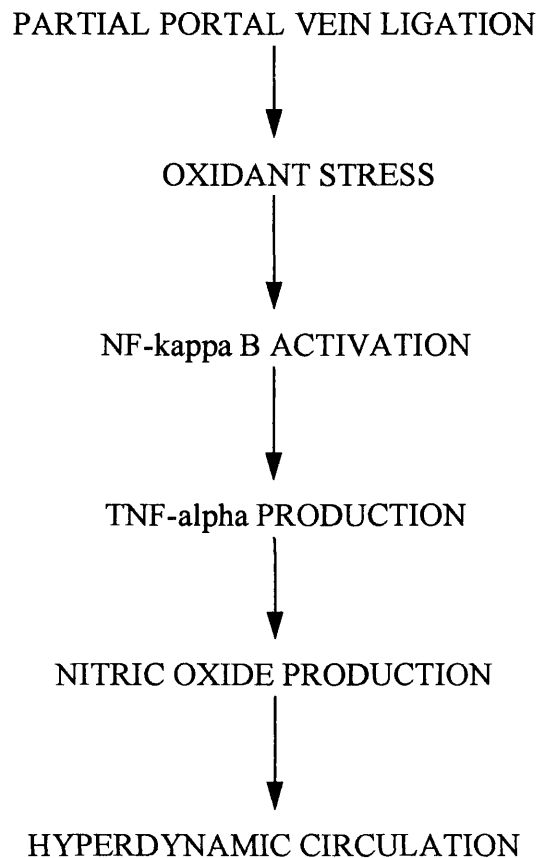
Figure 6. The Hypothesis

The diagram represents a possible sequence of events that occurs in this model to result in the hyperdynamic circulation. Another possibility is that oxidant stress is a downstream effect of TNF-α production and nitric oxide production. If oxidant stress is an important component in the generation of the circulatory changes in this model then this may eventually open alternative therapeutic pathways for the treatment of portal hypertension.

1.4.2 Aims of the thesis

In this thesis, the unifying hypothesis was tested in stages according to the mechanism shown in diagram (figure 6). The first stage was to demonstrate that oxidant stress occurred in the model. This involved measuring F_2-isoprostanes as biological markers of lipid peroxidation in liver, plasma and urine. The second stage was to demonstrate activation of the transcription factor NF-κB in this model. This involved the measurement of NF-κB in nuclear extracts from liver using an electromobility shift assay. The third stage involved measurement of plasma levels of the proinflammatory cytokine TNF-α and then plasma nitrite and nitrate as stable metabolism products of nitric oxide were measured. Haemodynamic studies were performed to demonstrate the hyperdynamic circulatory changes in the PPVL model. Finally, attempts were made to manipulate these changes pharmacologically. The pharmacological reagents used included the antioxidant N-acetylcysteine, the matrix metalloproteinase inhibitor BB1101, which is also known to inhibit TNF-α secretion, and an inhibitor of NF-κB activation, pyrrolidine dithiocarbamate.

CHAPTER 2: MATERIALS AND METHODS

2.1.1 Preparation of Animals.

Male Sprague Dawley rats weighing 280-330g were obtained from the comparative biology unit (CBU), Royal Free Hospital School of Medicine. All animals were housed in the CBU on a normal rodent chow diet (expanded SDSRM1, Witham, UK) with a light cycle of 12 hours on and 12 hours off, at a temperature of 19-23°C, and humidity of ~50%. The animals were housed in plastic cages except during 24 hour urine collections when they were placed in metabolic cages. The rats were allowed free access to food and water except during the urine collections when they had no access to food for 6 hours prior to and during the 24 hour collection period. Urine collections were made at baseline and on day 14 following PPVL or sham procedure. All animal experiments were conducted according to Home Office Guidelines.

Animals were used for either harvesting of blood and tissue or for haemodynamic studies. No animals were used in more than one study since blood and tissue variables may have been altered by recurrent injection of the thermal indicator during the haemodynamic studies.

All animals were sacrificed by exsanguination from the inferior vena cava on day 14 under deep terminal anesthesia with intraperitoneal (i.p.) sodium pentobarbitone (Rhône Mérieux Ltd, Harlow, UK). The blood was collected into tubes containing EDTA and centrifuged at 4°C. The liver tissue was harvested and snap frozen in liquid nitrogen. All plasma, urine and liver specimens were stored at -70°C until analysis.

2.1.2 Induction of Portal Hypertension by Partial Portal Vein Ligation.

PPVL was carried out as previously described (Chojkier and Groszmann, 1981). In brief, following anaesthesia with intramuscular hypnorm (Janssen Pharmaceuticals, Oxford, UK) and i.p. diazepam (Dumex Ltd, Tring, UK), the portal vein was isolated and a ligature (Mersilk 3/0, Ethicon, Edinburgh, UK) was placed around the vein and a blunt-tipped 20-gauge needle lying alongside the portal vein. The needle was then removed to allow restricted re-expansion of the portal vein with a calibrated stenosis. A sham operation was performed using the same procedure with the exception that no ligature was placed around the portal vein.

2.1.3 Administration of Pharmacological Agents:

N-acetylcysteine: This was administered by twice daily intraperitoneal injection of 120mg/kg (Parvolex) with the volume made up to 0.6 ml with 0.9% saline. The dosing regime was determined by provisional studies in the laboratory. The dosing was commenced two days prior to surgery and continued until day 14. Twenty animals received N-acetylcysteine including 6 sham and 14 PPVL animals. Two of the PPVL animals died prematurely during the haemodynamic studies. No other adverse effects were observed.

BB-1101(2S-allyl-N^1-hydroxy-3R-isobutyl-N-(1S-methylcarbamoyl-2-phenylethyl)-succinamide): This agent was kindly donated by British Biotech for experimental work. This was suspended in a vehicle of 0.01% Tween-80 in phosphate buffered saline using a sonic probe and administered at a concentration of 2mg/kg. Although it is insoluble studies, performed by British Biotech, have shown that it does have biological activity. 22 animals received BB-1101 including 8 sham animals and 14 PPVL animals. Two of the six animals receiving BB-1101 treatment for tissue/blood harvesting died prematurely explaining why only 4 animals were studied in this group. One of the eight animals used for haemodynamic study also died prematurely.

Pyrrolidine Dithiocarbamate: This was obtained from Sigma and was dissolved in water (10 mg/ml). It was administered by gavage at a dose of 200 mg/kg. This dosing regime and mode of administration was used by Sakurai et al. Sakurai et al. 1996) and was found to effectively inhibit NF-κB activation in a model of experimental glomerulonephritis. 20 animals received treatment with pyrrolidine including 6 PPVL animals for blood/tissue harvesting, 8 PPVL animals for haemodynamic study and 6 sham animals for haemodynamic study. 2 PPVL animals died prematurely during the haemodynamic studies.

Placebo groups: These animals received 0.6 ml of 0.9% saline by intraperitoneal injection on a twice daily basis. Sixteen were used for haemodynamic study and two out of the eight PPVL animals died prematurely.

2.2 Demonstration of Lipid Peroxidation in the Experimental groups:

2.2.1.Measurement of urinary F_2-isoprostanes

Free F_2-isoprostanes in urine were extracted and quantified using stable isotope dilution gas chromatography mass spectrometry (GCMS) method as described by Morrow et al. with the exception that a 30m DB 1701 GC column (J and W Scientific Inc., Folsom, California, USA) was used (Morrow and Roberts, 2nd. 1994). The principle of the assay is to purify the F_2-isoprostanes by solid phase extraction and thin layer chromatography followed by conversion of F_2-isoprostanes which contain a ring structure, a carboxylic acid group and three hydroxyl groups in the specimen into a purified volatile form which is able to capture electrons and which is able to elute and be separated from other peaks by passage through the gas chromatography column of the mass spectrometer. It is therefore necessary to extract, purify and derivatise the molecule into this suitable form (figure 7).

Urine samples (0.5 ml) were spiked with 1ng of deuterated internal standard of [D4]-8-iso-PGF$_{2\alpha}$ and 5 ng of [D4]-PGF$_{2\alpha}$. The [D4]-8-iso-PGF$_{2\alpha}$ standard is identical to one of the major F_2-isoprostanes formed *in vivo* with the exception that it contains four deuterium atoms, and is distinguished from the corresponding F_2-isoprostane by virtue of being four mass units heavier. Since the deuterated 8-iso-PGF$_{2\alpha}$ is otherwise chemically identical to F_2-isoprostanes, then it will have similar losses and thus recovery through each of the extraction and derivatisation steps as the endogenous non-deuterated form. The [D4]-PGF$_{2\alpha}$ standard is added to help with the final identification of the peaks from the chromatogram. Following the addition of 5 ml of pH 3.0 water, samples were extracted on tC18 Sep Pak cartridges (3ml size) (Waters, Watford, UK). The cartridges were then washed with 6 ml of pH 3.0 water followed by heptane to elute polar and non-polar compounds before elution into liquid phase by 6 ml of heptane:ethyl acetate:methanol (40:50:10). Following evaporation under nitrogen the samples were resuspendend in 80 μl of ethanol and then 3 ml of ethyl acetate. Then the samples were then loaded onto a silica Sep Pak cartridge (3ml size) (Waters, Watford, UK) preconditioned with ethyl acetate before further washing with ethyl acetate. Isoprostanes were eluted with 8 ml of ethyl acetate:methanol (60:40), dried under nitrogen, and resuspended in 40 μl of chloroform:methanol (2:1).

silica Sep Pak

↓

thin layer chromatography 1

↓

Derivatisation of -COOH group to form PFBR ester

↓

thin layer chromatography 2

↓

silylation of -OH groups with BSTFA to form TMS derivative

↓

Gas chromatography mass spectometry
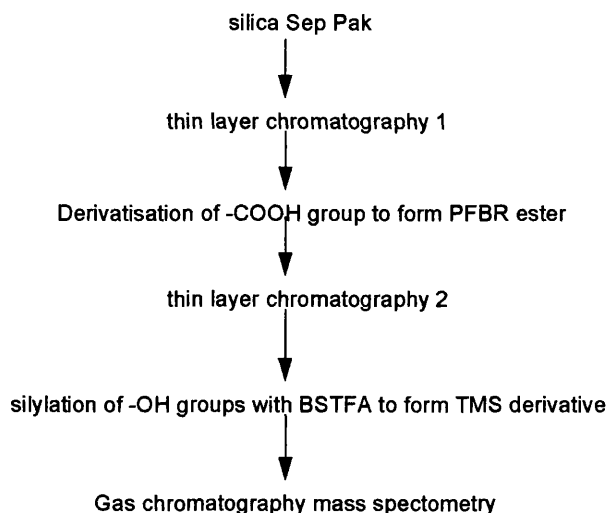
Figure 7.Outline of F_2-isoprostane Assay

The TLC plates (Whatman Silica Gel 60A, Linear K6D, 5 x 20 cm, 250 µm thick) were pre-run in methanol and then dried. Specimens were loaded onto each lane of the plate and 5 µg of $PGF_{2\alpha}$ was loaded onto a separate plate before placement of all plates into a TLC tank to determine the running positions of the F_2-isoprostanes and $PGF_{2\alpha}$ containing a mixture of chloroform:methanol:water:glacial acetic acid (86 ml:14 ml: 0.8 ml:1 ml). The presence of acid suppresses ionisation of the carboxylic acid group and facilitates chromatography. The solvent front was run to 13 cm and the position of the $PGF_{2\alpha}$ was confirmed on the standard plate by spraying the $PGF_{2\alpha}$-TLC plate with 10% phosphomolybdic acid in ethanol followed by heating the plate to approximately 100 to 150°C until a blue band developed. Previous studies in the laboratory had established that 8-iso-$PGF_{2\alpha}$ runs to ≈ 5.5 cm and $PGF_{2\alpha}$ runs to ≈ 5.8 cm (figure 8). The TLC lanes in which the samples were run were then scraped 1 cm above and 1.2 cm below the running position of the standard and collected into eppendorf tubes. Prostaglandins were then extracted from the silica by the addition of ethyl acetate:ethanol (50:50). After vortex mixing, shaking for sixty minutes and centrifugation, the supernatant, containing the extracted prostaglandins, were aspirated and dried under nitrogen. The carboxylic acid group was then derivatized to the pentafluorobenzyl ester by the addition of 20 µl of DIPEA (10% in acetonitrile) and 40 µl of PFBR (10% in acetonitrile) and the samples left at room temperature

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for 30 minutes before drying down under nitrogen. The DIPEA provides an alkaline but organic and volatile medium for the reaction. The PFBR group serves two purposes. It renders the molecules more volatile thus allowing passage down the GC column and the fluorine atoms in a five-membered ring structure provide an ideal electron capturing group. The PFBR ester of the F_2-isoprostanes were then purified by a second TLC step as above using chloroform:ethanol (93 ml: 7 ml). To determine the running position of the F_2-isoprostane-PFBR esters the isopropyl ester of the $PGF_{2\alpha}$ (5μg) was used. This has been shown in our laboratory to migrate to ≈ 3.4 cm compared with the 8-iso-$PGF_{2\alpha}$-PFBR ester which migrates to ≈ 3.2 cm (figure 9). Thus, samples were obtained by scraping 1 cm above and 1.2 cm below the running position of the ester collecting into eppendorf tubes, extracting from the silica using ethyl acetate:methanol (50:50) and centrifugation. The supernatant was aspirated and placed into a flat bottomed chromocol vial and dried under nitrogen. Samples were then derivatised with 10 μl of anhydrous DMF and 20 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA) to give the trimethylsilyl derivative. That is, each of the three hydroxyl groups had a trimethylsilyl group added. This step facilitates the passage of the derivatives down the GC column since the presence of the hydroxyl groups render the compound too polar. The specimens were then dried down under nitrogen, resuspended in 15 μl undecane and transferred to conical chromocol vials and 4 μl of sample was injected. Ions were then monitored at 569 and 573 (base ion -181) using a VG TRIO 1000 (Fisons, Manchester, UK) mass spectrometer coupled to a Carlo Erba GC 8000 Gas Chromatograph (Fisons, Manchester, UK) in negative CI mode using ammonia as the reagent gas. The GC gradient was 190° C for one minute followed by 20° C/minute until 300°C where it was maintained for 6 minutes. The injector was set at 250° C, interface at 280°C and the source at 200° CA typical mass spectrometry trace is shown in figure 10. The concentrations in each specimen were calculated from the tracing using the following equation:

$$\text{CONCENTRATION} = \frac{(\text{AREA OF SPECIMEN}) \times (\text{AMOUNT OF INTERNAL STANDARD ADDED})}{(\text{AREA OF DEUTERATED STANDARD}) \times (\text{VOLUME OF SPECIMEN})}$$

Historically, the height is used for measurement as described originally by Morrow et al. but as detection has become more sensitive with improvements in the purifcation steps the area under the peaks have been used.

This enabled the 24 hour excretion to be determined using the measured urinary volume excreted over the 24 hour period. Results were then corrected for body weight and expressed as $pg.day^{-1}g^{-1}$.

2.2.2 Measurement of Plasma F_2-isoprostanes

This method was similar to that employed for determination of urinary F_2-isoprostanes except less deuterated internal standard was added (0.4 ng of [D4]-8-iso $PGF_{2\alpha}$ and 2 ng of [D4]-$PGF_{2\alpha}$) and a single thin layer chromatography stage was employed following the derivatisation to the PFBR ester. This simpler extraction procedure reflects the fact that urine contains many potentially contaminating substances compared to plasma. Results were expressed as pg/ml.

2.2.3 Measurement of Tissue F_2-isoprostanes

To determine the component of membrane bound isoprostanes in liver it was necessary to homogenise a known weight of tissue (approximately 0.1-0.4 g). In liver tissue the F_2-isoprostanes are esterified and bound to lipids (predominantly membrane). It was therefore necessary to initially extract the lipids from homogenised liver into chloroform. The esterified F_2-isoprostanes were subsequently cleaved by base hydrolysis. Although this could be performed by phospholipase A_2, it was impractical. Therefore, a base hydrolysis was performed using 15% methanolic potassium hydroxide. This was a suitable alternative method as it is cheap, effective and the compounds are stable in the solution. Free F_2-isoprostanes were then extracted and derivatised as for plasma.
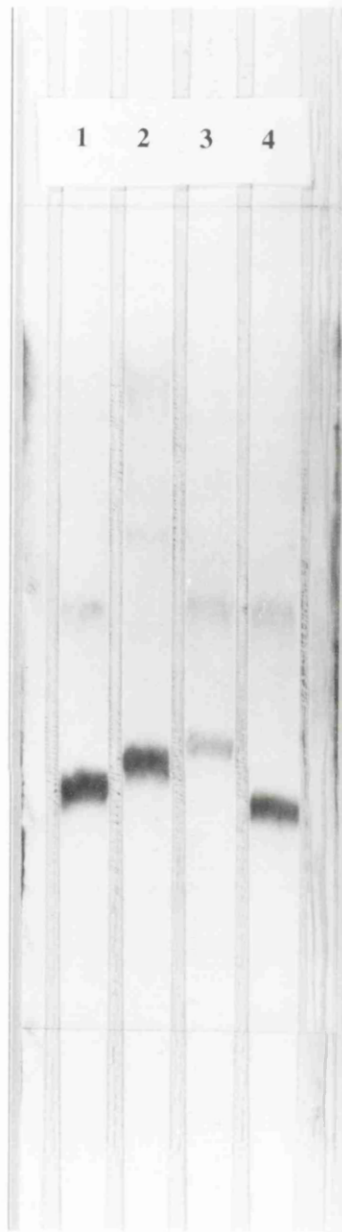
Figure 8. Thin Layer Chromatography Stage 1. This shows the running position of different standards following the first thin layer chromatography stage and development. The standards are as follows: 1) 9α-, 11β- $PGF_{2\alpha}$ 2) Isopropyl $PGF_{2\alpha}$ 3)$PGF_{2\alpha}$ 4) 8-iso $PGF_{2\alpha}$. During the assay the running standard used is $PGF_{2\alpha}$ which runs to approximately 5.8 cm and the isoprostanes (8-iso- $PGF_{2\alpha}$) run to approximately 5.5 cm. the specimen plates are therefore scraped 1 cm above and 1.2 cm below the running position of $PGF_{2\alpha}$.
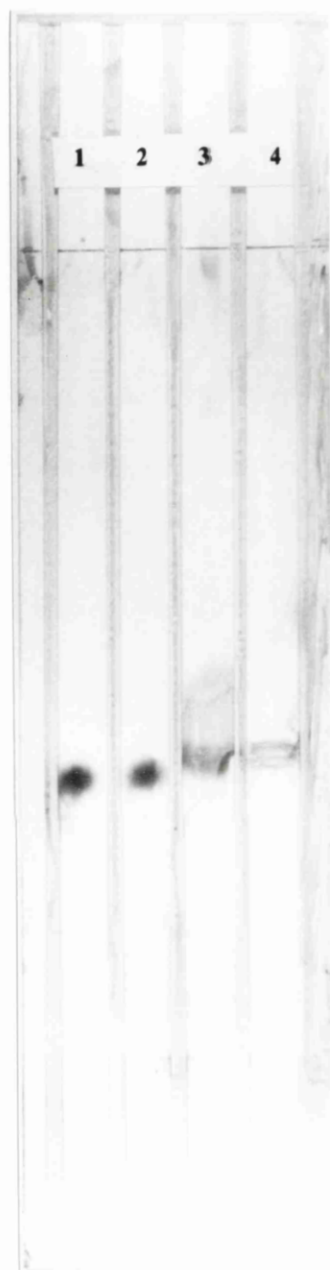
Figure 9. Thin Layer Chromatography Stage 2. This shows the running position of the PFBR ester derivative of 8-iso-PGF$_{2\alpha}$ (lanes 1 and 2) compared to the isopropyl ester of PGF$_{2\alpha}$ (lanes 3 and 4). During the assay the usual running position of the isopropyl ester is approximately 3.4 cm compared to the 8-iso-PGF$_{2\alpha}$ derivative which runs to approximately 3.2 cm. The specimen plates are therefore scraped 1cm above and 1.2 cm below the running position of the isopropyl PGF$_{2\alpha}$ standard.

Sample ID: N1322-Jan-1997In
Acquired 22-Jan-1997 at 23:38:5

BIMBI151   SIR of 2 Channels CI
                                                                        573.20
100   9.762   2.53e6
                                                      2409767   Height

%   9.435
             862890

                              9.668   9.892
                              211333   55257
2

BIMBI151   SIR of 2 Channels CI
                                                                        569.20
100   9.782   2.46e6
                                                      2362303   Height

■%   9.445
            1185180

                      9.542   9.682
                      27717   37215
3
9.350 9.400 9.450 9.500 9.550 9.600 9.650 9.700 9.750 9.800 9.850 9.900 9.950
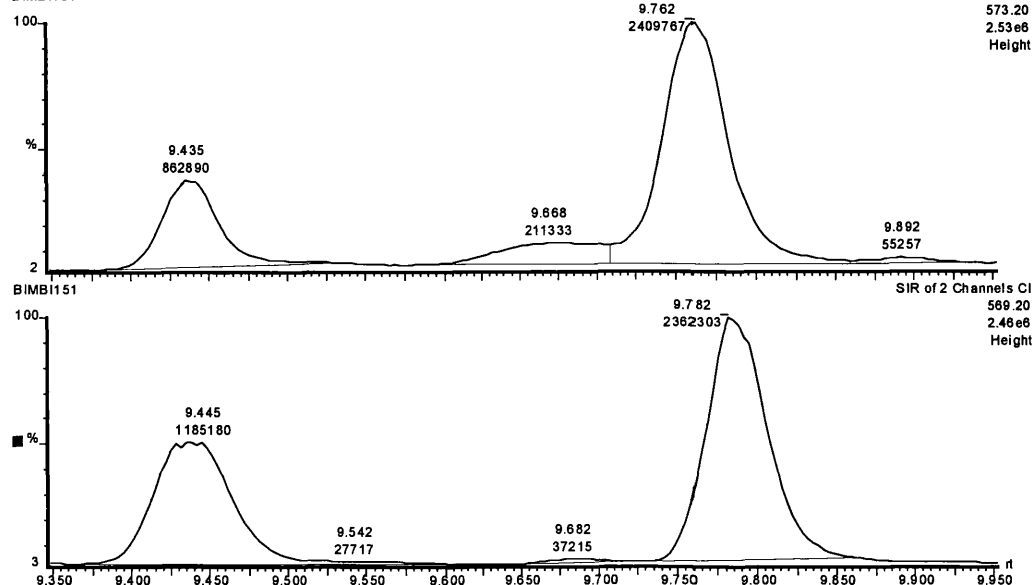
(a)

Sample ID: N1523-Jan-1997Inst
Acquired 23-Jan-1997 at 00:13:10

BIMBI153   SIR of 2 Channels CI-
                                                                        573.20
100   9.735   1.55e6
                                                      1461253   Height

%   9.412
             505536

                              9.635   9.652
                              33792   48647
4

BIMBI153   SIR of 2 Channels CI-
                                                                        569.20
100   9.765   3.03e6
                                                      2982313   Height

■%   9.415
            389356

                      9.665   9.889
                      25475   37333
1
9.350 9.400 9.450 9.500 9.550 9.600 9.650 9.700 9.750 9.800 9.850 9.900 9.950
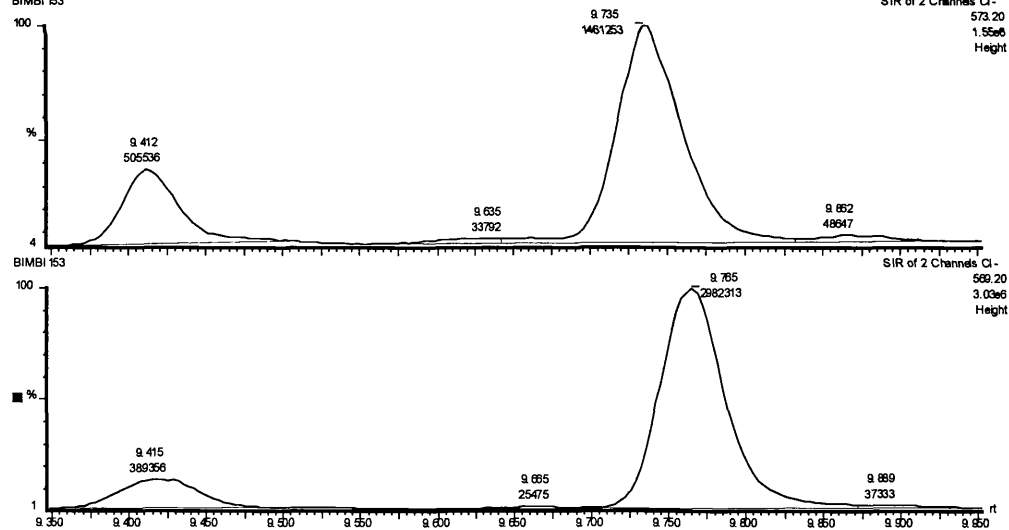
(b)

Figure 10. Mass Spectrometry Traces. Two examples are shown above. The upper panel on each trace corresponds to the peaks for 1 ng of deuterated 8-isoPGF$_{2\alpha}$ (left peak) and 5ng of deuterated PGF$_{2\alpha}$ (right peak) which were added to the specimen. The lower panel represents the unknown amount of 8-isoPGF$_{2\alpha}$ (left peak) and PGF$_{2\alpha}$(right peak) in the specimen which is calculated using the height (or area) in the formula described. Specimen (a) contains a greater amount of 8-isoPGF$_{2\alpha}$ compared to specimen (b).

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2.3 Demonstration of NF-κB in Liver Tissue from Experimental Groups:

NF-κB is normally present in the cytoplasm of cells. When NF-κB is activated the p50-p65 heterodimer migrates to the nucleus of cells and initiates transcription by binding to DNA. Therefore, extraction of nuclear protein and measurement of the p-50-p65 complex in the nuclei allows us to determine the extent of activation of NF-κB. The ability of the nuclear extract to bind a radiolabelled oligonucleotide consensus probe specific for NF-κB is determined by gel electrophoresis followed by autoradiography. This gives rise to bands on the gel which correspond to NF-κB but the position of these bands are confirmed using the supershift assay in which antibodies against the p-50 and p-65 subunits of the NF-κB molecule alter the mobility of the bands such that they occupy a different position. The outline of the whole assay is shown in figure 11. The three main stages of the assay are the nuclear extraction, the protein assay and finally the electromobility shift assay and the supershift assay and these will be described further.

2.3.1 Nuclear Extraction

The following solutions were made prior to nuclear extraction:

Buffer A :   10mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF

Buffer B: 0.1% Non-idet P-40 in buffer A

Buffer C: 20 mM HEPES, pH 7.9, 25% glycerol (v/v), 0.42 M NaCl, 1.5 mM
$MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 1 mM PMSF.

Liver tissue was treated using a modification of a method described by Manning *et al* (Manning et al. 1995). In brief, tissue samples of ~100 mg were rinsed with ice-cold Ca^{2+}- and Mg^{2+}-free phosphate buffered saline. The tissue samples were homogenized in 3 ml ice-cold buffer B using a Kontes tissue grind pestle (Vineland, New Jersey, USA). The homogenate was transferred to a polypropylene centrifuge tube and, after a 10 minute incubation period on ice, was centrifuged at 850 x g for 10 minutes at 4 °C. The cell pellet was then suspended in ice-cold buffer A, incubated on ice for 10 minutes and then recentrifuged as above. The crude nuclear pellet was rinsed again with buffer A, incubated for a further 10

minutes before recentrifugation as before. Following this the pellet was resuspended in 50-100 μl of buffer C and incubated on ice for 30 minutes. The nuclear protein was then extracted following centrifugation at 100,000 x g for 20 minutes at 4 °C.

2.3.2 Protein Assay

Protein concentrations in the nuclear extracts were determined using the Bradford assay (Biorad Laboratories, Hemel Hempstead, UK) with bovine serum albumin as a standard (Sigma, Poole, UK). A standard curve was prepared by adding increments of BSA from 0 to 20 μg made up to a final solution of 100 μl with water into 1.5 ml cuvettes. 5 μl samples of nuclear extracts and 95 μl of water were added to separate cuvettes in duplicate. To each cuvette 2 ml of diluted Biorad reagent (1 in 5) were added before mixing and then reading the absorbance at 543 nm. A standard curve was then prepared and this enabled the sample concentrations to be determined and diluted to a standard concentration of 0.5 mg/ml (figure 12).

Homogenise tissue

↓

Nuclear protein extraction

↓

Nuclear protein quantifiication

↓

Electrophoresis

↓ ⟶ Supershift Assay

Autoradiography

↓

Densitometry
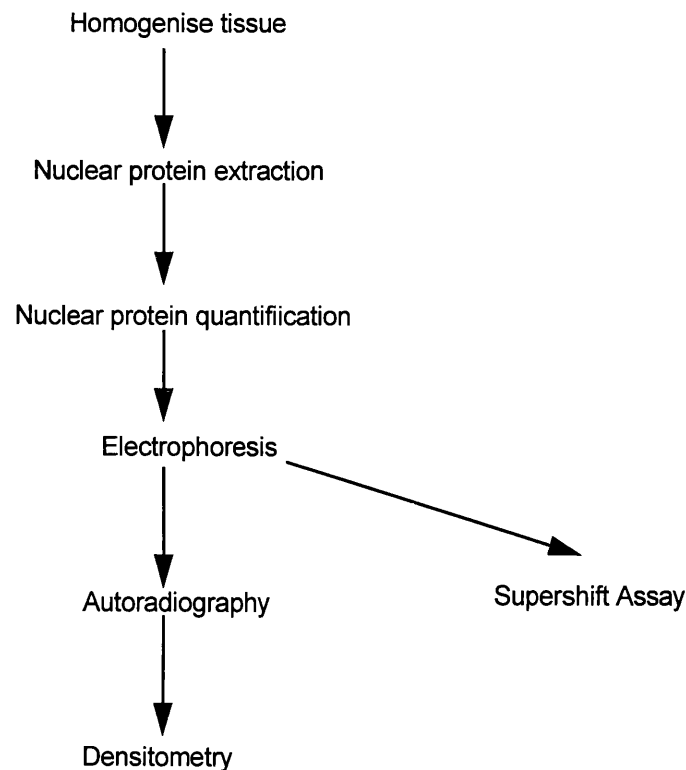
Figure 11. Outline of NF-κB Assay. This figure outlines the stages in the electromobility shift assay from whole tissue. The supershift assay is performed to confirm that the bands on the gel correspond to NF-κB.

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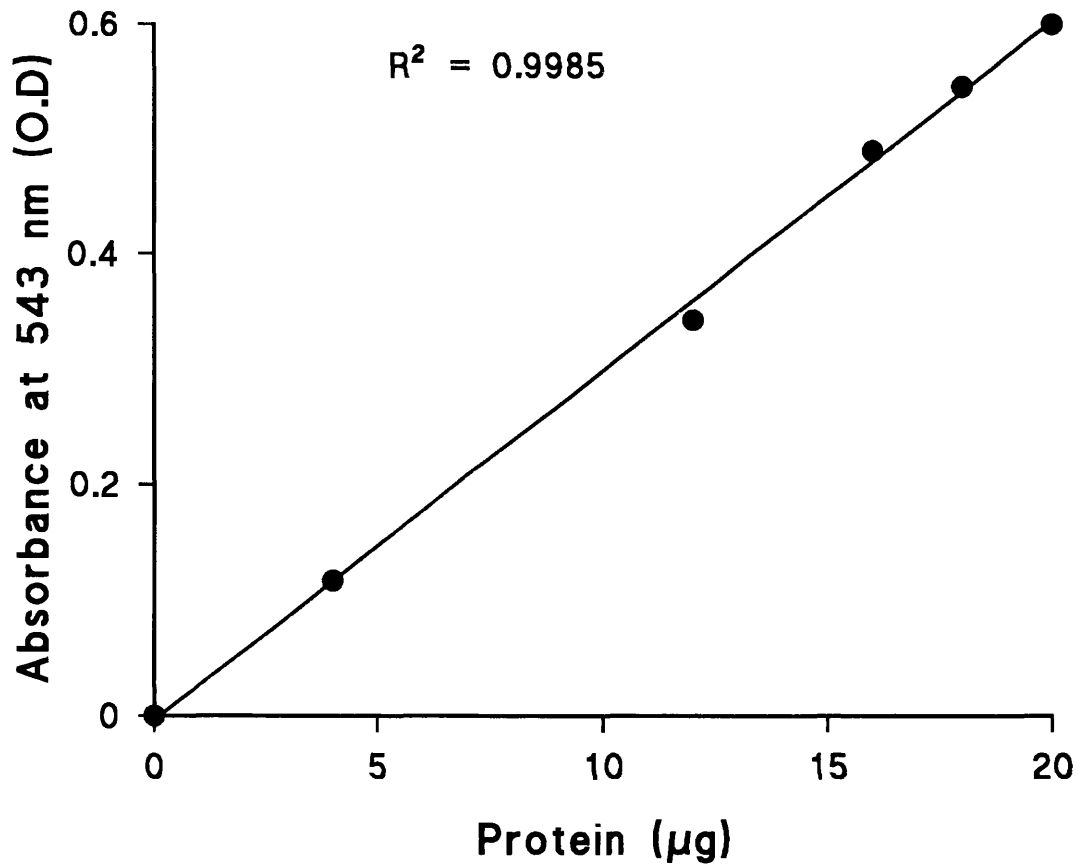
Figure 12. Standard curve for Protein Assay. Increments of BSA were made up to a final solution of 100 μl with water prior to the addition of 2ml of diluted Biorad reagent.

2.3.3 Electromobility Shift Assay and Supershift Assay

Antibodies cross-reactive to rat p50, p65 (relA), subunits of NF-κB were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). The double stranded NF-κB consensus oligonucleotide probe (5'-AGTTGA<u>GGGGACTTTCC</u>CAGGC-3') or the E-selectin NF-κB probe (5'-AGCTTAGA<u>GGGGATTTCC</u>GAGAGGA-3') were end labeled with $\gamma[^{32}P]ATP$ (10 µCi at 222 Tbq/mmol (Amersham International PLC, Buckinghamshire, UK). Binding reactions, containing 35 fmol ($\sim 1 \times 10^5$) of oligonucleotide and 3 µg of nuclear protein were conducted at room temperature for 20 minutes in a total volume of 10 µl binding buffer (10mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol (v/v), and 0.5 µg poly(dI:dC). Identity of the bands were confirmed by competition reactions in which unlabelled oligonucleotide was added 5 minutes before addition of radiolabelled probe. For supershift analysis, 1 µg of each antibody, p-50 or p-65 antibody, was added to the reaction mixtures immediately before addition of the radiolabeled probe (figure 13). Following the binding reactions, 1 µl of 10 x gel loading buffer was added and the reaction was subjected to non-denaturing 7% PAGE in low ionic strength buffer (45 mM Tris-borate, 1 mM EDTA) at 75 mV/8 mA for approximately 3 hours at 4°C. Gels were then vacuum dried and exposed to X-ray film (Hyperfilm MP, Amersham International PLC, Amersham, UK) at -70 °C. The density of the band corresponding to NF-κB on the gel was quantified by using an imaging densitometer (Biorad Laboratories, Hemel Hempstead, UK) and related to a constant HELA standard which was run with each gel. Results were expressed as optical density relative to one tenth of the optical density of the HELA standard (Rel.O.D). After I had prepared the nuclear extracts and protein assay, the electromobility shift assay and supershift assays were performed by Ms Radhika Anand in the host laboratory. In these series of experiments the supershift assays were performed on livers from rats which had undergone bile duct ligation instead of PPVL livers since these were being processed simultaneously by my co-workers. Since the purpose of the supershift assay was to confirm that the bands on the gel corresponded to NF-κB it was not felt necessary to repeat the supershift assay specifically for the PPVL livers since the gel band positions were identical.

SUPERSHIFT ASSAY
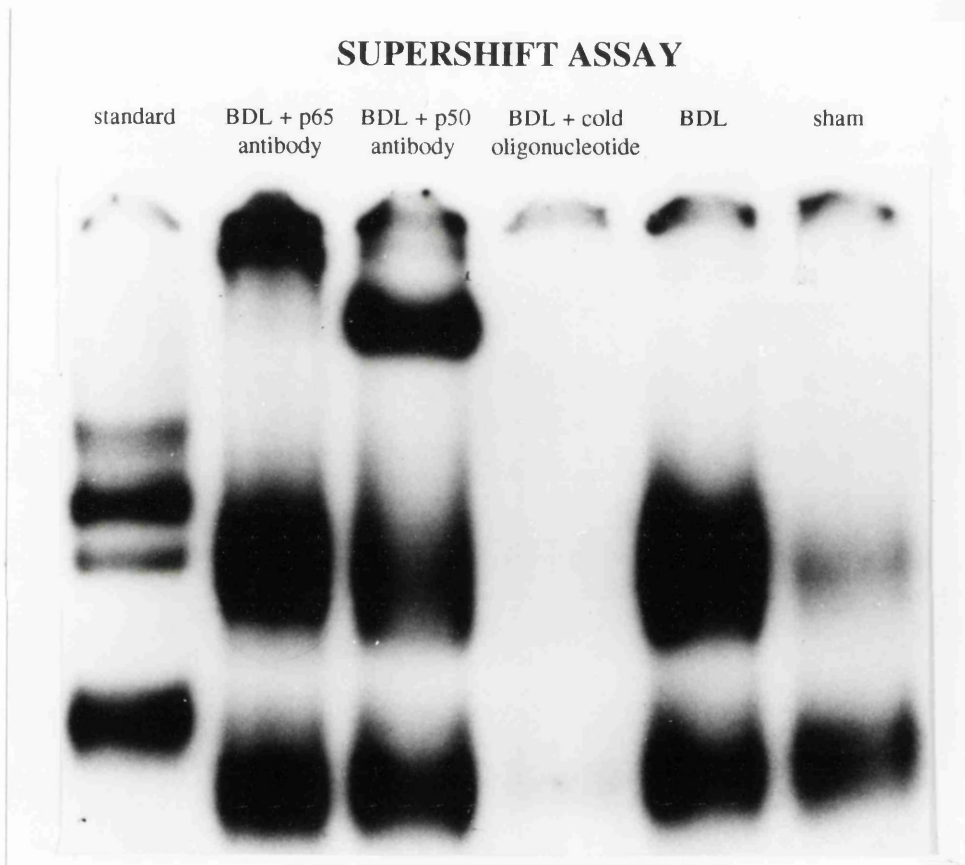
Figure 13. Characterisation of NF-κB complexes activated following surgery. NF-κB DNA-binding activity were measured in the nuclear extracts from liver by the EMSA using a ^{32}P-labelled NF-κB oligonucleotide probe. Competition experiments were performed on unlabelled NF-κB at 100x molar excess. In the supershift experiments antibodies for the p50 and p-65 were used at 1 μg per assay to provide confirmation that the band corresponds to NF-κB. This supershift assay was performed on rats undergoing bile duct ligation.

2.4 Demonstration of Tumour Necrosis Factor in Experimental Groups

Plasma TNF-α ELISA

TNF-α levels were measured in plasma specimens using two separate commercial ELISA kits. These were from Genzyme and later with an ultra-sensitive ELISA kit manufactured by Biosource International (Camarillo, California, USA). In principle an antibody specific for TNF-α is coated onto the wells of the microtitre strips. Samples are then pipetted into these wells. During the first incubation, the TNF-α binds to the immobilised antibody on one site. Following washing, a biotinylated secondary antibody specific for TNF-α is added. During this incubation, this antibody binds to the immobilised TNF-α captured during the first incubation. Following removal of the excess second biotinylated antibody, an enzyme Streptavidin-Peroxidase is added. This binds to complete the four member sandwich. After a third incubation and washing to remove all of the unbound enzyme, a substrate solution is added which is acted upon by the bound enzyme to produce colour. The intensity of the colour product is directly proportional to the concentration of TNF-α in the original specimen. The assays were performed by Dr Steve Holt in the host laboratory.

2.5 Demonstration of Nitrite/ Nitrate in Plasma from Experimental Groups

2.5.1 Griess reaction

Plasma and urinary levels of nitrite and nitrate were determined using a modification of Grisham's method (Grisham et al. 1996) in a microtitre assay. The principle of the Griess reaction is that nitrite reacts with Griess reagent to produce a red colour. At low nitrite concentrations, the colour intensity following the Griess reaction is proportional to the concentration of nitrite. Nitrate may also be reduced to nitrite using nitrate reductase prior to the reaction such that the total nitrite and nitrate concentration is measured.

Three solutions were made up prior to assay:

Solution A:	5.0 ml water,500 μl 1M HEPES buffer (pH 7.4)(Sigma) (kept at 4°C), 500 μl 0.1mM Flavin adenine dinucleotide, disodium salt (FAD: Sigma),1 ml of 1mM Nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt (NADPH;Sigma);
Solution B:	1ml of 100mM Pyruvic acid, sodium salt, Type II (Sigma). 0.5ml of 500 U/ml Lactate dehydrogenase (LDH; bovine muscle, Sigma); Aspergillus Nitrate reductase 2 U/ml (kept on ice until use)
Griess reagent:	(equal volumes of 0.2% (w/v) napthyleneethylenediamine and 2% (w/v) sulfanilamide in 5% (v/v) phosphoric acid.

70 μl of solution A was pipetted into each well before adding 40 μl of each test specimen. Each sample was run in duplicate. This included the sodium nitrate standards which were at a concentration of 0 to 160 μM. 10 μl of *Aspergillus* nitrate reductase was then added and incubated for 30 minutes at 37°C. Following this 15μl of solution B was added to oxidise any unreacted NADPH and incubated further for 10 minutes at 37°C. 200 μl of Griess reagent was then added and left at room temperature for 10 minutes. The absorbance of each sample was determined at 543 nm using a microtitre plate reader. Since all nitrate is converted to nitrite the assay measures total nitrite and nitrate.

As is apparent from the standard curve (figure 14) at concentrations below 20 μM the optical densities are quite low and therefore subjected to less accuracy.
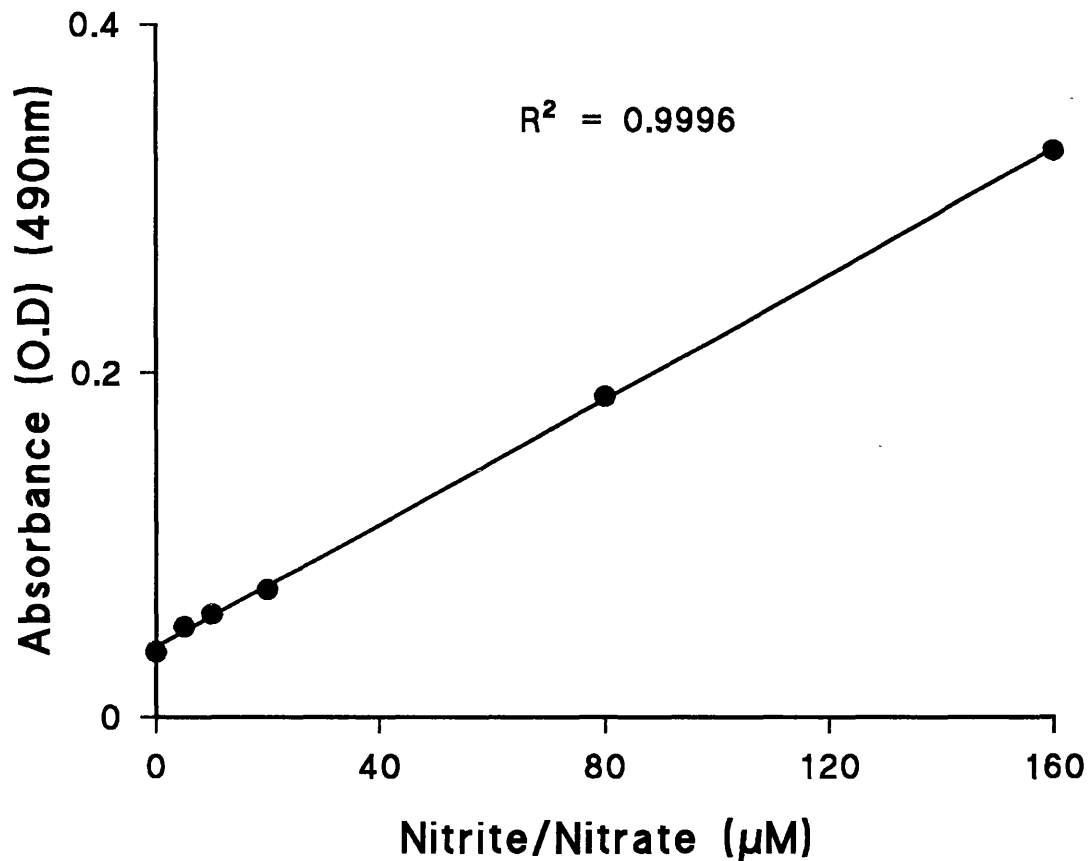
Figure 14. Standard curve for Total Nitrite/Nitrate levels using the Griess reaction. Sodium nitrate standards were used. Since all the nitrate is converted to nitrite by the nitrate reductase the assay measures total nitrite and nitrate.

2.5.2 Chemiluminescence

Since plasma concentrations, as determined by the Griess reaction were low (5-20 μM) and, since the colour of the plasma may have influenced the colorimetric assay, the results using this method were questionable. To confirm results obtained using the Griess reaction, a second method to measure plasma nitrite and nitrate levels was employed. This used nitric oxide released from nitrite and nitrate and detected by chemiluminescence using an nitric oxide analyzer (Sievers Research Inc, Boulder, Colorado, USA). Chemiluminescence is a process whereby a chemical reaction results in emission of light. The emission of light can be

enhanced and multiplied using a photomultiplier tube before detection. The nitric oxide analyser makes use of the following reaction between nitric oxide and ozone which occurs in gas phase:

$$NO + O_3 \rightarrow NO_2^* + O_2$$
$$NO_2^* \rightarrow NO_2 + \text{emitted light.}$$
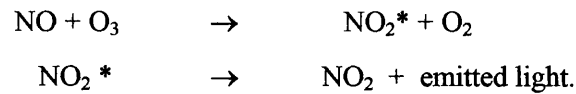
Both nitrite and nitrate were reduced to NO in refluxing vanadium chloride in hydrochloric acid. The NO produced was then purged from the refluxing solution by nitrogen and reacted with ozone before analysis by chemiluminescence (Ignarro et al. 1993). This is a highly sensitive and accurate method for detecting NO and the quantification of plasma nitrite and nitrate (Archer, 1993).

2.6 Haemodynamic Studies

2.6.1. Development and Validation of Thermodilution Method for Cardiac Output Measurement

Cardiac output was measured by thermodilution in which the flow rate is determined by injecting a known volume of a known thermal indicator and monitoring the temperature change using a thermistor. This method is based indirectly on the Fick principle. The budget for this thesis did not allow the purchase of a commercially available cardiac output computer for this purpose and therefore, a thermodilution technique for cardiac output measurement making use of the Maclab system was developed. This is an integrated system of computer hardware and software that is designed to record and analyse experimental data.

The development was performed in three stages:

1) Demonstration of Thermodilution Curves using the Maclab system

The first aim was to determine whether the Maclab computer would be able to detect rapid changes in temperature to produce a thermodilution curve. A Swan-Ganz catheter was obtained and connected to a Wheatstone bridge designed and made by Dr Roy Smith of the Department of Medical Physics, Royal Free Hospital and connected to a bridge amplifier which transmitted the thermal signal onto the computer monitor (figure 15). The thermistor was then placed into

the peripheral tubing of a cardiac output simulator with adjustable flow rates at similar temperatures to those of animals. Fine bore silicone tubing was connected to a pump, flow buffer and water bath as shown in figure 16. The water bath enabled the temperature of the flowing water within the tubing to be maintained at approximately 37°C. The pump was then adjusted to allow a wide range of flow rates to be assessed and the flow buffer was essentially placed within the circuit to allow the flow to be smooth. A thermal indicator was injected upstream of the thermistor to see if thermodilution curves could be produced by the thermistor which was connected to the Maclab system. Curves similar to those in figure 17 were displayed on the computer monitor confirming that rapid changes in temperature could be detected.

2)   Calculation of flow rates using a coefficient, k.

Following confirmation that the Maclab system was able to produce and display thermodilution curves the next stage was to demonstrate that flow rates could be determined from the curves using a coefficient, k.

Different flow rates were passed through the tubing and were measured directly by allowing the flowing water to pass into a measuring cylinder over one minute. A cold indicator at a temperature of approximately 15 degrees Celsius below that of the water within the tubing was injected upstream to the thermistor. This temperature change reflects that present for *in vivo* measurement (Animal core temperature, 37°C; room temperature, 22°C). This caused a typical thermodilution curve with a rapid upstroke and slow decay (figure 17). After calibrating the thermistor with numerous thermodilution curves at different known flow rates a coefficient, k, was obtained such that an unknown flow rate could be determined if the temperature of the perfusing fluid and the injectate were known from the equation.

$$\text{flow rate} = \frac{k. \text{ injectate vol. delta temp}}{\int \text{thermodilution curve}}$$

where k = coefficient for the thermistor

injectate vol. = volume of thermal indicator injected

delta temp = temperature difference between thermal indicator and perfusing fluid

∫thermodilution curve = area under thermodilution curve

Table 4 shows the calculated flow rates between 180 ml/min and 710 ml/min using the Swan-Ganz catheter. The calculated flow rate was determined from the equation using the mean coefficient value of 844 in the above equation. A curve was also plotted for the actual values versus the calculated values (figure 18).

Flow rate Ml/min	Temp diff. Degrees C	Volume ml	Integral Mv.s^{-2}	calc. factor	Coefficient	Calculated value ml/min
710	17	1	19.8	0.859	827	725
580	17	1	25.01	0.680	853	574
430	17	1	36.54	0.465	924	393
270	17	1	52.6	0.323	835	273
180	17	1	73.79	0.230	781	194
					mean coefficient = 844	

Table 4. Values calculated for flow rates compared with actual flow rates are shown. These were performed in a cardiac output simulator using a Swan-Ganz catheter.

3)   Modification and validation of the thermodilution method for use in rats

The final stage of the development was to produce a system which would allow measurement of cardiac output *in vivo* and more importantly to validate this method such that subtle changes in flow rates between different animals could be confidently measured. Thermistors were acquired from thermometrics and mounted in 22 gauge polyvinyl chloride catheter with an epoxy resin. The thermistor was then connected to a Wheatstone bridge and connected to a bridge amplifier which transmitted the thermal signal onto the computer monitor. The thermistor was then placed

into the peripheral tubing of the cardiac output flow simulator with adjustable flow rates and similar temperatures to those of small animals (figure 16).

This was tested extensively in the cardiac output simulator at two different flow rates which were more representative of the rat cardiac output values on three different days.

From this an intra-assay coefficient of variation of 2.9% ± 0.7%, and an inter-assay coefficient of variation of 4.9% ± 0.4% over the typical ranges for cardiac output in small animals (table 5). Following this validation it was felt that this thermodilution system was suitable to use in the anaesthetised rat.

	50ml/min				100ml/min			
	06/03/97	15/03/97	16/03/97	mean	03/03/97	06/03/97	16/03/97	mean
	55	52	53	**53**	96	106	101	**101**
	55	53	52	**53**	92	103	94	**96**
	51	53	55	**53**	97	104	92	**98**
	51	51	53	**52**	95	97	106	**99**
	50	50	53	**51**	98	99	95	**97**
	60	53	54	**56**	93	107	101	**100**
	52	57	53	**54**	98	103	94	**98**
	53	51	51	**52**	100	107	98	**102**
	49	58	49	**52**	108	98	96	**101**
	55	53	50	**53**	102	113	100	**105**
	55	54	50	**53**				
			51					
Mean	53	53	52		98	104	98	

Table 5. This demonstrates the intra and inter variability in calculated flow rates as determined using the cardiac output simulator. The simulator was set at 2 different flow rates and at least 10 values were obtained on three separate days. Each calculated flow rate was determined using the mean integral from three thermodilution curves. From these values the coefficient of variation (cv) was calculated by dividing the mean value by the standard deviation (Inter-cv = 4.9 % ± 0.4%, Intra-cv = 2.9% ± 0.7 %).

Maclab and Bridge Amplifier

Macintosh Computer

1
2
4
3
13 kΩ
13 kΩ
13 kΩ

thermistor probe

5
470 kΩ

8

7

Excitation voltage set to 5V

Ground

Pin out of 8 pin DIN connector
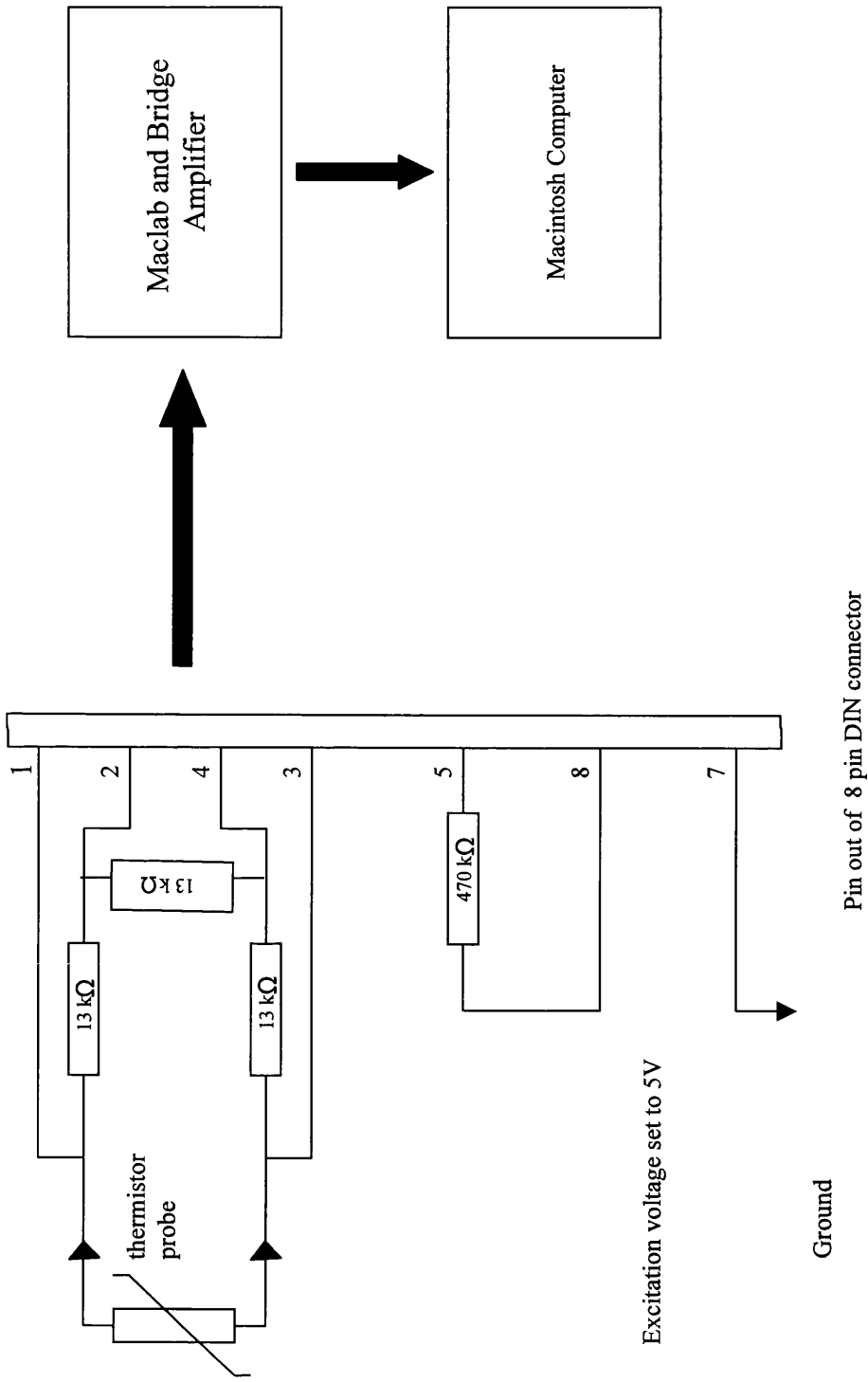
Figure 15. Connection of Thermistor to Maclab system and computer

Computer System

Injection site for thermal indicator

Flow buffer to smooth flow

Fine bore silicone tubing

Pump

Thermistor

Water bath at 37 °C
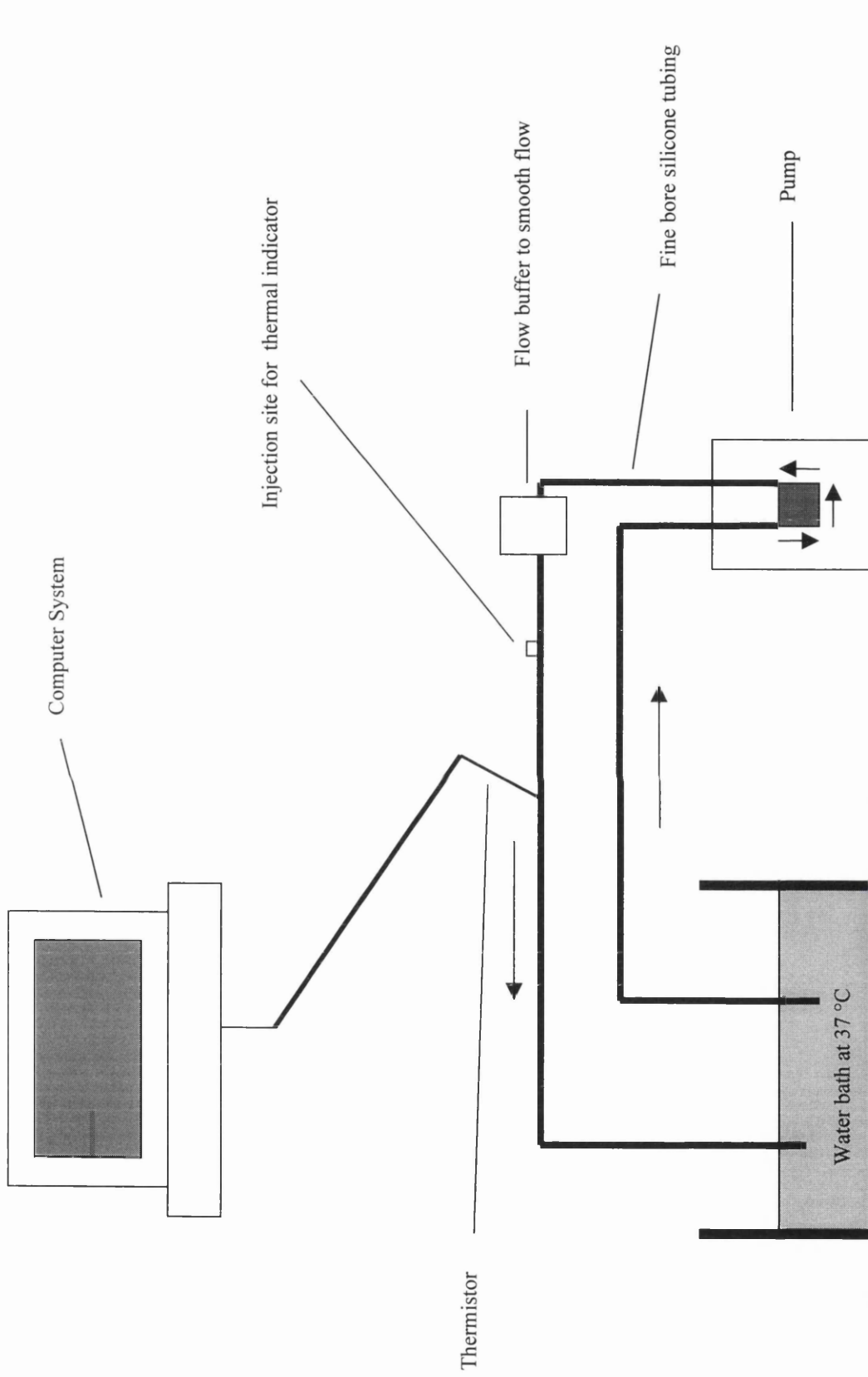
Figure 16 showing the set-up of cardiac output simulator to allow calibration of the thermistor. Thermodilution curves are obtained at different known flow rates as determined by direct measurement using a measuring cylinder at the end of the tubing.
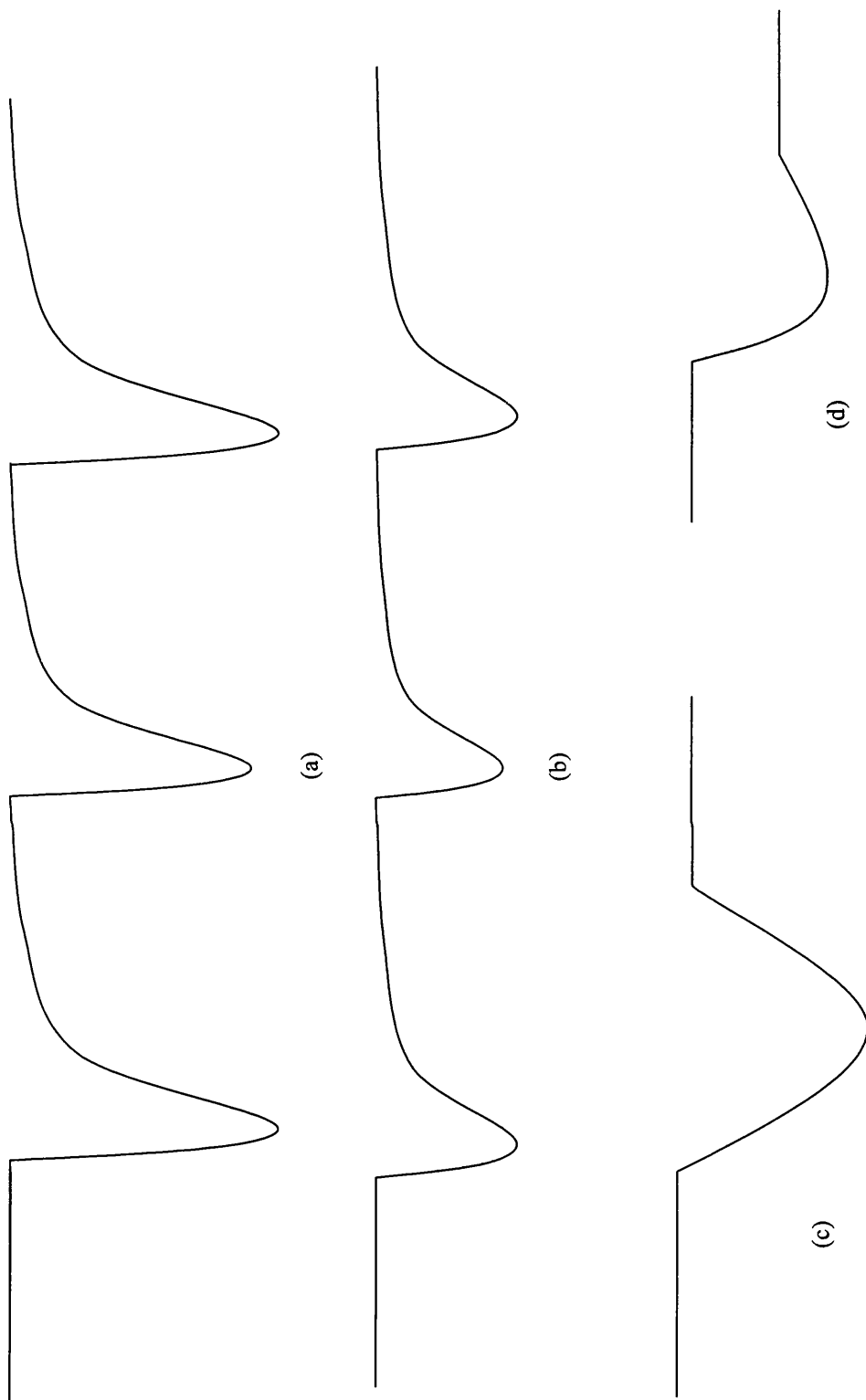
Figure 17. Thermodilution curves. A typical thermodilution curve should have a rapid downstroke with a slow steady return to baseline. (a) Large curves indicate a slower flow (b) smaller curves indicate a higher flow (c) indicates an abnormal curve with a sluggish downstroke which indicates that the thermistor is occluding the flow and is corrected by withdrawing the thermistor slightly. (d) indicates that the thermistor is not in far enough as the curve does not return to baseline due to pooling of the thermal indicator.
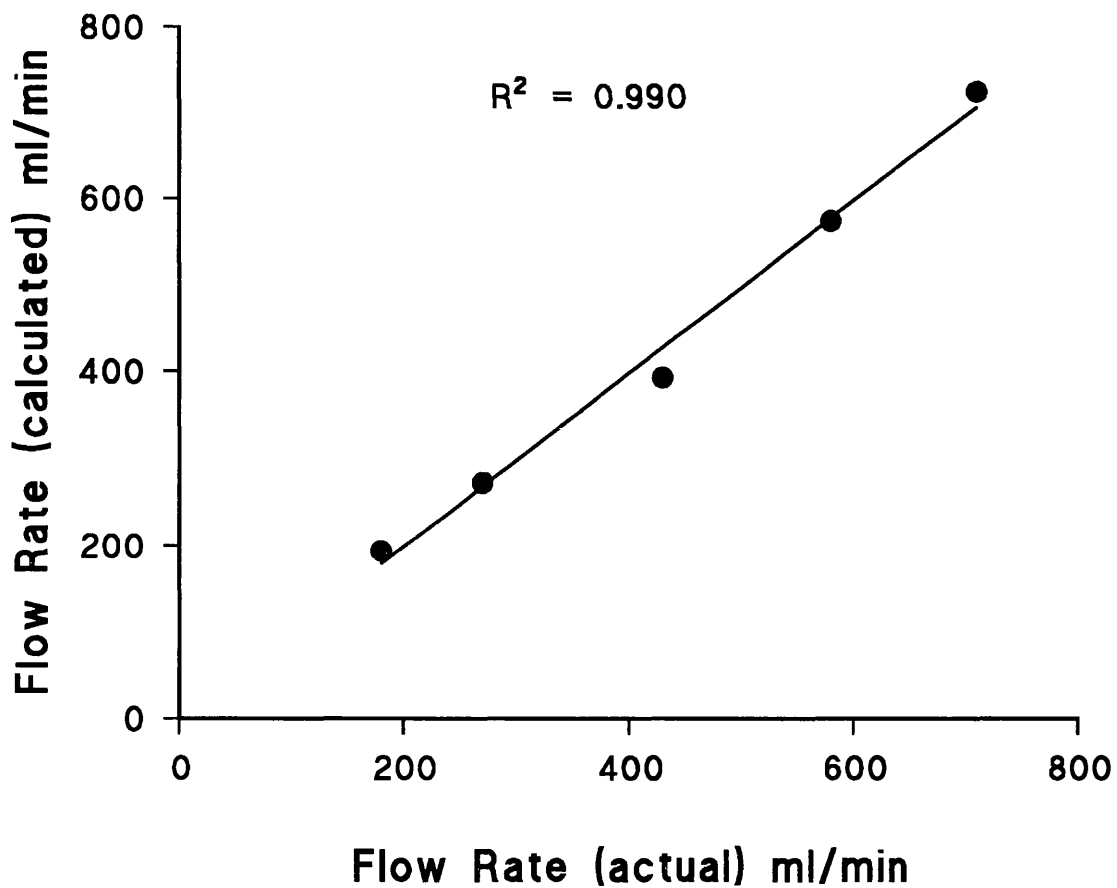
Figure 18. Graph showing calculated flow rates versus actual flow rates. Calculated flow rates were determined using the Maclab system on the cardiac flow simulator. Actual flow rates were determined by direct measurement into a measuring cylinder over a minute.

2.6.2 Haemodynamic Studies in Experimental Groups

Haemodynamic studies were performed in anaesthetised animals using sodium pentobarbitone (60 mg/kg) (figure 19).This has been shown by others to be a suitable anaesthetic since it has minimal effects on haemodynamics (Lee et al. 1985). To determine the cardiac output in small animals it was necessary to cannulate the external jugular vein with fine-bore tubing (PE-50) such that the tip was adjacent to the right atrium. The thermistor was placed in the carotid artery and advanced to the aortic arch. This gave the highest temperature reading on the thermistor trace. A thermal indicator was then injected into the jugular line and the thermistor trace was observed for the thermodilution curve. A typical curve had a rapid downstroke and slow return (figure 17). Atypical curves may show a slow downstroke and prolonged return, a sloping baseline or no curve at all. These could be corrected by placing the thermistor in a different position.

Mean arterial pressures were monitored by cannulation of the femoral artery with a 22 gauge Abbocath (Abbott laboratories). The femoral artery was carefully dissected free from the femoral vein and nerve before cannulation and the cannula was secured in place with mersilk ties. The cannula was connnected via tubing to a pressure transducer which was calibrated immediately prior to measurement with the zero reference point being the midportion of the rat (figure 19).

Portal pressure was measured by cannulation of the ileocolic vein, a tributary of the portal vein, with a 22 gauge Abbocath. The vein was carefully isolated in the mesentery before cannulation and once in place the cannula was tied in place. Again this was connected to a pressure transducer which was calibrated immediately before use.
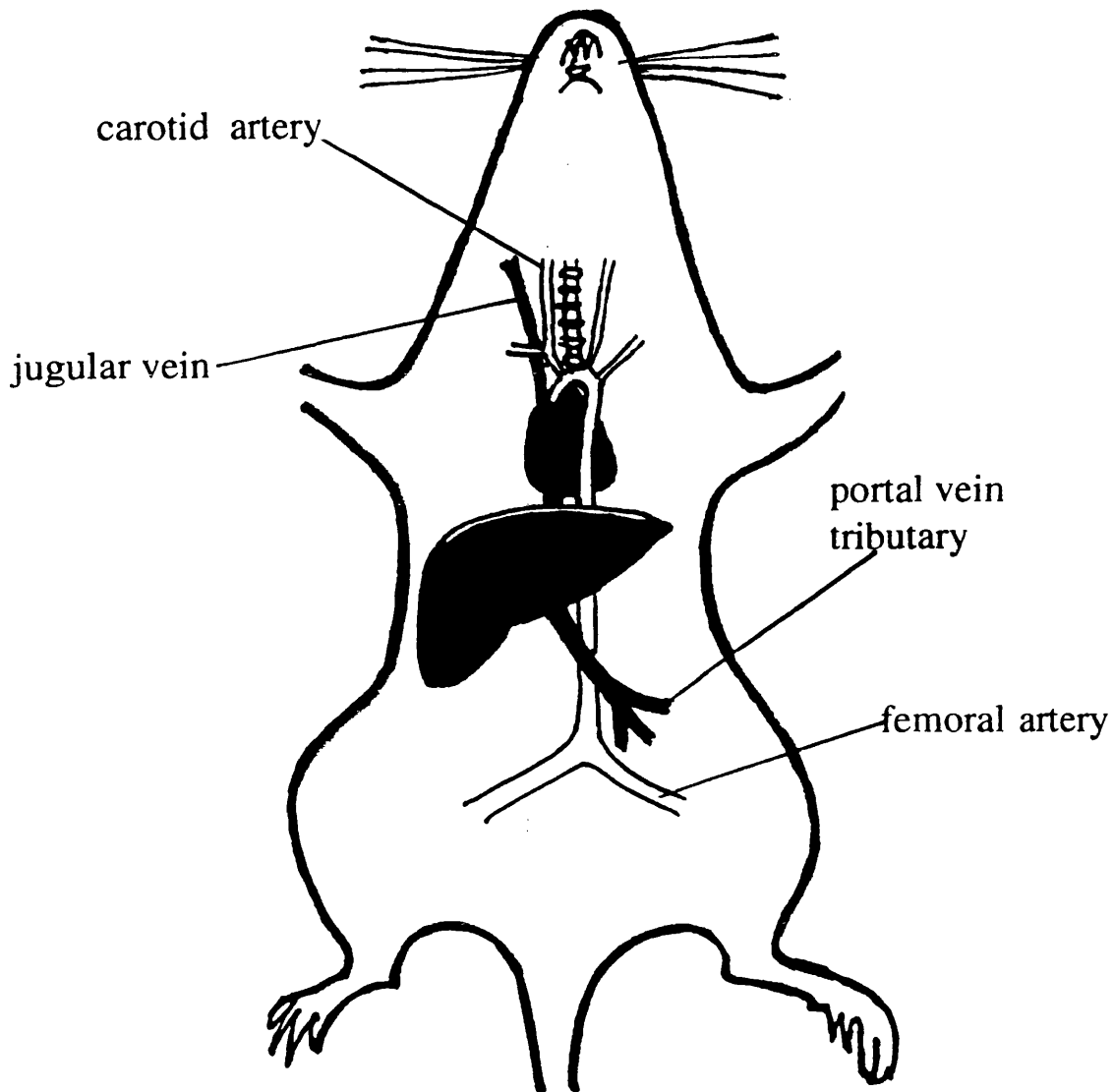
Figure 19. Animal undergoing Haemodynamic Studies. Cannulation of the jugular vein and carotid artery allowed cardiac output to be determined by thermodilution. Mean arterial pressure and portal pressure measurements were made by cannulation of the femoral artery and a tributary of the ileo-colic vein respectively. The core temperature of the animal was measured using a rectal thermometer and was maintained at 37 ± 0.5 °C by a heating pad.

2.7 Statistics

All of the observations are reported as mean ± SEM. Statistical analysis was performed using the unpaired student's t-test and p-values below 0.05 were regarded as statistically significant. When multiple comparisons were made between several groups an analysis of variance (ANOVA) was used to determine whether the variation between groups was significantly greater than expected by chance. If this was the case then a further statistical test was applied for specific comparisons (Bonferroni or Tukey-Kramer multiple comparison test).

CHAPTER 3: RESULTS

3.1   Measurement of F_2-isoprostanes in the Experimental Groups

Since the basis of the hypothesis is that oxidant stress is involved in the development of the hyperdynamic circulation it was necessary to demonstrate oxidant stress in the PPVL model and sham groups.

F_2-isoprostane levels were measured in the urine, plasma and liver to provide an index of lipid peroxidation in PPVL animals and compared them to sham operated animals. The initial study examined changes in the sequential 24 hour excretion of urinary F_2-isoprostanes from day 3 to day 8 and then at day 14 in PPVL animals. On the basis of these results, and published data, we then measured the F_2-isoprostanes excretion at day 14 in both sham and PPVL groups and examined the effects of N-acetylcysteine, BB-1101 and pyrrolidine.

Following purification by Sep Pak, thin layer chromatography and gas chromatography, the mass spectrometer detected a series of compounds with peaks which corresponding to a mass:charge (m/z) ratio of 569 and 573 in the selective ion monitoring mode (fig 10). The mass spectrometer does not discriminate between compounds that have a m/z ratio of 569 or 573. However, following the purification procedures which involve 3 chromatography steps (two thin layer chromatography and one gas chromatography), the level of impurities are low and the level of isoprostanes are concentrated. Therefore, the two peaks eluting at approximately 9.4 and 9.7 minutes with a m/z ratio of 569 and 573 corresponded to D_4-8-iso-$PGF_{2\alpha}$ and D_4-$PGF_{2\alpha}$ respectively. These elution times were pre-determined on pure standard specimens since a small day to day variation was observed.

1) Sequential excretion of F_2-isoprostane excretion in PPVL(day 4-8,14) compared to sham (day 1-4):

Following PPVL there was an increased urinary excretion of F_2-isoprostanes from day 5 onwards suggestive of increase lipid peroxidation (figure 20, table 6). In comparison, during the first four days after sham operation when one might expect to find an increased excretion of F_2-isoprostanes due to the trauma of surgery, there was no increase observed (table 6).

day after surgery	n-fold increase in F_2-isoprostanes	group number
day 3 post-PPVL	1.1 ± 0.4	5
day 4 post-PPVL	1.8 ± 0.4	7
day 5 post-PPVL	$2.4 \pm 0.7*$	6
day 6 post-PPVL	$2.8 \pm 0.3*$	8
day 7 post-PPVL	$2.7 \pm 0.5*$	5
day 8 post-PPVL	$3.4 \pm 0.5*$	8
day 14 post-PPVL	$3.9 \pm 0.8*$	5
day 1 post-sham	1.0 ± 0.2	4
day 2 post sham	0.8 ± 0.1	4
day 3 post sham	1.2 ± 0.2	4
day 4 post sham	1.5 ± 0.6	4

Table 6 showing the sequential urinary excretion of F_2-isoprostanes following PPVL or sham surgery. Following PPVL there was a significant increase in the excretion over baseline is observed from day 5 onwards whereas during the first four days after sham operation there was no significant increase. During these initial experiments I unfortunately omitted weighing the animals. Therefore the results are expressed as n-fold over baseline compared to actual values since there were observed differences in baseline values which were probably related to the animal weight. In the subsequent experiments the results were indexed to the animal weight (*$p < 0.05$). Comparisons were only made with baseline values and no comparisons should be made between different groups.
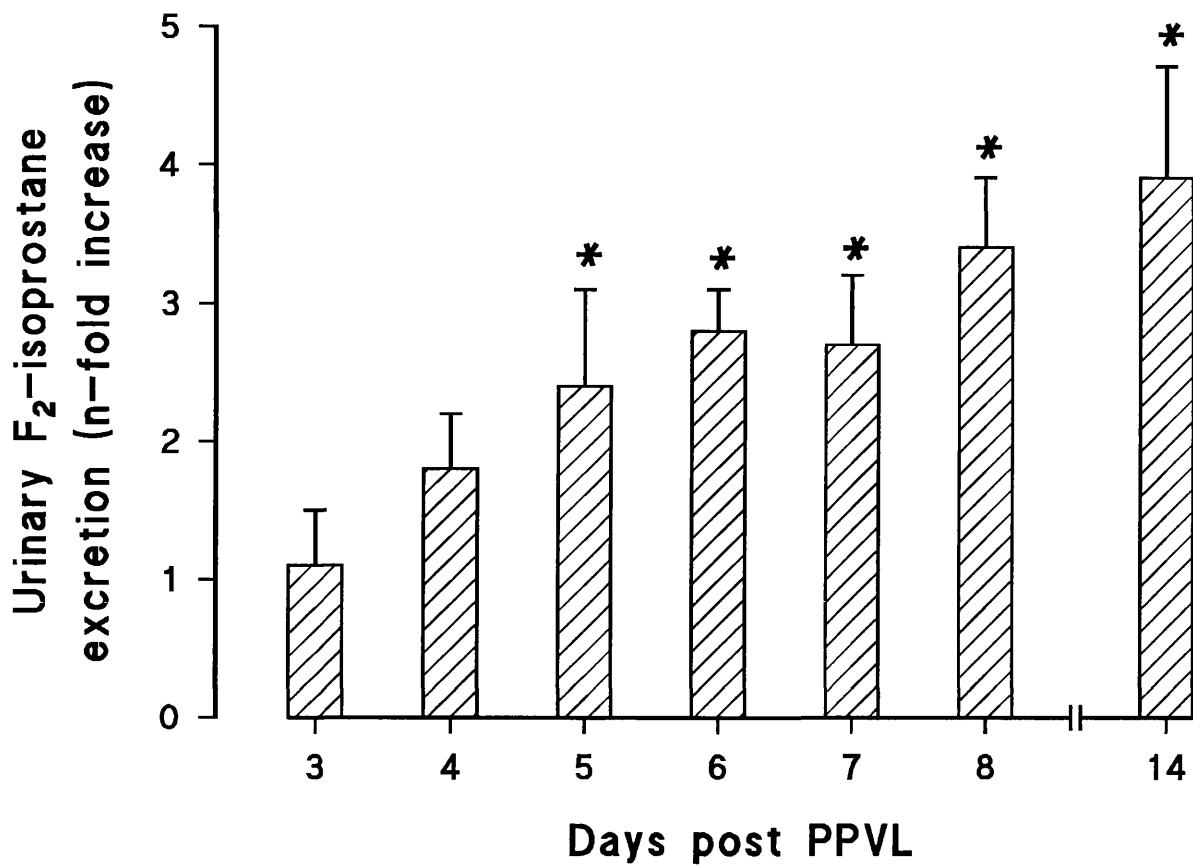
Figure 20. Sequential excretion of F_2-isoprostanes following PPVL. Urinary excretion of F_2-isoprostanes is significantly increased by day 5 following PPVL (*$p < 0.05$) when compared to baseline values. No comparisons should be made between groups studied on different days.

2) Urinary F₂-isprostane excretion in placebo-treated PPVL and sham operated rats

Having demonstrated the sequential changes following surgery, the next stage was to compare the two groups at day 14 with placebo treatment (table 7). There was no difference in the preoperative value (39 ± 5 pg/day/g versus 33 ± 5 pg/day/g) but, by day fourteen, urinary F_2-isoprostane excretion increased significantly following PPVL compared to sham animals (193 ± 37 pg/day/g versus 30 ± 5 pg/g/day), (figure 21).

group	Number	pre-op pg/day/g	post-op pg/day/g
pvl-placebo	5	39 ± 5	193 ± 37*
sham-placebo	5	33 ± 5	30 ± 5

Table 7 showing the pre-operative and post-operative 24 hour urinary excretion of F_2-isoprostanes. Following PPVL there was a significant increase in the urinary excretion of F_2-isoprostanes compared to baseline values and also to the post-operative sham value (*$p < 0.05$).

3) Urinary F₂-isoprostane excretion in NAC, BB-1101 and pyrrolidine-treated rats

Administration of N-acetylcysteine to PPVL animals prevented the increased excretion of urinary F_2-isoprostanes (79 ± 26 pg/day/g, $p < 0.05$). Treatment with pyrrolidine however, resulted in a significant increase in urinary excretion of F_2-isoprostanes (409 ± 26 pg/day/g, $p < 0.001$), (figure 22) but the increase with BB-1101 was insignificant. (265 ± 43 pg/day/g, $p > 0.05$). There were only 4 animals in the BB-1101 group as 2 died during the experimental period.

group	Number	Mean ± SEM pg/day/g
pvl-placebo	5	193 ± 37
pvl-NAC	5	79 ± 26*
pvl-pyrrol	6	409 ± 26*
pvl-BB1101	4	265 ± 43

Table 8 showing the 24 hour urinary excretion of F_2-isoprostanes in treated experimental groups. N-acetylcysteine prevented the increased excretion of F_2-isoprostanes following PPVL. Pyrrolidine and BB-1101 resulted in an increase in excretion when compared to PPVL-placebo but this was statistically significant only with pyrrolidine (*$p < 0.05$). For the ANOVA test and all the multiple comparisons for this study refer to figure 22.
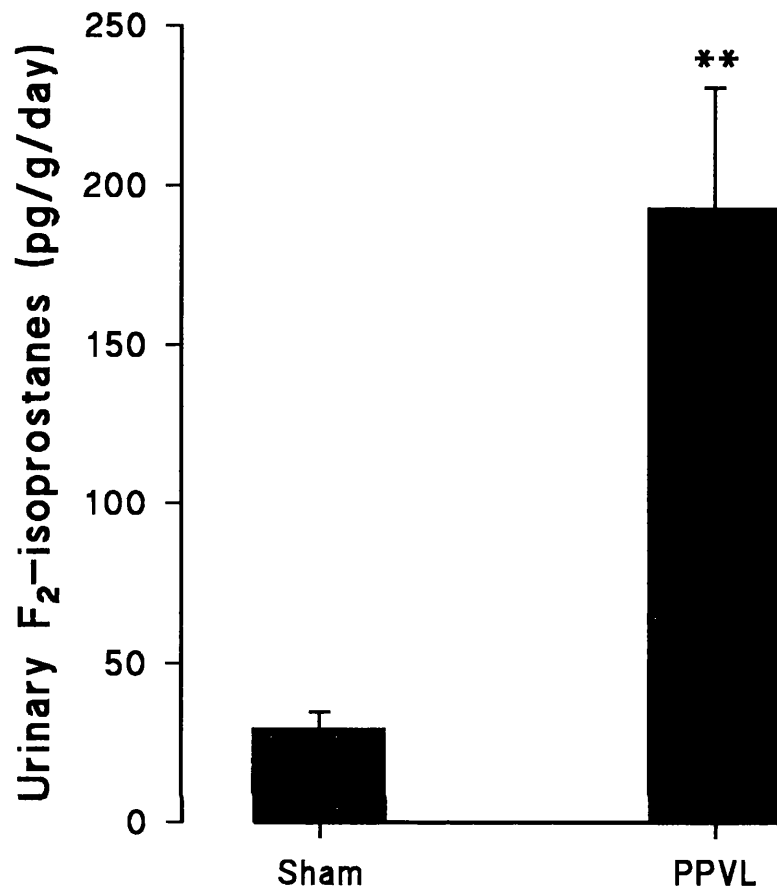
Figure 21. Urinary excretion of F_2-isoprostanes is significantly increased following PPVL compared to sham operated animals (** $p < 0.05$).
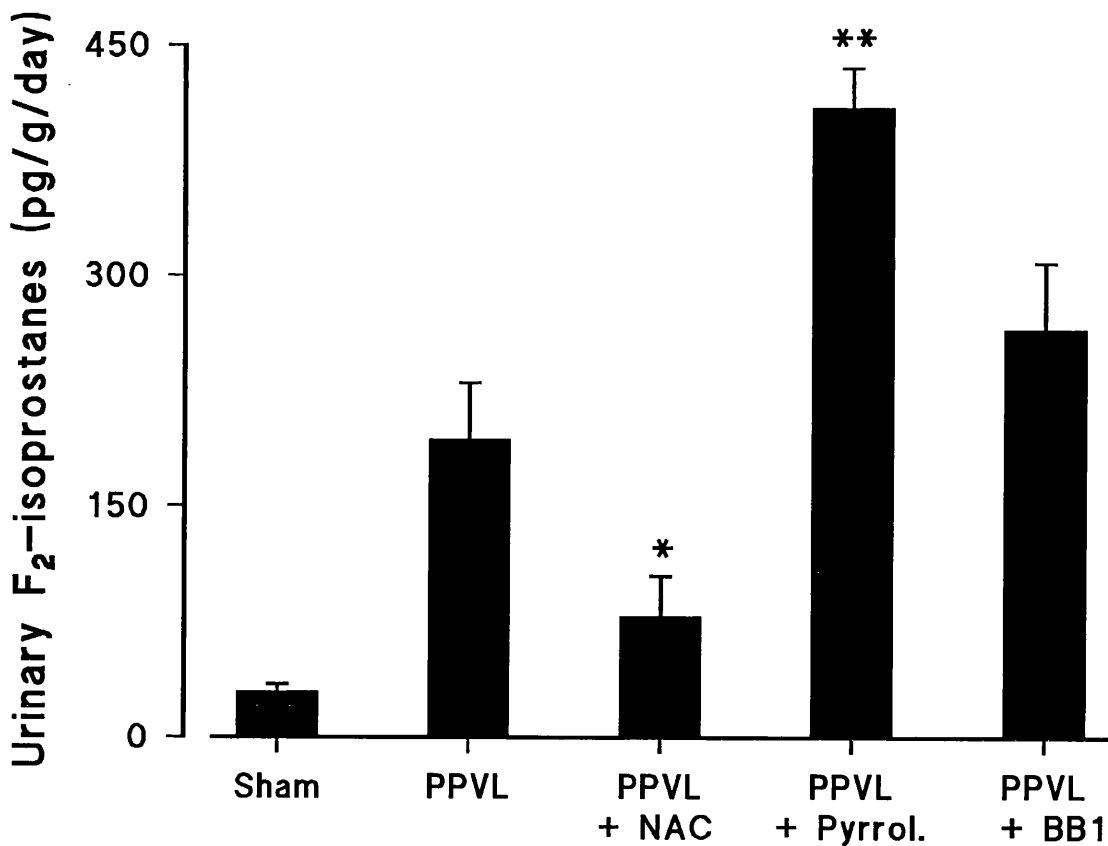
Figure 22. Urinary excretion of F_2-isoprostanes in study groups. Treatment with N-acetylcysteine resulted in a significantly decreased excretion compared to PPVL group (*p < 0.05). Pyrrolidine resulted in a greater excretion of urinary F_2-isoprostanes (**p <0.001). BB-1101 however had no significant effect. [ANOVA p-value for the above groups is < 0.001. Specific comparisons using the Tukey-Kramer test: sham vs PPVL+placebo p<0.01, sham vs PPVL+NAC: p>0.05, sham vs PPVL+Pyrrol: p<0.001, sham vs PPVL+BB1101: p<0.001, PPVL vs PPVL+NAC: p<0.05, PPVL vs PPVL+pyrrol: p<0.001, PPVL vs PPVL+BB1101: p>0.05, PPVL+NAC vs PPVL+pyrrol: p<0.001, PPVL+NAC vs PPVL+BB1101: p<0.01, PPVL+pyrrol vs PPVL+BB1101: p<0.05]

4) Plasma levels of F_2-isoprostanes in placebo-treated PPVL and sham operated animals:

Plasma levels and of F_2-isoprostanes however showed no significant differences between the PPVL and sham groups and N-acetylcysteine-treated group. (tables 9). They were not measured in animals treated with pyrrolidine or BB-1101.

Group	number	mean ± SEM (pg/ml)
sham	4	110 ± 22
pvl	5	126 ± 19
pvl-nac	5	122 ± 22

Table 9. This shows the results for plasma levels of F_2-isoprostanes. No significant differences were demonstrated between the three groups.

5) Liver tissue levels of F_2-isoprostanes in placebo-treated pvl and sham animals:

Tissue levels of F_2-isoprostanes in PPVL and sham operated rats showed no significant differences. Levels were not measured in N-acetylcysteine, BB-1101 and pyrrolidine-treated animals.

Group	number	mean +/- sem (ng/g)
pvl	n=4	3.6 +/- 0.5
sham	n=4	5.7 +/- 0.2

Table 10. Although liver tissue levels of F_2-isoprostanes show a difference between PPVL and sham operated groups, this was not significant.

The demonstration that urinary excretion of F_2-isoprostanes is increased by days 5 to 14 in the PPVL model is indicative of lipid peroxidation secondary to the formation of reactive oxygen species. This is the first demonstration of oxidant stress in this model. No increase was detected in the sham groups but the observation that N-acetylcysteine treatment prevented the rise in urinary excretion of F_2-isoprostanes confirmed the involvement of reactive oxygen species. N-acetylcysteine is a multifunctional antioxidant and the observation that it decreased urinary excretion of F_2-isoprostanes strongly suggests that this model is associated with the generation of reactive oxygen species.

BB-1101 and pyrrolidine administration both produced unexpected results. BB-1101 is an inhibitor of metalloproteinase as well as an inhibitor of TNF-α production. Since it is known that TNF-α can induce an oxidant stress involving reactive nitrogen species such as peroxynitrite anion, it could be predicted that inhibition of TNF-α production may decrease oxidant stress and lipid peroxidation. Pyrrolidine is an inhibitor of the transcription factor NF-κB as well as having antioxidant properties as an iron chelator. As with BB-1101, one would also predict that pyrrolidine would decrease lipid peroxidation. However, both treatments resulted in increased excretion of urinary F_2-isoprostanes after PPVL compared to placebo-treated rats but this was only significant in the pyrrolidine-treated group. (pvl-BB-1101: 265 ± 43 pg/day/g, $p > 0.05$; pvl-pyrrol: 409 ± 26 pg/day/g, $p < 0.001$; pvl-placebo: 193 ± 37).

3.2 Measurement of Nuclear Factor -kappa B activation in Experimental Groups

NF-κB is a protein heterodimer which is a key regulator of the inflammatory response in that it can initiate the transcription of a wide variety of key inflammatory proteins and cytokines including tumour necrosis factor-α (TNF-α). Since TNF-α is implicated in the aetiology of the hyperdynamic circulation, we measured activation of NF-κB in the liver. Having shown the presence of increased lipid peroxidation following PPVL and since reactive oxygen species can activate NF-κB, studies were performed to demonstrate the activation of liver NF-κB in the same experimental groups as for F_2-isoprostanes.

The supershift assay provided confirmation that the band on the gel corresponded to NF-κB. This was by binding with antibodies to the p-50 and p-65 subunits as well as cold oligonucleotide (figure 13).

PPVL was associated with activation of NF-κB as evidenced by naked eye inspection and measurement of relative optical density (3.4 ± 0.5 versus 1.6 ± 0.3 Rel.O.D), (figure 24). Previous studies using antioxidants *in vitro* have shown that NF-κB activation involves reactive oxygen species and is inhibited by N-acetylcysteine and pyrrolidine. To determine whether N-acetylcysteine or pyrrolidine could inhibit NF-κB activation *in vivo* both compounds were administered from before operation to day 14 post-operatively when the rats were sacrificed.

Treatment with N-acetylcysteine resulted in a significantly reduced optical density (1.2 ± 0.2 Rel.O.D) indicating that there was reduced NF-κB activation (figure 25,26). Pyrrolidine treatment also inhibited activation of NF-κB (pvl-pyrrolidine:0.85 ± 0.14 Rel.O.D), (figure 25,27). Treatment with BB-1101 had no statistical effect on NF-κB activation although lower optical density values were observed in this group compared to PPVL animals. (pvl-BB1101:2.45 ± 0.26 Rel O.D), (figure 25).

It is likely that the process of activation of NF-κB in this model is secondary to reactive oxygen species since treatment with N-acetylcysteine prevents this activation in a PPVL group. However, the process may not be as straightforward as this due to the findings from the BB-1101 and pyrrolidine groups which both also prevented the NF-κB activation despite the evidence of increased lipid peroxidation in these groups. Pyrrolidine is known to be a potent inhibitor of NF-κB and it may act by direct inhibition of NF-κB or by inhibiting oxidant stress. It could also act by preventing NF-κB from being activated despite the presence of increased lipid peroxidation. BB-1101 has two known actions: inhibition of metalloproteinase and also it inhibits the production of tumour necrosis factor-α. Since tumour necrosis factor itself can activate NF-κB then it is possible that BB-1101 may be preventing NF-κB activation by this method.
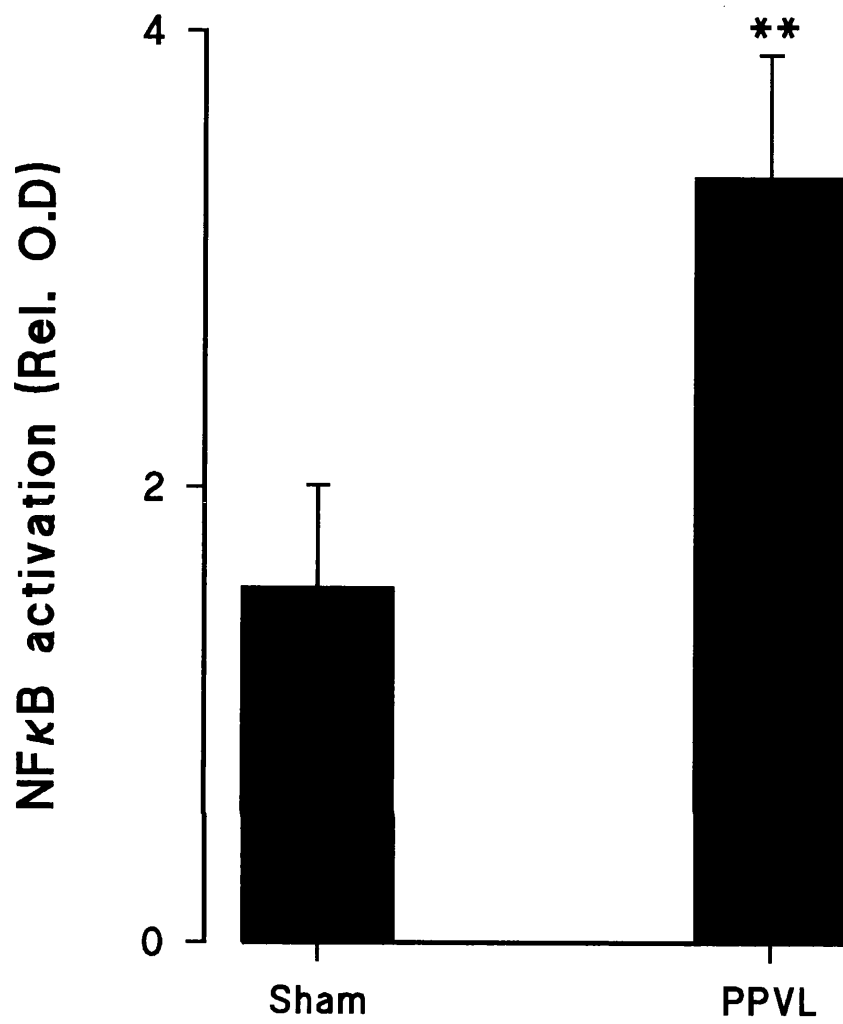
Figure 23. Hepatic activation of NF-κB. On day 14 after PPVL there was a significant increase in hepatic activation of NF-κB compared to sham operated animals (p < **0.02).
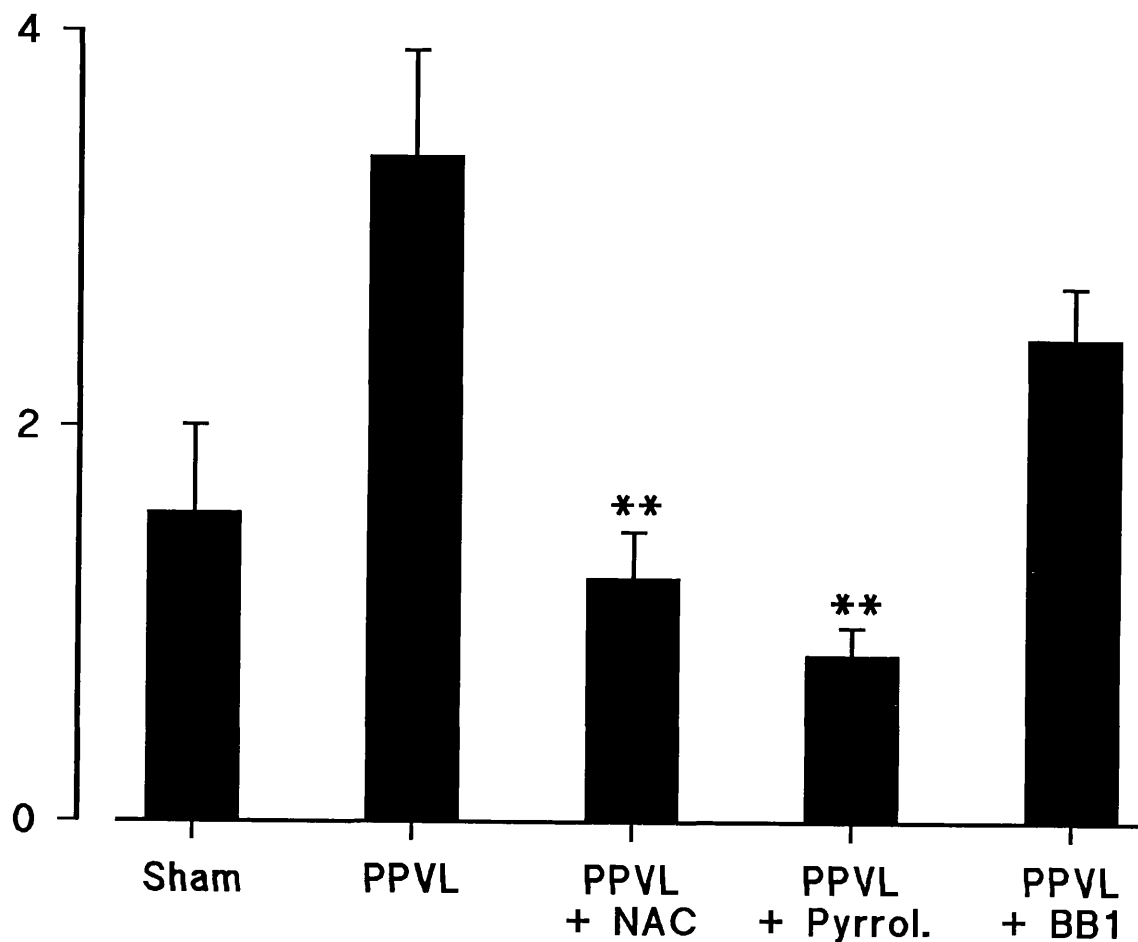
Figure 24. Hepatic activation of NF-κB in study groups. Treatment with N-acetylcysteine and pyrrolidine both resulted in a significantly reduced activation of hepatic NF-κB compared to placebo-treated animals (**$p < 0.05$). Treatment with BB-101 had no significant effect ($p>0.05$). [ANOVA p-value for the above groups is < 0.007. Specific comparisons using the Tukey-Kramer test: sham vs PPVL+placebo $p<0.05$, sham vs PPVL+NAC: $p>0.05$, sham vs PPVL+Pyrrol: $p>0.05$, sham vs PPVL+BB1101: $p>0.05$, PPVL vs PPVL+NAC: $p<0.05$, PPVL vs PPVL+pyrrol: $p<0.001$, PPVL vs PPVL+BB1101: $p>0.05$, PPVL+NAC vs PPVL+pyrrol: $p>0.05$, PPVL+NAC vs PPVL+BB1101: $p>0.05$, PPVL+pyrrol vs PPVL+BB1101: $p>0.05$]
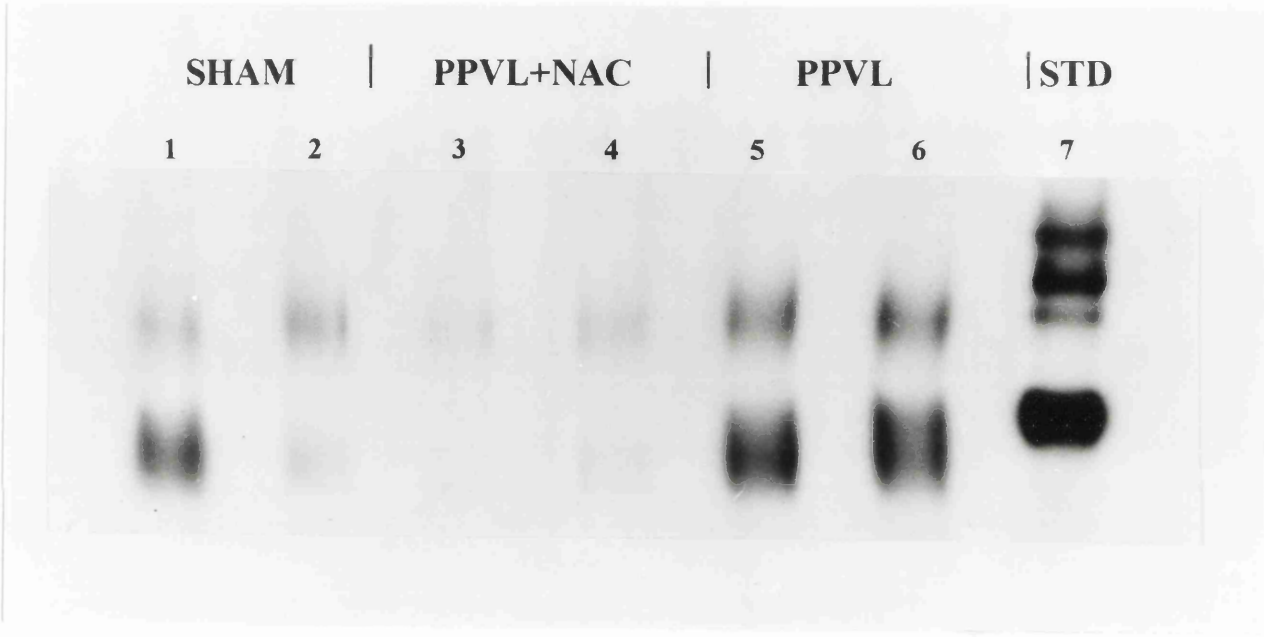
Figure 25. EMSA Gel autoradiograph. Naked eye inspection can confirm that there is an increased optical density of the band corresponding to NF-κB compared to sham operated animals and this increase can be prevented by administration of N-acetylcysteine.
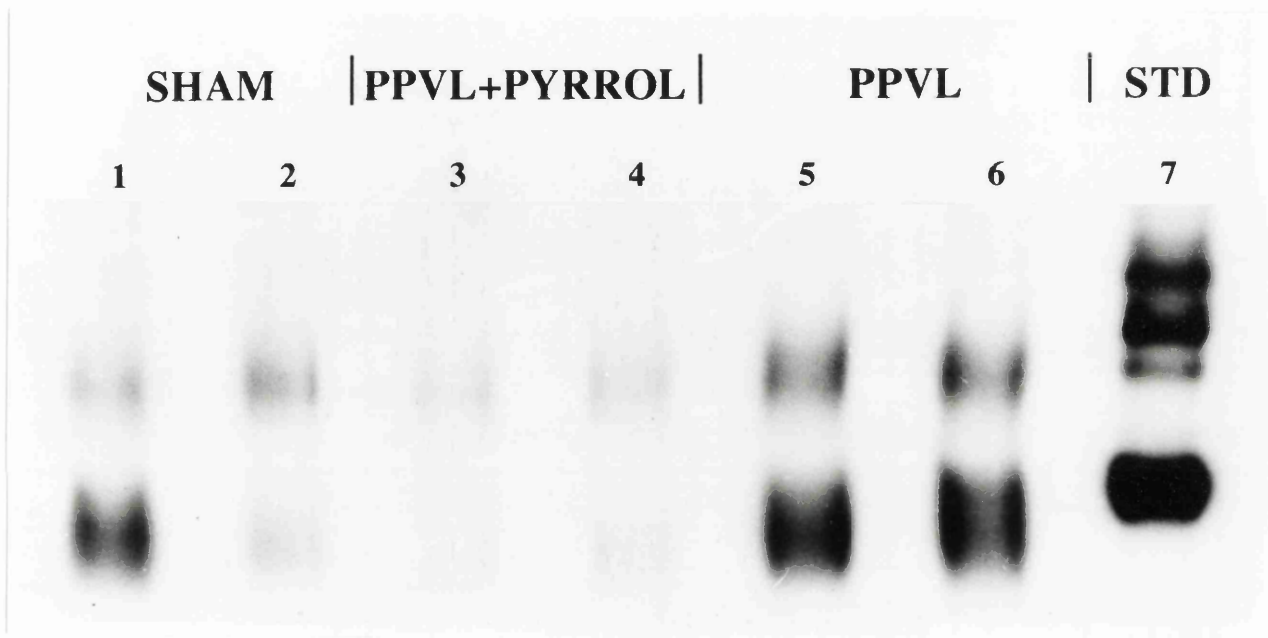
Figure 26. EMSA Gel autoradiograph. Animals undergoing PPVL and treated with pyrrolidine demonstrated reduced activation of hepatic NF-κB compared to placebo-treated PPVL animals.

3.3 Measurement of Plasma TNF-α Levels in Experimental Groups

Since TNF-α was shown to be a major contributor to the generation of the hyperdynamic circulation by Lopez-Talavera et al. plasma levels were determined in the experimental groups. It was felt to be necessary to measure plasma TNF-α levels in order to demonstrate the possible effects of different pharmacological reagents. In these studies the plasma levels of TNF-α were detected using two different ELISA kits. Initial experiments measured TNF-α levels following PPVL using a Genzyme ELISA kit.
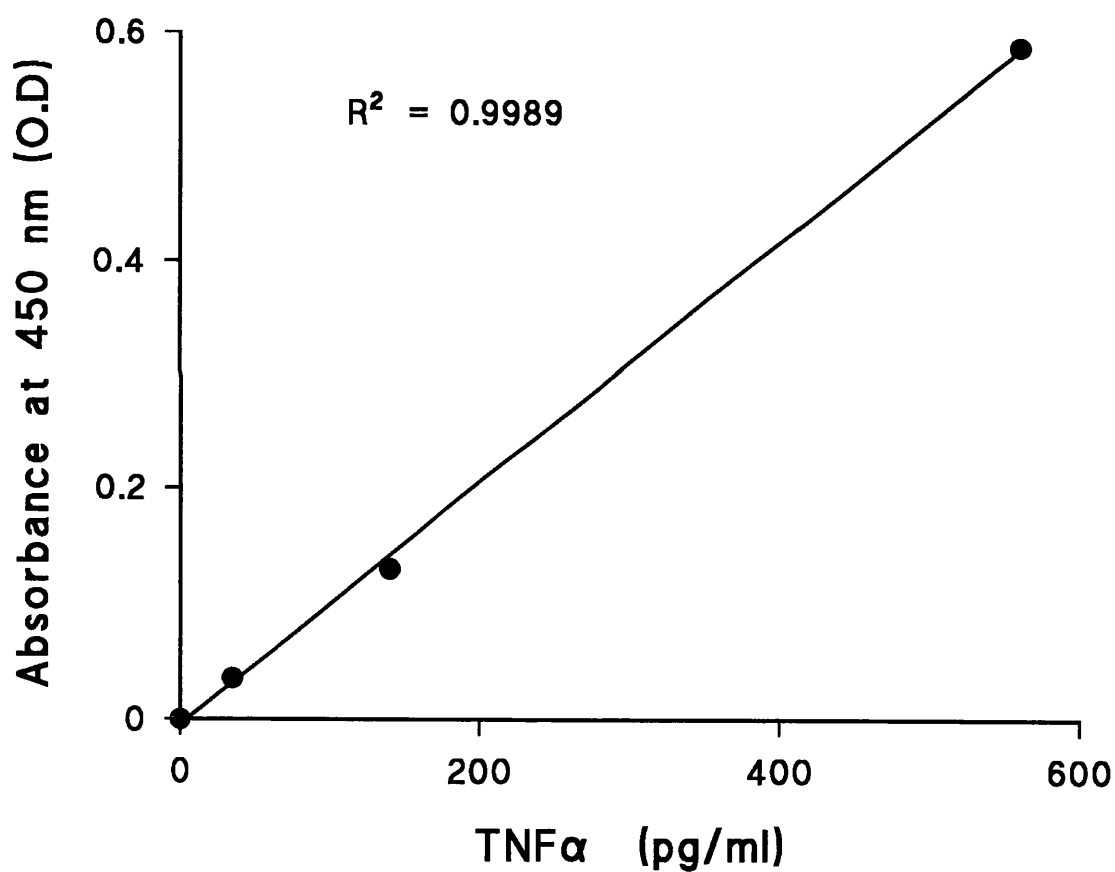
Figure 27. Standard Curve for TNF-α Measurement using the Genzyme ELISA assay.

day after PPVL	number per group	plasma TNF-α levels (pg/ml)
day 3	n=5	59.8 ± 12.3
day 4	n=4	39.0 ± 1.4
day 5	n=6	56.4 ± 4.8
day 7	n=5	47.8 ± 13.1
day 14	n=5	34.2 ± 13.0

Table 11 showing plasma TNF- levels as measured using the Genzyme ELISA

The values obtained using the Genzyme kit were between 34 and 60 pg/ml and the groups did not show statistical significance from day 3. Furthermore from the standard curve shown in figure 27 it can be seen that the values were in the lower part of the curve and therefore the concentrations detected in this region would be inaccurate. It was felt that the kit was more suitable for detecting large differences in TNF-α values instead of more subtle changes. For this reason a second ELISA kit manufactured by Serotec was used which was described as ultrasensitive and was able to detect lower values.

Using the ultrasensitive kit a small difference was observed between PPVL and sham operated rats but this was statistically insignificant (table 12, figure 29). PPVL rats treated with N-acetylcysteine and BB-1101 both resulted in lower TNF-α values compared to PPVL-placebo animals but again this was not statisically significant. (At the time of this study the pyrroldine specimens were not available for analysis. Since no significant differences were demonstrated between the other groups it was decided not to analyse the pyrrolidine group for plasma TNF-α levels). From this data we have been unable to demonstrate an effect of PPVL and drug treatment on plasma TNF-α levels.
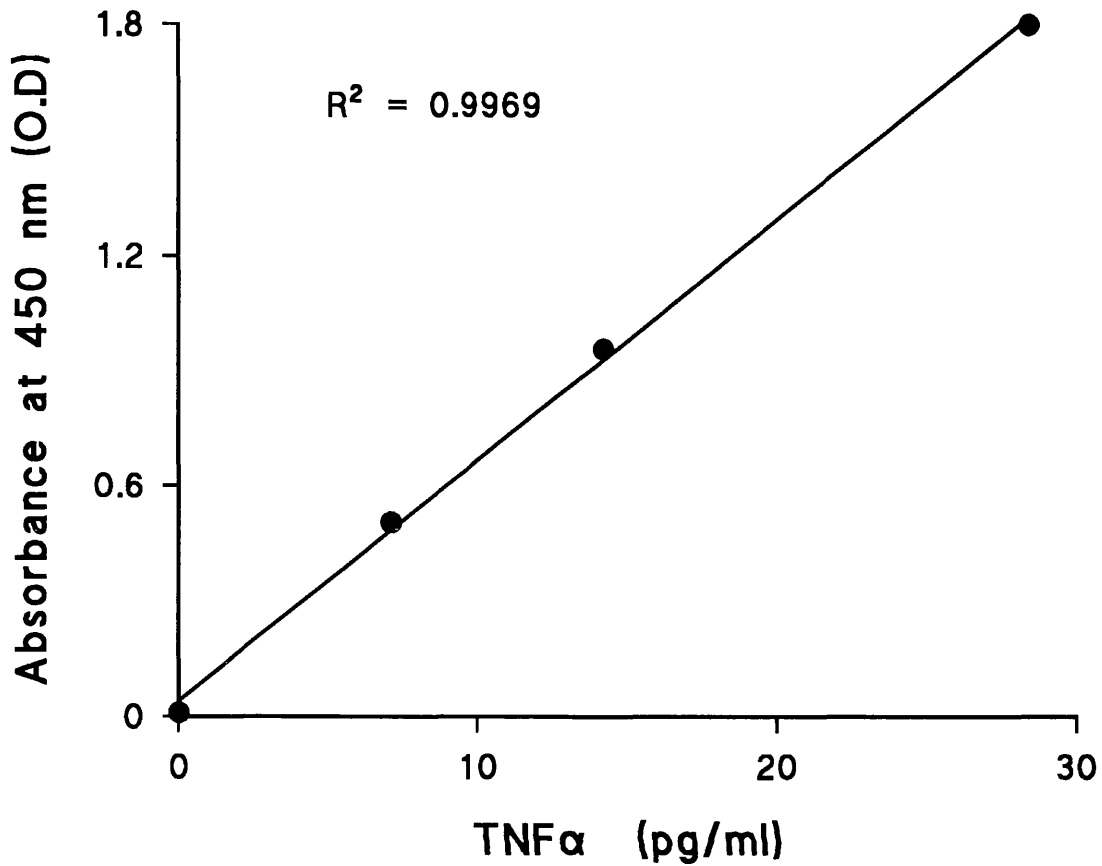
Figure 28. Standard Curve for TNF-α Measurement using the Serotec Ultra-sensitive assay.

group	Number per group	plasma TNF-α level (pg/ml)
PPVL	6	1.47 ± 0.66
sham	6	-0.35 ± 0.54
NAC	6	0.44 ± 0.35
BB101	5	0.80 ± 0.32

Table 12 showing the calculated plasma TNF-α levels using the ultra-sensitive ELISA kit
No significant differences were observed between the study groups.
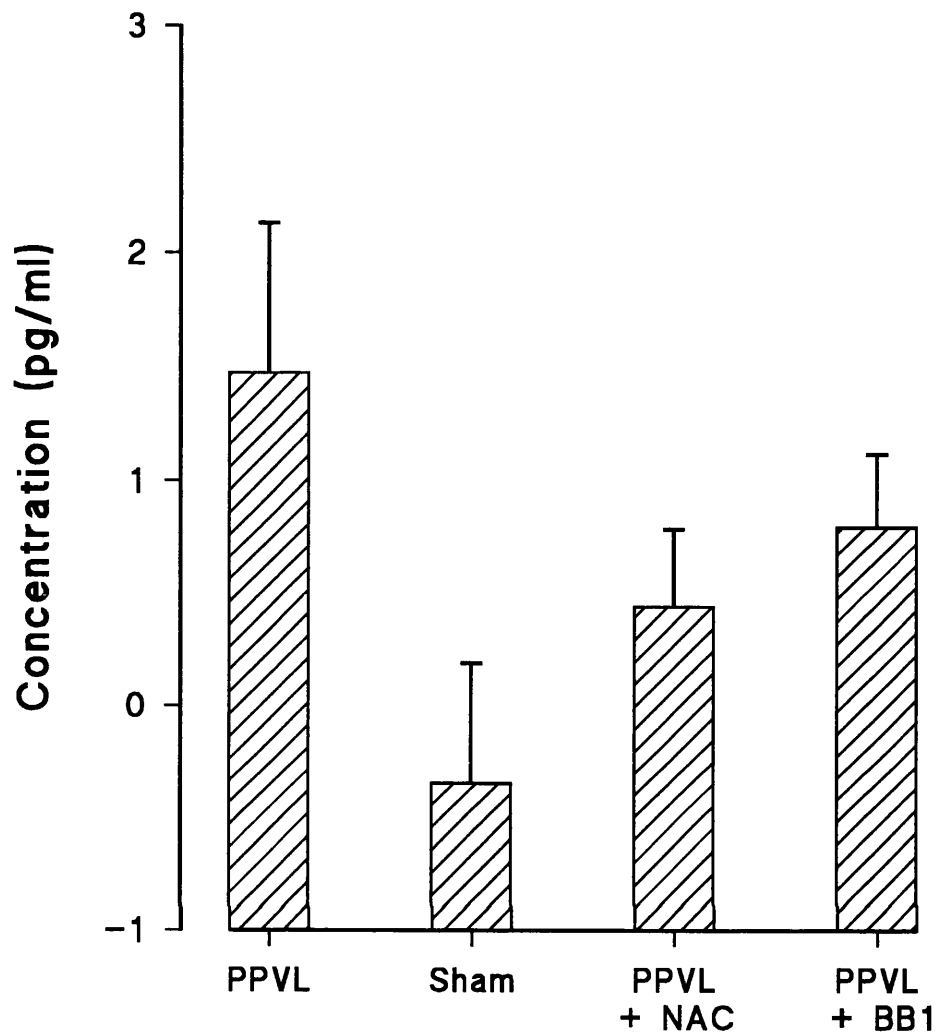
Figure 29. TNF-α Concentrations in study groups. A small difference was observed between PPVL and sham operated animals but this was statistically insignificant. Plasma values from PPVL animals treated with N-acetylcysteine and BB-1101 both resulted in lower values than placebo treated PPVL animals but again this was not statistically significant.

3.4   Measurement of Plasma Nitrite/Nitrate Levels from Experimental Groups

One of the proposed mediators of the hyperdynamic circulation is nitric oxide. This is a highly reactive, rapidly diffusible molecule. It was difficult to measure nitric oxide levels directly because of these characteristics. Instead the stable metabolic products of nitric oxide, namely nitrite and nitrate were measured in plasma.

Using the Griess reaction method plasma nitrite and nitrate levels following PPVL were greater than values obtained after sham operation although this was not statistically significant (PPVL: 28.0 ± 3.0 μM versus 21.1 ± 3.4 μM, p = 0.072). N-acetylcysteine treated animals undergoing PPVL had lower nitrate/nitrite concentration compared to placebo treated PPVL animals (18.4 ± 2.7 μM, p < 0.02).

group	Number	conc(μm)	sem	p-value vs. PPVL
PPVL	16	28.0	3.0	-
sham	13	21.1	3.4	0.072
NAC	11	18.4	2.7	0.011

Table 13 showing the plasma concentration of total nitrite and nitrate using the Griess reaction.

For similar reasons to those outlined for the Genzyme TNF-α assay and because of haemolysis in some of the samples it was decided to confirm the above data using a more sensitive chemiluminescence analyser which was recently acquired by the Department of Biochemistry at the Royal Free Hospital. Plasma specimens that had haemolysed would have interfered with the red colour generated on addition of the Griess reagent. However, using the Griess reaction a difference was observed between the PPVL and sham groups although this was not significant. N-acetylcysteine also appeared to have an effect in the PPVL group as it lowered the plasma nitrite and nitrate level.

Using the chemiluminescence method, PPVL was associated with elevated plasma nitrite and nitrate levels compared to sham operation suggestive of increase nitric oxide synthesis

(41.5 ± 6.0 μM versus 28.4 ± 2.0 μM, p < 0.05) (figure 31). Treatments with N-acetylcysteine and pyrrolidine to PPVL groups had no significant effects on nitrite and nitrate levels (NAC: 29.4 ± 2.8 μM, p > 0.05, Pyrrolidine: 66.2 ± 9.4 μM, p > 0.05). The BB-1101 group however resulted in elevated plasma nitrite and nitrate levels (BB-1101: 161.4 ± 62.3 μM, p < 0.001) (figure 32). In the BB-1101 group 2 animals died during the experimental period which prevented the measurement of 6 specimens in this group.

group	Conc (μm)	SEM	number	p-value (vs PPVL)
PPVL	41.5	6	11	-
sham	28.4	2	11	< 0.05
NAC	29.4	2.8	6	> 0.05
pyrrol	66.2	9.4	6	> 0.05
BB-1101	161.4	62.3	4	< 0.001

Table 14 showing the plasma nitrite and nitrate levels in the experimental groups as measured using chemiluminescence. Treatment with N-acetylcysteine and pyrrolidine had no significant effect but BB-1101 treatment increased plasma nitrite and nitrate levels when compared to placebo treated PPVL animals.

From the chemiluminescence studies it can be concluded that there was an elevated level of circulating nitric oxide following PPVL compared to sham since the stable metabolism products, nitrate and nitrite, were increased. N-acetylcysteine and pyrrolidine treatment had no significant effect on plasma nitrite and nitrate levels. However, BB-1101 treatment resulted in elevated circulating levels of nitrite and nitrate. The trends observed in plasma nitrite and nitrate levels for each group appeared to be compatible with the observed findings from urinary excretion of F_2-isoprostanes although it must be stressed that these were not significant when a multiple comparison statistical test was performed (Tukey-Kramer).
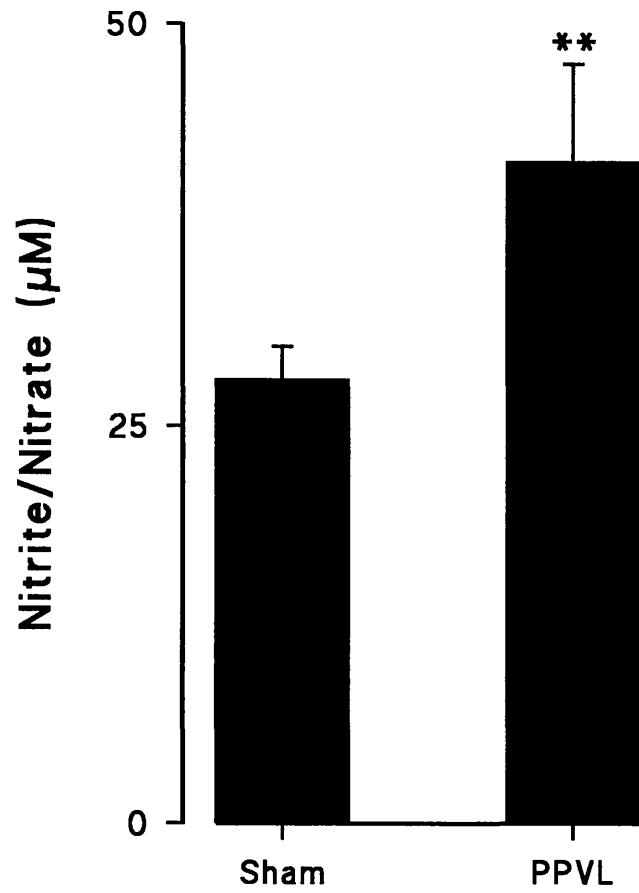
Figure 30. Plasma Nitrite/Nitrate levels measured using chemiluminescence are increased at day 14 following PPVL compared to sham operated animals (**$p < 0.03$).
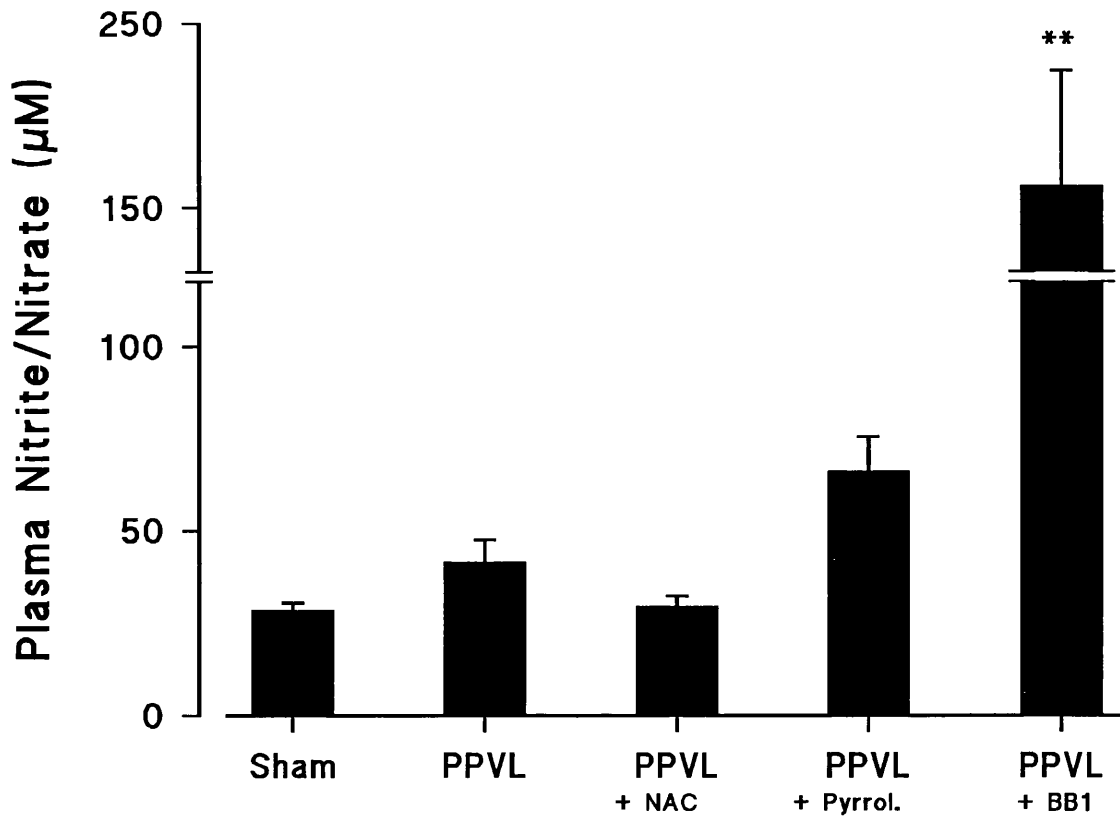
Figure 31. Plasma Nitrite/Nitrate levels in study groups. Although the ANOVA p-value for the above groups is < 0.0001 when comparisons were made using the Tukey-Kramer test the relation between sham vs PPVL-placebo became insignificant ($p>0.05$) (this was significant using the student's t-test). Other comparisons were as follows: sham vs PPVL+NAC: $p>0.05$, sham vs PPVL+Pyrrol: $p>0.05$, sham vs PPVL+BB1101: $p<0.001$, PPVL vs PPVL+NAC: $p>0.05$, PPVL vs PPVL+pyrrol: $p>0.05$, PPVL vs PPVL+BB1101: $p<0.001$, PPVL+NAC vs PPVL+pyrrol: $p>0.05$, PPVL+NAC vs PPVL+BB1101: $p<0.001$, PPVL+pyrrol vs PPVL+BB1101: $p<0.05$.

Haemodynamic Studies

Haemodynamic studies were performed in order to confirm that the model of partial portal vein ligation induced a hyperdynamic state and also to investigate the pharmacodynamic effects of the different drugs.

A. PPVL+placebo versus sham+placebo

Following PPVL there was a significant increase in cardiac output when compared with sham controls. (28.4 ± 1.5 vs. 18.8 ± 1.8 ml.min^{-1}.100g^{-1}, PPVL+placebo vs. sham+placebo respectively, P < 0.001). This was accompanied by a reduction of both mean arterial pressure (99 ± 5.1 vs. 125 ± 6.4 mmHg, P < 0.01) and decreased systemic vascular resistance index (3.5 ± 0.3 vs. 6.8 ± 0.6 mmHg.ml^{-1}.min^{-1}.100g, P < 0.001) indicative of a hyperdynamic circulation. Portal pressure increased following PPVL confirming the development of portal hypertension in this model (14.7 ± 1.1 vs. 8.7 ± 0.6 mmHg, PPVL+placebo vs. sham+placebo, P < 0.001), (figure 32).

B. Sham+placebo versus sham+NAC

In the sham groups, treatment with N-acetylcysteine had no significant effect on mean arterial pressure (MAP), systemic vascular resistance index (SVRI), cardiac index (CI) and portal pressure (PP) values in sham+NAC animals compared with placebo-treated sham controls (MAP:120 ± 3.3 vs. 125 ± 6.4 mmHg, P = NS, SVRI:7.3 ± 0.4 vs 6.8 ± 0.6 mmHg.ml^{-1}.min^{-1}.100g, P = NS, CI: 16.6 ± 1.3 vs. 18.8 ± 1.8 ml.min^{-1}.100g^{-1}, P = NS, PP: 7.0 ± 0.6 vs. 8.7 ± 0.6 mmHg, P= NS). (figure 33).
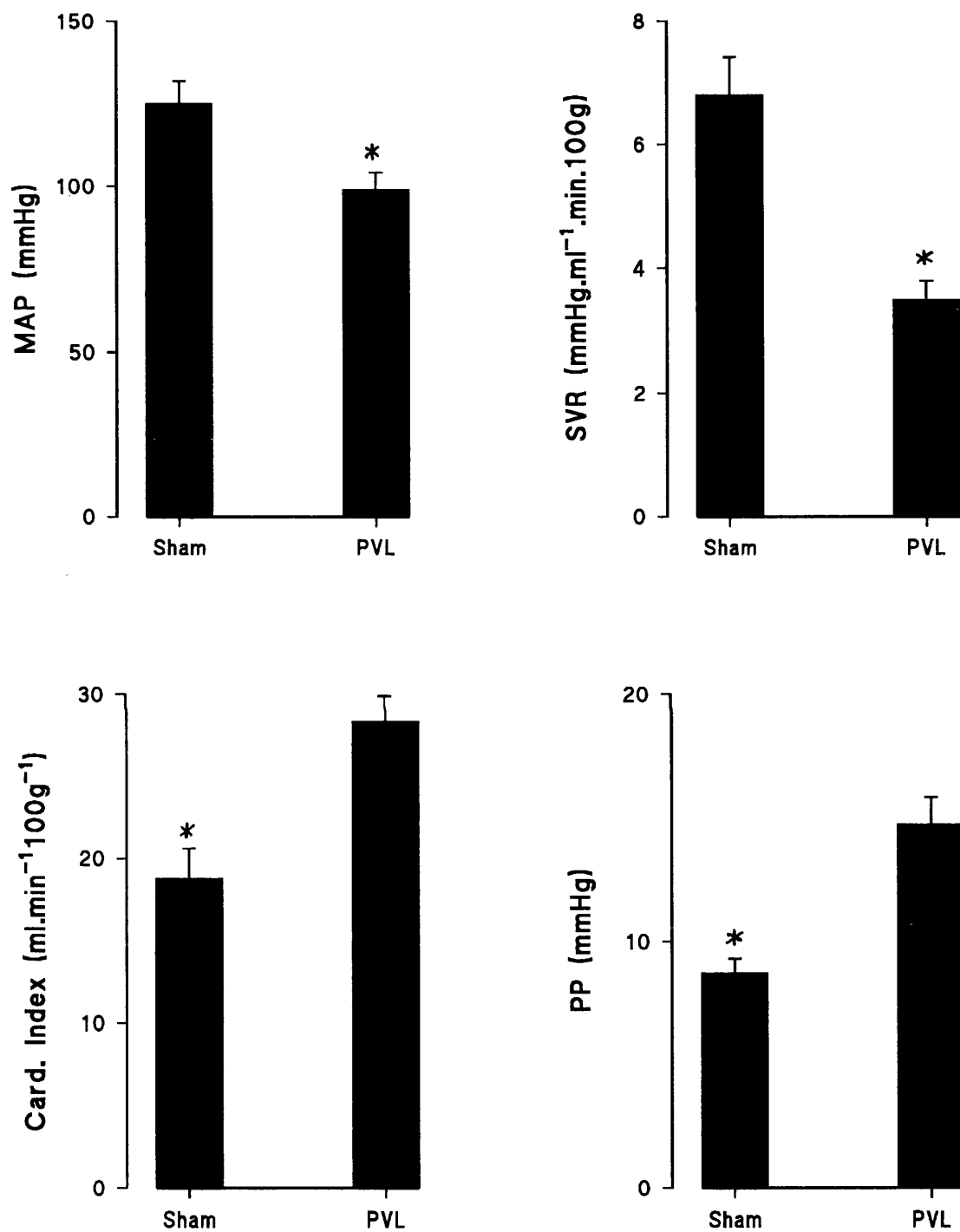
Figure 32. Haemodynamic studies. PPVL resulted in the development of a hyperdynamic circulation. This was associated with a significant reduction in systemic vasular resisitance and a significant increase in cardiac index. Portal pressure increased following PPVL(*p < 0.05).

C. PPVL+placebo versus PPVL+NAC

Chronic administration of N-acetylcysteine completely blocked the development of the hyperdynamic circulation following PPVL (figure 33). PPVL+NAC rats exhibited a higher mean arterial pressure compared with PPVL+placebo rats (109 ± 3 vs. 99 ± 5 mmHg, respectively) although this did not reach statistical significance (P = NS). This was associated with a significant increase in systemic vascular resistance index (6.3 ± 0.5 vs. 3.5 ± 0.3 mmHg.ml^{-1}.min^{-1}.100g, P < 0.01) and a lowering of cardiac index (17.6 ± 1.4 vs. 28.4 ± 1.5 ml.min^{-1}.100g^{-1}, P < 0.001), and a decrease of portal pressure (9.8 ± 0.3 vs. 14.7 ± 1.1 mmHg, P < 0.001). Following treatment with N-acetylcysteine, there was no significant difference between cardiac indices, mean arterial pressures, portal pressures and systemic vascular resistance indices in the PPVL+NAC and the sham+NAC groups. (figure 33).

D Sham+placebo versus sham+pyrrolidine

No significant differences were observed in cardiac index, mean arterial pressure, portal pressure and systemic vascular resistance between sham animals treated with placebo and sham animals treated with pyrrolidine respectively. (CI: 18.8 ± 1.8 ml.min^{-1}.100g^{-1} vs.17.6 ± 1.0 ml.min^{-1}.100g^{-1}, P=NS; MAP: 125 ± 6.4 mmHg vs. 125 ± 5.3 mmHg, P=NS; PP: 8.7 ± 0.6 mmHg vs. 7.9 ± 0.4 mmHg, P=NS and SVRI: 6.8 ± 0.6 mmHg.ml^{-1}.min^{-1}.100g vs. 7.2 ± 0.5 mmHg.ml^{-1}.min^{-1}.100g, P=NS), (figure 34).
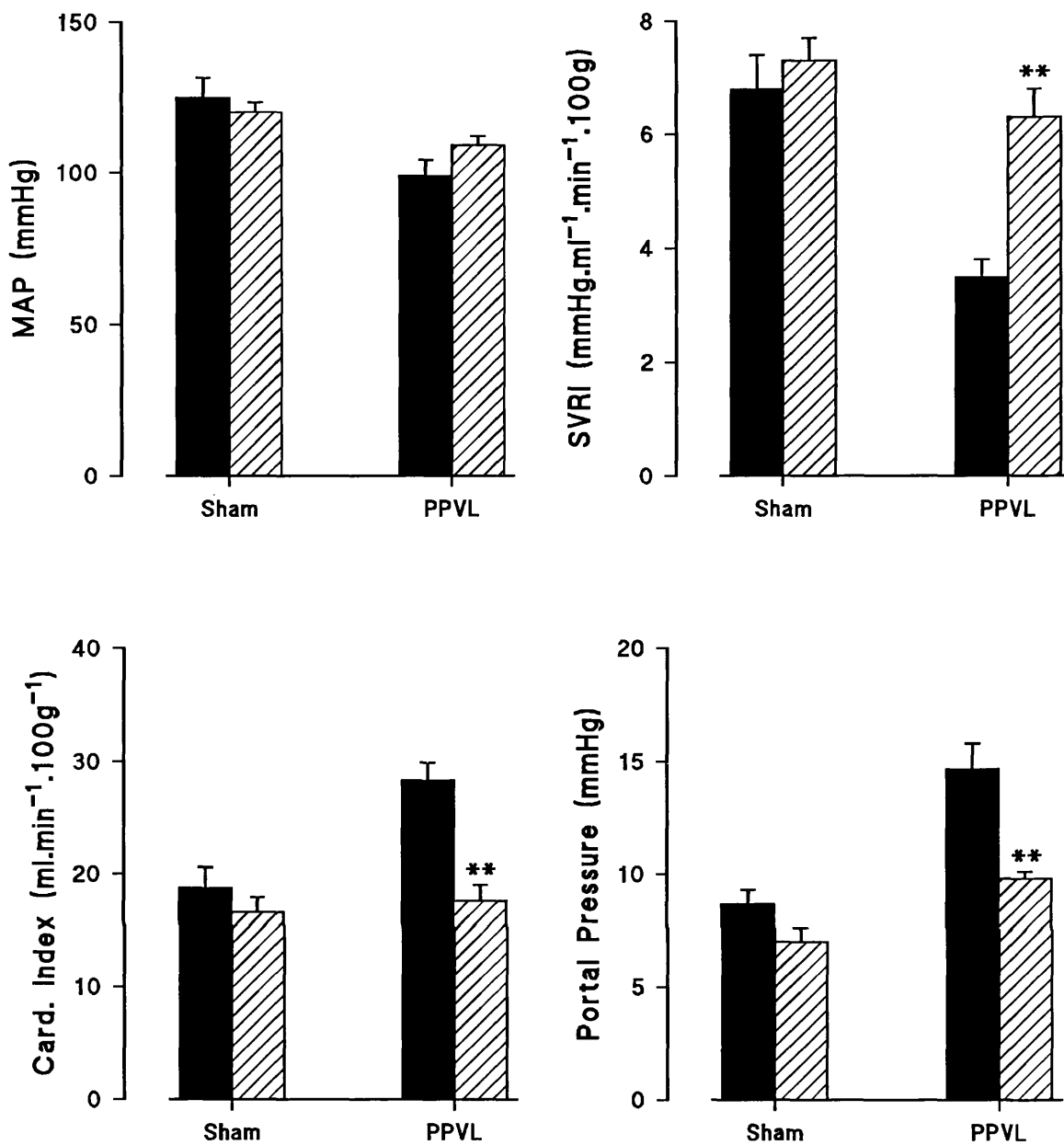
Figure 33. Haemodynamic effects of N-acetylcysteine (hatched bars) compared to placebo (solid bars). N-acetylcysteine treatment attenuated the hyperdynamic response and reduced portal pressure in PPVL animals (** $p < 0.005$). N-acetylcysteine also reduced portal pressure in sham operated animals (* $p < 0.05$). The ANOVA p-value was <0.005 and the Bonferroni test was used for multiple comparisons.
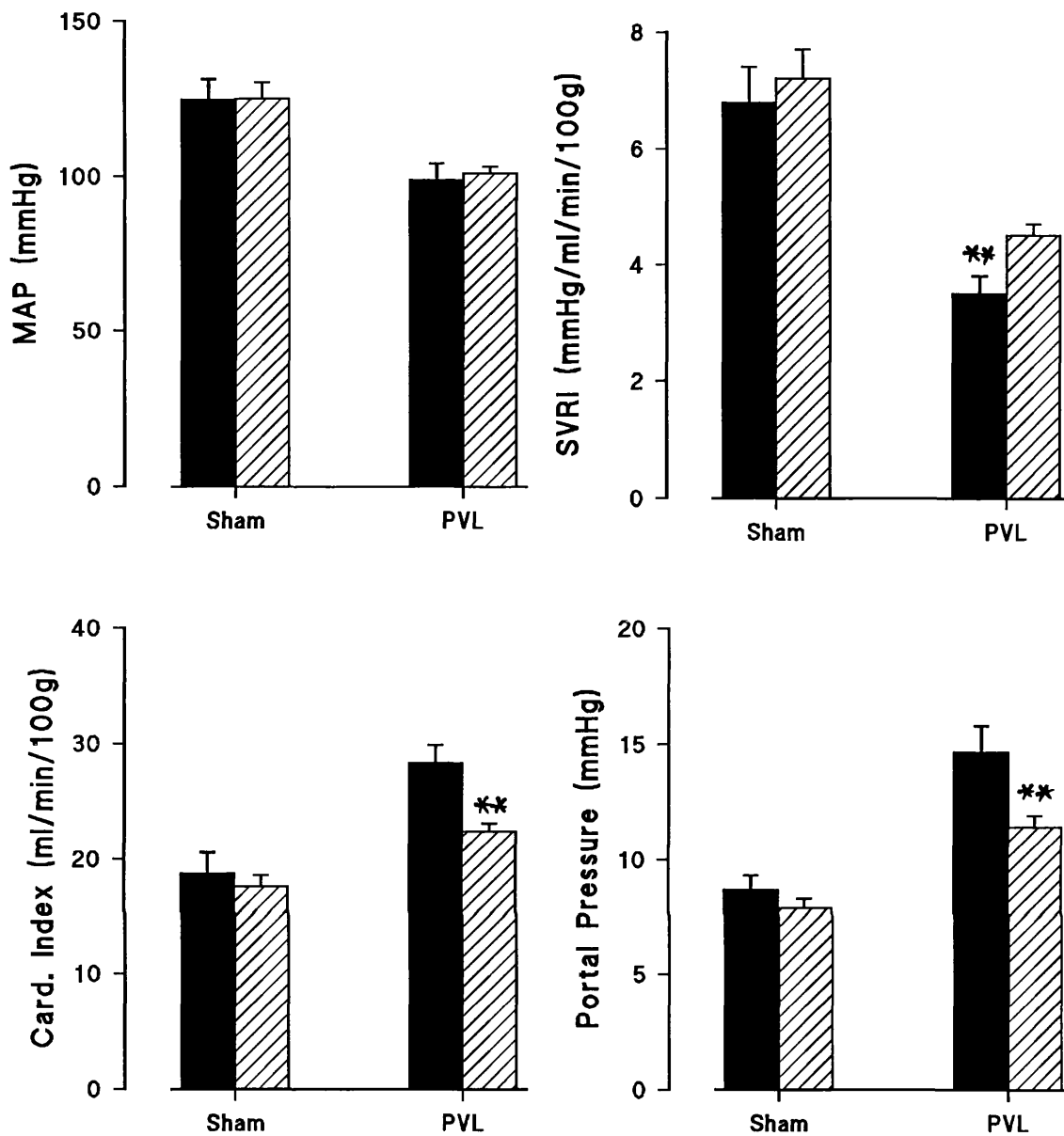
Figure 34. Haemodynamic effects of pyrrolidine (hatched bars) compared to placebo (solid bars). Pyrrolidine attenuated the hyperdynamic response and reduced portal pressure in PPVL animals (** p < 0.05). The ANOVA p-value was <0.005 and the Bonferroni test was used for multiple comparisons.

E   PPVL+placebo versus PPVL+pyrrolidine

Following PPVL animals treated with pyrrolidine demonstrated an attenuated hyperdynamic response (figure 34). Cardiac index and portal pressure were significantly reduced in pyrrolidine treated animals when compared to placebo treated animals (CI: 22.4 ± 0.7 ml.min^{-1}.100g^{-1} vs.28.4 ± 1.5 ml.min^{-1}.100g^{-1}, P< 0.05; PP:11.4 ± 0.5 mmHg vs. 14.7 ± 1.1 mmHg, P< 0.05). Systemic vascular resistance was increased by treatment with pyrrolidine but mean arterial pressure showed no significant difference when compared to placebo treated PPVL rats. (SVRI: 4.5 ± 0.2 mmHg.ml^{-1}.min^{-1}.100g vs. 3.5 ± 0.3 mmHg.ml^{-1}.min^{-1}.100g, P< 0.05; MAP: 101 ± 2.2 mmHg vs. 99 ± 5.1 mmHg, P=NS), (figure 34).

F   Sham + placebo versus Sham + BB-1101

In the sham groups, treatment with BB-1101 had no significant effect on mean arterial pressure (MAP), systemic vascular resistance (SVRI) and cardiac index (CI) values in sham+BB-1101 animals compared with placebo-treated controls (MAP:114 ± 4.7 vs. 125 ± 6.4 mmHg, P = NS, SVRI: 7.2 ± 0.3 vs. 6.8 ± 0.6 mmHg.ml^{-1}min^{-1}.100g, P = NS, CI:16.0 ± 0.9 vs. 18.8 ± 1.8 ml.min^{-1}.100g^{-1}, P = NS). There was however a small but significant decrease in the portal pressure between the sham+BB-1101 group and the sham + placebo group (6.0 ± 0.6 vs. 8.7 ± 0.6 mmHg, p < 0.05), (figure 35).

G   PPVL+placebo versus PPVL+BB-1101

BB-1101 administration resulted in the abrogation of the hyperdynamic circulation following PPVL (figure 36). Animals treated with BB-1101 had a significantly lower cardiac index and portal pressure compared to placebo-treated animals (CI: 18.0 ± 1.4 ml.min^{-1}.100g^{-1} vs. 28.4 ± 1.5 ml.min^{-1}.100g^{-1}, P < 0.001; PP: 10.3 ± 0.7 mmHg vs. 14.7 ± 1.1 mmHg, P < 0.001), whereas the systemic vascular resistance and mean arterial pressure were increased (although mean arterial pressure did not reach statistical significance), (SVRI: 6.3 ± 0.8 mmHg.ml^{-1}.min^{-1}.100g vs. 3.5 ± 0.3 mmHg.ml^{-1}.min^{-1}.100g, P < 0.01; MAP: 109 ± 6.4 mmHg vs. 99 ± 5.1 mmHg, P = NS), (figure 35).
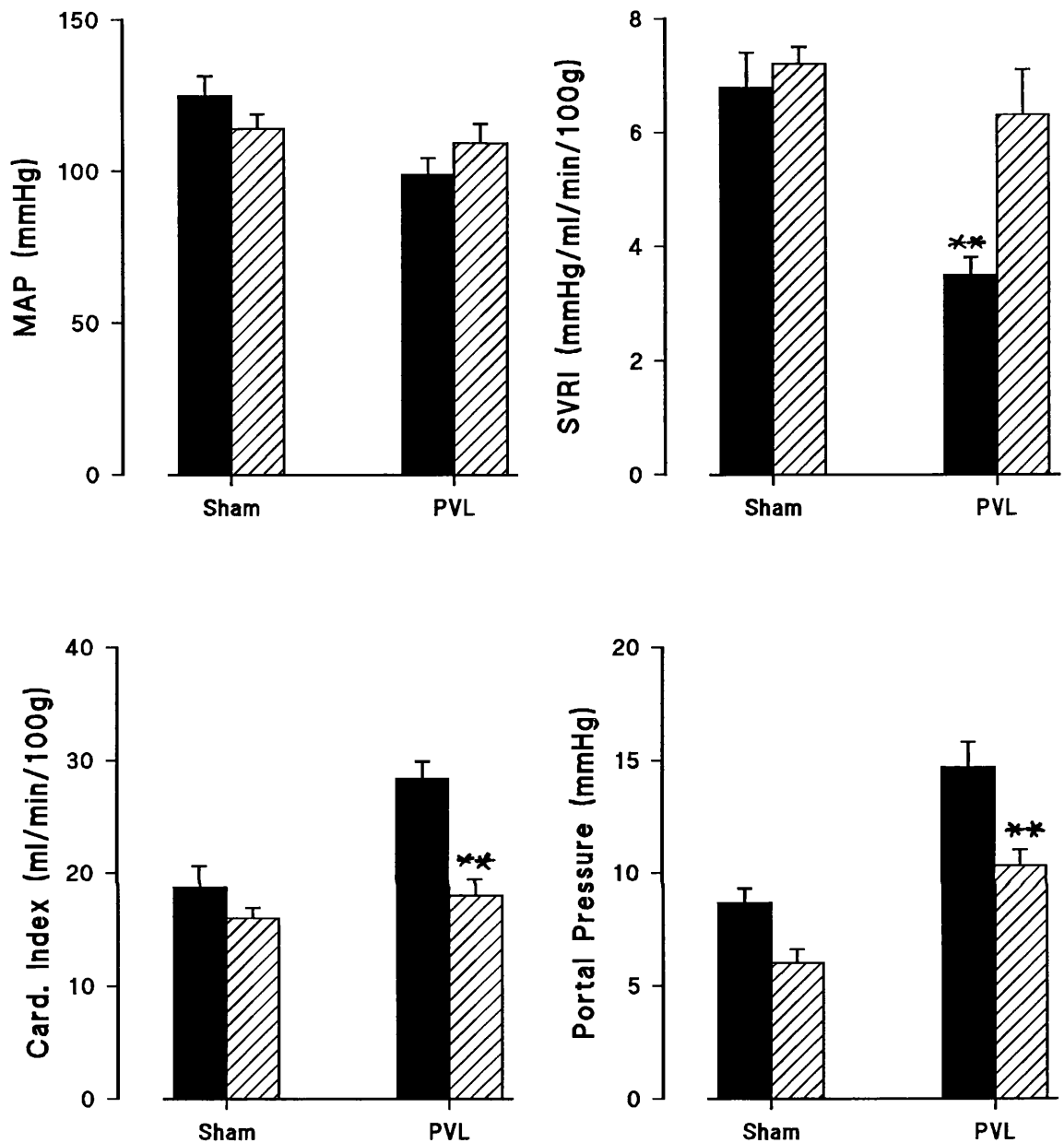
Figure 35. Haemodynamic effects of BB-1101 (hatched bars) compared to placebo (solid bars). BB-1101 treatment prevented the hyperdynamic response and reduced portal pressure in PPVL animals (** p < 0.05). The ANOVA p-value was <0.005 and the Bonferroni test was used for multiple comparisons.

study group	cardiac index (ml/min/100g)	mean arterial pressure (mmHg)	portal pressure (mmHg)	Systemic vascular resistance (mmHg/ml/min.100g)
sham-placebo (n=8)	18.8 ± 1.8	125 ± 6.4	8.7 ± 0.6	6.8 ± 0.6
PPVL-placebo (n=6)	28.4 ± 1.5	99 ± 5.1	14.7 ± 1.1	3.5 ± 0.3
sham-NAC (n=6)	16.6 ± 1.3	120 ± 3.3	7.0 ± 0.6	7.3 ± 0.4
pvl-NAC (n=6)	17.6 ± 1.4	109 ± 2.9	9.8 ± 0.3	6.3 ± 0.5
sham-BB-1101(n=8)	16.0 ± 0.9	114 ± 4.7	6.0 ± 0.6	7.2 ± 0.3
pvl-BB-1101 (n=7)	18.0 ± 1.4	109 ± 6.4	10.3 ± 0.7	6.3 ± 0.8
sham-pyrrol (n=6)	17.6 ± 1.0	125 ± 5.3	7.9 ± 0.4	7.2 ± 0.5
pvl-pyrrol (n=6)	22.4 ± 0.7	101 ± 2.2	11.4 ± 0.5	4.5 ± 0.2

Table 15. Summary of the Haemodynamic Studies

CHAPTER 4: GENERAL DISCUSSION

4.1 Discussion

Kowalski and Abelman first described the development of a hyperdynamic circulation in patients with chronic liver disease in 1953 (Kowalski and Abelman, 1953). It is associated with the development of portal hypertension and is believed to be an important determinant of many of the complications of chronic liver disease including variceal haemorrhage, portosystemic encephalopathy, ascites and renal failure. Therefore, the generation of the hyperdynamic circulation has been the subject of intense research.

In this thesis a model of pre-hepatic portal hypertension was studied. PPVL is a very useful model to use as it induces portal hypertension immediately and generates a hyperdynamic circulation after approximately 5 days which persists for several weeks. In addition to this there is minimal liver damage and a high degree of portosystemic shunting. As this model causes portal hypertension without liver damage the responses of different animals tend to be homogenous. This model is popular as it is straightforward to perform and is inexpensive. Therefore it has been studied extensively and the knowledge gained by other groups has been used in formulating the hypothesis to this work.

It is generally believed that the sequence of events leading to the development of the hyperdynamic circulation are that the liver disease results in abnormal vasoreactivity, with vasodilation and this leads to expansion of plasma volume which is critical to the development of the hyperdynamic circulation. The possible role played by several different mediators have been investigated including bile acids (Genecin et al. 1990a), glucagon (Benoit et al. 1986, Kravetz et al. 1988), adenosine (Murakami et al. 1996), endotoxin (Lee et al. 1996, Mehta et al. 1990), bacterial translocation (Garcia-Tsao et al. 1993) tumour necrosis factor (Lopez-Talavera et al. 1995, Lopez-Talavera et al. 1996), prostacyclin (Hamilton et al. 1982, Hamilton et al. 1981, Oberti et al. 1993) and nitric oxide. In cirrhosis the role of nitric oxide in the development is controversial as certain groups believe that a reduced sensitivity to vasoconstrictors, such as catecholamines, is important.

Several studies support the concept that increased formation of NO, is involved at least in part, in the vasodilatory changes that occur in animals with non-cirrhotic portal hypertension. These studies have demonstrated increased nitric oxide synthase (Cahill et al. 1996, Cahill et al. 1995, Niederberger et al. 1996) and increased production of nitrite and nitrate, the stable metabolism products of nitric oxide (Hori et al. 1995). Other studies have shown that inhibition of nitric oxide synthase or nitric oxide reverses the systemic hypotension and splanchnic vasodilatation in the PPVL model (Pilette et al. 1996a, Pilette et al. 1996b, Garcia-Pagan et al. 1994, Sogni et al. 1996, Sieber and Groszmann, 1992a, Sieber and Groszmann, 1992b, Lee et al. 1993). Induction of NO synthesis may be stimulated by endotoxin, TNF-α and interleukin-1 (Harbrecht et al. 1994, Hattori et al. 1996a). The observation that blocking the action of TNF-α using specific antibodies, prevents the development of the hyperdynamic circulation in PPVL rats demonstrates unequivocally that TNF-α or its downstream responses are key events in this process (Lopez-Talavera et al. 1995, Lopez-Talavera et al. 1996). TNF-α is produced principally by cells of the macrophage/monocyte lineage and plasma levels are elevated in the portal hypertensive rat (Lopez-Talavera et al. 1995). However, the factors which trigger the signal transduction pathways resulting in the production of TNF-α are unknown. The aim of identifying the signalling processes is to both increase our understanding of the disease pathophysiology and to develop therapeutic strategies to inhibit unwanted responses. These signal transduction pathways may involve the transcription factor NF-κB which can be activated by different mechanisms including viruses, phorbol esters, mitogens, ultra-violet light and, importantly, cytokines and reactive oxygen species. NF-κB activation is subject to redox regulation and reactive oxygen species are also involved in TNF-α signalling. Thus, NF-κB may be activated secondary to TNF-α or it may be involved in the induction of TNF-α. Therefore, we postulated that oxidant stress and the generation of reactive oxygen species may be an important factor in switching on this signal transduction pathway resulting in the synthesis of nitric oxide and the development of the hyperdynamic circulation in this model of portal hypertension. If this postulate is correct then the activation of NF-κB could be used as a potential therapeutic target in inhibiting the hyperdynamic response. We proposed that PPVL

would result in an oxidant stress which would alter the redox balance in cells resulting in NF-κB activation. This in turn would activate the signal transduction pathways for cytokines including TNF-α which may induce a further oxidant stress and activation of NF-κB, as well as the induction of nitric oxide synthesis. The net increased production of nitric oxide would thereby mediate the hyperdynamic response (figure 6). It remains unclear in this model as to whether the primary activator of NF-κB is oxidant stress or TNF-α. It is also unclear as to whether oxidant stress is a cause or a downstream effect of TNF-α activation.

To demonstrate the described sequence of events we initially determined whether oxidant stress occurred in this model. Urinary F_2-isoprostanes excretion was used as a marker of oxidant stress *in vivo*. These are prostaglandin-like compounds formed by the peroxidation of arachidonic acid and are formed independently of cyclooxygenase. Measurement of F_2-isoprostanes has emerged as one of the most reliable and specific approaches to assess lipid peroxidation *in vivo* (Roberts, 2nd and Morrow, 1994, Roberts, 2nd and Morrow, 1995). The demonstration that urinary F_2-isoprostanes are increased following PPVL is the first demonstration of oxidant stress in this model. Confirmation that ROS are formed in this model was the observation that N-acetylcysteine suppressed the increase of urinary F_2-isoprostane excretion in the treated PPVL group. N-acetylcysteine is a thiol-containing antioxidant which is used therapeutically in the treatment of paracetamol overdosage (Keays et al. 1991) and as an antimucolytic agent (Boman et al. 1983). It has also been used in heart failure after ischaemia-reperfusion (Qiu et al. 1990) and to protect the liver following hypothermic-reperfusion (Nakano et al. 1995). Although the precise mechanism of action of N-acetylcysteine is unclear, it is assumed to involve scavenging of ROS either directly as a thiol donor or indirectly by increasing glutathione synthesis, and detoxification of reactive oxygen species such as hydrogen peroxide.

F_2-isoprostane levels were also measured in plasma and liver. In the plasma level studies no significant differences were demonstrated between PPVL and sham operated animals. There was however a difference between the mean values of 16 pg/ml for the two groups. It could be argued that if this difference in mean values were correct then eventually a statistical difference may be observed if a much larger number of animals were studied. Using a statistical program

(Statmate, Graphpad, USA) it was calculated that approximately 125 animals in each group would have to be studied to demonstrate a difference of 16 pg/ml with a p-value of < 0.05 and 90% power assuming a standard deviation of approximately 40 pg/ml in each group. Clearly this hypothetical argument is based on extrapolating the results from a very small sample but it demonstrates that a very high number of animals would need to be studied. Therefore, it must be assumed that there is no significant difference between the groups. However, the fact that it is insignificant does not negate the findings from the urinary excretion of F_2-isoprostanes. It is generally accepted that urinary measurement of F_2-isoprostane levels over a set time period provides a more reliable index of overall lipid peroxidation than plasma levels and has been used in other studies (Reilly et al. 1996, Morrow et al.1992a). This is because urinary levels provide a time-integrated measurement of F_2-isoprostanes filtered and subsequently excreted by the kidney compared to the plasma F_2-isoprostane levels which provides a "snap-shot" image of circulating F_2-isoprostanes which are being continuously cleared from the body (Delanty et al. 1996).

Liver F_2-isoprostane levels were also measured in sham and PPVL animals. In the four animals studied in each group there was overall no statistical difference observed although again a trend was seen. Membrane-bound F_2-isoprostane levels were greater in the sham group compared to the PPVL group. This trend initially appears to be surprising since one may predict the opposite with increased levels in the PPVL group. However, it is important to realise that these represent membrane-bound F_2-isoprostanes which have not been cleaved from the membrane phospholipids. F_2-isoprostanes are formed from esterified arachidonic acid and exist as acyl moieties of membranes phospholipids. Unlike cyclooxygenase-derived prostaglandins which are formed *de novo* and are not stored, F_2-isoprostanes are released preformed from this membrane bound store. Oxidant stress is an ongoing process and occurs under physiological conditions resulting in the continued formation and storage of membrane-bound F_2-isoprostanes. However, in pathological conditions such as following PPVL F_2-isoprostanes will be formed at an increased rate. It is possible that under these conditions the enzyme phospholipase A_2 which cleaves the membrane phospholipids and releases F_2-isoprostanes could be upregulated in this

model resulting in a greater release of membrane bound F_2-isoprostanes from the liver (Morrow et al.1992b). This could explain the observed trend. Ideally more numbers in each group should be assessed to determine whether or not this trend becomes significant. Although no statistical difference was observed between sham and PPVL groups for plasma and liver tissue F_2-isoprostane levels it can still be concluded that this model is associated with increased lipid peroxidation by virtue of the urinary excretion results.

The mechanism of oxidant stress is unknown. The most likely explanation is that it is secondary to formation of TNF-α. TNF-α causes induction of nitric oxide synthase in a variety of cell types including hepatocytes, vascular smooth muscle cells and macrophages (Geng et al.1992, Adamson et al. 1991). Activation of macrophages or other inflammatory cell types by TNF-α or endotoxin also causes a respiratory burst and increased formation of superoxide (Bautista et al. 1991). The rate of reaction between nitric oxide and superoxide is rapid, and results in the formation of peroxynitrite (Beckman and Koppenol, 1996), which has previously been shown to initiate lipid peroxidation (Radi et al 1991) and the subsequent formation of F_2-isoprostanes (Moore et al. 1995). Furthermore peroxynitrite may rapidly decompose to produce hydroxyl radicals which would therefore cause further free radical injury (Beckman et al. 1990). Another possible explanation could be that the oxidant stress occurs secondary to an ischaemic-reperfusion-type process. Studies performed by Colombato et al. demonstrated that immediately after partial portal vein ligation there is a significant reduction in portal venous inflow compared to sham animals but by day 4 this is reversed such that the portal venous inflow is significantly greater than in sham operated animals (Colombato et al. 1992). The increase in portal venous inflow is as a result of sodium retention and plasma volume expansion caused by activation of the neurohumoral pathways (Albillos et al. 1992). As the liver has a dual afferent blood supply there is a degree of autoregulation of total liver blood flow. Therefore when the portal vein is constricted there is an autoregulatory compensation by the hepatic artery to maintain total hepatic flow. Nevertheless it is possible that the redox balance in relatively ischaemic areas of the liver or in the collateral circulation may be shifted in favour of oxidant stress. This may

explain why oxidant stress does not occur immediately after partial portal vein ligation but only following the plasma volume expansion and the increase observed in portal venous inflow.

Another explanation for the mechanism of oxidant stress could be as a result of bacterial translocation which has been demonstrated acutely. In these studies by Garcia-Tsao et al. rats undergoing PPVL were sacrificed at day 2 and were found to have a greater proportion of positive mesenteric lymph node cultures when compared to sham operated animals (Garcia-Tsao et al. 1993). However blood, liver and spleen cultures were negative. Fifteen days after the original surgery there were no differences between PPVL and sham animals in the mesenteric lymph node cultures. The mechanism by which bacterial translocation occurs acutely is probably by disruption of the intestinal mucosal barrier by acute venous congestion, oedema and ischaemia which are secondary to the acute stenosis of the portal vein. The bacterial translocation may result in priming of macrophages prior to a respiratory burst of activity involving the generation of reactive oxygen species.

A further PPVL group received treatment with pyrrolidine dithiocarbamate. This is a potent inhibitor of NF-κB activation. Pyrrolidine dithiocarbamate is thought to be an antioxidant and it is reported to have at least antioxidant properties (Schreck and Baeuerle, 1994). These are as a free radical scavenger and as a chelator for heavy metal ions which are needed for the catalytic decomposition of hydrogen peroxide to hydroxyl radicals. However, although these actions may contribute to the inhibitory effect on NF-κB activation it remains unclear as to the precise mechanism of inhibition by pyrrolidine dithiocarbamate like BB-1101. Treatment with pyrrolidine dithiocarbamate was associated with a significant increase in urinary excretion of F_2-isoprostanes compared to placebo treated-PPVL rats. This finding was opposite to the predicted observation implying that pyrrolidine dithiocarbamate increases lipid peroxidation. Recent work by one of our collaborators has questioned the antioxidant effects of pyrrolidine (Moellering et al. in press). They have demonstrated that pyrrolidine does not act as an antioxidant but acts by binding directly to the NF-κB molecule therefore preventing it from binding to target genes in the nucleus. Furthermore at high doses pyrrolidine acts as a pro-oxidant and also causes apoptosis. This may explain the observed results in the current study.

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The pharmacological effects of other agents were also investigated. BB-1101 is a drug which has been developed by British Biotech with a combination of effects including inhibition of matrix metalloproteinases and TNF-α. It has been shown to be beneficial in an animal model of multiple sclerosis by reducing the severity of disease and weight loss compared with a placebo treatment (Clements et al. 1997). It has also been shown to inhibit the release of TNF-α following *in vitro* and *in vivo* following LPS stimulation. Since TNF-α may induce an oxidant stress by its induction of NO which can combine with superoxide to form peroxynitrite, a highly reactive nitrogen species, it is possible that the peroxidation of arachidonic acid and thus formation of F_2-isoprostanes is secondary to production of this cytokine. If this were the case one would anticipate a reduction in oxidant stress by inhibiting TNF-α production using BB-1101. In fact an opposite observation was made and BB-1101 treatment resulted in increased excretion of F_2-isoprostanes indicative of increased oxidant stress. Therefore, under these conditions BB-1101 is behaving as a pro-oxidant. Clearly, more information needs to be obtained about its mechanism of action. Specifically, how it causes inhibition of TNF-α activation and what other actions it has.

The next stage was to study the activation of NF-κB. NF-κB is a nuclear transcription factor is a central regulator of inflammatory and immune reactions by virtue of the fact that it plays a crucial role in the regulation of numerous target genes. NF-κB exists as a protein heterodimer comprising of a p-50 and p-65 subunit (Baeuerle and Henkel, 1994). In most cells NF-κB activity is low or undetectable and is stored within the cytoplasm bound to an inhibitory protein, I-κB as its inactive form (Rice et al. 1993). When the cells are subjected to inflammatory stimuli such as phorbol esters, TNF-α or hydrogen peroxide, NF-κB becomes activated by dissociating from the inhibitory protein and translocating to the nucleus of the cell. In the nucleus it binds to promoter regions of target genes and promotes transcription of several proteins including TNF-α, IL-1, IL-6, IL-8, and inducible NOS (Spink et al. 1995). Thus activation of NF-κB may be central to both the transcription of TNF-α as well as the induction of nitric oxide synthase thereby increasing NO synthesis with the subsequent development of the hyperdynamic circulation. Several studies have demonstrated that activation of NF-κB can be

inhibited by N-acetylcysteine, pyrrolidine dithiocarbamate or other antioxidants *in vitro* (Hattori et al. 1996b, Kalebic et al. 1991, Mihm et al. 1991) but there had been no published studies using *in vivo* models when this thesis was commenced.

NF-κB activation was studied in liver since this was considered to be a likely site for activation. NF-κB was clearly activated in the liver following PPVL. To determine whether pharmacological agents inhibited NF-κB activation *in vivo*, groups of PPVL rats receiving treatment with N-acetylcysteine, pyrrolidine and BB-1101 were studied. The *in vitro* inhibitory effects of antioxidants have been previously mentioned but since TNF-α results in NF-κB activation then it could be postulated that inhibitors of TNF-α secretion may also result in suppressed NF-κB activation. Therefore, the effect of BB-1101 was also investigated.

The activation observed in PPVL liver was inhibited by treatment with N-acetylcysteine *in vivo* indicative of the involvement of reactive oxygen species in this process. In animals treated with pyrrolidine there was a significantly reduced activation of NF-κB compared with placebo-treated PPVL animals. However, the mechanism of reduced activation was likely to be by the pyrrolidine binding directly to NF-κB preventing it from binding to target genes (Moellering et al, in press). Treatment with the metalloproteinase inhibitor BB-1101 which is also an inhibitor of TNF-α secretion also showed a trend in inhibition of NF-κB activation although this did not reach statistical significance. Four animals were studied in this group since 2 died prematurely. However, it must be assumed that BB-1101 does not affect NF-κB activation.

Others groups have demonstrated increased TNF-α levels following PPVL compared to sham operation using a bioassay, although the range observed in these experiments was widespread (Lopez-Talavera et al. 1995). In the current studies we determined TNF-α levels using two commercially available ELISA kits. The Genzyme kit produced levels which were approximately ten-fold greater than values detected using the ultra-sensitive Serotec ELISA. Since both assays were run by the same person and kit standards were used in each assay a possible explanation for the discrepancy between the two results is that the specimen values

obtained are in the lower part of the standard curve and are therefore inaccurate. When TNF-α levels in the PPVL group were compared with sham animals using the ultrasensitive ELISA kit, the levels detected were again at the low end of the standard curve and were insufficiently high above background to determine a clear effect of N-acetylcysteine. This is consistent with the notion that TNF-α may exert a biological effect *in vivo* even if it is undetectable in the circulation (Lopez-Talavera et al. 1995). Furthermore, the lack of correlation between the plasma levels of TNF-α and the haemodynamic status has been described by several groups (Lopez-Talavera et al. 1995, Sakurai et al. 1993, Calandra et al. 1990). This is probably due to the fact that the TNF-α effect is amplified by a cascade of events which in turn are mediated by other cytokines and metabolic products. In addition to this the processes initiated by TNF-α may persist for a relatively long time after TNF-α is no longer present in the blood (Lopez-Talavera et al. 1995)

The stimulus causing macrophage activation and TNF-α secretion in this model is unknown. Endotoxaemia has not been observed in arterial samples obtained from rats that have undergone PPVL (Mehta et al. 1990, Lee et al. 1996). However, bacterial translocation has been observed in mesenteric lymph nodes (Garcia-Tsao et al. 1995, Sorell et al. 1993) as well as in blood cultures (Sorell et al. 1993) in this model. Thus, macrophages may have been activated within the lymph nodes even in the absence of circulating endotoxin. However, treatment with antibiotics has no influence on the systemic and splanchnic haemodynamics following PPVL suggesting that gut derived toxins are not important in this process (Mehta et al. 1990, Garcia-Tsao et al. 1995, Sorell et al. 1993, Lee et al. 1996).

It has been proposed that excessive NO mediates the generation of the hyperdynamic circulation of portal hypertension (Pizcueta et al. 1992, Garcia-Pagan et al. 1994, Pilette et al. 1996a). The observation in this study that plasma nitrite and nitrate are increased following PPVL support other data indicative of increased NO synthesis (Cahill et al. 1996, Hori et al. 1995). Increased synthesis of NO is presumably a consequence of TNF-α production which causes induction of nitric oxide synthase. However, in these studies we failed to demonstrate a significant effect of N-acetylcysteine and pyrrolidine on plasma nitrite and nitrate levels. What

was observed were trends which appeared to correspond with the urinary excretion values of F_2-isoprostanes. N-acetylcysteine showed a trend towards lower plasma nitrite and nitrate whereas pyrrolidine showed the opposite. The effect of treatment with BB-1101 caused an increased circulating plasma level of nitrite and nitrate compared to placebo-treated PPVL rats indicative of increased nitric oxide synthesis. This may explain why there was an increased urinary excretion of F_2-isoprostanes as the increased nitric oxide levels may have enhanced peroxynitrite production (Radi et al. 1991) and its subsequent effects on lipid peroxidation (Moore et al. 1995) One would therefore expect this drug to result in a worsening of the hyperdynamic circulation if this was mediated purely by nitric oxide. It therefore remains unclear as to whether nitric oxide is the key mediator of the hyperdynamic circulation or whether other mediators are just as important.

The initial observations that the oxidant stress and activation of NF-κB occur in the portal hypertensive rat and are inhibited by N-acetylcysteine, lead to the natural question as to whether long term administration of this drug could, by blocking the signalling pathways involved in cytokine activation, prevent the development of the hyperdynamic circulation. However, the effects of chronic administration of N-acetylcysteine have not previously been studied in models of portal hypertension or cirrhosis. It was also interesting to discover if other drugs that inhibit NF-κB and TNF-α could also abrogate the hyperdynamic circulation following PPVL since according to the hypothesis both proteins are postulated to have significant roles in the haemodynamic response. Pyrrolidine was used as it was shown to inhibit NF-κB in an experimental model of glomerulonephritis (Sakurai et al. 1996). BB-1101 was chosen as it was shown previously to have anti-TNF-α effects (Clements et al. 1997) although in the PPVL studies we failed to demonstrate anti-TNF-α effects.

The demonstration that N-acetylcysteine prevents the development of the hyperdynamic circulation as well as portal hypertension in the PPVL rat strongly suggests that oxidant stress is important in these changes in this experimental model. The findings that both pyrrolidine and BB-1101 prevents the hyperdynamic circulation are both interesting since both treatments

resulted in increased oxidant stress in this model. They may however help to identify the level at which oxidant stress generates the hyperdynamic state. Pyrrolidine resulted in increased F_2-isoprostane excretion but inhibited NF-κB. The expected downstream effects of this would be the reduced mediation of the hyperdynamic circulation by nitric oxide but in fact there were elevated levels of nitrite and nitrate indicative of increased nitric oxide. It is therefore likely that another mediator or factor is involved in the process. A similar discrepancy was observed in the BB-1101-treated group. There was a significantly higher urinary F_2-isoprostane excretion indicative of increased oxidant stress, reduced activation of NF-κB activation but increased circulating plasma nitrite and nitrate. This also implies that an additional mediator separate from nitric oxide is important in mediating the hyperdynamic circulation.

Currently, it is generally believed that the predominant site of vasodilatation involves the mesenteric vascular bed (Cahill et al. 1996, Ghosh and Baltimore, 1990) which will increase portal venous inflow. Lopez-Talavera and colleagues also observed that anti-TNF-α antibody administration and thalidomide treatment decreased portal pressure (Lopez-Talavera et al. 1995, Lopez-Talavera et al. 1996). In the current studies portal pressure was also decreased by chronic treatment with N-acetylcysteine, pyrrolidine and BB-1101. This suggests the hyperdynamic circulation is important in sustaining elevated portal pressure following PPVL, presumably by increasing portal venous inflow since the mechanical obstruction remains unaltered.

4.2   Criticisms and Possible Areas for Future Work

In these studies approximately 10% of the animals died during the experimental period. This either happened during the first three days after surgery or prematurely during the haemodynamic study. Post-mortem examinations were performed where possible and in the majority of the early cases it appeared that the cause of death was through peritonitis secondary to perforation of the bowel. This was likely to be caused by the intraperitoneal injections which were the route of administration for three of the four groups. There was a higher likelihood of causing peritonitis in the PPVL group since the bowel was oedematous and ischaemic during the early post-operative period. When the animals died during the haemodynamic studies it

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appeared that this was as a result of overdosage with sodium pentobarbitone despite taking the precautions of carefully weighing the animals and giving the appropriate dose by weight. The fact that animals died during the experimental period is of significance as it may be a source of bias. Animals with a greater morbidity may not survive long enough to be studied and an argument can be put forward that these animals are the important ones to study as they may reflect inadequacies of the treatments. Surprisingly, there is a paucity of published data relating to the mortality of this particular model.

Future work progressing from this thesis should pursue the role of antioxidant therapy in the abrogation of the hyperdynamic response. The first stage should involve studying other models of portal hypertension such as the bile duct ligated rat or carbon tetrachloride-induced cirrhotic rat. One study has already shown a reduced antioxidative capacity in liver mitochondria from bile duct ligated rats suggestive of an increased oxidant stress in these animals (Krahenbuhl et al.1995). A more recent study investigating the antioxidant enzyme status in treated bile duct ligated rats demonstrated as an incidental finding a significant reduction in portal pressure and spleen weight in chronic N-acetylcysteine-treated animals (Pastor et al. 1997). Unfortunately, no other haemodynamic measurements were made in this paper but it would be of considerable interest to determine if other haemodynamic variables were altered too. It is already known that the carbon tetrachloride model of cirrhosis is associated with significant lipid peroxidation (Morrow et al. 1992b) and activation of NF-κB which can be prevented by treatment with vitamin E (Liu et al. 1995). As the aim of this particular study was to establish a correlation between NF-κB activation and liver cell injury no haemodynamic measurements were made. However, a more recent study by Lopez-Talavera et al. demonstrated that the hyperdynamic circulation in carbon tetrachloride-induced cirrhotic rats could be ameliorated by the administration of tyrphostins (Lopez-Talavera et al. 1997). These are specific inhibitors of protein tyrosine kinase and they block the signalling effects induced by TNF-α and nitric oxide production. It would be very interesting to investigate whether antioxidant treatment has any

beneficial haemodynamic effects in this model since the mechanism of blocking the signal transduction pathways may be similar.

N-acetylcysteine and other antioxidants could be used separately or in combination in different models to find the ideal therapy for manipulation of the hyperdynamic circulation. In addition it would of clinical relevance to investigate the therapeutic effect of these drugs following the development of the abnormal circulatory changes rather than trying to prevent the changes from occurring as has been done in this thesis.

With respect to the current model it is important to identify another mediator or mediators which could have been manipulated by pharmacological treatment to abrogate the hyperdynamic response. It is highly likely that there are other mediators as both pyrrolidine and BB-1101 both abrogated the hyperdynamic response without reducing lipid peroxidation. Prostacyclin is a potential candidate as it has vasodilatory properties and levels have been shown to be increased in PPVL rats (Hamilton et al. 1981, Hamilton et al. 1982). Moreover, administration of indomethacin may attenuate the hyperdynamic circulation (Sitzmann et al. 1989, Sitzmann et al. 1991, Bruix et al 1985). An increase in prostacyclin production is secondary to increased activity of constitutive cyclooxygenase enzyme (COX-1) or by the expression of the inducible form of cyclooxygenase (COX-2). It should be noted that activity of COX-1 and the constitutive form of nitric oxide synthase (cNOS) can be stimulated by common physicochemical factors such as shear stress and neuropeptides (Hecker et al. 1993, De Nucci et al.1988) and that expression of the inducible isoenzymes can be activated by common proinflammatory agents such as endotoxin and cytokines (Masferrer et al.1992, Knowles et al. 1990). Other studies have shown that cyclo-oxygenase II expression is upregulated by reactive oxygen species (Feng et al. 1995). This suggests that prostacyclin production may be suppressed by antioxidants. It would have been an interesting study to measure prostacyclin levels in the study groups as this is possibly an important mediator in the mediation of the hyperdynamic circulation.

It has also been suggested that a humoral substance with vasoactive properties may mediate the hyperdynamic circulation. Such a substance would be produced by the gut and escape hepatic inactivation via intrahepatic or extrahepatic shunting such that it has elevated levels in portal hypertension. There are many substances which fulfill part or all of these criteria including histamine, 5-hydroxytryptamine, vasoactive intestinal polypeptide and glucagon. Among these glucagon has been studied most vigorously. This 29-amino acid peptide is secreted by the pancreatic-α cells and the oxyntic mucosa of the stomach and it is inactivated by the liver. It is elevated in patients and animals with portal hypertension (Marco et al. 1973, Sherwin et al. 1974, Ikeda et al. 1986). In addition to its well known effect on carbohydrate metabolism glucagon also has a vasodilatory effect and may be a cardiac inotrope at pharmacological doses (Silva et al. 1990). However at physiological doses its vasoactive properties are less clear (Sikuler et al. 1987 and Pizcueta et al. 1991). Whether glucagon is a mediator of the hyperdynamic circulation is controversial since the levels observed in portal hypertensive animals are a third of the levels which would induce haemodynamic changes in normal animals (Cerini et al. 1989, Pizcueta et al. 1990, Benoit et al. 1984). Also after a period the vasoactive effects of glucagon appear to diminish as a form of desensitisation occurs (Silva et al. 1990 and Lee et al. 1988). It remains possible that glucagon may be an important mediator in the induction of the hyperdynamic circulation but it is unlikely to be involved in its maintenance. However, it is possible that another gut-derived mediator may be of importance.

Before determining the identity of these additional elusive mediators it is clear that more information about the mechanism of action of the drugs used in these studies. The antioxidant action of pyrrolidine for example is being challenged and recent data suggests that it acts as a prooxidant (Moellering et al. in press). BB-1101 is a research drug and could have additional actions to its documented anti-TNF-α effects and inhibitory effects of matrix metalloproteinases. Furthermore it would have been ideal if the numbers in the BB-1101 group were increased to six since four precluded adequate statistical analysis. Additional information about the actions of these drugs could be achieved by altering the dose of the drugs administered and by studying other models of portal hypertension or by cell culture studies.

In this thesis I have demonstrated NF-κB activation only in whole liver. The reason why whole tissue was studied instead of individual cell types was due to concerns that activation of NF-κB may occur during the cell extraction process. Clearly it would be interesting to know which cell type or types are exhibiting NF-κB activation but unfortunately this was not studied during this period. Of particular interest would be whether the Kupffer cells are the predominant liver cell involved. Kupffer cells are the resident macrophages of the liver and account for 15 % of the total number of liver cells and 2.5 % of the cellular content of the liver (Kuiper et al. 1994). The normal function of Kupffer cells is to remove particulate and other foreign materials form portal blood by phagocytosis. When they are activated Kupffer cells produce ROS, cytokines and proteolytic enzymes and it is likely that this is secondary to activation of NF-κB.

NF-κB activation in other tissues and organs were not formally assessed. It would have been of interest to assess this in the superior mesenteric artery and portal vein since this was where there appeared to be the greatest flux in blood flow immediately after PPVL and in the subsequent period (Colombato et al. 1992). Another interesting question to answer would be whether other organs such as the kidneys and spleen were involved or whether the activation of NF-κB was limited purely to the liver.

As far as the role of TNF-α is concerned it would be useful to find a more reliable method of demonstrating TNF-α activity since the ELISA kits do have problems in detecting inactive and active TNF-α. An alternative means of demonstrating TNF-α would be by performing Northern blotting in extracted Kupffer cells to detect the degree of TNF-α mRNA. This too is unable to distinguish between the active and inactive forms of TNF-α but would merely show if there is upregulation of TNF-α mRNA transcription. The bioassay does overcome these problems to a certain extent as it detects only biologically active TNF-α. When TNF-α levels were determined by Lopez-Talavera using a bioassay they found that there was a 5-10 fold increase in biological activity of TNF-α following PPVL compared to sham operated

animals. However, it is important to realise that levels do not have to be increased for TNF-α to exert a biological effect as it may behave in a paracrine fashion. Furthermore the processes initiated by TNF-α, which include amplification by other cytokines and metabolic products, may persist for a relatively long period of time after TNF-α is no longer in the blood.

In this thesis haemodynamic responses have been evaluated in anaesthetised animals. These animals behave differently to unanaesthetised animals in that they have a lower cardiac output and the distribution of this cardiac output may be significantly different resulting in alterations of regional blood flows to different organs. Obviously the degree of these differences depends on the type of anaesthetic used as well as the dosage. In these studies we used sodium pentobarbitone which has previously been shown to maintain the hyperdynamic response in PPVL rats compared to sham operated rats. Although this reagent induces a significant reduction in the cardiac index it does not alter the regional blood flow to different organs and the splanchnic bed (Lee et al. 1985).

Although there are several methods available for determining cardiac output, in these studies I employed the thermodilution method. Other means of determining cardiac output include injection of radiolabelled microspheres (McDevitt et al. 1976, Malik et al 1976 and Ishise et al. 1990), injection of coloured microspheres (Coleman et al. 1974 and Matsunaga et al. 1980), injection of rubidium chloride (Mendell et al. 1971), use of electromagnetic or ultrasonic probes and the direct Fick principle. Radiolabelled microspheres pose problems with sample handling and disposal of radioactive substances. Furthermore, this method does not allow multiple measurements since the radiolabelled microspheres can only be injected once per animal unless different isotopes are used. In addition to this, the solutions used to inject the microspheres may exert a significant hypotensive effect (Stanek et al. 1983). Electromagnetic flow probes and ultrasonic Doppler flow probes are expensive and necessitate a thoracotomy on the animal for implantation around the aorta with the additional risk of damaging the nerve fibres which may be important in cardiac regulation. However, once in place these flow probes

allow continuous monitoring and repeated determinations to be made in unanaesthetised animals although only one animal can be studied at a time unless several probes are available. On the basis of these limitations and also the fact that the budget did not allow purchase of a cardiac output computer the thermodilution method using the Maclab computer system was developed. This gave values which were comparable to values obtained for cardiac output using other methods. However, this method also had its limitations. The main problem was of heat diffusion of the thermal indicator, which may have caused an overestimation of cardiac output especially in smaller animals (Kissling et al. 1993). Although this was reduced by minimal handling of the syringe and by keeping the thermal indicator at room temperature this source of error was inevitable. Another obvious problem was that repeat bolus injections of indicator influenced the cardiac output as well as the temperature of the animal (Mannesmann and Muller, 1980) as a function of volume and temperature of the injectate. Therefore, it was necessary to keep the number of bolus injections to a minimum and to continuously monitor the temperature of the animals. Finally, the fact that the animal had multiple cannulation of major vessels (carotid artery, jugular vein, femoral artery and a portal vein tributary) would itself cause an alteration in cardiac output and systemic vascular resistance. This source of error was unavoidable but since the animals undergoing haemodynamic studies were of similar weights it was expected that similar alterations would be seen in all the animals such that this error would not preclude comparisons in haemodynamic variables.

The eventual aim from these studies is to perform clinical trials investigating the effect of chronic administration of N-acetylcysteine, or other antioxidant therapy, in patients with chronic liver disease and to reduce the severity and complications of portal hypertension. The haemodynamic effects of N-acetylcysteine have already been evaluated in patients with liver disease. One study performed in patients with fulminant hepatic failure demonstrated that an acute injection of N-acetylcysteine caused a further reduction in systemic vascular resistance and an increase in cardiac index together with an increased oxygen delivery to liver tissue thereby accounting for its beneficial effect in preventing hepatic necrosis after paracetamol overdosage

(Harrison et al. 1991). In another acute study performed in stable cirrhotic patients similar haemodynamic effects were found with an increase in cardiac index and a reduction in systemic vascular resistance, following a 30 minute infusion of N-acetylcysteine, but this did not cause any benefit in oxygen delivery (Jones et al. 1994). It is interesting to note that in these two acute studies the haemodynamic effects of N-acetylcysteine are opposite to the findings in this thesis where N-acetylcysteine was administered chronically. A possible reason for this difference could be due to the presence of nitric oxide forming complexes with proteins. In the cirrhotic these nitric-oxide adducts such as nitrosoalbumin would circulate in the blood and the nitric oxide would be released by the administration of N-acetylcysteine when the thiol group binds to the protein causing nitric oxide displacement. If N-acetylcysteine is given acutely then this would result in a sudden release of nitric oxide from these protein adducts and cause significant vasodilatation as observed in the acute infusion study. The fact that the acute administration of N-acetylcysteine causes vasodilatation is not new since this is well known to reverse nitroglycerin tolerance (Torresi et al. 1985, Packer et al. 1987 and Pizzuli et al. 1997). If however, N-acetylcysteine is administered chronically then these nitric oxide-protein adducts may not be allowed to form since the thiol groups would prefentially bind to the proteins. This would explain the increase in systemic vascular resistance in the PPVL studies. In other words chronic administration of N-acetylcysteine prevents the formation of nitric oxide adducts which under other conditions act as a store for nitric oxide. Meanwhile, the nitric oxide produced would be immediately metabolised and rendered inactive on the endothelium.

It is therefore apparent that there are obvious differences between acute and chronic administration of N-acetylcysteine. The haemodynamic effects of chronic administration of N-acetylcysteine in cirrhotic patients are yet to be evaluated. Clearly this would be an interesting area for future work.

4.3   Conclusions

It can be concluded that formation of reactive oxygen species causing oxidant stress and activation of NF-κB may have an important role in the development of haemodynamic

abnormalities following PPVL. Whilst it is probably via a cytokine-dependent pathway this was not confirmed in these studies. Furthermore, the mediator may or not be nitric oxide. Since both pyrrolidine and BB-1101 also caused abrogation of the hyperdynamic response with an apparent increase in oxidant stress and nitric oxide production, it can also be concluded that factors other than oxidant stress and nitric oxide can cause these haemodynamic changes.

However, the observation that chronic administration of the antioxidant N-acetylcysteine to PPVL animals prevents the development of the hyperdynamic circulation and reverses portal hypertension opens a further avenue for potential therapy of portal hypertension.

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