PULMONARY AND RENAL NITRIC OXIDE METABOLISM FOLLOWING INFRARENAL AORTIC CROSS - CLAMP INDUCED ISCHAEMIA - REPERFUSION INJURY

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STATEMENT OF ORIGINALITY

The work on which this dissertation is based is my own independent work except where acknowledged

Mr Ravi Pararajasingam

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ABBREVIATIONS

< or >	less than or greater than
%	per cent
AAA	abdominal aortic aneurysm
ALI	acute lung injury
AOD	aortic occlusive disease
ARDS	adult respiratory distress syndrome
ATP	adenosine triphosphate
BAL	broncheoalveolar lavage
BSA	bovine serum albumin
cGMP	cyclic guanosine monophosphate
cNOS	constitutive nitric oxide synthase
COAD	chronic obstructive airway disease
CR	counts per minute
CT	computed tomography
DNA	deoxyribunucleic acid
DTPA	diethylene triamine penta-acetic acid
EDRF	endothelium derived relaxing factor
EDTA	ethylene diamine tetra-acetic acid
ELAM	endothelial leucocyte adhesion molecule
ELISA	enzyme linked immunoabsorbent assay
EVAR	endovascular abdominal aortic aneurysm repair
eNOS	endothelial nitric oxide synthase
Fe	iron
GFR	glomerular filtration rate
GMP	granulocyte membrane protein
GTN	glyceryl trinitrate
H2	histamine 2 receptor
H^+	hydrogen ion
Ig	immunoglobulin

IL-	interleukin
iNOS	inducible nitric oxide synthase
IRI	ischaemia-reperfusion injury
IVC	inferior vena cava
ICAM	intracellular adhesion molecule
K^+	pottasium ion
kDa	kilodalton
LECAM	lectin adhesion molecule
LDL	low density lipoprotein
L-NAME	NG L-arginine methyl ester
L-NMMA	NG monomethyl-L-arginine
LVEF	left ventricular ejection fraction
MBq	megabequerel
MI	myocardial infarction
MMP	matrix mettaloprotease
MOF	multiple organ failure
MPO	myeloperoxidase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
Na	sodium ion
NADPH	nicotinamide adenine dinucleotide
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2	nitrogen dioxide
NO_2^-	nitrite anion
NO_3^-	nitrate anion
NOC-18	Z-1-2Aminoethyl-(2 ammonioethyl) amino diazen-1-ium 1,2-diolate
NOS	nitric oxide synthase
O_2	oxygen
$\cdot O_2^-$	superoxide free radical

$^{\cdot}OH$	hydroxyl free radical
OH^{-}	hydroxyl anion
$ONOO^{-}$	peroxinitrite
PAF	platelet activating factor
PBS	phosphate buffered saline
PG	protaglandin
PTFE	polytetrafluoroethylene
RAAA	ruptured abdominal aortic aneurysm
RNA	ribonucleic acid
SIRS	systemic inflammatory response syndrome
SOD	superoxide dismutase
TA2	thromboxane A2
TBS	trisma buffered saline
Tc	technecium
TGF-β	tumor growth factor beta
TIMP	tissue inhibitors of metalloproteases
TNF-α	tumor necrosis factor alpha
UK	United Kingdom
USA	United States of America
XO	xanthine oxidase
1400W	3 aminomethyl benzyl acetamide

PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS THESIS

PRESENTATIONS TO LEARNED SOCIETIES

1. Renal nitric oxide metabolism following infrarenal aortic cross-clamp induced ischaemia-reperfusion injury.
   R Pararajasingam, SC Weight, PRF Bell, ML Nicholson and RD Sayers
   Surgical Research Society , Dublin, July 1998.

2. Influence of infrarenal aortic cross-clamp time on pulmonary nitric oxide metabolism.
   R Pararajasingam, SC Weight, PRF Bell, ML Nicholson and RD Sayers
   Vascular Prize session
   European Society for Cardiovascular Surgery, Paris, September 1998.

3. Renal nitric oxide metabolism following infrarenal aortic cross-clamp induced ischaemia-reperfusion injury.
   R Pararajasingam, SC Weight PRF Bell, ML Nicholson and RD Sayers
   Vascular Prize session
   European Society for Cardiovascular Surgery, Paris, September 1998.

4. Pulmonary nitric oxide metabolism following infrarenal aortic cross-clamp induced ischaemia-reperfusion injury.
   R Pararajasingam, SC Weight, PRF Bell, ML Nicholson and RD Sayers
   European Society for Vascular Surgery, Paris, September 1998.
   Winner: European Society Vascular Surgery Prize for Best Experimental Paper

5. Pulmonary nitric oxide metabolism following infrarenal aortic cross-clamp induced ischaemia-reperfusion injury.
   R Pararajasingam, SC Weight, PRF Bell, ML Nicholson and RD Sayers
   Midlands Vascular Society, Derby, March 1999.

6. Prevention of acute renal failure following aortic aneurysm repair by manipulation of of the endogenous renal nitric oxide response.
   R Pararajasingam, SC Weight, PRF Bell, ML Nicholson and RD Sayers
   European Society for Vascular Surgery, Copenhagen, September 1999.

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2. Influence of infrarenal aortic cross-clamp time on acute lung injury and endogenous pulmonary nitric oxide metabolism.
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2. Renal nitric oxide metabolism following experimental infrarenal aortic cross-clamp induced ischaemia reperfusion injury.
   R Pararajasingam, SC Weight, ML Nicholson, PRF Bell and RD Sayers
   British Journal of Surgery 1999, **86,** 795-799.

3. Pulmonary nitric oxide metabolism following infrarenal aortic cross-clamp induced ischaemia reperfusion injury.
   R Pararajasingam, SC Weight, ML Nicholson, PRF Bell and RD Sayers
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4. Prevention of acute renal failure following aortic aneurysm repair by manipulation of the endogenous nitric oxide response.
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CHAPTER ONE

Introduction

1.1 Abdominal aortic aneurysms

Abdominal aortic aneurysms (AAA) occur in about 3% of the population over the age of 50 years and AAA rupture accounts for 10000 deaths per annum in England and Wales (Office for national statistics, London 1995; Patel *et al.*, 1995). Although aneurysms have been recognised since the biblical ages, with reference to the diagnosis and treatment of traumatic aneurysms dating back to 2000BC, (Osler, 1905) successful surgical treatment has only been possible in the latter half of this century following the first successful AAA resection in 1951(Dubost *et al.*, 1952).

An arterial aneurysm is defined as a permanent localised dilation of an artery of more than 50% of the normal diameter of the artery in question (Johnston *et al.*, 1991). However, arteriomegaly or diffuse arterial enlargement are common among patients with aneurysms (Thomas, 1971), therefore a more practical definition of an aneurysm is a dilation of more than twice the size of the proximal artery. Although aneurysms may be classified according to their shape, size, structure, anatomical location, aetiology and underlying causative pathology, the most common type of aneurysm is the non-specific aneurysm which is often referred to as an atherosclerotic aneurysm and commonly affects the infrarenal aorta (Reed *et al.*, 1992) (Figure 1.1).

The prevalence of AAAs can be obtained from population based screening surveys and autopsy studies. In screening studies the prevalence of AAAs in English men varies from 1.3-12.7 %, depending on the age group screened and the criteria used to define AAAs (Collin *et al.*, 1988; Loh *et al.*, 1989; Lucarotti *et al.*, 1993; Scott *et al.*, 1995; Smith *et al.*, 1993). Similar prevalence data ranging from 4.1- 10.7% have been reported in North America and Western Europe (Wilmink & Quick, 1998). The prevalence from autopsy records varies from 1.4-4.3% in men and from 0.5-2.1% in women (Pleumeekers *et al.*, 1994). Abdominal aortic aneurysms are more prevalent among patients with co-existing vascular diseases. Five percent of patients with symptomatic coronary artery disease have an AAA, and 10% of patients with peripheral vascular disease have AAAs (Allardice *et al.*, 1988; Cabellon *et al.*, 1983). The prevalence of AAAs among patients with peripheral aneurysms has been found to be up to 50% (Anton *et al.*, 1986). Allen et al, (1987) reported a 5.3 % prevalence of AAA in hypertensive patients (Allen *et al.*, 1987).

The incidence of asymptomatic AAAs varies between 3.0 and 117.2 per 100,000 person years (Castleden & Mercer, 1985; Fowkes *et al.*, 1989; Melton *et al.*, 1984; Naylor *et al.*, 1988; Pleumeekers *et al.*, 1994). Most studies have reported a rise in the age related

incidence of AAAs in recent years which may be due to the increasing use of ultrasound scanning during investigation of other pathologies (Wilmink & Quick, 1998). The incidence of ruptured AAAs varies from 1 to 21 per 100,000 person years (Budd *et al.*, 1989; Castleden & Mercer, 1985; Drott *et al.*, 1992; Ingoldby *et al.*, 1986; Mealy & Salman, 1988; Thomas & Stewart, 1988). There has been an increase in the reported incidence of ruptured AAA, with one study reporting a sevenfold increase over 36 years. The age standardised mortality rate for ruptured AAA rose by 2.4% per year, which may represent a true increase in the incidence of ruptured AAA (Drott *et al.*, 1992).

The risk factors for AAAs are age, male sex, family history, previous vascular disease, hypertension and smoking. Wilmink et al (1998) have studied the influence of several co-existing risk factors for AAAs in published population based screening surveys (Table 1.1). The risk factors most strongly associated with AAA were male sex and smoking. Patients with peripheral vascular disease and cardiovascular disease were found to be twice as likely to have an AAA. Hypertension was found to be associated with a mildly increased risk of developing an AAA. Diabetes and cerebrovascular disease did not have a positive association with AAA (Wilmink & Quick, 1998).

Risk factor	Relative risk (95 % confidence intervals)
Male sex	**6.5 (5.9-7.2)**
Smoking	**2.9(2.6-3.3)**
Peripheral vascular disease	**2.4(2.0-2.9)**
Cardiovascular disease	**2.2(2.0-2.5)**
Cerebrovascular disease	**0.7(0.6-1.0)**
Hypertension	**1.5(1.4-1.6)**
Hypercholesterolaemia	**1.0(0.8-1.2)**
Diabetes mellitus	**1.0(0.8-1.2)**

Table 1.1: Risk factors for abdominal aortic aneurysm (Wilmink & Quick, 1998).

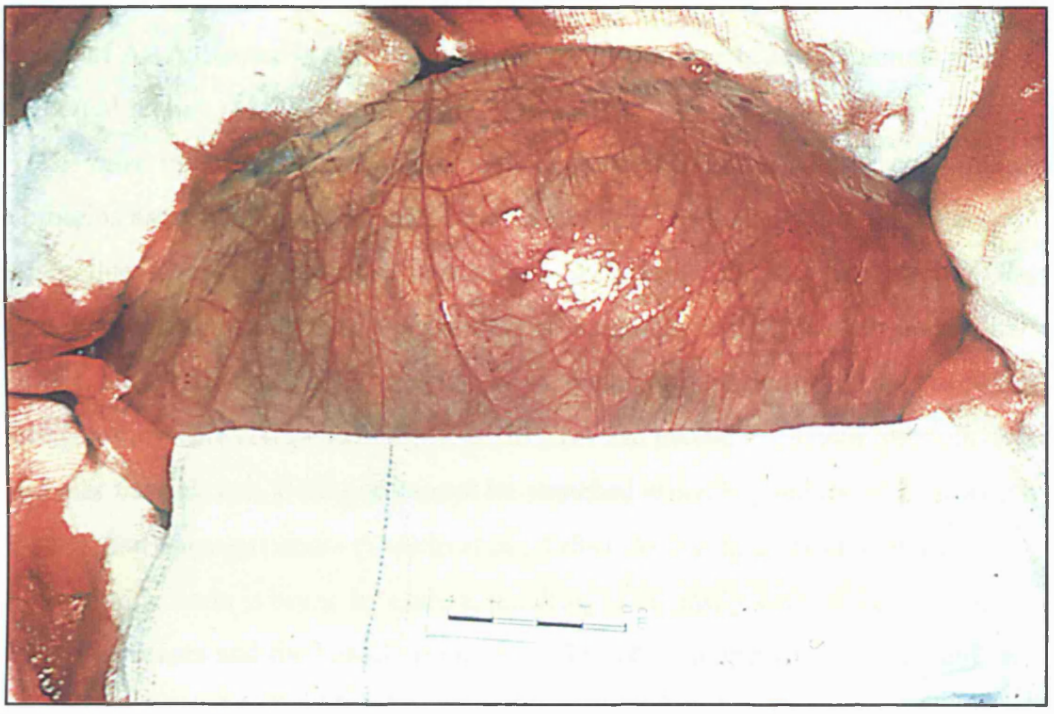


Figure 1.1 An abdominal aortic aneurysm before elective reconstruction

1.2 Pathogenesis of abdominal aortic aneurysms

Many factors may contribute to the development of aneurysms in the aortic wall, including genetic factors, depletion of elastin, proteolysis, inflammation, atherosclerosis and mechanical factors.

Although patients with AAAs are more likely to have relatives with AAAs no convincing link has been demonstrated between genetic factors and AAA. It seems that the inheritance of AAA disease is multifactorial, involving an interaction of genetic and environmental factors (MacSweeney *et al.*, 1994).

The most important structural elements of the aortic wall are elastin and collagen. These proteins are arranged in conjunction with smooth muscle cells in multiple concentric elastic lamellae, forming the basic structural unit of the aortic media (Wollinsky & Glagov, 1967). Elastin is easily stretched, it can double its length and spring back to its original dimensions, and is responsible for the elastic recoil of large arteries (Dobrin *et al.*, 1984). The collagen molecule comprises a triple helix structure giving it a tensile strength over 20 times greater than elastin. Collagen cannot be stretched much beyond its original length before structural damage occurs (Dobrin *et al.*, 1984). As the aortic wall stretches the initial load on the aorta is borne by elastin, resulting in an easily stretched elastic vessel. As the load increases and the vessel continues to stretch, collagen fibres uncoil and are progressively recruited as load bearing elements, so that the vessel becomes increasingly less distensible (MacSweeney *et al.*, 1994).

Studies on the aneurysm wall have demonstrated an increase in the collagen: elastin ratio compared to normal aortas. Elastin degradation appears to be important in the pathogenesis of aneurysm formation, and it has been shown that elastase activity is increased in the aortic wall of patients with aneurysms (Campa *et al.*, 1987).

Unlike elastin, collagen is continually synthesized throughout life and so collagen content reflects the net effect of synthesis and degradation (MacSweeney *et al.*, 1994). The increased collagen content of the aneurysmal aorta may be a compensatory effect of elastin degradation. Increased collagenolytic activity has been reported in the wall of the aneurysmal aorta (Butussil *et al.*, 1980) and it has been suggested that enzymatic destruction of collagen occurs before rupture of AAAs (Dobrin *et al.*, 1984).

Proteolysis is the enzymatic destruction of proteins. Proteolysis of structural components of the aortic wall leads to a weakened aortic wall and consequent aneurysm formation. Peripheral neutrophils and aortic smooth muscle cells of patients with AAAs

have been found to exhibit increased elastase activity (Cohen *et al.*, 1988). Vine and Powell (1991) found increased proteolytic activity in AAAs compared to normal aortas, suggesting destruction of the organised elastic lamellae may lead to formation of AAAs (Vine & Powell, 1991).

The matrix metalloproteases (MMPs) are a group of enzymes that selectively digest components of the extracellular matrix and have been implicated in AAA development. Their collective name is derived from the fact that their catalytic activity depends on the presence of zinc at the active site(Katsuda *et al.*, 1994; Senior *et al.*, 1991; Woessner, 1991). Elastin degradation by the MMPs causes weakening of the aortic wall, and there is now considerable evidence demonstrating increased MMP activity in the aortic wall of patients with AAAs compared to normal aortas (Elmore *et al.*, 1988; Grange *et al.*, 1997).

Abdominal aortic aneurysms often have some degree of chronic inflammatory infiltrate throughout the affected aortic wall (Crawford *et al.*, 1985; MacSweeney *et al.*, 1994; Sterpetti *et al.*, 1989). The inflammatory cells are a mixture of T-lymphocytes, B-lymphocytes and macrophages (Patel *et al.*, 1995). Evidence for the involvement of the inflammatory process in the pathogenesis of aneurysm formation comes mainly from experimental animal work. Inducing an inflammatory response in the rabbit carotid artery by applying calcium chloride to the adventitia can reliably create aneurysms (Gertz *et al.*, 1988). Infusion of elastase under high pressure into the aortic wall produces aneurysms in the rat aorta. In both models, aneurysms do not form after the initial insult but only after the development of the subsequent inflammatory process (Anidjar *et al.*, 1990). This suggests that inflammation and secondary inflammatory mediators may have a role in the pathogenesis of AAA (Carell *et al.*, 1999).

The observation of large atherosclerotic plaques inside AAAs and the risk factors that atherosclerosis and AAA disease share (especially smoking) has led many to believe that human AAAs may occur as a result of regression of an atheroscleotic plaque resulting in a weakened aortic media. Monkeys fed an atherogenic diet develop aortic plaques and subsequently changing to a low cholesterol diet results in plaque regression and aneurysm formation (Zairns *et al.*, 1990). In addition the infrarenal aorta has few vasa-vasorum, and the aortic media therefore depends on pressure filtration and diffusion from the aortic lumen to provide nutrition to the aortic wall. The combination of a thickened aortic intima due to atherosclerotic plaques and occlusion of the vasa-vasorum may lead to nutritional compromise and weakening of the aortic wall (Reed *et al.*, 1992).

The infrarenal aorta is subjected to arterial pulse pressure and shear stress. The pulse pressure increases as blood flows down the aorta because the aorta branches, tapers and is less compliant distally. The increased pulsatile stress in the distal abdominal aorta may cause fracturing of the elastic lamellae in the media, and cause the development of an aneurysm in the weakened wall (Henney *et al.*, 1993).

Serial ultrasound scans of screen detected small aneurysms show that aneurysm expansion rates are greater in patients who continue to smoke and are associated with increased levels of serum cotinine, a nicotine metabolite(MacSweeney *et al.*, 1994). Gaseous and blood-borne products of tobacco combustion contribute to inactivation of alpha-1-antitrypsin (Carell, 1986; George *et al.*, 1984). In the lung, this reduces the ability of alpha-1-antitrypsin to inhibit elastase and alters the normal balance between lung neutrophil elastase and antiprotease activity sufficiently to increase lung elastin degradation (Stockley, 1987). Smokers with AAA have a higher level of elastolytic activity and leucocyte granular elastolytic activity than non-smokers (Cannon & Read, 1982). Patients with chronic obstructive airways disease have been observed to have decreased elastin content in the lung and aorta. Lack of inhibitors of proteolytic enzymes are thought to be the cause of subsequent emphysema. This enzymatic imbalance may be involved in the pathogenesis of AAA in this group of patients (Stockley, 1987).

1.3 Natural history and diagnosis of abdominal aortic aneurysms

The natural history of AAAs is to dilate and rupture. Abdominal aortic aneurysms expand at an average rate of 4mm per year (Bergqvist *et al.*, 1994; Limet *et al.*, 1991). The risk of rupture is related to the size of the aneurysm, with aneurysms > 5.5cm having a significant risk of rupture. Aneurysms less than 5.5cm in diameter rupture less commonly, with annual rupture rates varying from 2.3-10%, depending on the initial size of the aneurysm (Glimaker *et al.*, 1991; Guirguis & Barber, 1991; Johansson *et al.*, 1990; Perko *et al.*, 1993). The five year survival of patients with aneurysms greater than 5.5cm in diameter who are not operated on is only 20% (Estes, 1950; Glimaker *et al.*, 1991; Szilagyi *et al.*, 1972).

Certain risk factors are associated with an increased risk of rupture. Cronnenwett et al identified diastolic blood pressure, initial anterior-posterior diameter and co-existing pulmonary disease as independent predictors of rupture (Cronenwett *et al.*, 1985). Strachan found that an increase in diastolic blood pressure by 10mmHg was associated with a 50%

increased risk of rupture. Smokers were also found to be 15 times more likely to rupture than non-smokers (Strachan, 1991). The presence of aortic blebs or blisters within the aortic wall may also be a sign of impending rupture (Faggioli *et al.*, 1994).

Most AAAs are asymptomatic until rupture occurs. Some AAAs are diagnosed incidentally during abdominal examination, radiological investigation or at laparotomy for other conditions (Collin, 1988). An increasing number of AAAs are detected by abdominal ultrasound examinations performed for non-vascular reasons (gall-stone disease, renal disease or prostatism) (Akersdjik *et al.*, 1991). Aneurysm expansion and stretching may result in abdominal or back pain, and patients presenting with these symptomatic non-ruptured aneurysms often proceed to urgent surgery. A small proportion of patients with symptomatic aneurysms have an inflammatory aneurysm (Goldstone *et al.*, 1978). Inflammatory aneurysms may present with a systemic illness and patients complain of general malaise, loss of appetite and loss of weight (Scott *et al.*, 1988). The inflammatory fibrosis may entrap the ureters resulting in ureteric obstruction and acute renal failure. The duodenum, left renal vein, inferior vena cava and iliac veins may also be adherent to the inflammatory mass surrounding the aneurysm, producing a variety of rare symptoms and making repair more technically difficult (Crawford *et al.*, 1985).

Rupture of an AAA results in certain death unless treated promptly. The classical presentation is abdominal pain and back pain, a pulsatile abdominal mass and hypovolaemic shock (Bannerjee, 1993; Hojer, 1992). Rupture most commonly occurs into the retroperitoneal space, containing the leak and allowing the patient time to reach hospital. Pressure created by the retroperitoneal haematoma can mimic a variety of symptoms which may be misdiagnosed as other acute abdominal conditions such as renal colic and pancreatitis (Bannerjee, 1993). Direct rupture into the peritoneal cavity results in rapid exsanguination and death, often before reaching hospital. Occasionally aneurysms erode and rupture directly into adjacent abdominal viscera or other tissues, resulting most commonly in an aorto-duodenal or aorto-caval fistula. The diagnosis is often made late in both situations and the prognosis very poor (Mianni *et al.*, 1994). Other rare presentations of AAAs include lower limb ischaemia due to peripheral embolisation of atheroma from the aneurysm sac and sudden thrombosis of the aneurysm (Johnston, 1994a).

In experienced hands ultrasound has a sensitivity approaching 100% in diagnosing AAAs. It is painless, quick, non-invasive and is currently the most common investigation used to confirm the diagnosis and measure the diameter of an AAA. It is also very useful

in the follow up of small AAAs (Ernst, 1993; Siegel & Cohan, 1994). The scan also provides information on the relationship of the AAA to the renal arteries and state of the iliac vessels. If the AAA is suprarenal it is important to define the involvement of the renal and mesenteric arteries as reconstruction of aneurysms involving these arteries is more complex and associated with greater morbidity. An angiogram or computed tomography (CT) scan can accurately define this relationship.

The development of endovascular aortic aneurysm repair demands more accurate pre-operative imaging and measurement of aneurysm dimensions. This may involve contrast enhanced computed tomography (CT) scanning, preferably with spiral acquisition and three dimensional reconstruction (Siegel & Cohan, 1994; Todd *et al.*, 1991), magnetic resonance imaging (MRI) or angiography with marker catheters to determine the sac length, diameter of the neck and minimum diameter and tortuosity of iliac arteries.

1.4 Management of abdominal aortic aneurysms

The natural history of abdominal aortic aneurysms is to increase in size and rupture. Data from historical series and patients unfit for surgery show that the five year survival of patients with AAAs larger than 5.5cm diameter who are not operated on is around 20% (Estes, 1950; Glimaker *et al.*, 1991; Szilagyi *et al.*, 1972). The mortality following elective surgery is only around 5% (Johnston, 1994b) whereas the mortality following surgery for a ruptured AAA has been reported to be 50%. However only 60% of patients with ruptured AAAs reach hospital alive, thus the overall mortality rate following rupture of an AAA is 90% (Drott *et al.*, 1992).

Patients with AAAs greater than 5.5cm in diameter should be offered elective repair to prevent rupture provided they are medically fit. At present UK surgeons follow up small (<5.5cm) aneurysms by serial ultrasound, reserving operative treatment for larger (>5.5cm) aneurysms and those that are expanding rapidly; usually >1cm in size between scans (Cronenwett *et al.*, 1990; Johansson *et al.*, 1990).

The UK small aneurysm trial has provided invaluable guidance on the operative management of small AAAs. In this study patients with AAAs between 3.0-3.9cm in diameter are followed up every 6 months by ultrasound scan. Fit patients with AAAs measuring between 4.0-5.5cm were randomised to early elective surgery or to 6 monthly ultrasound surveillance. Fit patients with aneurysms measuring between 5.0-5.5cm were randomised to early elective surgery or to 3 monthly ultrasound survellence. Aneurysms

greater than 5.5 cm were offered elective repair (The UK Small Aneurysm Trial Participants, 1998). Patients considered unfit for surgery have been kept under ultrasound surveillance to study aneurysm growth. The UK small aneurysm trial has also investigated the cost of treatment, the quality of life for each treatment modality and factors associated with the rapid growth of small aneurysms. Randomisation into this trial stopped in July 1996, and results of this study was recently published (The UK small aneurysm trial paricipants, 1998.) The data clearly demonstrated that there was no survival advantage associated with early elective repair of asymptomatic AAA <5.5cm in diameter. Ultrsound surveillence of these patients is a safe policy, which costs less than a policy of early elective surgery. A similar trial is running in the USA (Lederle *et al.*, 1994) and the results are eagerly awaited to see if they agree with the UK trial.

Several factors are associated with an increased operative risk in elective AAA repair. These include age > 80 years, the presence of coronary artery disease, severe respiratory disease, chronic renal impairment and cerebrovascular disease (Johnston, 1989; Roger *et al.*, 1989; White *et al.*, 1988). Thus all patients must undergo careful pre-operative assessment prior to elective AAA repair. This should include a full blood count, serum biochemistry, electrocardiogram, chest x-ray and an anaesthetic opinion. Any significant risk factors may require further investigation and correction.

Ischaemic heart disease remains the most prevalent risk factor among AAA patients, and is responsible for the majority of intra and post-operative deaths following elective surgery. In a large multicentre series of 680 elective AAA repairs Johnston et al (1989) reported a 4.8% mortality, with two thirds of these deaths related to cardiac complications. Patients who have had a myocardial infarction (MI) up to 6 months prior to any major surgery have a 55% incidence of post-operative MI compared to 6% among patients who are operated on 2 years following an MI (Smith, 1993). Patients with a left ventricular ejection fraction (LVEF) of less than 40% have been found to have a higher incidence of post-operative cardiac events (17%) compared to those with a LVEF greater than 40% (3.4%)(Pasternack *et al.*, 1984). Patients with significant ischaemic heart disease may require further investigation in the form of exercise electrocardiography, 24 hour cardiac monitoring and echocardiography to determine the ejection fraction. Cardiac function can usually be improved by medical therapy although some patients may require coronary angiography and angioplasty or coronary artery bypass grafting prior to AAA surgery.

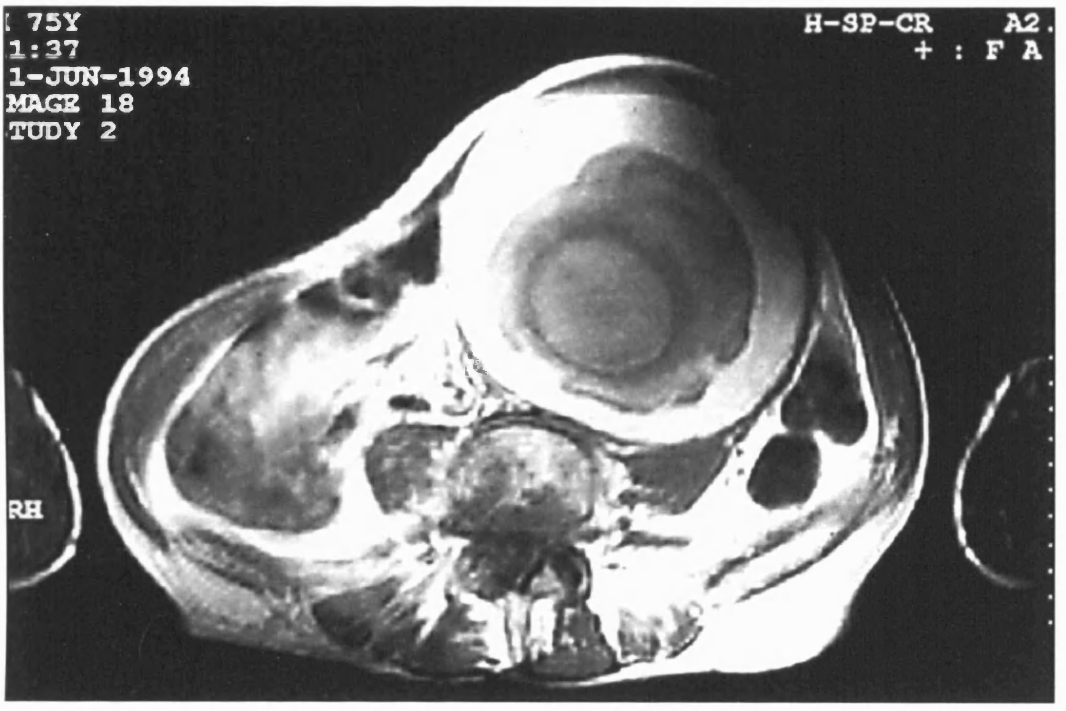


Figure 1.2 CT scan demonstrating an abdominal aortic aneurysm

Patients with significant pulmonary diseases such as COAD require assessment of respiratory function with formal lung function tests and arterial blood gases. Post-operative pulmonary complications may be reduced by pre-operative optimisation of respiratory function by stopping smoking, bronchodilators and physiotherapy but many may still develop post-operative respiratory failure requiring prolonged ventilatory support and tracheostomy with associated increased mortality (Sayers *et al.*, 1997).

Renal impairment exists in approximately 6% of patients undergoing elective AAA surgery and these patients are at risk of developing post-operative acute renal failure which has a high mortality. Patients with renal impairment (raised serum creatinine) should be investigated by a nephrologist to determine the cause (e.g. renal artery stenosis) and to optimise renal function. Patients with AAA and significant renal artery stenosis (RAS) can be treated by pre-operative renal angioplasty (Scoble, 1997) or combined AAA repair and renal revascularisation although the latter technique has a higher morbidity and mortality than AAA repair alone (Ghilardi *et al.*, 1992).

Abdominal aortic aneurysm repair is usually performed via a midline transperitoneal approach. The abdominal cavity can be explored for any co-existing pathology (e.g. gallstones) before the extent of the aneurysm, its upper and lower limits and the state of the iliac arteries are determined. The neck of the aneurysm is approached via reflection of the duodenum. The left renal vein may be divided to give better access to the neck. Some surgeons administer intravenous heparin at this stage. Vascular clamps are then applied and the aneurysm sac is opened. Thrombus is removed from the sac and bleeding from the lumbar arteries and inferior mesenteric artery (if patent) controlled with sutures. A dacron or PTFE graft of appropriate diameter is chosen and anastomosed to the proximal neck using the inlay technique. If the iliac arteries are not aneurysmal a tube graft is used and the distal anastomosis performed at the aortic bifurcation. If iliac aneurysms are present, they are usually opened anteriorly and the limbs of a bifurcated graft sutured to the combined orfices of the internal and external iliac arteries. Distal patency with good back flow is ensured before completing the distal anastomosis and releasing the clamps. Significant cardiovascular effects may occur at clamping and unclamping and this manoeuvre should be performed with full anaesthetic co-operation. A vasodilator is often given before clamping to lower systemic vascular resistance and fluid given before unclamping to raise the central venous pressure. The aim is to minimise blood pressure changes which may lead to coronary ischaemia and myocardial infarction. The aneurysm

sac is sutured around the graft to prevent adherence of the duodenum and small bowel to the anastomoses. Finally, haemostasis is checked and the sigmoid colon and feet inspected for viability before closing the abdomen.

Rupture is the most frequent and lethal complication of abdominal aortic aneurysm and accounts for 10,000 deaths per year in England and Wales (Department of Health Statistics, HMSO,1983). Despite advances in surgery and intensive care there has been little improvement in the mortality rate following rupture over the last two decades. Patients who present with the classic triad of sudden onset abdominal or back pain, hypotension and a pulsatile abdominal mass require urgent surgery to prevent death. Resuscitation with intravenous fluids and cross-matched blood should be commenced and the patient should be transferred immediately to theatre. If the diagnosis is in doubt then stable patients may be further assessed with ultrasound or CT scanning.

The abdomen is prepared and draped before induction of anaesthetic to preserve the effects of abdominal wall tamponade, which is lost with muscle relaxants. A long midline incision is made, and the diagnosis confirmed by the presence of an AAA with a retroperitoneal haematoma or free intraperitoneal blood (Figure 1.3). The priority is to gain proximal aortic control either at the infrarenal level or at the diaphragm. In a moribund patient this can be achieved with an aortic compressor, fist or retractor at the diaphragm or by rapid opening of the sac and introduction of a large balloon catheter into the proximal aorta. Once proximal control has been achieved, the patient may be further resuscitated with crystalloid, blood, fresh frozen plasma and platelets. After obtaining control of the proximal aorta, the iliac arteries are exposed and clamped and the aortic sac opened. The aortic reconstruction does not differ significantly from elective repair and a tube graft used whenever possible.

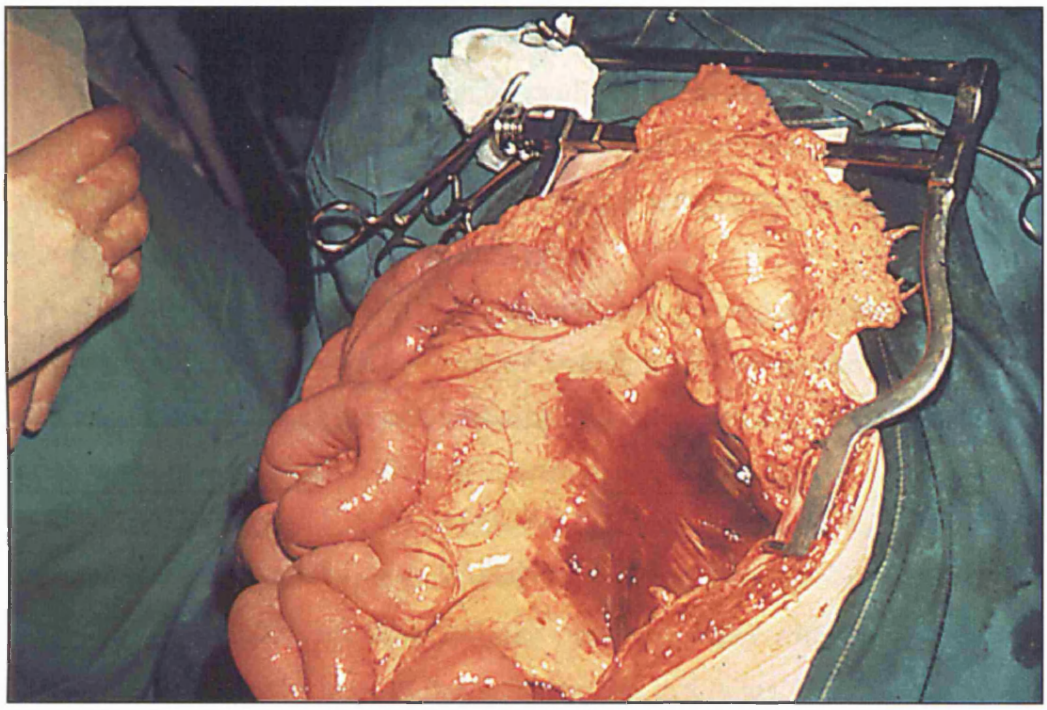


Figure 1.3 A ruptured abdominal aortic aneurysm with a contained retroperitoneal hematoma

Endovascular abdominal aortic aneurysm repair (EVAR) uses minimally invasive catheter techniques to introduce a graft-stent combination from a distant site to exclude the aneurysm sac from the arterial circulation by fixing a prosthetic graft within the aortic lumen. The first human endoluminal repair of an AAA was reported in 1991 (Parodi *et al.*, 1991), following his experiments in dogs. Many implants exist with combinations of tube or bifurcated grafts fixed in position with metallic stents, (self or balloon expanding) to anchor the graft material to the non-dilated aortic wall above and below the aneurysm (Woodburn *et al.*, 1998). Theoretical advantages of this technique include avoiding a long midline laparotomy and the consequent respiratory complications associated with the abdominal incision. Avoidance of an aortic cross-clamp reduces the cardiac stress, fluctuations in blood pressure and reperfusion injury.

Assessment of suitability for EVAR requires more precise pre-operative imaging and measurement of the aneurysm morphology including neck length and diameter, aneurysm length, and the diameter and tortuosity of the iliac vessels. The minimum parameters vary but only about 40-50% of AAAs are suitable for current EVAR techniques (Nasim *etal.,* 1998, Yusef *et al.,* 1995). Published data to date suggests that the peri-operative mortality rate following endoluminal AAA repair is similar to that reported for open repair. In addition there is a long 'learning curve' associated with this new technique (Woodburn *et al.*, 1998), and some of the complications that follow EVAR are unique to this technique. These include renal impairment secondary to administration of large quantities of contrast media (May *et al.*, 1997), fatal embolic events in the renal and lower limb vessels (Thompson *et al.*, 1997) due to manipulation of large catheters inside the sac, graft migration and endoleaks. Endoleaks occur where there is persistent blood flow outside the graft lumen but within the aneurysm sac, and are caused by poor fixation of the graft-stent combination to the aortic wall. The long term effects of endoleaks are unknown but the concern is of persistent AAA expansion and rupture (Woodburn *et al.*, 1998). The long term durability of endovascular devices is undergoing evaluation and a multicentre trial comparing EVAR and open AAA repair has begun.

1.5 Complications of AAA surgery

Intra-operative complications following elective AAA repair are uncommon and the intra-operative morbidity is very low. Troublesome venous bleeding from branches of the left renal vein at the neck of the aneurysm or bleeding from damage to the iliac veins

may occur during dissection. Anastomotic and suture line bleeding can also occur. Lower limb ischaemia may occur due to embolisation of debris into the distal circulation. The graft may have to be anastomosed more distally to the iliac or femoral arteries if the iliac arteries are diseased. Inflammatory aneurysms may present difficulty during dissection of the neck or distal end of the aneurysm, with the risk of damaging bowel and ureters.

Massive haemorrhage from inability to obtain proximal control at the neck of the aneurysm is the main intra-operative problem during surgery for ruptured AAA. Pannerton et al (1995) reported a 9.8% incidence of iatrogenic injury of a major artery or vein during surgery for a RAAA. In addition massive consumption of endogenous clotting factors and platelets in the retroperitoneal haematoma may lead to coagulopathy and further bleeding. Transfusion with large volumes of blood increases hypothermia, coagulopathy, and platelet dysfunction (Valeri *et al.*, 1987). Blood components including fresh frozen plasma, platelets and cryoprecipitate may be necessary to correct coagulopathy during and after surgery.

Intra-operative cardiac events which may be fatal occur more commonly during surgery for RAAA and are caused by hypotension, shock, aortic cross-clamping and declamping (Gelman, 1995). Studies have demonstrated that a long cross-clamp time and suprarenal aortic cross-clamping is associated with a worse outcome (Bauer *et al.*, 1993). Removal of the aortic cross-clamp may cause associated hypotension and reperfusion injury. This can lead to myocardial ischaemia especially in the presence of pre-existing coronary artery disease. Although this complication is rare during elective AAA surgery it remains a problem during surgery for RAAA (Bush *et al.*, 1997; Reiz *et al.*, 1979).

Atheromatous debris and clot can embolise from within the AAA sac during aortic dissection and manipulation. Acute lower limb ischaemia occurs in 0.6-9.5% of patients and is most common following emergency surgery (Imparato, 1983; Storm *et al.*, 1984). If backbleeding does not occur at the end of aortic repair, an embolectomy with a Fogarty baloon catheter should be performed.

The intra-operative mortality of RAAA is high. In a series of 112 patients, Pannerton (1995) reported that 15 (13.4%) died in the operating room; 12 from irreversible haemorrhagic shock and 3 from cardiac events.

Post-operative complications following AAA surgery may be divided into early (within 30 days) and late complications (months to years following surgery). Cardiac complications account for about two thirds of the early complications seen following

elective AAA surgery but respiratory, renal and multiple organ failure are common following emergency surgery.

Pre-operative assessment and optimisation of cardiac function should reduce the incidence of cardiac complications following elective AAA surgery. However the high prevalence of cardiac risk factors among these patients is reflected by the fact that cardiac events are responsible for about two thirds of the 30 day mortality reported following elective AAA surgery (Johnston, 1994b; Sayers *et al.*, 1997). Cardiac complications are much more common and contribute to the majority of deaths following surgery for RAAA. Acute coronary hypoperfusion associated with shock during a RAAA results in coronary ischaemia and contributes to major coronary events. Infrarenal aortic cross-clamping is associated with a sudden increase in blood pressure, further stressing the myocardium. Release of the cross-clamp results in sudden hypotension and the release of free-radicals into the circulation, which contribute to cardiac complications following RAAA repair (Panneton *et al.*, 1995, Johnston, 1994a; Sandison *et al.*, 1996).

Among patients who survive and are discharged from hospital following AAA surgery, cardiac causes continue to be the major cause of continued morbidity, with a 5 year heart related mortality rate of 14.3% compared to 6.4% in an age and sex matched population (Johnston, 1994b).

Respiratory complications are common following elective AAA surgery (Johnston, 1994b, Sayers *et al.*,1997). Traditionally post-operative atelectasis secondary to inadequate pain relief contributed to post-operative chest infections following elective AAA surgery. However the introduction of pre-operative anaesthetic assessment to identify patients with reduced lung function, advances in post-operative analgesia (including epidural analgesia and patient controlled analgesia) and the use of routine post-operative physiotherapy has reduced the incidence of pulmonary complications following elective AAA surgery.

Respiratory failure occurs in up to 50% of patients following surgery for a RAAA (Panneton *et al.*, 1995, Johnston, 1994a) with many patients requiring prolonged ventilation and/or a tracheostomy (Johnston, 1989; Panneton *et al.*, 1995; Sandison *et al.*, 1996). Shock, large blood transfusions and reperfusion injury excite a systemic inflammatory response, which in turn causes neutrophil infiltration and degranulation in the lung. This usually manifests itself in the early stages as pulmonary oedema of non-cardiac origin but can progress to the adult respiratory distress syndrome and multiple organ failure (Groeneveld *et al.*, 1997; Paterson *et al.*, 1989; Paterson *et al.*, 1989). The

aetiology of respiratory failure following RAAA surgery is usually a combination of all the factors discussed, and acute lung injury is often complicated by superimposed infection. Studies have demonstrated that development of respiratory failure following ruptured abdominal aortic aneurysm is associated with a significant mortality (Panneton *et al.*, 1995; Sayers *et al.*, 1997).

Acute renal failure is relatively uncommon following elective AAA surgery compared to a 25% incidence among patients who survive surgery for a RAAA. Acute renal failure complicating both elective and ruptured AAA surgery is associated with a 50-90% mortality (Abbott *et al.*, 1975; McCombs & Roberts, 1979; Porter *et al.*, 1966). Advanced age, ischaemic heart disease, occlusive renal artery disease and decreased functional renal mass contribute to this complication. Prevention of renal failure requires the recognition of risk factors, pre-operative preparation and careful operative monitoring and management. The pre-operative serum creatinine is an excellent screening test for renal impairment and should be measured in all patients prior to elective AAA surgery. Careful monitoring and maintenance of the intravascular volume is essential to prevent hypotension and low cardiac output during the operative period and a good diuresis should be established with intravenous hydration before aortic cross-clamping. Some clinicians use a low dose of dopamine to improve renovascular perfusion (Morrison, 1996). There is some evidence to support the beneficial effect of intravenous mannitol when it is given prophylactically 15 to 30 minutes prior to aortic cross-clamping (Nicholson *et al.*, 1996). Despite attention to adequate filling pressure and renal perfusion there is some impairment of glomerular filtration rate following elective AAA repair (Awad *et al.*, 1992).

About 25% of patients who survive surgery for a RAAA will develop renal failure. The main aetiological factor is ischaemic renal injury due to hypotension and shock (Miller & Meyers, 1987) and the mortality among these patients is very high. In a review of 65 patients referred to the regional renal unit at Leicester General Hospital over a 13-year period with renal failure following surgery for a RAAA we found that the overall mortality rate in this group of patients was 75%. We identified three factors that further increased mortality among these patients: a history of atherosclerotic disease (symptomatic ischaemic heart disease, cerebrovascular disease or peripheral vascular disease), the need for an additional intra-operative procedure (most commonly bilateral femoral embolectomy) or the development of an additional failing organ system. When these three factors were present the mortality increased to 90% (Singam *et al*, 1998).

Post-operative ileus is inevitable after all major abdominal surgery and is usually managed simply by intravenous fluids and nasogastric suction. Stress ulceration of the stomach or duodenum is a rare complication of elective AAA surgery and many intensive care units prescribe prophylactic H2 antagonists or sucralfate to protect against this complication. Left colonic ischaemia is also rare following elective surgery but may occur following extensive pelvic dissection required for bifurcated grafts which may disrupt the pelvic collateral blood supply to the colon.

Patients surviving surgery for a RAAA often have a prolonged post-operative ileus and are at risk of post-operative stress ulceration. Ischaemic colitis can also occur following RAAA and usually involves the sigmoid colon (Bast *et al.*, 1990; Ernst, 1983; Ernst *et al.*, 1978; Ernst *et al.*, 1976). Several factors may contribute to ischaemic colitis including pre-existing atheroma in the superior or inferior mesenteric artery, hypoperfusion, shock and pelvic dissection. Ischaemic colitis may lead to loss of the colonic mucosal barrier and results in bacterial translocation and subsequent endotoxaemia which may contribute to multiple organ failure.

A high index of suspicion for colonic ischaemia is required in patients following surgery for AAA and a colonoscopy or second look laporotomy may be necessary. Colonic ischaemia usually presents as bloody diarrhoea, fever, tachycardia and leucocytosis. Patchy mucosal changes at colonoscopy may be treated conservatively with hydration and antibiotics but extensive mucosal necrosis, signs of systemic toxicity and increasing abdominal tenderness are indications for re-operation. Other modalities which may have a role in peri-operative detection of sigmoid ischaemia include laser Doppler flow measurement of the mesenteric vessels at operation and sigmoid tonometry to detect changes in intramucosal pH (Soong *et al.*, 1994).

The post-operative mortality following elective AAA surgery is about 5% and two thirds of deaths are due to cardiac causes. Despite advances in resuscitation, anaesthesia and intensive care the post-operative mortality of patients reaching hospital alive following RAAA has not improved over the last 30 years. The mortality in this group of patients at 30 days remains around 50%.

Although many patients presenting to hospital with a ruptured AAA survive operation, they die post-operatively on the intensive care unit from coagulopathy, respiratory, renal and cardiac complications or multiple organ failure (MOF). The cause of MOF is unknown but recent work has focused on ischaemia-reperfusion injury and the

systemic inflammatory response which can compromise cardiac, pulmonary and renal function. Development of one failing organ system may in turn compromise further organ systems (Davies & Hagen, 1997). The details of the development of this syndrome will be discussed later in this chapter. Development of MOF following AAA surgery is associated with a mortality approaching 100%. Recent series have demonstrated that it is increasingly responsible for most of the post-operative mortality following ruptured abdominal aortic aneurysm(Huber *et al.*, 1995; Panneton *et al.*, 1995; Sandison *et al.*, 1996).

Graft infection is a rare complication of AAA surgery occurring in about 2% of patients and may present early or late (Earnshaw, 1991; Goldstone & Malone, 1974; Hoffert *et al.*, 1965; Lorentzen *et al.*, 1985; O'Hara *et al.*, 1986; Szilagi *et al.*, 1972). Patients undergoing operation for a RAAA may be at higher risk of graft infection due to possible decreased sterility of the operative field. An infected graft may present clinically as fever, leucocytosis, malaise, abdominal pain, an abdominal mass or gastro-intestinal bleeding (O'Hara *et al.*, 1986).

An upper gastrointestinal endoscopy is necessary to exclude peptic ulceration in patients presenting with gastro-intestinal bleeding. A CT scan of the abdomen or a gallium or indium labelled leucocyte scan may support the diagnosis with the presence of a fluid collection or gas around the graft on CT or concentration of indium or gallium around the graft on the labelled white cell scan.

The management of aortic graft infection remains controversial and includes conservative and radical approaches. Both are associated with a high morbidity and mortality. The former approach involves drainage of pus and necrotic tissue, lavage and placing gentamicin sponge or beads in the infected field or adjacent to the graft (Bailey *et al.*, 1987). The radical treatment consists of total graft excision, oversewing the aortic stump and extra-anatomical bypass. This approach is technically difficult and is associated with an early mortality of 24-45%. A major problem and cause of death is recurrent sepsis and secondary haemorrhage due to aortic stump leakage (O'Hara *et al.*, 1986; Yeager *et al.*, 1985). Another technique which has been used with some success is total graft excision and in-situ replacement with a rifampicin bonded prosthesis. The results are encouranging but the numbers treated so far are too small to comment on long term outcome (Naylor *et al.*, 1995). Late complications of AAA reconstruction include anastomotic aneurysms, chronic graft infection, aorto-enteric fistulae and late graft failure.

1.6 Late survival and quality of life

The long-term results for elective AAA treatment in terms of life expectancy and quality of life compared to the general population have been well documented with a 5 year survival rate of around 70% reported in several large series (Fielding *et al.*, 1981; Soreide *et al.*, 1982). Fielding et al (1981) followed up 243 patients and reported an overall 5 year survival of 64.8%; 68.1% in elective cases, 57.1% in ruptured cases and 66% in urgent non-ruptured cases. The major determinant of late survival after aneurysm repair is coronary heart disease. In a study of patients with coronary artery disease undergoing AAA repair Roger et al (1989) found 60% had either had a myocardial infarction or died at 5 years. These results contrast with patients with no obvious coronary heart disease or prior cardiac bypass surgery, who have a 70% survival at 5 years, a 60% survival at 8 years and only a 15% risk of a cardiac event.

The quality of life after AAA surgery has been investigated by several authors. Using objective quantifiable methods Rohrer et al (1988) demonstrated that the quality of life for survivors of RAAA was similar to that following elective aneurysm surgery. These findings were confirmed in a later study of patients surviving operation for ruptured AAA, comparing their quality of life with patients undergoing elective AAA surgery (VanRamshorst *et al.*, 1990). The good long term survival and quality of life in patents following successful elective and emergency AAA repair supports the current surgical approach.

1.7 Ischaemia-reperfusion injury

Lower torso ischaemia inevitably occurs following infrarenal aortic cross-clamping during AAA repair. Reperfusion of the ischaemic extremities with oxygenated blood results in ischaemia-reperfusion injury (IRI). Ischaemia-reperfusion injury can cause both local tissue damage in the organ subject to the insult and systemic damage in remote organs such as the lungs and kidneys. Parks and Granger (1986) demonstrated that 3 hours of ischaemia and one hour of reperfusion resulted in a greater severity of tissue damage than 4 hours of ischaemia alone in a feline model of intestinal ischaemia. Other early work showed that revascularisation of ischaemic limbs released K^+, H^+ and myoglobin into the circulation which resulted in impaired renal, cardiac and pulmonary function. Much of the damage following IRI is initiated by oxygen derived free radicals, generated by the xanthine oxidase pathway. Subsequent research has demonstrated that a cascade of inflammatory mediators are released into the circulation following IRI and these mediators result in activation of neutrophils, which can degranulate in local and remote sites (such as the lung and kidney) and cause organ damage (Figure 1.4).

Endogenous mechanisms exist to protect against IRI and hence the damage is self-limiting and may go unnoticed especially during elective vascular surgery. However in critically ill patients with ruptured AAA and hypovolaemic shock, the tissue extremities and mesenteric vasculature are ischaemic at presentation. Following resuscitation and repair of the RAAA these patients may be considered to have 'whole body' IRI which escapes local control mechanisms. Additional tissue insults may occur as a result of the combined effects of hypovolaemia, acidosis, hypothermia and blood transfusion which may lead to the systemic inflammatory response syndrome. This syndrome involves the systemic activation of pro-inflammatory cytokines, complement (and other humoral cascades) and neutrophils which cause further tissue injury and possible multiple organ failure. This chapter describes in detail the complex biochemical and pathophysiological changes in the microcirculation that accompany IRI, the local endogenous control mechanisms and the systemic inflammatory response.

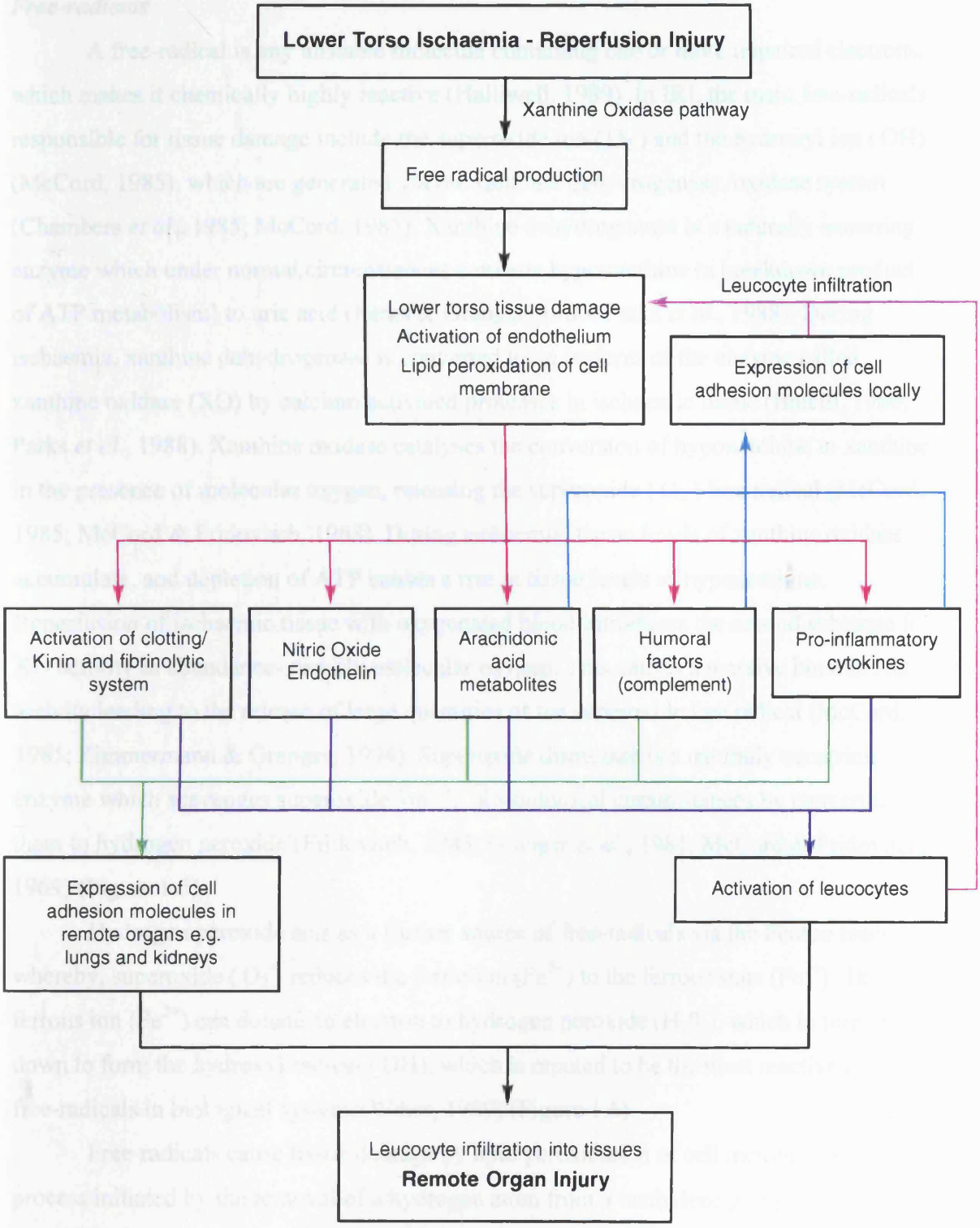


Figure 1.4 Lower torso ischaemia-reperfusion injury and remote organ damage

Free-radicals

A free-radical is any unstable molecule containing one or more unpaired electrons, which makes it chemically highly reactive (Halliwell, 1989). In IRI, the main free-radicals responsible for tissue damage include the superoxide ion ($\cdot O_2^-$) and the hydroxyl ion ($\cdot OH$) (McCord, 1985), which are generated via the xanthine dehydrogenase /oxidase system (Chambers *et al.*, 1985; McCord, 1985). Xanthine dehydrogenase is a naturally occurring enzyme which under normal circumstances converts hypoxanthine (a breakdown product of ATP metabolism) to uric acid (Parks & Granger, 1986; Parks *et al.*, 1988). During ischaemia, xanthine dehydrogenase is converted to an isoform of the enzyme called xanthine oxidase (XO) by calcium activated proteases in ischaemic tissue (Batelli, 1980; Parks *et al.*, 1988). Xanthine oxidase catalyses the conversion of hypoxanthine to xanthine in the presence of molecular oxygen, releasing the superoxide ($\cdot O_2^-$) free radical (McCord, 1985; McCord & Fridovitch, 1968). During ischaemia, tissue levels of xanthine oxidase accumulate, and depletion of ATP causes a rise in tissue levels of hypoxanthine. Reperfusion of ischaemic tissue with oxygenated blood introduces the second substrate for XO activity in abundance- namely molecular oxygen. This causes a massive burst in XO activity leading to the release of large quantities of the superoxide free radical (McCord, 1985; Zimmermann & Granger, 1994). Superoxide dismutase is a naturally occurring enzyme which scavenges superoxide ions in physiological circumstances by converting them to hydrogen peroxide (Fridovitch, 1983; Granger *et al.*, 1981; McCord & Fridovitch, 1969) (Figure 1.5).

Hydrogen peroxide acts as a further source of free-radicals via the Fenton reaction whereby, superoxide ($\cdot O_2^-$) reduces the ferric ion (Fe^{3+}) to the ferrous state (Fe^{2+}). The ferrous ion (Fe^{2+}) can donate an electron to hydrogen peroxide (H_2O_2), which in turn breaks down to form the hydroxyl radical ($\cdot OH$), which is reputed to be the most reactive of the free-radicals in biological systems(Weiss, 1989) (Figure 1.6).

Free-radicals cause tissue damage by lipid peroxidation of cell membranes; a process initiated by the removal of a hydrogen atom from a methylene group positioned between two unsaturated bonds of a lipid molecule. The result is a carbon centered lipid free-radical which in the presence of oxygen results in further lipid degeneration and consequent cell membrane damage and cell death. Proteins and DNA are also at risk from structural damage by free-radicals (Grace, 1994; Slater., 1984).

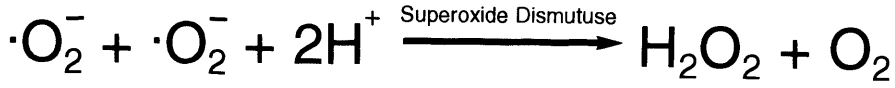


Figure 1.5 Formation of hydrogen peroxide

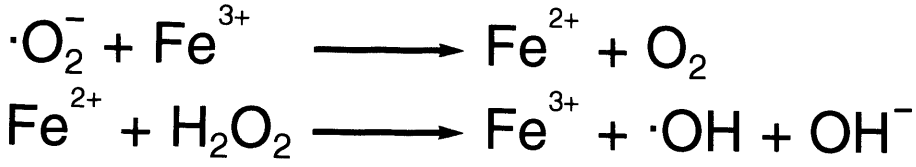


Figure 1.6 The Fenton reaction

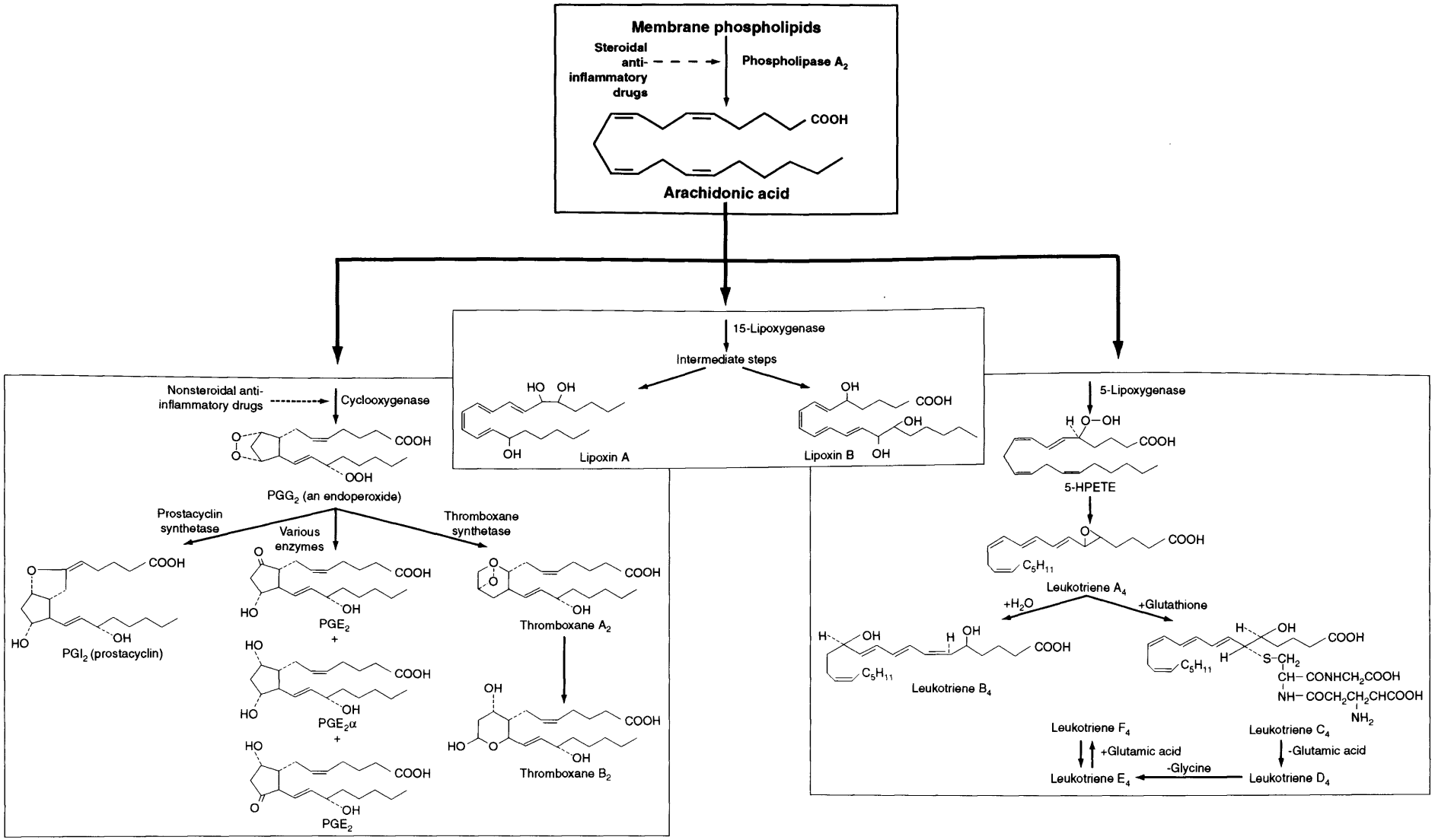


Figure 1.7 The biosynthesis of arachidonic acid metabolites

Lipid peroxidation of cell membranes also releases arachidonic acid, which is further metabolised to thromboxane, prostaglandins and leukotrienes (Homer-Vanniasinkam *et al.*, 1997). These arachidonic acid metabolites have many systemic properties including vasoconstriction, platelet aggregation (thromboxane), vasodilation (prostaglandins) and neutrophil chemotaxis (leukotrienes) (Figure 1.7).

Superoxide free radicals also react with endothelial cells to produce a series of changes that affect microcirculatory flow and encourage neutrophil-endothelial interaction. This occurs by the formation of inflammatory mediators, the expression of endothelial leucocyte adhesion molecules and inactivatation of endothelial derived nitric oxide (Granger *et al.*, 1993; Rubyani & Vanhoutte, 1986).

Leucocytes

In IRI leucocytes may cause local tissue injury and also remote organ injury after passing from the vascular compartment into the extracellular tissues through the endothelial barrier. Under normal physiological conditions blood flow is laminar and there is little contact between circulatory blood cells and the vascular endothelium. Following IRI, vasoconstriction occurs resulting in slow and turbulent blood flow. The heavier, white cells migrate to the periphery of the column of flowing blood, roll along the endothelium and some adhere, a process known as margination (Woolf, 1986). Leucocytes can then pass between the endothelial cells by diapedesis and infiltrate the tissues where they cause tissue damage. Neutrophil-endothelial interaction and binding is central to the process. The pro-inflammatory cytokines (tumour necrosis factor-α , interleukin-1 and interleukin-8), complement, platelet activating factor and free-radicals facilitate neutrophil-endothelial interaction by initiating endothelial cell expression of a group of endothelial leucocyte adhesion proteins, and activated neutrophils express complementary glycoprotein adhesion receptors. In order to aid this process, superoxide inactivates endothelial nitric oxide- an endogenous anti-adhesion molecule.

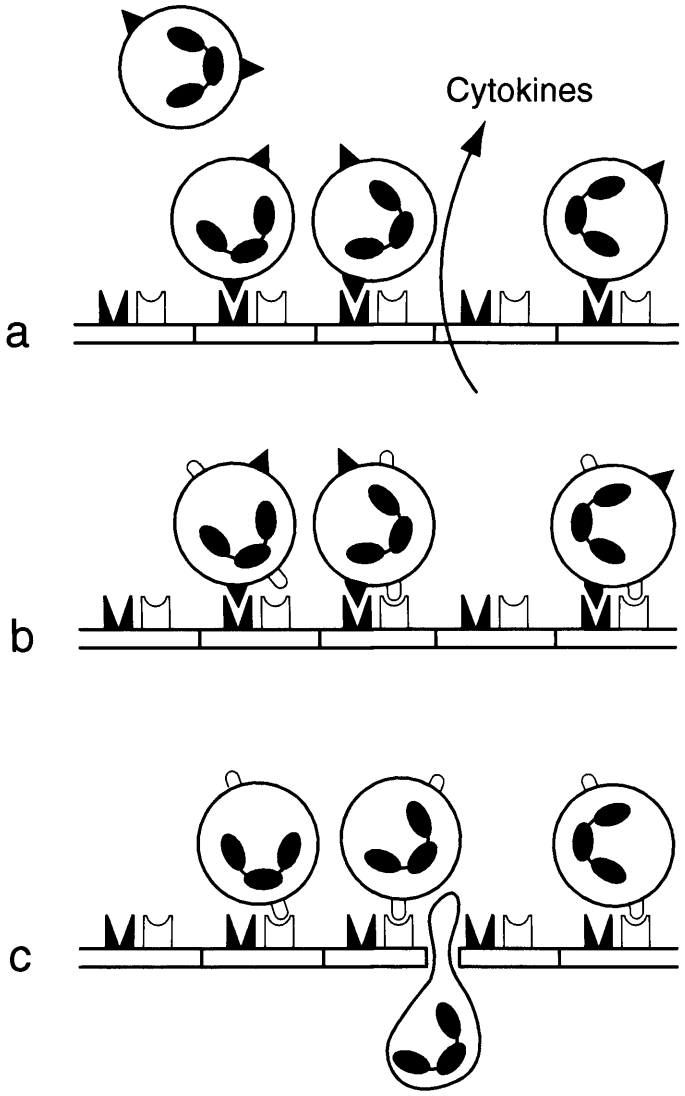
The neutrophil-endothelium interaction occurs in two stages. In the first stage endothelial cells activated by pro-inflammatory cytokines express a group of leucocyte adhesion proteins called selectins: namely endothelial leucocyte adhesion molecule (ELAM or E-selectin), intercellular adhesion molecule (ICAM or CD54) and granule membrane protein 140 (GMP140 or P-selectin)(Albeda, 1991; McEver, 1991; Schleimer & Rutledge, 1986). At the same time neutrophils express a group of surface proteins called

lectin adhesion molecules (LECAM), which can bind to ELAM (Windsor *et al.*, 1993). In the second stage of this process, inflammatory mediators (produced by the endothelium) stimulate bound neutrophils to express a group of cell surface glycoproteins called integrins. The most important integrins expressed by activated neutrophils include CD11b and CD 18 (Carlos & Harlam, 1990) which bind to ICAM while the LECAM receptors are rapidly shed. Then this integrin-selectin adhesion allows unopposed migration of the neutrophil between endothelial cells into the tissues via diapedesis (VonAdrian *et al.*, 1991). In addition, leucocytes are attracted to sites of inflammation by the chemotactic effect of several molecules including complement, leukotrienes and cytokines (Worthen *et al.*, 1989)(Figure 1.8).

Leucocytes cause tissue damage by two mechanisms, the generation of free-radicals (the respiratory burst) and the release of proteolytic enzymes. The respiratory burst describes the release of free-radicals and potent oxidising agents by leucocytes via a chemical reaction between O_2 and NADPH. Although intended as a defence mechanism against invading microorganisms, it can also cause severe tissue damage to the host. The enzyme reduced nicotinamide adenine dinucleotide oxidase is localized on the internal surface of the neutrophil lysosomal plasma membrane and transfers electrons from reduced nicotinamide adenine dinucleotide (NADPH) to molecular oxygen generating superoxide ions which destroy microorganisms and can also cause tissue damage (Lehrer *et al.*, 1988; McPhail *et al.*, 1976) (Figure 1.9).

In addition, neutrophil superoxide dismutase (SOD) can convert superoxide to hydrogen peroxide and neutrophil myeloperoxidase catalyses the conversion of hydrogen peroxide to hypochlorous acid (Weiss *et al.*, 1982) (Figure 1.10).

Hypochlorous acid is an oxidising and chlorinating agent which can react with primary amines to produce N-chloramines, which are also powerful oxidising agents. The cytotoxic effects of these oxidising agents are mediated through the oxidation of sulphyl groups, inactivation of haem proteins and cytochrome, and the degradation of proteins (Zimmermann & Granger, 1994). In addition, the cytosolic granules of neutrophils contain a powerful cocktail of proteolytic enzymes including elastase, gelatinase and collagenase which are released and digest the extracellular matrix (Anderson *et al.*, 1991). In IRI the end result of release of neutrophil derived free-radicals, oxidising agents and proteolytic enzymes is severe tissue damage.



a, Unstimulated neutrophils expressing lectin adhesion molecule (LECAM) ( ) are targetted and removed from the circulation by attachment to inflamed endotheliumexpressing endothelial cell leucocyte adhesion molecule (ELAM) ( ). b, Local chemical mediators activate the neutrophils with upregulation of cluster differentiation (CD)-11b-CD18 integrins ( ). Attachment to intercellular adhesion molecule receptors on the endothelium ( ) provides strong adhesive forces. c, Concomitant downregulation of LECAM on the neutrophil permits unopposed CD 18-dependent transendothelial migration.

Figure 1.8 Two-stepmigration of neutrophils across the endothelial cell layer
Windsor et al (1993)

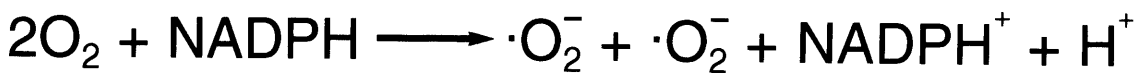


Figure 1.9 Generation of superoxide free-radicals by the respiratory burst

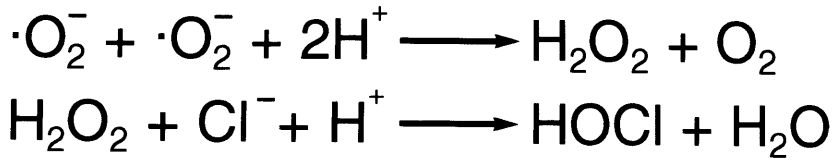


Figure 1.10 Generation of hypochlorous acid

The endothelium

The vascular endothelium is a single layer of cells lining the internal surface of all blood vessels. It is a complex organ with many functions including regulation of the microcirculation by release of a wide range of vasoactive factors. During IRI the endothelium is activated by free-radicals and produces several factors which facilitate neutrophil binding, chemotaxis and changes in the microcirculation (Figure1.4).

Prostaglandins (including prostacyclin, thromboxane and leukotriene) are a family of 20-carbon chain fatty acids derived from arachidonic acid which are released from the plasma membrane during inflammatory states. Free-radical induced injury to the endothelial cell plama membrane initiates the release of arachidonic acid via activation of the enzyme phospholipase A2. Arachidonic acid is metabolised to prostacyclins and thromboxane by the cyclooxygenase pathway and to leukotrienes via the lipoxygenase pathway (Figure 1.7)(Vane *et al.*, 1990). Prostacyclin is a powerful vasodilator, causes vascular smooth muscle relaxation and inhibits platelet aggregation. It may provide some protection during IRI but endogenous prostacyclin is unstable and its synthesis is impaired by smoking, diabetes, atherosclerosis and increasing age (Grace, 1994). Thromboxane A2 (TA2) is a potent vasoconstrictor and chemoattractant which stimulates platelet aggregation and facilitates neutrophil migration across activated endothelium (Spanguolo *et al.*, 1980; Vane *et al.*, 1990). TA2 levels increase following aortic cross-clamping, IRI and shock (Hechtman *et al.*, 1983; Paterson *et al.*, 1989). Leukotriene B4 (LB4) encourages neutrophil-endothelial interaction via binding to neutrophil receptors and stimulating neutrophil expression of CD 18 (Goldman *et al.*, 1990).

Nitric oxide (NO) is an important endogenous endothelium-derived mediator involved in the control of normal vascular tone; it is a powerful vasodilator, inhibits platelet aggregation and neutrophil adhesion. NO is formed as a by product of the conversion of L-arginine to L-citrulline by a vascular endothelial enzyme called nitric oxide synthase (NOS) (Davies *et al.*, 1995). Under physiological conditions basal release of NO occurs due to constitutive NOS. However under pathological conditions such as IRI, an inducible isoform of the enzyme (iNOS) causes massive NO release.

Following IRI, NO may have potentially beneficial and adverse effects. It may reduce poor capillary flow which occurs in the microcirculation due to capillary spasm and plugging of vessels with activated leucocytes. However, superoxide radicals produced during ischaemia-reperfusion injury can inactivate endogenous NO and thus promotes

endothelial-neutrophil interaction (Rubyani & Vanhoutte, 1986). In addition superoxide radicals also react with NO to form the peroxynitrite free radical ($ONOO^-$), which is also a precursor of the hydroxyl anion (Radi *et al.*, 1991) (Figure 1.11). The peroxynitrite and hydroxyl radicals are both highly toxic and capable of causing severe tissue damage; thus NO is a further source of potentially harmful free radicals.

Endothelin is the most potent vasoconstrictor known and also facilitates neutrophil-endothelial interaction. It is a family of peptides synthesised by vascular endothelial cells in response to hypoxia, thrombin, noradrenaline and transforming growth factor beta. During physiological states the balance between the opposing effects of endothelin and nitric oxide regulates vascular tone, but during IRI, plasma endothelin levels rise, and contribute to vascular constriction in the microcirculation (Brenner *et al.*, 1989; Miller *et al.*, 1989).

Platelet activating factor (PAF) is a phospholipid derivative produced by endothelial cells in response to injury. During IRI it causes leucocyte chemotaxis and encourages platelet aggregation and thrombus formation(Lewis *et al.*, 1988). The endothelium is also capable of activating complement and cytokines. These humoral factors are also produced in significant quantities by leucocytes and other cells in the reticulo-endothelial system.

Humoral factors

Humoral factors are molecules that are released into the bloodstream and exert their effects at distant sites. Ischaemia-reperfusion injury, haemorrhage and sepsis are all potent stimuli for the activation of a host of inflammatory humoral factors including complement, the coagulation cascade, fibrinolytic pathway, kinin systems and pro-inflammatory cytokines. Following IRI, free-radical induced local tissue damage activates factor XIII (Hageman factor), which is capable of activating these humoral pathways. The kinin system involves the conversion of kallikrein to bradykinin- a powerful vasodilator peptide which also increases vascular permeability. Activation of the coagulation cascade results in the conversion of fibrinogen to fibrin via thrombin. Formation of fibrin in the microcirculation traps platelets and neutrophils which impedes blood flow. Fibrin is broken down to fibrin degradation products (FDPs) by the fibrinolytic system which involves conversion of plasminogen to plasmin. Many of these peptides also cause increased vascular permeability and are neutrophil chemoattractants (Woolf, 1986).

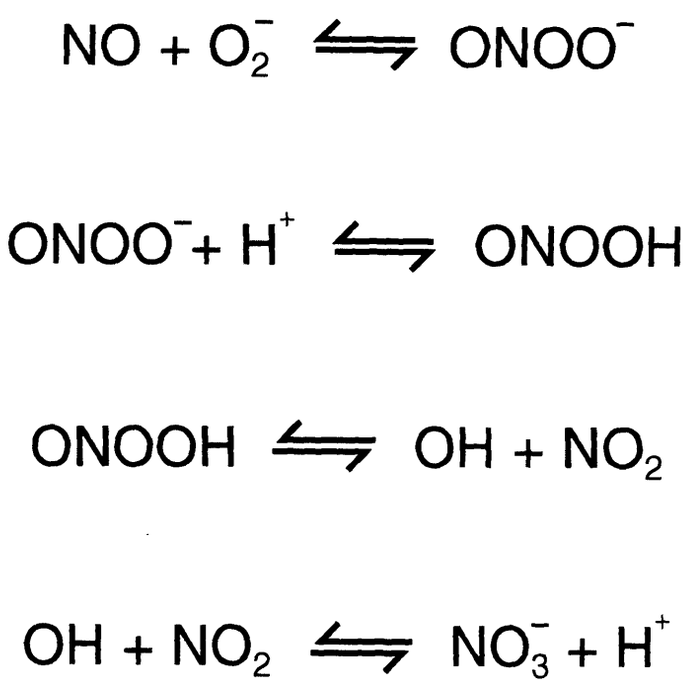


Figure 1.11 The generation of peroxynitrite and hydroxyl free-radicals

The complement system is a group of at least nine proteins which are involved in acute inflammation, phagocytosis, clotting and immune and hypersensitivity reactions. During physiological states, complement proteins circulate in the inactivated state, but reperfusion injury activates the complement cascade resulting in an increase in serum levels of the active peptides C3a and C5a (Bengston & Heidmann, 1986; Fosse & Mollnes, 1987). The activated complement proteins cause increased capillary permeability and vasodilation, and increased complement activity also enhances neutrophil chemotaxis, upregulates neutrophil CD18 receptor expression and promotes neutrophil free-radical production and degranulation (Rubin *et al.*, 1990).

Cytokines are a group of low molecular weight proteins produced by endothelial cells, leucocytes, hepatic Kuppfer cells and other cells involved in the inflammatory response. These molecules are responsible for coordination and communication in immune and inflammatory responses and include chemokines, interleukins, growth factors and interferons. Cytokines have a short duration of action and are very active at low concentrations. They have multiple effects that overlap between factors and are involved in the growth and differentiation of various cell types as well as the activation of cells to express specific proteins. During IRI they play an integral role in neutrophil activation, priming of the endothelium and neutrophil migration into tissues. The important pro-inflammatory cytokines which are seen following IRI include tumour necrosis factor-α (TNF-α), interleukin-1(IL-1), interleukin-6 (IL-6) and interleukin-8 (IL-8) (Baigrie *et al.*, 1993; Caty *et al.*, 1990; Sterpetti *et al.*, 1993).

Tumour necrosis factor-alpha is produced in response to IRI, shock, endotoxaemia and sepsis (Davies & Hagen, 1997; Debets *et al.*, 1989). TNF-α acts by binding to specific receptors on a wide variety of cells and its actions include neutrophil release from bone marrow and neutrophil activation and migration into the pulmonary tissues by upregulation of adhesion molecules. In addition TNF-α also stimulates neutrophil free-radical and protease production and endothelial cell synthesis of PAF (Gibbs *et al.*, 1990; Westlin *et al.*, 1990). TNF-α also promotes the differentiation and activation of monocytes and macrophages, stimulates the formation of acute phase proteins, activates the complement cascade and induces the release of IL-1 (Camussi *et al.*, 1987; Fulkerson *et al.*, 1996).

Interleukin-1 (IL-1) is released in parallel or in response to TNF-α production. It stimulates the formation of leucocytes and macrophages in the bone marrow by inducing granulocyte/macrophage colony stimulating factor (GM-CSF). IL-1 also increases

endothelial-leucocyte interactions by up-regulating adhesion molecules and potentiates leucocyte mediated damage by stimulating free-radical and lysosome release (Davies & Hagen, 1997; Westlin *et al.*, 1990). Interleukin–6 (IL-6) facilitates endothelial-neutrophil adhesion (Biffl *et al.*, 1994) and IL-6 and IL-8 act synergistically with TNF-α and IL-1 to modulate leucocyte mediated lung injury (Mullen *et al.*, 1992). IL-8 is produced by endothelial cells and is a potent leucocyte chemoattractant (Harada *et al.*, 1994).

Endotoxin is the lipopolysaccharide outer membrane of gram-negative bacteria which is released into the bloodstream by dying microrganisms. Animal studies have demonstrated an increase in plasma endotoxin levels following lower torso ischaemia (Yassin *et al.*, 1998) and transient endotoxaemia and increased intestinal permeability has been demonstrated in clinical studies in patients undergoing elective AAA repair (Roumen *et al.*, 1993; Welch *et al.*, 1995). Although the role of endotoxin in IRI is unclear, it may be important because it is a powerful activator of complement and pro-inflammatory cytokines.

1.8 Remote organ damage following ischaemia-reperfusion injury

Ischaemia-reperfusion may cause both local damage and distant injury. Pro-inflammatory cytokines activate neutrophils and prime the endothelium at distant sites. Neutrophil infiltration and degranulation in the lungs and kidneys may cause pulmonary and renal impairment. Although this may be a transient phenomenon following elective AAA surgery, it may be the cause of MOF following RAAA repair.

Ischaemia-reperfusion injury causes pulmonary damage either by the direct effects of free-radicals in the pulmonary circulation or by the effects of activated neutrophils which adhere to primed endothelial cells and enter the tissues. The pulmonary vascular bed contains between 50-60% of the total circulating neutrophil pool; thus the lungs are a major site of tissue damage in IRI (Boggs, 1967). Acute lung injury following IRI is characterised clinically by progressive hypoxia and non-cardiogenic pulmonary oedema (Anner *et al.*, 1987; Anner *et al.*, 1988) which may progress to the adult respiratory distress syndrome (ARDS) and respiratory failure (Fantini & Conte, 1995; Paterson *et al.*, 1989). Established ARDS has a 60% mortality rate. A large multicentre prospective study found that respiratory failure complicates only 8% of patients undergoing elective AAA surgery (Johnston, 1989) but 47% of patients undergoing surgery for a ruptured AAA (Johnston, 1994a). Advanced age, pre-existing lung disease, sepsis or the development of multiple

organ failure increases the mortality rate to 70-90% (Artigas *et al.*, 1991; Ashbaugh *et al.*, 1967; Sloane *et al.*, 1987).

The kidney is a highly oxygenated organ and thus particularly vulnerable to reperfusion injury. Acute renal failure may complicate both elective and emergency aortic aneurysm repair and is associated with a high mortality (Glimelius *et al.*, 1978; Heibert & Jacques, 1976; Mahado *et al.*, 1977).

The most important cause of renal injury following aortic surgery is decreased renal perfusion and studies have demonstrated that infrarenal aortic cross-clamping affects renal haemodynamics with a decrease in glomerular filtration rate and renal blood flow (Awad *et al.*, 1992; Gelman., 1995). Pre-existing renal impairment or further insults such as prolonged aortic cross-clamp time, blood loss, hypotension or acidosis increase the likelihood of acute renal failure. About 25% of patients who survive surgery for ruptured AAA develop acute renal failure, and the mortality in this group of patients is 75% (Singam *et al.*, 1998)

Ischaemia-reperfusion injury in the kidney is caused by free-radicals and the effects of activated neutrophils, which bind to activated endothelial cells and infiltrate the tissues. In addition activation of the renin-angiotensin system (which leads to vasoconstriction by angiotensin II) together with TA2 and endothelin increases vasoconstriction in the renal microcirculation and potentiates the effects if IRI.

1.9 Defence mechanisms against IRI

Several endogenous defence mechanisms exist to limit ischaemia-reperfusion injury and a number of therapeutic options have been found to be effective in experimental studies. These agents act in a variety of ways including free radical scavenging, inhibition of free-radical production, neutrophil inhibition, antioxidation, ischaemic preconditioning and hypothermia.

Free-radical scavengers react with free-radicals and inactivate them to prevent tissue injury. Superoxide dismutase is an endogenous enzyme that protects tissues against free-radical induced injury by converting superoxide to hydrogen peroxide, which decomposes to water and oxygen (Halliwell, 1989). Several agents including dimethylthiourea, dimethylsulphoxide, mercaptoproprionyl glycine, N-acetylcysteine, captopril and histidine have been shown to be free-radical scavengers under experimental conditions (Grace, 1994). The only free radical-scavenger that has been used effectively in

a clinical setting is manitol. In a study of elective AAA repair Nicholson et al (1996) demonstrated that administration of mannitol prior to infrarenal aortic cross-clamping significantly reduced renal glomerular damage as measured by urinary micro-proteinuria.

Inhibition of free-radical production involves use of enzyme –blocking drugs. Allopurinol is a structural analogue of hypoxanthine and competitively inhibits the enzyme xanthine oxidase to reduce production of superoxide. There is some clinical evidence demonstrating the benefit of low dose allopurinol in protecting against IRI during aortic surgery (Smeets *et al.*, 1995). Desferrioxamine is an iron-chelating agent which inhibits the Fenton reaction and reduces the production of hydroxyl radicals. Experimental animal work has demonstrated that desferrioxamine may improve myocardial function following IRI (Ambrosio *et al.*, 1987).

Neutrophil inhibition prevents neutrophil-endothelial interaction, and reduces neutrophil infiltration into tissues. Experimental work using monoclonal antibodies against neutrophil CD-11b/CD18, and endothelial cell adhesion molecules have reported some success in animal studies but have not been shown to be of any benefit in human studies (Simpson *et al.*, 1988). TGF-β inhibits neutrophil-endothelial adhesion (Lefer *et al.*, 1990) and adenosine inhibits free radical production in activated neutrophils via a receptor mediated system. Hetastarch, dextran and mannitol have also been shown to protect against ischaemia-reperfusion induced lung injury in animal models by preventing endothelial-leucocyte interactions (Nielson *et al.*, 1993; Paterson *et al.*, 1989; Schnell *et al.*, 1992). Heparin, a common anti-coagulant neutralises lysosomal proteases released by leucocytes and limits the activation of complement (Glimelius *et al.*, 1978; Heibert & Jacques, 1976; Mahado *et al.*, 1977).

Cytokines with predominantly anti-inflammatory actions have been identified. Interleukin-10 (IL-10) is one such cytokine that can inhibit TNF-α, IL-1 and IL-6 and is involved in tightly controlling the effects of IRI (Blackwell & Christman, 1996).

Antioxidants can prevent IRI induced tissue damage by inhibiting the production of further free-radicals. Several antioxidants including vitamin E, propranolol, calcium channel blockers, captopril and nafatrozam (Rubin *et al.*, 1996) have been shown to reduce IRI in experimental studies but there is no clinical evidence of their benefit.

Ischaemic preconditioning is a physical method of limiting ischaemia-reperfusion damage by exposure to a series of short ischaemic episodes followed by reperfusion that protect against further ischaemia-reperfusion injury (Walker & Yellon, 1992). The

mechanism of ischaemic preconditioning is not fully understood but the formation of adenosine during the ischaemic period may have a protective effect and administration of exogenous adenosine has been shown to have some beneficial effects in experimental work. It has also been shown that heat-shock proteins are involved in ischaemic preconditioning although the mechanism by which these stress proteins exert their protective effect has not been fully elucidated (Cophen & Downey, 1993; Karmazyn *et al.*, 1990).

1.10 The systemic inflammatory response syndrome

Ischaemia-reperfusion injury invariably follows aortic cross-clamping but most patients undergoing elective AAA repair are protected from the effects of IRI by endogenous defence mechanisms. However, in ruptured AAA, multiple tissue insults occur (e.g. shock, haemorrhage, hypothermia, massive blood transfusion and prolonged aortic cross-clamp time) and lead to loss of local control of IRI and the development of the systemic inflammatory response syndrome (SIRS). SIRS is defined clinically by the presence of two or more of the following conditions (a) temperature greater than 38°C or less than 36°C; (b) heart rate greater than 90 beats per minute; (c) respiratory rate greater than 20 breaths per minute or an arterial partial pressure of carbon dioxide lower than 4.3kPa and (d) white blood cell count greater than 12000 or less than 4000 cells per mm^3 or with more than 10 % immature forms (Bone *et al.*, 1992). Non-cardiogenic pulmonary oedema is often an early clinical manifestation of SIRS and many of these patients will go on to develop multiple organ failure (Davies & Hagen, 1997).

The pathogenesis of SIRS occurs in three stages. The first stage is a normal response to an insult such as aortic cross-clamping and ischaemia-reperfusion injury with the production of cytokines, an acute inflammatory response and activation of the reticuloendothelial system that leads to tissue repair and healing (Bone, 1996).

In the second stage of SIRS, small quantities of cytokines are released into the circulation and recruit neutrophils and macrophages which initiate an acute phase response. This response is tightly controlled and gradually down-regulated (by anti-inflammatory cytokines) while the initiating insult resolves. If the initial insult persists or multiple insults occur SIRS may develop with a large cytokine response. Sustained activation of the reticulo-endothelial system and generalised loss of microcirculatory integrity occurs which eventually results in multiple organ failure (Bone, 1996).

1.11 Inhibition of the systemic inflammatory response

Inhibition of the pro-inflammatory cytokine response with antibodies directed towards TNF-α, interleukins, leukotrienes and endotoxin has been shown to have some benefit in animal studies but these results are not reproducible in humans (Greenman *et al.*, 1991; Ziegler *et al.*, 1991; Fisher *et al.*, 1998). Prophylactic haemofiltration to reduce blood endotoxin and cytokine levels has been performed in patients at high risk of developing ARDS and multi-organ failure (Hirasawa *et al.*, 1996).

Thromboxane antagonists have been used in patients with acute lung injury and have produced encouraging results(Yu & Tomasa, 1993). Prostaglandin E1 (PGE 1) improved oxygenation and decreased venous admixture in patients with adult respiratory distress syndrome (Meyer *et al.*, 1998; Putensen *et al.*, 1998).

1.12 Nitric oxide

In 1980 Furchgott and Zawadski reported that acetylcholine-mediated relaxation of vascular rings was effected by a non-prostanoid, endothelium-derived relaxing factor (EDRF). Subsequent studies by Palmer (1987) and Ignarro (1987) demonstrated that EDRF was nitric oxide (NO) and that its production was dependent on the presence of L-arginine. The consequent explosion in NO research led to the naming of NO as 'molecule of the year' for 1992 by *Science*. Nitric oxide has been demonstrated to be an autocrine and paracrine cellular mediator involved in the maintenance of many physiological systems and derangements of its metabolism are found in many pathological conditions. Nitric oxide is unlike most other bioactive molecules, in that it is not stored, it does not require exocytosis to leave the cell and it does not act on specific extracellular receptors. Instead it is formed at a basal rate which can be augmented by external stimuli, it diffuses freely and binds to carrier compounds and acts on an intracellular receptor (Davies *et al.*, 1995).

1.13 Nitric oxide synthesis

Nitric oxide is formed as a byproduct of the conversion of L-arginine to L-citrulline by the group of enzymes called the nitric oxide synthases (NOSs) (Knowles & Moncada, 1994) which have molecular weights ranging from 130-150 kDa. They were initially named after the tissues in which they were first isolated, and are broadly divided into the constitutive and inducible isoforms. The constitutive isoforms were first isolated in

endothelial and neuronal tissue, and hence are named endothelial NOS (eNOS –135kD) and neuronal NOS (nNOS –150kD) respectively. Inducible NOS (iNOS –130kD) is readily expressed in many tissues when stimulated by pro-inflammatory mediators. Further analysis of NOS has divided this enzyme into four main isoforms displaying about 50% amino-acid sequence identity. Two of these are constitutive isoforms (cNOS) and two are inducible isoforms (iNOS)(Davies *et al.*, 1995) (Table 1.2). The constitutive isoforms of the enzyme are calcium dependent and are responsible for a continual, basal release of NO under physiological conditions, although this production can be upregulated. They are membrane bound and possibly linked to the endothelial cell cytoskeleton which accounts in part for the regulatory effect of endothelial NOS on vascular shear stress and flow. Other local mediators that regulate their action include acetylcholine, bradykinin and histamine.

Inducible NOS is a free cytosolic enzyme which is basally quiescent but once induced is capable of producing NO in amounts 1000 fold greater than cNOS (Fostermann *et al.*, 1994). It was initially believed that iNOS was calcium independent but this has recently been questioned. Recent work has demonstrated that iNOS contains calmodulin which is tightly bound and only requires low levels of calcium for activation and thus gives the impression that it is calcium independent (Cho *et al.*, 1992). All the isoforms of NOS also have the ability to produce superoxide particularly in the absence of L-arginine (Pou *et al.*, 1992). NO synthesis can be controlled by a negative feedback inhibition on NOS.

Form	Isoform	Principal source	? Basal no production	Endogenous inducers	Endogenous inhibitors
	Ia	Brain			
				ACh	
	Ib	EC		ATP	
Constitutive			Yes	ADP	ADMA
	Ic	PMN		Thrombin	
				SS	
	III	EC		AA	
	II			Cytokines	IL-4/IL-10
Inducible		Macrophage	No	Endotoxin	GC
	IV				

Abbreviations:

EC=	endothelial cells	ACh=	acetylcholine	ADMA=	asymmetric dimethyl arginine
PMN=	polymorphonuclear leucocytes	ATP=	adenosine 5'-triphosphate	IL-4=	interleukin-4
		ADP=	adenosine 5'-diphosphate	IL-10=	interleukin-10
pM=	ipicomolar	SS=	Shear stress	GC=	glucocorticoids
nM=	nanomolar	AA=	excitatory amino acids		

Table 1.2 Classification and properties of nitric oxide synthase
Weight et al (1998)

1.14 Nitric oxide metabolism

Nitric oxide is a small lipophilic molecule that is moderately soluble in water making it an ideal intra and intercellular messenger. Nitric oxide is a very reactive molecule, having a half-life of less than 30 seconds in solution. It readily binds and is inactivated by several pathways *in vivo.* Many of the physiological actions of NO occur by binding to the haem-iron complex of soluble guanylate cyclase, forming nitrosyl-haem. This in turn activates guanylate cyclase resulting in increased formation of cyclic guanosine monophosphate (cGMP), which leads to activation of cGMP dependent protein kinases (Henry *et al.*, 1993). This biological response is responsible for a myriad of physiological processes including smooth muscle relaxation, photo-transduction in the retina, enterotoxin related secretion of fluid into the intestine and inhibition of platelet aggregation (Murad, 1994).

Although endogenous NO is derived from a single pathway (the so-called L-arginine/NO pathway), its fate is more complicated and follows several routes (Figure 1.12). NO is a free-radical and its nitrogen atom can exist in multiple oxidative states, depending on the conditions of the surrounding microenvironment and on the concentration of NO in that medium (Gaston *et al.*, 1994). In oxygenated biological systems NO reacts with O_2 producing nitrogen dioxide (NO_2) and other oxidants as intermediates which in turn dissolve in solution to form nitrite (NO_2^-) as an end product. Nitrite will remain stable for a few hours in aqueous solution, but is rapidly converted in the blood to nitrate (NO_3^-) which is excreted by the kidneys. Nitrates and methaemaglobin form the major end products of the metabolic pathway for endogenously produced NO (Wennmalm *et al.*, 1993).

Nitric oxide readily reacts with the superoxide ion (O_2^-) to yield the peroxynitrite anion ($ONOO^-$). This was initially thought to play a beneficial role in scavenging superoxide but it is now known that the peroxynitrite anion can cause lipid peroxidation, nitrosation of several tyrosine molecules that regulate enzyme function and signal transduction, Na^+ channel inactivation and interactions with transitional metals. These interactions contribute to cell death (Radi *et al.*, 1991). Peroxynitrite also decomposes to yield the highly injurious hydroxyl ion (Beckman *et al.*, 1990) (Figure 1.11).

The rate of peroxynitrite formation depends on the product of superoxide and nitric oxide levels, it will increase 100 fold for every tenfold increase in superoxide and NO concentration. Thus, relatively small increases in rates of superoxide and NO production

may greatly increase peroxynitrite production to potentially cytotoxic levels (Beckman *et al.*, 1990).

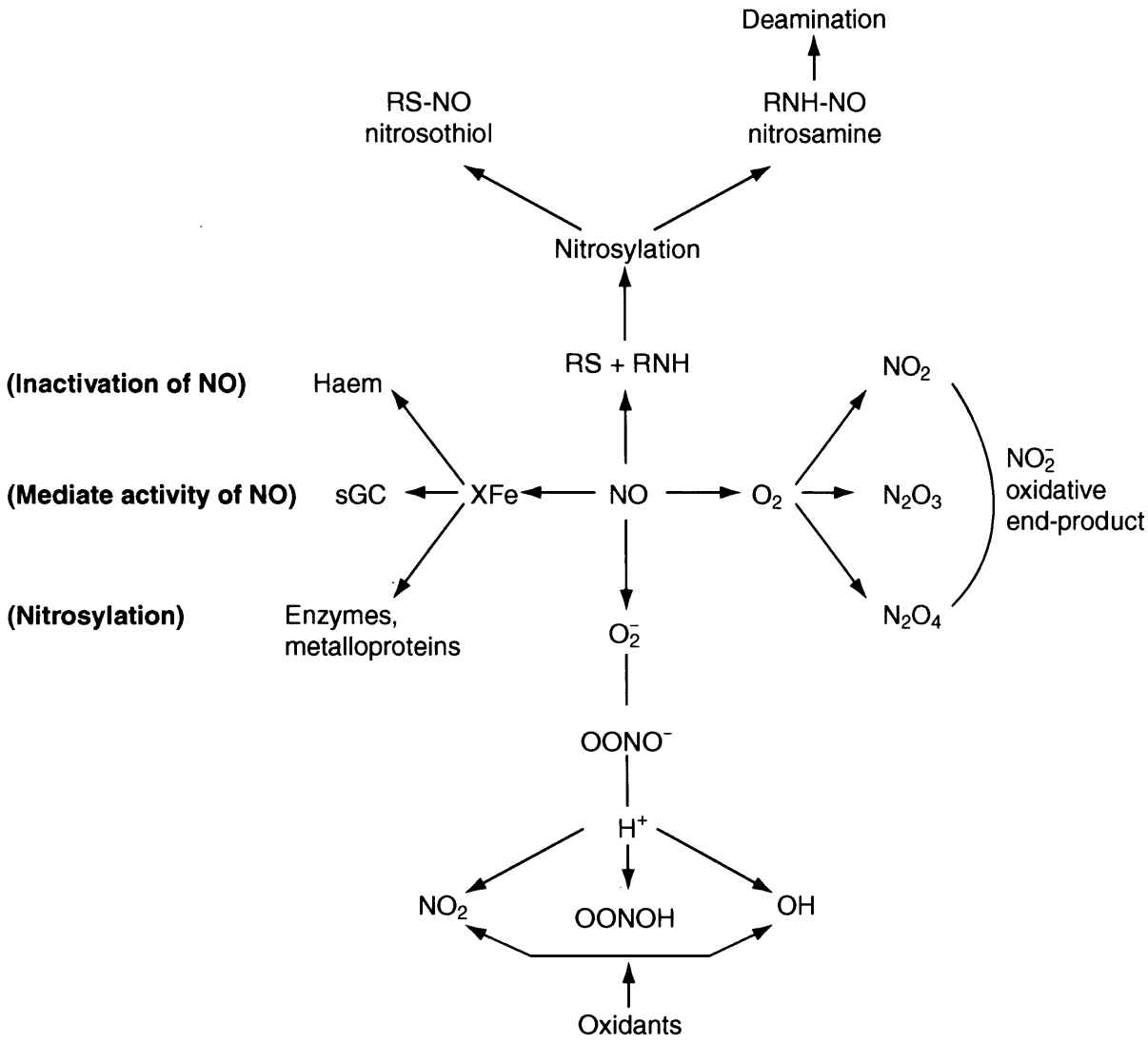
Nitric oxide has a high affinity for iron-containing compounds such as haem, non-haem metalloproteins and certain enzymes. NO is readily inactivated in blood through its interaction with haemoglobin. Under physiological conditions, NO will combine with protein bound thiol groups to form stable, biologically active S-nitrosyl compounds e.g. S-nitrosoalbumin and S-nitroso-L-cystine. These compounds are biologically active and are a secondary transport mechanism for NO (Keaney *et al.*, 1993; Stamler *et al.*, 1992).

A schematic representation of endogenous NO *in vitro* is illustrated in Figure 1.12

1.15 Modulation of NO metabolism

Interference at any step in the L-arginine / NO pathway can lead to inhibition of NO production. The massive explosion in NO research has led to many compounds that have been found to inhibit the nitric oxide synthases, act as NO donors and modulate NOS expression.

The guanido–substituted L-arginine analogues are an important group of competitive NOS inhibitors. The analogues used most commonly in research are NG-nitro-L-arginine methyl ester (L-NAME) and NG-monomethyl-L-arginine (L-NMMA). These two compounds competitively inhibit all isoforms of NOS, they act in a similar way and are orally active (Moncada *et al.*, 1997). The search for a specific inhibitor of iNOS led to the popular use of a guanidine compound-aminoguanidine in NO research. This compound is orally active. It is important to note that aminoguanidine is only relatively specific in its inhibitory actions on iNOS, and it does have some inhibitory actions on the other isoforms of NOS (Moncada *et al.*, 1997). There are now many new specific inhibitors of the various isoforms of NOS available for research use. One worthy of note is 1400W, which is a very tightly binding,specific,non-competitive inhibitor of iNOS which has little activity on the other NOS isoforms making it a valuable research tool. Another common method of specifically inhibiting the expression of iNOS is by the use of steroids. Glucocorticoids inhibit the synthesis and induction of iNOS, but not the activity of iNOS that has already been expressed. Steroids have no effect on expression and activity of the constitutive isoforms of NOS (Radomski *et al.*, 1990).



NO may react with O_2 forming nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3) or dinitrogen tetroxide (N_2O_4), and nitrate (NO^{2-}) is the major end product of this pathway. Secondly, NO may react with superoxide anion (O^{2-}) forming peroxynitrite ($OONO^-$) and peroxynitrous acid (OONOH) which are strong oxidizing agents. Thirdly, NO reacts with iron-containing compounds, metalloproteins and certain enzymes leading to nitrosylation of these compounds. Finally, through oxidation of NO to higher oxides of nitrogen it causes nitrosylation of thiols (RS-NO), or amines (RNH-NO), depending on the concentration of NO.

Figure 1.12 Schematic representation of the possible routes for nitric oxide (NO) fate.
Al - Ali et al (1998)

The clinical actions of nitrovasodilators are known to result from their ability to liberate NO and hence they are now classified as NO-donors. The componds used most commonly in clinical practice include glyceryl-trinitate (GTN) and sodium nitroprusside (Moncada *et al.*, 1997). They are often used in vascular surgery during aortic cross-clamping (to reduce the afterload on the heart), have a short half-life and marked effects on blood pressure. Ceratin nitrosylated componds such as S-nitroso-L-cystine and S-nitrosoglutathione may also be considered as biologically active carriers and donors of NO. These compounds have less marked effects on blood pressure, and also inhibit platelet aggregation (DeBelder *et al.*, 1995).

Finally NO may also be used directly as an inhaled gas. This has the advantage of acting directly on the airways and being readily inactivated by haemoglobin, hence having little systemic effect (Cuthbertson *et al.*, 1997).

1.16 Measurement of NO

The measurement of NO in biological samples is difficult because it is present in small quantities, is inactivated quickly and the end products readily disperse from their site of synthesis.

Direct measurement of NO is complex and expensive, requiring sophisticated detection equipment. The various methods include chemiluminescence where NO reacts with ozone and generates light (Chung & Fung, 1990), and electron paramagnetic resonance in which NO is trapped by nitroso compounds to form more stable compounds which give a signature spectrum when they return to a low energy state from a high energy one. When NO is trapped by haemoglobin the formation of methaemaglobin can be quantified spectrophotometrically by the resultant shift in absorbance from 433nm to 406nm (Ignarro *et al.*, 1987). Nitrosothioproline is another compound which traps NO, and changes in absorbance may be quantified in a mass spectrometer (Gustafsson *et al.*, 1991). Finally a porphyrin-based microsensor has been described with a detection threshold of 10^{-20}mols, allowing quantification of NO in single cells (Malinski & Taha, 1992).

Most experimental work published to date relies on indirect methods of quantifing NO by measurement of the stable metabolic end products and quantification of the NOSs and their activity. The measurement of total nitroxides (NO_2^- and NO_3^-) is a very popular method of indirectly quantifying NO synthesis. All the nitroxides in a given sample are converted to nitrite (NO_2^-) by the commercially available enzyme nitrate reductase. Nitrite

is measured simply in a diazotisation assay with Greiss reagent (0.1% napthylethylenediamine dihydrochloride in distilled water and 1 part 1% sulphanilamide in 5% concentrated phosphoric acid) and the resultant purple azo dye quantified spectrophotometrically (Gomez-Jimmenez *et al.*, 1995). Limitations of this assay include its lack of sensitivity and the fact that a significant quantity of NO produced combines with haemoglobin and other proteins.

A more sensitive marker of NO production is quantification of the activity of nitric oxide synthase. This can be performed by incubating a sample of tissue homogenate in a solution containing ^{3}H L-arginine for a set amount of time (e.g. 45 minutes) at 37^{o}C. At the end of this period, ^{3}H L-citrulline produced in the reaction mixture by tissue NOS, may be separated from the supernatant fluid by passage through an acidic ion exchange resin. L-arginine is basic and binds tightly to the resin while L-citrulline is neutral and remains in solution. The ^{3}H L-citrulline in the mixture may be quantified in a beta counter and is a measure of NO production in tissue homogenates (Thiemermann *et al.*, 1993). The limitation of this assay is that a sample of tissue is required .

Anti-bodies have now been raised to the various isoforms of NOS in different species and many of them are now commercially available. This allows the quantification of NOS in tissues by immunohistochemistry and western blot analysis. A complimentary nucleotide sequence (a probe) of the NOS RNA has been recently developed and can be used to detect expression of NOS RNA in cells by the reverse transcriptase-polymerase chain reaction (Luss *et al.*, 1996).

1.17 Nitric oxide in physiological states

Since its discovery, NO has been shown to have a role in many physiological processes including control of vascular tone as well as effects on cardiac, renal and pulmonary function.

Nitric oxide and the vascular endothelium

Endothelium-derived NO is involved in the regulation of blood vessel tone and the interaction of endothelium with leucocytes and platelets. The loss of endothelium interferes with regulation of vascular tone. There is a basal release of NO from vascular endothelial cells which can be stimulated and increased by many receptor–dependent and independent agonists. Basal NO release is controlled by a variety of physical, cellular and

humoral factors (Moncada *et al.*, 1991). NO has greater effects on venous than arterial tissues and this difference has been ascribed to either a relative lack of basal venous endothelial cell production or a relatively increased basal release of NO from arteries (Ignarro, 1989). Nitric oxide acts via stimulation of soluble guanylate cyclase in vascular smooth muscle cells, resulting in the formation of cyclic-GMP. Protein phosphorylation by the cyclic-GMP dependent kinases results in smooth muscle relaxation (Murad, 1994). Endothelial cells function as mechanosensors and transduce the physical forces produced by the flow of blood into biochemical signals to which the vessel wall can respond. Rapid responses are mediated in part by changes in NO production. Constitutive NOS is linked to the cytoskeleton and cell membrane, and the shear stress regulatory element of the cNOS gene allows the cells to respond with either upregulation or downregulation of NOS activity in response to altered external shear stresses. This may explain the phenomenon of flow-dependent vasodilation which is seen in both the macrocirculation and microcirculation (Davies & Tripathi, 1993).

Nitric oxide is one of several endothelium-derived thromboregulators, which are physiological substances that modulate the early phases of thrombus formation. NO reduces adhesion, activation and recruitment of platelets on the endothelial surface (Radomski *et al.*, 1987), and NO acts synergistically with prostaglandin I_2 to inhibit and actively mediate platelet disaggregation from the collagen fibres of the subendothelial matrix (Radomski *et al.*, 1990).

Nitric oxide is involved in leucocyte interactions with the vessel wall and can inhibit neutrophil aggregation (Kubes *et al.*, 1991). There is evidence to suggest that endothelial expression of adhesion molecules is upregulated in the presence of NOS inhibitors. Exogenous NO inhibits the adherence of mononuclear cells to endothelial cells, inhibits chemotaxis and inhibits the expression of monocyte chemotactic protein 1(Vidal *et al.*, 1992). Nitric oxide containing agents have been found to inhibit lysosomal enzyme release from neutrophils, and NO synthesis by activated neutrophils have been found to decreases superoxide production independent of its reactive capability with superoxide (Clancy *et al.*, 1992; McCall *et al.*, 1989).

Nitric oxide and pulmonary physiology

Nitric oxide is found at a concentration of 5-20 parts per billion in the exhaled air of healthy mammals (Barnes, 1993). Both cNOS and iNOS are expressed in the bronchial

epithelial cells, alveolar macrophages and the pulmonary vascular bed. Endogenous NO is involved in three important physiological functions in the lung- maintaining bronchial tone, regulating pulmonary blood flow and defence against invading microorganisms (Al-Ali & Howarth, 1998).

Within the tracheo-bronchopulmonary system, there are three main systems that control tone. The cholinergic system mediates bronchoconstriction and the adrenergic system mediates bronchodilation. In addition there is a non-adrenergic non-cholinergic pathway (NANC), which is more prominent in the proximal airways and these cells stain readily for NOS with immunohistochemical techniques. Nitric oxide is probably the prime neurotransmitter in these (nitrigenic) nerves and is the mediator of neurally mediated bronchodilation (Belvisi *et al.*, 1992; Rada *et al.*, 1993).

The low-pressure pulmonary vascular bed is regulated, at least in part by endothelial derived NO. Pulmonary arterial and venous endothelial cells are capable of generating NO (Iggarno *et al.*, 1987) and *in vitro* studies have demonstrated that inhibition of endothelium-dependent NO production by NOS inhibitors or by endothelial removal enhances the response to vasoconstrictor stimuli and increases pulmonary vascular resistance (Crawley *et al.*, 1990; Stamler *et al.*, 1994). Furthermore local infusion of L-NMMA into the segmental pulmonary arteries results in a dose-dependent decrease in local blood flow, which is reversed by acetylcholine in healthy conscious human adults. These observations suggest that NO is an endogenous nitrovasodilator which regulates the pulmonary vascular tone under basal conditions (Cooper *et al.*, 1996).

The respiratory system is directly exposed to the environment and acts as an immunological filter to airborne infection. There is evidence that NO synthesised in activated alveolar macrophages plays an important role in host defence against bacteria, viruses, fungi, parasites and tumour cells via peroxinitrite mediated toxicity (Albina & Reichner, 1995).

Nitric oxide and renal physiology

Nitric oxide synthesised by the constitutive isoforms of NOS plays a major role in the control of renal vascular tone. NO exerts a tonic vasodilatory effect on the afferent arteriole of superficial glomeruli, opposing angiotensin II- induced vasoconstriction (Kone, 1997). Angiotensin II and endothelin are both vasoconstrictors and dynamically oppose the vasodilatory action of endogenous NO to control glomerular blood flow (Ito *et al.*, 1991).

Endogenous NO preferentially adjusts the contractile responses of the preglomerular vessels to these vasoconstrictors. Intrarenal NOS blockade, at levels that do not alter systemic mean arterial pressure, results in a small increase in afferent arteriolar resistance, a small decrease in the glomerular ultrafiltration coefficient and a small decrease in glomerular filtration rate of superficial nephrons. Glomerular capillary pressure and efferent arteriolar resistance does not change until NOS blockade is high enough to increase the systemic blood pressure. In the juxtamedullary nephrons, NO plays a vasodilatory role in both the afferent and efferent arterioles. Generalised systemic NO inhibition leads to dose dependent increases in blood pressure and renal vascular resistance, a large fall in renal plasma flow and a fall in glomerular filtration rate (Bayliss & Qiu, 1996).

Nitric oxide influences sodium excretion and plays a physiological role in the control of sodium balance. NO has a direct tubular effect and inhibits sodium reabsorption in the collecting duct. Local adminisitration of NO inhibitors into the renal medullary interstitium selectively decreases papillary blood flow and lowers urinary sodium excretion (Bayliss & Qiu, 1996).

1.18 Nitric oxide in Pathological states

Atherosclerosis

Studies have demonstrated that atherosclerosis induces an early and selective impairment of endothelium-derived NO-mediated relaxation. In the coronary microcirculation, impairment of NO-mediated relaxation occurs before the early structural changes of atherosclerosis are seen; furthermore in experimental models of atherosclerosis using cholesterol enhanced diets, modulation of endothelium-derived relaxation occurs before any structural changes in the vessel wall occur (Harrison, 1993). The proposed mechanism is a decrease in NO release from the endothelium through alterations in certain receptor mediated functions and an associated interaction of NO with oxidized low density lipoproteins (LDL) leading to NO inactivation. Acute exposure of *in vitro* vessel ring preparations to LDL leads to a significant decrease in NO-mediated relaxation which can be prevented by LDL receptor antagonists (Rubyani, 1991).

Studies have also demonstrated that NO may be involved in the progression of atherosclerosis. Endothelium-derived relaxation can be preserved in hypercholesterolaemic

rabbits by an L-arginine supplemented diet, and this also results in a histological decrease in the formation of atheroma in major vessels (Cooke *et al.*, 1992). In the coronary circulation decreased NO-dependent vasodilation may be in part responsible for the pathophysiology of angina and myocardial infarction (Fuster *et al.*, 1992).

Inflammation and sepsis

There is evidence that NO plays a role in acute and chronic inflammation, the systemic inflammatory response syndrome and sepsis (Thiermermann, 1997). Peripheral blood monocytes, alveolar macrophages, Kupffer cells, neutrophils and smooth muscle cells are all capable of synthesising NO if stimulated (Nathan, 1992). Septic shock can be considered to be a cascade of cytokine mediated events resulting in hypoperfusion and organ dysfunction; and endotoxin and pro-inflammatory cytokines are powerful inducers of iNOS. In endotoxic shock, increased systemic NO production may cause hypotension and systemic inhibition of iNOS can increase systemic blood pressure and decrease cardiac output in these patients (Thiermermann, 1997).

In multiple organ failure, hepatocellular dysfunction occurs with raised levels of bilirubin and decreased levels of albumin. Hepatocyte dysfunction appears to be the result of cytokine-induced production of NO (through iNOS) and cytokine release from macrophages and Kupffer cells (Morris & Billar, 1994). Macrophages synthesise NO. In high concentrations NO may be responsible for the cytotoxic effects of macrophages on tumour cells and bacteria (Hibbs *et al.*, 1988; Nathan & Hibbs, 1991).

Non-specific immunity is also associated with the induction of iNOS (Kolb & Kolb-Bachofen, 1992; Morris & Billar, 1994), and anti-microbial and tumouricidal activity occurs following the induction of iNOS. The allogenic immune response as measured by the mixed lymphocyte response involves NO synthesis and inhibition of NOS results in an enhanced mixed lymphocyte response. In addition increased NO production is seen during graft rejection and graft versus host disease (Lancaster *et al.*, 1992).

Nitric oxide in pathological states of the lung

Acute hypoxia induces pulmonary vasoconstriction, and this pressor response is counteracted by NO release (Cremona *et al.*, 1991; Liu *et al.*, 1991). Inhaled NO has been shown to prevent and reverse the hypoxic pulmonary vasoconstrictor response in animal (Psion *et al.*, 1993) and human studies (Frostell *et al.*, 1993). Chronic hypoxia is an

important cause of pulmonary hypertension, and there is evidence that hypoxia markedly reduces NOS activity with a decrease in NO release (Gustafsson *et al.*, 1991). Pulmonary arterial rings from patients with COAD have marked impairment of endothelial-dependent relaxation in response to acetylcholine, with preserved and augmented responses to NO donors, suggesting that COAD patients have impaired synthesis and release of NO (Dinh-Xuan *et al.*, 1993). An immunocytochemistry study of pulmonary arteries demonstrated only weak staining for eNOS among patients with secondary pulmonary hypertension due to COAD (Giaid & Saleh, 1995).

The low levels of exhaled NO detected in normal subject are probably produced by the constitutive isoforms of NOS, while in inflammatory disorders of the respiratory tract there is evidence of increased NO production from iNOS. Asthmatic patients have significantly higher levels of exhaled NO together with increased expression of iNOS in the bronchial epithelium (Al-Ali & Howarth, 1998). Both NO levels and iNOS expression are reduced by corticosteroid therapy (Hamid *et al.*, 1993). Acute asthma attacks are associated with higher levels of exhaled NO and these levels fall within 48 hours of steroid therapy, coinciding with improvement in lung function (Massaro *et al.*, 1995). Stable asthmatics on inhaled steroids have lower levels of exhaled NO (Kharitonov *et al.*, 1996). These findings suggest that NO is a marker of airway inflammation.

Acute lung injury and non-cardiogenic pulmonary oedema may occur secondary to a variety of insults including shock, sepsis, lower torso ischaemia-reperfusion injury and massive blood transfusion (Bringham & Meyrick, 1986; Fantini & Conte, 1995; Malouf & Glanville, 1993). Although many patients recover from this insult, progression to the adult respiratory distress syndrome (ARDS) is associated with a high mortality. The pathogenesis of acute lung injury involves a systemic inflammatory response, with infiltration of leucocytes into lung tissue (Windsor *et al.*, 1993). Progression of the disease results in persistent hypoxia, alveolar collapse and consolidation, endothelial damage, superimposed infection, pulmonary hypertension and ventilation-perfusion mismatch (Artigas *et al.*, 1991). Patients with established ARDS have improved oxygenation with inhaled NO due to an improvement in ventilation-perfusion mismatch because NO selectively improves the perfusion of the areas of lung that are oxygenated (Artigas *et al.*, 1998).

Nitric oxide in renal diseases

Impaired NO synthesis in the kidney results in unopposed vasoconstrictor action and impaired sodium excretion. Studies of salt-loaded humans with mild essential hypertension suggest that salt-sensitive patients exhibit increased renal vascular resistance and impaired ability of the kidney to produce NO compared with salt-resistent hypertensive patients (Higashi *et al.*, 1996).

During glomerular inflammation, NO and cytokines from glomerular endothelial cells, mesangial cells and infiltrating macrophages can act together to sustain, promote or limit glomerular damage. Recent studies have implicated iNOS generated NO in several forms of glomerulonephritis. In humans, iNOS mRNA is consistently found in renal biopsies from patients with IgA nephropathy and mesangial proliferative glomerulonephritis. Immunohistochemical analysis has revealed that the cells expressing iNOS were predominantly infiltrating monocytes and macrophages, reflecting the inflammatory nature of these diseases (Kone, 1997).

Acute renal failure is associated with increased production of vasoconstricting agents including angiotensin II, thromboxane A2, cathecholamines and endothelin (Schramm *et al.*, 1996). These mediators decrease renal blood flow and glomerular filtration rate. Endogenous NO may act to preserve glomerular blood flow and maintain renal function in acute renal failure (Schramm *et al.*, 1996).

Ischaemia-reperfusion injury

Although NO plays a role in IRI there is disagreement on the endogenous NO response following IRI, not only between different organs but also in studies on the same organ. For example, a decrease in endogenous NO production was reported following IRI in skeletal muscle (Stenbergh *et al.*, 1992) and the coronary endothelium(Ma *et al.*, 1993) but an increase was reported in the lung (Ischiropolous *et al.*, 1995) and small bowel (Mueller *et al.*, 1994). Ma et al (1993) concluded that there was a decrease in NO production in the coronary endothelium following IRI but other studies have reached the opposite conclusion that NOS inhibitors may be protective following IRI.

There may be several explanations for this confusion. In some studies there has been a direct assay of NOS/NO or the stable metabolic end products wheares in others, NO donors and inhibitors were used to block or augment the endogenous response. The former

method is more reliable and appears to suggest that there is increased endogenous NO production following IRI.

Lower torso ischaemia-reperfusion injury generates a pro-inflammatory cytokine response (Thompson *et al.*, 1996) and this in turn may result in the generation of iNOS at remote organs such as the kidneys, lungs, heart and liver (Thiemermann *et al.*, 1993). The effects of NO following IRI have not been fully elucidated. NO may have a protective role in opposing the action of vasoconstrictors such as endothelin, angiotensin and thromboxane which are produced during ischaemia-reperfusion injury. It may also prevent adhesion of platelets and neutrophils to the endothelium (Kubes *et al.*, 1991; Radomski *et al.*, 1990). These effects may decrease the microcirculatory changes of poor flow and platelet plugging seen in ischaemia-reperfusion injury.

However superoxide radicals react with NO to form the peroxynitrite free radical ($ONOO^-$), which is also a precursor of the hydroxyl anion(Radi *et al.*, 1991) (Figure 1.11). The peroxynitrite and hydroxyl radicals are both capable of causing severe tissue damage thus NO is a further source of potentially harmful free-radicals. Furthermore NOS can generate the superoxide ion when there is insufficent L-arginine for NO production.

1.19 Current therapeutic uses of NO donors and inhibitors

Nitric oxide donors such as GTN and sodium nitroprusside are used clinically in the treatment of angina and hypertension. Inhaled nitric oxide is now established as a useful adjunct in the treatment of persistent pulmonary hypertension in the newborn, congenital heart disease and other paediatric disorders associated with hypoxia and pulmonary hypertension (Troncy *et al.*, 1997). In these conditions the foetal circulation is immature and the endogenous NO responses may be impaired.

Inhaled nitric oxide has also been used in the treatment of ARDS. In this condition inhaled NO may decrease the ventilation-perfusion mismatch and improve tissue oxygenation. Unfortunately the interim results of large clinical trials have not shown any decrease in mortality. The final results of these trials are keenly awaited (Artigas *et al.*, 1998).

Nitric oxide inhibition has been used in treatment of septic shock to antagonise the pro-inflammatory cytokine induced increase in iNOS activity. Increased iNOS activity partly contributes to hypotension in these patients (Thiermermann, 1997). Early reports of benefical haemodynamic effects of systemic L-NMMA stimulated a phase-1, escalating

dose study using L-NMMA in 32 patients with septic shock. In this study L-NMMA sustained blood pressure and enabled a reduction in vasopressor (noradrenaline) support. The cardiac index fell (possibly because of an increase in peripheral vascular resistance), and left ventricular function was well maintained (Thiermermann, 1997). There have been other studies on NOS inhibitors in septic shock and a multicentre clinical trial evaluating the effects of L-NMMA on the morbidity and mortality in patients with septic shock is ongoing.

1.20 Conclusions

Nitric oxide is a very important molecule which is involved in many physiological processes. In some disease states, it may have a protective role whilst in others it may be cytotoxic. Ischaemia-reperfusion injury is an important contributor to morbidity and mortality following aortic cross-clamping; nitric oxide may have both beneficial and harmful effects in this process. The aim of this thesis is to investigate the role of NO in lung and renal injury following aortic cross-clamping, and to explore pharmacological manipulation of this response.

CHAPTER TWO

Scope and design of the thesis

2.1 Introduction

2.2 Choosing a model of reperfusion injury

A rodent model of IRI induced acute lung injury

A rodent model of IRI induced acute renal injury

2.3 Measuring nitric oxide

2.4 Experimental work

Chapter 4: Investigation of the influence of infrarenal aortic cross-clamp time on pulmonary NO metabolism and acute lung injury

Chapter 5: Investigation of the influence of reperfusion time on pulmonary NOS activity and acute lung injury

Chapter 6: Investigation of the influence of aortic cross-clamp time on renal nitric oxide metabolism and glomerular filtration rate

Chapter 7: Can manipulation of NO metabolism decrease infrarenal aortic cross-clamp induced acute lung injury?

Chapter 8: Can manipulation of NO metabolism decrease renal impairment following infrarenal aortic cross-clamping?

2.1 Introduction

Abdominal aortic aneurysm remains an important disease in the western world being responsible for about 10,000 deaths per annum in England and Wales (Office for National Statistics, 1995). The overall mortality of patients with ruptured AAA in the community is 90% (Drott *et al.*, 1992). Among patients who survive transfer to hospital and subsequent operation, the 30 day mortality has remained at about 50% over the last 30 years. Much of this mortality is due to respiratory, renal and multi-organ failure (Sayers *et al.*, 1997). The cause of these complications may be multifactorial but recently there has been interest in the role of ischaemia-reperfusion injury (IRI). In the previous chapters the mechanisms by which IRI causes local and remote organ injury have been discussed. In this thesis I will investigate this process in the lungs and kidneys.

Non-cardiogenic pulmonary oedema associated with impairment of pulmonary function has been well documented following aortic cross-clamp induced lower torso IRI (Anner *et al.*, 1987; Anner *et al.*, 1988; Fantini & Conte, 1995; Paterson *et al.*, 1989; Paterson *et al.*, 1989). Although most patients recover from this insult, some progress to established adult respiratory distress syndrome (ARDS) which is associated with a very high mortality. In these patients a systemic inflammatory response is generated following lower torso IRI (Groeneveld *et al.*, 1997; Thompson *et al.*, 1996), and this activates leucocytes and primes the endothelium. Leucocytes infiltrate into the tissues, degranulate and cause tissue injury (Windsor *et al.*, 1993). Non-cardiogenic pulmonary oedema is often the first clinical sign of a systemic process which may progress to established ARDS and multiple organ failure particularly if further tissue insults occur (Davies & Hagen, 1997).

Post-operative impairment of renal-function and acute renal failure may also complicate AAA repair. Aortic cross-clamping is associated with marked changes in renal haemodynamics with a decrease in glomerular filtration rate and renal blood flow (Awad *et al.*, 1992; Gornick & Kjellstrand, 1983; O'Donnell *et al.*, 1989;Gelman, 1995). Ischaemia-reperfusion injury is also important in the pathogenesis of renal injury because the kidney is highly susceptible to damage by oxygen derived free-radical production. Furthermore, IRI is associated with the generation of many vasoconstrictive mediators including endothelin and thromboxane, which decrease effective renal plasma flow (Gelman, 1995).

It is also likely that NO plays an important role in the pathogenesis of renal and pulmonary injury following aortic cross-clamp induced lower torso IRI but its effects have not been fully studied. Nitric oxide inhibits neutrophil-endothelial interaction and

neutrophil infiltration into tissues. NO also prevents platelet aggregation and this may decrease poor microcirculatory flow following IRI. In addition NO is a powerful vasodilator and may counteract endogenous vasoconstrictors including thromboxane and endothelin. However during reperfusion NO can react with the superoxide ion to generate the peroxynitrite and hydroxyl free-radicals. These free-radicals are capable of causing severe tissue damage and hence any endogenous NO response may also contribute to end organ injury. In the previous chapters the role of NO metabolism in physiological and pathological states have been discussed. I have investigated the role of NO metabolism in renal and pulmonary injury following IRI induced aortic cross-clamping. In particular, I investigated the following questions:

1. **What effect does lower torso ischaemia-reperfusion injury have on pulmonary and renal NO metabolism?**
2. **Can manipulation of NO metabolism be used to counteract the injurious effects of lower torso IRI on the lungs and kidneys?**

2.2 Choosing a model of reperfusion injury

Human clinical studies of NO metabolism following infrarenal aortic surgery are difficult because it is not possible to obtain pulmonary and renal tissue samples during and after operation. Furthermore any clinical study must take into account the heterogenicity in age, sex, weight, comorbid factors, diabetes, renal impairment and current medication- all of which can alter the endogenous NO response. Therefore I have chosen to study IRI in two rodent models of aortic cross-clamping.

A model of IRI and lung injury has previously been described by Barry (1996) and I have modified it to allow investigation of NO metabolism. In addition, I have developed a new model to investigate aortic cross-clamp induced IRI and renal injury.

A rodent model of IRI induced acute lung injury

Barry et al (1996) described an elegant model of acute lung injury following infrarenal aortic cross-clamp induced IRI. Briefly adult rats were anaesthetised with halothane and subjected to a period of infrarenal aortic cross-clamping. Following the ischaemic period, the clamp was removed and the animal subjected to a period of reperfusion on a warming tray before being culled. At this point a sternotomy was

performed and part of the lung lavaged. Acute lung injury was quantified by the concentration of protein and nucleated cells in the lavage fluid as an index of pulmonary microvascular permeability. The remaining lung tissue was used for measurement of tissue mediators of IRI such as myeloperoxidase (Barry *et al.*, 1996), which is a haem containing enzyme found in neutrophils and an indicator of neutrophil infiltration and degranulation in the lung (Bradley *et al.*, 1982; Williams *et al.*, 1983). I have modified the above model by developing specific assays to measure NO metabolism in lung tissue, and to measure levels of pro-inflammatory cytokines in plasma.

A rodent model of IRI induced acute renal injury

Studies of IRI induced renal injury require measurement of glomerular filtration rate and renal biopsies to determine renal injury. Serum urea and creatinine levels are not sensitive indicators of renal damage as they only increase after at least half of the endogenous renal reserve is damaged (Morrison, 1996). Rodent models of renal pedicle IRI have been described (Jabolinski *et al.*, 1983; Shoskes *et al.*, 1997) for investigation of renal warm ischaemia during renal transplantation (Weight *et al.*, 1998).

I have developed a rodent model to study infrarenal aortic cross-clamp induced IRI and renal injury. Briefly, adult male Wistar rats were subjected to periods of infrarenal aortic occlusion followed by reperfusion under general anaesthesia. At the end of the reperfusion period a left nephrectomy was performed and the animal recovered. The harvested left kidney provided tissue for quantification of renal NOS activity and the function of the remaining kidney was measured using a ^{99}Tc DPTA isotope clearance technique on the second and seventh post-operative day (Nankivell *et al.*, 1992). At the end of this period the animal was culled and the remaining kidney harvested, and nitric oxide synthase activity measured.

2.3 Measuring nitric oxide

In chapter 1 various methods of measuring NO metabolism were described, which vary in reliability, reproducibility and cost. Direct methods of measuring NO are difficult, very expensive and not practical, therefore, I investigated two indirect methods of measuring NO metabolism- measuring total nitroxides (NO_2^- and NO_3^-) and measuring total NOS activity.

Measuring total nitroxides

Nitroxides (NO_2^- and NO_3^-) are amongst the stable end products of NO metabolism. The Greiss reagent assay is a simple method of measuring the total concentration of nitroxides. Briefly, all the nitroxides in a homogenised tissue sample are converted to nitrite (NO_2^-) by the enzyme nitrate reductase. Adding Greiss reagent to this sample results in the formation of a pink azo dye which can be quantified spectrophotometrically. Greiss reagent is a very popular method of measuring NO synthesis *in vitro* where nitrite and nitrate levels can accumulate and be measured easily. However I was concerned that this method may not be sensitive enough to detect differences *in vivo*, because NO is formed in very small quantities, and the end products of NO metabolism are not exclusively nitrites and nitrates. Therefore I used a second method of measuring NO activity via measurement of total NOS activity.

Measuring NOS activity

NO is formed by the enzyme nitric oxide synthase (NOS) as a byproduct of the conversion of L-arginine to L-citrulline. It is possible to measure total NOS activity by measuring conversion of radiolabelled ^{3}H L-arginine to ^{3}H L-citrulline. This assay involves incubating a tissue sample in a reaction mixture containing ^{3}H L-arginine for about 45 minutes. At the end of the incubation period an acidic ion exchange resin is added to the mixture. L-arginine is a basic amino acid and hence any residual ^{3}H L-arginine binds to the resin and the ^{3}H L-citrulline (which is neutral) remains in solution. The reaction mixture is now filtered and the quantity of ^{3}H L-citrulline in solution is proportional to the total NOS activity in the tissue sample. This method (the citrulline assay) had been the standard assay for measuring NOS activity in tissue samples in several studies of endotoxic shock and renal reperfusion injury (Cristol *et al.*, 1993; Luss *et al.*, 1996; Shoskes *et al.*, 1997; Thiemermann *et al.*, 1993).

There are 3 isoforms of NOS, the two constitutive isoforms (cNOS) and the inducible isoform (iNOS). In pathological states there is often a massive iNOS response. Therefore in order to study NO metabolism after IRI, it is important to quantify the iNOS response separate to total NOS activity. Some studies have measured iNOS using the citrulline assay with and without calcium in the reaction mixture, because the constitutive isoforms of NOS were thought to be calcium dependent whereas iNOS is calcium independent. However recent work has questioned whether iNOS is completely calcium independent (Cho *et al.*, 1992). Fortunately an antibody against rat iNOS which does not

react with rat cNOS is now commercially available. I therefore used the antibody to detect iNOS expression in tissue samples using western blot techniques (Luss *et al.*, 1996).

A detailed description of the animal model and subsequent assays are presented in Chapter 3.

2.4 Experimental work

Using the animal model and assays, I initially investigated the endogenous pulmonary and renal nitric oxide response following infrarenal aortic cross-clamp induced IRI. Then I investigated the effects of manipulating endogenous NOS metabolism by the use of specific NOS inhibitors and donors prior to infrarenal aortic cross-clamping. The agents used in these experiments were:

1. L-arginine (the substrate for NOS)
2. L-NMMA (a pan NOS inhibitor)
3. 1400W (a specific iNOS inhibitor)
4. Hydrocortisone (an inhibitor of iNOS expression)
5. NOC-18 (a slow release NO donor)

L-arginine is the substate for NOS. It has been extensively used as a NO donor in several experimental studies. L-arginine is a non-toxic water soluble molecule which is metabolised as an amino acid in physiological states. It is cheap and readlily availabe. Doses were titred according to previous experimental studies on male rats (Weight *et al.*,1999).

L-NMMA is a water soluble, non-toxic pan-NOS inhibitor. This molecule has been extensively used as a NO inhibitor in experimental work on septic shock. Doses were chosen in accordance to previous work on male rats and recommendations of the manufacturer (Calbiochem-Novabiochem, California; Thiermermann *et al.*, 1997)

1400W is a highly selective and tightly binding inhibitor of iNOS only. It is water soluble, and can be administered intravenously. It is a relatively new agent and doses were titered according to previous work on rats and manufacturer's reccomendations (Calboichem-Novabiochem, California; Garvey *et al.*, 1997, Thomsen *et al.*, 1997)

Hydrocortisone is a steriod. It inhibits the systemic inflammatory response and prevents upregulation of iNOS. Doses were titred according to previous work on male rats (Weight *et al.*, 1999)

NOC-18 is a very new and experimental NO donor. It was chosen because it has a very slow half-life of 3400 minutes and slowly releases NO. This confers it advantages over traditional NO donors such a Glyceryl Trinitrate and Sodium Nitroprusside, both of which rapidly release NO and cause major changes in blood pressure. Doses were titred according to previous experimental studies and manufacturer's reccomendations (Calbiochem-Novabiochem, California, Shibuta *et al.*, 1996).

Chapter 4: Investigation of the influence of infrarenal aortic cross-clamp time on pulmonary NO metabolism and acute lung injury

In this chapter I investigated the optimum ischaemic time required to produce quantifiable acute lung injury. Groups of male adult wistar rats (n=6) were subject to 30, 45 and 60 minutes of infrarenal aortic cross-clamping under halothane anaesthesia. A sham operated control group, which underwent laparotomy and exposure of the infrarenal aorta only was also studied. At the end of the ischaemic period the clamp was removed and the animals subjected to one hour of reperfusion before culling. The upper and middle lobes of the right lung were lavaged and the number of nucleated cells and protein concentration measured in the lavage fluid as an index of acute lung injury. The remaining lung tissue was stored for measurement of myeloperoxidase activity (as an index of neutrophil infiltration and degranulation in the lung), total NOS activity and lung wet:dry ratio (an index of lung oedema).

Chapter 5: Investigation of the influence of reperfusion time on pulmonary NOS activity and acute lung injury

In this chapter I investigated the influence of reperfusion time on the development of the pro-inflammatory cytokine response in acute lung injury. Animals were subjected to one hour of ischaemia, and culled after 0 minutes, 60 minutes and 120 minutes reperfusion. Plasma tumour necrosis factor-alpha (TNF-α) was measured to determine the pro-inflammatory cytokine response.

Chapter 6: Investigation of the influence of aortic cross-clamp time on renal nitric oxide metabolism and glomerular filtration rate

In this chapter I investigated the influence of infrarenal aortic cross-clamp time on endogenous renal NOS activity and glomerular filtration rate (GFR). Groups of male wistar rats (n=6) were subject to 30, 45 and 60 minutes of infrarenal aortic cross-clamping, followed by 1 hour of reperfusion. After this a left nephrectomy was performed and the animals recovered. The GFR in the remaining kidney was measured on the 2nd and 7th post-operative day using a ^{99}Tc DPTA clearance technique. The animals were then culled and the remaining kidney harvested. Renal NOS activity was measured in tissue samples removed on the first and seventh day.

Chapter 7: Can manipulation of NO metabolism decrease infrarenal aortic cross-clamp induced acute lung injury?

In this chapter I investigated the influence of manipulating NO metabolism on infrarenal aortic cross-clamp induced acute lung injury. A therapeutic dose of NO manipulating agents,(L-Arginine, L-NMMA, 1400W, hydrocortisone and NOC-18) was administered to animals 5 minutes before infrarenal aortic cross-clamping. The animals were subjected to 60 minutes of ischaemia and 120 minutes reperfusion and then culled. Acute lung injury was measured as previously described.

Chapter 8: Can manipulation of NO metabolism decrease renal impairment following infrarenal aortic cross-clamping?

In this chapter I investigated the influence of manipulating NO metabolism on IRI induced renal impairment. A therapeutic dose of NO manipulating agents (L-arginine, L-NMMA, 1400W, hydrocortisone and NOC-18) was administered to animals 5 minutes before infrarenal aortic cross-clamping. The animals were subjected to 60 minutes of ischaemia followed by 60 minutes of reperfusion. Then a left nephrectomy was performed and the animals recovered. The influence of manipulating the NOS response on glomerular filtration rate was measured on the 2nd and 7th day post-operatively using the ^{99}Tc DPTA clearance technique.

CHAPTER THREE

Materials and methods

3.1 Introduction

3.2 Experimental protocol for the rodent model of acute lung injury

Animal preparation and procedure for acute lung injury

Animal preparation and procedure for acute renal injury

Pharmacological manipulation of NO metabolism

3.3 Lung assays

Measurement of wet:dry lung ratio

Measurement of nucleated cells in lavage fluid

Measurement of brocheoalveolar lavage fluid protein concentration

Standard curves

Measurement of lung myeloperoxidase activity

Measurement of plasma TNF-α levels

3.4 Measurement of glomerular filtration rate

3.5 Measurement of nitric oxide

Measuring total nitroxides using Greiss reagent

Measurement of total NOS activity

Identifying the expression of iNOS

3.1 Introduction

In this chapter the experimental protocols and the materials used in my subsequent experiments will be described. A project licence under the Animals (Scientific Procedures) Act 1986 was granted (Licensee Professor ML Nicholson, University Department of Surgery, Leicester General Hospital, Reference number PPL 80/986) by the home office to allow the work in this thesis to be performed. In addition I received appropriate training in animal care, and attended a course for this purpose. I obtained a personal licence (PIL 80/6447) to work under the project licence in accordance with home office regulations. Adult male wistar rats weighing approximately 450-500g were used in all experiments. These were outbred from Charles River stock (Charles River, Margate, UK) and were then bred in house by the Biomedical Services Department (University of Leicester, UK). Following weaning, all animals were allowed free access to standard rat chow (UAR, Huntington, UK) and tap water *ad libitum*; and housed three to a cage. The animals were cared for in accordance with the Animals (Scientific Procedure) Act 1986 and all procedures carried out in line with the animal licence conditions.

3.2 Experimental protocol for the rodent model of acute lung injury

Animal preparation and procedure for acute lung injury

Groups of 6 adult male wistar rats weighing 450-500g were used for each arm of an experiment. Three animals were anaesthetised and operated on at the same time. Animals were placed individually in an anaesthetic box with 5% halothane (Zeneca, UK) in oxygen (BOC, UK) at 2 litres per minute flow. Following induction of anaesthesia, each rat was weighed, tail marked, placed supine on a heated mat to maintain body temperature and connected to an anaesthetic circuit (2% halothane in oxygen) via a face mask with a gas scavenger. One millilitre of sterile saline was injected subcutaneously into the nape of the neck each hour to replace ensuing surgical losses from evaporation. The abdomen was clipped and prepared with chlorhexidine in alcohol. A midline laparotomy was performed, the small bowel exteriorised and the exposure maintained with a self retaining retractor (Figure 3.1). A window was dissected in the retroperitoneum and the infrarenal aorta and inferior vena cava exposed. Sham operated control animals were subjected to exposure of the infrarenal aorta and inferior vena cava only. A 25G needle was inserted into the inferior vena cava and one millilitre (1ml) of either sterile saline or an NO manipulating agent infused. The needle was withdrawn and a small patch of Surgicell (Johnson & Johnson, UK) applied to the puncture site with light pressure to achieve haemostasis. Ten minutes

later a Diffenbach microvascular clamp (Bolton Instruments, UK) was used to occlude the infrarenal aorta (Figure 3.2). The bowel was then replaced in the peritoneal cavity and the abdomen closed loosely with a 3.0 vicryl (Ethicon, UK) suture for a variable ischaemic period. After this, the clamp was removed and the animals maintained under anaesthesia for a variable reperfusion time. After this, the animals were culled by rapid withdrawal of about 20 ml of blood from the aorta via a 14 G catheter (Abbot, Republic of Ireland). Blood was collected and immediately spun down at 13000g and a sample of plasma stored for subsequent analysis of TNF-α.

After confirmation of death a median sternotomy was performed. The left main bronchus and the right lower lobe bronchus were clamped. The trachea was cannulated and the right upper and middle lobes of the lung were lavaged three times with 2ml saline containing containing 0.07 mmol/l ethylene diamine tetra-acetic acid (EDTA) (Figure 3.3). The broncheoalveolar lavage fluid was collected in polystyrene tubes (Stardest, Germany) for subsequent measurement of the number of nucleated cells and protein concentration. Then the right lung hilum and the left lower lobe bronchus were clamped and the left upper lobe pulmonary vasculature was flushed with 50ml of saline via the right ventricle (Figure 3.4). The left upper lobe was immediately snap frozen in liquid nitrogen for subsequent measurement of myeloperoxidase activity. The remaining left lower lobe lung tissue was then removed and snap frozen in liquid nitrogen and stored at -80^{o} C for subsequent analysis of NOS. The unmanipulated right lower lobe was used to measure the wet:dry lung ratio.

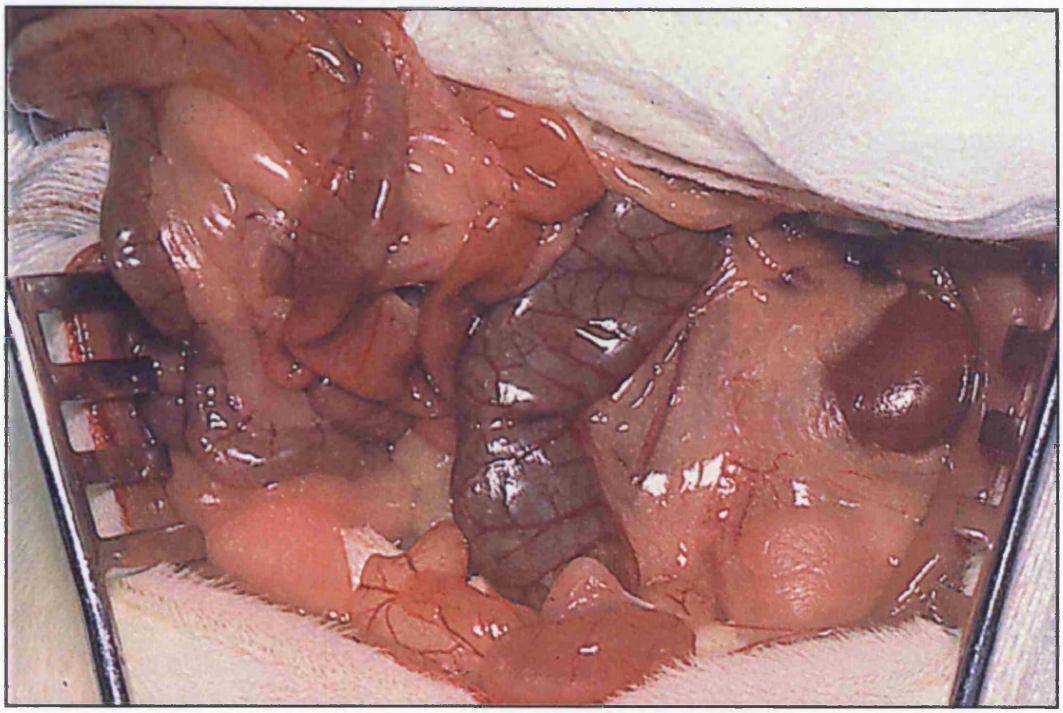


Figure 3.1 Approach to the retroperitoneum after externalising the small bowel

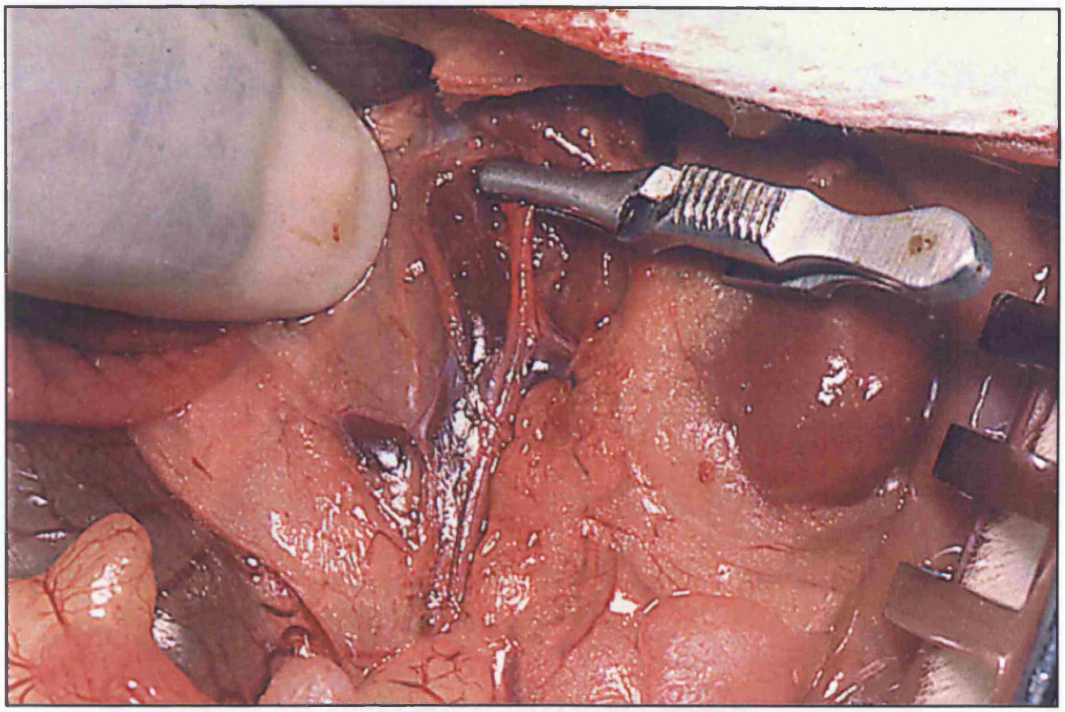


Figure 3.2 The inferior vena cava and infrarenal aorta exposed. A microvascular clamp placed at the level of the infrarenal aorta after 1ml of sterile saline or NO manipulating agent has been injected into the inferior vena cava

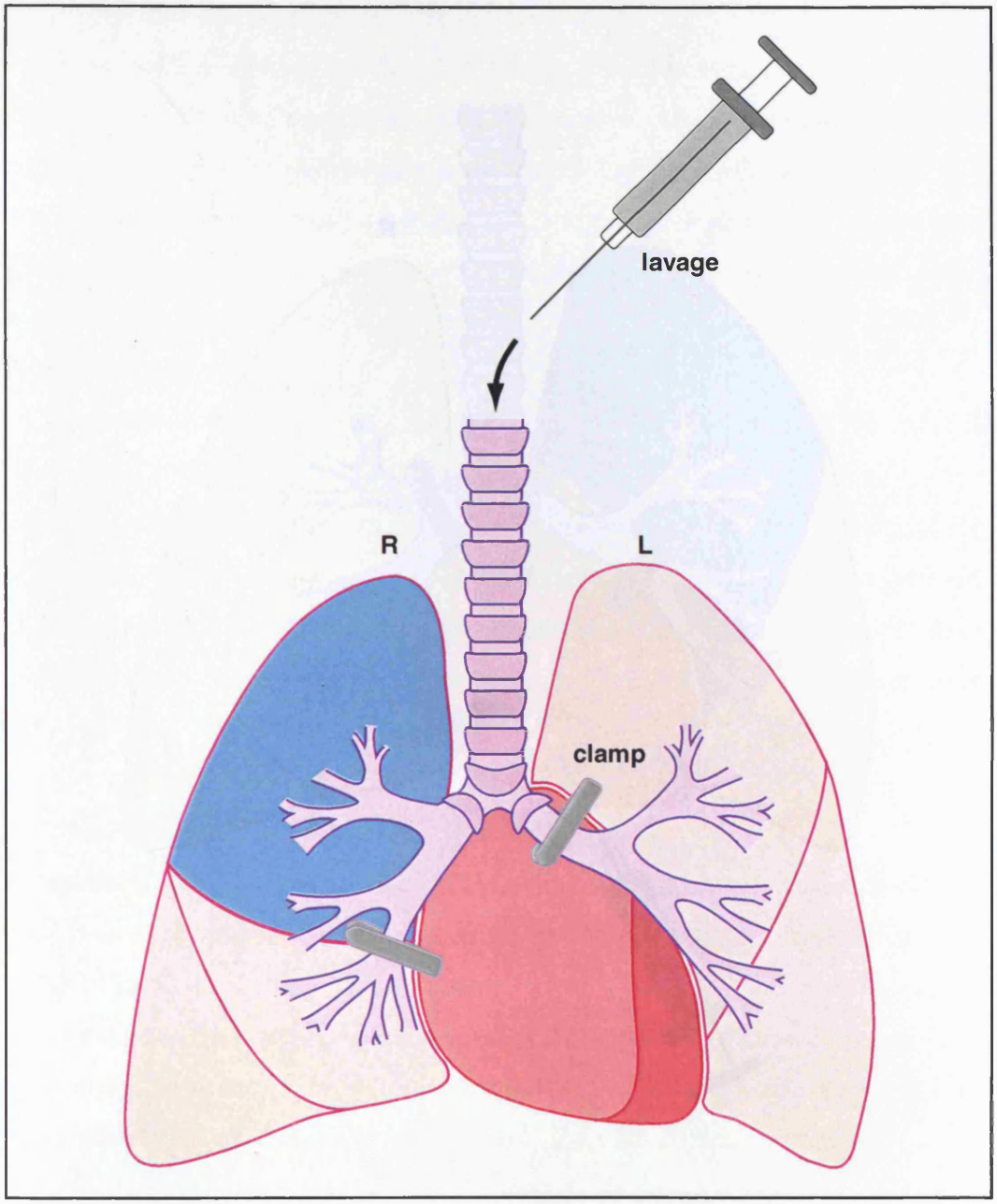


Figure 3.3 The left main bronchus and right lower lobe are clamped, the bronchus is cannulated and the right upper and middle lobes lavaged three times with 2ml saline containing 0.07ml EDTA

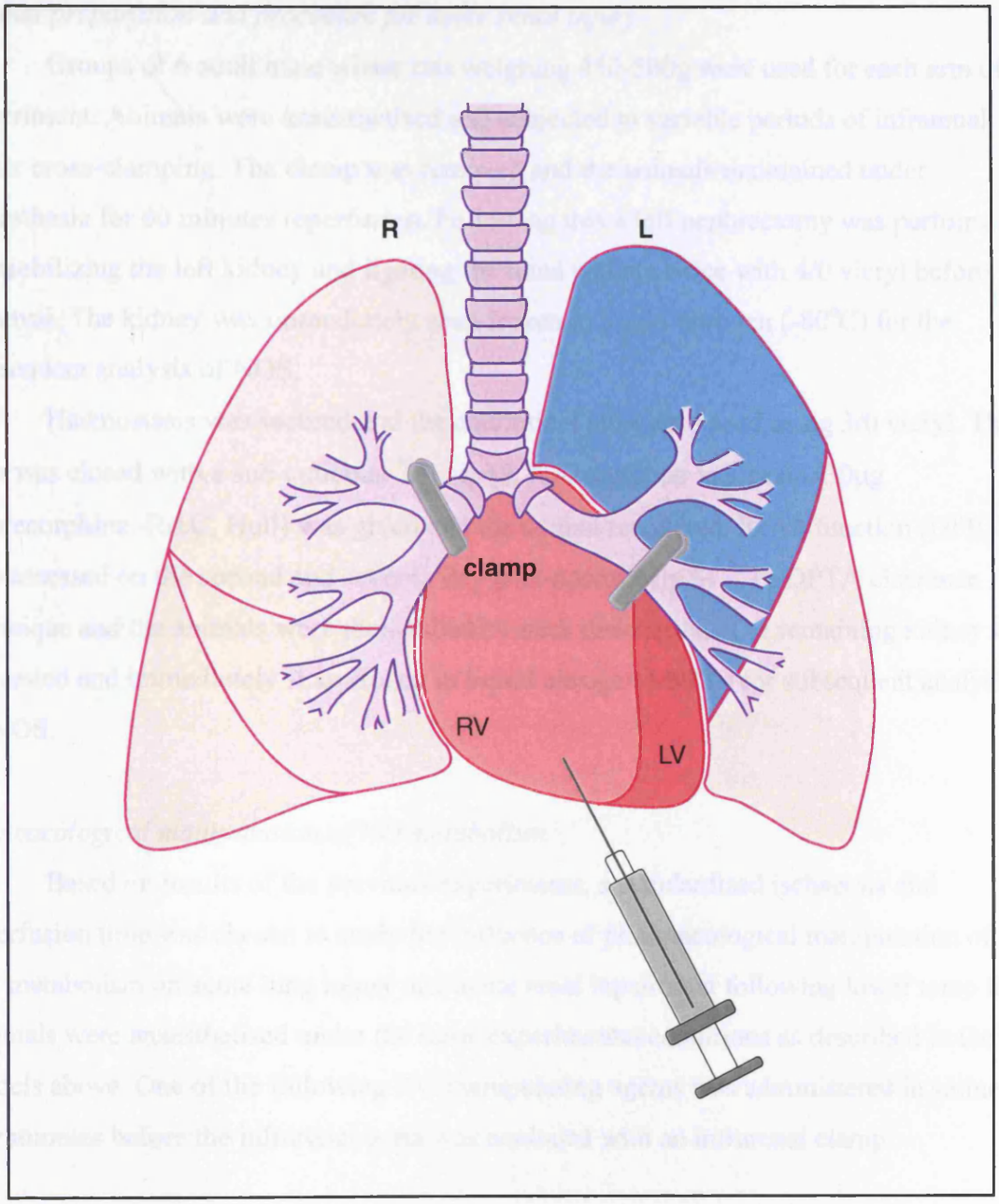


Figure 3.4 The right lung hilum and the left lower lobe bronchus were clamped and the left upper lobe pulmonary vasculature flushed with 50ml of saline via the right ventricle

Animal preparation and procedure for acute renal injury

Groups of 6 adult male wistar rats weighing 450-500g were used for each arm of an experiment. Animals were anaesthetised and subjected to variable periods of infrarenal aortic cross-clamping. The clamp was removed and the animals maintained under anaesthesia for 60 minutes reperfusion. Following this a left nephrectomy was performed by mobilizing the left kidney and ligating the renal pedicle twice with 4/0 vicryl before removal. The kidney was immediately snap frozen in liquid nitrogen (-80°C) for the subsequent analysis of NOS.

Haemostasis was secured and the abdominal muscles closed using 3/0 vicryl. The skin was closed with a sub-cuticular 3/0 vicryl. Intramuscular analgesia (30μg buprenorphine -R&C, Hull) was given and the animal recovered. Renal function (GFR) was assessed on the second and seventh day post-operatively by ^{99}Tc DPTA clearance technique and the animals were then culled by neck dislocation. The remaining kidney was harvested and immediately snap frozen in liquid nitrogen (-80°C) for subsequent analysis of NOS.

Phamacological manipulation of NO metabolism

Based on results of the previous experiments, a standardised ischaemia and reperfusion time was chosen to study the influence of pharmacological manipulation of NO metabolism on acute lung injury and acute renal inpairment following lower torso IRI. Animals were anaesthetised under the same experimental conditions as described in the models above. One of the following NO manipulating agents was administered in saline five minutes before the infrarenal aorta was occluded with an infrarenal clamp:

1. L arginine (the substrate for NOS)
2. L-NMMA (a pan NOS inhibitor)
3. 1400W(a specific iNOS inhibitor)
4. Hydrocortisone (an inhibitor of iNOS expression)
5. NOC-18(a slow release NO donor)

3.3 Lung assays

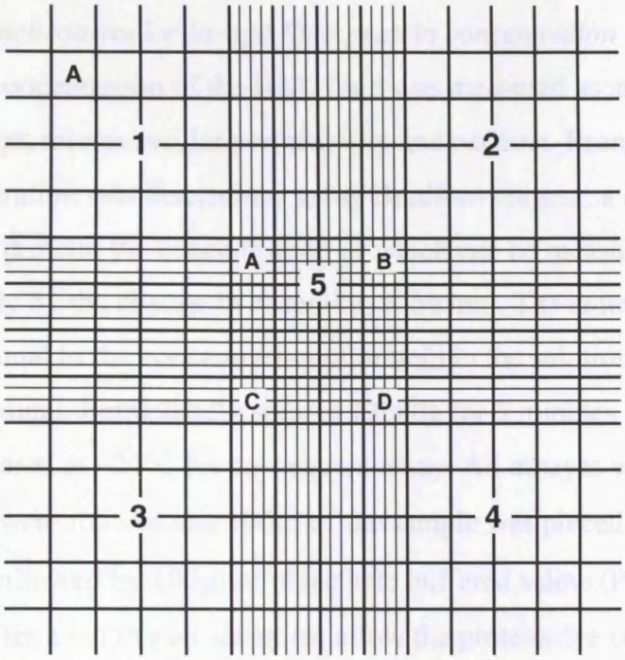
Measurement of wet:dry lung ratio

The wet to dry ratio was measured on a sample of lung tissue as an index of lung oedema. A sample of lung tissue was weighed before being placed in an oven at 60°C. The sample was serially weighed twice a day until there was no further decrease in weight of the sample. The original weight was divided by the final weight to give the wet:dry weight ratio.

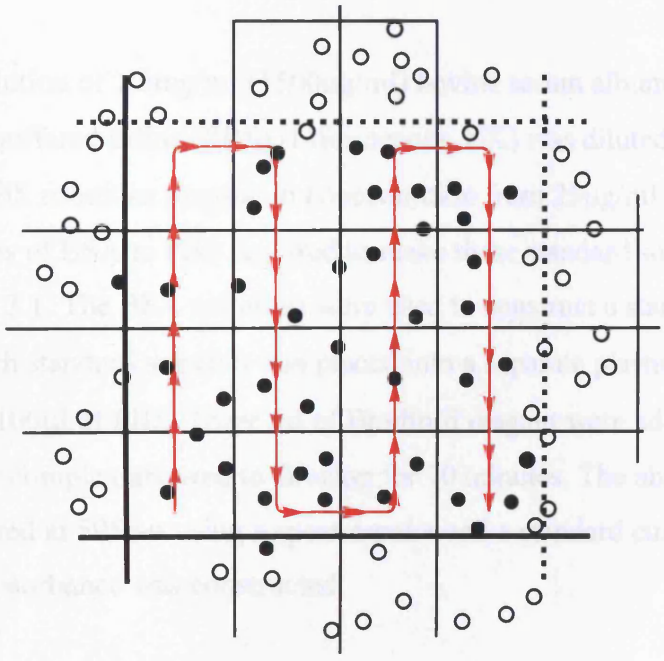
Measurement of nucleated cells in lavage fluid

The nucleated cell count in the BAL fluid was measured as an index of lung injury. Increased leucocyte migration into the lung combined with an increase in alveolar microvascular permeability and alveolar disruption following IRI would result in an increase in the number of nucleated cells in the BAL fluid. The nucleated cell count was performed in a Neubauer cell counting chamber using a similar method to that used to perform a manual leucocyte count in a whole blood sample. A sample of BAL fluid was diluted with a 1:1 ratio of diluting fluid and transferred to the counting chamber using a small capillary tube. The covered chamber was placed under a microscope and the 10X magnification eyepiece was used to focus on the central ruled area 5 of the counting chamber (Figure 3.5). Then using the 25X eyepiece, the nucleated cell count was quantified by counting the total number of nucleated cells in the four large corner squares of the ruled areas numbered 1, 2, 3 and 4 (Figure 3.5). The manual nucleated cell count was derived using the following formula:

$$\textbf{Nucleated cell count} = \frac{\textbf{Number of cells counted in square 1 – 4}}{\textbf{20}} \times 10^9/\text{litre}$$



The solid black lines are in fact triple lines. The white cells are counted in squares 1, 2, 3 and 4. Red cells and platelets are counted in squares 5A, B, C, D, E.



Counting sequence. A diagramatic representation of the white cell count, showing the sequence of counting which should be followed, the square shown is 1. The same sequence of counting would be followed for any squares 2, 3 and 4.

Figure 3.5 Improved Neubauer counting chamber

Measurement of broncheoalveolar lavage fluid protein concentration

The protein concentration of the BAL fluid was measured as an index of alveolar microvascular damage, microvascular permeability and oedema. Broncheoalveolar lavage fluid protein concentration was determined using Bradford reagent; a dye which forms a stable complex with protein, the concentration of which can be measured spectophotometrically by the change in absoption at 595nm. The amount of absorption produced is proportional to the concentration of protein in the solution. BAL fluid was collected from the animal, immediately spun at 13000g for 5 minutes and the supernatant was separated and stored at –20°C for subsequent assay. All assayes were performed at the same time. Samples were thawed and 100µl of the sample was placed into a clean disposable cuvette, followed by 100µl of phosphate buffered saline (PBS) and 3ml of Bradford reagent. After a ten minute delay (to allow the protein-dye complex to form) the absorption was measured at 595nm. The absorbance was then plotted onto a standard curve and the concentration of protein in the sample determined.

Standard curves

A stock solution of 1.5mg/ml (1500µg/ml) bovine serum albumin (BSA) (Sigma, UK) in phosphate buffered saline (PBS) (Lifesciences, UK) was diluted to produce standard BSA in PBS solutions ranging in concentration from 25µg/ml to 1500µg/ml. The equivalents volumes of BSA to PBS required to make these standard solutions are illustrated in Table 3.1. The BSA solutions were used to construct a standard concentration curve. 100µl of each standard solution was placed into a separate plastic disposable cuvette (Sigma, UK) with 100µl of PBS. Three ml of Bradford reagent were added to each cuvette and the protein dye complex allowed to develop for 10 minutes. The absorbance of each cuvette was measured at 595nm using a spectrometer and a standard curve of standard solutions against absorbance was constructed.

To make 10ml of final concentration of BSA solution (μg/ml)	Volume of 1500μl/ml BSA required (ml)	Volume of phosphate buffered saline required (ml)
1500	10	0
1400	9.33	0.67
1300	8.66	1.34
1200	8	2
1100	7.33	2.67
1000	6.66	3.34
900	6	4
800	5.33	4.67
700	4.66	5.34
600	4	6
500	3.33	6.67
400	2.66	7.34
300	2	8
200	1.33	8.67
100	0.66	9.34
50	0.33	9.67
25	0.16	9.84

Table 3.1: Serial dilutions required to make standard bovine serum albumin solutions in phosphate buffered saline.

BSA – Bovine serum albumin

Measurement of lung myeloperoxidase activity

Myeloperoxidase (MPO) is a haem containing enzyme which is found within the azurophil granules of neutrophils. It is an indicator of leucosequestration and leucocyte degranulation. Lung tissue myeloperoxidase activity was measured by its ability to form a chromogenic agent while decomposing hydrogen peroxide using the method described by Barry et al (1996). The assay sample was weighed and then homogenized in 5ml of 0.5% hexadecyltrimethyl ammonium bromide (Sigma, UK) in 50mmol pH 6 potassium phosphate buffer. The homogenate was snap frozen in liquid nitrogen, thawed twice and then centrifuged at 13000g for 5 minutes. The resulting supernatant was assayed spectrophotometrically for MPO activity by incubating 0.1ml of supernatant with 2.9ml of solution B. Solution B was prepared by dissolving 2.9 ml of O-dionisidine hydrochloride (Sigma, UK) in 90ml of distilled water and adding 10ml of 50mmol potassium phosphate buffer (pH 6) and hydrogen peroxide (final concentration 0.0005%). The absorbance at 460nm was recorded every minute for ten minutes (Philips PU/VIS spectrophotometer) and the highest reading recorded. The highest optical density measurement was used as an index of MPO activity and the results were expressed per gram of lung tissue.

Measurement of plasma TNF-α levels

Plasma TNF-α levels were measured as an index of the pro-inflammatory response using a commercially available rat specific enzyme-linked immunosorbent assay (ELISA) kit (Endogen, USA) containing a 96 well anti rat TNF-α antibody coated microtitre plate. Stored plasma samples were thawed to room temperature and 50μl of each sample was pipetted into a separate well. Each sample was assayed in duplicate. The plate was covered and incubated at room temperature for one hour, and then washed with a wash buffer three times. Following this, 50μl of a biotinylated secondary antibody was pipetted into each well and the plate incubated at room temperature for 2 hours. The plate was washed again with the wash buffer three times before 100μl of a streptavidin containing solution was added to each well and the plate incubated at room temperature for 30 minutes. The plate was then washed again with wash buffer three times before 100ul of a colour pigment forming substrate solution was added to each well and the pigment allowed to develop at room temperature for 30 minutes in the dark. The reaction was stopped with the stopping solution provided in the kit and the absorbance of the wells read using a microplate reader at 450nm. The results were calculated by constructing a standard curve using the standard

TNF-α solutions provided in the kit and plotting the sample absorbances against this standard curve.

3.4 Measurement of glomerular filtration rate

Glomerular filtration rate was measured as an index of renal impairment on the second and the seventh day post-operatively using a ^{99}TcDPTA clearance technique. The ^{99}TcDPTA was freshly prepared by the Department of Radiopharmacy, Leicester Royal Infirmary. Under sterile conditions, four aquilots of approximately 0.2ml 99mTc DPTA was suspended in 0.9% normal saline in 2ml syringes to give a final volume of 0.5ml with an activity of 20-25MBq. The activity was accurately measured in a gas ionisation calibrator (Capintec CR 10B), the time noted (T0) and the syringes transported to the animal laboratory at the Biomedical Services Unit (BSU) in a shielded lead container. The following protocol was followed:

1. Animals were lightly sedated in an anaesthetic box using 2% halothane in oxygen at 2 litres per minute flow.
2. The isotope was injected intraperitoneally (Figure 3.6), the time noted and the animals allowed to recover from the sedation.
3. An aliquot of ^{99}TcDPTA was used as a standard and injected into one litre (1000ml) of tap water in a volumetric flask.
4. The syringes (with needles attached) were immediately transported back to the Department of Radiopharmacy and the residual activity measured to allow the injected dose to be calculated after correction for decay.
5. The animals were lightly sedated again, and tail tip amputation performed 45 minutes after injection of ^{99}TcDPTA (T1). One ml of mixed rat blood was collected (Figure 3.7) in a 1.5 ml eppendorph (Stardest) which had been pretreated with 20μl of sodium heparin (5000U/ml) as an anticoagulant.
6. The eppendorphs were placed in a microcentrifuge and spun at 13000g for 5 minutes. 50μl of the resultant plasma was pipetted into a capped plastic gamma counter tube.
7. The volumetric flask containing the standard was inverted several times to ensure adequate mixing of the (heavy) ^{99}TcDPTA in the water and then a 50μl sample was pipetted into a plastic gamma counter tube and the time noted.

8. The procedure was repeated at 90 minutes post isotope injection (T2) although tail tip amputation was not required and tail tip blood easily obtained by removing the clot with a swab.
9. The plastic gamma counter tubes were immediately transferred to a gamma counter (Philips PW4800) which was programmed for ^{99}TcDPTA (programme 7). This calculated the average counts per minute (CR) over a total count time of 10 minutes per tube.
10. The GFR was calculated using the slope intercept method described by Nankivell et al (1992) (See Appendix 1).

6.5 Measurement of nitric oxide

Measuring total nitroxides using Greiss reagent

The end products of NO metabolism include both nitrate (NO_3^-) and nitrite (NO_2^-). The nitrate can be converted to nitrite by incubating the sample in a solution containing the enzyme nitrate reductase (Figure 3.8). A commercially available Greiss reagent assay kit was used to perform this assay. The kit included an assay buffer, nitrate reductase, a vial containing enzyme cofactors, nitrite standards, a 96 well microtitre plate and Greiss reagents 1 and 2.

Tissue samples (approximately 200mg) were homogenised in a 10:1 volume of PBS pH 7.4 (approximately 2ml). The samples were frozen and thawed twice in liquid nitrogen to facilitate cell lysis. The homogenate was centrifuged at 13000g for 5 minutes and the supernatnant transferred into a microfuge ultrafiltration device containing a 10kda molecular weight cutoff filter (Millipore,UK). The supernatant was centrifuged in the microfuge ultrafiltration device for 3 hours to remove larger protein molecules which intefere with the spectrophotometer reading. 80µl of the filtrate and 200µl of assay buffer were added to the blank wells. 10µl of reconstituted enzyme cofactor solution (required for nitrate reductase activity) are added to each well followed by 10µl of the reconstituted nitrate reductase solution. The plate was then covered and allowed to incubate at room

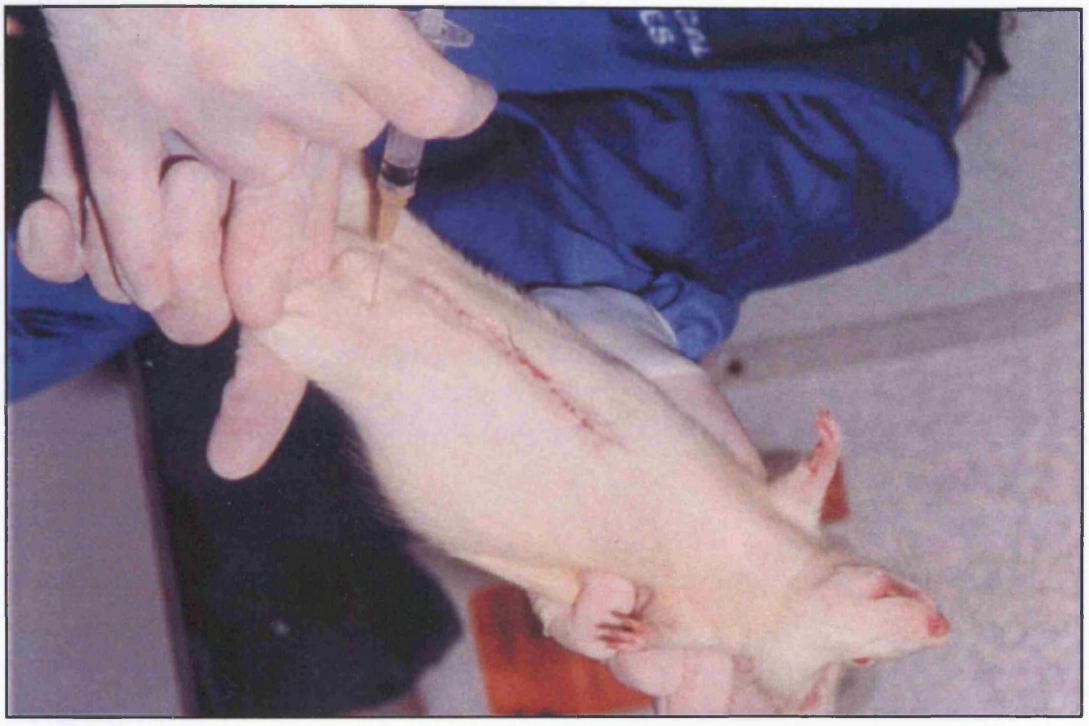


Figure 3.6 ^{99}Tc DPTA being injected intraperitoneally

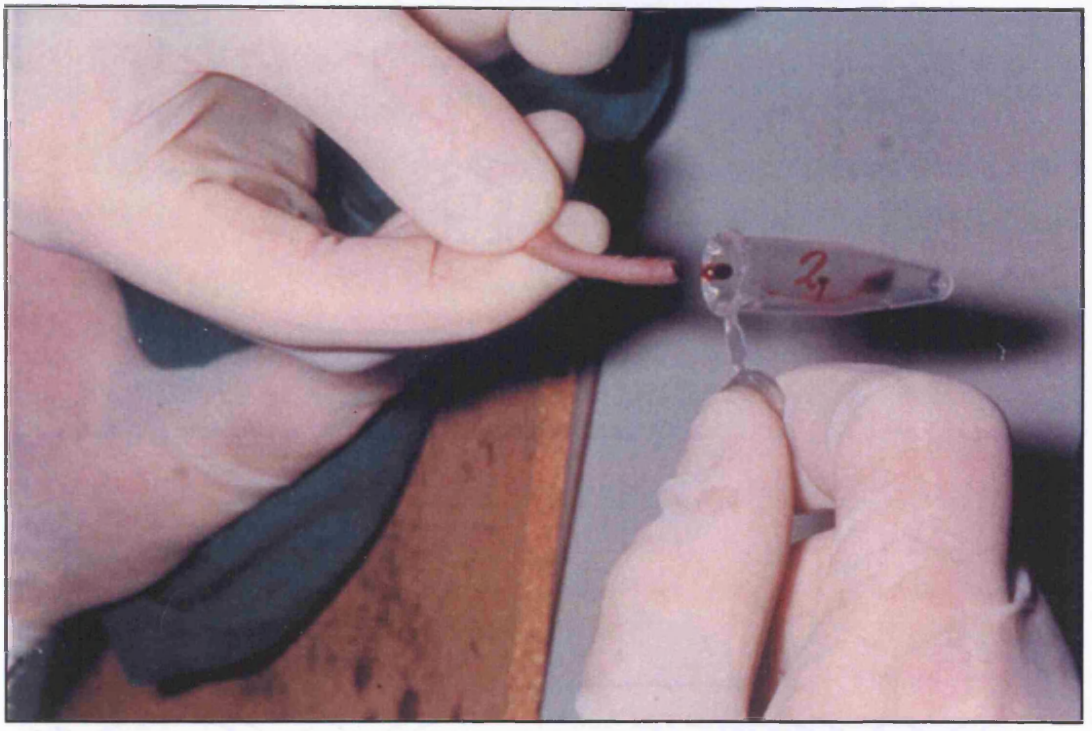


Figure 3.7 Tail tip amputation performed and rat blood collected

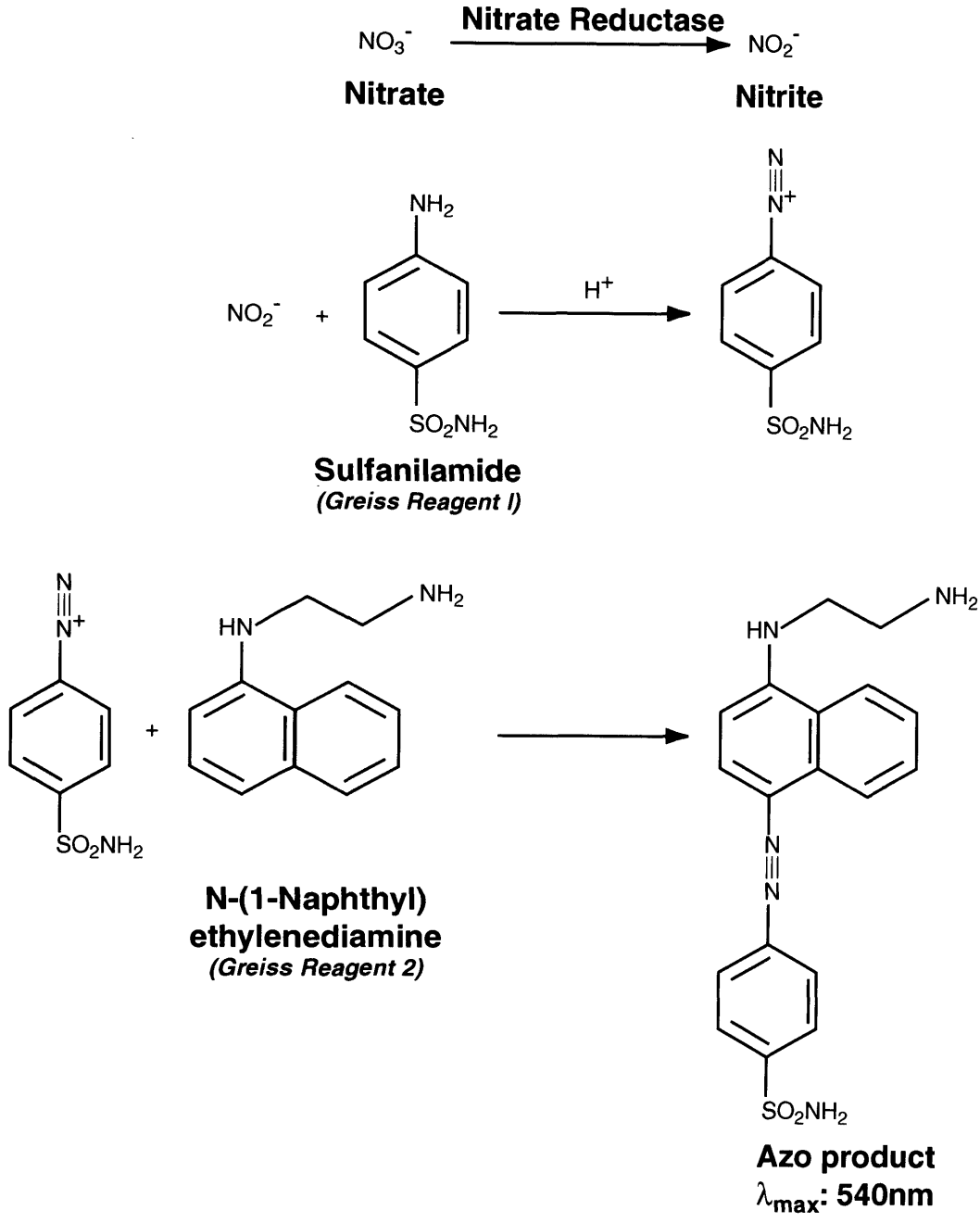


Figure 3.8 Biochemistry of the Greiss Reagent assay

temperature for 3 hours. At the end of the incubation period 50µl of Greiss reagent 1 was added followed by 50µl of Greiss reagent 2. The dye was allowed to develop for 10 minutes at room temperature and the absorbance read at 540nm using a microplate reader.

A standard curve was constructed using standard nitrite concentration samples provided in the kit and the concentration of nitrite in the tissue samples determined by plotting the absorbance against the standard curve.

Measurement of total NOS activity

Total NOS activity was measured in tissue homogenates by the conversion of ^{3}H L-arginine to ^{3}H L-citrulline. The ^{3}H L-citrulline formed at the end of the incubation period was separated from the ^{3}H L-arginine by adding an acidic ion exchange resin which binds all the basic L-arginine to the reaction mixture. This leaves all the neutral L-citrulline in solution. The amount of ^{3}H L-citrulline formed is a measure of the NOS activity in the tissue.

The following protocol was followed using a commercially available NOS assay kit (Stategene, USA), ^{3}H L-arginine (Amersham, UK) and a protease inhibitor cocktail (Sigma, UK).

1. Renal or lung tissue samples were homogenised in a buffer containing 25mM tris –HCl (pH 7.4), 1mM EDTA, 1mM etheleneglycol-bis (β- aminoethylether)-N,N,N,N-tetraacetic acid (EGTA) and an antiprotease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin-A, trans-epoxysuccinyl-L-leucylamido (4-guanido) butane (E-64), bestatin, leupeptin and apoprotinin (Sigma, UK).
2. 10µl homogenate (approximately 50µg protein) was incubated in the presence of 25µM cold L-arginine, 37kBq ^{3}H L-arginine (Amersham, UK), 25mM tris –HCL (pH 7.4), 3µM tetrahydrobiopterin, 1µM flavin adenine dinucleotide, 1µM flavin adenine mononucleotide, 2mM NADPH, 1mM calcium chloride and 0.1µM calmodulin (Alexis, UK) for 45 minutes at 37^{o} centigrade (Vials A). Control reactions were performed without NADPH and incubated in an ice bath (Vials B). All assays were performed in triplicate.
3. The reaction was stopped by dilution with cold 50mM N-2-hydroxyethylpiperazine – N-2-ethanesulfonic acid (HEPES) pH 5.5 and 5mM EDTA.

4. An acidic resin was applied to reaction mixtures to bind basic ^{3}H L-arginine, leaving neutral ^{3}H L-citrulline free in the reaction solution.
5. The reaction samples were filtered through spin cups and the filtrate transferred to scintillation vials. The radioactivity in the filtrate was quantified in a β counter and is a measure of ^{3}H L-citrulline formed from ^{3}H-L arginine.
6. The protein concentration in the sample homogenates was measured spectrophotometrically with Bradford reagent (Sigma, UK) using bovine serum albumin (Sigma, UK) as standard and the total nitric oxide synthase activity was expressed in picomoles of L-citrulline formed per mg of protein homogenate over a 45 min incubation period. (See Appendix 2)

Identifying the expression of iNOS

The expression of iNOS protein in pulmonary and renal tissue was detected by western blot analysis. Pulmonary or renal tissue was homogenised in a buffer containing antiprotease inhibitors as described above. The homogenate was centrifuged at 13000g for 5 minutes and the supernatant boiled for 10 minutes with gel loading buffer in a ratio of 1:1(Bio-Rad laboratories, UK). Equal amounts of the resultant sample was resolved by one dimensional gel electrophoresis (7.5% SDS-PAGE gel-Bio Rad Laboratories, UK). The proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories, UK) and nonspecific binding to the membrane was blocked by incubating the membrane in 5% bovine serum albumin solution in trisma buffered saline (TBS)-tween (Sigma, UK) for 1 hour. The membranes were washed twice in TBS-tween before incubation in an anti-iNOS specific polyclonal rabbit antibody (Calbiochem-Novabiochem, Calfornia) which does not cross-react with eNOS or nNOS for 1 hour. The membrane was then washed twice in TBS-tween before being incubated in an anti-rabbit goat antibody (Calbiochem-Novabiochem, California) conjugated with biotin. The membrane was washed twice in TBS-tween before being incubated with steptavidin conjugated to alkaline phosphatase (Calbiochem-Novabiochem, California). The membranes were finally washed in TBS-tween before being developed in western blue solution (Calbiochem-Novabiochem, California).

CHAPTER FOUR

Investigation of the influence of infrarenal aortic cross-clamp time on pulmonary nitric oxide metabolism and acute lung injury

4.1 Introduction

The experiments described in this chapter investigated the optimum aortic cross-clamp time required to produce acute lung injury. The determination of optimum cross-clamp time was an essential prerequisite for all subsequent experiments on IRI and NO metabolism. If the aortic cross-clamp time was too short, quantifiable changes in the lungs might not be detectable, whereas an unnecessarily long cross-clamp time might introduce the risks of a prolonged anaesthetic. The reperfusion time in these experiments was kept constant at 1 hour, although I have investigated optimum reperfusion times in Chapter 8.

The aim of this chapter was to investigate the optimum aortic cross-clamp induced ischaemia time required to produce quantifiable acute lung injury.

4.2 Methods

Groups of male wistar rats (n=6) weighing 450-500g were subjected to infrarenal aortic cross-clamp times of 30, 45 and 60 minutes. A sham operated control group was subjected to exposure of the infrarenal aorta only. At the end of the ischaemic period, the clamp was removed and the animals subjected to one hour of reperfusion. Following this the animals were culled by exsanguination. A median sternotomy was performed and the right upper and middle lung lobes lavaged with a solution containg 0.07mM EDTA. The nucleated cell count and the protein concentration of the broncheoalveolar lavage fluid was used as an index of acute lung injury. The pulmonary vasculature of the left upper lobe was flushed via the right ventricle as described in Chapter 6 and the lung tissue removed and snap frozen for subsequent measurement of myeloperoxidase activity. The right and left lower lobes were harvested and some was snap frozen for subsequent assays of NO metabolism and some used to measure the lung wet:dry ratio.

The following assays were performed:

1. Measurement of wet:dry lung ratio
2. Measurement of nucleated cell count of the BAL fluid
3. Measurement of BAL fluid protein concentration
4. Measurement of myeloperoxidase activity
5. Measurement of total NOS activity
6. Measurement of total nitroxides in lung tissue using Greiss reagent

Data sets were analysed using the Mann-Whitney U-test. Significance was accepted at a p value of < 0.05.

4.3 Results

The results are tabulated in Tables 4.1-4.2. There were no intraoperative deaths. An infrarenal aortic cross-clamp time of both 45 minutes and 60 minutes produced a significant increase in wet:dry lung ratio compared to control ($p<0.05$ at 45 minutes and $p<0.01$ at 60 minutes). An infrarenal aortic cross-clamp time of 30, 45 and 60 minutes produced a significant increase in the BAL nucleated cell count ($p<0.05$ at 30 minutes and $p<0.01$ at 45 and 60 minutes) and BAL protein concentration ($p<0.01$) at 30, 45 and 60 minutes versus control. Similarly an infrarenal aortic cross-clamp time of 30, 45 and 60 minutes produced a significant increase in pulmonary MPO and NOS activity compared to control animals ($p<0.01$). However there was no significant increase in the total nitroxides in pulmonary tissue (measured with Greiss reagent) subjected to 30, 45 or 60 minutes of infrarenal aortic cross-clamping compared to controls.

Ischaemic time (minutes)	Wet:dry lung ratio	BAL cell count ($x10^9$/litre)	BAL protein concentration (μg/ml)
Control	3.98 (3.9-4.65)	0.04 (0.01-0.07)	100 (62-120)
30	4.25 (4-4.65)	0.09 (0.03-0.14)*	217 (170-240)**
45	4.66 (3.98-4.94)*	0.22 (0.1-0.36)**	285 (230-365)**
60	4.71 (4.27-4.93)**	0.25 (0.11-0.41)**	382 (310-435)**

Table 4.1: The influence of infrarenal aortic cross-clamp time on lung wet:dry ratio, BAL cell count and BAL protein concentration.

(BAL= broncheoalveolar lavage, results are median and range, *p<0.05, **p<0.01 vs control Mann-Whitney U-test)

Ischaemic time (minutes)	Myeloperoxidase activity (units/gram)	NOS (picomoles/mg protein/45minutes)	Nitroxides (μm/litre)
Control	3.6 (2.5-4.2)	17.5 (12-23)	4.5 (2.5-6.5)
30	6.4 (5.3-7.1)**	259 (184-301)**	6.2 (4-7)
45	6.5 (5.7-8.1)**	317 (261-378)**	5.5 (4.5-8)
60	7 (6.1-7.8)**	365 (304-408)**	6 (5-8)

Table 4.2: The influence of infrarenal aortic cross-clamp time on pulmonary myeloperoxidase activity, NOS and nitroxides.

(NOS=total nitric oxide synthase activity, results are median and range, $p<0.01$ **vs control Mann-Whitney U-test)**

4.4 Discussion

The aim of the experiments described in this chapter was to investigate the optimum ischaemic time produced by infrarenal aortic cross-clamping that would lead to quantifiable acute lung injury. This would then allow subsequent investigation of the optimum reperfusion time and lead to a reliable model of ischaemia-reperfusion injury for investigation of the role of nitric oxide in this process and methods of pharmacological manipulation to ameliorate the injury. In these initial experiments, the rodent model that I developed proved to be reliable and allowed reproducible data to be obtained. There were no unforseen technical difficulties and no unexpected intra-operative deaths. For these initial experiments on optimum ischaemia time, I used a standard reperfusion period of 60 minutes but I went on to investigate optimum reperfusion times in the following chapter. In order to investigate acute lung injury, I developed a variety of assays to investigate different aspects of ischaemia-reperfusion injury in the lung. The assays that I used were: wet:dry ratio which measures pulmonary oedema, broncheoalveolar lavage nucleated cell count which measures leucocyte infiltration into the lung and alveolar disruption, brocheoalveolar lavage protein concentration which reflects the degree of alveolar microvascular permeability and pulmonary myeloperoxidase activity which is an additional measure of leucocyte infiltration into lung tissue. I measured nitric oxide activity in two ways: total pulmonary nitric oxide synthase activity was measured by the conversion of ^{3}H L-arginine to ^{3}H L-citrulline and total nitroxides (nitrite and nitrate) were measured by the Greiss assay. All of the assays proved to be robust and produced reliable and reproducible data exept the Greiss assay *(vide infra).*

These data showed that ischaemic times of 45 and 60 minutes produced significant acute lung injury which was reflected in the results of the wet:dry ratio ($p<0.05$ at 45 minutes and $p<0.01$ at 60 minutes Mann-Whitney U-test), BAL nucleated cell count, BAL protein concentration, and myeloperoxidase assays ($p<0.01$ Mann-Whitney U-test). Similarly, nitric oxide synthase activity was significantly raised with ischaemic times of 45 and 60 minutes ($p<0.01$ Mann-Whitney U-test). However, the Greiss assay which measured total pulmonary nitroxides did not show any significant differences with ischaemia times of 30, 45, and 60 minutes when compared to controls ($p<0.05$ Mann-Whitney U-test).

I encountered several technical difficulties with the Greiss assay which may have caused the unexpected results. Firstly, contamination of the lung homogenate samples with

excess protein may have impaired the ability of the spectrophotometer to read the concentration of the azo-dye complex formed between the Greiss reagent and nitrite. In order to overcome this problem, I attempted to remove excess protein by filtering the homogenates in a 10000 kilodalton cutoff filter spun in a centrifuge at 13000g. However, the large protein molecules often blocked the filter producing very little filtrate. After several attempts I found that lung tissue homogenised with 10 times weight to volume of buffer produced a homogenate that passed through the filter satisfactorily. However this manoeuvre led to a 10 fold dilution of the filtrate (and nitroxides) which may have been too dilute for detection by the Greiss assay.

Secondly, although the Greiss assay measures total nitroxides (nitrite and nitrate), there are other end products of nitric oxide metabolism which are not measured by the Greiss assay. Some nitric oxide is excreted in the expired air and some will combine with plasma proteins and haemoglobin and pass into the systemic circulation for excretion by the kidneys. It is therefore possible that the total nitroxides represent only a small percentage of metabolised nitric oxide which is not detectable by the relatively insensitive Greiss assay. I was concerned about these potential problems of the Greiss reagent while developing the assays to be used in these experiments and for these reasons I chose to also use the radioactive citrulline assay which proved to be very reliable. It is also possible that the failures of the Greiss assay may have been due to my own technical failures in the laboratory, therefore, I decideded to try the Greiss assay again in the following chapter on optimum reperfusion times before discarding it. It is interesting to note that although many researchers choose to measure total nitroxides with Greiss reagent in plasma samples (Barry *et al.*, 1996; Cristol *et al.*, 1993), there is little data on it's use in tissue homogenate samples. This may be due to similar technical difficulties experienced by others. Total NOS activity with the citrulline assay, however is commonly used in the measurement of NOS metabolism in tissue homogenates (Luss *et al.*, 1996; Shoskes *et al.*, 1997; Thiemermann *et al.*, 1993).

It is important to note that all other assays used in these initial experiments detected significant acute lung injury at both 45 and 60 minutes. However, the degree of acute lung injury was greater at 60 minutes of ischaemia and I therefore decided to use this ischaemic time as the optimum period for all subsequent experiments. In the next chapter I investigated the optimum reperfusion time to produce quantifiable acute lung injury using the optimum ischaemia time of 60 minutes determined in this chapter.

CHAPTER FIVE

Investigation of the influence of reperfusion time on pulmonary NOS activity and acute lung injury

5.1 Introduction

5.2 Methods

5.3 Results

5.4 Discussion

5.1 Introduction

In chapter 4 I investigated the optimum ischaemic time required to produce acute lung injury and demonstrated that 60 minutes of ischaemia followed by 60 minutes of reperfusion produced quantifiable lung damage. In this chapter, I investigated the optimum reperfusion period required to produce quantifiable acute lung injury using the optimum ischaemia time of 60 minutes and varying the reperfusion time.

In addition I investigated endogenous pulmonary NO metabolism and the systemic pro-inflammatory cytokine respones. Several studies have demonstrated that the reperfusion period of IRI may be associated with a systemic pro-inflammatory cytokine response. These cytokines activate leucocytes, facilitate leucocyte infiltration into pulmonary tissue and upregulate iNOS expression. To investigate this response I measured plasma tumour necrosis factor-alpha (TNF-α), a potent pro-inflammatory cytokine as an index of the pro-inflammatory cytokine response during the reperfusion period. In addition, iNOS is thought to be upregulated rapidly by alveolar macrophages and cells of the reticulo-endothelial system in IRI, leading to a large NO response. Therefore in addition to measurement of total NOS and total nitroxides, I also measured the expression of iNOS by western blot analysis.

The aim of this chapter was to investigate the optimum reperfusion time following infrarenal aortic cross-clamp induced ischaemia-reperfusion injury required to produce acute lung injury, and to investigate the pro-inflammatory cytokine response and pulmonary NOS metabolism during this period.

5.2 Methods

Groups of male wistar rats (n=6) weighing 450-500g were subjected to 60 minutes of infrarenal aortic cross-clamping. A sham operated control group was subjected to exposure of the infrarenal aorta only. At the end of the ischaemic period the clamp was removed and animals culled by exanguination at 0, 60 and 120 minutes of reperfusion respectively. Blood collected during exsanguination was immediately spun down and the plasma stored at –80°C for subsequent measurement of TNF-α. Then a median sternotomy was performed and the right upper and middle lung lobes lavaged with a solution containing 0.07mM EDTA. The nucleated cell count and the protein concentration of the resultant broncheoalveolar lavage fluid was used as an index of acute lung injury. The

pulmonary vasculature of the left upper lobe was flushed via the right ventricle as described in Chapter 6 and this tissue removed and snap frozen for the subsequent measurement of myeloperoxidase activity. The right and left lower lobes were harvested and part of this tissue was snap frozen for subsequent assays of NO metabolism and part was used to measure the lung wet:dry ratio.

The following assays were performed:

1. Measurement of wet:dry lung ratio
2. Measurement of nucleated cell count of the BAL fluid
3. Measurement of BAL fluid protein concentration
4. Measurement of myeloperoxidase activity
5. Measurement of total NOS activity
6. Measurement of total nitroxides in lung tissue using Greiss-reagent
7. Measurement of plasma TNF-α
8. Detection of iNOS expression in lung tissue by western blotting

5.3 Results

These data shows that an ischaemic period of 60 minutes alone without reperfusion did not produce significant acute lung injury as measured by wet:dry lung weight ratio or broncheoalveolar protein concentration compared to control animals (Table 5.1). There was, however significant increase in the BAL nucleated cell count ($p<0.05$), plasma TNF-α levels ($p<0.01$) and pulmonary NOS activity ($p<0.01$) in animals subjected to 60 minutes of ischaemia alone compared to control animals (Table 5.1, Table 5.2,). There was no significant difference in the pulmonary total nitroxide concentration of animals subjected to 60 minutes ischaemia alone compared to controls. Western blot analysis only detected trace quantities of iNOS in animals subjected to 60 minutes ischaemia alone and control animals.

Animals subjected to 60 minutes of lower torso ischaemia and 60 minutes and 120 minutes reperfusion demonstrated significant ALI as measured by wet:dry lung weight ratio ($p<0.01$), BAL protein concentration ($p<0.01$) and BAL nucleated cell count ($p<0.01$) compared to control animals (Table 5.1). Pulmonary MPO activity was significantly

increased ($p<0.01$) in animals subjected to 60 minutes of ischaemia and 60 minutes and 120 minutes of reperfusion compared to controls (Table 5.2).

There was a further increase in plasma TNF-α levels ($p<0.01$) in animals subjected to 60 minutes ischaemia followed by 60 minutes reperfusion and 60 min ischaemia followed by 120 minutes reperfusion (Table 5.2). Similarly, pulmonary NOS activity was significantly increased ($p<0.01$) in animals subjected to 60 minutes of ischaemia and 60 minutes or 120 minutes of reperfusion compared to controls (Table 5.2). The Greiss reagent assay did not produce a significant difference in pulmonary nitroxide levels in tissue homogenate among the four groups (Table 5.2). Western blot analysis demonstrated trace quantities of iNOS in control pulmonary tissue. Animals subjected to 60 minutes of ischaemia and 60 and 120 minutes of reperfusion demonstrated much clearer expression of iNOS in pulmonary tissue. Although these assays were not quantified there seemed to be more iNOS expression with the longer reperfusion period (Figure 5.1).

Ischaemia:reperfusion time (minutes)	Wet:dry lung ratio	BAL cell count ($x10^9$/litre)	BAL protein concentrtaion (μg/ml)
Control	4.07 (3.9-4.65)	0.04 (0.01-0.07)	100 (62-120)
60:0	4.19 (4.02-4.33)	0.1 (0.02-0.14)*	115 (100-160)
60:60	4.78 (4.27-4.93)**	0.25 (0.11-0.41)**	382 (310-485)**
60:120	4.91 (4.33-5.03)**	0.14 (0.07-0.29)**	395 (330-450)**

Table 5.1: The influence of ischaemia and reperfusion time on wet:dry lung ratio, BAL cell count and BAL protein concentration.

(BAL= broncheoalveolar lavage, results are medians and range, *p<0.05, **p<0.01 Mann-Whitney U-test vs control).

Ischaemia:reperf-usion time (minutes)	Myeloperoxidase activity (units / g)	TNF-α (picogrammes / ml)	NOS (picomoles / mg protein / 45min)	Nitroxides (μm/litre)
Control	3.6 (2.5-4.1)	<10	17 (12-29)	4.5 (2.5-7)
60:0	4.6 (3.6-5.4)	24 (18-35)**	61 (51-67)**	4.9 (4-7)
60:60	7 (6.1-7.8)**	53 (38-98)**	360 (304-409)**	6 (5-8)
60:120	7.4 (6.3-7.9)**	74 (45-100)**	410 (377-476)**	6.5 (5-8.5)

Table 5.2: The influence of ischaemia and reperfusion time on pulmonary myeloperoxidase activity, pulmonary NOS, pulmonary nitroxides and plasma TNF-α.

(TNF-α= tumour necrosis factor-alpha, NOS= nitric oxide synthase, results are medians and range, **p <0.01 Mann-Whitney U-test vs control).

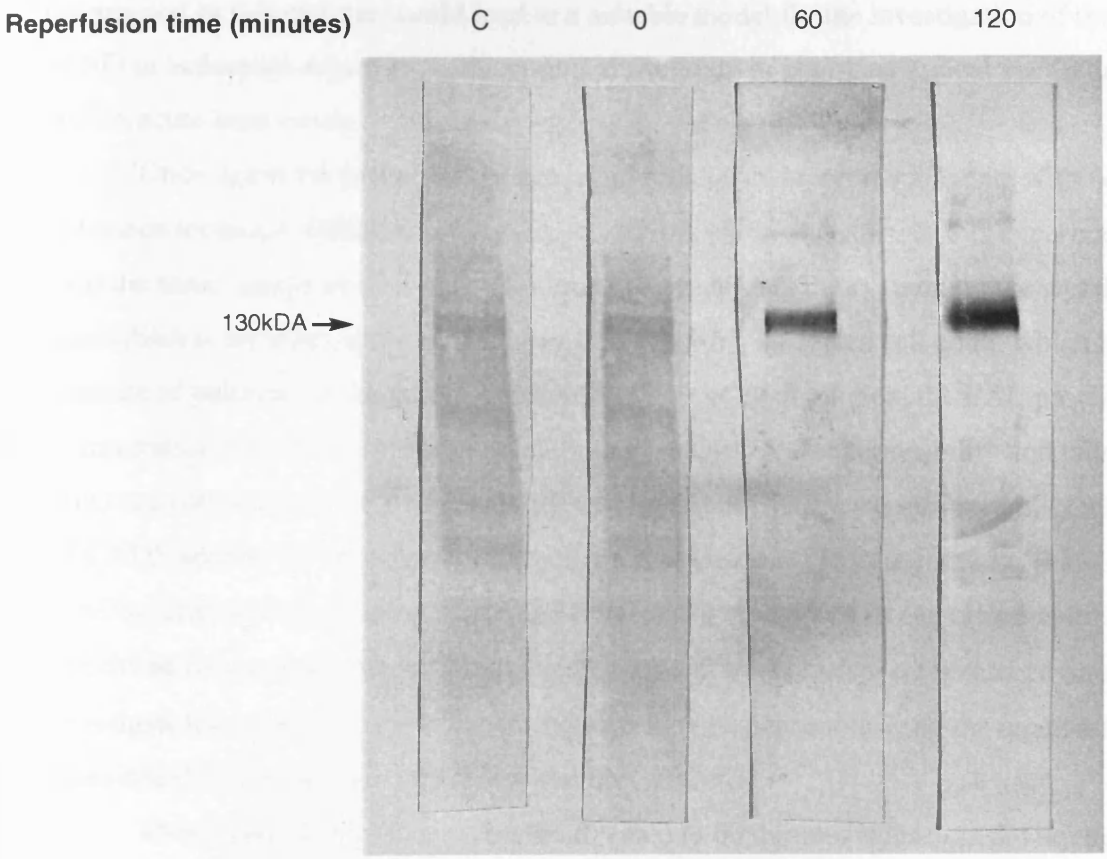


Figure 5.1 The influence of reperfusion time in minutes, on upregulation of the 130k Da iNOS protein
C= control sham operated animals

5.4 Discussion

The aim of the experiments described in this chapter was to investigate the optimum reperfusion time following 60 minutes of infrarenal aortic cross-clamping required to produce quantifiable acute lung injury. The combination of the optimum ischaemic time of 60 minutes determined in Chapter 4 and the optimum reperfusion time determined in this chapter would lead to a suitable model for the investigation of the role of NO in ischaemia-reperfusion injury and allow study of pharmacological manipulation of NO on acute lung injury.

Once again the rodent model produced reliable and reproducible data with no unforseen technical difficulties or unexpected intraoperative deaths. In this experiment I used the same assays as in Chapter 4 to quantify acute lung injury, namely the wet:dry ratio which is an index of pulmonary oedema, the BAL nucleated cell count which is a measure of pulmonary leucocyte infiltration and alveolar disruption, the BAL protein concentration which is a measure of pulmonary microvascular permeability and pulmonary MPO activity which is an additional index of pulmonary leucocyte infiltration. I measured total NOS activity by the measurement of the conversion of ^{3}H L-arginine to ^{3}H L-citrulline and total nitroxides (nitrite and nitrate) were measured by the Greiss assay. I introduced two new assays in this chapter: plasma TNF-α levels were measured in order to investigate the systemic pro-inflammatory cytokine response following the reperfusion phase and iNOS expression using western blot analysis.

These data showed that animals subjected to 60 minutes ischaemia did not develop significant acute lung injury measured by wet:dry lung ratio, BAL protein concentration and myeloperoxidase activity. However there was a significant increase in the BAL nucleated cell count in animals subjected to 60 minutes of ischaemia alone compared to control animals ($p<0.05$). After 60 minutes of ischaemia and 60 or 120 minutes of reperfusion there was significant acute lung injury compared to control animals ($p<0.01$) as measured by wet:dry lung ratio, BAL protein concentration, BAL nucleated cell count and pulmonary myeloperoxidase activity. This demonstrates that the reperfusion phase of IRI is important in the development of acute lung injury. The BAL nucleated cell count increased following 60 minutes of ischaemia alone which suggests that this process starts during the ischaemic phase but is accelerated during the reperfusion phase.

Plasma TNF-α levels were significantly increased in animals subjected to 60 minutes of ischaemia alone ($p<0.01$) and remained significantly elevated following 60 or

120 minutes of reperfusion ($p<0.01$). Again, this suggests that the pro-inflammatory cytokine response starts during the ischaemic period and is further magnified by reperfusion.

Similarly, pulmonary total NOS activity was significantly increased in animals subjected to 60 minutes of ischaemia alone ($p<0.01$) and remained elevated following 60 or 120 minutes of reperfusion ($p<0.01$). Western blot analysis demonstrated only trace quantities of iNOS in control animals and those subjected to 60 minutes of ischaemia alone but there was clear expession of iNOS after 60 or 120 minutes reperfusion. There were no significant differences in total nitroxides measured by Greiss reagent.

The data in these experiments demonstrate that the reperfusion period of infrarenal aortic cross-clamp induced IRI is associated with an acute lung injury and a large pulmonary total NOS response. The increase in the systemic pro-inflammatory cytokine response and the expression of iNOS following reperfusion suggests that the total NOS response is in part due to iNOS expression.

In these experiments acute lung injury was detected following 60 and 120 minutes of reperfusion, but the degree of injury was greater at 120 minutes using most assays. Therefore I decided to use a reperfusion time of 120 minutes for further studies on the role of pharmacological manipulation of the NO response in pulmonary IRI. In the next chapter I investigated the use of a rodent model of aortic cross-clamp induced ischaemia-reperfusion injury on renal injury.

CHAPTER SIX

Investigation of the influence of aortic cross-clamp time on renal nitric oxide metabolism and glomerular filtration rate

6.1 Introduction

6.2 Methods

6.3 Results

6.4 Discussion

6.1 Introduction

In Chapter 4 I investigated the optimum aortic cross-clamp induced ischaemia time required to produce quantifiable acute lung injury and found significant lung damage after 45 and 60 minutes. In Chapter 5 I investigated the optimum reperfusion time required to produce acute lung injury and found significant lung damage after 60 and 120 minutes. I also investigated the endogenous renal nitric oxide response and systemic cytokine effects. I was able to demonstrate a significant rise in circulating tumour necrosis factor-alpha (a pro-inflammatory cytokine) and detected iNOS in lung homogenates. However, the aim of this thesis was to study the role of nitric oxide in ischaemia-reperfusion injury in both the lungs and kidneys, and I have therefore investigated ischaemia-reperfusion injury in the kidney in this chapter.

In Chapter 3 I described a model of aortic cross-clamp induced ischaemia-reperfusion injury in the kidney whereby a nephrectomy was performed after the injury to study immediate renal injury and the second kidney harvested 7 days later to study progression of the injury. In order to use this model to study the role of nitric oxide in this process and the effects of pharmacological manipulation of the response, it was first necessary to determine the optimum ischaemic and reperfusion times required to produce quantifiable renal damage. This chapter investigates the influence of infrarenal aortic cross-clamp ischaemia time on renal injury and endogenous renal nitric oxide metabolism. In addition, I measured changes in renal function using glomerular filtration rate as opposed to serum urea and creatinine because changes in urea and creatinine do not occur until at least 50% of the functional renal reserve is lost and they are therefore insensitive measurements.

The aim of this chapter was to investigate the influence of infrarenal aortic cross-clamp time on endogenous renal nitric oxide metabolism and glomerular filtration rate.

6.2 Methods

Groups of male wistar rats (n=6) weighing 450-500g were anaesthetised with halothane and subjected to 30, 45 and 60 minutes of infrarenal aortic cross-clamping repectively. Control animals underwent laparotomy and exposure of the infrarenal aorta only. At the end of the ischaemic period the clamp was removed and the animal maintained

under anaesthesia for a further 1 hour reperfusion. Following this all animals underwent a left nephrectomy and the harvested kidney was immediately snap frozen in liquid nitrogen and stored at –80°C for subsequent assay of NOS activity. Animals were recovered after the administration of 30μg of intramuscular buprenorphine for post-operative analgesia. The glomerular filtration rate in the remaining right kidney was measured using a ^{99}TcDPTA clearance technique on the 2nd and 7th post-operative day. The animals were then culled and the remaining kidney harvested.

The following assays were performed:

1. Glomerular filtration rate on the 2nd and 7th post-operative day using a ^{99}TcDPTA clearance technique.
2. Total renal NOS activity (by the conversion of ^{3}H L-arginine to ^{3}H L-citrulline) in kidneys harvested on the 1st and 7th post-operative day.
3. Renal nitroxide levels on the 1st and 7th post-operative day using Greiss reagent.
4. Renal iNOS expression on the 1st and 7th day using western blot analysis.

6.3 Results

There were no unexpected intra-operative deaths. Results of GFR measurements are illustrated in Table 6.1. There was a significant impairment in GFR in animals subjected to 30, 45 and 60 minutes of cross-clamping compared to control animals on the second and seventh day ($p<0.05$).

Total renal NOS activity is tabulated in Table 6.2. Endogenous NOS activity measured in kidneys following 30 minutes, 45 minutes and 60 minutes of ischaemia and 1 hour of reperfusion was significantly increased compared to controls ($p<0.01$). Total renal NOS activity remained significantly elevated on the seventh day post-operatively in animals subjected to infrarenal aortic cross-clamping compared to controls ($p<0.01$).

Measurement of total nitroxides did not demonstrate any significant difference in the concentration of total nitrates and nitrites in renal tissue homogenates on the first or seventh day (Table 6.3).

Western blot analysis demonstrated that iNOS was not expressed in control kidneys on the first day and seventh day post-operatively. Kidneys removed on the first day following one hour of reperfusion did not express iNOS at all. All kidneys removed on the

seventh day following infrarenal aortic cross-clamping for 30, 45 and 60 minutes expressed iNOS strongly (Figure 6.1).

Cross-clamp time (minutes)	Glomerular filtration rate on day 2 (ml/minute)	Glomerular filtration rate on day 7 (ml/minute)
Control	2.25 (2.04-2.42)	2.33 (2.03-2.48)
30	1.62 (1.47-1.82)*	1.73 (1.65-1.83)*
45	1.53 (1.45-1.64)*	1.63 (1.54-1.69)*
60	1.45 (1.37-1.57)*	1.49 (1.44-1.63)*

Table 6.1: The influence of infrarenal aortic cross-clamp time on glomerular filtration rate on day 2 and day 7.

***p<0.05 vs control Mann-Whitney U-test.**

Cross-clamp time (minutes)	NOS activity on day 1 (picomoles L-citrulline/mg protein/45minutes)	NOS activity on day 7 (picomoles L-citrulline /mg protein/45minutes)
Control	13 (7.5-17)	12(9.5-15)
30	55 (43-67)**	49 (38-53)**
45	71 (55-89)**	57.5 (25-62)**
60	91 (69-116)**	71 (55-97)**

Table 6.2: The influence of infrarenal aortic cross-clamp time on renal NOS activity on day 2 and day 7.

NOS=nitric oxide synthase, **p<0.01 vs control Mann-Whitney U-test.

Cross-clamp time (minutes)	Nitroxides on day 1 (μmoles/litre)	Nitroxides on day 7 (μmoles/litre)
Control	4.3 (2.5-6)	4.2 (2-6)
30	4.4 (3.5-5.8)	4.4 (3.5-6.2)
45	5 (4.1-6)	4.4 (3.8-5.3)
60	5.1 (4.3-6.3)	5.2 (3.9-6.3)

Table 6.3: The influence of infrarenal aortic cross-clamp time on renal nitroxides on day 1 and day 7.

There were no significant differences compared to controls.

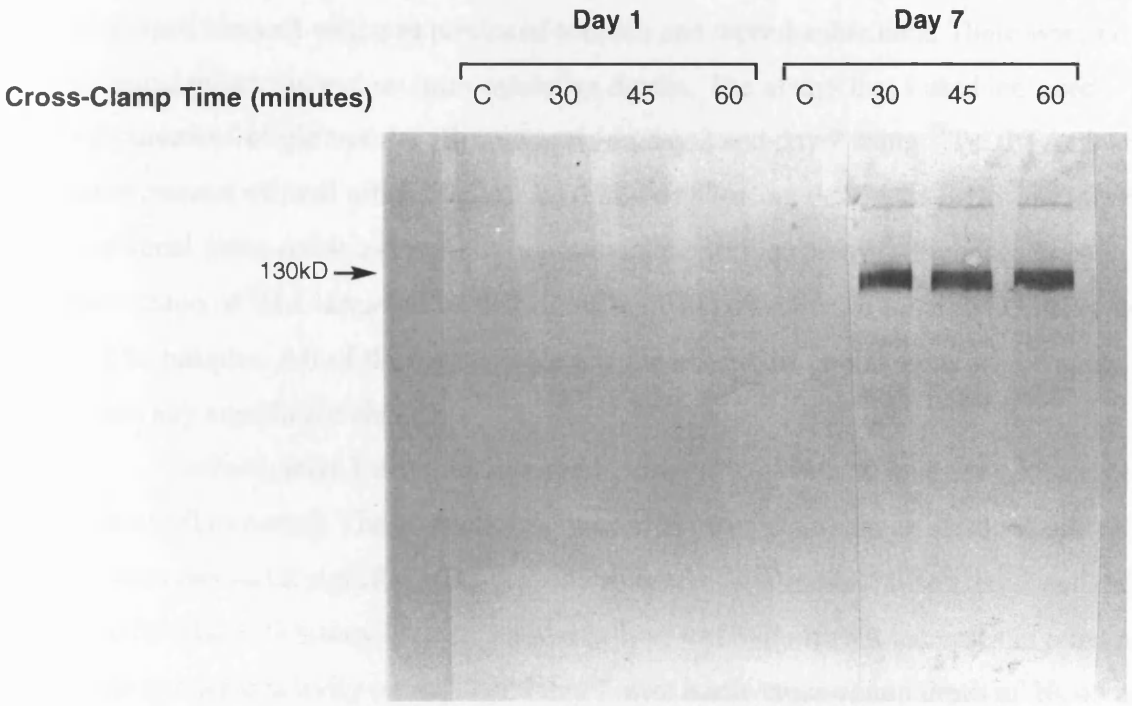


Figure 6.1 Western Blot demonstrating the influence of infranenal aortic cross-clamp time in minutes, on renal iNOS expression
C= control sham operated animals

6.4 Discussion

The aim of this Chapter was to investigate the optimum aortic cross-clamp ischaemia time required to produce quantifiable acute renal injury. The animal model of renal ischaemia-reperfusion injury following infrarenal aortic cross-clamping that I developed worked well and produced reliable and reproducible data. There were no technical problems and no intra-operative deaths. The assays that I used included measurement of glomerular filtration rate on day 2 and day 7 using ^{99}TcDPTA clearance, measurement of total nitroxides on day 1 and day 7 using the Greiss assay, measurement of total renal nitric oxide synthase levels using the citrulline assay (which measures conversion of ^{3}H L-arginine to ^{3}H L-citrulline) and detection of renal iNOS using western blot techniques. All of the assays were reliable except the Greiss assay which again did not detect any significant changes.

I investigated 3 different ischaemic times (30, 45 and 60 minutes with a reperfusion time of 60 minutes). These data show that aortic cross-clamp times of 30, 45 and 60 minutes produced significant decreases in glomerular filtration rate on day 2 and day 7 ($p<0.05$ Mann-Whitney U-test). Similarly there were significant increases in renal nitric oxide synthase activity on day 2 and day 7 with aortic cross-clamp times of 30, 45 and 60 minutes ($p<0.05$ Mann-Whitney U-test). Renal iNOS was not detected in kidneys harvested on day 1 but was strongly expressed in kidneys harvested on day 7 after aortic cross-clamp times of 30, 45 and 60 minutes.

The data from the experiments described in this Chapter allowed me to select an optimum aortic cross-clamp time of 60 minutes for the proposed investigations of pharmacological manipulation of the nitric oxide response. Although all 3 cross-clamp times produced significant changes in glomerular filtration rate, total renal nitric oxide synthase production and renal iNOS expression, these changes were greater after 60 minutes of ischaemia. Although I intended to investigate the influence of different reperfusion times on renal injury, the standard reperfusion time of 60 minutes (which proved to be the optimum time for pulmonary injury in Chapter 5) produced detectable changes in renal injury. In order to minimise usage of animals, I decided to use this reperfusion time of 60 minutes in the subsequent experiments on pharmacological manipulation of the nitric oxide response.

In conclusion, the data from the experiments described in Chapters 4, 5 and 6 show that an ischaemic time of 60 minutes and a reperfusion time of 60 minutes produced

significant and quantifiable pulmonary and renal injury in the model of aortic cross-clamp induced ischaemia-reperfusion injury. In addition, I demonstrated a significant rise in pro-inflammatory cytokine (tumour necrosis factor-alpha) and expression of iNOS in lung and renal tissue. In the next 2 Chapters I investigated the role of pharmacological manipulation of this response in an attempt to ameliorate the subsequent injury.

CHAPTER SEVEN

Can manipulation of NO metabolism decrease infrarenal aortic cross-clamp induced acute lung injury?

7.1 Introduction

7.2 Methods

7.3 Results

7.4 Discussion

7.1 Introduction

In chapters seven and eight I demonstrated that there is a significant acute lung injury associated with infrarenal aortic cross-clamp induced ischaemia-reperfusion injury. In addition there is a significant increase in the endogenous NO response during the reperfusion period with expression of iNOS. This endogenous NO response may be beneficial because NO will lead to lung microcirculatory changes such as vasodilation, and prevention of platelet aggregation. In addition NO prevents leucocyte-endothelial interaction which may in turn reduce leucocyte infiltration and degranulation. However, NO is the precursor of the toxic peroxynitrite and hydroxyl radicals formed in IRI, both of which are capable of further tissue injury and may exacerbate acute lung injury. Thus manipulation of the NO response in IRI may change the subsequent lung injury. In this chapter I manipulated the NO response by selective NO donors and inhibitors before infrarenal aortic cross-clamping in order to investigate if this ameliorated the acute lung injury. The agents used were:

- L-arginine - the substrate for NOS
- L-NMMA: NG-monomethyl-L-arginine - an inhibitor of all isoforms of NOS
- 1400W N: 3-aminomethyl benzyl acetamide - a highly selective inhibitor of iNOS
- Hydrocortisone-a non-specific inhibitor of the inflammatory response
- NOC-18: Z-1-2 Aminoethyl-N- (2 ammonioethyl) amino diazen-1-ium 1,2-diolate - a slow release NO donor.

The aim of this chapter was to investigate the effects of pharmacological manipulation of NO metabolism in infrarenal aortic cross-clamp induced ischaemia-reperfusion injury.

7.2 Methods

Groups of male wistar rats (n=6) weighing 450-500g were anaesthetised with halothane. The infrarenal aorta and IVC were exposed. One of the following agents was injected into the inferior vena cava 5 minutes before infrarenal aortic cross-clamping:

1. Control – Normal saline
2. L-arginine (300mg/kg)

3. L-NMMA (30mg/kg)
4. 1400W (10mg/kg)
5. Hydrocortisone (20mg/kg)
6. NOC-18 (0.5mg/kg)

At the end of the 60 minute ischaemic period, the clamp was removed and the animals subject to 2 hours of reperfusion. Following this the animals were culled by exsanguination. A median sternotomy was performed and the right upper and middle lung lobes lavaged with a solution containg 0.07mM EDTA. The pulmonary vasculature of the left upper lobe was flushed via the right ventricle as described in Chapter 6 and this tissue removed and snap frozen for subsequent measurement of myeloperoxidase activity. The right and left lower lobes were harvested and this tissue was used to measure the lung wet : dry ratio. The following assays were performed:

1. Measurement of wet:dry lung ratio – an index of lung oedema.
2. Measurement of BAL fluid protein concentration – an index of pulmonary microvascular permeability.
3. Measurement of myeloperoxidase activity – an index of pulmonary neutrophil infiltration.

7.3 Results

The results from these experiments are tabulated in Table 7.1. There was no significant changes in the wet:dry ratio in all groups (Table7.1). There was significantly reduced acute lung injury measured by BAL protein concentration in animals treated with hydrocortisone compared to controls ($p<0.01$). There was no significant difference in the BAL protein concentration in the animals subjected to NO manipulation compared to controls (Table 7.1). Animals treated with hydrocortisone had a significantly reduced pulmonary myeloperoxidase activity compared to controls ($p<0.01$). There was no significant difference in the myeloperoxidase activity in animals subjected to NO manipulating agents compared to controls (Table7.1).

Nitric oxide manipulating agent	Wet:dry lung ratio	Broncheoalveolar lavage protein concentration (μg/ml)	Myeloperoxidase activity (units/g)
Control	4.91 (4.3-5.03)	395 (330-450)	7.37 (6.27-7.98)
L-Arginine	4.57 (4.24-4.95)	401 (368-482)	7.39 (6.08-7.82)
L-NMMA	4.72 (4.08-4.85)	361 (288-420)	7.18 (6.63-8.10)
1400W	4.77 (4.14-4.94)	387 (310-408)	7.31 (6.37-7.98)
Hydrocortisone	4.39 (4.09-4.75)	225 (166-286)**	5.45 (4.54-6.70)**
NOC-18	4.76 (4.57-4.95)	383 (298-442)	7.17 (6.12-7.64)

Table 7.1: The influence of nitric oxide manipulating agents on pulmonary wet:dry ratio, broncheolar lavage protein concentration and myeloperoxidase activity.

****p<0.01 vs control Mann-Whitney U-test.**

7.4 Discussion

The aim of the experiments presented in this chapter was to investigate the effects of pharmacological manipulation of nitric oxide metabolism on aortic cross-clamp induced ischaemia-reperfusion pulmonary injury. If nitric oxide contributes to acute lung injury then pharmacological manipulation of the nitric oxide response by total or selective nitric oxide synthase inhibition might reduce or prevent lung injury. Alternatively, if endogenous nitric oxide prevents or ameliorates the lung injury, nitric oxide donors might facilitate this protective effect. These experiments were performed using the rodent model of aortic cross-clamp ischaemia-reperfusion lung injury already described with the optimum ischaemia and reperfusion periods. In addition, the inferior vena cava was exposed to allow administration of control (normal saline) or active pharmacological agents prior to aortic cross-clamping. Again, the model worked well and produced reliable and reproducible data with no unforeseen technical problems and no unexpected intra-operative deaths.

The following pharmacological agents were used to inhibit the nitric oxide response: L-NMMA a pan NOS inhibitor that inhibits all isoforms of NOS and 1400W a highly selective iNOS inhibitor (which has no effects on the constitutive isoforms of NOS). Similarly, the following agents were used as nitric oxide donors: L-arginine a simple amino acid which is the substrate for NOS and NOC-18 a water soluble slow release NO donor with a half-life of 2 days. I did not investigate the effects of traditional NO donors such as glyceryl trinitrate (GTN) or sodium nitroprusside because they often produce volatile changes in blood pressure and are administered via a continuous intravenous infusion. In addition, I studied the effects of hydrocortisone (an anti-inflammatory steroid) which inhibits the systemic inflammatory response in a non-specific manner. I used 3 assays to investigate acute lung injury: wet:dry lung ratio as an index of pulmonary oedema, bronchoalveolar lavage protein concentration as an index of lung microvascular permeability and myeloperoxidase as an index of pulmonary neutrophil infiltration.

These data show that hydrocortisone ameliorated the subsequent acute lung injury but none of the nitric oxide donors or inhibitors had any effect. The reasons for this are not clear and I will discuss them further in chapter 12. In the next chapter I investigated the role of pharmacological manipulation of the nitric oxide response on aortic cross-clamp induced renal ischaemia-reperfusion injury.

CHAPTER EIGHT

Can manipulation of NO metabolism decrease renal impairment following infrarenal aortic cross-clamping?

8.1 Introduction

8.2 Methods

8.3 Results

8.4 Discussion

8.1 Introduction

In chapter nine I demonstrated that infrarenal aortic cross-clamping is associated with significant post-operative impairment of glomerular filtration rate on the second and seventh post-operative day. I also demonstrated that there is a significant increase in the endogenous renal NO response following one hour of reperfusion, and this response remained elevated seven days post-operatively. Inducible NOS was detected in the kidneys of animals subjected to infrarenal aortic cross-clamping seven days post-operatively but not after one hour of reperfusion. These data suggest that the early NO response is due to the constitutive isoforms of NOS; with all three isoforms of NOS contributing to the late NO response on day 7. However, it is not clear if the endogenous NO response is beneficial, detrimental or just part of the kidney's response to IRI.

In this chapter I investigated if manipulation of the endogenous NO response with selective NO donors and inhibitors affected the impairment in renal function. The pharmacological agents I used were:

- L-arginine - the substrate for NOS
- L-NMMA: NG-monomethyl-L-arginine - an inhibitor of all isoforms of NOS
- 1400W: N-(3-Aminomethyl) benzyl acetamide - a highly selective inhibitor of iNOS
- Hydrocortisone-a non-specific inhibitor of the inflammatory response
- NOC-18: Z-1-2 Aminoethyl-N- (2 ammonioethyl) amino diazen-1-ium 1,2-diolate- a slow release NO donor).

The aim of this chapter was to investigate the effects of pharmacological manipulation of NO metabolism on infrarenal aortic cross-clamp induced renal injury.

8.2 Methods

Groups of male Wistar rats (n=6) weighing 450-500g were anaesthetised with halothane. The infrarenal aorta and inferior vena cava were exposed. One of the following agents was administered via the inferior vena cava 5 minutes before infrarenal aortic cross-clamping:

1. Control –Normal Saline
2. L-arginine (300mg/kg)
3. L-NMMA (30mg/kg)
4. 1400W (10mg/kg)
5. Hydrocortisone (20mg/kg)
6. NOC-18 (0.5mg/kg)

Following an ischaemic period of 60 minutes the clamp was removed and the animal maintained under anaesthesia for a further 1 hour reperfusion. Then a left nephrectomy was performed and animals allowed to recover after administration of an intramuscular dose of buprenorphine for post-operative analgesia. The glomerular filtration rate in the remaining right kidney was measured using the ^{99}TcDPTA clearance technique on the 2nd and 7th post-operative day.

8.3 Results

The results from this experiment are tabulated in Table 8.1. Treatment of animals with L-NMMA significantly impaired GFR ($p<0.01$) compared to controls on the second post-operative day and the seventh post-operative day ($p<0.05$). Hydrocortisone and 1400W had no significant effect on GFR on the second or seventh post-operative day (Table 8.1). L-arginine and NOC-18 had no influence on GFR on the second post-operative day but significantly improved GFR on the seventh post-operative day ($p<0.05$) compared to controls.

Nitric oxide manipulating agent	Glomerular filtration rate on day 2 (ml/minute)	Glomerular filtration rate on day 7 (ml/minute)
Control	1.50 (1.37-1.57)	1.49 (1.44-1.63)
L-arginine	1.62 (1.48-1.72)	1.67 (1.5-1.74)*
L-NMMA	1.16 (1.05-1.29)**	1.38 (1.23-1.56)*
1400W	1.48 (1.32-1.62)	1.51 (1.40-1.68)
Hydrocortisone	1.49 (1.41-1.60)	1.57 (1.41-1.67)
NOC-18	1.62 (1.52-1.72)	1.66 (1.57-1.75)*

Table 8.1: The influence of nitric oxide manipulating agents on glomerular filtration rate on day 2 and day 7.

*** $p<0.05$ and ** $p<0.01$ vs control Mann-Whitney U-test.**

8.4 Discussion

The aim of the experiments presented in this chapter was to investigate the effects of pharmacological manipulation of nitric oxide on infrarenal aortic cross-clamp induced renal injury. If NO contributes to renal impairment then pharmacological manipulation of the nitric oxide response by total or selective NOS inhibition might reduce or prevent impairment in GFR following infrarenal aortic cross-clamping. Alternatively, if endogenous NO prevents or ameliorates renal impairment, nitric oxide donors might facilitate this protective effect. The experiments were performed using the rodent model of infrarenal aortic cross-clamp induced renal injury with the optimum ischaemic time. The inferior vena cava was exposed to allow administration of control (normal saline) or active pharmacological agents prior to infrarenal aortic cross-clamping. Again, the model worked well and produced reproducible data with no unforeseen technical problems and no unexpected intra-operative deaths.

I investigated the influence of 2 NO inhibitors and 2 NO donors on post-operative renal function. L-NMMA, a pan NOS inhibitor inhibits all isoforms of NOS and 1400W, a highly selective iNOS inhibitor (which has no effects on the constitutive isoforms of NOS). L-arginine is a simple amino acid which is the substrate for NOS and NOC-18 is a water soluble slow release NO donor with a half-life of 2 days. I did not investigate the effects of other NO donors such as GTN or sodium nitroprusside because they have a very short half life, requiring a continuous intravenous infusion and often produce volatile changes in blood pressure. In addition, I studied the effects of hydrocortisone, (an anti-inflammatory steroid) which inhibits the systemic inflammatory response in a non-specific manner. I measured the GFR on the second and seventh post-operative day as an index of renal injury.

These data demonstrated that administration of the pan NOS inhibitor L-NMMA significantly impaired renal function on the second ($p<0.01$) and seventh ($p<0.05$) post-operative day. The highly selective iNOS inhibitor 1400W and hydrocortisone did not influence GFR significantly on the second or seventh post-operative day. This suggests that NO production from the constitutive isoforms of NOS may provide a protective effect against renal injury during the very early reperfusion period. Inducible NOS expression does not occur in the kidney during the early reperfusion period (Chapter 6) and this may explain why manipulation of iNOS did not make any difference.

The observation that hydrocortisone did not influence renal impairment following lower torso IRI but did significantly reduce acute lung injury following IRI suggests that the mechanism of injury may be different in the two organs. The lungs have a large surface area for gas exchange and are a route of entry into the body for airborne pathogens. As a result of this the lung has immunoprotective properties and is equipped with many inflammatory cells including neutrophils, mast cells and alveolar macrophages. The lungs are particularly sensitive to inflammatory states, in fact increased levels of exhaled NO have been found to be a very sensitive marker of exacerbation of asthma. A similar mechanism may make the lung particularly susceptible to the systemic inflammatory response and acute lung injury. Hydrocortisone has a general anti-inflammatory effect and this may explain its effect on reducing acute lung injury. The kidney however, primarily regulates blood volume and electrolyte balance and regulation of renal blood flow is critical to this process. Nitric oxide plays a very important role in the regulation of renal blood flow by its vasodilatory properties during physiological states. During ischaemia-reperfusion injury there may be an increase in plasma systemic vasoconstrictors including endothelin, thromboxane A2 and angiotensin resulting in a decrease in renal blood flow and GFR. This may explain why inhibiting the endogenous NOS response with L-NMMA resulted in impairment of GFR on the second and seventh post-operative day.

Animals treated with the NO donors L-arginine and NOC-18 prior to infrarenal aortic cross-clamping did not have a significant improvement in renal function on the second day. These agents did however significantly improve renal function by the seventh post-operative day ($p<0.05$). This suggests that treatment with a NO donor prior to infrarenal aortic cross-clamping may favorably improve the balance between vasoconstrictors and vasodilators on the renal vascular bed during the early reperfusion period, eventually protecting against renal impairment.

To conclude, these data suggest that the endogenous renal NO response following infrarenal aortic cross-clamp induced renal injury may have some protective function in maintaining renal blood flow and opposing the effects of systemic vasoconstrictors. Exogenous NO donors may have a role in renal protection following infrarenal aortic cross-clamp induced ischaemia-reperfusion injury.

CHAPTER NINE

Conclusions

9.1 Introduction

9.2 Experimental work

9.3 Discussion

9.4 Relavance to clinical work

9.5 Future work

9.1 Introduction

Abdominal aortic aneurysms are an important health issue in the United Kingdom. Elective surgical repair is the treatment of choice for patients with an AAA > 5.5cm diameter (UK Small Aneurysm Trial Participants, 1998) and has a low mortality of about 5% (Bradbury *et al.*, 1998; Johnston, 1994b). Surgical repair of a ruptured AAA is associated with a much higher post-operative mortality of around 50%. The cause of this mortality is probably related to the combined insults of infrarenal aortic cross-clamp induced ischaemia-reperfusion injury, hypoperfusion secondary to shock, acidosis, hypothermia, massive blood transfusion and infection (Bradbury *et al.*, 1997; Johnston, 1994a).

Acute lung injury and impaired pulmonary function has been well documented following infrarenal aortic cross-clamping and is caused by ischaemia-reperfusion injury. Infrarenal aortic cross-clamping also causes marked changes in renal haemodynamics with a decrease in GFR and renal blood flow. In addition IRI in the kidney may lead to progressive renal impairment and renal failure.

The aim of this thesis was to investigate pulmonary and renal nitric oxide metabolism following infrarenal aortic cross-clamp induced pulmonary and renal injury and the influence of pharmacological manipulation of the NO response on subsequent pulmonary and renal injury.

9.2 Experimental work

At present , human clinical studies on NO metabolism in the lungs and kidneys following infrarenal aortic cross-clamp induced IRI are not practical because it is not ethical to collect pulmonary and renal biopsies during the peri-operative period. Therefore I investigated renal and pulmonary IRI in two rodent models.

The model of acute lung injury used one simillar to that described by Barry et al (1996). Barry et al (1996) describe a model where the ischaemic period was 30 minutes followed by a reperfusion period of 120 minutes. There are variations in handling of eqipment, tissue and speed of operating among different scientists performing any experiment. I had to repeat the initial experiments to determine the best ischaemic and reperfusion times in my hands to produce a quantifiable acute lung injury. I also used this opportunity to study the endogenous pulmonary and renal NO response to varying degrees of ischaemic and reperfusion stress. The ideal ischaemic and reperfusion periods (in my

hands) were then used to study the effect of manipulating the NO response following infrarenal aortic cross-clamp induced IRI. I used a longer ischaemic period (60 minutes compared to 30 minutes) than Barry et al. I felt that any significant changes in acute lung injury caused by the NO manipulating agents would be more evident if the initial insult was larger.

The method chosen to cull the animals was different to that described by Barry et al. I found rapid exanguination was a more practical method as it gave me an opportunity to collect sufficent blood samples to measure any plasma mediators I would wish to measure in the future. All animals including controls were culled in a similar fashion and tissue samples immediately snap frozen in liqid nitrogen. In this way any inflence of the method of culling on NO metabolism was identical in all groups.

Several investigators have demonstrated that acute lung injury starts early in the reperfusion period (Abdih *et al.*, 1994; Barry *et al.*, 1996; Tassiopoulous *et al.*, 1998). In the clinical setting the full effects of reperfusion are often only noticed later as hypoxia secondary to non-cardiogenic pulmonary oedema. An additional group of animals subjected an ischaemic period of 60 minutes then recovered for 24-48 hours before being culled to measure acute lung injury may have enhanced this study. Furthermore a further set of experiments of pre-reperfusion dosing may have enhanced this work.

The assays used to measure acute lung injury and NOS activity worked well. I did have technical problems in measuring nitrate concentrations using the Greiss reagent. Future work should consider estimation of nitrotyrosine as a marker of NO metabolism

The model chosen to study renal injury was a modification of that used by Weight et al to study renal pedical IRI. Glomerular filtration rate is a sensitive and appropriate marker of renal impairment. The mechanism of renal impairment following lower torso IRI differs slightly between the lungs and kidneys. Infalmmatory cells are more involved in end organ damage in the lungs compard to the kidneys. The balance of vasoconstrictors to vasodilators in the renal vasculature following IRI has a large influence in post-reperfusion renal impairment. Hence it would have been interesting to see the effects of a vasodilator (such as prostacyclin) or an endothelin antagonist in addition to the experiments performed to try and manipulate renal NO metabolism.

9.3 Discussion

Experimental work on acute lung injury

Several other animals and human studies have demonstrated that acute lung injury occurs following lower torso ischaemia (Anner *et al.*, 1987; Anner *et al.*, 1988; Fantini & Conte, 1995; Paterson *et al.*, 1989; Paterson *et al.*, 1989) and that a systemic inflammatory response occurs following elective AAA surgery (Groeneveld *et al.*, 1997; Thompson *et al.*, 1996). This systemic inflammatory response primes the endothelium and activates leucocytes and in turn leads to leucocyte infiltration into lung tissue where they degranulate and cause acute lung injury.

There is only one previously study to date which has measured pulmonary NO generation following lower torso IRI (Tassiopolous *et al.*, 1997). In this study exhaled pulmonary NO was measured using a chemiluminescent method following IRI in a rodent model. Data in this study demonstrated that NO levels in exhaled air during the reperfusion period reached 50 times the baseline levels. A pro-inflammatory cytokine response (with increased TNF-α levels) was also demonstrated which upregulated iNOS expression.

There has been some data published to date on the influence of NO manipulating agents on the development of acute lung injury following pro-inflammatory states such as lower torso IRI, mesenteric IRI pancreatitis and endotoxin induced septic shock. Workers from the Royal College of Surgeons in Ireland demonstrated that inhibition of the endogenous NO response by L-NAME (a pan NOS inhbitor) made the acute lung injury worse suggesting that NO protected the lung against injury following lower torso IRI (Abdih *et al.*, 1994). This group also demonstrated that the NO donors GTN and sodium nitroprusside decreased the severity of the acute lung injury following lower torso IRI (Abdih *et al.*, 1994; Barry *et al.*, 1996). Other workers have demonstrated that NO donors may decrease acute lung injury following mesenteric artery ischaemia-reperfusion injury (Terada *et al.*, 1996), endotoxic shock(Bloomfield *et al.*, 1997; Galili *et al.*, 1997) and peritonitis(Fukatsu *et al.*, 1998) by its vasodilatory and anti-leucocyte adhesive properties.

However other workers have demonstrated that the endogenous NO response is a detremental response and inhibition of this response ameliorates acute lung injury. Aminoguanidine, a less specific iNOS inhibitor was found to protect against acute lung injury following lower torso IRI (Tassiopoulos *et al.*, 1998). In a study of acute lung injury following early pancreatitis, L-NMMA was found to reduce acute lung injury (Tsukuhara

et al., 1996). Aminoguanidine and L-NAME were found to decrease chemicaly induced acute lung injury (Cruz *et al.*, 1997). These studies demonstrate that although there has been a lot of work performed on NO manipulation to prevent acute lung injury, there remains some controversy on whether inhibition of NO or treatment with exogenous NO prevents development of acute lung injury. This must not be confused with the use of inhaled NO for established ARDS, where there is major ventilation / perfusion mismatch with alveolar collapse and superimposed infection. In this situation inhaled NO improves oxygenation by improving perfusion of ventilated areas. Inhaled NO therapy for established ARDS is currently undergoing randomised clinical trials; early results have not demonstrated any significant improvement in mortality among patients treated with inhaled NO (Artigas *et al.*, 1998).

Nitric oxide has two potential effects on the lung. It can prevent acute lung injury by the prevention of leucocyte adhesion or it can cause acute lung injury by generating peroxynitrite. It is possible that although the NO inhibitors I used prevented excess peroxynitrite production, this benefit was opposed by increased leucocyte infiltration and degranulation causing tissue damage by other free-radicals and proteases. Similarly, NO donors may have prevented leucocyte infiltration and degranulation but these benefits may have been opposed by increased peroxynitrite production. Finally, it is possible that NO metabolism is only a small part of the inflammatory process which involves leucocyte activation, expression of cell adhesion molecules and release of proteases and free-radicals by leucocytes in lung tissue. I found that steroids did decrease the acute lung injury. This may be because steroids are a general anti-inflammatory agent that inhibit the whole inflammatory process.

Although I demonstrated that steroids decreased acute lung injury I am very cautious to suggest that there may be a potential therapeutic role for steroids in preventing acute lung injury. Steroids also depress the immune response and may compromise patients with subclinical infection resulting in serious hazards to patients with a prosthetic aortic graft. Indeed after clinical trials in humans it is now established that there is no role for streoid therapy in established ARDS (Artigas *et al.*, 1998).

Experimental work on renal injury

There is no published work on the influence of lower torso IRI on renal NO metabolism. However , many workers have studied NO metabolism following renal pedicle ischaemia-reperfusion injury. There is evidence that renal pedicle occlusion followed by reperfusion results in upregulation of endogenous NO metabolism. The constitutive isoforms of NOS have been demonstrated to be responsible for the initial rise in endogenous renal NO production, with iNOS contributing to total NO production once it is upregulated (Cristol *et al.*, 1993; Shoskes *et al.*, 1997; Waz *et al.*, 1998; Weight *et al.*, 1998).

It is important to note that endogenous NO plays a vital role in the maintenance of renal blood flow and GFR during normal physiological states (Kone, 1997). During renal pedicle ischaemia-reperfusion injury several mechanisms contribute to renal impairment including free-radicals, infiltration of neutrophils and accumulation of systemic vasoconstrictors (Weight *et al.*, 1996). Several workers have demonstrated that administration of exogenous NO donors protect the kidney from renal pedicle occlusion induced IRI (Garcia-Criado *et al.*, 1998; Mashiach *et al.*, 1998; Schramm *et al.*, 1994).

Nitric oxide may protect the kidneys following ischaemia-reperfusion injury by several mechanisms Its anti-leucocyte adhesive properties may prevent leucocyte adhesion and infiltration into renal tissue, preventing further release of free-radicals and proteases (Garcia-Criado *et al.*, 1998; Lauriat & Linas, 1998). The vasodilatory properties of NO probably play a vital role in renal protection following ischaemia-reperfusion injury. During physiological states NO plays a major part in regulating renal blood flow. Following ischaemia-reperfusion injury several vasoconstrictors are prescent which decrease renal blood flow. These include angiotensin, thromboxane A2 and endothelin (Gelman, 1995). Nitric oxide opposes the effects of these vasoconstricors and preserve renal function. Many patients will have some degree of renal impairment following elective AAA surgery but this will not be clinically relavant as a large quantity of the renal reserve needs to be damaged before consistent changes in serum creatinine occur. However patients with a degree of renal impairment pre-operatively are at risk of developing acute renal failure. These patients may benefit from renal protection by NO donors. Furthermore 25% of patients undergoing surgery for a ruptured AAA develop acute renal failure, and treatment with a NO donor may protect the kidneys.

All these experiments have demonstrated that infrarenal aortic cross-clamp induced ischaemia-reperfusion injury causes an increase in the endogenous renal NO response. This is probably a protective response to counteract the effects of systemic vasoconstrictors formed following IRI. Pretreatment with a NO donor may benefit patients with pre-existing renal disease or those presenting with a ruptured AAA.

9.4 Relevence to clinical work

Although many patients develop acute lung injury following elective AAA repair, many recover completely from this process. Acute lung injury progressing to ARDS and respiratory failure is more common following surgery for ruptured AAA. In this thesis I did not demonstrate any benefit in pharmacological manipulation of NO metabolism to ameliorate acute lung injury following lower torso IRI as would occur during elective AAA repair. Although I demonstrated that hydrocortisone does reduce acute lung injury, this drug has potent immunosupressive effects and hence it may be potentially hazardous in patients following AAA surgery.

I clearly demonstrated that the endogenous renal NO response was protective. Inhibition of this response with the pan NOS inhibitor L-NMMA made renal function worse on the second and seventh post-operative day. Furthermore treatment with the NO donors L-arginine and NOC-18 improved renal function compared to control animals on the seventh post-operative day. NO donors may provide renal protection against IRI induced renal impairment. Although post-operative renal impairment occurs following elective AAA surgery, patients with normal pre-operative renal function usually have no clinical sequelae because of good renal reserve. However patients with a degree of pre-operative renal impairment, are at risk of developing post-operative renal failure. Treatment of this latter group of patients with a NO donor prior to infrarenal aortic cross-clamping may provide renal protection.

9.5 Future work

Respiratory failure complicates up to 47% of patients following surgery for a ruptured AAA compared to 8% of those following elective surgery (Johnston, 1994a; Johnston, 1994b). Similarly acute renal failure is much more common following surgery for a ruptured AAA and occurs in about 25% of patients.

Future work should address the problems of acute lung injury and renal impairment following surgery for a ruptured AAA. There is potential to modifying this rodent model to one including haemorrhagic shock and ischaemia-reperfusion to simulate the changes that occur following a ruptured AAA. Rats may be anaethetised with halothane and a catheter may be placed into the right carotid artery. Blood can be withdrawn slowly from this catheter and stored in a reservoir containing citrate anticoagulant while the blood pressure is monitored to simulate haemorrhage. After a period of haemorrhage the animals will undergo a laparotomy and infrarenal cross-clamping to simulate AAA repair. The blood withdrawn into the reservoir may then be infused back into the rat to simulate resuscitaion from a ruptured AAA. Following removal of the aortic cross-clamp and reperfusion injury animals can either be culled at this stage to study acute lung injury or the catheter may be removed from the carotid artery, the carotid artery tied off and the animals recovered. Renal function may be measured on the second and seventh post-operative day.

My data suggested that NO may be only part of a much larger process involved in acute lung injury, but did protect the kidneys following lower torso IRI. Future work should attempt to measure a wider range of endogenous mediators such as endothelins (powerful vasoconstrictors) and the arachidonic acid metabolites such as prostaglandins and thromboxane A2. Prostaglandins may have a protective role in the kidney and thromboxane A2 is known to be a vasoconstrictor. This should give us a broader understanding of the whole pro-inflammatory process following shock and whole body IRI. The influence of agonists or antagonists to these mediators can then be selectively manipulated and may lead to clinically beneficial results which may help the outcome of patients surviving surgery for a ruptured AAA.

Appendix 1: Measurement of glomerular filtration rate (GFR)

There are 3 methods of measuring GFR.

1. The classical method for the estimation of GFR uses the measurement of the rate of appearance of a test substance (e.g.inulin) in the urine over a specified time interval after establishing and maintaining constant plasma concentration using continuous intravenous infusion. The GFR is given by:

$$GFR = \frac{UV}{P}$$

U is the concentration of the test substance in the urine
P is the concentration of the test substance in the plasma
V is the urine flow rate during the test period

2. The second method is single injection of tracer with urine and plasma sampling After a single injection of a tracer, the plasma concentration will vary continuously with time, so that the clearance equation now only applies for a short time interval, δt, during which the plasma concentration , P, is reasonably constant. During this short time interval the portion of the urine volume produced is δV and the tracer has concentration U, as before. Then

$$GFR = \frac{U\delta V}{P\delta T}$$

Integrating the numerator and denominator gives:

$$GFR = \frac{UV}{\int_{t1}^{t2} P\delta t}.$$

Where V is the total urine volume produced during the sampling intervals from times t1 to t2.

3. The third method is the single injection of tracer with plasma sampling (which is used for neonates, children and small mammals) By considering the entire 'infinite' time interval from injection onwards and assuming that all of the tracer is eventually excreted and only via the renal route, then the total quantity of tracer excreted (UV) will be equal to the injected dose (I). Hence:

$$GFR = \frac{I}{\int_0^{\infty} P\delta t}.$$

If one assumes monoexponential kinetics are an acceptable description of plasma concentrtion versus time (that is $P = Ce^{-kt}$), then

$$\text{Clearance (GFR)} = \frac{Ik}{C_o}$$

Where I = injected activity of isotope

k = the exponential slope of the plasma clearance (per minute)

C_0 = the zero time intercept of the plasma clearance curve

I used this method to measure GFR

Protocol

The activities of a standard dose and the dose administered to the animal (20MBq) were measured in an ion chamber dose calibrator noting activity and time. The standard is diluted to 1000ml in a volumetric flask and the time of animal injection recorded. The syringe residues for the standard and animal dose were recorded.

For both the standard and animal dose:

Net dose = original count – residue count

Aliquots of the diluted standard, the two plasma samples (50μl each) and background are counted in the scintillation well and the net count rate is obtained by subtraction of the background count rate.

The injected activity (I) is given by:

$$I = \frac{\text{(Net standard count rate) x (net animal dose) x 1000 (for dilution)}}{\text{Net standard count}}$$

$$\text{GFR (50μl/min)} = \frac{Ik}{C_o}$$

$$\text{In ml /min} \qquad \text{GFR} = \frac{50\,Ik}{1000C_o}$$

where I is the injected activity (cpm), k is the exponential slope of the plasma clearance (per minute), and C_0 is the zero time intercept of the plasma clearance curve (cpm/50μl) (derived from extrapolation of the plasma clearance curve).

Appendix 2: The citrulline assay for measurement of nitric oxide synthase activity

Calculation of L-citrulline formed in reactions mixtures:

1. Vials A contained reaction mixtures, NADPH and tissue homogenate. They were incubated for 45 minutes at 37° C then passed through the resin to remove ^{3}H L-arginine.
2. Vials B contained reaction mixtures and tissue homogenate.They were incubated **in ice at 0°C** for 45 minutes before being passed through the resin to remove all ^{3}H L arginine.
3. Vials C contained reaction mixtures that were prepared **and not passed through the resin**, hence they represent the **total radioactivity** used in the experiment.
4. The total concentration of L-arginine in each reaction mixture was 0.000025M and the volume of each reaction mixture was 0.00004 l, hence the number of moles of L-arginine in each reaction mixture was 0.000025 x 0.00004 = 0.000000001 moles or 1000 picomoles of L-arginine.

The amount of L-citrulline formed (E) for a particular sample was calculated by the following formulae:

$$\mathbf{E = \frac{Average\ CPM\ Vials\ A - Average\ CPM\ Vials\ B}{Average\ CPM\ Vials\ C} \times D}$$

where D = [^{3}H L- arginine + L-arginine] x volume of reaction mixture
CPM = counts per minute in the β counter

The protein concentration in the tissue homogenate was measured using Bradford reagent and the results expressed as picomoles L-citrulline / mg protein / 45 min incubation.

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