A Model of the Hepatorenal Syndrome

A thesis submitted for the award of Doctor of Philosophy

By
Radhika S Anand

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Centre for Hepatology
Department of Medicine
Royal Free and University College Medical School
University of London
DEDICATION

I wish to dedicate my thesis to the loving memory of my father, Dr Nityanand, to my mother, Shanti and to Hassan, with love.
ABSTRACT

Renal failure occurs in approximately 55% of patients with acute liver injury. Hepatorenal syndrome (HRS) is the development of renal failure secondary to liver disease in the absence of significant renal pathology. Presently there are no suitable models for the study of HRS. Recently, the rat model of galactosamine-induced acute liver failure (galn-ALF) was found to develop a hyperdynamic circulation along with a disruption in the renal haemodynamics which are characteristics of HRS. The aim of the project was to investigate the suitability of the rat model of galn-ALF as a model for HRS and to determine the role vasoactive mediators play in the pathogenesis of renal failure in this model.

Characterisation of rats with galn-ALF showed that this was a suitable model for the study of HRS. The animals developed renal failure secondary to liver disease. The degree of renal impairment correlated with the degree of liver injury. The histology of the kidneys was normal and there was a reduction in the renal blood flow.

A reduction in renal blood flow is indicative of renal vasoconstriction. Endothelin-1 (ET-1), a potent vasoconstrictor is elevated in patients with HRS. In the galn-ALF rat model of HRS ET-1 was found to be elevated in the plasma. In vitro autoradiography revealed that the ET-1 receptor ET$_A$ was upregulated in the renal cortex. Using the mixed receptor antagonist Bosentan in this model of HRS, renal function was improved. This suggests that elevated levels of ET-1 play a role in the pathogenesis of renal failure.

Oxidant stress has also been found to play a role in renal failure. In the model there was marked lipid peroxidation. Oxidative stress was quantified by the measurement of F$_2$-isoprostanes. These compounds are biologically active and can themselves cause renal vasoconstriction. Treatment with the anti-oxidants N-acetylcysteine and α-lipoic acid conferred some protection against renal impairment but was not effective in inhibiting lipid peroxidation. This suggests that a mechanism other than lipid peroxidation is involved in the renal dysfunction.
HYPOTHESIS

This thesis tests the hypothesis that the rat with galactosamine-induced acute liver failure is a suitable model for the study of Hepatorenal Syndrome.
PUBLICATIONS

Published work from the thesis

Abstracts


Publications contributed to during the thesis


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I received valuable suggestions from my colleagues Dr Steven Holt, Dr Richard Marley and Mr Bimbi Fernando. Many thanks goes to Dr Michael Dashwood for assistance with the \textit{in vitro} autoradiography studies of the endothelin binding sites of the kidney, Pamela Milner for assistance with the studies on the ET-1 concentrations in the plasma and in the kidney and David Goodier for providing the liver function tests. I also wish to extend my thanks to my family and friends who have encouraged and supported this endeavour.

Finally, I would like to thank my husband Hassan and my mother, Shanti for their continuous support, patience and encouragement of this thesis.

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<th>Description</th>
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<tbody>
<tr>
<td>AcN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADH</td>
<td>Antidiuretic hormone</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>ALF</td>
<td>Acute liver failure</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>AP-1</td>
<td>Redox sensitive transcription factor</td>
</tr>
<tr>
<td>ARF</td>
<td>Acute renal failure</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate transaminase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDL</td>
<td>Bile duct ligation</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>BSTFA</td>
<td>N,O-bis(trimethylsilyl)fluoroacetamide</td>
</tr>
<tr>
<td>CBU</td>
<td>Comparative Biology Unit</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic 3',5'-Guanosine monophosphate</td>
</tr>
<tr>
<td>c-myc</td>
<td>Redox sensitive gene and proto-oncogene</td>
</tr>
<tr>
<td>COX</td>
<td>Cycloxygenase</td>
</tr>
<tr>
<td>Cr. Cl.</td>
<td>Creatinine clearance</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic blood pressure</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-di-isopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electromobility shift assay</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavine adenine dinucleotide</td>
</tr>
<tr>
<td>Fru-6-P</td>
<td>Fructose-6-phosphate</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>GCMS</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>Galn</td>
<td>D-Galactosamine</td>
</tr>
<tr>
<td>GalnAc-1-P</td>
<td>N-acetylgalactosamine-1-phosphate</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GRx</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced form of glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidised form of glutathione</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HRS</td>
<td>Hepatorenal syndrome</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor κB protein</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor κB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LA</td>
<td>α-Lipoic acid</td>
</tr>
<tr>
<td>LOOH</td>
<td>Lipid peroxide</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass charge ratio</td>
</tr>
<tr>
<td>ManNAc</td>
<td>N-acetylmannosamine</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NAC</td>
<td>N-Acetylcysteine</td>
</tr>
<tr>
<td>NeuNAc</td>
<td>N-acetylneuraminic acid</td>
</tr>
<tr>
<td>NFκxB</td>
<td>Nuclear factor κ B transcription factor</td>
</tr>
<tr>
<td>NOA</td>
<td>Nitric oxide analyser</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFBR</td>
<td>Pentafluorobenzylbromide</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
</tbody>
</table>
RAAS  Renin angiotensin aldosterone system
RNS  Reactive nitrogen species
RBF  Renal blood flow
SNS  Sympathetic nervous system
SOD  Superoxide dismutase
TBARS  Thiobarbituric acid reactive substances
TLC  Thin layer chromatography
TNF  Tumour necrosis factor
Tx  Thromboxane
UDP  Uridine diphosphate
UDP-Glc  Uridine 5'-diphosphate glucose
UMP  Uridine monophosphate
UTP  Uridine 5'-triphosphate
WD  Wilson disease
Chapter 1

General Introduction
1 General Introduction

1.1 Hepatorenal Syndrome (HRS)

1.1.1 DEFINITION AND EPIDEMIOLOGY

Patients with cirrhosis or acute liver injury, frequently develop renal failure which is often called hepatorenal syndrome (HRS) (Epstein 1987). However, HRS is frequently misdiagnosed as most cases of renal impairment in patients with liver disease are caused by sepsis, hypovolemia or drugs. Until recently, there has been a lack of consensus on the definition of HRS which has caused difficulty in the interpretation and comparison of studies in the field. The International Ascites Group in 1996 proposed new definitions and diagnostic criteria for HRS (Arroyo et al 1996).

HRS is defined as, “a syndrome that occurs in patients with chronic liver disease and advanced hepatic failure and with portal hypertension characterized by impaired renal function and marked abnormalities in the arterial circulation and activity of vasoactive systems. In the kidney there is marked renal vasoconstriction that results in low glomerular filtration rate. In the extra-renal circulation there is a predominance of arteriolar vasodilation, that results in reduction of systemic vascular resistance and arterial hypotension. A similar syndrome may also occur in the setting of acute liver failure” (Arroyo et al 1996).

Most research has concentrated on HRS in cirrhosis. The pathogenesis of HRS in cirrhosis is also pertinent to the factors involved in acute liver failure.

1.1.2 TEMPORAL RELATIONSHIPS OF RENAL FUNCTION ABNORMALITIES IN CIRRHOSIS

In the initial phases of cirrhosis when ascites is not present, systemic circulatory dysfunction is subtle and renal function is apparently normal. However, in this phase of pre-ascites, subtle renal function abnormalities exist. The most important and characteristic is a diminished renal capacity to excrete a sodium overload (Gines and
Rodes 1999). Progression of cirrhosis and increased portal hypertension is accompanied by more severe systemic circulatory dysfunction, and impaired renal ability to excrete sodium in conditions of regular sodium intake. This leads to fluid accumulation in the peritoneal cavity termed ascites.

In the early stages patients with cirrhosis have a normal GFR and are able to dilute their urine after a water load (Gines and Rodes 1999). However the patients develop progressive systemic vasodilation leading to a slight decrease in arterial blood pressure, and secondary activation of the renin-angiotensin-aldosterone system (RAAS) and the sympathetic nervous system (SNS) (Schrier et al 1988). The activation of these systems is a homeostatic response to maintain arterial blood pressure within a normal range and has a major role in the pathogenesis of sodium retention (Schrier et al 1988), in that activation of the RAAS results in overproduction of aldosterone which causes an increase in Na\(^+\) reabsorption (Bichet et al 1982b) (Gaudin et al 1991) (Henriksen et al 1988). The systemic vasodilation also results in hypersecretion of arginine vasopressin and this causes sodium and water retention.

With the progression of cirrhosis most patients develop severe impairment in their ability to excrete free water. This contributes to ascites formation and dilutional hyponatremia (which is a low plasma concentration of sodium secondary to nonosmotic retention of water) with a serum sodium level < 130mEq/l compared to normal sodium level of about 140mEq/l (Cardenas and Gines 1999). This latter condition develops despite the avid sodium retention of these patients because water is retained in excess of sodium and the plasma becomes hypo-osmolar (Cardenas and Gines 1999). Patients with ascites and dilutional hyponatraemia, have a decreased arterial blood pressure, marked sodium retention, moderate impairment of renal perfusion and GFR, increased plasma levels of vasopressin and high plasma levels of renin and adrenaline (indicative of activation of the RAAS and SNS) (Schrier et al 1988) (Wilkinson and Williams 1980) (Schroeder et al 1970). Dilutional hyponatremia indicates a severe derangement in systemic haemodynamics and these patients have a high risk of developing HRS (Cardenas and Gines 1999). HRS is the extreme manifestation of systemic vasodilation and activation of the SNS and RAAS and increased synthesis of various vasoactive mediators (Arroyo et al 1996).
1.1.3 DIAGNOSTIC CRITERIA

The reported incidence of HRS in patients with cirrhosis hospitalised for the treatment of ascites range between 7% and 15%. However, this percentage is greater in patients during end-stage liver disease (Gines et al, 1993).

There is no specific test that can be used to make a definitive diagnosis of HRS. Therefore, the diagnosis of this syndrome has been based for many years on the demonstration of a reduced GFR following exclusion of other causes of renal failure. The International Ascites Club recently proposed new criteria for the diagnosis of HRS.

<table>
<thead>
<tr>
<th>Major Criteria</th>
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<tbody>
<tr>
<td>1. Low GFR, indicated by serum creatinine $&gt; 130 \mu \text{mol/l}$ or 24 hour creatinine clearance $&lt; 40 \text{ml/min}$</td>
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<tr>
<td>2. Absence of shock, ongoing bacterial infection, and fluid losses and current treatment with nephrotoxic drugs</td>
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</tr>
<tr>
<td>3. No sustained improvement in renal function (decrease in serum creatinine to $\leq 130 \mu \text{mol/l}$ or increase in CrCl to $\geq 40 \text{ml/min}$) after diuretic withdrawal and expansion of plasma volume with e.g. 4.5% human albumin solution.</td>
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<tr>
<td>4. Proteinuria $&lt; 500 \text{mg/day}$ of protein and no ultrasonographic evidence of obstructive uropathy or parenchymal renal disease</td>
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</table>

All major criteria are necessary for the diagnosis of HRS.

Data from Arroyo et al. (Arroyo et al, 1996)

<table>
<thead>
<tr>
<th>Additional Criteria</th>
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</thead>
<tbody>
<tr>
<td>5. Urine volume $&lt; 500 \text{ml/day}$</td>
<td></td>
</tr>
<tr>
<td>6. Urine Na$^+$ $&lt; 10 \text{mEq/l}$</td>
<td></td>
</tr>
<tr>
<td>7. Urine osmolarity $&gt; \text{plasma osmolarity}$</td>
<td></td>
</tr>
<tr>
<td>8. Urine red blood cells $&lt; 50/ \text{high power field}$</td>
<td></td>
</tr>
<tr>
<td>9. Serum Na$^+$ concentration $&lt; 130 \text{mEq/l}$</td>
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Additional criteria are not necessary for the diagnosis, but provide supportive evidence.

Data from Arroyo et al. (Arroyo et al, 1996)

Table 1. Diagnostic Criteria of HRS According to the International Ascites Club
The first step in the diagnosis of HRS is the evaluation of GFR. Elevated serum creatinine level is specific for the detection of low GFR. However its sensitivity is poor in patients with cirrhosis because some patients with marked reduction of GFR may have normal or only slightly increased serum creatinine levels (Gines and Rodes 1999) (Caregaro et al. 1994). This has been attributed to the low endogenous production of creatinine caused by the poor nutritional status and decreased muscle mass. Blood urea nitrogen (BUN) levels are not accurate in the assessment of GFR in these patients. However, the cut off levels of serum creatinine and creatinine clearance chosen to identify HRS in the International Ascites Club criteria were 1.5mg/dL and 40mL/min, respectively (Arroyo et al. 1996). Creatinine clearance may overestimate GFR and requires an accurate 24-hour urine collection.

The second step in the diagnosis of HRS is to exclude other causes of renal failure, especially pre-renal failure secondary to volume depletion, acute tubular necrosis (ATN), sepsis, glomerulonephritis, and drug-induced nephrotoxicity.

Hypovolemia. Pre-renal failure secondary to hypovolemia (caused by e.g. diuretics or gastrointestinal bleeding) is characterised by reduced renal perfusion and low GFR as observed with HRS. However, in this case renal failure is reversible after administration of fluids to restore intravascular volume which is not the case in HRS (Sacerdoti et al. 1993).

ATN. The existence of shock before the development of the impairment in renal function precludes the diagnosis of HRS. In this setting the most common cause of renal failure is ATN.

Sepsis. Diagnosis of HRS is also precluded by active bacterial infection, such as spontaneous bacterial peritonitis (SBP) (Follo et al. 1994). Therefore, the diagnosis of HRS in patients with SBP should only be made after resolution of the infection.

Glomerulonephritis. The presence of significant proteinuria (urine protein > 500mg/day) and/or ultrasonographic abnormalities of the kidneys suggests the presence of glomerulonephritis.

Drugs. Drug induced causes of renal failure are excluded by inspection of the current drugs given and withdrawal of diuretics.
1.1.4 CLASSIFICATION OF HRS

HRS is now classified into type I and type II (Arroyo et al 1996). The classification was introduced to reflect the different clinical courses exhibited by patients. This classification also defines the patient groups studied and published.

Type I HRS, is characterized by a rapid and progressive increase in serum creatinine over a short period of time, measured in days. Renal failure is often associated with progressive reduction in urine volume, marked Na⁺ retention and hyponatremia. Serum potassium levels are frequently increased. Metabolic acidosis is uncommon despite the marked reduction in renal function. Patients with type I HRS have severe liver failure with jaundice, encephalopathy, and coagulopathy. It most commonly occurs in patients with alcoholic hepatitis with or without cirrhosis, and acute liver failure (Gines et al 1993).

Type II HRS is characterised by a moderate and stable reduction of GFR (serum creatinine is usually < 2 mg/dl). Type II HRS usually occurs in patients with diuretic resistant ascites (Gines et al 1993). The survival of patients with this condition is longer than that of patients with type I HRS, and may progress over months.

1.1.5 PATHOGENESIS

1.1.5.1 Introduction

HRS represents the extreme expression of the systemic circulatory dysfunction in cirrhosis or acute liver failure. The kidneys in HRS are structurally normal, and at least in the early part of the syndrome, tubular function is intact. This is reflected by avid sodium retention and oliguria. In fact, kidneys taken from patients with HRS and transplanted into patients with normal liver function show recovery of kidney function (Koppel et al 1969). The kidney function of patients who receive a transplanted liver were also found to recover (Gonwa et al 1995). It was demonstrated using pre- and post-mortem angiograms of a patient with HRS the presence of intense cortical vasoconstriction (Epstein et al 1970). The mechanism of vasoconstriction is not known and may involve increased synthesis of renal vasoconstrictors e.g. endothelin-1 (Moore et al 1992).
1.1.5.2 Factors affecting the pathogenesis of HRS
The pathogenesis of HRS involves three main factors. These are:
1. Circulatory dysfunction with peripheral vasodilation and reduced renal perfusion pressure
2. Maintenance of mean arterial pressure (MAP) by increased homeostatic activity
3. Increased synthesis of humoral and renal vasoactive mediators including the vasoactive diuretic and antidiuretic hormones and the renal autocoid systems.
These pathways are inter-related and are considered in turn.

1.1.5.2.1 Circulatory dysfunction causing reduced renal perfusion pressure
In healthy individuals the mean arterial pressure (MAP) is about 90 mmHg and the mean central venous pressure is between 5-8 mmHg. The autoregulatory system maintains a stable renal blood flow during changes in renal perfusion pressure (i.e. the mean arterial pressure minus the renal venous pressure) (Stein 1990) (Navar 1978). This regulatory system operates above a MAP of 70-74 mmHg and if it drops below 70-75 mmHg, renal blood flow (RBF) will decrease in direct proportion to renal perfusion pressure.

In decompensated liver disease and HRS, MAP is reduced, with the lowest values (typically 60-65 mmHg) observed in those developing HRS (Fernandez-Seara et al 1989). The renal response to decreased MAP is altered as a consequence of an activated sympathetic nervous system and increased synthesis of several renal and systemic vasoconstrictors. Animal studies have shown that this causes renal blood flow to become more pressure dependent due to a shift to the right in the autoregulatory curve (Persson et al 1990). Even a modest decrease in MAP may result in a marked fall in RBF and hence GFR (Van Roey and Moore 1996).

The renal venous pressure, normally less than 5mmHg, may be significantly increased in the presence of tense ascites or as a consequence of cirrhosis itself (Mullane and Gliedman 1966) (Cade et al 1987) (Henriksen and Ring-Larsen 1988) (Panos et al 1990). This increase in renal venous pressure coupled with decreased arterial pressure will cause a further decrease in RBF. Infusion of the vasoconstrictor substance 8-ornithine vasopressin (Sandoz, Basel) which increases blood pressure has been found to
also increase urine output, sodium excretion and GFR (Lenz et al 1991).

The most widely accepted mechanism for the reduction in effective blood volume is the “peripheral vasodilation hypothesis” (Schrier et al 1988). In this hypothesis, sinusoidal portal hypertension leads to splanchnic vasodilation (Benoit and Granger 1986) which may be caused by an increase in vasodilating substances such as, nitric oxide, glucagon, prostaglandins and vasoactive intestinal peptide (Sherwin et al 1974) (Silva et al 1990) (Sieber and Groszmann 1992). This splanchnic vasodilation leads to splanchnic pooling and underfilling of the arterial circulation. The underfilling is sensed by arterial baroreceptors, which stimulate the SNS and the renin-angiotensin-aldosterone system (RAAS). The increased activity of the SNS and RAAS results in hypersecretion of arginine vasopressin and this causes sodium and water retention. This situation has been observed in patients with established ascites.

1.1.5.2.2 Maintenance of MAP by increased homeostatic activity
To maintain the MAP when vasodilation occurs, homeostatic response mechanisms are activated. These neurohumoral response mechanisms are:
1. Activation of the renin-angiotensin-aldosterone system (RAAS)
2. Activation of the sympathetic nervous system (SNS)
3. Increased synthesis of vasopressin (antidiuretic hormone).
These neurohumoral mechanisms also result in the induction of renal vasoconstriction. This is because the renal vascular bed normally receives 25% of cardiac output and is an important regulatory site of blood pressure. Alteration of the normal renal autoregulatory response by the activation of these neurohumoral mechanisms by necessity contribute to the decreased renal blood flow observed in HRS.

1.1.5.2.2.1 Activation of the renin-angiotensin-aldosterone system
The RAAS is stimulated in 50-80% of patients with decompensated cirrhosis, and it is further elevated in patients with HRS (Schrier et al 1988) (Wilkinson and Williams 1980) (Schroeder et al 1970). Increased levels of angiotensin II protect renal function by selective vasoconstriction of the efferent glomerular arterioles, by increasing filtration fraction (Laragh et al 1963). In cirrhosis, inhibition of the stimulation of the RAAS by saralasin or captopril, which inhibit the angiotensin converting enzyme (ACE) and thus, angiotensin II, caused marked arterial hypotension and decreased GFR
(Schroeder et al 1976) (Cobden et al 1985) (Pariente et al 1985) (Wood et al 1985) (Gentilini et al 1993). However, in some cirrhotic patients infusion of angiotensin II improved the GFR, possibly because of increased arterial pressure.

1.1.5.2.2.2 Activation of the sympathetic nervous systems

In patients affected by liver cirrhosis there is an increase in activity of the sympathetic nervous system (SNS) (Henriksen and Ring-Larsen 1994) (Nicholls et al 1985) (Zambraski and DiBona 1988) (Bichet et al 1982b). This is shown by the increase in noradrenaline and urine and plasma catecholamine concentrations. The SNS can be stimulated in two different ways: 1) These include activation of pressure receptors in response to hypotension in the aortic arch and the carotid glomus and 2) activation of volume receptors in response to hypovolaemia in the atria. Activation of non-volume-dependent hepatic baroreceptors can also stimulate the SNS.

The activation of the SNS results in vasoconstriction of the outer renal cortex of the kidney and reductions in RBF and GFR. Renal vasoconstriction and the subsequent overproduction of renin activated through β-receptors results in an increased release of angiotensin II which also contributes to renal vasoconstriction. Activation of the SNS also results in the overproduction of aldosterone which causes increased Na⁺ reabsorption and thus a positive sodium balance (Bichet et al 1982b) (Ring-Larsen et al 1982) (Floras et al 1991).

The existence of a neural connection between the liver and the renal circulation, termed the hepatorenal reflex has been implicated in HRS for some years. It was demonstrated that a temporary lumbar sympathectomy was able to improve renal function in patients with HRS (Solis-Herruzo et al 1987). The hepatorenal reflex is elicited by an increase in hepatic pressure and/or certain amino acids in portal venous blood. It is transmitted by serotonin in the liver and possibly by noradrenaline in the kidney. It leads to a marked decrease in RBF, GFR and urinary flow rate (Lang et al 1991b) (Lang et al 1991a).

1.1.5.2.2.3 Increased synthesis of vasopressin

Antidiuretic hormone or vasopressin levels are elevated due to non-osmolar stimulation,
despite the frequent presence of hyponatraemia (Epstein et al 1984) (Bichet et al 1982a). Vasopressin causes vasoconstriction through V₁ receptors and renal tubular water retention through V₂ receptors in the medullary collecting ducts. This enhances volume expansion through water retention and helps to maintain mean arterial pressure.

1.1.5.2.3 Increased synthesis of humoral and renal vasoactive mediators

In HRS there is an increased synthesis of many humoral and renal vasoactive mediators, which can decrease or increase renal blood flow.

1.1.5.2.3.1 Humoral and Vasoactive Mediators

These vasoactive mediators are listed in Table 2.

<table>
<thead>
<tr>
<th>Renal vasodilators</th>
<th>Renal vasoconstrictors</th>
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<tbody>
<tr>
<td>Prostaglandin E₂</td>
<td>Cysteiny l leukotrienes</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>Kinin</td>
<td>Adenosine</td>
</tr>
<tr>
<td>Prostacyclin</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td></td>
<td>Angiotensin II</td>
</tr>
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<td></td>
<td>Isoprostanes</td>
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<table>
<thead>
<tr>
<th>Systemic vasodilators</th>
<th>Systemic vasoconstrictors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitric oxide</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>Atrial natriuretic peptide</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td></td>
<td>Arginine vasopressin</td>
</tr>
</tbody>
</table>

Table 2. Vasoactive substances and systems that may be influenced in the regulation of renal perfusion in patients with cirrhosis and ascites.

The presence of systemic hypotension is a consistent finding in HRS (Arroyo et al 1996). The initiating events are splanchnic vasodilation and portal hypertension with portosystemic shunting. With the additional effect of impaired liver clearance, splanchnic vasodilators enter the extrasplanchnic vasculature so further reducing systemic vascular resistence (Schrier et al 1988) (Gines et al 1997). Potent, locally acting vasoconstrictors such as F₂-isoprostanes, endothelins, thromboxanes and leukotrienes may also contribute to renal vasoconstriction (Arroyo et al 1999) (Epstein 1986) (Laffi et al 1997) (Moore et al 1992) (Zipser et al 1983). The splanchnic area, however, seems to escape the effect of vasoconstrictors and marked vasodilation persists. This is because of the existence of very potent local vasodilator stimuli e.g.

In advanced liver disease renal function is maintained within normal or near normal levels, despite the over-activity of vasoconstrictor systems by increased synthesis of the renal vasodilatory prostaglandins, PGE₂ and prostacyclin (PGI₂). The development of renal hypoperfusion leading to hepatorenal syndrome occurs when activation of vasoconstrictor systems cannot be counteracted by local vasodilator factors (Arroyo et al 1999) (Laffi et al 1994) (Badalamenti et al 1993) (Gentilini and Laffi 1992).

1.1.5.2.3.2 Mediators of vasodilation

Nitric oxide. Nitric oxide (NO) causes vasorelaxation and is synthesized by three isoforms of nitric oxide synthase (NOS), namely NOS 1, NOS 2, NOS 3 (originally termed neuronal NOS, inducible NOS and endothelial NOS, respectively) (Vallance and Moncada 1991) (Laffi et al 1995). Increased synthesis NO is induced by several stimuli including shear stress, or endotoxin-related cytokine expression (Groszmann 1994) (Bomzon and Blendis 1994) (Stark and Szurszewski 1992) (Hecker et al 1993). Cirrhotic patients who develop endotoxaemia have elevated levels of plasma nitrite and nitrate, indicative of increased NO production (Guarner et al 1993). In patients with HRS, NO may play an important direct role in the pathogenesis of peripheral vasodilation and renal vasoconstriction. Indeed, the administration of NO antagonists to rats with cirrhosis and portal hypertension results in a decrease in peripheral vasodilation and improve splanchnic blood flow (Pizcueta et al 1992) (Forrest et al 1995).

NO produced by the endothelium cannot be measured directly. However, NO reacts with its chemical environment to produce different redox forms. For example, NO can give rise to nitrosion (NO⁺), nitrite (NO₂⁻), nitrate (NO₃⁻), or peroxynitrite (ONO⁰⁻) ions. NO and its metabolites can form complexes with transition metal ions and thiols to form s-nitroso-iron complexes or S-nitrosothiols (Kelm 1999) (Jourd'Heuil et al...
The biological relevance of these compounds is not clear but they may play a major role in modulation of NO effects, both locally and distal to the site of production. Thus, any upregulation of NO either directly or via nitrosothiol generation, can cause both a reduction in mean arterial pressure and fall in GFR.

**Glucagon.** Plasma glucagon levels are elevated in many cirrhotic patients and act as a vasodilator. It also causes desensitization of the mesenteric circulation to catecholamines and angiotensin II (Mathie *et al* 1996) (Pak and Lee 1994). Glucagon induces increased production of cyclic AMP, which acts synergistically with endotoxin to induce NO synthase and thus NO release by vascular smooth muscle cells (Hirokawa *et al* 1994).

**Prostacyclin.** Prostacyclin (PGI$_2$) is a systemic vasodilator. In patients with decompensated cirrhosis urinary levels are elevated. The role PGI$_2$ plays as a vasodilator is controversial. Plasma levels were found to be elevated but not significantly enough to cause vasodilation in man (Guamer *et al* 1986b) (Moore *et al* 1991). However, it was also shown that administration of non-steroidal anti-inflammatory drugs (NSAIDS) to cirrhotics, results in haemodynamic changes suggesting that PGI$_2$ may facilitate vasodilation in cirrhosis (Bruix *et al* 1985).

**Endotoxaemia and cytokines.** Endotoxin levels are elevated in patients with decompensated liver disease and more so in patients with HRS. This is believed to be due to increased bacterial dislocation from the intestine to the liver and portosystemic shunting (Wilkinson *et al* 1976) (Triger 1991) (Bourgoignie and Valle 1988) (Lumsden *et al* 1988). Endotoxaemia may cause splanchnic vasodilation which may be mediated by cytokine and NO induction (Vallance and Moncada 1991). Infusion of lipopolysaccharide (LPS) into animals causes complement activation, an accumulation of neutrophils in the liver and renal dysfunction (Levy and Wexler 1984). The renal dysfunction that occurs can be blocked by both leukotriene and thromboxane antagonists. There are elevated circulatory levels of several cytokines including TNF$\alpha$ and IL-6, and further elevated in patients with alcoholic hepatitis and HRS (Sheron *et al* 1991) (Sheron *et al* 1993). Studies in the rat have shown that the systemic vasodilation observed in the partial portal vein ligated model is blocked by anti-TNF antibodies
The kallikrein-kininogen-kinin system. Tissue kallikrein is a serine protease present in many tissue that cleaves kininogen substrate to produce the potent vasodilating peptide kinin (Clements 1989) (Wolf et al 1999). Kinin is a peptide hormone that mediates a number of biological responses including increased local blood flow (Bhoola et al 1992). By binding to endothelial bradykinin B_2 receptors, kinin stimulates release of potent vasodilators including PGI_2 and NO (Hornig and Drexler 1997). Kinin has been found to cause vasodilation of glomerular vessels (Arima et al 1995) (Heller et al 1997).

Adenosine. Adenosine induces both splanchnic vasodilation (Lee et al 1992) and renal vasoconstrictor (Rossi et al 1988). There is little data on adenosine levels in liver disease.

1.1.5.2.3.3 Increased renal production of vasodilatory prostanoids

Renal prostaglandins play an important role in the preservation of renal function in many situations such as dehydration, congestive cardiac failure, shock or decompensated liver disease with elevated plasma levels of renin, angiotensin, noradrenaline or vasopressin. In decompensated liver disease urinary excretion PGE_2 and PGI_2 metabolites (i.e. 6-oxo-PGF_1α) are increased (Moore et al 1991) (Epstein 1986) (Laffi et al 1986) (Zipser et al 1979) (Rimola et al 1986) (Guarnier et al 1986a). The mechanism of increased PGI_2 synthesis is unknown. It is likely to be secondary to the elevated levels of the vasoconstrictors, many of which have been shown to cause prostaglandin formation in vivo and in vitro. Administration of cyclooxygenase inhibitors, nonsteroidal anti-inflammatory drugs (NSAIDs), to patients with ascites causes renal failure. This renal failure is reversed when NSAID treatment stops (Zipser et al 1979) (Boyer et al 1979). Some studies have suggested that HRS is caused by a deficiency of renal PGE_2 and PGI_2 (Zipser et al 1979). This is because urinary excretion of PGE_2 and 6-oxo-PGF_1α are low compared with patients with ascites but preserved renal function. Other studies have shown that in the presence of renal failure synthesis of PGI_2 is increased and urinary excretion of its metabolite is decreased. In patients with HRS, post-mortem immunohistochemical studies show PG
endoperoxide synthase is markedly decreased in medullary collecting tubules of patients with HRS, compared to patients with liver failure but normal renal function (Govindarajan et al 1987). There have been no further histochemical studies to confirm this and the mechanism is unknown.

1.1.5.2.3.4 Mediators of vasoconstriction in the renal autacoid system
Mediators of vasoconstriction in the renal autacoid system include thromboxane A\(_2\), F\(_2\)-isoprostanes, cysteinyl leukotrienes, platelet activating factor (PAF) and endothelins (Laffi et al 1996).

**Thromboxane A\(_2\) (TxA\(_2\)).** TxA\(_2\) production is increased during renal ischaemia and leads to further vasoconstriction and mesangial cell contraction (Imig et al 2000). Studies show that in both normal volunteers and groups of patients suffering from different types of liver and renal diseases including, compensated liver disease, liver disease with ascites, severe hepatitis, HRS and chronic renal failure, the ratio of the vasodilatory prostaglandins to the vasoconstricting TxA\(_2\) were similar (Moore et al 1991). Since the synthesis of renal PGI\(_2\) and TxA\(_2\) both increase to similar levels, it is not clear if TxA\(_2\) has a significant role in the renal pathogenesis in HRS. However, administration of the Tx synthase inhibitor, OKY 046 or a Tx receptor antagonist, ONO 3807 leads to a modest improvement in GFR and sodium excretion (Gentilini et al 1988) (Laffi et al 1992).

**F\(_2\)-isoprostanes.** Isoprostanes are prostaglandin-like compounds that are formed largely independently of the cyclooxygenase (COX) enzyme, by the action of free radicals on arachidonic acid (Morrow and Roberts 1996). F\(_2\)-isoprostanes are formed by lipid peroxidation. The major F\(_2\)-isoprostane formed in vivo, 8-iso-PGF\(_{2\alpha}\) is a potent renal vasoconstrictor. In HRS patients there is an increase in the synthesis of F\(_2\)-isoprostanes, indicative of increased lipid peroxidation (Morrow et al 1993). The synthesis of several mediators implicated in the pathogenesis of HRS are regulated through products of lipid peroxidation or through redox changes secondary to oxidant stress. The formation and biological activity of isoprostanes are explained in greater detail in Section 1.5.
Platelet activating factor (PAF). PAF is a potent aggregating agent and chemotactic factor for circulating white blood cells. PAF induces an increase in production of LTs and a decrease in GFR and RBF. Sodium excretion may also be decreased. In patients with HRS and in the presence of endotoxins, PAF may act as a co-factor in stimulating vasoconstriction in the renal cortex (Wang et al 1988). In patients with advanced cirrhosis with HRS, there is an increasing imbalance between these different products with a greater increase in the vasoconstricting metabolites. The release of PAF and LTs may contribute to the vasoconstriction and rapid deterioration of renal function seen in advanced cirrhosis with HRS (Gentilini and Laffi 1992).

Endothelin-1 (ET-1). Endothelin (ET) is intimately involved in both renal physiology and pathology. It has proved to be the most potent vasoconstrictor known in humans (Yanagisawa et al 1988b). Plasma ET-1 levels are increased in patients with decompensated liver disease (Moller et al 1993) (Uchihara et al 1992) and further elevated in patients with HRS (Moore et al 1992). The properties and actions of endothelin-1 are explained in greater detail in Section 1.4.
1.1.5.3 The Pathogenesis of HRS - Summary

In summary, the main pathways implicated in the development of HRS

Figure 1. Pathogenesis and possible mechanism of HRS in cirrhosis.

The peripheral arterial vasodilation theory for the development of HRS is currently the most accepted hypothesis (Schrier et al 1988). It proposes that renal hypoperfusion represents the extreme manifestation of underfilling of the arterial circulation secondary to a marked vasodilation of the splanchnic vascular bed. Arterial underfilling causes a decrease in effective arterial blood volume and results in progressive baro-receptor-mediated homeostatic activation of the endogenous vasoconstrictor systems such as the RAAS & SNS. Activation of the RAAS and SNS are early events in the development of sodium retention and ascites (Jimenez et al 1985). Increased secretion of arginine vasopressin (AVP) is a later event occurring when there is a severe impairment in circulatory function. HRS develops as an extension of the extreme progression of the decreased effective arterial blood volume and activation of vasoconstrictive mediators. SVR, systemic vascular resistance; SNS, sympathetic nervous system; RAAS, renin aldosterone angiotensin system; AVP, arginine vasopressin.
Postmortem angiography performed on the kidneys of five HRS patients disclosed a striking normalisation of the vascular abnormalities which had been observed prior to death. The peripheral vasculature filled completely, and the previously irregular vessels became smooth and regular. These findings provided evidence for the functional basis of the renal failure of HRS, operating though active renal vasoconstriction (Epstein et al 1970).

1.2 Animal Models of Liver Disease

Our understanding and treatment of liver diseases has been limited by the lack of satisfactory animal models. The animal models used for the study of liver disease can be divided into 2 main categories: 1) Surgical, including anhepatic, devascularization and bile duct ligation models and 2) hepatotoxic drug models such as carbon tetrachloride, acetaminophen (paracetamol) and galactosamine.

1.2.1 SURGICAL MODELS

1.2.1.1 Anhepatic Models
The anhepatic model is the surgical reduction of liver function by a one stage total hepatectomy (Filipponi et al 1999), with minimal trauma, minimal haemodynamic variations and no requirement for transfusion. The animals survive for a period of approximately 20 hours before developing cerebral oedema and brain death.

Although absolute liver failure results from total hepatectomy, there are many concerns about the validity of this model. The liver failure in this model is not reversible unless the animal is given a transplant. Also the liver failure differs in that there are no injured or necrotic liver cells within the circulation. The anhepatic model is thus quite different from the clinical condition in man caused by viruses or drugs where the liver is still perfused with blood and releases toxic levels of metabolites into the blood. The anhepatic model displays very little biochemical abnormality until 2 to 4 hours before death. This model is possibly suited for the study of a specific hepatic function (Mann 1921) (Freeman et al 1973) or the prelude to transplantation (Ringe et al 1988) rather
than to the study of acute/chronic liver disease.

1.2.1.2 Devascularization Models

There are three models commonly used, namely complete, graded devascularization and partial portal vein ligation. In a complete devascularization model a portacaval shunt and ligation of the hepatic artery is performed (Olafsson et al. 1995). This model is not reversible. Devascularization in the pig results in death after 6-12 hours (Hanid et al. 1979). This model has been useful in the study of intracranial pressure and cerebral edema associated with hepatic failure. In the graded devascularization models a portacaval shunt is performed but there is only a temporary hepatic occlusion. A potentially reversible graded devascularization model in the pig, has been described (De Groot et al. 1985). The partial portal vein ligation model is considered a useful model for the study of portal hypertension and the factors responsible (Fernando et al. 1998) (Garcia-Pagan et al. 1994) (Zhang et al. 2000).

1.2.1.3 Bile Duct Ligated Animals

Animals that have had ligation of their bile duct for up to 2 weeks are defined as being acute bile duct ligated (BDL). Those that have been ligated for longer than two weeks are defined as chronic models. Ligation of the common bile duct in rodents, dogs and primates provides a reproducible model for the study of liver cholestasis. This model is considered to be potentially useful for the study of the two common clinical conditions in humans, obstructive jaundice and Laennec's cirrhosis. After a period of 8-10 days in rats and 2-6 weeks in dogs following BDL the characteristic histological and biochemical changes of obstructive jaundice appear (Better and Massry 1972).

Obstructive jaundice is defined as retention of bile and bile components following extra- or intra-hepatic bile duct obstruction. In man, acute obstructive jaundice is associated with hypovolemia whereas chronic biliary obstruction with impaired liver function results in renal fluid retention and sodium salt retention. Investigations to study the effects of jaundice on renal handling of fluid and sodium have been performed extensively in dogs and rats after acute (short-term) or chronic (long-term) BDL. The acute BDL dog i.e. bile duct obstruction for 4 hours is analogous to acute biliary obstruction in man. There is an increase in RBF and GFR. There is also an increase in
fractional urinary sodium and water excretion, which caused a small but insignificant reduction in plasma volume (Levy and Finestone 1983).

The major disadvantage of the BDL model is that there is great species variability in response to the procedure. The degree of jaundice varies greatly between the species. This could be due to the difficulty of the interpretation of the published data obtained from BDL animal studies. The times at which the experiments were performed on the animals did not always correspond to the peaks of jaundice and of hepatocellular damage which can themselves vary from species to species. There are both quantitative and qualitative differences within and between species and the jaundice obtained following BDL. Also the duration and severity of the jaundice and hepatocellular damage is markedly influenced by whether the bile duct was ligated and divided between the ligatures, or ligated and resected. Furthermore many of the experiments were performed under anesthesia, which in itself can affect the function of the liver, kidney, heart and other organs (Fulop and Brazeau 1970) (Bloom et al 1976) (Gliedman et al 1970) (Mullane and Gliedman 1970) (Better and Massry 1972) (Hishida et al 1980) (Better et al 1980).

The haemodynamic changes of renal blood flow (RBF) and glomerular filtration rate (GFR) after BDL are also variable among the species. In dogs within 2 weeks of BDL, injection of saline was shown to have a blunted natriuretic response (Melman and Massry 1977). Increased sodium retention with ascites formation was observed in BDL rats of 10-14 days with no haemodynamic changes (Bank and Aynedjian 1975). Micropuncture on the anaesthetized rats showed an increase in sodium reabsorption in the superficial cortical nephrons. Total RBF was normal but the superficial single nephron blood flow was reduced. This suggests that the intra-renal blood flow distribution is greater in the deep cortical nephrons with increased medullary blood flow following BDL. The normal superficial single nephron GFR suggests an increased filtration fraction resulting from efferent arteriolar vasoconstriction. This by itself could enhance proximal tubular reabsorption of sodium and fluid by altering peritubular physical factors, i.e. decreased peritubular hydrostatic pressure and increased peritubular oncotic pressure.

A study on BDL rats of 4-7 days corresponding to the period of initial increased sodium
reabsorption and ascites formation has been reported (Yarger et al 1976). Curiously a decrease in plasma fluid volume with a small rise in haematocrit was observed despite the increase in sodium reabsorption. There was also a decrease in renal plasma flow (RPF) and GFR. The redistribution of intra-renal blood flow towards the renal medulla was associated with a reduction in blood flow in the nephrons. In BDL rats of 8-10 days duration blood pressure, cardiac output and systemic vascular resistance (SVR) were all normal and there were no changes in GFR or proximal tubular sodium reabsorption (Better et al 1980). However, a rise in renal vascular resistance (RVR) and a decrease in total RBF was observed. The plasma volume was increased but total blood volume was unaltered. Conscious rats 6 days after BDL showed a decrease in GFR and an increase in sodium excretion (Heidenreich et al 1987). However, they also found that fractional sodium and excretion rates were increased. This suggests that there is a decrease in proximal tubular reabsorption even though there is a contraction of the extracellular fluid volume (ECFV) that normally results in enhanced proximal tubular absorption. No sodium retention was found in rabbits 10 days after BDL (Hishida et al 1982). Comparison between studies are complicated and difficult since differences exist in the duration for which the animal is bile duct ligated, the nature of preparations and the different species examined.

Potential disadvantages with surgical models are their inability to recreate the inflammatory milieu that exists in acute liver failure and their reliance on surgical expertise.

1.2.2 HEPATOTOXIC DRUG MODELS

1.2.2.1 Carbon tetrachloride

Carbon tetrachloride (CCl₄) is a toxin that is administered by the intraarterial, intraportal, oral, intragastric or respiratory routes (van Leenhoff et al 1974) (Mourelle et al 1988). The reproducibility of this model has been found to be difficult but can be improved when phenobarbital is administered prior to the CCl₄ (van Leenhoff et al 1974). The toxic effect of CCl₄ is due to its metabolism into the trichloromethyl radical which initiates lipid peroxidation, protein and carbohydrate modification and DNA damage (Mourelle et al 1988). The resulting hepatocyte injury occurs principally around the central vein and does not resemble the histological features of human
massive liver cell necrosis. CCl₄ is not a specific hepatotoxin and damages other organs including the lungs and kidneys (McLean et al 1969) (Lopez-Novoa 1998) (Morrow et al 1992c).

1.2.2.2 Acetaminophen (Paracetamol)
An overdose of paracetamol may lead to acute liver failure in man. It has been difficult to reproduce a paracetamol animal model, since there is inconsistent toxicity from animal to animal (Miller et al 1976) (Henne-Bruns et al 1988). One of the reasons why this may be is that before the toxic damage of acetaminophen can occur, cellular glutathione (GSH) levels must be depleted to approximately 20% of their original levels (Mitchell et al 1973) (Jollow et al 1973). A reproducible model was produced when three doses of acetaminophen in DMSO were injected subcutaneously at 0, 9, and 24 hours in beagle dogs (Francavilla et al 1989).

1.2.2.3 Galactosamine
The galn-ALF rat model is a well-established model in which the pathogenesis of liver disease has been studied. It demonstrates a good dose-effect relationship within a wide range of galactosamine concentrations in a homogenous group of animals. This allows a high degree of reproducibility of both pathological and biochemical lesions. Most species respond to galactosamine administration in a very similar manner, although there is variation in susceptibility to a given dose (Keppler et al 1968) (Decker et al 1973). Galactosamine affects only the liver due to the action of enzymes found only in hepatocytes. The metabolism through which galactosamine causes liver injury is described in greater detail in Section 1.3.

1.2.2.4 Other Drug-Induced Models
Dimethylnitrosamine has been used in a number of studies (Sutherland et al 1977) but as it poses a clear hazard to staff its use can only be justified under strictly controlled circumstances. Massive liver necrosis has been produced in rats using anaesthetic agents, e.g. the halothane derivative, fluoroxyne (Harrison and Smith 1973), thioacetamide (Maddison et al 1987), the injection of heat-killed gram-positive anaerobes with a subsequent injection of lipopolysaccharide (Mizoguchi et al 1987) and the combination of hyperthermia and drugs (Miyazaki et al 1983). However, the use of these methods in large animals is limited because of the large number of animals needed
in studies to obtain conclusive data. Murine viral hepatitis has been used to test various forms of antiviral therapy (Farivar et al 1976), but the small size of mice poses problems in sequential monitoring of the response.

1.2.2.5 Genetic model
An inbred strain of Long-Evans rats (LEC rat) is another model used for the study of liver disease. This model was found to share similarities with patients who suffer from Wilson's disease (WD) (Miyoshi et al 1997). It has a mutation in the gene homologous to the human WD gene and shows many of the features of the disease. These include extensive copper deposition in the liver and kidney resulting in the development of renal abnormalities and decreased serum levels of ceruloplasmin (Bull et al 1993). The renal failure in this model may be due to the direct toxicity of the copper and not as a result of liver failure.

1.2.3 MAIN CRITERIA FOR ANIMAL MODELS OF LIVER DISEASE

An animal model of fulminant hepatic failure has six requirements. These are reversibility, reproducibility, death from liver failure, a therapeutic window, a large animal model and a minimum hazard to personnel (Terblanche et al 1975).

The first four requirements are the most important but the latter two are important if the model is to be used widely.

1. **Reversibility.** The hepatic failure produced in the model should be potentially reversible so that the animals can respond and survive if a suitable treatment were to be used.

2. **Reproducibility.** If the model is not treated there should be at least 80% mortality.

3. **Death from liver failure.** The liver injury should produce liver failure and death in the time period similar to that occurring clinically.

4. **Therapeutic window.** The time taken between administering the insult and the death of the animal should allow enough time for the treatment to be administered and the effects to be assessed. Also the animal should remain fit enough for the treatment to be administered.

5. **Large animal models.** A large animal model is preferred as the effects may be subsequently applied to man more directly. However, the rat and rabbit have been and...
are used extensively.

5. Minimal hazard to personnel. Any methods used should not present a risk to the laboratory personnel involved.

It has also been suggested that when trying to recreate the conditions seen in humans, it is important to choose a species with similar metabolic and physiological properties (Newsome et al. 2000). The response to surgery or toxic insult should be similar. It is of importance also that the development of animal models should comply with strict ethical and animal welfare standards.

There are two clear benefits of developing a model of HRS. First, it would allow for a greater understanding of the pathophysiological state, and second, it would permit the development and refinement of treatments for HRS.

1.3 The rat with galactosamine-induced acute liver failure

It was observed, by accident that rats which had received an intraperitoneal injection of D-galactosamine (galn), at a dose of 1.1g/kg suffered a rapid loss of liver glycogen, increased levels of liver specific enzymes in the blood and a reduction of plasma proteins (Keppler et al. 1968). The lesions observed in the livers of rats with galactosamine-induced liver failure (galn-ALF) resembled those observed in viral hepatitis. Galactosamine is an amino sugar. In animals it is found as a constituent of many heteroglycans although galactosamine is not detected as a free sugar in untreated animals. It is readily taken up by the liver (Maley et al. 1968) and metabolized.

1.3.1 METABOLISM OF GALACTOSAMINE

The metabolism of galactosamine in rat liver follows in its first steps the pathway of D-galactose and enters the pathways of D-glucosamine (Figure 2) (Keppler et al. 1970) (Maley et al. 1968) (Maley and Maley 1959) (Walker and Khan 1968). Formation of galactosamine-1-phosphate is catalysed by galactokinase (Walker and Khan 1968). At about one hour after administration of galactosamine, maximum levels of galactosamine-1-phosphate are reached. The formation of UDP-galactosamine results
from nonspecificity of the enzyme galactose-1-phosphate: UDP-glucose uridylyltransferase. The low affinity of this enzyme for the nonphysiological substrate and the limited supply of UDP-glucose favour the accumulation of galactosamine-1-phosphate. UDP-galactosamine is converted by UDP-galactose 4'-epimerase to UDP-glucosamine (Maley and Maley 1959). The latter compound does not serve as a uridylate donor in the uridylyltransferase reaction as does UDP-glucose which is regenerated in galactose metabolism. Thus, UDP-hexosamine formation functions as a trap mechanism for uridylate (Keppler and Decker 1969) (Keppler et al 1969) (Keppler et al 1970). UDP-N-acetylglucosamine and UDP-N-acetyl-galactosamine can be both derived from galactosamine (Maley and Maley 1959) and also accumulate after galactosamine administration (Keppler et al 1970).
Figure 2. The primary effects of D-galactosamine (galN) on the liver, and its dependence on uridine phosphate biosynthesis.

The primary effects of galactosamine in the liver lead to the formation and accumulation of UDP-derivatives of galactosamine, resulting in depletion of hepatic UTP, UDP-glucose and UDP-galactose. This causes the formation of primary biochemical lesions. Subsequent depression of uracil-nucleotide dependent biosynthesis of the macromolecules, ribonucleic acids, proteins, glycoproteins, glycolipids and glycogen produce secondary lesions causing organelle injury and finally necrosis of the liver cells (Decker and Keppler 1972).

Galactosamine hepatotoxicity results in a syndrome similar to fulminant hepatic failure in its morphological and functional features (Chojkier and Fierer 1985) (Keppler et al 1968). The liver injury caused is intimately connected with alterations in the structure and function of the plasma membrane due to impaired membrane glycoprotein synthesis (Bauer et al 1974) (Chojkier and Fierer 1985) (Bachmann et al 1977). The hepatic extracellular matrix is also altered. Fibronectin, collagen types I, III and IV and laminin increase, especially in areas of necrosis and along sinusoids. Extracellular matrix immunoreactivity reaches a maximum at 36 to 48 hours and decreases thereafter to pre-injury levels 3 weeks after galactosamine injection (Jonker et al 1992). A role for complement has been recently suggested when it was shown that, in the galactosamine-
injured rat, activated complement is fixed to the liver plasma membrane (Liehr et al 1978). There is also increased lipid peroxidation resulting in the inhibition of oxidative phosphorylation in the mitochondria (Padma and Setty 1997). Leukotriene B₄ synthesis is increased in the liver (Keppler et al 1985). Biochemical and structural changes in membrane lipid composition and enzyme activities have also been reported. The haemodynamic changes that follow galactosamine-induced liver failure include changes in intestinal blood flow and permeability (Kasravi et al 1996), an increase in cardiac output and a reduction in systemic vascular resistance (Makin et al 1997). Some of these animals also develop cerebral edema (Dixit and Chang 1987). Recently, it has been reported that galactosamine-induced liver injury also causes a reduction in renal blood flow (Javle et al 1998).

1.4 Endothelins

1.4.1 INTRODUCTION

The endothelin (ET) system is intimately involved in both renal physiology and pathology. It has proved to be the most potent vasoconstrictor known in humans (Yanagisawa et al 1988b). Three endothelin isoforms exist, ET-1, ET-2 and ET-3. Each one consists of a 21 amino acid peptide. ET-2 differs from ET-1 by two amino acids, and ET-3 by six (Yanagisawa et al 1988b) (Inoue et al 1989) (Yanagisawa et al 1988a) (Kimura et al 1989).

ET-1 is synthesized by the vascular endothelial cells of arteries and veins. Vascular endothelial cells do not produce ET-2 or ET-3. ET-2 appears to be produced almost exclusively by the intestine (Saida et al 1989). ET-1 is synthesized not only in the endothelium but also in the kidney, brain, lung and intestine. Both ET-1 and ET-3 are made in various renal cells including the epithelial cells of medullary and cortical collecting ducts but ET-1 is produced in a much greater quantity (Karet and Davenport 1996) (Kohan 1991).
Two receptors that specifically bind ETs have been isolated and cloned, ET\textsubscript{A} and ET\textsubscript{B}. ET\textsubscript{A} has a higher affinity for ET-1 and ET-2 and less for ET-3 (Arai \textit{et al} 1990) (Hosoda \textit{et al} 1992). ET\textsubscript{B} binds to all 3 isopeptides with near equal affinity.

1.4.2 ENDOTHELINS IN HRS

Patients with cirrhosis have increased levels of ET-1 in their plasma (Uemasu \textit{et al} 1992). Cirrhotic patients also showed a significantly elevated secretion of ET-1 and ET-3 by the liver and a corresponding renal extraction of ET-1 and ET-3 by the kidney (Gerbes \textit{et al} 1995). In patients with HRS, there is a further elevation in circulating plasma ET-1 concentrations (Moore \textit{et al} 1992) (Moller \textit{et al} 1993) (Schrader \textit{et al} 1990). It was also found that the level of ET-1 directly correlated with creatinine clearance (Moller \textit{et al} 1993). Normal human volunteers infused with levels of ET-1 similar to those found in HRS patients resulted in a significant decrease in GFR (Sorensen \textit{et al} 1994). Increased lipid peroxidation is known to occur in HRS (Morrow \textit{et al} 1993) and certain products of lipid peroxidation i.e. oxidised low density lipoprotein (ox-LDL) have been shown to induce ET-1 synthesis in vitro (Boulanger \textit{et al} 1992). It is not known, however whether lipid peroxidation and its subsequent metabolite production causes the increased production of ET-1. ET-1 increases the production of ANP, activates RAAS and partially blocks the water retaining effect of ADH (Vane \textit{et al} 1990).

1.4.3 ET RECEPTORS

Autoradiography shows that both ET\textsubscript{A} and ET\textsubscript{B} receptors are present in renal arteries and veins and on tubular epithelial cells (Karet \textit{et al} 1993) (Davenport \textit{et al} 1994b). ET\textsubscript{A} receptors are found on blood vessels throughout the kidney, particularly arcuate arteries and veins at the corticomedullary junction. ET\textsubscript{A} receptors found in vascular smooth muscle cells mediate vasoconstriction and cell proliferation. ET\textsubscript{B} receptors are concentrated in the medulla on nonvascular structures such as tubules and collecting ducts (Chow \textit{et al} 1995) (Terada \textit{et al} 1992). ET\textsubscript{B} receptors on endothelial cells mediate vasodilation via nitric oxide (Hirata \textit{et al} 1993). ET\textsubscript{B} receptors in rat mesangial cells initiate a positive autocrine loop by which ET-1 regulates expression of its own gene (Iwasaki \textit{et al} 1995).
Tissues that produce ET-1 express specific binding sites, suggesting a local rather than circulating hormone-type regulation. Specific binding sites have been identified in several fetal and adult organs, including lung, heart, brain and kidney (Benigni 1995). In rat cardiac atrium ET\textsubscript{A} is the major receptor type while in brain the ET\textsubscript{B} receptor seems predominant. The relative abundance of A and B receptors in the kidney depends on species; the ET\textsubscript{A}/ET\textsubscript{B} ratio approaches unity in rats but in man ET\textsubscript{B} is more predominant (Benigni 1995).

1.4.4 ET RECEPTOR ANTAGONISTS

ET-1 is released by serial cleavage of two precursor molecules, preproET and big ET. The enzymes responsible have not yet been fully characterized. Therapeutic attempts to block the deleterious effect of ET-1 are therefore at present directed towards receptor antagonism. A series of peptide receptor antagonists selective for ET\textsubscript{A} (i.e. BQ 123) or ET\textsubscript{B} (BQ 788) or non-selective for ET\textsubscript{A} and ET\textsubscript{B} (i.e. PD 145065) were developed (Ishikawa \textit{et al} 1994). Rat models of acute renal failure induced by ischaemia/reperfusion injury showed an improvement in renal function with the use of receptor antagonists such as BQ-123 (Banyu) and SB 209670 (SmithKline Beecham) (Mino \textit{et al} 1992) (Kusumoto \textit{et al} 1994) (Clozel \textit{et al} 1993) (Gellai \textit{et al} 1994) (Gellai \textit{et al} 1995).

ET receptor antagonists under clinical development include BQ123, SB 209670 and Bosentan (Table 4). In healthy volunteers, infusion of BQ-123 (an ET\textsubscript{A} receptor antagonist) into the brachial artery caused forearm vasodilation (Haynes and Webb 1994) and infusion of TAK-044 (an ET\textsubscript{A}/ET\textsubscript{B} receptor antagonist) caused sustained vasodilation and lowered peripheral vascular resistance and blood pressure (Haynes \textit{et al} 1996) suggesting a fundamental physiological role for endogenously generated ET-1. Patients with chronic heart failure treated with ACE inhibitors increased forearm blood flow after local infusion of BQ123 (Love \textit{et al} 1996). Bosentan administered by intravenous infusion at 100mg followed 1 hour later by 200mg lowered pulmonary artery pressure and vascular resistance in patients with chronic heart failure and increased cardiac index (Kioński \textit{et al} 1995). Hypertensive patients given Bosentan showed a lowering in blood pressure with no changes in heart rate and without
activation of SNS or RAAS (Krum et al 1998). Bosentan also prevented cyclosporin-induced renal vasoconstriction without effects on the systemic blood pressure (Binet et al 2000).

<table>
<thead>
<tr>
<th>Compound (Company)</th>
<th>Type of Antagonist</th>
<th>Clinical Studies</th>
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<tbody>
<tr>
<td>BQ 123 (Banyu)</td>
<td>ET&lt;sub&gt;A&lt;/sub&gt;</td>
<td>In chronic heart failure, increased forearm blood flow (Love et al 1996)</td>
</tr>
</tbody>
</table>
| Bosentan (Roche)  | ET<sub>A</sub>/ET<sub>B</sub> | In chronic heart failure, lowered pulmonary artery pressure & vascular resistance & increased cardiac index (Kiowski et al 1995)  
In acute cyclosporin nephrotoxicity, decreased renal vasoconstriction (Binet et al 2000).  
In essential hypertension, lowered blood pressure (Krum et al 1998). |
| TAK-044 (Takeda)  | ET<sub>A</sub>/ET<sub>B</sub> | Decreased vascular resistance and blood pressure in healthy volunteers (Haynes and Webb 1994). |

Table 3. Summary of ET receptor antagonists under clinical development.

1.4.5 ENDOTHELINS AND THE KIDNEY

Endothelins are produced in endothelial, mesangial, glomerular epithelial and tubular epithelial cells.

1.4.5.1 Endothelial cells

Endothelial cells produce and secrete ET-1 but not ET-3 (Shiba et al 1992). ET-1 is released predominantly into the interstitium, where it can then interact with vascular smooth muscle, mesangial, epithelial and also endothelial cells.

The release of ET-1 is stimulated by vasoactive agents such as bradykinin, angiotensin II, vasopressin (AVP) and adrenaline (Yanagisawa et al 1988b) (Marsden et al 1991) (Imai et al 1992). ET-1 release is also stimulated by inflammatory agents such as IL-1, TGFβ, TNFα (Kurihara et al 1989) (Lamas et al 1992) (Yoshizumi et al 1990), thrombin, ET-1, ET-3, the calcium ionophore A23187, erythropoietin and phorbol ester (Yanagisawa et al 1988b) (Carlini et al 1993) (Yokokawa et al 1991) (Saijonmaa et al
ET-1 production by endothelial cells was found to be inhibited by oxidized low density lipoprotein, atrial natriuretic peptides and nitrates (Jougasaki et al 1992) (Hu et al 1992) (Kohno et al 1991) (Kohno et al 1992b) (Boulanger and Luscher 1991). Shear stress can either stimulate (Yanagisawa et al 1988b) or inhibit (Sharefkin et al 1991) ET-1 production from endothelial cells.

1.4.5.2 Mesangial cells
Cultured mesangial cells synthesise and secrete ET-1 (Kohan 1992). The factors that stimulate mesangial cell ET-1 production include angiotensin II, thrombin, arginine vasopressin (AVP), platelet derived growth factor (PDGF), TNFα, TGFβ, TxA2, and phorbol esters (Kohan 1992) (Kohno et al 1992a) (Fukunaga et al 1991a) (Sakamoto et al 1992) (Zoja et al 1991). ET-1 production in mesangial cells is thought to be stimulated by activation of protein kinase C and inhibited by activation of protein kinase A or cyclic 3',5'-guanosine monophosphate (cGMP).

1.4.5.3 Glomerular Epithelial Cells
Rat glomerular epithelial cells synthesize and release both ET-1 and ET-3 (Kasinath et al 1992).

1.4.5.4 Tubular Epithelial Cells
Studies conducted on renal epithelial cell lines (Kosaka et al 1989) (Ohta et al 1990), cultured tubule cells (Kohan and Fiedorek, Jr. 1991) (Kohan 1991) and micro-dissected tubules (Kohno et al 1991) (Ujiie et al 1992) (Uchida et al 1992) have shown that the collecting duct, and in particular the inner medullary collecting duct (IMCD) is the major site of ET-1 production in the nephron (Kitamura et al 1989). Factors regulating ET synthesis in the collecting duct are not well characterised. TGFβ may increase ET-1 synthesis (Ujiie et al 1992) and hypertonicity was found to inhibit the synthesis and release of ET-1 (Kohan and Padilla 1993) in cultured IMCD cells.

1.4.6 SIGNAL TRANSDUCTION PATHWAYS AND ENDOTHELIN
There is now accumulating evidence to suggest that ET-1 mediates its effect in different nephron segments, as well as the renal cells, via a multiplicity of intracellular signaling
pathways. ET-1 activates phosphotidylinositol-specific phospholipase C (PI-PLC) in a variety of renal cells including mesangial cells (MC) (Simonson et al 1989), cortical collecting duct cells (CCD) (Korbmacher et al 1993), inner medullary collecting duct cells (IMCD) (Cassals et al 1990) and renal medullary interstitial cells (RMIC) (Wilkes et al 1991a). PI-PLC, is thought to be linked to both ET_A and ET_B receptors (Chow et al 1995) (Terada et al 1992).

ET stimulation of eicosanoid production, especially prostaglandin E_2 (PGE_2) has been reported in many renal cell types including MC (Fukunaga et al 1991b) (Uchida and Ballermann 1992), IMCD (Cassals et al 1990) (Fukunaga et al 1991b) and RMIC (Barnett et al 1994). ET activates PLA_2 in a [Ca^{2+}] dependent manner suggesting that cytosolic PLA_2 (cPLA_2) is activated (Clark et al 1991) (Sharp et al 1991). The mechanism in which cPLA_2 is coupled to the ET receptor has yet to be determined.

ET-1 also activates phosphatidylcholine phospholipase D (PC-PLD) and phosphatidylcholine phospholipase C (PC-PLC) in both MC (Baldi et al 1994) and RMIC (Friedlaender et al 1993). PC-PLD activation by ET-1 in RMIC appears to be under the dual regulation of PKC and [Ca^{2+}], (Friedlaender et al 1993). It is important to note that ET-1 activates the hydrolysis of PC by PC-PLC to yield phosphorylcholine and diacylglycerol (DAG), important mediators in intracellular signalling (Exton 1990).

In intact renal cells ET-1 stimulates tyrosine phosphorylation of proteins (Force et al 1991). The mechanism whereby the ligand-activated ET-1 receptor enhances tyrosine phosphorylation is at present not well understood. Both protein kinase C-dependant and independent pathways have been identified. Also ET-1 enhances phosphorylation of at least four protein bands in IMCD cells including a 145kDa band corresponding to PLC_\gamma (Hart et al 1992) and in MDCK cells a 60kDa band corresponding to pp60^{c-src} (Mirza et al 1994). In mesangial cells ET stimulates mitogen activated protein kinase (MAP kinase) (Wang et al 1994). Both p42 and p44 MAP kinases were stimulated with similar dose responses, but dissimilar kinetics. The effect appeared to be mediated via the ET_A receptor.
Figure 4. Putative Effects of Endothelin-1 (ET-1) binding to receptors present on either vascular smooth muscle or endothelial cells.

Vasoconstriction induced by ET-1 involves activation of endothelin receptors on vascular smooth muscle. At low concentrations of ET-1 both $ET_a$ and $ET_b$ receptors contribute, while at higher concentrations of ET-1, $ET_a$ receptors appear to be more dominant. The vasodilation induced by endothelins is mediated by $ET_b$ receptors on endothelial cells linked to the formation of NO and PGI$_2$. L-arg, L-arginine; NOS, nitric oxide synthase; COX, cyclooxygenase; AA, arachidonic acid; NO, nitric oxide; PGI$_2$, prostaglandin I$_2$; ECE, endothelin converting enzyme; cAMP, cyclic 3’,5’-adenosine monophosphate; cGMP, cyclic 3’,5’-guanosine monophosphate.
1.4.7 PHYSIOLOGICAL ACTIONS OF ENDOTHELIN IN THE KIDNEY

In the kidney, ET-1 is involved in the control of renal blood flow, reabsorption of water and sodium, and acid-base balance. The renal vasculature is up to ten times more sensitive to the vasoconstrictive effects of ET-1 as compared to other vascular beds (Pernow et al 1989) (Madeddu et al 1989) (Cornet et al 1990).

Low doses of ET-1 administered intravenously produce a transient vasodilation due to stimulation of NO, PGI$_2$, and PGE$_2$ release followed by long lasting vasoconstriction (Figure 4) (Harris et al 1991) (Claria et al 1991) (Tsuchiya et al 1990) (Yokokawa et al 1989) (Katoh et al 1990) (Denton and Anderson 1990). However, high concentrations of ET induces vasoconstriction in the kidney, which consequently increases renal vascular resistance and reduces the renal blood flow and glomerular filtration rate (GFR) (Cairns et al 1989) (Badr et al 1989) (Firth et al 1988) (Lopez-Farre et al 1989) (Miller et al 1989). The above reports suggest that ET-1 infused over a range of 0.1 to 10pmol/min into the renal artery resulted in a dose dependent decrease in GFR and an increase in RVR. However at lower doses of 0.1 to 1pmol/min there maybe some renal vasodilation.

Infusion of ET-1 into the renal artery of anaesthetized rabbits (Evans et al 1998) results in a decrease in renal blood flow, cortical perfusion and glomerular filtration rate and also a reduction in urinary flow, sodium excretion, fractional sodium and lithium excretion from the proximal tubule (Miller et al 1989). These findings suggest that ET-1 promotes the reabsorption of fluid and sodium in the proximal tubule. ET-1 may modulate the GFR through stimulation of mesangial cell contraction, which decreases the surface area of the glomerular capillary bed and lowers the glomerular ultrafiltration coefficient (K$_f$) (King et al 1989) (Badr et al 1989) (Orita et al 1989). However, in the medulla, the activation of ET$_B$ receptors causes natriuresis and diuresis. When big ET-1 is injected into the femoral artery of rats, it causes natriuresis and diuresis. This effect is not blocked by the ET$_A$ receptor-selective antagonist BQ-123, suggesting that ET-1 is acting via activation of ET$_B$ receptors at the level of the medullary collecting duct. The mechanism of diuresis is linked to natriuresis and to an ET-mediated inhibition of vasopressin's action in the inner medullary collecting epithelium. ET-1 lowers water
permeability six-fold in vasopressin-stimulated cells (Edwards et al 1993). In vivo, this would reduce water reuptake in the duct and increase urinary water loss.

1.4.8 THE ROLE OF ENDOTHELIN IN KIDNEY PATHOLOGY

The endothelin system has been implicated in several forms of renal disease. When human ET-1 is over-expressed in mice it leads to interstitial fibrosis, glomerulosclerosis and declining renal function (Hocher et al 1997). In the remnant kidney rat, the concentration of urinary ET-1 excreted directly correlates with the degree of chronic renal failure (Orisio et al 1993). In partially nephrectomized rats, a model of progressive renal disease, infusion of the ETA receptor antagonist FR 139317, one week after the nephrectomy decreases proteinuria and improves survival (Benigni et al 1993). In the isolated perfused rat kidney, the ETA-selective antagonist BQ-123 improves glomerular filtration rate and net tubular reabsorption, two indices of renal function (Chan et al 1994). BQ-123 also improved the survival of rats with induced ischemic acute renal failure, possibly by maintaining plasma potassium levels (Gellai et al 1994). In rats with acute renal failure treatment with the ETA antagonist PD156707, resulted in a decrease in the infiltration of monocytes and macrophages and also a decrease in matrix accumulation as there were fewer myofibroblasts present (Forbes et al 1999). Induction with glycerol of rhabdomyolysis in rats resulted in a 22% increase in plasma ET-1 concentration. When treated, Bosentan prevented the decrease in creatinine clearance, the increase in proteinuria and ATN produced by the glycerol (Karam et al 1995).

The vasoconstrictor action of ET is modified by several factors including, atrial natriuretic peptide, eicosanoids, platelet-activating factor, the renin-angiotensin system, nitric oxide and calcium channel antagonists. ET-1 leads to increased plasma concentrations of atrial natriuretic peptide and aldosterone (Miller et al 1989). ANP acts in a negative feed back system and ameliorates the vasoconstrictive effects of ET-1 (Perico et al 1990) (Suzuki et al 1991). Prostanoids (PGE2 and PGl2) also reduce the vasoconstriction effect of ET-1 (Perico et al 1990) (Trybulec et al 1991) (Miura et al 1991) (Cao and Banks 1990b,Chou et al 1990) while PAF may play a part in eliciting the vasoconstriction (Lopez-Farre et al 1991).
ET-1 attenuates renin release from isolated glomeruli, juxtaglomerular cells and kidney slices \textit{in vitro} (Matsumura \textit{et al} 1989) (Rakugi \textit{et al} 1988) (Takagi \textit{et al} 1988). The \textit{in vitro} effect on renin release may be overshadowed by the indirect \textit{in vivo} effects of ET, decreasing renal blood flow and the GFR and increasing blood pressure, peripheral resistance, and plasma renin activity. Inhibition of angiotensin II-converting enzyme resulted in the reduction of ET-1-induced vasoconstriction (Banks 1990) (Madeddu \textit{et al} 1991) (Cao and Banks 1990a). This effect may be associated with an interaction between ET-1 and the kallikrein/kinin system (Madeddu \textit{et al} 1991).

Nitric oxide may be an important modifier of the haemodynamic effects of ET-1. When nitric oxide production is inhibited by N\textsuperscript{\textalpha}-monomethyl-l-arginine monoacetate, ET-1 and ET-3 do not cause renal vasodilation and the ensuing vasoconstriction is more severe (Yamashita \textit{et al} 1991) (Madeddu \textit{et al} 1991) (Hirata \textit{et al} 1991). There is conflicting data as to whether calcium channels are involved in mediating the vasoconstrictive effect of ET-1. The calcium channel blockers nicardipine ameliorates the vasoconstrictive effect of ET-1 (Katoh \textit{et al} 1990) but verapamil and magnesium had no effect (Cao and Banks 1990c). In the split hydronephrotic rat kidney when nifedipine is added the ET-1 induced afferent arteriole constriction is blocked suggesting ET-1 acts though the Ca channels (Loutzenhiser \textit{et al} 1990). Nitrendipine had no affect on the same vessel in the same type of preparation (Fretschner \textit{et al} 1991).

### 1.5 Oxidative stress

#### 1.5.1 INTRODUCTION

In recent years, a substantial body of evidence has developed supporting a key role for free radicals in many fundamental cellular reactions, suggesting that oxidative stress might be important in the pathophysiology of common diseases including atherosclerosis, chronic renal failure, and diabetes mellitus (Knight 1998). Oxidative stress is the imbalance between free radical generation and free radical scavenging reactions. Free radicals are molecules with an unpaired electron (Halliwell and Gutteridge 2000). Many radicals are highly reactive and can either donate an electron to or extract an electron from other molecules and behave as reductants or
oxidants. This high reactivity results in the majority of radicals having a very short half-life (10^{-6} seconds or less) in biological systems (Halliwell and Gutteridge 2000).

1.5.2 SOURCES OF FREE RADICAL PRODUCTION

Free radical formation in the body occurs by several mechanisms involving both endogenous and environmental factors (Table 4).

<table>
<thead>
<tr>
<th>Sources of free radical production</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endogenous sources</strong></td>
</tr>
<tr>
<td>Mitochondrial leakage (Becker <em>et al</em> 1999)</td>
</tr>
<tr>
<td>Respiratory/oxidative burst—the host defence system (Curnutte and Babior 1987)</td>
</tr>
<tr>
<td>Enzyme reactions (Chance <em>et al</em> 1979)</td>
</tr>
<tr>
<td><strong>Environmental sources</strong></td>
</tr>
<tr>
<td>Cigarette smoke (Pourcelot <em>et al</em> 1999)</td>
</tr>
<tr>
<td>Pollutants including ozone and nitrogen dioxide (Cross <em>et al</em> 1994)</td>
</tr>
<tr>
<td>(Kelly <em>et al</em> 1995)</td>
</tr>
<tr>
<td>(Kelly and Tetley 1997)</td>
</tr>
<tr>
<td>UV light (McCaughan, Jr. 1999)</td>
</tr>
<tr>
<td>Xenobiotics (Kapiotis <em>et al</em> 1997)</td>
</tr>
</tbody>
</table>

Table 4. Examples of endogenous and environmental sources of free radical production.

1.5.3 FORMATION OF REACTIVE OXYGEN SPECIES

The most important free radicals in biological systems are derived from oxygen, and include e.g. superoxide (O_2^•-) and the hydroxyl radical (OH•). The hydroxyl radical (OH•) is the most reactive and damaging of the free radicals and is probably the final mediator of most free radical induced tissue damage (Lloyd *et al* 1997). Hydrogen peroxide and O_2^•- are believed to exert most of their pathological effects by generation of hydroxyl radicals (Table 5). The OH• reacts with extremely high rate constants with almost every type of molecule found in living cells including sugars, amino acids, lipids and nucleotides.
O$_2^\cdot$ is produced by the addition of a single electron to oxygen, and several mechanisms exist by which superoxide can be produced in vivo (Halliwell and Gutteridge 1992) (Table 5). Hydrogen peroxide can be generated when oxygen is reduced (Table 5). Hydrogen peroxide is also often generated in biological systems via the production of superoxide. Two superoxide molecules can react together through a dismutation reaction to form hydrogen peroxide and oxygen.

The generation of the OH$\cdot$ is formed by O$_2^\cdot$ reacting with two hydrogen ions to produce hydrogen peroxide together with the reduction of transition metal ions in particular iron and copper ions (Stohs and Bagchi 1995). The hydrogen peroxide and the reduced metal ion then react to form the OH$\cdot$ (Table 5). Hydroxyl radicals are also generated when superoxide reacts directly with hydrogen peroxide (Table 5).

Several enzymatic reactions, including those catalysed by glycolate oxidase and D-amino acid oxidase might produce hydrogen peroxide directly. (Chance et al 1979). H$_2$O$_2$ has the ability to cross cell membranes freely, which superoxide cannot do (Halliwell and Gutteridge 1990). Therefore hydrogen peroxide formed in one location may diffuse a considerable distance before decomposing to yield the highly reactive hydroxyl radical which is likely to mediate most of the toxic effects ascribed to hydrogen peroxide. Hydrogen peroxide thus, acts as a conduit to transmit free radical induced damage across cell compartments and between cells.
Formation of hydroxyl radical (OH•) and superoxide (O2•−)

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2 + e− → O2•−</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O2•− + 2H+ → H2O2 + O2</td>
<td>Superoxide</td>
</tr>
<tr>
<td>2O2•− + 2H+ → H2O2 + O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>2O2•− + 2H+ → H2O2 + O2</td>
<td>Superoxide</td>
</tr>
<tr>
<td>O2•− + Fe3+ → Fe2+ + O2</td>
<td>Superoxide</td>
</tr>
<tr>
<td>H2O2 + Fe2+ → OH• + OH− + Fe3+</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>O2•− + H2O2 → OH• + OH− + O2</td>
<td>Superoxide</td>
</tr>
</tbody>
</table>

Table 5. Formation of hydroxyl radical (OH•) and superoxide (O2•−).

1.5.4 REACTIVE OXYGEN SPECIES AS MEDIATORS OF SIGNAL TRANSDUCTION PATHWAYS

Redox (reduction-oxidation) reactions that generate ROS have been identified as important chemical mediators of signal transduction processes.

All cell types can generate ROS, the formation of which is stimulated by cytokines, growth factors and hormones, e.g. interleukin-1β (IL-1β) (Tolando et al 2000), interleukin 6 (IL-6) (Sung et al 2000), interleukin 3 (IL-3) (Sattler et al 1999), tumour necrosis factor-α (TNF-α) (Forsberg et al 2001), angiotensin II (ANGII) (Zafari et al 1998) (Ushio-Fukai et al 1999) platelet derived growth factor (PDGF) (Sundaresan et al 1995), nerve growth factor (NGF) (Suzukawa et al 2000), transforming growth factor-β1 (TGF-β1) (Thanhnickal et al 1993), granulocyte macrophage colony stimulating factor (GM-CSF) (Sattler et al 1999), and fibroblast growth factor (FGF-2). It is now recognised that ROS form part of the wide array of cell signalling pathways.
As well as activating different components of the signal transduction pathways, ROS also activate transcription. The immediate early genes (which are genes that are induced even in the absence of protein synthesis) c-fos, c-myc, c-jun, and β-actin are induced rapidly by ROS, possibly through the induction of DNA strand breaks (Amstad et al 1992). These genes encode transcription factors which participate in the induction of cell growth, differentiation and development. Low doses of ROS can stimulate the growth of fibroblasts and epithelial cells in culture (Murrell et al 1990). Thus, free radicals may play a role in inflammation by promoting fibrosis and wound healing. This may also take place via an indirect route of free radical attack on lipids producing peroxidation products, which themselves promote collagen synthesis (Chojkier et al 1989).

ROS may directly alter and regulate the activity of transcription factors. Redox-regulation has been reported for several transcription factors including nuclear factor κ-B (NFκB) (Schreck et al 1992) (Schreck et al 1991), activator protein 1 (AP-1) (Abate et al 1990) (Okuno et al 1993), Sp-1 (Wu et al 1996b), c-Myb (Guehmann et al 1992), p53 (Rainwater et al 1995), egr-1 (Nose and Ohba 1996), and hypoxia-inducible factor 1α (Hif-1α) (De Flora et al 1995).

1.5.5 REACTIVE OXYGEN SPECIES AND CELL INJURY

Free radicals are reactive molecules because of the presence of unpaired electrons. All of the major classes of biomolecules, lipids, proteins, carbohydrates and nucleic acids may react with free radicals. This results in lipid peroxidation, carbohydrate and protein modification and DNA strand breaks (Spear and Aust 1995) (Hall et al 1996) (Halliwell and Aruoma 1991).
1.5.6 LIPID PEROXIDATION

Lipid peroxidation is perhaps the most studied consequence of free radical attack.

Figure 5. Mechanism of lipid peroxidation.

Reactive free radicals e.g. the hydroxyl radical, have the capacity to abstract a hydrogen atom (H•) from a methylene group (-CH₂-) from fatty acids, leaving behind an unpaired electron on the carbon (-•CH-).
The resulting lipid peroxides are reasonably stable compounds, but their decomposition can be catalysed by transition metals and metal complexes producing alkoxyl and peroxyl radicals, which can stimulate further lipid peroxidation. Disrupted tissue is more susceptible to lipid peroxidation (Gutteridge et al 1995). Lipid peroxidation can have profound effects on cellular function. Extensive peroxidation in cell membranes will result in changes in fluidity, increased permeability, a decrease in membrane potential and eventually membrane rupture (Porter et al 1984) (Porter et al 1990).

1.5.7 PROTEIN MODIFICATION

The modification of proteins through oxidative stress has recently become a field of great interest. Oxidation of proteins can result in the amino acid residues being oxidised to alcohols and carbonyl groups (Stadtman 1992) (Davies et al 1999) and the formation of protein-protein cross-links. Also to the oxidation of polypeptide backbones causing protein fragmentation (Stadtman and Oliver 1991). Glycation products are formed by a non-enzymatic reaction of reducing sugars i.e. glucose and fructose acting on proteins (Brownlee et al 1988) and further oxidation results in advanced glycation end products (AGE) (Thorpe and Baynes 1996). AGE interaction with RAGE (receptors for AGE), a multiligand member of the immunoglobulin superfamily of cell surface molecules (Neeper et al 1992) (Schmidt et al 1992), results in triggering of cellular signal transduction pathways such as NFXB (Yan et al 1994) and p21^ras MAPK cascade activation (Lander et al 1997). AGEs accumulate in disorders such as diabetes, renal failure, and amyloidosis (Brownlee et al 1988) (Miyata et al 1996).

1.5.8 DEFENCES AGAINST REACTIVE OXYGEN SPECIES

Free radical production occurs in all animal cells and several different forms of defence against oxidative stress have evolved. The two main categories of such defences are those whose role is to prevent the generation of free radicals and those that intercept any that are generated (Cotgreave et al 1988). They exist in both the aqueous and membrane compartments of cells and may or may not be enzymes.
The preventative defences against oxidative stress includes highly efficient electron transfer and sequestration of transition metal ions. Iron, for example, is held tightly bound to special proteins such as transferrin and ferritin (Halliwell and Gutteridge 1984) (Halliwell et al 1992).

Defences against oxidative stress that intercept or “scavenge” free radicals include superoxide dismutase (SOD). SOD catalyzes the dismutation of $O_2^{•−}$ to yield $H_2O_2$ and $O_2$ (Sherman et al 1983) (Ho and Crapo 1988) (Hjalmarsson et al 1994). In cell membranes, the best characterised and possibly the most important is α-tocopherol, the major member of the vitamin E family (Burton et al 1983). α-tocopherol is termed a “chain-breaking” antioxidant because it functions to intercept lipid peroxyl radicals (LOO•) and so terminate lipid peroxidation chain reactions. The resultant tocopheryl radical is relatively stable and in normal circumstances, insufficiently reactive to initiate lipid peroxidation itself, an essential criterion of a good antioxidant (Burton et al 1983).

Glutathione is the major intracellular antioxidant and is present at high concentrations of about 5mM (Chance et al 1979). Reduced glutathione (GSH) plays a central physiological role in maintaining the body’s homeostasis and in protecting cells against oxidants, toxins, DNA-damaging agents and carcinogens of either exogenous or endogenous source (Meister et al 1988). The large GSH molecule however is not transported efficiently into cells. The reduced form of glutathione plays a part in detoxification by reacting with $H_2O_2$ and organic peroxides i.e. fatty acid hydroperoxides cleaved from membrane phospholipids by a phospholipase (Wendel 1980). The reaction is catalysed by glutathione peroxidase which is present in the cytosol and mitochondria. The oxidised form, GSSG can be reduced back to GSH by the enzyme glutathione reductase (GRx) utilising NADPH produced via the pentose shunt (Meister et al 1988). Glutathione is involved in reducing oxidized vitamin E and vitamin C in a detoxifying process to prevent lipid peroxidation (Burton and Ingold 1986).

Catalase is an enzyme which also safely decomposes peroxides. Catalase is mainly located in peroxisomes and acts upon hydrogen peroxide. In the aqueous phase other compounds act as free radical scavengers. Ascorbic acid (vitamin C) is an important
antioxidant both within cells and in the plasma (Stocker and Frei 1991). Uric acid in plasma (Stocker and Frei 1991) and glutathione in cell cytosol (Cotgreave et al. 1988) also possess strong radical scavenging properties.

**Defence mechanisms against reactive oxygen species**

<table>
<thead>
<tr>
<th></th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SOD</strong></td>
<td>$2O_2^- \cdot + 2H^+ \rightarrow H_2O_2 + O_2$</td>
</tr>
<tr>
<td></td>
<td>Superoxide</td>
</tr>
<tr>
<td></td>
<td>$\text{Hydrogen peroxide}$</td>
</tr>
<tr>
<td><strong>LOO• + α-tocopherol-OH → LOOH + α-tocopherol-O•</strong></td>
<td>Lipid peroxyl radicals</td>
</tr>
<tr>
<td><strong>Catalase</strong></td>
<td>$2H_2O_2 \rightarrow 2H_2O + O_2$</td>
</tr>
<tr>
<td><strong>GPx</strong></td>
<td>$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$</td>
</tr>
<tr>
<td><strong>GPx</strong></td>
<td>$2GSH + R-OH \rightarrow GSSG + H_2O + ROH$</td>
</tr>
<tr>
<td><strong>GRx</strong></td>
<td>$\text{GSSG} + \text{NADPH} + H^+ \rightarrow 2\text{GSH} + \text{NADP}^+$</td>
</tr>
</tbody>
</table>

Table 6. Examples of the defence mechanisms against reactive oxygen species

### 1.5.9 THERAPEUTIC ANTIOXIDANT AGENTS

Much research is being conducted to find effective antioxidant compounds which can be administered for treatment or prevention of free radical-mediated tissue damage. Antioxidant therapies being used clinically include recombinant enzymes such as SOD and/or catalase (Turrens et al. 1984) (Tang et al. 1993) (Walther et al. 1995) as well as N-acetylated-cysteine (NAC) (Smilkstein et al. 1988) (Rasmussen and Glennow 1988) (Jackson et al. 1984) (Behr et al. 1997) (Holt et al. 1999a) and α-lipoic acid. Antioxidants investigated in this thesis were NAC and α-lipoic acid (Femiano et al. 2002).
**N-acetyl-L-cysteine (NAC)**

NAC acts as a glutathione regenerator. It is readily deacylated in cells to yield L-cysteine promoting intracellular GSH synthesis (De Flora et al 1995). NAC has been used successfully to treat hepatic and renal failure caused by glutathione depletion secondary to acetaminophen overdose (Smilkstein et al 1988). It has an extensive history as a mucolytic and has been used in pulmonary diseases including chronic bronchitis (Rasmussen and Glennow 1988) (Jackson et al 1984) and fibrosing alveolitis (Behr et al 1997). NAC was also used successfully to improve renal function in patients with early HRS (Holt et al 1999a).

The mechanism of action of NAC includes the restoration of reduced and total glutathione levels in lung cell fluid (Behr et al 1997). NAC has been demonstrated to have heavy metal chelating capacities for toxic metals, as well as for copper, zinc, and boron (Kelly 1998). Several studies support evidence that NAC increases glutathione levels in vivo and in vitro (De Flora et al 1985) (Hoffer et al 1996) (Corcoran and Wong 1986) (Bernard et al 1997). There is also evidence NAC may boost cellular immunity directly (Kinscherf et al 1994). Response to interferon in hepatitis C patients previously unresponsive to four months of alpha-interferon was improved with NAC (Beloqui et al 1993). NAC inhibits activation of NFκB and the expression of a variety of pro-inflammatory cytokines, such as TNFα (Staal et al 1995).

**Lipoic acid (LA)**

Alpha-lipoic acid is a dithiol antioxidant found primarily in the mitochondria where it acts as a coenzyme in the α-keto-acid dehydrogenase complex (Rodwell 1996). It is a low molecular weight substance, present in the diet, absorbed from the gut and readily passes through the blood-brain barrier (Packer et al 1995). Lipoic acid has antioxidant activity in both lipid and non-lipid components and can act in membranes of the brain and nervous tissue, lymphoid tissue, and in most other cell types (Packer et al 1997). Both alpha-lipoic acid and its reduced form dihydrolipoate can scavenge a wide variety of reactive oxygen species, including superoxide and peroxyl radicals formed during lipid peroxidation, and is able to regenerate ascorbate and tocopherol and to increase glutathione concentrations (Packer et al 1997).
1.5.10 \textbf{F}_2\text{-ISOPROSTANE FORMATION BY FREE RADICALS}

\(\text{F}_2\text{-isoprostanes}\) are prostaglandin like compounds that are formed largely independently of the cyclooxygenase (COX) enzyme, by the action of free radicals on arachidonic acid (Morrow and Roberts 1996). Free radical attack on arachidonic acid causes lipid peroxidation, resulting in a number of isomers of prostaglandin \(\text{F}_2\alpha\). Like \(\text{PGF}_{2\alpha}\) these isomers consist of a cyclopentane ring and so are termed \(\text{F}_2\text{-isoprostanes}\) (Figure 6).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{The structure of \(\text{PGF}_{2\alpha}\) and 8-iso \(\text{PGF}_{2\alpha}\) showing the F ring structure.}
\end{figure}

Four main \(\text{F}_2\text{-isoprostane}\) isomers are formed by the lipid peroxidation of arachidonic acid (AA). Each isomer has eight racemic diastereoisomers. Thus there are 32 different \(\text{F}_2\text{-isoprostane}\) compounds (Morrow and Roberts 1996). Each isomer also contains the F ring cyclopentane structure but they differ in their tail structure (Awad \textit{et al} 1996). The position of the side-chain hydroxyl group is counted from the carboxylic acid group (C1) (Taber \textit{et al} 1997). The major \(\text{F}_2\text{-isoprostane}\) formed in vivo under conditions of oxidative stress is 8-iso\(\text{PGF}_{2\alpha}\) (15-\(\text{F}_2\alpha\)-IsoP) (Morrow \textit{et al} 1994). 8-iso\(\text{PGF}_{2\alpha}\) is found in urine and plasma (Roberts \textit{et al} 1996). \(\text{F}_2\text{-isoprostanes}\) are formed in situ in membrane phospholipids (Morrow \textit{et al} 1992a) (Morrow \textit{et al} 1998) and low density lipoproteins (Lynch \textit{et al} 1994) by free radical catalysed peroxidation of...
arachidonic acid. The resulting isoprostane remains esterified to the plasma membrane phospholipid which causes disruption in the plasma membrane structure. The esterified isoprostane is then cleaved by hydrolysis from the parent phospholipid by the action of a phospholipase or similar enzyme system to release free isoprostane and restore membrane integrity.

Following generation and release of $F_2$-isoprostanes into the circulation they are metabolised by the liver and eliminated in the urine. In a study conducted with a single human volunteer, 75% of the original radioactivity was found in the urine 4.5h after infusion of the parent compound, with the primary metabolite being 2,3-dinor-5,6-dihydro-PGF$_{2\alpha}$ (Roberts et al 1996). In the rabbit it was shown that a bolus injection of 8-iso-PGF$_{2\alpha}$ was rapidly distributed in the circulation, with a half-life of distribution of $\sim$1 min, and then was rapidly eliminated, with a half-life of $\sim$ 4 min (Basu 1998).

**1.5.11 BIOLOGICAL ACTIVITY OF ISOPROSTANES**

$F_2$-isoprostanes are biologically active. Administration of isoprostanes into the rat (low nanomolar range) produces a potent renal vasoconstriction that reduces glomerular filtration rate (GFR) and renal blood flow (RBF) by 40 to 45% (Takahashi et al 1992).

In rabbits and rats $F_2$-isoprostanes are potent pulmonary artery vasoconstrictors and they cause bronchial constriction in the rat lung (Kang et al 1993) (Banerjee et al 1992). In addition, $F_2$-isoprostanes have been shown to induce a significant release of ET from bovine aortic endothelial cells (Michael et al 1997).

A feature of the biological activity of isoprostanes is that the vasoconstrictive effects are blocked by the thromboxane A$_2$/ prostaglandin H$_2$ receptor antagonist (SQ29548) suggesting that isoprostanes may be acting on a thromboxane-type receptor (Takahashi et al 1992). The biological actions of 8-isoPGF$_{2\alpha}$ may result from interaction with this receptor which may be specific to $F_2$-isoprostanes (Fukunaga et al 1997) (Longmire et al 1994).
1.5.12 ISOPROSTANES AS MARKERS FOR OXIDANT STRESS

Elevated generation of F$_2$-isoprostanes has been reported in a variety of syndromes putatively associated with oxidant stress. These include coronary ischaemia-reperfusion syndromes (Delanty et al 1997) (Reilly et al 1997), Alzheimer’s disease (Pratico et al 1998c) (Montine et al 1998) adult respiratory distress syndrome and chronic obstructive pulmonary disease (Pratico et al 1998b). There is some evidence that F$_2$-isoprostane generation may increase with age (Wang et al 1995). Cigarette smoking (Pratico et al 1998a) (Bachi et al 1996) (Reilly et al 1996) (Morrow et al 1995) and alcohol have been shown to increase isoprostane generation (Meagher et al 1999) (Aleynik et al 1998).

One of the greatest impediments in the field of free radical research has been the lack of reliable methods to assess oxidant stress status in humans (Pryor and Godber 1991). The thiobarbituric acid test is the test most oftenly used at present to determine lipid peroxidation. In this assay the sample is heated with thiobarbituric acid and the resulting pink chromophore produced is measured at 532nm. The assay depends on the reaction between malondialdehyde (MDA) and thiobarbituric acid. However, thiobarbituric acid is highly reactive and also a large percentage of the MDA that reacts with the thiobarbituric acid is actually formed during sample preparation and from reaction conditions. TBARS are not very reproducible or reliable. The measurement of isoprostanes provides a more reliable and accurate approach for the assessment of oxidant stress in vivo both in animal models of oxidant injury and humans (Morrow and Roberts 1997). Reliable techniques using the GC-MS have been developed enabling F$_2$-isoprostanes to be measured in all biological material including plasma, urine, cerebrospinal fluid and tissue (Morrow and Roberts 1994).

F$_2$-isoprostanes found in urine are free isoprostanes as they have already been hydrolysed from the parent phospholipids in vivo. Free F$_2$-isoprostanes are also found in plasma at low concentrations. F$_2$-isoprostanes found in tissue and in plasma are termed esterified F$_2$-isoprostanes and their measurement can be used to localise the site of lipid peroxidation, provided that they are formed at a rate that exceeds hydrolysis (Morrow et al 1992a).
Some groups have found that small amounts of F$_2$-isoprostanes are synthesized in a COX-dependent manner in vascular smooth muscle (Jourdan et al 1999) (Jourdan et al 1997), endothelium (Watkins et al 1999), platelets (Klein et al 1997) (Pratico et al 1998a) (Pratico et al 1995) (Pratico et al 1996), monocytes (Patrignani et al 1996) (Pratico and FitzGerald 1996) (Pratico et al 1997), macrophages (Sautebin et al 1999) (Vacchiano et al 1998) and mesangial cells (Klein et al 1997). However, it is not entirely clear whether the isoprostanes are direct products of COX or arise secondarily to an overflow of COX-derived reactive oxygen species. Some recent data (Watkins et al 1999) suggest the latter may be the case. 8-iso-PGF$_2$α production in endothelial cells was shown to be COX dependent, in that it could be inhibited by indomethacin or aspirin and also sensitive to catalase but not to superoxide dismutase, suggesting that hydrogen peroxide originating from COX was responsible.

### 1.5.13 NUCLEAR FACTOR KAPPA B (NFκB)

NFκB is a collective name for dimeric transcription factors comprising of members of the Rel family of DNA-binding proteins that recognise a common sequence motif 5′-GGG(A/G)NN(T/C)(T/C)CC-3′, where N is any base. Five members of the mammalian Rel family are known: RelA (p65), RelB, c-Rel, NFκB1 (p50) and NFκB2 (p52) (Reviewed in (Baldwin, Jr. 1996) (Ghosh et al 1998)). Any homo- or heterodimer is considered NFκB, although the most commonly found in activated cells, RelA/NFκB1 (p65/p50) heterodimer, is considered the classic NFκB. p50 binds with high affinity to DNA and recognises the decameric sequence 5′-GGGACCTTTCC-3′ and variations thereof.

In most cells, NFκB resides in a latent form in the cytoplasm which can be activated by a great variety of agents including cytokines, viral transactivator proteins, activators of protein kinases and reactive oxygen species (Figure 7). It is thought that NFκB can be activated following the binding of TNFα to a phosphatidylycerol-specific phospholipase C (PLC) which hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP$_2$) to inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG). DAG activates NFκB via the stimulation of protein kinase C (PKC) (Schutze et al 1992). A change in the redox state due to reactive oxygen species is another pathway for the activation of NFκB.
(Schreck et al 1992) (Schreck et al 1991). The inducible form of NFκB is stabilised by the inhibitory subunit IκB (Baeuerle and Baltimore 1988). In complex with IκB, NFκB is a heterotrimer composed of IκB-α (37kDa) or IκB-β (43kDa), p50 and p65 (Zabel and Baeuerle 1990).

On stimulation of the cell, IκB kinases (IKK-α and IKK-β) activity are stimulated and phosphorylate serine residues of IκB-α (S32/S36) and IκB-β (S19/S37) (Song et al 1997) (DiDonato et al 1997) (Mercurio et al 1997). Phosphorylated IκB is ubiquitinated and degraded. NFκB translocates to the nucleus and initiates the transcription of numerous cellular genes that are involved in immune and stress responses, inflammation, and apoptosis (Reviewed in (Baeuerle and Henkel 1994) (Baeuerle and Baltimore 1996) (Barnes and Karin 1997) (Baichwal and Baeuerle 1997). The proposed mechanism of NFκB turnover results from resynthesis of IκBα after the IκBα gene is transactivated by active NFκB. Newly synthesized IκBα reassociates with nuclear NFκB causing its dissociation from DNA and shuttling back into the cytoplasm. In another mechanism of turnover in hepatocytes, NFκB is degraded by ATP-dependent proteolysis involving nuclear proteosomes that result in the transient appearance of truncated forms of NFκB such as p50/p35, which still have the capacity to bind DNA but are missing the transactivation domain (Taub 1996).
Figure 7. Activation of NFκB by oxidant stress.

A change in redox state by either ROS or receptor mediated activation of IκB kinase (IKK) occurs in the cytosol resulting in the phosphorylation and ubiquination of IκB and its dissociation from the p65-p50 heterodimer. The dimer translocates to the nucleus where it localises to enhancer sequences and results in the upregulation of gene expression.
1.6 Summary, hypothesis and aims

HRS is the development of renal failure in liver disease where there is no apparent anatomical or pathological cause for the renal failure. These patients also characteristically have a hyperdynamic systemic circulation and altered renal haemodynamics. This is because the imbalance between vasodilating and vasoconstricting forces are abnormal with elevated levels of endothelium-derived substances such as nitric oxide and endothelins.

At present there are no suitable models for the study of HRS. Recently, however the galn-ALF rat model was shown to develop a hyperdynamic circulation along with a decrease in renal blood flow and the development of renal failure (Javle et al 1998). However, no data on either the mechanism of renal failure or histological data on whether renal injury occurs were given.

1.6.1 HYPOTHESIS

In this thesis the hypothesis under investigation is that (a) the galn-ALF rat model is a suitable model for the study of HRS and (b) elevated vasoactive factors play a role in the development of renal failure in HRS.

1.6.2 AIMS

1. To determine the suitability of the galn-ALF rat model as a model for HRS by:
   i. Optimising the conditions under which the rats will develop liver injury and renal failure without a high mortality rate.
   ii. Determining whether there was a correlation between the degree of liver injury and renal impairment.
   iii. Investigating the renal histology of the kidney in rats with galn-ALF.
   iv. Establishing that galactosamine is specifically a hepatotoxin and does not have a nephrotoxic effect on the kidney.
   v. Determining the haemodynamic alterations in the galn-ALF rat model by measuring the mean arterial blood pressure and the renal blood flow.
2. To investigate the role of endothelin-1 in the renal function of the galn-ALF rat model by determining:
   i. The level of endothelin-1 in the plasma
   ii. The regional synthesis of endothelin-1 in the kidney
   iii. The distribution of the endothelin-1 receptors, ET\textsubscript{A} and ET\textsubscript{B} in the kidney
   iv. The effect of Bosentan, an endothelin-1 mixed receptor antagonist on renal function.

3. To investigate the role of oxidative stress in the renal dysfunction of the galn-ALF rat model by determining:
   i. The concentrations of F\textsubscript{2}-isoprostanes in the plasma and urine
   ii. The effect of the antioxidants, N-acetylcysteine and α-lipoic acid have on renal function
Chapter 2

Characterisation of the Biochemical and Pathological features of Renal failure in rats with Galactosamine-induced acute liver failure
2 Characterisation of the biochemical and pathological features of renal failure in rats with galn-ALF

2.1 Introduction

Renal failure occurs in 40-80% of patients with end stage liver disease or acute liver injury (Shear et al 1965). The development of renal failure in the absence of clinical, anatomical or pathological causes of renal failure is termed the Hepatorenal syndrome (HRS) (Epstein 1987). Classically, HRS is associated with end stage cirrhosis and is now termed type II HRS. However, it is widely recognised that renal failure occurs spontaneously in 50% of patients with acute liver failure (Ring-Larsen and Palazzo 1981) (Wilkinson et al 1974). Recognition that HRS may develop in acute liver failure is now included within the most recent definition of HRS and is termed type I HRS (Arroyo et al 1996). Type I HRS is characterised by a rapidly progressive reduction of renal function as defined by doubling of the initial serum creatinine to a level greater than 2.5 mg/dL or a 50% reduction in the initial 24 hour creatinine clearance to a level lower than 20mL/min in less than two weeks. Type I HRS is most frequently observed in patients with alcoholic hepatitis and in cirrhotics with ascites whose hepatic function deteriorates rapidly due to the development of a serious bacterial infection or gastrointestinal haemorrhage. The development of type I hepatorenal syndrome carries an ominous prognosis with an 80% mortality at 2 weeks and only 10% of patients surviving more than 3 months (Gines et al 1993). Spontaneous recovery of renal function is rare and usually follows the improvement of liver function (Gines et al 1993). Death results from a combination of hepatic and renal failure, as well as the precipitating cause of the syndrome.

One of the pathophysiological hallmarks of HRS is that there are relatively few histological changes in the kidneys. In the early part of the syndrome, at least, tubular function is intact, as reflected by avid sodium retention and oliguria. The functional renal failure is secondary to liver failure. It has been known for many years that patients with severe liver failure have a reduction in renal blood flow, indicative of renal vasoconstriction, and that this is most marked in those patients who develop HRS (Guarner et al 1987) (Epstein et al 1970).
Many studies have investigated the mechanism of galactosamine-induced hepatoxicity, which in rats causes a syndrome similar to acute liver failure (Keppler et al 1968). Following a single injection of high dose galactosamine, rats develop acute liver failure with development of a hyperdynamic circulation (Makin et al 1997). Some of these animals also develop cerebral oedema (Dixit and Chang 1987). Recently, it was reported that galactosamine-induced liver injury is associated with a decrease in renal blood flow and the development of renal failure (Javle et al 1998). However, no data on either the mechanism of renal failure or histological data were given.

At present there are no suitable models for the study of HRS. The suitability of the galn-ALF rat as a model for HRS was investigated by determining firstly, whether the renal failure was secondary to liver failure and secondly whether the histology of the kidney was normal and finally the changes in renal haemodynamics.

2.2 Materials and Methods

2.2.1 MATERIALS

D(+)galactosamine hydrochloride, Dulbecco’s modified eagle’s medium (DMEM), penicillin, streptomycin, glutamine, trypsin-EDTA and phosphate buffered saline tablets were purchased from Sigma-Aldrich Company Ltd. (Poole, UK). Culture plastic-ware was from Marathon Laboratory Supplies (London, UK); all flasks were fitted with a vented cap (0.2μm filter). Unless stated all other reagents were purchased from Sigma-Aldrich.

2.2.2 ANIMALS

All animal experiments were conducted according to Home Office guidelines under the Animals in Scientific Procedures Act 1986. Male Sprague Dawley rats (body weight 250-300g) were obtained from the Comparative Biology Unit, Royal Free Hospital School of Medicine. All animals were housed in the Comparative Biology Unit on a standard diet, with a light cycle of 12 hours on and 12 hours off, at a temperature of 19
to 23°C, at a humidity of 50%.

2.2.3 EXPERIMENTAL DESIGN

Rats were placed individually in polycarbonate metabolic cages with free access to food and water. The number of rats was limited to 12 for each experiment by the number of metabolic cages available. Baseline urine samples were collected 24 hours prior to the study and every 24 hours following galactosamine (gaN) or saline (150mM NaCl) injection. Animals were sacrificed by exanguination under anaesthesia (60mg/kg Sagatal i.p.), 48 hours following galactosamine or saline injection. Blood was obtained by cardiac puncture, collected either into EDTA containing tubes or allowed to coagulate in plain tubes, centrifuged at 800g for 10 mins and the plasma stored at -80°C until analysis. Liver and kidney tissue were collected for histology. No evidence of ascites was seen on opening of the abdominal cavity.

For the haemodynamic studies (described below), galn-ALF in rats (n=8) and sham controls (n=8) were studied. These rats were also placed in the polycarbonate metabolic cages with free access to food and water and 24 hour urine samples were collected. Tissue and blood samples were not harvested from the rats used in the haemodynamic studies.

2.2.4 TREATMENTS

Galactosamine was injected intra-peritoneally (i.p.) at 1.1g galactosamine/kg rat. The galactosamine stock solution was made at a concentration of 300mg/ml in saline, pH 6.8 (Chirito et al 1979). Sham controls were injected (i.p.) with a corresponding volume of saline (150mM NaCl), approximately 1.1ml.
2.2.5 PRELIMINARY STUDIES OF GALN-ALF IN RATS

To establish the correct dose of galactosamine that would result in acute liver injury and renal failure, without the animal dying, a dose response of galactosamine was performed. Animals were injected with 0.55g/kg (n=4), 0.75g/kg (n=6), 1.1g/kg (n=6) and 1.3g/kg (n=4) galactosamine and sacrificed 48 hours later. Sham controls were injected with saline (n=4).

The time taken for the onset of liver and renal failure to occur was determined by injecting 1.1g/kg galactosamine and sacrificing the animals at 24 hours (n=6), 48 hours (n=6) and 72 hours (n=6). Sham controls were injected with saline (n=6) and sacrificed after 72 hours. Blood and tissue were collected from all the animals.

2.2.6 RELATIONSHIP OF GALACTOSAMINE-INDUCED LIVER INJURY AND RENAL FAILURE

To determine if varying degrees of liver injury correlated with renal impairment, the effect of different doses of galactosamine on liver and renal function was investigated.

2.2.7 BIOCHEMICAL TESTS

Aminotransferases. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and are the two enzymes most widely measured as markers of hepatocyte disruption. Both are found in a variety of tissues, but ALT activities are lower in extrahepatic tissue and therefore an increase in plasma ALT activity is a greater indication of liver disease.

Plasma bilirubin concentration. Increased plasma conjugated bilirubin leads to jaundice and gives indications of underlying liver/biliary tract disease. In cases of conjugated hyperbilirubinaemia, the concentrations of bilirubin exceeds 50μmol/L (upper limit of normal reference range is 25μmol/L).
Plasma albumin. Low albumin levels are an indication of decreased liver synthesis. The relative long half-life of albumin of 21 days means that serum albumin is often normal in acute hepatitis, and is of greater value in assessing the severity of chronic liver disease.

Blood was collected from the inferior vena cava into ethylenediaminetetra-acetic acid (EDTA) or plain tubes and centrifuged at 800g for 10 mins at 4°C and the plasma or serum stored at -80°C. Serum and urine creatinine was measured by a kinetic colorimetric method (Hitachi Auto-analyser, Japan). This method involves converting bilirubin to biliverdin with potassium hexacyanoferrate (III), and uses a dual wavelength absorption method, eliminating interference from bilirubin. Creatinine clearance was calculated from urine and serum creatinine values. Liver function tests including the concentrations of alanine and aspartate transaminases (ALT and AST respectively), albumin and bilirubin in the plasma as well as plasma and urine sodium were determined by the auto-analyser (Hitachi, UK). Urine osmolality was measured as the depression of the freezing point (Advanced Micro Osmometer 3300, Advanced Instruments Inc., Massachusetts, USA), and plasma osmolality was calculated from the serum sodium, potassium and urea concentration.

Creatinine clearance, plasma osmolarity, osmolar clearance and free water clearances were calculated as shown below:

\[
\text{Creatinine clearance} = \frac{\text{urine volume (ml)} \times \text{urine creatinine (\(\mu\)M)}}{\text{plasma creatinine (\(\mu\)M)} \times \text{time (mins)}}
\]

\[
\text{Plasma osmolarity} = 2\text{Na}^+ + 2\text{K}^+ + \text{glucose(\(\mu\)M)} + \text{urea(\(\mu\)M)}
\]

\[\text{(mosmoles/kg)}\]

where \(\text{Na}^+\) and \(\text{K}^+\) represent the mM concentrations of sodium and potassium and are doubled to account for their anion equivalents in plasma.

\[
\text{Osmolar Clearance (C_{osm})} = \frac{\text{urine osmolarity(mosmoles/kg)} \times \text{urine vol/day (ml/day)}}{\text{plasma osmolarity(mosmoles/kg)}}
\]

\[\text{(ml/day)}\]

Free water clearance \((C_{H2O})\) \(\text{(ml/day)}\) = \(V - C_{osm}\)

where \(V\) is 24 hour urine volume, and results expressed as ml/day.
2.2.8 HISTOLOGICAL STUDY OF THE LIVER AND KIDNEY OF RATS INJECTED WITH 1.1G/KG GALACTOSAMINE FOR 48 HOURS

The optimum dose of galactosamine required to induce acute liver injury and renal failure in the rat was 1.1g/kg galactosamine at 48 hours (Section 2.3.1). The liver and kidney samples collected from animals obtained under these conditions were then examined by light and electron microscopy.

2.2.9 LIGHT MICROSCOPY OF LIVER AND KIDNEY TISSUE

Liver and kidney was fixed in formalin and embedded in paraffin blocks. Sections 3μm thick were cut and stained with haematoxylin and eosin.

2.2.10 ELECTRON MICROSCOPY OF LIVER AND KIDNEY TISSUE

2.2.10.1 Reagents

Reagents for microscopy were obtained from Merck Ltd. unless otherwise indicated.

Reynolds lead citrate: Equal volumes (15mls each) of 0.06mM lead nitrate and 0.09mM tri-sodium citrate were combined and the resulting precipitate dissolved with 8mls of 1M NaOH and diluted to 50mls with distilled water.

Cubes of tissue less than 2mm in thickness were cut from the kidney and fixed in 1% paraformaldehyde, 1.5% glutaraldehyde (TAAB Laboratories Equipment Ltd., Reading) in phosphate buffer saline for 2 hours. The preparations were then washed with several changes of phosphate buffered saline and post-fixed for 1½ hours using 1% osmium tetroxide dissolved in 1.5% potassium ferricyanide. The kidney specimens were washed using distilled water and dehydrated with graded alcohols from 30% to 100%, left in 50% alcohol/ 50% Lemix (TAAB) epoxy resin mixture overnight, and then infiltrated with 100% Lemix resin for a minimum of 6 hours. Finally they were embedded in fresh Lemix resin and polymerised at 70°C over-night.
Semi-thin (1μm) sections were cut using glass knives on a Reichert-Jung ultracut microtome, collected on glass microscope slides and stained using 1% toluidene blue/0.2% pyronine (both from Raymond Lamb, London) in 1% borax stain (sodium tetraborate). Ultra-thin sections were cut using a diamond knife (Diatome) and collected on 200HS, 3.05mm copper grids (Gilder, Grantham). The ultra-thin sections were stained with saturated uranyl acetate in 50% ethanol (TAAB) for 5 minutes and then by Reynold’s lead citrate for 5 minutes. The sections were viewed and photographed using a Philips 201 transmission electron microscope.

2.2.11 CULTURING OF CELLS

Two cell lines, HepG2 (human liver cells) and LLCPK1 (porcine kidney cells) were cultured. Both cell lines were obtained from The European Collection of Animal Cell Cultures. The cells were cultured in DMEM supplemented with 10% heat-inactivated foetal calf serum, streptomycin (70μmol/L) and penicillin (70μmol/L). The cells were grown in a 75 cm² flask in a humidified atmosphere at 37°C with 5% CO₂ and 95% air.

2.2.11.1 Subculturing of cells

Upon reaching confluency, the cells were passaged by washing with PBS, followed by a brief trypsinisation (2 min at 37°C with trypsin/EDTA solution) and then resuspended in DMEM. The cells were harvested by centrifugation at 300 g for 5 min and finally resuspended in fresh medium. The single cell suspension was reseeded (at a 1:3 split) in a 75 cm² flask.

2.2.11.2 Cell counting by trypan blue exclusion assay

Trypan blue is used in this dye exclusion procedure to enable counting of both viable cells (which do not take up the dye) and non-viable cells, those with compromised/permeable cell membranes, which take up the dye. A 30μl aliquot of single cell suspension was mixed with an equal volume of 0.4% (w/v) Trypan Blue solution. Cell viability was determined in a haemocytometer and cover-slip. The chambers were filled with the cell/stain mixture and the viable cells counted under an inverted light microscope (using the x 10 objective lens). The mean cell count of viable cells per 1mm square with a volume of 10⁻⁴ cm³, from a minimum of 8 squares counted, was
used in the calculation below to give the concentration of cells per ml in the original cell suspension:

\[
\text{Mean cell count per square x dilution factor (of 2) x } 10^4 = \text{ viable cell count per ml}
\]

To determine % cell viability in the suspension, both viable and non-viable cells were counted and the viable cell count expressed as a % of the total cell count.

### 2.2.11.3 Cell cryopreservation

Confluent cells from a 75 cm\(^2\) flask were harvested and centrifuged at 300 g for 5 min. The pellet was resuspended in 1ml of pre-chilled DMEM containing 10% (v/v) dimethylsulphoxide (DMSO) which is a cryoprotective agent that lowers the freezing point and allows for a slow cooling rate to limit the damage caused by freezing. The whole mixture was immediately transferred to a cryovial and frozen in the vapour phase of a liquid nitrogen tank by slowly lowering the vial nearer to the liquid over a 3 hour period. When required, cells were rapidly thawed by immersing the bottom of the vial in a 37°C water bath and the vial contents immediately added to an excess of DMEM, mixed and centrifuged at 300g for 5 min. The pellet was resuspended in fresh complete medium and transferred to a 75cm\(^2\) flask. Cells adhered to form a confluent monolayer overnight and were subcultured as described previously in section 2.2.11.1.

### 2.2.12 DETERMINATION OF TOXICITY OF GALACTOSAMINE ON TUBULAR CELLS

To determine whether galactosamine had any direct nephrotoxic effect, cultured renal tubular cells were incubated with galactosamine, and cell injury quantified by measuring release of LDH (Berry et al 1991) (Wu et al 1996a). LLCPK\(_1\) cells were cultured in 6 well plates (0.5x10\(^6\) cells/well) overnight at 37°C. Before the experiment the wells were carefully washed twice with PBS. Fresh culture medium without foetal calf serum was added. Galactosamine was dissolved in saline and added at a final concentration of 10μM to 50mM to the cells. Following incubation for 24 hours, 100μl of medium was collected from each well. A total cell lysate was prepared by collecting the cells in the medium, sonicating and centrifuging at 800g for 10mins. The
supernatants and the total cell lysate were tested for LDH activity using an LDH
diagnostic kit (Sigma). The LDH of the total cell lysate was used for calculating the
maximal percentage leakage of LDH (Berry et al 1991) (Wu et al 1996a). The cell
viability after the 24 hour incubation with galactosamine was assessed by trypan blue
exclusion. As a control HepG2 cells (human hepatocytes) were tested for LDH leakage
under the same conditions.

2.2.12.1 Measurement of the percentage leakage of LDH
The percentage leakage of LDH was evaluated using an LDH diagnostic kit
(Sigma). Briefly, all samples and the LDH reagent were maintained at 30°C. In a
cuvette, 50μl of sample was added to 1ml of LDH reagent, mixed, incubated for 30
seconds and the absorbance read at 340nm. This reading is INITIAL A. Further
readings were taken 30 seconds later and again at 60 seconds. The absorbance reading
taken at 60 seconds is FINAL A. INITIAL A is subtracted from FINAL A to obtain
absorbance change per minute (ΔA per min). To determine LDH activity the
calculation is:

\[
LDH \text{ Activity (U/L)} = \frac{ΔA \text{ per min} \times TV \times 1000}{6.22 \times SV \times LP}
\]

Where:

- ΔA per min = Change in absorbance per minute at 340nm
- TV = Total reaction mixture volume (1.05ml)
- SV = Sample volume (0.05ml)
- 6.22 = Millimolar absorptivity of NADH at 340nm
- LP = Lightpath (1cm)
- 1000 = Conversion factor for units per ml to units per litre

2.2.13 HAEMODYNAMICS: MEASUREMENT OF THE MEAN ARTERIAL
BLOOD PRESSURE AND RENAL BLOOD FLOW

2.2.13.1 Measurement of the mean arterial blood pressure (MAP)
Rats were anaesthetised with Inactin i.p. (60mg/kg) as this anaesthetic has been shown
to cause minimal changes to cardiovascular physiology. After induction of anaesthesia,
rats were secured in dorsal recumbency on a heating pad maintained at 36-37°C. 95%
O₂ was delivered via a facemask. The left femoral artery was isolated, by making an
inguinal incision in the leg and removing the fatty tissue by blunt dissection. The femoral artery lies between the femoral nerve lying rostral and the vein lying caudal to the artery. A ligature was tied at the distal end of the femoral artery. Then two ligatures were looped under the artery proximally. The distal ligature was pulled to make the artery taut and the proximal loops were pulled to prevent blood gushing out while a small incision was made close to the proximal loops. A small 22G Abbocath cannula (Abbott, Republic of Ireland) was inserted into the artery and then tied to keep in place with the proximally looped ligatures. There were no significant bleeding complications in any animal. To measure the mean arterial blood pressure the cannula was connected to a pressure transducer and recorded using a MacLab system (ADI Instruments). In the right leg the femoral vein was isolated, cannulated in the standard way and connected to an infusion pump delivering saline at 5ml/hr while the animal had no laparotomy and then 15ml/hr when the abdomen was open. This was to replace fluid losses. During pressure readings the saline was discontinued at a three-way tap.

2.2.13.2 Measurement of the renal arterial blood flow (RBF)
A ventral midline abdominal skin incision was made. The abdominal incision was extended into the abdominal cavity. The rate of the saline infusion pump was increased to 15ml/hour. The intestinal tract of the animal were carefully deflected to the rat’s right, wrapped in a wet swab and covered in cling film. This exposed the left kidney and the major renal vessels leading to the kidney. The fatty tissue around the renal artery and vein was removed by blunt dissection. The renal vein was first identified, as the renal vein is larger than the renal artery and therefore easier to identify. The renal artery which is more cranial and deeper than the vein was identified. The renal artery was then isolated and mobilised by gently clearing the fatty tissue away from the artery. The removal of as much fatty tissue as possible is important so that the Doppler flow probe (Transonic animal research flowmeters, T106/T206 series. Transonic Systems Inc, NY USA) (Welch et al 1995) could make proper acoustic coupling with the artery. The Doppler probe with lubricating gel was gently placed around the artery so that the artery lay in the bottom of the probe. Readings of 30 seconds were taken every 2 minutes for 30 mins. The mean arterial pressure and renal blood flow were then calculated and averaged over this time period. Renal blood flow results are expressed as ml/min/100g and represent the value for a single kidney. Haematocrit measurements were taken at the start and end of the study. Results showed that there was no
significant difference. The protocol for the dissection of the animals was from Transonic Systems Inc, NY, USA and the dissections were supervised under Mr Bimbi Fernando, a Surgical Officer at The Royal Free Hospital.

2.2.14 STATISTICAL ANALYSIS

Data from each experiment was recorded in Microsoft Excel 97 (Microsoft Corp., USA) spreadsheets and compared using statistical analysis tools in Excel. All the results are expressed as mean ± sem.

Student t-tests were used as the data was considered to follow a normal (Gaussian) distribution. The variant implemented by Microsoft Excel that assumes unequal variances was used based on the different sem values of the sample groups. Paired t-tests were used when two measurement were compared on the same animals and unpaired t-tests were used when comparing two different groups of animals. The limit of significance was taken as P < 0.05. Spearman rank test was used to determine whether there was a correlation between the concentration of ALT and serum creatinine.
2.3 Results

2.3.1 PRELIMINARY STUDIES

Liver injury, assessed by the concentration of alanine transaminase (ALT) in the plasma increased in a dose dependent manner with galactosamine. The ALT increased from 54 ± 3.8 IU/litre in the sham controls to 5571 ± 1327 IU/litre (p<0.001), 6352 ± 829 IU/litre (p<0.001) and 11,042 ± 2000 IU/litre (p<0.001) in rats injected with 0.55g/kg, 0.75g/kg and 1.1g/kg galactosamine respectively (Figure 8.A). However, rats injected with 1.3g/kg galactosamine all died.

Renal function was assessed by creatinine clearance and plasma creatinine levels. Creatinine clearance decreased from 1.06 ± 0.06 ml/min in sham controls to 0.65 ± 0.08 ml/min (p<0.01), 0.56 ± 0.07 ml/min (p<0.01) and 0.46 ± 0.04 ml/min (p<0.01) in animals injected with 0.55g/kg, 0.75g/kg and 1.1g/kg galactosamine, respectively (Figure 8.B). The plasma creatinine levels increased from, 35.5 ± 0.9 μM in sham controls to 54.3 ± 2.9 μM (p<0.01), 58.3 ± 2.2 μM (p<0.01) and 61.0 ± 2.5 μM (p<0.01) (Figure 8.C).
Animals were injected with galactosamine at 0.55 (n=4), 0.75 (n=6), 1.1 (n=6) and 1.3 g/kg (n=4) galactosamine. Sham controls were injected with saline (n=4). Animals were sacrificed after 48 hours. In each experiment the total number of rats was 12. Total numbers being obtained from 2 sets of experiments. The results showed that liver injury (A) and renal impairment (B & C) increased in a dose dependent manner. The alanine transaminases (ALT) were (54 ± 3.8, 5571 ± 1327, 6352 ± 829, 11042 ± 2000) IU/litre; the creatinine clearances were (1.06 ± 0.06, 0.65 ± 0.08, 0.56 ± 0.07, 0.46 ± 0.04) ml/min; and the plasma creatinines were (35.5 ± 0.9, 54.3 ± 2.9, 58.3 ± 2.2, 61.0 ± 2.5) μM for sham controls and those given (0.55, 0.75 and 1.1) g/kg galactosamine respectively. Values are mean ± sem. Data is significantly different from baseline, * (P < 0.05).
The dose of 1.1g/kg galactosamine was then used to determine the time taken, 24, 48 or 72 hours for the onset of liver disease and renal failure to occur. This dose was used as it allowed the rats to develop severe liver injury and renal failure but the animals showed low morbidity and mortality. The animals sacrificed at 24 hours had not developed liver injury or renal failure. Their ALT was 177 ± 5.8 IU/litre, the creatinine clearance was 1.0 ± 0.05 ml/min and plasma creatinine 38 ± 1.2 μM compared to the sham controls which were 58 ± 5.8 IU/litre, 1.16 ± 0.16 ml/min and 35.0 ± 0.6 μM respectively. Those sacrificed at 48 hours had developed severe liver injury and renal failure, with reproducible results. The ALT was 6246 ± 490 IU/litre, with a creatinine clearance of 0.51 ± 0.02 ml/min and serum creatinine 60 ± 2.2 μM (Figure 9.A, B & C). However, those animals that were to be sacrificed at 72 hours all died.
Figure 9 (A, B & C). The determination of the duration for the use of galactosamine.

Animals were injected with 1.1g/kg galactosamine and sacrificed after 24 hours (n=6), 48 hours (n=6) and 72 hours (n=6). Sham controls were injected with saline (n=6) and sacrificed after 72 hours. In each experiment the total number of rats was 12. Total numbers being obtained from 2 sets of experiments. The results showed that those animals sacrificed after 24 hours did not develop liver injury (A) or renal failure (B & C), those sacrificed after 48 hours developed severe liver injury (A) and renal failure (B & C) and those to be sacrificed at 72 hours all died before sacrifice. The alanine transaminases (ALT) were (58 ± 5.8, 177 ± 5.8, 6246 ± 490) IU/litre; the creatinine clearances were (1.16 ± 0.16, 1.0 ± 0.05, 51 ± 0.02) ml/min; and the plasma creatinines were (35 ± 0.6, 38 ± 1.2, 60 ± 2.2) μM for sham controls and those sacrificed after 24 and 48 hours respectively. Values are mean ± sem. Data is significantly different from baseline, * (P < 0.05).
To assess the effects of galactosamine-induced liver failure on the ability of the kidneys to concentrate the urine, a cohort of 8 animals was studied before and after the onset of liver failure, and data analysed using a paired t test. Although urine osmolarity fell in the rats with galn-ALF, it was not significantly different from the sham control group. The urine osmolarity in the sham control group was 1477 ± 258 mosmoles/kg water and in the galn-ALF group after the onset of acute liver failure was 1009 ± 116 mosmoles/kg water (p > 0.05). Indeed, the kidneys retained the ability to concentrate the urine in rats with galn-ALF, and the Urine:Plasma osmolality ratio was 3.1± 0.4 in rats with acute liver failure, and 4.9 ± 0.9 prior to the injection of galactosamine, equating to an osmolar clearance of 36.8 ± 2.6 and 27.6 ± 3.9 ml/day (p > 0.05) respectively. Moreover, the free water clearance was virtually identical before (-20.3 ± 4 ml/day) and after the onset of acute liver failure (-22.0 ± 1.9 ml/day) (p=NS). There was a small and non-significant increase in urine volume in rats with galn-ALF compared with sham controls, 10.3 ± 0.5 cm³ and 8.6 ± 1.1cm³ respectively (p > 0.05). These data confirm that the renal tubular function is intact in the animals that develop acute liver and renal failure.

2.3.2 RELATIONSHIP OF GALACTOSAMINE-INDUCED LIVER INJURY AND RENAL FAILURE

To determine if varying degrees of liver injury correlated with renal impairment, the effect of the different doses and the different exposure times of galactosamine on liver and renal function was investigated. As shown in Figure 10, the degree of liver injury (plasma ALT activity) correlated with the degree of renal failure (serum creatinine) for the 29 animals investigated (R= 0.75, P < 0.05). Results suggest that the severity of renal failure is proportional to the degree of liver injury. The data also shows that some rats given 1.1g/kg galactosamine developed much less severe liver injury and had lower serum creatinine.
The model conditions were therefore set at a dosage of 1.1g/kg galactosamine for 48 hours. These conditions were also used successfully by other groups (Javle et al 1998).

2.3.3 HISTOLOGICAL STUDY OF THE LIVER AND KIDNEY AT THE DETERMINED DOSAGE OF 1.1G/KG GALACTOSAMINE 48 HOURS AFTER ADMINISTRATION

Histological studies of the kidney and liver of sham controls and those injected with 1.1g/kg and sacrificed after 48 hours were examined by light and electron microscopy.

Histological examination by light microscopy of hematoxylin and eosin stained sections of liver confirmed the development of massive hepatocellular necrosis in those animals given galactosamine. Histological examination of the kidneys by hematoxylin and eosin staining showed no abnormality of the renal cortex or medulla in the animals injected with galactosamine (Figures 11.A & B).

Electron microscopy of the kidney tissue revealed that the glomeruli were normal. However, in the proximal tubules the vacuolar system was more prominent, with larger apical vacuoles as well as more prominent vacuoles with flocculent proteinaceous material towards the base of the epithelial cells (Figure 11.C & D).
is a system of vacuoles forming part of the endosome / primary lysozyme / secondary lysozyme system designed to perform the complex cast of endocytosis of macromolecules including proteins from the filtrate. Secondary lysozymes also contribute to autolysosomes of the cells. In the animals injected with galactosamine these vacuoles were more prominent, although the significance of this is not apparent.

Light micrograph (original magnification x 25) of kidney from (A) sham controls and (B) rats with galn-ALF injected with 1.1g/kg galactosamine, at 48hr. Kidney sections were fixed in formalin, paraffin-embedded and stained with haematoxylin and eosin. The kidneys showed no abnormality of the renal cortex or medulla in the animals with galn-ALF. n=6 in both groups. G, glomerulus containing mesangial cells; BC, Bowmans’s capsule; PT, proximal tubules; DT, distal tubule.

Electron micrograph (original magnification x 7500) of (C) kidney from sham controls and (D) kidney from rats with galn-ALF injected with 1.1g/kg galactosamine, at 48hr. Kidney sections were fixed in glutaraldehyde and stained with uranyl acetate and Reynold's lead citrate. In the animals injected with galactosamine the glomeruli were normal. However, in the proximal tubules the vacuolar system was slightly more prominent, with larger apical vacuoles as well as more prominent vacuoles with flocculent proteinaceous material towards the base of the epithelial cells. \( n=6 \) in both groups. N, nucleus; L, lysosome; M, mitochondrion; V, vacuole.
2.3.4 TOXICITY OF GALACTOSAMINE IN CELL CULTURE

![Graph A: Effect of D-galactosamine concentration on HepG2 human hepatoma cells.](image)

![Graph B: Effect of D-galactosamine concentration on LLC-PK1 porcine kidney tubule cells.](image)

**Figure 12.** The effect of different concentrations of galactosamine on (A) HepG2 human hepatoma cells and (B) LLC-PK1 porcine kidney tubule cells. The different cell types were each incubated for 24 hours at 37°C with (0.01, 0.1, 1.0, 10 & 50) mM galactosamine. The LDH released into the supernatant was measured and expressed as a percentage of the total LDH in the cell. Galactosamine showed to be a hepatotoxin, causing the release of LDH in a dose dependent manner. It had no effect on the kidney tubule cells. The data represents the mean ± sem from 3 experiments.
Galactosamine was toxic to HepG2 cells (Figure 12.A) and cytotoxicity was dose dependent. Galactosamine however was not toxic to LLCPK₁ (Figure 12.B). The percentage leakage of LDH in the controls was 9.5% compared to 10.6% in cells treated with 50mM galactosamine. After administration of galactosamine the cells they were tested for viability by exclusion of trypan blue. This revealed < 5% staining indicating viability of the cultured cells in excess of 95%. This together with the relative absence of histological damage effectively excludes a nephrotoxic insult by galactosamine.

2.3.5 HAEMODYNAMICS: MEASUREMENT OF THE MEAN ARTERIAL BLOOD PRESSURE AND RENAL BLOOD FLOW

There was a reduction in the mean arterial pressure in the animals injected with galactosamine compared to sham controls (102 ± 3.8 vs 117 ± 5.9 mmHg, Table 6) but the difference was not significant. There was, however, a significant reduction of renal blood flow compared with sham controls (1.71 ± 0.21 vs 3.0 ± 0.3 ml/min) in rats with galn-ALF at 48 hours post injection (P < 0.0001). When corrected for body mass for rats with galn-ALF and sham controls these correspond to values of 0.49ml/min/100g and 0.83ml/min/100g respectively. Since the overall contribution of a single kidney to total blood flow was unknown, all results are expressed as for a single kidney. Thus, assuming renal blood flow is equal in both kidneys, total renal blood flow was approximately 5.4ml/min (1.66ml/min/100g) in normal animals versus 3.4ml/min (0.98ml/min/100g) in those injected with galactosamine. The renal vascular resistance (mean arterial pressure/ renal blood flow) was significantly increased in the rats with galn-ALF which may be due to increased renal vasoconstriction.
Table 6. Circulatory and renal haemodynamic studies on galn-ALF in rats and sham controls.

Changes in the circulatory and renal haemodynamics 48hr after 1.1g/kg galactosamine injection were measured. Results showed that there was a reduction in mean arterial pressure (MAP) but the change was not significant. The renal blood flow (RBF) and renal vascular resistance (RVR) were significantly decreased and increased respectively in rats with galactosamine-induced liver and renal failure. Values are mean ± sem. Significance values: Arterial pressure of sham controls to animals with galn-ALF, P > 0.05; Renal blood flow and renal vascular resistance of sham controls to rats with galn-ALF, ***P < 0.0001. Con (n=8), GalN (n=7).
2.4 Discussion

In this study the results showed that rats developing galN-ALF had a significant reduction in creatinine clearance and an increase in serum creatinine indicative of renal dysfunction. However, despite the development of renal failure, histologically the kidneys were normal. Moreover, there was no electron microscopic evidence of acute tubular necrosis. The only abnormality observed on electron microscopy examination in the kidney of the animals with galN-ALF was a minor increase in vacuolation in the proximal tubules. Galactosamine was found to be toxic to HepG2 cells but had no effect on LLCPK1 cells. There was a moderate reduction in MAP and a significant decrease in RBF.

The cause and significance of the vacuolation in the kidneys are not yet known. In patients with functional renal failure electron microscopy studies have previously demonstrated proximal tubular necrosis, tubulorrhexis, and mitochondrial dark bodies (Mandal et al 1982) although these changes were considerably more extensive than those observed in this study. Indeed, in one of the original descriptions of this model, it was stated that there was no histological evidence of tissue damage in organs other than the liver (Keppler et al 1968). The small but not statistically significant increase in urinary volume observed following the onset of acute liver failure might suggest that mild tubular injury has occurred, but this is not evident on light microscopy. The presence of increased vacuolation in the proximal tubules observed by electron microscopy could possibly affect the renal function but there is no evidence for this. The kidneys, however, retained the ability to concentrate urine with a three fold concentration above plasma osmolarity and there was no significant change in free water clearance. The observation that urinary sodium excretion decreased in animals with acute liver and renal failure suggests that tubular function is preserved. It may also be due to decreased food intake by animals with liver failure although the animals in this study always ate and had food in their digestive tract. It was difficult to assess food intake as the rat chow is given as a powder and an undetermined proportion is displaced by the rats into the metabolic cages. However, there was no significant fall in animal weight during the 48 hours post galactosamine injection.
The absence of any direct toxic effect of galactosamine on cultured LLCPK₁ cells, a renal tubular cell line, also suggests that galactosamine is not directly nephrotoxic and all available published evidence suggest galactosamine is primarily hepatotoxic. Moreover, concentrations of galactosamine employed in the *in vitro* studies (up to 50mM) far exceed those used in the *in vivo* studies, which are known to be directly toxic to hepatocytes in culture (Wu *et al* 1996a) and to the HepG₂ cell line which was employed this study.

The observation that there was a marked reduction in renal blood flow in this model confirms the findings of Javle and colleagues (Javle *et al* 1998). The reduction in renal blood flow occurs despite an increase in cardiac output and systemic vasodilation, which were also observed in other studies performed in rats with galn-ALF (Makin *et al* 1997). The reduction in renal blood flow and development of a hyperdynamic circulation in this model parallels similar changes observed in patients with acute liver failure who develop renal failure (Ring-Larsen and Palazzo 1981) (Guarnier *et al* 1987) (Epstein *et al* 1977). The reduction in renal blood flow occurred independent of any significant change in arterial blood pressure, and was secondary to renal vasoconstriction. Reduced renal perfusion, under normal circumstances is accompanied by a decrease in salt and water excretion. In this study, there was a small but insignificant increase in urine output, such that fractional excretion of sodium was increased suggesting that there may be some disruption in tubular integrity. RVR was increased in the rats with galn-ALF, indicative of renal vasoconstriction. Systemic infusions of ET-1 caused a marked increase in RVR and a decline in RBF and GFR (Sorensen *et al* 1994). Infusion of ET into the renal artery of the rat decreased RBF GFR and urine volume. MAP did not change implicating a direct action of the peptide on the kidney (Katoh *et al* 1990).

At present there are no reliable models of HRS. Models of renal dysfunction secondary to liver disease include the bile duct ligated rat, rabbit, dog, and baboon (Bloom *et al* 1976) (Gliedman *et al* 1970) (Mullane and Gliedman 1970) (Better and Massry 1972) (Hishida *et al* 1980) (Better *et al* 1980) in which renal blood flow is decreased. In these BDL models there are great species variability in response to the procedure. The degree of jaundice (Bloom *et al* 1976) (Gliedman *et al* 1970) (Mullane and Gliedman 1970) (Better and Massry 1972) (Hishida *et al* 1980) (Better *et al* 1980) and the

Studies on kidney function have been carried out in CCl₄ induced cirrhotic rats. These animals develop cirrhosis after 2-3 months of chronic administration of carbon tetrachloride and subsequently develop ascites with salt and water retention and a slight decrease in GFR (Martin et al 1998b). The CCl₄ model in many respects resembles that of cirrhosis, with salt and water retention, and modest changes in renal function. In this model, however, there is extensive peroxidation of renal lipids. There is a 50 fold increase in the levels of esterified F₂ isoprostanes in the kidney, indicative of lipid peroxidation (Morrow et al 1992c). Although the lipid peroxidation is acute, the renal dysfunction observed in this model may be due to renal injury and not secondary to liver injury.

The GalN model shares many similarities with patients with acute liver failure. These include the development of renal failure secondary to liver injury and the development of a hyperdynamic circulation. As in many patients with acute HRS the histology of the kidney is apparently normal with only very minor alterations such as a slight increase in vacuolation. The model differs from patients with acute HRS in that there is increased urine output rather than oliguria that usually occurs in patients with acute HRS. It is now accepted that patients with acute liver failure together with renal failure have HRS, which has been defined at a consensus conference held at the American Association for the Study of Liver Disease in 1994 as type I HRS (Arroyo et al 1996).

In summary, the current study demonstrates that the galN-ALF model shares many of the characteristics needed to be regarded as a model of type I HRS. Renal failure appears to be secondary to liver failure in that the kidneys are histologically normal and renal blood flow is decreased.
Chapter 3

The Role of Endothelin-1 in the Renal Dysfunction of the Galactosamine-induced acute liver failure Model of Hepatorenal Syndrome
3 The role of endothelin-1 in the renal dysfunction of the galn-ALF model of HRS

3.1 Introduction

Plasma ET-1 concentrations are elevated in patients with severe liver disease and these levels are highest in patients with acute liver failure and HRS (Moore et al 1992) (Uemasu et al 1992) (Moller et al 1993). Ten of eleven patients with HRS had underlying acute alcoholic hepatitis with cirrhosis or acute liver failure. In this study there was a nine fold elevation of plasma ET-1 concentrations in those developing HRS.

Endothelin 1 (ET-1) was initially identified by Yanagisawa et al. and is one of the most potent vasoconstrictors in biological systems (Yanagisawa et al 1988b). Infusion of ET-1 causes renal vasoconstriction and a reduction in GFR (Firth et al 1988) (Cairns et al 1989) (Lopez-Farre et al 1989). Subsequent studies in human volunteers demonstrated that increasing the plasma concentration of ET-1 from basal levels to approximately 10pmol/l caused a significant reduction in renal blood flow, GFR, sodium excretion, and urinary flow rate (Sorensen et al 1994) demonstrating that circulating ET-1 can affect renal function.

Various cells within the kidney, including the vascular endothelial, tubular epithelial, and mesangial cells either express mRNA for ET-1 or can synthesise ET-1 (Uchida et al 1992) (Sakamoto et al 1990) and ET-1 may therefore have a paracrine or autocrine effect.

There are two types of ET-1 receptors, ET\textsubscript{A} and ET\textsubscript{B}. Autoradiography shows that both ET\textsubscript{A} and ET\textsubscript{B} receptors are present in renal arteries and veins and on tubular epithelial cells in human kidney (Karet et al 1993) (Davenport et al 1994b). ET\textsubscript{A} receptors are mainly localized to blood vessels throughout the kidney, particularly arcuate arteries and veins at the corticomedullary junction. The ET\textsubscript{A} receptors found in vascular smooth muscle cells mediate vasoconstriction and cell proliferation. ET\textsubscript{B} receptors are concentrated in the medulla on nonvascular structures such as tubules and collecting ducts (Chow et al 1995) (Terada et al 1992). ET\textsubscript{B} receptors on endothelial cells mediate
vasodilation via nitric oxide (Hirata et al 1993). ET_B receptor initiates a positive autocrine loop by which ET-1 regulates expression of its own gene (Iwasaki et al 1995). In the renal cortex of the rat there is an equal distribution of both ET_A and ET_B receptors whereas ET_B receptors predominate (70%) in the renal medulla (Nambi et al 1992) (Kohan et al 1992).

It was found that exogenous ET-1 and its analogues elicit initial depressor and vasodilator effects by activating ET_B receptors. The subsequent pressor and vasoconstrictor actions of these peptides may involve both ET_A and ET_B receptors (Hiley et al 1989) (Randall 1991) (Bigaud and Pelton 1992) (Clozel et al 1992) (Moreland et al 1992) (Cristol et al 1993) (McMurdo et al 1993) (Warner et al 1993)). These observations suggest that a non-selective ET_A and ET_B receptor antagonist would be more useful than an ET_A receptor antagonist in the study of pathophysiological conditions in which impairment of regional blood flow was due to endogenous endothelin(s). Clozel et al. (Clozel et al 1993) demonstrated the ability of the non-selective ET_A and ET_B receptor antagonist, Bosentan, to inhibit the decrease in renal blood flow following renal ischaemia. The haemodynamic changes of ET-1, ET-2 and ET-3 which are mediated through ET_A and/or ET_B receptors were also effectively antagonised by Bosentan (Gardiner et al 1994).

It has been known for many years that patients with severe liver failure have a reduction in renal blood flow, indicative of renal vasoconstriction, and that this is most marked in those patients who develop HRS (Guarner et al 1987) (Epstein et al 1970). However, studies have demonstrated that other factors must be involved since the decrease in glomerular filtration rate (GFR) is not always proportional to renal blood flow (Ring-Larsen 1977). This suggests that events independent of renal vasoconstriction may be involved in the pathogenesis of renal failure. For example, this may be due to a decrease in the filtration fraction or a decrease in the glomerular capillary ultrafiltration coefficient ($K_f$). For this reason, several studies have focused on mediators that can cause both renal vasoconstriction and contraction of mesangial cells, and thus a reduction in both renal blood flow and $K_f$ (Badr et al 1989).

At the start of this project, no studies had been published on the synthesis of ET-1 or the distribution of the ET_A and ET_B receptors in the kidney in liver disease. Although there
are interspecies variation, it has been determined that the galn-ALF rat model offers a reliable model of HRS, in which an initial investigation into the role of ET-1 in the renal pathogenesis of HRS may be studied.

The aim was to investigate the ET-1 system in the galn-ALF rat model of HRS. Firstly ET-1 levels in the plasma and urine were determined in this model. Subsequently the local synthesis of ET-1 and the distribution of $ET_A$ and $ET_B$ in the different regions of the kidney in rats with galn-ALF and sham controls was measured. Renal function and haemodynamics in this model were further investigated using Bosentan.

3.2 Materials and Methods

3.2.1 MATERIALS

All reagents were purchased from Sigma, Poole, Dorset UK, unless otherwise stated.

3.2.2 ANIMALS

The animals were housed as described in Chapter 2 (2.2.2).

3.2.3 EXPERIMENTAL DESIGN

Rats were placed individually in polycarbonate metabolic cages with free access to food and water. The number of animals per experiment was limited to 12 by the number of cages available. Baseline urine samples were collected 24 hours prior to the study and every 24hr following galactosamine (galn) or saline injection. Animals were sacrificed by exanguination under anaesthesia (60mg/kg Sagatal i.p.), 48 hours following galactosamine or saline injection. Bosentan was administered daily by gavage. Blood samples were either collected into EDTA containing tubes or allowed to coagulate in plain tubes, centrifuged at 800g for 10 mins and the plasma stored at -80°C until analysis. Liver and kidney tissue were collected. No evidence of ascites was seen on opening of the abdominal cavity.
The animals were divided into five groups by treatment. Group 1 (control group, n=12) was given 1ml saline i.p. Group 2 (galn-ALF group, n=12) was administered 1.1g/kg galactosamine i.p. Group 3 (Bosentan controls, n=4) received Bosentan p.o and saline i.p. Group 4 (Bosentan pre-galactosamine, n=8) received Bosentan p.o. 24 hours prior to galactosamine i.p. Group 5 (Bosentan post-galactosamine, n=12) received Bosentan 24 hours after galactosamine.

For the measurements of endothelin-1 synthesis in the different sections of the kidney a separate group of rats was used and in each of the sham control and galn-ALF group, 5 animals were used. A further group of rats was used for the \textit{in vitro} autoradiography studies to determine the distribution of ET$_A$ and ET$_B$ receptors. In each of the sham control and galn-ALF groups, 6 animals were used. These rats were placed in the polycarbonate metabolic cages with free access to food and water and 24 hour urine samples were collected. Blood and urine biochemistry was obtained. Whole kidneys were collected for studies on the regional synthesis of ET-1 and the autoradiography studies.

For the haemodynamic studies (described below) 4 sham controls and 4 rats with galn-ALF were used. The data from this set of animals was combined with the data from the initial haemodynamic studies described in Chapter 2. Therefore the total number of animals for each of the sham controls and the galn-ALF group, n = 12. These rats were also placed in the polycarbonate metabolic cages with free access to food and water and 24 hour urine samples were collected. Tissue and blood samples were not harvested from the rats used in the haemodynamic studies.

3.2.4 TREATMENTS

The sham controls and rats with galn-ALF were prepared as described in Chapter 2 (2.2.4). Bosentan (Ro 47-0203/029), a combined ET$_A$ and ET$_B$ receptor antagonist (Clozel et al 1994) (a gift from Martine Clozel, Actelion Ltd., Allschwil, Switzerland) was administered daily by gavage (p.o) at 100mg/kg in a 5% gum arabic suspension. Sham controls for this treatment received 1ml of 5% gum arabic solution alone. The animals were sacrificed 48 hours after receiving the galactosamine injection.
3.2.5 BIOCHEMICAL STUDIES

The biochemistry of the blood and urine were analyzed as described in Chapter 2 (2.2.6).

3.2.6 ENDOTHELIN-1 MEASUREMENT IN PLASMA

Endothelin was extracted from the plasma of sham controls (n=7) and rats with galn-ALF (n=8) using Amersham International’s AmprepTM 500mg C2 columns (Amersham International, UK). Endothelin-1 was quantified using the Biotrak endothelin-1 inhibition enzyme-linked immunosorbent assay (ELISA) system (Amersham International, UK). Briefly, 1ml of plasma was acidified with 0.25ml 2M hydrochloric acid and loaded onto the column. The column was washed with 2ml of 80% methanol in water and the eluent dried under nitrogen. The pellet was reconstituted in an assay buffer. Endothelin standards (in assay buffer) and the extracted endothelin samples were incubated overnight at 4°C in microtitre wells pre-coated with anti-ET-1 antibody. The plates were washed and the ET-1 bound to the wells was detected with horseradish peroxidase labelled Fab’ fragment of ET-1 antibody conjugate. The amount of peroxidase bound was determined by the addition of enzyme substrate, which developed into a blue colour. The reaction was stopped by adding sulphuric acid, and the Absorbance read at 450nm.

3.2.7 ENDOTHELIN-1 MEASUREMENT IN KIDNEY

A separate group of rats was used for the measurement of endothelin-1 in the kidney and in vitro autoradiography studies. They were treated exactly as before i.e. the sham controls were injected with saline and the experimental group injected with 1.1g/kg galactosamine. The animals were sacrificed after 48 hours. To measure the regional concentration of ET-1, fresh kidneys were dissected into the cortex, inner medulla, outer medulla and pelvis in sham controls (n=6) and rats with galn-ALF (n=6). Sections were immediately frozen in liquid nitrogen until ready for analysis. Weighed samples were boiled in 0.5M acetic acid for 15 minutes, homogenized and centrifuged for 15 minutes.
at 3500g. The supernatants were collected and quantified using an enzyme-linked immunosorbent assay (ELISA) (Bodin et al 1995). ET-1 was coated onto microELISA plates (Beckman, UK) at a concentration of 0.015μg/ml and rabbit antiserum to human ET-1 (CRB, Northwich, UK) was used at a final dilution of 1:25000. The antiserum to ET-1 cross-reacted, 7% with big ET 39, 15% with ET-2 and 100% with ET-3. The protein content in each sample was measured using the Bradford method (Bradford 1976).

3.2.8 IN VITRO AUTORADIOGRAPHY OF KIDNEY SECTIONS

The in vitro autoradiography technique was used to determine the ET-1 binding sites (Dashwood et al 1991) for the kidney (Karet et al 1993). Kidneys were removed from sham controls (n=6) and the rats with galn-ALF (n=6) following anaesthesia and sliced longitudinally in half. Each kidney block included cortex and medulla. The kidney was mounted on cork discs in the embedding medium for frozen tissue, Tissue-Tek O.C.T. compound (Miles Inc., USA), snap-frozen in isopentane pre-chilled in liquid nitrogen and stored at -80°C. Sections 10μm thick were cut on a cryostat and serial sections were thaw mounted onto gelatinized microscope slides. The slide mounted tissue was preincubated for 15 minutes at 22°C in 50mM Tris HCl buffer, pH 7.4 to reduce endogenous peptide levels. The slides were then transferred to the Tris HCl buffer which included 5mM MgCl₂, 0.2% bovine serum albumin (BDH, Poole, UK) and 100 kallikrein inhibiting units/ ml aprotinin (Sigma Chemical Co., Poole, UK). The ETₐ binding sites were identified by incubating the sections with the radioligand [¹²⁵I]-PD151242 (Davenport et al 1994a) and ETₐ with [¹²⁵I]-BQ3020 (Molenaar et al 1992). 100 to 150pmol/L of each compound was used (specific activity 2000Ci/mmol, A.I., UK). The degree of non-specific binding for each radioligand was determined by incubating alternate slides in the presence of 1μM unlabelled ET-1 (Bachem Fine Chemicals, Basel, Switzerland). The sections were washed twice for 10 minutes at 4°C in Tris HCl buffer, dipped in glass-distilled water (4°C), blotted and dried in a stream of cold air. Following incubation the slide mounted sections were placed in X-ray cassettes and exposed to Hyperfilm 3H (A.I. UK) for up to 21 days at 4°C. Receptor binding was assessed densitometrically on a Kontron VIDAS imaging system (Kontron Ltd, Thames, Oxfordshire, U.K) and binding calculated from curves generated by [¹²⁵I]
microscales (Amersham International) that were co-exposed with slide mounted tissue.

3.2.9 HAEMODYNAMICS: EFFECT OF BOSENTAN ON MEAN ARTERIAL PRESSURE AND RENAL BLOOD FLOW IN RATS WITH GALN-ALF

For the haemodynamic studies, the procedure was conducted as described in Chapter 2 (2.2.13). To examine the direct effect of Bosentan on renal blood flow and mean arterial pressure in rats with galn-ALF, Ro 47-0203/001, which is the water soluble sodium salt of Bosentan suitable for intravenous use was infused as a dose of 30mg/kg over 15 mins. There were no significant changes in the hematocrit over the 1 hour period.

3.2.10 STATISTICAL ANALYSIS

Data from each experiment was recorded in Microsoft Excel 97 (Microsoft Corp., USA) spreadsheets and compared using statistical analysis tools in Excel. All the results are expressed as mean ± sem. Statistical analysis was performed using the Student’s unpaired and paired t-tests and the limit of significance taken as P < 0.05, unless otherwise stated.
3.3 RESULTS

3.3.1 EFFECT OF BOSENTAN ON LIVER FUNCTION IN RATS WITH GALN-ALF

Liver injury in rats administered 1.1g/kg galactosamine 48 hours previously was very reproducible. In both the sham control group and the galn-ALF group n=12. All rats given 1.1g/kg galactosamine developed liver injury by 48 hours. This was confirmed by an increase in plasma bilirubin from 0.8 ± 0.1 to 148 ± 6 μmol/litre (P < 0.001) and in alanine transaminase (ALT) from 63 ± 4 to 8857 ± 1057 IU/litre (P < 0.001) in sham controls and rats with galn-ALF respectively (Table 6). Plasma albumin also decreased from 31 ± 1.0 to 28 ± 0.7 g/litre (P < 0.05) in rats with galn-ALF. Treatment of rats with galn-ALF with Bosentan, both prior to or after injection of galactosamine had no significant effect on the severity of liver injury as assessed by ALT or albumin concentrations at 48 hours. The bilirubin decreased in the rats with galn-ALF treated with Bosentan 24 hours after the galactosamine injection but the value still remained elevated compared to the sham control values (Table 7).
Table 7. Serum biochemistry at 48 hours after injection of galactosamine

Rats injected with galactosamine developed severe liver injury with significantly elevated levels of bilirubin, alanine transaminase (ALT) and albumin compared to sham controls. Rats treated with bosentan either 24hr prior to or 24hr post galactosamine injection had a similar degree of liver injury. Groups of rats: Control, Galactosamine (GalN), Bosentan + saline (Bos control), Bosentan given 24hr prior to GalN injection (Bos pre-GalN), Bosentan given 24hr after GalN injection (Bos post-GalN). Values are mean ± sem. *p< 0.05 compared with sham controls, †p< 0.05 compared with the GalN group.

3.3.2 EFFECT OF GALACTOSAMINE ON RENAL FUNCTION AND SODIUM EXCRETION

Renal failure secondary to liver failure in rats injected with 1.1g/kg galactosamine 48 hours previously was also very reproducible. The sham controls and the rats with galn-ALF had similar baseline renal function. Following galactosamine, there was a 50% decrease in creatinine clearance (1.03 ± 0.06 to 0.54 ± 0.03 ml/min, p< 0.001), (Figure 13.A), together with an increase in serum creatinine (38 ± 1 to 66 ± 2 μM, p < 0.01), (Figure 13.B), and serum urea (5.3 ± 0.2 to 9.8 ± 0.8 mmol/l, p< 0.01), (Figure 13.C). Urinary sodium excretion decreased from 1.24 ± 0.18 to 0.80 ± 0.08 mmoles/day, p < 0.05), compared to the sham controls which remained unchanged (from 1.20 ± 0.05 to 1.20 ± 0.07 mmoles/day), (Figure 13.D). Fractional sodium excretion however, increased from 0.56 ± 0.03% in sham controls to 0.78 ± 0.07% in
those injected with galactosamine (p < 0.05), and was accompanied by significant increase in urine volume (11.7 ± 2.2 vs 16.3 ± 2.5 ml/day) (p<0.05).

![Graphs showing changes in creatinine clearance, plasma creatinine, plasma urea, and sodium excretion after injection with galactosamine.](image)

Figure 13. Changes in the levels of (A) creatinine clearance, (B) plasma creatinine, (C) plasma urea and (D) sodium excretion, 48 hours after injection with galactosamine (1.1g/kg).

For sham controls vs galn-ALF group the creatinine clearances were (1.03 ± 0.06 vs 0.54 ± 0.03) ml/min; the plasma creatinines were (38.1 ± 1 vs 66 ± 2) μM; the serum ureas were (5.3 ± 0.2 vs 9.8 ± 0.8) mmole/l; urinary sodium excretions were (1.20 ± 0.05 [baseline] to 120 ± 0.07 vs 1.24 ± 0.18 [baseline] to 0.80 ± 0.08) mmole/day. Sham controls n=8 and Galn-ALF n=8. Values are mean ± sem. Data is significantly different from baseline ** (P < 0.05).
3.3.3 PLASMA ENDOTHELIN-1 CONCENTRATIONS

Plasma endothelin-1 concentrations were significantly increased in rats with galn-ALF (n=8) compared to sham controls (n=7) at 44 ± 8 and 20 ± 4 fmol/ml respectively, (p < 0.05), (Figure 14).

![Figure 14. Plasma endothelin-1 concentrations.](image)

Changes in plasma endothelin-1 in rats injected with saline (n=7) and galactosamine (n=8). Values are mean ± sem. ** Data is significantly different from sham control levels p < 0.05.

3.3.4 RENAL CONCENTRATION AND DISTRIBUTION OF ENDOTHELIN-1

To determine whether there was increased synthesis of ET-1 by the kidneys, freshly harvested kidneys were micro-dissected into their component regions, namely cortex, outer medulla, inner medulla and pelvis. 6 sham controls and 6 rats with galn-ALF were studied. There was, however, no significant difference between the ET-1 levels present in the different regions of the kidney in animals with galn-ALF compared with sham controls although there were clearly regional differences in the formation of ET-1 (Table 8).
Kidney Region | ET-1 concentration Control (n=6) (pmol/g) | ET-1 concentration GalN (n=6) (pmol/g)  
--- | --- | ---  
Cortex | 517 ± 66 | 508 ± 54  
Outer Medulla | 349 ± 82 | 396 ± 55  
Inner Medulla | 1697 ± 286 | 1612 ± 134  
Pelvis | 845 ± 144 | 1085 ± 125  

Table 8. Regional distribution of ET-1 levels in the kidneys of sham controls and rats with galN-ALF.  

Values are given as mean ± sem /g of wet tissue. There were no significant differences between the 2 groups, although there were clearly regional differences in the formation of ET-1.

3.3.5 EXPRESSION OF ET\textsubscript{A} AND ET\textsubscript{B} RECEPTORS IN THE KIDNEY

The expression of the ET\textsubscript{A} and ET\textsubscript{B} receptors in the kidney was determined by autoradiography (Figures 15 and Figure 16 respectively). In both groups of animals there was a greater binding of ET\textsubscript{A} receptor ligand in the inner medulla and pelvis compared to other kidney regions (Figures 15.A & C). The distribution of ET\textsubscript{B} binding was predominantly in the pelvis and the outer cortex in both groups of animals (Figures 16.A & C). ET\textsubscript{A} and ET\textsubscript{B} receptor binding was quantified by densitometric analysis (Figures 15.E and 16.E respectively). A 20% increase in the expression of the ET\textsubscript{A} receptor was observed in the renal cortex of those animals injected with galactosamine (p < 0.05) (Figure 15). No further differences were observed in either ET\textsubscript{A} or ET\textsubscript{B} receptor binding in the other regions of the kidney.
Figure 15.A-D. Effect of acute liver failure on ET₄ receptor expression in the kidney.

Specific binding (total - non specific binding) of ET₄ selective radio-ligand [¹²⁵I]-PD151242 (Graph A-D) in the different sections of the kidney in saline (control) and galactosamine injected rats. Autoradiographs depicting total and non specific binding of [¹²⁵I]-PD151242 in sham controls and rats with galn-ALF.

Figure 15.E. Densitometric analysis of ET₄ receptor binding.

There was a 20% increase in the expression of ET₄ receptor in the renal cortex of those animals injected with galactosamine (Con CTX vs GalN CTX, **p < 0.05). n=5 in both groups. Values are mean ± sem. CTX, cortex; OM, outer medulla; IM, inner medulla; PEL, pelvis.
Figure 16.A-D. Effect of acute liver failure on ET\textsubscript{B} receptor expression in the kidney.

Specific binding (total - non specific binding) of ET\textsubscript{B} selective radio-ligand \[^{[125]}I\]-BQ3020 respectively (Graph E-H) in the different sections of the kidney in saline (control) and galactosamine injected rats. Autoradiographs depicting total and non specific binding of \[^{[125]}I\]-BQ3020 in sham controls and rats with galn-ALF.

![Image of autoradiographs](image)

Figure 16.E. Densitometric analysis of ET\textsubscript{B} receptor binding.

There was no difference in the different sections of the kidney between the sham control group and the group injected with galactosamine. \(n=5\) in both groups. Values are mean ± sem. CXT, cortex; OM, outer medulla; IM, inner medulla; PEL, pelvis.
3.3.6 EFFECT OF BOSENTAN ON RENAL FUNCTION AND SODIUM EXCRETION IN RATS WITH GALN-ALF

All groups of rats had similar baseline renal function. Administration of Bosentan to rats with galn-ALF significantly improved renal function. Creatinine clearance increased from $0.54 \pm 0.03$ ml/min to $0.78 \pm 0.04$ ml/min ($P < 0.05$) in the rats with galn-ALF pre-treated with Bosentan and from $0.54 \pm 0.03$ ml/min to $0.92 \pm 0.05$ ml/min ($P < 0.05$) in animals given Bosentan 24 hours after galactosamine injection (Figure 17.A). Plasma creatinine decreased from $66 \pm 2.0$ μM to $57 \pm 3.0$ μM in rats with galn-ALF pre-treated with Bosentan and from $66 \pm 2.0$ μM to $59 \pm 2.0$ μM in rats given Bosentan 24 hours after galactosamine injection. The improvement in plasma creatinine did not appear to be significant but this may be due to the increase in plasma creatinine in the Bosentan controls of $49 \pm 2.0$ μM compared to the saline controls of $38 \pm 0.8$ μM.

Sodium excretion was not improved in the rats with galn-ALF pre-treated with Bosentan (from $1.3 \pm 0.1$ to $0.9 \pm 0.1$ mmol/day, $P < 0.05$) but was normalised in rats with galn-ALF administered Bosentan 24 hours post galactosamine injection (from $1.3 \pm 0.1$ to $1.4 \pm 0.2$ mmol/day, $P > 0.05$) (Figure 17.B). Sodium excretion in controls treated with Bosentan decreased from $1.29 \pm 0.05$ to $1.06 \pm 0.06$ mmol/day, a finding previously described (Hocher et al 1995).
Figure 17. Effect of Bosentan on (A) creatinine clearance and (B) sodium excretion.

Creatinine clearance (A) decreased significantly in rats with acute liver failure (GalN) compared with sham controls (Con) (p< 0.001**). Treatment with Bosentan, either before (Pre GalN) or after (Post GalN) prevented the development of renal failure compared to galactosamine alone (p<0.05). Urinary sodium excretion (B) improved slightly in the rats injected with galactosamine and pretreated with bosentan and was normalised in rats injected with galactosamine and administered bosentan 24 hours post galactosamine injection. Groups of rats: Con (n=12), GalN (n=12), saline + Bosentan (n=4), Bosentan given 24hr prior to GalN injection (Pre GalN) (n=8), or Bosentan given 24hr after GalN injection (n=12) (Post GalN). Values are mean ± sem.
3.3.7 HAEMODYNAMICS: EFFECT OF BOSENTAN ON MEAN ARTERIAL PRESSURE AND RENAL BLOOD FLOW IN RATS WITH GALN-ALF

There was a significant reduction of renal blood flow in the rats with galn-ALF compared with sham controls (1.8 ± 0.2 vs 2.9 ± 0.2 ml/min) (P < 0.0001). When corrected for body mass they correspond to the values 0.52ml/min/100g and 0.83ml/min/100g for rats with galn-ALF and sham controls respectively. Since the overall contribution of a single kidney to total blood flow was unknown, all results are expressed as for a single kidney. Thus, assuming renal blood flow is equal in both kidneys, total renal blood flow was approximately 5.8ml/min (1.66ml/min/100g) in sham controls versus 3.6ml/min (1.04ml/min/100g) in those with galn-ALF. The differences in renal blood flow occurred even though mean arterial blood pressure was similar in both groups (115 ± 4 mmHg in sham control vs 106 ± 5 in rats with galn-ALF) (Table 9).

Infusion of Bosentan into the femoral vein of rats with galn-ALF did not significantly improve renal blood flow (1.8 ± 0.2 ml/min in rats with galn-ALF vs 2.0 ± 0.4 ml/min in rats with galn-ALF infused with Bosentan). When corrected for body mass, renal blood flow has a value of 0.78ml/min/100g with a total renal blood flow of 5.4ml/min (1.55ml/min/100g). The renal vascular resistance significantly increased in rats with galn-ALF compared with sham controls (68.7 ± 9.0 vs 43.9 ± 4.3 mmHg.ml⁻¹.min⁻¹). Intravenous administration of Bosentan into rats with galn-ALF had no effect on the renal vascular resistance (72.2 ± 14.8 mmHg.ml⁻¹.min⁻¹) (Table 9).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Arterial Pressure mmHg Mean ± sem</th>
<th>Renal Blood Flow ml/min Mean ± sem</th>
<th>Renal Vascular Resistance mmHg.ml⁻¹.min Mean ± sem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=12)</td>
<td>115 ± 3.8</td>
<td>2.9 ± 0.2</td>
<td>43.9 ± 4.3</td>
</tr>
<tr>
<td>GalN (n=12)</td>
<td>106 ± 5</td>
<td>1.8 ± 0.2⁸</td>
<td>68.7 ± 9.0⁸</td>
</tr>
<tr>
<td>Control + Bos (n=4)</td>
<td>113 ± 5</td>
<td>2.7 ± 0.5</td>
<td>46.0 ± 9.0</td>
</tr>
<tr>
<td>GalN + Bos (n=6)</td>
<td>118 ± 7</td>
<td>2.0 ± 0.4⁸</td>
<td>72.2 ± 14.8⁸</td>
</tr>
</tbody>
</table>

Table 9. The effect of Bosentan on the changes in circulatory and renal haemodynamics in rats with galn-ALF.

Values are mean ± sem. ⁸ p < 0.001, Sham controls vs Galn-ALF group.
3.4 Discussion

Plasma ET-1 concentrations increased by twofold in the animals with galN-ALF compared to the normal controls. In the different regions of the kidney there was no change in ET-1 concentrations between the two groups. A 20% increase in the expression of the ET$_A$ receptor was observed in the renal cortex of the animals with galN-ALF. There was no change in ET$_B$ expression. Bosentan normalised the renal functions in animals with galN-ALF.

It has been demonstrated that patients who develop HRS, particularly in the context of acute liver failure or alcoholic hepatitis, have increased circulating concentrations of ET-1 (Moore et al 1992). Further evidence to support a role for ET-1 in the pathogenesis was that administration of BQ123, an ET$_A$ selective antagonist, improved renal function in four patients with HRS (Soper et al 1996). Kramer et al. demonstrated that rats with acute biliary obstruction and renal dysfunction had increased circulating ET-1 concentrations, and that administration of Bosentan (a combined ET$_A$ and ET$_B$ receptor antagonists improved renal function (Kramer et al 1997).

In the animals with galN-ALF the twofold increase in plasma ET-1 and the significant upregulation of the ET$_A$ receptor in the renal cortex together with the findings that Bosentan ameliorates the development of renal failure, strongly suggests that ET-1 has an important role as a determinant of renal function in this model of HRS. Compelling data that seems to exclude the possibility that galactosamine is acting as a nephrotoxic agent are the observations that galactosamine up to the concentration of 50mM is not nephrotoxic to cultured LLCPK$_1$ cells, a renal tubular cell line (Figure 8) and that when Bosentan is given 24 hours following galactosamine, it still protects against the development of renal impairment (Figure 14). However, data from these studies do not exclude the possibility that galactosamine may cause mild renal tubular injury, which may be independent of that causing decreased GFR, although no evidence was found to support this possibility.

The mechanism(s) leading to increased circulating ET-1 concentrations are not well understood. ET-1 is synthesised by a multitude of cell types, including endothelial, hepatic stellate, mesangial, and a variety of smooth muscle cells including vascular
smooth muscle cells. My observation that tissue levels of ET-1 are highest in the renal medulla is consistent with previous studies (Wilkes et al 1991b). The mechanisms leading to increased synthesis of ET-1 in liver disease are under great investigation but it has been postulated to involve endotoxaemia, vasodilation and sheer stress, activation of hepatic stellate cells, and a response to hypovolaemia (Moore et al 1992) (Kramer et al 1997).

One surprising aspect of these studies was the observation that infusion of Bosentan did not acutely increase renal blood flow. This may be for one of two reasons. Firstly, ET-1 may bind avidly with its receptor, and is not easily or rapidly displaced by Bosentan at the doses used. Alternatively, it may be that ET-1 exerts its action on kidney function at the microvascular level, by causing contraction of mesangial cells within the glomeruli and thus decreasing the surface area available for glomerular filtration. Thus ET-1 may cause a reduction in GFR over and above that caused by the reduction in renal blood flow alone. This could dissociate the changes in renal blood flow from those of GFR.

In summary ET-1 appears to be important in the pathogenesis of renal failure as there is an increase in plasma ET-1 concentrations, upregulation of the ET<sub>A</sub> receptor in the renal cortex, and the administration of an ET-1 receptor antagonist improves renal function. These results suggest that ET-1 has a central role in the pathogenesis of the renal dysfunction that occurs in this model of HRS.
Chapter 4

The Role of Oxidative Stress in the Renal Dysfunction of the Galactosamine-induced acute liver failure model of Hepatorenal Syndrome
4 The role of reactive oxygen species in the renal dysfunction of the galn-ALF model of HRS

4.1 Introduction

It has been suggested that reactive oxygen species may be involved in the renal dysfunction of biliary obstruction (Bomzon et al 1997). A six-fold increase in serum thiobarbituric acid reactive substances (a measure of ROS) was observed 48 hours following acute bile duct ligation (Panozzo et al 1995b) (Panozzo et al 1995a). One of the major targets of free radical injury are unsaturated fatty acids, which undergo peroxidation to lipid hydroperoxides and a variety of decomposition products, such as aldehydes or isoprostanes.

Measurement of isoprostanes in the plasma and urine are considered to be one of the most reliable metabolic markers of lipid peroxidation in vivo. Isoprostanes initially identified by Morrow and Jackson (Morrow et al 1992c) are biologically active non-cyclooxygenase derived prostenoids. Measurement of isoprostanes has been used to measure lipid peroxidation in a number of models of free radical injury and to explore the role of free radicals in several disease processes in humans such as the hepatorenal syndrome (Morrow et al 1993), scleroderma (Stein et al 1996), and atherosclerosis (Gopaul et al 1994). They act as potent vasoconstrictors of mesangial cells and in other biological systems. Experimentally they have been shown to cause the release of ET-1 (Fukunaga et al 1995) leading to renal vasoconstriction via a thromboxane A2 like receptor (Takabashi et al 1992).

Patients with HRS may develop endotoxaemia which results in the induction of various pro-inflammatory cytokines including TNFα, IL-1β, IL-6, and γ-interferon (Burke et al 1993) (Khoruts et al 1991). These together with up-regulation of adhesion molecules, cause a marked influx of activated polymorphonuclear neutrophils into the liver, which are sources of reactive oxygen species (Levy and Ruebner 1967). The production of cytokines involves the activation of the redox-sensitive, ubiquitous nuclear transcription factor, nuclear factor κB (NFκB) (Schreck et al 1992) which activates the genes
involved in the inflammatory and immune response (Baeuerle and Henkel 1994). In the liver, endotoxaemia triggers the production of reactive oxygen and nitrogen species (Hewett et al 1992) which may cause lipid peroxidation and disturb the integrity of cellular membranes (Peavy and Fairchild Jr 1986). Thus, the measurement of NFκB in liver and kidney tissue may act as a marker of oxidative stress and lipid peroxidation.

There is evidence that an upregulation of nitric oxide (NO) synthesis occurs in chronic liver disease (Morales-Ruiz et al 1996) (Marley et al 1999) (Heller et al 2000). NO that is synthesized by iNOS is an important component of the nonspecific host defence against bacterial infections (Fang 1997). It has been shown that mice lacking a functional iNOS gene are more susceptible to infection with Staphylococcus aureus (McInnes et al 1998) and Leishmania (Wei et al 1995). To induce iNOS mRNA, activation of NFκB is essential (Diaz-Guerra et al 1996) (Kim et al 1997) (Xie et al 1994) (Kleinert et al 1996) (Taylor et al 1998) although probably not the only activation needed for full iNOS induction (Beck and Sterzel 1996) (Spitsin et al 1997). Rats with biliary cirrhosis were found to have increased levels of plasma nitrite/nitrate, increased expression of inducible nitric oxide synthase (iNOS) in the arteries and increased iNOS activity in the liver (Morales-Ruiz et al 1996) (Marley et al 1999).

The role of antioxidants as therapeutic agents has been well supported in the literature in numerous studies (Halliwell 2001) (Halliwell et al 2000) (Zafari and Harrison 2002). It was found that NAC improves the renal function in a model of acute cholestasis and renal failure (Holt et al 1999b). It was found that the ET-1 mixed receptor antagonist, Bosentan prevented the development of the post ischemic endothelial dysfunction in isolated rat heart (Richard et al 1994) (Hagar 1994) (Li et al 1995) (Szabo et al 1998). ROS have been implicated in the mechanism of post-ischaemic endothelial dysfunction (Mehta et al 1989) (Tsao et al 1990) (Dauber et al 1991) (Gross et al 1992), suggesting an endothelin-mediated production of ROS.

In studies reported in this thesis the effects of the antioxidants NAC and lipoic acid on renal function and the production of isoprostanes, an oxidant stress marker, were evaluated in the galn-ALF model of HRS. The effect Bosentan has on the production of isoprostanes. The induction of NFκB in the liver and kidney were investigated and NO
(nitrites/nitrates) production was also assessed.

4.2 Materials and Methods

4.2.1 MATERIALS
All reagents were purchased from Sigma, Poole, Dorset, UK., unless otherwise stated.

4.2.2 ANIMALS
Animals were housed as described in Chapter 2 (2.2.2).

4.2.3 EXPERIMENTAL DESIGN

Male rats (250–300g) were placed individually in polycarbonate metabolic cages with free access to food and water. Baseline urine samples were collected 24 hours prior to the study and every 24 hours following (Galn) or saline injection. Animals were sacrificed by exsanguination under anaesthesia (60mg/kg Sagatal i.p.) 48 hours after galactosamine or saline injection. Blood samples (serum and plasma) were immediately harvested and stored at -80°C. Liver and kidney tissue, snap frozen in liquid nitrogen were collected for the measurement of F_2-isoprostanes. Tissue was also taken for histology or immunostaining.

4.2.4 TREATMENTS

The sham controls and rats with galn-ALF were prepared as described in Chapter 2 (2.2.4). N-acetylcysteine (NAC) (Parvolex, Evans Medical Ltd, Surrey, U.K.) was administered by i.p. twice daily at a dose of 200mg/kg in 2ml of saline. The sham control group was given i.p. 1ml 0.9% saline. Lipoic acid (LA) (Sigma) was administered to the rats in their drinking water at a dose of 1g/litre 24 hours prior to the galactosamine injection and throughout the experiment.

For the NAC experiments, the animals were divided into five groups by treatment. Group 1, the sham control group, (n=12) was given saline i.p. Group 2, the
The galactosamine group (n=12) were administered galactosamine i.p. Group 3, the NAC controls (n=4) received saline and NAC by i.p. Group 4, the galactosamine and pre-NAC group (n=8) received the initial i.p. injection of NAC, 24 hours prior to galactosamine. Group 5, the galactosamine with NAC group (n=12) received the initial i.p. injection of NAC 24 hours after treatment with galactosamine.

In the lipoic acid experiments, the animals were divided into 3 groups. Group 1, the sham control group (n=12) was given saline i.p. Group 2, the galactosamine group (n=12) was administered galactosamine i.p. Group 3 was the galactosamine and lipoic acid group (n=6). These animals received the lipoic acid in their drinking water at a dose of 1g/litre throughout the experiment and 24 hours prior to the galactosamine injection.

4.2.5 BIOCHEMICAL STUDIES

The biochemistry of the blood and urine were analysed as described in Chapter 2 (2.2.7).

4.2.6 ANALYSIS AND MEASUREMENT OF ISOPROSTANES

Isoprostanes are quantified as free compounds using gas chromatography/ mass spectrometry. Thus, to measure levels of isoprostanes esterified to phospholipids, the phospholipids are first extracted from the tissue sample and then subjected to alkaline hydrolysis to release free isoprostanes. Tissue contains esterified isoprostanes, blood contains both esterified and free isoprostanes and urine contains free isoprostanes.

The procedure for the measurement of isoprostanes involves extraction, purification, derivatisation and quantification by gas chromatography mass spectroscopy (Morrow and Roberts 1994). Given the complexity of the isoprostane family the specific isoprostane 8-iso-PGF$_{2\alpha}$ which is the major isoprostane formed in vivo under conditions of oxidative stress and found in urine and plasma (Roberts et al 1996) was the prostenoid analysed in the samples.
4.2.6.1 General precautions

Biological samples contain low concentrations of eicosanoids. Thus an extensive purification procedure is required before they can be analysed by GC-MS. New disposable tubes were used in the assay to prevent any contamination and masking of the eicosanoids. The pH of samples should not exceed the limits of 3-10 as eicosanoids are chemically labile compounds and are susceptible to degradation. Also high temperatures can have a marked effect on eicosanoid recovery so samples should, where possible, not be heated to greater than 30°C.

4.2.6.2 Extraction of esterified isoprostanes

Tissue samples were snap frozen with a clamp in liquid nitrogen. Samples were stored at -70°C until ready to be assayed. To assay for isoprostanes 200-300mg of tissue was placed in a 50ml polypropylene tube kept on ice. 12mls of ice cold Folch solution [chloroform:methanol 2:1 containing 5mg/100ml butylated hydroxytoluene (BHT) which acts as an antioxidant] was added and the tissue homogenised with an electric homogeniser. The homogenates were vortexed, allowed to stand for 30 mins and centrifuged for 10 mins at 2,000g. Centrifugation resulted in three layers, the upper aqueous layer, the middle particulate layer and the lower organic layer containing the lipids and related compounds. The organic layer was carefully removed without disturbing the particulate layer and dried under nitrogen in a water bath at 30°C. Methanol (0.5mls containing BHT 5mg/100ml) was added, and vortexed to resuspend the lipids.

Base hydrolysis to release free isoprostanes was performed by adding 2mls of 15% potassium hydroxide and incubating the solution at 45°C for 1 hour. The pH was then corrected back to 3.0 with 1M HCl and the solution was diluted to 15mls with pH 3.0 water (very dilute acetic acid at pH 3.0). To the lipid solution the internal standards of 1ng [²H₄]8-iso-PGF₂α and 5ng [²H₄]PGF₂α (Cayman Chemical, MI, USA) were added.

4.2.6.3 Extraction and purification of free isoprostanes

A series of chromatography steps were performed to purify the free isoprostanes. The first step was solid phase chromatography followed by thin layer chromatography.
In solid phase chromatography, tC18 Sep-Pak columns (Waters Corporation, MA, USA) were used to remove water soluble and non-polar organic compounds. The columns were prepared by washing with 6mls of methanol followed by 6mls pH 3.0 water. The sample was then loaded onto the column and allowed to slowly pass through. The column was then re-washed with 6mls pH 3.0 water to remove water soluble compounds. At this pH the isoprostanes are uncharged and are bound to the solid phase. The column is washed with 6mls heptane to differentially remove the less polar lipids. The bound isoprostanes are eluted with 6mls heptane/ethyl acetate/methanol (40:50:10, v/v). The eluent was dried under nitrogen at 30°C in a water bath and the residue resuspended in 80μl ethanol and diluted with ethyl acetate. A further column purification step was performed with a silica column (Waters Corporation, MA, USA). The columns were prepared by washing with 6mls ethyl acetate. The sample was loaded onto the column and allowed to pass through slowly. The column was re-washed with 6mls ethyl acetate. The isoprostanes were eluted with ethyl acetate/methanol (60:40, v/v) and dried under nitrogen.

Isoprostanes from plasma samples were derivatized at this stage while those from urine required an extra TLC purification step. In TLC for urine chromatography plates (Whatman Silica Gel 60A, Linear K6D, 5 x 20cm, 250μm thick) were pre-conditioned in methanol for approximately one hour and then allowed to air dry. The chromatography tank was prepared one hour before to allow the tank to equilibrate with the TLC solvent mixture of chloroform/methanol/glacial acetic acid/water (86:14:1:0.8, v/v/v/v). The dried samples were resuspended in 40μl of chloroform/methanol (2:1, v/v), spotted onto the TLC plates and placed in the TLC solvent tank. A standard of 5μg PGF2α was spotted onto a separate plate and run at the same time as the samples. The plates were run approximately 13cm above the area of sample application and allowed to air dry for 5 minutes. The plate containing the PGF2α was sprayed with 10% phosphomolybdic acid in ethanol and placed on a hot plate to allow the standard to be visualised as a blue/black band.

An area of 1.5cm above and below the standard was scraped from the plates containing the samples onto wax paper and the silica transferred to microcentrifuge tubes. 900μl of ethyl acetate/methanol (50:50, v/v) was added, vortexed and left on a rotating wheel for
10 mins to extract the isoprostanes. The tubes were then centrifuged in a microcentrifuge and the organic layer containing the isoprostanes removed, placed in 1ml glass vials and dried under nitrogen.

**4.2.6.4 Derivative formation**

To form the pentafluorobenzyl (PFB) ester derivative 20µl of 10% (v/v) DIPEA (N,N-diisopropylethylamine) in acetonitrile and 40µl of 10% (v/v) PFBR (pentafluorobenzylbromide) in acetonitrile were added. This procedure was performed in the fume cupboard as the solutions were highly volatile and are toxic. The vials were capped and left at room temperature for 45 minutes and then dried under nitrogen. This procedure was repeated to ensure quantitative esterification. After the second esterification step, the reagents were dried under nitrogen and the residues resuspended in 40µl of chloroform/ methanol (2:1, v/v).

The samples were spotted onto the TLC plates and placed in the TLC solvent tank containing chloroform/ ethanol (93:7, v/v). A standard of 3µg of the methyl ester of PGF$_{2\alpha}$ is spotted onto a separate plate and chromatographed alongside the samples of PFB esters. The plates are run to approximately 13cm above the spotting area. The standard is visualised by spraying the plate with 10% phosphomolybdic acid in ethanol and placing it on a hot plate to allow the standard to develop as a dark blue band. The silica from the chromatographed plates are scraped 1cm above and below the standard into waxed paper, transferred to polypropylene tubes and 900µl ethyl acetate added. The tubes were placed on a rotating wheel for 5 minutes and then centrifuged in a microfuge at 12000 rpm for 5 minutes. The ethyl acetate was then transferred to clean tubes and dried under nitrogen. The methyl ester of PGF$_{2\alpha}$ is chromatographed as the standard rather than the PFB ester because any contamination of the sample being analysed with the methyl ester of PGF$_{2\alpha}$ will not interfere with the analysis owing to the fact that the isoprostanes are analysed as PFB esters.

The isoprostanes are then converted to trimethylsilyl (TMS) ether derivatives by adding 20µl N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 10µl anhydrous dimethylformamide (DMF) for 30 minutes under argon. The samples are then dried under nitrogen and resuspended in 10µl of anhydrous undecane. Each sample is then
transferred to a conical sampler vial and sealed under argon ready to be analysed by gas chromatography-mass spectrometry (GC-MS). A summary of the technique for the identification of F$_2$-isoprostanes in biological samples is depicted in Figure 18.

Selective ion monitoring was performed at m/z 569 for F$_2$-isoprostanes and at m/z 573 for the deuterated [H$_4$]PGF$_{2\alpha}$ internal standard. A chromatogram of 8-iso-PGF$_{2\alpha}$ and PGF$_{2\alpha}$ and the deuterated derivatives is depicted in Figure 19.
Figure 18. Summary of the technique for the identification of F₂-isoprostanes in biological samples.

The technique involves a series of extraction, purification, derivatisation and gas chromatography mass spectrometry procedures.
Figure 19. A GC-MS chromatogram of 8-iso-PGF\textsubscript{2\alpha} and PGF\textsubscript{2\alpha} and the deuterated derivatives.

Negative ion chemical ionisation with ammonia was used to determine the mass spectrum. Selective ion monitoring at 569 and 573 m/z was performed, as these were the masses of the predominant fragments of the undeuterated and deuterated forms of the isomers respectively. 8-iso-PGF\textsubscript{2\alpha} retention time is 8.336 and PGF\textsubscript{2\alpha} retention time is 8.486 for the undeuterated isomers with 8-iso-PGF\textsubscript{2\alpha} coming off the column first.

4.2.7 MEASUREMENT OF NF\textsubscript{κ}B ACTIVATION

The activation of NF\textsubscript{κ}B in the liver and kidney was assessed by the electromobility gel shift assay (EMSA) (Promega, Southampton, UK). NF\textsubscript{κ}B translocates from the cytoplasm into the nucleus upon activation. The principle of EMSA is to bind the NF\textsubscript{κ}B in the nuclear extracts to radiolabelled DNA. Gel electrophoresis is performed on the NF\textsubscript{κ}B-DNA complex and visualised by autoradiography. The NF\textsubscript{κ}B-
radiolabelled DNA complex migrates more slowly through a non-denaturing gel than the unbound radiolabelled DNA and it appears as a retarded band upon autoradiography. The EMSA performed was a modified method described by Manning et al. (Manning et al. 1995).

4.2.7.1 Preparation of nuclear extracts

Tissue samples (200 – 250mg) were rinsed three times in ice-cold Ca\(^{2+}\)- and Mg\(^{2+}\)-free phosphate buffered saline (PBS). The samples were homogenised in 3ml ice-cold Buffer A [10mM HEPES, pH 7.9, 1.5 mM MgCl\(_2\), 10mM KCl, 0.5mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Nonidet P-40] with a Dounce homogeniser. The homogenate was transferred to a polypropylene centrifuge tube, incubated on ice for 10 mins and then centrifuged at 850g for 10 min at 4°C. The cell pellet was suspended in 4ml ice-cold Buffer B (Buffer A minus NP-40) and centrifuged at 850g for 10 min. The crude nuclear pellet was rinsed once with buffer B and re-suspended in 150µl Buffer C (20mM HEPES, pH 7.9, 25% [v/v] glycerol, 0.42M NaCl, 1.5mM MgCl\(_2\), 0.2mM EDTA, 0.5mM DTT and 0.5mM PMSF) and then incubated on ice for 30 min. The suspension was centrifuged at 100,000g for 20 min at 4°C in a Kontron TFT 65.13 ultracentrifuge. The resulting supernatant containing the nuclear proteins was carefully removed. The protein concentrations of the nuclear extracts were determined using the Bradford assay (Bradford 1976) and diluted to a concentration of 1mg/ml.

4.2.7.2 Preparation of the gel

Polyacrylamide gels were prepared using the solutions shown in Table 10. The gels were 7% acrylamide and 3mm thick instead of 4% acrylamide and 1mm thick as suggested in the protocol. This was to enable a better separation to occur and more protein sample to be loaded onto the gel.
Reagents | Volume  
---|---
5 x TBE | 30ml  
Acrylamide/bisacrylamide solution (30%) | 69ml  
Double distilled water | 201ml  
Ammonium persulphate (10%) | 720μl  
TEMED | 300μl  

Table 10. Formulation for two large gels.  
*(Anachem Ltd., Luton, Bedfordshire).*

The acrylamide solution consisted of 30% (w/v) acrylamide/bisacrylamide (37.5:1) and the buffer used was TBE (Tris borate EDTA buffer pH 8.3). Ammonium persulphate (APS) provides the free radicals that drives the polymerisation of acrylamide and bisacrylamide. TEMED (*N, N, N, N*-tetramethylethylenediamine) accelerates the polymerisation of acrylamide and bisacrylamide by catalysing the formation of free radicals from APS. All reagents were purchased from Anachem Ltd., Bedfordshire.

The dual vertical polyacrylamide gel system (20.5cm x 20cm) from Anachem was used. The apparatus was assembled and the gel solution prepared using the volumes given in Table 10. The gel solution was carefully poured between the plates. Any bubbles formed in the gel solution were expelled from the surface by gently tapping the plates.

**4.2.7.3 Electromobility shift assay**

EMSA was performed according to the manufacture’s protocol (Promega Corp., WI, USA). Double stranded NFκB consensus oligonucleotide probe (5'-AGTTGAGGGGACTTTCCCAGGC-3') (Read *et al* 1994) was end-labelled with γ[^32]P]ATP (50μCi at 222TBq/mmol, Amersham Pharmacia Biotech UK Ltd, Buckinghamshire) (Sambrook *et al* 1989). This was achieved by incubating together for 10 min at 37°C, 2μl consensus oligonucleotide (1.75pmol/μl), 1μl T4 polynucleotide
kinase 10x buffer (700mM Tris-HCl, pH 7.6, 100mM MgCl₂, 50mM DTT), 1μl γ³²P]ATP (3000Ci/mmol at 10mCi/ml), 5μl double distilled water and 1μl T4 polynucleotide kinase (10u/μl). The reaction was stopped by the addition of 1μl of 0.5M EDTA. The volume was made up to 100μl by adding 89μl of TE buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA). The radiolabelled oligonucleotide was stored in the freezer at -20°C.

### 4.2.7.4 Preparation of the reactions

The binding reactions were prepared in sterile microtubes. The binding reaction mixture is as given in Table 11. For the positive control, HeLa nuclear extract (5mg/ml) was used (Table 11). In competition experiments, 1μl unlabelled oligonucleotide was added to the reaction mixture. For the “supershift” analysis 1μg of either anti-p50 or anti-p65 antibody (1mg/ml) (Promega, Southampton, UK) was added to the reaction mixture immediately after the addition of the radiolabelled probe. 2μl of gel shift binding 5x buffer [20% glycerol, 5mM MgCl₂, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, 50mM Tris-HCl, pH 7.5, 0.25mg/ml poly (dl-dC)poly(dl-dC)] was added in each reaction. The reactions were incubated at room temperature for 10 mins. ³²P-labelled NFκB consensus oligonucleotide probe (1μl) was added and the reactions incubated on ice for 1 hour. The reactions were stopped by the addition of 1μl of 10x gel loading buffer (250mM Tris-HCl, pH 7.5, 0.2% bromophenol blue, 0.2% xylene cyanol, 40% glycerol). The reactions were then subjected to electrophoresis on a 7% non-denaturing polyacrylamide gel. The gels were vacuum dried and exposed to X-ray film (Hyperfilm MP, Amersham Pharmacia Biotech UK Ltd, Buckinghamshire) at -80°C for between 15 to 24 hours.
<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th>Positive Control</th>
<th>Competition Mixture</th>
<th>Supershift</th>
</tr>
</thead>
<tbody>
<tr>
<td>6µl nuclear extract</td>
<td>2µl HeLa nuclear extract</td>
<td>6µl nuclear extract</td>
<td>6µl nuclear extract</td>
</tr>
<tr>
<td>2µl gel shift binding 5x buffer</td>
<td>2µl gel shift binding 5x buffer</td>
<td>2µl gel shift binding 5x buffer</td>
<td>2µl gel shift binding 5x buffer</td>
</tr>
<tr>
<td>1µl distilled H₂O</td>
<td>5µl distilled H₂O</td>
<td>1µl distilled H₂O</td>
<td>1µl distilled H₂O</td>
</tr>
</tbody>
</table>

Table 11. Binding reaction mixtures for EMSA.

4.2.8 NITRIC OXIDE ANALYSER

The concentration of NO in the samples was determined indirectly using the nitric oxide analyser (NOA) (Sievers Instruments, Analytix Limited, UK). A current is produced by a photomultiplier gas-phase chemiluminescent reaction.

4.2.8.1 Measurement of nitrate/nitrite

The method used for the measurement of NO was a modified method by Grisham et al. (Grisham et al. 1996). The method requires that NO₃⁻ be reduced to NO₂⁻ by a nitrate reductase. NO₂⁻ is then reduced to NO in the NOA by KI (I₂ + NO₂⁻ 4H⁺ → 2NO + I₂ + 2H₂O). Reduction of NO₃⁻ to NO₂⁻ is performed using Aspergillus nitrate reductase (Boehringer Mannheim, Indianapolis, IN, USA) with cofactors. The plasma sample (100µl) was added to 0.04 U/ml Aspergillus nitrate reductase along with its cofactors, 80µM nicotinamide adenine dinucleotide phosphate (NADPH), 8µM flavine adenine dinucleotide (FAD), 20mM Tris buffer (pH 7.6), in a total volume of 500µl. The cocktail was then incubated for 1 hour at room temperature. 30µl of the reaction mixture was then injected into the NOA. Care was taken to make sure all of the sample was injected into the reducing agent (1% wt/vol KI in acetic acid) in the NOA chamber. Injection of the sample into the reaction chamber results in the release of NO and in a chemiluminescent reaction which is recorded on the monitor as a curve. The area under the curve or the height of the curve is a measure of the amount of NO present.
in the sample. Prior to the sample being measured the NOA was calibrated for the measurement of nitrite and nitrate. Standards ranging from 1-100 μM NaNO₂ were measured in the NOA to produce a standard curve for nitrites. Standards ranging from 1-100 μM NaNO₃ were measured to produce a standard curve representing nitrates. The nitrate in the standard NaNO₃ solution is converted to nitrite by nitrate reductase. The amount of nitrate converted to nitrite in the sample by the nitrate reductase was determined using the calibration curve (Figure 20).

![Image of calibration curves for nitrites and nitrates]

Figure 20. Calibration curve of nitrites and nitrates.

Calibration of the NOA with the standard solutions of sodium nitrite and sodium nitrate showed that approximately 100% of the nitrate standards were converted to nitrite.

4.2.9 STATISTICAL ANALYSIS

Data from each experiment was recorded in Microsoft Excel 97 (Microsoft Corp., USA) spreadsheets and compared using statistical analysis tools in Excel. All the results are expressed as mean ± sem. Statistical analysis was performed using the Student’s unpaired and paired t-tests and the limit of significance taken as P < 0.05, unless otherwise stated.
4.3 Results

4.3.1 EFFECT OF N-ACETYLCYSTEINE AND LIPOIC ACID ON LIVER FUNCTION OF RATS WITH GALN-ALF

As described previously in section 3.3.1 rats injected with 1.1g/kg galactosamine, developed liver injury by 48 hours. This was confirmed by an increase in plasma bilirubin from 0.8 ± 0.3 to 143 ± 7 μmol/litre (P < 0.001) and in alanine transaminase (ALT) from 64 ± 4 to 8528 ± 1213 IU/litre (P < 0.001) in sham controls and rats with galn-ALF respectively (Table 1). Plasma albumin decreased from 30 ± 0.6 to 28 ± 0.9 g/litre (P < 0.05). Administration of NAC (i.p. twice daily 200mg/kg) 24 hours prior to injection of galactosamine showed a trend towards improvement of liver function at 48 hours. There was a decrease in plasma bilirubin but the ALT and albumin levels remained unchanged. The administration of NAC 24 hours after the galactosamine injection also showed a trend towards improvement of liver function at 48 hours. Plasma bilirubin and albumin both decreased but these values remained significantly elevated compared to sham control values. There was no change in the ALT levels in either group. The animals with galn-ALF that received lipoic acid (1g/litre in drinking water) showed a fall in serum bilirubin and albumin values but remained significantly elevated compared to sham control values (Table 12). ALT levels remained unchanged.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bilirubin $\mu$mol/litre</th>
<th>ALT $lU/litre$</th>
<th>AST $lU/litre$</th>
<th>Albumin G/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=12)</td>
<td>0.8 ± 0.3</td>
<td>64 ± 4.2</td>
<td>122 ± 4.8$^a$</td>
<td>30 ± 0.6</td>
</tr>
<tr>
<td>GalN (n=12)</td>
<td>143 ± 7$^a$</td>
<td>8528 ± 1213$^b$</td>
<td>7969 ± 1056$^a$</td>
<td>28 ± 0.9$^a$</td>
</tr>
<tr>
<td>NAC (n=4)</td>
<td>0.5 ± 0.0</td>
<td>49 ± 2</td>
<td>90 ± 7</td>
<td>29 ± 0.6</td>
</tr>
<tr>
<td>NAC pre-GalN (n=6)</td>
<td>90 ± 8$^{ab}$</td>
<td>7488 ± 905$^a$</td>
<td>7495 ± 1095$^a$</td>
<td>27 ± 1$^a$</td>
</tr>
<tr>
<td>NAC post-GalN (n=7)</td>
<td>81 ± 7$^{ab}$</td>
<td>6780 ± 619$^a$</td>
<td>6469 ± 642$^b$</td>
<td>24 ± 1$^{ab}$</td>
</tr>
<tr>
<td>LA pre-GalN (n=6)</td>
<td>79 ± 1$^{ab}$</td>
<td>5221 ± 1208$^a$</td>
<td>4799 ± 988$^a$</td>
<td>24 ± 0.9$^{ab}$</td>
</tr>
</tbody>
</table>

Table 12. Plasma hepatic biochemistry at sacrifice, 48 hours after galactosamine injection.

Groups of rats: Control, Galactosamine (GalN), NAC + saline (NAC control), NAC given 24 hours prior to GalN injection (NAC pre-GalN), NAC given 24 hours after the GalN injection (NAC post-GalN), LA given 24 hours prior to the GalN injection (LA + pre-GalN). Values are mean ± sem. $^a$p< 0.05 compared with sham controls, $^b$p< 0.05 compared with GalN group.

### 4.3.2 EFFECT OF N-ACETYLCYSTEINE AND LIPOIC ACID ON RENAL FUNCTION AND SODIUM EXCRETION IN RATS WITH GALN-ALF

All groups had similar baseline renal function before treatment. Creatinine clearance in sham controls was 1.04 ± 0.06 ml/min and this decreased to 0.56 ± 0.03 ml/min in rats with galn-ALF (P < 0.001). The administration of NAC to rats injected with galactosamine resulted in the normalisation of renal function. Creatinine clearance in the rats with galn-ALF treated with NAC 24 hours prior to the galactosamine injection significantly improved (0.96 ± 0.1 ml/min) and in the rats with galn-ALF treated with NAC 24 hours after the galactosamine injection remained normal (1.09 ± 0.09 ml/min) (Figure 21.A). NAC given to controls showed an elevation in creatinine clearance (1.43 ± 0.1 ml/min). Serum creatinine levels decreased following NAC suggesting an improved renal function. In sham controls serum creatinine was 38 ± 0.9 µM and significantly increased to 65 ± 2.2 µM in rats with galn-ALF. In rats with galn-ALF
treated with NAC 24 hours prior to the galactosamine injection and rats with glan-ALF treated with NAC 24 hours after the galactosamine injection serum creatinine decreased to 47 ± 3.0 µM and 44 ± 2.0 µM respectively (Figure 21.B).

NAC improved urinary sodium excretion in rats with glan-ALF. The sodium excretion was normalised from 1.16 ± 0.18 to 1.22 ± 0.13 mmol/day, (p = NS) in the rats with glan-ALF pre-treated with NAC and from 1.00 ± 0.1 to 1.16 ± 0.17 mmol/day, (p = NS) in the rats with glan-ALF given NAC 24 hours post galactosamine injection. In the rats with glan-ALF which did not receive NAC sodium excretion decreased from 1.34 ± 0.2 to 0.86 ± 0.11 mmol/day.
Figure 21. Effect of NAC on renal impairment in rats with galN-ALF.

Creatinine clearance (A), serum creatinine (B), and urinary sodium excretion (C) showed a normalisation of renal function in rats with galN-ALF on treatment with NAC. Values are given as mean ± sem. ** P < 0.05.
The addition of LA to the drinking water of galactosamine injected rats significantly improved their renal function. Serum creatinine and creatinine clearance were both normalised. Creatinine clearance increased from 0.56 ± 0.03 ml/min in rats with galn-ALF to 0.84 ± 0.07 ml/min in rats with galn-ALF treated with LA in their drinking water (Figure 22.A). Serum creatinine decreased from 65 ± 2.2 μM in rats with galn-ALF to 51 ± 2.5 μM in rats with galn-ALF given LA (Figure 22.B). Urine sodium excretion was however still depressed after LA (Figure 22.C).
Figure 22. Effect of LA on the renal impairment in rats with galn-ALF.

Creatinine clearance (A), serum creatinine (B) and showed an improvement in renal function in rats with galn-ALF given LA. Urinary sodium excretion (C) in the rats with galn-ALF given lipoic acid did not normalise after 48 hours. Values are given as mean ± sem. ** P < 0.05.

4.3.3 EFFECT OF GALACTOSAMINE ON THE PRODUCTION OF URINARY F₂-ISOPROSTANES

The urinary isoprostanes excretion increased by fifteen fold from 11.4 ± 0.5 ng/day in the sham controls to 170 ± 45 ng/day in the rats with galn-ALF (P < 0.001), indicative of lipid peroxidation (Figure 23).

Figure 23. Urinary F₂-isoprostanes excretion in sham controls and rats with galn-ALF.

F₂-isoprostanes increased significantly in the rats with galn-ALF (n=4) compared to the sham controls (n=4). Values are given as mean ± sem. ** P < 0.001.
4.3.4 EFFECT OF GALACTOSAMINE ON THE PRODUCTION OF PLASMA $F_2$-ISOPROSTANES

The plasma isoprostanes also showed a two to three fold increase from 55 ± 6 pg/ml in the sham controls to 134 ± 20 ng/day in the rats with galn-ALF ($P < 0.001$), which is indicative of lipid peroxidation (Figure 24).

![Figure 24](image)

Figure 24. Plasma $F_2$-isoprostanes in sham controls and rats with galn-ALF. $F_2$-isoprostanes increased significantly in the rats with galn-ALF ($n=6$) compared to the sham controls ($n=4$). Values are given as mean ± sem. ** $P < 0.01$.

4.3.5 PRODUCTION OF ESTERIFIED $F_2$-ISOPROSTANES IN THE LIVER AND THE KIDNEY

As the rats injected with galactosamine develop both acute liver injury and renal failure, the levels of $F_2$-isoprostanes were measured in both of these organs to determine the possible site of oxidative stress and lipid peroxidation. Results showed that the level of isoprostanes were elevated in the kidneys of the rats with galn-ALF (Figure 25.B). The level of isoprostanes in the livers of the rats with galn-ALF, although elevated did not show a significant increase compared to sham controls (Figure 25.A). The number of livers assayed needs to be increased to give a more representative value.
Figure 25. Esterified isoprostanes in (A) the liver and (B) the kidney in sham controls and rats with galn-ALF.

Isoprostane levels in the liver were 14.2 ± 2.2 ng/g in sham controls (n=4) and 17.5 ± 2.8 in rats with galn-ALF (n=5) (not significant). In the kidney, isoprostane levels were 3.0 ± 0.9 in sham controls (n=7) and 5.2 ± 0.2 in rats with galn-ALF (n=8) **p < 0.05. Values are given as mean ± sem.

4.3.6 EFFECT OF N-ACETYLCYSTEINE AND LIPOIC ACID ON THE PRODUCTION OF F₂-ISOPROSTANES IN PLASMA AND URINE

NAC and lipoic acid both improved the renal function in the rats with galn-ALF. Although the mechanisms through which NAC and lipoic acid act, are not fully understood F₂-isoprostanes were measured to determine whether either NAC or lipoic acid improved renal function by scavenging the reactive oxygen species and thus reducing oxidative stress. Treatment with NAC in the rats with galn-ALF resulted in an
increase in plasma isoprostane levels and in urinary isoprostanes excretion (Figure 26.A & B respectively). This may indicate that NAC increases the excretion of isoprostanes from the tissues perhaps by increasing the activity of phospholipases and thereby protecting cell membrane activity. The addition of LA to the drinking water before the injection of galactosamine resulted in a reduction in the plasma isoprostane levels from 214 ± 46 pg/ml in rats with galn-ALF to 162 ± 32 pg/ml in rats with galn-ALF treated with LA, although this reduction did not reach statistical significance (Figure 27.A). There was however a greater fall in the urinary isoprostanes. Isoprostane levels fell significantly (p < 0.05) from 155 ± 14 pg/ml in rats with galn-ALF to 97 ± 20 pg/ml in rats with galn-ALF given LA (Figure 27.B).

Figure 26.A. Effect of NAC on the production of plasma isoprostanes in rats with galn-ALF.

NAC resulted in an increase in the production of plasma isoprostanes in animals with galn-ALF. The levels of plasma isoprostane were, 55 ± 6 pg/ml for the sham controls (n=4), 134 ± 20 pg/ml for rats with galn-ALF (n=7), 50 ± 6 pg/ml for the rats given saline and NAC (n=4), 239 ± 66 pg/ml for the rats with galn-ALF given NAC 24 hours prior to the galactosamine injection (n=4) and 278 ± 22 pg/ml for the rats given NAC 24 hours after the galactosamine injection (n=4). Values are given as mean ± sem, **p < 0.05.
Figure 26.B. Effect of NAC on the production of urinary isoprostane excretion in rats with galn-ALF.

NAC had no effect on the production of urinary F2-isoprostane excretion in animals with galn-ALF. The levels of urinary isoprostane excreted were, 11.4 ± 0.49ng/day for the sham controls (n=4), 170.0 ± 45.4ng/day for the rats with galn-ALF (n=4), 8.3 ± 0.8ng/day for the rats given saline and NAC (n=4), 145.6 ± 38.8ng/day for the rats given NAC 24 hours prior to the galactosamine injection (n=5) and 215.3 ± 31.1ng/day for the rats given NAC 24 hours after the galactosamine injection (n=5). Values are given as mean ± sem, ** P < 0.05.

Figure 27.A. Effect of LA on the production of plasma isoprostanes in rats with galn-ALF.

Treatment with LA resulted in a reduction in the level of isoprostanes in the plasma of rats with galn-ALF. The decrease was not significant. The levels of plasma isoprostane were, 105 ± 1.5 pg/ml for the sham controls (n=8), 214 ± 46.3 pg/ml for the rats with galn-ALF (n=7) and 162 ± 32 pg/ml for the rats with galn-ALF fed LA in their water (n=6). Values are given as mean ± sem, ** P < 0.01 for Con vs GalN; * P < 0.05 for Con vs LA pre-GalN.
Figure 27.B. Effect of LA on the production of urinary isoprostane excretion in rats with galn-ALF.

LA resulted in a significant reduction of isoprostanes in the urine. The levels of urinary isoprostane excreted were, 11.5 ± 0.6 ng/day for the sham controls (n=8), 155.0 ± 13.65 ng/day for the rats with galn-ALF (n=7), and 97 ± 20 ng/day for the rats with galn-ALF fed LA in their water (n=6). Values are given as mean ± sem, ** P < 0.01.

4.3.7 EFFECT OF BOSENTAN ON THE PRODUCTION OF F₂-ISOPROSTANES IN PLASMA AND URINE

Bosentan improved the renal function in the rats with galn-ALF (Figures 17.A & B). (Figure 28.A & B respectively). The administration of Bosentan to rats with galn-ALF resulted in a reduction in the plasma isoprostane levels in rats with galn-ALF pre-treated with Bosentan and in rats with galn-ALF post-treated with Bosentan. The reductions did not reach statistical significance (Figure 28.A). There was also a reduction in the urinary isoprostanes in rats with galn-ALF on treatment with Bosentan (Figure 28.B), although these reductions did not reach statistical significance.
Figure 28.A. Effect of Bosentan on the production of plasma isoprostanes in rats with galN-ALF.

The levels of plasma isoprostane were, 70 ± 3.2 μ/ml for the sham controls (n=4), 154 ± 22 μ/ml for rats with galN-ALF (n=7), 82 ± 3 μ/ml for the rats given saline and Bosentan (n=4), 137 ± 22 μ/ml for the rats with galN-ALF given Bosentan 24 hours prior to the galactosamine injection (n=4) and 115 ± 15 μ/ml for the rats given Bosentan 24 hours after the galactosamine injection (n=4). Values are given as mean ± sem, **P < 0.05.

Figure 28.B. Effect of Bosentan on the production of urinary isoprostane excretion in rats with galN-ALF.

The levels of urinary isoprostane excreted were, 8.4 ± 0.5 ng/day for the sham controls (n=4), 140.0 ± 35.4 ng/day for the rats with galN-ALF (n=4), 7.6 ± 3 ng/day for the rats given saline and Bosentan (n=4), 118 ± 24 ng/day for the rats given NAC 24 hours prior to the galactosamine injection (n=4) and 98 ± 10 ng/day for the rats given Bosentan 24 hours after the galactosamine injection (n=4). Values are given as mean ± sem, **P < 0.05.
4.3.8 ACTIVATION OF NFκB IN LIVER AND KIDNEY TISSUE IN THE RAT WITH GALN-ALF

Identification of the NFκB-DNA-binding complex by EMSA was performed on the liver of a sham control (lane 1) and a rat with galn-ALF (lane 2). Characterisation of the NFκB-DNA-binding complex identified a p50-anti-p50 supershift complex (lane 4) and a p65-anti-p65 supershift complex (lane 5) (Figure 28). The p65-anti-p65 supershift complex did not give rise to a strong signal. The lack of a strong signal indicates that the p65 antibody was not effective in binding the p65 sub-unit in the nuclear extract.

Levels of activated NFκB in the liver increased in the rats with galn-ALF (Figure 29). In the kidney however the basal level of activated NFκB appears to be repressed in the rats with galn-ALF (Figure 30). This result is difficult to explain but it does demonstrate that galactosamine is not directly toxic to the kidney.
Figure 28. Characterisation of NFκB complexes following EMSA.

NFκB-DNA binding activity was measured in nuclear extracts from the liver of a sham control (lane 1) and a rat with galn-ALF (lane 2). The composition of the inducible DNA-binding complexes was analysed in separate reactions by either the addition of unlabelled oligonucleotide (lane 3) or the addition of 1μg of anti-p50 (lane 4) or anti-p65 antibody (lane 5). HeLa extract was used as the positive control standard (lane 6). The position of NFκB-DNA complex is marked, as are the positions of the p50-anti-p50 and p65-anti-p65 supershift complexes and the unbound radiolabelled probe.
Figure 29. Activation of NFκB in the liver of animals with galn-ALF.

Liver nuclear extracts from sham controls (Con) (lanes 5,6,7) and rats with galn-ALF (Galn) (lanes 1,2,3,4) were analysed by EMSA for NFκB. HeLa extract was used as the positive control standard (lane 8). The positions of NFκB-DNA complex and the unbound radiolabelled probe are indicated.
Figure 30. NFκB in the kidney of animals with galn-ALF.

Liver nuclear extracts from sham controls (Con) (lanes 5,6,7) and rats with galn-ALF (Galn) (lanes 1,2,3,4) were analysed by EMSA for NFκB. HeLa extract was used as the positive control standard (lane 8). The positions of NFκB-DNA complex and the unbound radiolabelled probe are indicated.
4.3.9 EFFECT OF GALACTOSAMINE ON THE PRODUCTION OF NITRIC OXIDE

Activation of NFkB induces transcription of iNOS mRNA. Measurement of the nitrates as an index of NO production was therefore measured in the model. The results showed that there was a significant increase in NO production from $44.7 \pm 4.0 \, \mu M$ in the sham controls to $56.1 \pm 3.3 \, \mu M$ in the animals with galn-ALF ($p < 0.05$) (Figure 31).

Figure 31. Levels of nitrate in the plasma of sham controls and rats with galn-ALF.

*Values are mean ± sem. * $p < 0.05$. 
4.4 Discussion

In the galN-ALF rat model of HRS there was an increase in the concentration levels of F_2-isoprostanes in the plasma by two and a half fold and the urine by fifteen fold. F_2-isoprostane concentrations were found to be elevated in the kidneys but not the livers of the animals with galN-ALF. On treating the animals with galN-ALF with lipoic acid and NAC, lipoic acid reduced the level of F_2-isoprostanes in the urine but NAC resulted in an increase. Both NAC and lipoic acid improved renal function in animals with galN-ALF. NFkB was activated in the livers of animals with galN-ALF compared to sham controls. However, in the kidney, NFkB was activated in the normal controls but expression was suppressed in the group with galN-ALF. Nitric oxide was elevated in the plasma by one and a half fold.

Reactive oxygen species have been implicated in the pathogenesis in a number of diseases (Knight 1998). The induction of oxidative damage in liver disease and renal failure is unknown. Potential mechanisms could include endotoxaemia or immune activation leading to neutrophil respiratory burst both of which have been shown to increase oxidative stress and subsequently impaired host defences against oxidative damage. The oxidant stress markers of lipid peroxidation, malondialdehyde and 4-hydroxynonenal have been found to increase by 3 fold in the liver of cirrhotic animals (Parola et al 1996). There was a decrease in the levels of hepatic and erythrocytic glutamine along with vitamin E and selenium levels and a reduction in the activity of hepatic glutathione peroxidase and glutathione-S-transferase (Singh et al 1992) (Panozzo et al 1995a) (Tsai et al 1997) (Gonzalez-Correa et al 1997).

Recently NAC was shown to result in the improvement of renal function in patients with HRS (Holt et al 1999a). However the mechanism through which this improvement occurred was unable to be investigated. In the galn-ALF model the effect of whether the antioxidants NAC and lipoic acid could improve renal function was investigated. The production of F_2-isoprostanes as a possible cause of the renal dysfunction in the rat with galn-ALF was studied. NAC is a widely used water soluble anti-oxidant with important hepatic and renal protective properties. It is readily taken into cells where it is converted to cysteine, the rate limiting substrate for glutathione

Lipoic acid is a low molecular weight thiol with important antioxidant functions which has been used in the treatment of diabetic peripheral and cardiac autonomic neuropathy (Packer *et al* 1997). It is readily absorbed and taken up into cells and tissues, where it is inter-converted with its reduced form, dihydrolipoic acid. These two forms of lipoic acid are capable of scavenging hydroxyl, hypochlorous and peroxynitrite radicals directly in the fluid phase, in addition to being able to regenerate vitamins C and E and cellular glutathione (Han *et al* 1995) (Packer *et al* 1995).

In the galn-ALF model of HRS, there was an increase in isoprostanes produced both in the plasma and urine. It has not yet been elucidated if the F₂-isoprostanes that are excreted into the urine originate primarily from local production in the kidney, as with prostaglandins (Frolich *et al* 1975) or from the circulation. Thus, when there is an increase in F₂-isoprostanes in the urine it is not yet established whether the lipid peroxidation is systemic or localised to the kidney. Studies were thus undertaken to define the site of production of the isoprostanes. Esterified isoprostanes were measured in liver and kidney tissue. The level of isoprostanes were elevated in the kidney of rats with galn-ALF suggesting a possible site for the formation of isoprostanes. No change was observed in the livers of the rats with galn-ALF but this does not necessarily mean that the livers from the HRS rats were not producing more F₂-isoprostanes than normal. Molecular modeling studies have shown potential deleterious effects of esterified F₂-isoprostanes on membrane function (Morrow *et al* 1992b), and rapid removal would be essential for the maintenance of a functional cell membrane in the presence of uncontrolled lipid peroxidation. It may be that in order to preserve the integrity of cell membranes phospholipase activity is increased so that esterified isoprostanes are not able to accumulate. Under oxidant stress phospholipase A₂ activity is upregulated (Chen *et al* 1996). Phospholipase A₂ activity is also found to increase in liver disease (Vishwanath *et al* 1996). BDL rats which had been administered with LPS showed elevated levels of F₂-isoprostanes in both the liver and the kidney one hour after LPS administration and after a couple of hours the levels of isoprostanes had returned to their baseline values (Harry *et al* 1999).
NAC did not decrease the formation of isoprostanes but indeed seemed to increase isoprostane formation. Lipoic acid, however was able to significantly reduce the level of isoprostanes excreted in the urine. Lipoic acid can scavenge superoxide and peroxyl radicals formed during lipid peroxidation (Packer et al 1995). Treatment with both NAC and lipoic acid resulted in significant improvements in renal function in the model so the production of F₂-isoprostanes may not be a primary cause of the renal dysfunction observed. However, oxidant stress could still play a role in causing renal dysfunction as both NAC and lipoic acid improved renal function.

NAC may not have inhibited lipid peroxidation in this model but it did have a marked protective effect on hepatic and renal function. Although improved hepatic function may explain some of the functional improvement in renal function, it is likely that other pathways are important. NAC has several modes of action. For example, NAC inhibits angiotensin-converting enzyme and thus decreases the formation of angiotensin II, a potent vasoconstrictor (Boesgaard et al 1993). NAC may have effects on the release or formation of nitric oxide. Infusion of NAC causes acute vasodilation in patients with liver disease (Jones et al 1994), an effect believed to be caused by the release of nitric oxide. Thus NAC may increase renal production of nitric oxide and in turn increase the glomerular capillary ultrafiltration coefficient and thus increase glomerular filtration rate. NAC also improves haemodynamics and oxygen transport in patients with acute liver failure (Harrison et al 1991) and could therefore have beneficial effects in other liver diseases. NAC is widely used in clinical practice in both Europe and the United States. It has been used successfully to treat hepatic and renal failure caused by glutathione depletion secondary to acetaminophen overdose (Smilkstein et al 1988). It also has an extensive history as a mucolytic and has been used in pulmonary diseases including chronic bronchitis (Rasmussen and Glennow 1988) (Jackson et al 1984) and fibrosing alveolitis (Behr et al 1997). It has been studied extensively at both high and low doses and has a good safety profile.

Bosentan, a mixed ETₐ and ETₐₐ inhibitor ameliorates the development of renal failure (Figure 17.A & B). Bosentan was also found to reduce the formation of isoprostanes in the rats with galn-ALF. Along with inhibiting the vasoconstrictive effects of ET-1, Bosentan also appears to prevent lipid peroxidation. Bosentan was found to prevent the

Nuclear factor -κB (NFκB) was elevated in the liver of the rat with galn-ALF. The inhibitory role of NAC on NFκB activation and the consequent downstream effects involved in cytokine signalling and apoptosis (Cossarizza et al 1995) has been well documented. Thus NAC may inhibit NFκB activation and the pro-inflammatory cytokine stimulation and also prevent apoptotic pathways. NFκB was also analysed in the kidney to determine whether NFκB was also up-regulated as in the liver. Surprisingly the kidney of sham controls expressed large amounts of NFκB which was inhibited or down-regulated in the model of HRS. Constitutive NFκB binding activity in nuclear extracts of cells without deliberate activation has been reported in a number of cells including the monocyte lineage (Griffin et al 1989) (Haas et al 1990) (Kaufman et al 1992) rat thymocytes and B lymphocytes (Sen and Baltimore 1986). Protein-DNA complexes of constitutive NFκB are similar in mobility to the LPS-induced NFκB and both are recognised by an antibody specific to the p50 subunit of NFκB. A constitutively expressed form of NFκB, composed of two p50 subunits, has also been described (Frankenberger et al 1994) (Slater et al 1995). The constitutive NFκB appears to be functionally active, since a low level of tumour necrosis factor (TNF) transcript is detectable in monocytes, and this level can be increased by blocking transcript degradation using cycloheximide (Frankenberger et al 1994). The activated expression of NFκB in normal rat kidney epithelial cells has also been reported. It was found also that in contrast to that observed in many other cell types, the process of activation was not directly responsive to oxidative stress but rather, was regulated via processes independent of cellular redox status. The apparent lack of effect of ROS-generating NFκB activation is somewhat surprising in light of the well documented role of ROS in signal transduction events underlying NFκB activation in most cell types, particularly those of the immune system (Schultz-Osthoff et al 1995). In the kidney of the rats with galn-ALF the basal level of activated NFκB appears to be repressed. The
increase in F₂-isoprostane formation, in the kidney of rats with galn-ALF suggests that the activation of NFkB may be stimulated through differing redox/free radical effects.

The levels of plasma nitrite/nitrate, which is a measure of nitric oxide production were also measured in the galn-ALF model of HRS and found to be elevated. This is indicative of increased expression of inducible NOS (iNOS) synthesis in the arteries. Endotoxaemia in cirrhotics induce NOS in peripheral blood vessels directly, or indirectly via cytokines (Busse and Mulsch 1990) (Fong et al 1990). Increased iNOS activity has been found in the liver of rats with cirrhosis (Morales-Ruiz et al 1996) (Marley et al 1999). The tissue origin of the iNOS stimulation was not determined in these experiments. The hyperdynamic circulation can be reversed by the NO inhibitor L-NMMA (Pizcueta et al 1992) (Garcia-Pagan et al 1994) (Pilette et al 1996). Thus it is thought that excessive production of NO may contribute to the hyperdynamic circulation (high cardiac output, low MAP and SVR) observed in cirrhosis.

In conclusion the galn-ALF model of HRS is associated with a marked increase in urinary and plasma F₂-isoprostanes indicative of an increase in oxidant injury. Renal function may be improved by NAC and lipoic acid although the mode of improvement appears to be different. The mode of action of this improvement may not be dependent on F₂-isoprostanes. Oxidant stress may still play a role in the renal dysfunction in the model as NAC and lipoic acid, both antioxidants, improved renal function. However the mechanism through which these antioxidants improve renal function is not the same, as lipoic acid lowered F₂-isoprostane levels whilst NAC did not.
Chapter 5

General Discussion
5 General Discussion

5.1 Overview

The work described in this thesis has defined a new model for the study of HRS. The suitability of the galn-ALF rat model for the study of HRS was investigated and the role that vasoactive mediators may play in the pathogenesis of HRS was studied.

This thesis demonstrates that in rats with galn-ALF the development of renal failure correlates with the degree of liver injury. There was a 50% decrease of creatinine clearance and an increase in plasma creatinine. Importantly, the renal failure that develops is functional in nature with an intact tubular function as evidenced by the ability of the kidneys to reabsorb sodium. Histologically the kidneys are normal and even by electron microscopic examination only revealed mild vacuolar changes. Renal blood flow is reduced and renal vascular resistance (RVR) is increased. The systemic arterial pressure is slightly decreased but not significantly compared to sham controls. These findings parallel the features of HRS which include a decrease in GFR, functional renal failure, low RBF, increased RVR and a decrease in mean arterial pressure.

In healthy individuals there is a balance between vasoconstrictors e.g. endothelin-1 (ET-1) and vasodilators e.g. nitric oxide (NO) as regulators of renal and systemic haemodynamics. ET-1 is released predominantly in an abluminal direction towards the underlying smooth muscle (Wagner et al 1992), the tissue concentration is likely to be sufficiently high to activate local receptors. Recent studies using inhibitors of ET synthesis or receptor antagonists suggest that ET-1 is released tonically to maintain basal systemic vascular resistance in humans (Haynes and Webb 1994). In this way, ET-1 might balance out the dilator effects of NO, which is also thought to be involved in maintaining the tone of the vasculature (Vallance et al 1989) (Haynes et al 1993). There are several possible links between the ET-1 and NO systems. It has been known for some time that ET-1 can stimulate the release of NO from endothelial cells via the ETB receptor (Yanagisawa et al 1988b) (Warner et al 1989) (de Nucci et al
1988) which accounts for the transient vasodilator actions of ET-1. Another connection between these systems suggests that NO may regulate ET-1 release because NOS inhibitors stimulate ET-1 release and NO donors inhibit ET-1 release from cultured endothelial cells (Boulanger and Lüscher 1990). Finally, it is well documented that NO can directly antagonise the constrictor actions of ET-1 in vascular smooth muscle (de Nucci et al 1988).

In patients with HRS the balance between vasodilators and vasoconstrictors is altered due to the development of conditions such as endotoxaemia and an increase in oxidative stress. Renal vasoconstriction and systemic hypotension develop. The plasma levels of numerous vasoactive mediators are elevated including a ten fold increase in plasma endothelin-1 (Moore et al 1992), a ten fold increase in nitric oxide (Guamer et al 1993) and a five fold increase in plasma F₂-isoprostanes (Morrow et al 1993). The galn-ALF model of HRS studied in this thesis was also found to have elevated levels of plasma endothelin-1 by two fold, nitric oxide by one and a half fold and F₂-isoprostanes by two and a half fold in the plasma and fifteen fold in the urine.

In response to various stimuli NO can be increased within minutes. ET-1 synthesis however, is regulated at the transcriptional level, with a resultant delay in release (Yanagisawa et al 1988b) (Boulanger and Lüscher 1990). NO has a short half-life, and its effect can be terminated quickly by cessation of release. In contrast, endothelial ET-1 binds to its receptors on smooth muscles irreversibly and its constrictor and pressor effects are of longer duration (Hirata et al 1988) (Yanagisawa et al 1988a). It is thought that the elevated levels of ET-1 may play a role in the renal vasoconstriction and the elevated levels of NO in part may be responsible for the systemic hypotension. Systemic infusions of ET-1 cause a marked increase in RVR and a decline in renal blood flow (RBF) and glomerular filtration rate (GFR) with no change in systemic arterial blood pressure (Sorensen et al 1994). This illustrates the high sensitivity of the renal vasculature to ET-1 when compared to the systemic vasculature (Takabatake et al 1992). The increase in RVR, following ET-1 infusion results from intense contraction of glomerular arterioles (afferent > efferent) and arcuate and interlobular arteries. Administration of ET-1 into the renal artery of the rat decreases RBF, GFR and urine volume. Mean arterial pressure (MAP) does not change implicating a direct action of the peptide on the kidney (Katoh et al 1990).
Doubling of plasma ET-1 levels by exogenous infusion causes a significant increase in peripheral and renal vascular resistance (RVR) without affecting mean arterial pressure (Lerman et al 1990). In the galn-ALF model of HRS described in this thesis the concentration of plasma ET-1 was doubled (Figure 14). Moreover, it has been reported that in some pathophysiological states elevated ET-1 plasma concentrations may activate high affinity ET receptors (Lerman et al 1991). The increase in concentration of plasma ET-1 in this model of HRS appeared to be concomitant with an upregulation of the ETA receptors in the renal cortex (Figure 15). This is consistent with other findings in renal diseases where both ET-1 production and binding are increased (Firth and Ratcliffe 1992) (Nambi et al 1993). ETA receptors are present on vascular smooth muscle and are responsible for the vasoconstrictor properties of ET-1 (Pollock 1997). A major function of the kidney is to maintain extracellular fluid volume (ECFV) by regulating the amount of Na\(^+\) reabsorbed and excreted and thus regulate mean arterial pressure. The role of ET-1 on renal Na\(^+\) handling is not fully understood. Systemic infusions of ET-1 decrease Na\(^+\) excretion in some studies (Goetz et al 1988) (Hirata et al 1989) (Miller et al 1989). Intrarenal infusions of ET-1 also caused a decrease in Na\(^+\) excretion (Katoh et al 1990) (Stacy et al 1990). In the model of HRS Na\(^+\) excretion was also found to decrease (Figure 17). Bosentan, a mixed receptor antagonist improved the renal function in this model of HRS. The GFR and Na\(^+\) excretion were normalised on treatment (Figure 17), suggesting that elevated levels of ET-1 are involved in the pathogenesis of renal failure in HRS.

Patients with cirrhosis, the RAAS is stimulated (Schrier et al 1988). The increased concentration of plasma Ang II protects renal function by selective vasoconstriction of the efferent glomerular arterioles, by increasing the filtration fraction (Laragh et al 1963) (Wilkinson and Williams 1980). In patients with HRS, the RAAS activity was found to be further elevated (Schroeder et al 1970). Ang II has been shown to stimulate ET-1 synthesis (Chua et al 1993) (Kohno et al 1992a) (Kawaguchi et al 1990). Ang II can also stimulate the production of superoxide (Rajagopalan et al 1996). When superoxide reacts with NO, peroxynitrite is produced (Pryor and Squadrito 1995). Peroxynitrite has a greater oxidative capacity than most other compounds (Pryor and Squadrito 1995) and is able to oxidise arachidonic acid and release F\(_2\)-isoprostanes, potent vasoconstrictors (Takahashi et al 1992) (Fukunaga et al 1993). F\(_2\)-isoprostanes

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are able to stimulate ET-1 release (Fukunaga et al 1995). Oxidative stress can also stimulate ET-1 release (Michael et al 1997). The antioxidants N-acetylcysteine and α-lipoic acid improved renal function in the model of HRS suggesting that oxidative stress may play a role in the pathogenesis of renal dysfunction in HRS. This is consistent with clinical data showing that NAC improves renal function in patients with HRS (Holt et al 1999a). α-lipoic acid was effective in improving renal function in the galn-ALF model of HRS. It also inhibited lipid peroxidation, as evidenced by suppressed formation of F2-isoprostanes. In the model of HRS, Bosentan, a mixed ETA and ETB inhibitor ameliorates the development of renal failure by inhibiting the vasoconstrictive effects of ET-1 and possibly by preventing oxidative stress. Bosentan has been used successfully clinically in alleviating a number of disorders. Hypertensive patients given Bosentan showed a lowering in blood pressure with no changes in heart rate and without activation of SNS or RAAS (Krum et al 1998). Bosentan also prevented cyclosporin-induced renal vasoconstriction without effects on the systemic blood pressure (Binet et al 2000).

This is the first demonstration in a model of type I HRS to show that ET-1 and oxidative stress play a role in renal dysfunction.
Figure 32. Renal pathophysiology in model of HRS.

Patients with HRS develop conditions such as endotoxaemia and an increase in oxidative stress. In the model of HRS there was an increase in plasma ET-1, F₂-isoprostanes and NO. In the kidney itself there was an upregulation of the ET₄ receptors and an increase in F₂-isoprostane formation resulting in renal vasoconstriction. There was a modest reduction in MAP but a marked decrease in RBF. The increase in NO is thought to cause systemic vasodilatation and the reduction in MAP. In the kidney, NO may react with O₂⁻ to produce ONOO⁻ resulting in reduced NO-mediated vasodilatation. GFR decreased and Na⁺ uptake was increased. Bosentan (Bos), N-acetylcysteine (NAC) and α-Lipoic acid (LA) all improved renal function in the model of HRS by reducing reactive oxygen species (ROS). Bosentan also improved renal function by inhibiting the binding of ET-1 to its receptors.
5.2 Future work

NAC is used extensively clinically as a mucolytic agent and has been used in pulmonary diseases including chronic bronchitis (Rasmussen and Glennow 1988) (Jackson et al 1984) and fibrosing alveolitis (Behr et al 1997). It is also the antidote of choice when treating paracetamol overdose as NAC increases the level of glutathione in the liver (Smilkstein et al 1988). Recently NAC has been successfully used to improve renal failure in patients with HRS (Holt et al 1999a). α-lipoic acid is also emerging as an effective form of therapy. Their mechanisms of action however have not been elucidated and do not appear to be identical. In the model of HRS described in this thesis the mechanism of action of NAC appears to be independent of any role as an inhibitor or modulator of lipid peroxidation. However, the action of α-lipoic acid in improving renal function may partially be the result of the inhibition of lipid peroxidation.

NO is elevated in both HRS and in the model of HRS. Much of the systemic vasodilatation seen in HRS is likely to be due to overproduction of nitric oxide. The cause for NO overproduction is not clear but may be related to elevated levels of endotoxin or cytokines, such as TNFα (Chu et al 1997) or produced in response to increased shear stress caused by the hyperdynamic circulation (Martin et al 1998a). In humans there are higher systemic plasma concentrations of nitrite and nitrate in cirrhotics compared to normals, with even higher levels in the portal circulation (Battista et al 1997). NO reacts in biological systems with oxygen, superoxide and transition metals to form reactive nitrogen species that can support nitrosation reactions with thiol groups to form S-nitrosothiols or lead to the formation of peroxynitrite which results in the nitration of amino acids such as tyrosine to form nitrotyrosine. It has been reported that there is increased nitrotyrosine formation in plasma and kidney during endotoxaemia and chronic hepatitis C (Wizemann et al 1994) (Bian et al 1999) (Garcia-Monzon et al 2000). It was also reported that in a rat model of biliary cirrhosis that the formation of plasma S-nitrosothiols was markedly increased and also upregulated during endotoxaemia and the levels of plasma and hepatic nitrotyrosine were also increased (Ottesen et al 2001).
Future investigations using the galn-ALF model of HRS could study further the mechanisms of action of these antioxidants by asking the question "do N-acetylcysteine and α-lipoic acid improve renal function in the model of HRS by inhibiting nitrosation and nitration reactions on proteins?" Experiments might include the measurement of nitrotyrosine and S-nitrosothiol levels in plasma and the liver and kidney in this model before and after the administration of N-acetylcysteine or α-lipoic acid. Changes in creatinine clearance, plasma creatinine levels and sodium absorption also would be monitored along with the renal and systemic haemodynamics. To obtain further evidence as to whether the vasoactive mediators ET-1, F₂-isoprostanes and NO play a major role in the functional renal failure observed in this model of HRS the liver and renal functions could be monitored after 48 hours of the galactosamine injection. It is hypothesised that plasma ET-1, F₂-isoprostanes and NO should decrease as renal function improves, if they are mediators of renal dysfunction in the model of HRS.

The prognosis of HRS is poor with a > 90% mortality. The functional renal failure in HRS greatly compromises patients with liver disease in for example surgery for liver transplantation. By understanding the mechanisms through which renal failure occurs more appropriate therapies and treatments may be developed to improve renal function in this perplexing syndrome.
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