Pleiotropic Effects of Erythropoietin

A thesis submitted to the University of London for the degree of Doctor of Philosophy

by

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Declaration

I confirm that the work presented in this thesis is solely the work of the author. Assistance with techniques including histopathology is fully acknowledged in the text.
Abstract

The haematopoietic growth factor Erythropoietin (EPO) is essential for the survival of erythroid progenitors to maturation and differentiation. It has been recognised that the EPO signalling pathway is also present in other tissues including the brain and vasculature, and is integral to the physiological response to ischaemia. Exogenous EPO was found to improve the outcome in animal models of stroke. The primary aim of this thesis was to examine whether erythropoietin was protective in a model of acute kidney injury, and to determine the mechanism by which EPO exerted this effect. In vitro experiments using HK-2 cells, a human tubular epithelial cell line, showed that EPO induced dose-dependent changes in cell number, and activated a number of intra-cellular signalling pathways. EPO reduced apoptotic cell death induced by nutrient starvation through the expression of anti-apoptotic proteins. A short-term model of ischaemia reperfusion was used to determine that EPO reduced the development of acute kidney injury, with a reduction in caspase activity and apoptosis. Longer models of ischaemia were then performed to confirm these findings, and showed that a pre-conditioning regime before the onset of the insult was also effective. In order to examine the mechanism of action, EPO was used in a model of cisplatin-induced kidney injury. EPO reduced apoptosis and caspase activation through the maintenance of mitochondrial membrane potential, inhibition of stress kinase signalling, and expression of XIAP and Bcl-XL. EPO also reduced the induction of oxidative stress and PARP-1 activity.

EPO was then given to animals exposed to cisplatin and confirmed the finding that pre-treatment with EPO significantly reduced cisplatin nephrotoxicity. Finally, EPO was used in a model of myocardial infarction and heart cells in culture to confirm that EPO plays a significant physiological role in cellular protection in multiple tissues.
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<tr>
<td>[...]</td>
<td>concentration</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AKI</td>
<td>acute kidney injury</td>
</tr>
<tr>
<td>BFU-E</td>
<td>burst forming unit – erythroid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAPS</td>
<td>N-cyclohexyl-3-aminopropanesulfonic acid</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
</tr>
<tr>
<td>CFU-E</td>
<td>colony forming unit – erythroid</td>
</tr>
<tr>
<td>CKD</td>
<td>chronic kidney disease</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotides (e.g. adenine, dATP)</td>
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<tr>
<td>EDTA</td>
<td>ethanolamine diamine tetra-acetic acid</td>
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<tr>
<td>EGTA</td>
<td>ethylene glycol tetra-acetic acid</td>
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<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunoabsorbant assay</td>
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<tr>
<td>ESRF</td>
<td>end stage renal failure</td>
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<td>EPO</td>
<td>erythropoietin</td>
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<td>EPO-R</td>
<td>erythropoietin receptor</td>
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<td>FCS</td>
<td>foetal calf serum</td>
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<td>haemoglobin</td>
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<td>horse radish peroxidase</td>
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<tr>
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<td>interleukin</td>
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<tr>
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<td>intravenous</td>
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<tr>
<td>JAK</td>
<td>janus kinase</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>n</td>
<td>number of samples or replicates</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
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<td>probability</td>
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<td>polyclarlamide gel electrophoresis</td>
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<td>phosphate buffered saline</td>
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<td>polymerase chain reaction</td>
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<td>phenylmethylsulfonyl fluoride</td>
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<td>reverse transcriptase PCR</td>
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<tr>
<td>sc</td>
<td>sub-cutaneous</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
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<tr>
<td>TBS-T</td>
<td>TBS containing 0.05% Tween 20</td>
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<td>TEMED</td>
<td>$N,N,N,N'$ - tetramethylene diamine</td>
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<tr>
<td>TMB</td>
<td>3, 3',5, 5'-tetramethylbenzidine</td>
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<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<tr>
<td>SE</td>
<td>standard error of the mean</td>
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<tr>
<td>SH2</td>
<td>Src- homology 2 domain</td>
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<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<td>UV</td>
<td>ultraviolet</td>
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<td>Symbol</td>
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<td>Da</td>
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<td>mU</td>
<td>milliunits</td>
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<td>w/v</td>
<td>weight per volume</td>
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<tr>
<td>v/v</td>
<td>volume per volume</td>
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<td>%</td>
<td>percent</td>
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Publications arising from the work


2: Abdelrahman M, Sharples EJ, McDonald MC, Collin M, Patel NS, Yaqoob MM, Thiemermann C. Erythropoietin attenuates the tissue injury associated with hemorrhagic shock and myocardial ischemia.


Seminars in Nephrology 26; 325-331 (2006)


Acknowledgements

The studies which comprise this thesis would not have been possible without the support and encouragement of my supervisor, Professor Magdi Yaqoob. I am thankful for the opportunity to work with him and his invaluable guidance over the last four years in research and in my clinical career progression. He remains an inspiration as a clinician-scientist. This work would not have been possible without the financial support from Kidney Research-UK through a clinical training fellowship.

I owe thanks to the staff at the Biological Services Unit and Professor Thiemermann’s laboratory, particularly Nimesh Patel, for their considerable efforts in teaching and assisting me with the animal experiments. I would also like to thank the many people who have given their time and expertise to teach and assist me in learning the laboratory techniques described in this thesis. Particular thanks go to David Allen and Steve Harwood for unstinting advice, help, and a bountiful supply of biscuits and tea.

Finally, the time and commitment to this work has only been possible due to the forgiving and caring support from my lovely wife, Caroline, and my family.
Chapter One: General Introduction
1.1 Acute Kidney Injury

Acute kidney injury (AKI), also known as acute renal failure, is a clinical syndrome characterised by the rapid deterioration of kidney function over a period of hours or days, resulting in the failure of the kidney to excrete nitrogenous waste products, and to maintain fluid and electrolyte homeostasis. AKI can result from multiple types of insult, including decreased renal perfusion without cellular injury; an ischaemic, toxic, or obstructive insult to the renal tubular epithelium; a tubulo-interstitial process with inflammation; or a primary reduction in the filtering capacity of the glomerulus [Lameire N, 2005].

1.1.1 Epidemiology of AKI

The incidence of AKI varies greatly depending on the clinical setting, the demographics of the patient population, and, in particular, the definition of renal insufficiency utilised. It is difficult to directly compare the many studies of the epidemiology and management of AKI due to the wide variation in definition used. This weakness has recently been addressed with the universal adoption of the term acute kidney injury (AKI) in preference to acute renal failure or acute tubular necrosis (ATN), indicating that kidney damage may occur in the absence of complete loss of function, and with the consensus development of the RIFLE classification (Risk, Injury and Failure) based on the degree of change in biochemical parameters, observed urine output, and two clinical outcome scores (Loss, End-stage), through the work of the Acute Kidney Injury Network [Kellum JA, 2002].

Hou et al demonstrated an overall incidence of AKI of 5% in a hospitalised population (109 of 2216 medical and surgical patients) using an increase in serum creatinine of > 44 µmol/L (0.5 mg/dl) above the measured baseline as the definition of AKI. AKI was associated with decreased renal perfusion (42%) or major surgery (18%) in the majority of cases. Predictors
of poor prognosis included a reduced urine output (<400 mL/24 hr) and relatively modest elevation in serum creatinine concentration [Hou SH, 1983]. In the late 1990s an updated study, using identical diagnostic criteria, observed that the frequency of hospital acquired AKI had increased to 7.2 % [Nash K, 2002]. In comparison, community acquired AKI present at the time of admission to hospital was identified in only 0.9 % of patients [Kaufman J, 1991].

In the modern era, the use of more stringent RIFLE classification has reduced the observed incidence of AKI. Liangos et al analysed data from the National Hospital Discharge survey, a nationally collected sample of approximately 330,000 inpatient admissions from 500 hospitals in the USA, and demonstrated a 1.9 % incidence of AKI in hospitalized patients. Patients with AKI were more likely to have the sepsis syndrome or other non-renal acute organ system dysfunction [Liangos O, 2006].

Sustained impairment of renal perfusion is the most common factor that predisposes patients to ischaemia-induced tubular injury, and is implicated in approximately 70 % of community acquired AKI, and 40 % of hospital-related events [Mehta RL, 2004]. Hospital acquired cases are more often multi-factorial, with an accumulation of multiple acute insults, including exposure to nephrotoxic agents such as radio-contrast agents, treatment with non-steroidal anti-inflammatory drugs (NSAIDs), and infection, often in the presence of cardiac dysfunction and reduced tissue perfusion. Mortality rates in AKI range from 7 % among patients admitted to a hospital with hypotension-related AKI, to more than 80 % in patients with post-surgical AKI [Nash K, 2002; Thakker CV, 2003].

The severity of kidney injury may determine the natural history and patient outcome in AKI. Few epidemiology studies have examined the association between small changes in serum creatinine and patient outcome. These population studies can only show an association
between AKI and mortality, especially in intensive therapy unit-based studies, because it is often part of the spectrum of multi-organ failure. However, published studies have demonstrated a consistently elevated relative risk associated with AKI despite adjustment for co-morbid conditions and severity of illness. Levy et al. compared 183 patients with radiocontrast-associated AKI and 174 age-matched patients who received similar radiocontrast loads without developing AKI. The mortality rate was 34% in patients with AKI versus 7% in those patients without AKI. Adjusting for differences in co-morbidity, the odds of death were increased 5.5-fold in the AKI group [Levy EM, 1996]. This increased relative risk of death was confirmed in a more heterogeneous cohort of patients, which may be a truer representation of the general population, from a study of 19201 admissions to a large urban hospital. This demonstrated that even a 50% increase in serum creatinine above baseline was associated with an increased mortality adjusted odds ratio of 5.8 (4.6-7.5), with similar increases (odds ratio 4.1 (3.1-5.5) in mortality observed with only a 28 µmol/L (0.3 mg/dl) increase in serum creatinine [Chertow GM, 2005].

This increase in mortality associated with even quite small changes in renal function may directly be related to the distant effects of renal ischaemia on other organ systems. Models of AKI have shown induction of a more general systemic inflammatory response, with apoptotic cell death in the myocardium and changes in cardiac function [Grigiryev DN, 2008], vascular dysfunction [Kelly KJ, 2003], and neutrophil aggregation in the lungs with associated inflammation [Awad AS, 2009]. The observed increase in mortality may, however, be indirectly related to AKI, however, as the risk factors for AKI are also risk factors for cardiovascular disease and mortality, and AKI occurs much more commonly in those with higher co-morbidity, including pre-existing renal impairment. Patients with relatively early stage CKD (eGFR 30-45 mL/min) have a 5-fold increased odds ratio of AKI when compared to matched controls [Hsu Y, 2008].
1.2 The Pathophysiology of Ischaemic AKI

In health, the kidneys receive 25% of cardiac output, but renal blood flow is not uniformly distributed within the renal parenchyma, so that tissue perfusion and oxygen consumption are highly heterogeneous - resulting in areas susceptible to reduced oxygen tension [O’Connor PM, 2006]. Tissue oxygen tension varies between 60 -100 mm Hg (6.6 – 13.3 kPa), obtained close to the cortical surface in micro-puncture studies, and 10-20 mm Hg (1.3-2.9 kPa) in the S3 segment of the proximal tubule [Welsh WJ, 2001]. The anatomical arrangement of pre-glomerular arterial and corresponding post-capillary venous vessels suggests a structural basis for this low medullary oxygen tension. This is confirmed by studies demonstrating the large anaerobic capacity of the outer-medullary region, chronic up-regulation of hypoxia-induced gene expression, and an increased susceptibility to hypoxic insult [Brezis M, 1989].

The kidney responds to alterations in renal perfusion pressure by a feedback loop to auto-regulate pre-glomerular blood flow and hence maintain glomerular filtration rate (GFR) within fairly narrow limits, even with mean arterial pressure as low as 80 mm Hg [Dworkin LD, 2003]. When the blood pressure falls, gradual dilatation of the pre-glomerular arterioles occurs, mediated by the angiotensin stimulated generation of vasodilating products of arachidonic acid (prostaglandin I₂) and nitric oxide [Baylis C, 1978]. As perfusion pressure falls further, concomitant vasoconstriction of the postglomerular arterioles, mainly under the influence of angiotensin II, maintains a constant glomerular capillary hydrostatic pressure. The postglomerular capillary bed, which perfuses the tubules, has diminished blood flow and pressure, but the tubules remain intact. Drugs which act on the prostaglandin system, such as non-steroidal anti-inflammatory drugs, or inhibit the angiotensin pathway, can interfere with the normal autoregulatory responses and increase the risk of impaired perfusion pressure and subsequent AKI [Abuelo JG, 2007].
Several pathways interact to maintain the relationship between systemic blood pressure and renal perfusion. Vasoconstriction of the efferent arteriole increases transglomerular resistance and hence GFR, but contributes to tubular hypo-perfusion.
Prolonged ischaemic insults, alone or in synergistic combination with nephrotoxins, overcome the autoregulatory systems in the kidney, and initiate epithelial and vascular cell injury, resulting in an extremely rapid decrease in glomerular filtration rate (GFR), appropriately referred to as the *initiation phase* of AKI [Sutton TA, 2002].

The initiation phase is immediately followed by the *extension phase* [Sutton TA, 2002], in which multiple interrelated events dependent on altered vascular function lead to worsening of epithelial and endothelial cell injury and subsequent cell death, primarily in the cortico-medullary region of the kidney. Established ischaemic AKI is associated with a reduction in renal perfusion by 30-50%, and there is evidence of selective reduction in blood supply to the outer medulla [Yamamoto T, 2002].

The *maintenance phase* represents a phase of stabilization of injury, and subsequent correcting events leading to cellular repair, division, and re-differentiation. This sets the stage for improved epithelial and endothelial cell function and recovery of GFR during the *recovery phase*. Correction of the initiating insult with appropriate therapy during the early initiation and extension phases of AKI may limit the degree of cellular injury and hence the duration of the maintenance phase, allowing more rapid onset of the recovery phase and hence preservation of kidney function.

### 1.2.1 Vascular dysfunction

Under physiological conditions, the oxygen tension of the kidney decreases from the outer cortex to the inner medulla [Breizis M, 1995]. After an ischaemic insult, total renal blood flow returns to normal, but marked, regional alterations occur, playing an important role in the extension phase of renal ischaemic injury [Yamamoto T, 2002]. Blood flow to the outer
medullary or cortico-medullary junction regions remains approximately 10% of normal during early reperfusion, leading to congestion due to interstitial oedema, red blood cell trapping, leukocyte adherence and extravasation.

Several lines of evidence indicate that endothelial dysfunction is a feature of the initiation phase of ischaemic renal injury. Firstly, ischaemia results in profound loss of the vasorelaxing effect of acetylcholine [Lieberthal W, 1989]. Vasorelaxation in response to stimuli generating endothelium-derived relaxing factor was also inhibited [Conger JD, 1995]. In addition, nitric oxide production in response to bradykinin was found to be suppressed in ischaemic kidneys [Noiri E, 1996]. Loss of normal endothelial function and altered NO homeostasis alters vascular homeostasis, leading to an increased thrombotic potential and reduced response to local and systemically derived vaso-active substances.

Intra-vital video-microscopy has proved useful for the study of blood flow in peri-tubular capillaries [Yamamoto T, 2002]. Reperfusion initiates an immediate partial recovery of blood flow after release of renal artery occlusion, followed by a profound and sustained flow reduction. There is evidence of retrograde flow in some vessels, and temporary loss of vessel patency. The pattern of glomerular microcirculation is similar to that of the peri-tubular capillaries; initial brief recovery of flow is followed by ‘no flow’. Restoration of flow in these capillary beds, however, is different. Glomerular circulation is re-established initially, while return of blood flow to peri-tubular capillaries is significantly delayed. So, endothelial injury and dysfunction have major roles in the ‘no-flow–reflow’ phenomenon in the early stages of ischaemic injury. This injury manifests structurally as loss of endothelial integrity, and functionally as defective endothelium-dependent vasorelaxation.

Inflammatory activation of the endothelial cell with upregulation of adhesion molecules, as well as injury to the endothelial cells leading to cell swelling and loss of patency of the
endothelial barrier, potentiates the interaction with leukocytes and platelets causing mechanical obstruction to the small vessels [Bonventre J, 2003]. Leukocytes are activated by a number of local factors, including cytokines, chemokines, eicosanoids and reactive oxygen species, which result in further upregulation of adhesion molecules, leukocyte recruitment and inflammation [Suwa T, 2001]. Leukocyte trafficking and the differential role of lymphocytes and mono-nuclear cells in the generation of reperfusion injury remains a controversial subject [De Greef KE, 1998; Park P, 2002]. Recent studies have suggested an important role of T-lymphocytes in the development of reperfusion injury, and in particular, the systemic inflammatory response [Ascon DB, 2006; Grigoryev DN, 2008].

The reduced perfusion and hypoxic conditions lead to deprivation of nutrients and loss of ATP in the endothelium. Injection of cultured endothelial cells that constitutively express high levels of endothelial nitric oxide synthase (eNOS) into rats subjected to renal ischaemia resulted in implantation of these cells into the vasculature and reduction in ischaemic kidney injury, highlighting the important role of endothelial cells and the vasculature in the protection from renal ischaemic injury [Brodsky SV, 2002]. Permanent damage to the peritubular capillaries occurs in rats subjected to prolonged renal ischaemia, and this may be associated with the development of tubulo-interstitial fibrosis, poor urine concentrating ability, and impaired recovery in the post-ischaemic kidney [Basile DP, 2001].

1.2.2 Tubular changes

In the commonly studied vascular clamp small animal models of renal artery occlusion, the S3 segment of the proximal tubule that traverses the outer-medullary region is extremely susceptible to ischaemic injury when compared to S1 and S2 segments or distal tubule [Ventatchalam M, 1978]. The sensitivity of the outer medulla derives from a combination
of its microvascular architecture, and a relatively low glycolytic capacity to generate ATP in
the setting of rapid ATP depletion resulting from impaired oxidative phosphorylation. The
medullary thick ascending limb of the loop of Henle, although situated in the same region,
does not undergo the same extent of cell death because there is a greater glycolytic capacity
to generate ATP under ischaemic conditions [Bonventre JV, 2002].

During the maintenance phase of AKI, large areas of severe local ischaemia may no longer
be present, but cell injury continues due to surrounding acute inflammation and the effects of
prior insults on intrinsic cellular responses such as apoptosis. The tubular cell is also a major
contributor to the local inflammatory response. Tubular epithelial cells produce a number of
pro-inflammatory cytokines, including TNF α and interleukin-6, chemotactic cytokines such
as RANTES and monocyte chemoattractant protein (MCP-1) in response to an ischaemic
insult [Rice J, 2002]. Tubular epithelial cells also contribute to leukocyte migration through
the local production of the CX3-family chemokine fractalkine (CX3CL1) [Chakravorty SJ,
2002].

The structural response to the tubular epithelium to ischaemic injury is multifaceted, and
includes loss of cell polarity and brush border, cell death, de-differentiation of surviving
cells, proliferation and restitution of a normal epithelium. Cellular ATP depletion leads to a
rapid disruption of the apical actin cytoskeleton and redistribution from the apical domain
and microvilli into the cytoplasm [Molitoris B, 1992]. The ensuing alterations in microvillar
structure leads to formation of membrane-bound, free floating extracellular vesicles, or
“blebs,” that are either internalised or lost into the tubular lumen. Brush border membrane
components that are released into the lumen contribute to the formation of casts and tubular
obstruction. These casts and vesicles that contain actin and actin depolymerising factor have
been detected in the urine in animal and human acute renal failure. Disruption of the actin
cytoskeleton also results in the loss of tight junctions and adherens junctions. Reduced
expression, re-distribution and abnormal aggregation of a number of key proteins that constitute these junctions have been documented after ischaemic injury, and this loss of tight junction may magnify the transtubular backleak of glomerular filtrate that is induced by tubular obstruction.

Ischaemia results in the early disruption of two basolateral polarised proteins, Na, K ATPase and adhesion molecule family of β1-integrins. The Na, K, ATPase is normally tethered to the spectrin-based basolateral cytoskeleton at the basolateral domain via the adapter protein ankyrin. The mislocated Na, K, ATPase remains bound to ankyrin but is devoid of spectrin, possibly due to either phosphorylation of spectrin or cleavage of spectrin by proteases such as calpain [Woroniecki, 2003]. The β1 integrins are normally polarised to the basal domain, where they mediate cell-substrate adhesions. Ischaemic injury leads to a redistribution of integrins to the apical membrane, with consequent detachment of viable cells from the basement membrane. There is good evidence for abnormal adhesion between these exfoliated cells within the tubular lumen, mediated by an interaction between apical integrin and the Arg-Gly-Asp (RGD) motif of integrin receptors. Administration of synthetic RGD compounds attenuates tubular obstruction and renal impairment in an animal model of renal ischaemia [Molina A, 2005].
Figure 1.2 Alterations in tubular cell structure after ischaemic acute renal failure.

The initiation phase leads to sub-lethal injury, with loss of brush border and disruption of cell polarity and the cytoskeleton. If the injury is alleviated at this stage, then complete recovery ensues. If not, cell death, desquamation, luminal obstruction and an inflammatory response occurs.
1.3 Cellular Mechanisms of Tubular cellular injury following ischaemia-reperfusion

1.3.1 ATP and GTP depletion

In the absence of normal mitochondrial oxidative phosphorylation, prolonged ischaemia leads to a rapid decrease in the level of available adenine nucleotide pool (ATP, ADP and AMP). Dephosphorylation of ATP and ADP lead to further catabolism of AMP to hypoxanthine and xanthine, which contribute to the generation of reactive oxygen species. Functional recovery in rabbits after ischaemic injury was correlated to the degree of ATP depletion and xanthine accumulation [Buhl MR, 1979]. Zager et al demonstrated regional differences in ATP depletion after 15 minutes of ischaemia, with the outer medulla most affected, but after 45 minutes these regional differences were no longer significant due to global reduction [Zager RA, 1990]. In vitro studies of chemical induced ATP depletion, using inhibitors of mitochondrial oxidative phosphorylation pathway such as antimycin A, have demonstrated an association between ATP depletion and activation of pro-inflammatory and cell death pathways, including Fas and Fas ligand up-regulation and caspase-8 activation [Feldenberg, 1999]. Partial ATP depletion leads to apoptotic cell death whereas more severe or total ATP depletion causes necrosis [Leibenthal W, 1998].

GTP depletion also occurs in association with ATP depletion in both in vitro and in vivo models of ischaemic injury [Daugher P, 2000]. Preferential depletion of GTP with maintained ATP induces apoptosis in cultured renal tubular cells. Administration of guanosine before the inducement of ischaemic renal injury maintains GTP regenerative capacity, leading to a significant reduction in the number of apoptotic tubular cells, and hence improved renal function [Kelly KJ, 2001]. GTP depletion may induce apoptosis via the activation of p53, and there is in vivo evidence that inhibition of p53 pathway also
protects the kidney from ischaemia reperfusion injury [Kelly KJ, 2003; reviewed by Dagher PC, 2004].

1.3.2 Increased cytosolic Ca\textsuperscript{2+} concentrations

Renal proximal tubular cells undergo a significant increase in free cytosolic ionised calcium concentration during the initial five minutes of hypoxia. The increase in Ca\textsuperscript{2+} precedes hypoxic membrane damage, and is reversible if re-oxygenation occurred before the cell underwent lethal injury [Wetzels JF, 1993]. ATP depletion leads to impaired calcium sequestration in the endoplasmic reticulum, as well as diminished extrusion into the extracellular space [Edelstein CL, 1997]. Increased cytosolic Ca\textsuperscript{2+} leads to uptake of Ca\textsuperscript{2+} into the mitochondria via the Ca\textsuperscript{2+} uniporter. In the presence of ATP depletion, mitochondrial Ca\textsuperscript{2+} overload leads to opening of mitochondrial permeability transition pore (mPTP) and permeabilization of the inner mitochondrial membrane, and the release of pro-apoptotic mitochondrial proteins, including cytochrome c.

Prolonged increases in cytosolic Ca\textsuperscript{2+} activate the Ca\textsuperscript{2+}-dependent cysteine protease calpain. Calpain activity is tightly regulated by its ubiquitously expressed endogenous inhibitor calpastatin. Calpains result in the proteolysis of a broad spectrum of cellular proteins and a distinguishing feature of their activity is their ability to confer limited cleavage of protein substrates into stable fragments rather than complete proteolytic digestion. Thus, calpain-mediated proteolysis represents a major pathway of post-translational modification that influences various aspects of cell physiology, including apoptosis, cell migration and cell proliferation [Sato M, 2001].

Renal proximal tubule epithelial cells constitutively express calpains, which are activated in response to toxic and hypoxic injuries. Inhibition of calpain activity using various
pharmaceutical inhibitors ameliorated necrotic cell death in proximal tubule cells subjected to hypoxic injury [Harriman JF, 2000], ATP depletion [Liu X, 2001] and reduced tubular dysfunction in rats subjected to ischaemia reperfusion injury [Chatterjee PK, 2001].

1.3.3 Poly (ADP-ribose) polymerase-1 (PARP-1)

PARP-1 plays a primary role in the process of poly (ADP)-ribosylation, in which posttranslational modification of nuclear proteins is activated in response to DNA damage. Activation of PARP-1 by DNA strand breaks results in the synthesis of poly (ADP-ribose) at the expense of NAD\(^+\) that is cleaved into ADP-ribose and nicotinamide. PARP-1 catalyzes the binding of the first ADP-ribose on acceptor proteins, primarily at glutamic residues, and subsequent catalyzes the elongation and branching reactions with additional ADP-ribose units. Decreased NAD\(^+\) levels cause an unbalanced NAD/NADH ratio, which, in turn, affects the activation of enzymes involved in glycolysis, the pentose shunt, and the Krebs cycle. Decreased NAD\(^+\) levels also drastically alter the redox state of cells. Because the cell attempts to restore NAD\(^+\) pools by recycling nicotinamide with 2 ATP molecules, excessive activation of PARP-1 depletes pools of intracellular NAD\(^+\) and ATP and, consequently, interferes with most, if not all, energy-dependent cellular processes [De Murcia J, 1997].

The switch between necrosis and apoptotic cell death may also be modulated by the degree of activation of PARP-1 since this decision seems to be determined by the cellular ATP levels. PARP-1 overactivation and poly(ADP-ribose) synthesis result in the heavy use of NAD\(^+\) and a reduced activity of NAD\(^+\)-dependent cellular processes, including ATP synthesis. Moreover, because NAD\(^+\) re-synthesis is done at the expense of ATP, the resulting ATP depletion is believed to induce cell death by necrosis. In apoptosis, PARP-1 is cleaved into two fragments of 89 kDa and 24 kDa (p89 and p24) by caspases-3 and -7 at the
DEVD site located in the NLS of PARP-1. The p89 fragment contains the catalytic site and the auto-modification domain, and p24 contains the DBD and zinc finger domains. The motive for PARP-1 cleavage is not yet defined, but it seems to be a key event in the execution phase of apoptosis, possibly by allowing preservation of the cellular ATP required to accomplish apoptosis, because this process is energy dependent, and therefore allows completion of apoptosis rather than necrosis.

In the kidney, PARP-1 activity contributes to ischaemia reperfusion injury in transplanted kidneys [O’Valle F, 2005], and inhibition of PARP-1 has been shown to reduce tubular cell death in vitro [Chatterjee PK, 1999], and in animal models of haemorrhagic shock [McDonald MC, 1999] and acute renal ischaemia [Chatterjee PK, 2000].

1.3.4 Oxidant Injury

There is now substantial evidence for the role of reactive oxygen species in the pathogenesis of AKI. During reperfusion, the conversion of accumulated hypoxanthine to xanthine, catalysed by xanthine oxidase, generates hydrogen peroxide and superoxide. In the presence of iron, hydrogen peroxide forms the highly reactive hydroxyl radical. Concomitantly, ischaemia induces nitric oxide synthase (iNOS) in tubular cells, and the NO that is generated interacts with superoxide to form peroxynitrite, which contributes to cellular injury via oxygen toxicity and nitrosative stress [Devarajan P, 2005]. Collectively, reactive oxygen species cause renal tubular epithelial cell death by oxidation of proteins, peroxidation of lipids, damage to DNA and induction of apoptosis via redox activation of the transcription factor NF-κB and the mitogen-activated stress kinase family [Finkel T, 1998].
In vitro studies have confirmed that reactive oxygen species are important mediators of necrotic cell death following ischaemic and toxic injury to tubular epithelial cells [Beckman JS, 1996]. Mitochondrial injury leads to uncoupling of oxidative phosphorylation which has two sequelae: impaired ATP production and increased superoxide generation. Lipid peroxidation of the membrane phospholipid bilayers and subcellular organelles is a major contributor to necrotic cell death. Several scavengers of reactive oxygen species, including superoxide dismutase, catalase, N-acetylcysteine) have been effective in animal models of ischaemic injury [Chatterjee PK, 2004], but human studies have failed to show similar benefit [Macedo E, 2006].

1.4 Alterations in Cell Viability and Mechanisms of cell death

Both experimental and human studies indicate that tubular epithelial cells can suffer one of three distinct fates after ischaemia. The majority of cells remain viable, suggesting that they escape injury, or are only sub-lethally injured and can undergo recovery. The remaining tubular epithelial cells display patchy, non-continuous areas of cell death that results from at least two pathological mechanisms – necrosis and apoptosis. These two forms of cell death co-exist, and the mode of individual cell death depends primarily on the severity of the insult and the resistance of the cell type.

Necrotic cell death is characterised by progressive loss of cytoplasmic membrane integrity, rapid influx of sodium and calcium ions and water, resulting in nuclear degeneration and cytoplasmic swelling. The latter feature leads to cellular fragmentation and release of lysosomal and granular contents into the extracellular space, with subsequent development of acute inflammation.
A distinction between these changes of necrotic cell death and the morphology of cells undergoing controlled non-inflammatory cell death was observed more than 50 years ago. The term apoptosis was applied to the characteristic morphological changes associated with programmed cell death [Kerr JF, 1972]. It is defined by cytoplasmic and nuclear shrinkage, chromatin margination and fragmentation, and division of the cell into multiple spherical bodies that retain membrane integrity. These apoptotic bodies are rapidly phagocytosed and degraded by macrophages and by surrounding epithelial cells [Lieberthal W, 1996]. Phagocytosis provides an efficient mechanism for the removal of dead cells without incurring any of the surrounding tissue inflammation and injury associated with necrotic cell death [Kerr JF, 1972] although in some circumstances apoptotic cells may undergo a process of secondary necrosis.

Recently, a third process resulting in cell death has also been described, and is termed autophagy [Tsujimoto Y, 2005]. Autophagy plays a role in normal tissue homeostasis and growth, but is also activated in certain circumstances, such as nutrient starvation, hypoxia and high temperatures. The cells undergo partial autodigestion that prolongs survival for a short time, but if these adverse conditions persist, the cell dies [Ohsumi Y, 2001]. Autophagosomes are double-membrane cytoplasmic vesicles that are designed to engulf various cellular constituents, including cytoplasmic organelles. These fuse with lysosomes to become autolysosomes, where sequestered cellular components are digested. Autophagy is now recognized to play an important role in ischaemia reperfusion injury in the kidney and heart [Suzuki C, 2008; Matsui Y, 2008]
1.4.1 Apoptotic cell death in Acute Kidney Injury

There is now increasing evidence indicating that apoptosis is a major mechanism of early tubular cell death in ischaemic AKI. Several animal models have demonstrated the presence of apoptotic tubular cells [Schumer M, 1992], and this was subsequently confirmed in human studies in kidney transplantation [Oberbauer R, 1999; Castaneda MP, 2003]. Nevertheless, the mechanistic role of apoptosis in the degree of renal dysfunction observed is not fully understood.

First, most estimates place the peak incidence of apoptosis at only approximately 3-5% of tubular cells after ischaemic injury, which arguably is insufficient to explain the degree of renal insufficiency. It is likely, however, that the degree of apoptotic cell death is underestimated, because it is a rapidly occurring event that is heterogeneous in distribution, and both tissue preparation and techniques to demonstrate apoptosis have limited specificity. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay, which adds labelled dUTP to multiple free DNA ends generated by activated endonucleases during cell death, has been widely used to determine the extent and localisation of apoptotic cell death, but can be criticized for its lack of sensitivity and specificity in discriminating among apoptotic and necrotic mechanisms of cell death [Kelly KJ, 2003].

Secondly, apoptosis is commonly encountered in the distal tubule, whereas loss of viable cells occurs in the proximal tubule. Distal tubular cells are more resistant to cell death, with some evidence that this resistance may be determined by persistent Akt phosphorylation and activation, possibly as a protective mechanism against the osmotic and hypoxic environment, and apoptosis may appear to be the dominant cause of cell death as necrosis is therefore prevented [Kroning R, 1999].
Thirdly, apoptosis is crucial to the physiological repair processes that remove damaged cells after injury, and therefore may be beneficial to the organ and the organism. This can be reconciled by the observation that apoptosis occurs in two-waves in animal models. The first wave is detectable by six hours, peaks at 24-36 hrs and rapidly diminishes. The second wave becomes apparent after 4 days, removing hyperplastic and unwanted cells, and is likely to play a role in the remodelling of the regenerating tubule.

1.4.2 The Caspase cascade

Apoptosis is characterised by the activation of a family of cysteine proteases called caspases, which participate in enzymatic cascades that terminate in cellular disassembly. This action can be initiated through mitochondrial-dependent and -independent pathways.

The genetic analyses of apoptosis in the nematode flatworm *Caenorhabditis elegans* led to the elucidation of genes that control this cellular suicide mechanism [Driscoll M, 1992]. The central components of apoptosis machinery in *C. elegans* are a troika of ced genes: *ced-3*, *ced-4* and *ced-9*, where *ced-3* protein is a cysteine protease responsible for the execution of the apoptotic program, *ced-4* protein is a pro-apoptotic adaptor molecule, and *ced-9* protein is an inhibitor of apoptosis [Hengartner 1992; Miura M, 1993]. Homologues of these genes have been clearly shown to regulate apoptosis in higher eukaryotes, albeit utilizing much more evolved and complex mechanisms. The mammalian homologues of the inhibitor of apoptosis *ced-9*, the B cell lymphoma-2 (Bcl-2) family members, are critical regulators of the mitochondrial step in apoptosis [Gross A, 1999].

All caspases are synthesized as inactive zymogens containing an N-terminal prodomain followed by a large 20 kDa subunit, p20, and a small 10 kDa subunit, p10. Depending on the
structure of the pro-domain and their function, caspases are typically divided into three major groups. The caspases with large prodomains are referred to as inflammatory caspases (group 1: caspase-1, -4, -5, -11, -12, -13, -14), initiator caspases (Group 2: caspases-2, -8, -9, -10), while caspases with a short prodomain of 20-30 amino acids are named effector caspases (Group 3: caspase-3, -6, -7). Cleavage of a procaspase at the specific Asp-X bonds results in the formation of the mature caspase, which comprises the heterotetramer p202-p102 and causes release of the prodomain. Each heterodimer is formed by hydrophobic interactions resulting in the formation of parallel β-sheets, composed of 6 anti-parallel β-strands [Stennicke HR, 1999].

All caspases share a number of distinct features. These include the catalytic triad residues, consisting of the active site Cys235, which is a part of the conserved QACXG pentapeptide sequence, His237 and the backbone carbonyl of residue -177 [Stennicke HR, 1999]. The striking feature of the caspases is their specificity for substrate cleavage after an asparate residue, which is unique among mammalian proteases, except for the serine protease granzyme B.

The initiator caspases form active catalytic dimers via association of their long prodomains with adapter molecules with selectivity for individual caspases. In contrast, executioner caspases become catalytically active only after cleavage by the active initiator caspases. Once activated, executioner caspases can cleave initiator caspases, which may not lead to their activation, but may serve to stabilize active initiator caspase dimers, or render them subject to regulation via cellular inhibitors of apoptosis. This hierarchy of caspase activation generates a cascade culminating in executioner mediated cleavage of multiple substrates including PARP-1, cytoskeleton proteins such as fodrin, actin and focal adhesion kinase, an array of phenotypic characteristics including cell blebbing, condensation and
fragmentation of chromatin, and re-distribution of lipids in the outer plasma membrane, ending in controlled cell death [Lavrik IN, 2005].

1.4.3 Caspase Activation in AKI

Several studies have documented activation of caspase molecules after ischaemia reperfusion in the kidney and post-hypoxic injury in renal proximal tubule epithelium. The expression of caspases-1, -2, -3, -6, -7, -8, and -9 has been characterized in rat kidneys at the mRNA level [Kaushal GP, 1998; Singh AB, 2002]. The expression and activity of caspases-1, -2, and -6 are altered in kidneys post-I/R. LLC-PK₁ and Madin-Darby canine kidney (MDCK) cells subjected to chemical hypoxia underwent apoptosis with a marked increase in activation of caspases-3 and -8. The activation of caspase-3 is accompanied by Bax translocation from cytosol to mitochondria and cytochrome c release from mitochondria.

Inhibition of caspases using a pancaspase inhibitor is shown to protect kidneys from ischaemic injury. The pancaspase inhibitor Z-Val-Ala-Asp(OMe)-CH₂F protected against ischaemic AKI in mice by inhibition of apoptosis and subsequent inflammation [Daemen M, 2001]. Inhibitors of specific caspases have also been demonstrated to alter the natural history of renal injury in a short-term model of I/R in the rat, although there is controversy over exact specificities [Chatterjee PK, 2005]. The results from these studies clearly demonstrate a role for caspases in I/R. However, the role of individual caspases contributing to the injury and inflammation post-renal injury cannot be discriminated from these studies because non-specific caspase inhibitors were utilized. The availability of more specific inhibitors of individual caspases will provide better clues as to the functions of individual caspases in renal I/R. RNA interference techniques have also been used to inhibit the expression and activity of specific caspases during ischaemia reperfusion injury in a murine model. RNA interference inhibition of caspase-3 and caspase-8 has been shown to reduce both
histological and biochemical markers of ischaemic injury, and significantly improved rates of animal survival [Zhang X, 2006].

Caspase-1 does not appear to play a direct role in the induction of apoptosis, but rather, is integral to the development of the acute inflammatory response and removal of pathogens [Scott AM, 2007]. The role of caspase-1 in ischaemia reperfusion injury has not been determined, as induction of injury in mice deficient for caspase-1 has furnished contrasting results. Results from one study indicated that caspase-deficient mice underwent less severe injury than their wild-type counterparts and that this was due to impaired interleukin-18 activation [Melnikov V, 2001], whereas a second group found no changes in the severity of the injury between control groups and caspase-1-deficient mice [Daemen MA, 2001].

1.4.4 Mechanism of Caspase activation: Extrinsic Pathway

Apoptosis can be initiated through several separate but interacting pathways. Extracellular signals are transmitted to the cell via the TNF superfamily of death receptors, the members of which are characterised by a conserved extracellular cysteine-rich motif, and include Fas, TNFR1, TNF-related apoptosis-inducing ligand (TRAIL). These receptors, generally composed of 3 identical polypeptide chains, have binding and signalling features in common as well as unique, individual characteristics. These death receptors have an intracellular death domain (DD) that activates cytosolic cysteine proteases by proteolysis, recruiting adapter proteins that also have death domains, including FADD and TRADD. The death effector domain of FADD binds to the prodomain of caspase-8, forming a complex termed the death-inducing signalling complex (DISC). The auto-catalytic activation of caspase-8 then activates a series of downstream caspses that result in cleavage of structural and regulatory intracellular proteins [Jin Z, 2005]. C-FLIP is a naturally occurring dominant negative antagonist of death receptor mediated caspase-8 activation, and contains two DED
and a defective caspase-like domain. C-FLIP can associate with DEDs of FADD and caspase-8, interfering with the recruitment of caspase-8.

The extrinsic pathway is activated by ischaemia reperfusion injury in the kidney. Mice treated with siRNA knockdown of caspase 8 expression were protected from ischaemic injury and had improved survival when compared to untreated animals [Zhang X, 2006].

1.4.5 Mechanism of Caspase activation: Intrinsic (mitochondrial) pathway

The apoptotic signal transduction pathways that are undertaken in response to an intrinsic signal such as DNA damage, oxidative stress and growth factor deprivation involve mitochondrial release of pro-apoptotic molecules. A critical regulatory role for the maintenance of mitochondrial membrane permeability has been suggested for proteins of the Bcl-2 family, which includes anti-apoptotic proteins Bcl-2 and Bcl-X_L [Walensky LD, 2006].

Permeabilization of the outer mitochondrial membrane is followed by release of pro-apoptotic proteins, usually present in the intermembrane space of these organelles, into the cytosol. Such effectors include cytochrome c, smac/DIABLO, HtrA2/OMI, the flavoprotein apoptosis-inducing factor (AIF) and endonuclease G. In the cytosol, cytochrome c interacts with the adapter protein Apaf-1 and induces a conformational change that allows stable binding of dATP/ATP, an event that drives the formation of a heptamer Apaf-1/cytochrome c complex, the apoptosome [Acehan D, 2002], which binds procaspase-9 via their homologous caspase recruitment domains (CARDs). Caspase-9 is activated by allostERIC change and dimerisation, rather than cleavage [Rodriguez J, 1999], which in turn activates the downstream effector caspase-3,-6 and -7 by proteolytic cleavage of inactive precursors.
The vital role of mitochondrial release of cytochrome c in stress induced apoptosis has been questioned. Initial reports on mice lacking either Apaf-1 [Cecconi 1998], or caspase-9 [Hakem 1998] supported the importance of cytochrome c in the intrinsic pathway: mice died near birth with enlarged brains, as do mice lacking caspase-3. Recent findings, however, rule out the essential role for Apaf-1 and caspase-9 in stress induced apoptosis in certain cell lines [Marsden VS, 2002]. Unlike Bcl-2 overexpression or loss of Bim, the absence of Apaf-1 or caspase-9 did not interfere with the development of mature lymphocytes, where programmed cell death is essential. Thus, in many cells, the apoptosome is dispensable for the initiation of stress induced apoptosis. Since the level of caspase activity is lower in Apaf-1 knockouts, the apoptosome must be an amplifier of the caspase cascade rather than a critical initiator of it [Marsden VS, 2002]. This amplification may be more important in some cell types than others, and in certain types of cellular stress leading to activation of the programmed cell death machinery.
Apoptotic cell death is initiated by activation of a caspase cascade via two distinct but communicating pathways: receptor mediated activation of caspase-8 and mitochondrial depolarisation causing release of cytochrome c which activates caspase-9. These intermediate caspases causes activation of the effector caspases-3, -6 and -7 which causes nuclear vacuolation and shrinking, disruption to cellular membrane, and ultimately cell death.
Mitochondria participate in inducing apoptosis after I/R through multiple changes, including generation of oxygen free radicals, calcium translocations, altered permeability transitions, and release of cytochrome c, apoptogenic factors, and Bcl-2 family members. Renal I/R in rats can induce mitochondrial swelling, rupture of inner and outer membranes, and release of Bcl-2 post-injury. A recent study investigated the proximate events that lead to mitochondrial permeability transition and release of cytochrome c after hypoxia/reoxygenation injury in kidney proximal tubular cells. A persistent respiratory defect occurs in complex I-dependent substrates during reoxygenation after hypoxia, and this defect is associated with condensed mitochondrial configuration and incomplete recovery of mitochondrial membrane potential. Amelioration of impaired substrate flux through mitochondrial complex I and ATP generation by α-ketoglutarate plus greatly improved mitochondrial function and cellular recovery. The identification of these upstream pathways of anaerobic metabolism and the possibility of metabolic manipulations to improve mitochondrial functions at an early stage may help to prevent irreversible mitochondrial damage in renal ischemia [Plotnikov EY, 2007].

Ischaemic renal injury is associated with a marked increase in the expression of the antiapoptotic Bcl-2 family of proteins, Bcl-2, Bcl-XL and the apoptotic protein Bax, in distal tubules and moderate increases in the proximal tubules [Chien CT, 2001]. The marked upregulation of the antiapoptotic proteins in the distal tubules may tip the balance in favour of cell survival, and this imbalance may be involved in its adaptive resistance to ischaemic injury. It can be hypothesised that this survival mechanism may allow the cells to produce growth factors that may aid in the protection and/or regeneration of the distal tubules by an autocrine mechanism and of the more vulnerable proximal tubules by a paracrine mechanism.

The relative expression of the Bcl-2 family of proteins in distal and proximal tubules subjected to oxidant injury in culture is similar to that seen in vivo. However, the expression
of Bcl-X<sub>L</sub> is decreased in proximal tubular cells and a translocation of Bcl-X<sub>L</sub> from the cytosol to the mitochondria is observed in the surviving distal tubule cells. No change in the sub-cellular distribution of Bax was observed in the surviving distal tubule cells, and it remained widely distributed in the cytosol. The expression of Bcl-2 or Bax was also unchanged in PTC post-oxidant injury [Chien CT, 2001]. It is unclear if the translocation of Bcl-X<sub>L</sub> plays a role in its protection from the oxidative injury. In a separate study, proximal tubules subjected to ATP depletion induced by hypoxic injury or impaired oxidative phosphorylation are shown to translocate Bax from the cytosol to the mitochondria. It is suggested that Bax may form pores in the mitochondrial outer membrane causing the release of cytochrome c from the mitochondrial intermembrane space and may activate apoptotic pathways [Wei Q, 2004].

1.4.6 Cellular inhibitors of apoptosis

The inhibitors of apoptosis (IAPs) family proteins includes eight mammalian family members, including X-linked inhibitor of apoptosis (XIAP), c-IAP1, c-IAP2, and ML-IAP/livin [Deveraux QL, 1999]. They act as a last line of defence to prevent cellular disassembly by binding to caspases by their BIR (Baculovirus IAP repeat) motif, which contains a conserved cysteine and histidine core sequence Cx2Cx6Wx3Dx5Hx6C [Dereraux QL, 1997; 1999] which may lead to ubiquitination of the caspases. XIAP, X-linked inhibitor of apoptosis, contains three BIR domains and has been shown to directly inhibit caspase-3, -7 and -9, but not caspase-1, -6, -8 or -10. This caspase selectivity could be due to the presence of conserved amino acid residues found in the BIR domains and the linker regions that separate them [Sun C, 1999]. Severe hypoxia up-regulates these endogenous inhibitors of apoptosis, particularly c-IAP2, and hypoxia-resistant epithelial cells which express increased c-IAP2 and Bcl-X<sub>L</sub> are more resistant to a second ischaemic or toxic insult [Dong Z, 2001; Dong Z, 2003].
The activity of IAPs is regulated by Smac/DIABLO, a structural homologue of the Drosophila proteins Reaper, Hid, and Grim [Du C, 2000]. Smac/DIABLO is released from mitochondria and inhibits IAPs, which facilitates caspase activation during apoptosis. Omi/HtrA2 has been recently identified as another modulator of IAP function. Omi/HtrA2 is a mitochondrial-located serine protease, which is released in the cytosol and inhibits IAPs by a mechanism similar to Smac [Suzuki Y, 2001].
1.5 Erythropoietin

1.5.1 The cell biology of erythropoietin

Erythropoietin (EPO) is a glycoprotein hormone with a molecular weight of 30.4 kDa. The gene for EPO, situated on chromosome 7q11-22, consists of five exons and four introns, encodes a protein precursor of 193 amino acids. During post-translational modification, which consists of cleavage of a 27 amino acid sequence, glycosylation of 3 N-linked (at Asn-24, Asn-38 and Asn-83) and one O-linked (ser-126) amino acids, the removal of Arginine residue (Arg-166) from the C-terminal end yields the final circulating EPO molecule comprising of 165 amino acids. The tertiary structure of erythropoietin is defined by four anti-parallel α-helices. EPO was successfully purified from the urine of patients with aplastic anaemia [Miyake T, 1977]. From tryptic fragments of this urinary EPO, DNA probes were synthesized for the isolation and cloning of the human EPO gene [Jacobs K, 1985]. The manufacture and application of recombinant human EPO molecule (rhEPO) to treat the anaemia of chronic renal failure followed shortly thereafter [Winearls CG, 1986; Eschbach JW, 1987].

In the healthy adult, EPO secretion is primarily from the kidney, in response to hypoxia, in order to maintain an optimal red cell mass to cope with normal tissue oxygen demand. The basal level of EPO secretion in the picomolar range maintains a plasma concentration equivalent to 15-25 mU/ L, although during periods of hypoxic stimulation plasma levels may increase by 50 -100 fold [Al-Huniti NH, 2004]. In the kidney, EPO production is restricted to a population of cells in the interstitium of the cortex and outer medulla. Immunohistochemical characterization utilizing SV40 labelled erythropoietin-producing transgenic mice demonstrated that EPO is produced in a population of fibroblast-like type I interstitial cells using light and electron microscopy [Maxwell PH, 1993].
Figure 1.4  Primary structure of erythropoietin, demonstrating cysteine sulphide cross-bridges
Recently, it has been discovered that EPO is additionally produced at several other sites, including the brain [Masuda S, 1994], the endometrium [Yokomizo R, 2002] and the epididymis [Kobayashi T, 2002]. The physiological role of EPO produced in these locations remains uncertain, but foetal production at multiple sites may be essential for normal development [Juul SE, 2004].

1.5.2 Regulation of EPO production

EPO gene expression is under the control of the oxygen-sensitive transcription factor hypoxia-inducible factor (HIF), which consists of a regulatory α-sub-unit (HIF-1α, -2α, and -3α) and the constitutively expressed sub-unit HIF-1β [Wang GI, 1993]. Both subunits are members of multi-protein families and belong to the extended family of basic helix-loop-helix PAS domain transcription factors. Low oxygen tension averts enzymatic prolyl-residue hydroxylation within a region of HIF-α called the oxygen-dependent degradation domain (ODD), by a family of prolyl-4-hydroxylases (PHD 1-3). In normoxia, the hydroxyl residue serves as a target for von-Hippel-Lindau (VHL)-dependent polyubiquitination and subsequent proteosomal degradation. Hypoxia prevents HIF degradation by inhibition of this oxygen-dependent pathway, leading to nuclear accumulation of HIF and DNA binding. The VHL gene product is the recognition component of a multiprotein E3 ubiquitin-ligase complex that captures HIF-α chains that have undergone enzymatic prolyl hydroxylation [Jaakkola P, 2001].

The HIF isoform that hypoxically regulates EPO production remains controversial. Although HIF-1 was initially purified from human hepatoma (HepB3) cells as the HIF molecule that bound to the 18-nucleotide fragment of the 3’ regulatory element containing the HRE, the regulation was found to be largely dependent on HIF-2, and not HIF-1, in studies utilizing a
siRNA knockdown approach [Warnecke C, 2004]. Hepatic production of EPO is suppressed in the livers from animals with Cre-LoxP-mediated inactivation of HIF-2, and complete absence of HIF-2 further suppressed physiological EPO concentrations [Rankin EB, 2007]. This may be due to preferential binding of HIF-2 to the HRE, although the expression of HIF-2 is variable, and this observed dominance may be cell type specific [Racliffe PJ, 2007].

In addition, prolyl hydroxylation requires 2-oxoglutarate as a co-factor because the hydroxylation reaction is coupled to the decarboxylation of 2-oxoglutarate to succinate, which accepts the remaining oxygen atom [Kivirikko K, 1998]. The prolyl-4-hydroxylase requires iron as a cofactor, and cobalt administration mimics the effect of hypoxia on HIF-1α activation. Cobalt administration to rats, via up-regulation of HIF-dependent proteins including EPO, VEGF and haem-oxygenase-1 (HO-1), diminished the degree of renal injury caused by ischemia-reperfusion [Matsumoto M, 2003], suggesting the HIF-dependent production of EPO may play an important role in ischaemic pre-conditioning. Evidence supporting the importance of EPO in ischaemic pre-conditioning comes from work in transgenic heterozygotic HIF -/+ mice, which express only small amounts of constitutively expressed HIF-1, and are resistant to an ischaemic pre-conditioning protocol [Cai Z, 2003]. These animals showed increased renal production of EPO which was significantly greater than the increase in other HIF-dependent hormones VEGF and HO-1.

HIF activation leads to the altered expression of a diverse range of cytokines and mediators that mediate the adaptive response to stress and ischaemia, including pro-angiogenesis hormone vascular endothelial growth factor (VEGF), glucose transporters (GLUT1) and glycolytic enzymes, iron metabolism (transferrin), and a variety of genes involved in cellular proliferation, differentiation and viability (IGF binding proteins, p21, cyclin G2 and caspase-9) [Iyer N, 1998].
Figure 1.5  Oxygen Sensing and EPO gene transcription.

Low oxygen tension averts enzymatic prolyl-residue hydroxylation within a region of HIF-1α called the oxygen-dependent degradation domain (ODD), by a family of prolyl-4-hydroxylases (PHD 1-3), which, in normoxia, serves as a target for von-Hippel-Lindau (VHL)-dependent polyubiquitination and subsequent proteosomal degradation, thereby preventing HIF degradation, leading to nuclear accumulation of HIF-1. In the presence of c-factor p300, HIF binds to the hypoxia response element (HRE) in the EPO promoter sequence and increases gene transcription.
1.5.3 EPO in Haematopoietic Tissues

Due to the natural destruction of aging erythrocytes in the reticuloendothelial system there is a need for continual production of new red blood cells throughout life. There are several stages of erythroid cell maturation and each step is dependent on the presence of numerous growth factors for survival, proliferation and differentiation [Kaushansky K, 2006]. The effects of erythropoietin on the erythroid components of bone marrow are mediated by binding to specific EPO receptors on erythroid precursors (intermediate-stage erythroid burst-forming units (BFU-E) and the erythroid colony-forming units (CFU-E), which have already differentiated from pluri-potent stem cells. Differentiation to this stage is not dependent on EPO, as EPO-R knock-out mice are incapable of erythropoiesis but have committed BFU-E and CFU-E in foetal liver tissue [Wu H, 1995]. Thus the expression of the EPO receptor is itself an essential part of the mechanism by which erythropoiesis is controlled. The expression of the erythropoietin receptor is dependent on the specific phase of differentiation, and in the absence of EPO, the erythroid progenitors undergo apoptotic cell death [Boyer SH, 1992].

EPO can efficiently support the proliferation of murine erythroid progenitor cells ex vivo, and induce entry into the cell cycle in dormant cells [Miller CP, 1999]. Using the EPO-dependent human erythroid progenitor cell line HCD-57, Silva et al showed that erythropoietin maintained their viability via repressing apoptosis by upregulating Bcl-X_L, an anti-apoptotic protein of the Bcl-2 family [Silva M, 1996]. To further highlight the importance of this pathway, Bcl-X_L null mice exhibit fetal liver haematopoietic defects and severe anaemia during embryogenesis [Wagner KU, 2000].
Before the cloning of the EPO receptor cDNA, investigators had used radiolabelled EPO to demonstrate specific binding to cells derived from the erythroid lineage [D’Andrea D, 1990]. These studies showed that approximately 200 EPO receptors were present on the cell surface of purified normal erythroid progenitors. This relatively low expression of cell surface receptors is characteristic of other receptors of haematopoietic cytokines such as G-CSF, GM-CSF and interleukin-3 [Groopman JE, 1989]. Affinity cross-linking experiments using radiolabelled EPO revealed two cross-linked EPO receptor complexes, and subsequent crystallization studies have confirmed receptor dimerisation [Matthews DJ, 1996].

The murine EPO receptor was cloned by transfecting pools of recombinant plasmids from a MEL cDNA library in to COS cells and screening for uptake of radiolabelled EPO by transfected cells [D’Andreau AD, 1989]. The cloned EPO receptor was a 507-amino acid polypeptide with a single membrane spanning domain, with extensive homology with the interleukin 2 receptor β chain [D’Andreau AD, 1990]. The extracellular domain of the receptor possesses a conserved domain of ~200 amino acids, which derives from the duplication of a 100 amino acid subdomain with a type III fibronectin structure [Bazan JF, 1990]. The EPO receptor exhibits a WSXWS sequence in the membrane-proximal subdomain and two pairs of cysteines in the first subdomain. The two pairs of cysteine are held by disulphide bonds. The WSXWS sequence is thought to be necessary for the correct folding and cell membrane stability [Yoshimura A, 1992].

Like all the receptors in the cytokine receptor superfamily, the EPO receptor does not possess endogenous tyrosine kinase activity, but binding of EPO to the extracellular portion of the receptor induces a conformational change, which brings JAK2 kinase into proximity,
allowing autophosphorylation and activation of JAK2 kinase domain (Figure 1.5). This induces rapid tyrosine phosphorylation of a number of proteins [Withuhn B, 1993]. Eight tyrosine residues located on the cytoplasmic domain of the EPO receptor are phosphorylated by JAK2, upon EPO stimulation, and these phosphorylated tyrosine residues are docking sites for proteins containing src-homology 2 (SH2) domains [Dusander-Fourt I, 1992].

The STAT (signal transducer and activator of transcription) pathway plays a major role in cytokine-induced signalling. EPO activates both STAT5A and STAT5B [Damen JE, 1993; Wakeo H, 1995]. The two first tyrosine residues in the intracellular domain of the EPO receptor (Y343 and Y401) are responsible for STAT5 binding and activation [Chin H, 1996]. The precise role of STAT5 activation induced by EPO is the subject of controversy. A correlation between STAT5 activation and cellular proliferation has been observed, but STAT5 activation may also play a role in erythroid differentiation [Chretien S, 1996].

A direct association between phosphatidylinositol 3-kinase (PI 3-kinase) and the EPO receptor has been shown, which involves the SH2 domains of the p85 subunit of the PI 3-kinase and tyrosine-479 of the EPO receptor [Damen J, 1995]. An alternative pathway for the activation of PI 3-kinase has been described, which involves the tyrosine phosphorylation of the adaptor protein IRS2 and its subsequent association with PI 3-kinase, which allows for the previous finding that cells expressing a truncated EPO receptor had PI 3-kinase activity [Verdier F, 1997]. PI 3-kinase leads to phosphorylation and activation of its downstream target, the serine/threonine kinase AKT, which in turn phosphorylates multiple targets including FKHRl1, and P70S6-kinase [Downward J, 1998]. Several studies have suggested that the PI 3-kinase/AKT pathway is the major pathway involved in the anti-apoptotic effects of EPO in erythroid progenitors [Haseyama Y, 1999] and also has a role in the proliferation and differentiation of endothelial progenitor cells [Bahlmann FH, 2003].
AKT is an important molecule in mammalian signalling to promote cell survival by inhibiting apoptotic cell death pathways. AKT is also able to induce protein synthesis pathways, and therefore is a key signalling step in general tissue growth. AKT regulates the cellular survival and metabolism pathways by binding and regulating many downstream effectors both directly and indirectly, including BAD, a pro-apoptotic protein of the Bcl-2 family. AKT phosphorylates BAD on Ser$^{136}$, which makes BAD dissociate from the Bcl-2/Bcl-X$_L$ complex and lose the pro-apoptotic function [Chong Z, 2005]. AKT also activates NF-κB via regulating IκB kinase (IKK), thus result in transcription of pro-survival genes which are up-regulated by NF-κB [Terrangi J, 2008].

Erythropoietin receptor signalling is terminated through receptor internalisation and proteosomal degradation [Verdier F, 2000], after 30-60 minutes of stimulation. Receptor activation and signalling causes the activation of protein tyrosine phosphatases, which de-phosphorylates JAK2 and inactivates its kinase function, with inactivation of STAT pathways [Klingmuller U, 1995]. Mutations in the EPO receptor that lead to decreased or absent de-phosphorylase activity, prolonging the stimulation signal produced by ligand binder are associated with familial erythrocytosis syndromes [Arcisoy MO, 1999].
Figure 1.6  Conformational change on ligand binding allows activation

EPO binds to the extracellular portion of the receptor, leading to structural rearrangement which allows proximity-dependent autophosphorylation of receptor associated JAK2 kinases, which phosphorylate tyrosine residues on the intracellular portion of the receptor, allowing SH-2 domain containing mediators, activating STAT5. The activated STAT5 homodimer translocates to the nucleus.
Studies of the tissue distribution of EPO and the EPO receptor in the developing human foetus have demonstrated the presence of mRNA for EPO and the receptor in virtually all major organs in the first two trimesters [Juul SE, 1998]. This leads to speculation that EPO acts in concert with somatic growth and developmental factors during early foetal life. Although mice with EPO gene knock-down were found to be neurologically normal [Maxwell PH, 1994], subsequent studies have demonstrated defects in cardiac structure and reduction in the expression of neuronal stem cells [Chen ZY 2007] in these animals. Latent expression in adult tissues may be up-regulated in disease states, particularly in the development of cancer. Indeed, an increasing number of cancer cell types have been shown to express a functional EPO receptor [Westenfelder C, 2000; Kumar SM, 2005], which may have implications for the therapeutic use of erythropoietin in certain clinical situations. Further work is required to fully answer the questions raised by these observations.

Mature endothelial cells also express EPO receptors (Epo-Rs) and EPO induces a proangiogenic response in cultivated mature endothelial cells, as evidenced by stimulation of endothelial cell proliferation, migration, endothelin-1 release, and increase in cytosolic-free calcium concentration [Chong Z, 2002]. A physiologic significance for the EPO-EPO-R signaling pathway has been demonstrated for the oestrogen-dependent cyclical angiogenesis in the uterus [Yasuda Y, 1998].

1.5.5 Use of EPO in Anaemia associated with end-stage renal failure

Anaemia develops in most patients with CKD because less erythropoietin is produced by the diseased kidneys [Erslev AJ, 1997]. The decline in haemoglobin (Hb) concentration may start at levels of creatinine clearance of around 70 mL/min among men and 50 mL/min among women, and progresses relentlessly. The management of anaemia before the
introduction of recombinant human erythropoietin was a struggle, with a combination of “good dialysis”, minimizing blood loss, parenteral iron and androgens, together with avoidance of excess blood tests, achieving average haemoglobins of approximately 8 g/dl. This left about 10% of patients dependent on blood transfusions and susceptible to the complications of iron overload. Following successful cloning of EPO, pilot studies followed very rapidly [Winearls C, 1986], leading to a number of successful multi-centre trials [Eschback JW 1989]. The doses required to reverse anaemia and maintain haemoglobin were found to be similar in American and UK studies [Bommer J, 1988; Eschbach JW, 1989].

The erythrokinetic studies performed on the first dialysis patients treated with r-HuEPO showed that the total red cell volume was below normal before treatment and increased after treatment. These increases in red cell volume were accompanied by reciprocal changes in plasma volume, so that total blood volume was not altered by treatment. Red cell survival in non-transfused patients was only modestly shortened before treatment and was not altered by treatment. Studies of the response of bone marrow erythroid progenitors to r-HuEPO treatment showed that after treatment, the number of bone marrow BFU-E had fallen to a mean of 24% of pre-treatment values, but there was no significant change in the number of CFU-E. The failure to change the number of CFU-E would have to be explained by self-renewal and replenishment from the BFU-E, keeping the size of this compartment constant [Reid CDL, 1988].

The best marker of benefit of the introduction of erythropoietin to clinical practice is the reduction in the need for regular blood transfusions [Sundal E, 1991]. The improvement in symptoms attributable to anaemia, and hence quality of life, is unequivocal, and confirmed in several randomised, placebo controlled trials [Auer J, 1990]. Associated improvements in
haemodynamic status due to correction of anaemia lead to reduction in progression of left ventricular hypertrophy, with a concomitant reduction in mortality.

The therapeutic use of EPO in these patients led to the identification of systemic and vascular effects that caused significant complications. EPO administration results in an increase in diastolic blood pressure of up to 10 mmHg in 10-20% of patients [Smith KJ, 2003]. The underlying mechanism of this effect is unclear, but a number of possibilities have been studied, including the induction of the vaso-constrictor endothelin-1 [Carlini RG, 1993], altered intracellular calcium concentration and a diminished response to NO [Marrero MB, 1998].

1.5.6 The novel role of Erythropoietin in the Nervous System

Both erythropoietin and the EPO receptor are functionally expressed in the nervous system of rodents, primates and humans. In the mouse, EPO is present in the hippocampus, capsula interna, cortex and mid-brain areas [Digicaylioglu, 1995]. In cultured rat cortical neurones, the expression of the EPO receptor has been demonstrated by immunostaining and RT-PCR [Morishita, 1997]. In humans, EPO and the EPO receptor are present in both astrocytes and neurones, although the level of expression varies according to gestation age, with reduced production after birth [Juul, 1999].

Hypoxia results in the induction of EPO in brain tissues. Accumulation of EPO mRNA and the EPO protein in response to hypoxia has been observed in cultured astrocytes [Marti HH, 1996]. EPO and the EPO receptor are also inducible in the hippocampal neurones, although in vivo models demonstrate a broader up-regulation during hypoxia [Bernaudin M, 1999].
Initial experiments have therefore examined the potential role of EPO in the nervous system during cerebral ischaemia. Infusion of EPO into the lateral ventricle of gerbils subjected to the occlusion of the common carotid arteries prevented ischaemia-induced learning disability and rescued hippocampal neurones from degeneration [Sakanaka M, 1998]. Multiple studies have now confirmed the beneficial effects of EPO administration in the course of ischaemic brain injury in vivo, using systemic administration to overcome the impracticalities of ventricular delivery systems [Sadamoto Y, 1998; Matsushita H, 2003; Grasso G, 2002].

Ischaemia-related investigations examining the potential of erythropoietin to prevent neuronal injury have also been extended to the spinal cord, peripheral nervous system and visual system, summarized in table 1.1.
<table>
<thead>
<tr>
<th>Animal studies</th>
<th>25–100 U i.p. or 0.2–25 U day-1 i.c.v. applied either pretreatment or post-treatment with injury in rats, mice or gerbils</th>
<th>Infarct volume, brain edema and neuronal apoptosis decreased; neuronal survival increased</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sakanaka M, 1998</td>
<td>5000 U i.p. or 2 ml intravitreal applied imm. or posttreatment</td>
<td>Photoreceptor and retinal ganglion cell apoptosis decreased</td>
</tr>
<tr>
<td>Retinal ischemia</td>
<td>1000–5000 U/Kg i.p. applied pre-treatment with injury in mice</td>
<td>Infarct volume and neuronal apoptosis decreased; caspase-3 activity decreased</td>
</tr>
<tr>
<td>Junk AK, 2002</td>
<td>100–5000 U/kg i.p. with injury in rats</td>
<td>Motor neuron apoptosis, inflammation and lipid peroxidation decreased; functional recovery improved</td>
</tr>
<tr>
<td>Neonatal hypoxic–ischemic brain injury</td>
<td>1000 U/kg i.p. applied 5 min after injury in rabbits</td>
<td>Neuronal death decreased; functional recovery and blood flow autoregulation improved</td>
</tr>
<tr>
<td>Aydin A, 2003</td>
<td>1000–5000 U/kg s.c. applied pre-treatment with injury in rats</td>
<td>Dorsal and ventral root ganglion cell injury decreased; recovery from mechanical allodynia improved</td>
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<tr>
<td>Spinal cord injury</td>
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<td>Gorio A, 2002; Celik M, 2002</td>
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<td>Subarachnoid hemorrhage</td>
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<td>Buemi M, 2000</td>
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<td>Peripheral nerve injury</td>
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<td>Campana W, 2003</td>
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<tr>
<td>Tissue culture studies</td>
<td></td>
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<tr>
<td>Anoxic injury</td>
<td>10 ng/mL applied pre-treatment with injury in ECs or hippocampal neurons</td>
<td>DNA fragmentation and PtdSer exposure decreased; cell survival increased</td>
</tr>
<tr>
<td>Chong ZZ, 2003</td>
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<tr>
<td>NO injury</td>
<td>10 ng/ml or 20 U/ml applied pre-treatment or imm. with injury in ECs or hippocampal neurons</td>
<td>DNA fragmentation, PtdSer exposure and NO production decreased; cell survival increased</td>
</tr>
<tr>
<td>Yamazaki M, 2005; Sakanaka M, 1998</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate toxicity</td>
<td>50 ng/ml or 3–300 pM/ml pre-treatment with injury in hippocampal, cortical and cerebellar neurons</td>
<td>Glutamate release decreased; neuronal survival increased</td>
</tr>
<tr>
<td>Yamazaki M, 2005</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.1** Therapeutic use of EPO in experimental models of brain injury
1.6 Aims

The evidence presented in the introduction has led to the hypothesis that the haematopoietic growth hormone Erythropoietin is functional in tissues outside the bone marrow, and that it exerts physiological effects to lessen injury induce by exposure to ischaemia, and to initiate the tissue healing process.

In order to test this hypothesis, the primary aim of this thesis is to establish the role and effects of the haematopoietic growth factor erythropoietin (EPO) on the course of ischaemia reperfusion injury in the kidney. In order to fully achieve this, it is essential to study the direct in vitro effects of EPO on proximal tubular epithelial cells, and examine the intracellular mechanisms which underpin the protective effects of EPO in these models. Finally this thesis will aim to extend the examination of the effects of EPO into different types of injury model, including other in vivo organ systems.
Chapter Two:

*In vitro* effects of Erythropoietin in a Human Proximal tubular epithelial cell line
2.1 Introduction

In erythroid precursor cells at the CFU-E stage, EPO activates a number of signalling pathways via engagement of its cell surface receptor, which reduce growth factor withdrawal-induced cell death, and hence allow proliferation and differentiation, as discussed in detail in section 1.5.4.

A direct effect of EPO on the viability and resistance to cell death of tubular epithelial cells would require cell surface expression of a functional EPO-R on this cell population. The identification of a functional EPO/ EPO-R system in the nervous system led to a range of studies demonstrating signalling effects and beneficial responses in a range of injury models, including hypoxia and toxic insults, described in section 1.5.7. At the time of starting the body of work described in this thesis, experiments utilizing both polymerase chain reaction mRNA expression and immunoblotting techniques had demonstrated that the EPO-R is present on human, mouse and rat proximal tubular epithelial cells, and that, in cultured proximal tubular epithelial cells, EPO binds to its cell surface receptor, and exerts effects on cellular proliferation in culture [Westenfelder C, 1999].

2.2 Aims

The aim of the experiments in this chapter was to examine the signalling pathways activated by EPO in a human proximal tubular epithelial cell line, concentrating on the pathways known to be EPO-responsive in erythroid progenitor cells, and determine the effects of EPO on cell survival in a basic in vitro injury model.
2.3 Materials and Methods

2.3.1 Proximal Tubule epithelial cell lines

HK-2 cells, a proximal tubule epithelial cell line (PTEC) derived from normal human kidney, were obtained from the ECACC (European collection of cell cultures). HK-2 cells were immortalised by transduction with human papilloma virus 16 (HPV-16) E6/E7 genes. The recombinant retrovirus vector pLXSN 16 E6/E7 containing these genes was used to transfect the ectotropic packaging cell line Psi-2. Subsequent viruses produced by the Psi-2 cells were used to transfect the amphotropic packaging cell line PA317. Viruses obtained from the PA317 cells were used to transduce primary proximal tubule epithelial cells. The pLXSN 16 E6/E7 confers resistance to neomycin. The cells retain a phenotype indicative of well differentiated proximal tubular epithelium, and express alkaline phosphatase, gamma glutyltranspeptidase, leucine aminopeptidase, α3, β1-integrins and fibronectin [Ryan MJ, 1994].

2.3.2 Cell Culture

HK-2 cells were cultured in Dulbecco’s Modified Eagles Medium Ham’s F12 with 10% v/v fetal calf serum (FCS) and antibiotics (benzylpenicillin 100 U/ mL, streptomycin 10 μg/ mL, amphotericin 2.5 μg/ mL (all Sigma-Aldrich Chemical Co., UK) [hereafter called standard medium] in sterile T75³ culture flasks (VWR, UK) and incubated at 37°C in a humidified atmosphere comprising of 95% air and 5% CO₂. When confluent, the cells were sub-cultured; the medium was removed and the cells washed with 10 mL of Phosphate Buffered Saline pH 7.4 (PBS). PBS was removed before the addition of 10 mL of 0.05% Trypsin (Sigma), which was then incubated at 37°C for approximately 10 minutes until the cells had
detached from the culture flask. The cells were then harvested by centrifugation at 800rpm for 5 minutes. The supernatant was removed with care to avoid disturbing the pelleted cells, which were re-suspended in 10 mL culture medium for a wash step. The cells were again harvested by centrifugation at 800 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in culture medium and aliquoted into fresh culture flasks at a cell to medium ratio of 1:3. Cells were used for all experiments between passage three and six, and fresh batches of cultured cells were developed at all times to maintain optimum cell conditions for experiments. Foetal calf serum contains a variety of growth hormones, at very small concentrations, and may include EPO, although the amount is not specified in the manufacturer’s information.

2.3.3 Cell Count

After being harvested, an aliquot of pelleted cells was resuspended in 10 mL of culture medium and a 1:1 dilution of the cell suspension made with 0.1% v/v of Trypan Blue. 100 µL of cell suspension-trypsin blue mixture was transferred to a haemocytometer chamber over which a cover slip was placed. Using one chamber of the haemocytometer, the numbers of dead and viable cells were counted in the middle 1mm² square and at least two of the four corner squares. Each square on the haemocytometer (with the cover slip in place) represents a total volume of 0.1mm³ (10⁻⁴ mm³). Therefore,

- **Live cells per mL** = mean number of unstained cells per square x 10⁴ x 2
- **Total cells** = cells per mL x volume
- **Cell viability** = \( \frac{\text{number of unstained cells} \times 100}{\text{Total number of cells}} \)
2.3.4 Cell freezing

After being harvested by centrifugation, pelleted cells were resuspended in 100 µL of cell culture medium, and then aliquots were added to cell culture freezing medium (10% DMSO, 90% culture medium) and transferred to a cryovial, which was placed in a polystyrene container at -80°C for 2 hours before being transferred to liquid nitrogen for long term storage.

2.3.5 Quantification of protein by BCA assay

The BCA assay is a detergent-compatible formulation, based on bicinchoninic acid (BCA), for the colorimetric detection and quantification of total protein. This method combines the well-known reduction of Cu$^{2+}$ to Cu$^{1+}$ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu$^{1+}$) using a reagent containing bicinchoninic acid [Smith PK, 1985]. The purple colour reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous cation. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2,000 µg/ ml).

Protein concentrations are determined with reference to a standard curve of known protein concentrations made up with bovine serum albumin. 100 µL of each known dilution of the standard curve was added to duplicate wells on a 96 well plate (8 wells). 100 µL from each protein sample was added to the plate. The assay reagent buffer was added to each well (200 µL), and the plate incubated at 37 °C for 30 minutes. Absorbance at 562 nm was measured in a plate reader and corrected for a blank well measurement.
2.3.6 Western Immunoblotting

Sub-confluent cells were incubated in culture medium with and without experimental factors for varying time points. At the end of the experiment, the incubation was stopped by washing the cells with ice-cold PBS. Cells were removed after scraping the flasks with a Teflon coated cell scraper, followed by centrifugation for 5 minutes at 1200 x g. Cell pellets were then lysed by the addition of ice-cold lysis buffer:

**Standard Lysis buffer**

- 20 nM Tris (pH 7.4)
- 150 mM NaCl
- 1% NP-40
- 0.5 % w/v Na deoxycholate
- 0.1 % SDS (Promega)

Protease inhibitors were added immediately prior to cell lysis at the following concentrations:

- 1 mM PMSF (10 mg/mL stock solution in isopropanol)
- 1 mM Na orthovanadate (100 mM stock solution)
- 30 μL of a 5-19 U/mL solution of aprotinin from bovine lung

For 1-5 x 10^6 cells, 100 μL of lysis buffer was typically used. This was left on ice for 15-20 minutes with regular vortexing and then centrifuged at 15000 x g for 15 minutes at 4°C. The protein content of the supernatant was measured using the bicinchoninic acid (BCA) protein quantification assay (Pierce) in a 96-well plate format, described in 2.3.5.
2.3.7 SDS-PAGE

For each individual gel, 20 - 40 µg of protein from each sample was electrophoresed on a polyacrylamide gel in the presence of SDS. The composition of the solutions for SDS-PAGE is shown below:

**Stacking gel**
- Protogel (30 % w/v acrylamide; 0.8% w/v bis-acrylamide) 0.33 mL
- Resolving buffer (1.44 M Tris-HCl; 0.384% SDS, pH 8.8) 0.63 mL
- 10 % Ammonium persulfate (APS) 12.5 µL
- H₂O 1.54 mL
- TEMED 2.5 µL

**Resolving gel**
- Protogel 1.67 mL
- Resolving buffer (0.5M Tris-HCl; 0.4% SDS, pH 6.8) 1.30 mL
- 10 % APS 50 µL
- H₂O 1.98 mL
- TEMED 5 µL

Samples were mixed with sample buffer (0.125M Tris-HCl (pH 6.8), 4% SDS, 20 % glycerol, 10% 2-mercaptoethanol), heated to 95°C for 5 minutes and placed on ice. 20 - 40 µL (corresponding to 20 - 40 µg of protein, equalised for the lowest protein content) per well was loaded onto the gel. Electrophoresis was carried out at 25 mA per gel for approximately 45 minutes at room temperature in a buffer containing 0.025 M Tris (pH 8.3), 0.192 M glycine, 0.1 % SDS.
In later experiments, the Invitrogen XCell mini-cell electrophoresis system was used for all immunoblotting. Pre-cast NuPAGE Bis-Tris gels were used (10% or graduated 4-12%), and gels were run at 200 V constant, in running buffer (MOPS 50 mM, Tris-HCl 50 mM, 0.1% SDS, EDTA 1mM (pH 7.7).

### 2.3.8 Immunoblotting and detection of target proteins

Acrylamide gels were carefully removed from the electrophoresis apparatus and laid on top of a piece of polyvinylidifluoride (PVDF) membrane (Immobilon P; Millipore, Sigma) which had been cut to size and pre-soaked in 100% methanol and dH2O. Air bubbles were smoothed out with a sterile glass rod and the gel and membrane sandwiched between two sponges soaked in transfer buffer (10 mM CAPS, pH 11.0; 10% methanol in dH2O). The sandwich was then placed in the transfer apparatus with the membrane facing the anode and electroblotting carried out at 400 mA for 1 hour at room temperature. Following electroblotting, the membrane was separated from the gel and any residual acrylamide washed off by soaking in TBS-T for 2 minutes. Non-specific sites on the membrane were blocked by incubation with 5% BSA in TBS-T overnight at 4°C. After washing in TBS-T for 5 minutes at room temperature, the membrane was probed with specific primary antibody (1:400- 1:2000 v/v in TBS-T + 1% BSA) for 2 hours at room temperature with constant agitation.

The membrane was then washed in TBS-T 3 times for 5 minutes each. The membrane was then incubated with polyclonal secondary IgG antibody conjugated to horse-radish peroxidase (1:2000 in TBS-T + 1% BSA) for 1 hour at room temperature with constant agitation. The membrane was washed as before. The protein band of interest was visualised by chemiluminescence (ECL chemiluminescence Detection system, Amersham, UK). Equal
volumes (~2 mL) of ECL solutions (A+B) were mixed and gently pipetted onto the membrane and left for 30 seconds without agitation. The solutions were then drained off and the membrane placed on a lint-free tissue to remove any excess solution. The membrane was immediately covered in cling-film and exposed to light-sensitive film (Kodak Biomax Light) for various times in the dark room. The film was developed using an X-ray developer.

2.3.9 Quantification of cell viability and proliferation with MTS assay

Cell viability and proliferation experiments were performed utilizing the novel tetrazolium compound (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and the electron coupling reagent, phenazine methosulfate (PMS). In the presence of PMS, MTS is chemically reduced by live cells into formazan, which is soluble in tissue culture medium, and the formation of formazan is directly proportional to the number of viable, metabolizing cells. Experiments were carried out in clear 96-well microplates, using 100 µL of phenol-red free standard culture medium per well. At the end of the experiment, 20 µL of the MTS reagent (CellTiter 96® Assay, Promega) was added to each well and gently mixed. The plate was returned to the incubator for 30 minutes at 37°C.

The absorbance of the formazan at was read at 492nm, and expressed as a percentage of absorbance from control cells. Values over 100% were taken as to be a measure of increased cell proliferation over control, although this could be confounded by experimental conditions that alter cellular metabolism.
2.3.10 Quantification of cell death by DNA fragmentation assay

Cell death was detected by a DNA fragmentation enzyme linked immunosorbent assay (ELISA), which quantifies the mono- and oligo-nucleosomes in the cytoplasmic fraction of cell lysates (Cell Death Detection ELISA PLUS, Roche Diagnostics, Lewes, UK). Cytosolic lysates extracted using digitonin buffer (10 μM Digitonin in PBS (pH 7.4)) were diluted 6-fold in kit lysate buffer. 20 μL of the diluted cell lysate per well was added to a streptavidin coated microplate. 80 μL of a mixture of anti-histone-biotin and anti-DNA-POD were added and the plate incubated for 2 hours at room temperature with gentle mixing on a microplate stirrer (300 rpm). During the incubation period, the anti-histone antibody binds to the histone component of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin coated microplate via its biotinylation. Additionally, the anti-DNA-POD antibody reacts with the DNA content of the nucleosomes. The plate is then washed 3 times with incubation buffer (250 μL/ well) before 100 μL of 2, 2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) solution is added to each well and the plate incubated at room temperature for 5-10 minutes on a plate-shaker (300 rpm).

The plate is read at 405 nm on a Dynex MRX II plate reader. DNA fragmentation is expressed as an enrichment factor from a time 0 control sample per mg of total cell lysate protein.

2.3.11 Transcription factor DNA- binding Assay by ELISA

Families of closely related homo- and hetero-dimer complexes that bind to DNA (transcription factors) regulate many of the global signalling pathways and are widely studied as disease targets. Traditionally, translocation of transcription factors was
determined by electrophoretic mobility shift assay (EMSA) using labeled specific oligonucleotides, but this technique is time-consuming and only semi-quantitative in nature. The DNA binding ELISA uses binding of a specific antibody and labeled secondary antibody to give more sensitive, highly quantitative data.

10 μg of nuclear protein extract was added to each well on a 96-well plate coated with an immobilized oligonucleotide containing a transcription factor-specific consensus sequence,

\[\text{STAT family (5' TTCCCGAA-3')}\]

with 30 μL buffer, and the plate was incubated at room temperature with gentle agitation for one hr. After washing, buffer containing a transcription factor-specific primary antibody (1/10000 dilution) was added to each well, and the plate incubated without agitation for one hour. The plate was washed 3 times, and then a HRP-conjugated secondary antibody was added. The plate was incubated for one hour, and then washed four times. 100 μL of developing buffer was then added to each well for up to 10 min, and absorbance read on a Dynax MRX II (450 nm with reference 650 nm). Values were expressed as optical density (n=3 for 3 independent experiments).

2.3.12 Cell Transfection and transfection of siRNA

RNA-mediated interference (RNAi) is a well-recognised pathway employed by most eukaryotes as a cellular line of defence directed against invading viral genomes or as a method to clear a cell of aberrant transcription products [Hamilton AJ, 1999]. Whilst the exact mechanism of successful RNAi-mediated gene silencing remains to be fully elucidated, this method has become an invaluable tool for analysis of gene function and
target validation. Cellular uptake of long double stranded RNA (dsRNA) has been shown to induce RNA interference in a diverse group of lower eukaryotic organisms. In 2001, Dr Tuschl et al showed that when short RNA duplexes (21-23 bases in length) were introduced into mammalian cells in vitro, sequence-specific inhibition of target mRNA was effected without inducing an interferon response [Elbashir SM, 2001].

2.3.13 Optimisation of siRNA transfection protocol for HK-2 cells

In order to determine the most effective protocol for siRNA transfection in HK-2 cells, a series of optimization experiments were performed. HK-2 cells were seeded in low density (50% confluence) into 6-well plates 24 hours before transfection. Control non-targeted siRNA labelled with fluorescein was used to assess transfection by fluorescent microscopy. Standard medium was removed from each well and replaced by 800 µL standard media without antibiotics. Three µL control siRNA (final concentration 100 nM) was mixed with serum-free medium and incubated at room temperature for 7 minutes. 3 µL Oligofectamine (Invitrogen), GeneJuice, Dimri-C, RNAiffect, and NEB reagent were mixed with serum-free medium and kept. The two reagents were then gently mixed by inversion and incubated at room temperature for 20 minutes. The volume was made up to 1200 µL with medium, and 200 µL per well was added gently and mixed by swirling.

The plate was incubated in the incubator overnight. The medium was removed and 2 drops of antifade reagent (plus DAPI nuclear stain) was added dropwise to each well and placed in a dark box. Fluorescence was examined under a microscope, and percentage of cells showing green fluorescence was calculated by counting total cell number by positive nuclear DAPI staining on two random high powered fields. The highest efficiency of transfection
achieved over a number of experiments was with the transfection reagent oligofectamine, illustrated in Figure 2.1, and this was adopted for all subsequent transfection experiments.

2.3.14 Statistical Analysis of Experiments

All experiments were performed in identical passaged cells in triplicate unless stated in the methods. All values described in the text and figures are expressed as the mean ± standard error for \( n \) (number of) observations. Each data point representing biochemical measurements was obtained from up to 10-12 separate animals. For histological scoring, caspase activity assays and apoptosis scoring, each data point represents analysis of kidneys taken from six individual animals.

All statistical analysis was performed using GraphPad Prism 3.01/ Instat 1.1 (GraphPad Software, San Diego, USA). Data for multiple group comparisons were analysed using one-way ANOVA followed by Dunnett post-hoc test, or Kruskal-Wallis ANOVA for non-parametric data. A \( P \) value of less than 0.05 was considered significant.
Figure 2.1  Quantification of siRNA transfection rate by fluorescent microscopy for labelled non-targeted control siRNA. Comparison was made with DAPI nuclear staining to calculate percentage transfection and amount of expression by fluorescence. All slides were analysed in a single session without adjustment of microscope settings. Images were downloaded from the PC and have not been altered.
2.4 Results

2.4.1 Expression of EPO receptor on HK-2 cells

The detection of the EPO-R in whole cell lysates was performed by immunoblotting with a validated specific polyclonal rabbit antibody to the C-terminal (Santa Cruz C-20) of the EPO-R, the specificity of which is not affected by ligand binding. Lysates were prepared from serum-starved UT-7 cells, a growth-factor dependent human erythroleukaemic cell line, known to widely express the EPO-R, to act as a positive control for immunoblotting [Dusanter-Fourt I, 1992].

Figure 2.2 shows a representative western blot, from three individual experiments, of whole cell lysates from HK-2 cells, showing two bands at approximately 68 and 55 kDa, corresponding to the EPO-R, with similarly sized bands identified in the UT-7 cell lane.
Figure 2.2 Immunoblot of EPO-R in two cell lines shows similar bands sized approximately 65-69 kDa. Representative images are shown from 3 separate experiments. Protein loading was confirmed by reprobing membrane for actin.
2.4.2 EPO induces proliferation in HK-2 cells which is dependent on presence of EPO receptor

HK-cells were seeded at $1 \times 10^5$ /mL in a 96 well plate in standard medium containing 10% FCS and incubated overnight. The medium was removed and stimulated with phenol-red free standard medium containing different concentrations of EPO (1-100 U/mL), and an MTS assay was performed after 24 hrs incubation. Experiments were repeated on three individual occasions, with 12 wells per experimental group.

At 24 hrs, administration of EPO caused a dose-dependent increase in cell number over control levels in the range of concentrations 1 - 50 U/mL (when compared to control cell, EPO 1 U/mL + 19% ± 0.5, EPO 10 U/mL + 33% ± 1, EPO 50 U/mL + 45% ± 1, $P<0.01$), shown in Figure 2.3.

To confirm that these effects of EPO on cellular proliferation were dependent on receptor-mediated cell signalling, expression of the EPO-R was inhibited by transfection of an EPO-R specific siRNA (Dharmacon). Cells were seeded in 96 well plates and transfected with EPO-R siRNA or a control, non-targeted siRNA using the established protocol described in 4.2.3. The medium was removed 24 hrs after addition of the transfection solution or control, and then refreshed with phenol-red free standard medium with and without EPO at maximal effective concentration determined from the previous experiment (50 U/mL). The plate was then incubated for 24 hrs, followed by a MTS assay. Control cells unstimulated by EPO demonstrated that there was no significant effect of the transfection process on cell viability ($P=ns$), and normal response to 24 hr incubation with EPO (when compared to un-stimulated cells, EPO 50 U/mL +17% ± 7; $P<0.01$). However, cells transfected with EPO-R siRNA showed a significant reduction in the response to EPO at 24 hrs when compared to non-transfected cells ($P<0.01$), shown in Figure 2.4.
Figure 2.3 MTS value for n=12 wells expressed as relative value to control cells. EPO induced proliferation in HK-2 cells is dose-dependent (P<0.001).
Figure 2.4  Loss of proliferation response to EPO in the absence of EPO-R following siRNA knockdown. MTS calculated for n=8 wells (3 separate experiments) and expressed as relative to control cells (P<0.01 EPO treatment in control cells).
2.4.3 EPO induces STAT activation and DNA binding

HK-2 cells were seeded at 50-75 % confluence in standard medium in 6-well plates and placed in an incubator overnight. The medium was removed, and the cells re-fed with standard medium without FCS. The cells were incubated for 6 hours, and then the medium was removed. Cells were stimulated with 1mL standard medium without FCS containing EPO (50 U/ mL) for 10 minutes. At the end of each experiment (n=6 wells for three separate experiments), cells were washed with ice-cold PBS, scraped with a Teflon cell scraper, and centrifuged at 600g at 4°C for 5 minutes. The supernatant was discarded and the cell pellet was snap-frozen in liquid nitrogen.

Protein extraction was performed, and 10 µg of fractional lysates, corrected by volume for assay determined protein concentration, were used to determine STAT activation in a commercial transcription factor DNA binding ELISA assay (TransAM, Active Motif, France). The activation for STAT3 and STAT5A was determined. All results were expressed as increase in DNA binding over the value obtained for control un-stimulated cells for three experiments.

After stimulation with EPO (50 U/ mL) for ten minutes, there was significant increase in activation of STAT3 and STAT5A (STAT3 expression + 47 ± 3 % P<0.01, STAT5A + 52 ± 2 % P<0.01 when compared to time zero controls), shown in Figure 2.5 and 2.6.
Figure 2.5  STAT3 activation by EPO at 10 minutes in HK-2 cells was measured by ELISA. DNA binding was expressed as multiple of control cells for n=3 experiments. EPO caused increase in STAT3 DNA binding (P<0.01).
Figure 2.6  STAT5A activation at ten minutes by EPO in HK-2 cells was measured by ELISA. DNA binding was expressed as multiple of control cells for n=3 experiments. EPO caused increase in STAT5A DNA binding (P<0.01).
2.4.4 EPO Phosphorylates key Intracellular Pathways

HK-2 cells were incubated overnight in serum free medium followed by stimulation with EPO (50 U/mL) for varying lengths of time, or EPO plus either UO126 (10 µM), AG490 (10 µM), or LY294002 (10 µM). Following lysis with Phospho-safe lysis buffer (Pearce Inc), equal amounts of protein were run on a 4-12% graduated SDS gel.

Membranes were probed with specific polyclonal antibodies for phosphorylated ERK1/2. Incubation with EPO caused increased phosphorylation of ERK 1/2 which was maximal at 15 minutes. The observed phosphorylation of ERK by EPO was completed abrogated by co-incubation with the MEKK1 inhibitor UO126.

Membranes were also probed with specific polyclonal antibodies for serine-473 phosphorylated AKT. Membranes were stripped and re-probed for total AKT. Incubation with EPO caused increased serine phosphorylation of AKT which was maximal at 60 minutes. The observed phosphorylation of AKT by EPO was completed abrogated by co-incubation with LY294002, confirming the role of PI-3 Kinase. AG490, a JAK2 inhibitor, also reduced the phosphorylation of AKT by EPO at 60 minutes. This confirms the dependence of EPO signalling pathways on JAK2 activity.

The previous experiment demonstrated that AG490, an inhibitor of JAK2 kinase activity, reduced the phosphorylation of AKT in response to EPO stimulation in HK-2 cells. In order to confirm this finding was due to the inhibition of EPO-R / JAK2 interaction, a similar experiment was performed 24 hours following transfection of JAK2 siRNA, according to established protocol described in 3.3.2.2. Inhibition of JAK2 expression completely abrogated the phosphorylation of AKT following EPO stimulation (Figure 2.9)
Figure 2.7  Immunoblot for Phospho-ERK 1/2

Serum-starved cells were stimulated with EPO for 5-15 minutes and then scraped on ice and underwent protein extraction. Immunoblots with a specific primary antibody for phospho-ERK were performed. (Figure is presentative of three separate experiments). Inhibiting MEK1 with pre-administration of UO126 markedly reduced ERK phosphorylation by EPO.
**Figure 2.8 AKT activation by EPO**

Immunoblots for phospho-AKT (serine 478) in HK-2 cells stimulated with EPO for 30-60 minutes. Maximum band density was observed in cells stimulated for 60 minutes. This increase in phosphorylation was reduced by the addition of a JAK2 inhibitor, confirming the dependence of EPO-R signalling on JAK2 activity, and completely prevented by LY294002, a reversible inhibitor of PI3-kinase.
Figure 2.9  Loss of AKT activation following inhibition of JAK2 expression

AKT phosphorylation (serine 473) in response to EPO stimulation is prevented in cells transfected with JAK2 siRNA. All groups exposed to EPO 50 U/mL for 30 minutes. Representative blot shown from three separate experiments.
2.4.6 EPO attenuates Apoptosis following Serum Starvation

Deprivation of FCS from the standard medium causes a progressive loss of cell viability over time in HK-2 cells. HK-2 cells were plated out in standard medium in 96-well plate. After 24 hr, the standard medium was removed, and 100 µL of phenol-red free medium was replaced without FCS except in control wells. EPO was added in a range of final concentrations to experimental groups. The cells were incubated in normal conditions for 30 hrs and an MTS assay was performed. Serum deprivation caused a significant reduction in cell viability (P< 0.01). Dose-response experiments (EPO 1 - 100 U/mL) reversed the reduction in cell viability caused by 30 hrs incubation in serum-deprived conditions (EPO 1 U/L P<0.01; EPO 10 P< 0.01; EPO 20 U/mL P< 0.001; EPO 50 U/mL, P< 0.001), shown in Figure 2.10.

In parallel, extracted cell pellets from the end of experimental procedures were lysed in digitonin buffer and a DNA fragmentation ELISA was performed. Serum starvation induced a significant increase in DNA fragmentation (control 1.0 ± 0.1; starvation 1.93 ± 0.48, P<0.01). EPO treatment reduced the increase in DNA fragmentation (+ EPO 1.13 ± 0.33, P<0.05), shown in Figure 2.11. The lysates in which DNA fragmentation was measured were also used to examine the expression of proteins that regulate apoptotic signalling by immunoblotting. Serum starvation for 24 hrs was associated with a reduction in the expression of anti-apoptotic proteins XIAP and Bcl-XL. EPO treatment was associated with increased expression of XIAP and Bcl-XL. Representative blots from these experiments are shown in Figure 2.12.
Figure 2.10  Cell viability following serum starvation with different concentrations of EPO was measured by MTS assay. At 30 hours, MTS values for treatment groups were expressed relative to cells exposed to serum starvation. Groups were compared by ANOVA.
Figure 2.11  EPO reduces serum deprivation induced DNA fragmentation, measured by ELISA. Values for three experiments were expressed relative to control cells cultured with standard FCS. EPO significantly reduced DNA fragmentation (P<0.01 by ANOVA).
Figure 2.12  EPO maintains expression of XIAP and Bcl-XL

Western blots on lysates from cells deprived of serum for 24 hours and cells deprived of serum for 24 hrs treated with EPO (50 IU/mL) were probed with primary antibodies for XIAP and Bcl-XL. Serum starvation reduced the expression of both anti-apoptotic proteins, but administration of EPO caused maintenance of expression associated with a reduction in apoptotic cell death.
2.5 Discussion

The data presented in this chapter demonstrate the signalling processes initiated by the engagement of EPO with its cell surface receptor in human proximal tubular epithelial cells. The presence of the receptor on the particular cell line studied in these experiments was confirmed by the presence of an appropriately-sized strong band by immunoblotting, and also, independently, by the observation that knockdown of EPO-R expression by RNA interference completely abrogated the cellular response previously observed upon EPO stimulation.

Two bands were observed in the western blot for the EPO-R, at 59 and 68 kDa approximately. The 68-kDa band was similar in size to that observed from the erythroblast cell line UT-7, known to highly express the EPO-R. There has been some debate concerning the size of the human EPO-R protein, which undergoes post-translational modification. Westenfelder et al, using the same commercial antibody, detected two EPO-R immunoreactive proteins in murine proximal tubular cells of approximately 68 and 90 kDa, respectively, and the 68 kDa protein was not observed in non-anaemic murine spleen suggesting that this represented the EPO-R [Westenfelder C, 1999]. The cloned murine EPO-R cDNA encodes a 55 kDa polypeptide, and the EPO-R is post-translationally modified by N-linked glycosylation and phosphorylation, increasing its mass to approximately 76 kDa [Sawyer ST, 1993].

The ability of EPO-R siRNA to reduce intracellular signalling molecules and abrogate cell proliferation in the experiments described is strong evidence that these cells express a functional EPO-R. Recently, the specificity of the EPO-R antibody used in these
experiments has been questioned [Elliot S, 2006], as a dominant band at approximately 70 kDa was observed in cells which do not express the EPO-R, and the observed band could be abrogated by knockdown of HSP70 expression by RNA interference. Further studies have confirmed that the C-20 antibody does identify the EPO-R, but may show cross-reactivity with other proteins. EPO-R expression should be confirmed by either mRNA studies or functional studies with RNA interference, as was performed in these experiments [Verdner F, 2005]. The development of more specific commercial antibodies against the EPO-R is urgently required.

The described experiments demonstrated that EPO did have direct effects on the proliferation and survival of tubular cells in a cellular injury model. The protection from injury afforded by EPO in proximal tubule epithelial cells was dependent on the activation of EPO-receptor induced signalling. In neuronal cells, studies utilizing soluble EPO receptors and anti-EPO antibodies as competitive inhibitors of EPO receptor activation have confirmed that the effects of EPO in vitro are mediated via the EPO receptor [Ruscher K, 2002]. However, the recent development of a carbamylated erythropoietin molecule, CEPO, that does not stimulate erythropoiesis but retain a neuronal protective capacity, has questioned the uniqueness of the erythropoietin/erythropoietin receptor interaction [Leist M, 2004]. These authors have suggested that EPO signalling in neuronal cells may be mediated through the interaction between the EPO receptor and a common β-subunit heteroreceptor [Brines M, 2004], although this observation has yet to be confirmed, and EPO retains its anti-apoptotic properties in cell lines that do not express the common β-subunit heteroreceptor [Um M, 2007].

Activation of JAK2 by EPO has been shown, in endothelial cells and neuronal cells, to lead to the activation of phosphatidylinositol-3 kinase (PI 3-kinase) and Akt phosphorylation. Once activated, Akt activates multiple targets with anti-apoptotic effects, including
phosphorylation of Bad, Bax, caspase-9 and GSK-3β, maintenance of mitochondrial membrane potential and preservation of glycolysis and ATP synthesis. Inhibition of PI 3-kinase inhibited the specific serine-473 phosphorylation of AKT induced by EPO. Therefore, one major mechanism by which EPO protects proximal tubule cells against injury is through the induction the EPO receptor-dependent activation of PI 3-kinase leading to phosphorylation and activation of its target Akt, and subsequent downstream pathways.

EPO inhibits apoptotic cell death in proximal tubule epithelial cells (as determined by DNA-fragmentation assay), and in higher doses, causes significant proliferation even in serum-free conditions. EPO administration was associated with the up-regulation of Bcl-XL and XIAP. Bcl-XL, a member of the B cell lymphoma-2 family, has been shown to determine resistance to hypoxic insults in vitro [Dong Z, 2003]. Taken together, these in vitro findings support the conclusion that EPO directly protects proximal tubule epithelial cells by (i) binding to the EPO receptor and inducing JAK-2 kinase activity, (ii) with PI3K leading to phosphorylation of Akt and its downstream mediators, and (iii) up-regulation of a series of anti-apoptotic proteins including Bcl-XL and XIAP. These experiments have demonstrated that EPO is functional in human proximal tubule cells, and led to the hypothesis that EPO would reduce tubular cell death in an animal model of kidney injury.
Chapter Three:

Effect of Erythropoietin Administration on a

Short term model of ischaemia reperfusion in the rat
3.1 Introduction

As discussed in section 1.2, the outcome of AKI is a function of the interaction of multiple cell types in the tubular and vascular compartments of the kidney that must ultimately be understood at the whole tissue level, and is not predictable from the study of the behaviour of a single cell type.

This complexity necessitates an approach utilizing whole animal studies to unravel the mechanisms of injury and develop therapeutic modalities. This has led to the development of "single insult" models of renal injury induced by ischaemia, toxins and radiocontrast agents. An established animal model of ischaemia-reperfusion injury, which as far as possible mimics the type of renal injury observed in clinical medicine, in particular kidney transplantation, has been developed in the laboratory. This short-term model of ischaemia-reperfusion injury in the rat has been intimately characterised and is highly reproducible [Williams P, 1997; Chatterjee PK, 2003].

This model of bilateral renal artery occlusion in the rat is characterized histologically by widespread necrosis of proximal tubules, the distribution and extent of which vary with the ischaemic interval. The distal nephron is less affected, with mild damage in thick limbs of the loops of Henle and focal apoptosis rather than widespread necrosis in the distal convoluted tubules and collecting ducts. The S3 segment of the proximal tubule, which has the lowest oxygen tension in health, is most severely affected with between 20-90% of these segments showing lethal injury [Shanley PF, 1985]. In human AKI, morphological injury is usually more subtle and focal, affecting both proximal and distal tubules, although comprehensive biopsy series from patients with ischaemic AKI are lacking [Rosen S, 2008].
However, there are some important similarities between the rat model of ischemia and human AKI, with loss of the brush border, a predilection for the most severe injury to occur in the proximal straight tubules (S3 segments), prominent apoptotic cell death in the early phase, and the presence of luminal cast formation. Functional similarities between human ischaemic AKI and the renal artery occlusion model of AKI include the severe reduction in GFR and the reversibility, in most cases, of tubular injury with recovery of renal function.

3.2 Aims

The aim of this study was to determine whether EPO would have a protective effect in a model of ischaemia reperfusion injury in the kidney, an organ which is prone to ischaemic damage, and which expresses the EPO-receptor [Westenfelder C, 1999]. EPO had been used in a study in the management of anaemia in AKI in rats. Although there was no benefit in that study with regard renal function, mortality was significantly lower in the EPO treated animals [Nemato T, 2001].

A protocol of bolus administration of a single dose of EPO was devised from the design of studies of EPO cellular protection in models of cerebral ischaemic injury [Sakanaka S, 1998]. The aims of the experiment were to discover the biochemical and histological effects of EPO in an in vivo setting, and whether EPO was effective if administered at different timings relative to the initiation of tissue reperfusion.
3.3 Methods

3.3.1 In vivo rat model of ischaemia reperfusion (Short term)

Male Wistar rats (200-300g) were used in this procedure. Rats were kept in normal day/night conditions and receive standard chow and water ad libitum. The protocol described has been approved by the U.K. Home Office and the care and use of animals is in accordance with the Guidelines in the Operation of the Animals (Scientific Procedures) Act 1986.

All rats were anesthetised with sodium thiopentone (Intraval® Sodium, 85 mg/kg i.p; Rhone Merieux Ltd., Essex, U.K.) and anaesthesia was maintained by supplementary infusions of sodium thiopentone. The fully anaesthetised rat is placed, on its back, onto a thermostatically controlled heating mat (Harvard Apparatus Ltd, Kent, UK) and body temperature was maintained at 38±1°C by means of a rectal probe attached to a homeothermic blanket. A tracheotomy was performed to maintain airway patency and to facilitate spontaneous respiration. The right carotid artery was cannulated (PP50, I.D. 0.58mm, Portex, Kent, U.K.) and connected to a pressure transducer (Senso-Nor 840, Horten, Norway) for the measurement of mean arterial blood pressure (MAP) and derivation of the heart rate (HR) from the pulse waveform. The right jugular vein was cannulated (PP25, I.D. 0.40mm; Portex) for the administration of drugs. A midline laparotomy was performed and the bladder was cannulated (PP90, I.D. 0.76mm; Portex). 0.5 mL of normal saline was administered via the venous line to replace lost fluid. The venous line was connected to a constant-rate infuser pump and saline administered at a rate of 8 mL/Kg/ hr throughout the experimental period.
Animals were allowed to stabilize for 30 minutes after completion of preparatory surgery. Using a cotton bud to carefully move away the spleen and small intestine on the right hand side of the abdomen, the right kidney and the pedicle (containing renal artery and vein) was exposed. The pedicle was isolated and clamped using a Differenbach’s bulldog artery clip. The intestine is replaced and the left kidney isolated and the pedicle clamped in the same way. Occlusion was verified visually by a change in the colour of the kidney to a paler shade and then to a darker (cyanotic) colour within minutes. The intestine was replaced into the peritoneum and the peritoneum closed with a small clip and covered with saline-soaked gauze. A total ischaemia time of 45 minutes was used for all experiments. The kidneys were carefully exposed, and the artery clamps removed with the aid of forceps to prevent tearing or localised injury to the renal pedicle. Reperfusion was confirmed by an immediate colour change in the kidney. The peritoneum was closed with a small clip and covered with saline-soaked gauze.

3.3.2 Biochemical analysis

At the end of the reperfusion period (6 hrs in all groups unless stated), blood (1 mL) samples were collected via the carotid artery into tubes containing serum gel. The samples were centrifuged (6000 g for 3 minutes) to separate the serum from which biochemical parameters, including creatinine, sodium and asparate transaminase (AST), a marker of tubular injury, were measured. All serum samples were analyzed within 24 hours of collection (Vetlab Services, Sussex, UK). Urine was collected throughout the reperfusion period and the volume recorded. Activity of urinary N-acetyl-β-D-glucosaminidase (NAG), a specific indicator of tubular damage [Bosomworth MP, 1999; Kotanko P, 2000] was also measured on snap frozen urine aliquots, performed by Dr Joaquim Chaves at the Clinica Medica e Diagnostico, Lisbon, Portugal.
3.3.3 Histological Evaluation

Kidneys were removed from rats at the end of the experimental period and were cut in sagittal section into two halves, which were fixed in immersion in 10% (wt/ vol) formaldehyde in phosphate buffered saline (PBS; 0.01M; pH 7.4) at room temperature for 24 hours. After dehydration using graded ethanol, pieces of kidney were embedded in Paraplast (Sherwood Medical, Mahwah, NJ) and cut in fine (8 μm) sections and mounted on glass slides. Sections were then deparaffinized with xylene, counterstained with hematoxylin and eosin, and viewed under a light microscope (Dialux 22, Leitz, Milan, Italy).

100 intersections were examined for each kidney and score from 0-3 was given for each tubular profile: 0 normal histology; 1, tubular cell swelling, brush border loss and nuclear condensation with up to 1/3 nuclear loss; 2, as for score 1, but greater than 1/3 and less than 2/3 tubular profiles showing nuclear loss; and 3, greater than 2/3 tubular profile shows nuclear loss. The histological score for each kidney was calculated by addition of all scores with a maximum score of 300, by two blinded histopathologists [Chatterjee PK, 2000]. Deparaffinized sections were also used for immunohistochemical analysis. Sections were incubated overnight with primary anti-caspase-3 antibody (1 in 10,000) in blocking buffer. After washing, secondary antibody was added for 30 minutes at 25°C, followed by washing. A HRP containing buffer was added for a further 30 minute incubation before the slides were counterstained with haematoxylin, dehydrated and mounted for review by a blinded pathologist and the author.

In addition randomly picked sections were quantitatively assessed for apoptotic nuclei, and graded for severity, extent of nuclear changes, and the presence of nuclear debris and
apoptotic blobs. These were scored by a blinded pathologist and the author for three high-powered fields per animal.

3.3.4 Preparation of tissue Homogenate for protein Analysis

Sections of experimental kidneys that had been snap-frozen in liquid nitrogen immediately after dissection, were weighed and approximately equal tissue samples by weight were used for homogenisation. The tissue block was cut into small pieces and placed in a sterile glass tissue homogeniser.

Buffer containing 25 mmol/L EDTA, 20 mmol/L EGTA, 10 mmol/L sodium hydroxide (pH 7.3) and Imidazole: HCl buffer containing 10 mmol/L mercaptoethanol (pH 7.3) was added according to dry tissue weight (1 mL / 250 mg tissue) and the tissue was homogenised with twenty equal strokes of the glass rod over 2 minutes. The suspension was aspirated by pipette into an eppendorf tube and then the samples were centrifuged at 14000g for 15 min at 4°C. The resulting supernatant was aspirated into pre-chilled 1 mL Eppendorff tubes. Each sample was aliquotted into several tubes to avoid repeated thaw/ freeze cycles, and samples were kept on ice throughout the procedure.

3.3.5 End-point Caspase Activity Assay

The activity of caspase-3 was measured using the substrate Ac-DEVD linked to a fluorogenic 7-amino, 4-methylcoumarin (AMC) molecule. For in vivo experiments, frozen samples of harvested kidneys were washed in ice-cold PBS, and homogenized in ice-cold buffer containing 25 mmol/L EDTA, 20 mmol/L EGTA, 63.2 mmol/L imidazole-HCl, 10 mmol/L 2-mercaptoethanol (pH 7.3). After centrifugation at 16000g for 15 minutes at 4°C, the supernatant was aspirated and the amount of protein quantified using the BCA assay.
50 µg cellular protein was incubated with 50 µM substrate in caspase assay buffer (213.5 mM HEPES, pH 7.5, 31.25% sucrose, and 0.3125% CHAPS) for 1 hour, and fluorescence was measured on a microplate reader (Fluostar Galaxy, BMG Laboratory Technologies, Aylesbury, UK), with excitation at 380 nm and emission set at 460 nm. For each sample, four replicates were assayed, with two replicates containing 50 µM of the caspase-3 inhibitor (Ac-DEVD-CHO), and the remaining pair of replicates containing similar volume of vehicle (DMSO).

The activities of caspase-8 and caspase-9 were determined in the same way, using

Caspase 8: substrate Ac-IETD-AMC  inhibitor: Ac-IETD-CHO
Caspase 9: substrate Ac-LEHD-AFC  inhibitor: Ac-LEHD-CHO

Fluorescence readings from wells containing inhibitor were subtracted from total fluorescence, and results calculated as nmol AMC/min/ per mg protein.

### 3.3.6 Experimental Design

The short term model of ischaemia reperfusion injury in the anaesthetised rat was used for all experiments described in this chapter. Experimental design was as below:

(1) Sham-operated rats (n=8)
(2) Sham-operated rats, EPO 300 U/Kg administered 30 minutes prior to sham clipping (n=8)
(3) Ischaemia-reperfusion (I/R), animals subjected to 45 minutes bilateral renal pedicle clamping, followed by 6 hours reperfusion. (n=12)
(4) I/R, EPO administered 30 minutes prior to bilateral renal pedicle clamping. (n=12)
(5) I/R, EPO administered 5 minutes prior to reperfusion. (n=13)

(6) I/R, EPO administered 30 minutes into the reperfusion period. (n=12).

3.4 Results

3.4.1 Effect of Ischaemia reperfusion and EPO on Haemodynamic Parameters

Continuous arterial blood pressure was recorded throughout the experimental process in all animals. Hourly mean blood pressure was calculated for all experimental groups to determine whether EPO had any adverse or beneficial effect on haemodynamic parameters.

Resting mean arterial blood pressure in all experimental groups after stabilization was 130-140 mm Hg ($P=ns$). There was no significant effect on EPO administration on mean arterial blood pressure in sham operated animals. Ischemia reperfusion was associated with a non-significant fall in blood pressure in early reperfusion (Figure 3.1). When comparing each group as a series of BP measurements, there was no significant difference between I/R and EPO treated animals (ANOVA with bonferrari).
Figure 3.1  Mean arterial Blood Pressure during the Experimental Period in all experiments groups. Mean BP was calculated from the arterial pressure trasducer measurements and recorded every 30 minutes. Mean BP over the whole experimental period was similar in all groups (P=ns, ANOVA).
3.4.2 Effect of EPO on Renal Dysfunction caused by I/R

Routine biochemical parameters of kidney function were analysed in all experimental groups, including serum and urine creatinine and sodium. In comparison with sham animals, 45 minutes of ischaemia led to significant increases in serum, urinary and histological markers of renal dysfunction and injury after 6 hours reperfusion.

Administration of EPO to sham-operated animals did not have any effect on markers of renal function despite an increase in urine flow rate (Figure 3.4). Rats that underwent renal I/R exhibited significant increases in the serum concentrations of creatinine compared with sham-operated animals (serum creatinine: sham 53 ± 2 µmol/L; I/R 217 ± 7, \( P<0.001 \)) (figure 3.2) and decreases in urine flow (sham animals 0.01 ± 0.0001 mL/hr; I/R 0.006 ± 0.001, \( P<0.001 \)) (figure 3.3) and creatinine clearance (sham animals 1.3 ± 0.1 mL/min; I/R 0.053 ± 0.016, \( P<0.001 \)) (figure 3.4), suggesting a significant degree of glomerular dysfunction.

In comparison with I/R animals, the administration of a single intravenous bolus of EPO (300 U/ kg), either pre-ischemia or just prior to the onset of reperfusion, produced a significant reduction in serum levels of creatinine (EPO pre I 144 ± 6; EPO post R 143 ± 7; EPO late 183 ± 8; \( P<0.01 \)) associated with maintenance of improved urine flow rate (EPO pre 0.022 ± 0.002; EPO post R 0.02 ± 0.002; EPO late 0.01 ± 0.007, \( P<0.01 \)) and creatinine clearance (EPO pre I 0.28 ± 0.05, EPO post R 0.31 ± 0.05, \( P<0.01 \)). EPO administration 30 minutes after the onset of reperfusion still afforded renoprotection by attenuating the fall in glomerular filtration rate, and hence calculated creatinine clearance (figure 3.4).
Figure 3.2  Serum Creatinine after six hours Reperfusion in all experimental groups. Creatinine in the I/R group was compared with all treatment groups by ANOVA.
Figure 3.3  Urine Flow rates following Ischaemia-Reperfusion were measured in all experimental groups. When compared with I/R injury, urine flow was significantly maintained in EPO treated animals (P<0.01 by ANOVA).
Figure 3.4 Calculated Creatinine Clearance following Reperfusion was calculated from urinary creatinine and urine flow in all experimental groups. Effect of treatment with EPO was compared against I/R by ANOVA. Pre-treatment and Pre-reperfusion treatment significantly maintained creatinine clearance (P>0.05 by ANOVA).
3.4.3 Effect of EPO on Biomarkers of Ischaemia-Reperfusion induced Tubular Injury

In comparison with values obtained from sham-operated animals, renal I/R produced a significant increase in urinary N-acetyl-β-D-glucosaminidase (NAG) activity (sham 8 ±2, I/R 22.7 ± 5.6, \( P < 0.05 \)), a specific marker of tubular dysfunction, corrected for urine volume. Serum concentrations of the enzyme aspartate transaminase (AST) was analysed as a marker of tubular injury induced by reperfusion [Chatterjee PK, 2000]. Fractional excretion of sodium, as a quantitative marker of tubular function was calculated from the formula:

\[
FE_{Na}(\%) = \frac{uNa [\mu mol/ L] \times urine \ flow [mL/ min]}{C_{Cl} [mL/min] \times sNa [\mu mol/ L] \times 100},
\]

I/R injury significantly increased sodium excretion (sham 1.3 ± 0.18 %; I/R 19.9 ± 5.9, \( P <0.01 \)), demonstrating a reduction in tubular conservation of sodium. EPO administration was associated with a significant reduction in urinary NAG activity (sham 8 ± 2, I/R 22 ± 5.6, EPO pre 9.9 ± 3.0, EPO post 7.9 ± 0.8, \( P <0.01 \)), shown in Figure 3.5, and preservation of tubular function, measured by maintenance of sodium excretion (EPO pre-reperfusion 7 ± 0.5 %, \( P <0.05 \)) shown in figure 3.6. There was insufficient sample to measure sodium excretin in the EPO late (30 mins) group.

Renal I/R also produced a significant increase in serum AST (sham 200 ± 30, I/R 1864 ± 232, \( P <0.01 \)). Administration of EPO caused a significant reduction in serum AST levels (EPO pre 522 ± 40, EPO post 607 ± 64, EPO late 1130 ± 177, \( P <0.01 \)), shown in Figure 3.7, suggesting a marked reduction in tubular injury and hence preserved tubular function post reperfusion-injury.
Figure 3.5   Tubular injury measured by urinary NAG concentration following renal I/R injury in the rat. Measurements were corrected to urine volume. Treatment groups were compared to I/R by ANOVA. EPO treatment was associated with significantly lower urine NAG (P<0.001 by ANOVA).
Figure 3.6 Tubular Functional reserve by Fractional Sodium Excretion following Reperfusion Injury. When compared with I/R injury, there was a significant decrease in the change in sodium excretion in EPO treated animals (P<0.05 by ANOVA).
Figure 3.7  AST, a marker of Tubular Reperfusion Injury at Six Hours in all Experimental Groups. Comparision with I/R injury, by ANOVA, showed a significant reduction in tubular injury with early EPO treatment (P<0.01 by ANOVA).
3.4.4 Effects of EPO on Histological Alterations Caused by Renal I/R

Renal I/R caused marked alterations in renal histology when compared with kidneys taken from sham-operated animals. Specifically, this included widespread degeneration of tubular architecture, tubular dilatation, cell swelling and necrosis, luminal congestion with loss of brush border and infiltration of polymorphonuclear neutrophils (PMNs) (Figure 3.7 A and B). In contrast, renal sections obtained from animals treated with EPO pre ischaemia, or pre-reperfusion, demonstrated marked reduction of the histological features of renal injury. EPO administration 30 minutes after the onset of reperfusion was still associated with a significant reduction in injury, but this reduction was less marked than in the other treatment groups (Figure 3.7 C and D). Scoring of histological changes is shown in Figure 3.9.

Initially, TUNEL staining was attempted to gain a qualitative score for apoptotic cell death. Unfortunately, there was very high background staining which did not allow adequate discrimination of positive staining. In view of this, proximal tubular cell apoptosis was examined on serial sections under high power field, randomly selected by a blinded pathologist and then scored by the author and the pathologist and scores combined.

Kidneys from animals subjected to renal I/R showed extensive nuclear changes consistent with apoptotic cell death (Figure 3.10). In comparison, kidneys from sham-operated animals had no evidence of apoptosis. EPO administration prior to ischaemia, and pre-reperfusion significantly reduced the extent of apoptotic cell death (p<0.05). EPO administration 30 minutes after the onset of reperfusion caused only a small reduction in the number of apoptotic cells, but there was a reduction in the incidence of necrotic cell death.
Figure 4.8  Histological Assessment of Renal Injury

Kidney sections taken from:

(A) sham operated rat
(B) renal ischemia-reperfusion. Renal sections from a rat subjected to renal I/R after administration of
(C) EPO pre-ischemia (30 min), and
(D) EPO post reperfusion (30 min)

Haematoxylin and eosin-stained kidney sections (magnification x150).

Figures are representative of at least three animals (n=5 for all groups).
Figure 3.9  Histological assessment of Renal Injury following Reperfusion (method described in section 3.3.3). Scores for treatment groups were compared with I/R by ANOVA.
Figure 3.10. Quantitative scoring of apoptotic nuclei in high-powered fields was used to determine the extent of apoptotic cell death. Measurements were combined from the author and a blinded pathologist. Four fields were examined for each specimen, and an average score taken. Scores were analysed by ANOVA.
3.4.5 Effect of EPO on Caspase-3 activity *in vivo*

Animals subjected to ischaemia reperfusion were sacrificed during the reperfusion period after two, four and six hours respectively. Kidney tissue was harvested and then snap-frozen in liquid nitrogen. Tissue samples (n=6) for each group were homogenised and caspase activity measured. When compared to immediately pre-reperfusion, there was a significant time-dependent increase in caspase-3 activity (0hr 118 ± 28, 4 hr 239 ± 21 \( P<0.01 \), 6 hr 282 ± 40 \( P<0.001 \)), shown in figure 3.11.

Caspase-3 activity (nmol/ min/ mg total protein at 37°C) was significantly increased in the homogenates of kidneys at 6 hours in kidneys subjected to renal I/R, when compared with sham-operated animals (p<0.01). The elevation in caspase-3 activity was significantly reduced by EPO administration, both pre-ischaemia (p<0.05) or just prior to reperfusion (p<0.05). EPO treatment 30 min after reperfusion was associated with a non-significant reduction in caspase-3 activity, consistent with the higher levels of apoptosis observed in these kidneys (figure 3.12).

Immunohistochemical staining, described in 3.3.3, using a specific antibody to the cleaved active fragment of caspase-3 confirmed these observations. There was widespread positive cytoplasmic and perinuclear staining in both cortical and medullary tubules following I/R (Figure 3.13 B, representative image (n=4 for all groups)), when compared to sham animals (Figure 3.13 A). The severity and distribution of staining was greatly reduced in animals receiving EPO either pre-ischemia (Figure 3.13 C) or pre-reperfusion, but only partially when EPO was administered later in reperfusion (Figure 3.13 D).
Figure 3.11  Time course of Caspase-3 activity following reperfusion from kidneys snap-frozen at time points after reperfusion. Six kidneys were analysed for caspase activity for each time point. There was a significant increase in caspase-3 activity in later time points (P<0.01 by ANOVA).
Figure 3.12 Tissue Caspase Activity following Six hours reperfusion in all Experimental groups. Kidneys from each group (n=6) were homogenised and caspase-3 activity measured. EPO treatment caused a significant decrease in caspase-3 activity at six hours (P<0.01 by ANOVA).
Figure 3.13 Immunohistochemistry for active caspase-3 in Representative Specimens in Experimental Groups – a. Sham, b. I/R, c. EPO pre-ischaemia, and d. EPO re-reperfusion (representative image from n=5 kidneys each group). Arrow shows cytoplasmic positive caspase-3 fragments in proximal tubule.
3.4.6 Effect of EPO on Caspase-9 and Caspase-8 activity in vivo

Having demonstrated that apoptotic cell death was reduced, and that this was mirrored by a significant decrease in the activation of the executioner caspase-3, the relative contributions of the extrinsic and intrinsic (mitochondrial) pathways in the activation of caspases-3 were examined. Both pathways have been shown to participate in ischaemia-reperfusion injury. Caspase-8 and caspase-9 activity was assayed in tissue homogenates from all experimental groups.

Following I/R, activity of caspase-9 was significantly increased when compared to sham-operated animals (sham 218 ± 20, I/R 300 ± 21, P<0.01). The increase caspase-9 was attenuated by EPO treatment (EPO pre-reperfusion 230 ± 7, P<0.05), shown in Figure 3.14.

When compared sham-operated animals, ischaemia reperfusion caused an increase in the activity of caspase-8 at six hours (sham 38 ± 5, I/R 144 ± 48, P<0.01). Treatment with EPO as a pre-treatment, or just prior to reperfusion significantly reduced the increase observed (EPO pre-ischaemia 48 ± 17, P<0.01; EPO pre-reperfusion 56 ± 38, P<0.01), shown in Figure 3.15.
Figure 3.14  Caspase 9 Activity in kidney tissue homogenates following I/R injury was analysed as described in 3.3.5. EPO treatment was associated with significantly lower caspase-9 activity following I/R (P<0.05 by ANOVA).
Figure 3.15  Caspase-8 Activity in kidney tissue homogenates following I/R injury showed a reduction in activity in EPO treated animals when compared to I/R (P<0.05 by ANOVA).
3.5 Discussion

Following from the results in chapter two, which showed direct effects of EPO on tubular epithelial cells in culture, the studies described in this chapter demonstrate that recombinant human EPO is able to reduce the degree of injury in the kidney following ischaemia-reperfusion (I/R) injury.

EPO reduced both the glomerular and tubular injury and dysfunction, assessed by both biochemical and histological parameters, caused by severe bilateral renal ischaemia and reperfusion in the rat. EPO reduced I/R injury when administered as a single intravenous bolus injection, either prior to the onset of ischaemia (30 min pre-treatment) or when given immediately before release of clamps (pre-reperfusion). Most notably for its potential use as a therapeutic agent in clinical settings, EPO even partially protected kidney function against I/R injury when given 30 min after the onset of reperfusion, although there was more severe histological damage and more apoptotic cells.

The degree and extent of injury induced by ischaemia in control animals in this model was consistent with previously published work [Chatterjee PK, 2000]. Morphological studies have clearly demonstrated the existence of both necrosis and apoptosis in animal models of AKI [Rana A, 2001], and there is recognition of the importance of apoptosis in the initiation phase of renal injury, and the presence of apoptotic tubular epithelial cells has been demonstrated in kidneys of humans with ischaemic AKI [Castaneda MP, 2003]. Chemical inhibition of the caspase cascade has been shown to protect the kidney in a similar model of ischaemia-reperfusion in the mouse [Daemen MA, 1999]. The reduction in renal injury by EPO was associated with a significant decrease in the number of apoptotic tubular epithelial cells, and a reduction in caspase-3 activity, which suggest that EPO may partially mediate its effects by acting directly on tubular epithelial cell resistance to cell death through the up-
regulation of anti-apoptotic mechanisms. The use of immunohistochemical techniques to
determine tubular localisation of the active fragment of caspase-3 was good specific
evidence to confirm the quantitative assessment of tubular apoptosis used in the absence of
TUNEL staining. There was also a reduction in the activities of caspase-8 and caspase-9 at
six hours post-reperfusion. I/R causes the upregulation of inflammatory cytokines including
TNF-α which lead to cell death through the receptor-mediated caspase-8 pathway. A
reduction in cell death and hence a reduction in inflammation and cytokine release following
EPO treatment may limit the pathways that induce further apoptosis by preventing cytokine-
dependent amplification of cellular injury.

A number of potential beneficial effects of EPO in the kidney may account for the organ
protection observed, which may not require direct effects on tubular cell death. An increase
in renal blood flow might contribute to or even account for the observed protective effects of
EPO. This is, however, unlikely, as there was no significant haemodynamic effect of acute
administration of EPO on the systemic circulation. Although EPO increased urine flow rate
in sham-operated rats, suggestive of a possible effect on cortical perfusion and intra-
glomerular pressure, creatinine clearances were similar between the sham operated groups,
and there was no difference in tubular function measured by fractional sodium excretion
induced by EPO in sham animals. This observation is consistent with the study by Huang et
al, which demonstrated no immediate effect on isolated cortical and papillary perfusion in
normal rats following acute EPO administration [Huang C, 1992].
Chapter Four:
Effect of Erythropoietin Administration on Recovery Models of
Ischaemia-Reperfusion Injury
4.1 Introduction

The experiments described in chapter three showed that EPO given at the time of reperfusion, or as an immediate pre-treatment 30 minutes before ischaemia, was effective in attenuating the degree of renal injury observed at six hours. In this model, the extent of injury at six hours is predictive of the maximum renal dysfunction at 24 hours, and the natural history of the model shows progressive recovery of function after 24 hours, although histological recovery occurs more slowly with the development of fibrosis [Williams P, 1997].

It was recognized in 1986 that brief periods of ischaemia render an organ resistant to subsequent more prolonged ischaemic period, known as ischaemic preconditioning. Four cycles of five minutes of coronary artery occlusion prior to 40 minutes occlusion reduced infarction size by 75% [Murry CE, 1986]. This protective effect was lost when three hours occlusion was applied, reinforcing the vital need for timely reperfusion to limit damage. Ischaemic preconditioning offers two “windows” of protection in time - “early” or “classical” providing protection immediately after the preconditioning stimulus, and “late” or “delayed” preconditioning being effective for up to 24 hours. Activation of HIF-dependent signaling pathways, including EPO, has been shown to play an important role in ischaemic preconditioning in the heart and kidney [Cai Z, 2003; Hill P, 2008]. Cobalt administration to rats, via up-regulation of HIF-dependent proteins including EPO, VEGF and haem-oxygenase-1 (HO-1), diminished the degree of renal injury caused by ischemia-reperfusion [Matsumoto M, 2003].

A pre-conditioning therapy would be attractive in clinical scenarios where renal dysfunction is predictable, such as cardiac surgery, repair of aortic aneurysm or organ transplantation. The mechanism of renal protection from pre-conditioning therapy is potentially different from that observed in the short term model with EPO administered at the time of
reperfusion. Preconditioning may allow the synthesis of new anti-apoptotic signalling molecules which mediate organ protection, and there is evidence that endogenous nitric oxide (NO), possibly derived from endothelial NO synthase, plays an important role in delayed conditioning [Bolli R, 1997]. When renal tubular epithelial cells are exposed to repeated hypoxic insults, a clone of more resistant cells are selected, which show increased expression of anti-apoptotic proteins, including Bcl-2 and particularly Bcl-XL [Brooks C, 2007]. Induced Bcl-XL overexpression reduces apoptosis in animal model of ischaemia-reperfusion [Chien CT, 2007], and the pre-conditioning expression of this anti-apoptotic pattern might be HIF-dependent [Chen N, 2008].

4.2 Aims

The aim of this study was to determine whether EPO would also have a protective effect in a longer, recovery model of ischaemia reperfusion injury in the kidney. The aims of the experiments were to confirm that the biochemical and histological effects of EPO observed in the early phase post-reperfusion, as described in chapter three, were still present during the maintenance and recovery phase of AKI. The recovery model also was used to determine the relative effects of a pre-conditioning regime during which EPO was administered for 3 days before ischaemic injury.
4.3 Methods and Materials

4.3.1 Recovery Model of Ischaemia Reperfusion in the anaethetised Rat

When compared to the short-term model used in chapter three, the recovery model requires that the incision is closed and the animal recovers and survives during the period of experimentation. Experience in the laboratory had previously shown that the large abdominal wound used in the short term model was associated with wound breakdown and infection leading to reduced animal survival. A dorsal approach leaves a sutured wound that is virtually impossible for the animal to disrupt with its teeth or claws. Metallic surgical staples were used rather than sutures for closure of the skin incision to avoid any potential for loosening or cutting.

Male Wistar rats (200-300g) were used in this procedure. Rats were kept in normal night/day conditions and receive standard chow and water *ad libitum*. The protocol described has been approved by the U.K. Home Office and the care and use of animals is in accordance with the *Guidelines in the Operation of the Animals (Scientific Procedures) Act 1986*. Animals were anaesthetised with halothane, and placed on a sterile mat headed by an overhead lamp. Fur was shaved over the dorsal spine bilaterally, and an incision in the skin and fascia was made. The abdominal wall was identified and opened with a small (< 1.0 cm incision). Each kidney was identified and mobilised on its pedicle by blunt dissection of the peri-renal fat and connective tissue. Once fully mobilised, the renal pedicle was clamped with a Differenbach’s bulldog artery clip. Cessation of renal perfusion was confirmed by the development of colour change in the kidney. The kidney was replaced in the abdominal cavity, and the incision temporarily closed with a clamp. At 45 minutes, the kidney was mobilized and the clamp removed. Immediate “pinking-up” of the kidney confirmed adequate reperfusion. Animals were given 5 mls intrabdominal saline and subcutaneous
analgesia (pethidine). The abdominal wall incision was closed with interrupted sutures and the skin was closed with staples. Animals were placed in a cage under a heating lamp and observed until anaesthetic had worn completely off. Animals were anaesthetized for terminal venesection, and then sacrificed at 24 hrs, 48 hrs and 72 hrs after the onset of reperfusion.

4.3.2 Murine Model of ischaemia-reperfusion Injury

Male C57BL/6J mice (Charles River, Milan, Italy) weighing 25 to 30 g were used to assess the role of EPO in the pathogenesis of renal I/R in the mouse. Mice were allowed access to food and water ad libitum and were cared for in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192), as well as with the European Economic Community regulations (O.J. of E.C. L358/1 12/18/1986). These experiments were performed with the technical assistance of Dr. N. Patel.

Mice were anesthetized using chloral hydrate (125 mg/kg, intraperitoneally) and core body temperature maintained at 37°C using a homoeothermic blanket. Mice were maintained under anaesthesia for the duration of ischaemia (i.e., 30 minutes). After performing a midline laparotomy, mice in I/R groups were subjected to bilateral renal ischemia for 30 minutes, during which the renal arteries and veins were occluded using microaneurysm clamps. The time of ischaemia chosen was based on that found to maximize reproducibility of renal functional impairment, while minimizing mortality in these animals. After the renal clamps were removed, the kidneys were observed for a further 5 minutes to ensure reflow after which 1 mL saline at 37°C was injected into the abdomen and the incision was sutured in two layers. Mice were then returned to their cages where they were allowed to recover from anaesthesia and observed for 24 hours. Sham-operated mice underwent identical surgical procedures to I/R mice except that microaneurysm clamps were not applied.
4.3.3 Experimental Design

The recovery model of ischaemia reperfusion was performed in the following experimental groups:

1) Sham operated (n=8)
2) Ischaemia reperfusion (n=12)
3) Ischaemia reperfusion – administered EPO 1000 U/Kg at reperfusion (n=10)
4) Ischaemia reperfusion – administered EPO 1000U / Kg two hours post-reperfusion (n=8)

Mice were divided into the following four groups in experiments involving the acute administration of EPO:

1) I/R group in which control mice underwent renal ischemia for 30 minutes followed by reperfusion for 24 hours (N = 11)
2) I/R EPO prererefusion group included mice that were administered EPO (1000 IU/kg subcutaneously bolus) 5 minutes prior to reperfusion (N = 10)
3) sham group were sham-operated mice, which were subjected to the surgical procedures described above, but were not subjected to renal I/R (N = 4)
4) sham EPO prererefusion group were mice that were treated identical to sham mice except for the administration of EPO (1000 IU/kg subcutaneously) 5 minutes prior to sham reperfusion (N = 4).

In the above experiments, the route of administration and dose of EPO were based on that of a previously reported experiment in the mouse. Mice, which did not receive EPO, were administered 8 mL/kg saline (vehicle for EPO) at equivalent time points (5 minutes prior to reperfusion). In experiments involving the chronic administration of EPO, mice were divided into the following four groups:
(1) I/R group included control mice, which underwent renal ischemia for 30 minutes followed by reperfusion for 24 hours (N = 6)

(2) I/R EPO pretreatment group mice were administered EPO (1000 IU/kg/day subcutaneously) for 3 days prior to I/R (N = 6)

(3) sham group were sham-operated mice, which were subjected to the surgical procedures described above, but were not subjected to renal I/R (N = 4)

(4) sham EPO pretreatment-group were mice that were treated identical to sham mice except for the administration of EPO (1000 IU/kg/day subcutaneously) for 3 days prior to sham I/R (N = 4).

Mice, which did not receive EPO, were administered 8 mL/kg/day saline (vehicle for EPO) at equivalent time points (72, 48, and 24 hours before operation).

4.3.4 Measurement of Tissue Myeloperoxidase Activity

Myeloperoxidase (MPO) activity in kidney samples was determined as an index of polymorphonuclear (PMN) leukocyte accumulation. Kidneys were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide and 10 mmol/L 3-(N-morpholino)-propane-sulfonic acid dissolved in 80 mmol/L sodium phosphate buffer (pH 7), and centrifuged for 30 minutes at 20,000g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (16 mmol/L) and 1 mmol/L hydrogen peroxide.

The rate of change in absorbance was measured by a spectrophotometer at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol peroxide/min at 37°C and was expressed in units per gram weight of wet tissue.
4.4 Results

4.4.1 Effect of EPO administration in a recovery model of ischaemia reperfusion injury.

Biochemical parameters were studied in animals subjected to I/R injury and groups were sacrificed at different time points between 24 – 72 hrs following recovery.

The natural history of I/R in this model showed a peak in serum creatinine at 24 hrs (sham animals (n=8) creatinine 34 ± 3, I/R (n=12) 193 ± 74, \( P<0.01 \)), which had returned to near normal levels by 96 hrs (Figure 4.1). EPO was administered as a single sub-cutaneous injection (1000 U/ Kg). EPO administered at the time of reperfusion significantly attenuated the rise in creatinine at 24 and 48 hrs (creatinine in EPO treated animals 24 hrs 65 ± 31, \( P<0.001 \)). When administered two hours after reperfusion, EPO therapy was associated with a similar reduction in the rise in creatinine (creatinine at 24 hrs 90 ± 35, \( P<0.01 \)), when compared to control animals.
Figure 4.1 Serum Creatinine following I/R injury in the Recovery Model was measured at 24, 48 and 72 hours. I/R caused a significant increase at 24 hours when compared to sham animals (P<0.01). EPO treatment prevented this increase (dashed line), and there was a significant reduction in creatinine at 24 hours even when EPO was administered 2 hours into reperfusion (P<0.05)
4.4.2 Effect of Administration of EPO on renal dysfunction in the Mouse

When compared to sham-operated mice, I/R caused a significant increase in the plasma levels of creatinine in control mice (serum creatinine: sham 18 ± 7 µmol/L; I/R 170 ± 6, \( P < 0.001 \)), suggesting a significant degree of renal dysfunction. Administration of EPO, at reperfusion (1000 IU/kg) or, as pre-conditioning (1000 IU/kg/day for 3 days prior to I/R), attenuated the rise in serum creatinine observed by I/R in control mice (EPO at reperfusion 128 ± 9; EPO pre-conditioning 70 ± 22, \( P < 0.01 \)) (Figure 4.2).

Histological examination of kidneys obtained from control mice subjected to I/R demonstrated a significant degree of renal injury. Specifically, kidneys obtained from these animals exhibited degeneration of tubular structure, tubular dilatation, swelling and necrosis, luminal congestion, and eosinophil infiltration. In contrast, renal sections obtained from mice treated with EPO acutely (1000 IU/kg prior to reperfusion) and chronically (1000 IU/kg/day prior to I/R) demonstrated a marked reduction in the severity of these histological features of renal injury, when compared with kidneys obtained from control mice subjected to I/R only (Figure 4.3).
Figure 4.2 Renal function at 24 hours in Mice subjected to I/R injury was significantly preserved in animals treated with EPO (P<0.05) at reperfusion, with greater protection with pre-conditioning regime (P<0.01, by ANOVA).
**Figure 4.3  Histological Analysis of Mouse kidneys subjected to I/R Injury**

Representative sections of kidney tissue from mice sacrificed 24 hours following I/R injury.

A  Sham operated animal
B  I/R injury
C  I/R injury with EPO administered at reperfusion
D  I/R injury after 3 days pre-conditioning with EPO (1000 U/ Kg)
4.4.3 Effect of EPO on renal inflammation caused by Ischaemia-Reperfusion

When compared to sham-operated mice, the kidneys obtained from control mice subjected to I/R demonstrated a significant increase in MPO activity (sham animals 3 ± 0.3; I/R 66 ± 9.8, \( P < 0.001 \)), suggesting increased PMN infiltration into renal tissues. The increase in the tissue level of MPO seen in mice administered EPO, either acutely (1000 IU/kg prior to reperfusion) or chronically (1000 IU/kg/day prior to I/R) was significantly smaller than those seen in control mice subjected to I/R (EPO pre-reperfusion 15.2 ± 8; EPO pre-conditioning 5.5 ± 1.7, both \( P < 0.01 \)), which in the case of chronic EPO treatment were similar to values obtained from sham-operated mice (figure 3.5). It should be noted that the chronic administration of EPO reduced the MPO levels to levels which were not different from those observed in sham-operated animals.
Figure 4.4  Tissue Myeloperoxidase Concentration following I/R Injury in the Mouse was measured as a marker of inflammation. I/R induced a significant rise in tissue MPO (P<0.01). EPO treatment prevented the development of inflammation following I/R (P<0.01 by ANOVA).
4.5 Discussion

In this series of experiments, EPO reduced the renal dysfunction and degree of injury caused by bilateral occlusion and reperfusion observed both at 24 and 48 hours after organ reperfusion. This beneficial response to EPO administration was observed in both rat and mouse models of ischaemia-reperfusion injury.

In mice subjected to renal I/R, EPO (1000 IU/kg given 5 minutes prior to reperfusion) significantly attenuated the renal dysfunction (increases in plasma creatinine) and characteristic histological signs of marked tubular injury. A significant reduction in polymorphonuclear neutrophil accumulation, measured by MPO assay was also observed. All this data confirmed a well-known pattern of renal dysfunction and injury caused by I/R of the kidney. Moreover, our findings are in agreement with the notion that renal ischaemia causes both renal and tubular dysfunction, as well as secondary inflammation.

Pre-conditioning pre-treatment of mice with EPO (1000 IU/kg/day for 3 days) also reduced the renal injury, dysfunction, and inflammation caused by bilateral renal artery occlusion in the mouse. Interestingly, the degree of protection afforded by this regimen with EPO was greater than the one afforded by the acute administration of a single dose of EPO at the time of reperfusion. There are several potential explanations for this finding. First, one could argue that the pre-treatment protocol has resulted in favourable systemic or local renal hemodynamic effects. However, although acute administration of EPO (in the rat) has no effect on blood pressure or cortical and pyramidal perfusion, repetitive administration of EPO for up to 1 week caused a small reduction in cortical blood flow [Huang C, 2001]. This would argue against EPO induced haemodynamic changes in glomerular filtration rate, but might contribute to the induction of delayed pre-conditioning pathways.
Secondly, as the half-life of EPO is 10 hours in the rodent, it is possible and likely that the repetitive administration of 1000 IU/kg subcutaneously per day for 3 days (pre-treatment protocol) resulted in a higher steady-state plasma concentration, when compared to the peak concentration achieved from a single dose administration prior to reperfusion (1000 IU/kg prior to reperfusion).

Thirdly, pre-treatment of mice with EPO for 3 days may well have resulted in the up-regulation of protective genes, including endothelial nitric oxide synthase (eNOS), manganese superoxide dismutase (Mn-SOD) and heat shock protein 70 (HSP70), which in turn could have contributed to the observed protective effect, mimicking the effects of delayed ischaemic preconditioning, which is protective in animal models of ischaemic myocardial and brain injury, and is mediated by the up-regulation of multiple factors, including EPO, vascular endothelial growth factor and haem-oxygenase-1, which improve tissue performance in response to metabolic stress under the control of the oxygen sensor hypoxia-inducible factor-1 (HIF-1). The effects of delayed preconditioning were lost in HIF-1+/- mice [Cai Z, 2003]. The crucial role of the down stream effects of EPO production in delayed preconditioning of brain injury has been demonstrated by the attenuation of protection observed with the use of a soluble EPO receptor, and in vitro, medium transfer from hypoxic and glucose-starved astrocytes to untreated neurons induced protection against hypoxia in neuronal cells, which was attenuated strongly by the application of a soluble EPO receptor and anti-EPO receptor antibodies [Ruscher K, 2002]. The main difference is that, unlike treatment at reperfusion, preconditioning with the pre-treatment protocol enables increased protein synthesis and not simply activation of existing protein messengers.

Fourth, the recent findings that bone marrow–derived stem cells contribute to the regeneration of proximal tubule epithelial cells following I/R injury also creates an attractive hypothesis that the protection observed with EPO pretreatment is partially due to an increase
in the mobilization and circulation of endothelial progenitor cells and possibly bone marrow–derived stem cells. It has been shown in mice that EPO treatment caused a significant increase in the percentage of proliferating lin-/sca-1+ stem cells in the bone marrow and mobilization of endothelial progenitor cells [Heesch C, 2003; Bahlmann FH, 2003]. There is evidence that the pretreatment protocol of EPO used here results in enhanced levels of circulating endothelial progenitor cells. Most notably, it has been suggested that the increase in circulating endothelial progenitor cells by EPO contributes to the beneficial effects of EPO in animal models of I/R. It is, however, not known whether enhanced circulating levels of endothelial cell progenitors protect the kidney against I/R injury or improve endothelial repair and hence recovery of renal function after an acute ischaemic event.

The effects of EPO in a mouse model of ischaemia reperfusion described in this chapter have demonstrated that the observed effects in the rat are confirmed in a different species, increasing the likelihood of these effects being applicable to human studies. This is an important observation, as other growth factors such as insulin-like growth factor-1 (IGF-1) that were effective in one animal model have subsequently been not shown similar results in studies in rats or human clinical trials [Hirschberg R, 1999].

The beneficial effect of EPO on the course of ischaemic AKI has now been confirmed in several other studies [Yang CW, 2003; Vesey DA, 2004]. Yang et al administered high dose EPO (5000 U/ Kg IP) 24 hrs before the induction of ischaemia, and observed a significant degree of renal protection, and suggested that EPO caused the up-regulation of heat-shock protein 70 (HSP70). Vesey et al used a less severe animal model (nephrectomy and unilateral renal ischaemia), and demonstrated a significant reduction in both necrotic and apoptotic cell death, particularly in the region of the cortico-medullary junction.
Having demonstrated that EPO prevents the renal injury induced by ischaemia reperfusion in an animal model, associated particularly with a significant reduction in the degree of apoptotic cell death, the next step was to determine whether EPO would have a direct effect on proximal tubular cells in vitro, using a range of injury models that stimulate the mechanisms of cellular injury induced by ischaemia-reperfusion.
Chapter Five:

Erythropoietin prevents Toxin-induced Apoptosis
5.1 Introduction

Cisplatin is a highly effective chemotherapeutic agent used in the treatment of a wide variety of solid tumours, particularly ovarian, head and neck, and testicular germ cell tumours [Boulikas T, 2003]. However, 25-35% of patients experience a significant decline in renal function after administration [Lebwohl D, 1998]. The nephrotoxicity is a cumulative, dose-dependent effect which may necessitate dose reduction or withdrawal of cisplatin treatment, and hence limiting its effectiveness in many patients. Despite this toxicity, cisplatin remains one of the most commonly used chemotherapy drugs due to its therapeutic efficacy. Several mechanisms have been proposed for cisplatin nephrotoxicity [Arany I, 2003].

In animal models of cisplatin nephrotoxicity, analysis of proximal tubular injury reveals both necrotic and apoptotic mechanisms of cell death [Zhou H, 1999], which predominately affects the S3 segment of the proximal tubule. *In vitro*, the morphological changes of apoptosis have been demonstrated in low-dose cisplatin treated mouse isolated proximal tubules [Lieberthal W, 1996]. In culture, cisplatin induces apoptosis in a porcine renal proximal tubular epithelial cell line (LLC-PK1 cells) via Bax translocation to the mitochondrial membrane, cytochrome c release, and the activation of caspase-9 and caspase-3 [Park MS, 2002].

In proximal tubule cells in culture, mitochondrial depolarisation leading to the release of mitochondrial pro-apoptotic molecules including cytochrome c, Omi/ HtrA2, Smac/DIABLO and apoptotic inducing factor (AIF) has been shown in response to cisplatin treatment [Seth R, 2005; Cilenti L, 2005]. Mitochondrial depolarisation may be induced through a number of pathways, including oxidative stress, activation of mitogen activated kinases including JNK, and DNA damage, leading to p53-dependent caspase-2 activation [Jiang M, 2004; Seth R, 2005]. However, the importance of DNA damage to cisplatin-induced cell death has been questioned. Cisplatin has been shown to activate the caspase
cascade in enucleated cells (cytoplasts) via the generation of superoxide. Lower doses of cisplatin, insufficient to cause apoptosis, may lead to DNA damage, p53 activation and replicative senescence [Berndtsson, 2007].

5.2 Aims

The data presented in the previous chapter shows that EPO induces several signalling pathways, following ligand binding to its specific cell surface receptor, in proximal tubular epithelial cells in vitro, and that the activation of these pathways increased the resistance of the cell against apoptosis cell death induced by several different stimuli. The aim of the experiments in this chapter was study the particular mechanisms that mediated this anti-apoptotic effect by utilizing cisplatin-induced apoptosis as a standard model of mitochondrial stress induced caspase-dependent cell death. A rat model of cisplatin nephrotoxicity was used to determine whether the effects observed in vitro were confirmed in an animal model.
5.3 Methods and Materials

5.3.1 Fluorescent Microscopy and JC-1 staining of live cells

Quantitative changes in mitochondrial membrane potential ($\Delta\psi_m$) in cells at the early stages of apoptosis were measured using the fluorescent dye JC-1 [De Lisa F, 1995]. This dye exists as a monomer with emission at 530 nm (green fluorescence) at low concentrations but forms J-aggregates with emission at 590 nm (red fluorescence) at high concentrations. Therefore, the relative fluorescence of JC-1 at different wavelengths can be considered as an indicator of a change in mitochondrial membrane polarization.

HK-2 cells were seeded at $1 \times 10^5$/mL in 6 well plates in standard medium. Cells were stimulated with cisplatin for 16 hours overnight, in the presence or absence of EPO (50 U/mL). Cells were washed with 1 mL PBS, and then 1 mL 4% paraformaldehyde was added to each well for 10 minutes. Cells were then washed twice with PBS, then 0.2% Triton-X was added. After a further wash step, 2 drops of signal enhancer was added, and the plate incubated for 30 minutes. The cells were washed in PBS, then the coverslip was transferred and placed in an inverted position on the centre of a microscope slide, and 2 drops of mounting buffer containing DAPI, a fluorescent dye which strongly binds nuclear DNA, was added, and the slides stored in the dark. Slides were examined on a Zeiss fluorescent microscope. Relative intensity of fluorescence was qualitatively determined by 2 independent observers for 3 high-powered fields. Representative images were saved.

5.3.2 JC-1 Quantitative Assay

The medium was removed, and replaced with phenol-red free medium containing JC-1 (10 $\mu$M), and incubated in the dark for 30 minutes. Fluorescence was measured at two
frequencies 530nm and 580 nm, and controlled for background reading in a blank (cell-free) well. Cisplatin induced a significant reduction in the ratio of fluorescence at 450/ 510 nm, when compared with control cells, demonstrating a decrease in red fluorescence due to loss of mitochondrial “j-aggregates” following depolarisation of the mitochondrial membrane.

Briefly cells were seeded at 1 x 10^6/mL. After different treatments, the cells were incubated with 10 μM JC-1 at 37 °C for 8 min, washed twice, and resuspended in 100μL PBS. Relative fluorescence intensities were monitored using the FluoroMax-2 fluorescence spectrofluorometer with 490 nm excitation/535 nm emission and 570 nm excitation/610 nm emission. The ΔΦ_m was expressed as the ratio of the fluorescence of J-aggregate (570 nm excitation/610 nm emission) to monomer (490 nm excitation/535 nm emission) forms of JC-1, controlled by substracting fluorescence detected from blank wells.

5.3.3 PARP Activity Assay

Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme with DNA nick sensor function. Upon binding to broken DNA, PARP becomes activated and cleaves NAD+ into nicotinamide and ADP-ribose. PARP then polymerises ADP-ribose on nuclear receptor proteins including histones, transcription factors and PARP itself. PARP has been implicated in DNA repair, cell cycle progression and transcriptional regulation. Excessive PARP activation, induced by oxidative stress, leads to the depletion of cellular NAD and ATP pools and results in cell dysfunction or necrotic cell death, discussed in section 1.3.3.

In a cellular ELISA system, cells grown in 96-well plate are incubated with biotinylated-NAD after permeabilization with digitonin. Activated PARP cleaves bio-NAD into nicotinamide and biotinylated ADP-ribose and hence poly-bio-ADP-ribose polymers that are detected by conjugation with a streptavidin-peroxidase and colorimetric peroxidase
substrate. 50 μL of Biotinylated-NAD+ with was added to all experimental wells of a 96-well plate, and the plate was incubated at 37°C for 30 minutes. The medium was aspirated and 200 μL of 95% ethanol, chilled to -20°C, was added to each well and followed by 20 min incubation at -20°C. The ethanol was aspirated and each well was treated with 200 μl 30% hydrogen peroxide solution in methanol. The plate was incubated for 15 minutes at room temperature. The plate was then washed and 200 μl of streptavidin-peroxidase conjugate added, and incubated for 25 minutes at room temperature. The plate was washed three times and 100 μl TMB substrate was added and incubated in the dark. The development of blue colour was measured on the Dynex II by absorbance at 605nm, compared to blank wells.

5.3.4 Quantification of intracellular reactive oxygen species by FACS

The cell permeable fluorogenic probe 2′7′-dichlorodihydrofluorescein diacetate (DCF-DA) was used to detect oxidative stress. DCF-DA diffuses freely across cell membranes and is hydrolyzed by non-specific cellular esterases to the non-fluorescent compound dichlorofluorescein (DCFH), which is predominately trapped within the cell. In the presence of ROS, DCFH rapidly undergoes one-electron oxidation to the highly fluorescent compound dichlorofluorescein (DCF). Cell exposed to experimental conditions were washed once in PBS and then incubated with DCF-DA (20 μM) for 30 minutes at 37 °C in the dark. The cells were then trypsinized in 0.25% trypsin and then pelleted by centrifugation at 2000 rpm for 5 minutes. Pellets were washed once in PBS, and after a further centrifugation step, were re-suspended in 500 μL PBS in FACS tubes. Samples were analysed on a Becton-Dickenson flow cytometer. Side and forward scatter were controlled and the cell population was gated. Single dimension histogram at the appropriate wavelength was measured and
gated for 10000 cells per sample. For three individual experiments, samples (n=8) were analysed and median fluorescence shift was calculated and analysed.

5.3.5 Rat Model of Cisplatin Nephrotoxicity

This study was carried out using twenty-four male Wistar rats (Tuck, Rayleigh, Essex, UK) that weighed 250 to 300 g and received standard diet and water ad libitum. Animals were cared for in accordance with the Home Office Guidance in the Operation of the Animals (Scientific Procedures) Act 1986 (HMSO, London, UK).

Animals were treated with a single intra-peritoneal bolus of cisplatin (7 mg/Kg) or an identical volume of carrier (DMSO) solution. On day 5, blood was taken from tail vein and the animal sacrificed under general anaesthesia using intraperitoneal injection of sinactin (Inactin®, Suzan Bennett, Natick, USA). The abdomen was opened and both kidneys were removed. They were promptly bisected and fixed in formaldehyde (15%) and then embedded in paraffin; sections were cut at 3 µm and stained with haematoxylin and eosin, PAS, and trichrome Masson with light green.

A pathologist carried out a semiquantitative analysis of the kidney sections in a blinded fashion. Glomeruli and vessels were normal. Changes observed were limited to the tubules, especially to the proximal straight S3 portion, the main site of cisplatin toxicity. Tubular lesions were graded as follows: 0=no damage; 1+=area of tubular epithelial cell swelling, vacuolization, necrosis, desquamation less than 50%; 2+=lesion areas grater than 50% with or without focal involvement of the S3 segment in the medullary rays; 3+=lesion areas 100% with diffuse involvement of the medullary rays.
5.4 Results

5.4.1 Cisplatin induces dose dependent increase in proximal tubular epithelial cell death which is attenuated by pre-administration of EPO

Cisplatin in DMSO was freshly prepared for each set of experiments. Human proximal tubule epithelial (HK-2) cells were incubated with increasing concentrations of cisplatin for 24 hrs, and then DNA fragmentation (Cell Death ELISA) and cell viability assays (MTS assay) were examined.

Cisplatin caused a reduction in cell viability (figure 5.1) which was associated with an increase in DNA fragmentation, as a marker of apoptosis (figure 5.2). These results indicate that the pro-apoptotic effects of cisplatin are dose-dependent, with the greatest amount of DNA fragmentation seen in cells treated with 50 µM cisplatin. Cells were pre-treated with varying concentrations of EPO (10-50 U/mL) for one hr before being exposed to 25-50 µM cisplatin for 24 hrs. EPO pre-treatment caused a dose-dependent increase in cell viability (when compared to control cells: CP 68% ± 5, CP with EPO 10 U/ml 81% ± 11, CP + EPO 30 86 ± 10, CP + EPO 50 91 ± 11; P<0.01) which was associated with a significant decrease in DNA fragmentation (when compared to control cells: CP 7.6 ± 1.2 fold increase in DNA fragmentation, CP + EPO 4.8 ± 0.5; \( P < 0.01 \)).
Figure 5.1  Cell Viability is preserved by EPO in dose dependent manner. An MTS assay was performed after 24 hours exposure to cisplatin. Cell viability was expressed relative to the MTS value of control cells (in 3 separate experiments). EPO maintained cell viability when compared to untreated cells (P<0.01 by ANOVA).
Figure 5.2  EPO pre-treatment reduces Cisplatin induced DNA fragmentation in an ELISA assay. Values for experimental groups were expressed relative to control cells for 3 experiments. EPO significantly abrogated the rise in apoptosis following cisplatin exposure (p<0.001 by ANOVA).
5.4.2 Cisplatin induces Apoptosis via the activation of multiple Caspases

To elucidate whether the activation of capase-3 was necessary for cisplatin-induced cell death, HK-2 cells were plated out in a 96-well plate in 100µL phenol-red free standard medium, and a modified *in situ* assay for caspase-3 activation was used. In this assay, total caspase activation at various time points during the experiment was determined by quantification of cumulative AMC fluorescence in small aliquots of experiment medium (see methods section 2.3.4).

Cisplatin (50µM) induced a significant time-dependent increase in caspase-3 activity between 8 and 24 hrs (Figure 5.3). Pre-treatment with EPO (50 U/ mL) for one hr significantly reduced the increase in caspase-3 activity induced by cisplatin at 24 hours (CP 4158 ± 532, CP + EPO 1789 ± 247; *P*<0.001). To determine whether caspase-3 activation was dependent on the mitochondrial stress pathway, lysates previously used to determine DNA fragmentation at 24 hrs were used to assay caspase-9 activity. There was a significant increase in caspase-9 activity induced by cisplatin at 24 hours (Figure 5.4), which was significantly reduced by pre-treatment with EPO (CP 314 ± 71, CP + EPO 165 ± 97, *P*<0.05). Park *et al.* have previously shown no activation of caspase-8 at 24 hours in cisplatin exposed proximal tubular epithelial cells [Park N, 2002]
Figure 5.3  Caspase-3 activity was measured at various time points up to 24 hours in cisplatin exposed cells (results from n=6 wells in 3 separate experiments). When compared to early time points, there was significant rise in caspase-3 activity at 24 hours, which was reduced by EPO treatment (P<0.01).
Figure 5.4  EPO reduces caspase-9 activity induced by cisplatin when measured at 24 hours following cisplatin exposure (P<0.01 by ANOVA).
5.4.3 EPO inhibits mitochondrial depolarisation induced by Cisplatin

The activation of caspase-9 requires the release of cytochrome c from depolarised mitochondria. The mitochondrial probe JC-1 was used to study changes in relative mitochondrial membrane potential. Initially, fluorescent microscopy was utilized to examine JC-1 uptake and staining in cisplatin treated cells, as described in 5.3.1. Cells were fixed and stained with JC-1 before microscopy. In a series of experiments (n=3), there was a qualitative difference in JC-1 staining in cisplatin treated cells, with an increase in cells with orange fluorescence in EPO treated cells, indicting preservation of mitochondrial J aggregates (figure 5.5).

In order to quantitatively assess the observed findings in these initial experiments, an in situ assay was developed, described in section 5.3.2. HK-2 cells, plated in a 96-well plate (16 wells for each experimental group), were incubated with cisplatin (50 µM) with and without pre-treatment with EPO (50 U/ mL), for 24 hours. Pre-treatment with EPO caused significant preservation of JC-1 ratio (Figure 5.6), indicating preservation of mitochondrial membrane potential (control cells 11.4 ± 0.8, CP 10.3 ± 1.3, CP + EPO 9.0 ± 1.7; P <0.05).
Figure 5.5  
**JC-1 staining by microscopy in Cisplatin exposed HK-2 cells**

Cells incubated with Cisplatin demonstrated reduced orange fluorescence in polarised mitochondria with a predominance of green fluorescence indicating dimerised cytoplasmic JC-1. EPO pre-treatment was associated with preservation of orange fluorescence activity from monomeric JC-1 in mitochondria.
Figure 5.6  EPO pre-treatment was associated with preserved JC-1 ratio when compared to Cisplatin exposed cells. JC-1 was added to cells at the end of the experiment and fluorescence measured at two wavelengths. The ratio was calculated from the difference between intensity at different wavelengths for n=8 wells per experiment (P<0.05 by ANOVA).
5.4.4 Cisplatin induced apoptosis is dependent on the translocation of pro-apoptotic Bax to mitochondria with down-regulation of Bcl-X<sub>L</sub>

Changes in mitochondrial membrane potential are dependent on the balance of pro- and anti-apoptotic proteins. HK-2 cells were exposed to cisplatin for 24 hours with and without pre-treatment with EPO. Cells were lysed in RIPA buffer and 20 µg cellular protein was electrophoresed on a pre-cast 4-12% graduated gel (Invitrogen, UK). After electroblotting, membranes were probed with specific antibody to Bcl-X<sub>L</sub> (Santa-Cruz). Cisplatin down-regulated the expression of Bcl-X<sub>L</sub>, but pre-incubation with EPO prevented the reduction in expression (Figure 5.7a representative blot). Cell fractionation was also performed, and cytoplasmic and mitochondrial expression of Bax was examined by western blotting. Cisplatin induced the translocation of Bax to the mitochondrial fraction (Figure 5.7b).

In order to confirm the role of Bax in cisplatin induced apoptosis, RNA interference was used to knock-down Bax expression in HK-2 cells before cisplatin exposure. HK-2 cells were plated at 1 x 10<sup>5</sup> cells/mL in 6 well plates, and transfected with Bax and control non-targeted siRNAs, as described in section 2.6. After 24 hrs, the medium was replaced with standard medium with cisplatin (50 µM) for a further 24 hrs. Western immunoblotting with a Bax polyclonal antibody was performed to determine total Bax levels and hence confirm reduced expression in siRNA knock-down.

Knock-down of Bax expression by transfection with Bax siRNA abrogated DNA fragmentation induced by cisplatin (Figure 5.8) to a similar degree to the cellular protection observed with EPO treated cells (control 15 ± 6, CP 70 ± 16, CP + EPO 40 ± 14, CP in Bax siRNA treated cells 33 ± 11; <i>P</i> <0.01), confirming that Bax is a vital mediator of the mitochondrial mechanism of apoptotic cell death induced by cisplatin.
Figure 5.7  Immunoblotting for pro- and anti-apoptotic molecules.

A  Immunoblot with Bcl-X<sub>L</sub> antibody. Representative blot from n=3 experiments. Cisplatin exposure reduced expression of Bcl-X<sub>L</sub>, which was maintained in EPO treated cells.

B  Individual blots with specific Bax antibody from cellular fractions. Cisplatin induced Bax shift into mitochondrial fraction when compared to control cells.
Figure 5.8  
Inhibition of Bax prevents cisplatin induced apoptosis.

A  
Immunoblot with Bax antibody confirms reduction in expression with siRNA.

B  
DNA fragmentation ELISA as measure of cell death shows significant increase in DNA fragmentation with cisplatin exposure, which was reduced by siRNA Bax knockdown and by EPO treatment (P<0.05, by ANOVA).
5.4.5 EPO maintains the expression of the inhibitor of apoptosis, XIAP

The expression of XIAP by western blotting was decreased after 24 hours exposure to cisplatin. This reduction is expression was prevented in EPO treated cells, shown in Figure 5.9, suggesting that maintenance of NFκB dependent anti-apoptotic signalling via XIAP is important to the anti-apoptotic effects of EPO in this model.

To confirm this, we transfected cells with XIAP siRNA, and then examined the amount of DNA fragmentation induced by cisplatin (50μM) in three separate experiments. Compared to cells transfected with non-targeted control siRNA (control 23 ± 1.9, CP 50 ± 0.4, EPO + CP 34 ± 5, P<0.01), there was a small increase in DNA fragmentation in control cells transfected with XIAP siRNA (control 27 ± 4, P=ns). There was significantly more apoptosis in cells exposed to cisplatin in which XIAP had been knocked down (CP 61 ± 2.6) (Figure 5.10). The protective effects of EPO pre-treatment were abrogated by transfection with XIAP siRNA (57 ± 4, P=ns when compared to CP), confirming this pathway as vital to the anti-apoptotic effects of EPO in proximal tubular epithelial cells.
Figure 5.9  Immunoblot of XIAP from lysates of cells exposed to cisplatin and EPO. Representative image from n=3 experiments. Equal protein loading was confirmed by re-probing with housekeeper protein.
Figure 5.10  Inhibition of XIAP prevents the beneficial effect of EPO in cisplatin induced apoptosis. A DNA fragmentation ELISA assay was used to determine cell death relative to control cells. Transfection with XIAP siRNA induced an increase in DNA fragmentation in control cells when compared to controls. The protection observed with EPO treatment in control transfected cells (P<0.01, ANOVA) was lost in XIAP siRNA transfected cells.
5.4.6 EPO reduces cisplatin induced oxidative stress and PARP activation

Incubation with cisplatin (10 μM-50 μM) caused a significant increase in DCF fluorescence, shown in Figure 5.11, indicating an increase in the generation of reactive oxygen species. Pre-incubation with EPO attenuated the mean increase in oxidative stress induced by cisplatin (control 26 ± 0.7, 10 μM cisplatin 47 ± 0.3, 10 μM + EPO 38 ± 3, 25 μM cisplatin 47.5 ± 0.2, 25 μM cisplatin + EPO 40.5 ± 1.5, P < 0.01).

DNA damage induces the activity of PARP-1, which contributes to ATP depletion and secondary necrosis cell death. PARP activity is increased in oxidative stress, and may contribute to the development of lethal cellular injury. PARP-1 activation in live cells was determined as described in 5.3.3. HK-2 cells were seeded in a 96-well plate in standard medium. Cells were pre-treated with EPO (50 U/mL) for one hour, then the medium was removed and replaced by 100 μL standard medium containing cisplatin (50 μM). Cisplatin exposed cells showed marked activation of PARP-1 (control 0.24 ± 0.07, CP 0.84 ± 0.4; P < 0.01), which was significantly reduced by EPO pre-treatment (CP + EPO 0.27 ± 0.04, P < 0.01 when compared to CP), shown in Figure 5.12.
Figure 5.11  DCF staining in cisplatin treated HK-2 cells caused a right-shift due to an increase in oxidative radical production which is reversed by EPO. Image downloaded from flow cytometry representative of n=4 experiments.
Figure 5.12 EPO reduces PARP-1 activation induced by cisplatin administration. PARP activity was measured by measurement of utilization of biotinylated NAD. When compared to control cells, cisplatin caused significant increase in PARP-1 activity, which was abrogated by EPO pre-treatment (P<0.01 by ANOVA).
5.4.7 EPO reduces Renal dysfunction in a model of cisplatin Nephrotoxicity

Cisplatin (7 mg/ Kg) IP induced significant renal dysfunction in male wistar rats (n=9) on day 5 (creatinine 115 ± 52 umol/l). Animals pre-administered EPO (1000 U/Kg) 24 hours before cisplatin exposure (n=8) showed significant reduction in serum creatinine at day 5 (creatinine 61 ± 36, \( P<0.05 \)). Daily EPO administration (300 U/Kg) commenced after cisplatin exposure did not demonstrate any significant improvement in serum creatinine at day 5 (n=6), consistent with previously published observations [Variza ND, 2004], shown in Figure 5.13.

EPO pre-administration was associated with preservation of normal kidney architecture on histological examination, shown in Figure 5.14, with reduced histological injury scored by two pathologists blinded to experimental grouping (sham animals 0; cisplatin + vehicle 145 ± 1; EPO pre-treatment + cisplatin 45 ± 4, \( P<0.01 \)).
Figure 5.13  Serum creatinine at day 5 following Cisplatin exposure in male Wistar Rats. Cisplatin caused renal dysfunction with a significant increase in serum creatinine at day 5 (P<0.01). Pre-treatment with EPO (n=8) reduced the degree of dysfunction at day 5 (P<0.05, ANOVA), whereas daily administration of EPO post-cisplatin exposure had no effect.
Figure 5.14  Histological assessment of Renal Injury induced by Cisplatin was assessed by two blinded pathologists. Histological severity scoring, described in 5.3.5, showed that cisplatin induced widespread changes in the kidney when compared to control sham animals (P<0.01), and this was reduced by pre-treatment with EPO (P<0.01, by ANOVA). Representative images from each group are shown.
5.5 Discussion

The evidence presented in this chapter confirmed the findings described in chapter four, demonstrating that EPO significantly abrogated the induction of apoptotic cell death. EPO prevented cisplatin induced tubular epithelial cell toxicity through the activation of a number of important cell survival pathways. The reduction in apoptosis, measured by DNA fragmentation, was accompanied by a reduction in caspase-9 and caspase-3 activity, and preservation of mitochondrial membrane potential.

Cisplatin induced the translocation of bax to the mitochondrial fraction, and associated with the down-regulation of the Bcl-2 family member Bcl-X\textsubscript{L}, promotes mitochondrial depolarisation. In healthy cells, bax is sequestered in the cytoplasm via an interaction with 14-3-3 proteins, but following cellular stress, activation of the mitogen-activated protein kinase c-jun N-terminal kinase (JNK) phosphorylates 14-3-3 protein, leading to dissociation of Bax and mitochondrial depolarisation [Tsuruta F, 2004]. Cisplatin has been shown to cause a sustained activation of JNK and p38 in cancer cells lines and proximal tubular epithelial cells in culture. Inhibition of JNK activation by either kinase inhibitors or transfection of dominant negative MEKK1 has been shown to reduce apoptotic cell death after 24 hours, although there is some conflicting evidence [Krillele D, 2003; Arany I, 2004].

A major pathway for the protective effects of EPO in cisplatin induced apoptosis in these experiments was the preservation of anti-apoptotic molecules including XIAP and Bcl-X\textsubscript{L}. XIAP expression was maintained by EPO, which may be due to several mechanisms, including increased transcription through NF-\kappaB activation, and AKT-dependent
phosphorylation of XIAP which renders it more resistant to ubiquitin-dependent degradation [Dan HC, 2004].

These studies have provided further elucidation of the molecular mechanisms by which EPO exerts its anti-apoptotic effects in the kidney. EPO binds to its cell surface receptor, leading to activation of two major signalling pathways – the non-genomic activation of the PI-3 K /AKT pathway, which leads to inactivation of a number of apoptotic mediators, and activation and nuclear translocation of the transcription factor NF-κB, which induces genomic expression of a wide range of anti-apoptotic mediators, particularly XIAP, which inhibit apoptosis at several levels through the inhibition of caspase-3, -7 and -9, and contributes to the down-regulation of JNK signalling.

When administered as a pre-treatment, EPO exerted a significant effect on the course of cisplatin nephrotoxicity in a rat model. These observations were consistent with previous work, which demonstrated that EPO given after exposure to cisplatin did not affect the degree of injury seen, but induces a more rapid recovery in kidney function with tubular regeneration and mitosis occurring earlier than in untreated animals [Varizi ND, 2001]. Following on from the findings in models of ischaemia reperfusion injury in the kidney described in chapters three and four, these experiments confirm that EPO upregulates signalling pathways that alter the physiological resistance to an ischaemic or toxin insult.

The beneficial effect of EPO demonstrated in these experiments has been confirmed in two recent studies using animal models of cisplatin nephrotoxicity [Salahudeen AK, 2008; Choi DE, 2009]. Salahudeen et al used an in vitro model of cisplatin induced apoptosis, and the protective effect was abrogated by the use of an inactive form of human EPO, and by the inhibition of JAK2 signalling with the inhibitor AG490. Darbopoietin (25 µg/ Kg) reduced apoptotic and necrotic cell death in a in vivo model of cisplatin toxicity, and this benefit was not reduced in control group that underwent venesection to control for changes in haemocrit.
Chapter Six:

Effects of Erythropoietin in the Heart
6.1 Introduction

Cardiovascular disease is the number one cause of mortality in the developed world, and is fast becoming the main global health concern. In the United Kingdom, 105842 people died as a result of coronary artery disease in 2005, of which 36323 were attributed to acute myocardial infarction [Government Statistics, 2008]. Coronary atherosclerosis is a chronic disease with stable and unstable periods. During unstable periods with activated inflammation in the vascular wall, myocardial ischaemia and infarction may be a consequence of an arterial occlusion, reduced flow within a stenosed vessel or acute thrombosis related to plaque rupture.

Coronary artery occlusion and resulting myocardial ischaemia can be reversed by surgical or pharmacological strategies to remove the obstruction and restore myocardial blood supply. Cell death occurs after a period of as little as twenty minutes, although it may take several hours before myocardial necrosis can be identified by macroscopic post-mortem examination. Long term outcome has been correlated with the degree of recannulation of the coronary artery lumen [Clements IP, 1993], and hence the degree of restoration of normal arterial blood flow and myocardial perfusion and oxygenation. Successful examples include the administration of thrombolytic agents, streptokinase and tissue plasminogen activator, which lyse the platelet thrombus, and percutaneous transluminal angioplasty, in which the artery is selectively cannulated with a wire and a balloon inflated across the occlusion to mechanically restore patency, which evidence has suggested is the superior primary therapy [Weaver WD, 1997]. Large clinical trials investigating the use of thrombolitics have shown reduction in infarct size and mortality, and improvement in left ventricular function, if carried out within 6 hours of the onset of symptoms [GISSI 1986, ISIS-2 1988]. Restoration
of blood flow or reperfusion is absolutely essential for the survival of the ischaemic myocardium. However, reperfusion is not without its own consequences, as animal models suggest that reperfusion injury accounts for up to 50% of the final size of a myocardial insult [Bolli R, 2004].

Myocardial reperfusion injury was first described by Jennings et al in a canine model with histological features of cell swelling, contracture of myofibrils, and disruption of the sarcolemma [Jennings RB, 1960]. The injury to the heart during reperfusion causes several types of cardiac dysfunction, which are analogous to the early phases of acute kidney injury; initially “myocardial stunning” denotes mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage and despite restoration of normal or near-normal coronary artery flow [Braunwald E, 1982]. The myocardium usually recovers from this reversible form of injury after several days or weeks, with ventricular remodelling around the area of infarction.

At a cellular level, myocardial cell death occurs through mechanisms that are similar to those in the kidney, including changes in intracellular calcium, oxidative stress and mitochondrial dysfunction and interruption of normal cellular metabolic processes. Recently, attention has been focused on the phenomenon of ischaemic preconditioning, in which repeated short periods of myocardial ischaemia lead to physiological changes which limit the degree of injury induced by subsequent prolonged ischaemia. Ischaemic late preconditioning is dependent on HIF-α signalling, and has been associated with increased circulating EPO produced in the kidney and reduction in infarction size [Cai Z, 2003].
6.2 Aims

In view of the protective effects observed with EPO in a rat model of renal ischaemia reperfusion, it was important to extend these observations into another organ, and the heart is an ideal model to study. Previous studies have suggested a role for EPO in foetal cardiac development [Wu H, 1999], and the receptor is present on foetal and neonatal ventricular myocytes [Juul SE, 1998], although the expression of the receptor in adult tissues is not known. However, cardiac tissue has a very large amount of endothelium which does express the EPO-R [Chong ZZ, 2003], which could be an important site of action of EPO during the physiological response to ischaemia reperfusion injury in the heart.
6.3 Methods

6.3.1 Rat ventricular myoblast cell (H9c2) culture

The H9c2 embryonal rat heart-derived cell line (Kimes and Brandt, 1976; Hescheler et al., 1991) was obtained from the American Type Culture Collection (CRL 1446) and cultured in growth medium comprising DMEM (containing 1000 mg/l D-glucose and 110 mg/l sodium pyruvate) supplemented with 10% heat-inactivated newborn calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown in an air/CO₂ (49:1) humidified incubator at 37°C. Sub-confluent (80%) cells were harvested and seeded into six-well tissue culture plates in 2 mL growth medium. The cells were allowed to adhere for 18 hours in an incubator at 37°C with 5% CO₂ in 95% air.

6.3.2 Myocardial Infarction in the anaesthetised rat

Male Wistar rats (200-250 g, Tuck, Rayleigh, Essex, U.K.) were anaesthetised with thiopentone sodium (Intraval®, 120 mg/kg i.p.; Rhone-Merrieux, Essex, U.K.). The rats were tracheotomised, intubated and ventilated with a Harvard ventilator (30% inspiratory oxygen concentration, 70 strokes/min, tidal volume: 8-10 ml/kg). Body temperature was maintained at 38±1°C. The right carotid artery was cannulated and connected to a pressure transducer (Spectramed, P23XL) to monitor mean arterial blood pressure (MAP). The right jugular vein was cannulated for the administration of drugs.

Subsequently, a lateral thoracotomy was performed and the heart was suspended in a temporary pericardial cradle. A snare occluder was placed around the left anterior
descending coronary artery (LAD). After completion of the surgical procedure the animals were allowed to stabilise for 30 min before LAD ligation. The coronary artery was occluded at time 0 by tightening of the occluder. This was associated with the typical haemodynamic (fall in MAP) changes of myocardial ischemia. After 25 min of acute myocardial ischaemia, the occluder was re-opened to allow reperfusion for 2 h. Heart rate (HR) and MAP were continuously recorded on a 4-channel Grass 7D polygraph recorder (Grass, Mass., U.S.A.).

6.3.3 Determination of infarction size and area of risk

The coronary artery was re-occluded at the end of the reperfusion period, and Evans Blue dye (1 ml of 2% w/v) was injected into the left ventricle, via the right carotid artery cannula, to distinguish between perfused and non-perfused sections of the heart. The Evans Blue solution stains the perfused myocardium, while the occluded vascular bed remains uncoloured, and defines the area at risk (AR). The animals were killed with an overdose of anaesthetic and the heart excised. It was sectioned into slices of 3-4 mm, the right ventricular wall was removed, and the AR (unstained) was separated from the non-ischaemic (blue) area.

The identified AR tissue was cut into small pieces and incubated with $p$-nitroblue tetrazolium (NBT, 0.5mg/ml) for 40 min at 37°C. In the presence of functioning dehydrogenase enzyme systems in surviving viable myocardium, NBT forms a dark blue formazan, whilst areas of necrosis lack dehydrogenase activity and therefore fail to stain. Pieces were separated according to staining and weighed to determine the amount of myocardial infarction as a percentage of the weight of the total AR tissue.
6.3.4 Experimental design for in vivo experiments

The experimental groups outlined below were studied according the protocol described in 6.2.2.

(1) MI - control group: (n=8) Animals subjected to 15 min LAD occlusion and at 1 min prior to reperfusion received vehicle control (saline, 1 mg/ Kg bolus iv)

(2) MI - EPO group: (n=12) Animals subjected to 15 min LAD occlusion at at 1 min prior to reperfusion received EPO (300 U/ Kg in saline bolus iv)

(3) Sham Group: (n=6) Animals were subjected to the surgical procedure, without coronary artery occlusion and reperfusion, treated with vehicle control (saline 1mg/ Kg bolus iv)
6.4 Results

6.4.1 Effect of EPO on H9C2 cell proliferation

Preliminary experiments were aimed at confirming that the EPO/ EPO-R system was functional in H9C2 cells. After harvesting and counting, H9C2 cells were plated at a density of 0.5 x 10^6 in a clear 96-well plate with 100 μL phenol-red free standard medium containing 10% FCS. EPO was added to a final concentration of 10 U/mL – 30 U/mL. After 24 hrs incubation, 20 μL of MTS solution was added to each well and the plate returned to the incubator for 30 min. Optical density was measured in the colorimeter after 30 minutes incubation, and results were calculated as percentage of value of control cell minus blank well correction. In each of 3 experiments, twelve wells were used for each experimental group.

When compared to control cells cultured in standard medium with 10% FCS, there was a significant increase in cell number in response to administration of EPO (when compared to control cells, EPO 10U/mL 25 % ± 7, EPO 30 U/mL 33 % ± 8, P<0.001). The response to erythropoietin was also dose-dependent, being maximal at 30 U/mL (Figure 6.1).
Figure 6.1   EPO induces proliferation above control in H9c2 cells. The MTS assay was used as an indirect measure of cellular proliferation. When compared to control cells, EPO caused an increase in cellular metabolism, consistent with an increase in cell number, in a dose-dependent manner (P<0.01 by ANOVA).
6.4.2 EPO maintains cell viability in serum deprived H9c2 cells

H9C2 cells were plated at a density of 0.5 x 10⁶ in a clear 96-well plate with 100 µL phenol-red free standard medium in the absence of FCS. EPO was added in increasing concentrations (1 U/mL – 10 U/mL – 30 U/mL – 100 U/mL). Cells were incubated for 24 hours, and then 20 µL MTS solution was added to each well. After 30 minutes, the plate was read, and cell viability calculated as a percentage of control value.

H9C2 cells deprived of FCS showed significant reduction in cell number after 24 hrs (figure 6.2). EPO significantly maintained cell viability in all doses above that observed in serum deprived conditions (serum free 0.37 ± 0.03, +EPO 10 U/ml 0.46 ± 0.04, +EPO 30 U/ml 0.48 ± 0.03, +EPO 50 U/ml 0.51 ± 0.026, +EPO 100 U/ml 0.50 ± 0.026, all P<0.001). The protection observed with EPO increased in a dose-dependent manner, although with highest dose (100 U/mL) there was no appreciable increase in effect over that seen in cells treated with 50 U/mL EPO.

The decrease in cell viability seen in serum deprived culture conditions was due to loss of cell numbers by apoptotic cell death, which is associated with an increase in activity of the serine protease caspase-3 (figure 6.3). Cell lysates were used to examine caspase-3 activity, as described in 2.2.4, to confirm that erythropoietin inhibits caspase-3 activation from a variety of insults. Serum deprivation induced a significant increase in caspases-3 activity (control 125 ± 83, serum deprivation 3068 ± 756, P<0.001), which was attenuated by EPO (30 U/mL) pre-administration (973 ± 599, P<0.01 when compared to serum deprivation).
Figure 6.2 EPO preserves cell numbers in serum deprived conditions, measured by MTS assay. Measurement of cell viability was calculated by optical density minus blank well scores. EPO improved cell viability in a dose-dependent manner (P<0.01 by ANOVA).
Figure 6.3    EPO inhibits caspase-3 activation in serum deprived conditions, measured by a end-point caspase assay. Serum starvation caused an increase in caspase-3 activity when compared to control (P<0.0001), which was significantly reduced by treatment with EPO (P<0.001).
6.4.4 EPO prevents oxidative stress induced apoptosis in H9C2 cells.

In order to mimic the type of injury observed during reperfusion, hydrogen peroxide was used as a model of oxidative stress in H9c2 cells. After harvesting and counting, H9C2 cells were plated at a density of $1 \times 10^6$ in a clear 6-well plate with 1 mL standard medium without FCS. Erythropoietin was added in final concentrations of 10 U/mL – 30 U/mL of medium and after 1 hr, hydrogen peroxide (final concentration 200 µM) was added, and the plates returned to the incubator for 24 hrs. The medium was removed and cells washed with 1 mL ice-cold PBS. The cells were scraped with Teflon cell scraper, and centrifuged at 400 g for 5 minutes at 4°C. Pellets were lysed in digitonin buffer, and DNA fragmentation was assessed in lysates using cell death detection ELISA, described in 2.3.10.

Hydrogen peroxide (200 µM) induced significant DNA fragmentation after 24 hrs in serum-starved H9C2 cells (Figure 6.4). Pre-incubation with EPO (30 U/mL) for 1 hr prior to hydrogen peroxide significantly reduced DNA fragmentation (control 1.0 ± 0.18, H2O2 1.97 ± 0.6, EPO + H2O2 1.1 ± 0.4, P<0.01).

The lysates from the previous experiment were subsequently used to examine caspase-3 activation. Hydrogen peroxide (200 µM) induced a significant increase in caspases-3 activity (Figure 6.5), consistent with the observed increase in DNA fragmentation (Figure 6.4). Pre-incubation with EPO (30 IU/mL) for 1hr was associated with a significant reduction of the increase in caspase-3 activity induced by hydrogen peroxide (control 126 ± 83, H2O2 1890 ± 304, EPO + H2O2 1333 ± 287, P<0.01).
Figure 6.4 EPO reduces Oxidative stress induced cell death, measured with DNA fragmentation ELISA. Hydrogen peroxide caused an increase in cell death, which was reduced by EPO treatment (P<0.01, by ANOVA).
Figure 6.5 Caspase-3 activity in hydrogen peroxide treated H9c2 cells was measured with an end-point caspase 3 assay. When compared to control cells, hydrogen peroxide induced caspase-3 activity (P<0.001), which was reduced by EPO treatment (P<0.01 by ANOVA).
6.4.6 Administration of EPO upon reperfusion reduces myocardial infarction size in the rat

The mean values for area at risk (AR) were similar in all animal groups studied and ranged from 49 ± 2 to 52 ± 2 % ($P>0.05$). In rats, which received vehicle alone, occlusion of the LAD for 25 min followed by reperfusion for 2 hours resulted in an infarct size of 64 ± 3% (n=9) of the AR, as shown in Figure 6.6.

When compared with vehicle, administration of EPO (300 IU/kg i.v. bolus administration at 1 min prior to the onset of reperfusion) caused a significant reduction in myocardial infarct size (Figure 6.7). Sham operation alone did not result in a significant degree of infarction in any of the animal groups studied (< 1 % of the AR). It should be noted that coronary artery occlusion and reperfusion caused a progressive fall in mean arterial blood pressure (from a baseline MAP of 111 ± 6 mm/Hg to 77 ± 5 mm/Hg at the end of the 2 hr reperfusion period), (when compared with sham-operated animals). Administration of EPO had no significant effect on blood pressure.
Figure 6.6 There was no difference in the Area at Risk in Myocardial infarction in the rat between the experimental groups, confirming that ischaemia was restricted to myocardium perfused via a single vessel.
Figure 6.7  EPO reduces infarction size in a rat model of myocardial infarction. The area of infarction was measured by NBT staining, and the percentage of the area at risk stained was calculated. Treatment with EPO significantly reduced the percentage area of infarction (P<0.05)
6.5 Discussion

Having shown that EPO was protective in a short term model of renal ischaemia-reperfusion injury, shown in Chapter three, and that this protection may be mediated by direct effects on intrinsic kidney cells, it was important to examine whether this effect was organ-specific, or whether EPO would also be effective in a broader spectrum of organs, particularly the heart.

For EPO to exert direct actions on cardiac myocytes, these cells must express the appropriate receptor. The EPO receptor has been demonstrated in cardiac tissues as shown in a recent study of adult mouse hearts [Cai Z, 2003]. The potential disadvantage of examining whole organ preparations is that it this does not exclude expression on non-myocyteic cells of the heart, particularly the endothelium, and cardiac fibroblasts. These issues are partially addressed by immunohistochemical staining and immunoblotting of neonatal rat ventricular cardiac myocytes, which has shown that these cells, at least, express the EPO receptor. A functional assessment was used in H9c2 cells, and there was a significant dose-dependent increase in cell number induced by EPO administration.

In the simplest experimental system showing cardioprotection by EPO, H9C2 cells were exposed to EPO and subjected to *in vitro* conditions that mimic the stresses experienced either during or following infarction. EPO reduced apoptotic cell death induced by growth factor withdrawal and oxidative stress. This was associated with a significant decrease in caspase-3 activity, demonstrating that these injury models in different cell types are dependent on similar signal transduction pathways.

In an established model of myocardial infarction, EPO administration at the time of reperfusion in a dose similar to that successfully utilized in the kidney injury model, described in chapter three, reduced the area of myocardial infarction significantly.
Several studies have been published, subsequent to our experiments, which confirm our finding that EPO protects the heart in animal models of ischaemia-reperfusion [Cai Z, 2003; Moon C, 2003; Calvillo L, 2003; Parsa CJ, 2004; Wright GL, 2004].

Parsa et al evaluated the actions of EPO (5000 units/kg body weight, intraperitoneal injection) in the adult rabbit heart. EPO was delivered at the time of coronary artery ligation and its effects studied at 3 days. Cardiac function was measured in vivo by micro-manometry both under basal conditions and in response to β-adrenergic stimulation. Whilst EPO treated hearts did not completely retain global function, peak left ventricular pressures and left ventricular relaxation were significantly improved when compared to non-EPO-treated animals subjected to infarct. Closer examination of the area at risk showed that EPO decreased the area of infarcted tissue.

Calvillo et al and Moon et al have also followed recovery in vivo for longer periods of time following EPO treatment. These studies have examined adult male rats. Calvillo et al delivered EPO (5000 units/kg body weight, intraperitoneal injection) in two protocols. First, the effects of EPO pre-treatment were evaluated by administration at 24 and 0.5 hrs prior to a 30 min ligation of the left anterior descending coronary artery. In the alternative protocol, EPO administration was initiated at the time of reperfusion when the suture was removed. In both protocols, animals subsequently received repeat doses of EPO every 24 hrs for the following 7 days, then haemodynamic measurements were made of cardiac function, and the hearts were examined histologically. Cell death was measured together with cardiac myocyte cross-sectional area as an estimate of the hypertrophic response following increased workload.

These studies showed less cardiac myocyte loss and a smaller increase in myocyte size in those animals treated with EPO prior to ischaemia reperfusion. These differences appeared sufficient to normalise haemodynamic function, specifically with ventricular wall stress
remaining normal. In all of these studies, it would appear that the initial actions of EPO to reduce the degree of apoptotic cell death was the major contributor to the reduction in initial infarct areas and would therefore the decrease in left ventricular dilatation.
Chapter Seven:

Summary
7.1 General Summary

The haematopoietic growth hormone EPO plays an essential but tightly regulated role in maintaining basal erythropoiesis, with the ability to increase production many times over in response to stress stimuli. The EPO gene is exquisitely controlled by the hypoxia inducible factor HIF in response to oxygen tension in the renal interstitium. Originally it was thought that EPO had a sole function in the survival, maturation and differentiation of erythroid progenitors. It has now been established that EPO is also present in many sites outside the bone marrow, including the vasculature, brain, and kidney and plays an important physiological role in the response of different tissues to ischaemia.

This thesis attempts to provide evidence to extend the effect of EPO to the response of the kidney and the heart to ischaemic injury, which is mediated by the interaction between EPO and its cell surface receptor, and the signalling pathways activated. The series of experiments described in these chapters show that EPO reduces apoptotic cell death and organ dysfunction in several models of kidney injury. Several approaches were made in an attempt to elucidate the mediators of this survival signal, and how this applies to organ systems in vivo. Whilst this thesis does not provide a complete picture of these mechanisms, it demonstrates that the growth hormone EPO is a potential therapeutic tool to lessen the degree of organ damage caused by ischaemia and other toxic insults.

The major findings of this thesis are:

- EPO administered either before the onset of ischaemia, or at the point of reperfusion, significantly reduced the degree of renal dysfunction observed in a standard model of severe ischaemia reperfusion in the rat by (i) biochemical analysis, (ii) preservation of urine volume and (iii) creatinine clearance.
• The reduction of functional injury was mirrored by preservation of (i) normal renal tubular architecture and (ii) tubular function and (iii) reduced markers of tubular and reperfusion injury.

• EPO reduced the activity of multiple caspases, and the quantity of apoptotic tubular cell death. Apoptosis was measured by the presence of classic histological changes of nuclear blebbing and condensation, and was accurately quantified by the use of immunohistochemistry with an antibody specific to the cleaved active fragment of the executioner caspase-3.

• The EPO-receptor was shown to be expressed on human proximal tubular epithelial cells by immunoblotting, and ligand binding and receptor activation was associated with a proliferative response and a dose-dependent increase in cell numbers. This response was dependent on the presence of the known EPO-receptor and its associated JAK2 kinase, as inhibition of expression by RNA interference completely abrogated the observed cellular responses.

• EPO activates several signalling pathways that contribute to the observed inhibition of apoptosis, including STATs and AKT. EPO was effective in preventing cell death in a number of injury models. In particular, cisplatin-induced apoptosis was utilized to show that EPO altered the development of oxidative stress and prevented PARP-1 activation. The effect on cisplatin induced injury was also confirmed in an animal model with improvements in biochemical and histological parameters at day five after a single dose of erythropoietin was administered as a pre-conditioning therapy.

• The final chapter of this thesis showed that EPO also had signalling effects and prevented cell death in cardiac cells in culture, and, importantly, significantly
reduced myocardial infarction size in a rat model of coronary artery occlusion. This demonstrated that EPO is functional in a number of cell types and organ systems.

These findings are important and act as compelling evidence to consider the extension of studies in clinical practice to the use of EPO in trials of acute kidney injury. Translation of early successes in animal studies to positive outcomes in human clinical trials in AKI has proven difficult, but EPO is an attractive agent, particularly as it is widely established as a highly effective therapeutic tool in the management of anaemia.

It is important to further elucidate the mechanism by which EPO exerts these observed effects. The development of EPO related peptides, and modified EPO molecules such as carbamylated EPO (CEPO), which appear to have similar effects to EPO on cell death in ischaemia reperfusion injury in several organ systems, have suggested that these effects are mediated by a different cell surface receptor. Future work to continue the work described in this thesis would include:

- Receptor interaction studies using fluorescent-labelled EPO receptor and CD131 to determine whether ligand binding with EPO and CEPO cause direct proximity and interaction of these receptor chains.

- Dose ranging studies to determine the optimum dose of EPO that is effective in animal models. This would allow for planning of human clinical trials with the smallest effective dose.
• Investigation of the regulation of EPO-receptor expression in health and post-injury. There is some evidence that EPO-receptor expression is upregulated early following ischaemia that might act to amplify the anti-apoptotic signal.

• Clinical studies in specific clinical areas with predictable degrees of renal injury, such as kidney transplantation, renal injury following cardiac surgery or contrast nephropathy.
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