FUNCTIONAL ROLE OF HAEM OXYGENASE-1 (HO-1) INDUCTION PRIOR TO HYPOTHERMIC STORAGE AND REPERFUSION IN RENAL TISSUE

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I dedicate this thesis to my Lord Jesus Christ,

for the strength He has given me throughout the years,

and to my dear husband (Lekan) and children (Genesis and LJ) for their support and encouragement. I would like to say a big thank to my sisters, my dad and Mama (Granma) for their help and prayers over the years.
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SUMMARY

In renal transplantation, delayed graft function and primary nonfunction are major sources of graft loss and morbidity. Since the shortage of donor organs is a major problem, it is imperative to develop methods capable of preserving organs after significant periods of warm and cold ischaemia, and at the same time minimise the reperfusion injury associated with transplantation. One approach is based on the stimulation of a specific inducible stress protein known as haem oxygenase-1 (HO-1), which may act by producing antioxidant, anti-inflammatory and cytoprotective molecules.

The aims of the present study were: 1) to establish methods for upregulation, identification and measurement of HO-1 in experimental models; 2) to investigate the role of HO-1 and its products, CO and bilirubin, in protecting renal tissues during hypothermic storage and subsequent reperfusion. 3) to search for novel “pharmacological preconditioning agents” that are capable of selectively activating the HO-1 gene in renal tissues. The effect of HO-1 induction on renal function by various inducers was investigated after hypothermic storage and reperfusion in a) an ex vivo system of isolated perfused kidney; b) an autograft transplant model; and c) in vitro cell culture. The results show that, following induction of HO-1 in renal tissue, the enzymatic activity was maintained during hypothermia, and some beneficial effects on renal function could be observed after storage and reperfusion. In cell cultures, novel inducers of HO-1 were identified which may prove useful in the future for the development of more effective preservation solutions.

In Chapter 1, a general introduction is given; methods are described in Chapter 2; experiments on the induction of HO-1 in renal tissue are reported in Chapter 3; in Chapters 4-6 results from studies on whole kidney perfusion in vitro and autotransplantation in vivo are presented; in Chapters 7 and 8, studies on novel inducers of HO-1 using renal cells in culture are reported; in Chapter 9 a general discussion is given.
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LIST OF ABBREVIATIONS

AMP       adenosine monophosphate
ATP       adenosine 5' triphosphate
Ca²⁺      calcium ion
cAMP      cyclic adenosine monophosphate
cGMP      cyclic guanidine monophosphate
CO₂       carbon monoxide
Fe²⁺      ferrous ion
Fe³⁺      ferric ion
γ-GT      gamma glutamyl transpeptidase
GFR       glomerular filtration rate
g         gram(s)
h         hour(s)
H⁺        hydrogen ion
H₂O₂      hydrogen peroxide
i.m.      intramuscular
i.p.      intraperitoneal
i.v.      intravenous
l         litre
M         molar
min       minute(s)
ml        millilitre
mmol      milimole
mol       moles
MW        molecular weight
n         number in a group
Na⁺       sodium ion
NADH      reduced nicotinamide adenine dinucleotide
NADP      oxidised nicotinamide adenine dinucleotide phosphate
NADPH     reduced nicotinamide adenine dinucleotide phosphate
nm         nanomoles
NO        nitric oxide
OD        optical density
O₂        oxygen gas
P         probability
pH        potential of hydrogen (acidity)
pO₂       oxygen partial pressure
sec       seconds
µl        microlitre
µM        micromolar
vs.       versus
w/v       weight/volume
1. INTRODUCTION

1.1 The Renal System

The kidney is the principal organ responsible for water and electrolytes homeostasis. The maintenance of homeostasis requires that any input into a system is balanced by an equivalent output. The urinary system provides mechanism by which excess water and electrolytes are eliminated from the body. Another major function of the urinary system is the excretion of toxic metabolite compounds such as urea and creatinine. The end product of these processes is known as urine.

1.2 Kidney Anatomy

A cross section through the kidney reveals that the organ is composed of two distinct regions, the cortex and the medulla (Figure 1.1). The cortex is the outer region that surrounds the inner region known as the medulla. The medulla is divided into conical regions, known as the renal pyramids: the base of each pyramid starts at the cortico-medullary border, and the apex ends in the papilla that merges to form the renal pelvis and then on to form the ureter. In humans, the renal pelvis is divided into two or three spaces- the major calyces-which in turn divide into further minor calyces. The walls of the calyces, pelvis and ureters are lined with smooth muscle that can contract to force urine towards the bladder by peristalsis.
1.2.1 The Nephron

The cortex and medulla are made up of nephrons, which are functional units of the kidney responsible for the actual purification and filtration of the blood. About one million nephrons are in the cortex of each kidney and each one consists of a renal corpuscle and a renal tubule that carry out the functions of the nephron. Two types of nephrons exist within the mammalian kidney, superficial cortical and juxtamedullary nephron. The superficial cortical nephrons are found in the cortex and have short loop of Henle. While, juxtamedullary nephrons arise from deep within the cortex next to the medulla, these nephrons tend to have long loop of Henle.
Two processes are performed in the nephron: osmoregulation and excretion. These processes involve the following steps: 1) Filtration of small molecules from blood plasma to form a filtrate; 2) selective reabsorption of water and other molecules from the filtrate, leaving excess waste materials to be excreted and 3) secretion of some excretory products directly from blood into the filtrate.

1.2.2 The renal corpuscle

The renal corpuscle in the nephron consists of two structures known as Bowman's capsule and glomerulus; these components are responsible for filtration of plasma. The Bowman's capsule contains the primary filtering device of the nephron, the glomerulus. Blood is transported into the
Bowman's capsule from the afferent arteriole (branching off the interlobular artery). Within the capsule, the blood is filtered through the glomerulus and then passes out via the efferent arteriole. Meanwhile, the filtered water and aqueous wastes are passed out of the Bowman's capsule into the proximal convoluted tubule. The glomerulus is a globular network of densely packed anatomising capillaries which invaginates the Bowan's Capsule. Water and soluble wastes pass through the semi-permeable membrane of the glomerulus, which is then excreted out of the Bowman's capsule as urine.

In the renal corpuscle, much of the water and small molecular weight constituents of plasma are filtered from the glomerular capillaries into the Bowman's space, and the glomerular filtrate passes into the renal tubule. Thus, the filtration barrier between the capillary lumen and Bowman's capsule space consists of capillary endothelium, the podocyte layer and their common basement membrane known as the glomerular basement membrane.

1.2.3 Renal Tubule

The renal tubule consists of three major part. It leaves the glomerular capsule as the elaborately coiled proximal convoluted tubule (PCT), makes a hairpin loop called the loop of Henle, and then winds and twists again as the distal convoluted tubule.

The collecting ducts, each of which receives urine from many nephrons, run through the medullary pyramids and give them their striped appearance. As the collecting ducts approach the renal pelvis, they fuse to form larger papillary ducts, which deliver urine into minor calyces via papillae of the pyramids.

The primary function of the tubule is the selective reabsorption of water, inorganic ions and other molecules from the glomerular filtrate. In addition, some inorganic ions are secreted directly from blood into the lumen of the tubule. The renal tubule has a convoluted shape and has four distinct zones, each of which has a different role in the tubular function.
1. The proximal convoluted tubule (PCT) is the longest, most convoluted section of the tubules and is responsible for reabsorption of approximately 75% of the ions and water, and glucose reabsorption into the peritubular capillary blood. PCT are confined to the renal cortex and make up the greater part of its bulk. The last part of the PCT, the pars recta, is straight and descends towards the medulla region becoming the loop of Henle.

2. The loop of Henle arises from the PCT as a straight thick walled tubule; here, it loops closely back upon itself abruptly becoming thicker walled (thick ascending limb) as it returns to the renal cortex. The length of the loop of Henle varies from short to long, depending of the location of the renal corpuscle of the particular nephron.

The corpuscle of the short-looped nephron tends to be located in the superficial and mid-cortical regions, with the loop extending very little beyond the cortico-medullary junction. Long-looped nephrons are mainly associated with juxtamedullary corpuscles; a small portion of the long loops almost reaches the tip of the renal papillae but successively greater numbers turn back at higher levels as necessitated by the tapering shape of the medullary junction.

The main function of the loop of Henle is to generate high osmotic pressure in the extracellular fluid of the renal medulla; the mechanism by which this is achieved is known as counter-current multiplier system.

The limb of the loop of Henle is closely associated with parallel wide capillary loops, known as the vasa recta, which arise from the efferent arterioles of glomeruli located, near the cortico-medullary junction. The vasa recta descends into the medulla and then loop back on themselves to drain into veins at the junction of the medulla and cortex.

3. The Distal convoluted tubule (DCT) is a continuation of the thick limb of the loop of Henle after its returns from the medulla. Shorter or lesser convoluted than the PCT, the DCT is responsible for reabsorption of sodium ions (Na+) by an active transport process which is controlled by the
adrenocortical hormone aldosterone. Sodium reabsorption is coupled with hydrogen (H+) and potassium (K+) ions into the DCT. Tubular reabsorption serves to facilitate the conservation of substrates which are essential to normal function.

4. The collecting tubule is the straight terminal portion of the nephron. Several collecting tubules converging to form a collecting duct. The collecting ducts descend through the cortex in parallel bundles called medullary rays progressively merging in the medulla to form a large duct of Bellini which opens at the tip of the renal papillae to discharge urine into the pelvicalyceal system. The collecting tubules and duct are not normally permeable to water; however, in the presence of antidiuretic hormone (ADH) secreted by the posterior pituitary, the collecting tubule and ducts become permeable to water which in turn is drawn out by osmotic pressure generated by the counter-current multiplier system, into the interstitial tissue of the medulla. From there water is returned to the general circulation via the vasa recta. The loop of Henle and ADH thus provide a mechanism for the production of urine which is hypertonic with respect to plasma.

1.2.4 Juxtaglomerular apparatus

One special ultrastructural feature of the nephron is the juxtaglomerular apparatus (JGA), which shows the inter-relationship between the distal tubule of a nephron and the renal corpuscle (Bowman's capsule and glomerulus). The JGA is comprised of an area of specialized epithelial cells in the thick ascending limb of the loop of Henle called the macula densa and specialized vascular cells known as mesangial cells at the afferent arteriole with a granular or secretory function. This is the region where the kidney controls the blood pressure. The granular cells secrete renin into the blood. This secretion is increased by a fall in Na+ load (i.e. a fall in [Na+]), prostaglandins, sympathetic nerve activity, circulating catecholamines and a fall in blood pressure. Renin secretion is decreased by a rise in chlorine ions [Cl-], angiotensin II, ADH and increased blood pressure. Blood pressure can
also be sensed by stretch receptors on the afferent arterioles. Another set of cells known as mesangial cells are positioned adjacent to the glomerular capillary basement membrane. Their part of their role is to contract and may modify the surface area of the capillaries available for filtration. The mesangial cells are also phagocytic and remove macromolecules that escape from the capillaries.

Figure 1.3  An illustration of the renal nephron adapted from Principles of Renal Physiology by Christopher J. Lote Third Edition

1.3 Renal Vasculature

Kidneys are well-supplied by blood vessels which benefit their important function in maintaining homeostasis (Figure 1.3). The entrance and exit of blood to each kidney is through a renal artery and renal vein which can be found alongside the ureter. The renal artery branches directly from the descending aorta and enters the kidney with the ureter at the renal pelvis. The renal artery in the kidney branches into the interlobar artery, the arcuate artery, the cortical radial artery and the afferent arteriole which flows into the glomerular capillaries. With the exception of a glomerular capillary system, the venous system has similar subdivisions, which
terminate as the renal vein leaves the kidney. A unique feature of the renal arterial blood supply is the flow through two series of capillary networks. These include the glomerular capillaries or the glomerulus and peritubular capillaries. Blood from the afferent arteriole enters the glomerulus and exit through the efferent arteriole. Unlike the peripheral capillary beds, the glomerular capillary bed begins and ends with an arteriole. Blood exiting the glomerulus through the efferent arteriole enters the peritubular capillaries, the second capillary network. Blood from the pertubular capillaries drains into a small venule which eventually joins the renal vein to enter the systemic circulation.

1.4 Renal processes

Urine formation and the simultaneous adjustment of blood composition involve three major processes:

1. Glomerular filtration
2. Tubular reabsorption
3. Tubular secretion

1.4.1 Glomerular Filtration

Urine formation begins with glomerular filtration. For the most part, filtration is a passive, non-selective process in which fluids and solutes are forced through a barrier by hydrostatic pressure. Urine formation begins with the process of glomerular filtration which is defined as the ultrafiltration of plasma water and its non-protein constituents from the blood in the glomerular capillaries into Bowman's capsule. The composition of the glomerular filtrate (or ultrafiltrate) is identical to plasma with respect to small molecules like amino acids, glucose and the electrolytes Na⁺, K⁺, HCO₃⁻, Cl⁻. The ultrafiltrate does not contain plasma protein, large molecules or any blood cells found in whole blood. The filtration barrier prevents the passage of large molecules and cells from blood in the glomerular capillaries.
into Bowman's capsule. The filtration barrier consist of three layers 1) the endothelium (endothelial cells of the glomerular capillaries), 2) the basement membrane, and 3) the epithelium of the Bowman's capsule.

The volume of plasma separated by ultrafiltration from the blood flowing through the glomerular capillary bed per unit time is known as the glomerular filtration rate (GFR).

1.4.1.1 Control of Glomerular Filtration Rate (GFR)

GFR normally is altered homeostatically to maintain a constant internal environment. If GFR is increased, then the elimination of water and solutes normally increases; in contrast, if GFR decreases then the elimination slows. GFR is controlled via three main mechanisms: a) intrinsic control (autoregulation), b) nerves (neural control) and c) hormones (renin-angiotensin system). Autoregulation occurs under ordinary conditions, glomerular pressure is regulated by the kidneys autoregulatory system. By adjusting its own resistance to blood flow, a process called renal autoregulation, the kidney can maintain a nearly constant glomerular filtration rate despite fluctuations in systemic arterial blood pressure.

Neural control may overcome autoregulation control of glomerular pressure during periods of extreme stress or emergency when it is necessary to shunt blood to other areas in the body and to reduce the loss of water and solutes through the renal system.

Hormones, such as the renin-angiotensin mechanism which will be described in detail below, respond to various stimuli. It also stimulates the adrenal cortex to release aldosterone which causes the renal tubules to reclaim more sodium ions from the filtrate.

1.4.2 Tubular Reabsorption

Reabsorption is a transepithelial process that begins as soon as the filtrate enters the proximal tubules. The tubule cells are connected by tight junctions
and, therefore, movement between cells is limited by the permeability of the tight junctions. The tight junctions in the proximal tubule are very permeable (termed leaky junctions) and are major avenues for passive water and solute transport. The tight junctions in the collecting ducts are impermeable to water and so any water transport that takes place has to be across the cells themselves. This is the movement of substances from the lumen of the renal tubule (across the epithelial cell) and into the peritubular capillaries (across the endothelial cell).

Tubular reabsorption serves to facilitate the conservation of substrates which are essential to normal function such as water, glucose, amino acids and electrolytes. The proximal tubule is the site where 65% - 85% of tubular reabsorption occurs. The nutrient glucose is reabsorbed from the proximal tubule into the peritubular capillary blood; as a result, valuable nutrients are not excreted into urine.

1.4.3 Tubular Secretion

Although urine formation occurs primarily by the filtration-reabsorption mechanism described above, an auxiliary mechanism, called tubular secretion, is also involved.

Secretion is the process by which substances move into urine in the distal and collecting tubule from the blood capillaries around these tubules. Substances secreted include H\(^+\), K\(^+\) and in addition some drugs are excreted by active transport out of blood into tubular urine. Organic cations and anions are also secreted from the peritubular capillaries into the lumen; this secretory mechanism enables the elimination of anions and cations from the body that are normally bound to plasma proteins and therefore not filtered to a great degree. Some examples of exogenous and endogenous anions and cations include organic anions prostaglandins, urate, oxalate, penicillin, saccharin, salicylates and organic cations acetylcholine, creatinine, epinephrine, histamine, norepinephrine and morphine.
The kidney can adjust the body’s acid-base balance to prevent blood disorders such as acidosis and alkalosis both of which can impair the function of the nervous system. For example, during the occurrence of acidosis, the kidney moves hydrogen ions to the urine through tubular secretion in the kidney. Hence, filtration reabsorption and secretion occur in different regions of the nephron to accomplish the function of urine formation as shown in Table 1.1 and Figure 1.4.

Table 1.1 Processes of urine formation

<table>
<thead>
<tr>
<th>PART OF NEPHRON</th>
<th>PROCESS IN URINE FORMATION</th>
<th>SUBSTANCES MOVED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerulus</td>
<td>Filtration</td>
<td>Water and solutes (e.g. Na⁺, glucose and other nutrients filtering out of glomeruli into Bowman’s capsules)</td>
</tr>
<tr>
<td>Proximal tubule</td>
<td>Reabsorption</td>
<td>Water and solutes</td>
</tr>
<tr>
<td>Loop of Henle</td>
<td>Reabsorption</td>
<td>Sodium and chloride ions</td>
</tr>
<tr>
<td>Distal and collecting tubules</td>
<td>Reabsorption, Secretion</td>
<td>Water, Na⁺, and Cl⁻ ions, Ammonia, K⁺ ions, H⁺ ions and drugs</td>
</tr>
</tbody>
</table>

Figure 1.4 Filtration of the blood in the fine structure of the kidneys. Image from Purves et al., Life: The Science of Biology, 4th Edition.
1.5 Hormones of the Kidney

1.5.1 Aldosterone

The hormone aldosterone produced by the adrenal glands interact with the kidney to regulate blood's sodium and potassium content. High amounts of aldosterone cause the nephron to reabsorb more sodium ions, more water, and fewer potassium ions; low levels of aldosterone have the reverse effect. The kidney response to aldosterone maintains the blood's salt level within the narrow range that is best for crucial physiological activities. Aldosterone also helps to regulate blood pressure. When the blood pressure starts to fall the kidney releases an enzyme called renin.

1.5.2 Renin-Angiotensin

Renin is a proteolytic enzyme secreted into the blood by the granular cells. It converts angiotensinogen into angiotensin I. An enzyme called angiotensin converting enzyme (ACE) converts angiotensin I to II. Angiotensin II is a powerful vasoconstrictor. It constricts the blood vessels and raises the peripheral resistance, thereby acting to restore blood pressure. Angiotensin II also increases the secretion of aldosterone leading to Na+ reabsorption.

1.5.3 Antidiuretic hormone (ADH)

The regulation of the amount of water contained in blood by the kidney is influenced by antidiuretic hormone (ADH), also called vasopressin. This hormone is produced in the hypothalamus and is stored in the nearby pituitary gland. Receptors in the brain monitor the blood's water concentration. When the amount of salt and other substances in the blood becomes too high, the pituitary gland releases ADH into the bloodstream. When it enters the kidney, ADH makes the walls of the renal tubules and collecting duct more permeable to water, so that more water is reabsorbed into the bloodstream.
1.5.4 Erythropoietin (EPO)

Several hormones are produced in the kidney including erythropoietin (EPO), a glycoprotein, which influences the production of red blood cells in the bone marrow. When the kidney detects that the number of red blood cells in the body is declining, it secretes erythropoietin. This hormone travels in the bloodstream to the bone marrow, stimulating the production and release of more red blood cells. Stimuli such as bleeding or moving to high altitudes (where oxygen is scarce) trigger the release of EPO.

1.5.5 Calcitriol

Vitamin D is absorbed into the body when eaten and transported to the skin for modification by sunlight radiation. The vitamin D obtained from animals is converted to what is called 7-dehydrocholesterol, while the plant substance becomes ergosterol. The animal origin hormone is the most metabolically active. The vitamin D precursor, 7-dehydrocholesterol, is modified in the liver (a hydroxyl group is added to the 25th carbon of the vitamin D3 molecule) thus forming 25-hydroxycholecalciferol. After this, ferol (often abbreviated 25-D3) circulates to the kidney for its final activation. Another hydroxyl group is added in the final activation to form 1,25 dihydroxycholecalciferol, also known as calcitriol.

Calcitriol has several important actions. It acts on the cells of the intestine to promote absorption of both calcium and phosphate, promotes release of calcium and phosphate from the bones where they are stored and causes the kidney not to excrete calcium and phosphate. In short, vitamin D is designed to increase the amount of calcium and phosphorus circulating in the bloodstream. Insufficient calcitriol prevents normal deposition of calcium in bone. In childhood, this produces the deformed bones characteristics of rickets; in adults, it results in weakness of bones known as osteomalacia.
1.6 Major Clinical Forms of Renal Disease

Diseases in the kidney range from mild infection to life-threatening kidney failure. The most common form of kidney disease is an inflammation of the kidney, called pyelonephritis.

1.6.1 Pyelonephritis

Pyelonephritis is inflammation of the renal pelvis, medulla and cortex. It often begins as a bacterial infection of the renal pelvis and then extends into the kidney itself. It can result from several types of bacteria, including Escherichia coli. Pyelonephritis can destroy nephron and renal corpuscles, but because the infection starts in the pelvis of the kidney, it affects the medulla more than the cortex. As a consequence, the ability of the kidney to concentrate urine is dramatically affected. Symptoms of pyelonephritis include fever, chills, and back pain. Antibiotics are usually given to fight the infection; if left untreated scarring will occur in the kidney and impair renal function.

1.6.2 Glomerular nephritis

Glomerular nephritis results from inflammation of the filtration membrane within the renal corpuscle. This condition may occur when the body's immune system is impaired. Antibodies and other substances form large particles in the bloodstream become trapped in the glomeruli. This results in inflammation and prevents the glomeruli from working properly. Glomerular nephritis is also characterized by an increased permeability of the filtration membrane and the accumulation of numerous white blood cells in the area of the filtration membrane. As a consequence, a high concentration of plasma proteins enters the filtrate along with numerous white blood cells. Plasma proteins in the filtrate increase the osmolarity of the filtrate, making urine volume greater than normal. Symptoms may include blood in the urine, swelling of body tissue, and the presence of protein in the urine, as determined by laboratory tests.
Glomerulonephritis often clears up without treatment. When treatment is necessary, it may include a special diet, immunosuppressant drugs, or plasmapheresis, a procedure that removes the portion of the blood that contains antibodies.

1.6.2.1 Acute glomerular nephritis

Acute glomerular nephritis often occurs 1 to 3 weeks after a severe bacterial infection such as streptococcal sore throat or scarlet fever. Antigen-antibody complexes associated with the disease become deposited in the filtration membrane and cause its inflammation. This acute inflammation normally subsides after several days.

1.6.2.2 Chronic glomerular nephritis

Chronic glomerular nephritis is long-term and usually progressive. The filtration membrane thickens and eventually is replaced by connective tissue. Although in the early stages chronic glomerular nephritis resembles the acute form, in the advanced stages many of the renal corpuscles are replaced by fibrous connective tissue, and the kidney eventually becomes non-functional.

1.6.3 Acute renal failure

Renal failure can result from any condition that interferes with kidney function. Acute renal failure occurs when kidney damage is extensive and leads to the accumulation of urea in the blood and acidosis. In complete renal failure, death occurs in 1 to 2 weeks. Acute renal failure can result from acute glomerular nephritis, or it can be caused by damage to or blockage of the renal tubules. Some poisons such as mercuric ions or carbon tetrachloride that are common to certain industrial processes cause necrosis of the nephron epithelium. If the damage does not interrupt the basement membranes surrounding the nephron, extensive regeneration can occur within 2 to 3 weeks. Severe ischaemia associated with circulatory shock resulting from sympathetic vasoconstriction of the renal blood vessels can also lead to necrosis of the epithelial cells of the nephron.
1.6.4 *Chronic renal failure*

Chronic renal failure results when so many nephrons are permanently damaged and those nephrons remaining functional cannot adequately compensate. Chronic renal failure can result from chronic glomerular nephritis, trauma to the kidneys, abscess of kidney tissue caused by congenital abnormalities, or tumours. Urinary tract obstruction by kidney stones, damage resulting from pyelonephritis, and severe arteriosclerosis of the renal arteries also cause degeneration of the kidney.

In chronic renal failure, GFR is dramatically reduced and the kidney is unable to excrete excess excretory products, including electrolytes and metabolic waste products. The accumulation of solutes in the body fluids causes water retention and oedema. Potassium levels in the extracellular fluid are elevated, and acidosis occurs because the distal convoluted tubules cannot excrete sufficient quantities of potassium and hydrogen ion. Acidosis, elevated potassium levels in the body fluids and the toxic effects of metabolic waste products cause confusion, coma, and finally death when chronic renal failure is severe.

Other common kidney disorders include kidney stones, which are small, crystallized substances, such as calcium, that form in the kidney or other parts of the urinary tract. Smaller kidney stones can pass out of the body on their own, although this can be painful. Larger stones may require surgery, or they may be broken into smaller pieces by a procedure known as ultrasonic lithotripsy.

The kidney may be harmed whenever injury or disease affects the rest of the body. For example diabetes mellitus (a disease caused by a malfunctioning pancreas that produces little or no insulin) can result in impaired blood flow through the kidneys. The bacteria that cause tuberculosis can travel from the lungs and infect the kidney. Injured muscles can release large amounts of protein into the bloodstream which may result in the destruction of red blood cells (haemolysis), blocking the nephron, drugs, including long-term of
some prescription medication as well as illegal drugs, can also cause kidney
damage.

1.6.5 Renal Replacement Therapy

When the kidneys fail, patients must have treatment otherwise they will die
within 3 months. There are approximately 32,000 kidney patients receiving
treatment in the UK. Approximately 50% undergo dialysis (see below) and
50% need a transplant, according to the National Kidney Research Fund
2000. Each year the percentage of new patients needing renal replacement
therapy increases by about 5%. Treatment of severe kidney disease may
include kidney dialysis, a procedure in which blood is circulated through a
machine that removes toxic wastes and excess fluid from the bloodstream.
Some patients use dialysis for a short time, while their kidneys recover from
injury or disease.

1.7 Haemodialysis and Peritoneal dialysis

Haemodialysis is usually carried out in hospital dialysis units (or in satellite
units for less dependent patients) where patients are attached to a machine
for 3-5 hours, three times every week. The machine is linked to the patient's
blood circulation and the blood is passed across a membrane (dialyser) where
the toxins pass through to the other side. Approximately 64% of patients are
currently undergoing haemodialysis treatment.

There are two types of Haemodialysis and Peritoneal dialysis. Haemodialysis
is when an artificial kidney known as a haemodialyser is used to remove
waste and extra chemicals and fluid from the body. During this procedure
patients are attached to a machine for 3-5 hours, three times every week.
Minor surgery is performed on the arm or leg, whereby an access into the
patient's blood vessels is created, to allow the flow of blood into the
haemodialyser. This access is made by joining an artery to a vein under the
skin to form a bigger blood vessel called a fistula. In situations where the
blood vessels are not adequate for a fistula, soft plastic tubes are used to join
an artery and a vein under the skin, this is known as a graft. Also occasionally, an access is made by the means of a narrow plastic tube, called a catheter, which is inserted into a large vein in the neck. This type of access may be temporary, but sometimes it is used for long-term treatment.

Peritoneal Dialysis (PD), is carried out by the patient at home using dialysis fluid, which is introduced into the body through a tube sited in the peritoneal cavity. This procedure needs to be carried out 3-4 times a day or sometimes overnight. During the treatment, the peritoneal cavity is slowly filled with a dialysate through the catheter and the extra fluid and waste products are drawn out form the arteries and veins in the peritoneal cavity. There are different types of peritoneal dialysis but the major ones are Continuous Ambulatory Peritoneal Dialysis (CAPD) or Continuous Cycling Peritoneal Dialysis (CPPD).

CAPD is the only type of dialysis that is done without a machine; usually the patient can do this by himself/herself at home or at work. The dialysate is usually in a bag which the patient connects to the catheter attached to the abdomen, and dialysate stays attached for approximately four to five hours before it drains back into the bag (a process known as exchange). While the dialysate is in the peritoneal cavity, the individual can go about their usual activities at work or at home.

CPPD is usually done at home using a special machine called a cycle. This is similar to CAPD except that a number of cycles (exchanges) occur. CAPD is more expensive and uses a machine to introduce and remove fluid, usually overnight, and is mainly used in children.

Although some patients respond well to dialysis, many do not. Children maintained on chronic dialysis do not grow at normal rates. Adults frequently give up their jobs and lose their roles as providers and heads of their families. For those patients who adapt poorly to dialysis, the only hope for a normal
life is a new kidney. In addition, longer waiting times on dialysis negatively impact post-transplant graft and patient survival (1).

1.8 Renal Transplantation
A surgical procedure to implant a healthy kidney into a patient with kidney disease or kidney failure is known as renal transplant (Figure 1.5). Donors can be from cadavers or volunteer blood-related donors; in Europe up to 85% of kidneys come from cadavers (2). The surgical procedure differs according to the age of the recipient. In adults, the renal graft is placed extraperitoneally in the iliac fossa, in small children it is placed retroperitoneally.

Figure 1.5 A schematic illustration of kidney transplantation

1.8.1 Current status of renal transplantation
In the year of 1999, 1,742 kidney transplant operations were performed nationally (UK and Ireland) although 5,056 people were actively waiting for a suitable organ for transplant. The
number of people waiting (as of 30 September 2000) has increased slightly since 1996, yet the number of transplant carried out during this period has reduced (3).

In the United States, kidney transplants are the second most common transplant operation with over 9000 cases per year (4). The patients are usually treated with dialysis until a kidney donor is found. The biggest problem facing patients awaiting transplant is the shortage of suitable organs available to meet the rise in demand.

The rise in demand is due to the fact that more patients survive renal disease long enough to need treatment. In addition to the growing requirement for organs, the need for a diversified donor pool continues to increase, as population projections for the coming decade include a larger proportion of racial and ethnic minorities. For kidney transplant, current tissue typing practices coupled with the greater likelihood that a match will come from a same-race donor are among the factors that contribute to longer waiting times for minorities.

Although there is a rise in demand, organ donation has remained low; hence, it is likely that the divergence between supply and demand continues. Organ donation has failed to increase due to an increase in HIV infection and a decrease in the number of deaths from motor accidents (5). Furthermore, the law requiring helmets for motorcyclists and imposing stricter penalties for drunk driving have reduced the number of deaths.

The overall waiting times for most organ types has increased with the longest median waiting times being for heart-lung and kidney registrants. The longer a patient with kidney disease remains on dialysis while waiting for a kidney transplant the more likely the patient is to die prematurely even after receiving a new kidney (1). Immediate transplant is the best treatment for kidney failure, but not all transplant grafts are successful.
1.8.2 Delayed Graft function

Delayed graft function (DGF) and primary non-function are major sources of morbidity, increased cost and graft loss after renal transplantation. Deficits in renal function occur due to the cumulative effects of cellular stresses that may be incurred prior the transplantation process. This procedure inevitably exposes the kidney to a period of warm and cold ischaemia. The insult to the kidney starts during the removal of the kidney from the donor and, if haemodynamic instability is present, then renal perfusion will be a compromised. After organ procurement, it takes time to perform tissue typing and cross matching: during this period, the organ is stored in preservation solutions kept on ice (cold ischaemia). The kidney is subjected to further cold storage while in transit from the donor to recipient. In addition, there is further injury occurring during vascular anastomoses between the allograft and the recipient (eventually leading to reperfusion).

After transplantation, renal function improves and later stabilizes in most recipients. Acute tubular necrosis (ATN) is one of the risks that may occur after transplantation; this happens when the graft develops progressive dysfunction after the initial few post transplant months and ultimately fails within a decade, despite the use of immunosuppressive drugs sufficient to prevent acute rejection.

Several factors, immunological and non-immunological, contribute to the final outcome of the renal graft. For example, inadequate immunosuppression, poor graft quality pretransplant, donor variability and the health of the recipient (diabetic or hypertension) can affect the success of the transplant.

Another major contributor to the eventual graft failure is the effect of cold storage and subsequent reperfusion of kidneys, commonly referred to as ischaemia-reperfusion injury (IRI). Ischaemia and reperfusion can be responsible for renal cell damage resulting either from hypoxia alone or the generation of reactive oxygen species (ROS) following reperfusion. It is well
known that ischaemic damage during kidney transplantation is responsible for a 20-30% of worldwide incidence of delayed graft function and may increase the incidence of acute rejection, as well as favour chronic transplant nephropathy (6;7).

1.9 Ischaemia and Reperfusion injury

Ischaemia is the cessation of blood flow or oxygen supply to an organ, while reperfusion involves the re-introduction of blood or oxygen. Reperfusion to a deprived area has significant deleterious effects, which is termed as ischaemia/reperfusion injury (IRI). Renal IRI involves a series of complex and interrelated biochemical pathways, resulting in injury to and the eventual death of renal cells due to necrosis or apoptosis.

Although reperfusion is essential for the survival of ischaemic tissue, reperfusion itself causes additional cell injury (reperfusion injury) which is attributed to calcium overload, neutrophil infiltration and the generation of reactive oxygen species (ROS) (8-10).

1.9.1 Ischaemia and renal tissue

The cellular damage sustained by the kidney during the process of transplantation has been shown to be influenced by cold ischaemia-reperfusion injury. Acute tubular necrosis (ATN) is known as the most common cause of early dysfunction. The frequency of ATN correlates with the degree of ischaemic damage and increases after prolonged cold storage or when the graft is retrieved from a non-heart-beating donor(11).

Ischaemia results in depletion of substrate required for cell metabolism, maintenance of cellular integrity and co-factors required for appropriate enzyme activity, plus it disables the cell from eliminating metabolic wastes. Within the kidney, this situation is particularly complex, as ischaemia itself can cause early irreversible damage, which appears to be mediated by ROS.
A series of cellular pathophysiological reactions occur during ischaemia, with the involvement of key elements briefly listed below.

1.9.1.1 Depletion Adenosine triphosphate (ATP)

Under normal physiological conditions, renal cells function primarily on aerobic metabolism; however, when cells become ischaemic due to lack of blood flow (perfusion) or oxygen, there is a rapid fall in adenosine triphosphate (ATP) levels and purine pool with consequent impairment of the energy status of the cell (10). During this event, oxidative phosphorylation is lost and reduction in ATP production occurs due to inhibition of the mitochondrial electron transfer. The Na/K ATPase pump is disabled; as a result, there is a free flow of calcium into the cell that destroys transmembrane gradients of most electrolytes, leading to cellular oedema and loss of membrane integrity. After these events, adenosine monophosphate (AMP), a product of ATP degradation, becomes the predominant nucleotide in the cells. The alteration in the redox state of mitochondrial respiratory chain promotes AMP catabolism leading to the accumulation of adenosine, inosine, and hypoxanthine.

1.9.1.2 Calcium

A failure in the function of ATP-dependent sodium/potassium and calcium pumps, which normally maintain high intracellular potassium levels, leads to a consequential sodium and calcium increase within the cell. Calcium influxes into the cell are further aggravated by the development of an acidic intracellular milieu that increases free calcium normally complexed at physiological pH. The excess of free calcium within the cells has many effects including activation of phospholipase A2 (PLA2) which disrupts the cell membrane, followed by mitochondrial damage. In addition, PLA2 activation leads to an alteration of the cytoskeleton with aberrant volume regulation and cellular oedema. Furthermore, PLA2 breaks down the cellular membrane phospholipids resulting in the release of free fatty acids. Subsequently, arachidonic acid (AA) accumulation is triggered in the ischaemic tissue,
enhancing the production of prostaglandins and leukotrienes as well as promoting oxygen free radical formation.

Arachidonic acid metabolites increase membrane permeability and oedema and promote vasoconstriction and platelet aggregation in the microcirculation. Furthermore, the excessive production of oxygen derived free radicals (OFR) or reactive oxygen species (ROS) can irreversibly damage protein and lipid components of the cellular membrane.

1.9.1.3 Reactive oxygen species (ROS)

Oxygen free radicals (ROS) are chemical molecules with one or more unpaired electrons. They possess a thermodynamic instability due to their electronic configuration which makes them highly reactive. In the kidney, ROS are mediated by various mechanisms, such as mitochondrial respiration, activated neutrophils, and xanthine oxidase activity. Both events might "prime" kidney for subsequent burst of ROS formation by resumption of mitochondrial respiration and stimulation of xanthine oxidase activity when oxygen is re-introduced during anastomosis. Furthermore, following reperfusion, neutrophils accumulate within the previously ischaemic tissue releasing large amounts of ROS.

The Fenton chemistry exemplifies the potential damaging effects originating by ROS in the presence of transition metals. This reaction was first described in 1876 by Fenton et al. who reported the oxidation of an organic compound (tartaric acid) in the presence of metals. The metal iron (Fe3+ to Fe2+) functions as a redox agent and Fenton catalyst (see Equation 1.1) hereby playing a central role in mediating reperfusion-associated oxidative tissue injury through its ability to catalyze hydroxyl radical formation from superoxide anion (O2•−) and hydrogen peroxide (H2O2).
Equation 1.1 The chemical pathway in the Fenton reaction

Furthermore, other inflammatory events occur in vivo upon reperfusion such as the circulation of activated polymorphonuclear neutrophils as a consequence of cytokine release, which results in overproduction of superoxide. The activated neutrophils adhere tightly to the capillary endothelium, thus mechanically blocking the blood flow a process known as the no re-flow phenomenon. In addition, there is vasoconstriction in microvasculature, due to a decrease in vasodilatory molecules during ischaemia, followed by the final outcome vascular congestion. When blood flow is re-introduced into the ischaemic tissue, the increased amount of hypoxanthine also leads to production of free radicals via xanthine oxidase and the Fenton reaction.

In summary, the excessive generation of ROS produced during ischaemia followed by reperfusion may lead to interaction with purines and pyrimidines and consequently causing DNA damage. Free radicals also promote sulfhydryl-mediated protein cross-linking generating disulfide bonds which inactivate functional and structural proteins essential for the survival of the cell. In addition, initiation of lipid peroxidation is also mediated by ROS, which alter the phospholipid balance and permeability characteristics of cellular membranes.

1.9.2 Reperfusion injury

Reperfusion occurs on the re-establishment of blood circulation; this either assists in the restoration of health or reversibly damages cells, but can also lead to cell death via apoptosis or necrosis. Upon reperfusion, ischaemic tissue is rapidly warmed and exposed to molecular oxygen. The accumulated
hypoxanthine is quickly oxidized to xanthine via the xanthine oxidase pathway which uses oxygen as an electron acceptor, yielding superoxide anion. A massive ROS release occurs via the Fenton reaction (Equation 1.1), known as the respiratory burst which leads to deleterious effects such as reperfusion injury.

1.10 Cellular and molecular responses to oxidative stress

In response to an increased production of ROS, various systems are induced to counteract the potential damaging effect of oxidative stress and restore cellular homeostasis. The defence system consists of various intracellular enzymes, free radicals scavengers and vitamins, collectively referred to as antioxidants. If during ischaemia/reperfusion injury the production of ROS overwhelms the antioxidant defence system, irreversible damage can occur and thus the development of cell dysfunction.
1.10.1 Antioxidants

An antioxidant as described by B. Halliwell (12) is a substance which when present at low concentrations compared to an oxidizable substrate significantly delays or prevents oxidation of that substance. The term “oxidizable substrate” includes almost everything found in living cells, including lipid, proteins, carbohydrates and DNA.

The primary function of an antioxidant is to scavenge ROS and help to attenuate the pro-oxidant state of the cell to maintain normal cellular homeostasis. Cellular antioxidant defence mechanisms can be classified into primary and secondary systems. The primary defences include familiar nutrients such as α-tocopheral (vitamin E), ascorbic acid (vitamin C), carotenoids (β-carotene, lycopene), thiols (glutathione, lipoic acid), ubiquinols, flavonoids and polyphenols (from herbs, teas, grape skins) and plasma proteins (albumin). Primary defence mechanisms are thought to interact directly with harmful ROS. Secondary defences are primarily involved in repair of already damaged proteins and lipids. The intracellular enzymatic systems acting as antioxidants include: 1) superoxide dismutase (SOD), which catalyses the conversion of superoxide to hydrogen peroxide; 2) catalase (CAT), which catalyses the reduction of hydrogen peroxide to water; and 3) ‘glutathione peroxidase’ (GP), which catalyses the reduction of organic hydroperoxide using reduced glutathione). Apart from these active enzymatic pathways that are constitutively expressed, there exists a battery of cytoprotective genes that can be selectively stimulated whenever cells sense an imbalance in the redox state and/or are under threat by increased oxidative stress. These inducible stress proteins, also known as “heat shock proteins”, constitute the secondary line of defence and can effectively intervene to counteract the negative effects mediated by ROS. The induction of stress proteins by to render the tissue more resistant to injurious metabolic processes has been recently viewed as a potential pharmacological strategy for the development of therapeutic approaches in the field of organ preservation and transplantation.
1.10.2. How to exploit the stress response to protect organs undergoing transplant

In the realm of clinical organ procurement and transplantation there is an urgent need to suppress ongoing injury from temperature-sensitive ischaemic mechanisms. As mentioned above, the increase in the expression of cytoprotective proteins may render tissues less susceptible to oxidant-mediated damage. This may be achieved by the manipulation of cellular metabolism through addition of drugs metabolites to the storage solution or by pharmacological preconditioning of the organ. This strategy may offer the potential for resuscitation of ischaemic kidneys before they are exposed to the rigors of normothermic blood reperfusion and for improving both immediate and long-term graft function. Ischaemia to the kidney triggers complex pathophysiologic responses, and the restoration of normal renal blood flow after an ischaemic insult may not prevent the development of processes that perpetuate tissue damage(10). Hence, it would be ideal to find a therapeutic approach to protect renal tissue from the damaging effects of ischaemia and reperfusion injury. One such approach might be the production of specific stress-response proteins to protect cells. Among these stress proteins, haem oxygenase appears to be an excellent candidate to provide tissues with beneficial effects.

1.1 Haem oxygenase

Haem oxygenase (HO), originally identified by Tenhunen et al. (13), is a microsomal enzyme involved in the degradation of haem. Cellular haem derived from ubiquitously disposed haem proteins such as cytochromes, nitric oxide synthases, haemoglobin, myoglobin and catalase is degraded by HO. This enzyme system oxidatively cleaves haem to biliverdin, forms carbon monoxide (CO), and releases iron (Fe) (Fig 1.7). Biliverdin is subsequently reduced to bilirubin by biliverdin reductase in the cytosol (14).

Two isoforms of haem have been extensively characterized (15): an inducible enzyme (HO-1) and a constitutive isoform (HO-2) (16). The primary structure
of HO·1 and HO·2 fragments of 91 amino acid residues show only 58% homology, but shares a region with 100% secondary structure homology.

A third isoform HO·3 (17) has been recently identified, shares 90% of the amino acid homology with HO·2 and is found in most organs, the function of this enzyme is still unknown and is currently under investigation.

Table 1.2 presents a summary of the three isoforms, HO·1, HO·2 and HO·3, which are products of separate genes (18;19). HO·1 is widely distributed in tissue and highly inducible in virtually all cells. HO·2 is constitutively active but not responsive to any of the inducers of HO·1 (18;20). Furthermore, HO·2 is mainly expressed in the brain and testes of mammals. In contrast, HO·3 is nearly devoid of catalytic capability and may function chiefly as a haem-sensing or a haem-binding protein (18).
Acetate + Glycine

Heme

Heme oxygenase

Biliverdin reductase

Bilirubin

Figure 1.7 The reaction sequence catalysed by Haem oxygenase. The final products of the reaction are CO, free iron and biliverdin which is converted into bilirubin.
Table 1.2 The Isozymes of Haem Oxygenase

<table>
<thead>
<tr>
<th></th>
<th>HO-1</th>
<th>HO-2</th>
<th>HO-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major function</td>
<td>Catalytic haem degradation</td>
<td>Catalytic haem degradation</td>
<td>Haem sensing/binding protein</td>
</tr>
<tr>
<td>Inducible</td>
<td>Highly inducible by many conditions</td>
<td>Constitutive</td>
<td>Uninducible</td>
</tr>
<tr>
<td>Localization</td>
<td>Ubiquitous, highest in spleen, liver and kidneys</td>
<td>Mainly in brain and testes</td>
<td>Ubiquitous, highest in the liver, prostate and kidneys</td>
</tr>
<tr>
<td>Molecular Mass (kD)</td>
<td>32-33</td>
<td>36</td>
<td>32-33</td>
</tr>
</tbody>
</table>

Muller and co-workers isolated and sequenced the rat HO gene (21). This gene consist of 6830 nucleotides and is organized into four introns and five exons. Blot hybridisation analysis of rat liver DNA suggested that there is a single gene for HO in rat genome. The promoter activity of its 5' flanking region was established in vitro using HeLa whole cell extract (21). Several potential binding sites for different transcription factors were found in this 5' flanking region. In addition, these same factors were found to regulate amino acid synthesis in yeast upon starvation, followed by subsequent expression of genes for heat shock protein and therefore, HO gene regulation seemed to be related to stress response.

Interestingly, the inducible isoform HO-1 has a heat shock regulatory element, and possibly many promoter elements. These elements are recognized by a variety of transcription factors, which once activated by the respective inducer cause transcription of the HO-1 gene. For example, consensus binding sites for nuclear factor-κB (NF-κB), activator protein-1 (AP-1), AP-2, and interleukin-6-responsive elements as well as other transcription factors have been reported in the promoter region of the HO-1 gene suggesting a potential role for these transacting factors in modulating HO-1 gene expression (22;23).

1.1.1 Heat Shock Response
A common reaction of cells to different forms of stress, such as oxidative stress, exposure to toxic compounds, heat and injury, leads to the production of heat shock proteins (Hsps). The induction of Hsps was first discovered in fruit flies following elevations in temperature (24;25). Cells given a non-lethal preshock at 42 °C subsequently survived an otherwise lethal exposure to 46 °C. The preshocked cells stopped the synthesis of the normal spectrum of proteins and began to synthesize HSP proteins at high level. Several studies have demonstrated that heat shock proteins produced in response to an initial stress seem to protect against subsequent unrelated stresses. Hence, after expression of the stress genes, cells become resistant to subsequent stresses. This phenomenon has been termed "crosstolerance." Furthermore, the expression of HSP proteins has been observed during clinical situations, under conditions of diminished oxygen delivery such as ischaemia (26). More importantly, hyperthermia-induced heat shock proteins provide protection from ischaemia/reperfusion injury in organ transplantation (27).

Since the expression of HSPs is part of the cellular defence system protecting cells from harmful conditions, the regulation of their expression may be useful as a preventative measure prior to surgical manipulations, such as organ transplantation.

1.1.2 Haem oxygenase-mediated protection

The inducible isoform (HO-1) is susceptible to up-regulation in different tissues by a diversity of stress-related agents and conditions, including ultraviolet radiations (28), hypoxia (29), ischaemia-reperfusion (30), nitric oxide (NO) donors (31-33), peroxynitrite (33) (34), and various haemoglobins (35) (36).

Heavy metals (lead, gold, nickel, mercury, lead, arsenic, platinum) have also been shown to induce haem oxygenase activity, this was first reported by Maines and Kappas in 1974 (37). For example, cobalt chloride induced hepatic haem oxygenase activity by up to 45-fold with a single dose of 250μg/kg (38). Another group, demonstrated that haem oxygenase activity in
the left and right ventricles and the right atrium of rabbit hearts increased after cobalt treatment to 135,135 and 200% of the control levels, respectively (39). In fact, numerous studies have shown that the potency of haem oxygenase induction varies considerably from one tissue to another (40).

Furthermore, ample evidence suggests that HO-1, the inducible isoform, possess cytoprotective effects therefore functions as an "antioxidant" molecule. The mechanism by which HO-1 mediates this protective function is believed to by via its by-products carbon monoxide, biliverdin and bilirubin. The proposed antioxidant role of HO-1 is based on some crucial experimental observations: 1) HO-1 gene expression is extremely sensitive to up-regulation by oxidative stress in a variety of mammalian tissues (41;42); 2) induction of HO-1 protein or transfection of cells with the HO-1 gene protects tissue against oxidant-mediated injury (43); (44) and 3) knock-out mice exhibit reduced stress defences when exposed to oxidative challenge (45). Hence, increasing evidence suggest that, in the various pathophysiological states, the HO pathway is a powerful protective cellular system.

1.1.3 Carbon monoxide

Carbon monoxide is a well-known toxic agent that interferes with oxygen delivery to cell and tissues. In fact, this gaseous molecule has a very high affinity for haemoglobin and once present in high amounts in the blood stream can severely prevent oxygen transport to tissues leading to the toxic effect of hypoxia. Therefore, it is not surprising that high concentrations of this poisonous molecule are lethal; however, several studies shed a new light on this deadly molecule.

Several studies have shown that CO at low concentrations mediates important physiological and cellular functions and exerts protective effects. Small amounts of CO are capable of stimulating guanyl cyclase (GC) to produce the second messenger cyclic guanyl monophosphate (cGMP) in a reaction that resembles the well-known activation of GC by nitric oxide (NO),
another important signalling molecule (46). The activation of GC by CO and NO is a common mediator in modulating smooth muscle cell relaxation and vasodilatation (47). Motterlini and co-worker reported that HO-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses in vivo, elegantly demonstrating the crucial role of the haem oxygenase/CO pathway in the regulation of blood pressure under stress conditions (48). Synder and colleagues demonstrated that CO generated from HO in the human body can regulate vasomotor tone as well as neurotransmission (49). These findings provided the first evidence that this simple gaseous molecule could impart critical biological functions similar to NO. Induction of HO-1 and the consequent increase of CO in vascular tissue has been shown to modulate vessel relaxation and this effect can be distinct from the one mediated by NO (50). Although the vasodilatory function mediated by CO is not as potent as the one elicited by NO, CO is chemically more stable and thus can accumulate in the cell with a longer half-life and might promote a long-lasting effect (47).

Furthermore there is evidence to suggest that the regulatory role of CO accounts for the anti-inflammatory effects of HO-1 expression in endothelial and smooth muscle cells. Interestingly, HO-1 homozygous null (−/−) mice exhibit a maladaptive response to chronic hypoxia, with the development of right ventricular infarcts and organised mural thrombi resulting in hypertension (51). This data suggest that chronic deficiency of HO-1 leads to severe vasoconstriction in the vessels resulting in hypertension. Therefore, CO may afford protection by inhibiting platelet aggregation (52) via cGMP production which promotes vasodilatation. In addition, low-dose of inhaled carbon monoxide reduces pulmonary vascular resistance during acute hypoxemia in adult sheep (53). Finally, our group recently reported on a series of transition metal carbonyls, termed carbon monoxide-releasing molecules (CO-RMs), that have the ability to liberate CO to elicit direct biological activities (54). Our group believes that these transition metal carbonyls could be utilized for the therapeutic delivery of CO to alleviate vascular- and immuno-related dysfunctions in the future.
1.1.4 Bilirubin/Biliverdin

Biliverdin is the most abundant endogenous antioxidants in mammalian tissue, accounting for the majority of the antioxidant activity in human serum (55). In the brain, bilirubin acts as a potent antioxidant by scavenging peroxyl radicals. In addition, bilirubin has been shown to be as efficient as α-tocopherol or vitamin E (56).

On the contrary, bilirubin is best known as a potential toxic agent that accumulates in the serum of neonates, causing jaundice. However, neonatal jaundice can also have positive protective effects on new-borns delivered into a non-sterile environment (57). In high concentrations, bilirubin accumulates in selected regions of the brain to elicit neurotoxicity associated with kernicterus (58).

Interestingly, administration of biliverdin to rats modulates lipopolysaccharide-induced P- and E-selectin expression in the vascular system, illustrating the ability of bilirubin as a modulator of inflammatory response regardless of the influence of HO-1, CO, and/or ferritin (59). Furthermore, administration of biliverdin provides protection in a rat model of ischaemic heart injury (57). In addition, our group obtained similar results by administration of bilirubin in another model of rat ischaemic heart injury (60).

In addition, the reduction product of biliverdin formed by the activity of biliverdin reductase (61) (62), scavenges oxygen free radicals and is as effective as the potent antioxidant tocopherol (63), (64), (65). In fact, it has been documented that in man, higher serum levels of bilirubin is associated with decreased risk for early familial coronary artery disease (66).

Finally, bilirubin and biliverdin do not only serve as potent antioxidants but are also well known to inhibit the inflammatory response (67). Bilirubin has been shown to inhibit interleukin-2 (IL-2) stimulated killing activity of
lymphocytes (68), IL-2 production and antibody-dependent and independent cell-mediated cytotoxicity (69).

1.1.5 Iron

Iron produced by the HO pathway is rapidly sequestered into the iron storage protein known as ferritin. At physiological levels, iron balance is maintained by homeostasis. This process requires a combination of transcriptional and post-transcriptional mechanisms responsible for the proteins involved in the transport, cellular uptake and fate of iron.

Iron in haem is necessary for the transport, binding, and release of oxygen; the ready availability of iron for incorporation to haem is essential to organismal survival. Iron is also essential for the function of enzymes that participate in numerous critical cellular processes, including the cell cycle, the reductive conversion of ribonucleotides to deoxyribonucleotides, electron transport, and others. Ferritin, by capturing and "buffering" the intracellular labile iron pool, plays a key role in maintaining iron homeostasis.

Iron released by HO activity regulates several genes, the iron storage protein ferritin, the iron transport protein transferrin, NO synthase and HO-1 (70;71) (72;73).

It is well established that only loosely bound iron ("free" iron) not transferrin-bound iron, can function as a catalyst. Iron is a potent catalyst for oxygen radical formation and lipid peroxidation (74). Iron has the ability to donate electrons for the generation of the superoxide radical and can participate in the generation of hydroxyl radicals via the Fenton reaction (Equation 1). The toxicity of iron in cellular systems is attributable in large part to its capacity to participate in the generation of such reactive species, which can directly damage DNA, lipids, and proteins, leading to profound cellular toxicity.

However, the potential toxic effect is reduced when iron is sequestered into ferritin complex, rendering iron catalytically inactive. Such sequestration
lowers the pro oxidant state of the cell. This was supported by Balla et al. (75) who demonstrated that induction of ferritin was cytoprotective in a model of oxidant stress, demonstrating that cytotoxicity was greatly reduced and occurred independently of HO-1 activity. Vile and Tyrrell (76) showed that ferritin levels increase in the presence of oxidative stress such as ultraviolet irradiation. The HO-1-dependent release of iron also resulted in the upregulation of ferritin, which might provide protection after irradiation. Eisenstein et al. (77) clearly showed that ferritin is increased in correlation with HO-1 and decreased with inhibition in HO-1 activity. Finally, Otterbein et al. (78), in a model of endotoxic shock, demonstrated that when iron is chelated by the exogenous iron chelator desferoxamin, no ferritin is induced and protection is ablated.

Several studies have shown that a decrease in the iron content in cells expressing HO-1 accounts for the anti-apoptotic effect seen in cerebellar granular neurons (79) and cultured fibroblasts (80-81). Other molecules that chelate intracellular free iron are also thought to prevent apoptosis, suggesting that upregulation of ferritin by HO-1 may have a similar effect in preventing apoptosis.

1.2 Oxidative Stress Response and Preconditioning

HO-1 plays a role in modulating tissue responses to injury in pathophysiologic states and a lot of attention has been focused on the biological antioxidant, anti-inflammatory and anti-apoptotic properties of its by-products. Therefore, the common conception that products of haem degradation are merely eliminated from tissues as toxic wastes has been disputed by recent evidence demonstrating that CO, bilirubin and ferritin act as crucial effector molecules. HO-1 induction is an adaptive and beneficial response to a wide variety of oxidant stimuli including ultraviolet radiation, haem, hydrogen peroxide, cytokines, growth factors, heavy metals and nitric oxide (82). Recent studies have shown that HO-1 induction prior to transplantation in the liver (83){Kato, Buelow, et al. 1999 1630 /id} and heart,
reduces graft rejection. Also in renal models it has been shown that HO-1 ameliorates nephrotoxic nephritis (84) and produces resistance against glomerular inflammation in rats (85).

So far there has been no clear evidence on the role of HO-1 induction prior to warm and cold ischaemia followed by renal transplantation. The induction of this stress protein may represent a protective response to oxidative stress during ischaemia/reperfusion injury; overexpression of HO-1 causes induction of ferritin synthesis which may confer cytoprotection by its iron-sequestering capability; production of carbon monoxide, an anti-apoptotic, anti-inflammatory and vasodilatory molecule. Production of biliverdin and bilirubin as powerful anti-oxidant and anti-inflammatory agents may also contribute significantly to the protective effects of HO-1 induction. Therefore, it is possible that "preconditioning" the kidney with a suitable agent which is specific for the induction of HO-1 may protect renal tissue subjected to prolonged hypothermic storage against ischaemia/reperfusion injury post transplantation.
1.3 Hypothesis

The hypothesis being tested is whether HO-1 induction prior to ischaemia-reperfusion in the kidney provides better preservation during cold storage and improves renal function at reperfusion.

1.4 Aims of the Project

The aims of this project are:

To establish methods for upregulation, identification and measurement of haem oxygenase-1 (HO-1) in experimental models.

To investigate the role of inducible haem oxygenase (HO-1) and its products, CO and bilirubin, in the protection of renal tissue during hypothermic storage and maintenance of post-ischaemic renal vascular function.

To search for novel “pharmacological preconditioning agents” able to selectively activate the HO-1 gene in renal tissues.
2. MATERIALS AND METHODS

2.1 Materials

All rabbit renal tissue were harvested from female New Zealand White rabbit (2.5-6 kg) supplied by Highgate Farms Lincolnshire UK. Rat kidney and heart were taken from Sprague Dawley rats, raised in the SPF (specific pathogen free) Unit of Northwick Park Hospital.

Anaesthetics used were: Hypnorm (Janssen Pharmaceuticals Ltd, Oxford, UK): diazepam (Phoenix Pharmaceuticals Ltd, Gloucester, UK).

2.1.1 Reagents

All the reagents used, unless otherwise stated were obtained from Sigma Chemical Company (Poole, Dorset, UK). Hemin (ferrisprotoporphyrin IX chloride) was purchased from Porphyrin Products Inc. (Logan, Utah, USA). Monoclonal and polyclonal HO-1 antibodies were from Stressgen (Victoria, Canada). Anti-Nrf2 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The electron microscope was a JEOL 1200EX STEM and the inverted light microscope was an Olympus CK.

2.1.2 Scientific instruments

The peristaltic pump was model 505U (Watson Marlow UK). The polygraph recorder Grass Model 7D and pressure transducer Model P23XL were supplied by Astra Med Inc. (UT, USA). The spectrophotometer was a Helios α (Unicam, USA), Versamax tunable microplate reader (Molecular Devices, California USA), biological oxygen monitor YSI Model 5300 (Yellow Spring Instruments, Ohio USA), cooled Incubator (Sanyo, Model MIR-153).
2.2 Surgical Procedure and Anaesthesia: kidney harvest techniques

2.2.1 Animals

Female New Zealand white rabbits (2-4 kg) supplied by Highgate Farm U.K and fed on a maintenance diet were utilized for all experiments. The animals were administered with hemin or vehicle. These experiments were carried out under licenses issued by the Home Office (UK) under the Animals (Scientific Procedures) Act, 1986.

2.2.2 Cold-storage/Preservation Solution

A modified University of Wisconsin Solution (UW) solution as used in the North Thames Regional Organ Retrieval program was used. The UW solution was produced and provided by the Royal Free Hospital pharmacy. The composition of the solution was as follows (w/v): 3.861% Lactobionic Acid, 0.585% Potassium Hydroxide B.P, 0.014% Allopurinol, 0.355% Potassium Acid Phosphate B.P, 0.262% Magnesium Sulphate B.P, 1.860% Raffinose Pentahydrate, 0.096% Glutathione (Reduced), 0.154% Sodium Hydroxide B.P, to 100% water for injection.

2.2.3 Nephrectomy

Kidneys were harvested under general anaesthesia induced by Hypnorn (fentanyl, 0.315 mg ml⁻¹, and fluanisone, 10mg ml⁻¹) at 0.3 mg kg⁻¹ bodyweight intramuscularly followed 5 minutes later by diazepam at 1 mg kg⁻¹ bodyweight intravenously. Anaesthesia was maintained with Hypnorn via an intravenous saline drip. Oxygen was administered at 0.5 L min⁻¹ via a face mask throughout the operative procedure. Intraoperative monitoring was carried out by means of an oximeter in conjunction with blood gas and arterial blood measurements. Both kidneys were exposed through a midline incision and carefully freed from peritoneal tissue and fat. Immediately after a kidney was harvested, the renal artery was cannulated and the blood was flushed out manually with 30 ml of UW solution at 4°C. The kidneys were
weighed and prepared (for the various protocols described above) or placed in a further 500 ml of UW solution at 4°C immediate use, 24 or 48 h cold-storage preservation.

2.3 Renal Mitochondria Isolation

Kidneys were harvested from female New Zealand White Rabbits (2-4kg) following the protocol described below. The kidneys were immersed in a high EDTA isolation medium (210 mM mannitol; 70 mM sucrose; 50 mM Tris; 10 mM EDTA (dipotassium salt); pH 7.2). All procedures were carried out at 4°C and the mitochondria were isolated from the cortex using the method described by (Sammut et al., 2000).

The tissue was finely minced and washed using isolation medium to remove blood and vascular tissue and homogenised using a glass-teflon Potter-type homogeniser. The crude homogenate was suspended in 50ml of isolation medium and centrifuged at 1000g for 10 minutes to eliminate the nuclear component. The supernatant was centrifuged at 10000g for an additional 10 minutes to obtain the first mitochondrial pellet, which was resuspended low EDTA buffer (225 mM mannitol; 72 mM sucrose; Tris 10mM; 100 μM EDTA) using a glass-teflon homogeniser. The homogenate was the made up to 50ml with low EDTA buffer and centrifuge at 10000g to obtain a pure mitochondrial pellet. Protein concentrations were determined using the Bio-Rad DC Protein Assay (Bo-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.).

2.3.1 Oxygen Consumption in the Mitochondria

The measurement of oxygen consumption rates was performed using a one ml capacity incubation chamber with a water jacket and a Clark electrode (Yellow Springs Instruments Co., Ohio, U.S.A.) fitted into the top of the chamber. The incubation medium was stirred constantly using an electromagnetic stirrer and magnetic stirring bar. The oxygen consumption studies were conducted at 30°C in respiration medium consisting: 100 mM
KCl: 75 mM mannitol; 25 mM sucrose, 10 mM phosphate Tris; 10 mM Tris-HCl and 50 μM EDTA; pH 7.4. Approximately 0.5 mg of mitochondrial protein (20 μl) and 0.5 mg fat-free BSA (10 μl) were incubated in a total volume of 1 ml. The concentrations of substrates used were 5mM glutamate (monopotassium salt) and 5mM malate (sodium salt) or 10 mM succinate (Tris Salt) and 5 μM rotenone.

State 3 respiration was initiated 1 min later by the addition of 300 nmol ADP (10 μl) to produce a final reaction.

Preparations stimulated by the addition of ADP were assessed for respiratory control by their ability to return to State 4 respiratory rate when all the ADP had been phosphorylated to form ATP (Chance & Williams, 1956). The post-ADP stimulated State 4 rate was used to represent all references to the State 4 parameters. The respiratory control index (RCI), defined as the ratio of the State 3 respiration rate to the recovery rate of State 4 respiration, provides a measure of the degree of coupling of mitochondria electron transport to ATP synthesis.

### 2.3.2 Measurements of Mitochondrial Complex Activity

Maximal mitochondrial complex activities were assayed spectrophotometrically at 30°C using an Uvikon model 940 (Kontron Instruments Ltd., Watford) in mitochondrial samples lysed by three cycles of freeze thawing after storage at -80°C. Complex I activity was measured as the rotenone sensitive rate of NADH oxidation (at 340 nm and 30°C) using a modification of the method of (Ragan et al., 1987). The reaction mixture contained: 25 mM potassium phosphate pH 7.2; 0.2 mM NADH; 10 mM MgCl₂; 1 mM KCN; 2.5 mg of fat-free BSA and mitochondrial samples (approximately 20 μg) in a final volume of 1ml. The reaction was initiated by the addition of CoQ1 (50 μM final volume) and read at 340 nm against a blank containing all the components except CoQ1. After five minutes, 10 μl
of 0.5 mM rotenone was added to the cuvettes and the inhibited rate measured for a further 5 minutes.

Complex II-III activity was measured as the antimycin A sensitive rate of cytochrome c reduction (at 550nm and 30°C) using the method of King (1967). The reaction mixtures contained: 100 mM potassium phosphate pH 7.4; 0.3 mM potassium EDTA; 1 mM KCN, 100 μM Cytochrome c and mitochondrial sample (approximately 20 μg) in a final volume of 1ml. The reaction was initiated by the addition of 20 μl of 1.0 M succinate. After 5-7 minutes of running the assay, 10 μl of antimycin A was added to test cuvettes and the inhibited rate measured for a further 5 minutes.

Mitochondrial Complex IV activity was measured as the rate of cytochrome c oxidation (at 550nm and 30°C) using the method of Wharton and Tzagoloff (1967). The reference cuvette contained: 10 mM potassium phosphate pH 7.0; 50 μM reduced cytochrome c and 1 mM potassium fericyanide in a final volume of 1 ml. The test cuvette contained: 10 mM potassium phosphate pH 7.0 and 50 μM reduced cytochrome c. The reaction was initiated by the addition of mitochondrial sample to the test cuvette. The first order constant (k) was calculated from the difference between the natural logarithms of the absorbance at t=0 and at three time point, 1, 2 and 3 minutes after adding the mitochondrial sample. The Mean of these calculated values was then taken to be (k) and the activity was expressed in k/min/mg protein.

Complex V activity by was measured as the oligomycin-sensitive rate of NADH oxidation at (340 nm and 30°C). The reaction mixture contained: 50 mM Tris/HCl buffer pH 8.0; 5 mM phosphoenol pyruvate; 2 mM KCN; 0.3 mM NADH, 100 mM KCl; 6 mM MgCl₂, 10 μM rotenone, 25 U/ml lactate dehydrogenase, 25 U/ml pyruvate kinase and mitochondrial sample (5-20 μg) in a final volume of 1ml. All components are added to both sample and reference cells. The reaction was initiated by the addition of 20 μl of 300 mM ATP to the sample cell. After the stimulated rate was measured for 8-10 minutes, 10 μl of oligomycin (5 μg/ml preparation in ethanol) was then
added to the test cuvette and the assay was left to run for a further 8·10 minutes.

2.4 Perfusion Circuit

2.4.1 Perfusion Solution

The constituent of the Krebs buffer served as the rabbit serum and the Hespan was used as the major colloid for maintenance of onconic pressure. The composition of the Krebs buffer solution was as follows (in mmol/liter) adjusted to pH 7.40: 155 Na⁺, 139 HCO₃⁻, 6.8 K⁺, 139 Cl⁻, 1.2 Mg²⁺, 2 Ca²⁺, 500ml DMEM (DULBECCO'S MOD EAGLE MEDIUM), 500ml Hespan by Fresenius (composition per litre: Hetastarch Hydroxyethyl Starch 60.0g/L, NaCl 90g/L pH 5.5, osmolarity approximately 3.10 mOsm/L) 100ml Gelofusine a plasma substitute (4g of protein/100ml) for protein excretion measurements, 900ml distilled of distilled water, 1g/litre of Creatinine for determination of glomerular filtration rate (GFR).

2.4.2 Isolated Perfused Rabbit Kidney System

After harvesting or at the end of the hypothermic storage, kidneys were weighed (8·10g) and perfused via the renal artery with warmed (37°C) and continuously gassed (95% O₂-5%CO₂) freshly prepared Kreb's buffer. The perfusion system (Plate 2.1) was constructed from Silicon tubing (1.6mm O/D, Watson Marlow) based on a system described for the ex vivo assessment of kidney function (86) with a recirculating volume of 1.5 litre. The solution was perfused in a closed thermostatically controlled circuit with the assistance of a peristaltic pump (Watson Marlow 505U) and the renal arterial pressure was maintained at 100mmHg by the hydrostatic pressure of a buffer column (HUGO SACHS ELECTRONIK). Arterial pressure was monitored with a pressure transducer (Model P23XL OHMEDA) attached to the side-arm of the renal artery cannula and the data was amplified (Grass Model 7D polygraph).
Thirty minutes after initiating perfusion, when a steady state had been achieved, urine and perfusate samples were collected at 10 minutes intervals from the ureter (Plate 2.2) and renal vein over an hour; for the determination of perfusion flow rate (PFR), glomerular filtration rate (GFR), gamma glutamyl transpeptidase activity, fractional reabsorption of sodium (FrNa), glucose and protein leakage.
Plate 2.1  Photograph of the isolate perfused rabbit kidney system
2.5 Renal Function Parameters

2.5.1 Glomerular Filtration Rate (GFR)

The GFR was calculated from urine and perfusate creatinine concentrations measured by the assay developed by Bartels et al. in 1972 by the relationship described below. Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed was directly proportional to the creatinine concentration.

\[
GFR = \frac{\text{Urine [creatinine] (mM) \times Urine flow (m/min)}}{\text{Perfusate [creatinine] (mM)}}
\]

GFR is the measure of the amount of blood filtered by the kidney and is an accurate gauge in rabbit kidney as shown by comparable results to inulin clearance over a range of plasma concentrations of both compounds.
Secretion of creatinine by the kidney tubules, which occurs in man, guinea pigs and rats, is not significant in rabbits.

2.5.2 Reabsorption of sodium and glucose

Tubular solute reabsorption was calculated as follows:

Filtered load = perfusate [solute] (mM) x GFR (ml/min)
Excreted load = urine [solute] (mM) x urine flow (ml/min)

Sodium (Na+) is the major cation ion of the extracellular (outside of the cell) fluids and the concentration inside the cell is only about 3.5% in comparison. The sodium content of the blood is a result of a balance between dietary intake and renal excretion; hence sodium is the major determinant of extracellular osmolality. Sodium was determined enzymatically via sodium dependent β-galactosidase activity with ONPG (O-nitrophenyl galactosidase) as substrate. The absorbance at 405 nm of the product ONPG was proportional to the sodium concentration and was measured using a biochemical analyser COBAS MIRA (ROCHE) (87) and the reabsorption was calculated as described above.

2.5.3 Proteinuria

Proteinuria was measured by comparison of the known protein concentration entering the renal vasculature (4g/L) with that of any protein appearing in the urine. Protein concentration were measured spectrophotometrically using the method of Lowry et al with bovine serum albumin (BSA) as a standard, the results were expressed as a percentage of the perfusate protein concentration.

2.5.4 Gamma-glutamyl transpeptidase

γ-glutamyl transpeptidase (γ-GT) is a lysosomal enzyme MW140,000, rich in tubular cell. It serves as evidence of tubular injury when found at high
concentration in serum or urine. The concentration of the enzyme was measured in urinary and vascular effluents using the biochemical analyser COBAS MIRA (ROCHE) according to the method of (88) for the $\gamma$-GT. The $\gamma$-GT catalyses the transfer of glutamic acid to acceptors like glycylglycine in this case, this process releases 4-amino-2-nitobenzoate which absorbs light at 405 nm. The increase in absorbance at this wavelength is directly related to the activity of $\gamma$-GT.

2.6 Histology: Electron Microscopy

2.6.1 Transmission Electron Microscopy

Tissue sections (approximately 3nm$^3$) from the cortex and medulla were cut from the kidney using a skin-graft blade and were placed in a solution of gluteraldehyde (3%) in phosphate buffer (0.1M) at room temperature for 2 h. They were then stored at 4°C until processed (up to 2 days). Specimens were removed from the fixing solution and washed in 0.1M phosphate buffer 3 times allowing 10 min for each wash. Tissues were then placed in a 1% solution of osmium tetroxide in 0.1M phosphate buffer for 60 min at room temperature. A further wash (0.1M phosphate buffer) was given prior to dehydration.

The fixed tissue sections were placed in acetone increasing in concentrations: from 25%-50% then to 75% and finally to 100%. Twenty minutes were allowed for each stage until specimens reached the 100% stage where they were given 4 changes of the liquid, each lasting 20 minutes.

Dehydrated specimens were placed in a 1:1 mixture Araldite/acetone in glass tubes overnight on a rotating mixer, without lids on the tubes to allow evaporation of the acetone. The specimens were placed in Araldite on a rotating mixer for 2 h then the process was repeated with fresh Araldite.

Specimens were placed in fresh Araldite and placed in oven 60°C over-night. Section were cut from the specimens embedded in araldite using an ultratome and were then stained with osmium tetroxide before mounting on standard copper grids for histological examination using transmission electron microscopy. Specimens were blindly examined and scored by cell-type on a scale of 0 to 3 (Table 2.1)
Table 2.1: Scoring system for histology analysis

<table>
<thead>
<tr>
<th>Score</th>
<th>Severity of Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>+</td>
<td>Minor</td>
</tr>
<tr>
<td>++</td>
<td>Moderate</td>
</tr>
<tr>
<td>+++</td>
<td>Severe</td>
</tr>
</tbody>
</table>

The cell types or structures examined were:

(i) In the cortex: proximal tubule, distal tubule, glomerulus, glomerular basement membrane, cortical mitochondria

(ii) In the medulla: medullary thick ascending tubule, mitochondria

2.7 Light Microscopy

The kidney samples were fixed in 10% formal saline for processing to paraffin wax, sections and stained with haematoxylin and eosin (H & E) for light microscope analysis.

Histopathological evaluation was carried out by histopathologist blinded to the experimental procedures. In each kidney section, a total of sixty high power fields (HPF) was examined for morphological changes in four histological regions of the kidney: cortex (proximal convoluted tubules), outer stripe of outer medulla (mainly the straight portion of the proximal convoluted tubules), inner stripe of outer medulla (mainly the thick ascending loops of Henle) and inner medulla (collecting ducts). These morphological changes included tubular cell vacuolisation, necrosis as manifested by nuclear pyknosis, karyorhexis and/or disruption of cell membranes and intratubular cell detachment. The extent of tubular damage was graded as mild if the above criteria were observed in < 1/3 of the tubule, moderate if affecting up to 2/3 of the tubule and severe if > 2/3 of the tubule.
shows evidence of tubular cell injury or necrosis. The total number of

tubules in one HPF was counted and structural damage was expressed
quantitatively as the percentage of damaged tubules / field of view.

2.8 Haem oxygenase Activity Assay

The original method for the measurement of haem oxygenase activity was
described by Tenhunen et al. (89). The modification of this, described by
Motterlini et al. (90), was used.

2.8.1 Preparation of liver microsomal fraction

Male Sprague-Dawley rats (250-300 g) were anaesthetised using fentanyl-
fluanisone (0.7 ml/kg i.m.) and diazepam (2.5 mg/kg i.p.). A mid-line
laparotomy was performed and the hepatic vein was isolated. This was
cannulated with a 22G catheter (Venflon®, BOC Ohmeda AB, Helsingborg,
Sweden) and the descending aorta was divided. The animal was perfused
with 50 ml of cold saline (0.9% NaCl) at 4 °C. Perfusion of the liver was
confirmed by blanching of the organ as blood was washed out. The liver was
removed, weighed, transferred to a 10 ml Potter-Elvehjem glass-teflon
homogeniser (Jencons Scientific Ltd., Leighton Buzzard, Bedfordshire, U.K.)
on ice, containing 5 volumes of cold buffer (0.25 M sucrose; 50 mM Tris·HCl;
pH 7.4; 4 °C) and finely homogenised at 2000-3000 rpm using an electric
rotor (Glas-Col®, Terre Haute, Indiana, U.S.A.). The homogenate was
transferred to a centrifuge tube and centrifuged at 27 000g, 4 °C for 20 min
in a swing-bucket rotor centrifuge (LE-80 Ultracentrifuge, Beckman
Instruments Inc., Palo Alto, California, U.S.A.). The supernatant was
removed, transferred to a clean tube and centrifuged at 105 000g, 4 °C for 90
min. The supernatant was discarded and the microsomal pellet was
resuspended in 1 ml of cold buffer (0.1 M KH$_2$PO$_4$; 2 mM MgCl$_2$; pH 7.4).
The protein concentration of the microsomal fraction was determined using
a commercial kit (Bio-Rad DC Protein Assay, Bio-Rad Laboratories Ltd.,
Hemel Hempstead, Hertfordshire, U.K.), based on the method originally
CHAPTER 2

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described by (Lowry et al., 1951). Aliquots of the microsomal fraction were transferred to 1.5 ml tubes and stored at -80 °C until needed.

This microsomal fraction was used in the biochemical assay for haem oxygenase activity. It provided biliverdin reductase to allow the conversion of biliverdin to bilirubin, the absorption spectrum of which is easier to measure. An excess of biliverdin reductase was used in each assay so that the only limiting factor was the concentration of haem oxygenase.

2.9 Biochemical activity assay

2.9.1 Preparation of rabbit kidney microsomal fraction

Kidneys were harvested under general anaesthesia induced by Hypnorm (refer to nephrectomy section) and flushed with cold UW solution. The kidney was weighed, transferred to a 10 ml Potter-Elvehjem glass-teflon homogeniser (Jencons Scientific Ltd., Leighton Buzzard, Bedfordshire, U.K.) on ice, containing 5 volumes of cold buffer (0.25 M sucrose; 50 mM Tris·HCl; pH 7.4; 4 °C) and finely homogenised at 2000-3000 rpm using an electric rotor (Glas-Col®, Terre Haute, Indiana, U.S.A.). The homogenate was transferred to a centrifuge tube and centrifuged at 12 500g, 4 °C for 10 min in a swing-bucket rotor centrifuge (LE-80 Ultracentrifuge, Beckman Instruments Inc., Palo Alto, California, U.S.A.). The supernatant was removed, transferred to a clean tube and centrifuged at 25 000g, 4 °C for 90 min. The supernatant was discarded and the kidney microsomal pellet was resuspended in 1 ml of cold buffer (0.1 M KH₂PO₄; 2 mM MgCl₂; pH 7.4). The samples was then stored in -80°C until measurements of haem oxygenase activity were performed.

2.9.2 Preparation of LLC-PK1 cell microsomal fraction

Following experimental incubation, porcine renal epithelial proximal tubule cells (LLC-PK1) were washed twice with 10 ml cold PBS (4 °C) and manually scraped from the bottom of the culture flask in 5 ml cold PBS.
using a policeman (Thomas Scientific Inc., Swedesboro, New Jersey, U.S.A.). The flask was washed with a further 5 ml cold PBS and the 10 ml total was transferred to a 14 ml centrifuge tube. This was centrifuged at 10 000g, 4 °C for 10 min in a swing-bucket rotor centrifuge (LE-80 Ultracentrifuge, Beckman Instruments Inc., Palo Alto, California, U.S.A.). The supernatant was discarded and the pellet was resuspended in 600 µl of cold buffer (100 mM KH₂PO₄; 2 mM MgCl₂; pH 7.4; 4 °C). The sample was then frozen at -80 °C until required for measurement of haem oxygenase activity or for 20 min, if activity was to be measured immediately.

### 2.9.3 Measurement of haem oxygenase activity

The frozen microsomes isolated from the LLC-PK1 cell were then thawed in a water bath at 37 °C for 5 min and returned to the freezer at -80 °C for 20 min. This was repeated twice and after the third thawing samples were sonicated (Ultrasonic Cleaner ME 4.6, Mettler Electronics Corp., Anaheim, California, U.S.A.) in ice-water for 15 sec, to ensure that cellular disruption was complete. In the case of the microsomes isolated from the rabbit kidney, the samples were allowed to defrost at room temperature.

One hundred microlitres of the sample was taken for protein determination. Protein concentration was determined using a commercial kit (Bio-Rad DC Protein Assay, Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.).

The following reagents were pre-warmed in a water bath at 37 °C for 5 min and then mixed in the order given in a 4 ml glass tube on ice:

1 mg/ml of microsomes plus
0.1 M KH₂PO₄ with 2 mM MgCl₂ total volume of 700 µl
2.3 mg liver microsomal fraction
50 µl 40 mM glucose-6-phosphate
5 µl 50 U/ml glucose-6-phosphate
20 µl 1 mM hemin
20 µl 40 mM NADPH

Each tube was vortexed briefly, sealed with Parafilm "M" (American National Can™, Greenwich, Connecticut, U.S.A.) and wrapped in aluminium foil to exclude light to prevent degradation of NADPH and bilirubin. The samples were incubated in a water-bath at 37 °C for 60 min. The reaction was terminated by the addition of 1 ml of chloroform and the tube was thoroughly vortexed. Samples were centrifuged in a swing-bucket rotor, bench-top centrifuge (MSE Mistral 2000, Thermo FI, Crawley, Surrey, U.K.) at 500g, room temperature for 10 min. Tubes were then briefly vortexed to just disturb the layers and centrifuged again as before. The lowest (chloroform) layer was transferred to a 1 ml quartz cuvette. Absorbance at 464 nm and 530 nm was determined against a blank containing only chloroform, using a spectrophotometer (Uvikon 810P Spectrophotometer, Kontron Instruments Ltd., Watford, Hertfordshire, U.K.). Haem oxygenase activity was calculated from the difference in absorbance at 530 and 464 nm (extinction coefficient, e = 40 mM·1 cm⁻¹) using the following equation:

\[
\frac{\text{AOD}}{40 \times 10^6} = \text{pmoles bilirubin / mg protein / h in 400 µl cell suspension}
\]

2.10 RNA Isolation

2.10.1 Maintenance of an RNase-free environment

In order to preserve an RNase-free environment the following precautions were taken: all glassware was cleaned and baked at 200 °C for 6 hours; all plastic ware was new and used specifically for RNA extraction; and all water was treated with diethyl pyrocarbonate (DEPC). To treat water with DEPC, distilled water was poured into previously baked glass bottles and diethyl pyrocarbonate (DEPC) was added (0.5 ml/litre). The water was vigorously shaken and left in an oven at 40 °C for 24 hours. The water was then autoclaved at 121 °C for 25 min to eliminate any residual DEPC.
The method used for RNA extraction for solid tissue was based on that originally described by Chomczynski & Sacchi (1987).

2.10.2 RNA extraction from Kidney Tissue

Kidney sections were collected and snap-frozen in liquid nitrogen (-170 °C). Samples were stored at -80 °C, until further processing. The frozen tissue was crushed and ground to a powder under liquid nitrogen using a mortar and pestle. The ground tissue was immediately added to 2 ml of GT buffer. Further processing of solid tissue and cell culture samples was the same, as described below.

Samples were transferred to 12 ml tubes and left on ice for 5 min. Then, 0.2 ml of 2 M sodium acetate (pH 4.5) was added, followed by 2.5 ml phenol (pH 4.3; saturated with 0.1 M citrate buffer) and finally 0.4 ml chloroform-isoamyl alcohol (in the ratio of 49:1 v/v). Tubes were then vortexed thoroughly to mix all layers and left on ice for 15 min. Samples were centrifuged at 10 000g for 20 min at 4 °C in a bench-top centrifuge (Avanti 30 Centrifuge, Beckman Instruments Inc., Palo Alto, California, U.S.A.). The top, aqueous layer was transferred into a clean tube and an equal volume (~ 2 ml) of cold (-20 °C) 2-propanol was added. The tube was inverted three times to mix the aqueous layer with the 2-propanol and then left for 2 hours at -20 °C. Samples were centrifuged at 10 000g for 30 min at 4 °C. The supernatant was discarded and the pellet resuspended in 300 µl GT buffer. To this, 300 µl of cold 2-propanol was added. The tube was inverted three times to mix the contents and left at -20 °C for 1 hour. Samples were centrifuged at 10 000g for 30 min at 4 °C. The supernatant was discarded and the pellet was washed by adding 500 µl of 75% ethanol. The tube was vortexed briefly to loosen the pellet and once again centrifuged at 10 000g for 15 min at 4 °C. The washing step was repeated and the sample re-centrifuged. The supernatant was discarded and the pellet was left to air-dry by inverting the tube and leaving it on the bench top for 30 min. The RNA pellet was dissolved in (20-60 ml) DEPC-treated water.
RNA concentration in each sample was determined spectrophotometrically. One ml of sample was diluted in 99 ml of DEPC-treated water and the absorbance against a DEPC-treated water blank was determined at 260 and 280 nm using a spectrophotometer (Helios a, Unicam Ltd., Cambridge, U.K.). Total RNA concentration of the sample was calculated according to the following equation:

$$\text{OD}_{260} \times 4 = \text{RNA mg/ml}$$

The quality of the RNA was determined by the ratio:

$$\frac{\text{OD}_{260}}{\text{OD}_{280}}$$

To check the quality of the RNA, 2 μl of the sample were mixed with 5 μl of loading dye (15% w/v Ficoll 400; 20% v/v glycerol; 0.5% w/v bromophenol blue; 0.5% w/v xylene cyanol) and loaded into the wells of a 1% agarose gel (1 g agarose in 100 ml 1x TBE buffer). Electrophoresis was performed in 1x TBE buffer (90 mM Tris; 90 mM boric acid; 2 mM EDTA; pH 8) containing ethidium bromide (0.5 mg/ml) at 90 V until the bromophenol blue marker band had migrated a distance of 5 cm. The gel was inspected using an ultraviolet transilluminator (UV Dual-intensity Transilluminator TM 20, UVP, Genetic Research Instrumentation Ltd., Dunmow, Essex, U.K.) to check the quality of the RNA. Photographs were taken when necessary using a Polaroid DS 34 camera with a DS H-8 hood (Polaroid UK Ltd., St. Albans, Hertfordshire, U.K.) and Polaroid film (Type 667) with a No. 15, deep yellow filter.

2.10.3 Northern Blot Analysis

This method was adapted from that published by Tyrrell & Basu-Modak (1994). Total RNA (10-20 μg/lane), extracted as above, was taken and 10 μl of denaturing buffer (1x MOPS buffer; 2.2 M formaldehyde; 50% v/v deionised formamide; 0.5 mg/ml ethidium bromide) added. The sample was heated in a water-bath at 65 °C for 10 min to denature the RNA. It was cooled rapidly on ice for 2 min and 2 μl of loading dye (50% v/v glycerol; 0.4% w/v bromophenol blue; 0.4% w/v xylene cyanol; 1 mM EDTA) was added.
Samples (10-20 μg RNA/lane) were loaded on a 1.3% denaturing agarose gel (1.3 g agarose-LE (Ambion Inc., Austin, Texas, U.S.A.) per 100 ml, 1x MOPS buffer, 2.2 M formaldehyde) which had been pre-run at 120 V for 10 min. Electrophoresis was performed in 1x MOPS buffer (20 mM 3-(N-morpholino)propanesulfonic acid; 5 mM sodium acetate; 1 mM EDTA; pH 6.0) containing ethidium bromide (0.5 μg/ml) at 120 V, until the bromophenol blue marker band had migrated a distance of 5 cm from the wells (Fig. 2.4.1). The gel was checked on an ultraviolet transilluminator (UV Dual-intensity Transilluminator TM 20, UVP, Genetic Research Instrumentation Ltd., Dunmow, Essex, U.K.) before being rinsed briefly in DEPC-treated water and then washed in Rinse Solution (75 mM NaOH; 100 mM NaCl) for 45 min on an orbital shaker, followed by a wash in 100 mM Tris-HCl (pH 7.5) for 60 min. The gel was blotted overnight by upward capillary action onto a positively charged nylon membrane (GeneScreen™, NEN® Research Products, Du Pont de Nemours & Co. Inc., Boston, Massachusetts, U.S.A.) using phosphate buffer (25 mM Na₂HPO₄; 25 mM NaH₂PO₄; pH 6.5). Transfer was checked by viewing the membrane and the gel on an ultraviolet transilluminator. The membrane was baked at 80 °C for 30 min, wrapped in Saran™ (Dow Chemical Co., U.S.A.) and stored at -20 °C until hybridisation.

The membrane was first prehybridised using 0.1 ml/cm² Rapid-hyb buffer (Amersham International Plc., Little Chalfont, Buckinghamshire, U.K.) for 1 hour at 65 °C in a rotisserie (SI 20H Hybridisation Oven/Shaker, Stuart Scientific Co. Ltd., Redhill, Surrey, U.K.). cDNA probes to the rat and human HO-1 genes (both kindly donated by Professor Shibahara, Tohoku University, Japan) were labelled with Redivue™ [α-32P]-dCTP (Amersham International plc., Little Chalfont, Buckinghamshire, U.K.) using a commercial nick translation kit (Boehringer Mannheim UK Ltd., Lewes, East Sussex, U.K.). Unincorporated nucleotides were removed using a commercial kit (QIAquick™ Nucleotide Removal Kit, Qiagen Ltd., Dorking, Surrey, U.K.). The labelled probe was added to the pre-hybridisation buffer.
and the membrane was hybridised for 24 hours at 65 °C in a rotisserie. The membrane was then washed in 2x SSC (0.3 M NaCl, 30 mM sodium citrate; pH 7.0)/0.1% w/v SDS for 20 min at room temperature; 1x SSC/0.1% SDS for 15 min at 65 °C and 0.1x SSC/0.1% SDS for 15 min at 65 °C in a rotisserie. The membrane was then laid down against radiographic film (BioMax MR, Eastman Kodak Co., Rochester, New York, U.S.A.) at 80 °C and exposed for 3-7 days. The film was developed and the image was scanned using a densitometer and the accompanying imaging software (Model GS-700 Imaging Densitometer and Molecular Analyst respectively, Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.).

The membrane was then re-probed for a control gene (either GAPDH or β-actin). The membrane was first stripped of the previous probe by heating in 0.1x SSC/0.1% SDS for 60 min in a boiling water bath. Prehybridisation and hybridisation, using cDNA probes for either rat GAPDH gene (kindly donated by Dr. S. Mohammed, Royal Free Hospital Medical School, London) or human β-actin gene (kindly donated by Hazel Truman, RAFT Institute of Plastic Surgery, Mount Vernon Hospital, Northwood, Middlesex, U.K.), were performed as previously described.

To determine changes in HO-1 gene expression, the densitometric value obtained from the film probed for HO-1 was normalized to its respective value from the film probed for the control gene (rat GAPDH or human β-actin), as described in Tyrrell & Basu-Modak. Briefly, the densitometric values for the control gene were averaged and the actual control gene value for each sample was divided by this mean to get a ratio. The actual densitometric value for the HO-1 gene of each sample was then multiplied by its calculated ratio to arrive at a corrected value for the HO-1 mRNA level. This was done to prevent variations in the loading of RNA samples onto the gel or in the spectrophotometric quantification of RNA from causing apparent changes in HO-1 gene expression.
2.11 Western Blot Analysis

Kidneys were homogenized according to the method described by (Ishizuka et al., 1997) in 10 volumes of 20mM Hepes (pH 7.5), 2 mM EDTA, 25mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM orthovandate, 1% Tween 20, 1 mM phenyl methylsulfonyl fluoride, 10 μg/ml aprotinin and centrifuged at 8,000 x g for 10 min at 4°C, and the supernatants were used as tissue extracts.

A 100 ml aliquot was taken for protein determination using a commercial kit (Bio-Rad DC Protein Assay, Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.). Samples were then diluted in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8; 10% v/v glycerol; 2% w/v SDS; 2.5% v/v b-mercaptoethanol; 0.06% w/v bromophenol blue) to a concentration of 1 mg/ml and heated at 90 °C for 10 min using a dry heating block (Thermostat 5320, Eppendorf, Netheler-Hinz-GmbH, Hamburg, Germany). Two hundred μg of protein for each sample was loaded onto a 12 cm 15% w/v SDS-polyacrylamide resolving gel with a 4% w/v SDS-polyacrylamide stacking gel. Electrophoresis of the samples was performed in running buffer (1x SDS PAGE Tank Buffer “Electran”, BDH Merck Ltd., Poole, Dorset, U.K.) at 180 V, until the bromophenol blue marker band had migrated the length of the gel, using a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.). The gel was washed in transfer buffer (running buffer containing 20% v/v methanol) and proteins were electrophoretically transferred onto a nitrocellulose membrane at 30 V, room temperature for 16 hours. Non-specific antibody binding was blocked with 3% w/v non-fat dried milk containing 0.05% v/v Tween 20 for 2 hours at room temperature on an orbital shaker. The membrane was washed in PBS (10 mM NaHPO₄; 138 mM NaCl; 2.7 mM KCl; pH 7.4) for 5 min before being probed with mouse anti-HO-1 monoclonal antibody (1/1000 dilution in TBS (50 mM Tris; 138 mM NaCl; 2.7 mM KCl; pH 7.4)) for 2 hours at room temperature. The membrane was then washed three times in PBS containing 0.05% v/v Tween 20 (PBST) before being incubated for 1 hour.
with biotinylated rabbit anti-mouse IgG antibody (DAKO) (1/1000 dilution in TBS). The membrane was washed three times in TBS prior to incubation for 1 hour with 1/1000 dilution of ExtrAvidin alkaline phosphatase (Sigma-Aldrich Co., Poole, UK). The membrane was washed again three times in PBST before incubation in substrate solution (0.1 M Tris-HCl, pH 8.2 containing 20 ml/ml of 20 mM naphthol AS-BI phosphate in dimethylformamide and 1 mg/ml Fast Red RC salt). The membrane was finally washed in distilled water before being scanned using a densitometer and the accompanying imaging software (Model GS-700 Imaging Densitometer and Molecular Analyst respectively, Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.).

2.12 Preparation of nuclear extract

LLC-PK1 cells were washed twice with PBS (1X). Cells were then harvested in 1 ml PBS and centrifuged at 3,000 rpm for 3 min at 4 °C. The cell pellet was carefully resuspended in 200 µl of cold Buffer A consisting of 10 mM HEPES (pH=7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 µM DTT, and complete protease inhibitor cocktail (Roche, Mannheim, Germany). The pellet was then incubated on ice for 15 minutes to allow the cells to swell. After this time, 15 µl of 10% NP-40 was added and the tube was vortexed for 10 s. The homogenate was then centrifuged at 3,000 rpm for 3 min at 4 °C. The resulting nuclear pellet was resuspended in 30 µl of cold Buffer B consisting of 20 mM HEPES (pH=7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 µM DTT, and protease inhibitors. The pellet was then incubated on ice for 15 min and vortexed for 10-15 s every 2 min. The nuclear extract was finally centrifuged at 13,000 rpm for 5 min at 4 °C. The supernatant containing the nuclear proteins was loaded on a SDS-polyacrylamide gel and Western blot analysis as described above.
2.13 Cell culture

2.13.1 Culture and maintenance of cell lines

Porcine renal tubular epithelial cells known as LLC-PK1 cells (86121112) and rat kidney epithelial cells (NRK-52E) were purchased from American Tissue Culture Collection (Manassas, VA, USA), these cell line (Plate 2.3) was grown in specific culture medium, outlined in Table 2.2.

![Plate 2.3 Phase-contrast light micrograph of renal proximal epithelial cells (magnification: x 400)](image_url)

<table>
<thead>
<tr>
<th>Basal medium:</th>
<th>Minimal Essential Medium</th>
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<tr>
<td>Foetal Bovine Serum:</td>
<td>10 % (v/v)</td>
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<tr>
<td>Additives:</td>
<td>100 U/ml penicillin</td>
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<tr>
<td></td>
<td>0.1 mg/ml streptomycin</td>
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<td></td>
<td>2mM L-Glutamine</td>
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Cells were grown in 75 cm² tissue culture flasks (Costar, Cambridge, MA) and maintained in an incubator at 37°C in an atmosphere of 5% CO₂ balanced with air.

2.13.2 Preparation of cell culture reagents

Stock solutions or reagents were prepared freshly every day just prior to cell culture incubations. Stock solutions of both curcumin and CAPE were prepared by dissolving the compounds in ethanol.

2.13.3 Incubating cells with agents

Before each experiment, cells were washed with 10 ml DPBS at 37°C to remove cell debris, detached cells or unwanted chemical factors in the culture. Treatment agents were prepared as described in Section 2.13.2 and diluted in culture medium to the required final concentration for incubation with cells. 10 ml of incubation media was added to each flask which were returned to the incubator for the time-course of the experiment.

At the end of the incubation period the medium was aspirated and cells washed 2 x with 10 ml ice cold DPBS. Cells were then scraped from the flask in 5 ml DBPS with a plastic policeman (Thomas Scientific, Swedesboro, NJ) before being washed into a 12 ml centrifuge tube and centrifuged for 5 min at 1000 x g. The supernatant was discarded and the cell pellet re-suspended in a suitable volume of buffer (500μl PBS containing 2 mM MgCl₂ for haem oxygenase activity or 300μl PBS containing 1% (v/v) Triton X-100 for Western blot).

2.13.4 Cell viability assay: Lactate Dehydrogenase (LDH) Release

After treatment of cells with various agents, the supernatant was collected from each well and stored at 4°C. Cell monolayers were treated with lysing solution (1% Triton X-100) for 30 minutes at room temperature to lyse the cell membranes, and the lysate was collected. LDH activity was measured in
both the supernatant and the cell lysate fractions by using Cytotoxicity Assay Kit (Sigma-Aldrich Co., Poole, UK) following the manufacturers instruction. The procedure for determination of LDH is based on the reduction of pyruvate to lactate. The following reaction is catalysed by LDH:

$$\text{LDH}$$

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{Lactate} + \text{NAD}
\]

During reduction of pyruvate an equimolar amount of NADH is oxidized to NAD. The oxidation of NADH results in a decrease in the absorbance at 340nm. The rate of decrease in absorbance at 340nm is directly proportional to lactate dehydrogenase activity in the supernatant or cell lysate. The percentage of LDH release from the cell was determined using the formula:

\[
\text{Release (\%)} = \frac{\text{LDH activity in supernatant}}{\text{(LDH activity in supernatant + LDH activity in lysate)}}.
\]

2.13.5 Cell metabolism assay: the alamar blue method

Another method of determining cellular viability is the alamar blue cell viability assay (Serotec, UK). The alamar blue assay incorporates a fluorometric/colourmetric growth indicator based on detection of metabolic activity; however, the indicator used in the alamar blue assay both fluoresces and changes colour in response to chemical reduction of growth medium resulting from cell growth. Cells were treated as described in Section 2.13.1. The alamar blue stock solution was diluted 1:10 (v/v) according to the manufacturers' instructions in culture growth medium; 200 \(\mu\)l transferred into each well and left to incubate for 5 h in the incubator or until a colour change was seen. After incubation the absorption at 570 and 600 nm was read in a microtitre plate reader (VersaMax, Molecular Devices, UK). Relative metabolic activity was determined by subtracting the background absorbance measured at 600 nm from the absorbance at 570 nm.
2.14 Statistical Analysis

Differences among the groups were analysed by using one-way ANOVA (one-way analysis of variance) combined with the Bonferroni test. Values were expressed as a mean $\pm$ S.E.M. (standard error of the mean) and differences between groups were considered to be significant at $p < 0.05$. (ANOVA), determines the differences between means of groups, based on one independent variable. Interestingly, ANOVA determines if there are differences between groups, but does not describe these differences. This problem was solved or isolated by using multiple comparison procedures such as the Bonferroni analysis. This test compares each mean against all others and uses a stricter criterion for significant differences between groups.
3. EFFECT OF HEMIN, A POTENT INDUCER OF HAEM OXYGENASE-1 (HO-1), ON HEART AND KIDNEY MITOCHONDRIAL FUNCTION IN THE RAT

3.1 Introduction

The mitochondria are the main site of aerobic respiration, the major energy production centre in eukaryotes, responsible for the control of respiration and maintenance of cellular homeostasis. In this process, glucose is broken down in the cell's cytoplasm to form pyruvic acid, which is transported into the mitochondria. In a series of reactions, part of which is called the citric cycle or Krebs cycle, the pyruvic acid reacts with water to produce carbon dioxide and then hydrogen atoms. The protons and electrons obtained from the hydrogen atoms are eventually used for the production of adenosine triphosphate (ATP), through a series of oxidation-reduction steps. The ATP is transported to the cytoplasm of the cell, where it is used for virtually every energy-requiring reaction it performs.

The mitochondrial structure (Figure 3.1A) provides compartmentalization of mitochondrial metabolism and the outer membrane surrounds the central matrix space of the mitochondrion (Figure 3.1B). The outer membrane provides a permeability barrier to large cytosol molecules and separates the intermembrane space from the cytosol. The inner membrane consists of regions of inner boundary membrane that is parallel to the outer membrane. Portions of inner membrane invaginate into the matrix to form cristae, sites for ATP synthesis and electron transport chain. The electron transport chain contains four large protein-based Complexes that work in sequence to deliver electrons carried by the reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH$_2$) to oxygen (Figure 3.2). Each Complex consists of 1) a major enzyme that catalyses electron transfer, 2) a nonprotein organic group (prosthetic groups) that directly accepts and
releases electrons carried by the Complex, and 3) structural proteins that may contribute to movement of $H^+$ across the membrane. The system also contains two small, highly mobile carriers that shuttle electrons between major Complexes. One of these mobile carriers is cytochrome c, a relatively small protein with its own prosthetic group. The other is a nonprotein organic molecule, ubiquinone (also called coenzyme Q), which directly picks up and releases electrons. Three of the major Complexes (Complexes I, III, and IV) move $H^+$ across the membrane in response to electron flow. The mitochondrial electron transport system pumps $H^+$ from the matrix into the intermembrane space as electrons flow through the carriers from NADH or FADH$_2$ to oxygen.

Figure 3.1 Electron micrograph of isolated rat renal mitochondria showing multiplicity of cristae in their native configuration (A). Classical view of an isolated mitochondrion with major structures identified (B).
Figure 3.2: Schematic diagrams of the components of the electron transport chain.
The protons (H⁺) cannot return to the matrix except through one pathway, the enzyme FoF₁-ATPase (sometimes referred to as Complex V), which is embedded in the inner membrane. As the protons flow back into the matrix, ATPase adds a phosphate group to the molecule adenosine diphosphate (ADP) present in the matrix, resulting in the formation of adenosine triphosphate (ATP). Therefore, the proton gradient set up by the electron transport system provides energy by the FoF₁-ATPase, synthesizing ATP from ADP and phosphate. Furthermore, this extrusion of protons from the matrix to the intermembrane space generates the electrochemical gradient also known as the proton motive force. Most of the proton motive force is manifested in the form of mitochondrial membrane potential and the collapse of this membrane is associated with apoptosis (91). By definition (92) ATP is the major currency in the cellular economy, required to drive energy-requiring reactions in all living things, from bacteria to plants to man.

Hence, since the mitochondria play such an important role in the survival of the cell, it is not surprising that mitochondrial dysfunction plays a crucial role in pathological states as diverse as kidney disease, heart disease, stroke, cancer and neurodegenerative disease(93). Some of the factors which contribute to mitochondrial dysfunction are insufficient fuel supply (ATP), defective electron transport chain enzymes (Complexes I - IV) and lack of the electron carrier enzyme. Reduced oxygen availability, due to ischaemia and excessive membrane leakage of protons, results in insufficient mitochondrial inner membrane potential for ATP synthesis by the FoF₁-ATPase and mitochondrial DNA mutation.

The importance of examining the mitochondria in this study stems from the recognition that injury to this organelle, may inevitably lead to loss of cell viability and eventually cell death by apoptosis or necrosis (91). Therefore the regulation of oxygen consumption and mitochondria enzymatic activity is imperative, to provide the cell with a proper supply of ATP for cell survival during pathophysiological events. The regulatory role that mitochondria play in cell dysfunction and cell-death is further amplified by the ability of cellular
and intercellular messengers, which have the capacity to regulate cellular respiration and energy production, by binding to different sites of the electron transport chain.

A good example is nitric oxide (NO), a soluble, highly reactive gas formed by natural chemical and physical reactions in the atmosphere. It is also produced by certain animal and plant cells from the amino acid L-arginine. Because it is so small and diffusible, NO passes through cell membranes and is often used as a biological signal. Nitric oxide reacts in water with oxygen and its reactive intermediates to yield: (i) other radicals (e.g. NO2), (ii) moderately stable anions (NO2⁻), (iii) very stable anions (NO3⁻) and (iv) unstable peroxides (e.g. ONOO⁻). Nitric oxide (NO) is a ubiquitous signalling molecule implicated in neural transmission, immune response and vasodilatation by activation of guanylate cyclase (94).

Of major interest, are the recent findings highlighting the regulatory role of nitric oxide on mitochondrial oxygen consumption. Physiological concentrations of NO reversibly inhibits the mitochondrial enzyme cytochrome oxidase (Complex IV) in competition with oxygen (95-97). The first study to demonstrate reversible NO inhibition in mitochondria was carried in cultured astrocytes. Further studies, showed that mitochondrial function is also affected by NO reactive species (peroxynitrite, nitrogen dioxide and nitrosothiols) (98) which in some cases lead to irreversible inhibition of the respiratory chain, uncoupling, permeability transition, and cell death. In the isolated perfused heart, Cassina et al. (99) illustrated the effect of continuous influx of NO plus superoxide (O2⁻) which produced a steady state of ONOO⁻. This ONOO⁻ produces a strong inhibition of Complex I-II dependent oxygen consumption. However, ATPase activities, Complex IV-dependent respiration and cytochrome c oxidase activity were not inhibited. Another group (100) illustrated the effect of long exposure of cells (murine macrophage J774) to NO, which led to nitrosylation of thiol groups in Complex I, while glutathione transnitrosilates until its level drops to critical values. As long as glutathione
was replenished, the NO inhibition was reversed; however, once glutathione was completely exhausted, cellular respiration was blocked and NO was transformed from a physiological mediator into a pathological molecule. The reaction of superoxide (O2-) (101) and NO (102) is extremely rapid in vivo and results in the formation of highly reactive peroxynitrite anion (ONOO·), which can cause damage to the mitochondrial respiratory chain enzymes and uncouple mitochondria oxidative phosphorylation (103). In addition, NO binds to mitochondrial Complex I leading to the inhibition of mitochondrial phosphorylation and a decrease in ATP production (104). Therefore, the production or presence of high amounts of NO and related species can also have a deleterious effect on cell energy metabolism due to the production of reactive oxygen species, which can destroy cellular integrity.

On the contrary, inhibition by NO may regulate many cellular functions (105) for example, the regulation of ATP synthesis and formation of transmembrane potential. The presence of NO synthase in mitochondria under normal physiological conditions (106) suggests that, small physiological amounts of NO could be involved in cellular respiration by the regulation of cytochrome oxidase. Furthermore, NO regulates oxygen consumption of both NO-producing and the neighbouring cells (107), thus functioning as autoregulator or paracrine modulator of respiration.

It is interesting that cells and tissues exposed to NO-releasing agents or stimulated to augment endogenous NO production display a marked increase in HO-1 protein expression and haem oxygenase activity (108;109) (33;110). Although NO has been shown to reversibly/irreversibly inhibit mitochondrial respiration, no data is available on the effect of haem degradation products by haem oxygenase-1 (CO, iron and bilirubin) on mitochondrial respiration and ATP synthesis.

In previous studies, our group has shown that induced cardiac HO-1-derived activity ameliorates post-ischaemic myocardial dysfunction and preserves mitochondrial integrity upon reperfusion. Hence, we decided to examine the effect of HO-1 induction on mitochondrial function in the kidney. For initial
comparative purposes, the effect on kidney tissues was compared to the one elicited on heart tissues. This served as a guide for later studies in the in vivo rabbit model, which was the main experimental system utilized in this thesis.

3.2 Objective

The purpose of this study was (i) to investigate whether hemin treatment affects the oxygen uptake and ATP production (phosphorylation) in isolated mitochondria and (ii) to assess the effect of hemin treatment on the enzyme activity of Complexes I-IV of the electron transport chain.

3.3 Materials and Method

3.3.1 Preparation of reagents

Hemin (ferriprotoporphyrin IX chloride) was freshly prepared as stock solution prior to animal treatment by dissolving the compound in sterile 0.1 M NaOH and then adjusting the pH to 7.4 by addition of 0.01 M phosphate buffer. All other reagents were from Sigma Chemical (Dorset, UK).

3.3.2 Isolation of cardiac and renal mitochondria

Male Sprague-Dawley rats were treated with hemin (50 mg/kg) i.p. or sterile saline (control group). Kidneys and hearts were harvested 24 hours after hemin treatment and mitochondria were isolated from each organ. Tissues were then immersed in ice-cold isolation medium (225 mM mannitol, 75 mM sucrose, 10 mM Tris, 0.1 mM EDTA, and pH 7.2) and all the procedures were carried out at 4 °C. Mitochondria were isolated from the respective organ by homogenization and differential centrifugation. The protein content of the mitochondrial pellet was determined by using a kit based on a modified Lowry method. Detailed prescription of the protocol utilized for preparing isolated mitochondria can be found in Materials and Methods.
3.3.3 *HO-1* protein expression in renal and myocardial tissue

Rats were administered with hemin and the organs (kidney and heart) were harvested 24 hours after treatment. The organs were flushed with ice cold saline, and the tissues were processed as described in Materials and Methods for preparation of the microsomal fractions. HO-1 protein expression in the microsomes was then assessed by Western blot using a specific polyclonal antibody against HO-1 (see Materials and Methods).

3.3.4 Determination of mitochondrial oxygen consumption

Oxygen consumption of isolated mitochondria was measured using a water-jacketed Clark-type electrode (Yellow Spring Instruments, Yellow Spring, Ohio) connected to a chart recorder. All experiments were carried out at 30 °C using freshly isolated mitochondria resuspended in respiration medium which consisted of: 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 50 μM EDTA, 10 mM Tris-HCl, and 10 mM KH₂PO₄ (pH 7.4). Incubations with mitochondrial suspensions were performed in the presence of 0.5 mg bovine serum albumin. The addition of ADP causes a rapid uptake of oxygen, as ADP is converted to ATP. The active respiratory state is referred to as State 3 (+ADP), while the slower State (-ADP) is referred to as State 4 and respiration was measured after addition of 5 mM glutamate + 5 mM malate. State 3 respiration was initiated by addition of ADP and the respiratory control index (RCI) was calculated as a ratio between State 3 and State 4 respiration rates (Fig 3.3). Aliquots of isolated mitochondria were stored at -70 °C until mitochondrial enzyme Complex activities were measured.
3.3.5 Determination of mitochondrial Complex (I-IV) activities

Mitochondrial enzyme activities were measured spectrophotometrically at 30 °C in samples that were lysed by three cycles of freeze thawing and vortex mixing. Activities of respiratory chain Complex I-IV were determined as described in the Materials and Methods section.

3.3.6 Statistical analysis.

Differences in the data among the groups were analysed by using one-way ANOVA combined with the Bonferroni test. Values were expressed as a mean ± S.E.M. and differences between groups were considered to be significant at p < 0.05.

3.4 Results

3.4.1 Increased HO-1 expression in renal and myocardial tissues 24 hours after treatment with hemin

To determine the effect of hemin, HO-1 protein expression was determined in kidney and heart tissue. Male Sprague-Dawley rats were administered with
hemin (50 mg/kg) i.p. And the organs were removed 24 h after treatment. Kidney and heart tissues were flushed with cold saline and processed for Western blot analysis as described in Materials and Methods. The relative expression levels of HO-1 protein were analysed by a densitometer, as shown in Figure 3.4. Compared to control tissue, hemin treatment in both renal and myocardial tissue was accompanied by enhanced HO-1 expression. For this study, it was imperative to establish the presence of HO-1 protein in the respective tissues, especially in the kidney, the main organ of interest in later studies.

\[
\text{HO-1} \rightarrow
\]

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<thead>
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<th>Control</th>
<th>Treated</th>
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<tr>
<td>Kidney</td>
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<tr>
<td>Heart</td>
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Figure 3.4 Western blot analysis of HO-1 protein expression in kidney and heart tissue

Rats were injected with hemin (50mg/kg) i.p. and after 24 h the organs were harvested and flushed with cold saline. Microsomes were prepared from both the kidney and heart samples as described in Materials and Methods. The HO-1 protein was measured in the microsomal fraction of renal and myocardial tissue by Western blot using a polyclonal antibody.
3.4.2 Effect of hemin treatment on mitochondrial oxygen consumption and respiratory control index in kidney and heart tissues

Mitochondria were isolated from animals treated with hemin, 24 h after administration. The organs were removed, flushed with cold saline and mitochondria were isolated as described in Materials and Methods. The following mitochondrial functional parameters were measured: 1) oxygen consumption during oxidative phosphorylation (+ADP), the production of ATP from ADP (State 3), 2) the rate of oxygen consumption after the complete exhaustion of ADP (State 4), and 3) the respiratory control index (RCI) (State 3/State 4). In this study, the parameters of oxidative phosphorylation were obtained with NAD-linked substrates malate-glutamate. Compared to control tissues (p<0.05), mitochondrial oxygen consumption and rate of oxidative phosphorylation (State 3) was significantly reduced in hemin-treated kidney and heart mitochondria fractions. The rate of oxygen consumption was reduced by 50% during State 3, in comparison to respective control tissues, while State 4 remained unchanged. The effect of hemin treatment was very pronounced during State 3 (active respiration) as oxygen consumption was slower during the conversion of ADP to ATP (phosphorylation), in both renal and myocardial tissues (Figure 3.5). The slower oxygen consumption rate in hemin-treated mitochondria from both kidneys and hearts produced a significantly lower respiratory control index (Figure 3.6). Electron microscope analysis was employed to examine ultrastructural changes in the isolated mitochondria fractions (Plate 3.1); even after hemin-treatment mitochondrial integrity was still maintained in isolated renal and cardiac mitochondria.
Figure 3.5  Effect of hemin treatment on mitochondria oxygen consumption

Typical oxygraph tracing showing the rate of oxygen consumption during phosphorylation in mitochondria isolated from control (A) and hemin-treated (B) rat kidneys are shown. Similar tracing of oxygen consumption rate in mitochondria isolated from control (C) and hemin-treated (D) rat hearts are presented. Rats were injected with hemin (50mg/kg); after 24 h, organs were removed and flushed with cold saline. Mitochondria were isolated from both kidney and heart tissues, and the NAD-linked respiratory control index was measured using substrates glutamate and malate, as described in Materials and Methods.
Figure 3.6 Effect of hemin treatment on respiratory control index (RCI) in kidney and heart tissues

Rats were injected with hemin (50mg/kg) after 24 h, organs were removed and flushed with cold saline. Mitochondria were isolated from both kidney and heart tissues, and the NAD-linked respiratory control index was measured using substrates glutamate and malate, as described in Materials and Methods. Data represents (±SEM) n=6. *, P<0.05 vs. Control
Plate 3.1  Electron micrographs of isolated mitochondria pellets from kidney and heart tissues after hemin treatment

Rats were injected with hemin (50mg/kg); after 24 h, organs were removed and flushed with cold saline. Mitochondria were isolated from both kidney and heart tissues, and suspended in fixative for electron microscopy analysis. A: control kidney; B: control heart; C: hemin-treated kidney; D: hemin-treated heart
3.4.3 Effect of hemin treatment on Complex I-IV enzymatic activity in kidney and heart tissues

The enzymatic activity of mitochondrial Complexes was measured after 3 cycles of freezing and thawing of the mitochondrial fraction. This process lysed the outer mitochondrial membrane, facilitating measurements of enzyme activity for Complex I to IV. The effect of hemin treatment did not alter the NADH CoQ reductase (Complex I) activity obtained in both kidney and heart mitochondria (Figure 3.7). Activity remained unchanged in Complex II-III in both kidney and heart mitochondria after hemin treatment. Similarly, cytochrome oxidase (Complex IV) activities showed no significant difference when compared to control mitochondria. Hence, hemin treatment did not alter the enzyme activities of Complex I-IV. These results were intriguing, since the previous results (Figure 3.5-3.6), illustrated a reduction in oxygen consumption and oxidative phosphorylation after hemin treatment, indicating a possible defect or inhibition of the electron transport chain.

3.4.4 Effect of hemin treatment on oxygen consumption rate following exposure to high intensity light

Since hemin treatment reduces oxidative phosphorylation (Figure 3.5) without affecting enzyme activity of Complex I-IV, we aimed to establish the factor responsible for the reduction in oxygen consumption. Therefore, we hypothesized that carbon monoxide (CO), the product of the haem oxygenase pathway, was the factor responsible for reduction in oxygen consumption and phosphorylation. Clementi and co-workers (100) employed high intensity light to dissociate NO from the haemoprotein cytochrome oxidase, thereby reversing the inhibitory effect of NO. To confirm that CO inhibits cardiac mitochondrial oxygen consumption, mitochondria were exposed to high intensity light from a cold light source, during oxidative phosphorylation.
Interestingly, exposure of hemin-treated mitochondria to high intensity light restored the rate of oxygen consumption and phosphorylation, to levels comparable to control mitochondrial (Figure 3.8).

Figure 3.7 Effect of hemin treatment on Complex I-IV activity in rat kidneys and hearts

Rats were injected with hemin (50mg/kg); after 24 h, organs were removed and flushed with cold saline. Mitochondria were isolated from both kidney and heart tissues, and the enzymatic activity of Complexes I-IV was measured as described in Materials and Methods. A: Complex I activity; B: Complex II-III activity; C: Complex IV activity
Figure 3.8 Effect of high intensity light on oxygen consumption in hemin-treated mitochondria.

Oxygraph tracing showing the rate of oxygen consumption during phosphorylation in mitochondria isolated from hemin-treated heart (A). The effect of flashing high intensity light on oxygen consumption, of hemin-treated heart mitochondria is illustrated in (B). Rats were injected with hemin (50mg/kg); after 24 h, organs were removed and flushed with cold saline. Mitochondria were isolated from hemin-treated heart tissue, and the NAD-linked respiratory control index was measured using substrates glutamate and malate, as described in Materials and Methods.
3.5 Discussion

In mammalian cells, CO is endogenously generated by haem oxygenase (HO). Basal expression of HO-1 is either very low or absent, but can be elevated by chemical inducers such as hemin, pathophysiological conditions, oxidative stress or hypoxia. Under normal physiological conditions, low concentrations of CO exert different effect on physiological and cellular function. Neurologists and vascular biologist believe CO is important in cellular systems. For example, in the cardiovascular system, the substrate haem is readily available and localized in the vasculature and blood vessels; hence, there is a high capacity to generate CO ((111); (112). In addition, the pioneering work of Synder et al. (49) and Morita & Kourembanas (113), have clearly shown that CO generated from HO can regulate vasomotor tone as well as neurotransmission. These findings provide the first evidence that this simple gaseous molecule could have impact on critical biological functions and similar function to NO.

The circumstantial evidence indicating a role for CO in the vasculature was supported by direct detection of this gas in biological systems (114); this was also reinforced by measurements of haem oxygenase activity in endothelial and smooth muscle cells and by localization of haem oxygenase isoforms in this type of tissues. Haem oxygenase activity, determined as CO production, has been reported in rat and rabbit aortas as well as in the human inferior mesenteric artery (115-117). Similarly, immunohistochemistry analysis revealed the presence of HO-2 and HO-1 proteins in bovine pulmonary artery and vein, which correlated with haem oxygenase activity (118). In arteries, enzymatic activity was found in the adventitia and medial layer, and protein isoforms were localized in the vasa vasorum of the adventitia and throughout the smooth muscle cells in the medial layer. Increased haem oxygenase activity has been detected in aortic endothelial cells in culture after exposure to haem or various forms of haemoglobin (44;75;119). Haem oxygenase activity and CO production were also found in smooth muscle cells after exposure to low oxygen tension (29). This effect was associated with a significant increase in intracellular cGMP, which was abolished by a specific
haem oxygenase inhibitor but remained unaltered by blockade of the NO synthase pathway. These data demonstrate that vascular cells generate CO in normal conditions and have the ability to markedly increase the production of this gaseous molecule when the inducible haem oxygenase system (HO-1) is appropriately stimulated.

The very first evidence reporting the contribution of endogenously generated CO in the regulation of vascular function can be attributed to Suematsu and collaborators who designed experiments to elucidate whether haem oxygenase-derived CO can serve as an active vasorelaxant in the hepatic microcirculation (120; 121). Using a model of isolated liver perfusion, Suematsu and colleagues found that the CO flux, directly measured in the venous effluent by trapping the gas with myoglobin, is in the order of 0.7 nmol/min/gram of tissue. More recently, our group has demonstrated that aortic tissues expressing high levels of HO-1 release much more CO leading to an increased production of the second messenger cGMP (122) and making the vessels less susceptible to vasoconstriction. Similarly, induction of the HO-1/CO pathway in cardiovascular tissues results in suppression of the acute hypertensive responses mediated by NO synthase inhibitors and haemoglobin(123). These data clearly demonstrate that once the HO-1 system is maximally up-regulated, CO is an important endogenous regulator of vascular activities.

In co-culture system, vascular smooth muscle-derived CO after stimulation of the HO pathway exerts a paracrine effect on endothelial cells by increasing endothelial cell cGMP and decreasing expression of mitogens such as endothelin-1 and platelet-derived growth factor-B(113). Thus, CO via HO-1 activation may modulate cell-cell interaction and proliferation in vessel wall under hypoxic conditions.

In contrast to the beneficial effects of endogenously produced CO, high levels of CO are toxic, because of its strong affinity to haemoglobin and this condition is referred to as carbon monoxide poisoning. By binding avidly to
haemoglobin, CO replaces oxygen, induces general hypoxia, and impedes oxygen delivery to tissues. Furthermore, dissociation of carbon dioxide (CO₂) is impaired, thus producing an increase in blood CO₂ and removing the reflex stimulus to the respiratory centres of the brain. That would otherwise force the individual to breathe, more often to increase the blood oxygen levels. During CO poisoning, several reports have shown mitochondrial cytochrome c oxidase as a target site in human acute CO poisoning (124). Since over expression of HO produces increased CO level, we hypothesized that CO might bind to the terminal enzyme of the respiratory chain thereby modulating respiratory function.

In this study, we demonstrated the possible regulatory role of the HO/CO system on mitochondrial respiratory function. The following functional parameters of the mitochondrial respiratory chain were assessed to investigate the effect of HO-1 upregulation on mitochondrial function: (i) mitochondrial oxygen consumption; (ii) oxidative phosphorylation; (iii) respiratory control index; (iv) mitochondrial integrity and (v) enzyme activity of complexes I-IV.

The specific goal of this study was to examine the effect of HO pathway up-regulation on mitochondrial respiratory function. Administration of hemin in rats led to an increase in HO-1 protein expression in renal and cardiac tissue, which is usually followed by an increase in haem oxygenase enzyme activity(42). This data was not surprising as hemin is a potent inducer and a substrate for haem oxygenase pathway. And other investigators(35;84;125) have also used hemin as an inducer of HO-1.

Measurement of mitochondrial function showed that hemin treatment led to a decrease in State 3 during oxidative phosphorylation in renal and cardiac isolated mitochondria. Respiratory control index (RCI) (an index of electron transport chain coupling), was also reduced by 50% due to the decreased rate of oxygen consumption during State 3.
Electron microscopy analysis was used to investigate the mitochondria ultrastructure in both tissues after HO-1 up-regulation there was no visible sign of mitochondrial swelling or rupture membranes following hemin treatment. This is in agreement with previous study performed by Clark JE et al. showing maintenance of mitochondrial function in cardiac tissue after treatment with hemin (126). Furthermore, to detect any specific enzymatic induction, the activities of the individual complexes of the electron transport chain were investigated. The results show that HO-1 upregulation did not affect the enzyme activity of Complexes (I-IV). Hence, the upregulation of HO-1 did not affect enzyme activity of the respiratory chain complexes, but there was a significant reduction in oxidative phosphorylation and oxygen consumption, in both renal and cardiac mitochondria.

One important point is that, although hemin treatment reduced mitochondrial function, it did not totally inhibit respiration and this inhibition was reversible by light. Therefore, CO, like NO, might compete with oxygen to bind to mitochondrial haem-dependent proteins (127). Cytochrome oxidase in the respiratory chain is an expansible target e.g. its inhibition by CO has been shown to partially prevent oxidative phosphorylation. Like NO, CO might be a regulatory molecule involved in the regulation of oxygen consumption and oxidative phosphorylation. Clementi and co-workers (100) showed that NO results in a gradual and persistent inhibition of complex I. Notably, this inhibition was reversed by exposing the cells to high intensity light or by replenishment of intracellular reduced glutathione. Since NO and CO have similar biological actions, we hypothesize that CO, the product of the haem oxygenase pathway, may be responsible for the reduction in State 3 and RCI. Interestingly, bright light totally reversed cellular respiration to normal conditions in isolated mitochondria obtained from cardiac tissue, supporting the involvement of CO in the decline of mitochondrial function.
3.6 Conclusion

This study demonstrates an important role of the haem oxygenase system (HO-1/CO pathway) in the regulation of mitochondrial respiratory function. Specifically, the rate of oxygen utilization during oxidative phosphorylation, in isolated kidney and heart mitochondria is affected by up-regulation of the HO-1 system. Complex I-IV activities in isolated kidney or heart mitochondria were preserved in hemin-treated animals suggesting that the respiratory chain is not damaged by the HO-1 inducer. These findings illustrate that a product of haem degradation by haem oxygenase, presumably CO, may modulate mitochondrial oxygen consumption. Finally, haem oxygenase-1 induction via hemin treatment in some disease model, may be effective or beneficial, by regulation of mitochondrial function and maintenance of cellular anti-oxidant defence mechanisms.
4. HEMIN AN INDUCER OF HAEM OXYGENASE-1 (HO-1) IN RABBIT RENAL TISSUE

4.1 Introduction

Significant and increasing evidence indicates a close relationship between haem metabolism and kidney function. Haem functions as the prosthetic moiety for a number of haem-containing proteins with activities that are critical to vascular and renal function. These include soluble guanylate cyclase, nitric oxide synthase (NOS), and enzymes of the eicosanoid biosynthetic pathways, including cytochrome P-450 monooxygenases, thromboxane and prostacyclin synthases, and the haem-dependent cyclooxygenase. The capacity of haem biosynthesis in the kidney has been shown to be differentially distributed in a cortico-to-medullary gradient with greatest activity in the cortex and least in the medulla. Within the cortex, the activity is principally localized with the proximal tubule cells. Fluorescence studies showing greater porphyrin content of the proximal tubule cells and striking gradient of porphyrin fluorescence from cortex to medulla also support this view (128:129).

The regulation of haem degradation in the kidney appears to be readily responsive to the same influences which regulate haem oxygenase pathway in the liver. Therefore, the activity of renal haem oxygenase is increased by agents which induce this enzyme in the liver; likewise, the activity of bilirubin reductase is reduced by substances which inhibit the hepatic enzyme (130). Renal haem oxygenase is induced by a wide variety of trace metals; these include chromium, gold, manganese, iron, nickel, copper and zinc (131). Other factors, such as depletion of cellular glutathione content (132) or induction of haemolysis (133), have also been reported to increase renal haem oxygenase activity. In addition, haem oxygenase is also induced under diverse oxidative stress conditions and various substances (Table 4.1).
There is accumulating evidence that hemin (ferriprotoporphyrin IX chloride), which is the oxidized form of haem, is a potent inducer of haem oxygenase-1 (HO-1) gene expression and a substrate for haem oxygenase activity (134-137). Other groups have shown that hemin increases the amount of functional haem oxygenase mRNA and enzyme in cultured pig alveolar macrophages and rat liver. However, so far no one has investigated the effect of haem oxygenase induction in rabbit kidney by hemin. Therefore, it is essential that we understand the potential and efficacy of hemin as an inducer of HO-1 in rabbit renal tissue before we decide to use this inducer as a therapeutic agent for pre-conditioning renal tissue against oxidant-mediated damage.

Table 4.1 Conditions that induce haem oxygenase-1

<table>
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<tr>
<th>Conditions</th>
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<tr>
<td>Haem (138)</td>
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<tr>
<td>Other metalloporphyrins (139)</td>
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<tr>
<td>Transition metals (70)</td>
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<tr>
<td>Inflammatory cytokines (140)</td>
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<tr>
<td>Prostaglandins (141)</td>
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<tr>
<td>Phorbols esters (142)</td>
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<tr>
<td>Heat Shock (143)</td>
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<tr>
<td>Hydrogen peroxides (144)</td>
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<tr>
<td>Lipopolysaccharides (LPS) (145)</td>
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<tr>
<td>Sodium arsenite (144)</td>
</tr>
<tr>
<td>Nitric Oxide (146)</td>
</tr>
<tr>
<td>Hypoxia (126:147)</td>
</tr>
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4.2 Objective
The purpose of this study was to determine (i) the most effective route of hemin administration in order to maximize the induction of the HO-1 gene, (ii) the extent of HO-1 protein expression and enzyme activation, and (iii) how HO-1 activity is maintained after hypothermic storage of rabbit kidney.
4.3 Materials and Methods

4.3.1 Reagents

Hemin (ferriprotoporphyrin IX chloride) was purchased from Porphyrin Products (Logan, Utah, USA). Stock solutions of hemin were prepared freshly prior to animal treatment by dissolving the compound in sterile 0.1 M NaOH and then adjusting the pH to 7.4 by addition of 0.01 M phosphate buffer. All other reagents were purchased from Sigma Chemical (Dorset, UK).

4.3.2 Animal treatment and in vivo tissue methodology

The New Zealand White (NWZ) rabbits (2-4 kg) were injected with hemin via different modes: intraperitoneally (i.p.), intraperitoneally plus intravenously (i.p. + i.v.), subcutaneously and subcutaneously plus intraperitoneally (i.p. + subcutaneous). The kidneys were removed and flushed with cold University of Wisconsin (UW) solution 24 h after hemin administration as described in Materials and Methods. Kidneys were either processed immediately or stored in cold UW solution for 24 or 48 h. The renal tissue was then processed as described in Materials and Methods for the preparation of microsomal membrane. Haem oxygenase activity and protein expression in the microsomes were assessed by haem oxygenase activity assay and Western blot using a specific monoclonal antibody against HO-1 (as described in Materials and Methods). Northern blot analysis was performed on total RNA isolated from frozen section of renal tissue as described in Materials and Methods.

4.4 Results:

4.4.1 Haem oxygenase activity in rabbit renal tissue after intraperitoneal hemin treatment

New Zealand White rabbits were administered with hemin (50 mg/kg) i.p., the kidneys were removed 24 h after treatment and were flushed with cold
UW solution. The intraperitoneal (i.p.) hemin treatment slightly increased haem oxygenase activity, but there was no significant difference when compared to control (Figure 4.1). After 24 or 48 h hypothermic storage the hemin-treated kidneys exhibited a decrease in haem oxygenase activity in comparison to control.

Figure 4.1 Effect of hemin treatment (i.p.) and cold storage on renal haem oxygenase activity.

Rabbits were administered with hemin (i.p.) and kidneys were removed 24 h after treatment. Kidneys were then subjected to different periods (0, 24 or 48 h) of cold storage in UW solution prior to measurements of haem oxygenase activity as described in Methods and Materials. Haem oxygenase activity was measured in the unstored hemin-treated (i.p.) kidneys and compared to controls (untreated kidneys). Bars represent the mean ± S.E.M. of n = 6 independent experiments. * P<0.05 vs. controls.
4.4.2 Effect of intraperitoneal plus intravenous hemin administration on haem oxygenase activity in rabbit kidney

From previous data, we established that hemin treatment i.p was not very effective in significantly increasing haem oxygenase activity. Hence, we decided to administer hemin (50mg/kg) intraperitoneally plus intravenously (i.p. + i.v.). New Zealand White rabbits were administered with hemin (50 mg/kg) i.p. + i.v., the kidneys were removed 24 h after treatment and were flushed with cold saline. Haem oxygenase activity was significantly increased (p<0.001) in hemin-treated kidneys when compared to controls (Figure 4.2A). Specifically, there was a four fold increase in haem oxygenase activity when hemin was administered i.p. plus i.v. compared to i.p. only, which just led to a slight increase in haem oxygenase activity.

4.4.3 Effect of intraperitoneal plus intravenous hemin administration on haem oxygenase activity in rabbit kidney during hypothermic storage

Groups of rabbits (n =6) were injected with increasing doses of hemin i.p. + i.v. (5, 10 & 20 mg/kg). Kidneys were removed 24 h after the injection and flushed with cold saline. Hemin-treated kidney were either processed immediately or stored in cold UW solution for 24 or 48 h before microsomal fractions were isolated. Haem oxygenase activity increased significantly in a dose-dependent manner reaching a maximum with the dose of 20 mg/kg (Figure 4.2 B). Even after 24 h cold storage, haem oxygenase activity was still significantly high (p<0.05) in the groups that received 5 and 20 mg/kg of hemin, respectively. The group that received 10 mg/kg of hemin showed a decline in haem oxygenase activity after 24 h of hypothermic storage. However, after 48 h of hypothermic storage haem oxygenase activity in all the groups was comparable to control.
Figure 4.2  Effect of hemin-treatment (i.p. + i.v.) and cold storage on haem oxygenase activity.

(A) Rabbits were administered with hemin (i.p. + i.v.) and kidneys were removed 24 h after treatment. Haem oxygenase activity was measured in hemin-treated (i.v. + i.p.) kidneys and compared to controls (untreated kidneys). (B) In another set of experiments, rabbits were injected with different concentrations (5-20mg/kg) of hemin (i.p. + i.v.); kidneys were removed 24 h after treatment and subjected to different periods (0, 24 and 48 h) of cold storage in UW solution. Haem oxygenase activity was then measured in hemin-treated (i.p. + i.v.) kidneys and compared to controls (untreated kidneys). Bars represent the mean ± S.E.M. of n = 6 independent experiments. * P<0.05 vs. controls, *** P<0.001 vs. controls.
4.4.4 Time course of HO-1 mRNA in hemin-treated kidneys

To gain an understanding of the time-frame for kidney tissue response to hemin-treatment, we examined the HO-1 mRNA expression over time. New Zealand White rabbits were injected with hemin (i.p. + i.v.) and the kidneys were removed at 2, 4, 6, and 12 h, after administration. An untreated animal served as a control group. Total mRNA was isolated from the kidneys and analysed by Northern blot as described in Materials and Methods. The administration of hemin resulted in a time-dependent increase of HO-1 mRNA expression. After 2 h, HO-1 mRNA was clearly detectable, increasing after 4 h and peaking at 6 h (Figure 4.3 A). After 12 h the transcription of HO-1 mRNA was almost comparable to control indicating a gradual return of the HO pathway after the initial response to hemin. GAPDH, a housekeeping gene used as an internal control remained unchanged by hemin-treatment.

4.4.5 Effect of hemin on HO-1 protein expression

Five NWZ rabbits were injected with hemin via different modes; intraperitoneally (i.p.), intraperitoneally plus intravenously (i.p. + i.v.), subcutaneously and subcutaneously plus intraperitoneally (i.p. + subcutaneous). The kidneys were removed 24 h after administration, flushed and processed for Western blot analysis, as described in Materials and Methods. The induction of HO-1 was not restricted to changes at the mRNA level, but was also reflected by an increase in HO-1 protein as detected by Western blot (Figure 4.3 B). The control kidney was obtained from an untreated animal and when compared to the various modes of hemin administration hemin resulted in an enhanced expression of HO-1 protein.
Figure 4.3 Hemin pre-treatment stimulates HO-1 activation in rabbit renal tissue

(A) Rabbits were administered with hemin 50mg/kg (i.p. + i.v.); kidneys were removed at various time points (2, 4, 6 and 12 h) after the treatment and processed for Northern blots analysis according to Materials and Methods. (B) In a separate study, various modes of hemin administration (intraperitoneally (i.p.), intraperitoneally plus intravenously (i.p. + i.v.), subcutaneously, subcutaneously plus intraperitoneally (i.p. + subcutaneous)) were employed. Kidneys were removed 24 h after injection with 50 mg/kg hemin, flushed and then processed for Western blot as described in Materials and Methods.
4.5 Discussion

It is well known that administration of hemin induces the expression of HO-1 in a variety of tissue in vivo, as shown in the previous chapter for rat heart and kidney. Researchers in our group (126; 148; 149) have shown HO-1 mRNA and protein expression by hemin in rat heart, endothelium, skeletal muscles and vascular smooth muscles respectively. Aizawa et al. has recently reported that hemin-induced HO-1 upregulation is mainly in the tubular region of the kidney (120). In addition, several studies have used hemin as an inducer of HO-1 in various forms of renal disease where hemin pre-treatment ameliorated aspects of nephropathy induced by mercuric chloride (125), nephrotoxic nephritis (84), and glomerulonephritis (150). Therefore, we wanted to establish if hemin upregulates HO-1 in rabbit kidney and most importantly, if the overexpression is maintained after hypothermic storage in cold UW solution.

The data obtained showed an increase in HO-1 activity 24 h after administration of hemin in comparison to control. The time point for the removal of the kidney after hemin treatment was chosen on the basis of previous studies by our group, which demonstrated peak protein and enzyme activity of haem oxygenase 24 h after administration of hemin. The type of injection, whether i.p. only or a combination of i.p. + i.v., made a significant difference on haem oxygenase activation. The combination of i.p. + i.v. injection readily and significantly induced haem oxygenase activity by four-fold, while there was a minimal increase in haem oxygenase activity with i.p. injection. This result demonstrates that the route of administration influences the onset of haem oxygenase activity. The i.v. injection is the fastest way to get the inducer into the animal’s blood circulation and half of the dose (25 mg/kg) is directly injected into the vein; hence, the bioavailability of the drug would be equal to the amount injected. In addition, hemin will not be directly absorbed or metabolised by the liver, unlike the i.p. only injection which significantly increases hepatic haem oxygenase activity but not necessarily renal activity. However, we must also consider the problem of
toxicity related to i.v. injection, due to the rapid exposure of the vascular endothelium to the excessive amount of hemin. This is why we only injected 25mg/kg, half of the original dose.

Furthermore, the increase in haem oxygenase activity by hemin does not exclude the fact that some of the action by hemin is exerted on the other isoforms (HO-2 & HO-3) of haem oxygenase. In addition, although the kidney normally expresses significant HO-2 activity, it should be noted that inducers of HO-1 can increase activity of HO-1 to levels well above that of the constitutively expressed HO-2. Besides, HO-1 appears to be two times more reactive to some substrates than HO-2 (151).

Renal cold storage for transplantation is currently limited to 48 hours. To better understand the feasibility of maintaining elevated haem oxygenase activity during and after hypothermic storage, hemin-treated kidneys were stored for 24 or 48 hours in cold University of Wisconsin solution. Administration of hemin between 5 and 20 mg/kg showed a dose-dependent increase in haem oxygenase activity; in addition, this activity was maintained even after cold storage. After 24 h of cold storage haem oxygenase activity was significantly higher in hemin-treated kidneys when compared to control. This enzymatic activity, however, declined after 48 h hypothermic of storage.

The effectiveness of hemin was also confirmed in the present study using Northern blot, which demonstrated an increase in HO-1 mRNA expression. Hemin (injected i.p. + i.v.) increased functional levels of mRNA for HO-1 in the kidney suggesting that hemin acts at the transcriptional level to increase the amount of HO-1. HO-1 mRNA expression increased in a time-dependent fashion, reaching a maximum at 6 hours and declining after 12 hours with hemin treatment.

As mentioned earlier, hemin is well-known inducer of HO-1, but the method of administration has been diverse among investigators. Therefore, we decided to inject rabbits via different routes and investigate HO-1 protein expression. The results obtained prove that the following methods of hemin
administration; namely: intraperitoneally (i.p.), intraperitoneally plus intravenously (i.p. + i.v.), subcutaneously, subcutaneously plus intraperitoneally (i.p. + subcutaneous) were effective for the induction of HO-1 in renal tissue, as shown by Western blot. Although HO-1 protein was induced by all the various routes of administration, this does not necessarily mean that the HO activity would be the same. As previous results have shown i.p. injection is not as effective as i.p. + i.v. for HO activation.

HO-1 is considered to be one of the heat shock proteins (152) associated with oxidative stress in a variety of mammalian cells. Hence, the induction of HO-1 represents a protective response to oxidative stress. Similarly, overexpression of HO-1 may offer cellular protection against the deleterious effect of hypothermic storage and eventually ischaemia-reperfusion injury by the production of bilirubin/biliverdin (two powerful antioxidants) and carbon monoxide (CO) (a vasodilator)(153). Therefore, it is tempting to speculate that pre-induction of HO-1 may protect the kidney from subsequent oxidative injury. Hence, if we were to transplant these hemin-treated kidneys within the time frame of the haem oxygenase-1 enzyme activity (24h after hypothermic storage), we may be able to examine the effect of HO-1 induction after ischaemia/reperfusion injury or transplantation.

4.6 Conclusion

Haem oxygenase activity and protein expression of HO-1 were significantly increased in renal tissue by hemin. Hemin did not only act on the activity of haem oxygenase but also on the transcriptional level, with a time-dependent increase of HO-1 mRNA. Furthermore, haem oxygenase activity was affected by extended cold storage in UW solution, but still significantly increased after 24 hour of cold storage. These findings may provide a solution to alleviate the effects of reperfusion injury on kidneys which have been subjected to cold storage prior to transplantation.
CHAPTER 5

EX VIVO STUDIES

5. ASSESSMENT OF THE PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL EFFECTS OF HEMIN TREATMENT ON RABBIT KIDNEY USING THE ISOLATED PERFUSION SYSTEM

5.1 Introduction

Donated organs are maintained at low temperature (4°C) in clinical preservation solution, such as University of Wisconsin (UW) solution, prior to transplantation. During this period tissue typing and cross matching are performed. In some situations the organ may also be subjected to longer periods of cold ischaemia if the organ has been transported to a distant transplant centre.

The donated organ can be subjected to warm ischaemia which commences before removal of kidney from the donor if hemodynamic instability is present. Therefore, during procurement the harvested organ undergoes warm and cold ischaemia. This results in adverse effects such as oxygen deprivation, substrate depletion and waste product accumulation. Finally reperfusion of this organ may lead to additional deleterious effects resulting in further deterioration of cellular integrity and organ function(10).

Despite the pitfalls of cold preservation, hypothermic storage remains the most common technique in use. Preservation solutions, however, despite improvement do not guarantee long term graft survival. Several clinical studies (154;155) have shown a correlation between prolonged cold storage and delayed graft function (DGF). Previous studies have shown a 23% increase in the risk of DGF for every 6 hours of cold ischaemia (155). Hence, preservation solutions, despite improvements(156·158), remain an imperfect substitute and it is evident that best results are seen with the shortest preservation times.
The resumption of vascular supply to an organ that has been subjected to different periods of warm and cold ischaemia introduces a unique pathway known as ischaemia/reperfusion (I/R) injury. Whereas, the biochemical basis for ischaemia/reperfusion injury is complex, at least a component of such injury resides in the enhanced generation of reactive oxygen species (ROS). Oxidative stress as the ability to exacerbate tissue injury when coupled with reduced antioxidant capacity of tissues to effectively neutralize free radical mediated reactions. Therefore, the susceptibility of organs to ischaemia/reperfusion injury may be abrogated by the prior recruitment of a cytoprotective antioxidant response; one such response is the induction of the heat shock protein haem oxygenase 1 (HO-1). Recently, a study by Salahudeen et al. in cell culture illustrated that overexpression of haem oxygenase protects renal tubular cells against cold storage injury.

Haem oxygenase is involved in the antioxidant system by responding to cellular stresses and insults by the production of iron that is sequestered into ferritin, carbon monoxide and biliverdin/bilirubin. In this study we wanted to assess the effect of pre-treatment with hemin, a potent inducer of the enzyme haem oxygenase (HO-1) in the rabbit kidney as illustrated in Chapter 4 on renal function after hypothermic storage.

To examine the physiological effects of hemin in rabbit kidneys subjected to different periods of hypothermia ex vivo, the isolated perfused circuit was employed. This is a sensitive and reliable method for the measurements of renal function parameters. The isolated perfused (rabbit) kidney (IPRK) is also an appropriate system for studying potential nephrotoxicity and it is not influenced by extrarenal regulatory systems (for example, nervous, hormonal, and blood-borne factors). Compared with studies of the kidney in situ, renal function was maintained for only a relative short period of time (for this study up to an hour and thirty minutes) due to decline in renal function after a long period of ex vivo perfusion.
5.2 **Objective:**

These experiments were designed to (i) investigate the effects of two different methods of hemin administration: (intraperitoneal or intravenous plus intraperitoneal) on renal function; (ii) study the overall effect of hemin treatment on rabbit kidney physiology and pathophysiology *ex vivo* and (iii) assess the possible protective effect of HO-1 on hypothermic storage and reperfusion injury in rabbit kidneys.

5.3 **Methods:**

5.3.1 *Preparation of reagents*

Hemin (ferriprotoporphyrin IX chloride) was purchased from Porphyrin Products (Logan, Utah, USA). Stock solutions were prepared by solubilising hemin in PBS (pH 7.4) with the addition of 20μl of 2M NaOH: the solution was then sterilized through a 0.22μm filter before injecting it into animals. All other listed chemicals were of high purity and obtained from Sigma Aldrich.

5.3.2 *Animal Treatment with Hemin*

Three groups were studied: control group was injected with saline, the second group was injected with hemin (50 mg/kg) intraperitoneally (i.p.) and the third group received hemin both intravenously (25 mg/kg) and intraperitoneally (25 mg/kg) (i.p. + i.v.) (50mg/kg). Twenty four hours after the injection nephrectomy was performed as described in Materials and Methods. Kidneys were harvested and stored in UW solution at 4 °C for different time points (0, 24 and 48 h). For details of the surgical procedure and the isolated perfused system refer to the Methods and Materials section.

5.3.3 *Experimental groups*

Twelve NZW rabbit (2.5 kg) were used per group. Kidneys were either used immediately, stored for 24 hours or 48 hours in cold UW solution. In the
control group, animals received saline; the second group of animals received hemin i.p. (Hemin (i.p.)), and the last group received hemin i.p. +i.v. (Hemin (i.p. + i.v.)). Hence, the total number of kidneys used for the whole study was 48.

5.3.4 Isolated perfusion system

At the end of the nephrectomy or hypothermic storage kidneys were canulated and perfused continuously via the renal artery with warmed (37.5°C) and oxygenated (95% O₂-5% CO₂) freshly prepared Krebs solution (pH 7.4) containing the following (in mM): 155 Na⁺, 139 HCO₃, 6.8 K⁺, 139 Cl⁻, 1.2 Mg²⁺, 2 Ca²⁺, 500ml DMEM (DULBECCO'S MODIFIED EAGLE MEDIUM), 500ml Hespan from Fresenius (composition per litre: Hetastarch Hydroxyethyl Starch 60.0g/L, NaCl 90g/L pH 5.5, osmolarity approximately 3.10 mOsm/l) 100ml Gelofusine a plasma substitute (4g of protein/100ml) for protein excretion measurements, 900ml distilled of distilled water, 1g/l of creatinine for determination of glomerular filtration rate (GFR). Perfusion pressure was maintained at 100mmHg by the hydrostatic pressure of a buffer column.

5.3.5 Functional kidney parameters measurements

Renal function was monitored throughout the perfusion using creatinine clearance for the glomerular filtration rate (GFR). Renal vascular resistance (RVR) was measured by measuring the perfusate flow rate under standard conditions of arterial pressure. For this purpose, urine and perfusate samples were collected over an hour into separate 5 ml tubes at 10 minutes intervals after 30 minutes of experimental equilibration. Assessment of renal function was made by comparing markers of renal biochemistry and physiology in the hemin-treated kidneys to control kidneys. The indices of organ function were assessed by measuring the following parameter: 
1. Glomerular Filtration rate (GFR)
2. Tubular glucose reabsorption
3. Tubular sodium reabsorption
4. Urine flow rate
5. Perfusate flow rate (dependent upon vascular resistance in the constant perfusion pressure system, and equivalent to arterial flow rate)
6. Proteinuria (protein leakage)
7. Gamma glutamyl transpeptidase activity (γ-GT)

Glucose and sodium reabsorption were calculated from their respective urine and perfusate concentration ratio and urine volume, as described in Methods and Materials section.

5.3.6 Histology

For light microscopy (LM), kidneys were bisected along the long axis and fixed in 10% formalin and embedded in paraffin, followed by conventional haematoxylin and eosin (H & E) staining. Morphological examination was carried out as described in Materials and Methods. Analysis of cellular ultrastructure using electron microscopy was also performed on sections from the cortex and medulla as described in Materials and Methods. A grading scale of 0-3 (Table 2.1) was used for EM assessment.

5.3.7 Statistical analysis

Differences in the data among the groups were analysed by using one-way ANOVA combined with the Bonferroni test. Values were expressed as a mean ± S.E.M. and differences between groups were considered to be significant at P < 0.05.
5.4 Results

5.4.1 Glomerular filtration rate (GFR)

The effect of hemin on renal function was assessed on the rate of creatinine clearance in the kidney, a reliable method to approximate GFR. A significant reduction was observed within the control group after 48 h cold storage (CS) when compared to unstored kidneys (P <0.05) (Figure 5.1). Pre-treatment with hemin (i.p.) resulted in a significant (P <0.05) reduction of GFR in kidneys subjected to 24 h CS in comparison to control. In contrast to control, hemin treatment (i.p. +i.v.) produced, a significant reduction in GFR both in unstored and 24h CS kidneys (P <0.05).
Figure 5.1 Effect of cold storage and reperfusion on Glomerular filtration rate in isolated rabbit kidney.

Rabbits were administered hemin (i.p. or i.p. + i.v.) and kidneys were removed 24 h after treatment. Kidneys were then subjected to different periods (0, 24 or 48 h) of cold storage prior to reperfusion on the *ex vivo* system as described in Methods and Materials. Glomerular filtration rate was measured in the hemin-treated group and compared to controls (untreated kidney). Bars represent the mean ± S.E.M. of n = 6 experiments.* P <0.05 vs. 0 hour cold storage within the same group, † P <0.05 vs. controls.
5.4.2 Glucose and sodium reabsorption

Under normal physiological conditions the end product urine should be considered glucose-free. Glucose reabsorption remained unchanged with prolonged CS in control kidneys (Figure 5.2 A). In contrast, hemin treatment (i.p.) led to a significant decrease in the ability to reabsorb glucose after 24 and 48 h CS when compared to control (P <0.05). Hemin treatment (i.p. + i.v.) reduced glucose reabsorption significantly (P <0.05) only after 48 h CS, in contrast to control.

The proximal tubule of the nephron plays a major role in sodium reabsorption and production of concentration gradients that ultimately concentrate the urine. Hence, a reduction of sodium in urine reflects a loss in the ability of the renal tubule to concentrate urine. A significant (P <0.05) increase in sodium reabsorption in control kidneys was observed of 24 h CS but this increase declined with prolonged cold storage (48 h) Figure 5.2B. In comparison to control, sodium reabsorption was significantly (P <0.05) reduced after 24 h CS in hemin-treated (i.p.) kidneys. In contrast, unstored hemin-treated (i.p. + i.v.) kidneys illustrated a significant (P <0.05) increase in sodium reabsorption, in contrast to control.
Figure 5.2  Effect of cold storage and reperfusion on glucose and sodium reabsorption in isolated rabbit kidney

Rabbits were administered with hemin (i.p. or i.p. + i.v.) and kidneys were removed 24 h after treatment. Kidneys were then subjected to different periods (0, 24 or 48 h) of cold storage prior to reperfusion on the *ex vivo* system as described in Methods and Materials. Glucose and sodium were measured from urine and perfusate samples in the hemin-treated group and compared to controls (untreated kidney). Bars represent the mean ± S.E.M. of n = 6 experiments.* P<0.05 vs. 0 hour cold storage within the same group, † P<0.05 vs. controls. A: Glucose reabsorption (% of total glucose); B: Sodium reabsorption (% of total sodium)
5.4.3 Urine and perfusate flow rate

There was a significant (P <0.05) and gradual decrease in urine flow in control kidneys with prolonged hypothermic storage (Figure 5.3 A). A similar decrease in urine flow was also observed after 24 h CS in hemin-treated treated (i.p.) kidneys which was maintained after 48 CS. In contrast, unstored hemin-treated (i.p. + i.v.) displayed a significant (P <0.05) reduction in urine flow in comparison to control.

Hemin treatment (i.p.) lead to a significant (P <0.05) decrease in perfusate flow rate in unstored kidneys when compared to control (Figure 5.3 B). In contrast hemin treatment (i.p. + i.v.) lead to a significant (P <0.05) reduction not only in unstored but also after 24 h CS kidneys in comparison to control kidneys.
Rabbits were administered with hemin (i.p. or i.p. + i.v.) and kidneys were removed 24 h after treatment. Kidneys were then subjected to different periods (0, 24 or 48 h) of cold storage prior to reperfusion on the eX vivo system as described in Methods and Materials. Urine and perfusate flow rate were measured in the hemin-treated group and compared to controls (untreated kidney). Bars represent the mean ± S.E.M. of n = 6 experiments. * P<0.05 vs. 0 hour cold storage within the same group, † P<0.05 vs. controls. A: Urine flow (ml/min); B: Perfusate flow (ml/min)
5.4.4

Control kidneys only exhibited a slight increase in protein leakage with prolonged CS (Figure 5.4 A). Similar results were obtained for hemin treated (i.p.) kidneys, indicating that prolonged CS did not have a great impact on protein leakage. However, protein leakage was significantly (P <0.05) increased with hemin treatment (i.p. + i.v.) and increased with prolonged hypothermic storage (24 and 48 hour) when compared to control.

Generally, all experimental groups demonstrated a significant (P <0.05) and gradual increase in γ-GT activity with prolonged hypothermic storage (Figure 5.4 B). In addition, there was no significant difference between hemin treatment (i.p.) and control. However, in hemin-treated (i.p. + i.v.) kidneys, γ-GT activity increased significantly (P <0.05) after 24 and 48 hour CS.
Figure 5.4 Effect of cold storage and reperfusion on protein leakage and γ-glutamyl transpeptidase activity in isolated perfused rabbit kidney

Rabbits were administered with hemin (i.p. or i.p. + i.v.) and kidneys were removed 24 h after treatment. Kidneys were then subjected to different periods (0, 24 or 48 h) of cold storage prior to reperfusion on the *ex vivo* system as described in Methods and Materials. Protein leakage and gamma glutamyl transpeptidase activity were measured in urine and perfusate samples in the hemin-treated group and compared to controls (untreated kidney). Bars represent the mean ± S.E.M. of n = 6 experiments.* P<0.05 vs. 0 hour cold storage within the same group, † P<0.05 vs. controls. A: Protein leakage (% of total protein concentration); B: γ-glutamyl transpeptidase activity (Units/l)
5.5 Histological Analysis

5.5.1 Light microscopy analysis

At the end of the cold storage and *ex vivo* reperfusion, kidneys samples were fixed and embedded in paraffin for light microscopy analysis. Quantitative assessment was performed by light microscopy focusing on the cortex, convoluted and straight portion of proximal tubules, distal tubule (medullary thick ascending tubule (mTAT)) and collecting tubules. In this study, we observed significant changes with hypothermic storage and reperfusion mainly in the mTAT. This region has been shown to be susceptible to hypoxia and cold storage (162).

Morphological changes included tubular cell vacuolisation, necrosis as manifested by nuclear pyknosis, disruption of cell membranes and intratubular cell detachment. The extent of tubular damage was graded as mild to severe depending on the percentage of tubular damage. On the whole, there was no striking difference in the different regions of the kidney after *ex vivo* perfusion in the unstored hemin-treated kidneys when compared to control (Figure 5.5). After 24 h CS followed by *ex vivo* reperfusion, the percentage of damage tubules seen was significantly (P< 0.05) lower in mTAT of hemin-treated kidneys (i.p.) (Plate 5.1B) when compared to control kidneys (Plate 5.1 A) (Figure 5.6 C). In contrast, hemin treatment (i.p. + i.v.) did not reduce or increase the number of damaged tubules when compared to control after 24 h CS and *ex vivo* reperfusion.

In addition, hemin treatment (i.p.) significantly (P< 0.05) reduced the number of damaged mTAT when compared to control (Figure 5.7 C) after 48 h CS and *ex vivo* reperfusion. However, hemin treatment (i.p. + i.v.) (Plate 5.2 B) significantly (P< 0.05) increased the percentage of damage tubules in the cortex when compared to control (Plate 5.2 A) (Figure 5.7 A).
Figure 5.5  Light microscopy (LM) analysis of unstored kidneys after ex vivo reperfusion
Rabbits were administered with hemin (i.p. or i.p. + i.v.) and kidneys were removed 24 h after treatment. Tubular damage in harvested kidney was then assessed. Bars represent the mean ± S.E.M. of n = 6 experiments.* P<0.05 vs. controls. A: cortex (proximal convoluted tubules), B: outer stripe of outer medulla (mainly the straight portion of the proximal convoluted tubules), C: inner stripe of outer medulla (mainly the thick ascending loops of Henle) and D: inner medulla (collecting ducts)
Figure 5.6 Light microscopy (LM) analysis of kidneys after 24 hour cold storage in UW solution followed by ex vivo reperfusion

Rabbits were administered with hemin (i.p. or i.p. + i.v.) and kidneys were removed 24 h after treatment. Kidneys were then subjected to 24 hour cold storage in UW solution as described in Methods and Materials. Tubular damage in the isolated kidney was assessed. Bars represent the mean ± S.E.M. of n = 6 experiments.* P<0.05 vs. controls. A: cortex (proximal convoluted tubules), B: outer stripe of outer medulla (mainly the straight portion of the proximal convoluted tubules), C: inner stripe of outer medulla (mainly the thick ascending loops of Henle) and D: inner medulla (collecting ducts)
Plate 5.1 Light micrograph showing tubular injury in the medullary region after 24 hour cold storage in UW solution followed by ex vivo reperfusion (magnification x 200).

Plate 5.1 A Control group. The kidney was removed from the rabbit, stored for 24 h in cold UW solution followed by ex vivo reperfusion, processed and analysed for LM. Severe injury was evident in the inner stripe of the outer medullar (which includes the mTAT and collecting tubules). Morphological changes seen were nuclear pyknosis and the presence of intratubular cell debris.

Plate 5.1 B Hemin (i.p.) group. The kidney was removed from the rabbit 24 h after hemin (i.p.) treatment, stored for 24 h in cold UW solution followed by ex vivo reperfusion, processed and analysed for LM. Renal structure was maintained with mild intratubular casts and there was no sign of apoptosis or necrosis.
CHAPTER 5  

**EX VIVO STUDIES**

A  

Proximal Convoluted Tubule  

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Hemin (i.p.)</th>
<th>Hemin (i.p. + i.v.)</th>
</tr>
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<tbody>
<tr>
<td>% of damaged tubules per field of view</td>
<td>0</td>
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B  

Proximal Straight Tubule  

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</table>

C  

Medullary Thick Ascending Tubule  

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<tbody>
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<td>0</td>
<td>25</td>
<td>50</td>
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</table>

D  

Collecting Duct  

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<th>Hemin (i.p. + i.v.)</th>
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<tbody>
<tr>
<td>% of damaged tubules per field of view</td>
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<td>50</td>
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</table>

Figure 5.7  Light microscopy (LM) analysis of kidneys after 48 hour cold storage in UW solution followed by *ex vivo* reperfusion

Rabbits were administered with hemin (i.p. or i.p. + i.v.) and kidneys were removed 24 h after treatment. Kidneys were then subjected to 48 hour cold storage in UW solution as described in Methods and Materials. Tubular damage in the isolated kidney was assessed. Bars represent the mean ± S.E.M. of n = 6 experiments.* P<0.05 vs. controls. A: cortex (proximal convoluted tubules), B: outer stripe of outer medulla (mainly the straight portion of the proximal convoluted tubules), C: inner stripe of outer medulla (mainly the thick ascending loops of Henle) and D: inner medulla (collecting ducts).
Plate 5.2 A

Control group. The kidney was removed from the rabbit, stored for 48 h in cold UW solution followed by \textit{ex vivo} reperfusion, processed and analysed for LM. Severe intratubular casts with evidence of cell death in tubular cells can be seen in the proximal and distal tubules. These tubules are also surrounding by oedematous glomeruli.

Plate 5.2 B

Hemin (i.p. + i.v.) group. The kidney was removed from the rabbit 24 h after hemin (i.p. + i.v.) treatment, stored for 48 h in cold UW solution followed by \textit{ex vivo} reperfusion, processed and analysed for LM. Although there is evidence of intratubular casts the general ultrasturucture of the kidney is well maintained.
5.5.2 Electron microscopy analysis

Electron microscopy was performed on ex vivo perfused kidney sections. The cortex proximal and distal tubules, and medullary thick ascending tubule (mTAT) were scored for tubular damage according to the scoring system described in the Materials and Methods section.

The cortex proximal tubules of ex vivo perfused unstored kidneys of all the experimental groups suffered minor focal damage. In addition, no striking structural differences were seen in the cortex distal tubules and mTAT of all experimental groups (Plate 5.3 A to C). Interestingly, although these kidneys were not subjected to prolonged cold storage all groups exhibited moderate to severe injury in the mTAT after ex vivo reperfusion.

In kidneys subjected to 24 CS and ex vivo reperfusion, hemin-treated kidneys (i.p.) (Plate 5.4 C) illustrated moderate damage in the mTAT (Table 5.1) in comparison to control (Plate 5.4 A) or hemin-treated (i.p. + i.v.) kidneys (Plate 5.4 C) that exhibited severe damage. According to LM analysis, 48 CS plus ex vivo reperfusion lead to ultrastructural damage in all groups. However, EM analysis illustrated more damage in the mTAT of control (Plate 5.5 A) and hemin-treated (i.p. + i.v.) kidneys (Plate 5.5 B) in comparison to hemin-treated (i.p.) kidneys (Plate 5.5 C).
Table 5.1 Summary of data from electron microscopy (EM) analysis of renal tissues subjected to hypothermic storage and reperfusion.

<table>
<thead>
<tr>
<th></th>
<th>0 h CST HEMIN (i.p.)</th>
<th>0 h CST HEMIN (i.p. + i.v.)</th>
<th>24 h CST HEMIN (i.p.)</th>
<th>24 h CST HEMIN (i.p. + i.v.)</th>
<th>48 h CST HEMIN (i.p.)</th>
<th>48 h CST HEMIN (i.p. + i.v.)</th>
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<td>Cortex Proximal tubule</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Cortex Distal tubule</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Medulla Thick</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Ascending tubule</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Kidney sections were scored and rated as:

0 = no damage
+
++ = moderate damage
+++ = severe damage
Plates 5.3 Electron micrograph of thick ascending renal tubules of unstored kidneys followed by ex vivo reperfusion (magnification x 5000).

Plate 5.3 A Control group. The kidney was removed from the rabbit, followed by ex vivo reperfusion, processed and finally analysed for EM. Mitochondria are swollen, the apex cell area is broken with few cell debris.

Plate 5.3 B Hemin (i.p + i.v.) group. The kidney was removed from the rabbit 24 h after hemin (i.p. + i.v.) treatment followed by ex vivo reperfusion, processed and analysed by EM. The mitochondria are swollen, the apex cell area is disrupted, vacuolation and luminal debris are visible.

Plate 5.3 C Hemin (i.p.) group. The kidney was removed from the rabbit 24 h after hemin (i.p.) treatment followed by ex vivo reperfusion, processed and analysed by EM. Mitochondria are slightly swollen, there is some vacuolation but the lumen is still intact.
Plates 5.4 Electron micrograph of thick ascending renal tubules 24 hour after cold storage in UW solution followed by ex vivo reperfusion (magnification x 5000).

Plate 5.4 A Control group. The kidney was removed from the rabbit, stored for 24 h in cold UW solution followed by ex vivo reperfusion, processed and finally analysed for EM. Mitochondria are swollen with disrupted cristae, some vacuolation is present with loss of cellular material into the lumen leading to occlusion of the lumen.

Plate 5.4 B Hemin (i.p. + i.v.) group. The kidney was removed from the rabbit 24 h after hemin (i.p. + i.v.) treatment, stored for 24 h in cold UW solution followed by ex vivo reperfusion, processed and finally analysed for EM. Mitochondria structures appear to be well maintained, the apex cell area is slightly disrupted, some vacuolation but no luminal debris.

Plate 5.4 C Hemin (i.p.) group. The kidney was removed from the rabbit 24 h after hemin (i.p.) treatment, stored for 24 h in cold UW solution followed by ex vivo reperfusion, processed and finally analysed for EM. Mitochondria structures appear to be well maintained and no debris were present in the lumen.
Plate 5.5 A Control group. The kidney was removed from the rabbit, stored for 48 h in cold UW solution followed by *ex vivo* reperfusion, processed and finally analysed for EM. The mitochondria are slightly swollen, apex cell area disrupted, vacuolation is present with luminal debris.

Plate 5.5 B Hemin (i.p + i.v.) group. The kidney was removed from the rabbit 24 h after hemin (i.p. + i.v.) treatment, stored for 48h in cold UW solution followed by *ex vivo* reperfusion, processed and finally analysed for EM. The mitochondria are severely swollen and disrupted, the apex cell area is damaged; gross vacuolation and luminal debris are visible.

Plate 5.5 C Hemin (i.p.) group. The kidney was removed from the rabbit 24 h after hemin (i.p.) treatment, stored for 48h in cold UW solution followed by *ex vivo* reperfusion, processed and finally analysed for EM. Mitochondria are slightly swollen, the cell apex is broken with luminal debris, and the ultrastructure presents an oedematous appearance.

Plates 5.5 Electron micrograph of thick ascending renal tubules 48hour after cold storage in UW solution followed by *ex vivo* reperfusion (magnification x 5000)
5.6 Discussion:
In this study we assessed the possible benefits of upregulating the antioxidant pathway of HO-1 using hemin in rabbit kidneys prior to hypothermic storage and \textit{ex vivo} perfusion. The utilisation of the isolated perfused system enabled us to study the possible pathophysiological effects of hemin administration via two different modes (i.p. or i.p. plus i.v.) followed by hypothermic storage (0, 24 or 48 hours) on renal function \textit{ex vivo}. Warm (37°C) perfusion with an acellular solution supported oxidative metabolism of sufficient magnitude to restore urine flow \textit{ex vivo}. The parameters of renal function evaluated included GFR, urine flow rate, perfusate flow rate, sodium reabsorption, glucose reabsorption and indexes of tubular damage.

Although UW solution is clinically used as preservation solution, it only affords partial protection against cold storage (CS) injury. Even with improvements of preservation solutions by the addition of pharmacological agents lipid peroxidation and cell injury continues to occur in hypothermically stored organs. Oxygen delivery to hypothermically stored organs during reperfusion is known to be detrimental due to the generation of reactive oxygen metabolites such as superoxide anions, hydroxyl radicals and hydrogen peroxides. These molecules are generated during ischaemia followed by reperfusion and are known to have a deleterious effect on cellular constituents, such as proteins, lipids and DNA (164;165).

Electron microscopy studies illustrated injury in the mTAT of unstored kidneys which we speculate may be due the synergistic effect of surgery, warm ischaemia and \textit{ex vivo} reperfusion.

Renal kidney function was mainly impaired after exposure to cold storage of 24 h, and this deteriorated further with prolonged hypothermic storage of 48 h. The damage sustained during CS and \textit{ex vivo} reperfusion has been shown to be mediated by a variety of biochemical and molecular mechanisms which interact with vital structures and thereby impair the ability of the kidney to restore normal function(166). A critical mechanism mediated by membrane destruction is peroxidation of lipids subsequent to cellular stress (167) the cell
membrane structure for example virtually affects all aspects of cellular metabolism and organ function when damaged. Interestingly, studies by Gower et al. (168;169) demonstrated injury and lipid peroxidation in rabbit kidneys subjected to cold storage and ex vivo perfusion by the detection of lipid peroxidation products MDA and Schiff bases. Although we did not measure lipid peroxidation products in this study, we can speculate that decline in renal function with prolonged cold stored and reperfusion may be partially due to injury incurred at least in part as a results of cold storage and reoxygenation during reperfusion.

The glomerular filtration rate decreased in all groups with CS, this served as a reflection of glomerular damage as the kidney losses its ability to clear creatinine. Hemin treatment in general did not improve GFR with prolonged CS, especially administration of hemin (i.p. + i.v.) which gave rise to decreased glomerular filtration in both unstored and hypothermically stored kidneys. This correlated with protein leakage, which was exacerbated by hemin (i.p. + i.v.), hypothermic storage and reperfusion indicating that hemin pre-treatment intravenously may not be beneficial to renal function.

Glucose reabsorption was not affected by hypothermic storage in control kidneys. Hemin treatment (i.p), however, impaired tubular reabsorption of glucose with hypothermic storage; in contrast, administration of hemin (i.p + i.v) reduced reabsorption of glucose only after 48 hours of CS.

In this study, sodium reabsorption increased after 24 h cold storage in control kidneys then declined to basal levels after 48 hour CS. These results appear to be contradictory with other reports as prolonged CS is expected to result in the depletion of adenosine triphosphate stores which may compromise adenosine triphosphate pump (Na+-K+-ATPase) thereby resulting in decreased active transport of sodium into the proximal tubules. Alternatively one would have expected to see no change with increased hypothermic storage due to the large volume of perfusate (2 litre) used for ex vivo perfusion. Therefore, the measurements of sodium reabsorption using the IPRK system
may not be sensitive enough to pick up significant functional changes. Specifically, since sodium reabsorption is normally controlled by the hormone angiotensin II (a factor absence in this model) which stimulates the proximal tubule in the kidney to reabsorb sodium ions. Nevertheless, hemin treatment (i.p.) did not improve or maintain sodium reabsorption with hypothermic storage. In contrast, hemin treatment (i.p. + i.v.) led to an initial increase in sodium reabsorption in unstored kidneys which was followed by a declined with prolonged (48 h) hypothermic storage.

Intravenous plus intraperitoneal administration of hemin decreased urine flow rate in kidneys that were not stored in cold preservation solution. Intraperitoneal (i.p.) hemin-treated and control kidneys exhibited a decline in urine flow only after hypothermic storage.

There was a decline in perfusate flow rate due to administration of hemin generally in unstored kidneys, while control kidneys maintained perfusate flow even with prolonged hypothermic storage.

Insignificant protein leakage was evident after 24 or 48 CS in control and hemin-treated kidneys (i.p.), while, i.p. + i.v hemin treatment elevated protein leakage tremendously before and after hypothermic storage.

Gamma glutamyl transferase (γ-GT) activity was markedly increased in all groups with prolonged cold storage, however hemin treatment i.p. + i.v. exacerbated γ-GT activity after hypothermic storage, indicating that hemin treatment i.p. + i.v. is detrimental to renal function as high activity of γ-glutamyl transferase in pooled urine/perfusate samples occurs as a result of damage to kidney tubules and is often used for the evaluation of proximal tubule damage in experimental animals and humans (170).

Human kidneys subjected to prolonged cold storage are often associated with early graft non-function due to acute tubular necrosis of the proximal tubules and distal tubules (171). Hence, it is not surprising that hypothermic storage and reperfusion lead to histological changes in this study. Histological analysis of both light and electron microscopy clearly illustrate that hemin
administration (i.p.) maintained mTAT tubules (distal tubule) thereby protecting the kidney from severe postischaemic structural damage seen in controls. In contrast, hemin treatment (i.p. + i.v.) led to severe tubular damage in the cortex with prolonged cold storage and reperfusion.

It has been demonstrated that the administration of hemin can ameliorate I/R injury in various organs such as the heart and kidney (126) (172). The inducer of haem oxygenase-1 hemin (iron-protoporphyrin IX) is a versatile molecule in nature and serves as the prosthetic moiety for numerous haemoproteins involved in oxygen delivery and electron transfer. In the haem oxygenase pathway catalysed by HO-1, the protoporphyrin is converted into biliverdin, accompanied by release of carbon monoxide and liberation of iron in the ferrous form (Fe²⁺), a powerful pro-oxidant used in the Fenton reaction and other biochemical pathways to facilitate free radical formation. The potential toxicity of iron can be governed by absorption. Iron is absorbed in the ferrous state by cells of the intestinal mucosal. Normally gastric and intestinal secretions reduce Fe³⁺ to Fe²⁺ making available for absorption. However, in abundance, ferrous iron can have a major role in the initiation and propagation of lipid peroxidation by catalysing the conversion of superoxide to hydroxyl radicals. Furthermore, iron can directly catalyze lipid peroxidation, the oxidative reaction of polyunsaturated lipids, by removal of hydrogen atoms from the polyunsaturated fatty acids in the lipid bilayers of organelle membranes.

Hence, hemin can promote free radical formation and lipid peroxidation, resulting in cell damage and tissue injury (173) (174) (175). This is supported by studies by Zager et al. (176) who demonstrated acute inhibition of the HO-1 enzyme exerted protection against oxidant injury of kidney proximal tubules isolated from rats with rhabdomyolysis. Organ damage may arise when haem protein, such as myoglobin in muscle or haemoglobin in erythrocytes, escape from the intracellular space. Such haem mediated injury may result in renal vasoconstriction, tubular toxicity and luminal obstruction. During rhabdomyolysis the haem center of myoglobin initiates lipid peroxidation,
release of free iron with subsequent renal injury. Zager and co-workers proved that iron chelation by desferrioxamine was also protective, thus suggested that free iron liberated by HO-1 activity was a key determinant of haem cytotoxicity.

In summary, hemin administration can promote beneficial or detrimental effects as shown by numerous studies. These results show that hemin administration (especially intravenously) is detrimental, possibly due to the overload of iron in the kidney. As a result, the kidney has to filter large amounts hemin which may precipitate within tubules and the epithelium resulting in compromised tubular integrity, impaired glomerular and tubular function as seen from the various functional parameters measured in this study.

5.7 Conclusion

In summary, the results of this study provide objective data to substantiate that hemin treatment intravenously (i.p. + i.v.) is detrimental to the kidney function, which is strongly supported by increased activity of γ-GT, elevated levels of proteinuria and correlating histological findings. Intraperitoneal hemin-treatment (i.p.) however, does not protect the kidney during hypothermic storage and reperfusion injury nor exacerbates damage incurred during hypothermic storage when compared to control kidneys.

Although hemin treatment in this study did not protect the kidney against hypothermic storage and ex vivo reperfusion, further studies using different modes of administration or non-nephrotoxic inducers of HO-1 may ameliorate the effect of cold ischaemia/reperfusion injury in the isolated perfused system (IPK). Therefore, combinational treatment of low concentration of hemin serving as the substrate of haem oxygenase plus another natural non-toxic inducer of HO-1 such as curcumin an antioxidant may prove to be a better therapeutic challenge offering functional and histological protection against ischaemia/reperfusion injury.
Furthermore, this IPK model does not contain any blood components. Therefore, a model with blood perfusion would be important to establish the real pathophysiology of HO-1 upregulation and prolonged cold storage. A transplant model would represent almost the only investigative tool that would simulate situations close to clinical conditions and this would be investigated in the next chapter.
6. THE ROLE OF HAEM OXYGENASE-1 (HO-1) DURING COLD STORAGE AND RENAL AUTOGRAFT TRANSPLANTATION IN RABBIT

6.1 Introduction

Several clinical studies have shown that prolonged renal cold storage subsequently leads to delayed graft function or acute tubular necrosis post transplantation (6; 177). Preservation solutions also contribute to poor renal function (178). Therefore, procedures to decrease cold ischaemic injury are required to reduce the risk of graft failure.

One approach to improve immediate and long-term graft function would be the use of protective agents able to attenuate reperfusion injury. Scavengers of oxygen free radicals such as recombinant superoxide dismutase failed to decrease the need for haemodialysis after transplantation, but significantly diminished the incidence of acute rejection and increased the graft half-life (179). Other investigators, added pharmacologic additives, adenosine and reduced glutathione to UW solution in order to improve graft viability (157). However, during revascularization, these additive agents were rapidly depleted by the high amount of reactive oxygen species produced in the ischaemic tissue.

Although early restitution of blood flow to ischaemic tissue is essential to stop the progression of cellular injury associated with decreased oxygen and nutrient delivery, reperfusion of ischaemic tissue can also initiate complex series of pathologic events leading to cellular dysfunction and necrosis. This results in production of large amounts of reactive oxygen agents (superoxide anions, hydroxyl radicals, and hydrogen peroxides) produced by the re-entry of oxygenated-blood during reperfusion. Hence, prolonged periods of circulatory arrest accompanied with hypothermic storage followed by reperfusion may lead to diverse insults.
Recent studies have shown that ischaemia and reperfusion is one factor that contributes to non-immunological injury that occurs after transplantation. An example is acute tubular necrosis (ATN) known to develop as a result of both warm and cold ischaemia which increases both post-transplantation morbidity and the early loss of transplanted kidneys. Consequently, the improvement of quality preservation and development of new preservation strategies could be an important challenge.

Haem oxygenase-1 (HO-1) has been described as an inducible protein capable of cytoprotection. Recent studies have shown extended preservation of rat liver graft by induction of haem oxygenase-1 (180). The authors illustrated that heat preconditioning and, specifically, overexpression of HO-1 improved post-transplantation survival and graft function after prolonged cold preservation. Furthermore, another study by a different group illustrated that the induction of haem oxygenase-1 prevented ischaemia/reperfusion injury and improved long-term graft outcome in rat renal allografts (181).

In previous chapters we have shown that treatment with hemin a substrate of heoxygenase can (1) induce HO-1 upregulation in the kidneys; (2) maintain upregulated HO-1 activity during hypothermic storage (24 hours); (3) can be toxic in the kidney if used improperly and (4) failed to confer protection after hypothermic storage and ex vivo reperfusion. However, in the transplant situation, the influence of blood components such as neutrophils can play a significant role in reperfusion injury. It may be that HO-1 upregulation could ameliorate this pathological event.

Thus the aim of this chapter is to describe the effect of haem oxygenase-1 (HO-1) induction prior to hypothermic storage and autotransplantation. On in vivo recovery of renal function and structure after prolonged up to 48 hours hypothermic storage.
6.2 Objective

The purpose of this study was to investigate (i) whether HO·1 up-regulation in kidneys could result in protection from prolonged hypothermic injury and (ii) improve \textit{in vivo} renal function.

6.3 Materials and Methods

\hspace{0.5cm} \textit{6.3.1 Preparation of reagent}

Hemin (ferrisprotoporphyrin IX chloride) was purchased from Porphyrin Products (Logan, Utah, USA). Stock solutions were prepared by solubilising hemin in PBS (pH 7.4) with the addition of 20μl of 2M NaOH; the solution was then sterilized through a 0.22μm filter before injecting animal with 50mg /kg body weight. Rabbits were administered with either vehicle or hemin 50mg/kg (i.p. plus subcutaneous) 24 h prior to grafting of donor organ.

6.4 Surgical Procedure

\hspace{0.5cm} \textit{6.4.1 Experimental groups}

Experiments were performed on 36 NZW rabbits (2.5-4.0 kg) 5-6 animals were utilized per group. Animals received vehicle or hemin, 24 h prior to autografting of kidneys previously subjected to (0-48 hour cold storage (CS) in UW solution). The groups used for this study are shown in Table 6.1.
Table 6.1 Experimental groups in renal autotransplant study

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Treatment</th>
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<tr>
<td>Group 1A</td>
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</tr>
<tr>
<td>Group 1B</td>
<td>Hemin-treatment 24 hour prior to nephrectomy and immediate autotransplanted</td>
</tr>
<tr>
<td>Group 2A</td>
<td>Nephrectomy, 24 hour CS prior to autotransplantation</td>
</tr>
<tr>
<td>Group 2B</td>
<td>Nephrectomy, 24 hour CS and hemin-treatment 24 hour prior to autotransplantation</td>
</tr>
<tr>
<td>Group 3A</td>
<td>Nephrectomy, 48 hour CS prior to autotransplantation</td>
</tr>
<tr>
<td>Group 3B</td>
<td>Nephrectomy, 48 hour CS and hemin-treatment 24 hour prior to autotransplantation</td>
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6.4.2 Surgical Anaesthesia

All animal procedures were carried out according to Home Office regulation, Animal (Scientific Procedure) Act, 1986. All surgical procedures were carried out by Mr. C Shurey. For general anaesthesia rabbits were deeply sedated with an intra-muscular (i.m.) injection of Hypnorm (Fentanyl 0.315 mg/ml + Fluanisone 10 mg/ml) at 0.3 ml/kg intramuscular (i.m.) followed by a slow intravenous (i.v.) injection of Valium (Diazepam) five minutes later at 1.0 mg/kg. Intravenous bolus of 0.1 ml Hypnorm and 1mg of Diazepam was administered as necessary via an intravenous saline drip attached to a marginal ear vein for the maintenance of anaesthesia during the whole surgical procedure. Animals were supplied with 100% oxygen at 500ml/min via an open face mask throughout the surgical procedure.

6.4.3 Nephrectomy Procedures

The anaesthetized rabbit was shaved abdominally and dressed in previously autoclaved drapes. The right kidney was exposed through a mid-line abdominal incision. The intestines were gently displaced, externalised,
swathed in warm damp sterile swabs and supported on a sandbag raised slightly above the level of the peritoneum. The connective tissue surrounding the right kidney was carefully separated and the renal vessels divided and ligated as close to the aorta as possible using 4/0 silk thread. The renal artery was then cannulated with a 22- gauge cannula, and flushed with 30 ml ice-cold University of Wisconsin solution (UW; 0-2°C) from an ice-cooled bag suspended at a height of 1.5m. The maximum perfusion pressure (measured using a pressure-gauge) was therefore 90 mmHg.

The perfused kidney was plunged into a sterile glass beaker containing UW (0-2°C) and the beaker was surrounded by ice. The perfused kidney was then either autografted immediately into the donor rabbit or stored for 24 or 48 h within a closed polystyrene container packed with ice and held in a refrigerator prior to autografting. The storage times was thus maintained at a steady 0-2°C. The intestines were then gently replaced into the abdomen and the abdominal muscles were closed with interrupted 0 Vicryl suture whilst the skin was closed with interrupted 2/0 silk suture.

6.4.4 Autografting Procedures

After the storage period, kidneys were autografted into the donor rabbit. The rabbits were anaesthetized as described above, and the body cavity opened along the same mid-line incision. The left kidney was carefully dissected out from the connective tissue and removed after clamping the renal artery and vein proximal to the kidney using weighted bulldog clamps with protective rubber housing. The ureter was severed close to the kidney. The experimental graft (stored or unstored) was then autografted into the left renal bursa. The severed ends of the renal vein were carefully cleaned using isotonic saline to remove blood clots and procaine to prevent nervous or muscular spasm, and placed within the jaws of a double clamp. The ends were clipped with microsurgical scissors to ensure removal of tissue damaged during handling, and immobilized with stay sutures using 8/0
ethion suture under an operating microscope. The vein was then anastomosed using approximately 15 interrupted sutures to ensure strength, and the procedure repeated for the renal artery (with the same number of sutures). Upon reperfusion of autografted kidneys (by removal of clamps), recipients rabbits received a 1 ml bolus i.v. of frusemide and a 60 ml slow i.v. infusion of sodium chloride solution (0.9%). The bowel was then replaced in the abdominal cavity, abdominal muscles are closed with 0 Vicryl sutures taking care of the peritoneal lining, while the skin was closed with interrupted horizontal mattress 2/0 Silk sutures. Sixty ml of saline was administered subcutaneously (s.c.) along with 20mg frusemide i.m. and 10ml mannitol i.v. daily for 4 days post-operatively.

6.4.5 Post transplant management

Blood samples were withdrawn from the marginal ear vein into heparinised syringes before the nephrectomy procedure, grafting, on day 2 post-operatively and then on alternative days until the end of the experimental period (28 days). These blood samples were centrifuged at 3000 rpm for 3 minutes and the serum was store -20°C until assayed for creatinine and urea concentration as an indicator of kidney function.

Animals were visually assessed daily for good health and apparent kidney graft condition. Clinical signs of renal failure were taken as oscillating eyes movements, ataxia, diarrhoea, gelatinous urine and ultimately death. Animals deemed to be in poor condition with the unlikely chance of recovery were euthanized. Whilst animals that survived the experimental period of 28 days were finally euthanized at this time. The transplanted kidney was then excised and processed for histology.

6.4.6 Renal Histology

Sections from the cortex and medulla were obtained from the kidneys explanted and processed for electron microscopy (EM) analysis, as described
in Materials and Methods. A grading scale of 0·3 (Table 2.1) was used for EM assessment.

For light microscopy (LM) the kidneys were preserved in 10% buffered formalin dehydrated and then embedded in paraffin. Sections (3 µm) were cut and stained with haematoxylin and eosin. Histopathological evaluation was carried out by a clinical pathologist blinded to the experimental procedures. Each slide was assessed for morphology and quantified for injury according to the method adapted from Banff 97 working classification of renal allograft pathology(182). The Banff 97 scoring system was developed by a group of investigators for the standardization of renal allograft biopsy interpretation.

Each H & E section was examined for injury in the tubules, glomerulus and interstitium and in each region different factors that influenced the degree of injury (Table 6.1) were considered. To each parameter a semi quantitative score was assigned (0-3) reflecting the severity of injury (score 1, less than 10 % of the tissue section was involved; score 2, 10 - 50 %; score 3> 50 %).
Table 6.2  Summary of the criteria investigated using the modified Banff 97 scoring system for light microscopy (LM) analysis

<table>
<thead>
<tr>
<th>Tubules (T)</th>
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<tbody>
<tr>
<td>Epithelial vacuolisation</td>
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<tr>
<td>Dilatation</td>
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<td>Necrosis</td>
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<td>Casts</td>
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<tbody>
<tr>
<td>Number</td>
<td></td>
</tr>
<tr>
<td>Mesangial matrix</td>
<td></td>
</tr>
<tr>
<td>BM duplication</td>
<td></td>
</tr>
<tr>
<td>Sclerosis</td>
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<table>
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<th>Interstitium (I)</th>
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<tbody>
<tr>
<td>Inflammation</td>
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<tr>
<td>Oedema</td>
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<tr>
<td>Fibrin</td>
<td></td>
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<tr>
<td>Haemorrhage</td>
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<tr>
<td>Fibrosis</td>
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<tr>
<td>Infarction</td>
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6.5 Results

6.5.1 Renal function after transplantation

The effects of hemin and hypothermic storage on renal function are assessed by the measurements of serum creatinine and urea levels. Serum samples were collected on alternating days over a period of 24 days for the measurements of creatinine and urea.

Animals transplanted with control kidney grafts not exposed to hypothermia (Group1A) showed normal graft function after transplantation. This was supported by low levels of creatinine and urea obtained from their serum samples. However, at day 12 two animals showed slightly elevated levels but survived to term (Figure 6.1 and 6.2).
In contrast hemin-treated kidney autografts (Group 1B) produced high serum creatinine and urea levels in most animals resulting in the death of 2 animals at day 4 and 22 respectively.

Untreated kidneys cold stored for 24 hours followed by transplantation (Group 2A) resulted in extremely high levels of creatinine and urea. Interestingly 3 animals had high levels of creatinine and urea resulting in the death of 2 animals on day 2, followed by the death of the third animal by day 15. In summary only 1/5 animals survived to term in this group (Group 2A).
Figure 6.1 Effect of hemin treatment and cold storage on serum creatinine levels following kidney transplantation
Concentration of serum creatinine (μM) in Group 1A (unstored kidney immediately autotransplanted), 1B (hemin treatment 24 h prior to immediate graft autotransplant), 2A (graft autotransplant of 24 h cold stored kidney), 2B (hemin treatment 24 h prior to graft autotransplant of 24 h cold stored kidney), 3A (graft autotransplant of 48 h cold stored kidney) and 3B (hemin treatment 24 h prior to graft autotransplant of 48 h cold stored kidney). Discontinuation of a line denotes killing of animal. † Mortality crosses.
Figure 6.2 Effect of hemin treatment and cold storage on serum urea levels following kidney transplantation

Concentration of serum urea (µM) in Group 1A (unstored kidney immediately autotransplanted), 1B (hemin treatment 24 h prior to immediate graft autotransplant), 2A (graft autotransplant of 24 h cold stored kidney), 2B (hemin treatment 24 h prior to graft autotransplant of 24 h cold stored kidney), 3A (graft autotransplant of 48 h cold stored kidney) and 3B (hemin treatment 24 h prior to graft autotransplant of 48 h cold stored kidney). Discontinuation of a line denotes killing of animal.
6.5.2 *Effect of hemin treatment on graft survival*

Renal function in untreated and hemin-treated kidneys stored for 0, 24, or 48 hour before autografting was monitored by analysis of serum creatinine and urea levels (Figure 6.1 and 6.2). All animals displaying clinical symptoms of uraemia were killed immediately. Untreated kidneys (Group 1A) or hemin treatment (Group 1B) grafts were harvested flushed and transplanted (Figure 6.3A). In Group 1A all animals (n=6) survived to term, although while 5/6 hemin-treated kidney recipients survived to term. After 24 hours of cold storage, untreated kidney (Group 2A) or hemin treatment (Group 2B) were transplanted. Only 1/6 animals survived to term in Group 2A (Figure 6.3B), in contrast 5/6 animals in Group 2B survived to term. Hence, less than 17% of the control animals survived to term post-transplantation, while the hemin-treated group (Group 3B) had 83% of the animals survive to term (83% vs. 17% p<0.05). After 48 hour cold storage, all 5/6 animals died before the end of term in both control (Group 3A) and hemin-treated (Group 3B) (Figure 6.3C).
Figure 6.3 Percentage of Survival following kidney transplantation

Percentage of survival was calculated using the method of Kaplan and Meier. (A) Percentage of survival over 28 days in Group 1A (control kidney, flushed and immediately autotransplanted) and Group 1B (hemin-treated kidney, flushed and immediately autotransplanted). (B) Percentage of survival in Group 2A (control kidney, flushed and stored for 24 h prior to autotransplantation) and Group 2B (hemin-treated kidney, flushed and stored for 24 h, prior to autotransplantation). (C) Percentage of survival in Group 3A (control kidney, flushed and stored for 48 h prior to autotransplantation) and Group 3B (hemin-treated kidney, flushed and stored for 48 h prior to autotransplantation).
6.6 Histology

6.5.3 Electron microscopy analysis

Histological examinations of different renal structures obtained from renal grafts after euthanasia were similar in both experimental groups 1A and 1B (Table 6.3) showing no significant difference. Kidney sections obtained from Group 1A showed mild injury in the cortex proximal tubules with scattered vacuolisation (Plate 6.1), while the medullary thick ascending tubule showed severe injury (Plate 6.3). In comparison, the cortex proximal tubules in Group 1B suffered from luminal obstruction plus vacuolisation (Plate 6.2) while, the medullary thick ascending tubules suffered from oedema (Plate 6.4).

The nephron segments examined from untreated (Group 2A) and hemin-treated (Group 2B) kidneys subjected to 24 hour cold storage prior to autotransplantation suffered from mild to moderate injury (Table 6.3). The tubules examined in Group 2A (Plate 6.7 and 6.9) showed severe vacuolisation and tubular obstruction. In contrast hemin treatment (Group 2A) produced apical vacuolisation in the proximal tubule (Plate 6.6) and a reduced lumen in the medullary thick ascending tubule (Plate 6.8) in contrast to the completely disrupted lumen seen in Group 2A.

Evidence of necrosis was seen with prolonged cold ischaemia (48 hours) in the medullary thick ascending tubules obtained from Group 3A (Plate 6.11) and 3B (Plate 6.12). On the whole, tubular injury was evident in both experimental groups and no significant differences were observed. Finally minimal injury was seen in the glomeruli of all experimental groups or renal grafts observed.
Table 6.3 Summary of data from electron microscopy (EM) analysis of rabbit renal autografts

<table>
<thead>
<tr>
<th></th>
<th>Group 1A</th>
<th>Group 1B</th>
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<tbody>
<tr>
<td>Cortex Proximal tubule</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cortex Distal tubule</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Medulla Thick Ascending tubules</td>
<td>+++</td>
<td>++</td>
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<tr>
<td>Glomerulus</td>
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<th></th>
<th>Group 2A</th>
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<tbody>
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<td>+</td>
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<tr>
<td>Cortex Distal tubule</td>
<td>+</td>
<td>0</td>
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<tr>
<td>Medulla Thick Ascending tubules</td>
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<td>Glomerulus</td>
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<table>
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<tr>
<th></th>
<th>Group 3A</th>
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<tbody>
<tr>
<td>Cortex Proximal tubule</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Cortex Distal tubule</td>
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<td>Glomerulus</td>
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Kidney sections were scored and rated as:

- 0 = no damage
- + = minor focal damage
- ++ = moderate damage
- +++ = severe damage
Plate 6.1  Electron micrograph showing renal proximal tubule in Group 1A: control kidney immediately flushed and autotransplanted (magnification x 5000). Evidence of apical and basal vacuolisation.

Plate 6.2  Electron micrograph showing renal proximal tubule in Group 1B: hemin-treated kidney immediately flushed and autotransplanted (magnification x 5000). The cells appear to be oedematous and lumen is congestion.
Plate 6.3  Electron micrograph showing thick ascending tubule in Group 1A: control kidney immediately flushed and autotransplanted (magnification x 5000). The cells are swollen with disruption of cell apex and presence of luminal debris.

Plate 6.4  Electron micrograph showing thick ascending tubule in Group 1B: hemin-treated kidney immediately flushed and autotransplanted (magnification x 5000) The cells are swollen, disruption of cell apex and lumen is congested with cell debris.
Plate 6.5  Electron micrograph showing renal proximal tubule in Group 2A: control kidney, flushed and stored for 24 h prior to autotransplantation (magnification x 5000). The cells are swollen, the apex is disrupted, vacuolation and luminal debris are visible.

Plate 6.6  Electron micrograph showing renal proximal tubule in Group 2B; hemin-treated kidney, flushed and stored for 24 h prior to autotransplantation (magnification x 5000). Finger like projections in the lumen, evidence of apical vacuolisation plus dark deposit.
Plate 6.7 Electron micrograph showing thick ascending tubules in Group 2A: control kidney, flushed and stored for 24 h prior to autotransplantation (magnification x 5000). Gross cellular disruption due to vacuolisation, oedema, evidence of chromatin condensation, the apex is disrupted and the lumen is partially blocked by cell debris.

Plate 6.8 Electron micrograph showing thick ascending tubules in Group 2B: hemin-treated kidney, flushed and stored for 24 h prior to autotransplantation (magnification x 5000). Cell appears slightly oedematous, the apex is disrupted and the lumen is partially blocked by cell debris.
Plate 6.9  Electron micrograph showing renal proximal tubule in Group 3A: control kidney, flushed and stored for 48 h prior to autotransplantation. (magnification x 5000). Gross vacuolisation, disruption of brush border and cellular debris in the lumen.

Plate 6.10  Electron micrograph showing renal proximal tubule in Group 3B: hemin-treated kidney, flushed and stored for 48 h prior to autotransplantation (magnification x 5000). No abnormalities were detected.
Plate 6.11 Electron micrograph showing thick ascending tubules in Group 3A: control kidney, flushed and stored for 48 h prior to autotransplantation (magnification x 5000).
Evidence of gross cellular necrosis.

Plate 6.12 Electron micrograph showing thick ascending tubules in Group 3B: hemin-treated kidney, flushed and stored for 48 h prior to autotransplantation (magnification x 5000).
Cellular disruption due to focal vacuolisation, the apex is disrupted and the lumen is partially blocked by cell debris.
6.5.4 Light microscopy analysis

Detailed light microscopy analysis showed no significant difference in Group 1A and 1B (Table 6.4). The type of injury observed was moderate tubular cell death in cortico-medullary section of kidneys obtained from Group 1A (Plate 6.13). In contrast, there was evidence of minor vacuolisation in the cortico-medullary regions in Group 1B (Plate 6.14) no other severe tubular injury was observed.

The untreated and hemin-treated kidneys subjected to 24 hour cold storage prior to autotransplantation revealed some interesting features (Table 6.4). Group 2A (Plate 6.15) illustrated less injury in contrast to Group 2B (Plate 6.16). Severe tubular injury was observed in tubules and interstitium of Group 2B, there were also high number of intracellular apoptotic bodies present (Plate 6.16) indicating cell death. Although, the EM results obtained from Group 2B showed more damage, the animals in this group survived to term, in contrast to Group 2A.

After 48 hour cold storage and autotransplantation both groups Group3A (Plate 6.17) and 3B (Plate 6.18) displayed severe injury in all renal structures observed and gross necrosis as illustrate in Table 6.4.
Table 6.4  Summary of data from light microscopy (LM) analysis of rabbit autografts

<table>
<thead>
<tr>
<th>Group 1A</th>
<th>Group 1B</th>
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<tbody>
<tr>
<td>Tubules</td>
<td>++</td>
</tr>
<tr>
<td>Interstitium</td>
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<tr>
<td>Glomeruli</td>
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<td>Glomeruli</td>
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<tbody>
<tr>
<td>Tubules</td>
<td>+++</td>
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<tr>
<td>Interstitium</td>
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<tr>
<td>Glomeruli</td>
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</table>

Kidney sections were scored and rated as:

0 = no damage
+ = minor focal damage
++ = moderate damage
+++ = severe damage
Plate 6.13 Light micrograph of the cortico-medullary section in Group 1A; control kidney flushed and immediately autotransplanted (magnification x 400). Showing moderate cell death (as indicated by arrows) in the renal tubules.

Plate 6.14 Light micrograph of the cortico-medullary section in Group 1B; hemin treated kidney immediately autotransplanted (magnification x 400). Evidence of minor vacuolation (as indicated by arrows) in the renal tubules.
Plate 6.15 Light micrograph of the medullary region in Group 2A; control kidney, flushed and stored for 24 h prior to autotransplantation. (magnification x 400). Mild tubular dilation, minimal cell death with some luminal obstruction.

Plate 6.16 Light micrograph of the medullary region in Group 2B; hemin treated kidney, flushed and stored for 24 h prior to autotransplantation. (magnification x 200). Apoptotic bodies are visible (as indicated by arrows) and there is evidence of moderate to severe cell death in the renal tubules.
Plate 6.17 Light micrograph of the cortical region in Group 3A (control kidney, flushed and stored for 48 h prior to autotransplantation) (magnification x 400). Dilated renal tubules showing cytoplasmic vacoulation and detachment with degenerating nuclei.

Plate 6.18 Light micrograph of the cortical region in Group 3B; hemin treated kidney, flushed and stored for 48 h prior to autotransplantation. (magnification x 400). Showing changes similar to above (Group 2A)
6.6 Discussion

The results presented in this chapter show that prolonged ischaemic time leads to decreased rates of successful renal function. Warm ischaemia, followed by a period of cold preservation and reperfusion, is a major contributor to ischaemia/reperfusion (I/R) injury (183) (184). Warm ischaemia causes damage through a gradual process in which biochemical alterations lead to functional impairment, followed by structural changes (9).

Furthermore, it is well known that hypothermic storage greater than 24 hours increases the occurrence of delayed graft function, elevates postoperative serum creatinine levels, and increases rate of chronic graft nephropathy. Prolonged hypothermia is also an independent factor leading to decreased graft survival rates at both 1 and 5 years after renal transplantation.

Our plan was to introduce the new paradigm of "preconditioning" the kidney prior to hypothermic storage, thereby minimizing cold associated renal injury. To test this hypothesis, HO-1 was induced with hemin pre-treatment 24 hours prior to auto transplantation. In the previous chapter (Chapter 4) we established the time course of hemin-induced HO-1 expression and discovered that HO-1 upregulation could be maintained up to 24 hours in cold UW solution. Therefore, we speculated that hemin-treated kidneys transplanted within the time frame of elevated HO-1 activity may be able to alleviate cold associated ischaemia reperfusion injury after transplantation.

From the renal functional data, it is interesting to note that hemin treatment lead to high serum creatinine and urea concentration in the unstored auto-transplanted kidneys; however, all these animals survived to term. Also, although hemin treatment indicated possible poor renal function, histological finding both by light and electron microscopy did not find any significant differences in renal injury between the unstored untreated and
hemin-treated kidneys. The earliest changes detectable by electron microscopy were gross vacuolisation in the proximal tubule and disruption of cell apex in the medullary thick ascending tubules (mTAT) of the unstored control autotransplanted kidneys. The unstored hemin-treated kidneys illustrated similar feature in the mTAT while the proximal tubule had a blocked lumen with protein deposition. Hence, these results clearly show that warm ischaemia followed by reperfusion injury can be responsible for physiological and morphological damage seen after short period of ischaemia.

The outcome following 24 hour cold storage, autotransplantation and contralateral nephrectomy differed markedly. The recipients of control renal grafts subjected to 24 hour cold storage prior to auto transplantation developed progressive renal failure and almost all the animals died before 24 days with the exception of one. The survival corresponded to the renal function data which showed extremely high serum creatinine and urea levels in all of these animals. However, histological examinations showed less renal injury in the renal tubules of this group in comparison to the hemin treated grafts. In contrast, hemin-treated grafts survived longer, in fact 5 out of 6 hemin-treated graft recipients survived to the end of the study period. However, the light microscopy analysis of the hemin-treated grafts showed severe injury in the tubules and interstitium. In addition, these grafts illustrated severe abnormalities such as apoptotic bodies, evidence of DNA fragmentation and morphological injury to the cortico-medullary region of the renal grafts. The morphological injury observed in the grafts almost makes it impossible to believe that the animals could survive to term.

It is possible that HO·1 upregulation in this group has cytoprotective effects at subcellular levels, in the face of the generalised injury seen at the light microscopic level. Thus, this gives some hope that, if a less injurious agent for HO·1 upregulation could be determined, an improved preservation strategy could be possible.
Prolonged hypothermic storage (48 hours), followed by auto-transplantation resulted in immediate poor renal function and severe histological injury. Histological studies in experimental groups, showed tubular interstitial fibrosis, and patchy necrosis. No significant differences were found between control and hemin-treated renal graft function or survival. In addition, most of the animals in both experimental groups died within a week post-transplant.

Although, HO-1 upregulation by hemin improved graft survival after 24 hours cold storage, the histological data suggest that hemin treatment was nephrotoxic. The exacerbation of this cellular injury may likely be due to the increase in free iron released after HO-1 induction.

Other investigators have demonstrated the nephrotoxicity of haem in renal tissue. Nath and co-workers (172) confirmed haem-dependent injury as renal haem content increased in the model of rhabdomyolysis-induced acute renal failure. Haem injury was also demonstrated by Maines et al (185) in renal ischaemia reperfusion injury and nephrotoxic induced acute renal failure by Agarwal et al (74).

Even though, hemin is a substrate of haem oxygenase it also possesses pro-oxidant properties. Excess haem can damage renal tubules, cell membranes, mitochondria, cytoskeleton and the nucleus (172). Unlike other organs, the kidney seems to be extremely sensitive to haem overload, therefore it is important to choose the right inducer of haem oxygenase in relation to renal function.

6.7 Conclusion

Overexpression of HO-1 prior to 24 hour cold storage and autotransplantation increased survival in rabbits. The beneficial effect of HO-1 induction by hemin was only seen during survival studies but no histological protection was provided. This study clearly indicated the need to choose a better less injurious inducer of HO-1, that does not possess any haem-mediated cytotoxicity.
Finally, the limited availability of organs requires the quest to minimize deleterious factors such as prolonged cold storage which may lead to delayed graft function or loss after transplantation. To prolong graft survival and the overall success of renal transplant a new strategy is required. The use of a combinational therapy for the induction of HO-1, using low concentration of hemin as the substrate of haem oxygenase and a new non-toxic inducers such as curcumin or CAPE may provide a new break through. Therefore, there is a need to search for novel inducers of haem oxygenase without any nephrotoxic effects (see chapter 7).
7. THE SEARCH FOR NOVEL INDUCERS OF HAEM OXYGENASE (HO-1)

7.1 Introduction
The expression of haem oxygenase-1 (HO-1) can be markedly augmented in eukaryotes by a wide range of substances that cause a transient change in the cellular redox state. The importance of this protein in physiological and pathological states is underlined by the versatility of HO-1 inducers and the protective effects attributed to haem oxygenase products in conditions that are associated with moderate or severe cellular stress. We have recently shown that curcumin and caffeic acid phenethyl ester (CAPE), two plant-derived polyphenolic compounds, are potent inducers of HO-1 in vascular endothelial and neuronal cells (186;187) and we hypothesized that part of the pleiotropic and beneficial actions attributed to these and other chemically related natural substances could be explained by their intrinsic ability to maximally activate of the haem oxygenase pathway(186). In view of the increasing evidence corroborating the importance of CO and bilirubin to counteract cellular dysfunction in a variety of experimental models (146;188-191), the activation of HO-1 by natural compounds offers a great advantage for therapeutic purposes as curcumin and CAPE could become part of the human diet and be consumed daily as herbal supplements. Both curcumin and CAPE have been reported to possess both anti-tumor and anti-inflammatory properties (192) (193) as well as protection against vascular and neurodegenerative diseases (194). Notably, under severe hypoxic conditions, the potency of curcumin to increase endothelial HO-1 expression and consequently protect cells against oxidative stress is highly amplified (195). These data indicate that certain natural compounds can trigger the stimulation of intrinsic antioxidant defensive systems even when the oxygen availability is impaired, a feature that is common to several vascular dysfunctions and inflammatory-related conditions.
Unlike most "classical" HO-1 inducers, which are strictly dependent on their oxidant potential to transcriptionally activate HO-1 gene expression, curcumin and CAPE are known to possess also antioxidant as well as anti-tumor and anti-inflammatory properties (196-198). The electrophilic characteristic of curcumin and several other structurally-related polyphenolic compounds have been recently shown to induce the activities of Phase 2 detoxification enzymes which appear to be crucial in protection against carcinogenesis and oxidative stress (199:200). Among these defensive systems are γ-glutamylcysteine synthetase, glutathione S-transferases and NADP(H):quinone oxidoreductase. The coordinated induction of these cytoprotective genes is mediated through cis-regulatory DNA sequences located in the promoter or enhancer region, which are known as antioxidant responsive elements (AREs) or stress responsive elements (StREs). The consensus ARE/StRE resembles the Maf-recognition element and can be specifically bound by a combination of the basic leucin zipper (bZIP) transcriptional factors including Jun, Fos, Maf and Nrf2. Among them, Nrf2 has been demonstrated to play a central role in the transcriptional regulation of antioxidant and detoxifying genes.

Studies using Nrf2-deficient mice have confirmed their inability to express cytoprotective genes upon stimulation with carcinogens (201). In addition, cells in which the Nrf2 gene was deleted displayed a higher susceptibility to oxidant-mediated cell injury and death (202). The implication of Nrf2 as a potent positive regulator of the HO-1 gene and other detoxifying enzymes in response to various agents has been recently reported (203). However, a direct link between curcumin-mediated HO-1 induction and activation of Nrf2 expression remains to be examined.

7.2 Objectives
The purpose of this study was to (i) search for novel and safe inducers of HO-1 and (ii) to analyze the involvement of Nrf2 activation in HO-1 induction by curcumin and CAPE in renal epithelial cells.
7.3 Materials and Methods

7.3.1 Reagents
Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione), caffeic acid phenethyl ester (CAPE) and were obtained from Sigma (Dorset, UK). Stock solutions of curcumin and CAPE (5 mM) were prepared in ethanol. Tissue culture media and fetal bovine serum were obtained from GIBCO Invitrogen Corporation (Paisley, Scotland, UK). Polyclonal antibodies for HO-1 were from Stressgen Bioreagents (Yorkshire, UK). Anti-Nrf2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals reagents were obtained from Sigma (Dorset, UK).

7.3.2 Cell culture and experimental protocols.
Porcine renal epithelial proximal tubule cells (LLC-PK1) and rat kidney epithelial cells (NRK-52E) were purchased from American Tissue Culture Collection (Manassas, VA, USA). Cells were cultured using Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were grown in 75 cm² flasks and kept at 37 °C in a humidified atmosphere of air and 5% CO₂. Confluent LLC-PK1 cells were exposed to various concentrations of curcumin, CAPE or other phenolic compounds for different times. After each treatment, cells were harvested for the measurements of haem oxygenase activity, HO-1 and Nrf2 protein expression.

7.3.3 Haem oxygenase activity assay and Western blot for haem oxygenase-1 (HO-1)
Haem oxygenase activity and HO-1 protein expression were determined as described in Materials and Methods.
7.3.4 Preparation of nuclear extract and Western blot for Nrf2

Nuclear extraction was performed as described in Materials and Method. The supernatant containing the nuclear proteins was loaded on a SDS-polyacrylamide gel and Western blot analysis using Nrf2 antibodies (1:500 dilution) was performed as described in Materials and methods.

7.3.5 Statistical analysis

Differences in the data among the groups were analyzed by using one-way ANOVA combined with the Bonferroni test. Values were expressed as a mean ± S.E.M. and differences between groups were considered to be significant at \( p < 0.05 \).

7.4 Results

7.4.1 Effect of curcumin and CAPE on haem oxygenase activity and HO-1 expression in renal epithelial cells.

Curcumin caused a significant increase in haem oxygenase activity (Figure 7.1 A) and HO-1 protein expression (Figure 7.1 B) with maximal activation being observed at 15 \( \mu \text{M} \). Higher concentrations of the agent were gradually less effective and no statistically-significant change in both activity and protein were detected at 15 \( \mu \text{M} \) curcumin. In a similar fashion, CAPE also caused a significant increase in haem oxygenase activity (Figure 7.2 A) and HO-1 protein expression (Figure 7.2 B) with maximal activation being observed at 20 \( \mu \text{M} \). A higher concentration of the agent (30 \( \mu \text{M} \)) was less effective although still significantly higher compared to controls.
Figure 7.1 Effect of curcumin on haem oxygenase activity and HO-1 expression in renal epithelial cells.

LLC-PK1 cells were exposed to various concentrations of curcumin for 6 h. Haem oxygenase activity (A) and HO-1 protein expression (B) were measured as described in Materials and Methods. Each bar represents the mean ± S.E.M. of five independent experiments. * $p<0.05$ vs. 0 μM curcumin.
Figure 7.2  Effect of CAPE on haem oxygenase activity and HO-1 expression in renal epithelial cells

LLC-PK₁ cells were exposed to various concentrations of CAPE for 6 h. Haem oxygenase activity (A) and HO-1 protein expression (B) were measured as described in Materials and Methods. Each bar represents the mean ± S.E.M. of five independent experiments. * $p<0.05$ vs. 0 μM CAPE.
7.4.2 Curcumin and CAPE activate Nrf2 in renal epithelial cells (LLC-PK1)

LLC-PK1 cells were exposed to curcumin or CAPE at the final concentration of 15 or 30 μM (Figure 7.5). Western blot analysis was performed on nuclear extracts from cells treated for 3 and 6 h with curcumin or CAPE. Treatment with curcumin or CAPE caused a time-dependent increase in Nrf2 protein expression in the nuclear extracts. The positive control was represented by recombinant Nrf2 protein.

![Western blot analysis of Nrf2 protein expression](image)

**Figure 7.3** The effect of curcumin and CAPE on Nrf2 activation in renal proximal epithelial cells (LLC-PK1)

LLC-PK1 cells were exposed to curcumin or CAPE at the final concentration of 15 or 30 μM. Gel electrophoresis was performed on nuclear extracts from cells treated for 3 and 6 h with curcumin (A) or CAPE (B) and Western blot analysis using Nrf2 antibodies was carried out as described in Materials and Methods. The positive control (+ con) is represented by recombinant Nrf2 protein.
7.5 Discussion

Curcumin and caffeic acid phenethyl ester (CAPE) belong to a class of plant phenolics known as the hydroxycinnamic acids. Curcumin is a yellow spice extracted from the rhizome of *Curcuma longa* L. (turmeric), it is widely used for flavouring and colouring in foods. CAPE is obtained from propolis, a substance produced by the bark of conifer trees. These two naturally occurring phenolic are known to possess antioxidant and anti-inflammatory properties. Previous studies in our group demonstrated that these two plant-derived polyphenolic compounds possess the chemical features required to trigger the induction of antioxidant and defensive genes (Scapagnini *et al.*, 2002).

Many stress-induced proteins including HO-1 are regulated at the transcriptional level by consensus sequences for the antioxidant response element (ARE) found in their promoter regions. In addition, several reports have demonstrated a key role for Nrf2. A basic leucine zipper transcription factor that binds to a unique *cis*-acting regulatory sequence, termed the ARE. Nrf2 is an important transcription factor responsible for upregulating ARE-mediated gene expression (204) (203)(205:206). Hence, Nrf2 is essential for the constitutive and induced expression of many antioxidant genes involved in the phase II pathway.

Nrf2 activity is normally suppressed in the cytosol by specific binding to the chaperone Keap1 (207). Stimulation results in dissociation of Nrf2 from Keap1 and translocation to the nucleus where it forms heterodimers with other transcription factors. However, oxidants and electrophiles stimulate Nrf2 translocation and upregulation of ARE of phase 2 genes occurs. This mechanism of gene transcription leads to the synthesis of highly specialized proteins that efficiently protect mammalian cells from various forms of stress and, consequently, reduce the propensity of tissues and organisms to develop disease or malignancy (208).
Inducible proteins that require transcription via Nrf2 activation include drug metabolizing enzymes, such as \( \gamma \)-glutamylcysteine synthetase (209), glutathione S-transferases (210), NADP(H):quinone oxidoreductase(211) and anti-oxidant enzymes, such as HO-1 (212), which generates the antioxidant biliverdin and the signaling molecule CO.

In this Chapter, it is reported that curcumin and CAPE significantly increased haem oxygenase activity and protein expression in renal epithelial cells. In addition, Nrf2 protein was markedly increased in nuclear extracts of renal epithelial cells exposed to low concentrations of curcumin and CAPE. And more importantly, the translocation of Nrf2 protein into the nucleus following curcumin or CAPE treatment was associated with a marked increase in HO-1 expression and haem oxygenase activity levels.

Subsequent studies by Balogun E et al (data not shown) see Appendix demonstrated the obligatory role of ARE in HO-1 transcription by curcumin. Previous reports by Alam and co-workers have identified two enhancer regions, E1 and E2, located approximately 4 and 10 kbp upstream of the transcription initiation site of the \( ho-1 \) gene (213). Each enhancer contains at least three distinct AREs, which are essential for \( ho-1 \) gene activation in response to a variety of agents including hemin and heavy metal ions. We demonstrate here that deletion of both enhancers completely suppressed HO-1 induction by curcumin in renal epithelial cells and that each enhancer has the ability to respond to the effect elicited by the yellow pigment.
7.6 Conclusion

The data presented in this chapter show that the induction of HO-1 in renal epithelial cells by curcumin and CAPE maybe dependent on the regulation of the ARE/Nrf2 complex. Since curcumin has been reported to ameliorate oxidative stress-induced kidney injury in mice (214) and the present and previous studies confirm the high inducibility of HO-1 by curcumin human renal cells (215), it is feasible that plant-derived compounds could be seriously considered as promising pharmacological agents in the development of therapeutical approaches in the prevention or treatment of kidney diseases.
8. COLD STORAGE: HO-1 INDUCTION BY CURCUMIN IN RENAL PROXIMAL EPITHELIAL CELLS

8.1 Introduction

Prolonged cold storage is associated with poor renal function post-transplantation. Several studies have shown the role of reactive oxygen species (ROS) production during cold leading to organ injury and eventually graft failure. Green and co-workers demonstrated free radical induced damage in rabbit kidneys after simple hypothermic preservation and autotransplantation (216). Salahudeen's illustrated also that cold storage without reperfusion, can produce free radicals leading to necrosis but not apoptotic cell death in renal tubules (217). Furthermore, evidence of ROS-mediated injury has been provided in several cell types (218), where prominent lipid peroxidation during cold incubation and inhibition of cell injury by a number of antioxidants was demonstrated (219) (220). Hence, a possible protective mechanism to counteract these deleterious effects could be via the up-regulation of the antioxidant pathway haem oxygenase (HO-1).

Previous studies have shown that over-expression of HO-1 with cobalt protoporphyrin significantly decreased liver dysfunction and mortality in a transplant model of obese Zucker rats (221) and permitted longer cold storage periods of harvested livers before successful transplantation (27). Salahudeen and colleagues have also published that prior induction of HO-1 with hemin protects renal epithelial cells against the damage caused by cold storage (222), reinforcing the idea that manipulation of the HO-1 pathway could be beneficial to improve graft storage and function. However, these promising results were obtained by up-regulating HO-1 before the cold storage period and it remains to be determined if pharmacological treatment
of a (cadaver) donor in order to induce HO-1 before organ harvesting and preservation is feasible in a clinical setting. An ideal strategy to avoid these potential problems would be to stimulate expression of HO-1 during the storage period.

In the previous chapter we established that curcumin can act as a powerful and potent inducer of HO-1 in renal cells. Curcumin is a polyphenolic compound found in many food products such as fruits, vegetables, tea and wine (223) and has been shown to possess antioxidant, anti-carcinogenic and anti-inflammatory properties (224) (225).

In this chapter we extended our in vitro findings to evaluate the effect of curcumin treatment on porcine renal epithelial cells (LLC-PK1) during hypothermia under normoxic or hypoxic conditions. This model was developed to investigate the efficacy of HO-1 up-regulation during hypothermia and could serve as a powerful tool to dissect the biochemical events that occur during cold ischaemia.

8.2 Objective

To investigate 1) the efficacy of HO-1 induction with curcumin under hypothermic condition 2) the effect of curcumin pre-treatment and hypoxia under hypothermic conditions and 3) the possible protective effect of curcumin against oxidative stress during normoxic conditions.

8.3 Material and Methods

8.3.1 Reagents

Stock solutions of curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) (5mM) were prepared by dissolving the compound in ethanol. Stock solutions were prepared freshly before the experiment and diluted to 15μM in DMEM prior to cell culture incubations. Glucose oxidase (GOX) was prepared freshly and diluted to 1.2 U/ml in DMEM.
8.3.2 Cell culture and maintenance of cell lines

Porcine renal tubular epithelial cells (LLC-PK1) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were grown in 75 cm² tissue culture flasks at 37°C under an atmosphere of 95% air/5% CO₂ and passaged twice a week.

8.3.3 Cell culture and hypoxia

Confluent LLC-PK1 cells were treated with curcumin (15 μM), transferred to an air-tight chamber (Sanyo, Model MIR-153) and flushed with 95% N₂ and 5% CO₂. The gas was infused continuously into the chamber at a flow rate of 5 litres/min for the first 2 hours and at 1 litre/min for the following hours of incubation. Within the hypoxic chamber, cells were maintained either at a humidified atmosphere of 37°C or at various temperatures (37, 30, 20 and 10°C).

8.3.4 Cell culture and hypothermia

Confluent LLC-PK1 cells treated with curcumin (15 μM) were incubated either under hypoxic (atmosphere of 95% N₂/5% CO₂) or normoxic conditions (95% air/5% CO₂). The chamber was set at different temperatures (37, 30, 20 and 10°C) or programmed to gradual changes in temperature which consisted of: 3 hours at 37°C, followed by 1.5 hours at 20°C and 1.5 at 10°C.

8.3.5 Haem oxygenase activity assay and Western blot for haem oxygenase-1 (HO-1)

At the end of each incubation periods LLC-PK1 cells were collected for the analysis haem oxygenase activity and HO-1 protein expression as described in Materials and Methods.

8.3.6 Exposure of cells to an oxidant generating system
Curcumin-treated cells were incubated with or without GOX [1-2 U/ml] at various temperatures and conditions. Glucose oxidase (GOX), a flavoenzyme which catalyses the conversion of D-glucose to D-gluconolactone produces hydrogen peroxide at constant rate in vitro (see Equation 8.1). This system produces oxidative stress in vitro by the generation of reactive oxygen species (ROS).

\[
\begin{align*}
\text{I) } & \quad \beta\text{-D-glucose} + \text{GOX}_{\text{FAD}} \xleftrightarrow{} \text{GOX}_{\text{FADH}_2} + \delta\text{-D-gluconolactone} \\
\text{II) } & \quad \text{GOX}_{\text{FADH}_2} + \text{O}_2 \longrightarrow \text{GOX}_{\text{FAD}} + \text{H}_2\text{O}_2
\end{align*}
\]

Equation 8.1 The catabolic reaction of glucose oxidase (GOX)
1) D-glucose is oxidised to D-Gluconolactone producing a reduced form of the enzyme. 2) The reduced form then reacts with oxygen to produce hydrogen peroxide that is known to promote oxidative injury in vitro.

8.3.7 Cell viability assay

8.3.7.1 Alamar blue
Cell metabolism was determined by the alamar blue method. The alamar blue assay incorporates a fluorometric/colourmetric growth indicator based on detection of metabolic activity; however, the indicator used in the alamar blue assay both fluoresces and changes colour in response to chemical reduction of growth medium resulting from cell growth. Cells were treated as described in Section 2.13.1. The alamar blue stock solution was diluted 1:10 (v/v) according to the manufacturers' instructions in culture growth medium; 200 μl transferred into each well and left to incubate for 5 h in the incubator or until a colour change was seen. After incubation the absorption at 570 and 600 nm was read in a microtitre plate reader (VersaMax, Molecular Devices, UK). Relative metabolic activity was determined by subtracting the background absorbance measured at 600 nm from the absorbance at 570 nm.
8.3.7.2 Lactate Dehydrogenase (LDH) Release
A complimentary set of experiments were carried out to determine the possible cytoprotective or cytotoxic effect of curcumin. LLC-PK1 cells were seeded in 6-well tissue culture plates and allowed to grow for 2 days under normal conditions. Curcumin-treated cells were then incubated with or without glucose oxidase (GOX) at various temperatures and conditions. After the incubation period, the supernatant was collected from each well and stored at 4°C. Cell monolayers were treated with lysing solution (1% Triton X-100) for 30 minutes at room temperature to lyse the cell membranes, and the supernatant and cell lysate was collected. LDH activity was measured in both the supernatant and the cell lysate fractions by using Cytotoxicity Assay Kit (Sigma-Aldrich Co., Poole, UK) following the manufacturers instruction. The procedure for determination of LDH is based on the reduction of pyruvate to lactate. The following reaction is catalyzed by LDH:

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{LDH}} \text{L-Lactate} + \text{NAD}^+
\]

During reduction of pyruvate an equimolar amount of NADH is oxidized to NAD\(^+\). The oxidation of NADH results in a decrease in the absorbance at 340nm. The rate of decrease in absorbance at 340nm is directly proportional to lactate dehydrogenase activity in the supernatant or cell lysate. The percentage of LDH release from the cell was determined using the formula:

Release (%) = LDH activity in supernatant/(LDH activity in supernatant + LDH activity in lysate).

8.3.8 Statistical analysis
Differences in the data among the groups were analyzed by using one-way ANOVA combined with the Bonferroni test. Values were expressed as a mean ± S.E.M. and differences between groups were considered to be significant at \(p < 0.05\).
8.4 Results

8.4.1 Effect of temperature on curcumin-mediated HO-1 induction in renal epithelial cells

We were interested to analyse how the temperature of incubation of cells would affect the up-regulation of HO-1 by curcumin. Curcumin (15 µM) strongly enhanced haem oxygenase activity levels (Figure 8.1 A) and HO-1 protein expression (Figure 8.1 B) of porcine renal epithelial cells 6 h after incubation at 37 °C. However, the ability of curcumin to induce HO-1 was progressively lost as cells were exposed to the agent at lower temperatures. In fact, although higher than in control, haem oxygenase activity and HO-1 protein were already considerably reduced at 30 °C and did not change from control at 20 °C. At 10 °C, haem oxygenase activity was even lower than in control cells. Thus, consistent with the expected decrease in cell metabolism and protein synthesis normally observed at low temperatures, renal cells did not express HO-1 in response to curcumin when incubated at 20 or 10 °C.
Porcine renal epithelial cells (LLC-PK1) were incubated with 15μM curcumin (CUR) at various temperatures (37, 30, 20 and 10°C) for 6 h. After treatment, haem oxygenase activity (A) and HO-1 protein expression (B) were measured as described in Materials and Methods. Bars represent the mean ± S.E.M. of n = 6 independent experiments. * P <0.05 vs. control (CON); ***P <0.001 vs. CON

Figure 8.1 Effect of temperature on haem oxygenase activity and protein expression in renal epithelial cells (LLC-PK1) incubated with curcumin.
8.4.2 Effect of changes in temperature on curcumin-mediated HO-1 induction in renal epithelial cells

Since curcumin was unable to induce HO-1 at temperatures below 30 °C, we reasoned about an alternative approach we could employ in order to stimulate HO-1 expression and maintain it at low temperature. We then exposed cells to curcumin and gradually changed the temperature during the 6 h incubation time as follows: 37 °C for 3 h → 20 °C for 1.5 h → 10 °C for 1.5 h. Interestingly, these experimental conditions proved to be appropriate for augmenting haem oxygenase activity and HO-1 expression (Figure 8.2) in renal epithelial cells. Therefore, this protocol was adopted for all the following experiments.
Figure 8.2 Effect of gradual decrease in temperature on curcumin-mediated increase in haem oxygenase activation in renal epithelial cells (LLC-PK1)

Haem oxygenase activity in porcine renal epithelial cells (LLC-PK1) after incubation with 15μM curcumin (CUR) in conditions of gradual changes in temperature programmed as follows: 3 h at 37°C, followed by 1.5 h at 20°C and 1.5 h at 10°C. Haem oxygenase activity was measured at the end of the 6 h incubation period as described in Materials and Methods. Bars represent the mean ± S.E.M. of n = 6 independent experiments. ***P < 0.001 vs. control (CON). Inset: Western blot showing increased HO-1 protein expression in renal epithelial cells exposed to curcumin (15μM) for 6 h using the programmed change in temperature described above.
8.4.3 Effect of temperature on cell viability in renal epithelial cells (LLC-PK1) incubated with curcumin

Having established that curcumin up-regulated HO-1 during the gradual change in temperature (3 h at 37°C, followed by 1.5 h at 20°C and 1.5 h at 10°C), we wanted to determine whether these treatments affected the viability of cells.

LDH release from renal tubular cells is widely used as a marker of irreversible cell injury. The percentage of LDH release was used as measure of cell injury after the incubation periods. LDH release noticeably increased in curcumin treated cells at 37°C (Figure 8.3 A) but was not statistically significant from control. Curcumin-treated cells incubated under the programmed change in temperature (37°C → 20°C → 10°C) did not exhibit any evident cellular injury (Figure 8.3 B).

We also used a second method for the measurement of cell viability (Alamar blue) (Figure 8.4) and it was confirmed that the treatment with curcumin at 37°C or under the programmed change in temperature did not cause apparent cytotoxicity.
Porcine renal epithelial cells (LLC-PK1) were incubated for 6 h with 15µM curcumin (CUR) at 37°C (A) or using the programmed change in temperature (37°C → 20°C → 10°C) (B). Cell viability was measured by assessing total lactate dehydrogenase (LDH) release in the incubation medium and lysed cells as described in Materials and Methods. Bars represent the mean ± S.E.M. of n = 6 independent experiments.
Figure 8.4 Effect of temperature on cell viability in renal epithelial cells (LLC-PK1) incubated with curcumin
Porcine renal epithelial cells (LLC-PK1) were incubated for 6 h with 15μM curcumin (CUR) at 37°C (A) or using the programmed change in temperature (37°C → 20°C → 10°C) (B). Cell viability was measured with Almar blue at the end of the 6 h incubation period as described in Materials and Methods. Bars represent the mean ± S.E.M. of n = 6 independent experiments.
8.4.4 Effect of hypoxia and curcumin on HO-1 up-regulation at 37 °C or during the programmed change in temperature

Storage of organs before transplantation is characterized by low temperature and hypoxia. Therefore, we examined whether curcumin could still increase HO-1 expression when cells were incubated at 37 °C or during the programmed change in temperature under hypoxic conditions, in order to reproduce more closely the storage conditions. We have shown recently that exposure of endothelial cells to curcumin at low oxygen tension results in potentation of HO-1 induction when compared to curcumin or hypoxia alone (195). Renal epithelial cells responded analogously when incubated under the same experimental conditions at 37 °C (Figure 8.5 A). Notably, cells exhibited high HO-1 levels and haem oxygenase activity even when exposed to hypoxia for 6 h during the programmed change in temperature (Figure 8.5 B).
Figure 8.5  Effect of temperature and hypoxia on curcumin-mediated increase in heme oxygenase activation in renal epithelial cells (LLC-PK1).
Porcine renal epithelial cells (LLC-PK1) were incubated with 15μM curcumin (CUR) under hypoxic (HYP) conditions at 37°C (A) or using the programmed change in temperature (37°C → 20°C → 10°C) (B). Heme oxygenase activity was measured at the end of the 6 h incubation period as described in Materials and Methods. Bars represent the mean ± S.E.M. independent experiments of n = 6 * P <0.05 vs. control (CON); ***P <0.01 vs. CON.Inset A: Western blot showing increased HO-1 protein expression in renal epithelial cells exposed to curcumin (15μM) under hypoxic conditions at 37°C for 6 h. Inset B: Western blot showing increased HO-1 protein expression in renal epithelial cells exposed to curcumin (15μM) under hypoxic conditions using the programmed change in temperature described above.
8.4.5 Effect of curcumin treatment on glucose-mediated oxidative damage in renal epithelial cells (LLC-PK1)

Several research groups have shown that HO-1 induction ameliorates oxidative stress both in vivo and in vitro (139:174:226). Therefore, having established that HO-1 can be up-regulated by curcumin at 37°C, it was important to determine whether this inducible pathway could provide protection against oxidative stress. The experimental protocol was identical to previous experiments at 37°C and the results are displayed in Figure 8.6. Cells treated with 1-2 U/ml of GOX for 2 hours exhibited a significant and dramatic decrease in cell viability with increasing concentration of GOX (Figure 8.6 A). Interestingly, cells pre-treated with curcumin where resistant to the oxidative damage seen with GOX treatment, indicating a possible cytoprotective effect by curcumin treatment and induction of HO-1.
Figure 8.6  Effect of curcumin on glucose oxidase-mediated cell injury in renal epithelial cells (LLC-PK1)  

(A) Porcine renal epithelial cells (LLC-PK1) were incubated for 2 h with 1-2 U/ml glucose oxidase (GOX) at 37°C and cell injury was assessed as described in Materials and Methods. (B) Cells were pre-treated for 6 h with 15μM of curcumin (CUR) at 37°C followed by 2 h of incubation with fresh medium containing 1-2U/ml of GOX. Cells were then analysed for viability by Alamar blue as described in the protocol. Bars represent the mean ± S.E.M. of n = 6 independent experiments. ** P < 0.01 vs. control (CON); ***P < 0.001 vs. CON
8.5 Discussion

A dominant problem in renal transplantation is the possibility of renal injury during cold storage resulting in graft failure post transplantation. The production of ROS is one of the factors which contribute greatly to renal failure. In fact, oxidative stress occurs not only during cold storage, but also during reperfusion, and therefore a strategy is required to minimize cold-associated injury.

Several cell culture models have been employed to prove that cold storage induces ROS (227). Salahudeen and workers demonstrated that induction of HO-1 before cold storage by either hemin treatment or transient transfection can reduce cold-induced renal injury, possibly through the up-regulation of ferritin (222).

Using different animal models, Nath and colleagues demonstrated that HO-1 induction can minimize renal injury caused by oxidative stress (220:228:229). In addition, endothelial cell lines have been used to investigate the cytoprotective mechanism of prior up-regulation of HO-1, suggesting also that free iron mediated up-regulation of ferritin confers cytoresistance against oxidative injury (230) (187)(17). Although all these studies illustrate the possible cytoprotective effect of HO-1 up-regulation prior to oxidative stress both in vivo and in vitro, there are still major questions to address. Is it possible to apply these techniques or strategies to a clinical situation? Can we "precondition" the organ to up-regulate HO-1 during hypothermic storage prior to transplantation? These questions promoted us to examine whether curcumin could up-regulate HO-1 during hypothermic storage using an in vitro model. The present study was undertaken using LLC-PK1 cells, which are an analogue of proximal tubular epithelial cells, are well-characterized and have been used widely in toxin- and oxidant-related cell culture studies (231-234).

In our study curcumin strongly increased haem oxygenase activity and HO-1 expression in renal epithelial cells for 6 hours at 37°C. Interestingly,
incubation at lower temperatures such as 30, 20 and 10°C, decreased HO-1 activity and expression dramatically. In fact, a decrease in temperature as little as 7°C (incubation a 30°C) had a significant impact on haem oxygenase activity, and, although was higher than control, there was a decrease greater than 50% in haem oxygenase activity in comparison to cells incubated at 37°C. At 10 °C, haem oxygenase activity was even lower than control cells. These results were not surprising since a decrease in temperature leads to reduced cellular metabolism, hence renal cells did not respond to the curcumin treatment at low temperature.

We reasoned that an alternative approach would be to induce HO-1 at 37 °C and assess whether its expression could be kept high at lower temperatures. Cells were therefore incubated for 3 h at 37 °C, followed by a gradual reduction in temperature (1.5 h at 20 °C and 1.5 h at 10 °C). At the end of this incubation we determined that this specific treatment allowed for HO-1 up-regulation and maintenance at low temperature. These findings suggest that it is feasible to induce protective genes during storage of cells by changing the temperature conditions. Whether these conditions would be optimal for up-regulation of genes other than HO-1 remains, however, to be established.

Our findings also revealed that the treatment, although different from cold storage, did not result in major cytotoxicity. We determined the effect of temperature and curcumin treatment on cell injury by measuring LDH release and assessing cell viability with alamar blue. LDH was slightly elevated but not statistically significant in curcumin treated cells incubated at 37°C for 6 hours. In addition, this treatment did not affect cell metabolism as shown by alamar blue. Interestingly, no cellular injury was detected by either method in cells treated with curcumin and subjected to the programmed change in temperature. Therefore both treatment regimes with
curcumin at 37°C or programmed change in temperature did not result in any major epithelial cells injury.

In the clinical scenario, organs used for transplantation are subjected to hypothermia and hypoxia (i.e. ischaemia). In animal models, exposure of the brain, heart, or kidney to sublethal ischaemia induces tolerance for subsequent ischaemia, a phenomenon which has been termed 'preconditioning'. This tolerance or cytoresistance is attributable to the induction of heat shock proteins, such as HSP90 and HSP32 (HO-1). In the context of HO-1, our group has demonstrated that induction of the HO-1 pathway before the onset of ischaemia in isolated heart or hypoxia in cultured cardiomyocytes protects against the reperfusion (235) or the reoxygenation injury (236), respectively. Our group has also shown a time-dependent increase of haem oxygenase activity and protein expression in aortic endothelial cells exposed to severe hypoxia (110). In addition, further studies indicated that exposure of endothelial cells to curcumin at low oxygen tension resulted in very strong HO-1 induction when compared to curcumin or hypoxia alone (195). Similarly, in the present study induction of HO-1 was observed in renal cells treated with curcumin under hypoxic conditions at 37°C, as well as in curcumin treated cells under conditions of programmed change in temperature.

The presence of curcumin under hypoxic conditions in order to strongly stimulate HO-1 expression may be beneficial under different pathophysiological situations. Indeed, Yonehanna et al. demonstrated reduction in ATP content and increased free radical production under hypoxic conditions in LLC-PK1 cells (237) and another group, using the same cell line, also observed an increase in LDH release during hypoxia as an index of tubular injury (238).

Our findings indicate that curcumin exerts cytoprotective actions. In an oxidative stress model where glucose oxidase was employed as an oxidant-generating system, the viability of renal cells was considerably reduced with
increasing concentrations (1-2 U/ml) of glucose oxidase (after exposure of 2 hours). However, cells pre-treated with curcumin were resistant to this form of oxidative challenge.

8.6 Conclusion

In summary, this study demonstrates that the induction of HO-1 and the increase in haem oxygenase activity promoted by curcumin in renal epithelial cells are gradually reduced with a decrease in temperature from 37 to 10 °C under normoxic conditions. We established that a 3 h incubation period at 37 °C to allow for up-regulation of HO-1 followed by a gradual reduction to 20 and 10 °C is an optimal approach for the maintenance of HO-1 during hypothermic conditions. In addition, our data indicate that curcumin can up-regulate HO-1 under hypoxic conditions both at 37 °C and during the programme change in temperature. Finally, we showed that stimulation of HO-1 by curcumin results in protection against oxidative stress-mediated damage at 37 °C.

It must be stressed that, due to time limitation, the data presented and discussed in this chapter are the result of a short and preliminary investigation on the effect of temperature on curcumin-mediated haem oxygenase induction. However, we think these promising findings have important relevance for the fields of organ storage and transplantation and we hope they can serve as a good foundation for the exploitation of these novel ideas in organ and animal studies.
9. GENERAL DISCUSSION

The chapters in this thesis have been presented in chronological order. Experiments were determined in a logical manner, based on previous publications by our group and others. All the experimental models employed demonstrated and focused on the possible protective effect of HO-1 upregulation against oxidative stress and ischaemia/reperfusion injury and highlight possible future strategies for improving the viability of renal cells during hypothermic storage as well as kidney function after transplantation.

It is known that induction of HO-1 represent a protective response to oxidative stress caused by ischemia/reperfusion injury and other pathological states.

Therefore, it may be possible to “precondition” renal cells with a suitable agent that is specific for the induction of HO-1 in the kidney that may protect this organ from subsequent cold ischaemia and reperfusion injury. Hence, the studies in this Thesis addressed the hypothesis that induction of the haem oxygenase-1 prior to ischaemia-reperfusion in the kidney provides better preservation during cold storage and improves renal function at reperfusion.

The data presented in this Thesis substantiate the hypothesis that haem oxygenase-1, through the production of the potent antioxidant bilirubin, carbon monoxide a vasodilator and ferritin synthesis via iron, an iron detoxification mechanism may provide improved renal preservation and protection against ischaemia-reperfusion injury. Although, the products of HO-1 were not fully investigated, we discovered and established the urgent need to search for a safe inducer of HO-1 in renal tissue.
9.1 Assessment of methods for upregulation, identification and measurement of haem oxygenase-1 (HO-1) in experimental models.

There is a wide spectrum of HO-1 inducers available including heme, ultraviolet A radiation, hydrogen peroxide, heavy metals and NO donors (32;65:239:240), the use of these inducers generally result in a significant shift in cellular redox, followed by subsequent HO-1 induction. We employed a well known inducer of HO-1, hemin that not only serves as a substrate for HO-1 but actually stimulates HO-1 gene transcription both in vivo as illustrated in Chapter 4 in rabbit kidney and in cultured cells (241:242). Gonzales S. and co-workers recently studied the in vivo effect of hemin treatment on both brain oxidative stress and heme oxygenase-1 (HO-1) induction (243). The results indicated that the induction of heme oxygenase by hemin may be a general response to oxidant stress, by increasing bilirubin and ferritin levels and could therefore provide a major cellular defence mechanism against oxidative damage. Weber T et al (244) examined the effects of pre-treatment with hemin, on myocardial stunning and their result demonstrated that hemin pre-treatment attenuates myocardial stunning in conscious dogs.

We examined HO-1 gene expression in renal tissue with Northern blot analysis after hemin treatment in rabbit and HO-1 mRNA expression increased in a time-dependent fashion, reaching a maximum at 6 hours and declining after 12 hours with hemin treatment. Additional experiments were performed to establish the best method of HO-1 induction in rabbit kidney. A total dose of 50 mg/kg of hemin was administered via different modes namely: intraperitoneally (i.p.), intraperitoneally plus intravenously (i.p. + i.v.), subcutaneously, subcutaneously plus intraperitoneally (i.p. + subcutaneous). All methods of administration proved to be effective for the induction of HO-1 in renal tissue, as shown by Western blot in Chapter 4.

The pharmacological inducer of HO-1, hemin, also increased enzymatic activity of haem oxygenase. Most importantly, we discovered that although the levels of haem oxygenase activity can be affected by extended cold
storage in UW solution, the enzymatic activity in kidneys pre-treated with hemin remained significantly elevated after 24 hour of cold storage in UW solution. These results gave us important information on the stratagems to be utilized for improving the viability of tissues during hypothermic storage. That is, there might be a therapeutic window during the organ preservation procedure within which the potential deleterious effect of prolonged cold storage may be alleviated by increasing HO-1 activity prior to transplantation. At this point it needs to be emphasized that HO-1 is currently viewed as the classical prototype of an antioxidant defensive protein and that its role in counteracting the negative effects mediated by physical and metabolic stresses is strongly emerging. However, we cannot exclude that induction of other intracellular defensive systems can contribute to enhance the effectiveness of a "therapeutic window" required to increase the resistance of organs to the inevitable metabolic alterations occurring during hypothermic storage.

9.2 Role of inducible haem oxygenase (HO-1) in protection of renal tissue during hypothermic storage and maintenance of post-ischaemic renal vascular function.

It has been shown that in renal tissue activation of the HO-1 pathway ameliorates nephrotoxic nephritis (245) and confers resistance against glomerular inflammation in rats (246). Our group demonstrated that HO-1-derived bilirubin reduces post-ischemic cardiac injury in a model of isolated rat heart (60). Rat hearts were isolated and perfused according to the Langendorff technique to evaluate the recovery of myocardial function after 30 min of global ischaemia and 60 min of reperfusion. Upregulation of HO-1 by treatment of animals with hemin 24 hour before ischaemia ameliorated myocardial function and reduced infarct size in the isolated reperfused hearts. In line with our previous findings in the myocardium, we studied the effects of HO-1 induction by hemin treatment in the ex vivo kidney perfusion system as described in Chapter 5. This model was first described by Fuller et al in 1977 (247). Although, previous work in our group was
carried out in rats, we decided to use rabbit kidneys for the following reasons: (a) to eliminate problems of microsurgery encountered during renal transplantation and (b) for comparative purposes with the kidney transplantation model described in in Chapter 6.

The IPRK system was perfused with a cell free perfusate, hence hormones, neurons or blood-borne factors did not influence the kidneys. Nizet and co-workers demonstrated that perfusion with whole blood, frequently leads to vasoconstriction within minutes of reperfusion, rendering further perfusion impossible. This phenomenon of vasoconstriction was encountered and studied in isolated perfused dog kidney (248). Therefore, a cell free system was justified as appropriate, reliable and less cumbersome. Furthermore, we used a closed system, which allowed us to recirculate the perfusate and minimize cost. The kidney was perfused under controlled conditions and we were able to measure the following physiological parameters: glomerular filtration rate, tubular glucose reabsorption, tubular sodium reabsorption, urine flow rate, perfusate flow rate, proteinuria, gamma glutamyl transpeptidase activity (γ-GT). This system enabled us to study the effect of prolonged hypothermic storage, potential nephrotoxic effects of hemin treatment and physiological effects of HO-1 upregulation. The results obtained illustrate that hemin treatment intravenously is detrimental to the kidney function; this was evident by elevated levels of markers of glomerular and tubular injury such as proteinuria and of γ-GT activity. In contrast, hemin treatment (i.p.) did not protect or exacerbate renal function \textit{ex vivo} when compared to control kidneys.

In summary, the results of this study provide objective data to substantiate that hemin treatment intravenously (i.p. + i.v.) is detrimental to the kidney function, which is strongly supported by increased activity of γ-GT, elevated levels of proteinuria and correlating histological findings. Intraperitoneal hemin treatment (i.p.), however, does not protect the kidney during hypothermic storage and reperfusion injury nor exacerbates damage incurred during hypothermic storage when compared to control kidney.
Even though the outcome of the IPRK study was disappointing, the nephrotoxic effects of hemin were not surprising. We speculate that the accumulation of reactive iron released from the catalysis of heme may impart cellular cytotoxicity. Perhaps, the release of iron during heme catabolism reacts with hydrogen peroxide to form the hydroxyl radical, or decompose membrane lipid peroxides to yield alkoxy and peroxy radicals (249), species known to be involved in the initiation of lipid peroxidation chain reactions.

Interestingly, in additional experiments we also found that in vivo treatment with hemin affected mitochondrial respiration as illustrated in Chapter 3. The mitochondrion is a well known target of toxic agents; this cytotoxicity is known to inhibit cellular respiration secondary to inactivation of the electron transport chain components. Hence, measurements of mitochondrial complex activity and oxidative phosphorylation were undertaken to evaluate the effects of HO-1 upregulation on energy metabolism in rat heart and kidney. Although, the kidney was the main organ of interest in this study, we performed comparative studies in the heart as previous studies in our group have shown that upregulation of HO-1 protects against ischaemia-reperfusion injury in the rat heart (250).

The possible protection or pathogenesis of hemin treatment and HO-1 upregulation on mitochondrial function was investigated, to determine the degree of functional alteration on the electron transport chain and mitochondria oxygen consumption rate, hence ATP production. Mitochondrial function was measured by NAD+ and FAD-linked exogenous substrate (see Chapter 3). In addition, the activities of complexes I-IV in mitochondria were also determined. The rate of oxygen utilisation during oxidative phosphorylation, in the isolated mitochondria from hemin-treated animals was significantly reduced in the kidney and in the heart.

Our data are in line with several studies which show that hemin can promote free radical formation and lipid peroxidation, resulting in cell
damage and tissue injury (173;174) (175). These results indicate that products of heme degradation by HO-1 modulate mitochondria oxygen consumption and damage renal tissue. Therefore, the use of hemin in this ex-vivo model does not provide full protection and can not be considered as the ideal inducer of HO-1. Furthermore, the IPRK system lacks blood perfusion; this is another important factor in determining the physiological effects of hemin treatment in renal tissue after prolonged cold storage. The transplantation model is a perfect tool for studying the potential effects of hemin treatment/HO-1 upregulation in vivo before hypothermic storage and renal transplantation. In contrast, to the IPRK system or cell culture in which the elements of blood do not play any role, this transplant model offers the closest experimental simulation of ischaemia-reperfusion injury encountered in the clinical arena. The use of the autograft model abolishes interference from rejection process, making it possible to study the pathogenesis of hemin treatment, prolonged cold storage and ischaemia/reperfusion injury in isolation. However, in contrast to ex vivo and in vitro studies, this transplant study is considerably more complex, expensive and laborious. Hence, it is important to have a skilled and experienced surgeon to eliminate any additional negative factors that might contribute to poor graft survival post-transplantation.

In 1998, Hancock et al. (251) demonstrated that the induction of protective genes such as HO-1 protect allografts from chronic rejection. In addition, to transplant rejection, HO-1 also attenuated ischaemia-reperfusion injury that affects donor organ viability and subsequent transplantation (221)(252). Soares et al. (253) demonstrated that that the expression of the HO-1 gene is functionally associated with xenograft survival, and that rapid expression of HO-1 in cardiac xenografts can be essential to ensure long-term xenograft survival. Amersi et al (254) in 1999 demonstrated that overexpression of HO-1, by either cobalt protoporphyrin or gene transfer (using an adenoviral vector containing HO-1) attenuated ischaemia-reperfusion injury and prolonged graft survival after ischaemia followed by autotransplantation of fatty livers in the obese Zucker rat. Katori B et al.
(255) recently demonstrated that HO-1 overexpression protects rat hearts from cold ischemia/reperfusion injury via an anti-apoptotic pathway. Therefore, numerous investigators have reported on the beneficial effects of HO-1 overexpression prior to transplantation in various models.

To address the question properly and gain better understanding on the effect of hemin pre-treatment prior to hypothermic storage followed by subsequent reperfusion in vivo, we embarked upon a renal transplantation study. The autotransplant model was performed in New Zealand White rabbits which were treated with hemin. Twenty-four hours after hemin administration, the left kidney was first removed and flushed with cold University of Wisconsin (UW) solution. The kidneys were then stored for different time points (0, 24 or 48 hours) at 4°C and then autotransplanted, while the contralateral kidney was removed. The animals were allowed to live up to 28 days or euthanized as soon as it was apparent that the grafted kidney had failed. There was no significant difference between the hemin-treated and control autografts as animals of both groups survived to term. Kidney grafts, not exposed to hemin treatment showed severely reduced graft function, while hemin-treated autografts showed a significantly longer survival period when compared to control. This indicates that hemin confers protection up to 24 hours cold preservation, but not 48 hours as most animal did not survive by day 7 in both hemin-treated and control groups. Hence, in our hands hemin treatment in vivo might be beneficial provided that the heme oxygenase activity can be maintained during the cold storage. In fact, data from Chapter 4 revealed that following induction of HO-1, the activity of the enzyme is maintained for up to 24 hours of cold storage, after which it starts to decline; these results correlate with the outcome of the autograft experiments. Furthermore, histological analysis served as supporting evidence for the nephrotoxic effect of hemin, highlighting the fact that hemin is not a suitable agent for maximally activate HO-1 induction in the kidney without incurring into cellular damage. Although hemin-treated grafts survived longer after 24 hours of cold storage, we believe that this is due to the systemic effect of hemin treatment and HO-1 induction, rather
than HO-1 induction in the kidney alone. Despite the fact that several studies have shown that HO-1 induction by hemin is cytoprotective, we have found this inducer could be nephrotoxic at high concentrations. However, at this stage, we reasoned that low doses of hemin alongside agents that synergistically could potentiate the HO-1 pathway might result in the elimination of the toxic effects of hemin.

9.3 Search for novel “pharmacological preconditioning agents” able to selectively activate the HO-1 gene in renal tissues

Hemin serves as potential pro-oxidant molecule and as an essential substrate for heme oxygenase activity. Therefore, due to the toxic effects of hemin in renal tissue, we thought that the search for other inducers of HO-1 was extremely important. These inducers could either be used alone or in combination with non-toxic concentrations of hemin to maximally up-regulate this defensive system, furthermore this would be of great advantage for future clinical purposes. For this study, porcine renal epithelial proximal tubule (LLC-PK1) cells were employed. Unprecedented data from our laboratory revealed that low concentrations of curcumin, an antioxidant and anti-inflammatory agent, potently induces HO-1 mRNA, protein expression and activity in endothelial cells (187). Interestingly, curcumin and other bioflavonoids have been recently discovered as a new class of renoprotective agents (256). Therefore, curcumin and caffeic acid phenethyl ester (CAPE), are plant-derived polyphenolic compounds that have been shown to possess chemical features required to trigger the induction of antioxidant and defensive genes (186). Thus, curcumin and CAPE were tested for the induction of HO-1 in renal cells.

In Chapter 7, LLC-PK1 cells treated with curcumin or CAPE significantly increased HO-1 protein expression and activity in a concentration-dependent manner, with maximal activity being observed at 15 and 20 μM, respectively. To gain insight into the mechanism of action of these natural inducers of HO-1 at the transcriptional levels, we studied the transcription
factor NF-E2-related factor 2 (Nrf2). Nrf2 is a transcription factor and a key player in cellular response to oxidative stress. The activated Nrf2 protein binds to *cis*-acting elements in the promoters of target genes, called antioxidant response element (ARE) (257)(203), or electrophile response element(258). Subsequently, these target genes encode a series of cytoprotective proteins, including heme oxygenase 1 (HO-1)(203).

In Chapter 7, we illustrated that Nrf2 protein is markedly induced in renal epithelial cells exposed to low concentrations of curcumin or CAPE. In addition, Nrf2 and ARE-binding abilities of renal cell nuclear proteins were assessed by EMSA to determine whether curcumin or CAPE treatment enhances functional NRF2 activity. The results obtained illustrate that induction of HO-1 in renal epithelial cells by curcumin and CAPE are dependent on the regulation of the ARE/Nrf2 complex. Therefore, we speculate that these plant-derived compounds may serves as better inducers than hemin alone in activating HO-1 and preventing renal diseases such as ischaemia/reperfusion injury.

To extend our findings on the potential use of polyphenolic compounds as potential therapeutic agents in the context of organ preservation and transplantation, we finally carried out some experiments using curcumin as HO-1 inducer during hypothermic conditions. Renal tubule cells (LLC-PK1) were incubated with curcumin at different temperatures, ranging from 37 to 0°C. It was found that the HO-1 pathway could not be induced during hypothermia. Interestingly, there was a significant decline at 30 °C in the activity of the enzyme, which continued to gradually decrease as the temperature was lowered. Further experiments established that cells needed to be pre-incubated for at least 3 hours at 37°C in the presence of curcumin in order to observe an apparent HO-1 induction which was then maintained during hypothermia.

To investigate if overexpression of HO-1 via curcumin pre-treatment affords protection against oxidative challenge, renal cells were incubated with
glucose oxidase (GO), an enzyme that continuously generates hydrogen peroxide from glucose. Numerous studies have shown the oxidative cell injury caused by the generation of hydrogen peroxide (232:259). In Chapter 8 we show that cells pre-treated with curcumin become resistance to oxidative challenge. Thus, these data may have important implications in increasing the inherent cytoprotective system of kidneys prior to preservation and transplantation.

9.4 Conclusions, reflections and future perspective

The importance of heme oxygenase activity for normal physiology and survival was evident and described in the first human autopsy case of heme oxygenase (HO)-1 deficiency (260). The patient had persistent proteinuria and hematuria, with biochemical evidence of renal tubular injury. Tubular epithelial cells were injured, and massive deposition of iron and haptoglobin was detectable. Bowman's capsules were dilated significantly. This was the first report to show that HO-1 has critical roles in vivo in protecting renal tubuli, in addition to vascular endothelium, from oxidative injury. Our data provide further support for the importance of endogenous HO-1, as a cytoprotective enzyme, in the regulation of renal function and the mediation of pathophysiological stimuli leading to oxidative stress. Specifically, in the context of renal preservation and transplantation, our data indicate that the ability of renal cells to up-regulate HO-1 can be modulated by the use of hemin in combination with natural substances that could be useful for therapeutic purposes. Further studies specifically designed to explore how the HO-1 gene is regulated in conditions that simulate the preservation of organs and tissues will allow the development of new pharmacological treatments in the clinical setting of transplantation.
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is retarded only in the presence of Nrf2 antibodies, whereas it remains unaffected with members of the AP-1 family (Jun and Fos). In our system, we could not confirm that the nuclear factor-κB pathway contributes to transcriptional activation of HO-1 by curcumin, as partially shown in human renal proximal cells [39]. In addition, although ho-1 gene induction by cobalt in Chinese hamster ovary cells involves the formation of Nrf2-Maf heterodimers [25], we were unable to detect MafG protein in the curcumin-induced complex. The biological significance of Nrf2 dimerizing with small Maf proteins or different bZIP proteins has received little attention and remains to be fully investigated.

The experiments designed to determine a possible role of MAPK pathways in curcumin-mediated ho-1 gene induction showed that curcumin activates the ERK and p38 subfamilies, but not the JNK pathway. Additionally, the use of specific inhibitors for the MAPK subfamilies confirmed the involvement of p38, but not of the ERK or JNK pathways, in ho-1 induction mediated by curcumin. Taken together with the promoter-analysis data, these results are in agreement with a previous report showing that cadmium, a classical HO-1 inducer, promotes ho-1 gene expression in MCF-7 cells via sequential stimulation of the p38 kinase pathway and Nrf2 [24].

Collectively, the data presented in this paper are consistent with the specific requirement of AREs in the activation of protective genes by electrophilic substances [35] and highlight the fundamental role of the Nrf2/ARE complex in the regulation of HO-1 protein expression in conditions characterized by changes in the intracellular redox state [3]. Notably, a recent study reported that 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2), an α,β-unsaturated ketone containing two electrophilic carbons in the cyclopentenone ring, potently activates ho-1 gene in mouse hepatoma cells and that the mechanism of induction is mediated by AREs and the transcription factor Nrf2 [40]. As in the case of curcumin and CAPE, which exert a variety of beneficial actions, the potentiation of the JNK pathway and Nrf2 [24].

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with the ERK and p38 enzymes, phosphorylated c-Jun N-terminal kinase (JNK) was not detected in untreated or stimulated cells. The increase in the levels of phosphorylated ERK and p38 kinase was not due to a concomitant elevation in the amount of the respective enzymes.

**SB203580, a p38 Inhibitor, attenuates curcumin-stimulated E1 activity**

To address the role of individual MAPK pathways in ho-1 gene regulation by curcumin, we examined the effects of SB203580, PD908059 and SP600125, inhibitors of the p38, ERK and JNK pathways, respectively, on pE1-luc expression. Treatment of NRK-52E cells with PD908059 or SP600125 had no effect on basal or curcumin-stimulated pE1-luc expression (Figure 8). SB203580 also did not alter basal E1 activity, but reduced curcumin-dependent induction by 50%. Taken together with the MAPK-phosphorylation data, these results implicate the p38 pathway, but not the ERK or JNK pathways, in curcumin-mediated ho-1 gene induction.

**DISCUSSION**

The transcription factor Nrf2 is a member of the cap’n’collar family of basic leucine transcription factors and plays an essential role in the ARE-mediated expression of phase II detoxifying enzymes and stress-inducible genes [30]. The activity of Nrf2 is normally suppressed in the cytosol by specific binding to the chaperone Keapl [28]. However, upon stimulation by electrophilic agents or compounds that possess the ability to modify thiol groups [17], Keapl repression of Nrf2 activity is lost, allowing Nrf2 protein to translocate into the nucleus and potentiate the ARE response [28]. This mechanism of gene activation leads to the synthesis of highly specialized proteins that efficiently protect mammalian cells from various forms of stress and, consequently, reduce the propensity of tissues and organisms to develop disease or malignancy [31]. Inducible proteins that require transcription via Nrf2 activation include γ-glutamylcysteine synthetase [32,33], glutathione S-transferases [34], NADP(H):quinone oxidoreductase [35] and HO-1 [20], which generates the antioxidant biliverdin and the signalling molecule CO. The concerted defensive action of these and other ubiquitous enzymes against specific diseases is exemplified by very recent studies using Nrf2-deficient mice, which lack the inherent ability to respond to classical inducers of phase II enzymes and are highly susceptible to carcinogenesis [18]. Moreover, Nrf2-knockout mice develop lupus-like autoimmune nephritis [36] and may die of liver failure due to a decreased capability of hepatocytes in drug detoxification [37]. Thus a direct involvement of this redox-sensitive transcription factor in mediating the adaptive response of tissues against progression of cell dysfunction is currently emerging.

In the present study, we show that Nrf2 protein is markedly induced in renal epithelial cells exposed to low concentrations of curcumin and CAPE, two plant-derived polyphenolic compounds that possess the chemical features required to trigger the induction of antioxidative and defensive genes [5]. In addition, transfection studies confirm that curcumin exerts a significant stimulation of Nrf2 activity and that this effect is completely blocked by the native repressor of Nrf2, Keapl. The translocation of Nrf2 protein into the nucleus following curcumin or CAPE treatment was associated with stimulation of the ARE-binding activity as well as a marked increase in HO-1 expression and haem oxygenase activity levels. In view of the strong evidence demonstrating an essential physiological role for HO-1 and its products in the mitigation of vascular dysfunction and inflammatory states [8–10], these and our previous findings point to the potential pharmacological use of naturally occurring compounds in modulating haem oxygenase activity and function [4,5]. These data are also consistent with the recognized biological activity of a number of phytochemicals or extracts of edible plants which are being identified as potent inducers of phase II enzymes and are able to transduce the signal for transcription to AREs through a chemical interaction with Nrf2 proteins [17,31].

Our results emphasize the crucial role of Nrf2-mediated gene expression in response to natural substances that possess a peculiar chemical structure. The data presented here also support the critical role of Keapl in this response, as curcumin can significantly reverse the Keapl-mediated inhibition of Nrf2 activity. Thus it appears that curcumin promotes ho-1 gene expression by altering the Nrf2–Keapl interaction. Both curcumin and CAPE contain electrophilic α,β-unsaturated carbonyl groups which can react selectively with nucleophiles such as thiols, leading to formation of Michael adducts. In the case of curcumin, interaction with GSH has been reported [10] and kinetic studies in vivo have shown that the majority of orally administered curcumin is rapidly transformed into glutathione conjugates which can inhibit glutathione S-transferase activity [38]. Moreover, we have shown in cultured astrocytes that HO-1-mediated induction by curcumin and CAPE is associated with transient and marked changes in the intracellular GSH/GSSG ratio. Notably, Keapl contains several highly reactive cysteine residues; although the chemical modification that signals dissociation of the Keapl–Nrf2 complex is currently unknown, a specific modulation of thiols by electrophiles in Keapl might promote a conformational change that releases Nrf2 protein, thereby favouring its translocation to the nucleus [17]. Of major interest, and in line with this hypothesis, is that the potency of plant curcuminoids and other Michael-reaction acceptors as inducers of detoxifying enzymes depends on their specific reactivity with thiol groups [17]. The fact that the thiol donor N-acetyl-L-cysteine is unable to prevent the increase in haem oxygenase activity promoted by treatments with curcumin or CAPE [5] may be indicative of a unique chemical feature of these polyphenolic compounds in reacting with selective cysteines located in transcriptional factors or other proteins involved in signal-transduction pathways.

Our data demonstrate the obligatory role of the ARE in HO-1 transcription by curcumin. Previous studies have identified two enhancer regions, E1 and E2, located approx. 4 and 10 kb upstream of the transcription-initiation site of the ho-1 gene [26]. Each enhancer contains at least three distinct AREs, which are essential for ho-1 gene activation in response to a variety of agents, including haemin and heavy metal ions. We demonstrate here that deletion of both enhancers completely suppressed HO-1 induction by curcumin in renal epithelial cells and that each enhancer has the ability to respond to the effect elicited by the yellow pigment. The direct implication of Nrf2 in the regulation of the ho-1 gene via the ARE has been recently reported using Nrf2 dominant-negative mutants (Nrf2M). It was found that, in fibroblasts over-expressing Nrf2M, the accumulation of HO-1 mRNA was almost completely inhibited in response to ‘classic’ HO-1 inducers such as haemin, cadmium and arsenite [20]. From that study it also appears that Nrf2 plays a more prominent role than AP-1 in modulating ho-1 gene expression [20]. This is in line with the data presented here showing that, in renal cells exposed to curcumin, the migration of the DNA–protein complex in the EMSA reaction
Curcumin activates p38 kinase

To determine the role, if any, of MAPKs in curcumin-mediated hO-1 gene activation, we first examined the effect of curcumin on MAPK activities. MAPKs are activated by dual phosphorylation of threonine and tyrosine residues located in the "activation lip" of the conserved core kinase sequence, and the activated species can be detected by antibodies directed against phosphorylated peptides encompassing these residues. NRK-52E cells were treated with vehicle (ethanol) or 10 μM curcumin for up to 2 h, and cell extracts were analysed for phosphorylated and total MAPKs by Western blotting. Curcumin stimulated the activation of extracellular-signal-regulated kinase (ERK) 1 (p44), ERK2 (p42) and p38 in a time-dependent manner (Figure 7). Whereas appreciable stimulation of the activated ERK species was not observed until the last time point tested (2 h), increased phosphorylation of p38 (approx. 5-fold) was detected within 30 min after treatment with curcumin, and the amount of activated p38 remained above the basal level for up to 2 h. In contrast
against one or more members of these protein families were carried out to identify the protein constituent(s) within the curcumin-induced DNA–protein complex. The migration of this complex was quantitatively retarded using anti-Nrf2 IgG, but not with pan-Jun or pan-Fos antibodies that recognize all members within their respective families. Nrf2 does not form homodimers, but heterodimerizes with small Maf proteins. Although we have previously provided evidence for a role for Nrf2–MafG heterodimers in cobalt-mediated \( \text{ho-1} \) gene induction [25], MafG was not detected in the curcumin-induced complex. Taken together, these results support a role for Nrf2, but not for AP-1 proteins, in \( \text{ho-1} \) activation by curcumin. The identity of the Nrf2 dimerization partner in this response is not presently known, but does not appear to be MafG.

Figure 5  Curcumin and CAPE activate Nrf2 expression and stimulate ARE-binding activity in renal epithelial LLC-PK\(_1\) cells

LLC-PK\(_1\) cells were exposed to curcumin or CAPE at the final concentration of 15 or 30 \( \mu \text{M} \) to evaluate the expression of Nrf2 protein over time. As shown in Figures 5(A) and 5(B), treatment with CAPE or curcumin caused a significant dose- and time-dependent increase in Nrf2 protein expression in the nuclear extracts. Quantification of three independent Western blots showed that after 3 h exposure to 15 and 30 \( \mu \text{M} \) curcumin, Nrf2 expression increased 2.1 ± 0.7 and 4.4 ± 1.1-fold, respectively; in the presence of 15 and 30 \( \mu \text{M} \) CAPE, Nrf2 expression increased 3.1 ± 1.0 and 6.2 ± 1.5-fold, respectively. In addition, EMSA revealed that treatment of LLC-PK\(_1\) cells with curcumin or CAPE (15 \( \mu \text{M} \)) increases the amount of the Nrf2/ARE complex (see Figure 5C). Nrf2 in this complex was confirmed by antibody supershift analysis (results not shown).

Curcumin and CAPE activate Nrf2 expression and stimulate ARE-binding activity in renal epithelial cells (LLC-PK\(_1\))

LLC-PK\(_1\) cells were exposed to curcumin or CAPE at the final concentration of 15 or 30 \( \mu \text{M} \) to evaluate the expression of Nrf2 protein over time. As shown in Figures 5(A) and 5(B), treatment with CAPE or curcumin caused a significant dose- and time-dependent increase in Nrf2 protein expression in the nuclear extracts. Quantification of three independent Western blots showed that after 3 h exposure to 15 and 30 \( \mu \text{M} \) curcumin, Nrf2 expression increased 2.1 ± 0.7 and 4.4 ± 1.1-fold, respectively; in the presence of 15 and 30 \( \mu \text{M} \) CAPE, Nrf2 expression increased 3.1 ± 1.0 and 6.2 ± 1.5-fold, respectively. In addition, EMSA revealed that treatment of LLC-PK\(_1\) cells with curcumin or CAPE (15 \( \mu \text{M} \)) increases the amount of the Nrf2/ARE complex (see Figure 5C). Nrf2 in this complex was confirmed by antibody supershift analysis (results not shown).

Curcumin relieves Keapl inhibition of Nrf2

One model for the regulation of Nrf2 function stipulates that, under normal conditions, Nrf2 exists in an inactive, cytoplasmic localized state, in part or fully as a consequence of binding to the cytoskeleton-associated protein Keapl [28,29]. Upon cellular stimulation by xenobiotics, electrophiles or oxidative-stress-generating agents, the cytoplasmic-retention mechanism is inactivated and Nrf2 is transported to the nucleus, where it activates target-gene transcription. To examine whether curcumin modulates Nrf2–Keapl interaction, we carried out the experiment depicted in Figure 6. In these transfections, curcumin elicited a nearly 5-fold stimulation of the E1-regulated luciferase reporter activity (compare Figure 6, lanes a and b). Consistent with the proposed function of Keapl, co-expression of Keapl nearly abolished both basal and curcumin-dependent luciferase activity (Figure 6, lanes c and d). Conversely, ectopic expression of Nrf2 resulted in very high levels of luciferase activity (Figure 6, lane e), which was not further enhanced by curcumin (Figure 6, lane f), indicating maximal activation of E1 under these conditions and also supporting the idea that curcumin induces \( \text{ho-1} \) transcription via Nrf2. Importantly, Keapl almost completely inhibited Nrf2 trans-activation of E1 (Figure 6, lane g), and this response was partially mitigated by curcumin.
Activation of the mouse \( \text{ho-1} \) gene promoter by curcumin is mediated by the AREs

Induction of HO-1 by most agents is regulated primarily at the level of gene transcription [1]. To characterize the mechanism of HO-1 induction by curcumin, renal epithelial cells were transfected with various mouse \( \text{ho-1} \) gene promoter/luciferase fusion constructs and treated for 5 h with either vehicle or 10 \( \mu \text{M} \) curcumin (see the Materials and methods section). Curcumin stimulated the activity of the wild-type 15 kbp \( \text{ho-1} \) promoter by greater than 4-fold (Figure 3A). Deletion of both of the previously identified E1 (268 bp) and E2 (161 bp) enhancers \([\Delta \text{E}(1+2)]\) completely abolished induction, demonstrating that these regions are essential for this response. Interestingly, mutants containing only one enhancer were as responsive as the wild-type promoter, suggesting that, at least with respect to induction by curcumin, the enhancers are redundant. Consistent with this idea, in isolation both E1 and E2 can confer curcumin-responsiveness to the luciferase reporter gene (Figure 3B). Mutation of the ARE within the E1 or E2 enhancers abrogates this response. Furthermore, the ARE sequence by itself, but not its mutant counterpart, is capable of conferring curcumin-responsiveness. Together these results indicate that the ARE is both necessary and sufficient for induction of the \( \text{ho-1} \) gene by curcumin.

Curcumin stimulates ARE-binding activity in NRK cells

EMSA reactions using an ARE probe and whole-cell extracts from NRK cells were carried out to identify DNA-binding proteins potentially responsible for curcumin-mediated \( \text{ho-1} \) gene induction (see Figure 4). Several specific ARE–protein complexes of varying intensities were detected when using extracts from vehicle-treated cells (Figure 4, lane 1). Treatment of NRK cells with curcumin generated an apparently novel and slower-migrating complex (Figure 4, lane 7; marked by an arrow). The consensus core ARE sequence, YGCTGAGTCA, resembles the consensus binding sites for the activator protein 1 (AP-1; Jun–Jun or Jun–Fos dimers), Maf and cap’n’collar-bZIP families of transcription factors, which function as obligate dimers. Supershift EMSA reactions using antibodies directed
Where indicated, vehicle or mitogen-activated protein kinase (MAPK) inhibitors were added 30 min prior to the addition of curcumin. After a 5 h incubation period, cells were harvested for preparation of cellular extract and measurement of luciferase and β-galactosidase activities [27]. β-Galactosidase-normalized luciferase activities are presented.

EMSAs

These assays were carried out on both NRK-52E and LLC-PK1 cells. Confluent LLC-PK1 cells were treated in complete Dulbecco’s modified Eagle’s medium with 15 μM curcumin or CAPE for 3 or 6 h. NRK-52E cells were plated (4 x 10⁵ cells/100 mm plastic dish) and cultured in complete medium for 40–48 h, and then treated with vehicle or 10 μM curcumin in serum-free medium for 3 or 6 h. Whole-cell extracts were prepared and EMSA reactions carried out as described previously [25]. A 32P-labelled double-stranded oligonucleotide containing the sequence 5'-GATCTTTTATTGCTGAGTCATGGTTT-3' (core ARE underlined) was used as the probe in EMSA reactions. In antibody supershift assays, 1 μl (2 μg) of pre-immune IgG or specific rabbit polyclonal antibodies was added to the reaction mixture and incubated for 20 min at room temperature prior to electrophoresis. In the case of MafG, 1 μl of whole serum (pre-immune and anti-MafG) was used.

Western blot for MAPKs

NRK-52E cells were plated (5 x 10⁵ cells/60 mm plate) and cultured for 48 h. The culture media was replaced with serum-free medium and curcumin (10 μM) was added to individual plates at staggered time points so that all cells were exposed to serum-free medium for the same period. Cells were washed with cold PBS and lysed directly in 100 μl of 1 x electrophoresis sample buffer containing 2 mM EGTA and 50 mM NaF. Protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (Sigma). Samples of 20 μg were size-fractionated on 10% denaturing polyacrylamide gels and protein-blot analysis was carried out using the ECL Western Blotting System (Amersham Biosciences) according to the manufacturer’s recommendations. Antibodies to non-phosphorylated and phosphorylated MAPKs were obtained from New England Biolabs and used at dilutions and under conditions recommended by the manufacturer.

Statistical analysis

Differences in the data among the groups were analysed by using one-way ANOVA combined with the Bonferroni test. Values were expressed as means ± S.E.M. and differences between groups were considered to be significant at P < 0.05.

RESULTS

Effect of curcumin and CAPE on haem oxygenase activity and HO-1 expression in renal epithelial cells

Curcumin caused a significant increase in haem oxygenase activity, with a maximal value being observed at 15 μM (see Figure 1A). HO-1 protein expression was also significantly elevated by curcumin (Figure 1B); quantification of three independent Western blots showed that in the presence of 5, 10, 15, 20, 30 and 50 μM curcumin, HO-1 protein increased 1.4 ± 0.3, 1.8 ± 0.3, 5.6 ± 1.4, 12.4 ± 2.2, 12.5 ± 1.9 and 4.7 ± 1.0-fold, respectively. Thus at concentrations higher than 30 μM, curcumin appears to be less effective in stimulating haem oxygenase activity and HO-1 protein expression. In a similar fashion, CAPE also caused a significant increase in haem oxygenase activity (see Figure 2A) and HO-1 protein expression (Figure 2B). Quantification of three independent Western blots showed that...
Studies using Nrf2-deficient mice have confirmed their inability to express cytoprotective genes upon stimulation with carcinogens [18], and cells lacking the Nrf2 gene display a higher susceptibility to oxidant-mediated cell injury and death [19]. Nrf2 is a potent positive regulator of the hO-1 gene and other detoxifying enzymes [20]; however, a direct link between curcumin-mediated hO-1 induction and activation of Nrf2 expression via stimulation of ARE-binding activity remains to be examined. Here, we analysed the involvement of Nrf2 and ARE activation in hO-1 induction by curcumin and CAPE in renal epithelial cells.

MATERIALS AND METHODS

Chemicals and reagents
Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], CAPE and all the reagents for luciferase assays were obtained from Sigma (St. Louis, MO, U.S.A.). Stock solutions of curcumin and CAPE (5 mM) were prepared in ethanol. Tissue-culture media were from Life Technologies (Rockville, MD, U.S.A.) and fetal bovine serum was obtained from Technet (Herdon, VA, U.S.A.). Oligonucleotides were synthesized by IDT (Coralville, IA, U.S.A.). Radiolabelled nucleotides were obtained from NEN Life Science Products (Boston, MA, U.S.A.). Polyclonal antibodies for hO-1 were from Stressgen (Victoria, Canada). Anti-Nrf2 and all the other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All other chemicals were of reagent grade and obtained from Sigma unless otherwise specified.

Cell culture and experimental protocols
Porcine renal epithelial proximal tubule cells (LLC-PK1) and rat kidney epithelial cells (NRK-52E) were purchased from the American Tissue Culture Collection (Manassas, VA, U.S.A.). Cells were cultured using Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cells were grown in 75 cm^2 flasks and kept at 37 °C in a humidified atmosphere of air and 5 % CO_2. Confluent LLC-PK1 cells were exposed to various concentrations of curcumin, CAPE or other phenolic compounds for different times. After each treatment, cells were harvested for the measurements of haem oxygenase activity, hO-1 and Nrf2 protein expression and electrophoretic mobility shift assays (EMSSAs; see below).

Haem oxygenase activity assay and Western blot for hO-1
Haem oxygenase activity was determined at the end of each treatment using a modification of a method described previously by our group [21]. Briefly, harvested cells were subjected to three cycles of freeze–thawing and the suspension was added to a reaction mixture (1 ml final volume, pH 7.4) containing MgCl_2 (2 mM), NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 unit), 3 mg of rat liver cytosol and the substrate haemin (20 µM). The reaction was conducted at 37 °C in the dark for 1 h, terminated by the addition of 1 ml of chloroform, and the extracted bilirubin was measured by the difference in absorbance between 464 and 530 nm (ε = 40 mM^-1 cm^-1). The total protein content was determined using a Bio-Rad DC protein assay (Bio-Rad, Herts., U.K.) and haem oxygenase activity expressed as pmol of bilirubin/mg of protein per h.

Samples of cells were also analysed by the Western immunoblotting technique as described previously [22]. Briefly, 30 µg of protein was separated by SDS/PAGE, transferred overnight on to nitrocellulose membranes, and the non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-hO-1 antibody (1:1000 dilution in Tris-buffered saline, pH 7.4) for 2 h at room temperature. After three washes with PBS containing 0.05 % (v/v) Tween-20, blots were visualized using an amplified alkaline phosphatase kit from Sigma (Extra-3A) and the relative density of bands was analysed using an imaging densitometer (model GS-700; Bio-Rad).

Plasmids
Construction of plasmids pH0151uc, pH015ucΔE2, pH015ucΔ(E1 + E2), pE11uc and its corresponding mutant pE11ucM793, pE22uc and its corresponding mutant pE22ucMc45, and p3XStREMuc and its corresponding mutant p3XStREMuc22 has been described previously [23–26]. Plasmid pCMVβ-gal encodes Escherichia coli β-galactosidase and was used to normalize for variations in transfection efficiency. Expression plasmids for Nrf2 and Keap1 were kindly provided by Dr Stuart Orkin (Harvard Medical School and Howard Hughes Medical Institute, Boston, MA, U.S.A.) and Dr Masayuki Yamamoto (The Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Japan). The plasmid encoding Nrf2(29–597) was generated by deletion of 5' sequences up to the unique BglII site in the mouse Nrf2 cDNA.

Cell transfection and enzyme assays
NRK-52E cells were seeded in 12-well plates (1 x 10^6 cells/well), cultured for 20 h and transfected with a DNA mixture consisting of (per well) 100 ng of the appropriate luciferase construct, 50 ng of pCMVβ-gal and 100 ng of the appropriate empty vector or effector plasmid using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) according to the manufacturer's recommendations. Then, 24 h later, the transfection medium was removed and replaced with medium containing vehicle (ethanol) or 10 µM curcumin.
Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element

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The transcription factor Nrf2, which normally exists in an inactive state as a consequence of binding to a cytoskeleton-associated protein Keap1, can be activated by redox-dependent stimuli. Alteration of the Nrf2–Keap1 interaction enables Nrf2 to translocate to the nucleus, bind to the antioxidant-responsive element (ARE) and initiate the transcription of genes coding for detoxifying enzymes and cytoprotective proteins. This response is also triggered by a class of electrophilic compounds including polyphenols and plant-derived constituents. Recently, the natural antioxidants curcumin and caffeic acid phenethyl ester (CAPE) have been identified as potent inducers of haem oxygenase-1 (HO-1), a redox-sensitive inducible protein that provides protection against various forms of stress. Here, we show that in renal epithelial cells both curcumin and CAPE stimulate the expression of Nrf2 in a concentration- and time-dependent manner. This effect was associated with a significant increase in HO-1 protein expression and haem oxygenase activity. From several lines of investigation we also report that curcumin (and, by inference, CAPE) stimulates ho-1 gene activity by promoting inactivation of the Nrf2–Keap1 complex, leading to increased Nrf2 binding to the resident ho-1 AREs. Moreover, using antibodies and specific inhibitors of the mitogen-activated protein kinase (MAPK) pathways, we provide data implicating p38 MAPK in curcumin-mediated ho-1 induction. Taken together, these results demonstrate that induction of HO-1 by curcumin and CAPE requires the activation of the Nrf2/ARE pathway.

Key words: caffeic acid phenethyl ester, cytoprotection, haem oxygenase-1 regulation, plant-derived constituents.

INTRODUCTION

Haem oxygenase-1 (HO-1) is a ubiquitous and redox-sensitive inducible stress protein that degrades haem to CO, iron and biliverdin [1–3]. The importance of this protein in physiological and pathological states is underlined by the versatility of HO-1 inducers and the protective effects attributed to haem oxygenase products in conditions that are associated with moderate or severe cellular stress.

We have recently shown that curcumin and caffeic acid phenethyl ester (CAPE), two plant-derived polyphenolic compounds, are potent inducers of HO-1 in vascular endothelial and neuronal cells [4,5] and we hypothesized that part of the pleiotropic and beneficial actions attributed to these and other chemically related natural substances could be explained by their intrinsic ability to strongly activate the haem oxygenase pathway [5]. In view of the increasing evidence corroborating the importance of CO and bilirubin to counteract cellular dysfunction [6–10], the activation of HO-1 by natural compounds offers a great advantage for therapeutic purposes, as curcumin and CAPE could become part of the human diet and be consumed daily as herbal supplements. Both curcumin and CAPE exert a diversity of beneficial effects including inhibition of mutagenesis and chemically induced carcinogenesis [11,12], as well as prevention of vascular and neurodegenerative diseases [13,14]. A recent study revealed that under severe hypoxic conditions the potency of curcumin to increase endothelial HO-1 expression and consequently protect cells against oxidative stress is highly amplified [4]. Unlike most ‘classical’ HO-1 inducers, which are strictly dependent on their oxidant potential to transcriptionally activate ho-1 gene expression, curcumin and CAPE are known to possess also antioxidant as well as anti-tumour and anti-inflammatory properties [11,12,15,16]. By virtue of Michael reaction acceptor functionalities and its electrophilic characteristics, curcumin and several other structurally related polyphenolic compounds induce the activities of phase II detoxification enzymes, which appear to be crucial in protection against carcinogenesis and oxidative stress [17]. Among these defensive systems are γ-glutamylcysteine synthetase, glutathione S-transferases and NADP(H):quione oxidoreductase. The co-ordinated induction of these cytoprotective genes is mediated through cis-regulatory DNA sequences located in the promoter or enhancer region, which are known as antioxidant-responsive elements (AREs) or stress-responsive elements [the active sequences in the mouse ho-1 gene, referred to as the stress-responsive element (STRE), are structurally and functionally similar to the ARE, a more commonly used term: for simplicity, and to avoid confusion, the latter terminology will be used throughout the remainder of the article]. The consensus ARE resembles the Maf-recognition element and can be specifically bound by a combination of the basic-leucine zipper (bZIP) transcriptional factors including Jun, Fox, Maf and Nrf2. Among them, Nrf2 plays a central role in the transcriptional regulation of antioxidant and detoxifying genes.