Pharmacological Strategies to Reduce Ischemia/Reperfusion Injury in Kidney Transplantation

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Declaration

All of the written work herein is my own. Any contribution made by others to the experimental work is acknowledged in the text. This work has not previously been submitted for any other degree or qualification.

Ewen Harrison

October 2007

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EMH
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Abstract

Kidney transplantation is the gold-standard treatment for end-stage renal failure but despite many advances the incidence of graft failure remains high. Ischemia/reperfusion injury (IRI) contributes to the occurrence of delayed graft function and is consistently associated with poorer long-term outcome. A potential protective strategy involves the up-regulation of *heat shock*, or *stress proteins* (Hsps), a highly conserved group of intracellular chaperones. Heat shock protein expression is regulated by heat shock transcription factor 1 (HSF1), which itself is subject to a number of regulatory mechanisms. The influence of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway on HSF1 activation status was investigated. Despite effecting significant up-regulation of the PI3K/Akt pathway with insulin and insulin-like growth factor-1 (IGF-1), no change in the HSF1 trimerisation state, DNA-binding ability or nuclear localisation was demonstrated in the ACHN renal adenocarcinoma cell-line. Heat shock protein 70 (Hsp70) promoter activity, mRNA levels and protein expression were not influenced by alterations in PI3K/Akt activity.

However, following treatment with insulin, a 5-fold increase in heme oxygenase-1 (HO-1) mRNA and a 4-fold increase in protein expression were observed in ACHN cells; insulin-induced HO-1 expression was also demonstrated in mouse primary tubular epithelial cells. The induction of HO-1 in the ACHN cell-line was blocked by actinomycin D and cycloheximide and was abolished by the phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, but not by the inactive analogue, LY303511. The over-expression of a dominant-negative form of Akt abrogated the HO-1-

inducing effects of insulin, whereas cells transfected with a constitutively active Akt construct demonstrated an increase in HO-1 promoter activity and protein expression. The transcription factor NF-E2-related factor-2 (Nrf2) was found to translocate to the nucleus in a PI3K-dependent manner following insulin treatment. Pre-treatment with Nrf2 small-interfering RNA (siRNA) abolished insulin-induced HO-1 induction. Thus, insulin induces HO-1 mRNA and protein expression in renal cells in a PI3K/Akt and Nrf2 dependent manner.

The heat shock protein 90-binding agents (HBAs) geldanamycin and its analogues (17-AAG and 17-DMAG) are known to up-regulate Hsps and confer cellular protection. The ability of HBAs to protect the kidney was examined in a model relevant to transplantation. Hsp70 gene expression was increased 30 – 40-times in ACHN cells treated with HBAs and trimerisation and DNA-binding of HSF1 was demonstrated. A 3-fold and 2-fold increase in Hsp70 and Hsp27 protein expression, respectively, was found in ACHNs treated with HBAs. HBAs protected ACHN cells from an H_2O_2-mediated oxidative stress and HSF1 siRNA abrogated HBA-mediated Hsp induction and protection. *In vivo*, Hsp70 was up-regulated in the kidney, liver, lungs and heart of HBA-treated mice. This was associated with a functional and morphological renal protection from IRI. Therefore, HBAs mediate up-regulation of protective Hsps in mouse kidneys and are associated with reduced renal IRI and may be useful in reducing transplant-associated kidney injury.

Chapter 1 – Introduction

1.1 Overview

Kidney transplantation* is the gold-standard treatment for end-stage renal failure. Despite the great advances and clinical successes in the field, a significant rate of graft failure still exists with dire implications for the graft recipient. The physiological consequences of organ transplantation are complex. Many potential targets of intervention have been identified in the quest to improve outcome. This work seeks to discover strategies to reduce the injury sustained by the organ during the transplantation procedure and the period immediately thereafter, with the ultimate aim of introducing a clinical therapy that will reduce the incidence of graft loss. The thesis focuses on a group of protective proteins called *heat shock proteins* (Hsps) and how these may be harnessed to protect the transplanted organ.

In this introduction a number of arguments are presented to justify the work herein. There is a brief description indicating that the incidence of end-stage renal failure in the UK and other Western countries is increasing; that the best treatment, both to reduce patient morbidity/mortality and in economic terms, is renal transplantation; that despite being desirable, renal transplantation is often not possible due to a shortage of organs; that renal transplantation, despite its success, still has a significant failure rate; and that this graft failure, despite being due to a myriad of factors, may be considerably reduced by manoeuvres prior to, and during organ retrieval and storage. The pathophysiology of ischemia/reperfusion injury (IRI) is

* Transplantation: transferring an organ (or part of one) from one body to another, for the purpose of replacing the recipient's damaged or failing organ with a working one from the donor

considered and potential strategies to reduce the effect of this process. The potential of the Hsps in this regard are highlighted and discussed in more detail.

1.2 End-stage renal failure (ESRF)

1.2.1 Background

The kidneys are a pair of organs in the abdomen that function to excrete the waste products of metabolism, as well as maintaining acid-base balance, blood pressure and plasma volume. Kidney function is essential to life. Progressive and irreversible reduction in kidney function over time is termed *chronic renal failure* (CRF), or more recently *chronic kidney disease* (CKD), and is characterised by the gradual loss of nephrons, the functional unit of the kidney. While conservative management is successful in the early stages, some patients will progress to *end-stage (or established) renal failure* (ESRF). Prior to the introduction of the first effective replacement of renal function by Kolff and Berk in 1943, all patients with ESRF died (1).

1.2.2 Epidemiology and aetiology

The incidence of ESRF in the UK is rapidly increasing (Figure 1.1), making its effective diagnosis and management a priority for the healthcare profession. The number of adults commencing renal replacement therapy (RRT)[†] in 2004 was

[†] Renal replacement therapy: life supporting treatments for renal failure including hemodialysis, peritoneal dialysis, hemofiltration and renal transplantation.

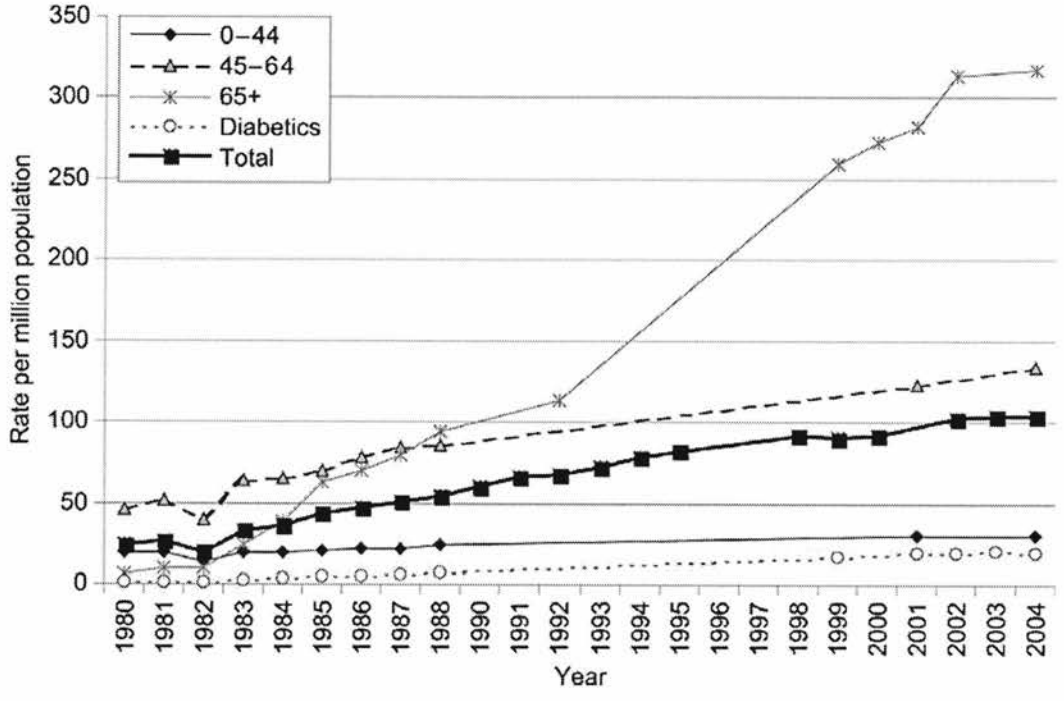

Figure 1.1 **Incidence of commencement of renal replacement therapy (per million population) in the UK 1980–2004.**
From UK Renal Registry Annual Report 2005 (2).

estimated to be 103 per million population (pmp), representing a 7% rise since 2000. The estimated prevalence of patients requiring RRT at the end of 2004 was 638 pmp. These trends are broadly in keeping with other Western countries (Table 1.1) and probably reflect the aging population and increasing incidence of vascular disease, diabetes and hypertension which are all important aetiological factors in the development of ESRF. The incidence also continues to rise in the paediatric population, though the aetiology of the disease in this group is different. Predictive models in both UK and US adult populations suggest that the prevalence of ESRF will continue to rise and is unlikely to reach a steady state for at least 25 years (Figure 1.2) (3,4). ESRF currently costs over 2% of the total NHS budget with this figure expected to rise (5). It is essential, therefore, that current treatments for renal failure are optimised and that research is performed to further delineate the pathological processes contributing to this disease. Any improvement in the cost-effective treatment of kidney disease is highly desirable.

1.2.3 Disease progression and early management

Chronic renal failure is a condition of degrees. Patients with mild disease may not realise they have it and will often have no symptoms. While some will never need RRT, the inexorable decline to ESRF is inescapable for many. Management initially focuses on general health advice (smoking cessation, weight loss, aerobic exercise, limiting sodium intake) and the avoidance of nephrotoxic drugs. Most patients will benefit from secondary prevention of cardiovascular disease (low-dose aspirin, lipid-lowering therapy, tight control of hypertension and possibly treatment with an ACE

Country	New patients commencing RRT (pmp)	Total patients receiving RRT (pmp)
United States	336	1403
Germany	184	919
Greece	163	812
Belgium (Dutch speaking)	160	855
Spain (Catalonia)	146	1022
Denmark	138	679
Italy	136	835
Austria	136	750
Sweden	124	735
New Zealand	119	655
Wales	105	641
Scotland	101	644
Netherlands	100	639
Australia	97	633
Norway	94	606
England	91	547

Table 1.1 **Number of patients commencing, and total patients receiving renal replacement therapy in 2001** (5).
pmp, per million population.

inhibitor) and all will require regular monitoring of renal function. Rigorous glycaemic control is required in diabetics. Yet despite these measures, the renal function of many patients will continue to decrease and symptoms may develop (fatigue, nausea, loss of appetite, breathlessness). Fluid and electrolyte disturbances will begin to occur and loss of renal hormonal function may result in anaemia, hyperphosphatemia, hypocalcaemia or hyperparathyroidism.

Renal replacement therapy includes hemodialysis, peritoneal dialysis, hemofiltration and renal transplantation. While dialysis is effective in mitigating the direct effects of renal failure, it is not without significant negative consequences. Renal transplantation has been shown to improve quality of life and reduce mortality, when compared with long-term dialysis treatment (6-8). Many of these early studies, however, did not control for the fact that transplant recipients are a highly selected subgroup of the dialysis population – patients placed on the waiting list for transplantation tend to be younger, in better health and of higher socioeconomic status than those not selected (9). However, in 1999 Wolfe *et al* demonstrated conclusively that in matched cohorts, long-term mortality was 48 – 82% lower in transplant recipients as compared with patients listed for, but who did not undergo, renal transplantation (10). Hence the current evidence clearly shows that renal transplantation is the best available treatment for ESRF.

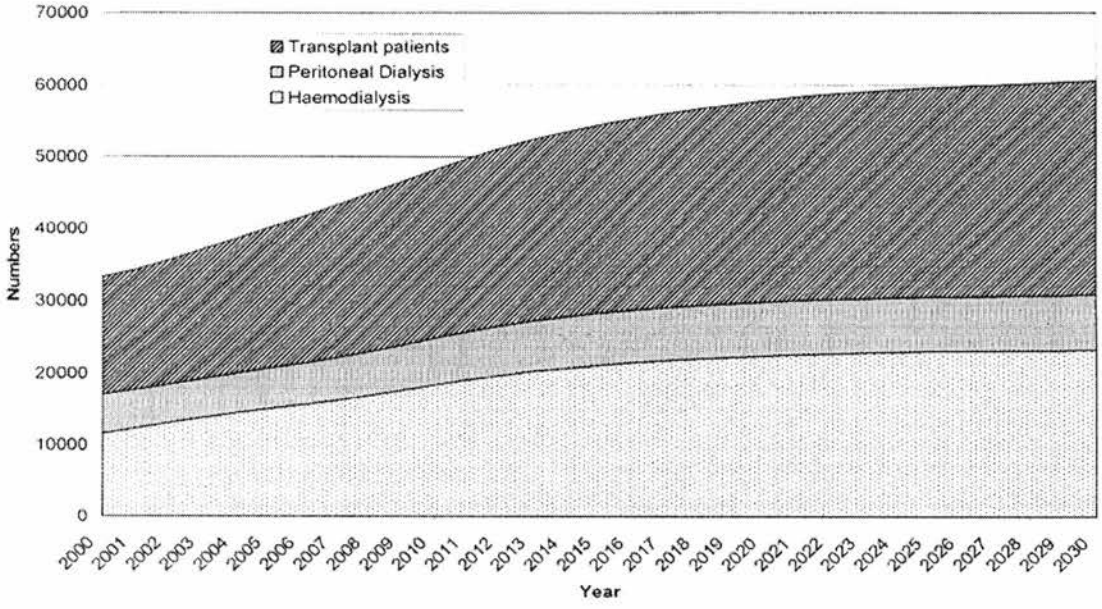

Figure 1.2 **Projected number of patients requiring renal replacement therapy from 2000 and 2030 in England.**
From Roderick et al (2004) (2).

1.3 Kidney transplantation

1.3.1 Historical context

The roots of modern solid organ transplantation lie in antiquity with the first references occurring in 2nd-century China, where Hua-To is reported to have replaced diseased organs with healthy ones (11). The legend of the 3rd-century Saints Cosmas and Damian describes the painless amputation of the ulcerated leg of a Christian verger, and its substitution with the healthy leg of a dead Moor (12).

The transition of transplantation from a spectacularly unsuccessful endeavour 60 years ago to the valuable clinical intervention of today has been dependent on two areas of work: the achievement of reliable techniques for organ preservation and revascularisation and the progressive understanding of the biological processes governing rejection.

The first reported human kidney allograft‡ was performed in 1936 by Yu Yu Voronoy, a Russian surgeon, who transplanted six human cadaveric kidneys without success (13). Further attempts in France and the US in the early 1950s were all unsuccessful. This incredibly discouraging phase in the history of transplantation ended in Boston on December 23 1954 when Murray, Merrill and Harrison removed a kidney from a healthy man, and transplanted into his uraemic identical twin brother. Despite no efforts being made to preserve the organ, it functioned immediately and the recipient lived for nearly 25 years (14).

‡ Allograft: transplantation of organs between members of the same species.

In 1943 Medawar demonstrated that rejection of skin allografts was an immunological phenomenon (15,16) and 10 years later, together with Billingham and Brent, showed that tolerance to skin allografts could be achieved by injecting foetal or newborn mice with lymphoid cells from an adult (17). These discoveries marked the beginnings of transplantation immunology and formed the basis of the development of immunosuppressive drugs essential to transplantation today.

Preservation of organs was initially performed by machine perfusion with cryoprecipitated plasma (18). In spite of this, simple cold storage after a vascular flush with a solution developed by Collins (19) was shown to yield superior results. Various preservation solutions are now available with University of Wisconsin solution being one of the most widely used (20).

1.3.2 Current state of transplantation

Despite the great successes of transplantation, it is with a certain despondency that one reviews the latest statistics compiled by UK Transplant, the regulatory body governing all solid organ transplantation in the UK (Figure 1.3). Despite the number of patients awaiting transplantation increasing from 5074 in 2003/04 to 5425 in 2004/05, the total number of cadaveric kidney transplants performed fell from 1388 to 1308 in the same period. During this time, 293 patients awaiting transplantation died and a further 420 were removed from the list, which often occurs when a patient becomes too unwell to undergo transplantation; this represents 7.8% of the total patients waiting and receiving organs for that period (Table 1.2). The reasons behind the scarcity of available organs are manifold but include positive factors, such as improvements in road safety and alterations in neurosurgical practice and less welcome factors, such as negative public attitudes towards donation.

A number of strategies have been adopted to attempt to reverse this trend. Increasing donation rate is possible and has been demonstrated in Spain, where a comprehensive donor management system has increased donation rates to 33 pmp, compared with 12.3 pmp (2004) in the UK (21). Living kidney donor programmes have also been very successful: the failure rate of organs procured in this way is significantly less compared with organs from deceased donors. The introduction of non-heart beating donor protocols and the use of marginal, or extended-criteria organs[§] has also gone someway to trying to reduce the gap between supply and demand. As technology

[§] Expanded-criteria donors: donors > 60 yrs *or* donors 50 – 59 yrs with two of the following characteristics – previous cerebrovascular accident, hypertension or elevated creatinine (United Network for Organ Sharing definition)

progresses many exciting interventions are being developed, such as stem cell-based therapies and xenotransplantation. However, these technologies are in their infancy and are unlikely to yield significant clinical benefit in the near future.

Xenotransplantation[**]

Using animals as a source of organs for transplantation is a potential solution to organ donors shortage (22). Xenotransplantation was first attempted in the 19th century, however it was not until the 1960s that serious study was given to the concept (23). Yet, despite concerted efforts over the last 50 years, results have been very poor. Swine kidneys transplanted into primates are subject to hyperacute rejection and acute humoral rejection (24). This is a result of antibodies directed against a specific epitope (galactose α1-3 galactose) on vascular endothelium. However, Phelps *et al* have developed an α-1,3-galactosyltransferase gene knockout pig that does not express the Gal epitope. When kidneys from these animals are transplanted into baboons, hyperacute rejection is avoided. However, the grafts are still subject to acute humoral and cellular rejection at 34 days with immunosuppression or 83 days when a tolerance-inducing protocol is used (25). The field, therefore, holds great potential but only if these great immunological barriers can be overcome.

[**] Xenotransplantation: (Xeno- from the Greek meaning "foreign") the transplantation of cells, tissues or organs from one species to another

Outcome	Total number (%)	
Remained active/suspended	6797	(75)
Transplanted	1578	(17)
Removed	420	(5)
Died	293	(3)
TOTAL	**9088**	

Table 1.2 **Kidney transplant in the UK 1 April 2004 – 31 March 2005.** (Data: UK Transplant)

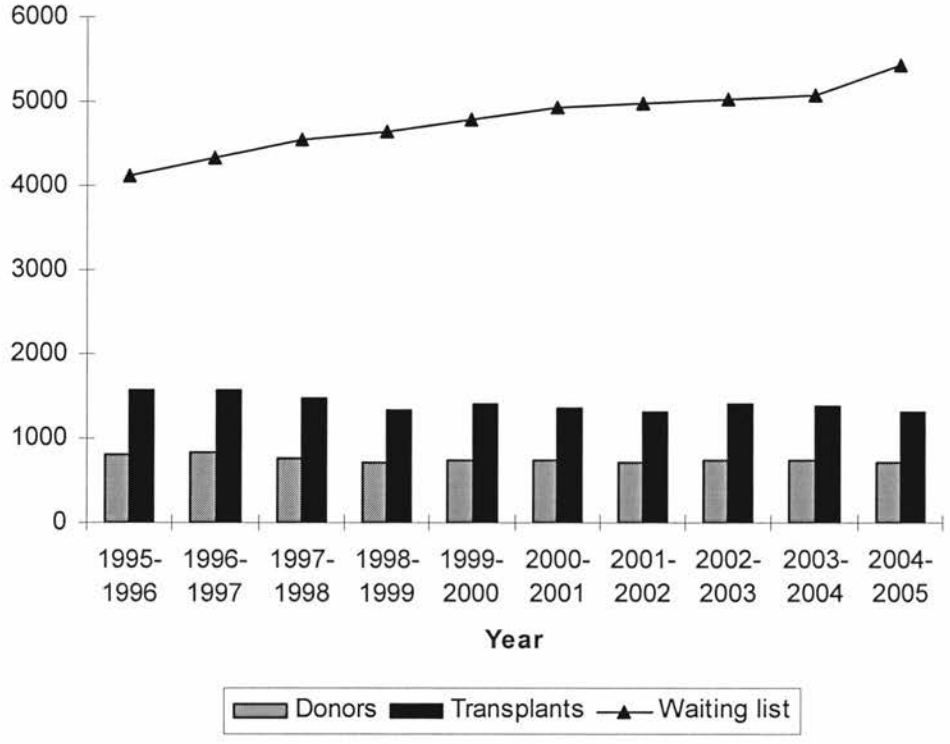

Figure 1.3 **Cadaveric kidney transplantation in the UK 1993 – 2004.**
Over the last 12 years, the number of organs available for transplantation remained static or decreased. Despite this, the number of patients with end-stage renal failure being listed for transplantation has increased year on year. (Data: UK Transplant)

1.3.3 Outcome after kidney transplantation

The success of transplantation in general and kidney transplantation specifically is without question. Better surgical technique and superior immunosuppression have contributed to year on year improvements in outcome (Figure 1.4A). The 3-year survival after any type of kidney transplantation is now 86%, compared with only 70% in the late 1980s. Even better results have been seen with living-donor grafts: in HLA-identical siblings the 3 year graft survival is 96% (Figure 1.4B), although living-donor grafts make up only 29% of UK kidney transplants as a whole (UK Transplant 2004/2005 data). With outcome appearing to improve so greatly, one may question whether efforts to try and improve it further are worthwhile? There are four good reasons why the figures quoted above do not tell the whole story:

1. Ischemia/reperfusion injury: a significant but modifiable insult that occurs at the time of transplant often with negative long-term consequences.
2. Functional outcomes: only 47% of grafts are still functioning at 10 years.
3. Marginal or expanded-criteria donors: the shortage of organs means kidneys that would have been discarded previously are now being used. These are associated with poorer outcome (Figure 1.4C).
4. Immunosuppression: improvements in outcome are as much a consequence of improved immunosuppression. However, complications are common, particularly infections and cancer.

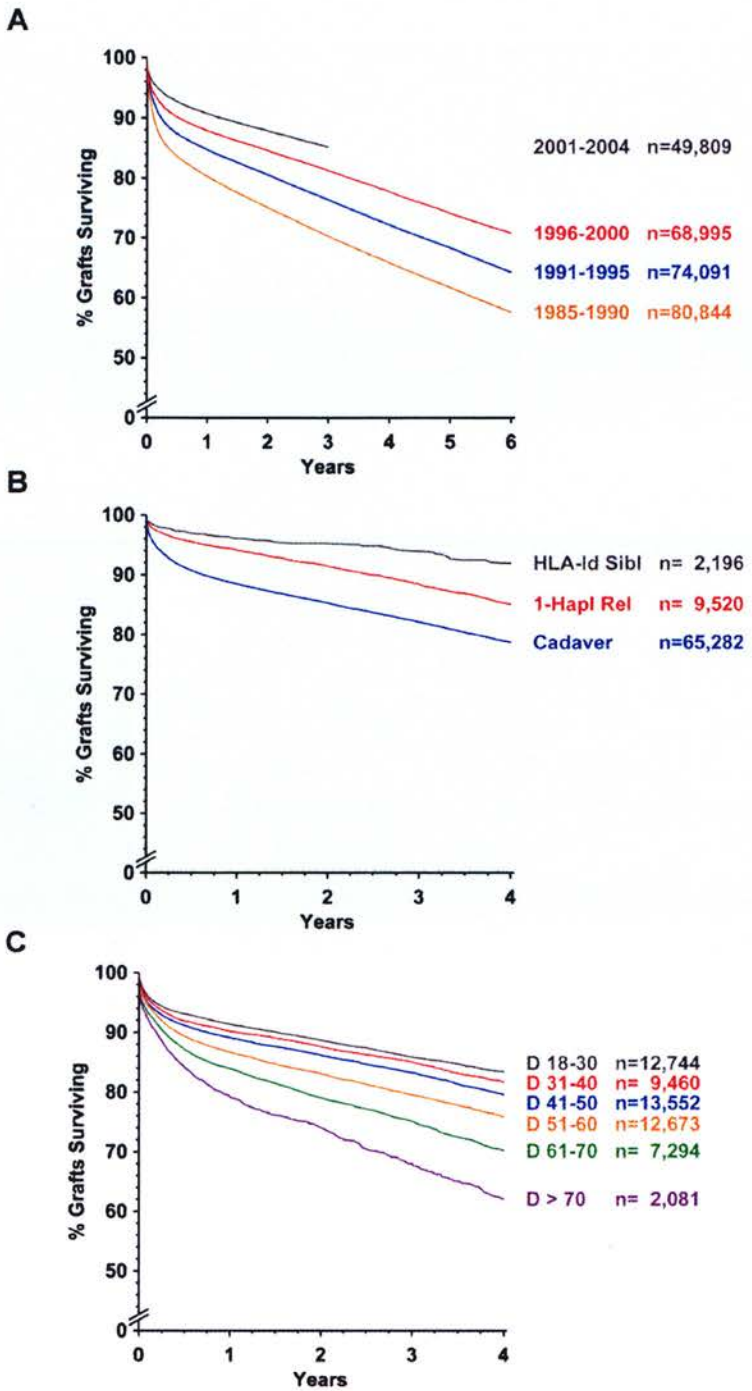

Figure 1.4 **Proportion of surviving kidney grafts by year (A), donor-recipient relationship (B) and donor age (C).**
(From Collaborative Transplantation Study, www.ctstransplant.org (members section, accessed 21 June 2006)

The last 40 years have seen a large number of studies analysing the risk factors contributing to negative outcomes in kidney transplantation. Patient populations are examined in a single-centre, multi-centre or registry settings, with multivariate analysis (linear, logistic or Cox regression) used to identify factors significantly related to outcome (usually graft failure). These risk factors can be grouped in five categories (adapted from (26)):

- Donor factors (tissue quality)
- Donor death circumstances (brain death/cardiovascular status)
- Preservation variables
- Donor-recipient immunological relationship
- Recipient factors

Before examining these categories further, two important points must be considered. Firstly, the majority of risk factors identified are not modifiable, i.e. it is not usually possible to improve donor tissue quality, the circumstances of brain death and pre-existing recipient morbidity. The impact of these variables can be influenced by matching donors and recipients carefully, but ultimately the effect of many of these factors is uncontrollable. Secondly, despite many of these factors appearing to be significant in small univariate analyses, many fail to achieve statistical significance when entered into a multivariate model of a large heterogeneous population.

Delayed graft function

An indicator of outcome that has been extensively studied (over 900 individual studies) is the occurrence of *delayed graft function* (DGF). DGF is most commonly defined as a requirement for dialysis within the first week post-transplantation. This definition has been modified in different studies, for instance excluding patients who only had one dialysis session for fluid overload or hyperkalaemia. Other studies have used alternative criteria, such as the pattern of serum creatinine in the first week following transplantation (27). The occurrence of DGF is consistently associated with poor outcome. When DGF and acute rejection occur together the outcome is even worse (28). Despite improvements in donor and recipient management, DGF rates have remained high, possibly due to increasing use of expanded-criteria donors (29). Most studies have found a direct correlation between DGF and long-term graft function and survival (Table 1.3). DGF represents the immediate impact of the sum of the five factors listed above, with some factors predicting in the incidence of DGF better than others. The risk factors for DGF commonly found to be significant are:

1. Cold ischemic time (CIT)[††].
2. Donor age.
3. High panel reactive antibody (PRA)[‡‡].
4. Recipient ethnicity.
5. Donor sex.

Again, it should be noted that other than CIT, none of the other risk factors are modifiable. CIT represents a combination of injury to the organ occurring during

[††] Cold ischemic time: time between retrieval of kidney from the donor and transplant into recipient.
[‡‡] Panel reactive antibody: a measure of pre-formed anti-human antibodies in recipient's blood.

cold storage and IRI. A number of strategies have been implemented to decrease cold ischemic time. These typically stipulate time limitations by which the organ has to be transplanted into the recipient (usually 24 h following retrieval). However, the detrimental effects occurring at a cellular level during cold storage and particularly as a result of IRI represent an attractive target for intervention. If these damaging effects could be lessened then it is possible that the incidence of DGF (with its intrinsic worsening of outcome) could be decreased. Additionally, it is possible that organs that would have been deemed unsuitable for use in the past become useable.

Author	Year	N	Data	DGF incidence	RF for DGF	Outcome (Graft survival)	Ref
Ojo	1985 – 1992	37 216	Registry	26.2%	CIT Donor age Recipient ethnicity	66% vs 53% at 5 yrs	(30)5)
Shoskes	1994 – 1997	27 096	Registry	22 – 27%	CIT Donor age	91% vs 75% at 1 yr	(31)d}
Sanfilippo	1977 – 1982	3800	Multi-centre	35%	CIT Donor ethnicity	40% vs 28% at 4 yrs	(32)7)
Boom	1983 – 1997	734	Single centre	23.2%	CIT Donor age Recipient MAP prior to graft Gender mismatch High PRA	NS but RF for suboptimal function at 1 year OR 1.68 (1.14 – 2.48)	(27)2)
McLaren	1985 – 1995	710	Single centre	27.3%	CIT Donor age High PRA	75% vs 56% at 5 yrs	(28)3)
Pfaff	1990 – 1995	586	Single centre	26.7%	CIT Donor age Cause of death	93.2% vs. 76.6% at 1 yr	(33)8)
Koning	1988 – 1993	547	Multi-centre	24%	CIT Donor age Recipient previous transplant Intra-op diuresis	74% vs. 64% at 4 yrs	(34)9)
Gentil	1990 – 2000	476	Single centre	36.8%	Donor age Vascular cause of death	80.5% vs. 70% at 5 yrs	(35)0)
Troppmann	-	457	Single centre	23%	CIT	NS	(36)1)
Bertoni	1991 – 1997	229	Multi-centre	Not stated	CIT Donor age Storage solution Gender mismatch PRA (26 – 50%)	90.9% vs. 69.3% at 1 yr	(37)2)
Pieringer	1993 – 1999	95	Single centre	48.4%	Dialysis time Pre-op BP Re-anastomosis time	84.4% vs. 74.2% (NS) at 3 yrs	(38)3)
Iglesias-Márquez	1997 – 1999	79	Single centre	31.6%	CIT Donor age Gender mismatch Non-traumatic death	100% vs 84% at 3 yrs	(39)4)

Table 1.3 **Non-comprehensive/representative list of studies analysing the incidence of delayed graft function (DGF) in cadaveric kidney allograft recipients.**

1.4 Ischemia/reperfusion injury

1.4.1 Overview

The process of organ transplantation, by its very nature, requires the disconnection of the blood supply of an organ followed by the restitution of that supply during the recipient procedure. The physiological consequence of this is termed ischemia/reperfusion injury (IRI). The ischemic injury to an organ is the sum of the periods of *warm ischemia* (prior to cooling and flushing with preservation fluid) and *cold ischemia* (the period during which the organ is stored on ice prior to revascularisation). Reperfusion injury begins at the point in the recipient procedure when the clamps are released and the blood supply to the organ is restored. The importance of IRI in transplantation cannot be over-emphasised. In the previous section, the contribution of cold ischemia to the occurrence of DGF and ultimately graft failure was demonstrated. CIT is a surrogate for the magnitude of IRI to the organ. Despite the great number of experiments that have been performed looking at IRI, it remains one of the least well described phenomena in transplantation. In recent years, the 'classical' understanding of IRI has gradually evolved to emphasise the specific roles of the inflammatory and immune responses (40,41). The classical division of the pathological consequences of 'ischemia' and 'reperfusion' is becoming irrelevant, but still serves the purpose of illustrating the different processes that occur (Figure 1.5).

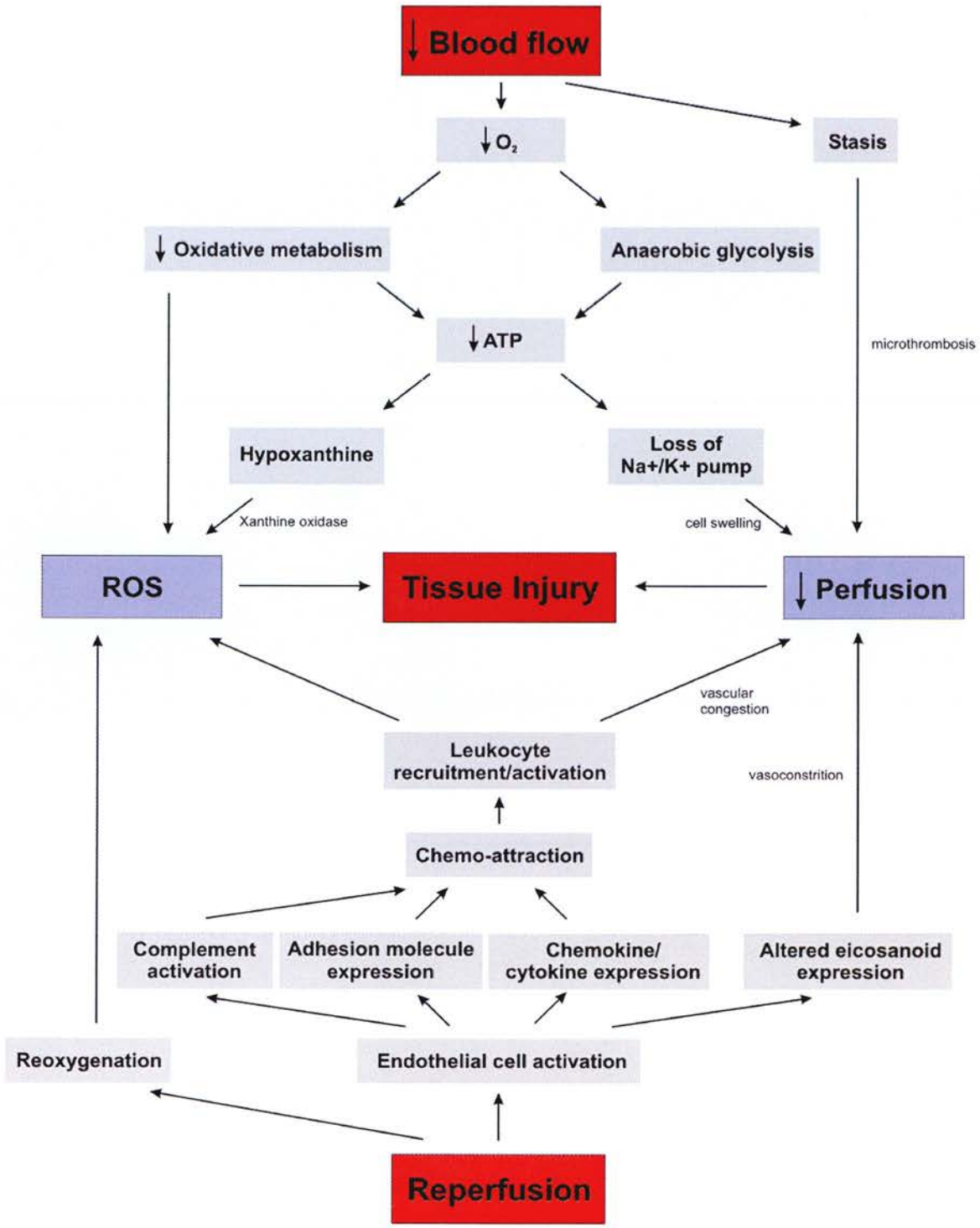

Figure 1.5 A classical representation of the mechanisms contributing to tissue injury following IRI.

1.4.2 Physiological renal hypoxia

Although the kidney receives 25% of the cardiac output of a resting adult, it is very susceptible to ischemic injury. Why does this contradiction exist? A major function of the kidney in land mammals is the conservation of water. The concentration of urine is dependent on a gradient of osmolality between the renal cortex and medulla established by the well described countercurrent mechanism. The increased medullary tonicity resulting from this process is inversely proportional to medullary blood flow – put simply, greater blood flow would 'wash away' solute thus abolishing the osmotic gradient essential for urinary concentration. To compound this, re-absorption along the medullary thick ascending limb of Henle is an active process requiring oxygen. The majority of renal blood-flow is directed to the cortex which has a partial pressure of oxygen around 50 mm Hg, compared with 10 – 20 mm Hg in the medulla. Medullary hypoxia, therefore, is the inevitable consequence of efficient urinary concentration, but leaves the kidney susceptible to ischemic injury should any alteration in blood-flow or oxygen delivery occur (42).

1.4.3 IRI – the classical understanding

The primary effect of diminished blood supply to an organ is hypoxia (Figure 1.5). Depletion of energy-rich phosphates (ATP) results in a failure to maintain ion balances across cell membranes resulting in intracellular accumulation of water. This cell swelling, together with microthrombosis, contributes to decreased capillary

perfusion and has been implicated in the 'no reflow' phenomenon*, exacerbating the already present hypoxia. *Endothelial activation* is a poorly defined term for the response to injury of the vascular endothelium, which includes increased thrombogenicity and a rapid up-regulation and surface expression of various adhesion molecules including intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM) and the selectins. These molecules facilitate the recruitment of leukocytes allowing binding to the vascular endothelium, further compromising microvascular blood flow. Leukocyte-endothelial interaction occur predominantly in the outer medulla, leading to the vascular congestion in this area evident after IRI (40). The adherent leukocytes release reactive oxygen species (ROS), a variety of cytokines and contribute to the activation of complement (44), further exacerbating the inflammatory reaction and tissue injury.

1.4.4 Role of ROS

Reactive oxygen species (ROS) are generated when molecular oxygen is reduced to water and are central to the pathogenesis of IRI. ROS are produced by endothelial cells and leukocytes during IRI (45). Small quantities occur in mitochondria during normal respiration while an ischemic episode is associated with significant changes in mitochondrial function. Impairment of oxidative metabolism at the level of the electron transfer chain results in 'electron leak' leading to the formation of superoxide radicals ($O_2\bullet^-$). Hydroxyl radicals (OH•) can also be formed via the Fenton reaction. The efficacy of antioxidant mechanisms, including mitochondrial

* No-reflow phenomenon: the inability to achieve myocardial reperfusion after removal of a coronary artery occlusion (43). Possible mechanisms include capillary vasospasm, oxygen free radical mediated injury, neutrophil and erythrocyte plugging and endothelial cell oedema secondary to IRI.

superoxide dismutase, is decreased and depletion of important substrates like glutathione occurs. Xanthine oxidase is also a significant source of ROS. Anaerobic respiration results in accumulation of hypoxanthine which cannot be converted to xanthine without oxygen. On reperfusion, the reaction proceeds with formation of ROS. *Lipid peroxidation* describes the oxidative degradation of lipids whereby free radicals "steal" electrons from lipids within cell membranes resulting in damage. Under physiological conditions, a number of anti-oxidant mechanisms act to negate the effects of ROS (46), including heme oxygenase-1 (HO-1; see section 1.6.3, page 53 and chapter 5, page 110) and mitochondrial superoxide dismutase. However, during IRI these mechanisms are typically overwhelmed.

Targeting ROS as a strategy for reducing IRI in transplantation is attractive and has been popular. A number of agents have been used experimentally but few have been examined in the clinical setting (see section 1.5, page 41). Although high concentrations of ROS result in cell damage, low concentrations may have a role as stress signalling molecules (47). Thus, even if complete elimination of ROS was possible, it may be undesirable as it would inhibit protective responses.

1.4.5 Role of adhesion molecules and inflammatory cells

Endothelial activation results in the up-regulation and surface expression of the selectins and the intracellular adhesion molecules (ICAMs) (48). The former act to slow and tether the rolling leukocyte, while the latter provide firm adhesion leading

to diapedesis[†]. Anti-E- and anti-P-selectin antibodies protect rat kidneys from IRI (49) and p-selectin glycoprotein ligand-1 is also protective in this model (50,51). Fuller *et al* demonstrated that the mode of protection following selectin-inhibition was an improvement in renal mircorperfusion, rather than the prevention of leukocyte extravasation which was similar in control and treated groups (50). Singbartl *et al* went on to demonstrate that platelet p-selectin inhibition, and not endothelial p-selectin, was important in reducing injury (52).

ICAM-1 blockade is protective in mice (53) and rats (54). In human trials, the use of anti-ICAM-1 was shown initially to reduce DGF (55), but was later found in a randomised multi-centre trial to be of no benefit (56). It should be noted that in both these trials, the drug was given to the recipient after organ implantation and not the donor prior to IRI.

Leukocytes are activated by cytokines and ROS, resulting in up-regulation of surface adhesion molecules on the leukocytes, which interact with endothelial adhesion molecules (40). The relative role of different leukocyte populations in mediating kidney injury following ischemia is the subject of great debate. Neutrophils have been studied most widely, yet their role remains the most controversial. Neutrophils are readily identifiable following ischemic injury in animal models, yet few are seen in biopsies from human kidneys subject to acute renal failure (48). Myeloperoxidase (MPO) activity following ischemic injury has been attributed by a number of authors as evidence of neutrophil infiltration (57), yet histological evidence remains scant

[†] Diapedesis: One step of extravasion, diapedesis refers specifically to the penetration of the vessel wall. Other steps incude the adhesion to the wall and the migration once through the wall.

(58) and MPO activity is also associated with monocytes/macrophages (59). However, if neutrophil infiltration is blocked, kidney injury is significantly reduced (49,52,60), although the specificity of effect in such experiments has been questioned (40). In balance, it is likely that the neutrophil plays a modest role as an effector cell in early IRI, also contributing to poor microvascular blood flow.

The increasing appreciation of the role of the T-cell in IRI has led some to pronounce that IRI is a "T-cell disease" (61). CD4/CD8 knockout and T-cell deficient (*nu/nu*)[‡] mice are protected from renal IRI, in the latter the injury being restored by adoptive transfer of T-cells (62,63). The mechanisms by which T-cells mediate IRI remain unclear, although it has been suggested that endothelial cells may act as antigen-presenting cells providing co-stimulatory signals to circulating T-cells (61).

1.4.6 Other mechanisms

A number of other important mechanisms exist in the pathogenesis of IRI, but are not examined directly in this work.

Complement. The activation of the complement cascade occurs in IRI and is associated with apoptosis. Inhibition of both C5 (64) and C3 (65) have been shown to reduce IRI in the mouse.

[‡] *nu/nu* mice: mice homozygous for the *nu/nu* spontaneous mutation exhibit abnormal hair growth and defective development of the thymic epithelium and are , therefore, athymic due to a developmental failure of the thymus.

Vasospasm. Alteration in the vascular perfusion of an organ is a common feature of IRI and contributes to disease progression. Severe injury to the proximal tubule results in altered eicosanoid expression. Excess thromboxane and decreased prostacyclin result in vasoconstriction and poorer tissue perfusion (66). Endothelin has also been proposed to have an important role: blockade of the endothelin receptor is protective in rat renal IRI (67) and patients with DGF have high serum levels of endothelin-1 (68).

Cytokines/chemokines. Endothelial cells, leukocytes and proximal tubular epithelial cells all express pro-inflammatory cytokines in response to IRI. Pro-inflammatory cytokines include tissue necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) and transforming growth factor-β (TGF-β) act to enhance the inflammatory response taking place. Chemotactic cytokines (chemokines) including monocyte chemoattractant protein-1 (MCP-1), IL-8 and regulated upon activation normal T-cell expressed and secreted (RANTES) are expressed contributing to the inflammatory cell response to IRI (40).

1.5 Strategies to reduce IRI

1.5.1 Overview

As has been shown, IRI is a multifactorial condition and a number of targets are available as potential targets for intervention, some of which have already been mentioned. As was discussed in section 1.3.3 (page 27), the incidence of DGF is closely associated with the degree of IRI. Of the five factors that contribute to DGF, in the context of IRI only three are modifiable:

- Donor death circumstances (and particularly cardiovascular status)
- Preservation variables
- Recipient factors

Donor death circumstances

The management of the donor after the diagnosis of brainstem death, and particularly maintaining haemodynamic stability, has been neglected at times in the past. There are a number of reasons for this including: a lack of active management after brainstem death diagnosis; referring physicians unaware of optimal donor management; a lack of reliable data on optimal donor management; delay in referral resulting in cardiovascular instability; and ethical concerns in treating the deceased donor. Maintaining cardiovascular stability in the donor patient is paramount and a loss of cardiac output prior to preservation is associated with poorer outcome in some series (69). Accurate fluid management and the judicious use of inotropes is beneficial (70). Brain stem death is associated with a loss of the pituitary–

hypothalamic axis leading to diabetes insipidus in 70 – 80% of brain-dead patients (71). Treatment with fluid replacement and desmopressin helps to maintain serum electrolyte levels. Replacement of insulin, triiodothyronine (T3) and cortisol helps to maintain normal organ function. A large, retrospective study demonstrated a significant increase in the number of organs transplanted in donors treated with methylprednisolone, vasopressin and T3/thyroxine (72).

Preservation factors

This study focuses on strategies to treat the donor prior to commencement of the multi-organ retrieval procedure. This is dealt with in detail in the next section. Other preservation variables are of the organ itself. A great deal of work has been done in the development of the preservation solutions used to flush and store organs in prior to implantation. These are balanced to maintain cellular homeostasis, provide energy and reduce the effect of ROS. University of Wisconsin solution has emerged as the standard (20). The method of preservation has also been examined by comparing cold storage with pulsatile machine perfusion. With improvements in technology the perfusion technique may be shown to be of benefit, but in a recent large registry study no benefit was demonstrated over cold storage (73).

Recipient management

The most important modifiable factor that has been shown to be of benefit in the recipient is adequate hydration. The administration of human albumin solution to expand the intravascular fluid volume was associated with a decreased incidence of DGF (74)

1.5.2 Preconditioning

The concept of treating an organ in order to protect it, prior to a known impending injury is termed *preconditioning*. It was first described by Murry *et al* in 1986 in the heart and has since been shown to apply in numerous circumstances (75). Many different strategies have been explored, which can be classified as *physical* or *pharmacological.*

Physical

Physical manoeuvres that can confer protection include hyperthermia, ischemia and oxidative stress. Whole-body or organ hyperthermia has been shown to be of benefit in a number of models relevant to transplantation (76-83) but is unlikely to be practical in the clinical setting. Hyperthermia is associated with induction of Hsps, which are believed to confer cellular protection. This thesis explores the up-regulation of Hsps and the potential cellular protection conferred in detail; Hsps are discussed further in section 1.6 (page 51).

Ischemic preconditioning (IP) is the most widely studied form of preconditioning. It can be used in different forms of surgery where an organ is to be subjected to a period of prolonged ischemia. Figure 1.6 illustrates examples of the different forms of IP that have been described. Organ transplantation is particularly suited to IP given that it is logistically relatively straight forward to render an organ ischemic for a short period of time prior to putting it in cold storage.

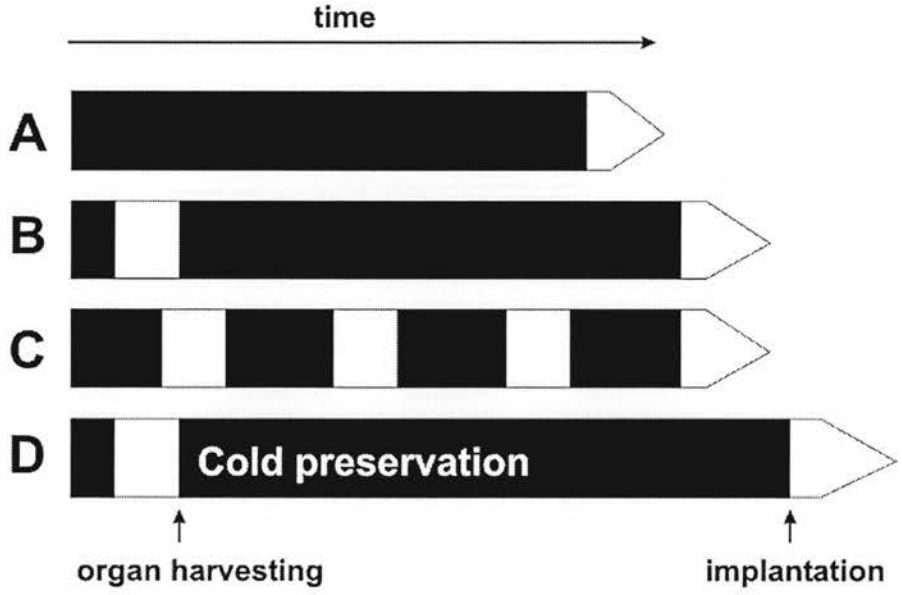

Figure 1.6 **Ischemic preconditioning strategies in surgery.**
Continuous ischemia (A), ischemic preconditioning (B), intermittent ischemia (C) and ischemic preconditioning as applied to solid organ transplantation (D). *Ischemia*, black; *reperfusion*, white. (Re-produced from Selzner *et al* (84))

In the kidney, results following IP have been inconsistent and appear to depend on the exact timing of the insult and the species. Initial studies focussed on whether renal artery occlusion prior to a second ischemic insult resulted in a poorer outcome. The investigators were surprised to find that it did not, and indeed 'a modicum of protection appeared to exist' (85,86) (Table 1.4). Islam *et al* found no benefit from 20 or 40 min renal artery obstruction in the rat, followed by a further ischemic insult 30 min later (87). A follow-up study from the same group identified a benefit when the second insult was performed only 5 min after the initial ischemic insult (88). It should be noted that any benefits or otherwise found in these studies cannot be attributed to processes requiring protein translation, given the short time periods examined. The most significant findings in the subject are from Park *et al*, who demonstrated protection from 30 min bilateral ischemia 8 and 15 days after a 30 min initial ischemic insult (89). In a follow-up study, the protection afforded by a 30 min ischemia insult persisted for 12 weeks, with improved post-ischemic renal function, less leukocyte infiltration and reduced post-ischemic disruption of the actin cytoskeleton noted (90). IP in larger animals has been less successful and has not been shown to be of benefit in dogs (91) and pigs (92), although the numbers in these studies were possibly too small to show a statistically significant effect.

Ischemic preconditioning has been performed in humans with success (Table 1.5). Clavien *et al* demonstrated the protective effects of IP in liver resection with inflow obstruction, finding serum levels of aspartate transferase and alanine transferase were reduced by more than twofold in the IP group versus controls, together with a reduction in the levels of apoptosis, although there was no significant change in the

Author	Year	Species	Preconditioning	Subsequent insult	Ref
Zager	1984	Rat	BO	IR	(85)
Zager	1985	Rat	BO	IR	(86)
Yoshioka	1990	Rat	BO	IR	(93)
Zager	1994	Rat	UO/BO	IR	(94)
Islam*	1997	Rat	UO	IR	(87)
Cochrane	1999	Rat	BO	IR	(95)
Riera	1999	Rat	Unknown	IR	(96)
Toosy	1999	Rat	UO	IR	(88)
Behrends	2000	Pig	UO	IR	(92)
Park	2001	Mouse	BO	IR	(89)
Torras	2002	Rat	BO	Isograft	(97)
Kosieradzki†	2003	Dog	UO	IR/Allograft	(91)
Park	2003	Mouse	BO	IR	(90)
Fuller	2005	Rat	UO	Isograft	(98)
Joo	2006	Mouse	BO	IR	(99)

Table 1.4 **Ischemic preconditioning in the kidney.**
BO, bilateral occlusion. * no benefit shown in this study, but the following report by Toosy *et al* from the same group showed a benefit. † no benefit shown. UO, unilateral occlusion; BO, bilateral occlusion; IR, ischemic reperfusion.

Author	Year	Organ	Setting	Study design	Ref
Jenkins	1997	Heart	CABG	RCT	(100)
Chen	1999	Lung	Lung resection	RCT	(101)
Clavien	2000	Liver	Liver resection	RCT	(102)
Nuzzo	2002	Liver	Liver resection	RCT	(103)
Yang	2002	Lung	Lung resection	RCT	(104)
Clavien	2003	Liver	Liver resection	Large RCT	(105)
Azoulay	2005	Liver	Liver transplantation	RCT	(106)
Koneru	2005	Liver	Liver transplantation	RCT	(107)
Jassem	2005	Liver	Liver transplantation	RCT	(108)
Cescon	2006	Liver	Liver transplantation	RCT	(109)
Amador	2007	Liver	Liver transplantation	RCT	(110)
Koneru	2007	Liver	Liver transplantation	RCT	(111)

Table 1.5 **Ischemic preconditioning in humans.**
RCT, randomised controlled trial; CABG, coronary artery bypass grafting

caspase 3 and 8 activity (105). The same group went on to show benefit in 100 consecutive liver resection patients randomised to IP or control (102). A prospective randomised clinical trial of the safety and efficacy of IP in deceased donor liver transplantation found that the procedure did contribute to a negative outcome, but did not demonstrate any benefit with the conservative protocol used (112).

An interesting continuation of IP is the remarkable observation that ischemia at a site remote to the organ in question has also been shown to be protective. For instance, subjecting the liver to a short ischemic episode has been shown to protect the kidney (113). Even effecting limb ischemia using a tourniquet has been shown to protect distant organs. This was shown in a pig model of myocardial infarction with four 5 min cycles of lower limb ischemia; the same study demonstrated a reduction in endothelial dysfunction in a human forearm IR model (114). Most excitingly for the field of transplantation, four cycles of 10 min IR of the hind limb of rats was shown to reduce the serum transaminase rise resulting from partial hepatic ischemia of the left lobes for 45 min followed by 240 min of reperfusion. This protection was associated with an induction of the small heat shock protein heme oxygenase-1 (HO-1), and was abolished with the HO-1 inhibitor zinc-protoporphyrin IX (ZnPP). In humans, an initial study found four 5 min cycles of lower limb IR to be protective in children undergoing cardiac surgery (115).

Pharmacological

Although ischemic preconditioning has shown to be efficacious in many situations, it may not always be practical to carry out and may carry unknown negative effects.

The quest for a pharmacological means of inducing similar protective effects promises much. Many different agents have been tried, although not as many in the kidney model as in that of the liver (Table 1.6) (84). As discussed in section 1.4.4 (page 36), reactive oxidant species are thought to have a fundamental role in the pathogenesis of IRI. A number of different antioxidants have been shown to be of benefit in models of IRI, yet none have entered clinical practice, with the exception of some constituents of preservation solutions (116-132). Anti-inflammatory agents have also been used at every level of the inflammatory response: adhesion molecule inhibition (49-51), inhibition of neutrophil infiltration (133), cytokine inhibition (134) and leukocyte activation (135). Apoptotic pathways have been targeted with caspase inhibitors (136). Of significant interest is the up-regulation of heat shock proteins, which will be discussed in the next section. This is associated with heating the organ (80-82) and with a number of pharmacological mediators of protection (137-141).

Ethics

Preconditioning presents ethical dilemmas which require to be fully considered and discussed within wider society. A paper exploring these ideas has recently been published (142) and is included in appendix V (page 288).

Mechanism	Drug	Species	Insult	Effector system	Ref
Anti-oxidant	Allopurinol	Rat	IRI	Anti-oxidant	(116,117)
	CoPP	Rat	Isograft	HO-1	(118-120)
	SnPP	Rat	IRI	HO-1	(121)
	CO	Rat	Isograft	CO	(122,123)
	CO-RM	Rabbit	Isolated perfusion	CO	(124)
	Bilirubin	Rat	IRI	Anti-oxidant	(125)
	Ghrelin	Mouse	IRI	IGF-1/GH	(143)
	N-acetylcysteine + SNP/phosphormidon	Rat Dog	IRI IRI	Anti-oxidant NO donor/ Endothlin1 inhibitor	(126) (127)
	Ascorbic acid	Dog	Autograft	Anti-oxidant	(128)
	α-tocophherol	Rat	IRI	Anti-oxidant	(129-131)
	Superoxide dismutase	Rat	IRI	Anti-oxidant	(132)
Anti-inflammatory	Pentoxifylline	Rabbit	IRI	TNFα inhibition	(134)
	Mycophenolate mofetil	Rat	IRI	Reducing inflammation	(135)
	Bimosiamose	Rat	Isograft	Selectin inhibition	(144)
	P-selectin glycoprotein ligand-1	Rat	IRI	Selectin inhibition	(50,51)
	alpha-1-acid glycoprotein	Mouse	IRI	Decreased neutrophil in	(133)
Hsp up-regulation	Geranylgeranylacetone	Rat	IRI	Hsp70	(137)
	FK506/Cyclosporine	Rat	IRI	Hsp70	(138,139)
	Erythropoeitin	Rat	IRI	Hsp70	(140)
	1,25-dihydroxyvitamin D3	Rat	IRI	Hsp70	(141)
Apoptosis	Caspase-1 inhibitor	Rat	IRI	Caspase-1	(136)
Unknown*	Insulin	Rat	IRI	-	(145)

Table 1.6 **Pharmacological agents reported to confer protection against IRI in kidneys.**
* see chapter 5. CO-RM, carbon monoxide-releasing molecule; HO-1, heme oxygenase-1; CO, carbon monoxide; SNP, sodium nitroprusside; SnPP, tin-protoporphyrin IX; CoPP, cobalt protoporphyrin; IRI, ischemia/reperfusion injury

1.6 The heat shock protein response

1.6.1 Overview

Heat shock, or *stress proteins* (Hsps) are a highly conserved group of intracellular chaperones that are expressed when a cell is exposed to one of an array of metabolic insults including heat (146), oxidative stress (147), hypoxia (148), ischemia (149), osmotic stress (150) and heavy metals (151). Increased Hsp expression following stress is associated with a tolerance to a subsequent injury that would otherwise be lethal (152). Hsp up-regulation has been associated with cytoprotection in many models of cellular stress and has great potential for future clinical intervention (153). One area of promise is solid organ transplantation (Table 1.7). Heat-induced expression of stress proteins has been associated with protection in rat models of kidney (80,81), liver (76,154) and lung (79) transplantation. Increased Hsp70 expression by gene transfer has improved outcome in rat lung isografts (155) and reduced IRI injury following rat heart transplantation (156). Much interest has been shown lately in the small heat shock protein, HO-1 (also known as Hsp32) (157), which has been associated with an improvement in outcome in heat-treated rats subjected to liver (76,158) and kidney transplantation (119). HO-1 gene transfer has been associated with improved outcome in kidney transplantation (159) and chemical up-regulation of HO-1 protects livers from IRI (160).

Author	Year	Species	Stimulus	Organ	Model	Protein	Ref
Perdrizent	1993	Swine	Heat	Kidney	Allograft	Hsp72	(78)
Hiratsuka	1998	Rat	Heat	Lung	Isograft	Hsp70	(79)
Hiratsuka	1999	Rat	Gene transfer	Lung	Isograft	Hsp72	(155)
Jayakumar	2000	Rat	Gene transfer	Heart	Isograft/ Ex-vivo	Hsp72	(156)
Matsumoto	2001	Rat	Heat	Liver	Isograft	Hsp72	(154)
Lin	2001	Rat	Heat	Liver	IR	Hsp72	(77)
Fudaba	2001	Rat	GGA*	Liver	Isograft	Hsp72/90	(161)
Redaelli	2001	Rat	Heat	Kidney	Isograft	Hsp72/HO-1	(80)
Redaelli	2002	Rat	Heat	Kidney	Isograft	Hsp72/HO-1	(81)
Redaelli	2002	Rat	Heat	Liver	Isograft	HO-1	(158)
Wagner	2003	Rat	Heat	Kidney	Isograft	HO-1	(82)
Yamagami	2003	Rat	Heat	Liver	IR	Hsp72/HO-1	(83)
Mokuno	2004	Rat	Heat	Liver	Isograft	Hsp72/90/HO-1	(76)
Patel	2004	Human	Ischemia	Liver	IR	HO-1	(162)
Suzuki	2005	Rat	GGA*	Kidney	IR	Hsp70	(137)
Jo	2006	Rat	Heat	Kidney	IR	Hsp70	(163)

Table 1.7 **Protection in transplantation-relevant models associated with Hsp up-regulation.**
* GGA, geranylgeranylacetone.

1.6.2 Heat shock proteins

The first description of a response associated with stress was in 1962 when Ritossa reported the occurrence of 'chromosomal puffs' in *drosophila busckii* subjected to mild heating, sodium salicylate or dinitrophenol (164). This heat shock response occurred very quickly and was associated with RNA synthesis (165,166). With the advancement of laboratory techniques, a group of proteins were identified that were up-regulated in association with the stress (167). These were termed *heat shock proteins* (168). Hsps of different molecular weights exist with Hsp27, Hsp40, Hsp60, Hsp70 and Hsp90 being the most widely studied. A group of related proteins, termed the glucose-regulated proteins (Grps), are homologues found in the endoplasmic reticulum of cells. These were discovered to be induced when cells were starved of glucose, but are also induced following heat and other stresses.

Initial work in the mid-70s was carried out exclusively in *drosophila.* When researchers began investigating the Hsp response in other organisms it was apparent that it was highly conserved across species (169). Another interesting feature, identified in 1975 by Gerner and Schneider, was that HeLa cells subjected to a non-lethal heat shock were protected against a subsequent heat stress (152).

1.6.3 Heat shock protein function

It is an important observation that Hsps are well conserved across virtually all organisms and suggests that that Hsp function is essential to cell survival. It has long been appreciated that protein folding is determined by protein structure, i.e. amino

acid sequence (170). However, in order for protein folding to occur accurately other proteins, termed chaperones, are required to assist proper conformational arrangement (171,172). Hsps have been identified in fulfilling this chaperone function (173). Hsps are present in physiological conditions, but a number are only expressed during stress. The function of Hsps in general has been found to be more wide-ranging than just assisting in protein folding (Table 1.8) and specific heat shock proteins have been identified in having particular roles. HO-1 has been shown to have antioxidant and anti-inflammatory functions (see Chapter 5) (174); Hsp25/27 inhibits actin polymerisation (175,176); Hsp60 is important in mitochondrial protein folding and assembly (177); the Hsp70 family may have a role in protein trafficking (178) and degradation (179); and Hsp90 takes part in multi-chaperone complexes (see chapters 6, 7 and 9). In models of cellular protection involving Hsps, it is often unclear which Hsp function is acting to reduce the injury, but it is highly likely to be a combination of a number of effects.

State	Function
Normal	Assisting protein folding Protein trafficking Complex formation Protein degradation
Stress	Repair damaged protein Prevent aggregation damaged protein Targeting lethal damaged protein for degradation Inhibition of apoptosis Cytoskeleton stabilisation Immunological functions

Table 1.8 **Functions of heat shock proteins in physiological and stress conditions.** Adapted from Kelly K. Heat Shock (Stress Response) Proteins and Renal Ischemia/Reperfusion Injury (180).

1.6.4 Heat shock protein regulation

The heat shock protein response is regulated by heat shock transcription factor-1 (HSF1). In order to achieve transcriptional activation, HSF1 must form a homotrimer, move to the nucleus and become hyperphosphorylated (181). The mechanisms that control this transactivation continue to be delineated but important components appear to include the repressive phosphorylation by a number of kinase cascades (explored further in chapter 4) and the formation of a repressive multi-chaperone complex (discussed in chapters 6).

Heat shock proteins, therefore, are strongly associated with cellular protection and up-regulation in the clinical setting promises significant benefits. Transplantation necessitates inflicting an injury on an organ, but the planned nature of this insult presents opportunity to act to reduce the magnitude of this injury. This thesis will examine potential mechanisms by which Hsps can be induced and any associated protective effect in the kidney.

Chapter 2 – Aims

The principal aim of this study:

To identify new strategies of protection utilising the heat shock/stress protein response that can be applied to ischemia/reperfusion injury in the kidney.

Within this overall aim:

1. To increase the understanding of the repressive phosphorylation of heat shock transcription factor-1 (HSF1) and how this might be reversed to stimulate the stress protein response.

2. To increase the understanding of the repressive multi-chaperone complex and how this might be manipulated to stimulate the stress protein response.

3. To increase the understanding of heme oxygenase-1 (HO-1) regulation and how it may contribute to protection from ischemia/reperfusion injury.

Chapter 3 – Materials and Methods

3.1.1 Materials

All reagents were obtained from Sigma-Aldrich Co. Ltd. (Poole, Dorset, UK) unless otherwise stated. GSK3, Nrf2 and lamin A/C antibodies were obtained from Santa Cruz (Wembley, UK); Hsp90, Hsp70, Hsp27, Hsp25 and HO-1 from Stressgen (Victoria, BC, Canada); β-actin antibody from BD Biosciences (San Diego, CA, USA); phospho-GSK3β (ser9) (pGSK3β), phospho-ERK1/ERK2 MAPK (Thr202/Tyr204) (E10) monoclonal (p-ERK1/2), ERK1/ERK2 MAPK (total-ERK1/2), phospho-p38 MAPK (Thr180/Tyr182) (28B10) monoclonal (p-p38), p38 MAP kinase (5F11) monoclonal (total-p38) antibodies from New England Biolabs (Hitchin, Hertfordshire, UK).

3.1.2 Cell culture

Renal adenocarcinoma cells (ACHN) (European Collection of Cell Cultures, Porton Down, UK) were maintained in Dulbecco's modified Eagle's medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and non-essential amino acids (5%). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air. Cells were passaged regularly and all experiments were performed with sub-confluent cultures. Stocks of cells were stored in liquid nitrogen following suspension in 10% DMSO in FCS. Following 10-12 passages, working cell cultures were discarded and new ones established from stock cells.

3.1.3 Plasmid DNA amplification

The various plasmids used were grown up in the following manner.

Mini-broth preparation

5 µg of plasmid DNA was added to 50 µl transformation competent E. coli. After 30 min on ice, tubes were heat shocked at 42 °C for 45 seconds and returned immediately to ice for 2 min. 450 µl of SOC medium was added and reactions incubated at 37 °C with vigorous shaking for 1 h. Reactions were diluted 1/10 with SOC medium and 100 µl plated onto LB agar plates which were incubated overnight at 37 °C. White colonies were 'picked' from the plates, transferred to 3 ml LB broth containing 50 µg/ml ampicillin and incubated overnight at 37 °C with shaking.

Mini-prep

1.5 ml mini-broth was centrifuged at 13 000 g for 2 min. The pellet was resuspended in 100 µl TE buffer (pH 8.0). To this was added 200 µl 0.2N NaOH/1% sodium dodecyl sulphate (SDS), followed immediately by 150 µl 3M potassium acetate/11.5% acetic acid, followed immediately by 400 µl phenol/chloroform (1:1). This was centrifuged at 13 000 g for 5 min at 4 °C and the upper phase transferred to a new tube. 200 µl isopropanol was added and following further centrifugation at 13 000 g for 10 min at 4 °C, the supernatant was removed and discarded. The pellet was washed in 100 µl cold 70% ethanol, recovered by centrifugation, air-dried and resuspended in TE buffer with RNAse (100 µg/ml), which was incubated 37 °C for 5 min. An digestion with appropriate restriction enzymes was then performed to confirm presence of plasmid DNA.

Maxi-broth preparation

The remaining 1.5 ml of mini-broth was added to 250 ml LB medium and incubated overnight at 37 °C with vigorous shaking. Bacterial cells were harvested by centrifugation at 6000 g for 15 min.

Maxi-prep

Plasmid DNA was extracted bacteria using the Endofree plasmid maxi kit (Qiagen, Crawley, UK) as per the manufacturer's instructions. The yield was determined by spectrophotometry.

3.1.4 Western blot

Whole cells extracts were produced by scraping cells in ice-cold RIPA buffer (typically 150 µl for a 6-well plate) with protease inhibitors (Sigmafast protease inhibitor tablets). Extracts were centrifuged at 10 000g for 10 min and the supernatent kept. Total protein concentration was determined by the Lowrie method (BioRad, Hemel Hempstead, UK). Cytoplasmic/nuclear lysates were prepared using Gobert's method (182). Proteins were separated by SDS-PAGE: typically, a 10% Tris/HCl gels was made with 3.96 ml dH_2O, 2.5 ml 1.5M Tris-HCl (pH 8.8), 100 µl 10% SDS, 3.33 ml Acrylamide/Bis 30% stock (BioRad), 100 µl 10% ammonium persulphate and 10 µl TEMED. A stacking gel was placed on top of this (see Appendix 1, page Appendix I – Buffers278). 20 µg of protein was used per minigel well. Gels were run for 40 min at 200V. Transfer was to nitrocellulose membranes (BioRad) was performed with electroblotting (1 h at 80 mA). The membranes were

soaked in blocking buffer (TBS, 0.05% Tween 20, 5% non-fat milk) followed by blocking buffer containing primary antibody. After washing, the membranes were exposed to a horseradish peroxidase-conjugated secondary anti-mouse (Upstate, Milton Keynes, UK) or anti-rabbit (Santa Cruz) antibody and were used at a concentration of 1:5000. Enhanced chemiluminescence reagent (Amersham, Chalfont St Giles, UK) was used followed by development using autoradiography. Membranes were stripped by washing in Restore Western Blot Stripping Buffer (Pierce, Rockford, IL, US) for 15 min at room temperature and equality of loading confirmed by probing for β-actin for whole-cell extracts, and lamin A/C for nuclear extracts.

3.1.5 DNA mobility shift assay

[$\gamma^{32}P$]ATP method

Forward and reverse oligonucleotides specific for the sequence being analysed (as detailed in the relevant chapters) were obtained (TAGN, Newcastle, UK). Heat shock elements (HSE) forward 5' ATCTCGGCTGGAATATTCCCGACCTGGCAGCCGA and reverse 5' GATCTCGGCTGCCAGGTCGGGAATATTCCAGCCGA. These were annealed in 0.5M NaCl by heating at 95°C for 3 min, followed by 2 h at room temperature. To radiolabel, 50 ng double-stranded probe was added to 1 µl T4 polynucleotide kinase (T4 PNK) (Promega), 1 µl 10X PNK buffer, 3 µl water and 3 µl [$\gamma^{32}P$]ATP; the reaction mixture was incubated at 37 °C for 1 h. Excess unlabelled [$\gamma^{32}P$]ATP was removed using the QIAquick nucleotide removal kit (Qiagen) as per the manufacturers instructions. The binding reaction was performed in 20 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.05%

NP40, 10% glycerol (all final concentrations). 10 μg protein, around 0.25 ng (1 μl) labelled probe and 100 μg poly [d(I-C)] were added and incubated for 20 min at room temperature. Control reactions using 20X molar excess of unlabelled consensus sequence, unlabelled mutant probe or with the inclusion of a shift antibody specific to the protein being analysed were also performed as appropriate. Reactions were run on 5% TBE polyacrylamide gels (Biorad) at 200V, the length of time being dictated by the size of product sought. Gels were dried and exposed to x-ray film.

Digoxigenin method

Digoxigenin-labelled forward and reverse oligonucleotides were obtained (TAGN) and annealed as above. The binding reaction was performed in 20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM ammonium sulphate, 1 mM DTT, Tween 20 0.2% (w/v), 30 mM KCl (all final concentrations). 20 μg protein, around 0.04 ng digoxigenin-labelled probe, 50 μg poly [d(I-C)] and 5 μg poly L-lysine were added and incubated for 20 min at room temperature. Loading buffer with bromophenol blue (0.05X TBE buffer, 12% glycerol final concentrations) was added and samples run as above. Gels were transferred to positively charged PVDF membranes, oligonucleotides cross-linked by exposure to around 120 mJ UV light, blocked, probed with anti-digoxygenin antibody (Fab fragments conjugated with alkaline phosphatase), treated with chemiluminescent substrate (CSPD) and exposed to x-ray film.

3.1.6 RNA isolation and fluorescence detection real-time polymerase chain reaction

RNA extraction and purification was performed using a TRIzol (Invitrogen, Paisley, UK). RNA samples were treated with DNAse and then run as a template for a standard PCR reaction using β-actin primers to exclude the presence of contaminating DNA. RNA was then reverse transcribed to cDNA using avian myeloblastosis virus reverse transcriptase (Promega, Southampton, UK) and random decamers (Ambion, Huntingdon, UK). Fluorescence-detected real time PCR was then performed using specific primers and TAMRA labelled FAM-probe. The following primer and probes were designed: Human Hsp70: Forward, 5' CAGAGTGCTGCCAAAAACTC; reverse 5' CCTAAGGCTTTCCTCTTGCAAA; Probe: 6-FAM-CTGGAGGCCCATGTCTTCCATGTGA-TAMRA. Mouse Hsp70: Forward, 5'-GGTGGTGCAGTCCGACATG; reverse, 5'-TTGGGCTTGTCGCCGT; Probe: 6-FAM-CACTGGCCCTTCCAGGTGGTGAA-TAMRA. A standard reaction contained Taqman universal master mix 12.5µl (Applied Biosystems, Warrington, UK), primer probe/mix 7µl (primers 25µM, Probe 5µM), 18S primer/probe mix 1.25µl, and water 1.75µl) and cDNA template 2.5µl. Samples were run on an ABI Prism 7700 Sequence Detection System and analysed using Sequence Detector 7.1 (Applied Biosystems).

3.1.7 Transfections

Akt expression constructs (Upstate) are based on the pUSEamp vector. The activated form (Akt-myr) contains an N-terminal myristoylation sequence targeting Akt to the

plasma membrane. The dominant-negative form (Akt-K179M) contains a methionine for lysine substitution at residue 179 abolishing Akt kinase activity. Wild-type form (Akt-WT) contains the unaltered Akt sequence and an empty vector (pUSEamp) was used as a control. The HO-1 luciferase reporter construct (pHOGL3/11.6) was a kind gift of Dr A Agarwal (University of Alabama, USA). The heat shock protein 70-β-galactosidase (Hsp70-β-gal) reporter construct was a kind gift from WJ Welch (UCSF, USA). The HIF1 reporter construct (pHRE-luc) was a kind gift of Professor Esumi (National Cancer Center Research Institute East, Japan). Transfection efficiency was controlled by co-transfecting with a β-galactosidase (pSV-β-gal) or a luciferase (pGL3-luc) expressing control vector (Promega). Transient transfections were performed using Fugene (Roche, Lewes, East Sussex, UK) at a 6:1 ratio reagent to DNA. Experiments on transfected cells were performed 24 – 48 h later.

3.1.8 Luciferase/β-galactosidase assay

Cells were co-transfected with the appropriate reporter vector and control vector and treated as per experimental protocol the following day. Cells were lysed with reporter lysis buffer (Promega) after which 20 μg lysate was combined with 50 μg luciferase assay reagent and the resulting light emission measured on a luminometer (Fluoroskan Ascent Fl, Thermo Electron, Basingstoke, UK). The remaining lysate (80 μg) was combined with β-galactosidase assay 2X buffer and following incubation at 37°C for 4 h was read at 420 nm on a spectrophotometer (Ultraspec 2000, Pharmacia Biotech).

3.1.9 Cell viability assays

MTT assay

Cells were treated as per experimental protocol in 96-well plates and recovered in 100 µl medium per well. 20 µl of 3-(4,5-dimethylthiazole-2-yl)-2,5-dipenyltetrazolium bromide (MTT) (5 mg/ml) was added for 2 h. Wells were then carefully emptied and resulting tetrazolium crystals were dissolved in 10 % SDS (pH3.0). Samples were analysed on a plate reader (test: 570nm; reference: 630nm), decreasing optical density reflecting a decreasing number of viable cells. Hydrogen peroxide was typically used as a stressor at a concentration that resulted in 50% cellular death (Figure 3.1).

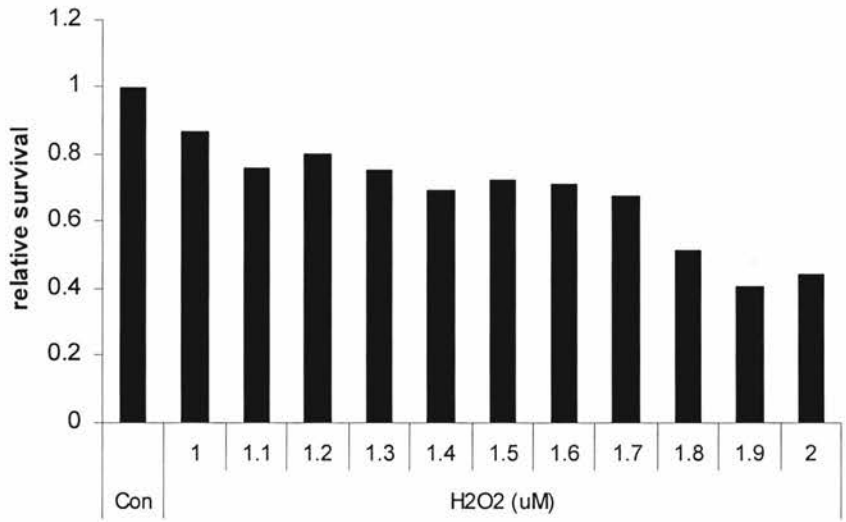

Figure 3.1 **Relative survival of ACHN cells following H_2O_2 treatment.**

Cells were exposed to increasing concentrations of H_2O_2 for 16 h. MTT was added for 2 h and the resulting tetrazolium crystals dissolved in SDS. Samples were analysed on a plate reader.

3.1.10 Immunohistochemistry

Tissue was fixed in methacarn (70% methanol, 20% chloroform, 10% glacial acetic acid) for 24 h before being mounted in paraffin. Sections were cut and mounted on SuperFrost slides (Fisher Scientific, Pittsburgh, PA, US). Sections were de-waxed in xylene for 10 min and rehydrated through decreasing concentrations of alcohol into distilled water. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min followed by washing in distilled water. Antigen retrieval was performed by microwaving sections in 10 mM citrate buffer (pH 6.0) for 15 min. Slides were cooled and transferred to TBS. Sections were blocked in 10% normal serum of the species the secondary antibody was raised in for 20 min. Primary antibody in 10% normal serum was then applied for 1 h at room temperature or overnight at 4 °C. After washing in TBS the sections were exposed to peroxidase-conjugated secondary antibody for 30 min. After washing in TBS for 5 min, sections were exposed to peroxidase substrate solution (3,3-diaminobenzidine tetrahydrochloride, DAB) (DAKO, Glostrup, Denmark). Following counterstaining with haematoxylin, sections were dehydrated through increasing concentrations of alcohol to xylene, before a cover-slip was mounted. Sections were then examined with light-microscopy. Appropriate primary antibody-only and secondary antibody-only controls were always performed to ensure no non-specific staining was taking place.

3.1.11 Immunofluorescence

Cells were cultured in chambered slides and treated as per experimental protocol. They were subsequently fixed with methanol and blocked in 10% normal serum of

the species the secondary antibody was raised in for 20 min. Primary antibody in 10% normal serum was then applied for 1 h at room temperature or overnight at 4 °C. After washing in TBS the sections were exposed to FITC-conjugated secondary antibody for 30 min, and typically counterstained with Hoechst prior to mounting. Fields were visualised with a Leica DM IRB microscope fitted with a Hamamatsu camera. Images were stored using the Leica Improvision software. Primary antibody only and secondary antibody only groups were always included as controls.

3.1.12 RNA interference

Cells were seeded in 6-well plates and transfected the following day with the appropriate siRNA (Santa Cruz) or control siRNA as per manufacturer's protocol. 48 h later transfected cells were treated and lysed. Adequacy of effect was ascertained with Western blot analysis with anti-Nrf2 antibody.

3.1.13 Statistical analyses

Data are presented as means and standard error of the mean (S.E.M.). Statistical comparisons for parametric continuous data were made using Student's t-test, one-way analysis of variance (ANOVA) and two-way ANOVA without interaction (using the Tukey post hoc correction for multiple comparisons). Non-parametric data was compared using the Mann-Whitney U test. All comparisons were performed on SPSS v14.0 (SPSS, Chicago IL, USA).

3.2 Firefly luciferase terminally degraded by mild heat exposure: an underappreciated phenomenon with implications for transfection efficiency control

3.2.1 Introduction

The use of reporter vectors is a rapid and highly sensitive method of investigating factors regulating gene expression (183). The promoter region of the gene of interest is cloned upstream of a sequence encoding the reporter enzyme; the resulting protein expression is proportional to gene activity and can be easily and reliably determined. In order to control for potential variation in transfection efficiency, it is common practice to co-transfect the reporter vector with a control vector, constitutively expressing a second distinct reporter enzyme under a viral promoter. For this second vector to function as an accurate control, enzyme expression must be independent of the experimental treatment and depend only on the number of cells that took up the plasmid DNA (184).

Heat shock proteins are highly conserved intracellular chaperones and are expressed when a cell is exposed to a stress (173). While investigating the induction of heat shock protein 70 (Hsp70) following heat exposure in renal adenocarcinoma cells (ACHN), an Hsp70B β-galactosidase reporter vector (Stressgen Biotechnologies Corp) was used. It was necessary to control for transfection efficiency so cells were co-transfected with the pGL3 luciferase control vector (Promega), which contains the *luc+* gene and expresses firefly luciferase under the control of the simian virus 40 (SV40) promoter. It was observed that luciferase activity in groups transfected with

the pGL3 vector and subjected to a mild heat exposure was much lower than expected.

In order to investigate whether this observation was an effect of heat on transcription we co-transfected the pGL3 vector with the pSV β-galactosidase control vector (Promega), also under the control of the SV40 promoter. The following day, groups were exposed to a mild heat induced stress (43 °C) for increasing lengths of time up to 45 min, after which all remaining groups were returned to 37 °C and recovered for further increasing time intervals. Additional examination involved exposing recombinant firefly luciferase (Sigma, Poole, UK) to a series of mild heat exposures for increasing lengths of time. All experiments were independently repeated 3 times.

3.2.2 Results and discussion

Cells transfected with both the pGL3 luciferase vector and the pSV β-galactosidase control and subjected to mild heat exposure demonstrated a marked reduction in luciferase activity compared with β-galactosidase activity, which remained unchanged (Figure 3.2). This occurred despite both enzymes being under the control of the same SV40 promoter, suggesting the effect was independent of transcription. The reduction was related to the length of heat exposure and a statistically significant difference between the pGL3 luciferase group and the pSV β -galactosidase control was observed following 30 min of heat exposure (Mann-Whitney U $p<0.05$). Luciferase activity increased during recovery and the difference between the groups

ceased to be statistically significant at 2 h, and remained so until the end of the experiment at 24 h (Figure 3.2B).

On exposing recombinant firefly luciferase to varying temperatures (Figure 3.3) a time and temperature dependent response was observed with luciferase activity decreasing markedly over time and with increasing temperature.

Firefly luciferase is the most commonly used bioluminescent reporter (185). Despite the sensitivity and convenience of the enzyme assay inherent problems exist, particularly relating to thermostability, both *in vivo* and *in vitro* (186). Improved heat tolerance of luciferase has been demonstrated using mutagenesis to achieve a single amino acid substitution in both the Japanese Firefly (*Luciola cruciata and Luciola lateralis*) (187) and the North American Firefly (*Photinus pyralis*) (188). Modifications of the luciferase gene have improved performance in other areas, including elimination of peroxisomal translocation (185); however, thermostability is not a feature of current commercial versions. Nguyen and colleagues have previously demonstrated the sensitivity of luciferase to heat. Inconsistent with our findings, his data demonstrates a significant decrease in β -galactosidase activity in mouse-derived cells exposed to heat, although no loss of activity was seen in *in vitro* studies. However, our model used a human cell-line, protein translation was not abolished with cycloheximide and cells were heated to 43 °C, not 45 °C. These factors may explain the difference in findings.

In conclusion, when a luciferase *reporter* vector is used to determine gene expression in cells exposed to mild heat, the activity of the gene in question may be underestimated. When, however, a luciferase *control* vector is used in cells exposed to heat, transfection efficiency may be under estimated, and so gene expression overestimated between groups.

These data demonstrate the propensity of firefly luciferase to become inactive following mild heat exposure. The pGL3 control vector should be used with caution in protocols that involve exposure to a mild heat insult. These findings have been published and the paper is included in Appendix V (page 288).

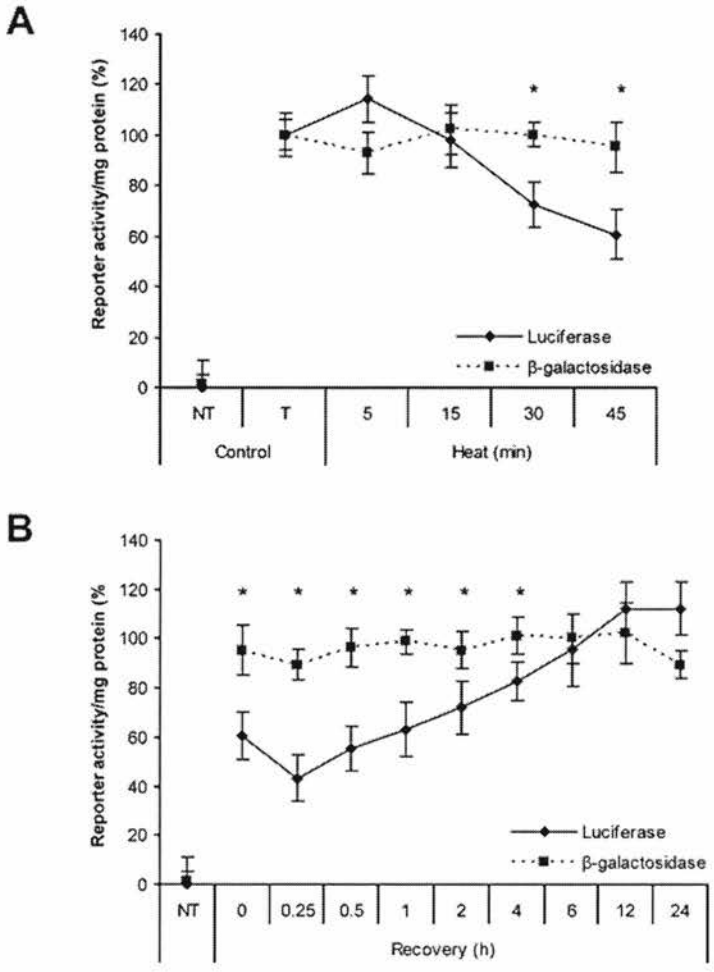

Figure 3.2 **Luciferase and β-galactosidase activity following mild heat exposure.** (A) The pGL3 luciferase and pSV β-galactosidase control vectors were co-transfected into a renal adenocarcinoma cell-line (ACHN) using the Fugene transfection reagent (Roche), as per the manufacturers protocol. The following day, groups were heated to 43 °C for varying lengths of time. Whole cell extracts were prepared with reporter lysis buffer (RLB). A protein assay was performed (Lowrie method; BioRad) and luciferase activity determined using the luciferase assay system (Promega). β-galactosidase activity was determined using the colorimetric β-galactosidase assay system (Promega). NT, non-transfected control; T, transfected control; * Mann-Whitney U $p<0.05$. (B) Cells were transfected as above and heated to 43 °C for 45 min 24 h later. Cells were lysed in RLB and luciferase and β-galactosidase activity were determined. * Mann-Whitney U $p<0.05$.

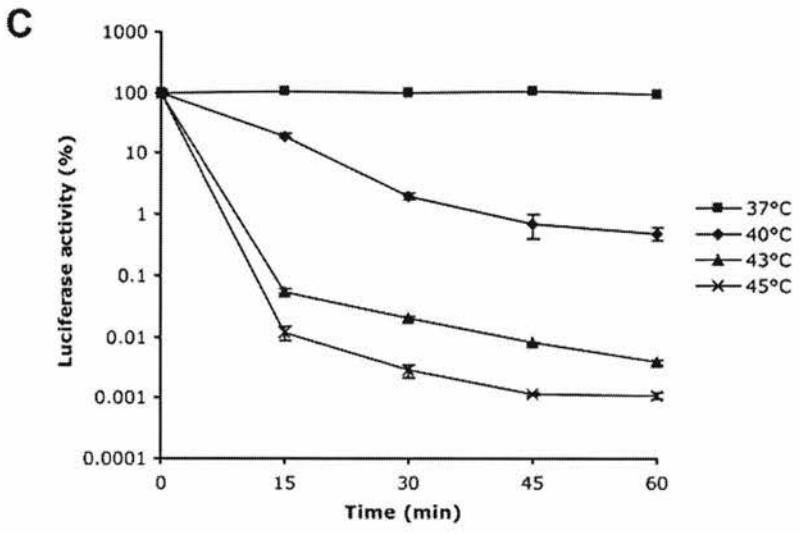

Figure 3.3 **Activity of recombinant firefly luciferase (Sigma) following mild heat exposure.**
1 μg of firefly luciferase was dissolved in distilled water (1 μg/μl) and made up to 20 μl with PBS. Samples were placed in a thermocycler (Techne PHC3) and heated for the times specified. Luciferase activity was determined using the luciferase assay system (Promega).

Chapter 4 – Heat shock transcription factor-1 regulation through the PI3K/Akt axis

4.1 Introduction

4.1.1 Background

As discussed in section 1.6, the induction of Hsps in both *in vivo* and *in vitro* models of cell stress has been shown to confer cytoprotective benefit. In particular, in animal models of transplantation Hsps have been shown to improve survival, both in the transplanted organ and the animal itself (Table 1.7). A clear understanding of the molecular mechanisms underlying this response is fundamental to the success of potential clinical interventions. This chapter focuses on the regulation of Hsps by heat shock transcription factor-1 (HSF1) and examines a potential mechanism of activation. In this introduction evidence will be presented that forms the basis of a hypothetical mechanism by which HSF1 is activated. It will be contended that the inducible stress protein response is mediated exclusively by HSF1; that repression of HSF1 occurs following phosphorylation of certain serine residues; that glycogen synthase kinase 3β (GSK3β) mediates the most important component of this repression; that GSK3β can be inactivated through activation of the phosphatidylinositol 3-kinase (PI3K)/Akt cascade; and that PI3K/Akt is activated by insulin-like growth factor-1 (IGF-1) and insulin. This description can be followed with the aid of Figures 4.1 and 4.3.

4.1.2 Heat shock transcription factor-1

Heat shock protein expression is regulated by members of the heat shock transcription factor (HSF) family[§]. Following the isolation of a single *hsf* gene in *Saccharomyces cerevisiae* (189,190) four members of the HSF family have been identified in vertebrates (191-194). Of these, HSF1 and HSF-3 (exclusively avian) are activated in response to classical stress stimuli; HSF-2 is not and appears to have a role in developmental regulation (195-198); HSF-4 is most recently described and awaits full characterisation (194).

In mammalian cells, the stress protein response is mediated exclusively by HSF1

The importance of HSF1 in the induction of Hsps in response to stress became clear from studies performed in the mid-90s. As Voellmy observes in a recent review (199), the inducible stress protein response (as distinct from constitutive Hsp expression) appears to be mediated by HSF1 alone in vertebrate cells[**]. In support of this view is the observation that although cultured embryonic cells derived from $HSF1^{-/-}$ mice do express constitutive Hsps, the Hsp response *to stress* is completely abolished (200). Likewise, Zhang *et al* reported elimination of stress-inducible Hsp70 and Hsp25 in mouse fibroblasts derived from $HSF1^{-/-}$ mice (201). In mice at least then, the expression of stress-induced Hsps can *only* occur following HSF1 activation. There have been no studies providing direct evidence of HSF1 exclusivity in mediating the stress protein response in human cells; however, convincing data

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[§] Hsp32/HO-1 is termed a heat shock protein but does not take part of the classical heat shock response. It is not regulated by the HSFs in humans, although does come under their control in other mammalian systems, including murine. It is regulated by a number of transcription factors including AP-1, AP-2, NFκB and Nrf2. This is dealt with in more detail in chapter 5.

[**] excluding avian cells where HSF1 and HSF3 both contribute to stress resistance

exists suggesting HSF-2 does not respond to heat shock in humans (or any other eukaryote) (202), that HSF-3 is exclusively avian (193) and that human HSF-4 appears transcriptionally inactive (194). It is reasonable to assume, therefore, that in human models of stress, Hsp gene induction is HSF1-dependent.

Analysing HSF1 structure as a means of elucidating function

The activation of HSF1 in response to stress is multi-faceted but there is compelling evidence that it occurs in a predominately post-translational setting (203). A number of conditions require to be fulfilled for HSF1 to become transcriptionally active (Figure 4.1)

1. Homotrimerisation[††]
2. Nuclear localisation
3. DNA-binding
4. Loss of transcriptional repression

These four events can be seen to occur prior to specific *Hsp* gene activation following exposure of a cell to the appropriate stimulus. Through systematic mutagenesis experiments, the functional contribution of different HSF1 protein domains has been determined (Figure 4.2). Two hydrophobic repeat sequences have been identified that are likely to contribute to the repression of trimerisation (HR-B/C). A further hydrophobic repeat sequence is necessary for trimerisation to occur (HR-A).

[††] Homotrimerisaton: the formation of a reaction product containing three identical molecules (in this case, three HSF1 molecules)

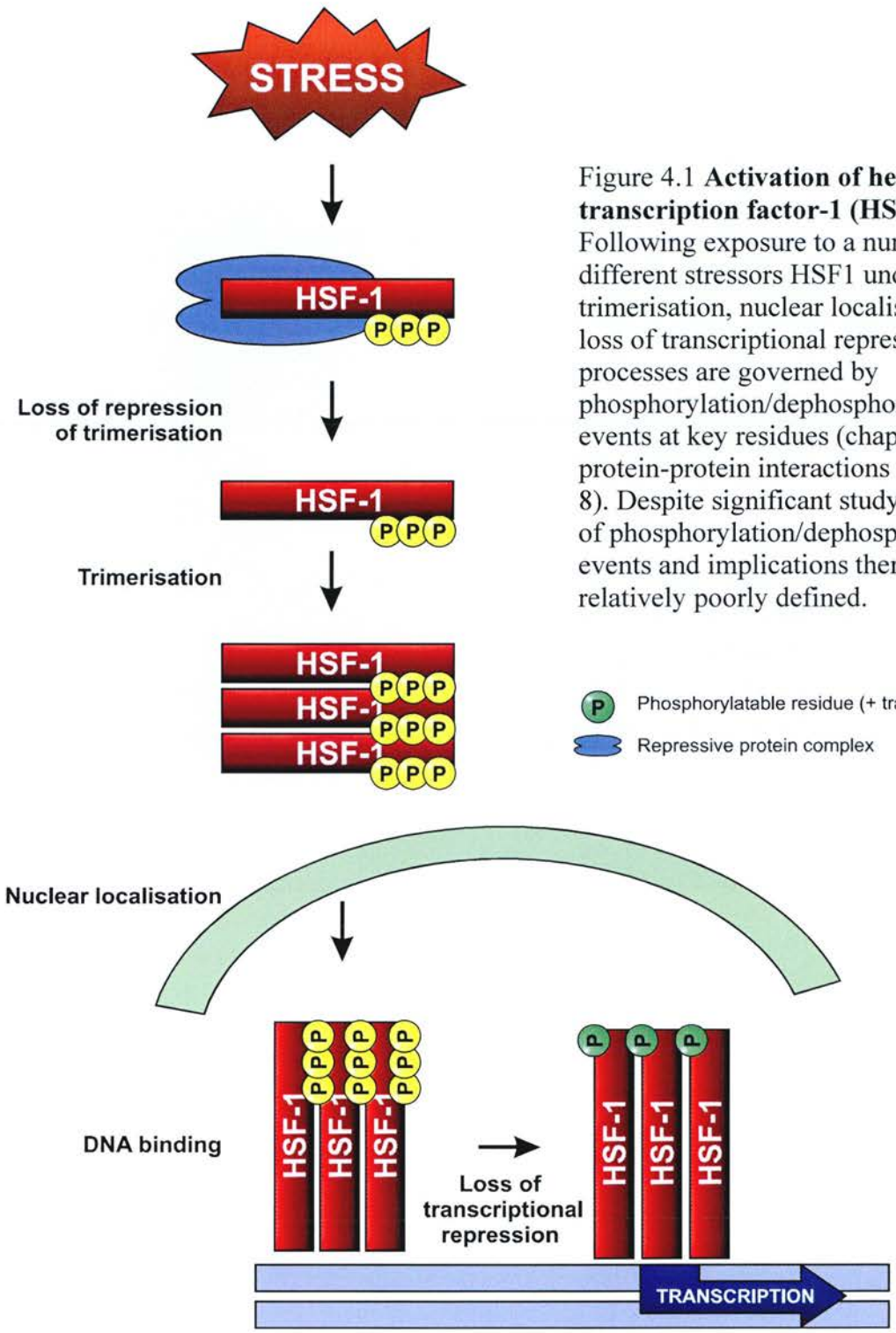


Figure 4.1 **Activation of heat shock transcription factor-1 (HSF1).** Following exposure to a number of different stressors HSF1 undergoes trimerisation, nuclear localisation and loss of transcriptional repression. These processes are governed by phosphorylation/dephosphorylation events at key residues (chapter 4) and protein-protein interactions (chapters 6 – 8). Despite significant study, the kinetics of phosphorylation/dephosphorylation events and implications thereof remain relatively poorly defined.

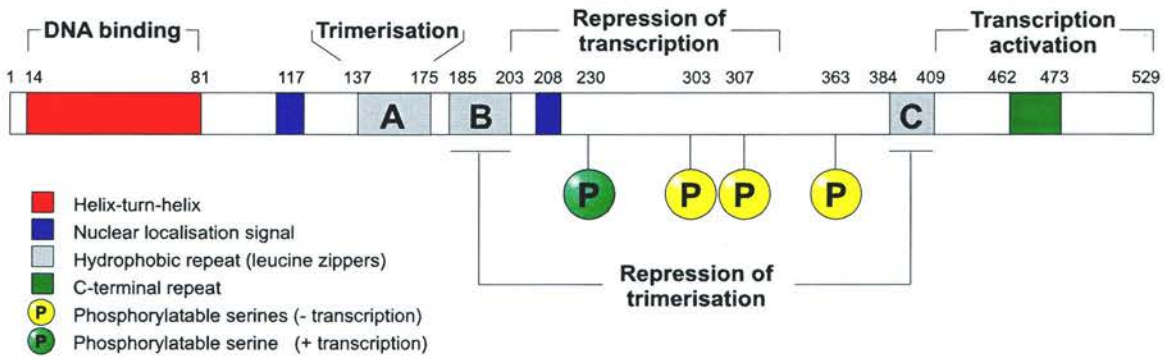


Figure 4.2 **Structure of human HSF1 with functional corollaries.** (after Voellmy 2004 (199)).

The nuclear localisation signal exists in the region of amino acids (aa) 117 and 208. An amino-terminal DNA-binding helix- loop-helix interacts with so called heat shock elements (HSE) upstream of the specific *Hsp* gene. The region from aa 203 to around 330 functions as a repressor of transcription, while aa 409 to 529 facilitates transcription activation.

Although the four requirements for transcriptional activation are distinct and correspond to specific regions of the HSF1 structure, it is unlikely that homotrimerisation can be uncoupled from DNA-binding, and by implication nuclear localisation (204). Consequently, any event that effects trimerisation is likely to result in HSF1 DNA-binding. Nonetheless, DNA-binding does not imply transcriptional activation, the latter requiring further steps in order to occur. This is most apparent in cells treated with salicylate, where HSF1 trimerisation and DNA-binding occurs, but *Hsp* gene activation does not (205).

Attempts to rationalise the events required for homotrimerisation/DNA-binding and transcriptional activation have fallen short. As yet, a cohesive description of HSF1 activation does not exist and while great progress has been made much remains unclear. During the four step activation process (in which monomeric HSF1 becomes trimerised and transcriptionally active), three global alterations in the HSF1 state have been observed:

1. Loss of a repressive multi-chaperone complex.
2. Dephosphorylation of 'repressive' residues.
3. Hyperphosphorylation of 'facilitative' residues.

Repression of trimerisation through interactions between HSF1 and one or more other proteins has become increasingly evident over recent years: the multi-chaperone complex theory. Additionally, a number of phosphorylation/dephosphorylation events alter both HSF1 DNA-binding ability and transcriptional activity. Crucially, the relationship between these alterations in HSF1 state and each step in the four step activation process remain obscure.

The importance of the multi-chaperone complex in HSF1 regulation is now recognised and will be discussed and investigated in detail in chapters 6 – 9. This chapter will focus on the role of phosphorylation/dephosphorylation events in mediating HSF1 regulation. Specifically, I will examine one putative mechanism by which transcriptional repression of HSF1 is maintained.

Phosphorylation at certain serine residues represses HSF1 activation

Phosphorylation of HSF1 alters its transcriptional activity (203): phosphorylation of certain residues represses transcription while phosphorylation at other sites increases transcriptional competence. One of the most important factors in altering HSF1 activity by phosphorylation is GSK3β.

Numerous factors have been reported to alter the activation state of HSF1, but contradictions exist (Table 4.1). Some studies describe transactivation of HSF1, while others report repression following up-regulation of the same factor e.g. PKC,

Kinase	Function	Reference
GSK-3	(-) transactivation	(206-211)
ERK	(-) transactivation	(206,209,210,212-215)
JNK	(-) transactivation	(216)
p38 MAPK	(-) transactivation	(214)
PKC	(-) transactivation	(210)
CaMKII	(+) transactivation	(217)
JNK	(+) transactivation	(218,219)
PKA	(+) transactivation	(220-222)
PKC	(+) transactivation	(220,221,223-227)
Rac1	(+) transactivation	(228-230)

Table 4.1 **Kinases reported to alter HSF1 activity.**
A myriad of kinases have been implicated in the regulation of HSF1. This table presents those reported to alter the transactivation state of HSF1 to date. Methodological problems exist in many of the reports and the picture remains confusing. Note that PKC and JNK have been described in some reports as being required for transactivation, but in others as being negative regulators of HSF1. GSK-3, glycogen synthase kinase-3; ERK, extra-cellular regulated kinase; JNK, c-JUN N-terminal kinase; p38 MAPK, p38 mitogen activated protein kinase; PKC, protein kinase C; CaMKII, calcium/calmodulin-dependent kinase II; PKA, protein kinase A.

JNK. While the possibility exists that the same kinase exerts different effects at different stages of HSF1 activation, it is more likely that these observations are mutually incompatible and methodological explanations will be found. Many reports describe alterations in HSF1 activity following up or down regulation of a given kinase, but most of this data is associative, with few demonstrating any causal link. Even fewer have examined the phosphorylation/dephosphorylation of specific residues in HSF1, arguably a more robust line of enquiry (Table 4.2).

One such study by Chu *et al* (209) identified the importance of two kinases in repressing HSF1 transcriptional activity. The extracellular signal-regulated kinases (ERK1/2) and GSK3β were shown to phosphorylate Ser-307 and Ser-303 respectively, both *in vitro* and *in vivo*, resulting in repression of transcriptional activity. Through a number of elegant mutagenesis experiments the authors concluded that in phosphorylating Ser-307, ERK1 primed subsequent phosphorylation at Ser-303 by GSK3β, a so called canonical mechanism. When Ser-303 was substituted for glycine (thus eliminating repressive phosphorylation at this site), the ability of this mutated HSF1 to activate a Hsp70 promoter reporter construct increased significantly. The same mutation at Ser-307 resulted in a less prominent increase in activity. Mutations at both Ser-303 and Ser-307 caused an increase in activity no greater than that seen with the Ser-303 mutation alone. So while over-expression of both kinases resulted in repression of HSF1, it seemed likely that ERK1 repression was GSK3β mediated. The importance of phosphorylation at Ser-303 and Ser-307 in the negative regulation of HSF1 was

Phosphorylation site	Kinase	Function	Reference
Ser-230	CaMKII	(+) transactivation	(217)
Ser-303	GSK3	(-) transactivation	(209-211)
Ser-307	ERK	(-) transactivation	(209,211,215)
Ser-363	PKC/JNK	(-) transactivation	(210,216)

Table 4.2 **Serine residues in HSF1 identified to be phosphorylated by specific kinases.**
There have been few reports examining phosphorylation/dephosphorylation at specific residues in HSF1. Four serine residues have been identified as targets for phosphorylation. CaMKII, calcium/calmodulin-dependent kinase II; GSK-3, glycogen synthase kinase-3; ERK, extra-cellular regulated kinase; PKC, protein kinase C; JNK, c-Jun N-terminal kinase.

substantiated by Kline and Morimoto (211) (who did not address the mechanisms by which this occurred) and by Knauf *et al* (215).

Moving to earlier events in HSF1 activation, further studies examined the ability of GSK3β to alter the DNA-binding ability of HSF1. In heat shock conditions, GSK3β inhibition by LiCl (a chemical inhibitor of GSK3β) or GSK-binding protein were found to increase HSF1 DNA-binding, as well as transcriptional activity in *Xenopus* oocytes (208). Conversely, when the cells were microinjected with GSK3β mRNA or active GSK3β, a significant reduction in DNA-binding (and transcriptional activity) was observed , suggesting a role for GSK3β beyond that of mere transcriptional repression. Additionally, He *et al* confirmed that ERK primes GSK3β phosphorylation and that over-expression of GSK3β facilitates the inactivation of activated HSF1 (206).

A contradictory report from Xia *et al* described the mapping of tryptic phosphopeptides of HSF1 from HeLa cells. This suggested that phosphorylation at Ser-307 was of greater significance in the negative regulation of HSF1 than phosphorylation at Ser-303 (231). This report was never substantiated and the inconsistency remains unresolved.

In a follow-up study by Chu *et al*, reference was made to Akt, an upstream inhibitor of GSK3β:

"[The fact that] overexpression of GSK3α or GSK3β directly represses HSF1 suggests a sub-population of HSF1 molecules may be constitutively phosphorylated at Ser-307 and that HSF1 may be directly regulated by the GSK3 pathway as well as indirectly through the [ERK1] pathway ... [Akt] is activated by heat shock and could potentially activate HSF1 through GSK inhibition (210)."

4.1.3 PI3K/Akt

GSK3 was originally discovered during the fraught race to describe the intracellular consequences of insulin receptor activation. GSK3 was found to phosphorylate and inactivate glycogen synthase, the enzyme catalysing the rate limiting step of glycogen synthesis (232). Insulin was later found to inactivate GSK3 (233,234), but the link between the insulin receptor and GSK3 remained elusive.

Akt was described simultaneously as the cellular homolog to the *v-Akt* oncogene of the AKT8 retrovirus (235), a serine/threonine kinase related to A- and C- protein kinase(s) (RAC-PK) (236), and as protein kinase B (PKB) (237). It was identified as the kinase responsible for GSK3 phosphorylation and inactivation – the missing link between extracellular insulin action and glycogen metabolism. It has since been found to have a diverse set of roles and is a 'key mediator of cell proliferation and survival' (238). Akt up-regulation has been implicated in cell survival in a number of different models of cell stress (239-242). Much of the initial interest in Akt derived from the fact it was activated by insulin and other growth factors in a manner dependent on phosphatidylinositol 3-kinase (PI3K) (243) (Figure 4.3). PI3K is activated by receptor tyrosine kinases following ligand binding and acts to

phosphorylate the membrane phospholipid, $PI(4,5)P_2$ to $PI(3,4,5)P_3$. This product regulates Akt in a complex manner which has taken a number of years to elucidate. Akt has a pleckstrin homogeny (PH) domain which allows it to bind directly to $PI(3,4,5)P_3$ with two distinct results: firstly, Akt is recruited to the plasma membrane allowing it to co-localise and interact with other proteins; secondly, it undergoes a conformational change revealing two sites which require to be phosphorylated for activation. The exact mechanism of this dual phosphorylation is still the subject of debate and is out with the scope of this discussion. Suffice to say that following PI3K activation, Akt is recruited to the cell membrane and activated.

GSK3β is phosphorylated and inactivated by Akt

GSK3β is an unusual signalling kinase in that it is fully active in unstimulated cells. It is inactivated by phosphorylation at the serine 9 residue. Ample evidence now exists that this is mediated by Akt (244). Many studies since the late 1990s have confirmed the insulin – PI3K – Akt – GSK3β axis (245-248).

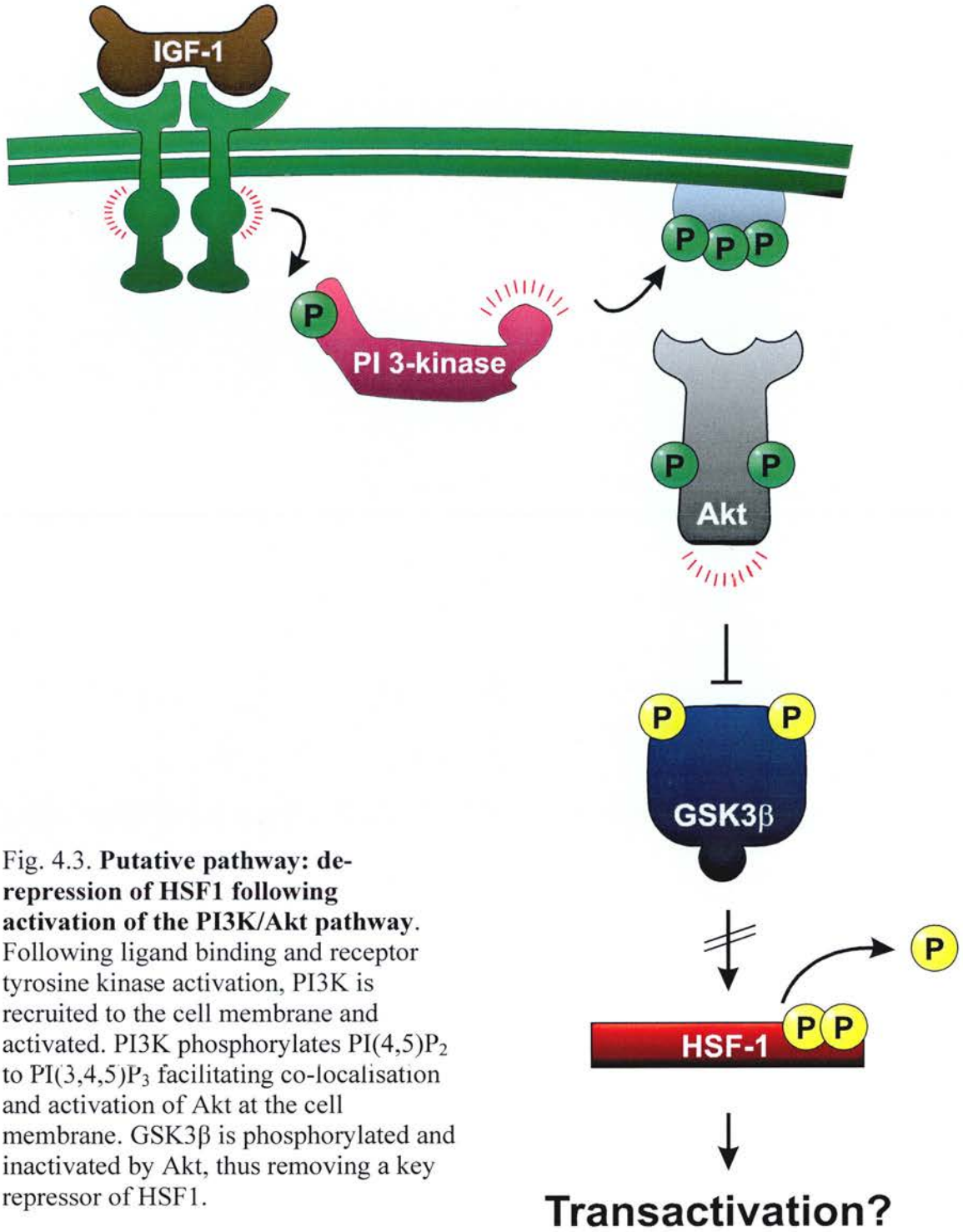


Fig. 4.3. **Putative pathway: de-repression of HSF1 following activation of the PI3K/Akt pathway.** Following ligand binding and receptor tyrosine kinase activation, PI3K is recruited to the cell membrane and activated. PI3K phosphorylates $PI(4,5)P_2$ to $PI(3,4,5)P_3$ facilitating co-localisation and activation of Akt at the cell membrane. GSK3β is phosphorylated and inactivated by Akt, thus removing a key repressor of HSF1.

GSK3β is the best target for modification of HSF1 activity by phosphorylation

In summary, significant evidence exists that GSK3β acts to repress HSF1 transcriptional activation in unstimulated cells, represses DNA-binding in cells exposed to heat shocked and accelerates the deactivation of HSF1. This is based on robust data from independent laboratories examining phosphorylation at specific HSF1 residues. Evidence of a significant role for ERK also exists, but it appears to act in a GSK3β dependent manner; thus, altering ERK and GSK3β activity would not be additive. Some data on specific phosphorylation by PKC and JNK is promising, but in contradiction to a significant number of other reports in the literature. Thus, GSK3β represented the best target for manipulation of HSF1 transactivation by phosphorylation. Inhibition of GSK3β through stimulation of the PI3K/Akt pathway was a potential mechanism by which HSF1 could be activated, stimulating the stress protein response without the requirement of classical stressors, with their inherent potential for toxicity. As the ultimate aim of this work was a clinical intervention, the investigation commenced using IGF-1 as an upstream activator of PI3K/Akt as it had been previously used in humans and had low side-effect profile (249).

4.2 Hypotheses

1. Activation of the PI3K/Akt pathway phosphorylates and inactivates GSK3β in ACHN cells.

2. De-repression of HSF1 at Ser-303 results in trimerisation and DNA-binding of HSF1.

3. Inactivation of GSK3β results in transactivation of HSF1 and induction of heat shock proteins.

4. Akt activation facilitates renal protection in a murine model of ischemia reperfusion injury.

4.3 Results

IGF-1 does not induce Hsp70 in ACHN cells

Cells were exposed to various concentrations of IGF-1 for 6 h (Figure 4.4A) and IGF-1 at a concentration of 100 μg/ml for various periods of time (Figure 4.4B/C). Western blots were performed on whole-cell lysates using antibody to inducible Hsp70. No induction of Hsp70 was observed following IGF-1 treatment. Western blots were performed for the stress proteins Hsp90, Grp94 and Hsp27 (Figure 4.4C). Similarly, no induction of these proteins was seen following IGF-1 treatment.

GSK3β phosphorylated at serine 9 in untreated cells

In order to determine whether IGF-1 treatment activated the PI3K/Akt axis, the phosphorylation state of GSK3β was determined. Cells were treated with IGF-1 (100 μg/ml) for various periods of time. Whole cell lysates were prepared and a Western blot performed using the phospho-specific antibody (Ser-9) for GSK3β (Figure 4.5A). It was found that GSK3β was phosphorylated in control cells, and the amount of pGSK3β present did not increase following IGF-1 treatment.

Culturing cells in serum free conditions for 16 h dephosphorylates GSK3β at Ser- 9; IGF-1 does not phosphorylate GSK3β at Ser- 9 in ACHN cells

Cells were plated out at a confluency of around 50% on day 1 in normal culture medium containing 10% fetal calf serum (FCS). The evening of day 2, the medium was changed to normal culture medium containing no FCS.

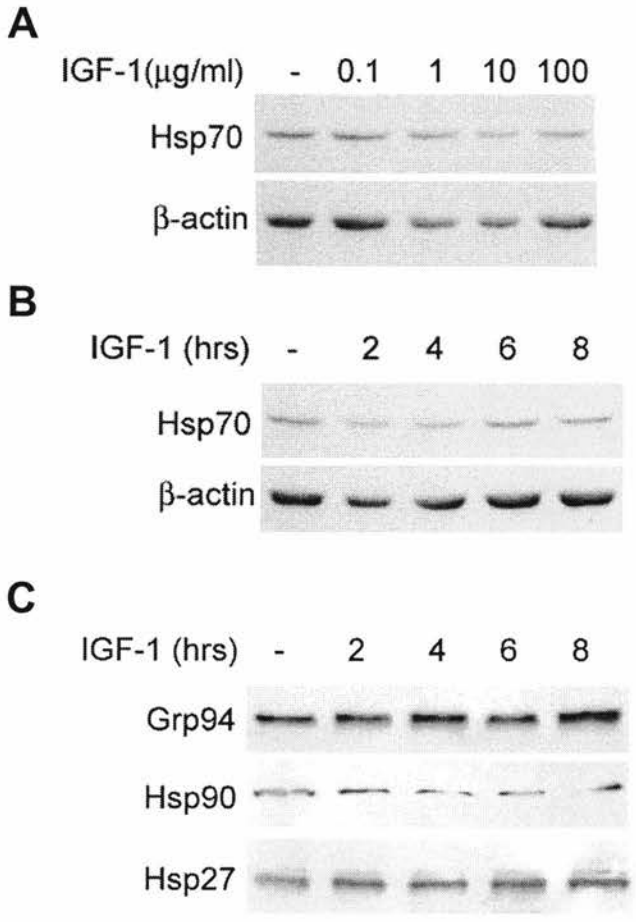


Figure 4.4 **Stress protein expression in ACHN cells following IGF-1 treatment.** ACHN cells were exposed to increasing doses of IGF-1 for 6 h (A) and IGF-1 (100 μg/ml) for various periods of time (B, C). Whole-cell lysates were prepared and analysed by Western blotting using antibody to Hsp70 (A, B) and antibody to Hsp90, Grp94 and Hsp27 (C). β-actin was used as a loading control.

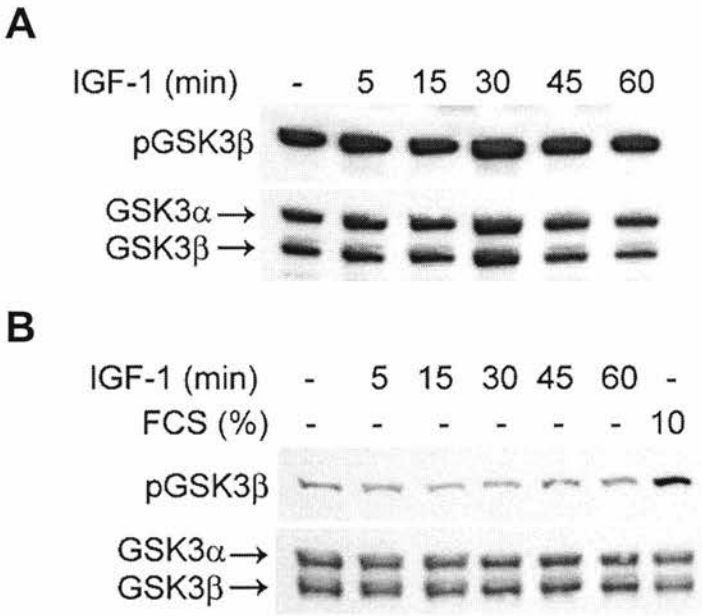


Figure 4.5 **Phosphorylation of GSK3β at Ser-9 following IGF-1 treatment in ACHN cells cultured in 10% FCS (A) and 0% FCS (B).**
Cells were exposed to IGF-1 (100 μg/ml) for increasing periods of time (A). Whole-cell lysates were prepared and analysed with Western blotting using antibody to pGSK3β with total GSK3β being used as a loading control. (B) Cells were plated out at a confluency of around 50% on day 1 in normal culture medium containing 10% fetal calf serum (FCS). The evening of day 2, the medium was changed to normal culture medium containing no FCS. Cells were treated with treated with IGF-1 (100 μg/ml) for increasing periods of time. Whole-cell lysates were prepared and analysed by Western blotting using antibody to pGSK3β with total GSK3β being used as a loading control.

Cells were treated with treated with IGF-1 (100 μg/ml) for various periods of time as before (Figure 4.5B). Phosphorylation at GSK3β Ser-9 was greatly reduced in cells cultured in 0% FCS. However, GSK3β was not phosphorylated at Ser-9 in response to IGF-1 treatment.

IGF-1 receptor expression low in ACHN cells, but insulin receptor expression significantly greater

To determine whether the absence of GSK3β phosphorylation in response to IGF-1 treatment was due to low expression of the IGF-1 receptor, immunofluorescence with an antibody to IGF-1Rα was performed on ACHN cells with HUH-7 cells (liver adenocarcinoma cell-line) used as a positive control (Figure 4.6). IGF-1Rα expression was found to be very low compared to expression in HUH-7 cells. As insulin is an alternative stimulator of the PI3K/Akt axis, the expression of the insulin receptor was also determined in ACHN cells. Immunofluorescence experiments were performed using an antibody to the insulin Rβ receptor protein with ACHN and HUH-7 cells. The expression of insulin Rβ was found to be high in both cells lines (Figure 4.7). Therefore, IGF-1Rα is poorly expressed on ACHN cells, while insulin Rβ expression in ACHN cells is comparable with the expression in HUH-7 cells.

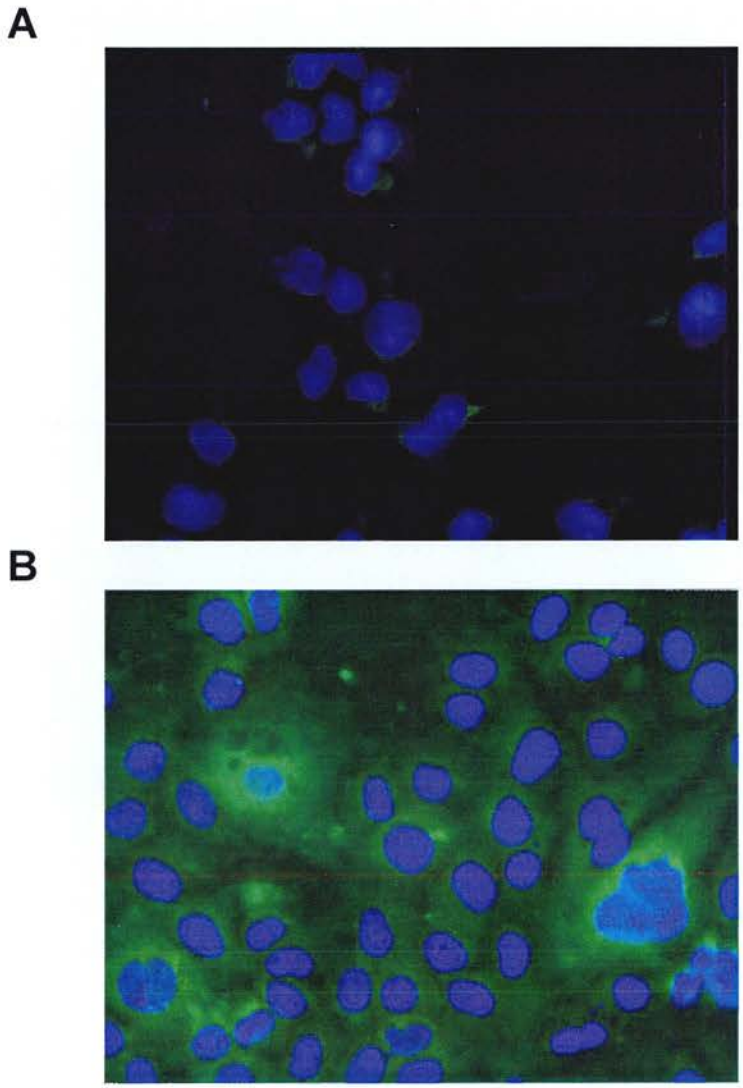


Figure 4.6 **IGF-1Rα expression in ACHN and HUH-7 cells lines.**
ACHN (A) and HUH-7 (B) cells were fixed with methanol, blocked with normal goat serum and exposed to the IGF-1Rα monoclonal antibody. After washing, cells were exposed to a secondary goat-anti-mouse FITC-conjugated antibody. Cells were counterstained with Hoechst. Fields were visualised with a Leica DM IRB microscope and images were stored using the Leica Improvision software. Primary antibody only and secondary antibody only groups were included as controls and on microscopy were found to have no staining.

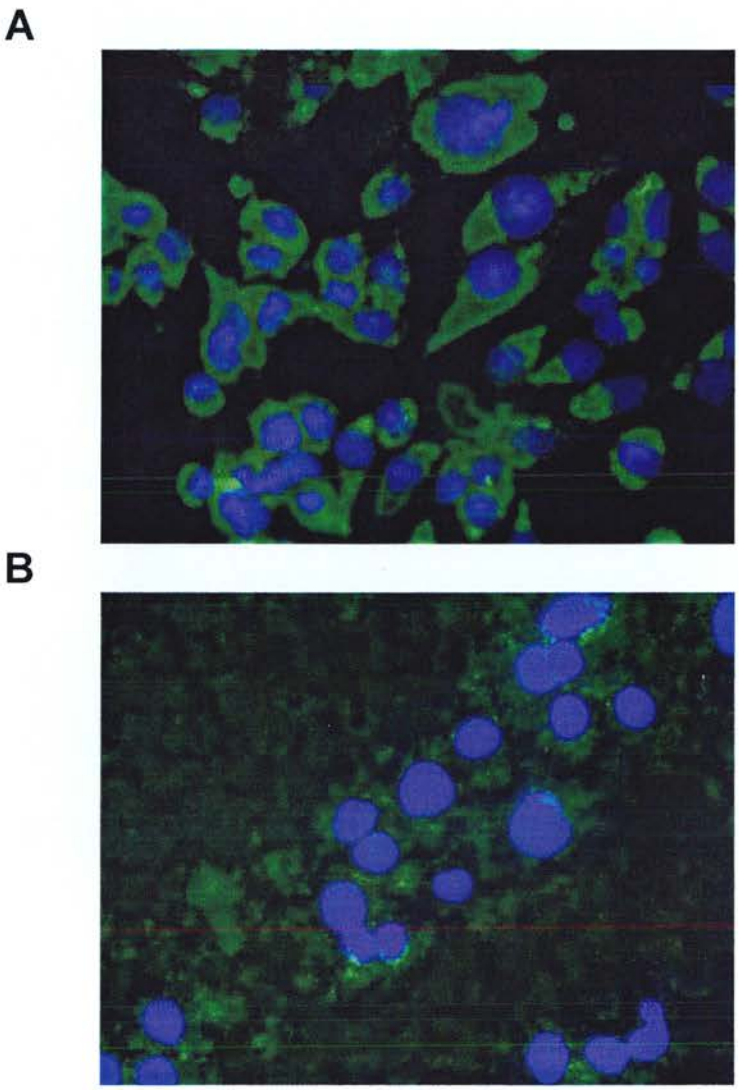


Figure 4.7 **Insulin Rβ expression in ACHN and HUH-7 cells lines.**
ACHN (A) and HUH-7 (B) cells were fixed with methanol, blocked with normal goat serum and exposed to the insulin Rβ rabbit polyclonal antibody. After washing, cells were exposed to a secondary goat-anti-rabbit FITC-conjugated antibody. Cells were counterstained with Hoechst. Fields were visualised with a Leica DM IRB microscope and images were stored using the Leica Improvision software. Primary antibody only and secondary antibody only groups were included as controls and on microscopy were found to have no staining.

Insulin phosphorylates GSK3β at Ser-9

ACHN cells were cultured as before in serum free conditions and treated with insulin (200 nM) for various periods of time. Phosphorylation of GSK3β at Ser-9 was found to increase very quickly in response to insulin treatment with maximal levels being attained within 15 min (Figure 4.8).

Serum deprivation in ACHN cells does not induce heat shock proteins

In order to ensure that serum-free cell culture did not itself induce the stress protein response, cells were cultured in serum-free conditions for 16 h after which whole-cell lysates were produced and Western blots performed using antibody to Grp94, Hsp90, Hsp70 and Hsp27 (Figure 4.9). No induction of these proteins was seen in cells incubated in 1%, 0.1%, 0% FCS for 16 h.

Insulin does not induce Hsp70 in ACHN cells

As in the IGF-1 experiments, cells were exposed to various concentrations of insulin for 6 h (Figure 4.10A) and insulin at a concentration of 200 nM for increasing periods of time (Figure 4.10B). Western blots were performed on whole-cell lysates with antibody to inducible Hsp70. No induction of Hsp70 was observed following insulin treatment.

Insulin does not cause nuclear localisation of HSF1, increase the DNA-binding ability of HSF1, nor induce trimerisation of HSF1 in serum-deprived ACHN cells

In order to determine whether insulin altered the oligomeric state or DNA-binding ability of HSF1, cells were treated with insulin (200 nM) for 2 h and nuclear

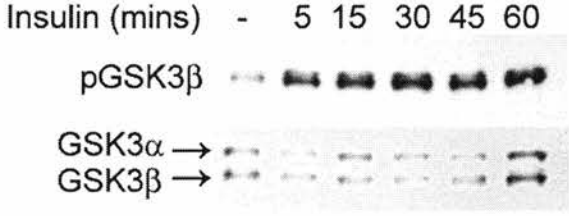


Figure 4.8 **Phosphorylation of GSK3β at serine 9 following insulin treatment in serum-free ACHN cells.**
Cells were plated out at a confluency of around 50% on day 1 in normal culture medium containing 10% fetal calf serum (FCS). The evening of day 2, the medium was changed to normal culture medium containing no FCS. Cells were treated with treated with insulin (200 nM) for increasing periods of time. Whole-cell lysates were prepared and analysed by Western blotting using antibody to pGSK3β with total GSK3β being used as a loading control.

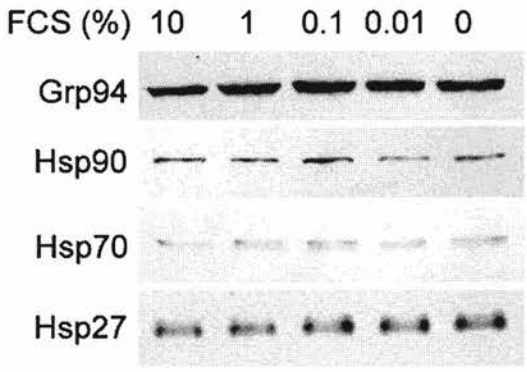


Figure 4.9 **Expression of stress proteins following serum deprivation in ACHN cells.**
Cells were cultured in medium containing varying concentrations of FCS for 16 h. Whole-cell lysates were prepared and analysed by Western blotting using antibody to Grp94, Hsp90, Hsp70 and Hsp27.

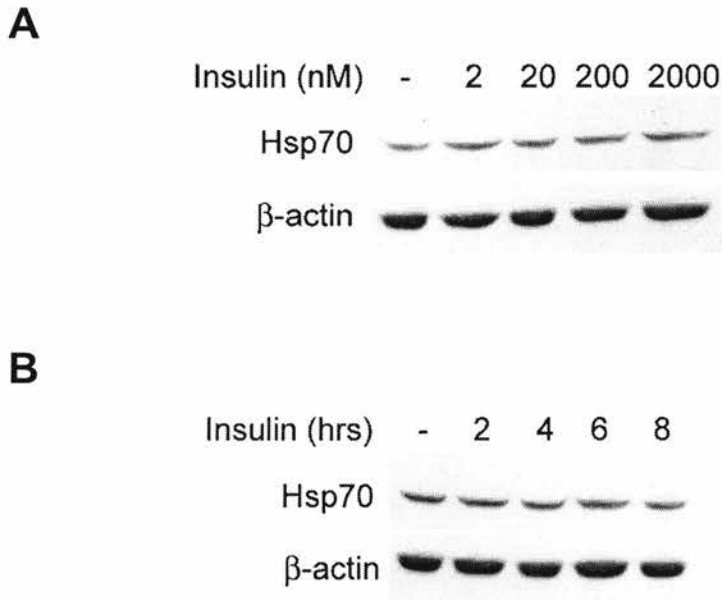


Figure 4.10 **Hsp70 protein expression following insulin treatment in serum deprived cells.**
ACHN cells were cultured in medium containing no FCS for 16 h, then exposed to insulin (200 nM) for various periods of time. Whole-cell lysates were prepared and analysed by Western blotting using antibody to Hsp70. β-actin was used as a loading control.

lysates prepared. A DNA-mobility shift assay was performed with [γ32P]ATP-labelled HSE oligonucleotides (Figure 4.11). No alteration in the oligomeric state or DNA-binding ability was seen following insulin treatment, when compared to controls subjected to heat treatment at 43 °C for 45 min. To determine whether insulin resulted in HSF1 nuclear localisation, the intracellular compartmentalisation of HSF1 was established. Cells were treated with insulin for 2 h, after which cytoplasmic and nuclear cell lysates were prepared and Western blot with antibody to HSF1 was performed (Figure 4.12). No change in the intracellular compartmentalisation was observed following insulin treatment.

Insulin does not increase Hsp70 induction following exposure to heat

Finally, to ascertain whether treatment with insulin alters the threshold of stress-induced Hsp70 induction, cells were treated with insulin (200 nM) for 30 min and incubated at 43°C for 45 min, followed by 4 h recovery (Figure 4.13). No alteration in Hsp70 expression was identified between those cells submitted to heat shock alone and those pre-treated with insulin.

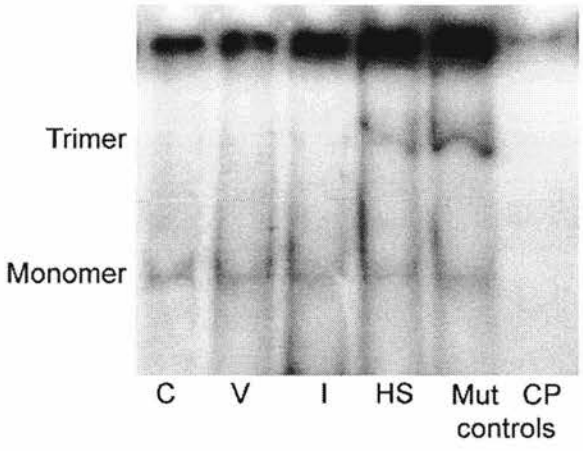


Figure 4.11 **HSF1 DNA-binding ability and oligomeric status following insulin treatment.**
Cells were cultured in serum-deprived conditions and treated with insulin (I) (200 nM) or vehicle (V) for 1.5 h; a further group was subjected to heating at 43 °C for 45 min. A DNA mobility shift assay was performed with [γ32P]ATP-labelled heat shock element consensus oligonucleotides. Excess unlabelled mutant (Mut) and consensus (CP) sequences were included as controls.

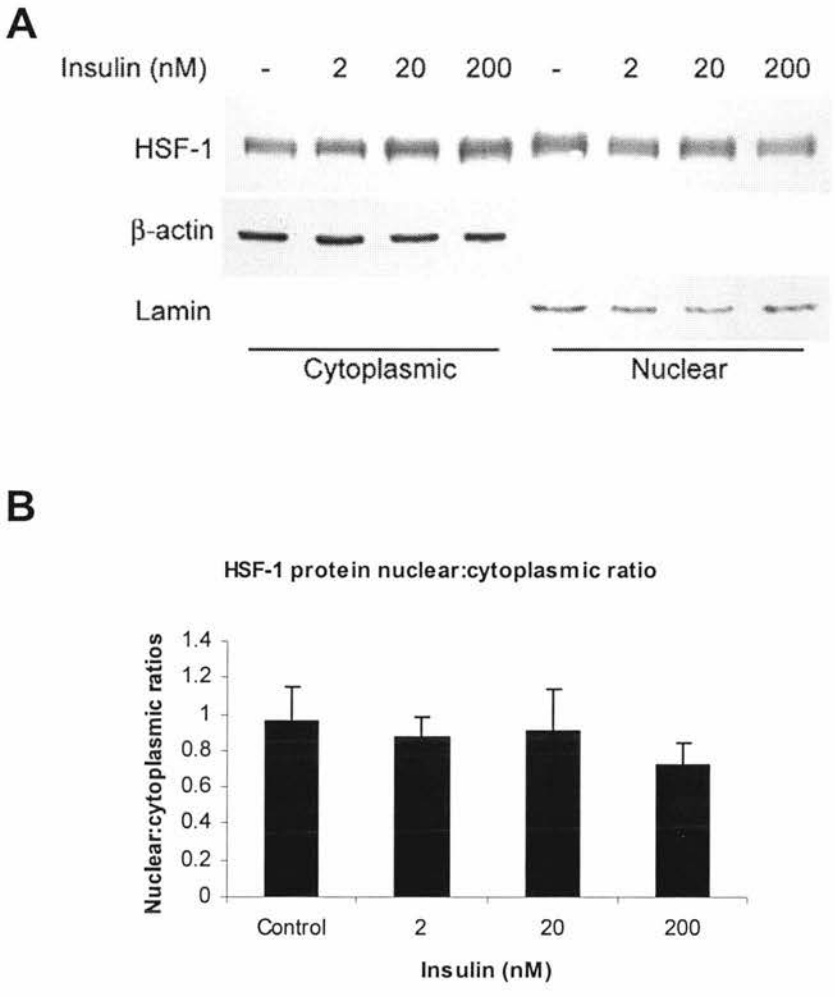


Figure 4.12 **HSF1 intracellular compartmentalisation following insulin treatment.**
Cells were cultured in serum-deprived conditions for 16 h and treated with insulin for 1.5 h. Nuclear and cytoplasmic lysates were prepared and analysed by Western blotting using antibody to HSF1, with loading control with β-actin for cytoplasmic extracts and lamin A/C for nuclear extracts. Band densitometry was ascertained using Quantity One software and nuclear:cytoplasmic ratios calculated. These are expressed graphically (B) with error bars representing S.E.M. over 3 independent experiments.

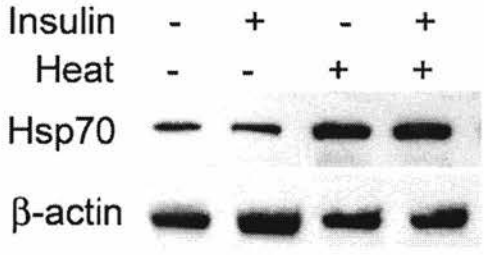


Figure 4.13 **Insulin does not alter Hsp70 expression following heat exposure.** Cells were treated with insulin (200 nM) for 30 min, incubated at 43°C for 45 min and recovered for 4 h. Whole-cell lysates were prepared and Western blotting performed with antibody to Hsp70. β-actin was included as a loading control.

4.4 Discussion

It was reported in 1985 that insulin induces Hsp70 in Hep3B/T2 cells, although the mechanism was not determined (250). Following the publication of data suggesting a regulatory role for GSK3β in HSF1 activation, it was hypothesised that insulin-induced Hsp70 induction may occur through the PI3K/Akt axis. With the ultimate goal of identifying a clinical strategy for inducing the protective stress protein response in kidneys IGF-1 was used. The rationale for this was that the alternative, insulin, has a plethora of physiological actions rendering its candidacy as an inducer of the stress protein response with no other side-effects impossible. IGF-1, on the other hand, is known to activate the PI3K/Akt axis in a similar manner to insulin, but has a lower side-effect profile (249). However, following treatment with IGF-1 no induction of the stress protein response was observed, bringing this hypothesis into question (Figure 4.4). There were a number of possibilities why the anticipated response did not occur and further investigation was warranted. First, it had to be demonstrated that GSK3β was phosphorylated at Ser-9 by IGF-1 as predicted. Western blots of IGF-1-treated cells revealed that unstimulated cells were maximally phosphorylated at Ser-9 on GSK3β (Figure 4.5A) and that IGF-1 treatment had no additional effect. Fetal calf serum contains a number of hormones and growth factors and it was hypothesised that one or more of these components was effecting constitutive phosphorylation of GSK3β. Indeed, depriving cells of FCS for 16 h resulted in dephosphorylation at the Ser-9 residue on GSK3β (Figure 4.5B) with no alteration in stress protein expression (Figure 4.10). Yet, treatment of cells with IGF-1 in serum-deprived conditions did not cause phosphorylation of GSK3β (Figure 4.5B). It had been shown by Ouban *et al*, when looking at the expression of the IGF-

1 receptor in various human cancers, that kidney adenocarcinoma expressed relatively little IGF-1 receptor (251). Indeed, no significant expression of IGF-1Rα was demonstrated in ACHN cells, confirming the findings of Ouban (Figure 4.6); however, high-levels of the insulin receptor (insulin Rβ) were demonstrated (Figure 4.7). In order to continue the investigation, insulin was used as an upstream activator of Akt. Despite demonstrating that insulin phosphorylated GSK3β, insulin treatment did not result in Hsp70 induction (Figure 4.10). This contradicts the findings of Ting (250) who demonstrated increased Hsp70 gene expression in response to insulin in a human hepatoma cell-line (Hep3B/T2). With the knowledge that HSF1 activation is a multi-step process and subject to a number of different layers of regulation, it was decided to pursue the hypothesis further. However, neither HSF1 nuclear localisation nor trimerisation were observed following insulin treatment (Figure 4.11 and 4.12). Altering GSK3β activity, therefore, did not alter the activation state of HSF1 in unstressed cells. To determine whether GSK3β repression lowered the threshold of HSF1 activation, cells were pre-treated cells with insulin and subjected to mild heat treatment. No difference in Hsp70 protein expression was seen in insulin-pre-treated cells subjected to heat compared to cells exposed to heat alone (Figure 4.13).

A number of issues are raised in analysing why in this part of the investigation the null-hypothesis was proven correct. Previous data from Ting *et al* showed Hsp70 induction following insulin treatment (250), but a number of methodological differences exist between this study and that presented here. The most obvious variation is the cell-line used: Hep3B/T2 cells, a human hepatoma cell-line. The concentration of insulin used by Ting *et al* was similar, but no details are provided of

the type of insulin (e.g. bovine, porcine, human recombinant) or if it was reconstituted in a particular manner (e.g. acidic conditions). Additionally, cells were serum starved for 6 days while in the present study cells were only deprived of serum over night. This time period was established following measurement of the phosphorylation status of GSK3β and showing that 16 h was sufficient for dephosphorylation. But if the mechanism of Hsp70 induction was different (i.e. GSK3β-independent) then 16 h may be insufficient to bring basal Hsp70 concentration down to a level where changes could be observed. Furthermore, insulin has been shown to induce reactive oxygen species in cells (252); while we did not find this in our cell model (see chapter 5), it is possible that this effect was occurring in Ting's model and was sufficient to induce a stress protein response. Ting *et al* did not provide any mechanistic data so further analysis is limited.

Data of excellent quality on the contribution of GSK3β and ERK1 to HSF1 repression is presented in two papers by Chu *et al* (209,210), however, a major flaw exists when taken in the context of this work. The data generated in these studies is based on the over-expression of recombinant HSF1 in cells, be that in wild-type or mutated forms. Intracellular HSF1 concentrations, therefore, are much greater in these models than would occur in physiological circumstances. Taken with the present understanding of HSF1 regulation, this would result in inhibitors of HSF1 (be those of a phosphorylation/dephosphorylation nature, or a multi-chaperone complex) being titrated out. Thus, over-expression of HSF1 is sufficient to activate HSF1 with no other stimulation (demonstrated aptly by the activation of an Hsp70 reporter following transfection of a wild-type HSF1 expressing construct alone (209)). In the

'physiological' model of the present study, therefore, the ratio of HSF1 to repressive systems is much lower than in the model used by Chu, presumably making HSF1 activation much more difficult. This observation is also true for the papers by Kline and Knauf (211,215).

Insulin has many physiological effects that could have acted to confound the present study. In particular, insulin is known to up-regulate ERK1 which has been implicated in the repression of HSF1 activity (206,209,210). The possibility exists, therefore, that ERK1 is activated by insulin and acts to repress HSF1. In the present study, it had been conjectured that as ERK1 action is GSK3β-dependent, turning off GSK3β would render ERK1 action redundant. Nevertheless, if GSK3β inactivation was incomplete, this could have contributed to the lack of response.

Although GSK3β represses HSF1 activation, this does not necessarily mean that removal of that repression will activate HSF1. Xavier *et al* demonstrated that GSK3β inhibition increased DNA-binding and transcriptional activation *in heat shock conditions* (208). But in cells pre-treated with insulin and heated (Figure 4.13), no increase in Hsp70 was observed. It is possible that the GSK3β inhibitors (lithium chloride and GSK-binding protein) used by Xavier *et al* were more effective than PI3K/Akt-mediated inhibition of GSK3β, or that they were having off-target effects. Also, it is not clear what the influence of cell choice (*Xenopus* oocytes) is on HSF1 activation. Lastly, the sensitivity of Hsp70 detection by Western blot was possibly not high enough to discern differences between insulin treated and control groups.

Whatever the reasons behind the failure to elicit Hsp induction via PI3K/Akt activation, it was clear that this was not a viable strategy for the induction of Hsps in models of transplantation. However, during the course of our investigation we noticed an interesting and unexpected effect: the small heat shock protein, heme oxygenase-1 (HO-1), was up-regulated following insulin treatment. HO-1 does not take part in the classical stress protein response and is controlled independently of HSF1 in humans (although not mice) (174). HO-1 is known to confer significant cellular protection, but up-regulation following insulin had not been previously described. The investigation of this response is the subject of the next chapter.

Chapter 5 – Heme oxygenase-1 induction through the PI3K/Akt pathway and the Nrf2 transcription factor

5.1 Introduction

5.1.1 Background

During the investigation described in chapter 4 of the possible effects of insulin on HSF1 activity, it was observed that heme oxygenase-1 (HO-1) protein was induced in response to insulin. HO-1, also known as Hsp32, is a heat shock protein but does not take part in the classical heat shock response in humans, i.e. it is not under the control of HSF1. Thus, a role for HO-1 was not predicted in the original hypothesis of heat shock protein induction in response to insulin/IGF-1. Insulin-mediated HO-1 induction has not been previously described and warranted further investigation.

5.1.2 Heme oxygenase-1

HO-1 catalyses the rate-limiting step in the degradation of heme to carbon monoxide (CO), free iron and biliverdin, which is immediately converted to bilirubin by bilverdin reductase (253) (Figure 5.1). At least two isoenzymes are known to exist: HO-1, which is strongly induced by its substrate heme and a number of stress stimuli including UV radiation and heavy metals; and constitutive HO-2 (254-256). The exact role of HO-1 in oxidative stress is not clear but it has been shown to be protective in a number of cell-types and animal models, by virtue of the products of

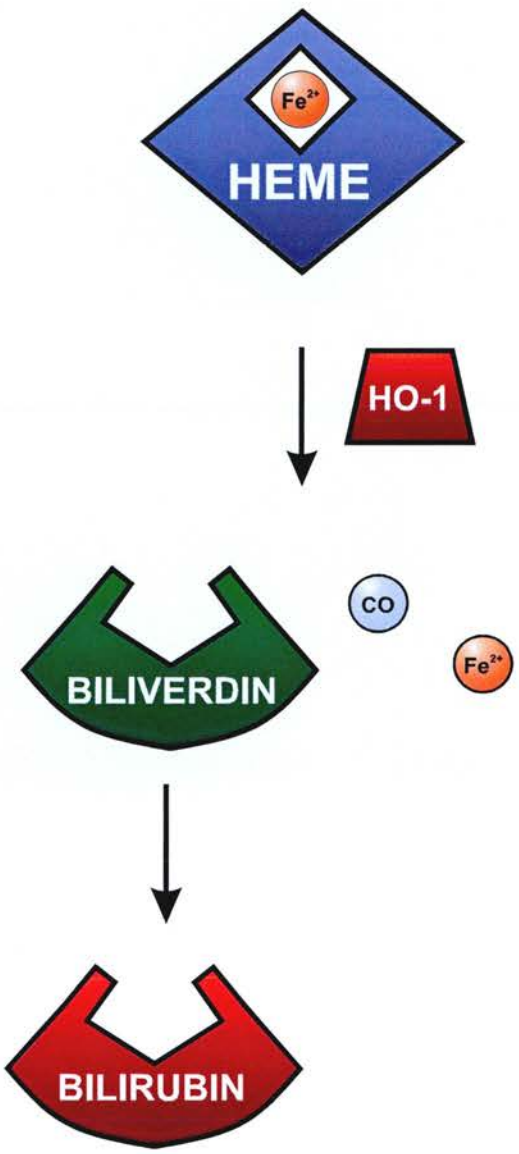


Figure 5.1 **Heme oxygenase-1 catalyses the rate limiting step of heme degradation.**
HO-1 acts to catalyse the breakdown of heme to the protective biliverdin (which is almost immediatcly converted to bilirubin), carbon monoxide and free iron. Although free iron can result in oxidative stress its release induces ferritin, an iron-sequestering protein which is also protective.

the reaction it catalyses (for recent review see (257)). Bilirubin is known to be a powerful antioxidant (258,259) and HO-derived bilirubin has been shown to provide protection in neuronal cells (260). CO was first demonstrated to be protective in a model of acute lung injury (261), subsequently in rodent cardiac (262,263) and renal transplantation models (122). Two important mechanisms of CO protection involving p38 mitogen-activated protein kinase (MAPK) and guanylyl cyclase have been identified, but these appear to be cell-type specific (257). Although HO-1 releases the pro-oxidant Fe^{2+}, this is associated with the rapid expression of the iron-sequestering protein ferritin, which is also known to be protective (264). It is generally accepted, therefore, that induction of heme degradation represents an adaptive response to oxidative insult.

Insulin is a polypeptide hormone that regulates glucose, lipid and protein metabolism and promotes cell growth and differentiation. On ligand binding, the insulin receptor tyrosine kinase initiates multiple signalling cascades including the activation of the phosphatidylinositol 3-kinase (PI3K) pathway and its downstream effectors (265). This pathway is a key signal transducer of many growth factors and cytokines and has been implicated in the regulation of cell growth, cell migration and cell survival (266). The protein kinase B/Akt family of serine/threonine kinases has been identified as an important target of PI3K in cell survival (239-242). Moreover, recent work has shown a direct link between the PI3K/Akt pathway and HO-1 regulation in PC12 cells (267,268). This may be through nuclear factor E2-related factor-2 (Nrf2), a member of the cap'n'collar family of basic leucine transcription factors and a well established regulator of HO-1 (269).

Author	Year	Species	Organ	Model	Stimulus	Ref
Soares	1998	Mouse to Rat	Heart	Xenograft	CVF/ Cyclosporin A	(263)
Amersi	1999	Rat	Liver	Ischemia/ ex-vivo	CoPP/ Ad-HO-1	(270)
Salahudeen	2001	Human	1° renal CL	Cold storage	Hemin	(271)
Kato	2001	Rat	Liver	Isograft	CoPP	(160)
Tullius	2002	Rat	Kidney	Isograft	CoPP	(118)
Redaelli	2002	Rat	Liver	Isograft	Heat	(158)
Katori	2002	Rat	Heart	Isograft	CoPP	(272)
Coito	2002	Rat	Liver	Isograft	Ad-HO-1	(273)
Amersi	2002	Rat	Liver	Ischemia/ ex-vivo	CO	(274)
Blydt-Hansen	2003	Rat	Kidney	Isograft	Ad-HO-1	(159)
Song	2003	Rat	Lung	Allograft	CO	(275)
Wagner	2003	Rat	Kidney	Isograft	Heat/CoPP	(82)
Zhang	2004	Mouse	Lung	IR	HO-1 siRNA	(276)
Patel	2004	Human	Liver	IR	Ischemia	(162)
Neto	2004	Rat	Kidney	Isograft	CO	(122)
Nakao	2004	Rat	Small bowel	Isograft	Biliverdin	(277)
Fondevila	2004	Rat	Liver	Ex-vivo Isograft	Biliverdin	(278)
Akamatsu	2004	Rat	Heart	Isograft	CoPP/ CO	(279)
Nakao	2005	Rat	Heart/ Kidney	Isograft	Biliverdin/ CO	(123)

Table 5.1 **Studies describing protective effects of HO-1 and products in transplantation.**
The beneficial effects of HO-1, CO and biliverdin/bilirubin have been studied extensively in many organ systems, including models of transplantation. Studies examining chronic allograft nephropathy have been excluded. CVF, cobra venom factor; CoPP, cobalt protoporphyrin; Ad-HO-1, adenoviral HO-1; CO, carbon monoxide; IR, ischemia/reperfusion.

In view of the beneficial effects of up-regulation of HO-1 in models of organ transplantation, it is important to identify signalling pathways involved in regulation of HO-1 gene expression. In this chapter, data is presented demonstrating a PI3K/Akt dependent induction of HO-1 following the administration of insulin to renal adenocarcinoma cells (ACHN). PI3K activity was necessary and sufficient for HO-1 induction and Nrf2 blockade was found to abolish the response. Supporting data illustrates similar insulin-induced HO-1 expression in mouse primary renal tubular epithelial cells.

5.2 Hypotheses

1. Insulin induces *de novo* HO-1 production.

2. HO-1 induction is dependent on a specific intracellular signalling pathway(s).

3. HO-1 induction is dependent on a specific transcription factor(s).

4. HO-1 induction facilitates renal protection in a murine model of ischemia reperfusion injury.

5.3 Methods and materials

Mouse primary tubular epithelial cell culture

The kidneys of 6 week old male BALB/c mice were removed in sterile conditions and placed in ice-cold HBSS containing penicillin (100 U/ml), streptomycin (100 µg/ml) and 1X antibody/antimycotic solution (Gibco). After decapsulation and bisection the medulla was removed and the cortices reduced with repeated incisions to 1 mm^3 pieces. Kidney pieces were incubated at 37 °C with HBSS containing collagenase type IV (0.5 mg/ml) and *DNase* (10 µg/ml) freshly prepared. Following confirmation of the presence of tubules, they were resuspended in DMEM-F12 with glutamax, penicillin (100 U/ml), streptomycin 100 µg/ml) (all Gibco), 1x insulin/transferrin/selenium, dexamethasone (35.7 ng/ml) and epidermal growth factor (25 ng/ml). Tubules were cultured in 6-well plates for 5 days or until 70% confluent. Culture conditions were then changed to DMEM-F12 with glutamax, penicillin, streptomycin and dexamethasone for 40 h, after which experiments were performed. Cells were cytokeratin positive and vimentin negative on immunocytochemistry.

5.4 Results

Insulin increases HO-1 expression in ACHN cells

Treatment of serum-deprived ACHN cells with increasing concentrations of human insulin resulted in a 4-fold induction of HO-1 after 6 h (Figure 5.2A). Maximal induction of HO-1 protein was achieved at concentrations of 200 nM insulin. A time course using insulin (200 nM) demonstrated accumulation of HO-1 after 2 h treatment (Figure 5.2B). HO-1 mRNA was found to increase over the same concentration range of insulin (Figure 5.2C) and achieved maximum induction after 2 h treatment with insulin (200 nM) (Figure 5.2D). HO-1 mRNA returned to resting levels after 16 h of treatment. To ensure HO-1 induction was not related to serum-deprivation, cells were cultured in medium containing different concentrations of fetal calf serum for 16 h (Figure 5.2E); no alteration in HO-1 protein expression was detected. To confirm that HO-1 accumulation was dependent on gene transcription, ACHN cells were pre-treated with actinomycin D (AD) followed by insulin (Figure 5.3A and C). Basal levels of HO-1 protein were reduced following AD and the HO-1 protein and mRNA response to insulin was abolished. Similarly, cycloheximide (CHX) was administered to establish the role of protein synthesis in insulin-induced HO-1 expression (Figure 5.3B and C). CHX abrogated HO-1 protein induction following insulin treatment but, in keeping with other studies, also eliminated HO-1 mRNA induction, suggesting protein translation is required to activate the HO-1 promoter (267,280,281).

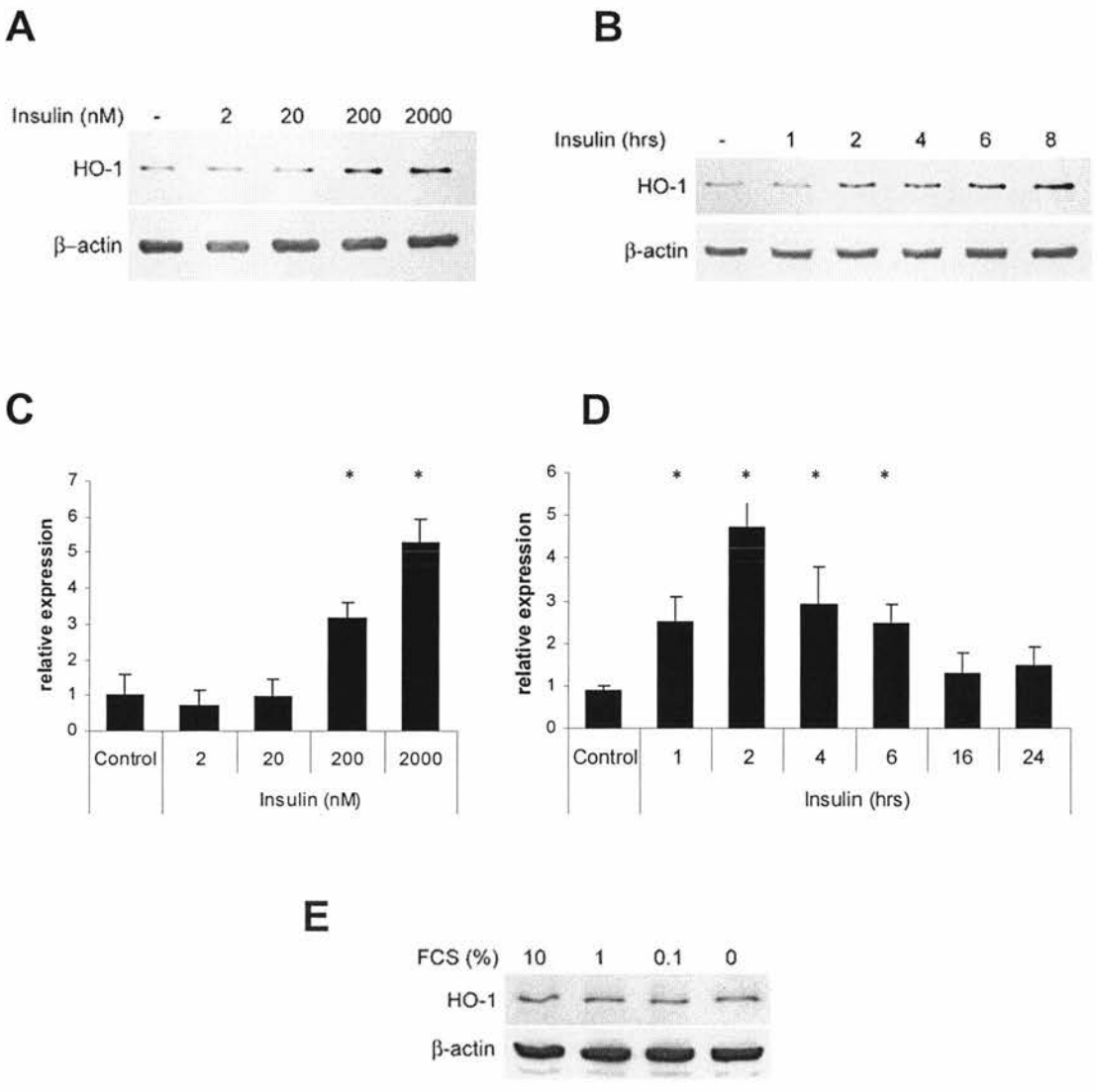


Figure 5.2 **Insulin stimulates HO-1 protein and mRNA accumulation in ACHN cells.**
Cells were serum-deprived for 16 h and treated with increasing concentrations of insulin for 6 h (A), 4 h (C) or insulin (200 nM) for various times (B, D). ACHN cells were cultured in medium supplemented with different concentrations of fetal calf serum (FCS) (E). Whole-cell lysates were prepared and analysed by Western blotting (A, B, E) using antibody to HO-1, with β-actin being indicated as loading control. mRNA extracts were prepared (C, D) using TRIzol and reverse transcribed to cDNA. Fluorescence-detected real time PCR was performed using HO-1 primers and probe with an 18s primer/probe control; results are expressed as mean relative expression ± S.E.M. of 3 independent experiments. * $p<0.05$ Mann-Whitney U test.

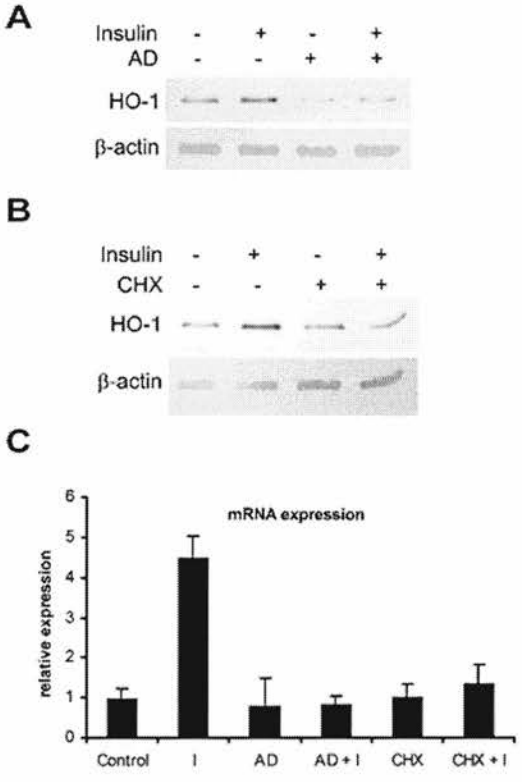


Figure 5.3 **Insulin stimulated HO-1 accumulation is transcription and translation dependent.**
Cells were serum-deprived for 16 h and pre-treated with actinomycin D (AD) (5 μg/ml) (A, C) or cycloheximide (CHX) (10 μg/ml) (B, C) for 30 min, followed by insulin (I) (200 nM) for 6 h (A, B) or 2 h (C). Whole-cell lysates were prepared and analysed by Western blotting (A, B) using antibody to HO-1, with β-actin being indicated as loading control. mRNA extracts were prepared (C) using TRIzol and reverse transcribed to cDNA. Fluorescence-detected real time PCR was performed using HO-1 primers and probe with an 18s primer/probe control; results are expressed as mean relative expression ± S.E.M. of 3 independent experiments.

Insulin increases HO-1 expression in mouse primary renal tubular epithelial cells

In order to ensure insulin-induced HO-1 expression was not a characteristic of transformed cells alone, mouse primary renal tubular epithelial cell cultures were prepared. These were treated in a similar manner with insulin (200 nM) for increasing periods of time (Figure 5.4). A robust induction of HO-1 protein was observed.

Insulin-mediated induction of HO-1 is PI3K dependent

In this model, phosphorylation GSK3β at Ser-9 was used as an indicator of PI3K/Akt axis activity. GSK3β phosphorylation was observed after 30 min of insulin at a concentration of 200 nM (Figure 5.5A). Following 30 min pre-treatment with the PI3K inhibitor LY294002 (Figure 5.5B), or its inactive analogue LY303511 (Figure 5.5C), ACHN cells were treated with insulin (200 nM) for 6 h to determine HO-1 protein accumulation and for 30 min to confirm GSK3β phosphorylation status. HO-1 was induced as expected following insulin, but this effect was abolished with increasing concentrations of LY294002. Following treatment with LY303511, HO-1 induction was not altered. LY294002-mediated reduction in GSK3β phosphorylation correlated with inhibition of insulin-induced HO-1 accumulation.

Akt activity is necessary and sufficient for HO-1 induction

48 h after transfection of ACHN cells with the pHOGL3/11.6 reporter construct and a constitutively-active Akt expressing construct (Akt-myr), an increase in luciferase activity was observed, representing a 6-fold increase in HO-1 promoter activity ($p<0.05$ ANOVA) (Figure 5.6A). Accumulation of HO-1 protein was also found

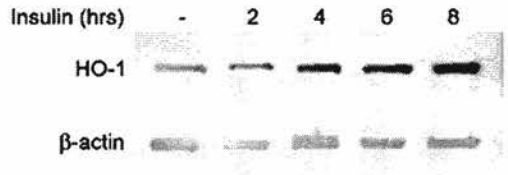


Figure 5.4 **Insulin stimulates HO-1 in mouse primary epithelial cells.**
Mouse primary renal tubular epithelial cells were prepared and treated with increasing concentrations of insulin. Whole-cell lysates were prepared and analysed by Western blotting using antibody to HO-1. β-actin is indicated as a loading control.

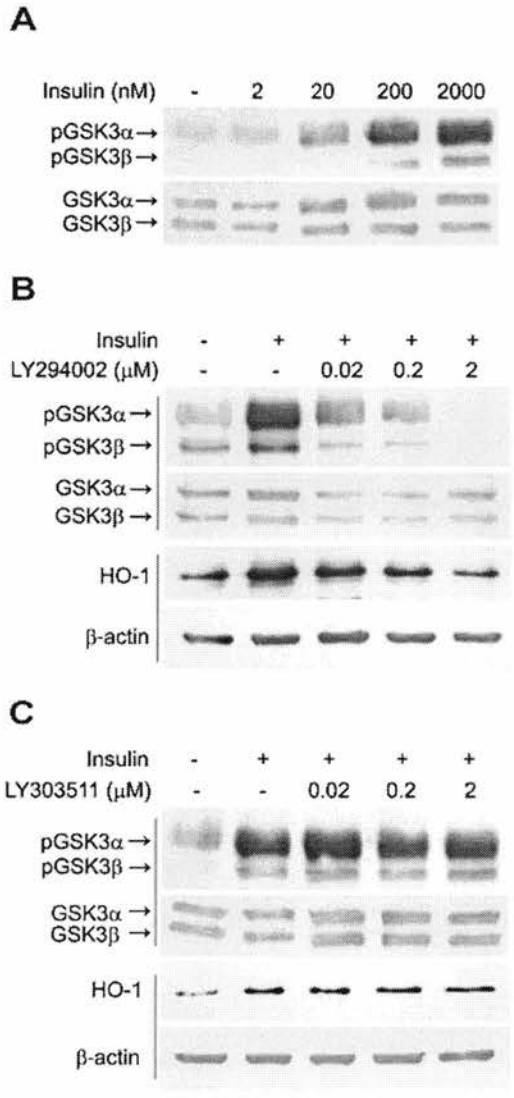


Figure 5.5 **Insulin stimulates HO-1 accumulation through a PI3K-dependent pathway.**
ACHN cells were serum-deprived for 16 h and treated with increasing concentrations of insulin (200 nM) for 30 min (A). Other groups were pre-treated with the PI3K inhibitor LY294002 (B), or its inactive analogue LY303511 (C) for 30 min, followed by insulin (200 nM) for 30 min to determine GSK3β phosphorylation status, and 6 h to determine HO-1 accumulation. Whole-cell lysates were prepared and analysed by Western blotting using phospho-specific antibody to GSK3α/β (ser 21/9) (pGSK3α/β) and antibody to total GSK3 as a loading control. As previously, antibody to HO-1 was used, with β-actin being indicated as loading control.

following transfection with either the Akt-myr or wild-type (Akt-WT) construct, in association with an expected increase in GSK3β phosphorylation (Figure 5.6B). Treating cells transfected with Akt-myr with insulin did not increase the HO-1 promoter activity (Figure 5.6A) over cells transfected alone, demonstrating that the effects of insulin and Akt over-expression on HO-1 accumulation are not additive. In cells transfected with a dominant-negative Akt expressing construct (Akt-K179M), and treated 48 h later with insulin, HO-1 promoter activity was found to increase slightly but this was not statistically significant (Figure 5.6A).

Insulin-mediated HO-1 accumulation is neither p38-MAPK nor ERK dependent

Insulin was found to phosphorylate p38-MAPK (Figure 5.7A) and ERK (Figure 5.7B) in a time-dependent manner. ACHN cells were then pre-treated with the p38-MAPK inhibitor, SB202190, or the MEK1 inhibitor, PD98059, and treated with insulin. Adequate inhibition of p38-MAPK was demonstrated by probing for phosphorylated Hsp27, a known downstream target of p38-MAPK (282) (Figure 5.7C). MEK1 inhibition was confirmed with blots for phosphorylated ERK1/2 (Figure 5.7D). In cells pre-treated with SB202190 or PD98059 and exposed to insulin, no decrease in the expected HO-1 accumulation was observed (Figure 5.7C and D), suggesting neither p38-MAPK nor ERK activity is required for insulin-induced HO-1 accumulation.

Insulin treatment fails to activate HIF1.

HO-1 is known to be regulated by hypoxia-inducible factor 1 (HIF1). To test whether insulin activated HIF1, ACHN cells were transfected with a pHRE-luc reporter

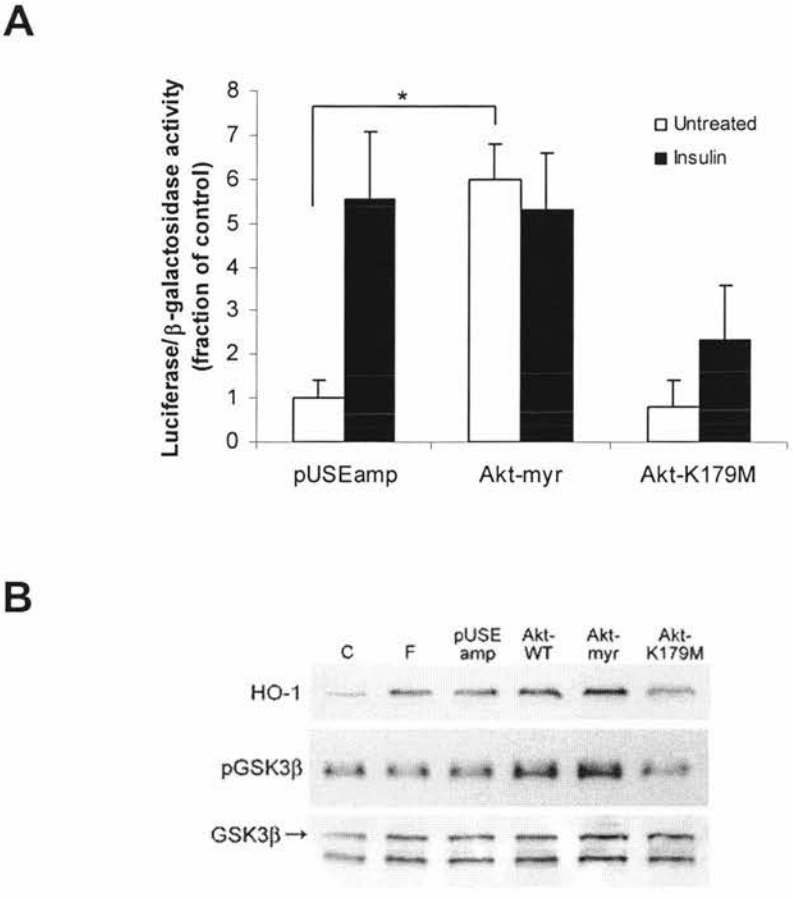


Figure 5.6 **Over-expressing active Akt causes HO-1 reporter activation.** (A) ACHN cells were triple-transfected with the pHOGL3/11.6 reporter construct, the pSV-β-galactosidase control construct and vectors expressing membrane-targeted active Akt (Akt-myr), dominant-negative Akt (Akt-K179M) or empty vector control (pUSE-amp). 48 h later cells were treated with insulin (200 nM) for 6 h and then lysed and luciferase and β-galactosidase assays performed. Results are expressed as luciferase activity per unit β-galactosidase activity ± S.E.M. of 4 independent experiments. * $p<0.05$ ANOVA. (B) ACHN cells were transfected with constructs expressing wild-type Akt (Akt-WT), membrane-targeted active Akt (Akt-myr), dominant-negative Akt (Akt-K179M) or empty vector control (pUSE-amp). 48 h later whole-cell lysates were produced and analysed by Western blotting using antibody to HO-1, phospho-specific antibody to GSK3β (ser-9) (pGSK3β) and antibody to total GSK3 as loading control. C, control; F, transfection agent alone.

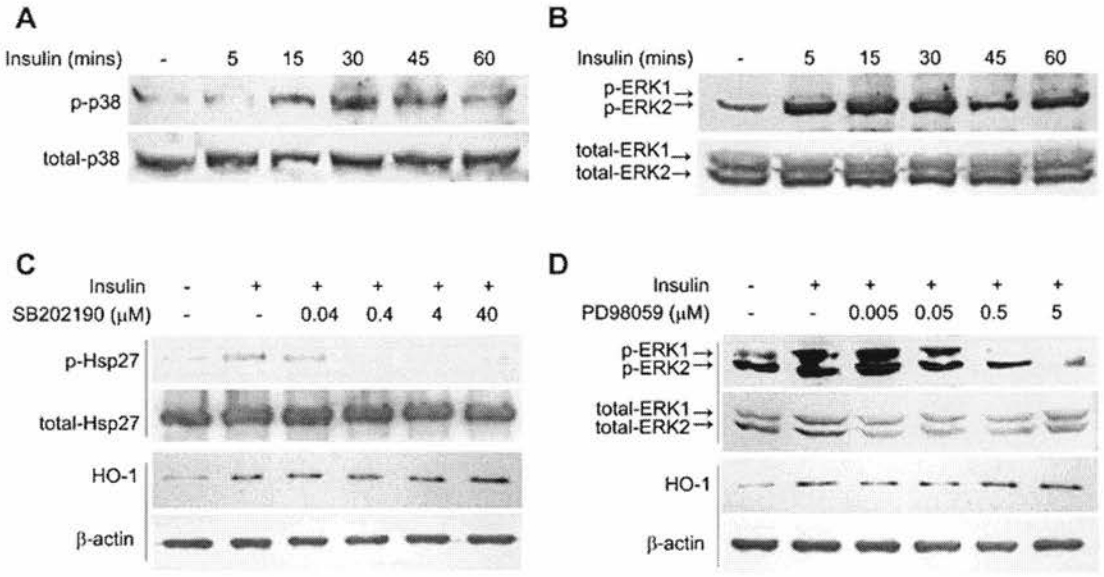


Figure 5.7 **p38-MAPK and ERK inhibition has no effect on insulin-induced HO-1 accumulation.**
(A, B) Cells were serum-deprived for 16 h and treated with insulin (200 nM) for various times. Whole-cell lysates were prepared and analysed by Western blotting using antibody to the phosphorylated form of p38 MAPK (Thr180/Tyr182) (p-p38) (A) and phosphorylated ERK1/2 MAPK (Thr202/Tyr204) (p-ERK1/2) (B) . Total-p38 (A) and total-ERK1/2 (B) are indicated as loading controls. (C, D) Cells were serum-deprived for 16 h and pre-treated with the p38-MAPK inhibitor, SB202190 (C), or the MEK1 inhibitor, PD98059 (D) for 30 min, after which insulin (200 nM) was added for 6 h. Whole-cell lysates were prepared and analysed by Western blotting using antibody to HO-1 and β-actin to control for protein loading. Adequacy of p38-MAPK inhibition was established with blots for phosphorylated Hsp27 (C). MEK1 inhibition was confirmed with blots for phosphorylated ERK1/2 (D).

construct (Figure 5.8). 48 h later cells were treated with increasing concentrations of insulin for 4 h, or treated with cobalt chloride (100 μM) for 4 h. No activation of pHRE-luc reporter was demonstrated.

Nrf2 translocates to the nucleus following insulin treatment

In ACHN cells treated with increasing concentrations of insulin for 1.5 h the nuclear fraction of Nrf2 was found to increase as the cytosolic component decreased (Figure 5.9A). Immunofluroescent labelling of Nrf2 revealed increased nuclear staining following insulin treatment (Figure 5.9B). Pre-treatment with LY294002 abolished nuclear accumulation of Nrf2 in response to insulin at doses previously shown to inhibit PI3K activity (Figure 5.9C); the inactive analogue, LY303511, had no effect on insulin-mediated Nrf2 nuclear accumulation (Figure 5.9D).

Insulin mediated HO-1 induction abolished by Nrf2 siRNA

ACHN cells were transfected with Nrf2 siRNA as per the manufacturer's instructions. 48 h later they were treated with insulin or the proteosome inhibitor MG132 (used as a positive control for Nrf2 accumulation) for 6 h. Cobalt chloride ($CoCl_2$) is a hypoxia-mimetic that activates the HO-1 promoter and was also used as a control. Groups treated with the Nrf2 siRNA demonstrated greatly reduced Nrf2 and HO-1 protein expression when compared with control siRNA treated groups (Figure 5.10). In Nrf2 siRNA groups treated with insulin, no HO-1 induction was observed; however, in Nrf2 siRNA groups treated with $CoCl_2$, HO-1 induction did occur, demonstrating Nrf2 activity is not a prerequisite for promoter activation. While nuclear localisation of Nrf2 following insulin was apparent, it was not clear whether insulin treatment resulted in increased total Nrf2. There was a suggestion on

Western blotting of whole-cell lysates that total cellular Nrf2 was increased following insulin, but on quantification of 3 independent blots no difference was demonstrated.

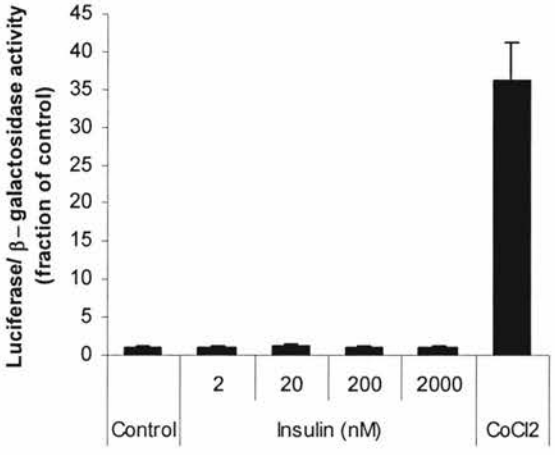


Figure 5.8 **Insulin treatment fails to activate HIF1.**
ACHN cells were transfected with a pHRE-luc reporter construct and the pSV-β-gal control vector. 48 h later cells were treated with increasing concentrations of insulin for 4 h, or treated with cobalt chloride (100 μM) for 4 h. Cells were then lysed and luciferase and β-galactosidase assays performed. Results are expressed as luciferase activity per unit β-galactosidase activity ± S.E.M. of 3 independent experiments.

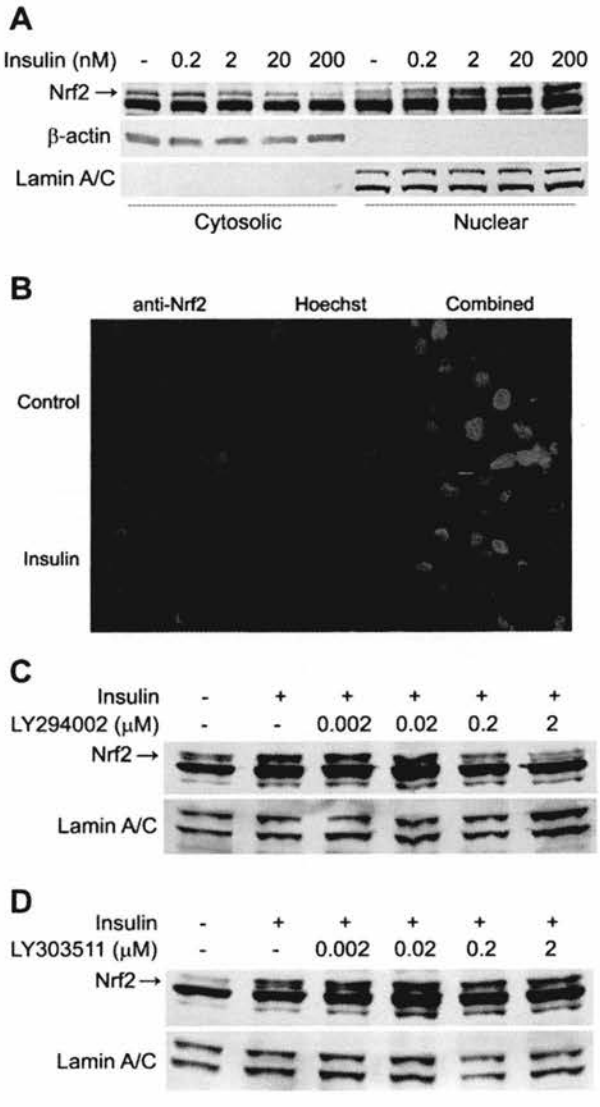


Figure 5.9 **Insulin treatment causes PI3K-sensitive nuclear migration of Nrf2.** (A) Cells were serum-deprived for 16 h and treated with increasing concentrations of insulin for 1.5 h. Nuclear and cytosolic lysates were prepared and analysed by Western blotting using antibody to Nrf2, with loading control with β-actin for cytosolic extracts and lamin A/C for nuclear extracts. (B) Cells were treated similarly with insulin (200 nM) for 1.5 h, prepared for immunofluorescence and treated with antibody to Nrf2, followed by Hoechst counterstaining. (C, D) Cells were serum-deprived for 16 h and pre-treated with the PI3K inhibitor LY294002 (C), or its inactive analogue LY303511 (D) for 30 min. Insulin (200 nM) was added for 1.5 h after which nuclear lysates were prepared and analysed by Western blotting using antibody to Nrf2, with lamin A/C used as a loading control.

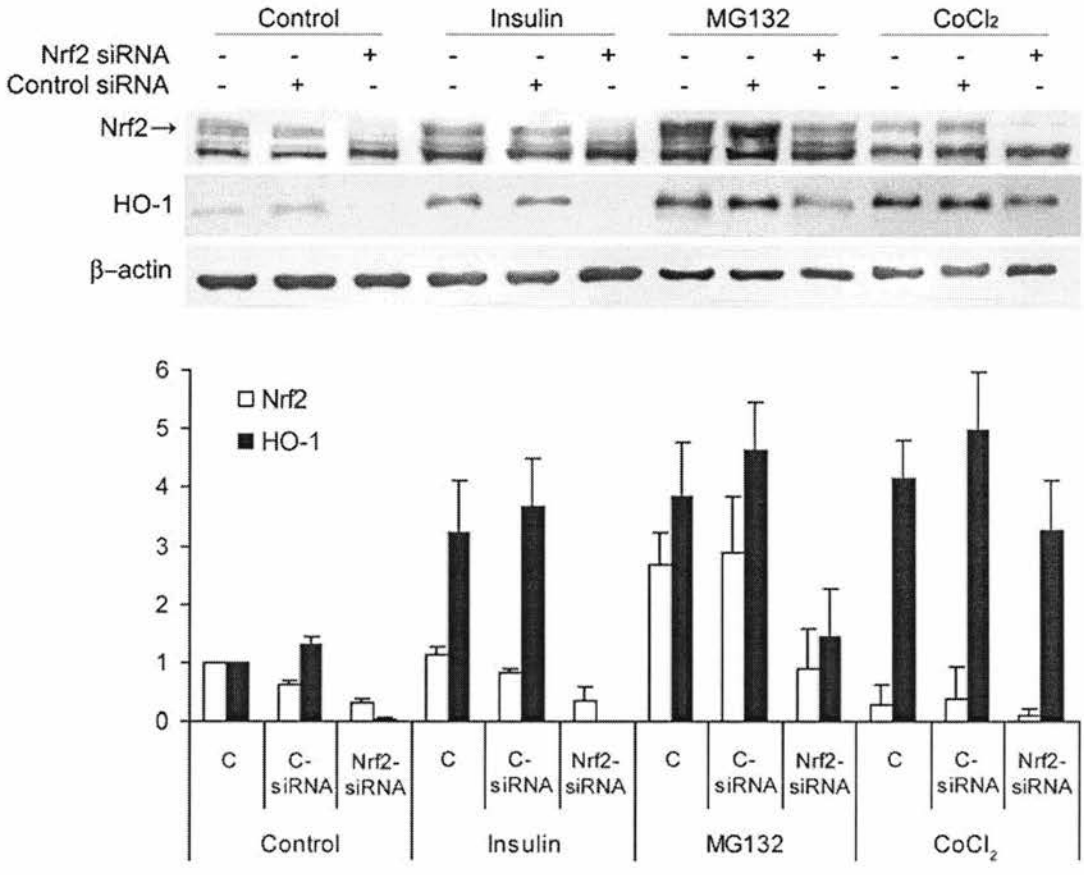


Figure 5.10 **Nrf2 silencing with siRNA prevents insulin-induced HO-1 accumulation.**
Cells were transfected with Nrf2 siRNA and 48 h later treated with insulin (200 nM), the proteosome inhibitor MG132 (20 μM) or cobalt chloride ($CoCl_2$) (1 mM) for 6 h. Whole-cell lysates were prepared for Western blotting using antibody to HO-1 and Nrf2 with β-actin used as a loading control. Optical densities of bands were quantified (Quantity One, Biorad). Bars represent the mean of 3 independent experiments with error bars representing S.E.M.

5.5 Discussion

In this chapter, direct evidence has been provided for a link between insulin activation of the PI3K/Akt cascade, Nrf2 transactivation and HO-1 induction. Insulin-induced HO-1 protein expression was sensitive to PI3K/Akt inhibition and Nrf2 gene silencing. The fold-increase in both HO-1 protein and mRNA in response to insulin was consistent, as well as being time and concentration dependent. The HO-1 response to insulin was also shown in mouse primary renal tubular epithelial cultures, demonstrating that the response is not limited to transformed cells alone.

The role of the PI3K/Akt pathway in the regulation of HO-1 has been the source of much interest lately. Data has been shown here demonstrating that insulin-induced HO-1 accumulation is sensitive to PI3K inhibition with LY294002. This is in keeping with results from other work demonstrating the importance of PI3K/Akt activation in HO-1 regulation following cell stimulation with nerve growth factor (NGF) (267), carnosol (268), hemin (283) and cadmium (284). Over-expression of active Akt alone was sufficient to mimic the effects of insulin on HO-1 expression in the model used here, adding weight to the suggestion that the effect of insulin on HO-1 is mediated predominantly or possibly exclusively, by the PI3K/Akt axis. Over-expressing membrane-targeted active Akt stimulated the HO-1 promoter, but significantly, adding insulin did not increase this activation. In contrast, Salinas *et al* report that although the basal level of HO-1 mRNA, measured by semi-quantitative reverse-transcriptase PCR, was higher in cells transfected with a membrane-targeted active Akt expressing construct, administration of NGF further increased this expression. This may indicate that NGF exhibits its effect through additional

mechanisms in comparison with insulin, though differences may be due to cell-type or transfection technique.

The exact role of the MAPK cascades in HO-1 regulation remains controversial. Inhibition of p38-MAPK reduces HO-1 expression following carnosol (268), diallyl sulfide (285) and cadmium (284), although an earlier report found that p38 inhibition had no effect on HO-1 mRNA expression following cadmium, arsenate or hemin (286). Data provided, however, shows that despite concentrations of insulin being sufficient to phosphorylate p38, inhibition of p38 did not alter insulin-induced HO-1 protein expression. Similarly, ERK inhibition did not impact on HO-1 expression following carnosol (268) or arsenite (287); however, ERK activity was required for HO-1 induction in HepG2 cells treated with diallyl sulfide (285) and LMH cells exposed to arsenite (288). It remains unclear why these disparities exist but it appears that p38 and ERK play a significant role in HO-1 regulation in some models, but not in others.

During this investigation, a number of different transcription factors were studied that may be involved in mediating the effect of insulin on HO-1 expression including hypoxia-inducible factor-1 (HIF1) and NF-E2-related factor 2 (Nrf2). The basic helix-loop-helix transcription factor HIF1 mediates essential homeostatic responses to reduced oxygen (289,290). HIF1 has been shown to mediate transcriptional activation of HO-1 in a rat model of hypoxia (291), rat renal medullary cells (292) and Chinese hamster ovary cells (293). In addition, an associative increase in HIF1 DNA-binding and HO-1 induction in a rat model of liver IRI (162). The relationship

between HIF1 and HO-1 induction in humans is less clear. Hypoxia has been shown to repress HO-1 mRNA expression in primary cultures of human umbilical vein endothelial cells, despite HIF1 transactivation. This reflects our observation that ACHN cells subjected to hypoxia demonstrate a decrease in HO-1 protein expression (data not shown). Controversial evidence exists linking PI3K activity with regulation of HIF1, both in hypoxic (294,295) and normoxic (296-300) conditions, although this appears to be cell-type specific (301,302). Insulin has been shown to up-regulate HIF1 directly through the PI3K/Akt pathway (300). However, despite all this, in our model HIF1 transactivation not seen following insulin treatment, as determined by a HIF1 luciferase reporter construct.

Nrf2 has been shown to regulate HO-1 (269) and is known to be under the influence of PI3K (268,283,303-306). Consistent with results presented here, insulin has previously been shown to cause nuclear localisation of Nrf2, although PI3K dependency was not investigated in that study (305). However, hemin has been shown to induce Nrf2 nuclear localisation in a PI3K sensitive manner (283). Using Nrf2 small-interfering RNA, dependence of basal HO-1 expression on Nrf2 activity has been shown: Nrf2 gene silencing practically abolished HO-1 expression. No HO-1 response was seen following insulin in Nrf2 siRNA treated cells, suggesting insulin-induced HO-1 expression has an absolute dependence on Nrf2 activity.

In this chapter, the ability of insulin to induce HO-1 in a PI3K/Akt and Nrf2 dependent manner has been demonstrated. Establishing mechanisms of HO-1

induction which can be implemented clinically is important for future organ protection strategies in transplantation.

Chapter 6 – Hsp90 inhibition as a means of stimulating the stress protein response in renal cells

6.1 Introduction

6.1.1 Background

In chapter 4, the regulation of HSF1 by GSK3β-mediated phosphorylation was examined. No influence on the activation state of HSF1 could be demonstrated by this mechanism in this cell model. One explanation for this could be the dominance of other regulatory systems and in particular the influence of the multi-chaperone complex in the repression of HSF1. In chapters 6 – 8, the role of this complex in HSF1 regulation will be examined, focusing on the contribution of heat shock protein 90 (Hsp90). In this introduction, the current evidence for regulation of HSF1 by the multi-chaperone complex is presented and Hsp90 is shown to be a component of intermediate and mature HSF1-protein complexes. The hypothesis presented contends that Hsp90 is the most important component of the HSF1 repressing multi-chaperone complex and that inhibition of Hsp90 results in HSF1 activation and stimulation of the stress protein response.

6.1.2 The multi-chaperone complex

Our current understanding of HSF1 regulation by the multi-chaperone complex stems from two distinct lines of enquiry:

1. Investigations based on the premise that classical stressors almost always give rise to increased quantities of unfolded proteins.
2. Work on steroid receptor regulation.

Unfolded proteins

As discussed in section 1.6 the diverse array of insults that stimulate the stress protein response share a common property: they adversely affect the proper conformation of proteins. A reasonable suggestion for the regulation of the stress protein response , therefore, is that unfolded proteins act as a collective proximal stimulus initiating the response; this had been proposed as far back as 1980 (307). The ability of unfolded proteins to stimulate the stress response was proven in an experiment where *Xenopus* oocytes were injected with an *hsp70* reporter construct and either denatured or native proteins – only the denatured proteins activated the *hsp70* promoter (308). Thus, while it was clear that unfolded proteins could stimulate the stress protein response, the mechanism by which cells 'sensed' the presence of these proteins remained unclear.

It had long been appreciated that a major function of stress proteins themselves, particularly Hsp70 and Hsp90, was to facilitate the correct folding of other proteins (see chapter 1 and review (151)). It was hypothesised that regulation of the stress

response may involve the interaction of a stress protein with HSF1 and unfolded proteins (Figure 6.1). If the stress protein acted to repress the activation state of HSF1 and was competitively bound by unfolded proteins, a regulatory mechanism would exist whereby HSF1 activation was sensitive to the cellular levels of unfolded proteins. Thus, stress protein expression would reflect the quantity of unfolded proteins present and, by inference, the magnitude of stress inflicted on the cell. Progress in the study of this hypothesis, however, was greatly hampered by the unstable nature of the protein-protein interactions required for such a mechanism to exist.

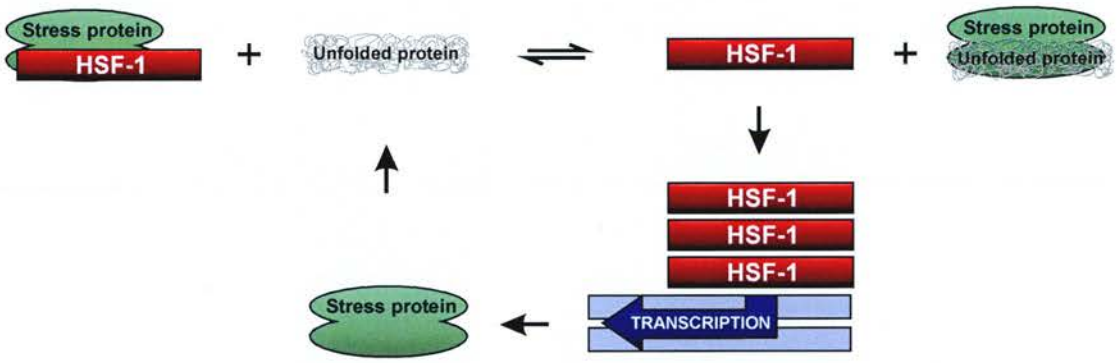


Figure 6.1 **Negative regulation of HSF1 by a stress protein: hypothesis circa 1980.**
The generation of unfolded proteins following metabolic insult was thought sufficient to stimulate the stress protein response. An attractive hypothesis for the regulation of such a response placed HSF1 in association with a repressive stress protein. Unfolded proteins would compete with HSF1 for stress protein binding, thus de-repressing HSF1 with resulting transactivation. An important component was the negative feedback loop: HSF1-mediated induction of the stress protein would only continue until sufficient stress protein had been synthesised or unfolded protein reduced to restore the equilibrium.

Steroid hormone receptors

The steroid hormone receptors (SHR) are a family of proteins that reside in the cytoplasm or nucleus of a cell and act as signal transducers for steroid hormone. Ligand binding of steroid hormone to the SHR results in dimerisation, nuclear translocation and transactivation of steroid-dependent genes (309). In the mid-80s, unstimulated SHR was found to take part in large multi-protein complexes that acted to repress transactivation and were later shown to be a prerequisite for ligand binding (310). Immunoprecipitation studies identified a number of component proteins including Hsp90 (311,312). Multi-chaperone complexes based on Hsp90 have now been shown to be essential for the correct function of a number of different proteins and signalling cascades (313). In 1993, while studying its ATPase activity, Nadeau *et al* demonstrated that Hsp90 could bind HSF *in vitro*.

The importance of Hsp90 in the HSF1 multi-chaperone complex

The year before Nadeau made the link between HSF and Hsp90, similar work had been published suggesting Hsp70 could associate with HSF (314). A number of studies pointed to Hsp70 as the hypothetical protein most likely to bind and repress HSF1, yet the evidence remained unconvincing (315-318). Although a further study demonstrated *in vitro* binding of HSF1 with Hsp90* (319), it was a number of years before Hsp90-HSF1 binding was finally demonstrated *in vivo* (320) (Table 6.1). Ali *et al* showed that Hsp90-HSF1 heterocomplexes existed in both unshocked and heat-shocked *Xenopus* oocyte nuclei and furthermore, that Hsp90 antibodies activated HSF1 DNA-binding (in the absence of heat shock) and that this effect was reversed

* Nair *et al* also demonstrated the ability of HSF to bind *in vitro* with a number of other proteins including Hsp70, p60, Fkbp51, Fkbp52, CyP40 and p23 (319)

by the subsequent injection of recombinant Hsp90 protein. For the first time in the 20 years since the hypothesis had been proposed, convincing evidence existed that HSF1 was regulated by a stress protein, Hsp90.

It was established from the work on steroid hormone receptors that other proteins were required in the formation of the complex as well as Hsp90. p23 stabilised the interaction between the mature Hsp90 complex and its substrate. Members of the immunophilin[†] family were also identified to form a necessary part of the complex, although the specifics of this remain unclear. Other proteins were also required in the formation of intermediate complexes including Hsp70 and the Hsp70/90 binding protein, Hop. At around the same time as Ali *et al* published the *in vivo* work on Hsp90-HSF1 interaction (320), Zou *et al* successfully demonstrated the importance of Hsp90 in repressing HSF1 activation in a novel *in vitro* model based on HeLa cell lystate (321). While immunodepletion of Hsp90 activated HSF1, immunodepletion of other proteins suspected of being involved (Hsp70, Hop, Hip, p23, Cyp40 or Hsp40) had no effect. In follow-up to the Ali *et al* paper, Bharadwaj *et al* (from the same group) showed that Hsp90 was not alone in associating with HSF1. In the *Xenopus* oocyte model they demonstrated, in a similar manner to SHR complexes, that p23 was also present in the HSF1-Hsp90 complex in stimulated and unstimulated cells (322). The immunophilin, Fkbp52, was also shown to be associated with Hsp90 only in stimulated cells. Accelerated recovery following heat shock was also seen following injection of purified Hsp90/70 and Hip/Hop. A

[†] Immunophilins: receptors for immunosuppressive drugs including cyclosporin A, FK506, and rapamycin. Cyclosporin A receptors are referred to as cyclophilins (CyP). FK506- and rapamycin-binding proteins are abbreviated to Fkbp.

Author	Year	System	HSF1 function (with Hsp90 bound)	Other proteins implicated	Ref
Nadeau	1993	*In vitro*	-	-	(323)
Nair	1996	*In vitro*	-	p23, Hsp70, various IPs*	(319)
Zou	1998	*In vitro/vivo*	(-) transactivation	-	(321)
Ali	1998	*In vivo*	(-) DNA-binding	-	(320)
Bharadwaj	1999	*In vivo*	(-) transactivation	p23, (Fkbp52)†	(322)
Guo	2001	*In vivo*	(-) transactivation	p23, (Fkbp52)†	(324)

Table 6.1 **Papers reporting HSF1-Hsp90 complex formation, functional implications and associated proteins.**
In 1993 the first description of HSF-Hsp90 interaction *in vitro* was published. It was not until 1998 that this observation was confirmed *in vivo*. A number of other proteins have been implicated in the multi-chaperone complex. * IPs, immunophilins: receptors for immunosuppressive drugs including cyclosporin A, FK506, and rapamycin. Cyclosporin A receptors are referred to as cyclophilins (CyP) and FK506- and rapamycin-binding proteins are abbreviated to Fkbp. Fkbp51, Fkbp52 and Cyp40 were all shown to interact with HSF1 *in vitro*. † Fkbp52 was shown by Bharadwaj and Guo to bind to HSF1 but only after heat shock. This association was not demonstrated in unstimulated cells.

delay in the recovery of HSF1 from heat shock was shown when antibodies to Hip/Hop and Fkbp51 (as well as Hsp90, p23 and Fkbp52) were injected, despite the fact neither Hip/Hop nor Fkbp51 could be co-immunoprecipitated with HSF1. Lastly, Guo *et al* (from the same group as Zou *et al*) showed that Hsp90 binds HSF1 in unstimulated cells, but this was vastly reduced following heat shock. Fkbp52 and p23 were shown to bind HSF1 in stimulated, but not unshocked cells.

What conclusions can be drawn from this body of work? The finding of Zou showing that p23 did not associate with Hsp90-HSF1 is likely to be erroneous and explained by differences in the model (and the artificial nature of the *in vitro* activation model). Work from the same group (Guo *et al*) confirmed associations between Hsp90-HSF1 and p23 and this is likely to be the case. It is clear that p23 and Hsp90 associate with HSF1 in both unstimulated *and* stimulated cells. Yet, the hypothesis relies on the fact that the repressive Hsp90 complex dissociates from HSF1 on stimulation of the cell resulting in activation of HSF1. The concept emerging is that complex inhibition is likely to exist at the level of the monomer *and* the trimer. This assertion is based on evidence that Hsp90-p23 interaction with HSF1 in the trimeric form has been identified. Also the immunophilin, Fkbp52, has been shown to be associated with the Hsp90-HSF1 heterocomplex only in stimulated (e.g. heat-shocked) cells, but not in unstimulated cells. It is likely, therefore, that this other complex acts to repress the trimeric form of HSF1 independently of monomeric repression. Figures 6.2 and 6.3 put this evidence together as a putative regulatory mechanism of HSF1 by the multi chaperone complex.

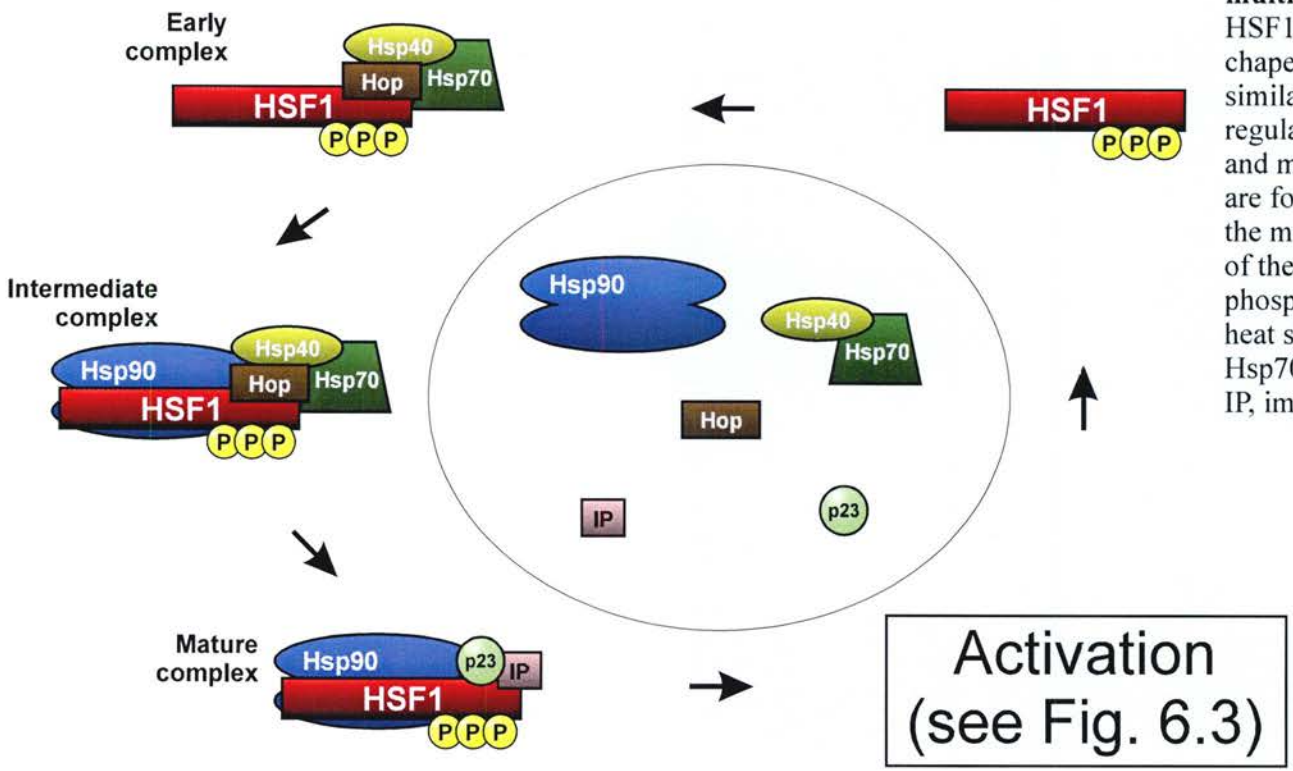


Fig. 6.2. **Formation of HSF1 multi-chaperone complex**. HSF1 regulation by a multi-chaperone complex is very similar to steroid receptor regulation. Early, intermediate and mature protein complexes are formed, with Hsp90 being the most important constituent of the mature complex. P, phosphorylatable serine; Hsp, heat shock protein; Hop, Hsp70/Hsp90 binding protein; IP, immunophilin.

Formation of HSF1 multi-chaperone complex: hypothesis

The mechanism of formation of the HSF1 multi-chaperone complex is still hypothetical and much is based on the current understanding of steroid receptor biology. Hsp70, Hsp40 and Hop form an early complex, Hop facilitating the binding of first Hsp70, then Hsp90 to form an intermediate complex. Hsp70, Hsp40 and Hop are substituted for p23 and (presumably) an immunophilin (313). Exposure to stress increases the molar-ratio of unfolded proteins to chaperones with the unfolded proteins associating with Hsp90. With fewer chaperones available for HSF1 binding, HSF1 trimerises and is transactivated resulting in transcription of heat shock proteins. With an increase in the availability of Hsps, HSF1 becomes complexed again, a negative feedback loop thereby being formed[‡].

[‡] HSF multi-chaperone complex: there is evidence that a multi-chaperone complex involving Hsp90, Fkbp52 and p23 acts to repress trimerised HSF1, i.e. two levels of regulation exist, one at the level of the monomer, the other at the trimer (322,324). For the purposes of this study, it does not matter whether Hsp90 is exerting greater influence on repression of monomeric or trimeric HSF1, the outcome is the same.

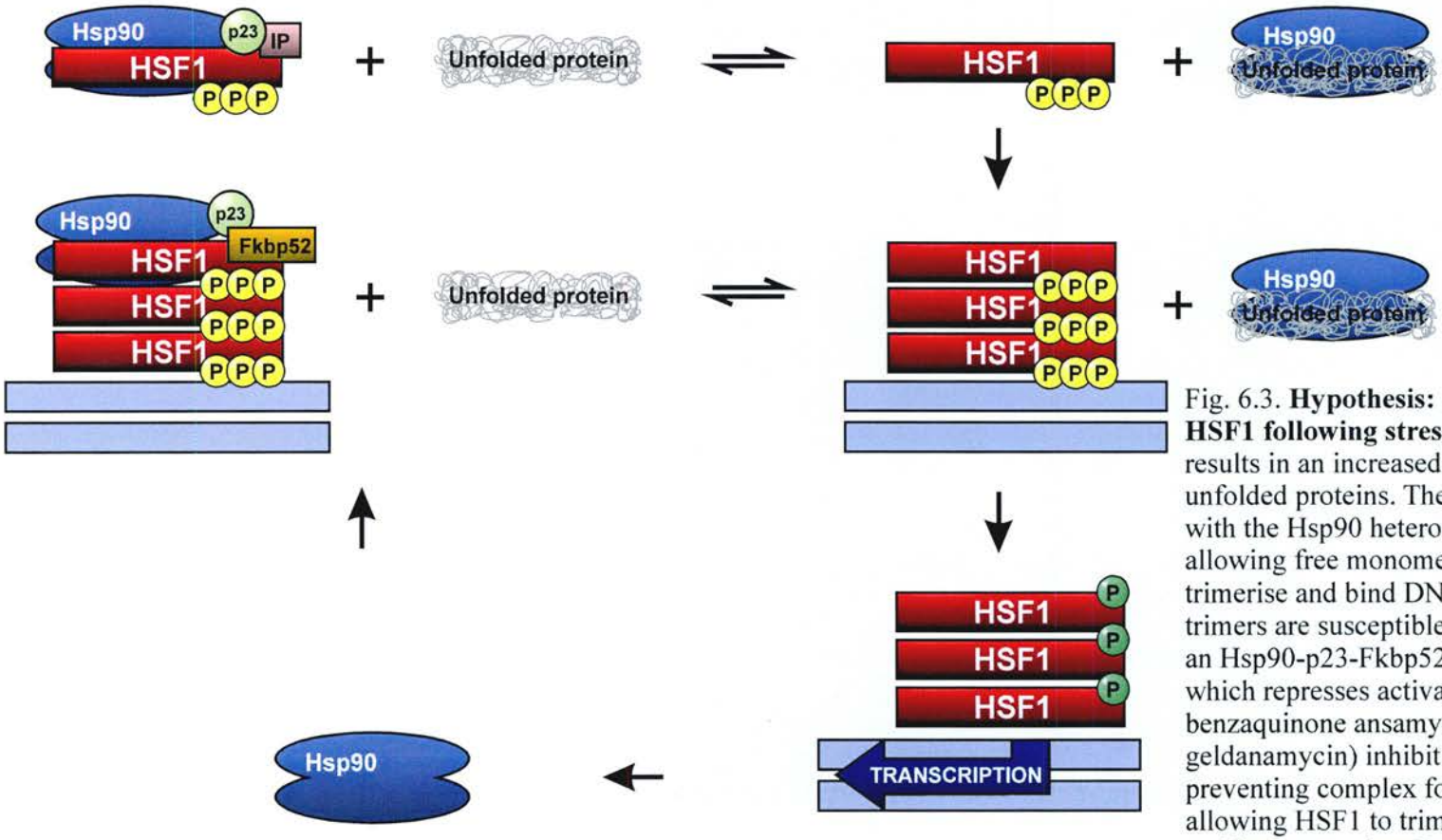


Fig. 6.3. **Hypothesis: activation of HSF1 following stress**. Cellular stress results in an increased concentration of unfolded proteins. These compete with the Hsp90 heterocomplex allowing free monomeric HSF1 to trimerise and bind DNA. On formation trimers are susceptible to capture by an Hsp90-p23-Fkbp52 complex, which represses activation. The benzaquinone ansamycins (including geldanamycin) inhibit Hsp90 preventing complex formation, thus allowing HSF1 to trimerise, bind DNA and become active. P, phosphorylatable serine; Hsp, heat shock protein; IP, immunophilin; Fkbp52, Fk506 binding protein;

6.1.3 Hsp90-binding agents (HBAs)

The identification of geldanamycin (GA) marked the first description of a group of drugs termed the benzoquinone ansamycins. It was isolated from *streptomyces hygroscopicus* in the early 1970s and was noted for its antiprotozoal activity (325,326). Herbimycin A was subsequently identified and found to have herbicidal properties (327,328).

Anti-neoplastic properties

The anti-tumour potential of the benzoquinone ansamycins was suggested in the late 1970s (329-332), but it was not until the mid-1980s that first herbimycin (333) and then GA (334) were shown to inhibit the malignant transformation of fibroblasts by the v-*Src* oncogene. Src kinase belongs to a family of non-receptor tyrosine kinases and the benzoquinone ansamycin effect was believed to be due to tyrosine kinase inhibition. It was, however, shown that Src formed a stable complex with Hsp90 and the benzoquinone ansamycins were specific Hsp90 inhibitors (335). Hsp90 inhibition prevented complex formation resulting in Src degradation by the ubiquitin-proteosome pathway. The benozoquinone ansamycins work by binding to the ATP-binding site of Hsp90, thus preventing its intrinsic ATPase activity (336) and have been shown to be very specific (335,337). Src is only one of a number of oncogenic proteins that require functioning Hsp90 to exert their effects. Hsp90 inhibition, therefore, is now the subject of trials assessing anti-neoplastic potential.

Cytoprotective properties

A number of studies have demonstrated cellular protection associated with HBA treatment, but none of these have been in a model relevant to transplantation (Table 6.2). Two studies have shown protection against IRI following HBA treatment (338,339). Lu *et al* also showed a GA-mediated reduction in co-immunoprecipitation of HSF1 with Hsp90 in brain tissue homogenates, an increase in HSE-binding of HSF in brain nuclear extracts and an increase in luciferase reporter gene transcription for the Hsp70 promoter in PC12 cells.

Given the beneficial effects of HBA-treatment in these models, it was hypothesised that HBAs could reduce the effects of IRI in renal transplantation. To investigate this further, the effects of GA in an *in vitro* renal cell model were examined.

Drug	Author	Year	Species	Type	Cell/organ	Model	Primary outcome	Ref
Herbimycin A	Hegde	1995	Rat	CL	Fibroblast (REF)	Heat	Absolute cell number	(340)
	Morris	1996	Rat	1°	Cardiomyocyte (RNC)	Heat/SI	Trypan blue exclusion/ LDH release	(341)
	Javadpour	1998	Rat	A	Lung	IR	Oedema/ neutrophil number	(338)
	Dinh	2002	Human	CL	Epithelial cells (REC)	Heat	Cell viability	(342)
	Sachidhanandam	2003	Rat	A	Whole animal	Heat	Hepatocyte apoptosis	(343)
Geldanamycin	Conde	1997	Rat	1°	Cardiomyocyte (RNC)	Heat/SI	CK/LDH release	(344)
	Xiao	1999	Mouse	CL	Hippocampal (HT22)	Glutamate	Apoptosis	(345)
	Pittet	2002	Rat	A	Lung	Haem. shock	Alveolar fluid clearance	(346)
	Lu	2002	Rat	A	Brain	IR	Infarct volume/apoptosis	(339)
	Xu	2003	Mouse	1°	Astrocyte	SI	Apoptosis	(347)
	Kiang	2006	Mouse	A	Jejunum	Haem. shock	Jejunal injury, ATP levels	(348)
Radicicol	Griffin	2004	Rat	1°	RNC	SI	CK release, cell viability (MTT)	(349)

Table 6.2 **Papers reporting cytoprotection associated with Hsp90-binding agent treatment.**
Reports are presented by drug-type and author. CL, immortalised cell-line; 1°, primary cell culture; A, animal; REF, rat embryo fibroblasts; RNC, rat neonatal cardiomyocytes; REC, retinal epithelial cells; IR, ischemia/reperfusion injury; CK, creatine kinase; LDH, lactate dehydrogenase; dep, deprivation; SI, simulated ischemia: typically, depriving cells of glucose and serum while increasing potassium, hydrogen and lactate concentrations with or without hypoxia; haem. shock, haemorrhagic shock.

6.2 Hypotheses

1. Geldanamycin-mediated Hsp90 inhibition induces HSF1 trimerisation and DNA-binding.

2. Geldanamycin-mediated Hsp90 inhibition induces the stress protein response in renal cells.

3. Hsp90 inhibition protects renal cells from oxidative stress.

6.3 Methods and materials

6.3.1 HSF1-GFP vector transfection

ACHN cells were prepared in 6-well plates and a transient transfection was performed 24 h later. A vector expressing green fluorescent protein under the control of an HSF-dependent promoter (HSF-GFP) was a kind gift of Mr Richard Morimoto (Northwestern University, IL, US) (350). The transfection agent fugene (Roche, Lewes, East Sussex, UK) was used at a 6:1 ratio of reagent to DNA. 24 h later transfected cells were treated with GA (2 μM) for 1.5 h. Cells were fixed with methanol, counterstained with Hoechst 33258 (0.2 mM) and visualised with a Leica DM IRB microscope fitted with a Hamamatsu camera. Images were stored using the Leica Improvision software.

6.4 Results

Geldanamycin induces Hsp70 in ACHN cells

ACHN cells were treated with GA (2 μM) for various times (Figure 6.4A). A 2.6 fold increase in Hsp70 was seen at 4 h and a 5 fold increase at 6 h. Hsp70 induction persisted for at least 8 h. No induction of Hsp70 was seen with vehicle (DMSO) alone. A dose response of GA showed a 5 fold induction of Hsp70 with 0.2 μM GA at 8 h (Figure 6.4B).

Geldanamycin-induced Hsp70 expression is transcription and translation dependent

To confirm that Hsp70 accumulation was dependent on gene transcription, ACHN cells were pre-treated with actinomycin D (AD) for 30 min, prior to 6 h treatment with GA (Figure 6.5). Basal levels of Hsp70 were slightly reduced following AD and the response to geldanamycin was abolished. Similarly, cycloheximide (CHX) was administered to establish the role of protein synthesis in Hsp70 expression following exposure GA for 6h. CHX completely abrogated Hsp70 induction following GA treatment.

Geldanamycin is not toxic to cells at concentrations that induce Hsp70

After 24 h of GA treatment cell viability was determined by MTT assay (Figure 6.6). No significant difference was seen between groups treated with increasing concentrations of GA and control groups.

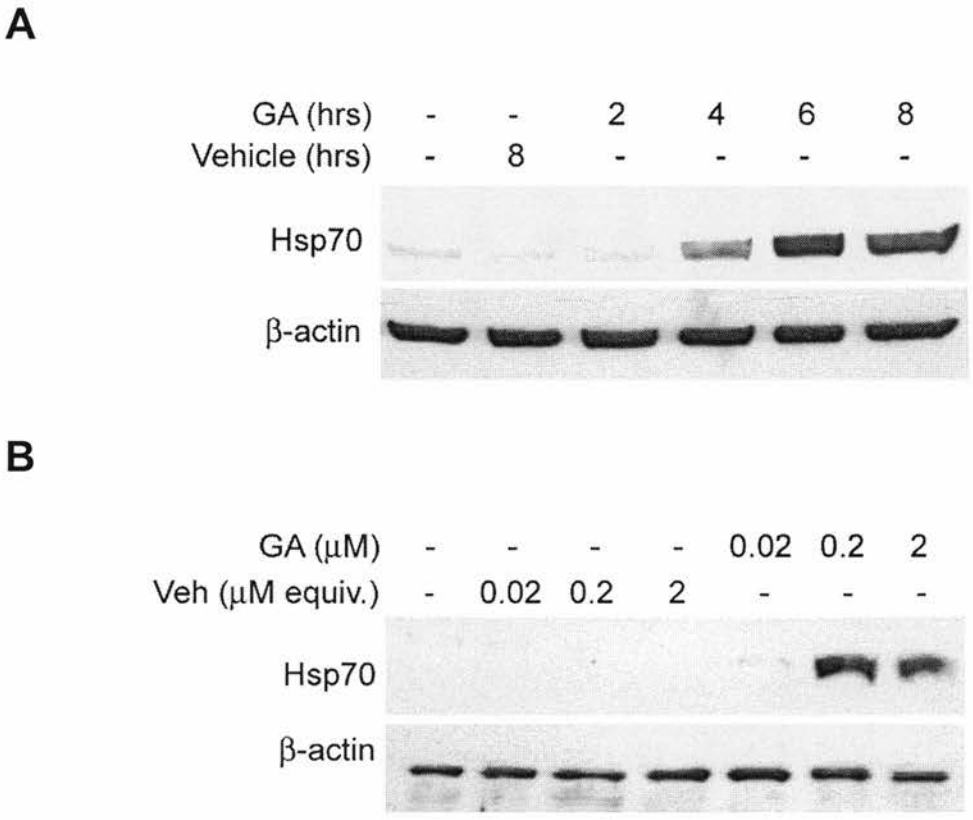


Figure 6.4 **Hsp70 expression in ACHN cells following geldanamycin treatment.** ACHN cells were exposed geldanamycin (GA) (2 μM) or vehicle (Veh) (DMSO) for various times (A) or to various concentrations of GA for 6 h (B). Whole-cell lysates were prepared and analysed by Western blotting using antibody to Hsp70. β-actin was used as a loading control.

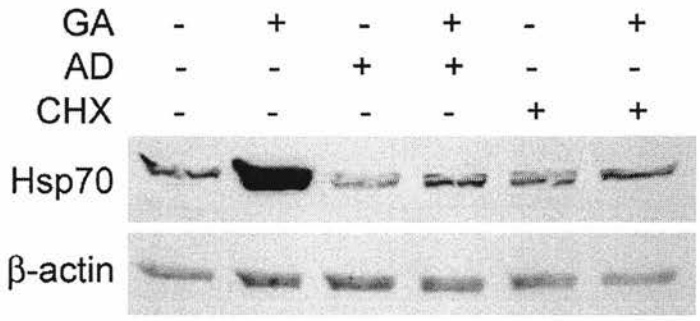


Figure 6.5 **Geldanamycin stimulated Hsp70 accumulation is transcription and translation dependent.**
ACHN cells were pre-treated with actinomycin D (5 μg/ml) (AD) or cycloheximide (10 μg/ml) (CHX) for 30 min, followed by GA (2 μM) for 6 h. Whole-cell lysates were prepared and analysed by Western blotting using antibody to Hsp70, with β-actin being indicated as loading control.

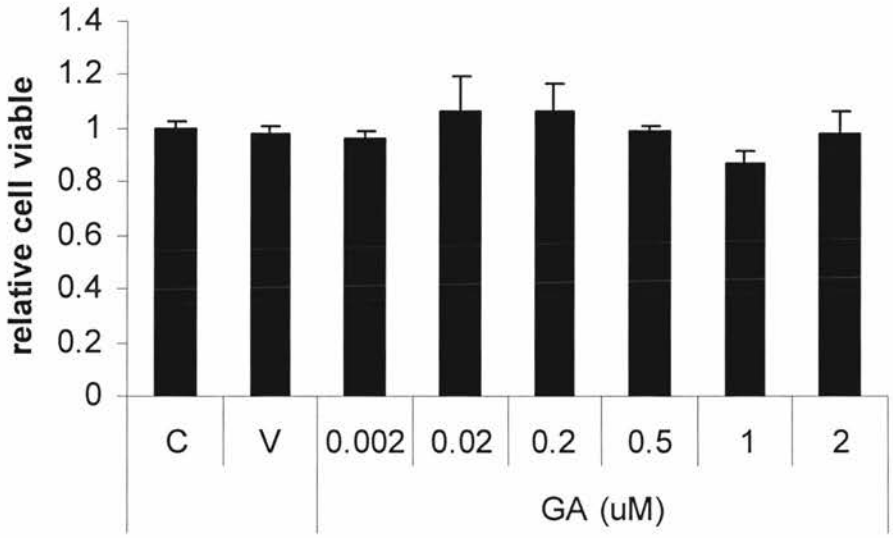


Figure 6.6 **Cell viability following geldanamycin treatment.**
ACHN cells were treated with various concentrations of GA for 24 h. MTT was added for 2 h and the resulting tetrazolium crystals dissolved in SDS. Samples were analysed on a plate reader and results are mean of 3 independent experiments expressed as a proportion of the control; error bars S.E.M.

Geldanamycin induces HSF1 trimerisation and DNA-binding

An increase in trimerised HSF1 was seen in cells treated with GA for 1.5 h which was similar to that observed in heat-treated cells (Figure 6.7). Specificity was confirmed with a band-shift seen when the binding reaction was run in the presence of anti-HSF1 antibody. The HSF1 bands disappeared when the reaction was run in the presence of a 20x molar-excess of unlabelled probe.

Geldanamycin induces HSF1 stress-granule formation

Nuclear localisation of HSF1 following GA treatment was confirmed in cells that had been transfected with an HSF1-GFP expressing construct (Figure 6.8). Nuclear stress-granule formation was seen in cells treated with GA, compared with diffuse cytoplasmic expression of HSF1 in control cells.

Geldanamycin activates an Hsp70 reporter construct

A reporter construct containing the luciferase gene under the control of five repeats of the heat shock elements (HSE; HSF1 binding site) portion of the Hsp70 promoter was co-transfected with the pSV-β-gal control vector into ACHN cells using the fugene transfection reagent (Figure 6.9). 24 h later cells were treated with GA (2 μM) for various times. A 2 fold increase was seen in promoter activity following GA was found after 4 h but did not achieve statistical significance after 3 independent repeats.

Geldanamycin stimulates Hsp70 mRNA accumulation in ACHN cells

ACHN cells were treated with geldanamycin (2 μM) for various times (Figure 6.10). Fluorescence detected real-time PCR was performed using an 18s primer/probe

control on whole-cell extracts. A 30 fold increase in Hsp70 gene expression was seen in cells treated with GA for 4 h.

Geldanamycin protects ACHN cells from oxidative damage

ACHN cells pre-treated with GA for 6 h were found to be protected from H_2O_2 (2 μM) when compared to untreated cells (Figure 6.11). In fact, there was a trend in GA treated groups to have a greater cell viability following H_2O_2 treatment than cells that had not received H_2O_2.

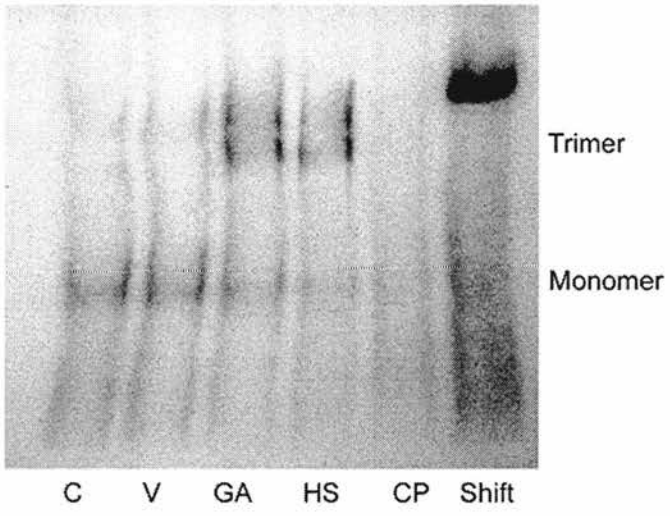


Figure 6.7 **HSF1 DNA-binding ability and oligomeric status following geldanamycin treatment.**
ACHN cells were treated with geldanamycin (GA) (2 μM) or vehicle (V) (DMSO) for 1.5 h; a further group was subjected to heating at 43 °C for 45 min and recovered for 45 min. Nuclear lysates were prepared and a DNA mobility shift assay performed using ^{32}P-labelled heat shock element consensus oligonucleotides. As controls, lysate from the GA group was run with either anti-HSF1 antibody (Shift) or excess unlabelled consensus oligonucleotides (CP).

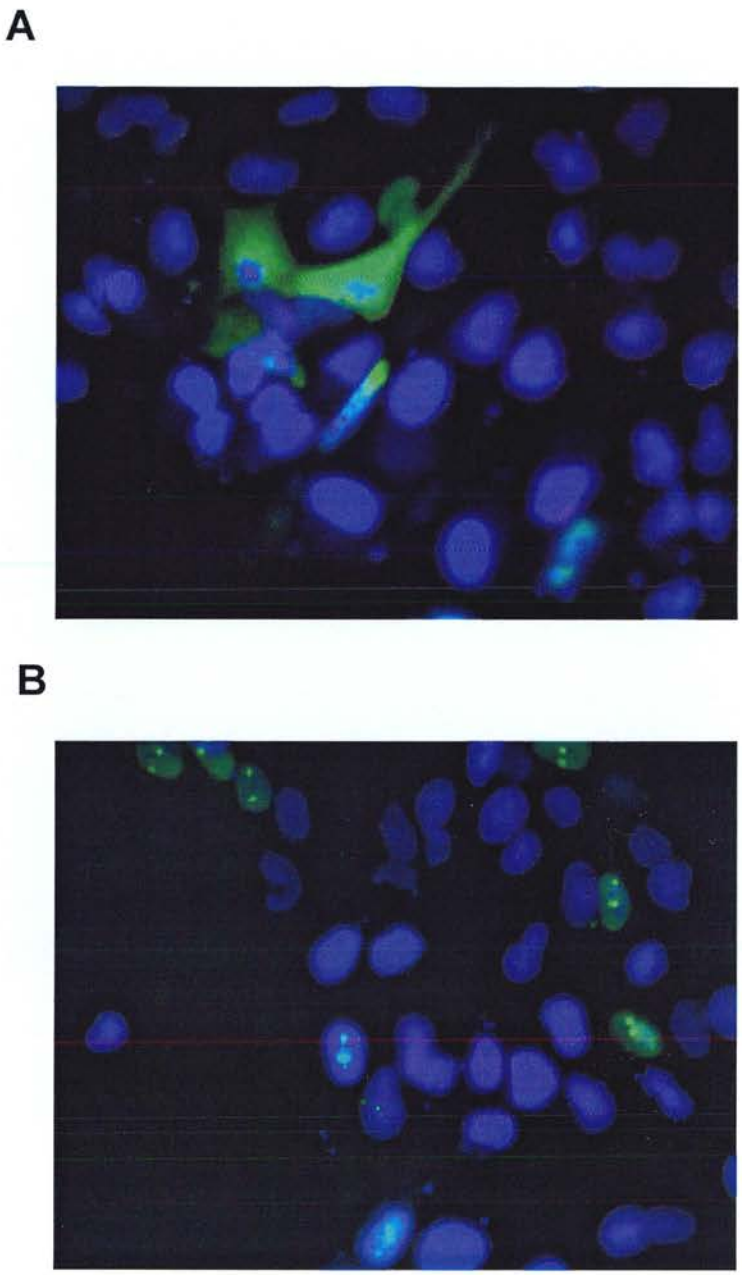


Figure 6.8 **Geldanamycin induces nuclear HSF1 stress-granules formation.** ACHN cells were transfected with the HSF1-GFP construct and 24 h later treated with GA (2 μM) for 1.5 h. Cells were fixed with methanol and counterstained with Hoechst. Fields were visualised with a Leica DM IRB microscope and images were stored using the Leica Improvision software.

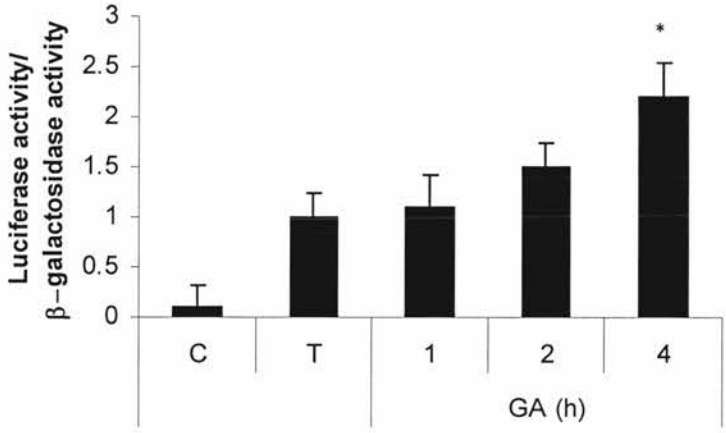


Figure 6.9 **Geldanamycin activates a reporter construct containing the Hsp70 promoter.**
ACHN cells were transfected with an Hsp70-luciferase reporter construct and the pSV-β-gal control vector. 24 h later cells were treated with GA (2 μM) for various periods of time. Cells were lysed and luciferase and β-galactosidase assays performed. Results are expressed as mean luciferase activity per unit β-galactosidase activity ± S.E.M. of 3 independent experiments. * $p<0.05$ Mann-Whitney U test. T, transfected.

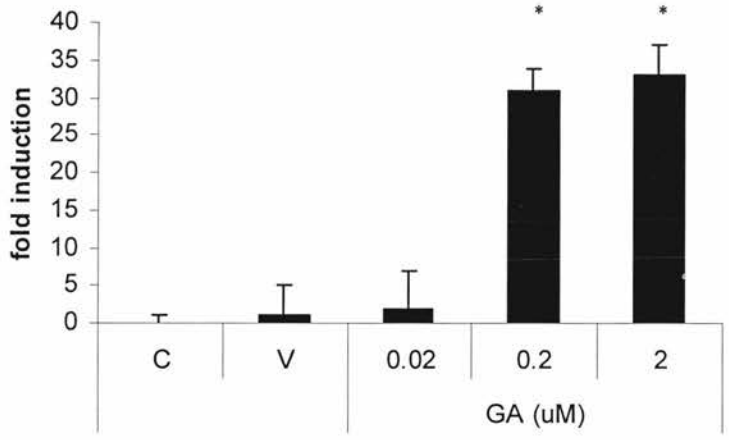


Figure 6.10 **Geldanamycin stimulates Hsp70 mRNA accumulation in ACHN cells.**
ACHN cells were treated with increasing concentrations of geldanamycin (GA) for 4 h. mRNA extracts were prepared using TRIzol and reverse transcribed to cDNA. Fluorescence-detected real time PCR was performed using Hsp70 primers and probe with a 18s primer/probe control; results are expressed as mean relative expression ± S.E.M. of 3 independent experiments. * $p<0.05$ Mann-Whitney U test.

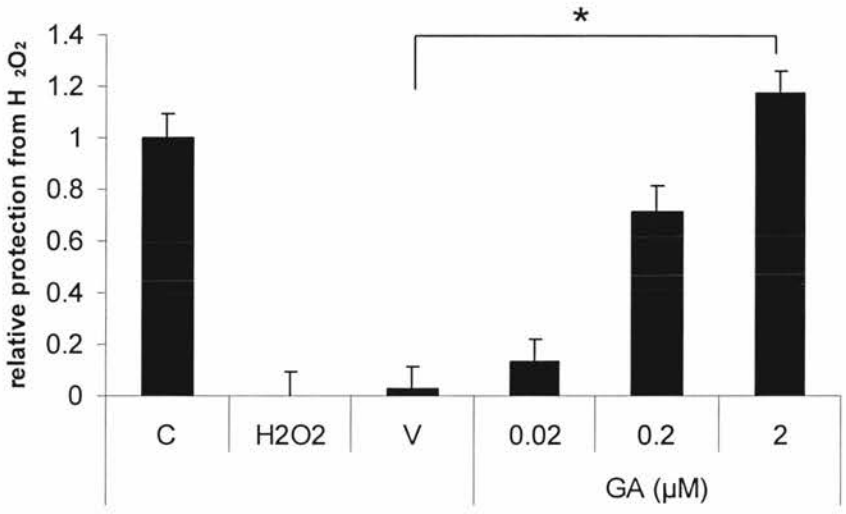


Figure 6.11 **Geldanamycin protects cells from H_2O_2-induced oxidative stress.** ACHN cells were treated with increasing concentrations of GA for 6 h after the medium was changed and included H_2O_2 (2 μM) for 16 h. MTT was added for 2 h and the resulting tetrazolium crystals dissolved in SDS. Samples were analysed on a plate reader (spectrophotometer) and results are the mean of 3 independent experiments expressed as the relative proportion of cells protected from H_2O_2; error bars S.E.M; * $p<0.05$ Mann-Whitney U test.

6.5 Discussion

In this chapter, it has been shown that treating a renal adenocarcinoma cell-line with the Hsp90-binding agent, geldanamycin, up-regulates Hsp70 mRNA and protein in a transcription- and translation-dependent manner. GA treatment was associated with HSF1 trimer formation, DNA-binding and stress-granule formation. GA was also shown to protect cells from H_2O_2-induced oxidative stress. These results confirm observations in other cell-types that treatment with HBAs is associated with Hsp induction and cellular protection. Hegde *et al* demonstrated Hsp28/72/73 and Grp94 induction following treatment with the HBA, herbimycin A, and that this was associated with cellular protection similar to that conferred by heat treatment (340). Morris *et al* also demonstrated Hsp70 induction following herbimycin A in cardiomyocytes, and although Hsp25/60/90 induction was not demonstrated, cells were protected in a model of simulated ischemia* (341). Javadpour *et al* showed decreased lung injury following infrarenal aortic clamping associated with Hsp70 induction following herbimycin A treatment (338). Dinh *et al* found herbimycin A protected human retinal epithelial cells from heat and was associated with increased Hsp70/90 mRNA expression on cDNA array (although no attempt was made to confirm this with real-time PCR) and Hsp70 protein induction (342). Sachidhanandam e*t al* demonstrated Hsp70 expression following herbimycin A treatment associated with lower levels of heat-induced hepatocyte apoptosis (343). Interestingly, the core body temperature (as measured by an implanted temperature sensor) of animals pre-treated with herbimycin A reached a lower maximum than

* Simulated ischemia (Morris): buffer simulates the extracellular milieu of myocardial ischemia, with concentrations of potassium, hydrogen, and lactate ions approximating *in vivo* conditions.

control animals, despite both groups of animals being placed in a Perspex container with tight temperature and humidity control.

A number of investigators have examined the ability of GA to protect cells and organs. Conde *et al* subjected cardiomyocytes to simulated ischemia[†] and demonstrated protection in GA treated groups associated with heat shock protein induction (344). Xiao demonstrated decreased apoptosis in GA pre-treated hippocampal cells subjected to oxidative stress (345). Pittet *et al* used a haemorrhagic model of lung injury to examine the benefits of GA pre-treatment or heat. Alveolar liquid clearance was significantly better in GA and heat-treated animals when compared with control groups (346). Lu *et al* infused GA directly into the middle cerebral artery, after which animals were subjected to 2 h of ischemia and 22 h of reperfusion (339). Decreased infarct volumes, fewer TUNEL-positive cells, reduced brain oedema and improved behavioural outcomes were shown in the GA treated groups. GA treatment was associated with HSF1 activation and Hsp70 and Hsp25 induction. Xu *et al* demonstrated protection of mature astrocytes from necrotic cell death and young astrocytes from apoptotic death with 8 h pre-treatment with GA (347). This was associated with increased Hsp70 protein expression. Kiang *et al* found GA inhibited hemorrhage-induced ATP loss in the jejunum, lung, heart, kidney, and brain of mice. Hsp70 gene transfer into intestinal epithelial cells promoted pyruvate dehydrogenase and ATP levels, whereas Hsp70 short-interfering RNA reduced them.

[†] Simulated ischemia (Conde): Hypotonic HBSS without glucose or serum, and made hypoxic for 4 – 6 h at 37 °C.

Fewer studies have been performed using the HBA, radicicol. Griffin *et al* demonstrated protection in rat cardiomyocytes subjected to simulated ischemia[‡] (349). This was also associated with Hsp induction.

While these studies are important and show strong a strong association between HBA treatment, Hsp induction and cellular/organ protection, little in the way of causal data has been reported. This is also true of the data presented in this chapter and is due in part to the lack of a specific chemical inhibitor of HSF1. A bioflavonoid, quercetin, has been widely used as an inhibitor of the heat shock response and may work by down-regulation of HSF1 (351). However, it is a very promiscuous inhibitor and has significant effects on a large number of kinases involved in different molecular mechanisms (Table 6.3). For this reason, published data based on its use must be treated with some scepticism. The development of short-interfering RNA (siRNA) has greatly aided study in this field and will be explored in the next chapter.

A criticism of this part of the study could be the choice of cell-line. The use of a renal adenocarcinoma cell-line may not provide a model that accurately reflects the physiological conditions encountered in a human kidney during renal transplantation. While the criticism is valid, the experiments act as a proof of principle prior to using the drugs in a mouse model of renal IRI.

[‡] Simulated ischemia (Griffin): buffer lacking glucose and Na_2HPO_4 bubbled for 30 min with argon and maintained at 0.03% O_2 for 16 h.

While these experiments were successful I was aware of the limitations of GA as a potential therapeutic intervention. In pre-clinical studies, significant hepatotoxicity had been found in dogs limiting its clinical application (352). However, analogues of the drug had been developed and became available during the period of this study. Similar Hsp90 inhibition had been demonstrated with these analogues, but lower degrees of toxicity and better bioavailability than GA. The ability of these agents to protect cells has not previously been studied. The next chapter examines these drugs and tests their ability to induce the stress protein response in cell culture, comparing their ability against that of GA.

Protein kinase	Kinase activity (% of control)
MKK1	94±3
MAPK2/ERK2	113±8
JNK1a1/SAPK1c	101±5
SAPK2a/p38	138±7
SAPK2b/p38β2	150±6
SAPK3/p38δ	132±1
SAPK4/p38α	103±3
MAPKAP-K1b	20±3
MAPKAP-K2	90±3
MSK1	37±3
PRAK	51±2
PKA	104±6
PKCα	70±1
PDK1	81±4
PKBα	99±2
SGK	35±0
S6K1	25±0
GSK3β	30±1
ROCK-II	55±2
AMPK	16±0
CK2	19±3
PHK	32±4
LCK	83±11
CHK1	56±1

Table 6.3 **Inhibition of protein kinases by quercetin.**
The effects of quercetin (20 μM) on a core panel of common kinases. From (353).

Chapter 7 – Analogues of geldanamycin (17-AAG and 17-DMAG) stimulate the stress response and protect renal cells from oxidative stress

7.1 Introduction

7.1.1 Background

In chapter 6, the efficacy of geldanamycin (GA) in stimulating the stress protein response through HSF1 inhibition was demonstrated. This is important proof-of-principle evidence, but the clinical use of GA is limited by toxicity. Having successfully demonstrated protection of renal cells with Hsp90 inhibition, the clinical potential of newly developed related drugs was examined. Modifications to GA have been attempted aiming to improve the bioavailability, reduce the side-effect profile, while maintaining potent Hsp90 inhibition.

7.1.2 Geldanamycin analogues

Two such drugs which fulfil these criteria have been developed by the US National Cancer Institute: 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-NN-dimethyl ethylene diamine-geldanamycin (17-DMAG).

17-AAG

When GA is treated with alkylamines bearing one displaceable hydrogen, it reacts to lose its 17-methoxy group to form 17-amino-17-demethoxy derivatives (354). The

anti-neoplastic properties of 17-AAG have been studied in pre-clinical, phase I (355-360) and phase II trials (361) (a further nine phase I and eight phase II trials are registered with the National Library for Medicine; www.clincialtrials.gov, accessed 16th March 2007). Few studies have specifically examined Hsp expression following 17-AAG treatment (Table 7.1). 17-AAG induces Hsp70 in colorectal cancer cells (362), primary glial cultures (363) and motor neurons (364,365), and a phase I clinical trial in patients with advanced solid tumours found Hsp70 induction in peripheral blood mononuclear cells (358).

17-DMAG

17-DMAG is another analogue of GA which is water soluble affording it excellent bioavailability and wide tissue distribution compared with 17AAG (366). X-ray crystallography suggests it has a much greater affinity for Hsp90 than 17-AAG (367) and pre-clinical trials suggest similar anti-neoplastic activity as 17-AAG (368). Six phase I clinical trials are registered for the treatment of advanced-stage solid tumours (www.clincialtrials.gov, accessed 16th March 2007) (369). As with 17-AAG, no reports exist at present looking at cellular protection associated with 17-DMAG treatment. Hsp70 induction in liver and kidney has been demonstrated in severe combined immunodeficiency (SCID) mice bearing breast cancer tumours treated with 17-DMAG (75 mg/kg IV) (370).

17-AAG and 17-DMAG both effect excellent Hsp90 inhibition *in vitro* and *in vivo* and cause significantly lower side-effects than GA in phase I (371) and pre-clinical trials (372), respectively. Unlike GA, the ability of 17-AAG or 17-DMAG to protect

cells has not been reported in the literature, either *in vitro* or *in vivo*. These drugs represent a potentially safe and efficacious way to stimulate the stress protein response conferring cellular protection that could be applied clinically. In this chapter, the ability of the analogues to do this *in vitro* was examined prior to moving into an animal model described in Chapters 8 and 9.

Drug	Author	Year	Species	Cell/organ	Protein	Ref
17-AAG	Clarke	2000	Human	Colorectal cancer cells	Hsp70	(362)
	Dello	2006	Mouse	Glial cells	Hsp70	(363)
	Waza	2005	Mouse	Motor neurons	Hsp40/70	(365)
	Batulan	2006	Mouse	Motor neurons	Hsp40/70	(364)
	Nowakowski	2006	Human	PBM cells	Hsp70	(358)
17-DMAG	Eiseman	2005	Mouse	Kidney/liver	Hsp70/90	(370)

Table 7.1 **Reports of heat shock protein induction following 17-AAG/17-DMAG treatment.**
PMB, peripheral blood mononuclear.

7.2 Hypotheses

1. 17-AAG and 17-DMAG cause Hsp90 inhibition and induce HSF1 trimerisation and DNA-binding.

2. 17-AAG- and 17-DMAG-mediated Hsp90 inhibition induces the stress protein response in renal cells.

3. HSF1 gene silencing abrogates the effects of 17-AAG and 17-DMAG.

4. Hsp90 inhibition protects renal cells from oxidative stress.

7.3 Methods and materials

7.3.1 Estimation of ROS production

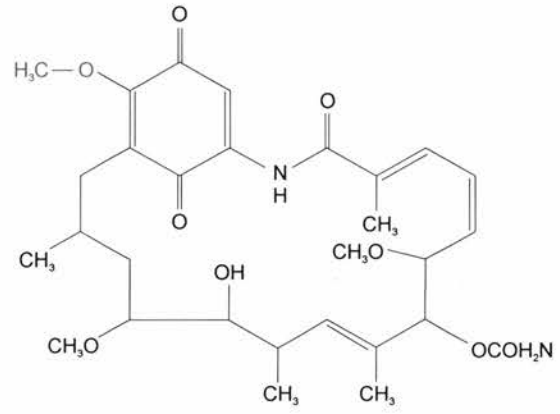
ACHN cells were grown to 90% confluence in a 96-well plate. Cells were then treated with GA, 17-AAG, 17-DMAG (all 2 μM) or glucose oxidase (0.1 unit/ml) for 1 h after which 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) (Molecular Probes, Invitrogen, Paisley, UK) (5 μM) was added. The plate was placed on a thermostatically controlled plate reader and read at 15 min intervals for 2 h (excitation 485 nm, emission 535 nm).

7.3.2 Annexin V/propidium iodide flow cytometry

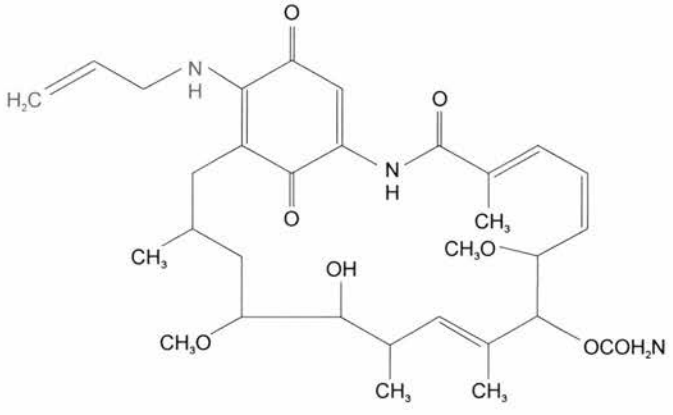
ACHN cells were treated with GA, 17-AAG and 17-DMAG (all 2 μM) for 8 h. After incubation, detached cells were collected and pooled with trypsinized adherent cells. Cells were centrifuged at 200 *g* for 5 min at 4 °C, and the supernatant removed. Cells were washed three times with ice-cold PBS buffer and re-suspended in 100 μl of binding buffer (10 mM Hepes, [pH 7.4], 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$). 5 μl of FITC-conjugated annexin V and 10 μl of propidium iodide were added and the cell suspension gently mixed and incubated in the dark on ice for 10 min. Samples were assayed for viable, apoptotic, and necrotic cells by FACS analysis (Coulter Epics XL-MCL, Beckman Coulter High Wycombe, Buckinghamshire, UK). Necrotic cells were defined as cells demonstrating positive staining for both FITC-conjugated annexin V and propidium iodide. Viable cells were not positive for either FITC-conjugated annexin V or propidium iodide.

Apoptotic cells were defined as cells exhibiting positive staining for FITC-conjugated annexin V and negative staining for propidium iodide. Fluorescence was measured on a double-parameter histogram, using logarithmic scales. For each tube, 5000 events were analyzed. No significant FITC staining was seen in control or HBA treated cells. Necrotic control cells stained positively for both PI and FITC.

Geldanamycin



17-AAG



17-DMAG

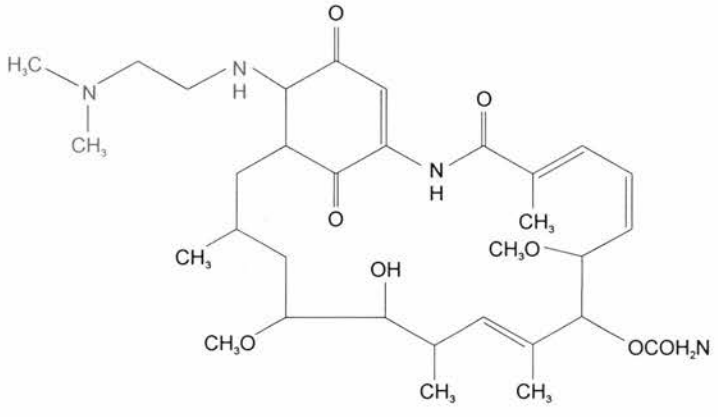


Figure 7.1 **Structural differences between geldanamycin analogues.**
Two analogues of GA have been synthesised with side-chain alterations at the 17-position (shown in red). Both analogues have better bioavailability and are less toxic in vivo than GA. 17-DMAG has the added advantage of being water-soluble. 17-AAG, 17-allylamino-17-demethoxygeldanamycin; 17-DMAG, 17-NN-dimethyl ethylene diamine-geldanamycin.

7.4 Results

HBAs induce Hsps in ACHN cells

The effects of different HBAs *in vitro* were examined in a human renal adenocarcinoma cell line (ACHN). A time course using GA, 17-AAG and 17-DMAG (all 2 μM) demonstrated a 3.5 fold increase in Hsp70 expression after 6 h (Figure 7.2A). Hsp90 was 3.4 fold greater 4 h after treatment with GA and 2.5 fold greater 4 h after 17-AAG or 17-DMAG. Maximal dose response of Hsp70 and Hsp90 was seen with HBAs at a concentration of 0.2 μM (Figure 7.2B). The small heat shock protein Hsp27 was not found to be up-regulated at these early time points, however, in 17-DMAG (2 μM) treated cells there was a 2 fold increase at 12 h, with the same increase at 16 h in cells treated with GA or 17-AAG (both 2 μM) (Figure 7.3). Hsp27 induction was demonstrated 12 h following 17-DMAG treatment and 16 h following GA and 17-AAG. HO-1 (Hsp32) expression was increased at 4 h by GA alone. HO-1 expression was not altered by 17-AAG or 17-DMAG. Cells treated for 1 h with HBAs demonstrated trimerisation of HSF1 on a DNA mobility shift assay (Figure 7.4). Hsp70 gene expression was increased 30, 35 and 42 fold, following 2 h treatment with GA, 17-AAG and 17-DMAG respectively (all 2 μM) (Figure 7.5).

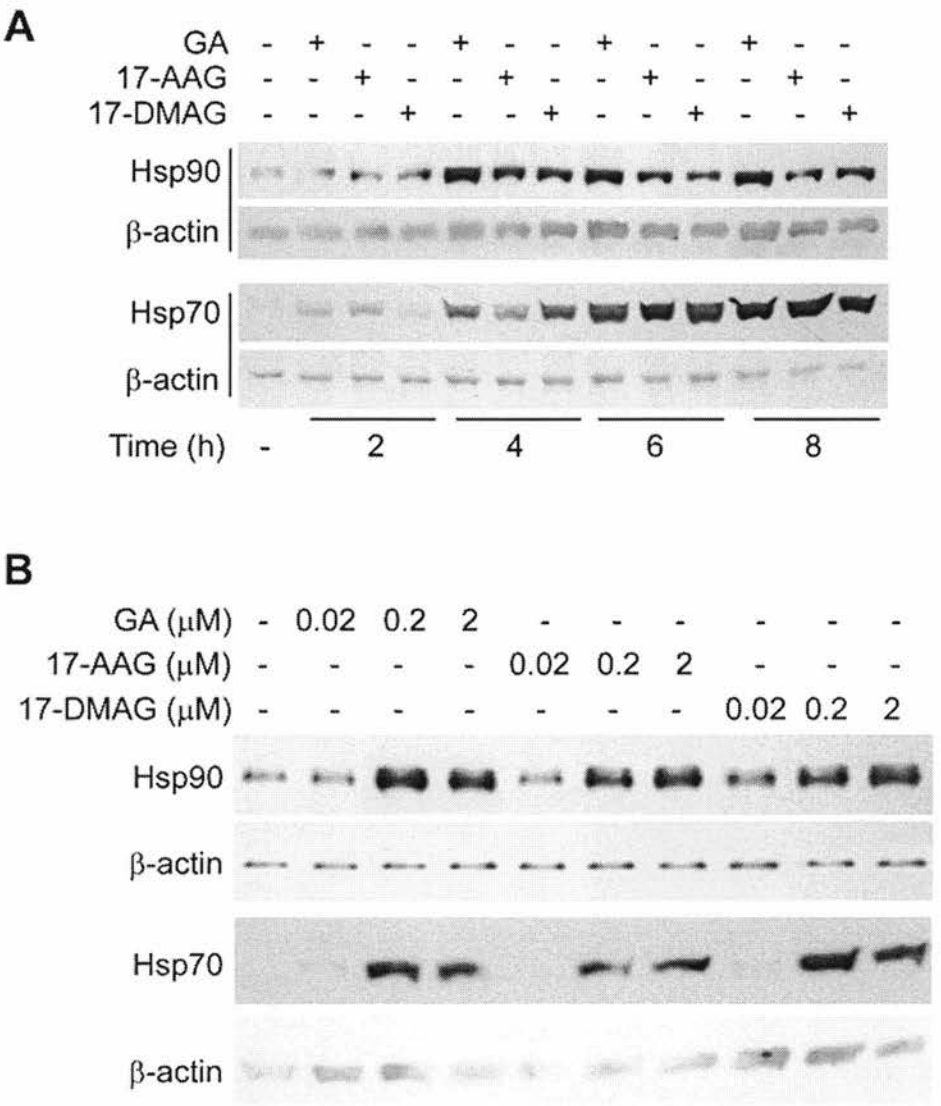


Figure 7.2 **Induction of Hsp90 and Hsp70 by geldanamycin, 17-AAG and 17-DMAG.**
ACHN cells were exposed to 2 μM GA, 17-AAG or 17-DMAG for various periods of time (A) and various doses of GA, 17-AAG or 17-DMAG for 8 h (B). Whole-cell lysates were prepared and analysed by Western blotting using antibody to Hsp90 and Hsp70. β-actin was used as a loading control.

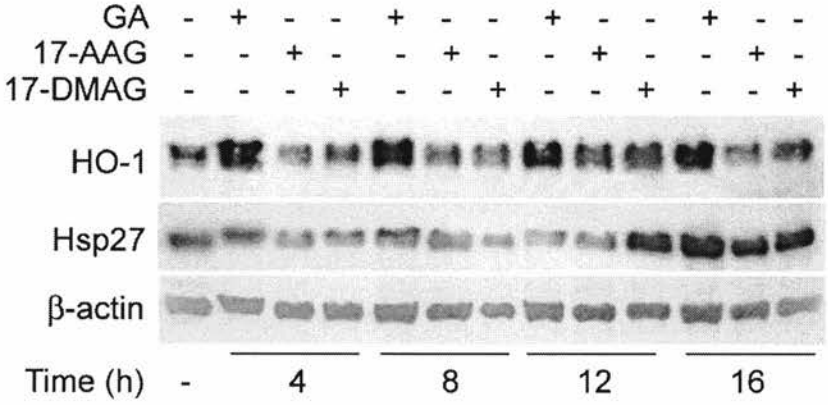


Figure 7.3 - **Induction of small Hsps by geldanamycin and analogues.**
ACHN cells were exposed to increasing doses of geldanamycin (GA), 17-AAG or 17-DMAG for periods up to 16 h. Whole-cell lysates were prepared and analysed by Western blotting using antibody to HO-1 (Hsp32) and Hsp27. β-actin was used as a loading control.

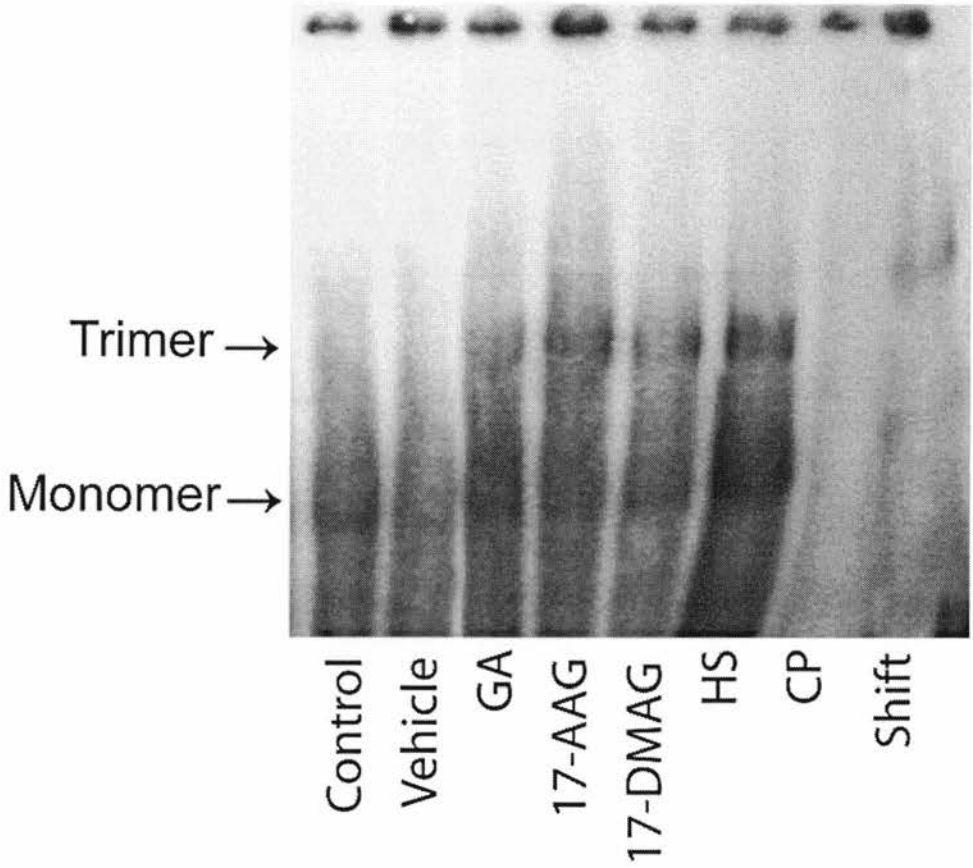


Figure 7.4 **HSF1 DNA-binding ability and oligomeric status HBA treatment.** ACHN cells were treated with geldanamycin (GA), 17-AAG, 17-DMAG (all 2 μM) or vehicle (V) (DMSO) for 1.5 h; a further group was subjected to heating at 43 °C for 45 min and recovered for 45 min (HS). Nuclear lysates were prepared and a DNA mobility shift assay performed using ^{32}P-labelled heat shock element consensus oligonucleotides. As controls, lysate from the HS group was run with anti-HSF1 antibody (Shift) or excess unlabelled consensus oligonucleotides (CP).

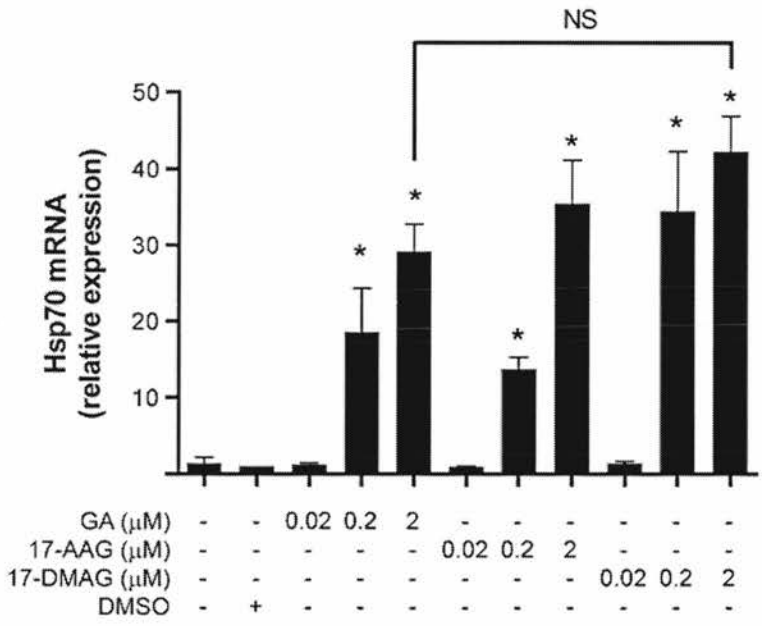


Figure 7.5 **Geldanamycin, 17-AAG and 17-DMAG stimulate Hsp70 mRNA accumulation in ACHN cells.**
Cells were treated with various concentrations of GA, 17-AAG or 17-DMAG for 4 h. mRNA extracts were prepared using TRIzol and reverse transcribed to cDNA. Fluorescence-detected real time PCR was performed using Hsp70 primers and probe with a 18s primer/probe control; results are expressed as mean relative expression ± S.E.M. of 3 independent experiments; NS, non-significant (ANOVA with Bonferroni correction).

Toxicity of HBAs on ACHN cells

To determine whether HBAs reduce cell viability, ACHN cells were treated with increasing concentrations of the drugs for 16 h (Figure 7.6). A trend was observed where GA reduced the cell viability; however, no statistical difference was demonstrated between GA and any other group (ANOVA).

HBA treatment does not cause reactive oxygen species accumulation in ACHN cells

To determine whether HBAs have a direct oxidant effect, reactive oxygen species (ROS) generation was measured. Cells were prepared in 96-well plates and treated with GA, 17-AAG or 17-DMAG. Following the addition of DCFDA, the resulting fluorescence was measured for 2 h (Figure 7.7). Cells treated with HBAs did not demonstrate any ROS production compared with control cells. Glucose oxidase treated cells were used as a positive control and exhibited significant ROS production.

HBA treatment does not result in significant apoptosis in ACHN cells

ACHN cells were treated with GA, 17-AAG and 17-DMAG (all 2 μM) for 8 h. Cells were treated with FITC-conjugated annexin V and propidium iodide (PI) and underwent flow cytometry (Figure 7.8). Annexin V positivity was not demonstrated in cells treated with HBAs, suggesting that early cell membrane changes associated with apoptosis were not present. Control necrotic cells were both PI and annexin V positive.

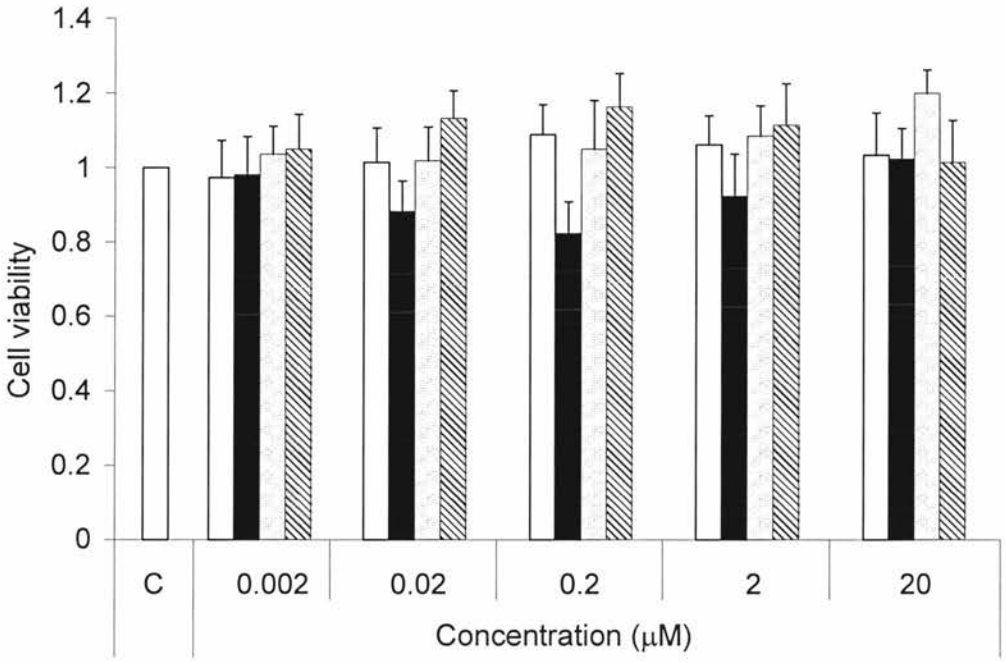


Figure 7.6 **Toxicity of geldanamycin, 17-AAG and 17-DMAG on ACHN cells.** Cells were treated with vehicle (DMSO) □, GA ■, 17-AAG ⬚ and 17-DMAG ▨ for 16 h. An MTT assay was performed and cell viability expressed as a fraction of control (C). Results represent mean of 6 independent experiments with S.E.M. indicated by error bars.

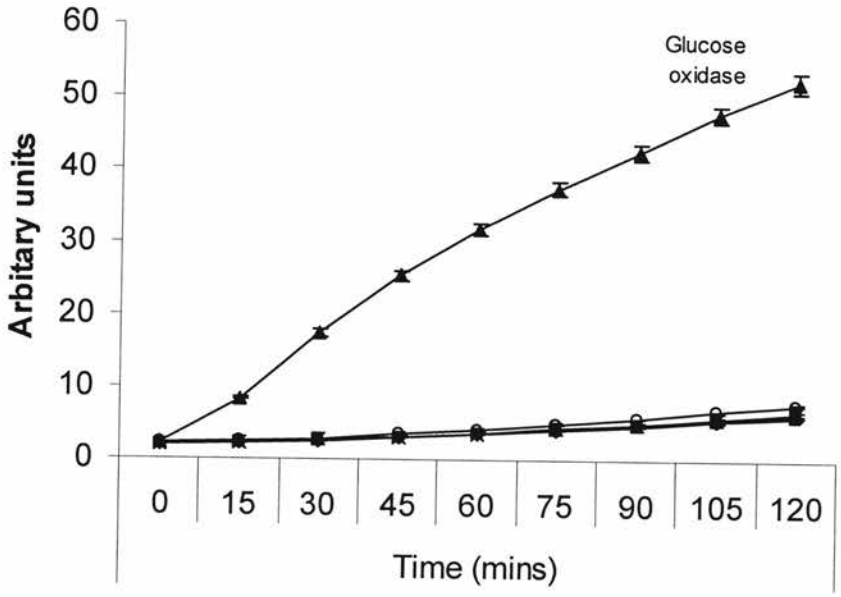


Figure 7.7 **Reactive oxygen species generation following treatment with geldanamycin, 17-AAG and 17-DMAG.**
ACHN cells were treated with DCFDA and geldanamycin (x), 17-AAG (□), 17-DMAG (○) or glucose oxidase (▲). Untreated cells (◊) and cells treated with DCFDA alone (■) were also included.

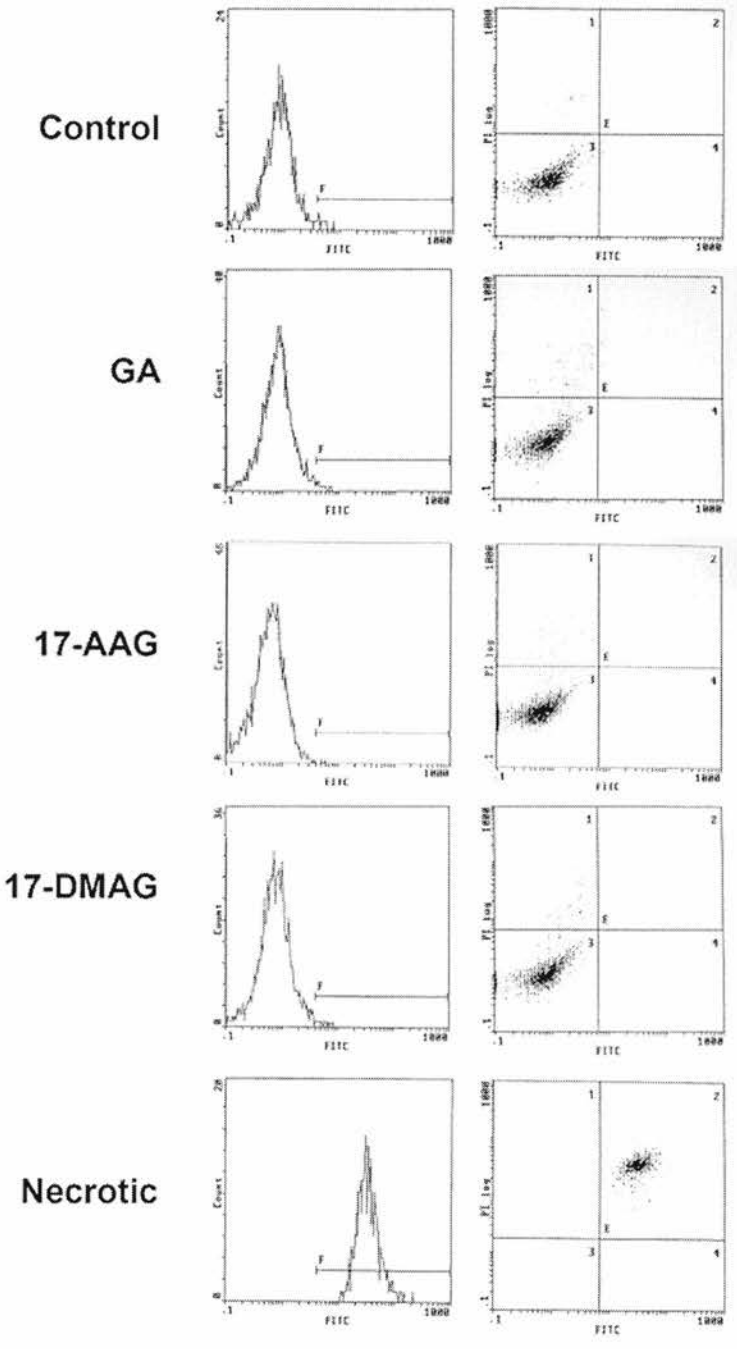


Figure 7.8 **Apoptosis following treatment with HBAs.**
ACHN cells were treated with GA, 17-AAG and 17-DMAG (all 2 μM) for 8 h. Necrotic cells were prepared by treating cells in a microwave for 15 s. After incubation, detached cells were collected and pooled with trypsinized adherent cells and combined with FITC-conjugated annexin V and propidium iodide (PI). Samples underwent FACS analysis with necrotic cells defined as demonstrating positive staining for both annexin V and PI and apoptotic cells defined as exhibiting positive staining for annexin V but negative staining for PI. Fluorescence was measured on a double-parameter histogram, using logarithmic scales.

HBAs protect ACHN cells from hydrogen peroxide (H_2O_2)-mediated oxidative stress

Cells were treated with GA, 17-AAG or 17-DMAG for 8 h, followed by H_2O_2 (2 μM) for 16 h. An MTT assay was performed and results expressed as a relative protection from H_2O_2. Pre-treatment with HBAs protected cells from a hydrogen peroxide (H_2O_2) mediated oxidative insult (Figure 7.9). This protection did not reach statistical significance after 8 h of GA (2 μM), but was significant in groups treated with 17-AAG ($p<0.05$) and 17-DMAG ($p<0.01$). 17-AAG and 17-DMAG treated cells demonstrated the same viability as control cells not treated with H_2O_2. Cell viability in the 17-DMAG group was not significantly greater than that of the GA group.

HSF1 siRNA abrogates HBA-mediated Hsp induction and protection

To determine whether HBA-mediated Hsp is HSF1 dependent, cells were transfected with HSF1 short-interfering RNA (siRNA). 48 h later cells were treated with HBAs and Hsp induction determined (Figure 7.10). An HSF1 Western blot confirmed adequate HSF1 silencing was consistently achieved. siRNA transfection resulted in 2.5-times increase in Hsp70 expression. However, when siRNA transfected cells were treated with HBAs a further 1.5-times increase in Hsp70 protein was found. This increase was abrogated when cells were transfected with HSF1 siRNA. The viability of cells transfected with siRNA and exposed to H_2O_2 was lower than those exposed to H_2O_2 alone (Figure 7.11). Comparable levels of protection were seen in transfected cells pre-treated with HBAs, prior to exposure to H_2O_2. Importantly, this protection was reduced in HSF siRNA cells pre-treated with HBAs and exposed to

H_2O_2; this did not achieve statistical significance in the GA group, but was significant in the 17-AAG and 17-DMAG groups ($p<0.01$).

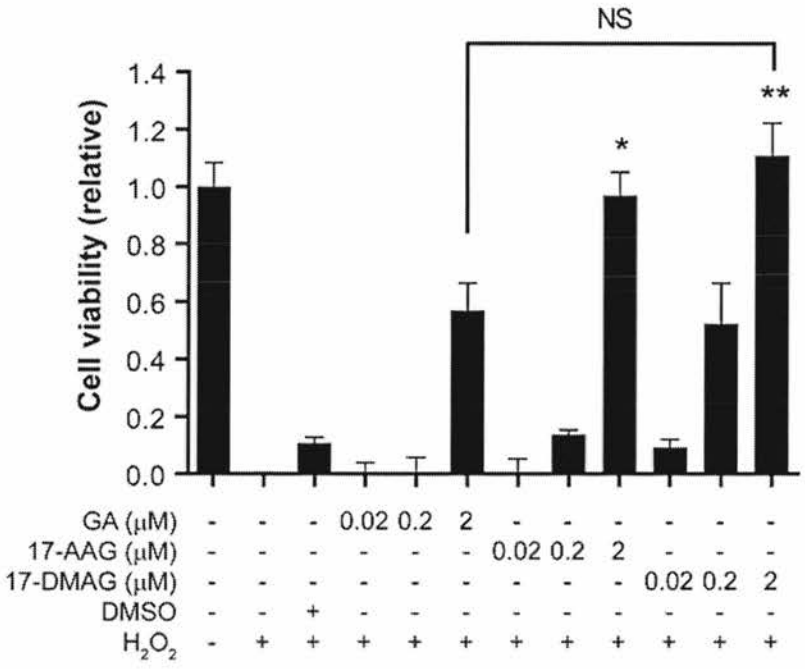


Figure 7.9 **Protection of ACHN cells from H_2O_2 induced oxidative stress.** ACHN cells treated with increasing concentrations of geldanamycin (GA), 17-AAG and 17-DMAG. Hydrogen peroxide (H_2O_2) (2 μM) was then added for 16 h. An MTT assay was performed and samples analysed on a plate reader. The results are the mean of 5 independent experiments expressed as the relative proportion of cells protected from H_2O_2; error bars S.E.M. C. control; V, vehicle. ** significantly different from vehicle ($p<0.01$; ANOVA with Tukey HSD correction); * significantly different ($p<0.05$; ANOVA with Tukey HSD correction); NS, not significantly different from vehicle.

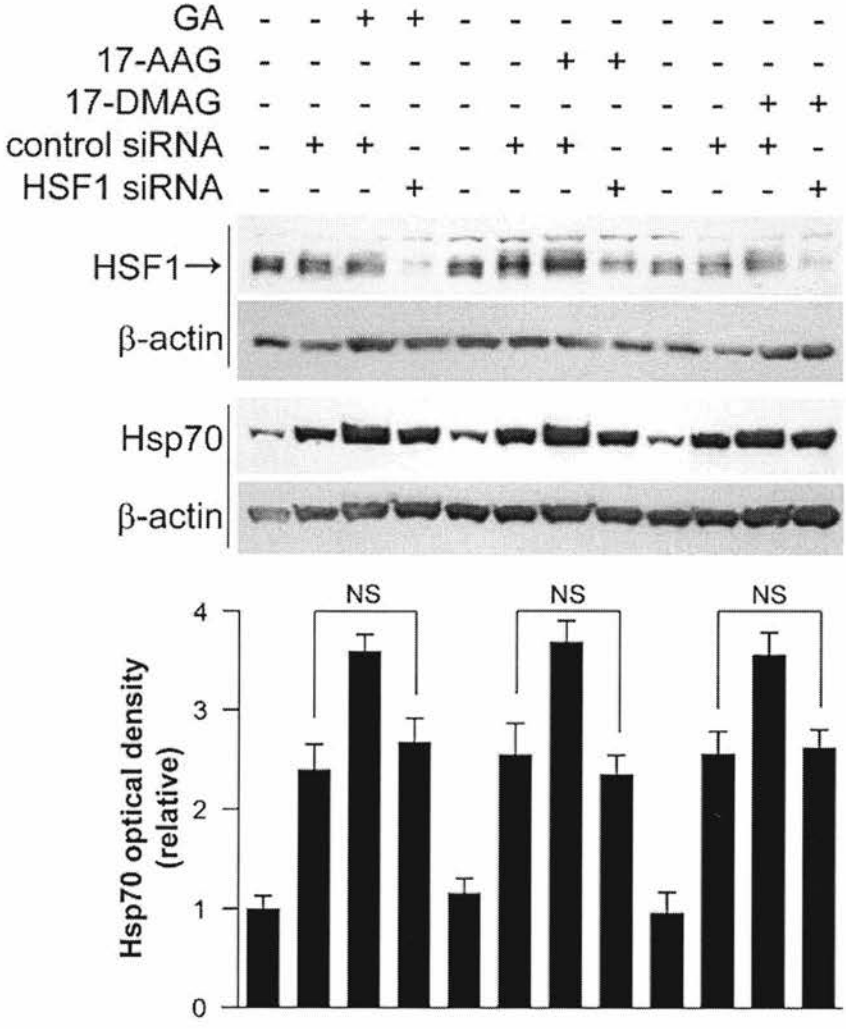


Figure 7.10 **Effect of HSF1 siRNA on Hsp70 expression following treatment with geldanamycin, 17-AAG or 17-DMAG.**
ACHN cells were transfected with siRNA target against HSF1, transfected with control siRNA, or not transfected. 48 h later cells were treated with 2μM GA, 17-AAG, or 17-DMAG for 8 h. Whole-cell lysates were prepared and analysed by Western blotting using antibody to HSF1 and Hsp70. β-actin was used as a loading control.

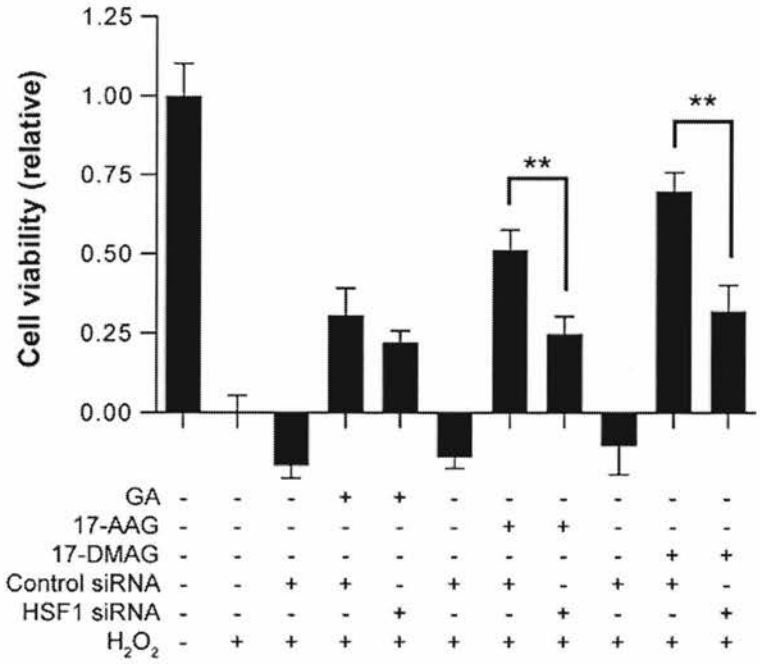


Figure 7.11 **HBA-mediated cellular protection following HSF1 siRNA treatment.** ACHN cells were transfected with HSF1 siRNA or control siRNA. 48 h later transfected cells were treated with GA, 17-AAG or 17-DMAG (all 2 μM) for 8 h, followed by H_2O_2 for 16 h. An MTT assay was performed with results expressed as the mean relative protection from H_2O_2 ± S.E.M. of 3 independent experiments. ** $p<0.01$ (ANOVA).

7.5 Discussion

In this chapter, it has been shown that treatment of renal cells with 17-AAG or 17-DMAG results in a robust up-regulation of Hsps, similar to the effect seen with geldanamycin treatment. Hsp70 mRNA expression following 17-DMAG showed a trend to greater expression when compared to GA treatment, although this did not achieve statistical significance. Similarly, 17-AAG and 17-DMAG both protected cells from H_2O_2, with 17-DMAG again tending to protect cells better (although a statistically significant difference between 17-DMAG and GA was not demonstrated).

When comparing cells treated with 17-AAG/17-DMAG and GA, the latter was found to have a small but measurable effect on cell viability (Figure 7.6). Related to this observation is the induction of heme oxygenase-1, seen following GA but not 17-AAG nor 17-DMAG (Figure 7.3). What differences exist between the drugs that could explain these findings? As has been discussed, pre-clinical trials of GA found significant hepatotoxicity which was not apparent with 17-AAG and 17-DMAG (352,372). One possible mechanism of toxicity is direct production of reactive oxygen species (ROS), a capacity common to all compounds containing a quinone group. Dikalov *et al* have shown that GA can generate superoxide (373) and Billecke *et al* have demonstrated that GA is capable of redox cycling* with nNOS (375). In the model presented here, 2 h treatment with 2 μM HBAs was not sufficient to show

* Redox-cycling: typically, a flavoenzyme mediates transfer of electrons from NADPH to the quinone chemically reducing it to the semiquinone. Subsequent electron transfer to oxygen from the semiquinone results in the formation of $O_2^{\cdot-}$. This redox cycle continues until the system becomes anaerobic at which time the oxygen radical production decreases and the semiquinone begins to accumulate to detectable levels (374).

a difference in the generation of ROS between treated and untreated groups. In the two studies cited, generation of ROS was apparent using high concentrations of GA (17 – 20 μM). Concentrations of less than 5 μM, as used here, resulted in much lower generation of ROS and a more recent study has confirmed this (376).

Even if the ROS production had been demonstrated, it would not explain differences between drugs as all contain a quinone ring and therefore should be equally capable of generating ROS. A more recent suggestion comes from an American Biotechnology firm that claims to have linked GA toxicity to an Hsp90-independent mechanism: inhibition of phosphoribosylaminoimidazole carboxylase, an enzyme involved in *de novo* purine biosynthesis (Serenex Inc., Durham, NC 27701; www.serenex.com). This effect was not observed in a number of unspecified analogues of GA, and so might explain the differences seen between GA and 17-AAG/17-DMAG. One might suppose that the mechanism of GA hepatotoxicity may be similar to that seen with azathioprine and 6-mercaptopurine, which also inhibit purine biosynthesis. These observations remain speculative as none of this work appears to have been published in the peer-reviewed literature.

HBA-mediated Hsp27 induction occurred significantly later than that of Hsp70 (Figure 7.3). The 12 – 16 h delay seen in Hsp27 up-regulation may indicate the presence of additional mechanisms to that of classical HSF1 activation. This contradicts previous data where, in rat forebrains, Hsp25 induction occurred concurrently with Hsp70 following GA injection to the lateral cerebral ventricles

(339), although in a different study no GA-mediated Hsp25 induction occurred in mouse striatal cells (377).

In an effort to establish whether the HBA-mediated Hsp expression was HSF1 dependent, cells were transfected with HSF1 siRNA. When establishing the Nrf2 siRNA knockdown model used in section 5.4, a low-grade non-specific activation of the stress protein response was observed. Although HO-1 was not greatly affected by this, the effect on Hsp70 was greater: Hsp70 was significantly up-regulated by the control siRNA. This effect was almost certainly due to the combination of the transfection technique and cell-line used, as others have used siRNA techniques to knockdown HSF1 with no induction of Hsps (378,379). Treating cells transfected with control siRNA with HBAs further increased Hsp70. Treatment with HSF1 siRNA resulted in almost complete abolition of HSF1 protein levels, and reduced Hsp70 levels to that of the control reaction. This would suggest that HBA-mediated Hsp70 induction occurs in an HSF1-dependent manner.

Similarly, in the oxidative stress model transfection with control siRNA resulted in greater cell death than cells treated with H_2O_2 alone (Figure 7.11). Cells were protected by HBA pre-treatment but transfection with HSF1 siRNA significantly reduced this protection ($p<0.01$). Interestingly, in HBA-treated HSF1 siRNA transfected cells exposed to H_2O_2, cell viability was not reduced to that of the control siRNA-transfected H_2O_2-treated cells. This may be a reflection of incomplete knockdown, but does raise the possibility of an HSF1-independent HBA-mediated protective effect.

Apoptosis following HBA treatment has received a great deal of attention in the context of cancer treatment. In neuroblastoma cell-lines, GA has been shown to induce apoptosis (380,381) although it has shown to cause differentiation in PC12 (381) and breast cancer cells (382). 17-AAG induces apoptosis in human acute myeloid leukemia cells (383), however, in a non-cancer cell-line (rat kidney epithelial cells) apoptosis was not increased by herbimycin A or GA (384). GA-mediated Hsp70 induction was associated with decreased apoptosis in doxorubicin treated apoptotic-prone cells and this effect was lost with Hsp70 siRNA treatment (21). On examining the effects of HBAs on apoptosis in ACHN cells, no significant apoptosis was seen at 24 h (Figure 7.10). As with many cancer cell-lines, ACHNs have been shown to have a greater resistance to apoptosis, for example following treatment with 5-fluorouracil or cisplatin (385), and as will be seen in the next chapter may not be reflect physiological conditions.

In summary, these data demonstrate that HBAs have the potential of imparting renal cellular protection through a stress protein-mediated mechanism which is independent of heme oxygenase-1. There was a trend towards greatest effect with 17-DMAG, although this did not always reach statistical significance. With this strong evidence of the benefit of HBAs *in vitro*, an *in vivo* model was developed in which potential protection could be tested following ischemia/reperfusion injury in the mouse kidney.

Chapter 8 – Development of a model of renal ischemia/reperfusion injury in the mouse

8.1 Background

In the previous chapter, it was demonstrated that Hsp induction and cellular protection occurred following treatment with GA, 17-AAG and 17-DMAG. In order to determine the potential clinical efficacy of these drugs, a model of renal IRI was developed. The objective was to impart a moderate renal injury in an animal model by the temporary interruption of the blood flow to one or both kidneys. The injury was required to be consistent, easily measured and translatable to human kidney transplantation.

8.2 Model development

8.2.1 Model

The mouse was determined to be the model of choice for a number of reasons:

- easy handling
- economical
- established model of IR injury
- allows for future work using transgenic animals

Models of renal IR injury in rodents have formed the basis of a number of previous publications (53,59,386-390). These reports proved useful but varied greatly in

technique and outcome. A significant amount of work was required to optimise the experimental conditions for this study.

The aim of the model was to inflict a moderate renal injury with tubular necrosis while minimising animal morbidity and mortality. Initially, a number of decisions had to be made:

- choice of strain?
- choice of anaesthetic?
- flank or midline incision?
- interruption of blood supply to one or both kidneys?
- clamp pedicle (i.e. renal vein and artery) or just artery?
- clamp time?
- recovery time prior to measurements?
- outcome measures?

Appropriate UK Home Office training and licensing were obtained prior to the start of the study and all work involving animals was conducted in accordance with the provisions of the UK Animals (Scientific Procedures) Act 1986. Development of the procedure itself began by observing a group performing mouse renal transplantation. The donor procedure in this model requires dissection of the renal pedicle in a very similar manner to that required to in the IRI models.

8.2.2 Strain

Different strains of mice have different susceptibilities to IRI, with NIH Swiss mice being more resistant than C57BL/6, and BALB/c mice (391). This is particularly important when comparing the phenotypic effects of specific gene deletions on different genetic backgrounds.

The BALB/c strain has been successfully used in a number of IRI studies. It is an inbred strain and therefore exhibits minimal genetic differences between batches. It is used as a general-purpose strain in many different disciplines and has good breeding performance and long reproductive life-span (Mouse Genome Informatics: http://www.informatics.jax.org).

8.2.3 Anaesthetic

Previous studies have found an intraperitoneal (IP) injectable anaesthetic provides sufficient time for the procedure to be performed, i.e. no volatile agent is required to prolong anaesthesia. The initial regime chosen was on the work of Flecknell *et al* (392):

- ketamine[†] 100 mg/kg IP
- xylazine[‡] 10 mg/kg IP

[†] Ketamine – dissociative agent. Effects: sedation, some analgesia, increased muscle tone. Side effects: severe respiratory depression, increased blood pressure, chronic use induces liver enzymes which decreases efficacy.

[‡] Xylazine – α_2 agonist. Effects: sedation, analgesia, muscle relaxation. Side effects: initial hypertension then hypotension, bradycardia.

During the pilot phase of the project, two unexplained deaths occurred in sham-operated animals, which were thought to be related to anaesthesia. The anaesthetic protocol was modified and no further anaesthetic-related deaths occurred during the rest of the study:

- ketamine 75 mg/kg IP
- medetomidine[§] 1 mg/kg IP
- atipamezole[**] 1 mg/kg IP to reverse medetomidine following procedure

It was predicted that significant fluid losses would occur during the procedure and these would have to be replaced. Each animal received:

- 0.9% saline 25 ml/kg SC to the scruff prior to and 2 h following the procedure.

Analgesia was given to all animals in the form of:

- buprenorphine[††] 0.05-0.1mg/kg SC

Buprenorphine has been shown to shorten recovery time following anaesthesia in mice and does not alter the magnitude of injury following IRI (393).

[§] Medetomidine – α_2 agonist. Effects/side-effects as for xylazine.
[**] Atipamezole - α_2 antagonist. Effects: reverses effects of medetomidine .
[††] Buprenorphine – partial μ-opioid receptor agonist and κ-opioid receptor antagonist. Effects: analgesia. Side effects: respiratory depression and other side-effects associated with opiates.

8.2.4 Procedure

A pilot study was undertaken to test the hypothesis that a consistent renal injury could be achieved in the mouse model of IRI. Modification of the initial model was required as described below. A midline incision was chosen for its ease, low blood loss and good access. In exploratory experiments, it was found that separating the renal artery and vein was technically difficult and impossible without the use of an operating microscope. An alternative approach would be to clamp the supra-renal aorta, but the associated injury delivered to the midgut, hindgut, pelvic organs and lower limbs was felt to be unacceptable. In the initial experiments, left renal pedicle occlusion alone was performed for various times (Table 8.1). During these experiments, two sham treated animals failed to recover from the anaesthetic. The reason for this was unclear but following advice from the Veterinary Service the anaesthetic protocol was changed as above. As part of this initial study, dose-response experiments were performed examining Hsp70 induction following treatment with Hsp90-binding agents. Renal Hsp expression following HBAs was variable and it was felt that baseline measurements of Hsp expression would be required to aid interpretation of the results of IRI experiments. The model was adapted to include removal of the right kidney during left renal pedicle clamping.

The purpose of model 2 was to identify the optimal left renal pedicle clamp time. Serum creatinine (SCr) was used as an initial surrogate marker for renal function. During these experiments it was apparent that significant unexplained variation in SCr in the IRI group existed (range 48 – 108 μmol/l) (Figure 8.1A). A number of measures were undertaken to reduce this variation and are detailed in section 8.3.

The result of this was a reduction in the variation of SCr to an acceptable level (Figure 8.1B). When a reliable injury could be delivered by the model the relationship between clamp time and SCr was established (Figure 8.2). As has been described in similar models, a sigmoid relationship was found. In order to achieve the greatest effect size the steepest part of the s-shaped curve was targeted: this was at around 30 min and this time was used for all subsequent experiments. No animals survived more than 60 min left renal pedicle occlusion with contralateral nephrectomy.

Model 3 was the protocol which was used for all subsequent experiments. The procedure performed can be followed in Figure 8.3. Briefly, mice received an IP injection of ketamine (75 mg/kg) (Vetalar; Pfizer, Sandwich, Kent, UK) and medetomidine (1 mg/kg) (Domitor; Pfizer) and a SC injection of 0.9% saline (25 ml/kg) and buprenorphine (0.1 mg/kg). A thermostatically controlled heated mat was used to maintain body temperature. The pedicle of the left kidney was dissected and occluded using an atraumatic vascular clamp (6 x 1 mm Micro Serrefine; Fine Science Tools, Linton, UK) for 30 min. Meanwhile, the right kidney was dissected and a titanium clip placed across the ureter and the renal pedicle (Hemoclip Plus; Weck Closure Systems, Research Triangle Park, NC 27709, USA), after which the kidney was removed as a control. Following removal of the left pedicle clamp, reperfusion was confirmed visually prior to closure of the incision with 4/0 silk to the rectus sheath and clips to the skin (Reflex 7 wound clip applicator; Fine Science Tools). The anaesthetic was reversed with atipamezole (1mg/kg) (Antisedan; Pfizer) and a further 25 ml/kg of 0.9% saline was given 2 h after the procedure. Animals

were recovered in an incubator at 25 °C or a metabolic cage (Techniplast, Exton, PA 19341, USA) depending on the protocol.

Model	N (per group)	Anaesthetic	Procedure	Clamp time	Outcome
Model 1	10	K&X	Left RPO	Variable	2 unexpected deaths + requirement for baseline tissue
Model 2	10	K&M	Left RPO + right nephrectomy	Variable	Unacceptable variation in degree of injury
Model 3	10	K&M	Left RPO + right nephrectomy	30 min	Final protocol

Table 8.1 **Renal ischemia/reperfusion model development.**
K, ketamine; X, xylazine; M, medetomidine; RPO, renal pedicle occlusion

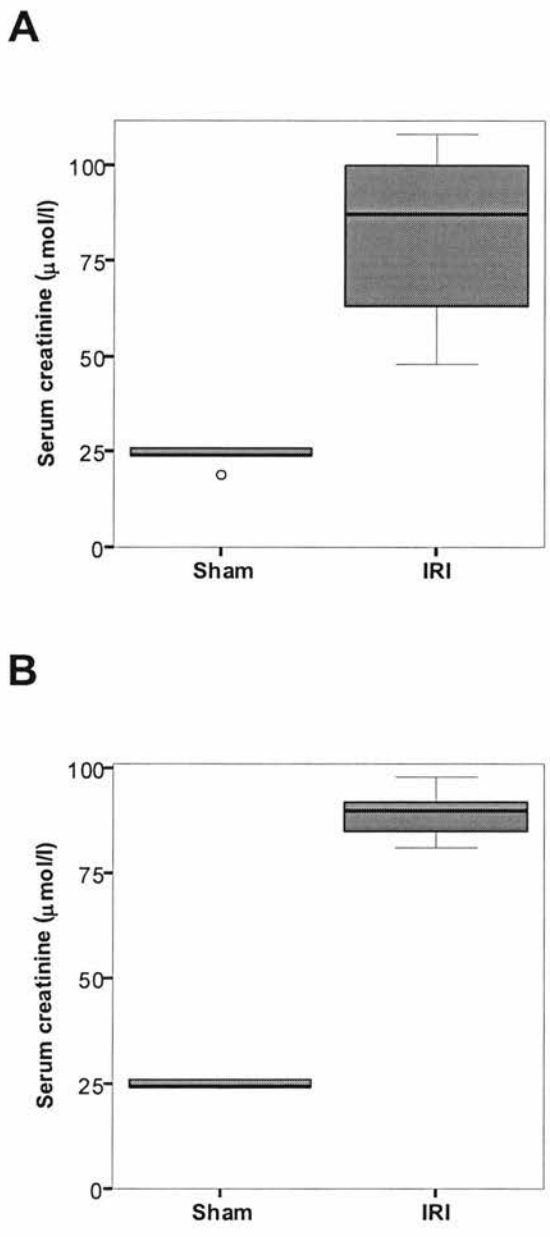


Figure 8.1 **Serum creatinine 24 h following IRI prior to (A) and after (B) variation reduction.**
Mice were subject to either 30 min left renal pedicle clamping or a sham procedure (abdomen opened for 30 min then closed). Serum creatinine at 24 h was determined (A). Following the introduction of a number of measures to reduce variation, the experiment was repeated (B). Horizontal line, mean; box, interquartile range; whisker, range.

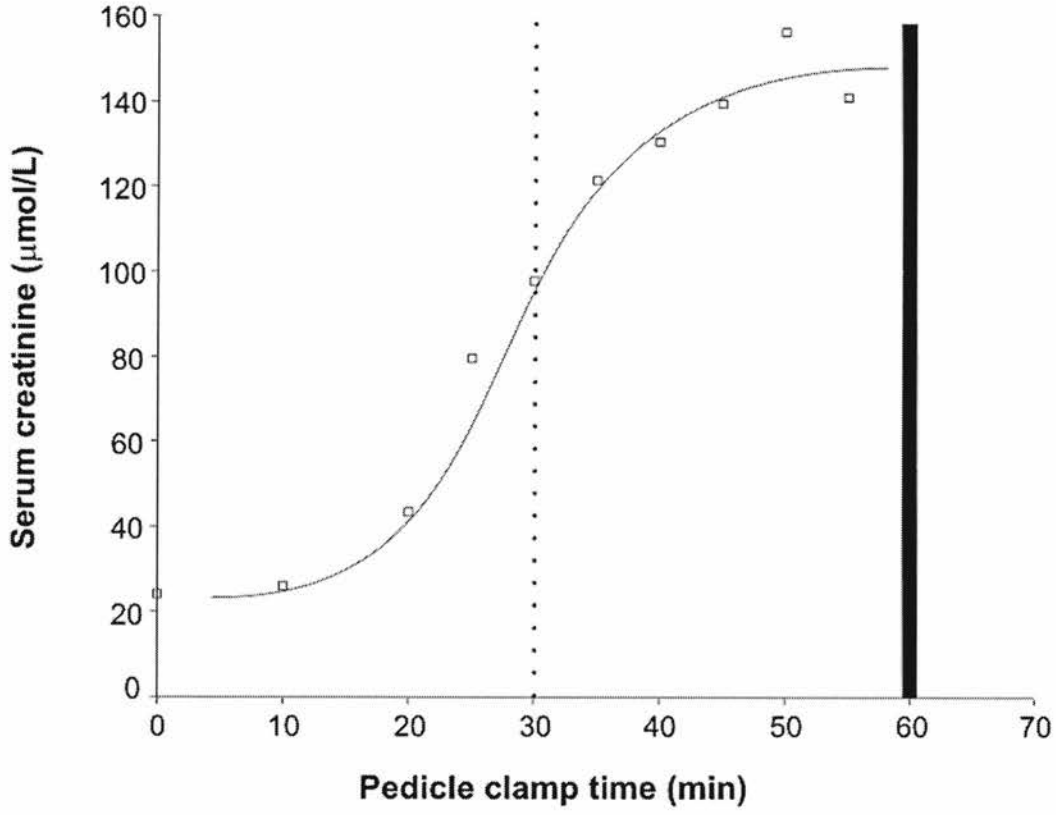


Figure 8.2 **Relationship between pedicle clamp time and serum creatinine in mouse IRI model.**
Mice were subject to left renal pedicle clamping for various times and 24 h later serum creatinine was determined. □ individual animals.

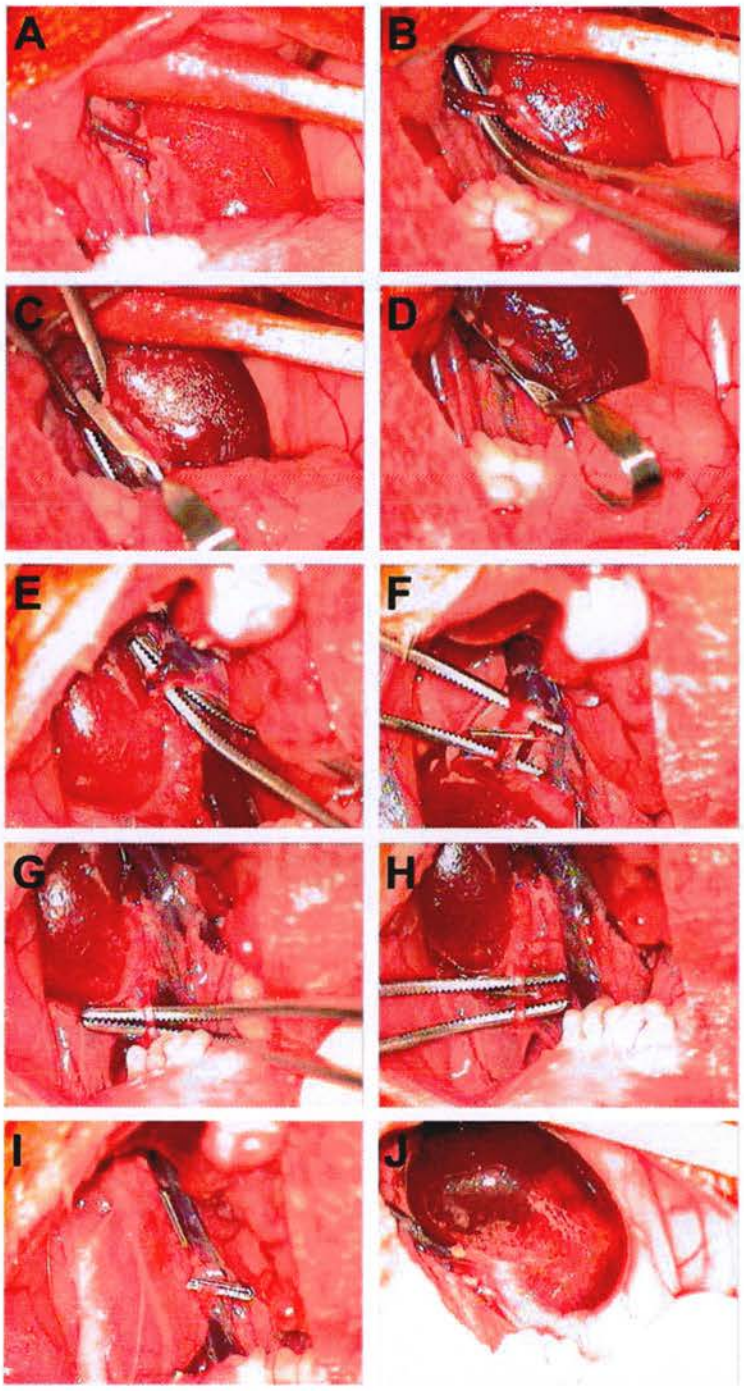


Figure 8.3 **IRI model in mouse.**
Left kidney exposed (A). Left renal pedicle dissected (B) and occluded (C, D). Right renal pedicle dissected (E) and clipped (F). Right ureter dissected (G) and clipped (H). Right kidney removed (I). Left renal occlusion removed (J).

8.2.4 Outcome measurements

Severity of injury following renal pedicle occlusion can be estimated by examining alterations in renal morphology or function.

Morphological outcomes following IRI

Histological changes in the kidney following ischemia are well described and in carefully controlled experiments correlate closely with measures of renal function (137). The degree of injury to the renal tubules can be determined following staining of sections with haematoxylin and eosin. As the magnitude of injury increases, the pattern of tubular damage progresses from dilatation and loss of the epithelial brush border to the detachment of epithelial cells and eventually full coagulative necrosis. With optimisation of the experimental procedure, a consistent renal morphological injury was produced (Figure 8.4). Other histological features of renal IRI were also noted including the presence of apoptotic bodies, hyaline casts and the infiltration of leukocytes (Figure 8.5). The presence or absence of these secondary features were not found to be good predictors of injury in our model. A semi-quantitative scoring system was adapted from (387) and developed. A method was sought that would continue to be predictive across the spectrum of mild to severe renal injury. Therefore, two scores were determined: the first based on the proportion of tubules exhibiting dilatation and/or a loss of the epithelial brush border (*tubular damage score,* TDS), the second based on the proportion of tubules with necrotic/detached cells (*tubular necrosis score,* TNS) (0, none; 1, less than 30%; 2, 30% to 70%; 3, more than 70%) (Figure 8.6). As can be seen in Figure 8.5, there is good correlation between the TDS/TNS and SCr (TDS R^2 0.76, $p<0.01$; TNS R^2 0.881, $p<0.01$).

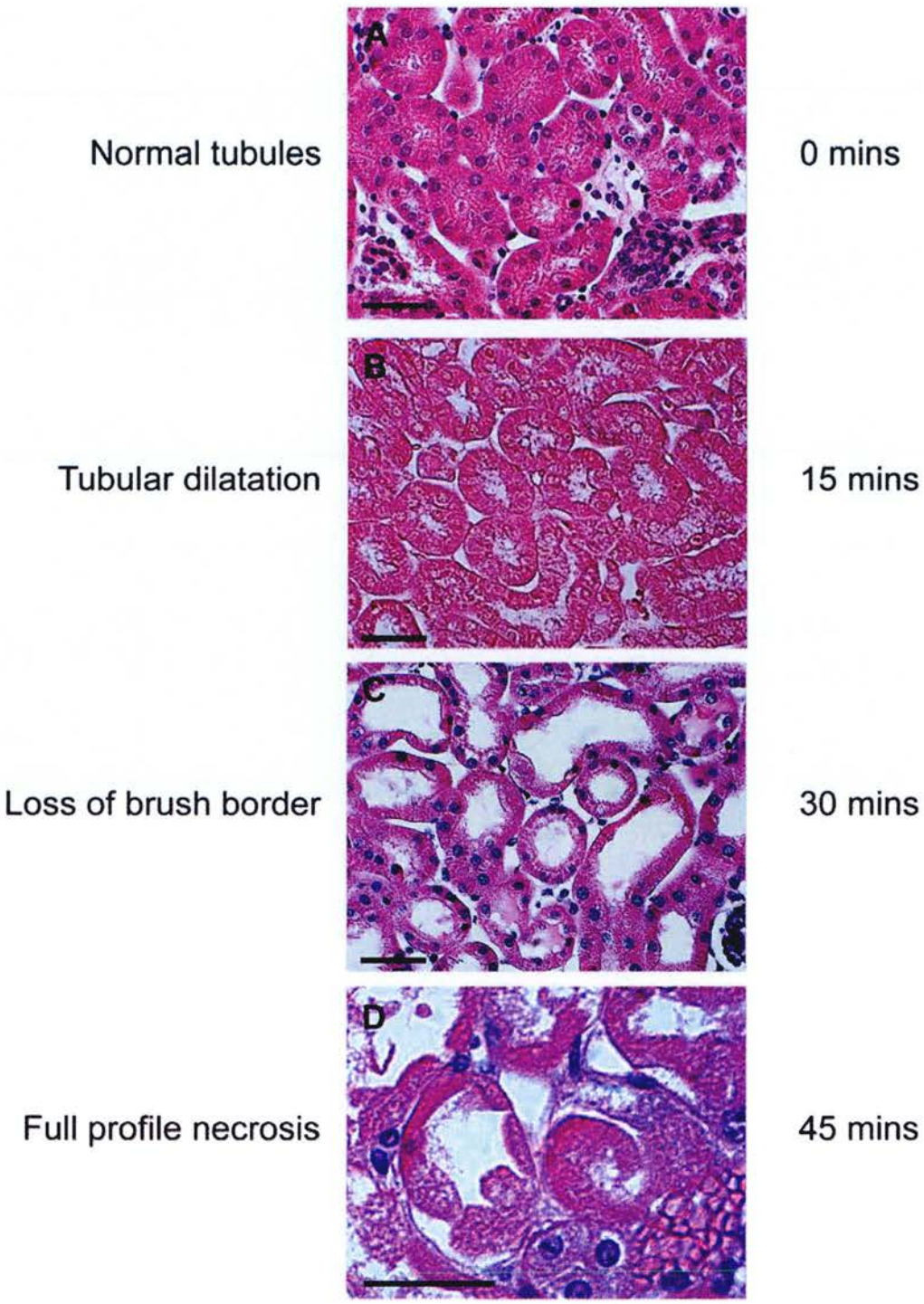


Figure 8.4 **Tubular injury 24 h following left renal pedicle clamping with indicative occlusion times.**
Scale bar = 50 μm.

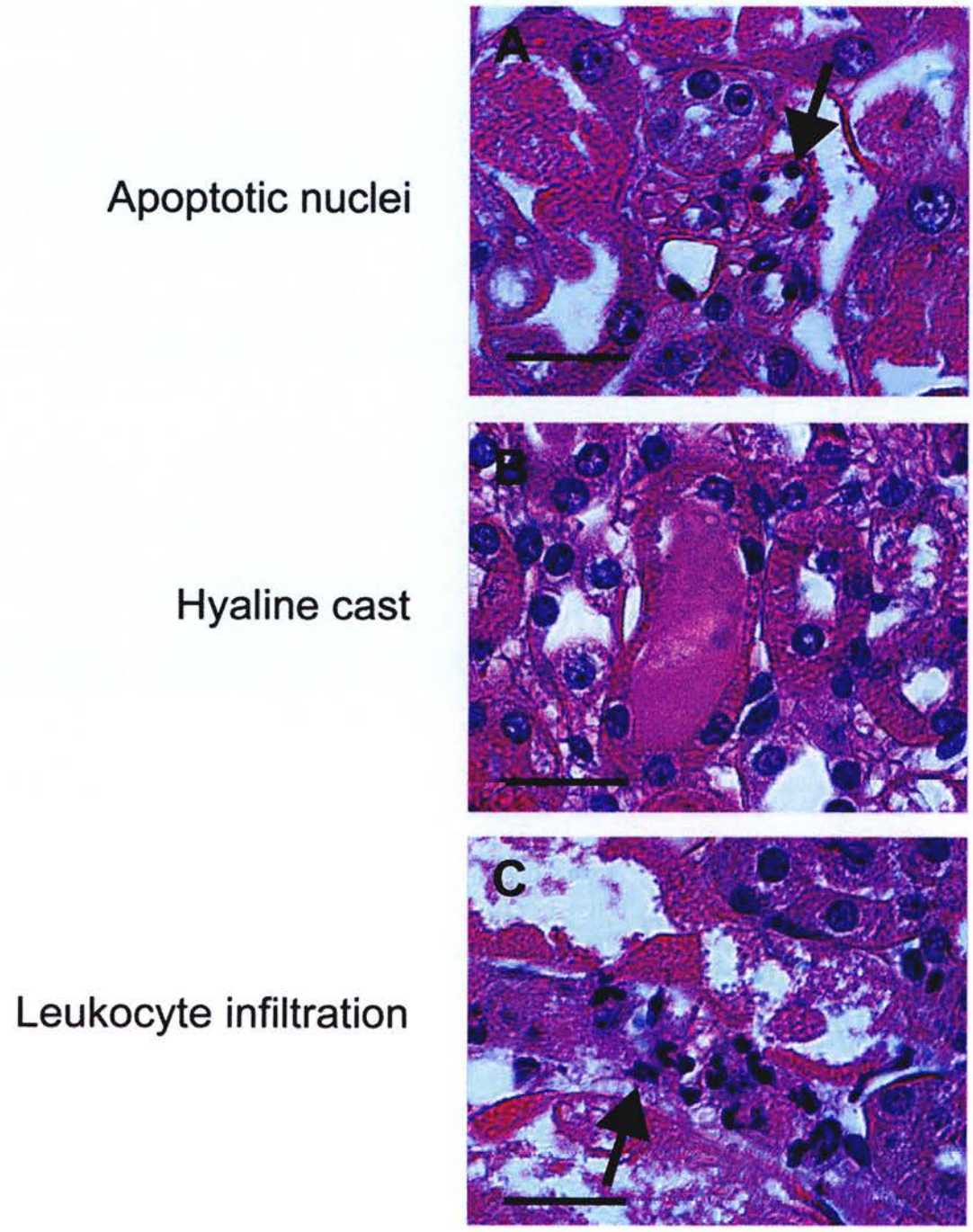


Figure 8.5 **Other histological features of renal IRI.**
Scale bar = 50 μm.

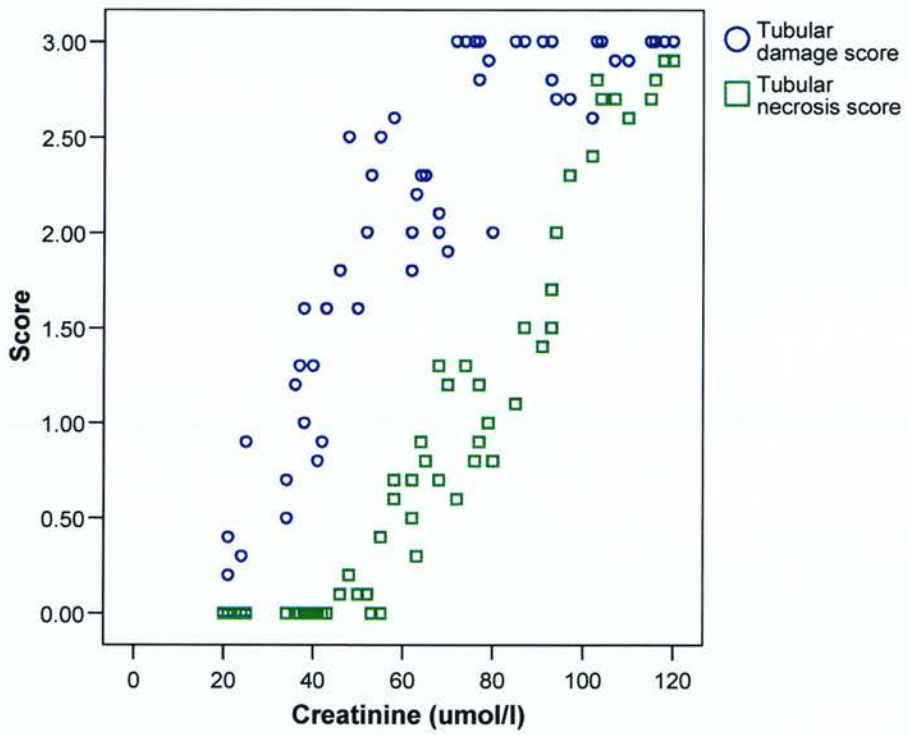


Figure 8.6 **Correlation between tubular damage score (TDS)/tubular necrosis score (TNS) and serum creatinine.**
Pooled data showing histological scores against serum creatinine. TDS R^2 0.76, $p<0.01$; TNS R^2 0.881, $p<0.01$.

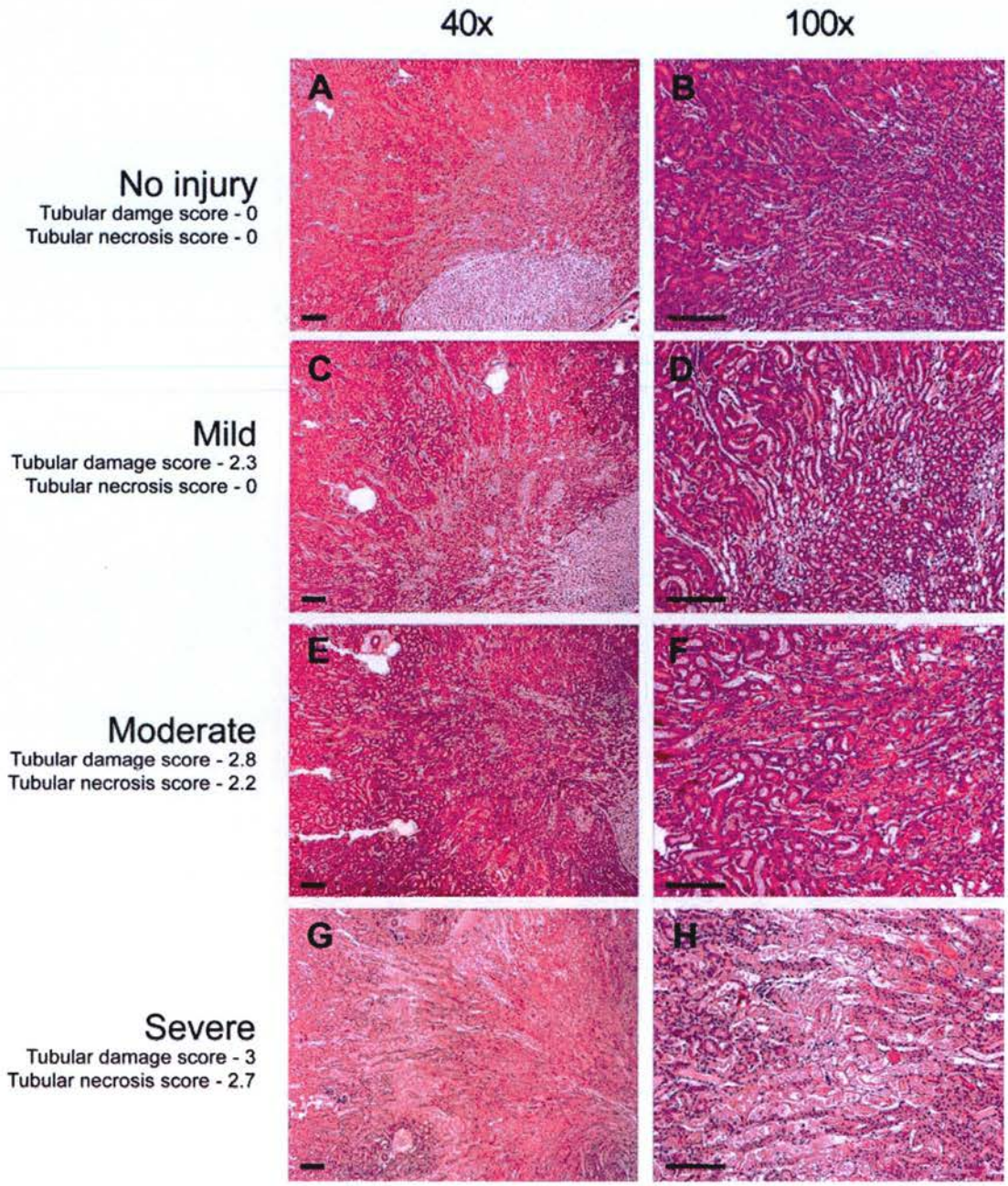


Figure 8.7 **Degrees of tubular injury 24 h following left renal pedicle clamping with typical tubular injury and tubular necrosis scores.**
Scale bar = 200 μm.

The TDS was better at predicting SCr in animals with a lesser injury, compared with the TNS which was a better predictor of SCr at greater magnitudes of injury. The sections were scored in a blinded manner by a consultant pathologist and me. Example sections of animals subjected to increasing degrees of injury can be seen in Figure 8.7 together with histological scores.

Function outcomes following IRI

Serum creatinine (SCr) and serum urea (SU) were measured as surrogate markers of glomerular filtration rate. Previous studies have demonstrated good correlation between SCr/SU and degree of injury. Measuring GFR by a more direct method such as FITC-conjugated inulin would have been a more direct alternative approach (394).

8.3 Controlling sources of variation

In initial experiments a great deal of variation was found in the SCr level following IRI. Controlling sources of variation proved invaluable in generating comparisons that were meaningful. Factors that contribute to unwanted variation can be described as fixed effects (Table 8.1) or random effects variables (Table 8.2). All fixed effects variables were accounted for in the design of the animal house and the management of the animals therein. There was no change in sex, strain, age or weight of animals during the experiments. Care was taken to ensure consistency between individual procedures performed within an experimental group. Animals were kept in a 12-h light/dark cycle, with free access to standard mouse chow and tap water.

More difficulty was met in controlling for random effects variables. A randomised block design was used to account for variation between batches of animals, social hierarchy within cages and time of year etc. Even after the procedure had been learned to a high standard, variation still occurred in animals that had an aberrant renal blood supply and animals where small amounts of bleeding occurred. In general, mice can tolerate a loss of 10% of their blood volume (around 0.2 ml), however, this had significant effects on the magnitude of IRI. Blood loss occurred less often with time but was carefully noted when it did. The most significant variable to control for was temperature. Small changes in temperature resulted in large differences in IRI. It was essential, therefore, that scrupulous attention was paid to reduce variation in the temperature of the animal. This meant controlling for the temperature of the room, the solutions, the length of time exposed and the temperature during recovery.

Fixed effects	Control
Animals	
Sex	All male
Strain	All BALB/c
Age	All 6 – 8 weeks
Weight	All 20 – 25 g
Procedure	
Ischemic injury time	Fixed for each set of experiments.
Time of cull	Animals culled at exact times after IR injury
Environmental conditions	
Bedding, feed etc.	All fixed

Table 8.2 **Fixed effect variables and controls in renal IRI model.**

Potential random effects	Control
Animals	
Genotypic and phenotypic variation.	Isogenic strain used to minimise this.
Differences in pain threshold/recovery time/fluid requirements after procedure.	Fixed volumes of subcutaneous fluids and analgesia administered per body weight animal. Some animals did recover more slowly which may have affected body temperature and fluid intake.
Variations in stock over time, different cage conditions.	Randomised block design used to account for these differences.
Procedure	
Learning curve	Early experiments affected, eliminated latterly.
Variation due to procedures being performed on different days.	Randomised block design used to account for these differences.
Aberrant renal blood supply.	Looked for during procedure and included in clamp if identified. Continued to be potential source of variation.
Blood loss.	The animals did not tolerate blood loss. An extremely accurate midline incision and careful dissection around the renal pedicle reduced blood loss to effectively zero in later experiments.
Temperature	
Temperature of solutions	All solutions warmed to 37 °C.
Temperature of heated mat	Thermostatically controlled, but significant variation existed.
Ambient temperature	Room temp fixed at 20 °C. Fairly constant.
Length of time exposed	Variation existed initially during learning curve, eliminated in latter experiments.
Temperature during recovery	In early experiments, animals recovered under heated lamp, but variation existed with different distances from lamp etc. Eliminated with use of incubator.

Table 8.3 **Random effect variables and controls in renal ischemia/reperfusion model.**

8.4 Experimental design

Mice were used at 6 – 8 weeks of age, were all male and were weight matched. Animals were caged in groups of 4 and a typical experiment would involve using the 4 animals for control, GA, 17-AAG and 17-DMAG. The cage was used as a blocking variable with two-way analysis of variance (without interaction). This removed variation that may occur between different cages/batches of animals. Residuals were found to be normally distributed and equal variances were found within each group.

Power calculation

A power calculation was performed to ensure adequate numbers were used in each group to maximise the opportunity of demonstrating statistically significant differences between treatments. A comparative study was used to estimate effect sizes for the potential reduction in serum urea and creatinine (137). Based on the reduction in urea and creatinine in treated animals subjected to 30 min bilateral renal IR injury, an effect size of 1.889 and 1.432 respectively was calculated (Table 8.4). This translated to a required sample size of between 8 and 12 animals per treatment group (395,396).

	Urea	Creatinine
Test significance level, α	0.05	0.05
Group 1 mean	53	1.13
Group 2 mean	20	0.60
Standard deviation	16.94	0.37
Effect size	1.889	1.432
Power (%)	90	90
n per group	8	12

Table 8.4 **Power calculation for group sizes**

Chapter 9 – Heat shock protein 90-binding agents in a murine model of ischemia/reperfusion injury

9.1 Introduction

In chapter 7, it was demonstrated that HBAs could protect renal cells from an H_2O_2-mediated stress in vitro. This protection was abrogated in cells that had been pre-treated with HSF1 siRNA, providing strong evidence that protection is HSF1 mediated. However, one weakness in this part of the study was the use of a transformed cell-line. It is possible that the responses observed in these cells do not reflect the resultant effect in the physiological environment encountered in the kidney during organ transplantation. As has been discussed, a major factor that influences outcome following organ transplantation is IRI. Using the model developed in the last chapter, the aim of the work described in this chapter was to determine whether Hsp90 inhibition could reduce the damage caused by renal IRI. Experimental work began by establishing whether treatment with HBAs induced Hsps in the mouse, and in which organs. With this information, the work proceeded to examine whether HBAs could protect kidneys from IRI.

9.2 Hypotheses

1. HBAs cause up-regulation of Hsps in the mouse kidney.

2. HBAs cause up-regulation in other transplantable organs in the mouse.

3. HBAs cause up-regulation of Hsps in areas of the kidney that are susceptible to IRI.

4. HBAs confer morphological and functional protection to the kidney following IRI.

9.3 Methods

9.3.1 Tissue collection

Under terminal general anaesthesia, blood was recovered by intra-cardiac puncture. This was centrifuged at 13 000 rpm/4 °C and the serum stored at -20 °C until analysis. The left kidney was divided in the transverse plane in two equal parts to ensure equivalent proportions of cortex and medulla with the superior portion snap-frozen in liquid nitrogen and the inferior portion being placed in methacarn (70% methanol, 20% chloroform, 10% glacial acetic acid). The liver, lungs and heart were then removed and placed in liquid nitrogen. All frozen tissue was maintained at -70 °C until analysis.

9.3.2 Hsp70 enzyme-linked immunosorbent assay (ELISA)

Hsp70 expression in different mouse tissue following treatment with HBAs was quantified using a commercially available Hsp70 ELISA kit (Stressgen) as per the manufacturer's instructions.

9.3.3 Serum urea and creatinine determination

Serum was analysed using an automated Olympus AU 2700 Clinical Chemistry System.

9.3.4 Caspase 3/7 ELISA

Caspase 3/7 activity in kidney tissue was measured using a Caspase-Glo 3/7 assay kit (Promega) with a modified protocol. The assay has previously been shown to detect caspase 3/7 activity in mouse liver (397). Briefly, whole kidney extracts were prepared as for the Hsp70 ELISA. Equal volumes of caspase 3/7 reagent and kidney extract were added to a white-walled 96-well plate and incubated at room temperature for 1 h. Luminescence was detected using a plate-reading luminometer (Fluoroskan Ascent Fl, Thermo Electron, Basingstoke, UK).

9.4 Results

HBAs induce heat shock proteins in mice

BALB/c mice were given an intraperitoneal injection of HBAs and sacrificed at various times. Organs were snap-frozen in liquid nitrogen and whole-organ lysates prepared. Neither Hsp90 nor HO-1 were induced by the HBAs in mice (Figure 9.1). A 2 – 3-fold increase in Hsp70 was seen following HBA administration. This was maximal at 6 h in mice treated with 17-DMAG, which was significantly earlier than in GA or 17-AAG treated groups. A 2 fold increase in Hsp25 was seen which again, occurred significantly earlier in the 17-DMAG group (6 h) compared with the GA and 17-AAG group. Hsp70 and Hsp25 induction was maintained in all groups until at least 24 h. An ELISA for Hsp70 was performed with whole-organ lysates and showed significant induction of Hsp70 in kidney, liver and lung following HBA administration (Figure 9.2A, B and C). A 3 fold increase was seen in liver which became statistically significant at 8 h, 16 h and 6 h for GA, 17-AAG and 17-DMAG respectively. A 2 – 3 fold increase in Hsp70 was found in lung which was significant at 6 h in 17-AAG and 17-DMAG groups; an increase occurred in animals treated with GA, but this did not reach significance. In lung there was a drop-off of Hsp70 levels between 8 and 16 h. In the heart, Hsp70 was found to be expressed in animals that had been treated with HBAs for 6 or 8 h (Figure 9.2D). Hsp70 levels at all other time points appeared to be less than the dynamic range of the test.

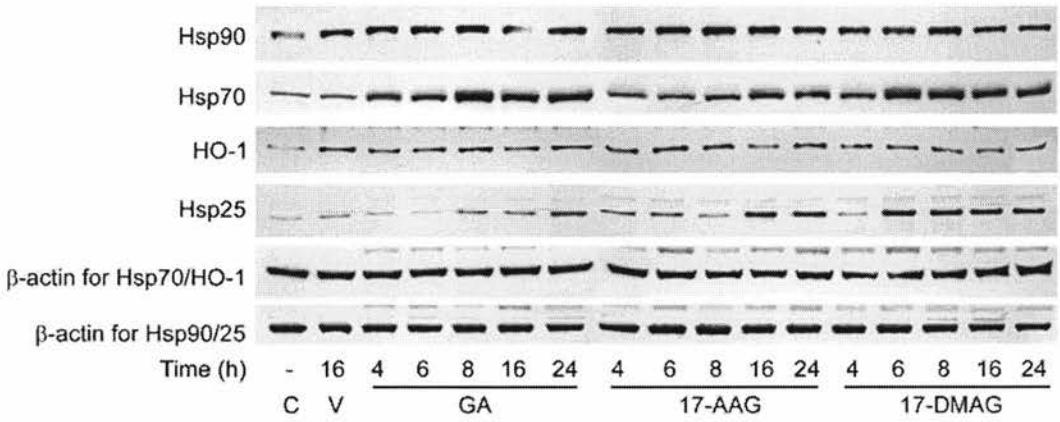


Figure 9.1 **Hsp expression in mouse kidney following HBA treatment.** BALB/c mice given an intraperitoneal injection of GA, 17-AAG, 17-DMAG (all 1 mg/kg) or vehicle and were sacrificed at various time points (n = 3/time-point). Whole-organ lysates were made and Western blots performed.

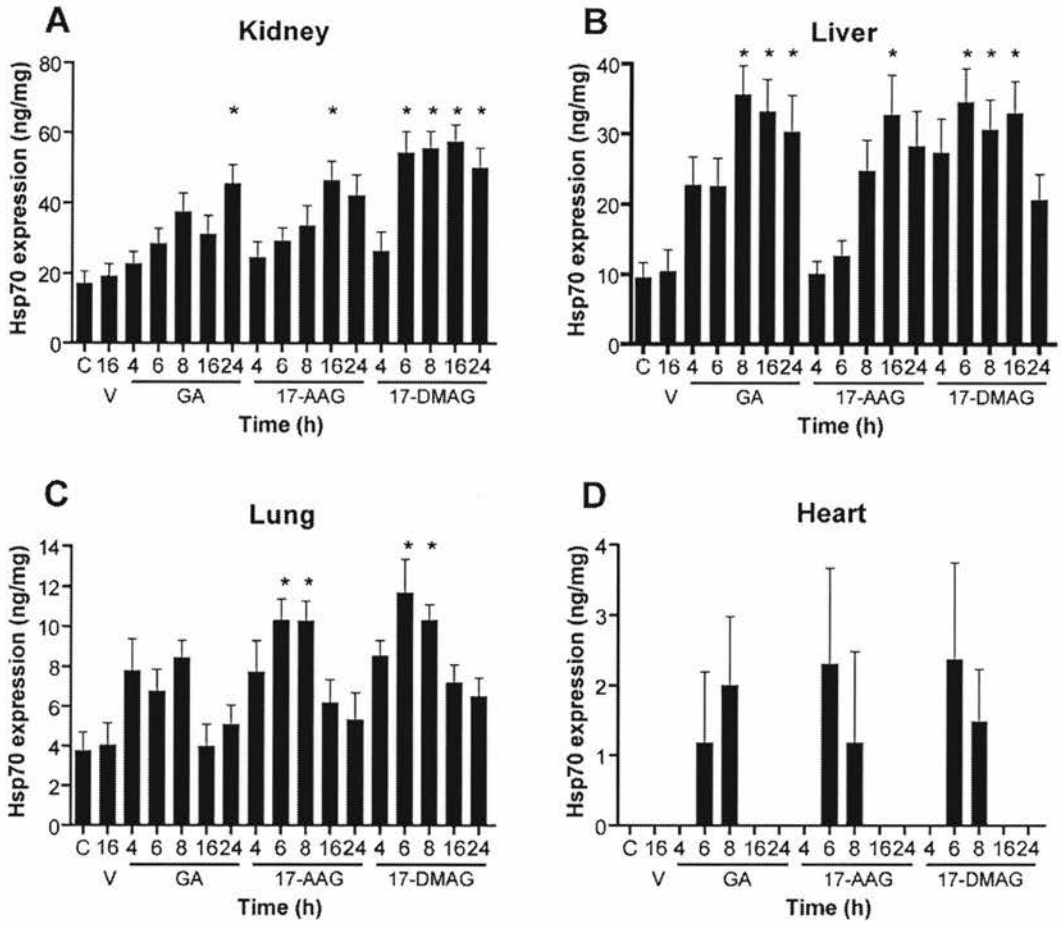


Figure 9.2 **Hsp expression in mouse kidney, liver, lung and heart following HBA treatment.**

BALB/c mice given an intraperitoneal injection of GA, 17-AAG, 17-DMAG (all 1 mg/kg) or vehicle and were sacrificed at various time points (n = 51). Kidney (A), liver (B), lung (C) and heart (D) were snap-frozen in liquid N_2. Whole-organ lysates were made and an Hsp70 ELISA performed. * $p<0.05$ (ANOVA).

HBAs induce Hsps in the cortex and outer medulla of the kidney

Immunohistochemistry was performed to localise the expression of Hsp70 and Hsp25. BALB/c mice were injected with 17-DMAG (Figure 4E – H and M – P) (1 mg/kg) or vehicle (Figure 4A – D and I – L). In vehicle-treated mice at 16 h, Hsp70 immunopositivity was maximal in the tubular epithelial cells of the inner medulla (large arrow; Figure 9.3B). Significant immunopositivity was also seen in the outer medulla (Figure 9.3A and D) with much lower levels in the cortex (Figure 9.3A and C). Following treatment with HBAs, Hsp70 immunopositivity was increased in the cortex (Figure 9.3E and G) but most strongly increased in the outer-stripe of the outer medulla (OSOM) (small arrow; Figure 9.3F and H).

Hsp25 was found to be expressed at lower levels than Hsp70 which is consistent with the Western blot findings (Figure 9.4). Basal expression was found to increase from the outer medulla, through the inner medulla, with maximal immunopositivity in the epithelial cells of the collecting ducts and ducts of Bellini in the renal papillae (Figure 9.5A). Immunopositivity was also strong in afferent and efferent arterioles (Figure 9.5B), vascular smooth muscle cells and endothelium (Figure 9.5C) and the urothelium (Figure 9.5D). Following treatment with HBAs, immunopositivity increased in the tubular epithelium of the OSOM (small arrow; Figure 9.4E and H), and to a lesser extent in the cortex (Figure 9.4G).

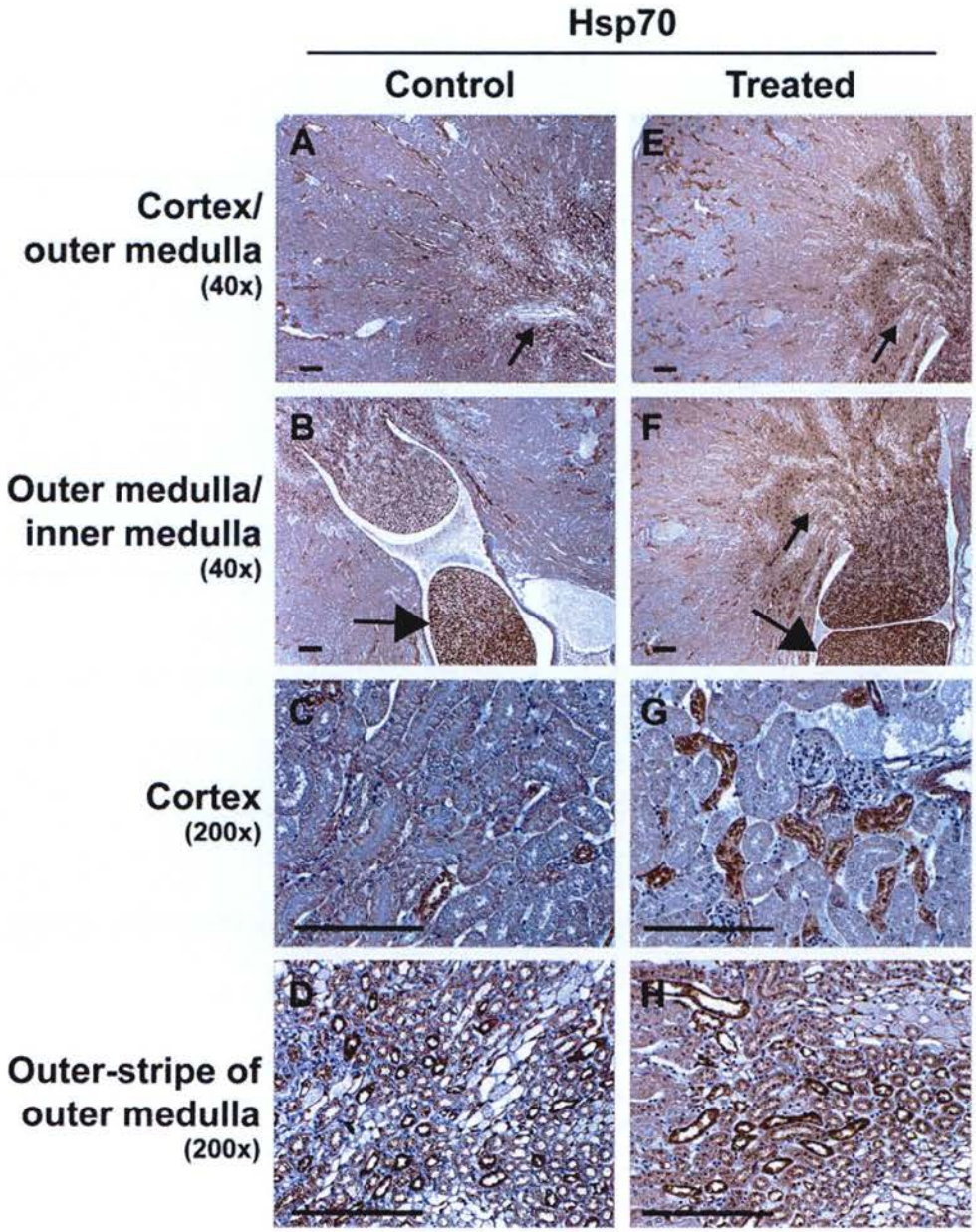


Figure 9.3 **Hsp70 expression in mouse kidneys following HBA treatment.** BALB/c mice were given an intraperitoneal injection of vehicle (A – D) or 17-DMAG (1mg/kg; E – H) and sacrificed at 16 h. Immunohistochemistry was performed using antibody to Hsp70 and visualised with DAB. Hsp70 basal immunopositivity was maximal in the inner medulla (large arrow; B) and maximum induction following HBA treatment was seen in the outer-stripe of the outer medulla (small arrow; E). Scale bar = 200 μm.

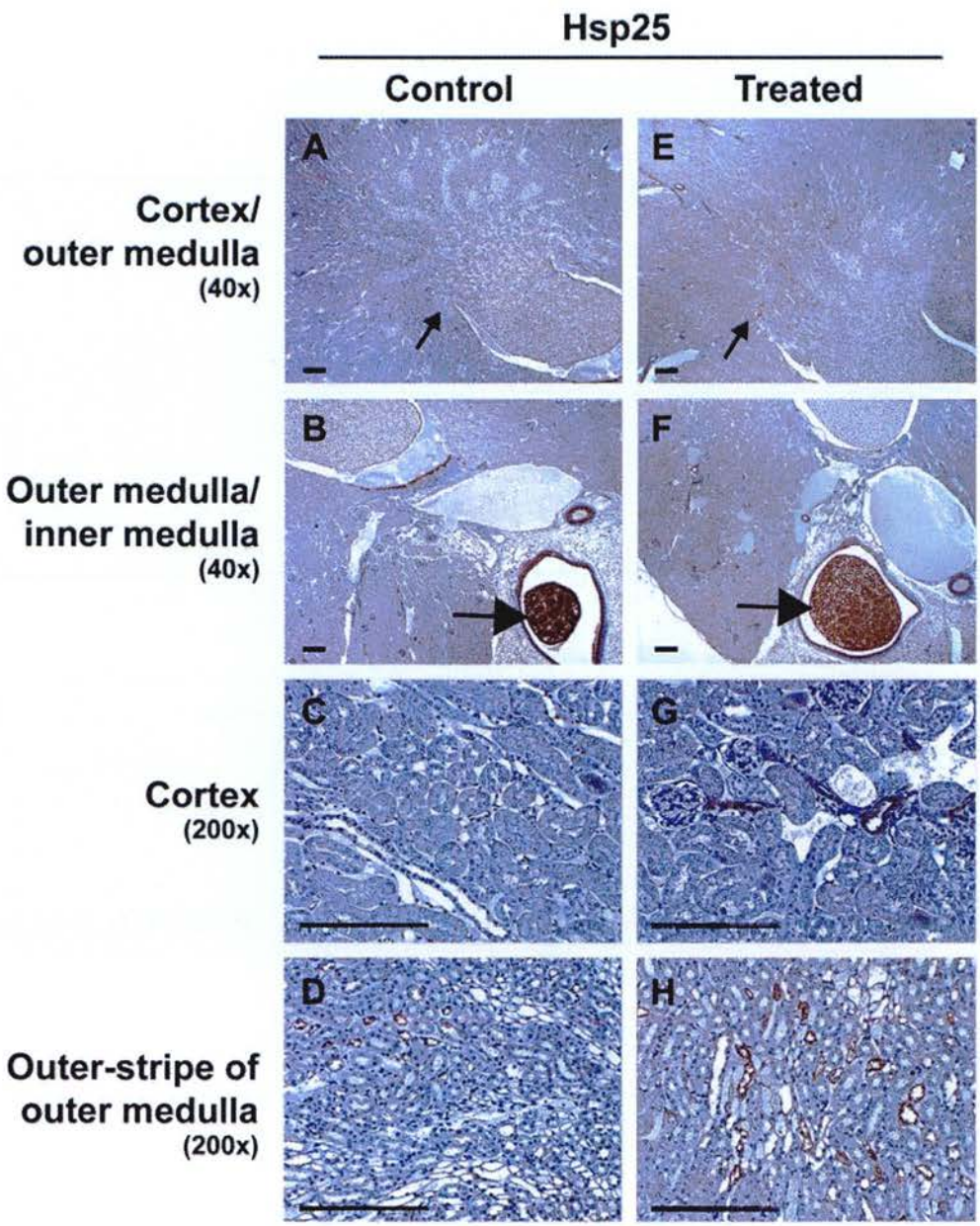


Figure 9.4 **Hsp25 expression in mouse kidneys following HBA treatment.** BALB/c mice were given an intraperitoneal injection of vehicle (A – D) or 17-DMAG (1mg/kg; E – H) and sacrificed at 16 h. Immunohistochemistry was performed using antibody to Hsp25 and visualised with DAB. Sections were counter-stained with haematoxylin. Hsp25 basal immunopositivity was maximal in the inner medulla (large arrow; B) and maximum induction following HBA treatment was seen in the outer-stripe of the outer medulla (small arrow; E). Scale bar = 200 μm.

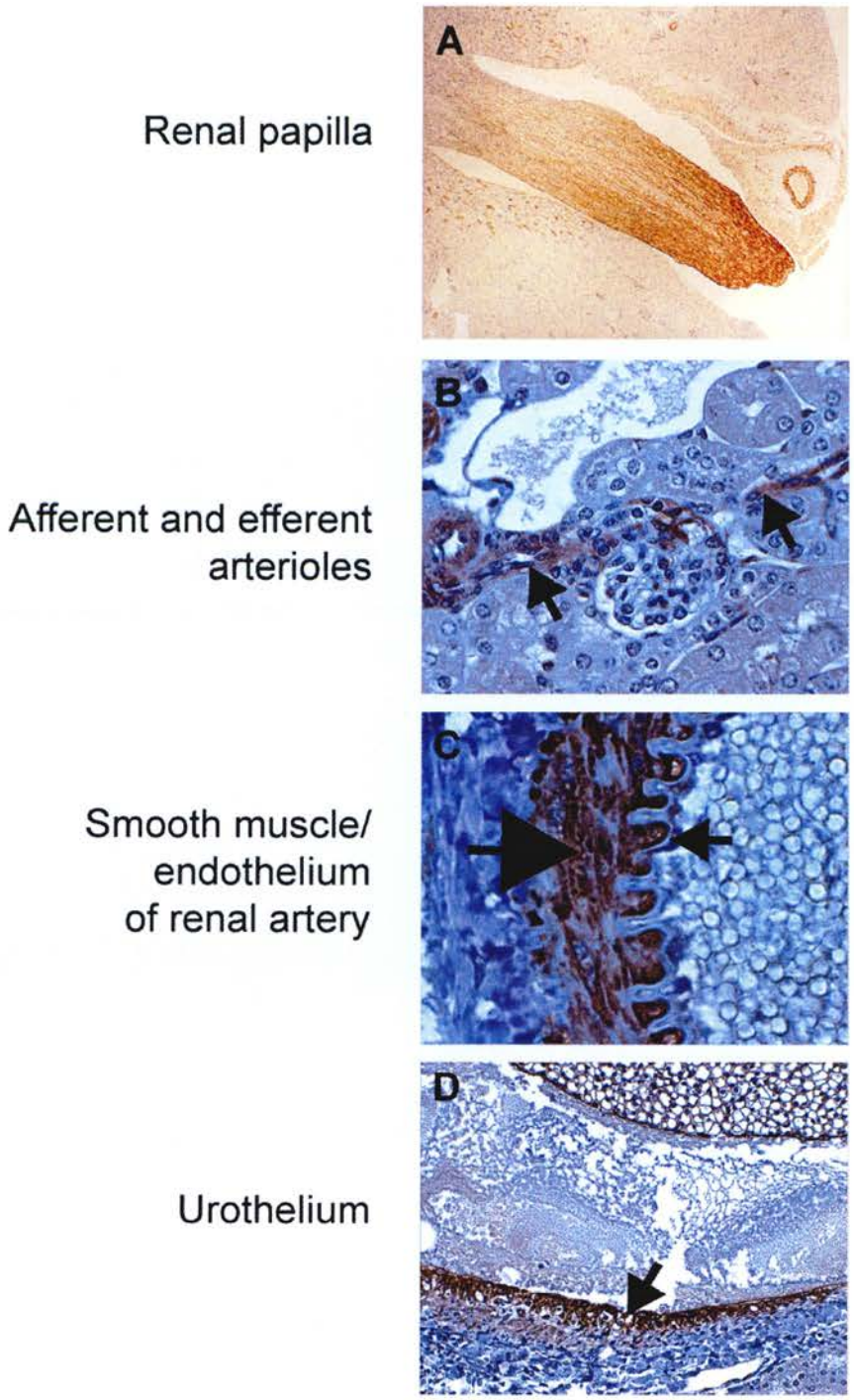


Figure 9.5 **Additional histological features of basal Hsp25 immunopositivity.** Immunohistochemistry was performed using antibody to Hsp25 and visualised with DAB on untreated mouse kidney. Sections were counter-stained with haematoxylin. Hsp25 basal immunopositivity increased from the medulla towards the end of the papilla (A); was apparent in afferent and efferent arterioles (B) and the smooth muscle (large arrow) and endothelium (small arrow) of the renal artery (C); and was also strong in urothelium (D).

HBAs do not cause liver damage within 1 week in the mouse

Given that the GA has been associated with hepatic toxicity in other models, the livers of mice treated with HBAs were examined. BALB/c mice were given an intraperitoneal injection of GA, 17-AAG and 17-DMAG (all 1mg/kg) and sacrificed 7 days later (Figure 9.6). Sections were examined and no evidence of liver injury was observed. There was no morphological difference in liver sections from animals treated with GA, 17-AAG or 17-DMAG.

HBAs reduce morphological renal damage following IRI

BALB/c mice were treated with GA, 17-AAG, 17-DMAG or vehicle. After 16 h, left renal artery pedicle clamping was performed for 30 min in combination with contralateral nephrectomy. 24 h later, animals were sacrificed and haematoxylin and eosin-stained kidney sections examined and scored by two independent observers (Figure 9.7A – D). Histological injury following left renal pedicle artery clamping was less severe in animals pre-treated with HBAs, particularly in the OSOM (small arrow; Figure 9.7A – D). This was scored in a semi-quantitative manner. Tubular damage score was reduced in mice treated with HBAs and was statistically significant in the 17-AAG and 17-DMAG groups (Figure 9.8A; $p<0.05$). Tubular necrosis score was reduced in mice treated with HBAs and was significant in all groups (Figure 9.8B; $p<0.01$).

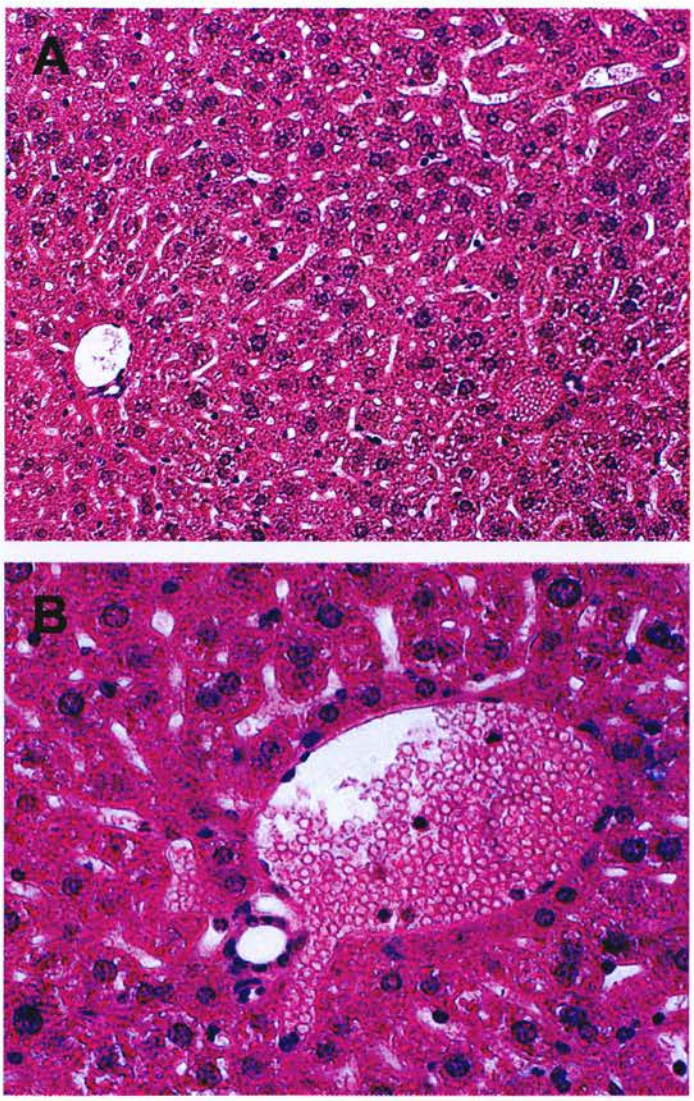


Figure 9.6 **Mouse liver 7 days after GA treatment.**
BALB/c mice were given an intraperitoneal injection of GA (1mg/kg) and sacrificed 7 days later. The liver was fixed and sections stained with haematoxylin and eosin. On examination of low power (A) and high power (B) fields, no evidence of liver injury was observed. These sections are also representative of liver sections taken 17-AAG and 17-DMAG treated mice.

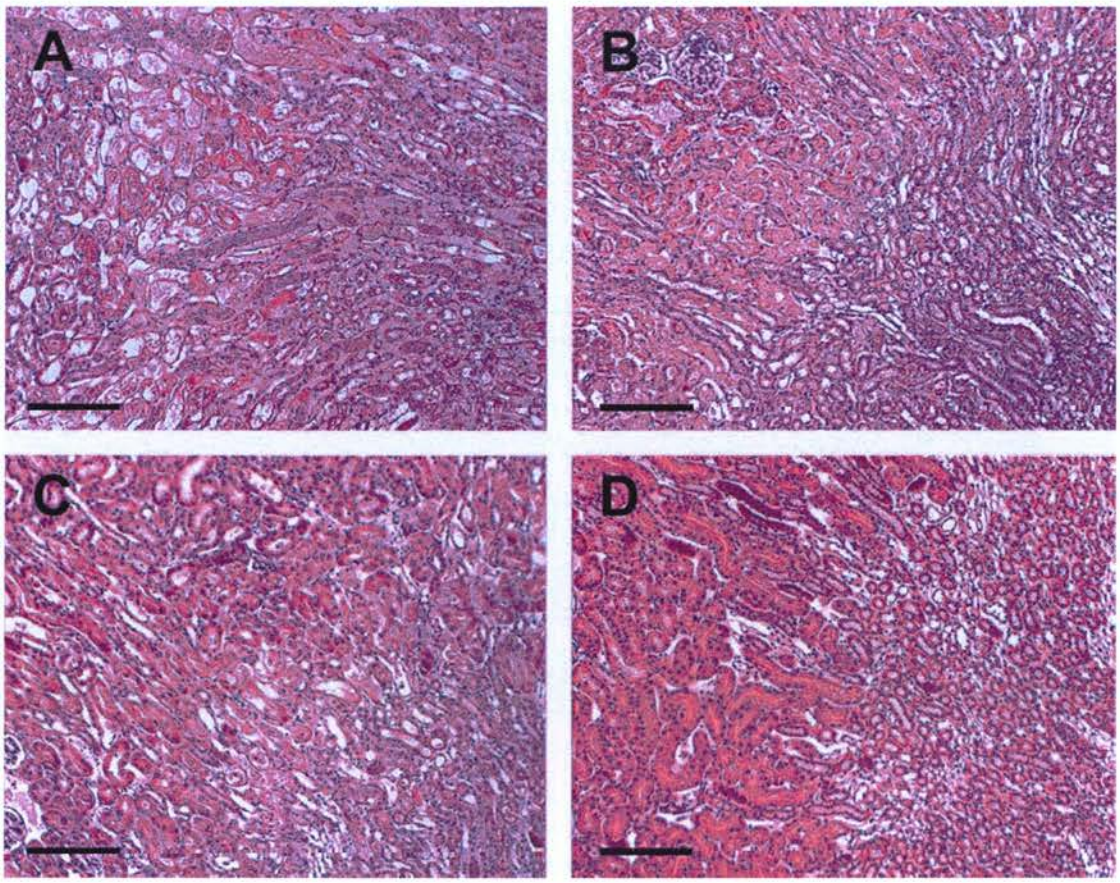


Figure 9.7 **Renal injury following treatment with HBAs and renal pedicle clamping.**
BALB/c mice (n = 50) were pre-treated with an intraperitoneal injection of vehicle (A), GA (B), 17-AAG (C) or 17-DMAG (D) (all 1mg/kg). After 16 h the left renal pedicle was clamped for 30 min during which the right kidney was removed. 24 h later the animals were sacrificed and the left kidney was placed immediately in methacarn. Sections were stained with haematoxylin and eosin and show the outer-stripe of the outer medulla. Scale bar = 200 μm.

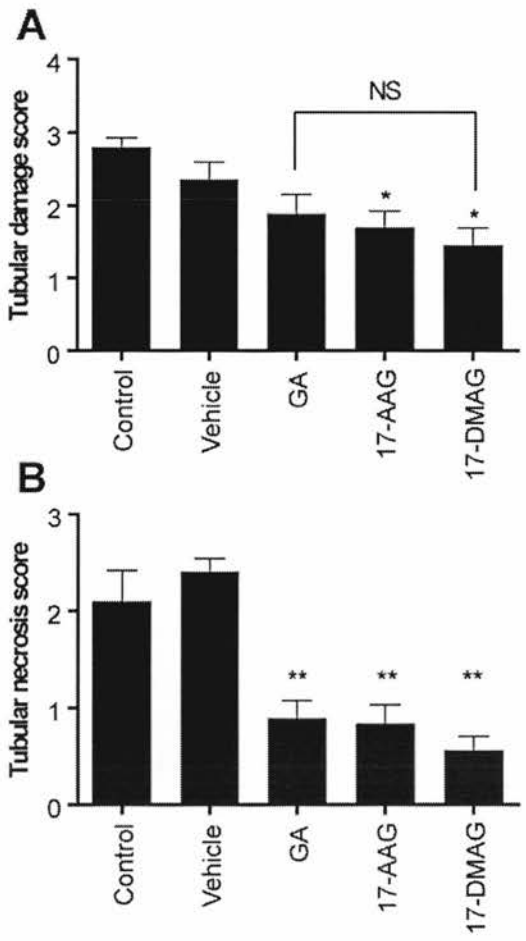


Figure 9.8 **Histological injury scores following treatment with HBAs and renal pedicle clamping.**
BALB/c mice (n = 50) were pre-treated with an intraperitoneal injection of vehicle (A), GA (B), 17-AAG (C) or 17-DMAG (D) (all 1mg/kg). After 16 h the left renal pedicle was clamped for 30 min during which the right kidney was removed. 24 h later the animals were sacrificed and the left kidney was placed immediately in methacarn. The tubular damage (E) and necrosis scores (F) were determined by two independent observers. * $p<0.05$, ** $p<0.01$ compared with vehicle (Mann-Whitney U test).

HBAs reduce functional renal damage following IRI

Serum urea and creatinine were significantly increased following 30 min IRI and contralateral nephrectomy (Figure 9.9A and B). In mice that were pre-treated with HBAs, both urea and creatinine were found to be lower 24 h after IRI than vehicle treated groups. This was significant in the 17-AAG ($p<0.05$) and 17-DMAG ($p<0.01$) groups. There was a significant difference in serum urea between those treated with GA and 17-DMAG treated groups ($p<0.05$).

HBAs alone increase caspase 3/7 proteolytic activity in mouse kidneys but effect a reduction in overall caspase 3/7 activity following IRI

As before, BALB/c mice were given an intraperitoneal injection of HBAs, sacrificed at various times and whole kidney lysates prepared. An increase in renal caspase 3/7 activity was seen after 6 h which became statistically significant for all HBAs at 24 h (Figure 9.10A). In animals treated with HBAs and subjected to IRI 16 h later, caspase 3/7 activity was reduced at 24 h, although this only reached statistical significance in the GA group.

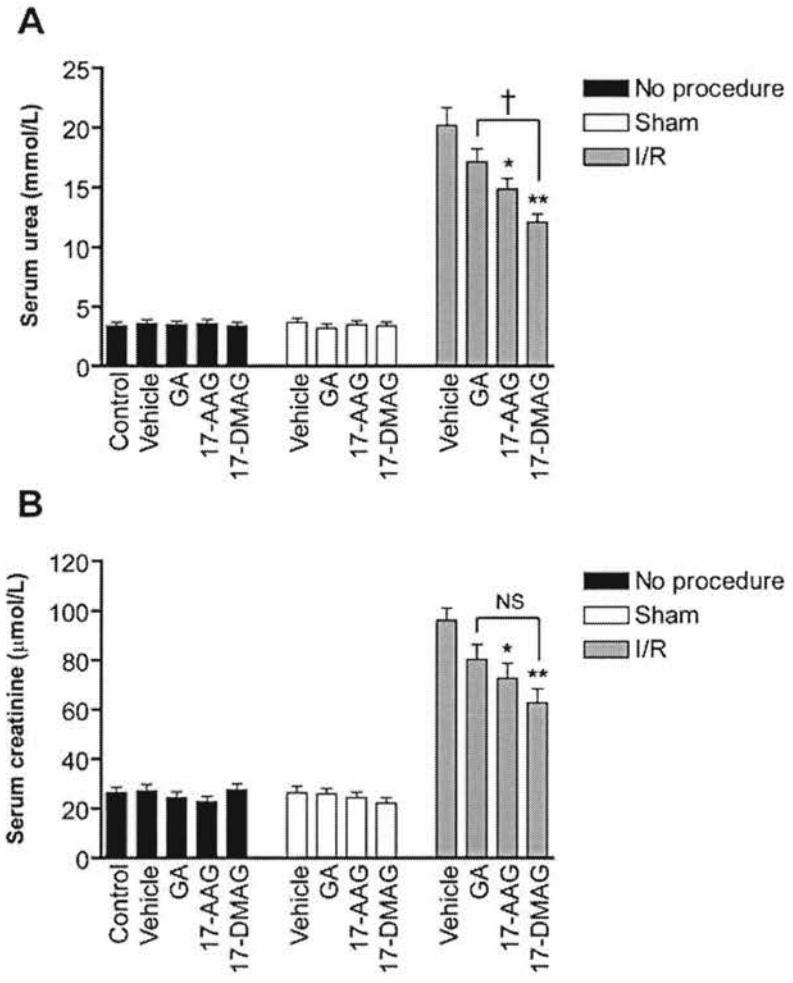


Figure 9.9 **Serum urea and creatinine following treatment with HBAs and renal pedicle clamping.**

BALB/c mice (n = 67)were pre-treated with an intraperitoneal injection of vehicle (A), GA (B), 17-AAG (C) or 17-DMAG (D) (all 1mg/kg). For animals undergoing no procedure, n = 3/treatment; sham, n = 3/treatment; IR, n = 10 / treatment.. After 16 h the left renal pedicle was obstructed for 30 min during which the right kidney was removed. 24 h later the animals were anaesthetised and blood was recovered by intracardiac puncture. Serum urea and creatinine were determined by auto-analyser. * $p<0.05$, ** $p<0.01$ compared with vehicle; † $p<0.05$ (ANOVA).

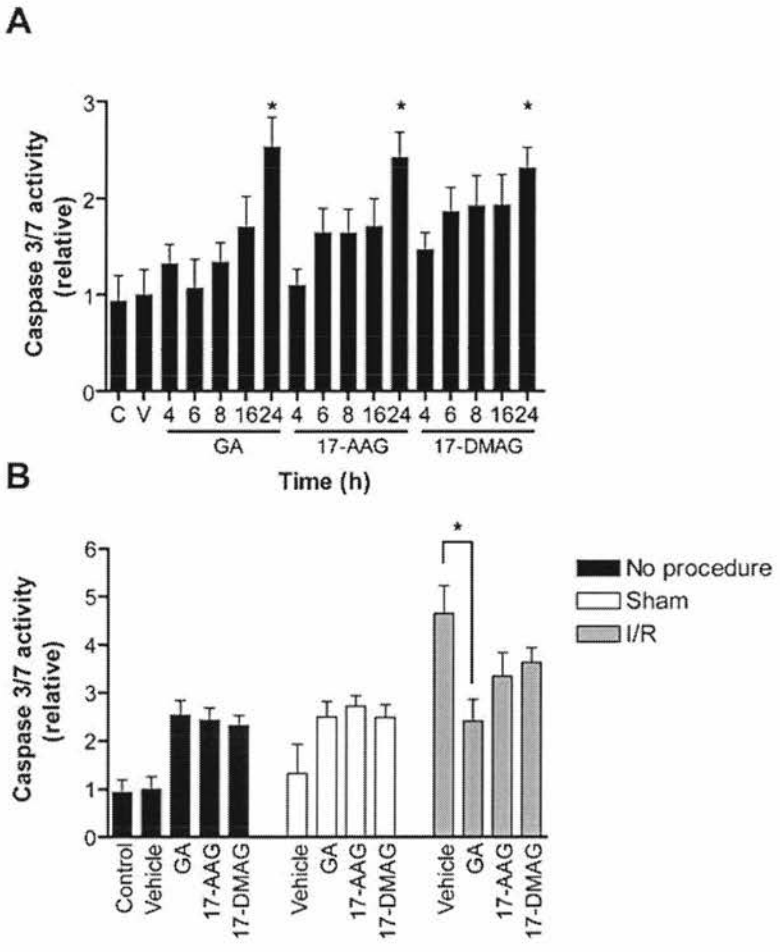


Figure 9.10 **Caspase 3/7 expression following treatment with HBAs with (B; n = 67) and without (A; n = 51) renal IRI.**
BALB/c mice were given an intraperitoneal injection of GA, 17-AAG, 17-DMAG (all 1 mg/kg) or vehicle and were sacrificed at various time points (A; n = 3/time point). In a separate set of experiments, mice treated with HBAs underwent left kidney pedicle clamping with right nephrectomy, or a sham procedure 16 h later. For mice undergoing no procedure, n = 3/treatment; sham, n = 3/treatment; IR, n = 10/treatment. After 24 h, the left kidney was snap-frozen in liquid N_2. Whole-organ lysates were made and a caspase 3/7 ELISA performed. * $p<0.05$ compared with vehicle (ANOVA).

9.5 Discussion

Hsps are known to reduce the effects of IRI in models of transplantation, yet practical strategies of up-regulation that may be implemented clinically remain elusive. In this chapter, it has been demonstrated that treatment with GA, 17-AAG or 17-DMAG results in increased expression of Hsp70 and Hsp25 in mouse kidneys, liver and lung and that this increased expression is associated with renal protection following renal IRI in a model which included contralateral nephrectomy.

In the mouse, Hsp70 and Hsp25 induction was significantly earlier following 17-DMAG than the other HBAs (Figure 9.1 and 9.2). 17-DMAG-treated animals subjected to IRI had a serum urea at 24 h that was significantly lower than animals pre-treated with GA (Figure 9.9A). As was discussed in the previous chapter, it is possible that differences in action at the molecular level exist between GA and 17-AAG/17-DMAG, but 17-DMAG is the only one of the three drugs that is water-soluble and improved pharmacokinetics may explain these differences (366).

The baseline distribution of Hsp70 and Hsp25 was found to be similar to previous descriptions: present in the renal cortex and outer medulla, but particularly abundant in the inner medulla (398,399) (Figure 9.3 and 9.4). Hsp25 staining has also been shown to be strong in the vascular endothelium and smooth muscle cells (400) (Figure 9.5). HBA treatment resulted in significant induction of Hsp70 in the cortex and but was most striking in the OSOM (Figure 9.3F). This is particularly significant as the OSOM is most susceptible to ischemic injury. The increase in Hsp25 was less

pronounced but still apparent, particularly in the outer-stripe of the outer medulla (Figure 9.4E and H).

As was discussed in the last chapter, apoptosis following HBA treatment appears to be cell-type dependent. Caspase 3/7 activity was used as a measure of apoptotic activity in whole - kidney homogenates with and without prior IRI. A 2.5 fold increase in caspase 3/7 activity was seen 24 h after IP injection of the HBAs. IRI increased caspase 3/7 activity in vehicle treated cells 5 fold, but pre-treatment with HBAs reduced this towards the levels of sham-treated animals. It may be that HBAs have a direct off-target effect on caspase but this is not clinically evident as increased cell injury *in vivo* or increased cell death *in vitro*. In any case, it is likely that the actual levels of apoptosis following HBA treatment *in vivo* are quite small, and are greatly outweighed by the benefits of Hsp induction.

Hsp70 induction was found in kidney, liver, lung and heart following HBA treatment. This is particularly significant in the context of the multi-organ donor, as the potential exists for protection to be conferred in all recovered organs. Concerns have been expressed that Hsp70 may be induced following brainstem death and therefore therapeutic up-regulation would provide little additional benefit. However, Hsp70 has been shown to be only basally expressed in the kidney prior to engraftment and hemodynamic instability in donors and the process of organ recovery were not found to produce a measurable increase in Hsp70 (401). This supports a view that therapeutic Hsp70 induction remains potentially beneficial.

These data demonstrate that HBAs have the potential of imparting protection to kidneys subjected to IRI through a stress protein-mediated mechanism. The effect size was greatest with 17-DMAG which is water soluble and has a favourable toxicity profile compared with GA. Increased expression of Hsps was evident in kidney, liver, lung and to a lesser extent heart, which is important considering the practical application in a clinical setting would require administration to potential multi-organ donors.

Chapter 10 – General discussion

This thesis has examined potential pharmacological strategies to stimulate the heat shock/stress protein response and protect organs from IRI. Data presented demonstrate that alteration of PI3K/Akt activity does not alone alter HSF1 activation state; that stimulation of the PI3K/Akt axis can induce HO-1 through activation of the Nrf2 transcription factor; that inhibition of Hsp90 activity alters HSF1 activation and induces Hsps; that Hsps can be up-regulated by Hsp90 inhibitors *in vivo*; and that Hsp90 inhibition protects cells *in vitro* and reduces IRI in mouse kidneys. Data from this thesis has been the subject of a number of presentations (Appendix IV, page 287) and has been published in peer-reviewed journals (Appendix V, page 288).

10.1 Potential weaknesses in hypotheses

10.1.1 Are there long-term benefits from reducing ischemic injury in renal transplantation?

The over-arching aim of this thesis was to utilise the heat shock/stress protein response to reduce the effects of IRI in kidney transplantation. Fundamental to this is the question: does increasing IRI correlate with a negative clinical outcome? The relationship between the cold ischemic time (CIT; a surrogate for IRI), delayed graft function and poorer long-term outcome is well established (Table 1.3). However, in the majority of the studies referenced, CIT is only considered as a binary variable: typically <12 h or >12 h. When the influence of CIT is compared between organs subjected to shorter ischemic times, the difference in outcome is less significant. If a

drug acts only to reduce the direct effects of IRI, it is interesting to consider how many "hours" of cold ischemic time might be saved by the intervention, i.e. would an organ pre-treated with a protective drug and subjected to 6 h of ischemia have a similar outcome to an un-treated organ made ischemic for, say, only 2 h? The most recent data on CIT comes from a large US registry study of living donors (n = 38,467) and examines CIT in 2 h strata (i.e. 0-2 h, 2-4 h, 4-6 h, 6-8 h) (402). Although a significant difference was demonstrated in the occurrence of DGF between the 0-2 h (4.7%) and 4-6 h (8.3%) groups, this did not influence 1 y serum creatinine levels. There was no difference in death-censored graft survival in any group with > 2 h CIT compared with the 0-2 h group (median survival 11 y). Therefore, in this group of patients, the added effect of IRI between 2 and 8 h has little or no influence on outcome. The caveats to this analysis are: 1) These are living donor grafts and are typically in excellent condition and subject to very low rates of DGF anyway, 2) As in the mouse model of IRI, the rate of change of the magnitude of injury between early time points is likely to be low (Figure 8.2), but will increase at later time points, e.g. 2 h CIT "saved" after 12 h maybe of greater benefit than 2 h saved before 12 h. It is interesting to speculate whether the use of a protective drug in living donors would be beneficial, but the answer is that they would probably not be. The potential side-effects of drugs in the living donor must also be considered.

Organs from deceased donors are still subjected to long periods of ischemia during transportation to well-matched recipients (the average CIT in the United Network for Organ Sharing register is 20 h and has not changed over the years). The influence on outcome of longer CITs is pronounced, particularly with CIT >30 h. Comparing

deceased donor organs subjected to <10 h cold ischemia, Salahudeen *et al* recently showed that 6-year graft survival was worse (but not significantly) in the 10-20 h CIT group (RR = 1.03, p = 0.79), 21-30 h CIT (RR = 1.12; p=0.27) and significantly worse for >30 h CIT (RR = 1.32, p<0.011) (403). It follows, therefore, that while the benefits of transportation to a good match still exist, this group represent an appropriate target for preconditioning.

The other group that preconditioning is likely to be beneficial in is the expanded-criteria donors. It is known that the outcome following transplantation from older donors and donors with cardiovascular co-morbidities is poorer (Figure 1.4C). Robust data is lacking, but it is hypothesised that expanded-criteria donor grafts are more sensitive to prolonged ischemia and the particular importance of reducing CIT has been emphasised (404). It follows, therefore, that these donor grafts are most likely to benefit from the reductions in IRI that may be possible with pharmacological intervention.

10.1.2 Are there problems associated with up-regulation of Hsps?

Another fundamental premise of this thesis is that the up-regulation of Hsps does not result in other negative effects. Concerns have been raised previously that Hsps may be associated allograft rejection (405). It has been suggested that Hsps are released from grafts during episodes of acute rejection and that augmented expression of Hsps in allografts accelerates rejection (406). Hsp70 expression has been shown to be increased in rat models of heart graft rejection and graft infiltrating lymphocytes proliferate in response to recombinant Hsp70 (407). Increased Hsp expression is

found in biopsies from rejecting human lungs grafts (408) and T-cells from rejected renal grafts respond to Hsp72 (409) (although the presence of Hsp-reactive T-cells does not necessarily reflect a response to injury: Hsp60 specific T-cells have been found in humans with remitting juvenile chronic arthritis suggesting that such reactivity may reflect an autoimmune regulatory mechanism (405,410)). However, much of the early work demonstrating a relationship between Hsp70 and dendritic cell maturation has been discredited and the results attributed to lipopolysaccharide (LPS) contamination of the recombinant Hsps used (411). Yet, a recent report has suggested that Hsp70 may be critical for the induction of autoimmune diabetes in an experimental murine model (412).

Further evidence linking Hsps to the innate immune response concerned the identification of Hsp70 as a ligand for toll-like receptor[‡‡] 2 and 4 (TLR2, TLR4) (413). It has lately been appreciated that the innate immune response plays an important part in the insult associated with IRI (41). In rat kidneys, IRI itself has been shown to induce TLR2 and TLR4 , mainly on renal tubular cells, associated with Hsp70 expression (414). In rats receiving liver isografts, increased TLR4 expression was seen in Kupffer cells (415) and TLR4 deficient mice were found to be less prone to IRI following hepatic ischemia (416). The absence of the TLR receptor signal adaptor protein MyD88 has been shown to prevent rejection in a minor antigen-mismatched skin allograft model due to reduced number of mature DCs leading to impaired generation of anti-graft-reactive T cells (417). In fully MHC mismatched murine skin and heart allograft models, rejection was shown to occur in

[‡‡] **Toll-like receptors**: a family of pattern recognition receptors that are activated by specific components of microbes and certain other molecules.

the absence of MyD88 but priming of naive recipient T cells by allogeneic DCs was diminished, although TH2 immunity remained intact (418).

The importance of the innate immune system in IRI is convincing but the role of Hsp70 remains unclear. A recent study aiming to clarify this showed that DCs could not be activated with low-endotoxin Hsp70, but could with LPS; Hsp70 levels were not found to rise in a highly mismatched murine skin graft model; and in $Hsp70^{-/-}$ mice acute rejection was not delayed, DC maturation impaired, or Th1 immune responses reduced during acute allograft rejection (419). This study concluded that Hsp70 did not play an essential role in acute allograft rejection this model.

10.1.3 Are heat shock proteins up-regulated in organ donors anyway?

It has been suggested that in the deceased donor, the physiological stress associated with 1) the pre-morbid injury, 2) the process of brainstem death and associated cardiovascular instability and 3) the procedure of organ retrieval, may up-regulate Hsps in donor grafts. If this were true, the benefit conferred by a pharmacological strategy to up-regulate Hsps is likely to be minimal. Mueller *et al* studied kidney biopsy specimens taken at the time of engraftment, ensuring that results reflected organ injury sustained prior to retrieval and that associated with warm and cold ischemia. Renal tubular expression of Hsp72 was low and not influenced by donor-, graft-, or procedure-related risk factors. Neither strength nor pattern of pre-transplant Hsp72 staining discriminated allografts with complicated post-transplant courses from those without complications. Moreover, low Hsp72 expression in pre-transplant

donor kidney biopsies did not predict DGF or acute rejection. These findings suggest that constitutive Hsp72 gene expression at the time of engraftment does not play a role in graft protection (401).

10.1.4 Are heat shock proteins degraded by cold storage?

Organs from deceased donor are placed in cold storage for varying periods of time following retrieval. If the up-regulation of heat shock proteins in donor kidneys is beneficial, one would hypothesise that this benefit would be maximal if Hsp up-regulation persisted through that period of cold storage up until the point of implantation. Although Hsps were not measured directly, Torras *et al* demonstrated that the effects of ischemic preconditioning persisted in rat renal isografts implanted after 5 h of cold-storage in Euro-Collins solution (97). Fuller *et al* also found that the beneficial effects of ischemic preconditioning persisted in syngeneic rat kidney grafts transplanted after 42 h cold storage (98). In human renal proximal tubular epithelial (HK-2) cells, Hsp27 and Hsp70 up-regulation following heat shock was shown to persist (and confer protection) after 4 and 16 h of cold-storage in University of Wisconsin solution (420). Finally, our laboratory have demonstrated that curcumin-mediated HO-1 induction in human hepatoma cells (HUH7s) is maintained after 18 h cold-storage in University of Wisconsin solution (421). Therefore, strong evidence exists in cell culture for the resistance of Hsp induction to the effects of cold storage, although a study has yet to be published which confirms this conclusively *in vivo*.

10.2 Potential weaknesses in study design

10.2.1 *In vitro* models

Most of the *in vitro* cell culture work has been performed in a renal adenocarcinoma cell-line. Observations made in immortalised cancer cell-lines sometimes differ from more physiological models. This model was chosen for ease of use with the knowledge that the study was going to be moved into a mouse model of IRI. In chapter 5, the observation that insulin induces HO-1 was confirmed in a mouse primary tubular epithelial cell model.

In chapter 4, the PI3K/Akt signalling pathway was manipulated in an effort to alter the activation state of HSF1. In hindsight, given the complexity and number of kinase pathways that potentially act to regulate this transcription factor, it was possibly naïve to hypothesise that alteration in HSF1 activity could be effected through the manipulation of just one. However, on review of the pre-existing literature the hypothesis was valid and worthy of exploration.

It could be argued that using insulin to alter HO-1 expression has limited clinical potential as insulin has many side-effects. However, the use of insulin is now advocated in the treatment of critically ill patients with severe sepsis (422). It remains unclear whether the reduction in mortality and morbidity from such treatment is related to the prevention of hyperglycaemia or directly to the infused insulin. It is possible that benefits are related to the up-regulation of HO-1 as demonstrated in this thesis. IGF-1 has been used in the treatment of burns patients (249) and remains a potential strategy for the up-regulation of HO-1.

10.2.2 *In vivo* models

Renal pedicle occlusion in the mouse produces a reliable model of IRI. This study only examined short-term outcome following IRI and work is on-going looking at outcome in the longer term.

Renal protection from IRI was demonstrated following administration of HBAs. It is desirable to confirm that this protective effect is occurring directly as a result of Hsp90 inhibition, HSF1 activation and Hsp up-regulation. Developing a model that produces reliable causal data is difficult. Using homologous recombination to knockout Hsp90 results in embryonic death, although it may be possible to develop a conditional model. Studying the effects of HBAs on IRI in an HSF1-null mouse would be useful.

IRI is only one aspect of transplantation and does not take into account the significant immunological insult present in an allograft model of transplantation. However, performing this study in an allograft model would be fraught with the technical difficulty of the model and be subject to greatly increased sources of variation. Demonstrating significant differences in outcome, therefore, would be very difficult.

Finally, although the use of HBAs to reduce IRI has been demonstrated in the mouse, it is necessary to show that these drugs are both safe and efficacious in the human prior to any clinical study in renal transplantation.

10.3 Future directions

This thesis has sought to examine the up-regulation of Hsps and associated benefits in models relevant to renal transplantation. Although many studies have shown the benefit of Hsp induction *in vitro* or in animal models, there has been little translation into the clinical setting and no accepted clinical strategies involving Hsps currently exist. This area has great potential to be taken forward into therapeutics, but urgent exploration in human trials is required before this can take place.

A number of exciting avenues of application are currently being explored. A recent study has demonstrated HO-1 induction using clinically accepted doses of isoflurane in rats with an associated protective effect against liver IRI (423). Protection was lost on treatment with the HO-1 inhibitor, tin protoporphyrin IX. The antioxidant and cytoprotective effects of bilirubin, CO, and ferritin have also been demonstrated experimentally, but potential toxicity has limited clinical application. Rinsing grafts with bilirubin ex vivo has been shown to have a similar effect to inducing HO-1 with hemin and has clinical potential (424). Inhaled CO and the use of CO-releasing molecules also hold great clinical promise (124,425).

Similarly, the induction of other Hsps has been shown to be beneficial in experimental models of transplantation (Table 1.7). Ischemic preconditioning has been demonstrated to have beneficial effects in some clinical trials (Table 1.5), but the exact mechanisms conferring benefit remain obscure. Remote ischemic preconditioning is particularly promising and with its ease of application, clinical trials should be forth-coming. Up-regulation of Hsp70 has been demonstrated

following treatment with 17-AAG in cancer patients, but no trial examining potential protection has been performed. In this work, 17-DMAG has been shown to have superior efficacy in Hsp induction. A phase 1 trial in healthy volunteers is essential to provide information on toxicity/side-effects and to measure Hsp expression prior to instigating a trial in transplantation patients.

It is unclear why there has been a reticence to examine promising pre-clinical strategies in human models. Apprehensions regarding the potential negative consequences of strategies applied in such a difficult group of patients, together with concerns over costs and clinical utility have no-doubt deterred investigators. However, if outcomes following transplantation are to be further improved, the important experimental findings in this field must be exploited to become valuable clinical interventions.

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Appendix I – Buffers

RIPA

- 1 X PBS
- 1% NONIDET P-40
- 0.5% SODIUM DEOXYCHOLATE
- 0.1% SDS
- Protease inhibitor – 1 tablet/10 ml

D-PBS (calcium and magnesium free)

- KCl 1.0 g
- KH_2PO_4 1.0 g
- NaCl 40.0 g
- Na_2HPO_4 5.75 g (anhydrous)
- H_2O to 5000 ml

Tissue homogenising buffer (THB)

- 50 mM Tris pH 7.4 2M stock
- 20 mM NaCl 5M stock
- 10 mM KCl 2M stock
- 1 mM EDTA 0.2M stock
- 1% SDS
- 0.1 mM DTT add fresh 1M stock
- Protease inhibitor 1 tablet/10 ml

Separating gel (0.375 M Tris, pH 8.8)

	15%	**12.5%**	**12%**	**10%**	**8%**	**7.5%**	**5%**	
Distilled H_2O	2.29	3.12	3.29	3.96	4.62	4.79	5.62	ml
1.5M Tris-HCl pH 8.8	2.5	2.5	2.5	2.5	2.5	2.5	2.5	ml
10% SDS	100	100	100	100	100	100	100	µl
Acrylamide/Bis 30% stock (Biorad)	5.0	4.17	4.0	3.33	2.67	2.5	1.67	ml
→								←
10% ammonium persulphate (fresh daily) (AMPS)	100	100	100	100	100	100	100	µl
TEMED	10	10	10	10	10	10	10	µl

Total monomer 10ml (enough for 2 gels)

Stacking gel 4% (0.125 M Tris, pH 6.8)

Distilled H_2O	5.98 ml
0.5M Tris-HCl, pH 6.8	2.5 ml
10% SDS	100 µl
Acrylamide/Bis (30% stock) (Biorad)	1.3 ml
→	
10% AMPS	100 µl
TEMED	20 µl

Sample loading buffer

	1X	5X (CONC)
Distilled H_2O	3.2 ml	0.5 ml
Tris-HCl, pH 6.8	1.0 ml 0.5M	2.5 ml 1M
Glycerol	0.8ml	4.0 ml
10% SDS	1.6 ml	0.8g
2-b-mercaptoethanol	0.4 ml	2.0 ml
0.05% bromophenol blue	1.0 ml	1.0 ml

Low molecular weight Western blot markers

Reconstitute in 200 µl loading buffer for markers (in fume hood)

Heat @ 95°C for 5 mins and aliquot - 5µl

High molecular weight Western blot markers

Reconstitute in 100 µl H_2O

Do not heat

Aliquot - 5 µl

Running buffer 5X

- Tris 30 g
- Glycine 144 g
- SDS 10 g

Dissolve in 1800mls dH_2O

pH to 8.3

Make up to 2 litres with dH_2O

Transfer buffer

- Glycine 5.86 g
- Tris 11.625 g
- SDS 0.75 g
- Methanol 400 ml

Dissolve and make up to 2 litres with dH_2O

10 X TBS

- Tris 121 g
- NaCl 438.5 g

Dissolve in 1800 ml dH_2O

pH to 7.4

Make up to 5 litres with dH_2O

Divide into 500ml bottles

10 X TBE

- Tris 107.8 g
- diNaEDTA.2H2O 7.44 g
- Boric acid ~55-60 g

Dissolve Tris and EDTA in ~800 ml H2O

Add boric acid to adjust pH to 8.3

Make up to 1 litre

5 X TBE

- Tris 53.9 g
- EDTA 3.72 g
- Boric acid 30 g boric acid

40 X TAE

- Tris 193.6 g
- Na acetate.3H2O 108.9 g
- diNaEDTA.2H2O 15.2 g

pH to 7.2 with acetic acid

Water to 1 litre

Methacarn

- 70% methanol
- 20% chloroform
- 10% glacial acetic acid

Appendix II – Abbreviations

1° NC, primary rat neonatal cardiomyocytes
17-AAG, 17-allylamino-17-demethoxygeldanamycin
17-DMAG, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin
AD, actinomycin D
CHX, cycloheximide
CK, creatine kinase
CL, cell-line
CO, carbon monoxide
CoPP, cobalt protoporphyrin
CO-RM, carbon monoxide-releasing molecule
Cyp, cyclophilin
DGF, delayed graft function
DTT, Dithiothreitol
EDTA, Ethylene diamine tetra acetic acid
ERK, extracellular signal-related kinase
FITC, fluorescein isothiocyanate
FKBP, FK506 binding protein
GA, geldanamycin
GGA, geranylgeranylacetone
GSK3β, glycogen synthase kinase 3β
HBA, heat shock protein 90-binding agent
HIF1, hypoxia inducible factor-1
HO-1, heme oxygenase-1
HSF1, heat shock transcription factor 1
Hsp, heat shock protein
ICAM-1, intracellular adhesion molecule-1
IGF-1, insulin-like growth factor-1
IL6, interleukin 6
IR, ischemia/reperfusion
IRI, ischemia/reperfusion injury
IP, immunophilin
K, ketamine
LDH, lactate dehydrogenase
M, medetomidine
MAPK, mitogen-activated protein kinase
MCP-1, monocyte chemoattractant protein-1
MPO, myeloperoxidase
MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-dipenyltetrazolium bromide
NF-E2, nuclear factor-erythroid 2
Nrf2, NF-E2-related factor 2
OSOM, outer-stripe of outer medulla
pGSK3β, phosphorylated glycogen synthase kinase 3β
PI, propidium iodide
PI3K, phosphatidylinositol 3-kinase

PKB, protein kinase B
RAC-PK, Related to A- and C-Protein Kinase
REF, rat embryonic fibroblasts
ROS, reactive oxygen species
RPO, renal pedicle occlusion
SHR, steroid hormone receptor
SI, simulated ischemia
siRNA, small/short-interfering ribonucleic acid
SNP, sodium nitroprusside
SnPP, tin-protoporphyrin IX;
TGFβ, transforming growth factor beta
TNFα, tumour necrosis factor alpha
VCAM, vascular cell adhesion molecule
X, xylazine
ZnPP, zinc protoporphyrin

Appendix III – Mouse ischemia/reperfusion protocol

Preparation

1. Sterilise instruments in autoclave (*quick unwrapped* cycle about 20 mins).
2. Alcohol wipe heated mat, turn on and stick rectangle of lab-top paper to it with masking tape/micropore.
3. Cut 4 pieces of micropore to stick feet.
4. Set magnifying light to correct distance and turn on.
5. Get instruments from autoclave and place vascular clips on piece of sterile towel so easy to pick-up.
6. Warm all fluids to 37 °C

Animal set-up

1. Weigh animal
2. Give anaesthetic (ketamine and medetomidine mix, reversed with atipamezole).
3. Place animal back in cage and replace top.
4. Once asleep (5 min) remove from cage and shave around 0.5 cm on either side of linea alba (keep shaving to a minimum to reduce heat loss).
5. Give 0.5 ml saline/PBS and 50 µl vetgesic to scruff
6. Place lacrilube on eyes

Procedure

1. Pick-up skin of abdo with toothed forceps just cranial to urethra.
2. Stab through skin with No. 11 blade still holding with forceps.
3. Open skin with tissue scissors.
4. Look for the very thin white line of the linea alba (keep to this to avoid bleeding).
5. Pick up linea alba with toothed forceps and open with carefully with No. 11 blade or scissors. Open as far as the xyphisternum .Watch out for the liver at the cranial aspect of the wound.
6. Place wire retractor.
7. Start with left kidney. With wet q-tip (stick with cotton wool at end) displace bowel to right. Put wet towel over bowel.
8. Jewellers forceps in my right hand and curved forceps in left to gently (very gently) pull fascia off renal pedicle. Grasp a small piece of fascia with one, and then pull against it with the other forcep. Do not pull on the vein itself.
9. When the fasica has been dissected, it is easy to place curved forceps around the back of the pedicle.
10. Pick up the vascular clip with the clip applicator.
11. Open forceps gently and place clip over pedicle
12. Displace bowel to left side and to the same to the right pedicle (when you get good the dissection only takes a minute or two.
13. Remove retractor and place moist towel over wound. Time accurately the clamp period.
14. After, remove clip.
15. Close linea alba with around a 4-0 silk/nylon (well anything really).

16. Clips to skin (titanium).

Post-procedure

1. Reverse anaesthetic.
2. Place in cage on paper towel in incubator (25 °C).
3. Administer further 0.5 ml saline/PBS around 2 hrs after procedure.

Appendix IV – Presentations of data from this thesis

ORAL PRESENTATIONS

Harrison EM, Ross JA, Wigmore SJ. **Pharmacological interventions to reduce ischemia/reperfusion injury in the kidney**. *Medical Research Scotland Open Day*, Edinburgh, May 2007.

Harrison EM, McNally SJ, Devey L, Garden OJ, Ross JA, Wigmore SJ. **Hsp90-binding agents improve outcome in kidney ischemia/reperfusion injury.** *Society of Academic and Research Surgery Conference*, Edinburgh, 2006

Harrison EM, Ross JA, Wigmore SJ. **Activating the PI3K/Akt pathway induces heme oxygenase-1: a novel target for organ protection.** *British Transplantation Society Annual Congress*, Belfast. April 2005.

Harrison EM, Ross JA, Wigmore SJ. **Organ protection: harnessing cell signalling in transplantation.** *British Transplantation Society Annual Congress*, Belfast. April 2005.

Harrison EM, McNally SJ, Sangster K, Ross JA, Garden OJ, Wigmore SJ. **Insulin induces heme oxygenase-1 via the PI 3-kinase/Akt pathway: potential preconditioning target in kidney transplantation.** *Society of Academic and Research Surgery Conference,* Newcastle, January 2005.

Harrison EM, Ross JA, Garden OJ, Wigmore, SJ. **Insulin induces HO-1 via the PI 3-kinase/Akt pathway: potential preconditioning target in kidney transplantation.** Chiene Medal Winner, School of Surgery Day, University of Edinburgh, November 2004

POSTER PRESENTATIONS

Harrison EM, McNally SJ, Ross JA, Garden OJ, Wigmore SJ. **Akt, but not MAPK, inhibition reduces heme oxygenase-1 expression in kidney cells.** *Scottish Society for Experimental Medicine*, Edinburgh, November 2004

Harrison EM, McNally SJ, Ross JA, Sangster K, Garden OJ, Wigmore SJ. **HO-1 expression in renal cells increased following PKB activation.** *MRC Cell Signalling Symposium*, Dundee, June 2004.

Harrison EM, Ross JA, Sangster K, Garden OJ, Wigmore, SJ. **Geldanamycin Protects Renal Cells from Oxidative Damage.** *British Transplantation Society Annual Congress*, Birmingham April 2004.

Appendix V – Publication of data from this thesis

Harrison EM, Sharpe Eva, Bellamy CO, McNally SJ, Devey, Luke, Ross JA, Garden OJ, Wigmore SJ. **Hsp90-binding agents protect renal cells from oxidative stress and reduce kidney ischemia/reperfusion injury.** Accepted with changes to American Journal of Physiology – Renal Physiology.

Harrison EM, McNally SJ, Devey L, Garden OJ, Ross JA, Wigmore SJ. **Insulin induces heme oxygenase-1 through the phosphatidylinositol 3-Kinase/Akt pathway and the Nrf2 transcription factor in renal adenocarcinoma cells.** *FEBS J.* 2006 Jun;**273(11)**:2345-56.

Harrison EM, Ross JA, Garden OJ, Wigmore SJ. **Firefly luciferase activity is reduced following mild heat exposure: implications for transfection efficiency control.** *J Immunol Methods.* 2006 Mar 20;**310(1-2)**:182-5.

McNally SJ, Harrison EM, Wigmore SJ. **Ethical considerations in the application of pre-conditioning to solid organ transplantation.** *J Med Ethics* 2005;**31**:631-634

Patel A, van de Poll MC, Greve JW, Buurman WA, Fearon KC, McNally SJ, Harrison EM, Ross JA, Garden OJ, Dejong CH and Wigmore SJ. **Early stress protein gene expression in a human model of ischemic preconditioning.** *Transplantation* 2004 Nov 27;**78(10)**:1479-87.

PUBLISHED ABSTRACTS

Harrison EM, McNally SJ, Sangster K, Ross JA, Garden OJ, Wigmore SJ. **Insulin induces heme oxygenase-1 via the PI 3-kinase/Akt pathway: potential preconditioning target in kidney transplantation.** *Br J Surg.* 2005; **92**:1302-1327

CLINICAL ETHICS

Ethical considerations in the application of preconditioning to solid organ transplantation

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J Med Ethics 2005;**31**:631–634. doi: 10.1136/jme.2004.011486

The shortage of organs for transplantation has led researchers to look for new techniques to expand the donor pool. Preconditioning strategies have the potential to protect organs from transplant associated injury or may improve the function of substandard organs so that they become suitable for transplantation. Translating this type of technology to the clinical setting raises ethical issues, particularly relating to the deceased donor. It is important that society has the opportunity to discuss the issues raised by implementation of preconditioning strategies before they are implemented rather than as a reaction to them.

Organ transplantation has been one of the great medical successes of our times. It has revolutionised the treatment of organ failure, often allowing the recipient to resume a normal lifestyle.[1] Every advance has led to an increased demand for organs for transplantation and despite major improvements in immunosuppression, organ preservation, and operating techniques, patients continue to die on waiting lists due to the shortage of donors.[2] The number of potential recipients in the United Kingdom awaiting organ transplantation has increased annually, reaching more than 7000 by March 2004.[2] New sources of organs, such as living donors, split livers, and non-heartbeating donors, have increased available organs to a degree; however, these developments have failed to keep pace with the decline in the available number of cadaveric organs. Novel therapies such as stem cell technology and bioartificial livers hold promise for the future, but have not yet fulfilled their potential. There remains, therefore, a requirement for solid organ transplantation that is unlikely to recede in the near future.

Preconditioning is a developing technique, which is currently making the transition from an experimental concept to a practical therapeutic option.[3] The basic premise is that treatment before a known injury occurs can be used to minimise the severity of that injury. This principle has widespread application in many areas of medicine, particularly in surgical disciplines.[4] Solid organ transplantation, with its attendant ischaemia/reperfusion injury, represents an area where preconditioning of the donor or the donated organs could make a great contribution,[5 6] potentially reducing primary non-function and early graft failure, and thus the requirement for retransplantation. There is also experimental evidence that preconditioning may allow organs that would currently be discarded to be used, thus expanding the organ pool. In this paper we discuss the practical and ethical issues involved in implementing preconditioning strategies in solid organ transplantation.

PRECONDITIONING

Practical implementation

The term preconditioning encompasses many different techniques, which all rely on harnessing aspects of the innate protective mechanisms that human cells use to survive stress. Broadly speaking there are two approaches to induce preconditioning: physical and pharmacological. Physical techniques include ischaemic preconditioning[7] and heat treatment.[8 9] Pharmacological techniques include the administration of drugs, cytokines, and gene transfer techniques.[10–12]

Pharmacological preconditioning, with drugs such as cyclosporin, would require the commencement of an additional drug infusion. This would be in addition to a range of infusions that the donor would already be receiving, and so would make little difference to the medical management of the donor. Heat preconditioning requires elevation of the body temperature by several degrees. This could be achieved by the use of warming blankets, increasing the temperature of inhaled gases with which the donor is ventilated, heating intravenous fluids, and possibly the use of heat lamps. Ischaemic preconditioning would be applied when the organs were being removed. This is achieved by commencing the retrieval operation as normal and then, immediately prior to perfusing the donor with cold preservation solution, clamping the blood vessels supplying the liver for ten minutes, and subsequently unclamping them for ten minutes to allow recovery. Perfusion with the cold preservation fluid would then be commenced and the remainder of the organ retrieval procedure performed as normal. Thus the practical implementation of preconditioning treatments ranges from the minor addition of a drug or a modification of operative technique to more interventional options such as whole body hyperthermia.

Clinical precedents

The optimum management of heartbeating organ donors currently involves the administration of a variety of drugs and infusions to maintain the organs in a suitable condition for transplantation. Preconditioning agents would be used to improve the target organs, rather than merely limiting the detrimental effects of brain stem death. Thus preconditioning crosses the boundary from maintenance to treatment, a fact which could give rise to ethical concerns. There are, however, several precedents for this type of active treatment. Hormonal resuscitation with triiodothyronine, vasopressin, and methylprednisolone is administered to heart transplant donors,[13] with the aim of correcting the endocrine imbalances that occur after brain stem death. These are active treatments, intended to increase the number of organs suitable for transplantation. They differ from preconditioning, however, in that they represent maintenance rather than therapeutic measures. Thus the boundary between donor maintenance, optimisation, and preconditioning can be considered indistinct.

Clinical application of preconditioning

There are several applications for preconditioning. Each individual setting requires different considerations with

regard to both the practical implementation of a preconditioning strategy, and the ethical issues it raises. Donor organs for transplantation currently come from three sources: living donors, brain stem dead heartbeating donors, and non-heartbeating donors. The application of preconditioning strategies raises both ethical and practical issues in each of these three donor groups. Similarly, different methods of preconditioning may be more or less acceptable to families of donors and any treatments used for preconditioning may require lack of objection or assent additional to that obtained for the actual donation.

ETHICAL CONSIDERATIONS

Lack of objection/assent

Consent for organ donation has been a contentious issue in the past. In the UK, even when the potential donor carries an organ donor card, the assent or lack of objection of the next of kin is sought.[14] The introduction of the Human Tissue Bill may change the emphasis of this process, giving greater priority to the autonomy of the donor. The provision of in depth details of the retrieval process required to explain a preconditioning strategy could potentially lead to a withdrawal of lack of objection to organ donation. Thus describing the preconditioning strategies, or asking for explicit assent to apply them, could potentially have a negative effect on donation rates. This issue must be considered in the light of potential benefits to society as a whole, of advancing science, and improving medical outcomes.

There is an alternative argument that preconditioning represents part of the normal process of organ harvesting and therefore should not require further assent or lack of objection beyond that associated with organ donation itself. Indeed, in his treatise on the ethics of organ donation, Price suggests that "where an individual has requested that his/her organs be used for transplantation after death, it seems correct to infer that permission is granted for procedures which form part of the routine preliminaries to transplantation without seriously compromising the patient in any way".[15]

Law and ethics

Even where there is no explicit authorisation of procedures for maintaining donors and their organs, they are not necessarily unlawful. Although explicit legislative provision to cover organ preconditioning techniques may be desirable, it is far more important that society endorses the concept that preconditioning strategies in donors to improve organ function and outcome are ethically acceptable and clinically warranted. This would avoid the difficulties that occurred with elective ventilation, which began in the UK in accordance with Home Office directives and agreement, but which has since been abandoned due to legal and ethical concerns.[16 17] Such legislation exists in Sweden, and it states: "Once it has been established that death has occurred, measures may be taken, if appropriate, in order to preserve organs or other biological material pending transplantation...Unless there are special circumstances, such measures should not continue beyond 24 hours" (Price,[15] p 170). With regard to organ procurement for transplantation, the UK Human Tissue Bill currently being considered by parliament states that "it shall be lawful...to take steps for the purpose of preserving that part [part of a body] for use in transplantation, and to retain the body for that purpose". However, this authorisation "shall only extend to the taking of the minimum steps necessary...and to the use of the least invasive procedure".[18] It is unclear whether such authorisation would extend to preconditioning techniques.

The timing of certain preconditioning treatments may be crucial, particularly with regard to agents that may require a significant length of time prior to harvest to provide the beneficial effect. Thus there may be the requirement to commence administration of the agent before lack of objection is obtained. Although this may be against normal principles, this type of approach has previously been approved by ethical committees and has been used in clinical trials on organ donors.[19] This is also analogous to the management required for uncontrolled non-heartbeating donors where, if the coroner agrees, cooling is commenced as soon as practicable, and then withdrawn if an objection to donation is expressed.[20] This is provided for in Dutch law, which declares that necessary measures to maintain the organ in a suitable condition for transplantation may be taken after death, so long as the procedure for obtaining the necessary consent has not been completed.[21]

Respect for the donor

The question of preconditioning having ethical issues derives from the observation that these manipulations will not benefit the individual directly in any way. There is indirect benefit, from the donor's volition to donate organs for transplantation being fulfilled, but is this enough?

Societal beneficence and justice require that the maximum good be obtained from the gifted organs. This could also be seen as an extension of autonomy (albeit posthumously), where respect for the wishes of the deceased to be an organ donor mandates that the transplant procedure be as effective and beneficial as possible. Thus it could be argued that there is a moral and ethical imperative to use preconditioning strategies if they improve the use of donated organs. Autonomy is difficult to establish once an individual has died, but this may become easier as the use of advance directives increases. As has been described in the debate on elective ventilation, however, the lack of public knowledge about specific treatments (such as elective ventilation) means that any impact of advance directives is likely to be limited (Price,[15] p 189).

The act of joining an organ donor register or carrying a donor card may be considered an indication of consent to organ donation, but it is not clear whether such consent would extend to preconditioning strategies. There is a need to establish whether preconditioning can be seen as no different from other medical interventions, such as the administration of heparin to donors, and also whether consent to organ donation implies consent to all techniques required to allow good use of the organs. As stated above, justice and societal beneficence require that the best possible use is made of the organs, and therefore that all beneficial manipulations are used.

It is conceivable, however, that the relative importance ascribed to such considerations might alter depending on the nature of the preconditioning strategy considered. It seems—for example, unlikely that many relatives would object to the deceased donor being given a drug to improve organ function but it is possible that they might raise objection if it were necessary to keep the donor ventilated for a prolonged period of time to allow the drug to take effect. Similarly, more interventional preconditioning techniques, such as whole body hyperthermia,[22] may be less acceptable to donor families.

SPECIAL CONSIDERATIONS

Non-heartbeating donation

In the controlled non-heartbeating donor, death is not diagnosed until cessation of cardiac function. In this setting interventions prior to death, either to optimise the donor or to precondition organs, may alter the process leading to death

and this is considered unacceptable. The guidelines currently being drawn up by the Intensive Care Society state quite clearly that "It is inappropriate to escalate current treatment, add new therapies—for example, inotropes, heparin, hormone replacement, or to undertake invasive interventions—for example, vascular cannulation before death for cold perfusion, to improve organ viability".[23] Preconditioning in this setting is only potentially possible after the diagnosis of death and an acceptable period (frequently 10 minutes) during which no intervention is made on the potential donor. In terms of practicality this means that only preconditioning strategies that involve perfusion of the deceased donor or involve ex vivo perfusion of organs would be acceptable in this context.

Living donation

Living donors provide the simplest model, where the donor is fully aware of the donor procedure and can provide detailed consent, with prolonged time for consideration and reflection. This situation therefore poses few ethical considerations not encompassed by fully informed consent. Although all organs donated for transplantation are considered precious, the act of living donation places additional responsibilities on the medical profession. Living donation of organs or tissues is one of the few circumstances in medicine where the donor undergoes a procedure with no physical benefit to themselves. In this context it is imperative that the risk to the donor is minimised. Similarly, because there is a risk to the donor, however small, it is generally accepted that the donated organ should have a reasonable chance of successful transplantation. Considering these issues, any preconditioning strategy applied to a living donor population must not only demonstrate benefit to the recipient but must also not place any additional burden or risk on the donor.

Multiorgan donors

The majority of organ donors in the UK donate more than one organ. This raises the question whether, if a preconditioning strategy had been demonstrated to confer a beneficial outcome for a particular organ, it would be acceptable to use such a treatment in a multiorgan donor if the effects on other organs were not known. The answer in this case would probably be negative and so research involved in the development of such strategies should consider effects on other organs that might be donated simultaneously in a clinical setting. If the effects of a treatment on other organs were not adverse, the question would then arise as to whether the potential recipients of other organs should be consulted and requested to give their consent to preconditioning being used. The issues of consent and collateral involvement become more prescient in the context of a clinical trial where the effect of a preconditioning strategy may be the subject of evaluation.

Recipients of preconditioned organs

In many circumstances where preconditioning would be used it may be impractical to obtain consent from the recipients in time to influence the application of the preconditioning intervention. There is concern therefore that if a recipient objects to receiving an organ that has undergone preconditioning this may subvert the normal informed consent process by placing coercion on the recipient. Thus the desire to receive a transplant may outweigh the concerns or objections of the recipient to receiving an organ from a research protocol. Adopting a utilitarian approach, it could be argued that preconditioning has the potential to improve the outcomes of transplantation and that society should support research which aims to advance science or improve outcomes in medicine. Following this argument to its practical application, the occasional refusal to receive a preconditioned organ would be offset by the perceived benefit to society as a whole. In practice perhaps the easiest way to deal with this issue would be to inform patients, at the time of addition to the waiting list, that they may be offered organs derived from trials of preconditioning protocols and that they should consider whether they would accept such an organ. They should also be informed that such protocols had received approval from local regional ethics committees and that such approval would not normally have been granted if there were issues over either safety or perceived benefit.

Preconditioning in a research setting

In common with other medical research, trials of preconditioning should abide by the principles enshrined within the Declaration of Helsinki.[24] Specifically there should be an adequate laboratory and animal research base to support human studies and these studies should have clear potential for benefit counterbalanced against the inherent risk of the intervention. The design of studies should be clearly set out and be undertaken by competent individuals. Studies should have been reviewed and approved by an independent committee—in the UK this would be the local or multicentre research ethics committee, as appropriate. Articles 10 and 11 of the Declaration of Helsinki deal with areas of research where preconditioning could raise ethical issues. Article 10 relates to the situation where an individual may be in a dependent relationship with the researcher, a possibility for the recipient of an organ used in a preconditioning study, and article 11 describes the case where the individual is legally incompetent—the situation of the deceased donor. These issues present specific problems for regional ethics committees, which often have to consider them without precedent for comparison or using guidelines derived from research in living subjects.

CONCLUSION

Preconditioning strategies have a great potential to improve outcome in solid organ transplantation and also to increase the availability of organs by allowing use of marginal donors. As with many issues in transplantation, there are few precedents to provide guidance on the practical implementation of such programmes. There are likely to be moves to integrate these treatments into organ procurement protocols in the near future. There is, therefore, a clear need to establish an ethical consensus on the acceptability of such treatments and the responsibilities of those involved in their administration to donors, their families, and recipients. It is hoped that transparency in such discussions will both preempt misunderstandings arising from perceptions of unnecessary intervention or experimentation on the deceased donor and provide an ethically acceptable framework upon which preconditioning strategies could be based.

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Insulin induces heme oxygenase-1 through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in renal cells

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Akt; heme oxygenase-1; insulin; kidney; transplantation

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Heme oxygenase-1 catalyzes the breakdown of heme and is protective in models of kidney transplantation. In this study we describe the induction of heme oxygenase-1 mRNA and protein by insulin. Following treatment with insulin, a five-fold increase in heme oxygenase-1 mRNA and a four-fold increase in protein expression were observed in renal adenocarcinoma cells; insulin-induced heme oxygenase-1 expression was also demonstrated in mouse primary tubular epithelial cells. The induction of heme oxygenase-1 in renal adenocarcinoma cells was blocked by actinomycin D and cycloheximide and was abolished by the phosphatidylinositol 3-kinase inhibitor, LY294002, but not by the inactive analog LY303511. Overexpressing a dominant-negative form of Akt abrogated the heme oxygenase-1-inducing effects of insulin, whereas cells transfected with a constitutively active Akt construct demonstrated an increase in heme oxygenase-1 promoter activity and protein expression. The transcription factor NF-E2-related factor-2 was found to translocate to the nucleus following insulin treatment in a phosphatidylinositol 3-kinase-dependent manner. Pretreatment with NF-E2-related factor-2 small-interfering RNA abolished insulin-induced heme oxygenase-1 induction. Insulin was also found to activate the mitogen-activated protein kinase cascades p38 and extracellular signal-related kinase; however, inhibition of these pathways with SB202190 and PD98059 did not alter insulin-induced heme oxygenase-1 expression. Thus, insulin induces heme oxygenase-1 mRNA and protein expression in renal cells in a phosphatidylinositol 3-kinase/Akt and NF-E2-related factor-2-dependent manner.

Cadaveric kidney transplantation is associated with substantial free radical injury as a consequence of cold storage and reperfusion of the organ [1,2]. This correlates with early organ dysfunction, which is associated with poorer long-term graft survival [3,4]. Strategies to reduce these effects and improve outcome are currently being sought [5].

Heme oxygenase catalyses the rate-limiting step in the degradation of heme to carbon monoxide (CO), free iron and biliverdin, which is immediately converted to bilirubin by bilverdin reductase [6]. At least two isoenzymes are known to exist: heme oxygenase-1 (HO-1), which is strongly induced by its substrate heme and a number of stress stimuli, including UV

Abbreviations
AD, actinomycin D; CHX, cycloheximide; ERK, extracellular signal-related kinase; GSK3β, glycogen synthase kinase 3β; HBSS, HANK's balanced salt solution; HIF-1, hypoxia-inducible factor-1; HO-1, heme oxygenase-1; HSF-1, heat shock transcription factor-1; HSP70, heat shock protein 70; MAPK, mitogen-activated protein kinase; MEK1, mitogen activated protein kinase kinase 1; NF-E2, nuclear factor-erythroid 2; NGF, nerve growth factor; Nrf2, NF-E2-related factor 2; pGSK3β, phosphorylated glycogen synthase kinase 3β; PI3K, phosphatidylinositol 3-kinase; siRNA, small-interfering ribonucleic acid.

radiation and heavy metals; and constitutive heme oxygenase-2 [7–9]. The exact role of HO-1 in oxidative stress is not clear, but it has been shown to be protective in a number of animal models of organ transplantation, including kidney [10], liver [11], heart [12] and small bowel [13], by virtue of the products of the reaction it catalyzes [14]. Bilirubin is known to be a powerful antioxidant [15,16], and HO-derived bilirubin has been shown to provide protection in neuronal cells [17]. CO was first demonstrated to be protective in a model of acute lung injury [18], and subsequently in rodent cardiac [19,20] and renal transplantation models [21]. Two important mechanisms of CO protection involving p38 mitogen-activated protein kinase (MAPK) and guanylyl cyclase have been identified, but these appear to be cell-type specific [14]. Although HO-1 releases the pro-oxidant Fe^{2+}, this is associated with the rapid expression of the iron-sequestering protein ferritin, which is also known to be protective [22]. It is generally accepted therefore that induction of heme degradation represents an adaptive response to oxidative insult.

Insulin is a polypeptide hormone that regulates glucose, lipid and protein metabolism and promotes cell growth and differentiation. On ligand binding, the insulin receptor tyrosine kinase initiates multiple signaling cascades, including activation of the phosphatidylinositol 3-kinase (PI3K) pathway and its downstream effectors [23]. This pathway is a key signal transducer of many growth factors and cytokines and has been implicated in the regulation of cell growth, cell migration and cell survival [24]. The protein kinase B/Akt family of serine/threonine kinases has been identified as an important target of PI3K in cell survival [25–28]. Moreover, recent work has shown a direct link between the PI3K/Akt pathway and HO-1 regulation in PC12 cells [29,30]. This may be through nuclear factor E2-related factor-2 (Nrf2), a member of the cap'n'collar family of basic leucine transcription factors and a well-established regulator of HO-1 [31].

In view of the beneficial effects of upregulation of HO-1 in models of organ transplantation, we wished to identify signaling pathways involved in regulation of HO-1 gene expression. This study presents data demonstrating PI3K/Akt-dependent induction of HO-1 following the administration of insulin to renal adenocarcinoma cells (ACHN). PI3K activity was necessary and sufficient for HO-1 induction, and Nrf2 blockade was found to abolish the response. Supporting data illustrate similar insulin-induced HO-1 expression in mouse primary renal tubular epithelial cells.

Results

Insulin increases HO-1 expression in ACHN cells

Treatment of serum-deprived ACHN cells with increasing concentrations of human insulin resulted in a four-fold induction of HO-1 after 6 h (Fig. 1A). Maximal induction of HO-1 protein was achieved at concentrations of 200 nM insulin. A time course experiment using insulin (200 nM) demonstrated accumulation of HO-1 after 2 h of treatment (Fig. 1B). HO-1 mRNA was found to increase over the same concentration range of insulin (Fig. 1C) and achieved maximum induction after 2 h of treatment with insulin (200 nM) (Fig. 1D). HO-1 mRNA returned to resting levels after 16 h of treatment. To ensure that HO-1 induction was not related to serum deprivation, cells were cultured in medium containing different concentrations of fetal bovine serum for 16 h (Fig. 1F); no alteration in HO-1 protein expression was detected. To confirm that HO-1 accumulation was dependent on gene transcription, ACHN cells were pretreated with actinomycin D (AD) followed by insulin (Fig. 2A,C). Basal levels of HO-1 protein were reduced following AD treatment, and the HO-1 protein and mRNA response to insulin was abolished. Similarly, cycloheximide (CHX) was administered to establish the role of protein synthesis in insulin-induced HO-1 expression (Fig. 2B,C). CHX abrogated HO-1 protein induction following insulin treatment but, in agreement with other studies, also eliminated HO-1 mRNA induction, suggesting that protein translation is required to activate the HO-1 promoter [29,32,33].

Insulin increases HO-1 expression in mouse primary renal tubular epithelial cells

In order to ensure that insulin-induced HO-1 expression was not a characteristic of transformed cells alone, mouse primary renal tubular epithelial cell cultures were prepared. These were treated in a similar manner with insulin (200 nM) for increasing periods of time (Fig. 1E). A robust induction of HO-1 protein was observed.

Insulin-mediated induction of HO-1 is PI3K dependent

In our model, phosphorylation of glycogen synthase kinase 1 (GSK3β) was used as an indicator of PI3K/Akt axis activity. GSK3β phosphorylation was observed after 30 min of insulin treatment at a concentration of 200 nM (Fig. 3A). Following 30 min of pretreatment

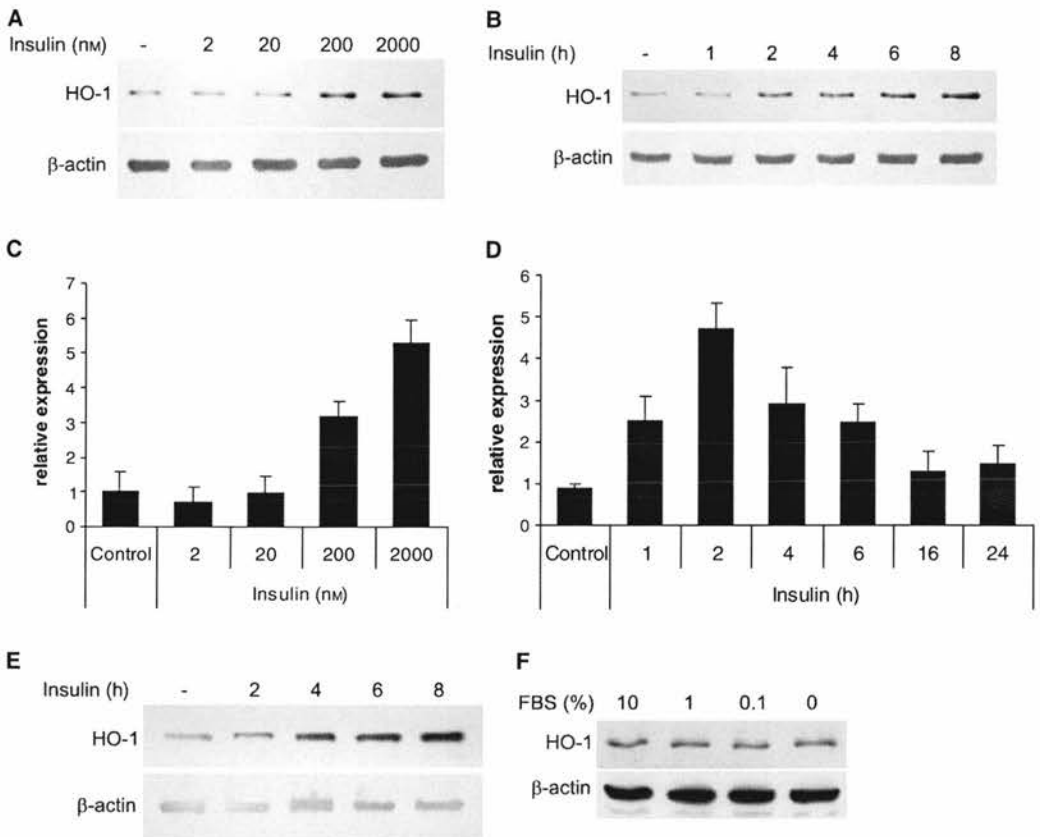

Fig. 1. Insulin stimulates heme oxygenase-1 (HO-1) protein and mRNA accumulation. Renal adenocarcinoma cells (ACHN) were serum-deprived for 16 h and treated with increasing concentrations of insulin for 6 h (A) or 4 h (C), or with insulin (200 nM) for various times (B, D). Mouse primary renal tubular epithelial cells were prepared and treated with increasing concentrations of insulin (E). ACHN cells were cultured in medium supplemented with different concentrations of fetal bovine serum (FBS) (F). Whole cell lysates were prepared and analysed by western blotting (A, B, E, F) using antibody to HO-1, with β-actin as loading control. mRNA extracts were prepared (C, D) using TRIzol and reverse transcribed to cDNA. Fluorescence detection real-time PCR was performed using HO-1 primers and probe with an 18S primer/probe control; results are expressed as mean relative expression ± SEM of three independent experiments.

with the PI3K inhibitor LY294002 (Fig. 3B), or its inactive analog LY303511 (Fig. 3C), ACHN cells were treated with insulin (200 nM) for 6 h to determine HO-1 protein accumulation and for 30 min to confirm GSK3β phosphorylation status. HO-1 was induced as expected following insulin treatment, but this effect was abolished with increasing concentrations of LY294002. Following treatment with LY303511, HO-1 induction was not altered. LY294002-mediated reduction in GSK3β phosphorylation correlated with inhibition of insulin-induced HO-1 accumulation.

Akt activity is necessary and sufficient for HO-1 induction

Forty-eight hours after transfection of ACHN cells with the pHOGL3/11.6 reporter construct and a constitutively active Akt-expressing construct (Akt-myr), an increase in luciferase activity was observed, representing a six-fold increase in HO-1 promoter activity ($P < 0.05$, ANOVA) (Fig. 4A). Accumulation of HO-1 protein was also found following transfection with either the Akt-myr or wild-type (Akt-WT) construct, in association with an expected increase in GSK3β phosphorylation (Fig. 4B). Treating cells transfected with Akt-myr with insulin did not increase the HO-1 promoter activity (Fig. 4A) over that of cells transfected alone, demonstrating that the effects of insulin and Akt overexpression on HO-1 accumulation are not additive. In cells transfected with a dominant-negative Akt-expressing construct (Akt-K179M), and treated 48 h later with insulin, HO-1 promoter activity was found to increase slightly but this was not statistically significant (Fig. 4A).

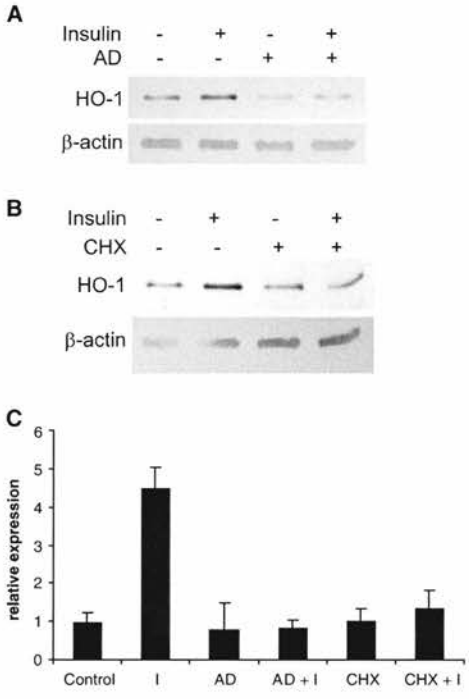

Fig. 2. Insulin-stimulated heme oxygenase-1 (HO-1) accumulation is transcription and translation dependent. Cells were serum-deprived for 16 h and pretreated with actinomycin D (AD) (5 $\mu g \cdot mL^{-1}$) (A, C) or cycloheximide (CHX) (10 $\mu g \cdot mL^{-1}$) (B, C) for 30 min, and then treated with insulin (I) (200 nM) for 6 h (A, B) or 2 h (C). Whole cell lysates were prepared and analysed by western blotting (A, B) using antibody to HO-1, with β-actin as loading control. mRNA extracts were prepared (C) using TRIzol and reverse transcribed to cDNA. Fluorescence detection real-time PCR was performed using HO-1 primers and probe with an 18S primer/probe control; results are expressed as mean relative expression ± SEM of three independent experiments.

Insulin-mediated HO-1 accumulation is neither p38-MAPK nor extracellular signal-related kinase (ERK) dependent

Insulin was found to phosphorylate p38-MAPK (Fig. 5A) and ERK (Fig. 5B) in a time-dependent manner. ACHN cells were then pretreated with the p38-MAPK inhibitor SB202190, or the mitogen-activated kinase kinase 1 (MEK1) inhibitor PD98059, and treated with insulin. Adequate inhibition of p38-MAPK was demonstrated by probing for phosphorylated Hsp27, a known downstream target of p38-MAPK [34] (Fig. 5C). MEK1 inhibition was confirmed with blots for phosphorylated ERK1/2 (Fig. 5D). In cells pretreated with SB202190 or PD98059 and exposed to insulin, no decrease in the expected HO-1 accumulation was observed (Fig. 5C,D), suggesting that neither p38-MAPK nor ERK activity is required for insulin-induced HO-1 accumulation.

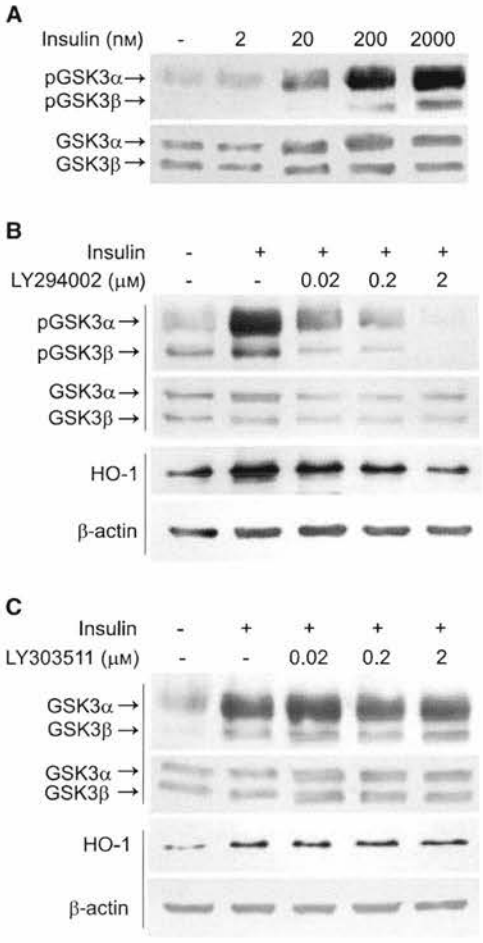

Fig. 3. Insulin stimulates heme oxygenase-1 (HO-1) accumulation through a phosphatidylinositol 3-kinase (PI3K)-dependent pathway. Renal adenocarcinoma (ACHN) cells were serum-deprived for 16 h and treated with increasing concentrations of insulin (200 nM) for 30 min (A). Other groups were pretreated with the PI3K inhibitor LY294002 (B), or its inactive analog LY303511 (C) for 30 min, and then treated with insulin (200 nM) for 30 min to determine glycogen synthase kinase 3β (GSK3β) phosphorylation status, and for 6 h to determine HO-1 accumulation. Whole cell lysates were prepared and analysed by western blotting using phospho-specific antibody to GSK3α/β (ser 21/9) (pGSK3α/β) and antibody to total GSK3 as a loading control. As previously, antibody to HO-1 was used, with β-actin as loading control.

Nrf2 translocates to the nucleus following insulin treatment

In ACHN cells treated with increasing concentrations of insulin for 1.5 h, the nuclear fraction of Nrf2 was found to increase as the cytosolic component decreased (Fig. 6A). Immunofluorescent labeling of Nrf2 revealed increased nuclear staining following insulin treatment (Fig. 6B). Pretreatment with LY294002 abolished

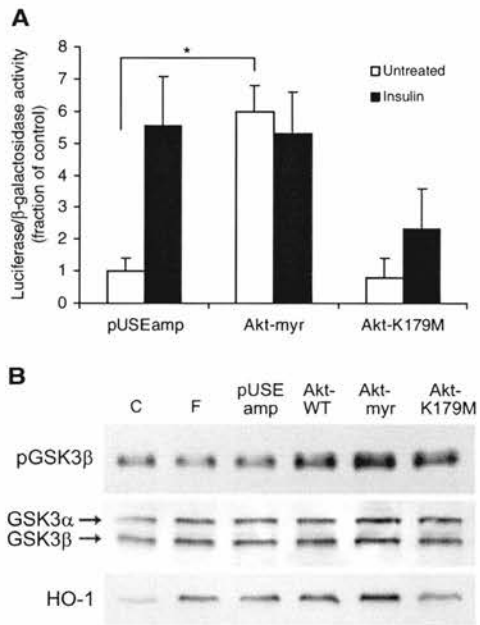

Fig. 4. Overexpression of active Akt causes heme oxygenase-1 (HO-1) reporter activation. (A) Renal adenocarinoma cells (ACHN) were triple-transfected with the pHOGL3/11.6 reporter construct, the pSV-β-galactosidase control construct and vectors expressing membrane-targeted active Akt (Akt-myr), dominant-negative Akt (Akt-K179M) or empty vector control (pUSE-amp). Forty-eight hours later, cells were treated with insulin (200 nM) for 6 h and then lysed in 100 μL of reporter lysis buffer, 20 μL of which was used for luciferase assay, the remainder being used for β-galactosidase assay. Results are expressed as luciferase activity per unit of β-galactosidase activity ± SEM of four independent experiments. $^*P < 0.05$, ANOVA. (B) ACHN cells were transfected with constructs expressing wild-type Akt (Akt-WT), membrane-targeted active Akt (Akt-myr), dominant-negative Akt (Akt-K179M) or empty vector control (pUSE-amp). Forty-eight hours later, whole cell lysates were produced and analyzed by western blotting using antibody to HO-1, phospho-specific antibody to GSK3β (ser 9) (pGSK3β) and antibody to total GSK3 as loading control. C, control; F, transfection agent alone.

nuclear accumulation of Nrf2 in response to insulin at doses previously shown to inhibit PI3K activity (Fig. 7C); the inactive analog, LY303511, had no effect on insulin-mediated Nrf2 nuclear accumulation (Fig. 7D).

Insulin mediated HO-1 induction is abolished by Nrf2 small-interfering RNA (siRNA)

ACHN cells were transfected with Nrf2 siRNA according to the manufacturer's instructions. Forty-eight hours later they were treated with insulin or the proteosome inhibitor MG132 (used as a positive control for Nrf2 accumulation) for 6 h. Cobalt chloride ($CoCl_2$), a hypoxia mimetic that activates the HO-1 promoter (data not shown), was also used as a control. Groups treated with the Nrf2 siRNA demonstrated greatly reduced Nrf2 and HO-1 protein expression when compared with control siRNA-treated groups (Fig. 7). In Nrf2 siRNA groups treated with insulin, no HO-1 induction was observed; however, in Nrf2 siRNA groups treated with $CoCl_2$, HO-1 induction did occur, demonstrating that Nrf2 activity is not a prerequisite for promoter activation. Although nuclear localization of Nrf2 following insulin treatment was apparent, it was not clear whether insulin treatment resulted in increased total Nrf2. There was a suggestion on western blotting of whole cell lysates that total cellular Nrf2 was increased following insulin treatment, but on quantification of three independent blots, no difference was demonstrated (Fig. 7).

Discussion

HO-1 is one of the most critical cytoprotective mechanisms activated during cellular stress, and clinically applicable pharmacological or gene-based strategies of induction need to be identified [35]. In the setting of organ transplantation, intervention to upregulate HO-1 could be directed at the donor, the harvested organ *ex vivo* or the recipient and would clearly need to be efficacious, be specific, lack side-effects and be easily deliverable to the organ in question. In this study, we have provided direct evidence of HO-1 induction by insulin through the PI3K/Akt cascade and the Nrf2 transcription factor in both transformed renal cells and primary mouse renal tubular epithelial cells. Insulin-induced HO-1 protein expression was sensitive to PI3K/Akt inhibition and Nrf2 gene silencing. The fold-increase in both HO-1 protein and mRNA in response to insulin was consistent, as well as being time and concentration dependent.

The role of the PI3K/Akt pathway in the regulation of HO-1 has been the source of much interest lately. Our data demonstrate that insulin-induced HO-1 accumulation is sensitive to PI3K inhibition with LY294002. This is in keeping with results from other work demonstrating the importance of PI3K/Akt activation in HO-1 regulation following cell stimulation with nerve growth factor (NGF) [29], carnosol [30], hemin [36] and cadmium [37]. Overexpression of active Akt alone was sufficient to mimic the effects of insulin on HO-1 expression in our model, adding weight to the suggestion that the effect of insulin on HO-1 is mediated predominantly, or possibly exclusively, by the PI3K/Akt axis. Overexpression of membrane-targeted active Akt stimulated the HO-1 promoter but, significantly, adding insulin did not increase this

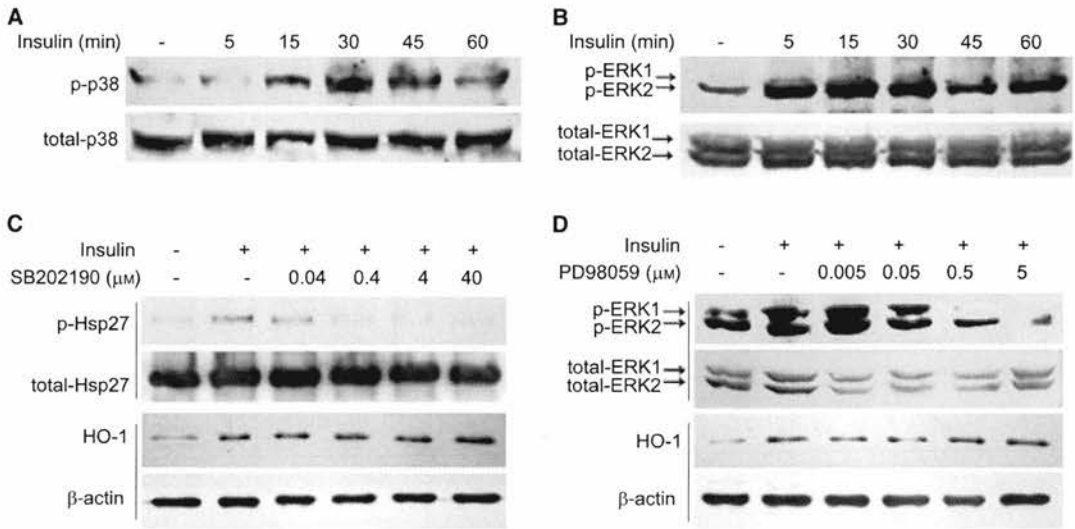

Fig. 5. p38 Mitogen-activated protein kinase (p38-MAPK) and extracellular signal-related kinase (ERK) inhibition has no effect on insulin-induced heme oxygenase-1 (HO-1) accumulation. (A, B) Cells were serum-deprived for 16 h and treated with insulin (200 nM) for various times. Whole cell lysates were prepared and analyzed by western blotting using antibody to the phosphorylated form of p38-MAPK (Thr180/Tyr182) (p-p38) (A) and phosphorylated ERK1/2 MAPK (Thr202/Tyr204) (p-ERK1/2) (B). Total p38 (A) and total ERK1/2 (B) were used as loading controls. (C, D) Cells were serum-deprived for 16 h and pretreated with the p38-MAPK inhibitor SB202190 (C) or the MEK1 inhibitor PD98059 (D) for 30 min, after which insulin (200 nM) was added (6 h). Whole cell lysates were prepared and analyzed by western blotting using antibody to HO-1 and β-actin to control for protein loading. Adequacy of p38-MAPK inhibition was established with blots for phosphorylated Hsp27 (C). MEK1 inhibition was confirmed with blots for phosphorylated ERK1/2 (D).

activation. In contrast, Salinas *et al.* reported that although the basal level of HO-1 mRNA, measured by semiquantitative RT-PCR, was higher in cells transfected with a membrane-targeted active Akt expressing construct, administration of NGF further increased this expression [29]. This may indicate that NGF exhibits its effect through additional mechanisms in comparison with insulin, although the differences may be due to cell type or transfection technique.

The exact role of the MAPK cascades in HO-1 regulation remains controversial. Inhibition of p38-MAPK reduces HO-1 expression following carnosol [30], diallyl sulfide [38] and cadmium [37] treatment, although an earlier study found that p38 inhibition had no effect on HO-1 mRNA expression following cadmium, arsenate or hemin [39] treatment. Our data, however, show that despite concentrations of insulin being sufficient to phosphorylate p38, inhibition of p38 did not alter insulin-induced HO-1 protein expression. In keeping with our results, ERK inhibition did not impact on HO-1 expression following carnosol [30] or arsenite [40] treatment; however, ERK activity was required for HO-1 induction in HepG2 cells treated with diallyl sulfide [38] and LMH cells exposed to arsenite [41]. It remains unclear why these disparities exist, but it appears that p38 and ERK play a significant role in HO-1 regulation in some models, but not in others.

During our investigation we studied a number of different transcription factors that may be involved in mediating the effect of insulin on HO-1 expression, including heat shock transcription factor-1 (HSF-1), hypoxia-inducible factor-1 (HIF-1) and NF-E2-related factor 2 (Nrf2). The PI3K/Akt pathway has been implicated in HSF-1 regulation by virtue of the repressive effects of the Akt target GSK3β on HSF-1 [42]. Although insulin treatment was sufficient to phosphorylate and deactivate GSK3β, this did not result in nuclear localization, trimerization or transactivation of HSF-1 (data not shown).

The basic helix–loop–helix transcription factor, hypoxia-inducible factor-1 (HIF-1), mediates essential homeostatic responses to reduced oxygen [43,44]. HIF-1 has been shown to mediate transcriptional activation of HO-1 in a rat model of hypoxia [45] and rat renal medullary cells [46]. In addition, we have previously reported an associative increase in HIF-1 DNA binding and HO-1 induction in a rat model of liver ischemia–reperfusion injury [47]. The relationship between HIF-1 and HO-1 induction in humans is less clear. Hypoxia has been shown to repress HO-1 mRNA expression in primary cultures of human umbilical vein endothelial cells despite HIF-1 transactivation, while $CoCl_2$, a known HIF-1 activator, was shown to induce expression [48]. This reflects our

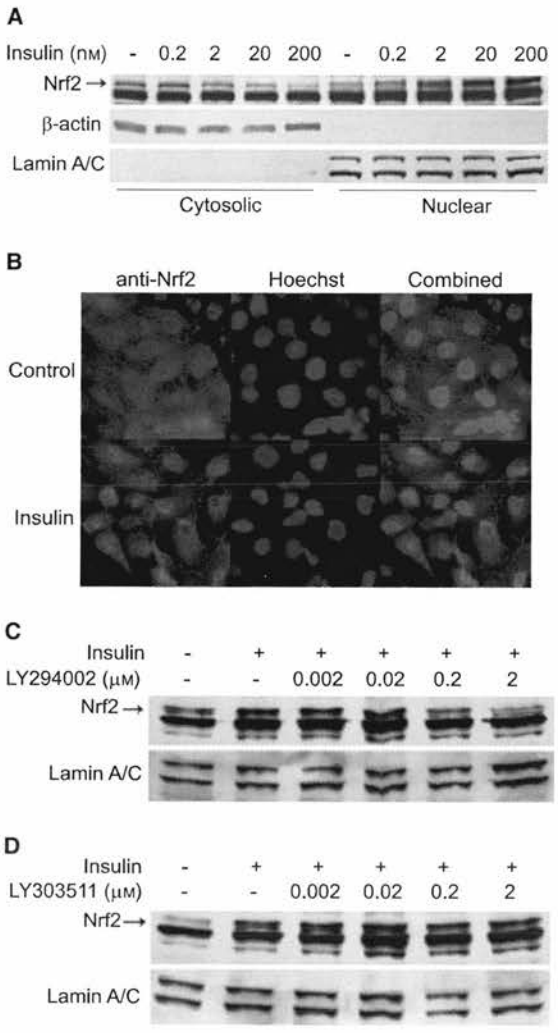

Fig. 6. Insulin treatment causes phosphatidylinositol 3-kinase (PI3K)-sensitive nuclear migration of NF-E2-related factor (Nrf2). (A) Cells were serum-deprived for 16 h and treated with increasing concentrations of insulin for 1.5 h. Nuclear and cytosolic lysates were prepared and analyzed by western blotting using antibody to Nrf2, with loading control with β-actin for cytosolic extracts and lamin A/C for nuclear extracts. (B) Cells were treated similarly with insulin (200 nM) for 1.5 h, prepared for immunofluorescence and treated with antibody to Nrf2, followed by Hoechst counterstaining. (C, D) Cells were serum-deprived for 16 h and pretreated with the PI3K inhibitor LY294002 (C) or its inactive analog LY303511 (D) for 30 min. Cells were treated with insulin (200 nM) for 1.5 h, after which nuclear lysates were prepared and analyzed by western blotting, using antibody to Nrf2, with lamin A/C loading control.

observation that ACHN cells subjected to hypoxia demonstrate a decrease in HO-1 protein expression (data not shown), while $CoCl_2$ induces HO-1 protein (Fig. 7). An explanation for this apparent contradiction may lie in the observation that in Chinese hamster ovary cells, HO-1 induction by hypoxia and $CoCl_2$ can occur in an HIF-1-independent manner; while $CoCl_2$ was shown to act in an Nrf2-dependent manner, hypoxia was not [49]. It is not clear how findings in these cells translate to other models, but our data would support this view: Nrf2 gene silencing resulted in a reduction in $CoCl_2$-mediated HO-1 expression. Yet some HO-1 induction was still apparent, possibly relating to HIF-1 activity, although this was not examined specifically. Controversial evidence exists linking PI3K activity with regulation of HIF-1, in both hypoxic [50,51] and normoxic [52–56] conditions, although this appears to be cell-type specific [57,58]. Insulin has been shown to upregulate HIF-1 directly through the PI3K/Akt pathway [56]. However, despite all this, in our model HIF-1 transactivation is not seen following insulin treatment, as determined by an HIF-1 luciferase reporter construct (data not shown).

Nrf2 has been shown to regulate HO-1 [31] and is known to be under the influence of PI3K [30,36,59–62]. Consistent with our results, insulin has previously been shown to cause nuclear localization of Nrf2, although PI3K dependency was not investigated in that study [61]. However, hemin has been shown to induce Nrf2 nuclear localization in a PI3K-sensitive manner [36]. Using Nrf2 siRNA, we have clearly shown the dependence of basal HO-1 expression on Nrf2 activity: Nrf2 gene silencing practically abolished HO-1 expression. However, the promoter could still be activated by $CoCl_2$ following Nrf2 gene silencing, although the mechanism by which this was occurring was not elucidated. No HO-1 response was seen following insulin treatment in Nrf2 siRNA-treated cells, suggesting that insulin-induced HO-1 expression has an absolute dependence on Nrf2 activity.

This report demonstrates the ability of insulin to induce HO-1 in a PI3K/Akt-dependent and Nrf2-dependent manner. HO-1 induction by PI3K/Akt or Nrf2 activation requires further delineation in models of transplantation and may represent an approach that can be implemented clinically as a future organ protection strategy.

Experimental procedures

Materials

All reagents were obtained from Sigma-Aldrich Co. Ltd (Poole, UK) unless otherwise stated. Antibodies to GSK3, Nrf2 and lamin A/C were obtained from Santa Cruz (Wembley, UK); antibodies to HO-1, phospho-Hsp27 (Ser78) and total Hsp27 were obtained from Stressgen

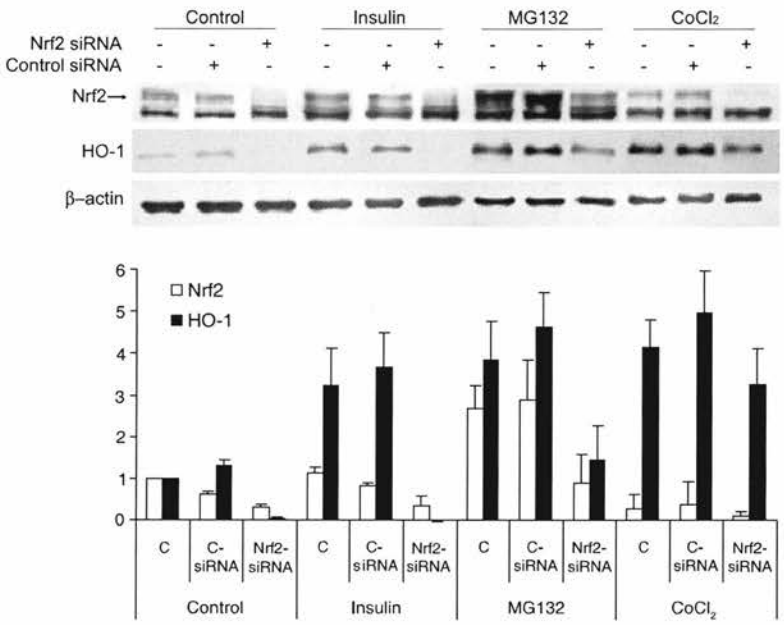

Fig. 7. NF-E2-related factor (Nrf2) silencing with small-interfering RNA (siRNA) prevents insulin-induced heme oxygenase-1 (HO-1) accumulation. Cells were transfected with Nrf2 siRNA and 48 h later treated with insulin (200 nM), the proteosome inhibitor MG132 (20 μM) or cobalt chloride ($CoCl_2$) for 6 h. Whole cell lysates were prepared for western blotting using antibody to HO-1 and Nrf2, with β-actin as loading control. Optical densities of bands were quantified (Quantity One, Bio-Rad). Bars represent the mean of three independent experiments, with error bars representing SEM.

(Victoria, BC, Canada); β-actin antibody was obtained from BD Biosciences (San Diego, CA, USA); phospho-GSK3β (ser9) (pGSK3β), phospho-GSK3α/β (ser21/9) (pGSK3α/β), phospho-ERK1/ERK2 MAPK (Thr202/Tyr204) (E10) monoclonal (p-ERK1/2), ERK1/ERK2 MAPK (total-ERK1/2), phospho-p38 MAPK (Thr180/Tyr182) (28B10) monoclonal (p-p38) and p38 MAP kinase (5F11) monoclonal (total p38) antibodies were obtained from New England Biolabs (Hitchin, Hertfordshire, UK).

Cell culture and transfections

Renal adenocarcinoma cells (ACHN) (European Collection of Cell Cultures, Porton Down, UK) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (50 $U \cdot mL^{-1}$), streptomycin (50 $\mu g \cdot mL^{-1}$) and nonessential amino acids (5%) (all Gibco, Paisley, UK). In experiments termed serum-deprived, cells were plated out on day 1 in DMEM with 10% fetal bovine serum. On the evening of day 2, the medium was changed to DMEM with 0% fetal bovine serum, and the experiment was performed on day 3. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. All experiments were performed with subconfluent cultures. Akt expression constructs (Upstate, Milton Keynes, UK) are based on the pUSEamp vector. The activated form (Akt-myr) contains an N-terminal myristoylation sequence targeting Akt to the plasma membrane. The dominant-negative form (Akt-K179M) contains a methionine for lysine substitution at residue 179 abolishing Akt kinase activity. The wild-type form (Akt-WT) contains the unaltered Akt sequence, and an empty vector (pUSE-amp) was used as a control. The HO-1 luciferase reporter construct (pHOGL3/11.6) was a kind gift from A. Agarwal (University of Alabama, Birmingham, AL, USA). The heat shock protein 70-β-galactosidase (HSP70-β-gal) reporter construct was a kind gift from W. J. Welch (University of California, San Francisco, CA, USA). The HIF-1 reporter construct (pHRE-luc) was a kind gift from H. Esumi (National Cancer Center Research Institute, Tokyo, Japan). Transfection efficiency was controlled by cotransfecting with a β-galactosidase (pSV-β-gal)-expressing or a luciferase (pGL3-luc)-expressing control vector (Promega, Southampton, UK). Transient transfections were performed using Fugene (Roche, Lewes, UK) at a 6 : 1 ratio of reagent to DNA. In dose-finding experiments using a construct constitutively expressing green fluorescent protein, the transfection efficiency was found to be 30–40%. Experiments on transfected cells were performed 24–48 h later.

Mouse primary tubular epithelial cell culture

The kidneys of 6-week-old male BALB/c mice were removed in sterile conditions and placed in ice-cold HANK's balanced salt solution (HBSS) containing penicillin (100 $U \cdot mL^{-1}$), streptomycin (100 $\mu g \cdot mL^{-1}$) (Gibco) and 1× antibody antimycotic solution. After decapsulation and bisection, the medulla was removed and the cortices were reduced with repeated incisions to 1 mm^3 pieces. Kidney pieces were incubated at 37 °C with HBSS containing freshly prepared collagenase type IV (0.5 $mg \cdot mL^{-1}$) and DNase (10 $\mu g \cdot mL^{-1}$). Following confirmation of the presence of tubules, they were resuspended

in DMEM-F12 with glutamax, penicillin (100 $U \cdot mL^{-1}$), streptomycin 100 $\mu g \cdot mL^{-1}$) (all Gibco), 1× insulin/transferrin/selenium, dexamethasone (35.7 $ng \cdot mL^{-1}$) and epidermal growth factor (25 $ng \cdot mL^{-1}$). Tubules were cultured in six-well plates for about 5 days until 70% confluent. Culture conditions were then changed to DMEM-F12 with glutamax, penicillin, streptomycin and dexamethasone for 40 h, after which experiments were performed. Cells were cytokeratin positive and vimentin negative on immunocytochemistry (data not shown). All experiments involving animals were conducted in accordance with the provisions of the UK Animals (Scientific Procedures) Act 1986.

Western blot

Whole cell extracts were produced using radioimmuno precipitation assay buffer with protease inhibitors and nuclear lysates using Gobert's method [63]. Proteins were separated by SDS/PAGE and transferred by electroblotting to nitrocellulose membranes (Bio-Rad, Hemel Hempstead, UK). The membranes were soaked in blocking buffer (NaCl/Tris, 0.05% Tween-20, 5% nonfat milk) followed by blocking buffer containing primary antibody. After washing, the membranes were exposed to horseradish peroxidase-conjugated secondary anti-mouse (Upstate) or anti-rabbit (Santa Cruz) and were used at a concentration of 1 : 5000. Enhanced chemiluminescence reagent (Amersham, Chalfont St Giles, UK, and Upstate) was used, with development using autoradiography. Equality of loading was confirmed by probing membranes for β-actin for whole cell extracts, and lamin A/C for nuclear extracts.

RNA isolation and fluorescence detection real-time PCR

RNA extraction and purification were performed using a TRIzol (Invitrogen, Paisley, UK). RNA samples were treated with DNase and then run as a template for a standard PCR reaction using β-actin primers to exclude the presence of contaminating DNA. RNA was then reverse transcribed to cDNA using avian myeloblastosis virus reverse transcriptase (Promega) and random decamers (Ambion, Huntingdon, UK). Fluorescence-detection real-time PCR was then performed using primers and probes specifically designed for human HO-1: forward primer 5′-AGGGTGATAG AAGAGGCCAAGA, reverse primer 5′-CAGCTCCTGCA ACTCCTCAA and TAMRA-labeled probe 6-FAM-TGC GTTCCTGCTCAACATCCAGCT-TAMRA. A standard reaction contained Taqman universal master mix 12.5 μL (Applied Biosystems, Warrington, UK), primer probe mix 7 μL (primers 25 μM, probe 5 μM), 18S primer probe mix 1.25 μL, water 1.75 μL and cDNA template 2.5 μL. Samples were run on an ABI Prism 7700 Sequence Detection System and analysed using Sequence Detector 7.1 (Applied Biosystems).

Luciferase/β-galactosidase assay

Cells were cotransfected with the appropriate reporter vector and control vector and treated as per the experimental protocol on the following day. Cells were lysed with reporter lysis buffer (Promega), after which 20 μL of lysate was combined with 50 μL of luciferase assay reagent and the resulting light emission measured on a luminometer (Fluoroskan Ascent Fl, Thermo Electron, Basingstoke, UK). The remaining lysate (80 μL) was combined with β-galactosidase assay 2× buffer and, following incubation at 37 °C for 4 h, was read at 420 nm on a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Chalfont St Giles, UK).

Immunofluorescence

Cells were cultured in chambered slides, treated as per the experimental protocol and fixed with methanol. Blocking with 10% normal goat serum in NaCl/Tris for 20 min was followed by primary antibody exposure (anti-Nrf2, 1 : 250 in 10% normal goat serum) for 1 h at room temperature. After being washed in NaCl/Tris, the sections were exposed to secondary antibody (alexa fluor 568 F(ab′)2 fragment of goat anti-rabbit IgG, 1 : 200 in 10% normal goat serum) (Invitrogen) for 30 min. Counterstaining with Hoechst 33258 (Sigma) was performed prior to mounting. Fields were visualized with a Leica DM IRB fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) and images taken with a digital camera. Primary antibody only and secondary antibody only groups were always included as controls.

RNA interference

Cells were seeded in six-well plates and transfected on the following day with Nrf2 siRNA (h) (Santa Cruz) or control siRNA according to the manufacturer's protocol. Forty-eight hours later, transfected cells were treated and lysed. Adequacy of effect was ascertained with western blot analysis with anti-Nrf2.

Statistical analysis

Data are presented as means and standard error of the mean (SEM). Statistical comparisons were made using one-way analysis of variance (ANOVA) with the Tukey post hoc correction for multiple comparisons using SPSS version 10.0 (SPSS, Chicago IL, USA).

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Technical note

Firefly luciferase terminally degraded by mild heat exposure: Implications for reporter assays

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Abstract

Luciferase reporter constructs are an accurate method of assessing gene promoter activity and vectors constitutively expressing luciferase are useful in quantifying transfection efficiency. Common methodologies for examining the induction of the heat shock (stress) response require exposure of cells transfected with luciferase-expressing vectors to a mild heat stress. Here we re-examine the under-recognised phenomenon that luciferase is exquisitely sensitive to small temperature changes. In cells subjected to mild heat exposure following transfection with both luciferase and β-galactosidase reporter vectors, a marked reduction in luciferase activity was observed compared with β-galactosidase activity. On exposing recombinant firefly luciferase to small increases in temperature in vitro, a time and temperature dependent decrease in luciferase activity was demonstrated. Loss of luciferase activity following mild heat exposure will result in misinterpretation of reporter activity. This vastly underappreciated effect is worthy of further emphasis and luciferase reporter vectors should be used with caution in protocols that involve exposure to temperatures outside the physiological range.

Keywords: Bioluminescence; Enzyme activity; Firefly luciferase; Heat; Heat shock proteins

1. Introduction

The use of reporter vectors is a rapid and highly sensitive method of investigating factors regulating gene expression (Schenborn and Groskreutz, 1999). The promoter region of the gene of interest is cloned upstream of a sequence encoding the reporter enzyme; the resulting protein expression is proportional to gene activity and can be easily and reliably determined. In order to control for potential variability in transfection efficiency, it is common practice to co-transfect the reporter vector with a control vector, constitutively expressing a second distinct reporter enzyme under a viral promoter. For this second vector to function as an accurate control, enzyme expression must be independent of the experimental treatment and depend only on the number of cells that took up the plasmid DNA (Ibrahim et al., 2000).

Heat shock proteins are highly conserved intracellular chaperones and have recently been identified as important immunoregulatory agents (Pockley, 2003). While investigating the induction of heat shock protein 70 (Hsp70) following heat exposure in renal adenocarcinoma cells (ACHN), we utilised an Hsp70B β-galactosidase reporter vector. It was necessary to control for transfection efficiency and thus cells were co-transfected with the pGL3 luciferase control vector,

Abbreviations: Hsp, heat shock protein; SV40, simian virus 40.

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which contains the *luc+* gene and expresses firefly luciferase under the control of the simian virus 40 (SV40) promoter. It was observed that luciferase activity in groups transfected with the pGL3 vector and subjected to a mild heat exposure was much lower than expected.

To investigate whether this observation was an effect of heat on transcription we co-transfected the pGL3 vector with the pSV β-galactosidase control vector, also under the control of the SV40 promoter. The following day, groups were exposed to a mild heat induced stress (43 °C) for increasing lengths of time up to 45 min, after which all remaining groups were returned to 37 °C and recovered for further increasing time intervals. Additional examination involved exposing recombinant firefly luciferase to a series of mild heat exposures for increasing lengths of time. All experiments were independently repeated 3 times.

2. Methods

2.1. Cell culture and transfection

Renal adenocarcinoma cells (ACHN) (European Collection of Cell Cultures, Porton Down, UK) were maintained in Dulbecco's modified Eagle's medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and non-essential amino acids (5%). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. Cells were passaged regularly and all experiments were performed with sub-confluent cultures. Luciferase (pGL3-*luc+*) and β-galactosidase (pSV-β-gal) expressing vectors were obtained from Promega, Southampton, UK. Transient transfections were performed using Fugene (Roche, Lewes, East Sussex, UK) at a 6 : 1 ratio reagent to DNA. Experiments on transfected cells were performed 24 h later.

2.2. Luciferase and β-galactosidase assay

Cells were lysed with reporter lysis buffer (Promega) and the protein concentration determined (Lowrie method; BioRad) after which 20 μl of lysate were combined with 50 μl luciferase assay reagent and the resulting light emission measured on a luminometer (Fluoroskan Ascent Fl; Thermo Electron, Basingstoke, UK). The remaining lysate (80 μl) was combined with β-galactosidase assay 2× buffer and, following incubation at 37 °C for 4 h, was read at 420 nm on a spectrophotometer (Ultraspec 2000; Pharmacia Biotech, now GE Healthcare, Chalfont St. Giles, UK).

2.3. In-vitro luciferase analysis

Firefly luciferase (Sigma) was dissolved in distilled water (1 μg/μl) and further diluted 1/20 in PBS. Samples were placed in a thermocycler (PCR Sprint Thermal Cycler, Thermo Electron) with the hot-lid facility turned off, for the specified times.

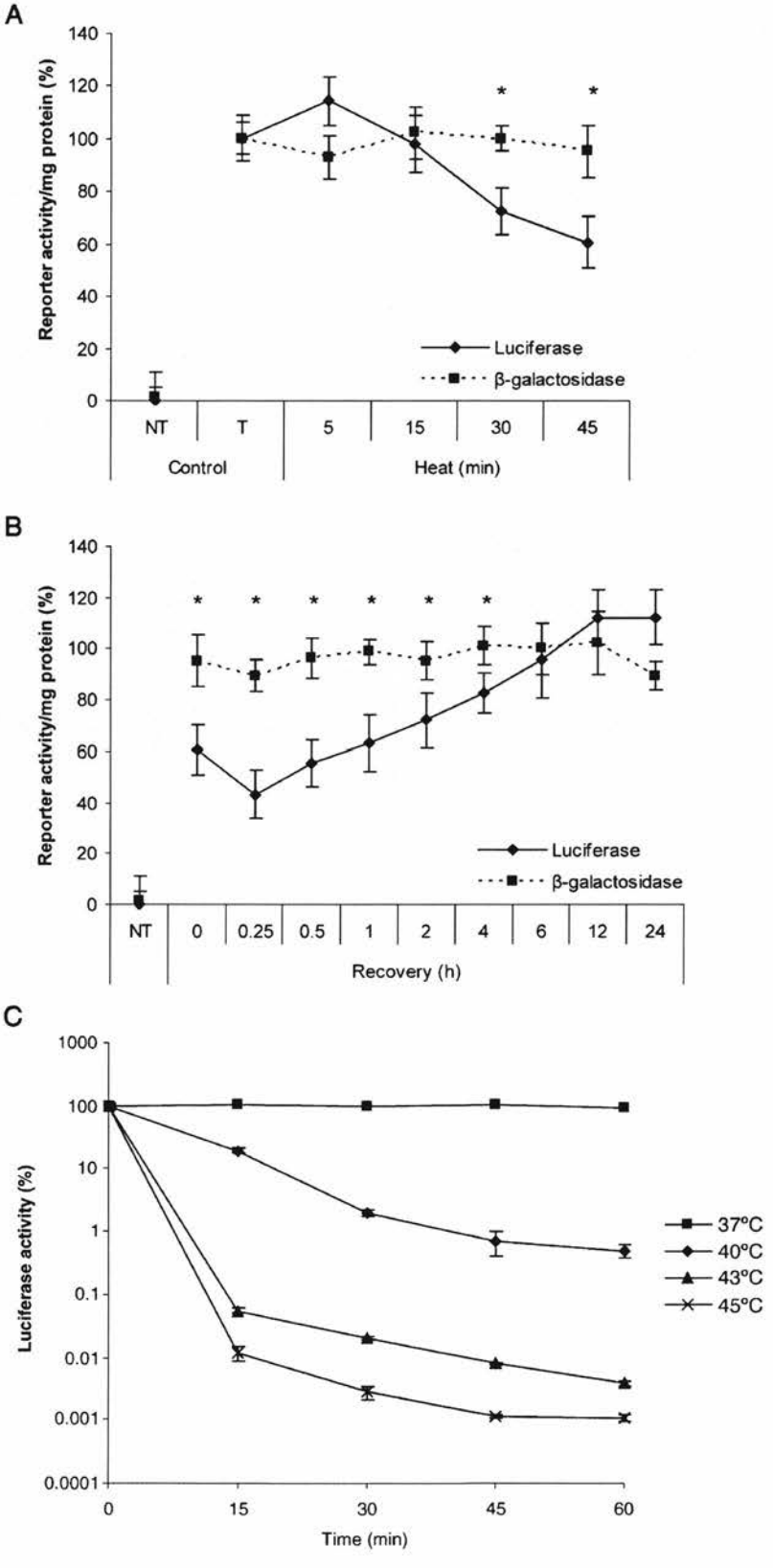
3. Results and discussion

Cells transfected with both the pGL3 luciferase vector and the pSV β-galactosidase control and subjected to mild heat exposure demonstrated a marked reduction in luciferase activity compared with β-galactosidase activity, which remained unchanged (Fig. 1A). This occurred despite both enzymes being under the control of the same SV40 promoter, suggesting that the effect was independent of transcription. The reduction was related to the length of heat exposure and a statistically significant difference between the pGL3 luciferase group and the pSV β-galactosidase control was observed following 30 min of heat exposure (Mann–Whitney U $p<0.05$). Luciferase activity increased during recovery but the difference between the groups was still statistically significant in the 4 h after the stimulus, the crucial period examined when measuring gene promoter activation (Fig. 1B).

On exposing recombinant firefly luciferase to various temperatures (Fig. 1C) a time and temperature dependent response was observed with luciferase activity decreasing markedly over time and with increasing temperature. An increase in temperature to 40 °C for 45 min was enough to reduce enzyme activity to less than 1% of control.

Firefly luciferase is the most commonly used bioluminescent reporter (Wood, 1998). Despite the sensitivity and convenience of the enzyme assay

Fig. 1. Luciferase and β-galactosidase activity following mild heat exposure. (A) The pGL3 luciferase and pSV β-galactosidase control vectors were co-transfected into a renal adenocarcinoma cell line (ACHN) using the Fugene transfection reagent. The following day, groups were heated to 43 °C for various lengths of time. Whole cell extracts were prepared with reporter lysis buffer (RLB), a protein assay was performed and luciferase and β-galactosidase activity determined. NT, non-transfected control; T, transfected control. (B) Cells were transfected as above and heated to 43 °C for 45 min 24 h later. Cells were lysed and luciferase and β-galactosidase activity were determined. (C) Firefly luciferase was diluted, placed in a thermocycler and heated for the times specified, after which luciferase activity was determined. * Mann–Whitney U $p<0.05$.


A
Reporter activity/mg protein (%)
140
120
100
80
60
40
20
0
*
*
Luciferase
β-galactosidase
NT
T
5
15
30
45
Control
Heat (min)
B
Reporter activity/mg protein (%)
140
120
100
80
60
40
20
0
* * * * * *
Luciferase
β-galactosidase
NT
0
0.25
0.5
1
2
4
6
12
24
Recovery (h)
C
Luciferase activity (%)
1000
100
10
1
0.1
0.01
0.001
0.0001
37°C
40°C
43°C
45°C
0
15
30
45
60
Time (min)

inherent problems exist, particularly relating to thermostability, both in vivo and in vitro (Nguyen et al., 1989). Improved heat tolerance of luciferase has been demonstrated using mutagenesis to achieve a single amino acid substitution in both the Japanese Firefly (*Luciola cruciata and Luciola lateralis*) (Kajiyama and Nakano, 1993) and the North American Firefly (*Photinus pyralis*) (White et al., 1996). Modifications of the luciferase gene have improved performance in other areas, including the elimination of peroxisomal translocation (Wood, 1998). However, thermostability is not a feature of current commercial versions since Nguyen et al. (1989) have previously demonstrated the sensitivity of luciferase to heat. Inconsistent with our findings, their data demonstrates a significant decrease in β-galactosidase activity in mouse-derived cells exposed to heat, although no loss of activity was seen in in vitro studies. However, our model used a human cell line, protein translation was not abolished with cyclohexamide and cells were heated to 43 °C, not 45 °C. These factors may explain the difference in findings.

In conclusion, when a luciferase *reporter* vector is used to determine gene expression in cells exposed to mild heat, the activity of the gene in question may be underestimated. When, however, a luciferase *control* vector is used in cells exposed to heat, transfection efficiency may be underestimated, and so gene expression overestimated between groups.

These data demonstrate the propensity of firefly luciferase to become inactive following mild heat exposure. The pGL3 control vector should be used with caution in protocols that involve exposure to a mild heat insult.

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