Induction of the stress response in hepatocytes as a protective mechanism and its use as a marker of toxicity in vivo and in vitro

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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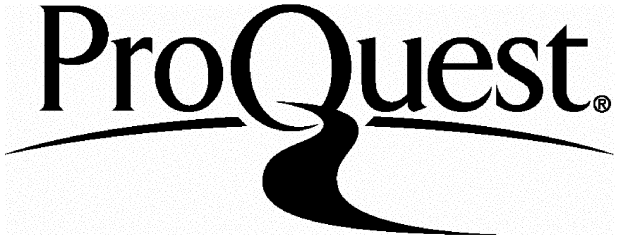
1998



Abstract

All prokaryotic and eukaryotic cells in response to stressful insults, such as chemical exposure or hyperthermic treatment upregulate a well conserved set of genes. These genes encode a family of proteins known as stress proteins whose induction is an adaptive response which serves to protect the cells by interacting with denatured or damaged proteins. The aim of the current thesis was to determine the effect of induction of stress proteins on the subsequent toxicity of chemicals in isolated hepatocytes. A further aim was to determine whether the induction of stress proteins following chemical exposure could be used as a sensitive marker of chemical exposure. These studies were performed in two hepatocyte models, hepatocyte monolayers and liver spheroids, and the hepatic effect was also determined *in vivo*.

Hyperthermic treatment of hepatocytes had a bi-phasic effect on the toxicity of subsequent chemical exposure. Exposure of cells to chemicals immediately following hyperthermic treatment resulted in a sensitisation of the cells to the toxin. If the cells were allowed a recovery period prior to chemical exposure the hepatocytes became tolerant to the toxic actions of the chemicals. The reasons for this are thought to be alterations in stress protein and GSH levels. The use of stress proteins as markers was problematic as hepatocyte monolayers had elevated basal stress protein levels resulting in poor induction following chemical exposure. However, liver spheroids had low basal stress protein levels which were significantly induced following chemical exposure to a greater extent than the response observed *in vivo*. However, in all situations only certain stress proteins were induced following exposure to specific chemicals. Differences in the activation of the transcription factor responsible for stress protein induction (HSF-1) were thought to be responsible for these differences. Therefore, measurement of these components of stress response may not be an all encompassing marker of toxicity but may be of use in determining the toxicity of certain chemicals. However, it does not rule out the possibility that other components of the stress response may be of use as such a marker.

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Acknowledgements

I would like to express my sincere thanks to the following;

Professor John Timbrell and Dr. Elisabeth George for their guidance, enthusiasm and support.

Dr Cathy Waterfield for her continuous technical assistance during the course of my work and Geraldine Hamilton for bringing the joys of spheroids to my attention.

Dan, Dave, Rob and everyone else in the Toxicology department for making those long evenings so bearable.

Carl, Denise, Janice, Pauline, Tracy at GlaxoWellcome for making my time there so enjoyable.

Finally Damian, Sarah and Stella for their support and encouragement during the writing up of this thesis.

Abbreviations

TCA	trichloroacetic acid
KH	Krebs Henseleit
ATP	adenosine triphosphate
GSH	glutathione (reduced form)
GSSG	glutathione (oxidised form)
EDTA	disodium ethylenediamine tetra acetate dihydrate
EGTA	ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N'-tetra-acetic acid
OPT	o-phthaldialdehyde
βNADH	β-nicotinamide adenine dinucleotide, reduced form
PBS	phosphate buffered saline
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
BSA	bovine serum albumin
Tween-20	polyoxyethylene sorbitan monolaurate
Tris	N-tris (hydroxymethyl) aminoethane
SSA	2-hydroxy-5-sulphobenzoic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
pHEMA	poly-2-hydroxyethylmethacrylate
FCS	foetal calf serum
FBS	foetal bovine serum
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
APS	ammonium persulphate
Menadione	2-methyl-1,4-naphthoquinone
Hsp	heat shock protein
HSF	heat shock factor
HSE	heat shock element

Chapter 1.

Introduction

1.1 Discovery of the cellular stress response

All prokaryotic and eukaryotic cells respond to elevated temperatures or other noxious stimuli by increasing the transcription of a set of genes encoding a range of proteins namely heat shock proteins. This response is generally known as the "heat shock response". The first observation of what was to be known as a heat shock response occurred in 1962 by Ferruccio Ritossa. He demonstrated that exposure of chromosomes isolated from the salivary glands of *Drosophila melanogaster* embryos to temperatures slightly above their ambient temperature caused the formation of "puffs" on the chromosomes. This new pattern of chromosome puffs was determined to represent specific transcription sites for the synthesis of heat shock proteins (Hsp's).

Further work investigating the effect of elevated temperatures on the cellular response showed that when the salivary glands of *Drosophila sp.* were exposed to sightly elevated temperatures the majority of protein synthesis within the cell was inhibited (Tissieres 1974). However, there were a discrete set of proteins that were in fact induced by the elevated temperatures, these were the so called "heat shock proteins". This discovery was then investigated further in chick embryos where large changes in protein synthesis in brain, lung, heart and liver tissues following heat shock were found (Vollemy 1982).

Following on from the work investigating the effect of elevated temperatures on heat shock proteins, work was also being performed to elucidate whether or not there were any other inducers of the heat shock response. The first demonstration of this was in 1978 by Hightower and Smith who showed that administration of the amino acid analogue canavanine was able to induce a heat shock response in chicken embryo cells in the absence of heat. It was therefore suggested, that the intracellular inducer of the heat shock response may be the accumulation of abnormally folded proteins within the cell (Hightower 1980). It was also postulated that the role of these induced heat shock proteins might involve recognition and removal of the damaged or misfolded proteins from the cell. Other inducers of the heat shock response include disulphuram (Levinson *et al.* 1978), 8-hydroxyquinoline, kethoxal bis(thiosemicarbazone), ortho-phenanthroline and thiosemicarbazide (Levinson *et al.* 1979).

The induction of the heat shock response by stimuli other than elevated temperatures was also demonstrated in bacteria where feeding of amino acid analogues caused the production of abnormally folded proteins and induction of the heat shock response (Goff and Goldberg 1985). It was suggested that whenever the proteolytic system of a cell was unable to cope with the increased intracellular load of denatured proteins, the stress response was induced. This theory was re-inforced by the demonstration that one of the proteins induced in the cells was a bacterial protease. This induction of the heat shock response was also demonstrated in animal cells following the addition of various amino acid analogues (Kelley and Schlesinger 1978).

One of the main breakthroughs in determining the induction triggers for the heat shock response occurred in 1986 when Anathan *et al.* demonstrated that injection of denatured proteins into frog oocytes containing an Hsp70/β galactosidase stress reporter gene construct caused the induction of the heat shock response. This gave conclusive evidence that an increase in the intracellular load of denatured proteins was sufficient to activate the heat shock response. It was therefore understood that exposure of a cell to elevated temperatures or adverse chemical stimuli caused the induction of a cellular defence mechanism known as the heat shock response. With the knowledge now that the heat shock response was induced by not just elevated temperatures but a wide range of chemicals the response was referred to as the stress response of a cell.

1.2 Biochemical and morphological changes following heat shock

Exposure of cells to temperatures 2-3°C above their ambient temperature causes a wide range of diverse biochemical and physiological changes within the cell. The main targets for heat induced damage to a cell include the plasma membrane, cytoskeletal components, respiratory metabolism, protein synthesis and DNA. One of the most striking changes following hyperthermia is an almost complete cessation of protein synthesis within the cell effectively keeping the cell in a growth arrested state. However as mentioned previously the exception to this are the family of stress proteins whose synthesis is actually up-regulated following heat shock.

The main biochemical effects of transient hyperthermia include an inhibition of plasma membrane Na/K ATP'ase function and an increase in inositol triphosphate (IP_3) levels. This effectively causes an increase in cystolic calcium levels as IP_3 causes the release of calcium from the sarcoplasmic reticulum. This increase in calcium and the effect on plasma membrane ionic channels effectively causes a decrease in ATP levels leading to an overall accumulation of unfolded or damaged proteins within the cell. Exposure of cells to elevated temperatures also affects the energy status of a cell, mitochondrial function becomes impaired with the mitochondria themselves taking on a swollen appearance. Due to this decrease in mitochondrial function the cell relies mainly on glycolytic energy metabolism and so levels of glycolytic enzymes are increased (Wijèweera *et al.* 1995, Welch 1992).

Other specific effects of hyperthermia include a decrease in the synthesis of cytokines such as interleukin-1β, a decrease in nitric oxide synthesis and an increase in prostaglandin synthesis. In hepatocytes, these effects are thought to be brought about by the inhibitory effect heat has on the nuclear factor-κB (NfκB) which regulates intracellular expression of cytokines (De Vera *et al.* 1996).

Morphologically, exposure of cells to elevated temperatures causes a severe alteration in the appearance of certain intracellular organelles. As mentioned previously, the mitochondria appear swollen but in addition to this the Golgi complex takes on a fragmented appearance. One of the most characteristic effects of heat is on the cytoskeletal network in a cell. Prior to heat shock intermediate filaments are arranged in a meshwork system but following heat shock they appear to localise to and surround the nucleus. Within the nucleus itself there is an increase in unprocessed forms of mRNA following heat shock in addition to a decrease in ribosomal RNA processing in the nucleolus and an accumulation of denatured pre-ribosomal components. Other nuclear structures that are especially thermally sensitive include the spliceosome and non-condensed chromatin (Welch and Suhan 1985, Welch 1992).

1.3 General structure and function of the stress protein family

Extensive work over the last 30 years has been carried out into the characterisation of the proteins involved in the stress response of a cell. The proteins and the corresponding genes have been isolated and extensively studied. To date a large family of stress proteins has been identified, these include, the glucose regulated proteins (GRP's), metallothionein, ubiquitin, cytochrome P450, heat shock proteins (Hsp's) and other oxidation specific stress proteins such as heme oxygenase.

1.3.1 Glucose regulated proteins

Glucose regulated proteins (GRP's) as their name suggests are induced mainly by glucose deprivation but are also induced by changes in intracellular Ca^{2+} levels, inhibitors of glycosylation, elevated temperatures and amino acid analogues (Hightower 1991). GRP's were found to have molecular weights of 78 and 94kDa and these two GRP's show a considerable structural sequence homology to Hsp70 and Hsp90 respectively. GRP78 is located in the lumen of endoplasmic reticulum (ER) where it is thought to interact with secretory proteins in an ATP-dependent manner and play a role in their assembly prior to secretion out of the cell. GRP78 is also known as BiP (immunoglobulin binding protein) as it was first known to interact with newly synthesised heavy and light chains of immunoglobulins in the ER before their assembly into the final immunoglobulin structure. GRP94 is also found in the ER and on the plasma membrane. Little is known about the function of GRP94, but it is known that it is able to bind to calcium within the ER and due to its location it is postulated that GRP94 is involved in protein synthesis and/or secretion (Welch 1992).

1.3.2 Metallothionein

Metallothioneins are small (approximately 6500Da), ubiquitous proteins rich in cysteine residues. They have the ability to bind metals such as copper and zinc under physiological conditions. In addition to this the metallothioneins are inducible by a wide number of metal ions (Zn^{2+}, Cu^{2+}, Cd^{2+}) as well as certain physiological stress conditions such as inflammation and endotoxic shock (Hernandez *et al.* 1996, Iszard *et al.* 1995). One of the primary functions of metallothionein is thought to be in the detoxification of heavy metals.

This is brought about by metallothionein binding to the metals thereby effectively preventing the metal from exerting its deleterious effects at its site of action. As well as having been shown to be protective against heavy metals metallothionein has also been shown to be protective against oxidative stress, ionising radiation, chemotherapeutic and alkylating agents (Moffatt *et al.* 1996). This protection is thought to be due to the ability of metallothionein to react with free radicals and electrophiles. Metallothionein has been shown to scavenge hydroxyl radicals *in vitro* and also functions as a superoxide dismutase thereby protecting against lipid peroxidation (Iszard *et al.* 1995).

1.3.3 Ubiquitin

Ubiquitin is a 76 amino acid protein which plays a role in the targeting of proteins for degradation. Exposure of cells to heat, chemical stress and denatured proteins all increase the synthesis of ubiquitin which then targets the damaged proteins for proteolytic degradation in an ATP dependant manner. The binding of ubiquitin to the damaged proteins covalently modifies the proteins thereby making them recognisable to the degradative enzyme known as the 26S proteasome or to the other major site of degradation, the lysosome (Hershko 1988, Hershko 1996, Pickart 1997, Welch 1992).

1.3.4 Cytochrome P450

The cytochrome P450 gene family encodes over 400 enzymes that catalyse the bio-transformation of a large number of substrates. They are mainly found in the liver where they are involved in the mono-oxygenation and hydroxylation of various xenobiotics in addition to numerous endogenous compounds. Although not classically thought of as stress proteins they do demonstrate some of the characteristics namely that they are involved in the de-toxification of a large number of compounds and are readily induced by a wide variety of chemicals. Cytochrome P450 enzymes are involved in phase I metabolism of compounds and some of the reactions catalysed by cytochrome P450 include aliphatic and aromatic hydroxylations, epoxidation, N-, O- and S- dealkylations, oxidative deaminations, N-, S- and phosphotionate oxidations, dehalogenations and alcohol oxidations. The overall stoicheometry of the reaction is as follows;

$$NADPH + H^+ + O_2 \xrightarrow{\text{cytochrome P450}} NADP^+ + H_2O + ROH$$

-where RH represents the an oxidisable substrate and ROH the hydroxylated metabolite

The newly hydroxylated metabolite can now undergo phase II metabolism and hence subsequent detoxification more readily. Cytochrome P450 enzymes are expressed constitutively but can be induced by various compounds. It is known that specific cytochrome P450 enzymes have specific inducers, for example cyp1A1 is induced by PAH and dioxins, cyp2B1 and 2B2 are induced by phenobarbital, cyp2E1 is induced by ethanol and cyp4A is induced by peroxisome proliferators. Therefore any alteration in the levels of individual isoforms of cytochrome P450 can therefore alter the toxicity of those compounds metabolised by that P450 isoform. It should also be noted however, that in addition to compound detoxification cytochrome P450 plays a major role in the metabolic activation of numerous toxicologically relevant compounds (Guengerich 1989, Gibson and Skett 1994).

1.3.5 Heat shock proteins

Heat shock proteins (Hsp's) consist of a large family of well conserved proteins ranging in molecular weight from 25kDa to 110kDa. The main groups of the stress proteins are the low molecular weight proteins ranging in size from 24-30kDa, stress proteins with molecular weights of 60, 70 and 90kDa and finally the high molecular weight (100-110kDa) stress proteins. These proteins are inducible by heat, a wide range of chemicals (for example cadmium, arsenite, lead, diethylmaleate, ethylene oxide and ethanol) and by certain physiological states such as inflammation and ischemia/reperfusion injury (Welch 1992, Gingalewski *et al.* 1996). The individual groups of heat shock proteins are discussed separately in greater detail below.

1.4 Structure and function of heat shock proteins

Early classification of the heat shock proteins was done on the approximate molecular weight of the proteins upon separation by SDS-PAGE, therefore the nomenclature of the stress proteins was derived by the size of the proteins in kD hence Hsp25, -32, -60, -72,-73, -90, -110 have molecular weights of 25kDa, 32kDa, 60kDa etc respectively. One of

the remarkable features of heat shock proteins is their striking sequence homology over a diverse range of species ranging from bacteria to humans. In some cases there is as much as 50% homology between certain heat shock proteins in bacteria and humans. This extreme conservation of a set of proteins would indicate that the heat shock proteins themselves have a pivotal role to play in the cellular homeostasis of all organisms. Those main roles that heat shock proteins play in organisms are as molecular chaperones, essentially aiding in the formation and folding of newly formed proteins, and as stress proteins effectively protecting the cells from adverse stimuli. The structure and function of the main groups of heat shock proteins are discussed below:

1.4.1 Heat shock protein 70 (Hsp70)

Analysis of the molecular structure of 70kDa stress protein family (Hsp70) showed that it contains a highly conserved N terminal ATP'ase domain of 45kDa (Flaherty 1990) and a less conserved 10kDa C terminal portion that is thought to be involved in peptide binding. The stress protein Hsp70 consists of two individual proteins; Hsp72 and Hsp73. Hsp73 is present in the cell constitutively and is therefore thought to have an essential role in the cellular processing of proteins. However, Hsp72 is only present in significant amounts following noxious stimuli and so it is believed that the main role of this protein is the protection of cells from stress. Therefore, from now on when the nomenclature Hsp70 is used it is referring to the two stress proteins Hsp72 and Hsp73.

Hsp70 is inducible by a wide variety of stressors including heavy metals (cadmium, mercury, lead), ozone, halothane and electro-magnetic fields (Goering *et al.* 1993b, Lin *et al.* 1994, Koundakjian *et al.* 1996, Wong *et al*, 1996) in numerous cell types. The characteristic scheme of events is that following a chemical insult the Hsp70, which normally resides in the cytosol, translocates and accumulates in the cells nucleus reaching a peak 2-6 hours post insult (Lin *et al.* 1994). The role of Hsp70 in stressed cells is to assist in the breaking up of protein aggregates and also to interact with unfolded proteins in such a way as to allow their refolding. The Hsp70 present in the cell also interacts with newly synthesised proteins which are unable to fold properly, effectively stabilising them (Beckmann *et al.* 1992). In addition to preventing necrotic cell death following chemical

insult, Hsp70 has also been shown to play a role in the prevention of programmed cell death following exposure of cells to inducers of apoptosis such as nutrient deprivation and tumour necrosis factor (TNF). Hsp70 is thought to prevent apotosis not by its interactions with damaged proteins but by preventing the activation of stress kinases such as JNK and p38 which are essential components in the signal transduction pathway leading to apoptosis (Gabai *et al.* 1995, Gabai *et al.* 1997).

In parallel to the role of protecting the cell from adverse stimuli the stress proteins also have an important physiological role to play as molecular chaperones. The term molecular chaperone was first applied to heat shock proteins by R.J. Ellis in 1987 and is currently defined as "a protein that binds to and stabilises an otherwise unstable conformer of another protein - and by controlled binding and release of the substrate protein, facilitates its correct fate *in vivo*; be it folding, oligomeric assembly, transport to a particular compartment, or controlled switching between active/inactive conformations" (Hendrick and Hartl 1995). Essentially, the molecular chaperones aid in the correct folding, assembly and transport of newly formed proteins.

Of all the heat shock proteins, Hsp70 has been the most extensively studied as a molecular chaperone. The role of Hsp70 as a molecular chaperone is thought mainly to be in its involvement in the membrane translocation of proteins and in the folding of newly formed proteins. In mitochondria, Hsp70 plays a vital role both in the import and export of proteins. In the cytosol most proteins interact with Hsp70, it is thought Hsp70 is involved in maintaining the unfolded state of newly formed polypeptides. This role of Hsp70 is vital for the mitochondrial import of proteins as tightly folded polypeptides are incapable of crossing the mitochondrial membrane (Martinus *et al.* 1995). In addition to this Hsp70 is also required for post translational protein translocation in the endoplasmic reticulum and mitochondria where Hsp70 is thought to interact with membrane associated components involved in protein translocation (Brodsky 1996). Evidence for this comes from studies in yeast where deletion of the yeast cystolic Hsp70 (Ssa1p) and disruption of the other cystolic Hsp70 causes an accumulation of both endoplasmic reticulum and mitochondrial precursor proteins. This accumulation of proteins was reversible as addition of Ssa1p to

the cell restored the translocation of the proteins. The exact mechanism of how Hsp70 aids in protein translocation is still under investigation. One of the theories is that Hsp70 binds to segments of the protein or interacts with imported regions of the peptide, then through thermal oscillations the protein is translocated passively with the aid of Hsp70 (Simon *et al.* 1992). The other model for protein translocation is that the Hsp70 present in the lumen acts as translocation motors effectively pulling the protein into the lumen with the subsequent hydrolysis of ATP (Glick 1996).

Another area in which Hsp70 has been shown to be involved is in the import of proteins to mammalian peroxisomes. Proteins of the Hsp70 family were found to be associated with peroxisomes where they were determined to be located on the outside of the peroxisomal membrane. Injection of antibodies into human fibroblast cells caused an inhibition of the import of proteins into peroxisomes, this inhibition could then be overcome by the addition of exogenous Hsp70 to the cells. The role of Hsp70 in peroxisomal import remains to be fully elucidated however it is thought that its role here does not involve the folding of proteins to be imported (Walton *et al.* 1994).

The role of Hsp70 in the folding of proteins is thought mainly to involve the inhibition of non-productive folding interactions and to modulate the state of macromolecular assemblies. The mechanism of Hsp70 induced protein folding involves the binding of ATP to the N terminal of Hsp70 which is pivotal to its role. Aiding Hsp70 in the folding of proteins are two other proteins DnaJ and GrpE. DnaJ is a 40kDa protein which can bind unfolded proteins and has the ability to interact with Hsp70; the other protein GrpE is a nucleotide exchange factor. The basic mechanism, as elucidated in *E. coli*, is that the unfolded proteins present in the cell interact with DnaJ which due to its intrinsic affinity then binds to DnaK (homologous to Hsp70 in eukaryotic cells). The unfolded protein-DnaJ complex then interacts with DnaK causing the hydrolysis of ATP. The resultant DnaJ-protein-DnaK-ADP complex is stable as when ADP is bound DnaK binds and releases peptides slowly, that is in contrast to when ATP is bound where DnaK binds and releases peptides rapidly. The resultant DnaJ-protein-DnaK-ADP complex is then targeted by the nucleotide exchange factor GrpE thereby causing the release of ADP from DnaK.

The binding of ATP to DnaK causes the folding and subsequent release of the protein and the release of DnaJ and GrpE (Hendrick and Hartl 1995). Although this is the general scheme of events in bacteria, it is still uncertain whether this represents an accurate version of events in eukaryotes as it is not known whether eukaryotic mitochondria contain a GrpE homologue.

1.4.2 Heat shock protein 32 (Hsp32/heme oxygenase)

The 32kDa heat shock protein (Hsp32) is the only heat shock protein present in the cell that has an essential enzymatic functional role to play. It was realised in 1989 by Keyse and Tyrrell that the Hsp32 induced by certain chemicals was in fact the enzyme heme oxygenase. Heme oxygenase (HO) is the essential microsomal enzyme involved in the catabolism of heme. HO cleaves the heme ring at the **α**-methylene bridge with the resultant formation of biliverdin and carbon monoxide, the biliverdin formed can then be further converted to the potent free radical scavenger bilirubin by the enzyme biliverdin reductase. The highest levels of HO are found in the spleen with detectable levels also found in the liver, bone marrow, testis and brain (Maines and Ewing 1996). There are two separate isoforms of HO, HO-1 and HO-2, which are coded for by separate genes. HO-1 corresponds to Hsp32 as it is inducible by adverse chemical stimuli, known inducers include UVA radiation, H_2O_2 and sodium arsenite (Keyse and Tyrrell 1989). HO-2 is present constitutively in the cell and is thought to be non-inducible.

The physiological role of HO-1 in normal cells is in the removal of excess heme from the cell and hence prevent the cell from oxidative damage. Contained within a heme molecule is chelated iron which is a potent pro-oxidant. In a cell, free iron is capable of producing excess amounts of deleterious hydroxyl radicals ($OH^{\bullet}$) by the catalysis of H_2O_2 via the Fenton reaction as shown in equation (1) (Pryor 1986).

$$H_2O_2 \xrightarrow{Fe^{2+}} OH^{\bullet} + OH^{\bullet} \qquad (1)$$

The HO-1 that is present is thus capable of decreasing the production of free radicals by the removal of excess heme and hence iron. As mentioned earlier, the catalysis of heme

by HO-1 is also capable of producing large amounts of bilirubin which due to its insolubility in water is tightly bound to albumin *in vivo*. The bound bilirubin is a potent chain breaking antioxidant capable of stopping highly damaging free radical induced lipid peroxidation by scavenging peroxyl radicals as shown in equation (2) (Stocker *et al.* 1987).

$$\text{Bilirubin} + 2\text{ROO}^{\bullet} \rightarrow \text{Biliverdin} + 2\text{ROOH} \qquad (2)$$

In addition to its role in cellular protection from excess heme HO is thought to play a vital role in the testis. HO is activated in germ cells during spermatogenesis with the highest levels found in the spermatocytes. In other tissues, such as bone marrow and spleen, HO is involved in the disposal of haemoglobin however this is not the case in spermatocytes. In these cells the main function of HO is thought to be involved in the degradation of heme that is contained within cytochrome *c* (Kurata *et al* 1993). There also appears to be pattern in the expression of HO that is intrinsically linked to sexual maturation. The function of HO in the maturing testis is not clear, but it is believed to be involved in the release of iron which is then thought to modulate iron responsive gene expression in the testis (Ewing and Maines 1995).

A wide number of stressors have been shown to induce HO-1 in various cell types, these include UVA radiation, hydrogen peroxide, cadmium, sodium arsenite and menadione (Taketani *et al.* 1989, Keyse and Tyrell 1987, Keyse and Tyrell 1989). However, the induction of HO-1 by heat shock is not entirely consistent as heat shock had no effect on the levels of HO-1 in human skin fibroblasts and human or murine melanoma cells (Keyse and Tyrell 1987, Caltabiano *et al.* 1986). Of the compounds that do induce HO-1, it appears that in general there are two main groups of inducers, these are agents that are oxidants or have the potential to generate reactive intermediates and those agents capable of modifying glutathione levels in the cell (Applegate *et al.* 1991). Depletion of cellular glutathione alone by compounds such as diethylmaleate have been shown to be sufficient to induce HO-1, but only when the depletion of intracellular GSH exceeded 80%, however the induction of HO-1 was triggered not by the depletion of GSH itself but by the

formation of a GSH conjugate (Freeman and Meredith 1989). Such a drastic decrease in cellular GSH levels would lead to increased levels of reactive oxygen species arising as a consequence of normal cellular metabolic processes. It would, therefore, appear that the essential pre-requisite for HO-1 induction is the formation of reactive oxygen species within the cell.

1.4.3 Heat shock protein 25 (Hsp25)

In addition to the larger families of heat shock proteins eukaryotic cells also have present small molecular weight heat shock proteins ranging in molecular weight from 16-30kDa depending on the species. Hsp25 in rodents is homologous to Hsp27 in human. It exists *in vivo* as a large structure with a molecular weight of approximately 400,000kDa in unstressed cells. However, following heat shock the protein translocates to the cells nucleus and forms structures of even larger molecular weights (estimated at $2x10^6$Da) (Welch 1992). Far less is known about the molecular chaperone functions of Hsp25. It is known, however, that unlike Hsp70 Hsp25 is not regulated by ATP but it has been shown to suppress protein aggregation and heat inactivation *in vitro* (Wynn *et al.* 1994). Hsp25 is induced by various stimuli including elevated temperatures, H_2O_2, arsenite, growth factors and calcium ionophores. One of the immediate effects following adverse stimuli is the phosphorylation of Hsp25 by a protein serine threonine kinase termed mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP-2) (Hayess and Benndorf 1997). The newly phosphorylated Hsp25 appears to have the ability to trap non-native protein aggregates thus forming stable protein complexes (Buchner 1996). Other proposed functions of Hsp25 include the protection of the polymerisation of actin filaments particularly during heat shock. The mechanism of this is thought to involve the newly phosphorylated Hsp25 binding to actin filaments thus allowing polymerisation to occur (Craig *et al.* 1994). *In vitro* Hsp25 acts to inhibit actin polymerisation but phosphorylation of Hsp25 causes an abolishment of its actin polymerisation inhibiting activity (Benndorf *et al.* 1994).

1.4.4 Other families of stress proteins

The remaining families of stress proteins consist primarily of proteins with molecular weights of 60, 90 and 100-110kDa. The 60kDa stress protein (Hsp60) is amongst one of the most studied heat shock proteins. Its unusual structure shows a 14 subunit ring shaped oligomer and usually associated with Hsp60 is a smaller co-protein Hsp10 that exists as seven-subunit rings. This co-protein is essential as Hsp60 alone will not prevent the formation of insoluble protein aggregation complexes following heat shock, however in the presence of Hsp10 this aggregation is prevented. The main function of Hsp60 is as a molecular chaperone primarily in the mitochondria where it binds non-native proteins in its central "hole", this complex then binds to Hsp10 and with the hydrolysis of ATP a native folded protein is formed (Hendrick and Hartl 1995, Craig *et al.* 1994).

The 90kDa stress protein (Hsp90) is one of the most abundant cytoplasmic cellular proteins where it constitutes 1-2% of the total protein in a cell, levels can also be further increased following heat shock. Structurally Hsp90 is a highly phosphorylated protein with an ATP binding site and in addition possesses autophosphorylating activity. Hsp90 has been shown to be inducible *in vitro* by compounds such as sodium arsenite and mercury in liver slices and splenocytes respectively (Wijèweera *et al.* 1995, Albers *et al.* 1996). In addition exposure of animals to cadmium, ethylene oxide or mercury have all been shown to increase levels of Hsp90 in the liver and kidney (Goering *et al.* 1993a, Katoh *et al.* 1991, Goering *et al.* 1993b). The newly induced Hsp90 is thought to be involved in the refolding of damaged proteins following chemical exposure. In addition to its chaperone role, Hsp90 appears to interact with specific cellular targets including progesterone and dioxin receptors, the Hsp90 bound to these receptors effectively inhibits the receptors activity until ligand binding (Craig *et al.* 1994, Whitelaw *et al.* 1995). Other targets of Hsp90 include caesin kinase II and tyrosine kinases to which Hsp90 binds and modulates the kinase activity of both positively and negatively depending on the individual kinase involved (Miyata and Yahara 1992, Wynn *et al.* 1994). The majority of the specific cellular targets of Hsp90, therefore, appear to be involved in signal transduction. It is thought Hsp90 has an essential regulatory role in this area but the precise mechanisms involved remain to be elucidated fully.

The final major group of stress proteins consist of the 100-110kDa stress proteins. These proteins are expressed constitutively at low levels but following induction they accumulate in the cells nucleolus. Studies have shown that Hsp110 is induced in the livers and kidneys of rats following administration of cadmium or mercury (Goering *et al.* 1992, 1993a, 1993b). This has also been demonstrated *in vitro* in H35 hepatoma cells where arsenite treatment was able to induce the synthesis of Hsp100 (Ovelgönne *et al.* 1995). The role of Hsp100-110 in cells is not entirely clear, but it is thought to be involved specifically in the disaggregation of large protein complexes following heat shock. These high molecular weight stress proteins are thought to have a specialised role in protecting the cell following extreme physical insults as cells grown with an absence of intracellular Hsp104 show a significant reduction in survival following exposure to extremes of temperature (Craig *et al.* 1994, de Pomerai 1996).

1.5 Physiological control of the stress response

Present in the cytosol and nucleus of eukaryotic cells is a transcriptional factor, heat shock factor (HSF), which is involved in the transcriptional activation of the genes involved in the stress response. Following heat shock or adverse chemical stimuli, the monomeric HSF present trimerises and binds to a region on the heat shock gene, the heat shock element (HSE), this binding causes an increased transcription of the gene and hence increased stress protein production. So far four heat shock factors have been characterised: HSF-1, HSF-2, HSF-3 and HSF-4. The most widely studied is HSF-1 which has been shown to be the factor primarily involved in increased heat shock protein gene expression following exposure to elevated temperatures and chemical stimuli. HSF-2 has been shown to be activated during haemin-induced differentiation of human K562 erythroleukaemia cells causing induction of Hsp70 and during mouse spermatogenesis where it is thought to regulate heat shock protein gene expression (Sarge *et al.* 1994). HSF-3 has also been shown to respond not only to heat stress (Martinus *et al.* 1995) but stressors that activate HSF-1 or HSF-2 do not activate HSF-3 (Morimoto 1993). Therefore, there would appear to be certain stimuli that activate the DNA binding activities of specific HSF's implying that the separate isotypes of HSF have different functional roles to play in the response of a cell to stress.

1.5.1 Structure of HSF-1

Structural analysis of HSF-1 has shown that the factor contains several distinct regions essential to its function. At the C-terminus of the protein is a domain involved in the transcriptional activation of heat shock genes, closely associated with this region is a regulatory domain which appears to regulate this transcriptional activation. When a cell is at an ambient temperature the regulatory domain serves to inhibit the transcriptional activation of heat shock protein genes by HSF (Newton *et al.* 1996). At the N-terminus there is a trimerisation domain consisting of an array of three intermolecular hydrophobic regions known as leucine zippers which are able to form hydrophobic coiled-coil interactions (figure 1.1). In higher eukaryotes HSF-1 possesses a fourth leucine zipper region at the C-terminus which is thought to interact with the leucine zippers at the N-terminus effectively preventing trimerisation of the inactive form of HSF-1. Next to the trimerisation domain at the N-terminus is a DNA binding region of approximately 100 amino acids which is thought to be involved directly in the binding of HSF-1 to the HSE on the heat shock genes. (Craig *et al.* 1994).

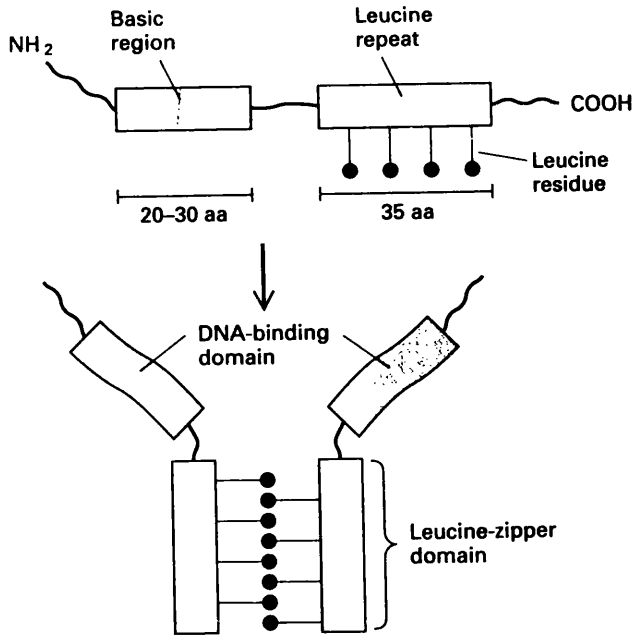


Figure 1.1 Proposed model showing the localisation and interactions of leucine zippers and DNA binding domains (Note this model is for C/EBP proteins, the localisation of the DNA binding and leucine zipper domains on HSF-1 are different). From Darnell 1990.

1.5.2 Involvement of HSF-1 in the stress response

In unstressed cells HSF-1 exists as a cytoplasmic monomer, it is thought that it is maintained in this state in part by the leucine zippers present at the N and C-termini but mainly by the interaction between HSF-1 and Hsp70. This was demonstrated in HeLa cells where following heat shock exogenous Hsp70 was shown to block the DNA binding activity of HSF-1, however this decrease in DNA binding activity was overcome by the addition of ATP (Abravaya *et al.* 1992). However, what is also known is that there is a pre-requisite for Hsp70 to be present in the cell as removal of all Hsp70 from the cell causes an inhibition of the trimerisation of HSF-1. The current picture is that a partially folded form of HSF-1 is bound to "negative regulatory element" which is thought to be a form of Hsp70 effectively inhibiting the activity of HSF-1. Following heat shock the level of denatured proteins increases in the cell which are then preferentially targeted by the Hsp70 bound to HSF-1. Therefore, Hsp70, in the presence of ATP, dissociates from HSF-1 which is then released in a conformation that allows trimerisation to occur (Schlesinger and Ryan 1993).

1.5.3 Interaction between HSF-1 and the heat shock element (HSE)

Once trimerised HSF-1 translocates to the cells nucleus where it binds to a promoter region on the heat shock gene, the heat shock element (HSE). The HSE is present on all heat shock genes and is located in the 5'-flanking sequences region on the gene. Bound to the HSE is another factor; constitutive HSE binding factor (CHBF), which is thought to act as a negative regulator of the HSE. The presence of a negative regulatory element explains the observation that binding of HSF-1 to the HSE does not always correlate with an increased transcription of Hsp70. One theory is that following a mild insult to a cell HSF-1 binds to the HSE, but CHBF does not dissociate from the HSE and, therefore no increase in Hsp70 is observed. However, following a severe insult HSF-1 again binds to the HSE, but in this case CHBF dissociates from the HSE thus allowing increased transcription of Hsp70. It is thought that the HSF-1 and CHBF binding sites on the HSE overlap and thus HSF-1 binding in the presence of CHBF is not capable of inducing Hsp70 (Liu *et al.* 1995) This might provide explanation for the observation that exposure of NIH

3T3 cells to oxidative stress activates HSF-1, but causes no induction of heat shock proteins (Bruce *et al.* 1993).

The trimerised HSF-1 is capable of binding to the HSE due to the presence of multiple $(GAAN)_n$ binding sites present on the DNA that appear to have approximately 3-fold symmetry. The trimeric DNA binding domains on the HSF-1 trimer then interact directly with these multiple $(GAAN)_n$ binding sites present on the HSE. (Craig *et al.* 1994, Okinaga and Shibhara 1993).

At the present time there are two known forms of HSE; HSE1 and HSE2. HSE1 is the main element that confers heat inducibility to the cell whereas the function of HSE2 is still unclear, but what is known is that alone HSE2 is unable to confer heat inducibilty to the cells. However, both HSE1 and HSE2 are required for maximal transcription of the heat shock genes and the two elements appear to work synergistically. HSF-1 recognises and binds to both HSE1 and HSE2, but there is also another, as yet unknown, specific constitutive factor present in the cells nuclei that recognises only HSE2 which also appears to play a role in the stress response (Konishi *et al.* 1995, Okinaga and Shibahara 1993).

Following binding of HSF-1 to the HSE1, phosphorylation of HSF-1 occurs and increased transcription of Hsp70 then follows. This has a negative feedback effect on the stress response, as the increased levels of Hsp70 not only interact with the denatured proteins present in the cell, but also interact with the active HSF-1 present. Therefore if any inactive HSF-1 is present bound to Hsp70, the bound Hsp70 will not dissociate due to the increased pool of free Hsp70 present in the cell. In addition to this, the newly formed Hsp70 binds to, and interacts with, any active HSF-1 present thereby accelerating its deactivation (Mosser *et al.* 1993, Rabindran *et al.* 1994). Hsp70 is thought to interact directly with the active phosphorylated form of HSF-1 and effectively de-phosphorylate HSF-1 perhaps by the activation of protein phosphatase-1 (Kim *et al.* 1995). A simplified scheme of events is shown in figure 1.2.

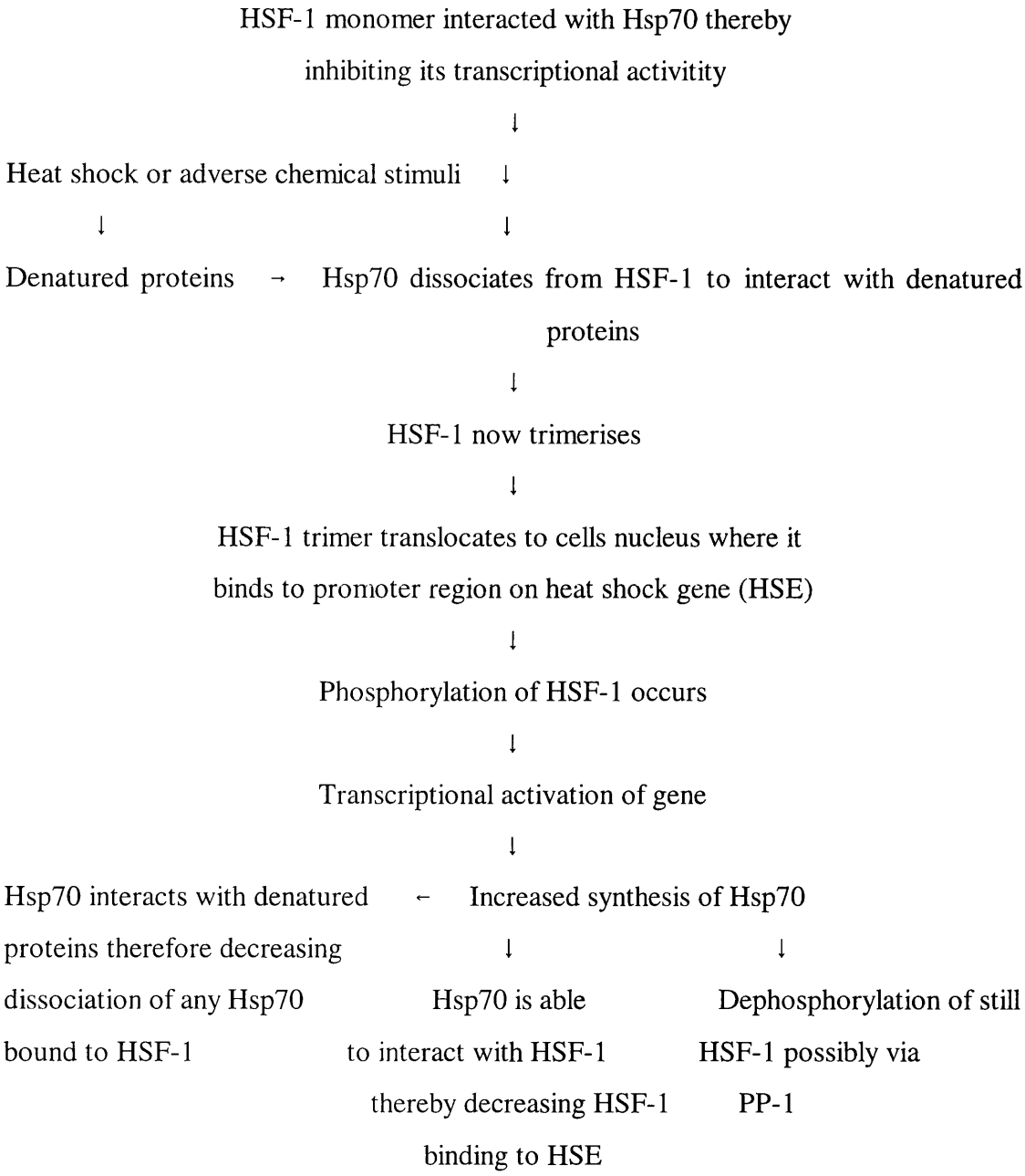


Figure 1.2. *Diagrammatical representation of the interactions between Hsp70, HSF-1 and HSE following a noxious stimuli.*

1.6 Thermotolerance

The phenomenon of thermotolerance was one of the first proposed functions of stress proteins. Cells exposed transiently to non-lethal increases in temperature acquire an increased resistance to a second normally lethal heat shock. In addition to this exposure of cells to a sub-lethal heat shock has also been shown to protect against subsequent toxic chemical exposure (Salminen *et al.* 1996). The basis for this protection is the elevated levels of stress proteins present when the second stressful stimulus is administered, be it either lethal heat shock or chemical exposure, as there is a correlation between the amount of stress proteins present and the degree of thermotolerance observed. It is now accepted that virtually every class of heat shock protein has been implicated to be involved in the phenomenon of thermotolerance (Schlesinger 1990). The fundamental aspects of thermotolerance can also be applied to chemical exposure as administration of sub-lethal doses of a toxic compound to a cell can protect it against subsequent exposure to normally lethal concentrations of the same compound (Wiegant *et al.* 1995). This has been shown to be the case with compounds such as sodium arsenite, ethanol and diamide, which all have the ability to induce both Hsp70 and Hsp25 (Kampinga *et al.* 1995). The phenomenon of thermotolerance also occurs physiologically during ischaemic preconditioning. It has been demonstrated that sub-lethal ischaemic periods prevent the oxidative damage normally associated in tissues following subsequent ischaemia and reperfusion. This protection from ischaemia/reperfusion injury has also been shown with pre-exposure of the tissues to sub-lethal increases in temperature. Increased levels of stress proteins have been implicated as the protective mechanism involved here as during a period of ischaemia/reperfusion oxygen free radicals are generated which are known inducers of the stress response (Hoshida *et al.* 1997). The elevated temperatures observed during infection have also been suggested to be important in conferring a state of physiological thermotolerance. During the immune response, the activation of monocytes by tumour necrosis factor (TNF) produces highly toxic free radical metabolites. Both Hsp70 and Hsp27 have been shown to confer some level of self protection to the monocytes themselves against the toxicity mediated by TNF (Jäättelä and Wissing 1993).

1.7 Use of the stress response as a biomarker of toxicity

There are a wide number of potential biochemical markers for determining the adverse effects of chemicals. Some of the classical general markers of biochemical toxicity include depletion of ATP, GSH levels and alterations in LDH leakage and trypan blue exclusion. More specific examples of these include the cytochrome P4501A subfamily which is induced by polycyclic aromatic hydrocarbons, polychlorinated biphenyls and dioxins. Other examples include the metallothioneins which are induced specifically by exposure to heavy metals. The use of these markers can give an indication of toxicity but their induction is obviously limited to a specific set of compounds or chemical species. The use of heat shock proteins as general markers of cellular damage or stress has, therefore, been of particular interest recently. There are several features of the stress response that make it feasible for use as a general marker of adverse chemical exposure. These include the observation that heat shock proteins are induced by a wide range of physical and chemical stressors, the main group of inducers are general toxic compounds with an ability to induce the stress response including ozone (Wong *et al.* 1996), selected pesticides (Bagchi *et al.* 1996), heavy metals (Shelton *et al.* 1986, Goering *et al.* 1993a), *n*-alcohols (Neuhaus-Steinmetz and Rensing 1997) and oxidative stress inducers (Donati *et al.* 1990). Other identified specific groups of inducers of the stress response include teratogens (Buzin *et al.* 1984) and low frequency electromagnetic fields (Koundakjian *et al.* 1996, Smith 1996).

There are also some other fundamental aspects of the stress response that reinforce the potential of heat shock proteins as markers of toxicity. Firstly, it is well established that with certain compounds induction of heat shock proteins occurs at chemical concentrations below the those required for toxicity (Goering *et al.* 1993a, Goering *et al.* 1993b). Secondly, the induction of heat shock proteins is rapid and easily measurable within 1-3 hours following chemical exposure (Blake *et al.* 1990). Finally, the observation that the heat shock response is one of the most widely conserved physiological responses across a wide range of organisms ranging from bacteria to man implies that any response in one organism could be extrapolated to another with relative ease (Boorstein *et al.* 1994,

Rensing and Maier 1994). These observations have thus supported the investigation of the use of heat shock proteins as markers of impending toxicity in cells.

1.8 Use of liver spheroids as an in vitro hepatocyte model

1.8.1 Development of long term primary hepatocyte cultures

In these investigations three models of hepatocytes were used: suspensions, monolayers and spheroids. Hepatocyte suspensions consist of freshly isolated cells maintained in rotating flasks for up to 4-5 hours. Hepatocyte monolayers consisted of the culture of freshly isolated hepatocytes on collagen-coated culture dishes. Monolayers are one of the most widely used *in vitro* models of hepatotoxicity. However, their use is limited by the fact that in culture hepatocytes lose many of their liver specific functions and only remain functionally viable for 3-4 days. Secretion of albumin and levels of gluconeogenesis fall rapidly within hepatocyte monolayers as do the levels of the phase I and phase II metabolising enzymes particularly several of the cytochrome P450 enzymes (Rogiers *et al.* 1995). Structurally, hepatocytes *in vivo* maintain a three dimensional lobular structure however once they form monolayers attached to collagen-coated culture dishes they adopt a flattened two dimensional structure. This change in the cells architecture causes a decrease in cell to cell contact and thus may affect certain functional cell processes.

Some of the measures used to overcome these problems associated with hepatocyte monolayers include addition of nutrients, trace elements, hormones and growth factors to the media in addition to the use of different media (Enat *at al.* 1984). Addition of exogenous chemicals such as DMSO to monolayers has been shown to preserve the morphological characteristics of the cells for up to 43 days although the addition of exogenous chemicals to the culture system is obviously undesirable (Isom *et al* 1985). The attachment of hepatocytes to an extracellular matrix in culture such as collagen, laminin or fibronectin were shown also to improve the maintenance of liver specific functions and extend the lifetime of hepatocytes in culture (Enat *et al.* 1984, Ben-Ze'ev *et al.* 1988). However, even with the use of all the above factors there was little significant overall improvement in the long term culture of hepatocytes. The main improvements for the long term culture of hepatocytes came with development of different hepatocyte models such

as the co-culture of hepatocytes, the use of collagen sandwiches and the generation of liver spheroids.

Co-culture of hepatocytes is normally performed by culturing hepatocytes with a non-parenchymal cell type such as liver epithelial cells. Co-culture of hepatocytes enables the cells to maintain, to some degree, their three dimensional structure and also many of their liver specific functions of the hepatocytes remain viable for many weeks. Long term secretion (up to 20 days) of two acute phase reactants, **α**-1 acid glycoprotein and the third component of complement (C3), was obtained by co-culturing hepatocytes with an epithelial liver cell type. Hepatocytes cultured alone showed a marked decrease in the secretion of these two proteins (Lebreton *et al.* 1986). However, one of the problems of using co-cultures in toxicological research is the uncertainty as to what effect the other cell type may have on the response of hepatocytes to an exogenous chemical. In addition to this, the measurement of DNA and protein levels for biochemical measurement normalisation in the culture would be affected due to the presence of the second cell type.

The culture of hepatocytes in a collagen sandwich involves plating the cells out onto a layer of collagen and then adding a second layer of collagen onto the top of the cells within 24 hours. This second layer of collagen prevents the cells from flattening out and so they retain their three dimensional structure to some degree. Culture of the cells by this method allows the hepatocytes to remain viable for many weeks whilst retaining many of their liver specific functions during this time. Levels of albumin mRNA in hepatocytes from collagen sandwiches were found to approximate to levels found from hepatocytes *in vivo* (Dunn *et al.* 1992). One practical constraint of this model is that the hepatocytes are effectively trapped between two layers of collagen, therefore for biochemical analysis of the hepatocytes, the collagen has to be enzymatically removed in order to get access to the cells. The other approach to the long term culture of hepatocytes is with the use of liver spheroids.

1.8.2 Development of liver spheroids

For the effective formation of liver spheroids hepatocytes in culture have to remain in suspension and not attach to the culture dishes. This is accomplished by the use of non-adherent substrata such as liver derived proteoglycans, polyurethane, poly-2-hydroxyethylmethacrylate (pHEMA) or the use of positively-charged surfaces. The use of these substrata means that the cell to dish interactions will be minimised and hence the cell to cell interactions will be maximised. The frequency of the cell to cell interactions can be further maximised by culturing of the hepatocytes on a rotating platform as this effectively increases the number of collisions between cells.

The development of spheroids is governed by the frequency of collision of the cells and the biological properties of the interacting cell surfaces. Hepatocytes cultured on positively charged surfaces appear initially to form monolayers that retract to form small clumps of cells that remain attached to the culture plates. However, over the next 24 hours these clumps grow progressively larger to form a multi-layered mass of cells as more hepatocytes interact with them. Within 72 hours these masses of cells have grown further and formed spheroids which now detach from the culture plates and remain in suspension (Peshwa *et al.* 1996). When hepatocytes are cultured on pHEMA coated plates there is a different profile to the formation of spheroids. It appears here that the formation of spheroids is a two step process. The initial stage is termed the "recognition phase" where the cells in suspension interact to form loose aggregates held together by weak bonds. This aggregation of hepatocytes is temperature independent and is not affected by metabolic inhibitors. This first phase is thought to involve cell recognition and the direct interactions between molecules present on the cell surfaces. The second phase is termed the "compaction phase" where the weak bonds formed in the first phase are replaced by much stronger bonds. This phase is Ca^{2+} dependent and requires metabolic energy and is thought to involve the formation of specialised junctions between the hepatocytes (Landry and Freyer 1984, Landry *et al.* 1985). The precise nature of the interactions between the individual hepatocytes in the spheroids remains to be understood fully.

1.8.3 Biochemical characteristics of liver spheroids

The metabolising capacities of spheroids has been well investigated (Schilter *et al.*1993) where measurement of ethoxycoumarin-O-deethylase (ECOD) and glutathione S-transferase (GST) were used as markers for phase I and phase II reactions respectively. Over a period of 14 days basal levels of ECOD and GST were measured in liver spheroids indicating that both phase I and II reactions were maintained during this period. Investigations into the levels of specific cytochrome P450 isozymes showed levels of CYP1A1/2 and CYP2B/1 were close to levels found in freshly isolated cells. However, the levels of CYP3A1/2, CYP4A1/2/3, CYP2C6 and CYP2C11 were found to be decreased by day 8 in culture albeit to differing extents (Ammann *et al.* 1997). Expression of albumin synthesis in spheroids was preserved, albumin mRNA was expressed in spheroids up to day 10 of culture and beyond whereas in contrast by day 10 albumin mRNA was undetectable in hepatocyte monolayers (Tamura *et al.* 1995, Roberts and Soames 1993). This increased expression of albumin mRNA resulted in an increased secretion of albumin which was found to increase over the first 6 days of culture after which time it remained constant for up to 60 days. In comparison, albumin secretion from monolayers was found to decrease after day 4 of culture (Koide *et al.*1989, Tong *et al.* 1990). Comparative metabolic studies of spheroids and hepatocytes have shown that upon addition of ammonia to the cells the spheroids remove ammonia and synthesise urea at levels significantly greater than those found in hepatocyte monolayers (Naruse *et al.* 1996). In addition to this, spheroids were also seen to maintain expression of the peroxisome proliferator-activated receptor (PPAR) whereas PPAR expression in monolayers was virtually undetectable (Roberts and Soames 1993). It would appear, therefore, that hepatocytes contained in liver spheroids have biochemical characteristics more reminiscent of those found in hepatocytes *in vivo* thanthose hepatocytes found in monolayers.

1.8.4 Structure of liver spheroids

As mentioned earlier, hepatocytes in liver spheroids maintain a more *in vivo* like structure than hepatocyte monolayers. Due to this three dimensional structure spheroids maintain extensive cell to cell contacts with microvilli abundantly present. Also present on the

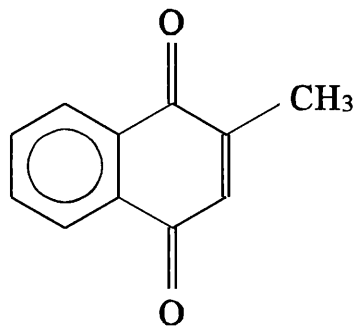
surface of the spheroids are small openings of approximately 2-4μM in diameter which are thought to represent openings for bile canalicular structures present in the spheroids (Peshwa *et al.* 1996). Bile canaliculi were found to be present in spheroids and use of the fluorescent organic anion fluorescein diacetate showed that spheroids possess an active transport mechanism from the sinusoidal membrane to the bile canalicular membrane (Yumoto *et al.* 1996). Ultrastructure of the spheroids shows numerous mitochondria and rough and smooth endoplasmic reticulum present. Glycogen granules, peroxisomes and lipid bodies were also present in the cytosol. The presence of microvilli and the accumulation and secretion of vesicles from the spheroids would imply that hepatocyte membrane polarity was maintained as indeed is cell polarity. This is in comparison to hepatocyte monolayers that show decreased levels of rough and smooth endoplasmic reticulum with smaller mitochondria also observed (Yuasa *et al.* 1993). These morphological observations clearly show that the structure of liver spheroids closely resembles that found in hepatocytes *in vivo*.

1.9 Compound used in the studies

To investigate induction of the stress response in the various hepatocyte models a number of compounds were used. The compounds chosen were menadione, hydrazine hydrate, A23187 and cadmium chloride. All the compounds have differing mechanisms of action and are described in detail in the following section.

1.9.1 Menadione

Menadione (2-methyl-1, 4-naphthoquinone) is one of the most common compounds used to generate oxidative stress experimentally. This is due to the fact that menadione is a redox cycling toxin. Menadione can undergo two different metabolism steps, the first involves metabolism via a NAD(P)H-quinone oxidoreductase (DT-diaphorase)-catalysed two electron reduction with the resultant formation of a hydroquinone. The formation of the hydroquinone is thought to represent a detoxification process as the

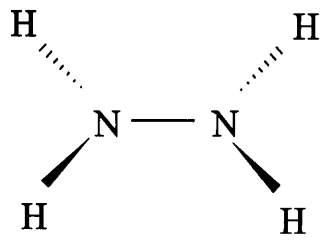


newly formed hydroquinone can then be further metabolised to glucuronide and sulphate conjugates (Gant *et al.* 1986, Miller *et al.* 1986). The second pathway for the metabolism of menadione involves a one electron reduction catalysed by flavoenzymes such as NADPH-cytochrome P450 reductase or NADH-cytochrome b_5 reductase to form semiquinone radicals. This semiquinone radical can then reoxidise in the presence of oxygen back to menadione with the concurrent formation of the superoxide free radical ($O_2^{\cdot-}$). The formation of the $O_2^{\cdot-}$ radical by itself is not particularly damaging, however it has the potential to interact with other chemical species including H_2O_2 resulting in the formation of the highly reactive and damaging hydroxyl radical ($OH^{\cdot}$). In addition to this $O_2^{\cdot-}$ can interact with nitric oxide ($NO^{\cdot}$) and form either $OH^{\cdot}$ or a nitronium ion (NO^{2+}) which is a powerful nitrating agent able to attack aromatic amino acid residues in proteins.

Evidence for the role of oxidative stress in the toxicity of menadione involves the depletion of GSH, the concurrent formation of GSSG and the subsequent oxidation of GSH by GSH peroxidase. Other metabolic alterations induced by menadione include the oxidation and depletion of acid soluble and protein thiols, the arylation of nucleophiles, disruption of calcium homeostasis and the formation of DNA strand breaks and subsequent DNA fragmentation (Fischer-Nielsen *et al.* 1995, Gant *et al.* 1988, Malorni *et al.* 1991). ATP depletion has also been implicated in the toxicity of menadione as fructose protects against menadione toxicity suggesting that mitochondrial function is impaired and thus contributes to cell death (Mehendale *et al.* 1994). Cytoskeletal effects of menadione include a characteristic blebbing seen following exposure which is thought to be due to the oxidation of thiol groups in actin microfilaments and an increase in cystolic calcium levels. This effectively causes a redistribution of cytoskeletal and membrane proteins and subsequent dissociation of these proteins from the plasma membrane thus causing areas of weakness and hence bleb formation (Malorni *et al.* 1991).

1.9.2 Hydrazine

Hydrazine (N_2H_2) is widely used in the chemical industry where it is utilised as a fuel and propellant in aircraft and rockets, it is also minor metabolite of some drugs namely isoniazid and hydralazine. Hydrazine has been shown previously to be toxic *in vivo* where a single dose has been shown to cause fatty liver (Scales *et al.* 1982) as well as liver necrosis but only after repeated dosing (Patrick and Back 1965). Following single dosing *in vivo* hydrazine produces characteristic mid-zonal fat accumulation in the liver but there is no evidence of accompanying necrosis. Liver triglyceride levels were found to increase following hydrazine dosing with decreases in ATP and GSH levels also observed, there was not however a concurrent increase in GSSG (Timbrell *et al.* 1982).

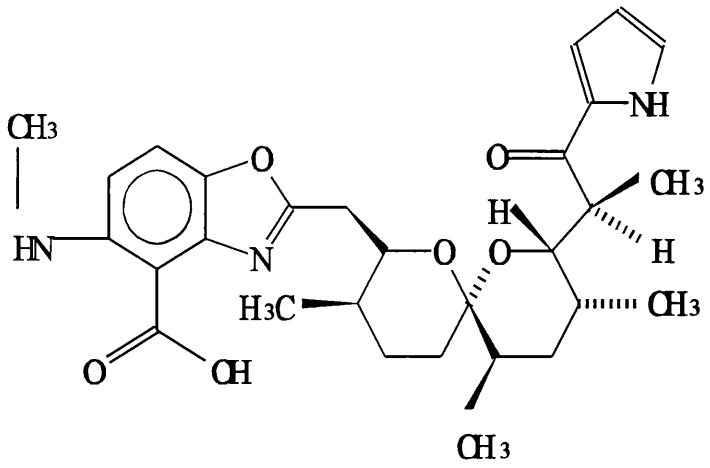


In addition to this hydrazine has been shown to be cytotoxic both in hepatocyte suspensions (Waterfield *et al.* 1994) as well as in rat hepatocyte cultures (Ghatineh and Timbrell 1994). *In vitro* hydrazine exposure was found to decrease protein synthesis initially with decreases also observed in ATP and GSH levels. Unlike the effect of hydrazine *in vivo* there was no increase in triglyceride levels in hepatocytes. However this may be due to the fact that the *in vitro* models used were inappropriate for the measurement of triglyceride synthesis (Waterfield *et al.* 1997).

The precise mechanism of hydrazine toxicity remains to be elucidated fully, however it has been shown that *in vivo* the most characteristic effect of hydrazine exposure is liver steatosis. The underlying cause of this is thought to involve inhibition of protein synthesis resulting in impaired transport of triglycerides out of hepatocytes (Waterfield *et al.* 1997). Depletion of ATP and GSH levels was also observed in hepatocytes exposed to hydrazine however this is thought to represent late cellular events and not the underlying cause of toxicity.

1.9.3 Calcimycin (A23187)

Calcimycin (A23187) is a compound widely used to investigate calcium mediated cell death. Following exposure of cells to A23187 intracellular calcium levels rise rapidly thus activating many deleterious calcium dependent processes such as Ca^{2+} activated neutral proteases and calponins which have the ability to cleave the cell cytoskeleton causing blebbing and loss of cell viability (Nicotera *et al.* 1986). The increase in free calcium levels also causes the excess production of phospholipase A_2 and nitric oxide synthase both of which have the capability to produce large amounts of damaging free radicals.



The exact mechanism of A23187 induced cell death remains to be elucidated fully. A23187 disrupts the plasma membrane permeability to Ca^{2+} and thus causes a Ca^{2+} shift depending on the Ca^{2+} gradients intra- and extracellularly (Matsuda 1991). A23187 is also a well known uncoupler of mitochondrial oxidative phosphorylation. Intracellularly, fructose has been shown to protect against A23187 induced cell death but only if oligomycin is present (Bronk *et al.* 1993), therefore implying that A23187 is acting as a mitochondrial uncoupler and causing bioenergetic cell death rather than Ca^{2+} mediated cell death. There is no possibility that this mitochondrial damage is causing an increase in cystolic Ca^{2+} levels as mitochondrial Ca^{2+} levels roughly approximate to cystolic Ca^{2+} levels (Mehendale *et al.* 1994). Other findings on the mechanism of A23187 induced cell death may specifically involve calmodulin and protein kinase C as inhibitors of these caused a decrease in A23187 mediated cell injury (Matsuda 1991).

1.9.4 Cadmium chloride

The liver is a well known target organ for cadmium toxicity, following either acute or chronic administration of cadmium the liver accumulates significant amounts of cadmium. However, the precise mechanism of toxicity remains to be elucidated fully in hepatocytes. Administration of $CdCl_2$ to rats is known to induce heme oxygenase (Hsp32), an enzyme which cleaves heme to form biliverdin, a known anti-oxidant. The activity of other anti-oxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase are also decreased following $CdCl_2$ administration thereby implying oxidative stress in cadmium toxicity (Ossola and Tomaro 1995). Further evidence for this comes from studies in primary hepatocytes showing that administration of cadmium causes an increase in the generation of reactive oxygen species and lipid peroxidation (Funakoshi *et al.* 1997). In addition to generation of reactive oxygen species cadmium has well known affinity for binding thiols and has been shown to interact with protein sulphydryls causing alterations in cytoskeletal organisation and this effect is thought also to be an underlying cause of cadmium toxicity (Li *et al.* 1994).

In addition to exerting deleterious effects in the liver cadmium is a well established testicular and nephro-toxin where it has numerous effects. One of the main reasons for cadmiums widespread toxicity is its ability to react with numerous ligands including thiols, phosphates, chlorides, carboxyls and amino groups. Another effect of cadmium is its substitution for zinc at the molecular level. This interference with zinc homeostasis has wide ranging adverse effects due to the large number of zinc-containing enzymes and transcription factors present which are inhibited by cadmium substitution (Li *et al.* 1994)

Another well known effect of cadmium is to disrupt ion transport across cell membranes (Blazka and Shaike 1991) and it is thought that this effect may also underlie the toxic action of cadmium. Administration of $CdCl_2$ to hepatocytes causes a marked decrease in intracellular pH, this decrease in pH is then thought to have a deleterious effect on mitochondrial function. Specifically the interaction of cadmium with the HCO_3^-/Cl^- exchange was shown to be of importance as addition of the HCO_3^-/Cl^- inhibitor probenecid partially prevented the intracellular acidosis following cadmium exposure (Koizumi *et al.*

1995).

Cadmium also has a profound effect on calcium in the cell. The cellular effects of cadmium include mobilisation of inositol triphosphate and hence release of calcium from the sarcoplasmic reticulum and inhibition of the Ca^{2+}-ATP'ases of the nucleolus and sarcoplasmic reticulum. The ionic radius of Cd^{2+} is 0.97Å which is very similar to that of Ca^{2+} (0.99Å) and because of this fact its thought that calcium channels are an important route of cadmium transport. Cadmium has also been shown to be a competitive inhibitor of Ca^{2+} uptake into cells thereby implying that it blocks receptor operated Ca^{2+} channels in hepatocytes (Souza *et al.* 1996).

Another putative cellular target of cadmium is the Na/K-ATP'ase which cadmium is thought to inhibit. This was suggested by the measurement of electrolyte ions in freeze dried cryosections of rat hepatocytes by energy dispersive electron probe X ray microanalysis. Administration of cadmium to the cells causes a significant increase in cytoplasmic Na^+ levels with a concurrent decrease in K^+levels. The inhibition of the Na/K-ATP'ase may be due to a depletion of the cellular ATP (Zierold 1997). It is therefore thought that the another fundamental aspect of Cd^{2+} toxicity is a perturbation of the cells ionic balance and pH which is then thought to bring about lipid peroxidation by the generation of reactive oxygen species.

1.10 Aims of the investigations

The general aim of these studies was to investigate the role of heat shock proteins in hepatocytes as protective agents and as markers of toxicity. Initially this would involve determination of the protective nature of induction of the stress response of hepatocytes *in vitro* against subsequent chemical exposure of a range of compounds. Following on from this it was hoped to determine the relevance and sensitivity of measurement of heat shock proteins as markers of toxicity in hepatocytes both *in vivo* and *in vitro* when compared to other well established biochemical markers of toxicity. *In vitro* this was to be performed in hepatocyte monolayers initially and then a long term hepatocyte model was to be developed as a model for studying the stress response. It was hoped to

determine if there was any difference in the stress response in short and long term hepatocyte cultures following chemical exposure. In addition to this the toxicity of the compounds could be investigated *in vivo* and in the two different *in vitro* hepatocyte models.

Chapter 2.

Materials and methods

2.1 Materials

Trypan blue, bovine albumin, collagenase (type I), luciferase, EDTA, EGTA, OPT, βNADH, pyruvic acid, insulin, hydrocortisone hemisuccinate, PBS, BSA, Tween-20, Kodak X-OMAT-AR film, GBX developer, GBX fixer, hydrazine hydrate (100%), $CdCl_2$ (98%), A23187, menadione, L-leucine, sulphosalicylic acid, Trizma base, 2-mercaptoethanol, DTNB, collagen, Triton X-100, HEPES, ATP, GSH, bovine-γ-globulin, H1777 hepatocyte medium and p-HEMA were all obtained from Sigma Chemical Company, Poole, Dorset UK. "Repelcoat" water repellant, TCA, NaOH, acetic acid, methanol, glycerol, $MgCl_2$. $6H_2O$, $NaCl_2$, KH_2PO_4, KCl, $MgSO_4$. $7H_2O$, $CaCl_2$. $2H_2O$, $NaHCO_3$, Na_2HPO_4. $2H_2O$ and Na_2HAsO_4. $7H_2O$ were all obtained from BDH Merck Ltd., Poole, Dorset, UK. Brilliant blue R250, Bradford protein determination kit, 30% acrylamide/bis (37.5:1), TEMED, APS, SDS and glycine were all obtained from Bio-Rad, Hemel Hempstead, Herts, UK. Williams E media, Williams E media (with Glutamax-1), glutamine, gentamycin, trypsin EDTA, Earles balanced salt solution, sodium bicarbonate, foetal calf serum (US origin) and foetal bovine serum were all supplied from Gibco Life Technologies, Paisley, UK. Monoclonal anti-Hsp70/Hsc70, polyclonal anti-Hsp25, polyclonal anti-Hsp32 (heme oxygenase-1), polyclonal anti-Heat Shock Transcription Factor-1, recombinant purified Hsp25, Hsp32 and Hsp70 were obtained from Stressgen Biotechnologies Corporation, Victoria, BC, Canada. Mouse, rat and rabbit secondary antibodies conjugated with horseradish peroxidase were obtained from DAKO Ltd., High Wycombe, Bucks, UK. Coomassie® Plus Protein Assay Reagent was obtained from Pierce, Chester, UK. Rainbow™ molecular weight standards (low range), ECL detection reagents, Hyperfilm ECL and Hybond ECL were obtained from Amersham Life Sciences, Little Chalfont, Bucks, UK. ^{3}H-leucine was obtained from NEN Life Science Products, Hounslow, UK. Neutral buffered formalin was obtained from Pioneer Research Biochemicals, Colchester, Essex, UK. Cetyldimethylethylammonium bromide (99%) was obtained from Acros Organics, New Jersey, USA. Hypnovel was obtained from Janssen Animal Health. Hypnorm was obtained from Roche, Welwyn Garden City, UK. Soluscint A was obtained from National Diagnostics, Hessle, Hull, UK.

2.2 *In vitro* hepatocyte suspension studies

2.2.1 Preparation of isolated hepatocytes

The isolation of hepatocytes was performed by the two step collagenase perfusion method. This method is based on the pre-perfusion of the liver with Ca^{2+} free buffer followed by enzymatic digestion of the liver with collagenase (Moldeus *et al.* 1978). Calcium plays a pivotal role in the process as pre-perfusion with Ca^{2+} free buffer essentially aids in the dispersion of the hepatocytes following collagenase perfusion. The reason for this is thought to involve a conformational change in the intercellular matrix that favours hepatocyte dispersion in particular the washout of a Ca^{2+}-dependent adhesion factor. It is thought that this Ca^{2+} adhesion factor may be the central plaque within desmosomes and removal of this plaque material causes the hemidesmosomes to move apart (Seglen 1975). However since collagenase is a Ca^{2+} dependent enzyme Ca^{2+} must be re-introduced to the system for effective dispersion to occur.

2.2.2.1 Perfusion apparatus

The apparatus used for the isolation of hepatocytes was composed of a recirculating system of buffer flowing through a blunt ended cannula. The perfusion buffers comprised two solutions, Hank I and Hank II, both maintained at 37°C in 250ml tall form beakers (see appendix 1 for composition of hepatocyte isolation buffers). Flow rate was maintained so that approximately 2 drops/second were emerging from the cannula. The solutions were gassed continuously with $95\%O_2/5\%CO_2$. A schematic diagram of the perfusion apparatus is shown in figure 2.1.

2.2.1.2 Surgical procedure

Adult male Han Wistar rats (GlaxoWellcome bred, weighing 180-260g) were anaesthetised with Hypnorm/Hypnovel (1:1:2 H_2O, 3.3ml/kg, ip). The thoracic and abdominal areas were swabbed with 70% ethanol and the abdominal cavity opened with a U shaped incision. The intestinal viscera was displaced to the right and the hepatic portal vein exposed. Two ligatures, approximately 10cm in length, were placed under the portal vein using curved forceps and tied loosely. The portal vein was then nicked with sprung scissors as far down the vessel away from the liver as was feasible and the

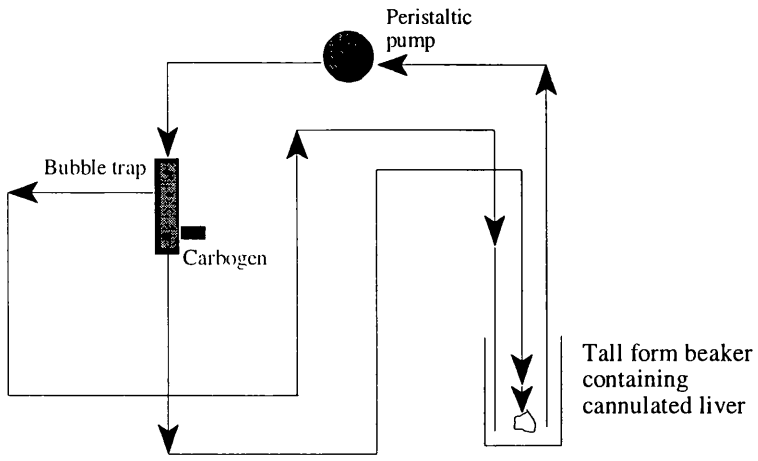


Figure 2.1. Schematic representation of the apparatus used for the perfusion of an isolated rat liver for subsequent hepatocyte isolation

cannula inserted. Once the vessel was successfully cannulated, as indicated by the blanching of the entire liver, the ligatures were tied securely around the cannula. The diaphragm, hepatic artery and vein were cut to kill the animal and the liver dissected out and placed in a tall form beaker containing 150ml Hank I buffer and perfused for 5 minutes. The flow from the cannula was adjusted so that there were 2 drops/second Hank I buffer emerging from the liver. The liver was then transferred to another beaker this time containing 100ml Hank II buffer with 50mg collagenase previously added. The liver was perfused with Hank II/collagense buffer for approximately 7-8 minutes.

Once sufficient digestion of the liver had occurred, as indicated by the "spongy" appearance of the liver, it was removed from the cannula and transferred to a vessel containing 50ml Krebs-Henseleit buffer containing albumin (KHAlb, see appendix 1 for composition). The Glisson membrane on the liver was removed with forceps and the lobes gently agitated to release the hepatocytes from the liver. The resultant cell suspension was filtered and transferred to four 20ml centrifuge tubes. The suspension was spun at 250rpm at 4°C for 2 minutes. The supernatant was aspirated off and the hepatocyte pellet resuspended in KH buffer and centrifuged again at 250rpm and 4°C for 2 minutes. This washing procedure was repeated once more in order to remove any dead cells or cellular debris and the final hepatocyte pellet was re-suspended in 20ml KH buffer.

2.2.1.3 Assessment of cellular viability and density

Estimation of cell density and viability was performed using the Trypan blue exclusion test. Trypan blue is taken up by dead cells which thus appear blue under the microscope, live cells exclude trypan blue. To 450µl 0.4% Trypan blue (see appendix 1) 50µl of the cell suspension was added and counted on an Improved Neubauer haemocytometer. The number of live cells and total number of cells were counted in 25 large squares in the haemocytometer. Viability of the isolated cells was calculated as follows;

$$\% \text{ Viability} = \frac{\text{number of live cells}}{\text{total number of cells}} \times 100$$

Viability of the cells was regularly in the range of 85-95%. Any isolations which gave a viability of less than 75% were discarded.

The density of the viable cells was calculated as follows;

Density = (viable cells/25 large squares x 10^5) cells/ml

Once counted the cells were diluted down with KH buffer (4°C) to a concentration of $1x10^6$ cells/ml.

2.2.1.4 Suspension of hepatocytes

Once diluted down to the appropriate concentration the hepatocytes were incubated in round bottomed 100ml flasks that had been previously treated with 'Repelcoat' water repellant. The rotating flasks were maintained at 37°C with a water bath and 95 %air/5% CO_2 was supplied via a bubble trap. The hepatocytes were pre-incubated for 30 minutes prior to any chemical or physical treatments.

2.2.2 Sample preparation

For biochemical analysis aliquots of cells (0.5ml) were taken from the flasks at the relevant timepoints. The samples were briefly centrifuged and 100µl supernatant removed and stored at 4°C for determination of LDH. To one aliquot 0.5ml 6.5% TCA was added, the sample vortexed and then spun at 13,000rpm for 5 minutes. The supernatant was

removed and stored at -80°C until analysed for GSH. To the other aliquot 0.5ml 10% TCA was added, again the sample was vortexed and spun at 13,000rpm for 5 minutes. The supernatant was removed and stored at -80°C until analysed for ATP.

2.2.3 Biochemical analysis

2.2.3.1 ATP determination

The ATP extracted from the hepatocytes with 10% TCA was detected by a bioluminescence assay based upon the method of Stanley and Williams (1969). The method relies upon the reaction of luciferase extract with ATP. The generalised scheme of events is as follows;

$$\text{Luciferase (E)} + \text{Luciferin } (LH_2) + \text{ATP} \Rightarrow E.LH_2AMP + PPi$$

$$E.LH_2AMP + O_2 \Rightarrow E + CO_2 + \text{AMP} + \text{oxyluciferin} + \text{LIGHT}$$

Therefore, the amount of light produced is proportional to the amount of ATP present in the cell. The light produced was detected using a Thorn-EMI photon detection system connected to an Amstrad PC1512 computer. The temperature of the detection system was maintained at -25°C in order to decrease the background reading.

To 75mm plastic tubes 2ml ATP buffer and 10µl sample or standard was added and the reaction started with the addition of 100µl Lantern extract. The sample was then vortexed and read 15 seconds following the addition of the Lantern extract (see appendix 2 for buffer compositions and standard curve).

2.2.3.2 GSH determination (fluorometric method)

This method of GSH measurement of Hissin and Hilf (1976) was designed specifically for the measurement of intracellular GSH in isolated hepatocytes. The fluorescent reagent o-phthalaldehyde reacts with GSH forming a highly fluorescent product that is activated at 350nm and has an emission peak at 420nm.

In 5ml plastic tubes 75µl of the TCA samples or standards was added to 2775µl phosphate/EDTA buffer + NaOH. OPT (150µl) was added to the standards or samples which were then vortexed and left to stand at room temperature for 25 minutes. After this

time, the fluorescence was read at wavelengths of 350nm excitation and 420nm emission on a Perkin Elmer LS3 fluorescence spectrophotometer (see appendix 3 for buffer compositions and standard curve).

2.2.3.3 Lactate dehydrogenase (LDH) determination

When cell membrane integrity is compromised the cytostolic enzyme LDH leaks from the hepatocytes into the suspension medium. The assay for LDH is based on the modification of Bergmeyer (1965) and relies on the reduction of pyruvate to lactate in the presence of LDH. The level of LDH in the medium was determined by measurement of the decrease in absorbance at 340nm when NADH was oxidised to NAD^+ as shown below;

$$\text{pyruvate} + \text{NADH} + H^+ \overset{\text{LDH}}{\leftrightarrows} \text{L-lactate} + NAD^+$$

To a 10µl sample of medium 290µl LDH buffer was added and the reaction monitored kinetically at 340nm for 5 minutes in a Labsystems Multiskan 96-well plate reader with the data analysed using Labsystems Genesis software (see appendix 5 for buffer compositions).

2.3 Hepatocyte monolayer studies

2.3.1 Preparation of type I collagen

Hepatocytes require an adherent substratum for optimal culture. In these studies hepatocytes were cultured on plates coated with rat tail collagen. Male Han Wistar rat tails were removed and stored at -20°C until use. Upon use the tails were defrosted and swabbed with 70% ethanol and the skin removed from the tails by cutting and stripping the skin towards the tip. The tails were then cut into several pieces prior to the removal of the tendons with forceps. The tendons were removed from the tails and air dried in a laminar flow cabinet for 3 hours and then separated into the individual fibres by gentle rubbing. The collagen fibres were weighed and 250ml sterile 0.1% acetic acid/g fibres added and stirred overnight at room temperature. The resulting stock collagen solution

was centrifuged at 1000rpm for 10 minutes to remove any undissolved material and the supernatant stored at 4°C until use.

2.3.2 Collagen coating of culture dishes

The stock collagen solution was diluted 1:15 with sterile 0.1% acetic acid. For 33mm culture dishes 400µl diluted collagen solution was added to the plates (approximately 40µl/cm^2) and swirled around to ensure the entire surface of the dish was coated. The dishes were air dried overnight in a laminar flow cabinet with the lids left ajar and then stored at 4°C.

2.3.3 Culture of hepatocyte monolayers

Hepatocytes were isolated as described in section 2.2.1, however once counted the cells were diluted down with Williams E medium (500ml Williams E media containing 1µM insulin, 0.1mM hydrocortisone hemisuccinate, 2mM glutamine and 60µg/ml gentamycin) to a concentration of 1x10^6 cells/ml. The cells were plated out onto 33mm diameter sterile culture dishes, 2ml medium being added to each plate thereby giving a final cell density of 2x10^6 cells per dish. The culture dishes were previously coated with rat tail collagen. Once plated, the cells were incubated at 37°C in a humidified atmosphere of 5%CO_2/95% air. The medium was aspirated off the cells and fresh medium added two hours after plating in order to remove any unattached or dead cells and any cellular debris. All sterile work was performed in a Class II cabinet.

2.3.4 Sampling of hepatocyte monolayers

The culture plates were removed at the relevant timepoints and placed on ice. The medium was removed, aliquoted and stored at 4°C for LDH determination. The cells were washed twice with 2ml PBS (4°C) and 1ml 6.5% trichloroacetic acid (TCA) added to each plate and left for 10 minutes at 4°C. The TCA was removed, aliquoted (500µl) and stored at -80°C for determination of ATP and GSH. The remaining protein present on the plate was dissolved in 1ml 1M NaOH and subsequently stored at 4°C for total protein determination. For determination of heat shock protein levels in the cells the culture dishes were removed at the relevant timepoints and 1ml trypsin/EDTA added to each well and incubated at 37°C for a few minutes. The hepatocytes were removed with the aid of a

rubber policeman and spun down for a few seconds to sediment the cells. The supernatant was removed and the pellet resuspended in 100µl Laemmeli buffer (see appendix 7 for composition), the samples sonicated for 5 seconds at 15 microns amplitude and bromophenol blue and 2-mercaptoethanol were added to give final concentrations of 0.005% and 5% respectively. The samples were heated to 100°C for 5 minutes and stored at -80°C until required for SDS-PAGE. LDH, ATP and GSH levels in the samples were measured by the methods described previously in sections 2.2.3.1-2.2.3.3.

2.3.5 Biochemical analysis

2.3.5.1 Total protein determination

Total protein was determined by the Bradford method using a Coomassie Brilliant Blue G-250 solution. Upon addition of protein to the solution the dye binds to the protein resulting in a colour change from reddish brown to blue. This binding of the protein causes a shift in the absorbance from 465nm to 595nm. Initial dilutions of the samples were made with H_2O to ensure that protein concentrations in the sample were contained within the linear part of the standard curve. To 150µl sample or standard 150µl Coomassie Protein Plus Reagent was added. The colour was allowed to develop for a few minutes and the absorbencies read at 595nm on a Labsystems Multiskan 96-well plate reader (see appendix 8 for reagents and standard curve).

2.3.5.2 Detection and analysis of heat shock protein levels in cell lysates

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to separate and estimate the size of proteins in a sample. Separation by SDS-PAGE is based on the molecular weight of the protein. SDS in the gel and sample buffer bind to the proteins and breaks all non-covalent bonds whilst 2-mercaptoethanol in the sample buffer breaks all di-sulphide bonds. The resultant SDS-protein complex is negatively charged and thus moves towards the anode in an electrical field at a rate proportional to the logarithm of its molecular weight. Western blotting of the protein samples effectively transfers all the separated proteins present in a gel to a nitrocellulose membrane. The protein of interest can then be detected by using a primary antibody raised against the specific protein. The amount of protein can be quantified by the use of a secondary peroxidase conjugated antibody which can oxidise a substrate thereby resulting in the

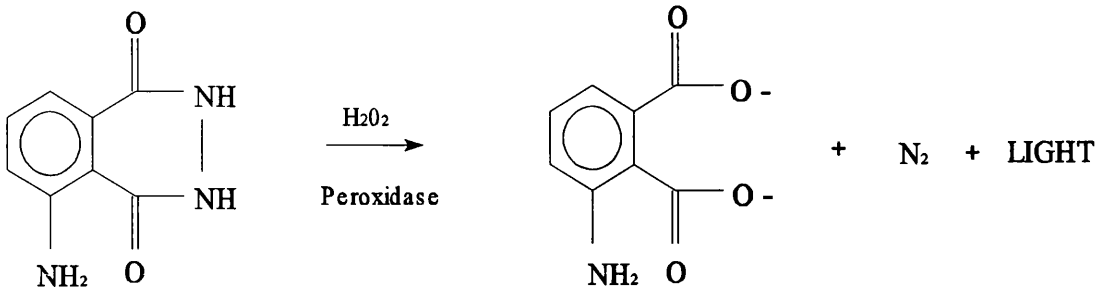
release of light.

The mini-PROTEAN II cell was assembled following manufacturers instructions. The proteins in the cell lysate were separated initially using SDS-PAGE with a 12.5% resolving and 5% stacking mini-gel (see appendix 6 for gel composition) (Laemmeli 1970), the lower buffer chamber was filled with approximately 200ml 1 x electrophoresis buffer (see appendix 7 for buffer compositions). Equal amounts of protein were loaded in each well. Separation of the proteins was accompanied by concurrent separation of prestained Rainbow molecular weight standards (low molecular weight range; 14.3-220kDa) and native purified heat shock protein (0.5µg) of interest. Electrophoresis was performed for 1 hour at 50V and 100V for 1½ and 2 hours depending on which heat shock protein was being investigated.

Following separation the proteins were electrophoretically transferred onto Hybond-ECL nitrocellulose membranes. Western blotting was carried out at 100V for 1hour 10 minutes with the buffer chamber containing 1 x Western blotting buffer at 4°C (see appendix 7). Once the proteins had been transferred to the nitrocellulose the membranes were incubated overnight in PBS containing 0.5% BSA in order to block all non specific binding sites of the immunoglobulin.

The membranes were washed 3 times in 0.1% Tween-20 for 5 minutes. Following this the membranes were incubated with a primary monoclonal antibody raised against the heat shock protein of interest for 1½ hours. In these investigations the antibodies used were mouse anti-Hsp72/73 monoclonal (clone N27F3-4 SPA-820), rabbit anti-HO-1 (Hsp32 SPA-895) and rabbit anti-Hsp25 polyclonal (SPA-801). The samples were additionally probed with a mouse anti-Hsp72 monoclonal antibody (clone C92F3A-5 SPA-810) but no reactivity was found in any of the samples. Removal of excess primary antibody was performed by washing the membranes 3 times in 0.1% Tween 20 in PBS with each wash lasting 10 minutes. The membranes were then incubated with a secondary antibody consisting of rabbit anti-mouse IgG or murine anti-rabbit IgG conjugated with horseradish peroxidase for 1 hour. Membranes were then washed 3 times in 0.3% and 3 times in 0.1% Tween-20, each wash lasting 5 minutes. All washes and incubations were carried out at

room temperature on a shaking platform. The antibody-protein complexes were visualised using an enhanced chemiluminescence(ECL) detection system used according to the manufacturers instructions and detected on Kodak X-OMAT-AR film or Hyperfilm ECL. The detection system was based on the conversion of Luminol to 3-aminophthalate by horseradish peroxidase in the presence of H_2O_2 with the subsequent emittance of light. The reaction is shown below:



Following a range of exposure times the films were developed using conventional X-ray developer and fixing solutions and left to dry. The individual bands representing the individual heat shock proteins were identified by the location of the purified protein of interest and quantified by image analysis densitometry (PC Image Plus software).

2.3.5.3 Staining of polyacrylamide gels

Following Western blotting of the proteins the gels were stained with Brilliant Blue to ensure equal loading of protein and efficient transfer of the proteins to the nitrocellulose during Western blotting had occurred. Following Western blotting gels were stained overnight with Brilliant Blue stain (0.1% w/v Brilliant Blue R-250, 25% v/v methanol, 5% v/v acetic acid). Destaining was carried out the following day, the destain solution consisted of 25% methanol and 5% acetic acid. Regular washes of the gels with destain solution allowed any residual proteins present in the gels to be visualised.

2.3.5.4 Determination of protein synthesis

This assay was based on the incorporation of ^{3}H-leucine into acid precipitable proteins in the hepatocytes which accounts for the majority of proteins present within hepatocytes. At the relevant timepoints hepatocytes were pre-incubated with 1μCi/ml ^{3}H-leucine and

5mM leucine for the 1 hour prior to each time-point. The cells were then removed from the culture plates by the addition of 1ml Trypsin/EDTA and gentle scraping with a rubber policeman. The cells were spun down for a few seconds to sediment the hepatocytes and the medium was removed. The cells were resuspended in 0.5ml 10% TCA (4°C). Protein was precipitated by centrifugation at 13,500g for 5 minutes at 4°C and the supernatant discarded. The protein pellet was then washed five times with 1ml aliquots of 10% TCA (4°C) by repeated centrifugation (13,500g, 5 minutes, 4°C) and resuspension in TCA. The protein pellet was then dissolved in 0.5ml 1M NaOH by heating to 50°C for 1 hour. 200µl aliquots were then placed in scintillation vials and 4ml Soluscint A added to the vials. The remaining 100µl NaOH was used to measure total protein by the Coomassie method. The vials were counted for 15 minutes on a Beckman LS6000IC scintillation counter with NaOH used as a blank and the results expressed as $dpm.mg^{-1}$ protein.

2.4 *In vivo* studies

2.4.1 Animal husbandry

Male Han Wistar rats (GlaxoWellcome) weighing between 210-260g were acclimatised in communal cages for 7 days following delivery. Animals were given free access to tap water and solid diet (Bantin and Kingman). Lighting was controlled to give a regular 12 hour light dark cycle (07:00 lights on, 19:00 lights off) and room temperature was maintained at 21 ± 2°C. For each study animals were housed in four communal boxes with each containing five rats.

2.4.2 Post mortem procedure

At the relevant time-points animals were anaesthetised with 3.3ml/kg Hypnorm/Hypnovel (1:1:2 H_2O), the abdomen was swabbed with 70% ethanol and opened with a U-shaped incision. The intestinal viscera was displaced to the right and the abdominal aorta identified. Animals were exsanguinated via the abdominal aorta and the blood placed into Microtainers (Beckton Dickinson) for serum separation. The samples were allowed to coagulate for at least 45 minutes and then spun for 30 seconds at 13,500rpm. The samples were stored at -80°C until required for serum analysis. The liver was removed, weighed

and the left lobe divided into four samples. Two samples were placed into liquid nitrogen and stored at -80°C for analysis of heat shock protein and GSH levels. One sample was placed into 10.5% (v/v) phosphate buffered formalin (pH 7.2) (PBF) for histological processing. The final liver sample was placed into liquid nitrogen and freeze clamped with a mortar and pestle, the resultant powder was transferred to pre-weighed vials containing 4ml 0.2M sulphosalicylic acid and stored at -80°C for determination of ATP levels. The testes were also removed and weighed. One testis was placed into liquid nitrogen and stored at -80°C, the remaining testis was placed into 10.5% PBF.

2.4.3 Sample preparation

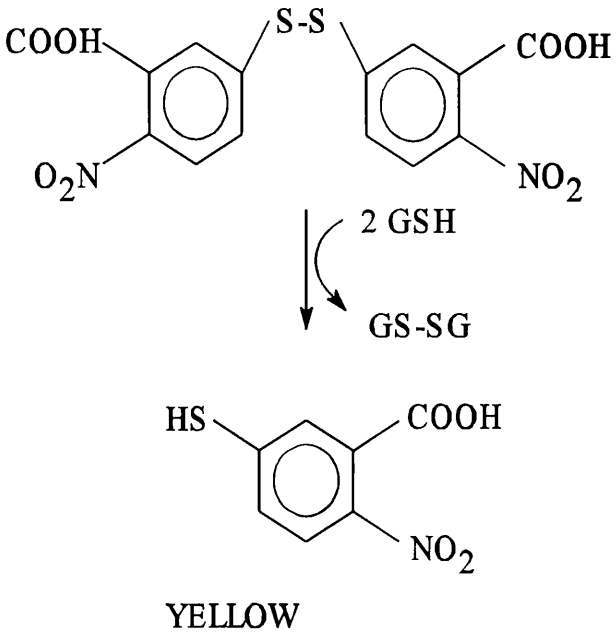
For determination of liver heat shock protein levels approximately 0.3g liver was homogenised on ice in 10mM Tris-HCl buffer pH 7.4 (12ml buffer/g^{-1} tissue). The resultant homogenate was centrifuged at 16,000g for 30 minutes (4°C), an aliquot of supernatant was removed for protein determination by the Coomasie method whilst the remainder was stored at -80°C until required for SDS-PAGE. Upon use the supernatant samples were diluted 5-fold with Laemmeli buffer. Bromomphenol blue and 2-mercaptoethanol were added to give final concentrations of 0.005% and 5% respectively and the samples heated to 100°C for 5 minutes. Determination of heat shock protein levels in the other tissues sampled was performed as described for liver samples with the exception that the testis was homogenised whole in 3.5ml 10mM Tris-HCl buffer pH7.4. GSH levels in the liver samples were analysed by homogenising on ice a sample of the left liver lobe (approximately 0.4g) in 4ml 0.2M sulphosalicylic acid (SSA, 4°C). The resultant homogenate was centrifuged at 4000rpm for 10 minutes (4°C) and the supernatant analysed for GSH (see 2.4.4.1).

2.4.4 Biochemical analysis

2.4.4.1 GSH (non protein sulphydryl) determination (spectrophotometric method)

This procedure for GSH measurement is based upon the method of Ellman (1959) and is specifically designed for measurement of GSH in tissue homogenates. The assay was based upon the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) with sulphydryl groups (-SH) present in the GSH molecule. However this reaction is not specific for GSH as DTNB reacts with any free -SH groups present in the sample. Therefore the results

express the amount of total non-protein sulphydryl groups (TNPS) present. DTNB is a disulphide chromogen and is readily reduced by suphydryl containing compounds. The resultant formation of the reduced chromogen was measured on a Shimadzu MPS-2000 spectrophotometer at a wavelength of 412nm. The reaction is summarised below:



To 0.25ml homogenate supernatant or GSH standard 0.25ml pH 7.4 buffer was added and vortexed. To this 4.5ml pH8.0 buffer was added and again vortexed. To all samples and standards 50µl DTNB was added and the samples left at room temperature for 15 minutes. The absorbance was then read at 412nm. (see appendix 4 for buffer composition and standard curve)

2.4.4.2 Serum chemistry

Levels of serum ALP, AST, ALT, albumin, urea, creatinine, triglycerides, cholesterol, sodium, potassium, chloride, calcium, phosphate, glucose and LDH were determined on a BM/Hitachi System 795 automatic analyser using the appropriate kits (Boehringer Mannheim GmbH Diagnostics) according to the manufacturers instructions.

2.5 Liver spheroid studies

All research concerning the use of liver spheroids was carried out in the GlaxoWellcome *In Vitro* Models Group, University of Hertfordshire, Hatfield, Herts.

2.5.1 Culture of liver spheroids

2.5.1.1 Plating of 6-well plates

a. Collagen

A stock solution of 2mg/ml rat tail collagen was made up in sterile H_2O and diluted down with sterile H_2O to a working concentration of 0.05mg/ml. 2ml of this solution was added to each well in a sterile 6-well plate (Falcon) and allowed to dry overnight at 37°C after which time the collagen was removed and the wells washed with 2ml sterile PBS. The plates were stored at 4°C until required for use.

b. p-HEMA

6.12g poly-2-hydroxyethylmethacrylate (p-HEMA) was dissolved in 232ml 100% ethanol and 13ml sterile H_2O (2% p-HEMA in 95% ethanol) with constant stirring and gentle heating over a period of at least 5 hours. The resultant solution was centrifuged at 1000rpm for 10 minutes in order to sediment any undissolved material. 2ml of the supernatant was added to each well in a sterile 6-well plate and the plate allowed to dry overnight at room temperature in a Class II laminar flow cabinet. Once dried the plates were stored at 4°C until use.

2.5.1.2 Surgical procedure

Male Charles River rats (aged 8-12 weeks, University of Hertfordshire) were sacrificed by cervical dislocation, the abdominal cavity swabbed with 70% ethanol and opened with a U-shaped incision. The liver was removed from the animal ensuring the intestine was not cut during the procedure. The median and right lobes were cut free of the liver and cannulae inserted into the major blood vessels supplying the lobes. The cannulae were connected to a non-circulating perfusion apparatus with the flow rate set at 10ml/minute. The apparatus consisted of three flasks of buffer maintained at 37°C and gassed continuously with 95% air/5% CO_2. Flask 1 contained chelating buffer, flask 2 contained perfusion buffer and flask 3 contained collagenase buffer.

The general perfusion buffer consisted of;

435ml sterile UHQ water

50ml Earles Balanced Salt Solution x10

15ml 7.5% sodium bicarbonate

The pH was adjusted to 7.4

The individual perfusion buffers were as follows;

Chelating buffer (Flask 1)

490ml perfusion buffer

10ml 25mM EGTA

Perfusion buffer (Flask 2)

500ml general perfusion buffer

Collagenase buffer (Flask 3)

100ml perfusion buffer

200µl 1M $CaCl_2$

50mg collagenase (type 1)

Upon successful cannulation the liver lobes blanched immediately. The lobes were perfused with chelating buffer from flask 1 for 10 minutes. After this perfusion buffer from flask 2 was perfused for 5 minutes. Collagenase buffer from flask 3 was then perfused and recirculated for approximately 30 minutes or until the liver lobes appeared completely digested.

The digested lobes were transferred to a sterile Petri dish and Sigma H-1777 Hepatocyte medium (500ml H-1777 medium, 10ml foetal calf serum [US origin], 5ml 200mM glutamine, 2.5ml 10mg/ml gentamycin) added. The lobes were agitated to release the hepatocytes which were then filtered and transferred to sterile 50ml centrifuge tubes. The cells were centrifuged at 350rpm for 3 minutes after which time the media was aspirated off and the cells resuspended in fresh media. The cells were spun again to remove any dead cells or cellular debris and finally resuspended in a known volume of medium.

2.5.1.3 Assessment of cellular viability and density

To count the cells 200µl media containing the hepatocytes was mixed with 200µl 0.4%

Trypan blue and the resultant solution counted on a Improved Neubauer haemocytometer. Two of the larger squares were counted with the number of viable cells noted along with the total number of cells. The value of the total count of viable cells in the two squares gave the hepatocyte stock density value expressed as [$x10^4$cells/ml]. Any preparations which gave a viability of less than 80% were discarded as poor spheroid formation was observed with viabilities of less than this. In order to determine the dilution factor for the stock hepatocytes the following equation was used:

$$\text{Volume of stock solution (ml)} = \frac{\text{volume to be made (ml)} \times \text{required density}}{\text{stock density}}$$

The volume to be made depended on how many plates were required and the required density of cells was $0.5x10^6$cells/ml. The hepatocytes were diluted down to $0.5x10^6$ cells/ml with Sigma H-1777 medium and 2ml of this solution was added to each p-HEMA coated well. The plates were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2 on an orbital shaker set at 90rpm.

When primary hepatocytes were required in the same study the stock solution of cells was centrifuged again at 350rpm for 2 minutes. The hepatocytes were then resuspended in an identical volume of Williams E (with Glutamax-1) medium containing 10% foetal bovine serum and 2.5ml 10mg/ml gentamycin. 2ml of this solution was added to each collagen coated well and incubated at 37°C in a humidified atmosphere of 95%air/5%CO_2 for 2 hours. The medium was then aspirated off the cells which were now attached to the collagen and fresh Williams E medium containing only 2.5ml 10mg/ml gentamycin was added.

2.5.1.4 Changing of spheroid media

Twenty four hours after the hepatocytes were plated out for spheroid formation the medium was changed. At this the time the hepatocytes had clumped together to form large cellular masses. The plates were tilted to allow the clumped cells to sediment, the medium was aspirated off and fresh medium added. To separate the clumped hepatocytes the medium and cells were agitated using a sterile pasteur pipette until no large cellular masses

were visible. Medium was then changed every two days by the same method with the exception that the cells were not agitated during subsequent washes.

2.5.2 Sampling of spheroids

Two wells of spheroids were pooled into centrifuge tubes and centrifuged for 10 seconds at 350rpm to sediment the spheroids. The medium was removed, aliquoted and stored at -20°C for determination of albumin secretion and at 4°C for LDH determination. Once the medium was removed 2ml 6.5% TCA (4°C) was added to the cells and the sample sonicated for 10 seconds at 20 microns amplitude in order to disrupt the spheroidal structure. The tubes were then stored at 4°C for 30 minutes after which time the samples were centrifuged at 2000rpm for 10 minutes (4°C). The supernatant was removed and stored at -80°C for determination of ATP and GSH levels. The pellet was dissolved in 5ml 1M NaOH and stored at -20°C for protein determination. For analysis of heat shock protein levels in the samples the spheroids were transferred to centrifuge tubes and allowed to settle. The media was removed and the spheroids resuspended in 150µl Laemmeli buffer and sonicated for 5 seconds at 20 microns amplitude. 50µl of the resultant solution was removed and stored at -20°C for protein determination. To the remaining sample bromophenol blue and 2-mercaptoethanol were added to give final concentrations of 0.005% and 5% respectively. The sample was then heated to 100°C for 5 minutes and stored at -80°C until required for SDS-PAGE.

2.5.3 Biochemical analysis

2.5.3.1 LDH determination

Leakage of LDH into the medium was measured by the same assay as described in section 2.2.3.3. 200µl of medium was placed into sample vials and LDH levels were determined on a Cobas Fara II centrifugal autoanalyser using an LDH diagnostic kit (Roche Diagnostics) according to the manufacturers instructions.

2.5.3.2 Albumin secretion

Measurement of the secretion of albumin into the medium was measured on a Cobas Fara II centrifugal autoanalyser using a Tina-quant albumin immunoturbidometric test kit (Boehringer Mannheim) according to the manufacturers instructions.

2.5.3.3 ATP measurement

ATP levels within the spheroids were measured by the bioluminescence assay described previously in section 2.2.3.1. To 2ml ATP buffer 10µl sample or standard was added and the solutions vortexed. 200µl of this solution was added to each well and 10µl Luciferase extract added and the luminescence measured on a Labsytems Luminoskan 96-well plate reader.

2.5.3.4 Measurement of total protein

Total protein was determined using the Bio-Rad protein assay kit based on the Bradford method of protein measurement as described in section 2.3.5.1. The stock assay reagent was diluted five fold with H_20 and filtered through Whatman No. 1 filter paper to give the working solution. To 200µl of this working solution 10µl sample or standard was added and the resultant colour development was measured at a wavelength of 595nm on an ICN Flow MS212 microplate reader with EIA3 software (see appendix 9 for reagent compositions and standard curve).

2.5.3.5 GSH measurement

GSH levels were measured by the method described previously in section 2.2.3.2 with the exception that levels of GSH in the TCA extracts were determined using a Cobas Fara II centrifugal autoanalyser.

2.5.4 Sampling and analysis of spheroids for light microscopy

The spheroids to be sampled were placed into centrifuge tubes and the medium removed, the cells were then washed with 2ml sterile PBS. Spheroids were fixed for a minimum of 24 hours in 10% buffered formalin at room temperature. The samples for light microscopy were processed as follows by the Pathology group, GlaxoWellcome, Ware. The sample was placed in 1% agar and allowed to set for a minimum of 2 hours. The solidified agar block was then sliced into 3-4 transverse sections which were further fixed for 24 hours in 10% buffered formalin. The sample was then dehydrated through a series of solvents from 70% industrial methylated spirits to xylene. The samples were than embedded in paraffin wax and sections of approximately 3-4µm in thickness were cut from the wax on a microtome. The sections were stained with haematoxylin and eosin in addition to

staining with Oil Red O.

2.5.6 Sampling and analysis of hepatocyte monolayers for light microscopy

Thermanox coverslips were placed into the wells of 6-well culture plates and 2ml 0.05mg/ml collagen solution added to each well. The coverslips were left overnight at 37°C after which time the wells and coverslips were washed with 2ml sterile PBS. Hepatocytes were cultured on the collagen coated Thermanox coverslips in 6-well plates at a density of $0.5x10^6$ cells/ml, 2ml being added to each well. The coverslips were removed at the appropriate timepoints and fixed in 10% buffered formalin for at least 24 hours. The coverslips were cut into 2 pieces with one half being stained with haematoxylin and eosin and the other with Oil Red O.

2.6 Heat shock factor studies

2.6.1 Isolation of hepatocyte nuclei

A 10ml aliquot of hepatocytes in suspension at a density of $1x10^6$ cells/ml was taken and centrifuged at 2000rpm for 10 seconds in order to sediment the cells. The pellet was resuspended in 3ml cell lysis buffer (10% cetyldimethylethylammonium bromide, 0.5% Triton X-100, 2mM $MgCl_2.6H_2O$, 15mM $NaCl_2$, 5mM KH_2PO_4, pH7.4), vortexed and left for 10 minutes at room temperature. The cell lysis suspension was centrifuged at 1100g (4°C) for 10 minutes to sediment the isolated nuclei. An aliquot of the supernatant was removed for protein determination by the Coomassie method and for analysis of non-nuclear levels of HSF-1. The nuclear pellet was resuspended in 200µl Laemmeli buffer and sonicated (10 seconds, 15 microns amplitude) to release the nuclear contents. 2-mercaptoethanol (5%) and bromophenol blue (0.005%) were added and the samples heated to 100°C for 5 minutes. The samples were stored at -80°C for a maximum of two weeks.

2.6.2 Determination of HSF-1 levels in vitro and in vivo

HSF-1 levels were determined from hepatocytes in suspension, monolayer cultures, spheroids and from *in vivo* samples taken to elucidate whether there was any difference

in the sensitivity of HSF-1 activation when compared to heat shock protein induction following chemical stimuli. Analysis of HSF-1 levels was performed by SDS-PAGE, the samples were separated on a 4% stacking gel and a 7.5% resolving gel (see appendix 6 for composition) as described previously in chapter 2.3.7. The nitrocellulose membranes were probed with a rabbit polyclonal antibody directed against HSF-1 (SPA-901 Stressgen Biotechnologies Corp. Vancouver, Canada) which recognises both the active (95kDa) and inactive form of HSF-1 (85kDa).

2.7 Statistical analysis

To assess statistical significance in the in vitro experiments the data was analysed initially by using a one way analysis of variance (ANOVA) which was performed to determine whether the differences among a number of sample means were large enough to convince that at least two of the corresponding population means were different. This was then followed by a Dunnett's comparison test (Dunnett 1964). Dunnett's test was used to test for differences between the mean of one control (reference) population and those of each other population ie comparison of treated groups of cells with one control group. Dunnett's test was also used to determine significance in the animal studies where multiple treatment groups were compared to a single control group. A p value of equal to or less 0.05 was determined to be significant.

Chapter 3.

The protective nature of heat shock proteins in hepatocytes

3.1 Introduction

It is well documented that a transient increase in a cells temperature effectively protects the cell from subsequent exposure to elevated temperatures that would normally prove lethal (Subjeck and Shyy 1986). The fundamental aspects of this phenomenon known as thermotolerance are discussed in greater detail in chapter 1.6. The phenomenon of thermotolerance also applies to chemical exposure where exposure of cells to sub-lethal concentrations of a toxin effectively protects the cell from subsequent exposure to normally toxic concentrations of the same compound. The basis of thermo- and chemical-tolerance is thought to be the induction of heat shock proteins. The elevated levels of the stress proteins induced by elevated temperatures or chemical exposure effectively protect the cell from further noxious insults. These phenomena have been demonstrated in numerous cell lines (Steels *et al.* 1992), however there is little data concerning thermotolerance in hepatocytes. This series of experiments was performed to investigate whether elevated temperatures could confer a degree of protection to isolated hepatocytes exposed to various hepatotoxic compounds. The compounds chosen in these investigations were hydrazine, cadmium chloride and calcimycin (A23187), all of which have differing mechanisms of action. Hydrazines' mechanism of action is thought to involve inhibition of protein synthesis and depletion of cellular ATP, cadmium is known to induce oxidative stress in cells as it is a redox cycling toxin and calcimycin causes calcium mediated cell death (Nicotera *et al.* 1986, Malorni *et al.* 1991, Waterfield *et al.* 1997). These compounds were chosen to investigate whether the mechanism of action of a compound affected the degree of protection observed following heat shock., the mechanisms of action of the compounds are discussed in greater detail in chapter 1.9.

3.2 Effect of transient increases in temperature on the subsequent toxicity of hydrazine in hepatocyte suspensions.

3.2.1 Aims of study

The main aim of this study was to determine whether or not pre-exposure of isolated hepatocytes transiently to elevated temperatures would confer a degree of protection to

the cells following exposure to a hepatotoxin. This was performed by exposing the hepatocytes transiently to elevated temperatures and then assessing the subsequent toxicity of increasing concentrations of hydrazine in heat shocked and non-heat shocked cells. Hydrazine is a well known hepatotoxin both *in vivo* and *in vitro* and its mechanism of action is described in greater detail in chapter 1.9.2.

3.2.2 Methods

Hepatocytes were isolated as described in chapter 2.2 and incubated for 30 minutes at 37°C in round bottomed flasks, the flasks were then transferred to an identical water bath maintained at 43°C and incubated for one hour. Following this the cells were re-incubated at 37°C for 3 hours during which time the hepatocytes were exposed to increasing concentrations of hydrazine. One set of control flasks, containing non-heat shocked cells, was maintained at 37°C for the one hour incubation period and then exposed to identical concentrations of hydrazine. The concentrations of hydrazine used were 0, 5, 10, 15 and 20mM. Following exposure to hydrazine samples were taken at 1, 2 and 3 hours post dose for measurement of LDH leakage and cell viability by trypan blue exclusion. Sampling and biochemical analysis in this study and in the following studies was performed as described in chapter 2.2.3.

3.2.3 Results

Exposure of control hepatocytes to hydrazine produced dose dependent toxicity as indicated by changes in LDH leakage and cell viability. There was no change in LDH leakage from control cells or from those exposed to 5mM hydrazine at any timepoint. Concentrations of 10, 15 and 20mM hydrazine produced significant increases in LDH leakage and concurrent decreases in cell viability when compared to control cells. Heat shocking the hepatocytes appeared to have no effect on the subsequent exposure to hydrazine, it was found that incubating the hepatocytes at 43°C slightly increased their susceptibility to hydrazine. There did appear to be a trend in the LDH and viability results showing an increase in the toxicity of hydrazine in heat shocked cells when compared to non-heat shocked cells particularly at three hours post dose. There was however no significant difference between the leakage of LDH and the measurement of cell viability from non-heat shocked and heat shocked cells following exposure to hydrazine (tables

3.1 and 3.2).

3.2.4 Discussion

The main aim of this study and of all subsequent studies in this chapter was to determine whether or not the transient exposure of rat hepatocytes to elevated temperatures or non-toxic concentrations of a toxin affected the subsequent toxicity of a hepatotoxin. In this first study the effect of transiently elevated temperatures on the toxicity of hydrazine was investigated. Hydrazine was shown to cause dose dependent toxicity over the three hour duration of the experiment as indicated by the decrease in cell viability and concurrent increase in LDH leakage. The toxicity data in non-heat shocked cells presented here correlates well with previous studies investigating the effect of hydrazine on isolated hepatocytes where concentrations of 12 and 16mM hydrazine were shown to significantly increase LDH leakage following a three hour incubation with hydrazine (Waterfield *et al.* 1997). In the data presented here LDH leakage was determined to be elevated following administration of 10 and 15mM hydrazine for three hours.

There was no difference in the toxicity of hydrazine between heat shocked and non-heat shocked cells. However the viability and LDH results showed a slight increase in susceptibility of heat shocked cells to hydrazine. The reasons as to why heat shocking the cells failed to confer a degree of protection to the cells is due to the timing of the second stressor. Initially after hyperthermic treatment there is a decrease in the levels of Hsp70 due to the presence of heat damaged structures. This effectively causes an induction of the stress response but an increase in newly synthesised Hsp70 does not occur for a number of hours. Thus exposure of a cell to a toxin immediately following heat shock results not in an decrease of the compounds toxicity but in a effective increase in toxicity due to the depleted cellular free pool of Hsp70. This initial phenomenon of thermosensitisation is usually demonstrated by utilisation of a step-down dosing protocol where following an initial heat treatment the cells are exposed to a lower hyperthermic treatment (Delpino *et al.* 1992, Schamart *et al.* 1992). Thermosensitisation can also be demonstrated with toxic chemicals such as sodium arsenite or cadmium chloride where administration of concentrations of the chemical which normally fail to induce a stress response actually do so in those cells that have been previously treated with higher

a) Control cells

Time	Dose of hydrazine (mM)									
	0		5		10		15		20	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	74.8	8.2	77.1	3.7	81.2	1.9	77.5	5.7	80.1	4.0
1	77.5	1.1	70.8	5.1	65.4	8.7	71.3	7.0	69.9	6.1
2	70.7	9.7	65.1	9.1	58.6	6.2	49.1	1.7	11.9	3.9
3	68.4	4.2	61.9	7.0	43.7	1.7	0	0	0	0

b) Heat shocked cells

Time	Dose of hydrazine (mM)									
	0		5		10		15		20	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	79.1	10.1	77.5	7.4	76.7	1.1	74.2	4.1	77.0	6.9
1	72.5	6.7	72.3	9.7	73.3	9.1	69.9	4.0	73.7	0.9
2	76.8	2.1	69.8	13.1	64.3	4.1	54.1	2.9	25.4	4.7
3	70.8	11.1	59.5	12.4	30.3	4.9	0	0	0	0

Table 3.1 Effect of prior heat shock on the viability of hepatocyte suspensions following exposure to increasing concentrations of hydrazine. Table a. shows the viabilities from control cells. Table b. shows the viabilities of those cells that were previously exposed to 43°C for 30 mins. Values are expressed as % viability as determined by trypan blue exclusion. n=3.

a) Control cells

Time	Dose of hydrazine (mM)									
	0		5		10		15		20	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	20.7	1.4	24.7	1.8	20.7	4.0	21.2	1.8	21.1	0.5
1	21.1	3.2	30.8	4.1	23.1	2.7	28.4	3.7	23.4	3.1
2	25.4	2.9	29.7	2.8	26.2	3.1	36.4	6.2	42.9	6.4
3	31.1	1.7	31.8	1.9	44.4	6.2	64.7	4.9	60.9	4.2

b) Heat shocked cells

Time	Dose of hydrazine (mM)									
	0		5		10		15		20	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	19.7	2.1	18.4	1.6	22.9	3.1	22.4	0.8	24.1	2.7
1	22.6	0.6	20.7	3.0	23.4	0.9	27.3	4.7	29.9	5.1
2	26.4	1.1	26.6	2.1	32.7	4.8	42.3	5.9	58.3	8.1
3	34.6	0.2	33.3	4.1	54.3	3.2	68.2	10.7	74.5	9.8

Table 3.2 Effect of prior heat shock on LDH leakage from hepatocyte suspensions following exposure to increasing concentrations of hydrazine. Table a. shows the LDH leakage from control cells. Table b. shows the LDH leakage from those cells that were previously exposed to 43°C for 30 minutes. Values are expressed as % of total LDH. n=3.

concentrations of arsenite or cadmium (Ovelgönne *et al.* 1995b, Wiegant *et al.* 1998). The increase in cell sensitivity is thought to be due to a number of reasons, firstly exposure of cells to elevated temperatures is known to cause a drop in ATP levels (Welch 1992) and one of the early events in hydrazine toxicity is a depletion of ATP (Waterfield *et al.* 1997). Hence in those heat shocked cells ATP levels would be at lower levels than in control cells when exposed to hydrazine thereby increasing the susceptibility of the heat shocked cells to hydrazine. In addition to affecting ATP levels heat shock interferes with many biochemical processes and physical structures within the cell (see chapter 1.2), these changes could in theory make a cell immediately more susceptible to chemical exposure. Another theory is that following heat shock the levels of denatured proteins increases and so the stress response is initiated, administration of a toxic compound immediately following heat shock interferes with the process of protein renaturation and hence the signal for induction of the stress response remains. The timing of administration of the second stressor would also appear to be of importance as following exposure to elevated temperatures it takes a number of hours for the stress proteins to be maximally expressed (Blake *et al.* 1990). Therefore exposure of cells to a toxin immediately following heat shock would mean that the induction of stress proteins would not be maximal and so be unable to confer a degree of protection to the cells. The timing of the administration of the second stressor was investigated further in the following study with the compound menadione.

3.3 Effect of transient increases in temperature on the subsequent toxicity of menadione in hepatocyte suspensions.

3.3.1 Aims of the study

Following on from the previous study it was decided to repeat the study with menadione, a compound known to generate oxidative stress intracellularly, to determine whether heat shocking the cells alters in any way the toxicity of this compound in hepatocytes. In this investigation two separate studies were performed, the first one involved exposing the cells to the toxin immediately following heat shock and the other involved heat shocking the cells, allowing them to recover for two hours and then exposing them to the toxin.

This was done in order to determine whether a recovery phase was required in order for the cell to synthesise the heat shock proteins to sufficient levels in order to confer a degree of protection to the cell. The toxin used in these experiments was the redox cycling toxin menadione whose mechanism of action is discussed in depth in chapter 1.9.1.

3.3.2 Methods

Hepatocytes were isolated as described previously in chapter 2.2. The cells were subsequently pre-incubated for 30 minutes as previously, in this study it was decided to incubate the cells at 43°C for 30 minutes and then expose the hepatocytes to increasing doses of menadione. In this case a comparative study was done, one study involved exposing the cells to menadione immediately after heat shock, the other study involved incubating the cells for two hours following heat shock and then exposing to the same concentrations of menadione. Exposure to menadione was, in both cases, for 3 hours and the concentrations used were 0, 50, 100, 150 and 200μM. Samples were again taken at 1, 2 and 3 hours post dose for LDH, GSH, ATP and cell viability measurements as described previously in chapter 2.2.

3.3.3 Results

In those cells exposed to menadione immediately following heat shock measurement of cell viability and LDH leakage over the 3 hours showed that heat shocking the cells conferred no decrease in susceptibility of the cells to menadione when compared to hepatocytes which did not undergo heat shock. Comparison of LDH leakage from heat shocked and control hepatocytes showed that following heat shock the cells showed an increased susceptibility to menadione as LDH leakage from heat shocked cells was significantly higher than from control cells following administration of 100 and 150μM menadione. The viability results also show no protection from menadione toxicity following heat shock but again reinforce the observation of an increased susceptibility to menadione in heat shocked cells as there is a significant difference between the viabilities of control and heat shocked cells following administration of again 100 and 150μM menadione (tables 3.3 and 3.4).

Exposure of the cells to menadione after a two hour recovery period following heat shock

a) Control cells

Time	Dose of menadione (μM)									
	0		50		100		150		200	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	77.7	9.2	80.3	5.6	81.2	12.1	80.7	11.1	82.5	4.8
1	83.8	3.9	83.6	8.9	77.7	3.5	73.7	5.6	68.5	5.8
2	73.5	5.9	81.6	10.8	75.3	6.5	34.7	9.0	17.1	2.0
3	71.7	10.1	75.7	4.0	62.9	19.1	0	0	0	0

b) Heat shocked cells

Time	Dose of menadione (μM)									
	0		50		100		150		200	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	82.8	6.9	82.8	3.8	83.3	6.0	80.1	8.1	79.4	6.5
1	74.4	8.9	65.7	8.9	56.6	8.1	60.9	10.3	51.2	8.2
2	73.8	12.5	53.7	12.9	28.1a	15.2	16.8a	5.6	3.3a	1.9
3	70.3	6.9	51.4	14.1	0a	0	0	0	0	0

Table 3.3 Effect of prior heat shock on the viability of hepatocyte suspensions following exposure to increasing concentrations of menadione. Table a. shows the viabilities of control cells. Table b. shows the viabilities of those cells that were previously exposed to 43°C for 30 minutes. Values are expressed as % viability as determined by trypan blue exclusion. n=3. Letters indicate values significantly different from corresponding control values using the Dunnett's t-test (a $P<0.05$)

a) Control cells

Time	Dose of menadione (μM)									
	0		50		100		150		200	
mins	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	18.4	2.0	17.4	0.5	20.9	3.2	19.9	1.8	20.0	3.0
60	16.3	3.9	18.4	3.9	22.2	1.3	22.5	3.1	22.1	1.2
120	19.2	0.9	17.9	1.2	24.4	0.9	38.3	4.2	46.6	10.2
180	21.8	6.2	20.4	3.2	22.3	4.1	73.1	2.9	73.1	5.4

b) Heat shocked cells

Time	Dose of menadione (μM)									
	0		50		100		150		200	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	22.9	2.1	25.3	1.9	20.1	2.6	19.2	0.5	18.9	2.2
1	27.4	1.0	23.9	2.3	27.5	3.1	25.1	1.8	23.6	0.9
2	27.1	4.6	27.1	4.0	49.6a	8.9	58.6a	6.4	69.7	8.5
3	36.1	9.2	28.8	3.5	71.1a	12.1	70.5	8.1	69.9	6.1

Table 3.4 Effect of prior heat shock on LDH leakage from hepatocyte suspensions following exposure to increasing concentrations of menadione. Table a. shows the LDH leakage from control cells. Table b. Shows the LDH leakage from those cells that were previously exposed to 43°C for 30 minutes. Values are expressed as % of total LDH. n=3. Letters indicate values significantly different from corresponding control values using the Dunnett's t-test (a $P<0.05$)

showed a different profile of sensitivity, in this case heat shock conferred a slight decrease in the susceptibility of the cells to menadione. There was no increase in the hepatocytes susceptibility to menadione as was found in the previous study. Measurement of LDH leakage and ATP levels showed no significant differences between control and heat shocked cells following exposure to menadione. GSH levels were the first parameter to be decreased following administration of menadione. Comparison of GSH levels in heat shocked and control cells showed that following administration of 50μM menadione levels of GSH were significantly higher in heat shocked cells at 1, 2 and 3 hours post dose compared to levels in control cells. However, this difference in GSH levels was not reflected in cell viability with the exception at 3 hours post dose where the heat shocked cells showed significantly higher viabilities when compared to non-heat shocked cells. (tables 3.5, 3.6, 3.7 and 3.8).

3.3.4 Discussion

Following on from the previous study, this study investigated the toxicity of menadione in heat shocked and non-heat shocked hepatocytes. The timing of the second stressor was investigated by comparing the toxicity of menadione in cells immediately following heat shock and in those cells which were allowed a two hour recovery period following heat shock prior to menadione exposure. The compound menadione was chosen in this investigation as its mechanism of action is relatively well understood and is known to induce a stress response in cells due to its oxidative capacity.

When the hepatocytes were exposed to increasing concentrations of menadione immediately following heat shock there was a slight increase in the susceptibility of heat shocked cells to menadione. This was demonstrated by significant difference in the leakage of LDH and viability of heat shocked and control cells following administration of 100 and 150μM menadione. This again re-inforces the results from chapter 3.1, but to a more significant extent, in that the cells appear thermosensitive to the toxicity of menadione immediately following heat shock presumably for the same reasons as discussed in chapter 3.1.4. The results generated when the cells were allowed a recovery period of two hours following heat shock prior to menadione exposure showed a different profile of toxicity. In this case heat shocking the cells appeared to confer a degree of

a) Control cells

Time	Dose of menadione (μM)									
	0		50		100		150		200	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	83.8	7.1	83.6	4.2	80.4	6.0	79.4	8.1	83.1	4.9
1	82.8	9.2	80.2	6.3	57.2	10.7	55.7	6.7	57.9	5.0
2	71.1	3.7	71.9	4.0	52.1	12.6	25.6	1.9	10.6	0.8
3	75.6	11.1	57.9	7.5	48.6	16.8	0	0	0	0

b) Heat shocked cells

Time	Dose of menadione (μM)									
	0		50		100		150		200	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	85.6	12.1	83.3	6.9	84.8	8.2	85.1	11.0	85.1	7.4
1	82.0	9.1	81.7	5.8	73.9	4.6	66.9	7.2	67.4	9.2
2	81.2	6.0	77.4	9.9	64.2	7.4	26.3	4.1	16.2	2.7
3	79.5	9.0	76.1a	8.1	54.6	9.1	1.7	0.1	1.4	0.2

Table 3.5 Effect of prior heat shock on the viability of hepatocyte suspensions following exposure to increasing concentrations of menadione. Table a. shows the viabilities of control cells. Table b. shows the viabilities of those cells that were previously exposed to 43°C for 30 minutes and allowed to recover for 2 hours. Values are expressed as % viability as determined by trypan blue exclusion. n=3. Letters indicate values significantly different from control values using the Dunnett's t-test (a $P<0.05$)

a) Control cells

Time	Dose of menadione (μM)									
	0		50		100		150		200	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	21.9	2.0	27.2	3.0	28.2	3.1	28.2	1.9	23.1	4.1
1	24.5	4.0	28.2	1.8	32.7	1.8	33.0	1.3	28.5	1.9
2	28.4	3.7	29.0	1.5	38.0	2.5	49.7	5.0	50.7	4.0
3	31.9	1.8	33.2	4.1	48.2	5.1	81.3	3.7	70.9	8.1

b) Heat shocked cells.

Time	Dose of menadione (μM)									
	0		50		100		150		200	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	19.3	2.1	21.2	1.9	22.7	2.8	25.1	2.5	27.0	1.4
1	23.0	3.0	21.7	2.0	25.0	1.9	30.3	4.0	33.8	3.7
2	25.0	0.8	24.2	3.9	35.9	4.2	60.9	3.9	68.1	4.1
3	27.9	1.2	25.3	1.6	50.7	8.7	78.7	5.2	80.0	10.2

Table 3.6 Effect of prior heat shock on the leakage of LDH from hepatocyte suspensions following exposure to increasing concentrations of menadione. Table a. shows the LDH leakage from control cells. Table b. shows the LDH leakage from those cells previously exposed to 43°C for 30 minutes and allowed to recover for 2 hours. Values are expressed as % of total LDH. n=3.

a) Control cells

Time	Dose of menadione (μM)									
	0		50		100		150		200	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	14.1	1.6	13.0	1.1	11.9	0.8	14.7	1.2	12.0	2.0
1	15.0	1.4	13.5	1.0	12.2	1.3	12.5	1.6	6.6	0.8
2	13.9	0.6	10.3	1.8	12.5	1.6	0.8	0.2	0.8	0.1
3	13.0	0.5	12.0	1.3	6.8	0.7	0.5	0.1	0.4	0.2

b) Heat shocked cells.

Time	Dose of menadione (μM)									
	0		50		100		150		200	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	16.2	2.0	15.6	0.9	13.2	0.5	13.1	1.9	12.3	1.5
1	13.6	0.8	14.7	1.4	14.5	1.4	10.4	0.8	5.1	0.3
2	14.4	1.2	14.5	0.6	9.8	1.4	0.6	0.1	0.6	0.1
3	10.5	1.1	13.3	0.4	6.9	0.3	0.5	0.2	0.5	0.03

Table 3.7 Effect of prior heat shock on the ATP levels of hepatocyte suspensions following exposure to increasing concentrations of menadione. Table a. shows the ATP levels from control cells. Table b. shows the ATP levels of those cells that were previously exposed to 43°C for 30 minutes and allowed to recover for 2 hours. Values are expressed as nmol ATP/10^6 cells. n=3.

a) Control cells

Time	Dose of menadione (μM)									
	0		50		100		150		200	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	31.0	1.3	34.3	4.2	28.1	1.9	31.1	2.9	39.9	4.8
1	31.6	2.9	14.1	0.8	5.5	1.2	4.5	0.2	6.2	0.9
2	30.0	3.0	15.2	1.9	4.9	0.7	3.8	0.6	4.0	0.3
3	28.7	0.9	13.1	2.2	4.0	0.2	2.4	0.1	2.3	0.2

b) Heat shocked cells

Time	Dose of menadione (μM)									
	0		50		100		150		200	
hrs	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	26.5	3.6	32.1	1.7	31.0	2.9	29.6	3.7	36.2	2.4
1	30.2	1.5	20.8a	2.5	7.6	1.1	3.2	0.6	5.9	0.2
2	27.3	2.6	24.0a	1.6	4.8	0.3	3.0	0.6	4.5	0.7
3	21.3	3.1	18.5a	2.7	5.5	0.7	2.9	0.2	3.4	0.1

Table 3.8 Effect of prior heat shock on the GSH levels of hepatocyte suspensions following exposure to increasing concentrations of menadione. Table a. shows the GSH levels from control cells. Table b. shows the GSH levels of those cells that were previously exposed to 43°C for 30 minutes and allowed to recover for 2 hours. Values are expressed as nmol GSH/10^6 cells. n=3. Letters indicate values significantly different from corresponding control values using the Dunnett's t-test (a $P<0.05$)

protection to the cells following exposure to menadione. This was evident in the viability of the heat shocked cells but particularly in the menadione induced depletion of GSH. Depletion of GSH is pivotal to the mechanism of action of menadione (see chapter 1.9.1) and consequently the first biochemical parameter to be altered in control cells following menadione exposure was GSH levels which were found to be depleted within 60 minutes of exposure to 50μM menadione. However, GSH levels in those cells that were heat shocked were significantly higher than in control cells at all timepoints following administration of 50μM menadione. This maintenance of GSH levels in heat shocked cells is probably the underlying cause of the increased resistance of the cells to menadione. These experiments with menadione show the dual effect of heat shock on the sensitivity of the cell to subsequent chemical exposure. Initially, the cells become thermosensitive presumably for the same reasons as discussed in the previous section and following this the cells experience a period of thermotolerance. The basis of the thermotolerance observed here was that exposure of the cells to elevated temperature caused the induction of the stress response. Consequently, during the following two hour recovery period heat shock proteins were synthesised within the cell to levels that were able to confer a degree of protection when the cells were exposed to menadione.

3.4 Effect of transient increases in temperature on the subsequent toxicity of A23187 in hepatocyte suspensions.

3.4.1 Aim of the study

The main aim of this experiment was to determine whether heat shocking the cells was able to decrease the subsequent toxicity of A23187 in isolated hepatocytes. The mechanism of action of A23187 is discussed in chapter 1.9.3, but briefly, it is thought to involve calcium mediated cell death. A23187 was chosen due to its rapid onset of action which thus allowed a shorter treatment time to be used than with the other compunds used previously. This in turn allowed the use of a longer recovery period following heat shock without encountering the viability problems normally associated with keeping isolated hepatocytes in suspension for many hours. In addition to measuring cell viability parameters, it was decided to measure the levels of two stress proteins Hsp25 and

Hsp72/3 immediately following heat shock and at four hours post-heat shock. Since it was shown in the previous study that those cells that were allowed a recovery period following heat shock demonstrated a slight decrease in susceptibility to the toxicity of menadione it was decided to investigate whether increasing the recovery period actually increased the degree of protection afforded to the cells.

3.4.2 Methods

Hepatocytes were isolated as described in chapter 2.2 and pre-incubated for 30 minutes. Following this the cells to be heat shocked were incubated at 43°C for 30 minutes and the cells were then allowed to recover at 37°C for 4 hours, control cells were incubated at 37°C for the duration of the experiment. The cells were then exposed to increasing concentrations of A23187 for 30 minutes. The concentrations of A23187 used were 0, 1, 3, 4 and 6μM with samples taken at timepoints 0 and 30 minutes post dose for LDH leakage, cell viability, Hsp25 and Hsp72/3 measurement.

3.4.3 Results

Exposure of hepatocytes to A23187 caused a rapid dose dependent decrease in cell viability and subsequent increase in LDH leakage with 6μM A23187 being overtly toxic within 30 minutes of exposure. Measurement of cellular viability showed that heat shocking the cells appeared to show no statistically significant decrease in the toxicity of A23187 when compared to its toxicity in control cells. The viability data were re-inforced by the LDH results which appeared to show no decrease in toxicity of A23187 in heat shocked cells (tables 3.9 and 3.10). Therefore, it appears that heat shocking the hepatocytes appeared to have no significant effect on the toxicity of A23187 in the cells. Measurement of cellular Hsp25 levels showed only a slight induction four hours following incubation of hepatocytes at 43°C for 30 minutes. There was, however, no induction as expected immediately following incubation at 43°C for 30 minutes (figure 3.1). Attempts to measure changes in Hsp72/3 were unsuccessful due to problems with the specificity fo the antibody.

3.4.4 Discussion

This study aimed to investigate whether extension of the recovery time following heat

a) Control cells

Time	Dose of A23187 (μM)									
	0		1		3		4		6	
mins	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	85.8	6.5	78.0	6.6	74.6	5.3	72.9	8.8	73.6	6.7
30	79.7	3.1	68.2	4.8	45.9	10.2	16.3	2.9	1.7	1.1

b) Heat shocked cells

Time	Dose of A23187 (μM)									
	0		1		3		4		6	
mins	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	75.0	11.2	82.4	4.1	82.1	9.5	75.8	5.1	78.3	8.2
30	81.3	6.2	76.1	9.1	64.5	7.2	25.4	3.1	10.0	3.1

Table 3.9 Effect of prior heat shock on the viability of hepatocyte suspensions following exposure to increasing concentrations of A23187. Table a. shows the viabilities from control cells. Table b. shows the viabilities of those cells that were previously exposed to 43°C for 30 minutes and allowed to recover for 2 hours. Values are expressed as % viability as determined by trypan blue exclusion. n=3.

a) Control cells

	Dose of A23187 (μM)									
Time	0		1		3		4		6	
mins	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	24.3	3.1	18.2	2.1	24.3	0.8	19.6	1.6	22.0	2.8
30	21.1	2.3	24.0	1.0	36.8	4.1	68.0	9.2	78.9	5.6

b) Heat shocked cells

	Dose of A23187 (μM)									
Time	0		1		3		4		6	
mins	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	19.8	2.4	20.9	1.6	23.6	1.1	25.2	2.9	18.9	1.2
30	23.5	1.3	21.1	2.8	32.1	1.6	59.6	6.2	72.1	3.5

Table 3.10 Effect of prior heat shock on LDH leakage from hepatocyte suspensions following exposure to increasing concentrations of A23187. Table a. shows the LDH leakage from control cells. Table b. shows the viabilities from those cells that were previously exposed to 43°C for 30 minutes and allowed to recover for 2 hours. Values are expressed as % of total LDH. n=3.

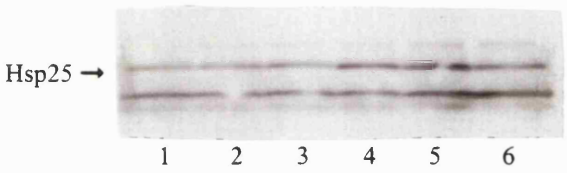


Figure 3.1 Typical Western blot showing the effect of exposure of hepatocyte suspensions to 43°C on the levels of Hsp25. Lanes 1 and 2 are from control cells, lane 3 shows levels of Hsp25 in cells exposed to 43°C for 30 minutes. Lanes 4, 5 and 6 show the level of Hsp25 in hepatocytes exposed to 43°C for 30 minutes and allowed to recover for 2 hours. Solid arrow represents Hsp25.

shocking the cells was able to confer a greater degree of protection to the cells against A23187 toxicity. The basis behind this was that by extending the recovery time the synthesis of heat shock proteins could be maximised and so in theory should increase the degree of protection. Measurement of the stress protein, Hsp25, showed levels were elevated when the hepatocytes were exposed to A23187 but there was no clear protection afforded to those cells with elevated stress protein levels. There was no significant difference in cell viability or LDH leakage between heat shocked or control cells following exposure to A23187. Therefore, the question that has to be asked is: Why does heat shocking the cells confer a slight degree of protection against menadione toxicity but not A23187 toxicity? The reasons for this are unclear, A23187 has been previously shown to inhibit the induction of Hsp70 by heat shock, this is thought to be brought about by a modulatory effect on the phosphorylation of the heat shock transcription factor, HSF-1 (Elia *et al.* 1996). However, for this to occur A23187 has to be present during the initial heat shock as the addition of A23187 post heat shock had no inhibitory effect on Hsp70 levels. Therefore, this is unable to explain the lack of resistance of cells to A23187 following heat shock. One of the theories could be that due to the wide range of deleterious effects that A21387 is known to cause the induction of heat shock proteins may not be able to protect the cells from the wide ranging effects. Changes in intracellular Ca^{2+} levels induced by A23187 cause the perturbation of many cellular processes, a large majority of which are potentially deleterious. These include the induction calcium dependant degradative enzymes including proteases, kinases, phospholipases and nitric oxide synthase as well as several immediate early genes including c-fos, c-jun and c-myc all of which have the potential to cause cell death (Trump and Berezesky 1995). It would seem unlikely, therefore, that the induction of heat shock proteins are able to confer a significant degree of protection against such wide ranging toxic effects. In addition to causing necrotic cell death calcium ionophores have the ability to induce apototic cell death, however, this would not appear to be the case with the cells studied here. Increased levels of Hsp70 have been shown to inhibit apototic cell death (Gabai *et al.* 1996) and since elevated levels of Hsp70 were detected in hepatocytes following heat shock it would follow that A23187-induced apototic cell death would be inhibited as a result. Therefore, it would appear that due to the mechanism of action of A23187 toxicity induction of the stress response in hepatocytes is unable to exert a protective effect against A23187

toxicity.

3.5 Effect of pre-exposure of hepatocyte monolayers to sub-threshold concentrations of hydrazine on the subsequent toxicity of hydrazine

3.5.1 Aims of the study

The initial aim of this study was to determine the toxicity of hydrazine in hepatocyte monolsyers when compared to its toxicity in hepatocyte suspensions used previously. Considering thermotolerance had been observed previously in hepatocyte suspensions it was also decided to investigate whether the phenomenon was observable in hepatocyte monolayers and, if so, to what degree did it occur. Hepatocyte monolayers were used in these investigations as the length of time the cells could be dosed and then allowed to recover could be increased greatly. In this case, instead of investigating the effect of elevated temperatures of cellular sensitivity it was decided to determine whether self-tolerance was evident in the cells. Self-tolerance is the phenomenon where exposure of a cell to low concentrations of a toxin effectively protects it from subsequent expoosure. This was performed by investigating the effect of pre-exposure of hepatocytes to a sub-threshold concentration of hydrazine on the subsequent exposure of the cells to a relatively toxic concentration of hydrazine. Prior to the re-exposure study it was decided to perform a dose ranging study to determine the toxicity of hydrazine in hepatocyte monolayers.

3.5.2 Methods

3.5.2.a Dose ranging study

Hepatocytes were isolated and cultured for 20 hours as described in chapter 2.3 and then exposed to 14mM hydrazine for 6 hours. The cells were then analysed to determine the effect of hydrazine on GSH, ATP levels and LDH leakage. Sampling and biochemical analysis of hepatocyte monolayers was carried out as described in chapter 2.3.5.

3.5.2.b Hydrazine re-exposure study

Hepatocytes were isolated and cultured for 20 hours as described in chapter 2.3 and then

exposed to 0.1mM hydrazine for 4 hours. After 4 hours exposure the media was changed so that the cells were then exposed to 14mM hydrazine for 8 hours. The control groups consisted of cells not exposed to hydrazine at any concentration, cells exposed to only 0.1mM hydrazine from 0- 4 hours only and cells exposed to only 14mM hydrazine from 4-12 hours only. Samples were taken throughout the 12 hour duration of the experiment and analysed for LDH leakage, GSH levels, ATP levels and Hsp25 and Hsp72/3 levels.

3.5.3 Results

3.5.3.a Initial dosing study

Measurement of LDH leakage at six hours post dose showed leakage increased 2-fold following exposure to 14mM hydrazine when compared to leakage from control cells. ATP and GSH levels were decreased in hepatocytes exposed to 14mM hydrazine for six hours (figure 3.2).

3.5.3.b Hydrazine re-exposure study

In control cells not exposed to any concentration of hydrazine levels of GSH and ATP were fairly constant over the 12 hours duration of the experiment. LDH leakage did not change significantly throughout the duration of the 12 hours.

Those cells that were exposed to 0.1mM hydrazine for four hours showed no increase in leakage of LDH over the subsequent 8 hours. GSH levels were slightly, but significantly, decreased following exposure to 0.1mM hydrazine compared to levels in control cells at 4-8 hours but levels recovered to control-like values by 10 hours. ATP levels showed a significant decrease following exposure to 0.1mM hydrazine compared to levels in control cells at 4 and 6 hours but again by 10 hours levels had recovered to control like values.

Exposure of hepatocytes to 14mM hydrazine at 4 hours caused a significant decrease in ATP and GSH levels within 2 hours. Levels recovered slightly over the next 6 hours but still remained significantly decreased compared to levels in control cells. Leakage of LDH was also found to be increased at 8 hours following exposure to 14mM hydrazine with leakage remaining elevated for the duration of the experiment.

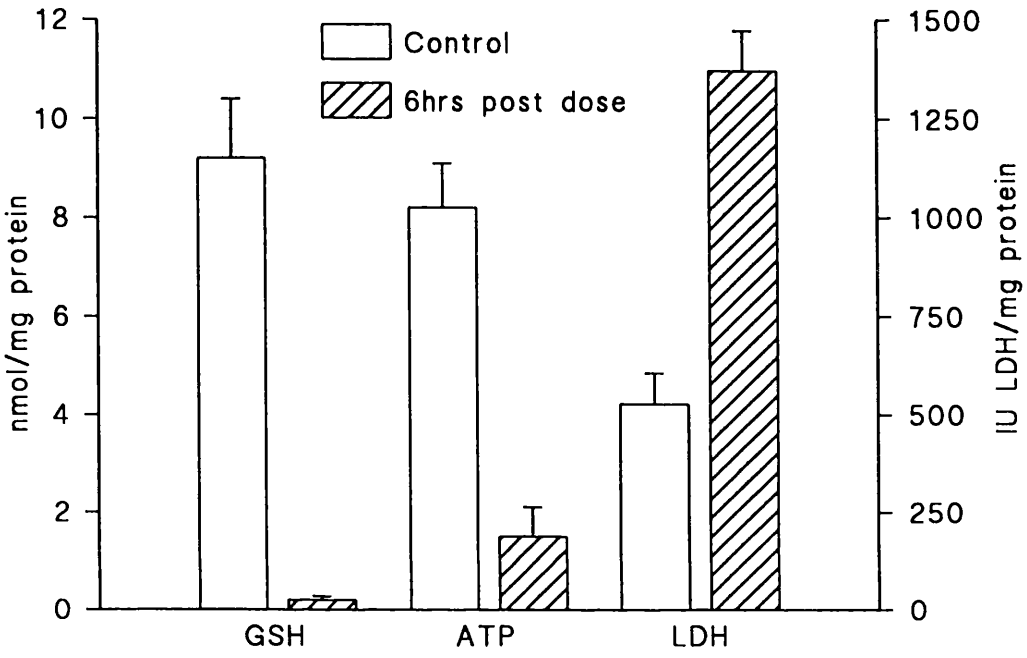


Figure 3.2 Toxicity of hydrazine in hepatocyte monolayers. Hepatocytes were exposed to 14mM hydrazine for 6 hours after which time levels of ATP, GSH and LDH leakage were determined. ATP and GSH values are expressed as nmol/mg protein and LDH leakage is expressed as IU LDH/mg protein. Values shown are the mean of three experiments ± SEM.

The effect of pre-exposure to 0.1mM hydrazine on the toxicity of 14mM hydrazine was investigated. The initial data appeared to show that 14mM hydrazine was toxic to the cells even after pre-exposure to 0.1mM hydrazine. However, at 6 hours levels of ATP and GSH in those cells exposed to both 0.1mM and 14mM hydrazine were significantly higher when compared to levels in those cells that had been exposed to 14mM hydrazine alone. At 6 hours levels of ATP and GSH had fallen to approximately 30% of control in those cells exposed to both 0.1 and 14mM hydrazine. In contrast, levels of ATP and GSH had fallen to approximately 5-10% of control in those cells exposed solely to 14mM hydrazine. However, at all other timepoints there was no significant difference in the levels of ATP or GSH in those cells exposed to 0.1mM and 14mM hydrazine and those cells exposed to 14mM hydrazine alone. There was no significant difference between the leakage of LDH from the two sets of cells at any of the timepoints (figures 3.3, 3.4 and 3.5).

Measurement of Hsp25 levels showed that there was a slight increase in the levels of this stress protein following exposure to 0.1mM hydrazine. This increase was maximal 6-8 hours following exposure to 0.1mM after which time levels began to fall back to control values. However, when the hepatocytes were exposed to both 0.1 and 14mM hydrazine a different pattern emerged. Following exposure to 0.1mM hydrazine levels of Hsp25 were slightly induced, however within 2 hours of exposure to 14mM hydrazine levels had fallen significantly. Within 4 hours levels of Hsp25 had fallen to almost zero and showed no recovery throughout the remaining 4 hours (figure 3.6). The Hsp25 shows two distinct bands here, Hsp25 and an un-identified band at approximately 20kD. This second band may represent alternative isofroms of Hsp25 that are detected due to the lack of specificity of the antibody. The other alternative is that the second band represents a dregradation product of Hsp25 that is detected by the antibody. The same membranes were probed for Hsp72/3, but unfortunately due to problems with the antibodies specificity, measurement of the protein in this study was not possible even though a different batch of antibody was used in this study than was used previously in study 3.4.

3.5.4 Discussion

This study in hepatocyte monolayers was performed to investigate whether the

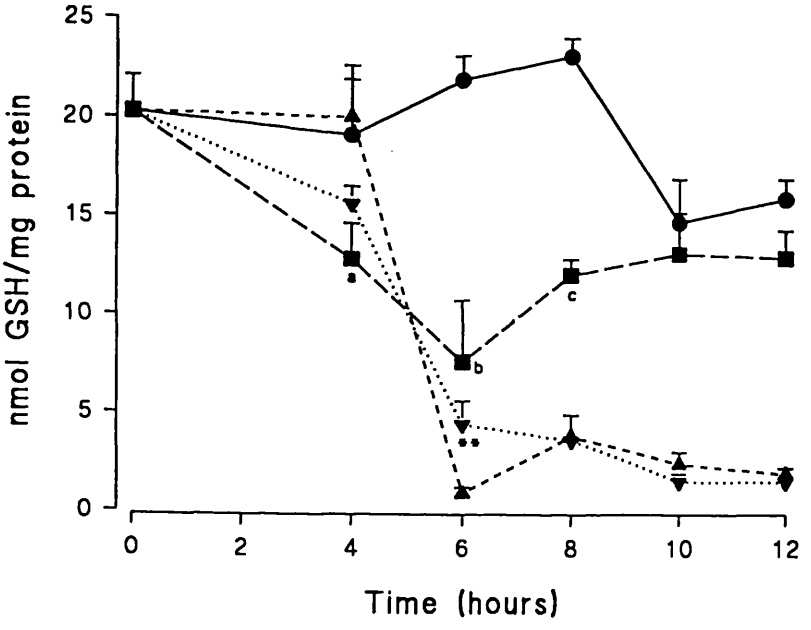


Figure 3.3 Effect of pre-exposure of hepatocyte monolayers to sub-lethal concentrations (0.1mM) of hydrazine on the alterations in GSH levels induced by subsequent exposure to 14mM hydrazine Values are expressed as mean nmol/mg protein ± SEM. n=3. ●-control, ■-cells exposed to 0.1mM hydrazine 0-4hrs, ▲-cells exposed to 14mM hydrazine 4-12hrs, ▼-cells exposed to both 0.1mM hydrazine (0-4hrs) and 14mM hydrazine (4-12hrs). Letters indicate values significantly different from corresponding control values using the Dunnett's t-test (^{a} $p<0.05$, ^{b} $p<0.01$, ^{c} $p<0.001$). Asterix indicate values significantly different from corresponding 14mM values using the Dunnett's t-test (** $p<0.01$).

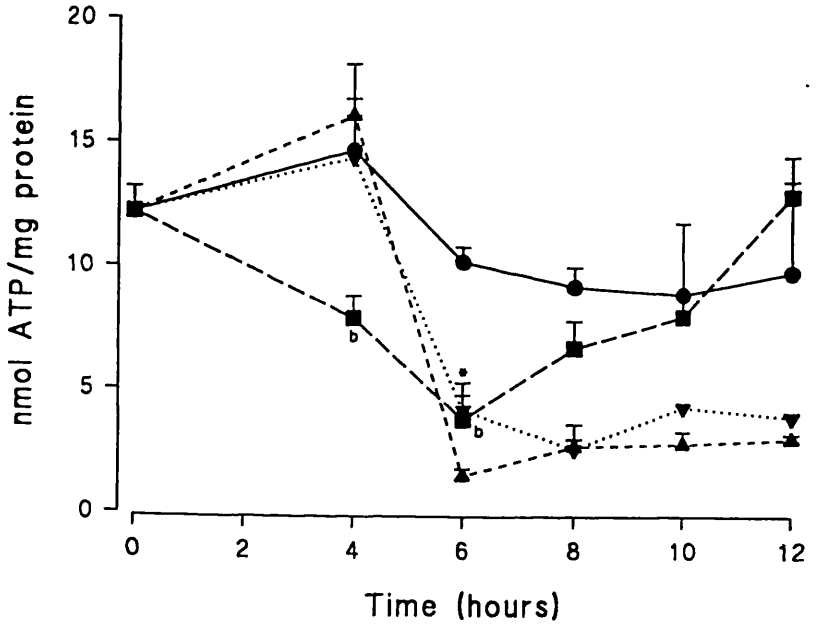


Figure 3.4 Effect of pre-exposure of hepatocyte monolayers to sub-lethal concentrations (0.1mM) of hydrazine on the alterations in ATP levels induced by subsequent exposure to 14mM hydrazine Values are expressed as mean nmol/mg protein ± SEM. n=3. ●-control, ■-cells exposed to 0.1mM hydrazine 0-4hrs, ▲-cells exposed to 14mM hydrazine 4-12hrs, ▼-cells exposed to both 0.1mM hydrazine (0-4hrs) and 14mM hydrazine (4-12hrs). Letters indicate values significantly different from corresponding control values using the Dunnett's t-test (^{b} $p<0.01$). Asterix indicate values significantly different from corresponding 14mM values using the Dunnett's t-test (* $p<0.05$).

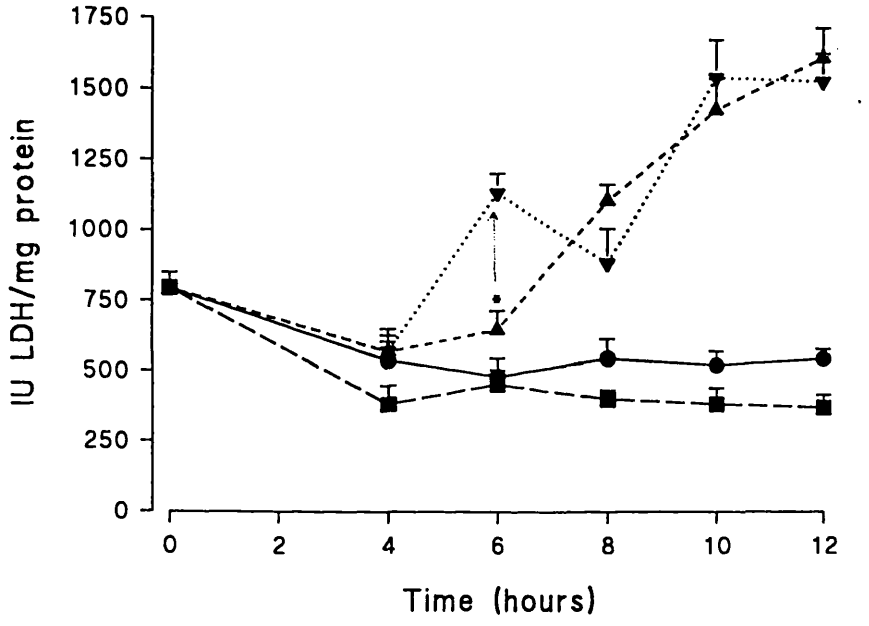


Figure 3.5. Effect of pre-exposure of hepatocyte monolayers to sub-lethal concentrations (0.1mM) of hydrazine on the alterations in LDH leakage induced by subsequent exposure to 14mM hydrazine Values are expressed as mean IU/l/mg protein ± SEM. n=3. ●-control, ■-cells exposed to 0.1mM hydrazine at 0-4hrs, ▲-cells exposed to 14mM hydrazine 4-12hrs, ▼-cells exposed to both 0.1mM hydrazine (0-4hrs) and 14mM hydrazine (4-12hrs). Asterix indicate values significantly different from corresponding 14mM hydrazine values using the Dunnett's t-test (* $p<0.05$)

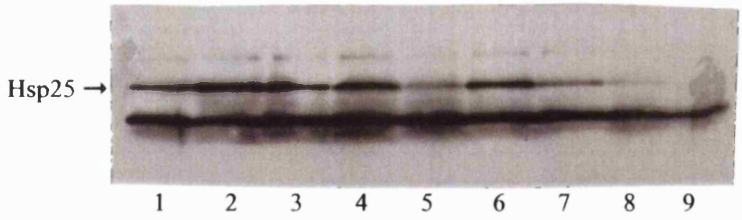


Figure 3.6 Typical Western blot showing the effect of hydrazine on Hsp25 levels in hepatocyte monolayers. Lanes as follows; Lane 1, 0hr; lane 2, 2hr; lane 3, 4hr; lane 4, 6hr; lane 5, 8hr following dosing with 0.1mM hydrazine. Lane 6, 0hr; lane 7, 2hr; lane 8, 4hr, lane 9, 6hr following dosing with both 0.1mM and 14mM hydrazine. Solid arrow represents Hsp25.

phenomenon of self-tolerance was evident in the cells. This was performed by administration of a sub-threshold concentration of hydrazine to the cells prior to exposure to a toxic concentration of the same compound to determine whether the toxicity of the second stressor is altered by administration of the first stressor. Measurement of late toxic events such as LDH leakage showed no difference between the two sets of cells, however there was a significant difference between the levels of ATP and GSH in those cells that had been pre-exposed to the low concentration of hydrazine and those that were only exposed to the high hydrazine concentration. This difference, however, was only noticeable at six hours, at all other timepoints there was no significant different difference between the two sets of cells. In the hepatocytes exposed to the high hydrazine concentration between 4-6 hours, however, prior exposure to 0.1mM hydrazine at 0-4 hours depleted the levels of GSH within the cell. Therefore, the toxicity of 14mM hydrazine would be expected to be accentuated, but this was not observed as the toxicity of 14mM hydrazine was decreased at six hours. The reason as to why tolerance was observed only at six hours is probably due to the toxicity of the second increased concentration of hydrazine. Presumably the four hour exposure to 0.1mM hydrazine initiated the stress response in the hepatocytes leading to elevated levels of stress proteins, this was confirmed by the observation of elevated levels of Hsp25 which were maximal at 6-8 hours. Following subsequent exposure to the high concentration of hydrazine, this elevation of stress proteins would confer a degree of protection to the cells. However, after two hours exposure to the high concentration of hydrazine the toxic effects of this dose would appear to outweigh the protective nature of the stress proteins and no further protection was observed. This hypothesis was re-inforced by the LDH results showing that the increase in LDH following exposure to 14mM hydrazine was time dependent implying that it takes a number of hours for hydrazine to exert its full toxic effects in hepatocyte monolayers. This time dependent toxicity of hydrazine has been shown previously where levels of ATP, GSH, NADH and NADPH were shown to decrease over a 3-4 hour period in isolated rat hepatocytes following exposure to hydrazine (Ghatineh *et al.* 1992). In summary, the exposure of hepatocyte monolayers to low concentrations of hydrazine prevents the deleterious biochemical changes observed with hydrazine toxicity. However, the effects are only observable within a small concentration and time-window. This phenomenon of self-tolerance has been demonstrated previously in

numerous cells however this is the first observation of self-tolerance using the compound hydrazine in cultured rat hepatocytes.

3.6 Conclusions

In conclusion, both thermotolerance and thermosensitivity are observable in hepatocytes. However, the degree of protection afforded to cells following heat shock or chemical exposure against subsequent chemical exposure appears to depend upon the mechanism of action of the compound used. Thermosensitivity is thought to be due to heat-induced biochemical changes within the cell such as alterations in ATP and GSH levels which subsequently enhance the toxicity of certain chemicals. The change from the cell becoming thermosensitive to thermotolerant appears to occur over a number of hours. The reasons for this conversion from a sensitive to a tolerant state is due to a number of reasons including the induction of the stress response, stabilisation of heat-damaged structures and restoration of biochemical processes within the cell. The degree of protection afforded to the cell during a period of thermotolerance would appear to be dependent on the compound used. Exposure of cells to those compounds with relatively specific toxic effects such as menadione appear to show a higher degree of protection when compared to those compounds that exert a wide range of toxic effects such as A23187. One of the other putative mechanisms that may underlie the phenomena of both thermotolerance and thermosensitivity is a change in intracellular GSH levels. Mitchell *et al.* (1983) have shown previously that GSH levels were elevated during heat-induced thermotolerance, however, during heat-induced thermosensitisation levels of GSH were found to be depleted. This was demonstrated in chapter 3.2 where levels of GSH in heat shocked hepatocytes were determined to be significantly higher than in non-heat shocked cells and consequently the toxicity of menadione was decreased in the heat shocked cells. Glutathione participates in a wide number of processes including the detoxification of reactive oxygen species and various heavy metals thereby protecting the cell from toxic chemicals and oxidative damage (Singhal *et al.* 1987, Cotgreave *et al.* 1988). Therefore, changes in intracellular GSH levels would obviously alter the toxicity of those compounds that utilise GSH in their detoxification process. Of all the compounds used in the previous

studies, menadione is the compound with which GSH has been shown to interact directly either by oxidation or conjugation with menadione itself, therefore, as demonstrated here, any changes in GSH levels would alter the toxicity of this compound (Miller *et al.* 1986) The compounds hydrazine and A23187 also both affect GSH levels although this effect is not a fundamental aspect of the compounds toxicity but rather a late toxic event, therefore any alterations in cellular GSH levels would not alter the toxicity of these compounds to the extent observed with menadione. In summary both thermosensitisation and thermotolerance are observable in hepatocytes, the degree and change in cellular sensitivity being dependent upon the nature and timing of the first and second stressor.

Chapter 4.

The use of heat shock proteins as markers of toxicity in hepatocyte monolayers

4.1 Introduction

The measurement of heat shock proteins or the stress response has been postulated for use as a potential marker of toxicity. Hepatocyte monolayers are widely used in toxicity studies but there is no work, to our knowledge, detailing the relevance of heat shock proteins as markers of toxicity in this *in vitro* system. The studies described here investigated whether hepatocyte monolayers were a suitable model for studying the stress response and whether it could be induced by hepatotoxic compounds. The use of the stress response as a marker of toxicity has been postulated for a number of reasons. Firstly, the induction of the response occurs at chemical concentrations below those required for toxicity (Goering *et al.* 1993a, b) and this induction is rapid and easily measurable (Blake *et al.* 1990). In addition to this the stress response is a widely conserved response across a diverse range of species, therefore, extrapolation from one species to another could be done with relative ease (Boorstein *et al.* 1994, Rensing and Maier 1994). The compounds hydrazine hydrate and cadmium chloride were used in this study, hydrazine has been shown previously to be toxic in rat hepatocyte monolayers (Ghatineh and Timbrell 1994) but its effect on the stress response in these cells is unknown. Cadmium is a well known inducer of the stress response *in vitro* and as such was used as a positive control (Goering *et al.* 1993a,b). Using these two hepatotoxic compounds it was hoped to determine whether the induction of heat shock proteins could be used as an early indicator of toxicity. In order to determine the sensitivity of the induction of the stress response levels of heat shock proteins were compared directly to other well established markers of toxicity such as LDH leakage, ATP and GSH levels.

4.2 Effect of hepatocyte cell density on cellular biochemical parameters

4.2.1 Aims of study

Before an extensive study was carried out into the investigation of heat shock proteins as markers of toxicity in cultured hepatocytes it was decided to investigate whether different cell densities during culture had any effect on various cellular biochemical measurements and cell viability. Therefore, the main aim of this preliminary study was to determine the

optimal cell density for hepatocyte culture that would most favour toxicity studies.

4.2.2 Methods

Hepatocytes were isolated as described in chapter 2.3 and diluted with Williams E medium to three different concentrations; 0.5, 1 and $2x10^6$ cells/ml. Aliquots (2ml) of each cell density was added to collagen-coated 33mm culture plates. After two hours the cells were washed with fresh medium to remove any unbound or dead cells and cultured for 16 hours. After 16 hours the plates were removed and sampled for LDH leakage, ATP and GSH levels, as described in chapter 2.3, to determine if differing cell densities affected any of these parameters.

4.2.3 Results and discussion

Analysis of the cells after 16 hours in culture showed that those plates incubated at a cell density $0.5x10^6$ cells/ml showed high levels of ATP and GSH present although the cells did not form a confluent monolayer. In comparison to the cells at higher densities, leakage of LDH was slightly raised at this cell density. Analysis of the cells seeded at a density of $1x10^6$ cells/ml showed that levels of ATP, GSH and leakage of LDH were all decreased when compared to those cells seeded at $0.5x10^6$ cell/ml. However, at this concentration the hepatocytes did appear to form a confluent monolayer within 16 hours. Examination of those cells seeded at a density of $2x10^6$ cells/ml showed that the hepatocytes did not form a confluent monolayer as it appeared the cells were stacked upon one another. In addition to this levels of ATP and GSH were seen to be severely decreased when compared to measurements from the other cell densities (table 4.1). Therefore with the biochemical measurements obtained and visual analysis of the cells it was decided to use a concentration of $0.75x10^6$ cells/ml as the working concentration in the hepatocyte monolayer studies.

4.3 Effect of hydrazine on the stress response in hepatocyte monolayers

4.3.1 Aims of the study

Hepatocyte monolayers are a widely used *in vitro* model in hepatotoxicity studies,

Table 4.1 Effect of hepatocyte cell density on biochemical parameters of viability after 16 hours in culture

Cell density ($x10^6$ cells/ml)	Protein (mg)	GSH (nmol/mg protein)	ATP (nmol/mg protein)	LDH leakage (IU LDH/mg protein)
0.5	0.745 ± 0.052	31.6 ± 4.7	17.3 ± 1.9	1782.1 ± 207.3
1	0.955 ± 0.021	26.4 ± 2.7	9.6 ± 0.7	1404.6 ± 87.5
2	0.441 ± 0.088	10.3 ± 3.1	4.8 ± 0.9	1011.0 ± 129.1

Values are expressed as mean of three experiments ± SEM.

however, there has been little work on the sensitivity of the induction of heat shock proteins as markers of toxicity in these cells when compared to other well established markers of toxicity. This study, therefore, aimed to determine whether hydrazine was able to induce a stress response in hepatocyte monolayers, this was assessed by measurement of the two stress proteins Hsp25 and Hsp72/3 in addition to other biochemical parameters of viability. Comparison of the induction of a stress response and the biochemical changes following hydrazine exposure would hopefully allow determination of the sensitivity of the stress response as a marker of toxicity when compared to other biochemical indicators of toxicity namely LDH leakage, GSH levels, ATP levels and protein synthesis.

4.3.2 Methods

Hepatocytes were isolated and cultured as described in chapter 2.3 and diluted down to a working concentration of 0.75×10^6 cells/ml. Once in culture the cells were incubated for a further 16 hours following the first initial washing prior to exposure to the toxin. The compound that was to be investigated in this study was hydrazine hydrate. The medium was removed and replaced with media containing the concentration of compound to be studied. The concentrations of hydrazine hydrate used were 0, 0.5, 1, 5, 10, 20mM. The hepatocytes were exposed to the compound for two hours after which time the medium was removed, the cells washed twice with 2ml sterile PBS and fresh medium added. There were two plates allocated per hydrazine concentration at each timepoint. Samples were taken for LDH leakage, GSH, ATP measurement, protein synthesis and heat shock protein analysis at 0, 2, 4, 6, 8, 10 hours post dose as descibed previously in chapter 2.3. The heat shock protein samples in this study and in studies 4.3, 4.4 and 4.5 were probed with rabbit polyclonal anti-Hsp25 and mouse monoclonal anti-Hsp72/3 antibodies. Hepatocytes were exposed to the compound between the timepoints 0 and 2 hours.

4.3.3 Results

4.3.3.1 Leakage of LDH

Leakage of intracellular LDH was measured as an indicator of cytotoxicity. In control cells there was no change in leakage of LDH over an 8 hour period. Concentrations of 1, 5, 10 and 20mM hydrazine caused up to a 2-fold increase in LDH leakage after 2 hours exposure but these were found not to be statistically significant. At 4 hours post dose

leakage of LDH returned to control values and there was no further increase throughout the rest of the duration of the experiment (figure 4.1).

4.3.3.2 ATP Levels

ATP levels showed a dose dependent decrease with increasing concentrations of hydrazine (figure 4.2). Control cells maintained their ATP levels relatively constant throughout the entire timecourse of the experiment. Exposure of hepatocytes to 10 and 20mM hydrazine for 2 hours caused significant decreases in ATP levels at 2 and 4 hours post dose respectively and levels remained significantly decreased until 10 hours despite removal of the toxicant from the cells at 2 hours. Levels of ATP in hepatocytes also fell slightly following administration of 0.5, 1 and 5mM hydrazine for 2 hours although this was determined to be not significant.

4.3.3.3 GSH Levels

Increasing concentrations of hydrazine caused a dose dependent decrease in intracellular GSH levels (figure 4.3). Levels were decreased significantly 10 hours following dosing with all concentrations of hydrazine despite removal of the toxicant at 2 hours. GSH levels in control cells remained constant throughout the course of the experiment whereas at all hydrazine concentrations GSH levels were significantly depleted at at least one timepoint during the course of the experiment. Levels were significantly decreased at concentrations of 10 and 20mM hydrazine initially after 2 hours exposure and remained decreased throughout the timecourse of the experiment. There appeared to be no recovery in the levels of GSH at any of the concentrations over the duration of the experiment following exposure to hydrazine.

4.3.3.4 Heat shock protein levels

Densitometric analysis of levels of Hsp25 in hepatocytes showed that in control cells levels remained fairly constant over the timecourse of the experiment. There was a slight but not significant increase in the levels of Hsp25 at 4 and 6 hours post dose compared to control cells following administration of 0.5 and 1mM hydrazine. However, at all other timepoints and concentrations the levels of Hsp25 remained relatively constant with the exception of those cells exposed to 20mM hydrazine. Exposure of hepatocytes to 20mM

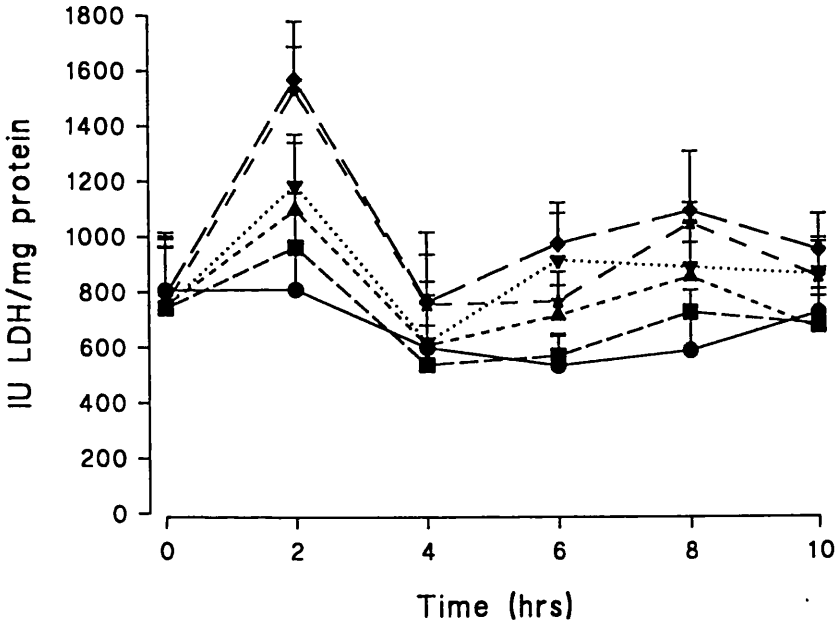


Figure 4.1 Effect of increasing concentrations of hydrazine on LDH leakage from hepatocyte monolayers. Values are expressed as mean IU LDH/mg protein ± SEM. n=3. ●-0mM, ■-0.5mM, ▲-1mM, ▼-5mM, ◆-10mM, ★-20mM hydrazine.

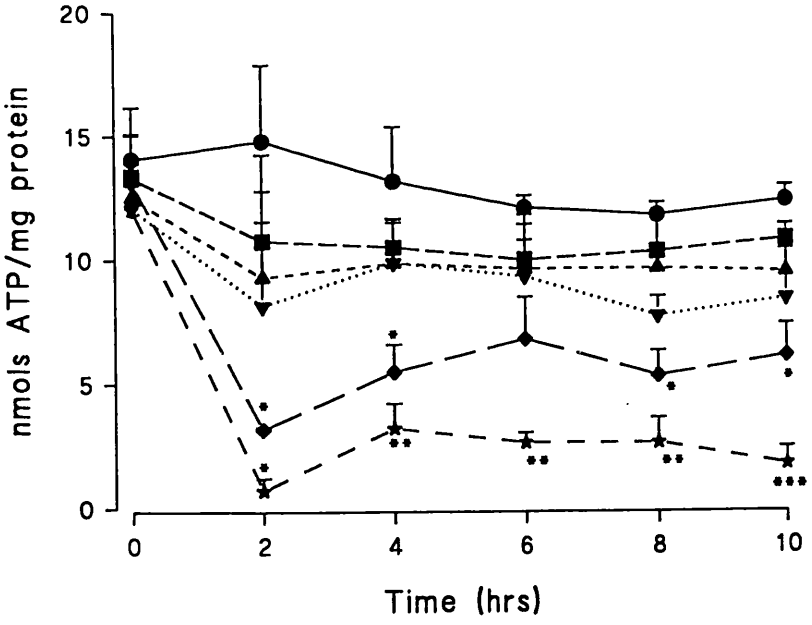


Figure 4.2 Effect of increasing concentrations of hydrazine on ATP levels in hepatocyte monolayers. Values are expressed as mean nmol ATP/mg protein ± SEM. n=3. ●-0mM, ■-0.5mM, ▲-1mM, ▼-5mM, ◆-10mM, ★-20mM hydrazine. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*p<0.05, **p<0.01, ***p<0.001)

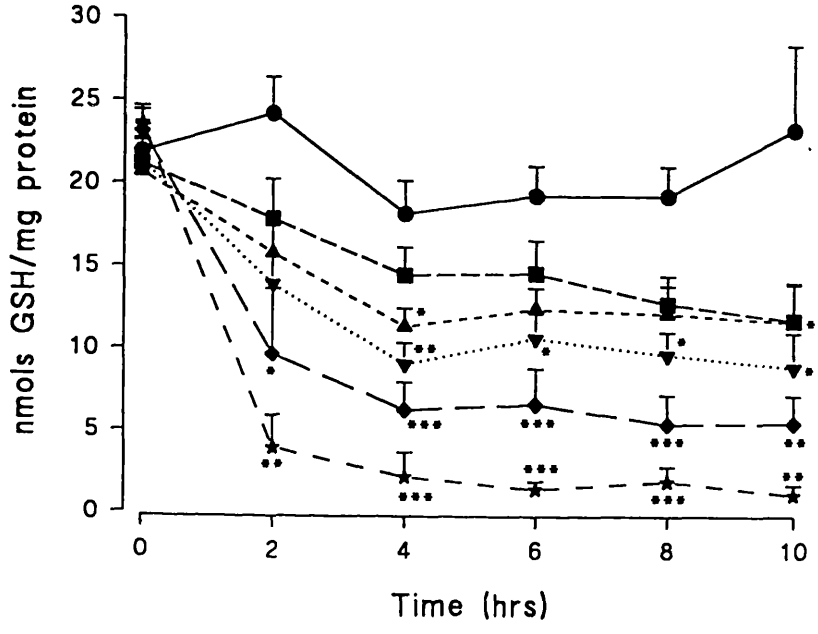


Figure 4.3 Effect of increasing concentrations of hydrazine on GSH levels in hepatocyte monolayers. Values are expressed as mean nmol GSH/mg protein ± SEM. n=3. ●-0mM, ■-0.5mM, ▲-1mM, ▼-5mM, ◆-10mM, ★-20mM hydrazine. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

hydrazine for 2 hours caused the levels of Hsp25 to be decreased by approximately 40% at 4, 6 and 8 and hours post dose although this was not significant. However, interpretation of the data accurately is hampered due to the large variance between replicates (figure 4.4).

Exposure of hepatocytes to hydrazine caused no clear changes in the synthesis of Hsp72/3. Levels of Hsp72/3 did not significantly increase from control values at any concentration of hydrazine at any of the timepoints throughout the timecourse of the experiment. There was a slight trend in the results showing an increase in the level of Hsp72/3 at 4 and 6 hours post dose following exposure to 1 and 5mM hydrazine although this was not significant (figure 4.5).

4.3.3.5 Protein synthesis

Levels of protein synthesis were determined in the hour prior to each timepoint by incubating the cells with ^{3}H-leucine for one hour prior to the timepoint. Exposure of hepatocytes to a range of hydrazine concentrations produced no significant decreases in levels of protein synthesis. There was a 35-45% decrease in the levels of protein synthesis within 2 hours following exposure to 5, 10 and 20mM hydrazine but this was found not to be significant. Another trend that was apparent following dosing with 20mM hydrazine was that levels of protein synthesis had approximately doubled by 4 hours post dose. This rebound increase was sustained until 10 hours post dose when the level of protein synthesis was determined to be significantly higher than levels in control cells (table 4.2).

4.3.4 Discussion

These studies were designed to investigate whether the stress response in hepatocytes monolayers could be used as an early indicator of toxicity. The induction of the stress response was measured by studying the levels of the two stress proteins Hsp25 and Hsp72/3 in addition to measurement of other general biochemical indicators of toxicity. These two heat shock proteins are both stress inducible to varying extents and so theoretically should be induced when a cell is exposed to a hepatotoxin. The effect of hydrazine on heat shock protein levels within hepatocytes has not been studied previously however there are numerous studies investigating the effect of various toxic chemicals on heat shock protein levels *in vitro*. However, the majority of these studies have however

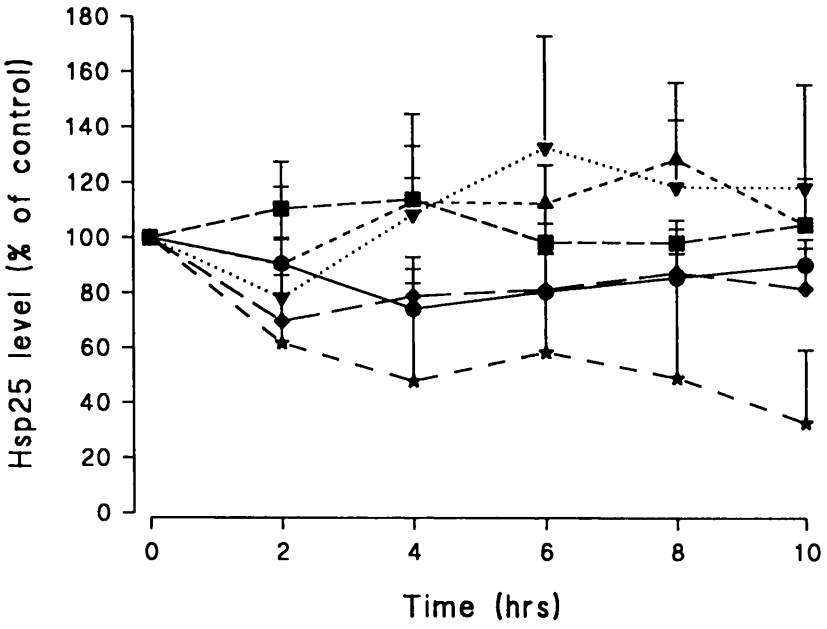


Figure 4.4 Effect of increasing concentrations of hydrazine on Hsp25 levels in hepatocyte monolayers. Values are expressed as mean % of control ± SEM. n=3. ●-0mM, ■-0.5mM, ▲-1mM, ▼-5mM, ◆-10mM, ★-20mM hydrazine.

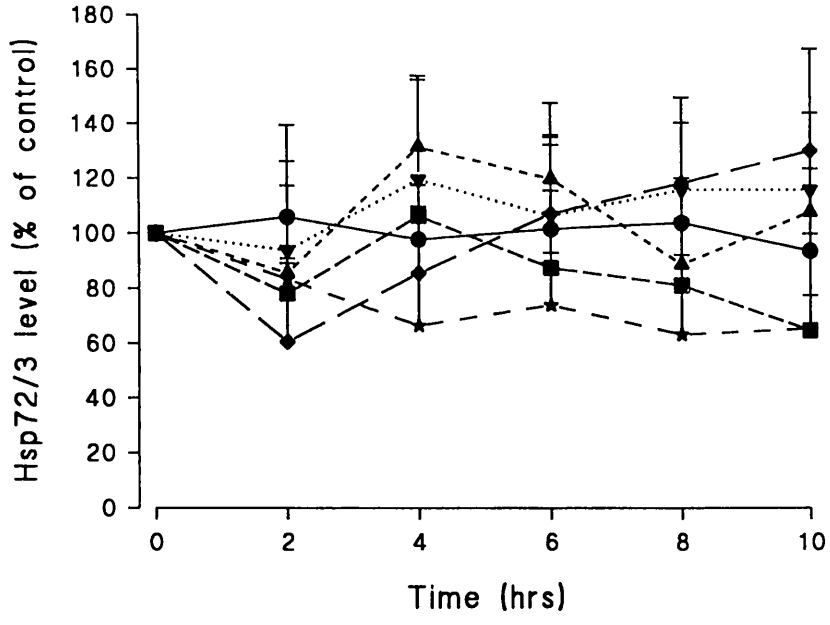


Figure 4.5 Effect of increasing concentrations of hydrazine on Hsp72/3 levels in hepatocyte monolayers. Values are expressed as mean % of control ± SEM. n=3. ●-0mM, ■-0.5mM, ▲-1mM, ▼-5mM, ◆-10mM, ★-20mM hydrazine.

Table 4.2 Effect of increasing concentrations of hydrazine on cellular protein synthesis in hepatocytes monolayers.

	Dose of hydrazine					
Time (hrs)	0mM	0.5mM	1mM	5mM	10mM	20mM
0	349.4 ± 30.4	394.0 ± 11.4	514.6 ± 177.8	368.9 ± 45.5	395.4 ± 96.4	411.7 ± 121.2
2	404.0 ± 56.2	359.3 ± 39.8	382.5 ± 101.0	255.1 ± 105.7	183.7 ± 89.5	266.1 ± 84.3
4	381.7 ± 54.1	442.4 ± 29.3	568.6 ± 117.8	444.9 ± 104.9	449.4 ± 41.3	700.7 ± 62.9
6	317.9 ± 83.2	297.0 ± 82.6	241.0 ± 76.8	259.8 ± 25.6	442.4 ± 188.7	721.7 ± 260.0
8	274.0 ± 26.1	320.9 ± 90.0	320.9 ± 90.1	465.3 ± 38.8	505.6 ± 224.0	1338.0 ± 624.
10	276.6 ± 96.5	271.6 ± 108.5	271.7 ± 108.5	438.4 ± 92.4	273.4 ± 25.4	1279.8 ± 241[c]

Values are expressed as mean DPM/mg protein ± SEM. n=3

Letters indicate values signifcantly different from corresponding control values (10hrs) using the Dunnett's t-test (c $P<0.001$)

been performed in hepatoma cell lines such as Reuber H35 or HepG2 cells (Cairo *et al.* 1989, Ovelgönne *et al.* 1995, Wiegant *et al.* 1995, Salminen *et al.* 1996).

Exposure of hepatocytes for two hours to increasing concentrations of hydrazine caused dose dependent toxicity as observed by changes in various cellular parameters. The biochemical parameter most sensitive to hydrazine exposure was GSH levels which were significantly decreased following exposure to a concentration of 0.5mM hydrazine. Work previously done investigating the effect of hydrazine on GSH levels in hepatocyte suspensions showed that concentrations of 4mM hydrazine were required to significantly deplete GSH levels. Furthermore, concentrations of 20mM hydrazine were required to deplete GSH levels in hepatocyte monolayers following a four hour exposure to the compound (Timbrell 1994). However, the previous experiments studied the effect of continuous exposure of hepatocytes to hydrazine. Therefore, it appears that the data presented here shows that cultured hepatocytes exposed to hydrazine for two hours and then allowed to recover exhibit an increased sensitivity to hydrazine-induced GSH depletion when compared to continuous exposure. Increasing concentrations of hydrazine produced dose dependent decreases in ATP levels and concurrent increases in LDH leakage following administration of 10 and 20mM hydrazine. Those data presented here concerning the effect of hydrazine on LDH leakage and ATP levels are comparable to previous data where it has been shown that a concentration of 16mM hydrazine over a period of four hours was required to deplete ATP levels significantly and increase in LDH leakage from hepatocyte monolayers (Ghatineh and Timbrell 1994). Protein synthesis is one of the most sensitive markers of hydrazine toxicity in hepatocyte suspensions and monolayers (Waterfield *et al.* 1997). However, in the studies concentrations of 10 and 20mM hydrazine decreased protein synthesis by approximately 50% although this was determined to be not significant. A possible explanation for the lack of effect of hydrazine on protein synthesis in the current study may be an insufficient uptake of ^{3}H-leucine into the hepatocytes. Ghatineh and Timbrell (1994) reported previously that levels ^{3}H uptake in control hepatocytes were $20 \pm 2 \times 10^{3}$ dpm/mg protein whereas levels of uptake in control cells in these studies approximated to 0.5×10^{3} dpm/mg protein. The reasons for this apparent discrepancy are unclear, but obviously with such low control uptake values any inhibition of protein synthesis by hydrazine would be hard to measure. The

observation that levels of protein synthesis increased 3-fold at 8 and 10 hours following administration of 20mM hydrazine would imply that the cell is not irreversibly damaged by exposure to 20mM hydrazine for 2 hours. Even though levels of ATP or GSH show no recovery by 10 hours the cell appears able to synthesise new proteins once hydrazine has been removed from the medium.

Hepatocyte monolayers were exposed to a complete dose range of hydrazine ranging from non toxic to acutely toxic as observed by the changes in the indicators of viability such as GSH or ATP levels. However even over this range of concentrations hydrazine did not alter levels of Hsp25 or Hsp72/3 in the hepatocytes. The question, therefore, has to be asked as to why hydrazine did not induce these stress proteins in this study? This is of particular interest when the results from chapter 3.5.3b are included which showed slightly increased levels of Hsp25 following administration of 0.1mM hydrazine albeit the dosing regime was different from the one employed here. Though hydrazine, in this study, does not induce Hsp25 or Hsp72/3 it does not rule out the possibility that hydrazine may selectively induce another family of heat shock proteins not studied here for example Hsp60 or Hsp90. Another possibility is that hydrazine may interfere with the induction of heat shock proteins, one possibility is that hydrazine may interfere with the transcription or translation of heat shock protein mRNA: although there is no evidence for hydrazine having this effect it cannot be ruled out as such. One of the characteristics of a compound that induces a stress response is its ability to damage or denature proteins and this is thought to be one of the triggers of the stress response. Therefore the possibility is that hydrazine does not damage cellular proteins and is therefore unable to induce a stress response, even though this theory is reinforced by the mechanism of action, it does not rule out that damaged proteins may be generated as a result of the toxic biochemical effects of hydrazine and so induce a stress response. The reasons for the apparent lack of induction of Hsp25 and Hsp72/3 are discussed further in chapter 4.4.4 with reference to the results generated in chapter 4.4.

In summary the results presented here show that hydrazine does not cause the induction of the two stress proteins Hsp25 and Hsp72/3 in hepatocyte monolayers. However, it cannot be ruled out that hydrazine is interfering with the induction of the stress response

or is in fact inducing stress proteins other than the ones measured here.

4.4 Effect of cadmium chloride on the stress response in hepatocyte monolayers

4.4.1 Aims of the study

Following on from the previous study it was decided to investigate the effect of cadmium chloride on hepatocyte monolayers, cadmium is a well known inducer of the stress response in well established hepatocyte cell lines such as HepG2 cells (Salminen *et al.* 1996). Considering that hydrazine failed to induce a stress response in hepatocyte monolayers cadmium was used to investigate whether these cells were capable of inducing stress proteins. In addition to measuring levels of stress proteins other biochemical parameters were measured to determine how sensitive heat shock proteins were, if at all, in predicting impending toxicity when compared to the other markers of toxicity.

4.4.2 Methods

Hepatocytes were isolated and cultured as described in chapter 2.3. Once in culture the cells were incubated for a further 16 hours following the initial washing prior to exposure. The compound used in this study was cadmium chloride. The medium was removed and replaced with media containing the concentration of compound to be studied. The concentrations of cadmium chloride used were 0, 5, 10, 30, 100, 300μM. The hepatocytes were exposed to the compound for two hours after which time the medium was removed, the cells washed twice with 2ml sterile PBS and fresh medium added. There were two plates allocated per cadmium concentration at each timepoint. Samples were taken for biochemical analysis at 0, 2, 4, 6, 8, 10 hours post dose and analysed for LDH leakage, ATP, GSH levels, Hsp25, Hsp72/3 and protein synthesis. Hepatocytes were exposed to cadmium chloride between the timepoints 0 and 2 hours.

4.4.3 Results

4.4.3.1 Leakage of LDH

Leakage of LDH from control cells and from those dosed with 5 and 10μM $CdCl_2$ did not change over the time course of the experiment. The first significant increase in LDH

leakage was observed 10 hours following administration of 30μM $CdCl_2$ (figure 4.6). Exposure of hepatocytes to concentrations of 100μM and 300μM $CdCl_2$ for 2 hours produced significant increases in leakage of LDH after exposure was terminated. Concentrations of 5 and 10μM $CdCl_2$ caused no change in LDH leakage throughout the duration of the experiment.

4.4.3.2 ATP levels

Increasing concentrations of $CdCl_2$ caused a dose dependent decrease in ATP levels. Control levels of ATP fell by approximately 25% over the timecourse of the experiment. There was no decrease in ATP levels in those cell dosed with 5μM $CdCl_2$, however levels of ATP fell gradually over 10 hours following dosing with 10 and 30μM $CdCl_2$ with levels significantly decreased 2 hours following exposure to 100 and 300μM $CdCl_2$ (figure 4.7). There was no recovery in ATP levels observed following removal of $CdCl_2$ from the cells.

4.4.3.3 GSH levels

Levels of GSH in cells exposed for two hours to 5 and 10μM $CdCl_2$ remained constant during the experiment as did the levels in control cells (figure 4.8). Concentrations of 30, 100 and 300μM produced dose and time dependent decreases in GSH levels. GSH levels were decreased significantly 6 hours post dose following administration of 30μM $CdCl_2$. Concentrations of 100 and 300μM $CdCl_2$ produced significant decreases in GSH levels at 6 and 2 hours respectively. There was no recovery in the levels of depleted GSH at these concentrations.

4.4.3.4 Heat shock protein levels

$CdCl_2$ did not significantly increase levels of Hsp25 at any timepoint or at any concentration when compared to control cells. Indeed concentrations of 30, 100 and 300μM $CdCl_2$ caused a rapid decline in the levels of Hsp25 within the cells. Levels of Hsp25 had already fallen to zero following exposure to 300μM $CdCl_2$ for two hours (figure 4.9).

Measurement of Hsp72/3 levels following dosing with $CdCl_2$ appeared to show a dual effect depending on the dose used (figure 4.10). Following administration of 5μM $CdCl_2$ Hsp72/3 levels were significantly increased from control values at 4 hours and remained

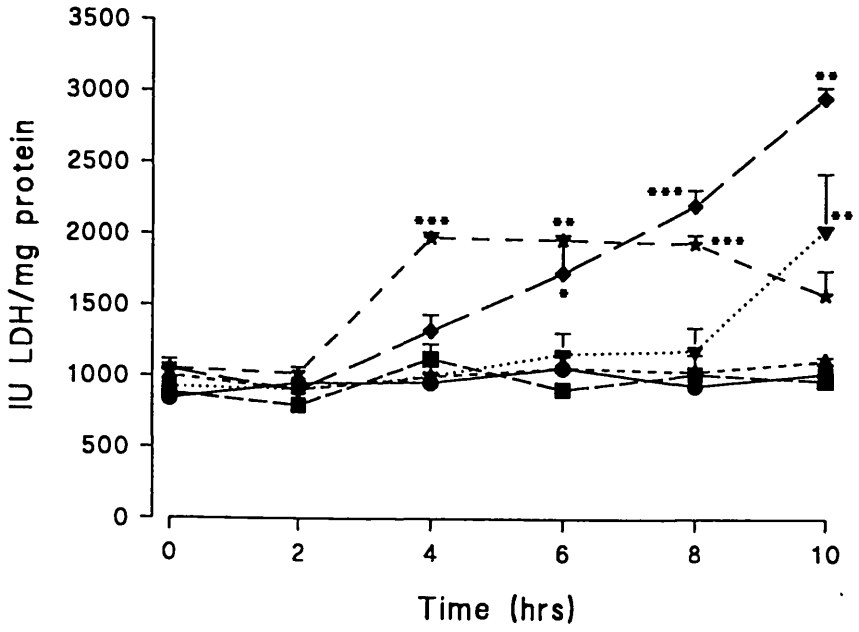


Figure 4.6 Effect of increasing concentrations of cadmium chloride on LDH leakage from hepatocyte monolayers. Values are expressed as mean IU LDH/mg protein ± SEM. n=3. ●-0μM, ■-5μM, ▲-10μM, ▼-30μM, ◆-100μM, ★-300μM $CdCl_2$. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*p<0.05, **p<0.01, ***p<0.001)

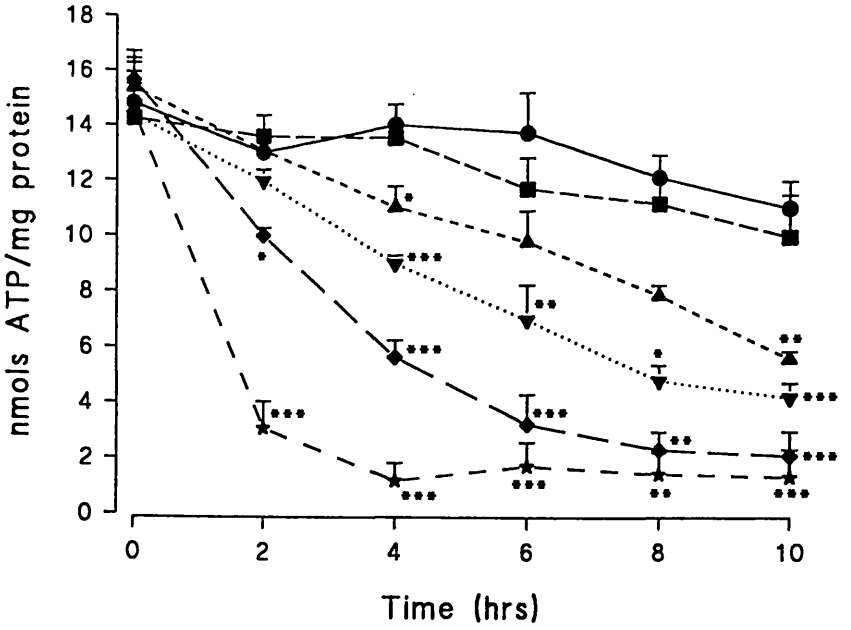


Figure 4.7 Effect of increasing concentrations of cadmium chloride on ATP levels in hepatocyte monolayers. Values are expressed as mean nmol ATP/mg protein ± SEM. n=3. ●-0μM, ■-5μM, ▲-10μM, ▼-30μM, ◆-100μM, ★-300μM $CdCl_2$. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*p<0.05, **p<0.01, ***p<0.001)

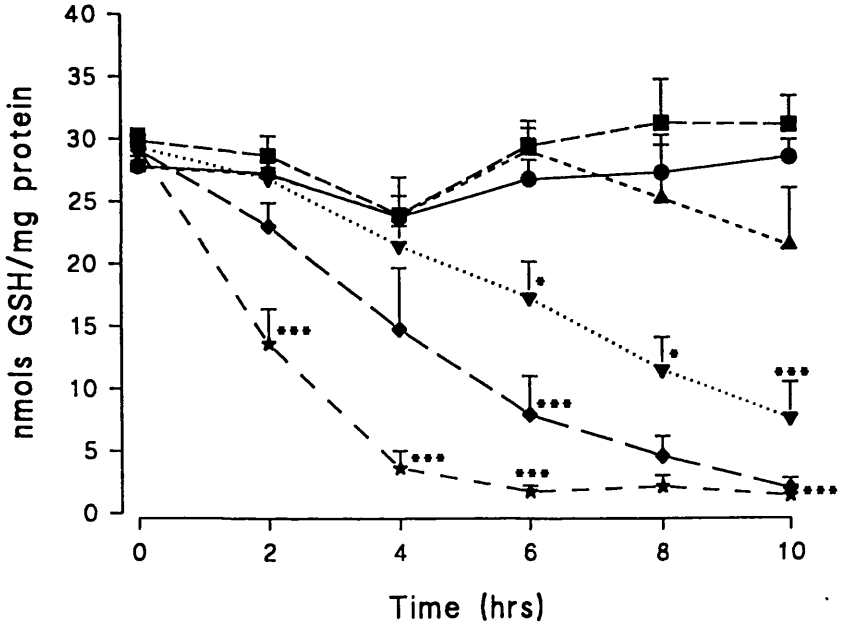


Figure 4.8 Effect of increasing concentrations of cadmium chloride on GSH levels in hepatocyte monolayers. Values are expressed as mean nmol GSH/mg protein ± SEM. n=3. ●-0μM, ■-5μM, ▲-10μM, ▼-30μM, ◆-100μM, ★-300μM $CdCl_2$. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*p<0.05, **p<0.01, ***p<0.001)

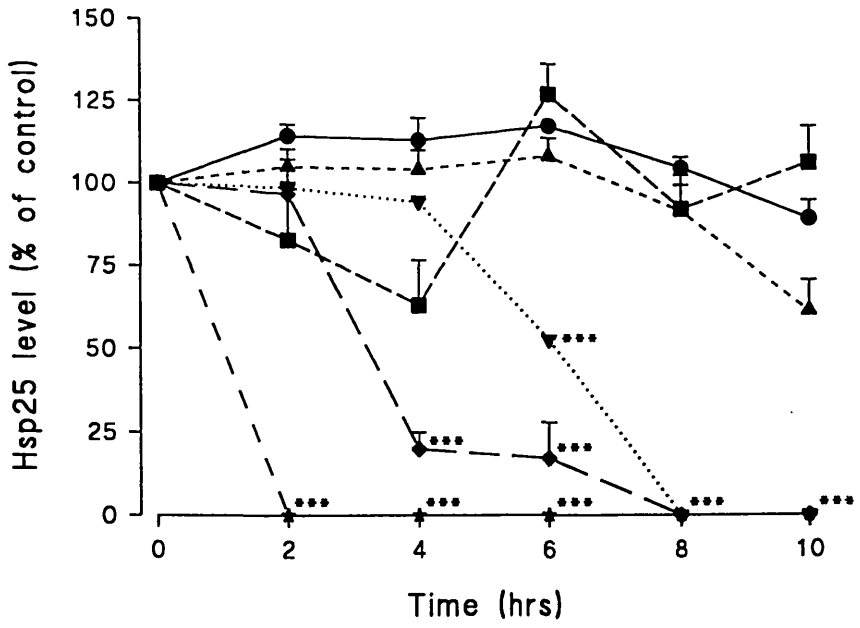


Figure 4.9 Effect of increasing concentrations of cadmium chloride on Hsp25 levels in hepatocyte monolayers. Values are expressed as mean % of control ± SEM. n=3. ●-0μM, ■-5μM, ▲-10μM, ▼-30μM, ◆-100μM, ★-300μM $CdCl_2$. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*p<0.05, **p<0.01, ***p<0.001)

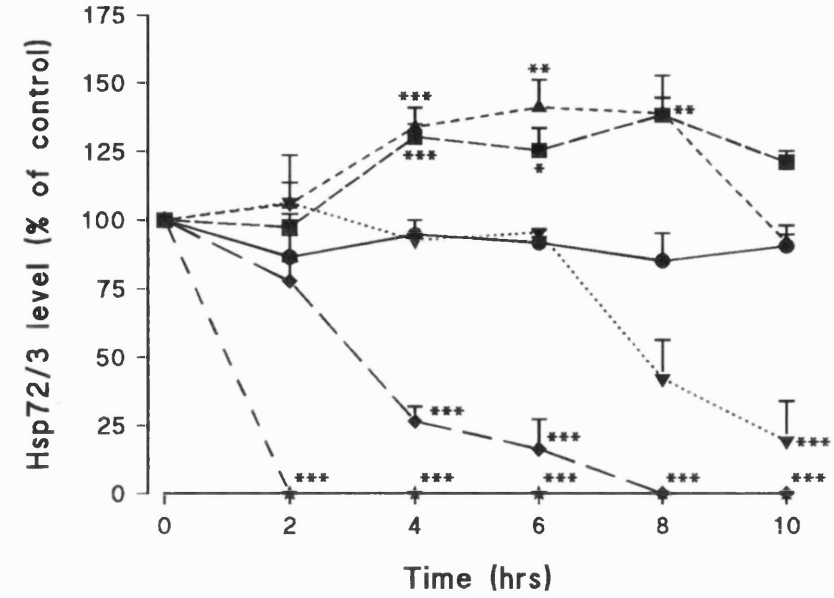
significantly increased until 8 hours post dose. Hsp72/3 levels were also significantly increased at 4 hours following dosing with 10μM $CdCl_2$ and remained significantly increased at 6 and 8 hours. However, at 8 hours levels had approximately returned to control values. Levels of Hsp72/3 remained relatively unchanged following dosing with 30μM $CdCl_2$ until 8 hours when levels decreased rapidly and significantly to approximately 20% of control value at 10 hours. Exposure to 100 and 300μM $CdCl_2$ caused a rapid decrease in levels of Hsp72/3 to zero within 8 hours

4.4.3.5 Protein synthesis

As in chapter 4.3.3.5 protein synthesis was determined in the cells in the one hour prior to each timepoint. Exposure of primary hepatocytes to cadmium chloride caused a significant decrease in levels of protein synthesis following administration of 10 and 30μM $CdCl_2$ 4 hours post dose. Levels of protein synthesis were also found to be decreased 2 hours following administration of 100 and 300μM $CdCl_2$ although this was found to be not significant. There was no change in protein synthesis following dosing with 5μM $CdCl_2$ (table 4.3).

4.4.4 Discussion

Cadmium chloride produced dose dependent toxicity in hepatocytes as indicated by changes in the cellular parameters. The initial changes within the cell following administration of $CdCl_2$ was a significant increase in the levels of Hsp72/3 after the cells were dosed with 5μM $CdCl_2$. Levels of Hsp72/3 were also significantly increased at 10μM however by this concentration levels of ATP had already begun to fall significantly. Following administration of 30μM $CdCl_2$ there were obvious signs of toxicity in the cells as indicated by the large decreases in ATP, GSH and Hsp25 levels. Therefore, it would appear here that changes in Hsp72/3 levels were the most sensitive indicator of toxicity. This is in contrast to Hsp25 levels which were not significantly increased by any $CdCl_2$ concentrations. Previous work detailing the effect of $CdCl_2$ on heat shock protein levels in Reuber H35 hepatoma cells showed that concentrations up to 30μM $CdCl_2$ for two hours failed to increase the synthesis of Hsp68, Hsp70, Hsp84 or Hsp100 (Ovelgönne et al., 1995a). Additionally, concentrations over and above 50μM $CdCl_2$ were only able to induce a stress response in Reuber H35 cells as measured by increases in two heat shock



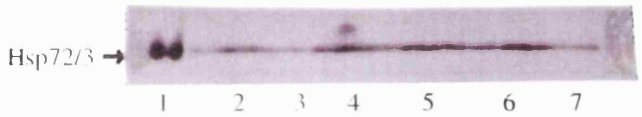


Figure 4.10 Effect of increasing concentrations of cadmium chloride on Hsp72/3 levels in hepatocyte monolayers. Values are expressed as mean % of control ± SEM. n=3. ●-0μM, ■-5μM, ▲-10μM, ▼-30μM, ◆-100μM, ★-300μM $CdCl_2$. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*p<0.05, **p<0.01, ***p<0.001.) Bottom panel shows a typical Western blot generated when hepatocytes were exposed to 10μM $CdCl_2$ for 2 hours and then allowed to recover. Lanes as follows; Lane 1, Hsp72/3 standard; lane 2, 0hr; lane 3, 2hr; lane 4, 4hr; lane 5, 6hr; lane 6, 8hr; lane 7, 10hr post dose. Solid arrow represents Hsp72/3.

Table 4.3 Effect of increasing concentrations of cadmium chloride on cellular protein synthesis in hepatocytes monolayers.

	Dose of cadmium chloride					
Time (hrs)	0μM	5μM	10μM	30μM	100μM	300μM
0	228.1 ± 29.1	379.9 ± 79.4	407.9 ± 41.7	469.8 ± 18.2	326.5 ± 18.2	366.8 ± 44.7
2	251.8 ± 38.2	259.6 ± 42.8	262.1 ± 63.0	248.2 ± 23.4	182.3 ± 32.2	187.7 ± 58.9
4	344.9 ± 61.2	292.4 ± 21.0	149.5 ± 17[b]	203.3 ±56.4[a]	282.8 ± 13.3	243.4 ± 38.0
6	465.2 ± 58.6	253.1 ± 85.9	169.3 ± 30.2	199.3 ± 43.6	337.7 ± 123.	315.0 ± 140.
8	322.1 ± 28.6	172.1 ± 23.9	249.8 ± 35.4	304.9 ± 15.4	371.6 ± 68.7	263.7 ± 77.9
10	395.5 ± 12.6	362.6 ± 22.5	417.3 ± 71.2	398.4 ± 67.2	400.3 ± 40.1	455.2 ± 56.5

Values are expressed as mean DPM/mg protein ± SEM. n=3

Letters indicate values signifcantly different from corresponding control values (10hrs) using the Dunnett's t-test (a $P<0.05$, b $P<0.01$)

proteins Hsp68 and Hsp84 (Ovelgönne *et al.* 1995). It would, therefore, appear here that the induction of the stress response in hepatocyte monolayers following exposure to $CdCl_2$ occurs at much lower chemical concentrations than observed in H35 hepatoma cells. In hepatocyte monolayers increasing concentrations of $CdCl_2$ decreases both the levels of Hsp25 and Hsp72/3 within the cells, administration of 300μM $CdCl_2$ to these cells caused the levels of these two stress proteins to fall to zero within two hours of exposure. This is in contrast to the effect of 300μM $CdCl_2$ in Reuber H35 cells which caused a significant elevation of Hsp70 levels as well as Hsp68, Hsp84 and Hsp100 following a two hour exposure (Ovelgönne *et al.* 1995a) again highlighting the difference in the sensitivity of the stress response in the two different cell types. The reason for the difference in the sensitivity of the stress response in cell lines when compared to hepatocyte monolayers may lie in the relative toxicity of cadmium in the two cell types. A review of the literature concerning the toxicity of cadmium in hepatoma H35 and HepG2 cells (Ovelgönne *et al.* 1995, Ovelgönne *et al.* 1995a, Wiegant *et al.* 1995, Salminen *et al.* 1996) appears to show that the toxicity of cadmium in these cell lines is markedly less than in hepatocyte monolayers. One explaination for this difference could be due to differences in cadmium uptake in the two hepatocyte models. Concentrations of approximately 100μM $CdCl_2$ appears to be the threshold for toxicity in H35 cells as demonstrated by minor changes in protein synthesis. In hepatocyte monolayers this concentration was demonstrated to be overtly toxic to these cells. It would, therefore, follow that considering the threshold for cadmium toxicity is higher in cell lines the accumulation of damaged proteins, a known effect of cadmium exposure, would occur at higher chemical concentrations than in hepatocyte monolayers. Thus the initiation of a stress response would occur at higher cadmium concentrations in HepG2 or H35 cells than in hepatocyte monolayers.

The apparent selective induction of Hsp72/3 by $CdCl_2$ and not hydrazine remains unclear, however one theory is that the mechanism of action of the compound may determine its ability to induce heat shock proteins. With respect to Hsp72/3 its induction and levels are closely related to ATP levels within the cells. Following heat shock or chemical exposure Hsp72/3 binds to denatured or damaged proteins and aids in the refolding of these damaged proteins. Once refolded Hsp72/3 dissociates from these proteins to target other damaged protein. This dissociation, however, requires the hydrolysis of ATP. If ATP

levels are depleted Hsp72/3 will be unable to disassociate from the denatured proteins and will, therefore, be unable to target other damaged proteins which may be present, therefore the intracellular load of damaged proteins will increase again putting demand on the intracellular levels of any free Hsp72/3 still present within the cell (Abravaya *et al.* 1992, Martinus *et al.* 1995). Furthermore, a decrease in ATP in the cell will in itself cause an increase in denatured proteins within that cell thereby further increasing the demand on the already depleted levels of free Hsp72/3. The mechanism of action of cadmium is relatively well understood (see chapter 1.9.4). Although the exact mechanism of hydrazine toxicity is unknown it is thought that ATP depletion (Preece *et al.*, 1990) or inhibition of protein synthesis (Ghatineh and Timbrell 1994) underlie the toxicity of hydrazine. The previous study showed that following administration of the lowest hydrazine concentrations (0.5 and 1mM), levels of ATP had fallen to 73% and 63% of the control values respectively within two hours of exposure. This is in contrast to ATP levels in those cells exposed to the lowest concentrations of $CdCl_2$ (5 and 10μM) where two hours after exposure ATP levels were unchanged from control cells. Two hours after exposure these concentrations of $CdCl_2$ (5 and 10μM) induced Hsp72/3 but these concentrations of hydrazine (0.5 and 1mM) had no effect on the levels of Hsp72/3. This may in part provide explanation as to why hydrazine failed to induce Hsp72/3 whereas $CdCl_2$ did. However at later timepoints following exposure to $CdCl_2$ levels of Hsp72/3 remained induced when ATP levels were decreased by 62% of control levels. Therefore, this may indicate that the ATP levels within a cell immediately after an initial insult are most important in determining the level of Hsp72/3 induction.

One of the characteristics of a stress-inducing compound is its ability of a compound to cause denaturation or damage to cellular proteins (Neuhaus-Steinmetz and Rensing 1997). The term proteotoxic has been coined to describe agents that cause damage to proteins (Hightower 1991). One of the fundamental aspects of cadmium toxicity is its ability to interact with protein sulphydryls (Li *et al.* 1994) and its ability to generate reactive oxygen species (Funakoshi *et al.* 1997). Both effects have the ability to damage intracellular proteins and so induce a stress response within the cell. However, even though the exact mechanism of toxicity of hydrazine is not completely understood it is not thought to interact directly with and damage cellular proteins. Therefore, hydrazine would at first

sight fall into the group of compounds that fail to elicit a stress response when administered to cells, i.e. it not proteotoxic, but it is known to have effects on cellular protein syntheis. However, one effect of hydrazine that is noticeable is an early depletion of ATP within the cell and an inhibition of protein synthesis. The effects, particularly the depletion of ATP, could cause an increase in the intracellular load of denatured proteins and so in theory should cause the induction of a stress response. However, no induction of Hsp25 or Hsp72/3 was observed following administration of a wide range of hydrazine concentrations.

The demonstration that $CdCl_2$ induces Hsp72/3 but not Hsp25 highlights again the possibility of selective induction of specific groups of heat shock proteins by a certain compound. It may, therefore, be feasible to utilise heat shock proteins as markers of toxicity, but as demonstrated clearly here the major problem is that not all compounds induce similar heat shock proteins. This study has shown that $CdCl_2$ induces Hsp72/3 at chemical concentrations where no other biochemical parameter measured here is altered. However $CdCl_2$ did not induce Hsp25 even at those concentrations that caused marked toxicity. The mechanism behind stressor-specific activation remains to be completely understood. In these studies cadmium induces the synthesis of Hsp72/3, this induction requires the binding of the heat shock transcription factor (HSF) to the nuclear heat shock element (HSE). This interaction between HSF and the HSE is essential to the induction of all stress proteins. However in this case the binding of HSF to the HSE results in the selective induction of Hsp72/3 but not Hsp25 therefore there must be an additional level of control in the induction of individual stress proteins. Following exposure to cadmium, there is a strong correlation between phosphorylation of and activation of HSF, however phosphorylation of the HSF activation domain is not an essential pre-requisite of HSF activation (Newton *et al.* 1996). Thus exposure of a cell to stressors initiates the binding of HSF to the HSE but induction of specific heat shock proteins may be altered at the level of phosphorylation thereby governing which heat shock proteins genes are transcribed and whether this transcriptional control is positive or negative regulated. The possibility that additional cofactors binding and interacting with the HSE's may modulate the stress response of a cell cannot be excluded. There are so far two HSE's known, i.e. HSE1 and HSE2, differences in the effects of phosphorylation of transcription factors and

binding sites for cofactors may be of importance in the individual transcription of heat shock proteins. Further work is still required to clarify the exact molecular mechanisms behind the individual induction of heat shock proteins.

In summary, the induction of the heat shock protein Hsp72/3 is the first response of hepatocyte monolayers following exposure to cadmium. Thus from these results, measurement of the stress response may be a useful parameter for use as a marker to predict the impending toxicity of certain chemicals. Care has to be taken, however, when using the stress response as a marker of toxicity as clearly demonstrated here certain compounds have the ability to induce specific individual heat shock proteins. With consideration that the levels of Hsp72/3 were elevated with the lowest concentration of cadmium used it was decided to try and determine at what chemical concentration cadmium caused an initial induction of Hsp72/3.

4.5 Effect of low concentrations of cadmium chloride on the stress response in hepatocyte monolayers

4.5.1 Aim of the study

The previous study demonstrated that concentrations of 5 and 10μM $CdCl_2$ significantly increased levels of Hsp72/3 at 4 to 8 hours post dose. Concentrations above 10μM $CdCl_2$ failed to induce Hsp72/3 and at these higher concentrations the hepatocytes showed obvious signs of toxicity. This study was, therefore, designed to determine whether lower concentrations of $CdCl_2$ were able to induce Hsp72/3 in hepatocyte monolayers.

4.5.2 Methods

Hepatocytes were isolated and cultured as described in chapter 2.3. In culture the cells were incubated for a further 16 hours following the initial washing prior to exposure. The medium was removed and replaced with media containing cadmium chloride at concentrations of 0, 0.1, 1 or 2.5μM. The hepatocytes were exposed to the compound for 2 hours after which time the medium was removed, the cells washed twice with 2ml sterile PBS and fresh medium added. There were two plates allocated per cadmium

concentration at each time-point. Samples were taken for LDH leakage, protein synthesis and Hsp72/3 determination at 0, 2, 4, 6, 8, 10 hours post dose. Hepatocytes were exposed to the compounds between the time-points 0 and 2 hours.

4.5.3 Results

4.5.3.1 LDH leakage

Administration of $CdCl_2$ at concentrations of 0.1, 1 and 2.5μM to hepatocytes did not alter the leakage of LDH when compared to control cells at any time-points during the course of the experiment (table 4.4).

4.5.3.2 Hsp72/3 level

Low concentrations of $CdCl_2$ were used in attempt to determine a threshold concentration of $CdCl_2$ that would cause induction of Hsp72/3. However levels of Hsp72/3 were unchanged over an 8 hour period following exposure to 0.1, 1 and 2.5μM $CdCl_2$ for two hours when compared with levels in control cells (table 4.5).

4.5.3.3 Protein synthesis

No changes in the levels of protein synthesis were observed in the hepatocytes following dosing with any of the concentrations of cadmium chloride used at any timepoint (table 4.6).

4.5.4 Discussion

In consideration of the results initially with $CdCl_2$, in particular the induction of Hsp72/3 following administration of concentrations of 5 and 10μM $CdCl_2$, it was decided to use lower concentrations of $CdCl_2$. Only Hsp72/3 levels, protein synthesis and LDH leakage were measured as all other parameters would be unaffected by such low concentrations of $CdCl_2$. The rationale behind this was to determine the threshold concentration of $CdCl_2$ which would cause induction of Hsp72/3. However, levels of Hsp72/3 were unaffected by any of the concentrations of $CdCl_2$ used here. Therefore it would appear that there is a very narrow concentration window for Hsp72/3 induction: a concentration of 5μM $CdCl_2$ causes significant induction whereas 2.5μM does not affect Hsp72/3 levels. A explanation for this apparent narrow window of induction may be that the levels of

Table 4.4 Effect of increasing concentrations of cadmium chloride on LDH leakage from hepatocyte monolayers

	Dose of cadmium chloride			
Time (hrs)	0μM	0.1μM	1μM	2.5μM
0	731.3 ± 88.3	629.0 ± 48.5	811.6 ± 170.3	742.3 ± 75.6
2	727.0 ± 134.5	840.0 ± 11.5	598.6 ± 146.0	813.3 ± 87.2
4	865.6 ± 94.7	675.6 ± 132.8	821.1 ± 146.6	633.9 ± 103.3
6	728.3 ± 118.9	715.0 ± 77.4	608.6 ± 131.6	768.7 ± 81.4
8	737.0 ± 171.5	703.3 ± 131.1	621.0 ± 151.8	748.5 ± 154.7
10	648.6 ± 89.5	810.0 ± 139.7	642.0 ± 149.5	757.7 ± 87.2

Values are expressed as mean IU LDH/mg protein ± SEM. n=3.

Table 4.5 Effect of increasing concentrations of cadmium chloride on Hsp72/3 levels in hepatocyte monolayers

	Dose of cadmium chloride			
Time (hrs)	0μM	0.1μM	1μM	2.5μM
0	100.0 ± 0.0	100.0 ±0.0	100.0 ± 0.0	100.0 ± 0.0
2	105.1 ± 5.9	99.5 ± 6.0	106.8 ± 2.2	95.1 ± 5.4
4	100.7 ± 1.6	94.6 ± 4.6	102.8 ± 4.2	88.7 ± 8.9
6	107.2 ± 6.4	92.8 ± 8.5	102.3 ± 2.8	87.3 ± 11.1
8	97.7 ± 1.0	91.7 ± 9.4	110.1 ± 6.5	103.4 ± 1.0
10	98.0 ± 1.4	96.2 ± 7.6	105.6 ± 8.3	91.6 ± 6.7

Values are expressed as mean % change from control Hsp72/3 level ± SEM. n=3.

Table 4.6 Effect of increasing concentrations of cadmium chloride on levels of cellular protein synthesis in hepatocyte monolayers.

	Dose of cadmium chloride			
Time (hrs)	0μM	0.1μM	1μM	2.5μM
0	116.1 ± 34.1	109.9 ± 34.1	93.1 ± 19.6	108.1 ± 24.8
2	129.6 ± 2.9	150.3 ± 2.9	135.8 ± 8.1	119.0 ± 19.1
4	161.8 ± 23.7	164.9 ± 10.4	183.7 ± 17.9	166.3 ± 29.4
6	188.1 ± 16.7	185.8 ± 20.8	201.2 ± 23.7	168.7 ± 17.3
8	196.6 ± 12.7	220.1 ± 42.1	202.0 ± 42.1	151.9 ± 8.1
10	214.1 ± 49.1	228.3 ± 27.7	228.0 ± 47.4	184.0 ± 15.0

Values are expressed as mean DPM/mg protein ± SEM. n=3

damaged proteins present after low concentrations of cadmium are administered are insufficient in causing adequate dissociation of Hsp70 from the HSF. Hsp70 bound to the HSF effectively inhibits the transcriptional activity of the HSF however after an insult Hsp70 dissociates from the HSF to target the damaged protein thus allowing transcription to occur. Therefore, the concentrations of cadmium used here failed to generate enough damaged proteins to cause sufficient dissociation of Hsp70 from the HSF. Another explanation for this is that these low concentrations of cadmium do in fact cause the dissociation of Hsp70 from the HSF which then translocates to the cells nucleus where it binds to the HSE. However also bound on the HSE is another factor, the constitutive HSE binding factor (CHBF) which acts as a negative regulator of the HSE (Liu *et al.* 1995). The low doses of cadmium are unable to cause the dissociation of CHBF from the HSE thus the binding of HSF to the HSE will not result in the increased transcription of heat shock proteins due to the presence of the inhibitory effects of the CHBF.

4.6 Determination of the basal stress response during the culture of hepatocyte monolayers

4.6.1 Aim of the study

As shown previously in study 4.3, $CdCl_2$ was able to induce Hsp72/3 but failed to induce Hsp25 and hydrazine did not increase levels of either proteins. The induction of Hsp72/3 by $CdCl_2$ was to a much lesser extent that has been observed in other cells such as HepG2 and so it was decided to investigate the basal levels of Hsp72/3 and Hsp25 during the total culture time of the hepatocytes to see if any variation was observed. The basal levels of the stress proteins during the culture of the hepatocytes were compared with levels found in the livers of freshly anaesthetised rats which were taken to represent control levels of the stress proteins.

4.6.2 Methods

Hepatocytes were isolated as described in chapter 2.3, however before the liver was cannulated a small piece of the right liver lobe was removed and placed into liquid nitrogen and then stored at -80°C. The sample of liver, once defrosted, was homogenised

in Tris-HCl pH7.4 (12ml buffer per gram tissue) on ice and 1ml of the resultant homogenate spun at 16,000g for 30 minutes at 4°C. The supernatant was removed, an aliquot removed for protein analysis, and the remainder was stored at -80°C until analysis for heat shock proteins. Upon dilution of the cells down to the working concentration of $0.75x10^6$ cells/ml an aliquot was taken for determination of heat shock protein levels in freshly isolated hepatocytes. The cells were then plated out as described previously and sampled for heat shock proteins at 6, 22, 30, 46 and 54 hours post cannulation. The cells were sampled for Hsp25, Hsp72/3 and LDH leakage as described previously.

4.6.3 Results

4.6.3.1 LDH leakage

LDH leakage from the hepatocytes was constant over the time course of the experiment but there was however a slight but not significant increase in leakage at the 54 hour timepoint (figure 4.11).

4.6.3.2 Hsp72/3 levels

The results for Hsp72/3 appear to show a significant decrease in levels at time-point 0 when compared to levels in liver samples taken from anaesthetised rats (hereafter referred to as *in vivo* levels), time-point 0 represents levels within freshly isolated hepatocytes and sampling of these cells occurred approximately 30 minutes after the liver sample was taken. This is in contrast to the levels of Hsp72/3 present in hepatocytes 6, 22 and 30 hours post cannulation where compared to *in vivo* levels there was almost a 3 fold induction of Hsp72/3. At 46 hours post cannulation levels of Hsp72/3 began to decline to what approximated to levels found *in vivo*, however at 54 hours levels were significantly decreased (figure 4.12).

4.6.3.3 Hsp25 levels

The results for Hsp25 show that there is a similar pattern of induction as observed with Hsp72/3 albeit with a different dynamic range. At 30 minutes following cannulation of the liver there is a highly significant decrease in levels of this protein found in the isolated hepatocytes when compared to levels found *in vivo*. This is again in contrast to levels at 6, 22 and 30 hours following cannulation where levels of Hsp25 increased to above what

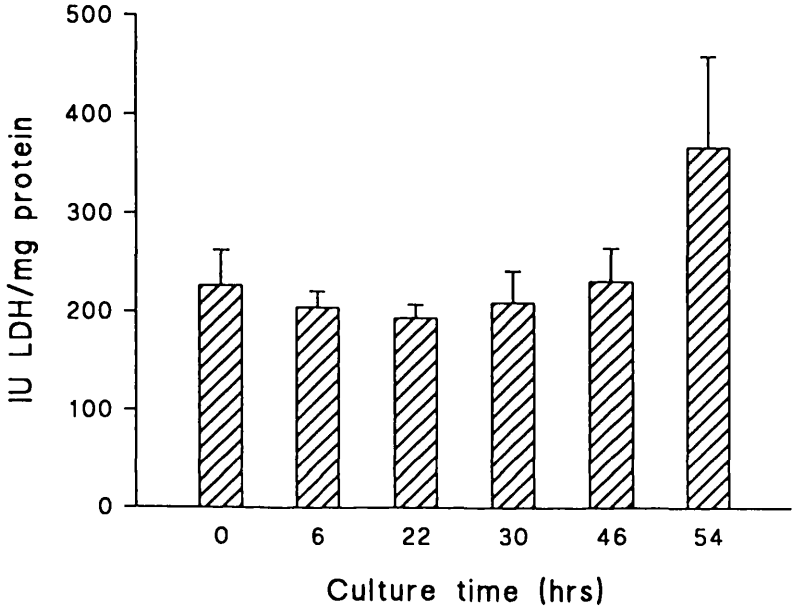


Figure 4.11 Basal leakage of LDH from hepatocyte monolayers over a 54 hour period. Values are expressed as mean IU LDH/mg protein ± SEM. n=4.

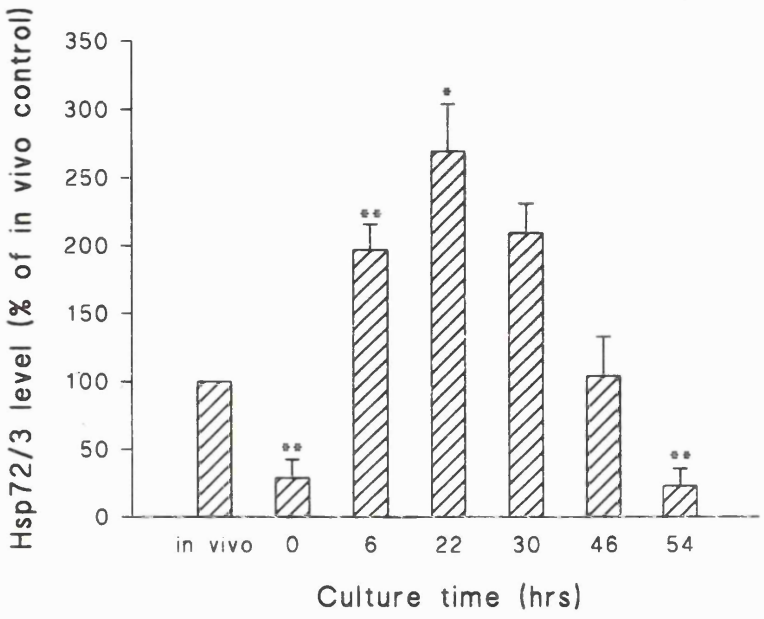


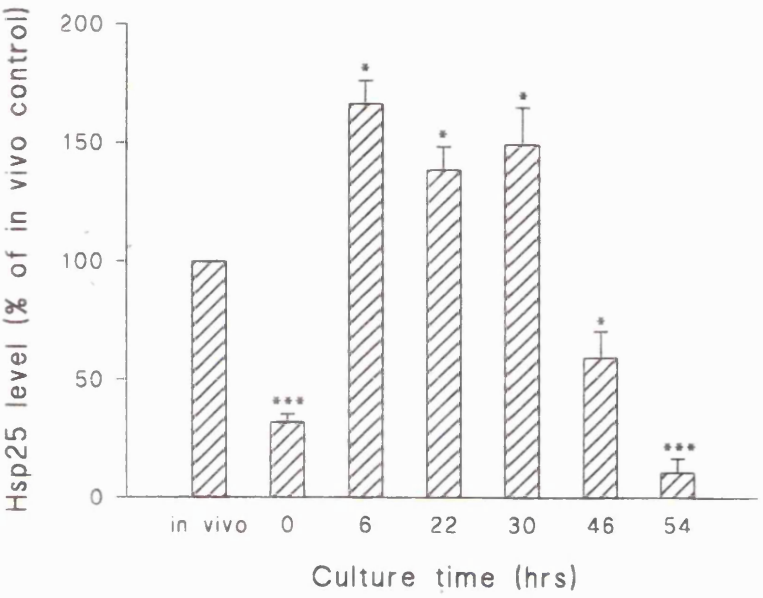
Figure 4.12 Basal levels of Hsp72/3 in hepatocytes monolayers over a 54 hour period. Timepoint in vivo refers to levels of Hsp72/3 in a sample of undigested liver, timepoint 0 refers to levels of Hsp72/3 in freshly isolated hepatocytes. Values are expressed as % of in vivo control ± SEM. n=4. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Bottom panel shows a typical Western blot showing basal levels of Hsp72/3 in hepatocytes over 54 hours. Lanes as follows; Lane 1, in vivo; lane 2, 0hr; lane 3, 6hr; lane 4, 22hr; lane 5, 30hr; lane 6, 46hr; lane 7, 54hr post cannulation. Solid arrow represents Hsp72/3.

was found *in vivo* albeit to a lesser extent than Hsp72/3. At 46 hours post cannulation levels of Hsp25 had fallen to approximately 60% of those levels found *in vivo*. As with Hsp72/3 levels of Hsp25 at 54 hours had again fallen significantly below those levels found *in vivo* (figure 4.13).

4.6.4 Discussion

Leakage of LDH did not change significantly during the time-course of the experiment, however it appears that at the final tim-epoint there was a slight increase in LDH leakage thereby implying that the cells were becoming slightly compromised. Levels of Hsp72/3 fell rapidly, within 30 minutes of cannulation, which may indicate that this decrease is due to the process of cannulation, collagenase perfusion and subsequent washing of the cells. Such a drastic, rapid fall in the levels of Hsp25 and Hsp72/3 might imply that the isolation of the cells may be interfering with the measurement of the stress proteins. It appears that the hepatocyte isolation procedure causes stress to the cells as levels of Hsp72/3 and Hsp25 were significantly increased 6 hours post cannulation. The source for this stress is no doubt the isolation procedure of the hepatocytes, enzymatic digestion with collagenase would damage the cells during isolation and the subsequent washing of the cells would exacerbate any such damage. Isolation of hepatocytes has been shown previously to affect the levels of hepatic biochemical parameters namely numerous isoenzymes of cytochrome P-450 m RNAs (Padgham and Paine 1993). Elevated levels of Hsp70 mRNA has been reported in primary cultures of hepatocytes previously (Van Remmen *et al.* 1996). However, here levels were only elevated after 48 hours in culture thereby indicating that the isolation procedure for the hepatocytes is not a cause of the elevated Hsp70 mRNA. This is in disagreement with our findings which indicated that the isolation procedure was mainly responsible for the elevated stress proteins during hepatocyte culture.

However, in addition to the effect of hepatocyte isolation the sustained increase in the levels of Hsp72/3 and Hsp25 over the following 30 hours would indicate that in culture the primary hepatocytes are under a continuous state of stress. The stress response in cells is a transient event and it is known that following a single insult, levels of Hsp72/3 return to values close to control values some 18-24 hours later (Goering *et al.* 1993). Therefore, it would expected that the levels of the stress proteins would return to control values



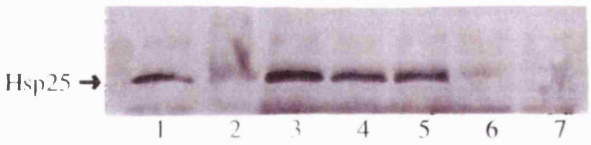


Figure 4.13 Basal levels of Hsp25 in hepatocyte monolayers over a 54 hour period. Timepoint in vivo refers to levels of Hsp25 in a sample of undigested liver, timepoint 0 refers to levels of Hsp25 in freshly isolated hepatocytes. Values are expressed as % of in vivo control ± SEM. n=4. *Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Bottom panel shows a typical Western blot showing basal levels of Hsp25 in hepatocytes over 54 hours. Lanes as follows; Lane 1, in vivo; lane 2, 0hr; lane 3, 6hr; lane 4, 22hr; lane 5, 30hr; lane 6, 46hr; lane 7, 54hr post cannulation. Solid arrow represents Hsp25.

within 24 hours of isolation but as observed levels remained elevated after 30 hours in culture. Thus this would imply that the increased levels of these stress proteins in primary hepatocytes are due not only to the isolation procedure but also to a sustained state of stress whilst in culture. The possible causes of this stress could be changes in cellular structure following isolation, preparation of hepatocytes using collagenase causes the dissociation of cell-cell contacts and the removal from the extracellular matrix (Wright and Paine 1992). This alteration in protein interactions might be a physiological trigger for induction of stress proteins as heat shock proteins are involved in protein interactions when acting as molecular chaperones. Another explanation that has been suggested for the elevated levels of stress proteins observed during hepatocyte culture is the increased formation of free radicals and reactive oxygen species (Van Remmen *et al.* 1996). However, our findings do not support this conclusively as if there was increased reactive oxygen formation, levels of GSH would be expected to be decreased during culture time and levels of GSH remained constant in control hepatocytes during culture (see chapters 4.3.3.3 and 4.4.3.3).

The reason why $CdCl_2$ and hydrazine failed to elicit a large stress response in hepatocyte monolayers may be due to the increased basal levels of the stress proteins studied. It is possible that since the cells are under a state of stress whilst in culture exposing them to a second stressful stimulus, in this case $CdCl_2$ or hydrazine, may not be able to elicit a full characteristic stress response. This may be due to the reponse being masked by the already induced levels of Hsp72/3 and Hsp25 present in the cell. In the previous studies (4.2 and 4.3) the hepatocytes were exposed to hydrazine or cadmium chloride after 18 hours in culture thus coinciding with the maximal basal expression of Hsp72/3 and Hsp25.

Finally, the decrease in levels at 54 hours following cannulation may also indicate that the cells are beginning to become compromised and are beginning to lose integrity. The significant decrease in levels of Hsp25 at the final two timepoints also indicates that the cells are in effect becoming compromised. This is reinforced by the significant decrease in Hsp72/3 levels and the slight increase in LDH leakage at the final timepoint. In summary because of the fluctuating basal levels of heat shock proteins the use of primary hepatocytes as a model for studying accurately the effect of chemicals on the stress

response may not be feasible.

4.7 Conclusions

Overall this study showed that hydrazine failed to cause the induction of Hsp25 or Hsp72 in hepatocyte monolayers at any concentration whereas cadmium caused the induction of only Hsp72/3 in the cells at a concentration when no other parameter of toxicity was altered. However, measurement of the basal levels of the two stress proteins during hepatocyte culture showed that the levels of Hsp25 and Hsp72/3 were elevated implying the cells were under a state of stress whilst in culture. In conclusion, therefore the use of heat shock proteins as markers of toxicity in hepatocyte monolayers is questionable. One of the major problems associated with the model is that the expression of several of the proteins involved in the stress response are altered when the hepatocytes are in culture. This alteration of basal stress protein levels would alter the stress response of a cell following exposure to a noxious stimulus. Whether or not other individual stress proteins, other than Hsp25 or Hsp72/3, are elevated during culture is not known but from the basis of these results presented here it is probable that other stress proteins would be elevated during the culture of hepatocytes. These alterations in basal stress protein levels would mean that any stress response in hepatocyte monolayers observed following chemical exposure would have to be viewed with care when attempting to compare with any response *in vivo*. Another consequence of the elevated stress proteins in primary hepatocytes that has to be considered is the effect on the relative toxicity of compounds in primary hepatocytes compared to the compounds toxicity *in vivo* or in cell lines. Stress proteins are known to protect a cell from adverse stimuli and so it would follow that the toxicity of certain chemicals would be decreased in those cells with elevated stress protein levels. Therefore, the extrapolation of toxicity data from hepatocyte monolayers to the liver *in vivo* has to be done with care particularly with those chemicals that are known to interact with stress response of a cell. However, to complicate matters further comparison of the toxicity of cadmium in hepatocyte monolayers (chapter 4.3.3) to the toxicity in HepG2 or H35 hepatoma cells showed the toxicity of cadmium was markedly less in the hepatoma cells. It could be speculated that this is opposite of what would be expected as

the elevated stress proteins in hepatocyte monolayers would be thought to protect the cells from cadmium toxicity, therefore the elevated levels of stress proteins in the cells would appear not to have a significant effect on the toxicity of cadmium.

On a more positive note is the observation that following cadmium exposure the first biochemical parameter to be altered in hepatocyte monolayers was an elevation of Hsp72/3 levels. This would imply that heat shock proteins do have the potential to be early biomarkers of impending cellular toxicity. However, the induction of Hsp72/3 appears to be stressor specific as exposure of hepatocytes to hydrazine did not alter the levels of Hsp72/3 and both cadmium and hydrazine failed to increase the levels of Hsp25. The reasons for the selective induction of heat shock proteins by specific chemicals are as yet unclear. Considering there are multiple members of the HSF gene family, namely HSF-1, HSF-2, HSF-3 and HSF-4, it does seem feasible that different factors play a role in the response of a cell to different forms of stress. The kinetics of stress induced DNA binding of HSF-1 and HSF-3 show different profiles, HSF-1 trimerises and translocates to the cells nucleus rapidly whereas HSF-3 responds to stress slowly and additionally these two factors show different thresholds in their responses to stress (Tanabe *et al.* 1997). This raises the possibility of selective induction of individual transcription factors which may underlie the stressor-specific induction of separate heat shock proteins. However, until the exact mechanisms behind the induction of specific heat shock proteins are elucidated the use of individual heat shock proteins as markers of toxicity particularly in hepatocyte monolayers is limited.

Chapter 5.

The use of heat shock proteins as markers of hepatic and testicular toxicity *in vivo*

5.1 Introduction

In the previous investigations it was determined that exposure of hepatocytes *in vitro* to cadmium increased the expression of Hsp72/3 in the cells whereas hydrazine exposure failed to affect levels of either Hsp25 or Hsp72/3. However, the investigations were problematic due to the fact that the hepatocytes were under a state of stress whilst in culture and so the induction of Hsp72/3 by cadmium was increased by, at most, 50% above *in vivo* control levels. It was therefore decided to investigate what the stress response was in hepatocytes *in vivo* when rats were exposed to hydrazine or cadmium by measurement again of the two stress proteins, Hsp25 and Hsp72/3. Hydrazine has been shown previously to be hepatotoxic *in vivo* with adverse biochemical changes observed following administration of doses of 5-10mg/kg to rats (Waterfield *et al.* 1997). There is, however, no information concerning the effect of hydrazine on the stress response *in vivo.* Cadmium is known to induce liver heat shock protein levels following administration of doses of 1-2mg/kg to rats with biochemical signs of toxicity observed following dosing with 2mg/kg $CdCl_2$ (Goering *et al.* 1993b).

In addition to measurement of the two stress proteins, Hsp25 and Hsp72/3, it was also decided to measure levels of the 32kDa heat shock protein (Hsp32). It is now recognised that Hsp32 is in fact the enzyme heme oxygenase (HO) which plays an essential role in the catabolism of heme with the resultant formation of biliverdin which is then further converted to the known antioxidant bilirubin (Taketani *et al.* 1989). There are two isozymes of heme oxygenase, HO-1 and HO-2, HO-1 is heat inducible whereas HO-2 is thought to be present in the cell constitutively (Maines 1988). The physiological antioxidant role of heme oxygenase, in addition to its other functions, has been discussed in greater detail in chapter 1.4.2. It is known that heme oxygenase is present in many tissues including the liver, and it is known that administration of cadmium to rats causes an induction of hepatic heme oxygenase (Ossola and Tomaro 1995). One of the tissues that contains the highest levels of heme oxygenase is the testis where it is thought to be involved in the maintenance of conditions required for spermatogenesis (Ewing and Maines 1995) and in addition to this the heme oxygenase gene has been shown to be activated at the spermatogonia stage of spermatogenesis (Kurata *et al.* 1993). Induction

of testicular heme oxygenase levels in Sertoli and Leydig cells has been demonstrated in rats following thermal stress and this increase in heme oxygenase is thought to play a role in the protection of spermatogenic cells during stress (Maines and Ewing 1996). Therefore, it was decided to determine the effects of chemical exposure on testicular heme oxygenase levels in addition to investigating hepatic heme oxygenase levels

By measurement of stress protein levels in the liver and testis of rats exposed to either hydrazine or cadmium it was hoped to elucidate whether there was a different profile between the induction of the stress response in hepatocytes *in vitro* and in hepatocytes *in vivo*. It was hoped, therefore, to determine whether the induction of heat shock proteins could be used as sensitive markers of toxicity both in the liver and testes. Measurement of other biochemical parameters of toxicity would also indicate how sensitive the heat shock proteins would be as potential markers of toxicity. This would also hopefully provide an understanding as to whether the stress response represents an early cellular response which may relate to the hepatic injury induced by the compounds used.

5.2 Determination of the distribution of heat shock proteins in individual liver lobes

5.2.1 Aims of the study

Prior to an *in vivo* study investigating the levels of heat shock proteins in the liver and testis a preliminary study was performed. The main aim of this preliminary study was to investigate whether the different lobes of the liver would have different basal levels of heat shock proteins. Therefore, the five individual rat liver lobes were analysed for their individual levels of the stress proteins Hsp72/3 and Hsp25.

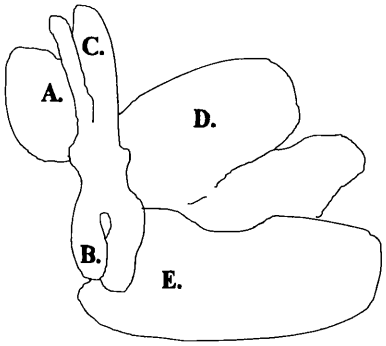
5.2.2 Methods

Male Han Wistar rats (GlaxoWellcome bred) were anaesthetised with Hypnorm/Hypnovel (1:1:2 H_2O, 3.3ml/kg i.p). The abdominal cavity was swabbed with 70% ethanol and opened with a U-shaped incision and the liver removed. The individual liver lobes were excised separately and placed into liquid nitrogen and stored -80°C until analysis. Upon use the liver lobes were defrosted and homogenised on ice in Tris-HCl pH7.4 (12ml

buffer/g tissue) and the resultant homogenates spun at 16,000g for 30 minutes at 4°C. An aliquot of supernatant was taken for protein determination by the Coomassie method. Supernatants were stored at -80°C until analysed for Hsp25 and Hsp72/3 levels. Upon use the supernatants were diluted five fold with Laemmeli loading buffer, bromophenol blue and 2-mercaptoethanol were added to give final concentrations of 0.005% and 5% respectively. The sample was heated to 100°C for 5 minutes before undergoing SDS-PAGE as described previously in chapter 2.

Liver lobes were classified as following when viewed form the ventral posterior position;

Lobe A; Right (I)
Lobe B; Posterior or caudate
Lobe C; Right (II)
Lobe D; Median
Lobe E; Left



5.2.3 Results

Analysis of the levels of Hsp72/3 in the separate lobes of the liver showed that there was no significant difference in the levels of this protein between the five separate liver lobes. With respect to Hsp25 the results showed that there was a significant difference in the levels of this protein between the individual lobes. The lowest levels of Hsp25 were found in the right (I) lobe of the liver and the highest levels found in the posterior/caudate lobe, increased levels of Hsp25 were also found in the median and right (II) lobes (see table 5.1). Due to the difference of heat shock protein levels between the individual liver lobes it was decided that in the following *in vivo* studies when the liver was sampled for stress proteins the left lobe was used.

Table 5.1 Comparison of the levels of the two stress proteins, Hsp72/3 and Hsp25, in individual rat liver lobes. Bottom panel shows a typical Western blot generated when individual liver lobes were probed for Hsp72/3 and Hsp25.

Liver lobe	Optical density units of Hsp72/3	SEM	Optical density units of Hsp25	SEM
Right (I) [A]	861.3	54.1	454.1 [abc]	50.0
Left [E]	797.8	66.3	521.1 [abc]	20.5
Median [D]	815.3	99.5	647.0 [c]	38.5
Right (II) [C]	922.0	77.4	731.8	94.0
Post./caudate [B]	925.0	80.4	854.7	74.9

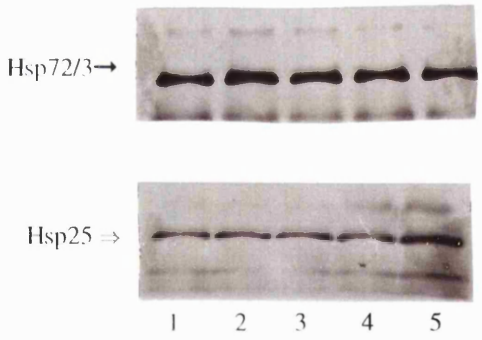
Values are expressed as arbitrary optical density units and are the mean of four experiments

Letters indicate values significantly different from corresponding values using the Dunnett's t-test

[a] significantly different from median lobe

[b] significantly different from right (II) lobe

[c] significantly different from posterior/caudate lobe



Typical Western blots showing levels of Hsp72/3 and Hsp25 in individual rat liver lobes. Lanes as follows; Lane 1, right (I) lobe; lane 2, left lobe; lane 3, median lobe; lane 4, right (II) lobe; lane 5, posterior/caudate lobe. Open arrow represents Hsp25, solid arrow represents Hsp72/3.

5.3 Effect of hydrazine hydrate on liver and testicular heat shock protein levels and liver biochemical parameters

5.3.1 Aims of study

The main aim of this study was to determine whether administration of hydrazine to rats was able to induce a stress response in the livers and testis of treated animals. This would allow a direct comparison to be made with the effect of hydrazine on hepatocytes *in vitro*. Considering in chapter 4.2 hydrazine had no effect on Hsp25 or Hsp72/3 levels in hepatocyte monolayers this study was performed to elucidate whether the lack of induction *in vitro* was due to the problems associated with the *in vitro* model in that the basal stress protein levels were elevated during culture. In addition to measurement of Hsp72/3 and Hsp25, levels of the stress protein Hsp32 (heme oxygenase-1) levels were also measured in the liver and testes samples. There is no data concerning the effect of hydrazine administration on stress protein levels *in vivo*. As mentioned in the previous chapter, if hydrazine was causing the induction of stress proteins this increase in levels might have been undetectable in hepatocyte monolayers due to the elevated basal stress protein levels, therefore administration of the compound *in vivo* should determine what the actual response of hepatocytes is following exposure to hydrazine. In addition to this it was also hoped to determine that if there was a change in the stress protein levels in the tissues whether or not this change was occurring before or after other biochemical indicators of toxicity were altered in order to establish whether heat shock proteins were more feasible markers of toxicity *in vivo*.

5.3.2 Methods and dosing

Male Han Wistar rats (GlaxoWellcome bred) were dosed with 0, 1, 2.5, 5, and 10mg/kg hydrazine free base in 1ml/kg sterile H_2O. All dosing was done *via* the i.p route of administration. Four rats were dosed per group. Animals were sacrificed 6 hours post dose for post mortem as described previously in chapter 2.4. In this study and study 5.3 the liver samples were probed with anti-Hsp25 and anti-Hsp72/3 antibodies and a rabbit polyclonal anti-Hsp32 (HO-1) antibody. The testis samples were probed with anti-Hsp72/3 and anti-Hsp32 (HO-1) antibodies.

5.3.3 Results

5.3.3.1 Liver ATP levels

Six hours following exposure to 1 and 2.5mg/kg hydrazine liver ATP levels were slightly depleted. Dosing with 10mg/kg hydrazine caused levels of ATP to fall significantly in the liver. There was no effect on liver ATP levels following administration of 5mg/kg hydrazine (figure 5.1).

5.3.3.2 Liver GSH levels

Liver GSH levels were determined to be slightly decreased following administration of 1, 5 and 10mg/kg hydrazine. This was found to be significant only at the 5mg/kg dosed group. There was no noticeable effect on liver GSH levels following dosing with 2.5mg/kg (figure 5.2).

5.3.3.3 Serum chemistry

Serum levels of albumin, bilirubin, urea, creatinine, triglycerides, cholesterol and LDH were found to be unchanged in all dosed groups compared to controls (data not shown). Levels of serum AST were determined to be significantly decreased following administration of 2.5, 5 and 10mg/kg hydrazine (figure 5.3). Serum ALT levels were unchanged following administration of 1 and 2.5mg/kg hydrazine, however levels were significantly decreased following dosing with 5 and 10mg/kg hydrazine (figure 5.4).

5.3.3.4 Liver heat shock protein levels

Hydrazine, at any concentration, did not alter levels of Hsp72/3 in the liver when compared to levels of Hsp72/3 in control livers. Measurement of liver Hsp25 levels showed that administration of hydrazine had no effect on the levels of this stress protein (figures 5.5 and 5.6). Upon probing the membranes with anti-Hsp32 no signal was observed indicating that basal liver levels of Hsp32 were below the detection range of the antibody used and levels of Hsp32 were unchanged following administration of hydrazine.

5.3.3.5 Testicular heat shock protein levels

Hydrazine, at any concentration, had no effect on the testicular levels of the two stress

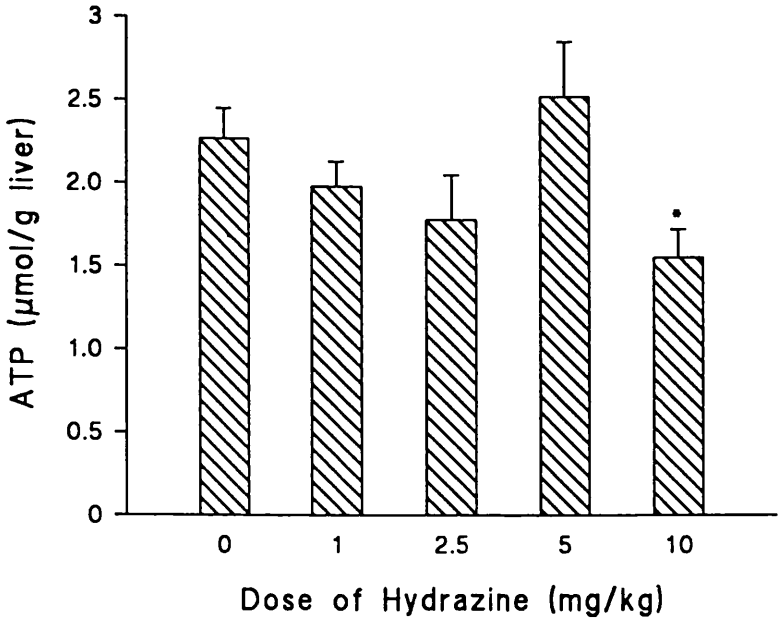


Figure 5.1 Effect of in vivo administration of hydrazine on liver ATP levels 6 hours post dose. Values are expressed as mean µmol ATP/g^{-1} liver ± SEM. n=4. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*p<0.05)

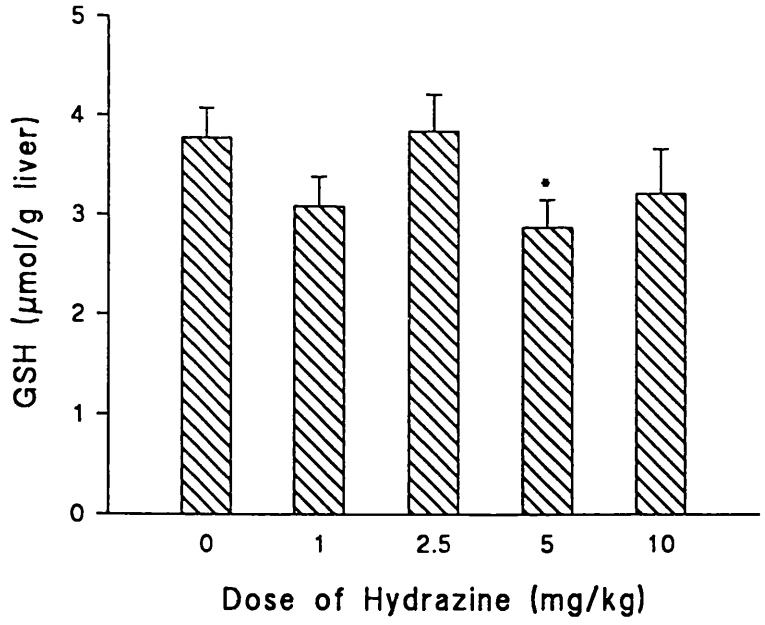


Figure 5.2 Effect of in vivo administration of hydrazine on liver GSH levels 6 hours post dose. Values are expressed as mean µmol GSH/g^{-1} liver ± SEM. n=4. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*p<0.05)

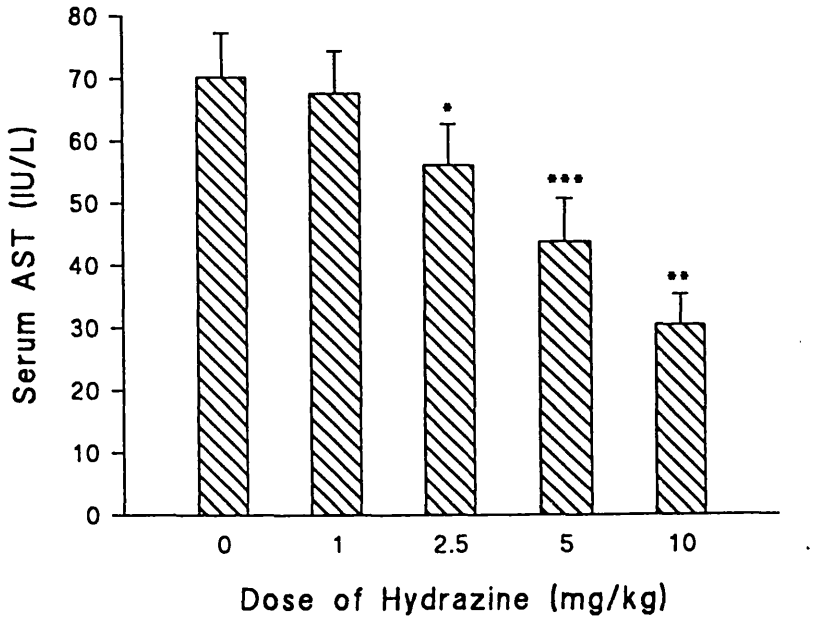


Figure 5.3 Effect of in vivo administration of hydrazine on serum AST levels 6 hours post dose. Values are expressed as mean IU/l ± SEM. n=4. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*p<0.05, **p<0.01, ***p<0.001)

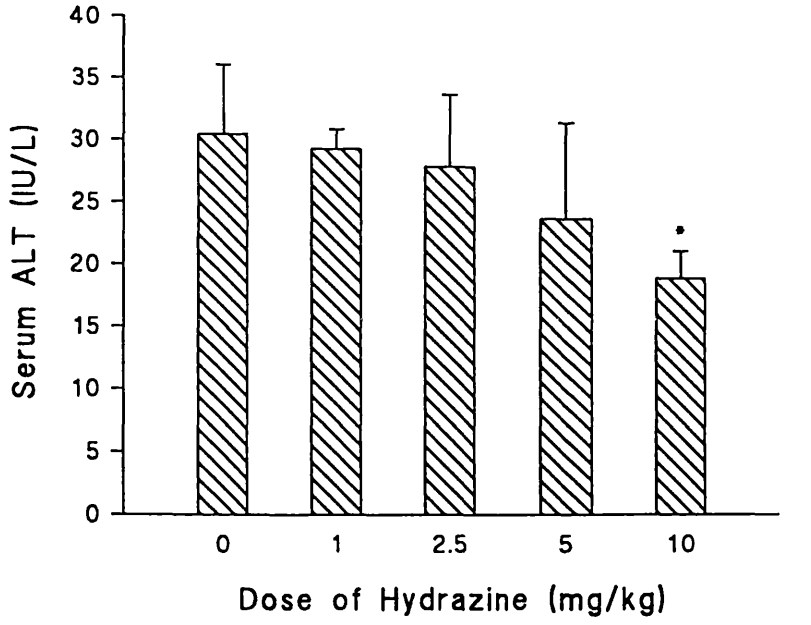


Figure 5.4 Effect of in vivo administration of hydrazine on serum ALT levels 6 hours post dose. Values are expressed as mean IU/l ± SEM. n=4. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*p<0.05)

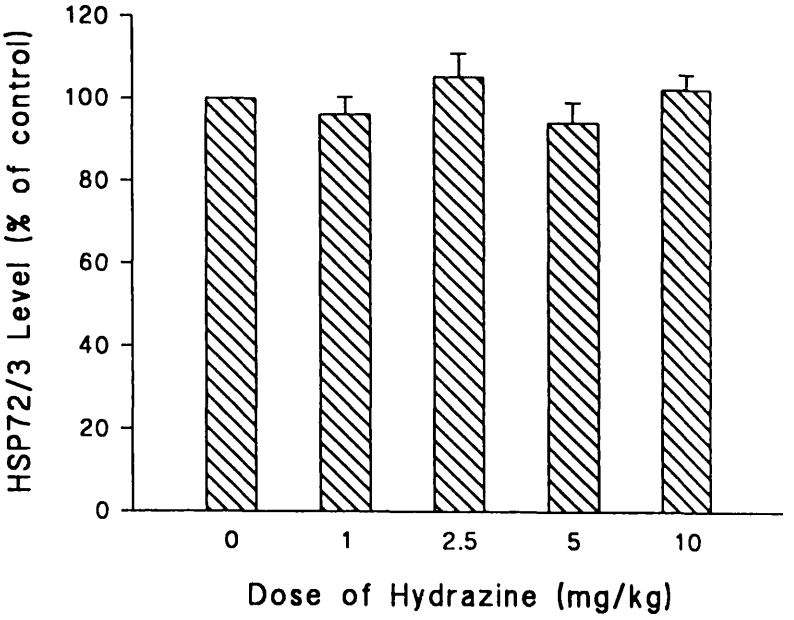


Figure 5.5 Effect of in vivo administration of hydrazine on liver Hsp72/3 levels 6 hours post dose. Values are expressed as mean % change from control ± SEM. n=4.

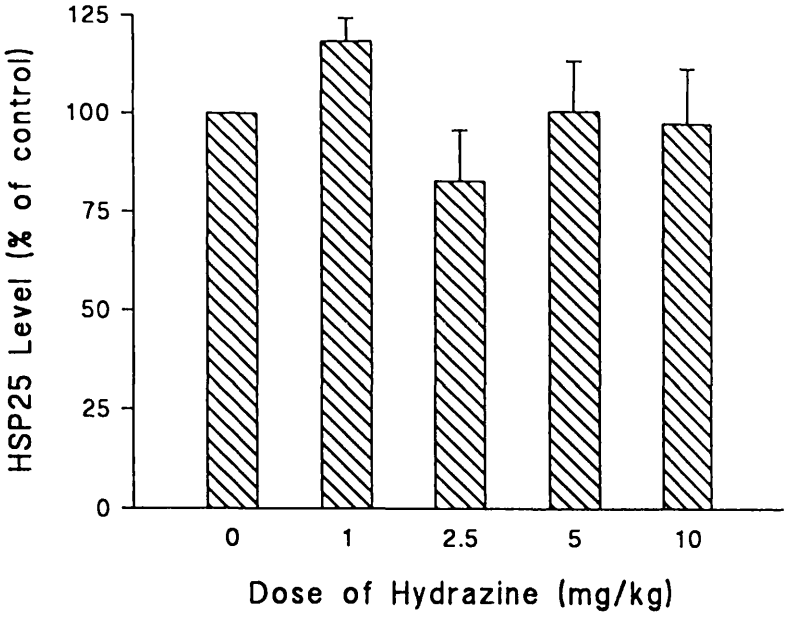


Figure 5.6 Effect of in vivo administration of hydrazine on liver Hsp25 levels 6 hours post dose. Values are expressed as mean % change from control ± SEM. n=4.

proteins Hsp32 (HO-1) and Hsp72/3 (figures 5.7 and 5.8).

5.3.4 Discussion

Following administration of hydrazine to rats the only biochemical effects of hydrazine observed were a significant decrease in liver GSH and ATP levels following dosing with 5 and 10mg/kg hydrazine respectively and, in addition to this there was a significant decrease in serum AST and ALT levels following dosing with 2.5-10mg/kg hydrazine. The depletion of GSH observed here occurred at much lower doses than determined previously, Jenner and Timbrell (1994) reported that liver levels of GSH were significantly reduced six hours following dosing with 60mg/kg hydrazine. The hydrazine-induced depletion of GSH is not accompanied by a concurrent increase in oxidised glutathione (Timbrell *et al.* 1982). It is therefore possible that hydrazine interferes with the synthesis of glutathione, this may be brought about by interactions with the enzymes required for GSH synthesis (Moloney and Prough 1983). Following administration of 5mg/kg hydrazine *in vivo* the plasma concentration of hydrazine approximates to 0.075mM (Waterfield *et al.* 1997). Comparison of the results generated here and those from chapter 4.3.3 showed that hydrazine *in vitro* significantly decreased hepatocyte GSH levels at a concentration of 5mM. Therefore, the concentration of hydrazine required to deplete GSH significantly *in vitro* is approximately 60-fold higher than that *in vivo*.

The depletion of hepatic ATP occurred following dosing with 10mg/kg hydrazine, this compares well with data presented by Waterfield *et al.* (1997) which showed initial decreases in ATP levels following dosing with 10mg/kg hydrazine. It would appear that this dose of hydrazine is the threshold dose for ATP depletion as Preece *et al.* (1990) demonstrated that following administration of 10mg/kg hydrazine to rats hepatic ATP levels were unchanged and the initial depletion in ATP levels was observed following dosing with 20mg/kg hydrazine. The mechanisms behind hydrazine induced ATP depletion are unclear but there is evidence that hydrazine affects mitochondrial function adversely possibly by the uncoupling of oxidative phosphorylation. In addition to this hydrazine may also affect ATP synthesis by depletion of essential tricarboxylic acid cycle precursors and intermediates (Ghatineh *et al.* 1992). Again comparison of the *in vivo* data presented here and the *in vitro* data in chapter 4.3.3 shows that there is a substantial

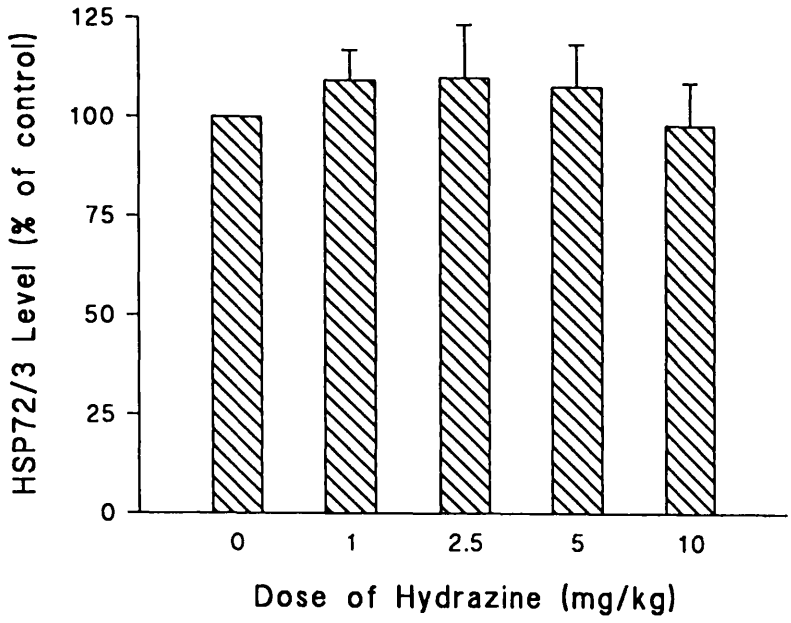


Figure 5.7 Effect of in vivo administration of hydrazine on testicular Hsp72/3 levels 6 hours post dose. Values are expressed as mean % change from control ± SEM. n=4.

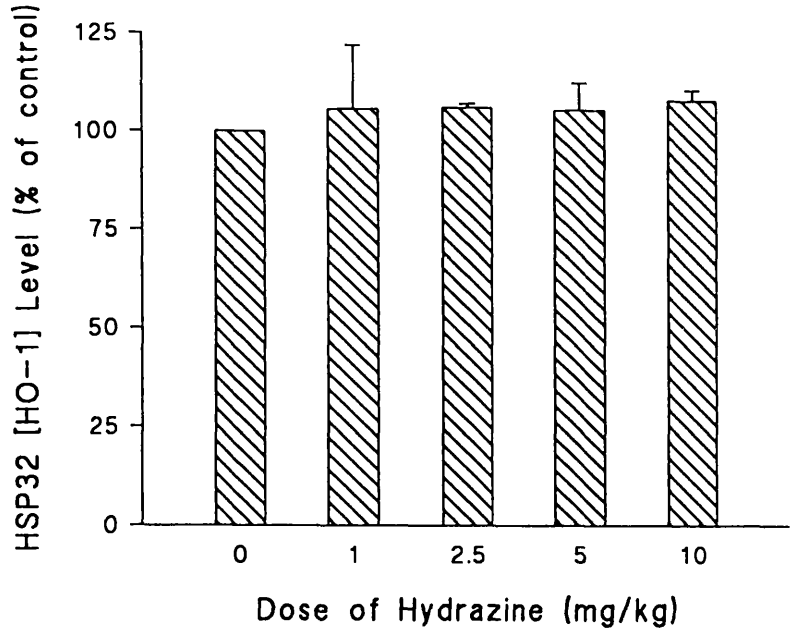


Figure 5.8 Effect of in vivo administration of hydrazine on testicular Hsp32 (HO-1) levels 6 hours post dose. Values are expressed as mean % change from control ± SEM. n=4.

difference in the sensitivity to hydrazine induced ATP depletion. *In vitro* hydrazine caused the depletion of ATP levels at a concentration of approximately 20mM whereas *in vivo* a plasma concentration of 0.1mM hydrazine was required to deplete hepatocyte ATP levels (equivalent to 10mg/kg hydrazine [Waterfield *et al.* 1997]), therefore as with the effects on GSH levels the concentration of hydrazine required to deplete ATP significantly *in vitro* is approximately 40-fold higher than that *in vivo*.

Exposure of rats to hydrazine failed to induce stress protein levels in the liver or testis. This is in accordance to the results generated in chapter 4.3.3 which showed that hydrazine *in vitro* had no effect on stress protein levels in hepatocyte monolayers. One of the possibilities in those results was that since there were elevated basal stress protein levels during the culture of hepatocytes hydrazine may be inducing a stress response but it was subsequently being masked by the elevated basal levels already present. However, the results from these *in vivo* studies would indicate that there was no induction of stress proteins in hepatocyte monolayers. Hydrazine did not alter levels of Hsp32 in the liver or testis of exposed animals, levels of Hsp32 in the liver were below the threshold of detection, but Hsp32 was measurable in the testis samples. This is in agreement with Maines (1988) who stated basal Hsp32 levels in rat testis were approximately 2-fold greater than levels determined in rat liver. However, even though hydrazine failed to induce levels of Hsp72/3, Hsp32 or Hsp25 *in vivo,* the possibility that it is affecting the levels of other stress proteins cannot be ruled out. In addition to this it also possible that hydrazine is inhibiting the synthesis of stress proteins, this argument is re-inforced by the observation that hydrazine is known to inhibit protein synthesis in hepatocytes at low concentrations (Ghatineh and Timbrell 1994).

In summary, hydrazine failed to induce the levels of stress proteins, Hsp25, Hsp32 and Hsp72/3, in hepatic or testicular tissues following exposure *in vivo* this, however, does not conclusively prove that hydrazine is unable to elicit a stress response *in vivo*. Comparison of the biochemical toxicity of hydrazine *in vivo* with the *in vitro* toxicity of hydrazine clearly showed there was a large difference. The reasons for this difference are unclear and any explanation is hampered by the lack of knowledge of the precise mechanism of action underlying the toxicity of hydrazine.

5.4 Effect of cadmium chloride on liver and testicular heat shock protein levels and liver biochemical parameters

5.4.1 Aims of the study

This study was essentially a repeat of study 5.2 with the exception that the compound used here was cadmium chloride. Again the aims of this study were to elucidate the *in vivo* expression of stress proteins in the liver and testis following cadmium exposure thereby allowing a direct comparison to be made with the results generated from the effect of cadmium *in vitro*. Similarly other biochemical parameters of toxicity were measured to determine the sensitivity of the stress response when compared to other well established indicators of toxicity.

5.4.2 Methods and dosing

In this study male Han Wistar rats (GlaxoWellcome bred) were dosed with 0, 0.1, 0.5, 1, and 2mg/kg Cd^{2+} in 1ml/kg sterile H_2O. All dosing was done via the i.p route of administration as described previously in chapter 2.4. Four rats were dosed per group. All animals were sacrificed 6 hours post dose for post mortem as described previously in chapter 2.4. The liver samples were probed with anti-Hsp25 and anti-Hsp72/3 antibodies and a rabbit polyclonal anti-Hsp32 (HO-1) antibody. The testis samples were probed with anti-Hsp72/3 and anti-Hsp32 (HO-1) antibodies

5.4.3 Results

5.4.3.1 Liver ATP levels

Administration of 0.5, 1 and 2mg/kg $CdCl_2$ caused slight decreases in liver GSH levels when compared to control levels, this decrease was determined to be significant following dosing with 2mg/kg $CdCl_2$ (figure 5.9).

5.4.3.2 Liver GSH levels

Cadmium chloride, at any dose, did not significantly alter liver levels of GSH when compared to levels in control samples (figure 5.10).

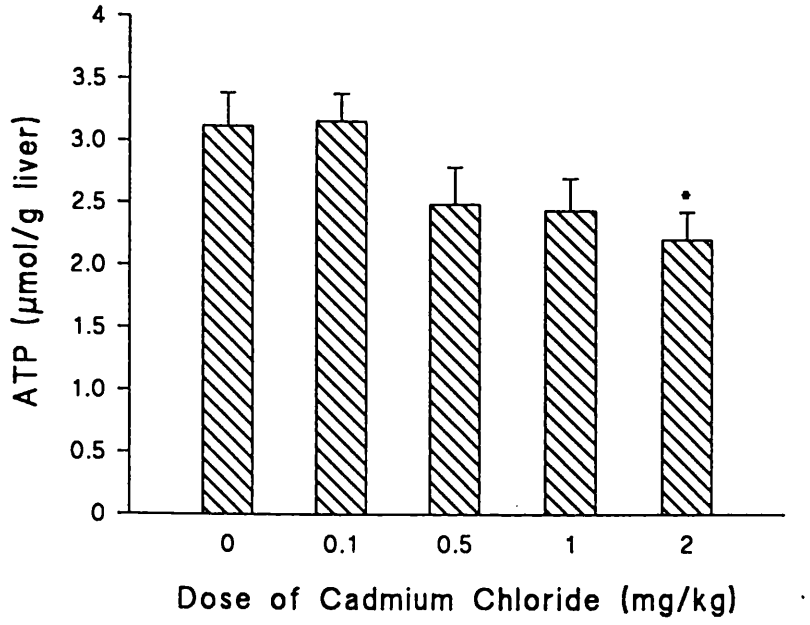


Figure 5.9 Effect of in vivo administration of cadmium chloride on liver ATP levels 6 hours post dose. Values are expressed as mean µmol ATP/g^{-1} liver ± SEM. n=4. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*$p<0.05$)

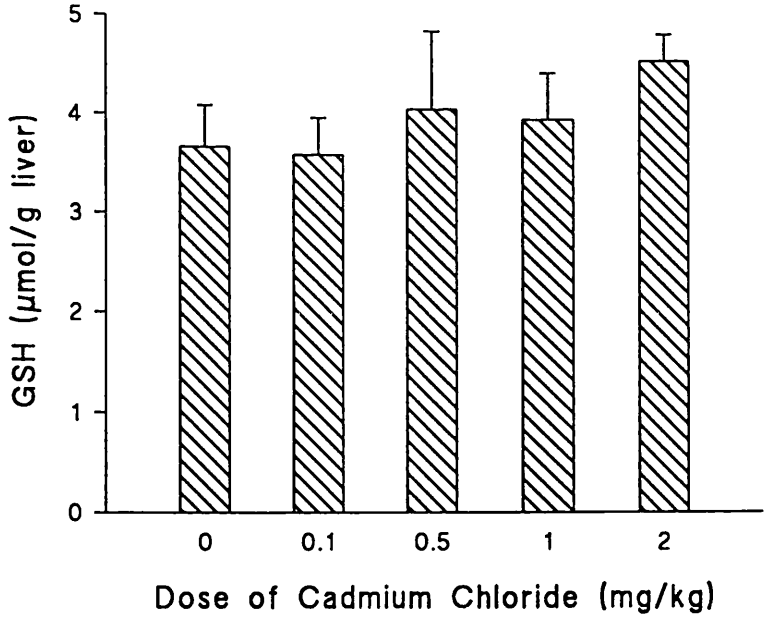


Figure 5.10 Effect of in vivo administration of cadmium chloride on liver GSH levels 6 hours post dose. Values are expressed as mean µmol GSH/g^{-1} liver ± SEM. n=4.

5.4.3.3 Serum chemistry

Serum levels of ALT, AST, albumin, bilirubin, urea, creatinine, triglycerides, cholesterol and LDH were found to be unchanged in all dosed groups compared to controls (figures 5.11 and 5.12, other data not shown).

5.4.3.4 Liver heat shock protein levels

Following administration of $CdCl_2$ there was a dose dependant increase in the liver levels of Hsp72/3. Dosing with 0.1 and 0.5mg/kg $CdCl_2$ produced slight increases in the levels of Hsp72/3 but the increase was not significant. However dosing with 1 and 2mg/kg $CdCl_2$ produced significant increases in the levels of Hsp72/3. Liver levels of Hsp25 were unchanged from control levels following administration of any dose of $CdCl_2$. Measurement of Hsp32 in the liver showed that in control livers the levels of this stress protein were undetectable, because of this the subsequent results for levels Hsp32 were expressed as the optical density values obtained and not expressed as % of control levels. In control livers and those dosed with 0.1 and 0.5mg/kg the levels of Hsp32 were undetectable, however following dosing with 1mg/kg there was a slight increase in the levels of this protein although this was not evident in all the dosed animals. There was a large significant induction of Hsp32 following dosing with 2mg/kg $CdCl_2$ (figures 5.13, 5.14 and 5.15).

5.4.3.5 Testicular heat shock protein levels

Levels of testicular Hsp72/3 were increased following dosing with $CdCl_2$. This increase, of approximately 40-60%, was observed at all concentrations of $CdCl_2$ but was determined only to be significant at the 2mg/kg dosed group. Basal levels of Hsp32 in the testis were much higher when compared to basal levels in the liver. Dosing with $CdCl_2$ caused a large significant induction in the levels of Hsp32 of approximately 3-4 fold. This increase was observed initially following dosing with 0.1mg/kg with levels significantly elevated at all doses of $CdCl_2$ up to 2mg/kg (figures 5.16 and 5.17).

5.4.4 Discussion

Cadmium has been shown previously to induce the levels of Hsp72/3 in hepatocytes *in vitro*, this study demonstrates that cadmium *in vivo* produces similar alterations to the

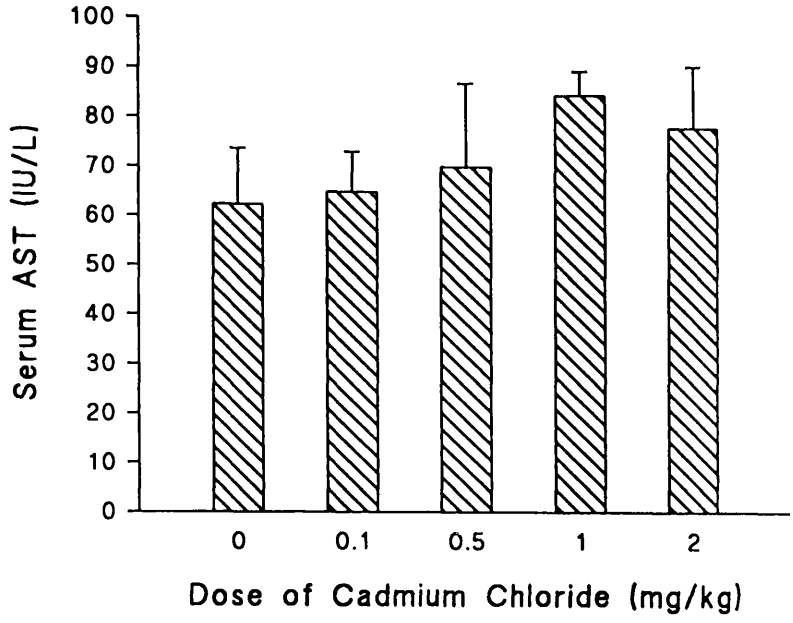


Figure 5.11 Effect of in vivo administration of cadmium chloride on serum AST levels 6 hours post dose. Values are expressed as mean IU/l ± SEM. n=4.

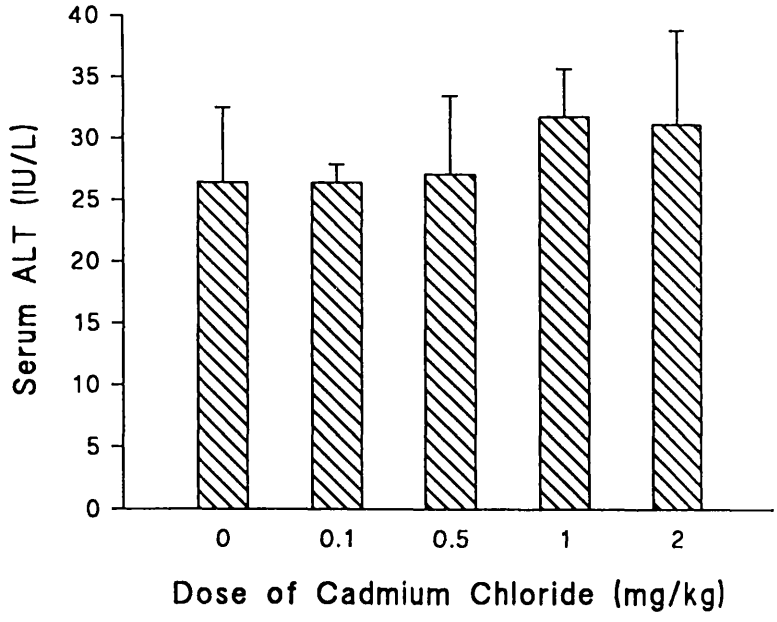


Figure 5.12 Effect of in vivo administration of cadmium chloride on serum ALT levels 6 hours post dose. Values are expressed as mean IU/l ± SEM. n=4.

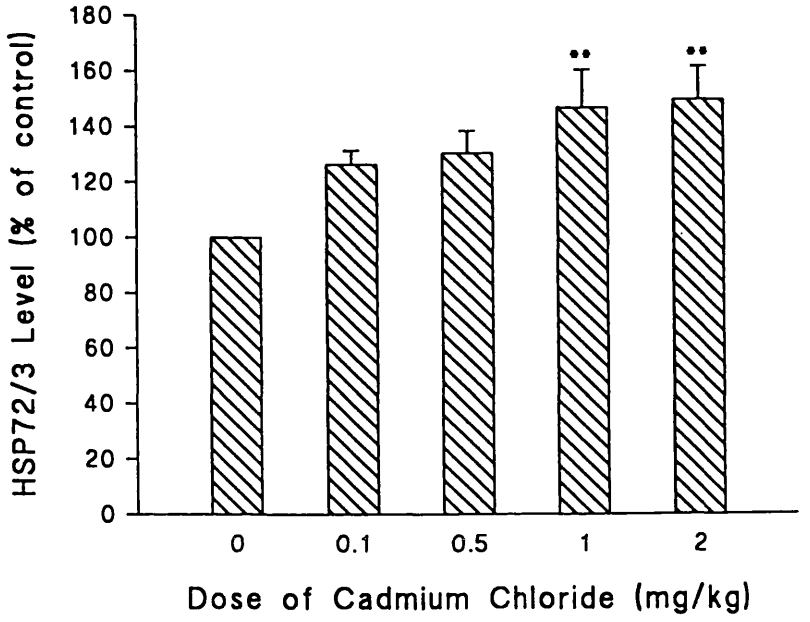


Figure 5.13 Effect of in vivo administration of cadmium chloride on liver Hsp72/3 levels 6 hours post dose. Values are expressed as mean % change from control ± SEM. n=4. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (**p<0.01)

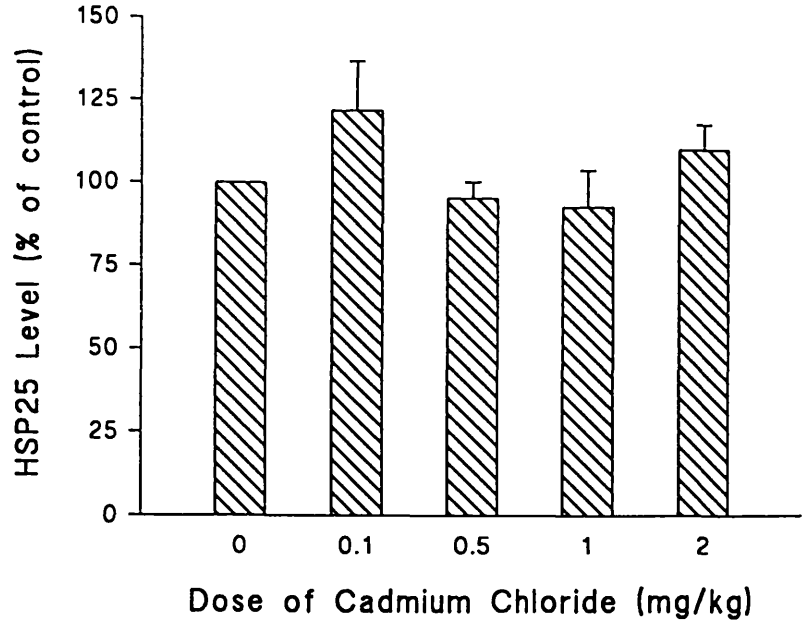
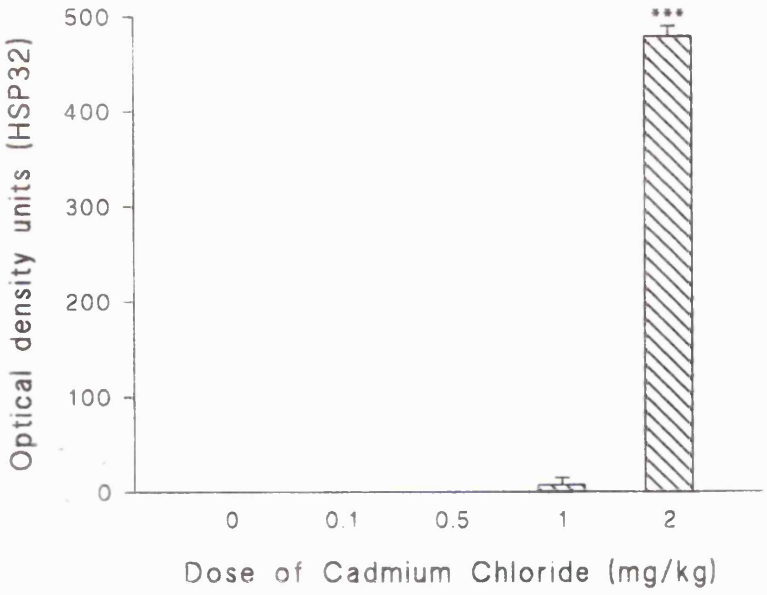


Figure 5.14 Effect of in vivo administration of cadmium chloride on liver Hsp25 levels 6 hours post dose. Values are expressed as mean % change from control ± SEM. n=4.



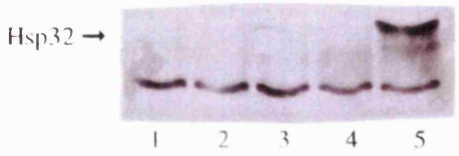


Figure 5.15 Effect of in vivo administration of cadmium chloride on liver Hsp32 (HO-1) levels 6 hours post dose. Values are expressed as mean arbitrary optical density units (due to protein being undetectable in control samples) ± SEM, n=4. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (***$p<0.001$). Bottom panel shows a typical Western blot of the effect of $CdCl_2$ on liver Hsp32 levels 6 hours post dose, lanes as follows; Lane 1, 0mg/kg; lane 2, 0.1mg/kg; lane 3, 0.5mg/kg; lane 4, 1mg/kg; lane 5, 2mg/kg $CdCl_2$. Solid arrow represents Hsp32 (HO-1).

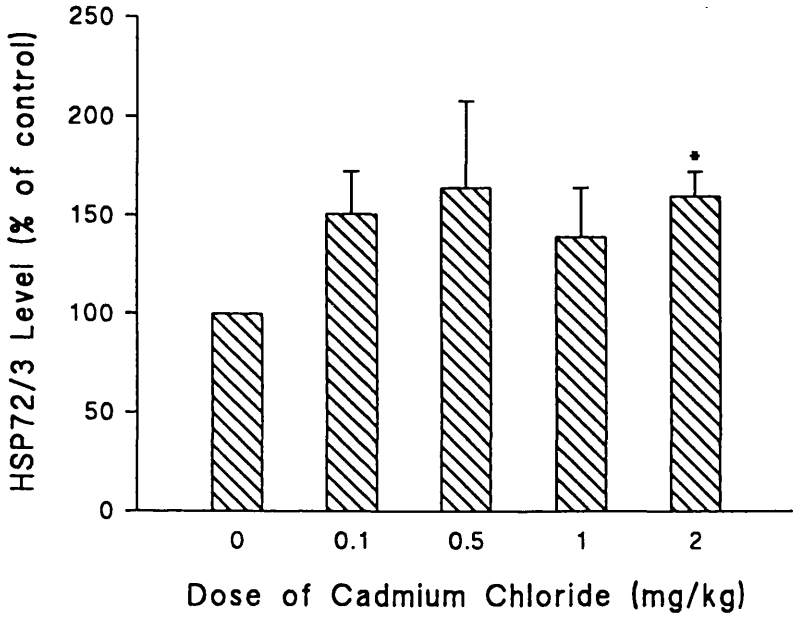
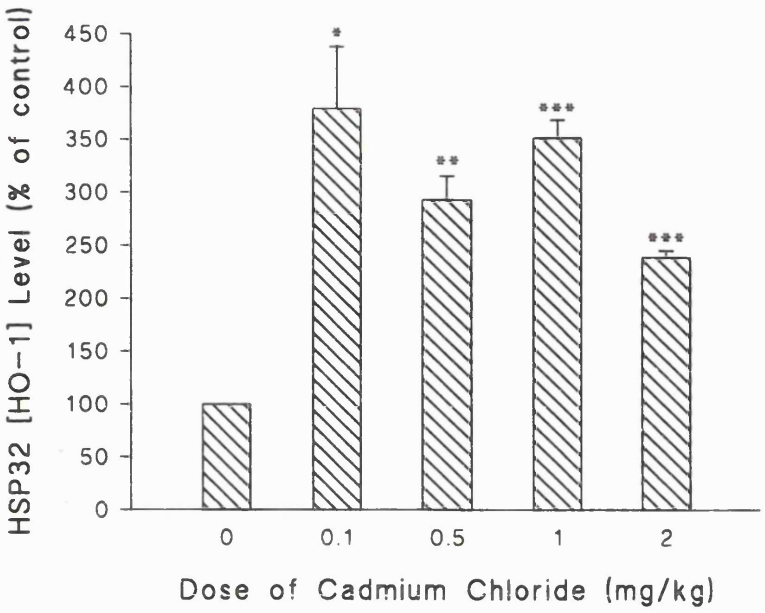


Figure 5.16 Effect of in vivo administration of cadmium chloride on testicular Hsp72/3 levels 6 hours post dose. Values are expressed as mean % change from control ± SEM. n=4. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*$p<0.05$)



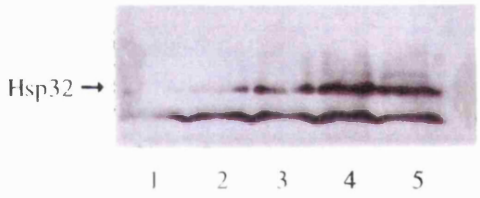


Figure 5.17 Effect of in vivo administration of cadmium chloride on testicular Hsp32 (HO-1) levels 6 hours post dose. Values are expressed as mean % change from control ± SEM. n=4. *Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Bottom panel shows a typical Western blot of the effect of $CdCl_2$ on liver Hsp32 levels 6 hours post dose. lanes as follows: Lane 1, 0mg/kg; lane 2, 0.1mg/kg; lane 3, 0.5mg/kg; lane 4, 1mg/kg; lane 5, 2mg/kg $CdCl_2$. Solid arrow represents Hsp32 (HO-1).

levels of stress proteins. As with the results from hepatocyte monolayers cadmium had no effect on hepatic Hsp25 levels. However, exposure of rats to cadmium chloride caused the induction of Hsp72/3 in the liver following administration of doses of 1 and 2 mg/kg. This result is in agreement with previous findings of Goering *et al.* (1993b) who demonstrated elevated hepatic Hsp72/3 levels following exposure to cadmium chloride. Comparison of the *in vivo* data presented here with results from *in vitro* studies, in chapter 4.4, is problematic as it is known that there is an approximate 10-fold accumulation of cadmium in hepatocytes *in vivo* (Goering *et al.* 1993b. Sauer *et al.* 1997). In order to overcome this problem it was decided to directly compare the intracellular load of cadmium in the hepatocytes *in vivo* and *in vitro*. The uptake of cadmium into hepatocyte *in vitro* is time and dose-dependent and linear up to concentrations of 20μM up to 20 hours, however it has been demonstrated that two hours following administration of 3μM $CdCl_2$ the intracellular load of cadmium is approximately 0.5nmol Cd/g (Hellström-Lindahl and Oskarsson 1989). Therefore, comparison of the intracellular load of cadmium in hepatocytes *in vivo* and *in vitro* would allow a direct comparison of the toxicity of cadmium *in vivo* and *in vitro*. Levels of Hsp72/3 in hepatocyte monolayers were significantly increased when the intracellular cadmium load was estimated to approximately be 0.83-1.66μmols Cd/g (equivalent to 5-10μM $CdCl_2$ [estimated from Hellström-Lindahl and Oskarsson 1989]). This is in comparison to the effect of cadmium *in vivo* where it was determined that Hsp72/3 levels were elevated when the estimated load of cadmium was approximately 0.069-0.138μmols Cd/g (equivalent to 1-2mg/kg $CdCl_2$) (estimated from Goering *et al.* 1993b). Therefore, the intracellular concentration of cadmium required to induce Hsp72/3 is substantially lower in hepatocytes *in vivo* when compared to the concentration required in hepatocyte monolayers. However, it should be noted that comparisons of cadmium concentrations *in vivo* and *in vitro* might not directly relate to toxicity observed *in vivo* and *in vitro* due to differences in the intracellular binding of the metal.

Testicular toxicity is a common characteristic of acute cadmium intoxication. Following cadmium exposure the toxicity consists of necrotic and apoptotic damage. The mechanism of cell death appears to depend on the dose of cadmium used, low doses of cadmium (approximately 1mg/kg) appear to induce testicular apoptosis whereas higher doses

(2.5mg/kg) induce extensive necrosis in germinal cells (Jones *et al.* 1997, Sauer *et al.* 1997). The role of heat shock proteins in apoptotic cell death is just beginning to be understood but it is known that elevated heat shock proteins protects cells from apototic cell death. The mechanisms that are thought to be involved include the protection of chromatin from proteases and nucleases involved in apoptotic cell death, in addition to this elevated levels of heat shock proteins have been shown to prevent the activation of stress kinases, such as JNK and p38, which are involved in apoptosis (Gabai *et al.* 1995, Gabai *et al.* 1997).

The effect of cadmium on Hsp32 levels in hepatic tissues demonstrated that doses of less than 2mg/kg had no effect on Hsp32. Ossola and Tomaro (1995) showed levels of hepatic Hsp32 were elevated following administration of $CdCl_2$ but this was at a relatively high dose of 100μmol $CdCl_2$/kg (equivalent to 10.8mg/kg). However, at this dose alterations in GSH levels were observed indicative of oxidative stress. This significant depletion in GSH implies that the dose of $CdCl_2$ used was relatively toxic and so the induction of Hsp32 was most likely due to the generation of reactive oxygen species within the cell. However, the results presented here demonstrate that levels of Hsp72/3 and Hsp32 were significantly elevated at doses of $CdCl_2$ which had no effect on any of the other biochemical parameters measured. The induction of Hsp32 (HO-1) in hepatic and testicular tissues by cadmium is of particular interest as the heme oxygenase gene contains a region which confers the cadmium-mediated induction of the gene. The 10-base pair sequence, TGCTAGATTT, present on the gene is required for cadmium-mediated inducibility of heme oxygenase; this mechanism of induction of heme oxygenase is different from the induction mediated by hemin or sodium arsenite exposure (Takeda *et al.* 1994). Therefore the induction of heme oxygenase following cadmium exposure must be of particular physiological importance. There are two possibilities for the importance of the cadmium-mediated heme oxygenase induction. Firstly, the oxidative stress induced by cadmium will be ameliorated by the elevated levels of heme oxygenase present due to the increased formation of the antioxidant bilirubin (Ossola and Tomaro 1995). The second possibility is that considering the biological function of the minute amounts of cadmium normally present in humans has yet to be determined it is feasible that cadmium, by its ability to bind to free sulphydryl groups and nucleic acids, may act as a physiological

mediator in the signal transduction pathway leading to the induction of heme oxygenase (Vallee and Ulmer 1972). Therefore, exposure of cells to cadmium will in effect cause an enhancement of the physiological signal transduction system leading to the increased formation of heme oxygenase hence conferring an increased level of protection.

The lack of effect of cadmium on hepatic GSH levels is confusing as it is known that cadmium causes an increase in the formation of reactive oxygen species (Funakoshi *et al.* 1997) and so GSH levels should be decreased particularly at doses which were able to decrease ATP levels. In vitro cadmium depletes ATP and GSH levels in hepatocytes at approximately the same concentration but this was not observed *in vivo*. The induction of Hsp32 (HO-1) in certain cell types is thought to be due to a decrease in cellular GSH levels (Applegate *et al.* 1990). However, it can be concluded that this is not the case *in vitro* as Hsp32 levels were elevated at doses which did not modify hepatic GSH levels.

The fact that levels of Hsp32 and Hsp72/3 were elevated in testicular and hepatic tissues at doses which did not affect any other biochemical parameters gives credence to the use of heat shock proteins as markers of toxicity. However, the elevation of stress proteins appears to be stress protein specific following cadmium exposure. Levels of hepatic Hsp25 and Hsp32 and testicular Hsp72/3 were only altered at doses which affected other biochemical parameters. The alterations in these stress proteins would, therefore, appear to be a result of the toxicity of cadmium in comparison to the changes in the other stress proteins which putatively play a role in the pathogenesis of cadmium toxicity.

5.5 Conclusions

This present study was designed to determine the effect of hydrazine and cadmium on the stress response in hepatic and testicular tissues in rats and in doing so allow correlations to be made between the *in vivo* and *in vitro* effects of the two compounds. The results suggest that alterations in the synthesis of stress proteins may be of use as early markers of cell injury. However, there are a number of considerations that must be addressed. Firstly, the alterations in stress protein levels were stressor specific in that cadmium

induced alterations in certain stress proteins whereas hydrazine did not alter the synthesis of any of the stress proteins measured. Secondly, the response to cadmium is stress protein specific, exposure of rats to cadmium caused the elevation of Hsp72/3 and Hsp32 but did not alter levels of Hsp25. Finally, some of the alterations in stress protein levels following chemical exposure are organ specific, doses of cadmium that elevated testicular Hsp32 levels failed to alter levels of hepatic Hsp32. However, bearing these caveats in mind the measurement of stress protein synthesis *in vivo* does appear to have potential as a sensitive marker of toxicity.

Comparison of the *in vivo* toxicity of the compounds used here with the toxicity previously determined *in vitro* demonstrates large differences in sensitivity. When comparisons between *in vivo* and *in vitro* toxicity are determined certain considerations must be taken into account namely that the *in vivo* system is a dynamic one in that hydrazine is being continuously supplied to the liver *via* the blood and in addition the compounds are subjected to metabolism and protein binding. The dosing regime used *in vitro*, ie exposure to the compound for two hours and recovery, was chosen to avoid exposing the hepatocytes continually to the compound. However, even with this different dosing method used there were still large differences in the toxicity of the compounds *in vivo* and *in vitro*. With respect to hydrazine the biochemical effects *in vivo* were observed at lower concentrations than those used *in vitro*. However, comparison of plasma hydrazine concentration *in vivo* with the concentration of hydrazine in hepatocyte media *in vitro* may not be as accurate as comparison of the sub-cellular concentrations of hydrazine, but these are as yet unknown.

Direct comparison of the toxicity of cadmium *in vivo* with its toxicity *in vitro* is hampered by the different uptake of cadmium into hepatocytes *in vivo* and *in vitro*. Exposure of hepatocytes *in vivo* to cadmium results in an accumulation of cadmium in the cytoplasm and nuclei of the cells, however over time the cadmium is translocated to the cytoplasm where it is bound to metallothionein. This is in contrast to the accumulation of cadmium in hepatocytes *in vitro* where it was found that the distribution of cadmium in the cytoplasm and nucleus was dependent on the dose of cadmium used. With increasing cadmium concentrations the proportion found in the nucleus was elevated where it was

determined to be bound to nuclear material (Bayersmann and Hechtenberg 1997). This difference in cadmium distribution in hepatocytes may be one of the reasons for difference in hepatotoxicity of cadmium *in vivo* and *in vitro*. Therefore, as with hydrazine, for a more accurate picture of the comparative toxicity of cadmium *in vivo* and *in vitro* the sub-cellular localisation of cadmium should be determined instead of, as in this study, comparison of the total intracellular load of cadmium.

The study of alterations in stress protein levels *in vivo* following chemical exposure has to be done with care due to the fact that stress protein levels can be altered as part of the normal physiology of a cell. Of particular interest here is the fact that the induction of Hsp70 synthesis and mRNA were significantly lower in hepatocytes isolated from old rats (22-28 months old) when compared to levels in hepatocytes isolated from young rats (4-7 months old). However this age related decline in Hsp70 synthesis was reversible by caloric restriction (Heydari *et al.* 1993). This raises the possibility that investigations into the use of stress proteins as markers of toxicity in older rats will yield little response as many compounds that induce a stress response in younger rats will not do so in rats of an older age. Other problems associated with the *in vivo* study of stress proteins includes the possibility of elevated levels of basal stress proteins due to the handling of the animals prior to and during the investigations which may cause inaccurate responses after exposure to chemicals so raising the possibility of false positive responses being generated. In addition to this, due to the physiological role of stress proteins, elevated levels may not always represent a response to an adverse stimuli but may be due to changes in environmental conditions or particularly changes in steroid hormone levels which have been shown to induce levels of stress proteins in animals (Fuqua *et al.* 1989). However, careful design of *in vivo* studies with the use of appropriate controls should minimise any such variations and generation of any false positive results.

In conclusion, both compounds used here showed an increased toxicity in hepatocytes *in vivo* when compared to the effects *in vitro*. Exposure of rats to hydrazine caused no alterations in the synthesis of stress proteins in hepatic or testicular tissues, this is in comparison to the effect of cadmium which produced organ- and stress protein-specific changes in the synthesis of heat shock proteins in hepatic and testicular tissues prior to any

biochemical toxic effect of cadmium.

Chapter 6.

Investigations into the stress response in liver spheroids

6.1 Introduction

The development of long term hepatocyte models was initiated due to the problems associated with primary hepatocyte monolayer cultures namely their limited lifespan and the loss of liver specific functions. One long term *in vitro* hepatocyte model which is being investigated to overcome these problems is the use of liver spheroids. Spheroids are formed when hepatocytes are cultured on a non-adherent substratum thereby causing the cells to remain in suspension. Culture of the cells on a rotary shaker causes increased cell to cell contact and hence increased spheroid formation. The structure and biochemical characteristics of liver spheroids have been discussed previously in chapter 1.8. This series of experiments was performed to investigate the development and use of spheroids as a hepatocyte model for studying the stress response. This would, therefore, allow a direct comparison to be made between long and short term hepatocyte cultures in their response to hepatotoxins. With spheroids maintaining more *in vivo* like characteristics than primary hepatocyte cultures it was hoped that the stress response in the spheroids would be a more accurate representation of the *in vivo* response. Initially, these studies looked at growth of spheroids both morphologically and biochemically. In order to directly compare the two hepatocyte models of primary hepatocyte and liver spheroids it was decided to again investigate the basal profile of stress proteins during spheroid development and determine the effect of hydrazine and cadmium on the stress response in addition to other biochemical markers of toxicity. These are the first investigations into the use of the stress response in liver spheroids as a marker of toxicity following chemical exposure.

6.2 Study of the basal morphology of spheroids

6.2.1 Aim of study

The initial aim of this study was to investigate, morphologically and histologically, the formation of multicellular spheroids from single hepatocytes over a 15 day period. The viability of the individual hepatocytes was also studied throughout this 15 day period.

6.2.2 Methods

Hepatocytes were isolated as described previously in chapter 2.5 and cultured on a rotary shaker at a density of $0.5x10^6$ cells/ml. The cells were studied morphologically with a light microscope on a daily basis, trypan blue was added to a sample of spheroids each day to determine the viabilities of the cells during the growth of the spheroids. On day 6 of culture samples of spheroids were taken for histological analysis to determine intracellular structure and viability. Determination of the optimal culture conditions and sampling methods for spheroids was performed by Hamilton (1998).

6.2.3 Results and discussion

Within 24 hours the hepatocytes had formed large irregular cellular masses. Upon dispersion of these masses, aggregates of 3 to 5 cells in diameter were visible within 24 hours. Over the next 6 days the spheroids grew in size from approximately 30 cells on day 3 to about 60-100 by about day 6 of culture with their overall structure becoming more regular and compact (figures 6.1 and 6.2). The growth of the spheroids appeared to be due to merging and fusion of smaller spheroids to form larger spheroids and was not due to cell division. Staining of the spheroids over this growth period showed that the hepatocytes contained within the spheroids were viable and any non-viable cells present were not incorporated into the spheroids. The non-viable cells were eventually removed when the spheroids were washed and the medium changed.

By day 6 spheroid growth stopped and the majority of the cultures consisted of spheroids of approximately the same size. Figure 6.3 shows a typical section through a day 6 rat spheroid. Histological analysis of the spheroids showed that within the spheroids hepatocytes maintained their normal morphology as would be seen with hepatocytes in vivo. There was little evidence of degeneration or necrosis in the spheroids. However, discrete cytoplasmic vacuolation was visible typical of that seen with fat accumulation. Staining of the spheroids with Oil Red O showed evidence of cytoplasmic fat accumulation (figure 6.4). Another characteristic of the spheroids that was observed included the presence of a layer of flattened hepatocytes on the periphery of the spheroids.

Analysis of the larger spheroids showed centres which were necrotic which was probably

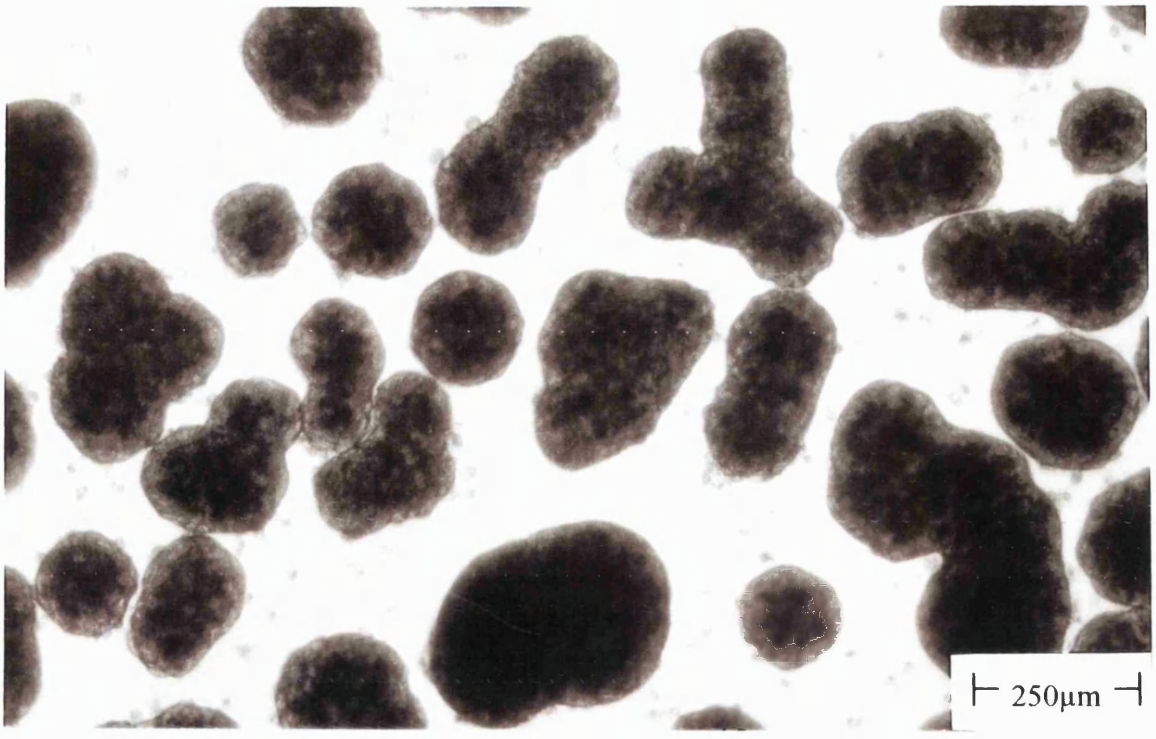


Figure 6.1 Adult rat spheroids after 3 days in culture

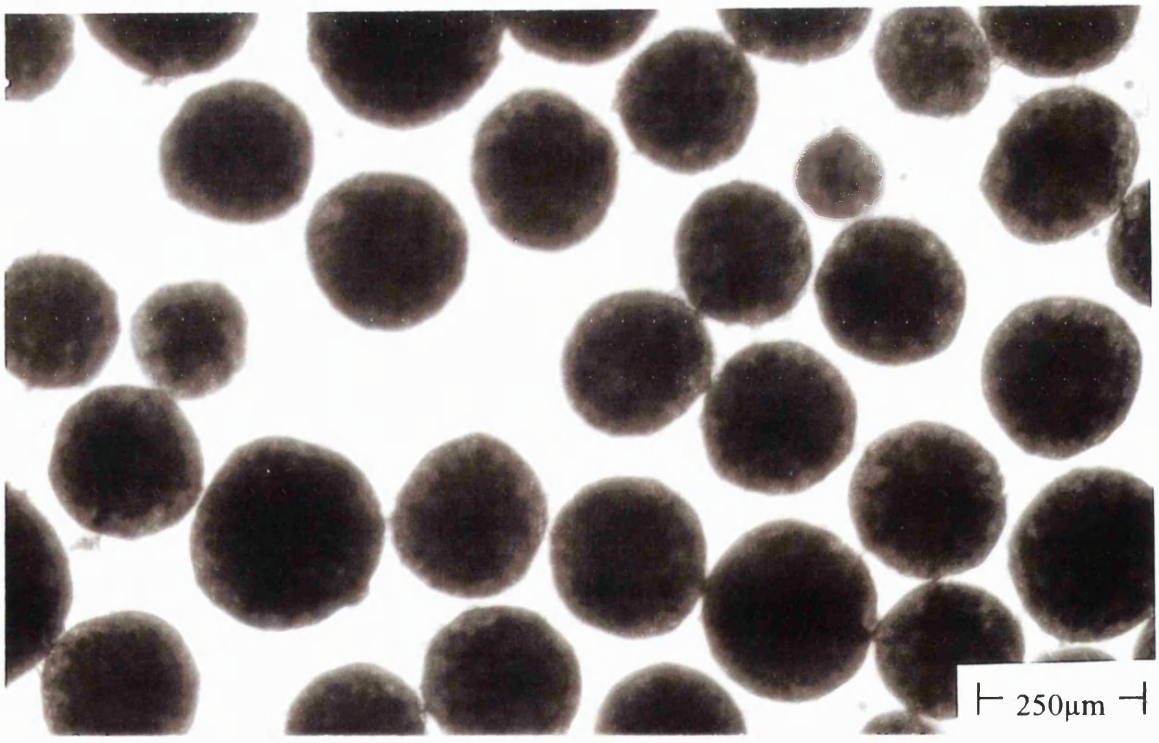


Figure 6.2 Adult rat spheroids after 6 days in culture

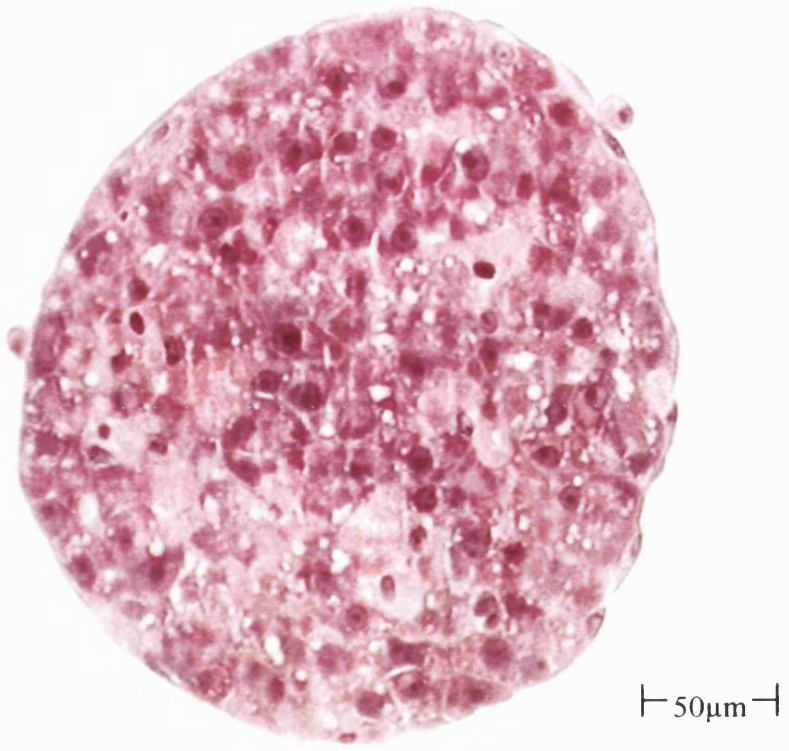


Figure 6.3 LM section of adult rat spheroids after 6 days in culture, stained with haematoxylin and eosin (x20 objective)

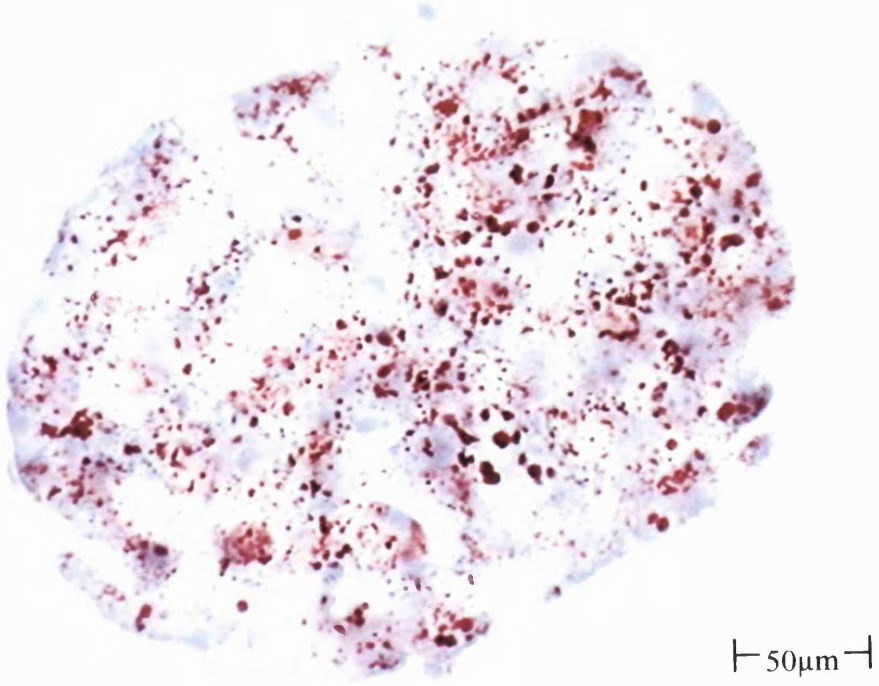


Figure 6.4 LM section of adult rat spheroids after 6 days in culture, stained with Oil Red O(x20 objective)

due to lack of oxygen and nutrients diffusing through the spheroids to the hepatocytes in the centre. This would probably explain the apparent cessation of spheroid growth by about day 6. Spheroids were maintained for 15 days in the initial studies and showed consistent preservation of spheroidal structure and viability throughout this period.

6.3 Investigations into the basal biochemical parameters during spheroid growth

6.3.1 Aim of study

The main aim of this study was to determine the basal levels of GSH, ATP levels and albumin secretion from spheroids over a 15 day period. This would then allow an assessment of the viability of the spheroids and allow a direct comparison to be made to the biochemical measurements taken from primary hepatocytes during culture.

6.3.2 Methods

Hepatocytes were isolated and cultured for spheroid development as described in chapter 2.5. Samples were taken from freshly isolated hepatocytes and from developing spheroids every couple of days and analysed for levels of GSH, ATP and albumin secretion over a 15 day period.

6.3.3 Results

6.3.3.1 GSH levels

GSH levels were determined every few days in culture. On day 1 levels of GSH in spheroids were 11nmol/mg protein and even though throughout the 15 day period GSH levels fluctuated greatly the general trend was that spheroids maintained their GSH levels well. By day 15 of culture GSH levels in spheroids were increased approximately 2-fold when compared to levels on day 1 (table 6.1).

6.3.3.2 Albumin secretion

Secretion of albumin was also measured as an indicator of cellular integrity. Levels of albumin secretion were initially low in spheroids however within 7 days levels had increased 4-fold to approximately 20μg albumin/mg protein. These levels were maintained

by the spheroids after 15 days in culture. As with GSH the levels of albumin secretion fluctuated greatly over the culture time which may be due to fluctuations in the overall functional viability of the spheroids and not due to changes in secretion levels (table 6.1).

6.3.3.3 ATP levels

ATP levels were low for some reason in both spheroids and primary hepatocytes. In the case of spheroids levels of ATP showed an apparent decrease in the first 3 days of culture when compared to levels in freshly isolated hepatocytes. However, levels of ATP increased 4-fold by day 6 of culture and remained increased up to day 15 when ATP levels in spheroids were increased approximately 2-fold when compared to levels in freshly isolated hepatocytes (table 6.1).

6.3.4 Discussion

Measurement of biochemical parameters over the 15 day period of spheroid development showed levels of ATP, GSH and secretion of albumin varied a large amount but overall all parameters were elevated by day 15 of culture when compared to levels in freshly isolated hepatocytes. The variation observed particularly in the levels of GSH and albumin secretion over the 15 day period probably represents differences in the quality of spheroids between individual wells and not actual differences in GSH levels or albumin secretion. These levels of GSH and albumin secretion correlate well with previously published data showing maintenance of these biochemical parameters in spheroids over a 13 day period (Juillerat *et al.* 1997). However, spheroid ATP levels were approximately 3-4 fold lower than values described previously by Juillerat *et al.* (1997). The reasons for this discrepancy are unclear, although ATP levels in the spheroids in this study were much lower than expected they did show an increase over the 15 day period. Considering ATP, GSH and albumin secretion are commonly viewed as indicators of cellular viability the results indicate that during culture the spheroids remained viable over the 15 day period.

Table 6.1 Basal levels of ATP, GSH and albumin secretion during spheroid development over a 15 day period

Day	GSH nmol/mg protein	SEM	Albumin secretion µg/mg protein/24hr	SEM	ATP nmol/mg protein	SEM
0	8.75	3.9	NA	NA	1.45	0.50
1	11.11	3.1	5.67	2.5	0.54	0.06
3	7.6	2.8	1.35	0.8	0.66	0.32
6	16.45	0.4	8.07	3.9	1.94	0.37
8	10.29	3.1	19.93	13.4	1.17	0.72
10	16.35	1.9	24.21	4.1	1.79	0.29
13	6.50	2.4	12.18	1.9	1.19	0.25
15	23.95	2.4	25.35	4.2	2.85	0.19

Values are expressed as mean of four experiments.

NA; not applicable

6.4. Investigations into the basal stress response during spheroid growth

6.4.1 Aim of study

In parallel to the study investigating the basal biochemical parameters during spheroid growth it was also decided to determine the basal levels of the stress proteins Hsp72/3 and Hsp25 over a 13 day period during spheroid development. This would then allow comparison with the basal levels of these stress proteins in primary hepatocyte cultures generated in chapter 4.6 to determine whether a similar profile would be observed.

6.4.2 Methods

Hepatocytes were isolated and cultured to allow spheroid development to occur as described in chapter 2.5. A sample of rat liver was taken and analysed for basal levels of Hsp25 and Hsp72/3, as described previously in chapter 4.5.2, which are, hereafter, referred to as *in vivo* levels. Samples were then taken from freshly isolated hepatocytes and from spheroids every 2-3 days during culture and analysed for levels of Hsp25 and Hsp72/3. Samples in this study, and in all following studies were probed with anti-Hsp25 and anti-Hsp72/3 antibodies. Samples were not probed for Hsp32 due to specificity problems with the antibody.

6.4.3 Results

6.4.3.1 Hsp72/3 levels

Hsp72/3 levels were increased 3-fold in freshly isolated hepatocytes and in those spheroids sampled on day 1 of culture compared to levels determined *in vivo*. However, by day 3 levels had decreased to those seen in *in vivo* samples. From day 3 to day 13 of culture the levels of Hsp72/3 remained consistent with no apparent decrease or increase observed. (figure 6.5)

6.4.3.2 Hsp25 levels

In contrast to Hsp72/3 levels of Hsp25 showed a different basal pattern of induction over the 13 day period. Levels of Hsp25 in spheroids up to day 6 in culture were unchanged from levels determined *in vivo*. However by day 8 there was a 2-fold significant increase in Hsp25 levels. This significant increase continued on day 13 when levels of Hsp25 were

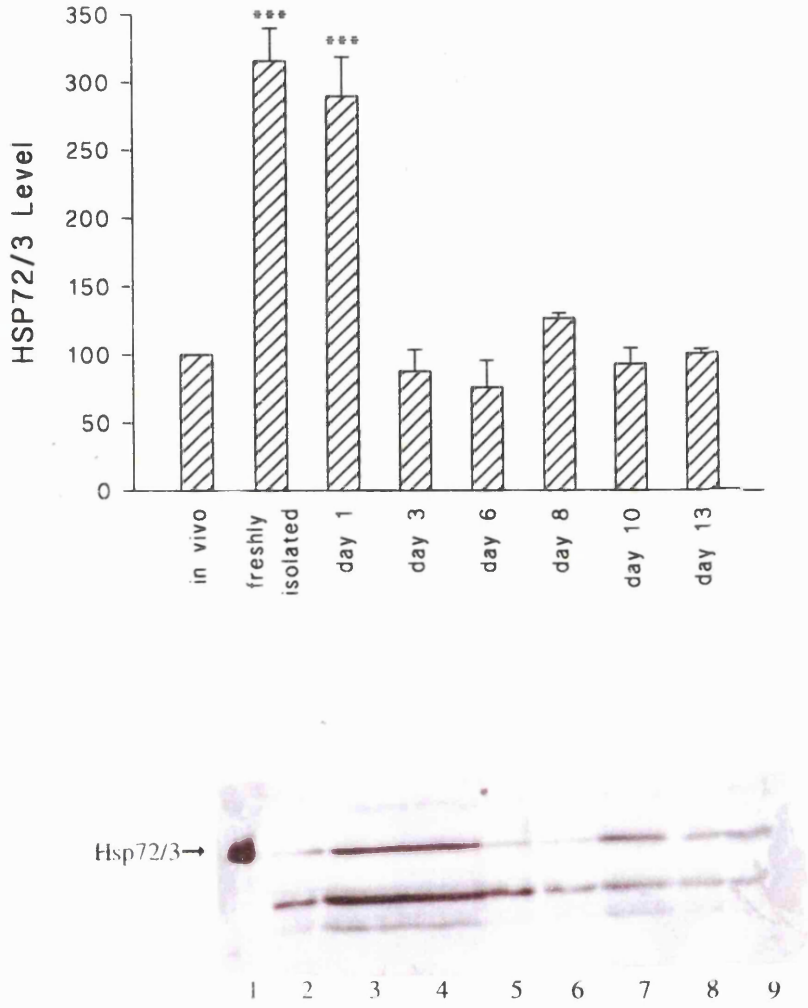


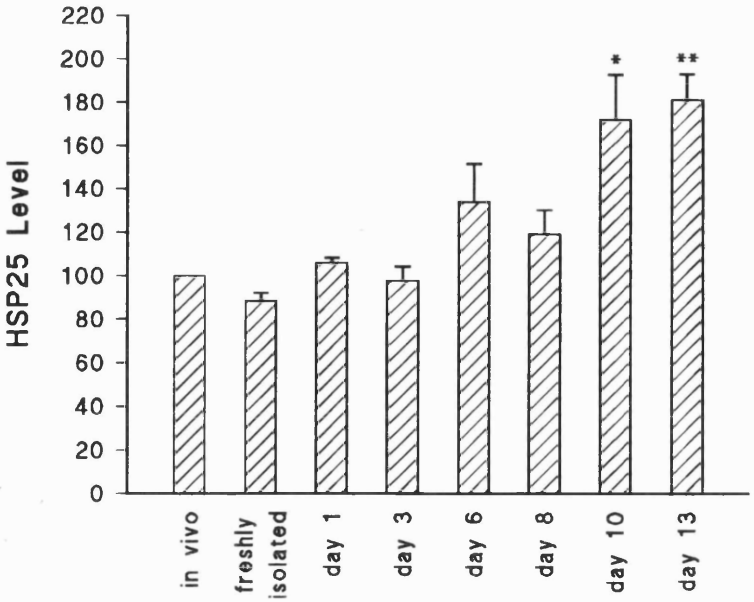
Figure 6.5 Basal levels of Hsp72/3 in liver spheroids during culture over a 13 day period. Timepoint in vivo refers to levels of Hsp72/3 in a sample of undigested liver, freshly isolated refers to levels of Hsp72/3 in freshly isolated hepatocytes. Values are expressed as % of in vivo control ± SEM. n=4. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (***P<0.001). Bottom panel shows a typical corresponding Western blot generated, lanes as follows; Lane 1, Hsp72/3 standard; lane 2, in vivo; lane 3, freshly isolated; lane 4, day 1; lane 5, day 3; lane 6, day 6; lane 7, day 8; lane 8, day 10; lane 9, day 13 of culture. Solid arrow represents Hsp72/3.

found to be increased 2-fold compared to *in vivo* levels (figure 6.6).

6.4.4 Discussion

Basal levels of Hsp72/3 and Hsp25 showed completely different profiles during the 13-day period of spheroid development when compared to levels observed previously in hepatocyte monolayers in chapter 4.6. Levels of Hsp72/3 increased significantly in freshly isolated hepatocytes and in spheroids on day 1 of culture thereby implying that the isolation of hepatocytes is causing damage to the cells. However, this data is in contrast to the levels of Hsp25 in spheroids on day 1 of culture and in freshly isolated hepatocytes which show no change. Comparison of these data with the results from chapter 4.6 which investigated the basal levels of Hsp72/3 and Hsp25 in primary hepatocytes raises some interesting questions. Firstly why were the levels of Hsp72/3 and Hsp25 significantly decreased in freshly isolated hepatocytes in chapter 4.6, but the results presented here showed no change in Hsp25 levels and additionally in this case Hsp72/3 levels were significantly elevated in freshly isolated hepatocytes. The reasons for this may be due to the different isolation procedures used for the hepatocytes, for the isolation of hepatocytes for monolayer culture the rats were anaesthetised and the liver cannulated in situ whereas when hepatocytes were required for spheroid development the animals were sacrificed prior to the removal of the liver and the individual liver lobes were cannulated ex-situ. The second procedure took considerably longer for the complete isolation of hepatocytes and so may be a more stressful procedure which could in theory explain the elevated levels of Hsp72/3 in the freshly isolated hepatocytes used for spheroids. However if this was the case you would expect levels of Hsp25 to be elevated as well but this was not observed. Another possibility is that the isolation of hepatocytes by either method does not in itself cause the induction of Hsp25 and the induction observed in chapter 4.6 is due to other reasons. One of these could be due to the use of anaesthetics in chapter 4.6 which may alter levels of Hsp25 in the cells and the fact that anaesthetics were not used here during the isolation procedure may explain why levels of Hsp25 were not elevated in freshly isolated hepatocytes.

Basal levels of Hsp72/3 returned to control values by day 3 of culture and remained so until day 13 of culture in comparison to Hsp25 levels which remained unchanged until day



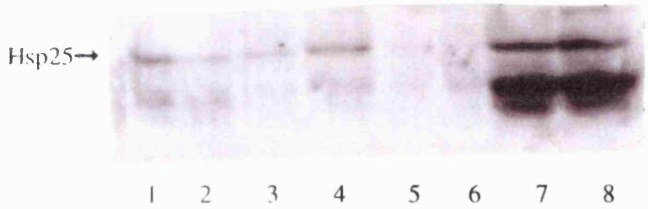


Figure 6.6 Basal levels of Hsp25 in liver spheroids during culture over a 13 day period. Timepoint in vivo refers to levels of Hsp25 in a sample of undigested liver, freshly isolated refers to levels of Hsp25 in freshly isolated hepatocytes. Values are expressed as % of in vivo control ± SEM. n=4. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*P<0.05, **P<0.01). Bottom panel shows a typical corresponding Western blot generated, lanes as follows; Lane 1, in vivo; lane 2, freshly isolated; lane 3, day 1; lane 4, day 3; lane 5, day 6; lane 6, day 8; lane 7, day 10; lane 8, day 13 of culture. Solid arrow represents Hsp25.

8 of culture after which time levels of Hsp25 were significantly elevated. These results imply that from day 3 to day 8 of culture the spheroids are under no stress whilst in culture. There is no evidence of oxidative stress in the cells during culture as levels of Hsp72/3 are unchanged from *in vivo* levels. The reasons why spheroids maintain control levels of stress proteins during culture up to day 8 and hepatocyte monolayers show elevated levels may be due to physical differences between the two culture models. Hepatocytes within the spheroids are very similar to hepatocytes found *in vivo* as they show extensive cell-cell contacts and tight and gap junctions are observed with structural integrity maintained throughout the spheroids (Roberts and Soames 1993). In addition to this, the hepatocytes show secretion of liver specific proteins and exhibit relatively well preserved levels of cytochrome P450 (Tong *et al.* 1992, Amman and Maier 1997). This is in comparison to primary monolayers which show a flattened morphology and thereby lose many of the characteristics of hepatocytes *in vivo*. Primary hepatocytes show a compacted cell morphology with smaller mitochondria present in addition to intermediate filaments being present (Yuasa *et al.* 1993). The fact that there is little discernible difference between hepatocytes *in vivo* and hepatocytes contained within liver spheroids probably explains why basal stress proteins remain at *in vivo* like values up to day 6 of culture.

The increased levels of Hsp25 from day 10 of culture onwards is harder to explain, little is known about the function of Hsp25 and so it hard to correlate the induction of Hsp25 with a certain cellular state. However, what is known is that Hsp25 is involved in the intracellular organisation of actin filaments (Benndorf *et al.* 1994). Following phosphorylation of Hsp25 it associates with actin filaments thus allowing actin polymerisation to occur (Craig *et al.* 1994). In addition to this it is known that the levels of low molecular weight stress proteins are developmentally regulated (Sanders 1993). With this knowledge it can be speculated that the increased levels of Hsp25 may be the result of two physiological states. The first possibility could be that after 10 days in culture the spheroids are exhibiting signs of cytoskeletal stress, this could be due to maintenance of the spheroidal structure itself or due to cytoskeletal damage incurred when the spheroids collide whilst in culture. This damage to the cytoskeleton would therefore require re-organisation of many microfilaments for repair purposes and so levels of Hsp25

are elevated. The other possibility is that the increased levels of Hsp25 is not in response to a stressful stimuli but due to the re-organisation of the cytoskeletal network required for spheroid culture. It is known that during the culture of spheroids single hepatocytes form multicellular spheroids, this formation requires the extensive re-organisation of many intracellular structures to form new cell-cell contacts. One of the structures that would be altered is the cytoskeletal network and so it is feasible to acknowledge that changes in actin polymerisation would occur during spheroid development and so levels of Hsp25 would vary during the spheroids development. However this structural re-organisation usually occurs from day 3 onwards but Hsp25 is only elevated from day 10. Therefore, the elevation of Hsp25 from day 10 may represent adverse changes to cytoskeletal structures during the culture of spheroids.

In summary, the basal levels of the stress protein Hsp72/3 are elevated following hepatocyte isolation but during the development of spheroids levels return to control values. However after day 8 of culture levels of Hsp25 began to increase significantly. The reasons for this are unclear but may represent a response to cellular stress or be part of the development process for spheroids. With consideration that the basal stress proteins were stable between day 3 and day 8 it was decided to perform any toxicity studies investigating the response of cells to chemical stimuli on day 6 of culture.

6.5 Investigations into the basal cellular parameters in hepatocyte monolayers

6.5.1 Aim of study

In parallel to the basal biochemical measurements in spheroids measurements of ATP levels, GSH levels , Hsp25, Hsp72/3 and albumin secretion were also taken from primary hepatocytes in culture over a 76 hour period. This was performed to allow direct comparisons to be made with the basal biochemical measurements taken from spheroids during culture and to ensure that the use of a different strain of rat or different isolation procedure did not alter the basal levels of heat shock proteins as observed in chapter 4.6.

6.5.2 Methods

Hepatocytes were isolated and cultured for the culture of hepatocyte monolayers as described in chapter 2.6. Samples were taken initially from undigested liver and freshly isolated hepatocytes and subsequently from hepatocyte monolayers twice daily for four days. Samples were analysed for ATP, GSH, albumin secretion, Hsp25 and Hsp72/3.

6.5.3 Results

Levels of GSH and ATP did not alter significantly over the time-course of the experiment. Measurement of the secretion of albumin, a liver specific marker, showed that levels initially were high. However, within 28 hours secretion of albumin into the medium had decreased by 70% when compared to initial levels. This decrease continued over the duration of the experiment with levels approaching zero within 70 hours of culture (table 6.2). The levels of Hsp72/3 and Hsp25 were determined in hepatocyte monolayers over a 76 hour period. These results were consistent with the previous study in chapter 4.6 in that levels of Hsp72/3 showed an apparent decrease in freshly isolated hepatocytes with levels showing a 2-fold increase over the next 30 hours but by 54 hours levels were significantly decreased. The pattern of induction of Hsp25 was almost identical to that found with Hsp72/3 in that there was an initial decrease in freshly isolated hepatocytes followed by an increase in levels over the next 30 hours and finally levels were significantly decreased by 54 hours. The levels of these stress proteins then decreased over the remaining duration of the experiment with levels further decreased at 76 hours (data not shown). The results referring toalterations in stress protein levels were identical to the results investigating basal heat shock protein levels in hepatocyte monolayers in chapter 2.6.

6.5.4 Discussion

This study was performed mainly to determine whether the use of a different strain of rat or the use of a different hepatocyte isolation procedure had an effect on the basal levels of Hsp25 and Hsp72/3 in hepatocyte monolayers when compared to the levels determined previously in chapter 4.6. The results showed that there was no difference in the basal levels of Hsp25 and Hsp72/3 between these results and those from chapter 4.6, therefore the use of a different strain of rat or hepatocyte isolation procedure has no effect on basal

Table 6.2 Basal levels of GSH, ATP levels and albumin secretion over 76 hours in hepatocyte monolayers

Hours in culture	GSH nmol/mg protein	SEM	Albumin secretion µg/mg protein/24hr	SEM	ATP nmol/mg protein	SEM
0	8.75	3.9	NA	NA	1.45	0.5
4	6.87	1.2	25.47	3.0	2.68	1.4
22	8.62	2.0	18.23	2.7	2.60	0.5
28	10.37	2.0	7.17	4.4	2.67	0.6
46	13.87	4.0	8.71	1.9	3.23	0.9
52	15.59	3.1	11.19	10.2	4.41	1.2
70	8.08	1.0	0.43[a]	0.3	2.56	0.6
76	10.04	1.9	1.44[a]	1.4	5.24	1.8

Values are expressed as mean of four experiments.

Letters indicate values significantly different from corresponding control values using the Dunnett's t-test (a $P<0.001$)

NA; not applicable.

stress protein levels in hepatocyte monolayers. These findings are contradictory with some of the conclusions in chapter 6.4 as to the lack of induction of Hsp25 in spheroids on day 1 of culture. The reason for this then was postulated to be the different hepatocyte isolation procedure but as demonstrated here the identical isolation procedure elevated Hsp25 levels hepatocyte monolayers. Therefore it appears that when hepatocytes were isolated for monolayer culture levels of Hsp25 were elevated on day 1 of culture, however when the identical hepatocytes were cultured for spheroid development there was no increase in Hsp25 levels observed on day 1 of culture.

The reasons for this discrepancy between the two hepatocyte models could be explained by differences in culture methods such as the use of different media or the addition of foetal calf serum to spheroids whilst in culture. However, one of the most likely reasons is due to the structural differences between spheroids and primary hepatocyte monolayers. Once in culture primary hepatocytes attach to the collagen coated surfaces and in doing so adopt a flattened shape as opposed to the usual three dimensional hepatocyte structure. This change in hepatocyte structure would clearly cause an alteration in the hepatocytes cytoskeletal structure and as mentioned previously Hsp25 is thought to be involved in the regulation of the cytoskeleton. Therefore it seem feasible that the change in the hepatocytes structure may be causing the induction of Hsp25. This is in comparison to hepatocytes used for spheroid development. Here the hepatocytes retain their original three dimensional change and so less alterations are theoretically made to their cytoskeleton. This would explain the observation that in the first few days of culture Hsp25 levels were not elevated in those hepatocytes that were cultured as spheroids.

6.6 Preliminary dosing study with hydrazine and cadmium in spheroids

6.6.1 Aim of study

Work investigating the toxicity of compounds in spheroids had shown that the toxicity of certain compounds was markedly less in spheroids when compared to results from studies utilising hepatocytes monolayers (personal communication G. A. Hamilton). One of the reasons for this may include problems of accessibility of the compound to those

hepatocytes situated deep within the spheroids. For this reason a preliminary dosing study was performed to determine the toxicity of hydrazine and cadmium chloride in spheroids.

6.6.2 Methods

Hepatocytes were isolated and cultured for spheroid development as in chapter 2.5. Day 6 spheroids were exposed to either hydrazine at concentrations of 0, 20, 50 and 100mM or cadmium chloride at concentrations of 0, 0.3, 1 and 3mM. Spheroids were exposed to the compounds for 2 hours, washed with sterile PBS and fresh medium added. Samples were then taken for LDH and GSH measurement over the next 78 hours.

6.6.3 Results

Concentrations of 20 and 50mM hydrazine had no effect on LDH leakage over 78 hours with the exception that at 48 hours post dose administration of 50mM hydrazine caused a significant increase in LDH leakage. Dosing with 100mM caused a slight but significant increase in LDH leakage 30 hours post dose which remained slightly elevated until 78 hours (table 6.3). Exposure of spheroids for two hours to hydrazine at any concentration did not decrease levels of GSH at any point during the experiment (table 6.4). Examination of the spheroids by light microscopy showed that there was no obvious difference in morphology between control and hydrazine dosed spheroids.

Exposure of spheroids to cadmium chloride for two hours caused a significant increase in LDH leakage first observed 2 hours post dose following administration of 1 and 3mM cadmium. Leakage of LDH remained significantly increased throughout the duration of the experiment following dosing with 0.3, 1 and 3mM cadmium (table 6.5). Spheroid GSH levels were significantly decreased following administration of 1 and 3mM $CdCl_2$ 4 hours post dose but levels appeared to show a recovery to control values over the following 79 hours (table 6.6). All the dosed spheroids appeared blebbed and showed changes in spheroid morphology indicative of necrotic changes.

With the initial results of hydrazine showing no overt signs of toxicity to the spheroids it was decided to repeat the study using higher concentrations of hydrazine. Measurements of LDH and GSH were taken at 2 and 6 hours post dose only. The concentrations used

Table 6.3 Effect of increasing concentrations of hydrazine on LDH leakage from liver spheroids

	Dose of hydrazine			
Hours post dose	0mM	20mM	50mM	100mM
0	80.7 ± 6.4	40.1 ± 5.9	58.6 ± 6.2	59.2 ± 6.6
2	40.5 ± 3.2	47.9 ± 3.5	40.2 ± 1.5	56.2 ± 2.5
4	43.4 ± 4.0	23.2 ± 2.0	37.7 ± 1.5	49.5 ± 5.4
6	29.7 ± 2.3	48.7 ± 5.2	19.6 ± 4.0	26.7 ± 2.6
24	148.9 ± 19.4	73.3 ± 4.9	98.1 ± 6.1	64.8 ± 6.8
30	57.2 ± 5.5	53.8 ± 2.6	89.0 ± 4.1	241.5 ± 25.0[c]
48	57.9 ± 4.9	51.6 ± 4.2	92.1 ± 4.7[a]	125.7 ± 12.1 [c]
55	104.2 ± 11.1	82.8 ± 11.4	140.0 ± 11.5	208.9 ± 21.0[b]
72	88.0 ± 6.6	84.7 ± 8.7	131.7 ± 15.6	181.7 ± 20.9[b]
79	215.0 ± 31.2	121.2 ± 29.1	192.7 ± 9.7	304.8 ± 26.1[a]

Values are expressed as mean IU LDH leakage/mg protein ± SEM, n=3.

Letters indicate values significantly different from corresponding control values using the Dunnett's t-test:

[a] significantly ($p<0.05$) different from control cells

[b] significantly ($p<0.01$) different from control cells

[c] significantly ($p<0.001$) different from control cells

Table 6.4 Effect of increasing concentrations of hydrazine on GSH levels in liver spheroids

	Dose of hydrazine			
Hours post dose	0mM	20mM	50mM	100mM
4	17.2 ± 3.0	15.5 ± 0.9	19.5 ± 2.1	16.5 ± 1.4
6	ND	ND	ND	ND
30	10.5 ± 1.6	10.5 ± 0.7	6.2 ± 1.8	11.3 ± 1.0
55	21.0 ± 1.4	23.7 ± 2.8	18.9 ± 1.3	20.9 ± 0.8
79	43.7 ± 5.7	35.1 ± 6.1	39.6 ± 3.2	38.3 ± 4.7

Values are expressed as mean GSH (nmol/mg protein) ± SEM. n=3. ND; not determined.

Table 6.5 Effect of increasing concentrations of cadmium chloride on LDH leakage from liver spheroids

	Dose of cadmium chloride			
Hours post dose	0mM	0.3mM	1mM	3mM
0	45.6 ± 3.7	38.5 ± 5.1	34.1 ± 4.0	30.7 ± 2.9
2	16.7 ± 2.4	30.3 ± 1.8	127.0 ± 8.2[c]	235.2 ± 35.2[c]
4	24.5 ± 4.0	114.8 ± 2.6[c]	347.6 ± 54.3[c]	512.9 ± 21.0[c]
6	29.1 ± 1.2	254.6 ± 16.7[c]	275.2 ± 37.8[c]	376.3 ± 41.7[c]
24	75.4 ± 4.9	602.9 ± 78.9[c]	439.6 ± 21.6[c]	527.7 ± 46.7[c]
31	94.4 ± 14.8	748.6 ± 56.8[c]	627.7 ± 89.6[c]	926.6 ± 104.3[c]
48	116.1 ± 10.3	832.2 ± 107.3[c]	743.3 ± 37.6[c]	590.4 ± 89.7[c]
55	223.2 ± 17.3	1294.9 ± 157.2[c]	1130.9 ± 146.5[c]	1093.0 ± 79.3[c]
72	294.2 ± 28.6	1034.9 ± 128.4[c]	947.5 ± 68.4[c]	696.5 ± 52.1[c]
79	256.3 ± 14.8	953.3 ± 59.7[c]	843.2 ± 101.9[c]	685.2 ± 89.3[c]

Values are expressed as mean IU LDH leakage/mg protein ± SEM, n=3.

Letters indicate values significantly different from corresponding control values using the Dunnett's t-test (a $P<0.001$)

Table 6.6 Effect of increasing concentrations of cadmium chloride on GSH levels in liver spheroids

	Dose of cadmium chloride			
Hours post dose	0mM	0.3mM	1mM	3mM
4	9.9 ± 1.2	8.7 ± 0.7	6.5 ± 0.4[a]	5.8 ± 0.7[a]
6	9.2 ± 0.9	7.8 ± 1.1	7.5 ± 1.0	6.9 ± 0.8
30	19.7 ± 2.3	19.5 ± 1.4	21.2 ± 1.1	20.9 ± 3.1
55	30.2 ± 1.7	33.1 ± 2.7	33.6 ± 3.0	36.9 ± 4.6
79	13.3 ± 1.3	13.8 ± 1.3	14.7 ± 2.1	15.0 ± 0.7

Values are expressed as mean GSH (nmol/mg protein) ± SEM. n=3.

Letters indicate values significantly different from corresponding control values using the Dunnett's t-test (a $P<0.05$)

were 0, 50, 100, 200, 500mM hydrazine. As shown previously concentrations up to 100mM had no effect on LDH leakage at 2 or 6 hours post dose. Concentrations of 200 and 500mM caused large increases in LDH leakage at 2 hours but had only a small effect at 6 hours. However, concentrations of hydrazine up to 500mM did not decrease spheroid GSH levels at any timepoint during the experiment (table 6.7).

Examination of the spheroids microscopically showed that those spheroids exposed to concentrations of 50, 100 and 200mM hydrazine did not appear different from control spheroids. However those spheroids exposed to 500mM hydrazine had clumped together to form one large multicellular aggregate which when stained with trypan blue was shown to consist mainly of dead cells.

6.6.4 Discussion

In a preliminary dosing study to determine the toxicity of these compounds in spheroids, hydrazine was determined to be slightly toxic 30 hours following administration of concentrations of 100mM hydrazine to the spheroids as observed by increases in LDH leakage. There were, however, no changes in GSH levels following administration of hydrazine at any concentration. Comparison of the data with the toxicity of hydrazine in hepatocyte monolayers showed that spheroids were relatively resistant to hydrazine toxicity. Exposure of hepatocyte monolayers to 20mM hydrazine caused to GSH levels to fall to near zero within four hours of exposure but as determined here concentrations up to 100mM hydrazine had no effect on spheroid GSH levels (see table 6.4). The reasons for this difference in toxicity is not clear, there is the possibility that due to the size of the spheroids diffusion of the compound to hepatocytes deep within the spheroid is restricted. However, if this was the case then diffusion of nutrients or oxygen to the same cells would be restricted as well thereby implying that those cells deep within the spheroids would not contribute to the evaluation of the hydrazines toxicity. Other criteria that may affect hydrazines toxicity include the use of foetal calf serum in the spheroids medium, hydrazine may bind to plasma proteins present in the medium, hence decreasing its toxicity. However, this does not explain the observation that at concentrations up to 500mM hydrazine GSH levels were not decreased in the spheroids even though LDH leakage was significantly increased and the morphology indicated overt toxicity. One possibility that

Table 6.7 Effect of increasing concentrations of hydrazine on LDH leakage and GSH levels in liver spheroids

Dose of hydrazine	Hours post dose	LDH (IU/l/mg)	SEM	GSH (nmol/mg)	SEM
0mM	2	39.3	2.2	22.5	1.3
	6	31.4	2.4	21.6	1.4
50mM	2	46.4	4.9	22.1	1.6
	6	42.5	8.7	21.0	3.7
100mM	2	85.6[a]	7.8	21.8	2.7
	6	52.7[a]	4.2	19.2	1.0
200mM	2	823.8[c]	98.7	20.3	1.9
	6	95.1[b]	8.3	18.6	1.4
500mM	2	1159.8[c]	60.9	22.1	3.1
	6	67.3[a]	7.8	30.2	4.2

Values are expressed as the mean of three experiments

.Letters indicate values significantly different from corresponding control values using the Dunnett's t-test

[[a] ($p<0.05$), [b] ($p<0.01$), [c] ($p<0.001$)]

was investigated was that hydrazine may have been interfering with the assay for GSH. However this was not the case as this concentration of hydrazine had no effect on the GSH standard curve when added to the samples. Even though hydrazine at concentrations up to 500mM did not deplete GSH levels in the spheroids it was decided to use concentrations up to 100mM hydrazine in the following studies investigating the effect of hydrazine on the stress response in spheroids. It was also hoped, by measurement of other markers of toxicity such as ATP levels and albumin secretion, to determine a more accurate picture of hydrazines toxicity in spheroids.

The toxicity of cadmium was similar in spheroids to that in hepatocyte monolayers. LDH leakage was significantly increased following administration of 300μM cadmium and as observed from the results presented in chapter 4.4 cadmium, at this concentration, significantly increased LDH leakage from hepatocyte monolayers. The cadmium induced GSH depletion was only evident at concentrations above 1mM and this decrease was only evident 4 hours post dose. In contrast to the LDH results, cadmium appears much less effective at depleting GSH levels in spheroids when compared to its effects in hepatocyte monolayers. Concentrations of 300μM cadmium were shown to decrease GSH levels to near zero within 4 hours of exposure in hepatocyte monolayers (chapter 4.4). The reason for this apparent difference in cadmium induced GSH depletion between the two hepatocyte models are not clear, levels of GSH in spheroids prior to cadmium exposure were approximately 30% lower than the levels in hepatocyte monolayers prior to cadmium exposure. Therefore, in theory spheroids should show an increased susceptibility to cadmium but this was not the case. With consideration that LDH leakage was significantly increased following administration of the lowest concentration of cadmium used here it was decided, in the following investigation, to use concentrations of 5, 30 and 100μM to determine the effect of cadmium on the stress response in liver spheroids.

6.7 Effect of cadmium chloride on the stress response in liver spheroids

6.7.1 Aim of study

The main aim of this study was to determine whether heat shock proteins could be used as early indicators of toxicity in liver spheroids. Other markers of toxicity were also measured to determine the sensitivity of heat shock proteins as markers of toxicity. The results from this study would also show whether there was any biochemical difference in the response of spheroids to cadmium and the response of hepatocyte monolayers previously determined in section 4.3.

6.7.2 Methods

Hepatocytes were isolated and cultured for spheroid development as described in chapter 2.5. Spheroids on day 6 of culture were exposed to 0, 5, 30 and 100μM cadmium chloride for 2 hours and sampled at 0, 2, 6. 24, 30, 48, 56 and 72 hours post dose. Samples were taken for LDH, GSH, ATP, albumin secretion, Hsp25 and Hsp72/3. The spheroids were exposed to the compound between the timepoints 0 and 2 hours.

6.7.3 Results

6.7.3.1 LDH leakage

Leakage of LDH was significantly increased compared with control spheroids 6 hours following administration of 30 and 100μM $CdCl_2$. At 24 hours post exposure leakage of LDH was significantly increased from all spheroids that were exposed to $CdCl_2$ and this increase was maintained until 72 hours post dose (figure 6.7).

6.7.3.2 GSH levels

Measurement of GSH levels showed that the only significant decrease observed in GSH levels was seen 6 hours following administration of 100μM $CdCl_2$. Interestingly GSH levels were found to significantly increased at 24 and 48 hours following exposure to 5, 30 and 100μM $CdCl_2$ (figure 6.8).

6.7.3.3 ATP levels

Following administration of $CdCl_2$ levels of ATP were unchanged from values in control

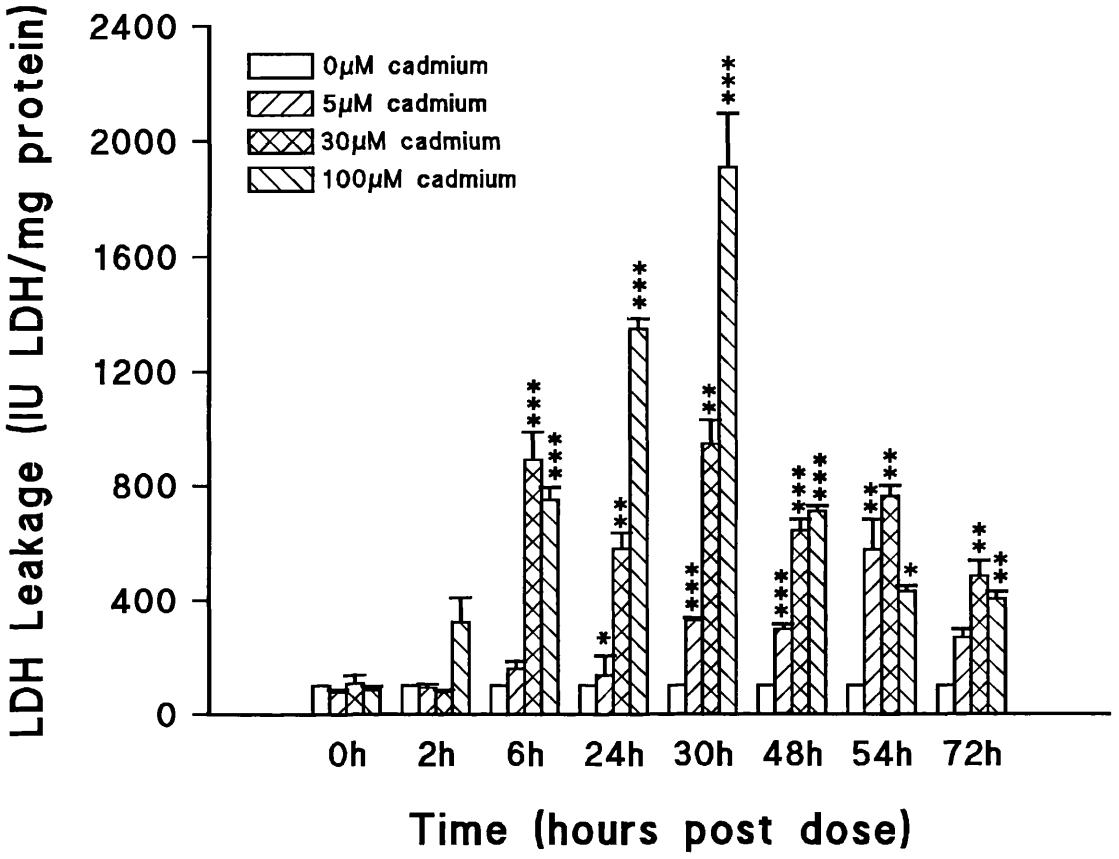


Figure 6.7 Effect of increasing concentrations of cadmium chloride on LDH leakage from liver spheroids. Values are expressed as mean IU LDH/mg protein ± SEM. n=3. Asterix indicate values significantly different from corresponding control values using the Dunnett's t-test (*P<0.05, **P<0.01, ***P<0.001)

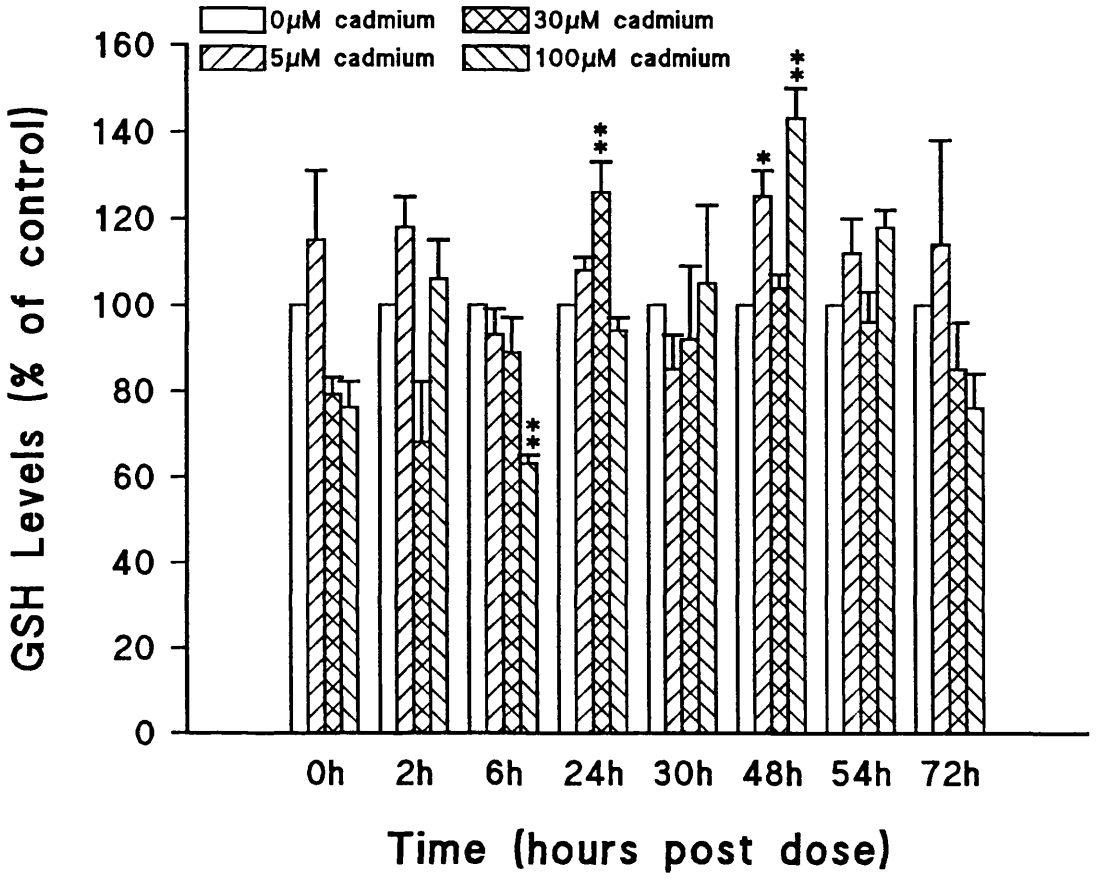


Figure 6.8 Effect of increasing concentrations of cadmium chloride on GSH levels in liver spheroids. Values are expressed as mean % of control ± SEM. n=3. Asterix indictae values significantly different from corresponding control (*P<0.05, **P<0.01).

spheroids, there was a trend showing an increase in levels at 30 hours following dosing with 100μM $CdCl_2$ although this was not significant (figure 6.9).

6.7.3.4 Albumin secretion

Secretion of albumin was not affected at any timepoint following dosing with 5μM $CdCl_2$. Following administration of 30μM $CdCl_2$ levels of albumin secretion were depressed throughout the timecourse of the experiment which was significant at 24 and 72 hours. Similarly, with 100μM levels were again depressed when compared to control levels with a significant decrease first observed at 6 hours post dose. Levels remained decreased throughout the timecourse of the experiment following administration of 100μM $CdCl_2$ (figure 6.10).

6.7.3.5 Heat shock protein levels

Measurement of Hsp25 levels in $CdCl_2$ treated spheroids showed that levels of this stress protein remained unchanged following administration of any concentration at any timepoint throughout the timecourse of the experiment (figure 6.11).

Hsp72/3 levels increased 2-fold at 2 and 6 hours following administration 5μM $CdCl_2$ although this was not found to be significant. However levels of Hsp72/3 increased 5 to 6-fold at 6 and 24 hours following dosing with 30μM $CdCl_2$, levels remained slightly elevated until 48 hours post dose. Concentrations of 100μM $CdCl_2$ caused no increase in levels of Hsp72/3, 30 hours post dose levels were found to be significantly decreased from control and remained so until the end of the experiment (figure 6.12).

6.7.4 Discussion

This study investigated the effect of cadmium on the stress response in liver spheroids and in the process allowed the comparsion of the toxicity of cadmium in spheroids with the toxicity of cadmium in hepatocyte monolayers. Toxicity was initially observed at 6 hours post dose with elevations in LDH leakage determined with concurrent decreases in GSH levels and albumin secretion. Over the remaining duration of the experiment LDH leakage remained elevated and albumin secretion was decreased but GSH levels showed a significant increase above control levels at 24 and 48 hours whereas ATP levels remained unchanged. The rebound increase in GSH levels implies the spheroids are metabolically

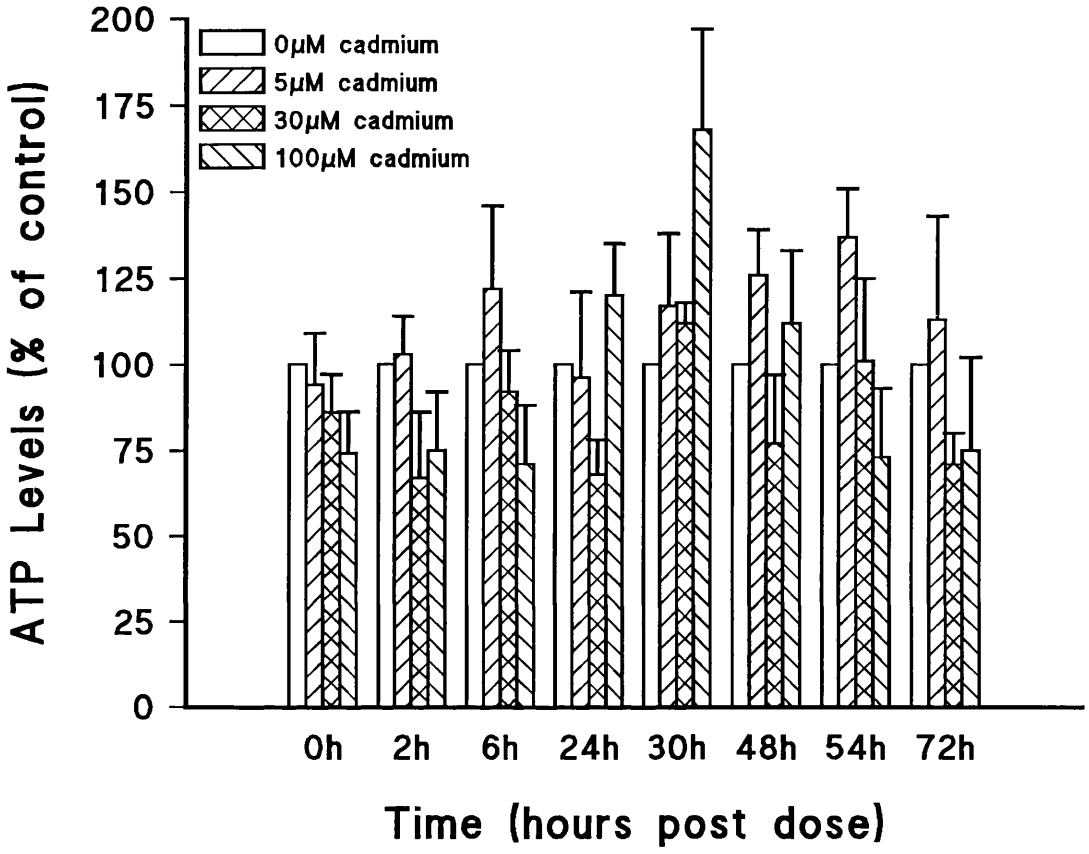


Figure 6.9 Effect of increasing concentrations of cadmium chloride on ATP levels in liver spheroids. Values are expressed as mean % of control ± SEM. n=3.

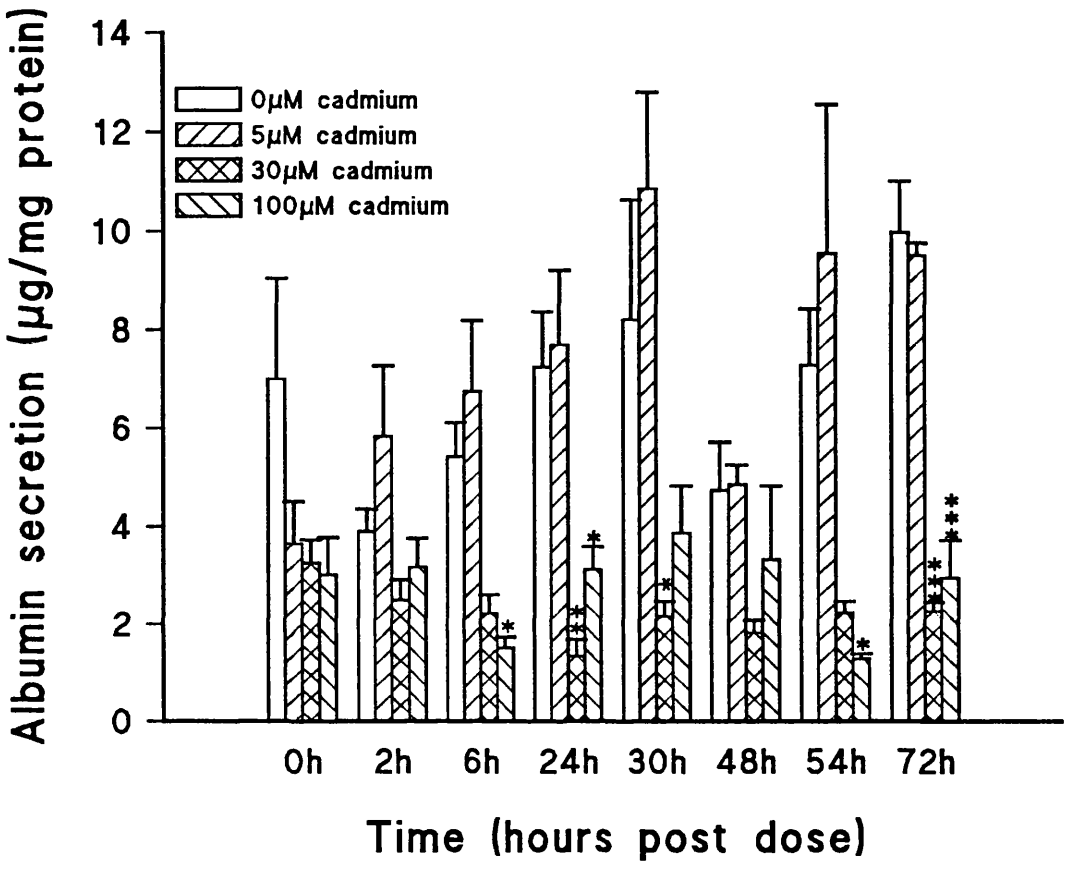


Figure 6.10 Effect of increasing concentrations of cadmium chloride on albumin secretion from liver spheroids. Values are expressed as mean µg albumin/mg protein ± SEM. n=3. Asterix indicate values significantly different from corresponding control using Dunnett's t-test (*P<0.05, **P<0.01).

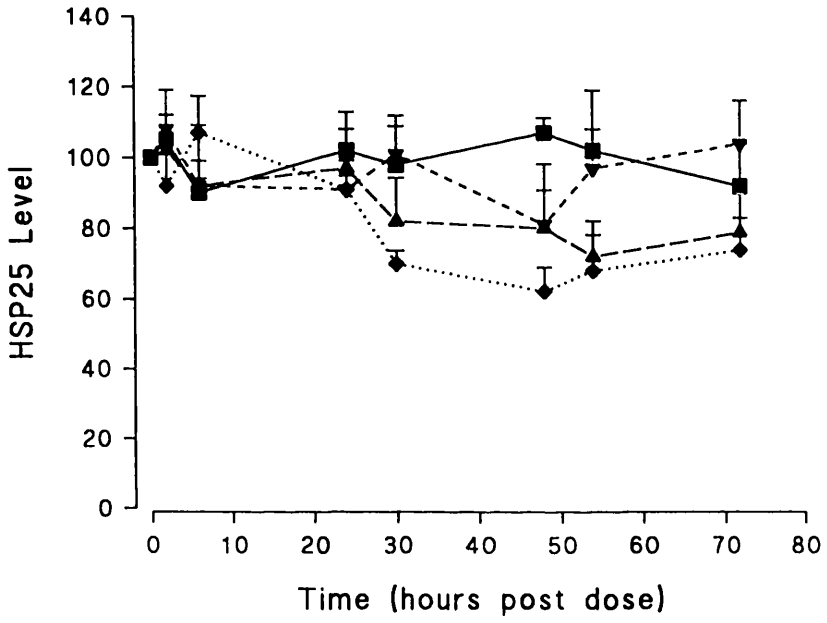
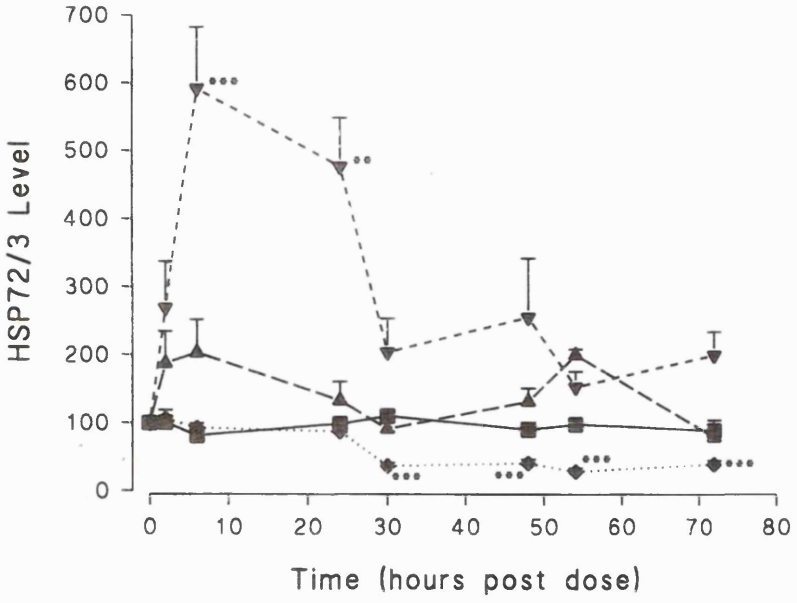


Figure 6.11 Effect of increasing concentrations of cadmium chloride on Hsp25 levels in liver spheroids. Values are expressed as mean % of control ± SEM. n=3. ■-0μM, ▲-5μM, ▼-30μM, ◆-100μM $CdCl_2$.



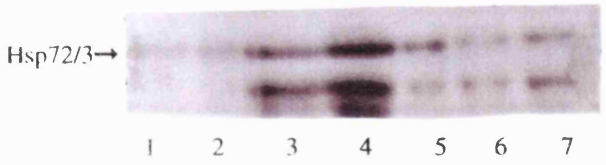


Figure 6.12 Effect of increasing concentrations of cadmium chloride on Hsp72/3 levels in liver spheroids. Values are expressed as mean % of control ± SEM. n=3. ■-0μM, ▲-5μM, ▼-30μM, ◆-100μM $CdCl_2$. Asterix indicate values significantly different from corresponding control using Dunnett's t-test (**P<0.01, ***P<0.001). Bottom panel shows a typical Western blot of the effect of 30μM $CdCl_2$ on Hsp72/3 levels in hepatocytes. Lanes as follows; Lane 1, 0hr; lane 2, 2hr; lane 3, 6hr; lane 4, 24hr; lane 5, 30hr; lane 6, 48hr; lane 7, 54 hr post dose. Solid arrow represents Hsp72/3.

viable as they are capable of synthesising GSH but this is contradicted by the alterations in LDH leakage and albumin secretion. Therefore, depending on which cellular parameter is examined following cadmium exposure the spheroids appear to show either no adverse effects or show signs of overt toxicity. The alterations in heat shock protein levels following cadmium exposure does not clarify matters any further, levels of Hsp25 were not decreased by high cadmium concentrations thereby implying that cadmium is non-toxic. This is in contrast to levels of Hsp72/3 which were significantly decreased at the highest concentration of cadmium used thereby indicating its toxicity. Comparison of the toxicity of cadmium in hepatocyte monolayers previously showed that following administration of 100μM cadmium decreased levels of ATP, GSH, Hsp25 and Hsp70 to zero within 8 hours of exposure. It would thus appear that spheroids, compared to hepatocyte monolayers, are relatively resistant to cadmium toxicity.

The stress response of liver spheroids to cadmium exposure showed that there was a large induction of Hsp72/3. The magnitude of the Hsp72/3 response in spheroids was 12-fold higher than that observed in hepatocyte monolayers following cadmium exposure. The reason for this difference is probably due to the fact that the basal levels of Hsp72/3 in spheroids were unchanged from control and so when the spheroids were exposed to cadmium a full stress response could be elicited. This is in comparison to the response in hepatocyte monolayers where the cells were exposed to cadmium when the basal levels of Hsp72/3 were elevated and so the cells were unable to respond fully. However, on a more negative note the first response of the hepatocyte monolayers following cadmium exposure was a significant elevation of Hsp72/3 levels but this was not the case with the response in spheroids. Here, the increase in Hsp72/3 levels occurred at the same time as the elevation in the leakage of LDH. Therefore, it appears that even though the magnitude of the stress response in spheroids to cadmium exposure is increased when compared to the response in hepatocyte monolayers the sensitivity of the response, in comparison to other endpoints, is decreased.

6.8 Effect of hydrazine hydrate on the stress response in liver spheroids

6.8.1 Aim of study

As hepatocyte monolayers failed to show a stress response following hydrazine administration, it was decided to investigate the effect of hydrazine on the Hsp25 and Hsp72/3 levels in liver spheroids. Measurement of other markers of toxicity, such as LDH leakage and GSH levels, were also measured to determine the sensitivity, if at all, of heat shock proteins as markers of toxicity. The results from this study would also show whether there was any biochemical difference in the response of spheroids to hydrazine and the response of primary hepatocytes previously determined in section 4.3.2.

6.8.2 Methods

Hepatocytes were isolated and cultured for spheroid formation as described in chapter 2.5. Spheroids on day 6 of culture were exposed to 0, 10, 50 and 100mM hydrazine hydrate for 2 hours and sampled at 0, 2, 6 and 24 hours post dose. Samples were taken for LDH, GSH, ATP, albumin secretion, Hsp25 and Hsp72/3. Spheroids were exposed to hydrazine between the timepoints 0 and 2 hours.

6.8.3 Results

6.8.3.1 LDH leakage

There was no change in leakage of LDH from hydrazine exposed spheroids at 2h or 6hr following dosing with hydrazine, the only significant increase in LDH leakage was observed 24 hours following dosing with 100mM hydrazine (figure 6.13).

6.8.3.2 ATP levels

ATP levels were found to be significantly decreased 2 hours following dosing with 10, 50 and 100mM hydrazine. Levels of ATP were decreased 50% two hours following dosing with 10mM hydrazine. By 6 hours post dose levels in those spheroids dosed with all concentrations of hydrazine appeared to show some recovery towards the values observed in control cells. At 24 hours post dose levels of ATP in those spheroids exposed to 10 and 50mM hydrazine were slightly elevated above control values. This is in contrast to spheroids exposed to 100mM hydrazine where levels of ATP still remained decreased

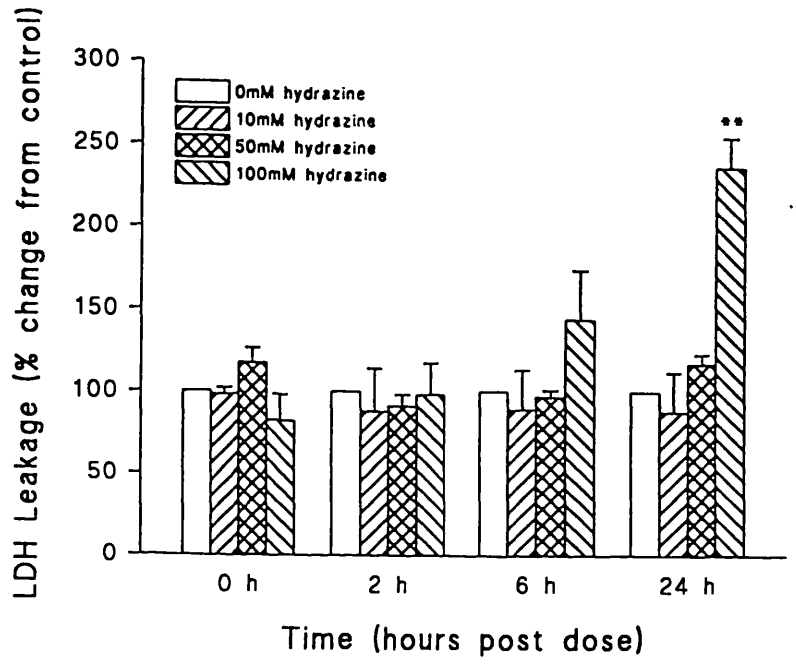


Figure 6.13 Effect of increasing concentrations of hydrazine on LDH leakage from liver spheroids. Values are expressed as mean % of control ± SEM. n=3. Asterix indicate values signifivantly different from corresponding control using Dunnett's t-test (**P<0.01)

(figure 6.14).

6.8.3.3 GSH levels

There was no change in GSH levels following administration of 10, 50 or 100mM hydrazine at any of the timepoints (figure 6.15).

6.8.3.4 Albumin secretion

Secretion of albumin was decreased 2 hours following dosing with 10, 50 and 100mM hydrazine. This decrease was found to be significant at only concentrations of 10 and 100mM hydrazine. Levels of albumin secretion appeared to show recovery at 6 and 24 hours with values returning approximately to control levels at 6 and 24 hours post dose (figure 6.16).

6.8.3.5 Heat shock protein levels

Levels of Hsp72/3 were measured over the initial 6 hours and it was determined that hydrazine at any concentration did not alter levels of Hsp72/3 within the spheroids when compared to the control levels (figure 6.17).

Levels of Hsp25 were slightly but significantly increased at 6 hours following dosing with 50 and 100mM hydrazine. Levels were unchanged at 2 hours post dose when compared with control levels (figure 6.18).

6.8.4 Discussion

Following administration of hydrazine to liver spheroids the first biochemical effects observed were a depletion of ATP and a decrease in the secretion of albumin. This decrease in ATP occurred at a time when there were no other overt signs of cytotoxicity, apart from a decrease in albumin secretion. This biochemical response to hydrazine is characteristic of the hepatic response in rats treated with hydrazine (Waterfield *et al.* 1997) as there is a reduction in liver ATP following hydrazine administration without overt cytotoxicity. The biochemical response in hepatocyte monolayers following hydrazine administration, as detailed in chapter 4.3, is a simultaneous decrease in ATP and GSH levels. Therefore, it would appear that the response of spheroids to hydrazine closely resembles the response of hepatocytes *in vivo* following administration of hydrazine.

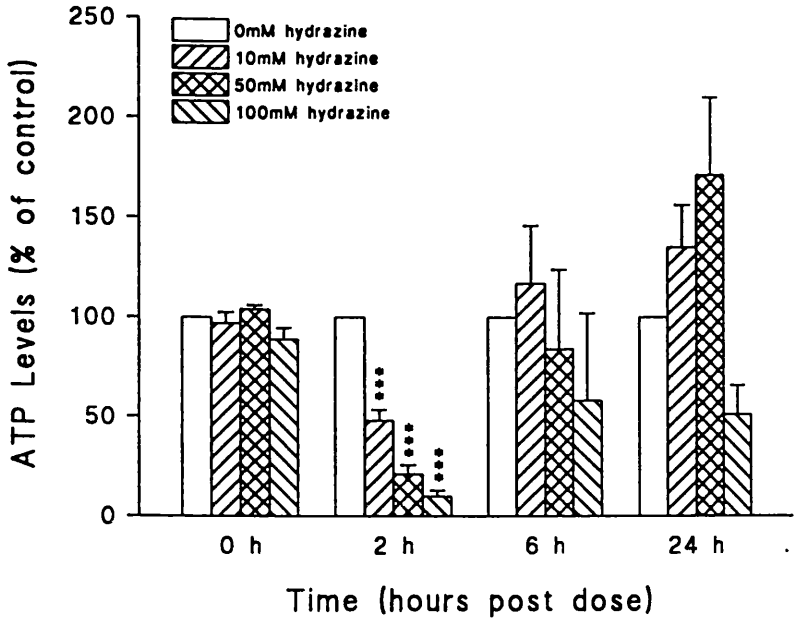


Figure 6.14 Effect of increasing concentrations of hydrazine on ATP levels in liver spheroids. Values are expressed as mean % of control ± SEM. n=3. Asterix indicate values significantly different from corresponding control (***P<0.001).

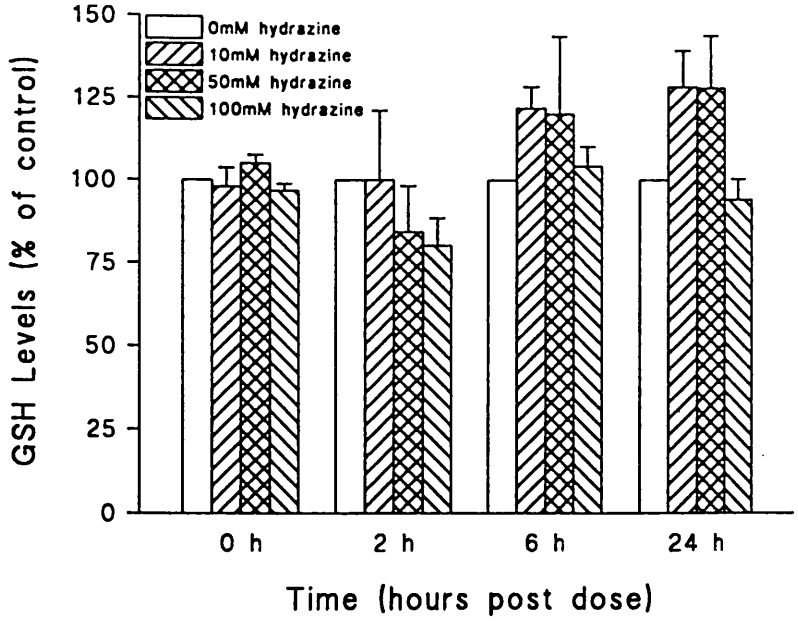


Figure 6.15 Effect of increasing concentrations of hydrazine on GSH levels in liver spheroids. Values are expressed as mean % of control ± SEM. n=3.

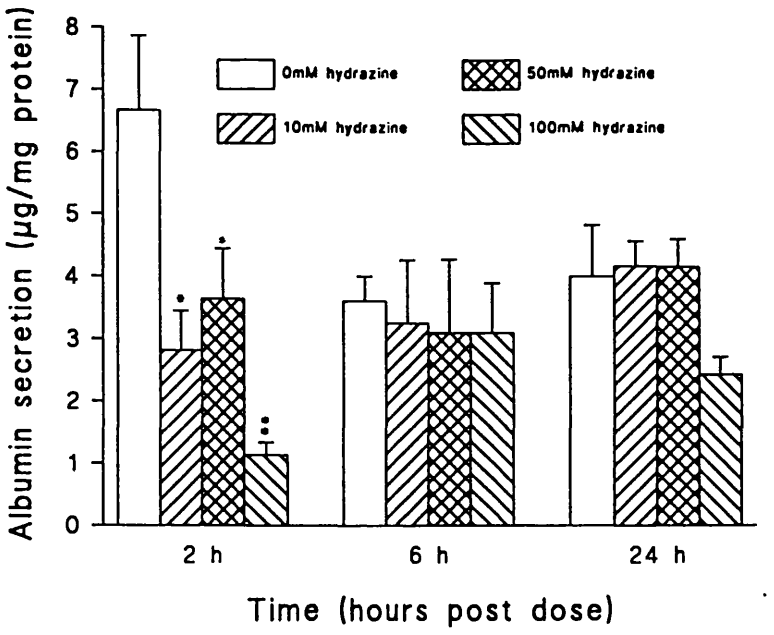


Figure 6.16 Effect of increasing concentrations of hydrazine on albumin secretion from liver spheroids. Values are expressed as mean µg albumin/mg protein ± SEM. n=3. Asterix indicate values significantly different from corresponding control (*P<0.05, **P<0.01).

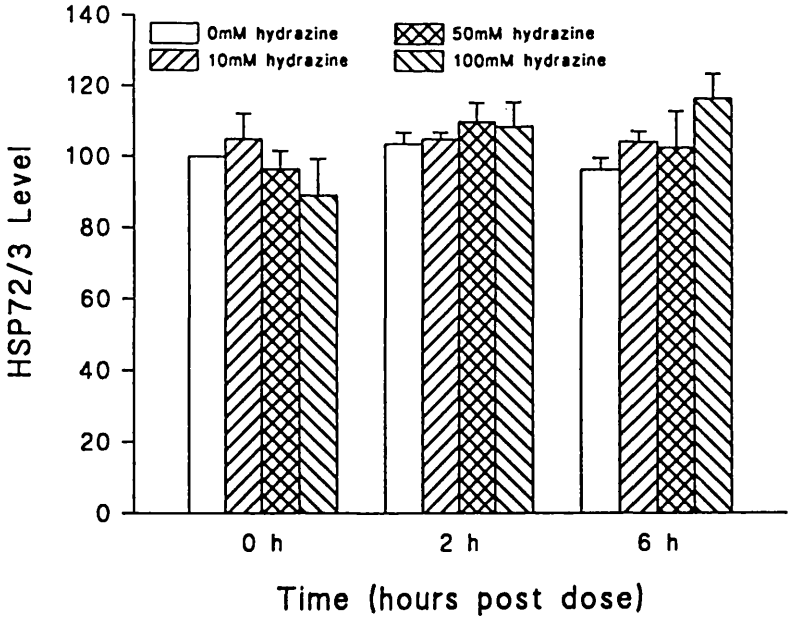


Figure 6.17 Effect of increasing concentrations of hydrazine on Hsp72/3 levels in liver spheroids. Values are expressed as mean % of control ± SEM. n=3.

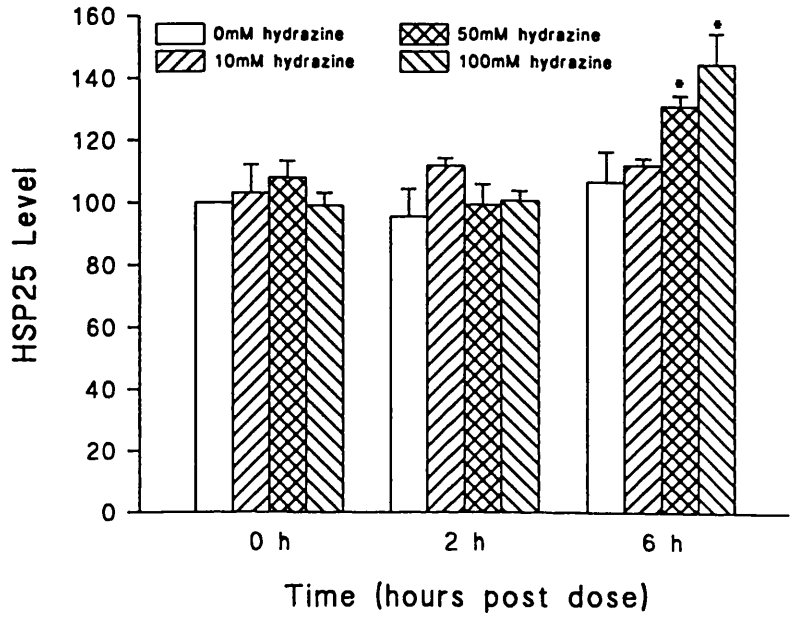


Figure 6.18 Effect of increasing concentrations of hydrazine on Hsp25 levels in liver spheroids. Values are expressed as mean % of control ± SEM. n=3. Asterix indicate values significantly different from corresponding control (*P<0.05).

Comparison of the response of spheroids to hydrazine with the response of hepatocyte monolayers again shows, as with cadmium, that the spheroids appear less sensitive to the toxic effects of hydrazine. Even though ATP levels were depleted within two hours of hydrazine exposure levels show a recovery to control values even after administration of concentrations up to 100mM hydrazine. The same is also true for the decreases in albumin secretion observed which showed a complete recovery within 6 hours of exposure to hydrazine. The elevation of LDH leakage at 24 hours appears to show a delayed toxic effect of hydrazine, however this result is not re-inforced by the other biochemical parameters which show no adverse changes at 24 hours. It would therefore appear that hydrazine is slightly toxic to liver spheroids within two hours of exposure but the cells appear, biochemically, to fully recover from any such insult within six hours of exposure.

Even though there was no alteration in Hsp72/3 levels hydrazine was shown to slightly, but significantly, increase levels of Hsp25 at 6 hours post dose. This is the first observation of hydrazine affecting stress protein levels in hepatocytes as it had no effect on stress protein levels *in vivo* or in hepatocyte monolayers. The lack of an effect in primary hepatocytes can most likely be explained by the elevated basal levels of Hsp25 during culture which would have obscured any response of the cell to hydrazine. However, the fact that hydrazine failed to elevate levels of Hsp25 *in vivo* does shed some doubt over the result generated in spheroids. It may be that hydrazine is able to increase levels of Hsp25 but the magnitude of induction is very small and the time-window of any induction is very narrow and because of this the *in vivo* hydrazine investigation in chapter 5.3 failed to determine any effect on Hsp25 levels.

In conclusion, within two hours of exposure to hydrazine the spheroids show biochemical changes indicative of cytotoxicity, however within six hours the biochemical changes observed at two hours had been reversed and the cells appeared to show no biochemical effects of toxicity. Hydrazine failed to elevate levels of Hsp72/3 but did slightly, but significantly, increase levels of Hsp25 24 hours post dose.

6.9 Histological study of the effect of hydrazine on spheroids and hepatocyte monolayers

6.9.1 Aim of study

Considering the biochemical measurements determined previously in section 6.8 showed that hydrazine exposure, even at relatively high concentrations, did not produce biochemical changes indicative of overt toxicity after 2 hours post dose it was, therefore decided to investigate histologically the effect of hydrazine on the hepatocytes within the spheroids.

6.9.2 Methods

Hepatocytes were isolated and cultured for development of spheroids and culture of monolayers as described in chapter 2.5. Spheroids on day 6 of culture and monolayers after 16 hours of culture were exposed to 100mM hydrazine for 2 hours after which time the hydrazine was removed and fresh medium added. Samples were taken at 2 hours and 6 hours post dose for histological staining with haematoxylin and eosin (H&E) and Oil Red O stain as described previously in chapters 2.5.4 and 2.5.5.

6.9.3 Results

In control spheroids there was little evidence of cellular degeneration or necrosis, however discrete vacuolation was visible which is normally associated with fat accumulation (figures 6.19, 6.22 and 6.25).Exposure of spheroids to 100mM hydrazine for 2 hours caused the spheroids to take on a ragged appearance and the hepatocytes within showed a loss of cellular definition and in addition a lack of cohesiveness (figures 6.20, 6.23 and 6.26). In some cases, cellular degeneration and necrosis was visible. However, the degree of cytoplasmic vacuolation was comparable to that seen in the control spheroids. Staining with Oil Red O showed no appreciable difference between the degree of cytoplasmic fat accumulation in treated cells compared to control cells. Examination of the spheroids that had been exposed to 100mM hydrazine for 2 hours and allowed to recover for 4 hours showed that the appearance was appreciably more viable than compared to those spheroids that were analysed directly after exposure. Even in the presence of some cellular degeneration the spheroids showed retention of cell cohesiveness. The hepatocytes within

the spheroids appeared to have a swollen appearance with only a small number of necrotic cells present. Again the degree of cytoplasmic vacuolation present was comparable to that seen in control spheroids. Staining with Oil Red O again failed to show any difference in cytoplasmic fat accumulation between control and treated spheroids (figures 6.21, 6.24 and 6.27)

Control hepatocyte monolayers consisted of a base monolayer of well defined mononuclear hepatocytes arranged in confluent, plump cords forming a trabecular network. A small number of dead or degenerating cells were present but these cells were detached from the substratum and tended to lie above the confluent cells. Following administration of hydrazine for two hours to hepatocyte monolayers, there was an increase in the number of dead and degenerating cells resulting in a less confluent appearance of the monolayer. These cells were removed from the monolayer whilst the remaining cells appeared slightly smaller and more basophilic. Analysis of hepatocyte monolayers at 6 hours post dose showed there was a further increase in the number of dead and degenerating cells that were removed from the monolayer resulting in a further loss of confluence. Those cells still present in the basal monolayer, however, showed similar morphology to control cells. There was no difference in the degree of fat accumulation between the remaining viable cells and the control cells at 2 or 6 hours post dose.

6.9.4 Discussion

The histological results presented here correlate well with previous biochemical data examining the effect of hydrazine exposure on liver spheroids. At two hours post dose there was a significant decrease in ATP levels and albumin secretion from spheroids and this was reflected in the histology showing an increase in cell degeneration. Even though necrosis was observed in spheroids two hours post dose it does seem contradictory that there was no elevation in LDH observed at this timepoint. It may be that the hepatocytes contained deep within the spheroids were necrotic but due to diffusion limitations the LDH that was leaked from the cells may have not released into the medium immediately. Again the recovery of the biochemical parameters observed at six hours post dose was reflected in the recovery of histology of the hepatocytes as there was less cellular

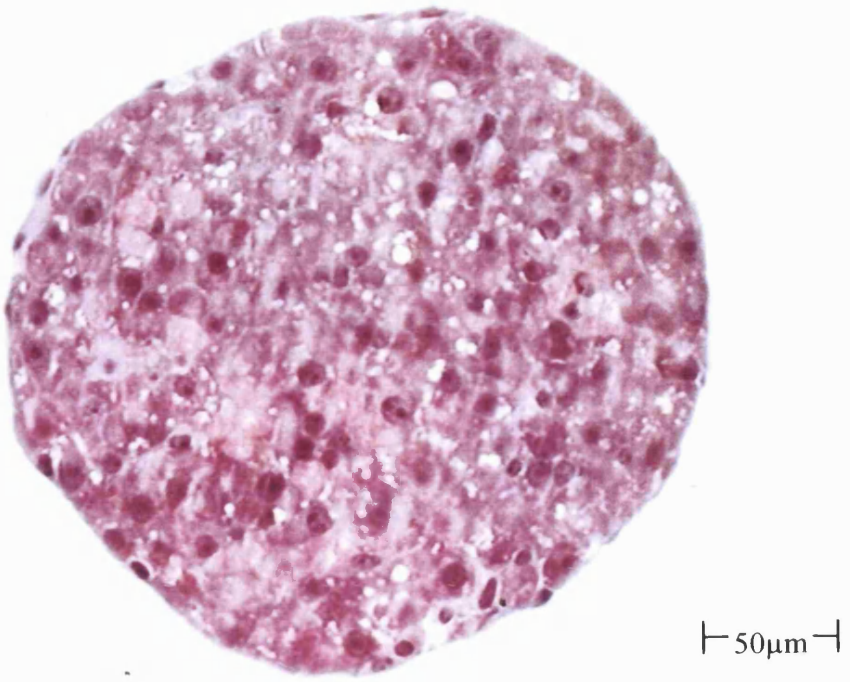


Figure 6.19 LM section of adult rat day 6 control spheroids stained with haematoxylin and eosin (x20 objective)

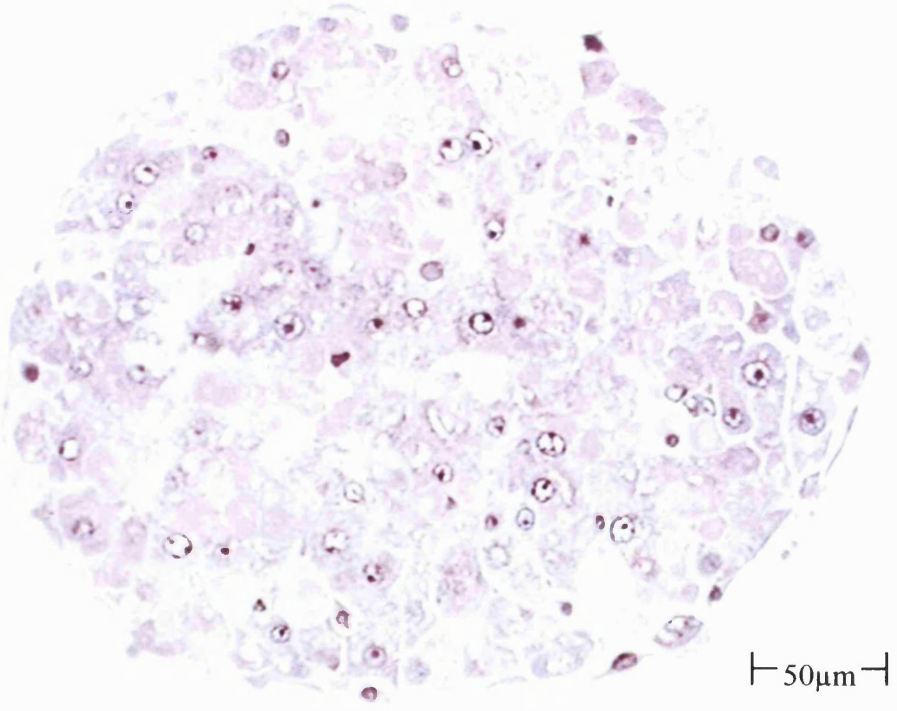


Figure 6.20 LM section of adult rat day 6 spheroids dosed with 100mM hydrazine for 2 hours, stained with haematoxylin and eosin (x20 objective)

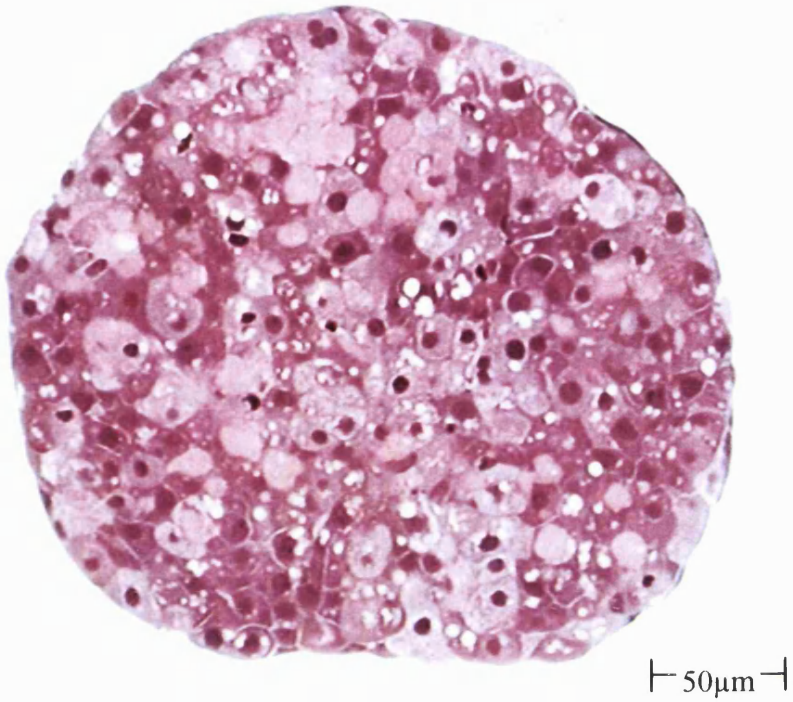


Figure 6.21 LM section of adult rat day 6 spheroids dosed with 100mM hydrazine for 2 hours and allowed to recover for 4 hours, stained with haematoxylin and eosin (x20 objective)

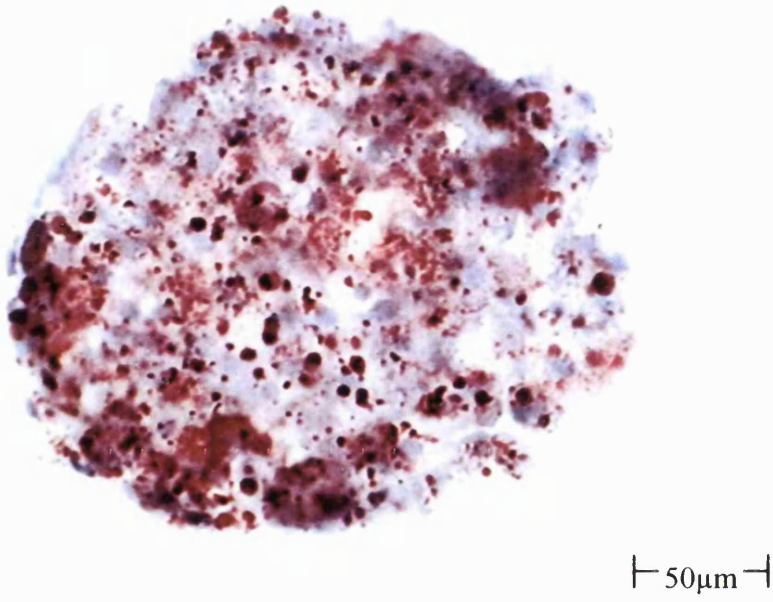


Figure 6.22 LM section of adult rat day 6 control spheroids stained with Oil Red O (x20 objective)

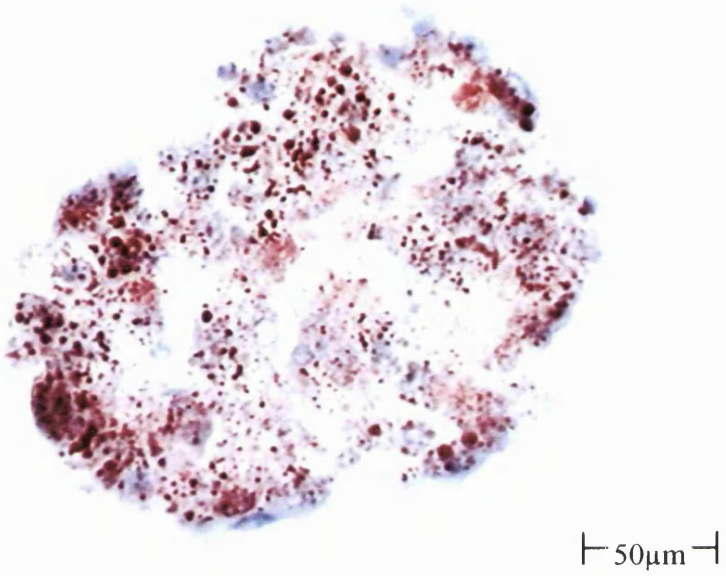


Figure 6.23 LM section of adult rat day 6 spheroids dosed with 100mM hydrazine for 2 hours, stained with Oil Red O (x20 objective)

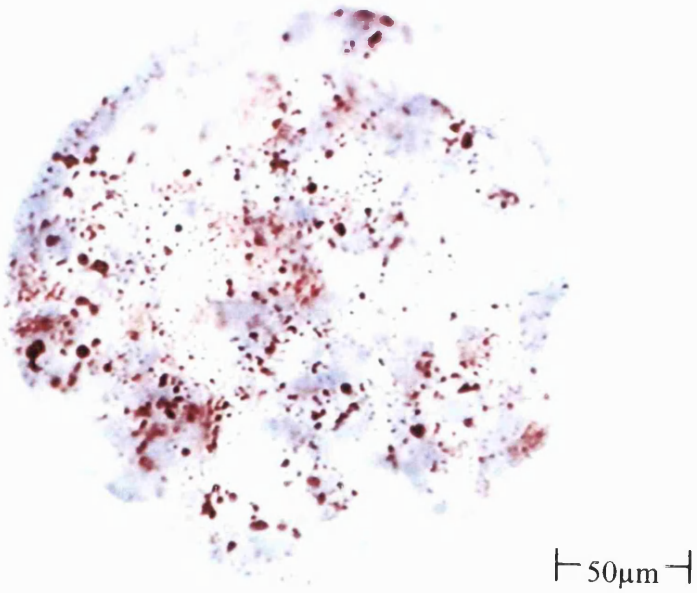


Figure 6.24 LM section of adult rat day 6 spheroids dosed with 100mM hydrazine for 2 hours and allowed to recover for 4 hours, stained with Oil Red O (x20 objective)

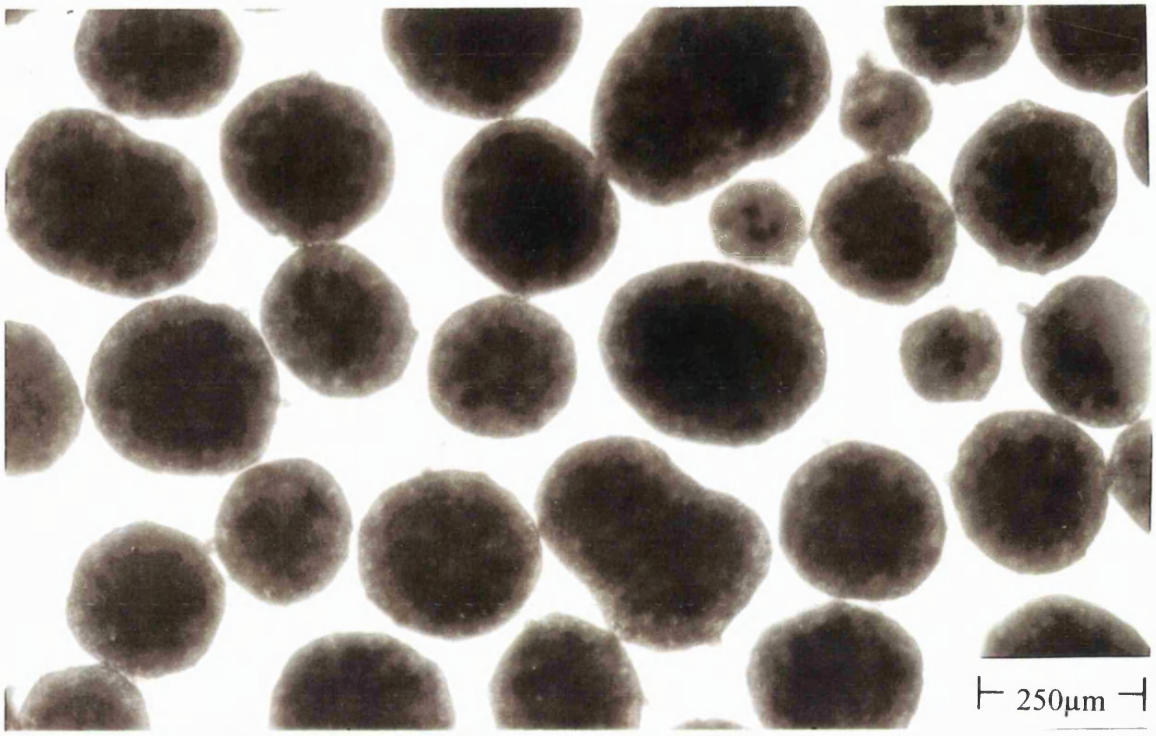


Figure 6.25 Adult rat day 6 control spheroids (x20 objective)

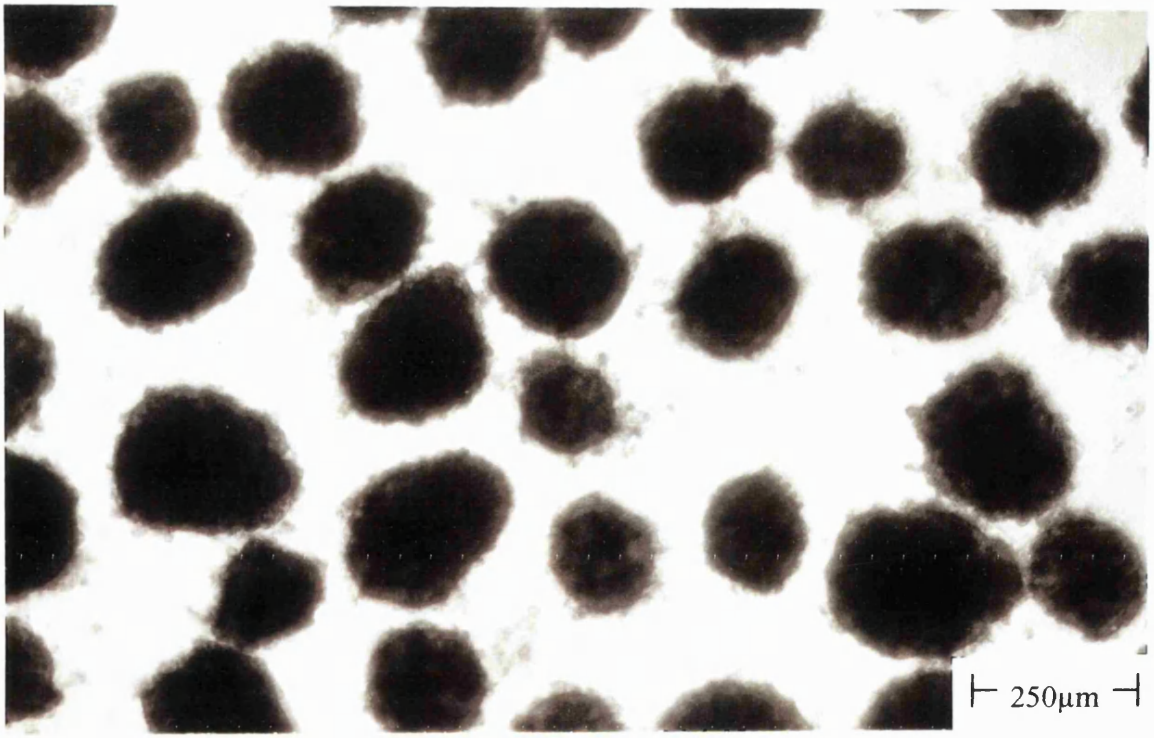


Figure 6.26 Adult rat day 6 spheroids dosed with 100mM hydrazine for 2 hours (x20 objective)

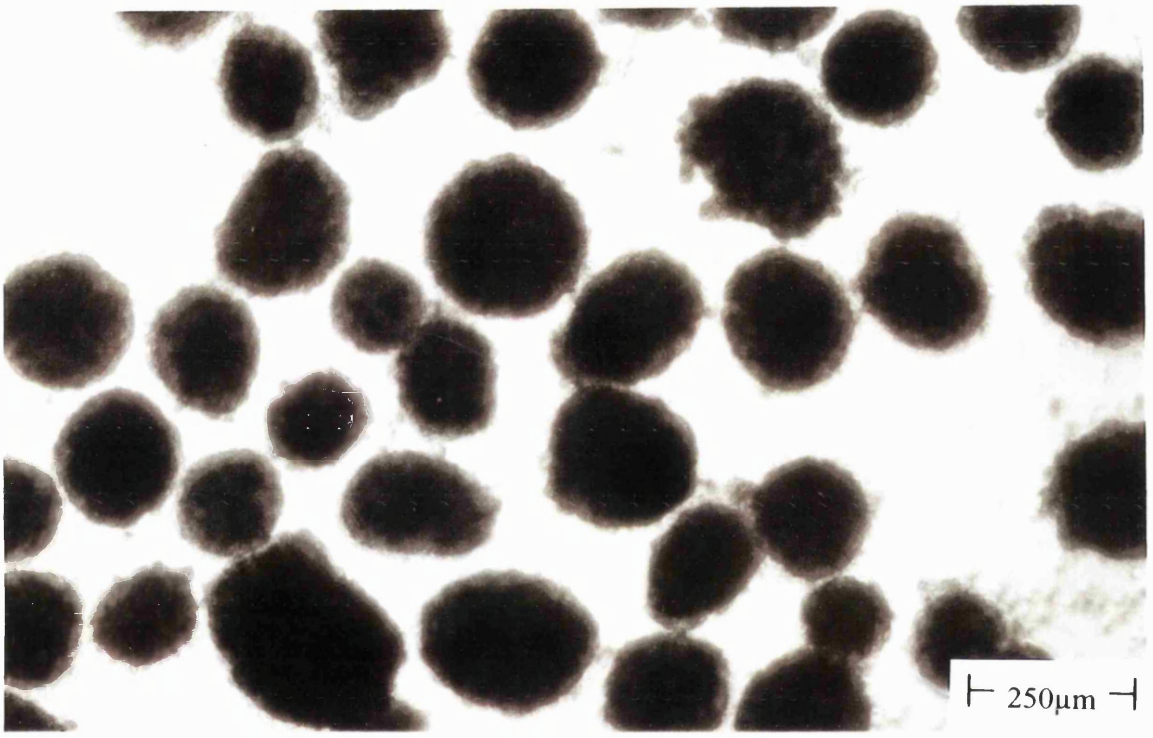


Figure 6.27 Adult rat day 6 spheroids dosed with 100mM hydrazine for 2 hours and allowed to recover for 4 hours (x20 objective)

degeneration than at two hours post dose and overall the hepatocytes appeared generally more viable. This again gives weight to the argument that the elevation of LDH leakage observed in the previous experiment at 24 hours post dose was due to the toxicity of hydrazine within the first two hours of exposure and not due to some delayed toxic effect of hydrazine. In comparison to the effect of hydrazine on the histology of spheroids the toxic effect of hydrazine in hepatocyte monolayers was determined to be non-reversible as there were appreciably more necrotic cells at six hours post dose when compared to two hours post dose, this was even after hydrazine had been removed from the medium four hours previously. Referring back to the biochemical data obtained when hepatocyte monolayers were exposed to hydrazine in chapter 4.3 it was observed that there was no recovery in the biochemical parameters following hydrazine exposure, this is in contrast to the biochemical effects of hydrazine in spheroids where parameters showed a complete recovery. Therefore, it would appear that the histological effects of hydrazine in spheroids and hepatocyte monolayers reflect the biochemical toxicity data in that they both show the reversible nature of hydrazines' toxicity in liver spheroids and its non-reversible nature in hepatocyte monolayers.

6.10 Conclusions

The main aims of this set of experiments was to develop the use of liver spheroids as an *in vitro* model for studying the stress response, this would allow direct comparisons to be made with the stress response in primary hepatocytes and also determine the relative toxicities of compounds in the two different hepatocyte models. Measurement of basal biochemical measurements and stress protein levels during spheroid development showed that it had the potential to be a good *in vitro* model for studying the stress response as it had low basal stress protein levels during culture. This potential was realised when cadmium administration was shown to induce levels of Hsp72/3 to such an extent that the magnitude of the response was 12-fold greater in spheroids than in primary hepatocytes. Hydrazine elevated levels of Hsp25 slightly in spheroids, an observation that was not detected in hepatocyte monolayers and following administration to rats *in vivo*.

The biochemical toxicity of the cadmium and hydrazine in spheroids was generally less pronounced than that observed in hepatocyte monolayers , the reasons for this may be due to the physical and functional differences between the two hepatocyte models. However unlike hepatocyte monolayers the spheroids appear to show reversible toxicity as they are more capable of recovering from chemical exposure. This reversible toxicity of hydrazine in spheroids was also demonstrated in the histology of the spheroids which showed a recovery when hydrazine was removed. This is in contrast to hepatocyte monolayers which showed degenerative histology even after hydrazine was removed from the system. In addition, the biochemical response of the spheroids to the toxicants, particularly hydrazine, was determined to be more *in vivo* like in nature than the response of hepatocyte monolayers.

One of the problems with the use of spheroids is that following chemical exposure the spheroids probably consist of hepatocytes that have different states of viability depending on the location of the cells. This is noticeable in the larger spheroids which have necrotic centres. Therefore, this would effectively mean that any biochemical measurements taken from the spheroids give you an average response of all the indivdual hepatocytes contained within the spheroids. However, overall the use of spheroids provides an *in vitro* model that allows reliable measurements of the stress response and the response of the cells to the toxicants appears to show a more *in vivo* like response when compared to the response in hepatocyte monolayers.

Chapter 7.

Investigations into the involvement of HSF-1 in the stress response *in vitro* and *in vivo*

7.1 Introduction

Present in the cytosol of all unstressed cells is a transcription factor, the heat shock transcription factor (HSF) which is involved in the transcriptional activation of the genes involved in the stress response. The precise details of the structure and function of HSF are described in full detail in chapter 1.5. In unstressed cells, the HSF is kept in an inactive state by binding with Hsp70. However, upon chemical exposure or hyperthermic treatment the levels of denatured or damaged proteins rise and so the Hsp70 dissociates from HSF and preferentially targets these proteins. This dissociation allows the trimerisation of HSF which then translocates to the cell nucleus where it binds to a promoter region on the heat shock gene, the heat shock element (HSE). This binding of HSF to HSE causes the increased transcription of the genes encoding heat shock proteins. The results presented in the previous chapters demonstrated that, with the exception of spheroids, exposure of hepatocytes *in vivo* or *in vitro* to hydrazine failed to alter levels of Hsp25 and Hsp72/3. However, it cannot be ruled out that hydrazine was affecting levels of other stress proteins. This study was designed to investigate whether the compounds used in this study altered the location or levels of the transcription factor necessary for heat shock protein expression, the heat shock factor (HSF). Investigations into the levels of HSF would hopefully elucidate whether hydrazine, although being toxic, was not capable of inducing a stress response in hepatocytes or instead the compound was inhibiting the synthesis of heat shock proteins. It was, therefore, decided to measure changes in levels of HSF both *in vitro* and *in vivo* following exposure to hydrazine or cadmium to determine whether the compounds affect cellular levels of HSF. It was hoped that alterations in the activation or location of HSF may be utilised as sensitive general markers of toxicity as in theory these alterations take place prior to the synthesis of stress proteins. Following a stressful insult the translocation of HSF into the cell nucleus occurs within one hour of the insult. Because of this, it is possible to measure changes in HSF levels in suspensions of hepatocytes without encountering the viability problems normally associated with maintaining hepatocytes in suspension for long periods of time. This would eliminate the problem associated with the use of hepatocyte monolayers in chapter 4.5 in that the basal levels of heat shock proteins were elevated at the time of chemical exposure. So far four HSF's have been characterised, HSF-1, HSF-2, HSF-3 and HSF-4.

The most extensively studied has been HSF-1 which is the primary factor involved in increased stress gene expression following chemical exposure. Therefore, in these investigations the effect of hydrazine and cadmium will be investigated with respect to changes in the levels and activation of cellular HSF-1. In the initial experiments the intracellular distribution of HSF-1 was investigated by determining the nuclear and non-nuclear levels of HSF-1 thus, the levels of nuclear HSF-1 express the levels of activated HSF-1 present in the cell. However in the other hepatocyte experiments the total cellular levels of HSF-1 were determined. Since the antibody used in these investigations detects both the inactive and active form of HSF then any increase in total HSF-1 therefore implies activation of the gene.

7.2 Effect of hydrazine hydrate and cadmium chloride on the sub-cellular location of HSF-1 in hepatocyte suspensions

7.2.1 Aim of study

This study involved the administration of hydrazine to hepatocyte suspensions in order to hopefully elucidate whether hydrazine was inducing a stress response in hepatocytes resulting in the induction of stress proteins other then Hsp72/3 or Hsp25. This should be measureable as changes in the induction of HSF-1. Since following a chemical insult HSF-1 is translocated into the cell nucleus it was decided to determine nuclear and non-nuclear levels of HSF-1 in isolated hepatocytes following hydrazine exposure. Cadmium chloride has been previously shown to induce a stress response in hepatocytes. This study was performed to elucidate whether or not there was any difference in sensitivity between the induction of stress proteins and the translocation of HSF-1 to the cells nucleus following cadmium exposure. This would allow determination of whether measurement of the change in cellular HSF-1 levels would be a more accurate marker of toxicity than measurement of individual heat shock proteins.

7.2.2 Methods

Hepatocytes were isolated as described in sections 2.2 and incubated in rotating round-bottomed flasks maintained at 37 °C at a density of $1x10^6$ cells/ml in KH buffer. The cells

were pre-incubated for 30 minutes and then exposed to increasing concentrations of hydrazine or cadmium chloride for two hours. The concentrations of hydrazine used were 0, 0.1, 1, 5, 10mM and the concentrations of cadmium chloride used were 0, 0.1, 1, 5 and 10μM. The hepatocytes were sampled for LDH and HSF-1 at two hours post dose and nuclear and non-nuclear levels of HSF-1 determined as described in chapter 2.6. In this study and all subsequent studies in this chapter, the samples were probed with a rabbit anti-HSF-1 polyclonal antibody which binds to both the active and inactive forms of HSF-1. The isolation of hepatocyte nuclei and detection of HSF-1 in the samples is described in chapter 2.6.

7.2.3 Results

7.2.3.1 LDH leakage

LDH leakage was initially determined from hydrazine exposed cells. Leakage of LDH from hepatocytes exposed to concentrations of 0.1, 1 and 5mM hydrazine for two hours was unchanged from leakage of LDH from control cells. However there was a slight but not significant increase in LDH leakage from those hepatocytes exposed to 10mM hydrazine for two hours (figure 7.1).

Exposure of hepatocytes to $CdCl_2$ for two hours caused a dose dependent increase in the leakage of LDH from the hepatocytes. This increase was first observed following administration of 5μM $CdCl_2$ where there was approximately 25% increase in LDH leakage when compared to leakage from control cells. Exposure of hepatocytes to 10μM $CdCl_2$ for two hours caused a significant increase in LDH leakage (figure 7.2).

7.2.3.2 Nuclear HSF-1 levels

Determination of HSF-1 levels in hepatocytes exposed to hydrazine showed that this compound did not affect the nuclear levels of HSF-1 (figure 7.3).

Exposure of hepatocytes to $CdCl_2$ caused an accumulation of HSF-1 in the nucleus of dosed hepatocytes. The accumulation of HSF-1 in the nucleus was shown to be dose dependent with significant increases in nuclear levels of HSF-1 observed following administration of 1, 5 and 10μM $CdCl_2$. The maximal increase in nuclear HSF-1 levels was an approximate 2.5-fold increase compared to levels in control cells following administration of 10μM $CdCl_2$ (figure 7.5).

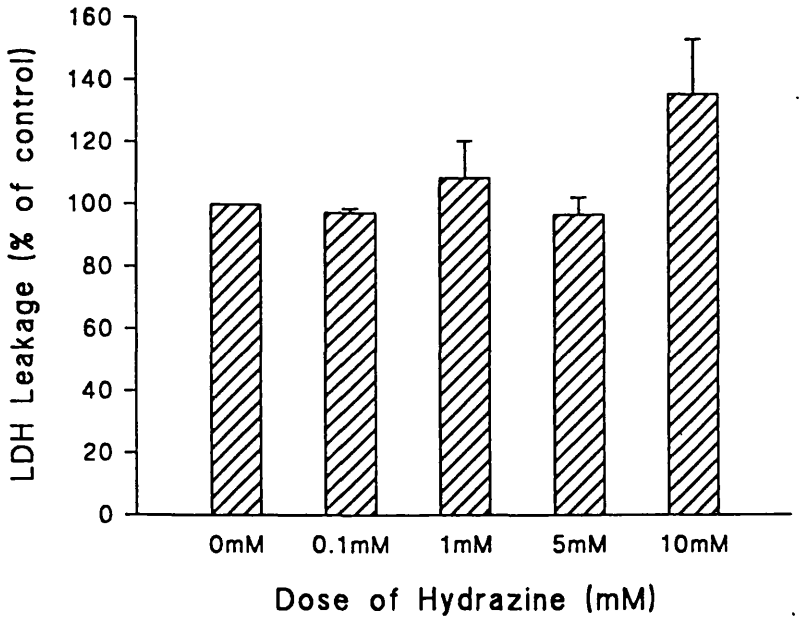


Figure 7.1 Effect of increasing concentrations of hydrazine on LDH leakage from isolated hepatocytes in suspension. Values are expressed as mean % of control ± SEM. n=4.

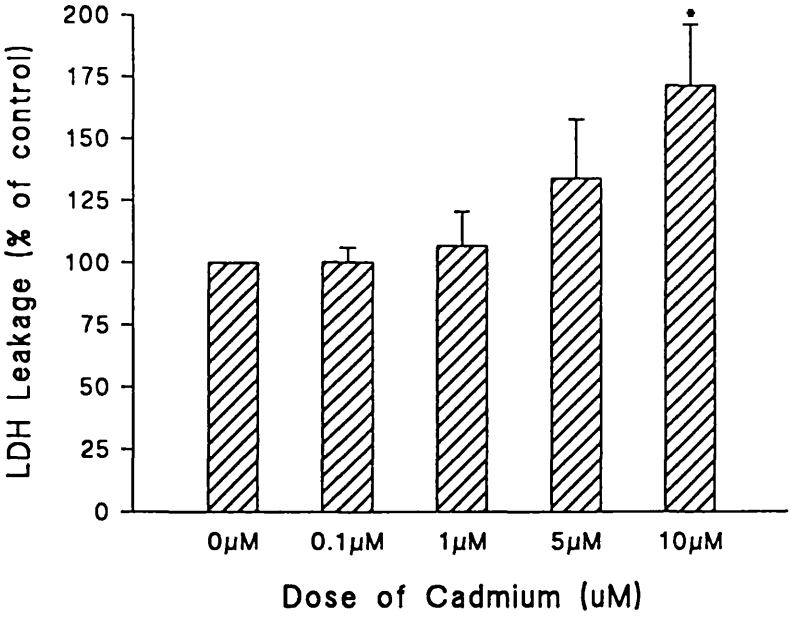


Figure 7.2 Effect of increasing concentrations of cadmium chloride on LDH leakage from isolated hepatocytes in suspension. Values are expressed as mean % of control ± SEM. n=4. Asterix indicate values that are significantly different from corresponding control values using Dunnett's t-test (*$p<0.05$)

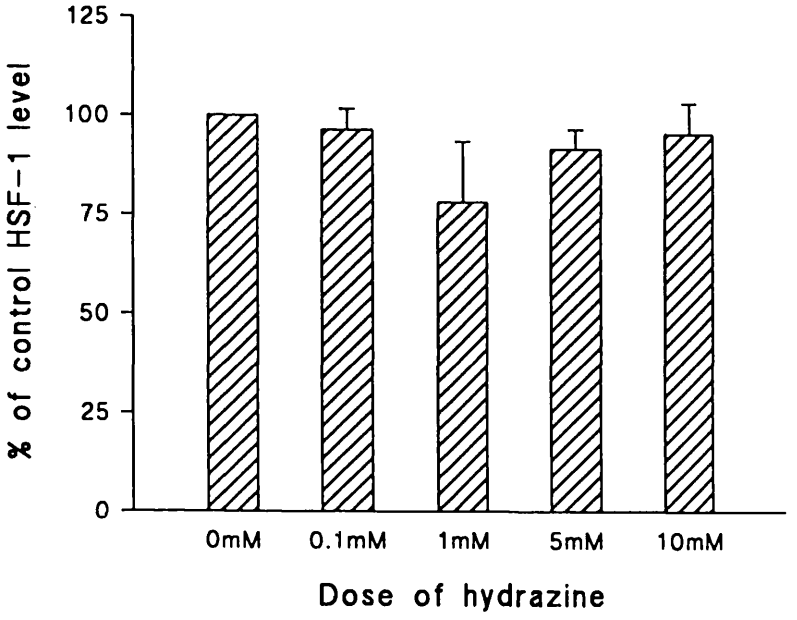


Figure 7.3 Effect of increasing concentrations of hydrazine on nuclear HSF-1 levels in isolated hepatocytes in suspension. Values are expressed as mean % of control ± SEM. n=4.

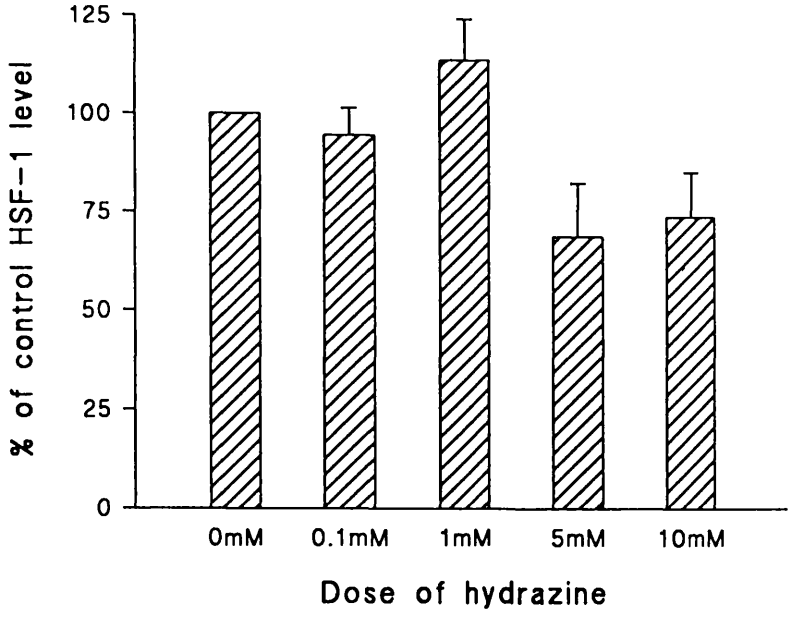


Figure 7.4 Effect of increasing concentrations of hydrazine on non-nuclear HSF-1 levels in isolated hepatocytes in suspension. Values are expressed as mean % of control ± SEM. n=4.

7.2.3.3 Non-nuclear HSF-1 levels

As with the nuclear HSF-1 levels, administration of hydrazine to hepatocytes in suspension had no effect on the non-nuclear levels of HSF-1 at any concentration (figure 7.4).

In contrast to nuclear HSF-1 levels administration of $CdCl_2$ to hepatocytes caused a dose dependent decrease in the non-nuclear levels of HSF-1. There was no effect at 0.1μM $CdCl_2$ but a concentration of 1μM $CdCl_2$ caused a 50% decrease in the non-nuclear levels of HSF-1. The non-nuclear levels of HSF-1 were further decreased to approximately 30% of control following administration of 5 and 10μM $CdCl_2$ (figure 7.6).

7.2.4 Discussion

Of the two compounds studied in these investigations cadmium was the only one which affected the activation and location of HSF-1. Hydrazine failed to alter the localisation of HSF-1 in isolated hepatocytes in suspension thereby implying that although the compound is toxic to these cells it does not cause the induction of a stress response. This is in agreement with the previous investigations in hepatocyte monolayers and in hepatocytes *in vivo* following hydrazine exposure which showed no effect on the levels of Hsp25 or Hsp72/3. Cadmium is known to cause the activation of HSF-1 (Liu *et al.* 1995, Ovelgönne *et al.* 1995) as would be expected of a compound known to cause the induction of heat shock proteins. This study demonstrated that exposure of hepatocyte suspensions to cadmium causes the accumulation of HSF-1 within the nuclei of exposed cells, this accumulation was rapid and dose dependent. The accumulation was accompanied by a concurrent decrease in the non-nuclear levels of HSF-1. These results represent the recognised scheme of events following a stressful insult in that HSF-1 translocates to the cells nucleus following an insult where it binds to the HSE causing the increased transcription of stress proteins. This results presented previously demonstrated changes in the localisation of the activated form of HSF-1 following exposure to either cadmium or hydrazine. However, when the total cellular levels of HSF-1 are analysed following exposure to either cadmium or hydrazine a different pattern of induction appears (table 7.1). Considering hydrazine had no effect on the sub-cellular localisation of HSF-1 it is as expected that it had no effect on total cellular levels of HSF-1. Cadmium only caused a significant increase in HSF-1 levels following administration of 10μM

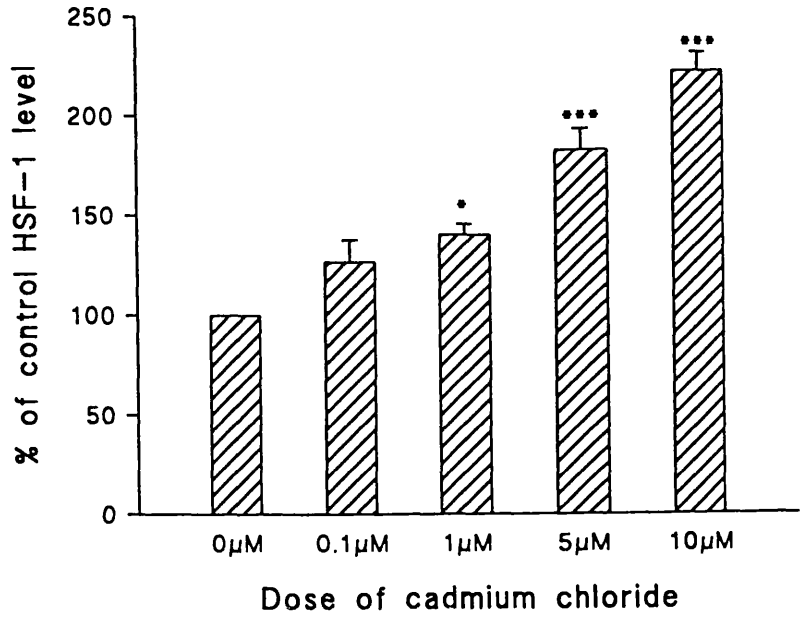


Figure 7.5 Effect of increasing concentrations of cadmium chloride on nuclear HSF-1 levels in isolated hepatocytes in suspension. Values are expressed as mean % of control ± SEM. n=4. Asterix indicate values that are significantly different from corresponding control values using Dunnett's t-test (*p<0.05, ***p<0.001)

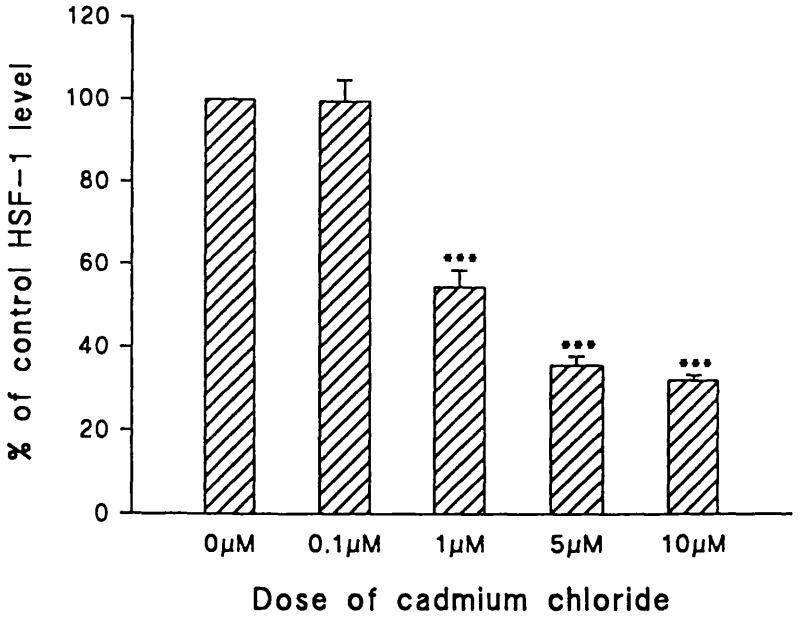


Figure 7.6 Effect of increasing concentrations of cadmium chloride on non-nuclear HSF-1 levels in isolated hepatocytes in suspension. Values are expressed as mean % of control ± SEM. n=4. Asterix indicate values that are significantly different from corresponding control values using Dunnett's t-test (***p<0.001)

$CdCl_2$, all other concentrations did not affect total levels of HSF-1. This is in contrast to nuclear levels of HSF-1 which were significantly elevated following administration of concentrations of 1μM $CdCl_2$ and above.

Table 7.1 Effect of cadmium or hydrazine on total HSF-1 levels in hepatocyte suspensions at two hours post-dose.

Conc. of cadmium (μM)	% of control HSF-1	SEM	Conc. of hydrazine (mM)	% of control HSF-1	SEM
0	100	0	0	100	0
0.1	112.5	8.1	0.1	95.4	6.1
1	97.5	4.5	1	95.9	12.9
5	109.5	5.7	5	83.1	9.5
10	127.5*	5.2	10	86.1	9.6

Asterix indicates values significantly different from the corresponding control using the Dunnett's t-test (*P<0.05)

Even though cadmium causes an accumulation of activated nuclear HSF-1 it only causes the induction of Hsp72/3 and not Hsp25 therefore there must be an additional level of control as to the induction of specific heat shock proteins. This is highlighted by the fact that HSF-1 activation and induction of Hsp25 are unrelated, the possible mechanisms for this include selective phosphorylation of HSF-1 or the involvement of additional factors required for selective induction of heat shock proteins.

The results presented here demonstrate that, with the two compounds used here, measurement of the activation of HSF-1 may be useful as an indicator of cellular stress and toxicity. However as with the measurement of individual heat shock proteins the changes in HSF-1 activation was stressor-specific as hydrazine failed to alter the localisation or activation of HSF-1. Comparison of the changes in HSF-1 activation following cadmium exposure with the changes in heat shock protein levels in the other hepatocyte models demonstrates that the changes in HSF-1 activation were occurring at chemical concentrations below those that were altering the levels of heat shock proteins.

Therefore, in hepatocyte suspensions, measurement of the changes in HSF-1 location may represent a more sensitive marker of toxicity when compared to changes in individual heat shock proteins in other models.

7.3 Effect of hydrazine hydrate and cadmium chloride on total HSF-1 levels in hepatocyte monolayers

7.3.1 Aim of study

Studies performed previously in chapter 4 demonstrated that exposure of hepatocyte monolayers to hydrazine failed to elevate level of Hsp25 or Hsp72/3. However exposure of the cells to cadmium caused the induction of Hsp72/3 in hepatocytes. The previous investigation showed that exposure of hepatocyte suspensions to hydrazine failed to cause the activation of HSF-1. This study was performed therefore to determine whether exposure of hepatocyte monolayers to hydrazine produced a similar response as that observed in isolated hepatocytes. In addition by investigating the effect of hydrazine on hepatocyte monolayers it was also hoped to determine whether hydrazine was in fact causing the induction of other stress proteins other than Hsp25 or Hsp72/3 and whether any lack of stress protein induction was due to inhibition of the synthesis of heat shock proteins following HSF-1 activation. With respect to the effect of cadmium on HSF-1 levels in hepatocyte monolayers it was hoped to determine whether or not changes in HSF-1 levels precede the changes observed in stress protein levels following exposure to cadmium that were determined previously in chapter 4.4. In doing so it was hoped to determine whether changes in the levels of HSF-1 may have potential as a more general marker of toxicity in hepatocyte monolayers. As opposed to the previous investigation total levels of activated HSF-1 were measured as the amount of nuclear material recovered from hepatocyte monolayers was insufficent to measure nuclear levels of HSF-1.

7.3.2 Methods

Hepatocytes were isolated and cultured as described in section 2.3. An aliquot for HSF-1 analysis was initially taken from freshly isolated hepatocytes. The cells were cultured for

16 hours in Williams E medium and then exposed to either hydrazine or cadmium chloride. The concentrations of hydrazine used were 0, 1, 5, 10mM and the concentrations of cadmium chloride used were 0, 1, 5 and 10μM. The hepatocytes were exposed to hydrazine or cadmium for two hours after which time the compound was removed, the cells washed twice with 2ml sterile PBS and fresh Williams E medium added. Samples for LDH and HSF-1 analysis were taken prior to dosing and at two and six hours post dose and prepared for SDS-PAGE as described previously in section 2.3.5 and 2.6.

7.3.3 Results

7.3.3.1 LDH leakage

Within two hours of exposure of hepatocyte monolayers to hydrazine LDH leakage was significantly increased when compared to control cells following administration of 5mM hydrazine. However there was no change in LDH leakage from those cells exposed to 1 or 10mM hydrazine. At six hours post dose there was no significant difference in the levels of LDH leakage between the dosed cells and the control cells (figure 7.7). At two hours post dose there was no difference in the levels of LDH leakage from control cells and those dosed with $CdCl_2$ at any concentration. At six hours post dose there was no difference in the levels of LDH leakage from the cells dosed with $CdCl_2$ and control cells (figure 7.8).

7.3.3.2 Total HSF-1 levels

At both two and six hours post dose there was no difference in the levels of total HSF-1 in control cells and in those exposed to any concentration of hydrazine (figure 7.9). Exposure of hepatocyte monolayers to $CdCl_2$, at any concentration, did not alter the levels of total HSF-1 when compared to levels in control cells (figure 7.10).

7.3.4 Discussion

The effect of hydrazine on HSF-1 activation in hepatocyte monolayers is in agreement with the results presented previously in isolated hepatocyte suspensions in that, although hydrazine is a known hepatotoxin it is unable to induce a stress response in hepatocytes. In contrast to the results generated in the previous experiments cadmium, in hepatocyte monolayers, failed to cause the activation of HSF-1. This result is of particular interest as

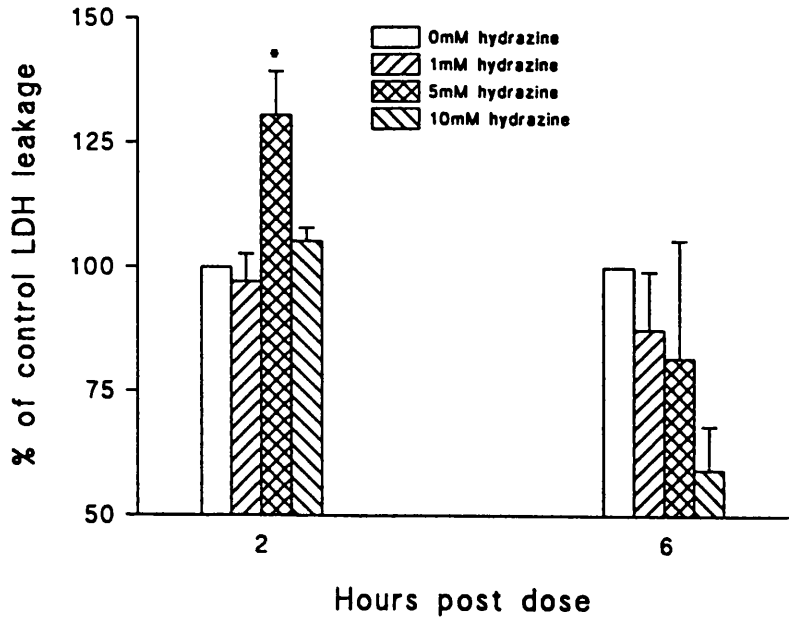


Figure 7.7 Effect of increasing concentrations of hydrazine on LDH leakage from hepatocyte monolayers. Values are expressed as mean % of control ± SEM. n=3. Asterix indicate values that are significantly different from corresponding control values using Dunnett's t-test (*p<0.05)

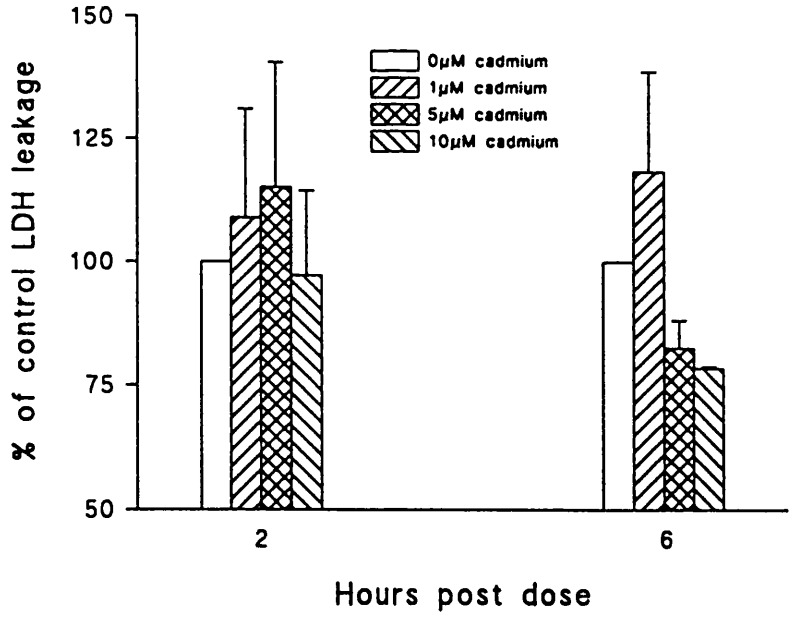


Figure 7.8 Effect of increasing concentrations of cadmium chloride on LDH leakage from hepatocyte monolayers. Values are expressed as mean % of control ± SEM. n=3.

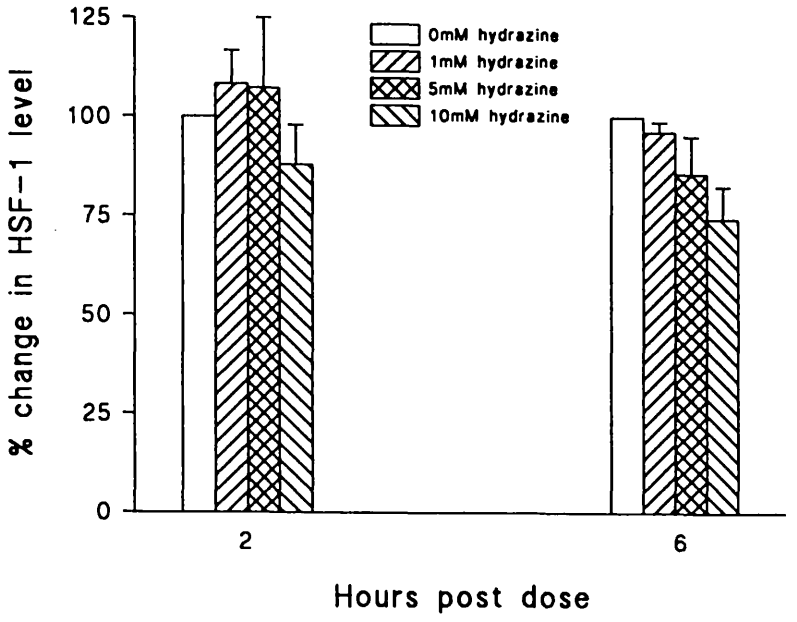


Figure 7.9 Effect of increasing concentrations of hydrazine on total HSF-1 levels in hepatocyte monolayers. Values are expressed as mean % of control ± SEM. n=3.

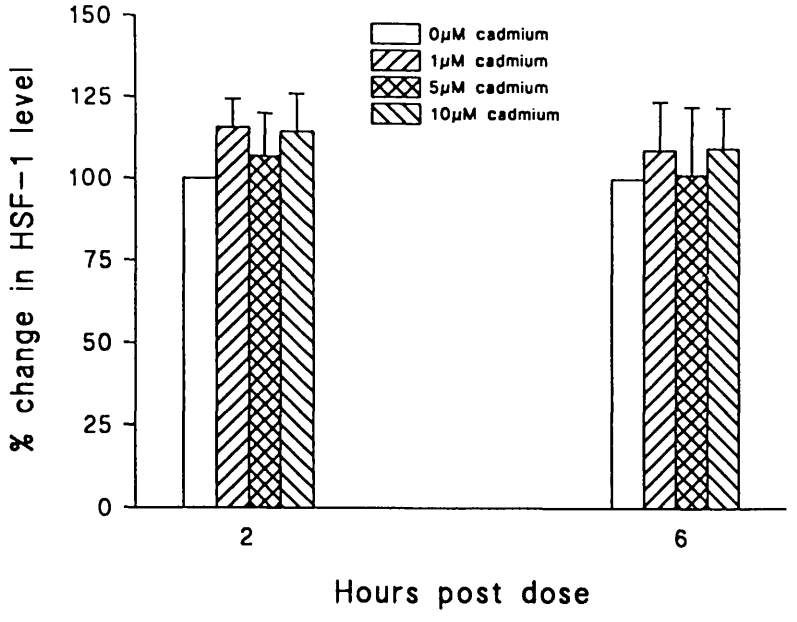


Figure 7.10 Effect of increasing concentrations of cadmium chloride on total HSF-1 levels in hepatocyte monolayers. Values are expressed as mean % of control ± SEM. n=3.

cadmium has been shown previously in chapter 4.4 to elevate the levels of Hsp72/3 in hepatocyte monolayers at concentrations of 5 and 10μM. It would therefore appear from these results presented here that cadmium is causing the induction of stress proteins in hepatocyte monolayers without causing the activation of HSF-1, an occurrence which most likely does not accurately represent the physiological situation. The situation is most likely explained by comparison of the observations determined previously in chapter 4, specifically the elevated basal stress protein levels observed during the culture of hepatocytes. Levels of HSF-1 activation were determined in freshly isolated hepatocytes and these levels of were compared directly to the levels of activation in the control samples taken at the two and six hour timepoints during the study investigating the effect of hydrazine or cadmium on HSF-1 levels. It was determined that levels of HSF-1 activation were approximately 3-fold higher in hepatocyte monolayer samples taken at two and six hours when compared to the levels of HSF-1 activation in freshly isolated hepatocytes (data not shown). This is to be expected as levels of the two stress proteins, Hsp25 and Hsp72/3, were elevated at identical timepoints. It may be that even though exposure of hepatocyte monolayers to cadmium causes the slight induction of Hsp72/3 any changes in the activation of HSF-1 will be unmeasurable since HSF-1 would have been activated as the basal stress protein levels were elevated. Therefore, small changes in stress protein levels would have been measurable following cadmium exposure but the concurrent changes in HSF-1 activation would have been undetectable. The results presented here further re-inforce the observation that the use of hepatocyte monolayers as model for studying the stress response as a markers of toxicity is fundamentally flawed due to the induction of the stress response during the culture of hepatocytes.

7.4 Effect of *in vivo* administration of hydrazine hydrate and cadmium chloride on total hepatic HSF-1 levels

7.4.1 Aim of study

This study was performed to determine whether administration of hydrazine or cadmium caused a change in the levels of HSF-1 in the livers of exposed rats. This was performed in order to allow a direct comparison of the levels of Hsp25 and Hsp72/3 levels following

hydrazine or cadmium exposure with the levels of HSF-1 to investigate whether the induction of HSF-1 occurred at different chemical concentrations to the changes observed in stress protein levels. Again in doing so it was hoped to elucidate whether any changes in HSF-1 levels were occurring in the hepatocytes of hydrazine exposed rats or as in the case of HSF-1 levels in isolated or hepatocyte monolayers, HSF-1 levels were unchanged following hydrazine exposure. In this investigation total levels of activated HSF-1 were measured as the amount of nuclear material recovered from the *in vivo* samples taken was insufficent to measure nuclear levels of HSF-1.

7.4.2 Methods

The levels of HSF-1 were determined in the samples of liver taken from the study investigating the effect of hydrazine and cadmium on heat shock protein levels as described in section 2.4. The effect of 0, 1, 2.5, 5 and 10mg/kg hydrazine free base and 0, 0.1, 0.5, 1 and 2mg/kg Cd^{2+} ip on total liver HSF-1 levels six hours post dose was therefore investigated.

7.4.3 Results

7.4.3.1 Effect of hydrazine on total liver HSF-1 levels

Administration of hydrazine to rats did not alter the total levels of HSF-1 in the livers of exposed animals when compared to levels in untreated rats (figure 7.11).

7.4.3.2 Effect of cadmium chloride on total liver HSF-1 levels

Administration of cadmium chloride to rats caused a dose dependent accumulation of HSF-1 in the livers of treated animals. Doses of 0.1 and 0.5mg/kg $CdCl_2$ produced approximately a 25% increase in the total levels of HSF-1 when compared to control levels in untreated livers. This increase in total HSF-1 was further accentuated following administration of 1 and 2mg/kg $CdCl_2$ where a significant 2-fold increase in the levels of HSF-1 was observed (figure 7.12).

7.4.4 Discussion

As with the effect of hydrazine in hepatocyte suspensions and monolayers exposure of rats to hydrazine failed to alter the activation of HSF-1 in hepatic tissues. This further re-

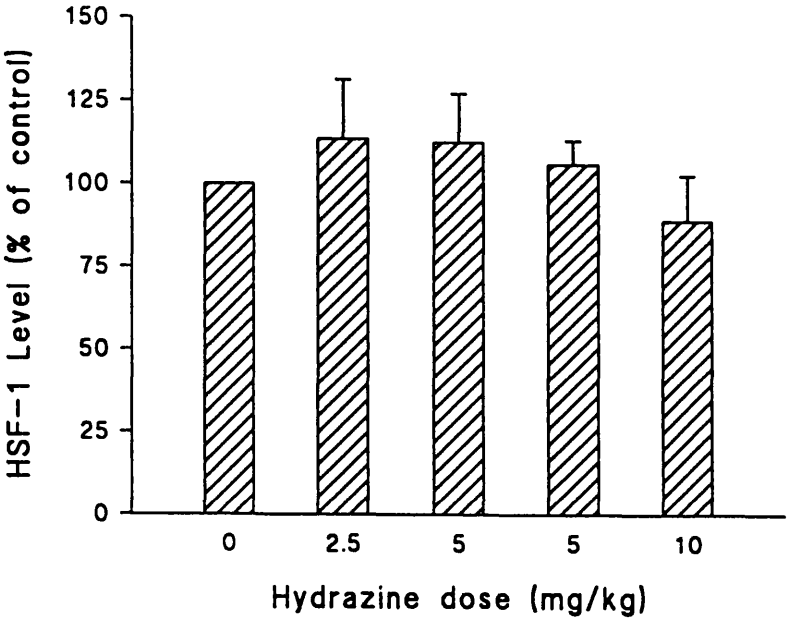


Figure 7.11 Effect of increasing concentrations of hydrazine on total liver HSF-1 levels 6 hours post dose. Values are expressed as mean % of control ± SEM. n=4.

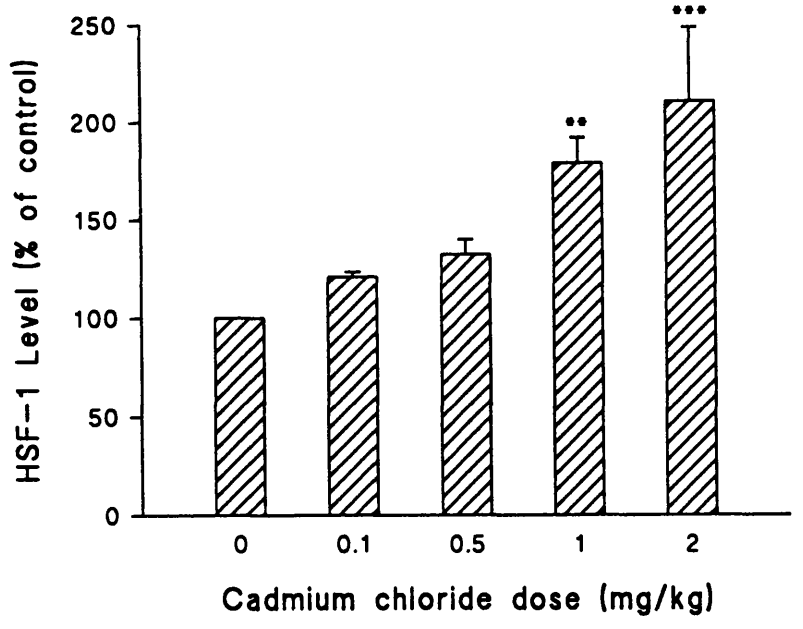


Figure 7.12 Effect of increasing concentrations of cadmium chloride on total liver HSF-1 levels 6 hours post dose. Values are expressed as mean % of control ± SEM. n=4. Asterix indicate values that are significantly different from corresponding control values using Dunnett's t-test (**p<0.01, ***p<0.001)

inforces the observation that although hydrazine is hepatotoxic it is incapable of inducing a stress response in hepatocytes *in vivo* or *in vitro*. Exposure of rats to cadmium caused the activation of HSF-1 at doses of 1 and 2mg/kg, these doses were identical to the ones shown previously in chapter 5.4 to cause the elevation of Hsp72/3 in hepatic tissues. Therefore, as opposed to the biochemical changes in hepatocyte suspensions following cadmium exposure the changes in HSF-1 activation *in vivo* were occurring at the same chemical concentrations that altered the induction of specific heat shock proteins. The measurement of HSF-1 activation as a marker of toxicity here is again no more sensitive an indicator of toxicity as measurement of changes in individual heat shock proteins. The observation again that HSF-1 activation occurs without the concurrent induction of Hsp25 in hepatic tissues again demonstrates that there must be an additional level of control involved in the induction of specific heat shock proteins. The results presented here, therefore, demonstrate that changes in HSF-1 activation occur at the same chemical concentrations which induce individual heat shock proteins.

7.5 Effect of hydrazine hydrate and cadmium chloride on total HSF-1 levels in liver spheroids

7.5.1 Aim of study

Investigations in chapter 6 showed that cadmium caused significant induction of Hsp70 in liver spheroids and additionally hydrazine exposure caused the elevation of Hsp25. This study was designed to determine whether changes in HSF-1 activation were occurring prior to changes in stress protein levels and so be may be more feasible as a marker of cadmium induced damage in spheroids. The observation in chapter 6 that exposure of spheroids to hydrazine elevated levels of Hsp25 was viewed with a certain amount of scepticism as all previous studied had showed hydrazine had no effect on Hsp25 levels in hepatocytes. This investigation therefore was to elucidate whether hydrazine was in fact causing the induction of Hsp25 by measurement of HSF-1 levels, in addition measurement of HSF-1 levels in spheroids following cadmium or hydrazine exposure would allow a direct comparison to be made with the changes observed in the other hepatocyte models used in the previous investigations. In this investigation, total levels of activated HSF-1

were measured as the amount of nuclear material recovered from spheroids was insufficent to measure nuclear levels of HSF-1.

7.5.2 Methods

Spheroids were isolated and cultured as described in chapter 2.5. A sample of freshly isolated hepatocytes was taken for HSF-1 analysis and then on day six of culture spheroids were exposed to either hydrazine or cadmium chloride. The doses of hydrazine used were 0, 10, 50, 100mM and the cadmium chloride doses were 0, 5, 10 and 30μM. The spheroids were exposed to the compound for two hours after which time the compound was removed, the spheroids washed twice with sterile PBS and fresh H1777 medium added. Samples were taken prior to dosing in addition to two and six hours post dose and prepared for SDS-PAGE as described previously in section 2.5.

7.5.3 Results

7.5.3.1 Effect of hydrazine on spheroid HSF-1 levels

Following exposure of spheroids to hydrazine for two hours there was no change observed in the total level of HSF-1 at any concentration. However at six hours post dose the levels of HSF-1 had increased 2-fold following administration of 10 and 50mM hydrazine, there was little change in the levels of HSF-1 in those spheroids dosed with 100mM hydrazine (figure 7.13).

7.5.3.2 Effect of cadmium on spheroid HSF-1 levels

Within two hours of exposure to $CdCl_2$ total levels of activated HSF-1 increased in liver spheroids. This increase was observed following administration of 5, 10 and 30μM $CdCl_2$. Determination of the significance of the increase showed that at all concentrations the increase was not significant, however this was due to the fact that one set of spheroids appeared to show no increase in HSF-1 levels whereas the other two sets of cells showed almost a 2-fold increase in the levels of HSF-1 following administration of all concentrations of $CdCl_2$. At six hours post dose there was no change in the levels of HSF-1 in any of the $CdCl_2$ exposed cells compared to the control cells (figure 7.14). Comparison of the levels of HSF-1 in spheroids on day six of culture and the levels of HSF-1 in freshly isolated hepatocytes showed no difference in levels (data not shown).

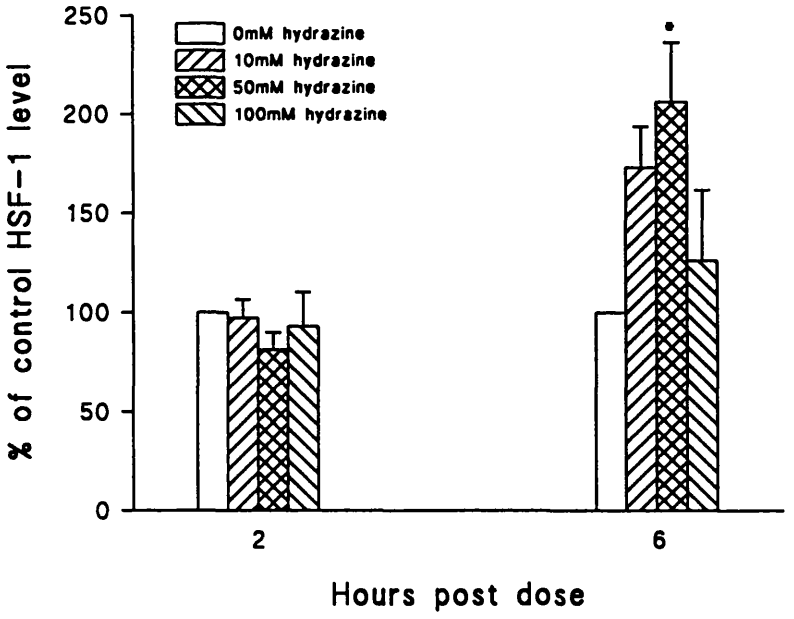


Figure 7.13 Effect of increasing concentrations of hydrazine on total HSF-1 levels in liver spheroids. Values are expressed as mean % of control ± SEM. n=4. Asterix indicate values that are significantly different from corresponding control values using Dunnett's t-test (*p<0.05)

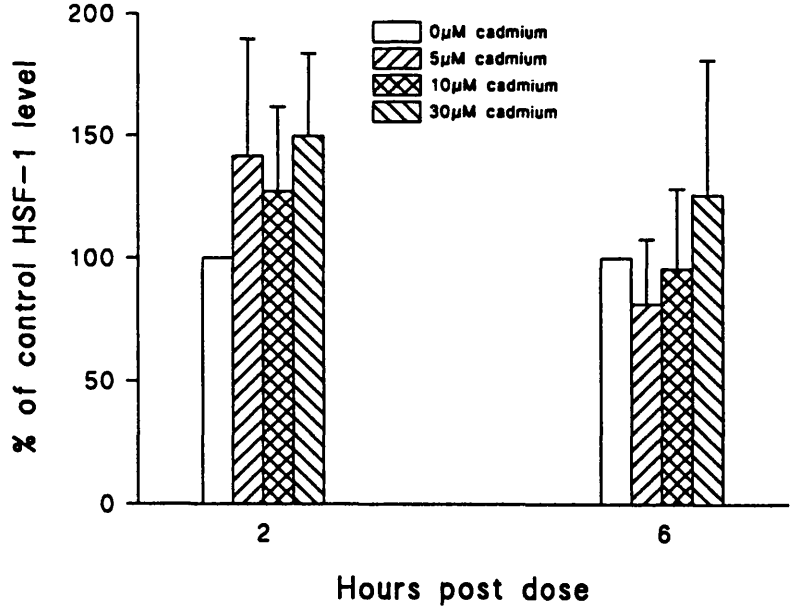


Figure 7.14 Effect of increasing concentrations of cadmium chloride on total HSF-1 levels in liver spheroids. Values are expressed as mean % of control ± SEM. n=3.

7.5.4 Discussion

In contrast to all those data presented previously in this chapter, hydrazine was shown to elevate the levels of activated HSF-1 in liver spheroids six hours post dose. The reasons for this are unclear, but the results do correlate well with those presented previously in chapter 6.8 which demonstrated that levels of Hsp25 were elevated six hours following dosing with hydrazine. It was unknown then as to whether or not this a true effect of hydrazine as previous data from hepatocyte monolayers and hepatocytes *in vivo* demonstrated that hydrazine had no effect on Hsp25 levels in these cells. Additionally in spheroids HSF-1 activation was shown to correlate with Hsp25 induction, this is in contrast to the result in hepatocyte monolayers where HSF-1 activation and Hsp25 induction are unrelated. Therefore, with consideration of the results presented here it would appear that hydrazine does elevate the levels of Hsp25 in liver spheroids. However, comparison of the concentrations of hydrazine required in spheroids to induce Hsp25 and those required to alter the level of activated HSF-1 show that HSF-1 was activated at concentrations which did not cause the induction of Hsp25. Levels of Hsp25 were only elevated in those spheroids exposed to concentrations of hydrazine of 100mM. However, levels of activated HSF-1 were shown to be elevated in those spheroids exposed to 10 and 50mM hydrazine whereas levels were unchanged following dosing with 100mM hydrazine. This pattern of heat shock protein induction and HSF-1 activation is different from that observed previously with respect to cadmium, exposure of hepatocytes to cadmium causes the induction of heat shock proteins and activation of HSF-1 at generally the same concentration. Hydrazine, however, causes the activation of HSF-1 at chemical concentrations that do not affect levels of Hsp25 or Hsp72/3. This means, therefore, that hydrazine may be causing the induction of stress proteins other than Hsp25 or Hsp72/3 at these concentrations or it may be that even though hydrazine activates HSF-1 it fails to increase levels of heat shock proteins. This second possibility is feasible as it has been shown previously that exposure of NIH-3T3 cells to certain compounds causes the activation of HSF-1 but again fails to increase the levels of heat shock proteins (Bruce *et al.* 1993). This is the first observation that hydrazine causes modulation of the stress response in hepatocytes, however the fact that this response only occurs in liver spheroids would imply that the control of the stress response in spheroids is different to that in hepatocyte monolayers or hepatocytes *in vivo*.

Exposure of liver spheroids to cadmium caused no significant changes in the levels of HSF-1 activation, however these data were not consistent across all the samples of spheroids taken. One set of spheroids sampled showed no change in the levels of HSF-1 activation following cadmium exposure, however, in the other samples there was a 2-fold increase in the levels of activated HSF-1. This result demonstrated that, as with some of the results in chapter 6, the response of liver spheroids to chemical exposure was not always consistent. This difference in sensitivity has been attributed to differences in spheroid viability between the individual samples. Comparison of the changes in HSF-1 activation with the changes in heat shock protein levels in spheroids following cadmium exposure demonstrates some close similarities. It has been shown previously in chapter 6.7, that, within two hours of exposure of spheroids to $CdCl_2$ at a concentration of 5μM there was a 2-fold non-significant elevation in the levels of Hsp72/3, this closely resembles the changes in activated HSF-1 levels which showed a 2-fold increase in two samples of spheroids following exposure to 5μM $CdCl_2$. Again this re-inforces the some of the previous observations that changes in HSF-1 activation were occurring at the same chemical concentrations which altered the levels of individual heat shock proteins.

7.6 Conclusions

One of the main aims of these investigations was to determine whether changes in the activation of HSF-1 could be used as a general sensitive marker of toxicity in hepatocytes. This would determine whether measurement of the activator of heat shock proteins was a more acccurate general marker of toxicity than measurement of the indivdual heat shock proteins, however the answer to this question was not as clear as hoped. With respect to cadmium it appeared that changes in the activation of HSF-1 were occurring at a chemical concentration equivalent to those which caused the induction of Hsp72/3. Therefore, measurement of the changes in the activation of HSF-1 in hepatocytes following cadmium exposure is no more sensitive a marker of toxicity than measurement of the levels of Hsp72/3. However, this is not the case when the changes in the localisation of HSF-1 are measured following cadmium exposure in hepatocyte suspensions. Here, a significant accumulation of HSF-1 in the nuclei of exposed cells was observed at concentrations

below those required for stress protein induction. With respect to hydrazine the picture is more complex. Hydrazine does not affect the activation of HSF-1 in hepatocytes in suspension, in monolayers nor in hepatocytes *in vivo*. This was as expected as levels of Hsp72/3 or Hsp25 were unaltered in the all the previous hepatocyte models following hydrazine exposure. But it was shown that hydrazine did affect the activation of HSF-1 in liver spheroids, this activation occurred at chemical concentrations which did not alter the levels of Hsp25 in the spheroids. Therefore, it would apear that measurement of HSF-1 activation does appear to be a more sensitive indicator of toxicity following hydrazine exposure than measurement of indivdual stress protein levels, but this is only true for the response in liver spheroids.

If the response of liver spheroids to hydrazine is different from all the other hepatocyte models used it is plausible that the control of the stress response is different in liver spheroids. Considering that exposure of hepatocytes *in vivo* did not result in the activation of HSF-1 it is feasible to assume that the response of liver spheroids does not represent the true physiological situation. It may be that during the culture of hepatocytes for spheroid development the cells undergo some transformation that essentially means once the spheroids were exposed to hydrazine HSF-1 was activated and elevated levels of Hsp25 expressed. This theory is re-inforced by results from chapter 6.4 which showed that basal levels of Hsp25 were elevated during the culture of spheroids, therefore it would appear that the control of Hsp25 induction during spheroid culture is different from that in hepatocytes *in vivo*. Hsp25 is known to be involved in the organisation of actin filaments (Benndorf *et al.* 1994) and as such the elevated basal levels of Hsp25 during spheroid culture imply that the cytoskeleton may be altered in some way possibly as a result of the structural re-organisation of spheroids during spheroid culture. This alteration of the cytoskeleton may effectively be making it susceptible to the toxic action of hydrazine and as such exposure of the spheroids to hydrazine results in cytoskeletal damage and hence induction of Hsp25. As the cytoskeleton is in its normal state in hepatocytes in situ, then exposure of these hepatocytes *in vivo* to hydrazine does not result in the induction of Hsp25, as is the case.

Exposure of hepatocytes to cadmium results in the activation of HSF-1 and the concurrent

induction of Hsp72/3, however exposure of spheroids to low concentrations of hydrazine effectively causes the activation of HSF-1 without the induction of Hsp25 or Hsp72/3. There are several explanations for this observation. Firstly, hydrazine may be activating HSF-1 at these concentrations and is in fact causing the induction of heat shock proteins other than Hsp25 or Hsp72/3. Another possibility, is that hydrazine activates HSF-1 but fails to elevate heat shock proteins in the cells, this situation has been demonstrated previously *in vitro* where compounds known to generate oxidative stress fail to increase levels of heat shock proteins following the activation of HSF-1 (Bruce *et al.* 1993). However, there is no evidence that hydrazine generates oxidative stress in hepatocytes. The final possibility is that at these low concentrations of hydrazine, the damage inflicted to the cells is sufficient to cause the activation of HSF-1 but not sufficient enough to cause the induction of heat shock proteins. The mechanism for this may involve a factor bound to the heat shock element (HSE), the constitutive HSE binding factor (CHBF), this factor is present on the HSE where it is thought to act as a negative regulator and is discussed in greater detail in chapter 1.5.3. For increased transcription of heat shock proteins the CHBF is required to dissociate from the HSE (Kim *et al.* 1995, Liu *et al.* 1995). Following exposure of spheroids to low concentrations of hydrazine HSF-1 is activated which subsequently binds to HSE, however the damage inflicted to the cells is insufficent to cause the dissociation of CHBF from HSE and so levels of Hsp25 are not elevated. It is only as the concentrations of hydrazine increase that the damage to the cells becomes severe enough to cause the dissociation of CHBF from HSE with the subsequent induction of Hsp25.

Stevens *et al.* (1995) demonstrated that the induction of Hsp70 following HSF-1 activation by certain compounds was intimately linked with their ability to deplete cellular GSH levels. However those data presented in this chapter are in disagreement with these findings as it has been demonstrated that exposure of hepatocytes to both hydrazine and cadmium causes the activation of HSF-1 and induction of heat shock proteins at chemical concentrations which do not cause any depletion in cellular GSH levels. The converse is particularly true with reference to hydrazine in that it depletes GSH levels significantly in primary hepatocytes but fails to cause the activation of HSF-1 in these cells. Therefore, the precise mechanism behind the hydrazine induced activation of HSF-1 in liver spheroids

remains to be elucidated.

One of the initial biochemical events that occurs in hepatocytes following hydrazine exposure is an inhibition of cellular protein synthesis (Waterfield *et al.* 1997). The question therefore has to be asked as whether this effect of hydrazine affects the stress response of cell? It is known that exposure of cells to protein synthesis inhibitors such as cyclohexamide or puromycin suppresses the activation of HSF-1, but only when the inhibitors are present in the cell prior to the stressful insult, if the inhibitors are added at the time of insult to the cell no inhibition of HSF-1 is observed (Baler *et al.* 1992). This inhibition is thought to be due to the fact that cyclohexamide pre-treatment inhibits the synthesis of nascent polypeptides which require the association of Hsp70 for folding and transport and so the levels of unbound Hsp70 increase. Therefore, a stressful insult will not cause the activation of HSF-1 as newly synthesised Hsp70 is not required due to the elevated levels of unbound Hsp70. So how does this situation relate to hydrazine exposure in hepatocytes? Exposure of hepatocytes to hydrazine will initially cause the inhibition of protein synthesis which is subsequently followed by other biochemical perturbations, these biochemical changes should cause the induction of the stress response but as explained above inhibition of protein synthesis of hydrazine may elevate basal levels of Hsp70 thus the activation of HSF-1 is not required. However, this theory is not re-inforced by observations in previous experiments, for example exposure of hepatocytes for two hours does not result in consistent inhibition of protein synthesis and additionally exposure of spheroids to hydrazine results in the activation of HSF-1.

In summary, measurement of HSF-1 activation or accumulation following chemical exposure is not as consistent a marker of toxicity as was hoped. Exposure of hepatocytes to cadmium results in the nuclear accumulation of HSF-1, but only in hepatocyte suspensions are the concentrations below those required to induce Hsp72/3. In spheroids and *in vivo* HSF-1 activation occurs at the same chemical concentration which alter levels of Hsp72/3. Additionally the activation of HSF-1 is different in hepatocyte monolayers than in all of the other hepatocyte models. Hydrazine does not activate HSF-1 in hepatocytes, with the exception in spheroids, even when the concentrations are toxic. Some of the possible reasons for this situation are detailed above. Therefore, more

information is required as to the differential molecular control of the stress response in the individual hepatocyte models to elucidate whether changes in HSF-1 activation may be used as marker of toxicity.

Chapter 8.

Final discussion

8.1 Thermo- and chemico-sensitisation/tolerance in hepatocyte suspensions and monolayers

One of the main aims of these investigations was to observe whether in hepatocytes, like numerous other cell types, the phenomenon of thermotolerance occurs. The results demonstrated that not only thermotolerance but also thermosensitisation occurs in hepatocytes, albeit only at specific timepoints. This bi-phasic change in cellular sensitivity has been demonstrated in other cell types following hyperthermic treatment or chemical exposure (Delpino *et al.* 1992, Wiegant *et al.* 1997). The results presented previously in chapter 3 demonstrated that exposure of hepatocytes to a hepatotoxin immediately following heat shock resulted in a slight increase of the compounds toxicity. However, if the same cells were exposed to the toxin a number of hours following heat shock it resulted in a decrease of the compounds toxicity, the phenomenon known as thermotolerance. In addition to demonstrating thermo-tolerance and -sensitivity hepatocytes also exhibited chemical tolerance in that exposure of the cells to a sub-threshold concentration of a compound altered the subsequent toxicity of the compound. In these studies exposure of hepatocyte monolayers to low concentrations of hydrazine decreased slightly the subsequent toxicity of high concentrations of hydrazine, but again, only at specific timepoints. The mechanisms behind these bi-phasic changes in the cells' sensitivity, particularly those underlying thermosensitisation, are not entirely understood. Thermosensitisation is usually demonstrated by a protocol known as step-down heating which consists of a brief severe hyperthermic treatment immediately followed by chronic exposure to a lower hyperthermic treatment. This sequence results in a synergistic interaction between the two hyperthermic treatments so that the resultant cell death due to the chronic hyperthermic treatment is greater than if the treatment was given alone. This characteristic pattern of cell death also occurs, as demonstrated in the experiments described in chapter 3, when for the second treatment the cells were chronically exposed to toxins instead of a hyperthermic treatment. The reasons for this thermosensitive effect are usually based around the two-step model of hyperthermic cell death in that the first step involves the formation of non-lethal lesions and in the second step one of these lesions is converted into a lethal event (Delpino *et al.* 1992). However, although this may explain the changes in cellular sensitivity observed following hyperthermic treatments it does not clearly explain the observations that the sensitivity to chemical exposure is

altered by a previous hyperthermic treatment. This may be due to the possibility that hyperthermic treatment and chemical exposure may have differing mechanisms of toxicity. A possible explanation for this lies in the fact that the initial hyperthermic treatment induces a stress response. During the initial hyperthermic treatment the levels of damaged proteins increase which are then targeted by the existing pool of free Hsp70. Prior to Hsp70 binding to the damaged proteins it must first dissociate from HSF, this dissociation results in the increased transcription of Hsp70. However, depending what tissue is studied, it takes a number of hours for the levels of heat shock protein mRNA to increase (Blake *et al.* 1990). Therefore, there would appear to be a time immediately following the initial hyperthermic treatment when the levels of free Hsp70 in the cell would effectively be depleted. Considering that Hsp70 is known to play a protective role against noxious stimuli is seems feasible that chemical exposure during this period would result in an increase of toxicity of the compound thus resulting in a thermosensitive effect.

Following the transient period of thermosensitisation associated after a hyperthermic treatment, the cell effectively becomes thermotolerant. The development of thermotolerance in cells is most commonly associated with the elevation of protective stress protein levels, however changes in GSH have also been implicated in the development of not only thermotolerance but also thermoresistance (Mitchell *et al.* 1983, Steel *et al.* 1992). The results in chapter 3 (fig. 3.3) showed that levels of GSH in heat shocked cells were significantly higher when compared to levels in non-heat shocked cells following hydrazine exposure thereby implying the role of GSH in thermotolerance may be of importance. There is evidence that there may be a link between induction of the stress response and GSH levels as it is known that modulation of GSH levels effects cellular stress protein levels (Freeman and Meredith 1989). Cells produce potentially damaging reactive oxygen species continually as a consequence of electron transport and cell signalling, however, normally the cells store of GSH effectively removes these species. However, during thermosensitisation one of the observations is a decrease in GSH and so these species, and those induced by the hyperthermic treatment, are not effectively removed so causing damage to various cellular components. This damage causes induction of the stress response and so again provides an explanation of the bi-phasic change in cellular sensitivity observed following hyperthermic treatment. However, it

would appear that the changes in cellular GSH levels are only effective in causing the induction of specific heat shock proteins as a decrease in cellular GSH levels is known to be a direct inducer of Hsp32 (Rizzardini *et al.* 1994). Alterations in cellular GSH levels have been shown to have no effect on other stress proteins namely Hsp70 and Hsp90 (Katoh *et al.* 1991). These results clearly demonstrate that there is individual control over the induction of specific stress proteins following hyperthermic treatment. Overall it would appear that following an initial hyperthermic treatment the free pools of GSH and Hsp72/3 are decreased, these changes effectively lead to an increase in the sensitivity of the cell to subsequent chemical or hyperthermic treatment. Within a number of hours the depletion of GSH causes the induction of Hsp32 and additionally there is increased transcription of other stress proteins, namely Hsp72/3, due to thermal damage to the cells. This increase in cellular stress protein levels protects the cell from further chemical or hyperthermic treatment leading to a state of thermotolerance.

Due to the protective state induced when a cell is thermotolerant it has been proposed that modulation of the response has potential investigative or therapeutic applications. However, modulation of the stress response is problematic as it is difficult to induce a cellular stress response, particularly by hyperthermic treatment, without causing overt damage to the cells as by its nature the stress response is induced when a cell undergoes a potentially damaging noxious stimuli. Recently a group of compounds known as 20 S proteasome inhibitors have been developed, one group of these are the peptide aldehydes (carbobenzoxyl-leucinyl-leucinyl-leucinal [MG132]) which have been shown to inhibit the degradation of proteins by the ubiquitin-proteasome pathway. These compounds therefore cause an accumulation of abnormal proteins within the cell and have been shown to elevate stress protein levels in the cytosol and endoplasmic reticulum and induce a thermotolerant state in the cell without affecting levels of normal protein synthesis or protein secretion (Bush *et al.* 1997). These observations raise the possibility for selective inducers of stress response as therapeutic agents that have the potential to protect the cell from injury, however much work is still required to determine whether proteasome inhibitors have any deleterious effects on cellular function. However, these compounds do raise the potential for therapeutic chemical manipulation of the stress response although as yet the specificity of the compounds remains to be fully determined.

8.2 Comparison of hydrazine and cadmium toxicity in hepatocytes in vitro and liver in vivo

The nature of the experiments in these studies was to investigate the effects of compounds on the stress response in hepatocytes *in vitro* and in the liver *in vivo*, however, these data produced allowed an investigation into the relative toxicity of the compounds in the different hepatocyte models, namely hepatocyte monolayers, liver spheroids and liver *in vivo*. The toxicity data showing the concentrations of hydrazine required to alter biochemical parameters in hepatocyte monolayers, liver spheroids and liver *in vivo* are shown in table 8.1. It is clear from these results that the concentration of hydrazine required *in vitro* to alter certain biochemical parameters is approximately 100-fold higher than the concentration required *in vivo* to cause similar effects. There are a number of possibilities for the large differences in hydrazine toxicity observed in hepatocytes *in vitro* and *in vivo*. Firstly, there may be problems with the uptake of hydrazine into hepatocyte monolayers or hydrazine may be being lost from the culture medium but this is unlikely as the compound is only present in the media for two hours. One of the other possibilities that may underlie the difference in toxicity *in vivo* and *in vitro* is the lack of other cell types in hepatocyte monolayers when compared to the organisation of hepatocytes *in vivo*. It is well known that Kupffer cells *in vivo* play an important role in the toxicity of certain compounds, Sipes *et al.* (1996) demonstrated that modification of function of Kupffer cells subsequently altered the toxicity of certain hepatotoxic compounds such as acetaminophen and allyl alcohol. The absence of Kupffer cells in hepatocyte monolayers may therefore be a factor in the difference in hepatotoxicity of compounds *in vivo* and *in vitro*, although the contribution of Kupffer cells to the toxicity of hydrazine remains unknown. One of the obvious biochemical differences between hepatocytes *in vivo* and *in vitro*, that is specifically known to affect certain compounds whose toxicity is based upon metabolic activation such as carbon tetrachloride and acetaminophen, is the difference between cytochrome P450 levels in hepatocytes *in vitro* and those *in vivo*. However this is an unlikely cause for the difference in toxicity observed *in vivo* and *in vitro* as it has been shown that the toxicity of hydrazine is not dependent on metabolic activation *in vitro* (Delaney and Timbrell 1995).

Table 8.1. Summary of data showing threshold concentrations of hydrazine required to significantly alter biochemical parameters *in vivo* and *in vitro*

	Hepatocytes *in vitro*		Liver *in vivo*	
Biochemical parameter	Hepatocyte monolayers	Liver spheroids	Dose[a]	Plasma concentration[b]
LDH	>20mM	↑ 100mM (24hrs)	>10mg/kg	>0.1mM
ATP	↓ 10mM (2hrs)	↓ 10mM (2hrs)	↓ 10mg/kg	0.1mM
GSH	↓ 1mM (4hrs)	>500mM	↓ 5mg/kg	0.075mM
Hsp25	NE	↑ 50mM (6hrs)	NE	-
Hsp72/3	NE	NE	NE	-

↑; signifcantly increased, ↓; significantly decreased

[a]; all *in vivo* values were taken at six hours post dose.

[b]; plasma concentrations taken from Waterfield *et al.* 1997

Values in ( ) refer to time in hours post dose at which the significant effect was first observed.

NE; no significant effects observed.

Overall these data show that the biochemical changes observed in hepatocyte monolayers following hydrazine exposure does not accurately represent the biochemical situation *in vivo*. The exact reasons for this discrepancy are unclear and investigative studies are hampered by a lack of precise knowledge of the hepatotoxic action of hydrazine.

Comparison of the biochemical toxicological data concerning the toxicity of cadmium in hepatocyte monolayers and the liver *in vivo* (table 8.2) demonstrates that the concentrations of cadmium required to alter biochemical measurement in monolayers were approximately 10-fold higher than those required *in vivo*. One of the reasons for this apparent difference in toxicity may be due to the different uptake profile of cadmium in hepatocytes *in vivo* than in hepatocyte monolayers. Cadmium accumulates in the cytoplasm in hepatocytes *in vivo* whereas following exposure cadmium accumulates in the nuclei of hepatocyte in culture (Stoll *et al.* 1976, Din and Frazier 1985). This difference in cadmium localisation may have profound effects on the toxicity as cadmium has pronounced effects on gene expression. The effects of cadmium on gene regulation include the induction of metallothionein, induction of glutathione synthesis, induction of stress proteins and induction of the tumour suppressor gene p53 (Bayersmann and Hachtenberg 1997). Upregulation of these genes has a protective effect to the cell; metallothionein and glutathione are both involved in detoxification processes, stress proteins are known to exert protective effects and the protein p53 is involved in cell proliferation. Therefore, due to the elevated levels of cadmium found in the nuclei of primary hepatocytes the upregulation of those genes known to have a protective effect on the cell would be greater than that observed *in vivo* as nuclear cadmium levels are lower in hepatocytes *in vivo*. This could provide an explanation why cadmium was effectively less toxic in hepatocyte monolayers than *in vivo*. However, in the studies here cadmium was not shown to significantly elevate total cellular GSH in hepatocyte monolayers but an effect on the other genes cannot be ruled out. What also has to be taken into account is that, in addition to its beneficial effects, the accumulation of cadmium in a cells' nucleus is known to have deleterious effects as cadmium is known to induce DNA conformational aberrations, DNA strand breaks and chromosomal aberrations albeit at relatively high cytotoxic concentrations. Therefore, overall the contribution of cadmium uptake and

Table 8.2. Summary of data showing threshold concentrations of cadmium required to significantly alter biochemical parameters *in vivo* and *in vitro*

	Hepatocytes *in vitro*		Liver *in vivo*	
Biochemical parameter	Hepatocyte monolayers	Liver spheroids	Dose[a]	Equivalent *in vitro* concentration[b]
LDH	↑ 30μM (6hrs)	↑ 30μM (6hrs)	>2mg/kg	>1.2μM
ATP	↓ 10μM (4hrs)	>100μM	↓ 2mg/kg	1.2μM
GSH	↓ 30μM (6hrs)	↓ 100μM (6hrs)	>2mg/kg	>1.2μM
Hsp25	↓ 30μM (6hrs)	NE	NE	-
Hsp72/3	↑ 5μM (4hrs)	↑ 30μM (6hrs)	↑ 1mg/kg	0.6μM

↑; signifcantly increased, ↓; significantly decreased

[a]; all *in vivo* values were taken at six hours post dose.

[b]; equivalent *in vitro* concentrations refers to the approximate concentration of cadmium required *in vitro* to produce the same intracellular hepatocyte load of cadmium as the*in vivo* dose used (estimated from data from Hellström-Lindahl and Oskarsson 1989 and Goering *et al.* 1993b)

Values in ( ) refer to time in hours post dose at which the significant effect was first observed.

NE; no significant effects observed.

localisation on the toxicity of cadmium in hepatocyte monolayers, when compared to its effects in the liver *in vivo*, cannot be ruled out due to the profound effects cadmium has on gene regulation.

The effect of the localisation and accumulation of cadmium on cellular gene regulation may in some way explain the difference cadmium had on the levels of stress protein induction in liver spheroids when compared to its effects in the liver *in vivo*. Studies investigating the effect of cadmium Hsp72/3 levels in spheroids and liver *in vivo* determined that the maximal induction of this stress protein was approximately 4-fold higher in liver spheroids when compared to the response in the liver *in vivo*. This discrepancy may be explained by the fact that the cadmium accumulation in the nuclei of spheroids caused a greater induction of Hsp72/3 when compared to the effect in the liver *in vivo* where the cadmium would be localised to the cytoplasmic compartment. However as there is no data concerning the distribution of cadmium in liver spheroids this theory assumes that the uptake and distribution of cadmium in the hepatocytes contained within liver spheroids is similar to that observed in hepatocyte monolayers but this may not necessarily be the case.

Comparison of the biochemical toxicity *in vivo* of cadmium with the toxicity *in vitro* also demonstrates a marked difference. Hepatic ATP levels *in vivo* were decreased when the intracellular hepatocyte cadmium load was estimated to be 0.138µmols Cd/g (equivalent to 2mg/kg) [cadmium load data calculated from results presented by Goering *et al.* 1993b. showing cadmium tissue concentrations following exposure]. This is in comparison to the effect of cadmium on ATP levels in hepatocytes where it was determined previously in chapter 4.5 that levels were initially decreased when the intracellular cadmium load was estimated to be 4.98µmols Cd/g (equivalent to 30µM $CdCl_2$). One of the possible reasons for this difference in toxicity may be that hepatocyte monolayers do not contain Kupffer cells whereas the liver consists of a mixture of hepatocytes and Kupffer cells. Evidence for the contribution of Kupffer cells towards cadmium toxicity comes from studies using the Kupffer cell inhibitor gadolinium chloride which inhibits their activity. Inhibition of Kupffer cell activity by gadolinium chloride significantly modified $CdCl_2$ induced acute hepatotoxicity (Sauer *et al* 1997). Therefore the lack of Kupffer cells in hepatocyte

monolayers may effectively decrease the hepatotoxicity of cadmium *in vitro* when compared to its hepatotoxic effect *in vivo*.

Comparison of the toxicity of hydrazine and cadmium in the two different hepatocyte models, monolayers and liver spheroids, showed that generally the spheroids were less sensitive to the toxic actions of both of the compounds used. The possible reasons have been discussed previously but briefly they are thought due to be the physical and biochemical differences between the different culture methods. The three-dimensional structure of liver spheroids is in comparison to the flattened two-dimensional structure of primary hepatocytes, this difference in cell shape would require the re-organisation of the network intermediate filaments in hepatocyte monolayers, an effect which may have an effect on the cells' ability to respond to a toxin. In addition to this it has been demonstrated that the cell aggregation observed during spheroid formation is of particular importance for the continuous expression of liver-specific functions in the cells (Yuasa *et al* . 1993). The expression of liver-specific proteins in liver spheroids is at a much higher level when compared to the expression of similar proteins in hepatocyte monolayers, expression of some of these proteins has the effect of decreasing the toxicity of certain chemicals following exposure and so the increased protein levels have the potential to alter the toxicity of certain chemicals in liver spheroids (Tong *et al.* 1992, Roberts and Soames1993, Schilter *et al.* 1993, Tamura *et al.* 1995, Ammann and Maier 1997, Juillerat *et al.* 1997). Therefore, when comparisons are made between the sensitivity of these two hepatocyte culture methods these observations imply that changes occurring during the primary culture of hepatocytes effectively makes the cells increasingly susceptible to chemical exposure.

The use of spheroids has been proposed to be of use in the development of bioartificial livers in the treatment of patients with severe liver failure (Naruse *et al.* 1996), however there is still controversy as to whether spheroid formation in the bioreactors can meet the clinical requirements required of such a product. The use of spheroids has been proposed in such a model due to the fact that these cells maintain many of their liver specific functions during culture. However, results in chapter 6 demonstrated that during the culture of liver spheroids levels of the stress protein Hsp25 were found to be elevated

after approximately 8 days in culture. Although the reasons behind this elevation are unknown it does imply that the cellular state of spheroids is perturbed in some way during culture which would imply that the cells are under some kind of stress whilst in culture. It is feasible that as a consequence of this elevation of Hsp25 certain cellular functions may be impaired. If the expression of certain stress proteins is altered during spheroid culture it seems feasible that the expression of other cellular proteins may be altered which could result in an impairment of normal physiological function but whether these changes have any effect on the function of liver spheroids in bioreactors remains to be determined.

One of the most obvious differences between the two different hepatocyte models used in these investigations was the difference in the basal level of stress proteins during culture. In the experiments with cadmium or hydrazine the basal stress protein levels in hepatocyte monolayers were significantly elevated when the cells were exposed to the compound whereas in spheroids the levels were unchanged from control. It would be expected that this would alter the toxicity of the chemicals in that due to the protective nature of stress proteins the elevated basal levels would confer a degree of protection to the hepatocyte monolayers against any chemical exposure. However, comparison of the biochemical data (tables 8.1 and 8.2) demonstrates that, with particular relevance to certain parameters such ATP and GSH levels, hepatocyte monolayers are more sensitive to cadmium or hydrazine exposure than liver spheroids. The possibility may be that the induction of the stress response, instead of conferring a degree of protection to the cells, may in fact make a cell more susceptible to chemical exposure. This was proposed by Hightower and Ryan (1997) who suggested that the induction of a stress response may compromise the cells due to the metabolic output associated with induction of stress proteins. As the stress response is essentially transient in nature any continuous expression of stress proteins would be at great metabolic expense to the cell. Therefore any chemical exposure during this metabolically challenged state would in effect cause a greater degree of damage to the cells as they would be unable to mount a substantial protective response. This theory may underlie the increased toxicity observed in hepatocyte monolayers as basal levels of stress proteins were substantially elevated in these cells and so these cells would be unable to mount an effective defence mechanism as liver spheroids, however, any deleterious effects would be offset to a degree by the elevated stress protein levels

themselves. However, direct comparisons of this kind between the two different hepatocyte models are problematic due to the physical and biochemical differences between the models discussed previously which also may have an effect on the toxicity of compounds.

8.3 Use of the stress response in hepatocytes as a marker of toxicity

Exposure of cells to heat shock results in an apparent cessation of cellular protein synthesis, that is with the exception for heat shock proteins whose synthesis is up-regulated following heat shock. The reason for this difference is thought to be due to differences in the translational initiation of normal mRNA and heat shock protein mRNA. One effect of heat shock is the decrease in the factors involved in the recruitment of mRNA to the ribosome, the initiation factors of the eIF-4 group as heat shock has been shown to decrease the activity of certain of these factors by decreasing their activity or inactivation of the kinases responsible for their phosphorylation. However, it has been demonstrated that translation of heat shock mRNA has a low requirement for the activity of the initiation factors eIF-4E and eIF-4γ and so the heat induced inhibitory effect on the factors will not result in an inhibition of heat shock protein synthesis (Joshi-Barve *et al.* 1992). It was hoped that this induction of heat shock proteins could be utilised as markers of toxicity.

However, the results presented here demonstrate that overall the induction of heat shock proteins or activation of HSF-1 does not always correlate well with the toxicity observed. Nevertheless in certain cases exposure of hepatocytes to cadmium resulted in heat shock protein induction which did correlate well with the toxicity observed. However, even in these cases the drawback was that only specific stress proteins were induced. In certain cases following cadmium exposure HSF-1 was determined to be activated and increased levels of Hsp70 were observed but Hsp25 levels were unchanged. Therefore, from these results it would appear that the activation of HSF-1 and induction of Hsp25 are not directly linked. Additionally this effect has also been demonstrated for Hsp60 which itself was not induced following cadmium exposure (Ovelgönne *et al.* 1995). Thus, following the activation of HSF-1 it appears that HSF-1 interacts only with the heat shock element (HSE) located on the Hsp70 gene and does not target the HSE's located on Hsp25 or

Hsp60 genes. Therefore, there must be an additional level of control in the transcriptional activation of stress proteins, one of these additional levels may be attributable to the phosphorylation of HSF-1 or the involvement of additional factors. Phosphorylation is known to play an important role in the activation of HSF-1 and so it may be hypothesised that differential phosphorylation of HSF-1 may be a second level of control in stress protein induction in that it is involved in the specific targeting of HSF-1 to individual stress proteins. However, the precise molecular mechanisms underlying selective stress protein induction following chemical exposure remain to be fully understood. Another possibility for the selective induction of specific stress proteins is the involvement of other isoforms of HSF other than HSF-1 as the functional differences and activation kinetics between different HSFs remain relatively unknown. The activation of HSF-3 has been demonstrated to induce stress protein transcription following prolonged severe stress treatments by either chemical or hyperthermic treatment (Tanabe *et al.* 1997). This raises the possibility that individual HSFs have different activation thresholds depending on the nature of the insult and as such may represent another level of control for stress protein transcription. Other possibilities whose contribution to the specificity of heat shock protein induction remains to be determined include the involvement of other, as yet, unidentified HSFs or HSEs.

In addition to the problems associated with the stress response being protein-specific there is another problem associated with the use of heat shock proteins as markers of toxicity in that the induction of the stress response is stressor specific. This was demonstrated in the investigations in hepatocyte monolayers where exposure to cadmium induced a stress response but exposure to hydrazine failed to do so. This difference in induction is thought to be due to the mechanism of action of the compounds and the ability to interact with cellular proteins is thought to be one of the most important factors in triggering a stress response. Although cadmium is known to interact with cellular proteins, hydrazine is not thought to interact directly with them but appears to interfere with protein synthesis. However, although hydrazine does not appear to be a direct inducer of the stress response secondary changes in certain biochemical parameters such as GSH levels would effectively cause an increase in free radical induced protein damage which should be effective in inducing such a response but this was not the case in these investigations. However, even

though certain biochemical changes caused by a compound may induce a stress response other biochemical changes may be detrimental to induction of any such response. Depletion of cellular ATP levels effectively causes an inhibition of the dissociation of Hsp70 from denatured proteins that is required for maintenance of the stress response, therefore depletion of cellular ATP will effectively inhibit a cell from mounting a full stress response following chemical exposure. These examples demonstrate how changes in the mechanism of action of a compound can have dramatic effects on induction of the stress response and as such a pre-determined knowledge of the mechanism of action of a compound is essential for any such investigations on its induction of the stress response.

The use of stress proteins as markers of toxicity has some general problems associated with them due to the fact that many of the stress proteins have an essential physiological role to play. Levels of stress proteins have been shown to be altered at varying ages both *in vivo* and *in vitro* and in addition levels were also changed following calorific restriction in rats (Heydari *et al.* 1993). This would therefore mean that the toxicity of certain compounds would be altered due to the changes in stress protein levels and additionally the use of older rats for studying the induction of heat shock proteins as markers of toxicity would be of limited use. Furthermore, the use of stress proteins as markers would be tissue or cell specific as different tissues appear to have differing control mechanisms for stress protein induction as following heat shock there is discordant expression of stress protein mRNAs between individual tissues (Blake *et al.* 1990). Again with consideration that stress proteins are intimately involved with many cellular processes levels are obviously going to change depending on environmental and physical conditions and as a result it would be difficult to reduce inter-lab variation. The generation of false positive results would be a problem additionally as certain physiological states are known to cause induction of stress proteins, in particular increased levels of circulatory cytokines such as prostaglandin A_1 or arachidonic acid (Amici *et al.* 1992, Jurivich *et al.* 1994) and so any study investigating the use of heat shock proteins would have to be controlled with care to avoid any such results. With these issues in mind it would appear that at this moment the use of stress protein induction as a predictive tool in toxicity screening has to be approached with caution.

8.4 Future directions of stress protein research

Even with all these associated problems there are several areas of research that have been proposed to have potential in elucidating whether measurement of the stress response could be used as a general marker of toxicity. One of the main areas of interest in stress protein measurement is the application of transgenic technology to the investigation of stress proteins where foreign reporter genes are placed under the control of heat shock gene promoters. The advantage of using reporter genes is that an easily quantified protein product is rapidly secreted out of the cells, this is in contrast to the direct measurement of intracellular stress protein levels which in comparison is a relatively cumbersome procedure. Fischbach *et al.* 1993 expressed a transfected human growth hormone gene sequence driven by the human Hsp70 promoter in NIH/3T3 cells and used the system to rank the relative toxicity of a range of metals. Generally the secretion of human growth hormone correlated well with other markers of toxicity such as LDH leakage and neutral red uptake, however there were a number of metals that were overtly toxic that did not induce the Hsp70 promoter even when relatively high concentrations were used. This selective non-induction of Hsp70 by overtly toxic compounds was also demonstrated in HepG2 hepatoma cells where chloramphenicol acetyl transferase (CAT) expression was placed under the control of the Hsp70 promoter (Todd *et al.* 1995). Although some compounds caused the induction of Hsp70 there were certain compounds (phorbol mystirate acetate, all *trans*-retinoic acid, dioxin, benzo[*a*]pyrene) that although being toxic to the cells did not induce Hsp70. These results highlight the problem again associated with measurement of a single stress protein promoter in that although the compound may be toxic it may be inducing stress proteins other than Hsp70. Therefore the advantages of reporter genes in the flexibility they provide over normal stress protein measurement is counteracted by the fact that it still only gives stressor specific responses depending on which stress gene promoter has been used.

The application of two-dimensional electrophoresis (2DE) has been proposed to be of use as it has been hoped that chemical exposure produces characteristic patterns of individual stress proteins. In support of this hypothesis the locations of the major stress proteins (Hsp25, Hsp32, Hsp60, Hsp72/3 and Hsp90) have been mapped in human and rodent liver homogenates (Witzmann *et al.* 1995). However as of yet there is little data

concerning the effect of compounds on the 2DE pattern of stress proteins. Exposure of rats to perfluoro-*n*-octanoic acid and perfluoro-*n*-decanoic acid resulted in the selective dose dependent induction of Hsp60 and Grp75. (Witzmann *et al.* 1996). Much work remains to be done to determine whether measurement of the changes in the location of stress proteins following 2DE has potential as a specific marker of toxicity. Vogt *et al.* 1996 took the procedure further in that following 2DE the proteins were electroblotted and the resulting peptides were subsequently separated by high performance liquid chromatography (HPLC) and detected by on-line electrospray mass spectrometry (LC/MS). This technique allowed the specific identification and measurement of individual stress proteins and, following a chemical insult, allowed a sensitive identification of the modified forms of stress proteins in cells. Utilisation of these techniques would no doubt give a great deal of information on the molecular changes in stress proteins following chemical exposure and so in theory the procedure would have great potential as a sensitive marker of toxicity. As changes in the modified forms of stress proteins could be measured at great sensitivity work is still required to determine whether there are stressor specific alterations observed in the modifications of stress protein sequences. Additionally these methods do have potential due to the fact that they can detect changes in the complete family of stress proteins so far known and as such would have the greatest potential in detecting the widest range of adverse chemicals. However, the problems encountered during the work presented previously, particularly the stressor specific induction of stress proteins, would also become apparent with the use of both 2DE and transgenic stress gene technology. Some of these problems could possibly be overcome by moving away from individual stress protein measurement as markers of toxicity and focusing on the cellular events prior to the changes in stress protein levels.

The precursor to stress protein elevation is the activation of the heat shock transcription factor (HSF-1) and so it would seem feasible to measure HSF-1 activation following chemical exposure to determine whether the activation of HSF-1 correlates with the observed toxicity. The results of these investigations were presented in chapter 7. The measurement of HSF-1 activation did not prove to be a general, accurate indicator of cellular toxicity as exposure of hepatocyte monolayers to cadmium resulted in elevation of Hsp70 levels but HSF-1 did not appear to be activated. However on the positive side

HSF-1 was shown to be activated in hepatocyte suspensions following cadmium exposure and also exposure of spheroids to hydrazine resulted in HSF-1 activation. Overall it would therefore appear that HSF-1 activation does not always correlate well with stress protein elevation and subsequent toxicity. Other shortcomings of measurement of HSF-1 activation in these type of studies include the observations that exposure of cells to certain toxins such as menadione or H_2O_2 activated HSF-1 but there was no subsequent elevation of stress protein levels although only a limited number of individual stress proteins were measured (Bruce *et al.* 1993). With consideration that overall changes in HSF-1 activation do not correlate well with stress protein induction a possible specific aspect of HSF-1 activation which is of particular interest as a marker of toxicity is the phosphorylation of HSF-1 which appears to be an integral aspect in the activation of HSF-1.

Certain cellular regulators such as HSF-1 have been shown to be hyperphosphorylated upon activation by heat and other stressors and this stress induced phosphorylation also appears to correlate well with induction of stress proteins (Amici *et al.* 1992, Jurivich *et al.* 1994, Xia and Voellmy 1997). Additionally exposure of cells to compounds such as indomethacin and sodium salicylate result in the activation of HSF-1 and its subsequent binding to the Hsp70 HSE yet transcription of Hsp70 was not induced. However it was determined that in this case the activated HSF was not inducibly-phosphorylated whereas the control heat shocked HSF was determined to be phosphorylated (Cotto *et al.* 1996, Lee *et al.* 1995). This would imply that the phosphorylation of HSF may be one of the most specific changes following stressful insult and as such may represent one of the most accurate cellular changes for studying the stress response as a marker of toxicity. However recently evidence has come to light that exposure of cells to certain stressors, particularly elevated hydrostatic pressures, increased levels of Hsp70 mRNA and protein without the prior phosphorylation and activation of HSF-1 or the transcriptional induction of Hsp70 gene (Kaarniranta *et al.* 1998). It was suggested that, in this case, the induction of Hsp70 is as a result of mRNA stabilisation rather than its induction and if this is the case it represents a novel pathway for stress protein induction although it cannot be ruled out that the increased hydrostatic pressure may be affecting the other isoforms of HSF other than HSF-1. However these results highlight the information that is still required to determine whether the phosphorylation of HSF-1 has the potential as an accurate marker

of toxicity. In conclusion even though the measurement of stress gene induction may not be a general marker of toxicity it does not rule out the possibility of certain molecular changes in the cascade of events leading to stress protein induction may be of use in a battery of *in vitro* tests for sensitively determining the toxicity of certain chemicals.

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Appendices

Appendix 1. Solutions for isolation of hepatocytes

a) Stock buffers

i. 80.45g $NaCl_2$

ii. 5.5g KCl

iii. 14.98g KH_2PO_4

iv. 13.7g $MgSO_4.7H_2O$

v. 8.82g $CaCl_2.2H_2O$

vi. 4.85g $NaHCO_3$

-all solutions made up in 500ml UHQ H_20 and stable at 4°C.

b) Krebs-Henseleit (KH) x 2

-the following volumes of the above buffers were used;

i. 50ml

ii. 37.5ml

iii. 6.25ml

iv. 12.5ml

v. 25ml

+ 196ml UHQ H_2O.

- the solution was gassed for 10-15 minutes with 95%O_2 / 5% CO_2 before addition of 250ml solution vi.

This resultant solution was stable for 4 weeks at 4°C.

c) 5.88% $CaCl_2$

5.88g $CaCl_2.2H_2O$ in 100ml UHQ H_2O.

d) 0.4% Trypan blue

0.4g trypan blue in 100ml 0.9% $NaCl_2$

-solution was filtered (0.22µm membrane) and stored at -20°C.

e) Hanks x 10

i. 80.0g $NaCl_2$

ii. 4.0g KCl

iii. 2.0g $MgSO_4.7H_2O$

iv. 0.6g $Na_2HPO_4.2H_2O$

v. 0.6g KH_2PO_4

-made up to 1 litre with UHQ H_2O. Stable 4-6 weeks at 4°C.

Working solutions;

I) KH + HEPES

300ml KH x 2

300ml UHQ H_2O

1.8g HEPES

HEPES was dissolved and the solution gassed with 95%O_2/5%CO_2 for 5 minutes. To 200ml of this solution 2g bovine albumin was added (KH+Alb), the remaining solution contains no albumin (KH). The pH of both solutions was adjusted to 7.4.

II) Hank x 1

50ml Hank x10 solution

450ml UHQ H_2O

1.05g $NaHCO_3$

1.5g HEPES

HEPES and $HaHCO_3$ was dissolved and the solution gassed with 95%O_2 /5%CO_2 for 5 minutes. The solution was then divided as thus;

Hank 1	Hank 2
300ml Hank x 1	100ml Hank x 1
68.4mg EGTA	1ml 5.88ml $CaCl_2$
2g bovine albumin	50mg collagenase

The pH of both solutions was adjusted to 7.4.

Appendix 2. Solutions for determination of ATP

a) ATP buffer

$MgSO_4.7H_2O$	19.72g/l
KH_2PO_4	1.36g/l
$Na_2HAsO_4.7H_2O$	31.20g/l

- before use the buffer were mixed in 1:1:1 quantities and the pH adjusted to 7.4.

b) Luciferase

Firefly lantern extract (Sigma FLE-250) was resuspended in 5ml H_2O. The solution was spun at 1000rpm for 5minutes at 4°C to sediment any insoluble matter. The supernatant was left in the vial for up to 15 minutes in order to increase luciferase activity. The solution was stable at 4°C for a 3-4 hours when protected from light.

Standard curve for ATP

6mg of ATP was made up in 10ml 10% TCA which gave a 1mM ATP solution. 400µl of this solution was added to 10ml 10% TCA giving a working solution of 40µM ATP.

nmol/ml ATP	µl 40µM ATP	µl 10% TCA
0	0	800
5	100	700
10	200	600
15	300	500
20	400	400
30	600	200
40	800	0

Appendix 3. Solutions for fluorometric determination of GSH

a) Phosphate / EDTA buffer

13.6g KH_2PO_4

1.86g EDTA

- made up to 1 litre with H_2O
- adjust pH to 8.0, stable at 4 °C.

b) o-phthaldialdehyde (OPT)

10mg OPT in 10ml methanol

-solution was be stored in darkness at room temperature and used within 30 minutes of preparation

c) Trichloroacetic acid (TCA) 6.5%

32.5g TCA in 500ml H_2O

d) GSH standard

10mg GSH in 10ml 6.5% TCA → 1mg/ml GSH

100µl 1mg/ml GSH in 10ml 6.5% TCA → 10µg/ml GSH

e) Phosphate/EDTA + NaOH

500ml Phosphate/EDTA + 5.45ml 1M NaOH

Standard curve for GSH

ng GSH	µl 10µg/ml GSH	µl 6.5% TCA
0	0	75
25	2.5	72.5
50	5	70
100	10	65
250	25	50
500	50	25
750	75	0

Appendix 4. Solutions for spectrophotometric determination of GSH

a) Phosphate buffer (pH 7.4)

13.6g KH_2PO_4 in 1 litre H_2O

- adjust pH to 7.4.

b) Phosphate buffer (pH 8.0)

13.6g KH_2PO_4 in 1 litre H_2O

- adjust pH to 8.0

c) Sulphosalicylic acid (SSA)

40g SSA in 1 litre H_2O

d) 5.5'-dithiobis-(2-nitrobenzoic acid) [DTNB]

39.6mg DTNB in 10ml phosphate buffer pH 7.4

- stable at 4 °C

e) GSH standard

30.7mg GSH in 10ml pH7.4 phosphate buffer

- made up fresh and stored on ice

Standard curve for GSH

mM GSH	ml 10mM GSH	ml SSA
0	0	0.25
0.2	0.005	0.245
0.4	0.01	0.24
0.6	0.15	0.235
0.8	0.02	0.23
1.0	0.025	0.225

Appendix 5. Solutions for the determination of LDH leakage

Phosphate buffer

3.4g KH_2PO_4 in 500ml H_2O

- pH adjusted to 7.4, stable at 4° C

Working solution;

100ml phosphate buffer containing 3.75mg sodium pyruvate and 22.2mg β-nicotinamide adenine dinucleotide, reduced form (β-NADH).

Appendix 6. Composition of SDS-polyacrylamide gels

	Stacking gel	**Resolving gel**	
	4%	**7.5%**	**12%**
30% Acrylamide/Bis	3.3ml	6.25ml	10ml
UHQ H_2O	15ml	12.125ml	8.375ml
0.5M Tris-HCl pH 6.8	6.3ml	-	-
1.5M Tris-HCl pH 8.8	-	6.25ml	6.25ml
TEMED	25µl	12.5µl	12.5µl
10% APS	125µl	125µl	125µl

- APS; ammonium persulphate
- TEMED; N,N,N',N'-Tetra-methyl-ethylenediamine

Appendix 7. Electrophoresis and Western Blotting buffers

a) Electrophoresis buffer x10

30g Trizma base

1g sodium dodecyl sulphate (SDS)

144g glycine

- all dissolved in 1 litre H_2O
- for 1x electrophoresis buffer 100ml 10x diluted in 900ml H_2O

b) Western blotting buffer x10

144.1g glycine

30.3g Trizma base

- all dissolved in 1 litre H_2O

- for 1x Western blotting buffer 100ml 10x diluted in 200ml methanol, made up to 1 litre with distilled H_2O

c) Laemelli buffer

0.485g Tris (equivalent to 40mM Tris)

10ml glycerol

2g sodium dodecyl sulphate

- adjust pH to 6.8 and make up to 100ml with UHQ H_2O

Appendix 8. Total protein standard curve

0.625ml of 2mg/ml bovine serum albumin (BSA) was made up to 50ml with H_2O to give a working solution of 25µg/ml.

BSA standard curve;

µg/ml BSA	ml 25µg/ml BSA	ml H_2O
0	0	1.25
2.5	0.125	1.125
5.0	0.25	1.0
7.5	0.375	0.875
10.0	0.50	0.75
15.0	0.75	0.50
20.0	1.0	0.25
25.0	1.25	0

Appendix 9 Total protein standard curve (Bio-Rad assay)

6.0mg bovine gamma globulin (BGG)was dissolved in 10ml 1M NaOH to give a stock solution of 0.6mg/ml and the standard curve made up as follows;

BGG standard curve;

mg/ml BGG	ml 0.6mg/ml BGG	ml 1M NaOH
0	0	0.60
0.1	0.10	0.50
0.2	0.20	0.40
0.3	0.30	0.30
0.4	0.40	0.20
0.5	0.50	0.10
0.6	0.60	0