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An investigation into the sensitivity of heat shock proteins as markers of cellular damage: a comparative study of hydrazine and cadmium chloride in primary rat hepatocyte cultures

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Stress proteins have been proposed as markers of toxicity. This study investigated the sensitivity and specificity of stress proteins as markers of toxicity in primary hepatocyte cultures following exposure to two compounds, hydrazine and cadmium chloride ($CdCl_2$). Hepatocytes were exposed to increasing concentrations of hydrazine and $CdCl_2$ for 2 h and levels of the heat shock proteins HSP72/3, and HSP25 measured. In addition to this, ATP and GSH levels and LDH leakage were measured over the following 8 h. The results show that increasing concentrations of hydrazine caused dose-dependent decreases in ATP and GSH levels over 8 h. There was no change in the levels of HSP25 or HSP72/3 over that period. $CdCl_2$ was found to significantly induce HSP72/3 at a concentration of 5 μM when no other biochemical parameter was altered, levels were also elevated following administration of 10 μM $CdCl_2$ but ATP levels were found to be decreased at this concentration. Levels of HSP25 were not increased following $CdCl_2$ exposure at any concentration. Higher concentrations of $CdCl_2$ produced significant increases in LDH leakage and depletion of intracellular levels of ATP and GSH. In addition to this levels of HSP25 and HSP72/3 were reduced to zero following administration of high concentrations of $CdCl_2$. In this study hydrazine does not induce either of the stress proteins studied here whereas $CdCl_2$ exposure causes the induction of HSP72/3 but not HSP25. However it was determined that during the culture of primary hepatocytes basal levels of HSP25 and HSP72/3 were significantly increased when compared with levels determined *in vivo*. The results suggest that stress proteins may have the potential to be sensitive markers of toxicity in primary hepatocytes; however, the induction of individual stress proteins appears to be dependent upon the compound used. The apparent non-induction of the stress response by hydrazine and minor induction by $CdCl_2$ might be explained by the fact that whilst in culture the hepatocytes are under a continuous state of stress and therefore may not be able to elicit a full stress response following a chemical insult.

Keywords: HSP25, HSP72/3, hepatocytes, hydrazine, cadmium chloride.

Introduction

Exposure of cells to heat and other metabolic stressors such as heavy metals (cadmium [Cd^{2+}], mercury, lead), various toxic chemicals (arsenite, diethylmaleate), infection, UV irradiation and ischaemia (Nover 1991) causes an increase in the synthesis of a family of well conserved proteins referred to as heat shock proteins (HSPs). These proteins consist of several sub-groups (HSP60, HSP70 and HSP90 for example), some of which are present in low amounts in normal cells where they are thought to function as molecular chaperones essentially aiding in the folding and assembly of newly formed proteins. However in stressed

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cells newly synthesized HSPs interact with partially denatured proteins either restoring them to their native conformation or, in the case of proteins too damaged to refold, aiding in their proteolysis. In an unstressed cell the total HSP content is approximately 1–2% of the cells' total protein although this can increase to up to 20% when the cells are stressed (Donati *et al.* 1990).

The compounds used in this study were hydrazine and cadmium chloride. Hydrazine has been shown previously to be toxic *in vivo* where a single dose has been shown to cause fatty liver (Scales and Timbrell 1982) as well as liver necrosis in some cases (Patrick and Back 1965). In addition to this, hydrazine has been shown to be cytotoxic in both rat hepatocyte suspensions (Waterfield *et al.* 1993) and cultured rat hepatocytes (Ghatineh and Timbrell 1993). The exact mechanism of hydrazine-induced cell death remains to be fully elucidated, however it is known that the cytotoxicity of hydrazine involves the depletion of cellular ATP and inhibition of protein synthesis. Also it interferes with various other metabolic processes causing changes in levels of NADH, NADPH and succinate dehydrogenase for example (Ghatineh *et al.* 1992).

Cadmium is both nephrotoxic and hepatotoxic (Morselt 1991) where the major effect of cadmium on proteins is to induce structural changes by its substitution for zinc and its interaction with vicinal thiol groups (Vallee and Ulmer 1972). Cadmium is a known inducer of the heat shock response both *in vivo* and *in vitro* (Donati *et al.* 1990, Goering *et al.* 1993, Ovelgönne *et al.* 1995).

It has thus been proposed that HSPs may be utilized as markers of toxicity as there have been a large number of findings demonstrating correlations between toxicity and induction of stress proteins (Hansen *et al.* 1988, Pipkin *et al.* 1988, Gonzalez *et al.* 1989, Aoki *et al.* 1990, Deaton *et al.* 1990, Cochrane *et al.* 1991, Low-Friedrich *et al.* 1991). HSPs may prove feasible as early indicators of cellular damage as it is known that induction of the heat shock response occurs at much lower chemical concentrations than those required to induce toxicity (Goering *et al.* 1990). In addition to this the induction of the heat shock response is rapid with significant increases in HSP levels measurable within 2 h of exposure (Blake *et al.* 1990, Goering *et al.* 1993). However, there is no work in primary hepatocytes detailing the sensitivity of these HSPs as markers of toxicity compared with other well established markers of cellular damage. This study was thus performed to determine whether HSPs may be used as early indicators of cellular damage in primary hepatocyte cultures using the compounds cadmium chloride, a known inducer of HSPs, and hydrazine. Levels of HSPs were compared directly to other well established indicators of toxicity such as LDH leakage, ATP levels and GSH levels to determine the sensitivity of HSPs as markers of cellular damage.

Materials and methods

Chemicals

Hydrazine hydrate (100%), cadmium chloride (99%) and all other chemicals were, unless otherwise specified, from Sigma Chemical Co. (Poole, UK).

Preparation of primary hepatocyte cultures

Rat hepatocytes were isolated as described by the two step collagenase perfusion method of Moldeus *et al.* (1975) from male Han–Wistar rats (approximate weight 250 g). Prior to collagenase perfusion a sample of the left liver lobe was taken and homogenized in Tris–HCl (pH 7·4) buffer for analysis of *in vivo* HSP levels. The isolated hepatocytes were then washed in sterile Williams E medium (ICN

Biomedicals Inc., Costa Mesa, CA) containing 1 μM insulin, 0·1 mM hydrocortisone hemisuccinate, 2 mM glutamine (ICN Biomedicals Inc., Costa Mesa, CA) and 10 mg ml^{-1} gentamycin (Gibco/Life Technologies Inc., Gaithersburg, MD). Once washed the cells were diluted down with medium to a concentration of $0{\cdot}75 \times 10^6$ cells ml^{-1} and an aliquot of 1×10^6 cells taken for determination of LDH leakage and HSP levels. The cells were plated out onto 33 mm diameter sterile culture dishes, 2 ml medium being added thereby giving a final cell density of $1{\cdot}5 \times 10^6$ cells per dish. The culture dishes were previously coated with rat tail collagen solution to give a final coating of approximately 4–8 μg collagen cm^{-2}. Once plated the cells were incubated at 37°C in a humidified atmosphere of 5%CO_2/95%O_2. The medium was changed initially 2 h after plating in order to remove any unbound or dead cells and any cell debris.

Treatment of primary hepatocytes

After the medium had been changed to remove any dead cells the hepatocytes were incubated for a further 16 h, after which time the medium was replaced with medium containing the dose of compound to be studied. For this study the compounds used were hydrazine and cadmium chloride. The doses of hydrazine used were 0, 0·5, 1, 5, 10 and 20 mM, the doses of cadmium chloride used were 0, 5, 10, 30, 100, 300 μM and 0, 0·1, 1, 2·5 μM. The cells were exposed to the compound for 2 h after which time the medium was rinsed off and the cells washed three times with sterile PBS and the original medium returned. Samples were then taken for biochemical analysis at 0, 2, 4, 6, 8 and 10 h post-dosing, the cells being exposed to the compound between the time points 0 and 2 h. Two plates of cells were allocated for each dose at each timepoint. In addition to the samples taken from the dosed cells, samples were also taken from untreated hepatocytes for determination of basal HSP levels during the culture time. Samples were taken for HSP analysis from freshly isolated hepatocytes and after 6, 22, 30, 46 and 54 h in culture.

Preparation of cell lysates

The culture dishes were removed from the incubator at the allotted time points and placed on ice. One hundred microlitres of medium was taken from each dish for LDH analysis and the plates were washed once with 2 ml ice cold phosphate buffered saline (PBS). To the plates 0·5 ml Trypsin–EDTA (Gibco/Life Technologies Inc., Gaithersburg, MD) solution was added to remove the cells which were then centrifuged for 3 min at 4°C and the cell pellet resuspended in 100 μl lysate buffer (40 mM Tris pH 6·8, 2% sodium dodecyl sulphate and 10% glycerol) on ice. The cells were then sonicated for approximately 10 s to ensure disruption of the cells following which 50 μl of cell lysate was removed for protein determination. To the remaining 50 μl cell lysate β-mercaptoethanol and bromophenol blue were added to give final concentrations of 5% and 0·002% respectively. The sample was boiled for 5 min after which it was transferred to storage at –80°C until HSP analysis.

LDH determination

Leakage of the cytosolic enzyme LDH into the medium was used as a marker of cell death. The assay is based on the spectroscopic disappearance of NADH which is oxidized to NAD^+ by LDH. The method was previously described in Bergmeyer *et al.* (1965).

ATP and reduced glutathione (GSH) determination

The culture plates were removed at the relevant time points and placed on ice. The medium was removed and the cells washed twice with 2 ml PBS (4°C) following which 1 ml 6·5% trichloroacetic acid (TCA) was added to each plate and left for 10 min. The TCA was removed and aliquots stored at –80°C until analysis for ATP and GSH. Once the TCA had been removed 1 ml 1 M NaOH was added to each plate and left for 30 min in order to dissolve the residual protein on the plate. Levels of GSH in the TCA were determined by the *o*-phthaldialdehyde detection method for acid soluble thiols (Hissin and Hilf 1976). ATP levels were analysed using the luciferase linked bioluminescence assay (Stanley and Williams 1968).

Protein determination

Total protein in the cell lysates and NaOH extracts was determined by the Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL) using bovine serum albumin (BSA) as the protein standard.

Detection and analysis of HSP levels in cell lysates

The proteins in the cell lysate were separated initially using sodium dodecyl sulphate polyacrylamide gel electrophoresis using a 12·5% resolving and 5% stacking mini-gel in a Mini Protean II

(Bio-Rad, UK). Equal amounts of protein were added to each well. Separation of the proteins was accompanied by concurrent separation of prestained Rainbow molecular weight standards (Amersham Life Science, UK) and native purified HSP of interest (Stressgen Biotechnologies Inc., Vancouver, Canada). Following separation the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Life Science, UK). Once the proteins had been transferred to the nitrocellulose membranes 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 incubated with a primary antibody derived against proteins from the 25- or 72/3-kDa heat shock family (Stressgen Biotechnologies Inc. Vancouver Canada). The secondary antibody consisted of rabbit anti-mouse IgG or murine anti-rabbit IgG conjugated with horseradish peroxidase (DAKO Ltd, UK). The protein antibody complex was detected using ECL reagents (Amersham Life Science, UK) and visualized on X-ray film (Kodak X-OMAT AR). The results were quantified by image analysis densitometry (PC Image Plus software).

Statistical analysis

Statistical significance was determined using Dunnett's test for multiple comparisons with one control with p values of less than 0·05 considered significant.

Results

Effect of increasing concentrations of hydrazine and cadmium chloride on various cellular parameters

Leakage of LDH: Leakage of intracellular LDH was used as an indication of the cytotoxicity of hydrazine. In control cells there was no change in leakage of LDH over an 8-h period. Concentrations of 1, 5, 10 and 20 mM hydrazine caused a slight increase in leakage of LDH at 2 h but these were not statistically significant (figure 1(a)).

Leakage of LDH from control cells and from those dosed with 5 and 10 μM $CdCl_2$ did not change over the time course of the experiment (figure 1(b)). Exposure of hepatocytes to concentrations of 30 μM, 100 μM and 300 μM $CdCl_2$ produced significant increases in leakage of LDH.

ATP levels: ATP levels showed a dose dependent decrease with increasing concentrations of hydrazine (figure 2(a)). Control cells maintained their ATP at relatively constant levels throughout the entire timecourse of the experiment. Concentrations of 10 and 20 mM hydrazine caused significant decreases in ATP levels at 2 h which remained decreased throughout the experiment.

Increasing concentrations of $CdCl_2$ caused a dose-dependent decrease in ATP levels. Levels of ATP fell over 10 h following dosing with 10 and 30 μM $CdCl_2$ but after exposure to 100 and 300 μM levels were significantly decreased at 2 h. After exposure to 300 μM $CdCl_2$ levels of ATP approximated to zero 4 h post-dose. Additionally by 4 h, levels were significantly decreased by 10 μM and all $CdCl_2$ concentrations above this (figure 2(b)).

*GSH level*s: Increasing concentrations of hydrazine caused a dose-dependent decrease in intracellular GSH levels (figure 3(a)). Levels were decreased significantly at 10 h following dosing with all concentrations of hydrazine. GSH levels in control cells remained constant throughout the course of the experiment. Levels were significantly decreased at concentrations of 10 and 20 mM hydrazine initially at 2 h and remained decreased throughout the timecourse of the experiment.

Levels of GSH in control cells remained constant during the experiment as did the levels in those cells exposed to 5 and 10 μM $CdCl_2$. Concentrations of 30, 100 and 300 μM produced dose- and time- dependent decreases in GSH levels. GSH levels were initially decreased significantly at 6 h following administration of 30 μM

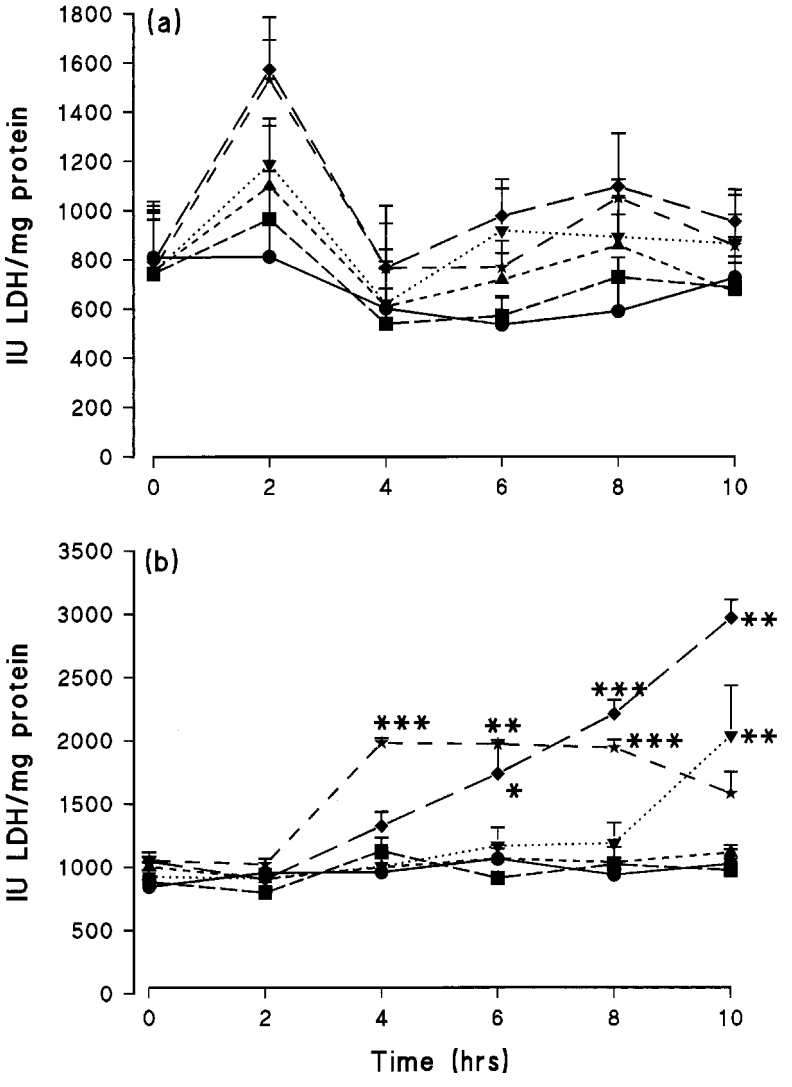

Figure 1. LDH leakage. (a) Effect of increasing concentrations of hydrazine on LDH leakage from primary hepatocyte cultures, -●- 0 mM hydrazine, -■- 0·5 mM hydrazine, - - 1 mM hydrazine, - - 5 mM hydrazine, -◆- 10 mM hydrazine, -★- 20 mM hydrazine. (b) Effect of increasing concentrations of $CdCl_2$ on LDH leakage from primary hepatocyte cultures, -●- 0 μM $CdCl_2$, -■- 5 μM $CdCl_2$, - - 10 μM $CdCl_2$, - - 30 μM $CdCl_2$, -◆- 100 μM $CdCl_2$, -★- 300 μM $CdCl_2$. Values are means±SEM, $n = 3$. * $p < 0{\cdot}05$; ** $p < 0{\cdot}01$; *** $p < 0{\cdot}001$; Dunnetts test for multiple comparisons with a single control group.

$CdCl_2$ and all higher concentrations. There was no recovery in the levels of depleted GSH at any of these concentrations (figure 3(b)).

HSP25 levels: Densitometric analysis of levels of HSP25 in the hepatocytes showed that in control cells levels remained fairly constant over the timecourse of the experiment. There was no induction of HSP25 in the cells following dosing with any concentration of hydrazine. In those cells exposed to 20 mM hydrazine there was a decrease in levels of HSP25 by approximately 40% at 4, 6, 8 and 10 h but this was determined not to be statistically significant (data n

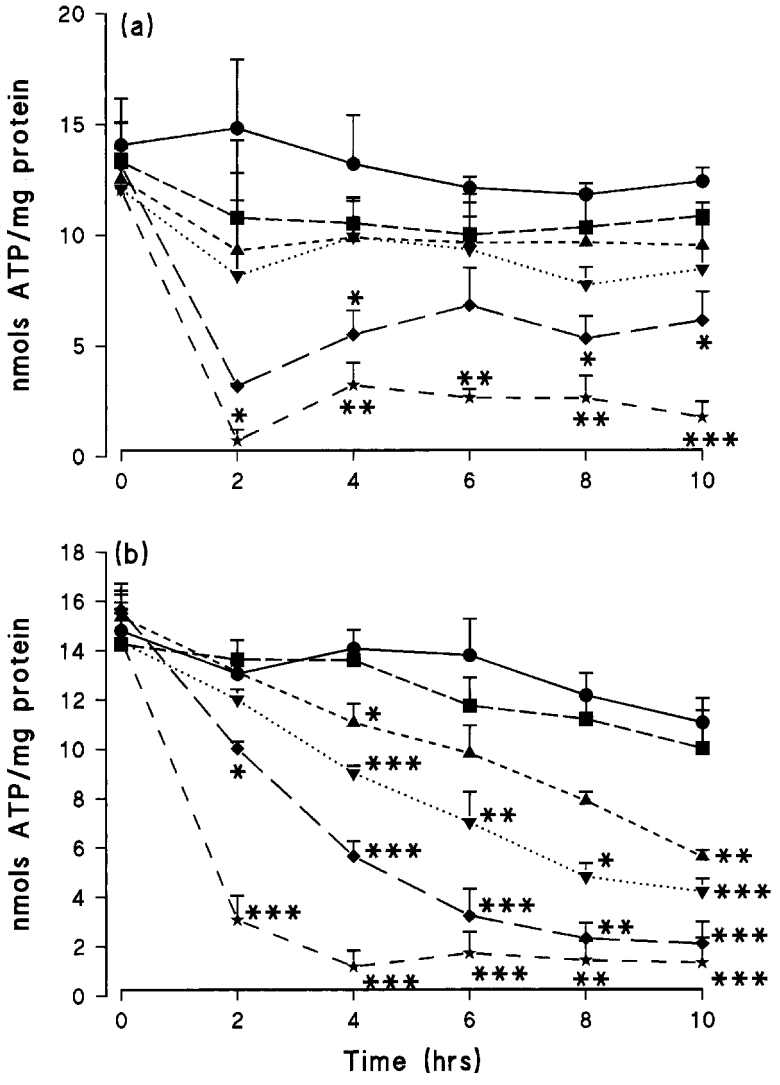

Figure 2. ATP levels. (a) Effect of increasing concentrations of hydrazine on ATP levels in primary hepatocyte cultures, -●- 0 mM hydrazine, -■- 0·5 mM hydrazine, - - 1 mM hydrazine, - - 5 mM hydrazine, -◆- 10 mM hydrazine, -★- 20 mM hydrazine. (b) Effect of increasing concentrations of $CdCl_2$ on ATP levels in primary hepatocyte cultures, -●- 0 μM $CdCl_2$, -■- 5 μM $CdCl_2$, - - 10 μM $CdCl_2$, - - 30 μM $CdCl_2$, -◆- 100 μM $CdCl_2$, -★- 300 μM $CdCl_2$. Values are means±SEM, $n = 3$. * $p < 0{\cdot}05$; ** $p < 0{\cdot}01$; *** $p < 0{\cdot}001$; Dunnetts test for multiple comparisons with a single control group.

$CdCl_2$, at any concentration, did not significantly increase levels of HSP25 at any time point when compared with control cells. Concentrations of 30, 100 and 300 μM $CdCl_2$ caused a rapid decline in the levels of HSP25 within the cells. At 2 h, levels of HSP25 had already fallen to zero following administration of 300 μM $CdCl_2$ (figure 4).

HSP72/3 levels: Exposure of heptocytes to hydrazine caused no clear changes in the synthesis of HSP72/3. Levels of HSP72/3 did not significantly increase from control values following administration of any concentration of hydrazine at any of the time points throughout the timecourse of the experiment (d

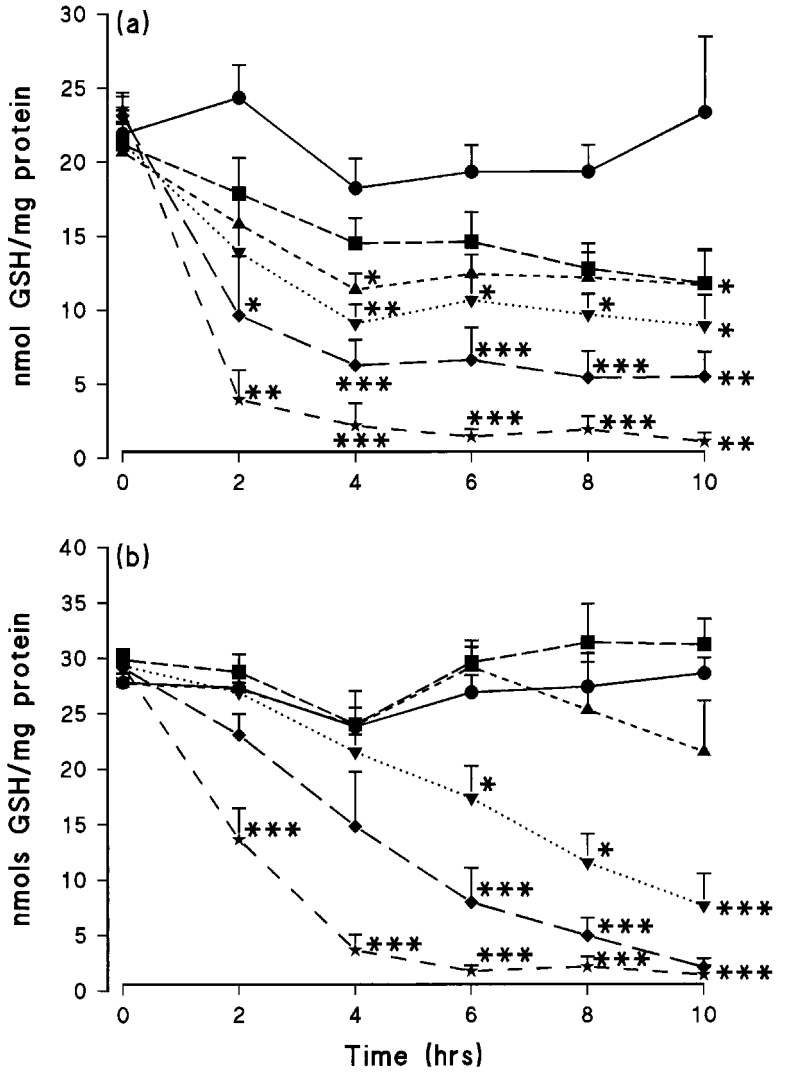

Figure 3. GSH levels. (a) Effect of increasing concentrations of hydrazine on GSH levels in primary hepatocyte cultures, -●- 0 mM hydrazine, -■- 0·5 mM hydrazine, - - 1 mM hydrazine, - - 5 mM hydrazine, -◆- 10 mM hydrazine, -★- 20 mM hydrazine. (b) Effect of increasing concentrations of $CdCl_2$ on GSH levels in primary hepatocyte cultures, -●- 0 μM $CdCl_2$, -■- 5 μM $CdCl_2$, - - 10 μM $CdCl_2$, - - 30 μM $CdCl_2$, -◆- 100 μM $CdCl_2$, -★- 300 μM $CdCl_2$. Values are means ± SEM, $n = 3$. * $p < 0{\cdot}05$; ** $p < 0{\cdot}01$; *** $p < 0{\cdot}001$; Dunnetts test for multiple comparisons with a single control group.

Measurement of HSP72/3 following dosing with $CdCl_2$ appeared to show a dual effect on HSP72/3 levels depending on the dose used (figure 4(b) and figure 7(a)). Following administration of 5 and 10 μM $CdCl_2$ HSP72/3 levels were significantly increased from control values at 4 h and remained significantly increased until 8 h. Following dosing with 30 μM $CdCl_2$ levels of HSP72/3 remained constant until 8 h when levels began to decline. Concentrations of 100 and 300 μM $CdCl_2$ also caused a rapid decrease in the levels of HSP72/3 at 4 and 2 h respectively.

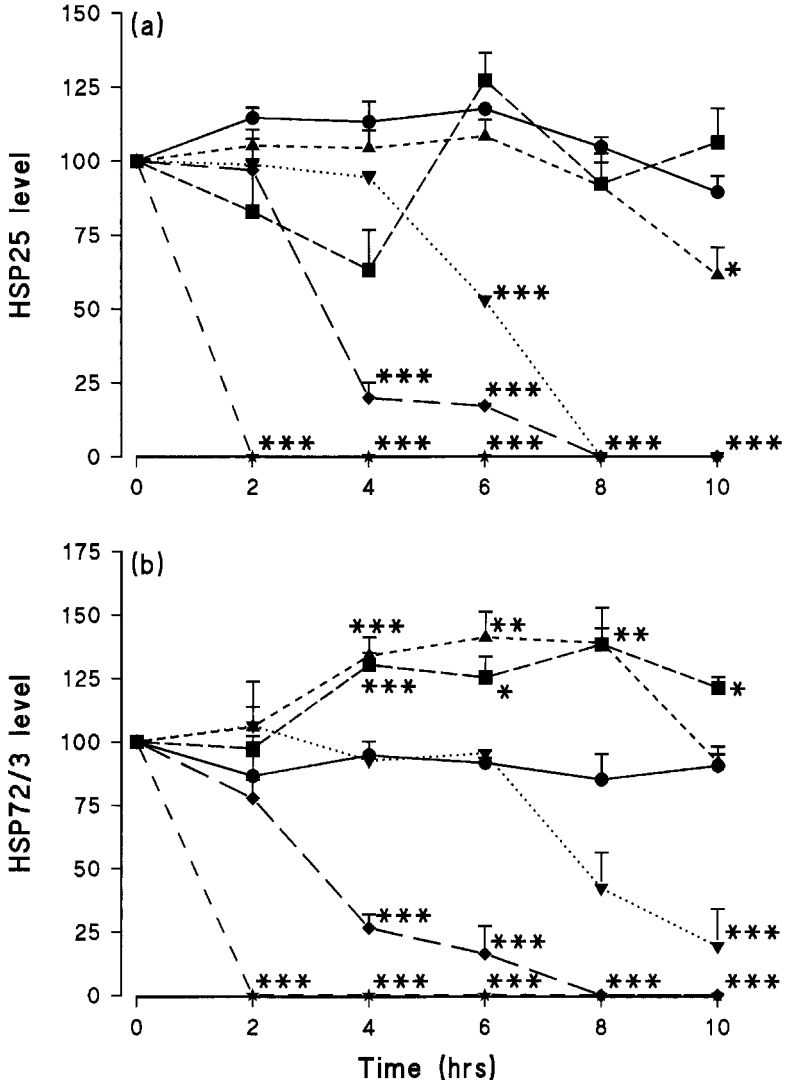

Figure 4. Heat shock protein levels. (a) Effect of increasing concentrations of $CdCl_2$ on HSP25 levels in primary hepatocyte cultures. (b) Effect of increasing concentrations of $CdCl_2$ on HSP72/3 levels in primary hepatocyte cultures, -●- 0 μM $CdCl_2$, -■- 5 μM $CdCl_2$, - - 10 μM $CdCl_2$, - - 30 μM $CdCl_2$, -◆- 100 μM $CdCl_2$, -★- 300 μM $CdCl_2$. Values are mean percent change from control ±SEM, $n = 3$. * $p < 0{\cdot}05$; ** $p < 0{\cdot}01$; *** $p < 0{\cdot}001$; Dunnetts test for multiple comparisons with a single control group.

Effect of increasing low level concentrations of cadmium chloride on various cellular parameters

LDH leakage: Concentrations of 0·1, 1 and 2·5 μM $CdCl_2$ did not alter leakage of LDH compared with control cells at any of the time points during the course of the experiment (data not shown).

HSP72/3 levels: Use of low concentrations of $CdCl_2$ was an attempt to determine a threshold concentration of $CdCl_2$ that would cause induction of HSP72/3. Levels of HSP72/3 were unchanged from control cells following administration of 0·1, 1 and 2·5 μM $CdCl_2$ (data not shown).

Basal levels of HSP72/3 and HSP25 during hepatocyte culture: Levels of HSP72/3 in freshly isolated hepatocytes were significantly decreased when compared with *in vivo* levels, however over the following 30 h levels of this stress protein increased more than two-fold above what was found *in vivo*. At 46 h levels of HSP72/3 had returned approximately to *in vivo* levels but at 54 h levels were found subsequently to be significantly decreased (figure 5 and figure 7(c)).

Basal levels of HSP25 in primary hepatocytes during culture showed a similar trend to HSP72/3. In freshly isolated hepatocytes levels of HSP25 were determined to be significantly decreased but as with HSP72/3 there was a significant increase in the levels of this protein over the next 30 h. Similarly at 46 and 54 h levels of HSP25 had fallen significantly below those levels found *in vivo* (figure 6 and figure 7(b).

Discussion

This study was designed to investigate whether the stress response in primary cultures of hepatocytes may be used as an early indicator of toxicity. The induction of the stress response was measured by studying the levels of HSP25 and HSP72/3 within the cells. These two HSPs are both stress inducible to varying extents and so theoretically should be induced when a cell is exposed to noxious stimuli such as hydrazine or cadmium chloride which are both hepatotoxic. The effect of hydrazine on HSP levels within hepatocytes has not been studied previously, however there are numerous studies investigating the effect of cadmium chloride on HSP levels *in vitro*. The majority of these studies have however been performed in hepatoma cell lines such as Reuber H35.

Exposure of hepatocytes for 2 h to increasing concentrations of hydrazine caused dose-dependent toxicity as observed by changes in various cellular

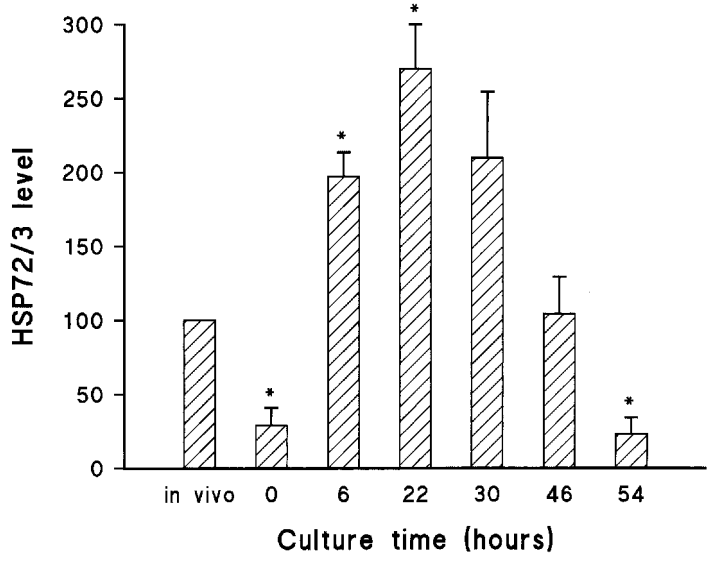

Figure 5. Basal levels of HSP72/3 during culture of primary hepatocytes over a 54 h period. 'in vivo' refers to levels determined from a fresh liver sample. Values at zero time are levels determined from freshly isolated hepatocytes. Values are expressed as percent of *in vivo* control HSP72/3 level± SEM, $n = 4$. *$p<0{\cdot}05$ compared with *in vivo* levels.

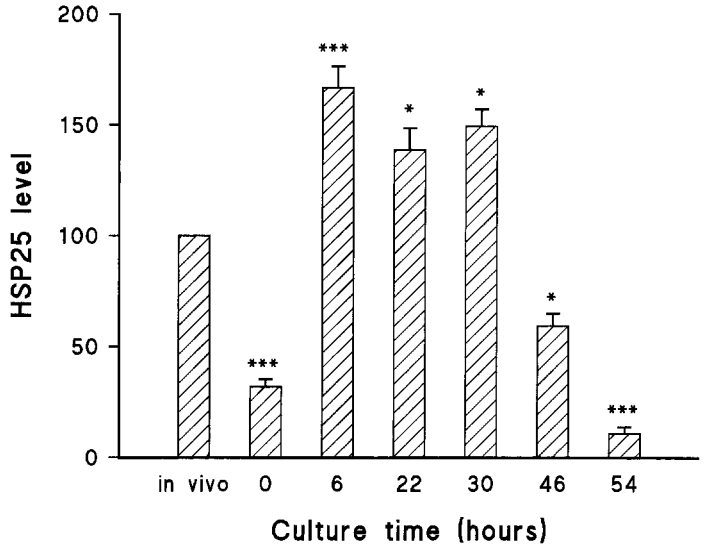

Figure 6. Basal levels of HSP25 during culture of primary hepatocytes over a 54 h period. *'in vivo'* refers to levels determined from a fresh liver sample. Values at zero time are levels determined from freshly isolated hepatocytes. Values are expressed as % of *in vivo* control HSP25 level ± SEM, $n = 4$. * $p < 0{\cdot}05$; *** $p < 0{\cdot}001$ compared with *in vivo* levels.

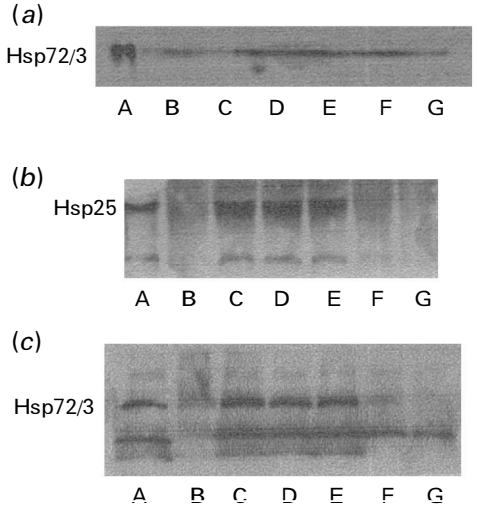

Figure 7. Representative Western blots. (a) Effect of 10 μM $CdCl_2$ on HSP72/3 levels in rat hepatocytes. Lane A, HSP72/3 standard; B, 0 h; C, 2 h; D, 4 h; E, 6 h; F, 8 h; G, 10 h post-dose. (b) Basal levels of HSP25 during culture of rat hepatocytes. Lane A, *in vivo* level; B, 0 h; C, 6 h; D, 22 h; E, 30 h; F, 46 h ; G, 54 h culture time. (c) Basal levels of HSP72/3 during culture of rat hepatocytes. Lane A, *in vivo* level; B, 0 h; C, 6 h; D, 22 h; E, 30 h; F, 46 h ; G, 54 h culture time.

parameters. The first biochemical measurement affected by hydrazine was GSH levels which were significantly decreased following exposure to a concentration of 0·5 mM hydrazine. Work previously done investigating the effect of hydrazine on GSH levels in hepatocytes in suspension and primary hepatocytes showed that much higher concentrations of hydrazine were required to

(Timbrell 1994, Timbrell and Waterfield 1996). The previous experiments studied the effect of continuous exposure of hepatocytes to hydrazine. Therefore it appears that the data presented here show that cultured hepatocytes exposed to hydrazine for 2 h and then allowed to recover show an increased sensitivity to hydrazine-induced GSH depletion. Primary hepatocytes 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 hydrazine did not alter levels of HSP25 or HSP72/3 in the hepatocytes.

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 5 μM $CdCl_2$. Levels of HSP72/3 were also significantly increased at 10 μM, however by this concentration levels of ATP had already begun to fall significantly. Following administration of 30 μM $CdCl_2$ 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 HSP levels in Reuber H35 hepatoma cells showed that concentrations up to 30 μM $CdCl_2$ for 2 h failed to increase the synthesis of HSP68, HSP70, HSP84 or HSP100 (Ovelgönne *et al.* 1995a). It would therefore appear here that the increase in HSP72/3 in primary hepatocyte cultures following exposure to $CdCl_2$ occurs at much lower concentrations than those observed in other cells. This is further highlighted by the response of Reuber H35 cells to a dose of 300 μM $CdCl_2$ which was found to induce HSP70 levels (Ovelgönne *et al.* 1995) whereas in primary hepatocytes such a dose is acutely toxic with levels of HSP70 reduced to zero.

In consideration of the results initially with $CdCl_2$, in particular the induction of HSP72/3 following administration of 5 and 10 μM, it was decided to use lower concentrations of $CdCl_2$; 0·1, 1 and 2·5 μM. Only HSP72/3 levels 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 5 μM $CdCl_2$ causes significant induction whereas 2·5 μM does not affect HSP72/3 levels.

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 HSPs. With respect to HSP72/3 its induction and level are closely related to ATP levels within the cells. Once HSP72 has bound to denatured or damaged proteins the dissociation of HSP72 from these proteins requires ATP. Therefore if ATP levels are depleted HSP72 will remain bound to denatured proteins and will 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 free HSP72 within the cell (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. Although the exact mechanism of hydrazine toxicity is unknown it is thought that ATP depletion (Preece *et al*. 1990) or inhibition of protein synthesis may be involved (Ghatineh and Timbrell 1994). Following administration of concentrations of 0·5 and 1 mM hydrazine, levels of ATP had fallen to 73% and 63% of the control values respectively within 2 h of exposure. This is in contrast to ATP levels in those cells exposed to 5 and 10 μM $CdCl_2$ where 2 h after exposure ATP levels were unchanged from control cells. This may in part explain why hydrazine failed to induce HSP72/3 whereas $CdCl_2$ did. However at later time points following exposure to $CdCl_2$, HSP72/3 remained induced when ATP levels were decreased by 38%. Therefore this may indicate that the ATP levels within a cell immediately after an initial insult are more important than the levels of ATP later in determining the level of HSP72/3 induction.

The reason why $CdCl_2$ and hydrazine failed to elicit a large stress response in primary hepatocytes may be due to the increased basal levels of the stress proteins studied. It would appear that the isolation of hepatocytes is a potential cause of stress to the cells as the levels of both stress proteins are significantly increased 6 h post-isolation. The sustained increase in the levels of these two proteins over the following 30 h would indicate that in culture the primary hepatocytes are under a continuous state of stress. One possible source of stress to the cells whilst in culture could be the generation of reactive oxygen species due to an atmosphere of 95% O_2 being used, however the observation that GSH levels were unchanged in control cells during culture implies that the hepatocytes were not exposed to oxidative stress to any significant amount whilst in culture. The stress response in cells is a transient event and it is known that following a single insult, levels of HSP72/3 *in vivo* return to values close to control values some 18–24 h later (Goering *et al*. 1993). This would imply that the increased levels of these stress proteins in primary hepatocytes is due not to the isolation procedure but to a sustained state of stress whilst in culture. 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, they may not be able to elicit a full characteristic stress response. Or there is the possibility that the compounds used in this study are eliciting a stress response but such a response is being masked by the already induced levels of HSP72/3 and HSP25 in the cells. The apparent decrease observed in the levels of HSP72/3 and HSP25 at 54 h is most likely due to a decrease in the metabolic viablity of the cells in culture.

In summary, 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. Exposure of hepatocytes to hydrazine caused dose-dependent changes in several biochemical parameters apart from HSP72/3 and HSP25. Levels of these proteins remained unchanged after administration of hydrazine. Though hydrazine does not induce HSP25 or HSP72/3 it does not rule out the possibility that hydrazine may selectively induce another family of HSPs not studied here, for example HSP60 or HSP90. The demonstration that $CdCl_2$ induces HSP72/3 but not HSP25 highlights again the possibility of selective induction of specific groups of HSPs by a certain compound. It may therefore be feasible to utilize HSPs as markers of toxicity, but, as demonstrated clearly here, the major problem is that not all compounds induce similar HSPs. Therefore the use of primary hepatocytes as

an *in vitro* model for studying the stress response has to be carefully considered as it has to be acknowledged that whilst in culture hepatocytes appear already under a state of stress.

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