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The use of stress proteins as a biomarker of sub-lethal toxicity: induction of heat shock protein 70 by 2-isobutyl piperidine and transition metals at sub-lethal concentrations

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The stress response is a highly conserved reaction to various physical, chemical and biological stimuli. The ubiquity of the response occurring across taxonomic classes has identified heat shock proteins as potential biomarkers. In this study using the neutral red assay, silver stained one-dimensional SDS–PAGE, Western blotting and ELISA, the use of heat shock proteins as biomarkers of sub-lethal toxicity was examined. Hsp70 was induced in the mouse connective tissue cell line (L929) at sub-lethal concentrations for three transition metals (cadmium, mercury and copper) and for 2-isobutyl piperidine, a novel compound whose chemical structure is similar to a toxin found in the Colorado potato beetle (*Leptinotarsa decemlineata*). Hsp70 induction was found to increase in a dose-dependent fashion. Expression of other potentially interfering proteins was found to decrease with increasing toxin concentration. The induction of hsp70 at sub-lethal concentrations by the transition metals and 2-isobutyl piperidine demonstrates the potential of hsp70 as a biomarker of sub-lethal toxicity.

Keywords: heat shock proteins, sub-lethal toxicity, transition metals.

Introduction

Increased expression of heat shock proteins, as a response to a variety of stimuli, provides the potential for them to be used as biomarkers of chemical toxicity. The stress response is a reaction of eucaryotic and prokaryotic cells to disparate chemical and physical stress conditions (Ballinger and Pardue 1983). The stress response is ubiquitous, with analogous sets of heat shock proteins being induced in a wide variety of cell types (Levinson *et al*. 1980, Anderson *et al*. 1982, Kothary *et al*. 1984, Craig 1985, Hightower and Renfro 1988). The adverse conditions that induce cellular stress include hyperthermia, hypoxia, irradiation and various chemical traumas (Zatloukal *et al*. 1988). They operate by inducing the activity of a small set of genes, thereby expressing a novel set of polypeptides (Ashburner and Bonner 1979, DiDomenico *et al*. 1982). Repression of normal protein synthesis is a common feature of the response of the affected cells (Anderson *et al*. 1982, Ballinger and Pardue 1983, Kothary *et al*. 1984, Welsh 1993). Although the stress genes are essentially quiescent under normal conditions, the rapid induction of heat shock proteins suggests that these genes are maintained in a vigilant state ready for immediate transcription (Kothary *et al*. 1984). In *Drosophila* hsp70 is the most abundant heat shock protein and is encoded at two

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cytological locations, 87a and 87c (Craig *et al.* 1982). Molecular weights vary from 70 to 75 kDa depending on gel concentration, markers and species (Mirault *et al.* 1978). The inducible form of heat shock protein 70 is not constitutive in most cells and is one of the most inducible of all heat shock proteins (Witzmann *et al.* 1995). Induction of heat shock proteins has been correlated with the ability of cells to survive further assault that would be lethal in the absence of a previous similar shock (Walsh and Crabb 1989).

One major obstacle to the study of toxicology has been the difficulty in differentiating primary cellular responses to stress, a period before lethal damage has occurred, and secondary responses, which are symptomatic of cellular damage and necrosis. The stress response is a primary protective reaction by cells and therefore its use as a biomarker of exposure to contaminants is worthy of consideration. A major heat shock protein, 'hsp70', is a member of a group of proteins called molecular chaperones. Under normal conditions these proteins are involved in protein folding and assembly (Sanders 1993). However, under adverse conditions heat shock proteins function as a cellular defence mechanism, making the analysis of these proteins a possible biomarker of sub-lethal toxicity. Under conditions of survivable attack, the synthesis of heat shock proteins can be increased quickly from very low proportions to a level at which they are the major proteins produced by the cell (Anderson *et al.* 1982). This unusual feature of increasing the concentration of the heat shock proteins while reducing the concentration of other proteins that could potentially interfere with an assay, adds to the suitability of heat shock proteins as general indicators of toxicity. Research focusing on the relationship between the stress response and exposure to environmental contamination has the potential to make major contributions in the monitoring of environmental toxicology (Sanders 1993) and human toxicology (Low-Friedrich *et al.* 1991). In this study, we investigate the potential of heat shock protein 70 as an indicator of sub-lethal environmental toxicity. Three transition series metals and 2-isobutyl piperidine, a toxin similar in structure to the Colorado potato beetle toxin (*Leptinotarsa decemlineata*), were investigated. The chemical characteristics of this novel compound suggested it was toxic. Using electrophoretic separation, Western blotting and ELISA techniques subtle variations in the levels of heat shock proteins were assessed.

Materials and methods

Chemicals and reagents

Dulbecco's modified Eagles medium, HEPES buffer, sodium bicarbonate buffer, L- glutamine, non-essential amino acids, foetal bovine serum, penicillin/streptomycin, trypsin and phosphate buffered saline were all cell culture grade and purchased from Biowhitaker (Biowhitaker Inc., 8830, Biggs Ford Road, M16, Walkersville, MD 21793-0027). Monoclonal anti-hsp70 mouse IgG_1 clone C92F3A-5 was purchased from Stressgen Biotechnologies Ltd, Glanford Avenue, Victoria, Canada. Goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate, BCIP/NBT tablets (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium), polyvinylindene difiuoride membrane (PVDF), high range (36–205 kDa) molecular weight markers, sodium dodecyl sulphate, Tris–HCl, acrylamide (specially purified), *N,N'*-methylene bisacrylamide ('Electran'), mercaptoethanol, ammonium persulphate (Ultragrade), bovine serum albumin, *N,N,N,N'*-tetramethylethylenediamine (Ultragrade), sodium azide and glycine were all of electrophoresis grade, bromophenol blue, trichloroacetic acid, sulphosalicylic acid, silver methylamine, *p*-nitrophenyl phosphate, Folin reagent, Kodak hypo clearing reagent, Tween-20, Triton X-100, diethanolamine were purchased from Sigma Aldrich, Poole, Dorset, UK. Cadmium chloride, mercury chloride, copper sulphate, magnesium chloride, acetone, neutral red, formaldehyde, acetic acid, calcium chloride, glycerol, ethanol, sodium acetate, sodium hydroxide, sodium bicarbonate, sodium potassium tartrate, citric acid, sodium thiosulphate and sodium chloride were purchased from BDH Chemicals, Poole, Dorset, UK. All chemicals were of Analar grade unless otherwise stated.

Apparatus

SDS–PAGE was performed on an 'ATTO' vertical electrophoresis unit (ATTO AE-6220) and Western blotting was performed on an 'ATTO' semi-dry electroblotter. ATTO Corporation, 2-3, Hongo 7-chrome, Bunkyo-Ku, Tokyo 113, Japan. The power supply Consort E773 (0–500 V range) was from Consort nv, Parklann 36, B2300 Turnhout, Belgium. The Anthos 2010 plate reading spectrophotometer was from Anthos Labtec Instruments GmbH. CREAM Densitometric software was purchased from Kem-en-Tec, A/S, Haraldsgrade 68, Copenhagen, DK-2100.

Cell culture

Mouse connective tissue (L929) cells were grown at 37 °C in a humidified incubator containing 5% CO_2 and propagated in Dulbecco's modified Eagles medium (pH 7.3) with 10% (v/v) foetal bovine serum. For concentration response experiments the cells were plated into 96-well cell culture plates and incubated at equal densities high enough to ensure exponential growth within 24 h. Exponentially growing cultures were used for all experiments (Doyle *et al*. 1993).

Preparation of test solutions

Cadmium stock solution (100 mg l^{-1} $CdCl_2.2.5H_20$), mercury stock solution, (100 mg l^{-1} $HgCl_2$ anhydrous), and copper stock solution (1000 mg l^{-1} $CuSO_4.5H_20$) were prepared in distilled water and filter-sterilized (0.22 μm). The respective metal solutions were further diluted into the appropriate culture medium at a final concentration of 10% stock solution for testing. 2-Isobutyl piperidine was dissolved 1:4 in acetone (10%), and filter-sterilized (0.22 μm) (Werner and Nagel 1997). Metal concentrations were verified by atomic absorption spectroscopy according to the standard procedure (APHA 1990).

Neutral red assay

The neutral red assay was carried out according to the procedure of Borenfreund and Puerner (1984). After 24 h incubation, the toxin-amended medium was removed from the 96-well plate and the cells re-incubated for 3 h in 100 μl of a sterile solution of 40 μg ml^{-1} neutral red, prepared in Dulbecco's modified Eagles medium (the neutral red is incubated in medium for 24 h to allow crystal formation and the crystals are then removed by centrifugation 1500 *g* for 10 min). The medium was decanted and the cells washed with 100 μl of 1% formaldehyde/10% calcium chloride solution. The neutral red dye was eluted from the cells using a 1% acetic acid/50% ethanol solution. To increase the efficiency of the elution process the plates were agitated on a plate shaker for 15 min. The optical density of each well was measured on a plate-reading spectrophotometer at 492 nm. The optical density was expressed as a percentage of the control well values (Borenfreund and Puerner 1984).

Protein determination

After 24 h incubation, the toxin-amended medium was removed from the 96-well plate and the cells washed three times with PBSA. Protein concentrations were determined by the Lowry method (Lowry *et al*. 1951). Cells were lysed with 0.05 N NaOH (Hightower 1980) (5 μl per well); lysates from eight replicate wells were combined. An aliquot from the combined cell lysate (20 μl) was mixed with 20 μl of 2 N NaOH and hydrolysed at 100 °C for 10 min in a boiling water bath. After cooling to room temperature freshly mixed complex-forming reagent (2% (w/v) Na_2CO_3, 1% (w/v) $CuSO_4.5H_2O$ and 2% (w/v) sodium potassium tartrate; mixed 100:1:1, v/v/v respectively) was added for 10 min (200 μl). Using a vortex mixer, Folin reagent was added (20 μl) and the mixture was left to stand at room temperature for 30–60 min. Absorbance was read at 750 nm.

Preparation of cell lysates

Analytical SDS–PAGE was performed using a modified procedure of Laemmli (1970). The appropriate aliquots of cell lysates were determined from the Lowry assay to allow equal protein loads to be separated by electrophoresis. Double strength sample buffer, containing 4% (w/v) SDS, 40% (v/v) glycerol, 10% (v/v) mercaptoethanol, and 125 mmol l^{-1} Tris–HCl; pH 6.8, was mixed with an equal aliquot of the combined cell lysate. Cell lysates were heated to 90 °C for 5 min then allowed to cool (Marshall 1984a).

Gel preparation

Polyacrylamide gradient electrophoresis gels (4–22.5% (w/v); 10 cm × 10 cm × 2 mm) were prepared using a Pharmacia gradient maker according to the manufacturer's instructions. Sample wells were prepared by using an acrylic comb template. Each well was filled with electrophoresis buffer containing bromophenol blue (250 μl of aqueous 0.1% BPB/10ml buffer) (Marshall 1984a).

Electrophoresis

Twenty µl of cell lysate was loaded through the BPB with a Hamilton syringe. The gels, two per tank, were carefully immersed in precooled (4 °C) electrophoresis buffer (25 mmol l^{-1} Tris–HCl containing 200 mmol l^{-1} glycine and 0.1% (w/v) SDS) (Gorg *et al.* 1981). The electrophoresis was run, without a stacking gel, at 50 mA/gel for approximately 3 h, progression was monitored by viewing the BPB dye 'front' (Marshall 1984a). Molecular weight standards (36–200 kDa) were co-migrated on each gel.

Staining

The electrophoretic gels were fixed overnight in 10% trichloroacetic acid and 5% sulphosalicylic acid (200 ml/gel). The proteins were detected by staining in alkaline silver nitrate using a modified procedure of Marshall (1984b). The gels were scanned on a CREAM densitometer. Protein bands were assessed using the protein band peak area.

Western blotting

Western blotting was carried out according to the method of Towbin *et al.* (1979). Following SDS–PAGE, proteins were transferred to PVDF membranes using the ATTO semi-dry transfer cell for 1 h at 112 mA in transfer buffer (25 mM Tris–HCl, 192 mM glycine, 20% methanol, pH 8.3). The membranes were blocked using blocking buffer (10 mM Tris–HCl, 150 mM NaCl containing 5% BSA, pH 7.3) for 24 h and were subsequently probed with mouse anti-hsp70 antibody (diluted 1 µg ml^{-1} in blocking buffer, pH 7.3) for 2 h. The membrane was washed five times in washing buffer (10 mM Tris–HCl, 150 mM NaCl containing 0.1% Tween-20). The membrane was probed with the goat anti-mouse alkaline phosphatase conjugate secondary antibody (diluted 1 µg ml^{-1} in blocking buffer, pH 7.3) for 2 h. The membrane was washed five times in washing buffer and the proteins visualized using alkaline phosphatase substrate BCIP/NBT (one tablet in 10 ml distilled water).

ELISA

Equal protein loads of cell lysates were bound to the ELISA plate overnight at 37 °C in a humidified incubator. ELISA plates were covered with cling film to reduce evaporation. The plates were washed three times with washing buffer (10 mM Tris–HCl, 150 mM NaCl containing 0.1% Tween-20, pH 7.3). To prevent non specific binding by the antibodies the plates were blocked using buffer containing (10 mM Tris–HCl, 150 mM NaCl, 5% BSA, pH 7.3) for 2 h and were subsequently probed with mouse anti-hsp70 antibody for 2 h (1 µg ml^{-1} in blocking buffer). The plates were washed three times in washing buffer (10 mM Tris–HCl, 150 mM NaCl containing 0.1% Tween-20). The plates were probed with the goat anti-mouse alkaline phosphatase conjugate secondary antibody for 2 h (1 µg ml^{-1} in blocking buffer). The absorbances of individual wells were read using a plate reading spectrophotometer at 405 nm after 30 min incubation with 10 mg *p*-nitrophenyl phosphate in 10 ml of 10 mM diethanolamine (pH 9.5) containing 0.5 mM $MgCl_2$.

Data handling

For the neutral red assay the optical density of the dye for each test concentration was expressed as a percentage of the control well values and reported as percentage cell mortality (Borenfreund and Puerner 1984). Percentage cell mortalities for replicate tests ($n = 31$) were averaged and plotted against toxin concentrations to generate dose–response curves. Variability was estimated by calculating the coefficient of variation (%CV) and was plotted as *y*-axis error bars. The median lethal concentration was determined by linear regression (Shopsis and Sathe 1984) and taken as the concentration that resulted in 50% inhibition of cell viability (Trevan 1927). The no-effect level was taken as the concentration whose cell mortality did not differ significantly from the control (Rand and Petrocelli 1985) as determined by Student's *t*-test ($p = 0.05$). For the stress–response assay molecular weight markers were included in each gel to allow accurate measurement of molecular weight. The CREAM densitometer calculated the molecular weights of the unknown sample proteins based on the relative mobility (R_f) of the molecular weight markers (Kem-en-Tec 1990). Changes in the level of individual protein bands were assessed using the protein bands' peak area. The peak area of hsp70 for each test concentration was expressed as a percentage of the control peak area and reported as percentage induction. Percentage inductions of heat shock protein 70 for replicate tests ($n = 8$) were averaged and plotted against toxin concentrations to generate stress response curves. Variability was estimated using the standard error of the mean (SEM). To account for variation in the technique the lowest observed effective concentration was determined by Student's *t*-test ($p = 0.05$).

Results

The median lethal concentration (LC_{50}) for each transition metal tested (cadmium, mercury and copper) was determined using the neutral red assay. The

Figure 1. Dose–response curves for the mouse connective tissue cell line (L929) to cadmium, mercury and copper.

LC_{50} value was calculated from the dose–response curves (figure 1). The LC_{50} values for the metals cadmium, mercury and copper were 2.33 mg l^{-1}, 3.1 mg $l^{-1}$and 40 mg l^{-1}respectively. Based on the LC_{50} cadmium was found to be 1.3 times more toxic than mercury and 17 times more toxic than copper for the L929 cell line. The no-effect levels were established from the dose–response curves and taken as the concentration that caused no apparent cell mortality. The metal concentrations for cadmium, mercury and copper, which demonstrated no significant cell mortality, were $\leq$ 1.5 mg l^{-1}, 1.5 mg $l^{-1}$and 25 mg l^{-1}respectively.

Figure 2(A, lane 1) illustrates the untreated, one-dimensional, protein pattern

of the mouse connective tissue cell line (L929). The gel gradient is generated to obtain the optimal separation at approximately 50–100 kDa, without obscuring the separation of other, potentially interesting proteins. Approximately, 25–30 different proteins could easily be resolved. The coefficient of variation (%CV) for the separation of replicate ($n = 48$) control samples was 5 %. To determine the effect of the test metals on the cell line, L929 cells in their exponential phase of growth were exposed for 24 h to metal concentrations ranging from sub-lethal to lethal as assessed by the neutral red assay. Figure 2(A) clearly demonstrates the induction of a 70 kDa protein and the reduction of other proteins. This 70 kDa protein was identified as the inducible isoform of heat shock protein 70 by Western blotting using a monoclonal mouse IgG anti-hsp70 antibody specific for inducible hsp70 (figure 2(B)). At metal concentrations above the LC_{50} the induction of hsp70 is the dominant cellular protein however, increasing the concentration of metal above the LC_{50} results in hsp70 production levelling off (figure 2(A and B, lanes 10–12). The reproducibility of this assay was established using the standard error of the mean (SEM) at a concentration of toxin equal to the LC_{50} (table 1).

The induction of hsp70 by metallic stress at sub-lethal concentrations is clearly evident (figure 2(A and B, lanes 2–4). The stress response had a 42 %, 28 % and 40 % greater response than the neutral red assay at the no-effect level, identifying hsp70 induction at concentrations that were 1.5, 1.6 and 2 times lower than the median lethal concentration for cadmium, copper and mercury respectively. The lowest observed effective concentration (LOEC) to show significant development of hsp70 as determined by the Student's *t*-test ($p = 0.05$) for cadmium, mercury and copper were 0.5 mg l^{-1}, 0.5 mg l^{-1} and 5.0 mg l^{-1} respectively. The stress response identified hsp70 induction at the lowest observed effective concentration at concentrations that were three times lower than the no-effect level for mercury and cadmium and five times lower for copper.

Figure 3 clearly demonstrates the usefulness of the stress protein assay. For the three metals tested the stress measured by the hsp70 induction assay showed a greater response than the neutral red assay. As hsp70 induction begins to decline above the LC_{50} value the magnitude of the response is less pronounced. The significant induction of hsp70 at the no-effect level and lowest observed effective concentrations where no cell mortality was measured demonstrates the sensitivity of the assay identifying sub-lethal toxicity.

The median lethal concentration and no-effect levels were established from the dose–response curves for 2-isobutyl piperidine using the neutral red assay. For 2-isobutyl piperidine the LC_{50} and no-effect levels were 0.6 % and 0.25% respectively. Figures 4 and 5 and table 2 demonstrate induction of hsp70 at sub-lethal concentrations for 2-isobutyl piperidine.

For the L929 cell line using ELISA the three toxins had similar dose-dependent increases in hsp70 induction as found when assayed by one-dimensional electrophoresis (figure 6). At the LC_{50}, hsp70 induction had an increased response over that found using the neutral red assay of 25 % for both cadmium and mercury and 18 % for copper. At the no-effect level the heat shock response assay identified hsp70 induction at concentrations that were 1.5, 1.6 and 2 times lower than the median lethal concentration for cadmium, copper and mercury respectively. At the lowest observed effective concentration the heat shock response assay identified hsp70 induction at concentrations that were three times lower than the no-effect level for mercury and cadmium and five times lower for copper.

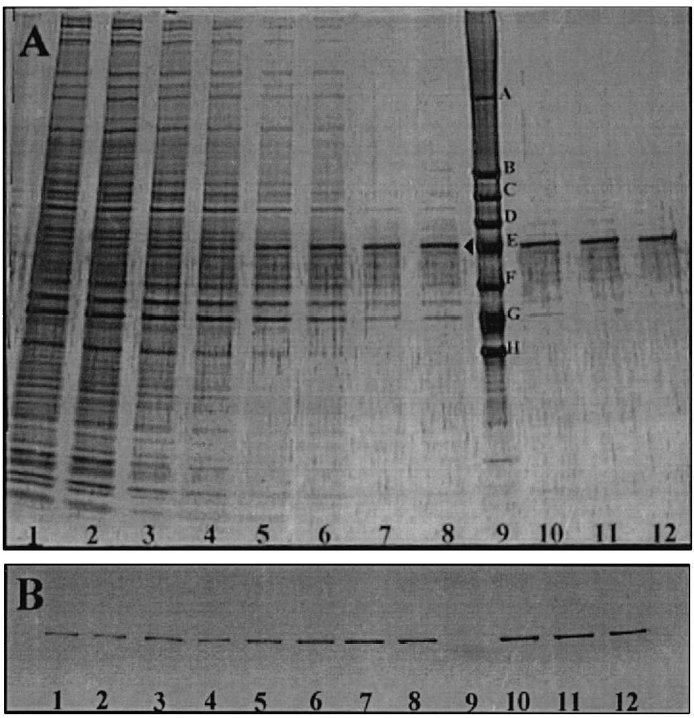

Figure 2. One-dimensional SDS–PAGE separation of mouse connective tissue cell line (L929) exposed to increasing concentrations of cadmium.

Table 1. Hsp 70 induction at the LC_{50} value for the mouse connective tissue cell line (L929).

Toxin	LC_{50} (mg l^{-1})	Hsp70 Induction (%)	SEM
Cadmium	2.33	68	3.45
Mercury	3.1	53	4.18
Copper	40	69	4.48

The median lethal concentration was estimated from these dose–response curves established using the neutral red assay. The peak area of hsp70 for each test concentration was expressed as a percentage of the control peak area and reported as percentage induction. Variability was estimated using the standard error of the mean.

Discussion

The neutral red assay was originally developed by Borenfreund and Puerner (Borenfreund and Puerner 1984) as a rapid, simple and economic assay for the assessment of cell viability. The neutral red assay compares favourably with other cytotoxicity assays, morphology (Borenfreund and Puerner 1985, Hunt *et al.* 1987), Kenacid blue (Riddell *et al.* 1986, Hunt *et al.* 1987), total protein (Babich and Borenfreund 1987), MTT and ATP determination (Benford and Good 1987) and cloning efficiency (Hunt *et al.* 1987). In addition, the neutral red assay has shown a strong correlation with the 'Draize Ocular irritancy test' (Borenfreund and Borrero 1984). The assay has been used extensively for the rapid screening of potential toxins, establishing narrower concentration ranges to facilitate further toxicity testing (Babich and Borenfreund 1987, Hunt *et al.* 1987). In this study, the neutral

Figure 3. Comparison of cell mortality and hsp70 induction for the L929 cell line exposed to cadmium, mercury and copper. ● cell mortality, ■ hsp 70 induction.

red assay was used to establish the median lethal concentration (LC_{50}) and the no-effect concentration from dose–response curves for cadmium, mercury and copper. The three metals were ranked based on their LC_{50} values, cadmium being the most toxic, and copper the least. Our rankings correlate well with published toxicity data (Verma *et al*. 1981, Clothier *et al*. 1988).

The induction of hsp70 by metallic stress has been extensively reported (Levinson *et al*. 1980, Fischbach *et al*. 1993, Ryan and Hightower 1994, Wollgiehn and Neumann 1995). Under identical test conditions, as in the neutral red assay, cells were exposed to metallic stresses at concentrations ranging from sub-lethal to lethal. The reduction in normal protein synthesis with increasing metallic stress is

Table 2. Hsp 70 induction at the lowest observed effective concentrations (L.O.E.C.) for the mouse connective tissue cell line (L929).

Toxin	LOEC	Hsp70 induction (%)	SEM
Cadmium	0.5	15	5.33
Mercury	0.5	23	5.70
Copper	5	11	3.69
2-Isobutyl piperidine	0.125 %	35	

The lowest observed effective concentration was estimated from dose–response curves established using the neutral red assay. The peak area of hsp70 for each test concentration was expressed as a percentage of the control peak area and reported as percentage induction. Variability was estimated using the standard error of the mean. To account for variation in the technique the lowest observed effective concentration was determined by the Student T-test (p=0.05).

Table 3. Hsp70 induction as determined by ELISA for the L929 cell line.

Toxin	LC_{50} %	NEL %	LOEC %
Cadmium	67	49	22
Mercury	66	28	24
Copper	61	35	15

The median lethal concentration, no-effect level and lowest observed effective concentrations were estimated from dose–response curves established using the neutral red assay. To account for variation in the technique the no-effect level and lowest observed effective concentration were determined by the Student T-test (p=0.05). Heat shock protein 70 induction was determined by ELISA. The optical density of each well was expressed as a percentage of the control well values and reported as percentage induction.

dramatic (figure 2(A)). The translation of normal proteins ceases as the cell concentrates on producing protective heat shock proteins (DiDomenico *et al.* 1982, Ballinger and Pardue 1983, Kothary *et al.* 1984). Figure 2(A) demonstrates a dose-dependent increase in a 70 kDa protein. This 70 kDa protein was identified as the inducible isoform of heat shock protein 70 by Western blotting using a monoclonal mouse IgG anti-hsp70 antibody specific for inducible hsp70 (figure 2(B)). No cross-reactivity was observed with other proteins, molecular weight markers were included as negative controls to ensure non-specific binding did not occur. Although the inducible form of hsp70 is not constitutively found in most cells, low levels were observed in the control samples. It is not known whether its presence was due to adverse conditions in culturing or sampling or if the inducible form of hsp70 is found normally in the L929 cell line. Kothary *et al.* (1984) using Western blotting also observed inducible hsp70 in control samples. From table 1 it can be seen that at the LC_{50} hsp70 induction for cadmium, mercury and copper was 68%, 53% and 69% respectively. At the LC_{50}, hsp70 is the predominant protein being produced. Above the LC_{50}, hsp70 induction begins to plateau (figure 2(A and (B)). At these concentrations the metals may begin to inhibit heat shock protein synthesis and denature hsp70 itself (Fischbach *et al.* 1993).

Figure 2(A and B) also demonstrates the sub-lethal induction of hsp70. The stress response identified hsp70 induction below the no-effect level and at substantial concentrations ranging from 28 to 42 % above control levels, measuring sub-lethal toxicity at concentrations as much as two times lower than the LC_{50} value. The sensitivity of the stress response assay to identify sub-lethal toxicity was assessed by examining hsp70 induction at the lowest observed effective

Figure 4. Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to 2-isobutyl piperidine. ● cell mortality, ■ hsp 70 induction.

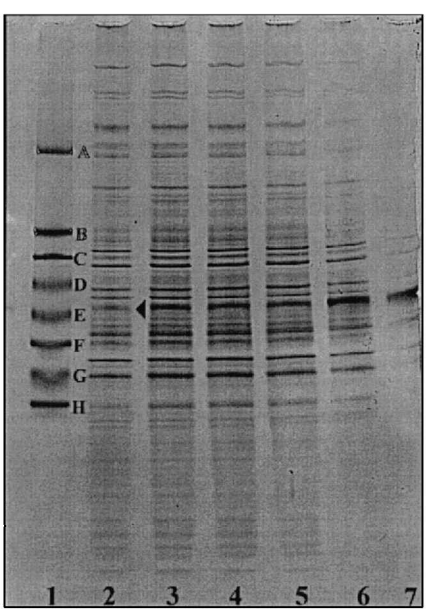

Figure 5. One-dimensional SDS–PAGE separation of mouse connective tissue cell line (L929) exposed to increasing concentrations of 2-isobutyl piperidine.

Figure 6. Stress–response curves for the L929 cell line exposed to cadmium, mercury and copper as determined by ELISA.

concentration. No cell mortality was identified by the neutral red dye assay at these concentrations, which is approximately five, six and eight times lower than the LC_{50} for cadmium, mercury and copper respectively, demonstrating the sensitivity of this assay. The high levels of induction at the lowest concentrations tested ranging from 11 to 23% (table 2) above the control indicate the sensitivity of the stress response assay for measuring sub-lethal toxicity. The stress response identified at the lowest observed effective concentration sub-lethal toxicity at concentrations five times lower than the no-effect level and as much as eight times lower than the LC_{50} value. The ability of the stress response to identify sub-lethal toxicity

demonstrates its potential as a tool in sub-lethal toxicity testing. The ubiquity of hsp70 and its conservation throughout evolution makes it an ideal general indicator of stress, a Tier 1 biomarker, (Sanders 1990).

To confirm the results we found for hsp70 with exposure to cadmium, copper and mercury, a novel compound was tested. The compound 2-isobutyl piperidine was synthesized by colleagues at the University of Gdansk in Poland. The chemical structure is similar to a toxin found in the Colorado potato beetle (*Leptinotarsa decemlineata*). Toxicity data were established using the neutral red assay. The median lethal concentration and no-effect level were identified as 0.6 % and 0.25 % respectively. One-dimensional SDS–PAGE separation showed the induction of hsp70 in a dose-dependent fashion. At the LC_{50}, hsp70 induction was at 54 % above that of the control. The lowest observed effective concentration (0.125 %) showed a 35 % induction in hsp70, no cell mortality was apparent at this concentration which was almost five times lower than the LC_{50}. 2-Isobutyl piperidine is a novel compound, thus comparative data for its toxicity or ability to induce heat shock proteins were not available.

Silver stained SDS–PAGE produces a wealth of information with regard to the total protein profile, however it is time consuming, technically demanding and labour intensive to perform. For routine application, induction of a single heat shock protein may provide sufficient information avoiding the necessity to analyse the total heat shock protein profile. Heat shock protein 70 is suggested as a suitable candidate, being highly conserved and, typically, the most abundant heat shock protein induced (Sanders 1993). The single band isolated by the mouse anti-hsp70 monoclonal antibody using Western blotting indicates the results generated using the ELISA technique are for the inducible isoform of hsp70. The results of the ELISA assay (figure 6) were found to be very similar to those of the SDS–PAGE separations. The slightly higher induction of hsp70 below the LC_{50} value may indicate that the ELISA assay is more sensitive at these concentrations, avoiding the potential interference from other proteins and the background stain associated with SDS–PAGE separations. The highly conserved nature of the heat shock response throughout evolution benefits this assay allowing single antibodies to be used across taxonomic classes thus reducing the expense. The potential for automation of the ELISA technique makes the ELISA heat shock protein assay very attractive and viable for routine application.

Conclusion

The stress response is a highly conserved cellular reaction to various stimuli. The ubiquity of the response occurring across taxonomic classes has identified heat shock proteins as potential biomarkers. The induction of hsp70 occurs in a dose-dependent manner with a decrease in potentially interfering normal protein. The assay is sensitive, identifying significant hsp70 induction far below the LC_{50}, suggesting the hsp70 assay as a valuable biomarker. In this study it was shown that 2-isobutyl piperidine and transition metals elicit significant hsp70 induction at concentrations below the detection limits of the neutral red assay. Based on these findings it is proposed that the hsp70 assay is a good general indicator of stress, i.e. a Tier 1 biomarker. The potential for automation of the ELISA technique makes the ELISA heat shock protein assay very attractive and viable for routine application.

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