Induction of hsp 70 in HepG2 Cells in Response to Hepatotoxicants

WILLIAM F. SALMINEN, JR.,* RICHARD VOELLMY, AND STEPHEN M. ROBERTS*†

Departments of *Pharmacology and Therapeutics and †Physiological Sciences, J. Hillis Miller Health Science Center, University of Florida, Gainesville, Florida 32601 and Department of Biochemistry and Molecular Biology, University of Miami, Miami, Florida 33124

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The objective of this study was to determine if a variety of hepatotoxicants could induce the level of heat shock protein 70I, and whether or not elevated levels of heat shock proteins (hsp's) could provide cytoprotection from those hepatotoxicants. Exposure of HepG2 cells to cytotoxic concentrations of bromobenzene, cadmium, cyclophosphamide, or diethylaminoethylamine increased the level of hsp 70I protein and mRNA, while carbon tetrachloride and cocaine had no effect on hsp 70I or mRNA levels. To determine if induction of hsp 70I might afford protection against cytotoxicity, HepG2 cells were given a prior sublethal heat shock (sub-LHS) (43°C for 1 hr) to induce hsp's and then challenged 24 hr later with the hepatotoxicants. Sub-LHS pretreatment diminished toxicity from bromobenzene, cadmium, cyclophosphamide, or diethylaminoethylamine, but not carbon tetrachloride or cocaine. In cells treated with [14C]-carbon tetrachloride or [3H]cocaine, no detectable covalent binding to proteins was observed; whereas, [14C]-bromobenzene treatment resulted in substantial covalent binding to cellular protein. The apparent absence of formation of reactive metabolite adducted proteins from cocaine and carbon tetrachloride may explain why no hsp 70I induction was observed with these agents. The correlation between hepatotoxicant induction of hsp 70I and cytoprotection afforded by sub-LHS pretreatment suggests that hsp 70I induction may represent an important cellular defense mechanism in the liver. © 1996 Academic Press, Inc.

Prokaryotic and eukaryotic cells respond to a variety of stresses by enhancing the transcription of a specific set of genes that encode heat shock proteins (hsp’s). In eukaryotic cells, increased levels of hsp50 occur as a result of the activation of a heat shock transcription factor (HSF) that is normally in an inactive form [reviewed by Voellmy (1994)], apparently as a heterooligomer with hsp 70 (Baler, 1992; Wu et al., 1994). Stresses including heat shock cause protein unfolding, and nonnative proteins have a higher affinity for hsp 70 than native proteins (Flynn et al., 1989). Titration of hsp 70 through such binding may cause the release of hsp 70 from HSF, allowing HSF to assemble DNA binding homotrimers (Baler, 1992; Westwood et al., 1992). The HSF homotrimers bind to promoters of hsp genes and, after a further stress-induced activation step, become competent to enhance the transcription of the genes (Zuo et al., 1995). After removal of the stress, HSF returns to an inactive state, and transcription of hsp genes decreases at a rate that depends on the severity of the stress (Zuo et al., 1995).

The binding of hsp 70 to damaged proteins is believed to assist in preventing their aggregation and promoting correct refolding (“molecular chaperoning”), as well as facilitating their degradation (Parsell and Lindquist, 1994). As such, hsp 70 and other stress proteins represent an important mechanism by which cells prevent accumulation of aberrant proteins. Further, experimental manipulations that result in elevated levels of hsp’s (e.g., thermal treatment or use of an inducible expression vector) have been observed to result in cytoprotection from a variety of subsequent stresses such as a lethal heat shock (Li et al., 1991; Kampinga et al., 1995; Parsell and Lindquist, 1994). These observations suggest that induction of hsp 70 and other hsp’s may represent an important cellular defense mechanism against proteotoxicity from a variety of stressors.

 Among the stressors that have been demonstrated to result in hsp induction is a rather extensive list of chemical toxicants (Goering et al., 1993; Nover, 1991; Levinson et al., 1980). The number and variety of toxicants shown, in one experimental system or another, to result in increased expression of hsp’s have led some to suggest that hsp induction may be a universal response to cytotoxicity (e.g., Sanders, 1993; Anderson et al., 1987; Blom et al., 1992). However, a recent study of stress protein induction in rat hepatoma cells in response to four chemical agents (viz., arsenite, cadmium, dinitrophenol, and ethanol) found marked differences among these agents in the ability to induce specific stress proteins. In fact, cytotoxic concentrations of two of these toxicants (dinitrophenol and ethanol) failed to induce any of the battery of hsp’s examined. The results of this study suggest that hsp induction in response to chemical toxicants may be more complex than originally envisioned, with some, but not all, toxicants producing proteotoxicity triggering an...
Lactate dehydrogenase (LDH) leakage assay. Co., St. Louis, MO) were dissolved in culture medium to the desired concentration indirectly by placing a measured amount into a 9-mm diameter flask. Preliminary experiments found that adding toxicants quickly volatilized from the vessel, and an equilibrium between the headspace and culture medium was achieved within 30 min. A series of experiments were conducted to determine the appropriate amount to add to the vessel to achieve the desired concentration in the culture medium. With the flask sealed, the concentration of carbon tetrachloride or bromobenzene in the culture medium decreased no more than 25% over 24 hr at 37°C.

Gas chromatography. To measure carbon tetrachloride and bromobenzene concentrations in the culture medium, 1 ml of culture medium was added to 2 ml of pentane. One hundred microliters of a 20-μg/ml trichloroethylene solution was added to each sample to serve as an internal standard. The mixture was vortexed for 1 min and centrifuged at 3000g for 10 min to separate the layers. A 3-μl aliquot of the top (pentane) layer was injected onto an Econo-cap SE-54 column, 15 m × 0.54 mm ID (Alltech, Deerfield, IL). The following conditions were used: helium carrier gas flow rate of 5 ml/min, nitrogen makeup gas flow rate of 50 ml/min, injector port temperature at 110°C, and detector temperature at 200°C. The oven temperature was 50°C for carbon tetrachloride analysis and 120°C for bromobenzene analysis. The eluted compounds were detected by an electron capture detector. Carbon tetrachloride and bromobenzene were quantitated using standard curves prepared from culture medium spiked with known amounts of reference compound.

Polycrylamide gel electrophoresis. Cells were homogenized in sample buffer [0.05 M Tris(hydroxymethyl)aminomethane (Tris-HCl, pH 6.8), 2% sodium dodecyl sulfate, 10 mM diithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride]. Each sample was boiled for 5 min, passed through a 22-ga. needle three times to shear DNA, and stored at −80°C until use. Twenty micrograms of protein from each sample were aliquoted to separate tubes, and bromphenol blue was added to a final concentration of 0.0025%. Each aliquot was boiled for 5 min, loaded onto separate lanes of a 10% SDS-PAGE gel, and resolved by electrophoresis (Laemmli, 1970).

Protein blotting and immunostaining. Proteins separated by SDS-PAGE were immediately blotted to supported nitrocellulose (Bio-Rad Laboratories, Hercules, CA) using a semidry blotting apparatus (Millipore, Bedford, MA) and one-half strength Towbin buffer (10 mM Tris-base, 25 mM glycine, and 10% methanol). Blotting was done at 320 mA for 1.5 hr. Upon completion, the membrane was blocked in TBS (20 mM Tris-HCl, pH 7.5, 500 mM sodium chloride) containing 3% gelatin. The membrane was washed twice for 5 min each in TTBS (TBS containing 0.05% polyoxyethyl- nenedimethylanhydroxylorant without monosaccharate) and probed with an antibody specific for hsp 70I (Stressgen, Victoria, BC, Canada) diluted 1:1000 dilution in TTBS containing 1% gelatin. Incubation was for 18 hr at 24°C with continuous shaking. The membrane was washed twice for 5 min each with TTBS and then a goat anti-mouse alkaline phosphatase-conjugated antibody (Bio-Rad Laboratories, Hercules, CA) diluted 1:3000 dilution in TTBS containing 1% gelatin was incubated with the membrane for 1 hr at 24°C with continuous shaking. Next, the membrane was washed twice for 5 min each in TTBS and once for 5 min in TBS. The colorimetric substrate BCIP/NBT (100 mM Tris-base, pH 9.5, 165 μg/ml 5-bromo-4-chloro-3-indolyl phosphate, 330 mg/ml nitro blue tetrazolium, 100 mM sodium chloride, 5 mM magnesium chloride) was added to the membrane to localize antibody binding.

Lactate dehydrogenase (LDH) leakage assay. Two hundred microliters of culture medium from each plate of cells were removed and the amount of LDH activity present determined spectrophotometrically using the LDH assay (Sigma Chemical Co., St. Louis, MO). All samples were analyzed at 340 nm using a kinetic microplate reader (Molecular Devices, Menlo Park, CA). The cells were then lysed by adding Triton X-100 to the culture medium to a final concentration of 0.7%. Two hundred microliters of the culture medium containing the lysed cells were removed and the amount of LDH activity determined. The percent of LDH released into the culture medium during incubation was determined by dividing the culture medium-only LDH activity by the total LDH activity (i.e., after cell lysis). The percent of LDH leakage was used as an indicator of cell viability.
**RESULTS**

In initial experiments, the time course of hsp 70I induction in HepG2 cells was determined using the classical hsp in-
induced cell lethality from carbon tetrachloride or cocaine (Fig. 4B).

One mechanism by which the hepatotoxicants in this study might stimulate hsp 70I induction is through adduction of proteins by reactive metabolites. Among the hepatotoxicants tested, carbon tetrachloride, cocaine, diethylnitrosamine, cyclophosphamide, and bromobenzene, each produce reactive metabolites in vivo (Evans, 1983; Sipes and Gandolfi, 1982; Hanzlik et al., 1989; Kanekal et al., 1992; Osterman-Golkar and Bergmark, 1988; Plaa, 1993). In order to interpret the apparent absence of hsp 70I induction by cocaine and carbon tetrachloride in HepG2 cells in the context of this potential mechanism, it was important to establish whether or not reactive metabolites were, or were not, being formed under the incubation conditions employed. To test this, the formation of reactive metabolites was evaluated through measurement of covalent (i.e., irreversible) binding to proteins following incubation with radiolabeled [14C]carbon tetrachloride, [14C]bromobenzene, or [3H]cocaine. As shown in Table 1, among these three hepatotoxicants, only bromobenzene produced detectable binding to protein.

**DISCUSSION**

A common theme among agents that induce the heat shock response is the ability to disrupt protein homeostasis (Ananthan et al., 1986; Hightower, 1991). Many studies have focused on the induction of hsp 70I since it appears to be

![Fig. 2](image1.png)

**FIG. 2.** Scanning densitometry analysis of the level hsp 70I in HepG2 cells 24 hr after hepatotoxicant treatment. Cells were exposed for 24 hr to various hepatotoxicants at the indicated concentrations. Protein was resolved on a 10% SDS-PAGE gel, and hsp 70I detected by Western blotting using a monoclonal antibody specific for hsp 70I. A goat anti-mouse IgG antibody conjugated with alkaline phosphatase was used to detect the primary antibody binding. DEN = diethylnitrosamine; Cd = cadmium acetate; BB = bromobenzene; CCl₄ = carbon tetrachloride; CP = cyclophosphamide. Values represent mean ± SEM (n = 3 plates). * denotes significantly different from control by Student Neuman–Keul’s post hoc test using p < 0.05.

may provide protection from other types of subsequent stresses. It has been inferred, but not shown, that the induction of hsp’s is responsible for this effect. It is possible that the induction of hsp 70I during exposure to some of the hepatotoxicants tested is a cytoprotective response of the cell; however, it may also be a by-product of cellular damage and serve no cytoprotective function. To examine these possibilities, the effect of a sub-LHS on hepatotoxicant potency was measured. Cells were subjected to sub-LHS, followed 24 hr later by exposure to hepatotoxicant. The effect of sub-LHS on hepatotoxicant-induced cell lethality, as measured by LDH leakage, is shown in Figs. 4A and B. As a positive control, sub-LHS pretreatment diminished the cell mortality from LHS (46.5°C for 2 hr) exposure, as expected. Sub-LHS also significantly diminished the cytotoxicity of diethylamino, cadmium acetate, bromobenzene, and cyclophosphamide (Figs. 4A and B). Interestingly, sub-LHS pretreatment caused no significant change in the hepatotoxicant-

![Fig. 3](image2.png)

**FIG. 3.** Northern blot showing the level of hsp 70I mRNA in HepG2 cells 24 hr after hepatotoxicant treatment. Cells were exposed for 24 hr to various hepatotoxicants at the indicated concentrations. Total RNA was isolated, resolved, and blotted as described in Methods. hsp 70I mRNA was detected by hybridizing the immobilized RNA with digoxigenin-labeled hsp 70I DNA. Hybridizing probe was located using an anti-digoxigenin antibody and a chemiluminescent substrate. The blot was stripped and reprobed with a digoxigenin-labeled β-actin probe. Sub-LHS = sub-lethal heat shock (43°C for 1 hr); DEN = diethylnitrosamine; Cd = cadmium acetate; BB = bromobenzene; CCl₄ = carbon tetrachloride; CP = cyclophosphamide.
FIG. 4. (a and b) The effect of a 24-hr prior sub-lethal heat shock (sub-LHS) (43°C for 1 hr) on hepatotoxicant-induced cell lethality. Cells were heated in a water bath maintained at 43 ± 0.1°C. Twenty-four hours after the sub-LHS, hepatotoxicants were incubated with the cells for an additional 24 hr and the percent of LDH activity released into the culture medium was determined. LHS = lethal heat shock (46.5°C for 2 hr); DEN = diethylnitrosamine; Cd = cadmium acetate; BB = bromobenzene; CCl₄ = carbon tetrachloride; CP = cyclophosphamide. Values represent mean ± SEM (n = 5 plates). * denotes significantly different from control by Student Neuman–Keul’s post hoc test using p < 0.05.

universally induced during stress. Consistent with recent reports by other investigators, however, the present study suggests there are exceptions to this rule, even though cell injury occurs (Wiegant et al., 1994; Mirkes et al., 1994; Goodman and Sloviter, 1993).

### TABLE 1
**Irreversible Binding of Bromobenzene, Carbon Tetrachloride, or Cocaine to Protein in HepG2 Cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration (hr)</th>
<th>pmol bound per μg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 mM Bromobenzene</td>
<td>4</td>
<td>13.36 ± 6.66</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>20.78 ± 5.67</td>
</tr>
<tr>
<td>0.8 mM Carbon tetrachloride</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>ND</td>
</tr>
<tr>
<td>5 mM Cocaine</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>ND</td>
</tr>
</tbody>
</table>

Note. HepG2 cells were treated with [¹⁴C]bromobenzene, [¹⁴C]carbon tetrachloride, or [³H]cocaine (1 μCi/plate) at the indicated concentrations. As a control, incidental binding was measured using the same culture exposure regimen in cells pretreated with trichloroacetic acid (6% w/v). Cells were harvested at the indicated times and washed extensively as described in Methods. ND = no detectable irreversible binding observed. Values represent mean ± SEM (n = 3 plates) after subtracting incidental binding.

A strong correlation between hepatotoxicant induction of hsp 70I and the ability of sub-LHS pretreatment to provide protection from those hepatotoxicants was observed. Diethylnitrosamine, cadmium acetate, bromobenzene, and cyclophosphamide induced hsp 70I and showed decreased cytotoxicity in cells pretreated with sub-LHS. In contrast, no change in the level of hsp 70I was evident after either carbon tetrachloride or cocaine treatment, nor was their cytotoxicity diminished in cells pretreated with sub-LHS. In this study, the extent of hsp 70I induction was evaluated at only one time point and, as a consequence, the relative ability of the various hepatotoxicants to increase hsp 70I levels cannot be determined with confidence. To the extent that the 24-hr data reflect overall induction, however, the magnitude of induction of hsp 70I appears to be correlated with the ability of a prior sub-LHS to provide protection from the hepatotoxicants. Diethylnitrosamine and cadmium strongly induced hsp 70I and showed the greatest decrease in cytotoxicity in cells given a prior sub-LHS. Cyclophosphamide and bromobenzene mildly induced hsp 70I at 24 hr and showed only minimal decreases in cytotoxicity. The apparent correlation between the ability of an hepatotoxicant to increase hsp 70I levels and cytoprotection afforded by elevated levels of hsp’s argues that hsp 70I may play an important role in providing cytoprotection from some hepatotoxicants. Information re-
garding similar correlations for other toxicants is extremely limited. Li and coworkers, however, in a study of hsp induction and cytotoxicity of a series of membrane-active agents (viz., solvents and local anesthetics), also found a strong correlation between the ability to stimulate hsp synthesis and cytoprotection afforded by prior hsp induction in Chinese hamster cells (Hahn et al., 1985).

Since carbon tetrachloride and cocaine did not induce hsp 70I and showed no decrease in cytotoxicity in cells pretreated with sub-LHS, it was necessary to investigate the possibility that the concentrations of toxicants used may have precluded the expression of hsp 70I through inhibition of transcription, translation, or some other means. To address these questions, Northern blots measuring hsp 70I mRNA were used to determine if transcription of the hsp 70I gene was inhibited by the toxicants. In addition, the effect of lower hepatotoxicant concentrations on the level of hsp 70I was measured. Neither of these experimental approaches indicated that carbon tetrachloride or cocaine were preventing the expression of hsp 70I at the concentrations used. The levels of hsp 70I mRNA after carbon tetrachloride or cocaine treatment were, in fact, the same as in untreated cells. These observations suggest that neither carbon tetrachloride nor cocaine is producing cellular changes triggering hsp induction (in contrast to the other toxicants), or that they are inhibiting expression prior to, or at a level of, transcription of the hsp 70I gene.

Induction of hsp’s is believed to be triggered by denatured proteins (Ananthan et al., 1986; Baler et al., 1992). Many hepatotoxicants are metabolized to reactive intermediates that bind cellular proteins, and it is possible that the adducted proteins are recognized by hsp’s as nonnative, thereby triggering induction. This type of mechanism has been proposed by Chen et al. (1992) for hsp induction by nephrotoxic cysteine conjugates. Among the toxicants tested in the present study, all are capable of producing reactive metabolites that bind to protein in vivo, with the exception of cadmium, which can bind to proteins directly through interaction with sulphhydryls (Jacobson and Turner, 1980). Assuming that each of the toxicants also bound to protein in the HepG2 model system, the absence of hsp 70I induction following exposure of HepG2 cells to cytotoxic concentrations of carbon tetrachloride and cocaine appeared initially to argue against a reactive metabolite binding mechanism. Subsequent experiments, however, served to reinforce a correlation between covalent binding and hsp induction. Though HepG2 cells have been reported to metabolize many toxicants through cytochrome P-450-dependent mixed function oxidation (Belisario et al., 1991), reactive metabolite binding was found to be absent in the HepG2 cells during the carbon tetrachloride and cocaine exposures. Bromobenzene concentrations that induced hsp 70I expression in the HepG2 cells, on the other hand, resulted in readily detectable covalent binding to proteins. Thus, HepG2 cells may be incapable of bioactivating carbon tetrachloride and cocaine. It is worthwhile noting that in mice, where administration of cocaine and carbon tetrachloride results in significant covalent binding, hsp 70I induction has been observed (unpublished data).

In conclusion, experiments conducted using the HepG2 human hepatoma cell line and a battery of hepatotoxicant chemicals indicate that cytotoxicity is not always accompanied by hsp 70I induction. Most of the toxicants tested produced at least moderate induction, but some appeared to be completely ineffective. It is logical to suspect that differences in induction reflect differences in interactions between the toxicants and cellular proteins, such as through the formation of reactive metabolites, although this has not yet been clearly demonstrated. Perhaps one of the most interesting observations was the strong apparent correlation between the ability of the hepatotoxicants to induce hsp 70I and the extent of cytoprotection against their effects afforded by sub-LHS pretreatment. This suggests that, at least for some hepatotoxicants, hsp 70I induction may be an important cellular defense mechanism.

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REFERENCES


