

Expression of *hsp 27*, *hsp 60*, *hsc 70*, and *hsp 70* Stress Response Genes in Cultured Human Urothelial Cells (UROtsa) Exposed to Lethal and Sublethal Concentrations of Sodium Arsenite

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The stress response is one mechanism that the bladder urothelium could potentially employ to protect itself from cellular damage after exposure to arsenic and, in so doing, influence the shape of the dose–response curve at low concentrations of exposure to this environmental pollutant. In the present study, we used the cultured human urothelial cell line UROtsa, a model of human urothelium, to determine the expression of heat shock proteins *hsp 27*, *hsp 60*, *hsc 70*, and *hsp 70* after acute and extended exposure of the cells to lethal and sublethal levels of sodium arsenite (NaAsO₂). Acute exposure was modeled by exposing confluent cultures of UROtsa cells to 100 μM NaAsO₂ for 4 hr followed by a 48-hr recovery period. Extended exposure was modeled by exposing confluent UROtsa cells to 1, 4, and 8 μM NaAsO₂ for 16 days, with the highest concentration producing cell death by 4 days of exposure. The expression of *hsp 27*, *hsp 60*, *hsc 70*, and *hsp 70* mRNA and protein was determined by reverse-transcription polymerase chain reaction and Western analysis. Cell viability was determined by the MTT [(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The results demonstrated that the expression of *hsp 27*, *hsp 60*, and *hsc 70* mRNA and protein were not consistently increased by either acute or extended exposure to NaAsO₂. In contrast, *hsp 70* expression was induced by NaAsO₂ after both acute and extended exposure. The degree and duration of the induction of the *hsp 70* protein in the extended time course of exposure to NaAsO₂ correlated directly with UROtsa cell cytotoxicity. The substantial level of basal expression of *hsp 27*, *hsp 60*, and *hsc 70* shown previously in human bladder urothelium, coupled with the inducible expression of *hsp 70*, could provide the human urothelium with a mechanism to withstand and recover from a low level of arsenite exposure. **Key words:** arsenic, arsenite, bladder, bladder cancer, bladder cell line, heat shock proteins, *hsp*, immortalization, stress response, urothelium. *Environ Health Perspect* 110:1225–1232 (2002). [Online 16 October 2002] <http://ehpnet1.niehs.nih.gov/docs/2002/110p1225-1232rossi/abstract.html>

Inorganic arsenic is a metallic element distributed throughout the earth's crust. Humans can be exposed to high levels of arsenic mainly through inhalation of contaminated dusts in occupational settings or by ingestion of drinking water contaminated with arsenic from naturally occurring sources. Despite limited findings showing arsenic to be a carcinogen in animal testing, there is extensive human epidemiologic evidence that trivalent and pentavalent forms of arsenic cause characteristic skin alterations, including hyperkeratosis and skin cancer (Steinmaus et al. 2000). Epidemiologic evidence also shows a strong association between arsenic ingestion from drinking water and the development of bladder cancer in Taiwan (Chiou et al. 1995), Argentina (Hopenhayn-Rich et al. 1996), Chile (Smith et al. 1998), and Japan (Tsuda et al. 1995). Subsequently, arsenic has been ranked first in priority in a list of the top 20 hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR 1997) and the U.S. Environmental Protection Agency (U.S. EPA; ATSDR 1997). Over 350,000 people in the United States consume drinking water containing arsenic in excess of 50 μg/L, the past U.S. EPA arsenic water standard, and an additional 2.5 million people

drink water containing in excess of 25 μg/L arsenic (Karagas et al. 1998).

Research that links drinking water arsenic to internal cancers has not been without controversy (Jager and Ostrosky-Wegman 1997), and the use of this research to estimate cancer risks at low doses and to establish a potentially costly new arsenic drinking water standard has received aggressive debate (Steinmaus et al. 2000). This controversy has centered on the possibility that the current U.S. EPA cancer slope factor (CSF) for arsenic may actually overpredict cases at relatively low exposure levels (Valberg et al. 1998). The debate centers on whether the CSF should be calculated assuming a standard linear dose–response relationship or whether a nonlinear or sublinear dose response may in fact be more appropriate. The available epidemiologic data provide empirical evidence for both linear and nonlinear associations. The mechanism underlying arsenic carcinogenicity is presently unknown, and elucidation of the mechanism of arsenic carcinogenicity could be an important factor in establishing the shape of a dose–response curve and the assessment of cancer risk at low levels of exposure. As recently reviewed (Kitchin 2001), proposed mechanisms of arsenic carcinogenesis include those that are

predominantly genotoxic (e.g., chromosomal abnormalities, oxidative stress, and gene amplification) and others more nongenotoxic (e.g., altered growth factors, enhanced cell proliferation and promotion of carcinogenesis, and altered DNA repair). As stated in this review, the present lack of knowledge of the modes of carcinogenic action for arsenic and the true shape of the dose–response curve at low concentrations negatively affects the process of regulating environmental arsenic exposure.

Our goal in the present study was to advance the cultured human urothelial cell line UROtsa as a model and adjunct for studies of arsenic carcinogenesis in the human bladder. A cell culture model of human urothelium is needed because there have been difficulties in demonstrating that arsenic is a carcinogen using animal models. The UROtsa cell line was derived from normal human urothelium lining the ureter and was immortalized using simian virus 40 (SV40) large T-antigen (Petzoldt et al. 1994, 1995). After immortalization, the cells did not acquire characteristics of neoplastic transformation as demonstrated by lack of colony formation in soft agar and lack of growth of tumors in nude mice. These cells, originally grown in serum-containing growth medium, remained undifferentiated and displayed few of the properties expected of human urothelium. Recently, we developed a serum-free growth medium for the UROtsa cell line that allowed expression of many of the structural features expected of human urothelium (Rossi et al. 2001). The cells also retained the basal expression of the metallothionein and heat shock proteins known to occur for *in vivo* human urothelium and could be readily transfected with expression vectors.

In the present study, we used the UROtsa cell line to determine the expression of several of the heat shock proteins after acute and extended exposure of the cells to lethal and sublethal levels of sodium arsenite (NaAsO₂).

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These proteins are participants in the heat shock (stress) response of the cell, a response that is widely recognized and accepted as a major factor in the cell's ability to protect against and recover from environmental insult, both physical and chemical (for reviews, see Craig et al. 1994; Georgopoulos and Welch 1993; Macario 1995; Schlesinger 1994). The examination of the *in vitro* response of these cells to arsenite was motivated by the possibility that these rapidly inducible proteins could potentially affect the dose–response curve at low levels of *in vivo* exposure to arsenite. We used combinations of acute and extended arsenite exposure to determine if induction of the stress–response proteins was transient even in the continued presence of arsenite.

Materials and Methods

Cell culture. Stock cultures of the UROtsa cell line were maintained in 75-cm² tissue culture flasks in serum-free growth medium as described by Rossi et al. (2001). Briefly, the serum-free growth medium was composed of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 supplemented with selenium (5 ng/mL), insulin (5 µg/mL), transferrin (5 µg/mL), hydrocortisone (36 ng/mL), triiodothyronine (4 pg/mL), and epidermal growth factor (10 ng/mL). The cells were fed fresh growth medium every 3 days, and at confluence (normally 6–12 days after subculture), the cells were subcultured at a 1:4 ratio using trypsin (0.05%):EDTA (0.02%). For use in experimental protocols, cells were subcultured at a 1:4 ratio, allowed to reach confluence (12 days after subculture), and then used in the described experimental protocols.

Preliminary experiments were performed to determine the conditions of acute exposure to heat and NaAsO₂ that were near to, but below, a level that produced cell death. This resulted in the following experimental protocols: for heat shock, exposure of the UROtsa cells to 42.5°C for 1 hr followed by a recovery period of 48 hr at 37°C; for NaAsO₂, exposure to 100 µM NaAsO₂ for 4 hr followed by a recovery period of 48 hr in NaAsO₂-free media. Preliminary experiments were also performed to determine the approximate

concentrations of NaAsO₂ that would result in UROtsa cell toxicity over a 16-day period of exposure. From this preliminary determination, three concentrations of NaAsO₂ were then chosen for experimental use (1.0, 4.0, or 8.0 µM) such that over the 16-day time course, one concentration would result in no cell death and another would result in significant cell death early in the time course.

Cell viability, isolation of total RNA, and RT-PCR. Cell viability, as an indicator of cytotoxicity, was determined by measuring the capacity of the UROtsa cells to reduce MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (Denizot and Lang 1986). Briefly, a 5-mg/mL solution of MTT (Sigma Chemical Co., St. Louis, MO) was prepared in DMEM, filtered through a 0.2-µm filter, and stored at –20°C in 1-mL aliquots. Cell viability was determined on cells grown in six-well plates (35-mm diameter wells), and each well contained 2 mL of growth media. MTT solution (40 µL) was added to each well, followed by incubation at 37°C for 4 hr in a 5% CO₂:95% air atmosphere. The cells were washed twice with 2 mL phosphate-buffered saline and 1 mL acidic propanol (0.1 N HCl in absolute propanol) added to each well. The absorbance was determined at 570 nm using a plate reader with acidic propanol as the blank. The MTT reduction was linear with time over the 4-hr incubation period.

Total RNA was isolated according to the protocol supplied with TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) as described previously (Garrett et al. 1998). The concentration and purity of samples were determined using spectrophotometer scan in the ultraviolet region and ethidium bromide (EtBr) visualization of intact 18S and 28S RNA bands after agarose gel electrophoresis. The methods and primers used for the reverse-transcription polymerase chain reaction (RT-PCR) analysis of mRNA representing the *hsp 27*, *hsp 60*, *hsc 70*, *hsp 70A*, *hsp 70B*, *hsp 70C*, and glyceraldehyde 3-phosphate dehydrogenase (*g3pdh*) genes have been described previously by this laboratory (Somji et al. 1999a, 1999d, 2000; Kim et al. 2001). Heat

shock protein mRNAs were determined by RT-PCR cycles as follows: *hsp 27* mRNA at 20 cycles; *hsp 60* at 35 cycles, *hsc 70* mRNA at 35 cycles, *hsp 70* at 35 cycles, and *g3pdh*, at 30 cycles. We used an input of 500 ng total RNA in all the RT-PCR reactions. The final PCR products were electrophoresed on 2% agarose gels containing EtBr along with DNA markers. For reactions in the linear region, integrated optical densities (IOD) of the samples were obtained by input of the EtBr fluorescent image using a Kodak DCS 420 CCD camera (Kodak, Rochester, NY) into a Dell workstation (Dell, Austin, TX) configured with KS400 software (Zeiss, Thornwood, NY). The resulting IOD values were used to generate a relative IOD that is the ratio of the respective heat shock protein reaction product to that of the housekeeping gene *g3pdh*.

Western analysis. The determination of the *hsp 27*, *hsp 60*, *hsc 70*, and *hsp 70* proteins by Western analysis has been described previously (Somji et al. 1999a, 1999d, 2000; Kim et al. 2001). We used the following primary antibodies to detect proteins: mouse monoclonal antibody (SPA-800; StressGen, Victoria, BC, Canada) to detect *hsp 27*; mouse monoclonal antibody (STM-806; StressGen) to detect *hsp 60*; rat monoclonal antibody (SPA-815; StressGen) to detect *hsc 70*; and mouse monoclonal antibody (SPA-810; StressGen) to detect *hsp 70*. For reactions in the linear range of analysis, IOD of the samples were obtained by input of the image using a Kodak DCS 420 CCD camera into a Dell workstation configured with KS400 software.

Analysis of *hsp 27* phosphoisoforms. Proteins were extracted from cell monolayers with 9 M urea, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 2% ampholines, 5% β-mercaptoethanol, and 2% Triton X-100. The proteins were focused on 4% polyacrylamide capillary tube gels containing 9 M urea, 1.5% 5/7 Biolyte, and 0.5% 3/10 Biolyte ampholines (Biorad, Hercules, CA). Capillary tube gels containing focused proteins were placed at the top of 12% polyacrylamide slab minigels, followed by separation of proteins in the second dimension. Resolved proteins were

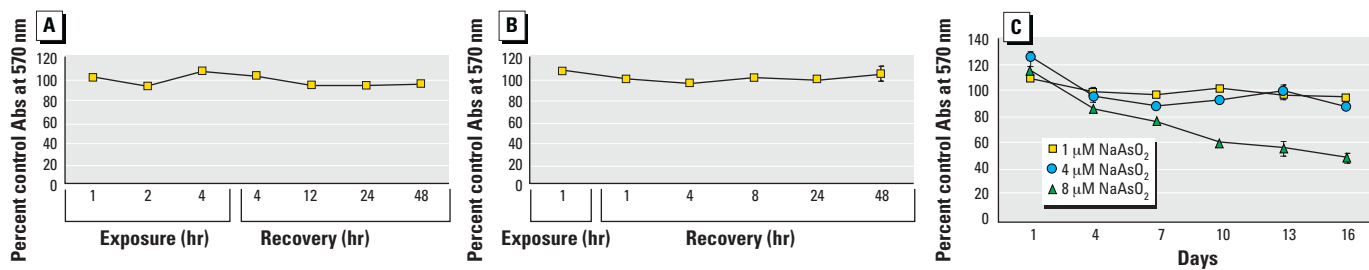


Figure 1. Viability of UROtsa cells exposed to NaAsO₂ and heat shock. (A) UROtsa cells exposed to 100 µM NaAsO₂ for 4 hr followed by a recovery period of 48 hr in NaAsO₂-free media. (B) UROtsa cells exposed to 42.5°C for 1 hr followed by a 48-hr recovery period at 37°C. (C) UROtsa cells exposed to NaAsO₂ for 16 days. Cell viability was determined by the MTT assay, and all determinations were in triplicate. Values shown are the percentage of the mean absorbance (Abs) of treated cells divided by the mean absorbance of the control cells for each triplicate determination.

electrotransferred onto polyvinylidene difluoride membranes (Biorad). We detected hsp 27 phosphoisoforms using procedures identical to those described for Western analysis.

Statistical analysis. All experiments were performed in triplicate. The relative IOD values are shown as the mean SEM. Statistical analyses were performed using Systat software (Systat Software Inc., Richmond, CA) using separate variance *t*-tests, analysis of variance with Tukey post hoc testing, and Kruskal-Wallis statistic with Dunnett's test. Unless otherwise stated, the level of significance was 0.05.

Results

Expression of hsp 27, hsp 60, hsc 70, and hsp 70 mRNA and protein in UROtsa cells acutely exposed to NaAsO₂.

Acute exposure of mammalian cells to elevated temperature or NaAsO₂ is the classic method to define the cellular response to physical or chemical stress, respectively. The effect of NaAsO₂ on the expression of hsp 27, hsp 60, hsc 70, and hsp 70 was determined by exposing confluent UROtsa cells to 100 μM NaAsO₂ for 4 hr followed by a 48-hr recovery period. The MTT assay showed that this concentration of NaAsO₂ caused no cytotoxicity to the UROtsa cells over the time course of the experiment (Figure 1A). The expression of hsp 27 mRNA was determined by comparing the IOD values

of hsp 27 mRNA with those of the housekeeping gene *g3pdh*, yielding the relative IODs at each analysis point over the time course of NaAsO₂ treatment and recovery (Figure 2A). Acute exposure to NaAsO₂ had only a marginal effect on the level of hsp 27 mRNA, with only two time points showing a small, but significant ($p < 0.05$), increase in hsp 27 mRNA. These increases were very modest compared with the control and with that expected historically for the stress response. The marginal induction of hsp 27 mRNA by NaAsO₂ was reinforced by the finding that NaAsO₂ caused no significant increase in hsp 27 protein at any point in the time course (Figure 2B). An analysis of the phosphoisoforms of hsp 27 demonstrated that NaAsO₂ treatment had no effect on the phosphorylation of hsp 27 and that the hsp 27 protein was present as a mixture of the unphosphorylated and singly phosphorylated isoforms similar to that found in control cells (data not shown).

Identical total RNA and protein samples were used to determine the expression of hsp 60 mRNA and protein in UROtsa cells exposed to NaAsO₂. Exposure of the UROtsa cells to NaAsO₂ resulted in a modest, but significant, increase in the expression of both hsp 60 mRNA and protein (Figure 3A,B). The hsp 60 mRNA increased to a maximum 3-fold elevation at 4 and 8 hr into the recovery period

and returned to control values by the end of the time course (Figure 3A). The hsp 60 protein also increased significantly and attained a maximal 2-fold elevation early in the recovery period before returning to control values by the end of the time course (Figure 3B).

These samples were then analyzed for the expression of hsc 70 mRNA and protein. Hsc 70 mRNA increased after 4 hr of NaAsO₂ exposure and at 4 and 8 hr after NaAsO₂ removal (Figure 4A). However, these modest increases in hsc 70 mRNA did not translate into an increase in hsc 70 protein, the level of which was constant throughout the time course of exposure and recovery (Figure 4B). In contrast to the constitutive hsc 70 family member, protein for the inducible family member hsp 70 was markedly increased by exposure to 100 μM NaAsO₂ (Figure 5A). The hsp 70 protein was undetectable both in control cells and in cells during the initial 4 hr of exposure to NaAsO₂. The hsp 70 protein was prominent in the treated cells by 4 hr into the recovery period and reached a maximum expression between 8 and 16 hr after removal of NaAsO₂. The hsp 70 protein remained elevated at the end of the 48-hr recovery period.

The hsp 70 protein is encoded by several genes, three of which (*hsp 70A*, *hsp 70B*, and *hsp 70C*) have been sequenced and identified, and these can be determined using RT-PCR technology (Somji et al. 1999d; Kim et al. 2001). Expression of mRNA for all three of these genes was markedly elevated after exposure to 100 μM NaAsO₂ (Figure 5B). The expression of hsp 70A mRNA was barely detectable in control cells, but was significantly elevated after a 1-hr exposure to NaAsO₂, reaching maximal elevation 4–8 hr into the recovery period (Figure 5B). The hsp 70A mRNA returned to control values within 16 hr of NaAsO₂ removal from the cells. The expression of hsp 70B mRNA was detected only after 4 hr of exposure to and recovery from 100 μM NaAsO₂ (Figure 5B). The hsp 70B mRNA was not detected in control cells or at any other time points in the NaAsO₂-treated cells. At the two time points when hsp 70B mRNA was detected, it was at similar relative levels of expression as found for hsp 70A mRNA. The expression of hsp 70C was also absent in control cells but showed a marked increase at 2 and 4 hr after NaAsO₂ exposure and at 4 and 8 hr after NaAsO₂ removal, thereafter returning to undetectable levels (Figure 5B). The mRNA for the *hsp 70B* and *hsp 70C* genes was undetectable in control cells at 40 cycles of the PCR with a 500 ng total RNA input (data not shown).

Expression of hsp 27, hsp 60, hsc 70, and hsp 70 mRNA and protein in UROtsa cells acutely exposed to heat shock. The effect of heat shock on the expression of hsp 27 was determined by exposing confluent UROtsa

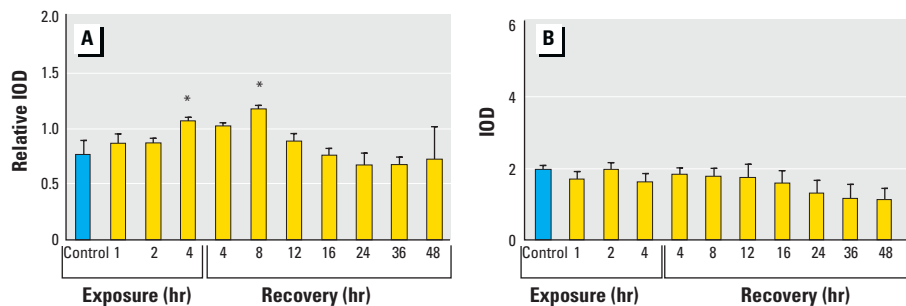


Figure 2. Expression of hsp 27 mRNA and protein for UROtsa cells after acute exposure to 100 μM NaAsO₂. (A) Relative expression of hsp 27 mRNA (mean ± SEM) compared with the *g3pdh* housekeeping gene. (B) Expression of hsp 27 protein as determined by Western analysis. The mean IOD values (± SEM) were calculated from three individual measurements for each time point.

*Significantly different compared with the control at a level of 0.05.

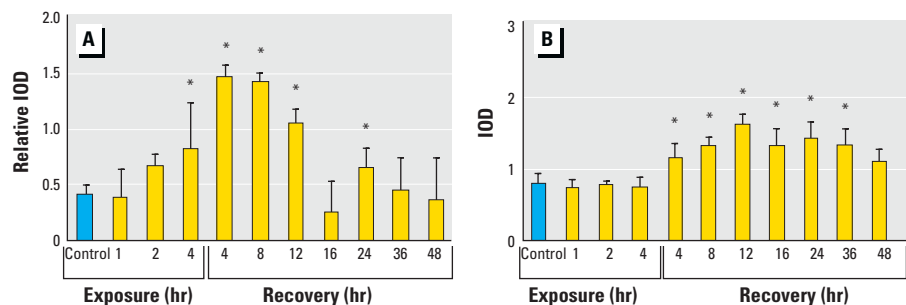


Figure 3. Expression of hsp 60 mRNA and protein for UROtsa cells after acute exposure to 100 μM NaAsO₂. (A) Relative expression of hsp 60 mRNA (mean ± SEM) compared with the *g3pdh* housekeeping gene. (B) Expression of hsp 60 protein as determined by Western analysis. The mean IOD values (± SEM) were calculated from three individual measurements for each time point.

*Significantly different compared with control at a level of 0.05.

cells to an elevated temperature of 42.5°C for 1 hr followed by a recovery period of 48 hr at 37°C. Similar to the effect found for acute NaAsO₂ treatment, heat shock had only a marginal effect on the level of hsp 27 mRNA or protein, with only one time point showing a significant increase in hsp 27 mRNA and two time points showing a small increase in hsp 27 protein (Figure 6A,B). These increases were very modest compared with the control and with that expected historically for the stress response. Heat shock also had no effect on the phosphorylation of hsp 27 (data not shown).

The response of hsp 60 to heat shock was also similar to that found previously for acute chemical stress. Exposure of the UROtsa cells to heat resulted in a significant increase in the expression of both hsp 60 mRNA and protein (Figure 6C,D). The hsp 60 mRNA increased to a maximum 3-fold elevation 2 hr after heat shock and returned to control values by the end of the time course (Figure 6C). The hsp 60 protein also increased, attaining a maximal 2-fold elevation 12 hr into the recovery period and remaining slightly elevated at several later time points throughout the recovery period (Figure 6D). The similarity in response of the UROtsa cells to acute chemical stress and heat shock also extended to hsc 70 and hsp 70, the constitutive and inducible members of the 70-kDa heat shock family (Figure 6E,F). The hsc 70 mRNA increased immediately after the heat shock and remained moderately elevated throughout the 48-hr recovery period (Figure 6E). These modest increases in hsc 70 mRNA, identical to those found for acute NaAsO₂ treatment, did not translate into an increase in hsc 70 protein, the level of which was constant throughout the time course of heat shock and recovery (Figure 6F). Heat shock resulted in an elevation of mRNA, similar to that found for NaAsO₂ treatment, for all three of the inducible heat shock genes, *hsp 70A*, *hsp 70B*, and *hsp 70C* (Figure 6G). Induction occurred within 1 hr after heat shock, peaked between 1 and 4 hr of the recovery period, and returned to control or undetectable levels by the end of the time course. Likewise, the hsp 70 protein was undetectable in control cells, was induced by 8 hr into the recovery period, and remained elevated for the remainder of the time course (Figure 6H).

Expression of hsp 27, hsp 60, hsc 70 and hsp 70 mRNA and protein in UROtsa cells exposed to NaAsO₂ for 16 days. The expression of hsp 27, hsp 60, hsc 70, and hsp 70 mRNA and protein was also determined when the UROtsa cells were exposed to NaAsO₂ for an extended time course, rather than the acute exposure classically used to define the stress response. This extended exposure was modeled by exposing the UROtsa cells continuously to three levels of NaAsO₂ over a 16-day time course. Three concentrations of NaAsO₂

were used: 1.0 μM, which produced no cell death over the 16-day time course; 4.0 μM, which also produced no cell death over the 16-day time course; and 8.0 μM, which produced significant levels of cell death 8 days into the 16-day time course (Figure 1C). The respective mRNA and proteins were determined at days 1, 4, 7, 10, 13, and 16, with the effects of NaAsO₂ on detectable expression of heat shock protein normalized to a control value of 1.0 for data presentation.

None of the heat shock proteins analyzed showed a significant difference in expression levels in control cells over the 16-day time course (data not shown). For hsp 27 there was no change in the expression of mRNA over the initial 13 days of the time course for all three concentrations of NaAsO₂ (Figure 7A). On the last day of exposure, there was a significant reduction in hsp 27 mRNA in a dose-dependent manner for all three concentrations of NaAsO₂. The expression of hsp 27 protein was also reduced by NaAsO₂ exposure, with significant reductions in expression occurring after 7 days of exposure to the highest dosage of NaAsO₂ and on the last day of the time course for the intermediate exposure level (Figure 7B). The level of hsp 27 protein was not altered for cells exposed to the lowest level of NaAsO₂ over the 16-day period.

For hsp 60, there was no change in the expression of mRNA except for two points of

the entire 16-day time course (Figure 7C). These changes were modest and consisted of increased mRNA expression on day 7 for cells exposed to the highest level of NaAsO₂ and on day 13 for cells exposed to the intermediate level of NaAsO₂. The expression of the hsp 60 protein was not altered by exposure of the cells to either the low or intermediate level of NaAsO₂ (Figure 7D). There was a modest increase in hsp 60 protein expression on days 10, 13, and 16 of the time course for cells exposed to the highest level of NaAsO₂. For hsc 70, there was no difference in the expression of mRNA or protein for any of the three exposures to NaAsO₂ over the 16 day period of exposure (Figure 7E,F).

For hsp 70, the expression of mRNA markedly differed between the *hsp 70A* gene and the *hsp 70B*, and *hsp 70C* genes. For the *hsp 70B* and *hsp 70C* genes, no expression of mRNA could be detected in control or NaAsO₂-treated cells at any point of the time course (data not shown). The cutoff value for expression was a 40-cycle RT-PCR primed with 500 ng of total RNA. In contrast, mRNA for the *hsp 70A* gene was found in control cells (at 35 cycles of PCR) at all points of the time course, and expression was not significantly different among the controls at any point of the time course (data not shown). Treatment with NaAsO₂ caused a modest increase in expression of hsp 70A mRNA in the cells at all

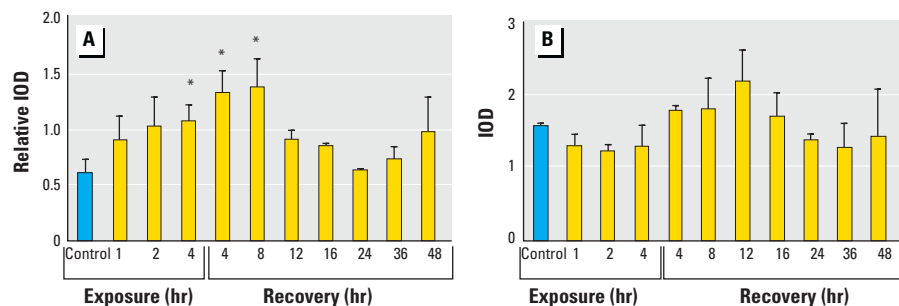


Figure 4. Expression of hsc 70 mRNA and protein for UROtsa cells after acute exposure to 100 μM NaAsO₂. (A) Relative expression of hsc 70 mRNA (mean ± SEM) compared with the *g3pdh* housekeeping gene. (B) Expression of hsc 70 protein as determined by Western analysis.

*Significantly different compared with control at a level of 0.05.

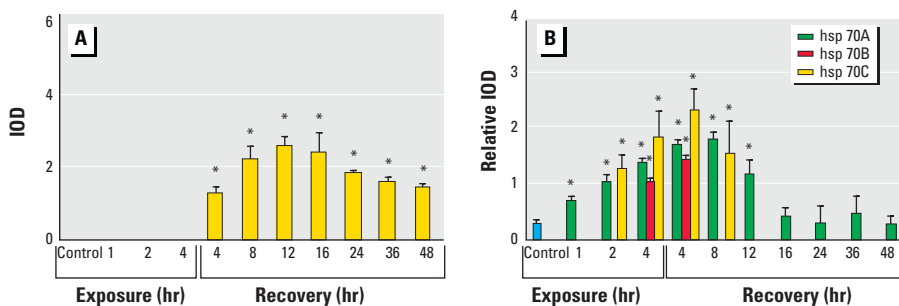


Figure 5. Expression of hsp 70 protein and mRNA for UROtsa cells after acute exposure to 100 μM NaAsO₂. (A) Expression of hsc 70 protein as determined by Western analysis. (B) Relative expression of hsp 70A, hsp 70B, and hsp 70C mRNA (mean ± SEM) compared with the *g3pdh* housekeeping gene. Samples were performed in triplicate.

*Significantly different compared with control at a level of 0.05.

three levels of exposure, with the most pronounced elevations being at the highest exposure level (Figure 7G). The hsp 70 protein was not detectable in control cells at any point of the time course using total protein loads of up to 10 μg in the Western analysis (data not shown). In contrast, hsp 70 protein was clearly expressed in a dose-dependent manner in UROtsa cells exposed to NaAsO_2 (Figure 7H). There was a high level of hsp 70 protein expression at the highest exposure (8 μM) of NaAsO_2 starting at day 1 and extending through the end of the time course. The levels

of hsp 70 protein expression were proportionately less at the lower concentrations, with elevations at the beginning and middle of the time course and a return to control values by 16 days of exposure. The levels of the hsp 70 protein were 2–3-fold higher relative to that of hsc 70 protein in UROtsa cells exposed to the highest level of NaAsO_2 and equal to hsc 70 at the intermediate level of exposure.

The light-level morphology of the UROtsa cells was also monitored over time at the highest level of exposure to NaAsO_2 . As described previously, the UROtsa cells grown

on serum-free growth medium appear on light microscopic examination as a cell monolayer with raised areas of cells in three-dimensional structures (Rossi et al. 2001). A combination of routine ultrastructural examination and freeze-fracture analysis was used to show that the overall morphology of the UROtsa cells was most consistent with that found in the intermediate layers of *in situ* urothelium. The light-level morphology of the confluent UROtsa cells not exposed to NaAsO_2 was constant over the 16-day time course (Figure 8A, day 1; Figure 8B, day 16). For the cells exposed to 8 μM NaAsO_2 , the earliest sign of toxicity occurred on day 3, when monolayer cells adjacent to the three-dimensional raised areas underwent a limited loss of cell density (Figure 8C, arrow). This was followed on day 4 by a more pronounced loss of monolayer density near the raised areas and detachment of cells from the growth surface (Figure 8D, arrow). By day 7, there was appreciable cell death over all areas of the culture, and cells were actively detaching from the growth surface (Figure 8E, arrow). At day 16 of exposure, there was a general destruction of the monolayer and raised areas, and no population of UROtsa cells appeared resistant to the effects of NaAsO_2 (Figure 8F).

Discussion

The stress response is one mechanism that the bladder urothelium could potentially employ to protect itself from cellular damage after exposure to arsenic and, in so doing, influence the shape of the dose–response curve at low-level exposures to this environmental pollutant. The most obvious feature of the stress response (or heat shock response, as originally named because of its discovery after heat treatment) consists of acute alterations in cellular gene expression defined by a cessation of normally occurring protein synthesis and induction of the synthesis of heat shock proteins after exposure of cells to stress (Bernstam and Nriagu 2000; Craig et al. 1994; Georgopoulos and Welch 1993; Macario 1995; Schlesinger 1994). When the stress passes, the heat shock response is down-regulated, normal protein synthesis is reestablished, and the cell recovers from the stressful event. Alternatively, if the level of stress is too great to be attenuated by the stress response, cell death can ensue by necrosis and/or apoptosis. Because of these general attributes, it has been assumed that the stress-response proteins can protect and allow recovery of cells from low levels of toxicant exposure but are ineffective at higher levels of exposure that overwhelm the stress-response mechanism.

The heat shock proteins are a large superfamily of proteins with molecular weights ranging from 8 to 170 kDa, with the members referred to as hsp 27, hsp 60, hsc 70 (constitutive form), hsp 70 (inducible form),

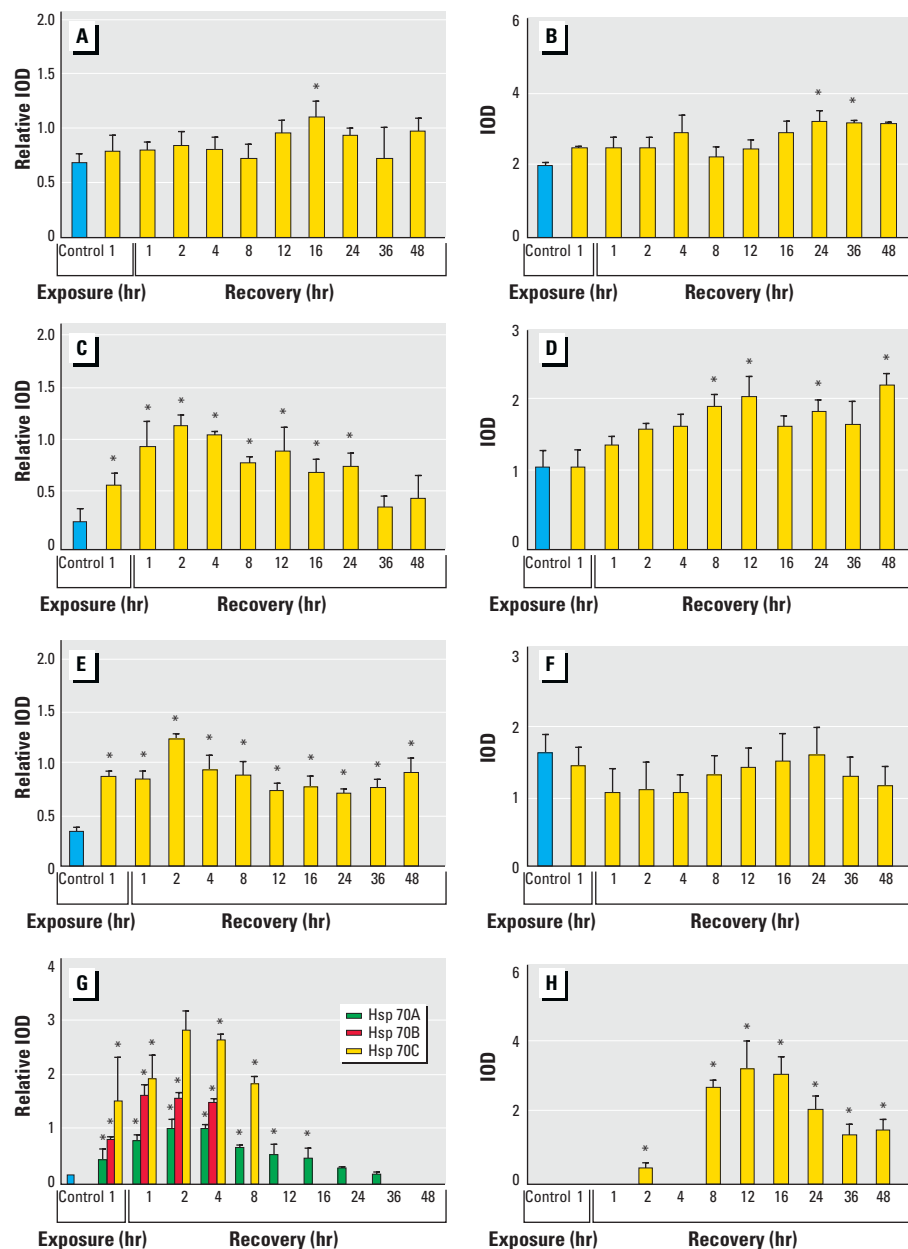


Figure 6. Relative expression of mRNA (mean \pm SEM) compared with the *g3pdh* housekeeping gene and expression of protein determined by Western analysis and expressed as the mean IOD values (\pm SEM) from three individual blots for each time point for UROtsa cells after heat shock and recovery. (A) Hsp 27 mRNA. (B) Hsp 27 protein. (C) Hsp 60 mRNA. (D) Hsp 60 protein. (E) Hsc 70 mRNA. (F) Hsc 70 protein. (G) Hsp 70A, B, and C mRNA. (H) Hsp 70 protein.

*Significantly different compared with control at a level of 0.05.

and hsp 90 being the proteins classically identified to be induced as a result of heat treatment of mammalian cells (Macario 1995). Although widely studied in cell culture lines and other organ systems, there have been very few studies that assess the expression of the stress proteins in the human bladder. To begin to address this deficiency, we defined the expression of hsp 27, hsp 60, hsc 70, and hsp 70 in the human urinary bladder through localization of expression using immunostaining and gene expression analysis using a combination of RT-PCR and Western analysis (Somji et al. 1998, 1999b, 1999c). This technology also allowed us to demonstrate that the

basal expression patterns of hsp 27, hsp 60, hsc 70, and hsp 70 in the UROtsa cell line are very similar to those of human urothelium (Rossi et al. 2001). Having defined a similarity between basal expression *in vivo* and *in vitro*, we used the UROtsa cell line in the present study to determine if the induction of the stress response by NaAsO₂ and heat would be similar to that expected from the large literature base that exists for these proteins.

When the UROtsa cells were exposed acutely to either heat or NaAsO₂, there was no induction in the levels of hsp 27 mRNA or protein, or any alteration in the steady-state phosphorylation status of the protein. This is

in contrast to what would be expected from the literature, where induction of hsp 27 mRNA and protein is commonly documented after acute treatment with either agent (Ciocca et al. 1993; Clark and Muchowski 2000). This lack of induction of hsp 27 by the UROtsa cells was not due to a laboratory-based problem in experimental design because other studies in the laboratory have shown hsp 27 to be induced by both heat and NaAsO₂ under identical conditions. Using mortal human proximal tubule (HPT) cell cultures as a model, both hsp 27 mRNA and protein were shown to be readily induced by heat shock, NaAsO₂, or CdCl₂ (Somji et al. 1999a). The inductions were rapid after treatment with the stimulus and extended well into the recovery period. Maximal inductions of 6–10-fold for hsp 27 mRNA and 4–7-fold for the hsp 27 protein were obtained depending on the stimulus (Somji et al. 1999a). Similarly, HK-2 cells, an immortal cell line derived from HPT cells by transfection with the human papillomavirus *E₆E₇* genes, were also shown to increase hsp 27 mRNA and protein after acute exposure to heat shock, NaAsO₂, or CdCl₂ (Kim et al. 2001). In these studies, the induction of hsp 27 in the immortalized cells was less than in the mortal cells, but induction of hsp 27 did occur after all three treatments. That hsp 27 is inducible in the *in situ* proximal tubule has also been shown in several studies where hsp 27 levels in the rat kidney were monitored during and after recovery from renal ischemia (Aufrecht et al. 1998; Schober et al. 1997, 1998).

In the previous studies comparing mortal and immortalized proximal tubule cells, the decreased induction of hsp 27 in the immortalized HK-2 cells was potentially explained by the fact that the HK-2 cells had very high levels of basal hsp 27 compared with the mortal cell line (Kim et al. 2001). The argument could be made that hsp 27 was already “partially induced” in the immortal HK-2 cells and that treatment with NaAsO₂ only completed the process of maximal induction. Such an argument could also be advanced for the UROtsa cells and the bladder urothelium because both have been shown to have high basal levels of hsp 27 expression. In the normal bladder, the immunoreactivity of hsp 27 was shown to be intense in the urothelium compared with many of the other cell types of the human bladder (Somji et al. 1998). Also, hsp 27 mRNA was shown to be highly expressed in preparations from the normal human bladder that contained, in addition to the RNA from the urothelium, RNA from other sections of the bladder that had low levels of hsp 27 immunoreactivity. In the UROtsa cell line, hsp 27 mRNA was highly expressed, with mRNA being detected 10–15 cycles earlier in the PCR than were the *g3pdh*

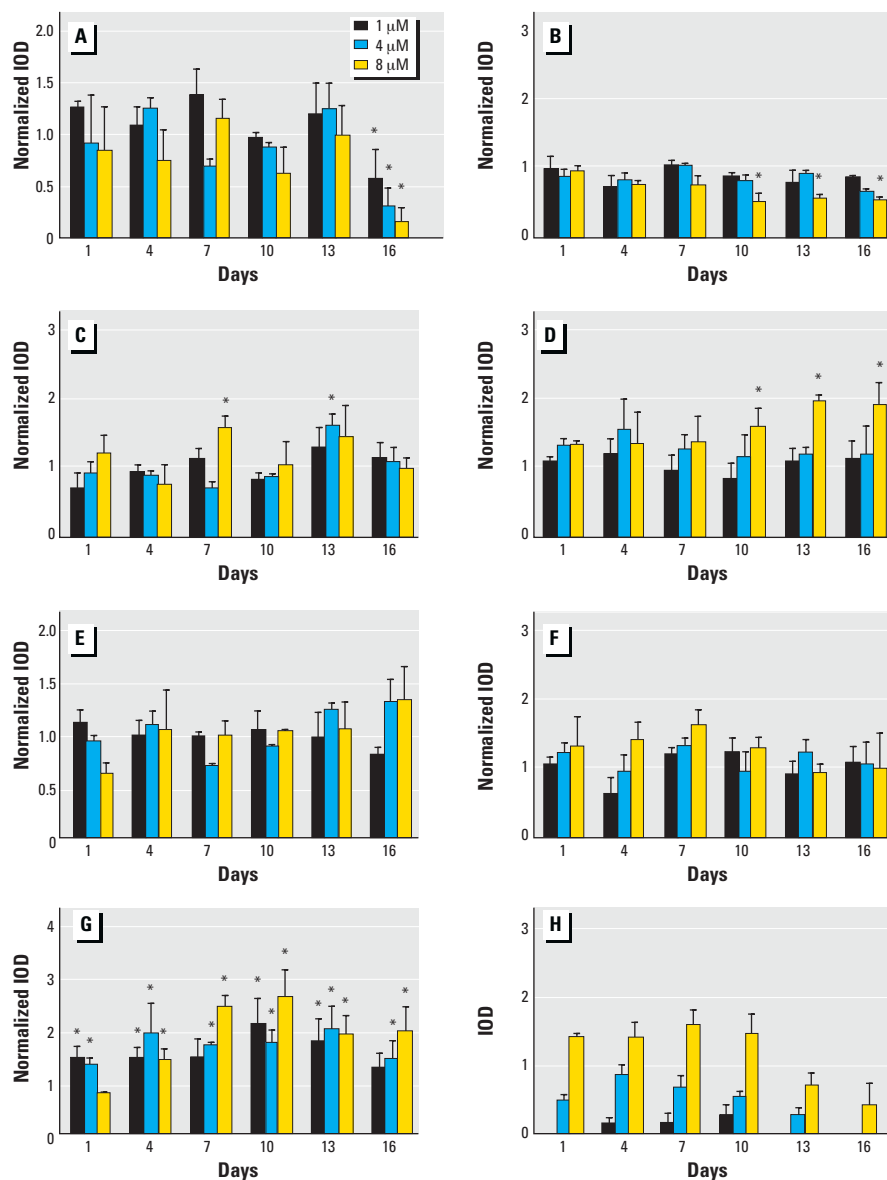


Figure 7. Relative expression of mRNA (mean \pm SEM) compared with the *g3pdh* housekeeping gene and expression of protein determined by Western analysis and expressed as normalized IOD (the mean \pm SEM IOD value from three independent blots normalized to that of the control at each time point) for UROtsa cells exposed to 1.0, 4.0, and 8.0 μ M NaAsO₂ for 16 days. (A) Hsp 27 mRNA. (B) Hsp 27 protein. (C) Hsp 60 mRNA. (D) Hsp 60 protein. (E) Hsc 70 mRNA. (F) Hsc 70 protein. (G) Hsp 70A mRNA. (H) Hsp 70 protein.

*Significantly different compared with control at a level of 0.05.

housekeeping gene (Rossi et al. 2001). Hsp 27 protein was also detectable on Western blots at lower inputs of total protein than were used in similar analyses of other cell types. However, there is only limited evidence to support the concept that the basal level of stress gene expression influences the ability of the *hsp 27*, or other heat shock genes, to be induced by a given stimulus.

The finding that hsp 60 mRNA and protein were modestly induced after acute exposure of the UROtsa cells to either heat or NaAsO₂ provides indirect support for the concept that the basal level of expression can influence the level of gene induction in these cells. Compared with hsp 27, the basal level of hsp 60 expression in the *in situ* human bladder was low in all the cell types comprising the bladder (Somji et al. 1999c). The urothelium, which had the highest expression of all cell types, showed only weak to moderate staining for hsp 60, and total RNA from the bladder showed expression of hsp 60 mRNA well below that of the *g3pdh* housekeeping gene. A similar pattern of hsp 60 basal expression was also shown for the UROtsa cell line, with hsp 60 mRNA expression being below that of the housekeeping gene (Rossi et al. 2001). Although the literature detailing hsp 60 induction as a result of physical and chemical stress is not as extensive as for some other stress proteins, the available literature supports the current findings that hsp 60 generally responds to stressful stimuli by an induction of gene expression (Bukau and Horwich 1998). Furthermore, parallel studies by this laboratory using HPT cells have shown hsp 60 to be readily induced by both heat and NaAsO₂ under identical conditions used for the UROtsa cells (Somji et al. 2000).

The expression of hsp 70 mRNA and protein was markedly induced after acute exposure of the UROtsa cells to heat or NaAsO₂ exactly as predicted from the literature (Bukau and Horwich 1998; Craig et al. 1994; Georgopoulos and Welch 1993; Macario 1995; Schlesinger 1994). In the UROtsa cells, the hsp 70 protein was undetectable both in control cells and during the initial 4 hr of exposure to NaAsO₂, but by 4 hr into the recovery period hsp 70 protein was prominent in the treated cells and reached a maximum expression between 8 and 16 hr after removal of NaAsO₂.

This response was similar to that previously noted by this laboratory when HPT cells were exposed to NaAsO₂ under similar conditions (Somji et al. 1999d). The hsp 70 protein is encoded by several genes, three of which (*hsp 70A*, *hsp 70B*, and *hsp 70C*) have been sequenced and identified, and these can be determined using RT-PCR technology (Kim et al. 2001; Somji et al. 1999d). The expression of mRNA for all three of these genes was markedly elevated in the UROtsa cells after exposure to 100 μM NaAsO₂, and the expression patterns were similar to those found previously in the identical assessment of HPT cells. Only mRNA for the *hsp 70A* gene could be detected in control cells, and assuming equal rates of translation into protein, this gene would likely be responsible for the majority of hsp 70 protein found in the UROtsa cells. The lack of basal expression of hsp 70 in the UROtsa cells was also in general agreement with that found for the *in situ* human bladder,

where a low level of hsp 70 expression, localized to the nucleus, was noted in cells of the basal layer of the urothelium but not in the more stratified, differentiated layers of urothelial cells (Somji et al. 1999b). The finding that hsc 70 expression was unaffected by acute exposure to heat or NaAsO₂ in the UROtsa cells was the response expected from the literature. Hsc 70 is the constitutive member of the *hsp 70* gene family and is normally not induced by stressful stimuli. Assuming that the findings in cell culture predict the response of human bladder urothelium *in vivo*, these observations suggest that the *hsp 27* gene in the human bladder urothelium is not induced by acute exposure to arsenite, whereas, the *hsp 60*, *hsc 70*, and *hsp 70* genes respond in the classical manner defined by the literature.

The majority of studies on the stress response employ only an acute time course of exposure to the stressful stimuli. Only a few studies have examined the expression of hsp 27, hsp 60, hsc 70, and hsp 70 when cells are continually exposed to stressful stimuli over an extended period. In previous studies by this laboratory using HPT cells, an extended time course was modeled by continually exposing the cells to lethal and sublethal concentrations of CdCl₂ for 16 days (Somji et al. 1999a, 1999d, 2000). After the expected increases during and after the acute period of exposure, the mRNAs and proteins for all four heat shock proteins had returned to, or remained at (hsc 70), control values within 48 hr of initial exposure and remained at or below control levels for the remaining 16 days of the time course. During this 16-day period, fresh growth medium containing the lethal and sublethal concentrations of CdCl₂ was provided to the cells every 3 days. Thus, in the HPT cell culture model, hsp 27, hsp 60, and hsp 70 all returned to control values after an acute response to exposure and remained at these control levels, even in the continued presence of Cd²⁺ concentrations that elicit cell lethality later in the time course.

The expression of hsp 27, hsp 60, and hsc 70 in the UROtsa cells exposed to lethal and sublethal levels of NaAsO₂ for 16 days was similar to that of the HPT cells. The expression of hsp 27 was not increased in the UROtsa cells continuously exposed to lethal and sublethal concentrations of NaAsO₂ for 16 days. Hsp 27 protein was reduced in the UROtsa

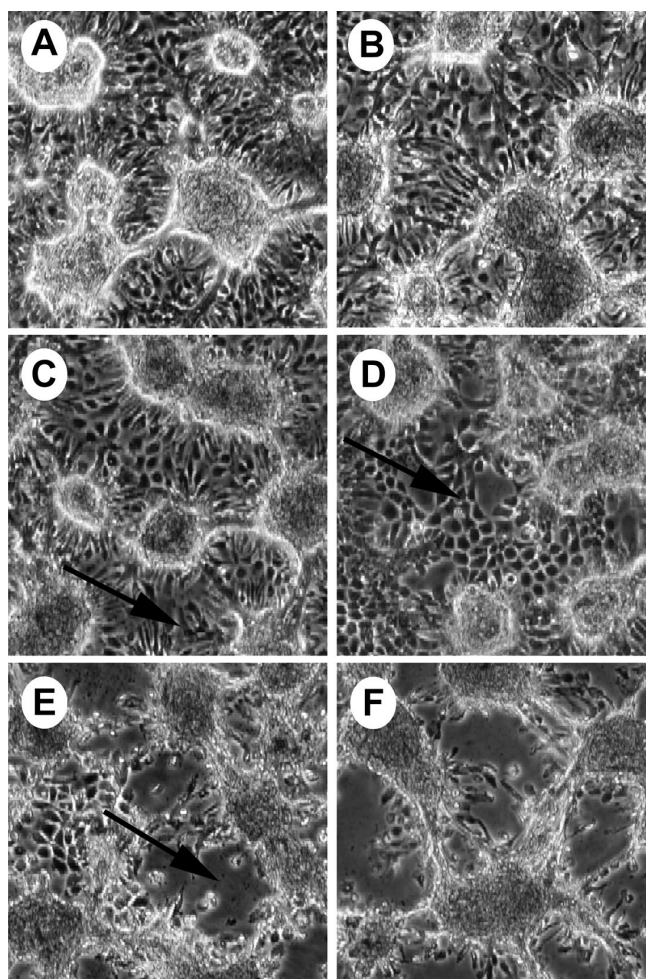


Figure 8. Phase-contrast photomicrographs of confluent UROtsa cells. Control (untreated) UROtsa cells at (A) 1 day and (B) 16 days. UROtsa cells exposed to 8.0 μM NaAsO₂ for (C) 3 days (arrow indicates areas of initial toxicity-induced change); (D) 4 days (arrow indicates a general thinning of the monolayer); (E) 7 days (arrow indicates the appearance of open areas); (F) 16 days (shows a general destruction of the monolayer and three-dimensional areas). Magnification: 100×.

cells exposed to lethal levels of NaAsO₂; however, these reductions occurred only at late points of the time course that were associated with reductions in cell viability. The expression of hsp 60 was also not increased in the UROtsa cells continuously exposed to sublethal concentrations of NaAsO₂ for 16 days. There was a small, but significant, increase in hsp 60 protein in the UROtsa cells exposed to lethal levels of NaAsO₂, but these increases were also associated with reductions in cell viability. The expression of hsc 70 by the UROtsa cells was not influenced by extended exposure to NaAsO₂.

In contrast, the expression of hsp 70 was markedly increased in UROtsa cells exposed to NaAsO₂ for 16 days. The most interesting feature of this increase was that it correlated with the cytotoxicity of NaAsO₂ exposure. When the UROtsa cells were exposed to 1.0 μM NaAsO₂ for 16 days, a level of exposure that produced no loss of cell viability, there was only a very modest increase in the hsp 70 protein. Under these conditions, there was a small proportionate increase in hsp 70 protein over the initial 10 days of exposure and then a return to undetectable control levels for the remainder of the time course. When the UROtsa cells were exposed to 4.0 μM NaAsO₂ for 16 days, a level that also produced no loss of cell viability, there was a proportionately larger increase in hsp 70 protein level. The largest increase occurred early in the time course, when hsp 70 levels were 5–10-fold higher compared with the 1.0 μM dose. The increase also extended longer into the time course, but hsp 70 protein did return to an undetectable level by the end of the time course. When the UROtsa cells were exposed to 8.0 μM NaAsO₂, a level that produced a significant loss of cell viability by day 7 of the time course, there was a further large increase in the level of hsp 70 protein. The increase in hsp 70 was maximal on the first day of the time course and remained at this highly elevated level for 10 days before decreasing over the last 6 days of the time course. One interpretation of this pattern of expression would be that hsp 70 is increased during periods of NaAsO₂-induced cellular damage, and if damage can be attenuated, hsp 70 returns to control values. If damage is not attenuated, hsp 70 remains at elevated levels preceding and during the period of NaAsO₂-induced cytotoxicity.

The present study, combined with past studies on the human bladder and the derived UROtsa cell line (Rossi et al. 2001; Somji et al. 1998, 1999b, 1999c), provides an overview of four components of the heat shock/stress response of the human bladder urothelium. It was demonstrated that hsp 27 has a high level

of basal expression in the urothelium of both the *in situ* human bladder and the UROtsa-derived cell line. It was also shown using the cell culture model that hsp 27 expression was not induced on exposure to the classic physical and chemical inducers of the stress response, heat and NaAsO₂. For hsp 60, a moderate level of basal expression was demonstrated in both the *in situ* urothelium and the UROtsa-derived cell line, and acute (but not extended) exposure to NaAsO₂ was a moderate inducer of both hsp 60 mRNA and protein for the UROtsa cells. The hsc 70 family member was shown to have a high level of basal expression in the *in situ* urothelium and a high to moderate expression in the UROtsa cell line. As would be expected for the constitutive hsc 70 family member, neither heat nor NaAsO₂ induced the expression of this protein. The hsp 70 protein was not found in either *in situ* urothelium or the UROtsa cell line under basal conditions. However, its expression undergoes a rapid induction after acute treatment of the UROtsa cells with either heat or NaAsO₂. The hsp 70 protein is also induced over an extended time course of exposure to NaAsO₂, with the degree and duration of induction correlating directly with cell cytotoxicity. The substantial level of basal expression of hsp 27 and hsc 70, coupled with the inducible expression of hsp 60 and hsp 70, could provide the human urothelium with a mechanism to withstand and recover from a low level of arsenite exposure.

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