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# Heat shock protein 70 as an indicator of early lung injury caused by exposure to arsenic

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## Abstract

Heat shock proteins (HSPs) are a family of highly conserved proteins that are induced by a number of stresses including toxic metals. Heat shock proteins expression has been reported to be an early and sensitive biomarker of cell stress. Arsenic is a naturally occurring metal that exists widely in the environment and is used in several industries. Exposure to arsenic is associated with the development of pulmonary cancers. We monitored changes in Hsp70 and markers of oxidative injury induced by arsenic in human pulmonary epithelial cells (BEAS-2B). Hsp70 protein, mRNA and reactive oxygen species (ROS) generation were measured after exposing cells to arsenic as markers of injury. Hsp70 protein expression showed significant 7.9-fold and 31.5-fold increase using Western blotting and ELISA assay, respectively, at a 50 $\mu$M As(III) with a 12 h exposure and an 12 h recovery time. Hsp70A and Hsp70B mRNA expression showed a two-fold increase and Hsp70C mRNA expression showed a six-fold increase. As(III)-induced Hsp70 protein expression was inhibited significantly by catalase and NAC, indicating mediation of ROS in Hsp70 expression. Intracellular glutathione (GSH) was significantly depleted by As(III) exposure. Lipid peroxidation by-product, 8-isoprostane, was increased six-fold at 24 h exposure to 20 $\mu$M As(III). Electron spin resonance and confocal microscope studies also showed As(III)-stimulated ROS generation. These results suggest that cellular injury by arsenic is mediated through ROS generation resulting in the expression of Hsp70. It is possible that Hsp70 may prove to be a sensitive biomarker for arsenic exposure with other markers of oxidative stress in human serum. (Mol Cell Biochem **277:** 153–164, 2005)

*Key words*: arsenic, biomarker, Hsp70, lung injury, reactive oxygen species, oxidative stress

## Introduction

Arsenic is a naturally occurring element widely distributed in the soil, water and food [1–5]. The International Agency for Research on Cancer (IARC) and other investigators have concluded from available epidemiologic and experimental data that arsenic is a human carcinogen [1–5]. Arsenic exposure causes many diseases, such as, cancer of the lung, skin and bladder, and cardiac abnormalities [1–9]. Increased incidence of urinary bladder, kidney, and liver cancer have also been reported in populations consuming arsenic-contaminated drinking water in Argentina, Chile, India, Japan, and Taiwan [10–18]. The National Institute for Occupational Safety and Health (NIOSH) estimated that approximately 1.5 million industrial workers are potentially exposed to arsenic in different manufacturing and processing occupations [16]. Occupational exposure to arsenic occurs in smelting of ores for cobalt, gold, lead, nickel, zinc and other metals. Exposure to gallium arsenide in microelectronic industry, wood preserving industry, pesticide industry, and agriculture are other major sources of occupational exposure. Anthropogenic emissions of arsenic on a global basis are estimated to be 23,600 tons per year [4]. Chronic exposure to high levels of arsenic mainly through food and water has been shown to be associated with increased incidence of skin and urinary cancers. Occupational and environmental exposure to arsenic

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also causes characteristic skin cancers and pulmonary cancers in exposed population [3].

Arsenic exists in many forms. Inorganic trivalent and pentavalent forms are the most important types causing human diseases [16, 19]. The molecular mechanisms of arsenic toxicity leading to carcinogenesis are not yet fully understood. Current evidence indicates that arsenic-induced ROS act on signaling pathways, promoting cell proliferation rather than causing direct DNA damage [20–22]. ROS-induced by toxic metals are capable of causing an oxidative stress that can damage or modulate a variety of cellular macromolecules and mechanisms [23, 24]. Arsenic-induced ROS generated during cellular reactions include hydrogen peroxide, superoxide anion, singlet oxygen, and hydroxyl radical, which in turn activate or inactivate cellular molecular mechanisms involved in protecting the cells from death, which might lead to carcinogenesis [25–29].

Heat shock proteins (HSP) are highly conserved stress-response proteins which play a central role in cellular repair and adaptation to stress [30, 31]. It was shown that ROS are powerful inducers of HSPs [32, 33]. These proteins play a critical role in maintenance of cells in a normal homeostatic state and the recovery of cells from adverse injury [30]. Heat shock protein 70 (Hsp70) is an important member of stress-induced proteins, whose expression is upregulated when the cell or organism is placed under stressful conditions [34]. The role of Hsp70 has been studied in a variety of clinically relevant animal models or conditions, such as hyperthermia, hypoxia, exposure to toxic chemicals, inflammation, apoptosis, and cancer. In human epithelial cells, inducible Hsp70 is expressed under basal conditions and is upregulated in response to stresses, such as toxic agents, metals, heat, and cold [35]. Expression of Hsp70 has been proposed as a potential marker for the presence of deleterious agents in soils [36], seaweed [37], mussels, and fish tissue [38]. Also, Hsp70 has been proposed to be a sensitive indicator of cadmium- and nickel-induced metal toxicity in the cultured human cells [39].

The aim of this investigation was to analyze the mechanisms involved in Hsp70 induction by arsenic, using human bronchial epithelial cells. Therefore, in this study, the relationship of arsenic exposure and the levels of Hsp70 expression were investigated in correlation with ROS generated and other markers of cell injury caused by ROS. Also an attempt was made to determine whether arsenic-induced molecular and cellular mechanisms correlate with corresponding changes in the secretion of Hsp70.

## Materials and methods

*Reagents*

Arsenic(III) was purchased from Aldrich (Milwaukee, WI), and catalase, superoxide dismutase (SOD), sodium formate (SF), NADPH, and *N*-acetyl-cysteine (NAC) were obtained from Sigma (St. Louis, MO). DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide) was purchased from Alexis Biochemicals (San Diego, CA). $H_2$DCFDA and dihydroethidium were purchased from Molecular Probes (Eugene, OR).

*Cell culture*

BEAS-2B cells (ATCC, Rockville, MD) were grown to 95% confluence at 37 °C in a 5% $CO_2$ atmosphere using Dulbecco's modified Eagles' growth medium (DMEM) supplemented with 5% FBS and penicillin (100 units/ml)–streptomycin (100 $\mu$g/ml). Medium was changed every 2–3 days and cells were subcultured using trypsin-EDTA at confluence. Exposure of BEAS-2B cells to arsenic in culture was performed in the presence of 0.1% serum. After 6 h or 12 h arsenic exposure, the cells were placed in fresh medium containing 5% serum for various periods (0–12 h).

*Cytotoxicity and proliferation test*

The effects of arsenic treatments on the cytotoxicity of cells were determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay [40], according to the manufacturer's protocol (Roche, Indianapolis, IN).

*Western blotting*

Cells were washed three times with PBS and harvested by scraping. The cells were sedimented by centrifugation. The supernatant was aspirated, and cells resuspended in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM $Na_2$EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM $\beta$-glycerophosphate, 1 mM $Na_3VO_4$, and 1 $\mu$g/ml leupeptin) at pH 7.5, containing protease inhibitors (Roche, Indianapolis, IN). After a brief sonication, lysed cells were centrifuged at 12,000 g for 10 min at 4 °C, and supernatants were transferred to new tubes and stored at −70 °C. The concentration of protein in the samples was determined by the DC protein assay (Bio-Rad, Hercules, CA). Hsp70 protein was separated from 30 $\mu$g aliquots of samples using 10% SDS-PAGE and then transferred onto 0.2 $\mu$m nitrocellulose membranes (Bio-Rad, Hercules, CA). The membrane blots were incubated with a blocking buffer (non-fat milk 5% (w/v) in Tris buffered saline and 0.1% Tween 20) for 1 h, and then incubated with AP conjugated mouse monoclonal antibodies specific for Hsp70 (StressGen, Victoria, BC, Canada) in the blocking buffer for 1 h at room temperature. Antibody was diluted 1:10,000 in the blocking buffer. After three washes in buffer, the blots were incubated with color developing reagent (Bio-Rad, Hercules, CA). To test the effect of antioxidants

and other reagents on As(III)-induced Hsp70 protein expression, BEAS-2B cells were exposed to 50 $\mu$M As(III) with or without antioxidants for 6 h followed by a 12 h recovery and harvested for Western blotting as described earlier.

*Hsp70 ELISA*

An enzyme-linked immunosorbent assay (StressGen, Victoria, BC, Canada) was used to determine the relative expression of Hsp70 protein in cell lysates. Cell lysates were prepared according to manufacturer's protocol, using extraction buffer containing protease inhibitors. One hundred microliters of total protein samples (0.025 $\mu$g of total proteins/$\mu$l) were added to the microassay plate. After the completion of the color developing reaction, the absorbance of samples was measured at 450 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The Hsp70 concentrations were calculated from the constructed standard curve.

*Isolation of total RNA and RT-PCR*

To determine the expression of Hsp70A, Hsp70B, and Hsp70C, 500 ng of total RNA was reverse transcribed with random hexamer primers. The samples were reverse transcribed for 15 min at 42 °C, followed by a 5 min denaturation step at 99 °C, using a DNA thermal cycler (Hybaid, Middlesex, UK). The resulting cDNA was amplified in 100 $\mu$l reaction by PCR (30 cycles) with commercially available oligonucleotides specific for Hsp70A, Hsp70B, or Hsp70C (StressGen, Victoria, BC, Canada). Hsp70 primers specific for Hsp A, Hsp B, or Hsp C were (forward and reverse, respectively): 5′TGTTCCGTTTCCAGCCCCCAA3′ and 5′GGGCTTGTCTCCGTCGTTGAT3′ (STM-506) for 70A; 5′CTCCAGCATCCGACAAGAAGC3′ and 5′ACGGTGTTGTGGGGGTTCAGG3′ (STM-507) for 70B; and 5′TTGAGGAGGTGGATTAGGGGC3′ and 5′AGCCTTTGTAG TGTTTTCGCC3′ (STM-508) for 70C. Primers for the determination of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) housekeeping gene expression were purchased commercially (Clontech, Palo Alto, CA). Polymerase chain reaction cycles consisted of 2 min initial step at 95 °C followed by 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C with a final elongation step for 15 min at 72 °C. The final PCR products were electrophoresed on 2% agarose gels containing ethidium bromide along with DNA markers. Optical densities of the samples were obtained by ethidium bromide fluorescent imaging into an ImageQuant 5.1 (Molecular Dynamics, Piscataway, NJ), using a Stratagene Eagle Eye II (La Jolla, CA). The amount of Hsp70 mRNA expressed was calculated by the ratio of the Hsp70 reaction product to that of the housekeeping gene, G3PDH [41].

*Intracellular glutathione assay*

Glutathione (GSH) assay kit (Cayman chemical, Ann Arbor, MI) was used for the quantification of cellular GSH. This method utilizes a carefully optimized enzymatic recycling using glutathione reductase. Briefly, cells were harvested by scraping and were sonicated briefly. After centrifugation (10,000 $\times$ $g$ for 15 min at 4 °C), the supernatant was deproteinated for assaying according to the manufacturer's protocol. Fifty microliters of samples were added to the microplate. After the completion of the color developing reaction, the absorbance of samples was measured at 405 nm using a microplate spectrophotometer. The GSH concentrations were calculated from the constructed standard curve.

*ESR measurements*

Electron spin resonance (ESR) was used to detect short-lived free radical intermediates. Short-lived free radical intermediates were spin trapped with DMPO present in the reaction medium. Electron spin resonance measurements were carried out using a Bruker EMX ESR spectrometer (Bruker Instruments Inc., Billerica, MA) and a flat-cell assembly [42, 43]. Hyperfine couplings were measured (0.1 Gauss) directly from magnetic field separation, using $K_3CrO_8$ and 1,1-diphenyl-2-picrylhydrazyl as standards. All the measurements were carried out at room temperature at 50 mW microwave power, 2 G modulation amplitude and a magnetic field of 3490 $\pm$ 50 G. Three scans were integrated for all the samples, and the scaling and analysis of the spectra were made using an Acquist program (Bruker Instruments Inc., Billerica, MA).

BEAS-2B cells ($1 \times 10^6$) were mixed with 200 mM DMPO, and 100 $\mu$M As(III) in a final volume of 1 ml. Reactants were incubated for 10 min at 37 °C and transferred to a flat cell for ESR measurements.

*Lipid peroxidation measurements*

A lipid peroxidation by-product, 8-isoprostane, was measured in the supernatant as a marker of oxidative injury to cells exposed to arsenic. The isoprostanes are produced by non-enzymatic random oxidation of tissue phospholipids by oxygen radicals. The method used for isoprostane assay is a competitive enzyme-linked assay for determining 8-isoprostane in biological samples, using an 8-isoprostane-acetylcholineserase (AChE) conjugate for a limited number of 8-isoprostane-specific rabbit antiserum sites. The ELISA method (Cayman, Ann Arbor, MI) was used, according to manufacturer's protocol, to determine time- and concentration-dependent lipid peroxidation induced by As(III). After exposure to arsenic, the media were analyzed

for 8-isoprostane in duplicate aliquots of 50 $\mu$l of samples. Samples were exposed for 18 h in plates coated with antibody and then treated with substrate for 1 h for color development. Absorbance of color developed was read at 410 nm, using a microplate spectrophotometer with standards and controls treated similarly. The concentration of 8-isoprostane produced was calculated from a standard curve.

### *Intracellular $H_2O_2$ and $^{\bullet}O_2^-$ detection by confocal microscopy*

Intracellular ROS production was tested using $H_2$DCFDA for $H_2O_2$ and dihydroethidium for $^{\bullet}O_2{}^-$, respectively [43, 44]. BEAS-2B cells ($1 \times 10^5$ per well) were seeded onto a glass coverslip in the wells of 24-well plate for 24 h. The cells were exposed to As(III) for 1 h in the presence of $H_2$DCFDA (25 $\mu$M) or dihydroethidium (10 $\mu$M). The cells were then washed with PBS, fixed with 10% buffered formalin for 10 min, and mounted on glass slides using Prolong Antifade (Molecular Probes, Eugene, OR). Slides were observed using a Zeiss LSM 510 (Carl Zeiss Inc., Thornwood, NY) laser scanning confocal microscope.

### *Total nitric oxide ($NO^{\bullet}$) generation assay*

Quantitative nitric oxide detection kit (Stressgen, Victoria, BC, Canada) was used to determine the total nitrogen oxides in tissue culture media. Most of the $NO^{\bullet}$ is oxidized to two stable breakdown products, nitrite ($NO_2^-$) and nitrate ($NO_3^-$). In this assay, the concentration of nitrate was used as a quantitative measure of NO production. BEAS-2B cells ($1 \times 10^6$ per well) were seeded in the wells of 6-well plate for 12 h. Cells were exposed to As(III) with concentrations of 0–50 $\mu$M for 12 h in tissue culture media. At the termination of exposure, the media (1:2 dilution with reaction buffer) were used for quantitative determination of $NO^{\bullet}$ according to manufacturer's protocol. Briefly, 50 $\mu$l of standards and samples were added to the microassay plate. Twenty-five microliters of NADH and nitrate reductase were added into all the wells, and then Griess Reagents I and II were added to the appropriate wells. The reaction was read at 560 nm, using a microplate spectrophotometer.

### *Statistical analysis*

Statistical analysis of data was undertaken, using the Sigma Stat (Jandel Scientific Software, San Rafael, CA) statistical program. Data presented are the means ± S.E. of values compared and analyzed using a one-way ANOVA. Student's *t*-test was used to assess the significance of differences between two groups and values of $p < 0.05$ were considered statistically significant.

## Results

### *Cytotoxicity of As(III) to BEAS-2B cells*

As(III) is believed to induce oxidative stress, cell injury and other molecular changes associated with apoptosis and cell proliferation. To determine the cytotoxic effects and to select optimal concentrations without causing significant cytotoxicity to As(III) in human bronchial epithelial cell line BEAS-2B, we performed a MTT assay with different concentrations of arsenic for different time periods (Fig. 1). Cytotoxicity resulting from As(III) increased in a dose- and time-dependent manner during 3-day exposure period. Exposure of cells to 50 $\mu$M concentrations of As(III) for 6 h decreased cell viability markedly. On 12 h exposure, the cytotoxicity was significant and apparent even at lower concentrations of 10 and 20 $\mu$M of As(III). On 24 h exposure, the survival rate declined to 70, 44 and 19% at 10, 20, and 50 $\mu$M concentrations of As(III), respectively. After 48 h exposure, the survival rate further decreased to 32, 17, and 5% in 10, 20, 50 $\mu$M concentrations of As(III), respectively, compared to control. On exposure to 72 h, almost all of the cells were killed in the presence of As(III) compared to control. The analysis of cytotoxicity data indicated a decrease in cell viability beginning at 6 h exposure to arsenic. Cytotoxicity continued to increase substantially at days 2 and 3. The rate of cell proliferation was also determined by optical microscopy using the conventional hemocytometer (data not shown). The results of these studies were very similar to that of MTT data correlating the

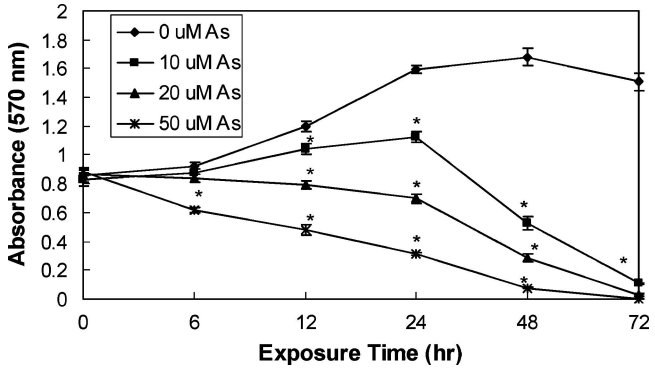


*Fig. 1.* Cytotoxicity of As(III) to BEAS-2B cells determined by the MTT assay. BEAS-2B cells were seeded in 96-well plates at $2 \times 10^4$ cells per well and incubated for 24 h. The medium was changed, and cells were exposed in triplicate to different As(III) (0–50 $\mu$M) concentrations for 3 days. The optical density was measured at 570 nm, and values presented represent the mean ± S.E. of five assay wells. Asterisk in superscript indicates a significant decrease of cell viability from control ($p < 0.05$).

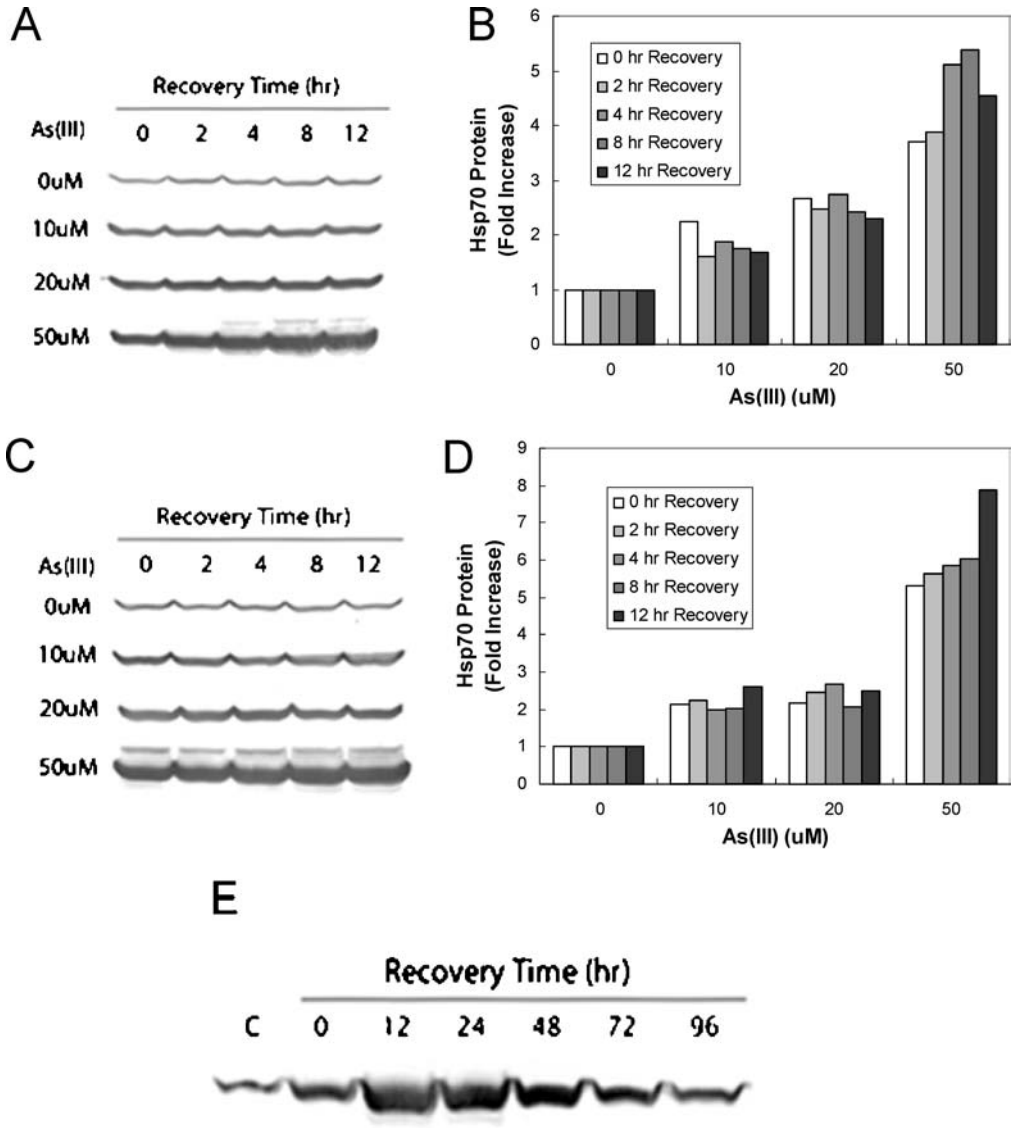


*Fig. 2.* Hsp70 protein detection by Western blotting during recovery periods. BEAS-2B cells were exposed to As(III) for two different times, 6 and 12 h, followed by recovery with fresh media for five different times periods (0–12 h) as indicated. Western blot of Hsp70 protein following As(III) exposure for 6 h and recovery periods (A) and densitometry graph (B). Western blot of Hsp70 protein following As(III) exposure for 12 h and recovery periods (C) and densitometry graph (D). (E) Cells were exposed to As(III) (50 $\mu$M) for 6 h, followed by 0–96 h recovery periods. Cells were harvested and 30 $\mu$g of total protein samples separated by 10% SDS-PAGE were transferred onto 0.2 $\mu$m nitrocellulose membranes. Western blotting was performed with the anti-Hsp70 monoclonal antibody.

functional status of the cells in response to arsenic toxicity. Therefore, this dose- and time-dependent cytotoxicity was taken as optimal in consideration in all further studies.

*Expression of Hsp70 protein was increased in a dose-dependent manner by As(III) exposure*

The expression of Hsp70 protein was determined after exposing BEAS-2B cells to As(III) for 6 and 12 h, followed by recovery periods (0–12 h). Western blot analysis showed a significant increase in Hsp70 protein with all the As(III) concentrations used (Fig. 2). Hsp70 protein increased approximately 1.7-, 2.3- and 4.5-fold at 10, 20, and 50 $\mu$M As(III) concentrations, respectively, following a 6 h exposure and 12 h recovery period (Fig. 2A and B). Protein expression also increased approximately 2.6-, 2.5-, and 7.9-fold at 10, 20, and 50 $\mu$M concentrations of As(III), respectively, following a 12 h exposure and 12 h recovery period (Fig. 2C and D). The maximum accumulation of Hsp70 was observed

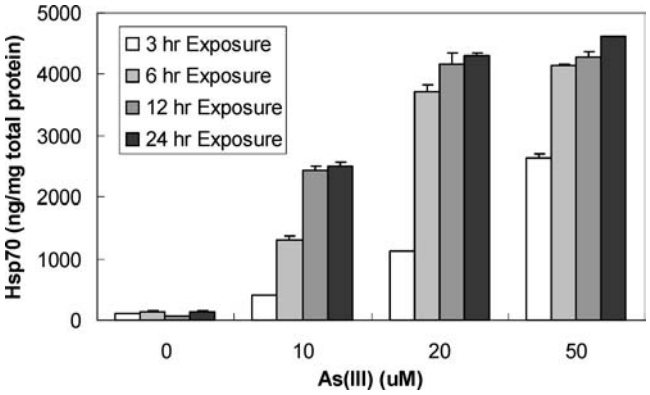


*Fig. 3.* Hsp70 protein detection by ELISA. BEAS-2B cells were exposed to As(III) for four different times, 3, 6, 12, and 24 h, followed by recovery with fresh media for 12 h. Cells were harvested and the total protein samples (0.025 $\mu$g/$\mu$l) were tested by Hsp70 ELISA kit. The absorbance of samples was measured at 450 nm, using a microplate spectrophotometer. The values of Hsp70 concentrations (ng/mg total protein) of each sample presented represent the mean ± S.E. of six assay wells from two independent experiments.

at 50 $\mu$M As(III) exposure. The overall Hsp70 protein expression level was higher in the 12 h As(III) exposed cells. To determine whether the Hsp70 protein continued to accumulate over longer recovery periods, cells were recovered for 0–96 h after 50 $\mu$M As(III) exposure for 6 h (Fig. 2E). The maximum accumulation was achieved at 12 and 24 h recovery periods (over 4.5-fold compared to control) followed by decrease but the accumulation was still higher in the 96 h recovery time compared to control. The Hsp70 ELISA assay was also performed to quantify the expression of Hsp70 and to verify a similar dose response with As(III) exposure (Fig. 3). Hsp70 protein expression increased approximately 3.4-, 9.2-, and 22.7-fold at 10, 20, and 50 $\mu$M As(III) concentrations, respectively, in the 3 h exposure and 12 h recovery period. In the 6 h exposure and 12 h recovery time, the expression increased 9.4-, 26.6-, and 29.6-fold at 10, 20, and 50 $\mu$M As(III) concentrations, respectively. In the 12 h exposure and 12 h recovery time, the Hsp70 protein expression further increased 17.9-, 30.7-, and 31.5-fold at 10, 20, and 50 $\mu$M As(III) concentrations, respectively. In the 24 h exposure and 12 h recovery time, the protein expression continue to increase moderately and was 18.7-, 32.1-, and 34.5-fold at 10, 20, and 50 $\mu$M As(III) concentrations, respectively (Fig. 3).

The protein expression of Hsp70 increased in a dose-dependent manner in the cells exposed to As(III). However, there was no dose-dependent effect at higher concentrations of As(III) in the 6, 12, and 24 h exposure. Time-dependent Hsp70 protein expressions were also observed in 3–12 h exposure at 10 $\mu$M As(III) concentration and in 3–6 h exposure at 20 and 50 $\mu$M As(III) concentrations. Dose- and time-dependent Hsp70 expression was not observed at higher concentrations of As(III) (20–50 $\mu$M) and in the longer exposure time periods (12–24 h). This is probably because maximum amount of Hsp70 protein was already synthesized at a certain point and prolonged exposure to As(III) caused cell toxicity and impaired synthesis.

### *Hsp70 mRNA expression in BEAS-2B cells exposed to As(III)*

The Hsp70 family of proteins is encoded by several genes, including Hsp70A, Hsp70B, and Hsp70C genes [45]. To investigate the effects of As(III) exposure on the expression of these three Hsp mRNAs in BEAS-2B cell, we analyzed the expression of mRNA of the three isoforms after a 6 h exposure and a recovery period of 0–12 h. The results for the expression of mRNA generated for the Hsp70A, Hsp70B, and Hsp70C genes are presented from a typical agarose gel experiment (Fig. 4A). Graphic illustration of the mean values from three independent experiments on the expression of these Hsp genes, compared to mRNA from the G3PDH housekeeping gene, is presented in Fig. 4B–D. The analysis showed that message for each Hsp70 gene had a differential expression pattern when the cells were exposed to As(III). Message for the Hsp70A isoform was highly expressed in all As(III) concentrations tested. The Hsp70A mRNA expression was increased approximately 2.7-, 2.2-, and 1.9-fold at 10, 20, and 50 $\mu$M As(III) concentrations, respectively, in the 0 h recovery period (Fig. 4B). During the recovery periods of 4–12 h, the Hsp70A mRNA expression decreased toward the control level. Hsp70B mRNA expression increased approximately 2.4-fold at 10 $\mu$M concentration of As(III) and did not increase further at a higher 50 $\mu$M concentration of As(III) (Fig. 4C). At 4–12 h of recovery, the Hsp70B mRNA expression returned to the control level except in cells treated with the 50 $\mu$M concentration of As(III). The expression pattern of Hsp70C mRNA was similar to that of Hsp70A mRNA. The Hsp70C mRNA expression increased approximately 4.8-, 5.2-, and 6.4-fold at 10, 20, and 50 $\mu$M As(III) concentrations, respectively, after a 0 h recovery period (Fig. 4D). Hsp70C mRNA returned to the control level by 8 h post exposure at 10, and 20 $\mu$M As(III) but remained elevated during the recovery period after a 50 $\mu$M As(III) treatment. From these results it is apparent that the expression of mRNA of Hsp70A, Hsp70B, and Hsp70C isoforms are increased rapidly in response to As(III) exposure at zero recovery period.

### *Effect of antioxidants and other reagents on As(III)-induced Hsp70 protein expression by Western blotting*

We hypothesized that As(III)-induced Hsp70 expression is mediated through ROS and that antioxidants may suppress

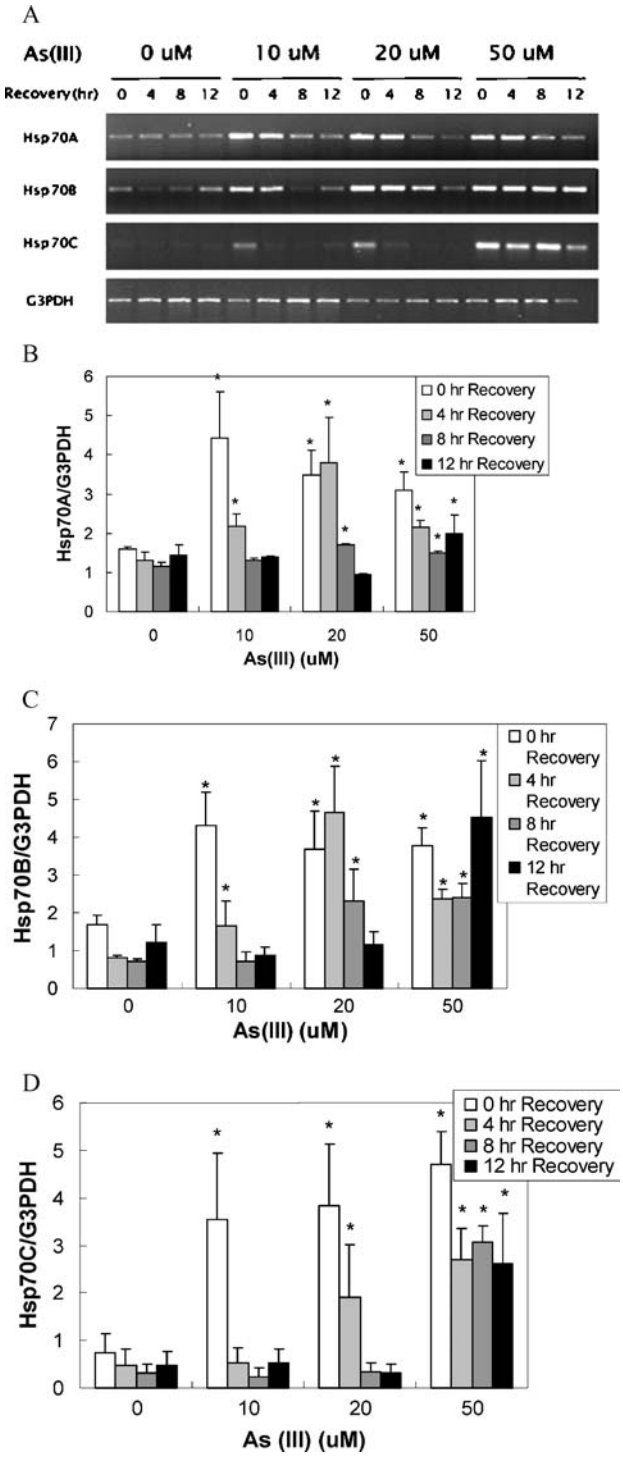


*Fig. 4.* RT-PCR analysis of mRNA from BEAS-2B cells exposed to As(III). BEAS-2B cells were exposed to 0–50 $\mu$M As(III) for 6 h, followed by a 0–12 h recovery period. (A) A representative agarose gel picture from one of the experiments used for data analysis. The values of Hsp70A (B), Hsp70B (C), and Hsp70C (D) mRNA expression presented represent mean ± S.E. of relative integrated optical density of bands divided by that for the housekeeping gene, G3PDH, from three independent experiments. Asterisk in superscript indicates a significant increase from control ($p < 0.05$).


*Fig. 5.* Effect of antioxidants and other reagents on As(III)-induced Hsp70 protein expression by Western blotting. BEAS-2B cells were exposed to 50 $\mu$M As(III) with or without antioxidants for 6 h. Western blotting was performed as those described in the legend to Fig. 3. The concentrations of the antioxidants and other reagents used were: catalase, 2000 units/ml; superoxide dismutase (SOD), 2000 units/ml; *N*-acetylcysteine (NAC), 10 mM; sodium formate (SF), 50 mM.

the expression of Hsp. To test this hypothesis, the effects of antioxidants and other reagents on As(III)-induced Hsp70 expression were investigated. BEAS-2B cells were exposed to 50 $\mu$M As(III) along with antioxidants for 6 h and the expression of Hsp70 was tested. The concentrations of the antioxidants and other reagents used were: catalase, 2000 units/ml; SOD, 2000 units/ml; *N*-acetylcysteine (NAC), 10 mM; SF, 50 mM. The effects of antioxidants and other reagents on As(III)-induced Hsp70 expression are shown in Fig. 5. Catalase, a $H_2O_2$-scavenging enzyme, inhibited As(III)-induced Hsp70 protein expression by 40%. SOD, a $^{\bullet}O_2^-$ radical scavenger that generates $H_2O_2$, and SF, a $^{\bullet}OH$ radical scavenger had some inhibitory effect approximately 28 and 34%, respectively, on the induction of Hsp70 protein. NAC, a thiol-containing general antioxidant, completely inhibited the induction of Hsp70 protein to the control level. These results suggests that ROS play a role in As(III)-induced Hsp70 protein expression.

### *Detection of intracellular GSH level in the cells exposed to As(III)*

Glutathione is a sulfhydryl antioxidant abundant in the cytosol of cells. Although synthesis and utilization of GSH are tightly controlled, exposure to toxic substances and oxidative stress are capable of depleting GSH from the cells and tissues [46]. Our data showed that the amount of intracellular GSH level was significantly decreased to 81, 79, and 69% at 10, 20, and 50 $\mu$M concentrations of As(III) in 6 h exposure to BEAS-2B cells, respectively (Fig. 6). This data indicates that As(III) can deplete intracellular GSH level resulting in increased oxidative stress. Addition of NAC in the cells exposed to As(III) replenished the GSH level (data not shown).

### *Generation of hydroxyl radicals by As(III)*

In an effort to investigate the mechanisms by which As(III) induces ROS generation, we exposed BEAS-2B cells to

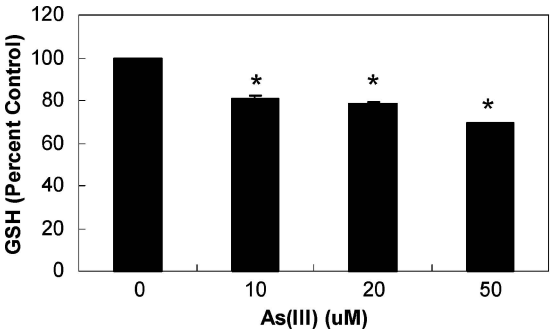


*Fig. 6.* Detection of GSH level in the cells exposed to As(III). BEAS-2B cells were exposed to As(III) for 6 h and the cell lysates were tested for the quantification of intracellular GSH. The optical density was measured at 405 nm and values presented represent the mean ± S.E. of six assay wells from two independent experiments. Asterisk in superscript indicates a significant percent decrease of GSH from control ($p < 0.05$).

100 $\mu$M of As(III) for 10 min in the presence of DMPO and phosphate buffer at 37 °C with and without •OH radical scavengers. ESR spectra, recorded 10 min after the initiation of reaction at a pH 7.4 in phosphate buffer containing 200 mM DMPO and 100 $\mu$M As(III), showed a typical 1:2:2:1 quartet with hyperfine splittings of $a_H = a_N = 14.9$ G. Based on these splitting constants of hydrogen and nitrogen, the signal was assigned to a DMPO/•OH adduct, demonstrating the generation of •OH radicals. Figure 7 shows that DMPO with cells, or DMPO alone failed to produce the •OH signal, while As(III) with DMPO and cells produced •OH radicals. Addition of catalase, a $H_2O_2$ decomposing enzyme, inhibited •OH generation substantially implying that $H_2O_2$ was produced in the As(III)-treated cells. Addition of SF, a •OH radical scavenger, decreased the signal intensity. Preincubation of cells with NAC, a thiol-containing general antioxidant before As(III) exposure, inhibited the •OH signal intensity almost completely. However, addition of NADPH, a cellular reductant, significantly increased the •OH signal. These data suggest that As(III) can produce ROS, such as $H_2O_2$ and •OH, as a result of reduction reactions within cells.

### *Lipid peroxidation and total NO• generation in BEAS-2B cells exposed to As(III)*

To test the generation of ROS, a lipid peroxidation by-product produced by cells in response to As(III) was analyzed immediately after a 24 h exposure. Isoprostanes are produced by the oxidation of phospholipids by oxygen radicals, and it is considered an ideal marker of oxidative stress. Analysis of conditioned media from cells exposed to As(III) showed dose-dependent, significant increases in the levels of 8-isoprostane (Fig. 8). The 8-isoprostane production increased approximately 1.9-, 3.1-, and 6.6-fold at 5, 10, and 20 $\mu$M As(III)

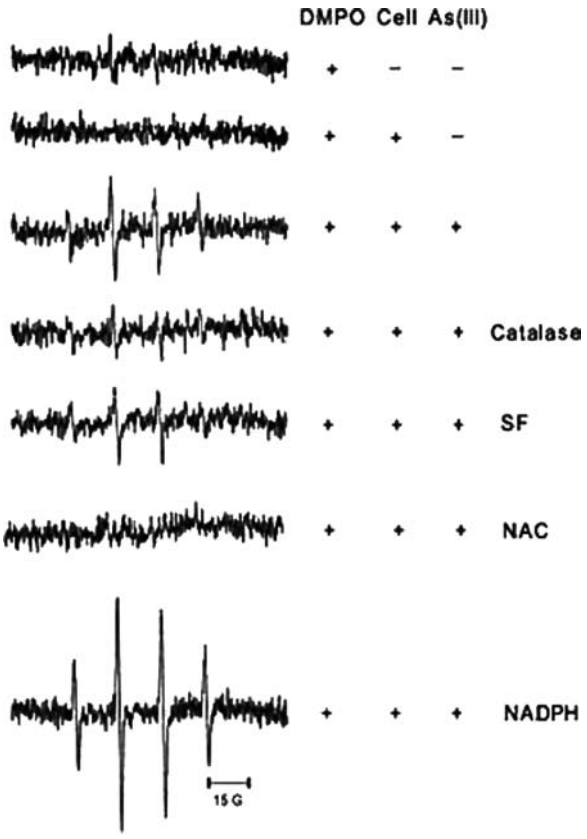


*Fig. 7.* ESR spectra generated by DMPO-•OH adducts obtained from BEAS-2B cells treated with As(III). BEAS-2B cells ($1 \times 10^6$) were incubated with 200 mM of DMPO, and 100 $\mu$M of As(III) for 10 min at 37 °C. The spectra were integrated by three scans. The spectrometer settings were: receiver gain, $6.32 \times 10^4$; time constant, 40.960 ms; modulation amplitude, 1.0 G; scan time, 41.943 s; magnetic field, 3490 ± 50 G. The concentrations of the enzymes and reagents used were: catalase, 2000 units/ml; sodium formate (SF), 500 mM; NADPH, 5 mM; NAC, 10 mM.

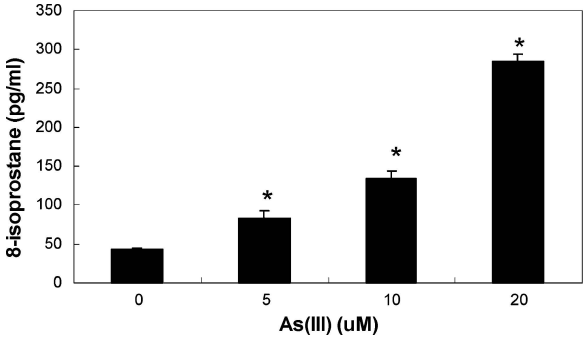


*Fig. 8.* Lipid peroxidation detection by 8-isoprostane ELISA method. Cells were exposed to As(III) for various concentrations (0–20 $\mu$M) for 24 h in the 6-well plate with 1 ml culture media. Immediately after exposure, the media were directly used for 8-isoprostane generation according to the manufacturer's protocol. The optical density was measured at 410 nm, using a microplate spectrophotometer, and values presented represent the mean ± S.E. of six assay wells from two independent experiments. Asterisk in superscript indicates a significant increase of 8-isoprostane generation from control ($p < 0.05$).

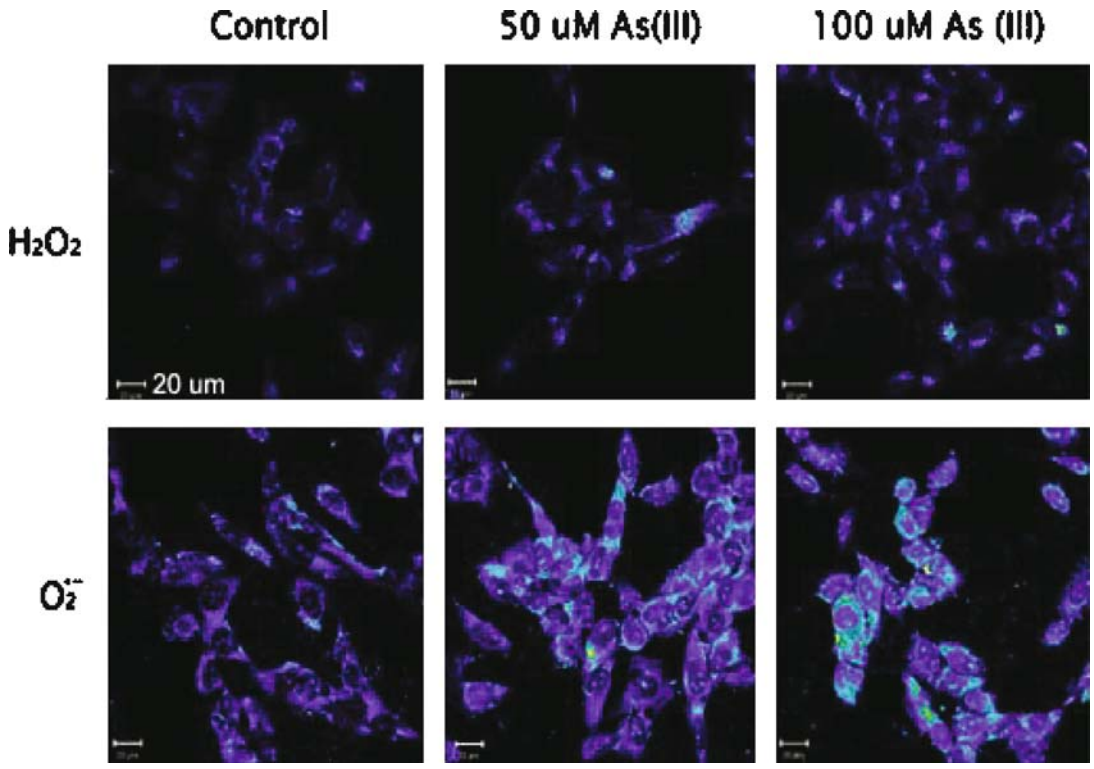


*Fig. 9.* Detection of intracellular $H_2O_2$ and $^{\bullet}O_2^-$ generation by exposure to As(III) using confocal microscopy. BEAS-2B cells ($1 \times 10^6$) were exposed with either 50 or 100 $\mu$M of As(III) in the presence of $H_2$DCFDA (25 $\mu$M) or dihydroethidium (10 $\mu$M) for 1 h. The cells were washed with PBS, fixed with 10% buffered formalin for 10 min, and mounted on glass slides. The images were captured with a laser scanning confocal microscope. The bright green, yellow and red areas in the cells represent oxidized DCFHDA and dihydroethidium indicating the intracellular generation and localization of $H_2O_2$ and $^{\bullet}O_2^-$ in BEAS-2B cells.

concentrations, respectively. However, total nitric oxide measured as nitrite and nitrate were not detected in the cells exposed to As(III) indicating that reactive nitrogen species are likely not involved in As(III)-induced cell toxicity in this cell system (data not shown).

*Detection of intracellular ROS generation by exposure to As(III) using confocal microscopy*

Generation of ROS by As(III) was further confirmed by confocal microscopy. BEAS-2B cells were exposed with As(III) in the presence of $H_2$DCFDA, a specific fluorescent dye for the detection of $H_2O_2$, or dihydroethidium, a specific fluorescent dye for the detection of $^{\bullet}O_2^-$. Images are displayed in pseudocolor where blue indicates areas of low intensity with increasing high-intensity areas in green < yellow < red and < white in a graded level. In the presence of increasing concentrations of As(III) the intensity of fluorescence increased significantly to green and yellow. To document the high intensity gradient levels of $H_2O_2$ and $^{\bullet}O_2^-$ generated cells were exposed to 50 and 100 $\mu$M of As(III) and results obtained are displayed (Fig. 9). These studies suggest that ROS are generated within the cell and $^{\bullet}O_2^-$ is generated in greater levels compared to $H_2O_2$.

## Discussion

It is well established that exposure to trivalent and pentavalent arsenic through occupational and environmental sources causes many diseases, including cancer of the lung, skin, liver, kidney and bladder [7]. Although many are known, inhalation is one of the major routes of exposure, and the lung is a frequent target for arsenic toxicity and carcinogenicity. The toxic mechanisms of interactions for arsenic include, but are not limited to, oxidative stress, mutation, gene amplification, DNA methylation, inhibition of DNA repair, uncoupling of oxidative phosphorylation or by combination with thiol groups on the active sites of enzymes and cellular proteins [25, 47]. Also, it is proposed that arsenic generates ROS and free radicals, such as hydrogen peroxide, hydroxyl radical, nitric oxide, or superoxide anion, and all of these reactive species are assumed to be involved in the stress response induced by arsenic [48].

Heat shock proteins are known to be highly conserved and ubiquitous molecules found in prokaryotic and eukaryotic cells throughout the evolution. They provide a defensive mechanism for protection of cells from adverse conditions. They have a wide range of housekeeping and cytoprotective roles and are released into the extracellular milieu in adverse conditions, upon injury to cells, or during necrosis. The generation of HSP is usually transient except under prolonged and sustained damaging conditions. Because homeostasis of HSP is important for intracellular functions, synthesis is regulated at the expression level through the repression of heat shock gene [49]. However, a growing number of reports suggest that, ROS and RNS are involved in the regulation Hsp70, which may be through the activation of the JAK/STAT pathway [50]. In this regard, heavy metals have the potential to generate $^{\bullet}OH$ through a Fenton-like reaction, which may induce oxidative stress resulting in the upregulation of HSP [31, 51, 52].

In the present study, we investigated the effects of arsenic on Hsp70 protein expression and the cellular and molecular mechanisms involved. The aim of this study was to explore whether Hsp70 is a sensitive biomarker for arsenic-induced cellular stress or perturbations and to determine the time course of this response to injury. The results demonstrate that exposure of trivalent arsenic to the human bronchial epithelial cell line, BEAS-2B, induces a significant expression of Hsp70 protein and mRNA in a time- and dose-dependent manner. Hsp70 protein and its isoforms were highly expressed in a relatively short time (after a 6 h exposure) and the protein accumulation rapidly increased during a 12 h recovery period following exposure. This implies that Hsp70 and its isoforms may be potential biomarkers for monitoring cellular damages induced by arsenic with other markers of oxidative injury to provide an excellent fingerprint for the early stages of injury. The augmented generation of ROS appears to have a profound influence in the induction of HSP. This is also supported by the direct involvement of ROS in the induction of HSP and the protective roles of antioxidant enzymes during heat-induced stress in yeast cells [53].

There are at least three highly homologous Hsp70 genes. They are Hsp70A, Hsp70B, and Hsp70C [54]. These three isoforms are believed to be translated to yield a single Hsp70 protein. In this study, we investigated the differential expression of these three isoforms. Hsp70A, Hsp70B, and Hsp70C mRNA was over-expressed after a 6 h exposure to arsenic with the expression decreasing during the 12 h recovery periods. This implies that Hsp70 mRNA expression is largely dependent on the transcriptional regulation of the early toxic effect of As(III) exposure, but the protein expression was probably regulated at the translational level in the later recovery stage of cells. Our RT-PCR data showed that Hsp70 mRNA levels increase in an on/off manner while protein levels increase in a dose-dependent manner. It is probably because of the regulation at the translational level and post-translational signaling mechanisms. Also, it is assumed that the accumulation of Hsp70 protein remained upregulated until the protein turnover gained a faster rate than the synthesis of new protein.

As stated earlier, arsenic is a well known ROS inducer, and ROS are powerful inducers of HSP. In this context, we investigated the relationship between the Hsp70 protein expression and the production of ROS and oxidative damage. The Hsp70 protein expression was decreased when the BEAS-2B cells were co-exposed to arsenic and catalase, a $H_2O_2$-scavenging enzyme, NAC, a thiol-containing general antioxidant, SOD, a $^{\bullet}O_2^-$ radical scavenger that generates $H_2O_2$, and SF, a $^{\bullet}OH$ radical scavenger. The results of these investigations indicate that ROS are important mediators of As(III)-induced stress protein expression. Compared to other reagents, NAC showed a more powerful inhibitory effect on HSP expression, indicating potential binding of As(III) with thiol groups on the active sites of enzymes or cellular proteins. Our data also showed that intracellular GSH, a thiol-containing antioxidant, was significantly decreased by arsenic exposure in a dose-dependent manner. These data suggest that depletion of antioxidant system causes oxidative stress that may lead to increase of HSP expression. The present study shows that As(III) is also able to generate ROS inside of cells, while RNS was not increased. The significant increase of lipid peroxidation is a strong evidence, for oxidative stress in response to As(III). As(III)-mediated ROS generation was further confirmed by fluorescence staining of As(III)-exposed cells and ESR spin trapping studies. Both $^{\bullet}O_2^-$ and $H_2O_2$ were highly increased in As(III)-exposed cells as measured by confocal microscopy. By ESR spectroscopy and spin trapping $^{\bullet}OH$ generation was confirmed as the final end product of As(III) exposure to BEAS-2B cells. These findings suggest that Hsp70 is a sensitive biomarker of cellular stress or perturbations induced by arsenic. ROS appears to play a crucial role in arsenic-induced Hsp70 expression.

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