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## Glutathione Protects Cells against Arsenite-Induced Toxicity

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### Abstract

To understand the role of glutathione (GSH) in the protection of cells from arsenite toxicity, we studied the mechanism of apoptotic cell death in cells genetically unable to synthesize GSH (GCS-2 cells). Arsenite stimulated an increase in protein ubiquitination in GCS-2 cells while the wild type cells were unaffected. Arsenite treatment increased lipid peroxidation and induced ubiquitination of molecular chaperone Hsp90 and impaired its ability to bind cochaperone p50<sup>Cdc-37</sup> and client proteins Plk-1 and Cdk-4 in GCS-2 cells. Treatment with arsenite also partially inhibited proteasome activity in GCS-2 cells. In these cells stably transfected with GFP<sup>u</sup> (a reporter consisting of a short degron fused to the COOH-terminus of GFP), intracellular fluorescence increased, suggesting the accumulation of GFP aggregates. GCS-2 cells underwent apoptosis accompanied by release of cytochrome C into the cytoplasm. Taken together, these data suggest that a possible mechanism of arsenite-induced apoptosis is the accumulation of ubiquitinated proteins and impairment of the protein degradative pathway. Further, protection from arsenite-induced ubiquitination is mediated by GSH and to a lesser extent by available reducing equivalents in the cells.

### Keywords

Glutathione; arsenite; proteasome; ubiquitination; Hsp90

### Introduction

Environmental exposure to arsenite is linked to increased risk of cardiovascular disorders and cancer of the bladder, lung, and skin [1,2]. Arsenic is also an important cytotoxin, exposure to which results in cell death and apoptosis [3]. However, little is known about the molecular mechanisms of arsenite-induced cellular toxicity; furthermore, a better understanding of the modulation of arsenic toxicity would contribute to our knowledge of the basic principles of cellular toxicity and might lead to better therapeutic strategies.

Thiol-containing compounds are a central component of many biologically significant reactions. The most abundant intracellular non-protein thiol, GSH, is found in millimolar concentrations in most cells [4,5]. GSH has been implicated in the protection of cells against cytotoxins and in the metabolism of xenobiotics, including arsenite, through the formation of GSH conjugates [6–9]. A number of proteins with regulatory functions such as NFκB, AP-1,

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JAK/STATs, EGF receptor, and Hsps are sensitive to the cellular redox environment [10,11]. Antioxidants such as GSH regulate these proteins by modulating the redox state of specific thiol residues of target proteins including transcription factors, stress kinases, and caspases [10,11].

Oxidative stress contributes to cellular structural changes resulting in the misfolding of proteins. Chaperones and cochaperones are pivotal for stability and function of a large group of client proteins including protein kinases, mutated p53, cyclin D-associated Cdk-4, and Cdk-6 [12,13]. Hsp90 is one of the most abundant and important chaperones that assists in protein folding and protects newly synthesized proteins from catabolism [14,15]. Recruitment of protein kinase clients to the Hsp90 chaperone involves the cochaperone p50<sup>cdc37</sup> acting as a scaffold, binding protein kinases via its N-terminal domain and Hsp90 via its C-terminal region [16,17].

Protein ubiquitination, the covalent modification of proteins by the addition of ubiquitin, is a mechanism by which damaged proteins are marked for subsequent degradation by the 26S proteasome [18,19]. In the ubiquitin-proteasome pathway, the sequential actions of E1, the ubiquitin-activating enzyme, E2, the ubiquitin conjugating enzyme (UBC), and E3, the ubiquitin-protein ligase, serve to attach the ubiquitin moiety (most often polyubiquitin chains) to acceptor lysine residues of protein substrates, which are in turn targeted for degradation by the 26S proteasome [20,21]. The ability to degrade and regulate levels of intracellular proteins has made the proteasome an important molecule in the regulation of diverse biological processes including cell cycle progression, cell signaling, and apoptosis [18,22].

Programmed cell death eliminates harmful or damaged cells in a highly regulated manner. Various extracellular and intracellular stimuli can trigger this process, and once initiated the signal is transduced through a series of protein-protein interactions. There is accumulating evidence that supports a close relationship between the ubiquitin-proteasome pathway and the apoptotic machinery [18,22]. Protein degradation mediated by polyubiquitination regulates the steady-state expression levels of many of the apoptosis regulators [22]. In most cell lines, proteasome inhibitors trigger apoptosis [23].

To investigate the role of GSH in arsenite-induced cytotoxicity, we have developed cell lines from 3.5 day post-coitus (dpc) embryos from both wild-type and  $\gamma$ -glutamyl cysteine ligase catalytic subunit (Gclc)-deficient mice [24]. GCS-2 (Gclc-deficient cells) grown in 2.5 mM GSH for 48 h have ~2.4% GSH levels of wild type (BDC-1) cells. This value drops to the limit of detection after GSH has been withdrawn for 24 h. We have used GCS-2 cells as a model system to elucidate the molecular mechanisms underlying arsenite toxicity under low GSH conditions. Results presented here demonstrate that exposure of GSH-depleted GCS-2 cells to low concentrations of arsenite lead to polyubiquitination of molecular chaperone Hsp90, degradation of Hsp90 and its client proteins, impaired proteasomal function leading to accumulation of protein-ubiquitin conjugates and apoptosis. These results support the concept that GSH serves to protect cells against arsenite-induced toxicity.

## Materials and Methods

### Reagents

Polyclonal antibodies to Hsp90, Hsp70, Hsp40, Hsp105, and Hsp27, cytochrome C, p70 S6 kinase, p90 S6 kinases 1, 2, and 3 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against ubiquitin and ubiquitin activating enzyme-E1 were from StressGen Biotechnologies (Victoria, British Columbia, Canada). Antibodies against phospho ERK, phospho p38 MAPK, and phospho JNK were purchased from Cell Signal Technologies (Beverly, MA). Secondary horseradish peroxidase-conjugated antibodies were from Bio-Rad

Laboratories (Richmond, CA).  $\alpha$ - $^{32}\text{P}$  dATP was purchased from Perkin-Elmer Life Sciences (Boston, MA).

### Cell Lines and Culture Conditions

All cell culture studies used M15 complete medium and cultures were done essentially as described earlier [24]. All GSH-dependent GCS-2 cells were maintained in medium containing 2.5 mM GSH and NAC-dependent GCS-2 cell lines were cultured in medium supplemented with 2 mM NAC. BDC-1 cells (wild type controls) were maintained in medium without NAC or GSH.

### Measurement of Thiols

Thiols were measured using the high-performance liquid chromatography-electrochemical detection method of Kleinman and Richie [25] with minor modifications.

### Treatment of Cells

Sodium arsenite was purchased from Sigma (St. Louis, MO). A 100  $\mu\text{M}$  stock solution was prepared in PBS, pH 7.4 and was used at final concentrations ranging from 0–2  $\mu\text{M}$ . Briefly, the GCS-2 cells were seeded at  $1.5 \times 10^6$  cells per 10 cm dish, allowed to grow for 48 h either in the presence of 2.5 mM GSH or 2 mM NAC, depleted off GSH or NAC for 24 h, and treated with various concentrations of arsenite for up to 21 h. BDC-1 cells were treated under similar conditions as GCS-2 cells except that they were grown in the absence of NAC or GSH.

### Cytotoxicity Assay

Cell viability, as an indicator of cytotoxicity, was determined by determining the capacity of BDC-1 and GCS-2 cells to reduce MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan [26]. Briefly, a 5mg/ml solution of MTT (Sigma Chemical Co., St. Louis, MO) was prepared, filtered through a 0.2  $\mu\text{m}$  filter, and used in the assays. Cell viability was determined on cells grown in 24-well plates. After arsenite treatment under appropriate conditions, MTT solution (40  $\mu\text{l}$ ) was added to each well, followed by incubation at 37°C for 4 h in a 5%  $\text{CO}_2$ :95% air atmosphere. The cells were washed twice in phosphate-buffered saline and added acidic propanol (0.1 N HCl in absolute propanol) 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 a 4 h incubation period.

### Lipid Peroxidation Assay

Lipid peroxidation, as an indicator of oxidative stress, was measured in BDC-1 and GCS-2 cells using an assay kit supplied by Oxford Biomedical Research, Oxford, MI according to the manufacturer's directions using 1, 1, 3, 3-tetramethoxypropane (TMOP) as the standard. The assay is based on the reaction of the chromogenic reagent, N-methyl-2-phenylindole with malondialdehyde (MDA) at 45°C. The resulting chromophore has maximal absorbance at 586 nm. Data are expressed as pmol MDA formed/mg protein.

### RNA Isolation and Northern Blot Analysis

Total RNAs were isolated from cultured cells using TRIzol reagent (InvitrogenLife Technologies, Carlsbad, CA) according to manufacturer's instructions. Fifteen  $\mu\text{g}$  of total RNA from cells after various treatments were electrophoresed on 1% agarose and 2.2 M formaldehyde gels, transferred onto a Zeta-Probe nylon membrane (Bio-Rad), and hybridized to a 410-bp  $^{32}\text{P}$ -labeled cDNA probe corresponding to Hsp90 or with a 456-bp probe corresponding to Hsp70 cDNA. The blot was then stripped and re-probed with  $\beta$ -actin cDNA.

## Proteasome Assay

The proteasomal activities of total cellular homogenates were measured using the proteasome specific substrate LLVY-AMC (Biomol, Plymouth Meeting, PA). This substrate is specifically used to assay the chymotryptic activity of the proteasome. Homogenates were prepared in the proteasome assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, and 0.5% NP-40) from the cells treated with and without arsenite. Reactions (100  $\mu$ l) containing homogenate and the proteasome specific substrate were prepared in quadruplicate. Assays were done in 96-well plates and the fluorescence was measured with excitation  $\lambda$ 360/emission  $\lambda$ 460 using SpectraMax Gemini Fluorometer (Molecular Devices Corp. Sunnyvale, CA).

## DNA Laddering Assay

For nonradioactive DNA laddering detection, DNA from treated and untreated cells was extracted in DNA isolation buffer (50 mM Tris-HC, 0.05% SDS, 0.1% Triton-X-100, 50  $\mu$ g/ml RNase, 50  $\mu$ g/ml proteinase K, and 25 mM EDTA), phenol/chloroform extracted, and ethanol precipitated. DNA (10  $\mu$ g) was then analyzed on 1% agarose gels containing ethidium bromide (0.1  $\mu$ g/ml) and visualized by UV transillumination.

## Immunoprecipitation and Western Blot Analysis

Cells were washed twice with ice-cold PBS, and harvested in cell lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM disodium EDTA, 1% sodium deoxycholate) containing the protease inhibitors (Roche Biochemicals, Indianapolis, IN). Cells were lysed by sonication and the protein supernatants were clarified by centrifugation at 25,000 X g for 10 min at 4 °C. Protein concentration was determined by the BCA protein assay reagent kit (Pierce, Rockford, IL). Hsp90 and p50<sup>cdc-37</sup> from clarified lysates were immunoprecipitated with 1  $\mu$ g of specific polyclonal antibody against Hsp90 or p50<sup>cdc-37</sup> (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and 20  $\mu$ l of protein A/G plus agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 2h at 4°C. Immunoprecipitated proteins were washed under stringent conditions, resolved on 7.5% SDS-polyacrylamide gels, transferred on to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ), and analyzed by western blotting. For immunoblotting of whole cell lysates, clarified cellular lysates were resolved on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and analyzed by immunoblotting using various antibodies. The ECL blots were developed according to the manufacturer's instructions.

## Transfection

BDC-1 and GCS-2 cells were plated at a density of 5 X 10<sup>4</sup> cells per well in 6-well plates 24 h prior to transfection. The cells were transfected using the FuGENE transfection reagent (Roche) with 1 mg of either control plasmid containing neomycin or pGFP<sup>u</sup> containing neomycin. After 24 h of transfection, the cells were selected with 800 $\mu$ g/ml G-418 until single clones were recovered. Clones were further expanded and GFP fluorescence was monitored with and without arsenite treatment by confocal microscopy.

## Results

### Arsenite-Induced Cytotoxicity in GCS-2 Cells

It has been well documented that GSH is the major non-protein thiol that regulates the intracellular redox balance [4,5] and that arsenite promotes apoptosis [3]. Availability of embryonic cell lines derived from Gclc<sup>-/-</sup> mice with little/no GSH (24) allowed us to examine the role of GSH depletion in arsenite-induced cytotoxicity. Arsenite dose response survival rates were compared between BDC-1 cells and GSH-deprived GCS-2 cells after 21 h of treatment (Fig. 1). GCS-2 cells exhibited decreased viability as the arsenite dose increased to

2  $\mu\text{M}$  whereas BDC-1 cells were unaffected. The  $\text{ED}_{50}$  for GCS-2 cells was 1  $\mu\text{M}$  whereas for BDC-1 cells (data not shown) it was 24  $\mu\text{M}$ . To determine if other antioxidants could substitute for GSH, we rescued GCS-2 cells in the presence of thiol antioxidant NAC and examined arsenite-induced cytotoxicity. Because NAC is known not to complex with arsenite [27], we cultured GCS-2 cells in the presence of NAC for 48 h and divided them into two groups: one group was grown for an additional 24 h in NAC and treated with arsenite for 21 h. In a parallel experiment, NAC was withdrawn from GCS-2 cells for 24 h and treated with arsenite in the absence of NAC for 21 h. GCS-2 cells treated with arsenite in the absence of NAC showed decreased viability rates similar to GCS-2 cells deprived of GSH, while cells exposed to arsenite in the presence of NAC exhibited intermediate survival rates (Fig. 1). These data suggest GCS-2 cells are much more susceptible to arsenite toxicity and that it is a function of the levels of intracellular GSH and NAC could only partially protect these cells from arsenite-induced cell death in the absence of GSH.

### Arsenite-Induced Ubiquitination of Cellular Proteins in GSH-depleted GCS-2 Cells

The role of GSH in arsenite-induced ubiquitination of proteins was investigated in GCS-2 cells that contain little or no GSH compared to BDC-1 cells. GCS-2 cells were grown for 48 hours in the presence of GSH, followed by depletion of GSH for 24 hours, and treatment with various concentrations of arsenite (0 to 2  $\mu\text{M}$ ) for up to 24 h. Exposure of GCS-2 cells to low concentrations of arsenite ubiquitinated the proteins in a dose-dependent manner, while BDC-1 cells remained unaffected (Fig. 2A). Untreated GCS-2 cells did not show ubiquitination indicating that the absence of GSH alone is not sufficient to increase protein damage by ubiquitination. The GCS-2 cells had 20-fold higher levels of ubiquitinated proteins compared to wild-type cells at low concentrations of arsenite (Fig. 2B). Ubiquitination was time-dependent and steadily increased over the course of the experiment (Fig. 2C).

We also treated GCS-2 cells grown in the presence of NAC for 48 h with arsenite for 21 h. In a parallel experiment, NAC was withdrawn for 24 h from GCS-2 cells that were grown in the presence of NAC for 48 h and treated with arsenite in the absence of NAC for 21 h. GCS-2 cells that were exposed to arsenite in the presence of NAC ubiquitinated proteins to half the extent as the cells in the absence of NAC (Fig. 2D). This finding indicates that NAC partially protects cells against arsenite-induced ubiquitination in the absence of GSH.

### Arsenite Induces Changes in Gene Expression in GCS-2 Cells

To detect changes in gene expression, we analyzed protein levels and phosphorylation status by immunoblotting. We examined five representative categories of proteins: MAP kinases, transcription factors, heat shock proteins, ubiquitin pathway enzymes, and ribosomal S6 kinases (Table 1). Total protein levels of MAP kinases remained unchanged upon arsenite exposure. We found increased phosphorylation of ERK1/2, JNK1/2, and p38 MAP kinase in the GCS-2 cells, while BDC-1 cells did not show any obvious changes. We examined five heat shock proteins that function as molecular chaperones and found that only Hsp90 protein levels were decreased. This finding suggests that Hsp90 is involved in arsenite-induced toxicity (see below). Because we found increased ubiquitination of total proteins in GCS-2 cells (Fig. 2), we examined changes in ubiquitin pathway enzymes including ubiquitin activating enzyme (E1), some of the ubiquitin conjugating enzymes (E2), and representative members of the ubiquitin ligase family (E3) and found no detectable changes (Table 1). We also examined the phosphorylation status of ribosomal S6 kinases under our conditions and found no detectable differences between GSH-competent and GSH-deficient cells.

### Exposure to Arsenite Downregulates Hsp90 at Both mRNA and Protein Levels

We further examined changes in Hsp90 levels and found that exposure to 0.5  $\mu\text{M}$  arsenite downregulated Hsp90 steady state total RNA levels by ~ 2-fold in GCS-2 cells while Hsp70

RNA levels were unaffected (Fig. 3A). Immunoblotting demonstrated that in GCS-2 cells, Hsp90 protein levels decreased to about 40% of the wild-type levels at 21–24 h (Fig. 3B). In Fig. 2B, it appears that Hsp90 protein levels are lower in the untreated GCS-2 cells than in untreated BDC-1 cells. Because only 2 out of 5 experiments appeared to show a decrease in Hsp90 expression in GCS-2 cells, we quantified signal intensities by scanning densitometry. We found that these changes were statistically not significant, with a  $p \leq 0.19$  (Student's *t* test). Under the same conditions, Hsp70 protein levels did not change.

### Arsenite Downregulates Hsp90 by Ubiquitination in GSH-Depleted GCS-2 Cells

In the GCS-2 cells, there was a marked diminution in the cellular content of Hsp90 protein upon treatment with low concentrations of arsenite (Fig. 4A). Hsp90 was immunoprecipitated from the cytosol with an Hsp90-specific polyclonal antibody and analyzed by immunoblot with an anti-ubiquitin antibody. In GCS-2 cells treated with arsenite, there are bands in the high molecular weight region (>90 kDa) ranging in size from 100 to 250 kDa. These data indicate that Hsp90 is polyubiquitinated in these cells after arsenite treatment (Fig. 4B). There was no evidence of Hsp90 ubiquitination in BDC-1 cells under the same conditions (Fig. 4B). These findings suggest that low/absent levels of GSH and exposure to arsenite result in Hsp90 ubiquitination in GCS-2 cells.

### Arsenite Affects Hsp90 Binding with its Cochaperone

We examined if ubiquitination of Hsp90 affects its ability to function as a chaperone, as analyzed by its ability to bind its major cochaperone p50<sup>cdc-37</sup> [16,17]. Cytosolic extracts from arsenite-treated and untreated BDC-1 and GCS-2 cells were immunoprecipitated with anti-Hsp90 antibody. Proteins were separated by SDS-PAGE and analyzed by immunoblot with p50<sup>cdc-37</sup>-specific antibody. We found that the treatment with arsenite diminished the interaction of Hsp90 with p50<sup>cdc-37</sup> by ~ 50% in GCS-2 cells whereas it was unaffected in BDC-1 cells (Fig. 4C). We next asked if the ubiquitination of Hsp90 and its reduced levels also destabilized its client proteins, leading to deficiencies in multiple unrelated mechanisms. We studied polo-like kinase-1 (Plk-1), a prominent regulator of cellular events such as spindle formation, chromosome segregation, and cytokinesis [28]. Cdk4 is also assisted in folding and subsequently stabilized by Hsp90 [29]. Both Plk-1 and Cdk-4 diminished after treatment with 0.5  $\mu$ M arsenite in GCS-2 cells but remained unaltered in BDC-1 cells (Figs. 4E and 4F). Thus arsenite causes ubiquitination of Hsp90 and downregulates Hsp90 client proteins in GCS-2 cells under low/absent GSH conditions.

### Arsenite Impairs Proteasome Function in GCS-2 Cells

The ubiquitin-proteasome pathway is the primary route for degrading ubiquitin-tagged proteins and some non-ubiquitinated proteins [20]. Since ubiquitinated proteins accumulate in arsenite-treated GCS-2 cells, we assayed proteasome function in these cells in comparison with BDC-1 cells. Proteasome activity was measured using a fluorogenic substrate of the chymotrypsin-like peptidase of the proteasome. We found that in the GCS-2 cells treated with 0.5  $\mu$ M arsenite, proteasome activity decreased 25–30% compared to untreated GCS-2 and BDC-1 cells, and BDC-1 cells treated with arsenite (Fig. 5A).

To investigate the specific relationship between aggregation of ubiquitin-protein conjugates and proteasome function, we transfected a reporter construct consisting of a short degron oligonucleotide sequence (corresponding peptide sequence ACKNWFSSLSHFVIHL) fused to GFP-C1 plasmid (GFP<sup>u</sup>) into BDC-1 and GCS-2 cells. GFP<sup>u</sup> is normally unstable [half-life  $t_{1/2} = 20$ –30 min] compared to GFP ( $t_{1/2} > 10$  h) transfected GFP<sup>u</sup> were cultured for 48 h, GSH was withdrawn for 24 h, and the cells were treated with 0.5  $\mu$ M arsenite for 21 h and monitored for fluorescence. GFP<sup>u</sup> fluorescence was not observed in BDC-1 cells or in GCS-2 cells without arsenite treatment (Fig. 5B, C, and D).

In contrast, GCS-2 cells treated with 0.5  $\mu\text{M}$  arsenite for 21 h showed increased GFP<sup>u</sup> fluorescence suggesting that GFP<sup>u</sup> accumulated intracellularly as aggregates (Fig. 5E).

### Arsenite-Induced Lipid Peroxidation in GCS-2 Cells

Lipid peroxidation is a well-established mechanism of cellular injury and is used as an indicator of oxidative stress in cells and tissues [31]. MDA formation was determined in GCS-2 cells that were treated with arsenite for 21 h after GSH depletion for 24 h (Fig. 6A). There was ~10-fold increase in MDA formation in GCS-2 cells treated with arsenite compared to BDC-1 cells treated with arsenite.

To determine if NAC protects GCS-2 cells against arsenite-induced oxidative stress, we exposed cells grown in the presence of NAC and cells that were depleted of NAC for 24 h, to arsenite for 21 h. Cells that were treated with arsenite in the presence of NAC had ~ half the MDA levels compared to cells exposed to arsenite in the absence of NAC suggesting that NAC provides partial protection against arsenite-induced toxicity (Fig. 6B).

### Release of Cytochrome C into Cytosol in GSH-depleted GCS-2 Cells

Release of cytochrome C into the cytosol is indicative of mitochondrial damage [32]. We evaluated cytochrome C release into the cytosol as a function of time and arsenite treatment in BDC-1 and GCS-2 cells. Cytosolic fractions of BDC-1 and GCS-2 cells were assayed for the presence of cytochrome C by immunoblot with anti-cytochrome C antibody. While there was no measurable cytochrome C present in the untreated or arsenite-treated BDC-1 cells, cytochrome C was readily detectable in the cytoplasm of untreated GSH-depleted GCS-2 cells (Fig. 7A) and the levels increased after arsenite treatment by approximately 2-fold at 21-24 h in the GCS-2 cells. This result suggests that these cells are poised to undergo apoptotic cell death and arsenite tips the balance toward apoptosis (Fig. 7).

### Arsenite Induces Apoptosis in GCS-2 Cells

To determine if arsenite induces apoptosis in our system, we exposed GSH-deficient GCS-2 cells to varying concentrations of arsenite for 21 h, and analyzed extracted DNA. BDC-1 cells showed no signs of DNA fragmentation, whereas GCS-2 cells showed a dose-dependent increase in DNA laddering at concentrations  $\geq 0.5 \mu\text{M}$  arsenite (Fig. 7B). While no DNA laddering was obvious up to 7 h of arsenite treatment, DNA fragmentation became evident at 21 h at 0.5  $\mu\text{M}$  arsenite treatment (Fig. 7C). Thus, the absence of GSH and the accompanying exposure to arsenite induce internucleosomal DNA fragmentation in arsenite-treated GCS-2 cells.

## Discussion

In this study, we have taken advantage of Gclc-deficient GCS-2 cells as an experimental system to assess the effects of very low/absent levels of GSH on arsenite-induced toxicity, protein ubiquitination and apoptosis. Our results are in general agreement with our previous results (24) and the recently reported arsenite toxicity studies performed on mouse embryo fibroblasts derived from glutamate-cysteine ligase modifier subunit-deficient (Gclm<sup>-/-</sup>) mice that have GSH levels ranging from 9–16% of the wild type levels [Fig. 1 and ref. 33]. The ED<sub>50</sub> for GCS-2 cells was 1  $\mu\text{M}$  (Fig. 1), whereas for BDC-1 cells, it was 24  $\mu\text{M}$  (data not shown). These data suggest that GCS-2 cells are very sensitive to arsenite and the cytotoxicity of arsenite in these cells directly correlates with the intracellular levels of GSH.

Intracellular GSH also plays a vital role not only in the transport of arsenic and its methylated derivatives but also in arsenic metabolism especially in the detoxification of monomethylarsonic acid (MMA; refs. 8,9). At present it is not clear if because of lack of GSH

in GCS-2 cells, they can convert inorganic arsenite into methylated arsenicals. Without a ready source of methylated arsenicals, it is likely that all of the cytotoxic effects of arsenic in these cells are attributable to inorganic arsenic species.

Although accumulation of ubiquitin protein conjugates has been shown to occur with a number of stressors, a direct correlation between the availability of GSH in the cell and the accumulation of high molecular protein aggregates has not been established [34–37]. Bredfeldt et al observed that reduction in GSH levels could cause the accumulation of ubiquitinated proteins in UROtsa cells even though the actual level of GSH after GSH depletion by the Gcl inhibitor DL-buthionine-(R,S)-sulfoximine (BSO) in these cells was not documented [38]. Our findings demonstrate that GSH, and to a lesser extent NAC, can protect cells against arsenite-induced protein ubiquitination (Fig. 2). In this context, we also note that although endogenous cysteine concentrations in GSH and NAC-rescued cells are comparable [24], our data indicate that NAC could only partially reverse the ubiquitination of proteins in the GCS-2 cells (Fig. 2D). This result suggests that even though NAC can support cell growth as well as GSH does, it cannot perform all the functions of GSH in the cell.

In the absence of GSH as an anti-oxidant, vicinal dithiol-specific SH reagent such as arsenite could react directly with the highly conserved Cys589/590 pair in Hsp90 impairing its chaperone activity [39]. Withdrawal of GSH and treatment with arsenite downregulated molecular chaperone Hsp90 at the transcriptional level and by ubiquitination, diminished its ability to bind cochaperone p50<sup>Cdc37</sup>, and destabilized Hsp90 client proteins Plk-1 and Cdk-4 in GCS-2 cells (Fig. 4). How does arsenic-induced down-regulation of Hsp90 gene expression result in cytotoxicity and cell death? Molecular chaperone Hsp90 is a central molecule in the proper folding of proteins. Inhibition of Hsp90 and its ubiquitination by arsenite could lead to the formation misfolded polypeptides which not only lose their function but they may also form toxic species, including oligomers or larger aggregates. These aggregates may be prevented from reaching their cellular destination due to retention and/or degradation. This increased retention and accumulation of insoluble aggregates may be further exacerbated by arsenite-induced partial inhibition of the proteasome.

Enhanced ubiquitination of Hsp90 and diminution of the cellular content of its client proteins have also been noted in cancer cells after exposure to the signal transduction inhibitor hypericin [12]. Whereas arsenite mainly induces apoptosis, hypericin causes mitotic cell death [12]. However, in both cases, Hsp90 inactivation by ubiquitination is a potential mechanism culminating in cell death.

Hsp90, p50<sup>Cdc37</sup>, and Plk-1 are all known to be over-expressed in certain types of cancers [28,40]. In fact, treatment of tumors with chemotherapeutic agents such as geldanamycin and radicicol, inhibitors of Hsp90, is known to induce significant tumor cytotoxicity [14,41]. Unlike geldanamycin and radicicol, which inactivate Hsp90 through interaction with the ADP/ATP binding pocket, arsenite exerts its cytotoxic effect through Hsp90 ubiquitination [12,41].

Arsenite not only enhanced the accumulation of protein-ubiquitin conjugates, but also inhibited proteasome function by about 25% in GSH-deficient GCS-2 cells, thereby providing a mechanism for reduced degradation of the ubiquitinated proteins (Fig. 5). Blocking proteasome function by proteasome-specific inhibitors leads to intracellular accumulation of ubiquitin-conjugated proteins [42].

Oxidative stress has been attributed as an important aspect of arsenite toxicity [31]. In the present study, exposure to arsenite induced cellular lipid peroxidation in both wild type and GSH-deficient cells (Fig. 6). However, more substantial increases in MDA were observed after arsenite treatment in GCS-2 cells that were depleted of GSH or NAC suggesting an increased



oxidative stress in the GCS-2 cells. This increased oxidative stress in GCS-2 cells could enhance protein ubiquitination and apoptosis.

Our data indicate that untreated GCS-2 cells had detectable levels of cytochrome C in the cytosol, which increased upon exposure to arsenite (Fig. 7A). However we did not detect DNA laddering at the same time point (Fig. 7C). These findings concur with our earlier observations that GSH depletion is sufficient to cause cytochrome C release into the cytosol [32]. Cytochrome C release increased with the cell's commitment to irreversible apoptosis since we observed DNA fragmentation in the GCS-2 cells only at 21 h after arsenite treatment (Fig. 7C). Most previous studies on the induction of apoptosis in normal cells involved acute exposure to concentrations of arsenite ranging from 40–400  $\mu\text{M}$  [3]. However, others have observed that the mechanism of resistance of tumor cells to the chemotherapeutic effects of arsenic is the up-regulation of GSH, and GSH depletion by (BSO) restored arsenic sensitivity [43,44]. JNK activation along with down-regulation of GSH may be an important mechanism by which arsenite induces apoptosis in tumor cells [44]. This is consistent with our finding of JNK activation in GSH-deficient GCS-2 cells (Table 1) may tip the balance toward apoptosis. Recently, Fernandez et al. have observed that  $\text{As}_2\text{O}_3$  (6  $\mu\text{M}$ ) caused apoptosis preferentially in U-937 human promonocytic cells and this effect was potentiated by depletion of GSH induced by BSO [45]. In addition, there are reports that suggest that mitochondrial function is affected by oxidative stress [46–48]. In a GSH compromised state, arsenite could alter mitochondrial membrane permeability via a direct effect on the adenine nucleotide translocator [47] and perturbation of mitochondrial permeability transition pore [46,48] resulting in apoptosis.

In summary, under conditions of reduced GSH availability, the cytotoxicity of arsenic may be viewed in terms of protein ubiquitination and Hsp90 inactivation, leading to destabilization of Hsp90 client proteins. These changes, along with partial inhibition of the proteasome, lead to intracellular aggregation of high molecular weight ubiquitinated protein conjugates. Ultimately damaged proteins could accumulate and induce cell death (Fig. 8). Further exploration into the role of cellular redox status might provide clues to the underlying mechanism of Hsp90-mediated signal transduction and posttranslational regulation of its client proteins.

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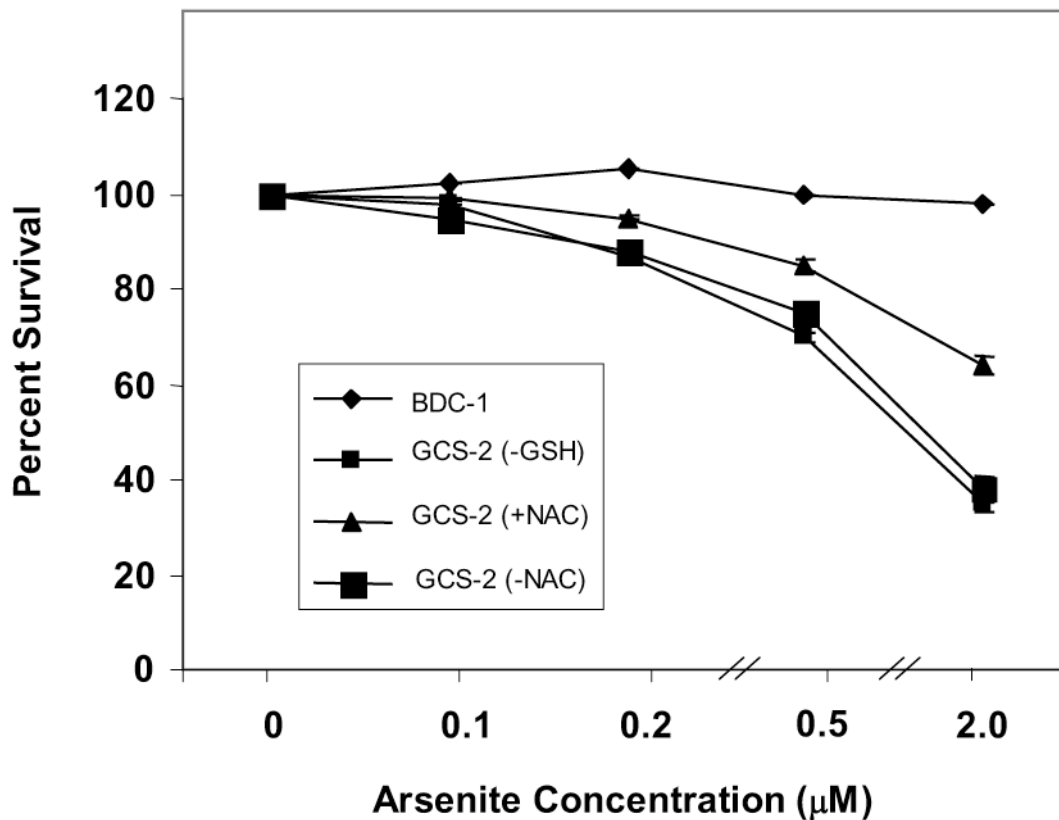
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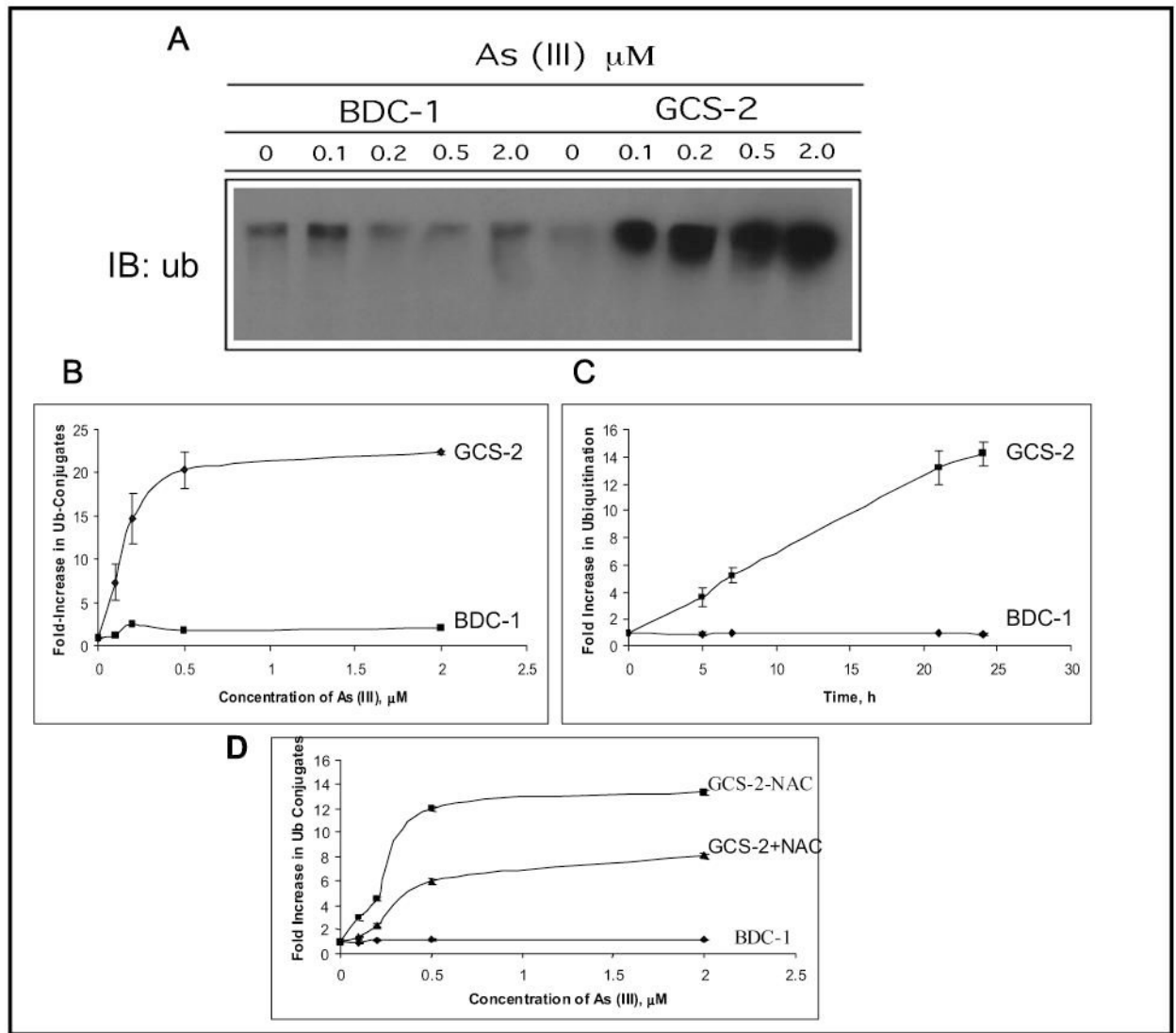
## Abbreviations

<b>Cdk-4</b>	cyclin dependent kinase-4
<b>Gcl</b>	glutamate cysteine ligase
<b>GSH</b>	glutathione
<b>Hsp90</b>	heat shock protein 90
<b>MDA</b>	malondialdehyde
<b>NAC</b>	N-acetyl cysteine
<b>Plk-1</b>	polo-like kinase-1
<b>SDS-PAGE</b>	sodium dodecylsulfate polyacrylamide gel electrophoresis



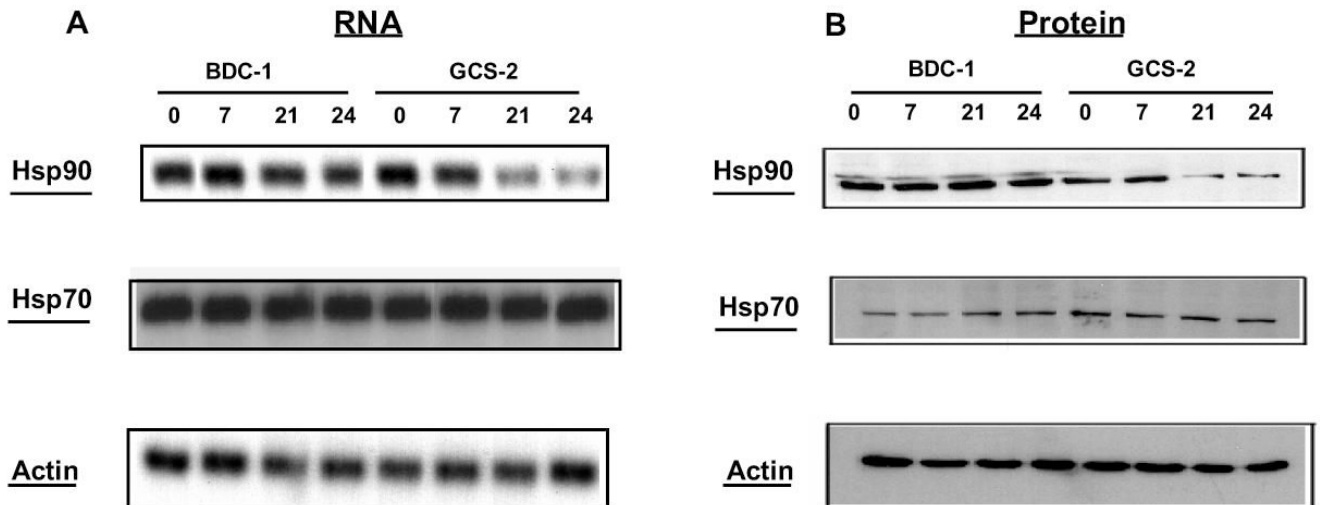
**Figure 1.**

Determination of the cytotoxicity of arsenite in GCS-2 cells. Dose response to arsenite-induced cytotoxicity in GCS-2 and BDC-1 cells. Cytotoxicity was measured using the MTT assay as described in “Methods”. A fixed number of GCS-2 cells were allowed to grow in 24-well plates in the presence of 2.5 mM GSH or 2 mM NAC for 48 h. GSH was withdrawn for 24 h and both BDC-1 and GCS-2 cells were treated with varying concentrations of arsenite for 21 h. NAC-dependent cells were divided into two groups: One group was grown for an additional 24 h in NAC and treated with different concentrations of arsenite in the presence of NAC while NAC was withdrawn from the other group for 24 h and the cells were treated with varying concentrations of arsenite for 21 h, followed by the addition of 40 μl of MTT reagent to each well. The plates were incubated for 4 h at 37°C and read on a plate reader at 570 nm. Results are presented as mean ± SEM.

**Figure 2.**

Effect of arsenite on protein ubiquitination in BDC-1 and GCS-2 cells. (A) Dose dependence of arsenite-induced ubiquitination in BDC-1 and GCS-2 cells. GCS-2 cells were plated at  $1.5 \times 10^6$  cells/dish in the presence of GSH for 48 h. GSH was withdrawn from GCS-2 cells for 24 h and both cell types were treated with various concentrations of arsenite for 21 h. Cell extracts were prepared and 10  $\mu\text{g}$  total protein was separated by SDS-PAGE and analyzed by immunoblot with polyclonal antibody against ubiquitin. (B) Quantification of the ubiquitin-protein conjugates by densitometry. Blots from (A) were then quantified to measure the high molecular weight ubiquitin-protein conjugates using a densitometer. Each point is an average of three independent determinations. (C) Time-dependent increase in the high molecular weight conjugates at 0.5  $\mu\text{M}$  arsenite treatment. The experimental conditions used were as described in A, except cells were treated for various time points with 0.5  $\mu\text{M}$  arsenite. The blots were quantified as described in B. (D) Effect of NAC on arsenite-induced ubiquitination in BDC-1 and GCS-2 cells. GCS-2 cells were grown in the presence of 2 mM NAC for 48 h and divided into two groups: one group was grown in NAC for an additional 24 h in the presence of NAC and treated with 0.5  $\mu\text{M}$  arsenite for 21 h. In a parallel experiment, NAC was withdrawn

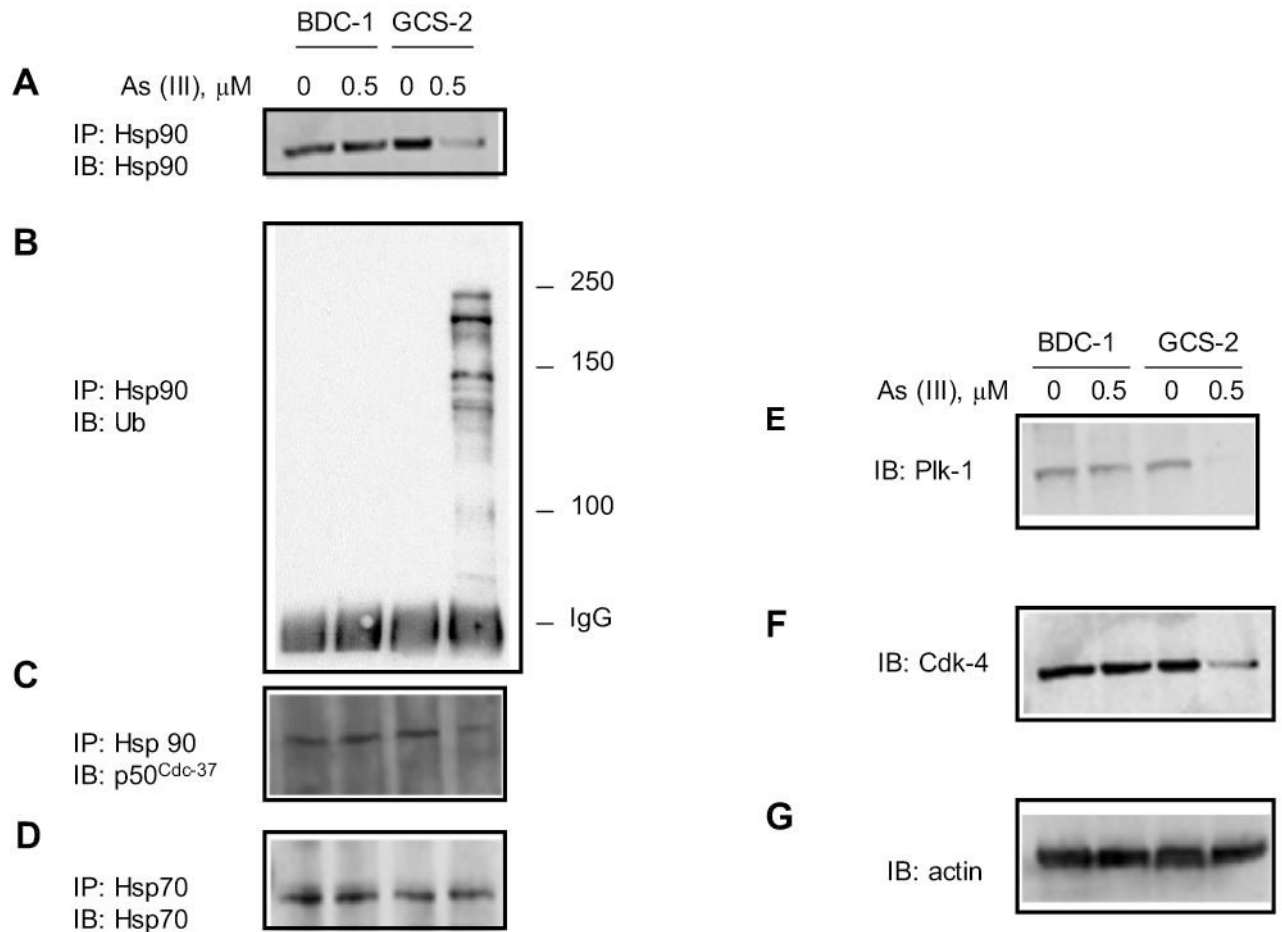
for 24 h from GCS-2 cells that were grown in the presence of NAC for 48 h and the cells were treated with various concentrations of arsenite for 21 h in the absence of NAC. Fold increase in ubiquitin conjugates were quantified as described in Fig. 2B.



**Figure 3.**

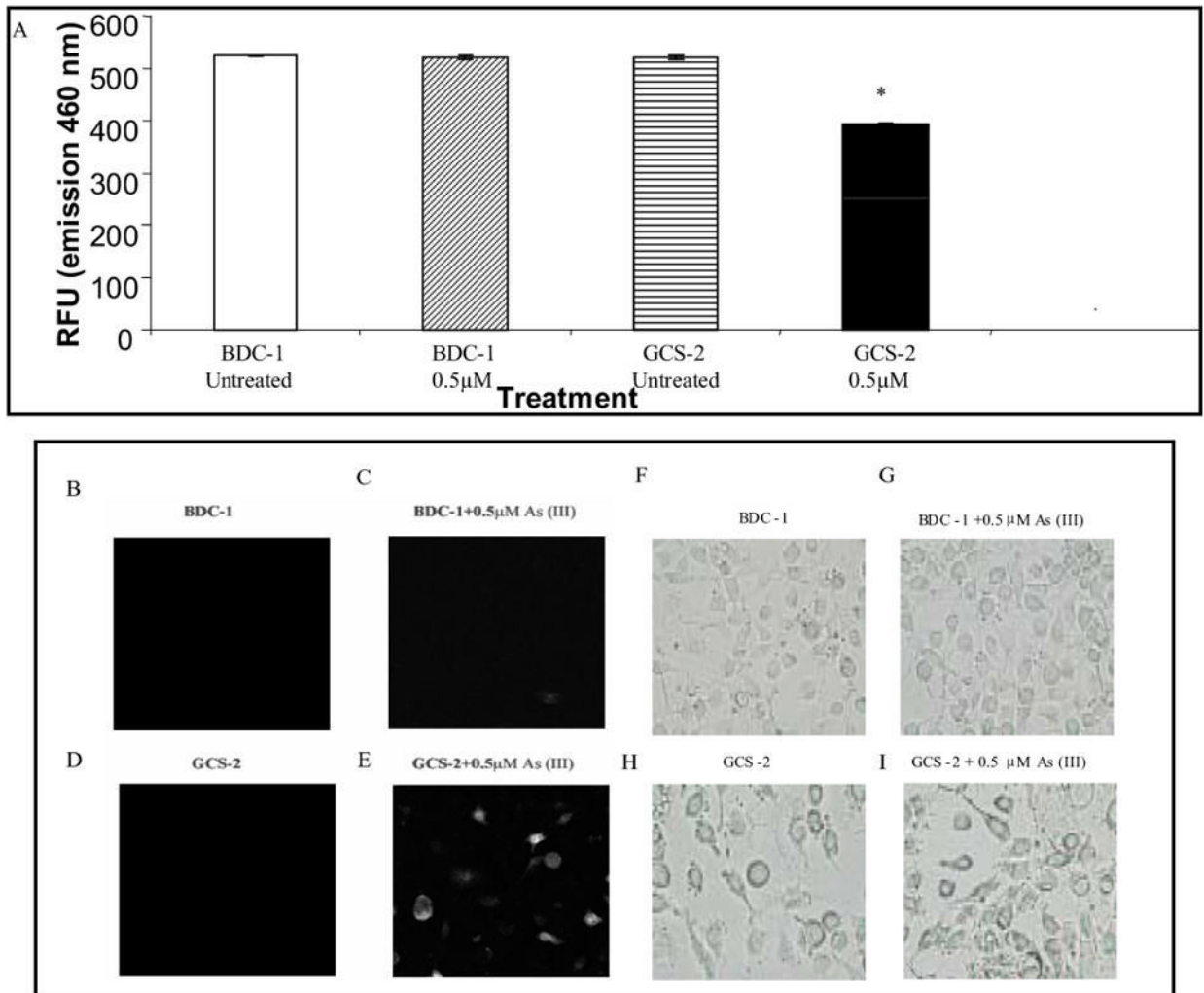
Determination of the effects of arsenite on Hsp90 and Hsp70 mRNA and protein levels. A. Northern blot analysis of Hsp90 and Hsp70. GCS-2 and BDC-1 cells were incubated with 0.5 μM arsenite for the indicated times and total RNA was isolated. 15 μg of RNA was analyzed by northern blot with Hsp90 (top) cDNA probe. The Hsp90 membrane was stripped and reprobbed with Hsp70 (middle) cDNA probe. Hsp70 membrane was stripped and reprobbed with actin (bottom) cDNA probe to verify equal loading. Incubation times in hours are indicated along the top. B. Analysis of Hsp90 and Hsp70 protein levels. Cells were treated as in A, and 50 μg of cytosolic extract was separated by SDS-PAGE and analyzed by immunoblot with Hsp90-specific (top) or Hsp70-specific (middle) antibody. Equivalent loading was verified with an anti-actin (bottom) antibody.





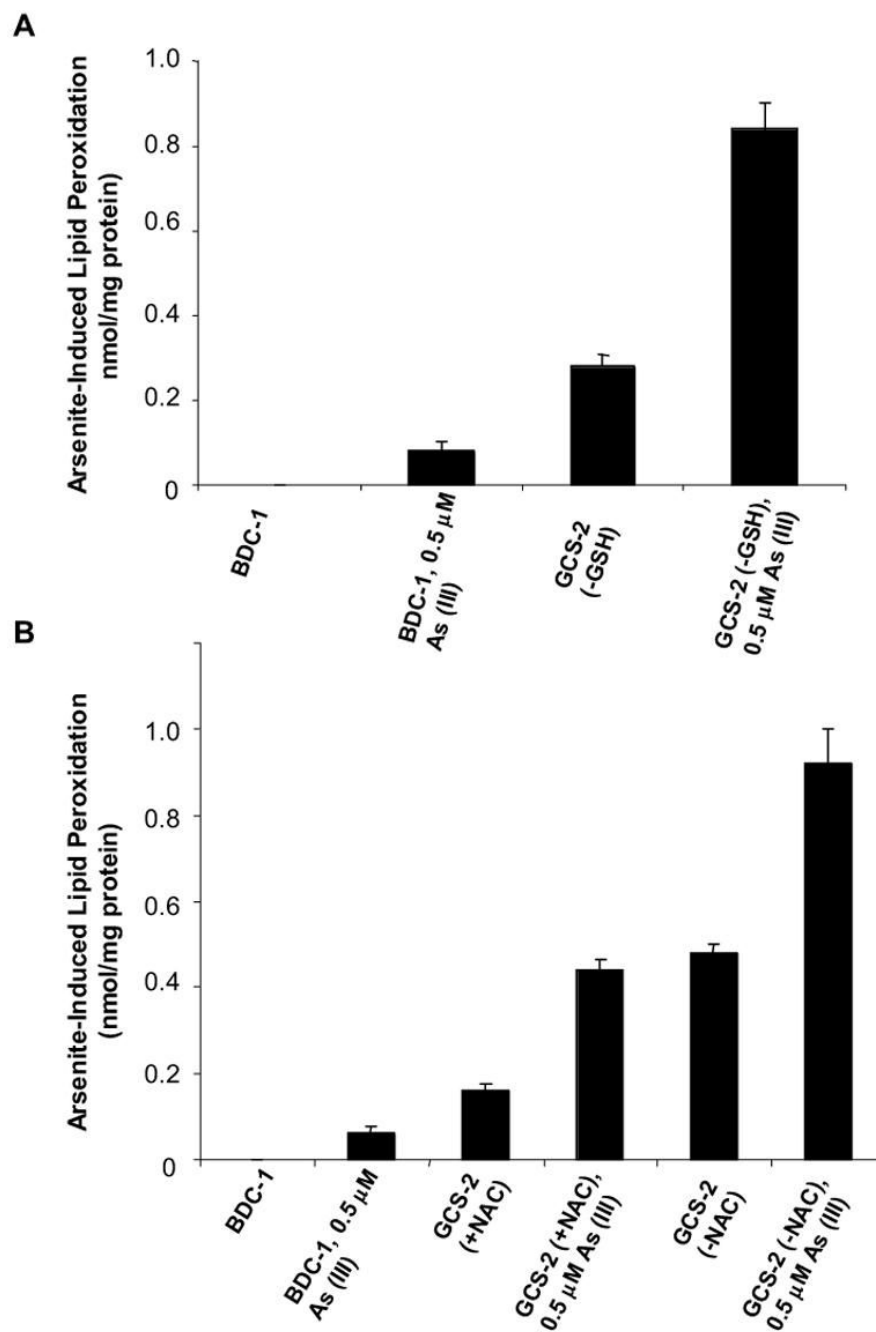
**Figure 4.**

Effect of arsenite on Hsp90 expression, ubiquitination, and association with co-chaperone and client proteins. (A) Effect of arsenite on the steady-state levels of Hsp90. Hsp90 was immunoprecipitated from total cell lysates with anti-Hsp90 antibody and immunoblotted with the same antibody. (B) Effect of arsenite on Hsp90 ubiquitination in GCS-2 cells. Hsp90 was immunoprecipitated with anti-Hsp90, separated by SDS-PAGE and analyzed by immunoblot with anti-ubiquitin antibody. (C) Effect of arsenite on Hsp90 association with its co-chaperone p50<sup>Cdc37</sup>. Cell lysates were immunoprecipitated with anti-Hsp90 antibody and blotted with anti-p50<sup>Cdc37</sup> antibody. (D) Effect of arsenite on steady-state protein levels of Hsp70. Cell lysates were immunoprecipitated with anti-Hsp70 antibody and immunoblotted with the same antibody. This also serves as a loading control. (E) Effect of arsenite on Plk-1 expression in GCS-2 cells. Total cell lysates were immunoblotted with anti-Plk-1 antibody. (F) Effect of arsenite on cyclin D-associated cdk-4 expression in GCS-2 cells. Plk-1 membrane was stripped and immunoblotted using anti-cdk-4 antibody. (G) Cdk-4 membrane was stripped and reprobed with actin-specific antibody to control for protein loading.



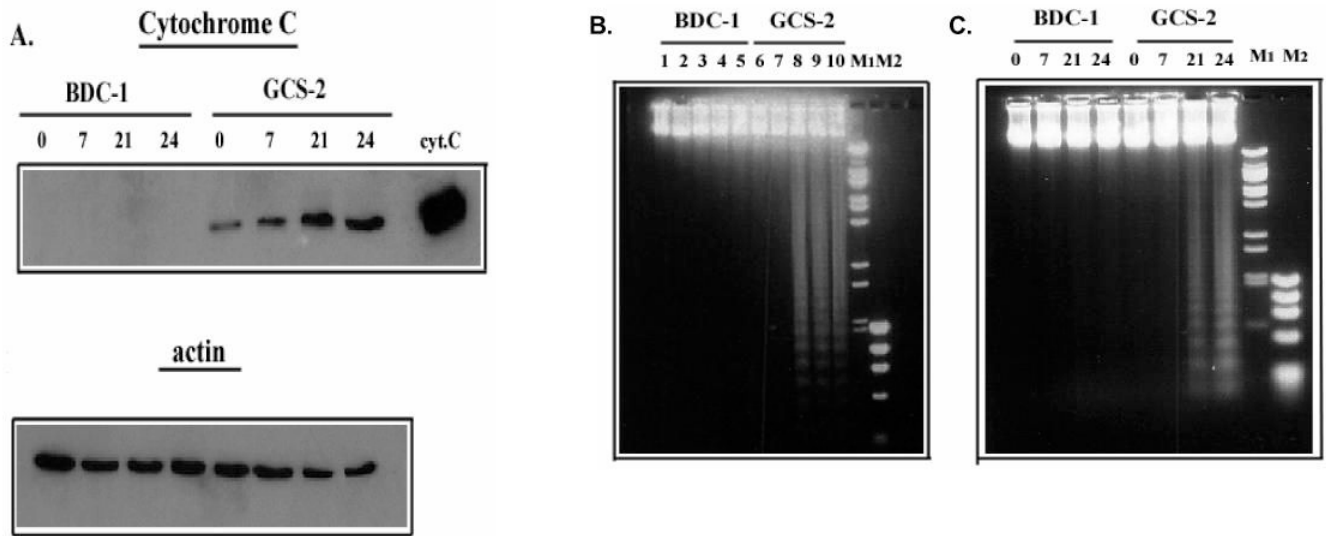
**Figure 5.**

Effect of arsenite on proteasome function in GCS-2 cells. (A) *In vitro* functional assay for proteasome activity. The chymotrypsin-like peptidase activity of the proteasome was assayed with fluorogenic substrate succinyl-leu-leu-val-tyr-AMC. Cells were treated with arsenite, washed, lysed, and assayed for chymotrypsin activity. Values are presented as mean  $\pm$  SEM for 5 determinations.  $p \leq 0.001$ ; RFU, relative fluorescence unit. (B) *In vivo* functional assay for the proteasome. BDC-1 and GCS-2 cells were transfected with pGFP<sup>u</sup> containing neomycin as described under “Materials and Methods”. Stably transfected cells were isolated, treated with and without arsenite and 21 h later the cells were assayed for intracellular fluorescence by confocal microscopy. B, D, F, and H are untreated cells; C, E, G, and I are cells treated with 0.5  $\mu$ M arsenite. B-E is confocal micrographs of GFP<sup>u</sup> expression in cells. F-H is the corresponding light micrographs.



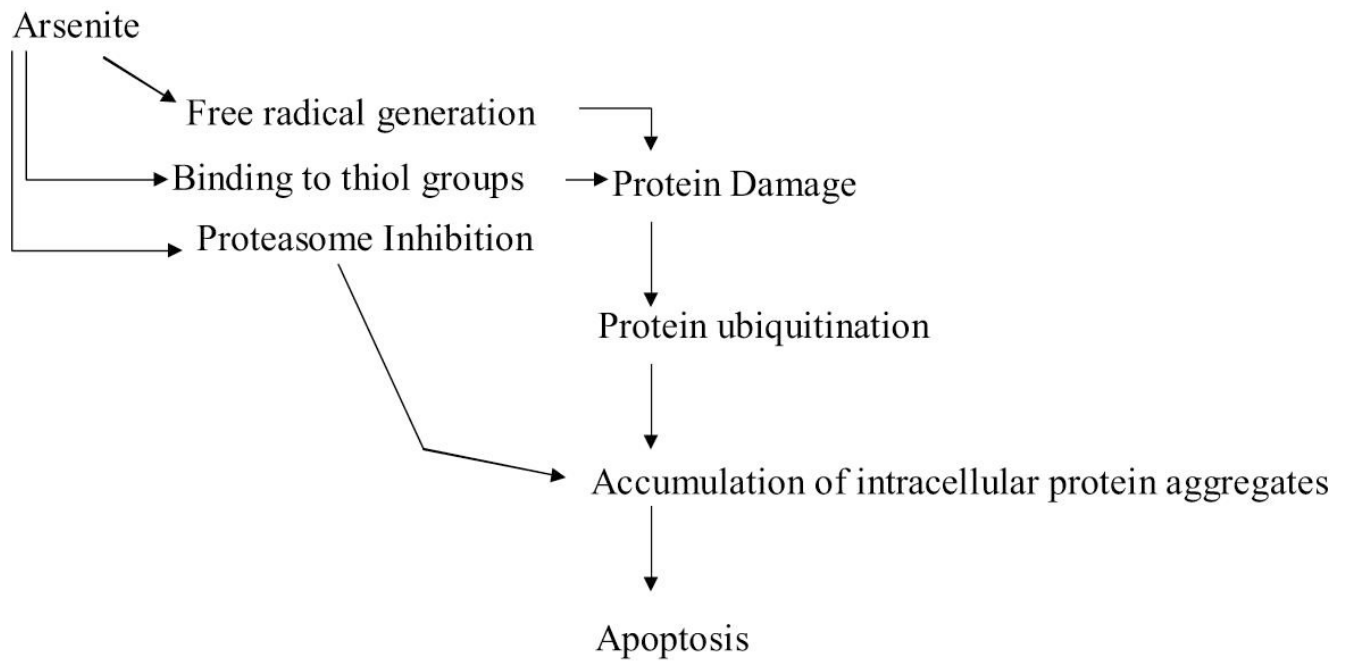
**Figure 6.**

Effect of arsenite on lipid peroxidation in GCS-2 cells. (A) Arsenite-induced lipid peroxidation in BDC-1 and GSH-dependent GCS-2 cells. Experimental conditions are as described in Fig. 1 except that the cells were treated with 0.5  $\mu$ M arsenite for 21 h. The amount of MDA formed in untreated BDC-1 cells was below the limits of detection. (B) Arsenite-induced lipid peroxidation in BDC-1 and NAC-dependent GCS-2 cells. The results in Fig. 6A and 6B are presented as mean  $\pm$  SEM for 3 determinations.



**Figure 7.**

Effect of arsenite on cytochrome C release in BDC-1 and GCS-2 cells. (A) Time-course of the effect of arsenite on the release of cytochrome C in BDC-1 and GCS-2 cells. Cells were treated with 0.5  $\mu$ M arsenite for the indicated times, and cytosolic extracts were separated by SDS-PAGE and immunoblotted with cytochrome C-specific antibody. Commercially available cytochrome C served as the positive control. The membrane was stripped and reprobed with an anti-actin antibody to ensure equal loading. (B) Dose response of arsenite-induced apoptosis in GCS-2 cells. Cells were treated with various concentrations of arsenite for 21 h, and DNA was isolated and analyzed on 1% agarose gels. Lanes 1 and 6, untreated; lanes 2 and 7, 0.2  $\mu$ M; lanes 3 and 8, 0.5  $\mu$ M; lanes 4 and 9, 1  $\mu$ M; lanes 5 and 10, 2  $\mu$ M arsenite treated. (C) Time-course of arsenite-induced apoptosis in GCS-2 cells. Cells were treated with 0.5  $\mu$ M arsenite for various time points and DNA was isolated and analyzed as described in "Methods".



**Figure 8.** Schematic representation of events induced by arsenite in the absence of GSH culminating in apoptotic cell death.

**Table 1**  
Arsenite-Induced Changes in Phosphorylation Status and Gene Expression in GCS-2 cells

Proteins	Phosphorylation	Changes in protein levels
<b>MAP Kinases</b>		
ERK1/2	Increased	No change
JNK1/2	Increased	No change
p38 MAPK	Increased	No change
<b>Transcription Factors</b>		
ATF-2	Increased	No change
C-Jun	Increased	No change
<b>Heat Shock Proteins</b>		
Hsp105	No change	No change
Hsp90	No change	Decreased
Hsp70	No change	No change
Hsp40	No change	No change
Hsp27	No change	No change
<b>Ubiquitin Pathway Enzymes</b>		
Ubiquitin activating enzyme (E1)	ND*	No change
Ubiquitin conjugating enzymes (E2)		
UBC-4	ND	No change
UBC-8	ND	No change
UBC-9	ND	No change
Ubiquitin Ligase (E3)		
E6-AP	ND	No change
Smurf-1	ND	No change
Smurf-2	ND	No change
NEDD-4	ND	No change
<b>Ribosomal S6 Kinases</b>		
p70 S6 kinase	No change	No change
p90 S6 kinases 1, 2, and 3	No change	No change

\* ND- not determined