

Sodium Arsenite-Induced Stress-Related Gene Expression in Normal Human Epidermal, HaCaT, and HEL30 Keratinocytes

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Arsenic is a carcinogen that poses a significant health risk in humans. Based on evidence that arsenic has differential effects on human, rodent, normal, and transformed cells, these studies addressed the relative merits of using normal human epidermal keratinocytes (NHEK) and immortalized human (HaCaT) and mouse (HEL30) keratinocytes when examining stress-induced gene expression that may contribute to carcinogenesis. We hypothesize that redox-related gene expression is differentially modulated by arsenic in normal versus immortalized keratinocytes. To test the hypothesis, we exposed keratinocytes to sodium arsenite for 4 or 24 hr, at which time *serine threonine kinase-25 (stk25)* and *nicotine adenine dinucleotide phosphate [nad(p)h] quinone oxidoreductase* gene expression were measured. The effect of glutathione reduction on arsenite-induced cytotoxicity and gene expression in NHEK also was evaluated by addition of L-buthionine-[S,R]-sulfoximine (BSO) to culture media. Results indicate the term LC₅₀ for arsenite is approximately 10–15 μM in NHEK and HEL30 keratinocytes and 30 μM in HaCaT keratinocytes. Compared with HaCaT and HEL30 keratinocytes, a nontoxic concentration of arsenite (2.5 μM) increases *stk25* and *nad(p)h quinone oxidoreductase* gene expression in NHEK, an effect partially attenuated by BSO. These data indicate that NHEK and HaCaT/HEL30 keratinocytes have similar sensitivities toward arsenite-induced cytotoxicity but unique gene expression responses. They also suggest that arsenite modulates gene expression in NHEK involved in cellular signaling and other aspects of intermediary metabolism that may contribute to the carcinogenic process. **Key words:** arsenite, glutathione, keratinocyte, redox, toxicity. *Environ Health Perspect* 110(suppl 5):761–766 (2002).

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Understanding toxicant effects on epidermal keratinocytes is critical because disturbances in keratinocyte function are responsible for the pathogenesis of many skin diseases such as psoriasis and skin cancer (1,2). Skin cancer is a common manifestation of arsenic exposure, and keratinocytes are a primary target of arsenic *in vivo* (3–9). Both primary and immortalized keratinocytes have been used in studies examining arsenic-induced skin cancer and epidermal toxicity. However, data derived from experiments using immortalized keratinocytes may be complicated by clonal variability, variations between passage, altered antioxidant complement, immortalization-specific gene expression, and alterations in differentiation capacity—variables not encountered to the same degree with primary keratinocytes.

Mechanisms proposed to be involved in the development of arsenic-induced cancer in skin and other tissues include altered DNA methylation (10–12), DNA repair/replication disturbances (13,14), clastogenicity and aneuploidy (15), dysregulated cell proliferation (10,16), and generation of oxidative stress (17,18). Arsenic-induced oxidative stress or redox disturbance (17,19–21) contributes to DNA damage (22–24), chromosomal aberrations (25), and protein expression alterations (22,26). However, its role in arsenic carcinogenesis is not completely understood. Glutathione

(GSH)-dependent enzymes are not significantly sensitive to arsenicals (27), but these enzymes play a significant role in attenuating the damaging effects of oxidative stress. It is conceivable that short- and long-term changes in GSH-dependent and stress-related mRNA/protein expression, modulated by arsenic, could abnormally affect cellular viability, phase I/II metabolism, and other GSH-mediated regulatory processes, and could be important in the development of arsenic-induced cancer.

This study was undertaken to determine the merits of using normal human epidermal keratinocytes (NHEK) and immortalized human (HaCaT) and mouse (HEL30) keratinocytes when examining effects of arsenite that may contribute to the development of cancer. Specifically, this study is designed to evaluate the effect of short-term arsenite exposure on keratinocyte viability under standard culture conditions and examine the ability of arsenite to modulate stress/redox-related gene expression in NHEK and HaCaT and HEL30 keratinocytes. It is proposed that NHEK display increased sensitivity to the gene-modulating effects of arsenite compared with HaCaT and HEL30 keratinocytes. The resulting data indicate that arsenite affects cellular viability in NHEK and HaCaT and HEL30 keratinocytes to a similar degree. However, arsenite elevates NHEK stress-related gene expression above

that seen in HaCaT and HEL30 keratinocytes. This study will help define the importance of using primary or immortal keratinocytes in experiments designed to elucidate the toxic and carcinogenic mode of action of arsenic in skin, as well as allow for the selection of appropriate *in vitro* models to evaluate dermatotoxicity.

Materials and Methods

Media and Reagents

Culture media for immortalized keratinocyte cultures. Rich media for mammalian cell culture (RPMI-1640), fetal calf serum (FCS), and L-glutamine were purchased from Life Technologies (Rockville, MD, USA).

Culture media for normal human keratinocytes. Keratinocyte basal medium (KBM-2) and growth supplements were obtained from BioWhittaker/Clonetics (Walkersville, MD, USA). Trypsin–EDTA, penicillin, streptomycin, and neutral red solution were obtained from Life Technologies. Sodium-m-arsenite and L-buthionine-[S,R]-sulfoximine (BSO) were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Tissue Culture

Adult NHEK (BioWhittaker/Clonetics) were cultured (passage ≤ 6) in low calcium concentration (0.15 mM) KBM-2 supplemented with epidermal growth factor (5 ng/mL) and bovine pituitary extract (50 mg/mL). The spontaneously immortalized human keratinocyte cell line HaCaT was donated by H. Hamadeh (National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA) and was cultured in RPMI-1640, penicillin 50,000 UI/L, streptomycin 50 mg/L, and

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10% FCS. Immortalized HEL30 keratinocytes were a gift from E. Corsini (Laboratory of Toxicology, Institute of Pharmacological Sciences, University of Milan, Milan, Italy) and were cultured similar to HaCaT keratinocytes. Cells were incubated at 37°C in a CO₂-enriched atmosphere (5%), and for all experiments, arsenite treatment was carried out at approximately 50–70% confluence and during logarithmic growth.

Cytotoxicity Determination

Keratinocytes were seeded into 96-well plates (2,500 cells/well), grown to approximately 50–70% confluence (2–3 days), and treated with 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, or 100 μM sodium arsenite in KBM-2 without bovine pituitary extract/hydrocortisone (NHEK), or RPMI-1640 (HaCaT and HEL30) (in triplicate). Viability was assessed 24 hr later by incubation with 50 μg/mL neutral red for 3 hr at 37°C. Cells were fixed in formaldehyde/CaCl₂, and dye taken up by viable cells was extracted with ethanol/acetic acid prior to absorbency determination at 570 nm using a Dynex microplate reader and Revelation software (Thermo Lab Systems, Chantilly, VA, USA). GSH-depletion experiments were performed by addition of BSO (100 μM) to culture media 24 hr prior to and during treatment with arsenite. Two hundred fifty micromolar BSO produces similar effects on arsenite-induced cytotoxicity to those seen at 100 μM.

Northern Blot Analysis

Keratinocytes were seeded into 150-mm² tissue culture dishes in KBM-2 (NHEK) or RPMI-1640 (HaCaT and HEL30) and grown to 50–70% confluence, then the medium was replaced with KBM-2 without bovine pituitary extract/hydrocortisone (NHEK) or RPMI-1640 (HaCaT and HEL30). Following a 1-hr acclimation at 37°C, sodium arsenite was added to a final concentration of 0, 0.005, or 2.5 μM for either 4 or 24 hr. Total RNA was isolated at each time point using the Qiagen RNeasy miniprep system (Qiagen, Valencia, CA, USA), and 10- to 20-μg aliquots were fractionated by formaldehyde–agarose gel electrophoresis. After transfer to nylon membranes, RNA was hybridized to ³²P-labeled probes for human *serine/threonine kinase-25* (*stk25*) (GenBank accession no. AA043533) or *nicotine adenine dinucleotide phosphate [nad(p)h quinone oxidoreductase* (GenBank accession no. H25860), all prepared using random primers methodology (Life Technologies). A ³²P-labeled probe for mouse *nad(p)h quinone oxidoreductase* (GenBank accession no. BC004579) was used for HEL30 *nad(p)h quinone oxidoreductase* detection. Human/mouse homology is approximately 89% (*stk25*) at the nucleotide level. Band

intensity was quantified using a Molecular Dynamics phosphoimaging system and Image Quant software (Molecular Dynamics, Piscataway, NJ, USA), and values obtained for arsenite-treated samples were graphed as percent of time-matched controls (no arsenite). Northern blot data presented in each figure are representative of an experiment replicated 2 times.

Data and Statistical Analysis

When appropriate, analysis of variance (ANOVA) was performed. Significance was determined using Dunnett's test ($p < 0.05$).

Results

Arsenite-induced cytotoxicity was quantified using neutral red uptake in NHEK and HaCaT and HEL30 keratinocytes. Figure 1 shows the median lethal concentration (LC₅₀) for arsenite in NHEK and HEL30 cells to be approximately 10–15 μM after 24-hr exposure, whereas the LC₅₀ for HaCaT keratinocytes is approximately 30 μM. Cytotoxicity measured at 48 hr is similar to that at 24 hr in NHEK and HaCaT and HEL30 keratinocytes and indicates that arsenite concentrations ≤ 3 μM are nontoxic (data not shown).

Arsenic-induced stress has been examined in keratinocytes; however, arsenite-modulated stress-related gene expression is not well characterized in NHEK. Our laboratory previously

determined that NHEK treated with arsenite display alterations in stress/redox-related gene expression (28). Two of these genes, *stk25* and *nad(p)h quinone oxidoreductase*, were chosen to evaluate if physiologically relevant concentrations of arsenite (low and high nontoxic concentrations, 0.005 or 2.5 μM, respectively) regulate stress/redox-related gene expression differentially between NHEK and HaCaT and HEL30 keratinocytes. Figures 2 and 3 are representative Northern blots examining gene expression at time points consistent with arsenite-induced immediate early gene expression, cytokine induction, and effects on cell division. Figure 2A indicates that NHEK *nad(p)h quinone oxidoreductase* expression is elevated at 24 hr of arsenite exposure (2.5 μM) approximately 3.4-fold but remains near baseline at 4 hr (1.3-fold). Arsenite at the same concentration results in a minimal increase in *nad(p)h quinone oxidoreductase* expression in HaCaT keratinocytes (1.2-fold at 4 and 24 hr) (Figure 2B), and in HEL30 keratinocytes 1.1- and 1.5-fold changes are observed at 4 and 24 hr, respectively (Figure 2C). Arsenite-induced stress-related gene expression is demonstrated further in Figure 3. Expression of *stk25* in NHEK is elevated approximately 2-fold (4 hr) and 2.7-fold (24 hr) at 2.5 μM arsenite (Figure 3A), whereas in HaCaT keratinocytes it remains near baseline at 4 and 24 hr (1.4- and 1.2-fold, respectively) (Figure 3B). Figure 3C

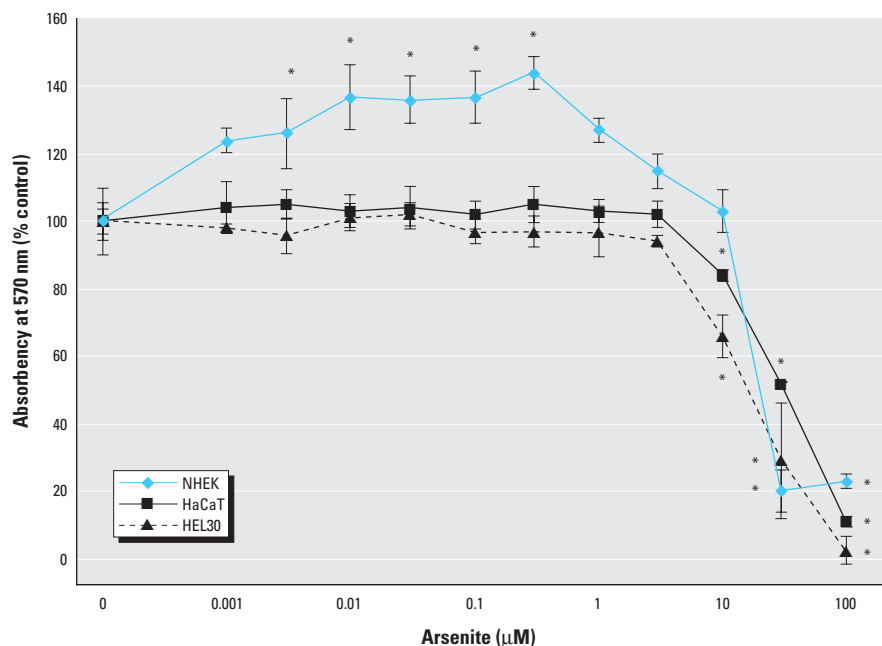


Figure 1. Effect of sodium arsenite on cellular viability in NHEK and HaCaT and HEL30 keratinocytes. Cells were seeded into 96-well plates (2,500 cells/well) and grown to approximately 50% confluence. The indicated concentrations of sodium arsenite were added in triplicate and cells were allowed to incubate for 24 hr. Medium containing arsenite was removed and replaced with fresh medium containing neutral red (50 μg/mL) for 3 hr. The cells were fixed, neutral red was extracted, and dye absorbency was quantified at 570 nm using a microplate reader. Each point represents the mean of three samples. Error bars denote SEM, and asterisk (*) indicates a statistically significant difference ($p < 0.05$) from control (no arsenite) as determined by ANOVA. Data are representative of an experiment replicated a minimum of 3 times.

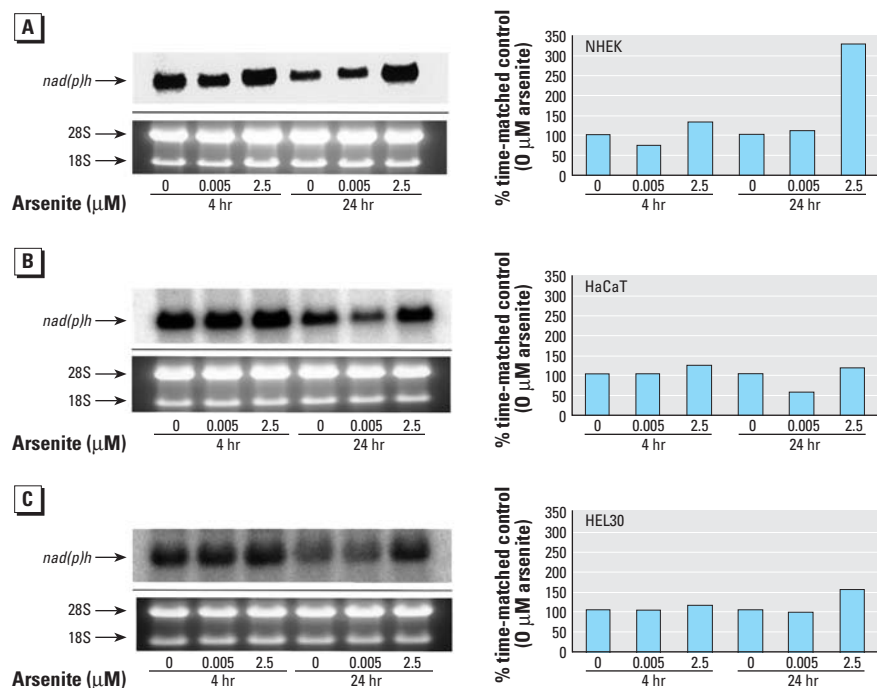


Figure 2. Arsenite-modulated *nad(p)h* quinone oxidoreductase expression in NHEK and HaCaT and HEL30 keratinocytes. Northern blot analysis of 20 μg total RNA from NHEK or HaCaT or HEL30 keratinocytes exposed to arsenite for 4 or 24 hr and probed for *nad(p)h* quinone oxidoreductase. Band intensity was digitized using Image Quant software (Molecular Dynamics). (A) NHEK. (B) HaCaT keratinocytes. (C) HEL30 keratinocytes. Ribosomal ribonucleic acid (rRNA) bands (28 and 18S) from an ethidium bromide stained-gel indicate equivalent loading of RNA. Bar graphs indicate percent increase above time-matched control (0 μM arsenite).

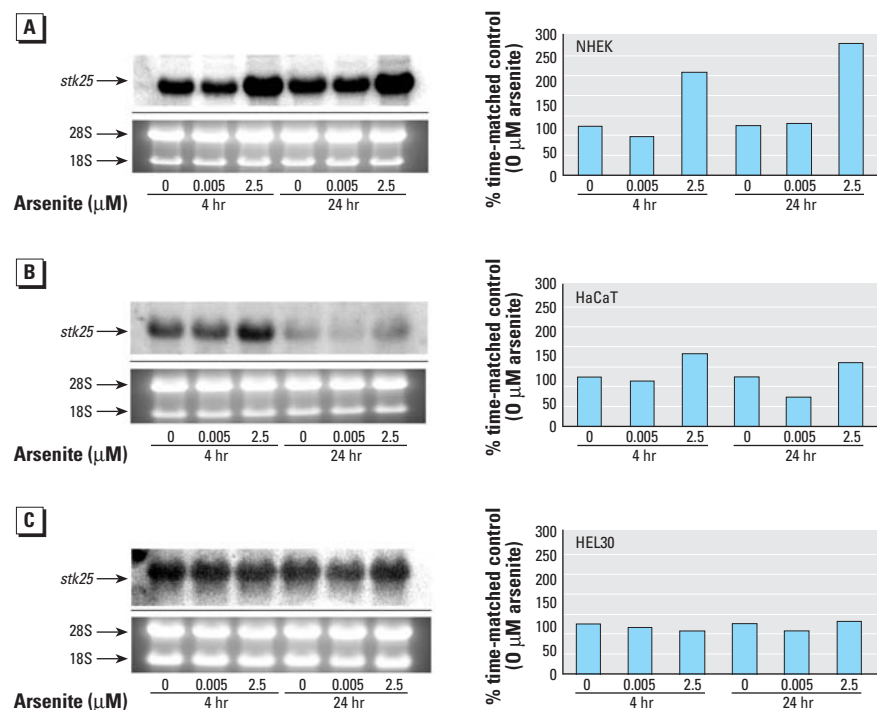


Figure 3. Arsenite-modulated *stk25* expression in NHEK and HaCaT and HEL30 keratinocytes; Northern blot analysis of 20 μg total RNA from NHEK and HaCaT and HEL30 keratinocytes exposed to arsenite for 4 or 24 hr and probed for *stk25*. Band intensity was digitized using Image Quant software (Molecular Dynamics). (A) NHEK. (B) HaCaT keratinocytes. (C) HEL30 keratinocytes. rRNA bands (28 and 18S) from an ethidium bromide-stained gel indicate equivalent loading of RNA. Bar graphs indicate percent increase above time-matched control (0 μM arsenite).

reveals that in HEL30 keratinocytes there is no change in *stk25* expression at 24 hr and a slight reduction at 4 hr (0.9-fold) after exposure to 2.5 μM arsenite.

GSH attenuates the damaging effects of arsenite in numerous biological systems (26,29,30), and reduction in cellular GSH exacerbates cellular responses to redox-regulating agents (31,32) and arsenic (33,34). Figure 4 demonstrates that the addition of 100 μM BSO to culture medium shifts the LC_{50} of arsenite 10-fold to approximately 1 μM in NHEK, suggesting that a reduction in GSH increases the potency of arsenite. Similar effects were observed in HaCaT and HEL30 keratinocytes (data not shown). BSO has no effect on NHEK viability at arsenite concentrations ≤ 0.3 μM , suggesting that low concentrations of arsenite do not produce cytotoxicity under conditions where the level of cellular GSH is compromised. To determine if a reduction in GSH influences arsenite-induced *nad(p)h* quinone oxidoreductase and *stk25* expression, NHEK were exposed to 0.005 or 2.5 μM arsenite and BSO as described in "Materials and Methods." Data in Figure 5 indicate that BSO treatment does not result in the induction of *nad(p)h* quinone oxidoreductase or *stk25* expression at 0.005 μM arsenite. However, Figure 5A shows that the gene-inducing effect of 2.5 μM arsenite is attenuated when used in combination with BSO, resulting in a 0.4- and 2.6-fold change in *nad(p)h* quinone oxidoreductase expression at 4 and 24 hr, respectively. (Compare with 1.3- and 3.4-fold from control NHEK, cross-hatched bars.) Figure 5B reveals that *stk25* induction at 2.5 μM arsenite also is attenuated after BSO treatment (1.4- and 1.8-fold at 4 and 24 hr, respectively; compare with 2- and 2.7-fold from control NHEK, cross-hatched bars).

Discussion

NHEK and HaCaT and HEL30 keratinocytes are epidermal cells used to characterize the effects of arsenic on proliferative gene expression (5,35), cytokine and growth factor expression (6,8,36), and the role reactive oxygen species (ROS) play in epidermal dysfunction and signal transduction modulation (37–39). Although well suited with respect to target tissue, these cells present unique phenotypes that may display different responses to oxidative stress-inducing agents like arsenic. NHEK are resistant to transformation when grown under normal conditions *in vitro*, have a finite lifespan, and retain many physiological characteristics observed *in vivo* (e.g., differentiation response) (40). The human HaCaT cell line displays a transformed phenotype *in vitro* but remains non-tumorigenic (41). Despite an unlimited growth potential, HaCaTs, similar to normal

human keratinocytes, can form a structured and differentiated architecture when transplanted into nude mice, and they also express differentiation-specific keratins and other markers (e.g., involucrin and flaggrin) (41). The HEL30 cell line is derived from the spontaneous transformation of murine keratinocytes grown *in vitro* (42–46) and is tumorigenic and invasive (47).

Arsenic is cytotoxic to normal human keratinocytes (35), fibroblasts (48,49), lymphocytes (50), hepatocytes (51), and epidermoid carcinoma cells (52). In a majority of these studies, short-term exposure to trivalent arsenicals results in significant cytotoxicity at concentrations between 1 and 10 μM . In our study, arsenite-induced cytotoxicity was dose dependent and resulted in a short-term

LC₅₀ of approximately 10–15 μM in NHEK and HEL30 keratinocytes and 30 μM in HaCaT keratinocytes. NHEK and HEL30 keratinocytes present a similar sensitivity toward arsenite-induced cytotoxicity that is hard to resolve because rodent cells generally have increased antioxidant capabilities compared with human cells (49,53). HaCaT keratinocytes display alterations in antioxidant enzyme levels (54), and this may partially explain the increased LC₅₀ for arsenite in this cell type.

We chose to examine the expression of two genes regulated by extra and intracellular stress (e.g., *stk25* and *nad(p)h* quinone oxidoreductase) at two nontoxic concentrations of arsenite (0.005 and 2.5 μM). Ste20-homologous proteins (e.g., STK25) are implicated as important transducers of signals from the p21 family of GTPases (55,56), can be activated by cellular stress, and are important mediators of oxidant-mediated signal transduction (57,58). In this study arsenite robustly induced NHEK *stk25* expression, an effect more profound in primary cells, and an effect attenuated by reducing GSH level with BSO. Whether the ability of NHEK to respond so robustly compared with HaCaT or HEL30 keratinocytes is a detoxification mechanism preserved in primary cells or a prelude to immortalization is unknown at present. However, the elevated baseline *stk25* expression observed in HaCaT and HEL30 keratinocytes would support the notion that this alteration is associated with immortalization. Along with previously defined effects of arsenic on mitogen and stress-related signal transduction (7,10,16,33,59), these data implicate STK25 in the transduction of arsenite-mediated stress signals.

NAD(P)H quinone oxidoreductase is a flavoprotein that catalyzes the reduction of quinones, quinone imines, and azo dyes, thereby protecting cells against free radical and ROS-mediated mutagenicity and carcinogenicity (60–62). Elevated NAD(P)H quinone oxidoreductase activity and gene expression are observed in both preneoplastic tissues and established tumors (63,64) and are induced by a variety of compounds including planar aromatic hydrocarbons, phenolic antioxidants, tumor promoters, and hydrogen peroxide (61). In our study, *nad(p)h* quinone oxidoreductase expression was dramatically increased in NHEK after exposure to a nontoxic concentration of arsenite (approximately 4-fold at 2.5 μM) compared with HaCaT and HEL30 keratinocytes. Interestingly, a similar trend in baseline and arsenite-induced *nad(p)h* quinone oxidoreductase expression in both HaCaT and HEL30 keratinocytes was observed, suggesting an immortalization/preneoplastic effect on gene expression. The mechanism(s) involved in

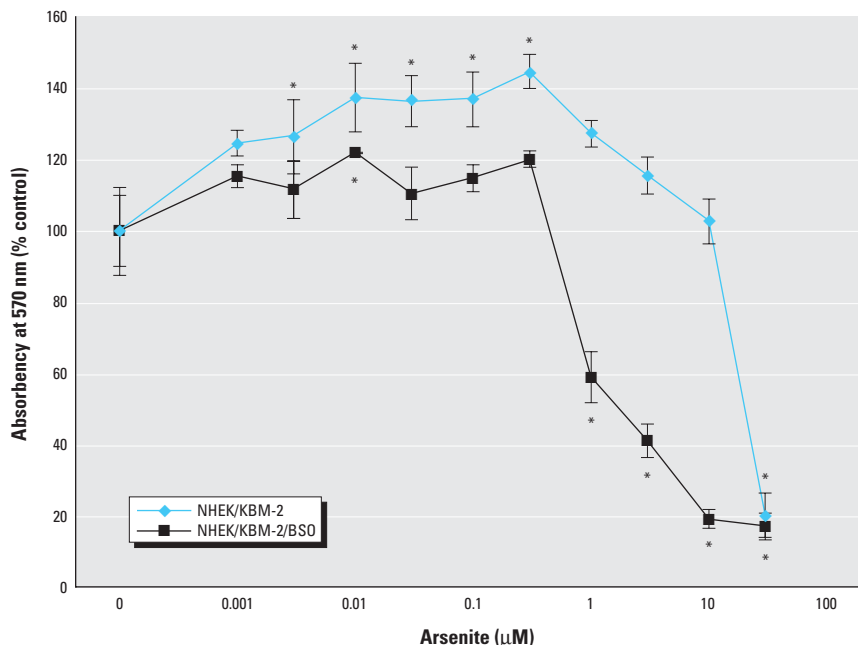


Figure 4. Effect of BSO on arsenite-induced cytotoxicity in NHEK. Cells were prepared as in Figure 1 with the exception that BSO (100 μM) in fresh culture medium was added to cells 24 hr prior to arsenite treatment. After removal of medium, the indicated concentrations of arsenite in fresh medium containing BSO (100 μM) were added to triplicate wells, and cells were allowed to incubate for an additional 24 hr. Cellular viability was determined as in Figure 1. Each point represents the mean of three samples. Error bars denote SEM, and asterisk (*) indicates a statistically significant difference ($p < 0.05$) from control (no arsenite) as determined by ANOVA. Data are representative of an experiment replicated a minimum of 3 times.

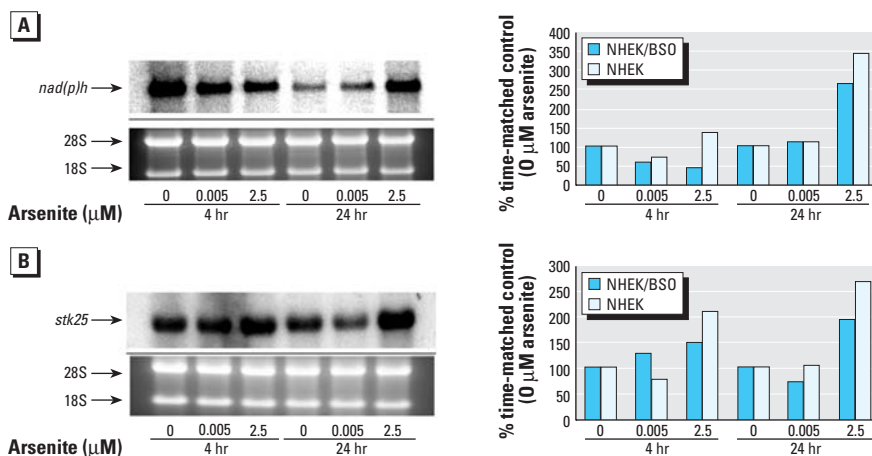


Figure 5. Effect of BSO treatment on arsenite-induced *nad(p)h* quinone oxidoreductase and *stk25* expression in NHEK; Northern blot analysis of 10 μg total RNA from NHEK pretreated with 100 μM BSO for 24 hr, exposed to arsenite for 4 or 24 hr, and probed for *nad(p)h* quinone oxidoreductase or *stk25*. Band intensity was digitized using Image Quant software (Molecular Dynamics). (A) *nad(p)h* quinone oxidoreductase. (B) *stk25*. rRNA bands (28 and 18S) from an ethidium bromide-stained gel indicate equivalent loading of RNA. Bar graphs indicate percent increase above time-matched control (0 μM arsenite). Black bars = BSO-treated NHEK and cross-hatched bars = data from control NHEK presented previously in Figures 2A and 3A.

arsenite-mediated *nad(p)h* quinone oxidoreductase induction in NHEK is currently unknown. However, oxidative stress activates activator protein-1 (AP-1) in human keratinocytes and other cells (65,66) and may contribute to elevated *nad(p)h* quinone oxidoreductase (known to be regulated by AP-1 and nuclear factor kappa B [NF-κB]) expression after arsenite exposure. This notion is further supported by arsenic-induced modulation of *c-jun* and *c-fos* *in vitro* and *in vivo* (67–69) and NF-κB activity *in vitro* (70,71).

We proposed that reducing cellular GSH levels with a specific inhibitor of GSH synthesis (BSO) could exacerbate the gene expression-modifying effects of arsenite on NHEK. However, in our experiments, treatment of NHEK with BSO attenuated the effect of arsenite on *nad(p)h* quinone oxidoreductase and *stk25* expression. The mechanism(s) that resulted in this effect is unknown but could be due in part to general cellular toxicity (after concurrent arsenite and BSO exposure) that disallows a normal gene expression response. In support of this, arsenite in combination with BSO increased cytotoxicity in the cell line HepG2, yet cellular GSH remained at the same level after BSO treatment alone (72). This supports the contention that general cellular toxicity may attenuate gene expression responses independent of GSH level.

In summary, the results of this study indicate that NHEK stress/redox-related gene expression is induced after nontoxic arsenite exposure. The ability of arsenite to modulate stress/redox-related gene expression will need to be incorporated into mechanism-based models for arsenic risk assessment. This will be important when assessing the potential health effects of arsenic exposure and in formulating regulatory guidelines regarding human exposure to this toxicant and carcinogen.

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