Journal of Cellular Biochemistry 87:29–38 (2002)

Arsenic Induces Oxidative Stress and Activates Stress Gene Expressions in Cultured Lung Epithelial Cells

Muyao Li,[1] Jiu-Feng Cai,[2] and Jen-Fu Chiu[2,3]*

[1]Department of Medicine, University of Vermont College of Medicine, Burlington, Vermont 05405
[2]Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05405
[3]Institute of Molecular Biology and Open Laboratory of Chemical Biology of the Institute of Molecular Technology for Drug Discovery and Synthesis, University of Hong Kong, Hong Kong, China

Abstract Chronic exposure to low levels of arsenic can cause lung cancer. However, the cellular and molecular mechanisms for lung cell transformation in response to arsenic are not known. These studies investigated the hypothesis that low levels of arsenic increase intracellular oxidant levels, promote production of mitogenic transcription factors and antioxidant enzymes. Initially, arsenic decreased GSH cellular level and rapidly increased to 280% of GSH level in nonexposed lung cells in 24 h. Buthionine sulfoximine (BSO) potentiated the arsenic toxicity of lung epithelial cells (LEC). Exposure of LEC to 5 μM arsenite cause time-dependent increase in γ-glutamylcysteine synthetase (γ-GCS) expression. Our data demonstrated that arsenic induced the heavy subunit of γ-GCS (γ-GCS-HS) mRNA levels as early as 4 h as compared to the control level. It significantly increased (sixfolds) γ-GCS-HS mRNA expression after 8 h of treatment. The activation of AP-1 transcription factors may also play a regulatory role in this process. Significant elevations in c-fos and c-jun mRNA levels were observed within 30 min after exposure to arsenic and by enhancement of AP-1 DNA binding activity and transactivation activity. Responsiveness of LEC to oxidative stress caused by arsenic exposure was further evaluated with mobility shift assay involving redox-sensitive transcription factor NF-κB. The specificity of binding was verified by an antibody-supershift. The NF-κB DNA binding activities increased more than twofold 30 min after exposure to arsenic and returned to control levels after 4 h of treatment. It remains to be determined whether NF-κB plays a role in the As-induced apoptosis or alternatively in attempting to protect the cells from As-induced cell death by upregulating the expression of resistance factors. J. Cell. Biochem. 87: 29–38, 2002.

Key words: arsenic; lung cells; oxidative stress; glutamycysteine synthetase; c-fos; c-jun; NF-KB

Environmental carcinogens have been postulated to be an important etiological factor for the majority of human cancer [IARC, 1982]. Arsenic, a known human carcinogen and teratogen, is widely found in nature and is distributed throughout the environment by water, food, and air. Epidemiological studies have shown that chronic exposure to arsenic can result in an increased incidence of cancer of the lung, skin, bladder, and liver [IARC, 1987; Bates et al., 1992; Chen et al., 1992; Nriagu, 1994; Waalkes, 1995; Schwartz, 1997]. Despite a wealth of epidemiological and clinical data, its carcinogenic mechanism so far remains unclear.

For reasons that are not clear, arsenic is not carcinogenic in rodent models [Wang and Rossman, 1996]. In the absence of animal models, in vitro studies become particularly important in providing information on the carcinogenic mechanisms of arsenic. Reactive oxygen species (ROS) have been implicated in the pathogenesis of cancer. Oxidative stress can be involved in either initiation, promotion, or progression [Guyton and Kensler, 1993; Kaidobad et al.,

Abbreviation used: AP-1, activator protein-1; BSO, buthionine sulfoximine; CAT, chloramphenicol acetyltransferase; DHRh-123, dihydrorhodamine-123; EMSA, electrophoretic gel mobility shift assay; γ-GCS, γ-glutamylcysteine synthetase; γ-GCS-HS, heavy chain of γ-glutamylcysteine synthetase; GSH, glutathione; GST, glutathione S-transferase; LEC, rat lung epithelial cell; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor κB; Rh-123$^+$, rhodamine-123$^+$; ROS, reactive oxygen species.

Grant sponsor: University of Hong Kong; Grant number: 10204004/39815/43700/301/01. Grant sponsor: AOE Scheme of Hong Kong University Grants Committee.

*Correspondence to: Jen-Fu Chiu, Institute of Molecular Biology, University of Hong Kong, Hong Kong.

Received 4 June 2002; Accepted 14 June 2002

DOI 10.1002/jcb.10269

1997]. Several recent studies suggest that the genotoxicity of arsenic may be mediated by ROS. Although the lung is one of the major organs that affected by arsenic, little information is presently available for the production of ROS in arsenic-exposed lung cells.

Inhalation of metals, such as arsenic, beryllium, cadmium, chromium, nickel, and vanadium, may promote directly and indirectly enhanced generation of ROS in pulmonary tissue [Ding et al., 2000]. ROS generation can cause specific molecular changes resulting in activation or inactivation of transcription factors that may alter gene expression leading to cell proliferation, differentiation, and carcinogenesis. The mechanisms responsible for arsenic-related lung pathogenesis are not well understood. Arsenic does not act through classic genotoxic and mutagenic mechanisms, as do other metals such as cadmium or chromium [Simeonova and Luster, 2000], but oxidative damage is believed to play a major role. Recently, Hei et al. [1998] have shown that arsenic is indeed mutagenic to endogenous genes in mammalian cells and that it induces mostly large multilocus deletions that are mediated through ROS. These data are consistent with the recent demonstration that antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and particularly catalase reduces the incidence of arsenite-induced chromosomal aberrations and sister chromatid exchanges in cultured mammalian cells [Nordenson and Beckman, 1991; Wang and Huang, 1991].

The mechanism responsible for the production of ROS in arsenic-exposed lung cells is not fully understood. Glutathione is the major thiol compounds in the lung cells that maintain redox homeostasis and cope with the ROS produced during oxidative stress. Current evidence indicates that ROS can act as signaling messengers to activate transcription factors and induce gene expression. Activator protein-1 (AP-1) and nuclear factor κB (NF-κB) are examples of transcription factors that are sensitive to changes in cellular redox state and are capable of binding to a specific response element present in the 5′-flanking region of genes that regulate cell differentiation, proliferation, and cell death [Barchowsky et al., 1996; Cavigelli et al., 1996]. For example, AP-1 protein complexes bind to 5′-flanking region of genes, including γ-glutamylcysteine synthetase (γ-GCS), the first enzyme in the glutathione synthesis, and glutathione S-transferases (GSTs) that catalyze conjugation of glutathione (GSH) to electrophiles. Stress of various types and the subsequent restoration of cellular homeostasis after insults often lead to the activation or silencing of genes encoding for regulatory transcription factors, acute-phase proteins, antioxidant enzymes, and structural proteins [Muller et al., 1997; Dalton et al., 1999].

In this study, using a well-characterized rat alveolar epithelial cell line, we designed to examine the hypothesis that arsenic-mediated oxidant stress stimulates γ-GCS gene expression in cultured lung cells, and to explore potential mechanisms that may be involved in regulation of γ-GCS altered gene expression.

METHODS AND EXPERIMENTAL PROCEDURES

Cell Line and Culture Condition

Rat lung epithelial cell (LEC) line used in this investigation was isolated and characterized by Li et al. [1983]. Cells were routinely grown at 37°C in 95% air/5% CO_2 using F-12 nutrient supplemented with 2 mmol/L glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO-BRL, Grand Isle, NY), and 10% newborn bovine serum (JRH Bioscience, Lenexa, KS). Unless otherwise stated, cells were grown to 75% confluence and changed to fresh complete medium 18–24 h prior to use in experiments.

Glutathione Analysis

Intracellular levels of glutathione were determined as described in our previous article [Shukla et al., 2000a]. Briefly, following extraction of the cells with 3.5% perchloric acid, a high-pressure liquid chromatography (HPLC) assay with *o*-phthalaldehyde precolumn derivatization was used to measure total soluble glutathione. In another set of experiments, cultured cells were treated with buthionine sulfoximine (BSO, Sigma, St. Louis, MO) at concentrations and times described in the text and figure legend. Stock solution of BSO was filtered and sterilized prior to use.

Determination of ROS Formation

Intracellular ROS generation was measured by oxidation of dihydrorhodamine-123 (DHRh-123) to rhodamine-123$^+$ (Rh-123$^+$), as described previously [Shukla et al., 2000b]. To perform the analysis, cells were washed once with Hank's balanced salt solution (HBSS) (GIBCO-BRL) and then were incubated for varying times, in

the presence or absence of arsenite, with 2 μmole/L DHRh-123 (Molecular Probes, Eugene, OR). Following incubation, the cells were rinsed with HBSS and subsequently evaluated with a Perkin Elmer spectrofluorometer using excitation and emission wavelengths of 500 and 530 nm, respectively.

Electrophoretic Gel Mobility Shift Assay (EMSA)

EMSA were used to demonstrate activation and translocation of proteins that bind to specific consensus DNA sequences for AP-1 and NF-κB as described previously [Shukla et al., 2000c]. The binding site for the AP-1 protein complex (i.e., 5′-AGCATGAGTCAGACACCTCTGGC-3′) or for the NF-κB protein complex (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were labeled using T_4 polynucleotide kinase (Boehringer-Mannheim) and [γ-^{32}P]ATP (4,000 Ci/mmol, ICN Costa Mesa, CA). After incubation of nuclear protein (5 μg) with 0.5 ng of labeled probe, the reaction mixture was resolved on a nondenaturing polyacrylamide gel. Afterward, the gel was dried, autoradiographed, and radioactivity was measured with PhosphoImager (Bio-Rad, Hercules, CA). Supershift assays were performed with affinity purified, polyclonal antibody to p50 (Santa Cruz Biotechnology, Santa Cruz, CA). Nuclear extracts were incubated with labeled probe as above, then incubated for an additional hour with 1.0 μg of the antibody.

Northern Blotting

Northern blotting analysis was conducted according to the procedure of Shukla et al. [2000a]. Total cellular RNA was extracted from cell monolayers with 1 ml of RNA-STAT-60 (Tel-Test, Inc., Friendswood, TX) per 100-mm dish, following the manufacturer's instructions. RNA was electrophorecticlly separated on agarose gel and blotted to Zeta-probe GT nylon membrane (Bio-Rad Laboratories, Richmond, CA). A DNA segment for γ-GCS was cut from pCRII-MGCS-Z plasmid and was labeled with [^{32}P]dCTP using random primer labeling kit (Gibco-BRL) and used for hybridization probes. Hybridization signals were detected using Biomax MS autoradiography film (Eastman Kodak Co., Rochester, NY) and quantitated using a Bio-Rad GS-250 PhosphoImager (Bio-Rad, Hercules, CA).

For determining the expressions of c-jun and c-fos, c-fos and c-jun cDNA plasmids were used as hybridization probes as described in our previous paper [Zhang et al., 1991]. A 190-bp fragment of the entire 7S cDNA sequence (280 bp from plasmid pA6) was used to detect 7S RNA for internal control for Northern hybridization.

Statistical Analysis

Statistical comparisons were performed by unpaired *t*-tests or analysis of variance using Student–Newman–Keuls to adjust for multiple comparisons. When variability between groups differed, logarithmic transformations of data were used for the analysis. The level of significance was set at $P < 0.05$.

RESULTS

Oxidative Stress Induced by Arsenic Exposure

A principal intracellular defense of mammalian cells against low levels of oxidants is glutathione peroxidase catalyzed reaction of oxidants with GSH. GSH is a thiol peptide and antioxidant that plays an important role in many xenobiotic detoxification reactions including arsenic detoxification. Cellular toxicity is inversely related to intracellular GSH levels and is exacerbated by GSH depletion. Arsenite is directly thiol-reactive. Several reports described that prolonged arsenite treatment results in GSH depletion [Royall and Ishiropoulos, 1993]. The effect of arsenic treatment on the change in lung cellular redox status was examined. As shown in Table I, a significant decrease (31.3%) in GSH was observed in the first 15 min after the treatment of LEC cells with 5 μM sodium arsenite. Recovery was rapid, as evidenced by the return of GSH to baseline levels at 1 h. Cellular

TABLE I. Effect of Arsenite on Glutathione Status in Rat Lung Epithelial Cells

Time (h)	n	Total glutathione (nmol/mg protein)
0.00	4	29.75 ± 2.5
0.25	4	20.45 ± 2.1
0.50	4	24.15 ± 1.4
1.00	4	27.31 ± 3.2
2.00	3	36.45 ± 4.9
4.00	4	45.55 ± 5.9
8.00	3	57.22 ± 5.4
12.00	4	67.79 ± 7.9
24.00	4	83.81 ± 9.1

LEC cells were grown to 75% confluence, changed to fresh complete medium for 24 h, and then treated with 5 μM sodium arsenite for the times designated. Results are expressed as the mean ± SEM, where n = 3–4.

GSH continuously rose and reached 280% of GSH level in nonexposed LEC cells by 24 h.

DHRh-123 was used to determine intracellular production of oxidants in response to arsenic treatment. LEC cells were incubated in the presence of DHRh-123 and 5 μM of sodium arsenite for 0, 10, 20, 40, and 60 min. The intensity of $Rh\text{-}123^{+}$, formed by the interaction of DHRh-123 with intracellular oxidants such as hydrogen peroxide, was subsequently quantified using spectrofluorometry. As shown in Table II, significant increases of $Rh\text{-}123^{+}$ fluorescence were detected 20 min after addition of arsenite. Comparing to control cells, the production rate of intracellular oxidants was significantly faster in As-treated cells. For example, As-treated cells accumulated almost threefolds more fluorescent product than control cells after 1 h treatment.

To examine the effect of altered GSH levels on arsenic cytotoxicity, LEC cells were also treated with BSO. The effect of BSO pretreatment on cellular GSH level and cytotoxicity of arsenite is shown in Figure 1A,B. The treatment with 50 μM BSO decreased cellular GSH level in a near-linear manner within 24 h (Fig. 1A). Next, we determined whether GSH depletion affects the sensitivity of LEC to the cytotoxicity of arsenite. As shown in Figure 1B, BSO potentiated the arsenic cytotoxicity of LEC. By itself, it had only a minimal cytotoxic effect (data not shown).

As-Induced Changes in γ-GCS Expression

The rapid recovery of GSH levels after the initial depletion, followed by the substantial rise in GSH at later time points after arsenic treatment, indicated the possibility that the gene for the rate-limiting enzyme in GSH synthesis may have been induced. Northern hybridization experiments were therefore used to test this hypothesis. We determined whether the steady-state mRNA levels of the catalytic (heavy) subunit of γ-GCS were increased in response to arsenic treatment. As shown in Figure 2, arsenic induced the heavy subunit of γ-GCS (γ-GCS-HS) mRNA levels as early as 4 h as compared to the control level. It significantly increased (sixfolds after normalization by L32 RNA content) γ-GCS-HS mRNA expression after 8 h of treatment.

TABLE II. Production of Oxidants by Arsenite in LEC Cells

	Relative fluorescence intensity	
Time (min)	Control	As-treated
0	1.0	1.0
10	1.2 ± 0.3	1.7 ± 0.4
20	1.5 ± 0.2	2.5 ± 0.6
40	1.9 ± 0.4	4.8 ± 0.7
60	2.3 ± 0.6	6.3 ± 1.1

LEC cells were grown to 75% confluence, changed to fresh complete medium for 24 h, then were exposed to 5 μM sodium arsenite and 2 μmole/L DHRh-123 for 0, 10, 20, 40, or 60 min. Data are expressed as the mean ± SEM, where n = 3–4.

As-Induced Changes in the Expression of Immediate-Early Genes and AP-1 Binding Activity

It is known that the 5′-flanking region of promoters for γ-GCS-HS gene has response element for AP-1, a transcription factor composed of either Fos/Jun heterodimers or Jun/Jun homodimers. We reasoned that induction of AP-1 in response to arsenic and subsequent binding of this transcription factor to AP-1 motifs in the γ-GCS promoter could explain the coordinate upregulation of these enzymes.

To explore the potential association between AP-1 and the induction of this GSH-producing enzyme, time-dependent changes in mRNA levels for *c-fos* and *c-jun* following the addition of 5 μM sodium arsenite were measured. As shown in Figure 3, significant elevations in *c-fos* and *c-jun* mRNA levels were observed within 30 min after arsenic exposure. Arsenite treatment caused a 3.1-fold induction in *c-fos* mRNA. We then determined whether the transcriptional activation of the *c-fos* and *c-jun* genes by arsenic resulted in the formation of a functional AP-1 complex. To accomplish this, we used EMSA to compare the ability of nuclear proteins, isolated from As-treated and control cells, to bind to AP-1 consensus sequence. Figure 4 shows that arsenic clearly increased the binding activity of the nuclear extract to the oligonucleotide probe of AP-1 binding site after treatment with arsenic for 2 h, and peaked at 4 h post-treatment. To test whether induced AP-1 protein is functional, LEC were transfected with APCAT reporter gene, which contains AP-1 binding sequence in the α-fetoprotein promoter region, fused to the structural gene coding for bacterial chloramphenicol acetyltransferase (CAT) [Zhang et al., 1991]. CAT activity was measured by the CAT-ELISA method, using the CAT-ELISA measuring kit (Promega). Results were expressed as the ratio of activity, with or without tumor promoter treatment, or fold induction. Shown in Figure 5 is arsenic induced

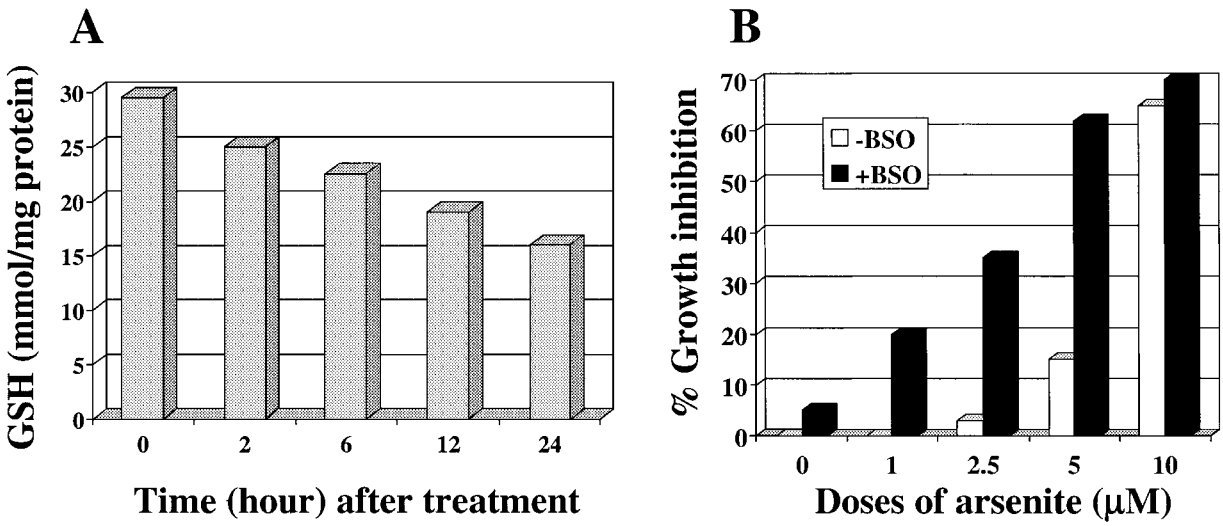

Fig. 1. (**A**) Depletion of intracellular GSH levels by treatment with 50 μM of BSO in LEC cells for 6, 12, or 24 h. Each point represents the mean ± SEM (n=3). (**B**) Effect of BSO pretreatment on arsenite-induced cytotoxicity in LEC cells. LEC cells were pretreated with 0 or 50 μM BSO for 24 h. Cells in each treatment groups were then exposed to 0, 1, 2.5, 5.0, or 10 μM sodium arsenite for 24 h.

CAT gene expression 48 h after treatment of transfected cells with 5 μM sodium arsenite.

Arsenic-Induced NF-κB Binding Activities

Activation of NF-κB binding is highly responsive to stress stimuli. These stimuli increase nuclear translocation of proteins that bind genomic κB elements. Therefore, responsiveness of LEC to oxidative stress caused by arsenic exposure was further evaluated with mobility shift assays involving redox-sensitive transcription factor NF-κB. Nuclear extracts were prepared from cells exposed to either 0 or 20 μM of sodium arsenite at 30 min, 1, 2, and 4 h. The specificity of binding was verified by an antibody-supershift. As shown in Figure 6A,B (autoradiogram and quantitative results from densitometric scanning, respectively), nuclear extracts prepared from cells exposed to As for 30 min, 1, and 2 h had approximately double the NF-κB DNA binding activities, compared to extracts from control cells. The NF-κB binding

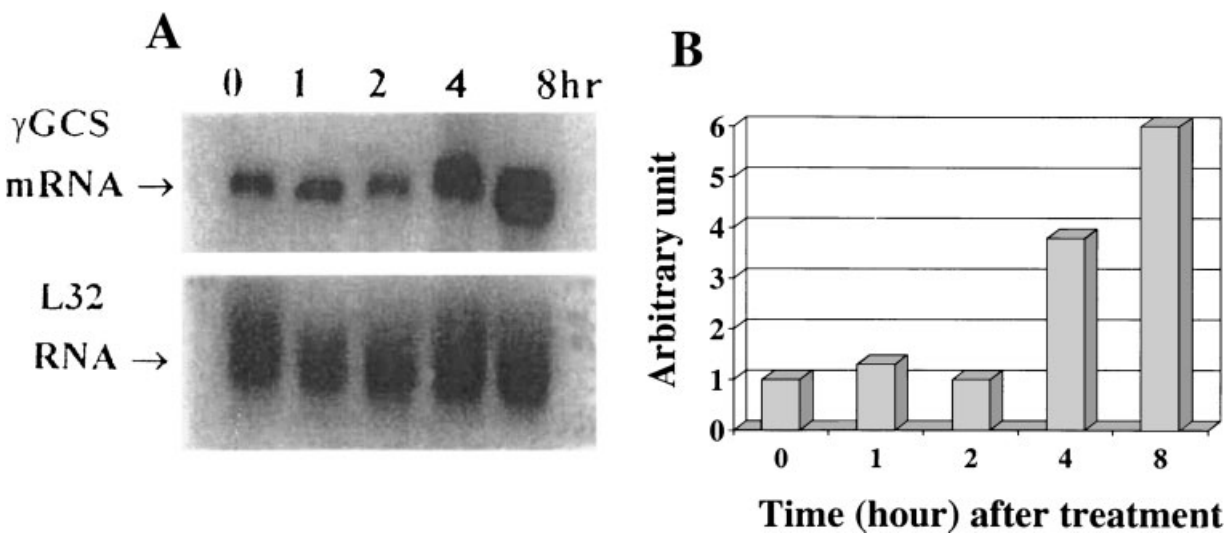

Fig. 2. (**A**) Northern blot analysis of steady state levels of γGCS mRNA in the cells treated with 5 μM sodium arsenite for 0, 0.5, 1, 2, or 4 h. (**B**) Right panel depicts the quantitative hybridization data normalized for differences in RNA loading using L32 ribosomal RNA as an internal standard.

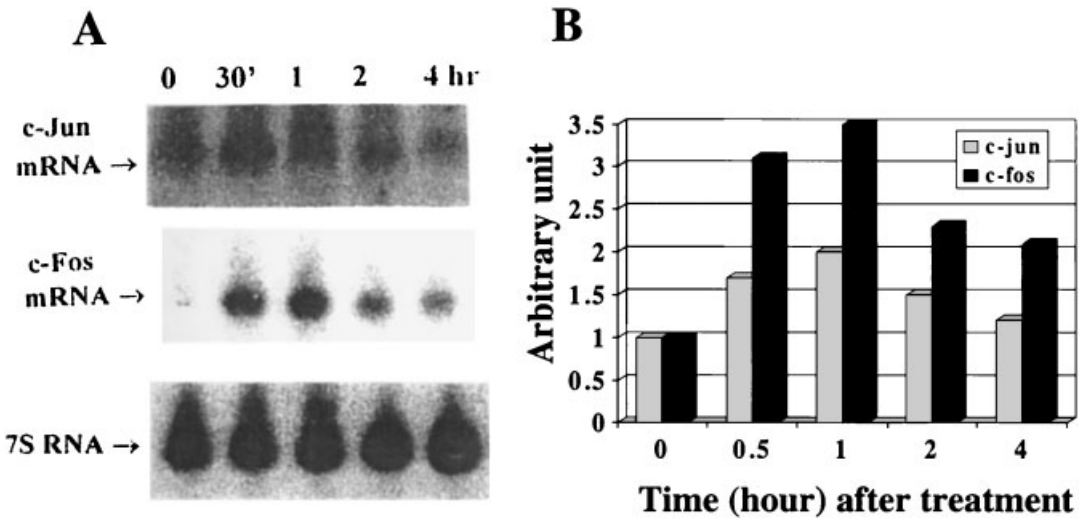

Fig. 3. (**A**) Northern blot analysis of steady state levels of c-Jun and c-Fos mRNA in the cells treated with 5 μM sodium arsenite for 0, 0.5, 1, 2, or 4 h. (**B**) Right panels depict the quantitative hybridization data normalized for differences in RNA loading using 7S ribosomal RNA for an internal standard.

activity returned to control levels after 4 h of treatment with arsenic. Supershift analysis with antibodies specific to the p50 subunits of NF-κB was used to identify the proteins bound in response to arsenite. The mobility of the binding complexes are further retarded by the antibodies indicating that p65/p50 heterodimers and possibly p50 homodimers accumulate in the nucleus following treatment with arsenite. It remains to be determined whether NF-κB plays a role in the As-induced apoptosis or alternatively in attempting to protect the cells from As-induced cell death by upregulating the expression of resistance factors.

Antioxidants Prevent Increased Nuclear Levels of NF-κB in Response to Arsenite

Translocation of NF-κB in response to most, but not all stimuli, involves an oxidant-sensitive regulatory step [Anderson et al., 1994; Suzuki et al., 1994; Thanos and Maniatis, 1995]. To demonstrate that oxidants or reaction with thiols is required for activating NF-κB in response to arsenite, LEC cells were incubated

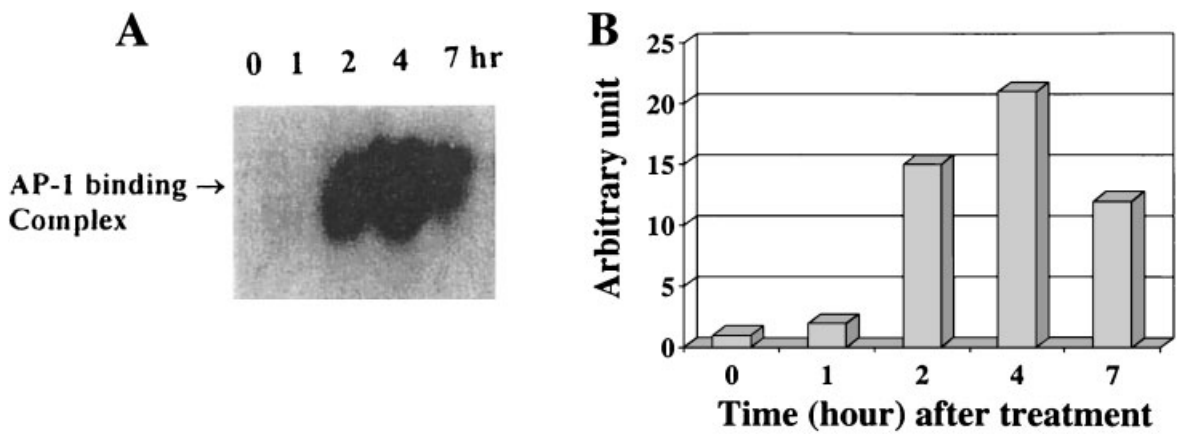

Fig. 4. (**A**) Induction of AP-1 binding activity in LEC cells exposed to 5 μM sodium arsenite for 0, 2, 4, or 6 h. Nuclear protein (5 μg) from cells treated with arsenite for various time periods was used to analyze for DNA binding activity using a ^{32}P-end-labeled, double-stranded, oligonucleotide consensus sequence for AP-1. Protein–DNA complexes were resolved by nondenaturing gel electrophoresis, visualized by autoradiography, and quantified by densitometer. (**B**) panel shows the quantitative data. (**A**) panel is a representative autoradiogram showing the retardation of the labeled probe by nuclear proteins (shown by an arrowhead).

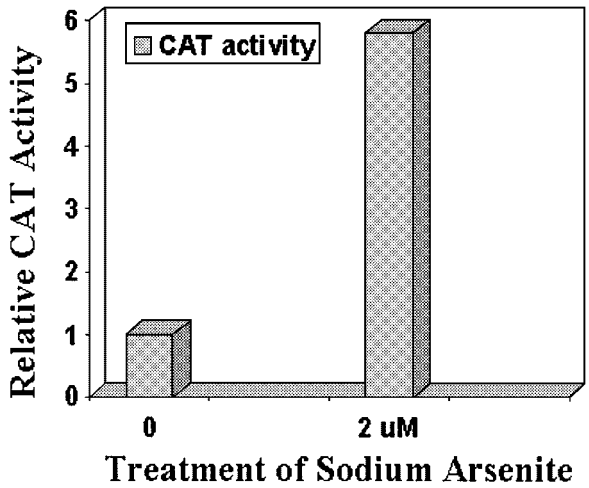

Fig. 5. Functional analysis of AP-1 induction by arsenite using transient transfection of pAFPCAT as a reporter gene. The CAT activity was determined by CAT-ELISA measuring kit (Promega) as described by manufacturer.

in complete medium supplemented with 5 mM *N*-acetyl-L-cysteine (NAC, Sigma) for 18–24 h prior to adding arsenite for an additional hour. Incubation with NAC only slightly reduced basal κB binding. However, pretreatment with antioxidant significantly reduced arsenite-induced NF-κB binding (Fig. 7). These data combined with those presented in Figure 1 indicated that increased nuclear levels of NF-κB in response to arsenite are initiated by or require critical oxidant-sensitive signaling.

DISCUSSION

Normally, intracellular redox status is tightly controlled by cellular GSH, and remains relatively constant unless these cells are subjected to oxidizing conditions. The mechanism of redox homeostasis regulates the level of ROS formed continually as side products of reactions involving electron transfers during oxidative phosphorylation. High levels of intracellular oxidants are deleterious and cytotoxic. Therefore basal levels of ROS are carefully modulated by specific genes that protect cells against oxidant injury or participate in cellular repair processes [Arrigo, 1999; Dalton et al., 1999].

The data presented in the present study are the first demonstration that arsenite can initiate lung cell signaling and activation of transcription factors, AP-1 and NF-κB. This activation follows the same time course as that for oxidant formation and is prevented by antioxidant. These findings suggest that oxidation of thiols is required for signaling in response to arsenite.

The first indicator of arsenic-induced oxidant stress occurred within 15–30 min after the

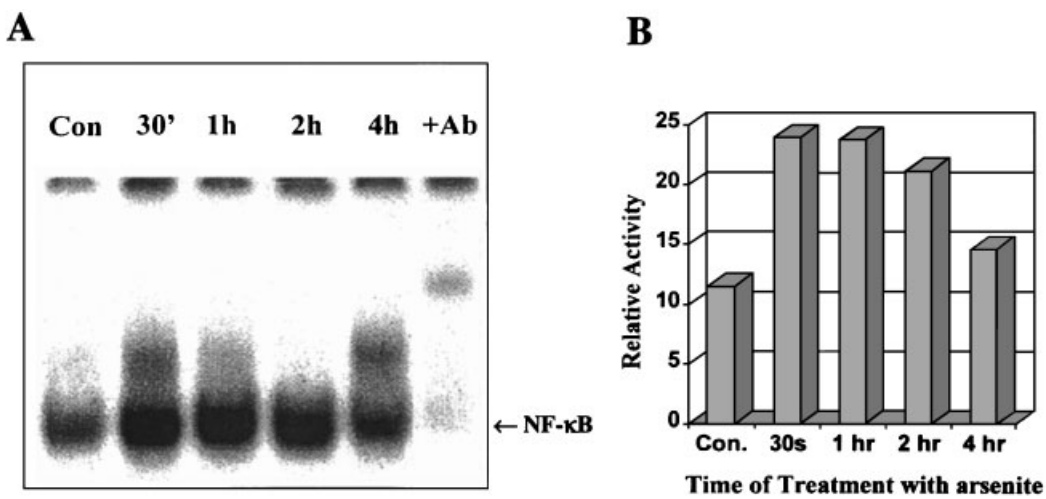

Fig. 6. (**A**) Induction of NF-κB binding activity in LEC cells exposed to 5 μM sodium arsenite for 0, 0.5, 1, 2, or 4 h. Nuclear protein (5 μg) from cells treated with arsenite for various time periods was used to analyze for DNA binding activity using a ^{32}P-end-labeled, double-stranded, oligonucleotide consensus sequence for NF-κB. Protein–DNA complexes were resolved by nondenaturing gel electrophoresis, visualized by autoradiography, and quantified by densitometer. (**B**) Right panel shows the quantitative data. Left panel is a representative autoradiogram showing the retardation of the labeled probe by nuclear proteins (shown by an arrowhead). Supershift analysis was used to identify the NF-κB binding proteins in the retarded complex. Monoclonal antibody to p50 subunit of NF-κB was added to the incubation mixture for an additional hour. The arrow designated SS (supershift) represents the additional retardation in migration caused by the interaction of the NF-κB DNA complex with an antibody to p50 subunit.

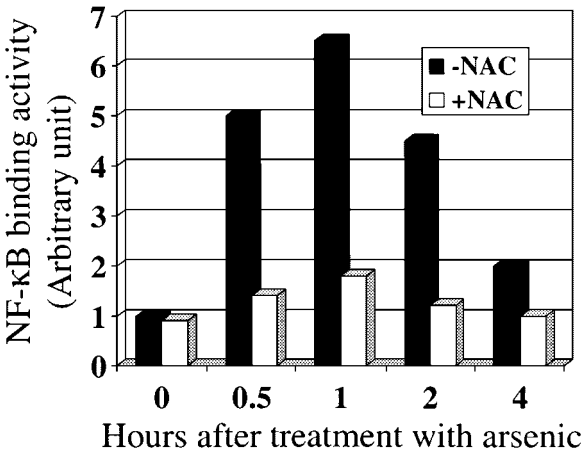

Fig. 7. Suppression of arsenite-induced NF-κB activation in NAC-pretreated LEC cells. Cells were incubated with 5 mM NAC for 24 h prior to adding 5 μM sodium arsenite for 0, 0.5, 1, 2, or 4 h. The NF-κB binding activity was analyzed by EMSA as described in Figure 6. The densities of binding bands were quantified by densitometry.

treatment of arsenic and was in the form of a transient decrease in lung cellular GSH content. The changes of redox status induced by arsenic could have resulted from the direct interaction of GSH with intracellular oxidants, or/and have been due to inhibition of GSSG reductase that serves to regenerate GSH. Soon thereafter, an increased level of ROS in arsenic-treated cells was detected by the probe DHRh-123 and quantified by spectrofluorometry. Recently Barchowsky et al. [1996] demonstrated that there was no depletion of GSH in response to 5 μM arsenite in porcine aortic endothelial cells. The contradictory results may be due to (1) Barchowsky et al. did not study earlier time point and (2) different cell lines may have different response.

In the present study, we demonstrated that exposure of LEC to arsenic increased the levels of intracellular oxidants that subsequently leads to induction of GSH synthesis by enhancement of redox-sensitive transcription factor AP-1 and in turn, upregulation of γ-GCS enzyme activity. γ-GCS is the first and rate-limiting enzyme in the de novo GSH snthesis pathway. It is a heterodimer, consisting of a catalytic (heavy) subunit and a regulatory (light) subunit [Griffith, 1999]. Very diverse chemical agents are know to induce γ-GCS subunit gene expression, but all of these compounds have in common the ability to generate an oxidant stress. For example, this study and our previous data [Shukla et al., 2000a] demonstrated that exposure of LEC to arsenic or cadmium, upregulated the expression of γ-GCS-HS gene, which was accompanied by increased level of intracellular oxidants. We have recently reported that the γ-GCS-LS gene is also transcriptionally activated by cadmium [Shukla et al., 2000a]. Induction kinetics for the heavy and light subunits appears to be identical.

The mechanism governing the coordinate induction of heavy and light subunits of γ-GCS gene by arsenic and cadmium remains to be fully elucidated. However, regulation could be achieved, at least in part, through a common response element found in the 5′-flanking region of each gene. It is conceivable that the coordinate induction of γ-GCS isoenzymes by cadmium and arsenic could be associated with activation of the transcription factor AP-1, and the subsequent binding of AP-1 to specific sites within promoters of these genes [Hayes and McLellan, 1999].

Several lines of evidence derived from the current study support the possible involvement of AP-1 in this regulatory process. We found that the expressions of *c-fos* and *c-jun* were rapidly and transiently activated by arsenic in LEC. The increase in *c-fos* and *c-jun* transcripts occurred within 30 min, coincident with the earliest sign of arsenic-mediated oxidant stress. Using EMSA, we were then able to demonstrate not only that AP-1 binding activity was enhanced in nuclear extracts derived from arsenic-exposed cells but also that it was maximal prior to the time when upregulation of γ-GCS expression was first detected (see Figs. 2 and 3).

In addition to activation of the AP-1 transcription complex, arsenite also increased NF-κB binding activity, supporting the observation that acute arsenic induces oxidative stress and activates NF-κB in cells [Muller et al., 1997; Kaltreider et al., 1999; Tully et al., 2000]. The carcinogenic property of arsenic could be explained by arsenite-stimulated transcription factors, such as AP-1 and NF-κB that subsequently stimulate proliferative genes [La Rosa et al., 1994; Janssen et al., 1995]. The data in the current study indicate for the first time that environmentally relevant concentrations of arsenite initiate lung cell signaling. This signaling is initiated by cellular oxidation that activates pathways leading to increased levels of active nuclear transcription factors. Increased binding of transcription proteins to genomic

κB and AP-1 sites may induce a mitogenic response.

In conclusion, we have shown in this report that exposure of LEC to arsenite causes a transient depletion of GSH which, in turn, leads to an increased production and accumulation ROS. In order to maintain redox homeostasis in the cells and to cope with the excess of ROS produced during this arsenic-mediated oxidant stress, the γ-GCS gene expression was activated. The molecular mechanisms by which the arsenic-induced gene expression will require further investigation. However, results derived from current studies suggested that the mechanisms responsible for this event may involve arsenic-induced transcriptional activation of the *c-jun* and *c-fos* genes. The subsequent binding of the transcription factor AP-1 complex to the AP-1 consensus sequence at promoter region located in the 5′ end of the γ-GCS gene, ultimately causing the upregulation of its expression.

ACKNOWLEDGMENTS

This investigation was partially supported by a seed funding from the University of Hong Kong. The authors thank Mr. Andy Tao Yin Lau for his assistance in preparation of this manuscript.

REFERENCES

Anderson MT, Staal FJ, Gitler C, Herzenberg LA. 1994. Separation of oxidant-initiated and redox-regulated steps in the NF-κB signal transduction pathway. Proc Natl Acad Sci 91:11527–11531.

Arrigo AP. 1999. Gene expression and the thio redox state. Free Radic Biol Med 27:936–944.

Barchowsky A, Dudek EJ, Treadwell MD, Wetterhahn KE. 1996. Arsenic induces oxidant stress and NF-κ B activation in cultured aortic endothelial cells. Free Radic Biol Med 21:783–790.

Bates MN, Smith AH, Hopenhayn-Rich C. 1992. Arsenic ingestion and internal cancers: A review. Am J Epidemiol 135:462–476.

Cavigelli M, Li WW, Lin A, Su B, Yushioka K, Karin M. 1996. The tumor promoter arsenite stimulate AP-1 activity by inhibiting a JNK phosphatase. EMBO J 15: 6269–6279.

Chen CJ, Chen CW, Wu MM, Kuo TL. 1992. Cancer potential in liver, lung, bladder and kidney due to ingested inorganic arsenic in drinking water. Br J Cancer 66:888–892.

Dalton TP, Shertzer HG, Puga A. 1999. Regulation of gene expression by reactive oxygen. Annu Rev Pharmacol Toxicol 39:67–101.

Ding M, Shi X, Castranova V, Vallyathan V. 2000. Predisposing factors in occupational lung cancer: Inorganic minerals and chromium. J Environ Pathol Toxicol Oncol 19:129–138.

Griffith OW. 1999. Biological and pharmacological regulation of mammalian glutathione synthesis. Free Radic Biol Med 27:922–935.

Guyton KZ, Kensler TW. 1993. Oxidative mechanisms in carcinogenesis. Br Med Bull 49:523–544.

Hayes JD, McLellan LI. 1999. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radic Res 31:273–300.

Hei TK, Liu SX, Waldren C. 1998. Mutageneicity of arsenic in mammalian cells: Role of reactive oxygen species. Proc Natl Acad Sci USA 95:8103–8107.

International Agency for Research on Cancer. 1982. IARC Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Suppl 4, 50–51.

International Agency for Research on Cancer. 1987. IARC Monograph on the Evaluation of Carcinogenic Risk to Humans—Overall Evaluations of Carcinogenicity: An Update of IARC Monographs 1 to 42 (IARC, Lyon, France). Suppl 7, pp 100–106.

Janssen YM, Barchowsky A, Treadwell M, Driscoll KE, Mossman BT. 1995. Asbestos induces nuclear factor kappa B (NF-κ B) DNA-binding activity and NF-kappa B-dependent gene expression in tracheal epithelial cells. Proc Natl Acad Sci 92:8458–8462.

Kaidobad I, Yong Xia Jay L, Der Zweier Sollott SJCJ, Fearon ER, Maiterayee S, Finkel T, Goldschmidt-Clermont PJ. 1997. Mitogenic signaling mediated by oxidants in RAS-transformed fibroblasts. Science 275:1649–1652.

Kaltreider RC, Pesce CA, Ihnat MA, Lariviere JP, Hamilton JW. 1999. Differential effects of arsenic(III) and chromium(V) on nuclear transcription factor binding. Mol Carcinog 25:219–229.

La Rosa FA, Pierce JW, Sonenshein GE. 1994. Differential regulation of the c-myc oncogene promoter by the NF-kappa B rel family of transcription factors. Mol Cell Biol 14:1039–1044.

Li AP, Hahan F, Zamora P, Shimizu RW, Henderson RF, Brooks AL, Richards R. 1983. Characterization of a lung epithelial cell strain with potential applications in toxicological studies. Toxicology 27:257–272.

Muller JM, Rupec RA, Baeuerle PA. 1997. Study of gene regulation by NF-κB and AP-1 in response to reactive oxygen intermediates. Methods 11:301–312.

Nordenson L, Beckman L. 1991. Is the genotoxic effect of arsenic mediated by oxygen free radicals? Hum Hered 41:71–73.

Nriagu JO. 1994. Arsenic in the environment, part II. Human health and ecosystem effects. New York: Wiley .

Royall JA, Ishiropoulos H. 1993. Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch Biochem Biophys 302:348–355.

Schwartz RA. 1997. Arsenic and the skin. Int J Dermatol 36:241–250.

Shukla GS, Shukla A, Potts RJ, Osier M, Hart BA, Chiu JF. 2000a. Cadmium-mediated oxidative stress in alveolar epithelial cells induces the expression of γ-glutamylcysteine synthetase catalytic subunit and glutathione S-transferase α and π isoforms: Potential role of activator protein-1. Cell Biol Toxicol 16:347–362.

Shukla GS, Chiu JF, Hart BA. 2000b. Enhanced expression of pulmonary γ-glutamylcysteine synthetase heavy subunit in rats exposed to cadmium aerosols. Toxicol Appl Pharmacol 163:249–259.

Shukla GS, Chiu JF, Hart BA. 2000c. Cadmium-induced elevations in the gene expression of the regulatory subunit of γ-reglutamylcysteine synthetase in rat lung and alveolar epithelial cells. Toxicology 151:45–54.

Simeonova PP, Luster MI. 2000. Mechanisms of arsenic carcinogenicity: Genetic or epigenetic mechanisms? J Environ Pathol Toxicol Oncol 19:281–286.

Suzuki YJ, Mizuno M, Packer L. 1994. Signal transduction for nuclear factor-kappa B activation. Proposed location of antioxidant-inhibitable step. J Immunol 153:5008–5015.

Thanos D, Maniatis T. 1995. NF-κB: A lesson in family value. Cell 80:529–532.

Tully DB, Collins BJ, Overstreet JD, Smith CS, Dinse GE, Mumtaz MM, Chapin RE. 2000. Effects of arsenic, cadmium, chromium, and lead on gene expression regulated by a battery of different promoters in recombinant HepG2 cells. Toxicol Appl Pharmacol 168:79–90.

Waalkes MP. 1995. In: Coyer RA, Klaassen CD, Waaikes MP, editors. Metal toxicology. New York: Academic. pp 54–56.

Wang TS, Huang H. 1991. Active oxygen species are involved in the induction of micronuclei by arsenite in XRS-5 cells. Mutagenesis 9:253–257.

Wang Z, Rossman TG. 1996. In: Cheng P, editor. Toxicology of Metals. Boca Raton, FL: CRC. pp 221–229.

Zhang XK, Dong JM, Chiu JF. 1991. Regulation of α-fetoprotein gene expression by antagonism between AP-1 and glucocorticoid receptor at their overlapping binding site. J Biol Chem 266:8248–8254.