

Arsenic Alters the Function of the Glucocorticoid Receptor as a Transcription Factor

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Chronic human exposure to nonovertly toxic doses of arsenic is associated with an increased risk of cancer. Although its carcinogenic mechanism is still unknown, arsenic does not directly cause DNA damage or mutations and is therefore thought to act principally as a co-mutagen, co-carcinogen, and/or tumor promoter. Previous studies in our laboratory demonstrated that effects of low-dose arsenic (III) (arsenite) on expression of the hormone-regulated phosphoenolpyruvate carboxykinase (PEPCK) gene were strongly associated with the glucocorticoid receptor (GR)-mediated regulatory pathway. We therefore examined specifically the effects of arsenite on the biochemical function of GR in hormone-responsive H4IIE rat hepatoma cells. Completely noncytotoxic arsenite treatments (0.3–3.3 μM) significantly decreased dexamethasone-induced expression of transiently transfected luciferase constructs containing either an intact hormone-responsive promoter from the mammalian PEPCK gene or two tandem glucocorticoid response elements (GRE). Western blotting and confocal microscopy of a green fluorescent protein-tagged-GR fusion protein demonstrated that arsenite pretreatment did not block the normal dexamethasone-induced nuclear translocation of GR. These data indicate that nontoxic doses of arsenite can interact directly with GR complexes and selectively inhibit GR-mediated transcription, which is associated with altered nuclear function rather than a decrease in hormone-induced GR activation or nuclear translocation. **Key words** arsenic, carcinogenesis, endocrine disruptor, gene regulation, glucocorticoid receptor, metal, transcription factor. *Environ Health Perspect* 109:245–251 (2001). [Online 26 February 2001]

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Arsenic, considered a human carcinogen (1–4), is present in high concentrations at many toxic waste sites through disposal of arsenic-containing compounds from industrial and mining practices. In addition, arsenic can accumulate in groundwater and well water from natural sources. Certain geological formations contain high levels of arsenic that can easily leach into groundwater and find their way into wells and other public water supplies (5). This has been a major problem in certain parts of the world including areas of Taiwan, South America, India, and Pakistan. Epidemiological studies of these populations have demonstrated a significant increase in the risk of lung, skin, liver, bladder, and other cancers associated with high levels of arsenic in drinking water (1–4), and most of the world's arsenic standards are based on risk assessment models of these data from high-exposure populations. More recently it has become apparent that regions of the United States have similar sources of natural arsenic that can contribute to elevated drinking-water levels, including areas of New Hampshire, Michigan, Nevada, and California (6,7). For example, in New Hampshire, where 40% of the population drinks from private wells, as much as 8% of the population (one-fifth of all private well users) is exposed to arsenic levels between the current standard of 50 ppb (0.67 μM) and the U.S. Environmental Protection Agency's (EPA's) new proposed

standard of 10 ppb (0.14 μM), and many wells have arsenic concentrations in the range of 100–800 ppb (6,7). Thus, understanding the cellular changes that occur in this range of exposures that may contribute to carcinogenesis is important for both theoretical and practical reasons.

As one way to assess its biological mechanism of action, we have examined the effects of arsenite on gene expression. Our laboratory has previously shown that a single, low, nonovertly toxic dose of arsenite can significantly and preferentially alter both the basal and inducible mRNA expression of the model hormone-inducible phosphoenolpyruvate carboxykinase (PEPCK) gene, both in whole-animal and in cell-culture models (8). These same treatments had no effect on expression of noninducible or constitutively expressed genes in these same systems (8). An important mechanism for altering gene expression in response to both endogenous and exogenous signals, including toxins, is altering nuclear transcription factor activities either directly or via specific cell-signaling pathways that regulate them. These effects were examined in a rat hepatoma cell line (H4IIE), previously shown to be sensitive to arsenite-induced alterations in gene expression (8). The PEPCK gene is primarily transcriptionally regulated, and its protein product, the cytosolic PEPCK enzyme, is the rate-limiting step in gluconeogenesis (9,10). The regulation of this

gene has been well characterized at both the physiological and molecular levels (10), and it is therefore an excellent model for examining mechanisms of gene regulation and their perturbation by toxic agents. Previous work in our laboratory demonstrated that toxin effects on PEPCK mRNA expression were primarily a result of changes in gene transcription rates (11). Effects of arsenic on PEPCK expression may also provide important clues as to how these toxic metals perturb homeostatic mechanisms, which may contribute to their overall toxicity.

Previous studies by Imai and co-workers (12) have shown that the first approximately 600 base pairs (bp) of the PEPCK promoter is sufficient to provide tissue-specific hormone regulation. Examining a luciferase construct under the transcriptional control of a 679 bp region of the proximal rat PEPCK promoter (positions –592 to +87), we have shown that arsenite treatments alter both basal and hormone-induced luciferase expression when stably integrated into H4IIE cells (8). This transgene responded normally to induction by the synthetic glucocorticoid, dexamethasone (Dex), and this induction was blocked by pretreatment of the cells with a nontoxic dose of arsenite, similar to the effects of arsenite on native PEPCK in this system (8). Site-directed mutagenesis of the glucocorticoid response unit essentially abolished both the Dex

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response and the arsenite effect, suggesting a direct role for the glucocorticoid receptor (GR) and transcription in the arsenite response. Sutherland et al. (13) showed that arsenite treatments reduced the responsiveness of both the native PEPCK gene and a stably integrated, full-length PEPCK-chloramphenicol-acetyl transferase (CAT) construct to a combination treatment of Dex and cAMP. These effects were primarily a result of changes in mRNA expression (13). These data suggest a possible role of GR in arsenite-induced alterations in gene expression. We were therefore interested in determining whether nontoxic levels of arsenite altered the function/activity of GR. We report that arsenite treatments can reduce GR transcriptional function significantly, while not interfering with the normal hormone-induced nuclear translocation of this receptor.

Methods

Plasmid constructs. The PEPCK-luciferase (Luc) construct contains the region from -592 (*NheI* restriction site) to +87 (*BglII* restriction site) of the rat proximal PEPCK promoter ligated into the pGL3-basic vector (Promega, Madison, WI), as previously described (8). The GRE2-Luc construct was generated by removing two tyrosine amino-transferase (TAT) glucocorticoid response elements (GRE) from a pXP2 GRE(2)-Luc construct (14) (a generous gift from J. Bodwell, Dartmouth Medical School) with *BamHI* and *KpnI* restriction enzyme digestion. This region was then ligated into the *KpnI* and *BglII* sites of a pGL3-promoter vector (Promega). All plasmids were grown in *Escherichia coli* JM109 strain. Plasmid DNA was purified using Qiagen columns (Qiagen, Valencia, CA), as per the manufacturer's protocol. Plasmids containing inserts were sequenced with an ABI PRISM DyeDeoxy Terminator Cycle Sequencing Kit (PE-BioSystems, Foster City, CA) using RVPrimer3 (upstream) and GLPrimer2 (downstream) (Promega). We checked all sequences against GenBank sequences using the Blast sequence analysis program to ensure specificity. We used a pGL3-control luciferase vector, which contains SV40 enhancer and promoter regions, to determine the effect of our treatments on the parent backbone (Promega).

Cell culture treatments and transfections. H4IIE rat hepatoma cells were cultured as previously described (8,15). All chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Arsenic(III) was administered as sodium arsenite (NaAsO_2) at the indicated doses in water. Stock solutions of Dex and RU-486 were generated at 0.1 mM in water or ethanol, respectively,

and stored at -20°C . Before cell treatments, stocks were diluted in water and added to the cells at the indicated concentrations. For transfection assays, cells were plated into Falcon six-well culture dishes (Becton Dickinson, Lincoln Park, NJ) at 5×10^5 cells per well. The following day, we performed transfections using LipofectAMINE-PLUS reagent (Gibco-BRL, Grand Island, NY), as per the manufacturer's protocol. Briefly, 2 μg of vector DNA were added to cells in 10 μL lipofectamine and 8 μL Plus reagent diluted in 200 μL incomplete culture media. After a 3-hr incubation, we adjusted serum levels to 3% by adding completed media, and the cells were allowed to recover overnight. Chemical treatments were performed the following day in a 1% serum-containing media and incubated overnight (except in experiments represented in Figure 5, in which cells were treated and incubated for 4–10 hr before protein isolation). Cell lysates were generated after two cold washes in phosphate-buffered saline (PBS) by addition of 400 μL of a $1\times$ lysis buffer (Promega) and scraping. Lysates were cleared of debris by a 4°C centrifugation at $12,500 \times g_{\text{avg}}$ for 2 min. Supernatants were saved and stored at -70°C for at least 30 min before analysis. We performed luciferase assays using 45 μL of the lysates and 100 μL of the luciferin reagent (Promega) with an integration over 5 sec on an EG&G Berthold luminometer (Oak Ridge, TN).

Protein isolation. We isolated cytoplasmic and nuclear protein as described previously (15,16). Briefly, after chemical treatments, cells were washed with ice-cold PBS, and then a modified Garrison's buffer [20 mM Tris-HCl, 2 mM EDTA, 1 mM EGTA, 0.1% digitonin with 0.01 mM protease inhibitor combination dithiothreitol (DTT), L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK), leupeptin, soybean trypsin inhibitor, and benzamide] was added. The cells were shaken gently at room temperature for 5 min and then scraped and spun at 4°C for $13,600 \times g_{\text{avg}}$ for 5 min, and supernatant was isolated as cytosolic protein fraction. The remaining pellet was then processed for nuclear extracts by the procedure of Dignam (17) as modified by Janssen et al. (18). Briefly, the pellet was washed with 1.0 mL of buffer A (10 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, and 0.01 mM of the protease inhibitor combination) and then spun at $13,600 \times g_{\text{avg}}$ for 2 min at 4°C . Nuclei were resuspended in 40 μL of buffer C (20 mM HEPES, 0.42 M NaCl, 1 mM EDTA, 0.1 mM EGTA, and 0.01 mM of the protease inhibitor combination) by shaking 15 min in a cold room. Samples were spun at $13,600 \times g_{\text{avg}}$ for 5 min at 4°C to

pellet the nucleic acids. The supernatant was removed and combined with 60 μL of Buffer D [20 mM HEPES, 20% (v/v) glycerol, 1 mM EDTA, 0.1 mM EGTA, 1% Nonidet-P40, and 0.01 mM of the protease inhibitor combination] for the nuclear protein extracts. Protein concentrations were determined by a standard absorbance assay [bicinchoninic acid (BCA) assay; Pierce Chemical Co., Rockford, IL] (8,16).

Total cellular lysates. After chemical treatments, cells were washed with ice-cold PBS/EDTA. Whole-cell lysates were generated in 800 μL of an SDS lysis buffer (2% SDS, 62.5 mM Tris, pH 6.8, 10% glycerol, 0.01 mM DTT, and protease inhibitors). Flasks were gently rocked for 5 min in the cold room, and lysates were collected by scraping cells into 2-mL tubes followed by sonication (12 times with 1-sec pulses in the cold room). We calculated the final volume and added an appropriate amount of $1\times$ Laemmli sample buffer (Bio-Rad, Hercules, CA) containing 5% 2-mercaptoethanol to each tube and boiled samples for 2 min. Samples were stored at -20°C until protein analysis was performed.

GR immunoprecipitation. After chemical treatments, cells were washed with ice-cold PBS/EDTA, and then 1 mL of ice-cold lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 0.2% NP-40, 2 mM EGTA, 2 mM EDTA, and protease inhibitors) was added to each flask. The flasks were shaken gently at 4°C for 30 min by slow rocking in the cold room and then scraped and centrifuged at 4°C for $13,600 \times g_{\text{avg}}$ for 10 min to clear debris. The supernatant was incubated with 10 μL (20 μg) of an agarose-conjugated polyclonal GR antibody or a control IgG antibody (Santa Cruz Bioreagents, Santa Cruz, CA). The samples were then gently shaken for 2 hr at 4°C by rocking in the cold room. The immunoprecipitated fraction was purified from the remaining proteins through centrifugation at $3,000 \times g_{\text{avg}}$ for 2 min. The pellets were then resuspended and washed three times in lysis buffer to obtain a clean fraction of purified GR. Proteins were solubilized by the addition of 60 μL of a $1\times$ Laemmli sample buffer containing 5% 2-mercaptoethanol and boiled for 90 sec.

Western blot analysis. We determined cytoplasmic and nuclear GR protein levels using a GR-specific monoclonal antibody, FIGR (a generous gift from J. Bodwell, Dartmouth Medical School). Briefly, protein samples (20 μg) were diluted 2:1 in Laemmli sample buffer (Bio-Rad) containing 2-mercaptoethanol and boiled for 90 sec. Samples were loaded and electrophoresed on a 4–15% gradient SDS-polyacrylamide gel (Bio-Rad) for 1.5 hr (45 mA) and electroblotted to

Immobilon-P membranes (Millipore, Bedford, MA) for 17–19 hr at 70 mA in a cold room. Membranes were blocked for 6–8 hr in 5% Carnation Instant Nonfat Dry Milk in PBS containing 0.3% Tween (CIMPT). Primary antibody, FIGR (5 $\mu\text{g}/\text{mL}$) or vinculin (1:400; Sigma), was added to blots and incubated overnight at 4°C. Following washes, the secondary, horseradish peroxidase-conjugated, anti-mouse antibody [1:5,000 (cytosolic) or 1:100,000 (nuclear); Sigma] was added for 30–45 min at room temperature in 2% CIMPT. Bands were visualized using the ECL system (Amersham, Buckinghamshire, England) for cytoplasmic proteins and Supersignal West Femto Maximal Sensitivity Substrate (Pierce Chemical Company) for nuclear proteins. To control for loading and transfer differences between the lanes, we standardized protein levels to the constitutively expressed protein vinculin by re-probing the membranes and visualization with the ECL system.

Confocal microscopy. Cells were plated onto glass cover slips in six-well plates at 5×10^5 cells per well. Transfections were performed as described above using 2 μg of a full-length human GR-green fluorescent protein (GFP) fusion protein expression vector (19) (a generous gift of I. Macara). After chemical treatments, cover slips were washed twice with cold PBS and fixed using 4% formaldehyde solution for 30 min at room temperature. After washing, DNA was stained with propidium iodide, and RNA was digested with RNase A for 30 min at 37°C. Cover slips were inversely mounted on slides using Valap (1:1:1 vasoline, lanolin, and paraffin wax). Slides were kept dark until imaging on a Bio-Rad MRC 1024 krypton/argon laser system using the 488 nm and 568 nm lines for excitation of GFP. We used Adobe Photoshop v. 4.0 for image conversion (Adobe Systems, Inc., San Jose, CA).

Statistical analysis. We analyzed data for statistical significance by analysis of variance, Student's *t*-test, and linear regression, where appropriate, using Instat v. 2.0 software and Prism v. 2.0b software programs (Graphpad Software Inc., San Diego, CA).

Results

Arsenite inhibits basal and Dex-stimulated luciferase expression of a PEPCK promoter-driven construct. We conducted initial experiments to characterize the hormone responsiveness of the transiently transfected PEPCK-Luc construct in this system. After transfection, the cells were treated with increasing doses (2.5–100 nM) of the synthetic glucocorticoid Dex. As shown in Figure 1, Dex produced a dose-dependent increase in luciferase expression with a

maximal expression of about 8-fold at 100 nM. Subsequent experiments with this construct used 50 nM Dex, which induced 70–80% of the maximal stimulation (Figure 1). We then examined the effect of arsenite on basal and inducible expression of this construct. After transfection, cells were treated with 3.3 mM arsenite alone or followed 2 hr later by 50 nM Dex. Previous studies using H4IIE cells had shown that this dose of arsenite causes little or no cytotoxicity [$\leq 5\%$ decrease in survival as measured by a colony forming assay (8)]. Dex treatment alone induced the expression of this construct to about 6.5-fold above control (Figure 2). Arsenite treatment (18 hr) reduced basal luciferase expression by 50%, and a 2-hr arsenite pretreatment almost completely abolished Dex-inducible expression (Figure 2). These results suggested that arsenite may alter PEPCK gene expression by suppressing GR-dependent regulation of this promoter.

Arsenite inhibits Dex-stimulated luciferase expression of a GRE promoter-driven construct. To examine directly the possible role of GR in the effects described above, we used a luciferase construct under the control of two tandem GRE elements from the rat TAT gene. We chose the TAT GRE sequences because the PEPCK GREs have a noncanonical sequence and can drive transcription only weakly in the absence of their adjacent accessory factor sites (20). The TAT GREs, which differ from the consensus GRE sequence by only one base, can act as independent transcriptional enhancers (21). This construct responded to Dex induction in a dose-dependent manner that was similar to that of the PEPCK-Luc construct (Figure 1). We used a dose of 50 nM Dex in subsequent

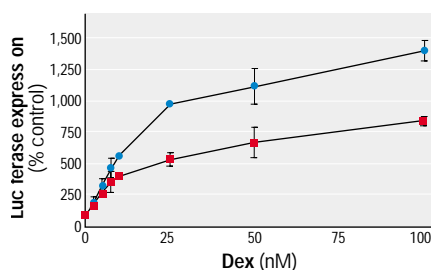


Figure 1. Effect of Dex on the expression of PEPCK-Luc and GRE2-Luc. H4IIE cells were transfected with either the PEPCK-Luc construct (squares) or GRE2-Luc construct (circles) as described in “Methods,” and then treated for 18 hr with increasing doses (2.5–100 nM) of Dex. Luciferase activity was measured in total cell lysates and data were expressed as a percentage of the transfected, solvent-treated control value. The mean relative light units for control and background were 4,429 and 92 for the PEPCK-Luc construct and 18,227 and 84 for the GRE2-Luc construct, respectively. Each data point represents the mean \pm SD of values from 3 to 18 individually transfected cultures from 1 to 6 independent experiments.

experiments, which produced a 70–80% (11-fold) of maximal hormone response (Figure 1). A 2-hr arsenite pretreatment significantly suppressed Dex induction of this construct in a dose-dependent manner ($r^2 = 0.90$) between 0.3 and 3.3 μM (Figure 3A). The highest arsenite dose (3.3 μM) caused $> 50\%$ suppression in hormone inducible expression (Figure 3A). This effect appears to be mediated exclusively by GR, because the GR-specific competitive antagonist, RU-486, completely blocked the hormone responsiveness of this construct (Figure 3A). To determine the time dependence of this suppression, cells were either pretreated with arsenite for 2, 4, or 6 hr before treatment with Dex or treated simultaneously with arsenite and Dex. As shown in Figure 3B, all of these treatments led to similar levels of suppression in Dex-inducible expression, suggesting that the effects of arsenite on GR function are both rapid and sustained. Arsenite and Dex treatments had no effect on expression of the pGL3-control luciferase construct containing the same promoter region with no GREs (Figure 4). This indicated that the effects of arsenite on Dex-inducible luciferase expression derived not from a general decrease in transcription or translation of luciferase in these cells, but rather from a GRE-mediated transcriptional effect.

Because these initial luciferase expression experiments were conducted overnight, we were concerned that these arsenite effects may have been a result of altered GR protein turnover rather than a specific alteration in GR transcriptional function. Therefore, we examined GR levels in arsenite- and Dex-treated cells and also repeated the luciferase

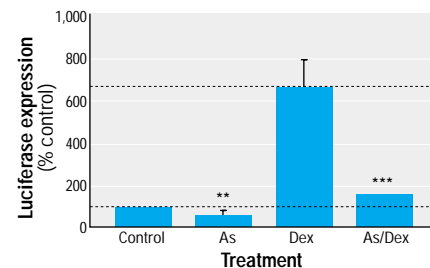


Figure 2. Effect of arsenite on basal and hormone-inducible expression of PEPCK-Luc. After transfection of the H4IIE cells with the PEPCK-Luc construct, cells were treated for 18 hr with 3.3 μM arsenite (As), 18 hr with 50 nM Dex, or treated with arsenite 2 hr before an 18-hr Dex treatment (As/Dex). Luciferase activity was measured in total cell lysates and data were expressed as a percentage of the transfected, solvent-treated control value. The mean relative light units for control and background were 15,257 and 102, respectively. Each bar represents the mean \pm SD of values from 3 to 12 individually transfected cultures from 1 to 4 independent experiments.

** $p < 0.01$. *** $p < 0.001$.

expression experiments using a much shorter induction period. Initial experiments were performed to determine the time course for hormone-induced luciferase expression in response to a 50 nM Dex. As shown in Figure 5A, Dex treatment significantly increased luciferase expression by as early as 4 hr, and a 6-hr treatment caused an approximately 4-fold induction. We then examined the effects of a 3.3 μ M arsenite pre-, post-, or simultaneous with treatment on a 6-hr Dex stimulation of this construct. As shown in Figure 5B, arsenite caused an approximately 50% suppression in Dex induction when administered either simultaneously with Dex or as a 2-hr pretreatment. This is essentially the same result as was observed in the overnight Dex treatments (Figure 3). Arsenite was also able to suppress hormone induction by approximately 50% when given 2 hr after Dex induction had been initiated (Figure 5B), even though a 2-hr Dex treatment alone causes maximal nuclear translocation of GR and gene activation. Therefore, arsenite can specifically alter GR-dependent nuclear gene regulation in intact cells at very low, environmentally relevant concentrations and disrupt GR function even after Dex-induced activation of GR.

Arsenite does not alter total cellular GR protein levels. The protein half-life of GR is about 8 hr in culture (22). Upon Dex stimulation, the half-life of GR protein was reduced to about 3 hr (22), and levels were decreased by approximately 70% relative to control (23). Therefore, we examined the effect of arsenite treatments on total cellular GR levels, which might lead to altered cellular function. Following treatments of 2–6 hr with either 3.3 μ M arsenite or 100 nM Dex, whole-cell lysates were generated and GR protein levels were determined. Arsenite treatments did not alter the total cellular GR protein levels, but Dex treatments rapidly

(2–4 hr) and significantly (50%) decreased these levels (Figure 6A). This was not a general decrease in total cellular protein levels or discrepancy between the samples because vinculin levels were virtually unchanged in these samples (Figure 6). Next, we examined the effect of a 2-hr 3.3 μ M arsenite pretreatment on the hormone-induced down-regulation of GR. Dex treatments rapidly and significantly decrease GR protein levels (Figure 6B). This degradation was not altered by the arsenite pretreatment (Figure 6B). Therefore, the mechanism by which arsenite decreases GR function does not appear to function through altered GR protein turnover.

Arsenite does not alter Dex-induced nuclear translocation of GR. To determine the basis for these inhibitory effects, we examined whether arsenite could block the hormone-dependent activation and nuclear translocation of GR as part of its mechanism

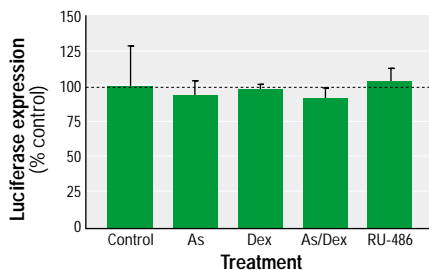


Figure 4. Effect of arsenite on expression of pGL3-control vector. After transfection of H4IIE cells with the pGL3-control construct, cells were treated with 3.3 μ M arsenite, 50 nM Dex, and 100 nM RU-486 as described in Figure 3. Luciferase activity was measured in total cell lysates and data were expressed as a percentage of the transfected, solvent-treated control value. The mean relative light units for control and background were 219,963 and 110, respectively. Each bar represents the mean + SD of values from 3 to 6 individually transfected cultures from 1 to 2 independent experiments.

of action. Simons and co-workers (24–28) reported that the hormone- and DNA-binding ability of immuno-purified GR *in vitro* could be decreased by the addition of arsenite. They postulated that arsenite interacts with critical sulfhydryls on GR to mediate this effect. This response appeared to be highly specific for GR because a similar effect was not observed by arsenite on the closely related mineralocorticoid and progesterone receptors, which lack these sulfhydryls (24,25,29). However, the effects on GR were observed only at concentrations of 10 μ M arsenite or higher in their *in vitro* system, and it was not clear whether similar effects would be observed in intact cells at lower, nontoxic doses. To examine this, we initially performed Western blot analysis with cytosolic and nuclear protein fractions from arsenite- and Dex-treated cells. As shown in Figure 7, control cells demonstrated an abundance of GR in the cytosol and little or no detectable GR in the nuclear fraction. Dex treatment alone caused an almost total disappearance of detectable GR from the cytosolic pool and the appearance of GR in the nuclear pool.

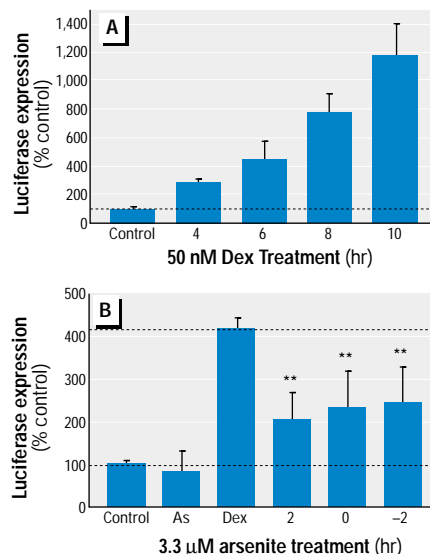


Figure 5. Effect of short-term arsenite treatment on hormone-inducible expression of GRE2-Luc. After transfection of H4IIE cells with GRE2-Luc construct, cells were treated for 6 hr with 3.3 μ M arsenite, for 6 hr with 50 nM Dex alone, or for 6 hr with Dex in combination with arsenite at the pre- and post-treatment times shown. (A) Time dependence of luciferase expression using a 50 nM Dex treatment for 4–10 hr as shown. (B) Effect of 3.3 μ M arsenite pre-, post-, and simultaneous treatments on 6-hr, 50 nM Dex stimulation. Luciferase activity was measured in total cell lysates and data were expressed as a percent of the transfected, solvent-treated control value. The mean relative light units for control and background were 60,244 and 109, respectively. Each bar represents the mean + SD of values from 3 to 12 individually transfected cultures from 1 to 4 independent experiments. ** $p < 0.01$.

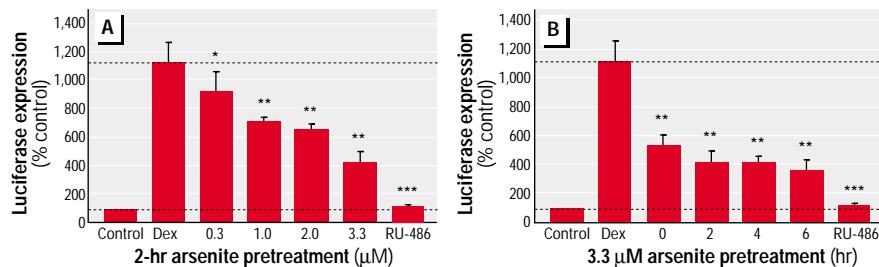


Figure 3. Effect of arsenite on hormone-inducible expression of GRE2-Luc. After transfection of H4IIE cells with the GRE2-Luc construct, cells were treated with arsenite, Dex, and RU-486 at doses and times indicated. (A) Dose dependence of arsenite effect, using a 2-hr pretreatment of arsenite or 100 nM RU-486 before an 18-hr Dex treatment as shown (linear regression of dose–response data, $r^2 = 0.90$). (B) Time dependence of arsenite pretreatment effect, using 3.3 μ M arsenite and pretreatment times shown, or 2-hr pretreatment with 100 nM RU-486, before an 18-hr Dex treatment. Luciferase activity was measured in total cell lysates and data were expressed as a percentage of the transfected, solvent-treated control value. The mean relative light units for control and background were 13,713 and 102, respectively. Each bar represents the mean + SD of values from 3 to 18 individually transfected cultures from 1 to 6 independent experiments.

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

However, a 2-hr arsenite pretreatment had no discernible effect on the translocation of GR from the cytosolic to the nuclear fraction in this assay (Figure 7).

There is always concern in such cell fractionation studies about the ability to cleanly separate cytosolic and nuclear fractions. As an alternative means of examining this phenomenon, we used a GFP-tagged-GR fusion protein and examined the effects of arsenite on GR cellular localization in intact cells using confocal microscopy. Cells were transfected with an expression vector containing the full-length human GR cDNA fused to GFP under the control of the cytomegalovirus promoter (19) (a generous gift from I. Macara). This GFP-GR fusion protein has been characterized previously and was shown to respond to hormone treatment in a similar fashion and to be regulated in a way similar to that of the native GR protein (19). As was observed in the Western blots with native GR, after transient transfection the GFP-GR was localized predominantly in the cytosol in control (untreated) cells (Figures 8A, 8D, and 9), and arsenite treatment alone had little or no effect on this distribution (Figure 9). After Dex stimulation, most GFP-GR was localized to the nucleus (Figures 8B, 8E, and 9), and this translocation was not altered detectably by arsenite pretreatment (Figures 8C, 8F, and 9). These results demonstrate that arsenite does not appreciably alter the normal distribution or the Dex-induced nuclear translocation of GR in these cells. In summary, these data demonstrate that low-dose arsenite is able to

specifically alter the nuclear function of GR independent of cytosolic hormone binding or nuclear translocation.

Discussion

In this study, we demonstrated that noncytotoxic doses of arsenite significantly reduced the expression of PEPCK promoter- and GRE-driven luciferase constructs. This appeared to involve alterations in the nuclear function of GR as a transcription factor, because there were no effects on hormone-stimulated translocation of GR from the cytosol to the nucleus, yet arsenite was able to block GR-dependent induction of gene expression even when administered 2 hr after Dex stimulation. We used the H4IIE rat hepatoma cell model system for these studies because it maintains an intact GR-mediated PEPCK induction pathway and exhibits a robust Dex response (10,30,31). In addition, the liver represents a relevant biological target of arsenic exposure, because it is a first-pass organ and is directly associated with various arsenic-related human disease states such as diabetes (4,32) and cancer (1–4).

Glucocorticoids induce numerous cellular and physiological effects that are mediated predominantly through their interaction with the cytosolic steroid hormone receptor GR. GR, a member of the nuclear receptor superfamily, mediates glucose homeostasis, immune modulation, cellular growth and differentiation, and numerous other physiological responses in a wide variety of tissues (33–36). Unlike many other sex steroid receptors that are localized predominantly to the

nucleus, GR is normally sequestered in a pre-active state in the cytosol, bound in a complex that includes multiple heat shock proteins (HSP56, 70, and 90) (33–35,37–40). Upon steroid binding, GR conformation is altered, unmasking a nuclear localization signaling motif and a DNA-binding domain. This leads to the translocation of the ligand-bound GR to the nucleus in a form that can interact with DNA (37,38,41). Once in the nucleus, GR binds as a homodimer in a head-to-head manner to its *cis*-acting DNA recognition element, the GRE (consensus GRE half-site, TGTTCT). GR also has been shown to participate through protein-protein interactions with other cofactors (co-activators/co-repressors), leading to either positive or negative effects on transcription of specific glucocorticoid-responsive genes (37,39–45).

Arsenic-induced alterations in GR function may play an important role in the mechanism of arsenic carcinogenesis. Lung and skin are two of the primary targets in humans for arsenic-induced increases in cancer risk after systemic environmental exposure. GR has been suggested to play a fundamental role in carcinogenesis in both lung and skin in experimental animal models. Glucocorticoids have long been known to suppress tumor promotion in the mouse two-stage skin cancer model (46). This appears to be primarily a result of glucocorticoid-mediated effects on both cell differentiation and suppression of cell proliferation (46). Glucocorticoid cotreatment with a phorbol ester tumor promoter can suppress the early but not the later stages of tumor promotion and progression

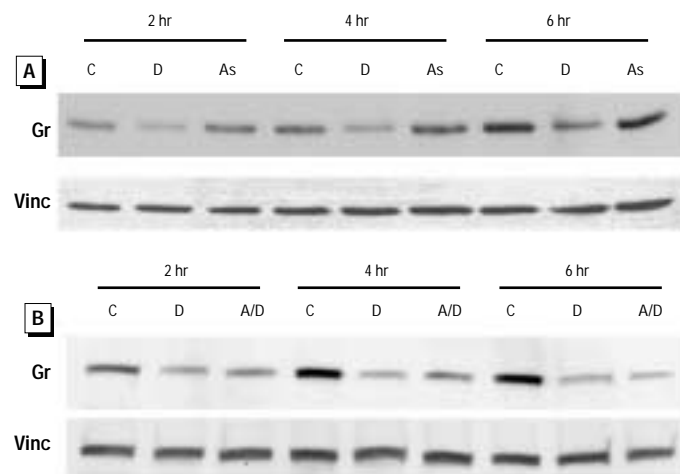


Figure 6. Effect of arsenite on total cellular GR protein. Abbreviations: A/D, Arsenite/Dex. As, Arsenite. C, Control. D, Dex. Vinc, vinculin. Cells were treated for 2–6 hr with 3.3 μ M arsenite or 100 nM Dex alone or arsenite given 2 hr before treatment with 100 nM Dex. Total cellular protein extracts were isolated and Western blot analysis performed with an antiGR antibody using 20 μ g of protein as described in "Methods." (A) Effect of 3.3 μ M arsenite treatment on total cellular GR levels. (B) Effect of a 2-hr, 3.3 μ M arsenite pretreatment on 100 nM Dex induced GR down-regulation. Digitized scans of representative gels are shown for each treatment condition. Each experiment was repeated twice with similar results. GR protein levels were compared in each sample with an internal control, vinculin, as indicated.

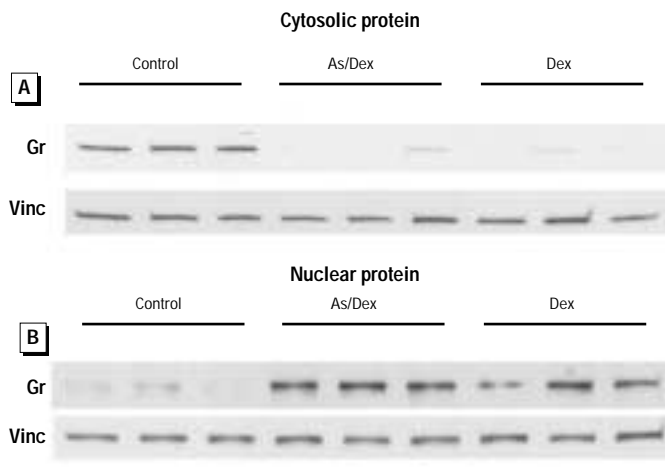


Figure 7. Effect of arsenite on hormone-induced nuclear localization of GR. Cells were pretreated for 2 hr with 3.3 μ M arsenite before a 2-hr treatment with 100 nM Dex. Nuclear and cytosolic protein fractions were isolated from three independently treated cultures per treatment and Western blot analysis performed with an anti-GR antibody using 20 μ g of protein, as described in "Methods." GR protein levels were compared in each sample with an internal control, vinculin (Vinc), as indicated.

in this system (47,48). A progressive loss of hormone responsiveness was observed in later stages of skin cancer in this model, which was associated with both a decreased GR expression and altered GR function. In the earlier stages, decreased response was associated with a decrease in expression of GR mRNA. In contrast, the later-stage tumors had normal levels of GR mRNA and protein, but the receptor was defective in signaling a hormone response even though the GR gene appeared to be intact (47,48).

In similar work examining a glucocorticoid-refractory lung tumor cell line, Parks et al. (49) found that the GR mRNA transcript in these cells was improperly spliced, leading to a GR protein that specifically lacked the hormone-binding domain. Several groups have shown that mouse lung tumor development can also be blocked by glucocorticoids (50–52). The synthetic glucocorticoid budesonide almost completely blocked benzo(a)pyrene-induced lung tumors in female A/J mice when administered in the diet (52) or as an aerosol to the lung (51). Others have demonstrated that the growth of various human and mouse lung tumor

cell lines can be inhibited by glucocorticoids (53–56), and this effect can be blocked by the GR antagonist RU-486 (57), whereas cell lines lacking GR do not respond to glucocorticoid-mediated growth suppression (53–56). Dex has also been shown to induce differentiation and surfactant production in type 2 alveolar cells (58).

Collectively, these results suggest that GR mediates suppression of tumor promotion in skin and lung by suppressing cell growth and inducing differentiation and, conversely, that down-regulation of GR or loss of function of GR is permissive to tumor growth. Similarly, arsenic exposure is associated with increased risk of liver cancer (2,3) and can also lead to a diabetes-like condition (4,32) that may involve disruption of GR-mediated glucose homeostasis in the liver and other organs. Thus, if environmentally relevant doses of arsenic are able to suppress the normal function of GR as a mediator of gene regulation, as suggested by our results, we hypothesize that this may contribute to its ability to promote tumorigenesis and contribute to other pathophysiological states in these tissues. This unique mechanism would suggest

further that arsenic may be able to act synergistically with other toxic and carcinogenic agents to increase disease risk, which is supported by epidemiological data that indicate a synergistic increase from cigarette smoking and exposure to arsenic (2,3). Because arsenic contamination of drinking water is widespread in the United States and elsewhere, and it is usually found in combination with many other toxic chemicals at most Superfund and other toxic waste sites, these combined exposures may represent a significant human health risk.

The mechanism by which arsenite inhibits GR-dependent transcription appears to involve nuclear events rather than alterations in steroid-induced nuclear translocation. Thus, arsenite may represent a new class of endocrine disruptors that may act by altering downstream receptor function rather than by direct competition of hormone binding. The arsenite effects appear to be highly specific for GR-mediated gene expression because arsenite blocked Dex-inducible expression of PEPCK but had no effect on the cAMP-stimulated expression of PEPCK or PEPCK-Luc or of a purely CRE-driven luciferase construct (59). We hypothesize that arsenite binds to and causes allosteric alterations in GR or GR-containing complexes. This in turn perturbs GR complex interactions with other proteins, such as other transcription factors and/or members of the initiation complex, that are critical for GR-regulated induced gene transcription (37,40–45,60). We are currently investigating whether this is directly mediated by arsenite binding to GR and/or GR complex partners. However, the arsenite binding data strongly suggest that direct binding to GR plays a role in these effects. Whether arsenite has similar effects on other members of the steroid receptor family, such as the estrogen and progesterone receptors, at these low doses in intact cells remains to be determined and will provide important information in assessing the overall role of arsenic-induced health effects.

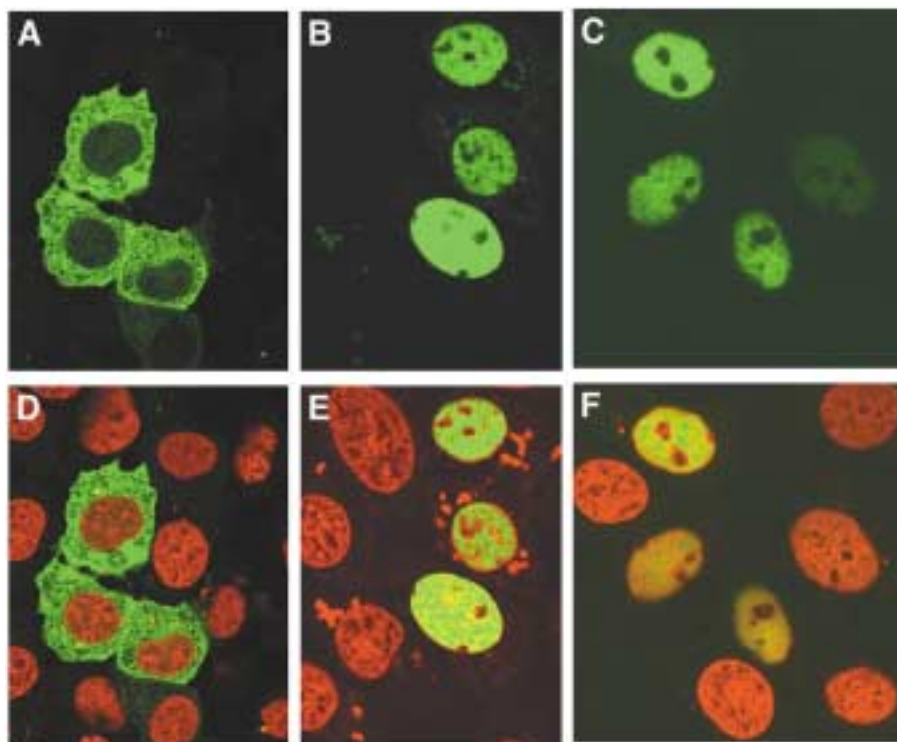


Figure 8. Effect of arsenite on hormone-induced nuclear translocation of a GFP-GR. After transfection of H4IIE cells with the GFP-GR construct, cells were pretreated for 2 hr with 3.3 μ M arsenite before a 2-hr treatment with 100 nM Dex. Cells were then stained and analyzed by confocal microscopy as described in "Methods." This figure shows representative images of control cells: transfected, solvent treated, which demonstrated a predominantly cytosolic localization of GR (A,D); cells treated with 100 nM Dex alone for 2 hr, which demonstrated essentially complete nuclear localization of GR (B,E); and cells pretreated with 3.3 μ M arsenite for 2 hr followed by 100 nM Dex for 2 hr, which also demonstrated essentially complete nuclear localization of GR similar to Dex alone (C,F). The upper panels (A–C) demonstrate GFP fluorescence (green channel); the lower panels (D–F) combine both the GFP and propidium iodide (DNA) fluorescence (green and red channels).

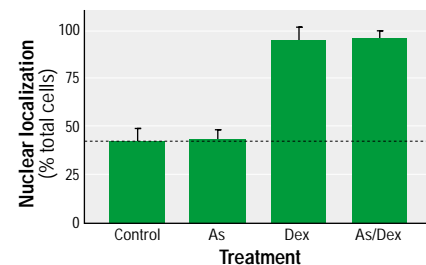


Figure 9. Graphical summary of the percentage of cells with nuclear staining for each treatment. Bars represent the mean + SD of the nuclear percent GFP staining of 50 cells using two slides per treatment in two independent experiments.

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