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Monomethylated trivalent arsenic species disrupt steroid receptor interactions with their DNA response elements at non-cytotoxic cellular concentrations

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Abstract

Arsenic (As) is considered a top environmental chemical of human health because it has been linked to adverse health effects including cancer, diabetes, cardiovascular disease, and reproductive and developmental problems. In several cell culture and animal models, As acts as an endocrine disruptor, which may underlie many of its health effects. Previous work showed that steroid receptor (SR)-driven gene expression is disrupted in cells treated with inorganic As (arsenite, iAs^{+3}). In those studies, low iAs^{+3} concentrations (0.1–0.7 μM) stimulated hormone-inducible transcription, whereas somewhat higher but still non-cytotoxic levels (1–3 μM) inhibited transcription. This investigation focuses on the mechanisms underlying these inhibitory effects and evaluates the role of methylated trivalent As metabolites on SR function. Recent evidence suggests that, compared with iAs , methylated forms may have distinct biochemical effects. Here, fluorescence polarization (FP) experiments utilizing purified, hormone-bound human glucocorticoid (GR) and progesterone receptor (PR) have demonstrated that neither inorganic (iAs^{+3}) nor dimethylated (DMA^{+3}) species of trivalent As affect receptor interactions with glucocorticoid DNA response elements (GREs). However, monomethylated forms (monomethylarsenite, MMA^{+3} and monomethylarsonic diglutathione, MADG) strongly inhibit GR-GRE and PR-GRE binding. Additionally, speciation studies of iAs^{+3} -treated H4IIE rat hepatoma cells show that, under treatment conditions that cause inhibition of hormone-inducible gene transcription, the intracellular concentration of MADG is sufficient to inhibit GR-GRE and PR-GRE interactions *in vivo*. These results indicate that arsenic's inhibitory endocrine disruption effects are probably caused in part by methylated metabolites' disruption of SR ability to bind DNA response elements that are crucial to hormone-driven gene transcription.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

SUPPORTING INFORMATION

Supporting information can be found in the online version of this article.

Keywords

monomethylarsenite; arsenic; arsenite; dimethylarsenite; arsenite triglutathione; monomethylarsonic diglutathione; arsenic speciation; glucocorticoid receptor; progesterone receptor; fluorescence polarization; zinc finger; DNA binding; endocrine disruption; hepatoma cells

INTRODUCTION

Chronic ingestion of inorganic arsenic (iAs) has been linked to significant effects on human health. These increased risks include several types of cancer (lung, liver, skin, kidney and bladder), type 2 diabetes, vascular disease, cardiovascular disease, and reproductive and developmental problems (Abernathy *et al.*, 1999; ATSDR, 1999; Simons *et al.*, 1990). Although there is justifiable concern about exposure to As from Superfund sites, other toxic sites and other anthropogenic sources, the principal concern for human exposure is via ingestion of groundwater containing excess As from natural geological sources. In the US, As-contaminated wells are particularly prevalent in the Northeast, Southwest and Rocky Mountains. Many other parts of the world have similar or higher levels of As contamination of groundwater including Southeast Asia and regions of Europe, Africa and South America. Worldwide there may be hundreds of millions of individuals exposed to excess As in drinking water (Nordstrom, 2002). More recently, it has also become apparent that As in food, such as rice, may represent a significant source of exposure to bioavailable As, as well (Jackson *et al.*, 2012). The US EPA recently reduced the maximum contaminant level of As in public drinking water supplies from 50 to 10 ppb (0.67 to 0.13 μM) because of these health concerns. However, private wells in the US and abroad (which are exempted from the regulatory guidelines that apply to public water supplies) and various foods remain significant sources of exposure.

In the environment, inorganic As exists in two valence states: trivalent arsenite (As^{+3}) or pentavalent arsenate (As^{+5}). In solution, these two forms are readily interconverted, depending on redox status and other solution conditions. In most biological systems, the two forms also interconvert both extra- and intracellularly depending on pH, redox and other conditions, and, therefore, exposure to either form results in similar biological effects. However, As^{+3} is typically more toxic than As^{+5} in cell culture, at least in part owing to differences in uptake (Zangi and Filella, 2012). In solution and at physiological pH, iAs^{+3} is complexed with oxygen (as arsenite) and is neutral (and isostructural with glycerol), so arsenite uses the aquaglyceroporin channels (Liu *et al.*, 2002) to readily enter the cell (and may also enter the cell via diffusion because it is uncharged). Like iAs^{+3} , iAs^{+5} is complexed with oxygen (as arsenate), but arsenate is charged (and isostructural with phosphate and sulfate). Thus, arsenate may not freely diffuse into the cell, and it enters via the so-called non-specific anion transporter and other phosphate transport systems (Andrew *et al.*, 2003), in competition with the high concentration of phosphate in serum (Druwe and Vaillancourt, 2010). Thus, As^{+3} is more readily taken up into the cell, in comparison with As^{+5} (Druwe and Vaillancourt, 2010; Zangi and Filella, 2012).

The extent of intracellular conversion from As^{+3} to As^{+5} is controversial and appears to depend on specific cell types and the relative redox capacity and potential. A liver cell, normally with ~5–10 mM reduced glutathione, is a strongly reducing environment, and, in fact, very little As^{+5} is seen in these cells (Bacquart *et al.*, 2010; Del Razo *et al.*, 2001; Watanabe *et al.*, 2011). Instead, most As species in liver cells are present as reduced, trivalent glutathione complexes (Kala *et al.*, 2000). Inorganic As can also be methylated *in vivo*, to form the organic forms monomethylarsenite (MMA^{+3}) and dimethylarsenite

(DMA⁺), among others. In particular, the monomethylated As⁺ form (MMA⁺) has been found to be more toxic on a molar basis than inorganic As (Petrick *et al.*, 2001; Styblo *et al.*, 2000).

Previous work showed (Bodwell *et al.*, 2004, 2006) that iAs⁺ treatment of rat liver hepatoma cells results in endocrine disruption of hormone-mediated gene expression by the glucocorticoid receptor (GR) and other steroid receptors (SRs). This endocrine disruption, also demonstrated in several different animal and cell culture models, with all types of steroid receptors, and with various endogenous and exogenous hormone-driven genes (Davey *et al.*, 2007, 2008; Hamilton *et al.*, 1998; Kaltreider *et al.*, 2001; Shaw *et al.*, 2007), may be a major mechanism underlying arsenic's effects on human health. The dose response for this disruption is biphasic. Treatment with low levels of iAs⁺ (0.05–0.7 μ M) results in a significant (*c.* 2- to 2.5-fold) enhancement of hormone-stimulated, GR-mediated gene expression; however, slightly higher (1–3 μ M), but still non-cytotoxic, levels results in almost complete inhibition. In the rat hepatoma line EDR-3, the glucocorticoid, progesterone, mineralcorticoid and androgen receptors all demonstrated very similar, biphasic responses to treatment with iAs⁺ (Bodwell *et al.*, 2006).

These receptors are members of the steroid receptor superfamily that exist predominantly as multicomponent complexes of immunophilins, heat shock proteins and other factors. This complex promotes proper SR conformation, efficient ligand binding and interactions with machinery necessary for transport to the nucleus (Murphy, 2005). Once in the nucleus, activated SRs normally associate as homodimers with specific gene regulatory sequences. All of these receptors utilize the same regulatory sequence used in this study, the glucocorticoid response element (GRE) (Cairns *et al.*, 1991; Perlmann *et al.*, 1990). Once associated with the GRE, SRs recruit various coactivators and transcription factors, allowing expression of hormone-responsive genes.

All the SRs, affected by iAs⁺, have a similar modular structure with ligand binding and ligand-dependent activation domains in the C-terminal region and ligand-independent activation domains in the N-terminal region. Whole-cell hormone binding assays have shown that As does not alter the GR hormone binding capacity (Bodwell *et al.*, 2006); As caused no changes in the amount of intracellular steroid or in the binding of steroid to GR within the cell. Thus, the ligand binding domain is probably not involved in the mechanism underlying arsenic's endocrine disruption. It is the central DNA binding domain (DBD) that is the most homologous (90–94%) among these receptors. The biphasic response to As remains intact even after the deletion of the N-terminal region or the C-terminal region (Bodwell *et al.*, 2004) suggesting that the DBD is strongly associated with the response to As. All of these DBDs share a common structure with two zinc (Zn) fingers coordinated to eight of the 10 cysteines within the DBD. Our previous studies (Bodwell *et al.*, 2004) demonstrated that the two free cysteines in the DBD are not involved in the response to As. These studies also demonstrated that mutations that alter the conformation of the dimerization domain (D-loop) to a constitutively DNA-bound conformation actually enhance arsenic's inhibitory effect on gene transcription. Taken together, these results point to involvement of the SR DBD region in the response to As and led us to consider whether As alters the level of GR binding to GRE DNA.

Although previous work demonstrated that treatment of purified SRs with iAs⁺ did not affect SR-GRE binding (Bodwell *et al.*, 2006), it remained possible that arsenic's methylated metabolites might interfere with this binding and as such could be a mechanism underlying the inhibition of SR-driven gene transcription by moderate levels of As. This study addresses this question of the role of methylated As metabolites in arsenic's endocrine disruption.

MATERIAL AND METHODS

Caution: *As* is classified as a known human carcinogen (Abernathy *et al.*, 1999; ATSDR, 1999). All arsenic compounds must be handled as highly hazardous chemicals.

Reagents, buffers, vectors and cell culture

These reagents, procedures and recipes have been previously detailed in (Bodwell *et al.*, 2004, 2006).

Preparation of H4IIE-G2T/Luc cell line

H4IIE, a rat hepatoma cell line, was transfected with the G2T reporter vector [a luciferase reporter gene controlled by tandem GREs (Bodwell *et al.*, 2004)] and pZEOSV (Zeocin resistance) at a ratio of 10:1 (22 µg DNA total). Transformation was performed in a BTX ECM830 at 190 volts (80 ms time constant) in a 0.4-cm cuvette with a total of 400 µl of cells (3E6) and media containing 2.5% fortified calf serum (Sigma Chemical, St. Louis, MO, USA) without antibiotics. After 24 h, transfected cells were selected with 500 µg Zeocin ml⁻¹ to obtain dexamethasone-responsive clones and further subclones were subsequently obtained by serial dilution.

As treatment

H4IIE-G2T/Luc cells (~80% confluent) were treated with the indicated levels of iAs⁺³ for ~24 h, as described previously (Bodwell *et al.*, 2004, 2006). Cells treated with 0, 1, 2 or 3 µM iAs⁺³, along with 2 nM dexamethasone (Dex), were cultured in 24-well plates for 24 h, and luciferase expression was determined as in (Bodwell *et al.*, 2004, 2006). Cells used to determine intracellular As metabolite levels were grown in twelve 15-cm tissue culture-treated Petri dishes. Half of the plates were treated with 1.7 µM iAs⁺³ for 24 h, whereas the remainder served as buffer controls. After As treatment, cells were washed with cold phosphate-buffered saline (PBS) and then scraped into 5 ml of cold PBS and all six plates from each treatment pooled and centrifuged (3 min, 1000 g, 2 °C). The cell pellet was resuspended with 2 ml of cold PBS and divided into two pre-weighed 2-ml centrifuge tubes and centrifuged as above. All liquid was carefully aspirated, and the tube was re-weighed to obtain the pellet weight. The pellet was processed as described in the next section.

Determination of intracellular arsenite metabolite levels

The cell pellets prepared above contained 6.94×10^8 cells g⁻¹, and the cell volume (determined from cytocrit analysis) was 1.29 ± 0.03 nl per cell under these culture conditions. Previous researchers (Warskulat *et al.*, 1997) also determined the total cellular volume of H4IIE cells using flow resistance cytometry and obtained a volume of 1.09 nl per cell. Use of a factor of 0.8 to approximate the accessible water space or intracellular volume, as was used in (Warskulat *et al.*, 1997), a value of 1.03 nl per cell was obtained – in good agreement with the Warskulat *et al.* value of 8784 nl per cell (Warskulat *et al.*, 1997). Using the weight of the cells processed and the intracellular volume from which the As metabolites were released, the intracellular As levels were determined and expressed as molar concentrations.

The weighted cell pellets from the previous section were dispersed by repeated pipetting (10×) with 5 volumes of lysis solution [cold 0.05% Triton with 5mM reduced glutathione (GSH) in 1% formic acid that had been degassed]. Tubes were gassed with nitrogen, capped, put on ice and immediately transported (~20 min) to the Trace Element Analysis Core for further processing and analysis. At the core facility, tubes were transferred to an anaerobic chamber and spun at 12 000 g at ambient temperature. Supernatants were removed and an

equal volume of degassed 1% formic acid with 5 mM GSH was added to give sufficient sample volume for analysis. Samples remained in the anaerobic chamber until immediately before analysis (1–4 h).

As–GSH complexes were synthesized 24 h before analysis. Sodium arsenite (Sigma-Aldrich), monosodium acid methane arsonate (Chem Services, West Chester, PA, USA) and cacodylic acid sodium salt (Sigma-Aldrich) (10 mg of each chemical) were placed into separate 50-ml PTFE tubes (Savillex), to which were added a 10-fold molar excess of reduced L-glutathione (GSH; Sigma Aldrich). Tubes were placed inside an anaerobic hood and 10 ml of N₂-purged ultrapure water (> 18.2 MΩ cm⁻¹) produced by Purelab Pluswater purifier (US Filter, Snellville, GA) was added to each tube, along with a stir bar. Solutions of each As complex (arsenite triglutathione, ATG; monomethylarsonic-diglutathione, MADG; and dimethylarsonic-glutathione, DMAG) were then stirred overnight in the anaerobic hood.

Accurate determination of As concentrations in stock solutions were determined by diluting each stock 10 000 times by volume in 5 ml of 1% HNO₃ (Fisher Scientific, Pittsburg, PA), followed by analysis with solution mode inductively coupled plasma mass spectrometry (ICP-MS). Composite standards of the three As complexes were prepared immediately prior to use, by volume by pipetting 50 µl of stock into 5 ml of a solution of freshly prepared and degassed 0.025% Triton X-100, 1% formic acid, 10% PBS buffer and 5 mM GSH.

Solutions of cell lysates were maintained in 5 mM GSH/1% formic acid under anaerobic conditions to keep As complexes stable until analysis (< 4 h after lysing) (Yehiayan *et al.*, 2009). Determination of As glutathione complexes was achieved by high-performance liquid chromatography (Agilent LC 1120; Agilent Technologies, Santa Clara, CA) coupled with a quadrupole ICP-MS (Agilent 7500C; Agilent Technologies), using a method similar to Yehiayan *et al.* (2009). Complexes were separated on a Waters Spherisorb C8 column (150 × 4.6 mm; 5-µm particle size). A gradient elution program of 1% formic acid and 5% acetonitrile, ramped to 15% acetonitrile over 10 min was applied, with a flow rate of 1 ml min⁻¹. Eluents were purged immediately prior to use, and the column compartment was cooled to ~10°C. The mobile phase was introduced to the ICP-MS via a Micromist nebulizer (Glass Expansion, Pocasset, MA, USA), and 3% O₂ (99.99% Air Gas, Salem, NH) was introduced to the nebulizer gas. Ions at m/z 75 and 77 were monitored for the analyte (⁷⁵As) and chloride interferences (⁴⁰Ar³⁵Cl and ⁴⁰Ar³⁷Cl). Concentrations of ATG, MADG and DMAG in samples were quantified by standard addition, where a 0.75-ml aliquot of each sample was analyzed once as-is, and a second 0.75-ml aliquot was spiked with 0.04 ml of a composite standard (10 µM ATG, MADG and DMAG). A chromatogram of As speciation of a cell lysate is given in Supplementary Fig. 1 and depicts the three As–GSH complexes: ATG, MADG and DMAG. Dissociated As species and a broad band, identified as an oxidation product of DMAG (Raab *et al.*, 2004; Yehiayan *et al.*, 2009), were also detected. Concentrations of these non-analyte species were estimated using the mean sensitivity (peak area/known concentration of As in standard solutions of ATG and MADG [DMAG sensitivity was not included as this species is prone to decomposition]). Because ICP-MS measures only As owing to decomposition of compounds/complexes by the plasma, the sensitivity of different As species is very consistent: the relative percent difference between ATG and MADG sensitivity was just 6%. Column recovery was calculated as the sum of all As species (including unidentified species), compared with a separate measure of total As in the cell lysate solutions determined by solution mode ICP-MS using external calibration.

Synthesis of As metabolites

MMA⁺³ and DMA⁺³ were a generous gift from Drs Dean Wilcox, Anne Spuches and Colette Quinn (Department of Chemistry, Dartmouth College, Hanover, NH, USA).

Preparation of MMA^{+3} , which involves the synthesis methyl-diiodoarsinous acid, MeAsI_2 , which forms MMA^{+3} when dissolved in aqueous solution, has been described previously (Spuches *et al.*, 2005). The preparation of DMA^{+3} , which involves the synthesis of dimethyl-diiodoarsinous acid, Me_2AsI_2 , which forms DMA^{+3} when dissolved in aqueous solution, was prepared according to the method of Burrows and Turner (1920). MADG was prepared by incubating MMA^{+3} ($< 10 \mu\text{M}$) with 1 mM GSH prior to adding receptor for fluorescence polarization (FP) analysis.

Purification of hPR and hGR

hPR and hGR were purified as described previously (Bodwell *et al.*, 2006), with hormone ligand (triamcinolone acetonide for GR and R5020 for PR) bound to the receptors' ligand binding domains. hGR was purified with the addition of 1 mM glutathione (GSH) and 7.5 mM CHAPS detergent whereas the hPR preparation contained neither GSH nor CHAPS. Purity ($>90\%$) and concentration were determined as previously described (Bodwell *et al.*, 2006).

Fluorescent Oligonucleotide Duplexes

The PR/GR response element (GRE) from the TAT gene promoter (Jantzen *et al.*, 1987) was used for the fluorescein-labeled FITC-GT probe, which contains a single GRE. Oligonucleotide duplexes of FITC-GT were prepared as described previously (Bodwell *et al.*, 2006).

Fluorescence Polarization

FP was performed essentially as described by Bodwell *et al.* (2006). Samples contained final concentrations of 1 nM FITC-GT duplex, 10 mM Tris, pH 7.8, 10% glycerol, 0.34 mg ml^{-1} BSA, 1 mM MgCl_2 , 150 mM NaCl, glutathione (GSH; used where indicated), 7.5 mM CHAPS detergent (used for all hGR but not for hPR experiments), hPR or hGR, plus 0–10 μM inorganic sodium arsenite (iAs^{+3}) or 0–2.03 μM monomethylarsenite (CH_3AsI_2 ; MMA^{3+}) or 0–10 μM dimethylarsenite [$(\text{CH}_3)_2\text{AsI}$; DMA^{3+}] or 0–2.03 μM monomethylarsonic diglutathione [$\text{CH}_3\text{As}(\text{GS})_2$; MADG] or 0–20 μM potassium iodide (KI). Samples were mixed at $20.5 \pm 1.5^\circ\text{C}$. In each experiment, hPR and hGR were added at a single concentration, equivalent to that able to bind ~60% of the FITC-GT DNA probe, and a battery of concentrations of As compounds or KI was added to the samples. For testing the effects of arsenic compounds or KI, receptors were incubated with As or KI and other buffer components for 20 min at $20.5 \pm 1.5^\circ\text{C}$ before the addition of the FITC-GT duplexes. The FP function of the Molecular Devices Spectramax M5 microplate reader was used to measure binding of the receptors to FITC-GT at room temperature ($20.5 \pm 1.5^\circ\text{C}$) and at equilibrium (equilibrium binding conditions are described in Bodwell *et al.*, 2006). Each experiment was performed two or three times with triplicate samples per treatment.

RESULTS

GR gene expression is readily inhibited by low micromolar levels of inorganic arsenite in H4IIE-G2T/Luc hepatoma cells

Our previous studies (Bodwell *et al.*, 2004, 2006) demonstrated that gene expression mediated through the steroid receptors, GR, PR and MR, was substantially inhibited (up to 90%) by treatment of cells with 1–2.7 μM iAs^{+3} . This study focuses on a potential mechanism that could provide an explanation for the iAs^{+3} inhibitory effects of SR gene expression in cells able to methylate iAs^{+3} and to maintain it in the trivalent state.

Previous studies (Bodwell *et al.*, 2004, 2006) utilized the EDR-3 hepatoma line, which does not contain functional GR, PR or MR. Although the EDR-3 cells were convenient for determining the effect of iAs^{+3} on various SRs, their use required transient transfection of vectors encoding both the SR and the reporter gene into the cell. In order to streamline the analysis, eliminate transfection variation, and utilize an endogenous SR (GR), we generated a stable cell line from the H4IIE hepatoma line, which has a similar lineage as the EDR-3 cell line. The new cell line, designated H4IIE-G2T/Luc, has a stably integrated luciferase gene under the control of two tandem GREs. As H4IIE-G2T/Luc has ~50 000 to 75 000 native and functional GRs per cell, it displays a dose-dependent increase in luciferase expression in response to increasing levels of glucocorticoids such as Dex (data not shown) as expected (Kaltreider *et al.*, 2001). Dex (2 nM) produced an 11.2-fold increase over the no-Dex control.

Treatment of H4IIE-G2t/Luc cells with iAs^{+3} (Fig. 1) reduced luciferase expression mediated by 2 nM Dex by 36% at 1 μM , by 73% at 2 μM and by 87% by 3 μM . Figure 1 is a representative data set; repeat experiments with 2 nM Dex and with lower and higher concentrations of Dex gave similar results (data not shown). These levels of inhibition of GR-mediated gene expression in the H4IIE-G2T/Luc cells are very consistent with the level of inhibition obtained using the EDR-3 system (Bodwell *et al.*, 2004, 2006). The standard deviation (SD) of the luciferase expression of the 0 μM As control samples, prior to normalization, was 4.9% of the mean value of triplicates (mean $\pm$ SD = 2439773 $\pm$ 118736 RLU mg^{-1}). Treatment with iAs^{+3} had no effect on the basal (0 nM Dex) levels of luciferase expression (data not shown). Previously, it was shown that these doses of iAs^{+3} are completely non-cytotoxic to H4IIE cells (Kaltreider *et al.*, 2001). Additionally, cytotoxicity assays (based upon trypan blue exclusion) with the stably-transfected H4IIE-G2T/Luc cell line indicate that iAs^{+3} doses of 6 μM (highest dose tested) or lower do not alter cell viability (data not shown). Intracellular levels of As^{+3} metabolites for this cell line were determined as described in subsequent sections.

Estimation of the concentrations of As metabolites produced in the H4IIE-G2T/Luc cell line

The key hypothesis of this study is that organic metabolites of iAs are responsible for altering binding of SRs to their GREs, resulting in suppression of hormone-mediated gene expression. If this hypothesis is true, the hepatoma cell line utilized above would be required to produce measurable levels of these iAs^{+3} metabolites. Thus, speciation analysis of H4IIE-G2t/Luc cells treated with iAs^{+3} was performed. Cells treated with 1.7 μM iAs^{+3} were found to concentrate As, and to produce significant amounts of monomethylarsonic diglutathione, MADG (3.1 μM) (Fig. 2).

Liver and hepatoma cells are known to metabolize iAs^{+3} into both mono- and dimethylated forms, but speciation results are usually not presented in a way that allows an estimation of the concentration of these metabolites within the cell. These cells have considerable reducing potential because they contain mM levels of GSH and other thiol compounds. Thus, intact cells would be expected to harbor predominantly trivalent forms of As (Dopp *et al.*, 2010) (rather than pentavalent), and this has been confirmed by a number of previous studies that have demonstrated that in the human hepatocarcinoma cell line, HepG2, the predominant As species are trivalent (Bacquart *et al.*, 2010; Watanabe *et al.*, 2011). However, during lysis of the cells, metabolites could be exposed to a much a more oxidative atmosphere, opening the possibility for conversion to arsenate forms. Thus, this conversion was minimized by inclusion of reduced GSH in all solutions, use of solutions purged of oxygen and use of an anaerobic glove box where possible.

After entry into cells with high GSH levels, iAs^{+3} is rapidly converted to the triglutathione complex ATG (Dopp *et al.*, 2010). ATG can then be methylated to form MADG and then

further methylated to DMAG. The extent of methylation was analyzed using reverse phase chromatography coupled with ICP-MS (Yehiayan *et al.*, 2009) to determine the intracellular levels of ATG, MADG and DMAG in the H4IIE-G2T/Luc cells (see Material and Methods).

The level of total soluble As (Fig. 2, 'Total As', $24.7 \pm 0.9 \mu\text{M}$) in the $1.7\text{-}\mu\text{M}$ iAs^{+3} -treated H4IIE-G2T/Luc cells points out an interesting outcome of this analysis, that H4IIE-G2T/Luc cells actually concentrate iAs^{+3} and its metabolites to a level ~15 times higher than the extracellular iAs^{+3} concentrations. A similar effect was noted in a mouse model system in which organ accumulation of iAs^{+3} and its metabolites was noted in the liver, kidney, lung and bladder (Kenyon *et al.*, 2008). Differences between that mouse system and ours and the way in which the analysis was performed and expressed preclude any direct comparison of the two data sets.

In the lysed cells that were treated with $1.7 \mu\text{M}$ iAs^{+3} , $1.2 \pm 0.3 \mu\text{M}$ ATG, $3.1 \pm 0.2 \mu\text{M}$ MADG and $4.8 \pm 1.2 \mu\text{M}$ DMAG were determined, showing that significant methylation occurs in the cell. Column recovery of As was $100 \pm 10\%$. DMAG is the least stable of the As-GSH complexes and produces a broad band that adds to the baseline of ATG (Supplemental Figure 1 Raab *et al.*, 2004; Yehiayan *et al.*, 2009). The robust recovery of ATG and MADG suggests that we are correctly detecting these species while underestimating DMAG. This is consistent with previous studies (Yehiayan *et al.*, 2009) that estimated half-lives, after removal from an anaerobic environment, of 35 min, 37 min and 55 min for ATG, MADG and DMAG, respectively. These half-life values suggest that, under our chromatographic conditions [0.1% formic acid = pH 2.7, and a 15-min run time (Yehiayan *et al.* 2009)], decomposition or interconversion will be minimal. The relatively long half-life seen for DMAG (Yehiayan *et al.*, 2009) would suggest that on-column oxidation issues may be leading to the enhanced degradation that we observe for that species.

The sum of the As-GSH species ($9.1 \pm 2.8 \mu\text{M}$) accounted for ~37% of the total soluble As in treated cells. A number of potential factors account for the remaining As. These include the degradation/oxidation of the DMAG (Raab *et al.*, 2004; Yehiayan *et al.*, 2009) (Supplementary Fig. 1), resulting in loss of signal and its underestimation; dissociation of GSH from the complexes; complexation of As to other thiol-containing compounds such as thioredoxin or lipoic acid, present in the lysates. In addition, active metabolites such as MADG could be constantly reacting with target compounds in the cell, some of which would be soluble and included in the total As pool. Nevertheless, MADG, the analyte of interest, was present as a third of the GSH-complexed As in the liver cell and at a high level ($3.1 \pm 0.2 \mu\text{M}$), verifying that this species is produced at sufficient rates in the cell to cause inhibition of the SR-GRE interaction (see below). Furthermore, the MADG concentrations determined here may be a conservative estimate because some dissociation and demethylation may have occurred during cell lysis, extraction and analysis. For comparison, previous researchers (Kala *et al.*, 2000) found ATG and MADG (which were not separated in their study) to account for 80–90% of As in rat bile, but were not able to detect DMAG.

Effect of As metabolites on the interaction of GR and PR with the GRE DNA

Much of our previous work has suggested that the SR DBD is involved in the mechanism of the As effect on gene transcription (Bodwell *et al.*, 2004, 2006). As a logical target for iAs^{+3} was the direct disruption of the SR-GRE interaction, it was initially surprising when fluorescence polarization studies (Bodwell *et al.*, 2006) clearly showed that iAs^{+3} has no effect on the interaction of either PR or GR with the GRE.

Recently, the Wilcox lab demonstrated (Spuches and Wilcox, 2008), using isothermal titration calorimetry, circular dichroism and other techniques, that, although iAs^{+3} has no

effect on the Zn-stabilized conformation of the GR DBD, the monomethylated metabolite MMA^{+3} readily disrupts the Zn-stabilized structure, thus reverting the DBD structure to the random coil conformation seen in the absence of Zn. Disruption of DBD structure by this magnitude would preclude any specific interactions between the DBD and GRE. If such a process occurs in the hepatoma cells such that the conformation the SR DBD is compromised by trivalent metabolites, then this would provide an explanation for at least a portion of the inhibitory effects of As on SR gene expression.

The H4IIE-G2T/Luc hepatoma cell line metabolizes iAs into MADG at appreciable intracellular levels (Fig. 2). Thus, experiments were performed to determine whether these individual metabolites affect the interaction of the intact, hormone-bound, full-length GR or PR with a GRE. FP methods optimized in prior As studies (Bodwell *et al.*, 2006) were utilized. FP is a method for quantifying binding in solution. When a small fluorescent molecule, which in this case is a DNA oligonucleotide duplex containing a GRE sequence (FITC-GT), is in solution, it tumbles rapidly. When the fluorophore (FITC) is excited by plane-polarized (vertical) light, the fluorescent light is emitted mainly in other planes, i.e. is depolarized, owing to the rapid motion of this small molecule. When a large molecule such as hGR binds the fluorescent FITC-GT DNA, the fluorophore is now part of a much larger, slow-tumbling complex. Consequently, more emitted fluorescence light remains polarized (vertical). The portion of emitted fluorescence that remains in the vertical plane is expressed as milli-Polarization units (mP). Higher mP values are measured when more binding (FITC-GT to SRs) occurs.

Using FP analysis, a fixed level of highly purified, ligand-bound human PR (hPR) was tested for its ability to bind to its GRE in the presence of a wide range of MMA^{+3} and MADG concentrations. Both MMA^{+3} and MADG strongly inhibited (Fig. 3) the PR-GRE interaction. Clearly MMA^{+3} is the more reactive metabolic product, with an IC_{50} of $\sim 0.02 \mu\text{M}$, but the PR-GRE interaction was also effectively inhibited by the glutathione conjugated form, MADG, with an IC_{50} of $0.5 \mu\text{M}$.

The hPR preparation was very stable and did not need any reducing or stabilizing agents during the purification. However, in order to obtain fully functional human GR (hGR), the additional presence of CHAPS and GSH was required for optimal purification and storage (see (Bodwell *et al.*, 2006)). Owing to the presence of residual GSH (0.125 mM), it was not possible to obtain a reliable estimate of the effect of MMA^{+3} on hGR because an unknown fraction of the residual GSH participated in forming MADG. Therefore, only the effects of MADG are shown for the hormone-bound GR in Fig. 3. It is interesting that the hGR also had an IC_{50} of $0.5 \mu\text{M}$ for MADG, just as was seen with the PR. Central to the argument that MADG reacts with SR-DBD and inhibits function is that there is a sufficient intracellular level of MADG. As shown above in Fig. 2, intracellular MADG levels were $\sim 3 \mu\text{M}$, which would be more than sufficient to cause inhibition of the SR-GRE interaction (Fig. 3) for both GR and PR (see Discussion).

DMA^{+3} , iAs^{+3} , and KI do not alter the interaction of SRs with the GRE

Experiments were performed with the dimethylated arsenic metabolite DMA^{+3} in place of MMA^{+3} or MADG in identical equilibrium experiments, using the same incubation times with the receptor prior to and after the addition of FITC-GT. DMA^{+3} had no significant effect on the interaction of hGR with its GRE even at very high concentrations (10 to $20 \mu\text{M}$, Fig. 4). Also included in Fig. 4, for comparison purposes only, are previously published data (Bodwell *et al.*, 2006) with iAs^{+3} . Similar incubation experiments were performed with potassium iodide (KI) in order to confirm that the binding inhibition by MMA^{+3} is as a result of the MMA^{+3} itself and not to the accompanying iodide ion from the preparation of

MMA³⁺, CH₃AsI₂, used in this study. Figure 4 shows that there was no effect of iodide on GRE binding.

DISCUSSION

This study is the first to demonstrate that two principal trivalent metabolites of iAs, MMA⁺³ and MADG, are able to inhibit the interaction of full-length, ligand-bound and functional SRs with their cognate DNA response element and to do so at concentrations well within the range of those present inside cells exposed to non-cytotoxic extracellular levels of iAs⁺³.

There has been no prior direct analysis of As speciation in H4IIE or EDR-3 cells, but there are data on other liver-derived cells, especially the hepatocarcinoma line HepG2. When HepG2 cells are treated with iAs⁺³, the As is converted to methylated species and remains predominantly trivalent (Bacquart *et al.*, 2010; Del Razo *et al.*, 2001; Watanabe *et al.*, 2011). This appears to be the case even when cytotoxic doses are applied (Bacquart *et al.*, 2010), suggesting that there is sufficient reducing power within liver-derived cells to maintain the trivalent state even in the presence of high intracellular levels of As. X-ray absorption near-edge structure (u-XANES) (Bacquart *et al.*, 2010) has also revealed that As remains in the trivalent state in the cytosol, nucleus and mitochondrial compartments although, in one of 10 cells analyzed, there was a mixture of tri- and pentavalent As in the nuclear compartment. Thus, it appears that the trivalent state is maintained predominantly throughout the entire cell.

The level of MADG present in H4IIE-G2T/Luc cells (~3 μM; Fig. 2) is clearly sufficient to account for the observed *in vitro* reduction in binding of GR and PR with a GRE. The IC₅₀ for this effect with MADG for both hGR and hPR was ~0.5 μM (Fig. 3). Thus, an intracellular concentration of ~3 μM might be expected to produce an even greater inhibition of gene expression than that shown in Fig. 1. Considering the diverse nature of the FP- and cell-based assays systems, the results are in good agreement, but there are a number of possibilities for this slight discordance. The As speciation analysis treats the intracellular volume as one compartment with equal distribution throughout. However, neither the site of MADG production nor the degree to which MADG is evenly distributed throughout the compartments of the cell is known. It is also not known where the reaction of MADG with SRs occurs and whether the MADG would migrate fully from its site(s) of formation to the site of its GR interaction (which could be either in the cytoplasm or in the nucleus). In addition, the FP analysis used purified GR and PR, but within the cell these receptors have a complicated mechanism of action that includes association with a number other molecules including heat shock proteins, other chaperone proteins, and immunophilins. It is possible that either the presence of these proteins alters (increases) the IC₅₀ or that MADG only reacts with the receptors that are dissociated from these proteins. Thus the reaction might be compounded by kinetic effects and require a higher intracellular concentration to get the same level of inhibition.

The results on MMA⁺³ inhibition on GRE binding presented in this study used full-length and functional SR, but a number of studies using isolated fragments of SRs and other Zn finger proteins also support the findings in this study. An elegant study (Spuches and Wilcox, 2008) demonstrated that both As⁺³ and MMA⁺³ bind to the apo-GR DNA binding domain (apo-GR DBD, i.e. the DBD stripped of its normal 2 Zn atoms). The native DBD (Zn₂ GR DBD) normally contains two Zn atoms that bind with two different affinities. The binding affinity of MMA⁺³, unlike that of iAs⁺³, is high enough that it could displace (at least partially) the lower-affinity Zn atom. In fact, CD studies subsequently demonstrated that only MMA⁺³ (and not iAs⁺³) was able to disrupt the Zn-stabilized conformation of the Zn₂ GR DBD. The destabilized structure of the MMA⁺³-treated Zn₂ GR DBD is random-

coil-like in nature, much like the apo-GR DBD, and would not be able to support specifically binding to the GRE. These data exactly parallel the FP data (Fig. 3) utilizing full-length, functional PR and GR, in that only MMA⁺³ and MADG (not iAs⁺³) are able to disrupt the interaction of these SRs with the GRE. Although structural data for the full-length SRs in the presence of MMA⁺³ is lacking, it is likely that the same mechanism detected by the Wilcox group for the isolated DBD is also occurring with the full-length receptors; i.e., the MMA⁺³ and MADG are disrupting the conformation of the DBD and preventing the interaction with the GRE.

Likewise, another research group (Piatek *et al.* 2008) found that MMA⁺³, but not iAs⁺³, could displace Zn from the Zn finger domain of xeroderma pigmentosum protein A (XPA), a key enzyme involved in DNA repair. The XPA Zn finger is the same type as found in SRs (C4), in that the Zn is bound by four cysteines. Others (Zhou *et al.*, 2011) have noted that the DNA repair protein PARP-1, which is a C3H1 (3 cysteines/1 histidine) Zn finger structure, will also bind iAs⁺³ to the free cysteines in the apo form. They also showed that addition of iAs⁺³ to the human keratinocyte cell line (HaCaT) decreases the Zn content of isolated PARP-1 and XPA proteins, suggesting decreased function. As this occurred in cells, it is not known if iAs⁺³ or a metabolite such as MMA⁺³/MADG was responsible for the loss of Zn in the PARP-1 and XPA proteins. Another group found that MMA⁺³ inhibits the function of PARP-1 in HeLa S3 cells at low, non-cytotoxic doses which are 10 times lower than the concentrations of iAs⁺³ needed to produce the same effects (Walter *et al.*, 2007). These researchers also found that both MMA⁺³ and iAs⁺³ inhibit activity of isolated recombinant PARP-1 protein, with a greater degree inhibition caused by the methylated compound (Walter *et al.*, 2007).

The glucocorticoid receptor signaling pathway crosstalks to many other pathways and is necessary for maintaining cellular and systemic homeostasis. Recently, an elegant study showed that arsenic-induced birth defects could be prevented via antagonist blockade of the glucocorticoid pathway (Ahir *et al.*, 2013).

These data support the hypothesis that in cells (such as liver) that are capable of methylating iAs⁺³ and that have sufficient reducing power (GSH levels) to maintain these species in the trivalent state, MADG will be produced at levels sufficient to disrupt C4 (or C3H1) Zn finger structures, thereby compromising function in a number of important systems including hormone-mediated gene transcription and DNA repair. Disruption of these systems would be expected to contribute to pathophysiological consequences that, in turn, would lead to the serious health consequences observed in As-exposed populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Treatment of H4IIE-G2t/Luc cells with inorganic arsenite (iAs³⁺) inhibits glucocorticoid receptor (GR)-mediated gene transcription. H4IIE-G2T/Luc cells are rat hepatoma cells that contain stably integrated luciferase under the control of a tandem glucocorticoid receptor response element (GRE) promoter. Cells were treated with iAs³⁺ (0, 1, 2 or 3 μM) and 2 nM dexamethasone (Dex) for 24 h prior to luciferase determination. The dataset was normalized to its 0 μM As/ 2 nM Dex control. Data values represent mean ± standard deviation (SD), of triplicate samples. Statistical significance was evaluated with Prism software (Graphpad, San Diego, CA, USA), and difference from 0 μM As in a one-way ANOVA, followed by Tukey's post-test, is reported as *** = $P < 0.001$.

Figure 2.

Estimation of intracellular levels of inorganic arsenite (iAs^{+3}) metabolites in H4IIE-G2T/Luc cells. H4IIE-G2T/Luc cells were treated for 24 h with $1.7 \mu M iAs^{+3}$. Using conditions to minimize oxidative effects (see Material and Methods), cells were processed in the presence of 5 mM glutathione (GSH), and both total As and metabolites with GSH were determined. From the number of cells processed, the cellular volume of the cells and estimation of the water accessible space, an intracellular concentration for each metabolite [arsenite triglutathione (ATG), monomethylarsonic diglutathione (MADG) and dimethylarsonic glutathione (DMAG)] and total As was calculated. Data are presented as the mean of duplicates $\pm$ standard deviation (SD) (see Material and Methods). Sum of species is the summation of all the metabolites determined. Total As is a separate determination, for monitoring the total concentration of all species of As in the cell.

Figure 3.

Monomethylarsenite (MMA⁺³) and monomethylarsonic diglutathione (MADG) inhibit human GR (hGR) and hPR binding to glucocorticoid receptor response element (GRE) DNA response elements. 1 nM FITC-GT in fluorescence polarization (FP) buffer plus the indicated final concentrations of MMA⁺³ or MADG were incubated with a fixed level (~50 nM) of hPR or hGR (MADG only), and FP was measured at equilibrium as described in Material and Methods. The no arsenic (As) controls were set at 1 and represent ~60% of maximal binding to the GRE. 'Change in polarization (normalized)' represents the difference between polarization values measured with and without added receptor, with the differences normalized to the highest change in polarization value on a given experimental day. Data points represent the average of two or three independent experiments per receptor/treatment combination, $\pm$ standard deviation (SD). (Three replicates were measured for each data point within each independent experiment.) Statistical significance was evaluated with Prism software (Graphpad, San Diego, CA, USA), and difference from 0 μ M As in a one-way ANOVA, followed by Tukey's post test, is reported as *** = $P < 0.001$.

Figure 4.

Dimethylarsenite (DMA⁺³), inorganic arsenite (iAs⁺³) and KI have no effect on human GR (hGR) binding to its glucocorticoid receptor response element (GRE) DNA response element. Experimental conditions were as described for Fig. 3, except that the hGR was incubated with iAs⁺³, DMA⁺³ or KI at the indicated concentrations. No statistically significant differences from 0 μM controls were found (one-way ANOVA with Tukey's post test).