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Original Contribution

As(III) transcriptionally activates the *gadd45a* gene via the formation of H_2O_2

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Abstract

Arsenic is a ubiquitous environmental contaminant associated with increased risks of human cancers of the skin, lung, bladder, and prostate. Intriguingly, it is also used to treat certain types of leukemia. It has recently been suggested that these paradoxic effects may be mediated by arsenic's ability to simultaneously activate DNA damage and apoptotic and transformation pathways. Here, we investigate the effects of arsenic exposure on the induction of the growth arrest and DNA damage protein 45α (GADD45α), which is thought to play roles in apoptosis, DNA damage response, and cell cycle arrest. We found that arsenic transcriptionally activates the *gadd45α* promoter located in a 153-bp region between −234 and −81, relative to the transcriptional start site. In addition, this transcriptional induction was abrogated in the presence of H_2O_2 scavengers, suggesting a role for H_2O_2 in the transcriptional control of the *gadd45a* gene through a Fenton-like free radical mechanism.

Keywords: GADD45α; Arsenite; As(III); Arsenic; BEAS-2B; DNA damage; Cell cycle control

Introduction

Inorganic arsenic is a ubiquitous environmental contaminant associated with a multitude of adverse health effects, including hyperkeratosis, hyperpigmentation, diabetes mellitus, cardiovascular disease, reproductive defects, and cancers of several tissues including liver, bladder, skin, lung, kidney, and prostate [1–17]. The most common route of arsenic exposure is the ingestion of contaminated drinking water, which has been documented in many countries at concentrations as high as 3.4 mg/L, more than 3000 times the World Health Organization recommended limits of 10 μg/L [18–23]. In the United States, more than 56 million Americans are estimated to have been exposed to drinking water containing arsenic concentrations that elevate the fatal cancer risk [24].

Although arsenic is poorly mutagenic in rodent and bacterial models, it has been shown to cause DNA adduct formation, DNA–protein cross-linking, and formation of reactive oxygen species (ROS) in vitro [25–27]. In addition, arsenic has been shown to induce an assortment of signaling events including NF-κB activation, AP-1 activation, c-myc

Abbreviations: GADD45α, growth arrest and DNA damage-inducible protein 45 alpha; As(III), arsenite; tss, transcriptional start site; DNA, deoxyribonucleic acid; ROS, reactive oxygen species; NF-κB, nuclear factor κB; AP-1, activator protein 1; ERK, extracellular signal-regulated kinase; G1/S, gap 1/synthesis phase; G2/M, gap 2/mitosis phase; MMS, methylmethane sulfonate; UV, ultraviolet irradiation; IR, ionizing radiation; FHRE, forkhead response elements; PBS, phosphate-buffered saline; PVDF, Polyvinylidene fluoride; TBS–Tween, Tris-buffered saline; SE, standard error of the mean; ESR, electron spin resonance; G, Gauss; DPPH, diphenyl-2-picrylhydrazyl; GPx1, glutathione peroxidase 1; SOD1, superoxide dismutase 1; cDNA, complementary DNA; EGFP, enhanced green fluorescence protein; qRT-PCR, quantitative real-time polymerase chain reaction; RNA, ribonucleic acid; rRNA, ribosomal ribonucleic acid; C_t, threshold cycle; $^{\bullet}OH$, hydroxyl radical; $O_2^{\bullet-}$, superoxide radical; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger ribonucleic acid; MBF-1, metal binding factor 1; Egr-1, early growth response gene 1; Egr-2, early growth response gene 2; Egr-3, early growth response gene 3; Yap1, yeast activator protein 1; gadd153, growth arrest and DNA damage-inducible protein 153.

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overexpression, involucrin repression, and extracellular signal-regulated kinase (ERK) activation, which are involved in such diverse processes as DNA repair, cellular differentiation, cell cycle arrest, and malignant transformation [28–32]. Together, these findings suggest that arsenic may be exerting its carcinogenic effects through an epigenetic mechanism.

Cell cycle dysregulation and/or perturbation are major mechanisms of carcinogenesis. While the signaling pathways involved in the G_1/S phase checkpoint have been well examined, much less is known about the signaling events that occur at the G_2/M phase checkpoint. Previous data from our lab and others have suggested that arsenic treatment can induce G_2/M phase growth arrest in several different cell lines [33–36]. A major player in G_2/M arrest is the growth-arrest and DNA-damage-inducible protein 45α (GADD45α), which is induced by a variety of stress-induced signals including methyl methanesulfonate (MMS), ultraviolet (UV) irradiation, ionizing radiation (IR), sodium arsenite exposure, and serum starvation [37–42]. In addition, GADD45α is proposed to play roles in chromatin remodeling and apoptosis [43,44].

Transcription of the *gadd45a* gene is thought to be controlled by both the promoter and the third intron regions and seems to be differentially activated depending on the stimulus [45]. For example, in response to IR, the p53 transcription factor binds to its consensus sequence site in the third intron of the *gadd45a* gene and subsequently interacts with the WT1 transcription factor to enhance gene expression [46]. However, several groups have also suggested that after MMS and UV exposure, the ubiquitous Oct-1 transcription factor binds the promoter region between −101 and −82 bp relative to the transcriptional start site (tss) of the *gadd45a* gene with the help of an unidentified cofactor to activate *gadd45a* transcription in a p53-independent manner [47–50]. Recently, the forkhead transcription factor family member FOXO3a has also been shown to bind to the forkhead response elements (FHREs) located in the *gadd45a* gene promoter region at −505, −377, and −803 in response to UV, resulting in transcriptional activation [51]. Thus, *gadd45a* gene regulation is complex and may involve many different signaling pathways.

Since GADD45α is a general stress response protein, we examined the effects of ROS produced in response to As(III) exposure on *gadd45a* gene regulation. We used a human bronchial epithelial airway cell line, BEAS-2B, to examine these effects because the lung is a target of arsenic-induced carcinogenesis, particularly in occupational settings [7]. Our results show that As(III) transcriptionally induces *gadd45a* gene expression from its promoter region via a free radical mechanism involving the formation of H_2O_2.

Materials and methods

Cell culture and media

The BEAS-2B human lung epithelial cell line was obtained from the American Type Culture Collection (Manasses, VA) and maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 5% fetal bovine serum (Invitrogen/Gibco BRL, Carlsbad, CA), 1 unit/mL penicillin, 1 μg/mL streptomycin, and 300 μg/mL L-glutamine (Sigma). BEAS-2B cells were passaged when confluency was reached, usually every 4–5 days.

Western blotting

BEAS-2B cells were grown in six-well tissue culture plates to a density of approximately 1×10^6 cells per well and treated with concentrations of $AsCl_3$ (Sigma) ranging from 0 to 50 μM over a time period of 0–24 h. Cells were lysed with the MPER mammalian lysis reagent (Pierce Biotechnology, Inc., Rockford, IL) and subsequently analyzed for total protein concentration using a BCA Protein Assay kit (Pierce Biotechnology, Inc.); 20 μg of total protein for each sample were used to perform electrophoresis on 4–20% precast gradient gels (Invitrogen). Membranes were incubated with a 1:200 dilution of GADD45α (H-165) primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 5% milk buffer overnight at 4 °C on a rocking platform. For β-actin primary antibody (Sigma) and GAPDH primary antibody (Santa Cruz Biotechnology, Inc.), dilutions of 1:2000 and 1:200, respectively, were used. For anti-rabbit or anti-mouse IgG AP-linked secondary antibody (Cell Signaling Technology, Beverly, MA), a 1:1000 dilution was used. CDP-Star Reagent (New England BioLabs, Inc., Beverly, MA) was used for visualization of protein abundance.

Oxygen consumption

A Gilson 5/6 Oxygraph (Gilson Medical Electronics, Inc., Middleton, WI) was used to perform oxygen consumption studies. BEAS-2B cells were trypsinized and washed with 1× PBS (Invivtrogen/Gibco BRL) and 4×10^6 cells were resuspended in a final volume of 1 mL of PBS; 1 mM $AsCl_3$ (Sigma) was added to each aliquot and allowed to incubate for 20 min at room temperature. Oxygen consumption rates were calculated using the equation

$$\text{nmol } O_2 \text{ consumed/min/}10^6 \text{ cells} = \frac{(\text{BP} - 47)\Delta\text{B}(1.835)}{(\text{m})(\text{t})(\text{n})},$$

where BP is atmospheric pressure in mm Hg, ΔB is number of vertical blocks traversed on the oxygraph chart paper (rise of experimental slope), m is slope of O_2 standards (409 in this case), t is time in min used to determine ΔB (run of experimental slope), and n is $n \times 10^6$ cells used in the assay. Three replicates of three different samples were averaged together to determine mean O_2 consumption and error bars equal ± standard error of the mean (SE).

Electron spin resonance (ESR)

BEAS-2B cells (1×10^6) were suspended in 500 μL of 1× PBS (Invitrogen/Gibco BRL) and treated with 1 mM $AsCl_3$

(Sigma) and/or 2000 U/mL catalase isolated from bovine liver (Sigma). All ESR measurements were conducted using a Bruker EMX spectrometer (Bruker Instruments Inc., Billerica, MA) and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reference standards. The relative radical concentration was estimated by multiplying half of the peak height by $(\Delta H_{pp})^2$, where ΔH_{pp} represents peak to peak width. The Acquisit program was used for data acquisitions and analyses. Reactants were mixed in test tubes in a final volume of 1.0 mL. The reaction mixture was then transferred to a flat cell for ESR measurement. The concentrations given in the figure legends are final concentrations. Experiments were performed at room temperature and under ambient air. Statistical analysis was performed using Student's *t* test and error bars equal ± SE.

Construction of GPx1–EGFP and SOD1–EGFP fusion constructs

pcDNA3.1(+)/GPx1 and pcDNA3/SOD1 constructs were graciously provided to our laboratory by Dr. Larry Oberley's lab at the University of Iowa [52,53]. The GPx1 cDNA was removed from the pcDNA3.1 construct using a *BamHI* (Promega Life Sciences, Madison, WI) and *HindIII* (Promega Life Sciences) double enzymatic digest. The SOD1 cDNA was removed from the pcDNA3 constuct using a *KpnI* (Promega Life Sciences) and *ApaI* (Promega Life Sciences) double enzymatic digest. Both GPx1 and SOD1 cDNA fragments were isolated from an agarose gel and ligated into a pEGFP-C3 (BD Biosciences–Clontech, Palo Alto, CA) vector in frame with the EGFP protein. Contents of both vectors were verified by DNA sequencing (Biotech Core, Inc., Mountain View, CA).

Transient transfections

Transient transfections were performed using SuperFect Transfection Reagent (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Briefly, cells were plated at 50% confluency, and DNA/SuperFect complexes were allowed to enter BEAS-2B cells for 3 h. DNA complexes were removed and fresh growth medium was added. Transfection efficiencies of the pEGFP–C3, pEGFP–C3/GPx1, and pEGFP–C3/SOD1 were calculated by counting EGFP(+) cells with a confocal microscope and determined to be roughly 8–10% for all three plasmids.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from approximately 1×10^6 BEAS-2B cells using an RNeasy Mini Kit (Qiagen, Inc.). Total RNA was quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quantitect SYBR green one-step qRT-PCR kits (Qiagen, Inc.) were used to set up qRT-PCRs using 10 ng of total RNA as a template. qRT-PCR experiments were performed on an ABI 7700 qRT-PCR machine (Applied Biosystems, Foster City, CA). Validated GADD45α primer sets were obtained from Qiagen, Inc. and 18S rRNA primer set controls were obtained from Ambion, Inc. (Austin, TX). Each sample was replicated three times per plate and an average ΔC_t value was calculated. Relative quantitation of GADD45α mRNA was calculated using the $\Delta\Delta C_t$ method. Error bars represent ± SE.

Dual-luciferase reporter assay

BEAS-2B cells were applied to 12-well tissue culture plates at approximately 50% confluency and transiently transfected with either a full-length or a deleted GADD45α promoter–firefly luciferase construct (kind gifts from Dr. Dennis Bruemmer at the University of California–Los Angeles, CA) or a GADD45α intron 3–firefly luciferase construct (a kind gift from Dr. Albert J. Fornace, Jr. at Harvard University, Cambridge, MA) [54,55]. In addition, each sample was cotransfected with a pRL-TK *Renilla* luciferase vector (Promega Life Sciences) underneath the constitutive control of the herpes simplex virus thymidine kinase promoter to provide a measure of transfection efficiency for normalization. Constructs were allowed to express for at least 60 h followed by a 12-h treatment with 25 μM $AsCl_3$ (Sigma). Then 100 μL of firefly luciferase substrate was injected into each well of a Lumitrac 200 96-well plate (USA Scientific, Inc., Ocala, FL) containing 20 μL of cell lysate. Firefly luciferase units were measured by an EG&G Berthold Microplate Luminometer MicroLumat Plus with an automatic injector (EG&G Berthold, Germany). WinGlow software (EG&G Berthold) was used to obtain readings with a 2.0-s delay and a 10.0-s measurement of luciferase activity and expressed in luciferase units. Luciferase substrates were obtained from the Dual-Luciferase Reporter Assay kit (Promega Life Sciences). Three replicates were assayed per experiment and the ratio of inducible firefly luciferase activity to basal *Renilla* luciferase activity

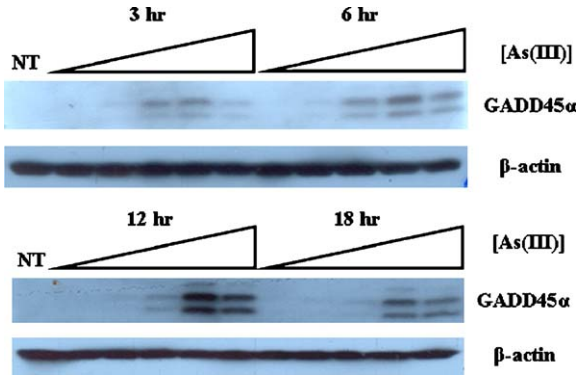


Fig. 1. As(III) (25 μM) induces maximal GADD45α expression at 12 h. A dose/time course Western blot of As(III)-exposed BEAS-2B cells show maximal GADD45α induction after 12 h of treatment. BEAS-2B cells were exposed to As(III) concentrations of 0, 3.25, 6.25, 12.5, 25, and 50 μM; 20 μg of total protein was loaded into each lane and GADD45α expression was detected with a polyclonal antibody. Data shown are representative of three independent experiments.

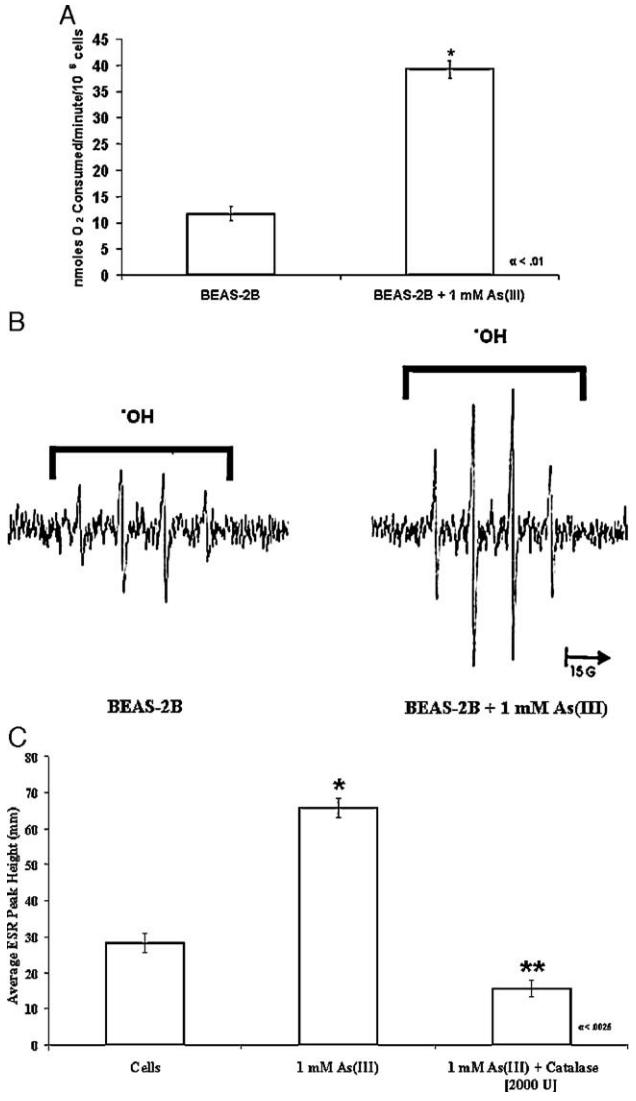


Fig. 2. (A) As(III) exposure increases O_2 consumption in BEAS-2B cells. Aliquots of 4 × 10^6 BEAS-2B cells were injected into a Gilson 5/6 Oxygraph and the rate of oxygen consumption was measured with or without a 20-min exposure to 1 mM As(III). O_2 consumption in the presence of As (III) was increased approximately threefold. Asterisk indicates a statistically significant increase compared to control. Data shown are averages of three independent experiments. (B) As(III) induces the formation of •OH in BEAS-2B cells. Formation of •OH was measured by ESR spectroscopy in aliquots of 1 × 10^6 BEAS-2B cells with or without exposure to 1 mM As (III). Shown are examples of spectra of control BEAS-2B cells (left) and As (III)-exposed BEAS-2B cells (right) exhibiting the characteristic •OH ESR fingerprint. (C) Graphical representation of ESR peak height. ESR peak heights were measured and quantified as described under Materials and methods. As(III)-exposed BEAS-2B cells showed a 2.5-fold increase in the amount of •OH formation which was abrogated by the addition of 2000 units/mL of catalase, confirming the identity of •OH. Single asterisk indicate a statistically significant increase in •OH formation compared to control cells. Double asterisks indicate a statistically significant decrease in •OH formation compared to As(III)-treated cells. Data shown are averages of three independent experiments.

was averaged for each group. Fold induction was calculated by dividing the average ratios of As(III)-treated samples by the average ratios of samples left untreated. Error bars represent ± SE and statistical significance was calculated using Student's *t* test. Data shown are representative of three independent experiments.

Results

As(III) exposure induces expression of the GADD45α protein in a time- and dose-dependent manner

Because GADD45α expression has been observed in response to many stress conditions including UV irradiation, serum starvation, and alkylating agents, we investigated the effects of As(III) exposure on its induction [32,37,38]. To simulate airway exposure to arsenic, an immortalized nontumorigenic BEAS-2B human lung airway epithelial cell line was used for these experiments. Western blot analysis of GADD45α in response to increasing concentrations of $AsCl_3$ revealed a time- and dose-dependent increase in protein abundance (Fig. 1), whereas equal concentrations of NaCl had no effect (data not shown). β-Actin abundance remained unaffected throughout the dose and time course of As(III) exposure. Densitometry readings, normalized to β-Actin expression, confirmed that GADD45α protein abundance increased more than 100-fold over untreated BEAS-2B cells 12 h after 25 μM As(III) treatment (data not shown). This result demonstrates that GADD45α protein is induced in BEAS-2B cells in response to As(III) exposure in a dose-dependent manner.

As(III) exposure increases oxygen consumption and •OH production in BEAS-2B cells

Since As(III) has been shown to exert mutagenic effects via ROS production in several cellular systems, it is possible that the signaling events induced by As(III), specifically GADD45α induction, are also mediated by a ROS mechanism [56]. To address this possibility, BEAS-2B cells were exposed to 1 mM As(III) for 20 min and the rate of oxygen consumption was measured by a Gilson 5/6 Oxygraph. As(III)-exposed BEAS-2B cells showed approximately a threefold increase in the amount of oxygen consumed in vitro (Fig. 2A), suggesting that a free

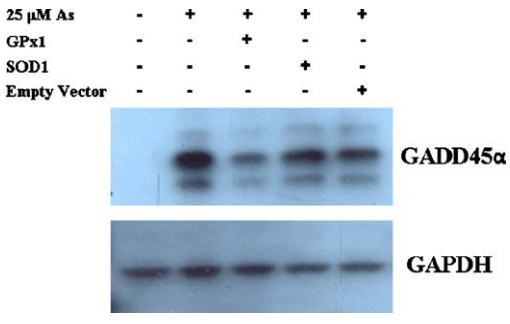


Fig. 3. Overexpression of the H_2O_2 scavenging enzyme GPx1 reduces GADD45α protein expression. BEAS-2B cells were transiently transfected with a GPx1, SOD1, or C3 empty vector and assayed for changes in GADD45α expression via Western blot. Removal of H_2O_2 by GPx1 overexpression decreased GADD45α expression by approximately 50%. Data shown are representative of three independent experiments.

radical mechanism may be occurring in BEAS-2B cells upon As (III) stimulation.

To determine the identity of the radicals produced, we employed electron spin resonance spectroscopy. Representative ESR spectra are displayed in Fig. 2B. A basal level of $^{\bullet}OH$, signified by its 1-2-2-1 relative peak height fingerprint, is produced by normal BEAS-2B cells and was expected as a consequence of normal respiration; however, after a 20-min exposure to 1 mM As(III), the $^{\bullet}OH$ signal significantly increased (Fig. 2B). Spectral results were quantified by peak height measurements in Fig. 2C, where a 2.5-fold increase in $^{\bullet}OH$ production was observed after As(III) exposure compared to normal respiring cells. The $^{\bullet}OH$ signal was attenuated by the addition of 2000 units/mL of catalase, showing that H_2O_2 is a precursor of $^{\bullet}OH$. These data indicate that $^{\bullet}OH$ is a major free radical formed in the BEAS-2B cell line in response to As(III) exposure.

GADD45α protein abundance decreases in response to As(III) in the presence of the H_2O_2 scavenger GPx1

Because As(III) produced $^{\bullet}OH$ in the BEAS-2B cell line and GADD45α is induced in response to many stressors, we hypothesized that its induction may occur through an oxidative stress mechanism. To assess the role of ROS in GADD45α induction, we constructed glutathione peroxidase 1 (GPx1)–EGFP and superoxide dismutase 1 (SOD1)–EGFP overexpression/fusion vectors and examined their effects on GADD45α protein abundance. The GPx1 enzyme is a H_2O_2 scavenger, whereas the SOD1 enzyme is a $O_2^{\bullet-}$ scavenger. GPx1–EGFP, SOD1–EGFP, and empty vector pEGFP–C3 constructs were transiently transfected into BEAS-2B cells. At 48 h after transfection, cells were treated with 25 μM As(III) for 12 h and subjected to Western blot analysis. Overexpression of the GPx1–EGFP fusion construct reduced GADD45α protein abundance by at least 50% (Fig. 3) as measured by densitometry and normalized to GAPDH expression (data not shown). Empty vector and SOD1 construct overexpression did not affect GADD45α expression (Fig. 3), implying that the formation of H_2O_2 is partially responsible for the induction of the GADD45α protein in response to As(III).

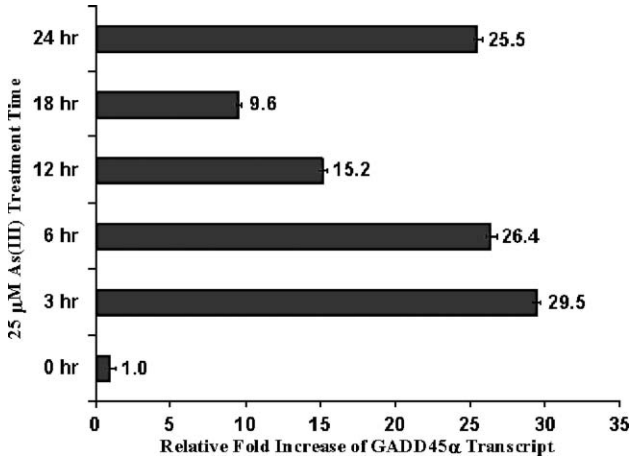


Fig. 4. As(III) exposure increases GADD45α transcript abundance. Relative qRT-PCR was employed to measure the abundance of the GADD45α transcript using the 0-h time point as a calibrator. Transcript abundance increased approximately 30-fold after a 3-h As(III) treatment. Data shown are representative of three independent experiments.

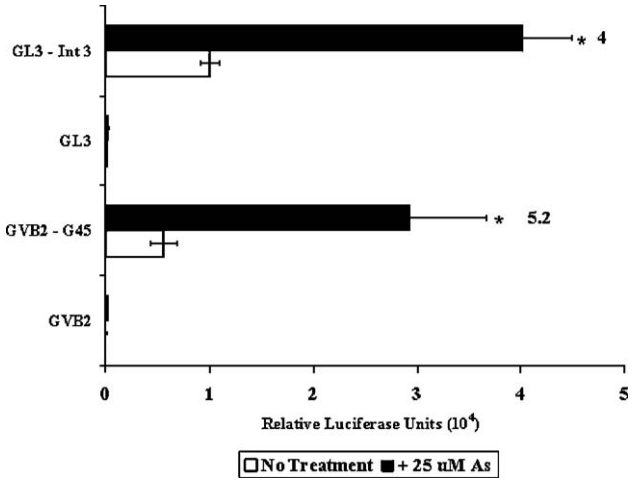


Fig. 5. As(III) activates both the GADD45α promoter region and the intron 3 region. A dual-luciferase reporter system was used to determine the activation of the GADD45α promoter–luciferase (G45) and the intron 3–luciferase (Int3) constructs. Constructs were transiently transfected into BEAS-2B cells and luciferase activity was normalized to an internal transfection control; 5.2- and 4-fold increases in the G45 and Int3 luciferase activity, respectively, were observed in As(III)-exposed samples compared to the untreated control samples. Data shown are representative of three independent experiments.

As(III) exposure increases GADD45α mRNA abundance

To determine how As(III) exposure induces the GADD45α protein, we examined GADD45α transcript abundance at several time points using qRT-PCR. A concentration of 25 μM As(III) was chosen to obtain a maximal effect. Relative quantification of GADD45α mRNA was performed using the

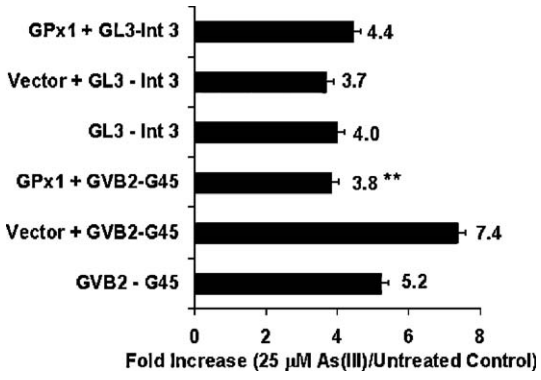
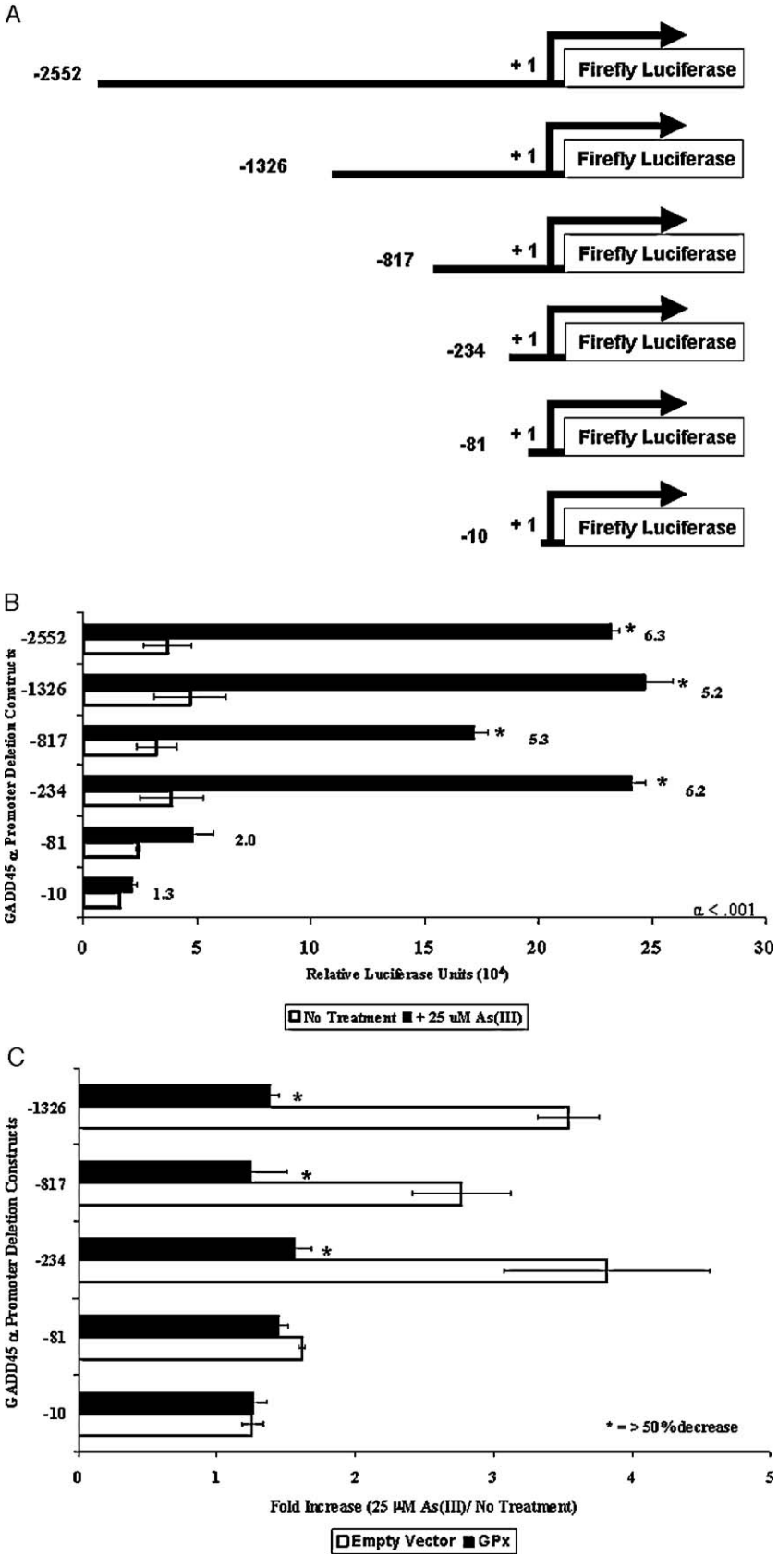


Fig. 6. Overexpression of GPx1 reduces GADD45α promoter activation but not intron 3 activation. G45 and Int3 luciferase constructs were transiently cotransfected with either an empty vector or the vector overexpressing the GPx1 protein and exposed to 25 μM As(III) or left untreated. GPx1 overexpression decreased the G45 promoter–luciferase activity by 50% but seemed to have no effect on Int3 activity. Data shown are representative of three independent experiments.


A
-2552
+1
Firefly Luciferase
-1326
+1
Firefly Luciferase
-817
+1
Firefly Luciferase
-234
+1
Firefly Luciferase
-81
+1
Firefly Luciferase
-10
+1
Firefly Luciferase
B
GADD45 α Promoter Deletion Constructs
-2552
-1326
-817
-234
-81
-10
6.3
5.2
5.3
6.2
2.0
1.3
α < .001
0
5
10
15
20
25
30
Relative Luciferase Units (10⁴)
No Treatment
+ 25 uM As(III)
C
GADD45 α Promoter Deletion Constructs
-1326
-817
-234
-81
-10
* = > 50% decrease
0
1
2
3
4
5
Fold Increase (25 μM As(III)/ No Treatment)
Empty Vector
GPx

0 h time point as a calibration value. As shown in Fig. 4, GADD45α mRNA increases almost 30-fold after 3 h of treatment. Although GADD45α mRNA slightly decreased during the following three time points, it began to increase again at the 24-h time point, mimicking the GADD45α protein expression pattern (see Fig. 1). Therefore, we reasoned that the *gadd45α* gene could be transcriptionally induced by As(III) exposure.

As(III) activates transcription of gadd45α through both the promoter region and the third intron region

To establish whether the increase in mRNA abundance resulted from transcriptional activation or as a consequence of increased mRNA stability, we examined the effects of As(III) on induction of the *gadd45α* promoter and intron 3 regions. GADD45α promoter–luciferase and intron 3–luciferase transcriptional fusion constructs were transiently transfected into BEAS-2B cells, subjected to As(III) treatment for 12 h, and assayed for luciferase activity. The GADD45α promoter construct showed a fivefold increase in luciferase activity in response to 25 μM As(III) (Fig. 5). Similarly, the GADD45α intron 3 construct exhibited a fourfold increase in activity (Fig. 5). Neither of the control constructs containing the luciferase gene alone (GVB2 or GL3) showed any discernible difference between the untreated samples and the As(III)-exposed samples, illustrating that both the promoter and the intron 3 regions of the *gadd45a* gene are activated by As(III) exposure.

Since H_2O_2 was involved in As(III)-induced GADD45a protein levels, we examined the effects of H_2O_2 on the transcriptional activation of *gadd45a*. GPx1 or a control vector was cotransfected with the aforementioned GADD45α promoter and intron 3 constructs. GPx1 overexpression decreased promoter–luciferase activity by 50% compared to transfection with the empty vector (Fig. 6). Surprisingly, the control vector itself slightly increased luciferase activity in the *gadd45a* promoter construct. The reason for this phenomenon is unclear. In contrast, the activity of the intron 3 construct after As(III) exposure remained largely unaffected (Fig. 6). These results suggest a role for H_2O_2 in the transcriptional activation of the *gadd45a* promoter region.

As(III) activation of the GADD45α promoter region occurs between −81 and −234 relative to the tss

Given that the GADD45α promoter–luciferase transcriptional fusion construct was activated by the presence of H_2O_2, we wanted to identify which portion of the promoter region was responsible for the activation. Six GADD45α promoter–luciferase deletion constructs, named for the length of the promoter region with regard to the transcriptional start site (+1),

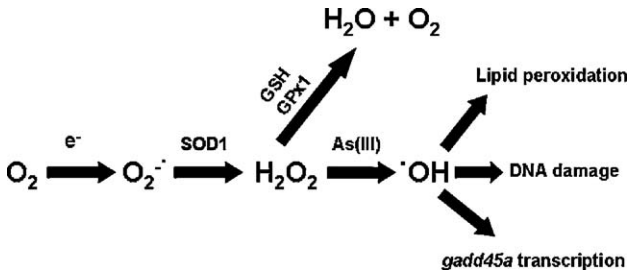


Fig. 8. Proposed ROS pathway involving arsenic. The proposed ROS pathway responsible for the activation of the *gadd45a* promoter region is a Fenton-like reaction in which molecular oxygen accepts an electron, forming $O_2^{\bullet -}$, and is then converted to H_2O_2 by superoxide dismutase. H_2O_2 is then further broken down into water and molecular oxygen, unless the presence of a transition metal, such as As(III), is present. Excess As(III) can then react with H_2O_2 and produce $^{\bullet}OH$, which leads to DNA damage, lipid peroxidation, and activation of cellular signaling pathways, including the induction of *gadd45a* transcription.

were transfected into BEAS-2B cells and exposed to 25 μM As (III) for 12 h (Fig. 7A). A five- to sixfold increase in luciferase activity was observed in all constructs containing at least 234 bp upstream of the +1 site (Fig. 7B). A further deletion of approximately 150 bp (to −81) eliminated most of the luciferase activity. Putative transcription factor binding sites located in this region include consensus sequences for AP-1, MBF-1, Egr-1, Egr-2, Egr-3, and WT1 (-KTS) [57].

We subsequently examined the effects of As(III)-induced H_2O_2 formation on the induction of the promoter deletion constructs. Again, GPx1 overexpression and the removal of H_2O_2 decreased luciferase induction by approximately 50% in all constructs showing more than fivefold induction (Fig. 7C). No change was seen in the smaller constructs. These results suggest that the 153-bp region located between −234 and −81 contains an As(III)-responsive region that is sensitive to the production of H_2O_2.

Discussion

The findings presented here suggest that As(III) exposure induces the transcriptional activation of the *gadd45a* gene through a Fenton-like free radical mechanism (Fig. 8). The Fenton reaction, which is usually associated with an excess of Fe(II), occurs when a transition metal is oxidized during its interaction with H_2O_2, producing an oxidized metal and two molecules of $^{\bullet}OH$. Our data show that As(III) treatment increases the amount of molecular oxygen consumed by BEAS-2B cells, indicating that more oxygen is available for conversion to $O_2^{\bullet -}$ and subsequent formation of H_2O_2. In addition, ESR data show that the relative amount of $^{\bullet}OH$ increases in response to As(III) exposure, which is alleviated by treatment with the H_2O_2 scaven ger enzyme catalase. The removal of H_2O_2 from the intracellular environment by GPx1 overexpression decreases *gadd45a*

Fig. 7. GADD45α promoter activation occurs between the −234 and the −81 regions. (A) Schematic diagram of GADD45α promoter–luciferase deletion constructs. (B) Effects of As(III) on the activation of GADD45α promoter–luciferase deletion constructs were examined after a 12 h treatment period with 25 μM As(III). The full-length (−2552), −1326, −817, and −234 constructs all retained an induction of approximately five to six fold when exposed to arsenic, whereas the −81 construct showed a loss of activation by As(III). (C) Activation of the full-length, −1326, −817, and −234 constructs was reduced by 50% in GPx1-overexpressing cell lysates. Data shown are representative of three independent experiments.

promoter–luciferase activity and GADD45α protein expression, further supporting a role for H_2O_2 in GADD45α induction. Surprisingly, treatment with H_2O_2 alone only weakly induced GADD45α protein expression compared to equal concentrations of As(III) treatment, whereas cotreatment with H_2O_2 and As(III) enhanced GADD45α protein induction (data not shown). Collectively, these results suggest that the formation of $^{\bullet}OH$ through a Fenton-like reaction is at least partially responsible for the induction of *gadd45a* transcription and protein expression in response to As(III) exposure.

The induction of *gadd45a* transcription by the production of H_2O_2 and/or $^{\bullet}OH$ and each molecule's subsequent intracellular activities may partially account for the wide variety of chemical and physical inducers of GADD45α protein expression. IR and UV irradiation, both potent inducers of GADD45α expression, also induce $^{\bullet}OH$ formation [37,58]. Additionally, generation of H_2O_2 and $^{\bullet}OH$ via the Fenton reaction can induce the formation of apurinic sites, the same type of DNA lesion induced by another potent GADD45α inducer, MMS [37,59]. These data suggest that H_2O_2 and/or $^{\bullet}OH$ production may be a key regulator of GADD45α transcriptional induction.

Because BEAS-2B cells contain a p53 protein that has been inactivated by expression of the E6 viral protein, *gadd45a* transcriptional activation by As(III) occurs through a p53-independent mechanism in these cells [60]. Based on the experimental data generated in this study, we have identified several other candidate transcription factors that may be involved in *gadd45a* transcription. One major candidate is the AP-1 transcription factor, since it has previously been identified as an As(III)-activated protein [61,62]. In addition, the yeast homolog of the human AP-1 protein, Yap1, contains a redox-regulated domain consisting of disulfide bonds that can mask or expose its nuclear localization sequence, depending on the redox status of the cell [63]. These findings make AP-1 an ideal candidate for ROS-mediated transcriptional activation. Moreover, the 153-bp region of the *gadd45a* promoter–luciferase deletion constructs affected by antioxidant overexpression boasts four possible AP-1 consensus binding sequences [57]. Finally, AP-1 has also been identified as the transcription factor responsible for increasing expression of the DNA-damage-response gene *gadd153* after As(III) exposure, suggesting that H_2O_2 production and redox status may be a general mechanism for the transcriptional activation of DNA damage-response genes [64].

Alternatively, the early growth response 1 (Egr-1) transcription factor may also be involved in H_2O_2-mediated transcriptional activation of *gadd45a*. Egr-1 has recently been shown to induce *gadd45a* transcription in response to UV irradiation [65]. Furthermore, Egr-1 induction occurs 3 h after 25 μM As(III) treatment, coincidental with the increase in *gadd45a* mRNA abundance shown in Fig. 4 (D. Bhatia and F. Chen, unpublished data). Testing to determine the effects of Egr-1 on *gadd45a* transcriptional activation in response to As(III) exposure is underway in our laboratory.

In conclusion, we have shown that the *gadd45a* gene is transcriptionally induced by As(III) exposure through the formation of H_2O_2. This induction occurs in the promoter region of the *gadd45a* gene and the redox-responsive element is located in the −234 to −81 bp region. Future work will focus on the identification of the transcription factor(s) responsible for the oxidative-stress-dependent transcriptional induction of *gadd45a*.

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