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Original Contributions

Methylated pentavalent arsenic metabolites are bifunctional inducers, as they induce cytochrome P450 1A1 and NAD(P)H:quinone oxidoreductase through AhR- and Nrf2-dependent mechanisms

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ABSTRACT

Activation of the aryl hydrocarbon receptor (AhR) ultimately leads to the induction of the carcinogen-activating enzyme cytochrome P450 1A1 (CYP1A1), and activation of the nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) in addition to the AhR pathway induces the expression of the NADP(H):quinone oxidoreductase (NQO1). Therefore, the aim of this study was to examine the effect of As(III) pentavalent metabolites, MMA(V), DMA(V), and TMA(V), on AhR and Nrf2 activation and on the expression of their prototypical downstream targets *CYP1A1* and *NQO1,* respectively. Our results showed that treatment of HepG2 cells with MMA(V), DMA(V), or TMA(V) in the absence and presence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or sulforaphane significantly induced both *CYP1A1* and *NQO1* at the mRNA, protein, and catalytic activity levels. Furthermore, these metabolites increased the AhR-dependent XRE-driven and the Nrf2-dependent ARE-driven luciferase reporter activities, which coincided with increased nuclear accumulation of both transcription factors. However, none of these metabolites were shown to be AhR ligands. The induction of *CYP1A1* by these metabolites seems to be ligand-independent, possibly through a decrease in HSP90 protein expression levels. The metabolites also increased ROS production, which was significantly higher than that produced by As(III). Upon knockdown of AhR and Nrf2 the MMA(V)-, DMA(V)-, and TMA(V)-mediated induction of both CYP1A1 and NQO1 proteins was significantly decreased. In conclusion, this study demonstrates for the first time that methylated pentavalent arsenic metabolites are bifunctional inducers, as they increase CYP1A1 by activating the AhR/XRE signaling pathway and they increase NQO1 by activating the Nrf2/ARE signaling pathway in addition to the AhR/XRE pathway.

The term “bifunctional inducer” was originally introduced by Prochaska and Talalay in 1988 [1]. Bifunctional inducer refers to compounds that are able to induce phase I aryl hydrocarbon hydroxylases and the phase II metabolizing enzyme NADP(H):quinone oxidoreductase (NQO1) by activating the aryl hydrocarbon receptor (AhR)/xenobiotic-responsive element (XRE) signaling pathway [1]. However, it has been recently accepted that bifunctional inducers activate both XRE- and antioxidant-responsive element (ARE)-driven genes, whereas monofunctional inducers activate only ARE-driven genes [2]. The hydrophobic AhR inducers enter the cell by diffusion and bind to the AhR ligand-binding domain. Upon ligand binding, the AhR–ligand complex dissociates from the cytoplasmic complex, which comprises two molecules of heat shock protein-90 (HSP90), the 23-kDa heat shock protein, and a 43-kDa protein known as the AhR-inhibitory protein or hepatitis B virus X-associated protein 2 [3], and translocates to the nucleus where it associates with its heterodimer the aryl hydrocarbon receptor nuclear translocator [3]. The whole complex then acts as a transcription factor that binds to a specific DNA recognition sequence, termed the XRE, located in the promoter region of a number of xenobiotic metabolizing enzymes, including four phase I enzymes (cytochrome P450 1A1 (CYP1A1), CYP1A2, CYP1B1, and

Abbreviations: AhR, aryl hydrocarbon receptor; ARE, antioxidant-responsive element; As(III), arsenite; CYP1A1, cytochrome P450 1A1; NQO1, NADP(H):quinone oxidoreductase; Nrf2, nuclear factor-erythroid 2 p45-related factor 2; SUL, sulforaphane; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic-responsive element; MMA(V), monomethylarsonic acid; DMA(V), dimethylarsinic acid; TMA(V), trimethylarsine oxide

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CYP2S1) and four phase II enzymes (NQO1, glutathione *S*-transferase A1 (GSTA1), cytosolic aldehyde dehydrogenase-3 (ADH3), and UDP-glucuronosyltransferase 1A6 (UGT1A6)) [3,4].

As the XRE was identified to be the DNA motif that upregulates a battery of genes including phase I and phase II metabolizing enzymes, the ARE was identified to be the DNA motif that upregulates specific phase II genes such as *NQO1, GSTA1, UGT1A6,* and *ALDH3* through the nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)/ARE signaling pathway. Nrf2 is a redox-sensitive member of the cap'n' collar basic leucine zipper family of transcription factors [5]. In response to oxidative stress, Nrf2 dissociates from its cytoplasmic tethering polypeptide, Kelch-like ECH-associating protein 1 (Keap1), translocates to the nucleus, dimerizes with a small musculoaponeurotic fibrosarcoma protein, and thereafter binds to and activates the ARE [6]. The proximity of the XRE and ARE in rodent NQO1 promoters suggests a cross talk and functional overlap between the two signaling pathways [7,8].

Many studies have shown that bifunctional inducers, which activate both the XRE and the ARE signaling pathways, require a direct cross talk between the XRE- and the ARE-mediated pathways for the induction of phase II genes [8]. Of interest, it has been reported that the induction of *NQO1* by selective ARE inducers requires the presence of the AhR, suggesting a more direct cross talk between the XRE- and the ARE-mediated pathways [7]. Furthermore, it has been suggested that mouse Nrf2 is under the control of AhR, as AhR ligands increased Nrf2 mRNA transcript levels [7]. Inversely, it was demonstrated that the expression of AhR, and subsequently CYP1A1, is partially dependent on Nrf2 in Hepa 1c1c7 cells, implying that Nrf2 modulates AhR and its downstream targets [9]. A reduction in AhR mRNA levels in Nrf2-/- mouse livers compared to wild-type provides further support to this hypothesis [9,10]. Moreover, AhR mRNA levels were increased in Keap1-knockout mice, implying a direct effect of Nrf2 in regulating AhR [9].

Previous studies have investigated the effects of arsenic in the form of arsenite (As(III)) on AhR and Nrf2 activity in both human and mouse liver cells [11–13]. In addition, a few studies have examined the effects of As(III) in vivo using wild-type [14] or Nrf2$^{-/-}$ mice [12]. Despite these efforts, the effects of arsenic metabolites on AhR or Nrf2 activities have never been investigated previously.

As(III) is a worldwide environmental pollutant and a human carcinogen. It is well recognized that the carcinogenicity of As(III) is largely dependent on the methylation levels (monomethyl, dimethyl, and trimethyl) that result from its metabolism in mammals [15,16]. In humans, as in many mammals, As(III) is readily absorbed and distributed to a number of organs, such as the liver, where most of its metabolism takes place [17]. There are two possible mechanisms that have been proposed for the metabolism of As(III), biomethylation and glutathione conjugation [16]. Biomethylation is an enzymatic conversion of As(III) to mono-, di-, and trimethylated metabolites, which results in exposure to the parent compound and the methylated arsenicals. We have previously shown that treatment of HepG2 cells and mouse primary hepatocytes with As(III) causes an inhibition of CYP1A1 [14,18]. Furthermore, administration of As(III) to C57BL/6 mice caused an induction of CYP1A1 and NQO1 in mouse livers, suggesting a possible role for biomethylated As(III) metabolites in addition to the role of hemoglobin as an endogenous mediator [14]. Therefore, we hypothesized that the biomethylated arsenic metabolites, namely, monomethylarsonic acid (MMA(V)), dimethylarsinic acid (DMA(V)), and trimethylarsine oxide (TMA(V)), are bifunctional inducers, as they activate both AhR and Nrf2. Thus, the aim of this study was to investigate the effects of MMA(V), DMA(V), and TMA(V) on the prototypical downstream targets of AhR and Nrf2 activities, CYP1A1 and NQO1, respectively, and the possible underlying mechanisms, using human hepatoma HepG2 cells.

Materials and methods

Materials

2′,7′-Dichlorofluorescin diacetate, 7-ethoxyresorufin, fluorescamine, anti-goat IgG peroxidase secondary antibody, protease inhibitor cocktail, 2,6-dichlorophenolindophenol (DCPIP), dicoumarol, isothiocyanate sulforaphane (SUL), and Dulbecco's modified Eagle medium (DMEM) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). High-Capacity cDNA reverse transcription kit, SYBR Green SuperMix, and 96-well optical reaction plates with optical adhesive films were purchased from Applied Biosystems (Foster City, CA, USA). Real-time PCR primers were synthesized by Integrated DNA Technologies. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). Chemiluminescence Western blotting detection reagents were from GE Healthcare Life Sciences (Piscataway, NJ, USA). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA, USA). AhR goat polyclonal primary antibody, HSP90 mouse monoclonal primary antibody, CYP1A1 mouse polyclonal primary antibody, actin rabbit polyclonal primary antibodies, and anti-rabbit IgG peroxidase secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NQO1 rabbit polyclonal primary antibody was purchased from Abcam (Cambridge, MA, USA). Nrf2 mouse polyclonal primary antibody and anti-mouse IgG peroxidase secondary antibody were purchased from R&D Systems (Minneapolis, MN, USA). Human AhR and Nrf2 validated siRNA and primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). pRL-CMV plasmid and dual luciferase assay reagents were obtained from Promega (Madison, WI, USA). Human ARE-driven luciferase reporter plasmid was generously provided by Dr. S. Itoh (University of Toronto). Human AhR/pCMV6-AC-GFP plasmid encoding green fluorescence protein (GFP)-tagged AhR protein was purchased from Origene (Rockville, MD, USA), and human plasmid pcDNA3-EGFP-C4-Nrf2 (Plasmid #21549) encoding enhanced GFP (EGFP)-tagged Nrf2 protein was purchased from Addgene (Cambridge, MA, USA). All other chemicals were purchased from Fisher Scientific (Toronto, ON, Canada).

Cell culture

The HepG2 cell line, ATCC No. HB-8065 (Manassas, VA, USA), was maintained in DMEM with phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 20 μM L-glutamine, 100 IU/ml penicillin, 10 μg/ml streptomycin, 0.1 mM nonessential amino acids, and vitamin supplement solution. Cells were grown in 75-cm^2 cell culture flasks at 37 °C in a 5% CO_2 humidified incubator.

Chemical treatments

Cells were treated in serum-free medium with 5 μM MMA(V), DMA(V), and TMA(V) in the absence and presence of 1 nM TCDD or 5 μM SUL as described in the figure legends. TCDD and SUL were dissolved in dimethyl sulfoxide (DMSO) and maintained in DMSO at −20 °C until use. MMA(V), DMA(V), and TMA(V) (40 mM stocks) were prepared in double-deionized water. In all treatments, the DMSO concentration did not exceed 0.05% (v/v).

RNA extraction and cDNA synthesis

Six hours after incubation with the test compounds, cells were collected and total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and quantified