Toxicology and Applied Pharmacology 277 (2014) 21–29

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Monomethylarsonous acid inhibited endogenous cholesterol biosynthesis in human skin fibroblasts

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ARTICLE INFO

Article history:
Received 17 September 2013
Revised 23 February 2014
Accepted 28 February 2014
Available online 10 March 2014

Keywords:
Monomethylarsonous acid
Quantitative proteomics
SILAC
FASP
De novo cholesterol biosynthesis

ABSTRACT

Human exposure to arsenic in drinking water is a widespread public health concern, and such exposure is known to be associated with many human diseases. The detailed molecular mechanisms about how arsenic species contribute to the adverse human health effects, however, remain incompletely understood. Monomethylarsonous acid [MMA(III)] is a highly toxic and stable metabolite of inorganic arsenic. To exploit the mechanisms through which MMA(III) exerts its cytotoxic effect, we adopted a quantitative proteomic approach, by coupling stable isotope labeling by amino acids in cell culture (SILAC) with LC-MS/MS analysis, to examine the variation in the entire proteome of GM00637 human skin fibroblasts following acute MMA(III) exposure. Among the ~6500 unique proteins quantified, ~300 displayed significant changes in expression after exposure with 2 μM MMA(III) for 24 h. Subsequent analysis revealed the perturbation of *de novo* cholesterol biosynthesis, selenoprotein synthesis and Nrf2 pathways evoked by MMA(III) exposure. Particularly, MMA(III) treatment resulted in considerable down-regulation of several enzymes involved in cholesterol biosynthesis. In addition, real-time PCR analysis showed reduced mRNA levels of select genes in this pathway. Furthermore, MMA(III) exposure contributed to a distinct decline in cellular cholesterol content and significant growth inhibition of multiple cell lines, both of which could be restored by supplementation of cholesterol to the culture media. Collectively, the present study demonstrated that the cytotoxicity of MMA(III) may arise, at least in part, from the down-regulation of cholesterol biosynthesis enzymes and the resultant decrease of cellular cholesterol content.

Introduction

Arsenic compounds are well-known environmental toxicants. As an abundant element in the earth crust, the presence of high levels of arsenic in naturally contaminated drinking water poses a widespread public health problem worldwide (Mandal and Suzuki, 2002). In addition, anthropogenic activities involving the use of arsenicals as pesticides, rodenticides, and fungicides further exacerbate the situation (Collotta et al., 2013). Arsenic exposure was found to be associated with the development of respiratory irritations, dermal defects, neurological diseases, cardiovascular diseases and cancers (ATSDR, 2007). Many mechanisms have been proposed to account for the deleterious effects of arsenic species. In one mechanism, binding of As(III) with protein thiols is thought to disable critical proteins for regulating cell proliferation and metabolism (Kitchin and Wallace, 2008; Shen et al., 2013). Additionally, arsenic was proposed to elicit its cytotoxic effects through the induction of oxidative stress (Flora, 2011; Kitchin, 2001).

Among the different arsenic species, monomethylarsonous acid [MMA(III)], a metabolite of inorganic arsenic, possesses the highest degree of toxicity. Biomethylation is the major mechanism for the metabolism of inorganic arsenic to produce MMA(III), though the role of this transformation in arsenic toxicity is under debate. Traditionally, methylation is viewed as the primary detoxification process since organic arsenic compounds can be detected in the urinary excretion (Marafante et al., 1985). This, however, becomes questionable, as mounting evidence supports that organic arsenic compounds are more toxic than their inorganic counterparts both *in vitro* (Petrick et al., 2000) and *in vivo* (Petrick et al., 2001). Thus, a deep understanding of the modes of action for MMA(III) toxicity will help define the actual role of biomethylation in the toxification and detoxification of arsenic species.

Abbreviations: MMA(III), monomethylarsonous acid; SILAC, stable isotope labeling by amino acids in cell culture; LC-MS/MS, liquid chromatography–tandem mass spectrometry; FASP, filter-aided sample preparation; DMEM, Dulbecco's Modified Eagle's Medium; EMEM, Eagle's Minimum Essential Medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; IAA, iodoacetamide; FA, formic acid; GenMAPP, Gene Map Annotator and Pathway Profiler; FDR, false discovery rate; LDL, low-density lipoprotein; SREBP, sterol regulatory element binding protein; LXR, liver X receptor; RXR, retinoid X receptor; TrxR1, thioredoxin reductase; Sel M, selenoprotein M; MVA, mevalonic acid; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

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http://dx.doi.org/10.1016/j.taap.2014.02.020
0041-008X/

Previous research about arsenic has focused on issues ranging from the detection and speciation of arsenic compounds to the investigation of arsenic transport, metabolism, and toxicity (Le et al., 2004; Mandal and Suzuki, 2002; Watanabe and Hirano, 2013). Genome-wide microarray analysis of human urothelial cells exposed with MMA(III) unveiled alterations of several biological processes and pathways including response to oxidative stress, enhanced cellular proliferation, anti-apoptosis, MAPK signaling, as well as inflammation (Medeiros et al., 2012). Recent advances in mass spectrometry instrumentation and sample preparation methods provide the opportunity for conducting high-throughput and in-depth analysis of the alteration of the whole proteome (Aebersold and Mann, 2003). Therefore, we reason that assessing the changes in global protein expression upon MMA(III) exposure may offer a more complete picture about the mechanisms of its toxicity (Greenbaum et al., 2003).

In this study, we employed liquid chromatography–tandem mass spectrometry (LC–MS/MS), together with stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002) and filter-aided sample preparation (FASP) (Wisniewski et al., 2009), to examine the perturbation of cellular pathways induced by MMA(III) exposure. In total, we quantified approximately 6500 unique proteins, among which 198 and 105 were significantly increased and decreased, respectively, upon MMA(III) treatment. Notably, this study demonstrated, for the first time, that MMA(III) exposure resulted in down-regulation of multiple enzymes engaged in *de novo* cholesterol biosynthesis. This finding, in combination with other experiments, supports that MMA(III) may exert its cytotoxic effect partly by inhibiting *de novo* cholesterol biosynthesis.

Methods

Cell culture. All reagents unless otherwise stated were obtained from Sigma-Aldrich (St. Louis, MO), and all cell lines and cell culture reagents unless otherwise noted were from ATCC (Manassas, VA). MMA(III) and GM00637 cells were generously provided by Profs. X. Chris Le (University of Alberta) and Gerd P. Pfeifer (the City of Hope), respectively. GM00637 and HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, ATCC) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Grand Island, NY) and 100 IU/mL penicillin (ATCC). WM-266-4 cells were maintained under the above-mentioned conditions except that Eagle's Minimum Essential Medium (EMEM, ATCC) was used. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C, with subculture at every 1–2 days.

MTT assay of dose-dependent cell viability was conducted using Cell Proliferation Kit 1 (Roche, Basel, Switzerland) and absorbance was recorded by Victor 2 plate reader (Perkin Elmer, Waltham, MA, Fig. S1). Caspase 3 activity was examined by Western blot using caspase 3 antibody (Cell Signaling, Boston, MA) that detects the endogenous levels of full-length (35 kDa) and large fragments (17/19 kDa) of caspase-3 after cleavage at aspartic acid 175.

For SILAC labeling experiments, light lysine and arginine, or their stable isotope-labeled heavy counterparts ([$^{13}C_6$,$^{15}N_2$]-L-lysine and [$^{13}C_6$]-L-arginine, Sigma), were added to the DMEM medium without L-lysine and L-arginine (Thermo Scientific, Rockford, IL) at concentrations following ATCC formulation to give the complete light and heavy DMEM media, respectively. GM00637 cells were maintained in the complete light or heavy DMEM medium with dialyzed FBS (Life Technologies, Grand Island, NY) for more than 10 days, corresponding to 5 cell doublings, to enable complete stable isotope incorporation into cells.

Monomethylarsonous acid treatment and sample preparation. GM00637 cells, at a density of ~5×10^5 cells/mL in the light or, after complete heavy isotope incorporation, the heavy DMEM medium without dialyzed FBS, were exposed with 2 μM MMA(III) for 24 h. Cells were harvested by centrifugation at 300 ×*g* at 4 °C for 5 min, washed with ice-cold phosphate-buffered saline (PBS) for three times, and lysed with 4% sodium dodecyl sulfate (SDS). Subsequently, the mixture was heated at 95 °C for 5 min and centrifuged at 16,000 ×*g* at 4 °C for 5 min with supernatant collected. The total protein concentration was measured by Bicinchoninic Acid Kit for Protein Determination (Sigma). For the forward SILAC experiment, the light labeled, MMA(III)-treated cell lysate was mixed at 1:1 ratio (*w*/*w*) with the heavy labeled, control cell lysate (Fig. 1A), whereas the labeling and MMA(III) treatment were reversed in the reverse SILAC experiment.

Filter-aided sample preparation. The above equi-mass mixture of light and heavy lysates was reduced with dithiothreitol (DTT) and processed with the FASP procedure (Wisniewski et al., 2009) for the removal of detergents (SDS) and tryptic digestion, where 30 kDa Microcon filtration devices were used (Millipore, Billerica, MA). Briefly, 400 μg lysate was loaded onto the filtration devices, washed with 8 M urea twice, and centrifuged at 14,000 ×*g* for 15 min. After the centrifugation, the concentrates of proteins were alkylated with iodoacetamide (IAA), digested with trypsin (Promega, Madison, WI) at 37 °C overnight, and the resulting peptides were collected by centrifugation of the filter units at 14,000 ×*g* for 20 min.

Off-line strong cation exchange (SCX) fractionation and desalting. The above protein digest was reconstituted in 0.1% formic acid (FA) and loaded onto a PolySulfoethyl A SCX column (9.4 × 200 mm, 5 μm, 200 Å, PolyLC, Columbia, MD). Peptides were eluted with a linear gradient of 0–500 mM ammonium acetate in 0.1% FA over 90 min, and collected every 4.5 min for a total of 20 fractions. Each fraction of the above protein digest was desalted and purified by the OMIX C_{18} pipette tips (Agilent, Santa Clara, CA). Formic acid solution (0.1%) was added until the solution pH of the peptide sample reached ~4. Initially, the OMIX C_{18} tips were hydrated with CH_3CN/H_2O (1:1, *v*/*v*), followed by equilibration with 0.1% formic acid. Subsequently, peptide samples were loaded onto the C_{18} tip. After washing with 0.1% formic acid, bound peptides were eluted with CH_3CN/H_2O (1:1, *v*/*v*).

LC–MS and MS/MS. An LTQ-Orbitrap Velos mass spectrometer was utilized for the on-line LC-MS/MS analysis, which was equipped with a nanoelectrospray ionization source and coupled to an EASY n-LCII HPLC system (Thermo, San Jose, CA). The experimental conditions were the same as those described elsewhere (Guo et al., 2013).

Database search, statistical analysis and pathway analysis. Database search was conducted as previously published (Guo et al., 2013). Briefly, the protein identification and quantification were performed using Maxquant (Cox and Mann, 2008), Version 1.2.2.5 based on the International Protein Index database, version 3.68. To obtain reliable results, the quantification of the protein expression ratio was based on three independent SILAC labeling experiments, which contained two forward and one reverse SILAC labelings. Statistical analysis for the protein expression ratios to define the significantly changed proteins was performed using Perseus 4.0 (Cox and Mann, 2008), where the "Significant A" value for each logarithmized ratio was calculated (Fig. S3, Supporting Information). With a Benjamini–Hochberg false discovery rate (FDR) threshold of 0.05, proteins with expression ratios [MMA(III)-treated/control] being greater than 1.30 or less than 0.70 fold were considered significantly altered. Follow-up pathway analysis of significantly changed proteins was carried out with the use of Gene Map Annotator and Pathway Profiler (GenMAPP-CS) (Salomonis et al., 2007).

RNA extraction and quantitative real-time PCR analysis. Total RNA was extracted from MMA(III)-treated and control GM00637 cells using the Total RNA Kit 1 (VWR, Randor, PA), and reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI) and a poly(dT) primer. Subsequent qRT-PCR was conducted using iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA) and a Bio-Rad MyiQ thermal cycler. Gene-specific primers used for the analysis are listed in Table S1.