Toxicology and Applied Pharmacology 260 (2012) 241–249

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

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

The endoplasmic reticulum is a target organelle for trivalent dimethylarsinic acid (DMA^{III})-induced cytotoxicity

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ARTICLE INFO

Article history:
Received 19 November 2011
Revised 29 February 2012
Accepted 29 February 2012
Available online 8 March 2012

Keywords:
Arsenic
Arsenite
ER-stress
Dimethylarsinous acid
Reactive oxygen species

ABSTRACT

The purpose of present study was to characterize the endoplasmic reticulum stress and generation of ROS in rat liver RLC-16 cells by exposing to trivalent dimethylarsinous acid (DMA^{III}) and compared with that of trivalent arsenite (iAs^{III}) and monomethylarsonous acid (MMA^{III}). Protein kinase-like endoplasmic reticulum kinase (PERK) phosphorylation was significantly induced in cells exposed to DMA^{III}, while there was no change in phosphorylated PERK (P-PERK) detected in cells after exposure to iAs^{III} or MMA^{III}. The generation of reactive oxygen species (ROS) after DMA^{III} exposure was found to take place specifically in the endoplasmic reticulum (ER), while previous reports showed that ROS was generated in mitochondria following exposure to MMA^{III}. Meanwhile, cycloheximide (CHX) which is an inhibitor of protein biosynthesis strongly inhibited the DMA^{III}-induced intracellular ROS generation in the ER and the phosphorylation of PERK, suggesting the induction of ER stress probably occurs through the inhibition of the protein folding process. Activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP) mRNA were induced by all three arsenic species, however, evidence suggested that they might be induced by different pathways in the case of iAs^{III} and MMA^{III}. In addition, ER resident molecular chaperone glucose-regulated protein78 (GRP78) was not affected by trivalent arsenicals, while it was induced in positive control only at high concentration (Thapsigargin;Tg), suggesting the GRP78 is less sensitive to low levels of ER stress. In summary, our findings demonstrate that the endoplasmic reticulum is a target organelle for DMA^{III}-induced cytotoxicity.

Introduction

Human exposure to inorganic arsenic can lead to many health problems such as hyperkeratosis, pigmentation and blackfoot disease, as well as carcinogenesis in liver, lungs and urinary bladder (NRC, 1999, 2001). Although the mechanisms underlying arsenic-induced diseases and cancers are not precisely understood, the arsenic bound to proteins in organs/tissues (or cellular organelle), arsenic-induced DNA methylation changes as well as oxidative stress are considered to be the main contributors to arsenic induced carcinogenesis (Henkler et al., 2010). Furthermore, the oxidative stress is recently considered to be one of the major toxic mechanisms (Eblin et al., 2006; Kitchin and Conolly, 2010; Matés et al., 2010; Valko et al., 2006).

In mammals, inorganic arsenicals are known to be primarily taken up by the liver, and then transformed into mono- and dimethylated arsenicals through a series of methylation reactions by arsenic methyltransferase (AS3MT) (Naranmandura et al., 2007; Stýblo et al., 2002), finally excreted into urine as pentavalent methylated forms (Mandal et al., 2004; Suzuki et al., 2001; Vahter, 1994). Regarding the toxicity between the tri- and pentavalent arsenicals, the trivalent forms are demonstrated to be more cytotoxic and genotoxic than that of pentavalent forms (Kitchin and Conolly, 2010; Liu et al., 2001; Mass et al., 2001; Styblo et al., 1999). Although some of the pentavalent thiolated arsenic metabolites have shown to be much more toxic to cells in a similar manner as trivalent arsenicals are found in human and animal urine (Chilakapati et al., 2010; Naranmandura et al., 2009; Ochi et al., 2008; Suzuki et al., 2010), it has been demonstrated that toxicity of pentavalent thioarsenicals is mainly through their reduction to the trivalent form, dimethylarsinous acid (DMA^{III}) in cells (Naranmandura et al., 2011a). Thereby, trivalent arsenic species

Abbreviations: iAs^{III}, arsenite; iAs^{V}, arsenate; CHX, cycloheximide; DMA^{V}, dimethylarsinic acid; DMA^{III}, dimethylarsinous acid; ER, endoplasmic reticulum; MMA^{III}, monomethylarsonous acid; ROS, reactive oxygen species; HPLC, high performance liquid chromatography; ICP MS, inductively coupled argon plasma mass spectrometry.

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doi:10.1016/j.taap.2012.02.017

responsible for arsenic toxicity and carcinogenesis in the target organs need to be clearly identified.

A number of researchers have indicated that oxidative stress might be a main cause for cancer development in target organs in humans, because the generation of ROS is associated with DNA damage and deletion mutations (Liu et al., 2001; Rossman, 2003). Animal experiments have shown that arsenic can cause cancer in urinary bladder in the rat after long term exposure to dimethylarsinic acid (DMA^V) (Cohen et al., 2007; Wei et al., 1999), and the generation of ROS is suggested to play an important role in the carcinogenesis (Eblin et al., 2006; Kitchin and Conolly, 2010; Wei et al., 1999). On the other hand, 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative stress to DNA and a risk factor for cancer (Wu et al., 2004), was detected in arsenic-related human skin cancer, and in organs (e.g., liver, lungs) of arsenic-exposed animals (An et al., 2004; Yamanaka et al., 2004). In addition, in vitro experiments have indicated that the toxic hydroxyl radicals (OH•) can be generated in rat liver microsomes (i.e., including endoplasmic reticulum and golgi) by incubation with pentavalent DMA^V but not by either of these two pentvalaents, iAs^V or MMA^V, suggesting generation of ROS may be caused by the reduced form of DMA^V in microsomes (Yamanaka et al., 2004). These results indicate that the dimethylated arsenic is one of the most potent toxic forms and it may relate to cancer induction in humans.

Liver is one of the identified target organs for arsenic toxicity, as well as a prime site for the conversion of inorganic arsenic to methylated metabolites (Eblin et al., 2006; Kitchin, 2001; Kitchin and Conolly, 2010; NRC, 1999, 2001). Originally it was thought that these methylation reactions play a protective role, instead, now it has been shown that the methylated species, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), have more toxic effects as compared to inorganic arsenic (Kumagai and Sumi, 2007; Petrick et al., 2000). Our previous studies have shown that the mitochondria are major target organelle for arsenic intermediate metabolite, MMA^{III}, which inhibit the complexes II and IV of electron transfer chain (ETC) inducing reactive oxygen species (ROS) specifically in mitochondria (Naranmandura et al., 2011b). Although it has been confirmed that the intracellular ROS can also be potentially induced by exposing DMA^{III} in cells, but it is still unclear where (i.e., which organelles) the ROS are generated.

The endoplasmic reticulum (ER) is a membranous organelle with diverse functions, which facilitates the proper folding of newly synthesized proteins destined for secretion, cell surface or intracellular organelles, and it provides the cell with a Ca^{2+} reservoir (Høyer-Hansen and Jäättelä, 2007; Ron and Walter, 2007). Although various kinds of stimuli (e.g., Ca^{2+} flux across the ER membrane, glucose starvation, hypoxia or defective protein secretion/degradation) can impose severe stress on the ER, reactive oxygen species are considered to be one of the most important molecules for severely damaging this organelle (Huang et al., 2004; Kim et al., 2009; Szegezdi et al., 2006). On the other hand, the three unfolded protein response (UPR) branches (i.e., PERK, IRE1α and ATF6) are known to promote cell survival by reducing misfolded protein levels (i.e., ER-stress), when too many misfolded/unfolded proteins get accumulated in ER (Lin et al., 2007), especially, PERK, which in turn inactivate the translation initiation factor, eukaryotic initiation factor 2a (eIF2a), by phosphorylation, and induces expression of ER chaperone BiP (GRP78) to protect cells from ER stress (Hung et al., 2010; Lin et al., 2007).

It has been reported that the heavy metal such as lead (Pb) is able to induce ER stress by inhibiting protein folding, disrupting calcium homeostasis as well as inducing GRP78 aggregation (Shinkai et al., 2010). Recently, a few researchers have indicated that inorganic arsenic-induced cell death may be through the oxidative stress-regulated and endoplasmic reticulum stress-triggered signaling pathways (Hung et al., 2010; Tang et al., 2009). However, the effects of arsenic intermediate metabolites (i.e., MMA^{III} and DMA^{III}) on the induction of oxidative stress and ER-stress, after the exposure to inorganic arsenic, are still unclear and a little is known about the molecular mechanisms, toxicological and biological significance of these intermediate arsenic metabolites.

In the present study, we hypothesize that the dimethylated DMA^{III} can specifically induce endoplasmic reticulum (ER) stress as well as oxidative stress in cells simultaneously. To address these questions, the generation of reactive oxygen species (ROS) and PERK signaling pathway (e.g., expressions of ER stress marker proteins (phospho-PERK) and related genes such as ATF4, CHOP and GRP78) was determined by western blotting and real time PCR in rat liver RLC-16 cells after being exposed to trivalent DMA^{III} compared with that of iAs^{III} and MMA^{III}.

Material and methods

Reagents. All reagents were of analytical grade. Milli-Q water (Millipore) was used throughout. Trizma® HCl, Trizma® Base, cytochrome c oxidase assay kit, rotenone, malonic acid, antimycin A, diphenyleneiodonium chloride, 2,6-dichloroindophenol sodium salt hydrate (DCPIP), phenazene methosulfate, decylubiquinone, 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Nitric acid, L-cysteine, sodium arsenite (iAs^{III}) and dimethylarsinic acid [$(CH_3)_2AsO(OH)$] (DMA^V) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Monomethylarsonic acid (MMA^V) was obtained from Tri Chemicals (Yamanashi, Japan). Fluorescein was purchased from Tokyo Chemical Industry Co., LTD (Tokyo, Japan). The arsenic standard solution (1000 μg/mL) for ICP MS was purchased from SPEX CentiPrep (Metuchen, NJ, USA). Stock solutions of all arsenic compounds (10 mmol/L) were prepared from the respective standard compounds. All stock solutions were stored in the dark at 4 °C. Diluted standard solutions for analysis were prepared fresh daily.

Preparation of trivalent monomethylarsonous acid (MMA^{III}) and d dimethylarsinous acid (DMA^{III}). $DMA^{III}(Cys)$ and $MMA^{III}(Cys)_2$ were synthesized in the forms coordinated with cysteine by reducing DMA^V and MMA^V, respectively, with a 5 molar equivalent of L-cysteine in distilled water at 90 °C for 1 h, and then centrifuged at 3000 *g* for 5 min to obtain the supernatant. The trivalent forms in the reaction solutions (i.e., supernatant) were confirmed by comparison with those prepared from their iodide forms in distilled water under a nitrogen atmosphere by HPLC–ICP-MS on a gel filtration GS-220 HQ column. $DMA^{III}(Cys)$ and $MMA^{III}(Cys)_2$ were dissociated to DMA^{III} and MMA^{III}, respectively, under the present column conditions. The methyl protons were detected at 1.26 and 1.34 ppm in NMR data for $DMA^{III}(Cys)$ and $MMA^{III}(Cys)_2$, respectively, confirming methyl protons on trivalent arsenic (methyl protons in DMA^V and MMA^V were detected at 1.85 and 1.99 ppm, respectively) (Naranmandura et al., 2007). Purity of MMA^{III} (98%, with 2% of MMA^V) and DMA^{III} (95%, and with 5% of DMA^V) was confirmed by HPLC–ICP MS, and then immediately diluted in Dulbecco's modified Eagle's medium (DMEM) for the experiments.

Culture of RLC-16 cells. Rat liver-derived cell line, RLC-16 cells were obtained from the Riken Cell Bank (Tsukuba, Japan). Cells were seeded at a density of 1.0×10^6 in a 10 cm dish, and were maintained in low glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 mg/mL of streptomycin, at 37 °C under a 5% CO_2 atmosphere. After 24 h post-seeding, cultures were washed twice with phosphate-buffered saline (PBS), and then fresh medium (FBS free) was added, the cells were then treated with iAs^{III}, MMA^{III} and DMA^{III} for 3 h.